Molecular Basis of G6PD Deficiency in Hyperbilirubinemic Neonates in Middle Euphrates Province : Iraq

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Abstract

Background: Neonates G6PD deficiency screening has been recognized as an essential component of public health care in most developed and some Mediterranean countries. However, such screening is yet to be widely embraced in Iraq. More than 442 variants of G6PD have been identified by various molecular methods. The aim of the present study was to determine the normal values of G6PD and deficiency prevalence of this enzyme in male neonates and then determination of the type molecular variant of G6PD prevalence in Middle Euphrates Province of Iraq.

Objective: The objective of this study was to investigate the molecular basis of glucose-6-phosphate dehydrogenase (G6PD) deficiency in hyperbilirubinemic neonates in Middle Euphrates province of Iraq. Molecular methods (genomic DNA extraction, polymerase chain reaction and restriction fragment length polymorphism analysis) and then investigate the type of G6PD variant predominantly present have been performed.

Methods: The study included a total of 917 full-term male neonates which were divided into two groups:

The first group which include 704 neonates (76.8%) associated with severe hyperbilirubinemia were admitted in Middle Euphrates Province Teaching Hospitals of Maternity and Pediatrics during 1st Oct., 2007 to 12th July, 2008 with age ranged between 1 – 28 days, their total serum protein , TSB levels ≥ 15 mg/dl.

The second group which include 213 neonates (23.2%) with the same age ranged were used as control group, their TSB levels < 1 mg/dl. The blood sample taken from each neonate was divided into two aliquots: the first aliquot was used for the determination of total and serum conjugated bilirubin (TSB and SCB), and G6PD activity. The second aliquot was used for molecular analyses 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, severe G6PD deficiency was detected in 75 of 704 hyperbilirubinemic neonates included and their activity levels was significantly decreased ($P < 0.05$) to less than 10% of that found in control group. Therefore, the incidence of severe G6PD deficiency identified in Middle Euphrates Province of Iraq was 10.65%. TSB levels were markedly elevated to (≥ 15 mg/dl), whereas the mean ± SD values of SCB were significantly lower than that found in controls ($P < 0.05$) , SCB was undetectable in 32 of 75 (42.67%) of hyperbilirubinemic neonates 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 was detected by agarose gel electrophoresis and then amplified by PCR and finally was subjected to digestion by endonuclease restriction enzymes to create RFLP and to enable the detection

of mutation that caused G6PD deficiency. The majority of affected severe G6PD deficient neonates with hyperbilirubinemia in Middle Euphrates province – Iraq, were due to G6PD Med variant (C563T, Ser 188 Phe) , of such 67 of 75 neonates (89.3%) have this type of mutation, and 5 of 75 (6.67%) have G6PD A- variant (G202A ; A376G mutations), whereas only 3 of 75 (5.3%) remain unknown G6PD variants which require future molecular studies.

Conclusion: The predominant *G6PD gene* detected in hyperbilirubinemic neonate with severe G6PD deficiency in Middle Euphrates province was G6PD Med.variant.

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

الخلاصـــة

يعتبر مرض عوز نازعة هيدروجين الكلوكوز- 6- فوسفات (G6PD deficiency) من الأمراض المنتشرة في كافة دول العالم ومنها حوض البحر المتوسط ومن بينها العراق . وقد تم تشخيص أكثر من 442 نوع من أنماط الإنزيم (Variants) باستعمال عدد كبير من التقنيات الحيوية ومنها التقنيات الجزيئية Molecular analytical methods والتي تحدد الطفرات الوراثية التي تحدث في الجينات المسئولة عن التصنيع الحيوي للأنماط المختلفة من الإنزيم .

