Original Article

Validity of Naked Eye Single Tube Red Cell Osmotic Fragility Test in Screening of ß-Thalassemia Trait

S.M. Alinoor Islam[1], Md. Kamrul Ahsan Khan[2], Liakat Hossain [3], Sheikh Abdullah Al Mahmud[4], Md. Khairuzzaman[5], M. Ekhlasur Rahman[6].

ABSTRACT: Background: *Worldwide the hemoglobin disorders are the most common clinically serious single gene disorders and are often regarded as incurable and expensive to treat.* ***Objective:*** *To find out sensitivity, specificity and predictive values of Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT) for detection of carriers of ß-Thalassemia.* ***Methods:*** *This descriptive analytic type of study was carried out in the Department of Pediatrics of Sylhet MAG Osmani Medical College Hospital, Sylhet, from July 2006 to June 2008. A total of 70 samples, 35 cases and 35 suitably matched controls were enrolled. In this study, siblings, parents and first degree cousins of patients of β-thalassemia major who had Hemoglobin A2>3.6%+HbA on hemoglobin electrophoresis were selected randomly as cases and the same of whom had Hemoglobin A2 (2.5±0.2)+ HbA on hemoglobin electrophoresis were selected as controls. Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT) was done from the cases and controls.* ***Results:*** *Sensitivity and specificity of NESTROFT in the diagnosis of carriers of β-thalassemia were found 94.3 % and 88.6 % respectively. The positive predictive value and negative predictive value of NESTROFT in the diagnosis of carriers of β-thalassemia were found 89.1% and 94% respectively.* ***Conclusion:*** *NESTROFT is a valid screening test for detection of ß-thalassemia trait and NESTROFT may be used as an alternative to hemoglobin electrophoresis to detect ß-thalassemia trait in Bangladesh.*

Key words: *Hemoglobin disorders, ß-thalassemia trait, Hemoglobin electrophoresis, NESTROFT.*

(The Planet 2018; 2(2):4-11)

INTRODUCTION:

The inherited disorders of hemoglobin are the commonest single-gene disorders in man. They fall into three overlapping groups: structural variants, thalassemias characterized by reduced rate of synthesis of one or more globin chains, and conditions in which fetal hemoglobin synthesis persists

1. Assistant Professor (Pediatric Nephrology), Sheikh Sayera Khatun Medical College. Gopalgonj.
2. Assistant Professor (Neonatology), Sheikh Sayera Khatun Medical College, Gopalgonj.
3. Professor (Cardiology) and Principal, Sheikh Sayera Khatun Medical College, Gopalganj.
4. Assistant Professor (Pediatrics), Sheikh Sayera Khatun Medical College. Gopalgonj.
5. Assistant Professor (Pediatrics), Sheikh Sayera Khatun Medical College. Gopalgonj.
6. Professor and Head (Ex), Department of Pediatrics, Sylhet MAG Osmani Medical College Sylhet.

beyond the neonatal period; collectively known as hereditary persistence of fetal hemoglobin. Hemoglobin disorders are responsible for an extremely complex series of clinical phenotypes.[1]

The World Health Organization has suggested that about 5% of the world populations are carriers for different inherited disorders of hemoglobin. WHO reports also state that about 370,000 severely affected homozygotes or compound heterozygotes of thalassemia are born every year.[1,2,3] Although these conditions occur at their highest frequency in tropical regions, population migrations have ensured that they are now encountered in most countries.[4]

Thalassaemia and haemoglobinopathy are the most common inherited disorders among humans, and they represent a major public health problem in many areas of the world, including south-east Asia. The most important disorders are α-thalassaemia and β-thalassaemia. The prime targets of prevention and control of severe thalassaemia are homozygous α^0-thalassaemia, homozygous β-thalassaemia and β-thalassaemia–HbE disease.[5]

Thalassemia is one of the commonest inherited diseases in Bangladesh, having population of over 170 million. A conservative world health report has estimated that 3% are carriers of β–thalassemia and 4% are carriers of Haemoglobin-E disease in Bangladesh.[6] However, these estimates must be interpreted with caution since the data was mainly based on studies conducted in 1980, and a small number of non-representative samples obtained from treatment centers were analyzed. A study on the prevalence of thalassemia among 735 school children in Bangladesh, found the prevalence of β-thalassemia trait was 4.1% and Hb-E trait was 6.1%.[7]

