

Molecular Basis of G6PD Deficiency in Babylon : Iraq

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Abstract

Objective:

The objective of this study was to investigate the molecular basis of glucose-6-phosphate dehydrogenase (G6PD) genes in hyperbilirubinemic neonates in Babylon province of Iraq by using molecular methods (genomic DNA extraction, PCR and RFLP analysis) and then to investigate the type of G6PD variant predominantly present.

Methods:

The study included a total of 236 full-term male neonates, 183 of them were associated with severe hyperbilirubinemia which were admitted in Teaching Hospital of Pediatric and Maternity / Babylon during 1st, Oct., 2007 to 14th, July, 2008 with age ranged between 1 – 28 days, their TSB levels ≥ 15 mg/dl, while another 53 neonates were used as control group. The blood sample taken from each neonate was divided into two aliquots: the first aliquot was used for hemoglobin (Hb), total and conjugated bilirubin (TSB and SCB), G6PD activity. The second aliquot was used for molecular analysis including genomic DNA extraction and then application of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) protocols.

Results and Discussion:

Severe hyperbilirubinemic neonates were screened for erythrocyte G6PD enzyme activity measurements, severe G6PD deficiency was detected in 22 of the total 183 hyperbilirubinemic neonates included and their activity levels was significantly decreased ($P < 0.05$) to 0.34 ± 0.17 U/g Hb as compared with control value 10.02 ± 1.17 U/g Hb. The incidence of severe G6PD deficiency in neonatal hyperbilirubinemic neonates identified was 12.02 %. TSB levels were markedly elevated to (23.01 ± 5.0) mg/dl, whereas the control value was 0.75 ± 0.23 mg/dl. The mean \pm SD values of each of SCB and Hb were significantly lower than that found in controls ($P < 0.05$) and reached to 0.063 ± 0.036 mg/dl ; 13.32 ± 0.94 g/dl as compared with that found in control neonates 0.19 ± 0.11 mg/dl ; 15.91 ± 1.62 g/dl respectively. Conjugated bilirubin was undetectable in sera of 10 of 22 neonates (45.5%) with severe G6PD deficiency which imply a partial defect of bilirubin conjugation.

The molecular part of the study involved the extraction of genomic DNA from hyperbilirubinemic neonates with severe G6PD deficiency which detected by agarose electrophoresis and then amplified by PCR and finally was subjected to digestion by endonuclease restriction enzymes to create RFLP to enable the detection of mutation that caused G6PD deficiency. The overall majority of affected severe G6PD-deficient neonates with hyperbilirubinemia in Babylon province : Iraq were due to G6PD Med variant (C563T, Ser 188 Phe) in which 19 out of 22 (86.4%) have this type of gene mutation, and only one G6PD A- variant (4.55%) (G202A ; A376G mutations) was diagnosed, whereas 2 of 22 (9.1%) remain with unknown G6PD variants.

Conclusion:

The predominant *G6PD gene* detected in hyperbilirubinemic neonate with severe G6PD deficiency in Babylon province was G6PD Med.

Keywords : Hyperbilirubinemia, G6PD gene, Polymerase Chain Reaction , RFLP.

Molecular analytical methods	(G6PD deficiency)		Variants)
	n	%	
Molecular analytical methods	400	86.4%	Med
	42	9.1%	
Molecular analytical methods	2	4.55%	Unknown
	22	100%	
Molecular analytical methods	(G6PD Variants)		Variants)
	n	%	
Molecular analytical methods	236	86.4%	Med
	28 - 1	11.8%	
Molecular analytical methods	53	22.46%	Unknown
	183	77.54%	
Molecular analytical methods	(TSB Variants)		Variants)
	n	%	
Molecular analytical methods	22	100%	Med
	22	100%	
Molecular analytical methods	0.34 ± 0.17 IU/g Hb	100%	Med
	23.01 ± 5.0 mg/dl	100%	

	SCB		(12.02%)
	UGT1A1		
TSB		G6PD	(P<0.05)
			.15 mg/dl
	Molecular Analysis		G6PD
	Genomic DNA		
Roche		G6PD	
		Polymerase Chain Reaction	
G6PD			(RFLP)
			. Med and G6PD A-
G6PD			
G6PD A- (G202A ;			(86.4) % Med(C563T)
	(9.1 %)	(4.55%)	A376G)

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that catalyses the rate-limiting step in the pentose phosphate pathway, providing reducing power to all cells in the form of NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) ⁽¹⁾. NADPH enables cells to counterbalance oxidative stress that can be triggered by several oxidant agents, and to preserve the reduced form of glutathione and other sulfhydryl groups which was essential for the reduction of hydrogen peroxide and reactive oxygen species and the maintenance of hemoglobin and other red blood cell proteins in the reduced state (Figure-1-). By preserving and regenerating reduced forms of glutathione as well as promoting the stability of catalase, NADPH plays a major role in the

stability of cell to withstand oxidative stress, since red blood cells do not contain mitochondria, the pentose phosphate pathway is their only source of NADPH; therefore, defense against oxidative damage is dependent on G6PD activity ⁽²⁾.

