



## Original Article

# Utility of Hematological Profile along with HPLC in detection of Hemoglobinopathies and study its shortcomings

Authors

Dr Krupa Jog<sup>1</sup>, Dr Varsha Munj<sup>1</sup>, Dr Shruti Shetye<sup>2</sup>, Dr Nelishka Gomes<sup>2</sup>

Pathology Department, North District Hospital

\*Corresponding Author

Dr Krupa Jog

North District Hospital, Peddem, Mapusa, Goa, Pin 403507

## Abstract

**Introduction:** Hemoglobinopathies are a serious genetic health problem responsible for severe morbidity and mortality worldwide and in India.

**Aim & Objective:** To analyze the value and limitations of diagnostics like hematological profile, HPLC with parental study and ethnicity for detection of Hemoglobinopathies.

**Materials and Method:** A total of 2684 cases were screened in a District Hospital based population over a period of 3 years. HPLC was performed on D-10 Biorad analyser and hematological profile was done on 3 part Automated Hematology analyser.

**Observation:** 238 cases were identified with abnormal variants.  $\beta$  Thalassemia trait was commonest followed by HbS and HbE disease. Migration and settlement of populations from different parts of the country to Goa has resulted in diverse spectrum of hemoglobin disorders.

Borderline HbA<sub>2</sub>, elution of variants in common window, few rare variants, existence of silent mutations,  $\beta$  Thalassemia causes difficulty in interpretation and requires genetic study for confirmation.

**Conclusion:** HPLC, hematological profile, family study can help to detect hemoglobinopathies and can be used as a screening tool.

**Keywords:** HPLC, Hemoglobin variants, Borderline HPLC.

## Introduction

Hemoglobinopathies are the most common genetic disorders worldwide. They include Thalassemias and abnormal variant hemoglobins such as Hemoglobin S, Hemoglobin E, Hemoglobin D etc<sup>1</sup>. An estimated 7% of the world population carries an abnormal hemoglobin gene, while about 3 lac to 5 lac are born with significant

hemoglobin disorders<sup>1</sup>. In India  $\beta$  Thalassemia is prevalent across the country with 3-4% average frequency of carriers. HbS is prevalent in tribal populations of Southern, Central and Western states<sup>2</sup>. HbE is common in North Eastern states<sup>4</sup>. HbD is seen in people in Punjab<sup>3</sup>. The clinical spectrum of these disorders varies from asymptomatic conditions to serious disorders like

Thalassemia major and other major hemoglobinopathies requiring regular blood transfusion and extensive medical care. They are the major genetic cause of morbidity and mortality imposing a heavy psychological and social burden on the affected families.

Being recessively inherited disorder it manifests in children of healthy carrier couples<sup>1</sup>. Hence awareness of the disorder has to be created with carrier screening of asymptomatic  $\beta$  Thalassemia and other variant hemoglobinopathies using reliable laboratory methods and genetic counseling is the best possible strategy for prevention of these disorders<sup>3</sup>. Cation Exchange High Performance Liquid Chromatography is emerging as the method of choice for initial screening of Hb variants and for accurate quantification of HbA2 and HbF. The simplicity of automated systems with internal sample preparation, superior resolution, rapid assay time and accurate quantitative analysis of hemoglobin fractions make it an ideal methodology for the routine clinical laboratory. In this study authors have evaluated CBC parameters with HPLC and its limitations for picking up  $\beta$  Thalassemia and other hemoglobinopathies in the population sample tested at the District Hospital.

#### **Aim of the Study**

- 1) Detection of spectrum of hemoglobin disorders in study population comprising of local and migrant population.
- 2) To analyze hematological profile with HPLC in detection of hemoglobinopathies and study its limitations.

#### **Materials and Methods**

This is a prospective study carried out in the Department of Pathology at District Hospital, Goa over a period of 3 years from January 2017 to January 2020. A total of 2684 cases were screened using HPLC. These included cases of anaemia, suspected cases of hemoglobinopathies, siblings of patients with hemoglobinopathies and antenatal patients coming for hemoglobinopathy screening

in 1<sup>st</sup> trimester of pregnancy. 5ml blood was collected in EDTA bulb and red cell indices were analyzed on 3 part automated cell counter (Mindray 3600). The sample was analyzed on Biorad D10 HPLC analyzer for HbA2, HbF and other hemoglobin variants.

Chromatograms were studied and interpreted taking into consideration red cell indices, reticulocyte count and ethnic origin of the patient. D10 Hb testing system library of chromatograms was referred for reporting. The retention time of each peak and the area below the curve was used for quantification of various hemoglobin fractions. Acceptance criteria for a chromatogram are

- 1) Total area count of chromatogram should be in range of 1 million to 4 million.
- 2) Peak shape should be sharp and symmetrical. There should be no degradation peak at the end of the run.

