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Research Article

Antenatal Screening for Hemoglobinopathies with HPLC

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Abstract

Introduction: Hemoglobinopathies are the most commonly encountered monogenic disorders of blood in Southeast Asia and Indian subcontinent. Screening of individuals at increased risk of being carriers for thalassemia and hemoglobinopathies, can identify couples with a 25% risk of having a pregnancy with a significant genetic disorder, for which prenatal diagnosis is possible. This study is done to know the prevalence of hemoglobinopathies and variant of haemoglobin using cation exchange high performance liquid chromatography (CEHPLC).

Materials and Methods: 2 ml of venous blood was collected in EDTA vials from the pregnant mothers after informed consent. The blood was subjected to complete hemogram, peripheral blood smear and HPLC using Variant Hemoglobin Testing System (BioRad Laboratories). Beta Thalassemia short programme was used. Descriptive analysis was done and data is presented in numbers and percentages.

Results: 467 blood samples from various ethnic groups were evaluated. 70 (14.99%) samples showed features of hemoglobinopathies by HPLC. There were 46 (9.85%) cases of HbE heterozygous,12 (2.57%) cases of HbE homozygous, 9(1.93%) cases of Beta Thalassemia Trait, 2 (0.43%) cases of double heterozygous and 1 (0.21%) case of Hb-D Iran.

Conclusion: This study showed a high prevalence of hemoglobinopathies in antenatal mothers necessitating an appropriate screening strategy for antenatal mothers. We also concluded that HPLC is a sensitive technique for studying hemoglobinopathies during pregnancy and may be utilized for screening.

Keywords: Antenatal mothers, HPLC, Hemoglobinopathies

Introduction

Hemoglobinopathies, are mainly divided into three overlapping groups: structural variants, thalassemia's characterized by reduced rate of synthesis of one or more globin chains, and hereditary persistence of fetal haemoglobin in which fetal haemoglobin synthesis persists beyond the neonatal period.[1]

The clinical spectrum of hemoglobinopathies varies from asymptomatic conditions to serious disorders which require regular blood transfusions and extensive medical care. According to World Health Organization (WHO) 7% of world population is carrier for haemoglobin disorders.[2]

Around 1.5% of the world's population carries the beta thalassemia and 7,000 babies with beta thalassemia major are born every year.[3] Of the several abnormal hemoglobins so far identified, there are three variants - sickle cell (Hb S), haemoglobin E (Hb E) and haemoglobin D (Hb D), which

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are predominantly prevalent in India. There are regional variations for these structural variants of haemoglobin.[4]

About 1.1% of couples around the world are at risk for having children with a haemoglobin disorder, of which 2.7 per 1000 conceptions are actually affected. Hemoglobin disorders contribute to 3.4% of mortality in children aged less than five years worldwide.[5]

The majority of the population in the North Eastern states of India have a high incidence of ß-thalassemia that causes the development of Hb E/ß-thalassemia disease, which is a severe condition; producing serious effects.[6,7]

Children inheriting these β-thalassemia major syndromes most often have a severe disease and a transfusion dependent survival from early childhood. It is therefore important to accurately identify carriers of these disorders and offer the option of preventive measures by prenatal diagnosis to couples at risk of having a child with severe disease.[8]

It is well established that the incidence of HbE gene in the North Eastern region of India is one of the highest in the world. Different states of the North Eastern region show a variable incidence of HbE varying from 16.2% to 47.3%.[9]

Since data on the prevalence of hemoglobinopathies and thalassemia's is scarce in India and review of literature did not reveal any study related to hemoglobinopathies in Meghalaya a screening programme is necessary to be taken up in North East India especially Meghalaya.

With this background knowledge, Antenatal screening to know the prevalence of hemoglobinopathies in antenatal mothers with HPLC was undertaken.

