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Original Research Article

HPLC and Haemoglobinopathies: A Deep Dive into Indian Health Statistics

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Abstract:

Background: Haemoglobinopathies are genetic disorders that affect the structure or synthesis of haemoglobin, the oxygen-carrying protein in red blood cells. These disorders are prevalent in various regions of India and pose a significant public health challenge. Early detection and prevention are crucial to manage these conditions effectively.

Materials and Methods: A prospective study was conducted to diagnose and quantify the prevalence of haemoglobinopathies in India. 760 blood samples were collected from different regions across the country. High-performance liquid chromatography (HPLC) was used as a screening tool for this study. HPLC is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It is sensitive and reliable for identifying and quantifying different haemoglobin fractions.

Results: It was found that 32.5% of the samples had abnormal haemoglobin variants. The most common variant was beta thalassemia heterozygous, found in 19.47% of the samples. Other haemoglobinopathies, such as Hb E, Hb S, Hb D, and some rare variants were also detected.

Conclusion: This study concluded that HPLC is an effective screening tool for identifying and quantifying different haemoglobin fractions. However, it was also noted that molecular tests are necessary for confirmation due to the complexity of these genetic disorders. The importance of early detection and prevention strategies for managing haemoglobinopathies was emphasized, given their significant prevalence in India.

Keywords: Haemoglobinopathies, HPLC, beta thalassemia, HbE, Sickle Cell Disease.

Introduction

Haemoglobinopathies are single gene disorders that affecting either Haem synthesis or structure. [1] Haemoglobinopathies are divided into two broad categories - (a) hemoglobinopathies due to defect in the structure of haemoglobin (Hb), in which abnormal Hb is produced by substitution of one amino acid by another amino acid, that leads to formation of abnormal structural Hb variants such as haemoglobin S, C, D and E and (b) Haemoglobinopathies due to abnormal globin chain synthesis or absence of globin chain of normal haemoglobin, includes thalassemia's and its variants. [2]

Haemoglobin is consisting of four sub-units; each of sub-units is consists of one polypeptide globin chain and one heme group. The adult haemoglobin consists of two types of haemoglobin Hb A and Hb A2. Hb A consist of two alpha and two beta polypeptide globin chains. These chains have different sequence of amino-acids but the length is similar. Hb A2 consist of two alpha and two delta polypeptide globin chains. [3]

Thalassemia disorder is due to quantitative aberrations in globin chain synthesis. Absence of alpha-globin chain known as Alpha thalassemia while absence of beta globin chain known as Beta thalassemia. In a newborn, predominant type of Hb is fetal Haemoglobin (Hb F- alpha 2, gamma2), which is gradually decreases and reach adult levels (<1% of total Hb) as Hb A begin to be produced by the end of the first year of life. In beta thalassemia's transformation of Hb F to Hb A is disturbed due to defect in the synthesis of Hb A, this leads to level of Hb F to remain higher than normal. Thalassemia's are characterized by premature destruction of red cell precursors in the

marrow and extramedullary sites haemolytic anaemia, ineffective erythropoiesis, iron overload, and associated complications. [3]

Sickle cell disease occurs due to mutation in the gene at beta globin chain which replace valine from glutamic acid at the sixth amino acid of the beta-globin chain, that leads to formation of sickle haemoglobin (Hb S). This alters the red cell membrane and the normal flexible round red blood cell changes into an elongated, fragile, crescent shaped cell due to alteration of red cell membrane, that reduces the red cell life. [6] Sickled cells often form aggregates, causes microvascular obstruction by aggregation in hypoxic state, which leads to painful crisis, osteonecrosis, emboli and multi-organ damage. [3]

Estimated prevalence of Haemoglobinopathies in the world is 7% that makes it the most common genetic disorder worldwide [3]. Among all sickle cell and Thalassemia's are the most common hemoglobinopathies. [4] Approximately 60-70 million population worldwide affected with sickle cell disease. It was also estimated that approximately 15 million population worldwide affected with thalassemia with 240 million of population are carriers of beta-thalassemia [5]. The prevalence of beta-thalassemia in India ranged from 3-17% and other variant of haemoglobin disease such as Hb S was 4.3%, Hb D was 0.86%, and Hb E was 10.9% (prevalent mostly in North Eastern region of India). [6]

Haemoglobinopathies are a great challenge for affected families and the health care system. Early detection of haemoglobinopathies and characterization are very important so that affected families can be counselled and warned of associated serious complications such as recurrent infections, cirrhosis, pulmonary hypertension, endocrine failure, hepatobiliary cancers, arthropathies, iron overload, transfusion related complications and early death. [7] These cases require lifelong care. Early diagnosis, pre-natal/pre-marital screening and appropriate treatment is utmost important in the prevalent areas.

