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Original Article

Pattern of Hemoglobinopathies Among Non-Anemic Individuals from a Rural Tertiary Care Institution in West Bengal

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Abstract

Introduction: Inherited disorders of hemoglobin synthesis form the most common single gene defect in man. Genetic carrier screening plays the most important role in prevention of birth of baby with these entities. A normal hemoglobin concentration does not necessarily signify a normal hemoglobin structure. Hence it is important to screen for abnormal hemoglobin variants in non-anemic individuals. This study aims to find out the occurrence of hemoglobinopathies and ascertain the need for mass screening of hemoglobinopathies among non-anemic population of the northern districts of West Bengal, like, Darjeeling, Jalpaiguri, Kalimpong, Cooch Behar, Dakshin Dinajpur and Uttar Dinajpur.

Materials and methods: The study was carried out in the Department of Physiology in collaboration with the Thalassemia Control Unit (NBTCU) Department of Pathology, North Bengal Medical College (NBMCH). The study population comprised of all volunteers attending NBTCU and persons attending the camps conducted by NBTCU over a period of one year. Hemoglobin variants was analyzed by using High Performance Liquid Chromatography (HPLC) with beta-Thal short program (Bio-rad). Volunteers with normal hemoglobin level were included in the study. Persons with history of blood transfusion or on any medications were excluded from the study.

Results: 1101 volunteers were screened from June '2011 to May' 2012. Of these, 501 volunteers were found to be non-anemic. 61 volunteers (12.18%) were found to be having some form of an abnormal hemoglobin pattern, the commonest being HbE trait 8.58% (43/501) followed by ß thalassemia trait 1.99% (10/501) and HbE Disease 0.19% (1/501).

Conclusion: The present study has identified a comprehensive pattern of occurrence of hemoglobinopathies among non-anemic population of this region and emphasizes the importance of mass screening for hemoglobinopathies in non anemic individuals.

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Introduction

Hemoglobinopathies refers collectively to the clinical picture resulting from disorder of hemoglobin synthesis and may be grouped into 3 main categories: those owing to structural variants of hemoglobin, those owing to failure to synthesize one or more of the globin chains of hemoglobin at a normal rate as in Thalassemias and those due to Hereditary Persistence of Fetal Hemoglobin (HPFH) (1, 2).

Inherited hemoglobin disorders are the most common single gene defect in man (2). Thalassemia and other structural hemoglobinopathies are the major genetic disorders prevalent world over including India (1, 3). According to recent data collected through the Hereditary Disease Program of the World Health Organization (WHO), the carriers of hemoglobin disorders in the world are estimated to be 269 million (4). The cumulative gene frequency of hemoglobinopathies in India is 4.2% (2).

In common practice, persons with normal hemoglobin concentration are usually not considered to be tested for Hemoglobin variants. However, a large number of hemoglobinopathies especially heterozygotes for HbE hemogbinopathy and ß thalassemia minor subjects are clinically asymptomatic with a normal or only mildly reduced hemoglobin level (5). As a consequence, there is a high possibility of birth of one double-heterozygous state if partners are not adequately screened. Hence it is important to detect the occurrence of hemoglobin variants even with a normal hemoglobin concentration.

Hemoglobinopathies are among the common genetic disorders that are seen to occur all over the world. In India, prevalence of thalassemia varies between 2.3% to 9.2% with an average of 5.5% (6). HbE is the second most prevalent hemoglobinopathy after HbS (6) and having highest prevalence in South-East Asia (7). In India, it is prevalent in North-Eastern states (8) including northern districts of West Bengal (9).

Screening of individuals with normal hemoglobin level is useful for detection of asymptomatic hemoglobinopathies. As North Bengal Thalassaemia Control Unit (NBTCU) caters a large area of northern districts of West Bengal, detection of hemoglobin variant in voluntary screening reflects the occurrence of hemoglobin variant pattern in the general population of this region. An extensive literature search revealed that no similar study has been conducted from this region of India.

The present study was undertaken to find out the type and magnitude of occurrence of hemoglobin variants in non-anemic healthy volunteers in this zone.

Material and Methods

This cross-sectional, observational study was carried out in the department of Physiology in collaboration with the Thalassemia Control Unit of North Bengal Medical College (NBMCH). The study population comprised of all volunteers from the various districts of Darjeeling, Jalpaiguri, Kalimpong, Cooch Behar, Uttar and Dakshin Dinajpur attending NBTCU and persons attending the camps which were conducted in schools, colleges and similar institutes in Siliguri and surrounding regions.

The cut off value of hemoglobin for inclusion in our study, with respect to age and sex of the volunteers, is depicted in Table I (10, 11).

