

Role of HPLC (High Performance Liquid Chromatography) as a Tool For Detection of Hemoglobinopathies.

Dr. Ravi Shankar¹ Dr.Kamal Narayan Prasad² Dr. Bejoy Chand Banerjee³

1.Author- Tutor, Department of Pathology ,Patliputra Medical college ,Dhanbad i

2. Co Author- Junior Resident, Department of Pathology , Rajendra Institute of Medical Sciences,Ranch

3.Corresponding Author- Professor and Head of Department, Department of Pathology ,Patliputra Medical college ,Dhanbad

Corresponding Author: Dr. Ravi Shankar

Date of Submission: 19-07-2019

Date of acceptance: 05-08-2019

I. Introduction

The real debt of modern science in the field of Medicine , is the knowledge about the structure of haemoglobin. In 1959, Max Preutz determined, molecular structure of haemoglobin by X-ray crystallography.

The haemoglobin molecule is composed of four separate polypeptide chains of amino acids, two alpha chains and two beta chains, as well as four iron bearing heme groups that bind oxygen. The alpha chains are coded for by two similar genes on chromosome 16 ; the beta chains by a single gene on chromosome 11. Mutations and deletions in these genes cause one of the many hemoglobinopathies.

In 1910, James B. Herrick reported “peculiar ,elongated , sickle shaped red corpuscles” in “a case of severe anaemia”. The sickle cells he thought were freakish poikilocytes ,and , with considerable prescience ,he suggested that they were manifestation of a peculiar chemical or physical condition. In 1917, Emmel , noted that sickling occurred both in persons with severe anemia and in others who were apparently healthy, thus recognizing both sickle cell anaemia and sickle cell trait. Linus Pauling with Itano demonstrated an electrophoretically abnormal hemoglobin in sickle cell anemia, thus introducing the concept of molecular disease.

In 1925, Cooley and Lee separate from the complex of disorders of infancy and childhood that had been as Von Jaksch anemia a syndrome characterized by chronic , progressive anemia beginning early in life , with pronounced peripheral blood erythroblastosis, characteristic facies , splenomegaly, and a familial incidence. The patients were mainly of Mediterranean background which led to the introduction of the name Thalassemia derived from the Greek word for sea.

The inherited disorders of hemoglobin are the commonest single gene disorders in man . Inherited abnormalities of hemoglobin synthesis may be divided into two groups: the hemoglobinopathies and the thalassemia. The hemoglobinopathies are characterized by production of structurally defective hemoglobin variants due to abnormalities in the formation of globin moiety of the molecule. The thalassemia are characterized by reduced rate of production of normal haemoglobin due to absent or decreased one or more types of the normal polypeptide chains. Many are functionally normal therefore clinically silent.

Haemoglobinopathies and thalassemias are the most common single gene disorders in the world. Around 7 percent of population worldwide are carriers with more than 3,00,000 severely affected babies born every year. Worldwide, the Hb disorders are responsible for 3.4% mortality in children below 5 years of age.

Inherited disorders of haemoglobin are extremely common in Indian population ranging from near structurally normal haemoglobins to severe transfusion dependant haemoglobinopathies. The disorders of Hb frequently encountered in India include beta thalassemia, HbE - beta thalassemia, HbE, HbD and sickle cell anemia. The prevalence of thalassemias and haemoglobinopathies varies with geographic locations. It has been estimated that in India, 0.37/1000 fetuses have a haemoglobin disorder. The prevalence of beta thalassemia mutations is as high as 17% in some Indian populations.

Their detection is important epidemiologically and to prevent other more serious haemoglobinopathies in future generations . The inherited disorders of haemoglobin, particularly the β -thalassemias and their interaction with haemoglobin E (HbE) and haemoglobin S (HbS) are a considerable health problem in India and contribute significantly to morbidity and mortality. Earlier studies have shown that the overall prevalence of β -thalassemia is 3–4 % with an estimate of around 8,000 to 10,000 new births with major disease each year. Most of these children have a severe clinical presentation but are managed sub-optimally due to lack of financial resources in majority of the families. Thus preventing the birth of affected children is the best option for India. A prerequisite for this is the knowledge of the prevalence of β -thalassemia and other haemoglobinopathies.

