Journal Homepage: -www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI:10.21474/IJAR01/15584
DOI URL: http://dx.doi.org/10.21474/IJAR01/15584

*RESEARCH ARTICLE*

# STUDY OF SPECTRUM OF HEMOGLOBINOPATHIES IN ADULT AGE GROUP DIAGNOSED ON HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN TERTIARY CARE HOSPITAL

**Dr.Supriya Patil[1], Dr. J.P. Laddha[2], Dr. R.R. Soni[3], Dr. Ashish Tayde[4] and Dr. Ashish Patil[5]**

1. Junior Resident 3, Department of Pathology, Dr PDMMC Amravati.
2. Professor, Department of Pathology, Dr PDMMC Amravati.
3. Head of Department and Professor, Department of Pathology, Dr PDMMC Amravati.
4. Associate Professor, Department of Pathology, Dr PDMMC Amravati.
5. Assistant Professor, Department of Pediatric. GMC Yavatmal.

## Manuscript Info

***Manuscript History***
Received: 30 August 2022
Final Accepted: 30 September 2022
Published: October 2022

***Key words:-***
Hemoglobinopathy, High-Performance Liquid Chromatography (HPLC), Hemoglobin Variants

## Abstract

**Background:** Inherited abnormalities of hemoglobin synthesis (hemoglobinopathies) include a myriad of disorders ranging from thalassemia syndromes to structurally abnormal hemoglobin variants.

**Aims and Objectives**: To study spectrum of hemoglobinopathies in adult age group diagnosed on HPLC in tertiary care hospital.

**Materials and Methods**: The present study carried out in the deparment of pathology , DR PDMMC AMRAVATI over a period of 18 months from january 2021 to june 2022. A total of 800 cases were included in the present study. The age group of patients ranged from 19 years and above. HPLC , complete blood count, and peripheral smear examination were done in all the cases.

**Results and Discussion**: In the present study total 800 patients were included out of which 134 cases displayed abnormal hemoglobin fractions on HPLC. It was seen that majority of the patients in study were in the 19yrs to 30yrs. The most common hemoglobinopathy observed in the present study was sickle cell trait (51.50 %) followed by Beta Thalassemia trait (26.12 %) and Sickle cell disease (16.42 %).HbDpunjab trait was observed in 3.74 % cases. Sickle cell Disease + Beta thalassemia trait ,HbE trait, HbE homozygous were observed in 0.74% cases each.

**Conclusion**: Detection of β-thalassemia carriers and other structural variants will provide a preliminary insight to the prevalence of hemoglobinopathies in hospital based patients.Awareness can be created in an effort to prevent the birth of affected children.The present study is intended to contribute to the available data as the identification of these disorders is important epidemiologically and aids in prevention of more serious hemoglobin disorders.



**Corresponding Author:- Dr. Supriya Patil**
Address:- Junior Resident 3, Department of Pathology, Dr PDMMC Amravati.

## Introduction:-

Inherited abnormalities of hemoglobin synthesis (hemoglobinopathies) include a myriad of disorders ranging from thalassemia syndromes to structurally abnormal hemoglobin variants [(1)].
Hemoglobin comprises of four globin chains: fetal hemoglobin (Hb F) has two α and two gamma chains (α2γ2) and adult hemoglobin (Hb A) has two α and two β chains (α2β2). Genes in the α-globin and β-globin gene clusters (on chromosomes 16 and 11) control globin-chain production[(2)] .

Hemoglobin disorders can be broadly classified into two general categories: 1. Those in which there is a quantitative defect in the production of one of the globin subunits, either total absence or marked reduction. These are called the thalassemia syndromes. 2. Those in which there is a structural defect in one of the globin subunits resulting in a hemoglobinopathy[(3)].

Diseases affecting hemoglobin synthesis and function are extremely common worldwide. More than 1000 naturally occurring human hemoglobin variants have been discovered, mainly through their clinical and/or laboratory manifestations[(4)] .It is estimated that 7% of the world's population is affected by hemoglobinopathies[(5)]. Inherited hemoglobin disorders (sickle-cell disorders and thalassemias) were originally characteristic of the tropics and subtropics but are now common worldwide due to migration[(6-8)].

India has a high incidence of thalassemia. Certain communities in India, such as Sindhis and Punjabis from Northern India, Bhanushali's, Kutchis, Lohana's from Gujarat, Mahar's, Neobuddhist's, Koli's and Agri's from Maharashtra, Gowda's and Lingayat's from Karnataka etc. have a higher carrier rate[(9)].

