Asian Hematology Research Journal

Volume 7, Issue 1, Page 27-40, 2024; Article no.AHRJ.114951

Usefulness of High Performance Liquid Chromatography and Red Cell Indices as a Screening Tool for Diagnosing Haemoglobinopathies: A Retrospective Observational Study from North India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/114951

Original Research Article

Received: 25/01/2024
Accepted: 30/03/2024
Published: 02/04/2024

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ABSTRACT

Introduction: The World Health Organization estimates that seven percent of the global population is a carrier for disorders of haemoglobin with Thalassemia and haemoglobinopathies being the commonest genetic disorders of haemoglobin. As an initial screening method, Red Blood Cell indices like total count of Red Blood Cell (RBC), Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH), Mean Corpuscular haemoglobin concentration (MCHC) and Red Cell Distribution Width (RDW) can be utilized. High Performance Liquid Chromatography (HPLC) has surfaced as a powerful, excellent screening tool for direct identification of different haemoglobin variants.

Aim: This study aims at using HPLC to identify different haemoglobinopathies and find out the distribution of different red blood cell (RBC) indices in different haemoglobinopathies to determine their significance as screening test.

Materials and Methods: A retrospective study was carried out on 73,333 cases from January 2017 to October 2022 (5 years). EDTA samples were run on automated haematology analyser for red blood cell indices before doing HPLC and then analysed on the Bio-Rad Variant II CE-HPLC system with use of the Variant II-Thalassemia short program (Bio-Rad Laboratories) for determination of different fractions of haemoglobin level.

Results: 6242 (8.52%) cases of abnormal haemoglobin variants were recorded. The most prevalent haemoglobin (Hb) fraction was Beta Thalassemia trait (5.59%) followed by HbD Punjab Heterozygous, HbE Heterozygous and Sickle cell heterozygous. RBC indices were statistically significant between subjects with normal haemoglobin study and those with haemoglobipathies ($p<0.0001$). RBC indices were also statistically significant between beta Thalassemia trait and subjects with normal haemoglobin study ($p<0.0001$). Mean value of RDW is markedly increased in both Thalassemia syndrome (36.06±6.79) and Hb-E Beta Thalassemia (32.49±6.57). Mean total RBC count was highest in Beta Thalassemia trait (5.07±0.90) and lowest in Beta Thalassemia syndrome (2.71±1.10).

Conclusion: RBC indices can be utilized to screen Thalassemia and other haemoglobinopathies and used as a supportive test to High Performance Liquid Chromatography (HPLC)

Keywords: Haemoglobin (Hb); high performance liquid chromatography (HPLC); electrophoresis; red cell indices; haemoglobinopathies; thalassemia.

1. INTRODUCTION

Thalassemia and haemoglobinopathies are commonest genetic disorders of haemoglobin [1]. Thalassemia are characterized by a reduced rate of production of normal haemoglobin due to absent or decreased synthesis of one or more type of polypeptide chain. Haemoglobinopathies are characterized by the production of structurally defective haemoglobin due to abnormalities in the formation of globin moiety of the molecule [2].These hereditary disorders are major public health problem in many parts of the world including India. The clinical spectrum of the disorders varies from asymptomatic conditions to serious disorders like Thalassemia major that requires regular blood transfusions and extensive medical care. World Health Organization (WHO) figures estimates that 7% of world population is carrier for haemoglobin disorders [3,4].

RBC indices like total count of RBC, MCV, MCH and RDW can be utilized as an initial screening method to screen Thalassemia and haemoglobinopathies. High Performance Liquid Chromatography (HPLC) of haemoglobin has surfaced as a powerful, excellent screening tool for direct identification of different haemoglobin variants with a high degree of precision in the quantification of major and minor, normal and abnormal, haemoglobin fractions [5,6].

Aim: The aim of the study was to identify different hemoglobinopathies using Bio-Rad Variant II CE-HPLC system with use of the variant II-Thalassemia short programme (Bio-Rad Lab) and to find out distribution of different red blood cell (RBC) indices in different haemoglobinopathies and to determine their significance as screening test along with HPLC.

2. MATERIALS AND METHODS

A retrospective study was carried out at Regional Reference Laboratory, Metropolis Healthcare, Delhi, India, from January 2017 to October 2022

(5 years). Total number of 73,333 cases was included in the study.

Inclusion criteria: Patients with anaemia, abnormal CBC findings, antenatal check-up and those patients who had a family history of haemoglobinopathy were included in the study.

Exclusion criteria**:** Patients with history of blood transfusion within the last three months were excluded. A detailed clinical history and family history was obtained from each patient.

