# Identifying β-chain Variants of Hemoglobin: Royal Medical Services Experience

*Raida Oudat*(MD), Heba Abu Al-ruz* (Bsc), Tania Ogeilat* (MD), Eman AL-Mashaqba*(Bsc), Sana'a Saidat*(MD), Rawan AL-Hiari*(Bsc), Raeda Saada* (PhD).*

## ABSTRACT

**Objective**: β-chain Hemoglobin (Hb) variants are qualitative hereditary hemoglobinopathies that range clinically from silent carriers to transfusion-dependent anemia. The aim of this study was to determine the frequency of β-chain Hb variants at the Jordanian Royal Medical Services.

**Methods**: The laboratory electronic records of hemoglobinopathy investigations for patients from different regions of Jordan during the period between 2008 to 2019 were retrospectively reviewed. The tests were performed at Princess Iman Research and Laboratory Sciences Center and included complete blood counts and Bio-Rad Variant II High Performance Liquid Chromatography (HPLC) to detect and quantify normal and abnormal Hbs; Polymerase Chain Reaction (PCR)-based reverse dot blot hybridization (Vienna lab StripAssay) was used to detect the corresponding gene mutations behind the identified variants.

**Results**: Of the 31,700 samples investigated, 811 carried β-chain Hb variants. HbS was identified in 690 (540 heterozygous, 150 homozygous), HbC in 53 (43 heterozygous and 10 homozygous), and HbE in 32 (all of which were heterozygous). The corresponding mutations for HbS, HbC and HbE were identified on PCR. The remaining 36 samples carried HbO Arab, HbD-Punjab, and Hb Lepore with counts of 20, 10 and 6, respectively; these were identified via the HPLC method.

**Conclusion**: HbS is the most common β-chain Hb variant in Jordon, followed by HbC then HbE. HPLC and PCR are reliable methods for identification of such variants. The current study enhances the knowledge of the hematologist into common Hb variants in our region, which can lead to better disease control, management, and prevention.

**Abbreviations**: Hb = Hemoglobin, HPLC = High Performance Liquid Chromatography, PCR = Polymerase Chain Reaction.

**Key words:** β-chain hemoglobin variants, HPLC, PCR, Jordanian Royal Medical Services.

**RMS April 2021; 28(1): 10.12816/0058876**

## Introduction

Hemoglobin (Hb) is an intraerythrocytic oxygen-transporting protein consisting of heme and globin components. The globin part is a tetramer that comprises two α-like and two β-like

* From the department of Hematopathology, Princess Iman Research and Laboratory Sciences Center, Royal Medical Services. Amman, Jordan, Corresponding Author: Dr. Raida Oudat - **Email**: Droudat@hotmail.com.

polypeptide chains. A heme moiety is covalently-linked to each globin chain and functions to reversibly bind oxygen molecules in the lung to release it later in the tissues (1). The β gene cluster encodes a 146-amino acid polypeptide and is located on chromosome 11 (2).

Hb disorders are a group of hereditary genetic disorders that affect the amount of Hb produced or its structure or both. Quantitative genetic defects result in underproduction of normal globin chains with the ensuing thalassemia syndromes. On the other hand, qualitative defects are generally point mutations in the coding genes that result in different structural variants of α and β chains. One example of β-chain variants is the well-known HbS that causes red cell sickling, chronic hemolytic anemia, pain episodes and organ damage (3). Although most carrier traits of variants are clinically silent, the global carrier rate of a significant hemoglobin disorder is estimated to be 5.2% and may warrant genetic counseling (4).

High Performance Liquid Chromatography (HPLC) is the method of choice to screen for hemoglobinopathies due to the ease of sample preparation and the accurate resolution and quantification of abnormal Hb fractions (5). This method is quick, convenient, and more reproducible than conventional Hb electrophoretic methods (6,7).

Molecular analysis utilizing allele-specific Polymerase Chain Reaction (PCR) complements HPLC and allows for the identification of couples who are at risk for adverse outcomes in offspring in populations like Jordan (8).

This retrospective study focused on identifying the qualitative β-chain structural Hb variants at the Royal Medical Services in a large sample size using HPLC and PCR.

## METHODS

The prior approval of the ethics committee of the Jordanian royal medical services was obtained for this retrospective study. It included a total of 31,700 peripheral blood samples that were analyzed at the hematology department of Princess Iman Research and Laboratory Sciences Center in Amman, Jordan between 2008 and 2019. We were able to include a large sample size over a long period of time which enabled us to obtain more representative results and identify rare variants. No previous studies had been conducted on this subject.

Samples were received as a part of hemoglobinopathy investigation for patients from several hospitals of the royal medical services that serve large areas including the northern, central, and southern regions of Jordan.

