

Molecular identification of glucose-6-phosphate dehydrogenase (G6PD) detected in neonatal screening

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Abstract

Objective: To present a strategy to identify G6PD molecular variants detected in neonatal screening (NS). **Material and methods:** Series of incident cases of newborns (NB) with G6PD deficiency detected in the NS. Based on nuclear DNA, with the real-time polymerase chain reaction technology, the G202A, A376G, T968C and C563T G6PD-molecular variants were searched. **Results:** Of 21,619 assessed neonates, 41 cases were reactive to G6PD in the NS (rate of 189.6/100,000 screened NBs); the molecular G6PD variant was confirmed in 34 cases (rate of 157.3/100,000 screened NBs). The most common allele combination was G202A/A376G (G6PD ratio and mean activity: 0.460 and 1.72 ± 0.35 U/g of hemoglobin [Hb], respectively), followed by G202A (0.170 and 1.74 ± 0.27 U/g Hb) and by the A376G/T968C combination (ratio: 0.150 and 1.10 ± 0.44 U/g Hb). The T968C allelic variant showed lower enzyme activity than the rest (1.1 ± 0.4 ; $p = 0.02$). Two females were detected to have G6PD deficiency, with the G202A/A376G and G202A variants. **Conclusions:** This molecular approach allows for involved variants to be identified in up to 80% of the cases. African origin alleles were predominant. (Gac Med Mex. 2015;151:31-7)

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Introduction

Neonatal screening (NS) is a series of tests designed to carry out secondary prevention interventions through preclinical diagnosis of several inherited disorders, in compliance with a set of widely described and updated methodological criteria¹. Candidate diseases to be included in the NS vary according to the target population

where it will be applied, which is why every test requires validation within the particular clinical setting^{1,2}, as in the case of G6PD deficiency detection.

G6PD deficiency belongs to the group of inherited hemolytic anemias and its prevalence varies between populations. Currently, more than 400 G6PD variants are known and it shows an X chromosome-linked inheritance pattern, out of which nearly 140 single-nucleotide mutations (single nucleotide polymorphism [SNP]) are known³.

G6PD deficiency shows wide variability in its clinical expression, which ranges from asymptomatic presentations to those cases with hemolytic anemia and

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severe neonatal hyperbilirubinemia (NNH)³. Different mutations generate diverse concentrations of erythrocyte enzyme, with no proportional relationship between higher severity and clinical expression⁴. Most subjects affected by this deficiency are asymptomatic, and only when exposed to oxidant substances, such as some foods, medications or infectious events, hemolytic crises can be triggered⁵.

Global prevalence of G6PD is widely variable according to the geographic region and the analytical method employed⁵. The highest prevalence occurs in Sub Saharan regions, where it affects between 23 and 39% of the population. In Latin America, it varies according to the populational African ancestry⁴; in Mexico, it is reported to be 0.39-4.09% according to the geographic zone^{6,7}, whereas in indigenous groups it occurs in 0.28-6.22% of the studied population⁷. There are reports on the association of NNH and G6PD deficiency in neonates readmitted to the hospital for jaundice, with up to 47% of them having G6PD deficiency⁸. In our environment, there are isolated reports on the occurrence of G6PD deficiency, which ranges from 0.43-0.66% in term neonates without jaundice⁹ to 1.57% in cases with NNH¹⁰. In spite of its elevated prevalence, G6PD deficiency detection is not a mandatory test included in NS in Mexico, nor are there reports on clinical follow-up of G6PD deficiency cases detected in the community or in the neonatal period, which hinders estimating the clinical or epidemiological impact of this group of mutations, as well as the potential benefit of its primary prevention.

The purpose of this work is to present the results of a strategy to identify the molecular variants of G6PD in cases detected in the NS by using a non-invasive collection technique to obtain genomic material in the molecular diagnosis of G6PD deficiency, according to the requirements indicated in the genetic association studies appearing in the STREGA Declaration¹¹.