After obtaining informed consent, 2ml of fresh whole blood was collected in ethylene diamine tetra acetic acid (EDTA) vial, stored at ambient temperature, transported to the lab and analysed within 24 hours of collection. These samples were run on automated haematology analyser (Unicell DXH 800) for cell counts and red blood cell indices before doing HPLC. The samples were then analysed on the Bio-Rad Variant II CE-HPLC system with use of the Variant II-Thalassemia short program (Bio-Rad Laboratories) for determination of different fractions of haemoglobin level. With every batch of samples in BIO-RAD HPLC, two levels of controls (normal: HbF 1–2%, HbA2 1.8–3.2% and abnormal: HbF 5–10%, HbA2 4–6%) and the Hb A2/F calibrator were run. Concentration of Hb (%) was calculated by determining the area of a peak as a fraction of the total area of all Hb peaks seen on the CE-HPLC chromatogram. The retention time was measured in minutes. The RBC morphology was evaluated for correlation by examining the blood films stained by Leishman's stain under a binocular light microscope (Olympus CH20i).

2.1 Statistical Analysis

Data was analysed using “R Studio version 1.4.1103” and results were presented in tabular and graphical form. Descriptive analysis was used to obtain the frequency and percentage of haemoglobinopathies in this given Indian population. Chi Square test was used to determine the association of age group and gender with haemoglobinopathies and normal haemoglobin study. Unpaired T test was used to determine significant differences of various RBC indices including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), haemoglobin (Hb)%, RBC count, and red cell distribution width (RDW-CV) among the haemoglobinopathies and Normal Haemoglobin study.

A two tailed p value of <0.05 was considered to be statistically significant.

3. RESULTS

In the present study, 73333 subjects were screened for hemoglobinopathies. Out of this, maximum patients were between 19-30 yrs (56.96%) followed by 31-45 yrs (32.11%). Out of the total number of patients, 63,593 (86.72%) were females and males were 9,740 (13.28%) (Fig. 1).

Out of the total 73,333 patients screened for haemoglobinopathies, 6242 (8.52%) cases of haemoglobinopathies were recorded (Fig. 2).

It was observed that the most prevalent Hb fraction was Beta Thalassemia trait (5.59%) followed by HbD Punjab Heterozygous (0.7%), HbE Heterozygous (0.49%) and Sickle cell heterozygous (0.32%). Other less common but clinically significant haemoglobinopathies were detected as well. (Details in Table 1) 226 (0.30%) cases were undiagnosed which either had marginally raised HbF or HbA2.

Beta Thalassemia trait was the commonest haemoglobinopathy seen across both genders. Among females, this was followed by Hb D Punjab heterozygous, HbE heterozygous and sickle cell heterozygous. Among males, Beta Thalassemia trait was followed by sickle cell homozygous, HbE heterozygous and sickle cell heterozygous (Table 2).

Beta Thalassemia trait was seen to be the commonest Haemoglobinopathy amongst all age groups. The percentage of other variants was somewhat varied across the age groups with Beta Thalassemia syndrome showing a higher percentage in the 1-12 yrs age group whereas HbD Punjab showed a higher percentage in the 19-30 yrs and 31-45 yrs age group (Table 3).

Out of the total 63,593 females screened, only 6.93% females were diagnosed with Haemoglobinopathies and out of 9740 males, around 18.93% were diagnosed with haemoglobinopathies (Table 4).

