Original Article

Screening of Beta Thalassaemia

The Prevalence and Antenatal Screening of Beta Thalassaemia Trait in Pregnancy by naked Eye Single Tube Red Cell Osmotic Fragility Test

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ABSTRACT

Objectives: To evaluate the prevalence of Beta Thalassaemia Trait (BTT) detected by Naked Eye Single Tube Red cell Osmotic Fragility Test (NESTROFT). We also highlight the validity and importance of this test for antenatal screening of BTT to prevent incidence of Beta Thalassaemia major in our community as well as differentiating the BTT, BTT with coexisting iron deficiency anemia and only iron deficiency anemia in pregnancy.

Study Design: Experimental and observational study.

Place and Duration of Study: This study was conducted at Pathology and Gynae-Obs Out Patient departments of Peoples University of Medical & Health Sciences for Women (PUMHSW) Hospital, Shaheed Benazirabad from February 2013 to February 2014.

Materials and Methods: Total 461 pregnant women with their age ranged between 18 – 42 years including multigravida and primigravida as well as first trimester to second trimester of pregnancy were selected. The family history of thalassaemia and history of cousin marriages were noted. 4ml of anti-coagulated whole blood and 2ml of clotted blood samples were collected from each pregnant women and sent to the pathology department for NESTROFT testing, and later tested for Complete Blood Count (CBC) along with peripheral blood smear stained with Leishman's stained on the 2 to 3 slides as enhanced tool for BTT case finding while estimation of serum Ferritin were done from the clotted blood sample. 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 from the 2ml of clotted blood in NESTROFT positive cases.

Results: Out of total 461 pregnant women with their mean age 30 $\pm$ 12, 30 were diagnose with BTT, out of 18 (54.5%) women were married with their cousins, neither the family history of Beta thalassaemia major was present nor husband of any women was carrier of thalassaemia. NESTROFT showed sensitivity, specificity, positive and negative predictive values and efficiency of 84%, 98.6%, 82%, 99% and 93% respectively. 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 were 11.9 g/dl, 4.5 millions/cmm, 82 fl, 38.7%, 26.9 pg, 33.2 g/dl respectively were showed in cases of BTT and co-existent iron deficiency anemia in pregnancy. Coexistent iron deficiency anemia did not preclude diagnosis of beta thalassaemia major.

Conclusion: The prevalence of BTT in pregnant women is 6.5% and NESTROFT is a valuable, cost effective screening test for beta thalassaemia trait in pregnancy with cousin marriage ratio of 54.6%. The significant difference of hematological parameters in BTT alone, BTT coexistence iron deficiency anemia and iron deficiency anemia alone were founded in our study.

Key Words: Prevalence, Beta thalassaemia trait (BTT), Naked eye single tube red cell osmotic fragility test (NESTROFT), prenatal screening, and coexistent iron deficiency anemia.

INTRODUCTION

The thalassaemia are monogenetic autosomal recessive disorders of hemoglobin synthesis characterized by complete lack or reduced synthesis of either alpha chain or beta chain that are manufactured by genes located on chromosomes 16 & 11 respectively and each chain combine with heme of the hemoglobin molecules, Beta thalassaemia major among the thalassaemia in children causes morbidity due to severe type of hemolytic anemia required blood transfusion that increases the burden of health care delivery system in developing countries[1]. Currently 217 causative molecular defects have been described in the beta globin gene causing beta thalassaemia, about 20 genetic mutations account for 90% of beta globin genes in the world and in Pakistan 11 different beta globin genes due to the high ratio of consanguineous marriages in different ethnic groups such as IVS 1-5 (G →C), 619bp del, IVS 1-1 (G → T), Fr 8/9 (+G), Fr 41/42 (-CTTT), CD 30 (G → A), CD 15 (G -> A), IVS II-I (G -> A), Fr 16 (- C), Cap +1 (A -> G) and CD 5 (-CT) are detected[2]. The

