Detection of Hemoglobin Inherited Disorders in Hemoglobin Electrophoresis Sensitivity

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Abstract

Objective: To detect hemoglobinopathies such as β-thalassemia and sickle cell anemia cases using the hemoglobin electrophoresis technique in a population sample from Children's Hospital Lahore.

Material and Methods: This cross-sectional study was carried out from Mar 2023 to Mar 2024 at Thalassemia Department, Fatima Jinnah Medical College, Lahore. Eighty blood samples were drawn from subjects suspected of having hemoglobino-pathies and examined by Hb electrophoresis. The diagnosis of hemoglobinopathies was made based on hemoglobin electrophoresis, sickling tests, and family studies. Individuals with low hemoglobin concentrations accompanied by elevated abnormal hemoglobin percentages were included in this study. Fifty-eight cases of hemoglobinopathies were diagnosed, including 30 (37.5%) with β-thalassemia and 28 (35%) with sickle cell disease carriers.

Results: β-thalassemia and sickle cell anemia carriers were identified in the hematology section of Children's Hospital. Recent hemoglobin protein studies revealed the presence of different common defected hemoglobin types associated with these disorders, distributed as follows: 15 subjects (18.7%) were HbAS (sickle cell minor carriers), 8 (10%) were HbFS (sickle cell minor carriers), 7 (8.8%) were HbS (sickle cell disease), while thalassemic majors were: 9 (11.3%) with HbF and 12 (15%) with HbAF type. Thalassemia minor hemoglobin (HbA2) type represented 7 (8.8%). However, 22 subjects had normal Hb electrophoresis.

Conclusion: The investigations described below demonstrate a rapid and simple method that allows quantitative analysis of the proportions of various hemoglobin forms present. Hemoglobin gel electrophoresis is a simple and convenient technique for studying hereditary hemoglobinopathies at an alkaline pH (4.8 to 6.8). We suggest extending its usage to detect other hemoglobin disorders.

Keywords: Hemoglobin (Hb), Hemoglobinopathies, Sickle cell anemia, hemoglobin disorders, Sickling test.

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Introduction

For the diagnosis of hemoglobinopathies, hemoglobin electrophoresis is the basic technique identifying a diverse array of mutants (genetically different), which are affecting the three-dimensional structures and functionality of hemoglobin. These mutations may cause anemia to fatal diseases. Thalassemia and sickle cell disease represent the most frequent hemoglobinopathies. Hemoglobinopathies is a genetic disorder due to abnormality of hemoglobin and it constitutes several monogenic disorders. Due to these mutations, altered synthesis of hemoglobin causes structural changes in chains, ultimately in 3D folding of the hemoglobin, and leads to severe health conditions like hemolytic anemia, Sickle cell disease, Thalassemia, polycythemia, and erythrocytosis. Thalassemia syndrome is a series of genetic disorders in hemoglobin (Hb) synthesis, characterized by a reduced rate of production of one or more of the globin chains of hemoglobin. About 3%

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of the world population carries genes for β-thalassemia and 3000 mutants of hemoglobin[1]. The adult hemoglobin (HbA) is a tetrameric protein(α2β2) and two types of globin gene clusters are responsible for forming two different genes of a tetramer. β-thalassemias are autosomal recessive inherited blood disorders that result mainly from mutations that decrease (β+) or eliminate (β0) the production of β-globin. This leads to an excess of α-globin, which precipitates in developing erythroblasts, resulting in ineffective erythropoiesis.[2-3] Patients with β-thalassemia are characterized by hypochromic, hemolytic anemia, and dependence on blood transfusions to sustain life.[4]

β-thalassemia is widely distributed throughout the world, with considerable frequencies in the Eastern Mediterranean countries, including Iraq,[5] and the Arabian Gulf region.[6] It has been estimated that 3% of the world's population, or 200 million people, in addition to almost 150,000 affected individuals born annually, carry the β-thalassemia gene. There are several variants of thalassemia due to different structural changes in hemoglobin and each of them has a different severity level. This gene carries a wide spectrum of clinical manifestations, ranging from β-thalassemia intermedia to severe, transfusion-dependent β-thalassemia major. The clinically important feature of β-thalassemia is its interaction with other hemoglobinopathies, like sickle cell disease, in co-inheritance, which improves the hematologic parameters of heterozygous β-thalassemia.[7] β-thalassemia minor is also a type of thalassemia, in which the person is heterozygous for the trait, it inherits one normal and the other one is thalassemia variant gene. Usually, these people do not have any kind of the symptoms but sometimes mild anemia, microcytosis, hypochromia, and increased iron load could be observed. From their diet, the absorption of iron ions increased.

