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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROFILE OF PATIENTS IN VARIOUS HEMATOLOGICAL DISORDERS IN SOUTHERN RAJASTHAN – A 5 YEAR STUDY

Pathology

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ABSTRACT

Objectives: Our aim is to study the spectrum of various hemoglobinopathies, by calculating incidence according to age and gender of patients by the assessment of hematological parameters and HPLC findings. **Materials And Methods:** The study was conducted in Government Medical College Kota from Jan 2017 to Dec 2021 (5 years), to evaluate the spectrum of various hemoglobinopathies in 392 cases. All cases of microcytic hypochromic anaemia not responding to conventional treatment, clinically suspected cases of hemoglobinopathies and antenatal cases coming to the department for thalassemia screening were included in the study. Blood samples were collected in EDTA vials and investigated for laboratory investigations. HPLC was performed on BIORAD Variant II Beta Thalassemia Short Program. **Results:** In our study done on 392 cases, 80 cases were of hemoglobinopathies, 272 cases were comprising of anemias whereas 40 cases were normal. We observed female predominance (60%). β-thalassemia trait was the most prevalent (12.76%) followed by β-thalassemia intermedia (3.57%) and β-thalassemia major (1.53 %). The rest comprised of SA Heterozygous (1.27%), Sickle cell disease (1.02%) and HbE heterozygous (0.26%). Majority patients of thalassemia disorders belonged to 0-20 yrs and few cases >40 yrs. Pallor, splenomegaly and hemolytic facies were the most common symptoms. **Conclusion:** We concluded that various hematological parameters followed by HPLC has appeared to be appropriate for direct identification and sensitive quantification of major and minor, normal and abnormal, haemoglobin fractions. The present study reflects the magnitude of thalassemia and other hemoglobinopathies in a hospital based population. This type of study can definitely help to increase awareness among both health care givers and general population.

KEYWORDS

Hemoglobinopathies, Beta thalassemia major, HPLC- High performance Liquid Chromatography, sickle cell disease.

INTRODUCTION

Hemoglobinopathies are the group of genetic disorders of haemoglobin in which there is a quantitative or qualitative abnormal production or abnormal structure of haemoglobin molecule causing anemia world-wide. An estimated 7% of the world population, about 300,000 -500,000 are born annually with significant hemoglobin disorders.[1]

β- thalassemia and Sickle cell disease represents the most frequent hemoglobinopathies. Other hemoglobinopathies seen are HbE, HbD, HbC, HbF & HbH. As per information available from population based surveys, in India, data on prevalence of silent carriers for hemoglobinopathies like β thalassemia range from 2.9-4.6%. It is estimated that about 10000-15000 babies with Thalassemia major are born every year.[2]

Sickle cell anemia especially among the tribal population ranges from 1-40%,[3] while hemoglobin variants like HbE in eastern India can be as common as 3-50%.[4]

High Performance Liquid Chromatography (HPLC) has emerged as one of the best methods for early screening and detection of various hemoglobinopathies and it provides rapid and precise results. The use of HPLC technology in the clinical laboratory setting has increased 12.5-fold in the past 10 years.

The simplicity of the automated system with internal sample preparation, superior resolution, rapid assay time and accurate quantification of haemoglobin fractions makes this an ideal methodology for the routine clinical laboratory.

The exact data pertaining to the prevalence of anaemias and hemoglobinopathies in this region is scarce, hence this study was conducted with objectives of creating profile for cases of hemoglobinopathies coming at the Department of Pathology, Government medical College Kota, to find out the extent of burden of hemoglobinopathies in this region.