Material and Methods

The study population included already booked pregnant mothers, attending the antenatal clinic for routine antenatal check-up, irrespective of their gestational age, from January 2013 to June 2014 at NEIGRIHMS, Shillong. The study was done after obtaining the necessary clearance from the ethical committee of the institution. Those patients who refused treatment from the institute were excluded from the study. A written informed consent was taken from the willing participants. A detailed history including all personal, socioeconomic and ethnic details, clinical history, obstetric and blood transfusion history was taken.

Two ml of venous blood was collected in EDTA (ethylene diamine tetra acetic acid). Blood samples were subjected without delay to a complete blood count using automated 5 parts Sysmex Coulter machine. A peripheral blood smear was prepared and stained with Leishman's stain. The blood samples were stored at 4ºC and subjected to high performance liquid chromatography (HPLC) in batches within a week of collection.

For this study, Variant Haemoglobin Testing system (Bio-Rad Laboratories, Hercules, CA) was used to perform HPLC. It operates on the principle of HPLC and the column comprises of a small, (3.0x0.46) cm cation exchange cartridge. The β-thalassaemia short programme was used.

The cut-off values used for HbF, HbA_2 and HbA for the diagnosis of various hemoglobinopathies in antenatal mothers were shown in Table 1.[1]

Table 1.Levels of HbF and HbA2 for the diagnosis of common hemoglobinopathies in antenatal mothers[10]

Types of Hemoglobinopathies	Hb A2	Hb F
HbE heterozygous	30 %	< 1%
HbE homozygous	80-90%	Mild increase or normal
Double heterozygous (HbE-Beta Thal trait)	50-70 %	15-50%
Beta Thalassemia trait	4-7%	1-5%

Results

A total of 467 antenatal women were screened. Normal adult chromatogram shows primarily HbA, a small percentage of HbA2 (<3.5%) and traces of fetal Hb (<1%). Pregnancy per se has no influence on HbA_2 percentage but in 15-20% of women HbF percentage increases with level as high as 5% being observed.[10]

In the present study, 70 cases out of 467 antenatal mothers with prevalence of 14.99% displayed abnormal haemoglobin fraction on HPLC. HbE heterozygous is the commonest hemoglobinopathy found in our study with a prevalence of 9.85% (46 cases). There were 12 cases (2.57%) of HbE homozygous, 9 cases (1.93%) of β- thalassemia trait and 2 cases (0.43%) of double heterozygous for HbE and β-Thalassemia trait found in our study. We also diagnosed one case (0.21%) of Hb- D Iran (Figure 1).

We analysed the various RBC parameters and percentage of Hb fraction in various abnormal hemoglobinopathies encountered in this study. The following tables (Table 2 & 3) show haematological profile and Hb fractions in various hemoglobinopathies.

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Table 2.Hematological profile in pregnant mothers with various hemoglobinopathies

HPLC diagnosis	No. of patients	Hb (g/dl) ± SD	RBC (X10^{12}L) ± SD	PCV (%) ± SD	MCV (fl) ± SD	MCH (pg) ± SD	MCHC (g/dl) ± SD	RDW ± SD
HbE heterozygous	46	11.26 ± 1.39	4.33 ± 0.54	33.89 ± 3.63	78.30 ± 7.43	25.96 ± 2.52	33.01 ± 2.37	15.68 ± 2.30
HbE homozygous	12	9.68 ± 0.93	4.46 ± 0.72	28.71 ± 3.30	64.39 ± 5.70	21.69 ± 1.84	33.64 ± 1.93	17.07 ± 2.03
Beta-thalassemia trait	9	10.84 ± 2.48	4.03 ± 0.86	33.02 ± 6.60	84.08 ± 24.37	27.84 ± 9.20	32.59 ± 2.02	16.12 ± 2.95
Double heterozygous	2	5.95 ± 1.06	3.01 ± 0.27	18.95 ± 1.48	62 ± 1.41	17.5 ± 0.71	28 ± 1.41	31.55 ± 0.78
Hb-D Iran	1	7.4	3.4	24.3	71	21	29	17

