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Original article

Development of allelic discrimination assay to detect Mediterranean G6PD mutation and its linked inheritance with normal vision and/colorblindness loci for 4 generations among Egyptian and Emirati families

Lina Maloukh [a,1], Alagappan Kumarappan [b,1], Houssam El-Din El-Wakil [c], Fatima Al-Kamali [d], Fatma Gomma [e], Amin Akhondi [f], Rajya Lakshmi T.V. [g,*]

[a] Zayed University, College of Natural and Health Sciences, Abu Dhabi, Khalifa City, United Arab Emirates
[b] Al Qassimi Women's and Children's Hospital, Molecular Biology Laboratory (Pure Health), Wasit Street, Sharjah, United Arab Emirates
[c] Alexandria University, Faculty of Agriculture (Saba Basha), Alexandria, Egypt
[d] Dubai Health Authority, Dubai, United Arab Emirates
[e] Science Tribune Software House, Dubai, United Arab Emirates
[f] University of Modern Sciences, College of Biotechnology, Dubai, United Arab Emirates
[g] Beaconhouse International College, Science Department, Ontario, Canada

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ABSTRACT

G6PD deficiency c563T is the most common inherent blood disease among the Mediterranean populations and its molecular diagnosis is critical as the enzyme assay fails for heterozygous individuals. The purpose of the study is to estimate the ubiquity of the heterozygous G6PD Med (c563T) variants among Egyptians and UAE nationals living in Dubai. We validated two molecular methods, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and qPCR allelic discrimination assay for detection of G6PD Med variants. Among 100 screened individuals, G6PD c563T variants are 30% of whom 15% are carriers. Sanger sequencing validated the qPCR discrimination assays. In search of a phenotypic marker to detect G6PD heterozygous variants, inheritance of G6PD locus and red-green color vision genes is studied in 1 Egyptian and 2 Emirati families. Among the 3 families, G6PD is polymorphic, displaying 4 phenotypes: in phenotype-1, person is normal, in phenotype-2 the person has no G6PD deficiency but with deuteranopia/deuteranomaly, in phenotype-3 the person is G6PD Med variant with deuteranopia/deuteranomaly and in phenotype 4 the person is G6PD Med variant has normal vision. Based on the molecular analysis of G6PD and Ishihara vision test it can be concluded that the two mutations at the two loci arose independent of each other without any interaction (epistatic effect) between them. Following the pedigree analysis of the two genes for 4 generations it is presumed that it is infeasible to use "deuteranopia /deuteranomaly" as a phenotypic marker to detect G6PD c563T heterozygous individuals among the Egyptian populations.

1. Introduction

The long arm of human X chromosome is gene and CpG rich. Among the X linked genes at q28 telomere, G6PD encodes a metabolic enzyme crucial in the pentose phosphate pathway, provides NADPH that protects the red blood cells from reactive oxygen species (Nkhoma et al., 2009). G6PD gene is remarkable for its genetic diversity with 217 mutations of which 182 (83.9%) are single nucleotide mutations (Gómez-Manzo et al., 2016). Mediterranean mutation (Class II of G6PD), the most common variant among the Mediterranean populations, is a point mutation of C/T substitution

* Corresponding author.
E-mail addresses: Lina.maloukh@zu.ac.ae (L. Maloukh), alagappan@neoscience.ae (A. Kumarappan), Lakshmibeaconhouse@hotmail.com, v.lakshmi@ums.ae (R.L. T.V.).
[1] Both are the first authors.

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at nucleotide 563 in the gene, with a switch of serine/phenyl alanine at amino acid 188 in the G6PD enzyme (Al-Ali et al., 2002; Alfadhli et al., 2005; Al-Musawi et al., 2012; Karimi et al., 2003; Vulliamy et al., 1988).

Diagnosis of G6PD Med variants (c563T) in the heterozygous females gives rise two possible scenarios: (1) if the X chromosome with mutant allele is inactive, the female would have normal G6PD enzyme, with mutant allele eclipsed, (2) but if the X chromosome with normal allele is inactivated the woman would be G6PD enzyme deficient. Because of this complication, molecular techniques for flawless diagnosis of the G6PD Med variants are mandatory (Arnaout et al., 2011). The primary objective of the present study is to detect the Mediterranean heterozygous carriers (c563T) accurately through molecular techniques among UAE nationals and Egyptians inhabiting in Dubai (UAE).