The laboratory diagnosis of haemoglobinopathies can be achieved by a stepwise approach that can be start with a detailed clinical history, followed by complete haematologic evaluation [including complete blood count (CBC), haemoglobin level, reticulocyte count, and detailed peripheral film examination], followed by protein based analytic methods [alkaline and acid Hb-electrophoresis, and high-performance liquid chromatography (HPLC)]. Advanced nucleic acid based methods [such as polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR, and sequencing of genomic DNA can be used for confirmation of haemoglobinopathies[1,2].

Material and Methods

A prospective study was carried out from the samples collected from peripheral labs of various centres of India, stored at 4-8°C and were processed at tertiary centres over twelve months duration from 01-10-2023 to 31-08-2023. Total 760 blood samples were sent for suspected haemoglobinopathy work-up by CE-HPLC analyser (BioRad laboratories, California, USA). Samples mainly includes transfusion requiring adults, antenatal females, children and their siblings. There was no absolute exclusion standard applied, but the sampling was postponed for minimum of four weeks for the patients in need of blood transfusions or the sample was taken just prior to the following transfusion. Each patient gave their written consent prior to their sample was allowed to be used for research. After taking complete relevant clinical details from the patient, to obtain complete blood counts approximately two millilitres of blood sample were taken in an EDTA vial. Which includes Hb (haemoglobin), MCV (Mean corpuscular volume), MCH (Mean corpuscular haemoglobin), MCHC (Mean corpuscular haemoglobin concentration) & RDW (Red cell distribution width) and were analysed in automated cell counter (Sysmex XT 1800i; Kobe, Japan). Peripheral blood film was made from the samples and were stained by Leishman stain and examined. Suspected cases of iron deficiency anaemia were sent for serum iron studies that includes total iron binding capacity, serum iron levels, serum ferritin levels and transferrin saturation.

After that high performance liquid chromatography (HPLC) test was performed based upon the principles of cation exchange chromatography using the BIORAD VARIANTTM-II Hb typing system Biorad Variant using beta thalassemia short program (Biorad laboratories, California, USA). Hb A2/F calibrator was analysed at the start of every run. The different Hb variants were identified by using retention time windows (retention time= the time in minutes from sample injection to the

maximum point of the elution peak) which were specified for each of these variants like Hb E, S, D and C. Sickling test was performed in all patients in whom a S variant was detected in the S window. Suspected cases of Hb E were sent for electrophoresis for confirmation and suspected case of Hb D Iran sent for molecular diagnostic modality. There is no follow up was done after the initial investigation.

Result

Samples of 760 suspected patients from October 2022 to August 2023 were sent for HPLC analysis. Patients was between 2 days to 88 years of age, among those 412 was male and 348 females (Male: Female = 1: 0.84). (Shown in Table :1) Association of age with haemoglobinopathies was not identified but maximum cases were between 1 to 10 years of age. 513 (67.3%) cases were normal, and 247 cases (32.5%) cases was abnormal. Distribution of various haemoglobinopathies in the study population shown in Table :2 and Figure no. 1,2,3.

Table 1: Age and Sex distribution

Age	Male	Percentage	Female	Percentage	Total
0-10	227	55.09	138	39.66	365
11-20	41	9.95	44	12.64	85
21-30	77	18.69	102	29.31	179
31-40	35	8.5	38	10.92	73
41-50	16	3.88	14	4.02	30
51-60	9	2.18	5	1.44	14
61-70	5	1.22	4	1.15	9
71-80	2	0.49	2	0.57	4
81-90	0	0	1	0.29	1
Total	412	100	348	100	760

