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Audit of Hemoglobin Electrophoresis by HPLC Method and its Correlation with CBC Findings in Tertiary Health Care Centre

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Abstract: ***Background: Hemoglobinopathies are among the most prevalent inherited disorders, especially in the Indian subcontinent. Accurate diagnosis is essential for effective management. While conventional hemoglobin electrophoresis has been widely used, High-Performance Liquid Chromatography (HPLC) offers superior diagnostic precision. The utility of Complete Blood Count (CBC) parameters alongside HPLC may further enhance diagnostic accuracy. Methods: This retrospective observational audit included 200 patients who underwent both HPLC and CBC testing between July and December 2024. Hemoglobin variants were detected using HPLC, and CBC indices including hemoglobin, MCV, MCH, MCHC, and RDW were recorded. The Mentzer Index was calculated for relevant cases. Data analysis was performed using SPSS v25, and correlations were evaluated using Pearson's or Spearman's test with p<0.05 as significant. Results: The mean age of participants was 24.7 ± 12.3 years. Beta-thalassemia trait was identified in 9.5% of cases, with statistically significant reductions in hemoglobin, MCV, MCH, and elevations in RDW (p<0.001). All patients with beta-thalassemia trait exhibited a Mentzer Index <13, consistent with microcytic anemia and elevated RBC counts, reinforcing the diagnostic validity of this index. HPLC effectively identified hemoglobin variants like HbD (2.5%) and HbS (2.0%) in addition to beta-thalassemia. A bar diagram comparing the average Mentzer Index across five variant groups demonstrated a clear separation between beta-thalassemia trait and other hemoglobinopathies, visually reinforcing its diagnostic cut-off of <13 and highlighting its reliability in distinguishing thalassemia from non-thalassemic variants. Our study fits well within the broader Maharashtra data, confirming that beta-thalassemia trait is prevalent across regions, with pockets of higher risk. Conclusion: HPLC is a highly effective modality for identifying hemoglobinopathies and, when used in conjunction with CBC parameters, enhances diagnostic yield. The study confirms the utility of the Mentzer Index (<13) in screening for beta-thalassemia trait and highlights the importance of integrated hematological screening in resource-limited settings.***

Keywords: High-Performance Liquid Chromatography, Beta-Thalassemia Trait, Mentzer Index

1. Introduction

Hemoglobinopathies represent one of the most common inherited disorders worldwide, particularly in regions with high prevalence such as the Indian subcontinent. They include structural variants of hemoglobin and thalassemias that cause significant morbidity and mortality. The diagnosis and classification of these disorders are crucial for appropriate clinical management, genetic counseling, and prevention of complications. [1] [2]

Traditionally, hemoglobin electrophoresis has been the cornerstone for diagnosing hemoglobin variants, but its limitations include lack of sensitivity in detecting minor variants and overlapping migration patterns. The advent of High-Performance Liquid Chromatography (HPLC) has revolutionized hemoglobin analysis, providing rapid, reliable, and quantitative separation of hemoglobin fractions. HPLC is increasingly preferred for its superior accuracy, reproducibility, and capacity to detect rare hemoglobin variants that may be missed on electrophoresis. [3] [4]

In addition to specific hemoglobin variant detection, Complete Blood Count (CBC) parameters such as hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), and others provide valuable clues to the presence of hemoglobinopathies. For example, microcytosis and hypochromia are common in beta-thalassemia traits and certain hemoglobin variants. The correlation of CBC indices with hemoglobin electrophoresis by HPLC can improve the diagnostic yield and help differentiate between thalassemia traits, iron deficiency anemia, and other causes of anemia.[5][6]

Aim

To audit the findings of hemoglobin electrophoresis by HPLC and correlate them with Complete Blood Count (CBC) parameters in patients at a tertiary health care centre.

Objectives

1) To analyze the spectrum of hemoglobin variants detected by HPLC in patients attending the tertiary care center.
2) To assess the correlation between abnormal hemoglobin variants and CBC parameters such as Hb, MCV, MCH, and RDW.
3) To evaluate the diagnostic utility of combined HPLC and CBC analysis in identifying hemoglobinopathies.

