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Research Article

A CLINICO-HAEMATOLOGICAL STUDY OF HEMOGLOBIN E VARIANTS IN A TERTIARY CARE CENTRE IN NORTH INDIA

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A B S T R A C T

Haemoglobin E is the second most prevalent haemoglobinopathy worldwide after Hb S. It has highest prevalence in South-east Asian countries. In India it is prevalent in the north-eastern states. We have analysed the clinical and haematological profile of 55 cases of HbE variants presented for the first time in our clinical pathology department in the last eight years. The study was designed to find out the incidence of HbE disease in Haryana, to study the clinical profile of these patients and whether RBC indices obtained from automated cell counter can provide a clue to the diagnosis of HbE disease. High Performance liquid chromatography (HPLC) was used as a confirmatory diagnostic test for identification of HbE variants. It is important to distinguish various HbE disorders because of marked differences in clinical course among different genotypes. Hence, clinicians should consider this haemoglobinopathy in the differential diagnosis of anaemia with or without splenomegaly for accurate diagnosis and management.

INTRODUCTION

Haemoglobin E is an inherited autosomal recessive disease caused by substitution of glutamic acid by lysine at codon 26 in the beta (β)-globin protein chain of Hb. HbE disorders occur in homozygous (EE), heterozygous (AE), and compound heterozygous states (e.g. HbE co-inherits with beta thalassemia, HbS, HbC and other haemoglobin variants) with widely variable clinical phenotypes.[1]

It occurs primarily in South-east Asian populations where its prevalence reach upto 30-40%.[2]Now a days, it is prevalent all over the world as a result of endogamous marriages and increasing migration of people from one place to another. In India, HbE is mostly restricted to North East India, with an average allele frequency of 10.2%.[3]

This study was conducted in Pt.B.D.Sharma, Post Graduate Institute of Medical Sciences, Rohtak (Haryana) from August 2011 to August 2018 for analysis of clinical and haematological profile of Hb E variants. Study of this kind had never been done before in this region. This type of clinico-haematological screening, using automated blood cell counter and HPLC, followed by genetic counselling provides a cost effective measure in preventing HbE genetic disorder in future generation especially in the low socio-economic countries.

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MATERIALS AND METHODS

The present retrospective study was conducted in the Department of Clinical Pathology, Pt.B.D.Sharma, PGIMS, Rohtak for the period of eight years, from August 2011 to August 2018.

Case selection: Study included antenatal pregnant females brought for routine check-up as well as referred patients suspected of having thalassemia or other hemoglobinopathies. Patients of various religions, tribes, castes, and languages irrespective of their sex were included in this study, while patients with history of blood transfusion in previous 03months were excluded from the study. A total of 4,088 cases were screened from which patients with HbE disorders were selected for detailed clinical and haematological evaluation.

Clinical analysis: 55 patients diagnosed as HbE haemoglobinopathy were selected for the study population. Thorough information regarding age at presentation, family history, presenting symptoms and blood transfusion requirement were noted.

Hematological analysis: Two ml venous blood was collected in EDTA vaccutainer from each patient under aseptic precautions. Complete haemogram with RBC indices was performed on 5-part differential automated blood cell counter, model BC-5800 of Shenzhen Mindray Bio Medical Electronics, based on the principle of electric impedance. Red cell morphology was observed in peripheral blood film stained with Leishman's stain. Reticulocyte count was done using

supravital stainig with methylene blue and sickling test was done using freshly prepared 2% sodium metabisulfite solution. HPLC was done using VARIANT II beta-thalassemia short program (BIORAD Laboratories, India Pvt. Ltd.) based on the principle of ion-exchange chromatography. On HPLC, HbE and HbA2 has the same retention time, but it can be distinguished by its high concentration. If the HbA2/E peak is greater than 10% and HbA0 is present, the patient can be HbE-trait or E/beta positive. The patient is E/beta positive, if HBA0% is less than HBA2/E% and has a high HBF%, while, the patient is homozygous HbE/E, if the HBA2/E peak is greater than 10% and HBA0 is not present.[4]

