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Research Article

"Study of Pattern of Hemoglobinopathies Using High-Performance Liquid Chromatography in Neonates and Infants"

Dr. Yogita Bhansali[1], Dr. Savitri M Nerune[2*], Dr. Siddu Charki[3]

[1]M.D. Pathology, Department of Pathology, Shri B.M Patil Medical College, Hospital and Research Centre, BLDE (DU), Ashram Road, Vijayapura, Karnataka, India.
*[2]*DCP, DNB Pathology, Department of Pathology Shri B.M Patil Medical College, Hospital and Research Centre, BLDE (DU), Ashram Road, Vijayapura, Karnataka, India.*
[3]Department of Paediatrics, Shri B.M Patil Medical College, Hospital and Research Centre, BLDE (DU), Ashram Road, Vijayapura, Karnataka, India.

Abstract

Introduction: Hemoglobinopathies are a significant public health concern in many countries, including India. Hemoglobinopathies contribute significantly to high levels of morbidity and mortality by causing moderate to severe hemolytic anemia. So, it is critical to correctly identify neonates and infants carrying these abnormalities and avoid developing hemoglobinopathies in future pregnancies. Hence, screening and accurately identifying hemoglobin variants have become increasingly crucial in antenatal diagnosis and preventing Hb disorders.

Materials and methods: 119 cases were screened for hemoglobinopathies on the Bio-Rad D10 DM high-performance liquid chromatography (HPLC) system. The retention times, the proportion of the hemoglobin (%) and the peak characteristics for all hemoglobin fractions were recorded. Neonates and infants with Hb and MCV below the normal range for that age were included, and infants with a history of blood transfusion in the last three months were excluded from this study.

Results: Out of 119 subjects, 111(93.27 %) were diagnosed as normal, 4 (3.36%) were diagnosed as Beta-thalassemia homozygous, 3 (2.52 %) were detected as beta-thalassemia heterozygous and 1 (0.8%) was diagnosed as Hemoglobin J on HPLC study.

Conclusion: For the routine evaluation of hemoglobinopathies, HPLC is advantageous due to its superior resolution, rapid assay time, and precise quantification.

**Author for Correspondence: Email: savitri.nerune@bldedu.ac.in*

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INTRODUCTION

A tetramer composed of two pairs of globin chain forms a hemoglobin. Around 800 distinct types of hemoglobin exist. Hemoglobinopathies refer to conditions characterized by abnormalities in these proteins. [1] Hemoglobinopathies can be clinically classified based on nomenclature associated with alterations in the involved globin chain, following the most prevalent and pragmatic approaches.[1]

The two most prevalent non-communicable genetic disorders in India are hemoglobinopathies and thalassemia [2]. The high occurrence of genetic diseases and congenital anomalies in India is due to consanguineous marriages, which are prevalent among 60 to 70% of the population [2]. The incidence of monogenic disorders like alpha and beta thalassemia, sickle cell disease, glucose-6-phosphate dehydrogenase deficiency, albinism, cystic fibrosis, phenylketonuria, and hemophilia A and B is 1 in 81 births [2]. Specifically, for beta-thalassemia and sickle cell disease, the estimated rate of congenital abnormalities and genetic disorders is approximately 1 in 2700, equating to 16,700 births annually [2].

Thalassemia is an autosomal recessive disease. The World Health Organization (WHO) reports that approximately 5% of

the global population are carriers of genetic hemoglobin disorders. Each year, an estimated 100,000 cases of thalassemia major are born worldwide [3].

In India, the prevalence of β-thalassemia in the general population varies from 3.5% to 15% [4]. On average, the country harbors over twenty-five million thalassemia carriers, with β-thalassemia, HbE, and HbD-Punjab being the most common forms. India accounts for approximately 10% of all thalassemia births globally annually, resulting in around 10,000 new cases [5]. Consanguineous marriages are a crucial factor contributing to this elevated prevalence in India [5].

The prevalence rates of β-thalassemia trait and sickle cell anemia in India are approximately 3-17% and 1-44%, respectively. In Karnataka, the prevalence of β-thalassemia carriers is 2.16% [6,7].

According to the National Family Health Survey (NFHS III), iron deficiency anemia (IDA) is the predominant cause of anemia in the pediatric population, with a prevalence rate of approximately 70%, followed by thalassemia. Accurate diagnosis of these two conditions is essential to ensure appropriate therapeutic interventions for the affected child [8].

Due to the heterogeneous demographic makeup of Karnataka, which includes a wide range of ethnic groups, multiple genetic illnesses, including hemoglobinopathies, are present at the same time [9, 10]. Past population migrations and unions within endogamous populations have established the unique genetic profile of this region. The frequency and variety of hemoglobinopathies that have been reported reflect genetic variability [9, 10].

Sickle Cell Disease (SCD) is prevalent in Karnataka, especially among tribal populations and specific castes [9,10]. It is characterized by a mutation in the beta-globin gene, resulting in the synthesis of abnormal hemoglobin S (HbS) [9,10]. Research indicates considerable variability in the prevalence of the sickle cell trait among various regions and communities in the state [9,10].

