SSR Institute of International Journal of Life Sciences
ISSN (O): 2581-8740 | ISSN (P): 2581-8732
Manger *et al*., 2024
DOI: 10.21276/SSR-IIJLS.2024.10.4.35

Research Article

open access

Detection of Hemoglobinopathies and Hemoglobin Variants through HPLC in Northen Gujarat: A Study of 2500 Cases

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Received: 30 Mar 2024/ Revised: 01 May 2024/ Accepted: 15 Jun 2024

ABSTRACT

Background- The most prevalent inherited single-gene illnesses are known as hemoglobinopathies. Thorough detection of these conditions is crucial for preventing clinically severe hemoglobinopathies and thalassaemia major. Our goal was to find out how common thalassaemia and hemoglobinopathies were in both indoor and outdoor patients at a tertiary care hospital in Gujarat, India.

Methods- A total of 2500 cases were studied retrospectively between January 2018 and April 2021. Following the collection of a clinical history and family history, an automated three-part cell counter produced the entire hemogram report. Using the beta thalassaemia short program, samples using Bio-Rad Variant II were subjected to high-performance liquid chromatography (HPLC).

Results- Of the patients, 198 (7.92%) had aberrant haemoglobin fractions, while 2302 (92.08%) showed a normal Hb pattern. Among 105 patients (4.2%), the most prevalent anomaly was the β (beta) thalassaemia trait. Six instances (0.24%) had β thalassaemia major, and four cases (0.16%) had double heterozygous β thalassaemia and delta beta-thalassemia. Additional variations found include doubly heterozygous for HbC and thalassaemia and Hb Lepore, sickle-cell disease, sickle β thalassaemia, HbD-Punjab trait, Hb Iran trait, delta beta-thalassemia or hereditary persistence of foetal haemoglobin (HPFH) trait.

Conclusion- HPLC is a great tool for accurately diagnosing hemoglobinopathies and quantifying different aberrant haemoglobin fractions. This allows for early patient management.

Key-words: Hemoglobinopathy, Haemoglobin, High-performance liquid chromatography (HPLC), Thalassemia, β thalassaemia

INTRODUCTION

One of the most prevalent hereditary diseases is haemoglobin (Hb) synthesis abnormalities, which can be qualitative (variant Hbs) or quantitative (thalassaemia syndrome). Among these thalassaemia syndromes, beta thalassaemia major and some forms of alpha thalassaemia are extremely dangerous and contribute significantly to public health issues in India [1,2].

How to cite this article
Manger U, Panchal N, Patel V, Patel M, Shrivastav A. Detection of Hemoglobinopathies and Hemoglobin Variants through HPLC in Northen Gujarat: A Study of 2500 Cases. SSR Inst Int J Life Sci., 2024; 10(4): 6020-6027.

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According to the 2011 Census of India, the average prevalence of beta thalassaemia carriers is 3–4%, or 35–45 million carriers in our diversified community, which also includes 8% of the tribal population [3].

Hemoglobinopathies, encompassing a wide range of inherited hemoglobin disorders, are a significant global health concern. HPLC has emerged as a pivotal method for the accurate detection and diagnosis of hemoglobinopathies, offering advantages over traditional electrophoresis techniques [4,5] Studies have shown that HPLC is highly sensitive, specific, and capable of quantifying various hemoglobin variants with precision, making it a valuable tool in the identification of abnormal hemoglobin patterns [6]. Utilizing HPLC in the screening and diagnosis of hemoglobinopathies has revealed a variety of abnormal hemoglobin variants,

DOI: 10.21276/SSR-IIJLS.2024.10.4.35

such as sickle cell anemia, sickle cell trait, beta-thalassemia, and compound heterozygous states, highlighting the effectiveness of HPLC in characterizing different types of hemoglobin disorders [7].

An increasing number of normal and pathological Hb fractions are being separated and quantified using cation exchanges high-performance liquid chromatography (CE HPLC) [1]. Several hemoglobinopathies can be prevented and managed with the help of HPLC, which provides a trustworthy instrument for early, accurate detection [8]. Therefore, the present study investigated how common thalassaemia and hemoglobinopathies were in both indoor and outdoor patients at a tertiary care hospital in Gujarat, India.

MATERIALS AND METHODS

The study was conducted in the Department of Pathology, GMERS Medical College, Gandhinagar, Gujarat, India, between January 2018 and May 2021. A total of 2500 cases had their hemoglobinopathies and variations examined.

