ORIGINAL RESEARCH

Detection Of Haemoglobinopathies In Patients Of Anaemia Using High Performance Liquid Cromatography (HPLC) In Jabalpur District Hospital Madhya Pradesh: A Cross-Sectional Study

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ABSTRACT

Context: Hemoglobinopathies are the most common heterogeneous group of monogenetic disorder in the world and its prevalence varies with geographical regions. India is developing country and many studies show a significant burden of hemoglobinopathies in India. **Aim:** The aim of the present study was to check the prevalence of various hemoglobinopathies in anemic subjects using high-performance liquid chromatography (HPLC) method in Jabalpur region of Madhya Pradesh. **Settings and design:** The present study was conducted at the department of pathology on anemic patients referred from different outpatient department and Wards of the hospital and informed consent were taken from all participants. **Subjects and methods:** The present study included a total of 239 individuals. The HPLC test was performed using Bio-Rad D-10 analyzer during study period from October 2023 to March 2023. **Results:** Out of a total of 239 cases, we found 49 (20.50%) cases with abnormal hemoglobin fractions and (79.49%) cases free from hemoglobinopathies. Out of the total hemoglobinopathies detected 23 (46.93%) were male and 26 (53.06%) were female. The major abnormality detected was heterozygous sickle cell disorder with 34 (69.3%) cases, followed by Homozygous sickle cell disorders 6 (12.24%), Beta thalassemia trait 4 (8.16%), hereditary persistence of fetal hemoglobin 4 (8.16%),Hb J 2 (4.08%). **Conclusions:** In our study, we found a high prevalence of hemoglobinopathies among anemic subjects. The most common disorder detected was heterozygous sickle cell disorder. Most of the hemoglobinopathies found in our study could be accurately quantified by HPLC which is a rapid, sensitive, and reproducible method for the detection of different hemoglobinopathies.

Keywords: Hemoglobinopathies, high-performance liquid chromatography, sickle cell anemia, thalassemia.

INTRODUCTION

The prevalence of anaemia among six groups as per the National Family Health Survey 5 (2019-21), is 25.0 percent in men (15-49 years) and 57.0 percent in women (15-49 years).(1)Hemoglobinopathies are the commonest genetic disorders worldwide. Hemoglobinopathies are inherited disorders of red blood cells. An estimated 7% of the world population carry an abnormal haemoglobin gene, while about 300,000-500,000 are born annually with significant haemoglobin disorders. They consist of two major groups –Thalassemias and Sickle cell syndromes. Sickle cell syndromes are more frequent and constitute 70% ofaffected births world-wide, the rest are due to thalassemias.1(2)India hasthe largest number of children with Thalassemia major in the world – about 1 to 1.5 lakhs and almost 42million carriers of s (beta) thalassemia trait. About 10,000 - 15,000 babies with thalassemia major areborn every year. Sickle cell disease affects many communities in certain regions, such as central Indiaand States of Gujarat, Maharashtra and Kerala. The carrier frequency of the Sickle cell gene varies from1 to 35 % and hence there are a huge number of people with Sickle cell disease.(3)In India, β-Thalassemia is prevalent across the country, with an average frequency of carriers being3-4% 4,5,6. A higher frequency has been observed in certain communities, such as Sindhis, Punjabis,Gujarati's, Bengali's, Maher's, Koli's, Saraswats, Lohanas and Gaurs 6,7.

HbS is highly prevalent in the tribalpopulations of Southern, Central and Western states reaching as high as 48% in some communities8.HbE is common in the North Eastern states, and has a carrier frequency as high as 50%, in some areas.It is found in lower frequencies in the Eastern states of West Bengal, Bihar and Uttar Pradesh, while HbDis present in about 2% of people in Punjab.(4)(5) Hemoglobinopathy patients can benefit temporarily with nutritional supplementation and blood transfusions but long term outcome can be better if specific diagnosis is made and specific therapy or precautions are undertaken.(6)We carried out this study to determine frequency of haemoglobin disorders in patients who presented to us with moderate to severe anaemia with various manifestations and to determine which disorder manifests more severely.