MATERIALS AND METHODS

The cross sectional study was conducted in Government Medical College Kota from Jan 2017 to Dec 2021 (5 years), to evaluate the spectrum of various hemoglobinopathies in 392 cases. All cases of microcytic hypochromic anaemia not responding to conventional treatment, clinically suspected cases of hemoglobinopathies and antenatal cases coming to the department for thalassemia screening were included in the study. A signed consent form was taken from all the patients included in the study. Detailed clinical history including family history and different hematologic parameters such as complete blood count, reticulocyte count and peripheral smear examination were performed to confirm the diagnosis. Past history of any blood transfusion if present was noted.

Blood samples were collected in Ethylenediamine tetraacetic acid (EDTA) vacutainers using aseptic techniques. Then the samples were labeled using patient's name, registration number and date of collection. Samples were analysed using Sysmex XS-800i analyzer, Japan.

For each patient, a peripheral blood smear (Leishman stain) and reticulin smear was prepared and compared with HPLC findings.

HPLC was performed on BIORAD Variant II Beta Thalassemia Short Program to study various hemoglobinopathies and variants.

RESULTS

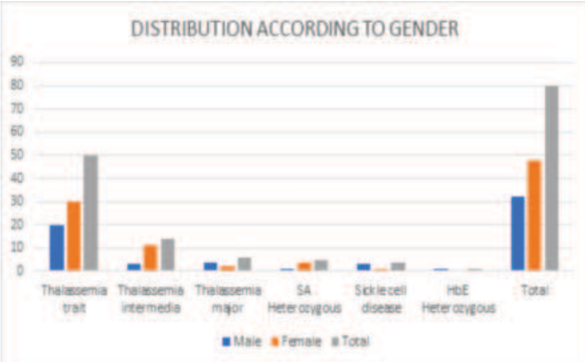
There were 392 cases included in the present cross -sectional study from Jan 2017 to Dec 2021. Clinical and haematological parameters were studied in all these cases

Following findings were observed-

Out of 80 cases, 50 suffered from thalassemia trait followed by thalassemia intermedia, 6 cases of thalassemia major and one case of HbE Heterozygous.

Table No. 1 – Incidence According To Diagnosis

Diagnosis	Frequency	Percentage
Thalassemia trait	50	12.76
Thalassemia intermedia	14	3.57
Thalassemia major	6	1.53
SA heterozygous	5	1.27
Sickle cell disease	4	1.02
HbE heterozygous	1	0.26
Anemias	272	69.38
Normal	40	10.21
Total	392	100



Graph no. 1– Shows Distribution Of Study Subjects According To Gender In Hemoglobinopathies

Studied cases showed female predominance. Out of 80, 48 were females and 32 were males. Female to male ratio 1.5:1

Age wise distribution showed majority patients of thalassemia disorders belonging to 0-20 yrs and few cases >40 yrs. Mean age was observed to be 25.8 yrs in thalassemia trait. Sickle cell disease has a mean age of 37.5 yrs.

Table No. 2 - Distribution Of Study Subjects According To Presence Of Various Clinical Findings

Diagnosis	Pallor		Splenomegaly		Hemolytic facies	
	No.	%	No.	%	No.	%
Thalassemia trait	40	80	0	0	0	0
Thalassemia intermedia	14	100	7	50	5	35.71
Thalassemia major	6	100	5	83.33	5	83.33
SA Heterozygous	4	80	3	60	0	0
Sickle cell disease	4	100	3	75	0	0
HbE heterozygous	1	100	1	100	0	0

According to clinical findings, all patients of thalassemia intermedia, thalassemia major, Sickle cell disease and HbE heterozygous showed pallor.

83.33 % patients with thalassemia major, 50% patients with thalassemia intermedia, 75 % patients with Sickle cell disease , 60% patients with SA Heterozygous and one patient of HbE trait showed splenomegaly.

Hemolytic facies were observed in 35.71 % patients of Thalassemia intermedia and 83.33 % patients of Thalassemia major.

Table No. 3- Mean Values According To Different Hemoglobin Profiles In Different Hemoglobinopathies.