Anemia is considered a major public health problem in India. Nutritional and non-nutritional factors causing anemia are widely prevalent in Jharkhand. In view of this it was considered worthwhile to analyze a large series of patients referred to a clinical diagnostic laboratory in RIMS to find the pattern of haemoglobinopathies and thalassemsias in the workup of anemia. So, this study was undertaken to know about the spectrum of different haemoglobinopathies in Jharkhand using HPLC.

High performance liquid chromatography (HPLC) is a simple, rapid, highly sensitive and specific method of detection of different Hb variants. Clinical history and findings of thorough hematologic evaluation, including complete blood count, reticulocyte count and red blood cell morphology are done to reach an accurate diagnosis. In some cases, family studies are also required to detect a particular Hb variant.

The knowledge of the common Hb variants encountered in a particular area is important for the formulation of specific diagnostic, genetic counseling, preventive and therapeutic strategies.

II. Materials And Methods

MATERIALS

This was a prospective study carried out in the Department of Pathology, Rajendra Institute of Medical Sciences (RIMS), Ranchi, from July 2013 to September 2014. Consecutive 200 patients referred by clinicians as a work up for anemia and for confirmation of clinically suspected cases of hemoglobinopathy or β thalassemia were analyzed on the Bio Rad Variant II HPLC system by β -thal short program. This included mainly anaemic person, antenatal cases and their family members. Cases with nutritional deficiency anaemia, where a co-existent thalassemia/ haemoglobinopathy was suspected, were also investigated.

Clinical aspect and lab investigations were correlated. Positive cases were further called for family screening. Written consent was taken from all patients for using their sample for research purpose. Initial hematologic investigations were done in all those cases including the Romanowsky staining for blood cell morphology. About 2-3 ml of blood sample was collected in EDTA vial and was analyzed in automated cell counter (Sysmex XT 2000i; Kobe, Japan) for complete blood counts. The sickling test was performed by using freshly prepared sodium metabisulphite solution as reducing agent in all cases. Samples were stored at 4-8 °C and were analyzed in batches within one week by HPLC.

This was a single time study and no follow up was done. Statistical methods like number, percentage and descriptive studies were applied for the present study. Since Jharkhand has a large population of tribals it was considered worthwhile to divide study subjects into non tribals and tribals.

INCLUSION CRITERIA

- Patients presenting with pallor and generalized weakness
- Patients with clinical suspicion of hemolytic anemia
- Patient with nutritional deficiency anaemia, where a co-existent hemoglobinopathy is suspected.
- Family members of these patients.
- Samples received from Referral centres and Primary Health centres suspected of hemolytic anaemia.

EXCLUSION CRITERIA

- In patients requiring blood transfusion sampling was deferred for at least 4 wk after or just before next transfusion.
- Non cooperative patients.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC; formerly referred to as **high-pressure liquid chromatography**), is a technique in **analytic chemistry** used to separate the components in a mixture, to identify each component, and to quantify each component.

It is a rapid method of screening of haemoglobin variants. Results are generally precise with high sensitivity and specificity. Our institute uses BIORAD VARIANT II with software CDM 5-1.

