



Özgün Araştırma / Original Article

## Prevalence of Various Hemoglobinopathies-An Experience from Tertiary Care Centre

Promil Jain<sup>1</sup>, Monika Gupta<sup>2</sup>, Shivani Dua<sup>3</sup>, Nisha Marwah<sup>4</sup>, Meenu Gill<sup>5</sup>, Rajeev Sen<sup>6</sup>

*1 Department of Pathology, Pt. B.D. Sharma PGIMS, Rohtak, Haryana, India ORCID: 0000-0002-6390-7465*

*2 Department of Pathology, Pt. B.D. Sharma PGIMS, Rohtak, Haryana, India ORCID: 0000-0003-3438-2943*

*3 Department of Pathology, Pt. B.D. Sharma PGIMS, Rohtak, Haryana, India ORCID: 0000-0002-4701-5934*

*4 Department of Pathology, Pt. B.D. Sharma PGIMS, Rohtak, Haryana, India ORCID: 0000-0002-7017-9785*

*5 Department of Pathology, Pt. B.D. Sharma PGIMS, Rohtak, Haryana, India ORCID: 0000-0001-5913-8918*

*6 Department of Pathology, Pt. B.D. Sharma PGIMS, Rohtak, Haryana, India ORCID: 0000-0001-7957-5613*

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### Abstract

**Objective:** To find out the prevalence of thalassemias and various other hemoglobinopathies in state of Haryana and to assess the role of High performance liquid chromatography (HPLC) for accurate and quick diagnosis of various hemoglobin disorders.

**Methods:** This study was conducted in Pt. B. D. Sharma, PGIMS, Rohtak from July 2010 to October 2016 for hemoglobin variant screening by HPLC of total of 4275 patients, selected through multistage random sampling technique.

**Results:** Among 4275 total cases surveyed, normal hemoglobin pattern was observed in 3862 (90.3%) and abnormal hemoglobin fractions on HPLC were detected in 413 (9.7%). Beta ( $\beta$ ) thalassemia trait was predominant abnormality found in a total of 318 cases (7.4%). There were 19 cases of  $\beta$ -thalassemia major and 24 of  $\beta$ -thalassemia intermedia. Other abnormal patterns found were HbE (trait in 11 and HbE disease in 6 cases), Hb S (2 cases each of sickle cell disease and sickle cell trait), 2 cases of high Hb F (1 case each of heterozygous and homozygous HPFH), 11 cases of Hb D Punjab along with 2 cases of Hb D Iran, 1 case of Hb C and 2 cases each of double heterozygotes for Hb E/  $\beta$ -thalassemia and Hb S/  $\beta$ -thalassemia.

**Conclusion:** There is high prevalence of hemoglobinopathies in this region and HPLC forms a rapid and accurate tool in early detection and management of various hemoglobinopathies.

**Keyword:** Hemoglobinopathies, Thalassemia, High Performance Liquid Chromatography.

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**Yazışma Adresi / Correspondence:** Promil Jain, Department of Pathology Pt. B.D. Sharma PGIMS, Rohtak-124001 Haryana India  
e-mail: jainpromil@gmail.com

## Hemoglobinopati Prevelansı- Bir Üçüncü Basamak Merkez Deneyimi

### Öz

**Amaç:** Haryana şehrindeki talasemi ve diğer hemoglobinopatilerin prevelansını ortaya çıkarmak ve bu hastalıkların hızlı ve doğru tanısında yüksek performans likid kromatografi (HPLC)'nin rolünü araştırmak.

**Yöntemler:** Bu çalışma çok dilimli random örnekleme metoduyla toplam 4275 hastanın HPLC cihazıyla varyantlarının gösterilmesi vasıtasıyla Temmuz-Ekim 2010 tarihleri arasında Pt. B. D. Sharma, PGIMS, Rohtak'da gerçekleştirilmiştir.