The normal and most common enzyme variant is designated as G6PD B+ . G6PD deficiency results from the inheritance of any one of a large number of the abnormalities of the structural gene that codes the amino acid sequence of the enzyme G6PD. G6PD deficiency was discovered in 1950s, and shown to be the cause of hemolytic effect of primaquine ⁽³⁾, hereditary genetic defect caused by mutations in the *G6PD* gene, resulting in protein variants with different levels of enzyme activity, that are associated with a wide range of biochemical and clinical phenotypes. G6PD deficiency is actually

the most common clinically important enzyme defect, not only in hematology, but also among all human diseases known⁽⁴⁾. The most common clinical manifestations are neonatal jaundice and acute hemolytic anemia, which in most patients is triggered by an exogenous agent⁽⁵⁾. The striking similarity between the areas where G6PD deficiency is common and *Plasmodium falciparum* malaria is endemic provides circumstantial evidence that G6PD deficiency confers resistance against malaria⁽⁶⁾. The highest frequencies are detected in Africa, Asia,

the Mediterranean region, and in the middle east; owing to recent migrations, however, the disorder is also found in North and South America and in northern European countries. Although the majority of people with this disease are asymptomatic, some of the clinical symptoms associated with deficiency are acute hemolytic anemia in association with infection or following the ingestion of some drugs or fava beans (favism), neonatal jaundice and in severe deficiency, chronic non-spherocytic hemolytic anemia (CNSHA)^(7,8).

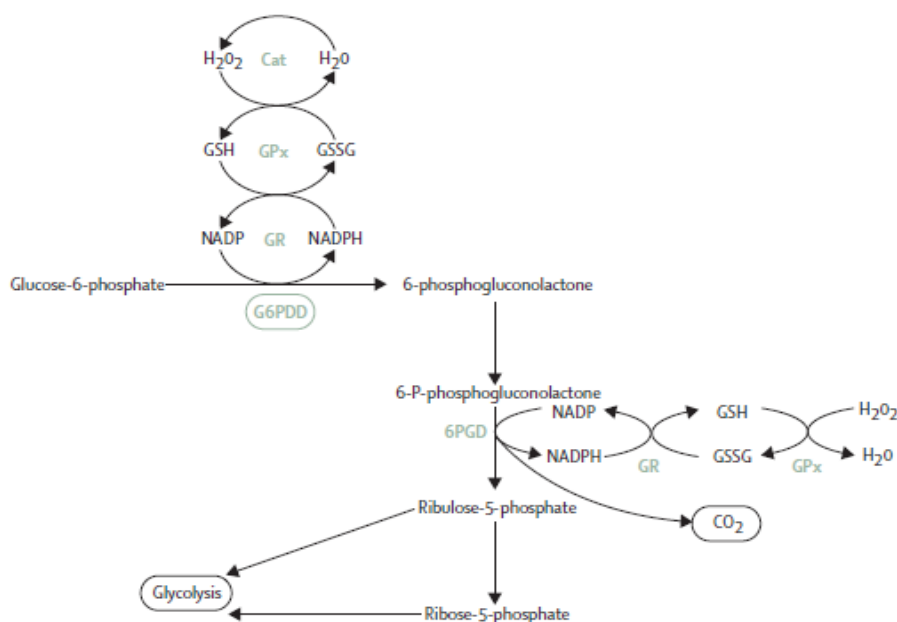


Figure-1- Pentose Phosphate Pathway⁽²⁾.

Any gene located on the X-chromosome is called an X-linked gene⁽⁹⁾. The *G6PD* gene was cloned in 1986 and is located at the telomeric region of the long arm of the X chromosome (band Xq28), close to the genes for hemophilia A, congenital dyskeratosis, and colour blindness (Fig.-2-)⁽¹⁰⁾. It consists of 13

exons and 12 introns and is 18.5 kb in length. ; it encodes 515 amino acids, and a GC-rich (more than 70%) promoter region. The active enzyme is composed variably of two or four identical 515 amino acid subunits; each monomer has a molecular weight of 59 kDa^(11,12).

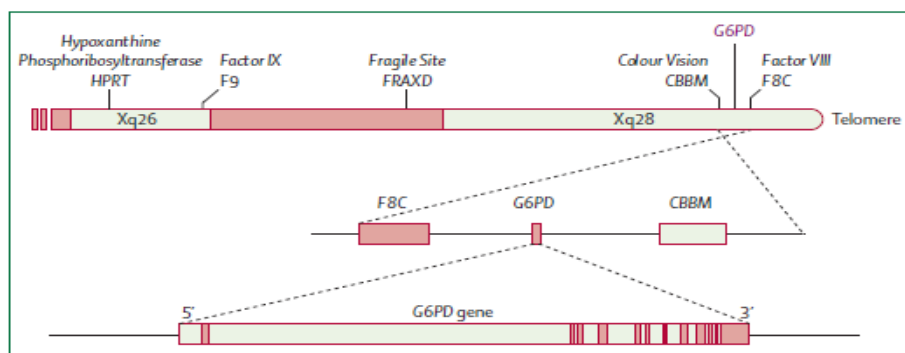


Figure-2- Location of G6PD gene on X chromosome

Deficient *G6PD* alleles are distributed worldwide; a conservative estimate is that at least 400 million people carry a mutation in the *G6PD* gene causing deficiency. The highest prevalence is reported in Africa, Southern Europe, the Middle East, Southeast Asia, and the central and Southern Pacific Islands; however, because of fairly recent migration, deficient alleles are nowadays quite prevalent in North and South America and in parts of Northern Europe⁽¹³⁾. For any given population, definition of the quantitative contribution of each allele to the overall prevalence of *G6PD* deficiency is still difficult, since epidemiological studies based on enzyme activity screening have been imprecise and have not extended to global coverage. In recent years, molecular analysis has been used to map the prevalence of *G6PD* deficiency. In most areas of high prevalence of *G6PD* deficiency, several polymorphic alleles are found and more than 442 variants of *G6PD* enzyme were investigated⁽¹⁴⁾. Tropical regions of Africa are one exception, where the African variant, *G6PD A-* accounts for about 90% of *G6PD* deficiency. *G6PD A-* is also frequent in North and South America, in the West Indies, and in areas where people of African origin are present. Moreover, *G6PD A-* is quite prevalent in Italy, the Canary Islands, Spain, and Portugal, and in the Middle East, including Iran, Egypt, and Lebanon⁽¹⁰⁾.

