Original Article

Beta Thalassaemia

Evaluation of Nacked Eye Single Tube Red Cell Osmotic Fragility Test for Screening of Beta Thalassaemia Trait

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ABSTRACT

Objective: The study aimed to evaluate the validity and significance of Nacked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT) for screening of beta thalassaemia trait (BTT) to reduce the incidence of birth of thalassaemic child in community.

Study Design: Cross sectional study

Place and Duration of Study: This study was conducted at Diagnostic and Research Lab in Pathology Department of Peoples University of Medical Health Sciences (PUMHSW) at Shaheed Benazirabad from January 2013 to December, 2013.

Materials and Methods: Total 504 subjects comprising 303 (60.3%) females and 201(39.7%) males with age ranging between 5 and 48 years and male to female ratio 1:1.5 were selected for this study. The family history of thalassaemia and history of cousin marriages were noted. EDTA anti-coagulated whole blood samples were collected for on-site NESTROFT testing, and later tested for Complete Blood Count (CBC) and serum Ferritin concentration at Diagnostic and Research Laboratory of Pathology Deptt. PUMHS. Screening for BTT was done on Naked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT) with 0.36% freshly prepared saline. The diagnosis of BTT was confirmed on automated Hemoglobin Electrophoresis at cellulose acetate alkaline pH. Red cell indices (automated Hematology cell counter cell-tac alpha) were assessed along with peripheral smear morphology (Leishman's stained slides) as enhanced tool for BTT case finding.

Results: Out of total 504 subjects 201 married women and 101 married men with their mean age (26.5 $\pm$ 21.5) years were selected. In this study, female to male ratio was 1.5:1 and among the total 302 married subjects, ratio of cousin marriages (60.4%) was noted. Neither any women were pregnant nor there was history of thalassaemia in their families. The laboratory parameters such as the mean values of hemoglobin g/dl, RBC count millions/cmm, PCV %, MCV fl, MCH pg, MCHC g/dl among these subjects respectively were 11.9 g/dl, 4.5 millions/cmm, 82 fl, 38.7%, 26.9 pg, 33.2 g/dl. Out of 504 samples, NESTROFT was positive in 21 (4.1%) and negative in 483 (95.9%). Out of all NESTROFT positive cases 15 (71.4%) were true positive confirmed on the hemoglobin electrophoresis with increased hemoglobin A2 level above 3.5 and remaining 6(28.6%) were false positive. Only 4 (1%) cases were false negative, then sensitivity, specificity, positive and negative predictive values and efficiency of NESTROFT were calculated 87%, 86%, 71%, 99% and 99.9 respectively.

Conclusion: The NESTROFT is a valuable, cost effective screening test for beta thalassaemia trait.

Key Words: Beta thalassaemia trait (BTT), Nacked eye single tube red cell osmotic fragility test (Nestroft), Screening.

INTRODUCTION

The two types of thalassaemia such as alpha and beta are autosomal recessive disorder of hemoglobin molecule caused by genetic defects like mutation or deletion leading to either complete lack of or decrease synthesis of either beta or alpha chains, if affected individuals carries abnormal beta gene from both parents they are called homozygous while individuals carrying abnormal beta gene from their mother or father they are called heterozygous carriers[1]. The patients with beta thalassaemia major or homozygous state present with severe type of anemia that appear at 06 month of age when fetal hemoglobin changes into the adult hemoglobin, hepatosplenomegaly, bone deformities while the patients with beta thalesemia minor or heterozygous carriers are asymptomatic and the diagnosis depends upon estimation of hemoglobin, red blood cell indices, examination of peripheral blood and bone marrow smears and detection of adult hemoglobin, hemoglobin A2 as well as fetal hemoglobin by hemoglobin electrophoresis[2]. The expenses of treatment of beta thalassaemia major are quite high amounting $2100 or 100000 per year for one thalassaemic child involving regular blood transfusion to correct anemia, iron chelation therapy to prevent iron overloading of vital organs and endocrine glands, splenectomy to reduce need of blood transfusion, bone marrow transplantation, gene therapy, recent advanced chemotherapy. As the monitoring & treatment of this disorder is expensive, it puts a socioeconomic burden on the families and ultimately on the state, therefore