Inclusion criteria- Patients presenting with symptoms suggestive of hemoglobinopathies, with a documented family history of hemoglobin disorders and who underwent HPLC testing for hemoglobin analysis were included in the study.

Exclusion criteria- Patients with recent blood transfusions before testing, with other known hematological disorders and who did not provide informed consent for participation were excluded from the study.

Methodology- Before performing HPLC to determine the Hb levels, samples were run through the three-part cell counter HEMAX-330. Peripheral smears, red blood cell (RBC) indices, and other analyses were also performed. The patient underwent additional screening using the sickling test and the solubility test. The tests were carried out using the HPLC-based BIO-RAD "VARIANT II" (beta thalassaemia short program). For every run, a Hb A2/F calibrator is analysed first. The permissible overall area was one to three million square feet. In every instance, blood transfusion history and pertinent family history were recorded. The program produces a printed report with the chromatogram and all the haemoglobin fractions eluted. The "windows" created from retention times (RT) are allocated to the integrated peaks [9]. Every haemoglobin fraction, the RT, the peak regions, and the values (%) of the various haemoglobins are displayed on the printed chromatogram. A peak is considered unknown if it elutes at a non-specified RT. From sample to result printing, each analytical cycle takes roughly 6.5 minutes.

Statistical Analysis- The statistical analysis was conducted using SPSS software. Descriptive statistics were employed to summarize the demographic and clinical characteristics of the patients. The results were presented as percentages and frequencies, providing a clear overview of the distribution of various hemoglobin variants identified through HPLC.

Ethical approval- Ethical approval for this study was obtained from the Institutional Review Board of the tertiary care hospital in Gujarat, India. Informed consent was secured from all participants before their inclusion in the research, ensuring adherence to ethical standards in medical research.

RESULTS

Table 1 indicates that 198 (7.92%) of these instances had aberrant haemoglobin fractions on HPLC. Table 2 displays a variety of laboratory parameters with varying la. We note that microcytosis and hypochromia with elevated RBC counts were the most common blood results in the current investigation. The main anomaly found was elevated haemoglobin A2. A threshold exceeding 3.9% was employed to diagnose beta thalassaemia trait [10]. For Hb A2, the retention period varied from 3.59 to 3.67 minutes. The number of cases diagnosed with beta thalassaemia trait was 105 (4.2%). The most common observations in peripheral blood were elevated RBC counts together with hypochromia and microcytosis. Six cases (0.24%) of beta thalassaemia major were found. There was a varied decrease in Hb A and an increase in Hb F levels. Patients with microcytic hypochromic anaemia, prominent anisopoikilocytosis, and many nucleated red blood cells were the symptoms of Thalassaemia in major instances.

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Table 1: Hemoglobin profiles in cases obtained on HPLC.

Distribution of cases	Number of cases (%)	HbA2 (Mean±SD)	HbF (Mean±SD)	Abnormal Hb peak
Normal	2302(92.08)			
β-Thal trait (mean±SD)	105(4.2)	0.64±0.3	4.65±0.7	
β -Thal major (mean±SD)	6(0.24)	83.2±10.7	7.5±3.1	
Thalassemia major or double heterozygous for beta thalassemia and delta beta thalassemia	4(0.16)	91.05	5.2	
Sickle cell trait (mean±SD)	51(2.04)	0.7±0.31	2.7±0.17	HBS 27.05±3.5
Homozygous Sickle cell disease (mean±SD)	19(0.76)	11.18±4.13	3.01±0.24	HBS 82.95±4.04
Sickle cell disease or double Heterozygous for HbS/b-thal	3(0.12)	14.4	4.2	HBS 76.8
HbD-Punjab trait (mean±SD)	3(0.12)	0.26±0.05	1.3±0.5	HBD 37.4±1.76
HbD-Iran trait (mean±SD)	2(0.08)	0.65±0.21	41.6±4.73	-
Double heterozygous for HBS and Thalassemia (mean±SD)	2(0.08)	14.3±1.62	4.65±0.63	HBS 35.4
Double heterozygous for HBC and Thalassemia or HBC and HB LEPORE	1(0.04)	0.9	9.9	HBC 87.1
Delta beta thalassemia trait or HPFH trait	2(0.08)	16.2	2.25	-

Table 2: Hematological parameters in different groups of hemoglobinopathies.

