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Spectrum of abnormal haemoglobin variant by cation exchange HPLC (D-10) in suspected population of haemoglobinopathy

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Abstract:

Inherited hemoglobin disorders include a broad spectrum of conditions, from thalassemia to structurally altered hemoglobin variants (hemoglobinopathies). Globally, it is estimated that 5% of people carry a potentially harmful hemoglobin gene. In India, the incidence of thalassemia traits ranges from 3% to 17%, while sickle cell disease affects between 1% to 44% of the population.

Certain populations in India show a relatively higher frequency of these disorders due to factors such as consanguinity, caste, and regional endogamy, which contribute to the public health burden. Hemoglobi- nopathies in India have a cumulative gene frequency of around 4.2%.

In some communities, the prevalence of Beta-Thalassemia mutations can reach up to 17%, while the frequency of hemoglobin D (Hb-D) is estimated to be approximately 1.1%. HPLC is a important screening tool for detection of various hemoglobinopathies. Molecular testing plays a crucial role in identifying hemoglobinopathies, especially in cases with abnormal hemoglobins like HbD, HbE, and HbS, where there may also be co-inheritance of beta-thalassemia mutations.

The present study was conducted to evaluate the diagnosis of various haemoglobinopathies using Cation exchange HPLC.

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The study was conducted at Bundelkhnad medical college in a duration of 18 months after obtaining ethical clearance from the IEC.

The findings consisted of Haemogloniopathies such as Hb D disease amd HbE disease along with sickle major sickle and Thalessemia trait.The findings were in concurrence with the peripheral smear along with the other pre tests used in case of suspicion of haemoglobinopathies.

The present study has similar findings as many other studies carried out over a period of time all across the world and especially in India.

We conclude that HPLC can be used for confirmation of the diagnosis of haemoglobiopathies although it is always better to cross check with mass spectrophotometer.

Introduction

The prevalence of thalassemia and other hemoglobinopathies varies significantly across different regions. In India, the prevalence of pathological hemoglobinopathies is estimated at 1.2 per 1,000 live births. [1]

The carrier frequency for hemoglobinopathies in India ranges between 3% and 17% in various populations. Cumulatively, the gene frequency of three abnormal hemoglobins sickle cell hemoglobin, hemoglobin D, and hemoglobin E is approximately 5.35%. [2]

Abnormal hemoglobin arises due to either a qualitative or quantitative defect in the hemoglobin molecule. Among the hemoglobinopathies in India, Beta-Thalassemia is the most common, followed by Sickle Cell Disease.

Aims And Objective

1) To correlate the socio-demographic distribution of various hemoglobinopathies on HPLC in Bundelkhand region of Madhya Pradash.

2) To compare Mentzer and Ricerca indices with HPLC findings in Thalassemia.

3) To compare peripheral blood smear and sickling test with HPLC in Sickle cell anemias.

4) To categorize rare hemoglobinopathies on HPLC in Bundelkhand region of Madhya Pradash.

MATERIAL AND METHOD

Study was conducted in the Department of Pathology, Bundelkhand medical college sagar Madhya pradash during July 2023 to december 2024 around 18 months. Study included around 197 patients presenting with anemia suspected of haemoglobinopathies indepartment of pathology.

INCLUSION AND EXCLUSION CRITERIA

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Inclusion criteria :-

All the anemic patients suspected of haemoglobinopathies above 1 year of age.

Exclusion criteria :-

Patients who have refused to give consent for study.

Patients with history of blood transfusion in past three months.

Newborns.

STUDY GROUP:

All the anemic patients suspected of haemoglobinopathies.

TYPE OF STUDY/STUDY DESIGN:

Observational study.

ETHICAL COMMITTEE APPROVAL:

Present study is conducted after approval from institutional ethical committee. (ethical committee no. - IEC/BMC/DHR/2023/13)

DURATION OF THE STUDY:

18 months after approval from Institutional Ethics Committee in Govt. Bundelkhand Medical College and Hospital, Sagar MP.