However, persons having history of blood transfusion within last two months, or receiving chemotherapy or receiving therapy for deficiency anemia, and whose peripheral blood smear showed any discrepancy between the findings of the automated cell counter and the peripheral blood film examination or the presence of an abnormal cell of any cell lineage, were excluded from the study. A total of 1101

TABLE I: Cut off value of hemoglobin with respect to age and sex.

Sex	*Age*	*Hb gm/dl*
Male	>14 yrs	≥13 gm/dl
Non Pregnant Female	>14 yrs	≥12 gm/dl
Pregnant Female	>14 yrs	≥11 gm/dl
Children	6–14 yrs	≥12 gm/dl

volunteers were screened over a period of one year from June, 2011 to May, 2012. Of these, a total of 501 non-anemic healthy volunteers, with none of the exclusion criteria as mentioned above, were included in the study group.

Permission to carry out investigation was obtained from the ethical committee. Detailed clinical and family history pertaining to hemoglobin disorders was obtained after having consent from the volunteers.

5 ml of venous blood from each volunteer was aseptically drawn either from the median cubital vein or from a prominent vein over the dorsal aspect of the hand. The blood was then immediately transferred to pre labelled vials, containing di-potassium salt of Ethylene diamine tetra- acetic acid (EDTA) as the anticoagulant and mixed thoroughly. In case of samples collected in camps, the vials were neatly packed in sample carriers and transported to NBTCU maintaining the cold chain. Blood camps contributed to 71.9% (792/1101) of the total samples collected. Of these 16 samples were found to be hemolysed at the centre and these were excluded from the study.

The blood sample was used for making the peripheral blood smear (done by Leishman stain), for obtaining the hemoglobin levels and other RBC parameters on the automated cell counter (SYSMEX XS-800*i*, Japan). The RBC parameters assessed included the RBC count, the Hematocrit (Hct), MCV, MCH, MCHC and RDW. The automated cell counter was calibrated using calibrator procured from SYSMEX Japan and daily controls were run using the control provided by the company itself. The same blood was later used for the screening of hemoglobinopathies using High Performance Liquid Chromatography (HPLC) by using beta-thalassemia short program Bio-Rad, (USA) where 'retention time' is used for separation of different hemoglobins. This technique is considered as one of the initial investigative procedures for hemoglobin variant analysis in laboratories analyzing large number of samples (1). The tests were done taking proper quality control measures including calibrators and controls. The HPLC machine works on the principle of cation exchange column chromatography (CE-HPLC). The instrument typically consists of a solvent reservoir, a pump, an injector, a chromatographic column, a data recorder, and a microprocessor. The "beta thal short program" is designed to separate and determine in 5 to 6 minutes the area percentages for HbA_2 and HbF and to provide qualitative determinations of hemoglobin variants. Only 5 µl of an EDTA blood sample taken in 1.0 ml of the lysis buffer (sodium phosphate) is required. Each sample takes 6½ minutes for analysis. The pump drives the sample (the mobile phase) through the injector-column-detector assembly and the data is processed as it passes through the microprocessor. The report is in the form of a 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 (12, 13). HbA_2 and HbE both have the same retention time of 3.30 to 3.90 minutes. The two are then interpreted based on their relative percentage. HbA_2 percentage in normal individuals ranges between 1.8 to 3.4%. It lies between 4.0 to 9.0% in persons with β thalassemia trait, 22 to 40% in persons with HbE trait and 70 to 90% in persons with HbE disease. A known calibrator for HbA_2 and HbF (procured from Bio-Rad USA) was run simultaneously with a response factor of 1.00±0.04. Moreover, controls (procured from Bio-Rad USA) were also run with each batch as the actual retention times are affected by the batch of buffer and column, the age of the column and the laboratory temperature (1).

Hemoglobin electrophoresisis is another effective method of separating abnormal hemoglobins. Hemoglobin being a negatively charged protein, in an electric field, migrates towards the anode. Most structural variants of hemoglobin separate due to surface charge differences, thus allowing identification of abnormal forms. Here the electrophoresis tanks are filled with TEB (Tris/EDTA/ Borate) buffer (pH 8.5) and the wicks are soaked and positioned in the tanks. Cellulose acetate membrane are soaked in TEB buffer for 5 minutes and blotted with absorbent paper (CAM Strip). Hemolysate is prepared from the sample and with the help of an applicator is applied approximately 2 cm from one end of the strip. CAM strip is then placed upside down in the tank so that the wicks are in contact with the buffer and the strip, such

that the application line is towards the cathode. 250 -350V power (with 1.5 mA current) is applied for 20 minutes or till adequate separation occurs. The strip is then removed and stained using Ponceau S stain which helps delineate the different structural hemoglobin variants. Electrophoresis using citrate agar at an acidic pH of 6.0 helps to distinguish between HbE, HbC and HbO which move similarly at an alkaline pH on cellulose acetate (1).

