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Spectrum of Sickle Cell Hemoglobinopathies Using High-Performance Liquid Chromatography as Diagnostic Tool: A Study from Western Odisha, India

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ABSTRACT

Background: Hemoglobinopathies, genetic disorders affecting haemoglobin, are prevalent globally, with a significant impact in India due to factors like consanguinity and regional endogamy. Sickle cell disease (SCD), the most common structural hemoglobinopathy globally, shows high prevalence in India, particularly in states like Odisha. Early diagnosis and management are crucial due to the severe clinical manifestations associated with these disorders. This study aimed to determine the prevalence of different sickle cell hemoglobinopathies in western Odisha and evaluate the diagnostic utility of high-performance liquid chromatography (HPLC) in identifying these variants.

Methods: A hospital-based prospective study analyzed 496 sickle slide test-positive patients over two years. Patients underwent thorough clinical evaluation, including CBC, red cell indices, and HPLC analysis using the Variant II Hemoglobin Testing System. Statistical analysis was performed using SPSS.

Results: The study revealed a predominance of sickle cell variants among both paediatric (47.36%) and adult populations, with Bargarh district contributing significantly. Chromatogram analysis identified various hemoglobin variants, highlighting the diagnostic precision of HPLC. Clinical symptoms varied by genotype, with significant differences in haematological and biochemical parameters observed across different hemoglobinopathies.

Conclusion: HPLC emerged as a reliable tool for diagnosing hemoglobin disorders, emphasizing its role in regional prevalence studies and guiding targeted management strategies. Further research is needed to explore clinical modifiers influencing disease severity and enhance therapeutic interventions.

Key-words: Sickle cell disease, hemoglobinopathies, high-performance liquid chromatography, diagnostic utility, India

INTRODUCTION

Hemoglobinopathies are a group of genetic disorders affecting hemoglobin, impacting 4.5% of the global population [1]. In India, the prevalence of β-thalassemia trait and sickle cell ranges from 3-17% and 1-44%, respectively, influenced by factors like kinship, caste, and area endogamy [2-4].

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Sickle cell disease, the most common structural hemoglobinopathy globally, shows the highest frequency in India in states like Odisha (9%), Assam (8.3%), Madhya Pradesh (7.4%), Uttar Pradesh (7.1%), Tamil Nadu (7.1%), and Gujarat (6.4%) [5]. Population migration has further globalized its prevalence. This disease includes homozygous states like sickle cell anemia (HbSS) and various compound heterozygous states such as sickle cell trait (HbAS), sickle cell β-thalassemia (HbSβ), sickle cell D Punjab (HbSD), HbSE, HbSC, and HbSLepore [6].

Sickle cell disease results from a mutation in the β-globin gene, replacing the sixth amino acid from glutamic acid to valine. This mutation causes deoxygenated HbS to polymerize into fibrous polymers, stiffening the RBC

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membrane and resulting in a sickle shape [7,8]. These cells, with altered, sticky membranes, adhere to the endothelium of small venules and capillaries, leading to microvascular occlusion and tissue ischemia, manifesting as vaso-occlusive crises. Haemolysis occurs as the spleen destroys the abnormal RBCs, with symptoms appearing before age one, including chronic haemolytic anaemia and developmental disorders [9,10].

Patients suffer from vaso-occlusive crises affecting various body parts and are highly susceptible to infections, notably by pneumococci, haemophilus, salmonellae, klebsiella, and mycoplasma. These infections can lead to sepsis, osteomyelitis, meningitis, and sometimes cardiac involvement, often resulting in death. Additionally, spleen crises, acute thoracic syndrome (ATS), and strokes cause significant morbidity and mortality [11]. These complications result in serious organ damage. In India, hemoglobinopathies are mostly diagnosed using conventional methods such as clinical and family history, red cell indices, complete blood counts, sickling tests, and hemoglobin electrophoresis. However, these methods have limitations in identifying hemoglobin variants with the same electrophoretic mobility and in diagnosing certain compound heterozygous states [12].

