Human Biology Review
www.humanbiologyjournal.com

Human Biology Review (ISSN 2277 4424) 11(3) 2022
Original scientific paper (Biswas et al., 178-188)
Revised and Accepted on June 14, 2022

Beta-Thalassemia and other abnormal Hemoglobin among the Bengalee of Southern West Bengal, India: A study of parametric evaluation of NESTROFT, CBC and HPLC

T. K. Biswas[1], S. Bhattacharya[2], D. K. Adak[3], B.C. Ray[4] and V.R. Rao[5]

Citation: Biswas TK, Bhattacharya S, Adak DK, Ray BC and Rao VR. 2022. Beta-Thalassemia and other abnormal Hemoglobin among the Bengalee of Southern West Bengal, India: A study of parametric evaluation of NESTROFT, CBC and HPLC. Human Biology Review, 11 (3), 178-188.

[1]Tapas Kumar Biswas, Anthropological Survey of India, 27 Jawaharlal Nehru Road, Kolkata.
E-mail: tapasbiswas2005@gmail.com
[2]Subhra Bhattacharya, Anthropological Survey of India, 27 Jawaharlal Nehru Road, Kolkata.
E-mail: subhra.ansi@gmail.com
[3]Dipak Kumar Adak, Anthropological Survey of India, 27 Jawaharlal Nehru Road, Kolkata.
E-mail: adakdipak@gmail.com
[4]B.C. Ray, Department of Chemistry, Jadavpur University, Jadavpur, Kolkata.
E-mail: drbidhanray@gmail.com
[5]V.R. Rao, Emeritus Medical Scientist, Osmania University, Hyderabad
E-mail: profraovr@gmail.com

Corresponding Author: Tapas Kumar Biswas, Anthropological Survey of India, 27 Jawaharlal Nehru Road, Kolkata. E-mail: tapasbiswas2005@gmail.com

ABSTRACT

Background: *Parametric evaluation of the NESTROFT (naked eye single tube red cell osmotic fragility test), CBC (complete blood count), and HPLC (high performance liquid chromatography) are the screening tools for detection of beta thalassemia trait and other abnormal hemoglobin.*
Design and setting: *Prospective study. Field camps in various schools and villages of Diamond Harbor, Block-II, South 24 Parganas district, West Bengal. A total of 1200 individuals were examined.*
Methods: *NESTROFT, CBC and HPLC to conform the beta thalassemia trait and other abnormal hemoglobinopathy screened Individuals.*
Results: *Percentage of normal, beta thalassemia trait, HbAE, HbEE and HPFH were found to be 85.16, 8.83, 5.58, 0.17 and 0.25 respectively. Mean corpuscular volume (MCV) <70 fl followed NESTROFT closely. Both MCH < 20gm/dl and MCV< 70fl differ significantly from normal value. NESTROFT in combination with MCV < 70 fl and MCH <20 gm/dl proved to pick up the trait or carrier status. However, the combination was not cost effective.*
Conclusion: *NESTROFT and CBC is sensitive, cost effective, rapid and reliable screening test for detection of beta thalassemia trait in a population. On the other hand, HPLC is very much important for the qualitative and quantitative estimation of HbA_2 value, which is also used for detection of beta thalassemia trait and other haemoglobinopathies.*

KEY WORDS: *Abnormal hemoglobin. Laboratory methods. Bengalee populations. West Bengal.*

INTRODUCTION

Thalassemia and other hemoglobinopathies are the most common monogenic disorders in human populations. Severity of the syndrome depends on the nature of mutation and degree of synthesis of beta globin gene. Carriers of thalassemia are apparently healthy and normal, but may have slight anemia. In alpha thalassemia, severe anemia begins even before birth and survival after the first few hours of life is rare. In children with beta thalassemia, symptoms appear in the first two years of life, which include paleness, headache, fatigue, irritability, failure to grow, shortness of breath etc. Besides these, unexpectedly slow development along with jaundice, enlarged spleen or liver or deformed bones can be the common signs of thalassemia.