Whole venous blood was drawn in Ethylenediaminetetraacetic Acid (EDTA) tubes for complete blood counts (Sysmex XE-2100, XN-1000, and Beckman Coulter systems). Normal range of Hb is 11.5-15.5g/dL for female adults, and 13.5-18 g/dL for males. The mean corpuscular volume (MCV) normal range is 76-96 fL. Ion-exchange HPLC (Variant II β Thalassemia Short Program, Bio-Rad) was performed on samples (either fresh or stored at 2-8 °C for a maximum of one week). HPLC is an automated system to separate and quantify normal HbS (HbA, HbA2, and HbF) and abnormal variants. Normal values for HbA2 is <3.5%, and for HbF is <1%. HPLC chromatograms demonstrate the efficient separation of HbS in different elution peaks depending on their Retention Time (RT), which starts with sample injection and ends with reaching the peak of Hb elution

window. This allows for a quantitative measure of each variant by calculating its peak area% as a fraction of the total area.

Our laboratory is accredited and well-trained and experienced laboratory technician usually follow the manufacturer's procedure; each run is preceded with calibrator Hb A2/F and two-level controls. Peaks were assigned to manufacturer-defined windows (Table I).

**Table I:** Analyte identification windows by Bio-Rad.

| Analyte name | Retention Time (minutes) | Window (minutes) |
|---|---|---|
| **F** | **1.10** | **0.98-1.22** |
| **P2** | **1.39** | **1.28-1.50** |
| **P3** | **1.70** | **1.50-1.90** |
| **A0** | **2.50** | **1.90-3.10** |
| **A2** | **3.60** | **3.30-3.90** |
| **D-WINDOW** | **4.10** | **3.90-4.30** |
| **S-WINDOW** | **4.50** | **4.30-4.70** |
| **C-WINDOW** | **5.10** | **4.90-5.30** |

RT, peak characteristics, and proportions were carefully examined for specific variant identification—especially for variants that elute in the same window. According to the manufacturer's instructions, specimens with HbA2 levels greater than 10% should be tested for the possible presence of hemoglobin variant interference. Other tests included May Grunwald Giemsa-stained peripheral blood smear examination and sickling tests for samples carrying the HbS variant.

The corresponding β-chain mutations were detected using the β globin StripAssay based on reverse dot blot hybridization (ViennaLab Diagnostics, GmbH), and the manufacturer's procedure was followed to simultaneously detect multiple targets (22 mutations). The Mediterranean StripAssay was used to detect HbS codon 6[A>T] and HbC codon 6[G>A]. Another strip tailored for Southeast Asia was utilized for HbE codon 26[G>A]. PCR amplification with biotinylated primers and labeled with streptavidin-alkaline phosphatase and color substrates was followed by hybridization to oligonucleotide probes in a parallel-line array.

Eligibility criteria for this study included patient age above 6 months. Sample criteria included both fresh samples and stored samples at 2-8 °C of a maximum of one week prior to analysis.

Frozen samples, old samples, samples of insufficient quantity (less than 3 ml), samples that were drawn in non-EDTA tubes, and samples that were stored at room temperature were all excluded. Quantitative defects (α and β thalassemia), cases with compound heterozygosity, patients who received recent blood transfusion (within one month) of drawing blood samples, and ethnic populations other than Jordanians were also excluded. Multiple follow up tests for the same patient were also excluded.

Hematological parameters, HPLC quantitative data, and molecular analysis results were retrieved from computerized database in our department. Microsoft Excel 2007 program was used to calculate means and standard deviations (SD) and descriptive data were reported as percentages of the total number of study recruits.

## RESULTS

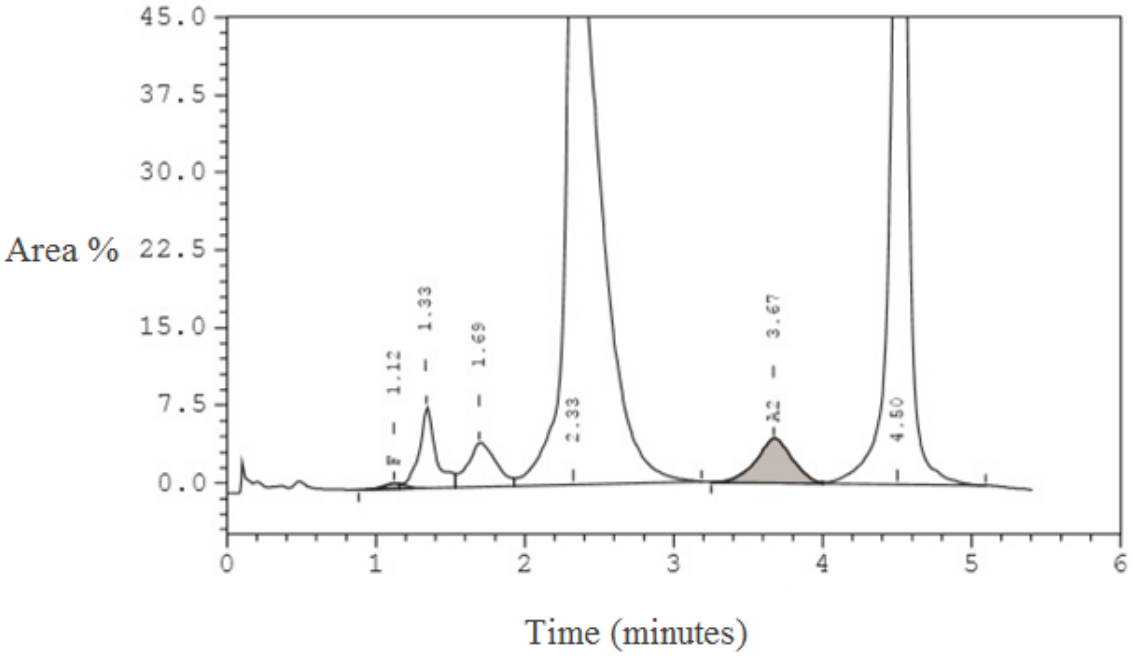
A total of 31,700 samples were screened for Hb disorders, and 811 were found to have β-chain structural variants. Of the 811, 438 (54%) were females and 373 (46%) were males. Their ages ranged from 6 months to 60 years (median 5 years).

