Original Research Article

Pattern distribution of abnormal hemoglobin variants by cation exchange High Performance Liquid Chromatography: a study of 9,116 subjects

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ABSTRACT

Background: The present study was conducted to identify pattern distribution of abnormal haemoglobin variants by using HPLC method in a tertiary care hospital, Surat, Gujrat, India.

Methods: A cross sectional study of one-year duration was conducted including 9,116 patients screened for the presence of abnormal haemoglobin variants. Blood samples were initially tested for solubility test and run on automated haemoglobin analyzer for complete haemogram. All the suspected and family study cases were processed for HPLC (Bio-Rad Variant II) for conclusive diagnosis. Patients with a history of recent blood transfusion of less than 3 months duration were excluded from the study.

Results: A total of 9,116 cases (1390 males, 7726 females) were included in the present study. The age group of patients ranged from 1 month to 95 years. Solubility test and complete haemogram were performed in all the cases. Out of the 9,116 cases, 8409(92.24%)cases had normal HPLC pattern. 492(5.40%)cases were diagnosed as sickle cell trait, 176(1.93%) cases as sickle cell disease, 29(0.32%) cases as β thalassaemia trait, 1(0.01%) case as β thalassaemia major, 2(0.02%)cases as Hb E heterozygous and 03 (0.07%) cases as Hb D Punjab heterozygous. One case of double heterozygous for Hb E-β thalassaemia was also found.

Conclusions: HPLC is a rapid, accurate and useful method for diagnosing haemoglobinopathies. It serves as an reliable tool in diagnosing the presence of abnormal haemoglobin variants in suspected cases on routine haematology in developing countries like India, where the resources for detection of haemoglobinopathies are limited. Early diagnosis may help in proper management of patients.

Keywords: Blood transfusion, Haemoglobinopathies, Haemogram, High performance liquid chromatography

INTRODUCTION

Haemoglobinopathies are the group of genetic disorders of haemoglobin in which there is quantitative or qualitative abnormal production or in the structure of haemoglobin molecule.[1] These hereditary disorders are major public health problem in many parts of the world including India.[1] Beta(β)-thalassaemia and sickle cell disease represents the most frequent haemoglobinopathies.[1,2,3,4]

The clinical spectrum of the disorders varies from asymptomatic conditions to serious disorders like Thalassaemia major and sickle cell disease that requires regular blood transfusions and extensive medical care.[1] The general incidence of thalassaemia trait and sickle cell haemoglobinopathy in India varies between 3-17% and 1-44% respectively.[5] But because of consanguinity, caste and area endogamy, some communities show a very high incidence, making the disease a major public health problem in this country.[2,3] These haemoglobinopathies

are common causes of morbidity and also exert burden on expenditure. To reduce the burden, accurate and reliable screening procedures should be there. Cation exchange high performance liquid chromatography has become the preferred technique suitable in Indian scenario, as it can detect most of the clinically significant variants. The simplicity of the automated system with internal sample preparation, superior resolution ,rapid assay time and accurate quantification of haemoglobin fractions makes this an ideal methodology for routine clinical laboratory.[5,6] Several studies in the literature have reported that Gujarat has higher frequency of β Thalassaemia and Sickle cell disease.[7-9] This study was done with the aim to identify pattern distribution of abnormal Hb variants in a tertiary care hospital, Surat, Gujrat India. This type of cross-sectional studies was essential to identify haematological abnormality in pre symptomatic period, preconceptional screening, screening of fetus to offer termination of pregnancy and to confirm a presumptive diagnosis.

METHODS

The Study design was a cross sectional study. The Study period was conducted at tertiary care hospital, Surat, Gujrat, India over a period of 1 year (01/01/2018 to 31/12/018). Study population is a total of 9,116 blood samples received from indoor and outdoor patients attending tertiary care hospital, Surat, Gujrat India.

Inclusion criteria

All clinically suspected cases of haemoglobinopathies and cases with positive family history of haemoglobinopathies from indoor and outdoor department were included in the study.

Exclusion criteria

Patients with history of blood transfusion within 3 months were excluded from the study.

