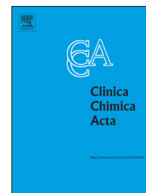
Clinica Chimica Acta xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim



Determination of optimal cutoff value to accurately identify glucose-6-phosphate dehydrogenase-deficient heterozygous female neonates

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ARTICLE INFO

Article history:
Received 23 December 2012
Received in revised form 28 April 2013
Accepted 1 May 2013
Available online xxxx

Keywords:
G6PD deficiency
Newborn screening
Cut-off value
Heterozygotes
Female

ABSTRACT

Background: Conventional screening tests to assess G6PD deficiency use a low cutoff value of 2.10 U/gHb which may not be adequate for detecting females with heterozygous deficiency. The aim of present study was to determine an appropriate cutoff value with increased sensitivity in identifying G6PD-deficient heterozygous females.

Methods: G6PD activity analysis was performed on 51,747 neonates using semi-quantitative fluorescent spot test. Neonates suspected with G6PD deficiency were further analyzed using quantitatively enzymatic assay and for common G6PD mutations. The cutoff values of G6PD activity were estimated using the receiver operating characteristic curve.

Results: Our results demonstrated that using 2.10 U/g Hb as a cutoff, the sensitivity of the assay to detect female neonates with G6PD heterozygous deficiency was 83.3%, as compared with 97.6% using 2.55 U/g Hb as a cutoff. The high cutoff identified 21% (8/38) of the female neonates with partial G6PD deficiency which were not detected with 2.10 U/g Hb. Our study found that high cutoffs, 2.35 and 2.55 U/g Hb, would increase assay's sensitivity to identify male and female G6PD deficiency neonates, respectively.

Conclusions: We established a reliable cutoff value of G6PD activity with increased sensitivity in identifying female newborns with partial G6PD deficiency.

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1. Introduction

Glucose-6-phosphate-dehydrogenase (G6PD) deficiency, one of the most common X-linked enzymopathy, may present with severe hemolytic episodes, extreme hyperbilirubinemia and life-threatening bilirubin encephalopathy [1–3]. Using the available screening tests, G6PD-deficient males can be accurately identified. However, females are more difficult to categorize because many in this group may be heterozygotes with phenotype overlap between normal homozygotes, heterozygotes, and deficient homozygotes [4,5]. Female heterozygotes should be detected as early as possible and treated as if they were G6PD totally deficient [6,7].

Neonatal screening for G6PD deficiency has been carried out in several countries for >30 years. The assays used in most screening programs are based on the semi-quantitative method described by Beutler et al. [8,9]. The method is rapid, simple, sensitive, and inexpensive. However, a considerable percentage of heterozygous females with partial G6PD deficiency cannot be detected by the method using a low cutoff value such as 2.10 U/gHb in many practices [7]. The detection level of diagnostic tests for clinical conditions that are amenable to interventional management is usually set to increase the sensitivity of the test to detect true positives for the condition while minimize the number of cases that are not affected. The cutoff value for identifying individuals affected in a population is best determined using receiver operating characteristic (ROC) curves generated from data using both affected and unaffected individuals of the population [10]. The aim of this study is to establish a more appropriate G6PD activity cutoff value, in order

Abbreviations: G6PD, glucose-6-phosphate-dehydrogenase; ROC, receiver operating characteristic; ARMS, amplification refractory mutation system.

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0009-8981/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.cca.2013.05.004

Please cite this article as: Miao J, et al, Determination of optimal cutoff value to accurately identify glucose-6-phosphate dehydrogenase-deficient heterozygous female neonates, Clin Chim Acta (2013), http://dx.doi.org/10.1016/j.cca.2013.05.004

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to improve sensitivity of identifying partial G6PD-deficient female neonates.

2. Materials and methods

2.1. Study design

A total of 51,747 (28,067 [54.2%] males; 23,680 [45.8%] females) neonates, born between March 2010 and February 2012 in Chongqing, China, were included in the study. The screening protocol was as follows: a heel capillary blood sample was collected from newborns between the 3rd and 5th day of life, adsorbed on Whatman 903 filter paper and mailed to our neonatal screening laboratory, where the modified fluorescent spot-test was applied to detect G6PD deficiency using the neonatal G6PD Kit (Wallac Oy, Turku, Finland). This technique is based on the evaluation of fluoresced reduced pyridine nucleotide (NADPH) when activated by UV light [11–13]. Using cutoff value (2.70 U/g Hb) recommended by the manufacturer, a second sample was requested from neonates with G6PD value <2.70 U/g Hb for G6PD gene mutations analysis. Meanwhile, we also measured G6PD levels using a previously described quantitative enzymatic assay. For the quantitative evaluation of G6PD activity, the improved G6PD Nitroblue tetrazolium (NBT) Quantification Ratio Kit (Micky, Guangzhou, China) was used. Those with G6PD/6PGD ratio <1.0 were considered as G6PD deficient [14,15]. The study was reviewed and approved by the Ethics Committee of Children's Hospital of Chongqing Medical University, and informed written consent was obtained from mothers before delivery.

