

ORIGINAL ARTICLE

## Why does the Iranian National Program of Screening Newborns for G6PD Enzyme Deficiency Miss a Large Number of Affected Infants?

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*Purpose:* G6PD enzyme deficiency is one of the most prevalent genetic disorders worldwide and it has high incidence rate in Northern provinces of Iran. It was observed that national neonatal screening for G6PD enzyme deficiency fails to detect all affected infants. In order to clarify the cause, this study has been done in Thalassemia Research Center, Sari, Iran. *Materials and Methods:* This was a diagnostic study. The newborns with parents of Mazandarani origin were enrolled. Cord blood from the placental side was collected and used for decolorization test, quantitative enzyme assay (QEA) and DNA study. A heel-prick sample collected on day 3–5 after birth was used for fluorescent spot test (FST). In male cases, QEA was considered as the gold standard. For females, DNA study was considered as the gold standard. Based on QEA test results, neonates with <20% and 20–60% of mean normal enzyme activity were considered as total deficient and partial deficient, respectively. *Results:* A total of 365 neonates (52.3% females and 47.7% males) were studied. According to FST, 13 male newborns had G6PD deficiency. No deficient female was detected. Decolorization test diagnosed 18 male and one female as G6PD deficient newborns. QEA diagnosed 19 males and 28 females with G6PD enzyme deficiency (26 partial, 2 total deficient cases). DNA analysis detected 14 males as hemizygote and 34 females as heterozygote. *Conclusion:* FST does not have the required sensitivity for newborn screening and QEA is recommended as the preferred method.

**Keywords** decolorization test, DNA analysis, fluorescent spot test, G6PD enzyme deficiency, neonatal screening, quantitative enzyme assay

### INTRODUCTION

According to the WHO, the rate of G6PD enzyme deficiency in Iran is 10–14.9% of the whole population [1], and the highest prevalence is reported from north and south of Iran (8.6–16.4% in Northern provinces, 12% in southern part (Shiraz) and 19.3% in southeastern of Iran) [2]. In countries with 3–5% prevalence rate of G6PD deficiency in male population, screening of all newborns for this X-linked enzyme deficiency is recommended [1], and previous reports from Iran confirms it to be above that range [3 and 4]. Since this deficiency is common in several developing countries, it is

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important to find simple and inexpensive laboratory methods to identify at risk persons, and for this purpose the fluorescent spot test (FST) described by Beutler has been extensively used in Iran [5–7].

National program for G6PD enzyme deficiency screening enrolls all live neonates since June 2006. Heel-prick blood samples in the 3rd to 5th postnatal days are collected, and FST is done through a universal protocol in all laboratories nationwide. A confirmatory test using venous blood by applying a quantitative enzyme assay (QEA; Baharafshan Institute of Research and Development, Iran) has to be done at 120 days after birth. In our previous work, we reported the results of three years screening of the newborns and concluded that through this approach a large number of infants are being missed [8]. In order to evaluate the sensitivity of FST comparing to Decolorization test, QEA method and also DNA analysis, this study was conducted in Thalassemia Research Center, Mazandaran, Iran. 40 45

## MATERIALS AND METHODS

This was a diagnostic study done from October 2012 until July 2013. Sampling was done in a private hospital in Sari. According to the inclusion criteria, the parents were asked whether both have been born in Mazandaran province and to consent for blood sample collection from their child. 50

The cord blood was collected after birth from the placental side and tested by Decolorization test, QEA, and DNA analysis. Then the newborn entered the ongoing screening program and was brought to the health center for heel-prick sampling on day 3–5 after birth. Results of the cord blood tests were compared to the results of the test on this sample. 55

### Decolorization Method

For semi-quantitative Decolorization method, a commercial kit (Saba Azmoon, Iran) was used. Twenty micro liters of fresh blood containing EDTA were mixed with 1 ml of distilled water, then 200  $\mu\text{L}$  of Tris buffer was added to the tubes, and the samples were shaken until the appearance of blue color. Then, 500  $\mu\text{L}$  of the solution were mixed with one drop of buffer A and after 20 min at 37°C, the red color appeared in enzyme sufficient cases, while in deficient cases the blue color did not change. 60 65

