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Study of Hematological Profile and High Performance Liquid Chromatography in Diagnosis of Hemoglobinopathies

[1]Swati, PG Resident (3rd year), Department of Pathology, Jhalawar Medical College, Jhalawar (RAJ.)

[2]Chetna Jain, Senior Prof. & Head, Department of Pathology, Jhalawar Medical College, Jhalawar (RAJ.)

[3]Madhu Sen, PG Resident (3rd year), Department of Pathology, Jhalawar Medical College, Jhalawar (RAJ.)

Corresponding Author: Swati, PG Resident (3rd year), Department of Pathology, Jhalawar Medical College, Jhalawar (RAJ.)

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Abstract

Objective: The aim of this study is to evaluate the clinico-hematological profile of thalassemia/ hemoglobinopathies.

Methods: In during the period of 1 year from June 2019 to May 2020, **104** patients fulfilling the inclusion & exclusion criteria, were included. A 5ml EDTA blood sample was collected. The sample was run on automated hematology analyser (Sysmex XN1000) for hemogram and red cell indices; PBF study was done and the same sample was analysed by High Performance Liquid Chromatography using variant II β-Thal short program.

Result: HPLC provides a superior resolution is automated and internal sample preparation is possible. 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: Our study concluded that RBC indices, HPLC finding, and family study are sufficient to detect and manage most of the hemoglobin variants prevalent in this country.

Keywords: CE-HPLC, Hemoglobinopathies

Introduction

Hemoglobinopathies are one of the major public health problems in India. Hemoglobinopathies can be either quantitative or qualitative[1]. WHO figures estimate that 5% of the world population is a carrier for hemoglobinopathies[2] and of these, thalassemia syndromes, particularly beta thalassemia major is serious and a major cause of morbidity. WHO figures state that about 370,000 severely affected homozygotes or compound heterozygotes of thalassemia are born every year[3]. The frequency of β-thalassemia in India ranges from 3.5 to 15% in general population[4]. Every year 10,000 children with thalassemia major are born in India, which constitutes 10% of the total numbers in the world.

Plethora of hemoglobin variants is prevalent in India owing to ethnic diversity of its population with minimal to major clinical significance. Being recessively inherited from the parents, the thalassemia and

thalassemic hemoglobinopathies pose serious health problem leading to severe morbidity and mortality in Indian population.

The aim of screening is to identify carriers of β thalassemia, as well as structural variants like Hb S and Hb E to identify couples at risk of having a child with β thalassemia major sickle cell disease, Hb S β thalassemia and Hb E β thalassemia. The compound heterozygous disorders (HbS D-Punjab, HbS E, HbS\ β thalassemia) or unusual variants (Hb D Iran, Hb J) are all clinically significant with varying degree of severity, making precise identification important. Cation exchange high-performance liquid chromatography (CE-HPLC) is used to separate and estimate various normal and abnormal Hb fractions and offers a definitive tool for early and accurate detection of hemoglobinopathies, thereby aiding in their prevention and management.

Aims And Objectives

- To evaluate the clinico-hematological profile of thalassemia / hemoglobinopathies.
- To assess the usefulness of HPLC in characterization of hemoglobin profile in thalassemia / hemoglobinopathies.
- To study the demographic factors like age and gender in cases of thalassemia / hemoglobinopathies.

Material and Methods

This prospective study was conducted at the Department of Pathology, Jhalawar Medical College and Hospital, Jhalawar (Rajasthan) during the period of 1 year from June 2019 to May 2020.