Diagnosis	Hb gm/dl		MCV fl		MCH pg		MCHC %		RBC Million/ cumm		Reticulocyte Count %		RDW SD		RDW-CV	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Thalassemia trait	9.18	2.05	65.36	10.53	21.68	8.99	30.76	9.93	4.54	1.26	1.77	0.97	46.02	18.49	19.95	4.37
Thalassemia intermedia	6.02	2.39	81.95	13.41	27.42	4.14	32.35	4.01	2.29	1.14	2.14	1.02	61.65	22.18	23.88	5.15
Thalassemia major	6.26	1.43	68.53	7.56	20.85	2.25	28.9	3.81	3.04	0.94	1.45	0.57	89.91	8.95	32.73	4.42
SA Heterozygous	11.46	2.36	82.24	13.22	26.9	5.32	31.64	3.92	4.22	0.84	1.3	0.4	52.16	23.13	18.48	7.21
Sickle cell disease	9.3	1.71	80.32	11.50	26.55	3.85	32.2	3.15	3.61	0.58	2.37	0.41	41.1	7.54	18.15	1.84
HbE Heterozygous	7.1	-	76.2	-	21.6	-	28.4	-	3.28	-	1.5	-	80.4	-	33.5	-

Mean Hb value was observed to range from 6.0 gm % (Thalassemia intermedia) to 11.4 gm % (SA Heterozygous) in different hemoglobinopathies. MCV count ranges from minimum in thalassemia trait (65.36 fl) to maximum (82.24 fl) in SA Heterozygous. RBC count was lowest in thalassemia intermedia (2.29) and highest in thalassemia trait (4.54). Reticulocyte count was observed minimum in SA Heterozygous (1.3%) and maximum in Sickle cell disease (2.37%). RDW-SD ranges from 41.1 (Sickle cell disease) to 89.91 (Thalassemia major). RDW-CV ranges from 18.15 (Sickle cell disease) to 33.5 (HbE Heterozygous).

Table No. 4- Mean Values According To Different Hemoglobin Profiles In Different Hemoglobinopathies .

Diagnosis	HbF		HbA		HbA2		HbS		HbE	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Thalassemia trait	2.45	3.26	80.56	4.04	5.59	1.06	-	-	-	-
Thalassemia intermedia	23.02	11.97	60.44	12.00	3.91	0.23	-	-	-	-
Thalassemia major	64.1	11.59	18.05	12.72	3.81	0.30	-	-	-	-
SA Heterozygous	0.88	0.07	57.62	3.32	3.42	0.34	32.42	2.83	-	-
Sickle cell disease	21.46	10.34	5.57	1.96	3.3	1.65	60.45	8.77	-	-
HbE Heterozygous	15.1	-	31.6	-	53.1	-	-	-	-	-

Mean HbF values range from 0.88 % (SA Heterozygous) to 64.1% (Thalassemia major). HbA levels range from 5.57 % (Sickle cell disease) to 80.56% (Thalassemia trait). HbA2 levels were in range 3.3 % (Sickle cell disease) to 53.1% (HbE Heterozygous). Values of HbS range from minimum of 32.42% (SA Heterozygous) to maximum 60.45% (Sickle cell disease). HbE showed a peak in HbA2 window with a value of 53.1%.

DISCUSSION

Inherited abnormalities of hemoglobin include a myriad of disorders ranging from thalassemia syndromes to structurally abnormal Hb variants. Detailed clinical history, complete hematologic evaluation along with newer ancillary techniques including High-performance liquid chromatography (HPLC) when used systematically can achieve laboratory diagnosis of thalassemia syndromes and hemoglobinopathies. Family studies can be of immense importance in diagnosing certain problematic cases.

Thus present study aimed to assess the usefulness of HPLC in characterization of hemoglobin profile in thalassemia syndrome and hemoglobinopathies and to correlate it with clinico-hematological features. We also compared our study findings with other studies.