To address these limitations, cation exchange high-performance liquid chromatography (CE-HPLC) is employed to separate and estimate various normal and abnormal Hb fractions. This method provides a definitive tool for early and accurate detection of hemoglobinopathies, aiding in their prevention and management [13,14]. Blood collected in dipotassium ethylenediamine tetraacetic acid (EDTA) vacutainers are analyzed using the Sysmex autoanalyzer for hemogram and red cell indices, and HPLC. The tests are performed using the BIO-RAD 'VARIANT II' instrument (beta thalassemia short program), which operates on the HPLC principle. Hb A2/F calibrator and two levels of controls (BIO-RAD) are analyzed at the start of each run. This process enables the identification of Hb variants and compound heterozygous states with a high degree of precision and reproducibility. To determine the prevalence of different sickle cell hemoglobinopathies in the western Odisha population and establish the diagnostic utility of HPLC in identifying the wide spectrum of sickle cell hemoglobinopathies.

MATERIALS AND METHODS

This hospital-based prospective study evaluated all cases of sickle slide test-positive patients who presented to the Pediatrics OPD & IPD, General Medicine OPD & IPD and Sickle Cell Institute at V.S.S. Institute of Medical Sciences and Research, Burla, over two years, from April 2022 to March 2024. The study aimed to assess the diagnostic utility of HPLC in identifying various sickle cell hemoglobinopathies and comparing them with conventional diagnostic methods.

Inclusion criteria- The inclusion criteria encompassed all sickle slide test-positive cases.

Exclusion criteria- The exclusion criteria included patients below one year of age due to the difficulty in interpreting HbF levels and those who had recently received blood transfusions, as HPLC cannot differentiate between the patient's cells and transfused cells.

During the study period, 496 cases of sickle slide-positive patients were enrolled. Cases were selected through simple random sampling from the Odisha Sickle Cell Project (NHM Odisha), which was initiated in April 2010 and includes 12 district sickle cell units across 12 western Odisha districts. Sickle slide test-positive cases from these units were further analysed using haemoglobin electrophoresis and HPLC. Patients diagnosed with sickle cell disease who met the inclusion and exclusion criteria were enrolled in the study after obtaining informed consent. Each patient's age, sex, chief complaints, history of present illness, history, family history, and previous treatment history were recorded. Thorough general and systemic examinations were conducted.

Methodology- Investigations included complete blood count (CBC), total and direct bilirubin, serum lactate dehydrogenase (LDH), serum creatinine, and hemoglobin variants estimation by HPLC. The CBC was performed using automated blood counters (Sysmex, KX-21), measuring parameters such as differential count, total leukocyte count, hemoglobin, total red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total platelet count, and absolute neutrophil count. Total and direct bilirubin levels were determined using the Bilirubin kit based on the modified Jendrassik and Grof method.

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The diagnosis of sickle cell disease was confirmed using a combination of sickling slide tests, hemoglobin electrophoresis, and HPLC. For the sickling slide test, a drop of whole blood was placed on a clean slide, covered with a dry, clean coverslip, and sealed with paraffin wax. After 24 hours, the slide was examined under a microscope for the presence of sickled erythrocytes. Hemoglobin electrophoresis was performed using a modified protocol by Lepp et al. (1978). EDTA mixed whole blood was washed with saline, and the hemolysate was prepared by centrifugation. The hemolysate was then subjected to agarose gel electrophoresis at 100-150 volts, and the results were interpreted based on electrophoretic patterns.

Quantification of hemoglobin variants was performed using the Variant II Hemoglobin Testing System (Bio-Rad) with the β-thalassemia short program. EDTA whole blood samples were diluted in a hemolysis solution and loaded into the instrument. The separated hemoglobin fractions were analysed based on their retention times, and the software provided a printed report with chromatograms showing the hemoglobin fractions eluted.

Statistical Analysis- Statistical analysis was performed using SPSS version 16.0, with categorical data compared using the Tukey-Kramer multiple comparison test of one-way analysis of variance (ANOVA). A p-value of <0.05 was considered statistically significant.