Both alpha and beta thalassemia are prevalent in Karnataka [9,10]. Beta thalassemia major, characterized by severe anemia and the necessity for frequent blood transfusions, presents a substantial health challenge [9,10]. The prevalence of thalassemia carriers is particularly noteworthy in certain districts, frequently associated with communities that have high rates of consanguineous marriages [9,10].

In addition to sickle cell disease (SCD) and thalassemia, hemoglobin variants like HbD and HbE have also been documented in Karnataka [9,10]. The prevalence of these variants is commonly linked to particular ethnic populations and geographic regions [9,10].

While numerous studies have reported on the prevalence of hemoglobinopathies in India, there is a need for more data specific to Karnataka. It is imperative to gather comprehensive, region-specific data to customize public health interventions effectively and cater to the distinctive requirements of the population [9,10].

The predominant approach most newborn screening programs adopt for the initial diagnosis of clinically significant hemoglobinopathies is High-Performance Liquid Chromatography (HPLC) [11].

This approach is preferred over alternative electrophoretic methods, such as citrate agar electrophoresis and cellulose acetate electrophoresis, among others [11], due to its higher sensitivity, lower labor intensity, and better suitability for screening large populations. Additionally, Hb variant measurement is achievable with HPLC [11]. Although the sensitivity of HPLC is excellent, results and interpretations may be affected by extreme prematurity or a recent history of blood transfusion [11].

The prompt and precise identification of hemoglobinopathies using HPLC screening in Karnataka has the potential to profoundly influence both clinical outcomes and public health [11]. Timely detection enables prompt interventions, including preventive therapies, regular supervision, and genetic guidance, all of which play a crucial role in managing hemoglobinopathies and enhancing the well-being of affected individuals [11]. Hemoglobinopathies present a substantial healthcare burden for impacted families and the healthcare system [11]. Early detection and treatment are essential for diminishing the morbidity and mortality linked to these conditions. This research endeavors to pinpoint trends that can guide public health strategies and healthcare regulations [11].

Aim and objective of the study

To investigate the diverse patterns of hemoglobinopathies in neonates and infants by applying high-performance liquid chromatography.

Materials and Methods

Study design: A cross-sectional study

Study setting: The study was conducted at the Central Laboratory of the Department of Pathology and the Department of Pediatrics, BLDE (Deemed to be University), Shri B.M. Patil Medical College, Hospital, and Research Centre in Vijayapura.

Study population: The participants of this study were neonates and infants who were admitted to the Department of Pediatrics at BLDE (Deemed to be University), Shri B. M. Patil Medical College, Hospital, and Research Centre in Vijayapura. The study period is scheduled from the 1st of September 2022 to the 30th of April 2024.

Sample size calculation was conducted using G*Power ver. 3.1.9.4 software. Anticipated Mean ± SD of the Hb S trait in neonates was 77.63 ± 2.30. The study indicated a required sample size of 119 with a 98% confidence level and a precision of 0.5.

Inclusion criteria:

Neonates and infants exhibiting hemoglobin and mean corpuscular volume (MCV) levels below the age-appropriate normal range.

Neonates with other inherited conditions such as G6PD deficiency, Galactosemia, Cystic fibrosis, and Phenylketonuria (PKU).

Exclusion criteria:

Infants who have received a blood transfusion in the past three months may produce negative results.

Sampling method: Random sampling method.

20 µL of blood was obtained from each neonate using the heel-prick method, while 2 mL of blood was collected from the infant in a vacutainer tube containing K2EDTA anticoagulant. High-performance liquid chromatography (HPLC) analysis was conducted using the Bio-Rad D10 DM analyser. In cases where the blood sample was insufficient, it was diluted with 1500 µL

of buffer wash until its color matched that of the provided calibrator sample.

Ethical consent: Written informed consent was obtained from the parents of each patient involved in the study.

Analysis plan: The collected data were imported into a Microsoft Excel spreadsheet, and statistical analysis was conducted using social science statistical software (Version 20). The outcomes were presented in the form of frequencies, percentages, and Mean ± Standard Deviation.

The standard report detailing the analysis of an HPLC sample

Kindly investigate the parameters specified in **Figure-1.**

a) The peak denoted as name F should be less than 2%.
b) Peak P2: It varies according to glycemic status, with up to 6% considered acceptable.
c) The peak which is denoted as P3, variation up to 6% is acceptable.
d) Peak A0 - refers to the non-glycated adult hemoglobin.
e) Peak name A2 -Normal falls within the 2 to 4% range.

