Modification to reporting of qualitative fluorescent spot test results improves detection of glucose-6-phosphate dehydrogenase (G6PD)-deficient heterozygote female newborns

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doi:10.1111/j.1751-553X.2011.01309.x

Received 27 September 2010; accepted for publication 25 January 2011

SUMMARY

Introduction: The glucose-6-phosphate dehydrogenase (G6PD) fluorescent spot test (FST) is a useful screening test for G6PD deficiency, but is unable to detect heterozygote G6PD-deficient females. We sought to identify whether reporting intermediate fluorescence in addition to absent and bright fluorescence on FST would improve identification of mildly deficient female heterozygotes.

Methods: A total of 1266 cord blood samples (705 male, 561 female) were screened for G6PD deficiency using FST (in-house method) and a quantitative enzyme assay. Fluorescence intensity of the FST was graded as either absent, intermediate or normal. Samples identified as showing absent or intermediate fluorescence on FST were analysed for the presence of G6PD mutations using TaqMan®SNP genotyping assays and direct nucleotide sequencing.

Results: Of the 1266 samples, 87 samples were found to be intermediate or deficient by FST (49 deficient, 38 intermediate). Of the 49 deficient samples, 48 had G6PD enzyme activity of $\geq 9.5$ U/g Hb and one sample had normal enzyme activity. All 38 intermediate samples were from females. Of these, 21 had G6PD activity of between 20% and 60%, and 17 samples showed normal G6PD activity. Twenty-seven of the 38 samples were available for mutation analysis of which 13 had normal G6PD activity. Eleven of the 13 samples with normal G6PD activity had identifiable G6PD mutations.

Conclusion: Glucose-6-phosphate dehydrogenase heterozygote females cannot be identified by FST if fluorescence is reported as absent or present. Distinguishing samples with intermediate fluorescence from absent and bright fluorescence improves detection of heterozygote females with mild G6PD deficiency. Mutational studies confirmed that 85% of intermediate samples with normal enzyme activity had identifiable G6PD mutations.
INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide. This X-linked inherited disorder most commonly affects persons of African, Asian, Mediterranean or Middle Eastern descent. G6PD catalyses nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form, NADPH in the pentose phosphate pathway. Because erythrocytes do not generate NADPH in any other way (Who Working Group, 1989), this deficiency increases the vulnerability of erythrocytes to oxidative stress. Clinical presentations are variable and include acute haemolytic anaemia, chronic haemolytic anaemia, neonatal hyperbilirubinaemia or absence of symptoms. In hemizygote deficient males, it is capable of causing neonatal hyperbilirubinaemia and haemolysis when subjects are exposed to oxidant agents. Females are also liable to haemolysis although the severity is variable (Meloni et al., 1983; Beutler, 2008).

Several tests are available for the detection of G6PD deficiency but only a few reliably detect heterozygous females. Molecular testing is available for diagnosis of G6PD deficiency in homozygous, hemizygous and heterozygous patients. The disadvantage of this method is that only a single mutation can be detected with one primer pair. Tests based on the measurement of NADPH production capacity of G6PD enzyme can be used for the diagnosis of all mutations of which the most frequently used are the fluorescence spot test (FST), the spectrophotometric assay and the cytochemical assay. These tests are based on the formation of fluorescence (the FST and the spectrophotometric assay) or colour (cytochemical assay) (Peters & Noorden Van, 2009). The FST based on the method proposed by Beutler (Beutler & Mitchell, 1968) is a simple, rapid and relatively cheap test for the identification of hemizygous males and homozygous females. However, this method has been described as being inappropriate for identification of heterozygote females (Reclos, Hatzidakis & Schulpis, 2000; Zaffanello et al., 2004). The qualitative nature of the test with results reported as either positive or negative fluorescence only allows for the detection of individuals with severe G6PD deficiency. In contrast, the spectrophotometric assay that quantitates enzyme activity shows better sensitivity for the identification of phenotypes in females ranging from severe deficient heterozygotes to mildly deficient heterozygotes (Wang et al., 2009). The cytochemical assay can detect all forms of G6PD deficiency in the red cells of patients. However, it is time-consuming, technically difficult and relatively expensive (Peters & Noorden Van, 2009).

