

ORIGINAL ARTICLE

Prevalence of Mediterranean Mutation of Glucose 6 Phosphate Dehydrogenase in Children of Malak Din Khel Subtribe of AfridiMohammad Amjid¹, Nazish Farooq^{2*}**ABSTRACT**

Objective: To determine the prevalence of Glucose 6 Phosphate Dehydrogenase (G6PD) deficiency in the Malak Din Khel subtribe, the biggest subtribe of Afridi, with expectations of newer findings in the area of medicine and research.

Study Design: Cross-sectional study.

Place and Duration of Study: This study was conducted in District Khyber from March 2018 to August 2018.

Materials and Methods: The sample size of this study was 177 preschool-going children of 4-5 years of age. Multistage cluster sampling was done. Only resident families of Khyber Agency were selected. Children of age 04 to 05 years were enrolled in the study after consent from a parent/ guardian. Children with major comorbidities, non-consenting parents/guardians, and children transfused in the last 24-48 hours were excluded from the study. 05 ml of blood was collected from each child. G6PD qualitative tests were performed in the field; rest of the tests were carried out later at Dabgari Garden lab. PCR was processed according to protocol. Data were analyzed by SPSS 26.0.

Results: A total sample size of 177 respondents were initially investigated through a qualitative test for G6PD deficiency. Among these, only 4(2.3%) respondents were found to be G6PD deficient, The PCR analysis depicted that among 177 respondents, 31(17.5%) were found to be mutant. Among these, 26(84%) were heterozygous, while 5(16%) respondents were found to be homozygous. The PCR analysis further revealed that among the 5 homozygous respondents, 3 were found to be G6PD deficient, as also depicted by the qualitative test. One of the homozygous respondents, although found deficient by qualitative test, showed no Mediterranean mutation.

Conclusion: Most studies done in Pakistan are hospital-based and the method for diagnosing is a qualitative test, but if the G6PD enzyme activity level is more than 30% qualitative test cannot pick the respondent as deficient. In our study, we ran qualitative tests on all respondents and only 2.3%, followed by PCR analysis for Mediterranean mutation of all samples, which showed that 17.5% were G6PD deficient. Further studies need to be conducted in the same manner to know the exact prevalence of G6PD.

Keywords: Anemic, Enzymopathy, Hemoglobinopathy, Hemolysis, Hemoglobin.

How to cite this: Amjid M, Farooq N. Prevalence of Mediterranean Mutation of Glucose 6 Phosphate Dehydrogenase (G6PD) in Children of Malak Din Khel Subtribe of Afridi. *Life and Science*. 2023; 4(3): 182-190. doi: <http://doi.org/10.37185/LnS.1.1.403>

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Funding Source: NIL; Conflict of Interest: NIL

Received: Mar 03, 2023; Revised: May 10, 2023

Accepted: Jun 15, 2023

Introduction

Glucose 6 Phosphate Dehydrogenase (G6PD) deficiency is a known hematological problem worldwide. It is the most common enzyme deficiency in humans.¹ Approximately 400 million people are affected by this enzymopathy around the world.²⁻⁴ The G6PD gene is on the long arm of X chromosome band 2, sub-band 8 (Xq28).⁵ About 50 years ago, this disease was discovered and attracted

scientists due its severe hemoglobinopathy effect.⁶ The disease is very common in Africa and the Arabian region, with the known prevalence of 32.5%.^{7,8}

G6PD is an enzyme of the pentose phosphate pathway, which converts glucose-6-phosphate into 6-phosphoglucono- δ -lactone and functions as the rate-limiting enzyme of the metabolic pathway.⁹⁻¹⁰