The most frequent variant was HbS (690 samples) constituting 85.1% of the abnormal variants and 2.2% of the total samples. These included 540 heterozygous (1.7% of total) and 150 homozygous (0.5% of total). The hematological profile of HbS heterozygous cases was either normal or showing mild anemia while homozygous cases showed severe anemia (mean Hb = 7.5±1.10 g /dL). HbS presented with a variant S-Window of 37.4%±3.7 and 72.0%±12.8 in heterozygous and homozygous cases respectively and a RT of 4.50 minutes; mean HbA2 was 3% and 3.4% in heterozygous and homozygous cases, respectively. HbF was raised in all patients of homozygous HbS with a mean of 13.7%±7.7 and normal in heterozygous ones (Figure 1 shows chromatograms for sickle cell trait).

**Figure 1:** A chromatogram of Sickle cell trait.

| Peak Name | Calibrated Area % | Area % | Retention Time (min) | Peak Area |
|---|---|---|---|---|
| F | 0.4 | --- | 1.12 | 12149 |
| P2 | --- | 3.4 | 1.33 | 128360 |
| P3 | --- | 3.0 | 1.69 | 114530 |
| Ao | --- | 51.9 | 2.33 | 1959301 |
| A2 | 4.3 | --- | 3.67 | 146916 |
| S-window | --- | 37.4 | 4.50 | 1411250 |

Total Area: 3772506

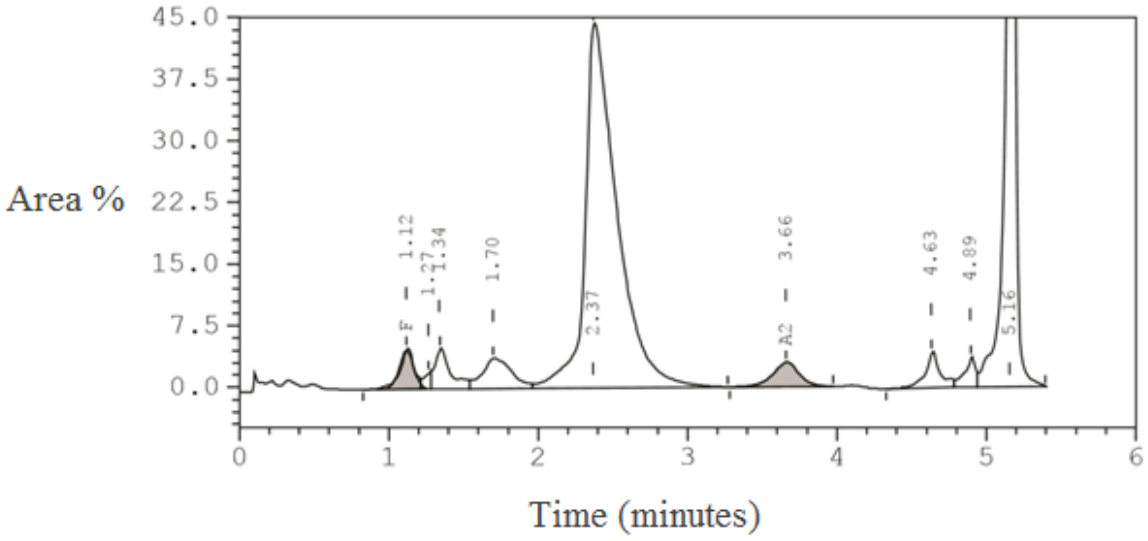



The next variant was HbC in 53 cases (0.17% of total, 6.5% of variants), 43 of these were heterozygous and 10 had the disease. These were associated with anemia in its homozygous forms (mean Hb = 11.7±2.2 g/dL) and slight microcytosis in traits. HbC presented with a variant C-Window of 36.3%±4.2 in heterozygous and 83%±3.5 in homozygous cases and RT of 5.16 minutes. Their mean HbA2 was 3.2% and 3.4% in heterozygous and homozygous cases, respectively. HbF was normal in both (Figure 2 shows chromatogram for HbC trait).