Hemoglobin electrophoresis, in both acidic and alkaline pH, and some tests depending upon physicochemical properties of variant hemoglobins like Alkali denaturation method for fetal hemoglobin and Sickling test for HbS were done as and when required.

Statistical Analysis was performed after collection of data which was compiled, charted in Microsoft excel and analyzed using the Mean, Median, Mode, Standard deviation.

Results

The age of volunteers ranged between 6 to 46 years (mean age of 20.54 years). The age wise details of volunteers has been shown in Table II. The total range, mean and standard deviation of hemoglobin with reference to male and female has been depicted in Table III.

TABLE II: Age wise details of volunteers.

Age in years	*Number of volunteers*
< 10	101
10 – < 20	267
20 – < 30	274
30 < 40	261
< 40	198

TABLE III: Total range, mean and standard deviation of hemoglobin with reference to male and female.

Sex	*Number*	*Range of Hb*	*Mean*	*Std Deviation*
Male	327	13 gm/dl to 17.3 gm/dl	13.8 gm/dl	1.008
Female	174	12 gm/dl to 17.0 gm/dl	13.6 gm/dl	1.046
Combined	501	12 gm/dl to 17.3 gm/dl	13.7 gm/dl	1.176

Of the 501 non-anemic volunteers a total of 61 volunteers (12.18%) were found to be having some form of an abnormal hemoglobin pattern (Figs. 1-4). Major abnormal hemoglobin patterns identified were HbE trait 8.58% (43/501), HbE Disease 0.19% (1/501) and ß thalassemia trait 1.99% (10/501) (Table IV). Among the non-anemic volunteers with abnormal hemoglobin pattern, Hb E trait was observed to be the commonest (70.49%), followed by β thal trait (16.39%). Only one case of Hb E disease (1.64%) was observed among the non-anemic volunteers (table 5). Not a single case of double heterozygous state of HbE hemoglobinopathy was observed in the present study.

TABLE IV: Frequency of abnormal pattern of hemoglobin detected in non-anemic population by HPLC using Beta-Thal short program.

Hemoglobin pattern	*Number of cases*	*Percentage*
HbE Trait	43	8.58
HbE Disease	01	0.19
B-Thal Trait	10	1.99
HbA_2 in Borderline Zone	07	1.39
Normal	440	87.82

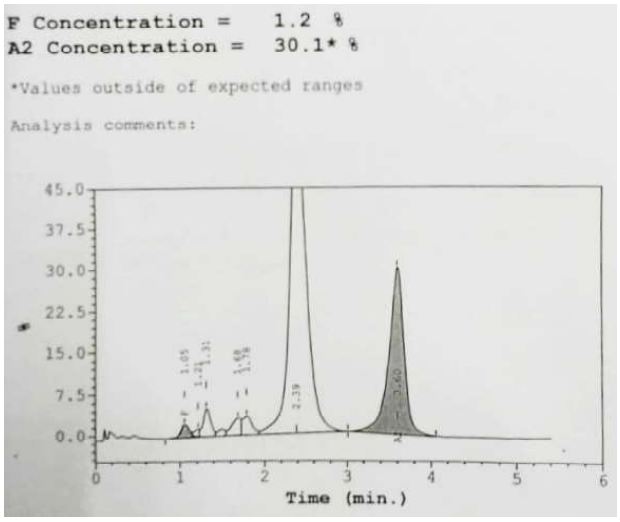

Fig. 1: HbE Trait with HbA2concentration of 30.1%.

HbA_2 was noted in borderline zone in 1.39% (7/501) which refers to a Hb A2 level between 3.5% to 4.0%. None were found to have any significant family history and hence they were all kept in a separate group and were advised serum iron, Vitamin B12, folic acid assay and Parental study along with mutation study.