This satisfies the energy requirements of the cells by upholding the level of the co-enzyme NADPH.^{10,11} The NADPH in turn keeps the supply of reduced glutathione in the cell.² Glutathione is consumed to mop up the free radicals that result from oxidative damage.^{8,12,13} The role of red cells as oxygen carriers, promotes them at significant risk of damage from oxidizing free radicals.^{11,14} Infections, some drugs and fava beans can result in oxidative stress.¹⁵ As a result, hemoglobin are damaged by oxidants; these damaged erythrocytes are phagocytosed and metabolized to bilirubin which clinically presents as jaundice.¹⁶

More than 400 variants (Mediterranean, ViangChan, Canton, Clombra, Mahidol, Chinese-5, Honiara, Sunderland, Orissa, Alabama, etc.) have been identified to date.¹⁷ On exon 6, a change from cytosine (C) to thymine (T) at base position 563 leads to the substitution of serine (Ser) with phenylalanine (Phe) at amino acid position 188. This leads to the most prevalent G6PD gene mutation in Asians, called the Mediterranean mutation.¹⁸

G6PD is a very common genetic problem affecting 7.5% population of the world. It was estimated in 1973 that almost 30 million people were deficient in G6PD globally.¹⁹ In Pakistan, the prevalence of G6PD deficiency has been reported from 2 to 8%.²⁰⁻²¹ This prevalence varies from region to region as; 2.5% in Lahore, 2.8% in Wah, 3.4% in Malakand, 4.3-5.8% in Rawalpindi, 6.2-7.5% in Karachi, and 7-8.6% in Peshawar.²⁰ This study aimed to determine the prevalence of Mediterranean mutation of glucose 6 phosphate dehydrogenase (G6PD) in children of Malak Din Khel subtribe of Afridi.

This study was taken for research purposes because G6PD is the commonest red cell enzymopathy worldwide. In Pakistan, G6PD deficiency is reported to be 2 to 8% in different studies.²⁰ Most of the studies done in Pakistan are institution based, while institution-based assessments usually give us false

figures about prevalence in the community. In different ethnic groups prevalence of G6PD deficiency is different and varies a lot. Afridi tribe is a large ethnic group that contains 08 subtribes and has never been screened for G6PD deficiency. So a study was carried out to find prevalence in the Malak Din Khel subtribe, the biggest subtribe of Afridi, with expectations of newer findings in the study area of medicine and research.

Materials and Methods

This study was conducted in District Khyber, which has an estimated total population of 546,730. Afridis are divided in 08 subtribes: Malakdin Khel, Zakha khel, Kuki khel, Adam khel, Qambar khel, Qamar khel, Aka khel and Sepah. As part of old traditions, each subtribe has their marked land containing villages for each subtribe in Khyber District. Malak Din Khel subtribe is the biggest subtribe of Afridi residing in 08 villages; Nala, Kohi, Surdhand, Mukha, Navya, Chargai dagarai, Chora and Maidan. This is a descriptive study. Multistage cluster sampling technique was utilized in randomly selected villages of Khyber District, densely populated by Malak Din Khel subtribe.

This study was carried out in three stages, in Hujra of local Malak, arrangements were done for G6PD qualitative test, door to door visits were done, children of visited houses were requested to come to malak's hujra and G6PD qualitative tests were carried out within 15 minutes after blood sampling. All blood samples were then transported for further testing to Bio Sciences Medical laboratory at Dabgari Gardens Peshawar. PCR for "G6PD Mediterranean mutation" was done in molecular laboratory of Institute of basic Medical Sciences, IBMS, KMU, Peshawar.

After approval from the ethical committee KMU Peshawar, the study was conducted from March 2018 – August 2018. The sample size of this study was 177 preschool-going children of 4-5 years of age. After approval from the ethical committee of KMU vide letter no DIR/KMU-EB/PM/000410, multistage cluster sampling was done. In the first stage (cluster) 04 villages were randomly selected on a draw basis, i.e., Nala, Navya, Chora, and Kohi. In 2nd stage (cluster), systematic random cluster sampling was done, and households were selected. A total sample

size of 177 children was divided by 04 villages with an outcome of 44, and children both male and female were selected from each village. Each village's first household to the right side of the Masjid was selected.

