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PCR-based allelic discrimination for glucose-6-phosphate dehydrogenase (G6PD) deficiency in Ugandan umbilical cord blood

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common X-linked disorder in the world. G6PD deficiency puts children at risk for hyperbilirubinemia and kernicterus during the newborn period and an increased risk of severe hemolysis after exposure to many anti-malarial medications. A laboratory diagnosis of G6PD deficiency is rare in the developing world due to limited resources. We developed a Taqman-based allele specific assay to rapidly determine rates of G6PD deficiency contributing alleles (G202A and A376G) in East Africa. We tested umbilical cord blood from 100 Ugandan newborns and found the overall allele frequency of G202A was 0.13 and A376G was 0.32. The overall incidence of G6PD A- (G202A/A376G) was 6%; all A- variants were males. There was no correlation between G6PD deficiency and umbilical cord blood hemoglobin, white blood count, platelet count, or other hematologic parameters. Allele specific PCR can serve as a rapid method to determine specific G6PD deficiency allele frequencies in a given population and as a diagnostic tool in a hospital setting in which laboratory resources are present.

Keywords

G6PD deficiency; Africa; Uganda; Allele specific PCR; Umbilical cord blood

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked disorder that affects as many as 400 million people worldwide, making it the most common human enzymatic defect[1]. G6PD is an enzyme that aids in processing carbohydrates into energy, and is a key enzyme in the oxidative pentose phosphate pathway converting nicotinamide adenine dinucleotide phosphate ($NADP^+$) to the reduced form, NADPH. NADPH is an important

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TCL conceived the study and wrote the manuscript. JH, DB, SN collected samples and CBC analysis. DF and MLC performed PCR reactions and data analyses. MLC, JAR, TCL performed data statistics and manuscript editing.

reducing agent that decreases oxidative stress and protects red blood cells from byproducts of metabolism. Exposure of G6PD deficient individuals to certain medications, typically anti-malarials, chemicals, fava beans, or other pro-oxidants can induce acute hemolytic anemia with resulting hematuria, jaundice, anemia and shock.

Due to the pattern of X-linked inheritance for G6PD, males are more often affected and symptomatic. Nevertheless, females can also be affected, especially if there is skewing of lyonization present in erythrocyte precursors[2]. One class of medications that can induce severe hemolysis in G6PD-deficient individuals is the 8-aminoquinolines, which are commonly used for malaria prophylaxis and treatment. Therefore, the identification of G6PD-deficient individuals in areas where malaria is endemic is critical for safe malarial prophylaxis and treatment [3]. Another crucial clinical problem is that of newborn hyperbilirubinemia due to G6PD hemolysis at birth, which, if left untreated, can lead to kernicterus[4,5].

Currently, most G6PD deficiency screening relies on the fluorescent spot test, which is inexpensive and easily performed, making it ideal for field application. The test is positive if a blood spot fails to fluoresce under ultraviolet light[6]. It is meant to identify hemizygous males and homozygous females at the G6PD locus, but is not sensitive for the identification of heterozygous females. Further, as results are only reported as positive or negative for fluorescence, it is only sensitive in detecting individuals with more moderate to severe G6PD deficiency (i.e., enzyme levels < 20% normal). Consequently, the test can result in false negatives for mild and moderate deficiencies[7]. African G6PD deficiency is typically of the A(-) variant (referred to as variant “A minus”). The G6PD A variant occurs with a single nucleotide polymorphism (SNP) at Asn126Asp (exon 5 376A>G; rs1050829). The variant alone causes no problem with enzymatic activity[8], however, when combined with the Val68Met SNP (exon 4 202 G>A; rs1050828), an unstable G6PD A- variant is produced[9,10]. Here, we describe the use of a TaqMan-based allelic discrimination assay to determine the frequency of G202A and A376G alleles in cord blood samples from Ugandan newborns. This method allows for rapid and specific determination of the G6PD deficiency specific alleles from dried blood spots.