Sample collection

Sample of the blood was collected from the subject by venepuncture method from antecubital vein with all aseptic measures. 2.5 ml of blood was collected in a clean vial containing Ethylene diamine tetra acetic acid (EDTA) and gently shaken to avoid clotting. Sample is stored at 2-8°C and the test is performed within 1 week of collection. Storage for longer duration leads to denaturation of haemoglobin. Haemolysate is prepared using lysis buffer (10 μ l blood in 1 ml lysis buffer.) Each sample takes 6.5 min for the result. The instrument is calibrated for HbA2/HbF using area percentage and retention time of HbA2 for which HbA2/F calibrator is provided which has been assigned values (in units of area percent of total hemoglobin) for both HbA2 and HbF and it is analyzed at the beginning of each run. The value more than 3.5% of A2 fraction of hemoglobin was

taken as cut off point for determining the β thalassemia trait and more than 10% was assumed to be hemoglobin E.

III. Results & Observation

The present cross sectional study on spectrum of hemaglobinopathies in Jharkhand was carried out in the department of pathology, Rajendra Institute of Medical Sciences, Ranchi. The period of study was from July 2013 to September 2014.

During this period total of 200 patients referred to our department for investigation of suspected thalassemia/hemoglobinopathies were analyzed by Bio Rad Variant II CE-HPLC using β -thal short program. Out of these 80 were found to have normal HPLC and 120 had one form or the other of hemoglobinopathies.

1. Gender distribution of the patients-

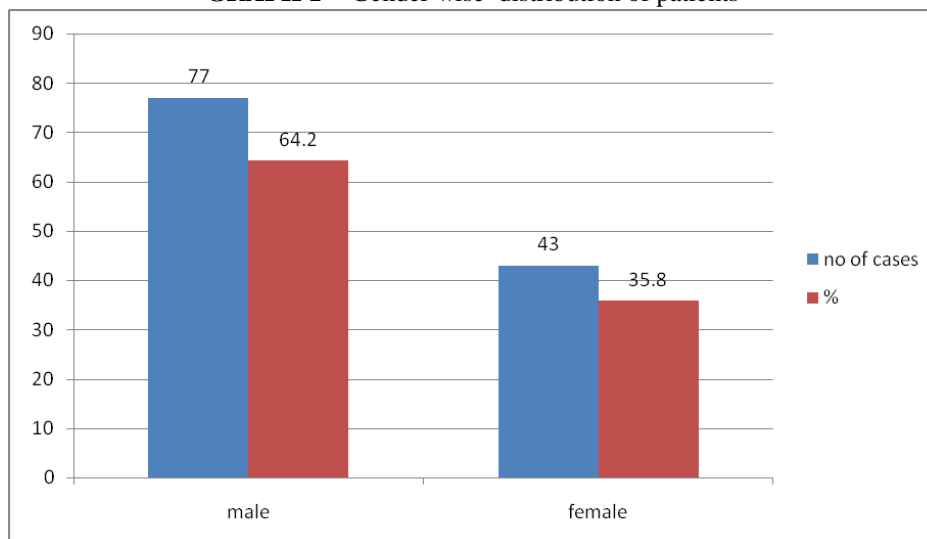
Gender distribution of patients studied is shown in table 1 and graph 1. The cases of abnormal haemoglobins included 77 males and 43 females.

TABLE 1-

Gender	No. of cases	Percentage (%)
Male	77	64.2
Female	43	35.8
Total	120	100

There is preponderance of males(64.2%) over females (35.8%).