Table 3.Hb fraction in various hemoglobinopathies

HPLC study	HbA%	HbF	HbA_2
HbE heterozygous	60.26 ± 9.91	1.27 ± 1.06	31.05 ± 8.55
HbE homozygous	4.6 ± 1.45	3.5 ± 1.03	85.08 ± 4.26
Beta-thalassemia trait	84.77 ± 1.51	2.36 ± 1.93	5.07 ± 0.96
Double heterozygous (HbE + BTT)	7.3 ± 1.84	20.4 ± 11.17	69.85 ± 12.52
Hb-D Iran*	38.1	2.7	51.3
* Single case			

HbE Heterozygous

HbE elutes in the A_2 window with retention time ranging from 3.3-3.9 min.[10] In our study all HbE heterozygous cases showed Hb ranging from 7.7 g/dl to 13.7 g/dl with a mean of 11.26 g/dl. Peripheral blood smears of most of the cases show normocytic normochromic to microcytic RBC. 14 cases presented with anaemia with Hb <11g/dl. 17 cases showed HbA_2 in the range of 23% to 28% which may be due to associated iron deficiency anaemia which is seen in 13 cases with low serum ferritin level. In other 4 cases out of 17, there was no association of iron deficiency anaemia. However, HbA_2 level was low in these cases which could be due to associated α-Thalassemia mutation which need further evaluation. In all HbE heterozygous cases HPLC showed mean HbA 61.44%, HbA_2 29.96% and HbF 1.20% (Figure 2).

Table 4.Hematological parameters of mothers having hemoglobinopathy with and without co-existent anemia

Haematological parameters	Hemoglobinopathy without anemia	Hemoglobinopathy with anemia	Hemoglobinopathy (all cases)
Hb	12.01 ± 0.91	9.21 ± 1.44	10.73 ± 1.83
PCV	35.71 ± 2.69	28.30 ± 4.1	32.32 ± 5.05
RBC	4.45 ± 0.56	4.05 ± 0.71	4.26 ± 0.66
MCV	81.34 ± 11.99	69.85 ± 9.57	76.09 ± 12.31
MCH	27.31 ± 4.23	22.59 ± 3.24	25.16 ± 4.46
MCHC	33.59 ± 1.32	31.99 ± 3.06	32.86 ± 2.41
RDW	14.94 ± 1.03	18.23 ± 4.46	16.44 ± 3.49

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Table 5.Ethnicity of the patients who attended antenatal clinic in NEIGRIHMS during the study period

Ethnicity	No. of patients	Percentage
*Khasi + Jaintia (Meghalaya tribes)	232	49.68%
*Garo (Meghalaya tribe)	20	4.28%
Other tribes (North Eats India)	82	17.56%
Non-tribal population	133	28.48%
Total	467	100%
* The tribal population of Meghalaya is composed of three major tribes belonging to Khasi, Jaintia and Garo-Hills districts, respectively.		

HbE Homozygous

In our study 12 cases of HbE homozygous was detected with Hb value ranging from 8.4 g/dl to 10.9 g/dl (mean 9.68 g/dl). In all the cases PBS showed evidence of haemolytic anaemia. The level of HbE ranged from 79.8 % to 90.7% with a mean of 84.63%. HbF ranged from 1.2% to 5.9% with a mean of 3.11% and HbA was 2.4 to 7.9 % with a mean of 4.60% (Figure 2).