Several hemoglobin variants which elute in same window were provisionally diagnosed by retention time and area percentage keeping in mind the ethnicity of the patient<sup>6</sup>. The results obtained were tabulated as frequency tables. All statistical analysis was performed using spss 16.0 software.

#### **Results**

2684 patients were tested. 238(8.87%) cases were identified presumptively based on retention time windows, area percents, geographical factors, ethnicity, clinical presentation and red cell indices. (Refer table 1 for hematological profile and HPLC results)

**Table 1: Hematological Profile and HPLC result**

Hemoglobinopathy	No of cases	Hemoglobin	RBC count	MCV	MCH	RDW	HbA	HbA2	HbF	Variant window	Percentage
β Thalassemia trait	142	9.5+/-2.1	4.6+/-1.5	68+/-10	19.6+/-2.2	17.8+/-2.5	89+/-2.5	4.8+/-0.6	1.3+/-1.4	-	59.7
β Thalassemia major	6	3.5+/-1.5	2.5+/-1.5	58+/-5.2	18+/-3.4	16.2+/-3.5	23.5+/-22.6	2.6+/-1.5	64+/-28.6	-	2.5
HbE trait	12	10.3+/-2.5	4.2+/-1.5	81+/-1.5	25.7+/-4.3	15.0+/-0.79	58.5+/-7.2	30+/-4.1	1.08+/-1.2	-	5.0
HbE disease	6	11.2+/-1.5	5.9+/-0.9	60+/-4.5	20.4+/-1.5	16.8+/-1.8	3.5+/-1.2	84+/-6.7	3.92+/-0.1	-	2.5
HbS trait	16	9.1+/-3.5	5.46+/-1.56	74+/-1.8	22+/-3.5	15.9+/-2.5	40.4+/-8.4	3.1+/-0.5	1.6+/-0.5	30.5+/-9.5	6.7
HbS disease	9	4.8+/-2.8	2.8+/-1.4	68+/-8.5	25.2+/-2.9	18.8+/-5.1	20.5+/-10.5	2.5+/-1.5	21.6+/-3.5	78.5+/-10.2	3.8
HbD	3	11.6+/-1.5	5.9+/-0.8	81+/-2.5	26.5+/-3.0	14.5+/-2.5	50.4+/-4.8	2.9+/-1.2	1.8+/-0.14	38+/-4.5	1.3
β thalassemia	5	9.2+/-2	4.8+/-2	66+/-10.8	29.6+/-1.5	15.6+/-3.6	50.9+/-2.2	3.3+/-0.6	1.2+/-0.5	-	2.1
Compound HbE with β Thalassemia	1	7.8+/-1.8	3.8+/-1.6	65+/-5.4	23+/-2.8	15.9+/-2.4	3.5+/-2.5	29.5+/-2.2	16.4+/-2.5	-	0.4
Compound HbS with β thalassemia	3	7.2+/-2.4	2.9+/-1.1	70+/-4.5	22.5+/-3.8	16.2+/-3.5	24.6+/-2.8	6.5+/-1.8	13.7+/-1.5	48+/-10.5	1.3
Compound HbE with β thalassemia	4	8.1+/-2.6	5.16+/-1.5	69+/-3.5	20.9+/-2.6	15.7+/-2.8	17.5+/-9.5	62.5+/-5.4	9.5+/-2.8	-	1.7
Compound HbS with β	3	6.8+/-1.2	3.5+/-1.8	74+/-2.8	28.6+/-2.2	17.6+/-2.1	3.8+/-1.2	38.2+/-0.5	5.5+/-1.9	42+/-5	1.3
HPFH	8	12+/-2.5	3.8+/-1.8	83+/-3.4	29.7+/-3.2	18.4+/-3.5	65+/-11.2	3.5+/-1.2	18+/-4.2	-	3.4
Borderline HPLC	17	10.5+/-2.8	4.2+/-1.1	62+/-8.6	19.2+/-3.5	19.5+/-2.8	90+/-2	3.8+/-0.2	1.3+/-0.4	-	7.1
P3 peak	3	9.2+/-3.5	4.5+/-2.1	63+/-3.5	19.8+/-3.4	18.1+/-1.7	86+/-5.8	1.8+/-0.3	1.2+/-0.5	12.1+/-1.5	1.3

