COMPARISON OF FLUORESCENT SPOT TEST AGAINST QUANTITATIVE ENZYME ASSAY FOR DETECTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

BY

DR SARAH ABDUL HALIM

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF PATHOLOGY (HAEMATOLOGY)

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LIST OF ABBREVIATIONS AND SYMBOLS

ATP	Adenosine Triphosphate
BioSensor1	careSTART^{TM} BioSensor1
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-Acetic Acid
FST	fluorescent spot test
G6PD	Glucose-6-Phosphate Dehydrogenase
GSSG	Glutathione Disulfide
GSH	Glutathione
GPX	Glutathione Peroxidase
HUSM	Hospital Universiti Sains Malaysia
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide Phosphate
NPV	Negative Predictive Value
PMS	Phenazine Methosulphate

PPV	Positive Predictive Value
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
Ru5P	Ribulose-5-phosphate
UGT	Uridine Diphosphate Glucuronosyltransferase 1A1
6PGD	6-phosphogluconate dehydrogenase

ABSTRAK

Objektif: Kekurangan enzim Glucose-6-Phosphate Dehydrogenase (G6PD) adalah penyakit kekurangan enzim yang paling biasa dijumpai. Sesetengah negara menggunakan ujian 'fluorescent spot test' (FST) sebagai kaedah utama untuk saringan bayi sejak tahun 1980. Walau bagaimanapun, FST mempunyai kekurangan yang tersendiri. Ujian enzim kuantitatif seperti ujian careSTART^{TM} Biosensor1 telah dibuktikan berupaya untuk mengatasi sebahagian daripada kekurangan ujian FST. Objektif kajian ini adalah untuk membandingkan prestasi antara ujian G6PD FST dan BioSensor1, dan mengesahkan julat rujukan tahap enzim G6PD untuk sampel darah tali pusat menggunakan BioSensor1.

Metodologi: Kajian ini adalah kajian 'cross-sectional' yang melibatkan 455 bayi yang dilahirkan di Hospital Universiti Sains Malaysia (Hospital USM), Kelantan, Malaysia di antara bulan Jun 2020 sehingga bulan Disember 2020. Dua mililiter darah tali pusat diambil di dalam botol EDTA untuk dianalisa menggunakan BioSensor1 dan darah tali pusat yang dikeringkan di atas kertas turas dianalisa menggunakan FST. Data demografik dan data kelahiran diambil daripada rekod di dewan bersalin. Data direkod dan dianalisis menggunakan aplikasi 'Statistical Package for the Social Software' (SPSS) versi 27. Nilai P kurang daripada 0.05 dianggap signifikan.

Keputusan: Tahap kepekaan FST adalah 91% manakala tahap kekhususan FST adalah 97% di senggatan 30% tahap aktiviti enzim G6PD. Ini dibandingkan dengan, di senggatan 60% tahap aktiviti enzim G6PD, tahap kepekaan menurun secara drastik kepada 29% dan tahap kekhusuan adalah 100%. Kelaziman kekurangan enzim G6PD adalah 5.1% untuk FST dan 17.8% untuk Biosensor. Ini menunjukkan perbezaan yang ketara di antara kedua-dua ujian ini ($p < 0.001$). Min tahap enzim G6PD untuk bayi matang adalah 6.84

U/gHb manakala untuk bayi pramatang adalah 6.63 U/gHb. Menggunakan ‘independent T-Test’, tiada perbezaan ketara di antara min tahap enzim G6PD untuk bayi matang dan pramatang. Tiada hubungan ketara yang dapat dikaitkan di antara umur kandungan, jenis kumpulan darah ibu dan berat kelahiran dengan min tahap enzim G6PD apabila diuji dengan ‘one way ANOVA test’.

Konklusi: FST mempunyai tahap kepekaan yang rendah pada senggatan 60% tahap aktiviti enzim G6PD. Tahap senggatan ini menunjukkan tahap pertengahan aktiviti enzim G6PD maka ujian FST terlepas saringan sebahagian individu yang mempunyai aktiviti enzim G6PD di tahap pertengahan. Dalam masa yang sama, kelaziman kekurangan G6PD enzim meningkat secara ketara dengan menggunakan kaedah BioSensor1. Umur kandungan, jenis kumpulan darah ibu dan berat kelahiran tidak mempunyai hubungkait yang ketara dengan tahap enzim G6PD. Di dalam fasiliti kesihatan yang mempunyai kekangan kewangan, adalah dinasihatkan untuk menggunakan kaedah enzim kuantitatif untuk menyaring bayi perempuan dan menggunakan FST untuk bayi lelaki terutamanya di kawasan di mana penyakit kekurangan enzim G6PD adalah berleluasa.