**Bulgular:** Toplam 4275 vakanın 3862'si (%90,3) normal bir hemoglobin paterni gösterirken, 413 vakada (%9,7) anormal hemoglobin fraksiyonları HPLC ile saptandı. Anormal vakaların 318'i (%7,4) beta ( $\beta$ ) talasemi idi. Vakaların 19'u  $\beta$ -talasemi major ve 24 'ü  $\beta$ -talasemi intermedia olarak tespit edildi. Diğer anormal durumların altısı HbE'li, ikisi HbS'li, ikisi HbF'li, 11'i HbD'li, biri HbC'li ve iki vakanın her biri Hb E/  $\beta$ -talasemi ve Hb S/  $\beta$ -talasemi heterozigot idi.

**Sonuç:** Bu bölgede yüksek hemoglobinopati prevelansı saptandı ve HPLC çeşitli hemoglobinopatilerin erken saptanmasında hızlı ve kesin bir yöntemdir.

**Anahtar kelimeler:** hemoglobinopatiler, talasemi, yüksek performans likid kromatografi

### INTRODUCTION

Hemoglobinopathies are disorders affecting the structure, function or production of hemoglobin. These conditions are usually inherited and range in severity from asymptomatic laboratory abnormalities to serious disorders like thalassemia major requiring regular blood transfusion and extensive medical care. Different forms may present as hemolytic anemia, erythrocytosis, cyanosis or vaso-occlusive stigmata<sup>1</sup>. Hemoglobinopathies are one of the major public health problems in India and can be quantitative (thalassemia syndrome) or qualitative (variant hemoglobinopathies)<sup>2</sup>. Of these hemoglobinopathies, thalassemia syndrome, particularly  $\beta$ -thalassemia major is a serious and common cause of morbidity. Moreover, social stigma associated with having thalassemia has significant psychosocial, financial and emotional impact on patients and their families.

According to WHO, 5% of the world population is carrier for hemoglobin disorders<sup>3</sup>. Out of which, the frequency of  $\beta$ -thalassemia in India ranges from 3.5% to 15% in different population groups.[4] Most institutes in India are using conventional methods for diagnosis of various hemoglobinopathies like correlating

fetal haemoglobin, complete blood count, red cell indices, clinical history, HbF estimation by alkali denaturation test, sickling test and Hb electrophoresis. But these methods have their own limitations including identification of variants and similar electrophoretic mobility in diagnosing few compound heterozygous states. However haemoglobin fraction analysis, cation exchange HPLC is superior in terms of quantifying HbF and HbA2 besides screening of other haemoglobin variants in a single, reproducible system which makes it excellent for screening of thalassemia and other haemoglobin variants and hemoglobinopathies.

Hemoglobinopathies screening programme with the help of HPLC help in awareness generation, carrier detection and genetic counselling and can create an effective database for the region. Hence, the study was planned to find out the prevalence of various hemoglobinopathies in state of Haryana and to assess the role of HPLC for an accurate and quick diagnosis of various hemoglobin disorders.

## METHODS

This study was conducted in department of Clinical Hematology in Pt. B. D. Sharma PGIMS, Rohtak, India. A total of 4275 patients/samples received from OPD and IPD of the institute for HPLC from July 2010 to October 2016 were studied for various hemoglobinopathies and variants. The study was approved by the ethical committee of the institute. These mainly included transfusion dependent children and adults, antenatal cases and their family members. In patients requiring blood transfusion, sampling was deferred to at least 4 weeks of transfusion.

A preinformed written consent for enrollment in this study was obtained. Complete clinical history including age of presentation, history of blood transfusion and relevant family history was taken in all cases. Three ml of venous blood sample from each patient was taken in EDTA vacutainers. The samples were first analysed in 5 part differential automated cell counter (BC-5800) of Shenzhen Mindray for complete blood count including Hb values, RBC mass and red cell indices (MCV, MCH, MCHC) before performing HPLC. Samples were stored in refrigerator at 4-8°C and analysed for HPLC in batches within one week on the patients whose hemoglobin was found to be less than 11 gm/dl. The tests were performed on BIORAD Variant II ( $\beta$ -thalassemia short programme), which utilises the principle of high performance liquid chromatography. Standard protocol for each run was followed. An HbA2/F calibrator and two levels of control- high and low, (BIORAD) were analyzed at the beginning of each run. The acceptable total area of each analysis should range between 1- million  $\mu$ Volt/second. The HPLC software delivers a printed report showing the chromatogram with all the hemoglobin fractions eluted, retention time, area of peaks and values in percentage of different hemoglobin components. Any peak eluting at a retention time that is not pre-defined is labelled as unknown. The integrated

peaks are assigned by manufacturer defined “windows” derived from specific retention time (RT). Retention time, of normal haemoglobin fraction and common variants, is the time that elapses from the sample injection to the apex of the elution peak<sup>5</sup>. (Table 1) The “windows” are established ranges in which common variants have been observed to elute using the Variant  $\beta$ -thalassemia short program. It takes about 6.5 minutes for each analytical cycle, from sampling to printing of results.