Statistical analysis: The results of the study were statistically analyzed using the Statistical Package for the Social Sciences (SPSS) version 20 (IBM Corp. SPSS statistics, in Armonk NY) for windows. Data were expressed as mean ± standard deviation and rangefor quantitative variables, numbers, and percentage. Comparison between levels of HbA2, HbF and RBC indices including RBC counts, Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Packed cell volume (PCV) and Red cell distribution width- coefficient variance (RDW-CV) was made using student t-test and Chi-square test whichever was appropriate. Correlation between levels of HbA2, HbF with RBC indices was analyzed using the Pearson's correlation test with an accompanying t value.

RESULTS

Relative Incidence: Among 4,088 cases screened, only 55cases (1.34%) were detected with hemoglobin E disorders in the last eight years. Based on HPLC, 09 patients were diagnosed as homozygous (HbE disease- HbE/E), 26 as heterozygous (HbE trait- HbA/E), 19 as HbE/β-thalassaemia and 01 as HbE/HbS (double heterozygotes). The relative incidence of HbE variants is depicted in Figure 1.

***Age and sex Distribution*:** Mean age of presentation ± SD with range of age of the study subjects was 18.1 years ± 11.64 (range 02 - 42 years) in HbE disease, 16.73 years ± 10.37 (range 01 – 36 years) in HbE trait, 10.78 years ± 8.30 (range 10 months- 28 years) in HbE-β thalassaemiaand 03 year in HbE/HbS, respectively. The age distribution of HbE variants is shown in Table 1.

Out of 55 patients, 20 were males (36.3%) and 35 females (63.6%) with a male: female ratio 1:1.75, this ratio may be spurious as antenatal pregnant females (10 out of 35 females, 28.57%) constituted the major group in this study.

Clinical Presentation: Out of 55 patients, 34 patients (61.81%) were symptomatic and 21 (38.18%) were asymptomatic. Majority of the patients presented with variable signs and symptoms of anaemia as shown in Table 2.09 out of 55 cases (16.3%) had positive family history.08 out of 19 cases (42.1%) of HbE-β thalassaemia had severe anaemia (Hb< 6.0 gm/dl) and had history of red cell transfusion (on demand) before presenting to our hospital. One such patient had undergone splenectomy with a beneficial response.

Peripheral blood film: Majority of the patients (42 out of 55, 76.3%) presented with microcytic picture with or without hypochromia. Variable degree of aniso-poikilocytosis (mild in HbE disease and HbE/HbS, moderate in HbE trait, and marked in HbE-β thalassaemia) was seen. Other findings observed were normoblastaemia, polychromasia, basophilic stippling and target cells. RBC morphology of a patient with HbE trait is shown in figure 2.

Bone marrow aspiration was done in two patients, who were admitted with a preliminary diagnosis of refractory anaemia, revealed micronormoblastic erythroid hyperplasia with increased iron stores, which were later on diagnosed with HbE-β thalassaemia on HPLC.

Reticulocyte counts were predominantly normal in HbE trait, normal to increased in HbE disease and increased in HbE- β thalassaemia and HbE/HbS.

Haematological values, their relevant RBC parameters and HPLC findings are given in Table 3, 4&5.

Chromatograms of some important variants are shown in Figures3, 4 & 5.

Statistical Analysis: There was negative correlation between HbA2/E peak values and RBC parameters like MCV and MCH with a t-value of -2.358 and -2.658 respectively. A positive correlation was found between HbA2/E peak values and RDW-CV (t-value 0.642) and no correlation was found with values of Hb level, RBC counts, PCV and MCHC.
There was negative correlation between HbF peak values and MCV with a t-value of -1.628, while no correlation was seen with values of Hb level, RBC counts, PCV, MCH, MCHC and RDW-CV.