2.2. Detection of G6PD gene variants

Genomic DNA was extracted from blood samples using TIANamp Blood DNA Kit (TIANGEN, Beijing, China). The oligonucleotide primers used in this study are shown in Table 1; the primer sequences were designed through complementation with mutated chain using the Primer 5.0 program (Premier Biosoft International, CA). G6PD gene variants were analyzed by the amplification refractory mutation system (ARMS)-based PCR, as previously described [12,13]. All mutant samples were confirmed by sequencing.

2.3. Determination the cutoff value of G6PD activity

The best cutoff value of G6PD activity was obtained from the ROC curve, which is the value that maximizes the classification of individuals as healthy or G6PD-deficient with greater sensitivity and specificity.

2.4. Statistical analysis

Analysis of the frequency distribution of G6PD values among the affected and unaffected newborns, ROC curve and statistics analyses were performed using SPSS version 19.0 (SPSS, Chicago, IL). Chi-square test was used to determine the frequency difference, and Student's *t*-test to evaluate the mean difference. A $p < 0.05$ was considered statistically significant.

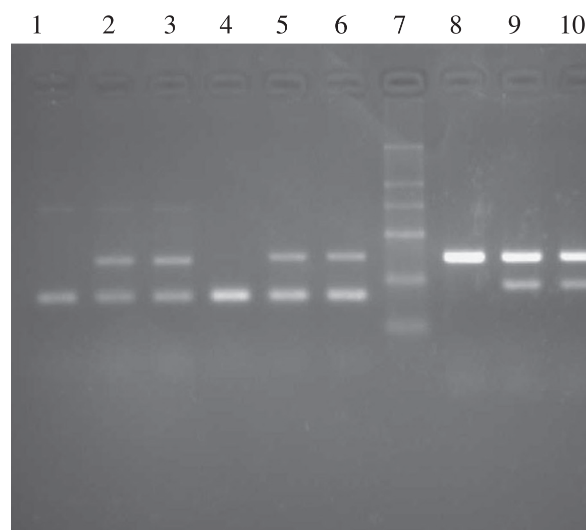

Fig. 1. Representative result from ARMS PCR analysis. Lanes 1, 4, 8 are internal controls showing 196 bp, 196 and 360 bp, respectively. Lanes 2, 5, 9 are the positive controls of 361, 345 and 226 in size, respectively. Lanes 3, 6, 10 are samples from the affected patients showing fragments of 361 bp, 345 and 226 bp corresponding to the mutations c.1388G>A, c.1376G>T and c.95A>G, respectively. Lane 7 is 100 bp molecular marker.

3. Results

3.1. Analysis of G6PD activities

Among the 51,747 neonates tested for G6PD activity, 332 cases were determined as suspected G6PD deficient using the fluorescence spot test. Of the 269 cases that were requested for a second sample for further analysis, 251 (221 males, 30 females) were found to be G6PD-deficient. The estimated prevalence was 7.87‰ in males, 1.26‰ in females and 4.85‰ among all newborns.

3.2. Determination of G6PD gene mutations using ARMS-PCR

DNA was isolated from all 269 neonates positive for G6PD deficiency. These samples were further analyzed by PCR for three most common G6PD mutations, c.1388G>A, c.1376G>T and c.95A>G in the Chinese populations [16]. Fig. 1 shows a representative gel image of the detection of the G6PD mutations using ARMS. In cases with c.1388G>A, c.1376G>T, and c.95A>G mutations, a 361-, 345- and 229-bp fragment was amplified, respectively.

Using the ARMS-PCR method, of the 269 cases, we identified 199 cases carrying one of the common G6PD mutations. Both c.1388G>A and c.1376G>T mutations were the predominant ones present among our cohort: c.1388G>A was present in 84 males and 16 females,

Table 1
Primer sequences, fragment sizes and annealing temperatures for PCR reactions for detecting G6PD mutations.

Mutations		Primer sequences	Fragment sizes (bp)	Annealing temperatures (°C)
c.1388G>A	L2	5′-GACCTGACCTACGGCAACAGATAC-3′	361	62
	M2	5′-GGTGCAGCAGTGGGGTGAAATTAT-3′		
c.1376G>T	L2	5′-GACCTGACCTACGGCAACAGATAC-3′	345	62
	M	5′-TGAAAATACGCCAGGCCTCAA −3′		
c.95A>G	L	5′-GTGTCACCCTGGTGTGAGACCC-3′	226	60
	M	5′-GCACCCATGATGATGAATTTGC-3′		
Internal control	4F	5′-TTGTCGGTCTCTCTGCTGGTCAGTG −3′	196	62
	4R	5′-CAAAGCCCTCACTCAAACATGAAGC −3		
	8F	5′-GTCCTTTACACACTTTACCTGTTGAG −3′	360	60
	8R	5′-GGCCTCATTCTCATGTTCTAATTAGT −3′		

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