**Figure 2:** A chromatogram of hemoglobin C trait.

| Peak Name | Calibrated Area % | Area % | Retention Time (min) | Peak Area |
|---|---|---|---|---|
| F | 2.7 | --- | 1.12 | 71899 |
| Unknown | --- | 0.5 | 1.27 | 16510 |
| P2 | --- | 2.6 | 1.34 | 80099 |
| P3 | --- | 3.6 | 1.70 | 112052 |
| Ao | --- | 48.3 | 2.37 | 1499718 |
| A2 | 3.0 | --- | 3.66 | 85138 |
| S-window | --- | 2.3 | 4.63 | 70526 |
| Unknown | --- | 1.4 | 4.89 | 44632 |
| C-window | --- | 36.3 | 5.16 | 1126508 |

Total Area: 3107083




The third variant was HbE with 32 cases (0.1% of tatal, 3.9% of variants), and all of which were traits. They exhibited mild microcytosis, normal Hb levels, and an HPLC migration pattern that elutes in the HbA2 window with values of 34.1%±3.8, RT 3.65 minutes, and normal HbF (Figure 3 shows chromatogram for HbE trait). Diagnosis of HbS, HbC, and HbE was confirmed by testing for their corresponding mutations by PCR following their preliminary detection by HPLC. Figure 4.

**Figure 3:** A chromatogram of Hemoglobin E trait.

| Peak Name | Calibrated Area % | Area % | Retention Time (min) | Peak Area |
|---|---|---|---|---|
| F | 0.9 | --- | 1.11 | 19191 |
| Unknown | --- | 0.4 | 1.27 | 11597 |
| P2 | --- | 2.1 | 1.35 | 54647 |
| Unknown | --- | 0.5 | 1.48 | 13453 |
| P3 | --- | 4.2 | 1.73 | 109130 |
| Ao | --- | 61.8 | 2.36 | 1605853 |
| A2 | 34.1* | --- | 3.65 | 785179 |

Total Area: 2599049

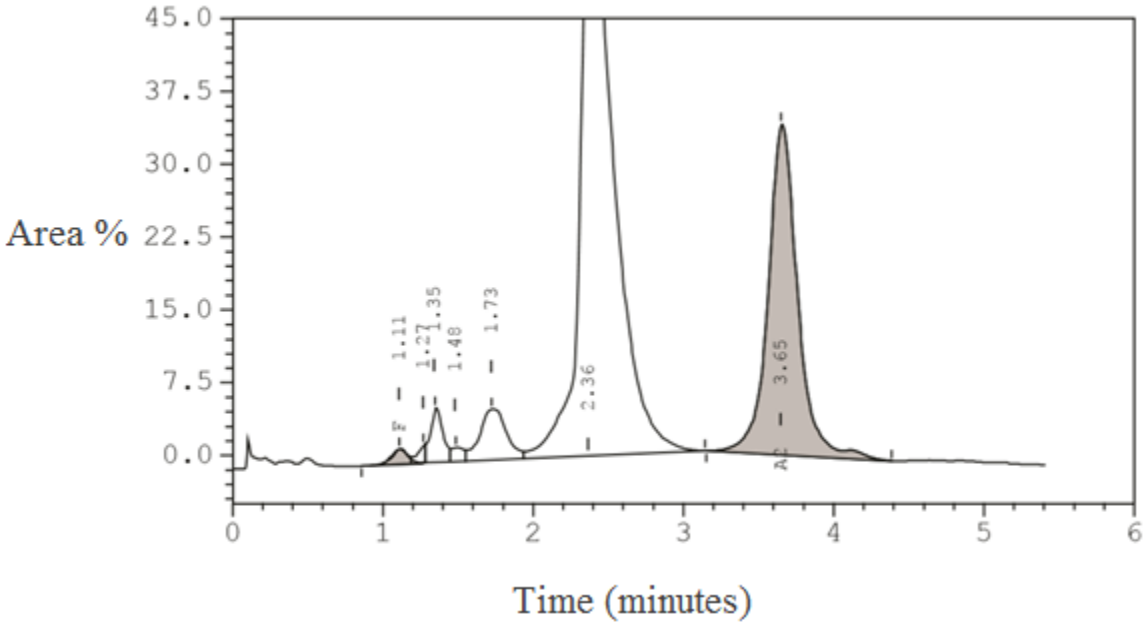
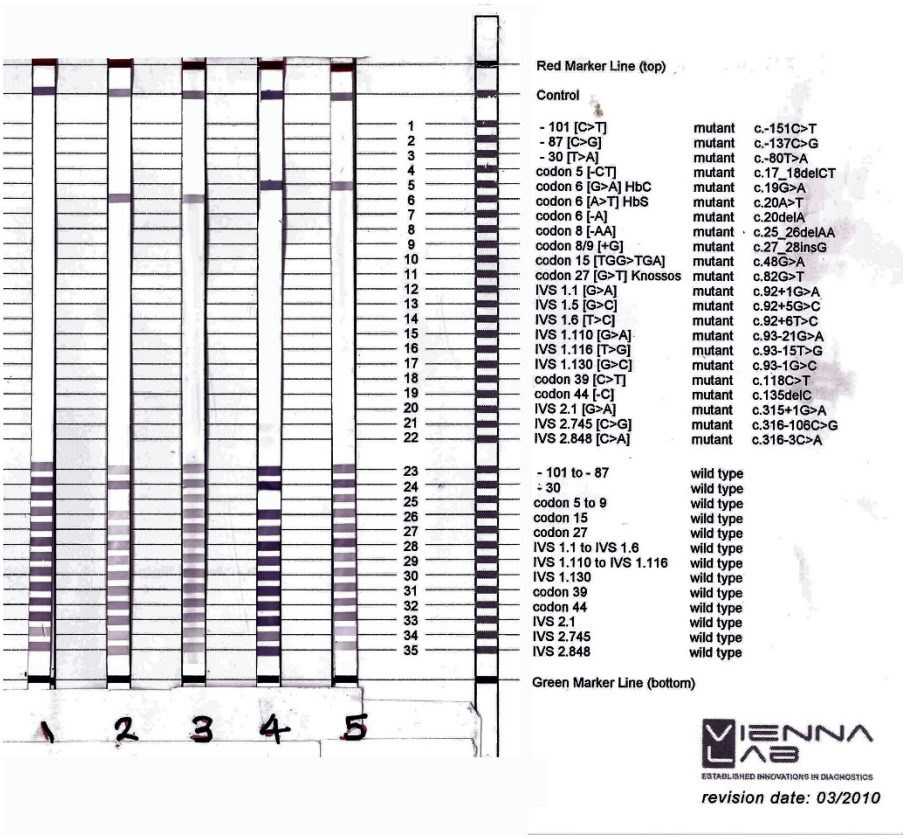



