Original Article

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The study of recent biochemical and pathological aspects of thalassemia

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Abstract

The prevalence of Beta thalassemia trait (BTT) varies from 1.0 to 14.9 % in different regions of India. Over 9000 Thalassemic children are born every year and an estimated annual average consumption of 27 units of blood and 4 lakhs worth of drugs are needed to manage each Beta Thalassemia patient according to recommended standards. The prevention of birth of Beta Thalassemia major children lies on effectively screening the carriers (BTT). The objective of the present study was to compare the effectiveness of various screening tests in predicting BTT in microcytic cases, who were later confirmed by HbA_2 levels on Cellulose acetate Electrophoresis. 500 cases with microcytosis (MCV < 80fl) based on coulter (Advia 60 – OT) were screened. The five DF's were evaluated for all cases to separate BTT and Non BTT cases. They are DF1 = MCV – [5 x Hb] – RBC, DF2 = MCV/RBC, DF3 = MCH/RBC DF4 = MCH x $(MCV)^2$ / 100 and DF5 = RBC counts. All the cases positive for at least two of the 5 DF's in the discrimination limits suggesting BTT were further screened with NESTROFT and confirmed for increased HbA_2 by electrophoresis and elution. 100 controls were included (MCV > 80 fl). DF's showed variable degree of sensitivity, specificity and positive predictive value. Mild anemia, RBC > 5 x 10^{12} / L, NESTROFT positivity with DF2 and DF3 were found to be the best combination after a multivariate logistic regression analysis to predict BTT.

Key words: Beta Thalassemia Trait; Discriminant functions; Electrophoresis; Microcytosis; NESTROFT

Introduction

β-Thalassemia is the commonest inherited haemoglobinopathy. A high prevalence of β-Thalassemia is found in populations the Mediterranean region and African continent. India, Pakistan, Bangladesh, Sri Lanka, china and Middle East record varying frequency of β-thalassemia [1]. Only in 1930's and 1940's did it become apparent that this disease, first described as severe anemia with bone changes and splenomegaly, results from a defective synthesis of one of the two globin chains, (∝ or β) that constitute adult haemoglobin. Most forms are inherited in a Mendelian recessive fashion from asymptomatic parents who are gene carriers, and who have one in four chance of having an affected child. The disease has tremendous variation, ranging from silent, asymptomatic carriers (BTT) to transfusion dependent patients, "Cooley's anemia" used to describe the severe form of the β-- globin deficiency [2]. In India, an estimated annual average consumption of 27 units of blood and 4,00,000 Rupees worth of drugs are needed to manage each β- Thalassemia patient according to the recommended standards. The most effective approach to reduce the burden of the society and reduce disease incidence is implementation of carrier screening programs offering genetic counselling, prenatal diagnosis and selective termination of affected fetuses [3].

Inheritance of one gene for β-Thalassemia results in BTT (also called thalassemia minor). Prevalence of β-Thalassemia trait [BTT] varies from 1.0-14.9% in various regions of India.1Correct identification of BTT by screening therefore assumes great important. Red cells in BTT are microcytic hypochromic usually with mild degree of anemia. There are however, other causes of microcytic hypochromic anemia, which are

observed in the same population with high frequency these include most commonly, iron deficiency anemia. So these patients are inappropriately administered with iron supplements and frank iron overload has been reported. It is clinically important to differentiate between these two anemias because each has an entirely different cause, treatment and prognosis. Screening for BTT therefore assumes importance in preventing the potential of homozygous offspring [4]. The diagnosis is made through evaluation of positive family history or during population screening [5]. BTT should be ruled out so as to prevent the occurrence of β thal major [6].

Materials and Methods

The study was carried out on patients undergoing investigations for anemia in the hematology central Lab, Department of Pathology, at Kempegowda Institute of Medical sciences, Bangalore, in the recent three years past. The cases included patients who attended the out patient and in patient departments. Total 500 cases were included in the present study. The inclusion criteria was cases with mean corpuscular volume (MCV) ≤ 80 fl [based on Advia 60-Open Tube (OT) 18, Automated Hematology System, Bayer]. The relevant personal history was obtained by personal interview with the patient when ever possible and from the hospital records as per the proforma. 100 controls were included in the present study. The control subjects were those with hemoglobin concentration, ESR and MCV in normal range (i.e. MCV>80fl). The exclusion criteria were pregnant women, those with recent blood transfusion and those with chronic inflammatory disorders. Ethical Clearance has been obtained from the Institutional Ethical Committee. Informed consent has been taken from the patients.

