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Prevalence of Three Gene Variants of G6PD Deficient Patients in Sulaimani Province

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Abstract

To detect the variants represents three mutations in Glucose-6-phosphate dehydrogenase (G6PD) gene of hemolytic anemic patients in Sulaimani governorate, tests revealed normal PCV, Hb, and G6PD activity ranges in the 10 subjects control sample, while patient's PCV ranged 10-44%, Hb 3.2-13.8 g/dl, and G6PD activity 0.35-8.7 Iu/gHb (2.9-74.3%). According to the G6PD activity, the severity of the anemia among the 40 patients was distributed as 6(15%) mild 25(62.5) moderate and 9(22.5) severe with no healthy. The Mediterranean variant among patients, as detected by PCR amplification of 545bp exons 6 & 7 and digestion by MboII endonuclease, prevalent 33(82.5%), The Chatham variant, as detected by amplification of 253bp exon 9 and digestion by BstXI, prevalent 2(5%), and Cosenza variant, as detected by amplification of 665bp exons 11-13 and digestion by Bsu36I (Eco81I) endonuclease, prevalent 4(10%), while 4(10%) did not revealed any of the three variants regarding that 3(7.5%) of the patients showed both Mediterranean and Cosenza. Out of 7 Mediterranean variant's females, 1 was homozygous. The 10 control subjects did not reveal any of the three variants.

Key words: G6PD, PCR amplification, Mediterranean, homozygous, variants

Introduction

Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency is the most common human genetic enzymopathy, affecting more than 400 million people worldwide (Bulliamy T. et al., 1997). G6PD is an essential enzyme to cell growth. Its deficiency causes favism and food or drug induced acute hemolytic anemia (Ewa Ja.-Sk., 1999), neonatal jaundice, and chronic non-spherocytic hemolytic anemia (Haung Ch.-Sh., 1995). G6PD is a cytoplasmic enzyme that catalyzes the first step in pentose phosphate pathway (conversion of glucose-6-phosphate to 6-phosphoglucono-δ-lactone) with the concomitant reduction of NADP+ to NADPH (Nicole Ch.J., 2000).

G6PD deficiency is a hereditary disease inherited through mutation in G6PD gene which is one of the best mapped in the human genome that spans 18.5 kb on the X chromosome (xq28) and contains 13 exons (Ewa Ja.-Sk., 1997) and encodes a protein (G6PD enzyme) composed

variably of two or four identical (515 amino acids) subunits, each monomer has a molecular weight of 59.26 kDa (Beutler E., 1994).

Polymorphisms of the G6PD gene are numerous, with G6PD deficiency due to single point mutations, deletions, insertions and, rarely, splicing variants. Approximately 200 G6PD variant alleles have been described related with geographical locations and races, 140 variants were characterized at the DNA level by 2002 (Beutler E & Bulliamy TJ, 2002) which are missense mutations leading to amino-acid substitutions and in a few cases base pair deletions that do not produce frame shifts (Gaskin et al., 2001).

The most common G6PD variants is Mediterranean variant (Bulliamy T. et al., 1997) which has cDNA nucleotide substitution at base position C563T (Ser 188 Phe), also G6PD-Chatham G1003A (Ala 335 Thr) and G6PD-Cosenza G1376C (Arg 459 Pro) are of clinical significance (Beutler E. et al., 1991).

G6PD variants are classified according to the demographic location, enzyme activity, electrophoretic mobility, and clinical manifestations. G6PD deficiency is very common in West Africa, Mediterranean, Middle East, and South East Asia. Mediterranean variant is the most severe variant (Kurdi-Haidar et al., 1990).

This study was aimed to detect the most prevalent mutations of G6PD gene in clinically diagnosed G6PD deficient patients in Sulaimani, and the frequency of each mutation through using DNA amplification technique by polymerase chain reaction (PCR) and digestion by restriction endonucleases.

Methods

Two ml of venous blood was collected in EDTA tubes from each of the 40 unrelated individuals (32 males and 8 females) that were clinically diagnosed as G6PD deficient patients (from beginning of Feb. 2009 to the end of Apr. 2009). Also, venous blood by the same method was collected from 10 apparently healthy people; whose histories do not have signs of G6PD deficiency.