Peak Name	Concentration	Area %	Retention Time (min)	Peak Area
F	0.3	---	1.04	4560
Unknown	---	0.9	1.19	17772
P2	---	3.8	1.30	72923
P3	---	4.1	1.70	78812
Ao	---	87.4	2.46	1682926
A2	3.2	---	3.56	67869

Total Area: 1,924,863

F Concentration = 0.3 %  
A2 Concentration = 3.2 %

Analysis comments:

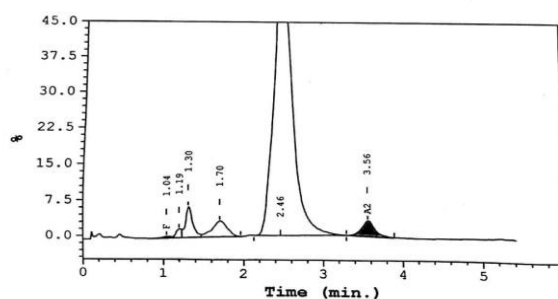


Figure 1: Normal chromatogram

## RESULTS

HPLC samples of 4275 patients of anemia were run and analysed, out of which 3313 (77.5%) were females and 962 (22.5%) were males, with female to male ratio of approximately 3.4:1. Number of female patients outnumbered males because of routinely advised HPLC screening in antenatal patients. Table 2 shows the sex distribution of total cases, reflecting the number of female and male patients who were tested for various hemoglobinopathies. Out of total 4275 cases, patient's age ranged from 3 months to around 80 years. (Table 3) Maximum

number of patients screened were antenatal mothers in the age group of 20-29 years. Family screening was done in most of the positive patients.

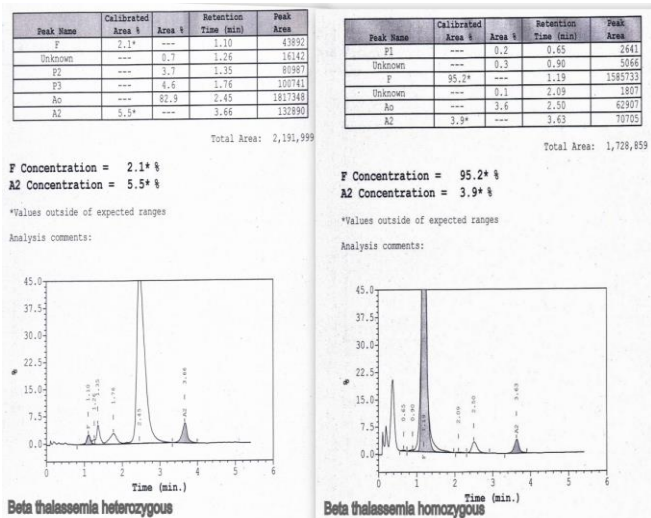


Figure 2: Chromatogram showing Beta thalassemia heterozygous and homozygous conditions