RESULTS

A total of 496 cases were analyzed for assessment of different spectra of hemoglobinopathy. Among these, 235 cases (47.36%) were pediatric patients aged 1-14 years, while the remaining 52.64% were adults. In the pediatric group, 95 (40.42%) were female and 140 (59.57%) were male. Male predominance was evident, especially among those with SS genotype (119 cases). In females, SS genotype was also predominant (83 cases) (Fig. 1).

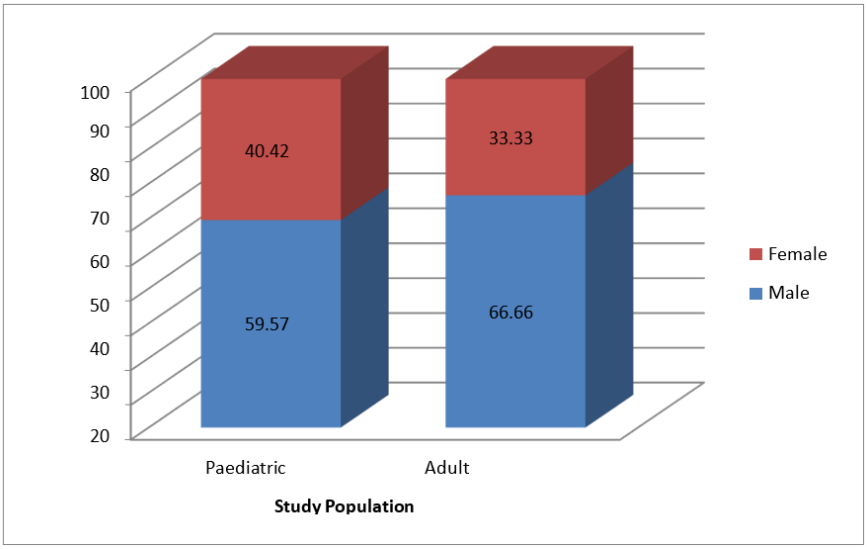

Fig. 1: Gender distribution

In the adult population, 66.66% were male and 33.33% were female. The distribution of genotypes varied, with SS being prevalent in both genders but with distinct variations across different genotypes. In the pediatric population, Bargarh district had the highest representation (81%), followed by Balangir (16.59%) and Sonepur (14.89%). Similarly, in adults, Bargarh district accounted for the highest percentage (25.28%), followed by Angul (12.26%) and Balangir (11.49%) (Table 1).

Analysis of 496 chromatograms revealed eight known windows (F, A0, A2, D, S, P1, P2, and P3) and additional unknown windows across different retention time ranges. Clinical symptoms varied among different genotypes, with predominant symptoms including arthralgia, vaso-occlusive crises (VOC), dactylitis, fever, pallor, seizures, growth retardation, and jaundice. Specific percentages for each symptom varied by genotype (Table 2).

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Table 1: District Wise Distribution of Cases

Name of the District	Pediatric population (n= 235) No (%)	Adult population (n= 261) No (%)	Total (n= 496) No (%)
Sambalpur	25 (10.63)	24 (9.19)	49 (9.87)
Bargarh	81 (34.46)	66 (25.28)	147 (29.63)
Jharsuguda	4 (1.7)	20 (7.66)	24 (4.83)
Balangir	39 (16.59)	30 (11.49)	69 (13.91)
Sundargarh	28 (11.91)	11 (4.21)	39 (7.86)
Sonepur	35 (14.89)	2 (0.76)	37 (7.45)
Nuapada	0	12 (4.59)	12 (2.41)
Angul	9 (3.82)	32 (12.26)	41 (8.26)
Koraput	4 (1.7)	2 (0.76)	6 (1.2)
Deogarh	0	2 (0.76)	2 (0.4)
Kalahandi	0	20 (7.66)	20 (4.03)
Boudh	0	10 (3.83)	10 (2.01)
Others	10 (4.25)	8 (3.06)	18 (3.62)

