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Research Article

Prevalence of haemoglobinopathies in anemic females

Promil Jain*, Vinod Kumar, Rajeev Sen, Shilpi Bhargava

Department of Pathology, Pt BD Sharma PGIMS, Rohtak, Haryana, India

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***Correspondence:**
Dr. Promil Jain,
E-mail: jainpromil@gmail.com

ABSTRACT

Background: The β-thalassemias and various other hemoglobinopathies are a major health problem in India. An accurate diagnosis of patients suffering from β-thalassemia syndrome and other hemoglobinopathies is important for prevention and management of major hemoglobin disorders. High-performance liquid chromatography (HPLC) is a highly sensitive and specific method for the accurate diagnosis of thalassemia and other hemoglobinopathies.
Methods: Present study was conducted on 1400 females of reproductive age group for screening of hemoglobinopathies in a rural block of north India. Patients were evaluated for presence of anemia with detailed hematological workup. Anemia cases were further screened by HPLC system for presence of any hemoglobinopathy.
Results: 855 subjects out of a total of 1400 were found to be anemic. 359 patients had normocytic normochromic anemia, 399 had microcytic hypochromic picture, 37 had macrocytic picture, while 60 patients had dimorphic anemia. 47 cases were detected positive for various types of hemoglobinopathies. 36 patients were positive for β-thalassemia trait/ heterozygous forming the major portion (76.5%), while one (2%) patient was β-thalassemia intermedia / homozygous and was non-transfusion dependent. 3 patients were positive for HbE heterozygous and hereditary persistence of fetal hemoglobin each, two for HbD Punjab, one each for HbS heterozygous and Hb Lepore.
Conclusions: The detection of various hemoglobinopathies is the key to diagnosis and proper treatment of various types of anemia. Antenatal screening should be an integral part of workup in pregnant females to prevent morbidity related to hemoglobinopathies like thalassemias.

Keywords: Anemia, Hemoglobinopathies, Thalassemic syndromes, High performance liquid chromatography

INTRODUCTION

Anemia is a group of disorders characterized by a quantitative or qualitative deficiency of the circulating erythrocytes. It is also defined as reduction in circulating hemoglobin (Hb) mass below the critical level. Anemia is a widespread health problem affecting more than 2 billion people worldwide, women and children being more affected. In India, anemia is the second most common cause of maternal deaths.[1]

Anemia is graded as mild (Hb-9-11 gm%), moderate (7-9 gm%), severe (4-7 gm%) and very severe (<4 gm%). Etiologic classification includes various causes that alter normal development of red blood cells (RBCs) and their hemoglobinization. Morphologically, anemias are classified using an orderly method based on cell size, cell shape, cell color etc. and provides a clue towards underlying etiology.[2]

Clinical syndrome resulting from disorders of hemoglobin synthesis are called hemoglobinopathies which could be due to structural variants like HbS or due to failure of synthesis of one or more of globin chain of hemoglobin like thalassemias or owing to failure to complete the neonatal switch from fetal hemoglobin to adult hemoglobin, i.e. hereditary persistence of fetal hemoglobin (HPFH).[3]

The thalassemia syndromes are heterogeneous group of disorders of hemoglobin synthesis due to inherited mutations that decrease the synthesis of either the α-globin or β-globin chains that compose adult hemoglobin, HbA ($\alpha 2\beta_2$).This leads to anemia, tissue hypoxia and red cell hemolysis related to the imbalance in globin chain synthesis.[4]

Thalassemias are divided into the α-, β-, δβ-,γβ-thalassemias, depending upon which globin chain is affected and produced in reduced amounts.[3] β-thalassemia and α-thalassemia are caused by deficient synthesis of β and α chains respectively. The hematologic consequences of diminished synthesis of any globin chain stem not only from hemoglobin deficiency but also from a relative excess of the other globin chain, particularly in β-thalassemia.[4]

The β-thalassemias and their interactions with structural Hb variants like HbE, HbD and HbS also produce thalassemic manifestations and are a major health problem in India.[5] These disorders cause anemia through two mechanisms: decreased red cell production, and decreased red cell lifespan.[4] An accurate diagnosis of patients suffering from β-thalassemia syndrome and other hemoglobinopathies is important for epidemiological studies as well as for management and prevention of major hemoglobin disorders.[6]