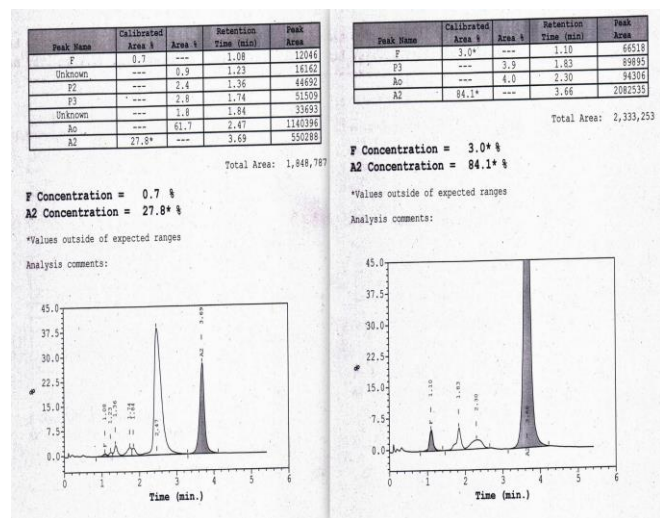


Figure 3: Chromatogram showing Hb E heterozygous and Hb E homozygous

Out of total of 4275 patients screened, 413 (9.7%) patients showed abnormal haemoglobin variants. Normal hemoglobin pattern was observed in 3862 cases. Figure1 shows chromatogram for normal hemoglobin pattern. Table 4 shows the pattern of various

hemoglobinopathies and thalassemia in our study. The major abnormality observed was high HbA2. A cut-off of over 3.9% was taken for diagnosis of  $\beta$ -thalassemia trait. A total of 318 cases (7.4%) of  $\beta$ -thalassemia trait were diagnosed. The retention time for HbA2 ranged between 3.30-3.90 minutes. Predominant peripheral blood findings were microcytosis and hypochromia with raised RBC counts. Few patients had borderline results as their chromatogram showed value of HbA2 between 3.5-3.9%. Most of them were antenatal mothers and were advised to get repeat HPLC screening done after correction of anemia as HbA2 levels are spuriously high in megaloblastic anemia. Moreover, screening of husband for HPLC was also advised.

$\beta$ -thalassemia homozygous was found in 43 cases, out of which 19 patients were diagnosed as  $\beta$ -thalassemia major, 24 were diagnosed as  $\beta$ -thalassemia intermedia. Clinically,  $\beta$ -thalassemia major patients presented within the first two years of life with severe anemia, requiring regular blood transfusion. Most of the cases had Hb F more than 90% at the time of diagnosis. Cases diagnosed with  $\beta$ -thalassemia intermedia had variable degree of anemia with anisopoikilocytosis and microcytic hypochromic (MCHC) blood picture. Hb F levels were raised with a variable reduction in HbA, but these patients were not transfusion dependent.

Eleven subjects were diagnosed as HbE heterozygous and 6 were homozygous for Hb E. (Figure 3) Hb E presents as raised peak in the A2 region with retention times ranging from 3.30-3.90 minutes. Hb E heterozygotes have on an average raised Hb E of 30% (usually less than 40%). Hemoglobin is slightly decreased to normal with microcytic hypochromic indices. Patients with homozygous Hb E have HbA2 > 60% and Hb F between 2-10%. In two of our Hb E homozygous patients Hb E was 84.1% (Hb 4.6 gm/dL) and 75% (Hb 7.6 gm/dL).

Peripheral blood picture was of microcytic hypochromic anemia with target cells.

HbD Punjab trait was seen in 11 patients on HPLC, which displayed a D-Window with variant percentage ranging between 30-45% and retention times of 3.90-4.30 minutes. (Figure 4) The blood counts of these patients were mostly normal.

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.0	0.96	658
F	0.4	---	1.06	6846
Unknown	---	0.8	1.22	13188
P2	---	2.0	1.34	34630
P3	---	2.6	1.70	45555
Unknown	---	2.3	2.08	39378
Ao	---	51.0	2.48	884684
A2	2.8	---	3.60	50872
D-window	---	38.0	4.06	658164

Total Area: 1,733,974

F Concentration = 0.4 %  
A2 Concentration = 2.8 %

Analysis comments:

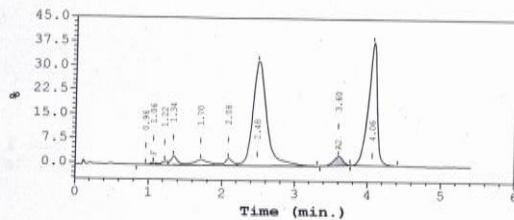


Figure 4: Chromatogram showing D window- Hb D heterozygote

HbS was present in 4 patients, with 2 each diagnosed as of sickle cell trait and sickle cell anemia. Sickling was seen in all 4 of these patients on performing sodium metabisulphite test. (Figure 5) In HbS homozygous patients, S-window is seen with HbS usually more than 50%, while HbS in heterozygous patients, HbS is between 30-40%. HbF is elevated in HbS homozygous patients.

