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PREVALENCE AND MOLECULAR CHARACTERIZATION OF G6PD VARIANTS AMONG SUBJECTIVELY HEALTHY ERITREAN ETHNIC GROUPS

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ABSTRACT

Introduction: **Glucose-6-phosphate dehydrogenase deficiency is the most prevalent X-linked enzymatic deficiency affecting more than 400 million people worldwide. To date, Eritrean population have never been genotyped for glucose-6-phosphte dehydrogenase (G6PD) variants. The aim of this study is to characterize and determine the prevalence of four common G6PD variants in subjectively healthy all nine Eritrean ethnic groups.**

Methods: **A total of 401 finger prick dried blood spot samples were collected onto 3 mm Whatman filter paper from volunteer unrelated subjectively healthy nine ethnic groups residing in all six zobas (regions) of Eritrea. Analysis was performed using polymerase chain reaction/restriction fragment length polymorphism for four common G6PD variants, namely, normal *G6PD B*, *G6PD A* (A376G), *G6PD A*$^{-}$ (G202A) and G6PD Mediterranean (C563T).**

Results: **Molecular studies performed on a total 401 subjectively healthy Eritrean ethnic groups showed that 352 (89.1%) carried the normal *G6PD B* allele and 43 (10.9%) had the *G6PD A* (A376G) variant. The median age of study participants was 33 years, and 75% of the study participants were under 46 years. No cases of *G6PD A*$^{-}$ and Mediterranean mutations were identified, leaving six samples uncharacterized.**

Conclusions: **The current study showed a high prevalence of normal *G6PD B* and *G6PD A* genotypes among subjectively healthy Eritrean ethnic groups.**

These findings are similar to those reported from neighboring countries, Ethiopia and Sudan and other parts of the world.

INTRODUCTION

Glucose-6-phosphate dehydrogenase deficiency (G6PDd) is an erythro-enzymopathy and one of the most common X-linked hereditary genetic defects. This deficiency occurs due to mutations in the G6PD gene and causes functional variants with many biochemical and clinical phenotypes. G6PDd affects about 400 million people worldwide (1) (2), and particularly those from the Mediterranean region and selected African and Asian countries wherein the incidence of G6PD deficiency may approach 60% of some populations (3). G6PDd is inherited in a sex linked fashion, being mainly expressed in hemizygous males and homozygous females, but in only a proportion of female heterozygotes (3). This Mendelian X-linked gene is one of the most highly polymorphic of the human genome with about 217 mutations responsible for functionally G6PDd variant (4) but the effect of each mutation on enzyme structure and function depends on the location of the substituted amino acid (5, 6).

Glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme in the hexose monophosphate (HMP) shunt pathway and catalyzes the regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and the production of ribose (7). The HMP is the sole source of NADPH to the red blood cell (RBC), which is necessary to protect the cell and its haemoglobin from oxidation in view of their role in oxygen transport. NADPH is critical for maintaining glutathione (GSH) in its reduced form, which is crucial for detoxification of reactive free radicals (ROS) and lipid hydro-peroxides during cellular oxidative stress, whether endogenous in origin or induced by drugs or environmental chemicals, collectively referred to as xenobiotics (8). Therefore, the primary effects of G6PD deficiency are haematological because the RBC has no alternative source of NADPH.

Most G6PD deficient individuals are asymptomatic and they are at risk for haemolysis due to antimalarial drug–primaquine administration, infections and ingestion of fava beans. The mechanism of RBC destruction is still poorly understood during haemlytic crisis but it is clear that oxidative damage causes denaturation and precipitation of haemoglobin leading to the formation of Heinz bodies, which trap and destroy the RBCs in the spleen. The symptom may vary from transient mild anaemia to acute anaemia, and thus the individuals suffer from back and abdominal pain, jaundice and haemoglobinuria, and transient splenomegaly (3).