The second most common variant is *G6PD* Mediterranean, *G6PD Med*, which is present in all countries surrounding the Mediterranean Sea, although it is also widespread in the Middle East, including Israel, where it accounts for almost all *G6PD* deficiency in Kurdish Jews, India, and Indonesia. In several populations, such as the countries around the Arabic Gulf, *G6PD A-* and *G6PD* Mediterranean coexist at polymorphic frequencies⁽¹⁵⁻¹⁷⁾.

The aim of the present study was to determine the mutations of *G6PD* gene in severe *G6PD* deficient neonates with hyperbilirubinemia in Babylon province of Iraq by using molecular techniques. In the first step, we screened *G6PD Med* variant because it is one of the most common *G6PD* variants. Then other mutation of *G6PD*, *G6PD A-* were also examined in this area.

Materials and Methods

The study included a total of 236 full-term male neonates, 183 of them were associated with severe hyperbilirubinemia which admitted in Teaching Hospital of Pediatric and Maternity / Babylon during 1st, Oct., 2007 to 14th, July, 2008 and another 53 neonates were used as control group with the same age ranged between 1 – 28 days. The blood sample taken from each neonate was divided into two aliquots: the first aliquot was used for Hb, total and conjugated serum bilirubin (TSB and SCB)⁽³²⁾ and *G6PD* activity

measurements. The second aliquot was used for molecular analysis including genomic DNA extraction and then application of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) protocols. All samples were screened to fluorescent spot test for G6PD detection, and then G6PD activity was measured in those neonates associated with severe G6PD deficiency. Activity of G6PD was measured quantitatively by using Sigma kit (345-B) at 37 °C by the kinetic method modified by Lohr and Waller⁽¹⁸⁾.

Genomic DNA was extracted and purified by salting out in several steps from 200 µl of EDTA blood sample

obtained from hyperbilirubinemic neonates with G6PD deficiency and control groups by using Roche High Pure PCR Template Preparation Kit, Germany and as described by Vogelstein and Gillespie⁽¹⁹⁾. After genomic DNA extraction, it has been subjected to electrophoresis through agarose gels which was detected by staining with the fluorescent dye ethidium bromide which is the most convenient and commonly used method to visualize DNA in agarose gels because it contains a tricyclic planar group that intercalates between the stacked bases of DNA and then visualized by illumination with UV light to confirm the presence and integrity of the extracted DNA^(20,21).

Table-1- Location of point mutations of G6PD variants under this study.

G6PD Variant	Exon No.	Base position	Base Change	Codon Change	Amino acid position	Amino acid substitution
Mediterranean	6	563	C → T	TCC → TTC	188	Ser → Phe
African, A-	4 5	202 376	G → A A → G	GTG → ATG AAT → GAT	68 126	Val → Met Asn → Asp

According to the common distribution of G6PD gene mutation in Middle East Countries, two mutations were chosen for the prevalence of molecular diagnosis and amplification with PCR in neonatal hyperbilirubinemia in Babylon Province which are Mediterranean (G6PD Med, 563 C → T) and African (G6PDA-, 202 G → A, 376 A → G)^(16,48). Three primer sets were chosen to amplify exons 6, 4, and 5 respectively. Table -1- shows some of the molecular characteristics of the three types of G6PD variants^(49,50).

The DNA region from the G6PD gene encompassing each point mutation was selectively amplified by PCR using specific oligonucleotide primers, followed by digestion with restriction enzyme. Digestion products were analyzed on 2% agarose gel⁽²⁰⁾. The sets of primers chosen

for PCR amplification of G6PD mutations were designed by CinnaGen Co.-Tehran / Iran as a lyophilized product of different picomolar (pM) concentrations which was adopted for primers resuspension to a final concentration of 25 pM/µl by deionized water to reach a final concentration of 25 pM/µl of suspension. The lyophilized primers were dissolved in 1 ml of distilled water or in 5 mM Tris-buffer, pH 7.5 as a stock solution and stored at -20 °C, then small aliquots were prepared to a final concentration of 25 pM/µl to avoid freezing and thawing and stored at -20 °C.

The most convenient option of digestion or cleavage of PCR products directly after DNA amplification reaction is the addition of a restriction enzyme directly to the reaction tube after completion of PCR

(22,23,51). These endonucleases which purchased from Fermentas Co. included:

- ***Mbo* II** stored with 10X buffer B which contain (10 mM Tris-HCl (pH 7.5 at 37 °C), 10 mM MgCl₂, and 0.1 mg/ml bovine serum albumin, BSA).

- ***Nla* III** supplied in a total 300 units in 1 ml vial with 10 X buffer G which contain (10 mM Tris-HCl pH 7.5 at 37°C), 10 mM MgCl₂, 50 mM NaCl, 0.1 mg/ml BSA).