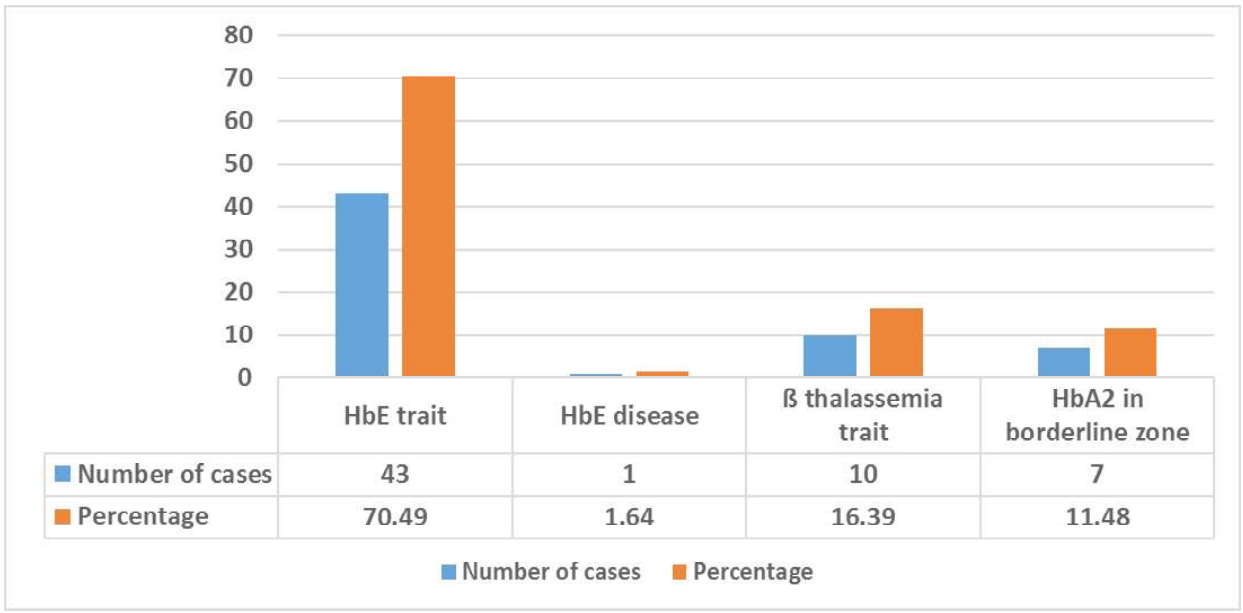

TABLE V : Frequency of abnormal Hemoglobin among all cases (n=61) of hemoglobinopathy.

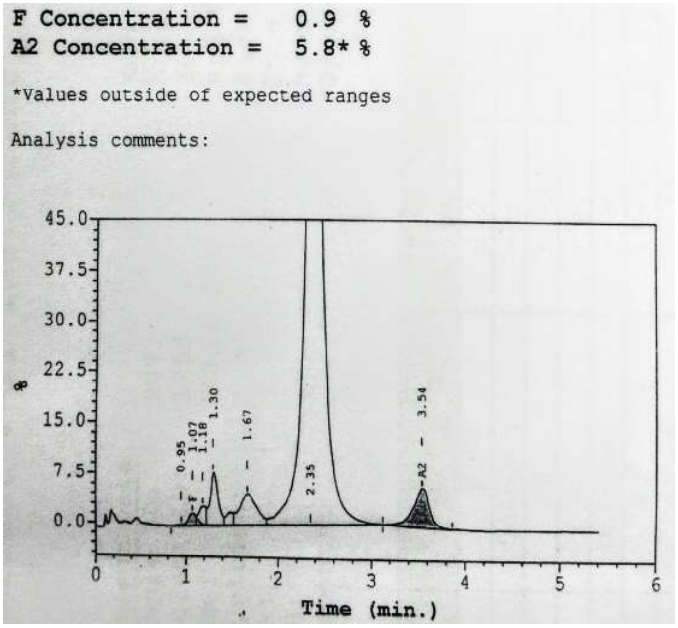

Fig. 2 : Beta Thal trait with HbA2concentration of 5.8%.

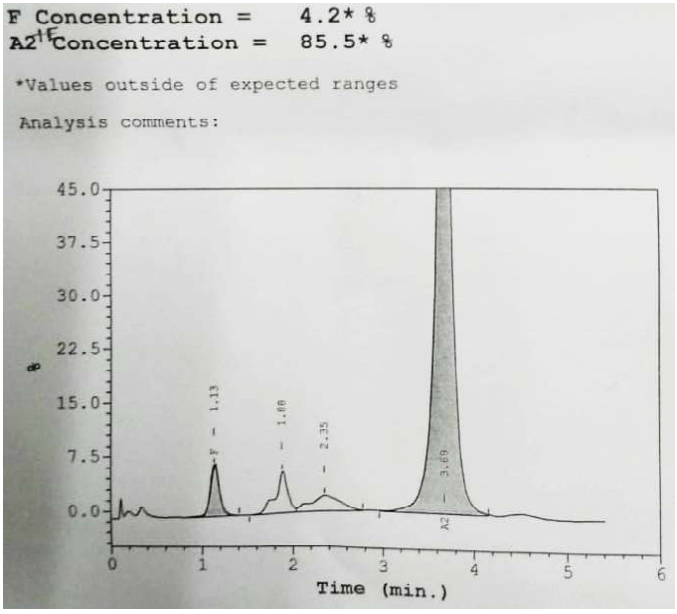

Fig. 3 : HbE Disease with HbA2concentration of 85.5%.

