Original Article

Beta-Thalassemia Carrier Detection by NESTROFT: An Answer in Rural Scenario?

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ABSTRACT

Background and Objectives: Beta-thalassemia continues to be a cause of significant burden to the society particularly in the poorer developing countries. Although sophisticated methods of screening have become available, a hunt for a cheap, rapid, objective screening method still remains elusive. Thus, the objectives are to study the validity of Naked-Eye-Single-Tube-Osmotic-Fragility-Test (NESTROFT) in detection of beta-thalassemia carrier state, to assess the prevalence of beta-thalassemia trait among antenatal mothers in the region and also to find out the effect of concurrent iron deficiency on the hematological parameters in these cases.

Materials and Methods: A total of 500 antenatal mothers in a rural tertiary care hospital were selected for the study. Their blood samples were subjected to NESTROFT, complete hemogram, reticulocyte counts and hemoglobin variant studies by electrophoresis and by high pressure liquid chromatography (HPLC). Serum ferritin estimation was done in cases diagnosed as beta-thalassemia trait. The results were analyzed statistically.

Results: A prevalence of 3.4% of beta-thalassemia trait and 0.6% of E-beta-thalassemia were observed among the study population. NESTROFT showed an overall sensitivity and specificity of 95% and 95.8% respectively in detection of heterozygous and double heterozygous states of beta-thalassemia. The various RBC indices were significantly (P <0.05) lower in carriers with concurrent iron deficiency. A co-existent iron deficiency did not preclude a diagnosis of beta-thalassemia carrier state.

Conclusions: NESTROFT appears to a valid test in rural setting with financial constraints. The hematological parameters in iron deficient beta-thalassemic carriers significantly differed from their iron replete counterparts but did not cause problem in diagnoses.

Keywords: Beta Thalassemia, Carrier States, Screening

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Introduction

The inherited hemoglobin (Hb) disorders are the most common single gene defect in man. The prevalence of hemoglobinopathies is on the rise worldwide. This is of special importance in developing countries where it increases the burden of health care delivery system. The abnormalities can either be quantitative (the thalassemia syndromes) or qualitative (the hemoglobin variants) or a combination of both. Of these, the thalassemia syndromes particularly the beta thalassemias and some alpha thalassemias are the major cause of morbidity (1). It is estimated that 1.5% of the world's population are carriers of beta thalassemia- that is, at least there are 80 million to 90 million people with an estimated 60,000 new cases being born each year. The South-east Asia region (which includes India, Thailand and Indonesia) accounts for 50% of world carriers (2). In India, nearly 30 million people are carriers of beta thalassemia and 7000 babies with beta-thalassemia major are born every year (3, 4). The carrier rate varies between 0 to 17% in different ethnic groups (5).

Among the hemoglobin variants, hemoglobin E (β-26 glutamine→lysine) is the hallmark of South-East Asia. It is the commonest hemoglobin variant in India with prevalence of 7-50% in Northeastern region and 1-2% in West Bengal (6). In the plains of the Darjeeling district, located in the northern part of West Bengal, the Rajbanshis form a major chunk of local inhabitants. The Rajbanshis are known to have a high prevalence of hemoglobin E mutation though there is no published data till date. Subjects having hemoglobin E, either in homozygous or heterozygous state are otherwise normal but the probability of combining with thalassemia trait can give rise to hemoglobin E-beta thalassemic babies. Hemoglobin E-beta thalassemia can manifest as thalassemia minor, intermedia or even the grievous thalassemia major.

As for treatment of thalassemia major patients till date remains a source of misery, burden and mostly disappointing, prevention plays the key role. As the hunt for an appropriate screening tool has been on for decades, Naked Eye Single Tube Osmotic Fragility Test (NESTROFT) has been variably looked upon as a simple, cheap, rapid, objective test with sensitivity as high as 99.8% in detection of thalassemia carriers in areas with high prevalence of this disease (7).

Here, we report an institution based study which was done to assess the utility of NESTROFT as a suitable screening procedure for carriers of beta-thalassemia trait among antenatal mothers attending the antenatal clinic of our hospital. The study also provided an assessment of the burden of thalassemia trait and other hemoglobinopathies in this region. The study also throws light on the effect of iron deficiency in the various hematological parameters among beta-thalassemia carriers.