In our study, out of all hemoglobinopathies, thalassemia trait was the commonest abnormality (12.76 %) followed by thalassemia intermedia (3.57 %) and thalassemia major (1.53 %). This was similar to the study done by Mondal et al. (2022).[6] Comparison studies of spectrum of hemoglobinopathies done with Khera R et al. (2015)[7] and Sachdev R et al (2010)[8] was found to be almost similar to our study showing thalassemia trait as the commonest hemoglobinopathy followed by thalassemia intermedia. The variation in the pattern of hemoglobinopathies can be attributed to the geographical area as well as difference in case material and sample size.

Regarding sex distribution (60%) were females and 32 (40%) were males thus showing female preponderance . Similar results were observed in a study done by Bhokare S et al. (2016)[9] where 187 cases consisted of 87 males (59.18%) and 100 females (40.81%). Female patients were found to outnumber males because routine antenatal check-ups included HPLC analysis of blood of pregnant women.

Our study showed cases of SA Heterozygous (1.27 %) and Sickle cell disease (1.02 %). Co-Inheritance of Haemoglobin variants with β – Thalassemia also seems to be very common. Hb E is prevalent in north eastern and eastern states of India, with majority of the patients with HbE gene (47.94%) of which HbE trait (25.48%) was the predominant variant followed by HbE disease (21.02%) (Baruah M et al.[10], 2014). Our study showed 1 male patient of HbE Heterozygous.

In present study, we observed that mean age to be (25.8 years) in thalassemia trait. Mean age of patients was observed to be minimum with Thalassemia intermedia (7.23 years). Sickle cell disease had a mean age of (37.5 years) and patient of HbE Heterozygous was (25 years) of age.

After comparing our study of age wise distribution, to the studies done by Balgir RS[11], Uddin MM et al, [12]and Mannan et al. [13] we observed that majority patients of thalassemia disorders belonged to childhood period 0-20 years, followed by reproductive age group 21-40 years and few cases >40 years.

Table No. 5- Comparative Studies Showing Age- Wise Distribution

Age group	Balgir R S [65]	Uddin et al[66]	Age group	Mannan et al[67]	Present study
0-15 yrs	38	56	0-20 yrs	68	35
16-45 yrs	57	39	21-40 yrs	26	39
>45 yrs	5	5	>40 yrs	6	6

β-thalassemia syndromes are the result of insufficient (β^{+}) or absent (β^{0}) production of β-globin chains. Hematological changes become manifest from between the ages of three months and six months onwards. Thalassemia major (severe homozygous or mixed heterozygous β-thalassemia) with long-term, transfusion-dependent anemia; untreated children die before the age of 10 (Olivieri NF)[14] . The patients suffering from beta- thalassemia major and Hb E/beta-thalassemia do not survive for more than 5 years without blood transfusion.

In our study, 3/4 patients with Sickle cell disease and SA Heterozygous, 5/6 with thalassemia major, 7/14 of thalassemia intermedia and one patient of HbE trait showed splenomegaly.

Pallor is usually the first presenting sign in thalassemia syndromes, accompanied by splenomegaly of variable size. In the present study all patients of thalassemia intermedia, thalassemia major, Sickle cell disease & HbE heterozygous showed pallor, 80% patients with thalassemia trait and SA Heterozygous showed pallor. Symptoms patients presented with were fatigue, bone pain, growth retardation and shortness of breath.

A study of the 110 cases done by Khera R et al. (2014)[15], 19 patients of thalassemia syndromes presented with pallor (21.8 %) and splenomegaly was present in 18 (22.9 %) cases (Thal major 6/6, Thal inter 2/5, HbH 3/4 HbE/thalassemia 6/8, HbS thalassemia 1/2).

The key to successful detection and characterisation of the haemoglobinopathies, particularly the thalassaemias, is the initial haematological data. We observed in the present study that mean Hb value was observed to range from 6.0 gm % (Thalassemia intermedia) to 11.4 gm % (SA heterozygous). Patients with thalassemia trait had mean Hb as 9.18 gm/dl (9.18±2.05).

