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ANALYSIS OF HEMOGLOBIN VARIANTS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN BETA THALASSEMIA (HPLC)

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ABSTRACT

The most prevalent genetic blood disorder in the world, beta-thalassemia is typified by base negotiation, or the minor omission or insertion of one or two nucleotides in the globin gene. These days, because of the mixing of the gene pool, this issue is not limited to any one ethical group of races; rather, each group reflects a unique set of mutations. A quick, sensitive, and accurate method for identifying aberrant hemoglobin fragments is High-Performance Liquid Chromatography (HPLC). A total of 55 cases of beta-thalassemia from Lahore and surrounding areas have been examined for vibrant hemoglobin variations. This is an observational (Prospective and Retrospective) study carried out in the Department of Pathology in CMH LMC & IoD. The study was performed on Agilent 1220 perpetuity LC (Agilent Technologies) a High-Performance Liquid Chromatography (HPLC) using EZChrom Elite for Beta-thalassemia. Abnormal hemoglobin variants were anatomized for 55 cases of Beta-thalassemia on High-Performance Liquid Chromatography (HPLC). There were about 18 cases of beta-thalassemia major and 37 cases of beta-thalassemia carriers. The frequency observed in our study was HbA1c (0.51), HbF (0.18), HbE (0.13), HbD (0.86), HbS (0.17), and HbA2(0.15). Automated High-Performance Liquid Chromatography is an applicable approach for the webbing and plausible identification of cases as well as carriers of Beta-thalassemia previous to DNA studies for a definitive opinion.

Keywords: Hemoglobinopathy, high-performance liquid chromatography (HPLC), thalassemia, Pakistan

INTRODUCTION

A diverse range of blood disorders known as beta-thalassemia is distinguished by a reduced or nonexistent production of β-globin chains. The disease may be caused by several genetic anomalies, the majority of which are mutations that affect the expression of the β-globin gene. [1]. The gene is located on the short arm of chromosome 11p15.5. In terms of clinical manifestation, beta-thalassemia can manifest as β° or β+, thalassemia intermedia (β+/β+ or β+/β°), or beta-thalassemia major (β°/β°). There are two types of beta-thalassemia based on phenotypic characteristics: β°- with no β globin chain synthesis and β+- with some beta globin chain synthesis.[2]. Thalassemia major, a severe transfusion-dependent anemia, is brought on by the homozygous

condition for β° (β°/β°). The mutations that produce beta-thalassemia are unique to each ethnic group [3,4]. Over 380 distinct kinds of mutations have been identified as the cause of this condition to date. The Mediterranean region, a portion of the Middle East, the Indian subcontinent, and South East Asia make up the thalassemia belt, which is where the illness is endemic. According to estimates, there are over 45 million beta-thalassemia carriers in South Asian nations including India, Sri Lanka, Bangladesh, and Pakistan, with a carrier rate of about 1:20. The condition causes severe morbidity and death in affected people, and treatment for those with beta-thalassemia major required frequent transfusion of blood and costly iron chelation therapy, which is not a suitable technique. Although Uttar Pradesh has a population of over 200 million people and is a multiethnic state, weddings most frequently occur within the same ethnic groupings. The data presented by various papers helps develop appropriate molecular screening methods for the benefit of thalassemic patients. High-Performance Liquid Chromatography is one method that offers a unique pattern of hemoglobin variation distribution in patients and carriers, making it simple to distinguish beta-thalassemia from other hemoglobinopathies.

The available research suggests that evaluation is necessary to prevent thalassemia from being born. In the city of Lahore and the surrounding regions, will be the official screening location for the presumed detection of beta-thalassemia carriers and sufferers. It will also offer molecular diagnostics and prenatal diagnosis. Due to its contribution to the eradication of a hereditary condition like thalassemia, this initiative will be helpful to society.

METHODOLOGY

Sample Collection Criteria

The calculated sample size is 55 cases, between the ages from 2 to 65 years old (18 patients and 37 carriers). Each patient's family members' 2ml of venous blood was drawn and placed in EDTA vials. All of the trial participants required blood transfusions. One of the patients was receiving iron chelation treatment on a regular basis, while another was receiving a transfusion for the first time. Age, sexual orientation, family background, and parent-child consanguinity were noted. Except for a small number of cases when transfusions were omitted at unpredictable intervals, most of these patients had regular transfusions. This 8-month prospective research was conducted at the CMH LMC and IOD in Lahore. There are no significant or minor flaws in the study that would offend human volunteers.