Material and methods

An observational, longitudinal, prospective, descriptive study was conducted from February 2008 on, when determination of G6PD activity was included in the NS. All NB at the Instituto Nacional de Perinatología, which is a tertiary care hospital for patients with high-risk pregnancies and that has a high prevalence of preterm births, were consecutively assessed in the screening. Neonates were included, regardless of gestational age and birth weight, hospitalized in general wards and special care units. Cases with early neonatal

death were deferred or excluded from the procedure, as well as those severely ill neonates when no blood sample was available for the baseline assessment.

Semiquantitative G6PD detection was performed at the post-natal period with a quick screening method, by means of a colorimetric technique (Neonatal G6PD Assay, Semiquantitative; Bio-Rad Laboratories) in blood samples soaked in a dry matrix (Güthrie card). The cut-off point to define G6PD deficiency was deemed as values ≤ 2.6 U/g Hb. Reactive cases in the NS were further assessed, along with their families, for the confirmatory molecular study. Clinical and hematological follow-up of the cases was maintained at least during the first year of life at the Pediatric Hematology outpatient clinic.

Exfoliative cytology material was obtained for DNA extraction by means of buccal mucosa smear with a Dacron polyester swab. In the case of the mothers, a sample of peripheral blood was obtained to subsequently extract nuclear DNA. In this report, the results of the fathers or siblings of the family are not included.

DNA was extracted using a commercial method (High Pure PCR Template Preparation Kit Roche®, Mannheim, Germany). The sample was stored at -70°C until its molecular study.

Given the large number of polymorphisms associated with G6PD existing in our country⁶, a strategy was established in order to optimize the confirmatory molecular study. The selection of G6PD polymorphisms was carried out by consulting the opinion of three national experts on the subject (R. Lisker, G. Vaca and B. Ibarra Cortés), who considered the 14 most frequently reported polymorphisms in our country^{5,6}; additionally, the criteria established by the World Health Organization were incorporated¹². For this report, a first block of four polymorphisms was selected: two of African origin (G202A and A376G) and two of European origin (C563T and T968C), with the highest national prevalence^{13,14}. Due to its accessibility for application in the clinical setting, the RT-PCR methodology was selected, using the hybridization probes format¹⁵. The designs for the different SNPs were made by TIB MOLBIOL (Eresburgstrasse, Berlin) (Table 1).

Since this is a condition with an X chromosome-linked inheritance mechanism, male gender cases identified by molecular tests were defined as mutated hemizygotes. The identified female gender cases were defined as mutated homozygotes or heterozygotes. Cases where the assessed polymorphisms were not identified were defined as wild type (WT).

The results are presented using descriptive statistics by means of frequencies, percentages, rates and proportions

Table 1. Oligonucleotide sequences of the primers and hybridization probes used in the determination of G6PD polymorphisms

Assessed polymorphism	Sequence	T _m (°C)
G202A H	5' TCAGgTggCTgTTCCg 3'	62.9
G202A R	5' CTCACTCTgTTTgCggAT 3'	59.9
G202A Sen	5' CCgAAAACACCTTCATCgTgggCT-FL 3'	67.4
G202A Anc	5' LC640-gCCCgTTCCCGCCTCACAgTggCTgACA-PH 3'	79.7
A376G F	5' TgTCTgTCTgTCCgTgTCTCC 3'	65.9
A376G R	5' ACTCgTgAATgTTCTTggTgA 3'	62.9
A376G Sen	5' gCgCCTCAACAgCCACATggATgCCCT-FL 3'	75.6
A376G Anc	5' LC640- ACCTggggTCACAggCCAACCgCCTCTT--PH 3'	75.5
C563T F	5' gTTCAAgggggTAACgCAG 3'	66.4
C563T R	5' CACCTCAgCACCATgAggTT 3'	66.5
C563T Sen	5' CATCTCCTCCCTgTTCC-FL 3'	49.4
C563T Anc	5' LC640-AggACCAgATCTACCgCATCgACCACTAC--PH 3'	66.2
T968C F	5' CCAgTACgTggggAAC 3'	58.8
T968C R	5' CAgTgCCCgCACAC 3'	61
T968C Sen	5' ggTCgTCCgggTACCCTTT-FL 3'	60.5
T968C Anc	5' LC640 gTggCCTCgCCCTCTCCATCg--PH 3'	69.6