Table 2: Haemoglobinopathy and Different Clinical Symptoms

Clinical symptoms	SS (%)	Sβ (%)	SD (%)	SE (%)
Arthralgia	11.71	19.6	11.76	0
VOC	5.4	17.64	11.76	36.84
Dactylitis	26.42	26.47	17.64	21.05
Fever	14.71	4.9	11.78	10.52
Pallor	12.01	7.84	23.52	15.78
Seizure	14.11	8.82	5.88	0
Growth Retardation	3.3	8.82	0	0
Jaundice	12.91	5.88	17.64	15.78

Significant differences were observed in hematological parameters such as total leukocyte count (TLC), red blood cell count (RBC), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) among different hemoglobinopathies (Table 3).

Table 3: Data Are Expressed in Mean ± Sd

Parameters	SS	SB	SD	SE	p-value
TLC ($\times 10^3 \mu l$)	10.42±3.91	11.35±5.42	11.78±5.59	9.1±1.64	0.004
RBC ($\times 10^6 \mu l$)	3.44±0.76	3±0.89	3.01±0.82	4.17±1.29	<0.0001
Hb (g/dl)	8.34±1.68	7.81± 1.74	7.69±1.54	8.35±2.06	0.016
PCV (%)	25.35±5.08	25.02±5.05	25.13±4.68	27.3±6.3	0.022
MCV (fl)	75.6±10.07	71.64±22.39	84.61±13.39	75.54±10.07	<0.0001
MCH (pg)	24.76±3.71	26.73± 3.54	26.67±3.48	25.3±4.04	<0.0001
MCHC (%)	32.56±2.42	31.88±2.5	31.76±2.64	33.16±1.78	0.018
TPC ($\times 10^6 \mu l$)	2.74± 1.08	2.83±1.06	2.75±1.05	2.44±1.18	0.547

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The concentration of various hemoglobin types (A0, F, A2, S, D) differed significantly among the studied groups, highlighting distinct patterns in each genotype (Table 4).

Table 4: Concentration Of Different Haemoglobin

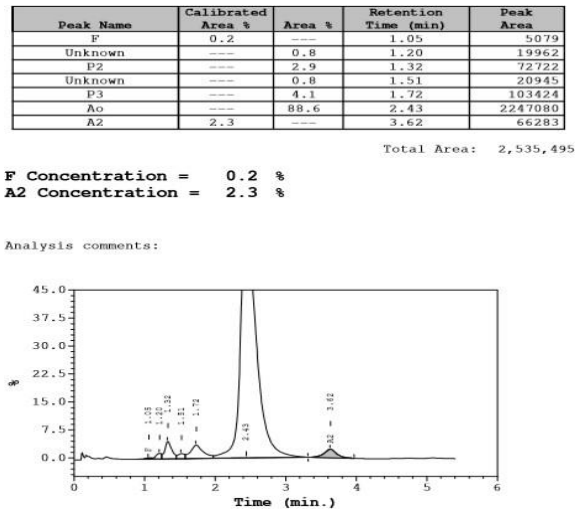
Type of Hemoglobin (%)	SS	SB	SD	SE
A0	6.38±4.52	3.71±0.89	2.73±0.38	2.71±0.33
F	20.54±6.56	17.23±1.51	18.48±4.65	10.57±3.95
A2	2.69±0.84	6.60±0.99	2.22±0.73	29.45±5.11
S	69.37±6.18	70.47±3.35	32.56±3.64	57.16±15.98
D	-	-	43.56±1.95	-

Biochemical indices including alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, and lactate dehydrogenase (LDH) showed significant differences across different hemoglobin variants (Table 5).