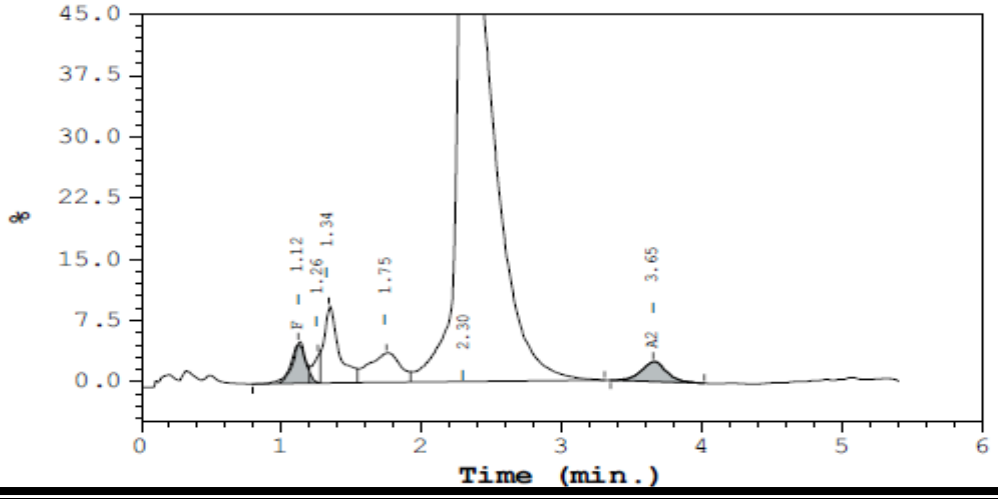
Figure- 1 shows the standard HPLC sample report

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.9	---	1.12	67645
Unknown	---	0.9	1.26	24879
P2	---	5.0	1.34	135024
P3	---	4.0	1.75	108817
Ao	---	85.4	2.30	2325508
A2	2.5	---	3.65	59904

Total Area: 2721778

F Concentration = .9 %
A2 Concentration = 2.5 %

Analysis comments:



Results

A total of 119 subjects underwent screening for hemoglobinopathies, with 65 (54.6%) being males and 54 (45.4%) females (Figure 2). Among the participants, the majority (94.2%) consisted of infants, accounting for 112 cases, while neonates represented a smaller proportion, with 7 cases (5.8%). **(Figure-2)**

Figure 2 shows age and gender distribution.

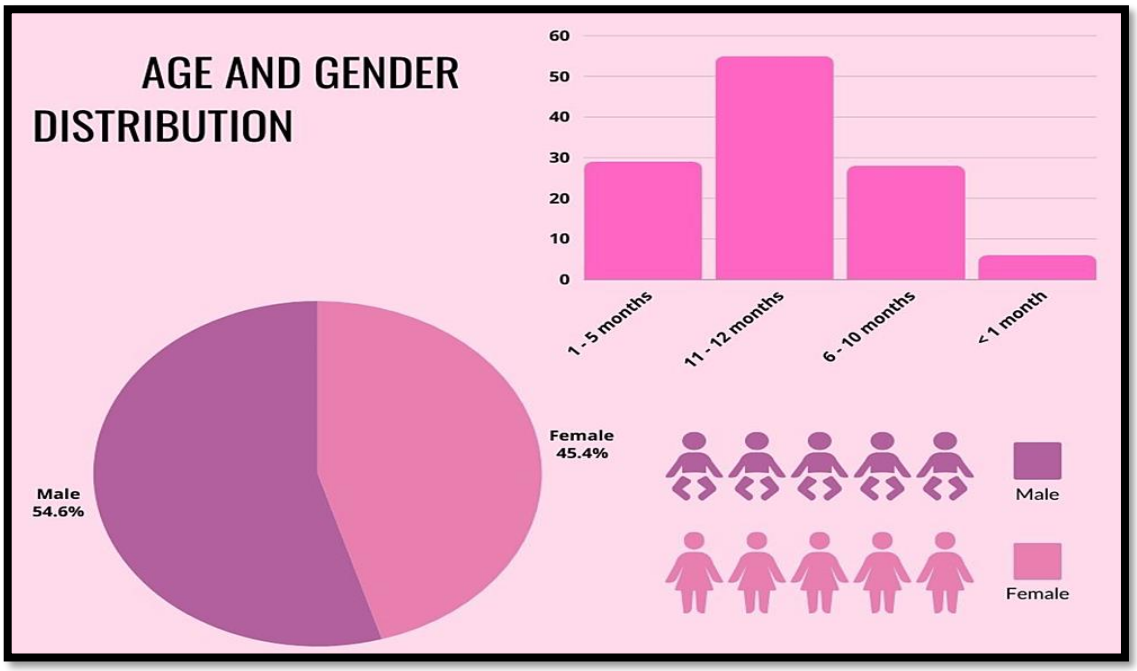
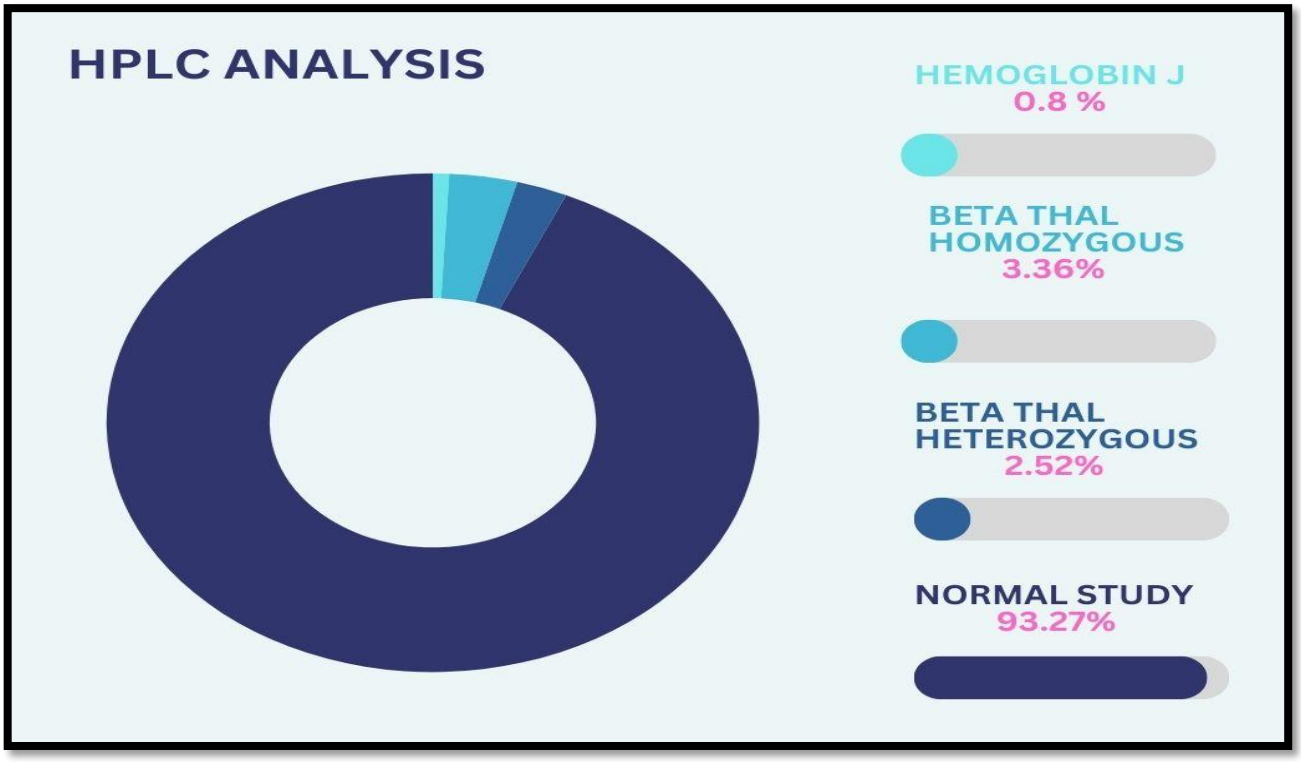