There is no national data about the number of thalassaemia patient in the country. Khan et. al.[7] estimated that existing thalassaemic patient in Bangladesh is about 1 lac and suspected total number of β-thalassaemia major and Hb-E β-thalassaemia born around 1040 and 6443 per year respectively in our country. Rahman et. al.[8] in a study conducted in Bangabandhu sheikh Mujib Medical University, in Bangladesh, found that, among the thalassemic patients 67% had HbE-β Thalassemia and 29% had β-Thalassemia major or intermedia. In another study, in Dinajpur Medical college hospital, Hasan et al. observed that among 60 patients, Hb E Trait was 41.67% and Hb E disease was 30% and Hb Eβ-thalassaemia and β-thalassaemia trait was 23.33%, 3.33%% respectively.[9] But according to Khan et al.[7] carrier status of Hb-E is 6.1% and as high as 41.7% in tribal school children in Bangladesh. Farhana et. al.[10] found 17.39% Hb E trait and 13.04% Hb E disease in their study done in Bangladesh Institute of Rehabilitation in Diabetes Endocrine and Metabolic disorder (BIRDEM). So, it is explicable that thalassaemia will be a emerging health burden for our country, if it is not prevented.

As these disorders are hereditary and incurable as well as expensive to treat, to avoid fatalities from untreated thalassaemia, the expense and difficulty of

providing optimum treatment for patients, which creates burden on patients, families and national health services, moreover, high frequency of the condition in some populations are the reasons why it is important to develop prevention programs. ß-Thalassemia is an ideal example of all genetic diseases in that, identification of carrier is possible. Identification of carriers of the thalassemia gene plays an important role in preventing this fatal but preventable disease. Any at-risk person can then offered reproductive choice and avoid the birth of an affected child.

The most effective approach to reduce the burden of the society and reduce the disease incidence is implementation of a carrier screening program, offering genetic counseling, prenatal diagnosis and selective termination of affected fetuses. Though there is a definite need for carrier screening in our country, it is hard to draw a consensus regarding the age group upon whom screening will be done. Due to lack of education and public awareness about the disease, even carrier status for a disease can become a stigma.

Various options available are: (i) Screening of school going children (ii) Screening of high risk communities (iii) Premarital screening (iv) Extended family screening-screening of relatives if there is a thalassemic child in a family and (v) Routine antenatal screening in early pregnancy, ideally between 10-12 weeks. The first three options are logistically not feasible in our country due to social and economic reasons. Extended family screening is acceptable and is being practiced to a certain extent, but with such a program we are likely to miss many carriers.[11] However, the technical facilities, infrastructure and financial resources available, affect both the strategy and the choice of method for carrier identification.

Different screening methods are available to detect carriers of β-thalassemia trait. In our country measurement of red cell indices and hemoglobin electrophoresis are in limited use. All laboratories do not have the facility of electronic cell counters to measure red cell indices and facilities for hemoglobin electrophoresis. Other option like high performance liquid chromatography needs high technologies and sophisticated equipment. Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT) is a sensitive, cost effective, rapid and reliable screening test for detection of β-thalassemia trait.[12] NESTROFT is being done in abroad extensively to detect carriers of β-thalassemia trait. NESTROFT was evaluated as a screening tool for detection of thalassemia trait in our country, where they recommended a further multicenter study.[13] So, this study was designed to find out 'Sensitivity, Specificity and Predictive Values of Naked Eye Single Tube Red Cell Osmotic Fragility Test (**NESTROFT**) for Detection of ß-Thalassemia Trait. In this study **NESTROFT** is evaluated against hemoglobin electrophoresis.

METERIALS AND METHODS:

This descriptive type of analytical study was carried out from July 2006 to June 2008 in the department of Pediatrics, Sylhet MAG Osmani Medical College Hospital (SOMC), Sylhet. All the siblings, parents and first degree cousins of patients of β-thalassemia

were the study population. Systematic random sampling technic were used and every second case was included in the study. A total of 35 cases and 35 suitably matched controls were enrolled.