Table 6.Prevalence of hemoglobinopathies as per the ethnicity in the present study

Ethnicity	No. of patients (out of 70)	Percentage
Meghalaya tribe (Khasi, Jaintia and Garo)	44	62.86%
Other tribes	10	14.28%
Non-tribal	16	22.86
Total	70	100%

Table 7.Prevalence of hemoglobinopathy in Meghalaya population (among Khasi, Jaintia and Garo tribe) in this study

Tribes	Total number	With hemoglobinopathy	Percentage
Khasi + Jaintia	232	33	14.22%
Garo	20	11	55%

β-Thalassemia Trait

9 cases of BTT was detected with Hb ranging from 7.6 g/dl to 14.7 g/dl (mean 10.84 g/dl) and RDW ranging from 12.9 to 22.3 (mean 16.12). RBC count ranges from 3.2 to5.6 (mean 4.03) with increased count noticed in two cases. PBS was normal in two cases with other cases showing anisopoikilocytosis, microcytosis, target cells, and polychromasia. HbF ranged from 0.2% to 5.9% with a mean of 2.36% and HbA was 83.2% to 86.4% with a mean of 84.7%. HbA_2 ranged from 4% to 6.6% with a mean of 5.07% (Figure 2).

Double Heterozygous for HbE and Beta Thalassemia Trait

HbE beta thalassemia, the compound heterozygous state of HbE and beta thalassemia, results in a variable clinical picture similar to that of homozygous beta thalassemia.[16] Two cases (0.43%) was diagnosed to be double heterozygous for HbE and β-Thalassemia trait with Hb ranging from 5.2 g/dl to 6.7 g/dl (mean 5.95g/dl). PBS showed marked anisopoikilocytosis, microcytosis, polychromasia and target cells. HbE ranged from 61% to 78.7 % (mean 69.85%) while HbF ranged from 12.5% to 28.3% (mean20.4%). The level of HbA was from 6% to 8.6%, with a mean of 7.3% (Figure 3).

Table 8.The prevalence of different types of hemoglobinopathy in tribes of Meghalaya in the present study

Ethnicity	HbE hetero	HbE homo	Beta Thal trait	HbD Iran	Double heterozygous (HbE + BTT)
Khasi + Jaintia	25	3	4	1	-
Garo	3	8	-	-	-

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HbD-Iran

One case was diagnosed as Hb D-Iran. It presented with raised A_2 peak of 51.3%, retention time of 3.92 minutes and peripheral smear showed microcytic hypochromic to normocytic RBC. Compared with Hb E heterozygous, Hb D Iran tends to have Hb A_2 more than 40% whereas Hb A/E has percentage of abnormal haemoglobin less than 40%. Family studies along with molecular studies were recommended for a definite diagnosis (Figure 3).

The comparative study of various haematological parameters of mothers having hemoglobinopathy with and without co-existent anemia is done which is shown in Table 4.

Ethnicity

In the present study, the ethnic background was also considered. It was a hospital-based study and the institute, being a tertiary care centre, catered to a mixed population of patients. The following table (Table 5) shows the ethnicity of the patients who attended Obstetrics and Gynaecology department for antenatal check-up during the study period

Since our institute is situated in the Khasi hills district, which has an admixture of the Khasis and Jaintias, there is a predominance of the Khasi and Jaintia patients over Garo patients.

Apart from tribes of Meghalaya, various other tribal populations from the neighbouring states of Meghalaya were also seen in the present study. It included tribes from Assam, Arunachal Pradesh, Manipur, Mizoram and Nagaland. The non-tribal population consisted of Assamese (non-tribal) Bengali, Bihari, Muslim, Nepali and few patients from other states of India.

We also evaluated the prevalence of hemoglobinopathies as per the ethnicity which is presented in Table 6.

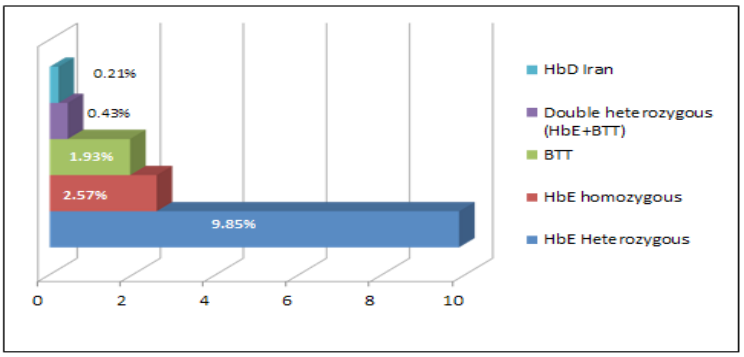

Figure 1.Histogram to show the various hemoglobinopathies found in the present study

