European Review for Medical and Pharmacological Sciences 2014; 18: 2821-2827

Identification of Mediterranean mutation in Egyptian favism patients

H.G. OSMAN, F.M. ZAHRAN[1], A.M.A. EL-SOKKARY, A. EL-SAID[2], A.M. SABRY

Biochemistry Division, Chemistry Department, Faculty of Science, Mansoura University, Mansoura, Egypt
[1]Biochemistry Division, Chemistry Department, Faculty of Science, Zagazig University, Zagazig, Egypt
[2]Genetic Engineering Department, Pediatrics Hospital, Mansoura University, Mansoura, Egypt

Abstract. – OBJECTIVE: Identify and screen the G6PD Mediterranean mutation in favism patients by applying A Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR).

PATIENTS AND METHODS: A total of 114 unrelated Egyptians patients were included in the present study; their ages ranged between (2-9) years with male to female ratio 4.5:1. G6PD activity was determined qualitatively from red cell hemolysate during attack. The G6PD Mediterranean mutation in patients has been identified by ARMS-PCR.

RESULTS: G6PD deficiency was detected in 87.7%, (n=100). The frequency of G6PD Mediterranean mutation was (94.7%), (n=108). The association between G6PD deficiency and Mediterranean mutation was a highly significant.

CONCLUSIONS: Glucose-6-phosphate dehydrogenase Mediterranean mutation is one of the most common mutations causing G6PD deficiency among Egyptian children with favism.

Key Words:
G6PD-Deficiency, Favism, Mediterranean mutation, ARMS PCR.

Introduction

Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway of carbohydrate metabolism[1]. G6PD deficiency is the most common human enzymopathy, affecting more than 400 million people worldwide. It may result in neonatal jaundice; drug induced hemolytic anemia, favism and chronic-non spherocytic hemolytic anemia. A clinical symptom of G6PD deficiency closely linked to drug induced haemolysis is the hemolytic anemia resulting from the ingestion of the fava bean[1,2]. Patients with favism are always G6PD deficient, but not all G6PD deficient individuals develop haemolysis when they ingest fava beans. It is assumed that some other factors, such as genetic and metabolism of the active ingredients in the beans, which causes oxidative damage in red blood cells, are involved[2]. A recent study has shown increased oxidative stress parameters and less antioxidant defense in favic patients compared to normal individuals[3]. The vast majority of cases of favism occur in individuals with severely deficient (class 2) variants of G6PD (e.g. Mediterranean and Cosenza) as well as class 3 variants (e.g. Chatham)[2].

The gene for G6PD containing 13 exons is located on the X-chromosome (Xq28)[4]. The active enzyme is composed of different proportions of two or four identical subunits of 515 amino acids with a GC-rich promoter typically of many housekeeping genes[5], each monomer has a molecular weight of 59 kDa. Glucose-6-phosphate dehydrogenase (G6PD) is a polymorphic enzyme encoded by the extremely X-linked (Xq28) genes of human and expressed in several tissues in the body[6]. There is a wide variation in the activity of G6PD in the individual red blood cells and, therefore, a number of cases can escape from the correct diagnosis[7]. The most important source of error is the presence of population of young red cells with high reticulocytic number showing an increase in G6PD activity, leading to false results. This condition is common after hemolytic attack, in patients affected by congenital non-spherical cell hemolytic anemia (CNSHA), and in cases of unbalanced X chromosome is disabled in heterozygous females. For all must be completed by the previous reasons, and DNA analysis for a definitive diagnosis[8].

The frequency of G6PD deficiency varies widely among world populations[9]. The prevalence of G6PD deficiency in Egypt has been re-

Corresponding Author: Ahmed M. A. El-Sokkary, Ph.D; e-mail: aelsokkary@mans.edu.eg

ported to be 4%-9.9%, which is relatively higher than some other countries of the Mediterranean[8,10]. G6PD Med mutation is a C-T transition in the position of one nucleotide (nt) 563, which led to Serine/phenylalanine replacement at amino acid position 188. G6PD Med is applied to a group of very similar but probably heterogeneous variants found in the Mediterranean region[8].

G6PD deficiency is known to have over 400 variant alleles, or different forms of the same gene. In Egypt, the only type of allele that exists is called the "Mediterranean" variant, among the population, whereas in other countries such as Japan there is a different variant with a different type of mutation prevalent within that population. This type of mutation is called the "Japan" variant[11]. The Mediterranean variant, found in Southern Europe, the Middle East and in India, is characterized by very low enzyme activity (0-10%) in RBCs using spectrophotometric and potentiometric methods[11].