Materials and Methods

The study population included already booked pregnant mothers attending the antenatal clinic of the hospital for routine antenatal check-up irrespective of their gestational age from a period of 12 months. The study was done after obtaining the necessary clearance from the ethical committee of the institution. The sample size was 500 pregnant women selected by simple random sampling. A detailed schedule including all personal, socioeconomic and ethnic details, personal history, clinical history, obstetric history etc. was filled up after taking proper informed consent of the subjects. Six ml of venous blood was collected in EDTA (ethylene diamine tetracetic acid) vials from the pregnant mothers

while additional 2 ml blood was allowed to clot in a separate vial. Two direct smears were also made in each case. The anticoagulated blood was used for performing NESTROFT, CBC(Complete Blood Counts), reticulocyte count, hemoglobin electrophoresis, hemoglobin A_2 and F estimation and HPLC (high pressure liquid chromatography). NESTROFT was done using 0.36% buffered saline. CBC was done using automated cell counter (Celly 70 version 3.xx Biocode Hycel, France). Peripheral blood smears were stained by Leishman's stain and reticulocyte count was done by new methylene blue. Hemoglobin electrophoresis was carried out on cellulose acetate membrane using TEB buffer, pH 8.6. Hb A_2 estimation was done following elution after electrophoresis on cellulose acetate, TEB buffer, pH 8.9. Hemoglobin F estimation was done by modified Betke's method. All the samples were run on BIORAD VARIANT (beta thalassemia short program) which functions on the principal of HPLC. Serum ferritin was done by the principle of microplate immunoenzymometric assay using ACCUBIND ELISA Microwells (Monobind Inc. Product Code: 2825-300) in suspected cases of heterozygous state of beta-thalassemias by the above screening procedures. A cut off Hb A_2 level of $\geq$ 3.6% was used for diagnosing thalassemia trait while values between 3.2% and 3.5 % were considered borderline. A serum ferritin level of <10ng/ml was taken as reflective of iron deficiency. The results were analyzed statistically by using SPSS version 16.0.

Results

A total of 500 antenatal mothers were screened for hemoglobinopathies by using NESTROFT, RBC indices (using criteria MCV $\leq$ 76fl and MCH $\leq$ 26 pg), hemoglobin electrophoresis with subsequent HbA_2 measurement and the HPLC. 17 cases (3.4%) of beta-thalassemia trait and 3 cases (0.6%) of E-beta-thalassemia were diagnosed among the study population. Out of 17 cases of beta-thalassemia trait, NESTROFT was positive in 16 cases. It showed 23 false positive and 1 false negative results (Table 1). Thus for detection of beta-thalassemia trait among antenatal women, the sensitivity and specificity of NESTROFT came to be 94.12% and 95.23% respectively. The positive predictive value, negative predictive value, percentage of false negatives and percentage of false positives were 41.02%, 99.78%, 5.88% and 4.78% respectively. The test also gave positive results for all the mothers having E-beta-thalassemia thus showing 100% sensitivity in the present study. Thus it attained an overall sensitivity of 95% and specificity of 95.8% in detection of heterozygous and double heterozygous states of beta-thalassemia. The test gave false positive results in 9 cases of hemoglobin E trait, 5 cases of homozygous hemoglobin E, 2 cases of iron deficiency anemia and 4 normal subjects (Table 2).

Only 1 case gave a borderline result (HbA_2 3.2%) on HPLC. It was NESTROFT negative and had a MCV of 110 fl. The case had no iron deficiency; the RBC morphology was overtly macrocytic and had low serum and RBC folate. A repeat HPLC following folate supplementation gave an HbA_2 level within the normal range. Seven out of 17 cases (41.11%) of beta-thalassemia suffered from co-existent iron efficiency as measured by serum ferritin. Out of 17 mothers, 3(17.64%) were Rajbanshis. A high incidence (58.8%) was noted among the scheduled caste and tribe category. 52.94% of the cases were illiterate. None of the cases had any organomegaly, history of blood transfusion or any history of thalassemic baby. The examination of the peripheral blood smear showed the presence of microcytic, hypochromic blood picture

with mild to moderate anisopoikilocytosis the degree being higher in cases of concurrent iron deficiency. Pencil shaped cells were noted in the later cases. No case presented with reticulocytosis. Basophilic stippling was noted in one case. The cases of beta-thalassemia trait with iron deficiency were more anemic with a mean hemoglobin level of 8.07±0.8 mg/dl compared to those without iron deficiency (mean hemoglobin level-10.09±0.65 mg/dl).