A 5ml EDTA blood sample was collected from all cases of microcytic hypochromic anaemia (MCV < 80 fl, MCH < 27 pg) not responding to conventional treatment, clinically suspected cases of thalassemia/hemoglobinopathy and transfusion requiring children, adults, antenatal cases and their family members in K2 EDTA vacutainers. After obtaining approval and clearance from the ethical committee, a total of **104** patients fulfilling the inclusion & exclusion criteria, were included in this study. Informed consent was taken from each patient. A detailed clinical history was taken and primary complaints were noted with emphasis on age at onset of disease, duration of disease, family history, failure to thrive and/or failure to gain weight and requirement of blood transfusions. A detailed physical examination was carried out. The following points were noted in particular: pallor, splenomegaly, presence of hemolytic facies and jaundice. Then the sample was run on automated hematology analyser (Sysmex XN1000) for hemogram and red cell indices; PBF study was done and the same sample was analysed by High Performance Liquid Chromatography using variant II β-Thal short program.

Statistical Analysis

Data was analyzed by appropriate statistical test using the SPSS software 20.0 version

Observations And Results: A total of 104 cases were included in the study. Clinical and haematological parameters were studied in all these cases. Following findings were observed:

Table 1: Distribution of study subjects according to gender

Gender	Frequency	Percentage
Male	57	54.81
Female	47	45.19
Total	104	100

Table 1 shows distribution of study subjects according to gender.54.81% patients were male subjects, whereas 45.19% were females.

Table 2: Incidence according to diagnosis

Diagnosis	Frequency	Percentage (Prevalence)
Thalassemia Trait	27	25.96
Thalassemia Major	7	6.73
Thalassemia Intermedia	5	4.81
HBS & B ThalassemiaHeterozygous	2	1.92
HBS Heterozygous	2	1.92
Sickle Cell Homozygous	2	1.92
Hb D Punjab Heterozygous	1	0.96
Hb E TRAIT	1	0.96
Δβ Thalassemia	1	0.96
Total Hemoglobinopathies	48	46.15
Normal	56	53.85

Table 2 shows incidence according to diagnosis. A total of 104 cases were studied, out of which 48 cases of thalassemic disorders and hemoglobinopathies were observed, whereas 56 patients were normal. Out of 48 cases, 27 were having thalassemia trait, followed by 7 cases of thalassemia major and 5 cases of thalassemia intermedia. Two cases of HbS & β thalassemia heterozygous, HbS heterozygous and sickle cell homozygous each. One cases each with Hb E trait and δβ thalassemia.

Table 3: Age of subjects in different thalassemic disorders and hemoglobinopathies

Diagnosis	Mean age		Age at onset	
	Mean	SD	Mean	SD
Thalassemia Trait	19.2	16.8	4.75	2.97
Thalassemia Major	0.99	0.07	0.38	0.05
Thalassemia Intermedia	5.14	4.81	7	-
Hbs & B ThalassemiaHeterozygous	29.5	5.96	-	-
Hbs Heterozygous	1.7	2.17	-	-
Sickle Cell Homozygous	16.5	6.01	-	-
HbD PunjabHeterozygous	0.3	-	-	-
Hb E Trait	18	-	-	-
δβ Thalassemia	2	-	-	-
chi square	9.812		1.843	
p-value	<0.05		<0.05	

Table 3 shows age of subjects in different thalassemic disorders and hemoglobinopathies. Mean age was evaluated for study subjects suffering with hemoglobinopathies and mean age at onset of disease.

Mean age of patients was observed to be 19.2yrs in thalassemia trait. Sickle cell homozygous has mean age of 16.5yrs. Patients in Hb E trait were around 18yrs of age and patients in δβ thalassemia group were 2yrs of age. Mean age of patients was observed to be minimum in patients with Hb D Punjab heterozygous (0.3yrs), followed by thalassemia major (0.99±0.07yrs), 1.7±2.17yrs in HbS heterozygous, 5.14±4.81yrs in thalassemia intermedia. HbS & β thalassemia heterozygous found in patients with mean age of 29.5yrs of age. Thalassemia trait patients has mean age of 4.75±2.97yrs and thalassemia major has mean age of 0.38±0.05yrs and a thalassemia intermedia has mean age of 7yrs at onset of disease.