**Figure 4:** B-Globin stripAssay Mediterranean mutations.

| Sample No. | Genotype |
|---|---|
| **1)** | Normal |
| **2)** | Homozygous for sickle cell anemia |
| **3)** | Heterozygous for sickle cell anemia |
| **4)** | Homozygous for HbC |
| **5)** | Heterozygous for HbC |




An additional 36 cases of rare variants encountered in the current study were distributed as follows: 20 carriers for HbO Arab, 10 carriers for HbD Punjab, and the remaining 6 carried Hb Lepore and thus comprising 2.5%, 1.2% and 0.8% of the identified variants, respectively. HbO Arab was identified with a variant percentage of 30%±2.2. The RT was 4.91 minutes with normal hematological indices. HbD Punjab displayed a D-Window with variant percentage of 35.2±1.1, RT of 4.15 minutes, and mostly normal hematological indices (Figure 4). Hb Lepore exhibited an HbA2-like HPLC pattern (13%±1.4) similar to HbE but with different RT of 3.37 minutes. HbF is slightly increased (3%±2.5) with mild anemia and hypochromia. Unfortunately, these three rare variants were preliminary diagnosed by HPLC alone because molecular testing for their specific mutations was not available at our center. All hematological parameters, quantitative HPLC data, and molecular characteristics are detailed in Tables II and III.

**Table II:** Variants' hematological profile and HPLC data expressed as mean values with standard deviation.

| Variant type | Hb level (g/dL) | Mean corpuscular volume (fL) | RT in minutes | Variant Hb % | HbA2 % | HbF% |
|---|---|---|---|---|---|---|
| ***HbS:*** *Heterozygous* | 12.8±1.6 | 76±7.1 | 4.50 | 37.4±3.7 | 3.0±0.2 | 0.7 ± 0.8 |
| *Homozygous* | 7.5±1.1 | 85.0±5.6 | | 72.0±12.8 | 3.4±0.8 | 13.7±7.7 |
| **HbC:** *Heterozygous* | 12.9±1.8 | 73.0±6.8 | 5.16 | 36.3±4.2 | 3.2±0.6 | 1.1±1.6 |
| *Homozygous* | 11.7±2.2 | 71.6±6.1 | | 83.0±3.5 | 3.4±0.4 | 2.0±1.4 |
| **HbE*** | 13.0±1.6 | 72.0±3.2 | 3.65 | 34.1±3.8 | 34.1±3.8** | 0.7±0.24 |
| **HbO Arab*** | 12.9±0,74 | 80.0±1.8 | 4.91 | 30.0±2.2 | 1.5±0.6 | 0.6±0.3 |
| **HbD Punjab*** | 12.7±0.65 | 76.0±2.5 | 4.15 | 35.2±1.1 | 1.5±0.31 | 1.0±0.6 |
| **Hb Lepore*** | 12.0±1.4 | 71.1±3.5 | 3.37 | 13.0±1.4 | 13.0±1.4** | 3.0±2.5 |

* These variants were all detected as heterozygous mutation.
** HbA2% is equal to the variant% because they co-elute in the same window on HPLC.