F: primer or first sense; R: primer or first anti-sense; Sen: sensor probe (5'LC Red 640, 3'ph: phosphate); Anc: anchor probe (3'FL: fluorescein); T_m: melting temperature.

of the presented incipient cohort. The frequency of identified cases is reported in rates per 100,000 live births (LB). The single-factor analysis of variance statistical parametric test or the non-parametric Kruskal-Wallis test (according to data distribution) were used for the comparison of mutated alleles and allelic combination with the activity percentage of the G6PD enzyme and total serum bilirubin levels with a statistically significant p-value of 0.05.

The protocol was approved by the Research Ethics Commission of the institute. The study was entirely financed with federal funds assigned to the project. The authors declare there are no conflicts of interest at all.

Results

A total of 1,209 NB (5.3%) were not included in the NS for several reasons, including early neonatal death, moved to other hospital, early hospital discharge and failure to assist to the outpatient clinic for neonatal metabolic screening.

The distribution of G6PD enzyme activity concentrations detected in the screening ranged from 0.2 to 2.6 U/g Hb.

Seven cases were reported as WT, all of them males; jaundice was detected in 3 cases; in 2 cases, the G6PD activity values approached the cutoff values. Two female neonates were identified to be reactive in the NS: one showed G6PD activity of 0.8 U/g Hb and had inherited the G202A/A376G allele combination from both parents. The second female case had G6PD activity of 2.5 U/g Hb, with heterozygous presence of the G202A allele, and none of the studied molecular variants was detected in her mother and it was not possible to obtain the blood sample of the father. The C563T mediterranean G6PD allelic variant was not identified in any case (Table 3).

The concentrations of the G6PD activity for the T968C, G202A and A376G allele variants show a dispersion of 1.1 ± 0.4 (0.50-1.80), 1.73 ± 0.3 (0.80-2.20) and 1.57 ± 0.46 (0.50-2.20), respectively, with statistically significant differences (Kruskal-Wallis, $p = 0.02$). Of the 41 initially reactive neonates, six cases had G6PD activity values of ≤ 1.0 U/g Hb (0.150 ratio); 29 additional cases showed values of 1.1 to 2.0 U/g Hb (0.700 ratio) and in the 6 remaining cases, concentrations of 2.1 to 2.6 U/g Hb (0.150 ratio) were observed.

Table 2. Incidence rate of G6PD deficiency cases detected in the NS

Assessment period	LB (22,828)	Screened (21,619)	% (94.7)	Cases		Rate x 100,000 LB	
				Reactive (n = 41)	Confirmed (n = 34)	Screen (189.6)	Confirmed (157.3)
From January to December 2008	5,110	4,927	96.4	6	4	121.8	81.2
From January to December 2009	4,772	4,528	94.8	9	9	198.7	198.7
From January to December 2010	4,189	4,083	97.5	10	7	244.9	171.4
From January to December 2011	4,229	4,131	97.7	8	6	193.6	145.2
From January to December 2012	4,528	3,950	87.2	8	8	202.5	202.5

The T968C allelic variant showed the highest ratio of G6PD enzyme activity < 1.0 U/g Hb cases (0.333 versus 0.037 and 0.120, respectively). There were no statistically significant differences in the occurrence of neonatal jaundice or in bilirubin serum concentration by allelic variant (Table 4).