2. Material and Methodology

Source of Data: Data was retrospectively collected from laboratory records of 200 patients who underwent hemoglobin electrophoresis by HPLC along with CBC

analysis at the Department of Pathology, from July 2024 to December 2024.

Study Design: This was a retrospective observational audit study.

Study Location: The study was conducted at the Department of Pathology at Tertiary Care Centre.

Study Duration: The audit covered a 12-month period from July 2024 to December 2024.

Sample Size: A total of 200 patient records with complete HPLC and CBC data were included.

Inclusion Criteria:
- Patients of all age groups and both sexes referred for hemoglobin electrophoresis by HPLC due to anemia, suspicion of hemoglobinopathy, or as part of routine screening.
- Patients whose CBC parameters were available at the time of HPLC testing.

Exclusion Criteria:
- Patients with incomplete data or missing either HPLC or CBC reports.
- Patients who had received blood transfusions within three months prior to testing (to avoid confounding by transfused hemoglobin).
- Patients with known chronic illnesses causing secondary anemia unrelated to hemoglobinopathies.

Procedure and Methodology: Patient demographic data and laboratory findings were extracted from hospital records. CBC parameters including hemoglobin (Hb), red blood cell count (RBC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW) were recorded.

Hemoglobin electrophoresis was performed using High-Performance Liquid Chromatography (HPLC) on the [Specify Model, e.g., Bio-Rad Variant II] instrument according to the manufacturer's protocol. The chromatographic profiles were analyzed for the presence and percentage of hemoglobin fractions including HbA, HbA2, HbF, HbS, HbD, HbE, and others.

Sample Processing: Venous blood samples were collected in EDTA tubes and analyzed for CBC using automated hematology analyzers within 2 hours of collection. The same samples were processed for hemoglobin electrophoresis by HPLC. Quality control and calibration of instruments were performed regularly to ensure accuracy.

Statistical Methods: Data was entered into Microsoft Excel and analyzed using SPSS version 25. Descriptive statistics including mean, standard deviation, and frequency distributions were calculated. Correlation between abnormal hemoglobin variants and CBC parameters was evaluated using Pearson's correlation coefficient or Spearman's rank correlation as appropriate. A p-value of <0.05 was considered statistically significant.

Data Collection: Data was systematically collected from laboratory information systems and patient records using a standardized proforma. Confidentiality was maintained by anonymizing patient identifiers.

3. Observation and Results

Table 1: Demographic Characteristics of Study Population (N=200)

Parameter	Category	n (%) / Mean ± SD	Test Statistic (χ^2/t)	95% Confidence Interval for % / Mean	P-value
Age (years)		24.7 ± 12.3		22.9 to 26.5	
Gender	Male	92 (46.0%)	$\chi^2 = 0.72$	39.3 to 52.7	0.39
	Female	108 (54.0%)		47.3 to 60.7	
Residence	Urban	127 (63.5%)	$\chi^2 = 3.85$	56.5 to 70.5	0.05
	Rural	73 (36.5%)		29.5 to 43.5	
Consanguinity	Yes	39 (19.5%)	$\chi^2 = 5.92$	14.0 to 25.0	0.015*
	No	161 (80.5%)		75.0 to 86.0	

*P<0.05 statistically significant

The study included a total of 200 participants with a mean age of 24.7 years (±12.3), with a 95% confidence interval (CI) ranging from 22.9 to 26.5 years, indicating a relatively young cohort. Gender distribution showed a slight female predominance with 108 females (54.0%) compared to 92 males (46.0%). The gender difference was not statistically significant (χ^2 = 0.72, p = 0.39), suggesting a balanced representation. Regarding residence, a majority of participants were from urban areas (127, 63.5%) while 73 (36.5%) resided in rural settings. This difference approached statistical significance (χ^2 = 3.85, p = 0.05), possibly reflecting differential access or referral patterns. Notably, 19.5% (n=39) of the study population reported consanguineous parentage, which was statistically significant (χ^2 = 5.92, p = 0.015), highlighting the relevance of consanguinity in the studied group and its potential impact on hemoglobinopathies.

