Journal of Pathology of Nepal (2021) Vol. **11**, 1803 - 1810

Journal of
PATHOLOGY
of Nepal
www.acpnepal.com

Original Article

Role of cation exchange high performance liquid chromatography in detection of hemoglobinopathies- a study of 500 cases in a tertiary care hospital.

Sneha Samir Babaria[1], Puja Bhavesh Jarwani[1], Sadhana Lalit Kothari[1], Shantibhai Patel[1]

[1]*Department of Pathology, GCSMC Hospital and Research Centre, Ahmedabad, Gujarat, India*

Keywords:

Cation Exchange High-Performance Liquid Chromatography; Hemoglobinopathies; Thalassemia;

ABSTRACT

Background: Abnormalities of hemoglobin synthesis are among the most common inherited disorders. Cation exchange high-performance liquid chromatography offers a reliable tool for early, accurate detection thereby aiding in the prevention and management of thalassemia major and various hemoglobinopathies.

Materials and methods: This was a retrospective study carried out in the Department of Pathology, GCSMC Hospital and Research center, Ahmedabad over six years from August 2013 to August 2019. 500 cases were studied for the identification of various hemoglobin disorders in patients referred for screening and detection of hemoglobinopathies.

Results: Abnormal hemoglobin fractions were seen in 104/500 (20.8%) cases. The β thalassemia trait was the predominant abnormality with a total of 69 cases (66.3%). β thalassemia major, β thalassemia intermedia, Hb D Punjab- β thalassemia, Acquired Hb F and Hereditary persistence of fetal hemoglobin/ δβ thalassemia trait was found in 1 case (0.96%) each. Sickle cell heterozygous was found in 9 cases (8.6%), Sickle cell homozygous in 5 cases (4.8%), and Sickle-ß thalassemia in 6 cases (5.8%). Other variants detected included Hb Q India heterozygous and Hb D Punjab heterozygous in 3 cases (2.9%) each and 2 cases (1.9%) of Hb E heterozygous and Hb J each.

Conclusions: Cation exchange high-performance liquid chromatography is an ideal and widely used methodology for routine clinical laboratory because of the simplicity of the automated system. The majority of the abnormal cases are diagnosed with it except a few inconclusive cases for which molecular and genetic studies are required.

Correspondence:

Dr. Puja Bhavesh Jarwani

Department of Pathology,

GCSMC Hospital and Research Centre, Ahmedabad, Gujarat, India

ORCID ID: 0000-0002-3852-2176

Email: pujajarwani@gmail.com

Received : June 18th 2020 ; **Accepted** : November 17th 2020

Citation: Babaria SS, Jarwani PB, Kothari SL, Patel S. Role of cation exchange high performance liquid chromatography in detection of hemoglobinopathies- a study of 500 cases in a tertiary care hospital. J Pathol Nep 2021;11:1803-10.DOI: 10.3126/jpn.v10i2.29511

INTRODUCTION

Abnormalities of hemoglobin (Hb) synthesis are among the most common inherited disorders of man. They are known as hemoglobinopathies and are classified as quantitative (due to reduced synthesis of globin polypeptide chains) which includes thalassemias and qualitative (due to structural alterations in globin polypeptide chains) which includes HbS, HbD, HbE, HbQ India, etc.[1] Thalassemias are the most common genetic disorders in the world, affecting nearly 200 million people worldwide. α thalassemia trait

DOI : 10.3126/jpn.v10i2.29511

occurs in 1–15% of persons of Mediterranean origin. β thalassemia has a 10–15% incidence in individuals from the Mediterranean and Southeast Asia.[2] These disorders can be heterozygous or homozygous. Various heterozygous states can become homozygous in newborns resulting in failure to thrive or serious morbidity. Also, certain 'Double Heterozygous' states can cause significant morbidity.[3]

In 1975, the expert group on "Abnormal hemoglobins and thalassemias" of the International Committee for Standardization in Hematology recommended two sets of investigations for the diagnosis of these disorders. Initial investigations included complete blood count (CBC), electrophoresis of the Hb at alkaline pH, sickling test, solubility test, and quantitation of HbA^2 and Hb F. If abnormal hemoglobin was detected or suspected on electrophoresis, the next set of investigations included electrophoresis of Hb at acid pH, electrophoresis of globin chains at acid and alkaline pH, isoelectric focusing, heat and isopropanol stability tests for unstable hemoglobins and tests for identifying hemoglobins with altered oxygen affinity. This resulted in a tedious array of tests to diagnose abnormal variants and hemoglobinopathies.[4] Nowadays, Cation exchange High-Performance Liquid Chromatography (CE-HPLC) offers a reliable tool for early, accurate detection thereby aiding in the prevention and management of various hemoglobinopathies.[5] The simplicity of the automated system with internal sample preparation, superior resolution, rapid assay time, and accurate quantification of Hb fractions makes this an ideal methodology for the routine clinical laboratory.[6,7]