إن أحد أهم أهداف هذه الدراسة هو تحديد نسبة انتشار المرض لدى حديثي الولادة من الذكور ذو النمو الجنيني المتكامل والمصابين بمرض اليرقان الولادي في محافظات إقليم الفرات الأوسط وتحديد الطفرات الوراثية في تتابع القواعد النتروجينية للجينات المسئولة عن تصنيع الأنماط المختلفة للإنزيم (G6PD Variants) والتي تسبب حدوث مرض اليرقان الولادي الحاد باستعمال الطرق الجزيئية. شملت الدراسة 917 عينة من الذكور حديثي الولادة للفترة من 1/ 10 /2007ولغاية 12/ 7 /2008 ولأعمار تراوحت ما بين 1 – 28 يوما حيث تم توزيعهم إلى مجموعتين اعتمادا على تركيز البيليروبين الكلي TSB وكما يلي:-

- المجموعة الأولى والتي شملت 704 (76.8%) حديث الولادة مصابين باليرقان الولادي وكان تركيز البيليروبين قد ارتفع وبشدة (TSB ≥15 mg /dl).
- المجموعة الثانية وهي مجموعة السيطرة والتي شملت 213 (23.2%) حديث الولادة وكان تركيز البيليروبين الكلي طبيعيا (TSB < 1 mg/dl) .

وتضمنت هذه الدراسة تحديد العوز الحاد للإنزيم على تركيز كل من البيليروبين الكلي TSBوالبيليروبين المقترن SCBفي مصل الدم. ولقد وجد أن نشـــــــــاط الإنزيم قد انخفض معنويا بشكل حاد في 75 حالة من حالات اليرقان الولادي حيث وصل إلى أقل من (10%) مقارنة مع المعدل الطبيعي لنشاط الإنزيم وان نسبة انتشار النقص الحاد لإنزيم الـ G6PD في منطقة الفرات الأوسط بالعراق هي (10.65%). بينما معدل الـ TSB قد ارتفع وبشدة إلى أكثر من 15mg/dl . وقد اقترن ذلك بانخفاض معنوي كبير في تركيز البيليروبين المقترن SCB وانعدامه في 32 من 75 (42.67%) حالة مصابة بالنقص الحاد في نشاط الإنزيم وهذا يوضح عدم حدوث عملية الاقتران للبيليروبين في خلايا الكبد لغـــــرض التخلص منه بسبب عدم نضج ميكانيكية الاقتران ووجود نقص في بعض الإنزيمات المسئولة عن ذلك ومنها إنزيم الـــ UGT1A1 . وقد وجد أن هناك ارتباط معنوي (P<0.05) سالب مابين نشاط الإنزيم المنخفض بشدة وتركيز البيليروبين الكلي TSB الذي ارتفع إلى أكثر من 15 mg/dl.

وقد تضمن المحور الجزيئي التحري عن الطفرات الوراثية للقواعد النتروجينية التي تحدث في الجينات المسئولة عن التصنيع الحيوي لإنزيم الـــ G6PD باستعمال التقنيات الجزيئية Molecular Analysis والتي تعتمد على استخلاص الحامض النووي منقوص الأوكسجين Genomic DNA الذي تم استخلاصه من دم الذكور حديثي الولادة والمصابين بالنقص الحاد في نشاط إنزيم الـــ G6PD باستعمال طقم خاص تم استيراده من شركة Roche الألمانية ومن ثم متابعة دراسة التحليل الجيني للطفرات الوراثية حيث أخضعت العينات المرضية مع مجموعة السيطرة إلى تفاعل التضاعف التسلسلي Polymerase Chain Reaction للحامض النووي بعدها تم استعمال طريقة الهضم بالأنزيمات المعتمدة (RFLP) للكشف عن الطفرات في الجينات المسئولة عن التصنيع الحيوي لنمطي الإنزيم G6PD Med and G6PD A- . وقد بينت الدراسة أن الطفرات الوراثية التي تحدث في الجينات المسئولة عن تصنيع الإنزيم الحيوي والتي سببت النقص الحاد لنشاط إنزيم الـ G6PD في مصل الدم لـ 75 حالة تم تشخيصها وباستعمال الطرق الجزيئية ومن ثم إحداث اليرقان الولادي في حديثي الولادة بمحافظات الفرات الأوسط العراقية كانت 67 حالة (89.3%) من النمط المتوسطي للإنزيم G6PD Med(C563T) بينما تم تشخيص 5 حالات فقط (6.67%) من النمط الأفريقي G6PD A- (G202A ; A376G) بينما بقيت 3 حالات أخرى (5.3%) لم يتم التعرف على النمط الإنزيمي لها.

