Original Article

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Hemoglobinopathies by HPLC: A 3 Year Study of 106,277 Cases

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ABSTRACT

Introduction: Hemoglobinopathies and thalassemias are the most common single gene disorders in the world. World Health Organization figures estimate that 5% of the world populations are carriers of a potentially pathological hemoglobin (Hb) gene. The general incidence of thalassemia trait and sickle cell anaemia in India varies between 3-17% and 1-44% respectively3 but because of consanguinity, caste and area endogamy, some communities show a very high incidence, making the disease a major public health problem in our country. Cation exchange high-performance liquid chromatography (CE-HPLC) is one of the best methods for screening, detection, and identification of various hemoglobinopathies.

Material & Method: A retrospective study was carried out from period of 2017 to 2019 with 106277 cases evaluated with an aim to identify various hemoglobinopathies seen in Indian population by high-performance liquid chromatography. Cases outside Indian geographical location were excluded from the study

Result: A total of 18,936(17.82%) cases with abnormal haemoglobin variants was reported in the study with 35 difference variants across India. Northeast India reported maximum abnormal hemoglobinopathies (50.16%). Beta Thalassemia Trait was the most common abnormal variant found.Such a high incidence emphasises premarital and prenatal screening for prevention of dangerous effects of hemoglobinopathies in the population.

Conclusion: CE-HPLC should be used for early detection and proper management of these haemoglobinopathies. The most common hemoglobinopathy observed in our study was Beta thalassemia trait followed by Sickle cell trait and then sickle cell disease. It was also observed that Northeast India had maximum abnormal hemoglobinopathies.

Keywords: *Hemoglobinopathies, Cation Exchange High-Performance Liquid Chromatography, Thalassemia,*

Introduction

Hemoglobinopathies and thalassemia's are the most common single gene disorders in the world. World Health Organization figures estimate that 5% of the world populations are carriers of a potentially pathological hemoglobin (Hb) gene. [1] Worldwide migration of human population, relatively higher frequency of consanguineous marriages in many of the high frequency countries, has equally contributed for the increased burden of hemoglobinopathies. They are a heterogeneous group of inherited disorders of hemoglobin (Hb) characterized by reduced or absent production of globin chains. It is the commonest single gene disorder in the world and causing a significant morbidity and mortality in India and abroad. [2]

Thalassemias and other structural haemoglobinopathies are the major genetic disorders prevalent in certain parts of the world including India. The general incidence of thalassemia trait and sickle cell anaemia in India varies between 3-17% and 1-44% respectively [3] but, because of consanguinity, caste and area endogamy some communities show a very high incidence, making the disease a major public health problem in our country.[3,4] In our country, without therapy, most thalassemia major patients cannot survive beyond the second decade of life, and mostly die from cardiomyopathy. Complications such as diabetes mellitus (DM) and hypothyroidism are also common. These patients also require recurrent blood transfusions and are thus, are exposed to the dangers of transfusion. Recurrent visits to the hospitals expose them to dangerous hospital acquired infections. Hence, these inherited disorders of hemoglobin synthesis are an important cause of morbidity and mortality in our country.

Cation exchange high-performance liquid chromatography (CE-HPLC) is one of the best methods for screening, detection, and identification of various hemoglobinopathies with rapid, reproducible, and precise results. It is a highly

sensitive, specific, quick but more expensive method for diagnosis.[5]

Alpha- and beta-thalassemia are the most common single-gene Hb disorders in the world.[5] These disorders, which were mainly confined to certain area, religion, cast, and tribe, are now widely prevalent worldwide.

Identification of specific mutations of rare variants of hemoglobin can be achieved by molecular studies. Our objective was to identify the prevalence of abnormal hemoglobinopathies in the study population and identify any rare variant of hemoglobin by gene sequencing.

The aim of the study was to identify different haemoglobinopathies by Bio-Rad Variant II CE-HPLC system with use of the Variant II-thalassemia short program (Bio-Rad Laboratories) at reference laboratory in Indian population.

Materials and Methods

This retrospective study was carried out at Global Reference Laboratory, Metropolis Healthcare, Mumbai, India, from 2017 to 2019. Cases outside Indian geographical location was excluded from the study. The number of male and female cases in our study was 26,385 and 79,892 respectively. The number of Pediatric cases accounted for 12,472 cases. Patients who came for a premarital check-up voluntarily, patients with abnormal CBC findings and those patients who had a family history of hemoglobinopathy were included in the study. However, patients with history of blood transfusion within the last 3 months and patients with inconsistent history of blood transfusion were excluded, except in cases of haemolytic anaemia with strong family history, smear findings and repeated transfusion history, HPLC finding of High HbF and HbA2 values were suggestive of Beta Thalassemia Syndrome. A detailed clinical history and family history was obtained from each patient. 2ml of fresh whole blood was collected in ethylene diamine tetra acetic acid (EDTA) bulbs, stored at ambient temperature, transported to the lab and analysed within 24 hours of collection. All the EDTA samples were collected at the peripheral centres of Metropolis Healthcare labs and processed at Global Referral Lab. Every specimen was subjected to complete blood count (CBC) by Unicel DXH 800 and then analyzed on the Bio-Rad Variant II CE-HPLC system with use of the Variant II-thalassemia short program (Bio-Rad Laboratories). Concentration of Hb (%) was calculated by determining the area of a peak as a fraction of the total area of all Hb peaks seen on the CE-HPLC chromatogram. The retention time was measured in minutes. Patient consents were taken at the time of blood collection.