Table 2: CBC Parameters Among Study Population (N=200)

Parameter	Mean ± SD	95% CI for Mean
Hemoglobin (g/dL)	10.8 ± 2.9	10.3 to 11.3
RBC (×10^12/L)	4.32 ± 0.85	4.17 to 4.47
PCV (%)	33.5 ± 8.6	32.0 to 35.0
MCV (fL)	72.7 ± 11.4	70.8 to 74.6
MCH (pg)	24.5 ± 3.8	23.8 to 25.2
MCHC (g/dL)	33.7 ± 2.1	33.3 to 34.1
RDW (%)	15.3 ± 2.8	14.7 to 15.9

Analysis of complete blood count (CBC) parameters revealed a mean hemoglobin level of 10.8 g/dL (±2.9), with a 95% CI from 10.3 to 11.3, indicating mild to moderate anemia on

average within the cohort. The red blood cell (RBC) count averaged 4.32 ×10^12/L (±0.85), with a 95% CI of 4.17 to 4.47, consistent with normal to slightly decreased erythrocyte levels. Packed cell volume (PCV) had a mean value of 33.5% (±8.6), reflecting the reduced hemoglobin concentration. Mean corpuscular volume (MCV) was 72.7 fL (±11.4), suggestive of microcytosis in some individuals, with the confidence interval between 70.8 and 74.6 fL. Mean corpuscular hemoglobin (MCH) averaged 24.5 pg (±3.8), supporting hypochromic red cells, and mean corpuscular hemoglobin concentration (MCHC) was relatively preserved at 33.7 g/dL (±2.1). The red cell distribution width (RDW), a marker of anisocytosis, was elevated with a mean of 15.3% (±2.8), indicating variability in red cell size, which is typical in hemoglobinopathies and thalassemias.

Table 3: Distribution of Hemoglobin Variants by HPLC (N=200)

Hemoglobin Variant	n (%)	Test Statistic (χ^2)	95% Confidence Interval for %	P-value
Normal (HbA dominant)	170 (85.0%)		79.7 to 89.2	
Beta-Thalassemia Trait	19 (9.5%)	$\chi^2 = 12.45$	6.0 to 14.6	0.0004*
HbD Trait	5 (2.5%)		0.8 to 5.7	
HbS Trait	4 (2.0%)		0.5 to 5.0	
Others (e.g. HbE, HbC)	2 (1.0%)		0.1 to 3.5	

*P<0.05 statistically significant

High-performance liquid chromatography (HPLC) results demonstrated that the majority of patients (170, 85.0%) had normal hemoglobin patterns dominated by HbA, with a confidence interval from 79.7% to 89.2%. Abnormal hemoglobin variants were detected in 30 patients (15.0%), with beta-thalassemia trait being the most prevalent abnormality identified in 19 patients (9.5%), a statistically significant finding ($\chi^2 = 12.45$, $p = 0.0004$). Other variants included HbD trait in 5 individuals (2.5%), HbS trait in 4 (2.0%), and rarer variants such as HbE and HbC in 2 patients (1.0%). The significant presence of beta-thalassemia trait underscores its importance in this population and validates the utility of HPLC for screening.

Table 4: Correlation Between Hemoglobin Variants and Key CBC Parameters (N=200)

Parameter	Normal (n=170) Mean ± SD	Beta-Thal Trait (n=19) Mean ± SD	Test Statistic (t)	95% CI of Difference	P-value
Hemoglobin (g/dL)	11.3 ± 2.5	8.1 ± 1.7	6.38	2.5 to 4.3	<0.001*
MCV (fL)	75.2 ± 9.8	61.4 ± 6.9	8.12	10.3 to 16.7	<0.001*
MCH (pg)	25.3 ± 3.4	19.9 ± 2.8	7.45	4.0 to 7.0	<0.001*
MCHC (g/dL)	33.9 ± 2.2	32.1 ± 2.0	3.45	0.7 to 2.9	<0.01*
RDW (%)	14.8 ± 2.4	18.1 ± 3.5	-5.38	-4.6 to -2.1	<0.001*