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. 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 was 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)].

Any gene located on the X-chromosome is called an X-liked gene[(8)]. 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. 1)[(9)].

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[(50)].

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[(10)].

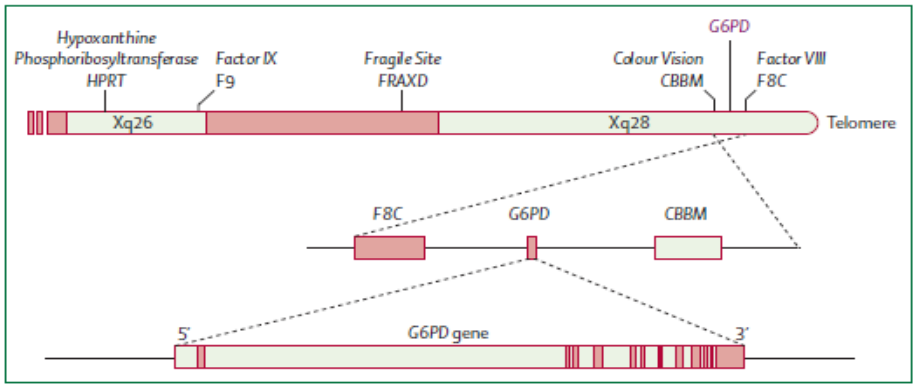


Figure 1. Location of G6PD gene on X chromosome[9].

In most areas of high prevalence of G6PD deficiency, several polymorphic alleles are found and more than 442 variants of G6PD enzyme were investigated[11]. 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[9].

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, India, and Indonesia. In several populations, such as the countries around the Arabic Gulf, *G6PD* A– and *G6PD* Mediterranean coexist at polymorphic frequencies [12].

The aim of the present study was to determine the mutations of G6PD gene in severe G6PD deficient hyperbilirubinemic neonates in Middle Euphrates 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-* was also examined in this area.

Materials and Methods

The study included a total of 917 full-term male neonates, 704 of them were associated with severe hyperbilirubinemia (TSB ≥ 15 mg/dl) who were admitted to each of:

1. Kerbala Pediatrics Teaching Hospital / Kerbala
2. Babylon Teaching Hospital of Maternity and Pediatrics / Babylon
3. Al-Zahra Teaching Hospital of Maternity and Pediatrics / Najef
4. Teaching Hospital of Maternity and Pediatrics / Diwaniyah

during 1st , Oct., 2007 to 12th , July, 2008 with age ranged between 1 – 28 days. Their TSB levels ≥ 15 mg/dl . Another 213 neonates were used as control group. The cord blood sample taken from each neonate was divided into two aliquots: the first aliquot was used for total and conjugated serum bilirubin (TSB and SCB) [13] 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 by fluorescent spot test for G6PD detection, and then G6PD activity was measured in those neonates whose associated with severe deficient G6PD. **A**ctivity of G6PD was measured quantitatively by using Sigma kit (345-B)

at 37 ^{o}C by the kinetic method modified by Lohr and Waller [14].

Genomic DNA was extracted and purified by salting out in several steps by using Roche High Pure PCR Template Preparation Kit, Germany and as described by Vogelstein and Gillespie[15]. After genomic DNA extraction , it has been subjected to electrophoresis through agarose gels which was detected by staining with the fluorescent dye ethidium bromide [16].

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 Middle Euphrates Province which are Mediterranean (G6PD Med ,563 C⟶T) and African (G6PDA− , 202 G⟶ A, 376 A⟶ G) [17]. 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 [18].