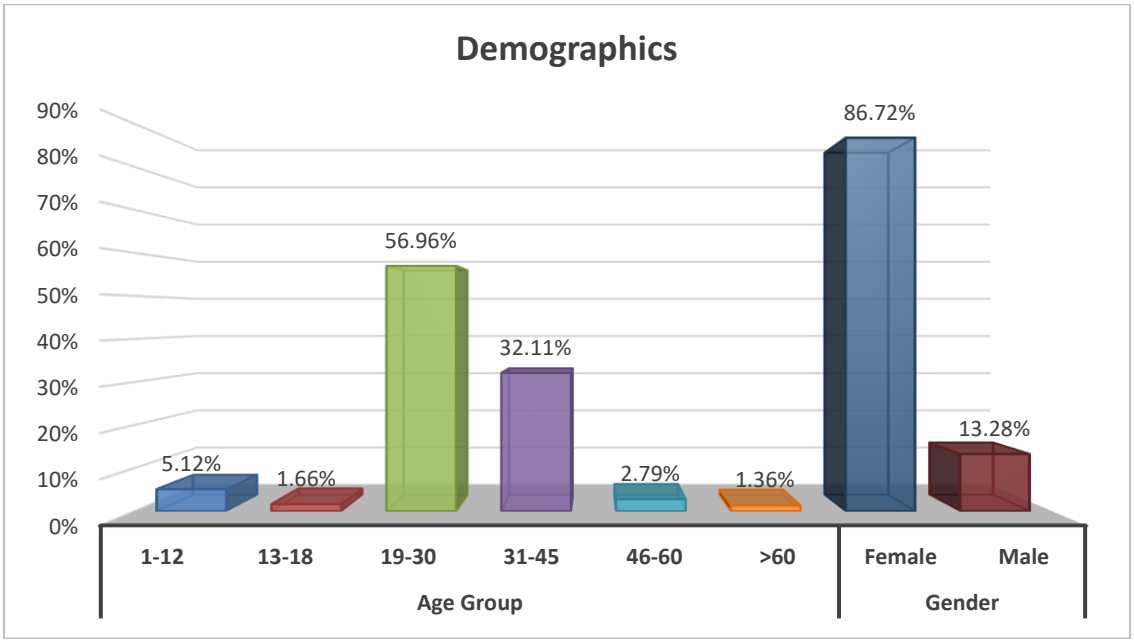


Fig. 1. Overall Demographic distribution

Table 1. Distribution of abnormal haemoglobin variants

Sr. No	Haemoglobinopathies	Frequency	Percentage
1	Beta Thalassemia Trait	4103	5.595%
2	HbD Punjab Heterozygous	553	0.754%
3	HbE Heterozygous	361	0.492%
4	Sickle cell disease Heterozygous	238	0.325%
5	Sickle cell disease Homozygous	162	0.221%
6	HbE Homozygous	144	0.196%
7	Beta Thalassemia Syndrome	138	0.188%
8	HbQ India Heterozygous	87	0.119%
9	Double Heterozygous for HbS and Beta Thalassemia	52	0.071%
10	Double Heterozygous for HbE and Beta Thalassemia	42	0.057%
11	HbJ meerut	40	0.055%
12	HbD Iran Heterozygous	38	0.052%
13	HbD Punjab Homozygous	19	0.026%
14	Double Heterozygous for HbD Punjab and Beta Thalassemia	18	0.025%
15	Hereditary Persistance Fetal Haemoglobin (HPFH)	13	0.018%
16	Double Heterozygous for HbS and HbD	3	0.004%
17	Delta Beta Thalassemia Trait	3	0.004%
18	Hb Lepore	3	0.004%
19	Double Heterozyous for HbD & HbE	2	0.003%
20	Delta Beta Thalassemia Homozygous	1	0.001%
21	Beta Chain Variant	1	0.001%
22	HbH Disease	1	0.001%
23	Undiagnosed	226	0.308%

Undiagnosed are HbA2 (n=98), Raised HbF (N=128 case)