Molecular analysis has revealed that G6PDd prevalence depends on the region and ethnic group and the G6PDd is highly prevalent in areas historically exposed to *Plasmodium* infections, in agreement of selection by malaria (9). This suggests that some G6PD deficient variants like *G6PD A*$^{-}$ and G6PD Mediterranean provide protection against malaria infections caused by *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*) (10). In sub-Saharan Africa, three variants occur with polymorphic frequencies greater than 1%:– wild type *G6PD B*, a non-deficient variant *G6PD A* and the deficient variant *G6PD A*$^{-}$ (11, 12). *G6PD A* is caused by a point mutation A376G in exon 5 while the *G6PD A*$^{-}$ deficient variant has one an A376G mutation plus a G202A mutation in exon 4 (13). The *G6PD A* variant is widely spread worldwide and resulted from an adenine to guanine mutation at nucleotide number 376, leading to a single amino acid substitution

of aspartic acid (GAU/GAC codons) to asparagine (AAU/AAC codons). The common mutation of *G6PD* A^- occurs at nucleotide number 202 and causes the replacement of a guanine by adenine nucleotide at amino acid number 68 substitution of valine (GUG codon) to methionine (AUG codon) (14). The G6PD Mediterranean (C563T) deficient variant occurs due to the replacement of cytosine (C) by thymine (T) at position 563 nucleotide in exon 6 and causes an acute haemolytic anaemia (15).

In Eritrea, there is little knowledge of G6PD deficiency. Eritrean population have never been genotyped for G6PD variants to date. Eritrea is located in the Horn of Africa and is bordered by the Red Sea, Ethiopia, Sudan and Djibouti. Administratively, Eritrea is divided into six zobas: Maekel, Anseba, Gash-Barka, Debub, Semienawi Keyih Bahri (Northern Red Sea) and Debubawi Keyih Bahri (Southern Red Sea). The country has a population of about 5,187,948 million people with nine recognized ethnic groups, which can be also classified by ethno-linguistic origins: Cushitic (Afar, Bilen, Hidareb and Saho), Nilotic (Kunama and Nara) and Semitic (Rashaida, Tigre and Tigrigna). Tigrigna is the first largest ethnic group, followed by Tigre whereas Rashaida is the smallest group in the country. In this study, the most common G6PD allelic variants were characterized and their prevalence in frequency determined using PCR-RFLP based method in subjectively healthy nine Eritrean ethnic groups residing in all six zobas (regions) of Eritrea.

Ethical Issues: Informed consent was obtained prior to blood sample collection from all participants or from parents for children aged less than 15 years. Ethical approval was obtained from the Health Research Proposal Review and Ethical Clearance Committee of Eritrea and the Ministry of Local Government of all six zobas of Eritrea. All molecular laboratory activities were performed with permission from the Department of Biological, Geological and Environmental Sciences Laboratory of Molecular Anthropology Centre for Genome Biology (BiGeA), University of Bologna, Italy.