Only resident families of Khyber Agency were selected. Children of age 04 to 05 years were enrolled in the study after consent from parent/guardian. Children were selected of the mother from Malak Din Khel subtribe of the Afridi tribe. Subsequently, children of Afridi families with mothers from non-Malak Din Khel subtribe, children with major comorbidities, non-consenting parents/guardians, and children transfused in the last 24-48 hours were excluded from the study.

A protocol was followed for blood sample collection by proper aseptic technique. 03 ml of blood was put in an EDTA tube, while 02 ml of blood was put in a jell tube. During blood collection, G6PD qualitative tests were performed in the field using "Biolab kit", while blood smears were prepared just after blood sample collection. After PCR optimization, all extracted DNA was processed on the basis of optimization for Polymerase Chain Reaction according to protocol, and readings were recorded for normal, homozygous, heterozygous Mediterranean mutations.

Clinical manifestation

Drug-induced hemolysis: In 1926, Mr. Cordes observed acute hemolysis in some patients treated with antimalarial drugs, but after about 30 years, the mechanism of acute hemolysis was understood, and the disease was recognized as G6PD deficiency.

Drugs that are contraindicated in G6PD deficiency

Anti-malarial	Primaquine
Analgesic/antipyretic	Aspirin
antibacterial agents	Chloramphenicol, ciprofloxacin
Sulphonamides	Sulfacetamide
Sulphapyridine	Dapsone thiazolesulfon
Sulphones	Sulphamethoxazole
Other sulfur-containing drugs	G l i b e n c l a m i d e

G6PD deficient malaria patients can be treated with halofantrine, Chloroquine, pyrimethamine, Quinine, and proguanil.²² Results showed that the 2 cases were G6PD deficient and highly concentrated

purified DNA (visible with the naked eye), respectively

Polymerase Chain Reaction (PCR) Optimization

Extracted DNA was processed for optimization,

The Tn specified primers for the G6PD

The mediterranean mutation was prepared using primer blast, the sequence of the primer was as follows:

Primer name and sequence

G6PD C563T forward normal (FN)

5'CCG GCT GTC CAA CCA CAT ATC 3'

G6PD C563T forward mutant (FM)

5'CCG GCT GTC CAA CCA CAT ATT 3'

G6PD C563T reverse (R)

5'CCA GCC TCC CAG GCG AGA 3'

Results

The significance of this chapter is to facilitate the understanding of the real outcomes of the research in a comprehensive manner. The mark of research is not merely assemblage of raw data but to epitomize the analyzed data in a systematic and meaningful form. For this intent, tabulation of the analyzed data and interpretation of results are included so that logical inferences may be deduced.

A total sample size of 177 respondents was initially investigated through a qualitative test for G6PD deficiency. Among these, only 4(2.3%) respondents were found to be G6PD deficient while all the remaining respondents were normal. The PCR analysis depicted that among 177 respondents, 31 (17.5%) were found to be mutant. Among these, 26(84%) were heterozygous, while 5(16%) respondents were found to be homozygous. The PCR analysis further revealed that among the 5 homozygous respondents, 3 were found to be G6PD deficient, as also depicted by the qualitative test. One of the homozygous respondents, although found deficient by qualitative test, showed no Mediterranean mutation. This might be due to the presence of recessive alleles or the epistatic phenomenon due to some other alleles. These heterozygous and homozygous respondents were investigated for various hematological and demographic attributes, to pinpoint the effect of Mediterranean mutation on these attributes. The details of all these attributes are given below.

Association of G6PD Mediterranean mutation with gender

The total sample size of 177 respondents comprised 109(61.6%) male and 68(38.4%) female children (Figure 1). Among the 109 males, 106(97.2%) were found normal and only 3(2.8%) had G6PD Mediterranean mutation. Similarly, among the 68

females 40(59%) were normal, 28(41%) had heterozygous. The chi-square test depicted a non-significant ($p=0.279$) association of gender with G6PD mutation, and the respondents were normally distributed irrespective of mutation categories. It clearly showed that the mutation rate had no association with the gender of respondents.