CONSENT:

Informed/ Written/audio consent will be obtained from the patients to participate in this study.

DATA COLLECTION:

Especially designed pre-structured proforma will be used for collecting the relevant data.

The data will be obtained from patient's history, blood investigations, Peripheral blood smear finding and cation - exchange HPLC finding.

Plan for statistical analysis of the study:

Analysis will be carried out by appropriate statistical methods.

- For quantitative data: SD (Standard Deviation) will be calculated.
- For qualitative data: Frequency and Percentage will be calculated.

Appropriate statistical test will be applied for hypothesis testing.

Python and Its libraries will be used for data analysis.

STUDY METHOD:

- The study will be conducted as per ICMR 2017 guidelines and after due approval from the Institutional Ethics Committee.

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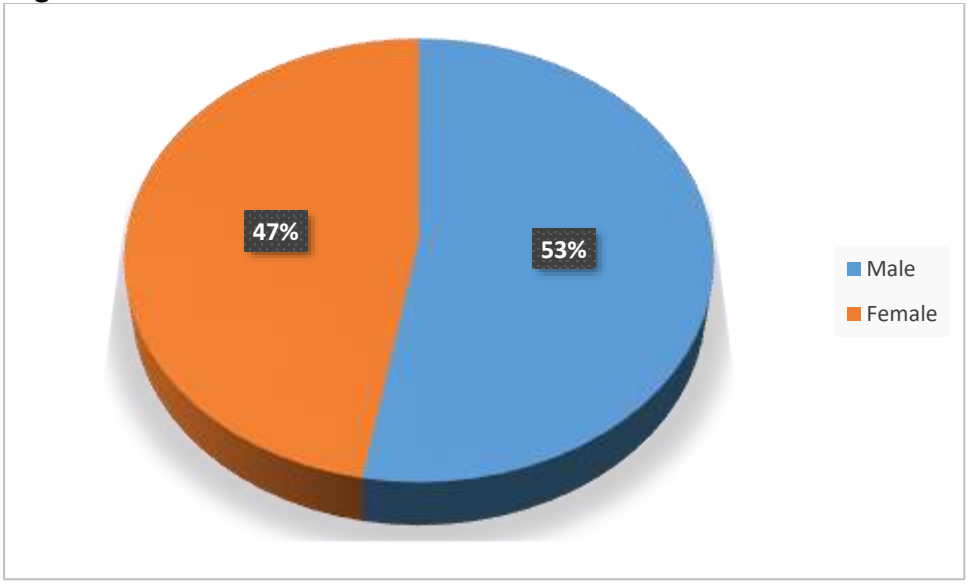
- Patients meeting the eligibility criteria will be enrolled in the study after obtaining a Informed/ Written/audio consent.
- Blood sample was withdrawn in EDTA and analyzed on cell counters (Mindray BC5300 or Mindray BC3600).
- The red cell indices noted and peripheral smear was evaluated. Screening tests - supravital staining (new methylene blue) for reticulocyte count, sickling test using 2% sodium metabisulphite for HbS done. Samples were then run-on BIO-RAD D10 HPLC.

RESULTS

A total of 197 cases were studied in this study between July 2023 to december 2024 . Of these 197 cases, 104 (52.79%) were male, and the remaining 93 (47.21%) were female.

Figure 1: Gender-wise distribution of the cases



Of these 197 cases, 98 (49.75%) were married, and the remaining 99 (50.25%) were unmarried. The data presented in Table 2 were insignificant, with a p-value of 0.94.