There are a few genetic studies describing the linkage disequilibrium between G6PD and the red green color vision genes. Wild G6PD allele is linked with the color vision genes in Sardinia and Turkish people (Filippi et al., 1977; Yücel et al., 1992), while the mutant allele of G6PD is similarly linked among Italian, Greek and Chinese populations (Filosa et al., 1993; Fraser et al., 1963; Wu et al., 1988). However, there are no reports of such linkage among the Mediterranean populations; in an attempt to maneuver red-green color blindness as phenotypic marker to detect the G6PD c563T Med carriers, 3 Arabian families (1 Egyptian and 2 Emirati) with G6PD deficiency are screened for their color vision; pedigrees are constructed for the three families up to 4 generations in the present study.

2. Materials and methods

2.1. Sample collection and genomic DNA extraction

Sterilized swabs (n = 100) were used to collect the cheek saliva samples from each person under aseptic conditions into sterile ethylene diamine tetra-acetic acid (EDTA) vials. Genomic DNA was extracted from all the samples with Qiagen genomic DNA isolation kit (lot No. 69581) following the manufacturer's instructions and the DNA is stored at -20^0C for further analysis.

2.2. Detection of G6PD c563T mutation by PCR-RFLP

PCR conditions and restriction enzyme digestion are as mentioned by Ezz El-Deen's group (Ezz-El-Deen et al., 2013). PCR reaction was set with specific primers to amplify the DNA for healthy and G6PD c563T variants (Table 1). PCR reaction mixture of 25 μl contained: 12.5 μl of 2X PCR Master mix, 1 μl of Forward Primer, 1 μl of Reverse Primer, 9.5 μl nuclease-free water, and 1 μl template DNA (60–80 ng/μl). PCR (T100 BioRad, USA) conditions were as follows: 1 cycle (94 °C for 3 min), 30 cycles (94 °C for 45 sec, at 62 °C for 45 sec, at 72 °C for 1 min) and 1 cycle at 72 °C for 7 min. Each PCR product was digested with *MboII* restriction enzyme (NEB, lot No. R0148S), 20 μl mixture contained 2 μl of the PCR amplicon, 1 μl of *MboII,* 2 μl of 10X buffer and 15 μl of nuclease free water; is set at 37°C for 30–45 min. The digested amplicons were visualized on 2% agarose gel electrophoresis with 100 bp marker. The 547 bp long amplicon from a healthy person upon *MboII* digestion yields 2 visible bands of 377 and 119bps; while those of Med variants yield bands of 277, 119 and 100 bps respectively (as shown in Figure-1).

2.3. Allelic discrimination assay

Taqman® allelic discrimination assay was performed in Bio-Rad CFX96 thermal cycler (CFX96 iCycler Touch™ system). The assays included unlabeled forward and reverse primers along with two fluorescent TaqMan® probes which were designed following GenBank Accession No: X55448 and Primer 3 online software (Rozen and Skaletsky, 2000). TaqMan® probe of wild allele was labeled with FAM reporter dye at 5′ end and quencher BHQ1-3′ at the 3′ end; the probe of the mutant allele (c563T) was with HEX reporter at 5′ end, and quencher BHQ1-3′ at the 3′ end (Table 1). The FAM probe specifically binds to the wild amplicon and HEX probe binds to the mutant amplicon which are generated during PCR amplification and each selectively reports the respective allele.

From the results of PCR-RFLP, the samples of 10 people (5 of the affected and 5 healthy) were further analyzed through qPCR. After conforming the results of the 10 people, cheek saliva samples of 3Arabian families (n = 26) were collected and were assayed through qPCR.

Each real time PCR reaction mixture of 20 μl contained: 10 μl 2X TaqMan® genotyping master mix (ABI Applied Biosystems), 1 μl of forward primer, 1 μl of reverse primer, 1 μl of each wild probe and/or mutant probe, 1 μl of genomic DNA (60–80 ng/μl) and 5 μl of nuclease-free water. The concentration of genomic DNA (60–80 ng/μl) was so chosen after standardization, as they did not affect melting profiles. The reaction mixtures were loaded in a 96 well Bio-Rad Real time PCR system, a sample of negative control (no DNA template with water), was also included. The PCR conditions were: 1 cycle of denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 30sec, and 60°C for 45sec and Melt curve at 50–85°C. PCR program was run on allelic discrimination setup and the results were analyzed through discrimination curves. After PCR amplification, in the healthy person with CC genotype, FAM will fluoresce showing red peaks, in the affected TT genotype HEX will fluoresce showing blue peaks and the heterozygous with both peaks on the screen. All qPCR reactions were done in triplicate and final quantification cycle (Cq) values were the means of 3 samples.