Materials and Methods

Study Population

Cord blood was collected from discarded placental umbilical cords from one hundred babies born at Mulago Hospital, Kampala, Uganda. This study and the use of cord blood were approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota and the Mulago Hospital, Kampala, Uganda. Approximately, 100 – 125 μl of umbilical cord blood was spotted on an individual QIAcard FTA One Spot (Qiagen Sciences, Inc., Germantown, MD) blood collection card for each newborn and allowed to dry for 1 hour. Cards were then placed in individual envelopes containing a MiniPax desiccant packet (Multisorb Technologies, Inc., Buffalo, NY) and were stored at ambient temperature until processed. Each blood sample was also analyzed for a complete blood count on a Nihon Kohden hematology analyzer.

Genotyping

Approximately 1/8th of each blood spot was removed with an individual sterile razor blade under sterile conditions and was placed in a 1.5 ml microcentrifuge tube.DNA was isolated utilizing the QIAamp DNA Mini Kit (Qiagen) per the manufacturer’s protocol for dried blood spots. DNA was eluted from the spin column in 25 μl of elution buffer twice and the eluents combined for a total of 50 μl. DNA samples were stored at −20°C for future use.

Polymerase chain reaction 5' nuclease allelic discrimination assay

Genomic DNA was amplified by TaqMan® polymerase chain reaction (PCR) assays. Pre-designed TaqMan SNP Genotyping Assays were ordered from Life Technologies (Grand Island, NY) for G6PD single nucleotide polymorphisms (SNPs) G202A rs1050828 (Assay ID C_2228686_20) and A376G rs1050829 (Assay ID C_2228694_20). Reactions were set up in 96-well microplates using TaqMan Universal PCR Master Mix (Life Technologies, Grand Island, NY) and SNP Genotyping Assay buffer for either SNP and 1 μl of stock DNA in a 25 μl total reaction volume

All TaqMan PCR reactions were cycled in a Perkin Elmer GeneAmp PCR System 9600 thermal cycler (Perkin Elmer/Life Technologies). For each assay, there were four no-template negative controls, two controls containing the wild-type allele, two heterozygous controls, and two controls containing the variant allele. Thermocycling was performed with an initial 50°C incubation for two minutes, followed by 95°C denaturation for ten minutes, then 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for one minute for the G202A SNP. For A376G, annealing was performed at 62°C. Results of the amplification reactions were assayed on an ABI Prism 7900HT Sequence Detection System (Life Technologies) using Sequence Detector Software (SDS), version 2.1.

Results and Discussion

We collected umbilical cord blood onto blood spot cards from the placentas of 100 newborns born at Mulago Hospital, Kampala, Uganda, including 50 males and 50 females. The range of ethnicities was represented largely by the Gandans (Table 1), which is expected given it is the most prevalent ethnicity in Uganda. Using previously collected samples that were known to be G6PD deficient, allele specific Taqman-based PCR reactions were optimized to discriminate the alleles of interest. Figure 1 shows an example of the results we obtained testing G202A. The data shows excellent discrimination of the allele based on the G202A SNP. For samples that gave an ambiguous result, a second PCR reaction produced interpretable data in every case. Less than 5% of the samples had to be run a second time.

Overall results of gene frequencies are shown in Table 2. The overall allele frequency of G202A was 0.13 and A376G was 0.32. G202A was found in 6/50 males (12%), while the heterozygous 202 AG was found in 14/50 females; no 202 AA homozygotes were found. For Hardy-Weinberg equilibrium, we would expect 1.44% of samples would be 202 AA, or, at most, 1 newborn. The A376G allele was found in 21/50 males (42%), while the heterozygous state (AG) was found in 14/50 females and the homozygous state (GG) in 5/50 females (10%). These frequencies nearly followed Hardy-Weinberg equilibrium, where we expect about 8 (17.6%) homozygous females would be 376 GG. The overall incidence of G6PD A- (G202A/A376G) was 6%, which is consistent with a recent report by De Niz et al where enzyme-based fluorescent spot screening was performed on 235 Ugandan children and a prevalence of moderate deficiency of 7.4% was found[11].

