Genetic studies on glucose-6-phosphate dehydrogenase deficiency in neonates suffering from hemolytic anemia

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Original Article

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ABSTRACT

Objective: To evaluate the association between G6PD mutations including Mediterranean mutation (exon 6 C563T), Mahidol mutation (exon 6 G487A), Chatham mutation (G1003A) and hemolytic anemia incidence in Saudi population.

Design: Cross-section study

Setting: Department of Hematology at the Maternity and Children's Hospital located in Jeddah, Saudi Arabia

Subjects: One hundred blood samples (50 males and 50 females) were collected from neonates

Intervention: Neonates aged from newborn to 12 months old

Main outcome measure: Cases were asked to answer a questionnaire. Quantitative evaluation of G6PD enzyme activity was performed using spectrophotometric method. Genotypes and allele frequencies were studied by PCR and RFLP for the three SNPs. Descriptive data were calculated as the mean ± standard deviation (SD). Association between clusters of genotypes was evaluated using Mann-Whitney and Chi-square tests.

Results: The Mediterranean mutation was the most common mutation detected in the male and female groups, whereas Chatham and Mahidol mutations were found in a fewer number of male samples. In total, out of 50 newborns, 22 males and 25 female subjects (44%) were with Mediterranean mutation; one male was Chatham with Mediterranean mutations (2%), one male had only Chatham mutation (2%) and others had Mahidol mutation (2%). Three females (heterozygous CT) belonged to class II G6PD variant and suffered from severe hemolytic anemia. The nucleotide 563T was the most frequent polymorphism observed followed by nucleotide 1003A and 487A.

Conclusion: Our finding strengthens the evidence for the association between G6PD deficiency and genetic variation of G6PD genes in the etiology of hemolytic anemia.

KEY WORDS: Chatham mutations, G6PD deficiency**,** hemolytic anemia, Jeddah, Mahidol mutations, Mediterranean mutations

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked, inherited enzymatic disorder. It is triggered by the mutations in the G6PD gene and results in protein variants with different levels of enzyme activity and are correlated with a varied range of biochemical and clinical phenotypes. The G6PD enzyme deficiency can be produced by a reduction in the number of enzyme molecules, a structural difference in the enzyme producing a qualitative change, or both. However, until now, their precise molecular basis has remained unknown. Data from several studies suggested that about a third of all male newborns with neonatal jaundice have a G6PD deficiency in them; however, the deficiency is less frequent in female neonates[1-3]. Most individuals suffering from G6PD deficiency are asymptomatic throughout their lifespan. The most common clinical manifestations are fatigue, back pain, neonatal jaundice and acute hemolysis anemia. In most of the patients, it is activated by an exogenous agent or when red blood cells are subjected to oxidative stress prompted by agents such as drugs, infection, or the ingestion of fava

beans[4,5]. G6PD deficiency does not appear to influence life expectancy, life quality, or affected individuals activity[6,7].

World Health Organization[8] grouped variants of G6PD deficiency in 5 different classes[8]. Variants can also be classified as sporadic or polymorphic[5]. Class I variants have severe enzyme deficiency and have chronic hemolytic anemia. Class II variants also have a severe enzyme deficiency, but there is usually only intermittent hemolysis. Class III variants have moderate enzyme deficiency with intermittent hemolysis, usually associated with infection or drugs. Class IV variants have no enzyme deficiency or hemolysis. Class V variants have increased enzyme activity. Classes IV and V are of no clinical significance.

Deficient G6PD alleles are distributed worldwide, a conservative estimate is that at least 400 million people carry a mutation in the G6PD gene causing a deficiency. High prevalence is reported from 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 North Europe[9].

The present study was designed to determine the frequency and spectrum of G6PD mutations in newborn patients in Jeddah, Saudi Arabia. The study will help to understand the relationship between G6PD deficiency and variation in the genes that code for G6PD to determine the role of inheritance and the mechanism of its operation in the etiology of hemolytic anemia.

MATERIALS AND METHODS

Sample collection and study subjects

The present study recruited a cohort of 100 participants with 50 hemolytic anemic patients (25 male and 25 female) and 50 normal/control participants (25 male, 25 female). The participants' age was in the range of 0-12 months old. The blood samples of participants were collected in the Department of Hematology at the Maternity and Children's Hospital located in Jeddah, Saudi Arabia and stored at 4 °C until further use. Written informed consent was obtained from all of the participants enrolled in the study. The study was approved by the Ethical Committee (Unit of Biomedical Ethics) (No. H-02-J-002) from Directorate of Health Affairs, Jeddah. The research has been conducted at the postgraduate studies laboratories, Biological Science Department, Faculty of Science, King Abdul Aziz University, Jeddah.