Figure- 3 shows distribution of normal cases and hemoglobinopathies.



Out of 119 subjects, 111 (93.27%) were diagnosed as normal, 4 (3.36%) were diagnosed as Beta-thalassemia homozygous, 3 (2.52%) were detected as beta-thalassemia heterozygous, and 1 (0.8%) was diagnosed as Hemoglobin J on HPLC study. **(Figure-3)**

The mean values for red blood cell count (RBC), hemoglobin (HB), and hematocrit (HCT) were significantly higher in the normal group at 3.99, 9.82 g/dL, and 29.37%, respectively, compared to 2.72, 6.00 g/dL, and 17.98% in the beta thalassemia homozygous group. Conversely, the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were lower in the thalassemia group at 67.18 fL and 22.38 pg, respectively, in contrast to the normal group with values of 74.18 fL and 23.14 pg. These results are summarized in **Table-1.**

Furthermore, the mean corpuscular hemoglobin concentration (MCHC) exhibited a higher value in the thalassemia group, measuring 33.25 g/dL, in contrast to 30.35 g/dL in the normal group. Moreover, the percentage of reticulocyte count and

absolute reticulocyte count demonstrated elevated erythropoiesis in thalassemia patients, with values of 3.97% and 71950 cells/μL respectively, compared to 2.39% and 56650 cells/μL in individuals without thalassemia.(summarized in **Table-1**)

The findings highlight the substantial hematological implications of beta thalassemia homozygous, characterized by diminished erythrocyte size and count and ineffective erythropoiesis despite increased reticulocyte production. This research offers insights into the diagnostic criteria necessary for managing and surveilling beta thalassemia homozygous.

In cases of Beta Thalassemia Heterozygous, the average RBC count was significantly higher at 4.50 compared to 3.99 in individuals with normal hemoglobin levels. Moreover, the average hemoglobin concentration (HB) was slightly lower at 9.47 g/dL in heterozygous cases compared to 9.82 g/dL in normal cases, although this difference is less pronounced than in the homozygous variant. Hematocrit levels were similar, with heterozygous cases having an average of 29.50%.

Furthermore, the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) exhibited lower values in the heterozygous group (67.17 fL and 21.70 pg, respectively) in comparison to the normal group (74.18 fL and 23.14 pg). Notably, the mean corpuscular hemoglobin concentration (MCHC) was higher in the heterozygous group at 32.13 g/dL.