Hemoglobin concentration was estimated according to Barbara J.B. and Imelda B. (2001).

In order to detect bite cells and blister cells blood smears were stained by Leishman's stain according to Barbara J.B. and SM Lewis (2001). In order to detect Heinz bodies blood smears were stained by methylene blue stain according to Barbara J.B. and Imelda B. (2001). Packed Cell Volume was estimated according to Barbara J.B. and Imelda B. (2001). G6PD activity in red blood cells was estimated according to Tietz N.W. (1999).

DNA was extracted using 'salting-out' technique according to Miller et al., 1988.

The population group comprises native Kurdish people. Primers for PCR amplification of the fragments that includes the site of mutation were chosen based on the G6PD gene sequence deposited in NCBI (accession number X55448) have been previously reported (Poggi et al. 1990).

The sequences of these primers are as follows:

F-Med 5'- ACTCCCCGAAGAGGGGT…-3' exons 6 and 7 encompassing C563T mutation
R-Med 5'- CCAGCCTCCCAGGAGAGA .-3'

F-Chat 5'-… CAAGGAGCCCATTCTCT …3' exon 9 encompassing G1003A mutation
R-Chat 5'-… TGCCTTGCTGGGCCTCG …3'

F-Cos 5'-… CTTCAACCCCGAGGAGT …3' exons 11-13 encompassing G1376C mutation
R-Cos 5'..GGGAAGGAGGGTGGCCGTG...3'

The PCR mixture contained 0.25µl (5u/µl) of Hot Start Taq DNA polymerase (0.025u/µl), 5µl 10X of PCR buffer (1X), 3µl 25Mm of MgCl2 (1.5mM), 1µl 10mM of dNTPs (0.2mM), 1µl 10µM of F-primer (0.2µM), 1µl 10µM of R-primer (0.2µM) and 2µl of DNA sample were used in a final volume of 50 µl.

Amplification of 545 bp Mediterranean (exons 6 & 7) include initial denaturation for 3 min. at 94°C, then 35 cycles: each cycle consists of 1 min., 30 sec., and 40 sec. at each of the following temperatures respectively 94°C, 57°C, and 72°C (according to primer manufacturer), and final extension for 5 min. at 72°C.

Amplification of 253 bp Chatham (exon 9) include initial denaturation for 3 min. at 94°C, then 30 cycles: each cycle consists of 1 min., 30 sec., and 20 sec. at each of the following temperatures respectively 94°C, 54°C, and 72°C (according to primer manufacturer), and final extension for 5 min. at 72°C.

Amplification of 665 bp Cosenza (exons 11-13) include initial denaturation for 3 min. at 94°C, then 36 cycles: each cycle consists of 1 min., 30 sec., and 50 sec. at each of the following temperatures respectively 94°C, 57.6°C, and 72°C (according to primer manufacturer), and final extension for 5 min. at 72°C. The amplification products were analyzed on 1% of agarose gel at 90 V, 100 mA, 9 W for 60 min.

For RFLP analysis, the PCR products were digested with either the restriction endonucleases. The amplification product (545bp) of Mediterranean primer was digested with MboII by mixing 10µl of PCR product; 16µl of DNase free water; 3µl of 10X buffer B and 1µl of MboII enzyme (5u/µl) (according to Fermentas manual), then incubated in waterbath for 60 min at 37°C. The restriction enzyme was inactivated for 20 min at 65 °C.

PCR product (253bp) of Chatham primer was digested with BstXI by mixing 10µl of PCR product; 16µl of DNase free water; 3µl of 10X buffer O and 1µl of BstXI enzyme (10u/µl) (according to Fermentas manual), then incubated in waterbath for 60 min at 55°C. The restriction enzyme was inactivated for 20 min at 80 °C. PCR product (665bp) of Cosenza primer was digested with Bsu36I (Eco81I) by mixing 10µl of PCR product; 16µl of DNase free water; 3µl of 10X buffer Tango and 1µl of Eco81I enzyme (10u/µl) (according to Fermentas manual) then incubated in waterbath for 60 min at 37°C. The restriction enzyme was inactivated for 20 min at 80 °C. The digestion products were analyzed on 2% of agarose gel at 80 V, 100 mA, 8 W for 60 min.