Determination of enzyme activity in G6PD

The activity of the G6PD enzyme was determined by using Glucose-6-Phosphate Dehydrogenase Assay Kit (Sigma – Aldrich, USA), according to the manufacturer's instructions. The final activity was determined by plotting a standard curve obtained by taking optical density (OD) at A450.

Extraction of DNA from blood sample and determination of the concentration

DNA was extracted from entire blood by using QIAamp DNA Blood Mini Kit (Qiagen Inc., USA) according to instructions given in the manual. The DNA concentration was detected by measuring the optical density. The DNA samples were diluted 1:200 in nuclease-free water in 1.5 ml microcentrifuge tube. The mixture was then transferred to 1 ml quartz cuvette and was quantified using spectrophotometric

analysis using 6800 UV/ Vis Spectro-photometer (Jenway, UK). The purity was of DNA sample was determined by calculating the ratio of absorbance at A260/ A280. Pure DNA sample should have absorbance ratio between 1.7-1.9.

PCR amplification and purification of amplicons

Amplification of exon

PCR amplification of exon 6 Mediterranean mutation, exon 6 of Mahidol mutation and exon 9 Chatham mutation gene was done by using the forward and the reverse primers given in Table 1. Briefly, DNA was amplified in a 50µl volume reaction containing 2µl (0.2 µg/µl) genomic DNA, 25 µl Hot Start Green Master Mix, 20.5µl nuclease-free water, and 1.25 µl of each respective primer. PCR amplification of DNA was set up with cycling conditions as described earlier by Jarullah *et al*, Sulaiman *et al*, and Gandomani *et al*, for exon 6 Mediterranean mutation, exon 6 of Mahidol mutation and exon 9 Chatham mutation gene respectively[10-12].

The final amplicon was electrophoresed on 3% agarose gel in a horizontal electrophoresis system (SCIE-PLAS, UK) and visualized in a gel documentation system. The amplicons were visualized under UV light and photographed using gel documentation system (Syngene, USA).

Purification and visualization of amplicons

Amplicons or PCR products were purified using QIAquick gel extraction Kit (Qiagen Inc., USA) according to manual's instruction. The final purified products were electrophoresed on 3% agarose and visualized under UV light and photographed using gel documentation system (Syngene, USA).

Genotyping of exon mutation

The genotypes for these SNPs were determined by restriction fragment length polymorphism (RFLP) procedure using different restriction enzyme for each exon mutation *i.e.*, 6 C/T Mediterranean mutation, 9 G/A Chatham mutation and 9 G/A Chatham mutation.

(a) exon 6 C/T Mediterranean mutation

The genotype of resulting amplicon of 547 bp was determined using the reaction of 15 µl of PCR product, 13µl of sterile deionized water and 2µl of 10X Buffer Tango were added in an Eppendorf tube and mixed thoroughly by pipetting. In the end 2µl of restriction enzyme, *Mbo* II was added to the reaction mixture. The tube was incubated for 24 hours at 37C followed by heat inactivation for 20 minutes at 65˚C. The genotypes were resolved on a 3% agarose gel electrophoresis.

(b) exon 6 G/A Mahidol mutation and exon 9 G/A Chatham mutation

The genotype of resulting amplicon of 104 bp and 208 bp of exon 6 G/A Mahidol mutation and exon 9 G/A Chatham mutation respectively was determined using the reaction of 10 µl of PCR product, 18µl of sterile deionized water and 2µl of 10X Buffer Tango added in an Eppendorf tube and mixed thoroughly by pipetting. In the end, 2µl of restriction enzyme, *Hin* IIII and *Bst*XI was added to the reaction mixture for exon

6 G/A Mahidol mutation and exon 9 G/A Chatham mutation respectively. The tube was incubated for four hours at 37 ˚C followed by heat inactivation for 20 minutes at 65 ˚C. The genotypes were resolved on a 3% agarose gel electrophoresis.

Statistical analysis

The final data was analyzed statistically using Statistical Package for Social Science v.16 (SPSS Inc., Chicago, IL, USA). Descriptive data were calculated as the mean ± standard deviation (SD). Association between clusters of genotype was evaluated using Mann-Whitney and Chi-square test (χ^2 test. 2-by-3 and 2-by-2 were applied to determine the association between genotypes and clinical groups and alleles in clinical groups respectively. To evaluate the relative risk and strength of association for the various genotypes or their combinations contingency analysis was used to calculate the odds ratio (OR) and risk ration (RR) at 95% confidence interval (CI). The p-value less than 0.05 was considered as statistical significance. To compare the observed and expected genotypes frequencies among the two group of participants (patient and normal) Hardy-Weinberg Equilibrium was applied. It was done by goodness-of-fit χ^2 test, with a degree of freedom value as one. Linkage disequilibrium[8] resulting from the association between the genotypes of exon 6 C/T and exon 9 G/A polymorphisms was assessed by the $\chi 2$ test. The linkage disequilibrium coefficient was calculated using an online server, Haplotype calculation online: (http://www.oege.org/software/cubex/)