Our study was in accordance with Jaskirat Singh et al. (2016)[52] who observed 35.71% patients of beta- thalassemia trait had Hb less than 9gm/ dl and 28.57% patients had Hb ranging between 7-9 gm/dl. In the study of Baruah M et al. (2014),[69] study population had Hb ranging from 1.0 g/dl to 10.9 g/dl with a mean of 8.9 g/dl.

Evaluation of other RBC indices is important. In our study, MCV count ranges from minimum (65.36 fl) in thalassemia trait to maximum (82.24 fl) in SA Heterozygous. MCH count ranges from minimum (20.85 pg) in Thalassemia major to maximum (26.9 pg) in SA heterozygous. As stated by Khera R et al.[7], it appeared that MCV and MCH were almost similar in thalassemia major, thalassemia intermedia and thalassemia trait cases. Jaskirat Singh et al.[16], observed that all patients had MCV less than 82 fl and MCH less than 27 pg. Low Hemoglobin, reduced MCV, MCH and raised RBC count suggest beta thalassaemia trait.

RBC count was lowest in thalassemia intermedia (2.29) and highest in thalassemia trait (4.54). Khera R et al.[7] observed that red cell count was high in thalassemia trait, similar to our study.
Reticulocyte count was observed minimum in SA Heterozygous (1.3%) and maximum in Sickle cell disease (2.37%).

RDW-SD ranges from 41.1 (Sickle cell disease) to 89.91 (Thalassemia major). RDW-CV ranges from 18.15 (Sickle cell disease) to 33.5 (HbE Heterozygous).

We observed clinical features and blood pictures in thalassemia syndromes and other hemoglobinopathies. Peripheral smear examination is essential to pick up such cases. Predominant peripheral blood findings in our study were microcytosis & hypochromia with raised RBC counts in thalassemia trait patients.

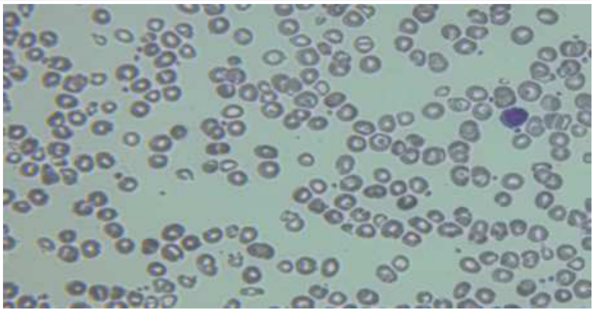


Figure 1: Hb E Heterozygous – Peripheral blood smear showing microcytic hypochromic picture and target cells in a case of Hb E Heterozygous.

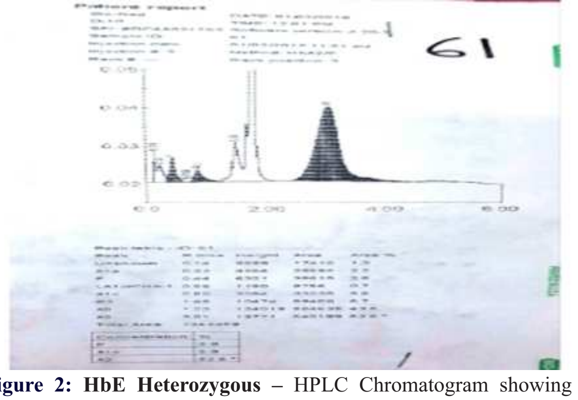


Figure 2: HbE Heterozygous – HPLC Chromatogram showing HbA2 – 52.6% (Hb E elutes in HbA2 window, and Hb F levels are normal indicating Hb E heterozygous.