The data were analyzed using “R Studio version 1.4.1103”. Descriptive analyses were made to obtain the frequency and Percentage of Haemoglobinopathy in this given Indian population.

Results

The study recorded 18,936 (17.82%) cases of abnormal hemoglobinopathies out of total of 106,277 cases. The most prevalent Hb fraction was Beta thalassemia trait (9.23%) followed by Sickle cell trait (2.52%) and sickle cell disease (1.48%).

Clinically significant variants of Hb (Hb J trait, Hb Q India, Hb H Disease, borderline lowA2 and borderline high A2, Hb D trait, Hb E trait and HbE disease) were found to be3.731%. Rare variants of Hb like Hb Austin, Hb Hope/ Hb Camden, Hb Hashron, Hb Hofu, Hb Melledgeville, Hb K-Woolwich, Hb Lepore, Hb Russ, Hb San Diego/ Hb North Manchester/ Hb Valletta, Hb Santa Clara, Hb Saurashtra, Hb Toulon, Hb TY Gard, Hb Tyne/ Hb Aubagne werefound to be 0.071%. They all were reported based on their retention time and concentration.

There were 3 cases which did not provide any conclusive results of any known haemoglobin variant. So, they were subjected to molecular analysis by Beta- globin gene sequencing. The results are provided in the table below.

It was observed that Northeast India had maximum abnormal hemoglobinopathies (50.16%), followed by Central India (30.02%), North India (25.31%), East India (21.18%), South India (20.44%) and least in West India (16.44%). Beta Thalassemia Trait being the most common abnormal variant across all regions. Complete detail about the different abnormal hemoglobinopathies variants has been given in able-3.

Discussion

Beta thalassemia trait formed the largest subgroup of abnormal haemoglobin (9.23%). The characteristic hematological findings in a typical case of beta thal trait include microcytosis with raised RBC counts. Haemoglobin is normal or slightly reduced. A cut off over 4% of Hb A2 was taken for diagnosis of BTT. A study by Santosh Mondal also shows similar findings of 4.60% of total cases reported as Beta thalassemia trait.[6]

Thalassemia major constituted approximately 0.49% of cases. Children with thalassemia tend to present within two years of life and are dependent on regular blood transfusions. History of recent blood transfusion must be sought along with correct age to aid in an accurate diagnosis. The high incidence of thalassemia trait calls for the need of antenatal screening and screening of marriageable age groups. This

Table 1: Abnormal Hemoglobin variants detected in our study.

Sr. no	Haemoglobinopathy	Frequency	Percentage
1	Beta Thalassemia Trait	9817	9.237
2	Sickle Cell Trait	2680	2.52
3	Sickle Cell Disease	1582	1.489
4	borderline low A2	1383	1.301
5	borderline high A2	657	0.618
6	HbD Trait	657	0.618
7	HbE Trait	563	0.53
8	Beta Thalassemia Syndrome	529	0.498
9	HbE Disease	354	0.333
10	HbJ Trait	219	0.206
11	HbD Disease / HbS-D Disease	181	0.17
12	HbQ India	107	0.101
13	Hereditary persistence of fetal hemoglobin	96	0.09
14	HbH disease	26	0.024
15	Hb Lepore	16	0.015
16	Hb Saurashtra	14	0.013
17	Hb Hashron	9	0.008
18	Hb Camden / Hb hope	7	0.007
19	Hb Santa Clara or Hb Ausitn or HbJ	7	0.007
20	Hb Hofu or Hb Melledgeville	4	0.004
21	Hb santa clara	4	0.004
22	Hb TY GARD	3	0.003
23	Double heterozygous for HbS and HbE	2	0.002
24	HB Hope	2	0.002
25	Hb K – Woolwich	2	0.002
26	HbC Trait	2	0.002
27	Double heterozygous for HbS and Hb Lepore	1	0.001
28	Double hetrozygous for HbD and HbE	1	0.001
29	Hb Austin	1	0.001
30	Hb HOFU	1	0.001
31	Hb Russ	1	0.001
32	Hb San Diego or Hb North Manchester or Hb Valletta	1	0.001
33	Hb Toulon	1	0.001
34	Hb Tyne or Hb Aubagne	1	0.001
35	HbC Disease	1	0.001