The diagnosis of hemoglobinopathies is made by a detailed clinical evaluation along with laboratory methods like a thorough hematological work-up, Hb Electrophoresis and recent methods such as Iso Electric Focussing (IEF) and High Performance Liquid Chromatography (HPLC). Also polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR techniques are used to detect specific mutations at the molecular level[(10)]. HPLC is less labour intensive with rapid turnaround time. With automation and quantitative power, it is a scientific and accurate technique for identification and quantification of normal and abnormal Hb fractions.

## Aims And Objectives:-

1. To study spectrum of hemoglobinopathies in adult age group diagnosed on HPLC in tertiary care hospital.
2. To make a presumptive diagnosis of commonly occurring hemoglobin variants.

## Methodology:-

**I) Study Design**
Cross sectional.

**II) Study Setting**
Study carried out at tertiary care centre.

**III) Study Population**
Patients with age more than 18 years included in study.

**IV) Sample Size**
Sample size is 800 .

**V) Sampling Technique**
Purposive sampling.

**VI) Study duration**
18 month from january 2021 to june 2022.

**VII) Inclusion Criteria And Exclusion Criteria**
**Inclusion Criteria**
1. Samples from patients of both genders above 18 years of age.

2. All cases with abnormal peripheral blood smear examination suspicious of hemoglobinopathies.
3. All cases with hematological parameters suggestive of hemolytic anaemia
4. Patients with positive family history of thalassemia.
5. Patients dependent on blood transfusion for hemolytic anemias.

**Exclusion Criteria**
1. Patients with history of blood transfusion in the last 1 month.
2. Patients with unknown peaks in their HPLC graph patterns which are not predefined and thus cannot be interpreted.

**Data Collection Procedure**
All adult age group samples sent in hematological laboratory for suspected hemoglobinopathies or thalassemia were taken for study. About 3 ml of samples were collected in EDTA bulb. Samples were run on automated hematology analyser. Peripheral blood smear, complete blood count(CBC) and red blood cell indices study have been done in all cases.Before processing for hplc sample tubes were to reach room temperature. No sample preparation was required. If abnormal tube type or if sample was less than 2ml then predilution of sample by taking 5ul of sample in 1.5 ml of wash/diluent was done.Whole blood specimen stored up to 4 days at 2-8 degree celcius or 1 day at room temperature before processing for hplc.

After hplc a chromatogram was obtained in all cases which shows all fractions of hemoglobin, retention time and peak areas and the values of fractions . These graph was analyse according to concentration of various hemoglobin and diagnosis of thalassemia or hemoglobinopathies were made. HbS presence was confirmed by sickling test using 2% sodium metabisulphite wherever required.

**Data Analysis**
Appropriate test sign applied. Data collected and entered in microsoftexcell and analysed by Statistical Packaging For Social Science.

## Observation And Results:-

In present study total 800 patients who were clinically suspected to have hemoglobinopathies were selected for study. Out of 800 cases , 134 (16.75%) cases were diagnosed to have one or other hemoglobinopathies.

In present study we distributed patients according to age group 19-30 years,31-40 years,41-50 years , 51-60 years and above 60 years . Most of the patients were in the age group of 19-30 years i.e. 87(64.93%) followed by 31-40 years i.e. 22(16.42%) .

Out of total 800 patients suspected to have hemoglobinopathies 600 were females and 200 were males . Out of 600 female patients ,100(16.66%) patients were found to have hemoglobinopathies . Out of 200 male patients , 34(17%) were diagnosed to have hemoglobinopathies . Overall distribution of patients according to gender was found to be same. In present study the overall number of female patients was more as most of the patients were came for screening purpose from ANC OPD/Wards.

Family history of hemoglobinopathy was observed in 90(67.16%) cases.

**Table No. I:-** Distribution Of Patients According To Hemoglobinopathies.

| **Hemoglobinopathy** | TOTAL | Percentage |
|---|---|---|
| Sickle trait | 69 | 51.50 % |
| Beta Thal trait | 35 | 26.12 % |
| Sickle disease | 22 | 16.42 % |
| HbD Punjab trait | 5 | 3.74 % |
| HbE homozygous | 1 | 0.74 % |
| HbE trait | 1 | 0.74 % |
| Sickle Disease + Beta thal trait | 1 | 0.74 % |
| Grand total | 134 | 100 % |

Out of 134 cases in our study 69(51.50%) patients were found to have sickle cell trait followed by beta thalassemia trait 35(26.12%) followed by sickle cell disease 22(16.42%) followed by HbD Punjab trait 5(3.74%) cases and 1 (0.74%) case each of HbE homozygous, HbE trait , Sickle Disease + Beta thal trait . In our region most common hemoglobinopathies was found to be sickle cell trait.