MATERIAL AND METHODS

The present cross-sectional study was conducted at the department of pathology on anemic patients referred from different outpatient department and Wards of the hospital and informed consent were taken from all participants.The present study included a total of 239 individuals. The HPLC test was performed using Bio-Rad D-10 analyzer during study period from October 2022 to March 2023.Individuals displaying irregularities in their complete blood count (CBC) indicators, including indications of microcytic hypochromic anaemia and haemoglobin levels below 12 g/dl, coupled with a noteworthy familial medical background, were considered for the research. Those who had received blood transfusions within the previous three months were not included. All participants gave their permission to participate, and the study received approval from our organization's ethics board.

Sample Collection

Peripheral blood samples were collected from all participants. Approximately "5 mL" of blood was drawn from each participant using venipuncture and collected in EDTA tubes. The samples were labelled, stored, and transported under standardized conditions to ensure sample integrity.

Hemoglobin Variant Analysis

The primary methodology employed for the presumptive identification of haemoglobin variants was high-performance liquid chromatography (HPLC). We used the Variant Hemoglobin Testing System(Variant II Beta Thalassemia Short Program, Bio-Rad Laboratories Inc., Bio-Rad D-10 analyser.) under the experimental conditions specified by the manufacturer.(7)

Procedure

1. Blood samples were first lysed using [specific lysing agent or procedure].
2. Hemolysates were then injected into the HPLC system.
3. Separation of hemoglobin variants was based on their differential retention times.
4. Chromatograms were generated and studied for peaks corresponding to various hemoglobin types.The identification of hemoglobin variants was made primarily based on retention time (RT) windows and area percent. Reference standards and controls were run alongside the samples to ensure the accuracy of the identified peaks.

Data Analysis

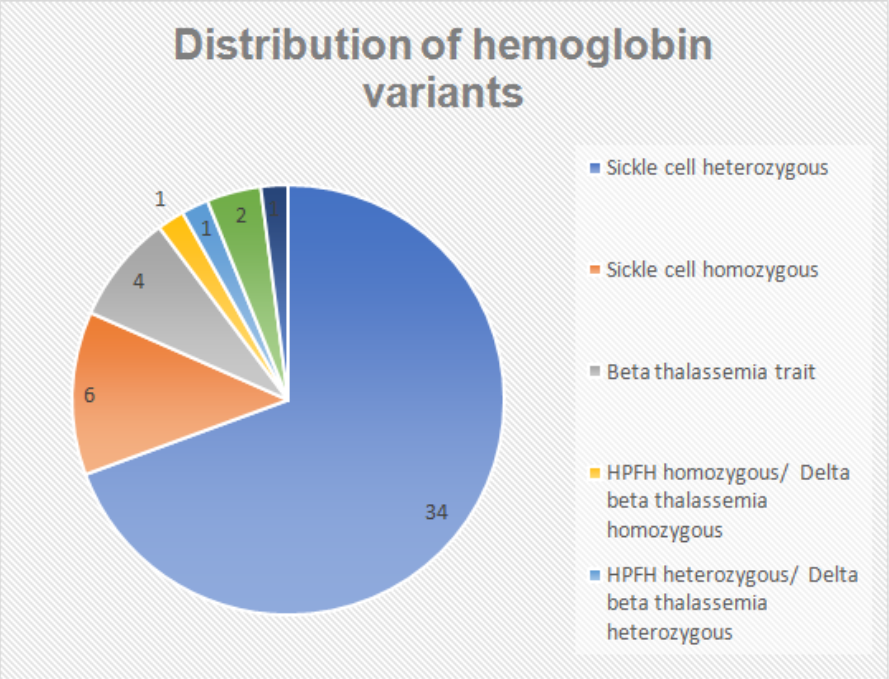
Data were recorded and then analyzed using Microsoft office 2021. Frequencies and percentages were used to represent the distribution of various hemoglobin variants. Additionally, chromatograms of notable variants were documented (Figures 1-6), and genotypes of specific structural variants tabulated (Table 1).