The mean MCH value of the cases of beta-thalassemia trait with iron deficiency was 18.89(±1.20) pg and their mean MCV was 60.06±2.4 fl (Table3). The comparison of CBC and Hb A, Hb A_2 and Hb F results between thalassemia carriers with and without iron deficiency are summarized in Table 4. In cases of beta-thalassemia trait with iron deficiency, the mean RBC count, mean MCV and mean MCH were significantly lower than those with beta-thalassemic carriers without iron deficiency (P <0.05). The overall Hb A_2 levels among thalassemia carriers ranged from 4.1% to 6.6% with a mean of 5.25±0.7% (Table 4).Though the levels were lower in cases of carriers with iron deficiency, the level did not preclude the diagnosis in our study. Three cases of double heterozygous state of E-betathalassemia were detected. Two of them were Muslims and 1 belonged to the Rajbanshi community. All of them were illiterate. Two mothers were multiparous and had history of previous blood transfusion including at the time of previous deliveries. The other one was nulliparous and had no history of blood transfusion. All the three cases were NESTROFT positive with the peripheral blood film showing microcytic, hypochromic blood picture with polychromasia, thin macrocytes, fragmented cells, moderate anisocytosis and target cells. Reticulocytosis was seen in 2 out of 3 cases.

Table 1- Table showing screening test results of NESTROFT

NESTROFT results	Diagnosis		Total
	Beta thalassemia trait	**Not Beta thalassemia trait**	
Positive	16 (True Positive)	23 (False Positive)	39
Negative	1(False Negative)	460 (True Negative)	461
Total	17	483	500

Table 2- Table showing the final diagnoses of NESTROFT +ve cases

Final diagnosis	Number of NESTROFT +ve cases (Total =39)
Beta thalassemia trait	16
E-beta thalassemia (double heterozygous)	3
Hemoglobin E trait (heterozygous)	9
Hemoglobin E (homozygous)	5
Iron deficiency anemia	2
Normal	4

Table 3-Table showing comparison of the mean value with standard deviations (Ranges given in parentheses) of various hematological parameters of BTT with and without co-existent iron deficiency anemia (IDA)

Hematological Parameters	Laboratory Diagnosis		
	BTT* without IDA**	**BTT with IDA**	**BTT (all cases)**
Hemoglobin (mg/dl)	**10.09±0.65** (9.1-11.4)	**8.07±0.8** (7.3-9.5)	**9.26±1.2** (7.3-11.4)
Hematocrit (%)	**30.1±3.14** (26.2-36.6)	**25.51±3.05** (21.3-30.4)	**28.2±3.8** (21.3-36.6)
RBC count (million/mm^3)	**4.94±0.37** (4.2-5.27)	**4.28±0.44** (3.9-4.9)	**4.67±0.51** (3.9-5.27)
MCV(fl)	**63.5±3.47** (59.1-69.6)	**60.06±2.4** (56.6-64.1)	**62.08±3.48** (56.6-69.6)
MCH(pg)	**20.44±1.18** (19-22.62)	**18.89±1.20** (16.4-20.0)	**19.8±1.4** (16.4-22.62)
MCHC(mg/dl)	**31.06±1.77** (29.5-35.8)	**31.34±2.08** (27.4-33)	**31.6±1.8** (27.4-35.8)
RDW(CV)	**14.06±0.88** (12.9-15.5)	**17.94±0.96** (16.2-18.8)	**15.65±2.16** (12.9-18.8)

* Beta thalassemia trait ** Iran deficiency auemia

Figures in bold indicate the mean value with standard deviations. Figures in parenthesis indicate the range of values.

Table 4- Relative percentages of various hemoglobins of BTT with and without Co-existent iron deficiency anemia (IDA)

Hematological Parameters	Laboratory Diagnosis		
	BTT* without IDA**	**BTT with IDA**	**BTT (all cases)**
Hemoglobin A2/E	**5.57±0.66** (4.3-6.6)	**4.8±0.55** (4.1-5.8)	**5.25±0.7** (4.1-6.6)
Hemoglobin F	**1.06±0.52** (0.3-1.8)	**1.5±1.5** (0.4-4.7)	**1.14±1.02** (0.3-4.7)
Hemoglobin A	**85.06±1.4** (82.4-86.9)	**85.6±0.9** (84.5-87.5)	**85.28±1.23** (82.4-87.5)

Figures in bold indicate the mean value with standard deviations. Figures in parenthesis indicate the range of values.