- ***Fok* I** supplied as a total activity which is 500 units in 1 ml vial with 10X buffer Tango which contain (33 mM Tris-acetate, pH 7.9 at 37 °C , 10 mM magnesium acetate , 66 mM potassium acetate, and 0.1 mg/ml BSA). All these enzymes were stable for short period at – 20 °C and at – 70 °C for long period (more than 30 days).

All DNA samples were screened for the C→T mutation at nucleotide 563, which is

characteristic of G6PD-Med, using F: 5'...CCCCGAAGAGGAATTCAAGGGG GT...3', R: 5'...GAAGAGTAGCCC-TCGAGGGTGACT...3' primers and PCR amplification followed by digestion by *Mbo* II restriction endonuclease. To check the activity of PCR component, two external control samples were used : water and positive control which produce negative and positive results respectively.

The PCR reaction conditions used for exon 6 amplification of G6PD-Med variant with two primer sets include denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 60 sec , annealing at 58 °C for 60 sec, extension at 72 °C for 60 sec, and final extension at 72 °C for 5 min. Amplification by using 5 unit of *Taq* DNA polymerase / µl in a final PCR volume used 25 µl as shown below in table-2-.

Table -2 – PCR components in a final volume of 25 µl used

Components	Final Concentration	Volume (µl)
Deionized water	-----	18.0
dNTP mixture, pH 7.0	10 mM of each	0.5
PCR buffer*	10X TBE buffer	2.5
MgCl ₂	50 mM	0.75
Primer F	10 pM/µl	0.5
Primer R	10 pM/µl	0.5
DNA extracted samples	0.5 – 1.0 µg/µl	2.0
<i>Taq</i> DNA polymerase	5 units/µl	0.25

*The PCR buffer contain 50 mM KCL, 10 mM Tris-HCl , pH 8.4 at room temperature.

The G6PD-Med mutation at the base position 563 creates one *Mbo* II site in exon 6 and 7 of the G6PD gene^(26, 27,28). The recommended digesting DNA with a 2-fold to 10-fold excess of enzyme in the total volume of 20 µl using 0.2-1.5 µg of DNA. The amplification product (10 µl) was digested with 5 - 10 U/1.0 µl of a restriction endonuclease enzyme *Mbo* II for 4 h at 37°C and the digestion products were analyzed on 2% agarose gel^(22-25,50).

The same samples which did not show G6PD-Med mutation were looked for G→A and A→G mutation at nucleotide 202 and 376 respectively which is characteristics of G6PD A- by using another two sets of specific primers and PCR reaction conditions, then the PCR amplification products were digested with another restriction endonucleases. The PCR amplification conditions for exon 4 of G6PD A- was performed by using another two primer sets 4F: 5'-

GTCTTCTGGGTCAGGGAT-3' and the reverse primer 4R: 5'-GGAGAAAGCTCTCTCTCC-3'.

Denaturation at 94 °C for 2 min was followed by 45 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 60 sec, and final extension at 72 °C for 4 min⁽²⁶⁾.

Amplification on exon 5 of G6PD A- was carried out by using another two primer sets 5F: 5'-CCTGTTCCCTCTGCCACA-3', and 5R: 5'-GGGGGTCTCAAGAAGTAC-3'.

Denaturation at 94 °C for 2 min was followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 60 sec, extension at 72 °C for 30 sec, and a final extension at 72 °C for 4 min.

The reaction conditions used for *Nla* III and *Fok* I restriction endonucleases were composed of the following : (1-2 µl of PCR product ; 1 µl of restriction enzyme buffer ; 0.2 µl of restriction enzyme and 6.8-7.8 µl D.W.), this components were mixed by pipetting, then the tube was closed and centrifuged for few seconds, then incubated at 37 °C overnight. Then proteinase was added and incubated at the same temperature for further 2 hr. This sample (10 µl) were applied to agarose gel electrophoresis 2%^(20,21).

After the digestion of both G6PD Med and A- variants with endonucleases were completed, their products with PCR amplification products (3 µl of PCR product plus 1 µl loading buffer) and the ladder markers were loaded and analyzed by agarose gel electrophoresis (2% agarose in 0.5X tris-borate ethylenediamine tetra

acetic acid (TBE) working buffer, and 0.5 µg/ml ethidium bromide) at 80 V and 95-100 mA for 40 min , then the bands were visualized on UV transluminator and then photographed by using photo documentation system.

Results and Discussions

G6PD deficiency occurs with increased frequency throughout Africa, Asia, the Mediterranean , and the Middle East. Consistent with previous studies from Iraq conducted by Amin-Zaki, et. al.⁽²⁹⁾ ; Hamamy and Saeed,⁽³⁰⁾ and Al-Naamah, et. al.⁽³¹⁾. The present study indicated that G6PD deficiency is common in Iraqi neonatal hyperbilirubinemia of Babylon province. Therefore, it is of interest to establish whether the incidence of G6PD deficiency is due to a single or multiple G6PD mutation variants.

Of 183 G6PD-deficient hyperbilirubinemic neonates was diagnosed, only 22 (12.02%) neonates of them were associated with severe G6PD-deficiency and their G6PD activity levels was markedly decreased to 0.34 ± 0.17 U/g Hb and reached to 0.92% of the G6PD activity levels found in control neonates 10.02 ± 1.17 U/g Hb. Bilirubin profile indicated that TSB levels were increased markedly and reached to 23.01 ± 5.0 mg/dl as compared with the control value 0.75 ± 0.23 mg/dl, while serum conjugated bilirubin (SCB) level was decreased and reached to 0.063 ± 0.036 mg/dl as compared with that found in control group 0.19 ± 0.11 mg/dl as shown in Table-3-.