Figure 2: Marital Status distribution of the cases

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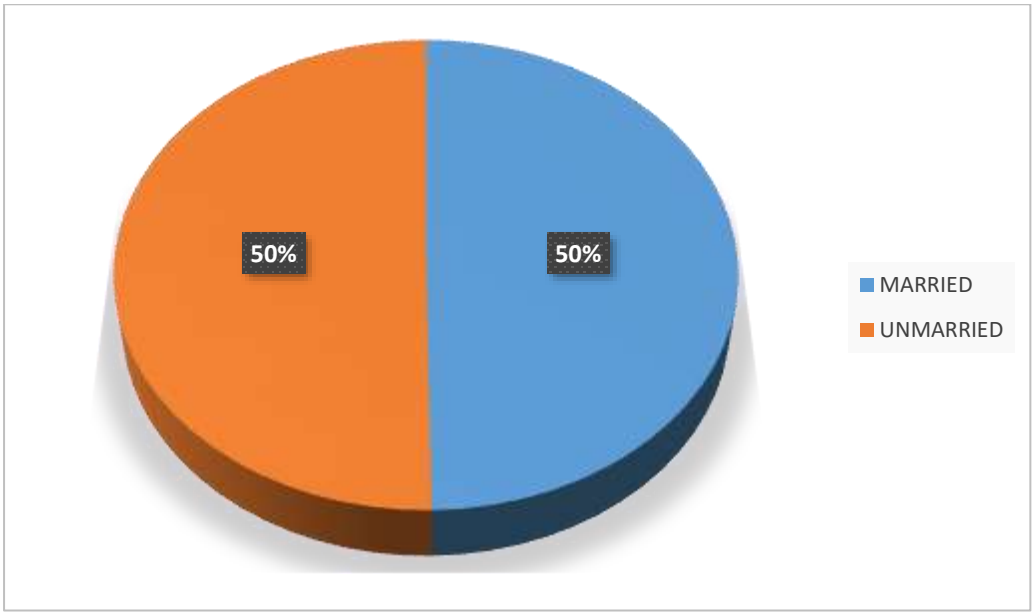

INFERENTIAL ANALYSIS

To evaluate the utility of Mentzer and Ricerca indices in predicting the diagnosis using HPLC, multi-class Logistic Regression was performed. The model score was 0.647. The Confusion Matrix is presented in Table 14.

Table 1: Confusion Matrix for Multi-class Logistic Regression using only Mentzer and Ricerca indices to predict HPLC diagnosis (N=17)

		Predicted Value		
		0	**1**	**2**
Truth Value	**0**	9	0	0
	1	4	2	0
	2	2	0	0

The heatmap for the Multi-class Logistic Regression is presented in Figure 3.

Heatmap for Multi-class Logistic Regression using Mentzer and Ricerca indices to predict HPLC diagnosis (N=17)

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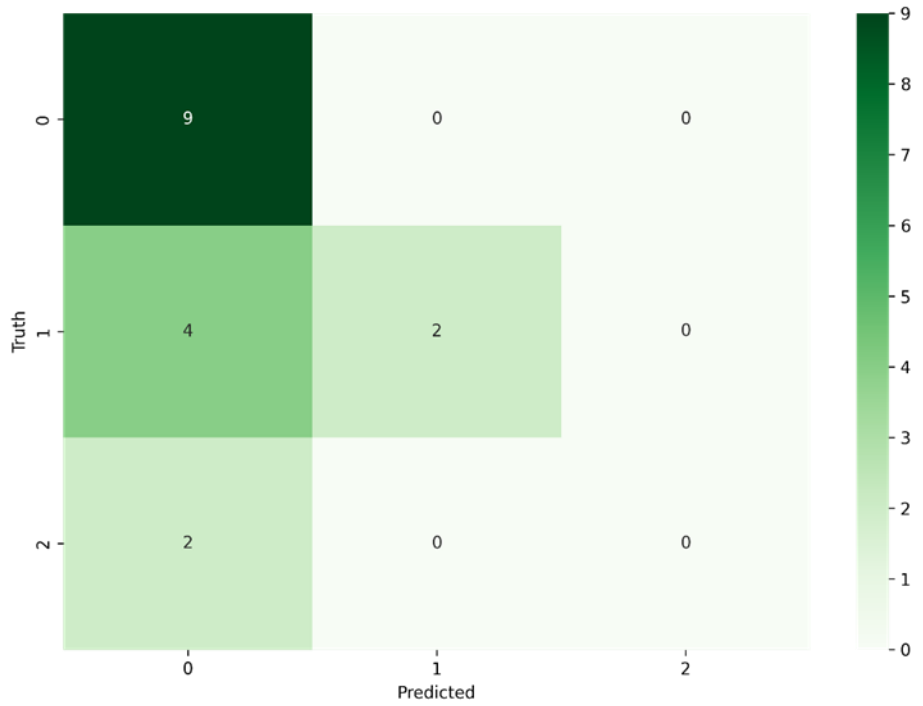