hematological consequence of beta thalassaemia major is life threatening severe type of anemia caused by diminished hemoglobin synthesis as a result of decreased beta chain synthesis of hemoglobin and ineffective erythropoeisis due to the excessive alpha chain that impair the normal erythropoiesis on one hand and on the other hand excessive alpha chain aggregates to form toxic products leading to hemolysis of Red Blood Cell[3]. The clinical and laboratory findings of Beta thalassaemia major includes, severe anemia that appear in infancy and childhood at the age of 6 months to 2 years than fetal hemoglobin change into the adult hemoglobin, growth retardation due to the bone deformity, hepatospleenomegally, iron overloading and suseptability to infections are other complication of disease, the diagnosis of beta thalassaemia major depends upon complete blood examination, estimation of fetal hemoglobin, adult hemoglobin A and hemoglobin A2 by hemoglobin electrophoresis as well as high performance liquid chromatography and DNA analysis of fetal cells during pregnancy[4]. The treatment of thalassaemia major remains a source of misery, burden and mostly disappointing; hence emphasis must shift from the treatment to the prevention of such births of children with beta thalassaemia major in the future, the most effective and feasible approach for solving this problem includes population education, mass screening, genetic counseling and prenatal diagnosis, is the only effective way of coping successfully with such a disease[5]. The 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 thalassaemia carriers in pregnancy as a prenatal screening in areas of high prevalence of this disease[6]. All pregnant women attending antenatal clinics can be screened for BTT at the time of their first antenatal visit. To identify pregnancies at risk of producing children with Beta thalassaemia major by testing there husbands for the identification of BTT and mild to moderate degree of hypochromic microcytic anemia is encountered in about 65-85% of carriers of BTT with or without iron deficiency anemia and iron therapy in BTT cases causes harmful effects because of iron overloading, therefore it is necessary to differentiate these cases to avoid unnecessary iron supplementation in pregnant women with carrier state of beta thalassaemia[7]. Hence, in our study we aim to evaluate prevalence and antenatal screening of beta thalassaemia trait by NESTROFT that is suitable screening procedure for carriers of beta-thalassaemia trait among antenatal mothers attending the gynae-obs department of our hospital. This study also highlights the differentiation of iron deficiency anemia alone, coexistence with BTT and BTT alone by the various hematological parameters among the pregnant women because unnecessary iron therapy in BTT cases causes iron overloading.

MATERIALS AND METHODS

A. Inclusion criteria

1. An experimental and observational study was conducted at Gynae-obs Out Patient and pathology departments of PUMHS from February 2013 to February 2014 on a samples of 461 pregnant women with all the three trimester of pregnancy and primigravida as well as multigravida coming from rural areas of districts Shaheed Benazirabad and other neighboring districts were selected. The ages of women range between 18 & 40 years and out of 461, 30 were diagnosis with BTT and 18 were married with their cousins hence ratio of cousin marriage was 56.6, the awareness regarding the thalassaemia was created by distributing pamphlets to the each pregnant lady and detailed history was filled about the any family member present with beta thalassaemia major, history of cousin marriages neither any women gives history of abortion nor any history of blood transfusion.

2. The six 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 thalassaemia 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-thalassaemia 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 ($HbA_2 > 3.5$ %).

B. Exclusion criteria: The patients with liver diseases and with other type of hemoglobinopathies were excluded from the study.

RESULTS

The characteristic features of 461 pregnant women were as follows. The mean age of these women was 26.5 ± 21.5 years while cousin ratio of 56.6 among them was observed. The multigravida and primigravida were 302 (65.5%), 159 (34.5%) respectively while gestational ages of these pregnant women such as 101 (22.0%) in first trimester, 139 (30.1%) in second trimester, 221 (47.9%) in third trimester was noted. 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 %, and microscopic examination of peripheral blood smears revealed a fairly hypochromic microcytic red cell picture with presence of target cells among the 18 pregnant women with BTT, 12 with BTT coexistence with iron deficiency anemia (IDA) respectively. Serum Ferritin level <15ug/dl was taken as cut off for IDA. Ferritin levels were found normal in BTT cases. Out of 461 samples, NESTROFT was positive in 30 and negative in 431 samples. Out of all NESTROFT positive cases, 24 were true positive ($HbA2 > 3.5$) while remaining 6 were false positive and false negative were observed in 5 subjects only. Sensitivity 89%, specificity 98%, positive predictive value 81% and negative predictive value 99% while efficiency of test was calculated to be 98.6% and overall prevalence of BTT among the pregnant women was 6.5 while coexistence iron deficiency anemia does not cause any problem in this study.

