

Comparison of detection of glucose-6-phosphate dehydrogenase deficiency using fluorescent spot test, enzyme assay and molecular method for prediction of severe neonatal hyperbilirubinaemia

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ABSTRACT

Introduction: This study aimed to compare the detection rates of glucose-6-phosphate dehydrogenase (G6PD) deficiency in neonates by fluorescent spot test (FST), enzyme assay and molecular methods, and to identify which method was a significant predictor of severe hyperbilirubinaemia.

Methods: 74 term infants of Chinese descent admitted with severe hyperbilirubinaemia (total serum bilirubin equal or greater than 300 micromol/L) and 125 healthy term infants born in the hospital without severe hyperbilirubinaemia were recruited into the study. Specimens of blood were collected from each infant for FST, G6PD enzyme assay and TaqMan[®] minor groove binder single nucleotide polymorphism genotyping assay.

Results: 26 (13.1 percent) infants were diagnosed to have G6PD deficiency by FST. They had significantly lower median enzyme levels (0.8 IU/g Hb, interquartile range [IQR] 0.4–4.3) than those diagnosed to be normal (12.0 IU/g Hb, IQR 10.3–15.8) (p-value is less than 0.0001). Based on the enzyme assay, 39 (19.6 percent) infants had G6PD deficiency at an enzyme cut-off level of less than 8.5 IU/g Hb. G6PD mutation was detected in 27 (13.6 percent) infants. Logistic regression analysis showed that the only significant predictors of severe hyperbilirubinaemia were G6PD deficiency based on a cut-off level of less than 8.5 IU/g Hb (adjusted odds ratio [OR] 5.3, 95 percent confidence interval [CI] 2.4–11.4; p-value is less than 0.0001) and exclusive breast-feeding

(adjusted OR 11.4, 95 percent CI 3.1–42.4; p-value is less than 0.0001). The gender and birth weight of infants, FST results, G6PD mutation and the actual G6PD enzyme levels were not significant predictors.

Conclusion: A G6PD enzyme level of less than 8.5 IU/g Hb is a significant predictor of severe hyperbilirubinaemia.

Keywords: glucose-6-phosphate dehydrogenase, fluorescent spot test, enzyme level, hyperbilirubinaemia, neonatal jaundice

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INTRODUCTION

Severe neonatal hyperbilirubinaemia with total serum bilirubin (TSB) \geq 300 μ mol/L is a common problem in Malaysia, with a reported incidence of 32.4 per 1,000 live births.⁽¹⁾ Glucose-6-phosphate dehydrogenase (G6PD) deficiency has been identified as one of the common causes of severe neonatal hyperbilirubinaemia in Malaysia. Despite this, in more than 40% of these infants, no underlying causes were identified.⁽¹⁾ Since its inception in the 1980s, the Malaysian G6PD newborn screening programme used the Beutler's modified fluorescent spot test (FST)⁽²⁾ for the detection of G6PD deficiency. This test, being a qualitative one, detects only cases with severe G6PD deficiency when the enzyme levels are $<$ 30% of the normal level.⁽³⁾ In view of this limitation, measurement of the G6PD enzyme has been used in some countries as the screening method.^(4,5) In recent years, with advances in molecular techniques, the detection of G6PD variants has become possible in many countries, although not yet as a routine screening method.^(6,7)

Our research questions were how much more the

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detection rates of G6PD deficiency in infants with severe hyperbilirubinaemia could be improved by measurement of their G6PD enzyme levels and by molecular techniques, and which of these were better predictors of infants developing severe hyperbilirubinaemia. To answer this question, this study aimed to compare the detection rates of G6PD deficiency between FST and the measurement of actual enzyme levels, and between FST and molecular methods, and to identify the significant predictors associated with severe hyperbilirubinaemia in neonates with G6PD deficiency.