The aim of this study is to identify the G6PD Mediterranean mutation in favism patients by Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR).

Patients and Methods

Patients

Totally 114 unrelated boys and girls (2-9 years), from pediatric hospitals, Zagazig and Mansoura Universities, were referred following acute anemia triggered by the ingestion of fava beans.

All cases and controls (120 children) were subjected to full history taking and clinical examination with special emphasis on history of fava bean ingestion, infection or drug intake, after taking a written formal consent from the patient's parents. Five ml of venous blood were obtained from each patient by a sterile venipuncture in an ethylenediaminetetraacetic acid (EDTA) vacutainer.

Methods

G6PD Assay

G6PD activity was determined from red cell hemolysate by qualitative method during the attack, and one month later according to method of Ells and Kirkman[12].

Mutation Analysis

DNA Extraction

Genomic DNA was extracted (according to manufacturer's instructions) from the peripheral blood using column method by Gene Jet™ Whole Blood Genomic DNA Purification Mini Kit (Cat No= #K0781, #K0782) (Waltham, MA, USA).

Detection of G6PD Mediterranean Mutation (563 C—T) by (ARMS) PCR[8]

Two forward allele-specific primers, within exon 6 of the G6PD gene, were designed as follows: ARMS M (5' CCG GCT GTC CAA CCA CAT ATT 3') complementary to the mutant allele and ARMS N (5' CCG GCT GTC CAA CCA CAT ATC 3') complementary to the normal one. These primers contained a specific base at their 3' terminus: C for normal DNA (563 C) and T for mutant DNA (563 T). An additional mismatch (C—-T) nucleotide upstream from 3' end was included. The J primer (5' CCA GCC TCC CAG GAG AGA 3')[11], used as the reverse primer. The amplification product was 442-bp DNA fragment.

DNA samples were amplified simultaneously in two parallel reactions with each primer set: (1) ARMS M and J to amplify the mutant allele; (2) ARMS N and J for the normal allele. The amplification mixture (50 µl) contained 25 µl of PCR Master Mix (Maxima TM Hot Start PCR k1051 containing TaqDNA polymerase in reaction buffer (Thermo Fisher Scientific, Wilmington, MA, USA), $MgCl_2$, and dNTPs), 2 µl (20 pmole/µl) of either Primer M or Primer N, 2 µl (20 pmole/µl) of the Primer J, 2 µl (20 pmole/µl) of each forward and reverse internal control primers, 5 µl of Template DNA, and sterile distilled water (12 µl) to a final volume of 50 µl.

The computerized thermocycler was programmed for the following conditions: an initial heat denaturation step at 95°C for 4 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. Each PCR run had a blank without genomic DNA, as a negative control. The amplification products were evaluated by electrophoresis on 2% agarose gel containing ethidium bromide, at 150 V for 20 minutes.

Statistical Analysis

Data were exported into a Microsoft Excel worksheet. Statistical analysis was conducted using SPSS 14 software (SPSS Inc., Chicago, IL,

USA)[13]. A preliminary analysis was done on the data provided, using the classical measures (e.g., mean, standard deviation (SD), frequencies, and percentages). Chi square test used to estimate differences in qualitative variables. A p value of ≤ 0.05 was considered statistically significant, and a p value of ≤ 0.01 was considered highly statistically significant.

Results

The studied group included 114 G6PD-deficient pediatric patients. Their ages ranged between (2-9) years; with a mean of (3.99 ± 1.2). The male to female ratio was 4.5:1 (Table I).

G6PD deficiency was detected in 100 patients (87.7%), while the other patients were clinically normal (12.3%). As for control group they were all clinically normal.

The G6PD Med (563 C/T) mutation was detected in 108 patients (94.7%). Among the 108 Mediterranean positive patients, there were 94 homozygote patients (91 homozygote male and 3 homozygote female) and 14 heterozygote female (Table II).

In control group G6PD Mediterranean mutation was found in 2 heterozygote females (1.7%) and the other 118 children were normal (98.3%) (Figure 1).

There was highly statistically significant distribution for Mediterranean mutation between patient and control groups according to Chi square test (Table III).

Table I. Characterization of control and patient groups.