The World Health Organization (WHO) has divided G6PD deficiency into five classes according to the level of enzyme activity in the red cells and clinical manifestations (Who Working Group, 1989). Class I includes severely deficient variants that are associated with chronic nonspherocytic haemolytic anaemia (CNSHA). Class II variants have <10% of residual enzyme activity but without CNSHA, Class III variants are moderately deficient (10–60% residual enzyme activity), Class IV variants have normal enzyme activity, and in Class V, the enzyme activity is increased. The common pathological variants are all in Classes II and III.

Simple screening methods for G6PD deficiency regardless of type are needed because, in principle, it is desirable to detect: (i) hemizygous males (and homozygous females) to advise them to take simple precautions to avoid an acute haemolytic crisis, (ii) newborn hemizygous males (and homozygous females) to ensure that neonatal jaundice is detected and treated early and (iii) heterozygous females to give specific advice about the care of their male newborn infants. G6PD variants with <20% residual enzyme activity are considered as severe deficiency while those with 20–60% of residual activity are classified as mild deficiency.

Reclos et al. (Reclos, Hatzidakis & Schulpis, 2000), in their study evaluating the ability of the semi-quantitative FST to detect partial deficiency of G6PD, claimed that a major drawback of the modified semi-quantitative method described by Beutler (Beutler et al., 1977) was its low cut-off limit (<2.1 U/g Hb) that could only discover those cases with a residual red cell G6PD activity level of <20% of normal, therefore missing the diagnoses of the partially deficient individuals. Many female heterozygotes may have mild to moderate reduction of red cell G6PD level, varying from 20% to 60% of residual enzyme activity. Their study showed that 24 partially deficient females identified as such by the qualitative method.
(10–60% of residual activity) were classified as normal by FST.

The prevalence of G6PD deficiency is high in southeast Asia. National newborn screening programmes are in operation in most countries within the region, predominantly utilizing the FST. In Malaysia, G6PD deficiency is common with an overall incidence of 3.1% among males and is an important cause of severe neonatal hyperbilirubinaemia (Ainoon et al., 2003a). A neonatal screening programme using the FST has been in place in all public hospitals since 1980.

The objective of our study was twofold. The first was to identify whether FST was as effective as quantitative enzyme assays in detecting male hemizygotes. The second was to ascertain whether reporting an additional category of intermediate (reduced) fluorescence in between positive (bright) and negative (absent) fluorescence on the FST would help in identifying heterozygote females with partial G6PD deficiency.

**MATERIALS AND METHODS**

One thousand three hundred and forty-three consecutive cord blood samples were obtained from newborns delivered at the University Malaya Medical Centre between April and October 2008. Seventy-seven were excluded from analysis as the sex of the newborn could not be verified from the hospital information system. The samples were collected into K2-EDTA tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) that were subsequently spotted and dried on filter paper (Whatman Ltd., Maidstone, UK) for analysis of G6PD activity by the semiquantitative FST on the same day of sample collection. Quantitative enzyme assay was carried out in parallel on the same sample using OSMMR2000-D (R&D Diagnostics Ltd., Papagos, Greece) according to manufacturer’s recommendations. Genomic DNA was extracted using QI Amp DNA Blood Mini Kit (Qiagen®, Valencia, CA, USA) for molecular analysis.

In our centre, neonatal screening is carried out using the FST based on the modified semiquantitative method described by Beutler et al. (1977). Commercial FST kits that grade the G6PD results as normal (presence of fluorescence) or deficient (no fluorescence) are available. However, we employ an in-house method that has been in place for more than 20 years. This procedure involves a three-scale reading when reporting results of FST: normal, intermediate or deficient.