GRAPH 1 - Gender wise distribution of patients



2. Age distribution of patients:

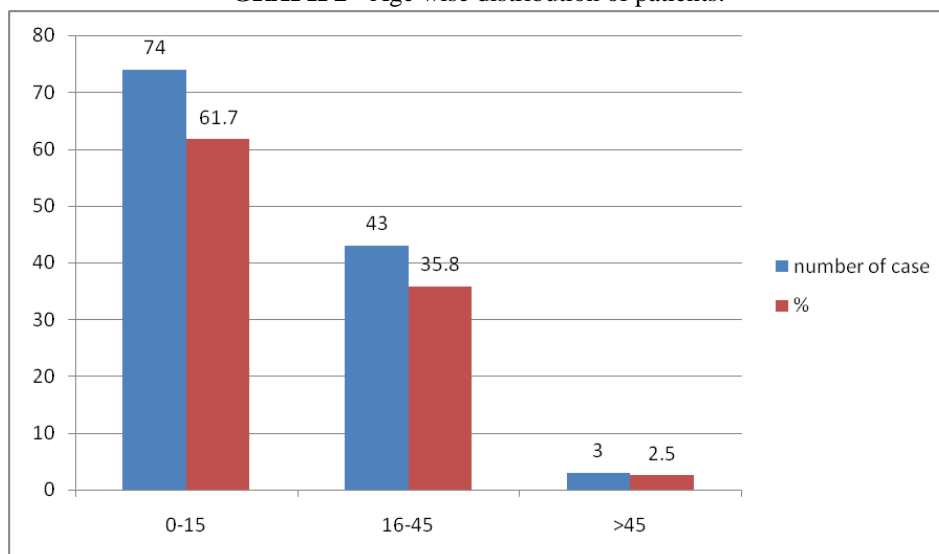
Age distribution of the patients studied is shown in table 2 and graph 2.

TABLE 2

Age in years	Number of cases	Percentage(%)
0- 15yrs	74	61.7
16-45 yrs	43	35.8
>45 yrs	3	2.5
Total	120	100

Increased incidence was found in children below 15 yrs of age .More than half of the cases of study were in age group below 15 yrs. (61.7%).

GRAPH 2 - Age wise distribution of patients:



3. Spectrum of hemoglobinopathies/thalassemia -

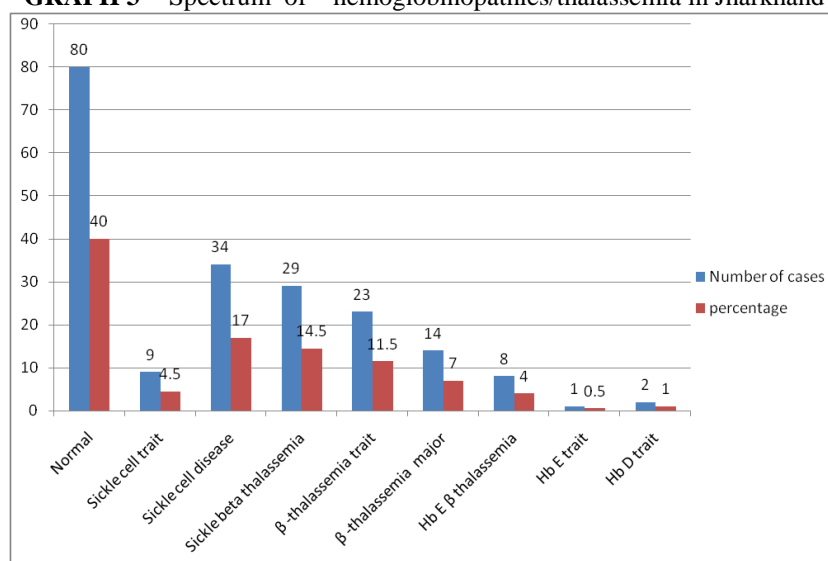
Spectrum of hemoglobinopathies/thalassemia in Jharkhand is shown in table 3 and graph 3.

TABLE 3

Sl no.	HPLC Finding	No. of cases	Percentage(%)
1.	Normal	80	40
2.	Sickle cell trait	9	4.5
3.	Sickle cell disease	34	17
4.	Sickle beta thalassemia	29	14.5
5.	β -thalassemia trait	23	11.5
6.	β - thalassemia major	14	7
7.	Hb E β thalassemia	8	4
8.	Hb E trait	1	.5
9.	Hb D trait	2	1
Total		200	100

Out 200 cases, 80 (40 %) were found normal and 120 (60%) had one form or the other of hemoglobinopathies. Sickle cell disease(17%) and sickle β thalassemia(14.5%) were the major hemoglobinopathies found . Few cases of HbE β thalassemia were found (4 %) .A single case of HbE trait and 2 cases of HbD trait were also found in the study.