Characteristic	Control	Patient
Number	120	114
Age (year) mean ± SD	4.29 ± 1.59	3.99 ± 1.20
Range	2-8	2-9
Sex		
Male	87 (72.5%)	93 (81.6%)
Female	33 (27.5%)	21 (18.4%)

Also in the present study it was found that there was a highly significant correlation (Figure 2) between G6PD deficiency and Mediterranean mutation (Table IV).

Patients' profiles are shown in (Table V) and data were expressed by Mean ± SD.

Discussion

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common hereditary disorder in humans and is found in people of Mediterranean, South-east Asian and African descent. The common clinical manifestations of G6PD deficiency are neonatal jaundice and acute hemolytic anemia triggered by certain drugs, infections or ingestion of fava beans. G6PD deficiency is a heterogeneous enzyme abnormality[14].

It was found that in the present study, G6PD deficiency was detected in 100 patients (87.7%) while the other patients were clinically normal

Table II. Frequency of polymorphism distribution between studied groups Cross tabulation.

Polymorphism		Group		Total
		Patient	Control	
Homozygote	Count	94	0	94
	% within polymorphism	100.0%	.0%	100.0%
	% within group	82.5%	.0%	40.2%
	% of Total	40.2%	.0%	40.2%
Heterozygote	Count	14	2	16
	% within polymorphism	87.5%	12.5%	100.0%
	% within group	12.3%	1.7%	6.8%
	% of Total	6.0%	.9%	6.8%
Normal	Count	6	118	124
	% within polymorphism	4.8%	95.2%	100.0%
	% within group	5.3%	98.3%	53.0%
	% of Total	2.6%	50.4%	53.0%
Total	Count	114	120	234
	% within polymorphism	48.7%	51.3%	100.0%
	% within group	100.0%	100.0%	100.0%
	% of Total	48.7%	51.3%	100.0%

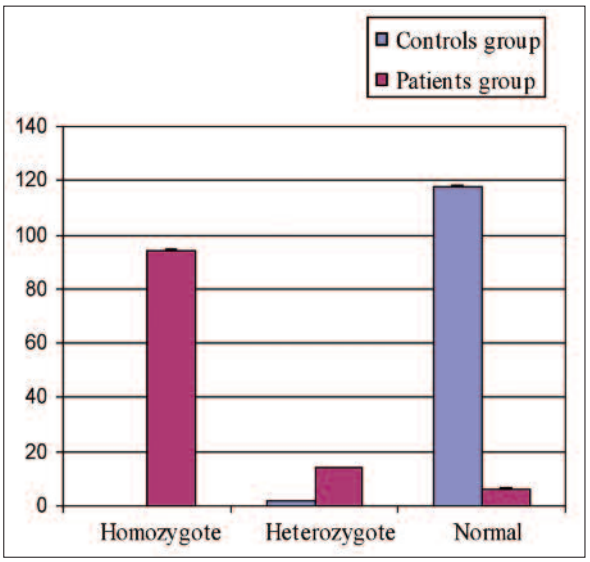

Figure 1. Frequencies of polymorphism distribution between patients and control groups.

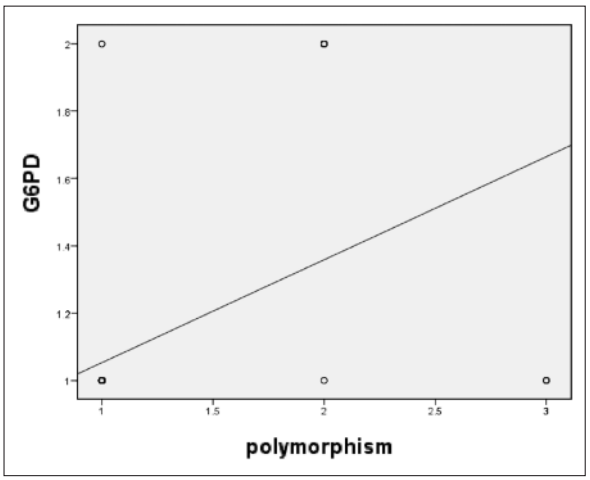

Figure 2. Positive Pearson correlation between G6PD deficiency and Mediterranean mutation.

