Original article:

An analysis of NESTROFT and Red cell indices in evaluating antenatal mothers for Beta Thalassemia trait

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Abstract

Background: The frequency of thalassemia trait is about 3% worldwide, while in developing countries like India, it is a major cause of burden on the health care system. Naked eye single tube red cell osmotic fragility test (NESTROFT) used in population screening for beta thalassemia trait. It is a simple, low cost, reliable and most suitable screening test for β BTT, with a sensitivity of 99.8% in regions with high prevalence rates. ***Material &Methods:*** The present study was conducted in the Department of Pathology and included a total of 174 antenatal cases, attending the Out Patient Department of Obstetrics and Gynecology of a tertiary health care center in New Delhi between June 2011 to January 2012. The aim of the study was to screen mothers antenatally for early detection of BTT and to test the validity of NESTROFT in detection of thalassemic carriers of this area. ***Results:*** 174 pregnant women attending antenatal clinics were screened for detecting hemoglobinopathy with the help of NESTROFT, red cell indices, haemoglobin electrophoresis and HPLC It was seen that only four cases were BTT while ten were of microcytic anaemia and two had normal red cell indices. ***Conclusion:*** antenatal diagnosis of BTT is an important screening programme to reduce the burden of birth of children suffering from thalassemia and other hemoglobinopathies.

Keywords: NESTROFT; Antenatal; Thalassemia

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Introduction

Hemoglobinopathy is a common genetic problem worldwide with the commonest inherited being thalassemia. In developing countries like India, it is a major cause of burden on the health care system. The frequency of thalassemia trait is about 3% worldwide.[1] In South-East Asia alone reside 50% of the thalassemic carriers of the world.[2] The incidence in India however is variable ranging from 3%-18% in north and 1.3% in southern India.[3]β Thalassemia Trait (BTT) is an asymptomatic condition resulting in microcytosis and mild naemia on the other hand β Thalassemia major is a much severe condition requiring lifelong blood transfusion. Thus, the birth of Thalassemia child places considerable strain, not only on the affected child and its family, but

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also on the community and nation at large.[4]Genetic counselling and prenatal diagnosis are effective methods to reduce the incidence of genetic disorders for which the population at risk needs to be identified. Varioushaematological investigations are available which are helpful in diagnosing BTT. However, due to cost restrictions, increase time consumption or cumbersome procedures requiring specialized equipment, implementing them for screening is not possible. Naked eye single tube red cell osmotic fragility test(NESTROFT) used in population screening for beta thalassemia trait, is a simple, low cost, reliable and most suitable screening testfor β BTT, with a sensitivity of 99.8% in regions with high prevalence rates.[5].Thus, antenatal diagnosis of BTT is an important screening programme to reduce the burden of birth of children suffering from thalassemia and other hemoglobinopathies. Regarding this problem, most studies have been conducted among urban population, and very few have been reported in the semi-urban/slum and rural population. The present study was conducted in such area of a medical college, situated in New Delhi. The aim of the study was to screen mothers antenatallyfor early detection of BTT and to test the validity of NESTROFT in detection of thalassemic carriers of this area. The objective was finding the prevalence of the BTT among the pregnant females attending the ante-natal clinics, by using the NESTROF test and to screen the pregnancies 'at risk' of delivering babies with Thalassaemia major and also making them aware of its consequences among pregnant women. The present study evaluates the efficacy of NESTROFTin detection of beta thalassemia trait in a semi-urban population of New Delhi.

Material and methods:

The present studywas conducted in the Department of Pathology and included a total of 174 antenatal cases, attending the Out Patient Department of Obstetrics and Gynecologyof a tertiary health care center in New Delhi between June 2011 to January 2012 and agreeing to give consent for the study. Our hospital caters predominantly to a semi urban / slum population.These patients attended the hospital for routine ante-natal check up in early pregnancy (First Trimester). A detailed clinical history of all the patients was taken.

Venous blood (4ml)was collected in ethylene diamine tetra acetic acid (EDTA) vials from pregnant mothers attending the antenatal clinics. The anticoagulated blood was used for performing haematological investigations including Complete Blood Counts (CBC), NESTROFT's, reticulocyte count(, haemoglobin electrophoresis and HPLC wherever indicated. Reticulocyte count was done with help of supravital stains (Brilliant Cresyl Blue) and count was done manually under the microscope by trained pathologists. Hemoglobin electrophoresis and HPLC was done using Variant 2 (Biorad) and D-10 (Biorad) respectively.