Hemoglobinopathies (n)	Hb(g/dl) mean±SD	RBC count±SD (million/cumm)	MCV(fl) mean±SD	MCH(pg) mean±SD	MCHC(g/dl) mean±SD
β-Thal trait (mean±SD)	11.3±1.8	5.6±0.79	64.5±7.6	20.3±2.8	30.7±3.5
β -Thal major (mean±SD)	3.5±1.07	1.85±0.83	53±5.3	19.0±3.2	35.7±1.02
Thalassemia major or double heterozygous for beta thalassemia and delta beta thalassemia	4.05±0.6	2.07±0.4	66.15±2.9	19.95±1.8	30.1±1.4
Sickle cell trait (mean±SD)	10.4±2.8	4.8±1.0	68.2±7.6	21.9±.50	31.9±1.5
Homozygous Sickle cell ds (mean±SD)	7.2±2.3	3.04±0.09	75.48±7.7	23.8±2.3	31.6±2.1
Sickle cell disease OR double Heterozygous for HbS/β-thal	7.9±2.2	3.60±1.05	69.2±3.4	22.8±2.4	32.2±1.6
HbD-Punjab trait	12.3±0.8	5.02±0.4	76.5±0.9	28.1±0.4	34.3±2.1
HbD-Iran trait (mean±SD)	13.2±0.4	4.12±0.8	94.6±0.3	32.1±0.6	33.9±.0.3

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Double heterozygous for HBS and Thalassemia (mean±SD)	7.3±3.5	3.015±1.4	75.8±2.3	24.3±.14	32.05±1.2
Double heterozygous for HBC and Thalassemia or HBC and HB LEPORE	7.0	4.21	51.0	16.7	32.9
Delta beta thalassemia trait or HPFH trait	4.7	1.65	81.0	28.3	34.8

Four cases were diagnosed as Thalassemia major or double heterozygous for beta-thalassemia and delta beta-thalassemia (Fig. 1a) with HBF value 91.05% and HBA2 level 5.2%, peripheral blood smear findings were microcytosis and hypochromia, raised RBC counts with nucleated RBC. Hb S homozygous 19 cases (0.76%) presented with a variant S-Window of 82.95±4.04 and retention time 4.38 minutes (Fig. 1b). Sickling solubility test was positive. Mean Hb was 7.2 gm/dl with target cells and a few irreversibly sickled cells in the peripheral smear. Hb F was raised to 11.18±4.13.

Three cases had high Hb A2 along with high Hb S, blood picture with target cells. These were diagnosed as sickle cell homozygous or double heterozygous for Hbs and beta thalassemia (Fig. 1c). Hb S heterozygous 51 cases (2.04%) presented with a variant S- window of 24.2-37% and retention time 4.34 minutes. The sickling solubility test was positive for mild anemia. Two cases were diagnosed as Delta beta thalassemia trait or HPFH trait with HBF value 16.2%. Family studies along with molecular studies are required for confirmation of diagnosis (Fig. 1d). Hb D- Punjab heterozygous constituted 3 (0.12%) cases. HPLC displayed a D Window with variant percentage ranging from 37.4±1.76% and retention times of 4.07-4.11 minutes. The blood counts were essentially normal (Fig. 1e). Double heterozygous for HbC and thalassemia or HbC and Hb Lepore constituted one case. Hb A2 was raised to 9.9%, HbC value 87.1% with moderate anemia. Two cases were diagnosed as Hb D-Iran. It presented with a raised A2 peak of 41.6±4.73%, retention time of 3.50 minutes and normal hematological parameters (Fig. 1f).

The figure illustrates the various hemoglobin fractions identified, including normal and abnormal peaks, along with the prevalence of specific hemoglobinopathies such as β-thalassemia trait, sickle cell disease, and HbD traits among the studied population.