2.4. DNA sequencing

After detecting the c563T mutant samples, a 50 μl reaction is set for PCR amplification with the primers used in qPCR reaction. The reaction mixture of 50 μl contained : 25 μl of Taq PCR Master Mix (Norgen Biotek, Canada), 1 μl of the DNA template (50–80 ng/ μl) from the mutant samples, 1 μl forward primer 5′- CTGGAACCG-

Table1
The primer sets and/or probes used in the PCR-RFLP and qPCR methods.

Name of Primer/probe	Sequence for c563T mutation	Length (nt)	Restriction enzyme
	PCR-RFLP		
Forward Primer	5′-ACTCCCGAAGAGGGGTTCAAGG-3′	22	*MboII (NEB)*
Reverse Primer	5′-CCAGCCTCCCAGGAGAGAGGAAG-3′	23	*MboII (NEB)*
	Allelic Discrimination Assay		
Forward Primer	5′- CTGGAACCGCATCATCGT −3′		–
Reverse primer	5′- GGTTCTGCACCATCTCCTT −3′		–
Probe – Wild type	5′-FAM-CAACCACATCTCCTCCCTGTTCC –BHQ1-3′		–
Probe-Mutant type	5′-HEX- CCAACCACATCTTCTCCCTGTTCC –BHQ1-3′		–

CATCATCGT −3′ (10 μM), 1 μl reverse primer 5′- GGTTCTGCAC-CATCTCCTT −3′ (10 μM) and 22 μl distilled water. The PCR conditions were as follows 1 cycle (94°C for 3 min), 35 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) and 1 cycle 72°C for 7 min. Amplified PCR products were checked on 1.5% agarose gel electrophoresis for the 150 bp bands; were sent to Macrogen (Seoul, South Korea) for DNA sequencing. The obtained forward and reverse sequences were aligned by using online Pairwise alignment tool and submitted to NCBI BLAST search for identification of the query sequences (www.ncbi.com) and submitted to GenBank are with Accession number- MT975591.

2.5. Pedigree analysis among the 3 Arabian families −1 Egyptian and 2 Emiratis

After screening the cheek saliva samples for c563T variant, fresh samples were collected from G6PD affected family members (n = 26) viz., grandparents, parents, children and grandchildren for 1 Egyptian and 2 Emirati families. The analysis was repeated for the samples employing real time PCR discrimination assay and Sanger sequencing. The Egyptian family members were thirteen, and those of Emirati were eight and five respectively.

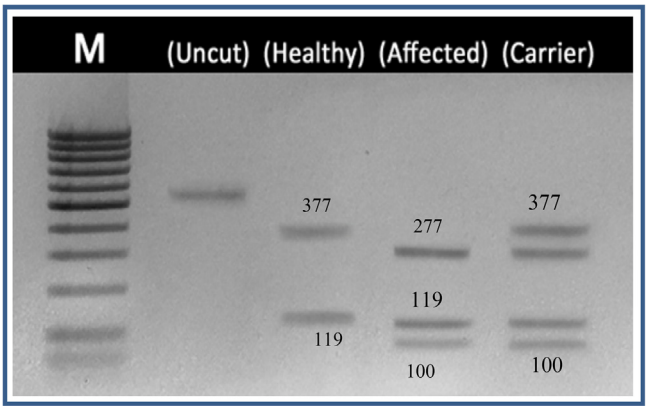


Fig. 1. PCR-RFLP (*MboII)* assay of healthy and c563T homozygous and heterozygous variants. Size marker (M) 50 bp.

2.6. Color blind test

Color vision was checked for the family members with Ishihara 38 plates test following the test instructions (Birch, 1985). Individuals who read 13 plates or less are red green color blind while those who read 17 or correctly were diagnosed to have normal vision; there were no individuals who read 14–16 plates correctly. The results were further confirmed by Ishihara 38 plate test done by Global health Bureau, (USAID) for the affected individuals.