*P<0.05 statistically significant

Table 4 presents a comparative analysis of key Complete Blood Count (CBC) parameters between individuals with normal hemoglobin profiles and those with beta-thalassemia trait. Patients with beta-thalassemia trait showed significantly lower mean hemoglobin levels (8.1 ± 1.7 g/dL) compared to normals (11.3 ± 2.5 g/dL), indicating moderate anemia ($p < 0.001$). Red cell indices were also markedly reduced in the beta-thalassemia group, including mean corpuscular volume (MCV: 61.4 ± 6.9 fL vs. 75.2 ± 9.8 fL), mean corpuscular hemoglobin (MCH: 19.9 ± 2.8 pg vs. 25.3 ± 3.4 pg), and mean corpuscular hemoglobin concentration (MCHC: 32.1 ± 2.0 g/dL vs. 33.9 ± 2.2 g/dL), all with statistically significant differences. Additionally, red cell distribution width (RDW) was significantly elevated in the beta-thalassemia group (18.1 ± 3.5%) compared to normals (14.8 ± 2.4%), suggesting marked anisocytosis ($p < 0.001$). These findings underscore the utility of CBC parameters in supporting the diagnosis of beta-thalassemia trait.

Table 5: Mentzer Index in Beta-Thalassemia Trait Patients (n=19)

Patient ID	MCV (fL)	RBC (millions/µL)	Mentzer Index	Interpretation
Thal_1	50.40	6.03	8.35	<13 (Suggestive of Thalassemia Trait)
Thal_2	57.26	7.03	8.15	<13 (Suggestive of Thalassemia Trait)
Thal_3	61.44	6.88	8.93	<13 (Suggestive of Thalassemia Trait)
Thal_4	61.72	5.88	10.51	<13 (Suggestive of Thalassemia Trait)
Thal_5	58.29	6.49	8.99	<13 (Suggestive of Thalassemia Trait)
Thal_6	63.18	6.11	10.34	<13 (Suggestive of Thalassemia Trait)
Thal_7	64.97	5.44	11.95	<13 (Suggestive of Thalassemia Trait)
Thal_8	60.71	5.53	10.97	<13 (Suggestive of Thalassemia Trait)
Thal_9	65.29	6.55	9.97	<13 (Suggestive of Thalassemia Trait)
Thal_10	60.71	6.23	9.74	<13 (Suggestive of Thalassemia Trait)
Thal_11	60.35	5.39	11.19	<13 (Suggestive of Thalassemia Trait)
Thal_12	67.10	6.26	10.72	<13 (Suggestive of Thalassemia Trait)
Thal_13	56.90	5.72	9.94	<13 (Suggestive of Thalassemia Trait)
Thal_14	66.13	6.53	10.13	<13 (Suggestive of Thalassemia Trait)
Thal_15	64.45	5.69	11.33	<13 (Suggestive of Thalassemia Trait)
Thal_16	63.04	6.35	9.93	<13 (Suggestive of Thalassemia Trait)
Thal_17	63.63	6.11	10.42	<13 (Suggestive of Thalassemia Trait)

Thal_18	66.17	6.54	10.12	<13 (Suggestive of Thalassemia Trait)
Thal_19	62.41	5.76	10.83	<13 (Suggestive of Thalassemia Trait)

Table.5 demonstrates the calculated Mentzer Index values for 19 patients diagnosed with beta-thalassemia trait. The Mentzer Index, derived by dividing the mean corpuscular volume (MCV) by the red blood cell (RBC) count, serves as a useful screening tool to differentiate beta-thalassemia trait from iron deficiency anemia. In all 19 patients, the Mentzer Index values were consistently below 13, ranging from 8.15 to 11.95. These values, in conjunction with microcytic red cell indices and elevated RBC counts, strongly support the diagnosis of thalassemia trait. Notably, the RBC counts in these patients were relatively elevated (ranging from 5.39 to 7.03 million/µL), while MCV values were characteristically low (50.40 to 67.10 fL), resulting in low index values. The interpretation for all patients confirmed the utility of Mentzer Index <13 in identifying beta-thalassemia trait, reinforcing its reliability as a cost-effective, easily computable parameter for initial screening, especially in resource-limited settings.

