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Detection of Glucose-6-Phosphate Dehydrogenase Deficiency in Heterozygous Saudi Female Neonates

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Abstract

Background: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzymopathy affecting 400 million people, globally. G6PD deficiency is an X-linked genetic condition, which is more likely to affect males than females. Heterozygous females go undetected in commonly used method. The aim of the study was to identify & validate female heterozygous neonates, missed in neonatal screening programs.

Methods: Blood samples were collected from 984 Saudi neonates (448 Male and 536 Female) in EDTA tubes for quantitative evaluation of G6PD enzyme activity. Quantitative evaluation was done by Sigma diagnostic kits (No. 345-UV). The reduction of nicotinamide adenine dinucleotide phosphate to nicotinamide adenine dinucleotide phosphate oxidase, reflecting G6PD activity was measured spectrophotometrically. Hemoglobin (Hb) was measured on the same sample. G6PD activity was recorded as U/g Hb. Samples identified as deficient with cutoff ≤6.6 U/gHb were subjected to molecular genotyping for common G6PD variants.

Results: Out of 448 male neonates, 47 (10.3%) were designated as G6PD deficient with average G6PD enzyme activity of 1.89 U/gHb. Females (536) showed continuum results. With ≤ 4.6 U/gHb cutoff, 14 (2.6%) female neonates were designated as G6PD deficient with average G6PD enzyme activity of 2.6 U/gHb, while with cutoff ≤ 6.6 U/gHb, 34 (6.3%) with average G6PD enzyme activity of 5.5 U/gHb were marked deficit. Additional neonates which were designated as deficit with cutoff ≤ 6.6 U/gHb showed presence of G6PD mutations, 18 (80%) showed G6PD Mediterranean, and 2 (20%) were identified a G6PD Aures.

Conclusion: Considerable amounts of partially deficient G6PD female heterozygous are missed, when ≤ 4.6 U/gHb cutoff is used to identify deficient female neonates, however, deficient males, hemizygotes were detected efficiently with ≤ 4.6 U/gHb as cutoff point. Higher reference value (≤ 6.6 U/gHb) is recommended for female neonates.

Keywords: G6PD deficiency; Female G6PD Heterozygous; Cutoff points

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common genetic enzymopathy, affecting millions of people worldwide [1-4]. Because G6PD deficiency is an X-linked condition, males may be either G6PD normal or G6PD deficient hemizygotes, whereas females may be, either normal homozygotes, deficient homozygotes, or heterozygotes. Using biochemical testing, identification of the deficit and normal male groups should be accurate and easy. Categorization of females, however, may be erroneous. In any female cell, only one X chromosome is active [5,6]. If X chromosome inactivation were random, 50% of the cells would be G6PD normal and 50% would be deficient. G6PD enzyme activity, representing both cell components, would be intermediate between normal and deficient levels. However, because X chromosome inactivation is frequently nonrandom, varying proportions of red blood cells may have either G6PD–normal or –deficient. As a result, female heterozygotes will have a continuum of G6PD activity results.

All screening test that have been devised and commercialised, typically detect males with ease, however, they do not detect heterozygous females with high efficiency. Heterozygous females are liable to haemolysis, although the severity is variable [7,8]. The most devastating potential complication of this in the newborn is an acute hemolytic crisis, causing extreme hyperbilirubinemia, which may result in acute bilirubin encephalopathy [9]. Neonatal screening for G6PD is long established in many countries, and the screening method most commonly used is the semi-quantitative method described by Beutler and Mitchell [10], or modification to this method [11,12].

In Saudi Arabia, the incidence of G6PD deficiency varies from one region to another, ranging from 8%-14% [13,14]. The objective of this study was to device a methodology by which the heterozygous female G6PD deficiency neonates are not missed in screening tests. These deficient female which are classified as normal are at high risk of an episodically haemolytic attack.

Materials and Methods

Cord blood samples from 984 Saudi neonates (448 males and 536

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females) were obtained from newborns delivered at King Abdulaziz University hospital, Jeddah Saudi Arabia. Consent was obtained from their parents, prior to conducting the research. The study was approved by the Biomedical Ethical Research Committee of the Faculty of Medicine of King Abdulaziz University (Reference No. 530-11). The blood samples were collected into K2-EDTA tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) that were subsequently stored at 4°C, and tested within 48 hours of collection. G6PD was measured quantitatively by using sigma quantitative kits, using Sigma glucose-6-phosphate dehydrogenase H control (Sigma Medical, USA). The kits use the chemical reaction described by Beutler et al. [15], with NADPH production measured at 340 nm in kinetic mode (change in optical density per minute).

Genomic DNA was extracted from blood leukocytes of the deficient neonates by standard methods (proteinase K digestion, phenol/isoamyl alcohol/chloroform extraction and ethanol precipitation) [16,17].

The most frequent glucose-6-phosphate dehydrogenase variants were determined by Restriction Fragment Length Polymorphism (RFLP). The G6PD Aures mutation is located in exon 3 (c.143T>C, p.Ile48Thr), G6PD A$^-$ mutations are located in exon 4 (c.202 G>A, p.Val68>Met) and exon 5 (c.376A>G, p.Asn126Asp), and G6PD Mediterranean is in exon 6 (c.563C>T, p.Ser188Phe). DNA fragments were amplified using primers as listed in table 1. For the reactions, 500 ng DNA were amplified on a GeneAmp® PCR System 2700 thermal cycler (Applied BioSystems, Foster City, CA, USA), with 1.25 U AmpliTaq® DNA Polymerase (Roche, New Jersey, NJ, USA) and 10 pmol of each primer, 0.2 mM of each dNTP, and 1.5 mM $MgCl_2$. The final buffer comprised 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-Cl, pH 8.8, 1.5 mM $MgCl_2$, 67 mM Na_2EDTA, pH 8.0, 70 µg BSA, and 10 mM β-mercaptoethanol. An initial denaturation step at 94°C for 5 minutes was followed by 35 cycles of denaturation at 94°C for 10 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension at 72°C for 7 minutes completed the cycling reaction. For RFLP studies, 18 µl of each PCR product was restricted, according to the manufacturer's recommendation, with 20 IU of each corresponding enzyme. Exon 3 was cleaved with BglII, exon 4 with NlaIII, exon 5 with FokI, and exon 6 with MboII.

