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HPLC in Characterization of Hemoglobinopathies: A single center based hematological study in a newly developed Government medical college in west Bengal, India.

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Abstract: *Detailed clinical history, complete hematologic evaluation along with newer ancillary techniques including High performance liquid chromatography (HPLC), when used systematically can achieve laboratory diagnosis of thalassemia syndromes and hemoglobinopathies. With this background knowledge we framed the present scientific study to assess prevalence of Hemoglobinopathies by HPLC among antenatal cases and their family members and transfusion requiring children and adults, according to clinical findings in a newly developing government run medical college in Uttar Dinajpur district of West Bengal. The study was carried out from May,2018 to April,2019 i,e, for twelve month period. HbA2 value of 3.3% to 3.8% was considered as borderline and a cut-off of over 3.9% was taken for diagnosis of β-thalassemia trait. we have found that a total 15.8% cases were within the age group of 2 yrs, who presented for HPLC screening. Also seen, 24.29 % cases were within the age group of >2-15 yrs and 41.12 % cases were within the age group of >15-25 yrs.18.69 % cases were within the age group of >25-60 years. Out of 107 cases studied in the first 12 month of development of a new medical college in a rural district of West Bengal,0.93% cases came out to be B thal major,4.67% cases came out to be B thal trait, Thal E homozygous/EB thal cases constituted 37.38% of total cases. E thal trait constituted 8.41% of all cases and 43.92% cases came out with normal HPLC study. The present findings show HPLC as an excellent, powerful diagnostic tool for the direct identification of haemoglobin variants with a high degree of precision in the quantification of normal and abnormal haemoglobin fractions.*

Key words : *HPLC, Hemoglobinopathies, Uttar Dinajpur HPLC in Characterization of Hemoglobinopathies: A single center based hematological study in a newly developed Government medical college in west Bengal, India.*

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I. Introduction

Thalassemia is an autosomal recessive disease. As per WHO estimate, 4.5% of world populations are carriers of hemoglobinopathies[1] .The largest concentration of Thalassemia patients are present in Sri Lanka, Bangladesh, North West India, Pakistan, Middle East Countries, Greece, and Italy[2]. In South East Asia, there are 40 million carriers of thalassemia gene, 50% of which are in India alone[3]. The mean prevalence of the carrier status in India is 3.3% (ranging from 1 to 17% in various communities). If a line is drawn between Mumbai and Kolkata on the Map of India, the region above the line has an incidence of 3 to 17%, whereas region below the line has incidence of less than 3%[2].It is estimated that every year approximately 1, 00, 000 children with thalassemia major are born all over the world[4]. With the birth rate of 22.8/1000 in India, it is estimated that there are about 9000 , to 10 000 cases being added every year[2]. Inherited abnormalities of hemoglobin (Hb) include a myriad of disorders ranging from thalassemia syndromes to structurally abnormal Hb variants/hemoglobinopathies. Detailed clinical history, complete hematologic evaluation along with newer ancillary techniques including High performance liquid chromatography (HPLC), when used systematically can achieve laboratory diagnosis of thalassemia syndromes and hemoglobinopathies[5]. Family studies can be of immense importance in diagnosing certain problematic cases[6].

Way back in 1975, the expert group on "Abnormal hemoglobins and thalassemias" of the International Committee for Standardization in Hematology recommended 2 sets of investigations for diagnosis of these disorders. The initial investigations included a complete blood count, electrophoresis of the hemoglobin at alkaline pH, tests for sickling and solubility and quantization of HbA2 and Hb F. If an abnormal hemoglobin was detected on electrophoresis or suspected, further testing included electrophoresis of Hb at acid pH, electrophoresis of globin chains at acid and alkaline pH, isoelectric focusing (IEF), heat and isopropanol stability tests for unstable hemoglobins and tests for identifying hemoglobins with altered oxygen affinity[7].Thalassemia and other hemoglobinopathies are genetic disorders of hemoglobin, which can be prevented by population screening and offering genetic counseling. In India it is estimated that there are over 25 million carriers of this disease[8]. Beta-thalassemia (β-thalassemia), sickle cell anemia, E-beta thalassemia and

hemoglobin D Punjab are the common hemoglobinopathies in the world including India. About 4.5% of world population is affected by hemoglobinopathies[9].Still there is no sufficient data from rural Bengal specially from the district of Uttar Dinajpur, West Bengal.

