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**Original Research Article**

# Detection of Hemoglobinopathies by HPLC in Patients with Microcytic Hypochromic Anemia

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**Abstract**

**Introduction:** Most commonly encountered disorders manifesting as microcytic hypochromic anemia are iron deficiency and thalassemia. These need to be accurately categorized epidemiologically and also to provide necessary counselling and avoid unnecessary iron overload. HPLC has emerged as one of the best methods for detection of hemoglobinopathies with rapid, precise and reproducible results.

**Aim and Objective:** To evaluate the role of HPLC in the diagnosis of hemoglobinopathies in cases of microcytic hypochromic anemia & to detect various rare variants of hemoglobinopathies.

**Materials & Methods:** This study was conducted in the Department of Pathology, M.K.C.G. Medical College, Berhampur from October 2019 to September 2021. Blood samples of 1260 patients with microcytic hypochromic anemia were collected and analyzed on the Bio-Rad Variant II HPLC system with use of the Variant II β -Thalassemia Short Program Reorder Pack (Bio-Rad Laboratories). An Hb A2/F calibrator and two levels of controls (BIORAD) were analyzed at the beginning of each run. The integrated peaks are assigned to manufacturer-defined "windows" derived from specific retention time (RT).

**Observations & Result:** Out of 1260, 737 (58.5%) cases displayed abnormal hemoglobin fractions on HPLC. Beta thalassemia trait (362 cases, 28.7%) was the most prevalent followed by Sickle cell trait (247 cases, 19.6%). Other hemoglobinopathies found were Sickle cell disease (5.26%), Beta Thalassemia homozygous and sickle-beta thalassemia double heterozygous (1.9% each) and HbE heterozygous (0.47%). There were also 3 cases of HbE-beta thalassemia, 2 cases each of HbS-HbE double heterozygous and 1 case of HbE homozygous.

**Discussion:** Thalassemia was the most common hemoglobinopathy detected in this study indicating the importance of early detection followed by genetic counselling for prevention of birth of thalassemia homozygous baby. Sickle cell cases were the next most prevalent indicating possible associated iron deficiency and/ or alpha thalassemia.

**Keywords:** Microcytic Hypocromic Anemia**,** Hemoglobinopathies, HPLC, beta thalassemia, sickle cell.

## Introduction

Anaemia is the commonest disorder of blood affecting populations of all countries of the world. Total cases of anemia globally were estimated to be 1.74 (1.72–1.76) billion in 2019 accounting for 22.8% of global population, increasing from 1.42 (1.41–1.43) billion in 1990. India roughly accounts for a quarter of all cases of anemia globally [1].

Morphologically, anemia are classified based on red blood cell (RBC) size into microcytic (< 80 fl), normocytic (80-96 fl) and macrocytic (> 96 fl) [2]. Most common anemia encountered in clinical practice is microcytic hypochromic anemia, various causes of which include iron deficiency, thalassemia & other variant hemoglobins, anemia of inflammation, sideroblastic anemia and lead poisoning [3]. An accurate diagnosis of microcytic hypochromic anemia is very important as it provides indication for iron supplementation in iron deficiency patients, helps in avoiding unnecessary iron overload in thalassemia traits & also for preventing severe & lethal forms of thalassemia syndromes [4,5].

Hemoglobinopathies are the commonest single gene disorders in the world. At least 7% of world population carries an abnormal hemoglobin gene, & about 1.1% of couples worldwide are at risk for having children with hemoglobin disorders [6]. Incidence of hemoglobinopathies differs in different parts of India [7-9].

Sickle cell anemia & thalassemia are the most frequently encountered hemoglobinopathies. Clinical symptoms vary from incidentally detected asymptomatic states to severe, lifelong transfusion dependent anemia with severely reduced life expectancy. It's extremely important, both epidemiologically as well as for prevention of economic & emotional burden of a chronic disease, to identify & properly manage these disorders [4]. There are many methods for estimation of different Hb fractions, one of the best being Cation exchange- high performance liquid chromatography (CE-HPLC). It's a simple and rapid automated system with reproducible and precise results [10]. Hemoglobinopathies are prevalent in southern Odisha as evidenced from the fact that there are 442 transfusion dependant hemoglobinopathy patients registered in Blood bank of MKCG Medical College and Hospital, Berhampur.