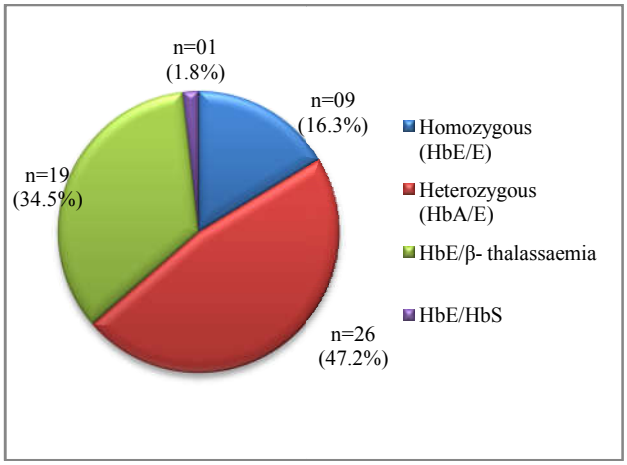

Figure 1 The relative incidence of HbE variants (n=55)

Table 1 Age distribution of HbE variants (n=55)

Age group in years	Total		HbE/E		HbA/E		HbE/β		HbE/HbS	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
0-10	24	43.6	03	33.34	09	34.61	11	57.89	01	100
11-20	11	20	02	22.23	06	23.07	03	15.78	-	-
21-30	17	30.9	03	33.34	09	34.61	05	26.31	-	-
31-40	02	3.63	-	-	02	7.69	-	-	-	-
41-50	01	1.81	01	11.12	-	-	-	-	-	-
Total	55	100	09	100	26	100	19	100	01	100

Table 2 Distribution of patients by clinical presentation of HE variants (n=55)

Clinical Presentation	Total		HbE/E		HbA/E		HbE/β		HbE/HbS	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Asymptomatic	21	38.18	04	44.45	14	53.84	03	15.78	--	
Symptomatic	34	61.81	05	55.56	12	46.15	16	84.21	01	100
Generalised weakness	25	73.52	03	60.00	09	75.00	12	75.00	01	
Pallor	19	55.88	02	40.00	07	58.33	09	56.25	01	
Exertional dyspnoea	05	14.70	--		02	16.66	03	18.75	-	

Palpitation	04	11.76	--		02	16.66	02	12.50	-
Pregnancy with anaemia	10	29.41	01	20.00	05	41.66	04	25.00	-
Failure to thrive	03	8.82	--		01	8.33	02	12.50	-
Jaundice	04	11.76	--		01	8.33	03	18.75	-
Splenomegaly	06	17.64	--		01	8.33	04	25.00	01
Fever	04	11.76	--		01	8.33	03	18.75	-
Recurrent infections	04	11.76	--		01	8.33	02	12.50	01
Delayed puberty	05	14.70	--		03	25.00	02	12.50	-
Thalassemicfacies	04	11.76	--		01	8.33	03	18.75	-

Table 3 Haematological values in HbE variants (n=55)

Haematological values	HbE/E (n=09)	HbA/E (n=26)	HbE/β (n=19)	HbE/HbS (n=01)
Hb (gm/dl)				
Mean±SD	9.27±1.839	11.27±0.857	6.22±1.628	8.2
Range	6.8 – 13.2	7.5 – 12.4	3.2 – 10.5	
Total RBC count(10^{12}/Lit)				
Mean±SD	4.24±0.549	4.85±0.369	3.05±1.368	3.50
Range	1.5 -5.4	2.3 – 5.3	1.0 – 3.8	
TotalWBCcount (109/Lit)				
Mean±SD	7.73±3.026	7.16±3.184	8.43±2.678	11.50
Range	4.8 – 10.5	2.5 – 5.3	2.0 – 13.4	
Platelet count (Lakhs/cmm)				
Mean±SD	3.24±0.781	2.81±0.87	2.15±1.27	1.8
Range	1.5 – 4.2	1.2 – 3.8	0.5 – 3.2	
Reticulocyte count (%)				
Mean±SD	3.16±0.52	0.86±0.42	4.27±0.25	3.5
Range	2.03 – 4.80	0.5 – 2.14	2.45 – 7.66	