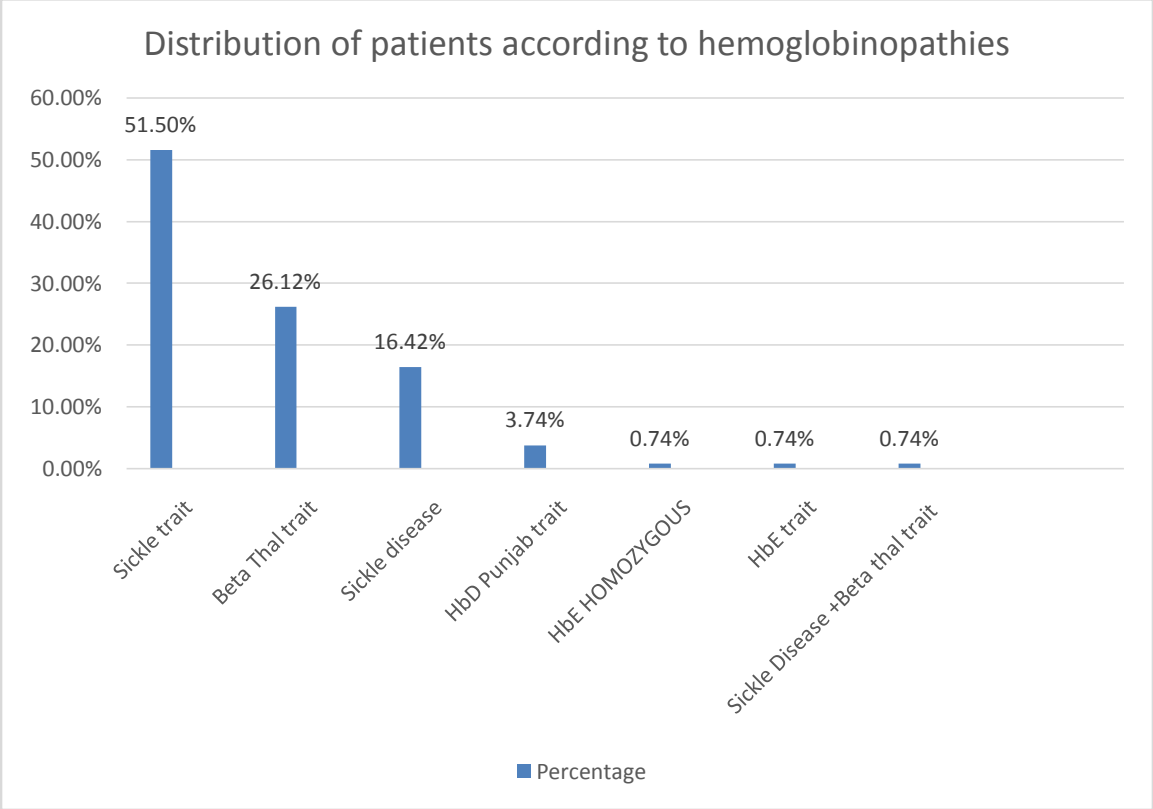


**Table No. II:-** Distribution Of Patients According To Category.

| CATEGORY | NO OF PATEINT | Percentage |
|---|---|---|
| SC | 47 | 35.07 % |
| OBC | 37 | 27.62 % |
| OPEN | 21 | 15.67 % |
| ST | 9 | 6.72 % |
| MUSLIM | 9 | 6.72 % |
| VJ | 6 | 4.47% |
| NT | 5 | 3.73 % |
| TOTAL | 134 | 100 % |

In present study out of total 134 positive cases 47(35.07%) cases were found to be SC category followed by 37(27.62%) cases found to be OBC category followed by Open category 21(15.67%).