### Aim and Objective

To evaluate the role of HPLC in the diagnosis of hemoglobinopathies in cases of microcytic hypochromic anemia & to detect various rare variants of hemoglobinopathies.

### Material and Methods

A cross-sectional study was conducted over a period of 2 years from October, 2019 – September, 2021 in the department of Pathology, M.K.C.G Medical College and Hospital, Berhampur, Odisha after obtaining necessary approval from the Institutional Ethical Committee.

### Inclusion criteria:

1. Valid informed consent.
2. All patients with microcytic hypochromic anemia (MCV< 80fL & MCH< 26pg for adults and age & gender specific ranges [11] for pediatric patients) and subjected to HPLC due to suspicion of existence of hemoglobinopathy.

### Exclusion criteria:

1. Lack of consent.
2. Patients with history of recent blood transfusions (within 3 months).

A total of 4935 patients were found to have microcytic hypochromic anaemia. Out of which, 1260 cases were subjected to HPLC and were included in the study group. A detailed clinical history, examination findings and other relevant investigation findings were recorded. CBC

was performed by Automated Hematology Analyzer (Sysmex XT 2000i). All samples

were assessed by the Variant Hemoglobin Testing System (Variant II Beta Thalassemia Short Program, Bio-Rad Laboratories Inc., Hercules, CA, USA) under the experimental conditions specified by the manufacturer. The integrated peaks elicited were assigned to manufacturer-defined "windows" derived from specific retention time (RT) [12].

Statistical data analysis was done on Microsoft Excel 2012. Continuous variables were expressed as mean SD. Categorical variables were expressed in frequencies and percentages. Data obtained was tabulated using version 22 of the statistical package for social sciences (SPSS).

## Observations

A total of 4935 patients were found to have microcytic hypochromic anemia; out of which, 1260 were suspected to have hemoglobinopathy and HPLC was done. 737(58.5%) cases were found to have abnormal hemoglobin pattern with the rest showing normal hemoglobin pattern. Of the 737(58.5%) cases with abnormal hemoglobin pattern, 423 (33.57%) were male & 314 (24.92%) were female. The age group of all cases ranged from 1 to 65 years with a mean of 17.44 years. Among the 737 cases of hemoglobinopathies, most belonged to the age group 1-10 years (31.2%) followed by 21-30 years (25.7%).

Normal adult chromatogram shows primarily HbA0 (mean 86.32%), a small percentage of HbA2 (mean 2.56%) & traces of HbF (mean 0.83%). P2 & P3 windows were normal.

Most common abnormal hemoglobin pattern observed was beta thalassemia trait with 362 (28.7%) cases. HbA2 levels of 3.9-9% are diagnostic of beta thalassemia trait in an asymptomatic individual with no or mild anemia. The mean Hb A2 in BTT cases in the present study was 4.91. Beta thalassemia major was diagnosed in 24 (1.9%) cases. The mean HbF & HbA2 were 85.55% & 3.95% respectively.

The 2nd most common abnormality in this study was sickle cell trait with 247 (19.6%) cases. HbS elutes in the S-window with a retention time of 4.12-4.42 min. The mean HbS in sickle cell trait cases was 32.26%, while mean HbA0 was 56.91% and mean HbF was 2.33%. In sickle cell disease (66 cases, 5.23%), mean HbF was 18.04% & mean HbS was 74.85%.

24 cases (1.9%) of HbS - beta Thalassemia double heterozygous were seen with mean HbA2, HbF & HbS of 4.45%, 13.84% &73.85% respectively. 2 cases of HbS-HbE double heterozygous were diagnosed, with mean HbA2 & HbS of 27.65% & 61% respectively.

HbE elutes in the A2 window with retention time ranging from 3.68 to 3.98 min. 6 cases (0.47%) of HbE heterozygous were seen with a mean HbE of 29.9%, HbA 55.31% and HbF of 3.58%. There was one case of HbE homozygous with 82.5% HbE. HbJ variant elutes in the P3 window with retention time of 1.60 – 1.90 min. There were two cases with mean HbJ of 23%.