**Table III:** HPLC Abnormal β chain variants: their numbers, genotypes, and phenotypes.

| Hemoglobin Variant Name | Number of cases | Causing mutation | Detected genotype | Phenotype |
|---|---|---|---|---|
| **HbS:** *Heterozygous* | 540 | $\beta^{6\ Glu \rightarrow Val}$ | $\beta\ \beta^{S}$ | Mild anemia (if any) |
| *Homozygous* | 150 | | $\beta^{S}\ \beta^{S}$ | Severe anemia |
| **HbC:** *Heterozygous* | 43 | $\beta^{6\ Glu \rightarrow Lys}$ | $\beta\ \beta^{C}$ | Asymptomatic with microcytosis |
| *Homozygous* | 10 | | $\beta^{C}\ \beta^{C}$ | Mild to moderate anemia with microcytosis |
| **HbE*** | 32 | $\beta^{26\ Glu \rightarrow Lys}$ | $\beta\ \beta^{E}$ | Asymptomatic, Microcytosis |
| **HbO Arab*** | 20 | $\beta^{121Glu \rightarrow Lys}$ | NP | Asymptomatic |
| **HbD Punjab*** | 10 | $\beta^{121Glu \rightarrow Gln}$ | NP | Asymptomatic |

| | | | | |
|---|---|---|---|---|
| Hb Lepore* | 6 | δ /β hybrid gene | NP | Asymptomatic or mild Anemia with mild microcytosis |

NP: Not performed.
*These variants were all detected as heterozygous.

## DISCUSSION

Most structural β-chain variants are clinically benign in their carrier states. Severe transfusion-dependent anemia, however, may arise in the homozygous states or when combined with other variants. Many variants used to be concentrated in malarial zones of Central Africa and spread around the world through population migration (9, 10). HPLC is an excellent method for precise identification and quantification of various hemoglobin fractions (11).

To the best of our knowledge, there are no previous large-scale studies of β-chain Hb variants using HPLC in Jordan. Abnormal β-chain variants were found in 2.6% of our total samples. This is a significant percentage, albeit less common than the reported 5.93% prevalence rate of β-thalassemia in Jordan (12). HbS was the most common variant followed by HbC and HbE. In contrast, the HbS was followed in frequency by HbE and HbD in Saudi Arabia (13). HbS was the predominant variant conforming to its national and global prevalence (12-16). Trait prevalence in Arab countries ranged from 0.3% by Nafei in Egypt to 26% in some regions of Saudi Arabia (17-19). Our rate was approximate to rates in Lebanon, UAE, Yemen, Tunisia, and Libya. (20-24). HbS trait cases were asymptomatic and either had a normal hemogram or mild anemia. Their mean MCV was low (76 fL) which was attributed upon further investigation to concomitant iron deficiency. HPLC results produced a mean Hb variant percentage of 37.4%±3.7 eluting in the S window and low HbF. PCR results were heterozygous for HbS codon 6[A>T]. On the other hand, all HbS disease cases presented with severe anemia (Hb mean of 7.5±1.10 g/dL), which may be attributed to chronic hemolysis as expected in this disorder. Their HPLC data showed higher HbS% and HbF%, and the PCR results were homozygous for the related mutation. Although compound heterozygous disorders of HbS with other β-chain variants were not included in the current study, they might precipitate a significant sickling disease and must be ruled out for the sake of management and genetic counseling.

The second most frequent Hb variant was HbC. This variant originated from West African countries and spread to North Africa, the Middle East and the Arabian Peninsula (25, 26). It is the third frequent variant worldwide, followed by HbD Punjab (27). The phenotype in trait cases was asymptomatic with mild microcytosis. Patients with HbC trait had no anemia, but only mild microcytosis. The disease cases presented with anemia, which was less severe than HbS disease (mean Hb = 11.7±2.2 g/dL). This mild hemolytic anemia is due decreased solubility and rhomboidal crystal formation (28). HbC codon 6[G>A] mutation was detected in one or both alleles.

Thirty-two cases were diagnosed as HbE trait; the third-most frequent variant in our study. HbE is the most common abnormal Hb in Southeast Asia and some parts of India. It is the second most prevalent worldwide (29). Trait and disease states are not associated with severe anemia unless combined with β thalassemia or HbS (30-32). In our analysis, HbE traits showed microcytosis without anemia. HPLC revealed a HbA2-like migration pattern. Suspicion of HbE arose from the high area percentage in the A2 window (mean of 34.1%±3.8). Hb variant percentage and RT are valuable indicators for differentiating Hbs that co-elute in the same window (11). Hb codon 26[G>A] was positive. Molecular analysis is of particular importance to rule out genetic modifiers that may alter the clinical course including α- thalassemia that may be hidden by HbE microcytosis (33, 34). However, HbE trait cases with significant microcytosis in our study were explained by iron deficiency which is commonly seen in Jordan.