MATERIALS AND METHODS

This was a retrospective study carried out in the Department of Pathology, GCSMC Hospital and Research center, a tertiary care hospital in Ahmedabad, over six years from August 2013 to August The study was approved by the Institutional Scientific and Ethical Committee. A total of 500 cases received for the screening of hemoglobinopathies were included in the study. Patients who had received recent blood transfusions were excluded from the study population as it can alter the level of various Hb variants. A complete switch to adult Hb occurs by 6 months of age after birth, so those below 6 months of age were excluded from the study population. All the demographic data including age, sex, clinical history, family history and history of blood transfusion were retrieved and collected. About 2.0 ml of whole blood was collected in EDTA vacutainers and stored at 2-8°C till further analysis. Statistical analysis was performed with Survey System software (version 11.0). The mean and standard deviation of hematological parameters: Hb, Red blood cell (RBC) count, Hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and Red cell distribution width (RDW) as well as of various hemoglobin fractions (HbF, HbA2, HbA, and HbS) were calculated. Categorical variables were expressed as frequencies and percentages. A 95% confidence interval was calculated for the various hemoglobinopathies. Mentzer index was calculated in cases of microcytic hypochromic anemia. It is a ratio of MCV in fL divided by the RBC count in millions per microliter. Specificity, sensitivity, and Youden index were also calculated in these cases. If the Youden index is not over 50%, then the test does not meet empirical benchmarks for being administered for diagnostic purposes.

About 2.0 ml of collected whole blood was used for CBC analysis to determine RBC indices including Hct, MCV, MCH, MCH concentration (MCHC), and RDW using an automated Hematology analyzer (SysmexXT2000i, Sysmex Corporation, Kobe, Japan). Peripheral blood smear (PBS) study and reticulocyte count were performed in all the cases.

HPLC was performed on BIO-RAD D-10TM (Dual program) manufactured by BIO-RAD laboratories, USA. BIO-RAD D-10TM utilizes the principle of CE-HPLC which has a negatively charged cartridge and acidic buffer. The acidic buffer has the property of changing Hb to positively charged molecules that interact with the negatively charged cartridge particles. Buffer with different ionic concentrations is pumped through the cartridge. Thus, those Hbs that are not removed by lower ionic strength buffers get removed with the higher ionic strength buffers and give us different retention/elution times. An HbA2/F/A1c calibrator was analyzed at the beginning of each run. The total area acceptable was between 1 to 4 million microvolts. second. The sample ratio was increased in the case of low total area and vice versa.[8] Specific defined windows are there from the manufacturer from specific retention time (RT) and integrated peaks are accordingly assigned. The RT is the time taken from the sample injection up to the apex of the elution peak. Established ranges of elution of common Hb variants are marked as "windows" (Table 1). RT not assigned comes as an unknown peak. Each sample takes 6.5 minutes for the result. Figure 1. displays normal chromatogram results showing RT, height, area, and area percentage. The cut-off of HbA2 for β-thalassemia trait (BTT) was taken at 4.0% along with reduced MCV (<80fl) and MCH (<27pg) levels with a relatively high RBC count and normal RDW.[9] Sample analysis was performed within 48 hours of sample collection.

Serum ferritin assay

Serum ferritin was assayed on Cobas e411 Immuno-assay Analyzer (Roche Diagnostics) using Elecsys Ferritin kit (Roche Diagnostics) following manufacturer's instructions to assess for iron status in all the cases to rule out iron deficiency anemia (IDA). Cut off value of iron deficiency was taken as 30 ng/ml in males and 15 ng/ml in females.

DOI : 10.3126/jpn.v10i2.29511

Sickling test

A sickling test was also performed (using sodium meta-bisulfite) when S-window was eluted in the sample in cases suspicious of sickle cell disease.

The final diagnosis was given in light of the RBC indices and keeping in mind the possible confounding factors. Borderline cases were advised for further hematological and biochemical tests depending on the observed values of different hemoglobin fractions.