As per literature, sickle cell trait cases are diagnosed when both HbA & HbS are present with HbA > HbS, but typically HbS value ranges from 38-45%. However, HbS values can be lower if there is co-inheritance of alpha Thalassemia. A comparison of CBC parameters was done between the sickle cell trait cases having HbS of 38-45% and those cases with lower HbS%. (Table-4) This table shows that while the differences in relation to hemoglobin & RDW were not significant, there were significant differences regarding RBC count, MCV & MCH.

## Discussion

Anemia is a public health problem not sparing any region of the world. According to the WHO, globally it affects1.62 billion

**Table 1: Spectrum of hemoglobinopathies in the present study**

| Hemoglobinopathies | Observed number of cases | % |
|---|---|---|
| β Thalassemia heterozygous | 362 | 28.7 |
| βThalassmia homozygous | 24 | 1.9 |
| HbS heterozygous | 247 | 19.6 |
| HbS homozygous | 66 | 5.23 |
| HbS + β Thalassemia double heterozygous | 24 | 1.9 |
| HbE heterozygous | 6 | 0.47 |
| HbE homozygous | 1 | 0.07 |
| HbE- β Thalassemia double heterozygous | 3 | 0.23 |
| HbJ Meerut | 2 | 0.15 |
| HbS-HbE double heterozygous | 2 | 0.15 |
| Normal | 523 | 41.5 |
| TOTAL | 1260 | 100 |

**Table 2: RBC indices in various hemoglobinopathies**

| Hemoglobin variants | HB (g/dL) | TRBC (million/µL) | MCV (fL) | MCH (pg) | RDW-CV (%) |
|---|---|---|---|---|---|
| β Thalassemia heterozygous | 10.24±2.47 | 4.94±1.3 | 62.75±6.88 | 19.79±2.26 | 18.5±3.88 |
| βThalassmia homozygous | 4.08±1.09 | 2.48±1.44 | 65.26±6.66 | 18.77±3.16 | 31.46±7.19 |
| HbS heterozygous | 10.21±2.36 | 4.81±1.02 | 66.63±8.74 | 21.57±3.26 | 18.06±4.81 |
| HbS homozygous | 7.83±2.23 | 3.40±1.14 | 70.03±6.4 | 22.82±2.0 | 19.75±3.7 |
| HbS + β Thalassemia double heterozygous | 8.35±2.27 | 3.89±1.1 | 64.4±4.47 | 21.35±1.68 | 20.54±4.47 |
| HbE heterozygous | 9.16±5.21 | 4.55±1.53 | 62.65±10.61 | 18.93±6.07 | 18.85±4.51 |
| HbE homozygous | 7.4 | 2.97 | 59.8 | 21.9 | 18.7 |
| HbE- β Thalassemia | 6.06±0.30 | 3.61±0.28 | 52.03±5.11 | 28.5±1.51 | 28.5±0.95 |
| HbJ Meerut | 8.5±0.14 | 3.8±0.02 | 66.25±0.77 | 22±0.7 | 17.25±0.49 |
| HbS-HbE double heterozygous | 8.25±0.21 | 3.82±0.07 | 66.6±0.28 | 22.15±0.49 | 24±0.42 |
| Normal | 8.95±3.06 | 4.36±1.20 | 66.46±8.34 | 20.36±3.57 | 18.79±4.84 |

