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STUDY OF HEMOGLOBINOPATHIES IN PATIENTS OF ANEMIA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) IN WESTERN INDIA

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ABSTRACT

Introduction: In India, major cause of anaemia is nutritional deficiencies which can be treated by medications. Hemoglobinopathies are the most common inherited red cell disorders worldwide. Most clinically significant hemoglobinopathies are inherited defects of the beta (β) globin chain of adult haemoglobin. Identification of these disorders is immensely important epidemiologically and for improved management protocols. Our aim is to detect the haemoglobin disorders in patients with anaemia and to assess the suitability of using high performance liquid chromatography (HPLC) routinely for screening patients with anaemia.

Methods: A total of 500 cases of patient's with anaemia having haemoglobin up to 11 gm% were studied for routine haematological investigations and by HPLC for diagnosing any abnormal haemoglobin disorder by BIO RAD 'VARIANT' analyser.

Results: Out of 500 cases of anaemia studied, 43 cases showed abnormal haemoglobin fractions on HPLC. Of these, 26 cases of the beta Thalassemia trait was the predominant abnormality (5.2%). There were 6 cases of sickle cell trait patients (1.2%), 4cases of high Hb F patients (0.8%) and 2 cases of Hb D Punjab heterozygous patients (0.4%). Other hemoglobinopathies were also identified in smaller proportions.

Conclusion: HPLC is very simple, accurate and superior technique in early detection of various haemoglobin disorders, which helps in early management of patients.

Keywords: Anaemia, Hemoglobinopathies, HPLC

INTRODUCTION

Hemoglobinopathies are the group of genetic disorders of haemoglobin in which there is a quantitative or qualitative abnormal production or structure of haemoglobin molecule.[1,2] These hereditary disorders are major public health problem in many parts of the world including India.[2] Beta (β)- thalassemia and sickle cell disease represents the most frequent hemoglobinopathies.[2,3,4,5] The clinical spectrum of the disorders varies from asymptomatic conditions to serious disorders like Thalassemia major that requires regular blood transfusions and extensive medical care.[2] World Health Organization (WHO) figures estimate that 5% of world popu-

lation is carrier for haemoglobin disorders. [5,6,7] Thalassemia major is a worldwide disease, but it more common in the Mediterranean region, the Middle East, the Asian subcontinent, and southeast Asia, as well as southwest Europe and central Africa.[4] The prevalence of beta Thalassemia trait and sickle cell in India varies between 3-17% and 1-44% respectively.[1,2] Sickle cell disease is a protean disorder caused by elevations of intraerythrocytic and total blood viscosity. Hypoxia induced gelation of haemoglobin S deforms the erythrocyte and its membrane and cause increased stickiness. It leads to haemolytic anaemia and acute vaso-occlusion. Organ damage occurs from recurrent erythrocyte sickling.[3]The cumulative gene frequency of the three most predominant abnormal hemoglobins, i.e. sickle cell, haemoglobin D and Haemoglobin E has been found to be 5.35% in India.[2]

As the curative treatment like bone marrow transplantation is costly and so, a prospective prevention through population screening and genetic counselling is the best possible strategy for prevention of these disorders. As the exact data pertaining to the prevalence of hemoglobinopathies in this region is scarce, we considered it important to find out the extent of burden of hemoglobinopathies in anaemic patients.

METHODS

In this study, target group adopted is, anaemic patients attending G.M.E.R.S. Medical College and Hospital, Sola, Ahmedabad. 2 ml EDTA Blood samples were collected in clinical haematology lab. Details of clinical examination, history of blood transfusion, family history and consent was taken in all cases. Patient's samples whose haemoglobin was up to 11gm/dl were selected for this study. Haemoglobin and Red Blood Cell indices were measured on automated - five part differential cell counter using well mixed anticoagulated blood. Peripheral blood smears examination and Reticulocyte count study was also done in all the patients. The results of haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell (RBC) count and red cell distribution width (RDW) was correlated with peripheral smear examination. All these samples were analysed for haemoglobin disorders by BIORAD 'VARIANT' HPLC machine (BETA THALASSEMIA SHORT PROGRAM). It utilizes the principle of high performance liquid chromatography (HPLC). An HbA2/F calibrator and two level controls were analysed at the beginning of each run. The total area acceptable was between- one million to three million. Sample ratio was increased in case of low total area and vice versa. The software delivers a printed report showing the chromatogram, with all the haemoglobin fractions eluted. The integrated peaks are assigned to manufacturer – defined "windows" derived from specific retention time (RT). This retention time is the time that elapses from the sample injection to the apex of the elution peak, of normal haemoglobin fraction and common variants.