Among 250 cases screened, 49 (19.6%) cases were detected with abnormal hemoglobin in this study. Presumptive identification of hemoglobin variants was made primarily by retention time (RT) windows and area percent. Distribution of hemoglobin variants identified is shown in Table 1.Chromatograms of some important variants are shown in figures (Figures 1, 2, 3, 4, 5, and 6).Genotypes of some structural variants are shown in Table 3.Sickle cell heterozygous was the most common hemoglobin variant (69.3%) detected in our study with elevated HbA2 level (>2.8%) and RT 4.12 min.Majority were asymptomatic and detected during carrier screening and family studies.Six cases of Sickel cell homozygus were detected. HbF was raised (21.8%), with variable HbA2 (0.8%). Cases of Sickle cell homozygus presented within 1st year of life. Parental study was done to find out carrier status and to confirm the diagnosis.

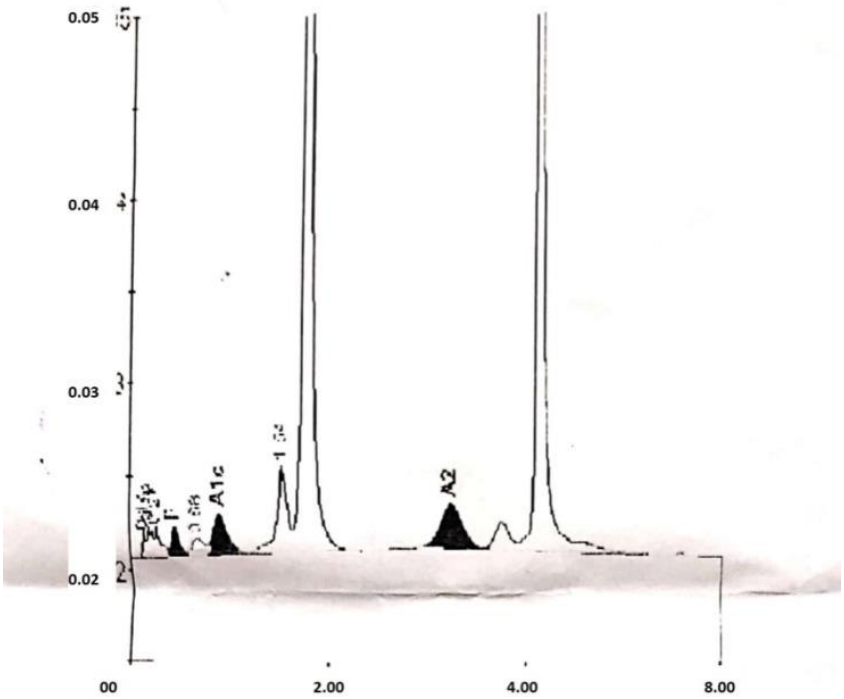
Table 1: Distribution of hemoglobin variants.

Hemoglobin Pattern	Male	Female	Total Cases
Sickle cell heterozygous	14 (28.5%)	20(40.8%)	34(69.3%)
Sickle cell homozygous	5(10.2%)	1(2.04%)	6(12.24%)
Beta thalassemia trait	2 (4.08%)	2 (4.08%)	4(8.16%)
HPFH homozygous/ Delta beta thalassemia homozygous	1 (2.04%)	-	1 (2.04%)
HPFH heterozygous/ Delta beta thalassemia heterozygous	-	1 (2.04%)	1 (2.04%)
Hb J	1 (2.04%)	1 (2.04%)	2(4.08%)

Double Heterozygous for sickle cell and beta thalassemia	-	1 (2.04%)	1 (2.04%)
Total	23(46.93%)	26(53.06%)	49(100%)



1: Chromatogram of Sickle cell heterozygous State showing elevated S window 36.8% (RT-4.12 min)