RESULTS

Demographic data and characteristics of the subjects

The study subjects were categorized according to their gender and as a patient (affected) and normal (control) participants. Fifty normal participants (control) with 25 males and 25 females and 50 patient participants with 25 males and 25 females were recruited for the study. The mean age for patient group was 3.001 months (SD ±3.45), and for normal group, it was 5.64 months (SD ±3.215). The *P*-value was .49; therefore, there was no statistically significant difference between patient and normal groups. The mean age for male participants was 5.3 months (SD ± 2.76) and 5.48 months (SD ±3.47) for the patient and normal months respectively. The mean age for female participants 6.96 months (SD ±3.91) and 5.8 months (SD ±2.99) for the patient and normal months respectively. There was no significant difference between the two groups in case of both male and female (Table 2). The hospital stays for the participants was 2-8 days with mean hospital stay days for 4.4 days (SD ±1.2 days). The mean bilirubin level for all participants was 18.3 mg/dl (SD ± 2.8). The treatment used for icteric patients was phototherapy in 30 neonates (68%) and exchange transfusions in 16 cases (72%). The mean bilirubin levels and hospital stay days showed no significant difference in the groups for different types of mutation.

Further, the G6PD deficiency was observed in 5 cases (50%) by the screening test and G6PD Mediterranean mutation in 40 cases (80%). The total frequency of G6PD deficiency among neonates with prolonged or high jaundice was 17.9%.

Classes of G6PD deficiency

The enzyme activity in blood samples was determined using Glucose 6 phosphate dehydrogenase assay kit. World Health Organization classified G6PD variants on the basis of the extent of the enzyme deficiency and the severity of hemolysis[8,13]. In the present study, the G6PD deficiency was determined for all the 100 participants. Twenty-six percent of participants were found to be with normal G6PD activity (Class IV), 24% suffered from increased enzyme activity (Class V) and 50 % had enzyme deficiency (less than 10% - 60%) (Class I, II and III patients). Among the 50 G6PD deficient participants 30 (60%) had a mild enzyme deficiency, 10 (20%) had a moderate deficiency and 10 (20%) had a distinct enzyme deficiency. As an expected class I G6PD variant was found in male participants (10%) only and no female participants were found to be suffering from severe enzyme deficiency and have chronic hemolytic anemia. The class II, III, IV and V G6PD variants were found in both male and female participants (Table 3).

Mutations and detection of nucleotides

Mutations

The most common mutation observed in male and female groups was Mediterranean mutation 563 C>T. The Mediterranean with G6PD Chatham mutation, G6PD Chatham 1003 G >A and Mahidol mutations 487 G>A were found mostly female and in some of the male participants. Out of 50 newborns, Mediterranean mutation 563 C>T was found in 22 (44%) males and 25 (50%) females participants, the 2 (4%) male participants with Chatham mutation G1003A and only one (2%) male participants with Mahidol mutation 487G>A.

Detection of nucleotide G6PD Med 563 C>T polymorphism

PCR amplification of G6PD gene was performed for all the 50 of each patient and controls samples. The G6PD-Med mutation 563 C>T at base site 563 produces a *Mbo* II site in exon 6 of the *G6PD* gene. A single 547 bp characteristic band for Med. mutation 563 C>T was observed. The control samples showed 60, 119 and 377 bp bands and the mutant samples showed 60, 100, 119 and 277 bp bands. In heterozygote samples, a 60, 100, 119, 277 and 377 bp bands were observed (Figure 1). The G6PD Mediterranean genotype constituted 40 cases (80 %) out of the total 50 deficient participants.

Detection of nucleotides G6PD Mahidol variant (487G>A) polymorphism

The samples (PCR product) contain a restriction site for the *Hind* III restriction enzyme; therefore, it was digested with the enzyme. Two bands of low molecular weight, 22bp and 82bp were observed indicating the presence of allele A (the flanking fragment of 22 bp was too small to detect on the gel). A single band of 104 bp was also observed indicating the presence of allele G which lacks the restriction site for *Hind* III enzyme (Figure 2).