MATERIALS AND METHODS

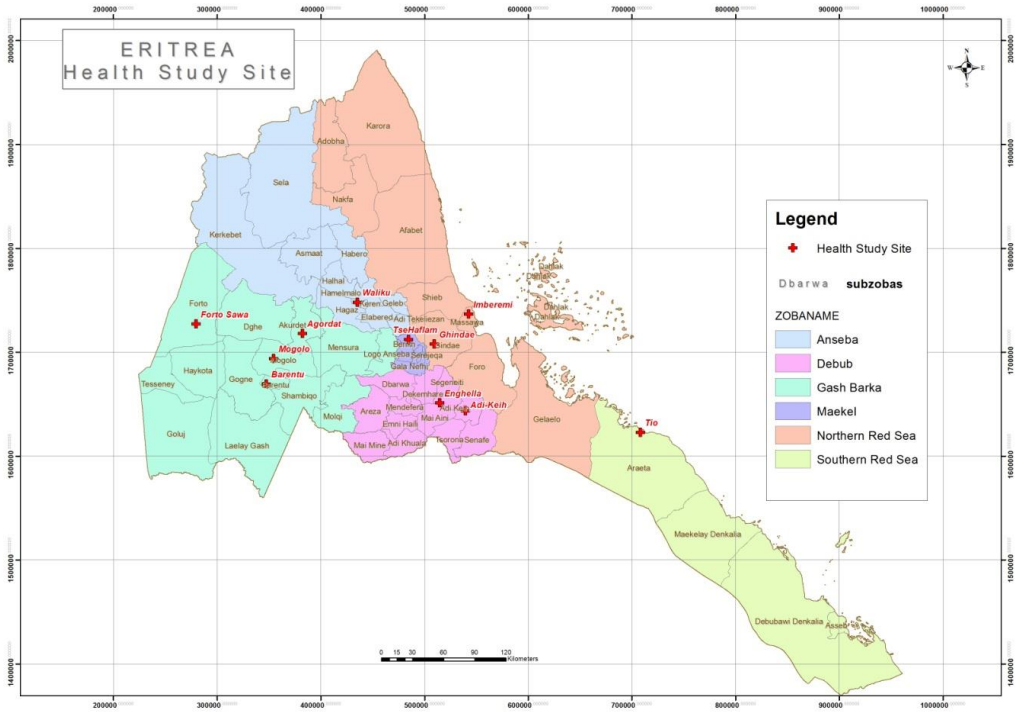
Study sites, study population and sampling techniques: This cross sectional study was undertaken at 11 health study centre located in all six zobas of Eritrea between August 2016 and November 2016. Study Sites were selected based on practical accessibility, resource availability and geography. The study population comprised of 401 subjectively healthy all nine Eritrean ethnic groups (201 females and 200 males) of all age groups. Sample selection was random based on probability proportional to size (PPS). To limit double sampling of genetically related individuals, a single individual per family was recruited. Individuals born from parent of two different ethnic groups were exclude from this study. The nature of the study was explained in Tigrigna and other local languages as deemed necessary, and focus group discussion was used to collect human blood sample. Local government administrators, medical doctors and health staff members assisted in the recruiting and collecting blood samples from the study participants.

Dried blood spot sample collection, packaging and shipping: A total of 401 dried blood spot (DBS) samples by a finger-prick onto Whatman 903™ filter paper card were collected by professional nurses and laboratory technicians from subjectively health Eritrean ethnic groups residing in all six zobas of Eritrea (Figure 1). After air-dried at room temperature, DBS

individually packaged in a small zip-lock plastic with two desiccant bags and transported to the National Health Laboratory (NHL), Asmara, Eritrea. Subsequently samples were transported to the Department of Biological, Geological and Environmental Sciences Laboratory of Molecular Anthropology, University of Bologna, Italy for molecular analysis.

Figure 1

Blood sample collection at 11 study sites located in all six zobas of Eritrea



Genomic DNA extraction from dried blood spot samples: Three punched out circles from each sample were placed in a 1.5 ml microcentrifuge. Genomic DNA was extracted from DBS samples using a QIAamp DNA blood Mini Kit (Qiagen, Germany) and eluted from the spin column in 50 µL of AE buffer. The extracted DNA was genotyped for common G6PD variants: *G6PD A* (376 A→G), *G6PD A⁻* (202 G→A), *G6PD B* (376A) and G6PD Mediterranean (563 C→T), whose mutations are located within the exons 4-6 of G6PD gene using PCR-RFLP based method.

Genomic DNA amplification by conventional PCR: All PCR reaction was carried out to a final volume of 25 µL, using the 5X Green GoTaq® Flexi Buffer, 10 µM of each primer, 10 mM of each dNTP, 5 units of GoTag® G2 Flexi DNA polymerase, 25 mM $MgCl_2$, 9.3 µL sterile water and 6 µL of isolated DNA template according to manufacturer's recommendations (Promega, Madison, WI). Three sets of primers were used as described by Tishkoff et al. (16) and thermal cycling conditions programmed for 34 cycles: initial denaturation at 94 °C for 2 minutes, denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute, extension at 72 °C for 30 seconds and a final extension step at 72°C for 5 minutes. PCR products were analyzed by 1% agarose gel electrophoresis stained with midori green dye in a Biometra Tgradient PCR machine.