The most common allele combination was G202A/A376G, with 19 neonates (0.460 ratio), out of which 18 males were mutated hemizygotes and one mutated homozygote female; mean enzyme concentration was 1.77 ± 0.41 U/g Hb (0.8 to 2.2 range). The second most common combination was G202A; in 8 cases, 7 mutated hemizygote boys and one mutated heterozygote girl (0.195 ratio), with a mean enzyme concentration of 1.83 ± 0.37 U/g Hb (1.30 to 2.50 range). The third variant was A376G/T968C with 6 cases, 6 mutated hemizygote boys (0.146 ratio) and enzyme concentration of 1.10 ± 0.44 U/g Hb (0.5 to 1.8 range). Finally, the WT variant occurred in 8 cases, all of them males (ratio of 0.195) and with enzyme concentration of 1.58 ± 0.87 U/g Hb (0.2 to 2.6 range). The frequency order in the allele combinations of the 39 assessed mothers was for the G202A/A376G allele, in 16 mutated heterozygote cases (ratio of 0.440). Total serum bilirubin was determined in 27 neonates, with no statistically significant difference being observed with regard to the allele combination (Table 5).

During neonatal evolution, 27 cases had indirect hyperbilirubinemia. Mean total serum bilirubin was 11.2 ± 4.1 mg/dl (range, 5.6-21); 16 cases required treatment with phototherapy (ratio, 0.600). Clinical follow-up of the cases was maintained from 2 to 30 months of age, providing with counselling and education on this condition. During pediatric follow-up, a single hemolytic crisis event associated with the consumption of broad beans was documented in one case, but no transfusional

management was required. The maternal study was performed in all cases; all mothers were Mexican for at least 3 previous generations, except for one mother who was born in the Republic of Cuba and her maternal grandmother was of African descent. Of the 39 mothers and 41 children (there were two mothers with two reactive children each), the involved G6PD molecular variant could not be identified in 9 cases in spite of having decreased enzyme activity estimates (data not shown).

Discussion

African ancestral origin alleles are predominant in the observed results; the G202A and A376G alleles together represent the predominant allelic frequency (0.896). About 14 genotypical variants have been identified in our environment^{16,17}. The data we present do not differ significantly from national reports, where the predominance of African origin in G6PD allelic variants is documented^{10,17}. In the report of mestizo-Mexican subjects, the G202A/A376G combination was observed to occur in 45% of the assessed cases and accounted for three times more than the cases with the A376G/T968C combination¹⁸. There are geographic differences associated with the predominance of the G6PD variants in the Mexican population; the G6PD B- or Mediterranean variant has been reported in neonates originating in northeastern Mexican Republic, whereas the African-origin A variant is predominant in the Gulf of Mexico region¹⁰.

The World Health Organization has proposed a classification of the G6PD variants according to biochemical and clinical characteristics. The allelic variants presented in this study belong to class II and III, with clinical data of moderate to severe neonatal jaundice and favism¹².

Table 3. Description of allelic variants identified in the mother-child binomial, enzymatic activity values and clinical expression