### G6PD Enzyme Activity Assay

For the determination of the level of G6PD enzyme activity, Baharafshan Institute of Research and Development commercial kit (Iran), that determines the exact level of G6PD enzyme activity expressed as unit per gram (U/g) of hemoglobin (Hb) was applied. According to the protocol, 50  $\mu\text{L}$  of blood samples was mixed with 1 mL of lysis buffer and then 50  $\mu\text{L}$  of prepared solution was mixed with 1mL of standby solution followed by photometric measurement of the kinetic reaction at 340nm (A1) at 37°C. 5 min later the photometric measurement was done again (A2) and  $\Delta A$  was calculated by subtracting A1 from A2. Using the following formula, G6PD activity was finally calculated as U/g Hb:

$$\text{G6PD enzyme activity (U/g Hb)} = \Delta A \frac{1350}{\text{Hb} \left( \frac{\text{g}}{\text{dl}} \right)}. \quad (1)$$

In order to figure out the mean normal G6PD enzyme activity by QEA, results of the neonates diagnosed by decolorization test as deficient were excluded, and the average result of the rest was considered as mean normal enzyme activity. Neonates with 70

<20% and 20–60% of that value were regarded as total deficient and partial deficient, respectively [9].

#### DNA Analysis Method

For molecular studies on nucleic acid, DNA was extracted from cord blood samples using standard protocols. Then, presence of three common G6PD gene mutations; Mediterranean (563 C→T), Chatham (1003 G→A), and Cosenza (1376 G→C) were evaluated by using polymerase chain reaction (PCR) method and specific restriction enzymes: *MboII*, *BstXI*, and *Bsu36I* enzymes. Enzymatically digested products were subjected to electrophoresis on 3% concentrated agarose gel.

#### 80 Fluorescent Spot Test

Fluorescent Spot Test was carried out for all cases, as part of the screening program in the reference lab (Mazandaran University of Medical Sciences, Sari, Iran). The sample was heel-prick sample of 5mm width, punched from filter papers. A commercial kit (Kimia Pajouhan, Iran) was used and the test was performed according to instruction provided by the manufacturer.

85 For male newborns, the quantitative enzyme assay (QEA) was considered as the gold standard for other conventional screening methods: fluorescent spot test (FST) and Decolorization test. As in some female carriers the QEA could be unreliable due to the phenomenon of lyonization, DNA analysis was considered as the gold standard.  
90 Sensitivity, specificity, positive predictive (PPV), and negative predictive value (NPV) of tests against the gold standard were calculated. The acquired data was analyzed by SPSS software V18 (I.B.M., U.S.A.).

## RESULTS

In this study, a total of 365 neonates, consisting of 191 females (52.3%) and 174 males (47.7%) were studied, and as mentioned in method part, all results were presented on a gender-specific basis. The mean G6PD enzyme activity of neonates with negative decolorization test results was  $48 \pm 11.7$  U/g Hb. Newborns with enzyme activity of <9.6 and 9.6–29.8 U/g Hb were considered as total and partial deficient, respectively.

#### Studies on Male Cases

100 According to FST, 13 male newborns had G6PD deficiency (7.5%, CI95%: 3.59–11.41); decolorization test diagnosed 18 subjects (10.3%, CI95%: 5.78–14.82); QEA identified 19 cases (10.9%, CI 95%: 6.27–15.53), and DNA analysis could detect 14 hemizygote deficient persons (8.0%, CI95%: 3.97–12.03). Table 1 shows the power of FST, decolorization test, and DNA analysis against QAE as the gold standard test for male  
105 subjects.

#### Studies on Female Cases

Among 191 female subjects, no one was identified as G6PD deficient by FST, while decolorization test detected two cases (1.1%, CI 95%: up to 2.48). Using QEA, 28 newborns were identified as G6PD deficient (14.6%, CI95%: 9.59–19.61), among which 26  
110 were partial and two were total deficient. These two cases were the same ones detected by decolorization test.