The commonly used investigative tools for the diagnosis of hemoglobinopathies are alkaline and acid electrophoresis and quantification by ion-exchange column chromatography for HbA_2, HbF quantification by alkali denaturation method. The identification of hemoglobin variants by these conventional methods is often presumptive, based on a characteristic electrophoretic mobility of the band and quantification. Definite identification usually requires DNA analysis or amino acid sequencing.[7]

High-performance liquid chromatography (HPLC) is a newer technique introduced for the accurate diagnosis of thalassemia and other hemoglobinopathies. It is a highly sensitive and specific method of diagnosis. HPLC offers the distinct advantage over classic hemoglobin electrophoresis as it can accurately identify and simultaneously quantitate abnormal hemoglobins. Specific elution windows are defined for HbA_2, HbF and other variant hemoglobins like HbS, D and C. This method has helped in identification of rare hemoglobins like HbQ India, HbJ Meerut, HbD Iran and various other unstable hemoglobins.[7] HPLC gives an accurate quantification of HbA_2 and HbF and detects large majority of Hb variants.[8]

The present study was conducted in females of reproductive age group (15-45 years) of rural block of north India with the aim to study prevalence of various hemoglobinopathies in the females of this age group.

METHODS

Present study was conducted as a pilot programme for screening of hemoglobinopathies in a rural block of north India. The total number of subjects included in the study was 1400.The target group comprised of females aged 15-45 years including pregnant females. A detailed hematological workup including complete hemogram with hemoglobin levels, RBC indices including MCV, MCH, MCHC, RBC Count, RDW-CV was performed on 5-part differential automated blood cell counter (BC-5800) of Shenzhen Mindray Bio Medical Electronics based on the principle of electrical Impedance.

The normal reference ranges taken as laboratory control included: Hb: 11-16 gm%, RBC Count: 3.5-5.5 x106/µl, Hct: 37-54%, MCV: 80-100 fl, MCH: 27-34 pg, MCHC: 32-36 gm/dl, RDW: 11-16%. Typing of anemia was done after peripheral blood smears were evaluated for red cell morphology and correlated with RBC indices. Morphologically anemias were classified as normocytic normochromic (MCV: 80-100 fl, MCH: 27-32pg), microcytic hypochromic (MCV<80fl, MCH<27 pg) and macrocytic anemia (if MCV>100 fl).

Anemia cases were further screened by high performance liquid chromatography (HPLC) Biorad (Variant II) system for presence of any hemoglobinopathy. Interpretation of HPLC results was done using various parameters (as described in company module) like flat baseline, total peak area, peak profile and shape, relative percentages of the hemoglobin fractions found after reviewing CBC data from 5-part cell counter to determine whether a variant was present.

β-Thalassemia syndromes are classified into 3 types; β-thalassemia major or intermedia (homozygous) in which HbF is elevated up to 90%, HbA is reduced with normal or increased HbA_2; β-thalassemia intermedia has similar findings but patient is not transfusion dependent. Patient with β-thalassemia minor/trait (heterozygous) has raised RBC count (>4.5 million/µl) and HbA_2 levels (4-9%) but HbF is normal (<2%). In patients with HbE hemoglobinopathy, it can be HbE homozygous (if HbF-2-10%, HbA2 >60%) or HbE heterozygous (if HbF <1%, HbA2 between 25-35%).

HbS (Sickle cell) hemoglobinopathy is either HbS homozygous (if HbF >5%, HbA2 <5%, HbS >50%) or HbS heterozygous (if HbF <1%, HbA2 <4%, HbS 30-40%). Patient is diagnosed as HbD Punjab heterozygous if there is D-window (HbD 30-40%) and HbF and HbA_2 are normal, as HbD Iran heterozygous if HbA_2 is between 40-48%, as a case of Hb Lepore trait if HbF <10% and HbA_2 is between 10-18%, as a case of hereditary persistence of fetal hemoglobin (HPFH) heterozygous if HbF is between 5-30% with normal HbA2. The results thus obtained were analyzed for presence of various hemoglobinopathies.

RESULTS

855 subjects out of a total of 1400 were found to be anemic. Mean hemoglobin of anemic subjects was 8.8 gm%. Morphological typing of anemia was done. 359 patients had normocytic normochromic anemia, 399 had microcytic hypochromic picture, 37 had macrocytic picture, while 60 patients had dimorphic anemia. 47 cases were detected positive for various types of hemoglobinopathies.

Table 1: MCV, MCH, MCHC, RDW-CV wise distribution of thalassemic cases.