Absolute reticulocyte counts were lower in heterozygous cases, with a mean of 42433 cells/μL. Reticulocyte percentages were also significantly lower at 0.96%, indicating reduced erythropoiesis activity compared to normal cases. Moreover, HBA2 levels were notably higher in heterozygous cases, averaging at 4.83, in contrast to 2.61 in normal cases, suggesting the genetic influence of thalassemia on hemoglobin synthesis **(Table 1).**

One case was identified as Hemoglobin J through HPLC analysis. In this case, the average red blood cell (RBC) count was elevated at 4.93, in contrast to the normal cases where it was 3.99. However, the hemoglobin (HB) level was lower at 8.80 g/dL in the Hemoglobin J case compared to 9.82 g/dL in normal cases, while the hematocrit (HCT) was higher at 33.40%.

(Refer to **Table 1**)

The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) values were lower for Hemoglobin J (67.70 fL, 17.80 pg and 26.30 g/dL respectively) compared to the normal group.

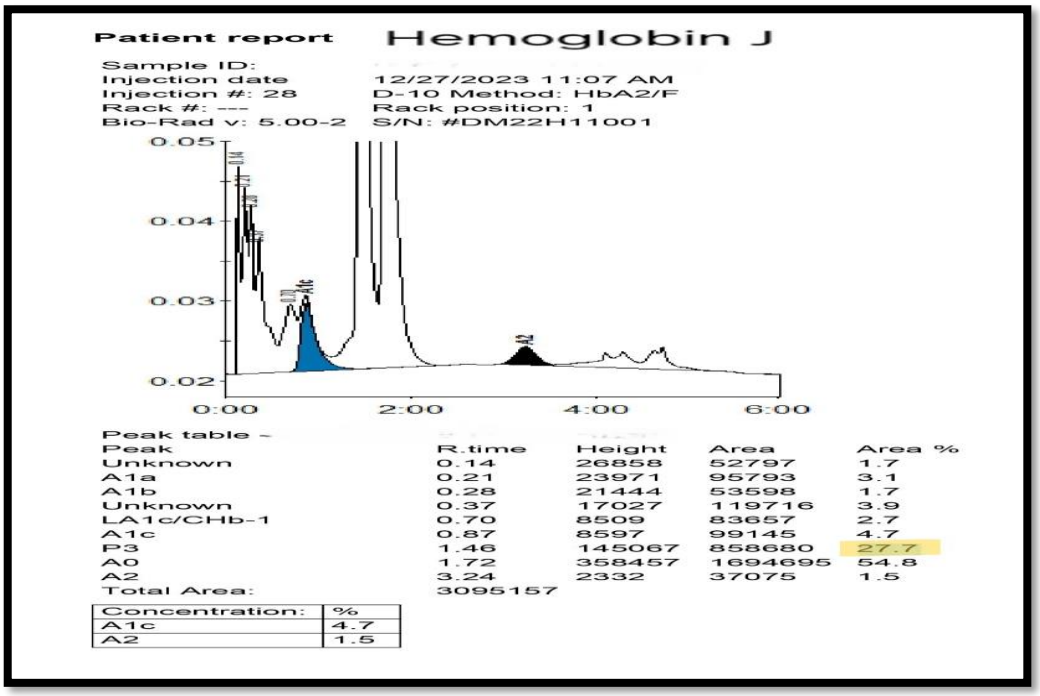
This comprehensive analysis highlights the distinctive hematological effects of Hemoglobin J, underscoring the necessity for specific diagnostic and therapeutic strategies for individuals affected by this condition.

Table-1 shows results of haematological and reticulocyte parameters with mean values and standard deviations (SD) for each parameter.

CASE TYPE	RBC	HB	HCT	MCV	MCH	MCHC	RETIC%	#RETIC	HBA0	HBA2	GENDER
NORMAL	3.99 ± 0.86	9.82 ± 9.89	29.37 ± 6.50	74.18 ± 16.07	23.14 ± 5.23	30.35 ± 3.17	2.39 ± 8.96	56649 ± 40743	73.89 ± 15.74	2.61 ± 0.76	M: F 61:50
BETA THALASSEMIA HOMOZYGOUS	2.72 ± 1.05	6.00 ± 2.16	17.98 ± 6.48	67.17 ± 6.57	22.38 ± 1.73	33.25 ± 1.16	3.96 ± 2.08	71950 ± 25483	14.45 ± 21.71	2.15 ± 0.90	M: F 1:3
BETA THALASSEMIA HETEROZYGOUS	4.50 ± 1.12	9.47 ± 1.32	29.50 ± 4.06	67.17 ± 12.02	21.70 ± 4.92	32.13 ± 1.76	0.96 ± 0.15	42433 ± 8428	79.37 ± 0.35	4.83 ± 0.58	M: F 3:0
HEMOGLOBIN J	4.93 ± N/A	8.80 ± N/A	33.40 ± N/A	67.70 ± N/A	17.80 ± N/A	26.30 ± N/A	0.91 ± N/A	44900 ± N/A	54.80 ± N/A	1.50 ± N/A	M: F 1:0