On an automated blood counter, hemoglobin, erythrocytes (RBC) counts, and red blood cell indices were calculated. A Mean Corpuscular Volume (MCV) & Mean Corpuscular Hemoglobin (MCH) 27pg and Erythrocytes count >5 million/l are the traditional red cell indicators for beta-thalassemia. HbA2, HbF, and other hemoglobin variants were investigated using the High-Performance Liquid Chromatography technology, which is used for the chromatographic separation of human hemoglobin. Agilent 1220 LC (Agilent Technologies), a High-Performance Liquid Chromatography system with EZChrom Elite, was used to analyze samples for beta-thalassemia. The beta-thalassemia kit (Gordion Diagnostik, Turkey) was used to analyze every sample. Agilent Technologies' 1220 LC found a chromatogram of the beta-thalassemia major cases identified by the EZChrom application [5,6].

High Performance Liquid Chromatography system Agilent 1220 LC employs double wavelength detection of 415nm & 600nm and is completely automated. From the manufacturer (Gordion Diagnostiks, Turkey), many elution techniques were provided, including particular columns, buffers, and software. The EZChrom Elite for beta-thalassemia has been developed to isolate and quantify aberrant hemoglobin by calculating the retention duration and area percent of HbA2, HbF, and other Hb variations. Elution takes place in the 3 x 0.46 cm nonporous Cation-Exchange column at a flow rate of 1.5 ml/min and analytical duration of 6.5 minutes using a gradient of buffers A & B with different ionic strengths and pHs. Each case's 5 l of whole blood was collected in an Eppendroff container, and 1 ml of lysis buffer was added to each. 10 l of the mixture was obtained and injected after 10 min of room temperature incubation[7].

By examining the HbF and HbA2 levels for beta-thalassemia, reports and chromato-graphs were examined and interpreted. Retention time and area % were analyzed for additional structural variations. A peak for HbA1c, HbD, HbC, HbE, HbS, and

HbA0 may be seen on each chromatogram. The manufacturer's instructions for variations' values were used to interpret the retention times for each hemoglobin variant[8].

RESULTS

Of 55 were included for the investigation, 27 (49%) were males and 28 (51%) were females Figure-1. Of whom 18 cases had Major β-thalassemia and 37 were carriers. The patients' family members served as the carriers. The investigation found aberrant hemoglobin variations in every patient that was examined. Retention duration and area % were used as a basis for assuming the identification of hemoglobin variations. However, additional variables were also included, including location, ethnicity, and clinical presentation. Table-I displays the frequency of hemoglobin variations discovered during analysis, while Table-II displays the pertinent Erythrocytes (RBC) properties of β- thalassemia majors and carriers.

Fig I

Gender Distribution

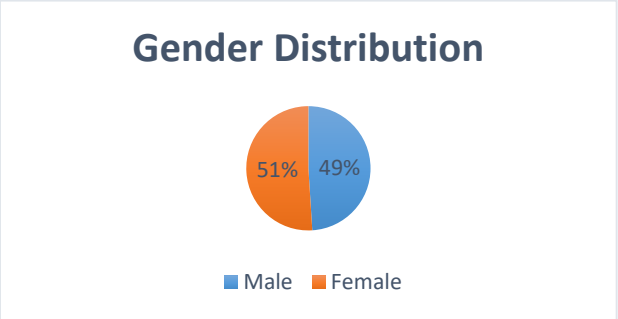

Table I:

Analysis revealed the frequency of hemoglobin variations

Hemoglobin variants	Number (%)
HbA_{1c}	10 (0.51)
HbF	37 (0.18)
HbA_0	24 (0.12)
HbE	27 (0.13)
HbA_2	31 (0.15)
HbD	17 (0.86)
HbS	27 (0.17)
HbC	23 (0.11)

Table II

shows the red blood cell count (RBC), hemoglobin (Hb), and red cell indices (MCV, MCH, and MCHC) in majors and carriers of β thalassemia.