Procedure

5ml of whole blood sample was collected in EDTA which can be stored at 2-8° C for maximum 7 days if processing is delayed. No preparation was required. Pre dilution required if sample was in a tube other than the recommended tube or there was less than 500 μl of sample in the tube. HbA2/F calibrator and normal and abnormal controls were analyzed at the beginning of each run on HPLC. Hemoglobin and RBC indices were measured on automated cell counter. Solubility test was performed on all the received samples. All the clinically suspected cases of haemoglobinopathies, solubility positive and grey zone samples were proceeded to be analysed by BIO-RAD VARIANT II machine for confirmative diagnosis. The underlying principle of HPLC is based on haemoglobin separation by an analytic cartridge in cation exchange HPLC using a pre-

programmed buffer gradient with increasing ionic strength to the cartridge. The separated fractions pass through dual wavelength detector, where absorbance of the sample component is measured at 415nm. Background noise is reduced with the use of a secondary wavelength at 690 nm. The absorbance data are transmitted from the detector to the PC and displayed by CDM as a real time chromatogram.

Statistical analysis

Qualitative data are presented as frequencies and percentages, and quantitative data were shown as mean. All the data were analysed using Microsoft excel 2013.

RESULTS

Out of 9116 cases, 8409 cases had normal and 707 cases had abnormal haemoglobin pattern. Presumptive identification of haemoglobin variants was made primarily by analyte identification windows as shown in Table 1; however geographical factors, ethnicity and clinical presentation were also taken into consideration.

Table 1: Analyte identification windows; window time and retention time of predefined parameters of BIO-RAD VARIANT II.

Peak name	Window(min)	Retention time(min)
F window	0.98-1.22	1.10
P2 window	1.28-1.50	1.39
P3 window	1.50-1.90	1.70
A0 window	1.90-3.10	2.50
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

In present study, overall patients age ranges from 1 months to 94 years. As shown in Table 2, Out of 9116 cases, 1390 cases (15.25%) were of male and 7726 cases (84.75%) were of female.

Table 2: Sex wise distribution of cases.

Gender	No. of cases	Percentage(%)
Male	1390	15.25
Female	7726	84.75
Total	9116	100

As described in Table 3, 8409 cases were diagnosed as Normal haemoglobin variant, 492 cases as Sickle cell trait, 176 cases as sickle cell disease, 29 cases as β Thalassaemia trait, 01 case as β Thalassaemia major, 06 cases as Haemoglobin D Punjab heterozygous, 02 cases as Haemoglobin E heterozygous. One case of double heterozygous for Hb E/β Thalassaemia was also found. Authors considered peak in S window (RT 4.30-4.70 mins) having Hb S value of 20-40% for diagnosis as

sickle cell trait and Hb S value >50% with mildly raised Hb F level for diagnosis of sickle cell disease. Sickle cell trait and sickle cell disease show high level of Hb A2 may be because of Hb S adducts. The most common disorder detected was sickle cell trait (Hb S heterozygous) with 492 cases. Most of the patients had low haemoglobin, reduced MCV, MCHC and raised RBC count. The mean haemoglobin S reported in chromatogram was 28.57%. Authors detected 176 cases of sickle cell disease with mean haemoglobin S reported in chromatogram was 78.3%. Parental study was advised to confirm the double heterozygous status. Solubility test was found to be positive in all these cases.

A total of 29 β Thalassaemia minor cases were diagnosed in this study. The major abnormality was the presence of elevated levels of Hb A2 with RT(3.30-3.90 mins). Authors considered Hb A2 value >3.5% as a cut off for diagnosis of β Thalassaemia minor. In this study, mean percentage of HbA2 was 4.8%.Most of the patients had low haemoglobin, reduced MCV, MCHC and raised RBC count. One case of β Thalassaemia major was diagnosed. Hb F was raised (91.4%) with variable HbA2 (3.8%). The present case was diagnosed within 1st year of life. Parental study was conducted to find out carrier status and to confirm diagnosis. 06 cases showed peak in the D window (RT 3.90-4.30 mins), indicating presence of structural variant haemoglobin D(Hb D) Punjab. Hb D value between 33-43% considered as a diagnosis of Hb D Punjab heterozygous. These cases showed reduced haemoglobin, MCV and MCH levels.

The Hb E elute into the HbA2 window but differentiated upon the retention time and Hb Fractions %. Authors considered HbA2 value <40% as diagnosis for heterozygous Hb E. In present study, authors found one case of Hb E heterozygous. Hb level was mildly reduced with raised RBC counts and reduced MCV, MCH values. One case of double heterozygous for Hb E/β Thalassaemia was also diagnosed showing peak in HbA2 window (64%)and raised Hb F level (19%). Again, parental study was carried out for confirmation.