3. Result

3.1. Detection of G6PD c563T variants by PCR-RFLP

The PCR amplified DNA (n = 100) with specific primers yielded a ~ 550 bp fragment could be visualized on 2% agarose gel (Fig. 1, uncut). The PCR product after restriction digestion with *MboII* for 45 mins, yielded 2–4 fragments in electrophoresed gel: for a normal person 2 visible bands of 377 bp and 119 bp (Fig. 1 healthy), for mutant males and for mutant homozygous females three bands viz, 277 bp, 119 bp and 100bps (Fig. 1 affected) and for heterozygous females (carriers) 4 fragments of 377 bp, 277 bp, 119 bp and 100 bp (Fig. 1 Carrier). As expected the mutation C →T creates an *MboII* site where the restriction enzyme cleaves the 377 bp DNA band into two bands of 277 bp and 100 bp. From a total of 100 samples, 70 are healthy, 30 are affected, and of who 15 are hemizygous and homozygous (male and female) while 15 of them are heterozygous carriers. The gel observed results of healthy and c563T variants are consistent with Sequencing, GenBank accession number MT975591 (see Fig. 2).

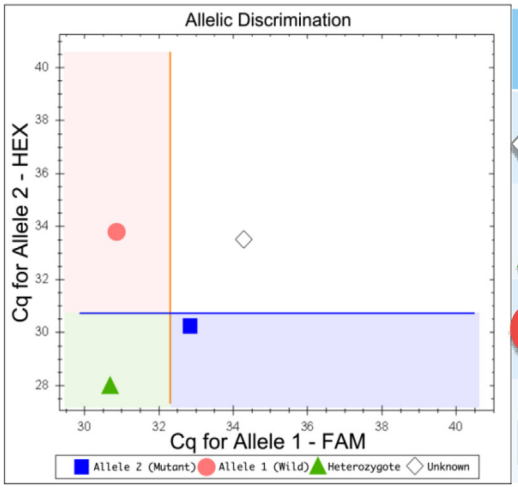
3.2. Detection of G6PD c563T variants by TaqMan® qPCR

The TaqMan® PCR results of the 10 samples are shown in figure-2 and those of the Arabian families in table-2.

3.3. Inheritance of c563T mutation and color blindness in the 3 families:

3.3.1. Inheritance of c563T mutation in Egyptian family (1)

The family included thirteen members over four generations from grandparents (P) to great- grandchildren (F3). The members are 2



#	SAMPLE	Cq 1	Cq 2	TYPE
◇	Negative Control	40	40	None
▲	G6PD **Carrier**	30.66	27.93	Heterozygote [CT]
●	G6PD **Normal**	34.27	33.51	Allele 1 (Wild) [CC]
■	G6PD **Affected**	32.9	30.35	Allele 2 (Mutant) [TT]

Fig. 2. Genotyping for G6PD c563T variants through TaqMan® qPCR. X-axis represents the relative fluorescent emission (Cq: quantification value) for the wild allele (CC) - the specific probe labeled with FAM reporter, while the Y-axis represents (Cq value) for the mutant allele (TT) - specific probe labeled reporter HEX. The three groups indicate three different genotypes of G6PD normal and variants; red indicates normal homozygous wild allele, green indicates heterozygous c563T variant, blue indicates homozygous TT affected and white is negative control.

parents in **(P),** 1 daughter **(F1)**, 2 grandchildren **(F2)** and 5 great-grandchildren **(F3).** Allelic discrimination assay qPCR of G6PD gene revealed 7 members healthy, 4 heterozygous carriers and 2 hemizygous (Table 2). The heterozygous carriers are grandmother, daughter (F1), granddaughters in F2 and F3 generations; while the hemizygous Med mutants are grandsons in F2 and F3 (Fig. 3).

