

Spectrum of Hemoglobin Variants: A Cross Sectional Study from North Indian State

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Abstract

Introduction:

Hemoglobinopathies, comprising thalassemia syndromes and structural hemoglobin variants are heterogeneous group of inherited monogenic blood disorders, characterized by reduced or absent production of globin chains in hemoglobin. Mutations in genes coding for globin proteins produce thalassemia syndromes and variant hemoglobins. Epidemiologically identification of these disorders are very important to prevent serious clinical implications.

Material and Methods:

A total of 2518 blood samples of anemic patients were processed by HPLC. Target group comprised -anemic patients with Mentzer's index < 13, antenatal cases, patients who were clinical suspects of abnormal hemoglobin and family members of transfusion dependent children with various hemoglobinopathies, enrolled in thalassemia clinic under a campaign for screening of thalassemia.

Results:

Out of 2,518 patients were processed by HPLC (from July 2017 to October 2020) in department of Clinical Pathology, PGIMS, Rohtak, Haryana, 1302 (51%) were females and 1216 (48.2%) were male subjects with age ranging from 5 months to 60 years. Among the studied samples, 1882 (74.7%) showed normal pattern and 647 (25.6%) revealed abnormal chromatograms. Among different hemoglobinopathies detected, prevalence of β - thalassemia trait (BTT) was the highest, constituting 447 (17%) cases followed by 44 (1.7%) cases of β - thalassemia intermedia and 34 (1.3%) cases of β thalassemia major. Along with the β - thalassemia, we also recorded cases of the HbS, HbE and HbD hemoglobinopathies.

Conclusion:

High incidence of β - thalassemia trait in the Indian subcontinent makes HPLC-based Hb variant analysis imperative. It also emphasizes the need of prenatal screening for prevention of potentially detrimental hemoglobinopathies in offsprings.

Keywords:

Hemoglobinopathies, thalassemia syndromes

Introduction

Hemoglobinopathies, comprising thalassemia syndromes and structural hemoglobin variants are heterogeneous group of inherited monogenic blood disorders. These are inherited disorders of hemoglobin characterized by reduced or absent production of globin chains. Mutations in genes coding for globin proteins produce thalassemia syndromes and variant hemoglobins. More than 200 β -thalassemia mutations have been recognized, occurring in a wide range of ethnic groups [1]. In these disorders, a significantly reduced rate of synthesis of one type of globin chain leads to unbalanced

chain synthesis, with an excess of abnormal globin chain contributing to the pathological effects, causing either damage to erythroid precursors and ineffective erythropoiesis or damage to mature erythrocytes and haemolytic anemia [2]. The two most common types of thalassemias are α and β -thalassemia, while others like $\delta\beta$ thalassemia or those caused by structural interactions between hemoglobin chains result in hemoglobinopathies like HbD, HbE, HbS, HbC etc. have low prevalence [2]. Globally over 3,00,000- 4,00,000 babies are born with severe form of thalassemia every year, while in India there is a huge burden of estimated 1,00,000 patients with thalassemia [3-4]. Although these are being extensively studied at the molecular and cellular level but due to diversity and heterogeneous distribution of these disorders, epidemiological data to assess the actual disease profile is limited, particularly in regions of high prevalence [5]. In India, the average prevalence of β thalassemia carriers is 3-4% which translates to 35 to 45 million carriers in our

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multi-ethnic and culturally and linguistically diverse population of 138 crore people [6-7]. Many government and non-government organizations are working towards screening and providing treatment including blood transfusion, drugs and even facilities like bone marrow transplantation. But for reaching out such patients, an effective screening programme further tailed with strong confirmatory testing techniques are required. For screening, clinical assessment, complete blood count along with red cell indices, solubility tests, sickling tests and Hb electrophoresis are required. Hb electrophoresis, has now been replaced by High Performance Liquid Chromatography (HPLC) at many centers as HPLC has high sensitivity and specificity for screening and detection of various hemoglobin variants along with precise quantification of normal and abnormal hemoglobin fractions like HbE, HbD, HbS and in identification of rare hemoglobinopathies like HbQ, HbJ Meerut [8]. It is very helpful tool to generate awareness, identification of carriers and predicting the actual burden of the disease. Thalassemia syndromes and structural hemoglobinopathies are chronic illnesses, clinical spectrum of which varies from asymptomatic carriers to serious disorders like Thalassemia Major requiring regular blood transfusion and extensive medical care. These disorders are life impairing and in some cases are life threatening and impose a heavy emotional and financial burden on families and the nation as a whole. But, these are preventable, if pre-marital screening is done mandatorily in antenatal mothers and in suspected population and carriers are identified in time, so as to avoid birth of severely affected children. This study was conducted to see the prevalence of various hemoglobinopathies in patients reporting at a tertiary care center of Haryana.