2.5ml of venous blood was collected into a tube containing K2 EDTA (1.5±0.25 mg/ml). Complete blood counts (CBC) were performed on all the samples as routine test on coulter (Advia 60-Open Tube (OT) 18, Automated Hematology System, Bayer) within two hours of sample collection. Cases with MCV ≤ 80fl were included in cases population (n=500) and those with MCV > 80fl, normal range of Hb and ESR were included in the control group. An 18 parameter analysis of blood was automatically obtained. The counting principle is based on impedance variation generated by passage of cells through the calibrated micro aperture [WBC = 80µm and RBC/ platelet = 50µm]. The peripheral blood smear examination was carried out for all cases.

Those cases positive for at least 2 of the discriminant functions were further screened by Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT) by standard method.

Table 1: Discrimination limits (cut-off points) which suggest BTT and NON BTT

	Discriminant Functions	**BTT**	**NON – BTT**
England and Fraser [7]	DF1=MCV-RBC-(5XHb)-3.4	<0	>0
Mentzer ratio	DF2=MCV/RBC	<13	>13
Srivastava ratio	DF3=MCH/RBC	<3.8	>3.8
Shine and Lal product	DF4 =$(MCV)^2$ /MCHX0.01	<1530	>1530
Klee	DF5=RBC Counts	>5X10^{12}/L	<5X10^{12}/L

Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT)

Positive (+ve) – The black is not visible, Negative (-ve) – The black line is clearly visible, Doubtful (+/-) – The black line is partially visible.

β thalassemia trait was confirmed by Cellulose acetate Hemoglobin Electrophoresis at 8.6 pH and quantization of HbA2 by elution Technique. HbA2 > 3.5 % were considered diagnostic for BTT.

Staining of blood smear: The blood smears were stained by Leishman's stain.

Reticulocyte count: The counting procedure should be appropriate to the number of the reticulocyes present. Very large number of cell have to be surveyed if a reasonably accurate count is to be obtained.

Statistical Methods: Chi-square and Fisher exact test have been used to test the significance of proportions of Lab parameters between BTT and Non-BTT diagnosed based on Electrophoresis. Student test (independent) has been used to find the significance of Mean values of Lab parameters and Discriminant functions between BTT and Non-BTT. The Odd Ratio has been used to find the strength of relationship of BTT and lab parameters. The Multivariate Logistic Regression has been used to find the significant predictors among lab parameters and Discriminant functions of BTT. The Statistical software namely SPSS 11.0 and Systat 8.0 were used for the analysis of the data and Microsoft word and Excel have been used to generate graphs, tables etc.

Table 2: Interpretation of HbA_2

Hb A_2 range (%)	Interpretation
3.5-7.0	β Thalassemia trait
>7.0	Exclude structural variant
	Repeat HbA_2 Estimation
	Rare β Thalassemia mutations
3.3 – 3.7	Severe IDA in β Thalassemia trait
	δβ Thalassemia trait
	Interaction of αβ Thalassemia
	Rare β Thalassemia mutations
	Presence of HbS, making accurate measurement difficult
	Analytical error. Repeat analysis.
2.0-3.3	Normal
	δβ Thalassemia (If Hb F elevated)
	α Thalassemia trait
<2.0	δβ Thalassemia (If Hb F elevated)
	α Thalassemia trait
	Hb H disease

Results:

Figure 1: Age distribution with Sex in cases with MCV <80fl

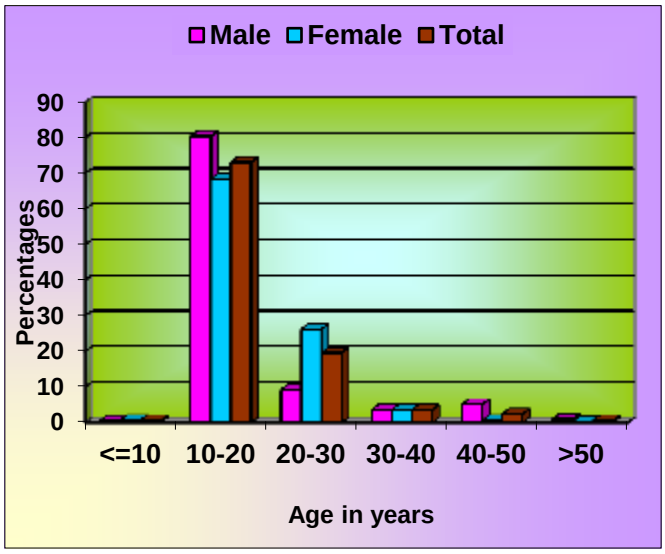

Table 3: Personal History in the study group MCV<80fl

Personal History	BTT		Non-BTT		Total	
	Number	%	Number	%	Number	%
Nil	19	55.88	260	55.79	279	63.8
Weakness	10	29.41	113	24.25	123	20.6
Fever	2	5.88	55	11.80	57	9.4
Cough	-	-	17	3.65	17	2.4
Rhinitis	1	2.94	5	1.07	6	0.2
URI	-	-	4	0.86	4	0.8
Icterus	1	2.94	-	-	1	0.2
Allergy	-	-	3	0.64	3	0.6
Joint pain	1	2.94	1	0.21	2	0.4
Loss of Appetite	-	-	8	1.72	8	1.6
Total	34	100.00	466	100.00	500	100.0