Distribution of different haemoglobinopathies was done on the basis of retention time, peak characteristic and haemoglobin percentage. Distribution of the haemoglobinopathies among studied group is shown in table 2. Most common haemoglobinopathy found was Beta thalassemia heterozygous that is 19.47% (148 cases) followed by Beta thalassemia homozygous in 37 cases (4.88%), Double heterozygous Beta thalassemia with Hb E Trait in 19 cases (2.5%), Sickle cell heterozygous in 10 cases (1.32%), Hb E heterozygous in 8 cases (1.05%), each Hb E homozygous, Hb D Punjab heterozygous and Lapore in 4 cases (0.53%), Double heterozygous Beta thalassemia with Hb D Punjab in 3 cases (0.39%), each Sickle cell homozygous, HPFH, Delta Beta heterozygous in 2 cases (0.26%), each Hb D Punjab homozygous, Double heterozygous Beta thalassemia with Delta Beta, Sickle cell trait with Hb D Punjab and Hb D Iran in 1 case (0.13%) among studied samples.

In this study high level of fetal Hb in F window (0.98-1.20 min) was found in Beta thalassemia homozygous (4.88%), Sickle cell homozygous/ disease (0.26%), Hereditary persistence of fetal haemoglobin (0.26%), Double heterozygous Beta thalassemia with Hb E Trait (2.5%), Double heterozygous Beta thalassemia with Hb D Punjab (0.39%), Double heterozygous Beta thalassemia with Delta Beta (0.13%), Delta Beta heterozygous (0.26%).

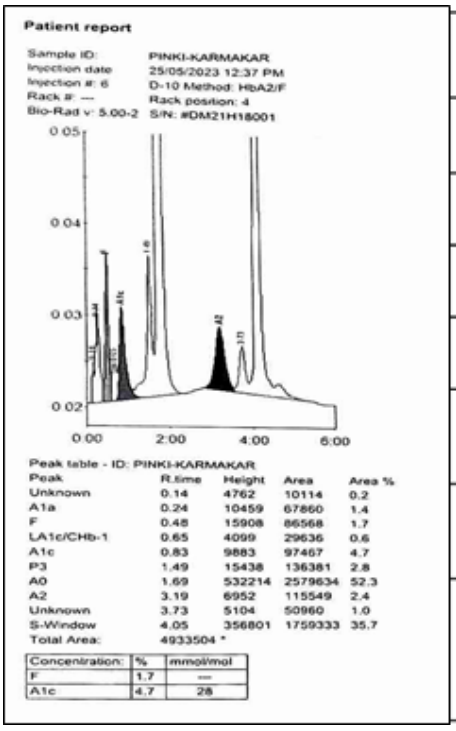
Hb E, Hb A2, Hb D Iran and Hb Lapore elute in the Hb A2/ Hb E window with the same retention time of 3.30 – 3.90 min, which includes Hb E heterozygous/ trait (1.05%), Hb E homozygous (0.53%), Double heterozygous Beta thalassemia with Hb E Trait (2.5%), Hb Lapore (0.53%), Hb D Iran (0.13%). Those were differentiated from each other on the basis of % of abnormal haemoglobin, family studies and clinical picture.

Hb D window with retention time of 3.9 – 4.25 min includes Hb D Punjab heterozygous (0.53%), Hb D Punjab homozygous (0.13%), Double heterozygous Beta thalassemia with Hb D Punjab (0.39%)

Hb S elutes in the Hb S window with the same retention time of 4.30 – 4.70 min, which includes Sickle cell heterozygous/ trait (1.32%), Sickle cell homozygous/disease (0.26%).

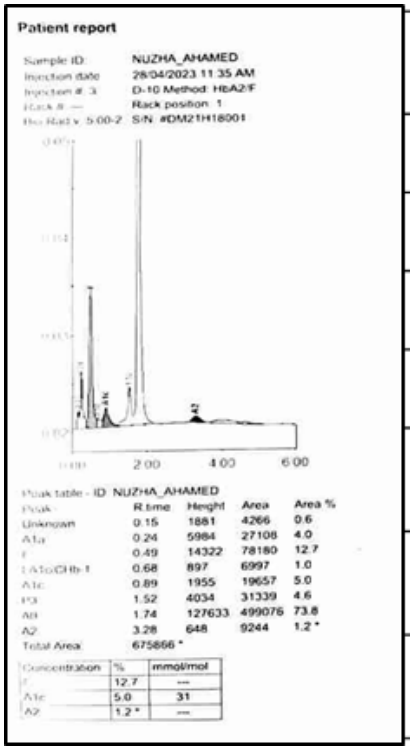
Table 2: Haemoglobin distribution pattern

