Chromatographic insights into hemoglobinopathies: spectrum analysis by high-performance liquid chromatography in a western Indian tertiary care hospital

Running title: Hemoglobinopathies by HPLC in a western part of India

Dr. Parineeta Shelke
Department of Pathology, Bharati Vidyapeeth (DTU) Medical College, Pune, Maharashtra, India, parineetashelke@gmail.com, ORCID Id – 0000-0002-3978-1137
Dr. Preeti Doshi
Department of Pathology, Bharati Vidyapeeth (DTU) Medical College, Pune, Maharashtra, India, prdoshi22@gmail.com, ORCID Id- 0000-0002-6454-5435
Dr. Amit Nisal
Department of Pathology, Bharati Vidyapeeth (DTU) Medical College, Pune, Maharashtra, India, dramitnisal@yahoo.com, ORCID Id – 0000-0002-8166-1614
Dr. Abdulrahaman Momin
Department of Biochemistry, Bharati Vidyapeeth (DTU) Medical College, Pune, Maharashtra, India, rahaman.momin@gmail.com, ORCID Id - 0000-0002-3913-5418
Dr. Ravindra Nimbargi
Department of Pathology, Bharati Vidyapeeth (DTU) Medical College, Pune, Maharashtra, India, nimbargiravindra@gamil.com, ORCID Id – 0000-0002-0496-7556

Corresponding author: Preeti Doshi
Email: prdoshi22@gmail.com
Tel: +919423005002
Address: Department of Pathology, Bharati Vidyapeeth (DTU) Medical College, Pune, Maharashtra, India

Abstract

Background: Hemoglobinopathies are a group of genetic disorders affecting the structure or production of hemoglobin. Two main categories are thalassemia syndromes and structural hemoglobin variants. Preventing inherited hemoglobinopathies has been recognized as an international health priority. These disorders can now be accurately diagnosed using HPLC. The present study aimed to study various hemoglobinopathies using HPLC from Western Maharashtra, India.

Methods: The cross-sectional study was conducted in Bharati Vidyapeeth (DTU) Medical College, Pune. During the study period of 3 years, 1455 specimens of either gender were analyzed for variant analysis using VARIANTTM II β-thalassemia Short Programme. The HPLC with ion exchange chromatography principle was used. The hematological parameters were estimated in every subject. The incidence of hemoglobinopathies, with its types, is presented.

Results: The age of the 1455 subjects included was 26.91 ± 7.06 years, of which the majority were females. The overall incidence of hemoglobinopathies was found to be 8.78%. The most prevalent condition was β-thalassemia minor, followed by β-thalassemia major, Hb S trait, and Hb S disease. There were two cases with heterozygous for Hb D Punjab, and one case was heterozygous for Hb E. Mentzer index was positively associated with Hb F and MCH, and negatively associated with hemoglobin, Hb A and RDWC levels.

Conclusion: The incidence of hemoglobinopathies in this region was reported to be 8.78%. The most common hemoglobinopathy reported was β thalassemia, of which most were heterozygous for the β thalassemia trait. The presence of Hb E increased the severity of anemia when present with β-thalassemia.

Keywords: Hemoglobinopathies; β-thalassemia; HPLC; Hb A2; Hb E

Introduction

Hemoglobinopathies are a class of genetic hemoglobin (Hb) disorders characterized by abnormal hemoglobin molecule production or structure (1). It includes both qualitative and quantitative globin synthesis disorders. Hemoglobinopathies are the most prevalent monogenic diseases and one of the major global health issues, with approximately 7% of the world's population being carriers (2). The Mediterranean region and sizable portions of Asia and Africa were where they were first discovered. They have spread from those areas all over the world due to international migration (2, 3) in India, leading to significant morbidity and mortality (4). Geographical differences affect the prevalence of thalassemia and hemoglobinopathies. According to estimates, 0.37 per 1,000 fetuses in India have Hb disorders (4). In the Indian subcontinent, hemoglobinopathy and thalassemia are the health issues. The prevalence of β-thalassemia trait in 59 ethnic groups ranged from 0 to 9.3%, according to Mohanty Det al. The overall prevalence of β-thalassemia trait was 2.78% and varied from 1.48 to 3.64% in different states of India (5).