ABSTRACT

Objectives: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy in the world. Some countries have practiced fluorescent spot test (FST) as a neonatal screening method since the 1980s. However, FST has its own limitations. Quantitative assays such as the careSTART^{TM} BioSensor1 have been demonstrated to be able to overcome some of these limitations. The objectives of this study are to compare the performance of FST with BioSensor1 in detecting G6PD deficiency in neonates and to verify the reference range of G6PD level for cord blood using BioSensor1.

Methods: This study was a cross sectional study involving 455 neonates born in Hospital Universiti Sains Malaysia (Hospital USM), Kelantan, Malaysia beginning June 2020 until December 2020. Two millilitres of cord blood were taken in EDTA bottles to be analysed with BioSensor1 and dried cord blood spots on filter paper were sent for FST to determine the levels of G6PD. Demographics data and birth characteristics were taken from labour room records. Data was recorded and analysed using the Statistical Package for the Social Software (SPSS) version 27. P-value less than 0.05 were considered as significant.

Results: The sensitivity of FST was 91% whilst its specificity was 97% at 30% cut-off G6PD activity level. In contrast, at 60% cut-off G6PD activity level, the sensitivity drastically decreased to 29% whilst the specificity was 100%. The overall prevalence of G6PD deficiency was 5.1% and 17.8% for FST and Biosensor1 respectively, demonstrating a drastic difference between the two tests ($p < 0.001$). The mean G6PD level for term neonates was 6.84 U/gHb whilst for preterm neonates was 6.63 U/gHb. Using independent T-Test, there was no significant difference in the mean G6PD levels

between term and preterm neonates. There was also no significant association between different gestational age groups, maternal blood group and birthweight with mean G6PD level when tested using one way ANOVA test.

Conclusions: FST has 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 our study. At the same time, the prevalence of G6PD deficiency significantly increased with the use of BioSensor1. Gestational age, birthweight and maternal blood group do not have significant association with G6PD level. In a budget constrained facility, it is recommended to use quantitative enzyme assay to screen female neonates and to use FST for male neonates especially in areas where G6PD deficiency is prevalent.

Keywords: G6PD deficiency, fluorescent spot test, BioSensor1, prevalence.

Chapter 1: Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy globally. It affects up to 400 million individuals with the highest prevalence of the disease being 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.[1] Individuals with G6PD deficiency are usually asymptomatic, however they can present with oxidative haemolysis after exposure to oxidative stress. G6PD deficiency can also cause neonatal jaundice.

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 reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) which provides reducing power to the red blood cells. Red blood cells do not contain mitochondria; hence the pentose phosphate pathway is the only source of NADPH.[1] NADPH production in the cells is important as it confers protection from oxidative stress, whilst also serving as electron donor for many enzymatic reactions.

The inheritance of G6PD deficiency follows X-linked pattern. [2] Because 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 either have normal gene expression, or are heterozygous (partial deficiency). Rarely, will there also be homozygous or have compound heterozygosity for two G6PD gene mutation. [2] 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, rendering these females to be susceptible to oxidative stress. [1]

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). The FST was introduced in Malaysia as a national screening test for G6PD deficiency in 1980. However, studies have shown that the FST can miss a significant proportion of females with intermediate levels of G6PD when compared with quantitative methods [3]. This is detrimental because even individuals with intermediate levels of G6PD can have haemolysis crisis. There are also several quantitative tests that are available for quantification of G6PD activity such as the spectrophotometric assay, which is the current gold standard, and point-of-care assay such as careSTART^{TM} BioSensor1 (BioSensor1). Quantitative enzyme assays can quantify the amount of G6PD activity either by normalization of haemoglobin or red blood cell count. BioSensor1 is a point-of-care assay that can measure the haemoglobin level with G6PD in a sample.