3.3.2. Inheritance of red-green color blindness

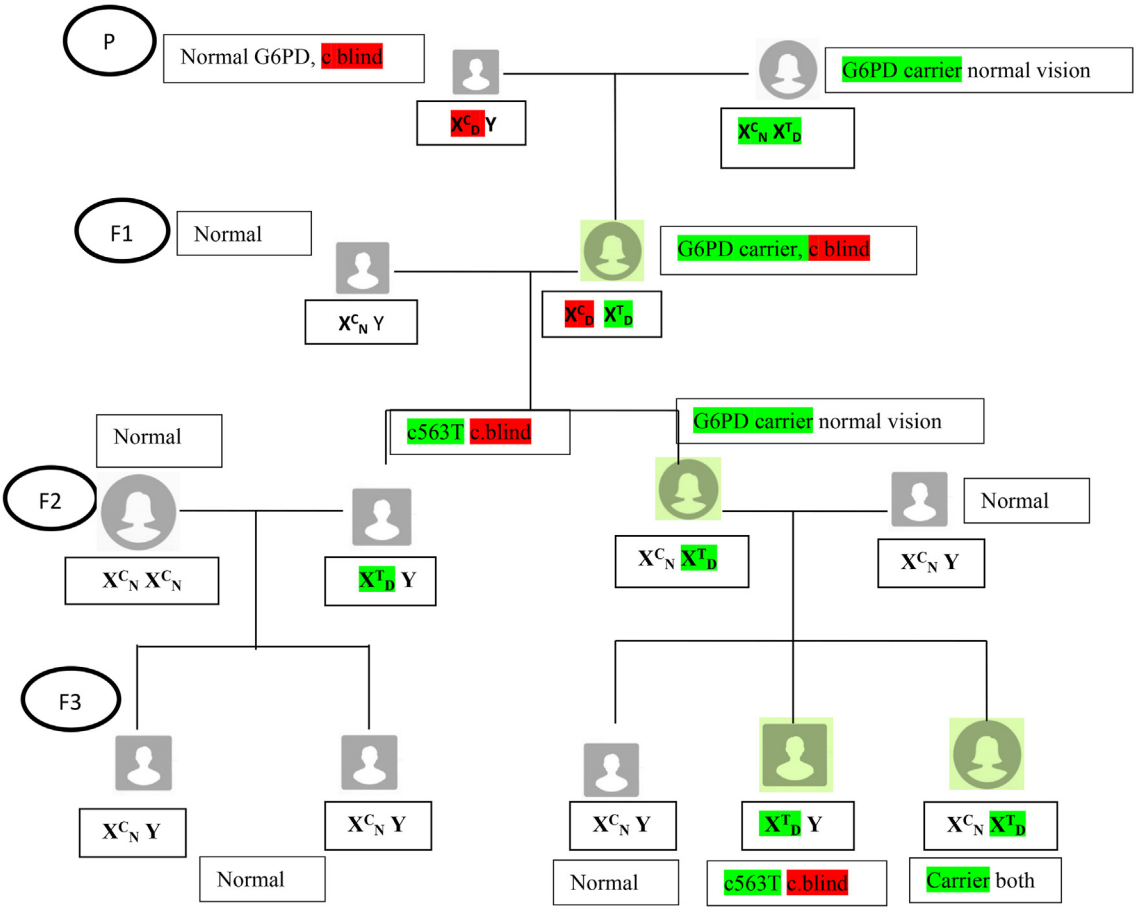
The Ishihara 38 plate test (Global health Bureau, USAID) of the family (n = 13) revealed that male parent (in P), daughter (in F1), grandson (in F2) and maternal great grandson (in F3) are Deuteranopes **(the result of great grandson in F3, Supplementary S-1).**

Pedigree analysis discloses that the father in P generation is color blind-deuteranope, his daughter (F1) is deuteranope, inherited the defective genes from both the parents. The deuteranope mother in F1, passed the defective gene to both her children (F2), son is deutan color blind, while daughter is normal visioned (carrier). The Deuteranope son married a normal visioned lady; his 2 sons (in F3) had normal vision. The carrier mother (in F2), passed the defective MW gene/s to two of three children in F3, son is deuteranope and the daughter is normal visioned (carrier, Fig. 3).

Based on the molecular results and Ishihara 38 plate test, 3 haplotypes could be deduced from the family members. In the haplotype −1 both genes G6PD and LW and MW are normal (males in F3), in haplotype-2, normal G6PD allele is linked with defective or anomalous MW gene/s (man in P), in haplotype-3, c563T allele is linked with defective or anomalous MW gene/s resulting in deuteranopia/deuteranomalous (male in F2 and F3), as shown in Table-3. The G6PD gene (normal/c563T variant) is coupled with MW opsin gene/s at Xq28 (normal/ deutan type) for 4 generations from P to F3 generations (Fig. 3).