Carriers of b-thalassemia have a near normal Hb level although they may be slightly anemic with hypochromic and microcytic red cells. In most population screening programmes an MCV of < 80fl and MCH of < 27 pg are generally used as cut off points for further screening. The hallmark of diagnosis for classical b-thalassemia carriers is a raised HbA2 varying between 3.5% and 4% depending on the method of estimation used. It has been shown in a recent study that while the MCH and HbA2 levels in b-thalassemia carriers are quite stable in samples stored at 4^{o}C for up to 3 wk, the MCV levels showed a progressive rise from 62.6 fl on day 1 to 84.0 fl on day 21, suggesting that MCV is not a good parameter if samples cannot be processed within a day[10] .

With this background knowledge we framed the present scientific study to assess prevalence of Hemoglobinopathies by HPLC among antenatal cases and their family members and transfusion requiring children and adults, according to clinical findings in a newly developing government run medical college in Uttar Dinajpur district of West Bengal.

II. Aim and Objective

The aim of the present study is to evaluate the role of cation exchange HPLC (CE-HPLC) in the diagnosis of Thalassemia syndromes/hemoglobinopathies and to correlate Hb profile in such cases with clinico-hematological features in a newly developing government run medical college of northern part of West Bengal.

III. Materials and methods

The study was carried out in the newly developed Govt.medical college from May,2018 to April,2019 i,e,for twelve month period. Hemoglobinopathy work-up were analyzed in persons including mainly referred from outpatient antenatal cases and their family members and transfusion requiring children and adults, and other patients department according to clinical findings. No absolute exclusion criteria were used but for patients requiring blood transfusions, sampling was deferred for at least 2 week after transfusion. Written consent was taken from all patients for using their sample for diagnostic purpose.

About 2 ml of blood sample was collected in EDTA vacutainers and was analyzed in automated cell counter (Sysmex XT-2000) for complete blood counts. Samples were stored at 4–8 deg C and were analyzed in batches within 1 week of collection.

Today CE – HPLC has become the method of choice for quantitation of Hb A2, Hb F and other Hb subtypes. One of the most versatile and widely used machine we used is the Variant Hemoglobin Testing System (BioRad Laboratories).

The Variant operates on the principle of HPLC and the column comprises of a small 3.0 x 0.46 cm cation exchange cartridge. The beta-thalassemia short programme is used for screening for b-thalassemia. Only 5 ml of an EDTA blood sample taken in 1.0 ml of the lysis buffer is required. Up to 80 samples can be simultaneously loaded in the auto sampling chamber and each sample takes 6 ½ minutes for analysis.

The samples are injected into the analysis stream and separated by the cation exchange cartridge using a phosphate ion gradient generated by mixing 2 buffers of different ionic strengths to elute the different hemoglobins. A dual wavelength filter photometer monitors the eluent from the cartridge as it passes through the photometer cell. Changes in optical density at 415 nm are measured. A secondary filter at 690 nm corrects the effects caused by mixing buffers of different ionic strengths. The data is processed and the report giving the chromatogram of time *vs* absorbance where the different peaks are identified in defined windows and their retention time, relative percentage and area are printed out. This automated HPLC provides a more efficient and accurate assessment of HbA2 and Hb F compared to manual methods.[11,12,13]

HbA2 value of 3.3% to 3.8% was considered as borderline and a cut-off of over 3.9% was taken for diagnosis of β-thalassemia trait[14,15,16].

IV. Results

Results of the present study is simply tabulated below.