Results

The exons 6 and 7 PCR amplicons of G6PD gene (545 bp) were digested by MboII restriction endonuclease, 33 out of 40 deficient samples (82.5%) were positive for Mediterranean variant, when ran on agarose gel electrophoresis while the control samples were not. Seven negative deficient samples as well as all healthy samples (controls) showed 4 fragments 27, 337, 61 and 120 bp whereas the positive samples for MboII typing showed 5 fragments, that represent the Mediterranean variant, which were 27, 103, 234, 61 and 120 bp(figure1).

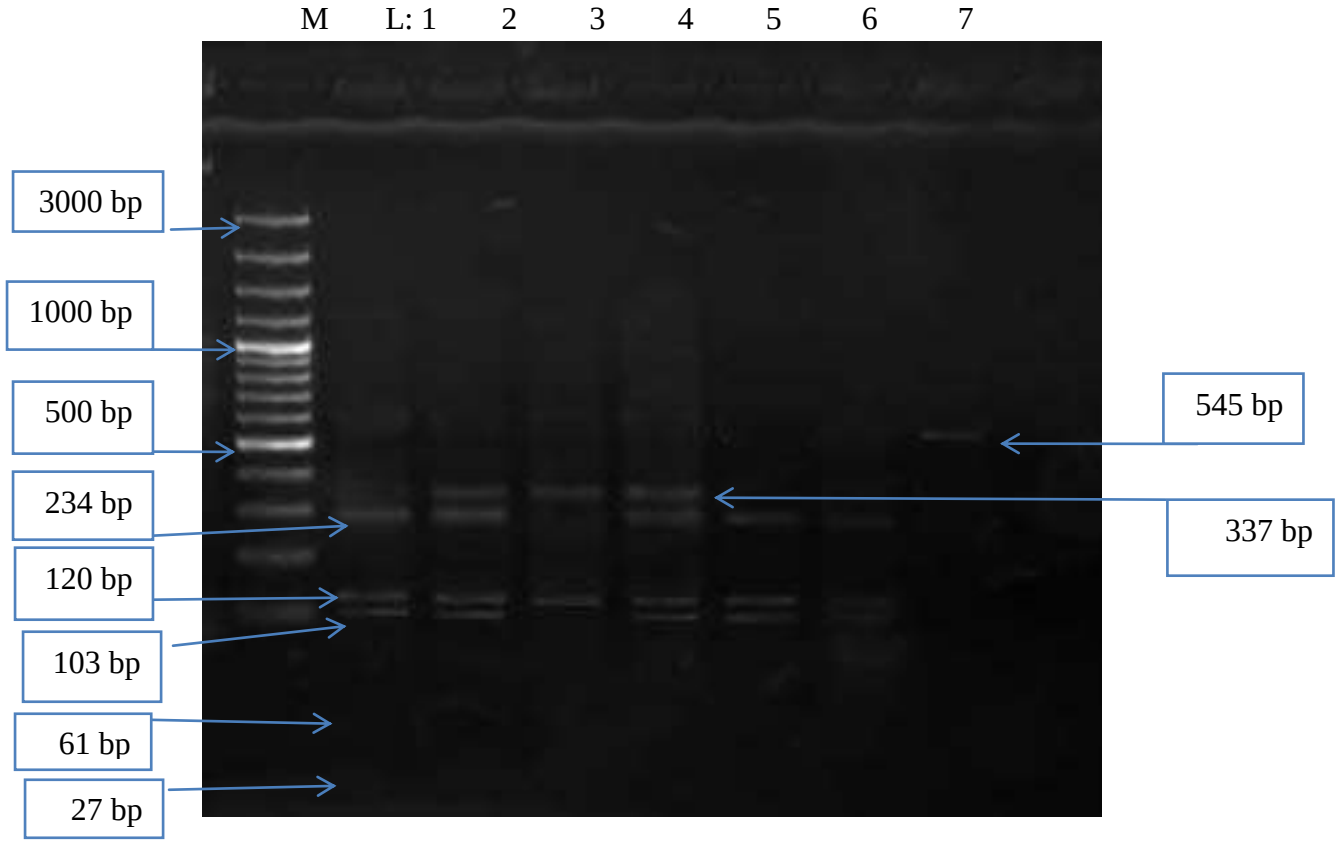

Figure 1: Agarose gel electrophoretic pattern of restriction digestion of exons 6 and 7 of G6PD gene by MboII endonuclease. Samples were run on 2% agarose gel, at 80 V, 100 mA, 8 W for 60 min. stained with Ethidium bromide.
M: DNA ladder marker (3 kb); Lanes 1, 6: G6PD Mediterranean mutation (hemizygous male); Lanes 2, 4: Mediterranean variant (heterozygous female); Lane 3: healthy sample (Control); Lane 5: Mediterranean variant (homozygous female); Lane 7: PCR amplification product (545 bp).

The exon 9 PCR amplicon of G6PD gene (253 bp) was digested by BstXI restriction endonuclease, 2 out of 40 deficient samples (5%) were positive for Chatham variant when ran

on agarose gel electrophoresis while the control samples were not. The 38 negative deficient samples as well as all healthy samples (controls) showed 2 fragments (77 and 176 bp) whereas

the positive samples for BstXI typing showed 3 fragments, that represent the Chatham variant, which were 77, 96 and 80 bp (Figure 2).