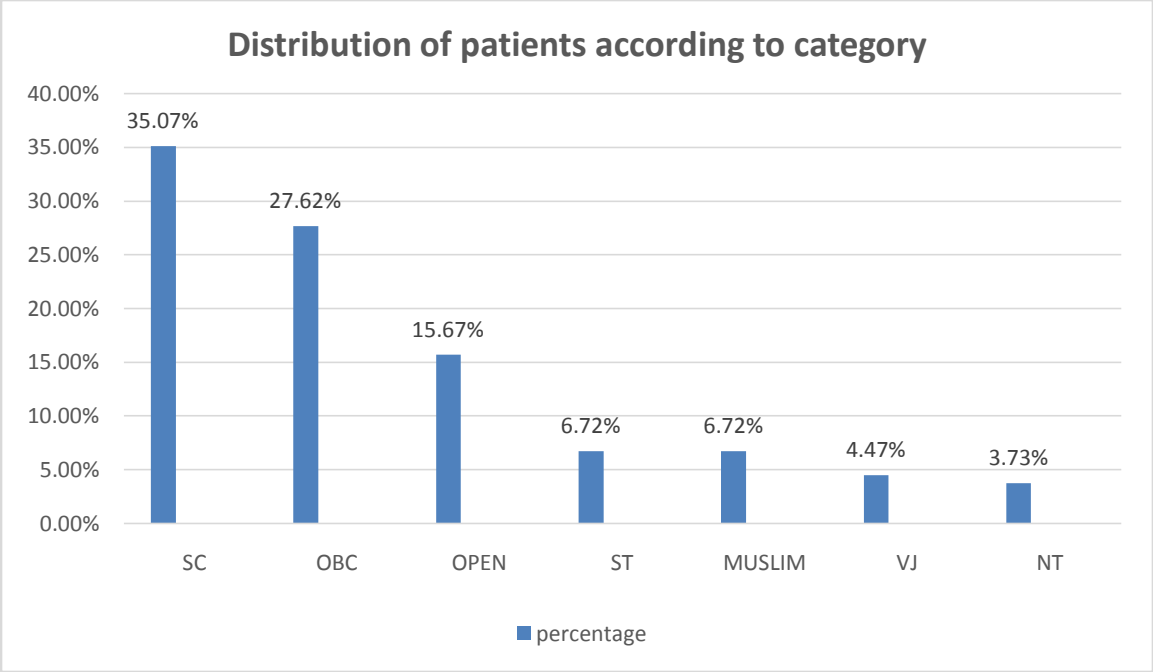


**Table No. III:-** Hemoglobin Profile In Each Case Obtained On HPLC.

| | | **HbA** | **HbA2** | **HbF** | **HbS** | **HbD Punjab** | **HbE trait** |
|---|---|---|---|---|---|---|---|
| Sickle trait | Mean | 61.07 | 3 | 6.7 | 22.9 | NA | NA |
| | SD | 32.91 | 2.97 | 9.47 | 14.84 | NA | NA |
| | Min | 37.8 | 0.9 | 0.00 | 12.4 | 0.00 | 0.00 |
| | Max | 84.34 | 5.1 | 13.4 | 33.4 | 0.00 | 0.00 |
| Beta Thal trait | Mean | 91.5 | 4.95 | 1.64 | 2.7 | NA | NA |
| | SD | 5.23 | 1.34 | 1.08 | 3.8 | NA | NA |
| | Min | 87.8 | 4 | 0.8 | 0.00 | 0.00 | 0.00 |
| | Max | 95.2 | 5.9 | 7.2 | 5.4 | 0.00 | 0.00 |
| Sickle disease | Mean | 22.65 | 3.15 | 15.95 | 64.6 | NA | NA |
| | SD | 24.40 | 3.18 | 22.56 | 29.27 | NA | NA |
| | Min | 5.4 | 0.9 | 0.00 | 43.9 | 0.00 | 0.00 |
| | Max | 39.9 | 5.4 | 31.9 | 85.3 | 0.00 | 0.00 |
| HbD Punjab trait | Mean | 59.75 | 2.95 | 0.9 | 15.95 | 27.8 | NA |
| | SD | 17.32 | 0.78 | 0.14 | 22.56 | 6.08 | NA |
| | Min | 47.5 | 2.4 | 0.8 | 0 | 23.5 | 0.00 |
| | Max | 72 | 3.5 | 1 | 31.9 | 32.1 | 0.00 |
| HbE Homozygous | Mean | NA | NA | NA | NA | NA | NA |
| | SD | NA | NA | NA | NA | NA | NA |
| | Min | 7.7 | 85.6 | 6.7 | 0.00 | 0.00 | 74.60 |
| | Max | 7.7 | 85.6 | 6.7 | 0.00 | 0.00 | 74.60 |
| HbE trait | Mean | NA | NA | NA | NA | NA | NA |
| | SD | NA | NA | NA | NA | NA | NA |
| | Min | 69.4 | 26.4 | 1 | 3.2 | 0.00 | A |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Max | 69.4 | 26.4 | 1 | 3.2 | 0.00 | A |
| Sickle Disease + Beta thal trait | Mean | NA | NA | NA | NA | NA | NA |
| | SD | NA | NA | NA | NA | NA | NA |
| | Min | 19 | 8.6 | 6.7 | 65.7 | 0.00 | 0.00 |
| | Max | 19 | 8.6 | 6.7 | 65.7 | 0.00 | 0.00 |