Table 2
Genotyping of 3 Arabian families through qPCR assay.

Family	No of members	Normal G6PD	Homozygous G6PD	Heterozygous G6PD	Hemizygous c563T
Egyptian	13	7 (53.8%)	–	4 (30.7%)	2 (15.3%)
Emirati	13	3(23%)	3 (23%)	4 (30.7%)	3 (23%)



In each generation, different colors for are used to distinguish the maternal X chromosome from the paternal X chromosome. X^{C} allele for normal G6PD and X^{T} allele for the c563T variant; X_N for normal vision, and X_D for color blindness Deuteranopia or Deuteranomaly.

Fig. 3. Pedigree of Egyptian family for 4 generations, showing the inheritance of G6PD/c563T and normal/red-green color vision genes.

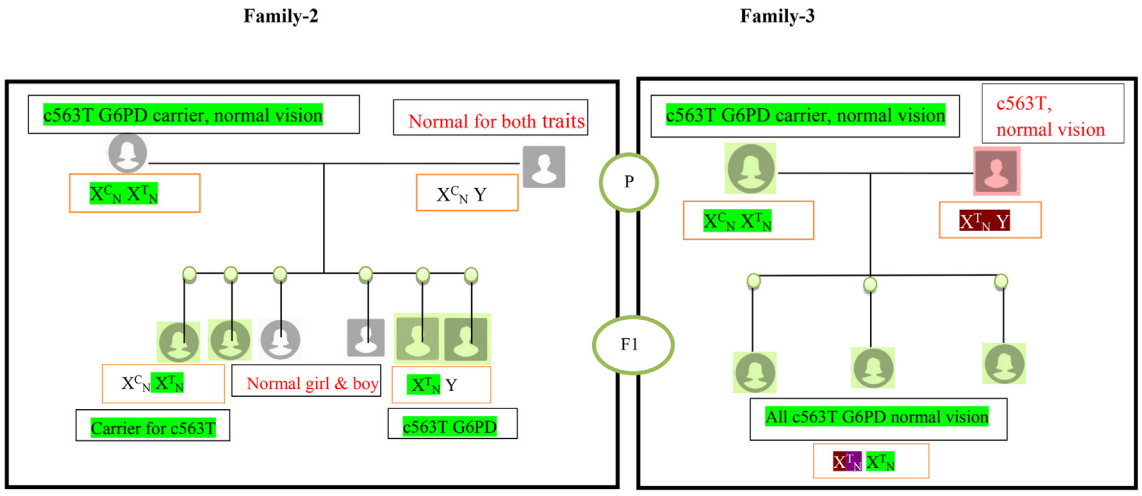


Fig. 4. Pedigree of the Emirati families showing the inheritance of G6PD/ c563T and normal/red-green color vision genes for two generations.

Table 3
Phenotype and deduced haplotype of G6PD/ c563T and normal/ red-green color blindness loci among the 3 Arabian families.

Family	Phenotypes	Deduced Haplotype	Linkage between G6PD and red-green loci
Egyptian	Phe-1 no G6PD deficiency, normal vision	$\mathbf{X^C_N}$	wild G6PD allele with normal LW and MW
„	Phe-2 no G6PD deficiency, deuteranopia	$\mathbf{X^C_D}$	wild G6PD allele with defective MW
„	Phe-3 Med variant, deuteranopia	$\mathbf{X^T_D}$	c563T with defective MW
Emirati	Phe-4 Med variant, normal vision	X^T_N	c563T with normal LW and MW

Phe = Phenotype.

3.3.3. Inheritance of c563T mutation in Emirati families:

In one Emirati family, the 8 members are father, mother and six children. qPCR allelic discrimination assay revealed the mother, 2 of the daughters are carriers and 2 of the sons are G6PD c563T affected (Fig. 4, family-2). qPCR assay of this family revealed the father and the 3 daughters are affected while the mother is carrier of G6PD c562T (Fig. 4 family-3). Sanger sequencing validated the G6PD c563T variants.

The genotyping of the 13 members of the 2 Emirati families is, 3 are healthy, 4 heterozygous carriers and 6 are affected (Table 2).