Material and Methods

The present study was conducted in the department of Clinical Pathology in Pt. B. D. Sharma PGIMS, Rohtak, India retrospectively over a period of 3 years and 2 months (July 2017 to October 2020). A total of 2518 blood samples of anemic patients were received in EDTA vacutainers from OPD and IPD of the institute. Target group comprised anemic patients with Mentzer's index <13, antenatal cases, patients who were clinical suspects of abnormal hemoglobin and family members of transfusion dependent children with various hemoglobinopathies, enrolled in thalassemia clinic under a campaign for screening of thalassemia.

Complete clinical history including age of presentation, history of jaundice, blood transfusion and relevant family history was taken in all the cases. Complete blood count was performed on 5-part differential cell counter along with peripheral blood smear examination.

HPLC analysis was done on EDTA samples to look for presence/absence of various hemoglobinopathies on BIORAD Variant II (β thalassemia short programme) which works on the principle of high performance liquid chromatography, that is separation of molecules based on one or more of three main characteristics- polarity, charge and size of molecules. Samples were stored at 2-8°C and were analysed in batches within one week.

HbA₂/HbF calibrators with normal and abnormal controls (two levels of control- high and low, (BIORAD)) are analyzed at the beginning of each run routinely. The acceptable total area of each analysis is between 1 million to 3 million μ Volt/second. Interpretation of HPLC chromatograms is done based on parameters like flat baseline, peak profile, peak shape, total peak area etc, as each chromatogram shows eluted Hb fractions, retention times, peaks of HbA₀, HbA₂, HbF, along with C window, D window, S window and two minor peaks, P2 and P3. The integrated peaks are assigned by manufacturer defined 'windows' derived from specific retention time (RT). Retention time of normal hemoglobin fraction and common variants is the time that elapses from the sample injection to the apex of the elution peak. The 'windows' are established ranges in which common variants have been observed to elute using the variant $\hat{\alpha}$ -thalassemia short program; for example, in- F window- HbF elution time is between 0.98-1.20 minutes; HbA₀ window- HbA elutes at 1.93-3.10 minutes; HbA₂ - HbE, HbD Iran, Hb Lepore elute at 3.30-3.90 minutes; HbS elutes at 4.30-4.70 minutes in HbS window and D window- HbD Punjab elutes at 3.9-4.25 minutes. It takes about 6.5 minutes for each analytical cycle, from sampling to printing of results.

Results

In our study samples from 2,518 patients were processed by HPLC (from July 2017 to October 2020) in department of Clinical Pathology, PGIMS, Rohtak, Haryana out of which 1302 (51%) were females and 1216 (48.2%) were male subjects with age ranging from 5 months to 60 years. Among the studied samples, 1882 (74.7%) showed normal pattern and 647 (25.6%) revealed abnormal chromatograms (Table 1).

Table 1: Distribution of normal and abnormal Hb patterns on chromatogram (n=2518)

Hb pattern	Number (%)
Normal	1882 (74.7%)
Abnormal	647 (25%)