Chromatograms of abnormal hemoglobin variants are shown in figure- 4,5,6. On HPLC, chromatogram of Hemoglobin J is showing P3 peak (27.7%) with retention time of 1.46 minutes. **(Figure-4)** Chromatogram of beta thalassemia heterozygous showing elevated hemoglobin A2 level (5.3%). **(Figure-5)** Chromatogram of beta thalassemia homozygous shows elevated hemoglobin F level (96%) and markedly reduced hemoglobin A0 levels (2.9%). **(Figure-6)**

Figure- 4: HPLC report of hemoglobin J variant showing P3 peak of 27.7% highlighted by yellow color.



Patient report — Hemoglobin J

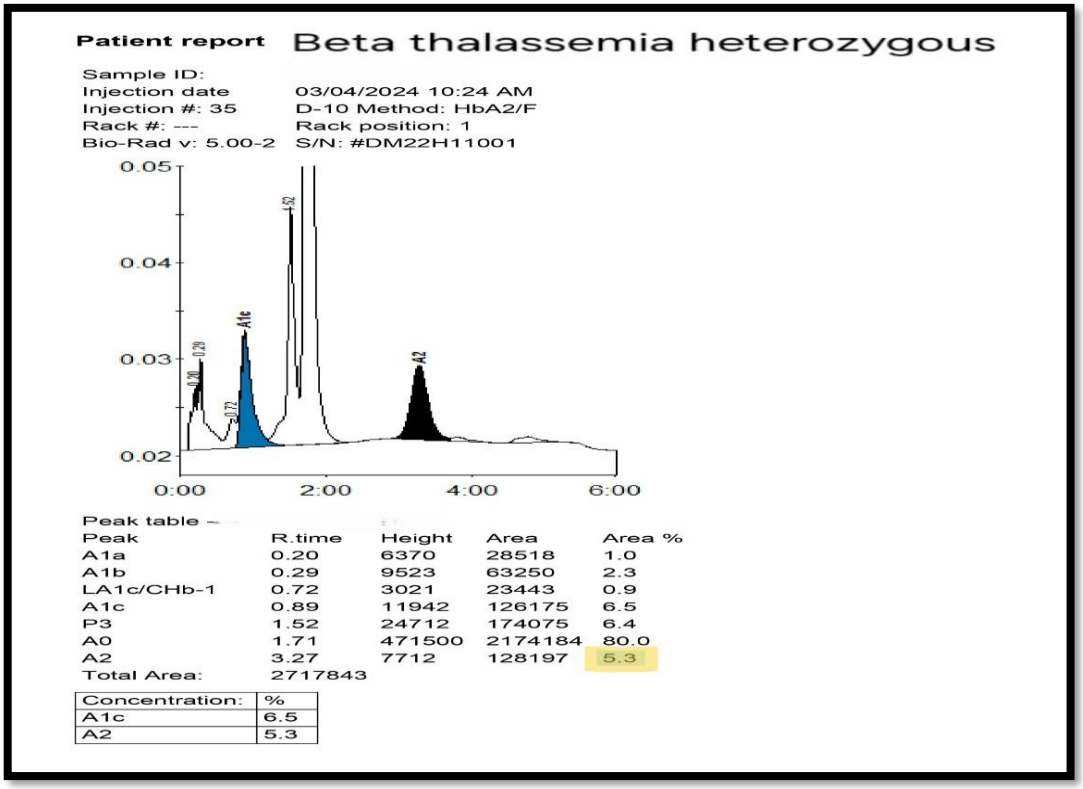
Sample ID:
Injection date 12/27/2023 11:07 AM
Injection #: 28 D-10 Method: HbA2/F
Rack #: --- Rack position: 1
Bio-Rad v: 5.00-2 S/N: #DM22H11001

Peak table –

Peak	R.time	Height	Area	Area %
Unknown	0.14	26858	52797	1.7
A1a	0.21	23971	95793	3.1
A1b	0.28	21444	53598	1.7
Unknown	0.37	17027	119716	3.9
LA1c/CHb-1	0.70	8509	83657	2.7
A1c	0.87	8597	99145	4.7
P3	1.46	145067	858680	27.7
A0	1.72	358457	1694695	54.8
A2	3.24	2332	37075	1.5
Total Area:		3095157		

Concentration:	%
A1c	4.7
A2	1.5

Figure-5: HPLC report of beta thalassemia heterozygous. Elevated A2 level is highlighted by yellow color.



