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Reproducibility of Naked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT) as a screening test for the detection of β thalassemia and Hemoglobin E traits with the variation of reading time

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Abstract:

Background: *Though 0.36% NaCl buffered solution has been used as an effective reagent for carrying out Naked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT) as a screening test for the detection of β thalassemia and Hemoglobin E traits, reading time for its interpretation is yet to be settled.*

Methods: *This cross -sectional analytic study was done at Department of Pediatric Hematology and Oncology, BSMMU from January 2006-June 2007. NESTROFT was performed in 3 separate tubes for each of the 100 subjects (with â thalassemia trait and HbE trait and normal hemoglobin) with 0.36% NaCl buffered solution and reading was taken at different time at 5-minute, 15-minuteand 30-minute. Results were recorded as positive and negative. Hb electrophoresis, Hemogram with RBC indices for each sample and iron profile of selected subjects were done.*

Results: *Among the 100 study subjects, 48 were carriers (18 were â thalassemia traits, 30 were Hb E traits). Age range was 1 to 52 yrs. Mean Hemoglobin concentration, MCV, MCH of both β thalassemia and Hb E carrier were significantly lower than normal subjects (p values <. 001). At 5-minute observation, NESTROFT was positive in 17 out of 18 â thalassemia traits with a sensitivity of 94.4%, 24 out of 30 HbE traits gave positive result with a sensitivity of 80%. Among the 52 normal subjects 35 gave negative result with a specificity of 67%. Positive predictive value and negative predictive value for detection of β thalassemia traits and HbE traits were 50% & 97% and 58% and 85%respectively. Result of NESTROFT interpreted at 15-minute and 30-minute did not show any change.*

Conclusion: *From this study it may be concluded that for interpretation of NESTROFT reading at 5-minute would be optimum.*

Keywords: *NESTROFT, β– thalassemia , Hemoglobin E traits, screening.*

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Introduction:

Thalassemia and hemoglobinopathies are the most common inherited disorders in human and they represent a global public health problem. Historically, thalassemia and hemoglobinopathies occur in highest frequency in tropical region along the falciparum malarial belt, because of population migration it is now in increasing trends in non endemic countries of the world.[2,3] World Health Organization (WHO) estimates that at least 5.2% (over 360 million) of world populations (and over 7% of pregnant women) carry significant Hb variants and over 330,000 affected babies are born annually and approximately 80% of these births occur in low or low-middle-income countries with unsatisfactory management facilities.[4,5] Thus birth of a thalassemic child poses a considerable physical and economic burden to the community and nation.[6,7] Therefore, screening program for haemoglobin disorders should be included in the basic health services in most countries.[4]

Naked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT) has been regarded suitable mass

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screening test as it is cheap cost effective, rapid and reliable screening test. [7,8] NESTROFT is the modified osmotic fragility test which is based upon the principle that thalassemic red cells are resistant to lysis when placed to hypotonic saline more than that of normal red cell. [9,10]

Osmotic fragility was the first method used for screening of thalassemia and was introduced as simple approach to detect thalassemia carrier in Rome by Silvestroni & Bianco in the 1940's.[2] They used 0.40% Tyrode's solution as a regent for the test.[11] Nastev et al. [12], Katamis et al.[11] and Chow et al.[10] did comparative study with different strength of NaCl solution (0.32%, 0.34%, 0.36%) and 0.40% Tyrode's solution and had standardized of using 0.36% NaCl solution in single tube and interpretation by visual inspection through the sample of a black line drawn on a white paper against which the tube is to be held. The result is recorded as *positive* if the black line is not visible, *negative* if black line is clearly visible and *doubtful* if the line appears blurred. Positive and doubtful results indicated that all the red cells in the tested sample have not undergone lysis in hypotonic saline and so presence of thalassemia carriers.[13] During the past decades many other authors had reported the efficacy of NESTROFT as a screening test for β thalassemia and Hb E carriers detection. [4,7,14-18] Maheswari et al.[19] in 1999 made observation at 20 minute and had reported sensitivity; specificity, positive predictive value and negative predictive value were 91%, 95%, 55% and 99 % respectively.