One patient each was diagnosed as heterozygous hereditary persistence of fetal haemoglobin (HPFH) and homozygous HPFH. Both of these patients were pregnant adult females who came for routine HPLC screening. They did not have any clinical symptoms. Other hemoglobinopathies identified were 2 cases of HbD Iran (Figure 6), 1 case of HbC and 2 cases

each of double heterozygotes for Hb E/ $\beta$ -thalassemia and Hb S/  $\beta$ -thalassemia.

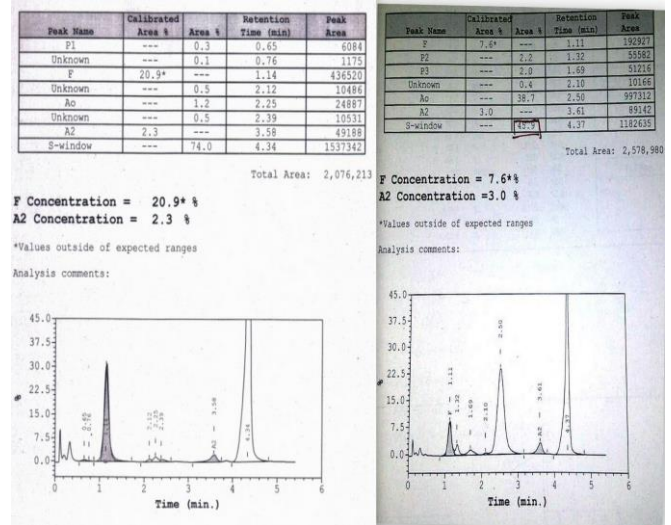


Figure 5: Showing chromatograms of Hb S homozygous

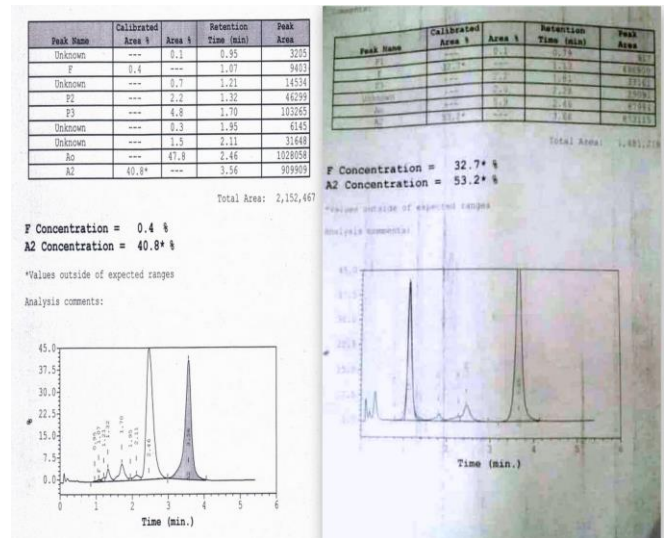


Figure 6: Chromatogram showing Hb D Iran heterozygous; HbE  $\beta$ -thalassemia

In addition, there were 11 cases which showed various abnormalities in the chromatograms, but these could not be assigned any diagnosis either due to lack of clinical data or the patients were lost and did not come to collect the report.



## DISCUSSION

Hemoglobinopathies are the most common inherited red cell disorders worldwide. These diseases pose serious medical, social and economic problems to the family and to the public<sup>2</sup>. Large numbers of severely affected patients represent an enormous human suffering for many families and they need intensive supportive therapy with little or no chances of being cured<sup>6</sup>.

The most common tools for diagnosis of hemoglobinopathies are gel electrophoresis, HbF estimation by alkali denaturation test and HbA<sub>2</sub> estimation by ion exchange column chromatography but cation exchange HPLC is emerging as one of the best methods for screening and detection of various hemoglobinopathies with rapid, reproducible and precise results<sup>7</sup>. It can accurately identify and quantify abnormal hemoglobins.