Figure 2: Frequency of Beta Thalassemia Traits in MCV<80fl cases

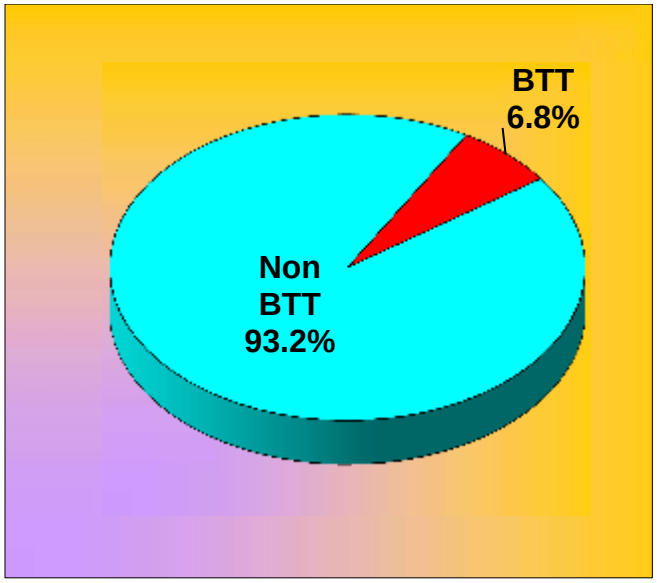

Figure 3: Association of Family History and Consanguinity with the presence of BTT

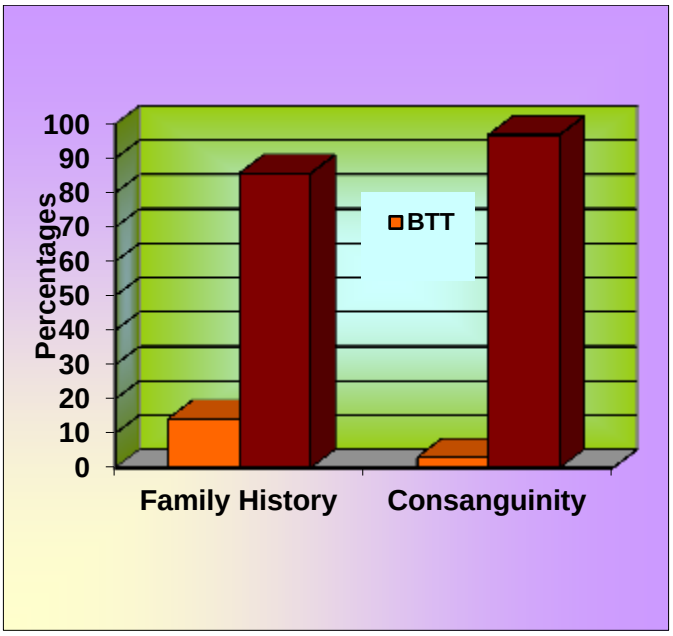

Table 4: Mean pattern of parameters between Presence/Absence of BTT

Parameters/Risk factors (Mean ± SD)	BTT (n=34)	Non-BTT (n=466)	P value
WBC (cells/mm^3)	8985.29±2384.56	9016.20±2945.33	0.952
RBC(x 10^{12} cells/ L)	5.76±1.16	4.82±0.75	0.000**
Hb(gms/dl)	11.01±2.06	11.21±1.99	0.579
HCT (%)	34.74±7.27	36.61±15.63	0.488
MCV(fl)	60.56±5.58	74.54±6.58	0.000**
MCH (pg)	18.78±3.45	23.39±2.91	0.000**
MCHC (gms/dl)	30.52±2.56	31.43±3.38	0.123
RDW (%)	16.33±2.28	15.99±2.58	0.469