Peak Table- ID: 8814749867

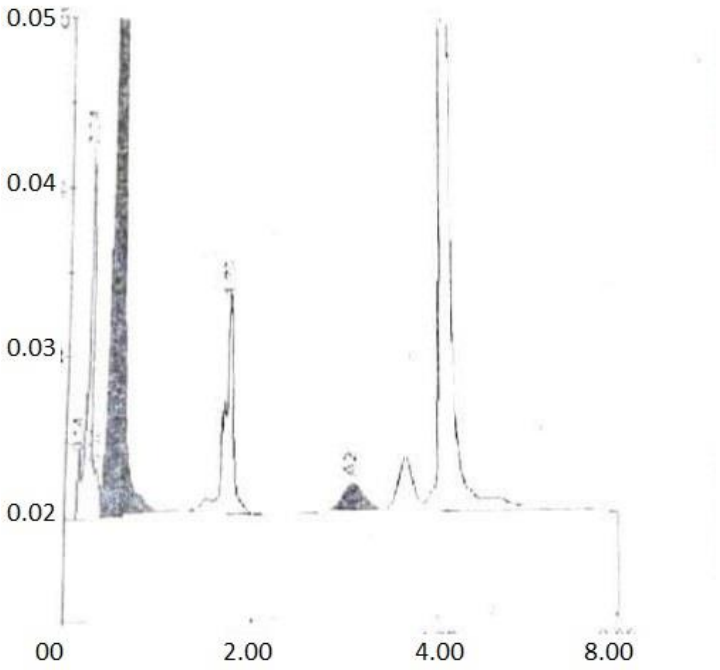
Peak	R. TIME	HEIGHT	AREA	AREA%
Unknown	0.14	1447	2531	0.2
A1a	0.19	1677	6854	0.6
A1b	0.27	1621	7116	0.7
F	0.46	1598	9162	1.0

LA1c/CHb-1	0.68	933	7704	0.7
A1c	0.91	2114	20612	4.9
P3	1.54	4637	33789	3.2
A0	1.76	147329	554749	51.8
A2	3.24	2234	33791	2.8
S-WINDOW	4.12	108503	393796	36.8
Total Area	1070104			

CONCENTRATION	%
F	1.0
A1c	4.9
A2	2.8

Sickle cell heterozygoushemoglobin variant detected in 34(69.3%)study subjects with elevated s. window 36.8% and RT 4.12 min.Majority were asymptomatic and detected during carrier screening and family studies.

2: Chromatogram of Sickle cell homozygous state showing elevated S window 68.7% (RT-4.08 min)



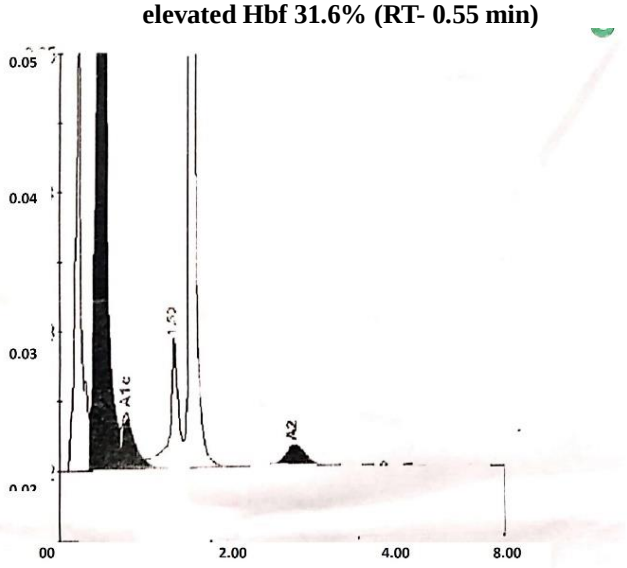
Peak Table- ID:8814774346

Peak	R.TIME	HEIGHT	AREA	AREA%
Unknown	0.14	3979	8544	0.5
A1a	0.24	21690	68071	4.1
A1b	0.33	2848	9662	0.6
F	0.53	43569	321065	21.8*
A0	1.75	13108	82132	5.0
A2	3.08	1482	24693	0.8
S-WINDOW	4.08	25898	4427032	68.7
Total Area	1641499			

CONCENTRATION	%
F	21.8*
A2	0.8*

Sickle cell homozygoushemoglobin variant detected in 4(8.16%) study subjects with elevated s. window 68.7% and RT 4.08 min.Majority were asymptomatic and detected during carrier screening and family studies.