Sickle cell disease (SCD) is a protean disorder caused by elevations of intra-erythrocytes and total blood viscosity. Hypoxia-induced gelation of hemoglobin S (HbS) deforms the erythrocyte and its membrane, causing massive cation loss and increased erythrocyte surface expression of adhesion molecule receptors.[8] This leads to hemolytic anemia and acute vasoocclusion, resulting in organ damage from recurrent erythrocyte sickling, chronic hemolysis, and progressive endothelial vasculopathy.[9]

Like other parts of the country, hemoglobinopathies are an expression of the β-globin gene, leading to changes in the rate of synthesis of β-globin chains of hemoglobin. In classic β-thalassemia major, life expectancy is shortened to 25-30 years on average, due to associated complications such as growth retardation,[10-11] diabetes mellitus, endocrine dysfunction, hypothyroidism, progressive failure, and cardiac complications.[12-20]

Hemoglobinopathies are an important problem in Nassiriyah province (in southern Iraq, with a population of around 1.5 million and over 400 registered patients). The emergence of these disorders is partially due to consanguineous marriages, which are common in this area. This article aims to use hemoglobin gel electrophoresis to detect some hemoglobinopathy forms. With the process of hemoglobin electrophoresis, hemoglobin components can be separated from each other on the basis of their charge and mass value. This approach may contribute to treatment and possibly prevent the transmission of the hemoglobin mutation by identifying carriers, as their offspring are at risk of inheriting the mutation.

Material and Methods

This cross-sectional study was carried out from Mar 2023 to Mar 2024 at Thalassemia Department, Fatima Jinnah Medical College, Lahore. After taking approval from ethical committee Reference No UCP/ORIC/EC/08 Dated 24-08-23. A total of 80 subjects with low hemoglobin levels (under 10mg/dl) suspected of having hemo-globinopathies and voluntary individuals with signs and symptoms of anemia, who attended hospitals, were recruited for this study. All were ethnic Arabs, with ages ranging between 1.2 and 34 years (median age of 7.8 years). They included 38 males and 42 females. 3 ml whole blood samples were collected in EDTA-coated tubes from patients during their hospital visits and used immediately. Hemoglobin level: The level of hemoglobin was checked using the Reflotron Plus (Roche, Germany) Roche diagnostic GmbH machine.

Complete blood count was determined using the Coulter Micro diff II machine according to the manufacturer's instructions, in addition to routine examination of peripheral blood films. A sickling test was performed by adding 50 µl of well-mixed whole blood to 4 ml of phosphate buffer/sodium hydrosulfite solution (one tube for each test and each control). The tube was covered with a cap or parafilm, mixed three or four times, and then incubated in the reading rack for 10 to 20 minutes at room temperature. A positive result was indicated by the presence of turbidity and the absence of visible lines on the reading rack. Electrophoresis of the hemoglobin solutions was carried out in an apparatus (HelloBio), utilizing the alkali denaturation technique as follows: Venous blood was drawn from fasting individuals into EDTA-treated vacuum tubes. About 100-200 µl of whole blood

was added to a tube with 10 ml saline and centri-fuged. 30 µl of the sediment was mixed with 130 µl of hemolyzing solution. Then, 5 µl of each hemolysate was applied across the slits and left for 20 to 30 seconds to allow absorption. The gel was placed in the tank with samples on the cathodic side and run at 200 volts for 20 minutes. The gel was dried completely with hot air (less than 60°C) and stained for 5 minutes with a protein staining solution. The film was destained for 5 minutes in three destaining solution baths and dried again with hot air. The results were analyzed, and the standards were evaluated and analyzed by software using HbAFSA2 and HbAFS protein.

Results

Low hemoglobin concentration is a common manifestation of anemia caused by many environmental factors, such as malnutrition and hemorrhagic conditions, or by hereditary factors, such as hereditary persistence of fetal hemoglobin (HPFH) and hemoglobinopathies (HbP). The normal ranges of Hemoglobin in adults and infants are represented in Table 1. In the case of hemoglobinopathies, the percentage of HbA, HbS, and HBA2 is disturbed due to mutation in the beta chain of hemoglobin. In this study, we found that the age of HbA2, HbAF, and HbAS carriers was older than HbF and HbS hemoglobin type carriers. The 70 unrelated blood samples derived from Iraqi β-thalassemia and sickle cell carriers were analyzed to elicit several forms of sickling and β-thalassemia hemoglobin protein patterns as follows. Table 2 shows the representative characteristics of Beta thalassemia and sickle cell disorders. Thalassemia gene carriers are characterized by the absence of the S form of hemoglobin (HbS), having a high concentration (70-90%) of the fetal form of hemoglobin (HbF), accompanied by an HbA2 hemoglobin pattern as low as normal (less than 1%) as shown in Figure 1, and the highest percentage of hypochromic and reticulocytes obtained by blood film monitoring. In Figure 2, the difference between the normal and variant of hemoglobin polypeptide is also indicated in which Hb-A has normal Glutamate at 6th position, while Hb-S, the variant of polypeptide indicating that valine replaced the glutamate at 6th position, represented in red color.