Stock solutions of oxidized 0.008 M l-glutathione, 0.0075 M B-NADP hydrate and 0.01 M D-glucose-6-phosphate disodium salt hydrate are made by reconstituting 0.098, 0.1148 and 0.0608 g, respectively, in 20 ml each of TRIS–HCL (pH 7.8) buffer. Stock solutions are stable for a period of 8 weeks at 4 °C. All reagents are obtained from Sigma-Aldrich, St Louis, MO, USA. The working reagent is prepared freshly on a daily basis for the FST by adding equal volumes of the above reconstituted reagents.

Freshly collected cord blood samples in K2-EDTA tubes are sent directly to the laboratory for G6PD screening. In the laboratory, the sample tube is mixed by inversion, and a strip of Whatman 1 filter paper is inserted into the tube so that approximately 1 cm of the strip is coated with blood. This labelled strip is dried in a conventional microwave oven at 70 °C for 4 min. A punched out disc (5 mm in diameter) is obtained from this labelled strip and placed into a tube containing 0.1 ml of working reagent. This tube is incubated at 37 °C in a water bath for 15 min. Using a haematocrit capillary tube (Hirschmann® Laborgerate GmbH & CO, Eberstadt, Germany), the solution is spotted onto Whatman 1 filter paper and dried for 15 min at 70 °C and subsequently viewed under UV lamp. Readings are subjectively visualized and graded by trained technicians who are periodically assessed for competence and concordance in grading results. These technicians were involved in the interpretation of all cord blood samples in this study and were not blinded to the sex of the patient. G6PD activity is deemed to be normal when the samples are brightly fluorescent, intermediate when the fluorescence intensity is diminished and deficient if there is no fluorescence (Figure 1). Repeat samples (heel prick) are obtained from all neonates with intermediate fluorescence and retested by FST as per our laboratory standard operating procedures. Repeat samples and samples from kernicteric babies (as stated in the request form) are centrifuged at 1610 \( g \) for 10 min prior to fluorescence testing.

Quantitative enzyme assay using OSMMR2000-D (R&D Diagnostics Ltd.) was carried out according to manufacturer’s instructions; 5 μl each of whole blood samples was pipetted into wells of a microplate (Plate
1) together with 75 μl of elution buffer. The plate was placed on a shaker for 20 min. In a second flat-bottomed microplate (Plate 2), 75 μl of reagent was added to each well, and the plate was slowly warmed to 37 °C. In Plate 2, 15 μl each of eluted sample from Plate 1 was added to the corresponding well. The contents were mixed well, and the plate was placed in a spectrophotometer, and a single reading was taken at 405 nm. Eighty microlitres of Colour Reagent Booster mixture (provided by manufacturer) was then added into each well and mixed well. The plate was transferred to a reader immediately, and two readings were taken for each sample at 0 and 12–15 min. In the interval between the two readings, the plate remained in the incubator. A commercial normal control was run together with samples in each plate. G6PD activity in U/g Hb was calculated based on the formula provided by the manufacturer.

The mean normal G6PD enzyme activity of the kit was calculated prior to this study from 1271 cord blood samples of G6PD normal males (686) and females (585). The average G6PD activity of our sample population was 15.9 U/g Hb. We used 3.2 U/g Hb (20% of residual activity) as the upper limit for total G6PD deficiency, and 9.5 U/g Hb (60% of residual activity) as the upper limit of mild G6PD deficiency (partial activity). In other words, severe deficiency was defined as <3.2 U/g Hb while mild deficiency was between 3.2 and 9.5 U/g Hb for both males and females.