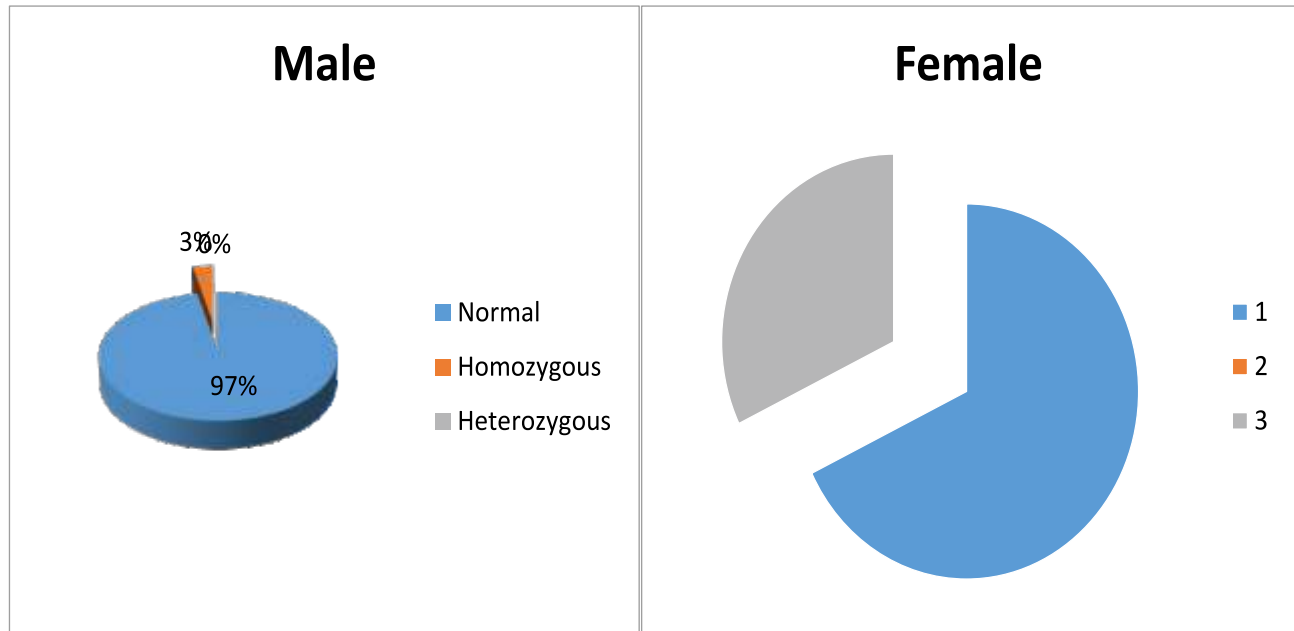


Fig 1: Frequency distribution of respondent's gender regarding G6PD mutation

The data regarding hemoglobin recorded in normal, heterozygous, and homozygous respondents are presented in Table 1. The mean hemoglobin values of these normal, heterozygous, and homozygous respondents were found to be 11.60, 12.51, and 11.54, respectively. One way analysis of variance depicted that there was a highly significant difference between normal and heterozygous

respondents for hemoglobin, showing that mutation has significantly influenced the hemoglobin in heterozygous individuals. However, the difference between normal with homozygous was non-significant. Similarly, the difference in hemoglobin between homozygous and heterozygous respondents was also non-significant.