The percentage of HbF was much higher in case of sickle cell trait and sickle cell disease with a mean value of 22.9% and 64.6% .

The percentage of HbA2 in case of β thalassemia trait was higher with a mean value of 4.95%.

**Table No. IV:-** Haematological Parameters Stratified According To Hemoglobinopathies In The Study Population.

| | | **Hb%** | **RBC count** | **RDW** | **MCV** | **MCH** | **MCHC** |
|---|---|---|---|---|---|---|---|
| Sickle trait | Mean | 7.9 | 2.79 | 25.9 | 74.35 | 29.25 | 30.75 |
| | SD | 5.37 | 2.41 | 18.80 | 50.98 | 14.49 | 15.48 |
| | Min | 4.1 | 1.08 | 12.6 | 38.3 | 19 | 19.8 |
| | Max | 11.7 | 4.5 | 39.2 | 110.4 | 39.5 | 41.7 |
| Beta Thal trait | Mean | 7.3 | 3.65 | 21.75 | 65.2 | 21.2 | 31.65 |
| | SD | 7.7 | 4.24 | 10.39 | 21.21 | 10.60 | 9.82 |
| | Min | 1.8 | 0.65 | 14.4 | 50.2 | 13.7 | 24.7 |
| | Max | 12.8 | 6.65 | 29.1 | 80.2 | 28.7 | 38.6 |
| Sickle disease | Mean | 7.75 | 3.47 | 18.3 | 83.55 | 29.55 | 35 |
| | SD | 6.71 | 2.27 | 5.52 | 29.06 | 12.79 | 5.9 |
| | Min | 3 | 1.87 | 14.4 | 63 | 20.5 | 30.8 |
| | Max | 12.5 | 5.08 | 22.2 | 104.1 | 38.6 | 39.2 |
| HbD Punjab trait | Mean | 12.5 | 4.36 | 26.25 | 82.35 | 28.55 | 34.05 |
| | SD | 0.98 | 0.54 | 13.93 | 3.18 | 1.48 | 0.77 |
| | Min | 11.8 | 3.98 | 16.4 | 80.1 | 27.5 | 33.5 |
| | Max | 13.2 | 4.75 | 36.10 | 84.6 | 29.6 | 34.6 |
| HbE Homozygous | Mean | NA | NA | NA | NA | NA | NA |
| | SD | NA | NA | NA | NA | NA | NA |
| | Min | 7.4 | 5.39 | 23 | 48.7 | 13.7 | 29.4 |
| | Max | 7.4 | 5.39 | 23 | 48.7 | 13.7 | 29.4 |
| HbE trait/HbLepore trait/HbD Iran trait | Mean | NA | NA | NA | NA | NA | NA |
| | SD | NA | NA | NA | NA | NA | NA |
| | Min | 10.2 | 3.89 | 16.7 | 68.2 | 26.7 | 38.9 |
| | Max | 10.2 | 3.89 | 16.7 | 68.2 | 26.7 | 38.9 |
| Sickle Disease + Beta thal trait | Mean | NA | NA | NA | NA | NA | NA |
| | SD | NA | NA | NA | NA | NA | NA |
| | Min | 10.1 | 3.33 | 18.1 | 75.2 | 25.5 | 32.1 |
| | Max | 10.1 | 3.33 | 18.1 | 75.2 | 25.5 | 32.1 |

Assessment of the hematological parameters of different hemoglobinopathies in the present study revealed the following findings:

Relatively normal RBC counts in β thalassemia trait, HbE trait, HbE homozygous disease, HbSβ thalassemia and Sickle cell disease. Slightly lower RBC count in sickle cell trait.
Low hemoglobin levels in all disorders except HbD Punjab, which showed normal hemoglobin levels.
Low MCV in all disorders except sickle cell disease,HbD Punjab, sickle cell disease+Betathal trait which showed normal MCV.
Low MCH in all disorders except HbDPunjab ,HbE Trait , sickle cell disease+Betathal trait , which showed normal MCH.
Low MCHC in all disorders except HbDPunjab ,HbE Trait , sickle cell disease+Betathal trait , which showed normal MCHC.
High RDW in all disorders except HbE Trait, which showed normal RDW.