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
Mediterranian	6	563	C→ T	TCC→TTC	188	Ser → Phe
African, A-	4	202	G → A	GTG→ATG	68	Val → Met
	5	376	A → G	AAT→GAT	126	Asn→ Asp

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[16]. 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. The lyophilized primers was dissolved in 1 ml of distilled water or in 5 mM Tris-buffer, pH 7.5 as a stock solution and stored at – 20 ^{o}C, then small aliquots were prepared to a final concentration of 25 pM/µl to avoid freezing and thawing and stored at – 20 ^{o}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. These endonucleases which purchased from Fermentas Co. included ***Mbo* II**, ***Nla* III** and ***Fok* I**. All these enzymes were stable for short period at – 20 ^{o}C and at – 70 ^{o}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.

The G6PD-Med mutation at the base position 563 creates one ***Mbo* II** site in exon 6 and 7 of the G6PD gene[22,23]**.** 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 [19,22].

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 [23].

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 ^{o}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%[16].

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.[24] ; Hamamy and Saeed,[25] and Al-Naamah, et. al.[26]. The present study indicated that G6PD deficiency is common in Iraqi hyperbilirubinemic neonates of Middle Euphrates 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 704 G6PD-deficient hyperbilirubinemic neonates diagnosed, only 75 (10.65%) neonates of them were associated with severe G6PD-deficiency and their G6PD activity levels was decreased and reached to less than 10% found in control neonates (see table-2-). Bilirubin profile indicated that TSB levels were markedly elevated while serum conjugated bilirubin (SCB) level was decreased as compared with the control group, as compared with that found in control group as shown in (tables-3 and 4).

These results confirm with other studies performed in Italy, and Taiwan which suggest that the G6PD–deficient neonates are at increased risk for hyperbilirubinemia[27]. Therefore, data presented in this study may probably suggest that severe neonatal hyperbilirubinemia may continuously cause a problem in this region of Iraq,

which showing 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 [28].

The data observed in this study 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.

Table 2. Incidence of severe G6PD deficiency in full-term hyperbilirubinemic neonates in Middle Euphrates Province Governorates of Iraq.

Middle Euphrates Province Governorate	Group I Healthy Control TSB < 1.0 mg/dl		Group II Full-term Neonates with Severe Hyperbilirubinemia , TSB ≥ 15 mg/dl			
	No.	G6PD activity Mean ± SD U/g Hb	Total No.	No. of Severe G6PD Deficient	G6PD activity Mean ± SD U/g Hb	% Incidence of severe G6PD deficiency
Kerbala	56	10.4 ± 1.78	197	18	0.56 ± 0.32	9.14%
Babylon	53	10.02 ±1.17	183	22	0.34 ± 0.17	12.02%
Najef	61	10.91 ±1.56	179	21	0.46 ± 0.41	11.7%
Diwaniyah	43	9.95 ± 1.76	145	14	0.41 ± 0.3	9.66%
Total	213		704	75		10.65%

Table 3. Sample Distribution and TSB levels in severe neonatal hyperbilirubinemia (TSB ≥ 15 mg/dl) with G6PD deficiency in Middle Euphrates Province Governorates of Iraq.

Middle Euphrates Province Governorate	Group I Healthy Control		Group II Full-term Neonates with Severe Hyperbilirubinemia TSB ≥ 15 mg/dl		
	No.	TSB levels Mean ±SD mg/dl	Total No.	No. of Neonates with Severe G6PD Deficiency	TSB level Mean ±SD mg/dl
Kerbala	56	0.57 ± 0.25	197	18	20.26 ± 4.96
Babylon	53	0.72 ±0.24	183	22	23.01 ± 5.0
Najef	61	0.58 ± 0.22	179	21	21.29 ± 5.43
Diwaniyah	43	0.65 ± 0.29	145	14	24.38 ± 6.53
Total	213		704	75	

Table 4. Serum conjugated bilirubin in normal and in hyperbilirubinemic neonates with severe G6PD deficiency in Middle Euphrates Province Governorates of Iraq.