Chi square statistical analysis was done in relation to mean age and age of onset according to thalassemic disorders and hemoglobinopathies and it was found to be statistically significant (p-value<0.05)

Table 4: Mean values according to haematological profile in different thalassemic disorders and hemoglobinopathies

Diagnosis	HB		MCV		MCH		MCHC		RBC Count		Reticulocyte Count		RDW SD		RDW CV		Statistical Analysis	
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	F-Statistics	P-Value
Thalassemia Trait	10.477	4.92	62.21	18.76	19.95	3.817	31.65	2.98	5.559	3.981	1.67	2.81	43.30	31.09	22.31	7.15	11.816	0.023*
Thalassemia Major	6.35	1.91	61.68	2.10	25.35	6.109	38.9	7.26	2.84	1.89	1.24	0.08	72.156	18.17	36.18	8.01	17.1881	0.045*
Thalassemia Intermedia	5.06	2.78	64.98	4.19	22.02	4.98	36.36	3.09	2.318	0.781	1.16	0.09	73	11.98	36.36	9.61	11.715	0.006*
Hbs & B Thalassemia Heterozygous	9.3	1.09	69.75	4.10	23.23	2.10	33.55	0.01	3.96	0.92	1.75	0.45	40.75	1.81	21.7	2.10	1.1981	0.024*
Hbs Heterozygous	3.7	1.0	71.45	8.16	23	4.98	32.84	7.10	1.58	0.18	0.9	0.1	43.35	2.19	27.25	4.19	23.181	0.005*
Sickle Cell Homozygous	4.05	1.67	71.15	3.19	28.8	1.16	40.55	1.81	1.76	0.02	2	1	63.1	18.71	32.8	7.19	12.918	0.001*
Hb D Punjab Heterozygous	6.9	-	83.7	-	70.3	-	83.2	-	0.99	-	1	-	107.3	-	32.27	-	-	-
Hb E Trait	14	-	72	-	23	-	32	-	6.1	-	1	-	35.2	-	18.3	-	-	-
Δβ Thalassemia	6.1	-	75	-	26	-	34.6	-	2.34	-	2.5	-	50	-	18.4	-	-	-

Table 4 shows mean values according to haematological profile in different thalassemic disorders and hemoglobinopathies. Mean Hb value was observed to range from 3.7gm% (HbS heterozygous) to 14gm% (Hb E trait) in different hemoglobinopathies. MCV count ranges from minimum in thalassemia major (61.68fl) to maximum (83.7fl) in Hb D Punjab heterozygous. MCH count ranges from minimum in thalassemia major (19.95pg) to maximum (70.3pg) in Hb D Punjab heterozygous. MCHC value was minimum in patients with thalassemia trait (31.65gm) and maximum in Hb D Punjab heterozygous (83.2gm). RBC count was lowest in Hb D Punjab heterozygous (0.99%) and highest in Hb E trait (6.1%). Reticulocyte count was observed minimum in HbS heterozygous (0.9%) and maximum in δβ thalassemia (2.5%). RDW

SD range from 35.2(Hb E trait) to 107.3(Hb D Punjab heterozygous). RDW CV range from 18.3(Hb E trait) to 36.36(thalassemia intermedia).

ANOVA statistical analysis was done to find relation between hemoglobinopathies and various parameters. Level of significance was observed to be significant (p-value<0.05).

Table 5: Mean values according to haemoglobin profile in different thalassemic disorders and hemoglobinopathies