when planning health facilities it is preferably needed to prevent the thalassaemia in developing countries like Pakistan and India rather than to treat[3]. The prevention of thalassaemia include awareness about the thalassaemia, education, premarital screening and genetic counseling, prenatal diagnosis, mass screening of population in rural areas of resources limited countries have great importance in highly prevalent regions including Greece, Italy, Saudi Arabia and Turkey[4]. For mass screening of beta thalassaemia trait in our rural areas of resource limited country, a valid and effective test is needed such as naked eye red cell osmotic fragility test (NESTROFT) initially described by Kattamis et al and this test is inexpensive, requires a small amount of blood, does not require sophisticated equipment as compared to the expensive, time consuming and difficult laboratory technique for the detection of beta thalassaemia trait not suitable for rural areas[5]. The diagnosis of beta thalassaemia is important not only for screening of beta thalassaemia major but it could be differentiated from iron deficiency anemia to prevent iron loading by giving iron supplementation in the beta thalassaemia trait, the useful laboratory test that differentiate the BTT from iron deficiency anemia are estimation of the hemoglobin concentration, RBC count and mean corpuscular volume along with hemoglobin A2 and serum ferritin levels and also NESTROFT could be used to differentiate these two types[6] due to the cousin marriages in our country, the couples refuse to testing for the screening of beta thalassaemia during pregnancy because of danger of occurrence of beta thalassaemia major in their coming children due to the un-education and un-awareness, hence premarital screening is important among the couples who are planning to marriage than the screening of couples during pregnancy[7].
The aim of our study is to evaluate the validity and effectiveness of NESTROFT test among the peoples living in the rural areas of districts Shaheed Benazirabad, Sanghar and Naushero Feroze for the screening of beta thalassaemia in rural setup. We also determine the importance of cousin marriages among the married couples.

MATERIALS AND METHODS

A. Inclusion criteria

1. An analytical and cross sectional study was conducted at diagnostic and research laboratory in pathology department of PUMHS from January 2013 to December 2013 on a samples of 504 subjects coming from rural areas of districts Shaheed Benazirabad and other neighboring districts. Total 504 subjects including 151 married couples and remaining 200 subjects were selected for this study. Out of 504 subjects, 293 were females and 211 were males, hence females to males ratio was 1.4:1 and among the married couples, 81 couples were present with cousin marriages.

2. The awareness regarding the thalassaemia was created by distributing pamphlets to the each people and detailed history was filled by each people about the any family member present with beta thalassaemia major as well as history of cousin marriages and any woman who was pregnant should be noted.

B. Exclusion criteria: The liver diseases, other type of hemoglobinopathies and pregnant women were excluded from this study because of limitation of nacked eye single tube red cell osmotic fragility test (Nestroft) in this study.

The Five ml of venous blood was taken from all these subjects, 3 ml of blood out of 5 ml was well mixed in quantity of 1.5 $\pm$ 0.2 mg/ml anticoagulant such as Ethylene Diamine Tetracetic acid from these and remaining 2ml blood was allowed to clot in separate tube. All the coagulated and anti coagulated samples of blood were send to the diagnostic and research laboratory in pathology department of PUMHS for the screening of beta thaleseema trait. The Nestroft was done using 0.36% buffered saline and hematological parameters such as hemoglobin g/dl, RBC indices (PCV, MCV, MCH & MCHC) were analyzed by Nihon kohden, estimation of hemoglobin A2 level in NESTROFT positive cases was carried out by hemoglobin electrophoresis on cellulose acetate membrane using TEB buffer, pH 8.6. Hb A2 estimation was done following elution after electrophoresis on cellulose acetate, TEB buffer, pH 8.9 for this test in these subjects within two hours of collection of anti clotted blood samples. Two to three peripheral blood smears were also made and stained by Leishman's stain in each case. 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-thalassaemias from the clotted blood samples. A cut off Hb A2 level of $\geq$ 3.6% was used for diagnosing thalassaemia trait and serum ferritin level of <10ug/dl was taken as cut off of iron deficiency. The results were analyzed statistically by using SPSS version 16.0.