**Table 3: Hemoglobin fractions in various hemoglobinopathies**

| Hemoglobin variants | HBA0% | HBA2% | HBF% | HBS% | P3% |
|---|---|---|---|---|---|
| β Thalassemia heterozygous | 83.86±2.52 | 4.91±0.39 | 1.2±1.38 | | |
| βThalassemia homozygous | 10.18±4.37 | 3.95±1.31 | 85.55±4.3 | | |
| HbS heterozygous | 56.91±5.08 | 2.91±0.4 | 2.33±5.32 | 32.26±5.63 | |
| HbS homozygous | 1.88±2.93 | 2.64±0.62 | 18.04±5.92 | 74.85±6.5 | |
| HbS + β Thalassemia double heterozygous | 5.75±5.33 | 4.45±0.66 | 13.84±7.58 | 73.85±7.45 | |
| HbE heterozygous | 55.31±5.18 | 29.9±5.61 | 3.58±2.81 | | |
| HbE homozygous | 2.9 | 82.5 | 2 | | |
| HbE- β Thalassemia | 8.26±0.96 | 64.1±2.98 | 17.1±6.31 | | |
| HbJ Meerut | 64.95±0.35 | 2.4±0.7 | 5.35±0.49 | | 23±0.7 |
| HbS-HbE double heterozygous | 5.5±0.98 | 27.65±0.21 | 3.15±0.21 | 61±0.98 | |
| Normal | 86.32±5.88 | 2.56±0.41 | 0.83±1.3 | | |

**Table 4: Comparison of CBC parameters among sickle cell trait cases**

| HbS(%) | Hb | TRBC | MCV | MCH | RDW |
|---|---|---|---|---|---|
| 20-38 | 10.13±2.36 | 4.88±1.02 | 69.64±5.96 | 22.20±2.18 | 18.26±4.83 |
| 38-45 | 10.59±2.35 | 4.45±0.96 | 72.01±7.69 | 23.75±2.00 | 17.06±4.59 |
| P-VALUE | 0.2535 Not significant | 0.0126 Significant | 0.0270 Significant | 0.0161 Significant | 0.141 Not significant |

people [13]. Microcytic hypochromic anemia is the most common form of anemia encountered in clinical practice. Various causes include iron deficiency and hemoglobinopathies [3].

Hemoglobinopathies are the group of genetic disorders of hemoglobin in which there is a quantitative or qualitative abnormal production of hemoglobin molecule. Appropriate laboratory tests are required for diagnosis of these disorders, among which HPLC is proven to be rapid, sensitive, specific, and reproducible [14,15].

A total of 4935 cases of microcytic hypochromic anemia were detected in the department of pathology, M.K.C.G. Medical College & Hospital. Out of these, HPLC was done in 1260 cases suspected to have hemoglobinopathies and they were included in the present study.

In total, 737 (58.5%) cases showed hemoglobinopathies while rest had normal hemoglobin patterns. This finding correlates with the study by Balgir *et al* [9]. in Odisha who found approximately 65% cases having hemoglobinopathies and Baruah *et al* [7]**.**

**Table 5: Incidence of various hemoglobinopathies**

| **Hemoglobinopathies** | **Alam *et al* [17] (n=331)** | **Balgir *et al* [9] (n=1015)** | **Philip *et al* [4] (n=4335)** | **Raman *et al* [18] (n=788)** | **Bhagora *et al* [16] (n=200)** | **Sarvaiya *et al*[22] (n=2035)** | **Present study (n=1260)** |
|---|---|---|---|---|---|---|---|
| **Sickle Heterozygous** | 15.4 | 29.8 | 1.24 | 15.1 | 3.5 | 4.71 | 19.6 |
| **Sickle homozygous** | 10.27 | 7.8 | 0.11 | 9.9 | 2.0 | 0.93 | 5.23 |
| **Beta thalassemia heterozygous** | 18.73 | 18.2 | 10.49 | 6.1 | 8.0 | 10.61 | 28.7 |
| **Beta thalassemia homozygous** | 2.11 | | 0.55 | 0.89 | 2.0 | - | 1.9 |
| **Beta thalassemia intermedia** | 1.21 | - | 0.46 | 1.02 | - | - | - |
| **Sickle Beta thalassemia** | 23.00 | 1.7 | 0.48 | 2.79 | 0.5 | 0.68 | 1.9 |
| **E homozygous** | 1.51 | 0.9 | 0.46 | 0.76 | - | 0.04 | 0.47 |
| **E heterozygous** | 0.91 | 0.3 | 0.85 | 0.12 | - | - | 0.07 |
| **E-B thalassemia** | 2.71 | 0.7 | 0.06 | 0.63 | 0.5 | 0.04 | 0.23 |
| **S-E double heterozygous** | - | - | - | - | - | - | 0.15 |
| **D heterozygous** | - | - | 0.20 | 0.12 | 0.5 | - | - |
| **J Meerut** | - | - | - | - | - | - | 0.15 |

in Assam found 59.11% hemoglobinopathies, while others found lesser number of hemoglobinopathies in their studies. This was possibly because the present study was a hospital-based study with study group comprising of patients with anemia suspected to have hemoglobinopathies, while other studies with lesser number of cases were population based.