Among different hemoglobinopathies detected, prevalence of β -thalassemia trait (BTT) was the highest, constituting 447 (17%) cases followed by 44 (1.7%) cases of β -thalassemia intermedia and 34 (1.3%) cases of β

thalassemia major. (Figure 1 a,b,c). Most of the β -Thalassemia trait patients presented with features of mild anemia and revealed microcytosis and hypochromia. On HPLC, in BTT HbA was between 90-93%, HbA₂ > 4% and Hb F is between 1-4%. These patients had normal to decreased Hb, decreased MCV and MCH and nearly normal MCHC. Thalassemia major patients were mostly less than one year of age and showed features of severe anemia, prominent microcytosis, hypochromia, decreased RBC indices and required regular blood transfusions. On HPLC HbA and HbA₂ were decreased with HbF upto 95%. Contrary to thalassemia major, patients with thalassemia intermedia presented with moderate severity of anemia and decreased RBC indices. On HPLC, HbF was mostly between 10-30% but in some cases was found to be raised upto 70-80% and these patients were not transfusion dependent.

Table 2: Distribution of Abnormal Hemoglobin among abnormal Chromatograms

Types of abnormal Hb	Number (%)
β thalassemia trait	447(17%)
β thalassemia intermedia	44(1.7%)
β thalassemia major	34(1.35%)
HbE heterozygous	29(1.14%)
HbE homozygous	4(0.15%)
β E double heterozygous	15(0.6%)
HbD Pb heterozygous	9(0.35%)
HbD β double heterozygous	1(0.03%)
HbS heterozygous	2(0.07%)
HbS β double heterozygous	3(0.11%)
δ β thalassemia	12(0.47%)
HPFH	1(0.03%)
HbJ Meerut	4(0.15%)
Hb Lepore	1(0.03%)

Along with the β -thalassemia, we also recorded cases of the HbS, HbE and HbD hemoglobinopathies. (Table 2) These included 29 (1.14%) cases of HbE heterozygous, 15 (0.6%) cases β - E double heterozygous and 04 (0.15%) cases of HbE Homozygous. (Figure 2 a,b,c). 09 (0.35%) cases were diagnosed as HbD Punjab heterozygous (Figure 3a) and 12 (0.47%) cases as $\delta\beta$ Thalassemia. 02 (0.07%) cases were of HbS heterozygous, 03 (0.11%) cases as HbS- β double heterozygous, HbJ Meerut constituted 4 cases (0.15%) (Figure 3b) and 1 case (0.03%) each was diagnosed as HbD β -Thalassemia double heterozygous, HPFH (Figure 3c) and Hb Lepore. Samples of patients with HbE homozygous show elution in HbA₂ window and is usually >60% and HbF between 2-10% and patients with HbE trait have HbA₂ between 15-30% and HbA between 70-80%. HPFH patients have increased HbF >95% with near total absence of HbA but these patients are asymptomatic despite almost undetectable HbA. 285 (11.4% of the total screened subjects) cases were advised repeat HPLC analysis. Most of these included anemic children with age less than one year having mildly raised HbF levels which could be normal for the age.

Discussion

Hemoglobinopathies like β -thalassemias and other structural variants like HbE, HbD, HbS etc producing thalassemic manifestations pose a significant health problem worldwide. Though these are not always life threatening, yet hamper normal life causing anemia and pose risk to the offsprings if the parents are carrying the mutations. So, to prevent any major haemoglobin disorders and to improve quality of life it becomes imperative to screen and diagnose the suspicious or at risk population

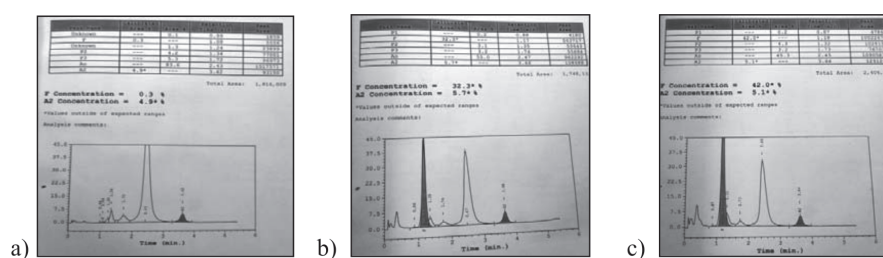


Figure 1: Chromatogram showing a) β -thalassemia trait, (b) Thalassemia intermedia, (c) Thalassemia major