Justifications for this study include being able to address the dilemma of services offered for detection of G6PD deficiency in the laboratory. On one hand, it is known that FST can miss heterozygote females but on the other hand, the use of spectrophotometric assays as screening test are laborious and costly. A cheaper, less laborious method is needed to address this dilemma. As BioSensor1 is a point-of-care assay with cheaper upfront costs, it is an attractive alternative to spectrophotometric assays.

This study aims to study the performance of FST and BioSensor1 in detecting G6PD deficiency with implications to future services. This study also aims to demonstrate

the difference in the detection rate of G6PD deficiency between the two methods, to verify the reference range for G6PD level in cord blood for BioSensor1 and lastly, to detect any significant difference in G6PD levels between term and premature neonates, which could potentially lead to the development of new reference range for premature neonates.

This study was a cross sectional study involving neonates born in Hospital Universiti Sains Malaysia (HUSM), with the sample collection period taken from June 2020 until December 2020. Dried cord blood spots taken with filter paper and two millilitres of cord blood were taken in ethylenediaminetetraacetic acid (EDTA) bottles at delivery. The dried cord blood spots were processed with FST whilst cord blood in EDTA bottles were processed with BioSensor1. Demographics data were obtained from labour room records. Data was recorded and analysed using the Statistical Package for the Social Software (SPSS) version 27.

This dissertation was arranged according to Format B (Manuscript ready format) according to guideline by Postgraduate Office, School of Medical Sciences (2016). The following chapter contains the objectives of the study. Chapter 3 is the manuscript entitled "Performance Comparison between Conventional Fluorescent Spot Test and Quantitative Assay in Detecting G6PD Deficiency and Practical Recommendations." that has already been submitted to Oman Medical Journal and is currently under review. Chapter 4 contains the study protocol that had been submitted and had obtained ethical approval from Jawatankuasa Etika Penyelidikan Manusia (JEPeM), Universiti Sains Malaysia. The appendices contain elaboration of methodology, additional literature review, additional results and additional discussion. The raw data is included in the the attached CD.

Chapter 2: Objectives

General Objectives

To study the performance of fluorescent spot test and BioSensor1 in detecting G6PD deficiency in neonates.

Specific Objectives

1. To compare the prevalence of G6PD deficiency in neonates born in Hospital USM using fluorescent spot test and BioSensor1

2. To compare mean G6PD levels using BioSensor1 in term and premature neonates.

3. To verify the reference range of G6PD level for cord blood using BioSensor1 in haematology lab Hospital USM.

Objectives 2 and 3 are not included in the proposed manuscript for publication and are discussed in the appendices.

Chapter 3: Manuscript

3.1 Title page

Title: Performance Comparison between Conventional Fluorescent Spot Test and Quantitative Assay in Detecting G6PD Deficiency and Practical Recommendations.

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3.2 Abstract

Objective*:* Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy in the world. Some countries in the Asia Pacific region have practiced conventional fluorescent spot test (FST) as a neonatal screening method since the 1980s. However, FST has its own limitations. Quantitative assays such as the careSTART^{TM} BioSensor1 (BioSensor1) has been demonstrated to be able to overcome some of the limitations of FST. It is important to 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 BioSensor1 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^{TM} BioSensor1 and at the same time, dried cord blood spots were sent for FST. Data was recorded and analysed using SPSS version 27. This study was carried out with the ethical approval of Human Research Ethics Committee (HREC) of Universiti Sains Malaysia.

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 drastic 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 our 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. It is recommended, in resource poor areas with high prevalence of G6PD deficiency, to use quantitative enzyme assay to screen female neonates and to use FST for male neonates.

Keywords: G6PD deficiency, fluorescent spot test, BioSensor1, prevalence, sensitivity, specificity.

3.3 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 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 reduced 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 generating 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. [2] NADPH is also needed to reduce GSSG and the sulfhydryl groups of some necessary proteins which protects against oxidative stress. If this protection against ROS are absent, the red blood cells can undergo oxidative haemolysis. [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. [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 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. [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 for quantification of 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^{TM} Bioensor1 and to analyse the difference in prevalence of G6PD deficiency in neonates between both methods.