Siblings, parents and first degree cousins of patients of β-thalassemia major who had Hemoglobin A2>3.6%+Hb A on hemoglobin electrophoresis, were included in the study as **Case**. Siblings, parents and first degree cousins of patients of β-thalassemia major who had Hemoglobin A2 (2.5±0.2)+HbA on hemoglobin electrophoresis, were enrolled as **controls.** Clinically diagnosed Iron deficiency anemia, and those who were transfused whole human blood during the last 4 week were excluded from the study.

When a patient was diagnosed as β-thalassemia major by Hb electrophoreis, his/her siblings, cousins and parents were subjected to perform Hb electrophoresis. Among them those who showed evidence of β-thalassemia were excluded. Those who were found to have β-thalassemia trait were enrolled randomly as cases. Those who were found to have normal Hb on Hb Electophoresis were enrolled randomly as controls. Naked eye single tube red cell osmotic fragility test (NESTROFT) was done from the cases and controls.

NESTROFT was performed by using 0.36% buffered saline solution. Two ml of the buffered solution was taken in one tube (10 cm x 1 cm diameter) and 2 ml distilled water was taken in another test tube. A drop of blood from each case or control was added to each tube and these test tubes were left undisturbed for half an hour at room temperature. Both the tubes were then shaken for 1-2 times and held against a white paper on which a thin black line was drawn. The line was clearly visible through the contents of the tube containing distilled water because all the Red Blood cells were hemolysed completely. When the line was similarly visible through the contents of the tube with the buffered saline, the test will be considered negative. That is Red Blood cells were completely hemolysed and patient had normal hemoglobin. When the line was not clearly visible, the test was considered positive, no hemolysis, less visibility (increased turbidity). Positive tests indicate lowered red cell osmotic fragility suggestive of ß-thalassemia trait. Hemoglobin electrophoresis report was taken as confirmatory for carrier detection of β-thalassaemia.

Informed consent was taken from all participants after proper explanation of the procedure. This study was approved by the ethical committee of Sylhet MAG Osmani Medical College Hospital. Instruction given by the BMRC (Bangladesh Medical Research Council) for experiment upon human research is followed. Data were collected by using a preformed structured questionnaire; and were processed and analyzed by using computer program SPSS-16 for Windows. All the known confounding variables were controlled appropriately.

RESULTS: A total of 70 subjects were screened. Subjects were distributed in 2 groups, 35 were β-thalassemia trait and

another 35 were controls. NESTROFT were applied for both groups.

Table1. Baseline characteristics of cases & controls

Parameters	Cases (n=35)	Controls (n=35)	p-value#
Mean age in years	(16.5± 13.65)	(15 ±12.54)	>0.05(NS)*
Sex	Male 19 Female 16	Male - 14 Female - 21	>0.05(NS)*
Consanguinity	7	5	>0.05(NS)*
Mild Anaemia	35	32	>0.05(NS)*
Siblings	7	9	>0.05(NS)*
Parents	11	12	>0.05(NS)*
Cousin	17	14	>0.05(NS)*

Chi-square test, *NS: not significant.

Cases and controls were matched. There are 19 (54.3%) male and 16 (45.7%) female in the case group; it was 14 (40%) male and 21 (60%) female in the control group. The difference between the groups did not vary statistically (p>0.5).

Table-2: Distribution of subjects according to Hemoglobin electrophoresis and NESTROFT

NESTROFT	Diagnosis		Total
	Cases	Controls	
Positive	33 (a)	4 (b)	37 (a + b)
Negative	2 (c)	31 (d)	33 (c + d)
Total	35 (a + c)	35 (b + d)	70 (a + b + c + d)

NESTROFT was positive in 37 subjects among them 33 were cases, diagnosed as carrier by hemoglobin electrophoresis (true positive) and 4 had normal result on hemoglobin electrophoresis (false positive). The test was negative in 33 subjects, among them 2 were diagnosed as carrier by hemoglobin electrophoresis (false negative) and 31 had normal hemoglobin on Hb electrophoresis (true negatives).

The letter "a" denotes those individuals found positive on the test who have the condition or disorder being studied (i.e., true positives). The group labeled "b" includes those who have a positive test result but who do not have the disease (i.e., false positives). Group "c" includes those with negative test results but who have the disease (i.e., false negatives). Finally, those with negative results who do not have the disease are included in group "d" (i.e., true negatives).

Evaluation of a screaning test:

Sensitivity:

Sensitivity = a / (a + c) X 100

The letter "a" denotes true positives=33.