Various evolutionary forces are found to regulate the frequency of a deleterious mutation in human populations. People of Mediterranean, Middle Eastern, African and Asian descent are at higher risk of carrying the genes for thalassemia, for which it is also called Mediterranean Anemia. This has a special importance in developing countries like India, where it increases the burden of health care delivery system. Every year more than 10,000 children with thalassemia major are born in India, which constitutes 10% of the total number in the world, and one out of every 8 carriers of thalassemia worldwide lives in India (WHO 2008**).**

Hemoglobinopathies are the most common, autosomal recessive disorder worldwide. 7% of the global population carry an abnormal hemoglobin gene. More than half a million affected children are born each year. Every year 1000 children with thalassaemia major are born in India, which constitute 10% of the total number in the world (Varawalla et al, 1991). The incidence of beta-thalassemia in different regions of India varies from 3% to 17 % with a mean prevalence of 4% (Balgir 1999). It is estimated that there are about 45 million carriers of the beta-thalassemia gene in India, while about 15,000 affected infants are born every year, contributing to about 10% of the total thalassemics born all over the world (Balgir 2000).

But the action programmes like intensive counseling may be instituted to the groups, which show high prevalence rate rather than general population to make the program not only cost-effective but cost-efficient as well. This is mainly because of the heterogeneity of beta-thalassemia and the absence of a single test to loosen all beta-thalassemia variants (Kattamis et al. 1981).

As the frequency of thalassemia carrier is increasing by the consanguinity and endogamous mating, it may be assumed that different communities in India are facing the problem at large scale. But the frequency and distribution of thalassemia among different community in India is less well-documented. Though some studies have reported the incidence of hemoglobinopathies among different communities of India, the results are scattered and limited to specific region. Hence, this study aims to

have a vivid picture regarding distribution and present situation of thalassemia among the Bengalee inhabited in different parts of Diamond Harbor district, Southern West Bengal.

MATERIAL AND METHODS

Area of study was selected in the Diamond Harbor Block of South 24 Parganas district, West Bengal. This area is inhabited by the Bengalee populations. Methods used for the study were NESTROFT, CBC and HPLC. Venous blood was collected from 1200 unrelated individuals through organized camps in the schools and villages in the said area. Prior consent was taken.

Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were first introduced by Wintrobe in 1981 to define the size (MCV) and hemoglobin content (MCH, MCHC) of red blood cells. "International Committee for Standardization in Hematology" recommended selecting methods for quantitative estimation of HbA_2 and for HbA_2 Reference Preparation. Determination of concurrently elevated levels of hemoglobin A_2 and F has become the most practical means to diagnose carriers of the ß-thalassemia gene (Rowley 1976). Methods for the quantization of hemoglobin A_2 include electrophoresis (Marengo-Rowe 1965) and anion-exchange column chromatography (Huisman et al 1975). Methods for the quantization of hemoglobin F include electrophoresis, alkali denaturation and radial immunodiffusion (RID). High performance liquid chromatography (HPLC), which can be a relatively fast and reproducible method, has been used for the determination of various hemoglobin including hemoglobins A_2 (Turpeinen 1986) and F.

The most commonly occurring hemoglobin variants include hemoglobins D, S, C and E (Fairbanks and Decker 1980). Presumptive identification of these hemoglobin variants is made using retention time windows, such as a "D-Window," "S-Window," and "C-Window." Hemoglobin E, the second most frequently occurring hemoglobin variant, elutes within the hemoglobin A_2 retention time window using the VARIANT ß-thalassemia Short Program. Differentiation from hemoglobin A_2 can be made with observation to the area percent calculation on the sample report. Hemoglobin E in heterozygous condition (phenotype AE) is typically present in a 30% to 35 % (Bunn and Forget 1986) range, refer to Interpretation of results.

This study is an outcome of the ongoing "Community Genetic Extension Program" (CGEP) of Anthropological Survey of India. Blood samples were collected during 2009 to 2014. Bengalee caste groups covered in the study are Paundra Kshatriya, Mashishya, Bagdi and others (Namasudra, Kaora, Sodgope, Jalia Kaiborta, Kaiborta, Muchi, Kumar, Kayastha and Brahmin etc.). Apart from this Bengalee Muslim population were also considered in the present study.