**Table 2 Age Sex distribution of Hemoglobinopathies**

Spectrum of Hemoglobinopathy	6-10 years		11-40 years		>40 years		Total		Percentage	
	M	F	M	F	M	F	M	F	M	F
β Thalassemia trait	28	33	26	38	6	11	60	82	42.3	57.5
β Thalassemia major	2	4	-	-	-	-	2	4	33.3	66.7
HbE trait	1	2	3	6	-	-	4	8	33.3	66.7
HbE disease	2	3	1	-	-	-	3	3	50	50
HbS trait	1	3	2	5	2	2	5	10	33.3	66.7
HbS disease	2	3	2	1	1	-	5	4	55.6	44.4
HbD	-	-	2	1	-	-	2	1	66.7	33.3
β thalassemia	1	1	1	2	-	-	2	3	40	60
Compound HbE with β Thalassemia	-	1	-	-	-	-	-	1	0	100
Compound HbS with β thalassemia	1	2	-	-	-	-	1	2	33.3	66.7
Compound HbE with β thalassemia	3	1	0	-	-	-	3	1	75	25
Compound HbS with β	2	1	1	-	-	-	3	1	75	25
HPFH	1	-	2	1	2	1	5	2	71.4	28.6
Borderline HPLC	2	4	5	6	-	-	7	10	41.2	58.8
Abnormal P3 peak	1	1	-	1	-	-	1	2	33.3	66.7

β Thalassemia was found to be the most common hemoglobin variant followed by sickle cell trait and HbE trait.

**Table 3: Geographic distribution of Hemoglobinopathies**

State	No of cases	Percentage
Goa	78	32.8
Maharashtra	64	26.9
Karnataka	49	20.6
West Bengal	4	1.7
Orissa	10	4.2
Jharkhand	8	3.4
Gujarat	9	3.8
Punjab	3	1.3
Uttar Pradesh	2	0.8
Bihar	2	0.8
Nepal	9	3.8

### Observation

- MCV between carriers (62.08fl) and normal cases (79.7fl) was statistically significant. MCV is the key indicator for diagnosis and screening.
- $\beta$  Thalassemia trait was a common hemoglobinopathy.
- MCH among hemoglobinopathies was lower than in normal cases (25.6pg) in most variants and significantly lower in  $\beta$  Thalassemia trait (21.35pg).
- RDW was normal in spite of low MCV and MCH in most carriers of  $\beta$  Thalassemia trait but cannot be used in all cases of hemoglobinopathies.
- Hemoglobinopathy traits presented with normal haemoglobin or mild decrease in haemoglobin.
- HbS disease and  $\beta$  Thalassemia major presented with severe anaemia.
- Diversity of Hemoglobinopathies was identified in multiethnic, multilingual population migrated and settled in Goa.
- Hemoglobinopathies were common in children and women in reproductive age group.
- Sick cell disease and trait were seen in tribal population migrated to Goa from Maharashtra, Gujarat and Jharkhand.
- HbE was seen in population migrated from North eastern states, Assam, West Bengal.
- $\beta$  Thalassemia was seen in migrants from Karnataka.
- HbD was seen in population migrated from Gujarat and Punjab.

### Discussion

In India there is great ethnic diversity with regional variations which is reflected by the presence of different hemoglobin variants in different ethnic groups. Due to migration of people for jobs, marriage, urban rural shifting there is a mixing of people from different regions along with marriages amongst the affected members. Many of the abnormal hemoglobin

variants are of little clinical significance in heterozygous form, but when combined with other variants or when present in homozygous state it may give rise to severe disease requiring lifelong treatment. Hence there is a need for screening method to detect different variants. HPLC has the advantage of quantifying HbF, HbA<sub>2</sub> along with the detection of other variants in a single screening test<sup>7</sup>.

Alkaline and Acid Electrophoresis is another method for detection of Hemoglobinopathies. Advantages of HPLC to Electrophoresis are as below<sup>8,9</sup>.

Features	HPLC	Electrophoresis
Quantification	Yes(objective)	No(subjective)
Automation	Present	Not present
Ease of operation	Easy	Manual procedure difficult for the operator.
Quality Control and Calibration	Available	Not optimised
Data Storage	Easy	Difficult
Data analysis	More information from single graph	Both Acid and alkaline electrophoresis to be run
Time taken	6 minutes	Few hours to few days

HPLC is a powerful tool for identification of the most clinically significant haemoglobin variants especially  $\beta$  Thalassemia trait. HPLC is sensitive, specific, reproducible, less time consuming, requires less technical specialization and is ideal for routine clinical laboratory with high workload. This study is carried out in the District Hospital in North Goa which receives samples from the hospital and peripheral Primary Health Centres, Community Health Centres and Urban Health Centres of the state. The laboratory also receives samples from tertiary care hospital of suspected patients/ siblings/family for screening of hemoglobinopathies. The study group were local Goans and migrant population from all over India and Nepal who visited the hospital for various ailments. Thalassemia being the major concern in this study, quantification of HbA<sub>2</sub>, HbF was of prime importance as facility for genetic testing is

not available. Parental study/ Sibling study with history of ethnic origin greatly helps in arriving at a conclusive diagnosis before referring patient for genetic testing. Apart from  $\beta$  Thalassemia common variants encountered were HbE, HbS, HbD as the common group.