3.4 Method

A cross sectional study was conducted amongst neonates born in Hospital USM, located in the state of Kelantan in the 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 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 intra uterine growth restriction. Two millilitres of cord blood were taken in EDTA bottles during delivery for quantitative enzyme activity measurement by careSTART^{TM} 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 FST 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 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 lack of or no fluorescence. For the test, 100μL of 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 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^{TM} BioSensor1 (WELLS BIO, INC. Korea). This method uses electrochemical method to measure the enzyme activity in a sample. It measures the electron transfer from NADPH into reduced NADPH by 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. Quality control level 1 and 2 were performed before analysis. Haemoglobin strip and G6PD strip were inserted at the designated spots on the analyser and 20 μL of blood was pipetted on each strip. G6PD activity is calculated automatically from the instrument. Reading from the analyser was then recorded onto the worksheet.

Data was recorded and analysed using SPSS version 27. For the reference range, 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 are categorised as deficient, 30%-60% as intermediate and >60% as normal, and these cut off values are derived from the available literature.[7] Sensitivity, specificity, positive predictive value, negative predictive value, and prevalence were

calculated for FST using the afore-mentioned cut-off value obtained from BioSensor1. 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.

3.5 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. Table 1 demonstrates the demographics data of our study sample.

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 2]

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 3]. The distribution of G6PD status, divided into deficient, intermediate, and normal level, differed when stratified by gender and gestational age as highlighted in table 4.

Figure 1 depicts the distribution of G6PD enzyme level (U/gHb) 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.

3.6 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%).[8] Our sample's demographic follows a similar distribution.

The diagnostic performances of FST in this study is outlined in Table 2. 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 is 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 one particular past study, the sensitivity of FST was 100% at <30% activity level but decreased to 65% at <70% activity level, whilst in another study,

the sensitivity of FST is 91.4% and the specificity was 99.9% at <30% activity. [9, 10] It is interesting that in the study by Thieleman et al, the reduction of sensitivity at <60% threshold was more drastic than in the earlier study. 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. A past study found that the PPV for FST when used in new-born cord blood samples was 97.7% (95 % CI: 96.9-98.5). [10] On the other hand, another study found that the PPV was 72.0% (95% CI: 50.6–87.9) at 30% cut-off value. [7] In this latter study, samples that were diagnosed as G6PD deficient by FST were mainly in the intermediate group when classified according to their G6PD enzyme activity. 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. [4,9] The prevalence of G6PD deficiency using Biosensor1, was higher compared to previous study that used spectrophotometer as quantitative G6PD enzyme assay, which could possibly be due to the fact that their study used a cut-off point (taken arbitrarily) of <20% as G6PD deficiency. [4] Past literature found that the prevalence

of G6PD deficiency increases to 9.8% when using quantitative assay as opposed to FST (1.3%).[4]

Data on the global prevalence of G6PD deficiency showed great heterogeneity. The prevalence of G6PD deficiency would differ according to ethnicity, locality and the method used to detect the deficiency. Globally, the estimated prevalence is 4.9%.[11] It is observed highest in Sub Saharan Africa (7.5%), followed by the Middle East (6.0%) and Asia (4.7%). The prevalence did not significantly differ within Europe (3.7%) and the Americas (3.4%).[11] The failure of semiquantitative screening test to detect heterozygous females would also contribute to the discrepancy in prevalence. In the past, based on data published in 1985 from the WHO Working Group, the global prevalence was 7.5% for carriers of mutant alleles and 3.4% with G6PD deficient phenotype. The 3.4% included all male hemizygotes, all female homozygotes and some female heterozygotes. The current estimated global prevalence of 4.9% is higher than the previous estimate which could reflect the range of phenotypic expression of female heterozygotes. [11] Fundamentally, different screening tests would yield different estimated prevalence. If DNA analysis were employed, all heterozygous females would be classified as G6PD deficient since DNA analysis detects the presence of the mutant allele. However, if semiquantitative tests were used, the percentage of activity for each patient will vary.

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. [12] A past study 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. [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.