Table 1 : Age wise distribution of cases presented for HPLC

Age(yrs)	Number of cases	% of total cases
Upto 2 yrs	17	15.88 %
>2-15 yrs	26	24.29 %
>15-25 yrs	44	41.12 %
>25-60 yrs	20	18.69 %

Table 2 : Sex wise distribution of cases presented for HPLC

Variable	Number of cases	% of total cases
Female	65	60.74 %
Male	42	39.25 %

Table 3 : Age & sex distribution of HPLC cases

Age	Sex wise distribution(Female/Male)	
Up to 2 yrs	10	07
>2-15 yrs	12	14
>15-25 yrs	34	10
>25-60 yrs	09	11
Total	65	42

Table 4 : Religion wise distribution of cases

Variable	Number of cases	% of cases
Muslim	42	39.25%
Hindu	65	60.74%

Table 5 :HPLC report wise distribution of cases

Diagnosis	No. of cases	% of cases	Hindu/Muslim distribution	
B thal major	01	.93 %	00	01
B thal trait	05	4.67 %	02	03
E homozygous/EB thal	40	37.38 %	22	20
E thal trait	09	8.41 %	05	04
Others	05	4.67 %	02	03
Normal HPLC study	47	43.92 %	34	13

Table 6 : Relevant laboratory parameters(average) in relation to normal and abnormal haemoglobin variants

HPLC diagnosis	No. of cases	Hb (gm/dl)	MCV (fl)	MCH (pg)	MCHC (%)	RBC (x106/µl)	HbA (%)	HbF (%)	HbA2 (%)
Normal	47	11.2	83.3	25.1	30.6	3.85	88	0.2	2.5
B thal trait	05	9.1	68.6	20.5	28.3	4.01	83.1	1.0	5.5
B thal major	01	5.4	74.9	23.3	31.1	2.41	39.2	52.2	3.7
E homozygous/EB thal	40	6.2	63.9	19.3	30.1	3.2	18.1	21.7	52.3
E thal trait	09	10.1	82.3	26.2	31.6	4.1	61.9	1.6	-

Table 7: Positive family history and transfusion history among the study cases

History	No. of cases	%
Positive family history	08	7.47
Positive blood transfusion history	29	27.10

V. Discussion

HPLC has been shown to be a sensitive, specific, and reproducible alternative to electrophoresis. With automation and quantitative power, it appears to be a sensitive and accurate technique for direct identification and quantification of normal and abnormal haemoglobin fractions[17,18] .

Different reports have addressed the precision of the retention times obtained with stored normal and abnormal samples. There are a few studies from India which evaluated and emphasized the role of HPLC for diagnosis of Thalassemia and various haemoglobinopathies[19,20] .

Hemoglobinopathies are a major health problem all over the world including India and Southeast Asian region[21]. In our study, in a rural medical college in a district town of north Bengal, which is very adjacent to Bangladesh also, we have found that a total 15.8% cases were within the age group of 2 yrs, who presented for HPLC screening. Also seen,24.29 % cases were within the age group of >2-15 yrs and 41.12 % cases were within the age group of >15-25 yrs.18.69 % cases were within the age group of >25-60 years.(Table1)

Table 2 shows that out of 107 cases underwent for HPLC study in first 12 month of a newly developed medical college, 65 were female and 42 male population.

Table 3 explains the age and sex wise distribution of HPLC cases. In the age group of 2 yrs,10 patients are female and 7 are male. Out of 26 patients in the age group of >2-15 yrs,12 are female and 14 are male. In the age group of >15-25 yrs, female and male numbers are 34 and 10 respectively.11male and 9 female constituted the age group of >25-60 years.

Table 4 shows local religion wise distribution of cases.39.2% cases are Muslim (42) and 60.74% are Hindu(65).

Due to socio-cultural practices, marriages in India are usually among individuals of the same caste or ethnic group and this makes it important to know the prevalence of β-thalassemia and also HbE in different ethnic groups[22].