3: Chromatogram of Delta beta thalassemia heterozygous State or HPFH heterozygous State showing elevated Hbf 31.6% (RT- 0.55 min)



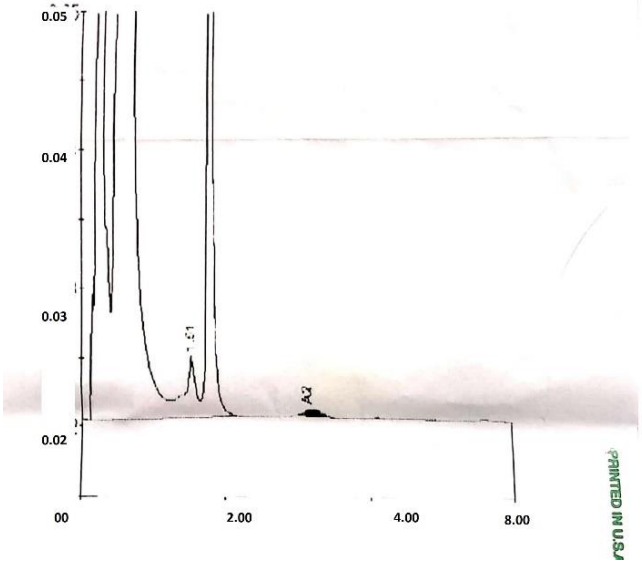
Peak Table- ID : 8814044139

Peak	R.TIME	HEIGHT	AREA	AREA%
A1a	0.25	33672	177332	11.3
F	0.55	53264	444149	31.6*
A1c	0.88	3590	43803	5.8
P3	1.50	9367	71926	4.6
A0	1.72	21867	804016	51.4
A2	3.11	1364	24299	0.8*
Total Area	1565524			

CONCENTRATION	%
F	31.6*
A1c	5.8
A2	0.8*

Delta beta thalassemia heterozygus state or HPFH Heterozygous state hemoglobin variant detected in 1(2.04%) study subjects with elevated HbF level 31.6% and RT 0.55 min.Majority were asymptomatic and detected during carrier screening and family studies.

4: Chromatogram of Delta beta thalassemia homozygous state or HPFH Homozygous state showing elevated LA1C/ CHb 61.6% (RT-0.63 min)