RESULTS

Out of a total of 500 cases, 209 (41.8%) were male and 291 (58.2%) were female. Out of 500 cases, 50 patients were part of antenatal screening (10.0%). Table 2 depicts the sex and age-wise distribution and the frequency of different types of hemoglobinopathies among the study population. Patients' age ranged from 6 months to 76 years. The majority of hemoglobinopathies were observed in the age group of 13–36 years (58.2%) followed by 28.1% in the age group of 6 months–12 years.

Various abnormal hemoglobin defects detected in the present study are shown in figure 2.

Out of the total 500 cases, 104 (20.8%) cases had abnormal hemoglobin fractions and out of these most common defect was β thalassemia trait (BTT; 66.3%; HbA2> 4%). In the antenatal group, six cases (12.0%) were detected with BTT, and one case with borderline HbA[2]. Most of the BTT cases were in the adolescent and adult age group (13-36 years), the majority of which were female while in the pediatric age group (6 months to 12 years) more cases were seen in males.

Various hematological parameters and Hb variants in different hemoglobinopathies are shown in Tables 3 and 4. Table 5 shows the PBS findings seen in various hemoglobinopathies observed in the present study. In cases of BTT, HbA2 RT was between 2.94 to 3.30 (fig. 3). The sensitivity and specificity of the Mentzer index for detection of BTT in cases of microcytic hypochromic anemia were (62.3% and 74.3%) respectively with the Youden index of 36.6%.

There was one case of beta-thalassemia intermedia, a ten year female with low Hb, marked splenomegaly, and HbF 6.10%. One case of beta-thalassemia major was observed in which there was severe anemia with HbF 76.3%. There were 19 cases with borderline HbA2 (HbA: 3.6-3.9%). The majority of cases showed moderate anemia and PBS showed macrocytosis, macroovalocytes, and anisopoikilocytosis. They had Vitamin B12 or folic acid deficiency. One of these cases was a male patient aged 36 years with normal Hb,

Table 1: Window time and retention time of predefined parameters of BIO-RAD D108

Peak name	Retention time (min)	Window (min)
F	0.52	0.42-0.62
A1c	0.84	0.80-0.90
P3	1.43	1.23-1.63
A0	1.7	1.55-1.85
A2	3.15	2.80-3.50
D	3.80	3.70-3.90
S	4.16	4.02-4.30
C	4.75	4.65-4.85

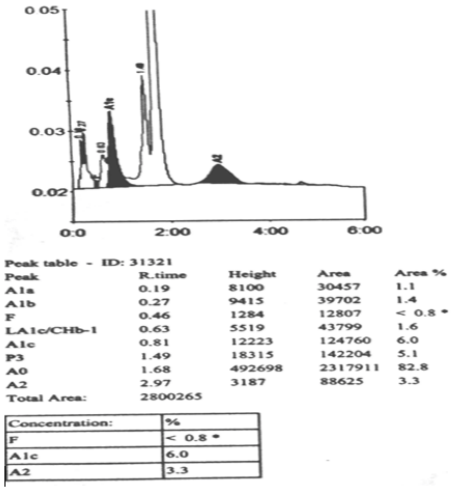

Figure 1: *Chromatogram showing normal Hemoglobin variants hemoglobin, CI=confidence interval*

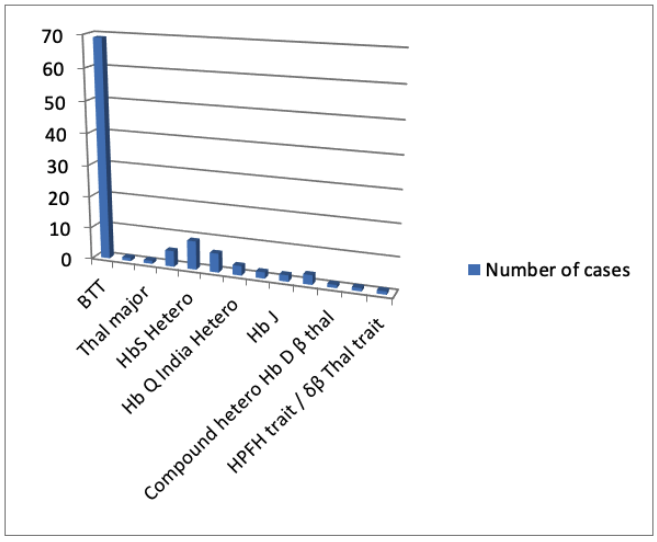

Figure 2: *Column chart of frequency of various Hemoglobinopathies in our study*
BTT: beta thalassemia trait, Thal: thalassemia, Homo: homozygous, Hetero: heterozygous

high RBC count and normal peripheral smear examination. Genetic study was advised to rule out rare mutations. Five cases (4.8%) of Sickle cell anemia (homozygous HbS) patients had low Hb, low RBC count, and high reticulocyte