Table 2: The results of Beta Globin gene sequencing of 3 unknown haemoglobin variant cases

Sr. No	HPLC window	Variant Hb concentration (%)	Retention time (min)	DNA interpretation	Mutation change
1	Unknown	41	2.18	Hb Regina	Heterozygous for C.289 C>G
2	Unknown	40.6	2.17	Hb Regina	Heterozygous for C.289 C>G
3	D S Unknown	11.1 15.5 16.8	4.18 4.36 4.76	Non conclusive	CAT-CAC Histidine HbS (A>T) Heterozygous

Table 3: Segregation of data based on geographical locations in India.

Abnormal Impression	Central India	East India	Northeast India	North India	South India	West India
Beta Thalassemia Syndrome	40(0.82%)	15(0.767%)	6(1.881%)	29(0.796%)	49(0.994%)	300(0.332%)
Beta Thalassemia Trait	520(10.66%)	172(8.789%)	25(7.837%)	464(12.737%)	452(9.166%)	8184(9.047%)
borderline high	46(0.94%)	12(0.614%)	2(0.627%)	30(0.823%)	22(0.446%)	545(0.602%)
borderline low	90(1.85%)	14(0.716%)	3(0.94%)	60(1.647%)	72(1.46%)	1144(1.265%)
double heterozygous for HbS and Hb Lepore	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.001%)
double heterozygous for HbS and HbE	0(0%)	1(0.051%)	0(0%)	0(0%)	0(0%)	0(0%)
Double hetrozygous for HbD and HbE	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.001%)
Hb Austin	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.001%)
Hb Camden / Hb hope	3(0.06%)	0(0%)	0(0%)	1(0.027%)	1(0.02%)	2(0.002%)
Hb Hashron	1(0.02%)	0(0%)	0(0%)	0(0%)	1(0.02%)	7(0.008%)
Hb HOFU	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.001%)
Hb Hofu or Hb melledgeville	0(0%)	1(0.051%)	0(0%)	0(0%)	1(0.02%)	2(0.002%)
HB Hope	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(0.002%)
Hb K - Woolwich	0(0%)	0(0%)	0(0%)	2(0.055%)	0(0%)	0(0%)
Hb Lepore	0(0%)	1(0.051%)	0(0%)	5(0.137%)	2(0.041%)	8(0.009%)
Hb Russ	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.001%)
Hb San Diego/ Hb North Manchester/ Hb Valletta	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.001%)
Hb santa clara	1(0.02%)	0(0%)	0(0%)	1(0.027%)	1(0.02%)	1(0.001%)
Hb Santa Clara/ Hb Ausitn/ HbJ	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	7(0.008%)
Hb Saurashtra	0(0%)	0(0%)	0(0%)	1(0.027%)	0(0%)	13(0.014%)
Hb Toulon	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.001%)
Hb TY GARD	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	3(0.003%)
Hb Tyne or Hb Aubagne	0(0%)	0(0%)	0(0%)	0(0%)	1(0.02%)	0(0%)
HbC Disease	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.001%)

Abnormal Impression	Central India	East India	Northeast India	North India	South India	West India
HbC Trait	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(0.002%)
HbD Disease / HbS-D Disease	23(0.47%)	5(0.256%)	0(0%)	23(0.631%)	21(0.426%)	109(0.12%)
HbD Trait	34(0.70%)	13(0.665%)	2(0.627%)	46(1.263%)	32(0.649%)	530(0.586%)
HbE Disease	18(0.37%)	64(3.274%)	56(17.555%)	49(1.345%)	40(0.811%)	127(0.14%)
HbE Trait	14(0.29%)	62(3.171%)	57(17.868%)	42(1.153%)	38(0.771%)	350(0.387%)
HbH disease	3(0.06%)	1(0.051%)	0(0%)	4(0.11%)	4(0.081%)	14(0.015%)
HbJ Trait	13(0.27%)	0(0%)	5(1.567%)	12(0.329%)	22(0.446%)	167(0.185%)
HbQ India	2(0.04%)	0(0%)	1(0.313%)	4(0.11%)	6(0.122%)	94(0.104%)
HbS trait	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(0.002%)
HPFH	9(.18%)	5(0.256%)	0(0%)	4(0.11%)	4(0.081%)	74(0.082%)
Sickle Cell Disease	359(7.36%)	28(1.432%)	1(0.313%)	80(2.196%)	122(2.474%)	992(1.097%)
Sickle Cell Trait	288(5.91%)	20(1.023%)	2(0.627%)	65(1.784%)	117(2.373%)	2186(2.417%)
No evidence	3413 (69.98%)	1541 (78.824%)	159 (49.843%)	2721 (74.691%)	3923 (79.558%)	75588 (83.559%)

can prevent the presence of Beta thalassemia major in their offspring's. Study by Santosh Mondal also shows similar findings of 1.66 % of total cases reported as Thalassemia major.[6]