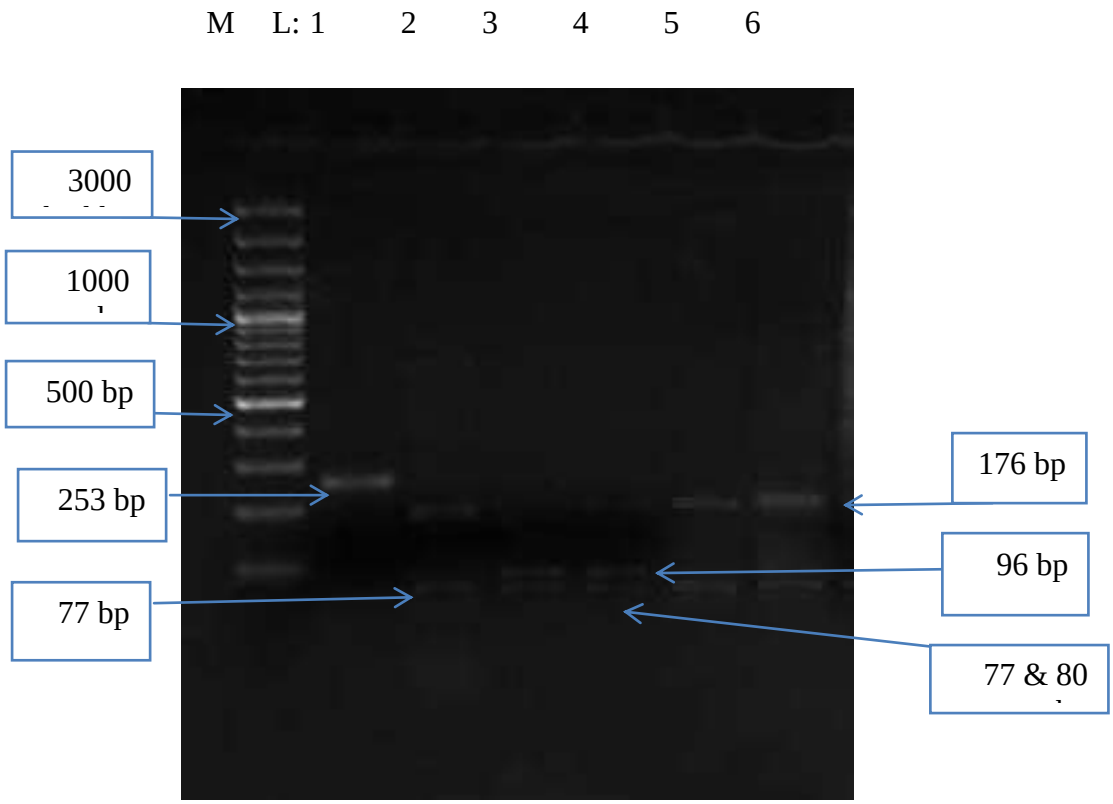

Figure 2: Agarose gel electrophoretic pattern of restriction digestion of exon 9 of G6PD gene by BstXI endonuclease. Samples were run on 2% agarose gel, at 80 V, 100 mA, 8 W for 60 min. stained with Ethidium bromide.
M: DNA ladder marker (3 kb); Lane 1: PCR amplification product (253 bp); Lane 2: healthy sample (control); Lanes 3, 4: Chatham variant (hemizygous male); Lanes 5, 6: BstXI negative variants.

The exons 11-13 PCR amplicons of G6PD gene (665 bp) were digested by Bsu36I restriction endonuclease, 4 out of 40 deficient samples (10%) were positive for Cosenza variant when ran on agarose gel electrophoresis while the control samples were not. The 36 negative deficient