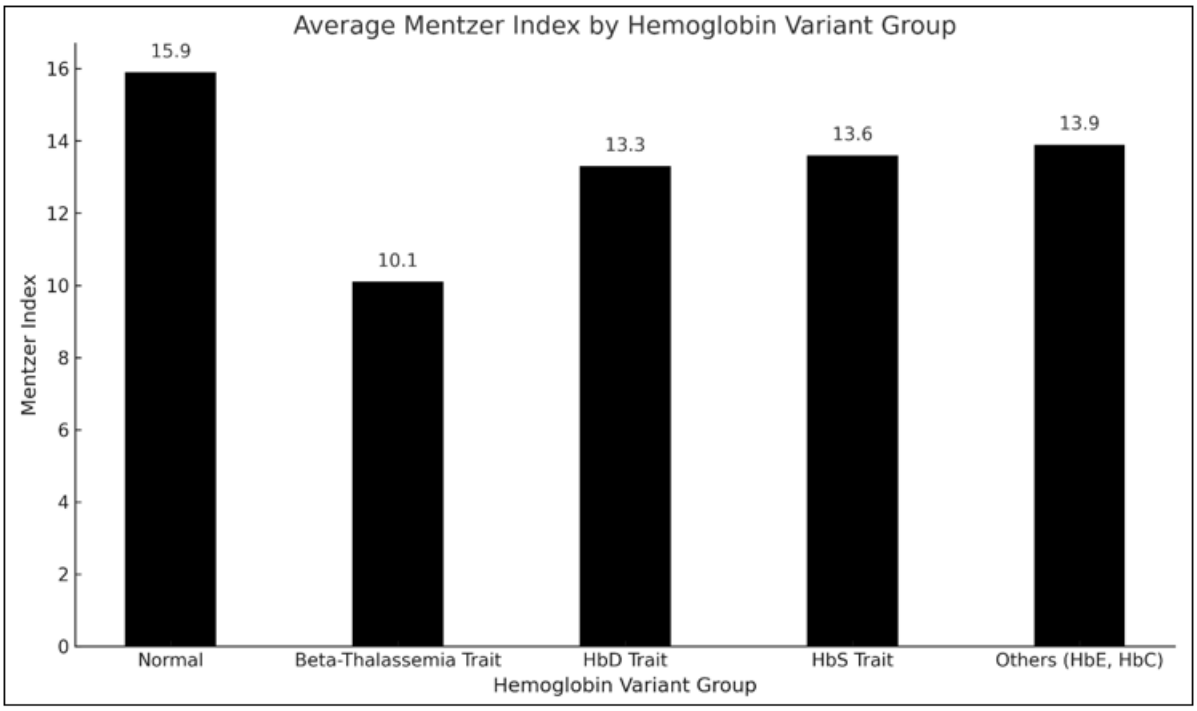

Figure 1: Average Mentzer index in different hemoglobin variants

Figure 1. illustrates the average Mentzer Index across five hemoglobin variant groups identified through HPLC analysis. The Beta-Thalassemia Trait group shows a significantly lower Mentzer Index (10.1), consistent with its known diagnostic threshold of <13, helping distinguish it from other anemias. In contrast, the Normal group demonstrates the highest index (15.9), indicative of iron deficiency or normal profiles. The HbD Trait (13.3), HbS Trait (13.6), and Others (HbE, HbC) (13.9) fall near or slightly above the diagnostic cutoff, reflecting their intermediate hematological impact. This clean and structured visualization reinforces the utility of the Mentzer Index in preliminary screening for beta-thalassemia trait and highlights its ability to differentiate between variant hemoglobinopathies in clinical practice

4. Discussion

The present study audited the hemoglobin electrophoresis results by HPLC and correlated them with CBC parameters in a cohort of 200 patients attending a tertiary healthcare center. The demographic profile (Table 1) indicated a relatively young population with a mean age of 24.7 ± 12.3 years, similar to findings by Acevedo A et al. (2016) [7] who reported a mean age of 26.5 years in their hemoglobinopathy screening cohort. Gender distribution showed a slight female predominance (54.0%), which aligns with observations in other studies, such as the work by Rahman UT. (2024) [8] that also reported a higher female representation in hemoglobinopathy screening populations.