* Significant at 5% ** Significant at 1% [a] Near Significant

Table 5: Mean pattern of Discriminant functions between Presence/Absence of BTT

Discriminant Functions (Mean ± SD)	BTT (n=34)	Non-BTT (n=466)	P value
DF1 MCV-RBC-(5 X Hb)-3.4	-0.26±11.38	10.28±7.99	P<0.001**
DF2 MCV/RBC	11.20±4.08	15.82±2.85	P<0.001**
DF3 MCH/RBC	3.58±1.77	4.99±1.12	P<0.001**
DF4 0.01 X MCH X (MCV)^2	714.83±226.45	1330.82±314.36	P<0.001**
DF5 RBC COUNTS	5.76±1.15	4.82±0.75	P<0.001**

* Significant at 5% ** Significant at 1% [a] Near Significant

Table 6: Association of Lab parameters with BTT

Lab Parameters	BTT (n=34)	Non-BTT (n=466)	Total (n=500)	P value (OR-BTT)
WBC (>11000)	6 (17.6)	67 (14.4)	73 (14.6)	0.616 (1.26)
RBC (>5 x10^{12})	28 (82.4)	195 (41.8)	223 (44.6)	P<0.001** (6.41)
Hemoglobin (<10)	15 (44.1)	110 (23.6)	125 (25.0)	0.008** (2.53)
HCT (<35)	16 (47.1)	197 (42.3)	213 (42.6)	0.720 (1.20)
MCH (<26.5)	32 (94.1)	406 (87.1)	438 (87.6)	0.409 (2.25)
MCHC (<31.8)	23 (67.6)	313 (67.2)	336 (67.2)	P>0.05 (0.99)
RDW (<15)	12 (35.3)	227 (48.7)	239 (47.8)	0.122 (0.57)
Inference	RBC and Hemoglobin are significantly and positively associated with the incidence of BTT (P<0.05)			

(Figures in parenthesis are percentages) * Significant at 5% ** Significant at 1% [a] Near Significant

A screening study consisting of 500 cases with red cell microcytosis (MCV <80fl) was designed so as to pick up possible Beta Thalassemia trait cases. The effectiveness of various lab parameters, Discriminant Functions and NESTROFT when compared to electrophoresis was investigated. 100 subjects with MCV> 80fl was taken as controls. Around 73.20% of the study population is in 10-20 age group followed by the 19.6% in the age group 20-30 years. The study population consisted of 61% females and 39% males.

Around 56% of BTT had no symptoms, 29.41% of BTT were associated with generalised weakness, 5.88% with fever, 2.94% with rhinitis, 2.94% with icterus, 2.94% with joint pain as shown in the table 3. The frequency of Beta Thalassemic traits was 6.8% among the patients with MCV<80fl as shown in figure 2.

Family history was positively associated with the BTT. Patients with Family history of BTT were 2.30 times more likely to have BTT, (p=0.393).

Mean RBC was significantly more in BTT (5.76 x1012) when compared to Non-BTT (4.82x1012), Mean hemoglobin (11.01 gram/dl) was mildly less in BTT cases when compared to non-BTT (11.21 gram/dl). Similarly, MCV, MCH and MCHC were significantly reduced in BTT cases when compared to non-BTT cases. RDW (16.33%) was mildly increased when compared to non-BTT as shown in table 4. Mean score of discriminant functions namely DF1, DF2, DF3 and DF4 were significantly reduced in BTT cases when compared to Non-BTT cases (P<0.001), DF5 was significantly raised in BTT cases when compared to Non-BTT cases (P<0.001).table 5

Cases of BTT were found to have a Hb of <10gms/dl, Red cell count of >5x1012/L. Therefore microcytic cases with Hb<10gms/dl and red cell count of>5x1012/L were found to have 2.53 times and 6.41 times more likelihood of having BTT respectively as shown in the table 6.

222 cases were positive for at least two of the five DF's in the limits, which suggest BTT. Hence 44.40% the cases for which MCV <80fl, electrophoresis was conducted. Patients with Nestroft Positivity were 141.17 times significantly more likely to have Increased HbA2 levels on electrophoresis.

The most sensitive DF was found to be DF4 with sensitivity of 97% however the specificity was lowest (9.57%). DF1, DF2 and DF3 showed sensitivity of 70.6%, 85.3% and 82.4%, specificity were 78.19%, 71.28% and 75.53% respectively. The discriminant functions DF1, DF2 and DF3 showed higher accuracy (77.03%, 73.42% and 76.58%) despite comparatively low positive predictive values. As the diagnostic values