In this view, this study has been designed to see the influence of various reading time with same sample on outcome of NESTROFT and to have an optimum reading time for interpretation of NESTROFT.

Methodology:

This cross-sectional analytic study was done in the department of Pediatric Hematology and Oncology, Bangabandhu Sheikh Mujib Medical University (BSMMU) , Dhaka, Bangladesh from January 2006 – June 2007. One hundred cases were recruited from volunteers attending Pediatric Hematology laboratory for screening and relatives of thalassemia syndromes being counseled for screening. These cases were evaluated by taking complete history regarding transfusion and systemic illness and thorough physical examination for exclusion of thalassemia syndrome. Known thalassemia syndrome and those who received blood transfusion within three months were excluded from the study. After then the study subjects and /or their guardians were given information about the motive of the study for providing written/ verbal consent.

From each subject 4 ml of venous blood was taken from ante cubital vein with all aseptic precaution in 5 ml syringe, kept into two Eppendorp tubes containing 100 µl EDTA (ethylene di amino tetra acetic acid) by 2 ml in each. From 1st tube hematologic analysis was done clinical pathology department of BSMMU using Sismax Hematology analyzers *(XT 2000i, Fluorescence Flow cytometry 6 part differential, 40 parameters analyzer, Manufacturer Sysmex Corporation, Country of origin Japan)* which provides full blood count including Hb%, hematocrit, Red cell count, total and differential count of white blood cell along with platelet count and calculated RBC indices like Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC).

From 2nd tube NESTROFT was done on the same day within 4 hours of collection of the sample without the knowledge of hematological analysis and electrophoresis report of cases and the rest of the sample was stored at 4^0C for Hb electrophoresis. For each case 2 ml of freshly prepared 0.36% buffered solution was taken in into each 3 test tubes (12x75) mm^2 with the marking of 5 min, 15 min and 30 min. Twenty micro litter (20µl) whole blood from 2nd eppendorp was added to the solution in each tube and then allowed to stand undisturbed at room temperature. Readings were taken at 5-minute, 15-minute and 30- minute. Two readings were taken for each tube first before and second after shaking the tube and were recorded as per the following table.

Interpretation was made by holding test tube against a sharp black line drawn on white paper placed on wall to check its visibility and the result were recorded as *positive* when the black line was not visible, *negative* when it was clearly visible and *doubtfu*l when it appeared blurred. For statistical calculation, both the positive and doubtful results were taken as positive.