Samples identified as deficient and intermediate on FST were subjected to molecular identification for common G6PD mutations. Because of the heterogeneity of G6PD variants in the local population, we chose a combined approach of single-nucleotide polymorphism (SNP) detection using real-time polymerase reaction (PCR) for identification of known common mutations and direct sequencing of selected regions of the G6PD gene for identifying uncommon or unidentified mutations. Oligonucleotide primers and probes were designed for fluorescent real-time detection of six of the common mutations found in this region (Table 1), Gaohe 95 A>G, Mediterranean 563 C>T, Coimbra 592 C>T, Viangchan 871 G>A, Canton 1376 G>T and Kaiping 1388 G>A. The Taqman SNP genotyping assay (Applied Biosystems, Foster City, CA, USA) was performed in a final reaction mixture of 15 μl, containing 7.5 μl genotyping master mix, 0.75 μl genotyping assay mix and 6.75 μl nuclease-free water. An initial hot-start of 10 min was followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. Amplification and real-time detection was performed on Rotorgene 3000 (Corbett Research, Mortlake, NSW, Australia), and the final results were analysed on the instrument’s software. Samples that did not exhibit any of the six mutations targeted by the allelic discrimination assay were subjected to direct sequencing for the region spanning exons 5, 6 and 7 using previously described methods (Poggi et al., 1990).

Statistical analysis was performed using The Statistical package for Social Science version 16.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Of the 1266 samples, 561 were from female newborns and 705 were from male newborns. FST identified 49 samples as deficient with no fluorescence (47 males and 2 females) and 38 samples as intermediate with diminished fluorescence (all females).

Eighteen of the 49 samples identified as deficient by FST had enzyme activity <3.2 U/g Hb (<20% of mean activity) which included one female. Thirty of the 49 samples had measured G6PD activity of between 3.3 and 9.5 U/g Hb (60% of mean activity) which also included one female. One sample had G6PD enzyme activity of more than 9.5 U/g Hb. Twenty-one of the 38 samples identified as intermediate with diminished fluorescence (all females).
Sixty-one of the 87 abnormal samples (deficient and intermediate) were available for molecular analysis. Of these, 34 samples (32 males and two females) were from the deficient group, and 27 were from the intermediate group. Of the 34 samples from the deficient group sent for molecular analysis, 31 samples including one female sample were found to have mutations. Twenty-eight of these mutations were identified by RQ-PCR and three by direct sequencing (exons 5, 6, 7). Ninety-four percent (30/32) of deficient males were shown to harbour a G6PD mutation. The female deficient sample had compound heterozygosity for two G6PD mutations (G6PD Canton and G6PD Kaiping). Fifteen G6PD-deficient samples did not have sufficient DNA material for mutational analysis.

Table 1. Forward and reverse primer sequences with reporter oligonucleotides labeled with fluorescent tags used for allelic discrimination assay of common glucose-6-phosphate dehydrogenase (G6PD) mutations in East Asia

<table>
<thead>
<tr>
<th>G6PD variant name</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Reporter 1</th>
<th>Reporter 2</th>
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<tbody>
<tr>
<td>Canton G1376T</td>
<td>GCCTCCCAAGCCATACTATGTC</td>
<td>GGGCTTCTCAGCTCAATCTG</td>
<td>VIC-CCTCAAGGAGCTG-NFQ</td>
<td>FAM-CCTCAAGGAGCTG-NFQ</td>
</tr>
<tr>
<td>Kaiping G388A</td>
<td>CGACGAGCTCGTGAGG</td>
<td>GGGCTTCTCAGCTCAATCTG</td>
<td>VIC-CCTCAAGGAGCTG-NFQ</td>
<td>FAM-CCTCAAGGAGCTG-NFQ</td>
</tr>
<tr>
<td>Viangchan G871A</td>
<td>CCATTCTCCTGCCCTTTTTC</td>
<td>GGGCTTCTCAGCTCAATCTG</td>
<td>VIC-CCTCAAGGAGCTG-NFQ</td>
<td>FAM-CCTCAAGGAGCTG-NFQ</td>
</tr>
<tr>
<td>Mediterranean C563T</td>
<td>CTTGCTCGCTGGCTGATGAT</td>
<td>ACCGATGCTCCATTTATGAT</td>
<td>VIC-TCGATGACATATTNFQ</td>
<td>FAM-TCGATGACATATTNFQ</td>
</tr>
<tr>
<td>Gaohe A95G</td>
<td>CAGGGCGATGCCTCCCAT</td>
<td>ACCGATGCTCCATTTATGAT</td>
<td>VIC-TCGATGACATATTNFQ</td>
<td>FAM-TCGATGACATATTNFQ</td>
</tr>
<tr>
<td>Coimbra C592T</td>
<td>CACCCACATCATCCTCCCTGGTC</td>
<td>CATCTCCTGCCCAGGTAATG</td>
<td>VIC-TCGATGACATATTNFQ</td>
<td>FAM-TCGATGACATATTNFQ</td>
</tr>
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</table>