METHODS

This observational study was carried out over a two-year period (from April 1, 2004 to March 30, 2006) in the neonatal intensive care unit and postnatal wards of the Hospital Universiti Kebangsaan Malaysia. The inclusion criteria for severely hyperbilirubinaemic newborns were term infants (gestation between 37 and 42 completed weeks), with at least one parent of Chinese ethnicity, and who were admitted for severe unconjugated hyperbilirubinaemia (TSB > 250 µmol/L during the first 48 hours of life, or TSB ≥ 300 µmol/L thereafter). The exclusion criteria were infants with conjugated hyperbilirubinaemia > 15% of TSB levels, age ≥ 28 days of life or gestation < 36 weeks. On randomly-selected days, healthy term infants of Chinese ethnicity born in the hospital were recruited as controls. Parental consent was obtained for all cases prior to participation in the study. The institute's Research and Ethics Committees approved the study protocol.

Upon admission, the following investigations were carried out on all severely hyperbilirubinaemic newborns: TSB level and fractionation, screening for G6PD deficiency using the World Health Organisation (WHO) qualitative FST,⁽⁸⁾ quantitation of G6PD enzyme level, and single nucleotide polymorphism (SNP) analysis of G6PD gene for the seven common variants (G1376T, G1388A, A95G, C1024T, G871A, G487A, C563T) detected previously in the Malaysian Chinese population.⁽⁹⁾

For infants recruited as controls (without severe hyperbilirubinaemia), the blood tests mentioned above were carried out similarly on their cord blood specimens. Upon discharge and during the first seven days of life, these infants were monitored via phone calls by the investigators. They were also followed-up by their visiting community nurses according to standard protocol for newborn care in Malaysia. Infants with severe jaundice were referred to the hospital and treated accordingly. Infants confirmed to have severe hyperbilirubinaemia were removed from this non-severely hyperbilirubinaemic group and included in the

severely hyperbilirubinaemic group.

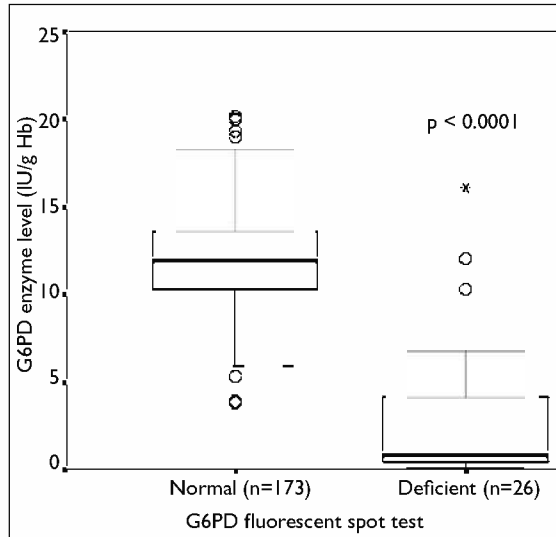
Serum bilirubin levels of infants during the study were measured by the diazo method using the Cobas Integra system (Roche, Switzerland).⁽¹⁰⁾ Quantitation of G6PD enzyme level in erythrocytes was determined by the ultraviolet method⁽¹¹⁾ using Randox glucose-6-phosphate dehydrogenase kits (Randox Laboratories Ltd, United Kingdom) and a spectrophotometer (Hitachi model 717, Japan). G6PD deficiency was diagnosed when an enzyme level was < 8.5 IU/g Hb, based on our previous study.⁽¹²⁾

Detection of G6PD variants was by real-time polymerase chain reaction (PCR) with TaqMan[®] minor groove binder (MGB) SNP genotyping assay (Applied Biosystems, Foster City, CA), using ABI PRISM SDS 7000 (Applied Biosystems, Foster City, CA).⁽¹³⁾ Oligonucleotide primers and probes for nucleotides G1376T, G1388A, A95G, C1024T, G871A, G487A and C563T were designed using Primer Express Software version 2.0 (Applied Biosystems, Foster City, CA). Genotyping assay was performed in a final reaction of 25 µL, which contained 2.5 µL of 2× TaqMan Universal Master Mix, 0.5 µL of 40× TaqMan SNP genotyping assay mix (Applied Biosystems) and 15 ng of genomic DNA in 12 µL of distilled water. The amplification conditions were 2 mins at 50°C for AmpErase uracil-N-glycosylase activity and 10 mins at 95°C for AmpliTaq Gold activation, followed by 40 cycles of 15 seconds at 95°C for denaturation and 1 min at 60°C for annealing and extension. The samples were run together with the non-template control in a 96-well plate. Allelic discrimination was performed on the post-PCR product. The fluorescence data of the post-PCR products were analysed directly using allelic discrimination software of the ABI Prism 7000 instruments and presented in an x-y scatter dot plot format. Each sample was verified by examining the PCR curve generated to eliminate false-positive results due to aberrant light emission. For confirmation of the real-time PCR findings, all samples were subjected to sequencing using primers sequence for G6PD exon 2–13 described previously.⁽¹⁴⁾