Restriction fragment length polymorphism (RFLP) technique: For RFLP studies, 20 µL

of each PCR product was restricted according to the manufacturer's recommendation with 10 units of each corresponding enzyme. The *G6PD A* (376A→G) variant was detected by *FokI* restriction enzyme. All digested samples showing positive (+) for A376G variant were further subjected to PCR amplification using primers for G202A, followed by digestion with *NIaIII* to detect *G6PD A⁻* mutation. Exon 6 was cleaved with *MboII* enzyme to detect the G6PD Mediterranean 563C→T mutation. All restriction endonucleases and reagents were sourced from New England Biolabs and used at optimal temperature and digestion period. RFLP digested products were analyzed through electrophoresis on 3% nusieve- agarose gels stained with midori green dye at the presence of 100 base pair (bp) DNA ladder. Gels visualized by ultraviolet light. The primers, amplified exons and restriction enzymes used for each of these reactions (Table 1).

Table 1

The primer sequences, exons amplified and the restriction enzymes used in the PCR-RFLP procedures to detect the common G6PD variants

G6PD variant	*Primer sequences	Amplified exon	Restriction enzyme	Fragment size	
				Normal	Mutated
A 376 A→G	F5′-CAAAGAGAGGGGCTGACATC-3′ R3′-CGAGGGTGGTGAGATACTGC-5′	5	FokI	342	173, 169
A⁻ 202 G→A	F5′-AACCACACACCTGTTCCCTC-3′ R3-CAAGACGGGAGAGATGGTCG-5′	3+4	NIaIII	320	207, 113
Med 563 C→T	5′-AGGTGGAGGAACTGACCTTG-3′. 5′-TGCAGCTGTGATCCTCACTC-3′	6	MboII	388, 352, 36	253, 99, 36

Statistical analysis: Data were analyzed using Statistical Package for Social Sciences (SPSS) (IBM SPSS Statistics for Windows for version 23.0) software. Categorical variables were presented as counts and percentages. Figure used to show the RFLP gel electrophoresis result. Pearson's Chi-square (χ^2) Test at 95% confidence level and bivariate logistic regression analysis were performed to investigate the level of statistical significant association at P-value <0.05.

RESULTS

A total of 401 volunteer, unrelated subjectively healthy all nine Eritrean ethnic groups residing in all six zobas of Eritrea were recruited for molecular characterization of four common G6PD variants (Figure 2). The median age of study participants was 33 years, and 75% of the study subjects were under 46 years. The total number of DBS samples collected from each ethnic group (Table 2).

Figure 2

Total number of dried blood spot samples collected from nine Eritrean ethnic groups

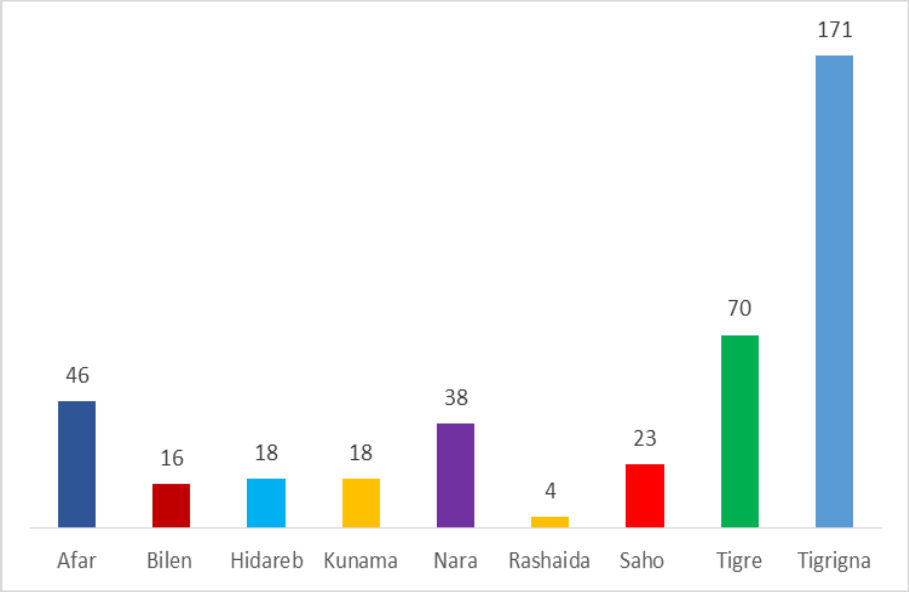


Table 2

Number and percentage of study samples collected from all six zobas of Eritrea

Zobas (Regions)	Sample (N*)	%
Anseba	16	4.0
Debub	28	7.0
Gash Barka	229	57.1
Maekel	22	5.5
Debubawi Keyih Bahri	46	11.6
Semienawi Keyih Bahri	60	15.0
Total	401	100%