The NESTROFT was done with freshly prepared 0.36% buffered saline from stock solution that was prepared in the form of 10% buffer saline at pH 7.4 with NaCl 90g, anhydrous Na_2HPO_4 13.65G and NaH_2PO_4 2.43g (can be stored in well stoppered bottle in refrigerator for 6 months). Working buffer was prepared fresh by putting 3.6ml of stock solution for 100 ml buffer (by adding distilled water). For NESTROFT testing, 20uL volume of EDTA anti-coagulated whole blood was pipetted out into a clean glass test tube (10x100mm) containing 4 mL of 0.36% freshly prepared buffered saline solution. Contents of tubes were mixed and left at room temperature for 20 minutes. After mixing again, tubes were read in a standardized light against sharp black lines drawn behind the tube at a standardized distance.

The results were recorded as "Negative" with clearly visible lines and "Positive" when lines were not visible and "Doubtful" when partially visible lines seen. The doubtful cases were also interpreted as positive result. The preliminary NESTROFT test result cards were issued to all participating subjects. Subjects with positive NESTROFT were counseled for follow up confirmation of BTT on Hb Electrophoresis at 8.6 Ph (HbA_2 > 3.5 %).

RESULTS

The socio demographic characteristic of 504 subjects enrolled for study were showed in table 1. The mean age 26.5 ± 21.5 years of these subjects composed of 202 unmarried and 302 /151 married couples including 293 (58.1%) females, 211 (41.9%) males with female to male 1.4:1 ratio was observed. Out of total 151 married couples, 81 couples were consanguineous marriages (53.6%) and 71 couples were non consanguineous.

Table No. 1: The socio demographic characteristic among all the subjects (n=504)

S.No.	Characteristics	Total Subject
1	Mean age	26.5 ± 21.5 years
2	Sex Females Males	293 (58.1%) 211 (41.9%) Female to male ratio 1.4:1
3	Marital status Married Unmarried	 302 (59.9%) 202 (40.1%)
4	Married couple Consanguineous Non Consanguineous	151 / 302 81 / 162 (53.6%) 70 / 140 (46.4%)
5	Education Illiterate Literate	 391 (75.2%) 113 (24.8)
6	Occupation Housewives and Labors Government Servants	 472 (87.8%) 32 (12.2%)
7	Socio economic status Upper middle class Poor and lower middle class	 12 (5.4%) 492 (94.6)

The significance difference of values of hematological parameters such as mean values of hemoglobin g/dl, Red Blood Count millions / cmm, Packed Cell Volume %, Mean Cell Volume fl, Mean Cell Hemoglobin pg, Mean Cell Hemoglobin Concentration g/dl, Red Cell Distribution width %, NESTROFT positivity and microscopic examination of peripheral blood smears revealed a fairly hypochromic microcytic red cell picture with presence of target cells among the 21, 182 subjects with BTT, iron deficiency anemia (IDA) respectively and normocytic normochromatic picture in 301 subjects with non BTT and Non IDA out of total 504 subjects were showed in table 2. Serum Ferritin level <15ug/dl was taken as cut off for IDA. Ferritin levels were found normal in BTT cases. Peripheral bloods smear morphology in BTT positive case. Out of 504 samples, Nestroft was positive in 21 (4.3%) and negative in 483 (95.70%) samples. Out of all nestroft positive cases 15 (3.3%) were true positive (HbA2 > 3.5) while remaining 6 were false positive and false negative were observed in 4 (1%) subjects only. Sensitivity 89%, specificity 98%, positive predictive value 71% and negative predictive value 99% while efficiency of test was calculated to be 97.2%.

Table No. 2: Hematological parameters and NESTROFT positivity among the subjects with the BTT and iron deficiency anemia as well as non BTT and non iron deficiency anemia (n=504)

Laboratory parameters	Iron deficiency anemia n=182	Beta Thalassaemia Trait n=21	Non BTT and Non Iron deficiency anemia n=301
Hemoglobin g/dl PCV % RBC count million/cmm MCV fl MCH pg MCHC g/dl RDW %	10.7 ± 2.6 31.3 ± 8.1 4.2 ± 0.3 68.2 ± 7.5 22.5 ± 3.1 29.8 ± 3.4 15.9 ± 2.8	11.2 ± 2.4 31.1 ± 6.7 5.5 ± 0.8 66.3 ± 6.8 21.2 ± 2.9 31.5 ± 3.5 14.1 ± 0.4	11.9 ± 2.7 35.1 ± 8.5 5.5 ± 0.9 82 ± 7.5 24.3 ± 1.9 33.8 ± 2.1 14.2 ± 0.3
PBS	Microcytic hypochromic Red Blood Cells (RBC) with presence of target cells.	Microcytic hypochromic Red Blood Cells (RBC) with presence of target cells.	Normocytic normo-chromic Red Blood Cells
NESTROFT	09 cases (4.9%) positive out of 182 (95.1%) cases	15 cases (71.4%) positive out of 21 cases	Negative in 474 (94%)