Table 7: Association of Discriminant function with BTT

Discriminant Functions	BTT (n=34)	Non-BTT (n=466)	Total (n=500)	P value
DF1(<0.0) MCV-RBC-(5 X Hb)-3.4	24 (70.59)	42 (9.01)	61 (12.2)	P<0.001**
DF2(<13.0) MCV/RBC	29 (85.3)	54 (11.6)	83 (16.6)	P<0.001**
DF3(<3.80) MCH/RBC	28 (82.35)	7 (1.5)	24 (4.8)	P<0.001**
DF4(<1530.0) 0.01XMCHX(MCV)^2	33 (97.05)	339 (72.7)	373 (74.6)	P<0.001**
DF5(>5.0) RBC COUNTS	28 (82.4)	197 (42.3)	225 (45.0)	P<0.001**
Inference	Picking rate of BTT is less with the use DF1when compared to other DF`s			

(Figures in parenthesis are percentages) ** Significant at 1%

Figure 4: Association of Nestroft and Electrophoresis in DF positive cases

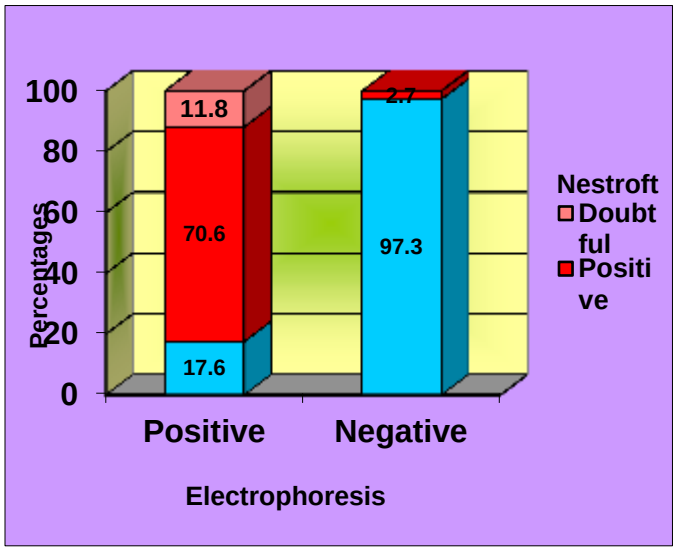

Table 8: Association of Peripheral Smear with BTT

Peripheral smear	BTT (n=34)	Non-BTT (n=466)	Total (n=500)	P value (OR-BTT)
1.Microcytic, Hypochromic with Anisopoikilocytosis	16 (47.1)	87 (18.7)	103 (20.6)	P<0.001** (3.87)
2.Microcytic, hypochromic with Mild Anisopoikilocytosis, target cells	9 (26.5)	15 (3.2)	24 (4.8)	P<0.001** (10.51)
3.Microcytic, Hypochromic Target cells, Polychromatophilic cells	2 (5.9)	2 (0.4)	4 (0.8)	0.025* (14.41)
4.Normocytic Hypochromic	2 (5.9)	90 (19.4)	92 (18.4)	0.049* (0.26)
5.Normocytic normochromic	4 (11.8)	265 (57.2)	269 (53.8)	P<0.001** (0.09)
6.Microcytic, hypochromic with Anisopoikilocytosis target cells & Basophilic Stippling	1 (2.9)	-	-	P<0.001**
Inference	Patients with Microcytic, hypochromic cells with Mild Anisopoikilocytosis, target cells were significantly more likely to have BTT (P<0.05).Also with presence of basophilic stippling(p<001) .Normocytic normochromic cells seen were subjective but coulter could pick them as microcytic.			

are obtained independently and will not take into account the interaction between the parameters. Hence multivariate logistic regression analysis was done to evaluate the best combination of DF's to screen cases of BTT. DF2 and DF3 were found to be a good combination (adjusted odd's ratio DF2=17.44 and DF3=8.51) in order of significant predictors to screen BTT, as per the multivariate logistic regression, which includes 4 discriminant functions in the model.

Table 9: Diagnostic values of Discriminant Functions with respect to Electrophoresis

Discriminant Functions	Diagnostic values in relation to Electrophoresis						
	Sensitivity	Specificity	FN	FP	PPV	NPV	Accuracy
DF1(<0.0) MCV-RBC-(5 X Hb)-3.4	70.59	78.19	44.12	21.81	36.92	93.63	77.03
DF2(<13.0) MCV/RBC	85.29	71.28	14.71	28.72	34.94	96.40	73.42
DF3(<3.80) MCH/RBC	82.35	75.53	50.00	3.72	37.84	95.95	76.58
DF4(<1530.0) 0.01 x MCH x $(MCV)^2$	97.05	9.57	2.95	90.43	16.67	94.74	22.97
DF5(>5.0) RBC COUNTS	82.35	10.11	17.65	89.89	14.21	76.00	21.17
NESTROFT	90.00	97.34	10.00	2.66	84.85	98.39	96.33