Consecutive number	Gender	G6PD activity (U/g HB)	Neonatal G6PD alleles	Maternal G6PD alleles	Jaundice/NNH
1	Male	1.9	WT	WT	No
2	Male	1.8	G202A/A376G	G202A/A376G	No
3	Male	1.6	G202A/A376G	G202A/A376G*	Yes/13.9
4	Male	2.7	G202A/A376G	G202A/A376G*	No
5	Male	1.0	A376G/T968C	A376G/T968C	Yes/10.6
6	Male	0.5	A376G/T968C	A376G/T968C	Yes/16.3
7	Male	2.4	WT	WT	No
8	Female	0.8	G202A/A376G	G202A/A376G	No
9	Male	1.4	G202A/A376G	G202A/A376G	No
10	Male	2.1	G202A/A376G	G202A/A376G	Yes/8.7
11	Male	1.7	G202A/A376G	G202A/A376G [†]	No
12	Male	1.9	G202A/A376G	G202A/A376G [†]	Yes/5.7
13	Male	1.2	G202A/A376G	G202A/A376G	Yes/17.6
14	Male	1.7	G202A/A376G	G202A/A376G	Yes/ND
15	Male	1.2	A376G/T968C	A376G/T968C	Yes/14.3
16	Male	0.8	A376G/T968C	A376G/T968C	No
17	Male	1.7	G202A/A376G	G202A/A376G	No
18	Male	2.2	G202A/A376G	G202A/A376G	Yes/12.4
19	Male	2.1	G202A	G202A	Yes/10.3
20	Male	2.0	G202A	G202A	Yes/7.1
21	Male	0.5	WT	WT	No
22	Male	1.7	G202A/A376G	G202A/A376G	Yes/11.4
23	Male	2.6	WT	WT	Yes/17.8
24	Male	1.3	WT	WT	No/8.2
25	Female	2.5	G202A	WT	Yes/8.1
26	Male	1.8*	G202A	G202A	No/5.6
27	Male	1.5	G202A	G202A	Yes/7.4
28	Male	2.2	G202A/A376G	G202A/A376G	Yes/10.9
29	Male	1.3	G202A	G202A	Yes/21.2
30	Male	1.6	WT	WT	No
31	Male	1.7	G202A	G202A	No
32	Male	1.8	G202A/A376G	G202A/A376G	No
33	Male	0.2	WT	WT	Yes/13.4
34	Male	1.8	G202A/A376G	G202A/A376G	Yes/6.1
35	Male	1.2	G202A/A376G	G202A/A376G	Yes/ND
36	Male	1.5	A376G/T968C	A376G/T968C	Yes/8.4
37	Male	2.0	G202A/A376G	ND	Yes/10.5
38	Male	1.4	G202A/A376G	G202A/A376G	Yes/6.8
39	Male	1.8	G202A	G202A	Yes/8.6
40	Male	1.8	G202A/A376G	G202A/A376G	Yes/12.4
41	Male	1.8	A376G/T968C	A376G/T968C	Yes/14.4

Non-mutated WT for the studied SNPs.

ND: not determined.

*,[†]: mother with two reactive cases.

Table 4. G6PD-deficiency allelic variants, enzyme concentration and total serum bilirubin

Variables		G6PD allele		
		T968C* (n = 6)	G202A (n = 27)	A376G (n = 25)
G6PD activity concentration (U/g Hb)*		1.1 ± 0.4 (0.50-1.80)	1.73 ± 0.3 (0.80-2.20)	1.57 ± 0.46 (0.50-2.20)
G6PD stratum (n/standard deviation)	< 1.0 (6/0.150)	0.333	0.037	0.120
	1.0-2.0 (29/0.700)	0.667	0.778	0.760
	2.0-2.6 (6/0.150)	0.000	0.185	0.120
Presence of jaundice (ratio)	No	0.167	0.296	0.280
	Yes	0.833	0.704	0.720
Serum bilirubin (mg/dl)	Mean, SD (range)	12.8 ± 3.2 (8.4-16.0)	10.4 ± 4.3 (5.6-21.0)	11.3 ± 3.5 (5.7-18.0)

SD: standard deviation.

*T968C allele versus all other alleles. Kruskal-Wallis test, 0.020 significance.

Molecular identification of the G6PD variants was accomplished in nearly 80% of the assessed cases, results that are consistent with reports by other authors for our country¹³. The remaining 20% of cases, where decreased G6PD activity is observed, may be due to the fact that we only studied 4 molecular variants, which is why increasing the number of G6PD-deficiency polymorphisms in the molecular strategy would be convenient.

The combined strategy of screening with a colorimetric test and confirming with a group of molecular variants targeted to the detection of the G6PD A- (G202A; A376G) African variants and the mediterranean variants (C563T) with other additional combinations has been reported in literature, identifying up to 90% of polymorphisms that cause the G6PD decrease detected in NS¹⁹.