Moderate to mild G6PD deficiency (WHO class III, 10-60% activity) is the most common form found in East Africa[1]. The genotypic A- variants we determined were also subject to the fluorescent spot test based on the method of Beutler et al, which detects G6PD deficiency especially well in males when enzymatic activity is less than 20%. All of the G6PD A- variants identified by PCR were males; they also showed markedly reduced G6PD activity using the fluorescent spot test. We found no females that were homozygous for the A- variant, which is an agreement with a report by Johnson et al who reported only 4 A- homozygous variants after genotyping 600 Ugandan children for the same G6PD alleles[12]. Our overall results were also consistent with a larger study by Carter et al who showed a

hemizygous prevalence of 7.2% for genotypic G6PD deficiency in males from a population of 2045 individuals representing six African countries[13].

In general, both variant alleles are needed to produce G6PD deficiency A- [14]. One drawback to using allelic discrimination in this population is that heterozygous females are difficult to determine, as there is no current method to determine if a given allele is present on the same X chromosome. Based on allele determination, we found that 15/50 females had the potential of being heterozygote for G6PD A-. Nevertheless, skewed lionization and variability in G6PD enzyme levels also hinder enzyme activity-based spot tests in determining heterozygous females[15-17].

We found no significant differences in hematologic parameters in the G6PD A- variants versus non-G6PD A- (Table 3). Furthermore, there were no differences in complete blood count parameters in G6PD A- variants (all males) compared to male newborns analyzed separately. The aforementioned study of G6PD deficiency in 2045 Africans also showed no significant effect of G6PD deficiency on hemoglobin level[13]. While G6PD deficiency puts a newborn at risk for hemolysis-associated hyperbilirubinemia[4,5,18], we were not able to follow our cohort of infants to determined occurrence of hyperbilirubinemia.

The allele specific technique described herein provides a rapid method to determine the genotype status of patients with G6PD deficiency. One advantage of the PCR technique is that it is rapid. Moreover, DNA can be easily isolated from blood spotted on filter cards, thus such a method allows for easy transport as well as prolonged storage time without refrigeration. The results generated provide definitive G6PD genotypes of patients (in the case of the male hemizygote).

Disadvantages are that it inherently costs more per test versus that of the fluorescent spot assay because of the specialization of the reagents and the use of a thermocycler capable of reading fluorescence. Though, because this technique uses an endpoint read, a stand alone fluorescent reader may be an alternative to a real-time thermocycler. The detection of G6PD molecular variants has been used in various populations, but choosing the relevant SNPs requires prior knowledge of variants that are most prevalent in a population. For example, some persons with G6PD deficiency will be missed if they have traveled from another region such as West Africa where G6PD deficient persons may have different variants than East Africans[19,20].

Knowledge of G6PD status can be beneficial to the health and well being of children for several reasons. First, because G6PD deficiency can lead to hyperbilirubinemia that results in devastating kernicterus, mothers can be proactive in seeking medical attention for infants who develop jaundice in the first few days of life. However, a positive clinical impact would only be relevant if the result of the molecular screen could be turned expediently after birth, preferably within 24 hours. Second, many G6PD deficient children being treated for malarial infection can develop hemoglobinuria, anemia, and jaundice both as a result of antimalarial treatment, but also as a manifestation of malarial infection itself. A DNA based test can determine if G6PD deficiency is present. Finally, unlike enzymatic activity spot tests, allelic discrimination methods would not be skewed by elevated G6PD levels present in reticulocytes after brisk hemolysis or necessarily confounded by blood transfusions from G6PD sufficient donors. Thus, allelic discrimination tests can provide accurate determination of the A- variant and aid in diagnosis[3,9].