Table 10: Multivariate Logistic regression for Beta Thalassemic trait

Model Parameters	Estimates of Multivariate Logistic Regression Model				
	B weight	SE	Wald	P value	Adj(OR)
DF1 (<0.0)	0.166	0.582	0.082	0.779	1.18
DF2 (<13.0)	2.86	0.596	22.974	0.000**	17.44
DF3 (<3.80)	2.14	0.591	13.117	0.000**	8.51
DF5 (>5.0)	0.355	0.613	0.335	0.162	1.43
Constant	-4.566	0.528	74.862	-	-

DF4 is omitted as it gives larger standard Error, therefore it is not statistically feasible to include in the model.

Table 11: Multivariate Logistic regression for Beta Thalassemic traits

Model Parameters	Estimates of Multivariate Logistic Regression Model				
	B weight	SE	Wald	P value	Adj (OR)
WBC (>11000)	-1.020	1.063	0.921	0.337	0.36
RBC (>5 x10^6)	2.669	1.196	4.981	0.026*	14.43
Hemoglobin (<10)	2.642	1.338	3.896	0.048*	14.04
HCT (<35)	-2.375	1.641	2.093	0.148	0.09
MCH (<26.5)	0.121	1.558	0.006	0.938	1.13
MCHC (<31.8)	-0.156	0.910	0.029	0.864	0.86
RDW (<15)	-0.863	0.737	1.369	0.242	0.42
NESTROFT (Positive)	6.619	1.176	31.683	0.000**	749.00
Constant	-6.891	4.467	2.379	-	-

Table 12: Mean HbA_2 levels in BTT/Non-BTT as per Electrophoresis

HbA2	Electrophoresis (n=222)	
	BTT (n=34)	Non-BTT (188)
Range	3.50-6.50	1.70-3.40
Mean ± SD	4.53 ± 0.65	2.54 ± 0.30
95% CI	4.31-4.78	2.49-2.58

Table 13: Mean pattern of Lab parameters between two groups of MCV

Parameters/Risk factors (Mean ± SD)	MCV <80 (n=500)	MCV >80 (n=100)	P value
WBC cells/mm^3	9014.08±2908.38	8084.00±1891.97	0.002**
RBC(x 10^6) cells/ mm^3	4.89±0.82	4.87±0.59	0.802
Hb gram/dl	11.15±2.02	12.65±1.29	0.000**
HCT %	36.48±15.21	41.21±4.75	0.002**
MCV fl	73.58±7.41	84.91±2.89	0.000**
MCH pg	23.09±3.15	26.08±1.47	0.000**
MCHC gram/dl	31.33±3.39	31.15±4.03	0.648
RDW %	16.02±2.56	14.15±1.84	0.000**

** Significant at 1%

Table 14: Association of BTT with MCV

Case-Control	MCV <80 (n=500)	MCV >80 (n=100)
BTT	34 (6.80)	-
Non-BTT	466 (93.20)	100
Total	500 (100.00)	100
Inference	Beta Thalasemic traits are significantly associated with MCV <80	

The Multivariate logistic regression included 8 parameters, namely, WBC, RBC, Hb, HCT, MCH, MCHC, RDW and NESTROFT, out of which NESTROFT, RBC and Hemoglobin are the significant predictors in order of BTT in term of Adjusted Odds ratio as shown in the table 11. Electrophoresis on cellulose acetate at pH 8.6 was performed on 222 cases after screening 500 microcytic cases by DF's and NESTROFT. In the present study 34 cases (n=222) were showing increased HbA2 in the range of 3.5-6.5% and mean 4.53 ± 0.65%. Among all the 222 samples which were subjected to electrophoresis, border line HbA2 (3.3 - 3.7%) levels were seen in 4 cases.

One case with 3.4% HbA2, two cases with 3.5% and one case with 3.7%. Electrophoresis was repeated on all four cases. After which 3 cases with HbA2 levels of 3.5% and were considered to be BTT. One case with HbA2 was followed up after oral iron therapy and repeat electrophoresis showed HbA2 levels, and one case of HbS – β thalassemia trait was diagnosed out of 34 BTT cases.

Statistical Methods:

Chi-square and Fisher exact test have been used to test the significance of proportions of Lab parameters between BTT and Non-BTT diagnosed based on Electrophoresis. Student test (independent) has been used to find the significance of Mean values of Lab parameters and Discriminant functions between BTT and Non-BTT. The Odd Ratio has been used to find the strength of relationship of BTT and lab parameters. The Multivariate Logistic Regression has been used to find the significant

predictors among lab parameters and Discriminant functions of BTT.