Upon including demographic data, age, gender, religion, and caste, with Mentzer and Ricerca indices to predict the diagnosis using HPLC, multi-class Logistic Regression was performed. The model score was 0.8823. The Confusion Matrix is presented in Table 15.

Table 2: Confusion Matrix for Multi-class Logistic Regression using Demographic data with Mentzer and Ricerca indices to predict HPLC diagnosis (N=17)

		Predicted Value		
		0	**1**	**2**
Truth Value	**0**	8	1	0
	1	1	5	0
	2	0	0	2

Note: 0 = B-THAL TRAIT; 1 = B-THAL INTERMEDIA; 2 = B-THAL MAJOR

The heatmap for the Multi-class Logistic Regression is presented in Figure 4.

Figure 4: Heatmap for Multi-class Logistic Regression using Demographic data with Mentzer and Ricerca indices to predict HPLC diagnosis (N=17)

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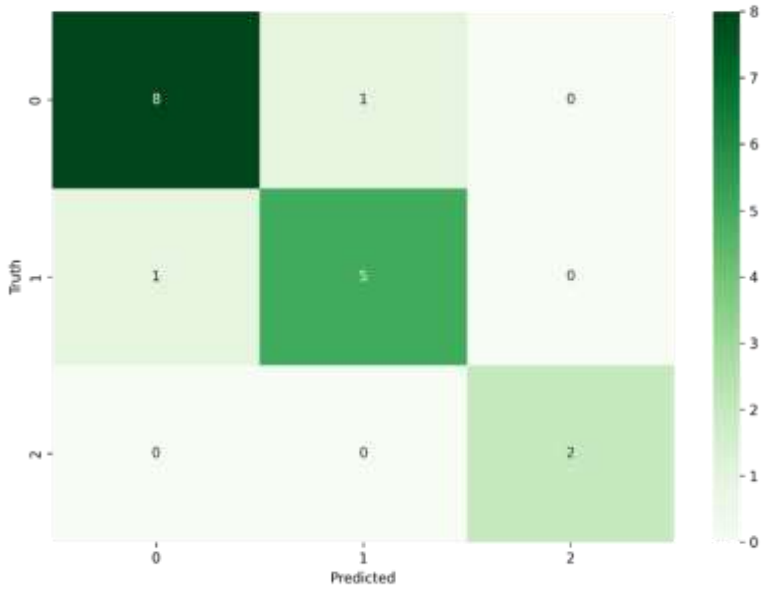

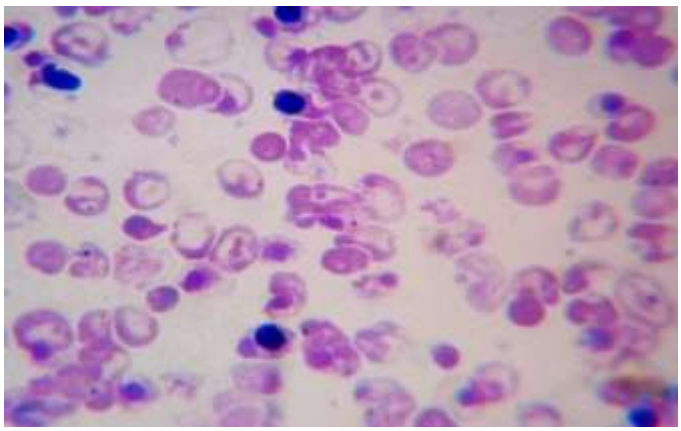

Figure 5:- A case of thalassaemia major with numerous nRBC's and target cells.