Based on DNA analysis, 34 females were detected as heterozygote for three mentioned mutations (17.8%, CI 95%: 12.38–23.22). Among these cases, G6PD Mediterranean mutation had the highest frequency (24 cases, 12.5%; CI 95%: 8–17), G6PD Chatham mutation had the second highest frequency (7 subjects, 3.7%; CI 95%:  
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TABLE 1 Power of FST, Decolorization Test, and DNA Test in Identification of G6PD Deficiency in Comparison with QEA as the Gold Standard in Male Newborns, Iran, 2013

Parameters	FST	Decolorization test	DNA test
True positive	13/19	18/19	14/19
True negative	155/155	155/155	155/155
False positive	0/155	0/155	0/155
False negative	6/19	1/19	5/19
Sensitivity	68%	95%	74%
Specificity	100%	100%	100%
Positive predictive value	100%	100%	100%
Negative predictive value	96%	99%	97%

1.0–6.4), and G6PD Cosenza mutation was detected in 3 cases (1.6%; CI 95%: up to 3.4).

Table 2 shows the power of QEA, Decolorization test, and FST in identification of G6PD deficiency against DNA analysis test as the gold standard in female newborns. QEA could identify 21 out of 34 heterozygote cases (59%) and in 13 other cases had G6PD enzyme activity above 29.8U/g Hb, and hence were categorized as negative. There were 7 neonates identified as G6PD deficient by QEA, who had none of the evaluated mutations.

## DISCUSSION

The study showed that FST could not detect any affected female newborn. This is an important weak point for this test. Although G6PD enzyme deficiency is a recessive X-linked disorder, not all affected persons are male. In female cases, in mutant homozygote (or compound heterozygote) and Turner syndrome cases that are carrier for G6PD mutations, G6PD enzyme deficiency occurs as severe as affected male cases. In female G6PD heterozygote subjects, the disease has clinical manifestation with various levels of severity, due to the phenomenon of Lyonization. In these cases the level of active G6PD enzyme in blood is high enough, and therefore these cases cannot be identified by FST as enzyme deficient, and in some cases by QEA as partial deficient, while the DNA test that is not based on the level of enzyme activity, can identify the affected individuals in a more precise manner.

Our study showed that up to 18% of female population is carrier of one of the three common mutations on G6PD gene. Although all of them are not susceptible for developing hemolysis, they are carriers of the mutations and are at risk of having affected children. Therefore, any test that can detect these cases is more suitable for screening.

TABLE 2 Power of QEA, Decolorization Test, and FST in Identification of G6PD Deficiency Compared to DNA Test as the Gold Standard in Female Newborns, Iran, 2013

Parameters	QEA	Decolorization test	FST
True positive	21/34	1/34	0/34
True negative	150/157	156/157	157/157
False positive	7/157	1/157	0/157
False negative	13/34	33/34	34/34
Sensitivity	62%	3%	0%
Specificity	96%	99%	100%
Positive predictive value	75%	50%	0%
Negative predictive value	92%	83%	82%

140 In our first report on 1000 newborns with the same method (FST) and kit evaluated in  
the current work, 3% of all affected newborns were female (CI95%: 1.94–4.06) [3]. In the  
national program after screening of 115,622 newborns (51.4% male), G6PD enzyme  
deficiency was found in 6.1% of the newborns (CI95%: 5.92–6.28%). Male:female ratio  
145 was calculated as 6.1:1, which means 86.1% of the affected infants were male and 13.9%  
were female. In the other words, one in every 25 boys and one in every 69 girls are at risk  
for the hemolysis [8]. Our study showed that a large proportion of the neonates (mostly  
partial deficient females) were missed during screening program, and the prevalence  
of affected children is higher than previous works.