There was a definite male preponderance in the present study (1.41: 1). A review of literature reveals a wide range of male to female ratio implying that there is no definite sex predilection in hemoglobinopathies. The maximum number of cases (230 cases, 31.2%) was seen in the 1 -10-year group, with 81.5% cases being less than 30 years of age. This indicates that most cases of hemoglobinopathies present at an early age while in later years, the detections are due to incidental findings or complications.

In the present study, beta thalassemia trait was most prevalent with 362 (28.7%) cases, followed by sickle cell trait (247 cases, 19.6%), sickle cell homozygous (66 cases, 5.23%) and beta thalassemia homozygous

& HbS- beta thalassemia double heterozygous (24 cases each, 1.9%). There were 7 (0.54%) hemoglobin E cases (6 heterozygous& 1 homozygous), as well as 3 cases of E β thalassemia and 2 cases each of Hbs- HbE double heterozygous & HbJ Meerut.

BTT was the most common abnormal hemoglobin variant seen in the present study (362 cases, 28.7%). Studies [3,16] previously conducted on microcytic hypochromic anemia patients had reported beta thalassemia trait followed by sickle cell trait to be the most common hemoglobin variants. However, studies done previously in Odisha [9,17,18] had revealed sickle cell syndromes to be the most common hemoglobinopathy. The higher incidence of BTT in the present study was presumably because only microcytic hypochromic anemia cases were taken in the study group.

The cases of BTT had characteristic microcytic hypochromic red cells with normal or slightly reduced Hb and raised RBC count. The mean HbA2 value was 4.91% with a SD of 0.39. Raised A2 level is the most important abnormal chromatogram finding helpful for diagnosis. However, conditions with borderline HbA2 need careful interpretation. The range for borderline HbA2 used in this study was 3.5-3.9%. Levels of HbA2 may be affected by deficiency anemia, milder forms of Thalassemia and a co-inheritance of delta thalassemia. Genetic studies are confirmatory and should be advised in all cases of diagnostic ambiguity [19].

Beta Thalassemia major was seen in 24 cases (1.89%). Almost all of them presented at an early age (<10 years) with severe symptoms, microcytic hypochromic RBCs and hemolytic blood picture. These cases had a mean Hb F of 85.55% with SD of 4.3.

The hematological profile and HPLC findings in cases of Thalassemia in this study correlated well with other studies done in Odisha [18]. and other places in India [4,20].

Various sickle cell syndromes accounted for 339 cases (26.75%). These included sickle cell trait (19.6%), sickle cell disease (5.23%), sickle beta thalassemia cases (1.9%) and two cases of HbS – HbE double heterozygous. HbS has been reported to be 38-45% in trait, 75–95% in homozygous HbS, and 60-90% in HbS-β thalassemias (depending on presence of $\beta^0$ or $\beta^+$) [21]. In the present study, it was 32.26% in trait, 74.85% in the homozygous forms, and 73.85% in HbS- β thalassemia.

Sickle cell homozygous cases in this study were mostly less than 20 year old and presented with severe pallor, some also had jaundice, fever and bone pain. These patients had mean hemoglobin of 7.83 g/dl with lesser reduction in MCV (70.03fL) compared to other cases in this study, sickling test was positive in all cases, mean of Hb S fraction was 74.85% with SD of 6.5 and Hb F was 18.04%. The slightly higher HbF level, and consequent reduction in clinical severity, usually seen in Indian subcontinent could possibly be due to the Saudi Arabia/ Indian haplotype prevalent in Indian populations [18].