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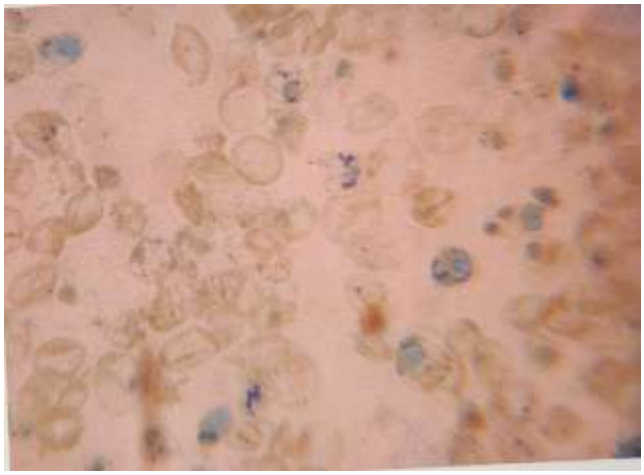
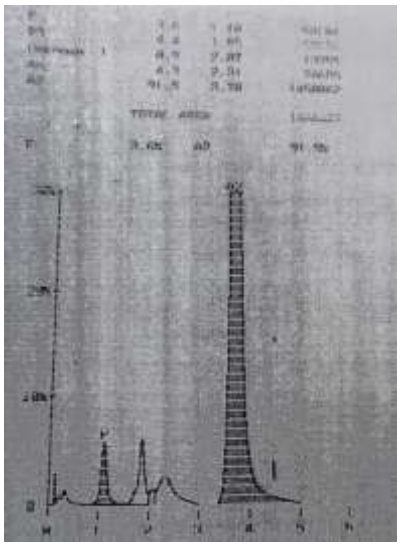


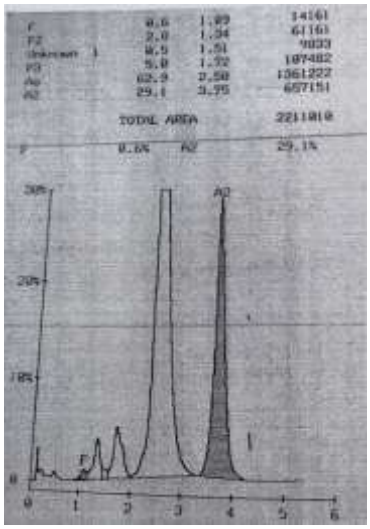
Figure 6:- A case of thalassaemia major with high reticulocyte count.

HPLC RESULT

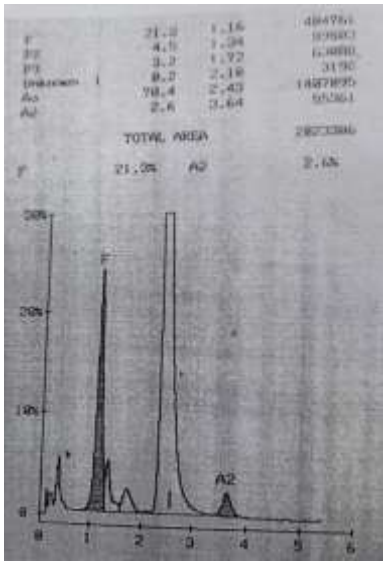
Chromatogram 1: HbD Iran Heterozygous



Chromatogram 2: HbE heterozygous



Chromatogram 3: Thalassaemia homozygous/ intermedia



Chromatogram 4: Sickle with β -Thalassaemia trait

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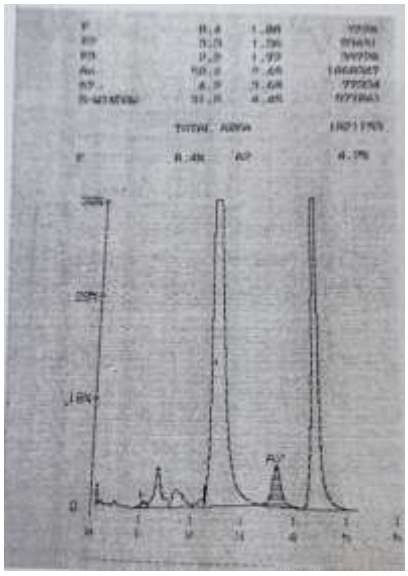