(12.3%). However, subjects of control group were all clinically normal. This may be due to the fact that; there is a wide individual variability in G6PD activity in RBCs. Therefore, a number of cases can escape the correct diagnosis[8]. The most important source of error is the presence of a young red cell population with a high reticulocytic number (8.02±1.11, $p < 0.001$) (Table V) showing an increases in G6PD activity giving rise to false-normal results[15]. Furthermore, patients with favism are always G6PD deficient, but not all G6PD-deficient individuals develop hemolysis when they ingest fava beans[16].

As biochemical characterization has lost its significance as a means of identifying G6PD variants; therefore, current molecular techniques are required to elucidate a structure function relationship[8]. Moreover, these molecular techniques are needed to confirm the molecular homogeneity of G6PD deficiency[9].

One hundred and fourteen G6PD-deficient unrelated patients were included in this study with a male to female ratio of 4.5:1 (Table I). This could be attributed to the fact that the G6PD deficiency is a sex-linked disease. Thus, a female would present clinically only if she were homozygous for the mutant gene or heterozygous with the preferential expression of the G6PD-deficient gene and X-inactivation of the normal gene or the presence of an enhancer gene would make the expression of the G6PD deficiency more likely[8].

The prevalence of G6PD Mediterranean mutation among the G6PD-deficient children in the present study was (94.7%). In other previous studies, prevalence in Egypt (60%) was reported by Hafez et al[17] However, Rizk et al[18] reported a lower frequency of such a mutation (28.6%) among G6PD-deficient Egyptian children. In consistent with the present study, other authors found a high prevalence of G6PD Mediterranean mutation among glucose-6-phosphate dehydrogenase deficient patients, as found by El-Gezeiry et al (43.6%)[11], while Arnaout et al stated (60%)[8].

Table III. Highly statistically significant distribution for Mediterranean mutation between patient and control groups according to Chi-Square test.

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	204.142[a]	2	< 0.001
Likelihood Ratio	264.135	2	< 0.001
Linear-by-Linear Association	195.968	1	< 0.001
N of Valid Cases	234		

a: 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.79.

Table IV. Significant correlation between G6PD deficiency and Mediterranean mutation.

		G6PD	Polymorphism
G6PD	Pearson Correlation	–	0.494**
	Sig. (2-tailed)		< 0.001
	N	114	114
Polymorphism	Pearson Correlation	0.494**	–
	Sig. (2-tailed)	< 0.001	
	N	114	114

**Correlation is significant at the 0.01 level (2-tailed).

The increased frequency of the G6PD Mediterranean mutation among the Egyptian G6PD-deficient patients in the present study relative to the studies in Egypt could be attributed to the larger scale of this study and the restriction of this investigation to patients presenting with favism, contrary to the other studies including more clinical variants. Therefore, different molecular researches should be performed covering the various regions of Egypt due to the complexity and the different ethnic origins of the Egyptian population. Another important factor contributing to such a difference in the prevalence was the use of a more sensitive and specific technique (ARMS-PCR) in the current study[8].

The frequencies of Mediterranean G6PD mutation among deficient individuals ranged between 71.4% and 84% for Eastern Saudi Arabia[6]. In Iraq according to Helmi et al[19] stated that the occurrence of the mutation was 54.8%. While a higher prevalence of 87.8% in the Kurdish population of Northern Iraq was reported according to Al-Allawi et al[20] In a recent work in Baghdad, G6PD Mediterranean mutation was detected in 74.3% of deficient males[21]. Frequency of G6PD deficiency varied worldwide possibly due to the patient selection criteria, the ethnical and geographical differences[10].

Furthermore, in this report few cases of favism patients (12.3%) have shown normal G6PD enzyme activity, were found to have G6PD Mediterranean mutation, this could be due to the false negative qualitative test that could occur when blood is obtained just after an acute hemolytic action. This could be explained by destruction of severely enzyme deficient RBCs during the hemolytic attack leaving normal RBCs behind[10]. These patients were found to be heterozygote females (Table II), where in heterozygote females, red cell mosaicism arising from random X-chromosome inactivation results in two populations of G6PD-deficient and G6PD-normal cells. The proportions of these two cell types can vary greatly, ranging from completely normal activity to complete deficiency[22].

On the other hand, six cases have shown to have enzymatic deficiency, but found to have no G6PD Mediterranean mutation. Hence, the absence of this mutation among the six cases of G6PD deficiency diagnosed qualitatively may necessitate molecular workup for detection of other gene mutations[10].

Table V. Clinical manifestations of patients.