3.3.3.1. Inheritance of red-green color blindness in the Emirati families:. The color vision test revealed all of them (n = 13) to have normal vision (Table-3). Based on the results of qPCR assay and Ishihara 38 plate color vision test, the pedigree of the two families could be deduced as shown in Fig. 4. G6PD c563T variant is coupled with normal MW opsin gene/s in the Xq28 for 2 generations (Table 3).

In each generation, different colors are used to distinguish the maternal X chromosome from the paternal X chromosome. $\mathbf{X^C}$ allele for normal G6PD and $\mathbf{X^T}$ allele for the c563T variant; $\mathbf{X_N}$ for normal vision, and $\mathbf{X_D}$ for color blindness Deuteranopia or Deuteranomaly.

4. Discussion

Over the last few decades, there has been a lot of information on G6PD variants among Arabic populations, when hemolytic anemic patients were usually screened. The common practice for the detection of G6PD variants is through enzyme assay and/or PCR-RFLP molecular method. In our study, we used two molecular techniques, between them we recommend the qPCR technique to detect homozygous, heterozygous and hemizygous Med individuals, as it is quicker, many samples could be analyzed simultaneously without the need of restriction enzyme and gel analysis.

Since G6PD deficiency is the most common blood disorder, newborn and young children with clinical symptoms are often tested for the enzyme efficiency. The Med variants are 33% among Egyptian neonates, while it is 62% in older children (Ezz-El-Deen et al., 2013; Arnaut et al., 2011), 95% of 21 Arabs (Kurdi-Haidar et al., 1990) and 50% of 1189 Greeks (Molou et al., 2014), Our test system established 30% Med variants included Egyptians and Emiratis of age groups 20–75 years. The differences in the per cent of Med variants in our study compared to the previous reports could be due to the reasons: 1) it included individuals of age groups up to 75 years 2) both sexes and 3) variation in the sample size.

Sequence polymorphisms and mRNA analysis were often used to designate the inactivated X chromosome in females. In this context, the silent mutation 1311C/T in exon 11 of G6PD gene was explored as a molecular marker which was common among 40% of all ethnic populations (Prchal et al., 1993). The wild nt 1311C and mutant 1311 T are observed in both G6PD-normal and deficient populations; hence the association of C1311T with G6PD Med mutations is studied in different populations (Moiz et al., 2009). The G6PD c563T variant is associated with the C1311T mutation in 28.6% and 77% among Egyptian children (Arnaout et al., 2011; Ezz-El-Deen et al., 2013) and 95.2% among Palestinians (Sirdah et al., 2012). However in such investigations, molecular analysis of both the regions (c563T and C1311T) had to be carried to determine the inactivated X chromosome; in search of an alternative marker, the red-green color blindness is explored to detect c563T variants in the current investigation.

In the present study, the pedigree analyses of the 3 Arabian families for the G6PD as well as the green gene are found to be dimorphic. The observed phenotypes and dimorphism are not

unique to Egyptian and Emirati families as they were detected among Turkish, Singaporeans and Thais. Phenotypes −2 and −4 were observed among Turkish men and women (Yücel et al., 1992) and Thai males (Soontarawirat et al., 2017), while phenotypes 2, 3 and 4 were documented among pre-enlistees men (Chan et al., 1992). However, in these studies G6PD deficiency was diagnosed through enzyme assay, neither Ishihara test was used to distinguish between the protan and deutan type vision defects. In the pedigree of the Egyptian family deuteranopia is not always associated with c563T mutation, hence this defective vision is not a useful phenotypic marker to detect the G6PD c563T variant. Soontarawirat's group (2018) opined that color blindness was not informative to detect the G6PD Mahidol variant among Thai people. Our results are similar to those observed among Thais, though the two mutations/variants of G6PD are different. Molecular characterization of the MW gene/s is needed to divulge the cause of the deuteranopia in the Egyptian family.

5. Conclusions

However, based on the molecular analysis of G6PD gene and Ishihara 38 plate color vision test, the following conclusions are drawn from 26 members of 3 Arabian families studied- 1. The two loci are polymorphic (dimorphic) 2. It is evident from the pedigree study that mutations occurred at the two loci independently without any epistatic effect 3. There is strong linkage disequilibrium between the two loci, were inherited up to 4 generations 4. Deuteranopia cannot be chosen as a phenotypic marker to detect G6PD Mediterranean mutation, warranting investigation in larger samples.

Declaration of Competing Interest

None.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.05.014.

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