Table 1: Effect of G6PD Mediterranean mutation on hemoglobin

G6PD mutation type	Mean Hb value	No of cases (n)	G6PD mutation type	SD	P value
Normal	11.60	146	Heterozygous	0.209	0.000
			Homozygous	0.628	1.000
Heterozygous	12.51	26	Normal	0.209	0.000
			Homozygous	0.630	0.379
Homozygous	11.54	5	Normal	0.628	1.000
			Heterozygous	0.630	0.379

Distribution of respondents regarding anemia status

The three mutation groups, i.e., normal, heterozygous and homozygous, were investigated for anemic status, and the data recorded are presented in (Figure 2). The figure showed that among the total respondents, 137(77.4%) were

normal, 22(12.4%) had mild, and 18(10.2%) had moderate anemia. Among the anemic respondents, 13(59.1%) were from normal group, 8(36.4%) were heterozygous and 1(4.5%) were found to be homozygous. Similarly, among the 18 moderate anemic respondents, 17(94.4%) were normal, and 1(5.6%) was homozygous.

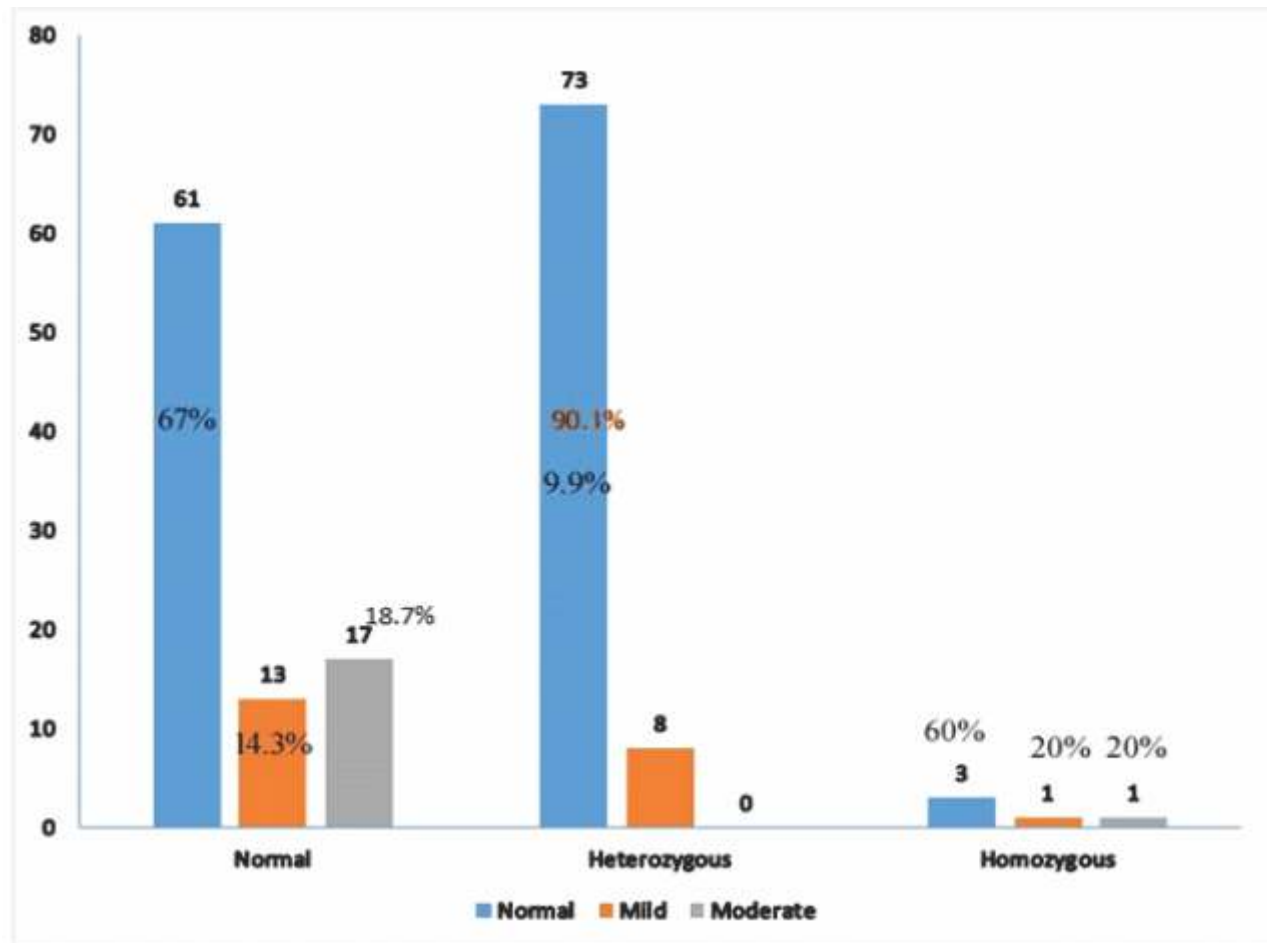


Fig 2: Frequency distribution of respondents in mutation groups regarding anemia status

Correlation of PCR analysis G6PD mutation and G6PD qualitative test (time in minutes)

The data pertaining to G6PD qualitative test as influenced by the G6PD mutation categories are presented in Table 2a (Supplementary). The statistical analysis revealed significant differences among the mutant groups for G6PD. The table clearly depicts that the homozygous group was significantly different from normal and heterozygous groups regarding the G6PD mutation. It explains that G6PD qualitative test was effective in homozygous respondents and can be used as a diagnostic tool. However, the normal and heterozygous groups were

statistically at par with each other. Maximum G6PD status difference of 51.80 minutes was recorded in the homozygous group, whereas the mean time (in minutes) of normal and heterozygous groups were 25.45 and 25.20.

Effect of G6PD on MCV

The data pertaining to mean values of MCV as influenced by G6PD are presented in Table 2b (Supplementary). The mean MCV values of normal, heterozygous, and homozygous respondents were found to be 87.71, 93.48 & 100.28, respectively. It is evident from the table that MCV was significantly affected in heterozygous ($p=0.000$) and homozygous