RESULTS

Distribution of NESTROFT among 1200 individuals is furnished in Table 1. In the category of NESTROFT positive percentage of male is 8.58, female is 9.67 and total is 18.25. In the category of NESTROFT negative male, female and total are 30.50, 31.0 and 61.50 respectively. On the other hand, in the NESTROFT suspected category, percentage of male, female and total is 10.67, 9.58 and 20.25 respectively.

Table 1: Percentage distribution of individuals according to NESTROFT

NESTROFT	Male	Percentage	Female	Percentage	Total	Percentage
N+	103	8.58	116	9.67	219	18.25
N-	366	30.50	372	31.00	738	61.50
N-	366	30.50	372	31.00	738	61.50
N-	366	30.50	372	31.00	738	61.50
N±	128	10.67	115	9.58	243	20.25
Total	597	49.75	603	50.25	1200	100.00

Note: N+ = NESTROFT positive, N- = NESTROFT negative, N± = NESTROFT suspected

Table 2: Percentage distribution of individuals according to CBC with cut off values

CBC cut off values	Male	Percentage*	Female	Percentage*	Total	Percentage*
RBC $>5.5\times10^6/\mu l$	130	10.83	73	6.08	203	16.92
MCV(fl) <70fl	74	6.17	114	9.50	188	15.67
MCH(pg) <20pg	64	5.33	93	7.75	157	13.08
Total	268	22.33	280	23.33	548	45.67

Note: 1. RBC-red blood corpuscles, MCV- mean corpuscular volume, MCH-mean concentration of hemoglobin. 2.. *Percentage values are calculated in terms of total (i.e. 1200) individuals

The percentage distribution of individuals with cut off values for complete blood count (CBC) parameters has been shown in Table 2. It is found that the percentages of individuals suspected for beta thalassemia are different for each parameter, with highest (16.92) for RBC compared to MCV (15.67) and MCH (13.08).

Number of individuals with HbA_2 value > 3.5% (BTT), 23.2- 34.5% (HbAE) and 77.7- 88.8% (HbEE), and HbF value 18.7- 21.3% (HPFH) have been depicted in Table 3. Only 2 cases (0.17%) of HbEE and 3 cases (0.25%) of HPFH were detected in the studied subjects. Whereas, 106 (8.83%) and 67 cases (5.58%) were beta thalassemia trait and Hb E carrier respectively.

Table 3: Percentage distribution of individuals according to HPLC with cut off values

HPLC	Male	Percentage*	Female	Percentage*	Total	Percentage*	Inference
HbA_2 (>3.5%)	47	3.92	59	4.91	106	8.83	BTT
HbA_2 (23.2-34.5%)	24	2.00	43	3.58	67	5.58	HbAE
HbA_2 (77.7-88.8%)	2	0.17	0	0	2	0.17	HbEE
HbF (18.7-21.3%)	1	0.08	2	0.16	3	0.25	HPFH

Note: 1. HbA_2 – hemoglobin fraction ($\alpha_2\delta_2$) in percentage, HbF – fetal hemoglobin fraction ($\alpha_2\gamma_2$) in percentage, HbAE – HbEE homozygote patient) and HPFH – hereditary persistence of fetal hemoglobin. 2. *Percentage values are calculated in terms of total (i.e., 1200) individuals

Frequency of beta-thalassemia trait and other abnormal hemoglobin are shown in Table 4 according to NESTROFT. In beta thalassemia trait category NESTROFT positive, NESTROFT negative and suspected cases are estimated to be 82 (77.36%), 2 (1.89%) and 22 (20.75%) respectively. But the picture is something different in case of HbE carrier, where NESTROFT negative is in first position, suspected in second position and NESTROFT positive in third position. In the detection of HbAE, NESTROFT is not so suitable test as a preliminary screening. In case of HbEE, NESTROFT positive are 100 percent. On the other hand, the HPFH carrier state shows 33.33 percent NESTROFT positive, 66.67 percent suspected and NESTROFT negative is nil. In case of normal individuals, NESTROFT negative is maximum (68.79%), NESTROFT positive is 11.35% and suspected is 19.86%. NESTROFT eliminate the maximum number of normal individuals. In case of HPFH one individual is NESTROFT positive and two individuals are NESTROFT suspected.