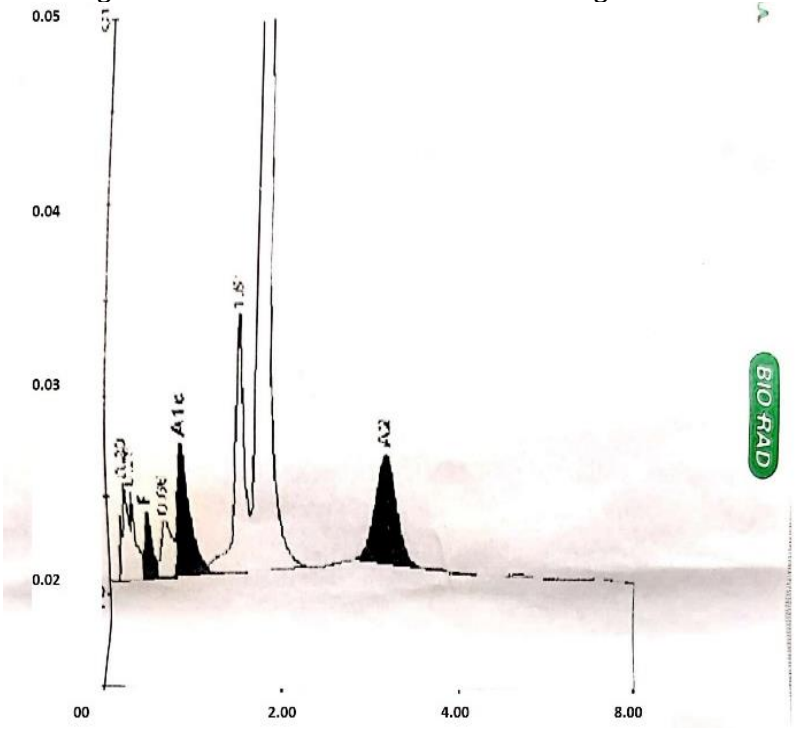
Peak Table- ID: 8814867342

Peak	R.TIME	HEIGHT	AREA	AREA%
A1b	0.26	71706	426763	16.4
LA1c/CHb-1	0.63	153908	1607660	61.6
P3	1.51	4578	50610	1.9
A0	1.74	147750	514250	19.7
A2	3.13	533	10242	0.1*
Total Area	2609526			

CONCENTRATION	%
A2	0.1*

Delta beta thalassemia homozygous state or HPFH Homozygous state hemoglobin variant detected in 1(2.04%) study subjects with elevated LA1c/CHb 61.6%and RT 0.63min.Majority were asymptomatic and detected during carrier screening and family studies.

5:Chromatogram of Beta thalassemia trait State showing elevated A2 5.4% (RT-3.16%)



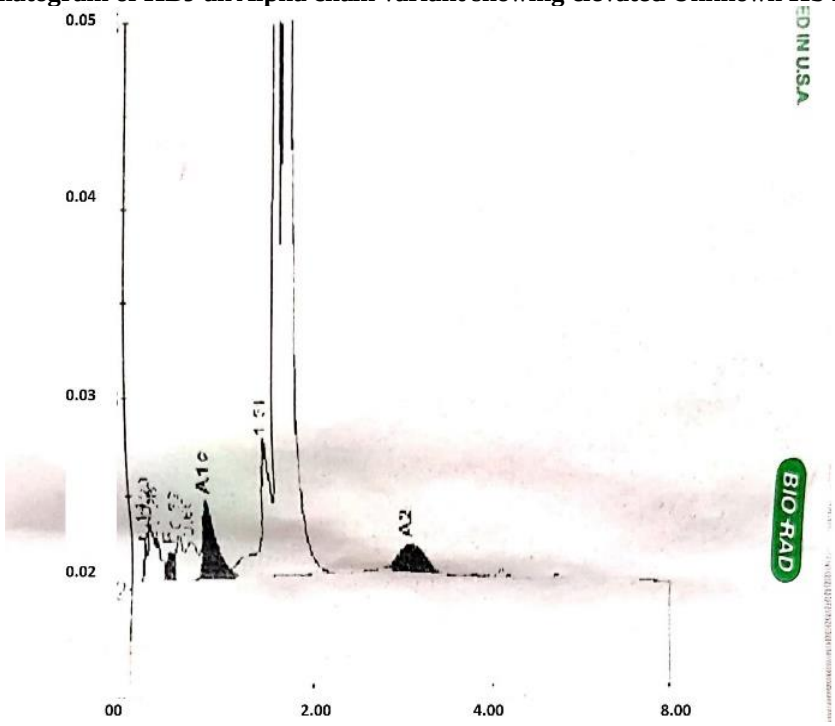
Peak Table- ID : 8815363118

Peak	R.TIME	HEIGHT	AREA	AREA%
A1a	0.20	4890	28033	1.6
A1b	0.28	4665	18207	1.1
F	0.46	3333415	19646	1.3
LA1c/CHb-1	0.66	2912	25308	1..5
A1c	0.86	65667	61650	5.5
P3	1.51	13107	96684	5.6
A0	1.75	346432	1381049	80.2
A2	3.16	5440	92196	5.4
Total Area	1722773			

CONCENTRATION	%
F	1.3
A1c	5.5
A2	5.4

Beta thalassemia trait Statehemoglobin variant detected in 4(8.16%) study subjects with elevated HbA2 level (>5.4%) and RT 3.16 min.Majority were asymptomatic and detected during carrier screening and family studies.