HbO Arab, HbD Punjab, and Hb Lepore are rare variants identified in our study (all of which were traits). Such traits are usually benign but can adversely interact with HbS to cause severe disease (34). HbO Arab was first identified in a Palestinian and an Egyptian Arabs. It is actually more common in Eastern Europe (35). Heterozygous mutations were mostly silent. HbD Punjab was named after the northwest Indian region of Punjab. It is the most prevalent variant in Kurdish people from Western Iran, and the fourth variant globally (36, 27). Carriers of HbD Punjab, like HbC, had only mild microcytosis. Hb Lepore is another variant that elutes in the A2 window and is the least common variant in our study. It is usually seen among people with Mediterranean descent. Trait cases are usually asymptomatic, and in our case, they exhibited mild microcytic anemia and a relatively high HbF 3.0±2.5 (37).

Most of our identified variants are benign when the mutation is in a single allele. However, some homozygous forms and some in *trans* combinations are serious conditions. Intra-familial marriages in Arab countries pose a risk for the inheritance of recessive genetic diseases including hemoglobinopathies. Early detection, premarital counseling, and family-oriented screening are crucial to prevent serious disorders in future offspring.

The limitations of this study include its retrospective nature which limited our access to clinical data and patients' contact information including residence that would have helped in determining the frequency of each variant in relation to the country region. Limitations also included the lack of similar previous studies in Jordan to compare our results with, and the lack of molecular analysis for certain mutations concerning rare variants in our study.

## CONCLUSION

The most common β-chain variants in Jordan were HbS followed by HbC and E. Rare variants including HbO Arab, HbD Punjab, and Hb Lepore were also encountered. The diagnosis depends on convenient, quick, and reliable tools that include the gold standard HPLC technique together with clinico-hematological and molecular characteristics. The early detection of abnormal variants is critical for proper patient evaluation and management including family and premarital screening programs and genetic counseling for at-risk marriages to avoid detrimental future disease. This study enhances the hematologist's knowledge about common β-chain Hb variants in our region, which can lead to better disease control, management, and prevention.

***Abbreviations:***

Hb = Hemoglobin.

HPLC = High Performance Liquid Chromatography.

PCR = Polymerase Chain Reaction.

RT = Retention Time.

## REFERENCES


1. **Mathur S, Schexneider K, Hutchison R.** Hematopoiesis. In: McPherson RA, Pincus MR. Henry's Clinical Diagnosis and Management by Laboratory Methods. 22nd ed. Philadelphia: Saunders Elsevier; 2011. P. 536-56.
2. **Elghetany M, Banki K.** Erythrocytic disorders. In: McPherson RA, Pincus MR. Henry's Clinical Diagnosis and Management by Laboratory Methods. 22nd ed. Philadelphia: Saunders Elsevier; 2011. P. 557-600.
3. **Forget BG, Bunn HF.** Classification of the disorders of hemoglobin. Cold Spring Harb Perspect Med. 2013; 3(2): a011684.
4. **Modell B, Darlison M.** Global epidemiology of haemoglobin disorders and derived service indicators. Bull World Health Organ. 2008; 86(6):480-7.
5. **Clarke G, Higgins T.** Laboratory Investigation of Hemoglobinopathies and Thalassemias: Review and Update. Clin Chem. 2000; 46(8):1284–90.
6. **Ou CN, Rognerud CL.** Diagnosis of hemoglobinopathies: electrophoresis vs HPLC. Clin Chim Acta. 2001; 313:187–194.
7. **Joutovsky A, Hadzi-Nesic J, Nardi M.** HPLC retention time as a diagnostic tool for hemoglobin variants and hemoglobinopathies: a study of 60000 samples in a clinical diagnostic laboratory. Clin Chem. 2004; 50:1736–1747.
8. **Sanchaisuriya K, Chunpanich S, Fucharoen G, Fucharoen S.** Multiplex allele-specific PCR assay for differential diagnosis of Hb S, Hb D-Punjab and Hb Tak. Clin Chim Acta. 2004 May; 343(1-2):129-34.
9. **Williams T, Weatherall D.** World distribution, population genetics, and health burden of the hemoglobinopathies. Cold Spring Harb Perspect Med. 2012; 2(9):a011692.
10. **Cavazzana M, Antoniani C, Miccio A.** Gene Therapy for β-Hemoglobinopathies. Mol Ther. 2017; 25(5):1142–54.
11. **Khera R, Singh T, Khuana N, Gupta N, Dubey AP.** HPLC in characterization of hemoglobin profile in thalassemia syndromes and hemoglobinopathies: a clinicohematological correlation. Indian J Hematol Blood Transfus. 2015; 31(1):110–115.
12. **Sunna E, Gharaibeh N, Knapp D, Bashir N.** Prevalence of hemoglobin S and beta-thalassemia in northern Jordan. J Obstet Gynaecol Res. 1996; 22:17–20.
13. **Mehdi SR, Al Dahmash BA.** Analysis of hemoglobin electrophoresis results and physicians investigative practices in Saudi Arabia. Indian J Hum Genet. 2013; 19(3):337-341.
14. **Herklotz R, Risch L, Huber AR.** Hemoglobinopathies—clinical symptoms and diagnosis of thalassemia and abnormal hemoglobins. Ther Umsch. 2006 Jan;63(1):35-46.
15. **Dobrila L, Zhu T, Zamfir D, Tarnawski M, Ciubotariu R, Albano M, et al.** Detection of hemoglobin (Hb) variants by HPLC screening in cord blood units (CBU) donated to the national cord blood program (NCBP). Blood 2016; 128 (22):2182.
16. **Kohne E.** Hemoglobinopathies clinical manifestations, diagnosis, and treatment. Dtsch Arztebl Int. 2011 Aug; 108(31-32):532–540.