Table 3: Pattern distribution of abnormal haemoglobin variants among study subjects.

Haemoglobin pattern	Male	Female	Total(n)	Percentage
Normal	1129	7280	8409	92.24
Sickle cell trait	143	349	492	5.40
Sickle cell disease	98	78	176	1.93
β Thalassaemia trait	17	12	29	0.32
β Thalassaemia major	00	01	1	0.01
Haemoglobin E heterozygous	00	02	2	0.02
Haemoglobin D Punjab heterozygous	03	03	6	0.07
E-β Thalassaemia	00	01	1	0.01
Total	1390	7726	9116	100

DISCUSSION

India is an ethnically diverse country with marked regional variation .This diversity is reflected in the presence of different haemoglobin variants in different ethnic groups. Due to migration, there is mixing of people from different regions. Many of these abnormal variants are of little clinical significance in heterozygous state, but when combined with other variants they may give rise to severe disease. Therefore, there is always a need for a screening method which can detect maximum variants. HPLC has the advantage of quantifying Hb F and HbA2 along with detecting other variants in a single screening test.

Besides HPLC, there are other analytical procedures used for detection of thalassaemia and haemoglobinopathies such as alkaline and acid electrophoresis, HbA2 quantification by ion exchange column chromatography and Hb F quantification by alkali denaturation and radial immunodiffusion.[10] Electrophoretic method does not separate all variants from each other and it is recommended by screening programs such that further tests should be carried out to confirm the presumed identify abnormal variant. None of the above-mentioned methods can detect multiple haemoglobin fractions in a single step procedure. HPLC has many advantages over these methods and over the past decades it has evolved as an excellent and powerful diagnostic tool for identification of most of the clinically significant Hb variants. HPLC is sensitive, specific, reproducible and less time consuming and requires less manpower. Hence, it is an ideal tool for a routine clinical laboratory with high workload.

In India, according to hospital-based study, average frequency of sickle cell gene is around 5%. The highest frequency of sickle cell gene in India is reported in Orissa (9%) followed by Assam (8.3%), Madhya Pradesh (7.4%), Uttar Pradesh (7.1%), Tamil Nadu (7.1%) and Gujarat (6.4%). The frequency of sickle cell gene (7.3%)

in this study is in concordance with the reported prevalence.[11,12] The distribution of β Thalassaemia is not uniform in the Indian subcontinent. The frequency of β Thalassaemia trait varies for 1-17% in. different population of India.[13-15] In this study, the frequency of β Thalassaemia trait is (0.31%.) Borderline HbA2 requires further investigations before reaching out on conclusion. Both iron deficiency and megaloblastic anemia may have an effect on level of HbA2. Iron deficiency anemia can mildly reduce, and megaloblastic anemia can mildly elevate HbA2 level.

HB E trait, homozygous Hb E disease(Hb EE) and double heterozygous Hb E trait and Hb EE are mild disorder. Detection of this variant is very important because when combined with thalassaemia or Hb S, it gives rise to moderate to severe anemia. In present study, the frequency of Hb E is 0.03% with one case diagnosed as double heterozygous for Hb E/β Thalassaemia.

Heterozygous Hb D Punjab mostly presented as asymptomatic heterozygous condition with normal haematological parameter. Hb D Punjab occurs with greatest prevalence that is 2% among Sikhs in Punjab and in Gujarat reported prevalence is 1%. In present study, the frequency of Hb D is 0.07%.

The findings in the present study show HPLC as an excellent, powerful diagnostic tool for the direct identification of Hb variants with a high degree of precision in the quantification of normal and abnormal haemoglobin fractions. With the integration of proper algorithms involving retention time, % Hb and peak characteristics, a clinical laboratory is capable of sidentifying 75% of common variants encounted without the need for confirmatory studies such as alkaline and acid electrophoresis.[10] In the light of family study, ethnicity, hemogram and clinical data, authors can detect most of the frequently occurring clinically significant variants by HPLC method alone.

CONCLUSION

To conclude, HPLC findings in correlation with hemogram and family study are sufficient to detect most of the haemoglobin variants prevalent in this study with few inconclusive cases require further genetics and molecular studies.

The present study conducted using HPLC reflects the magnitude of abnormal hemoglobin variants in a tertiary care hospital population which may be in fact the tip of an iceberg, but this type of study can definitely help to increase awareness among both health care givers and general population.

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