Table 3: *Comparison of Predictive Values (Bishop Score vs. Cervical Length)*

Hemoglobin	Chains	Normal % Adult	Normal % Neonate
HbA	A_2, β_2	96-98%	27%
HbF	A_2, γ_2	0.5-0.8%	70%
HbA2	A_2, δ_2	1.5-3.2%	0.3%

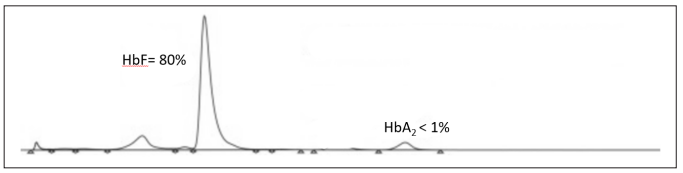

Figure 1: *HPLC of an adult thalassemia disease carrier*

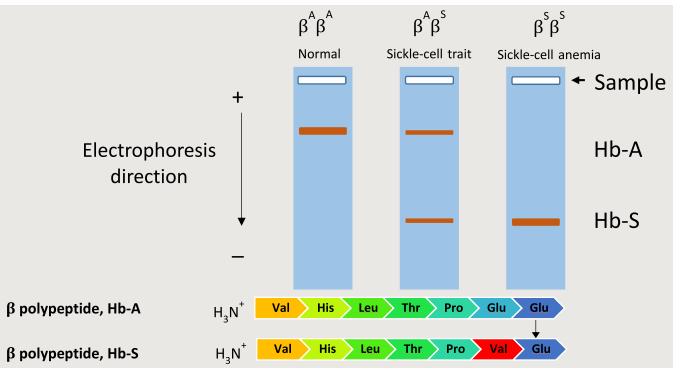

Figure 2: *Electrophoretic hemoglobin pattern of sickling trait*

Table 2: *Representative characteristics of Beta thalassemia and sickle cell disorders*

Hemoglobinopathies	Hb(AS)	Hb(S)	Hb(FS)	Hb(F)	Hb(AF)	Hb(A2)
N	15	7	8	9	12	7
Age (years)	28.3-34	2-28	1.8-5.2	1-2.8	2-6	2.5-7
Hb g/dl	10.04±1.35 8-2	9.3±1.5 7.8-12.2	8.3±1.8 6.2-12	5.3±1.4 3-8	7.4±1.6 5.5-10.8	9±1.3 6.8-10.8
Reticulocytes	2.67±1.06 1.2-4.06	1.83±0.53 1.96-3.2	3.74±1.19 2-5.4	6.1±1.9 4-10	3.3±0.79 2.2-4.8	2.90±0.87 2-3.88
Hb F (%)			48.2±8.19 33.6-60	86.62±5.8 79.8-93.2	40.15±6.77 28.8-48	
Hb A2	1.27±0.45 0.53-2.02	0.92±0.51 0.22-2.0	0.83±0.66 0.1-2.02	0.96±0.47 0.56-26	1.58±0.71 0.8-3.2	9.33±2.49 6.22-12.3
HbS	42.41±6.70 30.8-55	85.04±6.28 78.4-92.06	40.4±6.85 30.8-50.2			

Discussion

Hemoglobinopathies are the most common red blood cell genetic disorder, for its treatment it is necessary to recognize the mutations in hemoglobin chains. Abnormalities of hemoglobin are quite common, mostly it is detected in public by surveys and prevention programs laid for the treatment of hemoglobinopathy. For better confirmation of any abnormality in hemoglobin, at least two methods of detection are used. The more efficient methods of diagnosis are CE-HPLC (Cation exchange high-performance chromatography), CE (Capillary electrophoresis and IEF (isoelectric focusing). CE HPLC is used to diagnose abnormalities in adults as well as in children, it is able to differentiate between SS, AS, SD, S-β+-thal, AC, SC, and C-β+-thal, in blood. Red blood cells become more glycosylated in old age[21]. In the case of isoelectric focusing, variants of hemoglobin having different isoelectric points can be separated from each other at zero pH. In this way, different mutants can be identified so far.