Table 1: Manufacturer- assigned windows for Bio-Rad Variant II HPLC system [8]

Peak name	Retention Time, min
P1 window	0.63-0.85
F window	0.98-1.2
P2 window	1.24-1.40
P3 window	1.40-1.90
A_0 window	1.90-3.10
A_2 window	3.30-3.90
D window	3.90-4.30
S window	4.30-4.70
C window	4.90-5.30

Table 1 show "windows" of established ranges in which common variants have been observed to elute using the Variant beta – thalassemia short program. The printed chromatogram shows all the haemoglobin fractions eluted, the retention times, the areas of the peaks and the values (%) of different haemoglobin components. If a peak elutes at a retention time that is not pre-defined, it is labelled as an unknown. Each analytical cycle, from sampling to printing of results takes about 6.5 minutes.

RESULTS

As shown in table 2, total of 500 cases were studied. Out of these, 43 cases displayed abnormal haemoglobin fractions on HPLC.

The major abnormality observed in thalassemia cases was high Hb A2. A cut of over 3.9% was taken for diagnosis of Beta Thalassemia Trait. [9] A total of 26 (5.2%) of beta Thalassemia trait were diagnosed. The retention time for Hb A2 ranged between 3.30-3.90 minutes. Peripheral blood smear showed microcytosis, hypochromia and

target cells. In most of the patients RBC count was raised.

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Table no 4 shows that 46.14% patients of Beta thal trait were having Hb less than 9gm/dl and 53.86% patients have Hb between 9-11 gm/dl.

Table 2: Type of Hemoglobin pattern among study subjects

Hemoglobin pattern	Patient (%), n=500
Normal Hb Pattern	457 (91.4)
Beta thalassemia Trait(BTT)	26 (5.2)
Thalassemia major	1 (0.2)
Hb S Homozygous	0 (0)
Hb S Heterozygous	6 (1.2)
Hb D Punjab Heterozygous	2 (0.4)
Hb D Punjab+BTT	1 (0.2)
HPFH	1 (0.2)
HbE– Homozygous	1 (0.2)
HbE+BTT	1 (0.2)
Raised Hb F	3 (0.6)
HB-D Iran Heterozygous	1 (0.2)

Table 3: Haematological parameters in different group of hemoglobinopathies

Hemoglobinopathies (n)	Hb (g/dl) mean±SD	RBC count ±SD (million/cmm)	MCV(fl) mean±SD	MCH(pg) mean±SD	MCHC(g/dl) mean±SD
Beta thal trait(26)	8.3±2.5	4.4±1.3	59.5±6.1	19.4±3.09	32.92±3.7
Beta thal major (1)	2.1	0.85	62.73	24.82	39.56
Sickle cell trait(6)	8.2±1.4	4.27±0.43	61.01±5.2	19.25±2.03	32.13±2.2
Hb D Punjab heterozygous(2)	6.15±0.6	4.22±0.3	53.07±5.7	14.48±0.30	27.34±3.33
Hereditary persistence of fetalhemoglobin (HPFH)(1)	8.8	4.57	67	19.2	28.90
HbE Homozygous(1)	8.2	4.47	48	18.3	37.9
HbE+BTT(1)	4.1	2.41	55.61	17.02	30.60
Hb D Punjab +BTT(1)	10.8	4.26	74.52	25.34	34.01
Raised Hb F(3)	4.9±1.7	2±1.2	71.13±15.7	28.10±7.7	40,76±14.4
HB-D Iran (1)	7.2	3.43	62.73	21.02	33.51

Table 4: Values of haemoglobin and RBC indices in 26 beta thalassemia trait patients.