Discussion

Beta thalassemia is the commonest inherited hemoglobinopathy. Prevalence of beta thalassemia trait (BTT) varies from 1.0% - 14.9% in various regions of India. The classic heterozygote carrier of BTT is usually asymptomatic [1]. Though family history of thalassemia is important, a significant number of patients do not have previously affected family members [2].

The aim of this study was to evaluate the sensitivity & specificity of various cost-effective screening tools like Discriminant Functions & NESTROFT for detection of BTT before subjecting the suspected microcytosis cases for an expensive technique like hemoglobin electrophoresis and estimation of HbA2 which is confirmatory.

Microcytosis in routine hematology is encountered in about four well defined conditions. They are iron deficiency anemia (IDA), thalassemia minor, anemias of chronic disease and sideroblastic anemia. Among these, IDA and BTT are common causes of microcytosis. However in all cases of microcytosis, BTT should be ruled out so as to prevent the occurrence of β thal major [6]. In this direction, in the present study an algorithm was used to segregate cases of non-BTT and possible BTT by simple, cost effective screening tests. Therefore the use of red cell indices in the form of Discriminant functions and osmotic fragility test like NESTROFT were used.

The base of this algorithm was to screen cases of microcytosis. Nishi Madan et al found sensitivity of MCV < 80fl to be 98.21% and MCV<70fl to be 89.31% [1]. England and Fraser suggested that DF's should only be used when BTT or IDA are suspected in individuals with microcytosis and it is pointless to calculate DF's for individuals with normal MCV i.e. MCV >80fl [7]. It has been proposed that electronic measurement of MCV<80fl should be used as a screening test for BTT as per the BCSH General hematology task force guide lines for investigation of the α and β thalassemia traits [8]. Hence in the present study all the cases with MCV<80fl were screened for possibility of BTT. Various formulae (DF's) have been used to determine whether the blood count is more suggestive of BTT or IDA, however these DF's have been found to give a misleading results in pregnancy, hemodilution and after acute hemorrhage [7]. Hence the present study was carried on five hundred cases with MCV<80fl and 100 controls with MCV >80fl.The exclusion criteria were all pregnant women, cases with recent hemorrhage and chronic inflammatory conditions.

Age

Bolan et al in 2001 screened 14200 participants, with MCV≤80fl including 50% female & 50% male ranging from 14 to 54 years of age [9]. Lan, Yu Lung et al in 1997 carried out screening in 1800 high school students (10 -20 yrs) out of which 150 students had MCV <80fl with prevalence of 3.4% BTT [10]. The present study included 500 cases with MCV <80fl. Majority of patients in the present study were females (61%) and males composed 39%.Majority (73.20%) were in 10 – 20 years age group followed by 19.6% in the age group of 20-30 yrs, 3.6% in 30- 40 yrs, 2.4 % in 40-50 yrs range. This study included age ranging from 2 yrs to 52 yrs.

Frequency of BTT

The distribution of Beta thalassemia gene is not uniform in the Indian sub continent and therefore its varying frequency in different regions. Ambekar et al in 2001 studied 1291 subjects in Western Maharashtra for detection of BTT [11]. The criteria used for diagnosis of BTT were positive family history, Hb level below 10 g/dl, NESTROFT positivity & Hb A2 level more than 3.5 %. In the present study the frequency was 6.8%.

Laboratory parameters based on Coulter Readings (Advia 60 OPEN TUBE (`18)--BAYER)

Hemoglobin

Madan et al studied 337 BTT cases & 40 normal controls for which red cell indices and DF's were studied. They found mean Hb concentration was 11.6 ± 1.6 g/dl (P< 0.0001) as compared to controls [1]. Mohamed. et al found that mean hemoglobin of 11.30±1.45g/dL in BTT cases [12]. Das Gupta et al studied in 1994, 56 cases of BTT and 50 cases of controls and found mean Hb of 11.2± 1.4g/dL in BTT cases [13]. Khin Ei Han et al studied 133 BTT and 30 controls and found mean Hb of 11.5±1.6g/dL in BTT cases [14]. In the present study the mean Hb was 11.01±2.06g/dL in BTT cases and 12.65±1.29g/dL in controls. BTT cases generally have mild anemia with mild decrease in Hb value. So Hb was significantly less in BTT as compared to controls.