NESTROFT was performed using 0.36% buffered saline solution. Two ml of buffer solution was taken to which one drop of test blood sample was added,2ml distilled water was also taken in another test tube, to which also the test sample was added, both tubes were mixed well and left undisturbed for 30 min at room temperature. After half hour both tubes were shaken and then held against a white paper on which a thin blackline is drawn. Interpretation of the test was done by experienced pathologist. The black line should be clearly visible through the contents of the control tube.If the line was clearly visible through the contents of the tube labeled test, NESTROFT was considered negative. If the line was not clearly visible through the contents of the tube with 0.36% buffered saline, the test was considered positive.If the gestational age was less than 20 weeks, then the couple was counselled to undergo Hb electrophoresis to make a confirmatory diagnosis and subsequently, to make a prenatal diagnosis. If the gestational age was more than 20 weeks, the couple was counselled to undergo electrophoresis for the confirmation of the diagnosis and to make a prenatal diagnosis for the subsequent pregnancies. The diagnosis of iron deficiency anemia was made on basis of Hb values<11g/dl, MCV<80fl, increased RDW,and confirmed with serum ferritin<12µ/L AND serum iron<60mg/dl.

The results were tabulated and relevant statistical analysis was done.Variations of $P<0.05$ were considered significant. Test of significance chi square tests were used.

Results:

A total of 174 pregnant women attending antenatal clinics were screened for detecting hemoglobinopathy withthe help of NESTROFT, red cell indices, haemoglobin electrophoresis and HPLC. In the present study, majority of the females 82(47.12%) were in the age group 21-25 years while 138 (79.31%) were in the age group of 20-30 years (Figure 1).

All the 174 pregnant women were subjected to the NESTROFT test as a primary screening test, 16 were found to be positive and 158 negative. (Table 1)

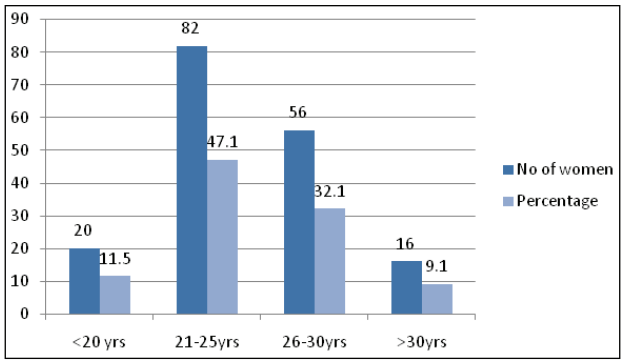

Figure 1: Age-wise distribution of the patients

Out of 174 subjects in the study, 78 (44.82%) had normal red cell indices while microcytosis (Mean Corpuscular Volume MCV<80fl) was seen in 96 (55.17%) of the women. Out of the women with microcytosis 4(2.29%) confirmed to be of BTT. 68 (73.91%) out of the 92 patients with microcytosis had decreased serum ferritin values while the rest 24 (26%) were not of IDA. (Table 1). Ferritin values vary according to pregnancy period due tohemodilution. and this fact was considered in our study.

Table 1: Screening test results of NESTROFT

Primary screening test NESTROFT	Number of patients	Percentage
Positive	16	9.19%
Negative	158	90.81%
Total	174	100%

Table 2:Age-wise distribution of cases according to diagnosis

Age (Yrs)	Normal	BTT	MHA/ IDA	Total
<20	7	-	13	20 (11.49%)
21-25	38	1	43	82 (47.12%)
26-30	24	1	31	56 (32.18)
>30	9	2	5	16 (9.19%)
	78(44.82%)	4 (2.29%)	92 (52.87%)	174

*MHA(Microcytic Hypochromic Anemia)On further evaluating the NESTROFT positive results with HPLC for diagnosis of BTT, only 4 cases were true positive with HbA2>3.5% and 12 had HbA2 < 3.5% and comprised the false positive group. Hence, for detecting the BTT, the sensitivity and specificity NESTROFT were 100% and 92.9% respectively in our study.A cut off Hb A2 level of ≥ 3.5% was used to confirm the diagnosis of thalassemia trait and values between 3.2% and 3.5 % were considered to be of borderline cases. Thus, the disease prevalence amongst the pregnant women who attended the ANC clinic at the HAHC hospital was 2.3% (with 95% CI of 0.63% to 5.78%)