N* = number of samples

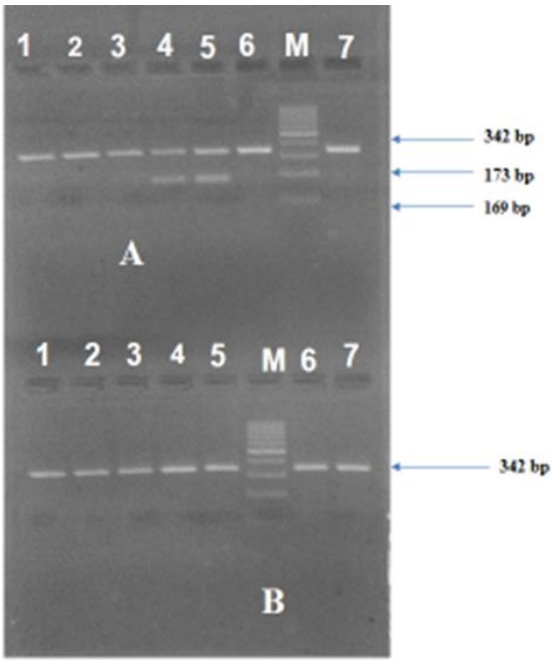
Molecular characterization and prevalence of common G6PD genotypes: Of the initial 401 DBS samples, 395 were successfully analyzed by PCR–RFLP techique, while the remaining six were excluded due to DNA amplification problems. Of 395 genotyped available results, 197 (49.9 %) were females and 198 (50.1 %) males. Molecular study results revealed that 89.1% (352/395) subjectively healthy Eritrean ethnic groups had the normal *G6PD B* genotype and 10.9% (43/395) individuals carried the *G6PD A* (A376G) variant. Of 43 individuals with *G6PD A* mutation, 28 were heterozygous females (AB type), one was homozygous female (AA type) and 14 were hemizygous males (A type). No cases of *G6PD A*$^{-}$ (G202A) and G6PD Mediterranean (C563T) mutations were detected in this study.

Molecular detection of common G6PD variants by 3% nusieve-agarose gel electrophoresis: After gel electrophoresis, the results (bands) of the unknown digested products of interest were interpreted by

comparing the gel photograph of the visible band of known fragment DNA size marker (100 bp). After *FokI* digestion and electrophoresed on 3% nusieve-agarose gel, the presence of *G6PD A* variant identified by reading the band at 342 bp, 173 bp, and 169 bp in heterozygous female and at 173 bp and 169 bp in hemizygous male/ homozygous female. Absence of *G6PD A* (A376G) variant was showed at 342 bp band (Figure 3). Individuals who lacked *FokI* and *NIaIII* restriction sites were classified as *G6PD B*.

Figure 3

RFLP results on 3% Nusieve-agarose gel. Lane (A) 1-3, 6-7; individuals without G6PD A mutation. Lane M: 100 bp size marker. Lanes 4-5: heterozygote females with G6PD A mutation. (B) Lanes 1-5, and 6-7: individuals without G6PD A mutation



The G6PD A (A376G) variant and sex: The highest proportion of individuals with *G6PD A* (A376G) variant was observed in females (14.7%) than and in males (7.1%) in this study. Bivariate logistic regression analysis was performed to investigate whether *G6PD A* (A376G) variant has relationship with sex, and a statistically significant association was observed (P=0.015<0.05) (Table 3).