There are different methodologies employed in the NS to identify G6PD-deficiency carriers, including enzyme semi-quantification using a colorimetric method, the fluorescence test, tandem mass spectrophotography or confirmatory molecular identification of *G6PD* gene mutations³.

It is important taking into account that preterm neonates can generate false negative results in the semi-quantitative detection of G6PD. The heterogeneity among G6PD global prevalence estimates has been assessed in a meta-analysis of 280 studies in 88 countries⁴. The high degree of heterogeneity also depends on the diversity of the methodologies used for analytical measurement of the enzyme. The magnitude of the global, regional or within-country variations on the prevalence of G6PD deficiency is important for public health,

Table 5. G6PD-deficiency allele combinations, enzyme concentration and total serum bilirubin

Allele combination	Enzyme activity (U/g Hb)*	Assessed cases	Serum bilirubin (mg/dl)†
G202A/A376G (n = 19/0.460)	1.77 ± 0.41 (0.80-2.60)	11	10.58 ± 3.6 (5.70-18.0)
G202A (n = 8/0.195)	1.83 ± 0.37 (1.30-2.50)	7	9.76 ± 5.2 (5.60-21.0)
WT (n = 8/0.195)	1.58 ± 0.87 (0.20-2.60)	4	13.2 ± 3.9 (8.20-18.0)
A376G/T968C (n = 6/0.146)	1.10 ± 0.44 (0.50-1.80)	5	12.79 ± 3.2 (8.40-16.0)
Total (n = 41)	1.65 ± 0.56 (0.20-2.60)	27	11.6 ± 4.1 (5.60-21.0)

Statistical test selected by asymmetric (non-Gaussian) distribution of data.

Values expressed as means, standard deviation, minimal and maximal rank.

*p-value = 0.020 obtained with the Kruskal-Wallis test.

particularly when programs are being planned to improve neonatal care and health².

The discussion on inclusion of new tests in NS is controversial due to its potential impact on the health system, ethical aspects for the informed consent, storage of the obtained samples or protection against discrimination²⁰, as well as due to specific issues such as the moment of sample-taking, the preterm birth condition, the effect of transfusions or parenteral nutrition on the NS results²¹.

The detection of G6PD deficiency is not a mandatory test in NS in Mexico. However, it was incorporated to the NS in our institution cover the study of those cases with indirect NNH and eventually neonatal hemolytic disease, whose etiology could not be clarified with the technological resources available in that moment. So, the initial step was to find out the prevalence of G6PD deficiency in the neonatal population, followed by the molecular strategy for its study and then assessing the clinical usefulness of the diagnostic test.

The usefulness of identifying G6PD-deficient neonates helps to recognize a genetic risk factor for the development of indirect hyperbilirubinemia in the neonatal period²².

Glucose-6-phosphate dehydrogenase produces sudden hemolysis episodes and associated with other risk factors can induce an exponential increase in bilirubin serum concentrations and produce neurological damage and, therefore, NNH should be regarded as the result of complex interactions between genes and the environment. Neonatal screening programs, added to neonatal medical care and parental education can be successful in limiting the severity of the disease²³.

G6PD is a well known risk factor for significant NNH. In the study of G6PD-deficient neonates, 11.1% showed high total bilirubin serum levels compared with those without this deficiency. In the case of NB requiring hospital re-admission for hiperbilirubinemia, G6PD-deficiency was detected to be associated in 47%. Nock et al. concluded that neonatal screening is useful for G6PD in American NB, and more useful in populations at higher risk for severe hiperbilirubinemia, such as Afro-American and Asian populations²⁴.

In conclusion, the strategy for the detection of G6PD-deficient subjects by means of a colorimetric test and confirmation using a RT-PCR-based molecular method allows for nearly 80% of deficient subjects to be identified. External validation of these results is needed by means of multi-center, collaborative trials.

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