Detection of nucleotides G6PD Chatham variant (1003G>A) polymorphism

The samples were digested with restriction enzyme *Bst* XI. Three bands of 30 bp, 78 bp and 100 bp were observed. That showed the presence of allele A as the sample contains two sites for the restriction

enzyme *Bst* XI (The flanking fragment of 23 bp was too small to detect on the gel). Two other bands of 78 and130 bp were also observed indicating the presence of allele G, as it contains only one site for restriction enzyme *Bst* XI. A single band of 208 bp of G6PD exon 9 Chatham variant (1003G>A) polymorphism was obtained (Figure 3).

Characteristics of subjects according to their *G6PD Med*. 563 C/T genotype

The frequency of each genotype of the G6PD Med. 563 C>T in patient and normal participants was determined. In case of normal participants, it was observed that 98% (n=49) were homozygous with CC genotype, 2% (n=1) were heterozygous with CT genotype, and 0.00% (n=0) was homozygous with TT genotype. While in case of patient participants it was observed that 0.00% (n= 0) were homozygous with CC genotype, 80% (n= 40) were heterozygous with CT genotype and 20% (n=10) were hemizygous TT genotype males. The p-value was 0.001, which showed there is a highly significant difference in alleles frequencies between patient and normal groups. The distribution of genotype was in the Hardy-Weinberg equilibrium. The stability of homozygotes and heterozygotes observed was as predicted by the Hardy-Weinberg equation from the allele frequencies, Goodness of fit X^2= 0.943, df = 1, *P*=.33 and Goodness of fit X^2= 0.00, df = 1, *P*=.94 in normal and patient subjects, respectively

The variation between groups among genotypes in Med 563 C/T SNP is shown in Table 4. The *P*-value was .05, which is statistically high, and therefore there is a significant difference in variation between patient and normal groups among genotypes. The association between genotype distribution and allele frequencies of the polymorphisms in the two groups (patient and normal) was observed and found to be highly significant (*P*=.00). It implies that majority of the patient group tend to be of CT genotype, and most of the normal group tend to be of CC genotype. The odds ratio (OR) and risk ration (RR) were 0.013 (93.5% CI: 0.20- 1.00) indicating no association among them. Therefore, a risk ratio cannot be calculated. Instead, the study identified the exposure status of a sample of cases and another of controls. The OR calculated from a case-control study can approximate a relative risk, but only when the disease is rare (say, up to around 5% in the sample, as is the case for many chronic conditions).

The Mediterranean variations between the groups among genotype showed a high significant difference with *P*-value 0.03 and 0.018 between classes of G6PD variants and healthy controls groups in male and female respectively. Twenty percent (n=10) of the patient suffering from chronic hemolytic anemic (class I variant) was male with hemizygote T, and no female patient with homozygote TT was found. Further, 32% of the male patient of class II variant was hemizygote C, whereas 50% of the female patient was heterozygote CT. All normal group participant was homozygote CC (Table 5).

Association between G6PD Med. 563 C/T polymorphism and incidence of hemolytic anemia

(a) Male participants

In patient group the Med. 563 C/T SNP, the distributions of genotype were 0% (n=0) in case of hemizygous C, 60% (n=6) hemizygous class II C, and 40% (n=2) hemizygous class I T. In normal group, 96% (n=24) hemizygous C, 4% (n=1) hemizygous class II C and 0% (n=0) hemizygous recessive T. The equilibrium of hemizygote C and T was found to be as predicated by the Hardy-Weinberg equation from

these allele frequencies in Med. 563 T/C SNP (X^2= 4.65, df = 1, *P*=.032) (X^2= 0.001, df =1, *P*=.918) in both patient and normal, in tandem. The variation between groups among genotype in both SNPs is shown in table 6. The association between genotype distribution and allele frequencies of the Med. 563 C/T polymorphism in the two inpatient and the normal group is shown in table 6. Also, there is a highly significant association between groups and genotype (*P*=.001) in Med. 563 T/C.

The Mediterranean variations between groups among genotype showed a highly significant difference (*P*-value .00) in variation between classes of G6PD variants and healthy controls groups in male groups. Twenty percent of the male patient was suffering from chronic hemolytic anemic (class I variant) with hemizygote T, and 32% were in class II variant were hemizygote C. All normal male group participants were hemizygote C. In Med. 563 C/T SNP, the frequency of the C and T alleles for the patient group were 60% and 40% respectively. The frequency in the normal group they were 98 % and 2% respectively (Table 6).