A significant public health issue in India is thalassemia and its interaction with structural Hb variants like Hb S and Hb E (6). Several analytical techniques, such as electrophoretic and chromatographic methods, have been developed for the analysis of human hemoglobin in a standard clinical laboratory. Red blood cell (RBC) count with erythrocyte indices and a hemoglobin test (hemoglobin electrophoresis and/or chromatography) are used in routine practice to diagnose hemoglobinopathies (2, 7).

In 1975, an expert panel on "Abnormal Hemoglobins and Thalassemias" convened by the International Committee for Standardization in Hematology recommended two panels of tests for making the diagnosis. Initial tests included a complete blood count, hemoglobin electrophoresis at an alkaline pH, sickling and solubility tests, and the measurement of HbA2 and Hb F (8). for both screening and confirmation of hemoglobinopathies, high-performance liquid chromatographic (HPLC) methods have been developed and offer quick, repeatable, and accurate results. (9 , 10)

The best method of preventing these disorders is prospective prevention through population screening and genetic counseling. The present study aimed to determine the incidence of hemoglobinopathies and the spectrum of different types of hemoglobinopathies in all patients referred for Hb HPLC because precise data on the prevalence of hemoglobinopathies in the Western region of Maharashtra have been lacking. Finding the prevalence of thalassemia and other hemoglobinopathies in ANC patients was one of the objectives.

Methods

This cross-sectional study was conducted in the department of Pathology of a tertiary care teaching hospital in Pune, Maharashtra from April 2019 to March 2022. Participants included patients with abnormal haemograms suggestive of hemolytic anemia and patients who voluntarily came in for a premarital checkup. Transfusion-dependent children and adults were also included. The study was approved by the institutional ethics committee (BVDUMC/IEC/131) after the inclusion of patients in the study consent form was signed. The work described has been carried out by the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

The whole blood specimens were collected in a vacuum collection tube containing ethylene diamine tetrachloride acetate (EDTA), and complete blood count, RBC indices, and reticulocyte count were estimated using DxH-800 fully automated hematology analyzer (Beckman Coulter, Inc. USA). The EDTA blood specimens were also used for Hb variant analysis, for various hemoglobinopathies and variants. The tests were performed on VARIANT^{TM} II β thalassemia short

program (Bio-Rad Laboratories, California, USA). The VARIANT^{TM} II β thalassemia short program utilizes principals of ion-exchange high-performance liquid chromatography (HPLC).
The positively charged Hb fractions were separated by high-performance liquid chromatography (HPLC) using ionic interactions with a negatively charged stationary phase in a chromatography column and then eluted by a mobile phase with phosphate buffers varying in pH and ionic strength. The positively charged Hb molecules that had been adsorbed were eluted from the column into the liquid phase at a rate proportional to their affinity for the stationary phase. The retention time of the hemoglobins was used to identify them, and the area under the peak in the elution profile was used to calculate their concentration (4,11). Single priming and calibration were performed with every run.