Indian subcontinent has diverse population due to large number of castes, communities and ethnic groups with each revealing different genetic traits. Different hemoglobinopathies are found concentrated in certain populations. A high frequency of HbS is predominantly found in tribal populations of central and southern part of India, while HbE is widely distributed in north eastern states and HbD is seen mostly in north India.  $\beta$ -thalassemia trait is variably distributed in almost every Indian population<sup>8</sup>. Highest frequency of  $\beta$ -thalassemia trait has been recorded from Gujarat (10-15%), followed by Sindh (10%), Punjab (6.5%), Tamil Nadu (8.4%) and Maharashtra<sup>9</sup>.

In our study all 4275 screened subjects belonged to the state of Haryana. We found that a total of 413 patients had various hemoglobinopathies;  $\beta$ -thalassemia trait formed the largest sub group of abnormal hemoglobin including 318 patients (7.4%). The results of present study were compared with those of other studies (Table 5).

Verma et al from a 2 year study in Uttar Pradesh found hemoglobinopathies in 12.1% of patients<sup>9</sup>. Similarly, in a 8 years study done in West Bengal on 90,210 patients, Mondal et al reported that 11.4% had hemoglobinopathies with  $\beta$ -thalassemia trait as most common hemoglobinopathy (4.29% in whole of the screened population)<sup>10</sup>. Similarly, Jain et al published their experience from a tertiary care hospital in West Bengal in 2012 and found 29.32% of screened population to be suffering from various hemoglobinopathies, out of which,  $\beta$ -thalassemia trait was commonest hemoglobinopathy (55.84%)<sup>11</sup>. In a study from Gujarat by Patel et al, there was a high frequency of  $\beta$ -thalassemia trait (20.37% in all 2022 patients screened)<sup>12</sup>. Madan et al from a screening program carried out in 11090 school children of Delhi and Mumbai found hemoglobinopathies in 5.25% of screened population and revealed that  $\beta$ -thalassemia trait was commonest (4.05%) followed by Hb D Punjab<sup>13</sup>. Findings in our study are consistent with findings of Sachdev et al and Bhalodia et al, who documented hemoglobinopathies in 12.5% and 8.6% population respectively and found that  $\beta$ -thalassemia trait formed predominant abnormality in 8.9% and 5.2% subjects respectively<sup>14,15</sup>. Mondal et al in a 10 year prospective HPLC study on 119,336 cases for prevalence of thalassemia and hemoglobinopathy in eastern India found that abnormalities were detected in 12.17% patients and beta thalassemia was commonest abnormality (in 4.6%)<sup>16</sup>.

Haritha et al in a study of 103 cases from Andhra Pradesh found hemoglobin disorders in 14.5% patients and most common hemoglobin fraction was Hb S (14%).<sup>[8]</sup> Ghosh et al in a study on 188 antenatal patients in Darjeeling found abnormal hemoglobin fractions in 50 patients (26.6%) and Hb E to be the commonest hemoglobinopathy (15.42%)<sup>17</sup>.

The high incidence of  $\beta$ -thalassemia trait in this region underscores the need for antenatal

screening for prevention of thalassemia major in the offspring. In this large study by using HPLC, we experienced that HPLC is an excellent, powerful diagnostic tool for the direct identification of Hb variants with a high degree for precision in the quantification of major and minor, normal and abnormal Hb fractions, yet it too has its own limitations like higher capital, reagent cost and considerable skill and experience required for interpretation of results. Another limitation is non-detection of alpha thalassemia by HPLC.

In a country like India, which carries remarkable diversity in frequency of thalassemia and other hemoglobinopathies, accurate micro mapping is important while estimating the disease burden for planning the preventive programmes.

### CONCLUSION

In view of high prevalence of hemoglobinopathies in this region, a routine premarital screening programme is needed for the identification and prevention of high risk marriages. HPLC forms a rapid, accurate and reproducible tool for early detection and management of hemoglobinopathies and variants. Our study had high incidence of  $\beta$ -thalassemia trait. Early detection of traits will prevent occurrence of thalassemia major in offspring. Detection of other variants becomes important due to complex interactions in cases with double heterozygous and homozygous states, which may lead to severe hematological abnormalities.

**Declaration of Conflicting Interests:** The authors declare that they have no conflict of interest.

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