Figure 2. Distribution of glucose-6-phosphate dehydrogenase enzyme activity among samples identified as deficient, intermediate, and normal fluorescence by FST classified according to gender. Boxes show the interquartile ranges; horizontal lines inside the boxes represent medians; I-bars represent highest and lowest values; circles represent outliers. * represents an extreme value. P-values were calculated based on comparison between groups 1 and 3 as well as groups 2 and 4 (1,3: t-test, P = 0.00 and 2,4: t-test, P = 0.00).
Of the 27 samples in the intermediate group, 24 were found to have G6PD mutations (22 by RQ-PCR and two samples by direct sequencing). No mutations were identified in three samples with either method. Of the 24 intermediate samples that had mutations, 13 had G6PD activity of more than 60% with the enzyme assay method. Eleven samples were insufficient for DNA analysis. Table 3 depicts the frequency of different G6PD mutations in FST-deficient and intermediate groups in our population.

**DISCUSSION**

The effective implementation of newborn screening for G6PD deficiency is important in preventing adverse morbidity arising from neonatal jaundice and kernicterus in countries with high prevalence of G6PD variants. The findings from our study concur with previous observations that FST is as effective as quantitative enzyme assays for the detection of hemizygous males (Ainoon et al., 2003a) where the authors found that all 26 cases of G6PD-deficient male neonates had G6PD enzyme activity of $\leq 20\%$. We further show that molecular identification using rapid real-time PCR methods supplemented by conventional sequencing is feasible for confirmation of G6PD variants. Ninety-four percent (30/32) of affected males were found to have G6PD mutations using a limited set of primers designed to detect common polymorphisms in the population (Ainoon et al., 2003b, 2004).

Although it is generally stated that fluorescence intensity cannot be visually graded, we have observed that samples showing diminished fluorescence can be easily discriminated from those without any fluorescence or those with bright fluorescence (Figure 1). Studies reporting FST results as negative or positive have indicated that the test is unable to detect heterozygotes (Reclos, Hatzidakis & Schulpis, 2000; Ainoon et al., 2003a; Zaffanello et al., 2004). However, we were able to identify most heterozygote females with our method of reporting fluorescence results as normal, intermediate or deficient. The incidence of G6PD deficiency among females when the three-level FST grading was used was 7.1% which better approximates the figure of 9.3% quoted by Ainoon et al.

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<th>Table 2. Semi-quantitative fluorescent spot test (FST) and quantitative glucose-6-phosphate dehydrogenase enzyme assay distribution among male and female cord blood samples</th>
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<tbody>
<tr>
<td><strong>Sex</strong></td>
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</tr>
<tr>
<td>Male</td>
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<td></td>
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<td>Female</td>
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<td></td>
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<td>Total</td>
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<tr>
<th>Table 3. Frequency of different glucose-6-phosphate dehydrogenase (G6PD) mutations in deficient and intermediate patients (FST)</th>
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<tr>
<td><strong>Type of G6PD mutations</strong></td>
</tr>
<tr>
<td><strong>FST</strong></td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>Deficient</td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td>Intermediate</td>
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</tbody>
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*The total number of alleles identified (32) exceeds the total number of samples with a detectable mutation (31) sent for analysis due to one of the samples showing compound heterozygosity for 1376GT/1388GA.