GRAPH 3 - Spectrum of hemoglobinopathies/thalassemia in Jharkhand



Normal adult chromatogram shows primarily HbA, a small percentage of HbA₂ (<3.5%) and traces of fetal Hb (<1%). Mean MCV was 80.3 fl, MCH 26.1 pg and red cell diameter width (RDW) was 16.7. HPLC showed mean HbA 87.3%, HbA₂ 2.8% and HbF 0.5%.

Sickle cell anemia (SS) was the predominant abnormality followed by HbS β thalassemia. HbS elutes in the S window with a retention time of 4.12-4.42 min. Hb in HbAS ranged from 1.1 to 14.4 g/dl with a mean of 6.4 g/dl. HbS comprised of a mean of 30.1% (range 15.4-43.3%) while HbA comprised of a mean of 57.1% (range 36.3-68.6%). HbF ranged from 5.0% to 12.4%.

In HbSS, Hb ranged from 1.4 to 12.6 g/dl with a mean of 6.2 g/dl with the PBS showing anisopoikilocytosis, target cells and sickle cells. HbSS was invariably associated with a raised HbF ranging from 4.3% to 47.6% (mean 19.0%).

In double heterozygotes of HbS and β thalassemia Hb ranged from 1.7 to 10.3 g/dl with a mean of 5.7 g/dl and the PBS showed a severe degree of anisopoikilocytosis with RDW ranging from 17.2 to 40.6 (mean 21.2). The level of HbS in these patients ranged from 42.8% to 86.7% (mean 69.3%), A₂ from 5.1% to 8.9% (mean 6.3%), HbA from 0.9% to 10.8% (mean 4.6%), and HbF from 8.3% to 45.0% (mean 18.9%).

HbA₂ levels of 4-9% are diagnostic of BTT in an asymptomatic individual with no or mild anemia. 23 patients were diagnosed to be of BTT. HbA₂ ranged from 4.0% to 8.5% with a mean of 5.3%.

As no laboratory tests could differentiate between beta thalassemia major and intermedia which is basically a clinical subdivision, it was decided to group both these groups together as beta thalassemia homozygous. The Hb ranged from 1.3 g/dl to 9.0 g/dl with the PBS showing marked degree of anisopoikilocytosis with hypochromia and polychromacia with target and tear drop cells and a raised RDW (19.7-34.2 mean 26.8). HbF levels ranged from 74.4% to 98.4% with a mean of 88.6%.

HbE beta thalassemia, the compound heterozygous state of HbE and beta thalassemia, results in a variable clinical picture similar to that of homozygous beta thalassemia. HbE ranged from 32.8% to 82.0% (mean 56.7%) while HbF ranged from 14.9% to 57.2% (mean: 30.7%). The level of HbA was from 0.4% to 8.7% with a mean of 3.8%. The PBS invariably showed anisopoikilocytosis with hypochromia and target cells depending on the severity of the anemia with the red cell indices showing a microcytic hypochromic anemia, and an RDW of 15.6-39.8 (mean 23.6). The HbE fraction ranged from 16.9% to 40.8% with a mean of 29.0%; HbA ranged from 55.7% to 70.9% with a mean of 59.6%.

In one patient of Hb E trait Hb% was 8.9 with HbF 1.3 and HbA₂ 30.2%.

HbD trait were detected in 2 patients. HbD elutes in the D-window with a retention time of 3.98-4.12 min. HbD in trait ranged from 19.5 to 40.2%.