There are over 400 G6PD variants with different clinical and biochemical properties, and more than 200 G6PD mutations have been reported. The majority of the G6PD mutations are from single nucleotide substitutions (which are effectively missense variants), and the rest are due to multiple mutations, deletions or introns affected mutations. [13] In Malaysia, different G6PD variants are seen amongst different races. For example, in ethnic Malays, G6PD Viangchan is the most common mutation, followed by G6PD Mediterranean and G6PD Mahidol.[14] However, these mutations have been characterized as having low G6PD levels (<10% of enzyme level), which contrast with our findings as in our study population, most G6PD deficient patients have enzyme levels of more than 30%, even though our ethnic Malays are the majority of the sample population. It is postulated that in Malay Kelantanese population, there might be other types of mutation that confer slightly higher G6PD level than the reference population used in the company's reference range. It is therefore proposed for future studies, to include molecular assays to help bridge this knowledge gap.

We observed that BioSensor1 can detect a higher proportion of neonates with intermediate level of G6PD deficiency (15.8%) as compared to FST (0.7%). 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 population 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. [15] Consequently, some heterozygous females have ratios that have high proportion of RBCs with normal G6PD enzyme level whilst some heterozygous females have high proportion of RBCs with decreased G6PD enzyme level. This implies that many heterozygous females will have enzyme level 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. This is in concordance with the trend of our results.

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 distribution 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. [15] 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, additionally, the implications go beyond the neonatal period. In the neonatal period, G6PD deficient and G6PD intermediate neonates are at higher risk of neonatal jaundice which subsequently may 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 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. [16] 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. [1 4 10]

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. [17] 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. [9] The advantages of using BioSensor1 as a point-of-care test include cheaper price and faster result availability. It is also easier to perform by minimally trained staff, compared to the spectrophotometer.

3.7 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 30% cut-off value its sensitivity is acceptable to correctly predict individual having G6PD deficiency. Hence, it is recommended, especially in a budget constrained facility where it is not feasible to perform quantitative assays on all samples, that for male neonates, it is acceptable to use FST as a screening method, but for female neonates, a quantitative assay would give a better predictor to their true G6PD status. It is also proposed that in the future, molecular study is performed in parallel to confirm the G6PD variants to bridge the knowledge gap between genotype and phenotype association.

3.8 Tables and Figures

Table 1: Study Sample Demographics (n=455)

Characteristics	Number of samples (n)	Frequency (%)
Ethnic Group		
Malay	443	97.4
Thai	4	0.9
Arab	3	0.7
Rohingya	3	0.7
Chinese	2	0.4
Gender		
Male	217	47.7
Female	238	52.3

Table 2: Performance of FST at specific G6PD cut-off value

Cut-off	G6PD activity (U/gHb)	Sensitivity(%) 95% CI	Specificity(%) 95% CI	Positive Predictive Value (95% CI)	Negative Predictive Value (95% CI)	Prevalence (95% CI)
30%	<2.8	91% (57-100)	97% (95-98)	43.5% (24-65)	99.8% (98-99)	2.4% (1.2-4.4)
60%	2.8 - 5.6	29% (19-40)	100% (98-100)	100% (82-100)	86.8% (83-89)	1.8% (1.4-2.2)

Cohen's κ agreement was determined to see the agreement between these two methods.

There was fair agreement between the two methods, $\kappa = 0.21$, $p < 0.001$

Table 3: Difference in prevalence of G6PD deficiency across groups.

Category	**Frequency (n)**			**BioSensor1**		**p-value**
				Normal	Deficient	
Male	218	**FST**	Normal	185	16	<0.001
			Deficient	0	17	
Female	237	**FST**	Normal	189	42	<0.001
			Deficient	0	6	
Term	371	**FST**	Normal	308	44	<0.001
			Deficient	0	19	
Preterm	84	**FST**	Normal	66	14	<0.001
			Deficient	0	4	

Table 4: Prevalence of G6PD stratified by gender and gestational age.