Results

A total of 1455 samples were received of either gender, of which 111 were males and 1344 were females. The mean age of the study population was 26.91 ± 7.06 years. All participants' samples were analyzed using HPLC. There was a total of 111 (7.60%) males and 1344 (92.40%) females (Table 1). The hematological parameters in the study population are depicted in Table 2. In the present study, the most common indication for HPLC was during the antenatal care period (719 (49.42%)); the other indications were suspected cases of thalassemia trait, presence of anemia which were ruled out for iron deficiency, patients with lower vitamin B12 levels, etc.
A total of 1455 patients were enrolled during the 3-year study period. Out of these, 128 (8.78%) cases were diagnosed with different types of hemoglobinopathies; the detailed distribution of hemoglobinopathy cases is depicted in Table 3. β-thalassemia homozygous and heterozygous were confirmed in 11 (0.76%) and 85 (5.84%) of cases. The borderline Hb A2 level was detected among 18 patients with inconclusive diagnoses. Heterozygous and homozygous sickle cell (Hb S) were detected in 9 (0.62%) and 2 (0.14%) of cases. Heterozygous Hb D Punjab was evident in 2 (0.14%) cases, while Hb E heterozygous in only one (0.07%) case. A low level of Hb A2 was seen in 2 (0.14%) of cases. The representative cases of hemoglobinopathies in the study population are presented in the form of chromatograms in Figure 1.
Among these patients with hemoglobinopathies, 38 came for ANC testing, therefore giving the incidence of 5.15% in ANC patients. Of which 25 cases were having β-thalassemia trait, four cases were heterozygotes for Hb S, one case had Hb D Punjab and eight cases were with borderline Hb A2 levels.

Discussion

HPLC is a sensitive, specific, and reproducible alternative to electrophoresis. It appeared to be an appropriate candidate for direct provisional identification and sensitive quantification of major and minor, normal and abnormal hemoglobin fractions with a high degree of precision. On the other hand, the technical performance of electrophoresis depends on various factors like hemoglobin concentration, amperage, running temperature, and length of electrophoresis run. These variables can affect the quality of separation and the relative positioning of the bands (10, 12).
The present prospective study conducted in Pune, i.e. western region of Maharashtra state, included 1455 cases for analysis of blood samples by HPLC, which gave incidence of hemoglobinopathies to be 8.78% (128/1455). The mean age of the patients included in the present study was 26.91 ± 7.06 years, of which most belonged to their 20s and 30s, with predominance of females. Among these patients with hemoglobinopathies, 77 patients had a Mentzer index of >13, which is indicative of iron deficiency anemia.