Discussion

A large number of haemoglobin variants prevalent in the population indicate that thalassaemias & haemoglobinopathies are not uncommon amongst our population. The inherited disorders of haemoglobin synthesis are one of the important public health problems in India.

Haemoglobinopathies are the most common disorders of haemoglobin. India is the home of several haemoglobin variants. The most common Haemoglobinopathy in this study is β- thalassaemia trait followed by

B-thal intermedia. The present study was conducted on 197 cases.

In the present study, out of 197 cases analyzed, 104 (52.79%) were male and 93 (47.21%) were female, indicating a slight male predominance. This finding aligns with the results reported by Archana Buch et al. who observed a higher proportion of males (61%) in their study population. [3] However, contrasting findings were reported by D. Mukhopadhyay et al., Atul Shrivastav et al., and Embong et al., [4,5,6] all of whom documented a female predominance in their respective studies. These variations may be attributed to differences in demographic profiles, sample sizes, and study settings.

In this study most frequently observed study population was 21-30 years of age group accounting for 30.96% (61) participants. This result is consistent with the study by D. Mukhopadhyay et al. where the average age of the study population was 22.9 years. A significant proportion of our study participants fell within the 20-30 year age range. This higher representation could be attributed to a greater propensity for individuals in this age group to seek hospital care for various health concerns.

Our study found that a majority of participants practiced Hinduism, a finding consistent with previous studies by D. Mukhopadhyay et al., T. Adeyemo et al., S. Raman et al. [4,7,8]

In our study conducted in the Bundelkhand region, Hindu participants constituted the

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majority. This is consistent with the demographic composition of the region, where the Hindu community forms a significant proportion of the population.

In our study, the most frequently reported clinical presentation was pallor, observed in 181 participants (91.88%). This finding is consistent with the observations of S. Raman et al., and Atul Shrivastava et al. Similarly, Rachna Khera et al. also reported pallor as often being the first clinical symptom in cases of thalassemia.[5,9]

In our study, out of 197 cases, 27 were diagnosed with HPLC, while the remaining cases were considered normal. The most common diagnosis among the HPLC cases was β-thalassemia trait, accounting for 33.33% (9 cases), followed by β-thalassemia intermedia at 22.22% (6 cases), and sickle cell trait at 11.11% (3 cases).

Our finding that beta thalassemia is the most common diagnosis aligns with the observations reported by Rachna Khera et al., D. Mukhopadhyay et al. and Atul Shrivastav et al. However, in contrast to our results, Sarojini Raman identified sickle cell disorder as the most prevalent condition in her study.[4,5,9]

In our study, the sickling test was positive in all six diagnosed cases of sickle cell anemia. This finding is consistent with the observations reported by T. Adeyemo et al., Murab Parul et al., S.V. Raman et al., and Atul Shrivastava et al., who also reported 100% positivity of the sickling test among confirmed cases.[7,8,9,10]

In studies conducted by T. Adeyemo et al., Murab Parul et al., S.V. Raman et al., and Atul Shrivastava et al., all cases that tested positive on the sickling test demonstrated a normocytic normochromic peripheral blood smear pattern. This consistent morphological finding supports the classical hematological presentation of sickle cell disorders in the absence of concurrent nutritional deficiencies or other causes of red cell morphological changes.[7,8,9,10]

In our study, out of six cases diagnosed with sickle cell anemia, four exhibited microcytic hypochromic peripheral blood smear (PBS) findings, while only two showed normocytic normochromic features. This variation in red cell morphology could be attributed to coexisting iron deficiency anemia, which is commonly observed in regions with a high burden of nutritional deficiencies or chronic disease. Iron deficiency can mask or alter the typical normocytic normochromic picture of sickle cell disease, leading to a microcytic hypochromic pattern. Therefore, the presence of microcytosis and hypochromia in the majority of our sickle cell cases may reflect overlapping nutritional anemia rather than pure hemoglobinopathy alone.