Fig.-1: *Work Station of NESTROFT*

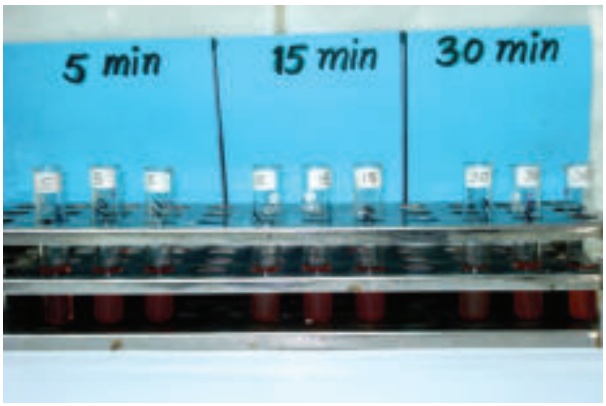

Figure.-: *NESTROFT stand showing observation on different reading time*

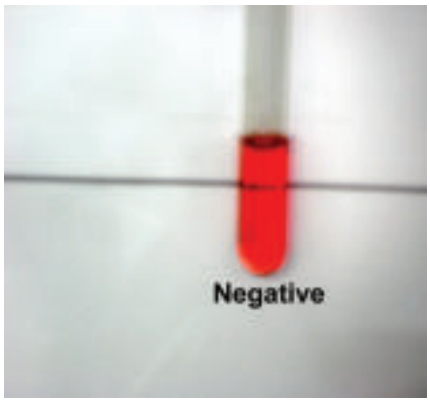

Figure 3

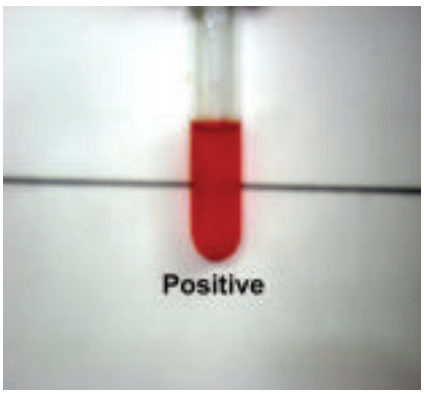

Figure 4

Additional 2 ml of blood was collected from 10 cases who were NESTROFT positive with normal hemoglobin pattern on electrophoresis but had low MCV<77fl and MCH<27pg value for estimation of serum ferritin. Two blood smears were prepared and dried in air during collection for blood film study. Peripheral blood films were examined under microscope for RBC morphology and checking the report given by electronic counter by the authors after Leishman stain following standard procedure.

Hemoglobin electrophoresis was done on each sample to see the variants of hemoglobin within 7 days of collection. The test was carried out by Beckman Coulter (made in USA) Paragon^{R} Hemoglobin (Hb) electrophoresis system KIT (P/N441780) by Hydra Gel (agar gel) electrophoresis in alkaline media following manufacturer's instruction. After electrophoresis the hemoglobin in the gel were immobilized to a fixative solution and the gel was then dried to a film. The hemoglobin pattern was then visualized by staining the film with a protein specific stain as per manufacturer's guidelines. Analysis of data was done by Statistical Package for Social Science (SPSS) version 10.0 was used.

Results

NESTROFT results of 100 samples at 5-minute, 15-minute and 30-minute observation were analyzed. Age of the study subjects ranged from 1 year to 52 years with a mean age 18.25 ±13.10 yrs. (Table I) Among the 100 cases 52 were male and 48 were female with male to female ratio was 1.08:1 (Figure-5). Study subjects were categorized as *â* thalassemia trait in 18 cases, HbE trait in 30 cases and 'Normal Subject' in 52 cases according to case definition (Figure-6).

Table I

Age distribution of the studied subjects (n 100)

Age groups(years)	Number	Percentage
1- 15	51	51 %
16-30	30	30 %
31-45	18	18%
46-60	1	1%

Hemoglobin concentration of 'Normal Subjects' was 12.16±2.77 gm/dl, of HbE trait was 12.01±3.49 gm /dl and of *â* thalassemia trait was 10.46±1.82 gm/dl. As compared to 'Normal Subjects' reduction of Hb level was significant in β thalassemia trait (p value 0.05) but not in HbE trait (p value 0.82). (Table II)

Highest level of Mean MCV was observed in 'Normal Subjects' (83.13±11.35fl) and lowest level was found in *â* thalassemia trait (64.98± 6.13fl) and in HbE trait it was 73.78±2.96fl. Reduction of MCV observed both in HbE trait and β thalassemia trait as compared to 'Normal Subjects' was highly significant (p value 0.00 & 0.00). (Table II)