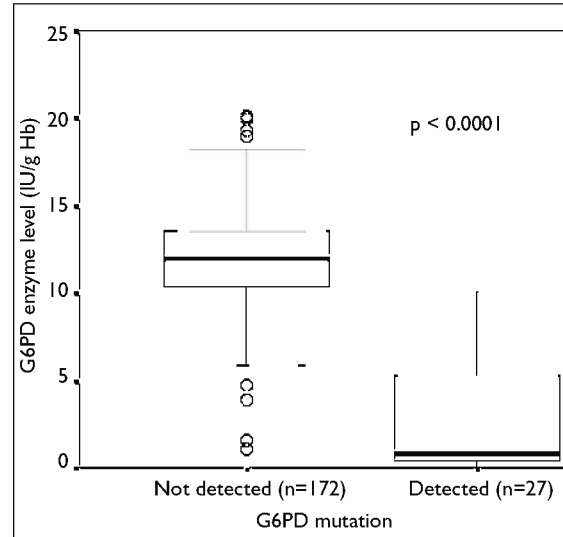
The Statistical Package for Social Science version 10.1 (SPSS Inc, Chicago, IL, USA) was used for analysis of the data. Student's *t*-test (unpaired) was used for analysis of continuous variables with normal distribution between groups and the Mann-Whitney U test for variables with a skewed distribution. The chi-square test (or Fisher's exact test for expected values of < 5) was used for analysis of categorical variables. Using severity of hyperbilirubinaemia as the dependent variable, and the gender of infants, birth weight, exclusive breast-feeding, G6PD deficiency based on a cut-off level of < 8.5 IU/g Hb, actual G6PD enzyme

Table I. Comparison of results of glucose-6-phosphate dehydrogenase (G6PD) enzyme levels, fluorescent spot test (FST) and detection of G6PD variants in a group of Malaysian Chinese term infants.

G6PD enzyme level (U/g Hb)	Results of FST	G6PD mutation detected		Total (n = 199)
		Yes (n = 27)	No (n = 172)	
< 8.5	Deficient	20	3	23
	Normal	6	10	16
≥ 8.5	Deficient	0	3	3
	Normal	1	156	157

**Fig. 1** Boxplot shows the relationship between glucose-6-phosphate dehydrogenase (G6PD) enzyme levels and results of the G6PD fluorescent spot test.

Boxes show the interquartile ranges; horizontal lines inside the boxes indicate medians; I-bars represent highest and lowest values; circles represent outliers; * represents an extreme value; and p-value was calculated based on a comparison between the two groups.

**Fig. 2** Boxplot shows the relationship between glucose-6-phosphate dehydrogenase (G6PD) enzyme levels and G6PD mutation.

Boxes show interquartile ranges; horizontal lines inside the boxes indicate medians; I-bars represent highest and lowest values; circles represent outliers; and p-value was calculated based on a comparison between the two groups.

level, results of FST and presence of G6PD mutation as independent variables, forward logistic regression analysis was carried out to identify the significant predictors associated with the development of severe hyperbilirubinaemia. p-values of < 0.05 were considered to be statistically significant.