Although traditionally a Mentzer Index <13 suggests thalassemia trait, consistent findings in our study and others (Mondal et al., Mukhopadhyay et al., Khera et al., Shrivastava et al.) indicate values >13 in some thalassemia trait patients. This may be attributed to co-existing iron deficiency, population-specific hematological parameters, and the limitations of Mentzer Index as a standalone diagnostic tool. Hence, relying solely on this index may lead to misclassification, underscoring the need for confirmatory tests like HPLC.[8,9,10,11]

In this study, the Ricerca Index was higher in thalassemia major and intermedia, consistent with findings by Mondal et al., Mukhopadhyay et al., Khera et al., and Shrivastava et al. This reflects more severe hematological abnormalities in advanced

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thalassemia and highlights the need for confirmatory tests alongside index-based screening.

Unlike studies by Gupta et al., Raman et al., and Buch et al. that showed male predominance, our study found more female cases of abnormal hemoglobinopathies. This may be due to increased screening in females, especially during antenatal visits, as well as regional healthcare-seeking patterns and sampling differences. Since hemoglobinopathies are autosomal, the variation is likely due to socio-demographic factors rather than a true biological difference.

My study findings are consistent with those of Archana Buch and Mondak, who reported maximum number of HPLC cases in the Sudhra community.
HPLC has been shown to be a sensitive, specific, and reproducible alternative to electrophoresis. With automation and quantitative power, it appears to be a sensitive and accurate technique for direct identification and quantification of normal and abnormal haemoglobin fractions. [3,11]
Particularly in our study other than β thalassaemias other type of haemoglobin variants were also diagnosed such as Hb S, E, C, D, Iran and intermedia.
Compare to traditional electrophoresis HPLC has following additional advantages:-
a) The analyzer is automated and thus utilizes less staff time and permit processing of large batches of samples.
b) Very small samples are sufficient for analysis which is especially useful in pediatrics work.
c) Quantification of normal and variant haemoglobins is available on every sample.
d) HPLC was also able to separately identify two haemoglobin variants of HbD family; HbD-Iran and HbD-Punjab. Both exhibited identical electrophoretic mobilities but eluted in A2 and D windows respectively on HPLC.
These situations are clinically important because HbD-Punjab produces a significant sickling disorder when present in a double heterozygous HbD- HbS form; whereas HbD-Iran is clinically benign.
The misdiagnosis of HbD-Iran as HbD-Punjab based solely on Hb- electrophoresis or as HbE based solely on Hb- HPLC, where the subtle difference in %Hb or retention time is disregarded, may lead to incorrect genetic counseling in addition to undue anxiety for the family.
One of the foremost limitations in HPLC technique is its cost and not availability in rural centres , a false high or low value of Hb A2 in case of megaloblastic anemia & iron deficiency anemia respectively and an invalid impression if there is a recent history of blood transfusion (within 3 months).
HPLC is a revolutional investigation which can easily take to remotest corner of the country & can be used as a screening as well as diagnostic modality for thalassaemia & Haemoglobinopathy.

Conclusion

This study provides a comprehensive overview of the demographic, clinical, and hematological profiles of patients evaluated for hemoglobinopathies using HPLC analysis. Among the 197 cases analyzed, 27 (13.7%) were diagnosed with abnormal hemoglobin patterns, with Beta Thalassemia Trait being the most common, followed by Beta Thalassemia Intermedia and Sickle Cell Trait.

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Demographic variables such as gender, marital status, religion, and caste showed no significant association with the occurrence of hemoglobinopathies in HPLC-positive cases. However, the age distribution, religious composition, caste, and clinical presentation (pallor) were significantly associated with the overall case population.

