



Variant Hemoglobin Spectrum By Cation Exchange High Performance Liquid Chromatography: A Study Of 2035 Subjects

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ABSTRACT

Introduction: Hemoglobinopathies and thalassemia are hereditary disorders of hemoglobin (Hb) affecting mankind at prevalent regional level. Automated cation exchange high performance liquid chromatography is being increasingly used as the initial diagnostic method for identifying normal and abnormal hemoglobin variants.

Methodology: Total 2035 sample received and studied. All samples run on cation exchange high performance liquid chromatography machine by BIO-RAD.

Results: Total 386(18.96%) cases had abnormal hemoglobin fractions. Beta thalassemia major cases were 17(0.83%). Beta thalassemia intermedia cases were 4(0.19%). Sickle cell trait (heterozygous Hb S) cases were 96(4.71%). Double heterozygous for sickle cell-beta thalassemia cases were 14(0.68%). There were 06 cases (0.29%) of Hb D heterozygous. There were one each case of Hb E heterozygous and Hb E-beta thalassemia (0.04% each). Hb F was raised in 7 adult cases (0.34%).

Conclusion: To conclude, cation exchange high performance liquid chromatography is less time consuming, cost effective, labor saving, reproducible, accurate, sensitive and specific method to detect hemoglobinopathies and thalassemia. Most of the abnormal cases are diagnosed with this method, with few inconclusive cases require further genetic and molecular workup.

Keywords: Haemoglobinopathies, Thalassemia, Cation Exchange High Performance Liquid Chromatography, Screening

Introduction

Hemoglobin (Hb) is a conjugated protein of molecular weight 64,000. Haem group is attached to two pairs of polypeptide globin chains. Haem group binding with each of these chains is important for oxygen carrying capacity of hemoglobin and also it stabilizes the hemoglobin molecule. There are also many naturally occurring variants of hemoglobin (more than 1000) which are genetically determined, most are harmless and some of them have serious clinical consequences^[1]. Hereditary disorders of hemoglobin result either from qualitative defect (structural alteration of a globin polypeptide chain) or quantitative defect (reduced synthesis of globin polypeptide chain). Examples of qualitative defects are Hb S, Hb D and Hb E etc. Examples of quantitative defects are alpha and beta thalassemia. Major causes of morbidity of all these are homozygous beta thalassemia and some alpha thalassemia^[2]. 5% of world population is a carrier for hemoglobin disorders as per world health organization report^[3]. In India 10,000 children every year are born suffering from thalassemia major, amounting to approximately 10% of total world numbers^[4]. In India individuals with beta thalassemia are 3.5-15% in total population^[5]. Almost 10 billion rupees per year is spent for thalassemia patients

in India. In India still the conventional methods are used in most of the places for diagnosing these defects. These conventional methods are family history, clinical features, hemoglobin level, red blood cell indices, red cell count, peripheral blood smear study, Hb A₂ quantification, Hb F quantification, cellulose acetate electrophoresis at alkaline pH for hemoglobin and sickling test. Various drawbacks and shortcomings of these methods are that Hb S, G, D, Q and Lepore have same mobility on electrophoresis and Hb A₂, C and E also have same mobility^[6]. Double heterozygous states also are difficult to diagnose^[6]. Clinical and laboratory features have low specificity.

Parents with various heterozygous states can lead to offspring with double heterozygous or homozygous defects. Automated cation exchange high performance liquid chromatography is being increasingly used as the initial diagnostic method for identifying various normal and abnormal hemoglobin^[7,8]. It is simple, easy, reliable, accurate yet cost effective method for early detection of normal and abnormal hemoglobin^[9,10].

Material & Methods

Total 2035 sample received and studied from January 2015 to August 2016 that were sent for hemoglobin variant analysis



in a tertiary care center in north Gujarat. Most of the cases were from North Gujarat and Kutch area of Gujarat with some were from Rajasthan, Madhya Pradesh, Uttar Pradesh and Bihar. Naked eye single tube red cell osmotic fragility test was done and observed for hemolysis. All the samples were run by automated hematology analyzer (cellenium triviron, abacus3 and abacus5) and hemoglobin values and red cell indices were noted. The whole blood samples were taken in K3 EDTA (Ethylene Diamine Tetraacetic Acid) anticoagulant containing vacutte. Anticoagulated whole blood samples were analyzed with BIO-RAD VARIANT II (beta thalassemia short program) machine by BIO-RAD laboratories, United States of America. It runs on cation exchange high performance liquid chromatography principle. On each run, one calibrator and two controls with one blank were added initially. Acceptable area was between 1-3 million. Ranges outside this area had been rejected. All the data regarding clinical history, history of blood transfusion were recorded. Chromatogram results of samples printed. Specific defined windows are there from manufacturer from specific retention time and integrated peaks are accordingly assigned [1]. The retention time is the time taken from the sample injection up to the apex of elution peak [1]. Established ranges of elution of common hemoglobin variants are marked as “windows” (Table 1). Chromatogram result shows retention time, area, area percentage and concentration. Retention time not assigned comes as an unknown. Each sample takes somewhat around 6 minutes for result.