Conclusion

Although a prior study utilized allele specific PCR to determine G6PD allleles in Malaysian newborns[7], ours is the first study to determine the most common alleles by Taqman-based

allele specific PCR accounting for the A- variant in East Africans. The blood collection and processing can be performed in a timely manner with results for hemizygous males easily interpretable. This method will be useful for population-based studies and as a diagnostic tool for clinicians in the developing world.

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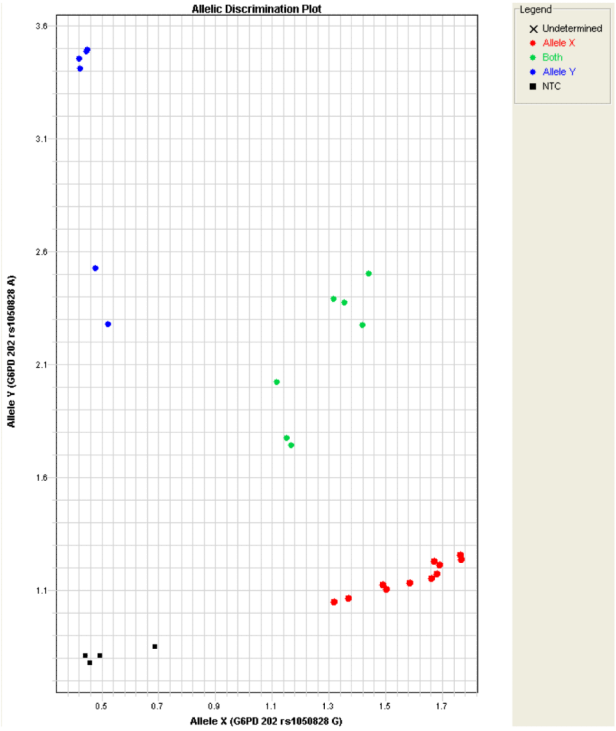

Figure 1. Examples of results from Taqman based allele specific PCR

The 4 black squares in the lower left corner represent negative controls (no template control reactions), the blue and red circles represent homo- or hemizygous individuals for either allele. The green circles represent heterozygous females.

Table 1

Characteristics of the study population.

Males	50
Females	50
Ethnicity	
Ganda	68
Nkoli	5
Lugbaro	4
Soga	4
Acholi	4
Other	15

Table 2

Results of allele specific PCR for G6PD G202A and A376G genotypes.

Allele	Males	Females
202 A	6	
202 AG		14
202 AA		0
376 G	21	
376 GA		22
376 GG		5
G202A/A376G	6	0

Table 3

Hematologic parameters from G6PD A- newborn cord blood and non A- newborns. WBC – white blood cells ($\times 10^3/mm^3$), RBC – red blood cells ($\times 10^6/mm^3$), HGB – hemoglobin (g/dl), HCT – hematocrit (%), MCV – mean cell volume (fl), MCH – mean cell hemoglobin (pg/cell), MCHC – mean cell hemoglobin concentration (g/dl), RDW – red cell distribution width (%), PLT – platelet count ($\times 10^3/mm^3$). P-values were determined from a Student's t-test.

	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	RDW	PLT
G6PD A- (male)	**13.7**	**4.3**	**15.2**	**43.9**	**103.0**	**35.8**	**34.7**	**16.1**	**304**
Non A- (male + female)	12.8	4.3	15.1	43.4	102.1	35.6	34.8	17.4	296
p-value	*0.74*	*0.91*	*0.97*	*0.83*	*0.83*	*0.91*	*0.93*	*0.14*	*0.78*
G6PD A- (male)	13.7	4.3	15.2	43.9	103.0	35.8	34.7	16.1	304
Non A- (male)	12.2	4.4	15.4	44.3	100.3	35.0	34.9	17.3	293
p-value	*0.56*	*0.45*	*0.71*	*0.88*	*0.44*	*0.54*	*0.76*	*0.20*	*0.73*