Table 3

Frequency of G6PD A (A376G) and G6PD B variants in subjectively healthy Eritrean ethnic groups with respect to gender

Category	Genotypes		Chi square value	P-value
	G6PD A	*G6PD B*		
Sex				
Female	29 (14.7%)	168 (85.3%)	5.957	0.015
Male	14 (7.1%)	184 (92.9%)		

95% CI = 95% confidence interval

The present study showed varying frequencies of *G6PD A* (A376G) variant with respect to ethnic groups and zobas. The highest frequency of this mutation was in Saho (31.6%), followed by Nara (23.7%), Kunama (22.2%), Bilen (12.5%), Afar (11.9%), Tigrigna (7.6%), Hidareb (5.6%), Tigre and Rashaida (4.1%). For the main analysis, Rashaida (n=4) has been grouped together with Tigre ethnic group (same ethno-linguistic origin and geographical location) because the sample was too small for adequate analysis. Bivariate (Chi-square) logistic regression analysis showed a statistical significant association between *G6PD A* (A376G) variant and ethnic groups (P=0.002<0.01) and ethno linguistic origins (P=0.001>0.01). Similar to ethnic groups, the prevalence in frequencies of *G6PD A* (A376G) variant varied among the six zobas, which ranged from 1.7% in Zoba Semienawi Keyih Bahri to 33.3% in Zoba Debub (33.3%), and the difference was statistically significant (P=0.001<0.01) (Table 4).

Table 4

Prevalence of G6PD A and normal G6PD B genotypes according to ethnic group, ethno linguistic origins and zobas

Categorical variables	n	Genotypes		χ^2	P-value
		G6PD B (376A)	*G6PD A(A376G)*		
Ethnicity					
Afar	42	37 (88.1%)	5 (11.9%)		
Bilen	16	14 (87.5%)	2 (12.5%)		
Hidareb	18	17 (94.4%)	1 (5.6%)		
Kunama	18	14 (77.8%)	4 (22.2%)	23.201	0.002
Nara	38	29 (76.3%)	9 (23.7%)		
Saho	19	13 (68.4%)	6 (31.6%)		
Tigre-Rashaida	74	71 (95.9%)	3 (4.1%)		
Tigrigna	170	157 (92.4%)	13 (7.6%)		
Ethno linguistic groups					
Cushitic	95	81 (85.3%)	14 (14.7%)		
Nilotic	56	43 (76.8%)	13 (23.2%)	14.938	0.001*
Semitic	244	228 (93.4%)	16 (6.6%)		
Zobas (Regions)					
Maekel	21	20 (95.2%)	1 (4.8%)		
Anseba	16	14 (87.5%)	2 (12.5%)		
Gash Barka	229	204 (89.1%)	25 (10.9%)	20.181	0.001*
Debub	27	18 (66.7%)	9 (33.3%)		
Semienawi Keyih Bahri	60	59 (98.3%)	1 (1.7%)		
Debubawi Keyih Bahri	42	37 (88.1%)	5 (11.9%)		

**Rashaida grouped together with Tigre ethnic group; 95% CI = 95% confidence level*

DISCUSSION

This is the first study of its kind performed to characterize and determine the prevalence of four common G6PD variants using DBS samples collected from volunteer, unrelated subjectively health all nine Eritrean ethnic groups residing in all six

zobas of Eritrea by PCR-RFLP based method. In the current study, 89.1% (352/395) individuals carried the normal *G6PD B* allele and 10.9% (43/395) individuals had the *G6PD A* variant. The result is in agreement with a previous study done in Burkina Faso, of which the prevalence of *G6PD B* allele was 74.5% (12) and among inhabitants of sub-Saharan Africa (Sierra Leone, Mende, Temne, Ghana, Fante, Ga, Cameroon, and Bakaka) ranged from 53.6–81.5% (18). Our finding was slightly higher compared to their studies and this could be probably due to molecular heterogeneity of the study populations.

G6PD A variant is grouped as non-deficient variant (12), because it has 60-90% normal activity compared to the wild type *G6PD B* allele. The overall prevalence, in frequency, of the *G6PD A* variant detected 10.9% in this study. A statistically significant association was seen (P>0.050). The result was in accordance with a previously study done in Ethiopia, in which the prevalence of *G6PD A* was 14% (18) while in Kurdish Iraq 10.9% (19). The similarities in frequencies of *G6PD A* variant in these populations could be probably due to the population structure. Of the 10.9% *G6PD A* variant, 7.1% were heterozygous females, 3.5% were hemizygous males and 0.3% (1/395) homozygous female and with statistically a significant association (P<0.05). This figure is relatively lower compared to the Burkina Faso report for gender, of which it was 27.6% in heterozygous females and 6% in homozygous males (12).