Table 4 Red cell indices in HbE variants (n=55)

Red cell indices	HbE/E (n=09)	HbA/E (n=26)	HbE/β (n=19)	HbE/HbS (n=01)
P.C.V (%)				
Mean±SD	28.1±7.28	35.48±1.158	19.28±5.318	24.2
Range	20.4 – 40.5	23.5 – 39.4	11.2 – 32.0	
M.C.V (fl)				
Mean±SD	62.49±5.151	70.24±5.151	66.18±9.933	69.4
Range	52.8 – 90.7	62.6 – 88.4	50.1 – 76.2	
M.C.H (pg)				
Mean±SD	21.73±2.629	23.17±3.85	19.71±3.07	22.6
Range	16.6 – 25.0	18.1 – 28.1	15.2 – 22.7	
M.C.H.C(gm/dl)				
Mean±SD	34.66±2.50	31.72±1.601	28.47±3.06	31.8
Range	28.9 – 38.5	24.2 – 36.2	20.9 – 34.2	
RDW-CV (%)				
Mean±SD	22.92±2.847	16.1±2.949	33.78±6.650	15.2
Range	13.2 – 26.4	12.7 – 21.2	21.4 – 39.4	

Table 5 HPLC indices in HbE variants (n=55)

HPLC indices	HbE/E (n=09)	HbA/E (n=26)	HbE/β (n=19)	HbE/HbS (n=01)
HBA0 (%)				
Mean±SD	3.578±1.265	64.146±4.609	11.742±5.357	1.2
Range	1.4 -8.3	54.2 – 73.6	2.2 – 20.8	
HBA2/E (%)				
Mean±SD	83.967±5.381	29.126±5.526	52.594±10.394	24.6
Range	79.5 – 92.4	21.7 – 43.0	36.3 – 73.1	
HBF (%)				
Mean±SD	3.223±0.957	0.657±0.391	25.184±10.123	8.4
Range	0.6 – 4.8	0.3 – 2.4	10.2 – 45.5	
Any other peak (%)	-	-	-	**HbS**-66.3

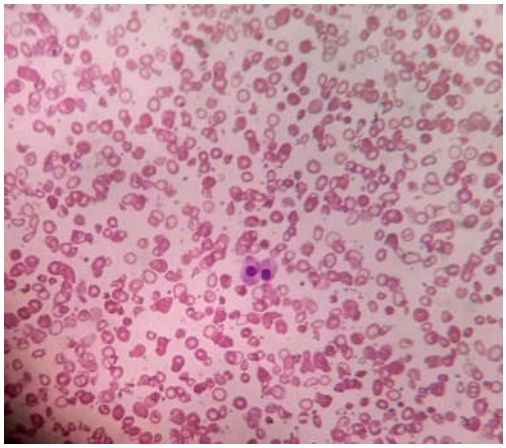

Figure 2 RBC morphology of a patient with HbE heterozygous with microcytosis, hypochromia, mild aniso-poikilocytosis, few target cells and nucleated RBC (Lieshman 40x)

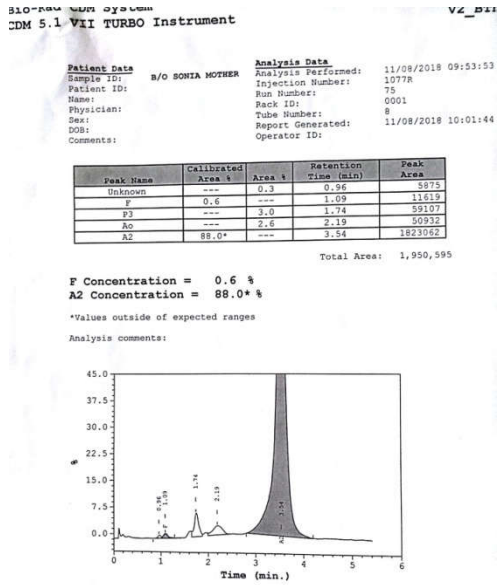

Figure 3 Chromatogram of HbE homozygous showing markedely elevated HbA2/E - 88.0%