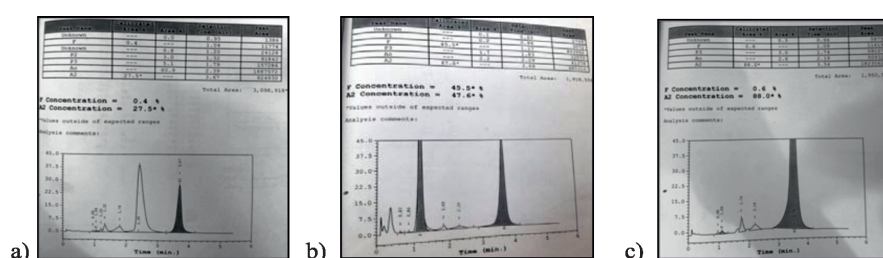


Figure 2: (a) Chromatogram showing HbE Trait, (b) HbE- β double heterozygous, (c) HbE Homozygous

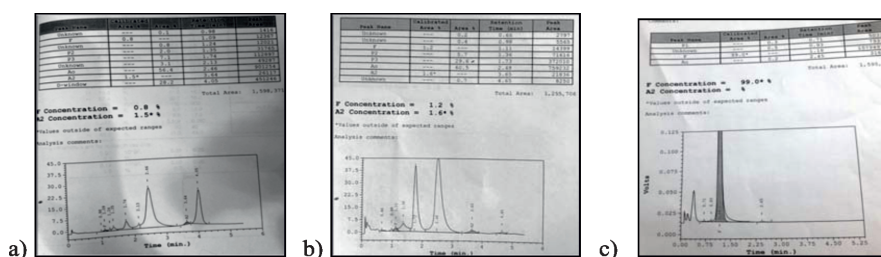


Figure 3: Chromatogram showing (a) HbD Punjab, (b) HbJ Meerut, (c) HPFH

for the hemoglobinopathies. Many investigative tools have been developed for the diagnosis of various hemoglobinopathies and HPLC has emerged to be the novel method for accurate detection of hemoglobinopathies with optimum results.

Variable genetic traits, ethnicity and diverse topography influence the presence and prevalence of different hemoglobinopathies all over Indian subcontinent. Results of our study showed 647 (25%) cases with abnormal pattern of haemoglobin on HPLC out of a total of 2518 cases, which were comparable with a study conducted by Ambedkar et al who reported the frequency of hemoglobinopathies in Western Maharashtra as 106 (26.5%) out of 400 subjects [9]. Similarly, Chopra et al revealed that out of 1032 participant, 258 (25%) cases had abnormal hemoglobin [10]. These studies were consistent with our study. However, Patel J et al conducted a study in Gujarat and found that out of 428 subjects, 153 (35.7%) had hemoglobinopathies [11] while another study from same authors in year 2012 found even higher prevalence (38.97%) [12]. But Modell and Petrou in their study showed lower prevalence i.e. 12% incidence of major hemoglobinopathy traits in Gujarat [13]. Similarly, Verma P et al showed that 143 (12.1%) cases out of 1180 subjects showed various hemoglobinopathies in Uttar Pradesh [14]. Also, Sachdev et al reported 327 cases (12.6%) hemoglobinopathies out of 2600 subjects [15]. Another study done in our institute on anemic females of reproductive age group in a rural population revealed prevalence of various hemoglobinopathies in 5.5% subjects [16]. Contrary to our result, a study of Bangladesh reported that, out of 600 screened individuals, 253 (42.2%) were found normal and 347 (57.8%) had one or the other form of hemoglobinopathies [17]. This frequency of hemoglobinopathies is overall higher in comparison to our findings. In our current study, the incidence of various hemoglobinopathies was higher probably because along with ante-natal mothers and anemic patients presenting in out-patient department, family members of patients those registered in thalassemia clinic were also screened, under a campaign.