The present study indicated that semi-quantitative approach of decolorization test  
150 on cord blood samples is more reliable than FST on heel-prick blood sample (sensi-  
tivity of 95% versus 68% in detecting male neonates) to identify G6PD deficient cases.  
However, Decolorization test missed 5% of the total deficient patients (male and fe-  
males), and like FST was unable to identify any of the partial deficient female patients.  
These results show that at least in male cases, decolorization test is more robust than  
155 FST in detecting affected individuals and in neonatal screening program; if sensitive  
QEA test cannot be performed, decolorization test is recommended as a substitute to  
that test.

QEA with cut off of 29.8 U/g Hb could identify around 60% of heterozygote female  
cases. About 40% of cases diagnosed as heterozygote by DNA analysis, were normal by  
160 QEA. Fortunately, this population is not at risk for hemolysis, due to the phenomenon  
of lyonization. On the other hand, six out of 21 total newborns that were diagnosed as  
total deficient by QEA (28% of all newborns), probably had mutations different than  
ones evaluated in the current study.

Cohan *et al.* evaluated the patients who were born after the start of screening in  
165 Fars province and admitted to the hospital with acute hemolysis (Favism). They used  
a quantitative spectrophotometric method with a Sigma kit (USA) for detecting the  
enzyme deficiency. They concluded that almost 80% of newborns hospitalized due to  
G6PD enzyme deficiency, have been falsely reported as negative by FST [10]. This re-  
sult is in harmony with ours, indicating that FST used in screening program misses  
170 large number of affected cases.

A work by Ainoon *et al.* showed that in Malay population of Malaysia, FST, the cho-  
sen approach for screening program, could detect only 7.5% of heterozygote cases,  
while QEA detected 53% (35 of 67) of heterozygote females (31 as partial deficient and  
4 as total deficient) [9]. In our study, no G6PD heterozygote female newborn was de-  
175 tected by FST, while QEA detected around 60% of heterozygote females. Comparing  
to our results, FST in our study could not detect any heterozygote patient, while in  
both studies QEA was able to identify large proportion of female heterozygote sub-  
jects. Hence, QEA is more suitable for screening program of G6PD enzyme deficiency.

Reclos *et al.* investigated the efficiency of screening program in detecting partial  
180 deficient patients using quantitative test as a gold standard in Greece. In that study,  
FST diagnosed 3.2% and quantitative method 5.5% of neonates as deficient. According  
to that study, FST as the means of screening program misses considerable amount of  
female neonates with G6PD partial deficiency. They recommended that FST should  
be replaced by a quantitative test [11].

185 Iranpour *et al.* reported the result of neonatal screening program in Isfahan, a cen-  
tral province of Iran [12]. They used a quantitative method with a commercial kit  
(GAMMA, Belgium) to measure the G6PD enzyme activity in 2501 newborns. The over-  
all frequency of G6PD enzyme deficiency was 3.2% with a male/female ratio of 5.5:1.  
The mean G6PD enzyme activity in neonates was  $35.1 \pm 12$  U/g Hb, and cut off of  
190 6.4 U/g Hb was determined for the detection of G6PD enzyme deficiency, regardless  
of being total or partial deficient. One may consider that cut-off values for quantitative

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assay must be determined in each and every population separately. This could explain the discrepancy of these cut-off values between our work and theirs.

In a similar study in Thailand, Nantakomol *et al.* compared the results of phenotypic tests (FST, methemoglobin reduction, QEA, and cytochemical test) with DNA analysis of 5 common mutations of G6PD gene [13]. In that study, the FST and methemoglobin reduction tests correctly diagnosed all male G6PD deficient and female total deficient individuals - who were homozygote double mutants - and 50% of heterozygote female cases. Although in that study the cytochemical test could detect heterozygote cases with higher specificity, QEA was able to detect 83% of all deficient cases. Parts of this report which addresses the FST results, is not in accordance with our findings. This may be explained by the possibility of variance in setting the tests and therefore different sensitivity of the FST setup.

The result of our study strongly suggests that FST used in screening program should be replaced by a more reliable quantitative test that could detect most of the partial newborns for G6PD deficiency.

### Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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