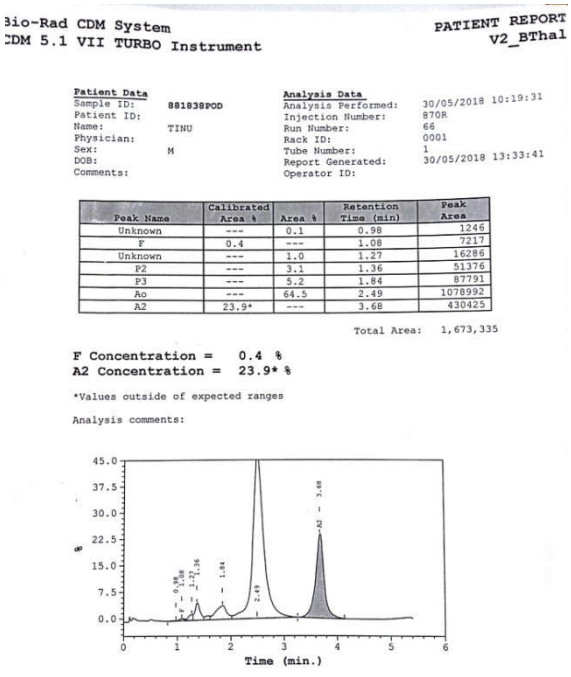

Figure 4 Chromatogram of HbE heterozygous showing elevated HbA2/E - 23.9%

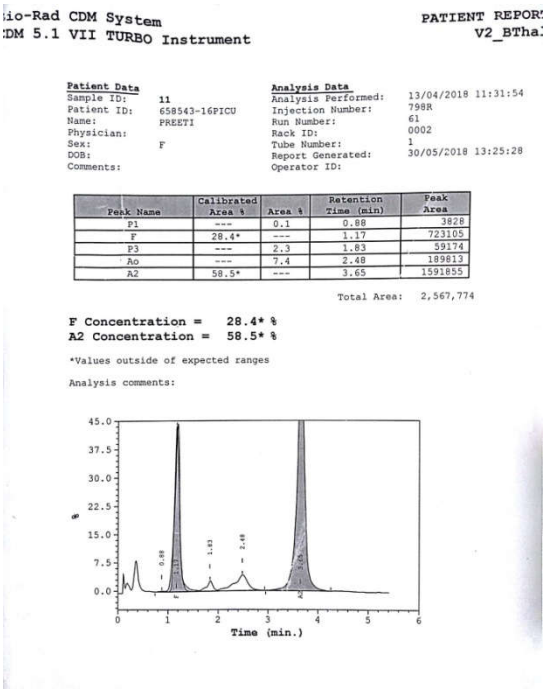

Figure 5 Chromatogram of HbE/β- thalassaemia showing elevated HbA2/E - 58.5% and elevated Hb F - 28.4%