We recorded 657 cases of borderline High Hb A2 and 1383 cases of borderline low Hb A2 in our study. These conditions are of significance and need careful interpretation as iron deficiency can lead to low Hb A2 and vitamin B12 and folic acid deficiency can lead to high Hb A2 levels and thus mask a thalassemia trait. DNA analysis is advised in such cases for a conclusive opinion. The importance of nutritional deficiencies on the levels of Hb A2 is associated with borderline high levels of 3.6% to 3.9% and borderline low levels of 1.5% to 1.8%. In such cases, we advised revaluation after treatment with haematinics.[7]

In our study, we reported, 2.52% as sickle cell trait and 1.48% as sickle cell disease. They are a group of autosomal recessive disorders caused by point mutation at the sixth position of in β chain, valine substituting glutamic acid. The resultant HbS has poor solubility in the deoxygenated state and can polymerise with the red cells. The red cells show a peculiar shape change because of polymer formation and become distorted and rigid, the so called 'sickle cell'. Sandeep Saha et al reported 4.41% of sickle cell trait cases in their study which showed findings like our study.[8]A study by Atul Shrivastava et al reported sickle cell disease cases as 1.17% which was similar to our study.[9]

Hb E disease and Hb E trait were the most common structural variants with raised peak in Hb A2 window. We reported a total of 563cases of Hb E trait and 354cases of Hb E disease accounting to 0.53% and 0.33% with mild elevation in HbF. HbE variant results from a beta chain mutation (β26 Glu→Lys) and tends to elute in A_2 window within the retention time ranging from 3.3 to 3.9 min.[7]G S Chopra et al found that the patients having Hb E trait and Hb E disease were mainly from Eastern – Coastal region and North-East India (West Bengal, Assam, Manipur and Nagaland) were this abnormal Hb E and variants are highly prevalent. Even we had similar findings in our study. [10]

Hb J presents as an elevated peak in P3 window on HPLC. P3 values upto 10% can denote degeneration of the sample and values above 15% with the retention time of 1.71 to 1.74 minutes were considered as Hb J which is most probably an Alpha thalassemia variant. Hb J is usually asymptomatic, or patients may present with mild anaemia. We recorded 219 cases in total which is 0.20% of total cases. A study by Warghade et al showed similar findings of 0.07%.[7]

We recorded 107cases of Hb Q India, making it 0.1% of all cases, which is again a rare Alpha thalassemia variant which presented as an unknown window (range 10 to 18%) at retention time of 4.74 plus/minus 0.10 minutes. It is caused by mutation AAG→GAG (Asp→His) in the position of codon 64 of the alpha-1 gene. A study by Warghade et al showed similar findings of 0.08%.[7]

We recorded 7 cases of Hb Hope/ Hb Camden. This hemoglobin variant has been shown to have normal stability and functional properties, and none of the affected individuals has shown evidence of hematologic disease.[11]

The total cases of HPFH (96 cases), Hb Lepore (16 cases) and Hb H (26 cases) were similar to the cases recorded by Mondal et al. [6] Hb Lepore, a delta beta variant elutes in A2 window with concentration of 10 to 15%. It shows a characteristic hump on the downward slope in comparison to HbE.The other rare variants which we recorded in our study were Hb Austin, Hb Hope/ Hb Camden, Hb Hashron, Hb Hofu, Hb Melledgeville, Hb K-Woolwich, Hb Lepore, Hb Russ, Hb San Diego/ Hb North Manchester/ Hb Valletta, Hb Santa Clara, Hb Saurashtra, Hb Toulon, Hb TY Gard, Hb Tyne/ Hb Aubagne and accounted for 0.071% of our total study population.

Conclusion

This is one of the few reports of a very large study of 1,06,277 cases on use of CE-HPLC for screening and identification of hemoglobin variants done at a referral laboratory. In India, anaemia is most caused by nutritional deficiency, but the differential diagnosis of abnormal haemoglobin variants/ haemoglobinopathies should also be kept in mind. We recorded a total of 18,936 cases with abnormal hemoglobin variants which is a significant number of cases. The most common hemoglobinopathy observed in our study was Beta thalassemia trait followed by Sickle cell trait and then sickle cell disease. It was also observed that North East India had maximum abnormal hemoglobinopathies followed by Central India, North India. Also 3 unknown hemoglobin variant cases were also observed which were subjected to Beta globin sequencing. Such a high incidence emphasises premarital and prenatal screening for prevention of dangerous effects of hemoglobinopathies in the population. CE-HPLC should be used for early detection and proper management of these haemoglobinopathies.

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