	Patients (%), n=26
Hemoglobin Value	
<7 gm/dl	6 (23.07)
7-9 gm/dl	6 (23.07)
9-10gm/dl	7 (26.92)
>10gm/dl	7 (26.92)
RBC Indices	
MCV	
<82 fl	26 (100)
82-92 fl	0 (0)
>92 fl	0 (0)
MCH	
<27pg	26 (100)
27-32 pg	0 (0)
>32 pg	0 (0)
MCHC	
<32 %	8 (30.7)
32-37%	18 (69.23)
RBC COUNT	
<3.8 million/cmm	7 (26.92)
3.8-4.8 million/cmm	8 (30.7)
>4.8 million/cmm	11 (42.3)

All patients were having MCV less than 82 fl. and MCH less than 27 pg. 69.23% patients were having MCHC more than 32%, and RBC count was more than 3.8 million/cmm. in 73.08%.patients.

Chromatogram of beta thalassemia major showed marked anaemia, anisopoikilocytosis, microcytic hypochromic anaemia and polychromasia with nucleated RBCs.

Six cases (1.2%) were sickle cell trait with variant S-Window ranging from 15-28% and retention time 4.30-4.70 minutes. These patients haemoglobin was between 6.2 to 10.6 gm/dl and showed mild anaemia and anisopoikilocytosis.

Two cases (0.4%) of Hb D Punjab heterozygous showed D-Window with variant percentage 33.0% and 35.10% and retention time of 4.156 and 4.137 minutes. These patients were anaemic with haemoglobin 6.6 and 5.7 gm/dl respectively and hypochromic microcytic blood picture. One patient was double heterozygous for HbD Punjab and beta thalassemia trait showed 65.7% variant D-Window and 4.2% Hb A2 with mild anaemia.

Hb-E variant included one case Hb E homozygous (0.2%) and one case double heterozygous for Hb-E and beta thalassemia trait (0.2%). Hb-E presents as raised peak in the A2 region with retention time of 3.810 and 3.737 minutes respectively. Case of Hb E homozygous has high Hb E of 74.6%, retention time 3.810 and haemoglobin 8.2 gm/dl. Case of Hb E beta thal trait double heterozygous had Hb E 69.6% and Hb F 15.2%, and4.1 gm/dl Haemoglobin. Both patients showed hypochromic microcytic blood picture with target cells. Hb-D Iran patients had Hb A2 43.2%, retention time 3.600, normal Hb F (Hb 7.2%gm/dl) with microcytic hypochromic anaemia. One patient presented with Hb F value 96%. This patient categorized in to Homozygous HPFH and advised for genetic studies for confirmation.

Three patients presented with normal Hb A2 and raised Hb F ranging from 6.5% to 24% with low Hb, MCV, and MCH. These patients' categorized as suspected cases of delta beta thal trait or heterozygous HPFH, as other reasons of temporary raised HbF are, pregnancy, leukemia, microcytic anaemia and some drugs. In such cases repeat HPLC study advised after six months and genetic studies for confirmation. [10]

DISCUSSION

Thalassemia and hemoglobinopathies are autosomal recessive inherited disorders, primarily affecting the globin moiety of the haemoglobin molecule. [11] Alpha and beta- Thalassemia are the commonest single-gene haemoglobin disorders in the world. [5,7,12,13] These disorders, which were mainly confined to certain areas, religions, casts and tribes particularly with endogamous norms of marriages, are now widely prevalent all over the world. This is because of ever increasing migration of people from one place to another and mixing of different communities through marriages. [11] The Indian population comprises numerous casts and tribal groups, each revealing different genetic traits. [14] Large numbers of severely affected patients represents an enormous human suffering for many families and they need intensive supportive therapy with little or no chances of being cured. [3]

Low haemoglobin concentration is a result of many factors such as malnutrition, haemorrhagic conditions or by hereditary factors such as hemoglobinopathies. [3,4] These facts help us in using new techniques for early detection, prevention and treatment of this disease.