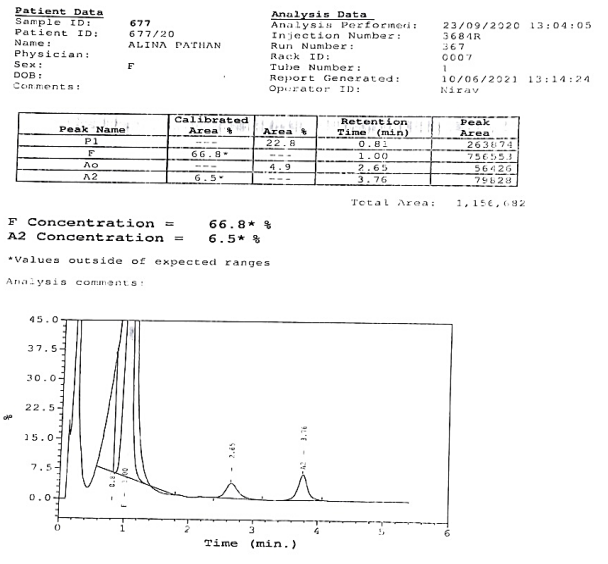


Fig. 1a: Thalassemia Major or Double heterozygous for thalassemia and delta beta thalassemia

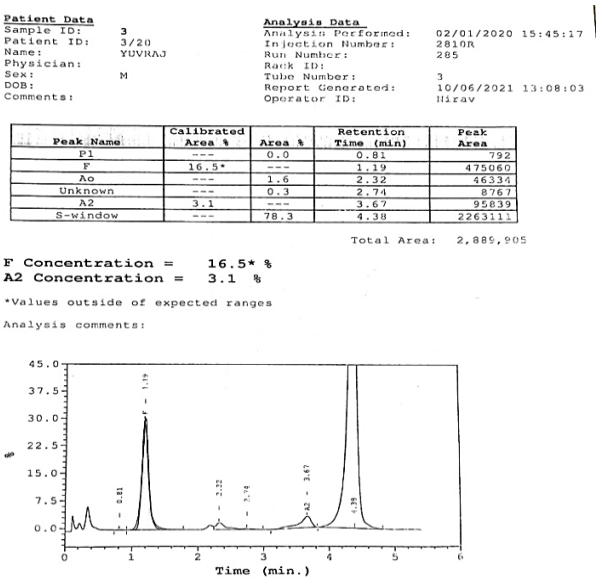
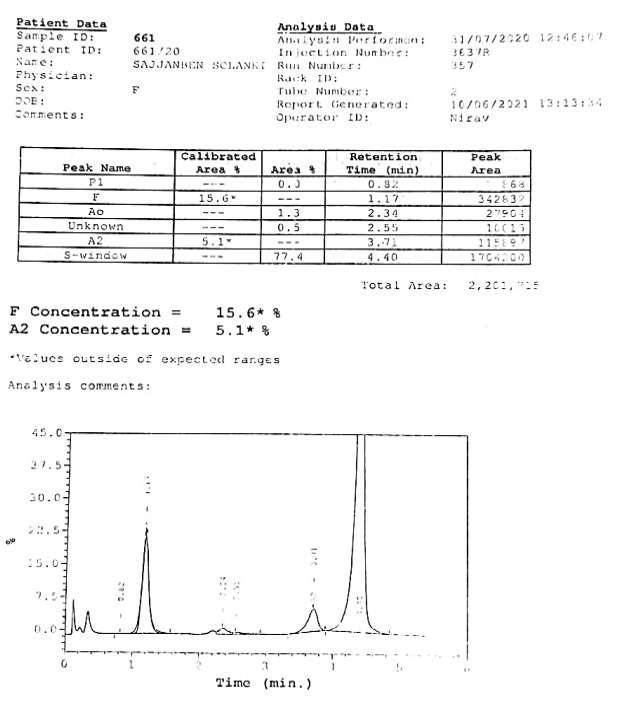


Fig. 1b: Sickle Cell Disease

DOI: 10.21276/SSR-IIJLS.2024.10.4.35



Patient Data
Sample ID: 661
Patient ID: 661/20
Name: SAJJANBEN SOLANKI
Physician:
Sex: F
DOB:
Comments:

Analysis Data
Analysis Performed: 31/07/2020 12:46:07
Injection Number: 3637R
Run Number: 357
Rack ID:
Tube Number: 2
Report Generated: 10/06/2021 13:13:34
Operator ID: Nirav

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.3	0.82	868
F	15.6*	---	1.17	342632
Ao	---	1.3	2.34	27904
Unknown	---	0.5	2.55	10013
A2	5.1*	---	3.71	115887
S-window	---	77.4	4.40	1704.00