The group labeled "b" includes false positives, b=4.

Group "c" includes those with negative test results but who have the disease (i.e., false negatives). c=2

Finally, those with negative results who do not have the disease are included in group "d" (i.e., true negatives). Here, d=31.

Sensitivity = a /(a + c) X 100 = 94.3%

Specificity:

Specificity = d/(b +d) X 100

The letter "a" denotes true positives, a=33.

The group labeled "b" includes false positives, b=4.

Group "c" includes false negatives, c=2

Finally, in group "d" includes true negatives. Here, d=31

Specificity = d/(b +d) X 100 = 88.6%

Positive predictive value:

Positive predictive value=a/(a+b)X100

The letter "a" denotes true positives, a=33.

The group labeled "b" includes false positives, b=4.

Group "c" includes false negatives, c=2

Finally, in group "d" includes true negatives. Here, d=31

Positive predictive value=a/ (a+b) X 100=89.1%

Negative predictive value:

Negative predictive value=d/(c+d) X 100

The letter "a" denotes true positives, a=33.

The group labeled "b" includes false positives, b=4.

Group "c" includes false negatives, c=2

Finally, in group "d" includes true negatives. Here, d=31

Negative predictive value=d/(c+d) X 100=94%

Sensitivity and Specificity of NESTROFT in the diagnosis of carriers of β-thalassemia were found 94.3 % and 88.6 % respectively. The Positive predictive value and Negative predictive value of NESTROFT in the diagnosis of carriers β-thalassemia were found 89.1 % and 94% respectively. Sensitivity, Specificity Positive predictive value and Negative predictive value of NESTROFT in the diagnosis of β-thalassemia carriers are found high.

DISCUSSION:

From the result of this study it was shown that NESTROFT was effective for screening of heterozygous β-thalassaemia. NESTROFT was successful in detecting 94.3% of subjects of β-thalassaemia trait with a specificity of 88.6%. The predictive value of a positive test was 89.1%, and the predictive value of a negative test was 94%.

The present study found the NESTROFT to be more sensitive though not as specific, which correlates with other studies. Manglani et al. found sensitivity of NESTROFT 94.4%, Mehta et al. 95% and Raghavan et al. 95.5%.[12,14,15] Mehta et al found the specificity of NESTROFT 82.1%.[14] While our study found specificity of 88.6%, which is lower to the findings of Kattarnis et al.; who found specificity of NESTROFT 91%.[16] The lower specificity found in the present study, may be due to high false positive rate in iron deficiency.

The negative predictive value of the test in this study was 94%. The result is comparable with other studies who reported values 96.5%, 97% and 97.6% by Thomas et al., Mehta et al., and Manglani et al. respectively.[17,14,12] The important point brought to the notice is that the presence of negative test almost rules out the possibility of β-thalassaemia trait in general

population. The application of this test for screening the cases before further investigation would reduce the workload on specialized laboratories. The positive predictive value of the test of this study is 89.1% which is quite comparable to other studies done by Raghavan et al. and Mehta et al., who reported values 70.5% and 73% respectively.[15,14] A lower positive predictive value suggested false positive results probably due to associated iron deficiency.

The cost of performing a single NESTROFT comes to less than taka five. It is easy to perform and much technical expertise is not required and no well-equipped laboratory is needed. The stock solution once made kept well in a stoppered bottle, thus can be used in field surveys. A single individual can perform 40 to 50 tests in an hour. Other screening strategies like MCV, MCH, RBC count, RDW and discriminant analyses based on red cell indices need electronic cell counter which is quite expensive. The confirmatory tests needed for diagnosis of β-thalassaemia trait are costly, laborious and time consuming. By excluding control subjects and thus restricting further investigation for the precise diagnosis to the small proportion of positive subjects, NESTROFT reduces the time, cost and labor. NESTROFT seems to be valuable as a single screening test in areas with limited laboratory facilities and economic resources as well as for mass screening of β-thalassaemia trait.

CONCLUSION AND RECOMMENDATION: The present study found NESTROFT to be both sensitive and reasonably specific with a high negative predictive value. Since the test is inexpensive, practical, suitable for field survey, it might be considered as the single screening test to detect β-thalassemia trait as an alternative to hemoglobin electrophoresis in areas with limited laboratory facilities and economic resources. However, multicenter study with large sample size is needed to recommend NESTROFT as a single screening test for detection of β-thalassaemia trait.