The use of Mentzer and Ricerca indices demonstrated moderate predictive ability for distinguishing types of Beta Thalassemia, with the logistic regression model yielding an accuracy of 64.7%. This suggests these indices can serve as useful, low-cost preliminary screening tools, especially in resource-limited settings. Targeted awareness and intervention programs are crucial for the early identification and management of inherited blood disorders such as thalassemia and sickle cell disease.

Limitation

Limitations of HPLC in Diagnosing Hemoglobinopathies:- High-Performance Liquid Chromatography (HPLC) is widely recognized as one of the most reliable and precise methods for identifying and quantifying hemoglobin variants. However, like any diagnostic technique, HPLC does have some limitations when it comes to diagnosing hemoglobinopathies and related blood disorders. Below are the key limitations:

Requirement for Specialized Equipment and Expertise: HPLC requires expensive instruments and highly specialized technical skills for operation, maintenance, and data interpretation. This can limit its availability in low-resource or remote settings, where the necessary infrastructure and trained personnel may not be present. The interpretation of results also demands expert knowledge to differentiate between closely related hemoglobin variants, which could lead to potential misinterpretation without skilled professionals.

Inability to Detect Low Concentrations of Hemoglobin Variants: HPLC may not be sensitive enough to detect low concentrations of hemoglobin variants, such as in heterozygous carriers of sickle cell trait or thalassemia. In these cases, the amount of abnormal hemoglobin might be insufficient to produce a detectable peak, leading to false negatives.

In conditions with very low levels of hemoglobin S (HbS) or hemoglobin F (HbF), these variants might not be accurately detected, especially in situations where the variant is mixed with normal hemoglobin (HbA).

Difficulty in Detecting Rare or Uncommon Hemoglobin Variants: While HPLC is excellent for detecting the most common hemoglobinopathies like sickle cell disease (HbSS) and thalassemia, it may have limitations in detecting rare or less common hemoglobin variants. Variants with atypical migration patterns may be missed or misclassified. Variants with similar elution profiles (e.g., HbD, HbE, and HbC) can be difficult to distinguish from each other using HPLC, as they may overlap in their migration on the chromatographic column.

Interference from High Hemoglobin Levels: In patients with high hemoglobin concentrations or polycythemia, the overlapping peaks of hemoglobins can complicate the accurate quantification and differentiation of specific hemoglobin variants. Falsely high readings may occur if there is an abnormal increase in certain hemoglobin fractions or interference due to the presence of other abnormal proteins.

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Cost and Accessibility: The high cost of HPLC systems and their maintenance can be prohibitive for many hospitals or laboratories, especially in developing countries or low-resource settings. This cost can limit widespread screening and diagnosis of hemoglobinopathies.

Moreover, the need for reagents, consumables, and calibration can add to the ongoing financial burden, making it less feasible for routine use in some regions.

Inability to Provide Molecular-Level Information: While HPLC is highly effective at detecting and quantifying hemoglobin variants, it does not provide detailed molecular information about the genetic mutations causing the hemoglobinopathies. For example, it cannot distinguish between the exact mutations responsible for hemoglobin S (sickle cell anemia) or hemoglobin C (HbC disease).

Molecular techniques like PCR or DNA sequencing are required to provide a definitive diagnosis of the genetic mutations associated with hemoglobinopathies.

Possible Variability in Results: The results from HPLC can be influenced by technical factors, such as sample preparation, column degradation, or equipment malfunction, which could lead to inaccurate results.

Minor variability in results can also occur based on the specific HPLC system or method used, which could cause differences in the peak patterns of hemoglobin fractions.

Limitations in Diagnosing Compound Heterozygous States: In patients with compound heterozygous states (e.g., co-inheritance of sickle cell disease with another hemoglobinopathy like thalassemia or HbC), HPLC may not always distinguish the combination of variants effectively, making it difficult to interpret complex cases without further confirmation through other tests, such as genetic analysis.

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