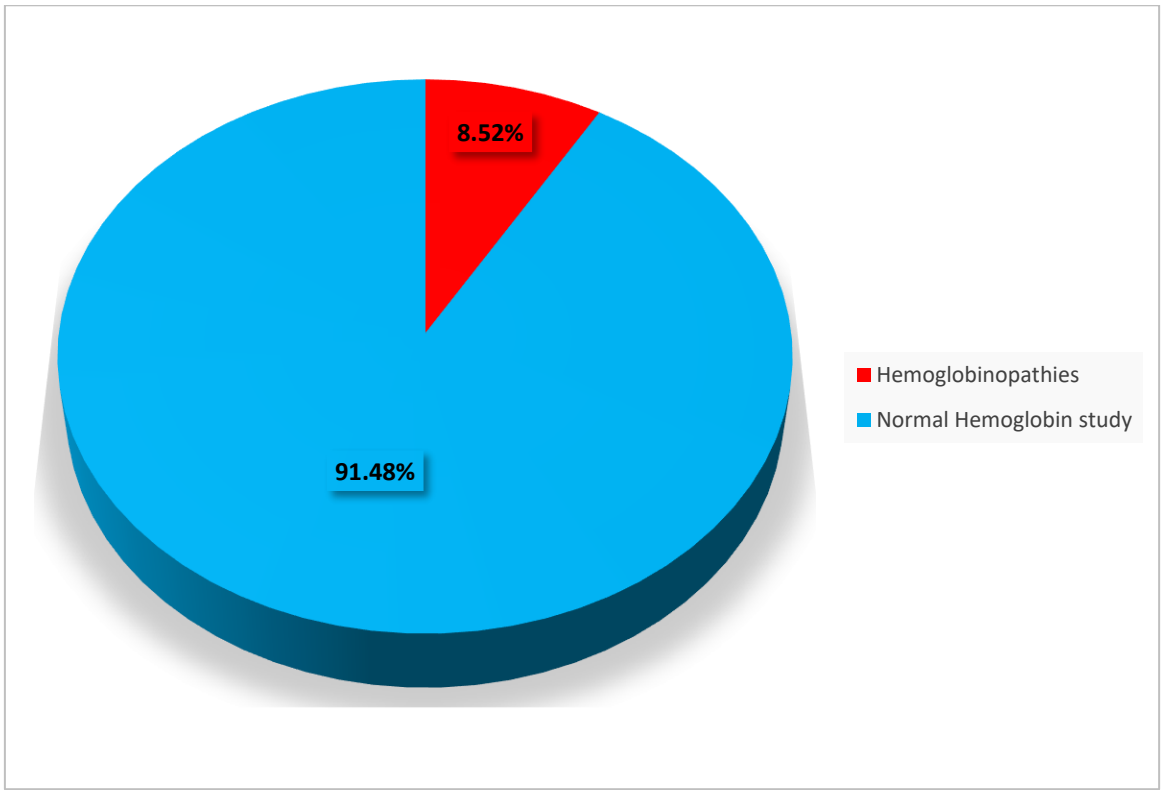


Fig. 2. Prevalence of Haemoglobinopathies

Table 2. Gender wise association of Haemoglobinopathies

Haemoglobinopathy	Female		Male	
	n	%	n	%
Beta Thalassemia Trait	2920	66.30%	1183	64.15%
HbD Punjab Heterozygous	472	10.72%	81	4.39%
HbE Heterozygous	274	6.22%	87	4.72%
Sickle cell disease Heterozygous	155	3.52%	83	4.50%
Sickle cell disease Homozygous	69	1.57%	93	5.04%
HbE Homozygous	79	1.79%	65	3.52%
Beta Thalassemia syndrome	57	1.29%	81	4.39%
Hb Q India Heterozygous	70	1.59%	17	0.92%
Double Heterozygous for HbS and Beta Thalassemia	27	0.61%	25	1.36%
Double Heterozygous for HbE and Beta Thalassemia	18	0.41%	24	1.30%
Hb J meerut	33	0.75%	7	0.38%
Hb D Iran heterozygous	30	0.68%	8	0.43%
HbD Punjab Homozygous	13	0.30%	6	0.33%
Double Heterozygous for HbD Punjab and Beta Thalassemia	10	0.23%	8	0.43%
Hereditary Persistance Fetal Haemoglobin	11	0.25%	2	0.11%
Delta beta Thalassemia trait	1	0.02%	2	0.11%
Double Heterozygous for HbS and HbD	1	0.02%	2	0.11%
Hb Lepore	0	0.00%	3	0.16%
Double Heterozyous for HbD & HbE	2	0.05%	0	0.00%
Beta Chain Variant	1	0.02%	0	0.00%
Delta Beta Thalassemia Homozygous	1	0.02%	0	0.00%
HbH Disease	1	0.02%	0	0.00%
Undiagnosed	159	3.61%	67	3.63%

Table 3. Age wise distribution of Haemoglobinopathies

Haemoglobinopathies	1-12		13-18		19-30		31-45		46-60		>60	
	n	%	n	%	n	%	n	%	n	%	n	%
Beta Thalassemia Trait	414	50.67%	146	57.25%	1951	68.58%	1087	65.56%	310	73.29%	195	78.00%
HbD Punjab Heterozygous	36	4.41%	11	4.31%	314	11.04%	168	10.13%	16	3.78%	8	3.20%
HbE Heterozygous	38	4.65%	14	5.49%	153	5.38%	116	7.00%	28	6.62%	12	4.80%
Sickle Cell Disease Heterozygous	35	4.28%	12	4.71%	106	3.73%	65	3.92%	13	3.07%	7	2.80%
Sickle Cell Disease Homozygous	46	5.63%	31	12.16%	55	1.93%	25	1.51%	2	0.47%	3	1.20%
HbE Homozygous	9	1.10%	7	2.75%	43	1.51%	51	3.08%	28	6.62%	6	2.40%
Beta Thalassemia Syndrome	124	15.18%	6	2.35%	5	0.18%	1	0.06%	1	0.24%	1	0.40%
HbQ India Heterozygous	4	0.49%	0	0.00%	44	1.55%	36	2.17%	0	0.00%	3	1.20%
Double Heterozygous for HbS and Beta Thalassemia	20	2.45%	6	2.35%	19	0.67%	7	0.42%	0	0.00%	0	0.00%
Double Heterozygous for HbE and Beta Thalassemia	22	2.69%	4	1.57%	8	0.28%	7	0.42%	1	0.24%	0	0.00%
HbJ meerut	3	0.37%	0	0.00%	21	0.74%	15	0.90%	1	0.24%	0	0.00%
HbD Iran Heterozygous	2	0.24%	0	0.00%	16	0.56%	16	0.97%	4	0.95%	0	0.00%
HbD Punjab Homozygous	0	0.00%	2	0.78%	5	0.18%	7	0.42%	3	0.71%	2	0.80%
Double Heterozygous for HbD Punjab and Beta Thalassemia	5	0.61%	3	1.18%	4	0.14%	4	0.24%	1	0.24%	1	0.40%
HPFH	0	0.00%	0	0.00%	6	0.21%	6	0.36%	1	0.24%	0	0.00%
Delta Beta Thalassemia Trait	2	0.24%	1	0.39%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Double Heterozygous for HbS and HbD	0	0.00%	0	0.00%	3	0.11%	0	0.00%	0	0.00%	0	0.00%
Hb Lepore	0	0.00%	0	0.00%	2	0.07%	0	0.00%	0	0.00%	1	0.40%
Double Heterozygous for HbD & HbE	0	0.00%	0	0.00%	0	0.00%	1	0.06%	1	0.24%	0	0.00%
Beta Chain Variant	0	0.00%	0	0.00%	0	0.00%	1	0.06%	0	0.00%	0	0.00%
Delta Beta Thalassemia Homozygous	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	1	0.40%
HbH Disease	1	0.12%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Undiagnosed	56	6.85%	12	4.71%	90	3.16%	45	2.71%	13	3.07%	10	4.00%