S. No.	Diagnosis	No. of cases	Percentage
1	Beta thalassemia heterozygous	148	19.47
2	Beta thalassemia homozygous	37	4.88
3	Hb E heterozygous/ trait	8	1.05
4	Hb E homozygous	4	0.53
5	Sickle cell heterozygous/ trait	10	1.32
6	Sickle cell homozygous/disease	2	0.26
7	Hb D Punjab heterozygous	4	0.53
8	Hb D Punjab homozygous	1	0.13
9	Hb Lapore	4	0.53
10	HPFH (Hereditary persistence of fetal haemoglobin)	2	0.26
11	Double heterozygous Beta thalassemia with Hb E Trait	19	2.5
12	Double heterozygous Beta thalassemia with Hb D Punjab	3	0.39
13	Double heterozygous Beta thalassemia with Delta Beta	1	0.13
14	Sickle cell trait with Hb D Punjab	1	0.13
15	Delta Beta heterozygous	2	0.26
16	Hb D Iran	1	0.13
17	Normal	513	67.5
	Total	760	100



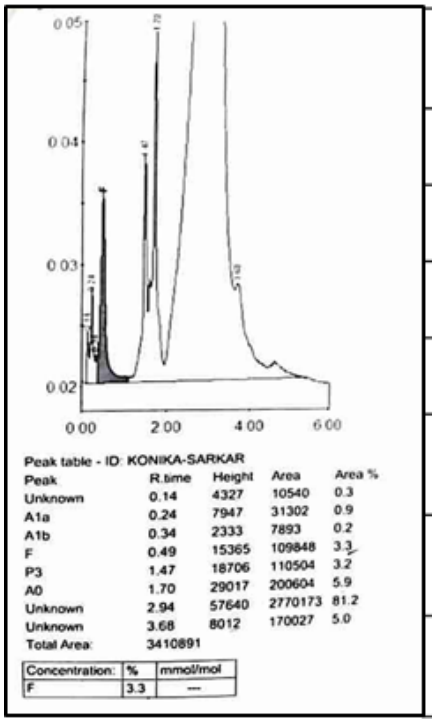
RBC	4.53X10^6/µL
HGB	13 g/dL
HCT	40 %
MCV	89.2 fL
MCH	28.7 pg
MCHC	32.2 g/dL
RDW-CV	14.1 %
RDW-SD	55.4 fL

Figure 1: Chromatogram suggestive of Sickle cell heterozygous along with CBC findings



RBC	3.68X10^6 /µL
HGB	5.4 g/dL
HCT	16.8 %
MCV	100.2 fL
MCH	32.5 pg
MCHC	32.4 g/dL
RDW-CV	20.2 %
RDW-SD	91.6 fL

Figure: 2 Chromatogram suggestive of Deta beta with CBC finding of vitamin b12 deficiency



RBC	4.26X10^6 /µL
HGB	9.3 g/dL
HCT	28.9 %
MCV	67.8 fL
MCH	21.9 pg
MCHC	32.3 g/dL
RDW-CV	15.6 %
RDW-SD	44.6 fL

Figure: 3 Chromatogram suggestive of double heterozygous for Hb D Iran.

Discussion

Hemoglobinopathy constitute major health problem in India. The prevalence of hemoglobinopathy is different in different part of India. [8,9,10] In this study sample was taken from various peripheral centres of India.