## Discussion:-

The present study included 134 patients. Jain BB et al [130] included 3832 cases. Chaudhury SR et al [131] included 14145 cases. Mondal SK et al [132] included 90210 cases. Mandal PK et al [133] included 50487 cases. Goswami BK et al [134] included 1872 cases. Mukhopadhyay D et al [135] included 10407 cases .Mondal SK et al [136] included 1193336 cases.

The present study consisted of more females (74.63%) than males (25.37%), whereas the studies conducted by Goswami et al [134] and Jain BB [130] et al showed almost equal distribution of males and females. Debasis et al [135] conducted a similar study where the population of females was more than males. This was due to the increased number of females who came for ante-natal checkups and diagnosed to have hemoglobin disorders.

The present study showed that maximum number of cases (81.34%) presented in the age group of 19-40 years which was concordant with a study conducted by Jain BB et al [130] (54.15%) and Gowswami et al [134] (32.2%).

**Table 1:-** Comparison Of Percentages Of Various Hemoglobinopathies With Different Studies.

| **Hemoglobinopathy** | **Present Study** | **Chaudhury et al** | **Mondal SK et al** | **Mondal et al** | **Debasis et al** | **Mandal et al** | **Gowswami et al** | **Jain BB et al** |
|---|---|---|---|---|---|---|---|---|
| Sickle cell Trait | 51.50 % | 01.65 | 03.11 | 04.02 | 02.50 | 04.84 | 1.1 | 2.14 |
| β thalassemia trait | 26.12 % | 41.69 | 37.76 | 37.52 | 38.37 | 56.91 | 17.8 | 55.84 |
| Sickle cell disease | 16.42 % | 00.10 | 02.63 | 03.33 | 00.50 | - | 0.2 | 0.54 |
| HbD Punjab | 3.74 % | 08.23 | 00.98 | - | 00.60 | 00.58 | - | 0.71 |
| HbE homozygous | 0.74 % | 00.55 | 02.80 | 03.50 | 02.12 | 00.44 | 25.3 | 0.62 |
| HbE Trait | 0.74 % | 21.30 | 24.80 | 23.45 | 34.60 | 23.91 | 34.4 | 15.70 |
| HbSβ thalassemia | 0.74 % | 04.13 | 02.13 | 01.81 | 00.27 | 01.28 | 3.4 | 1.07 |

All values are in percentages

The percentage of cases of sickle cell trait was calculated to be 51.50%. This was higher as compared to the studies conducted by Chaudhury et al [131], Mondal SK [136] et al, Mondal et al [132], Debasis et al [135], Mandal et al [133], Jain BB [130] et al, Goswami et al [134].

β thalassemia major cases were calculated to be 26.12% of the total number of cases. Chaudhury et al [131], Mondal SK et al [136], Mondal et al [132], Mandal et al [133] ,Debasis et al [135] and Jain BB [130] reported a

higher rates in this particular disease. Goswami at al [134] reported lesser percentages as compared to the present study.

The percentage of cases of sickle cell disease was calculated to be 16.42%. This was higher as compared to the studies conducted by Chaudhury et al [131], Mondal SK [136] et al, Mondal et al [132], Debasis et al [135], Mandal et al [133], Jain BB [130] et al and Goswami et al [134].

The percentage of cases of HbD Punjab was calculated to be 16.42%. This was higher as compared to the studies conducted by Chaudhury et al [131], Mondal SK [136] et al ,Debasis et al [135], Mandal et al [133], Jain BB [130] et al.

The percentage of cases of HbE homozygous was calculated to be 0.74%. This was lesser as compared to the studies conducted by Mondal SK [136] et al, Mondal et al [132], Debasis et al [135], Goswami et al [134]. Chaudhury et al [131], Mandal et al [133] , and Jain BB [130] reported a lower rates in this particular disease.