Results

Total 2035 samples received. Out of which 1186(58.28%) were male and 849(41.72%) were female. The overall sample patients age range was from 28 days to 74 years. The few upper age range was mostly grandparents of patients for parental screening. Total 386(18.96%) cases had abnormal hemoglobin fractions. Most common

defect was increased Hb A2. 3.9% was taken as a cutoff for beta thalassemia trait diagnosis (BTT). Total 216 cases were diagnosed with beta thalassemia trait (raised HbA2). Peripheral blood smear findings were microcytic hypochromic red blood cells, anisocytosis, and target cells. Red blood cell count is increase in most cases. The Hb A2 retention time was between 3.50 to 3.75 min. Table 1 shows retention times for all predefined windows. Mentzer index was less than 13 in 179 out of 216 cases. Mentzer index is a ratio of mean corpuscular volume in fL divided by the red blood cell count in Millions per micro Liter.

Beta thalassemia major cases were 17(0.83%). Beta thalassemia intermedia cases were 4(0.19%). All the thalassemia major cases were first presented in their first two years. Peripheral blood smear findings of these cases were severe anemia, severely microcytic and hypochromic red blood cells, moderate to severe anisopoikilocytosis, target cells and nucleated red blood cells.

Sickle cell anemia (homozygous Hb S) patients had Hb S range from 72-89% and total 19 (0.93%) cases were there. Sickle cell trait (heterozygous Hb S) cases were 96(4.71%). Hb S in them was from 32 to 38%. Double heterozygous for sickle cell-beta thalassemia cases were 14(0.68%).

There were 06 cases (0.29%) of Hb D heterozygous. D window is displayed in HPLC. Retention time was between 4.03 to 4.21 min. There were one each case of Hb E heterozygous and Hb E-beta thalassemia (0.04% each). There is a peak of Hb E in A2 region. Retention time was 3.58 min. Hb F was raised in 7 adult cases (0.34%). Provisional diagnosis of hereditary persistence of fetal Hb was made in each case with an advice for molecular confirmation. Various abnormal hemoglobin defects in our study are shown in table 2 and figure 1. Various hematological parameters are shown in table 3.

Table 1: Window time and retention time of predefined parameters of BIO-RAD VARIANT II.

Peak name	Window(min)	Retention time(min)
F window	1.00-1.30	1.15
P2 window*	1.30-1.60	1.45
P3 window*	1.60-1.90	1.75
A ₀ window	1.90-3.30	2.60
A2 window	3.30-3.90	3.60
D window	3.90-4.30	4.10
S window	4.30-4.70	4.50
C window	4.90-5.30	5.10

*P2 and P3 are associated with Hb A

Table 2: Sex wise distribution of hemoglobinopathies.