(a) Female participants

In case of patient group, 0% (n= 0) were homozygous CC and 100% (n=25) heterozygous CT. In normal group, 100% (n=25) were homozygous CC and 0% (n=0) were heterozygous CT. The balance of homozygote and heterozygote observed was as predicted by the Hardy-Weinberg equation from the allele frequencies, X^2= 25, df = 1, *P*=.000 and X^2= null, df = 1, *P*= null inpatient and normal group respectively. It implies that the population is at Hardy-Weinberg equilibrium. The variation between groups among genotype was shown in table 7. There was no significant difference in variation between the two groups among genotype (*P*=.55). The association between genotype distribution and allele frequencies in the two female's groups were shown in Table 7. The frequency for the patient group of the C and T alleles were 50% and 50% respectively. In normal group, the frequency of the C and T alleles were 100% and 0%, respectively. There was a strong significant difference in alleles frequencies between patient and normal groups (*P*=.000).

None (n=0) of the patient group participants were in class I variant with homozygote TT. All female patient group participants were in class II variant representing 100% (n=25) with heterozygote CT. In contrast, all (n=25) of normal female group participants were homozygote CC. (Table 7). In Med. 563 C/T SNP, the frequency of the C and T alleles for the patient group were 50% and 50%, respectively and for normal group, the frequency was 100% and 0% respectively.

DISCUSSION

G6PD deficiency is a genetic disorder that results in missing or defective enzymes (enzymopathy) affecting millions worldwide. It is also closely related to neonatal jaundice, favism and food or drug-induced acute hemolytic anemia[14]. Mutations in the G6PD gene may diminish functionality and/or stability of the G6PD enzyme leading to different levels of enzymatic activity and a wide range of biochemical and clinical manifestations. Since it is an X- linked gene the hemizygous males suffer from more severe hemolytic crises than heterozygous females who have variable proportions of G6PD normal and deficient erythrocytes[15]. The present study was thus conceptualized to estimate the prevalence of G6PD enzyme

deficiency in newborn male and female participants and the molecular defects of the glucose-6-phosphate dehydrogenase gene was characterized in deficient patients. Also, the association between G6PD mutations including Mediterranean mutation (exon 6 C563T), Mahidol mutation (exon 6 G487A) and Chatham mutation (G1003A) polymorphisms and incidence of hemolytic anemia.

We identified four different G6PD deficiency variants namely; G6PD Mediterranean, G6PD Mediterranean with Chatham, G6PD, and G6PD Chatham. We found that about 94% of the participants were suffering from the G6PD Mediterranean mutation. The G6PD Mediterranean, having C to T transition at nucleotide 563 of exon 6 with most prevalent allele has been reported from Mediterranean Middle East and India as well[16]. By 1988, around 400 variants of G6PD was documented. According to a study, G6PD Mediterranean mutation is the most common G6PD deficient variant in West Africa, Mediterranean, Middle East and Southeast Asia. Black people often have a mild deficiency, Orientals are more deficient and the Mediterranean is the most severe[17].

In 2011, another study from Jeddah done by Al-Jaouni *et al* reported high prevalence (89.1%) of G6PD Mediterranean mutation which is much closer to our study[18]. The data from the study of Al-Ali *et al* showed 84% of the participants had the G6PD Mediterranean and is the most common type of mutation in the Eastern Province of Saudi[19]**.** Our data were consistenet with the results obtained by Al-Ali *et al*, suggesting geographical location can be a determinant factor for the similarity. Various other studies from Saudi Arabia also reported G6PD Mediterranean mutation among G6PD deficient patients with a lower percentage. Gari *et al*, and Al Jaouni *et al*, reported 51.1% and 38.1% G6PD Mediterranean mutation among G6PD deficient patients respectively[18,20]. Another study of Al-Hassa and Al-Qatif areas of Saudi Arabia's Eastern province also showed G6PD Mediterranean as the most frequent variant, with a prevalence of 45.9% and 36.5% for these 2 areas[21]. Karadsheh, *et al,* reported 53.3% samples with the Mediterranean mutation in Jordan[22].

Further, the mutations observed in the male and female newborns with G6PD deficiency was not significantly different with Mediterranean mutation being the most common mutation in all population. High prevalence of Chatham mutation was observed in male than in female, which indicates that the frequency G6PD mutations is lower in female than in the male group. The females because of unequal X-chromosome inactivation, are functionally G6PD deficient[23]. In the present study, 10 (40%) females of class II and 15(60%) females of class III were affected; one tested positive for G6PD Med mutation (heterozygous) and the other had prolonged decoloration time. Similarly, high frequency (24.5%) of affected females was reported from Iran[24]. A study from UAE reported 5% of affected females[25].