Table 4: Beta-thalassemia trait and abnormal hemoglobin according to NESTROFT

Types of individuals	NESTROFT positive (N^+)	NESTROFT negative (N^-)	Suspected ($N^\pm$)	Total number of individuals
BTT	82 (77.36)	2 (1.89)	22 (20.75)	106 (8.83)
HbAE	18 (26.86)	27 (40.30)	22 (32.84)	67 (5.58)
HbEE	2 (100)	0	0	2 (0.17)
HPFH	1(33.33)	0	2 (66.67)	3 (0.25)
Normal	116 (11.35)	703 (68.79)	203 (19.86)	1022 (85.17)
Total	219 (18.25)	732 (61.00)	249 (20.75)	1200 (100.00)

Note: Figures in parenthesis indicate percentage values. Key: BTT= beta thalassaemia trait, HbAE = HbEheterozygous (HbE carrier), HbEE= HbEhomozygous (HbE Patient), HPFH= hereditary persistence of fetal hemoglobin (carrier state)

Table 5: Beta-thalassemia trait and abnormal hemoglobin according to CBC

Types of individuals	CBC positive(C^+)	CBC negative(C^-)	Total number of individuals
BTT	105 (99.06)	1(0.94)	106 (8.83)
HbAE	23 (34.33)	44 (65.67)	67 (5.58)
HbEE	2 (100.00)	0	2 (0.17)
HPFH	0	3(100.00)	3 (0.25)
Normal	66 (6.46)	956 (93.54)	1022 (85.17)
Total	196 (16.33)	1004 (83.67)	1200 (100.00)

Note: Figures in parenthesis indicate percentage values.Key: C^+ = indicative of carrier status and C^- =non-indicative of carrier status. C+= MCV<70±4 fl, MCH <20, RBC>5.5*10^6 and Hb<10gm/dl; C^- =MCV>70fl, MCH>20pg and RBC<5.5*10^6, Hb>10gm/dl

Table 5 describes the frequency of beta-thalassemia trait and other abnormal hemoglobin as obtained by complete blood count (CBC). In case of BTT, complete blood count predicted 99.06% positive. Here, CBC positive means MCV less than 70fl, MCH less than 20pg, hemoglobin is more than 11.5gm/dl and number of RBC count is greater than 5.5*10^6/µl. This stringent laboratory protocol follows this good quality of result. In case of HbE trait frequency of CBC negative (65.67%) is much higher than that of the CBC positive (34.33%). In this point the value of MCV ranges between 70.5fl to 74.5fl and value of MCH ranges between 20.6pg to 23.5pg.

Table 6: Beta-thalassemia trait and abnormal hemoglobin according to HPLC

Types of individuals	HPLC positive (V^+)	HPLC negative (V^-)	Total number of individuals
BTT	106 (100.00)	0	106 (8.83)
HbAE	67 (100.00)	0	67 (5.58)
HbEE	2 (100.00)	0	2 (0.17)
HPFH	0	3 (100.00)	3 (0.25)
Normal	0	1022 (100.00)	1022 (85.17)
Total	175 (14.58)	1025 (85.42)	1200 (100.00)

Note: Figures in parenthesis indicate percentage values
Key: V^+ = variant positive (HbA_2>3.5) and V^- = variant negative (HbA_2<3.5)

Table 6 describes the frequency of beta-thalassemia trait and other abnormal hemoglobin according to HPLC. In case of BTT, HPLC show 100% positive prediction. Side by side, HbAE and HbEE also show 100% positive prediction. But in case of HPFH and Normal, 100% is negative prediction.