The sickle cell trait patients were mostly in younger age group and had mild pallor or were mostly asymptomatic. These patients had mean hemoglobin of 10.21 g/dl and MCV of 66.63fL, sickling test was positive in all cases and Hb S fraction was 32.26% with SD of 5.63.

Bhagora *et al* [16] and Sarvaiya *et al* [22] also reported microcytic hypochromic blood picture in sickle cell cases, while several other studies [4,18,23] found normocytic normochromic RBCs. The microcytic hypochromic blood picture could be due to associated alpha thalassemia or iron deficiency which is highly prevalent in this geographic region.

High incidence of iron deficiency has been reported in patients with sickle cell disease

from India [18]. Mohanty *et al* [24] using the single criteria of ZPP/heme ratio (ZPP/heme) for diagnosis in Indian subjects reported iron deficiency in 67.7% of SCDs patients and 26.2% of sickle cell trait patients. Alpha thalassemia reduces the intracellular HbS level there by reducing HbS prompted cellular destruction which leads to diminished severity of disease [25,26]. In many countries, 3.7 kb alpha-globin gene deletion has been reported among SCA patients including India (32%), Brazil (29%) and in the UK among African Britons (34%) [27]. Some sickle cell trait cases showed presence of HbS < 38%, this was possibly because of simultaneous presence of alpha thalassemia. However, due to lack of facilities, molecular confirmation could not be done. A comparison of CBC parameters was done between the sickle cell trait cases having HbS in expected range (38-45%) and those cases with lower HbS%. The RBC counts were higher while both MCV and MCH values were lower ($p< 0.05$, statistically significant) in cases showing lower HbS values indicating the possible usefulness of these ubiquitously available CBC parameters in presumptive diagnosis of alpha thalassemia. However, another study incorporating molecular diagnosis of alpha thalassemia is required for validating these data.

The sickle beta thalassemia patients had clinical picture similar to sickle cell disease cases with major difference being less dependence on transfusion. These patients had mean hemoglobin of 8.35 g/dl, sickling test was positive in all cases and Hb S fraction was 73.85% with SD of 7.45 and mean HbA2 of 4.45%. Balgir *et al* [9] also described the clinical profile of 12 cases of sickle-beta thalassemia as similar to those of sickle cell disease. Two cases of HbS – HbE double heterozygous were detected showing microcytic hypochromic anemia (mean Hb- 8.25 g/dL), 61% Hb S and 27.65% Hb A2.

HbE elutes in the A2 window on HPLC[21] and is the commonest hemoglobin variant in Southeast Asia and second most prevalent in the World[7]. In the present study, the prevalence of HbE gene was 0.92% which is slightly lower than the finding in other studies conducted in Odisha (Raman *et al* [18] with 1.63% and Balgir *et al* [9] with 1.90%). Of the total 12 cases showing presence of HbE, there were 6 (0.47%) cases of Hb E heterozygous with mean Hb A2 fraction of 29.9% and mild microcytic hypochromic anemia. There was one case only of Hb E homozygous that had Hb A2 of 82.5% and severe microcytic hypochromic anemia. Most severe anemia (mean Hb of 6.06g/dL) was seen with Hb E- beta thalassemia cases (3 cases), who had mean Hb E fraction of 64.1% and Hb F of 17.1%.

Hb J Meerut elutes in P3 window.[21] There were 2 cases with mean hemoglobin concentration of 8.5 g/dL, variant hemoglobin fraction of 23% with SD of 0.7 and 5.35 mean Hb F.

## Conclusion

A high prevalence of hemoglobinopathies was found in this region, indicating the futility of indiscriminate iron supplementation in all cases of microcytic hypochromic anemia and the need of mass screening programs and genetic counselling.

This study again reiterates the fact that HPLC is an excellent, powerful diagnostic tool for the direct identification of Hb variants with a high degree for precision in the quantification of major and minor, normal and abnormal Hb fractions. But it has always been emphasized that interpretation of chromatograms must be done only after taking into consideration the clinical history, family history, complete blood count and findings of blood smear study.

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