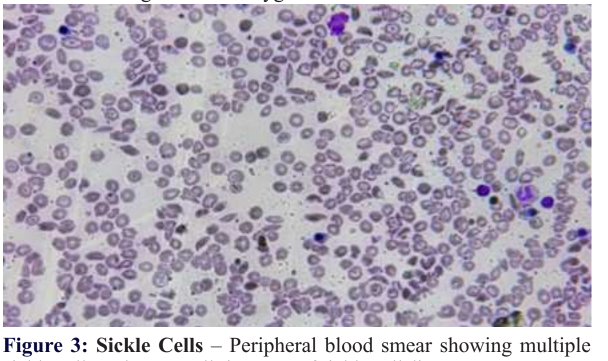


Figure 3: Sickle Cells – Peripheral blood smear showing multiple sickle cells and target cells in a case of sickle cell disease.

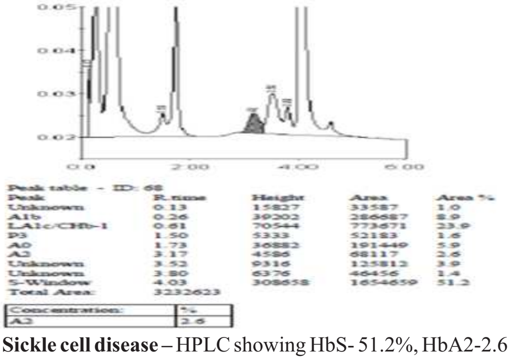


Figure 4. Sickle cell disease – HPLC showing HbS- 51.2%, HbA2-2.6 %

In our study, HbA2 levels were in range 3.3 (Sickle cell disease) to 53.1% (HbE heterozygous) where Hb E eluted in the HbA2 window. HbF was highest in thalassemia major (64.1%) .We observed values of HbS ranging from minimum (32.42%) in SA Heterozygous to maximum (60.45%) in Sickle cell disease.

HPLC helped us in detecting various heterozygous states. Though abnormal variants have less clinical significance, when combined with other variants, they can give rise to severe disease which highlights the significance of mass screening of the population.

Table No 6- Comparison Of HPLC Data With Other Studies.

Hemoglobinopathy	HbA	HbA2	HbF	HbS	HbE
Thalassemia trait [Khera R et al.]	83.6 ± 2.7	5.7 ± 0.9	1.5 ± 1.2	-	-
Present study	80.56±4.04	5.59±1.06	2.45±3.26		
Thalassemia intermedia [Khera R et al.]	30.4 ± 31.6	5.5 ± 2.6	62.0 ± 37	-	-
Present study	60.44±12.0	3.91±0.23	23.02±11.97		
Thalassemia major [Khera R et al.]	25.0 ± 22.7	2.8 ± 1.5	65.8 ± 29.8	-	-
Present study	18.05±12.7	3.81±0.30	64.1±11.59		
SA Heterozygous [Khera R et al.]	46.3	6.9	1.2	33.8	-
Present study	57.62±3.32	3.42±0.34	0.88±0.07	32.42±2.83	
HbE Heterozygous [Khera R et al.]	62.3 ± 2.7	-	0.96 ± 0.7	-	28.2 ± 5.0
Present study	31.6	53.1	15.1		

The data obtained from our study, for the screening of Thalassemia and Haemoglobin variants shows that, HPLC as an excellent, cost-effective methodology for mass screening and consequent reduction in the incidence of Thalassemia and Haemoglobinopathies. Hb variants that are eluted in the same window can be differentiated according to the retention time and the percentage of different hemoglobins obtained. It is important especially when the incidence of beta thalassemia traits is higher in developing counties like India, where there are limited resources available for early diagnosis.

CONCLUSION-

The high prevalence of Beta Thalassemia trait in India makes the screening programs for the above diseases, as a mandatory entity and HPLC has become an inevitable methodology, for rapid and definitive diagnosis. Screening and prevention at birth for either β thalassemia or haemoglobinopathies appears to be of utmost importance.

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