We also compared the relative frequencies of the class I mutations to the total number of G6PD mutations. The class I mutations affected the exon 6 (44%) and 9 (2%) more frequently, which encodes the regions that bind the enzyme substrate. This result was in agreement with that study conducted by Minucci *et al* in 2012 [26]. They reported that class I mutations affected exon 6, 10 and 13 which encodes the regions that bind the enzyme substrate, dimer interface, and NADP+ structural site, respectively[26]. It is interesting to note that class I mutations result in the more severe phenotype of G6PD deficiency such as chronic non-spherocytic hemolytic anemia. These mutations appear to be the most easily distinguishable in the wide-ranging[26]. Further, our findings showed that the percent of exchange transfusion was higher in

Mediterranean mutation as compared to other mutations; however, the difference was not statistically noteworthy. In Chatham mutation, the need of exchange transfusion and the clinical phenotype was milder. These findings support a common ancestry of the population and the theory of genetic drift throughout Mediterranean regions.

The results of screening test showed that there were 10 cases (40%) of G6PD Mediterranean deficiency type II and 15 cases (60%) of G6PD Mediterranean deficiency type III; hence, the overall frequency of G6PD deficiency among the neonates with prolonged or high jaundice was 17.9 %. Our results were in agreement with the similar study conducted by El-Menshay and Pao *et al*[27,28].Other regions of the world also reported G6PD deficiency among jaundiced neonates[29-31]. Also, the mean serum bilirubin level in all patients was not statistically significant for different types of mutation. Similarly, in an investigation done by Ainoon *et al*, no difference was found between the incidence of neonatal icer, the mean serum bilirubin level and the percent of newborns in need of phototherapy and duration of phototherapy between the two most common types of G6PD gene mutations[32].

The association of G6PD enzyme and its genetic variations, in the gene and thus human body plays an important role in susceptibility to hemolytic anemia. Therefore, it is recommended to perform the further investigation with large sample size and geographical area. Further evaluation of genetic variation in the G6PD regulatory region may fully explain the relationship between G6PD expression and susceptibility to hemolytic anemia.

CONCLUSION

Finally, we can conclude that molecular analysis of glucose-6-phosphate dehydrogenase deficiency conducted in Jeddah revealed a higher prevalence of G6PD Mediterranean (94 %), Mahidol (2 %) and Chatham (4 %) compared with other studies performed in Saudi Arabia. The presence of two mutations Med.563 C/T and Chatham 1003 G/A was detected in one patient causes class I of G6Pd deficiency. The nucleotide 563T is a most frequent polymorphism observed in Saudi Arabia followed by nucleotide 1003A and 487 A.

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Conflict of interest: I declare that there is no conflict of interest.

Author Contribution: Dr Sanaa Tork and Afnan Saad Ekhmimi evaluated the enzyme activity, in addition to DNA extraction and RFLP-PCR analysis. Data interpretation and article writing were carried out equally by the three authors.

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Table 1: Sets of primers, exons, restriction enzymes and PCR fragment size included in the study

Exon	**Enzyme**	**Forward 5'-3'**	**Reverse 5'-3'**	**Fragment size(bp)**
6	*Mbo* II	GCAGCTGTGATCC TCACTCC	GCAAGGTGGAGGAACTG ACC	547 bp
6	*Hind* III	GCGTCTGAATGAT GCAGCTCTGAT	CTCCACGATGATCGGTTC AAGC	104 bp
9	*Bst* XI	CAAGGAGCCCATT CTCTCCCTT	TTCTCCACATAGAGGACG ACGGCTGCCAAAGT	208 bp

Table 2: Mean and standard deviation of the subjects depending on age factor in both groups (Patient and Normal) for male and female gender

Gender	**Variable**	**Group**		***P*-value**
		Normal	**Patient**	
Male	*N*	25	25	1
n= 50	Age (Months)	5.48±3.47	5.3±2.76	
Female	*N*	25	25	.25
n= 50	Age (Months)	5.8±2.99	6.96±3.91	

Table 3: Blood samples (100) tested and categorized according to G6PD enzyme activity and the severity of hemolysis

Group	Residual enzyme activity (%)	Number of male samples	Number of female samples
Patient	Class I Severely deficient, chronic hemolytic anemia	10	0
	Class II 1-10% residual activity	6	10
	Class III 10-60% residual activity	9	15
Normal	Class IV 60-150%; normal activity	12	13
	Class V 150%; increased activity	12	12

Table 4: The association between the presence of *G6PD Med*. 563C/T polymorphism and the incidence of hemolytic anemia

SNP	Group	Mediterranean Genotype distribution			Allele frequency	HWE	p-value[s]	p-value[b]	p-value[s]
		CC	CT	TT					
563 C>T	Normal	(n=49) 98%	(n=1) 2%	(n= 0)	C: 99 (99%) T: 1 (1%)	0.943 *P*:0.33	0.05*	0.001**	0.00**
	Patient	(n=0)	(n= 40) 80%	(n=10) 20%	C: 40 (40%) T: 60 (60%)	0.000 *P*:0.94			

n: No. of the sample.