In a similar type of study Bhokare SB et al to prove the role of Hb A2 by HPLC, gave the prevalence of abnormal hemoglobin to be 37.4% out of 500 suspected anemia patients (13).
In the HPLC study by Mukhopadhyay D et al., among the total of 10,407 subjects, 8,898 (85.5%) were diagnosed as normal, 579 (5.6%) were identified as β-thalassemia trait (BTT), and 522 (5.0%) were found to be detected as HbE carriers. Ray GK et al. detected hemoglobinopathies in 50.2% of the 21,371 anemic patients, with beta-thalassemia and sickle cell hemoglobinopathies being the major types observed, among other included HbS gene in 52.48% cases, beta thalassemia in 54.06% and HbE hemoglobinopathies in 9.19% cases (12, 14).
Out of 128 (8.78%) patients diagnosed with different hemoglobinopathies, 85 (5.84%) were confirmed to be heterozygous for β thalassemia (Figure 1a). Among these patients, one patient had an increased level of Hb F, and one of the patients was compound heterozygous for Hb S (Figure ab). 18 (1.24%) of patients had borderline value for Hb A2, followed by 11 (0.76%) patients with β thalassemia homozygous (Figure 1c), of these 11, 1 patient each had presence of Hb E. 9 (0.62%) of cases were reported to have heterozygous Hb S (Figure 1d), among these patients eight patients were suggestive of presence of alpha thalassemia. Two of the patients had Hb S disease (homozygous) (Figure 1e), and there were two cases of Hb D Punjab (Figure 1f) and both were reported to be heterozygous. Two patients had low levels of Hb A2, which were suggestive of alpha thalassemia, and one case of heterozygous Hb E (Figure 1g).
The most common hemoglobinopathy reported by Bhokare SB et al. was sickle cell trait, followed by β thalassemia trait, Hb S+β thalassemia trait, and β thalassemia major, and similar to present study 1 case of Hb E, but in conjunction with thalassemia trait (13).
Ankur, K et al among a total of 2,789 patients, exhibited abnormalities in 30.8% of the cases (15). The most prevalent abnormality detected was β (beta) thalassemia heterozygous, followed by thalassemia homozygous, HbE heterozygous, and Sickle cell trait. Other variants identified included Eβ thalassemia, Hb D Punjab trait, HbE disease, sickle cell disease, Hb Lepore, hereditary persistence of fetal hemoglobin (HPFH), sickle-β thalassemia, and double heterozygosity for Beta Thalassemia & Hb D Punjab, along with various other combinations. Additionally, a rare case of Hb Burke was detected, accounting for only 0.04% of the cases.
Singh J et al. Among the 100 cases, 51 (51%) displayed abnormal hemoglobin fractions as detected by HPLC (16). Specifically, 42 cases (42%) were diagnosed as thalassemia trait, 4 cases (4%) as beta thalassemia major (with HbF levels exceeding 75%), 2 cases (2%) as HbE, and 3 cases (3%) as hereditary persistence of fetal hemoglobin (HPFH). Conversely, 49 cases demonstrated a normal HPLC pattern.
In patients with hemoglobinopathies, the mean level of Hb A2 was 5.01 ± 1.67%, ranging between 1.1 to 56.7%. The highest values, i.e., 26.3 and 56.7% of Hb A2, were reported in a patient with heterozygote for Hb E and in another patient diagnosed with homozygous β thalassemia along with Hb E disease, respectively. This indicates the severity of the condition if thalassemia and Hb E are present in combination in a patient.
Sickle cell disease is one of the most common genetic pathologies in the world. It is characterized by homozygous hemoglobin S (Hb S) or Hb S associated with other Hb variants (17, 18).
There is great clinical variation in the clinical manifestations between sickle cell disease patients; several factors are associated with the different presentations. Some determinants are already well established, such as genetic, clinical and laboratory factors, while others, such as psycho-social and nutritional factors, have been less well studied (17, 19-22).
Among the patients with the presence of heterozygous and homozygous Hb S, the mean percentage of Hb S was 43.73 ± 19.24%. Among the 11 β thalassemia homozygous patients, were having Hb

S disease with only 5.8 & 5.7% of Hb A, 79.8 & 68.7% of Hb S, 10.0 & 20.6 % of Hb F and 4.4 & 5% of Hb A2 values. The hemoglobin levels in these 2 patients were also low, i.e. 7.4 and 8.4 g/dl, with Mentzer index of >13. The 8 of 9 patients who were heterozygous for Hb S had mean Hb A, Hb F, and Hb A2 values of 58.03 ± 5.97, 1.1 ± 1.09, and 2.57 ± 0.17%, which was suggestive of the presence of alpha thalassemia in these patients.

Further, among the patients diagnosed with hemoglobinopathies, we found a positive association of Meltzer index with the levels of Hb F ($p=0.0010$), MCH ($p<0.0001$) and a Negative association with levels of hemoglobin ($p<0.0001$), Hb A ($p=0.0187$), RDWC ($p<0.0001$). One of the limitations of our study is that eight patients for whom we suspected alpha thalassemia were not ruled out as the present study was limited to HPLC.

Conclusion

In conclusion, this study conducted at a tertiary care teaching hospital in Pune revealed an incidence of hemoglobinopathies of 8.78% using the highly sensitive technique of HPLC. The most common hemoglobinopathy observed was β thalassemia, with a prevalence of 6.6%. Among the cases of β thalassemia, the majority exhibited β thalassemia trait, while a few presented with β thalassemia major. Notably, the presence of Hb E in patients was associated with severe anemia, characterized by higher Hb A2 levels. Furthermore, the coexistence of Hb E with β thalassemia resulted in an even more challenging clinical picture. These findings emphasize the clinical significance and implications of hemoglobinopathies, particularly β thalassemia and Hb E, and highlight the importance of early detection and appropriate management strategies for these conditions.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Ethics approvals

The study was approved by the institutional ethics committee (BVDUMC/IEC/131) and conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Bharati Vidyapeeth (DTU) Medical College, Pune, Maharashtra, India.