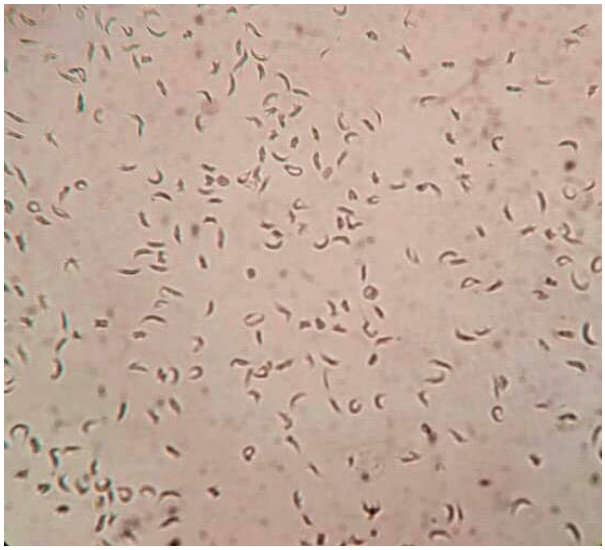

Figure 6 Positive sickling test in patient of HbE/HbS (2% sodium metabisulfite preparation, 40x)

DISCUSSION

HbE was first discovered by Itano, Bergren and Sturgeon in 1954 in a person of Guatemalan origin with Spanish and Hindu ancestry and was the 4th abnormal hemoglobin variant discovered after HbS, HbC and HbD2.[5] Prevalence is very high in South-east Asia, especially in Cambodia, Laos and Thailand. The borders of these countries are considered the "Hb E Triangle". This variation began as a response to the selective pressure of malaria.[1] In India, the highest incidence of HbE trait has been reported from West Bengal (3.9%), and it is also prevalent in Assam and Tripura states. HbE/beta-thalassaemia is the commonest of the thalassaemia syndrome in Myanmar.[6] Our study was conducted using HPLC, in a tertiary care hospital in Haryana, 4,088 cases were screened for eight years, and only 55 cases (1.34%) were detected with hemoglobin E disorder, it may reflect only the tip of an iceberg. But this type of study can definitely help to increase awareness among both health care providers and general population.

There are various diagnostic modalities to assess this haemoglobinopathy including evaluation of Hb level, RBC counts, various RBC parameters, HPLC, capillary electrophoresis and DNA analysis. HPLC is an excellent, cost-effective, rapid diagnostic tool, for detection of asymptomatic carriers by accurate quantificatication of HbA2 and HbF levels, because when combined with other variants they may give rise to severe disease.

According to a study by Vichinsky, individuals with HbE disease (homozygous) are usually completely asymptomatic, with very mild anaemia, microcytosis and targeting.[1]In our study, 05 out of 09 cases (55.56%) had signs and symptoms of anaemia like generalised weakness and pallor. Haematological features were slight decrease in haemoglobin level, low to normal RBC counts, normal to increased reticulocyte count, reduced PCV, MCV and MCH. MCHC was normal, RDW was increased. The WBC and platelet counts were normal for all patients. Peripheral blood film showed, microcytic hypochromic picture with target cells and mild aniso-poikilocytosis. HPLC revealed normal to slightly increased HbF (< 5%) and markedly increased HbA2/E levels (>79%).

Individuals with HbE trait (heterozygous) are usually asymptomatic, 14 out of 26 cases (53.84%) in our study were asymptomatic. 10 out of 26 cases (38.46%) presented with variable signs and symptoms of anaemia. Haematological features were normal to slight decrease in haemoglobin level, RBC, WBC, platelet counts, PCV, MCV, MCH and MCHC. Reticulocyte counts were consistently normal while RDW was normal to increased. Peripheral blood film showed, microcytic hypochromic picture with target cells and moderate aniso-poikilocytosis. Findings in the present study were comparable with the earlier studies done by Aggarwal*et al*[7] and Bhargava *et al*.[8] HPLC revealed normal HbF (< 2.5%) and increased HbA2/E levels (range 21-43%).