samples as well as all healthy samples (controls) showed the same amplicon (665 bp) without cutting whereas the positive samples for Bsu36I typing showed 2 fragments, that represent the Cosenza variant, which were 350 and 315 bp (Figure 3).

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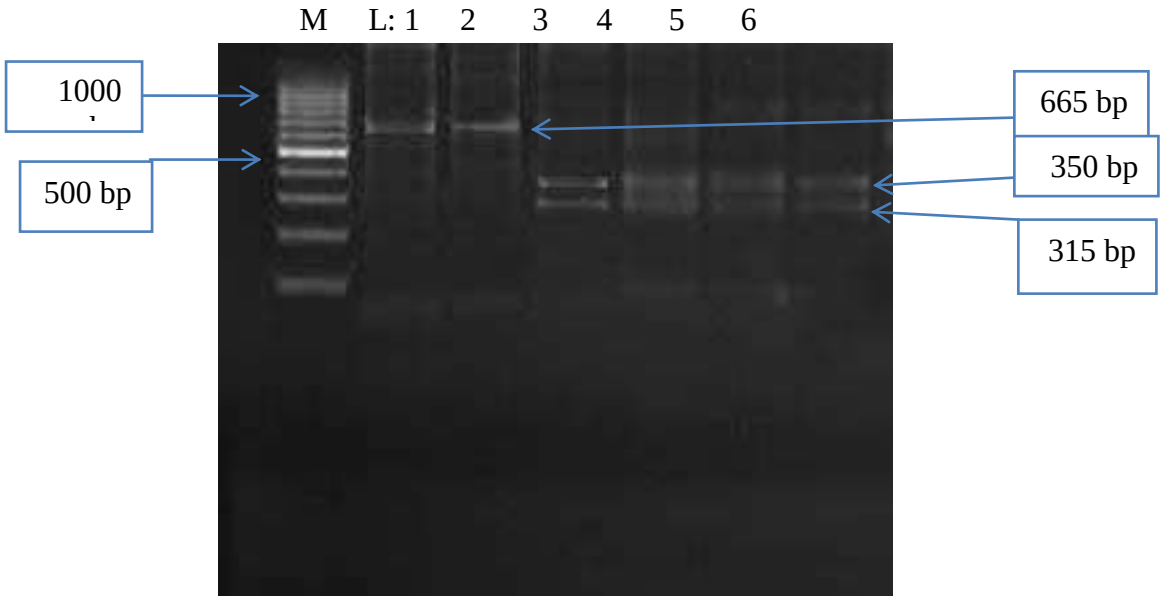

Figure 3: Agarose gel electrophoretic pattern of restriction digestion of exons 11-13 of G6PD gene by Bsu36I (Eco81I) endonuclease. Samples were run on 2% agarose gel, at 80 V, 100 mA, 8 W for 60 min. stained with Ethidium bromide.
M: DNA ladder marker (1 kb); Lane 1: PCR amplification product (665 bp); Lane 2: healthy sample (control); Lanes 3, 4, 5, 6: Cosenza variant (hemizygous male).