RESULTS

During the study period, 82 infants were admitted with severe hyperbilirubinaemia. Eight were excluded due to inadequate blood samples for DNA extraction and parental refusal to repeat blood sampling (n = 2), no parental consent (n = 1) or the investigators were not aware of their admission to the hospital (n = 5). Among the 74 severely hyperbilirubinaemic infants recruited, 71 had double Chinese parenthood, while one of the parents of the remaining three infants was non-Chinese (Burmese: 1, Malays: 2). Their median age when jaundice was detected was three (interquartile range [IQR] 3–4) days and

the median age of admission was 5 (IQR 4–8) days. On admission, 71 had TSB ≥ 300 (range 300–506) $\mu\text{mol/L}$ and three had TSB > 250 $\mu\text{mol/L}$ during the first 48 hours of life. No infants manifested signs of kernicterus.

Over a period of six months (between May 10, 2004 and October 31, 2005), 2,358 Chinese infants were born in the hospital. Of these, 233 (9.9%) were delivered on randomly-selected days for recruitment. However, 108 of these were excluded because of parental refusal to participate in the study (n = 5), discharge before parental consent was obtained (n = 76), no cord blood sample was available for analysis (n = 25) or severe hyperbilirubinaemia (n = 2). The remaining 125 infants were recruited into the group with no severe hyperbilirubinaemia: 77 (61.6%) of them developed mild to moderate jaundice at a median age of 4 (IQR 3–5) days with a mean serum of 230 $\mu\text{mol/L}$ (standard deviation [SD] 43) in the moderately jaundiced infants (n = 22); 48 infants developed very mild jaundice and serum bilirubin was not measured. These infants were discharged home

Table II. Comparison of clinical variables and glucose-6-phosphate dehydrogenase (G6PD) status based on molecular study, enzyme level and fluorescent screening test between term infants with and without severe hyperbilirubinaemia.

Variables	With severe hyperbilirubinaemia (n = 74)	No severe hyperbilirubinaemia (n = 125)	95% CI of difference between the means	Unadjusted OR (95% CI)	p-value
Mean (SD) birth weight (g)	3,015 (386)	3,064 (455)	-169-70	-	0.4
Median (IQR) G6PD enzyme level (IU/g Hb)	11.0 (7.8)	11.9 (3.0)	-	-	0.04*
G6PD enzyme level < 8.5 IU/g Hb (%)	26 (35.1)	13 (10.4)	-	4.7 (2.2-9.8)	< 0.0001*
G6PD deficiency detected by FST (%)	19 (25.7)	7 (5.6)	-	5.8 (2.3-14.7)	< 0.0001*
G6PD mutation (%)	19 (25.7)	8 (6.4)	-	5.1 (2.1-12.3)	< 0.0001*
Males (%)	45 (60.8)	68 (54.4)	-	1.3 (0.7-2.3)	0.5
Exclusive breast-feeding (%)	14 (18.9)	3 (2.4)	-	9.4 (2.6-34.0)	< 0.0001*

CI: confidence interval; SD: standard deviation; IQR: interquartile range; FST: fluorescent spot test; OR: odds ratio
* denotes statistical significance

within four days after birth and none developed severe hyperbilirubinaemia during the first month of life.

Among these 199 infants (74 with severe hyperbilirubinaemia, 125 without severe hyperbilirubinaemia) recruited, 113 (56.8%) were males. The mean birth weight of these 199 infants was 3,046 (SD 431) g. G6PD mutation was detected in 13.6% (27/199) of them. The median level of G6PD enzyme in all infants was 11.5 IU/g Hb (IQR 9.1-13.3). Based on a cut-off enzyme level of <8.5 IU/g Hb, 19.6% (39/199) were G6PD deficient (Table I). Based on the results of FST, only 13.1% (26/199) of them were G6PD deficient, 41.0% (16/39) of infants with G6PD enzyme levels of <8.5 IU/g Hb were not diagnosed to have a deficiency by the FST and 1.9% (3/160) with normal levels were falsely diagnosed to be G6PD deficient by FST. FST detected G6PD deficiency in only 74.1% (20/27) of infants with G6PD mutation. Although infants diagnosed to have G6PD deficiency by the FST had significantly lower median enzyme levels than those diagnosed to be normal (Fig. 1), 11.5% (3/26) of them had an enzyme level \geq 8.5 IU/g Hb. Among infants diagnosed to have normal G6PD by the FST, 9.2% (16/173) of them had an enzyme level of <8.5 IU/g Hb.