Diagnosis	HB F		P3		HB A		HBA2		HBS		HB D		HB C		Statistical Analysis	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	F-Statistics	P-Value
Thalassemia Trait	1.92	0.51	5.28	0.89	86.588	0.23	5.955	1.15	0.185	1.12	0	0	0	0	1.817	0.05*
Thalassemia Major	84.64	12.65	1.13	0.12	20.38	1.81	2.84	1.88	0	0	0	0	0	0	12.918	0.002*
Thalassemia Intermedia	51.52	11.60	3.34	1.51	39.64	10.02	4.16	0.91	0	0	0	0	0	0	4.180	0.001*
Hbs & B Thalassemia Heterozygous	19.3	2.83	1.1	0.1	15.45	4.29	4.3	0.1	63.2	1.1	0	0	0	0	10.009	0.043*
Hbs HETEROZYGOUS	6.95	0.91	4.3	1.81	54.95	9.91	3.85	0.98	31.5	0.89	0	0	0	0	1.910	0.007*
Sickle Cell Homozygous	14.5	2.5	0	0	9.85	2.10	2.6	0.2	70.7	1.3	0	0	0	0	1.005	0.01*
HB D Punjab Heterozygous	16.4	-	-	-	54.4	-	2.2	-	0	0	31.8	-	0	0	-	-
HB E Trait	0.8	-	4.3	-	70.9	-	23.5	-	0	0	0	0	0	0	-	-
Δβ Thalassemia	12.6	-	6.7	-	79.1	-	1.8	-	0	0	0	0	0	0	-	--

*p-value<0.05 is significant

Table no. 5 shows Mean values according to haemoglobin profile in different thalassemic disorders and hemoglobinopathies. Mean Hb F values range from 0.8% (Hb E trait) to 84.64% (thalassemia major). Hb A levels ranges from 9.85% (HbS homozygous) to 86.588% (thal trait). HbA2 levels were in range 1.8% (δβ thalassemia) to 23.5% (Hb E trait). Values of HbS range from minimum of 0.185% (thal. Trait) to maximum of 70.7% (Hb S homozygous). Hb D was found only in patients with Hb D Punjab, with mean value of 31.8%. No patients were observed with Hb C.

ANOVA statistical analysis was done to evaluate statistical level of significance and it was found to be statistically significant (p-value<0.05) between each hemoglobinopathy and various parameters.

Table 6: Correlation of splenomegaly and age with Hb F% in thalassemia major.

Variables	Splenomegaly	Age(years)
Hb F%		
Correlation coefficient	0.352	0.410
P value	0.439*	0.361#

* Point biserial correlation coefficient

Pearson correlation coefficient

Table 6: shows non-significant (p value: 0.439) moderately positive correlation (correlation coefficient: 0.352) between Hb F and splenomegaly in thalassemia major.

Table 7: Correlation of splenomegaly and age with Hb F% in thalassemia intermedia.

Variables	Splenomegaly	Age (years)
HB F%		
Correlation coefficient	-0.261	0.551
P value	0.672*	0.335#

Table 7: shows non-significant (p value: 0.672) negative correlation (correlation coefficient: -0.261) between Hb F and splenomegaly in thalassemia intermedia.