In this study, we found that the age of HbA2, HbAF, and HbAS carriers was older than HbF and HbS hemoglobin type carriers. The 70 unrelated blood samples derived from Iraqi β-thalassemia and sickle cell carriers were analyzed to elicit several forms of sickling and β-thalassemia hemoglobin protein patterns as follows. The presence of mild microcytosis, in the absence of iron deficiency, suggests the presence of β-thalassemia. These carriers or individuals with β-thalassemia trait are essentially normal, although they can usually be detected by screening red cell indices that demonstrate a reduced mean corpuscular volume (MCV) and reduced mean corpuscular hemoglobin value (MCHV). Gel electrophoresis precisely determines the elevation of hemoglobin A2 levels in thalassemia minor. Our result is consistent with other studies previously submitted in literature. This thalassemia intermediate pattern may result from the elevation of fetal hemoglobin (HbF) to around 30-50% as illustrated in thalassemia disease.

Hemoglobin gel electrophoresis may yield a pattern of 65% sickle hemoglobin and 34% F hemoglobin. The presence of normal (A) hemoglobin in combination with more than 90% S hemoglobin has so far only been encountered in sickle cell-thalassemia disease. Blood transfusions are required during the treatment of this disease.

This genetic disorder is accompanied by one normal gene and one sickle cell gene (HbS). It is an autosomal recessive disease, for an infected person it is necessary to be homozygous for the sickle cell genes. A protein with a negative charge, hemoglobin (Hb) is drawn to the anode pole of an electric current and separates from the positive pole as a result. The kind and intensity of the charge, in addition to the hemoglobin type's molecular weight, influence this movement. Currently, the hemoglobin molecule is known to be susceptible to over 300 genetic mutations, some of which have obvious clinical consequences and pose a serious risk to health, particularly in heterozygous conditions.[22]

Certain studies suggest that the fractional HbS content of sickle trait erythrocytes may influence the severity of certain clinical complications.[22] The amount of S hemoglobin in sickle cell trait carriers has been found to vary from 34 to 39%, without any apparent correlations to the severity of their clinical manifestations. We encountered several instances of low hemoglobin values in patients with a positive sickling test, caused by a variety of anemia superimposed on the sickling trait. Electrophoretic analysis of the hemoglobin of some individuals revealed that even in the absence of a positive sickle cell test by blood film test, erythrocytes may still contain small amounts of S hemoglobin, consistent with a study.[23] The reduction of the HbS level in the sickle cell trait associated with α-thalassemia can be explained by a greater affinity of BA than BS chains for α chains in limited supply.

Electrophoresis of hemolysates of patients with sickle cell anemia produced a characteristic pattern, which consisted of a major component of hemoglobin S showing variations from 70 to 91%, and usually with reciprocal values for F hemoglobin (data not shown). Fetal hemoglobin (HbF) is a major contributor to the remarkable phenotypic heterogeneity of sickle cell anemia and influences the levels of disease severity.[24] Since, in some patients with the disease, the minor component was less than 5% or even absent, quantitation of the fetal fraction from gel electrophoresis is necessary.

Our results indicated that SCA patients' blood films are characterized by a low rate of hypochromic and reticulocyte cells by microscopic diagnoses. By indirect investigations, we revealed that some anemia patients may have suffered from transient hemolysis due to pyogenic infection or occurrence of G6PD (glucose-6-phosphate dehydrogenase deficiency), but not due to heritable hemoglobin disorder. In those individuals, hemolysis ceased after appropriate treatment. It may be argued that the high consanguinity rate among homozygous

individuals may have affected the actual frequencies of hemoglobinopathies' emergence. The significant stimulus to carry out such studies was the demonstration that hemoglobin in hemoglobinopathy patients is electrophoretically different from normal adult hemoglobin, and their assessment does not require a complex apparatus and special skills necessary for common detection methods.

Conclusion

Hemoglobinopathies and hemoglobinopathy traits require comprehensive laboratory examination using CBC, blood cell morphology, and qualitative and quantitative electrophoresis. The results indicate the importance of quantifying HbA, HbF, and HbA2 to aid in the differential diagnosis of different types of hemoglobinopathies. This investigation narrates a rapid, efficient, and simple method that can quantitatively analyze the proportions of various hemoglobin variants present in the blood sample. Hemoglobin gel electrophoresis is a simple and convenient technique for studying hereditary hemoglobinopathies at an alkaline pH (4.8 to 6.8). We suggest extending its usage to detect other hemoglobin disorders.

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Conflict of Interest *None*

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Authors Contribution

SI: Conceptualization of Project
AA: Data Collection
SP: Literature Search
SZ: Statistical Analysis
SH: Drafting, Revision
AA: Writing of Manuscript