6: Chromatogram of HBJ an Alpha chain variant showing elevated Unknown Hb 20.5% (RT- 1.64 min)



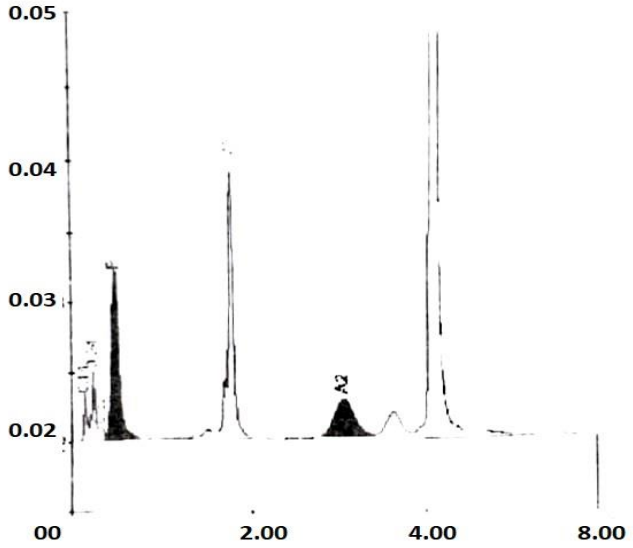
Peak Table- ID: 8814582226

Peak	R.TIME	HEIGHT	AREA	AREA%
Unknown	0.14	2243	4806	0.3
A1a	0.20	3196	13732	0.9
A1b	0.28	2721	10523	0.7
F	0.43	1621	10498	<0.8*
Unknown	0.52	2469	13139	0.9
LA1c/CHb-1	0.66	2054	16603	1.1
A1c	0.85	3939	41152	4.1
P3	1.51	7079	66734	4.5
Unknown	1.64	81299	301016	20.5
A0	1.76	246355	960227	65.5
A2	3.07	1334	28581	0.9*
Total Area	1467012			

CONCENTRATION	%
F	<0.8*
A1c	4.1
A2	0.9*

Hb J hemoglobin variant detected in 2(4.08%) study subjects with elevated unknown level 20.5% and RT 1.64min.Majority were asymptomatic and detected during carrier screening and family studies.

7: Chromatogram of Double Heterozygous for Sickle cell & Beta thalassemia state showing elevated S window 79.0 % (RT-4.08 min) & elevated A2 4.4% (RT- 3.05 min)



Peak Table- ID: 8814774922

Peak	R.TIME	HEIGHT	AREA	AREA%
Unknown	0.14	3469	8310	0.8
A1a	0.24	5098	12851	1.2
A1b	0.32	925	3448	0.3
F	0.47	12138	62763	6.6
A0	1.76	19401	89586	8.4
A2	3.05	2852	47693	4.4
S-WINDOW	4.08	207119	846122	79.0
Total Area	1070775			

CONCENTRATION	%
F	6.6
A2	4.4

One case had peak in S window (RT 4.08 min), indicating presence of hemoglobin S (HbS) and elevated A2 levels of 4.4%. Parental study was advised to confirm the double heterozygous status.

DISCUSSION

Our study aimed to investigate the prevalence and distribution of hemoglobinopathies among 250 screened individuals. The key findings were the detection of sickle cell heterozygous state as the predominant variant and the identification of other hemoglobin variants like Beta Thalassemia Trait, Delta Beta Thalassemia, HPFH, and Hb J variant.The high prevalence of sickle cell trait 34(69.3%) in our study aligns with findings from other studies in regions where malaria was or is endemic. Ambekar et al. found a prevalence of hemoglobinopathies in Western Maharashtra in 26.5% (106 out of 400) of the subjects studied.(8) Similarly, Chopra et al. discovered that 25% (258 out of 1032) of the participants had abnormal hemoglobin.(9)The protective effect of sickle cell trait against malaria might explain the higher prevalence in certain populations.(10)Mavis oppong et al reported the prevalence of sickling screening positive was 16.0% with an overall prevalence of sickle cell disorders being 2.0% in Ghana which is endemic to maleria.(11)The presentation of sickle cell homozygous cases within the first year of life is consistent with clinical observations that symptoms often begin between 4 to 6 months of age when fetalhemoglobin levels decrease.(12)The detection of Beta Thalassemia Trait (BTT) and the elevated HbA2 levels further reiterate the importance of carrier screening. Beta thalassemia trait is often asymptomatic but has implications for offspring if both parents are carriers.(13)Lastly, the potential double heterozygous state (indicated by a peak in the S window with elevated A2 levels) highlights the complexity of hemoglobinopathies and their inheritance patterns. Compound heterozygosity can present with variable phenotypes, sometimes more severe than either of the single mutations alone.(14)Our study underscores the importance of

carrier screening and family studies, as many of the detected variants were asymptomatic. Early detection can lead to better clinical management and informed reproductive choices.