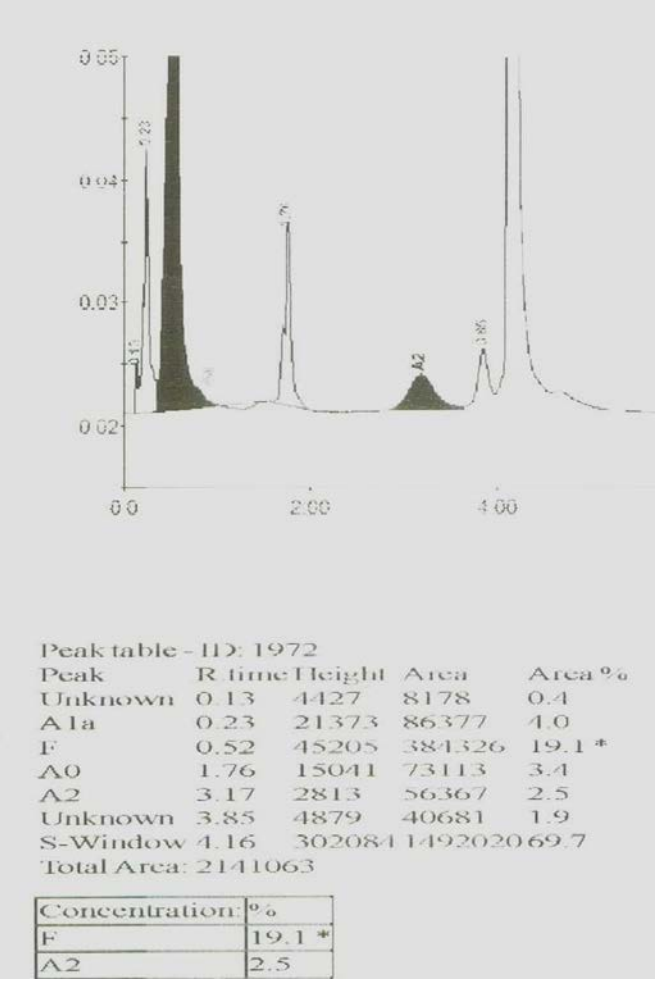

Figure 1: Sickle Cell Homozygous

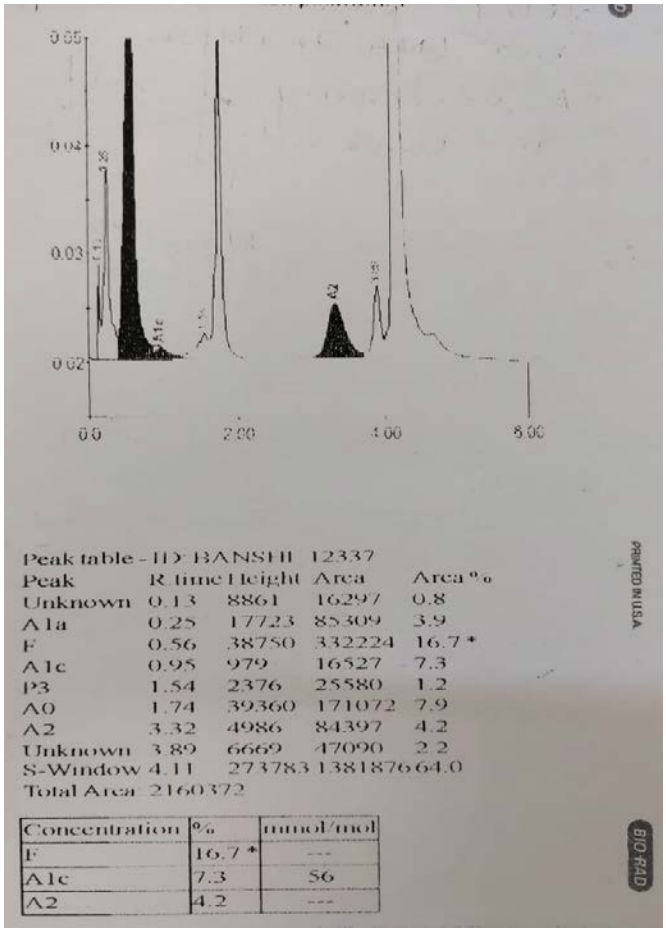

Figure 2: Hbs & B Thalassemia Heterozygous

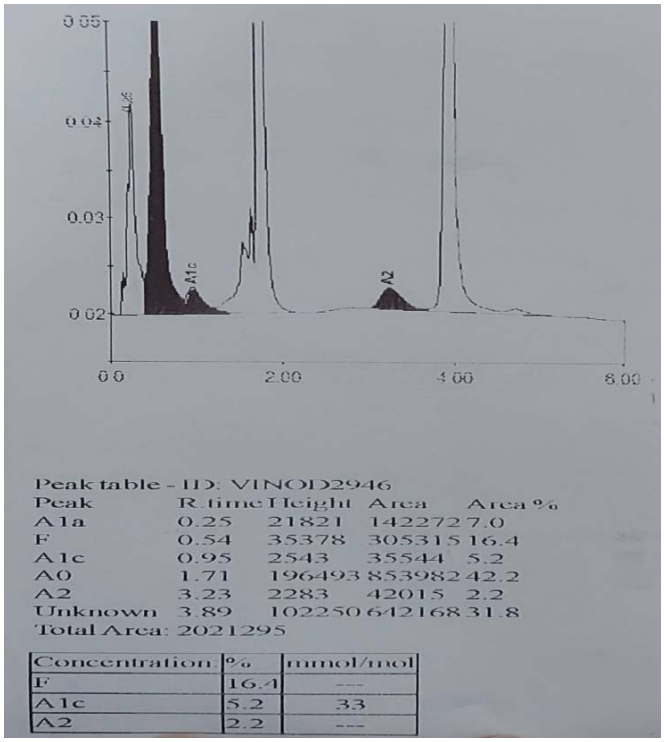

Figure 3: Hb D Punjab Heterozygous

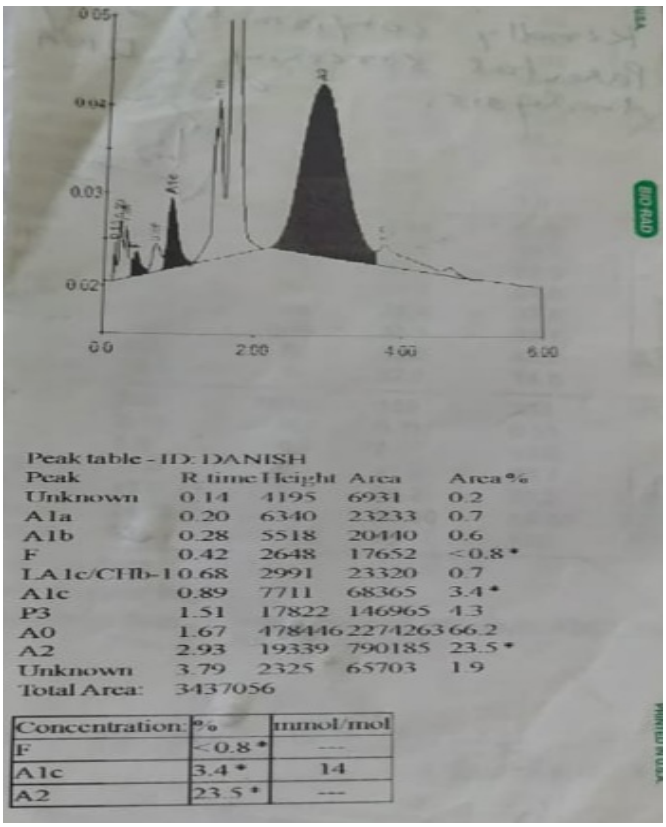

Figure 4: Hb E Heterozygous

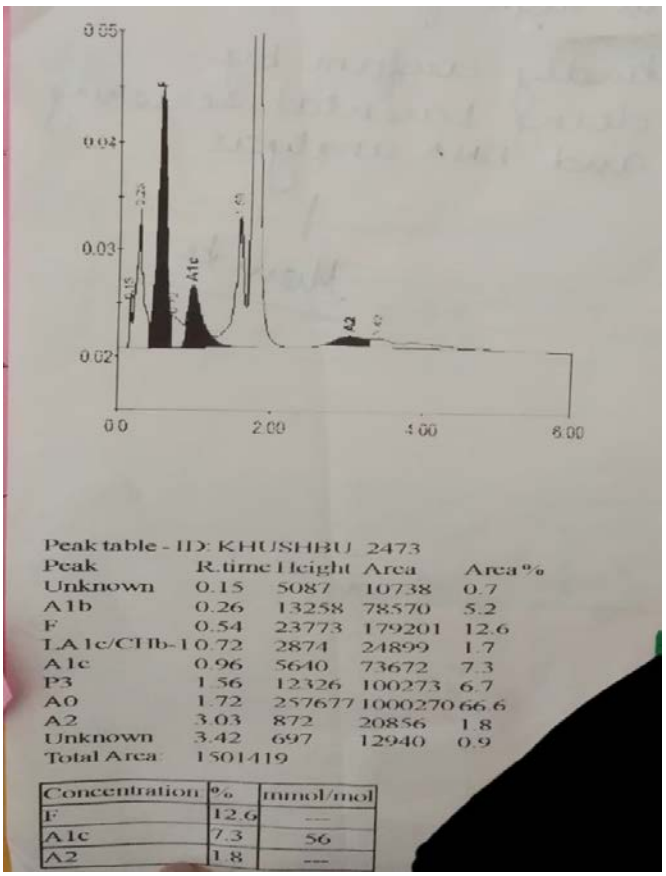

Figure 5: Δβ Thal Trai

Discussion

The present study was conducted to evaluate the clinico-hematological profile of thalassemia syndrome patients. The study also 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.

In present study, we observed that Mean age of patients was observed to be 19.2yrs in thalassemia trait. Sickle cell homozygous has mean age of 16.5yrs. Patients in Hb E trait were around 18yrs of age and patients in δβ THALASSEMIA group were 2yrs of age. Mean age of patients was observed to be minimum in patients with Hb D Punjab heterozygous (0.3yrs), followed by thalassemia major (0.99±0.07yrs), 1.7±2.17yrs in HbS heterozygous, 5.14±4.81yrs in thalassemia intermedia. Mean age in patients of HbS & β thalassemia heterozygous was found 29.5yrs.Thalassemia trait patients had mean age of 4.75±2.97yrs and thalassemia major has mean age of 0.38±0.05yrs and thalassemia intermedia has mean age of 7yrs at onset of disease. We observed