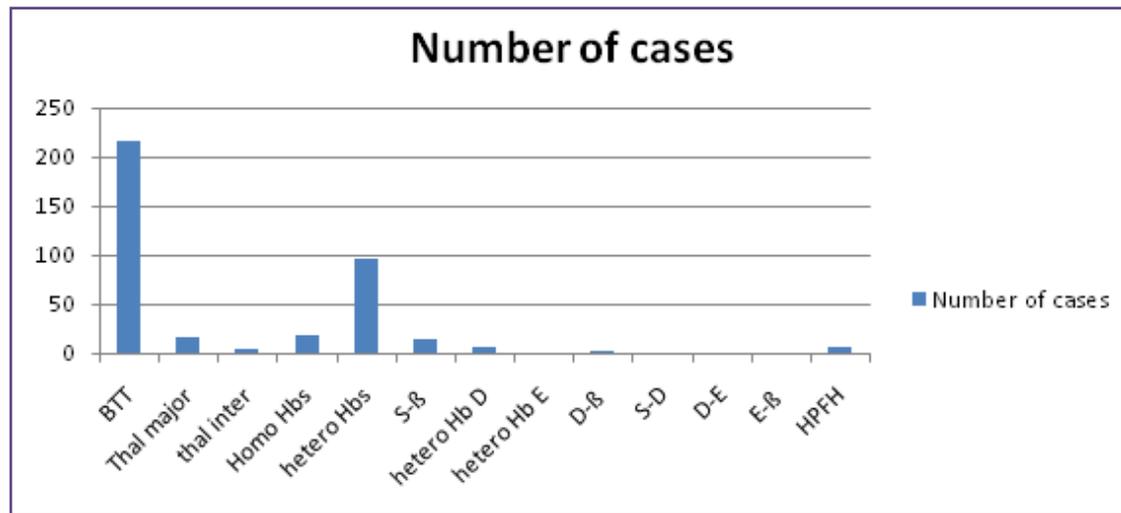
Hemoglobinopathies	Male	Female	Total (percentage)
Beta thalassemia trait	119	97	216(10.61%)
Beta thalassemia major	11	06	17(0.83%)
Thalassemia intermedia	01	03	04(0.19%)
Hb S homozygous	07	12	19(0.93%)
Hb S heterozygous	45	51	96(4.71%)
Hb S- beta thalassemia	08	06	14(0.68%)
Hb D heterozygous	04	02	06(0.29%)
Hb E heterozygous	01	00	01(0.04%)
Hb D-beta thalassemia	02	00	02(0.09%)
HbS-Hb D	01	00	01(0.04%)
Hb D-Hb E	01	00	01(0.04%)
Hb E-beta thallemia	00	01	01(0.04%)
HPFH	03	04	07(0.34%)
TOTAL	203	183	386(18.96%)

Table 3: Hematological parameters of normal and different hemoglobinopathies.

Parameter	Normal	BTT	Thal major	Hb S Homo.	Hb S Hetero.	Sickle- β thal	Hb D hetero.	HPFH
Hb(g/dl)	10.6 \pm 2.9	10.2 \pm 2.4	4.86 \pm 2.23	6.38 \pm 2.1	10.1 \pm 2.9	7.68 \pm 2.3	10.9 \pm 2.9	12.3 \pm 2.8
RBC	4.55 \pm 0.9	5.62 \pm 0.82	2.36 \pm 1.32	3.26 \pm 0.8	4.2 \pm 0.36	3.86 \pm 1.0	4.7 \pm 1.25	4.6 \pm 0.9
PCV	31.4 \pm 4.7	30.4 \pm 3.62	15.2 \pm 2.91	21.2 \pm 5.23	30.6 \pm 7.1	24.1 \pm 7.3	35.9 \pm 10.8	39.1 \pm 4.5
MCV	76.7 \pm 12.71	59.91 \pm 7.1	61.9 \pm 7.2	76.1 \pm 8.6	70.6 \pm 5.1	70.4 \pm 6.34	75.5 \pm 20.4	81.1 \pm 7.5
MCH	26.4 \pm 3.85	20.3 \pm 2.58	21.62 \pm 4.2	24.7 \pm 3.2	21.6 \pm 4.0	23.4 \pm 2.1	24.7 \pm 4.1	25.6 \pm 3.4
MCHC	31.21 \pm 2.1	30.4 \pm 1.98	31.1 \pm 4.62	31.8 \pm 2.3	30.9 \pm 2.3	32.4 \pm 1.4	32.4 \pm 2.4	31.5 \pm 2.3
RDW-CV	18 \pm 4.7	18.4 \pm 3.8	20.32 \pm 5.2	22.5 \pm 4.1	21.6 \pm 4.3	21.68 \pm 3.9	18.4 \pm 3.64	17.6 \pm 2.8
Hb A	89.12 \pm 4.65	86.3 \pm 4.3	20.4 \pm 19.8	5.4 \pm 2.3	58.1 \pm 4.3	6.3 \pm 2.21	51.68 \pm 5.27	85.2 \pm 3.5
Hb A2	2.63 \pm 0.61	5.15 \pm 0.72	3.4 \pm 1.2	3.54 \pm 0.86	3.4 \pm 0.9	5.58 \pm 1.21	2.4 \pm 0.38	2.7 \pm 0.51
Hb F	0.65 \pm 0.36	1.4 \pm 0.5	81.2 \pm 14.3	15.9 \pm 6.89	1.9 \pm 3.21	2.31 \pm 7.4	0.7 \pm 0.2	9.4 \pm 3.4
Hb S	-	-	-	74.5 \pm 8.4	32.6 \pm 5.6	69.4 \pm 8.93	-	-
Hb D	-	-	-	-	-	-	36.7 \pm 6.8	-

*Numbers are mean \pm standard deviation. Hb=hemoglobin, RBC=red cell count, PCV= packed cell volume, MCV= mean corpuscular volume, MCH= mean corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration, RDW= red cell distribution width, BTT= beta thalassemia trait, Thal major= beta thalassemia major, Homo=homozygous, Hetero=heterozygous, HPFH=hereditary persistence of fetal hemoglobin

Fig. 1: Column chart shows frequency of hemoglobinopathies.