Table 4. Demographic association of Haemoglobinopathies

Demographic	Result				
	Haemoglobinopathies		Normal Haemoglobin study		p value
	Frequency	Percentage	Frequency	Percentage	
Age Group					
1-12	817	21.78%	2934	78.22%	<0.0001
13-18	255	20.90%	965	79.10%	
19-30	2845	6.81%	38927	93.19%	
31-45	1658	7.04%	21886	92.96%	
46-60	423	20.64%	1626	79.36%	
>60	250	25.08%	747	74.92%	
Gender					
Female	4404	6.93%	59189	93.07%	<0.0001
Male	1844	18.93%	7896	81.07%	

Table 5. Association of Haemoglobinopathies with Red cell indices

	Haemoglobinopathies	Normal Haemoglobin study	p value
MCHC	30.73±1.94	31.79±2.06	<0.0001
MCH	21.62±4.23	27.07±4.33	<0.0001
MCV	70.12±11.62	84.80±10.85	<0.0001
RDW	18.79±4.77	16.86±3.73	<0.0001
Haemoglobin(Hb)	10.06±2.18	11.24±2.21	<0.0001
RBC	4.75±1.06	4.17±063	<0.0001

Table 6. Association of Beta Thalassemia trait with Red cell indices

	Beta Thalassemia Trait	Normal Haemoglobin study	p value
MCHC	30.41±1.52	31.79±2.06	<0.0001
MCH	19.94±2.46	27.07±4.33	<0.0001
MCV	65.54±7.35	84.80±10.85	<0.0001
RDW	18.31±3.05	16.86±3.73	<0.0001
Haemoglobin(Hb)	10.02±1.69	11.24±2.21	<0.0001
RBC	5.07±0.90	4.17±063	<0.0001

Table 7. Overall distribution of Red cell indices among various Haemoglobinopathies

Haemoglobinopathy	MCHC	MCH	MCV	RDW	Hb	RBC
No Evidence Of Haemoglobinopathy	31.79±2.06	27.07±4.32	84.81±10.85	16.86±3.72	11.24±2.20	4.17±0.63
Beta Thalassemia Trait	30.41±1.52	19.94±2.46	65.54±7.35	18.31±3.05	10.02±1.69	5.07±0.90
HbD Punjab Heterozygous	32.15±2.03	26.17±4.41	81.04±10.96	16.93±3.62	11.45±2.31	4.39±0.65
HbE Heterozygous	31.39±1.72	24.57±3.08	78.21±8.55	17.16±3.47	10.86±2.06	4.43±0.72
Sickle Cell Disease Heterozygous	31.44±1.80	25.20±4.20	79.92±11.24	17.39±4.4	11.11±2.45	4.44±0.87
Sickle Cell Disease Homozygous	31.27±2.01	26.55±4.49	84.76±12.26	20.40±3.85	8.91±2.01	3.43±0.89
HbE Homozygous	30.98±1.36	20.05±1.77	64.71±5.05	18.81±3.12	9.80±2.05	4.89±1.01
Beta Thalassemia syndrome	29.95±4.80	21.46±4.48	71.65±8.91	36.06±6.79	5.59±2.12	2.71±1.10
HbQ India Heterozygous	32.31±1.66	27.24±3.27	84.12±8.54	15.74±3.03	12.23±1.93	4.50±0.51
Double Heterozygous for HbS and Beta Thalassemia	30.42±1.38	23.05±3.79	75.64±11.30	21.99±4.06	8.51±2.04	3.76±0.91
Double Heterozygous for HbE and Beta Thalassemia	28.51±2.27	19.31±3.29	67.61±9.23	32.49±6.57	6.42±1.99	3.35±0.93
HbJ meerut	30.66±3.08	26.26±5.61	84.49±13.57	17.99±4.85	11.27±2.56	4.34±0.71
HbD Iran Heterozygous	32.15±2.22	26.37±3.95	81.73±8.81	16.17±2.60	11.42±2.24	4.31±0.46
HbD Punjab Homozygous	32.10±1.14	20.62±2.64	64.36±8.87	18.66±6.03	9.87±2.11	4.83±1.11
Double Heterozygous for HbD Punjab and Beta Thalassemia	31.08±1.43	20.33±2.67	65.6±9.91	22.12±6.53	8.87±2.38	4.48±1.44
HPFH	31.45±1.06	24.93±2.02	79.21±5.59	18.97±3.28	11.38±1.77	4.58±0.67
Double Heterozygous for HbS and HbD	32.03±0.86	28.1±5.60	87.67±16.35	27.73±16.7	8.83±4.84	3.45±2.43
Delta Beta Thalassemia Trait	31.57±0.81	26.2±4.71	83.17±15.98	23.7±4.56	8.3±0.53	3.23±0.63
Hb Lepore	30.7±0.89	23.47±4.28	76.13±11.51	18.2±3.30	10.97±3.29	4.94±2.07
Double Heterozygous for HbD & HbE	32.1±1.13	25.55±2.19	79.55±4.03	15.7±2.26	12.2±0.28	4.78±0.31
Undiagnosed	30.78±3.13	26.12±8.03	84.24±22.05	22.46±7.12	8.48±3.11	3.49±1.35