Parameter	Control subjects mean ± SD	Favism patients mean ± SD	*p* value
Hb (g/dl)	12.85 ± 1.72	3.88 ± 1.25	< 0.001
N	120	114	
Conjugated bilirubin (g/dl)	0.28 ± 0.01	0.13 ± 0.039	0.05
N	120	114	
Reticulocytes count (%)	0.90 ± 0.60	8.02 ± 1.11	< 0.001
N	120	114	

It was found that 2 female cases of control group were found to have the G6PD Mediterranean mutation (heterozygote), this may be due to X-inactivation of the mutant gene, or presence of an enhancer gene would make the expression of the G6PD deficiency less likely[23].

There was statistically significant distribution for Mediterranean mutation between patient and control groups according to Chi square test (Table III). Also, it was found that there were a significant correlation between G6PD deficiency and Mediterranean mutation (Figure 2, Table IV). Thus, it could be concluded that Mediterranean mutation is frequently distributed between Egyptians Favism patients. Sukumar et al[24] reported that G6PD Mediterranean mutation was associated with more severe clinical manifestation. This was identical with our findings, where in our study the favism patients have shown to have decreased conjugated bilirubin (0.13 ± 0.039, $p < 0.05$) and decreased hemoglobin levels, (3.88 ± 1.25, $p < 0.001$) compared to controls (0.28 ± 0.01) and (12.85 ± 1.72), respectively (Table V). These clinical manifestations of favism present distinctively with sudden onset of severe hemolytic anemia within 24 to 48 h ingestion of the fava beans[25].

Conclusions

The G6PD Mediterranean mutation is one of the most common mutations causing G6PD deficiency in Egyptian favim disease. Further molecular characterization of the genetic variants of G6PD is required to fully cover its molecular pattern in Egyptian patients. Biochemical characterization has lost its significance as a means of identifying variants. Fully characterized biochemical variants, therefore, need to be reanalyzed using molecular techniques to elucidate a structure–function relationship. Identification of mutations that cause pathology is of interest not only in the understanding of genetic disease but also for the information that can be provided concerning the corresponding normal function of the respective gene product.

Acknowledgements

The authors would like to thank all the medical staff of Pediatric Hospitals in both Mansoura and Zagazig Universities for providing help and assistance during the collection of samples.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Matsubara S, Takayama T, Iwasaki R, Komatsu N, Takizawa T. Enzyme-cytochemically detectable glucose-6-phosphate dehydrogenase in human villous macrophages (Hofbauer cells). Placenta 2001; 22: 882-885.
2) Noori-Daloii MR, Hajebrahimi Z, Najafi L, Mesbah-Namin SA, Mowjoodi A, Ganji SM, Yekaninejad MS, Sanati MH. A comprehensive study on the major mutations in glucose-6-phosphate dehydrogenase-deficient polymorphic variants identified in the coastal provinces of Caspian Sea in the north ofIran. Clin Biochem 2007; 40: 699-704.
3) Osman HG, Zahran FM, El-Sokkary AMA, Sabry AM. Oxidative stress and antioxidant defense in Egyptian favism patients. Eur Rev Med Pharmacol Sci 2013; 17: 1211-1217.
4) Nicol CJ, Zielenski J, Tsui L, Wells PG. An embryo protective role for glucose-6-phosphate dehydrogenase in developmental oxidative stress and chemical teratogenesis. FASEB J 2000; 14: 1-25.
5) Noori-Daloii MR, Soltanian S, Gangi SHM, Yousefi A, Hejazi S, Bani-Hashem A, Hitratfar S, Sanati M. Molecular identification of the most prevalent mutations of glucose-6-posphate dehydrogenase (G6PD) gene in deficient patients in Khorasan province of Iran. J Sci Islamic Republic Iran 2006; 17: 103-106.
6) Al-Ali AK, Al-Mustafa ZH, Al-Madan M, Qaw F, Al-Ateeq S. Molecular characterization of glucose-6-phosphate dehydrogenase deficiency in the Eastern Province of Saudi Arabia. Clin Chem Lab Med 2002; 40: 814-816.
7) World Medical Association. Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects, the 59th WMA General Assembly 2008; Seoul, South Korea.
8) Arnaout HH, El-Gharbawy NM, Shaheen IA, Afifi RA, Abd El-Dayem OY. Incidence and association of 563 C/T mediterranean and the silent 1311C/T G6PD mutations in G6PD-deficient Egyptian children. Lab Med 2011; 42: 355-360.
9) Gari MA, Chaudhary AG, Al-Qahtani MH, Abuzenadah AM, Waseem A, Banni H, Al-Sayes FM, Al-Harbi A, Lary S. Frequency of Mediterranean mutation among a group of Saudi G6PD patients in Western region-Jeddah. Int J Lab Hematol 2010; 32(1 Pt 2): 17-21.
10) Settin A, Al-Haggar M, Al-Baz R, Yousof H, Osman NA. Screening for G6PD Mediterranean mutation among Egyptian neonates with high or prolonged jaundice. Haema 2006; 9: 83-90.
11) El-Gezeiry D, El-Gendy W, El-Kaffash D, Hassab H, Moez BW. Comparative study for the detection of