17. **Hamamy HA, Al-Allawi NA.** Epidemiological profile of common haemoglobinopathies in Arab countries. J Community Genet. 2013; 4(2):147–167.
18. **Nafei A.** Prevalence of hemogolobinopathies and study of their genetics. Tanta Med J. 1992; 20(1):467–480
19. **El-Hazmi M, Warsy A.** The frequency of glucose-6-phosphate dehydrogenase phenotypes and sickle cell genes in Al-Qatif oasis. Ann Saudi Med. 1994; 14:491–494.
20. **Khoriaty E, Halaby R, Berro M, Sweid A, Abbas H, Inati A.** Incidence of sickle cell disease and other hemoglobin variants in 10,095 Lebanese neonates. PLoS One. 2014; 9(9):e105109.
21. **Al-Hosani H, Salah M, Osman HM, Farag HM, Anvery SM.** Incidence of haemoglobinopathies detected through neonatal screening in the United Arab Emirates. East Mediterr Health J. 2005; 11:300–07.
22. **Al-Nood H, Al-Ismail S, King L, May A.** Prevalence of the sickle cell gene in Yemen: a pilot study. Hemoglobin. 2004; 28:305–15.
23. **Fattoum S.** [Hemoglobinopathies in Tunisia. An updated review of the epidemiologic and molecular data]. Tunis Med. 2006; 84:687–96.
24. **Jain RC.** Haemoglobinopathies in Libya. J Trop Med Hyg. 1979; 82:128–32.
25. **Piel F, Howes R, Patil A, Nyangiri O, Gething P, Bhatt S, et al.** The distribution of haemoglobin C and its prevalence in newborns in Africa. Sci Rep. 2013; 3:1671.
26. **Ouzzif Z, El Maataoui A, Oukhedda N, Messaoudi N, Mikdam M, Abdellatifi M, Doghmi K.** Hemoglobinosis C in Morocco : A report of 111 cases. Tunis Med. 2017; 95(12):229-233.
27. **Rohlfing C, Hanson S, Weykamp C, Siebelder C, Higgins T, Molinaro R, et al.** Effects of hemoglobin C, D, E and S traits on measurements of hemoglobin A1c by twelve methods. Clin Chim Acta. 2016; 455:80-3.
28. **Charache S, Conley C, Waugh D, Ugoretz R, Spurrell J.** Pathogenesis of hemolytic anemia in homozygous hemoglobin C disease. J Clin Invest. 1967; 46(11):1795–1811.
29. **Warghade S, Britto J, Haryan R, Dalvi T, Bendre R, Chheda P, et al.** Prevalence of hemoglobin variants and hemoglobinopathies using cation-exchange high-performance liquid chromatography in central reference laboratory of India: A report of 65779 cases. J Lab Physicians. 2018; 10(1):73-79
30. **Dolai T, Dutta S, Bhattacharyya M, Ghosh M.** Prevalence of hemoglobinopathies in rural Bengal, India. Hemoglobin. 2012; 36:57–63.
31. **Nishad A, de Silva I, Perera H, Pathmeswaran A, Kastutiratne K, Premawardhena A.** Role of red cell distribution width in screening for Hb E trait in population screening for hemoglobin disorders. J Pediatr Hematol Oncol. 2014; 36(8):e490-2.
32. **Mohanty D, Colah R, Gorakshakar A, Patel R, Master D, Mahanta J, et al** Prevalence of β-thalassemia and other haemoglobinopathies in six cities in India: a multicentre study. J community genet. 2013; 4(1):33–42.
33. **Vichinsky E.** Hemoglobin and syndromes. Hematology Am Soc Hematol Educ Program. 2007:79-83.
34. **Bain B.** Haemoglobinopathy Diagnosis. 2nd ed. Chicester, United Kingdom: John Wiley and Sons Ltd; 2006. P .201-209.
35. **Steensma D, Hoyer J, Fairbanks V.** Hereditary red blood cell disorders in Middle Eastern patients. Mayo Clin Proc. 2001; 76:285-93.
36. **Rahimi Z, Muniz A, Mozafari H.** Abnormal hemoglobins among Kurdish population of Western Iran: hematological and molecular features. Mol Biol Rep. 2010; 37(1):51-7.
37. **Gonçalves I, Henriques A, Raimundo A, Picanço I, Reis A, Correia Junior E, et al.** Fetal hemoglobin elevation in Hb Lepore heterozygotes and its correlation with beta globin cluster linked determinants. Am J Hematol. 2002; 69(2):95-102.