Patient report — Beta thalassemia heterozygous

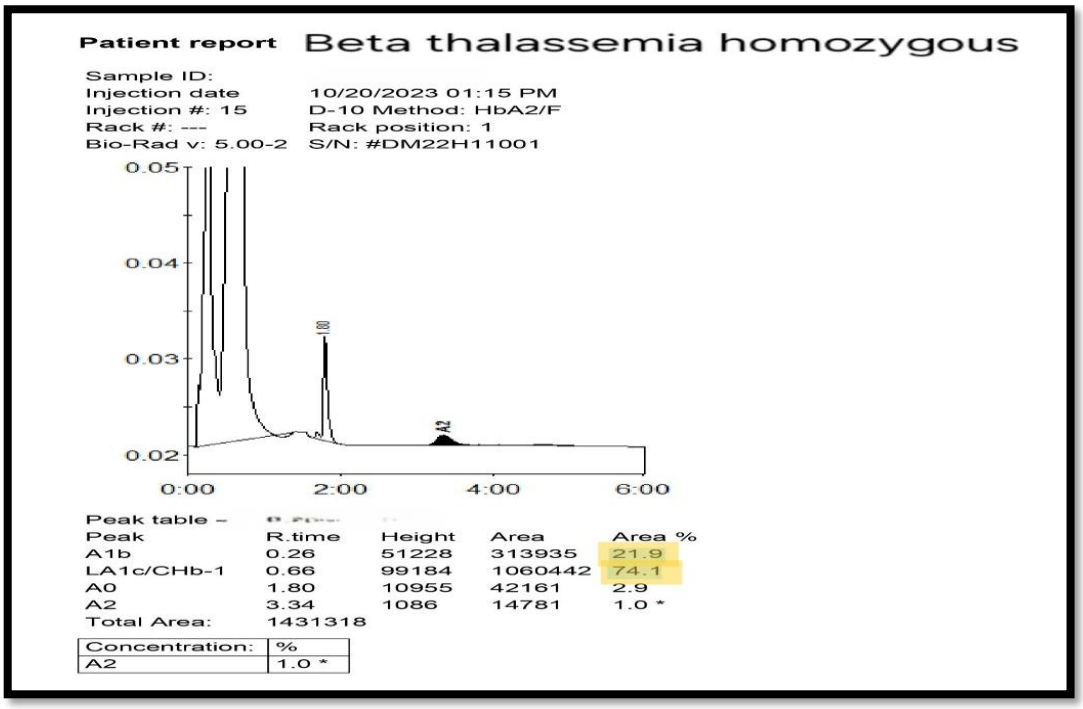
Sample ID:
Injection date 03/04/2024 10:24 AM
Injection #: 35 D-10 Method: HbA2/F
Rack #: --- Rack position: 1
Bio-Rad v: 5.00-2 S/N: #DM22H11001

Peak table –

Peak	R.time	Height	Area	Area %
A1a	0.20	6370	28518	1.0
A1b	0.29	9523	63250	2.3
LA1c/CHb-1	0.72	3021	23443	0.9
A1c	0.89	11942	126175	6.5
P3	1.52	24712	174075	6.4
A0	1.71	471500	2174184	80.0
A2	3.27	7712	128197	5.3
Total Area:		2717843		

Concentration:	%
A1c	6.5
A2	5.3

Figure-6: HPLC report of beta thalassemia homozygous highlighted by yellow color.



This detailed analysis emphasizes the unique hematological impact of these hemoglobinopathies, underlining the need for distinct diagnostic and therapeutic approaches for patients with these conditions.

Discussion

Hemoglobinopathies are hereditary disorders characterized by abnormal hemoglobin structure or production. These conditions are widespread globally and present substantial health burdens because of their persistent nature and severe complications (11). High-Performance Liquid Chromatography (HPLC) has become a valuable diagnostic instrument, especially beneficial for neonatal and infant screening, enabling timely identification and intervention (15). This research investigates the hemoglobinopathy patterns detected through HPLC in neonates and infants, juxtaposing our results with recent studies conducted in the last decade (15).

HPLC has emerged as the preferred method for diagnosing hemoglobinopathies due to its precision and ability to distinguish between different hemoglobin variants. This technique outperforms conventional electrophoretic methods by offering comprehensive quantification and discrimination of hemoglobin fractions, which are crucial for precise diagnosis and treatment. Nevertheless, HPLC is an expensive procedure that demands expertise, experience, and proficiency in result interpretation [15].

Despite its advantages, high-performance liquid chromatography (HPLC) has limitations. For instance, Hb E and Hb Lepore co-elute with Hb A2, posing challenges in accurately quantifying Hb A2. Likewise, Hb D Punjab and G-Philadelphia elute at identical retention times [12]. In such instances, capillary electrophoresis is employed with HPLC to differentiate these hemoglobins [12].

The World Health Organization (WHO) has reported that approximately 5% of the worldwide population are carriers of significant hemoglobin variants [16]. In India, the prevalence of β-thalassemia trait ranges from 3% to 17%, while the prevalence of sickle cell disease varies between 1% and 44%, depending on the region. Factors such as consanguineous marriages are known to contribute to these elevated prevalence rates [16].

The present study aimed to identify the pattern of hemoglobinopathies in neonates and infants utilizing High-Performance Liquid Chromatography (HPLC). The main findings are outlined below:

Normal Hemoglobin Patterns: In the study population, 93.27% displayed normal hemoglobin patterns, suggesting successful prenatal screening and genetic counseling initiatives.