(2003a) where a quantitative test was used for screening. On the rare occasion, a male cord blood sample has been identified as G6PD intermediate with diminished fluorescence. (During this study period, no male intermediate result was encountered). However, repeat heel prick samples had shown these patients to be deficient. The initial diminished fluorescence could have been because of an increase in reticulocytes or white blood cells with high G6PD activity. As stated earlier, repeat samples and samples from kernicteric babies are centrifuged at 1610 \( g \) for 10 min prior to fluorescence testing. This is to prevent variability in the outcome of the test where deficient samples are falsely interpreted as intermediate/normal. However, it is not feasible to implement this centrifugation step as part of routine G6PD screening by FST.

It can be argued that visually grading the FST as diminished fluorescence is operator dependent and may result in inclusion of false positives. However, in competency assessments, we have observed a high concordance showing perfect agreement among all technicians when reporting levels of fluorescence in the FST. Our study shows that although only 27/38 (71%) of female samples with intermediate fluorescence were available for molecular analysis, 24/27 (89%) of the analysed samples did show a mutation, indicating that most of the FST results reported as intermediate fluorescence were true positives. Statistical analysis also showed a significant difference between the female intermediate and female normal samples by quantitative analysis \( (P = 0.00) \) as illustrated in Figure 2.

Fifty-five of 61 intermediate and deficient samples were found to have a specific G6PD mutation. Six samples (three from the deficient and three from the intermediate group) showed no identifiable mutation either by PCR or by direct sequencing of exons 5, 6 and 7. It is possible that these six patients may have one of the other 140 reported G6PD mutations that we did not screen for (Cappellini & Fiorelli, 2008). One sample that was deficient by FST had normal G6PD activity by quantitative spectrophotometric assay. This could be a false-positive FST or a falsely high G6PD quantitation because of technical error. Unfortunately, there was insufficient sample to repeat the tests or for DNA analysis. Eighteen of the G6PD intermediate samples had normal G6PD enzyme activity by the quantitative method. Thirteen of 18 of these samples were available for DNA analysis, and 11 showed identifiable G6PD mutations, such as G6PD Viangchan (7), G6PD Mediterranean (2) and G6PD Canton (2). Two samples showed no mutations. Discordant results between the FST and quantitative enzyme assay activity could have arisen because of technical limitations of the quantitative assay. For example, bubble formation in the wells may lead to high absorption of light, and consequently, a G6PD deficient specimen could be misclassified as normal.

Detection of heterozygotes for G6PD deficiency poses special problems. Because of X inactivation, heterozygotes have two RBC populations. One of these populations consists of normal RBCs and the other of RBCs that are as deficient as those of a hemizygous male with the same deficient variant. On the average, half of the cells are normal and half are deficient. However, heterozygous females with extremely skewed X inactivation have activity ranging from hemizygote to normal. The detection of mildly deficient female heterozygotes may appear to be of less clinical importance than the identification of severely deficient males and homozygous females. Nevertheless, there are reports that suggest that deficient females are also at risk of developing severe hyperbilirubinaemia although these patients may have been those patients with RBC enzyme activity at the lower end of the spectrum (Kattamis, Kyriazakou & Chaidas, 1969; Sanna \textit{et al.}, 1979; Corchia \textit{et al.}, 1995; Wang \textit{et al.}, 2009). Thus, the FST and RBC enzyme activity measurements cannot be relied upon for the detection of heterozygotes. The cytochemical method, although a relatively complicated test, is the only reliable assay for definitive diagnosis of heterozygous females.

In conclusion, our study shows that the FST is effective in identifying deficient hemizygote males and is also able to identify mildly deficient heterozygote females, provided diminished fluorescence is distinguished as a separate category. The FST will continue to have an important role to play in screening for G6PD deficiency in view of its relatively good diagnostic efficiency coupled with its ease of use and low cost.
REFERENCES