Table 2: Sex and age-wise distribution and frequency of different types of hemoglobinopathies among the study population

Type of Hemoglobinopathies	Sex and age group in years								Total	Frequency
	6 mths-12 yrs		13-36 years		37-60 years		>60 years			
	M	F	M	F	M	F	M	F		
BTT	12	7	13	28	3	4	1	1	69	66.3(62.16-70.44)
ß thal intermedia	0	1	0	0	0	0	0	0	1	0.96(0.09-1.83)
ß thal major	0	1	0	0	0	0	0	0	1	0.96(0.09-1.83)
Sickle cell hetero	2	0	3	1	2	0	1	0	9	8.6(6.14-11.06)
Sickle cell homo	2	0	3	0	0	0	0	0	5	4.8(2.93-6.67)
Sickle- ß thal	1	0	3	2	0	0	0	0	6	5.8(3.75-7.85)
HbQ India hetero	2	0	1	0	0	0	0	0	3	2.9(1.43-4.37)
HbE hetero	0	0	0	1	1	0	0	0	2	1.9(0.7-3.1)
HbJ	0	1	0	1	0	0	0	0	2	1.9(0.7-3.1)
HbD Punjab hetero	0	0	2	1	0	0	0	0	3	2.9(1.43-4.37)
HbD Punjab- ß thal	0	0	0	0	0	0	0	1	1	0.96(0.09-1.83)
Acquired HbF	0	0	1	0	0	0	0	0	1	0.96(0.09-1.83)
HPFH Trait /δß ThalTrait	0	0	1	0	0	0	0	0	1	0.96(0.09-1.83)
Total	19	10	27	34	6	4	2	2	104	100
Total (both sexes)	29(27.9%)		61(58.7%)		10(9.6%)		4(3.8%)		104(100%)	

M: male, F: female, BTT: beta thalassemia trait, Thal: thalassemia, Homo: homozygous, Hetero: heterozygous, HPFH=hereditary persistence of fetal hemoglobin, CI=confidence interval

count. HbS was ranging from 59.1-75.9% (Fig. 4).

Nine cases of Sickle cell trait (6%) were found with HbS ranging from 21.5% to 35.0%. They had mild anemia and a high RBC count. Double heterozygous for sickle cell-β thalassemia cases were six (5.8%). They showed mild anemia and normal RBC count. The sickling test was positive in all the cases of sickle cell disease. Confirmation was also done by the parental study. There were three cases (2.9%) of HbQ India Heterozygous with an unknown peak at RT of 4.43 to 4.46 min and area amounting to 15.0 ± 5.0%. There was no anemia. Also, there were two cases of HbE heterozygous (1.9%). There was mild anemia.

Two cases showed elevated P3 peak with area 29.2± 3.1%, with RT 1.54 to 1.56 min, suggesting Hb J. There was no anemia but RBC was mildly increased. Iron studies and family studies were correlated. There were three cases of

Table 3: Hematological parameters in different hemoglobinopathies

Parameters	Hb (g/dL)	RBC ($\times 10^6$ cu.mm)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)
BTT	9.4 ± 2.5	4.8 ± 1.2	29.6 ± 7.4	63.9 ± 14.5	20.3 ± 5.4	31.8 ± 5.6	19.9 ± 5.2
ß thal intermedia	4.4	1.22	13.1	107.4	36.1	33.6	35.0
ß thal major	5.8	2.81	18.4	65.5	21	31.5	35.8
Hetero HbS	10.6 ± 2.8	5.0 ± 1.0	33.4 ± 7.0	69.3 ± 7.1	21.9 ±3.8	31.3 ± 2.89	17.7 ± 2.9
Homo HbS	8.0 ± 0.6	3.3 ± 0.3	23.8 ± 1.8	71.9 ± 8.2	24.1 ± 3.0	33.5 ± 1.0	20.6 ± 4.2
Sickle-ß Thal	9.6 ± 0.6	4.12 ± 0.3	28.7 ±2.7	69.9 ± 7.1	23.3 ± 2.0	33.4 ± 0.9	19.4 ± 2.8
HbQ India hetero	11.8 ± 2.7	4.5 ± 0.9	31.1 ± 9.2	76.4 ± 27.0	27.5 ± 11.7	38.5 ± 3.8	15.9 ±4.5
HbJ	12.9± 5.4	5.2±0.2	39.6±11.0	75.8±23.7	20.8±6.9	31.6±2.7	16.5±3.1
HbE hetero	9.0±3.0	3.4±1.7	26.8±9.6	82.4±12.1	27.7±4.6	33.5±0.7	20.1±8.8
HbD Punjab hetero	13.8± 2.5	44±0.6	40.4±6.8	91.7±6.9	31.2±2.5	34.1±0.5	13.6±0.9
HbD Punjab ß thal	9.0	4.70	28.9	61.5	19.1	31.1	16.3
Acquired HbF	5.7	1.57	16.5	105.1	36.3	34.5	19.4
HPFH/δß thal trait	13.2	5.27	40.7	77.2	25.0	32.4	16.6