Beta Thalassemia Homozygous: 3.36% of cases were diagnosed as beta-thalassemia homozygous. These cases exhibited significantly lower levels of red blood cells (RBC), hemoglobin (HB), and hematocrit (HCT) compared to normal cases, indicating severe anemia that necessitates frequent blood transfusions.

Beta Thalassemia Heterozygous accounted for 2.52% of cases diagnosed. Individuals with this condition exhibited lower hemoglobin levels but higher red blood cell counts than homozygous cases, suggesting a milder disease manifestation.

Hemoglobin J, a rare variant, was identified in 0.8% of the cases, exhibiting unique hematological parameters that necessitate specialized diagnostic and therapeutic interventions.

Patel et al. (11) analyzed 10,000 newborn samples using HPLC. The study revealed that 3.2% of the cases exhibited a prevalence of beta thalassemia homozygous, 2.3% showed a prevalence of beta thalassemia heterozygous, and 0.9% exhibited a prevalence of Hemoglobin J.

In a study conducted by Dolai T K et al. [13], the prevalence of β-Thalassemia trait was reported at 10.38%, Hb E (β26 (B8) GluàLys) trait at 4.30%, sickle cell trait at 1.12%, borderline

HbA2 value at 0.73%, low HbA2 at 0.68%, and Hb D trait at 0.37%.

Similarly, a study conducted by Tamhankar, P.M., et al. [17] reported a prevalence of 3.8% for beta thalassemia homozygous, 2.5% for beta thalassemia heterozygous, and 0.4% for Hemoglobin J. These findings align with the results of the current study.

S Rajan et al. [14] conducted a screening of 474 neonates who were referred for other inherited conditions. Among them, 20 babies exhibited hemoglobin variants, including 2 cases of alpha, 3 cases of beta thalassemia, 3 cases of sickle trait, 8 cases of Hb E, 1 case of Hb SC, 1 case of Hb S, and 1 case of Hb SS. This prevalence was lower than the current study, demonstrating a diverse case distribution.

In a study conducted by D Mukhopadhyay et al. involving 10,407 cases, it was found that 88.5% were classified as normal, 5.6% as beta-thalassemia homozygous, 5.0% as thalassemia heterozygous, 0.2% as Hemoglobin J, and 5% as Hb E heterozygous state [18]. The findings of this research indicated a higher prevalence of hemoglobinopathies compared to the current study, which could be attributed to a larger sample size and geographical variances.

Various studies collectively underscore the notable prevalence and diversity of hemoglobinopathies in different regions of India. They highlight the crucial role of sophisticated diagnostic techniques such as HPLC and underscore the significance of customized public health interventions to cater to the distinct requirements of diverse populations. Through the integration of focused screening with thorough genetic and hematological assessments, these studies offer a blueprint for enhancing the management of hemoglobinopathies and developing preventive strategies, with the ultimate goal of enhancing health outcomes in affected communities.

Conclusion

The present study emphasizes the crucial role of High-Performance Liquid Chromatography (HPLC) in diagnosing and characterizing hemoglobinopathies in neonates and infants. The comprehensive analysis of hematological parameters offers vital insights for clinical decision-making, and incorporating these results with recent research emphasizes the worldwide significance and implications of hemoglobinopathies.

As the progression of diagnostic capabilities and understanding of hemoglobinopathies evolves, it becomes crucial to establish inclusive screening initiatives and allocate resources to research endeavors aimed at enhancing the well-being of individuals impacted by these disorders. By integrating HPLC with state-of-the-art genetic methodologies, substantial advancements can be made in combating these incapacitating genetic conditions.

Limitations of the current study

Sample Size and Scope: The study's limited sample size of 119 cases and its regional focus on Karnataka, India, constrain the extent to which the findings can be generalized to broader populations.

Potential Sampling Bias: The selection of subjects from a particular geographic area and healthcare setting may not represent the general population, thereby introducing potential sampling bias.

Detection Limits of High-Performance Liquid Chromatography (HPLC): Although HPLC is known for its sensitivity, it may not be able to distinguish closely related hemoglobin variants such as Hb E and Hb Lepore from Hb A2 or Hb D Punjab from G Philadelphia.

Lack of Comprehensive Genetic Analysis: The research should have included in-depth genetic analysis, limiting our capacity to establish correlations between precise genetic mutations and clinical phenotypes and discover new mutations.

Cross-Sectional Design: The study's cross-sectional design hinders the evaluation of long-term clinical outcomes and the efficacy of early interventions over an extended period.

Recommendations

Regular screening for hemoglobinopathies in neonates and infants is crucial, especially in high-prevalence areas. High-performance liquid chromatography (HPLC) is used for accurate detection. Targeted testing focuses on infants with low hemoglobin and mean corpuscular volume levels. Careful interpretation of HPLC results is essential. Public health advocacy, ongoing research, and patient education can improve early detection and management.

Ethical approval

Approved by IEC Via Letter no. BLDE(DU)/IEC/675/2022-23, Dated 30-08-2022.

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