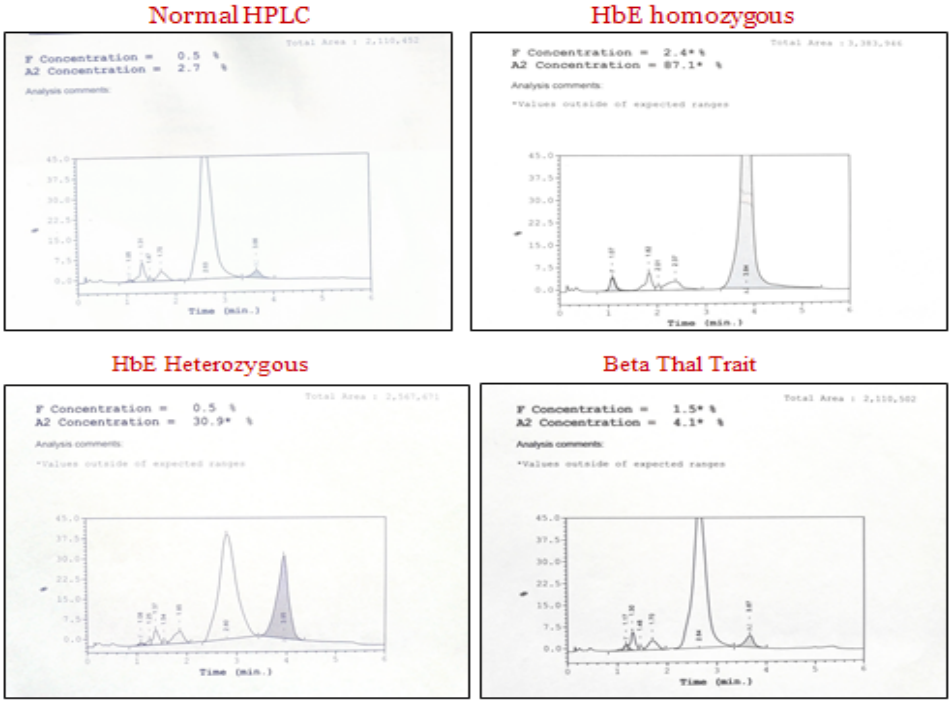

Figure 2.Various chromatograph of HPLC showing normal, HbE homozygous, HbE heterozygous and Beta Thal Trait

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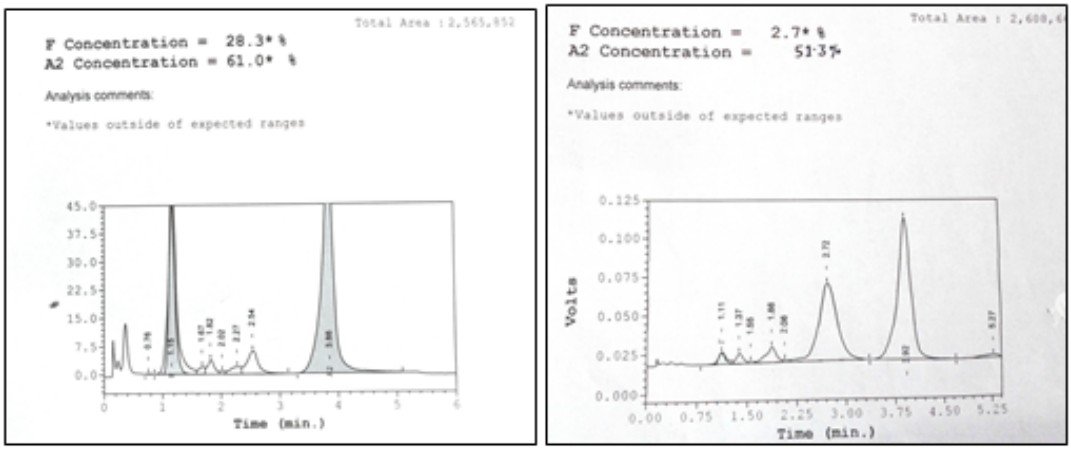