Table 7: Parametric evaluation of BTT screening by NESTROFT, CBC and HPLC (n=1200)

Derivation set												
	Positive (N^+)				Negative (N^-)				Suspected ($N^\pm$)			
NESTROFT	217 (18.08)				734 (61.17)				249 (20.75)			
CBC	C^+		C^-		C^+		C^-		C^+		C^-	
	126 (10.50)		91 (7.58)		16 (1.33)		718 (59.83)		54 (4.50)		195 (16.25)	
HPLC	Validation subset											
	V^+	V^-	V^+	V^-	V^+	V^-	V^+	V^-	V^+	V^-	V^+	V^-
	5 (0.42)	121 (10.08)	14 (1.17)	77 (6.42)	8 (0.67)	8 (0.67)	18 (1.50)	700 (58.33)	5 (0.42)	49 (4.08)	17 (1.42)	178 (4.83)

Figures in parenthesis indicate percentage values

Table 7 reveals the derivation set of NESTROFT, CBC and HPLC. Out of 1200 tested individuals NESTROFT positive is 217 (18.08%), negative is 734 (61.16%) and suspected is 249 (20.75%). Here, suspected means the NESTROFT practically shows transition state which may be positive or negative. Along the NESTROFT positive side, the CBC positive is 126 (10.50%), CBC negative is 91 (7.58%). On other side of NESTROFT negative, CBC positive is 16 (1.33) and CBC negative is 718 (59.83%). In case of suspected category CBC Positive is 54 (4.50) and CBC Negative is 195 (16.25%). HPLC shows that 82 (6.83%), 2 (0.17%), 1 (0.08%), 2 (0.17%), 18 (1.50%) and 1 (0.08%) are beta thalassemia trait and rest are negative.

Incidence of beta thalassaemia trait (BTT), HbAE, HPFH and HbEE:

Table 8 and Figure 1 reveal the prevalence of beta thalassemia trait, HbAE, HPFH and HbEE in the studied area. The percentage of normal, beta thalassemia trait, HbAE, HbEE and HPFH are 85.16, 8.83, 5.58, 0.17 and 0.25 respectively.

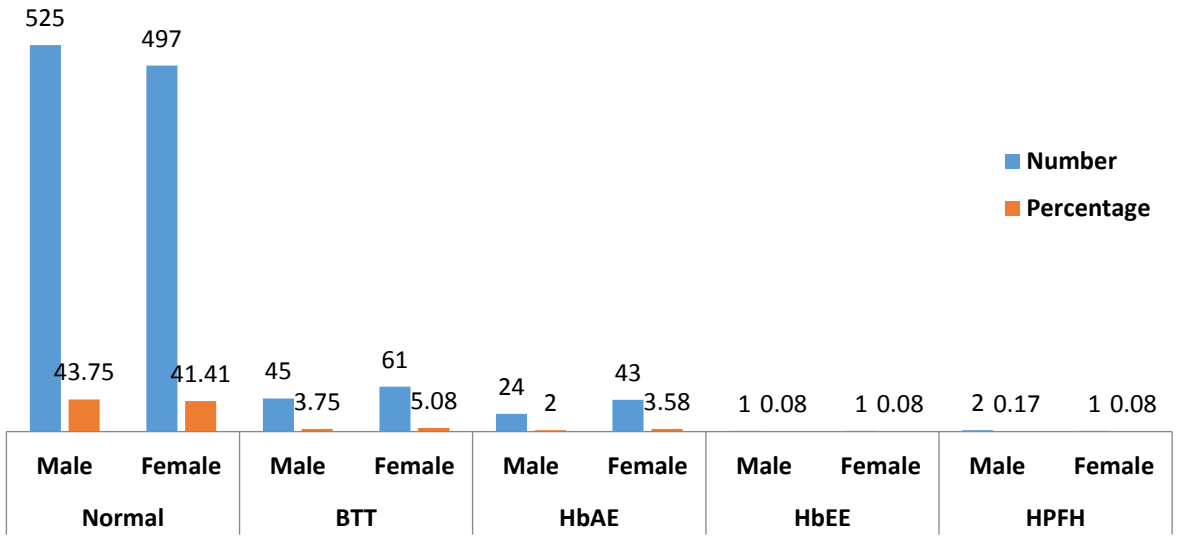
Table 8: Percentage of Normal, BTT, HbAE, HPFH and HbEE individual among the populatio

Note: * Percentage values are calculated in terms of total (i.e. 1200) individuals

Individuals	NORMAL		BTT		HbAE		HbEE		HPFH	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Number	525	497	45	61	24	43	1	1	2	1
Percentage*	43.75	41.41	3.75	5.08	2.00	3.58	0.08	0.08	0.17	0.08