Table 5: Biochemical Indices of Different Haemoglobin Variants

Biochemical Indices	SS	SB	SD	SE	P value
BIL (T) (mg%)	3.01±2.34	3.01±1.32	2.81±0.96	2.19±1.3	0.649
AST (I.U)	47.26±17.94	62.29±24.31	59±13.42	62.35±33.24	<0.0001
ALT (I.U)	28.21±22.06	29.82±11.45	32.23±11.29	38.46±17	0.250
ALP (I.U)	210.07±11.04	360±189.45	400±198	273±67	<0.0001
Urea (mg %)	47.95±10.94	43.12±23.79	43.44±23.21	44.174±12.94	0.034
Creatinin (mg %)	0.84±0.71	1.74±0.82	1.16±0.73	1.33±0.45	<0.0001
LDH (I.U)	1434±1364	1313.22±671	1451±625	1570±823	0.750

Based on chromatogram analysis and red cell indices, diagnoses included sickle cell trait (AS), homozygous sickle cell anemia (SS), and double heterozygous states such as sickle cell-beta thalassemia (Sβ-thal), HbSC, Hb SD Punjab, HbS-Lepore (Fig. 2a to 2g).



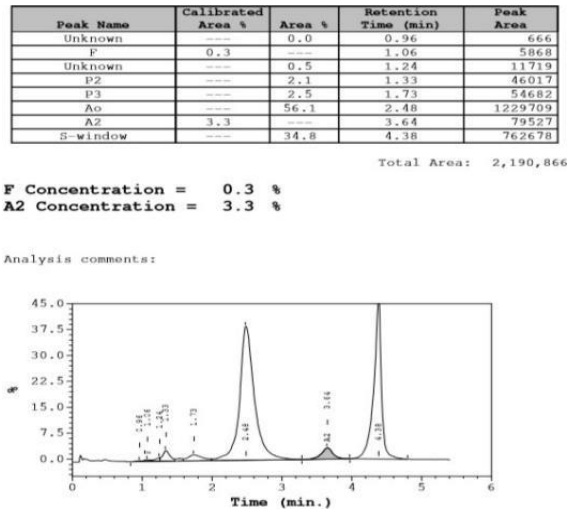
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	0.2	---	1.05	5079
Unknown	---	0.8	1.20	19962
P2	---	2.9	1.32	72722
Unknown	---	0.8	1.51	20945
P3	---	4.1	1.72	103424
Ao	---	88.6	2.43	2247080
A2	2.3	---	3.62	66283

Total Area: 2,535,495

F Concentration = 0.2 %
A2 Concentration = 2.3 %

Analysis comments:

Fig. 2a: Chromatogram of a healthy control (AA)



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.0	0.96	666
F	0.3	---	1.06	5868
Unknown	---	0.5	1.24	11719
P2	---	2.1	1.33	46017
P3	---	2.5	1.73	54682
Ao	---	56.1	2.48	1229709
A2	3.3	---	3.64	79527
S-window	---	34.8	4.38	762678

Total Area: 2,190,866

F Concentration = 0.3 %
A2 Concentration = 3.3 %

Analysis comments:

Fig. 2b: Chromatogram of heterozygous sickle cell anaemia (AS)

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Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	7.6*	---	1.11	179602
Ao	---	3.0	2.32	71328
A2	2.5	---	3.64	65896
S-window	---	86.6	4.37	2045045

Total Area: 2,361,87

F Concentration = 7.6* %
A2 Concentration = 2.5 %

*Values outside of expected ranges

Analysis comments:

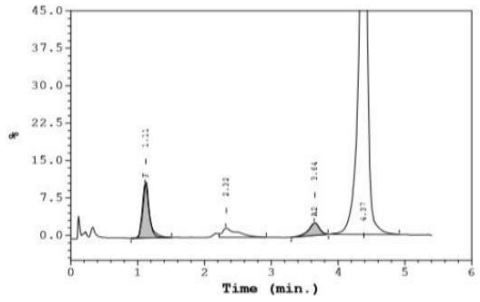

Fig. 2c: Chromatogram of homozygous sickle cell anaemia (SS)

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	12.1*	---	1.15	161288
Unknown	---	0.7	2.19	10193
Ao	---	1.5	2.34	21295
A2	5.2*	---	3.69	87401
S-window	---	80.3	4.40	1141067

Total Area: 1,421,244

F Concentration = 12.1* %
A2 Concentration = 5.2* %

*Values outside of expected ranges

Analysis comments:

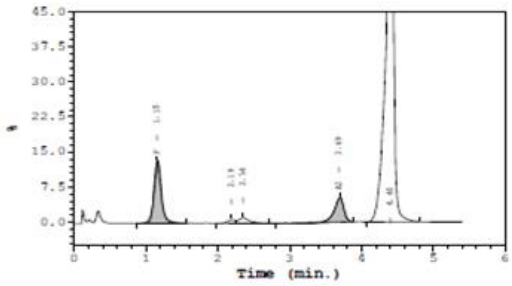

Fig. 2d: Chromatogram of Hb S-β Thalassemia

Patient Data
Sample ID: BHABANI KANTA BEHERA
Patient ID:
Name:
Physician:
Sex:
DOB:
Comments:

Analysis Data
Analysis Performed: 25/11/2017 16
Injection Number: 8662R
Run Number: 378
Rack ID:
Tube Number: 9
Report Generated: 25/11/2017 16
Operator ID: DR S SAHOO

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.0	0.86	766
F	8.0*	---	1.14	180237
Ao	---	1.7	2.36	39071
A2	2.8	---	3.64	61140
S-window	---	43.3	4.35	970800
Unknown	---	0.4	4.87	9463
C-window	---	43.7	5.13	979181

Total Area: 2,240,658

F Concentration = 8.0* %
A2 Concentration = 2.8 %

*Values outside of expected ranges

Analysis comments:

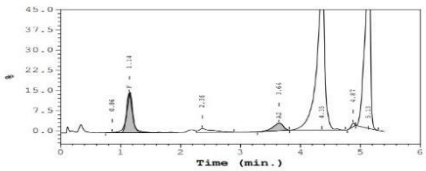

Fig. 2e: Chromatogram of HbSC

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	0.1	0.84	1090
F	30.1*	---	1.14	339306
Ao	---	2.4	2.12	28090
Unknown	---	0.9	2.30	10014
Unknown	---	1.2	2.91	14353
A2	1.9*	---	3.64	24748
D-window	---	41.6	4.07	485536
S-window	---	22.6	4.33	264238

Total Area: 1,167,374

F Concentration = 30.1* %
A2 Concentration = 1.9* %

*Values outside of expected ranges

Analysis comments:

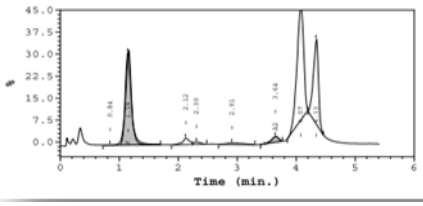

Fig. 2f: Chromatogram of SD Punjab

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	7.5*	---	1.12	159132
P2	---	0.4	1.28	9273
Unknown	---	0.2	1.60	3410
P3	---	0.3	1.75	7084
Unknown	---	1.1	2.16	24445
Ao	---	2.1	2.31	44758
Unknown	---	0.1	2.80	2354
A2	13.0*	---	3.52	304576
S-window	---	74.1	4.38	1589322

Total Area: 2,144,351

F Concentration = 7.5* %
A2 Concentration = 13.0* %

*Values outside of expected ranges

Analysis comments:

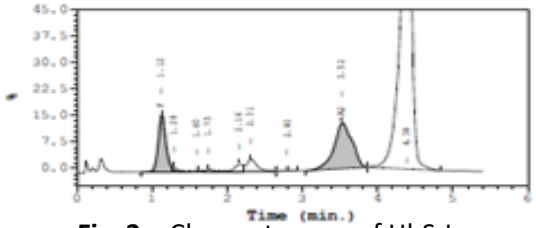

Fig. 2g: Chromatogram of HbS-Lepore

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DISCUSSION

A total of 496 cases, encompassing both pediatric and adult populations, were analyzed for the diagnosis of hemoglobin disorders in this study. Most cases originated from districts such as Bargarh, Sambalpur, and Jharsuguda, reflecting the regional dependence on the Sickle Cell Institute at VIMSAR, Burla, for healthcare needs. Pediatric cases predominantly came from the Department of Pediatrics, underscoring the importance of early diagnosis in childhood, which is crucial for effective disease management. This is particularly relevant given the poor pregnancy outcomes observed in women with sickle cell diseases, highlighting the significance of antenatal diagnosis for optimal case management [12-15].