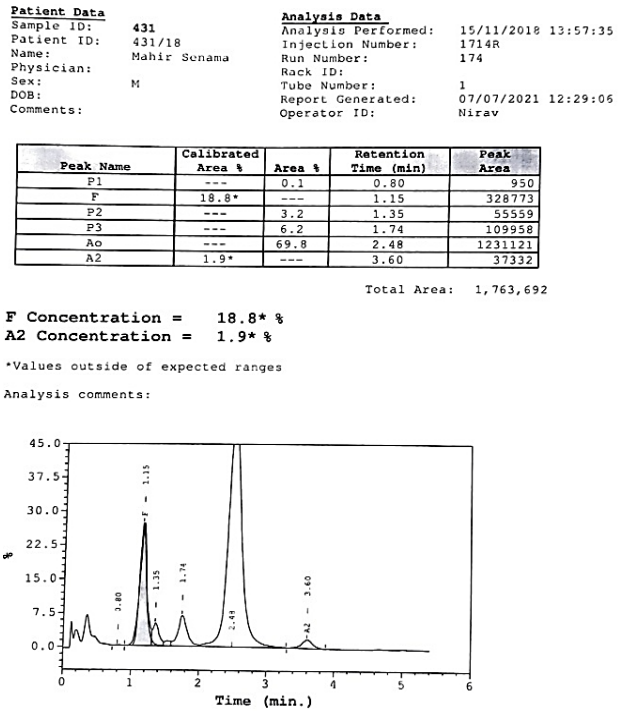
Total Area: 2,201,715

F Concentration = 15.6* %
A2 Concentration = 5.1* %

*Values outside of expected ranges

Analysis comments:

Fig. 1c: Sickle cell homozygous or double heterozygous for Hbs and beta thalassemia



Patient Data
Sample ID: 431
Patient ID: 431/18
Name: Mahir Senama
Physician:
Sex: M
DOB:
Comments:

Analysis Data
Analysis Performed: 15/11/2018 13:57:35
Injection Number: 1714R
Run Number: 174
Rack ID:
Tube Number: 1
Report Generated: 07/07/2021 12:29:06
Operator ID: Nirav

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.80	950
F	18.8*	---	1.15	328773
P2	---	3.2	1.35	55559
P3	---	6.2	1.74	109958
Ao	---	69.8	2.48	1231121
A2	1.9*	---	3.60	37332

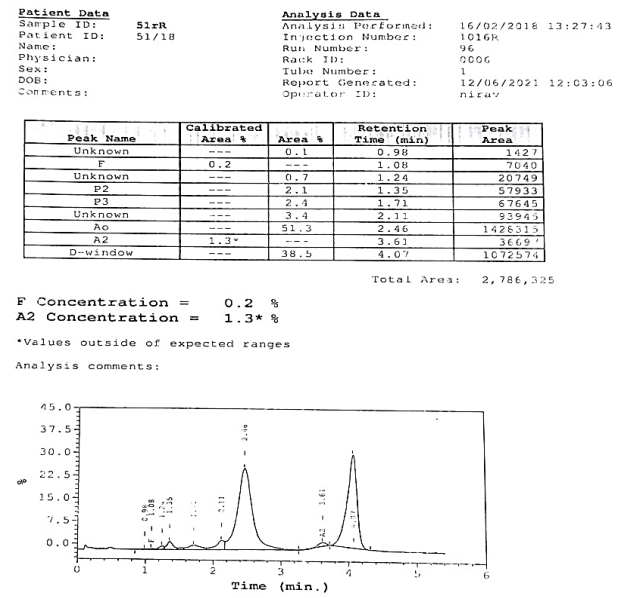
Total Area: 1,763,692

F Concentration = 18.8* %
A2 Concentration = 1.9* %

*Values outside of expected ranges

Analysis comments:

Fig. 1d: Delta beta thal trait or HPHF trait



Patient Data
Sample ID: 51rR
Patient ID: 51/18
Name:
Physician:
Sex:
DOB:
Comments:

Analysis Data
Analysis Performed: 16/02/2018 13:27:43
Injection Number: 1016R
Run Number: 96
Rack ID: 0006
Tube Number: 1
Report Generated: 12/06/2021 12:03:06
Operator ID: nirav

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.98	1427
F	0.2	---	1.08	7040
Unknown	---	0.7	1.24	20749
P2	---	2.1	1.35	57933
P3	---	2.4	1.71	67645
Unknown	---	3.4	2.11	93945
Ao	---	51.3	2.46	1428315
A2	1.3*	---	3.61	36697
D-window	---	38.5	4.07	1072574