Numbers are mean ± standard deviation. PCV: packed cell volume, BTT: beta thalassemia trait, Thal: thalassemia, Homo: homozygous, Hetero: heterozygous

Table 4: Value of hemoglobin variants in different hemoglobinopathies

Parameters	HbF (%)	HbA2 (%)	HbA (%)	HbS (fL)
BTT	2.3± 3.0	5.4 ± 0.9	82.0 ± 3.0	-
ß thal intermedia	6.10	3.30	76.1	-
ß thal major	76.3	1.5	4.80	-
Hetero HbS	<0.8	2.8 ± 0.9	60.3 ± 4.1	30.2 ± 4.6
Homo HbS	19.5± 6.3	3.0 ± 0.8	6.2 ± 0.8	70.3 ± 6.8
Sickle-ß Thal	15.5 ± 3.5	5.1 ± 0.7	13.8 ± 17.6	63.4 ± 15.4
HbQ India hetero	<0.8	2.5 ± 0.6	81.7 ± 6.1	-
HbJ	2.1±0.4	2.4± 0.6	53.2±5.3	-
HbE hetero	0.9	30.3± 3.6	59.6±4.5	-
HbD Punjab hetero	<0.8	2.4± 0.2	54.8±1.7	-
HbD Punjab ß thal	5.0	5.0	20.0	-
Acquired HbF	6.5	2.8	90.0	-
HPFH/δß thal trait	21.8	2.3	64.6	

BTT: beta thalassemia trait, Thal: thalassemia, Homo: homozygous, Hetero: heterozygous

Table 5: Peripheral smear findings in different hemoglobinopathies

Parameters	Microcytosis	Hypochromia	Macrocytosis	Aniso-Piokilocytosis	Basophilic stippling	Target cells	Sickle cells	Polychromasia	Normoblast
BTT	√	√	-	√	√	√	-	-	-
ß thal intermedia	√	√	√	√	-	√	-	√	√
ß thal major	√	√	-	√	-	√	-	√	√
Hetero HbS	√	√	-	√	-	-	-	-	-
Homo HbS	√	√	-	√	-	-	√	-	-
Sickle-ß Thal	√	√	-	√	-	√	√	-	-
HbQ India hetero	√	√	-	-	-	-	-	-	-
HbE hetero	√	√	-	√	-	-	-	-	-
HbJ	√	√	-	-	-	-	-	-	-
HbD Punjab hetero	-	-	-	-	-	-	-	-	-
HbD Punjab ß thal	√	√	-	-	-	-	-	-	-
Acquired HbF	-	-	√	-	-	-	-	-	-
HPFH/δß thal trait	√	√	-	√	-	√	-	-	-

BTT: beta thalassemia trait, Thal: thalassemia, Homo: homozygous, Hetero: heterozygous