Of the 27 infants with G6PD variants detected, 24 (17 males, 7 females) were homozygotes and three (all females) were heterozygotes. Infants with G6PD mutation had significantly lower levels of enzyme than those without mutation detected (Fig. 2). There was no significant difference in the proportion of infants with G6PD mutations between the two gender groups (males 15.0%, females 11.6%)($p = 0.5$). All except for one infant

with G6PD mutation had enzyme levels of <8.5 IU/g Hb. The infant, whose enzyme level was 10.1 IU/g Hb, was a female with a homozygous A95G variant. Of the seven G6PD variants reported previously among the Malaysian Chinese,⁽⁸⁾ only five (A95G, G871A, C1024T, G1376T, and G1388A) were detected in these infants. Among these five variants, G1388A and G1376T were the most common. No infant was found to have the variants G487A and C563T. One infant was detected to have the variant G392T, which has not been previously reported among the Malaysian Chinese. There were 13 infants with enzyme levels of <8.5 IU/g Hb (Table I) in whom no mutation was detected, based on normal results obtained in the PCR sequencing of exons 2-13 in the G6PD gene. The G6PD enzyme level of these 13 infants (median 7.1 IU/g Hb; IQR 5.3-8.4) was, however, significantly lower than the 159 infants with enzyme level \geq 8.5 IU/g Hb and no evidence of mutation (median 12.4; IQR 10.9-13.7) ($p < 0.00001$). Eight (61.5%) of these 13 infants developed severe hyperbilirubinaemia.

There was no significant difference in the gender distribution and mean birth weight between infants with or without severe hyperbilirubinaemia (Table II). However, infants with severe hyperbilirubinaemia had significantly lower G6PD enzyme levels ($p = 0.004$) and significantly higher proportions with G6PD deficiency detected by the enzyme level, FST and molecular methods. Compared to the FST and molecular methods, the G6PD enzyme cut-off of < 8.5 IU/g Hb detected a higher proportion of infants with G6PD deficiency in infants with severe hyperbilirubinaemia. A significantly higher proportion of the severely hyperbilirubinaemic

infants were on exclusive breast-feeding than those who were not severely hyperbilirubinaemic ($p < 0.0001$). Based on the blood grouping results, 17.6% ($n = 13$) of the severely hyperbilirubinaemic infants had group BO incompatibility with their mothers, 5.4% ($n = 4$) had group AO incompatibility and 1.4% ($n = 1$) had Rhesus incompatibility. However, the Coombs test of all these infants was negative. No infant in the not severely hyperbilirubinaemic group had maternal-foetal blood group incompatibility.

A significantly higher proportion of male infants with severe hyperbilirubinaemia had G6PD mutation ($n = 13$, 28.9%) than males without severe hyperbilirubinaemia ($n = 4$, 5.9%) ($p = 0.002$). Although a higher proportion of female infants with severe hyperbilirubinaemia had mutation ($n = 6$, 20.7%) than females without severe hyperbilirubinaemia ($n = 4$, 7.0%), the difference was not statistically significant ($p = 0.08$). Among the six female infants with G6PD variants who developed severe hyperbilirubinaemia, two were heterozygotes. Anaemia was not a common problem among the severely hyperbilirubinaemic infants as the median haemoglobin level on admission was 15.4 (IQR 14.1–16.6) g/dL. The median reticulocyte count of these infants was 2.2% (IQR 1.3–3.2; range 0.01–15.4), suggesting that haemolysis was a cause of severe hyperbilirubinaemia in some of the infants. Forward logistic regression analysis showed that the only significant predictors associated with the development of severe hyperbilirubinaemia were G6PD enzyme levels of < 8.5 IU/g Hb (adjusted OR 5.3, 95% CI 2.4–11.4; $p < 0.0001$) and exclusive breast-feeding (adjusted OR 11.4, 95% CI 3.1–42.4; $p < 0.001$). The gender and birth weight of infants, FST results, presence of G6PD mutation and the actual G6PD enzyme levels were not significant predictors.