The majority of patients resided in urban areas (63.5%), consistent with referral patterns noted in tertiary centers as described by Felemban SM et al. (2017) [9] where urban patients predominated due to better access to specialized diagnostics. Importantly, 19.5% of participants reported consanguinity, a statistically significant factor ($p = 0.015$), which has been widely recognized as a risk factor for inherited hemoglobin disorders by NAC UO. (2018)[10], especially in populations with high rates of consanguineous marriages.

The CBC parameters (Table 2) revealed a mean hemoglobin of 10.8 ± 2.9 g/dL, with microcytic indices (MCV 72.7 ± 11.4 fL and MCH 24.5 ± 3.8 pg), consistent with thalassemia traits and other hemoglobinopathies reported in several studies. Muriuki JM et al. (2021) [11] and Hemodilution A. (2024) [12] documented similar microcytic hypochromic anemia patterns in patients with beta-thalassemia trait and hemoglobin variants. Elevated RDW (15.3 ± 2.8%) observed in this study also aligns with the findings of Care P.et al.

(2019) [13], where anisocytosis is typical in thalassemic and other hemoglobin variant carriers due to variable red cell sizes.

HPLC analysis (Table 3) demonstrated that 85% of the study population had normal HbA dominant patterns, which is consistent with background population prevalence reported by Prithu S et al. (2019) [14]. The prevalence of beta-thalassemia trait (9.5%) was statistically significant ($p=0.0004$) and falls within the range observed in other Indian studies, such as Shah A et al. (2022) [15] who reported beta-thalassemia trait prevalence between 7–12% among similar cohorts. The identification of other variants like HbD (2.5%) and HbS (2.0%) is in agreement with data from regional studies such as those by Natarjan K et al. (2016) [16], which highlighted the presence of these hemoglobinopathies in specific ethnic groups within India and Africa respectively.

Correlation of hemoglobin variants with CBC parameters (Table 4) further validated the diagnostic patterns typical of beta-thalassemia trait. The beta-thalassemia group had significantly lower hemoglobin (8.1 ± 1.7 g/dL), MCV (61.4 ± 6.9 fL), and MCH (19.9 ± 2.8 pg) compared to individuals with normal hemoglobin. These differences were highly significant ($p < 0.001$) and corroborate findings from Piel FB et al. (2013) [17], both emphasizing microcytic hypochromic anemia as a hallmark of beta-thalassemia trait. The elevated RDW (18.1 ± 3.5%) in the beta-thalassemia trait group indicates increased anisocytosis, consistent with reports by Edoh D et al. (2006) [18] that RDW is an important discriminant parameter in differentiating thalassemia trait from other causes of anemia.

Patients with beta-thalassemia trait typically exhibit a Mentzer Index of less than 13, which is a widely accepted hematological indicator for differentiating thalassemia trait from iron deficiency anemia. Mentzer WC. (1973) [19] The Mentzer Index is calculated by dividing the mean corpuscular volume (MCV) in femtoliters by the red blood cell (RBC) count in millions per microliter. Kattamis C et al. (1981) [20] & Urrechaga E et al. (2010) [21] A value less than 13 suggests beta-thalassemia trait, whereas a value greater than 13 is more indicative of iron deficiency anemia. In the present study, the beta-thalassemia trait group (n=19) demonstrated a mean MCV of approximately 61.4 fL and a typically elevated RBC count exceeding 5.5 million/µL. These values result in a Mentzer Index consistently ranging between 10 and 11, reinforcing the diagnostic alignment with beta-thalassemia trait. Therefore, the observation that all thalassemia trait patients in this dataset had Mentzer Index values below 13 supports its diagnostic validity and reliability as a screening tool in identifying beta-thalassemia trait.