563 C----T G6PD mutationusing restriction enzyme assay and amplification and refractory mutation system (ARMS). Alexandria J Pediatr 2005; 19: 51-55.

12) Ells HA, Kirkman HN. A colorimetric method for assay of erythrocytic glucose-6-phosphate dehydrogenase. Proc Soc Exper Biol Med 1961; 106: 607-609.

13) Lvesque R. SPSS programming and Data Management: a guid for SPSS and SAS users, Fourth Edition, SPSS. inc., Chicago IL, 2007.

14) Luzzatto L, Mehta A, Vulliamy TJ. Glucose-6-phosphate dehydrogenase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic and Molecular Basis of Inherited Diseases, 8th ed. New York. McGraw-Hill, 2000; pp. 4517-4553.

15) Maffi D, Pasquino MT, Caprari P, Caforio MP, Cianciulli P, Sorrentino F, Cappabianca MP, Salvati AM. Identification of G6PD Mediterranean mutation by amplification refractory mutation system. Clin Chim Acta 2002; 321: 43-47.

16) Noori-Daloii MR, Daneshpajooh M. Molecular basis of G6PD deficiency current status and its perspective. Acta Med Iranica 2008; 46: 167-182.

17) Hafez MM, El Tombary U, Hawas S. Study of the 563 and 1311 mutations of the G6PD gene among Egyptian children with favism. Hum Antibodies 2001; 10: 33-34.

18) Rizk SH, Aziz M, Abdel-Ghany H. Frequency of 563 C-T Mediterranean mutation among a group of Egyptian pediatric patients. Lab Hematol 2000; 6: 127-131.

19) Hilmi FA, Al-Allawi NA, Rassam M, Al-Shamma G, Al-Hashimi A. Red cell glucose-6-phosphate dehydrogenase phenotypes in Iraq. East Mediterr Health J 2002; 8: 42-48.

20) Al-Allawi N, Eissa AA, Jubrael JM, Jamal SA, Hamamy H. Prevalence and molecular characterization of glucose-6-phosphate dehydrogenase deficient variants among the Kurdish population of Northern Iraq. BMC Blood Disord 2010; 10: 6.

21) Al-Musawi BMS, Al-Allawi N, Abdul-Majeed BA, Eissa AA, Jubrael JMS, Hamamy H. Molecular characterization of glucose-6-phosphate dehydrogenase deficient variants in Baghdad city - Iraq. BMC Blood Disord 2012; 12: 4.

22) Ainoon O, Alawiyah A, Yu YH, Cheong SK, Hamidah NH, Boo NY, Zaleha M. Semiquantitative screening test for G6PD deficiency detects severe deficiency but misses a substantial proportion of partially-deficient females. Southeast Asian J Trop Med Public Health 2003; 34: 405-414.

23) Abdulrazzaq YM, Micallef R, Qureshi M, Dawodu A, Ahmed I, Khidr A, Bastaki SM, Al-Khayat A, Bayoumi RA. Diversity in expression of glucose-6 phosphate dehydrogenase deficiency in females. Clin Genet 1999; 55: 13-19.

24) Sukumar S, Mukherjee MB, Colah RB, Mohanty D. Molecular basis of G6PD deficiency in India. Blood Cells Mol Dis 2004; 33: 141-145.

25) Meloni T, Forteleoni G, Dore A, Cutillo S. Favism and hemolytic anemia in glucose-6-phosphate dehydrogenase deficiency subjects in North Sardinia. Acta Haematol 1983; 70: 83-90.