HWE: Hardy- Weinberg equation

p – Value [a]: expressed as frequencies and were compared by Mann-Whitney test.

p-Value [b] corresponds to genotype distribution using 2-by-3 Chi-Square test.

p-Value [c] corresponds to allele frequency using 2-by-2 Chi-Square test.

** Highly significance (P< 0.05).

Table 5: Frequency of genotypes for both alleles in both groups (patient and normal) for *G6PD Med*. 563 C/T

Group	Class	Genotype	N	Frequency (%)	X^2	Alleles	N	Frequency (%)	*P*-value
Patient	Class I								
	Male	T	10	20.0	4.66	C	139	69.5	.010**
	Female	TT	0	0.0	5.55	T	61	30.5	
	Class II & III Male	C	16	32.0					
	Female	CT	25	50.0					
Normal	Class IV & V	C	24	48.0					
	Male	CC	25	50.0					
	Female								
	Total								
	Male		50	100%			200	100%	
	Female		50	100%					

Data were presented as a number of samples with frequency.

X^2 indicated a significant value in genotype data $P = 0.03$ and $P = 0.018$ in male and female, respectively.

Table 6: Association between the presence of *G6PD Med*. 563 C/T polymorphism and incidence of hemolytic anemia in male

SNP	Group	Mediterranean genotype distribution			Allele frequency	HWE	*P*-value[s]	*P*-value[b]	*P*-value[s]
		C	C Class II	T Class I					
563 C>T	Normal N=25	(n=24) 96%	(n=1) 4%	(n=0)	C: 49 98% T: 1 2%	0.918	.50	.001**	.00**
	Patient N=25	(n=0)	(n=15) 60%	(n=10) 40%	C: 15 60% T: 10 40%	0.032			

n: No. of the sample.

HWE: Hardy- Weinberg expectations.

p –value [a]: expressed as frequencies and were compared by Mann-Whitney test.

p-value [b] corresponds to genotype distribution using 2-by-3 Chi-Square test.

p-value [c] corresponds to allele frequency using 2-by-2 Chi-Square test.

** Highly significance (P< 0.05).

Table 7: The association between the presence of *G6PD Med*. 563 C/T polymorphism and incidence of hemolytic anemia in females

SNP	Group	Mediterranean Genotype distribution			Allele frequency	HWE	*P*-value	*P*-value[s]	*P*-value[s]
		CC	CT	TT					
563C>T	Normal (n=25)	(n= 25) 100%	(n= 0)	(n= 0)	C: 50 100%	Null	.50	Null	.00**
					T: 0 0%				
	Patient (n=25)	(n= 0)	(n= 25) 100%	(n= 0)	C: 25 50%	0.000			
					T: 25 50%				

n: No. of the sample.

HWE: Hardy- Weinberg expectations.

p –value [a]: expressed as frequencies and were compared by Mann-Whitney test.

p-value [b] corresponds to genotype distribution using 2-by-3 Chi-Square test.

p-value [c] corresponds to allele frequency using 2-by-2 Chi-Square test.