Lachant described HbE homozygous to have on an average of 1g/dl less haemoglobin concentration than that seen in heterozygotes; and MCV averages about 5-10 fl less than HbE trait.[9] Similar findings were observed in our study. We also noted the presence of HbA0 in all 09 cases of HbE disease, ranging from an insignificant 1.4 to as much as 8.3%. Normally, HbA0 is not expected in untransfused homozygous HbE disorders, it might have resulted from adducts of HbE falling in the Hb A peak. Adducts of sickle Hb causing raised Hb A2 has been reported in literature.[10]

HbE/β-thalassaemia is the most severe form of HbE disease. Clinical presentation is usually variable, from completely asymptomatic to transfusion dependence at an early age. Manifestations generally include refractory anemia, unexplained jaundice, splenomegaly, delayed puberty, growth retardation and over expansion of bone marrow cavity leading to facial deformity and defective tooth implantation. In addition these patients may have complications like iron overload, hypercoagulable states particularly (post-splenectomy), and cardiopulmonary diseases.[11]

In our study, 12 out 19 cases (63.1%) of HbE/β- thalassaemia presented with variable signs and symptoms of anaemia, the

rest were diagnosed incidentally while they were investigated for some other illness. Majority of the haematological parameters (Hb, RBC, PCV, MCV, MCH and MCHC) were reduced to a variable degree. Reticulocyte counts and RDW were markedly increased. WBC and platelet counts were normal to reduced. Peripheral blood film showed microcytic hypochromic picture, marked aniso-poikilocytosis, target cells, polychromasia, basophilic stipling and variable number of nucleated red cells. Similar findings were observed by Aggarwal*et al*[7] and Patne *et al*.[3] HPLC revealed elevated HbF (range 10-45.5%) and HbA2/E levels (range 36-73%). We could not differentiate HbE/β^0 from HbE/β^+ as samples were not analysed for β- thalassaemia mutations.

We observed a single case of HbE/HbS (double heterozygote). The patient was 03 years old, who presented with anaemia, mild splenomegaly and history of repeated upper respiratory tract infections. Peripheral blood film showed microcytic hypochromic picture with mild aniso-poikilocytosis, sickle cells were not seen but sickling test was positive (Figure 6). On HPLC, he was having increased HbS (66.3%), HbA2/E (24.6%) and HbF (8.4%). On family screening, his father turned out to be sickle cell trait and mother as HbE trait. Inter caste marriages has been reported into these compound heterozygous condition.[12]

A negative correlation has been found between levels of HbA2/E peak values and RBC indices including MCV and MCH, indicating that microcytic hypochromic picture is more common with elevated HbA2/E levels. A positive correlation was found between HbA2E levels and RDW-CV, which helps us in differentiating HbE/β- thalassaemiafrom β-thalassemia in which RDW is usually within normal range. Similar correlation was found in studies by Kishore *et al*,[13] Sharma *et al*[14] and Pani *et al*.[15]Iron deficiency, silent mutations and concomitant inheritance of alpha thalassemia also lowers the percentage of HbA2/E levels. But in our study both iron profile and genetic analysis were not done.

A negative correlation was found between HbF peak values and MCV, indicating marked microcytosis with elevated HbF levels, especially in HbE-β thalassaemia group, while Kishore *et al*[11] observed negative correlation between HbF level and Hb, RBC counts and MCV and Pani *et al* observed a positive correlation between HbF and MCV.

Strengths and Limitations

This is the first kind of study from this region of North-India, describing various HbE variants. Detailed clinico-haematological parameters were discussed. However, sample size was small (a total of 4,088 cases were screened from last eight years, out of which only 55 cases were detected with hemoglobin E disorder). The study was also limited by lack of iron studies and molecular analysis.

CONCLUSION

HbE disorders are a heterogeneous group of diseases that are rapidly increasing worldwide. Clinicians and pathologists should consider this in the differential diagnosis of microcytic hypochromic anaemia for early diagnosis and proper management. HPLC can successfully identify most of the haemoglobin E variants prevalent in this country. Genetic studies are indicated to confirm borderline cases and to detect silent carriers.

Compliance with Ethical Standards

Funding: No financial disclosure.

Conflict of interest: No conflict of interest exists.

Ethical approval: All procedures performed in this study involving human participants were in accordance with the ethical standards of the institution.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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