Table (1): Distribution of different G6PD deficiency variants among 40 deficient patients of Sulaimani province

Subjects	No. Of Subjects	Med° No. (%)	Chat° No. (%)	Cos° No. (%)	Med & Chat No. (%)	Med & Cos No. (%)	Chat & Cos No. (%)	-Ve
Males	**32**	**23(71.8)**	**2(6.25)**	**1(3.12)**	**-**	**3(9.3)**	**-**	**3(9.3)**
Females	**8**	**7*(87.5)**	**-**	**-**	**-**	**-**	**-**	**1(12.5)**
Total	**40**	**30(75)**	**2(5)**	**1(2.5)**	**-**	**3(7.5)**	**-**	**4(10)**

°-ve: No G6PD mutation for the three variants (Mediterranean, Chatham and Cosenza) was detected.
* Out of the 7 Mediterranean females, 1 was homozygous and 6 were heterozygous (Figure 4-6).

Discussion

The two bands 27 and 61 bp not appeared in figure 1 because of their small size, although the concentration of agarose gel was increased to 2% and the voltage was decreased to 80 V. In order to get these two small bands 10% acrylamide gel is recommended to use, but unfortunately it has no facility at that time.

It was clear that male patients showed hemizygosity with digestion of the mutant fragment and have no homologous wild allele. Females showed heterozygous pattern through appearance of the 6 fragments which encompass the wild fragment (337 bp) for the wild allele of one X-chromosome and the two fragments (103 and 234 bp) for the mutant allele of the other X-chromosome that result from digestion of the 337 bp fragment.

The homozygous females showed 5 fragments appeared in heterozygous except the wild fragment which appeared to be digested in both homologous X chromosomes. Heterozygous detection is entirely reliable although one X chromosome in females is inactive, it does not prevent the detection of its genes or their mutations. In fact the X inactivation may alter the methylation pattern of DNA on the inactive X chromosome (Fairbanks et al., 1980; Hirono A. & Beutler E., 1988), which leads to prevent transcription (Kurdi-Haidar et al., 1990) but does not affect its DNA detection.

In figure 2 the two bands 77 and 80 bp were appeared in one band because of their close size that the difference is only 3 bp. In order to resolve these two bands 12% acrylamide gel is recommended to use, but unfortunately it was unavailable. Also selecting the reverse primer in a more distant location can solve this problem. The two positive cases were male that represented hemizygosity and so there was no opportunity to detect heterozygous females.

In figure 3 like BstXI typing, the four patients were also males and so there was no opportunity to detect heterozygosity among females.

From the restriction endonuclease typing the prevalence of the three deficiency variants studied (Mediterranean, Chatham and Cosenza) distributed among males and females is shown in Table (3). The most prevalent variant was Mediterranean 75% followed by Chatham 5% and Cosenza alone 2.5%, while Cosenza appeared with Mediterranean for 7.5% of total patients. This result revealed that Cosenza (10%, alone and in combination with Mediterranean, not appeared in Table 3) is more frequent than Chatham variant regarding its frequency rather than its appearance alone, while the prevalence of Mediterranean variant alone and in combination with Cosenza was 82.5% (not appeared in Table 3). Out of 40 clinically diagnosed G6PD deficient, 4 were negative for the three variants. The 4 negative subjects have appeared to be mild deficient according to the G6PD activity. So either they are other variants or they are mild deficient due to physiological reasons leading to affect G6PD activity.

Cases of sporadic gene mutations occur in all populations, Mediterranean variant mostly affect Italian, Grecian, Spanish, Arabic and Kurdish Jews (Gregg X. T. & Prchal J. T., 2000). Different populations have different types of mutations, but within a specific population, common mutations are usually shared.

Most G6PD deficient individuals in the Middle East have the G6PD Mediterranean variant, also G6PD-Chatham, G6PD-Cosenza and other variants are found in some area (Luzzatto & Notaro, 2001).

So, the prevalence of Mediterranean variant alone in Sulaimani is common (75%, Table 3). The population of Sulaimani city is significantly pure Kurdish, but in general Mediterranean variant is the commonest G6PD deficiency variant in the nearest Kurdish involving countries such as Turkey 77% and Iran 69% (Noori-Daloii et al., 2006)

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