HbA2 is elevated in  $\beta$  Thalassemia carriers (3.5-6%). We faced problems with regards to cut off value of HbA2 for  $\beta$  Thalassemia trait. Each laboratory needs to establish its own normal reference ranges. Borderline HbA2 values (3.6-4.0%) could result due to mild  $\beta$  Thalassemia alleles or co-inheritance of  $\beta$  Thalassemia. Rangan et al used the term borderline HbA2 levels in the range 3.0 – 4.0% and found mutations in 32% people with HbA2 3.4 – 3.9 %<sup>10</sup>. Similar findings are described by Colha et al<sup>3</sup>. The values ranging between 3.5-4% should be reported with caution keeping in mind the red cell indices and silent  $\beta$  Thalassemia or coinheritance of  $\beta$  Thalassemia. Iron deficiency with  $\beta$  Thalassemia tends to reduce the HbA2 value<sup>11</sup>. Megaloblastic anaemia could falsely elevate HbA2 value<sup>11</sup>. Also presence of  $\beta$  Thalassemia with  $\beta$  Thalassemia can result in normal MCV and MCH values. A few cases with refractory microcytic hypochromic anaemia with normal, borderline or reduced HbA2 levels were found. These cases should be investigated for the presence of  $\beta$  Thalassemia or coinheritance with  $\beta$  Thalassemia gene as  $\beta$  Thalassemia is a common hemoglobinopathy in India<sup>12</sup>. Molecular genotyping of  $\beta$  Thalassemia helps to diagnose unexplained microcytosis and thus prevent unnecessary iron supplementation.<sup>11</sup>

HbE trait and HbEE are mild disorders. However detection of these is important because when combined with thalassemia or HbS it gives rise to moderate to severe disease.<sup>4,5</sup>

Parental HPLC study should be done in suspicious cases before reporting compound hemoglobinopathies. However DNA analysis is the confirmatory test for coinheritance of hemoglobinopathies.<sup>14</sup>

$\beta$  Thalassemia and HPFH constitutes a heterogeneous group of disorders characterized by

absent or reduced synthesis of adult hemoglobin and increased synthesis of fetal hemoglobin. HPFH is characterized by normal red cell morphology, normal MCV and MCH levels, normal hematocrit and heterocellular distribution of HbF<sup>16</sup>. While in  $\beta$  thalassemia trait the red cell morphology is abnormal, MCV and MCH levels are reduced, hematocrit is reduced and there is heterocellular distribution of HbF. HPFH is clinically asymptomatic but interaction of  $\beta$  thalassemia with  $\beta$  thalassemia can result in severe disorder<sup>17,18</sup>.

We had 2 cases of anaemia with HPLC producing peak at the start of integration but were lost to follow-up. Such peaks are suggestive of HbH<sup>19</sup>. HbA2 was decreased. 3 cases of elevated P3 peak in the range of retention time of 1.4 to 1.5 minutes with mild microcytic hypochromic anaemia were detected. HbA2 was reduced<sup>20</sup>. They are due to  $\alpha/\beta$  chain variant which needs genetic study for confirmation. They too were lost to follow up.

### Conclusion

Hematological profile, HPLC and family study are efficient tool to detect hemoglobinopathies. However there needs to be awareness of the limitations and problems associated with the diagnostic methods. Molecular studies are indicated to confirm borderline cases and detect silent carriers of  $\beta$  Thalassemia,  $\beta$  Thalassemia and detection of other rare variants.

The present study using haematological and HPLC parameters reflects the prevalence of hemoglobinopathies in a hospital base population. It may need a larger study to identify the magnitude of the problem in the state of Goa as not much data is available about the prevalence of hemoglobinopathies in the state and impact of migration. HPLC with red cell indices is a cost effective methodology which can be used for mass screening by primarily identifying carrier and avoiding marriage of carrier couples thereby preventing birth of affected child through prenatal diagnosis.



The state has a big challenge to control hemoglobinopathies. It needs to give special emphasis on awareness, prevention and control of this disorder by effective screening which is the need of the hour.

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