(0.016) individuals in comparison to normal respondents (Table 2b). In contrast, the homozygous and heterozygous individuals were non-significantly different from one another. As MCV was significantly influenced by the mutation and can be used as an important hematological attribute in mutant respondents.

Effect of G6PD on MCH

The mean values of MCH as affected by G6PD are given in Table 2c (Supplementary). The mean performance of the normal respondents was 24.85, heterozygous was 26.81, and homozygous was 28.46. The analysis of the variance of MCH in these groups showed highly significant ($p \leq 0.01$) differences between heterozygous and normal respondents. Similarly, the association of homozygous with normal was also significant ($p \leq 0.05$). The homozygous respondents differed non-significantly from heterozygous individuals. The MCH was also considerably affected by the mutation in both the mutant groups compared to normal respondents.

Effect of G6PD on MCHC

The respondents were also investigated for MCHC, and the data recorded for MCHC indicated that the mean values of normal, heterozygous, and homozygous were found to be 28.31, 28.73, and 28.42, respectively (Table 2d (Supplementary)). It was also observed that heterozygous respondents were significantly different from the normal respondents showing that this hematological parameter was also under the influence of mutation. In contrast, the difference between normal & homozygous and homozygous & heterozygous was non-significant.

Discussion

G6PD deficiency is an x-linked recessive disorder, and due to the x-linked inheritance pattern, most of the patients are male; however, it can also be induced in female carriers because of lyonization. The deficiency is mostly found in the Mediterranean region. Different studies reported pockets of disease in Asia and middle-east with 62% prevalence in Kurdish Jews²³ and 31% in Vietnam.²⁴ Similarly, in our country, the deficiency has been reported from 2 to 8%.^{20,21} This was the first scrutiny conducted to investigate the occurrence of Mediterranean mutation in G6PD deficient children of the Malak Din Khel subtribe of

Afridi. This study helped the preliminary evaluation of the frequency of the mutation related to G6PD in the municipality of this region of the country.