Limitations and Future Directions: Like all studies, ours had limitations, including [e.g., the reliance on retention time (RT) windows and area percent for presumptive identification, geographical constraints, limited sample size, etc.]. Future research could aim to use more comprehensive molecular methods, increase the sample size, or investigate the clinical implications of the identified variants in greater depth.

REFERENCES

1. Ministry of Health and Family Welfare. Anaemia Mukt Bharat; 2022 Feb 4.
2. World Health Organization, Thalassaemia International Federation. Management of Hemoglobin Disorders. Report of Joint WHO-TIF Meeting, Nicosia, Cyprus, 16-18 November 2007. Available from: http://www.who.int/genomics/WHO-TIF_genetics_final.pdf
3. Ministry of Health and Family Welfare. HB Pathies in India Report.
4. Mohanty D, Colah RB, Gorakshakar AC, Patel RZ, Master DC, Mahanta J, Sharma SK, Chaudhari U, Ghosh M, Das S, Britt RP, Singh S, Ross C, Jagannnathan L, Kaul R, Shukla DK, Muthuswamy V. Prevalence of β-thalassemia and other hemoglobinopathies in six cities. J Community Genet. 2013; 4:33-42.
5. Urade BP. Sickle Cell Gene Scenario in tribal India. J Health Med Inform. 2012. doi: 10.4172/2157-7420.1000114.
6. Memish ZA, Saeedi MY. Six-year outcome of the national premarital screening and genetic counselling program for sickle cell disease and β-thalassemia in Saudi Arabia. Ann Saudi Med. 2011; 31:229-235.
7. VARIANT II. b thalassemia short program instruction manual.
8. Ambekar SS, Phadke MA, Balpande DN, Mokashi GD, Khedkar VA, Banker MP. The Prevalence and heterogeneity of beta thalasemia mutations in the Western Maharashtra Population: A hospital-based study. IJHG. 2001;1(3):219-223.
9. Chopra GS, Nair V, Gupta PK. Spectrum of Haemoglobinopathies in a Tertiary Care Hospital of Armed Forces. Med J Armed Forces India. 2000 Oct;64(4):311-4. doi: 10.1016/S0377-1237(08)80005-6.
10. Aidoo M, Terlouw DJ, Kolczak MS, McElroy PD, terKuile FO, Kariuki S, Nahlen BL, Lal AA, Udhayakumar V. Protective effects of the sickle cell gene against malaria morbidity and mortality. Lancet. 2002 Apr 13;359(9314):1311-2. doi: 10.10.
11. Oppong M, Lamptey H, Kyei-Baafour E, Aculley B, Ofori EA, Tornyigah B, Kweku M, Ofori MF. Prevalence of sickle cell disorders and malaria infection in children aged 1-12 years in the Volta Region, Ghana: a community-based study. Malaria journal. 2020;19(1):426. doi: 10.1186/s12936-020-03500-5.
12. Akinsheye I, Alsultan A, Solovieff N, Ngo D, Baldwin CT, Sebastiani P, Chui DH, Steinberg MH. Fetalhemoglobin in sickle cell anemia. Blood. 2011;118(1):19–27. doi: 10.1182/blood-2011-03-325258.
13. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ. 2001;79(8):704-12. PMID: 11545326; PMCID: PMC2566499.
14. Steinberg MH, Sebastiani P. Genetic modifiers of sickle cell disease. Am J Hematol. 2012 Aug;87(8):795-803. doi: 10.1002/ajh.23232. PMID: 22641398; PMCID: PMC4562292