Table-3- G6PD activity levels and bilirubin profile in hyperbilirubinemic neonates with severe G6PD deficiency in Babylon province : Iraq.

Parameters	Control	Severe G6PD-Deficient hyperbilirubinemic neonates
	n= 53	n=22
G6PD activity, U/g Hb	10.02 ± 1.17	0.34 ± 0.17
TSB , mg/dl	0.75 ± 0.23	23.01 ± 5.0
SCB , mg/dl	0.19 ± 0.11	0.063 ± 0.036

These results confirm with other studies performed in Isreal, Italy, and Taiwan which suggest that the G6PD-deficient neonates are at increased risk for hyperbilirubinemia⁽⁵²⁾. Therefore, data presented in this study may probably suggest that severe neonatal hyperbilirubinemia may continuously cause of a problem in this region of Iraq, which show that those neonates with severe G6PD-deficiency who developed higher maximal TSB values had significantly lower SCB fractions than those who remained only moderately jaundiced. Serum bilirubin profile demonstrated in the subsequently hyperbilirubinemic with severe G6PD-deficient neonates (high TSB, with low SCB) is a reminiscent of that seen in conditions of partial deficiency of the bilirubin conjugating enzyme UDP-glucuronosyl transferase1 A1 (UGT1A1), such as Gilbert's Syndrome⁽⁵³⁾. The SCB was absent in 10 of 22 (45.5%) of G6PD-deficient hyperbilirubinemic neonates.

These data support functionally the concept of the gene interaction demonstrated between G6PD deficiency and the variant promoter for the gene encoding the bilirubin conjugated enzyme UGT1A1 and then diminished bilirubin conjugation ability. Gene variants is reported to be in association with an increased risk for neonatal hyperbilirubinemia include those of :

- (1) The red blood cell enzyme (*G6PD*)⁽⁵⁴⁾ ;
- (2) The hepatic bilirubin-conjugating enzyme UGT1A1⁽⁵⁵⁾ ;
- (3) The hepatic organic anion transporter polypeptide1 B1 (*OATP1B1*)⁽⁵⁶⁾.

G6PD gene variants may predispose to neonatal hyperbilirubinemia via either an acute hemolytic event with or without an identifiable environmental trigger or a low-grade hemolysis coupled with UGT1A1 gene polymorphisms⁽⁵⁷⁾. More recent findings suggested that gene polymorphisms of OATP1B1 a putative bilirubin transporter localized to the sinusoidal membrane of hepatocytes (i.e, the blood hepatocyte interface), may be a predispose to neonatal hyperbilirubinemia by possibly limiting hepatic bilirubin uptake⁽⁵⁸⁾. The primary site of the pathogenesis of the hyperbilirubinemia therefore appears to be localized to a deficiency in bilirubin conjugation. As a result, G6PD-deficient neonates who become hyperbilirubinemic have bilirubin conjugation ability which is even more inefficient than that of the physiological immaturity of conjugation normally found in neonates. Those with an excessively immature bilirubin eliminating capacity are more likely to develop hyperbilirubinemia than those with a more mature ability. This mechanism may exist to a certain extent in all neonates but may be exacerbated in the G6PD deficiency

state because of increased hemolysis and the resultant additional bilirubin load⁽⁵⁹⁾. Bhutani, et. al., in (1999) have demonstrate that measuring the TSB level and further testing (blood group, coombs and G6PD tests) at the time / or before infants are discharged from hospital is helpful in predicting which infants will experience severe hyperbilirubinemia and to evaluate the risk and to prevent it⁽⁶⁰⁾.

Results in this study also show that deficient bilirubin conjugation which was reflected by low SCB values measured, is a cardinal factor in the pathogenesis of G6PD deficiency associated with neonatal hyperbilirubinemia.

It is unknown at present time whether the previous observations related to hemolysis and bilirubin production⁽⁵⁹⁾, or the deficient serum conjugated bilirubin fractions described above are unique to Sephardic Jews with G6PD Med or whether they have global implications for the hundreds of millions of people worldwide estimated to have G6PD deficiency⁽⁵⁾. Additional study of the pathophysiology of this process may lead to improved therapeutic or prophylactic interventions in the clinical management of G6PD deficiency associated neonatal hyperbilirubinemia.

These results indicated that there is a significant negative correlation ($r = -0.203$, $P < 0.05$) between G6PD activity levels and TSB concentrations elevated in severe G6PD-deficient hyperbilirubinemic neonates with the $TSB \geq 15$ mg/dl but not in control individuals as shown in (Figure-3-).

The mechanism of the relationship between G6PD activity and neonatal hyperbilirubinemia is not clear. The presence of another genetic factors has been postulated in the pathogenesis of neonatal hyperbilirubinemia in G6PD deficiency. Kaplan, et. al., (1997) reported that UGT1A1 gene mutation, diminishing the activity of the conjugated enzyme UGT1A1, was associated with neonatal hyperbilirubinemia in G6PD deficiency⁽⁴²⁾. Weng, et. al., (2002) reported that the expression of heme oxygenase-1, a rate-limiting enzyme in the production of bilirubin and inducible under the exposure to oxidative stress, was increased in G6PD deficiency. Recent studies suggest that bilirubin was a strong endogenous antioxidant⁽⁶¹⁾. Therefore, it is reasonable to suggest that the neonatal hyper-bilirubinemia caused by increased heme oxygenase-1 in G6PD deficiency is the consequence of genetic interaction to compensate the decreased antioxidant activity. Therefore, the low levels of G6PD activity in male infants may play a role in the interaction of different genes, such as UGT1A1 and heme oxygenase-1, and subsequently aggregative the high TSB levels.