Though β -thalassemia trait is variably distributed in Indian population but as recorded by our study it is quite prevalent

in Haryana (17.67%). Similar study conducted previously in our institution by us had found that a total of 9.7% (413) patients had various hemoglobinopathies out of which β -thalassemia trait formed the largest sub-group of abnormal haemoglobin including 318 patients (7.4%) [18]. According to data from other studies, the highest frequency of β -thalassemia trait has been reported in Gujarat (10-15%), followed by Sindh (10%), Punjab (6.5%), Tamil Nadu (8.4%) and Maharashtra [19]. A study conducted in West Bengal total of 35,413 individuals were screened for hemoglobinopathies. β - Thalassemia trait was found in 10.38%, which is similar to our finding (13.3%) [20]. Prevalence of thalassemia major in present study was found to be 1.3% in Haryana (state in northern India) which is in concordance with overall distribution of thalassemia major in India.

Prevalence of Thalassemia intermedia was found to be 1.7% in current study, which is in concordance to study conducted by J Patel et al in Gujarat 0.23% [12]. Even a study conducted previously in our institute showed 0.56% prevalence of thalassemia intermedia [16].

According to Williams et al Hb E occurs at extremely high frequency in many countries of Asia [3]. In India, HbE disorders is commonly seen in north-eastern states and West Bengal [21,22]. Tribal communities constitute a major part of the Indian population and are particularly vulnerable to hemoglobinopathies [23]. Our study recorded prevalence of HbE disorders of approx 1.9% (4 homozygous, 29 heterozygous and 15 β - E double heterozygous cases) which is comparable with the study done by Balgir RS in a tribal population, i.e. Delki Kharia in Orissa, showing 1.4% prevalence of HbE disorders (10 traits and one disease case) [23]. However, another study conducted by Kishore B et al showed eleven cases (4.78%) of HbE disorders (4 cases with HbE trait, 3 cases with HbE disease and another 4 cases with β - E thalassemia) identified through HPLC among 230 patients suspected of having hemoglobinopathies [24].

Sickle cell disease is a protean disorder with incidence range in India varying within various communities from 1%-44% [25]. High frequency of HbS was predominantly found in a study from Chhattisgarh (33.3%) [26] but our study showed lower incidence of sickle cell disease (0.07%) with 2 (0.07%) cases of HbS heterozygous and 3 (0.11%) cases

of HbS β double heterozygous.

Frequency of other hemoglobinopathies like $\delta\beta$ thalassemia was 0.47% in current study which is similar to study conducted by J Patel et al (0.23-0.47%) [11]. Our study also detected 1 case each of HbF_H and Hb Lepore (0.03%) and 4 (0.15%) cases of Hb J Meerut which is comparable to the study conducted by Mondal S showing frequency of HbF_H, Hb Lepore and Hb J Meerut as 0.12%, 0.004% and 0.03% respectively [27]. Patients with HbF_H, Hb Lepore and Hb J Meerut hemoglobinopathy, are mostly asymptomatic or may present with mild to moderate anemia.

Hemoglobinopathies are an important cause of morbidity and mortality all over the world though they are not curable but these can be prevented by screening, genetic counselling and prenatal diagnosis. It has been emphasised in various Indian studies that prenatal diagnosis remains the most appropriate intervention to prevent the birth of homozygous children. This information would offer parents to undertake apt reproductive choices, and the pregnancy of non-immune hydrops fetalis could also be potentially avoided. Mandatory screening should be offered to pregnant females/individuals from a high-risk ethnic background and consanguineous marriages. The couples at risk should be counselled regarding the nature and severity of the disease and the implications of being carriers. Moreover, family members of thalassaemic patients should be mandatorily screened as there are increased chances of them to be carriers of various hemoglobinopathies.

Conclusion

This is the retrospective study (2518 cases) HPLC technique for screening, detection, and identification of Hb variants within a clinical laboratory setting of India. The data obtained from our study finds HPLC (β -thalassaemia short program) as an excellent diagnostic tool for screening and detection of Hb variants and identification of unknown Hb variants. High incidence of BTT in the Indian subcontinent makes HPLC-based Hb variant analysis imperative. It also emphasizes the need of prenatal screening for prevention of potentially detrimental hemoglobinopathies in offsprings. Moreover, detection of other variants becomes important due to complex interactions as in cases with double heterozygous and homozygous states, which may lead to severe haematological abnormalities. HPLC findings must be supplemented with hemogram, family/sibling studies, and molecular studies for further confirmation.

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