Middle Euphrates Province Governorate	Group I Healthy Control		Group II Neonates with Severe Hyperbilirubinemia TSB ≥ 15 mg/dl		
	No.	SCB levels Mean ±SD mg/dl	Total No.	No. of Neonates with Severe G6PD Deficiency	SCB level Mean ±SD mg/dl
Kerbala	56	0.15 ± 0.08	197	18	0.053 ± 0.046
Babylon	53	0.19 ± 0.11	183	22	0.063 ± 0.036
Najef	61	0.18 ± 0.07	179	21	0.081 ± 0.051
Diwaniyah	43	0.21 ± 0.09	145	14	0.094 ± 0.055
Total	213		704	75	

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*) [29] ;

(2) The hepatic bilirubin-conjugating enzyme UGT1A1 [30] ;

(3) The hepatic organic anion transporter polypeptide1 B1 (*OATP1B1*) [31].

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[32]. 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 predispose to neonatal hyperbilirubinemia by possibly limiting hepatic bilirubin uptake[33]. 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[34]. 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[35].

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[34], 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 ($P < 0.05$) between G6PD activity levels and TSB concentrations elevated in severe G6PD-deficient hyperbilirubinemic neonates with the TSB ≥ 15 mg/dl but not in control individuals **(see table 5)**.

Table 5. The correlation between G6PD activity levels and TSB conc. in severe G6PD-deficient hyperbilirubinemic neonates in Middle Euphrates Province Governorates.

Middle Euphrates Province Governorate	**Full-term Neonates with Severe Hyperbilirubinemia TSB ≥ 15 mg/dl**				
	No. of Severe G6PD Deficient	**G6PD activity Mean ± SD U/g Hb**	**TSB levels, mg/dl**	***P* Value**	**r Value**
Kerbala	18	0.56 ± 0.32	20.47 ± 3.12	< 0.05	- 0.551
Babylon	22	0.34 ± 0.17	23.01 ± 5.0	< 0.05	-0.203
Najef	21	0.46 ± 0.41	21.29 ± 5.43	< 0.05	-0.320
Diwaniyah	14	0.41 ± 0.3	24.38 ± 6.53	< 0.05	-0.367

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[(36)]. 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[(37)]. Therefore, it is reasonable to suggest that the neonatal hyperbilirubinemia 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 , widespread in the middle east, including Israel, where it accounts for almost all G6PD deficiency in Kurdish Jews, India, and Indonesia[(10, 45)]. In several populations, such as the countries around the Arabian and subsequently aggregative the high TSB levels.

Two G6PD variants were identified by using a molecular diagnostic methods, 67 neonates (89.3%) was diagnosed to be G6PD Med mutation in exon 6 (563C→T) (Ser188Phe) ; and 5 neonates (6.67%) 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) **(see table-6-)**.

In most areas of high prevalence of G6PD deficiency, several polymorphic alleles are found [(38)]. 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[(9,44)]. The second most common variant is *G6PD* Med, which is present in all countries surrounding the Mediterranean Sea although it is also Gulf, *G6PD* A– and *G6PD* Med coexist at polymorphic frequencies[(46)]. Other polymorphic variants are the Seattle and Union variants, which have been reported in southern Italy, Sardinia, Greece, the

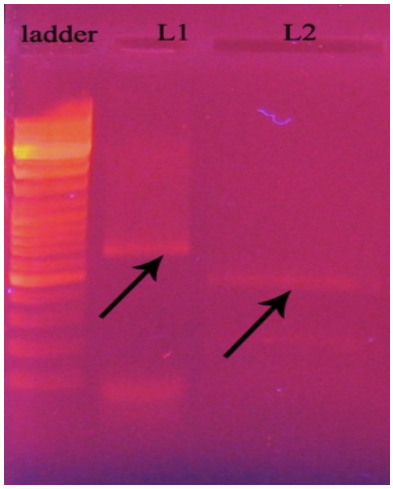
Canary Islands, Algeria, Germany, Ireland and China [47].

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 ^{o}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[16,19]. 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.

Molecular analysis by using thermo cycler device with agarose electrophoresis was accomplished for identification of the G6PD variants in 75 hyperbilirubinemic neonates with severe G6PD deficiency obtained from Middle Euphrates province and compared with that found in control samples.



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

Fig 2. 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. 2).

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.-2- 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-3-).