The RBC indices were analyzed against diagnosis, results were expressed as mean±SD (Table 5,6,7). Mean value of haemoglobin was lowest in patients with Beta Thalassemia syndrome (5.59±2.12) followed by double heterozygous for HbE and Beta Thalassemia (6.42±1.99). Mean total RBC count was highest in Beta Thalassemia trait (5.07±0.90) and lowest in Beta Thalassemia syndrome (2.71±1.10) 7). Mean value of RDW is markedly increased in both Thalassemia syndrome (36.06±6.79) and Hb-E Beta Thalassemia (32.49±6.57). Mean value of MCV is lowest is for HbE homozygous and HbD Punjab homozygous.

RBC indices showed a statistically significant difference between subjects with normal haemoglobin study and those with haemoglobipathies (Table 5).

RBC indices were statically significant between beta Thalassemia trait and subjects with normal haemoglobin study. (Table 6)

4. DISCUSSION

Thalassemia and other hemoglobinopathies are one of the major public health problems in countries such as India. The estimated number of persons with haemoglobinopathies is around 25 million in country such as India [7]. To screen Thalassemia and other haemoglobinopathies, HPLC has been proven to be sensitive, specific and reliable method in rapid detection and characterization of haemoglobin fraction with automated and analytical capacity [8,9,10]. HPLC is preferred due to its resolution, reproducibility and quantification, which can guide the further line of treatment. However, due to the lack of affordability, red blood cell indices may be used as the first level of screening in certain clinical set ups [5, 6]. Tyagi et al and R B Colah et al had further investigated the usefulness of HPLC in the Indian scenario with respect to population diversity and cost barriers [11, 12]. Tyagi et al in his study, has questioned the need of HPLC for screening of Thalassemia and hemoglobinopathies in the Indian laboratory setting, due to the expense incurred [11]. Recent studies done in the Indian population have established and recommended the usefulness and need of HPLC for accurately predicting hemoglobinopathies and its variants among Indians [13]. In the current study, the samples were screened for red blood cell indices post which HPLC technique was used. In our study, out of the total 73333 cases screened, 86.72% were females whereas males were only 13.28%. Out of this maximum were between 19-30 yrs (56.96%), the proportion of pediatric patients was minimal (5.12%). The prevalence of Thalassemia and other haemoglobinopathies was observed to be 8.52%. Singh et al studied 100 patients in a tertiary hospital in North India and observed that 51 patients had abnormal hemoglobin variants, with 42% having BetaThalassemia trait [13].The most common Hb abnormality detected in our study was also that of β Thalassemia trait (5.59%) [Fig. 3]. However, this percentage was lower as compared to the study by Singh et al, maybe due to the significant difference in data set studied (current study n=73333).

4.1 Retention Times and Proportions of Haemoglobin Variants

A cut off over 4% of HbA2 on HPLC was considered for diagnosis of Beta Thalassemia trait [14,15,16]

Several studies reveal that in most parts of India, β Thalassemia trait was the commonest Hb disorder. A study conducted by Madan N and coinvestigators showed the overall gene frequency of β Thalassemia trait reported in northern and western India was 4.60% [17], which was somewhat in accordance with our study as well.

In addition to the most commonly encountered Beta Thalassemia trait, Hb D Punjab heterozygous and HbE heterozygous are other common mutations that showed prevalence in our current study on the North Indian population, with prevalence of 0.7 % and 0.49 % respectively. Narang et al studied females in the reproductive age group (20years to 40 years) and found the prevalence of HbD Punjab heterozygous variant to be close to 17.8% [18]. Even in the current study, out of the total HbD cases seen, 472 cases were seen in females. Although rare, there have been some previous case reports on the co-existence of HbD heterozygous variants with Beta Thalassemia traits as well [19,20]. The prevalence and practice of consanguineous marriage may be a major contributor for this. These patients are at an increased risk of chronic anemia, and severe B12 deficiencies among other consequences as well. G S Chopra et al found the prevalence of Hb E disease to be 0.6%. They further found that Hb E disease is common in the Eastern – Coastal region and North-East India (West Bengal, Assam, Manipur and Nagaland) where

abnormal Hb E and variants are highly prevalent [21].

On HPLC, the separation between HbF, HbA2, HbA, S window and D window was clear. In Hb E Heterozygous, Hb E tends to elute in A2 window (Fig. 4) with retention time ranging from 3.3 to 3.9 minutes and in HbD Punjab heterozygous (Fig. 5), Hb D elutes in the D window with area percentage between 30-40% [14].