** Highly significance (P< 0.05).

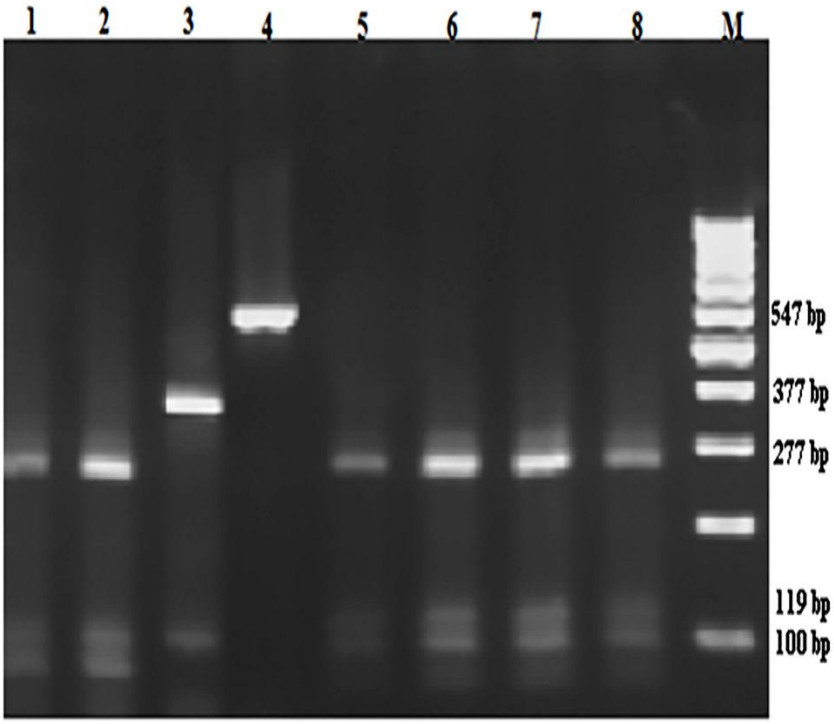

Figure 1: Agarose gel of digested products of 547 base pair fragment containing Mediterranean mutation 563 C>T. Lane 4 showed uncut PCR product. Lanes 1, 2, 5, 6, 7 and 8 showed Mediterranean mutation pattern (pieces of 277, 119, 100 and 60 bp resulted from cutting 547 base pairs fragment of samples with defective G6PD). Lane M indicated 100bp DNA marker. Lane 3 showed the pieces result from cutting 547 base pairs fragment of normal G6PD. In this lane, pieces of 377, 119 and 60 bp can be respectively seen.

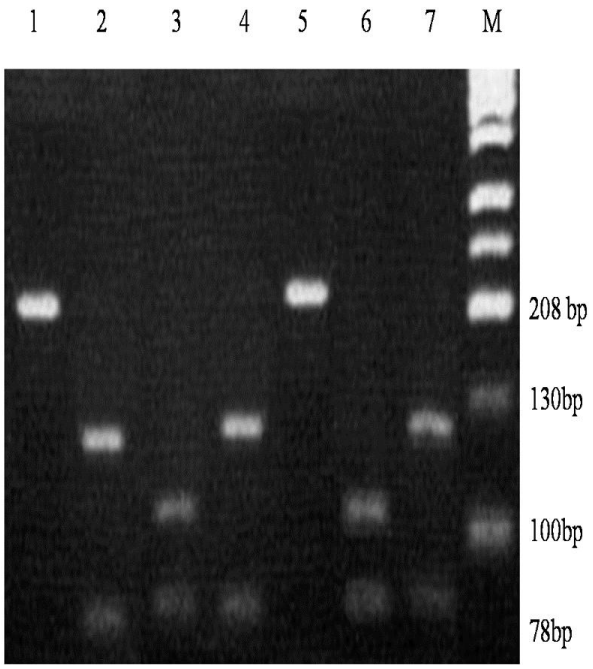

Figure 2: Agarose gel showing hemizygote patient for Mahidol mutation exon 6 variant of G6PD deficiency. Lane M: 100 bp DNA marker; Lanes 1, 4, 6: Mahidol homozygote (82 bp); Lane 3, 4: G6PD normal (Uncut PCR fragment: 104 bp); Lanes 5: G6PD normal/ Mahidol heterozygote (pieces of 104 and 82 bp).

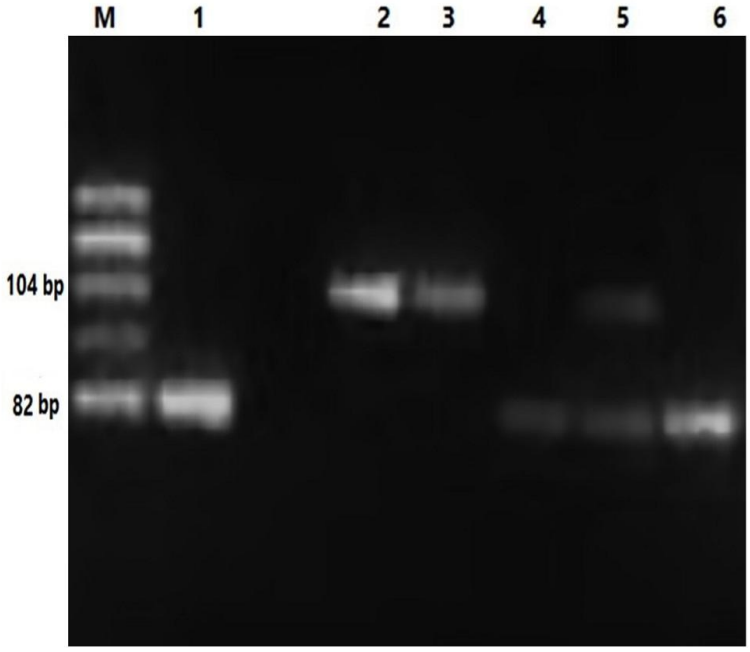

Figure 3: Agarose gel showing restriction digestion of PCR products related to G6PD Chatham mutation with *Bst*XI enzyme. Lane M, DNA marker (50 bp). Lane 1and 5 PCR product(208bp). Lane 3 and 6 the samples have Chatham mutation. Lane 2,4 and 7 without Chatham mutation.