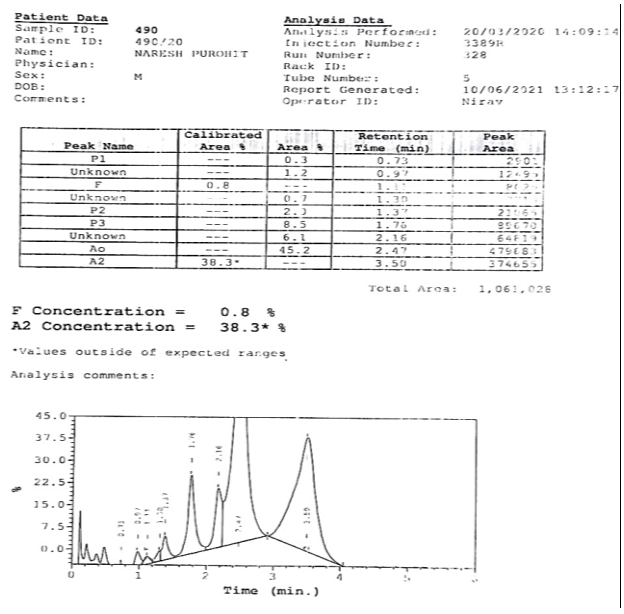
Total Area: 2,786,325

F Concentration = 0.2 %
A2 Concentration = 1.3* %

*Values outside of expected ranges

Analysis comments:

Fig. 1e: Hb D – Punjab trait



Patient Data
Sample ID: 490
Patient ID: 490/20
Name: NARESH PUROHIT
Physician:
Sex: M
DOB:
Comments:

Analysis Data
Analysis Performed: 20/01/2020 14:09:14
Injection Number: 3389R
Run Number: 328
Rack ID:
Tube Number: 5
Report Generated: 10/06/2021 13:12:17
Operator ID: Nirav

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.3	0.73	250
Unknown	---	1.2	0.97	12491
F	0.8	---	1.17	8423
Unknown	---	0.7	1.30	7417
P2	---	2.1	1.37	21164
P3	---	8.5	1.76	89170
Unknown	---	6.1	2.16	64119
Ao	---	45.2	2.47	479683
A2	38.3*	---	3.50	374655

Total Area: 1,061,028

F Concentration = 0.8 %
A2 Concentration = 38.3* %

*Values outside of expected ranges

Analysis comments:

Fig. 1f: Hb D- Iran trait

DISCUSSION

Hemoglobinopathies are prevalent illnesses that significantly affect several nations, including India. A class of congenital anaemias known as thalassaemia are characterized by quantitative abnormalities in one or more of the globin chains that make up normal haemoglobin. However, specific mutations lead to the production of structural variations at a lower rate (such as HbE and Hb Lepore) and hyper-unstable haemoglobin variants with a Thalassaemia phenotype (Hemiaglobinopathies, Thalassaemia) [11]. Previously limited to specific regions, faiths, social classes, and ethnic groups—especially those with consanguineous unions—these illnesses are now routinely observed worldwide. This is because different races have migrated there over time, resulting in a diversified population in terms of sociocultural backgrounds, linguistic languages, and ethnic backgrounds [12].

In our study conducted at a Gujarati tertiary care facility, we observed that the prevalence of abnormal

DOI: 10.21276/SSR-IIJLS.2024.10.4.35

hemoglobin disorders was notably lower at 7.92% compared to four other significant population-based investigations by Mondal *et al.* [13], Bhalodia *et al.* [14], Shrivastav *et al.* [15], and Sachdev *et al.* [8], which reported prevalences of 12.17%, 8.6%, 22.24%, and 12.18%, respectively. Among the various hemoglobin anomalies identified, β thalassemia trait emerged as the most frequent, accounting for 4.20% of cases in our investigation. This finding aligns with the results from Mondal *et al.* (4.60%) [13], Bhalodia *et al.* (5.20%) [14], Shrivastav *et al.* (11.55%) [15], and Sachdev *et al.* (8.90%) [8], where β thalassemia trait was also the predominant condition noted across studies. It is important to consider that iron deficiency can lead to reduced levels of HbA2, potentially masking the presence of β thalassemia trait. In contrast, insufficient vitamin B12 or folate levels may elevate HbA2 values, resulting in misdiagnosis of thalassemia traits in patients [13].