The percentage of cases of HbE trait was calculated to be 0.74%. This was lower as compared to the studies conducted by Chaudhury et al [131], Mondal SK [136] et al, Mondal et al [132], Debasis et al [135], Mandal et al [133], Jain BB [130] et al, Goswami et al [134].

The percentage of cases of HbSβ thalassemia was calculated to be 0.74%. This was lower as compared to the studies conducted by Chaudhury et al [131], Mondal SK [136] et al, Mondal et al [132], Mandal et al [133], Jain BB [130] et al, Goswami et al [134] and Mandal et al [133]reported lesser percentages as compared to the present study.

## Conclusion:-

The wide prevalence of thalassemias and hemoglobinopathies in India has been attributed to the sociocultural activities of marriages in the same castes and ethnic groups. These disorders which were mainly confined to certain geographic regions, religions, castes and tribes are now widely prevalent due to migration of various races over time, and thus creating more serious hemoglobin disorders.

HPLC forms a rapid, accurate and reproducible tool for early detection and management of hemoglobinopathies and variants. Continuous awareness programs, mass screening of the population especially childbearing age and school going children will help in early detection of hemoglobinopathies . This can in turn with proper genetic counseling help in reducing the morbidity and mortality.

Detection of β-thalassemia carriers and other structural variants will provide a preliminary insight to the prevalence of hemoglobinopathies in hospital based patients.Awareness can be created in an effort to prevent the birth of affected children.The present study is intended to contribute to the available data as the identification of these disorders is important epidemiologically and aids in prevention of more serious hemoglobin disorders.

## References:-

1. Sachdev R, Dam AR, Tyagi G. Detection of Hb variants and hemoglobinopathies in Indian population using HPLC: Report of 2600 cases. Indian J PatholMicrobiol 2010;53:57-62
2.Livingstone FB. Frequencies of haemoglobin variants. New York: Oxford University Press 1985.3. Forget BG and Bunn HF. Classification of the Disorders of Hemoglobin. Cold Spring HarbPerspect Med 2013;3: a011684.
4. Christopher S. Thom, Claire F. Dickson, David A. Gell, and Mitchell J. Weiss. Hemoglobin Variants: Biochemical Properties and Clinical Correlates Cold Spring HarbPerspect Med 2013;3:a011858.
5.Elisabeth Kohne. Hemoglobinopathies Clinical Manifestations, Diagnosis, and Treatment. DtschArztebl Int. 2011 Aug; 108(31-32): 532–540.6.Livingstone FB. Abnormal hemoglobins in human populations. Chicago: Aldine; 1967.
7.Weatherall DJ, Clegg JB. Distribution and population genetics of the thalassaemias. 4th ed. Oxford: Blackwell Science; 2001. Chapter 6.
8. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ 2001; 79: 704-12.

9. Maji SK, Mandal PK, Bera R, Dolai TK et al. The prevalence and characterization of β-thalassemia trait by high-performance liquid chromatography among the rural population in West Bengal, India. Thalassemia Reports 2014;4:2188.
10. Rao S, Kar R, Gupta SK, Chopra A, Saxena R. Spectrum of haemoglobinopathies diagnosed by cation exchange-HPLC & modulating effects of nutritional anemias from north India Indian J Med Res 2010;132:513-9.
11. Shrivastav A, Patel U, Joshi JR, Kaur A, Agnihotri AS. Study of hemoglobinopathies and Hb variants in population of Western India using HPLC: A report of 7,000 cases. J ApplHematol 2013;4:104-9
12. Ray GK, Jena RK. Spectrum of Hemoglobinopathies: A New Revelation in a Tertiary Care Hospital of Odisha. Indian J Hematol Blood Transfus. 2019 Jul;35(3):513-517.
13. Sharma N, Pant AD, Manandhar U. Spectrum of hemoglobinopathies in a tertiary care centre. JPN 2020;10: :1645-9.
14. Bhokare SB, Phulgirkar PP, Joshi AR, Bindu RS. Spectrum of hemoglobinopathies by high performance liquid chromatography with special reference to role of HbA2 levels at tertiary care centre. Int J Res Med Sci 2016;4:5269-76.
15. Mondal SK, Dasgupta S, Mondal S, Das N. Spectrum of thalassemias and hemoglobinopathies in West Bengal: A study of 90,210 cases by cation exchange high-performance liquid chromatography method over a period of 8 years. J ApplHematol 2014;5:91-5.