The frequency of *G6PD A* variant was higher in females (14.7%) than in males (7.1%) in the present study, the difference was statistically significant although a higher frequency of the deficient phenotype is expected in males than in females. Since the gene of the G6PD enzyme is located on the X-chromosome and males are hemizygous, single allele of the gene would need to express the deficient phenotype. Females have two X-chromosomes and they can have either normal, intermediate or deficient phenotype because of the random inactivation of X-chromosome. From this point of view, females seem to be more affected by *G6PD A* variant than males.

Furthermore, the highest proportion of individuals with *G6PD A* variant was observed in Saho ethnic group (31.6%) and Nara (23.7%), which in line with different studies showed the prevalence of *G6PD A* variant depends on the region and ethnicity (20). Additionally the proportion of *G6PD A* variant was 14.7% in Cushitic, 23.2% in Nilotic and 6.6% in Semitic, and a strong statistical significant association was observed (P<0.01). The prevalence of G6PD deficiency significantly varies among different countries and regions or even localities for several reasons (21, 22). In the current study, the highest prevalence, in frequency, of *G6PD A* (A376G) variant observed in Zoba Debub (33.3%) and in Zoba Anseba (12.5%). A statistically significant difference was observed (P<0.05). Our finding is also in line with the previous study report in Ethiopia, accounted for 0.0652 in Oromia, 0.063 in Benishangul Gumuz and 0.074 in Gambella regions (18) and 9.0% in the neighbouring Saudi Arabia (23). The differences of prevalence, in frequencies, of *G6PD A* variant among the zoba (regions) in this study could be explained due to the differing ethnic make-up of each region (24).

Deficient *G6PD A*$^{-}$ (G202A) variant that predominates in sub-Sahara Africa (25) was successfully genotyped in this study for 395 subjectively healthy Eritrean ethnic groups. Of the genotyped individuals, 0/395 carried this *G6PD A*$^{-}$ deficient variant, which is in accordance with the previous findings in north Sudan (26), Ethiopia (18) and Jeddah (27). However, some Arab African countries like Tunisia (63.6%) and Algeria (46%)

showed the highest frequency of *G6PD A⁻* deficient variant (28, 29). Absence of *G6PD A⁻* deficient variant in this study could be due to geographical location and molecular heterogeneity of the study populations. In addition, the Mediterranean (563 C→T) deficient variant was detected since previous studies have reported marked differences between regions in *G6PD* mutations (25). In agreement with previous reports from neighbouring countries, Sudan (26) and Ethiopia (30), none of G6PD Mediterranean (C563T) mutation was detected among the present study participants. In contrast, a previous study reported a 89.1%% presence of G6PD Mediterranean (C56T) variant in Jeddah (27). Absence of G6PD Mediterranean variant in this study is most likely due to non-occurrence of mutation here and/or lack of genetic flow from Mediterranean regions and northern African countries to this part of Africa. The results obtained in the present study indicate that the current study participants particularly residing in malaria endemic regions of Eritrea could be likely susceptible to malaria infections, but less at risk of haemolytic anaemia induced upon the exposure to certain drugs, infections and ingestion of fava beans (31, 32).

CONCLUSION

This the first study of its kind and molecular characterization of common G6PD allelic variants conducted in this study revealed the predominantly prevalence of *G6PD B* and *G6PD A* variants while the absence of *G6PD A⁻* and G6PD Mediterranean variants among subjectively healthy Eritrean ethnic group living in all six zobas of Eritrea. Similar findings are also reported from neighboring countries, Ethiopia and Sudan, and distant countries. Moreover, our findings contribute to filling the knowledge gaps regarding to molecular characterization and prevalence of common G6PD variants in this part of Africa. The finding supports the safe use of primaquine for transmission interruption of *Plasmodium falciparum* gametocyte and radial cure of *Plasmodium vivax* without G6PD testing in Eritrea.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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