Cat ion exchange HPLC is emerging as one of the best methods for screening and detection of various hemoglobinopathies with rapid, reproducible and precise results. [13] It has the advantage of quantifying Hb F and Hb A2 along with haemoglobin variant screening in single and highly reproducible system. 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. [8]

According to study done by Indian Red Cross society, Gujarat State Branch, prevalence of beta thal trait is 3.4% and sickle cell trait is 0.7% in Ahmedabad. [1] In our study slightly higher rate 5.2% for beta thal trait and 1.2% for sickle cell trait is found. This is because our target population is anaemic patients. Low Haemoglobin, MCV, MCH and raised RBC count points towards beta thal trait. It is confirmed in our study.

Table 5: Different studies showing values of hemoglobin and RBC indices in beta thal patients

Different studies	Hb (g/dl) mean ±SD	RBC count (million/cmm) ±SD	MCV (fl) mran±SD	MCH (pg) mean±SD	MCHC (g/dl) mean±SD
Joseph Philip et al.[7]	9.8±2.4	5.06±0.9	68.5±6.2	21.3±2.6	28.3±1.8
Fakher Rahim study[12]	9.53±1.43	-	62.9±5.3	20.03±1.80	-
Baruah et al [5]	7.9±3.4	3.6±1.5	71.1±11.1	22.4±4.3	31.2±3.3
Our study	8.3±2.5	4.4±1.3	59.5±6.1	19.4±3.09	32.92±3.7

Table no 5 show patients of beta thalassemia trait in different studies. In a study by Madan et al. in Delhi, MCV below 80 fl and a MCH below 27pg were found to be very sensitive markers in detection of beta thal trait, even in the presence of iron deficiency which is also comparable in our study. [15] In all above studies value of MCV is less than 80 fl and MCH is less than 27pg. Thus along with detailed peripheral blood picture study, values of hemoglobin and RBC indices also help in timely detection of beta thalassemia trait. It is very important in preventing birth of homozy-

gous thalassemia major child by genetic counselling regarding the nature of the disease. A thalassemia major child is dependent of regular blood transfusions to maintain life since early childhood. However safe blood is available only for a small fraction and most such patients die due to iron overload.

In our present study 6 cases (1.2%) of sickle cell trait detected in anaemic patients. Timely detection of sickle cell trait can be helpful in warning patients of the possible complications and the preventive measures to be taken. Prenatal or early post-natal diagnosis of sickle cell disease helps in institution of prompt therapy before the onset of serious complications of the disease. [7]

Identification of Hb variants is very important. Doubly heterozygous state of Hb E and Beta thalassemiais clinically characterized by thalassemia major. In our study one such patient was detected with severe anaemia (Hb-4 gm/dl). It is important to increase awareness of this rare disorder, in clinicians and patients to assist in prenatal diagnosis, genetic counselling and clinical management. [7]Hb D- Punjab trait is of no significance clinically, but when it associated with Hb S it causes mild haemolytic anaemia and co-inheritance with B^0thalassemia produces a mild thalassemic condition. Also association of Hb D and haematological malignancies has been reported. [16]

It is a common practice among clinicians that to give iron therapy in all anaemic patients. It can lead to unnecessary iron overload in patients of thalassemia syndrome or patients of other hemoglobin variants. In India premarital screening is still considered taboo. So the best approach would be to target those patients attending the haematology OPD, the antenatal population and extended family members. Person having positive report for carrier state should be counselled regarding the nature of the disease and implications of being carrier which help in preventing birth of child with homozygous inheritance of hemoglobinopathies.

CONCLUSION:

In our country major cause of anaemia is nutritional deficiencies which can be treated by medications. Abnormal hemoglobin as a cause of anaemia should also be considered, as morbidity and mortality is higher in homozygous conditions of hemoglobinopathies. HPLC is a rapid, accurate and reproducible tool for early detection and proper management of hemoglobinopathies and its variants. This is especially important in view of high incidence if beta thalassemia trait in developing county like India, where resources are limited. Out of 500 anaemia cases in our study 43 (8.6%) cases showed some abnormalities in hemoglobin by HPLC. Detail investigation of anaemia keeping in mind the possibilities of detecting abnormal haemoglobin is very much helpful in finding out more carriers of different hemoglobinopathies. Combined approach of primary and secondary prevention needs to be followed. It will prove to be cost effective by preventing the birth of child with genetic homozygous inheritance disease.

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