HPLC also separately identified two Hb variants of HbD family; HbD-Iran and HbD-Punjab. Where HbD- Iran elutes in A2 window whereas HbD Punjab elutes in D window. The HPLC findings were also significantly different between patients with heterozygous Hb D-Iran and Hb E; and those with heterozygous Hb D-Iran showed a higher peak area (40.3 vs. 26.7%) and a lower retention time (3.56 vs. 3.67 min)(Fig. 6)

In the present study, 226 cases were undiagnosed, of which 98 cases had borderline HbA2 (3.6%-3.9%). Rangan et al. [22] followed the term borderline for HbA2 levels between 3.0 and 4.0%. Colah et al. [23] made comparable observations. In a study by Khera et al HbA2 levels were found to be significantly lower in the iron deficient group as compared to iron replete group [6]. Colaco et al reported that 35.4% of individuals having borderline HbA2 carry a molecular defect, which warrants the necessity to investigate these cases at molecular level, particularly if partner is a carrier of beta – Thalassemia [24]. In a review by Thilakarathne et al the authors analysed multitude of factors affecting Hb A2 levels and thus diagnosis of beta thalassemia. They identified megaloblastic anemia, hyperthyroidism, antiretroviral therapy may lead to falsely increased HbA2 levels whereas Beta thalassemia when co-inherited with alpha thalassemia, iron deficiency anaemia, HPFH lead to falsely decreased HbA2 levels [25]. Careful interpretation and revaluation along with proper clinical history is warranted in such cases. In such cases, revaluation after treatment with hematinic is advised.

The cut-off for HbF was upto 2%. There were 128 cases with marginally high HbF (2-3%). All the cases were pregnant females with mildly raised HbF with normal RBC indices and no family history. Several studies have shown that pregnancy is the only known non-pathological condition in which HbF level can transiently increase in adults, either by feto-maternal transfusion or by physiologic expression [26].

Among children, majority of the cases of haemoglobinopathies were between the age group of 1-12 years and most common haemoglobinopathies were Beta Thalassemia trait and Beta Thalassemia syndrome respectively. As per the NHM Guidelines on Prevention and Control of Hemoglobinopathies in India, India has the largest number of children with Thalassemia major in the world [27]. One reason for this could be the practice of consanguineous marriage in certain parts of India. The lack of awareness of the need for pre-marital genetic counselling and antenatal counselling for prevention of genetic disorders in the unborn child could be another major cause. All such cases usually present early in life with severe anaemia, splenomegaly, and failure to thrive and are transfusion dependent. Among adults, majority of the cases were between 19-45 age group. Out of the total number of subjects, female were more than males. This can be attributed to the fact that females getting antenatal screening for abnormal haemoglobin. However, overall, only 6.93% of females were diagnosed with haemoglobinopathies and 18.93% of males were diagnosed with haemoglobinopathies as the number of males who were screened were less than females and only those were tested who had anaemia or any family history.

Sadiya S, et al concluded that RBC indices can be utilized for screening of Thalassemia and other haemoglobinopathies and are to be confirmed by HPLC method. However, they confirmed RBC indices of normal subjects with different haemoglobinopathies and they found most of the RBC indices were having a significant difference [28]. In contradiction to their study, Khondaker et al found no diagnostic significant role of RBC indices in Hb-E related haemoglobinopathies [29]. However, the study was limited to small sample size and only compared HbE trait with normal subjects. A study by Singh et al found the role of RBC count, MCV and MCH as a strong predictor of Beta Thalassemia trait. They evaluated a compendium of various discriminatory indices and red cell parameters to identify Beta Thalassemia trait [30].

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.00	1245
F	0.7	---	1.10	9875
Unknown	---	0.8	1.21	11361
P2	---	3.6	1.31	53977
P3	---	4.2	1.71	62815
Ao	---	85.1	2.42	1271332
A2	5.4*	---	3.66	83233

Total Area: 1,493,838

F Concentration = 0.7 %
A2 Concentration = 5.4* %

*Values outside of expected ranges

Analysis comments:

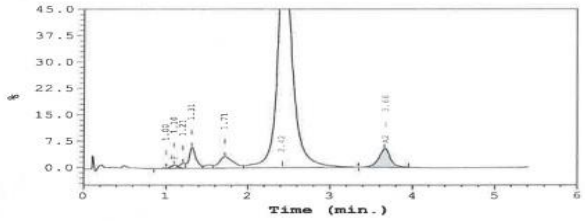


Fig. 3. Chromatogram of beta Thalassemia trait

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	1.5	---	1.09	24151
Unknown	---	1.0	1.19	14585
P2	---	3.1	1.30	47844
P3	---	2.7	1.70	41131
Unknown	---	2.4	1.80	35979
Ao	---	62.8	2.43	956600
A2	26.7*	---	3.67	404123