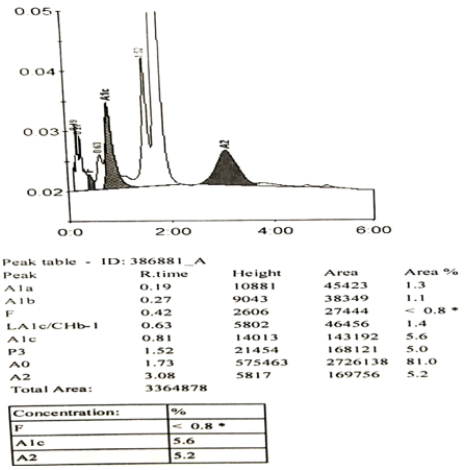

Figure 3: *Chromatogram of β Thalassemia trait*

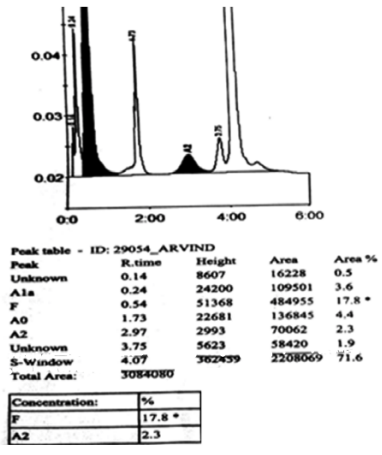

Figure 4: *Chromatogram of HbS*

HbD Punjab heterozygous. There was an unknown peak at RT of 3.86-3.88 min and area amounting to 35.9±1.2%. Hematological parameters were normal in all cases. There was one case of compound heterozygous for HbD and β-thalassemia. There was an unknown peak at 3.86 min and an occupying area of 70%. A parental study was done and a genetic study was advised for confirmation. One case was observed with HbF 6.5%. The rest of the Hb variants were within the normal range.

There was one case of 17 yr male having normal Hb (13.2g/dl), mildly reduced MCV (77.2fl), and MCH (25.0pg) with mildly raised RBC count (5.27×10^6 cu /mm) and near-normal RDW (16.6%) showing high HbF (21.8%), low HbA (64.6%) and normal HbA2(2.3%). The case was reported with the possibility of HPFH Trait or δβ-Thalassaemia Trait.

Few cases had chromatograms showing normal Hb variants but they showed low MCV, MCH, and high RBC count, so further evaluation by capillary electrophoresis and genetic study was advised.

DISCUSSION

Thalassemias are a group of hemolytic anemias that result from an inherited abnormality of globin gene production. The diagnosis of hemoglobinopathies and thalassemia is required to explain hematological abnormalities, identify an abnormality in the pre-symptomatic period, pre-conceptional screening, screening of fetus to offer termination of pregnancy and to confirm a presumptive diagnosis.[10]

Hemoglobin fraction analysis by CE-HPLC is a highly reproducible system making it an excellent technology to screen for Hb variants and thalassaemias, as it has the convenience of quantifying HbF and HbA2 along with hemoglobin variant screening in a single run. As compared to the bands obtained in electrophoresis, the chromatograms are easier to read and better in the resolution which helps to differentiate hemoglobin fractions eluting in a range very near to each other.

In our study, 104 (20.8%) cases had abnormal hemoglobin fractions and out of these most common defect was BTT (66.3%). A comparison of our results with other similar studies done throughout the country is given in Table 6.

Screening programs undertaken in different regions have shown that many ethnic groups like Sindhis, Kutchi, Bhanushalis, Lohanas, Punjabi Khatris and Aroras, Bengalees, some Muslim groups, and some tribal populations from Orissa and Gujarat have much higher prevalence rates than the average ranging from 4 to 17%. Some of these communities have greater awareness and are more receptive and amenable to undergo screening as they may have come across a thalassemia major child among their extended families or friends.[15]

In the present study, 10.0% of the cases were a part of antenatal screening out of which 12% of the cases turned out to be BTT and there was one case with borderline HbA2. This proves that antenatal screening is of great value to prevent potential offspring with thalassemia or any other hemoglobinopathy. Most of the hemoglobinopathies were observed in the age group of 6 months–36 years as most of the screening is done in early life, patients show symptoms at an early age and these findings were comparable with Jain BB et al.[16]

The sensitivity and specificity of the Mentzer index for detection of beta-thalassemia trait in cases of microcytic hypochromic anemia were 62.3% and 74.3% respectively with the Youden index of 36.6% suggesting that the Mentzer index is neither a sensitive nor a specific parameter to differentiate BTT from IDA.[17] This probably may be due to the presence of a large number of antenatal cases and cases from a lower socioeconomic class. As a tertiary care center, we have a large burden of such nutritionally deprived patients. So we cannot rely solely on the screening methods which were used in past like RBC count, MCV, MCH, RDW, PBS, and various hematological indexes to differentiate BTT from IDA.