Numerous studies have highlighted the uneven distribution of the beta thalassaemia gene across various ethnicities within the Indian subcontinent, with prevalence rates ranging from 1% to as high as 22%. This genetic variability is significant for understanding public health implications and tailoring screening programs. In addition to beta thalassaemia, sickle cell disease has emerged as a notable hemoglobinopathy in this region. Recent research indicates that sickle cell trait is present in approximately 2.04% of patients, while homozygous cases account for about 0.76%. Supporting these findings, a study by Shrivastav *et al.* [15] reported a slightly higher prevalence of sickle cell trait at 2.95% and homozygous cases at 1.17%. Other investigations, such as those conducted by Bhalodia *et al.* [14], found a lower prevalence of sickle cell trait at 1.2%, while Mondal *et al.* [13] reported an even more modest occurrence of 0.38%. In Ahmedabad specifically, research by the Gujarat State Branch of the Indian Red Cross Society noted a sickle cell trait incidence of 0.7%, alongside a beta thalassaemia trait prevalence of 3.4%. These findings underscore the importance of regional studies in mapping out genetic disorders and their implications for healthcare strategies across diverse populations in India [16].

In this investigation, a variety of hematological conditions were identified, showcasing the complexity and diversity of hemoglobinopathies. Among the cases examined, β-Thalassemia major was noted in six instances, indicating a significant prevalence of this severe form of thalassemia within the studied population. Additionally, there were four cases classified as Thalassemia major or double heterozygous for both beta-thalassemia and delta beta-thalassemia, highlighting the occurrence of co-inheritance of different thalassemic mutations. The investigation also revealed three cases of sickle cell disease or double heterozygous disease for both HbS and beta-thalassemia, which underscores the clinical implications of having multiple genetic variants affecting hemoglobin production. Furthermore, traits associated with HbD-Punjab and HbD-Iran were observed in three and two cases respectively, suggesting regional variations in hemoglobin disorders. The presence of double heterozygosity for both HbS and thalassemia was documented in two instances, while one case involved double heterozygosity for HBC and Thalassemia or HBC and HB Lepore. Lastly, two cases were identified as having either Delta beta thalassemia trait or HPFH trait, further emphasizing the genetic diversity present in this cohort.

HPLC is a quick, dependable, and precise method for identifying and quantifying various hemoglobin (Hb) fractions. However, it is crucial to note that HPLC has limitations; specifically, it cannot differentiate between normal HbA2 and the variants associated with β thalassemia or α thalassemia. When interpreting chromatograms produced by HPLC, one must also consider nutritional anemias, as variations in hemoglobin that elute at the same retention time cannot be distinguished from one another. Therefore, when HPLC results are inconclusive or insufficient for identifying specific hemoglobinopathies, additional molecular studies become necessary. Techniques such as polymerase chain reaction (PCR), amplification refractory mutation system (ARMS), and other comparable tests can be employed to identify specific mutations responsible for hemoglobin diseases. This multi-faceted approach ensures a comprehensive understanding of the patient's condition and aids in accurate diagnosis [17].

CONCLUSIONS

For the early diagnosis and treatment of hemoglobinopathies and variations, HPLC has proven to be a sensitive, precise, and reliable method. Given the prevalence of β thalassaemia trait in India, screening should be done both before and throughout pregnancy

DOI: 10.21276/SSR-IIJLS.2024.10.4.35

to prevent the delivery of children who have β thalassaemia major. The current community burden of thalassaemia may be eliminated in the future, or at least reduced, with greater knowledge and the use of this straightforward screening method. This study shows the extent of hemoglobinopathies in our hospitalized population, which may only be the tip of the iceberg. Nevertheless, research of this kind can contribute to raising awareness in the public and healthcare systems.

CONTRIBUTION OF AUTHORS

Research concept- Urvashi Manger, Mona Patel

Research design- Urvashi Manger, Mona Patel, Nital Panchal

Supervision- Mona Patel

Materials- Urvashi Manger, Vaidehi Patel, Atul Shrivastav

Data collection- Nital Panchal, Vaidehi Patel, Atul Shrivastav

Data analysis and Interpretation- Mona Patel, Vaidehi Patel, Atul Shrivastav

Literature search- Urvashi Manger, Vaidehi Patel, Atul Shrivastav

Writing article- Urvashi Manger, Nital Panchal, Atul Shrivastav

Critical review- Mona Patel, Nital Panchal, Vaidehi Patel, Atul Shrivastav

Article editing- Urvashi Manger, Mona Patel

Final approval- Mona Patel, Nital Panchal, Vaidehi Patel

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