MCV (fl) range	Positive cases of thalassemic syndrome	MCH (pg) range	Positive cases of thalassemic syndrome	MCHC gm/dl	Positive cases of thalassemic syndrome	RDW-CV (%)	Positive cases of thalassemic syndrome
<80	45	<27pg	44	<32	46	12-14	27
80-100	2	27-34	3	32-36	1	14-16	18
>100	0	>34	0	>36	0	16-18	2
Total	47		47				47

Microcytic picture was found in 45 positive cases of thalassemia, while 2 had normocytic picture. 44 patients had hypochromia while 3 had normochromic picture. 46 cases with thalassemia syndrome had MCHC<32 gm%. 27 patients with thalassemia syndrome had normal RDW-CV, but 18 patients had slightly raised RDW-CV, indicating concomitant presence of iron deficiency anemia (Table 1).

Table 2: Spectrum of various hemoglobinopathies with mean RBC indices.

Type of hemoglobinopathy	Total 47/855 (5.5%)	Hb (gm/dl)	MCV (fl)	MCH (pg)	MCHC (gm/dl)	RDW-CV (%)	RBCs (million/µl)
β-Thalassemia trait	36 (4.1%)	8.7	61.3	19.9	29.8	14.5	4.8
β-Thalassemia intermedia	1 (0.1%)	8.9	63.8	17.2	30.6	13.6	4.65
Hb E heterozygous	3 (0.4%)	6.4	68.6	22.4	29.4	15.9	4.89
Hb D	2 (0.2%)	8.4	62.3	23.4	30.3	15.8	5.28
Hb S	1 (0.1%)	7.1	62.5	19.5	27.6	13.5	5.26
Hb Lepore	1 (0.1%)	7.3	62.6	19.2	30.5	17.2	4.88
HPFH	3 (0.4%)	10.1	63.3	25.1	30.2	14.4	4.88

Out of a total of 855 anemic patients screened, 47 patients tested positive for various hemoglobinopathies, of which 36 patients were positive for β-thalassemia trait/ heterozygous forming the major portion (76.5%), while one (2%) patient was β-thalassemia intermedia/ homozygous and was non-transfusion dependent. 3 patients were positive for HbE heterozygous and hereditary persistence of fetal hemoglobin each, two for HbD Punjab, one each for HbS heterozygous and Hb Lepore. Mean values of various RBC indices are illustrated in Table 2.

DISCUSSION

Anemia is one of the world's most widespread health problems. It more commonly affects women and children worldwide. It is defined as reduction in circulating hemoglobin mass below the critical level. The normal hemoglobin (Hb) concentration in the body is between 12-14 gm%. WHO has accepted up to 11gm% as the normal hemoglobin level in pregnancy. However, in India and most of the other developing countries the lower limit is often accepted as10 gm%.[2]

Mean Hb of our patients was found to be 8.8 gm%. In a study done by Gupta et al mean Hb was found to be 9.3 gm%.[9] Lower hemoglobin in some of our cases could be due to associated iron deficiency (Table 3). The hemoglobinopathies, particularly β-thalassemias are a considerable health problem.[10] The identification of β-thalassemia trait is often based on characteristics like higher red cell count, reduced MCV, MCH along with raised levels of HbA_2.

Classical red cell indices for β-thalassemia trait are indicated by MCV<75 fl and MCH<27 pg. Mean MCV in

study group as a whole was 61.3 fl and mean MCH was 19.9 pg. Our findings of MCV, MCH, RDW-CV and RBC count were found to be consistent with findings of George et al (Table 3).[11] Microcytosis and hypochromia are commonly observed in the peripheral blood smear in patients with β-thalassemia trait. Tyagi et al and Gupta et al found microcytic hypochromic blood picture in all the cases of β-thalassemia trait.[9,12]

Table 3: Comparison of various RBC Indices by different authors.

Study	Mean Hb (gm%)	Mean MCV (fl)	Mean MCH (pg)	Mean RDW-CV (%)	Mean RBC Count (millions/ µl)
Gupta et al[9]	9.3	67.7	20.9	14.7	5.6
George et al[11]	10.0	64.5	21.3	15.3	5.7
Tyagi et al[12]	10.8	61.6	20.5	14.9	5.4
Present Study	8.8	61.3	19.9	14.5	4.8