*BTT= beta thalassemia trait, Thal major= beta thalassemia major, thal inter=thalassemia intermedia, Homo=homozygous, Hetero=heterozygous, s-β = sickle cell beta thalassemia, D-β =Hb D beta thallememia, S-D= sickle cell HbD, D-E= Hb D Hb E, E-β = Hb E beta thallememia, HPFH=hereditary persistence of fetal hemoglobin

Discussion

In India and Mediterranean belt, still thalassemias and hemoglobinopathies are very common causes of morbidity and also exert burden on expenditure. To reduce the burden accurate and reliable screening procedure should be there. The diagnosis of hemoglobinopathies and thalassemia is required to explain hematological abnormalities, identify abnormality in pre symptomatic period, preconceptional screening, screening of fetus to offer termination of pregnancy, and to confirm a presumptive diagnosis [12].

Naked eye single tube red cell osmotic fragility (NESTROFT) and red cell indices are helpful tests that aid in diagnosis but the result must have been supported by confirmatory test as suggested by Chakrabarti et al [13], Gorakshakar et al [14], Dangi et al [15] and other studies [16]. Yousafzai et al stated that NESTROFT is easy and simple but it is very much error prone, red cell indices also are lacking in sensitivity and specificity [16]. We have found in our study that sensitivity and specificity of mentzer index are 60.4 and 91%. Both of these are lacking in sensitivity and specificity so not used for diagnostic purpose exclusively.

So far, some of the common investigations done for thalassemia and hemoglobinopathies are Hb electrophoresis by cellulose acetate at alkaline pH, acid electrophoresis, Hb F quantification by alkali denaturation and Hb A2 quantification by chromatography. These all methods have certain limitations; these methods are totally dependent on the performer's expertise. So variability of the result is there. Even with the same performer, the bands are

not exactly positioned on the same area (reproducibility is low). To differentiate between hemoglobins that have same electrophoretic mobility is difficult and the control with known variant hemoglobin or multiple stored known controls is required to be run every time with patient's sample. It is very difficult to get a single control for comparison with all the variant hemoglobin in it [17]. It is also nearly impossible to run many samples in a single run. These methods are also time consuming. Compound heterozygous or double heterozygous states and unusual hemoglobin variants are all clinically important to diagnose, so exact identification of them is very important [18,19]. With single electrophoretic method none out of these variants can be identified precisely [20]. Cation exchange high performance liquid chromatography has high sensitivity, specificity and also is reproducible compared to hemoglobin electrophoresis [21].

Total 386 cases (18.96%) were diagnosed with some hemoglobin variants. Out of which beta thalassemia trait cases were 216(10.61%). This proves antenatal screening of value to prevent potential offspring with thalassemia major. Borderline Hb A2 requires further investigation before reaching out on conclusion. Studies have suggested both iron deficiency anemia and megaloblastic anemia may have an effect on level of Hb A2; iron deficiency anemia can mildly reduce and megaloblastic anemia can mildly increase [22,23,24,25].

Different studies by Sachdev et al [26], Rao et al [27], Dolai et al [28] and Mukhopadhyay et al [29] have taken 2600, 800, 35413 and 10407 cases respectively for their study.

Sachdev et al ^[26], Jain et al ^[30] and Mukhopadhyay et al ^[29] found 12.57%, 29.3 % and 14.5% abnormal hemoglobin fractions respectively. Sachdev et al ^[26], Rao et al ^[27], Dolai et al ^[28] and Mukhopadhyay et al ^[29] found beta thalassemia trait cases to be 8.9 %,18.1 %,10.38 % and 5.6 % respectively. Our findings are comparable to these different Indian studies.

Conclusion

To conclude, cation exchange high performance liquid chromatography is less time consuming, cost effective, labor saving, reproducible, accurate, sensitive and specific method to detect hemoglobinopathies and thalassemia. It should be used as a screening tool. Most of the abnormal cases are diagnosed with this method; with few inconclusive cases require genetic and molecular studies.

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