Table 3: Distribution of cases according to diagnosis on NESTROFT screening

	NESTROFT +	NESTROFT -	TOTAL
BTT	4	0	4
MHA/IDA	10	82	92
NORMAL	2	76	78
TOTAL	16	158	174

On evaluation of diagnosis NESTROFT positive and negative cases, it was seen that only four cases were BTT while ten were of microcytic anaemia and two had normal red cell indices. Of the 158 NESTROFT negative cases 51.9% were in the MHA category and 48.1 % in the normal red cell indices category, no false negative case was seen in the present study. The red cell parameters wereanalyzed for their mean values ± standard deviation depicted in Table 4. The P value to see statistical significance ofthe mean values was also further calculated , where P value <0.05 was taken to be statistically significant. All red cell parameters including Haemoglobin(Hb), Mean corpuscular volume, Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC) and Red Cell Distribution Width (RDW) were found to be statistically significant Pvalue< 0.05 while Red blood cell (RBC) count and Haematocrit values (HCT) were not statistically significant(P value >0.05).Reticulocyte counts were within normal range of adult.

Table 4: Mean Values of Various Red Cell Parameters

Red cell Parameter	BTT (Mean±SD)	NBTT (Mean±SD)	P value
Hb(gm/dl)	8.87±1.6	10.64±1.61	0.020
RBC count ($X10^6$)	4.33±0.70	3.95±0.51	0.301
HCT(%)	30.27±4.37	33.23±4.24	0.093
MCV(fl)	68.67±4.60	78.98±8.39	0.024
MCH(pg)	23.12±0.54	27.03±3.18	0.041
MCHC(%)	29.1±1.68	31.92±1.90	0.048
RDW(%)	26±4.12	39.76±15.5	0.003

Discussion:

The idenification of β thalassemia trait is important especially in developing countries where resources are limited and management expensive. Not only does the major form of the disease compromise quality of life it is an extra financial burden on the family.Also, as the red cell morphology in beta thalassemia trait is microcytic hypochromic; these patients are often misdiagnosed, as those suffering from iron deficiency anaemia and given unnecessary iron medication.[3] Hence, the aim of the present study was to screen the pregnant women attending the antenatal clinic of our hospitalby NESTROFT test to assess the burden of β thalassemia trait of antenatal mothers of this areaas well as offercounselling and prenatal diagnosis.

Beta thalassemia is the commonest inherited hemoglobinopathy, population screening has identified the prevalence of β-thalassemia carrier status to be as high as 17% in certain communities in India.[6]The classical heterozygous form ofBTT is usually asymptomatic. The family history of thalassemia is important however a significant number of patients do not have previouslyaffected family members.[7]The *prevalence* of BTT is known to vary from 1.0%-14.9% in various regions of India. [8] In the present study the disease prevalence was 2.3% of the screened 174 cases.The lesser number of women ready for screening in the present study as compared to other studies,[4,6]could be attributed to lesser awareness about hemoglobinopathies amongst the health-care professionals, pregnant women and their families. Majority of the studies were based on population including all ages and both sexes while our study was based on antenatal mothers.

The majority of the screened mothers (47.1%)were in the age group 21-25 years with the mean age of 24.72 years and SD ±3.76.This was in concordance to 60% women in this age group in a study by Parikh et al[6] However other authors have also reported a similar agegroup and a female preponderance in their studies [3,8].

The distribution of β thalassemia gene in the Indian subcontinent is not uniform possibly due to ethnic diversity, it exists in different regions with varying frequency, ranging from <1-17% with an average of 3% [9] Disease prevalence in the present study was 2.3% in pregnant women. A prevalence of 3.4% of β thalassemia carrier state, among antenatal women has been reported by Chakraborti et al.[10]and 3.1% by Sujatha et al [8] in previous studies.

Effective screening will continue to be the backbone of preventive strategies against BTTespecially in countries where prevalence is high and resources limited. Consequently, there is considerable emphasis on strategies to optimize the cost–benefit ratio of mass screening. [11] Naked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT) can be a very useful screening tool for BTT because of low cost and less technically advanced and easy result availability. It is based on the principle of decreased red cell osmotic fragility and increased resistance to osmotic lysis that occur due to altered shape and functioning of red cell.[12,8] The sensitivity and specificity of NESTROFT in detection of BTT in the present study were 100% and 92.9% respectively, in concordance with other authors, 95.2% sensitivity and 94.1% specificity[10] and 85.4% and 100%[3] Although considered to be an effective screening test, authors have argued that the success of NESTROFT in large-scale screening programmes is lessened because of the associated false-negative error rate.[11] Since the costs of treating β-thalassaemia major cases is highthat could result from the β -thalassaemia carriers missed by NESTROFT, it has been suggested that information from the haematological parameters output by automated cell counters should be combined with the results of NESTROFT to improve the yield from screening strategies for BTT.[11] In evaluating a potential screening programme, false- negative and false-positive error rates should be considered in addition to cost. The false negative results could not be accurately assessed due to limitations, the false