Author contribution

Written informed consent was taken from all participants after explaining the research objectives.

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Tables and figures

Table 1: Distribution of demographic parameters in study population (n=1455)

Demographic parameters		Mean ± SD, n (%)
Age (years)		26.91 ± 7.06
Gender	Male	111 (7.60%)
	Female	1344 (92.40%)

Table 2: Distribution of hematological parameters in study population (n=-1455)

Hematological parameters	**Mean**	**SD**
Mentzer index (MCV/RBCs)	19.41	7.43
Hemoglobin (g/dL)	11.10	2.06
RBC count	4.25	0.72
PCV	33.57	5.70
M.C.V	79.83	10.82
M.C.H.	26.36	4.52
M.C.H.C	32.91	1.72
RDW-CV	16.57	4.35
Hb A	85.01	8.05
Hb F	1.16	6.67
Hb A2	2.87	0.70

Table 3: Profile of hemoglobinopathies (total number of patients evaluated 1455, total number of patients detected to have hemoglobinopathies 128 (8.78%))

Profile of hemoglobinopathies	**Frequency (n)**	**Percentage (%)**
Normal	1325	91.07
Heterozygous for β thalassemia	85	5.84
Borderline HbA2 Value	18	1.24
Homozygous for β thalassemia.	11	0.76
Heterozygous for Hb S	9	0.62
Homozygous for Hb sickle	2	0.14
Heterozygous for Hb D Punjab	2	0.14
Low Hb A2	2	0.14
Heterozygous for Hb E	1	0.07
Grand Total	1455	100.00

Table 4: Assessment of hematological parameters in different groups of patients and normal subjects

Hematological parameters	Normal (n=1325)	Borderline HbA2 (n=18)	Homozygous for β thalassemia (n=11)	Heterozygous for β thalassemia (n=85)	Heterozygous for Hb Sickle (n=9)	Others (n=7)
Mentzer index	19.41 ± 4.75	36.36 ± 42.10	41.07 ± 24.19	14.72 ± 8.90	12.93 ± 2.29	19.76 ± 5.18
Hemoglobin	11.24 ± 2.00	10.17 ± 2.91	5.04 ± 2.27	9.95 ± 2.08	10.50 ± 1.88	9.59 ± 2.98
RBC Count	4.25 ± 0.59	3.57 ± 1.14	2.16 ± 0.88	4.91 ± 1.10	5.11 ± 0.56	3.89 ± 0.82
PCV	34.00 ± 5.33	30.45 ± 8.52	15.34 ± 6.24	31.53 ± 6.67	33.30 ± 5.15	28.92 ± 8.44
M.C.V	80.29 ± 9.86	89.04 ± 14.11	70.98 ± 3.20	64.93 ± 6.47	65.30 ± 8.43	74.10 ± 11.24
M.C.H.	26.54 ± 4.15	29.69 ± 4.70	22.70 ± 3.45	20.50 ± 2.09	20.61 ± 3.38	24.59 ± 4.48
M.C.H.C	32.92 ± 1.63	33.36 ± 0.89	31.94 ± 4.28	31.57 ± 0.80	31.46 ± 1.39	33.03 ± 1.33
RDW-CV	16.16 ± 3.72	17.75 ± 6.60	38.14 ± 4.75	18.34 ± 4.49	18.04 ± 3.36	20.29 ± 7.57
Hb A	86.23 ± 2.51	86.36 ± 1.39	6.48 ± 2.71	82.51 ± 4.45	58.67 ± 1.76	34.25 ± 30.55
Hb F	0.48 ± 2.53	0.36 ± 0.26	89.85 ± 1.32	1.77 ± 2.46	1.10 ± 0.77	9.72 ± 9.71
HB A2	2.69 ± 0.29	3.46 ± 0.09	3.66 ± 1.37	5.23 ± 2.36	2.51 ± 0.19	9.01 ± 17.93