Table No. 3: Nestroft result hemoglobin A2 and serum ferritin levels among the subject with BTT and Iron deficiency anemia. (n=203)

Types	Nestroft	Hemoglobin A2 %	Serum Ferritin (ug/dl)
BTT	Positive True 15 False 6 Negative True 483 False 4	4.8 ± 1.5 < 3.5	15 ± 2.1
IDA	Positive 9 (4.9%) Negative 173 ()	2.1 ± 0.9	10.2 ± 1.9

< = less than
BTT = Beta Thalassaemia Trait
IDA = Iron Deficiency Anemia
SD=Standard Deviation

Table No.4: Sensitivity, Specificity, positive and negative predictive values and efficiency of nestroft in prediction of BTT.

Sensitivity (%)	Specificity (%)	Positive predictive values (%)	Negative predictive values (%)	Efficiency of test (%)
78.8%	98.3%	71.0%	99.0%	98.9%

DISCUSSION

World Health Organization[8] reported that thalassaemias were significant health problem throughout the world including Mediterranean countries, Indian sub continent, Middle East and in Pakistan 5 – 8 % prevalent rate of beta thalassaemia trait with 8-10 million carriers as well as 6,000 children's were born each year, the frequency of thalassaemia increased due to the high ratio of cousin marriages. Abdullah[9] KN, et al reported that risk factors such as poverty, the high ratio of cousin marriages, poor health facilities and lack of education and thalassaemia control program in Pakistan, the frequency of beta thalassaemia was increased, hence bill about thalassaemia control program in our country has been put forward in the national assembly because treatment of thalassaemic child remained source of miseries including socio economic burden on the family and state. Due to the heterogeneity of beta thalassaemia trait because of increased ratio of cousin marriages in Pakistan, therefore prevention of beta thalassaemia major is not difficult task due to the simple, cheap required two rupees per head, easy to perform and adaptable for mass screening, coming close to an ideal screening test called NESTROFT testing in which exposure of RBC with 0.36% saline of subjects of BTT remained sensitive solution and provides accurate results compared to other concentrations of saline, in contrast to difficult laboratory tests that are time consuming and expensive as well as not suitable for mass screening of BTT required for screening as reported by Singh et al[10]. Recently published data concludes that NESTROFT can be effectively used as screening marker for detection of 96-100% β-Thalassaemia trait observed by Rakholia et al[11] and Mamtani et al[12]. According to a recent study conducted at PNS Shifa Hospital, Karachi, Pakistan, NESTROFT had a Positive Predictive Value of 85.38%, Negative Predictive Value of 97.66% and the diagnostic accuracy of NESTROFT was 94.6% correlating to internationally published data and Red Cell Osmotic Fragility Test could be used as a potential screening test for thalassaemia because microcytic red cells in thalassaemia have a low surface area to volume ratio and therefore resist lysis when placed in a hypotonic saline solution reported by Yazdani et al[13].

Other studies such as Niazi et al[14] and Eijaz et al[15] had proved that useful laboratory tests such as hematological parameters are utilized for the differentiation of beta thalassaemia trait from the iron deficiency anemia and the individuals with Hb A2 >3.5% were labelled beta thalassaemia trait while serum ferritin below 15.0 µg/l indicated iron deficiency. In our study with ratio of cousin marriage (53.6%), the diagnostic accuracy of Nestroft among the subjects with BTT were proved and sensitivity, specificity, positive and negative predictive values as well as efficiency of NESTROFT were 78.8%, 98.3%, 71.0%, 99.0% and 98.9% respectively. Our study were correlated with International as well as Pakistani study.

CONCLUSION

One tube Osmotic Fragility test is a cost effective and simple test that can be done in the field without any equipment or expertise. It is very sensitive for the screening of Beta thalassaemia carriers. The test has a high negative predictive value and OTOFT negative individuals can safely be declared free of thalassaemia.

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