Limitation: Sample size is very small and RBC-indices are not compared as another screening test. Raised Hb-A2 is an important diagnostic feature of β-thalassaemia trait. With concomitant severe iron deficiency, Hb-A2 level may fall, sometimes to normal range, thus making diagnosis of β-thalassaemia trait difficult. Hence Subjects of iron deficiency anemia require iron therapy to exclude possibility of beta-thalassaemia trait which was not done in this study.

REFERENCES:

1. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bulletin of the World Health Organization. 2001;79: 704–712.
2. Shivashankara AR, Jailkhani R, Kini A. Hemoglobinopathies in Dharwad, North Karnataka: a hospital-based study. Journal of Clinical and Diagnostic Research .2008; 2; 593-599.
3. Angastiniotis M, Modell B. Global epidemiology of hemoglobin disorders. Ann N Y Acad Sci.1998; 850:251-269
4. Weatherall DJ, Clegg TB, Higgs DR, Wood WG. The hemoglobinopathies. In: Scriver

CR, Beaudet AL, Sly WS, Valle D. (Eds.). The Metabolic and Molecular Bases of Inherited Diseases, 8th Edition. New York: McGraw Hill; 2001: 4571-4636.
5. Fucharoen G, Sanchaisuriya K, Sae-ung N, Dangwibul S, Fucharoen S. A simplified screening strategy for thalassaemia and haemoglobin E in rural communities in south-east Asia. Bulletin of the World Health Organization. 2004; 82(5): 364-372.
6. Organization WH et al. Management of haemoglobin disorders: report of a joint WHO-TIF meeting, Nicosia, Cyprus, 16-18 November 2007. 2008.
7. Khan WA, Banu B, Amin SK. Prevalence of ß-Thalassemia trait and Hb-E trait in Bangladeshi school children and health burden of thalassemia in our population. Dhaka Shishu (children) Hospital Journal. 2005; 21(1): 1-7.
8. Rahman SA, Jamal CY. Congenital Hemolytic Anemia in Bangladesh: Types and Clinical Manifestations. Indian Pediatrics. 2002; 39:574-577.
9. Hasan MK, Haque O, Rubaiyat KA, BarshanAD, Talukder SI.Clinical Presentation and Electrophoretic Patterns of Hereditary Haemoglobin Disorders in Adults, a Study at Dinajpur Medical College Hospital. Dinajpur Med Col J. 2013;6:167-71.
10. Farhana DT, Quamrun N, Subhagata C. J Bangladesh Coll Phys Surg. 2009; 27: 148-54.
11. Maheshwari M, Arora S, Kabra M, Menon PSN. Carrier screening and prenatal diagnosis of β-thalassaemia. Indian Pediatrics.1999; 36:1119-1125.
12. Manglani M, Lokeshwar MR, Vani VG, Bhatia N, Mhaskar V. 'NESTROFT'-an effective screening test for beta thalassemia trait. Indian Journal of Pediatrics. 1997; 34: 702-707.
13. Begum JA, Amin SK, Khan WA, Selimuzzaman M, Sharmin S, Hossain B. Evaluation of naked eye single tube red cell osmotic fragility test (NESTROFT) in detecting ß-Thalassemia trait. Dhaka Shishu Hospital Journal. 2005; 21(2): 44-48.
14. Mehta BC. NESTROFT: A screening test for beta thalassemia trait. Indian J Med Sc. 2002; 56: 537-44.
15. Raghavan K, Lokeshwar MR, Birewar N, Nigam V, Manglani MV, Raju NB. Evaluation of naked eye single tube red cell osmotic fragility test in detecting beta-thalassemia trait. Indian pediatrics.1991; 28(5): 469-72.
16. Kattamis C, Efremovt G, Pootrakul S. Effectiveness of one tube osmotic fragility screening in detecting β-thalassaemia trait. Journal of Medical Genetics.1981; 18:266-270.
17. Thomas S, Srivastava A, Jeyaseelan L, Dennison D, Chandy M. NESTROFT as a screening test for the detection of thalassemia and common haemoglobinopathies- An evaluation against a high performance liquid chromatographic method. Ind J Med Res. 1996; 104:194–7.