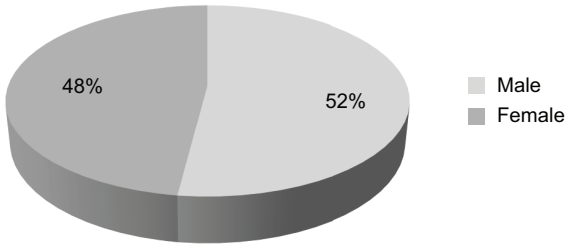

Fig.-5: *Sex distribution of cases (n=100)*

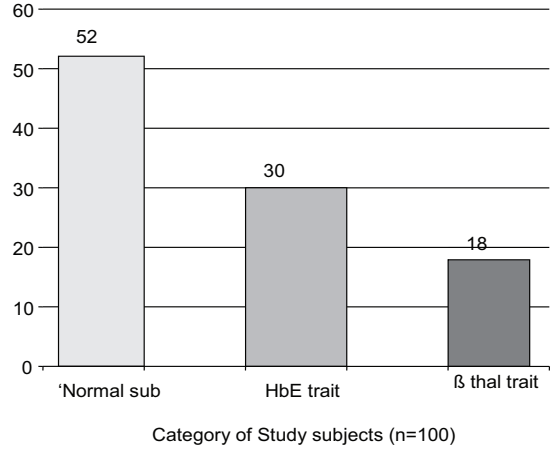

Fig.- 6 : *Category of study subjects on the basis of Hb electrophoresis*

Mean MCH (mean±SD) was highest in 'Normal Subjects'(25.11±4.49 pg) and lowest level was observed in *â* thalassemia trait (19.07±2.38 pg), level in Hb E trait was in intermediate place (21.92±2.96pg). Mean MCH was also found significantly low both in HbE trait and β thalassemia trait in comparison to Normal subjects (p value 0.001 & 0.00). Mean MCHC (mean±SD) of 'Normal Subjects' was 29.66 ±2.69, of HbE trait was 29.75±1.77 and of β thalassemia trait was 29.27±1.62. No statistical significant difference of MCHC found in traits as compared to'Normal Subjects' (p value 0.86 & 0.57). (Table II)

There was no change of interpretation of NESTROFT in different time of observation. All the positive results observed at 5 minute remain positive at 15 minute and 30 minute reading. Similarly, none of the negative results at 5-minute did show positive result on 15-minute or 30-minute observation. (Table III) Among 18 cases of *â* thalassemia trait 17 showed positive result, sensitivity was 94.4%, and 24 cases of HbE traits out of 30 showed positive result with a sensitivity of 80%. Among the 'Normal Subjects' the test was positive (false positive) in 17 cases and was negative in 35 (true negative) so specificity was found 67%. Positive Predictive value was 50% for *â* thalassemia trait and 58% for Hb E trait. Negative Predictive value was 97% β thalassemia trait and 85% for Hb E trait. (Table IV)

Serum ferritin level of 10 'Normal Subjects' who gave positive result on NESTROFT having low MCV <77fl & MCH <27pg. Serum ferritin of these patients was estimated for assessment of iron deficiency, which might cause positive result of NESTROFT. Among these 10 cases, 4 patients had low serum ferritin ≤12µg/L. Six cases had normal serum ferritin among which 4 had HbA_2 <2% and other 2 cases had borderline HbA2 (3.0-<3.5%). (Figure VII)

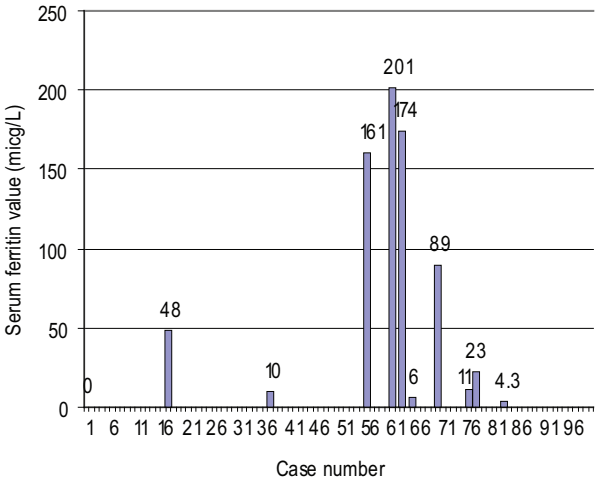

Figure 7: *Serum ferritin value of studied cases (n=10)*
**Numerical indicates study subject number

Table-II

Comparison of hematological values of study subjects (n=100)

Parameter	Normal Subjects	HbE trait	β thalassemia trait	t -test(df)	*p* value
Hemoglobin (mean± Sd)(gm/dl)	12.16 ±2.77	12.01 ± 3.49		0.22(80)	0.82
	12.16 ±2.77		10.46± 1.82	2.95 (45.35)	0.05*
MCV(mean± Sd)(fl)	83.13± 11. 35	73.78 ±7.79		4.14(80)	.000*
	83.13± 11. 35		64.98 ± 6.13	6.44(68)	.000*
MCH (mean± Sd)(pg)	25.11±4.49	21.40 2.99		3.59(80)	.001*
	25.11±4.49		19.07± 2.38	5.4(68)	.000*
MCHC(mean± Sd)	29.66±2.71	29.75±1.71		0.16(80)	0.86
	29.66±2.71		29.27± 1.62	0.57(68)	0.57