Key: BTT= beta thalassemia trait, HbAE=HbE heterozygous (HbE carrier) and HbEE= HbE homozygous (HbEpatient), HPFH= hereditary persistence of fetal hemoglobin

Fig. 1: Incidence of BTT, HbAE, HbEE and HPFH in the studied area



Different Categories of BTT and Hemoglobinopathies

DISCUSSION

In this mass screening protocol, we try to eliminate the normal individual through different techniques applying chemical technologies. In the first step, we screened the whole 1200 individual through NESTROFT. As a result, we got some individuals are positive, some are negative and rest are suspected cases (doubtful cases). Then we follow the second procedure CBC or FBC (complete blood count or full blood count) to retest all the 1200 individuals. Here, also we got the results of some cases

where CBC is positive, negative and suspected. But the interesting thing is that certain number of individuals is normal in both screening test NESTROFT and CBC/FBC. Then we conform that they are absolutely normal which is verified by the Variant-II, Hemoglobin testing System (Bio-Rad). This is globally considered as gold standard method which measure the hemoglobin fractions (like HbA/HbA_0, HbA_2, HbF etc.) following the method of Joutovsky et al. (2004). This Variant Machine is based on cation- exchange High Performance Liquid Chromatography. In this way we tested and retested the other categories of individual like negative and suspected cases. Similarly, they are also verified by the gold standard method i.e. Variant-II and conform which one are trait/carrier and which one are normal. Here, actual procedure is the elimination of normal individuals and detection of trait or carrier status of the subjects.

NESTROFT eliminate the maximum number of normal individuals. CBC is a stringent laboratory protocol which shows good quality of result. On the other hand, HPLC is accepted worldwide as gold standard method for hemoglobin testing system (Variant Machine; BIO-RAD) on the principle of high-performance liquid chromatography. We usually pick up the microcytosis and hypocromic individual which may be BTT or HbE directly through the positive indication of NESTROFT and CBC. Most of the cases, where CBC is positive their variant may be positive. In this study v+ means HbA_2 value more than 3.5%. And oppositely, v^- means HbA_2 value less than 3.5%. Here we also like to mention that c+ means CBC positive, considering the MCV value less than 70 and MCH less than 20. This standard protocol is followed in our laboratory. External laboratory control is maintained by EQAS (External Quality Assurance Services) Hematology, Bio-Rad laboratories, Irvine, CA92618, USA. Our laboratory reference number is 11770.

The highest prevalence of HbE is found in the periphery area of Assam. Bodo Kachari showed 64.5% frequency, the highest observed frequency of this mutation in the world (Krishnamurti and Lakshmanan 2000). The tribal population of adjoining Tripura (Das et al., 2000) followed it. The highest prevalence of HbE is found in the periphery of the area of distribution (Bodo-Kachari, a Tibeto Burman language group of upper Assam) and tribal population of adjoining Tripura (Das et al., 2000). Surprisingly the Bodo Kachari population has been reported to be 64.5%, the highest observed frequency of this mutation in the world (Krishnamurti and Lakshmanan 2000). The phenotype genotype interaction of Hb E is also variable in the population (Talukdar and Sharma 1994).

The highest frequency of beta thalassemia trait is reported in Gujarat, followed by Sindh, Punjab, Tamil Nadu, South India and Maharashtra (1.9%) as shown in Ambekar et al. (2001). Rao and Gorakshar (1990) have shown that the Gond of Maharashtra is a high-risk group for beta-thalassemia gene (2.83%). The result was supported by Gond and Gond related tribes of Madhya Pradesh (Gupta et al, 2003). In Rajasthan beta-thalassemia syndrome were encountered in 3.79% (Choubisa 1991). The

author has shown the higher incidence of mutant gene (HbS, HbD, HbE) among scheduled tribes (6.85%) like Bhil, Damor, Garasia and Mina as compared to scheduled castes (3.08%) and general castes (2.32%).

ACKNOWLEDGEMENTS

The present work is funded by the Anthropological Survey of India under the project "Human Genetics Extension Programme". The authors acknowledge the help and cooperation rendered by the subjects who took part in this study.

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