	Method	Deficient (activity <30% of normal)	Intermediate (activity between 30% to 60% of normal)	Normal (activity > 60% of normal)
Male	FST	16 (7.3%)	1 (0.5%)	201 (92.2%)
	BioSensor1	9 (4.1%)	24 (11.0%)	185 (84.9%)
Female	FST	4 (1.7%)	2 (0.8%)	231 (97.5%)
	BioSensor1	0 (0%)	48 (20.3%)	189 (79.7%)
Term	FST	16 (4.30%)	3 (0.80%)	352 (94.90%)
	BioSensor1	8 (2.20%)	55 (14.80%)	308 (83.00%)
Preterm	FST	4 (4.80%)	0 (0.00%)	80 (95.20%)
	BioSensor1	1 (1.20%)	17 (20.20%)	66 (78.60%)

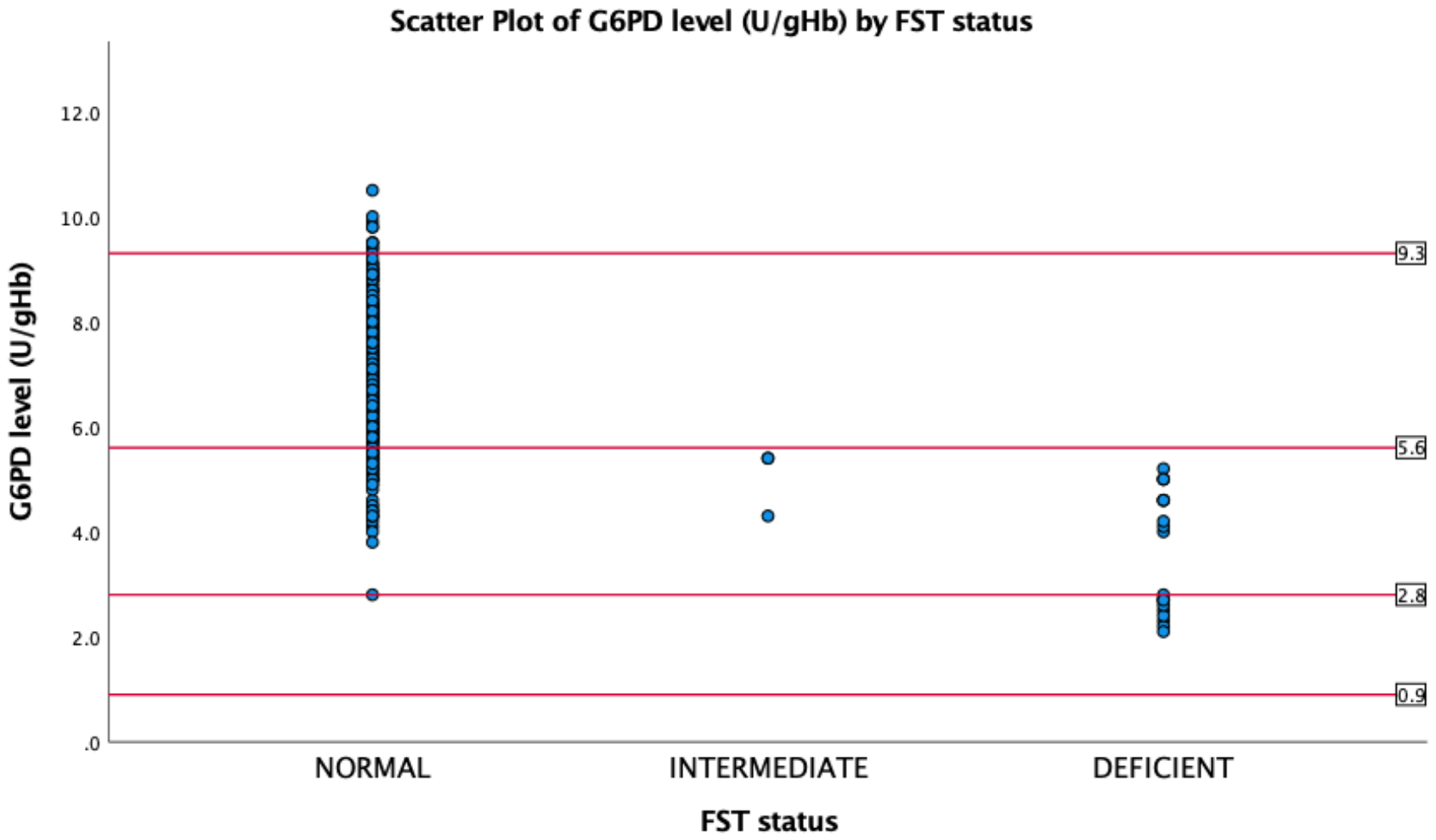

Figure 1: Distribution of G6PD level (U/gHb) according to FST status.