Table-III

Analysis of NESTROFT outcome depending on different reading time

Category of subjects	NESTROFT RESULT					
	5-minute reading		15-minute reading		30-minute reading	
	Positive N (%)	Negative N(%)	Positive N(%)	Negative N(%)	Positive N(%)	Negative N(%)
Normal Subjects(n=52)	17 (33)	35(67)	17(33)	35(67)	17(33)	35(67)
Hb E Trait (n=30)	24(80)	6(20)	24(80)	6(20)	24(80)	6(20)
Beta thalassemia Trait (n=18)	17(94.4)	1(5.6)	17(94.4)	1(5.6)	17(94.4)	1(5.6)
Total	58(58)	42(42)	58(58)	42(42)	58(58)	42(42)
	100	100	100			
			Sensitivity			
HbE traitBeta thalassemia trait		80%		80%		80%
		94.4%		94.4%		94.4%
SPecificity		67%		67%		67%
Positive predictive Value		58%		58%		58%
HbE traitBeta thalassemia trait		50%		50%		50%
Negative predictive value		85%		85%		85%
HbE traitBeta thalassemia trait		97%		97%		97%

Table IV

Statistical calculation of β thalassemia traits detected by NESTROFT

		β–*th\al traits*			
	+	-			
		(Normal subjects)			
NESTROFT	+	a	b	All with positive result	Positive predictive value
		17	b17	=(TP+FP)	=TP/(TP+FP)
		(TP)	(FP)	= 34	=17/34=0.50
	-	c	d	All with negative result	Negative predictive value
		1	35	=(TN+FN)	=TN/ (TN+FN)
		(FN)	(TN)	=36	=35/36
					=0.97
Total	18	52			

TP: True positive; TN: True negative; FP: False positive; FN: False negative

$$\text{Sensitivity: } = \frac{a}{a+c} = \frac{17}{18} = = 0.944$$

$$\text{Specificity: } = \frac{d}{b+d} = \frac{35}{52} = = 0.68$$

Table IV

Statistical calculation of β thalassemia traits detected by NESTROFT

		HbE traits			
	+	- (Normal subjects)			
NESTROFT	+	a 24 (TP)	b 17 (FP)	All with positive result =(TP+FP)= 41	Positive predictive value =TP/(TP+FP) =24/41 =0.58
	-	c 6 (FN)	d 35 (TN)	All with negative result =(TN+FN)=41	Negative predictive value =TN/ (TN+FN) =35/41 =0.85
Total	30	52			

TP: True positive; TN: True negative; FP: False positive; FN: False negative

$$\text{Sensitivity: } = \frac{a}{a+c} = \frac{24}{30} = = 0.80$$

$$\text{Specificity: } = \frac{d}{b+d} = \frac{35}{52} = = 0.68$$

Discussion

The present study evaluated the effect of 3 readings at 5 minute, 15 minute and 30 minute outcome of NESTROFT to get an optimum reading time. Mean age was 18.25 ±13.10 yrs and 51% of the cases were ≤15 yrs of age representing sample from children and adult group of people.

Here, Hb concentration of β thalassemia trait was significantly lower than that of 'Normal Subjects'. This finding was comparable with the findings of many other authors.[8,16] However, we did not find significant reduction of hemoglobin concentration in HbE trait as compared to 'Normal Subjects' (p value 0.82) which is consistent with the finding of other authors. [9,20]

Mean Corpuscular Hemoglobin (MCH) was lowest in β thalassemia trait also low in HbE trait. Reduction of MCH found both in HbE and β thalassemia traits was statistically significant as compared to that of 'Normal Subjects' (p value 0.001 & <0.001). This finding was comparable with that of Lanzkowsky and Vichinsky.[9,20] Modell and Berdoukas described that MCH level in thalassemia carriers had become low because of defective production of globin chain and it usually had varied from 18-25pg in different thalassemic trait).[21] Mannan et al found lower level of MCH in thalassemia and Hb E traits which was comparable to the finding of the present study.[16]