Double Heterozygous for HbE & BTT HbD- Iran

Figure 3.Various chromatograph of HPLC showing HbE & BTT, HbD - Iran

We observed that the majority (44 out of 70) of antenatal women with hemoglobinopathy belonged to the Meghalaya tribes. It was followed by the non-tribal population and other tribes. In the present study, it was observed that the study population was a mixed population and not truly representative of Meghalaya's indigenous tribal population. However, the prevalence and types of hemoglobinopathies in the major tribes of Meghalaya were evaluated separately in our study. The results are presented in Table 7 & 8 respectively.

We found that the prevalence of hemoglobinopathies was significantly high (55%) in the Garo tribe as compared to the Khasi & Jaintia tribes.

Discussion

Thalassemia and other structural hemoglobinopathies are major and important cause of morbidity and mortality worldwide which can be prevented by population screening, genetic counselling and prenatal diagnosis.[11]

Screening of individuals at increased risk of being carriers can identify couples with a 25% risk of having a pregnancy with a significant genetic disorder. Antenatal screening is the important step to identify women having the risk of producing a child affected with hemoglobinopathy.[1, 3, 8]

In this study a total of 467 antenatal mothers underwent complete hemogram and haemoglobin variant study by CE-HPLC method. Prevalence of hemoglobinopathies in our study is 14.99%. Altogether 5 different variants were detected which included HbE heterozygous, HbE homozygous, β-Thalassemia trait, Double heterozygous for HbE and β-Thalassemia trait, and HbD- Iran. Hemoglobinopathies are now widely prevalent all over the world. This is because of increasing migration of people and marriage among people from different communities. Hb E is most common in South East Asia and second most prevalent haemoglobin variant worldwide.[12]

In 1975, Sukumaran and a recent large study conducted in metropolitan cities of India had observed that ß-thalassemia is probably the commonest inherited haemoglobin disorder in the Indian subcontinent. Contrary to their results; we found that haemoglobin E was the commonest hemoglobinopathy with a prevalence of 9.85%.[13] In Tripura and Assam it was found to be 45.83% and 25.49 % respectively.[9, 11] Other studies conducted in central, north and west India found the prevalence of haemoglobin E to be 0.9%, 0 and 0.04% respectively.[13, 14]

The prevalence of haemoglobin E homozygous was found to be 2.57%. Sengupta B et al. found that the prevalence of Hb E homozygous in tribal population of Tripura to be 14.83%.[11] The prevalence of ß-thalassemia trait in our study was 1.93%. Madan N et al. found the overall gene frequency of ß-thalassemia trait in Delhi and Mumbai to be 5.47% and 2.68% respectively.[13] The carrier rate of β-thalassemia varies from 1 to 17 % in India with an average of 3-4 %. The overall prevalence of BTT was 3.38 % among antenatal women.[1] Conditions with borderline Hb A_2 need careful interpretation. Iron deficiency may lead to a low Hb A_2 and hence may mask a thalassemia trait whereas B12/folate deficiency may lead to slightly raised Hb A_2 leading to a false diagnosis of a trait. Careful evaluation of indices with iron profile will usually help in such cases. Similarly, milder forms of thalassemia or a co-inheritance of delta thalassemia may lead to borderline A_2 levels. Genetic studies should be advised in such cases for a conclusive opinion.[15]

The prevalence of compound heterozygous for ß-thalassemia and Hb E in the present study was found to be 0.43 %. Sengupta B et al. observed a rise in the incidence of compound heterozygous for ß-thalassemia and Hb E in Tripura, mainly among persons of Bengali/Bangladeshi origin. They pointed out that this may emerge as a future public health problem. They also emphasized the necessity of screening for compound heterozygous for ß-thalassemia

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and HbE in the population of North East India.[11] The two cases of compound heterozygous detected in our study were from non-tribal population.