DISCUSSION

Molecular characterisation of G6PD was previously done by the PCR restriction enzyme (RE) method. The present study was the first to use fluorescence-based SNP assay to detect the presence of G6PD variants. Previously, based on the PCR RE method, significant false positive results due to incomplete restriction enzyme digestion have been reported.^(15,16) Furthermore, compared to the TaqMan MGB SNP method, the PCR RE method is more time-consuming, especially for post-amplification steps, such as verifying the presence of PCR products by gel electrophoresis, restriction endonuclease digestion and restriction pattern visualisation by gel electrophoresis. The TaqMan MGB assay is convenient to do, particularly for high throughput screening, as it does not require extensive post-amplification

manipulation and genotyping results can be obtained within two hours. Due to its increased mismatch discrimination ability, a DNA probe conjugated into a MGB group allows increased specificity of the detection of variants. The TaqMan MGB assay is also designed to conform to standard thermocycling conditions (40 cycles containing a 60°C annealing and extension step), thus allowing it to be performed simultaneously with other assays, making it ideal for use in a clinical laboratory setting where numerous assays for different SNPs are being performed.

The present study confirms the results of previous studies that the qualitative FST detects G6PD deficiency only when the deficiency is severe.⁽⁹⁾ The FST tends to give a false negative result for mild to moderate deficiencies. This could possibly explain the misdiagnosis of some of the 44% of infants with severe hyperbilirubinaemia in Malaysia in whom no cause was detected.⁽¹⁾ Although the rate was not high, there were false positive results associated with the FST. The most plausible cause of this could be due to contamination of specimens in the laboratory.

The results of this study suggest that the G6PD enzyme assay should replace the FST test as a screening test in order to improve the detection rates of G6PD deficiency. This is because based on the G6PD enzyme cut-off level of 8.5 IU/g Hb, a significantly higher proportion of infants with severe hyperbilirubinaemia were identified to have G6PD deficiency than those without severe hyperbilirubinaemia. Logistic regression analysis showed that, after controlling for various potential confounders, this cut-off level was a significant predictor of infants developing severe hyperbilirubinaemia. The clinical implication of using the enzyme assay method for screening of G6PD deficiency is that more high-risk infants could be detected early for close monitoring to prevent the development of severe hyperbilirubinaemia and kernicterus.

Our study also confirms that five of the seven G6PD variants reported previously among Malaysian Chinese were found in the study infants.⁽⁹⁾ The two variants that were most commonly associated with severe hyperbilirubinaemia in this study were G1388A and G1376T. On the other hand, only one of the four infants with variant G871A was associated with severe hyperbilirubinaemia. Although only one of the 27 infants with G6PD mutation had a normal enzyme level (10.1 IU/g Hb), this female infant with homozygous A95G developed severe hyperbilirubinaemia. On the other hand, four males and three females with homozygous G6PD variants and one female heterozygote did not develop severe hyperbilirubinaemia, suggesting that the propensity to develop severe hyperbilirubinaemia may differ among the variants. This could be the underlying

explanation as to why the presence of the G6PD mutation was not a significant predictor of severe hyperbilirubinaemia. However, to confirm this theory, a much larger study series should be carried out. Furthermore, although none of the 13 infants with low G6PD enzyme level (below 8.5 IU/g Hb) were found to have any of the seven G6PD variants, there is still a possibility that other yet-to-be-identified variants causing their low enzyme levels were present.

This study also showed that both males and females with mutation were at risk of developing severe hyperbilirubinaemia. For females, both the homozygotes and heterozygotes were at risk. This again emphasises the need to perform G6PD enzyme assay and/or molecular analysis in infants of both genders with severe hyperbilirubinaemia in regions where FST continues to be used as the principle method of screening for G6PD deficiency, irrespective of their FST screening result.

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