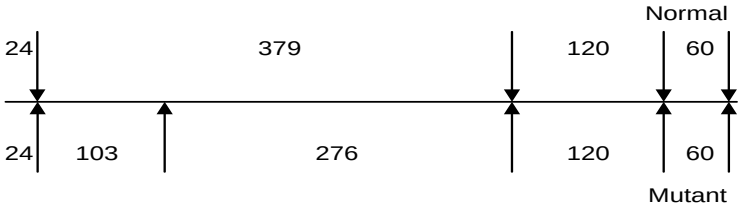


Fig. 3. Restriction sites for *Mbo* II in G6PD Med variant at exons 6 and 7.

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-6- and then PCR amplification followed by digestion by ***Mbo*** **II** restriction endonuclease.

The predominant G6PD mutation variant in hyperbilirubinemic neonates identified in Middle Euphrates Province in Iraq is G6PD Med. The severe G6PD genotype contain 67 of 75 cases (89.3%) of G6PD Med variant of the total severe G6PD deficient neonates detected. The incidence of G6PD mutations found in Middle Euphrates province and that reported in some neighboring countries was indicated in (Table-6 and 7-).

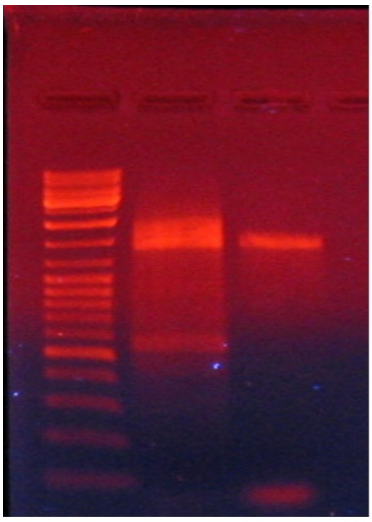
2. The same samples which did not show G6PD Med mutation were looked for nucleotide 202 G→A mutation at exon 4 and for nucleotide 376 A→G mutation at exon 5 which were characteristics for G6PD A- by using another two sets of specific primers and PCR reaction conditions, then the PCR amplification products 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 (Fig. 4).

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 7. 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 (89.3%) in Middle Euphrates Province : Iraq , followed by G6PD A− (202 G→A ; 376 A→G) mutations (6.67%).

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.

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 [(44)]. 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 [(41)]. 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 89.3% of the neonatal samples investigated.



Lane : 1 2 3

Fig. 4. 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.

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 [(36)].

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.

Table 6. Incidence of G6PD mutation variants identified in 75 hyperbilirubinemic neonates with severe G6PD deficiency in Middle Euphrates Province Governorates of Iraq by molecular methods.

G6PD Variants	**%**	**Mutation**	**Amplified Exon**	**Total Incidence %**	**Restriction endonuclease enzyme**	**Middle Euphrates Province Governorates**			
						Kerbala	**Babylon**	**Najef**	**Diwaniyah**
G6PD Med	67	563 C→T	6	89.3%	***Mbo* II**	94.4%	86.4%	90.5%	75.7%
G6PD A-	5	202 G→A 376 A→G	4 5	6.67%	***Nla* III** ***Fok* I**	5.56%	4.55%	4.8%	14.3%
Unknown G6PD mutation	3			5.3%		-------	9.1%	4.8%	------

Table 7. Prevalence of G6PD Med and African (A-) variants in some countries in the world.

Country	Percentage of G6PD variants %G6PD Med	%G6PD A-
Italy	80 - 84%	
India	82%	
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%

The other G6PD mutation detected in this study by molecular technique was G6PD A- mutation which was detected in only 5 neonate out of 75 (6.67%) patients of the 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%)[45].

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 [42,46]. 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 [49]. 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%).

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 [49]. 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 [48].

The data have shown that G6PD A- variant, which is predominant in Africa, is also present among 6.67% 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.

Conclusion

In conclusion, the most common deficient G6PD mutation variants in Middle Euphrates Province of Iraq based on molecular characterization, is G6PD Med which account of 89.3% of the severe G6PD-deficient hyperbilirubinemic

neonates and G6PD A- variant which constitutes 6.67% .

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