HPLC is sensitive and specific technique for identification and quantification of various haemoglobins fractions. But HPLC should be used as screening test only, for confirmation of particular hemoglobinopathy advance molecular test like PCR, amplification refractory mutation

system and other similar test should be used. [11,12]

In this study the prevalence of hemoglobinopathy was 32.5 %, that includes beta thalassemia homozygous, beta thalassemia heterozygous, other thalassemia syndromes (Hb E Thalassemia, Hb D Thalassemia, delta beta Thalassemia, Hereditary persistence of fetal haemoglobin), Hb E disorders, Sickle cell disorders and Hb D Iran. A study done by Bikash Mondal et. al. from west Bengal shows prevalence of hemoglobinopathy 27.35%. [13] Seema Rao et al. reported 30.9 % prevalence of hemoglobinopathy from north India region. [14] A study done by Ankur et al. at Rajasthan shows 31% prevalence of hemoglobinopathy. [15] 21371 cases studied by Gopal Krishan ray et al from Odisha out of which hemoglobinopathies cases was 50.2%. [16]

Beta thalassemia heterozygous was the most common (19.47%) haemoglobin abnormality detected in this study. Rao et al. reported β-thalassaemia trait most common (18.1% cases) Hb abnormality in north India. [14] Mondal et al. also reported β (beta) thalassemia trait was the most common abnormality found in 3870 (4.29%) patients from west Bengal. [17]

Another study from West Bengal done by Bikash Mondal et al. reported β-thalassemia heterozygous as a most common haemoglobinopathy (17.64%). [13] Ankur et al. reported most common Hb abnormality detected in the study from Rajasthan was β thalassemia trait (21%). [15] Singh et al. reported 14.27% cases of beta thalassemia trait from Maharashtra. [18] Jain et al. documented Beta thalassemia trait was predominant abnormality in 7.4% cases from Haryana. [19]. In a study from Gujarat by Patel et al, high frequency of β-thalassemia trait 20.37% was reported. [20] Beta thalassemia homozygous was found 4.88% in this study. In most of studies major abnormality detected was Beta thalassemia heterozygous/trait.

Distribution of different Haemoglobin variant vary in different parts of India. In this study, 1.05 % cases were found Hb E trait and 2.5% cases were Double heterozygous Beta thalassemia with Hb E Trait. Study conducted in north part of India by Rao et al 1.1% prevalence of Hb E trait. [14] Mondal et al found 2.68 % prevalence for Hb E trait. [17] Ankur et al detected 0.7% cases of HbE trait and 0.4% patients of Eβ thalassemia. [15] Singh et al. found 1.41% prevalence of Hb E in west region of India (Pune), among this 0.82% cases were Hb E trait. [18]

Frequency of sickle cell disorders are different in different part of India. In this study Hb S trait were found in 1.32% and Hb S disease 0.26% of cases. Study conducted by Rao et al from north India shows 1.4% prevalence of Hb S trait. [14] Study conducted by Ankur et al found HbS trait and HbS disease in 0.7% and 0.3% of cases. [15] Singh et al. reported 58 cases of sickle cell disorders which were further classified into sickle cell trait 36 (1.33%) and sickle cell disease 22 (0.82%) [18]

Hb D Punjab heterozygous /trait was found to be 0.53% in this study. Rao et al. shows prevalence of Hb D Punjab was 0.9%. [14] Study done by Jain et al. in Haryana found 11 cases of Hb D Punjab among 4275 cases. [19] Rao et al. reported 1.1% cases of Hb D Punjab trait a study done from north India. [14]

Other variant found in this study included, Hb E homozygous 4 (0.53%) cases, Hb Lapore 4 (0.53%) cases, Double heterozygous Beta thalassemia with Hb D Punjab 3 (0.39%) cases, HPFH (Hereditary persistence of fetal haemoglobin) 2 (0.26%) cases, Delta Beta heterozygous 2 (0.26%) cases.

Rare haemoglobin variant also found in the present study, such as Hb D Punjab homozygous 1 (0.26%) case, Double heterozygous Beta thalassemia with Delta Beta 1 (0.26%) case, Sickle cell trait with Hb D Punjab 1 (0.26%) case and Hb D Iran 1 (0.26%) case.

Conclusion

Prevalence of various haemoglobin disorder is different in various states of India. For detection of these haemoglobinopathies high performance liquid chromatography is sensitive and reliable diagnostic method. Beta thalassemia heterozygous/trait most prevalent haemoglobinopathy in the various states of India. Moreover, distribution of other common haemoglobinopathies is important to formulate appropriate therapeutic and preventive strategies. Early detection of haemoglobin disorders is important for better progression and prevention of transmission from parents to children. For detection of haemoglobinopathies high performance liquid chromatography is sensitive and reliable diagnostic method. However other advanced modalities are important for the confirmation of diagnosis.

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