positive results however were 6.8% in present study which was lower than 15.32% reported by Piplani et al.[3]The lower values in our study could be attributed to small sample size.Previous workers have also reported false positive rates ranging from 16.4% to 18.5%. [10,13,14,15]The positive and negative predictive values (PP, NPV) reflect the presence or absence of disease when a test is positive or negative. Although, we could not reliably calculate these values due to limitation of the sample size reflecting the true prevalence of the disease, the role of these values is highlighted in many reports,[4,10,3] a high negative predictive value of the test is important as it almost rules out the possibility of beta thalassemia trait in the population tested negative with a particular screening test (NESTROFT) hence a negative test can reliably rule out BTT.[4]The PPV on the other hand show a wide variation ranging from 33.6% to 100% with majority ranging between 51.9% to 73.1%.[3]

As has been suggested, haematologicalparameters by automated cell counters could be combined with the results of NESTROFT to improve the yield from screening test for BTT.[11]. However, authors suggest that red cell parameters have a limited use and can be influenced by several non genetic causes such as nutritional status and heritability only explains only ~50% of the variability in these parameters.[16] A comparison of the mean values of different parameters along with their P values was analyzed in the present and a significant statistical association was drawn between BTT and Non BTT all red cell parameters except red cell count and andhaematocrit values. However in a study by Patel et.al a significant association was seen in all red cell parameters.[4] Among the various parameters, the role of RDW is important but has limited specificity.[4] Values of MCV and MCH were significantly lower in BTT cases in our study in concordance with other studies[103] although it is recommended that MCH is used to screen thalassemia as this parameter is more stable than MCV. Nutritional anaemia commonly coexists both at a geographic and individual level, and can severely confound the red cell parameters. An increase in MCV in BTT patients has been attributed to possible concomitant Vitamin B12 or folic acid deficiency or could be due to significant intramedullary lysis of the red cells leading to a spillage of immature erythroblasts into peripheral circulation. [11]According to Sujatha et.al, RBC > 5 x 10^{12}/l and mildly decreased Hb were best combination for prediction of BTT. An increase in RBC count has also been observed in BTT patients by Pethe et.al [9]In the present study, lower hematocrit levels and mean Hb% could possibly be due to prevalence of anemia during pregnancy. The relative advantage of the haematological examination of peripheral blood over NESTROFT is unknownThe peripheral smear finding in both iron deficiency anemia and β-thalassemia are microcytic hypochromic, hence, often misdiagnosed and supplemented with unnecessary iron therapy. In countries such as India, it seems appropriate to includethe haematological investigation as a screening aid to NESTROFT for antenatal screening of BTT, because the screening services can be effectively integrated with the existing services for institutional antenatal care in primary, secondary or tertiary health care settings.[11]

Conclusion:

Thalassemia is better prevented than treated, promoting prevention and effective utilization of prenatal diagnosis still remains the goal. NESTROFT is a highly sensitive as well as specific test for detecting beta thalassemia trait.Besides being economical and easy to perform. It is an effective reliable screening test when employed alone. More so, red cell parameters can be an added advantage in screening and it seems appropriate to include these investigation as a screening aid to NESTROFT for antenatal screening of BTTThe significance of NESTROFT as a preliminary screening method for beta-thalassemia trait in resource limited areas with economic constraints in the provision of public health services, cannot be underestimated.Hence we propose the addition of NESTROFT for screening of BTT in pregnant women among the antenatal tests in resource restricted set up. Thus, antenatal diagnosis of BTT is an important screening programme to reduce the burden of birth of children suffering from thalassemia and other hemoglobinopathies.

Author contribution

SR was responsible for conception & drafting the manuscriptZSJ was for acquisition, analysis and interpretation of data for the work; SJ was responsible for editing the manuscript. She is the guarantor, final approval of the version. MJH was responsible for review of literature.MG is the referring clinician keeping track and follow up of the patients.

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