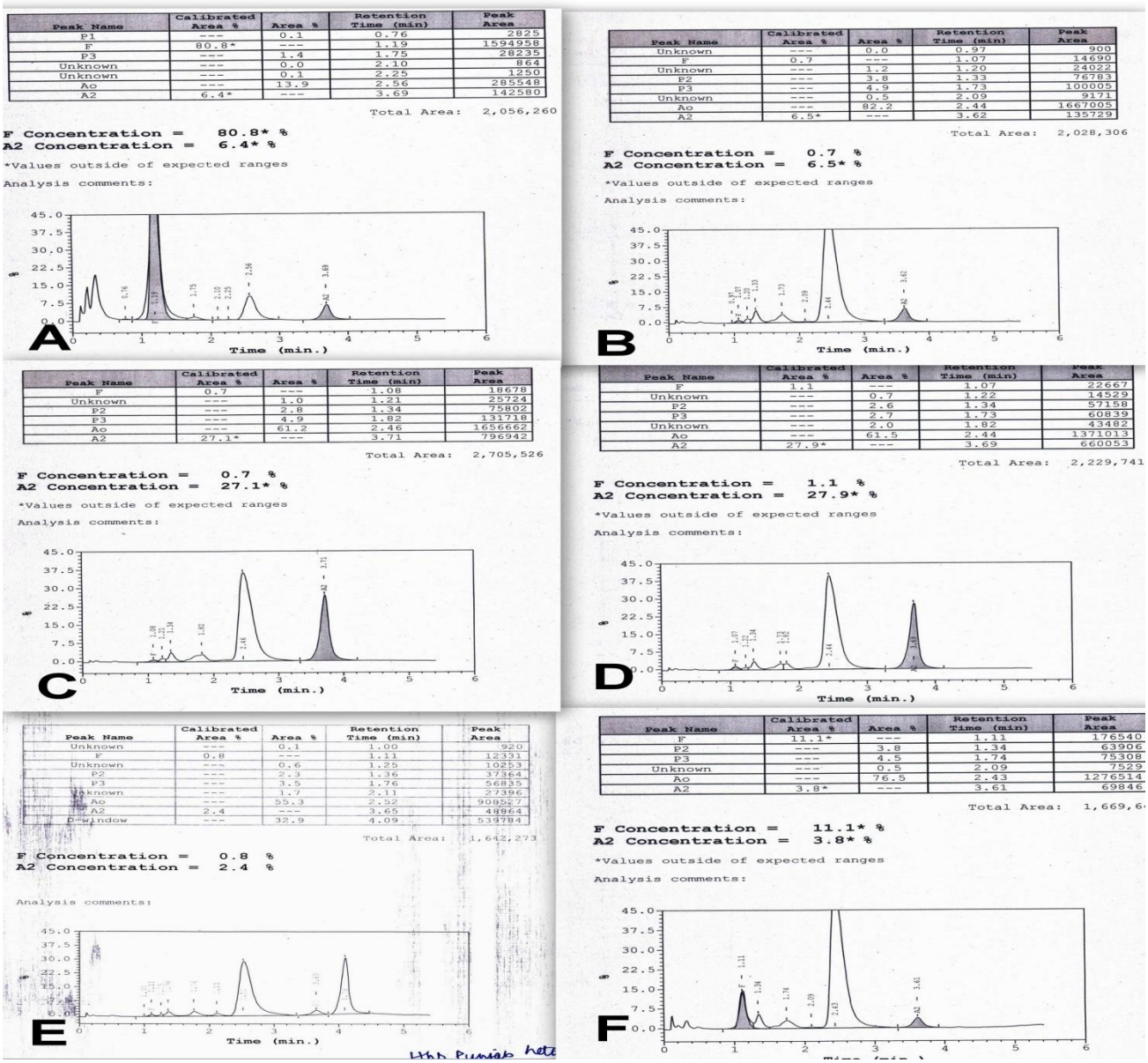

Figure 1: HPLC chromatograms: (A) β-Thalassemia Homozygous; (B) β-Thalassemia Heterozygous; (C and D) HbE heterozygous; (E) HbD Punjab heterozygous; (F) HPFH.

We also found microcytic hypochromic blood picture in all thalassemia trait cases. Degree of aniso-poikilocytosis also varied from none to mild to moderate degree (Table 3). In a study done by Sachdev et al on 2600 cases from North India, β-thalassemia trait was the predominant hemoglobinopathy found with a total of 232 cases (8.9%).[13] In another multicenter study done in six cities of India to determine the prevalence of hemoglobinopathies in different caste/ethnic groups using uniform methodology showed overall prevalence of β-thalassemia trait to be 2.78% and varied from 1.48 to 3.64% in different states, while the prevalence of β-thalassemia trait in 59 ethnic groups varied from 0 to 9.3 %.[14] Present study too revealed β- thalassemia trait in 4.1% of total anemic patients screened (Figure 1B).