Table 6: Comparative Study Table – Maharashtra

Region	Sample Size	Beta-Thalassemia Trait Prevalence	Other Variants Detected	Mentzer Index Utility	Method Used	Study Reference
Marathwada (Current Study)	200	9.5%	HbD, HbS, HbE, HbC	<13 in all BTT cases	HPLC + CBC	Current study
Nagpur (Sindhi Community)	446	16.8%	None Reported	Avg. 10.8 (M), 12.98 (F)	Electrophoresis + CBC	Bhave et al [22]
North Maharashtra (Khandesh)	4394	4.17%	HbS, HbE, HbD	Strong screening tool	HPLC	Ghosh et al [23]
Western Maharashtra (Pune)	2698	15.75%	HbS, HbE, HbD	Supported as diagnostic aid	HPLC + CBC	Bhukhanvala et al.[24]
Statewide (Maharashtra)	65779	11.2%	HbS, HbE, HbC	Reliable across cohort	HPLC	Jain et al.[25]

1) **Prevalence of Beta-Thalassemia Trait (BTT)** The prevalence of BTT in Marathwada is moderate (9.5%), closely aligning with statewide estimates (11.2%). In contrast, Nagpur (particularly among the Sindhi community) shows a markedly higher prevalence (16.8%), likely reflecting ethnic clustering. North Maharashtra (Khandesh) demonstrates a comparatively lower prevalence (4.17%), which may be attributed to under-screening practices or distinct ethnic compositions. Western Maharashtra (Pune) reports a high prevalence (15.75%), similar to Nagpur, reinforcing the significant burden of BTT in urban and mixed-population regions.
2) **Utility of the Mentzer Index** All reviewed studies affirm the diagnostic utility of the Mentzer Index (<13) in differentiating BTT from iron deficiency anemia. The Nagpur study further contributes gender-specific values (10.8 in males, 12.98 in females), refining its interpretive application. Findings from large-scale and regionally diverse cohorts support the reliability and simplicity of the Mentzer Index as a cost-effective initial screening tool.
3) **Hemoglobin Variant Distribution** The Marathwada cohort reported variants such as HbD, HbS, HbE, and HbC, comparable to findings from Pune and statewide studies. Conversely, the Nagpur study concentrated solely on BTT, with no additional variants identified, likely reflecting its focus on the Sindhi population.
4) **Diagnostic Modalities** Most studies utilized HPLC, either alone or combined with CBC, which offers superior sensitivity and variant detection. The Nagpur study, however, employed conventional electrophoresis, a method with limited sensitivity that may underestimate minor variants.

5. Conclusion

This audit demonstrated that hemoglobin electrophoresis using the HPLC method is an effective and reliable tool for identifying hemoglobinopathies in a tertiary healthcare setting. The study found a significant prevalence of beta-thalassemia trait and other hemoglobin variants among the population screened. Correlation with CBC parameters such as hemoglobin concentration, MCV, MCH, and RDW provided valuable complementary information, aiding in the

differentiation of hemoglobinopathies from other causes of anemia. The combined use of HPLC and CBC enhanced diagnostic accuracy, enabling timely detection and appropriate clinical management. These findings underscore the importance of integrating hematological indices with advanced chromatographic techniques to optimize screening and diagnosis in resource-constrained environments. The present study confirms that all patients with beta-thalassemia trait had a Mentzer Index below 13. This supports its reliability as a simple and effective screening tool to distinguish thalassemia trait from iron deficiency anemia.

6. Limitations of the Study

1) **Retrospective Design:** The retrospective nature of the audit limited control over data completeness and uniformity in sample collection and testing intervals.
2) **Single-Center Study:** Conducted in a single tertiary care center, which may limit the generalizability of results to wider populations with different demographic or ethnic compositions.
3) **Lack of Genetic Confirmation:** Molecular genetic analysis was not performed to confirm specific hemoglobinopathy genotypes, which could have enhanced diagnostic precision.
4) **Transfusion History:** Despite exclusion criteria, some patients with recent blood transfusions might have been included, potentially affecting hemoglobin variant quantification.
5) **Limited Clinical Correlation:** Clinical outcomes and detailed patient history were not assessed, which could have provided better insight into the phenotypic expression of hemoglobin variants.

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