Mean corpuscular volume (MCV) was found lowest in β thalassemia trait and lower in HbE trait. Reduction of MCV level in both the traits was significant as compared to 'Normal Subjects' (p value 0.00 & 0.00). Lower levels of MCV reported by Vichinsky[9], and Mannan[16] were comparable with our finding. Modell and Berdoukas described that the reduction of MCV in thalassemic red cells is due to reduced MCH that reduces intracellular oncotic pressure and fluid volume.[21]

MCHC level observed both in HbE trait and *â* thalassemia trait did not have any significant difference as compared to 'Normal Subjects' (p value was >0.5). Proportionate reduction of MCH and MCV did not affect MCHC. Maheshwari et al.[13] also describes that thalassemic traits in general have reduced MCV and MCH with normal or near normal MCHC.

The results of NESTROFT at 5-minute observation, 17 out of 18 cases of β thalassemia traits showed positive result with a sensitivity of 94.4%, which is comparable to the report by Kattamis et al.[11], Chow

et al.[10] and Mannan[16] who made observation at 5 minute with a range of sensitivity 91.5- 98.4%.

Among 30 HbE traits, 24 showed positive result with a sensitivity of 80%. This finding was consistent with that of Mannan1[16] who reported with a sensitivity of 84%. Fucharoen et al.[26], reported a sensitivity of 72% in detection of HbE trait by osmotic fragility test however they use 0.34% NaCl solution. Hundred percent sensitivity was reported by Raghavan et al.[14] and Thomas et al.[18] but their sample size was small. On the other hand, low sensitivity of the test in detection of HbE trait was reported by Kattamis et al.[11] (68.8 %) and Chow et al.[10] (54%).

Among the 52 'Normal Subjects', 35 cases gave negative (true negative) result of NESTROFT. The specificity was 67%, which was comparable with those of Manglani et al.[19], Thomas et al.[18] and Mannan[16]. Higher specificity 87%-91% reported by other authors.[10, 11, 14, 15]

Lower specificity reported by the authors was due to the fact that in their study, normal subjects were selected from hematology clinics and cases of iron deficiency anemia and á thalassemia carrier who might be giving positive result were not excluded. In our study, out of 17 normal subjects giving positive results 4(23%) were found iron deficient and another 4 (23%) had thalassemic RBC indices with normal serum ferritin needing evaluation for β thalassemia and 2 (11%) cases had borderline HbA2 (>3.0-<3.4%) with normal serum ferritin warrants assessment for silent thalassemia carriers by DNA testing.

Positive Predictive value of NESTROFT at 5-minute observation was 50% for β thalassemia trait and 58% for HbE trait. The negative predictive value was 97% for β thalassemia trait and 85% for HbE trait. Finding of the present series was comparable to the report of Mehta et al.13 and Begum et al. 14. On the other hand positive predictive values reported by Mangalani et al.7 was lower than that of our value but Kattamis et al.[11] and Thomas et al.18, reported higher positive predictive value. The high negative predictive value of NESTROFT observed in this study was comparable with most of the authors.[10,11,14,15]

The interpretation of NESTROFT result recorded at 15-minute and at 30-minute showed positive result for 17 (94.4%) cases out of 18 cases of *â* thalassemia trait, 24 (80%) cases out of 30 cases of HbE trait and negative result for 35 (67%) cases among 52 cases of 'Normal Subjects'. The calculated sensitivity, specificity, positive predictive value and negative predictive value of NESTROFT at 15-minute and 30-minute observation were same as those found at 5-minute reading.

So, it could be suggested from the study that increasing reading time for interpretation of NESTROFT is not necessary. Therefore 5-minute recording were assumed to be optimum for interpretation of the result of NESTROFT.

Conclusion:

It may be concluded that for interpretation of Naked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT), 5-minute reading time would be optimum.

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