The study found that 4/177 (2.3%) of the investigated respondents showed G6PD deficiency through qualitative tests. This overall occurrence was lower than the previously reported prevalence of the country, which was 3.4% in Malakand, 4.3-5.8% in Rawalpindi, 6.2-7.5% in Karachi, 7-8.6% in Peshawar regions, Wah (2.8%) and Lahore (2.5%).²¹ Similarly, in 2003, 200 patients were investigated at Khyber Teaching Hospital, and among these, 24 (12%) were found to be G6PD deficient.²⁵ The present investigation showed that the prevalence was slightly different from the previous findings in various parts of the country.

However, in the second phase of this study, 31(17.5%) respondents were found to be having G6PD Mediterranean mutation through PCR analysis. These G6PD Mediterranean mutants consist of 26 heterozygous and 5 homozygous individuals. These results are totally in contradiction to the previous studies carried out in various parts of the country which reported only 2-8% occurrence. This huge amount of variation might be because so far, no detailed study has been conducted and all the previous studies are either carried out only through a qualitative test or conducted in hospitals. In most of the previous studies, the respondents have only been investigated through G6PD qualitative test. Also, in the present study, the qualitative test depicted only 2.3% occurrence, but the detailed PCR analysis results (17.5%) were found to be more alarming. Thus it can be estimated that most of the respondents are at risk, and further detailed studies must be carried out to understand the actual picture of this alarming issue/concern.

Moreover, most of the previous studies have been conducted in hospitals such as in 2003, 200 patients were investigated at Khyber Teaching Hospital and among these 24 (12%) were found to be G6PD deficient.²⁵ It was also recorded that these respondents were brought to the hospital with complaints of jaundice which might be one of the symptoms of G6PD deficiency. It is thus concluded that qualitative tests and hospital studies very poorly demonstrate the actual position of this serious

threat because mainly diseased people visit hospitals and they do not represent the whole population for prevalence, thus detailed investigations in various ethnic groups at a larger level must be carried out.

In Asian countries, its prevalence has been reported in Indonesia, Thailand, Malaysia, Bangladesh, Pakistan and China.^{20-21,26-30} In those regions where the prevalence of G6PD deficiency is somewhat higher, the doctors and consultants must be aware of the G6PD status of the patients while they are treating the patients. They should prescribe with care the contraindicated drugs for G6PD deficiency. As this deficiency is associated with and caused by various types of mutation, which can further lead to hemolysis, therefore mutations at the molecular level must be determined.

In this study, a non-significant association of gender, weight, height and G6PD deficiency was observed however, on the initial qualitative test, G6PD deficiency was more in male in comparison to female. Study by Zahraa M et al. also reported the same non-significant association of G6PD with these parameters.

Our present findings confirmed that hemoglobin was significantly lower in homozygous and heterozygous individuals in comparison to normal respondents. This lower Hb in mutant respondents in comparison to normal could be attributed to the fact that G6PD deficient red blood cells have less viability than the normal red blood cells, even without the cells subjected to oxidative stress as reported by Zahraa M et al. confirmed our findings that Hb is low in G6PD deficient subjects.³⁰

The present findings explored that almost 22% of respondents were suffering from anemia, which might be because the hallmark of the G6PD deficiency is the destruction of RBCs on account of oxidative stress. G6PD deficiency might be one of the important causative factors of anemia induced by food and drugs. Thus, the donors must be first investigated for G6PD deficiency. A negative association of G6PD deficiency with hemoglobin was recorded, which is in close agreement with the previous findings of a study carried out by Ajlaan S, who also reported a negative correlation with hemoglobin.²⁹

Conclusion

G6PD deficiency is a serious condition in Pakistan, with a high prevalence 17.5% found in the Malak Din Khel subtribe of Afridi. Although most studies use qualitative tests, only 2.3% of respondents were deficient, and PCR analysis revealed 31.5% of participants were G6PD deficient. Further research is needed to determine the exact prevalence and to address the threat of G6PD in other ethnic groups. Early diagnosis and prevention are crucial for managing complications related to G6PD deficiency.

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