The chromatogram analysis of the 496 cases demonstrated that the retention times of all identified variants were consistent with the manufacturer's guidelines, ensuring the reliability of HPLC as a diagnostic tool despite occasional uncertainties in the percentage of unknown variants. The study identified several abnormal hemoglobin variants, including Sβ, SE, SC, and Sd Punjab, with sickle cell variants being the most prevalent, consistent with previous reports indicating a high incidence in the study area [16-18].

β-thalassemia emerged as the second most common hemoglobin disorder, diagnosed across various forms such as β-thalassemia trait, major, and co-inherited with other variants. This aligns with national prevalence estimates and underscores the clinical diversity observed within hemoglobinopathies in India [19]. The distribution of cases showed a significant proportion in the pediatric age group (47.36%), highlighting the vulnerability of younger populations to severe forms of hemoglobinopathies, corroborating findings from other studies [18-21].

District-wise analysis revealed that Bargarh district contributed the highest number of cases, with disparities observed in sample representation from districts like Boudh and Deogarh, potentially due to sample size limitations. Previous studies have noted similar regional patterns, reflecting geographic variations in hemoglobinopathy prevalence [20-21]. Furthermore, caste-wise distribution highlighted a predominant prevalence among the Kulta caste, possibly linked to historical practices of consanguineous marriages facilitating the persistence of the sickle cell gene within specific gene pools [22].

The co-occurrence of hemoglobin E, particularly in heterozygous states, in a region with high sickle cell prevalence is noteworthy due to its clinical implications. Cases of Hb SD-Punjab trait further underscored the complexity of hemoglobinopathies in the study population, necessitating comprehensive diagnostic approaches [23-25].

Clinical parameters such as microcytic hypochromic anemia, elevated platelet and leukocyte counts, and variations in hemoglobin levels were consistent across different hemoglobinopathy groups, reflecting the diverse clinical presentations associated with these disorders [23-26]. Symptomatic manifestations including arthralgia, vaso-occlusive crises, and growth retardation, were prevalent among specific variants, necessitating targeted management strategies tailored to individual clinical profiles [24-27].

Elevated alkaline phosphatase levels observed in sickle cell patients underscored the severity of bone involvement during crises, necessitating careful monitoring and management of bone complications [25-28]. Similarly, renal and hepatic dysfunction indicators highlighted the multisystem involvement in hemoglobinopathies, emphasizing the need for holistic management approaches addressing both hematologic and systemic manifestations [25-28].

CONCLUSIONS

In conclusion, HPLC (Variant II hemoglobin testing system) emerged as a reliable tool for the accurate and rapid diagnosis of hemoglobin disorders in this study. The high prevalence of sickle cell and β-thalassemia mutations in the study area underscores the importance of early diagnosis for effective disease management. Knowledge of regional hemoglobin variants and their clinical manifestations is crucial for guiding appropriate preventive and therapeutic interventions. However, further research incorporating larger datasets and exploring clinical modifiers influencing disease severity is warranted to enhance our understanding and management of hemoglobinopathies.

CONTRIBUTION OF AUTHORS

Research concept- Narendra Kumar Majhi, Binod Kumar Sahu

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Research design- Bhagyashree Khatua, Deepak Ranjan Bhol
Supervision- Alaka Sahu
Materials- Narendra Kumar Majhi, Binod Kumar Sahu
Data collection- Bhagyashree Khatua, Deepak Ranjan Bhol
Data analysis and Interpretation- Alaka Sahu
Literature search- Narendra Kumar Majhi, Binod Kumar Sahu
Writing article- Bhagyashree Khatua, Deepak Ranjan Bhol
Critical review- Alaka Sahu
Article editing- Narendra Kumar Majhi, Binod Kumar Sahu
Final approval- Alaka Sahu

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