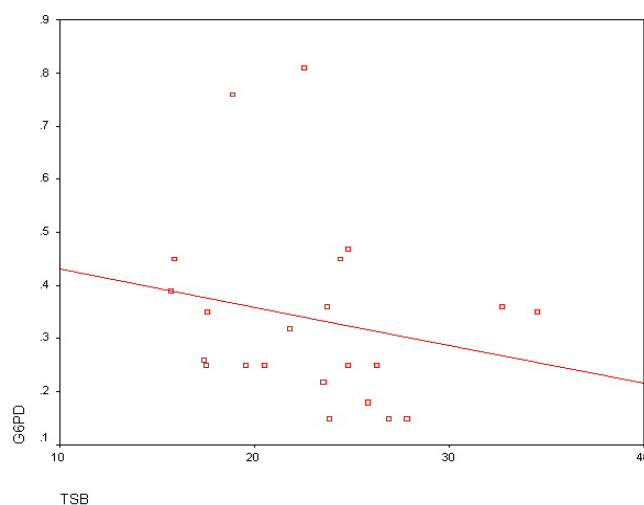


Fig. -3 – The correlation between G6PD activity level and TSB conc. in severe G6PD-deficient male hyperbilirubinemic neonates ($P < 0.05$; $r = - 0.203$)

Two G6PD variants were identified by using a molecular diagnostic methods. Nineteen neonates (86.4%) was diagnosed to be G6PD Med mutation in exon 6 (563C→T) (Ser188Phe) ; and only one neonates (4.55%) had G6PD African deficient variant (G6PD A-) in exons 4 and 5 (202 G →A) (Val68Met) and exon 5 (376 A→G)(Asn126Asp) ; no neonates has G6PD A+ mutation (202 G → A), while two G6PD-deficient neonates (9.1%) remain within unknown G6PD gene mutation variants.

In most areas of high prevalence of G6PD deficiency, several polymorphic alleles are found⁽³³⁾. Tropical regions of Africa are one exception, where the *G6PD* A- variant accounts for about 90% of G6PD deficiency. *G6PD* A- is also frequent in North and South America, in the West Indies, and in areas where people of African origin are present. Moreover, *G6PD* A- is quite prevalent in Italy, the Canary Islands, Spain, and Portugal, and in the middle east, including Iran, Egypt, and Lebanon^(10,34-36). The second most common variant is *G6PD* Med, which is present in all countries surrounding the Mediterranean Sea although it is also

widespread in the middle east, including Israel, where it accounts for almost all G6PD deficiency in Kurdish Jews, India, and Indonesia^(15-17, 37). In several populations, such as the countries around the Arabian Gulf, *G6PD* A- and *G6PD* Med coexist at polymorphic frequencies⁽³⁸⁾. Other polymorphic variants are the Seattle and Union variants, which have been reported in southern Italy, Sardinia, Greece, the Canary Islands, Algeria, Germany, Ireland and China⁽³⁹⁾.

Blood samples of normal and hyperbilirubinemic neonates with severe G6PD deficiency were subjected to genomic DNA extraction within 24-48 hours of aspiration. This method eliminates the need for organic solvent extractions and DNA precipitation, allowing for rapid purification of many samples simultaneously.

The concentration of genomic DNA extracted was determined and the band integrity were found to be different according to the amount of genomic DNA and its purity which depend upon the amount of WBCs in neonatal sample used. In addition, using fresh blood samples were found to be better than that stored at -

20 °C for several days, therefore, the genomic DNA should be applied as early as possible.

The genomic DNA extracted were subjected to electrophoresis through agarose gels which was detected by staining and then visualized by illumination with UV light to confirm the presence and integrity of the extracted DNA⁽²⁰⁻²²⁾. The visualization method of

DNA extracted was performed by staining with the fluorescent dye ethidium bromide which is the most convenient and commonly used method to visualize DNA in agarose gels. Figure -4- presented the agarose electrophoresis bands of genomic DNA extracted from normal and hyperbilirubinemic neonates used in this study.

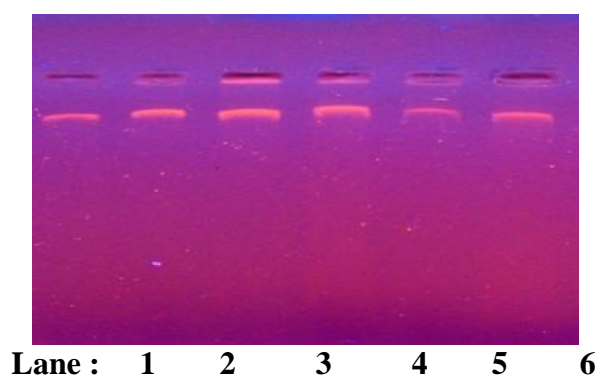
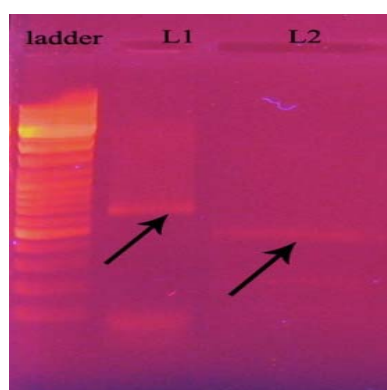


Figure -4- Agarose gel electrophoresis of genomic DNA extracted from control and hyperbilirubinemic neonates with severe G6PD deficiency. (1 -3 bands for normal ; 4-6 bands for severe G6PD-deficient neonates.