Table No.1: The characteristic features among the total screened prenatal mothers N = 461

Sr. No.	Characteristics	Total
1	Mean age	30 ± 12 years
2	Consanguinity Out of 30 women with BTT, 18 were married with their husband	56.6 %
3	**Gravida** Multigravida Primigravida	 302 (65.5%) 159 (34.5%)
4	**Gestational age** First trimester Second trimester Third trimester	 101 (22.0%) 139 (30.1%) 221 (47.9%)
5	Family history of thalassemic child	Nil
6	Carrier state of Thalassaemia among the husbands of pregnant women	Nil
7	Prevalence rate of BTT	6.5%

N = Number of screened prenatal mothers

Table No.2: Hematological parameters among the screened prenatal mothers with the BTT and BTT coexistence with iron deficiency anemia and iron deficiency anemia. N = 461

Hematological parameters	BTT with coexistence IDA N=12	Beta Thalassaemia Trait N=18	Iron deficiency anemia N=301
Hemoglobin g/dl	10.7 ± 2.6	11.2 ± 2.4	11.9 ± 2.7
PCV %	31.3 ± 8.1	31.1 ± 6.7	35.1 ± 8.5
RBC count million/cmm	4.2 ± 0.3	5.5 ± 0.8	5.5 ± 0.9
MCV fl	68.2 ± 7.5	66.3 ± 6.8	82 ± 7.5
MCH pg	22.5 ± 3.1	21.2 ± 2.9	24.3 ± 1.9
MCHC g/dl	29.8 ± 3.4	31.5 ± 3.5	33.8 ± 2.1
RDW %	15.9 ± 2.8	14.1 ± 0.4	14.2 ± 0.3
PBS	Microcytic hypochromic Red Blood Cells (RBC) with presence of target cells.		

N = Number of screened prenatal mothers

Table No.3: Nestroft results, hemoglobin A2 and Serum ferritin levels among the screened prenatal mothers with BTT, Iron deficiency anemia and coexistence BTT with iron deficiency anemia. N = 203

Laboratory Parameters	BTT N = 18	Coexistence IDA with BTT N = 12	Iron Deficiency Anemia N = 301
NESTROFT Positive	True 14 False 4	True 10 False 2	11 (3.6%)
Negative	True 456 False 5		290 (96.4%)
Hemoglobin A2 %	5.2 ± 1.6	4.9 ± 1.3	1.4 + 0.5
Serum Ferritin	15.2 ± 3.5ng/dl	8.1 ± 1.1ng/dl	7.9 ± 0.9 ng/dl

N = Number of screened pregnant women
BTT = Beta Thalassaemia Trait

BTT with coexistent IDA = Beta Thalassaemia Trait with Iron Deficiency Anemia
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 among the screened prenatal mothers.

Sensitivity (%)	Specificity (%)	Positive predictive values (%)	Negative predictive values (%)	Efficiency of test (%)
84%	98.6%	82%	99%	93%