It is interesting to note that among the tribes of Meghalaya, the Garo tribe showed a high percentage of hemoglobinopathy with a prevalence of 55% (11/20 cases). However, there are no earlier studies from Garo Hills district to compare our finding and the number of cases in our study is less for a definite conclusion. Thus, there is a clear need for a separate study to know the prevalence and pattern of hemoglobinopathies in the Garo Hills district of Meghalaya.

In our study we detected a single case of HbD-Iran in Khasi population. It presented with raised A_2 peak of 51.3% and retention time of 3.92 minutes. HbD-Iran in India is mainly seen in north western region. On HPLC, abnormal haemoglobin elutes in the A_2 window. Differentiation with Hb E on HPLC relies mainly on the fact that, in heterozygous states, Hb D Iran is usually more than 40% whereas Hb E is less than 40%. This finding should be further evaluated with family studies and the final confirmation is by molecular diagnosis. Phenotypic presentations of such cases are normal.[16]

In the present study, there was no case of sickle cell trait/ disease. Sengupta B et al. also did not report any case of Hb S from Tripura and Arunachal Pradesh.[11]

On comparing the haematological profile in pregnant mothers with hemoglobinopathy, lowest haemoglobin level with peripheral smear showing evidence of haemolytic anemia is seen in double heterozygous and HbE homozygous cases. This may be due to associated nutritional anemia or hemoglobinopathy itself. The results are in accordance with the study of Philip J et al. upon antenatal mothers in Maharashtra who observed lowest Hb, MCV and MCV in βThalassemia major followed by double heterozygous (for HbE and BTT) and HbE homozygous cases.[5]

The present study shows that the MCV and MCH levels were lower in hemoglobinopathy cases with coexistent anemia. The results are in accordance with the study of Chakrabarti I et al.[16] done on antenatal mothers who found that patients with β-Thalassemia trait with co-existent iron deficiency had haemoglobin, MCV and MCH levels significantly lower than those of beta-thalassemia only.

Careful and detailed complete blood count and peripheral blood smear examination (PBS) is very essential in the diagnosis of various haemoglobin disorders. In an α-thalassemia trait when the HPLC can be absolutely normal with perhaps a low normal or low Hb A2, careful correlation of the microcytosis which is unexplained by BTT or iron deficiency can lead us to suspect α-thalassemia disorder which can be confirmed by DNA analysis. BTT usually have a low mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) with a raised total RBC count. The PBS may show basophilic stippling and target cells which are more characteristic of β-thalassemia than iron deficiency anemia. Iron stores are normal/high in β-thalassemia. The PBS in sickle cell disorders will show sickle cells and the diagnosis can be confirmed by HPLC and by sickling test. In other hemoglobinopathies the blood picture can be non-specific and HPLC comes in as an excellent diagnostic tool for detection and quantification of several normal and abnormal hemoglobins.[5]

Conclusion

For accurate quantification of haemoglobin HPLC is an ideal methodology for routine clinical laboratory. In spite of highly reliable technique, considerable expertise is required to interpret the data produced. Many types of haemoglobin may have same retention times as normal hemoglobins or other variants and co-inheritance of different traits can further confuse the issue. Cases of borderline HbA_2 values between 3 and 3.9% should be carefully analysed as concomitant iron deficiency can lead to a low HbA_2 and mask a BTT. However, in our study we did not encounter any borderline case of beta- thalassemia trait.

Antenatal screening is necessary for early detection of Thalassemia traits which will prevent occurrence of Thalassemia major in off springs. Detection of other variants become important due to complex interaction in cases with double heterozygous and homozygous states. However, public awareness and a field study is necessary to understand the need for appropriate screening strategy for antenatal mothers in our region.

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Conflict of Interest: None

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