In present study we found one case of thalassemia homozygous/intermedia (Figure 1A) having hemoglobin 8.9 gm%. The mean Hb levels in various authors were 7.4 gm% to 7.5 gm%.[12,15] Such low Hb could be due to concurrent iron deficiency anemia.

Table 4: HPLC Chromatograms in table form.

Dagnosis	HbF (Peak Area)	HbA_0	HbA_2	Any other peak	Total area
A) β-Thalassemia Homozygous,	80.8% (1,594,958)	13.9% (285,548)	6.4% (142,580)	-	2,056,260
B) β-Thalassemia Heterozygous	0.7% (14,690)	82.2% (1,667,005)	6.5% (135,,729)	-	2,028,306
C) HbE heterozygous	0.7% (18,678)	61.2% (1,656,662)	27.1% (796,942)	-	2,705,526
D) HbE heterozygous	1.1% (22,667)	61.5% (1,371,013)	27.9% (660,053)	-	2,229,741
E) HbD Punjab heterozygous	0.8% (12,331)	55.3% (908,527)	2.4% (48,864)	D-window:32.9% (539,784)	1,642,273
F) HPFH	11.1% (!76,540)	76.5% (1,276,514)	3.8% (69,846)	-	1,669,643

In present study, we found 3 cases of HbE heterozygous (Figure 1C and D). HbE heterozygous are clinically normal with only minimal hematologic changes. Red cells are normocytic or slightly microcytic with minor morphologic changes such as target cell morphology. HbA_2 should be between 25-35% of total hemoglobin for the diagnosis of HbE heterozygous. Homozygotes for HbE have HbA_2>60%.

Patients with HbE hemoglobinopathy are usually asymptomatic with either normal hemoglobin levels or mild anemia in some cases. The peripheral smear shows microcytosis and increased target cells.[16] In present study, mean MCV of all the 3 patients of HbE was 68.6 fl and mean MCH was 22.4 pg. Our values were lower than one of the authors while in concordance with other studies.[17,18]

We found 2 cases of HbD Punjab heterozygous/trait (Figure 1E); having mean Hb 8.4 gm%, MCV 62.3 fl, MCH 23.4 pg, RBC Count 5.28 million/µl and RDW-CV 15.8%. On HPLC analysis we found concentration of HbD as 38.0% and 32.9%, HbA_2 -2.8% and 2.4% and HbF-0.4% and 0.8% in first and second patient respectively. Blood picture was microcytic-hypochromic in both. In the present study, one case of sickle cell heterozygous (HbS trait) was found. The MCV was 62.5 fl and MCH was 19.5 pg in our patient and is in concordance with Mohanty et al.[19] Blood picture in our case was microcytic-hypochromic. Sickle cell trait is rarely associated with clinical or hematological manifestations of significance. On HPLC analysis we found HbS concentration 36.5%, HbA_2-3.4%, HbF-1.3%. We found 1 case of Hb Lepore trait case was with hemoglobin 7.3 gm%, showing microcytic hypochromic PBF and reduced values of MCV (62.6 fl) and MCH (19.2 pg). RDW-CV was 17.2 % and RBC Count was 4.88 million/µl. Heterozygotes with Hb Lepore trait have abnormal hemoglobin in the HbA_2 retention time window, constituting usually 10-18% of the total hemoglobin.

On HPLC analysis we found HbA_2-12.1% and HbF-1.2 %. In the present study, we found 2 cases of hereditary persistence of fetal hemoglobin (HPFH) (Figure 1F) having mild anemia with Hb 10.1 gm % and microcytic hypochromic picture and reduced MCV and MCH. RDW-CV was 14.4% and RBC Count was 4.88 million/µl. HPLC findings showed HbA_2 to be 3.8 % and 3.0% and HbF was 11.1% and 15.0%.

To improve the sensitivity and specificity of our results we need to be aware of the possibility of β-thalassemia trait in combination with IDA, the two most common causes of microcytic hypochromic blood picture.

CONCLUSION

The detection of various hemoglobinopathies is the key to diagnosis and proper treatment of various types of anemia. Antenatal screening should be an integral part of workup in pregnant females to prevent morbidity related

to hemoglobinopathies like thalassemias. Furthermore study of prevalence of various hemoglobinopathies, which are endemic in certain areas, by HPLC helps in detection of rare variants like HbQ, HbD Punjab etc. This can help government to formulate several programmes for management of these diseases.

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