Total Area: 1,524,415

F Concentration = 1.5 %
A2 Concentration = 26.7* %

*Values outside of expected ranges

Analysis comments:

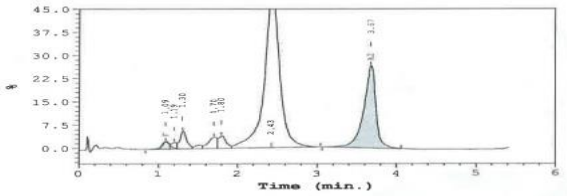


Fig. 4. Chromatogram for HbE Heterozygous

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.99	1469
F	0.2	---	1.07	5973
Unknown	---	1.0	1.19	24507
P2	---	2.0	1.29	49061
P3	---	2.5	1.67	59122
Unknown	---	3.4	2.02	81261
Ao	---	52.1	2.40	1254551
A2	1.4*	---	3.61	33631
D-window	---	37.3	4.09	897509

Total Area: 2,407,085

F Concentration = 0.2 %
A2 Concentration = 1.4* %

*Values outside of expected ranges

Analysis comments:

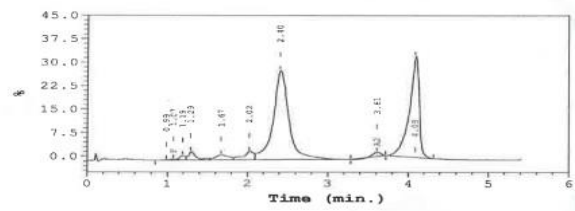
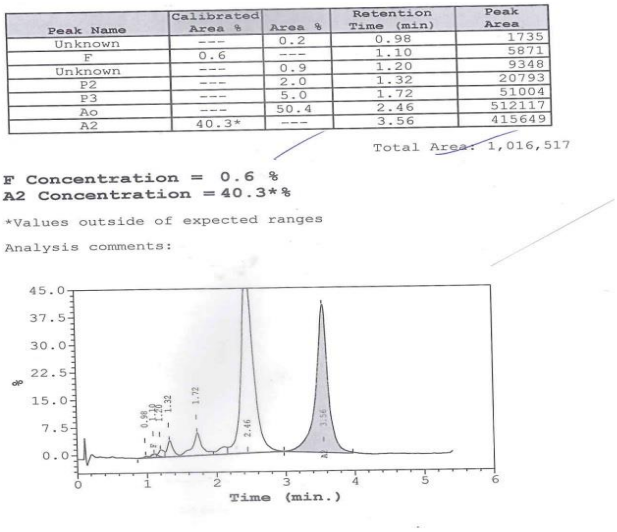


Fig. 5. Chromatogram for Hb D Punjab Heterozygous



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.2	0.98	1735
F	0.6	---	1.10	5871
Unknown	---	0.9	1.20	9348
P2	---	2.0	1.32	20793
P3	---	5.0	1.72	51004
Ao	---	50.4	2.46	512117
A2	40.3*	---	3.56	415649

Fig. 6. Chromatogram for Hb D-Iran

In comparison to the above studies, in the current study the RBC indices were analyzed against diagnosis and compared between normal subjects and those with haemoglobinopathies. In this study, RBC indices showed statistically significant difference between subjects with normal haemoglobin study and those with haemoglobipathies (Table 5, 6). RBC indices when analysed among different haemoglobinopathies also had significant difference. Mean total RBC count was highest in Beta Thalassemia trait and lowest in Beta Thalassemia syndrome (Table 7). This is also clinically apparent as all Beta Thalassemia trait shows compensatory hyperplasia. Mean value of RDW is markedly increased in both Thalassemia syndrome and Hb-E Beta Thalassemia (Table 7). Though anisopoikilocytosis (RDW) was moderate to severe in beta thalassemia syndrome and mild in thalassemia trait, still it appears difficult to differentiate thalassemia major and intermedia based on red cell morphology alone. Therefore, blood findings must be correlated with clinical picture. Henceforth we categorized all such cases as beta Thalassemia syndrome. Mean value of MCV is lowest is for HbE homozygous and HbD Punjab homozygous (Table 7). Corresponding to the fact that these haemoglobinothies have significant microcytosis.

5. CONCLUSION

Nutritional deficiencies is the leading cause of anaemia in India but anaemia caused by abnormal haemoglobin (Thalassemias and Haemoglobinopathies) should also be evaluated. Establishing services for premarital and preconception screening backed by genetic counselling services can assist in taking appropriate actions and raising awareness among the public.

High Performance Liquid Chromatography (HPLC) of haemoglobin is an efficient and reliable technique for easy detection of haemoglobin variants and characterization of normal and abnormal haemoglobin components with high level of precision. Additionally, RBC indices can be utilized to screen Thalassemia and other haemoglobinopathies and used as a supportive test to HPLC.

CONSENT

As per international standards or university standards, patient(s) written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standards or university standards written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/114951