Various normal and variant haemoglobins encountered were as follows :-

Haemoglobin variants with retention times in the F window (1.00-1.30 min): At least seven haemoglobin variants (four β - and three α -variants) are expected to elute in this window, all in quantities >10 per cent⁵. However, most of our patients with high Hb in the F window were mostly homozygous β -thalassaemia patients or double heterozygous β thalassaemia/haemoglobinopathy and sickle cell disorder patients. The haplotype of HbS gene that is prevalent in India is the Saudi Arabia/Indian haplotype. This haplotype is associated with higher levels of HbF which reduces the clinical severity of the disease.

Haemoglobin variants with retention times in the p2 window (1.30-1.60 min): HbA_{1c} elutes in the P2 window. No haemoglobin variants were detected in this window.

Haemoglobin variants with retention times in the p3 window (1.60-1.90 min): A previous study has found nine haemoglobin variants (four α - and five β -variants) with elution peaks in the P3 window⁶. No haemoglobin variants were detected in this window.

Haemoglobin variants with retention times in the A0 window (2.20-3.30 min): Six haemoglobin variants (two α - and four β -variants) have been reported in the A0 window⁶. No such variant was found in the present study.

Haemoglobin variants with retention times in the A2 window (3.68-3.90 min): Two haemoglobin variants had elution peaks in the A2 window- HbA₂ and HbE. The retention times and %Hb for HbA₂ (3.65 min) and HbE (3.73 min) were significantly different

Haemoglobin variants with retention times in the D window (3.90-4.12 min): Only one haemoglobin variant; HbD-Punjab, with retention times of 4.15 min, was identified in D window.

Haemoglobin variants with retention times in the S window (4.12-4.42 min): Only HbS variant, with retention times of 4.41 min, was seen in this window. A new abnormal haemoglobin Hb D Agri with 2 amino acid substitutions in the same β globin chain [β 9 (A6) Ser \rightarrow Tyr, β 121(GH-4) Glu \rightarrow Gln] has been reported in India which can be mistaken for HbS based on HPLC alone. However, a negative sickling and solubility test should raise a suspicion followed by molecular studies for confirmation.

Haemoglobin variants with retention times in the C window (4.88–5.18 min): No haemoglobin variant was detected in this window.

Effect of iron deficiency anaemia on HbA₂ levels - The patients with normal HPLC pattern (total 30 in number where iron deficiency was suspected based on clinical and haemogram findings) were subjected to iron studies. Patients were divided in two groups depending upon their iron profile. The levels of HbA₂ for 26 patients with IDA ($2.74 \pm 0.34\%$) was found to be significantly lower ($P=0.004$) than the 4 patients with normal iron profile ($3.03 \pm 0.26\%$). However; there was no significant difference in the levels of HbA₂ for 23 β -thalassaemia trait patients, 17 with concomitant IDA ($5.49 \pm 0.52\%$) and 6 without IDA ($5.09 \pm 0.58\%$).

Effect of megaloblastic anaemia on HbA₂ levels : The patients with normal HPLC were divided in two groups depending upon their MCV values (>110 fl) and confirmed by further testing with Vit B₁₂ and folate assay. The 19 patients with megaloblastic anaemia showed HbA₂ levels ($3.32 \pm 0.56\%$) which was significantly higher ($P<0.001$) than the HbA₂ levels ($2.89 \pm 0.37\%$) of normal cases with $MCV<110$ fl.

IV. Conclusion

The present study provides an overview of the burden and spectrum of hemoglobinopathies in this region of country. Along with sickle cell disorders, β -thalassaemia is also prevalent in this region. In this perspective, it is emphasized that a routine premarital screening program is needed for identification and prevention of high-risk marriages. Nevertheless, mass awareness, knowledge generation, and genetic counseling are still a vital requisite. It can be concluded from the study that HPLC is a versatile, reproducible chromatographic technique for the estimation of haemoglobinopathies/ thalassaemia.

Since this work was carried out in a limited period of time with a small number of cases, it is envisaged that a long term comprehensive study in this line will throw more light and help in formulating ways to prevent and for early diagnosis and treatment of different hemoglobinopathies prevalent in Jharkhand.

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