Table 5: Distribution of patients based on Mentzer index cut-off value of 13 among different hemoglobinopathies cases

Impression	Mentzer index category		
	<13	>13	Total
Borderline HbA2 Value	0	18	18
Heterozygous β thalassemia	48	37	85
Heterozygous for Hb D Punjab	0	2	2
Heterozygous for Hb E	0	1	1
Heterozygous for Hb S	5	4	9
Homozygous for β thalassemia	0	11	11
Homozygous for Hb sickle	0	2	2
Low Hb A2	0	2	2
Normal	57	1268	1325
Total	**110**	**1345**	**1455**

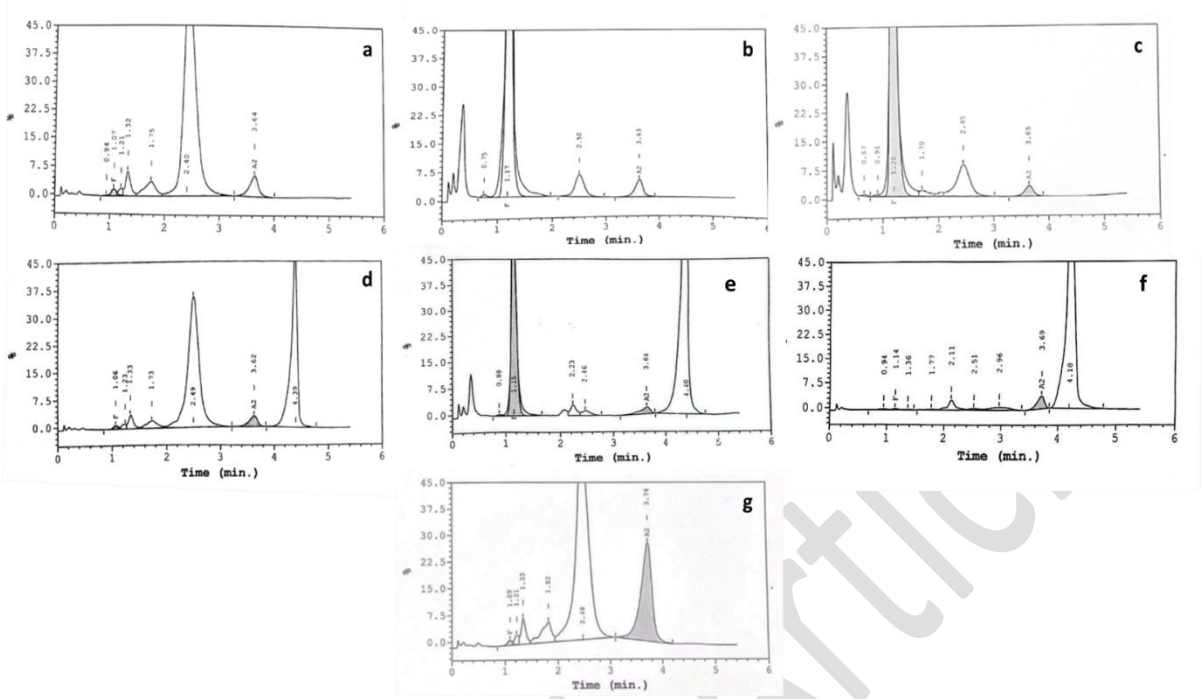

Figure 1: Electrophoretograms showing various hemoglobinopathies a) Heterozygous β thalassemia; b) Compound heterozygous with β thalassemia; c) Homozygous β-thalassemia; d) Heterozygous Hb S; e) Hb S disease (homozygous); f) Homozygous for Hb D Punjab; g) Heterozygous Hb E