Present study found relatively lower prevalence of hemoglobinopathies among Muslims and tribal's, in contrast to other studies. Other states noted higher prevalence of hemoglobinopathies among them[23, 24]. Assam recorded a higher morbidity load of hemoglobinopathies, due to incorporating various linguistic and ethnically diverse population and migrants[24]. Clustering of hemoglobinopathies was found in small pockets, attributable to small population size, caste endogamy, consanguinity, virtual lack of medical facilities and natural barriers like rivers, forests, etc[23.]. Migration of people and marriages between communities has led to its wide prevalence.

Table 5 explains the distribution of hemoglobinopathies among the cases studied. Out of 107 cases studied in the first 12 month of development of a new medical college in a rural district of West Bengal,0.93% cases came out to be B thal major(1 out of 107),4.67% cases came out to be B thal trait(5 out of 107 cases),Thal E homozygous/EB thal cases constituted 37.38% of total cases(40 out of 107 cases).E thal trait constituted 8.41% of all cases(9 out of 107 cases).43.92% cases came out with normal HPLC study.

The largest screening programme for Thalassemia using the HPLC system in the population had been carried out in Gujarat, India by the Indian Red Cross Society in Ahmedabad and other cities. From 2004 to 2010, they screened 370,117 subjects for carrier status, among whom there were 173,112 students, 45,000 youths and 8,377 pregnant women. Carrier rate has varied from 4.3% to 5.0%[25].

In different studies, the prevalence of β-thalassemia trait was around 6.61% making this the major hemoglobinopathy in West Bengal, India. The high prevalence rate of β-thalassemia trait is found in more than 60 countries with carrier population up to 150 million[26].

The distribution of the β-thalassemia gene is not uniform in Indian subcontinent[27]. The prevalence of β-thalassemia trait varies from 1% to 17% in different populations of India as revealed by many studies from India[28,29,30]. The prevalence of β-thalassemia trait was 5.5% in northern India, 2.7% in western India and the overall frequency was 4.05%[31]. Another tertiary center study from western India reported incidence of β-thalassemia trait was 11.55 %(28).A study on college, university students and pregnant women from different cities of eastern India (Kolkata, West Bengal), north east India (Dibrugarh, Assam) and southern India (Bangalore, Karnataka) showed prevalence of β-thalassemia carriers was 3.64%, 1.48% and 2.16%, respectively[32] . Another study with 1726 cases on hemoglobinopathies in tribal population of Eastern and Northeastern India, including 463 cases from West Bengal showed the prevalence of β-thalassemia carrier of 5.18%[33].

Worldwide, hemoglobin E thalassemia is one of the most important varieties of hemoglobinopathy[34].There were 1403 cases of HbE trait leading to a prevalence of 2.78% in a study population[34]. Since its classic description by Chernoff et al[35], it has been noted to be an important public health problem in the Indian subcontinent and Southeast Asia. In a recently published report of multicenter study conducted by the Indian Council of Medical Research including Kolkata in West Bengal in the east, Dibrugarh in Assam in the north east amongst six cities included in the study, done in tertiary care centers covering mostly city based population showed the prevalence of HbE trait as 3.92% in Kolkata and 23.90% in Dibrugarh[32] whereas the prevalence of HbE trait (8.41%) found in the present study conducted in rural areas was different than the earlier study[34]. The difference in prevalence of HbE trait between the studies reflects the population heterogeneity in the studies.

Recent published study from Kolkata showed only 39 cases (0.276%) of sickle cell trait amongst 14, 145 cases included in the study[36]. Earlier studies from West Bengal by Mohanty et al[37] and Dolai et al[38] showed a prevalence of HbD-Punjab trait of 0.2% and 0.37%, respectively. But in our small study we did not find any of such cases. This may again be explained by small number of study population and population heterogeneity(Table 5)

There are few studies on HPFH in India. Only a few cases have been reported from western India and Kolkata[39]. Rao et al.,[40] in a study of 800 samples, from north India reported one case each of Hb Lepore trait and HPFH trait. In a recent study from Kolkata, West Bengal, there was only one case of Hb Lepore trait and no HPFH reported. In our study also no case of HPFH and Hb Lapore was reported(Table 5)

The first case of HbE disease in India was reported by Chatterjee et al[41].Because of the extremely high frequency of HbE trait within the Indian subcontinent, it is very common for individuals in this region to inherit both hemoglobin E and β-thalassemia. Data collected over recent years indicate that HbE-β-thalassemia is causing an increasingly severe public health problem throughout the Indian subcontinent and parts of Southeast Asia[42].