Discussion

The highest prevalence of HbE has been reported in South-East Asia (14). In India HbE is found to be prevalent mostly in the north-eastern states (7, 9) mainly restricted to West Bengal, Assam, Nagaland, Manipur, Tripura and Meghalaya showing an average

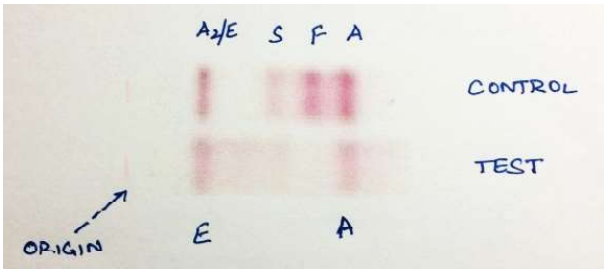

Fig. 4 : CAM electrophoresis HbE Trait.

allele frequency of 10.9% (15).

Study conducted by Deka R in Upper Assam found HbE to be the only variant hemoglobin occurring in appreciable frequencies in North East India (16). Studies done by De M et al (17) and Krisnamurty L (18) in the states of Assam and Tripura reveal a high prevalence of HbE syndromes of 63.45% and 64.5%, respectively.

Study conducted by Baruah et al, in Upper Assam found that the majority of the patients had the HbE gene (47.94%) of which HbE trait (25.48%) was the predominant variant followed by HbE disease (21.02%). BTT was detected in 3.48% of study population (19). A multicentre study conducted by Mohanty et al, in six cities of six states of India (Maharashtra, Gujarat, West Bengal, Assam, Karnataka and Punjab) showed that HbE is mainly seen in Assam (Dibrugarh) (23.9%) and in West Bengal (Kolkata) (3.92%) (20). Kalita et al, in her study of different types of hemoglobin in northeast India found HbE hemoglobinopathy to be the commonest entity (38%), HbE trait being 21% and HbE disease being 17% (21). Study conducted by Goswami et al, found that HbE trait was the most common entity (34.4%) followed by homozygous E (25.3%) in the Northern districts of West Bengal (9).

Contrary to the aforementioned studies, the present study was conducted only on non-anaemic volunteers, who were also found to have a high occurrence of HbE hemoglobinopathy (8.77%).

β thalassemia trait was the second most common abnormal hemoglobin pattern found in the non-anemic group (1.99%). In India nearly 30 million people are carriers of β thalassemia (22, 23) with a varying carrier rate of 1 to 3% in Southern India and 3% to 15% in Northern India (24, 25). A high incidence of β thalassemia and abnormal HbE in normal population of Eastern India including the heterogenous population of West Bengal has been reported (26, 27). Mandal et al, in their study on the present scenario of hemoglobionopathies in West Bengal, India, found the prevalence of β thalassemia trait to be 6.61% and that of HbE trait to be 2.78% (28).

The present study, carried out only on non-anemic volunteers, detected an occurrence of 1.99% of â thalalassemia trait.

HbE trait is found to be the largest sub-group (8.58%) of hemoglobinopathy, followed by â thalassemia trait (1.99%) (Table IV). HbE trait individuals are usually asymptomatic, having a normal hemoglobin concentration (4, 5). Keeping in mind the high prevalence of HbE hemoglobinopathy in the North Eastern parts of India and the more dreaded HbEβ Thalassemia, care must be taken that not a single case remains undetected during screening programs as well as in day to day practice.

Conclusions

61 out of the 501(12.18%) non anemic, clinically asymptomatic and hematologically silent volunteers were detected to have an abnormal hemoglobin variant. It can therefore be concluded that

1. Individuals with normal hemoglobin concentration may present with an abnormal hemoglobin pattern.

2. HbE trait, followed by β thal trait is the most common hemoglobin variant seen among the non-anemics in this region.

3. High incidence of HbE and β thalassemia trait in the normal population of this region indicates that hemoglobin variant screening for carrier detection is of utmost importance to reduce the burden of hemoglobinopathies, be it homozygotes or double heterozygotes, in this region and in India as a whole.

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