In our study, we found nineteen borderline cases with a mean HbA2 of 3.71%. It is found that megaloblastic anemia gives rise to a spuriously high HbA2. Borderline HbA2 requires further investigation before reaching a conclusion. There may be the presence of confounding factors that make the diagnosis more perplexing and labor-intensive. Mutation analysis should be offered to all at-risk couples with borderline HbA2, especially those with values between

Table 6: Comparison of the present study with other studies

Studies	Number of cases	% of Abnormal Hemoglobin fractions	% of ß Thalassemia Trait
Sarvaiya et al[11]	2035	18.96%	10.6%
Sachdev et al[12]	2600	12.57%	8.9%
Rao et al[13]	800	30.90%	18.1%
Mukhopadhyay et al[14]	10407	14.50%	5.6%
Present study	**500**	**20.60%**	**13.8%**

3.5% and 4.0% and microcytic hypochromic indices. Borderline HbA2 could result due to some mild beta-thalassemia alleles like the capsite (+1)(A→C) mutation or co-inheritance of delta thalassemia.

Sickle cell disease is found in the western, central, and eastern belts of India including states like Rajasthan, Gujarat, Maharashtra, Madhya Pradesh, Chhattisgarh, and Orissa. Most cases present as hemolytic anemia. Family history, vigilant PBS examination, sickling test, and Hb variant analysis by CE-HPLC are useful in such cases to diagnose sickle cell syndrome. On the D-10™ Hb testing system, HbS in Sickle cell trait ranges between 30-40%. For homozygous Sickle cell anemia, HbS ranges from 70-90% along with a variable HbF of 5-25%.[9] In the present study, a total of twenty cases of Sickle cell disease were detected in which nine cases were HbS heterozygous, 5 cases HbS homozygous, and 6 cases were of Sickle-Beta thalassemia. Since Hb S shares its electrophoretic and chromatographic properties with several variants, it is necessary in the absence of a familial history of HbS, to do a confirmatory test. A good compromise is to perform CE HPLC and CE, or acid agar electrophoresis.

Three cases (2.9%) of HbQ India heterozygous were found and all of them belonged to the Sindhi community. Chromatogram findings in HbQ India include an unknown peak at 4.45 ± 0.02 minute and ranges of 8-25 percentage.[9] In our study there was an unknown peak ranging from 4.43 to 4.46 minutes and ranges of 10-20%. Sukumaran et al also had similar findings.[18]

Hb E results from a beta chain mutation (β26 Glu→ Lys, GAG→AAG. It is the most common Hb variant in South East Asia and the second most prevalent Hb variant worldwide. HbE heterozygotes have about 30% HbE (usually less than 40%) which elutes in the HbA2 window (2.80-3.50 min) on HPLC. HbE in homozygotes accounts for 85-95%. The way to differentiate it from HbA2 is its percentage, the HbA2 fraction will never be >9%.[9] In the present study, two cases of HbE heterozygous with a mean HbA^2 level of 30.25% was detected.

There were two cases of HbJ variant with reduced MCV and MCH values. So this variant needs to be ruled out in cases of microcytic hypochromic blood picture. Recommendation for parental study and molecular studies should be done. In the present study, both the cases were from the same family, father, and son.

Hb D Punjab tends to have a normal phenotypic presentation. There is a mutation in the beta chain at β121 Glu→Gln (GAA-CAA).[9] We had detected Hb D Punjab heterozygous in 2.9% of cases. On alkaline electrophoresis, it migrates in the S/D region. On CE-HPLC, it elutes in the D-Window, separate from the HbS peak. We had one case of compound heterozygous for Hb D Punjab- β thalassemia and these patients tend to have mild anemia and are asymptomatic.

Hematological findings are variable in rare hemoglobinopathies like HbQ India, HbE, and HbJ variants and even normal in HbD Punjab heterozygous. Due to globalization and consanguineous marriages we are able to find cases that are not routinely prevalent in that particular area. So CE-HPLC is preferable in screening and diagnosis of various hemoglobinopathies.

One case of high HbF was observed in a 19-year-old male whose bone marrow examination suggested aplastic anemia. Flow cytometry was performed and turned out to be a case of Paroxysmal Nocturnal Hemoglobinuria. Finally, it was reported as a case of Acquired HbF syndrome due to erythroid stress.