Molecular analysis by using thermo cycler device with agarose electrophoresis was accomplished for identification of the G6PD variants in

22 hyperbilirubinemic neonates with severe G6PD deficiency obtained from Babylon province and compared with that found in control samples.



**Lane : DNA L 1(376 bp) L 2 (276 bp)
marker**

Fig-5- PCR and RFLP digestion product for G6PD Med mutation variant

The results of the current study reveal that G6PD mutation samples were amplified by PCR through the use of specific primers and it is shown that the PCR products are :

1. For G6PD Med mutation single band of 276 bp was observed as shown in Lane 2 (Fig. -5-).

The G6PD Med variant at base position 563 creates an *Mbo* II site in exon 6 of G6PD gene. The results of successful PCR amplification were treated by digestion with *Mbo* II

restriction enzyme. After the end of the digestion period, DNA bands were separated by agarose gel electrophoresis containing ethidium bromide and then visualized by photo documentation system. The normal sample showed 379 bp fragment (Fig.-5- lane1), whereas the mutant samples showed 103 bp and 276 bp fragments (L2) beside to 120, 60 and 24 bp fragments found in normal and pathological samples, see (Figure-6-).

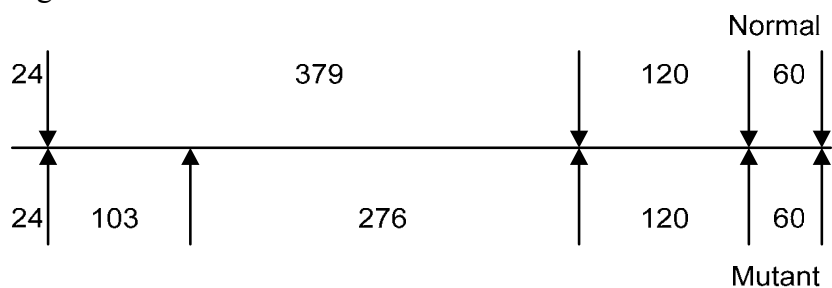


Fig. – 6- Restriction sites for *Mbo* II in G6PD Med variant at exons 6 and 7.

Three regions of G6PD gene were amplified from genomic DNA using the PCR method. All the genomic DNA samples were screened firstly for the C→T mutation at 563, which is characteristics of G6PD Med variant using the two sets of specific primers as indicated in Table-4- and then PCR amplification followed by digestion by *Mbo* II restriction endonuclease.

The predominant G6PD mutation variant in hyperbilirubinemic neonates identified in Babylon Province in Iraq is G6PD Med. The severe G6PD genotype contain 19 (86.4%) out of 22 neonates of G6PD Med variant of the total severe G6PD deficient neonates detected. The incidence of G6PD mutations found in

Babylon province and that reported in some neighboring countries was indicated in (Table-4 and 5-).

2. The same samples which did not show G6PD Med mutation were looked for nucleotide 202 G→A mutation at exon 4 and for nucleotide 376 A→G mutation at exon 5 which were characteristics for G6PD A- by using another two sets of specific primers and PCR reaction conditions, then the PCR amplification products were digested with another restriction endonucleases *Nla* III which digest G6PD gene at exon 4 followed by *Fok* I which digest G6PD gene at exon 5.

Table-4- Incidence of G6PD mutation variants identified in 22 hyperbilirubinemic neonates with severe G6PD deficiency in Babylon province : Iraq by molecular methods.

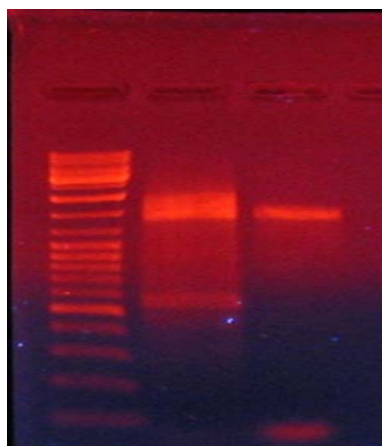
G6PD Variants	Mutation	Amplified Exons	Restriction endonuclease enzyme	No. of G6PD Variant	Incidence %
G6PD Med	563 C→T	6	<i>Mbo</i> II	19	86.4%
G6PD A-	202 G→A 376 A→G	4 5	<i>Nla</i> III <i>Fok</i> I	1	4.55%
Unknown G6PD variant				2	9.1%

Table-5- Prevalence of G6PD Med and African (A-) variants in some countries in the world.