non-significant (p value: 0.361) moderately positive correlation (correlation coefficient: 0.410) between Hb F and age (years) in thalassemia major, non-significant (p value: 0.335) moderately positive correlation (correlation coefficient: 0.551) between Hb F and age (years) in thalassemia intermedia, significant (p value: 0.0004) positive correlation (correlation coefficient: 0.631) between Hb A2 and age (years) in thalassemia trait, perfect positive correlation (correlation coefficient: 1) between age and Hb S in Hb S & β thalassemia heterozygous, HbS homozygous and HbS heterozygous (due to small sample size).

In our study we observed that Mean Hb F values range from 0.8 to 84.64%. Hb F was highest in thalassemia major and lowest in Hb E trait. **Trent RJ**[91] stated that a

slightly raised Hb F to 2–3% (normal is <1% in an adult) might indicate heterocellular HPFH or may be a subtle pointer to an underlying silent β thalassaemia. Hb F levels 5% and above is more likely to be due to δβ thalassaemia or HPFH (heterocellular or pancellular). In the case of δβ thalassaemia or deletional HPFH one would expect the HbA_2 level to be low. Hb A levels range from 9.85 to 86.588%. Hb A levels were maximum in patients with thalassemia trait and minimum in patients with sickle cell homozygous. A raised HbA_2 is the key parameter indicating the presence of β thalassaemia, as advocated by **Trent RJ[91].** HbA2 levels were in range 1.8 to 23.5%. Levels were minimum in patients with δβ thalassemia and maximum in Hb E trait. Similar to our study, **Rangan *et al*.[93]** used the term borderline with HbA2 levels of 3.0-4.0% and found mutations in 32% people with HbA2 3.4-3.9%. Similar findings were described by **Colah RB *et al*.** HbS range from minimum of 0.185% (thalassemia trait) to maximum of 70.7% (HbS homozygous). Hb D was found only in patients with Hb D Punjab with mean value of 31.8%.

Conclusion

Our study concluded that RBC indices, HPLC finding, and family study are sufficient to detect and manage most of the hemoglobin variants prevalent in this country. However, one has to be aware of the limitations and problems associated with the diagnostic methods to avoid false negative diagnosis in day to day practice. The present study was conducted using HPLC reflects the magnitude of thalassemia and hemoglobinopathies in a small hospital based population which may be in fact the tip of an iceberg, but this type of study can definitely help to increase awareness among both health care givers and general population.

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