DISCUSSION

According to the world health organization report, about 7% pregnant women were carrier of BTT and 1% couples were at risk for thalassaemia major among their coming off springs through out the world. Abdullah KN et al stated that risk factor such as consanguinity increasing the frequency of thalassaemia in Pakistan, hence prevention plays key role rather than the treatment of beta thalassaemia major that could be health burden on the health care delivery system of our country. They also founded safety of chorionic villus sampling as a diagnostic tool for pre-natal diagnosis in selected patients for the DNA analysis of fetal cells. Study conducted by Ou. Z et al[10] and Atulshrivast et al[11] detected elevated hemoglobin A2 as marker of beta thalassaemia trait in pregnancy by hemoglobin electrophoresis and high performance liquid chromatography. In contrast to these studies in which diagnosis of beta thalassaemia trait was made by expensive, time consuming required sophisticated instrument and expertise, Hafeez M et al[12] recommended NESTROFT for screening of beta-thalassaemia trait in pregnancy as a antenatal screening where there is high prevalence and constrained resources. They also founded similar ratio of cousin marriages in the various ethnic groups of peoples of South Punjab. In our study, total 461 antenatal mothers underwent NESTROFT, complete blood count, RBC indices and hemoglobin electrophoresis for estimation of hemoglobin A2 level among the NESTROFT positive cases. NESTROFT was true positive in 24 out of 30 thalassaemia carriers, 6 false positive, 5 false negative and 431 subjects were true negative, hence in our study, a 6.5% prevalence of beta thalassaemia carrier state among antenatal women was founded while specificity, sensitivity, predictive positive and negative values of NESTROFT among the total 461 antenatal mothers were 80 to 89.9% and ratio of cousin marriages such as 56.6% was founded among the pregnant women in our study. The same prevalence of BTT among the pregnant women were showed by Sinha *et al.*[13] and Sur D, Mukhopadhay SP[14]. The effectiveness of NESTROFT positivity among the antenatal mothers was 79% to 100% as observed by Chakrabarti et al[15] and Sirichotiyakul S et al[16] and these results were co-related with our study.

For the avoidance of unnecessary iron therapy in pregnant women with BTT, the differentiation of microcytic hypchromic anemia in BTT alone or with iron deficiency anemia or iron deficiency alone required. Hence in our study, the significant difference of the hematological parameters such as hemoglobin, the RBC counts, haematocrit, MCV, MCH, Red Cell Distribution Width, Serum ferritin and hemoglobin A2 levels were found among the pregnant women with beta-thalassaemia trait (BTT) alone or with iron deficiency Anemia (IDA) and Iron deficiency, however iron deficiency did not preclude a diagnosis of beta thalassaemia. The HB A2 levels were significantly high (mean HB A2 level 4.8 ± 0.55%) among the pregnant women with BTT alone or with IDA and HB A2 level in IDA alone was 1.2% in our study. While serum ferritin levels in these three cases were 15.2 $\pm$ 3.5ng/dl, 8.1 $\pm$ 1.1ng/dl, 7.9 $\pm$ 0.9 ng/dl respectively.

Sumera A et al[17] observed that NESTROFT was positively in 13% cases of Iron Deficiency Anemia while it remained negative in 87% cases of iron deficiency anemia and for differentiation between IDA, BTT with or without iron deficiency anemia, hematological parameters, serum ferritin and hemoglobin A2 levels were significantly different. They also observed sensitivity, specificity, positive and negative, predictive values such as 93%, 88%, 74% and 97% respectively. In our study NESTROFT was positive in 3.6% cases of iron deficiency anemia and negative in 96.4% cases of iron deficiency anemia and other laboratory parameters are accordingly with the above results for the differentiation of IDA, BTT alone and with the Iron deficiency anemia.

CONCLUSION

1. The prevalence of Beta Thalassaemia Trait among the pregnant women was 6.5% with 54.4% ratio of cousin marriages in our study. However no any husband of these pregnant women who were their cousins founded to be carriers, hence there was no risk of thalassaemia in their children.
2. in our study, the cost which was incurred in conducting the NESTROF test was only Rs. 1.50 per subject, which implied that the NESTROF test was a simple and a low cost screening tool which could be used for the identification of the carrier status of beta thalassaemia. It is useful for screening large populations, especially in the remote village areas and at the primary health care centers, where laboratory facilities are not available.
3. Along with screening of BTT in pregnancy, differentiation of BTT with or without iron deficiency anemia are essential because of the presence of microcytic hypochromic anemia in

these cases. The iron supplementation causes iron overloading in BTT cases, so therefore differentiation from iron deficiency anemia is necessary.

4. In Pakistan resulting genetic heterogencity in different ethnic groups due to the different beta genetic mutations supplemented by cousin marriages, the DNA analysis would be required for identification of BTT cases in our country to prevent birth of children with Beta thalassaemia major among the couples who were carriers of the beta thalassaemia. The DNA analysis is gold standard test used for the diagnosis of BTT cases but facilities in our countries are limited in contrast to developed countries like USA, Canada, England and Germany..

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