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 hemizygoites, whereas females may be, either normal homozygoites, deficient homozygoites, or heterozygoites. 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 are 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 heterozygoites 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 heterozygoites females with high efficiency. Heterozygoites 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 devise a methodology by which the heterozygoites 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 females) were subjected to molecular genotyping for common G6PD variants. Methodology was designed to identify female heterozygoites 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%) females 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 deficient 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 heterozygoites are missed, when ≤ 4.6 U/gHb cutoff is used to identify deficient female neonates, however, deficient males, hemizygoites 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 Heterozygoites; Cutoff points

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. Heterozygoite females go undetected in commonly used method. The aim of the study was to identify & validate female heterozygoites 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.

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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 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 cycle (Applied Biosystems, Foster City, CA, USA), with 1.25 U AmpliTaq®

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].

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

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Analyzed Region</th>
<th>Forward 5’→3’</th>
<th>Reverse 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFLP (Tn = 61°C)</td>
<td>Exon 3</td>
<td>GTGGAGGAGATGTATGATGATGATT</td>
<td>AGGGCAAGGCACAGCTTAA</td>
</tr>
<tr>
<td></td>
<td>Exon 4</td>
<td>TAAGTCTGTCGCTCGTCGTC</td>
<td>CGAGGTCGTCGTCGTC</td>
</tr>
<tr>
<td></td>
<td>Exon 5</td>
<td>CTGTGCTGTCGTCGTCCTGTCCTGTC</td>
<td>GAGGGCAAGGCCAGCAAGGCTT</td>
</tr>
<tr>
<td></td>
<td>Exon 6</td>
<td>GCAGCGTCTGATCTCCTACTCC</td>
<td>GCAAGGTGAGGAMCTGACC</td>
</tr>
</tbody>
</table>

Table 2: Glucose 6 Phosphate Dehydrogenase deficiency in neonates.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Total 984 (448M-536F)</th>
<th>Frequency</th>
<th>G6PD Activity Range</th>
<th>Mean G6PD Activity/U/gHb*</th>
<th>Ratio Male/Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>47(77%)</td>
<td>0.14-4.15</td>
<td>1.89 ± 1.45</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>≤6.6 U/gHb</td>
<td>14(23%)</td>
<td>0.12-4.5</td>
<td>2.6 ± 1.48</td>
<td>1:3.6</td>
</tr>
<tr>
<td>Female</td>
<td>&lt;4.6 U/gHb</td>
<td>34(34%)</td>
<td>0.12-6.6</td>
<td>5.5 ± 0.59</td>
<td>1:1.3</td>
</tr>
</tbody>
</table>

*Statistic was done using SPSS 16

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

<table>
<thead>
<tr>
<th>G6PD Variant</th>
<th>Number of neonates</th>
<th>Mutation</th>
<th>Amplified Exon</th>
<th>Enzyme</th>
<th>Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mediterranean</td>
<td>18 (90%)</td>
<td>563 C&gt;T</td>
<td>6+7</td>
<td>Mbo II</td>
<td>547</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>377, 119, 26, 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>143 T&gt;C</td>
<td>3+4</td>
<td>Mbo I</td>
<td>353</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>293,60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>353</td>
</tr>
</tbody>
</table>
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.

References