Ten microliters of the digestion products were size fractionated by electrophoresis through polyacrylamide gels (6%), stained with ethidium bromide (0.25 µg/ml), and analyzed by UV transillumination [18].

Results

A total of 984 Saudi neonates (448 males and 536 females) were screened for glucose 6 phosphate dehydrogenase deficiency. At ≤ 4.6 U/gHb cutoff, 46 male neonate and 14 females showed G6PD deficiency, whereas, with cutoff point as ≤ 6.6 U/gHb there were 34 deficient females' neonates (Table 2). Molecular studies were carried out on additional 20 deficient samples identified using ≤ 6.6 U/gHb cutoff. Eighteen out of twenty showed G6PD Mediterranean mutation (80%), while two (20%) had G6PD Aures variant (Table 3).

Discussion

Neonatal screening is long been established in Saudi Arabia, and there are many published data for the prevalence in different region, ranging from 8-14 percent [13,14]. However, most of the studies have used mass screening fluorescent method to report the prevalence rate in the region. G-6-PD deficiency is different from other diseases tested for mass screening programs, since most G-6-PD deficient individuals remain healthy, and lead perfectly normal lives. No immediate treatment is available or necessary, and only a small fraction will develop extreme hyperbilirubinemia.

Partly for these reasons, national G-6-PD screening programs have not been widely implemented. However, recent reports determining neonatal heterozygotes of increased hemolysis, hyperbilirubinemia, and even fatal bilirubin encephalopathy, suggest that some of these females may be at risk, and that it may be prudent to attempt to identify these individuals [19,20]. Because G6PD deficiency is an X-linked condition, males may be either G6PD normal or deficient hemizygotes, whereas females may be normal homozygotes, deficient homozygotes, or heterozygotes. These female heterozygous are at high risk of developing hemolytic episodes, even severe ones, which makes the detection of partially G6PD deficient females mandatory. Animal studies have also shown that increased prenatal and post natal deaths are in heterozygous G6PD deficient animals [21].

Analysis	Analyzed Region	Forward 5'→3'	Reverse 5'→3'
RFLP Analysis (T_m = 61°C)	Exon 3	GTGGAGGATGATGTATGTAGGT	AGGGCAGGGCACAGCTGTAA
	Exon 4	TACAGTCGTGCCCTGCCCT	CCGAAGCTGGCCATGCTGG
	Exon 5	CTGTGTGTGTGTCTGTCTGTC	GGAGGGCAACGGCAAGCCTT
	Exon 6	GCAGCTGTGATCCTCACTCC	GCAAGGTGGAGGAACTGACC

Table 1: Primers used for identification of glucose-6-phosphate dehydrogenase defects.

Gender Total 984 (448M-536F)	Frequency	G6PD Activity Range	Mean G6PD Activity U/gHb*	Ratio Male/Female
Male	47(77%)	0.14-4.15	1.89 ± 1.45	NA
Female <4.6 U/gHb	14(23%)	0.12-4.5	2.6 ± 1.48	1:3.6
Female<6.6 U/gHb.	34(34%)	0.12-6.6	5.5 ± 0.59	1:1.3

*Statistic was done using SPSS 16

Table 2: Glucose 6 Phosphate Dehydrogenase deficiency in neonates.

G6PD Variant	Number of neonates	Mutation	Amplified Exon	Enzyme		Fragment Size		
				Name	Restriction Site	Uncut	Normal	M Mutated
Mediterranean	18 (90%)	563 C->T	6+7	Mbo II	GAAGA/ CTTCT	547	377, 119, 26, 25	277, 119, 100, 26, 25
Aureus	2 (10%)	143 T->C	3+4	Mbo I	GATC/ CTAG	353	293,60	353

Table 3: Detection of mutation In 20 deficient heterozygous females identified with 6.6 U/gHb cutoff.

Anticipating that many heterozygous females may be missed, we changed our cutoff point from ≤ 4.6 U/gHb to ≤ 6.6 U/gHb, as a reference value set for designating G6PD deficient neonates. When ≤ 6.6 U/gHb is used as a cutoff point, the number of female deficient increased. Similar findings were reported by Reclos et al [22], in which authors have suggested the cutoff as ≤ 6.4 U/g hemoglobin. To confirm that these additional cases which were detected are actually deficient or not, we did the molecular studied on these samples. All the samples showed the presence G6PD deficient variants.

It was concluded that many undiagnosed partially G6PD deficient female are missed by commonly used screening method. Since female heterozygous can also develop sever hemolytic episodes, it is utmost important to classify the G6PD deficiency neonates with higher cutoff points. There is no reliable biochemical screening assay to detect G6PD heterozygous, since standard methods test both red cell populations in single sample. Only DNA analysis meets the requirement. In view of the cost effectiveness, we suggest to undertake the DNA analysis in samples, identified with revised cutoffs.

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