Total number of samples (N= 55)		
β-thalassemia Major (n=18) Mean ± 2 SD		**β -thalassemia** Carriers(n=37) Mean ± 2 SD
Hb	5.31 ± 0.23	11.23 ± 0.88 gms/dl
RBC	3.35 ± 0.48	4.91 ± 0.34 X 10^{12}/L
MCV	56.53 ± 2.08	76.32 ± 4.45 fl
MCH	15.44 ± 0.88	23.94 ± 0.18 pg
MCHC	26.02 ± 2.36	K30.83 ± 1.56 gms/dl

Table III:
Criteria used for presumptive screening of thalassemia

MCV (fL)	MCH (pg)	Hb Type	Interpretation
≥ 80	≥ 27	A_2A, Hb A_2 ≤ 3.5%	Normal hemoglobin type or non-clinically significant thalassemia
< 80	< 27	A_2A, Hb A_2 ≤ 3.5%	Normal hemoglobin type with low levels of MCV (< 80 fL) and MCH (< 27 pg)
< 80	< 27	A_2A, Hb A_2 3.6–8 %	Beta thalassemia trait with or without alpha thalassemia
< 80	< 27	A_2F	Beta thalassemia major with or without alpha thalassemia
< 80 or ≥ 80	< 27 ≥ 27	EA, Hb A_2 ≥ 25 %	Hb E trait
< 80	< 27	EE, Hb E ≥ 80%, Hb F ≤ 5 %	Hb E disease with or without alpha thalassemia
< 80	< 27	EFA	Beta thalassemia/Hb E disease with or without alpha thalassemia
< 80	< 27	A_2A Barts H or A_2A H	Suspected Hb H disease

Overall, our research article provides an overview of beta-thalassemia, its prevalence, importance of screening and diagnosis. The methods section includes details on sample collection, hematological parameters, and the use of High-Performance Liquid Chromatography for analysis. The results show the identification of aberrant hemoglobin variations in patients and carriers. In the major group of thalassemia, no clear link was found between HbF and other hematological markers and clinical outcomes.

DISCUSSION

High-Performance Liquid Chromatography (HPLC) has emerged as a rapid and reliable technique for the investigation of beta-thalassemia and hemoglobin (Hb) anomalies[9]. The cation-exchange approach, particularly using HPLC, has become the preferred method for determining both normal and aberrant Hb fractions[2]. To address this complexity, effective screening methods are imperative, and HPLC stands out by offering a comprehensive single-screening test, measuring HbF, HbA1c, and HbA0, while also detecting additional variants[5,7].

HPLC, known for its sensitivity, specificity, repeatability, and time efficiency, becomes an excellent approach for regular clinical laboratory analysis of thalassemia screening[7]. Given the focus on beta-thalassemia in this study, the quantification of HbA2 and HbF levels by HPLC was deemed crucial, particularly in a laboratory equipped for genetic research[10,11].

Indicators such as red blood cell count and indices, elevated HbA2 levels, and imbalanced globin chain synthesis are frequently used in diagnosing beta-thalassemia, emphasizing the importance of understanding this genetic disease through family investigations. Microcytosis, increased red blood cell numbers, and lower-than-normal hemoglobin levels are the hallmarks of the beta-thalassemia trait, the most common grouping of hemoglobin defects. Asian environments are frequently associated with a variety of mutations, such as IVS1-5(G-C), 619 bp deletion, IVS 1-1(GT), CD8/9(+G), CD41/42 (CTTT), CD15 (G-A), and CD30 (G-C)[4,12].

In this study, an HbA2 level greater than 15% was considered significant. Both beta-thalassemia major and carrier populations exhibited higher HbF frequencies, indicating the relevance of quantifying HbF and HbA2 for identifying homozygous beta-thalassemia variations. The presence of HbA0 and HbA2 characteristics in specific cases further emphasized the intricate interactions in double heterozygous and homozygous states, underlining the need for detecting additional variations[6]. According to current guidelines, an independent approach should be used to confirm abnormal variant Hbs. This is sensible practice, and it's usually inexpensive and simple (e.g., electrophoresis for certain peaks, sickling test for S-window peaks). Because the diagnosis affects prenatal testing, screening pregnant women is extremely crucial[7]

CONCLUSION

Elevations in HbA2 and HbF levels serve as defining features of classical beta-thalassemia, and the automated HPLC technique emerges as the preferred method for testing. The ability of HPLC to swiftly, accurately, and consistently manage thalassemia and hemoglobinopathies is particularly crucial given the high incidence of beta-thalassemia. Early diagnosis of thalassemia traits plays a pivotal role in preventing thalassemia major in progeny.

The complex interactions in heterozygous and homozygous areas, emphasize how important it is to find more variances and can result in serious hematological issues. Regular premarital screening, aimed at preventing high-risk marriages due to the prevalence of hemoglobin abnormalities, becomes imperative. The HPLC investigation presented in this study sheds light on the extent of hemoglobinopathies and thalassemia in a hospital-based limited population, catalyzing raising awareness among affected patients. While this study may represent just the tip of the iceberg, it undoubtedly contributes to the broader understanding of these conditions and the importance of proactive screening.

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Conflict of Interest
Authors declare no conflict of interest

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