* Beta thalassemia trait ** Iran deficiency auemia

Table 5 - Table showing comparison of performance of NESTROFT for detection of beta- thalassemia carriers

	Present Study (2006-07)	Reference 12 (%)	Reference 13 (%)	Reference 14 (%)	Reference 15 (%)
Sensitivity	94.12	91	97.7	97.1	97.6
Specificity	95.23	95	71.7	100	72.9
PPV	41.02	55	51.9	100	33.6
NPV	99.78	99	99.0	98	99.5

Discussion

A total of 500 antenatal mothers underwent NESTROFT, complete hemogram, and hemoglobin variant study by electrophoresis and HPLC method. The purpose of the study was to assess the burden of beta-thalassemia trait among antenatal mothers in this region and to test the validity of NESTROFT in detection of thalassemic carriers. Seventeen cases of beta-thalassemia trait were identified, thus showing a 3.4% prevalence of beta-thalassemia carrier state among antenatal women in this region. In 1994, Desai *et al.* (8) showed a beta-thalassemia carrier rate of 8.45% among antenatal mothers while a study in 2006 by Sinha *et al.* (9) showed the rate to be 5.8%. In a community based study in 3 districts of West Bengal namely Burdwan, 24 Parganas (North) and Midnapore (undivided) the prevalence rate of carrier state among antenatal mothers was 5.95% (10). In spite of intense search of literature no published data regarding thalassemia carrier prevalence could be found in Darjeeling district, located in the northern part of West Bengal where there is a heterogeneous mixture of Rajbanshis (the major ethnic groups in the plains of Darjeeling), various hill tribes (including Nepalis, Sikkimese, Bhutias, Gorkhas etc) and various other caste and creeds of Hindus as well as other religions like Muslims and Buddhists.

In our study, NESTROFT was able to pick up 16 out of 17 thalassemia carriers thus giving a sensitivity of 94.12% for identification of heterozygous beta- thalassemia trait. It gave only 1 false negative result and showed a specificity of 95.23%. It also gave positive results for all the three cases of double heterozygous states of E-betathalassemia with a sensitivity of 100%. Thus, it attained an overall sensitivity of 95% and specificity of 95.8% in detection of heterozygous and double heterozygous states of beta-thalassemia. A study by Thomas *et al.* (n=150) found that the sensitivity, specificity, PPV and NPV of NESTROFT in detecting heterozygous beta-thalassemia cases were 98.4%, 66.6%,81.5% and 96.5% respectively. In detecting other hemoglobinopathies, the sensitivity of NESTROFT was 100%, specificity was 66.6% and PPV and NPV were 69.5% and 100% respectively. The overall sensitivity, specificity PPV and NPV were 98.9%, 66.6%, 87% and 96.5% respectively. They showed that NESTROFT was very useful in picking up homozygous and heterozygous hemoglobin E and hemoglobin S disease as well as Hereditary Persistence of Fetal Hemoglobin (11). The most common cause of false positive result in our case was Hb E

since its prevalence is quite high in this area. The comparison of sensitivity, specificity, positive predictive value and negative predictive value of NESTROFT in our study with some other previous studies (12-16) also recommended NESTROFT for screening of beta-thalassemia trait where there is high prevalence and constrained resources. Comparison of hematological parameters among various hematological parameters among iron replete and iron deficient beta-thalassemic carriers showed interesting results. The present study shows that the MCV and MCH levels were significantly lower in cases of beta-thalassemia trait with coexistent iron deficiency than their iron-replete counterparts. The results are in accordance with the study of Madan *et al.* (17) who found that patients with beta-thalassemia trait with co-existent iron deficiency had hemoglobin level, MCV and MCH levels significantly lower than those of beta-thalassemia only. In our study, the RBC counts and hematocrit were also lower in cases of beta-thalassemia with iron deficiency. The hemoglobin A_2 levels were also significantly lower ($P<0.05$) in iron deficient carriers. This was in contradiction to the findings of Madan *et al.* (17). However, in consensus with their findings, iron deficiency did not preclude a diagnosis of beta-thalassemia carriers as in these cases also, the HBA_2 levels were significantly high (mean HBA_2 level 4.8±0.55%) with the lowest HBA_2 level 4.1%.

Conclusion

NESTROFT appears to be a valid test for detection of both double and single heterozygous states of beta-thalassemia among pregnant women. The high prevalence of Hb E and beta-thalassemia in this region underscores the need of larger community-based screening strategies in this region to estimate the burden of hemoglobinopathies. Our study also shows that all the RBC indices and HbA_2 were significantly lower in beta-thalassemia carriers with iron deficiency compared to their iron replete counterparts. Iron deficiency also did not appear to preclude a diagnosis of beta-thalassemia carrier in the present study population.

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