Country	Percentage of G6P variants	
	%G6PD Med	%G6PD A-
Italy	80 - 84%	
Oman	75%	5%
Jordan	53.3%	3.6%
Iraq	84 - 92.6%	0.3%
Iran	79.4%	1.2%
UAE	55%	1.8%
Algeria	23%	46%
Saudi Arabia	47- 84.2%	1.1- 4.6%
Kuwait	72.9 -74.2%	12.4 - 14.3%
Turkey	79 - 80%	
Egypt	52.6%	1.9%

G6PD Mediterranean and G6PD A- were the most commonly detected variants among individuals with G6PD deficiency in the Middle Eastern Gulf area as shown in Table-5-. Screening for the spectrum of G6PD mutations in hyperbilirubinemic

neonates of this study revealed that G6PD Mediterranean (563 C→T) is the most common mutation (86.4%) in Babylon : Iraq , followed by G6PD A- (202 G→A ; 376 A→G) mutations (4.55%).



Lane : 1 2 3

Fig. -7 - PCR product for G6PD A- mutation. Bands run on agarose gel 2%.

Lane 1 : DNA marker (50 bp) ; Lane 2, 3 : G6PD A- PCR product.

PCR is a technique used to amplify single piece of DNA many times. PCR has proven to be an extremely powerful tool in the field of molecular biology⁽⁴⁰⁾. DNA polymorphisms, identified as RFLPs, have proved to be invaluable tool in the study of the human genome, not least in providing markers for gene mapping and linkage analysis⁽²⁸⁾.

Based on biochemical and genetic analysis of a number of samples enrolled in multiple studies, it had been assumed that the common type present in the Eastern Province is G6PD Med. G6PD Med and G6PD A- are the most commonly detected variants among individuals with G6PD deficiency in the Middle Eastern Gulf area, including Iran. These results are consistent with findings among other Arabic populations in the region⁽²⁷⁾. The molecular basis was found to be due to a point mutation (C→T) at nucleotide 563 leading to serine to phenylalanine replacement at amino acid 188⁽⁴¹⁾. This was found to be associated with another but silent mutation at nucleotide 1311 in those from Mediterranean region and Middle East but not in those from India. This does not support the original concept of single

origin of this variant but suggests a possible independent origin of G6PD Med in Middle East and Europe from that of Indian subcontinent⁽³⁷⁾. Recently, no other variant has been reported for this population. However, studies in other parts of the world have shown that a gene flow due to population migration is common. The analysis of a large number of G6PD-deficient samples in this study revealed that G6PD Med accounted for 86.4% of the neonatal samples investigated.

Kaplan, et. al., in (1997) presented data suggesting that the coexistence of G6PD Med mutation type with the AT insertion polymorphism of the promoter of the UGT1A1 gene, which is associated with Gilbert syndrome in adult, is responsible for the development of neonatal hyperbilirubinemias⁽⁴²⁾. This is the most devastating clinical consequence of G6PD deficiency ; it can be severe and result in kernicterus or even death. It was also found that neither G6PD deficiency nor the polymorphism of UDP glucuronosyltransferase alone increased the incidence of neonatal hyperbilirubinemia, but in combination they did. They suggested that this gene interaction may serve as a paradigm of the

interaction of benign genetic polymorphisms in the causation of disease.

The other G6PD mutation detected in this study by molecular technique was G6PD A- mutation which was detected in only one neonate (4.55%) out of 22 hyperbilirubinemic neonates studied. This observation was nearly disagreed to that of Hilmi, in (1998) who mentioned that the frequency of G6PD A- has not reached polymorphic frequency (0.3%)⁽⁴³⁾.

G6PD A- mutation has been reported in Saudi Arabia with frequencies ranged from 0.2-5% in various regions of the Kingdom, while it was found in 3 (1.8%) out of 166 UAE nationals^(38,44). Furthermore, G6PD A- variant was not considered as the most common variant in neither of the above two Gulf states. However, in Algeria G6PD A- variant is the most common variant (46%) of G6PD deficiencies as determined by DNA studies⁽⁴⁵⁾. Screening for the spectrum of G6PD mutations in ethnic Kuwaitis revealed that G6PD Mediterranean (563 C→T) is the most common mutation (74.2%), followed by G6PD A- (202 G→A; 12.4%)⁽²⁷⁾.

Such differences in G6PD A- distribution between Arabian countries in Asia and those in Africa expected since G6PD A- is widely spread through Africa, and wherever there are immigrant population of African origin, and in tropical Africa G6PD A- accounts for about 90% of G6PD deficient cases unlike the situation in most areas of high G6PD deficient frequencies in which multiple polymorphic alleles are responsible for high prevalence rates⁽⁴⁶⁾. Despite the non accurate compared to molecular techniques, the biochemical methods have been utilized by most of the above mentioned studies to detect G6PD deficient variants. This fact might explain the little differences of the percentage of G6PD A- reported by the present study as biochemical methods may misdiagnose the

type of G6PD mutations with others. G6PD A-, but not G6PD A, was found to be present in Kuwaiti and Jordanian populations. A similar observation has been made for Oman⁽⁴⁷⁾.

The data have shown that G6PD A- variant, which is predominant in Africa, is also present among 4.55% of the G6PD-deficient full-term male hyperbilirubinemic neonates of this study. Moreover, it is also documented that G6PD A- is also commonly encountered outside Africa⁽³⁶⁾.

Conclusion

In conclusion, it appears that the most common deficient G6PD mutation variants in Babylon province of Iraq based on molecular characterization, is G6PD Med which account of 86.4% of the severe G6PD-deficient hyperbilirubinemic neonates and G6PD A- variant which constitutes 4.55%, while two hyperbilirubinemic neonates with G6PD-deficiency remain with unknown G6PD variants.

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