We had one case in which differential diagnosis of HPFH Trait or δβ-Thalassaemia trait was given, further parental study and mutational analysis was advised. The two groups of disorders are distinguished by the phenotype of heterozygous individuals. Heterozygotes of δβ-thalassemia mutations have 5% to 20% HbF, which is heterocellularly distributed in red cells, whereas heterozygotes of HPFH mutations have 17% to 30% HbF, with a pancellular distribution.[19]

CONCLUSIONS

CE-HPLC an ideal methodology for routine clinical laboratory and this has increased its use in the last decade. A concentrated effort towards education and awareness will continue to be needed in the complex and heterogeneous Indian population in both urban and rural regions in every state. Thus, in a country like India where a load of hemoglobinopathies could not be neglected, HPLC has given a bright ray of hope in identifying those silent cases which can contribute to significant morbidity and mortality if remained undiagnosed. The majority of the abnormal cases are diagnosed with it except few inconclusive cases for which complete characterization, including amino acid sequencing or genotyping by direct DNA analysis, is readily available from several reference laboratories and should be requested if clinical suspicion is high, or when HPLC fails to yield definitive answers.

Acknowledgement

We acknowledge the technical staff and doctors of the Indian Red Cross Society, Old Wadaj, Ahmedabad, Gujarat for their extended co-operation.

Conflict of interest: None

REFERENCES

1. Kutlar F. Diagnostic approach to hemoglobinopathies. Hemoglobin 2007;31:243-50. Crossref

2. Benz EJ. Disorders of Hemoglobin, In: Jameson JL, Kasper D , Longo DL, Fauci AS, Hauser SL, Loscalzo J. Harrison's Principle

of Internal Medicine. 20th edn. McGraw Hill Education: New York; 2018. pp 692.

3. Clarke GM, Higgins TN. Laboratory investigation of hemoglobinopathies and thalassemias: Review and Update. Clinical Chemistry 2000;46:1284–90. Crossref

4. Colah RB, Surve R, Sawant P, et al. HPLC Studies in Hemoglobinopathies. Indian J Pediatr 2007;74:657-62. Crossref

5. Beginners Guide to Liquid Chromatography. What Is HPLC (High Performance Liquid Chromatography? Website

6. Wild BJ, Stephens AD. The use of automated HPLC to detect and quantitate hemoglobins. Clin Lab Haematol 1997;19:171–6. Crossref

7. Ou CN, Rognerud CL. Diagnosis of hemoglobinopathies: Electrophoresis vs. HPLC. Clin Chim Acta 2001;313:187–94. Crossref

8. Instruction Manual. D-10TM Dual Program, Bio-Rad. Website

9. Library of Chromatograms, D10 TM Hemoglobin testing system. Bio-Rad laboratories.

10. Singh T. Hemolytic Anaemias, In: Atlas & Text of Hematology. 4thedn. Avichal Publishing Company: Delhi; 2018. pp 157.

11. Sarvaiya AN, Chauhan SC. Variant Hemoglobin spectrum By Cation Exchange High Performance Liquid Chromatography: A Study 0f 2035 Subjects. Annals of Pathology and Laboratory Medicine 2017;4:297-302. Crossref

12. Sachdev R, Dam AR, Tyagi G. Detection of Hb variants and hemoglobinopathies in Indian population using HPLC: report of 2600 cases. Indian J Pathol Microbiol 2010;53:57-62. Crossref

13. Rao S, Kar R, Gupta SK, Chopra A, et al. Spectrum of hemoglobinopathies diagnosed by Cation exchange-HPLC & modulating effects of nutritional deficiency anaemias from north India. Indian J Med Res 2010;132:513-9. DOI: 10.4103/0971-5916.73390

14. Sur D, Mukhopadhyay SP. Prevalence of thalassaemia trait in the state of West Bengal. J Indian Med Assoc 2006;104:11-5. PMid: 16850860

15. Colah R, Italia K, Gorakshakar A. Burden of thalassemia in India: The road map for control. Pediatric Hematology Oncology 2017;2:79-84. Crossref

16. Jain BB, Roy R, Ghosh S, et al. Screening for Thalassemia and Hemoglobinopathies in a tertiary Care Hospital of West Bengal: Implications for Population Screening. Indian Journal of Public Health 2012;56:297-300. Crossref

17. Bhushan R, Shukla S, Singh D, et al. Reliability of Different RBC Indices to Differentiate Between Beta Thalassemia Trait and Iron Deficiency Anemia During Antenatal Screening. World J Pathol 2018;7:14-20. Website

18. Sukumaran PK, Merchant SM, Desai MP, et al. Hemoglobin Q India (alpha 64 (E13) aspartic acid to histidine) associated with beta-thalassemia observed in three Sindhi families. J Med Genet 1972;9:436–42. Crossref

19. Mansoori H, Asad S, Rashid A, et al. Delta beta thalassemia: a rare hemoglobin variant. Blood Res 2016;51:213-4. Crossref