Among ethnic groups in which prevalence of HbE and β-thalassemia are known, there is a vast discrepancy between the reported number of cases of HbE/β-thalassemia and the number predicted by Hardy-Weinberg law[43].

HbE preponderance among hilly tribes and in Assam and Tripura regions hint a lineage simulation, acculturation and inter-caste marriages between Rajbanshis and these states. Earlier studies in blocks of Darjeeling district also found low prevalence of hemoglobinopathies among Nepali population. Being mostly converts from Rajbanshis, Muslims exhibit more preponderance for the disease due to intermixing and narrowing down of differences with Rajbanshis genetically[44].

Table 6 depicts Relevant laboratory parameters(average) in relation to normal and abnormal haemoglobin variants in details. Average of Hb%,RBC indices and % of different types of Hemoglobin were tabulated against different cases of hemoglobinopathies.

Present study noted that among subjects diagnosed anaemic, majority were girls (65.6%), a finding shared by other studies[45]. Adolescent girls need enlightenment on importance of scope of study. Presuming anemia in rural terrain as nutritional, a possibility exists that HbA2 values were interpreted as false low and hemoglobinopathy exists more[45].intergenerational life cycle approach, importance of balanced diet and awareness generation on pertinent issues. Differentiating between types of anemia was beyond

Table 7 depicted Positive family history and transfusion history among the study cases.7.47% cases showed positive family history and 27.1% cases showed positive history of blood transfusion .Hemoglobinopathies were also significantly associated with positive family history and history of consanguineous marriages, a finding similar to a pilot study conducted among antenatal women[46].

VI. Conclusion

The present findings show HPLC as an excellent, powerful diagnostic tool for the direct identification of haemoglobin variants with a high degree of precision in the quantification of normal and abnormal haemoglobin fractions.

CE-HPLC (beta-thal short program) may be a valuable tool in rapid diagnosis of a varied spectrum of haemoglobinopathies. Percentage of variant haemoglobin can provide important clues in differentiating variant haemoglobins eluting in the same window.

Iron deficiency anaemia causes significant lowering of HbA2 values. HbA2 in the borderline range needs further evaluation especially for silent mutations, and co-existing nutritional deficiency. Current guidelines require that abnormal variant Hbs should be confirmed by another independent technique. This is prudent practice, and in most cases (like a sickling test for S-window peaks, electrophoresis for others) cheap and easy. It is especially important while screening pregnant women as the diagnosis has implications for prenatal testing.

It is also suggested that more detailed information on frequency and economic data is required to provide evidence for the health burden posed by thalassemias in the developing world. The programme of prevention through carrier screening and prenatal diagnosis should receive the highest priority in the future, in order to reduce drastically the birth of affected children.

Diseases which are known to run in families and have high-risk of recurrence become a social stigma when nothing or very little is known about them. Illiterate populations are ignorant about the medical, social and financial burden of the disease. This further compounds the problem.

The present study conducted in a newly developed Government medical college catering rural population in West Bengal arguably reflects the different types of hemoglobinopathies prevalent in North Dinajpur area of the country.

Limitation of study : The primary limitation in this study was that the Hb variants were not fully confirmed by genetic sequencing. A further limitation exists in the form of sample bias. This was not a community based study. Patients who came to hospitals and their relatives are included in this study only. Furthermore this study was taken in hand in a newly developed Government run medical college. Still with all limitations this study, has started, providing a comprehensive database on the spectrum of hemoglobinopathies in North Dinajpur district of West Bengal,India.

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