Table 15: Mean pattern of various lab parameters in BTT in different studies

Study	No. of cases (n)	Hb(g/dl)	RBC ($\times 10^{12}$/l)	MCV fl	MCH pg	MCHC %	RDW %
Das Gupta et al (1994) [13]	N= 56	11.2±1.4	5.6±0.7	64.5±3.7	20±1.2	31.2±0.94	15.1±1.2
Mohamed.M et al (1999) [12]	N=382	11.3±1.45	5.45±0.71	64.81±4.72	20.75±1.64		16.06±0.97
Khin Ei Han et al (1992) [14]	N=133	11.5±1.6	5.9±1.0	62.7±12.1	19.9±3.5	29.3±2.2	
Nishi Madan et al (1999) [1]	N=337	11.6±1.6	5.56±0.76	64.7±4.8	20.6±3.6		
Present study(n=500)	**N=34**	**11.01±2.06**	**5.76±1.16**	**60.56±5.58**	**18.78±3.45**	**30.52±2.56**	**16.33±2.28**
Present study controls	**N=100**	**12.65±1.29**	**4.87±0.82**	**84.91±2.89**	**26.08±1.47**	**31.15±4.03**	**14.15±1.84**

Table 16: Comparison of sensitivity (Number of patients correctly classified as BTT) of BTT with other studies

Studies	Number of cases	Sensitivity (%)				
		DF1	DF2	DF3	DF4	DF5
1.George Klee et.al (1976) [18]	39	79.5	84.6	84.6	-	84.6
2.Khin Ei Han et.al (1992) [14]	133	62.4	71.4	66.9	88.7	79.0
3.Mamatha et.al (1997) [19]	1695	42.7	62.2	55.6	-	-
4.Das Gupta et.al (1997) [13]	56	-	60.0	-	-	-
5.Mohamed M et.al (1999) [12]	382	88.7	70.6	-	-	-
6. Nishi Madan et.al (1999) [1]	337	83.4	88.7	89.0	97.9	85.2
7. Present study (2006)	**500**	**70.6**	**85.3**	**82.4**	**97.1**	**82.3**

In conclusion, differentiation of BTT from non-BTT has important clinical implications in hematology and medicine. The present study demonstrates that a set of linear discriminant functions (DF2 and DF3) using routine haemogram data can effectively discriminate between BTT and non BTT.

NESTROFT

A positive test of NESTROFT indicates lowered red cell osmotic fragility, which is suggestive of BTT. Mehta BC et al in 1991 studied

131 cases and found sensitivity, specificity, PPV and NPV to be 99.2%, 75.8%, 69.3% and 99.5% respectively [20]. Raghavan et al in 1991 found sensitivity, specificity, PPV and NPV to be 95.5%, 87%, 70.5% and 98.3% [21]. Susanna Thomas et al in 1996 studied 137 cases and found sensitivity, specificity, PPV and NPV to be 98.7%, 66.6%, 87% and 96.5% [22]. Mamta et al in1997 studied 1695 cases and found sensitivity, specificity, PPV and NPV to be 94.4%, 64.2%, 35.3% and 97.6% [19]. Manjula et al in 1999 studied 1048 cases and found sensitivity, specificity, PPV and NPV to be 91%, 95%, 55% and 99% [3]. In the present study Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT) was carried on cases which showed DF's positive for BTT. The sensitivity was 90%, specificity was 97.34%, and PPV was 84.85% NPV was 98.39%.

.

Table 18: NESTROFT TEST

Study	No of cases	Sensitivity	Specificity	PPV	NPV
Manjula et al (1999) [3]	n=1048	91%	95%	55%	99%
Mamta Manglani et al (1997) [19]	n=1695	94.4%	64.2%	35.3%	97.6%
Mehta B C et al (1991) [20]	n=131	99.2%	75.8%	69.3%	99.5%
Susanna Thomas et al (1996) [22]	n=137	98.7%	66.6%	87%	96.5%
Raghavan et al (1991) [21]		95.5%	87.0%	70.5%	98.3%
Present study	n=222	90%	97.34%	84.85%	98.39%

PPV- positive predictive value, NPV-negative predictive value

Though the sensitivity is slightly lower (90%), the specificity is higher (97.34%) in the present study. The present study is comparable to Manjula et al [3] and Susanna et al [22]. The PPV in the present was 84.85% which is comparable to Susanna Thomas et al. All the studies including the present study show a higher NPV. The possible explanation given by Susanna Thomas et al is constant occurrence of IDA with BTT which needs conformation by HbA_2. Similarly from the present study we conclude as Susanna Thomas et al that NESTROFT can be used as a mass screening test which is cost effective, simple and rapid when combined with DF's.

Conclusion

Multivariate logistic regression analyses to evaluate the best combination of DFs to screen BTT were found to be DF2 & DF3 [adjusted Odds ratio of 17.44 and 8.51]. NESTROFT showed 90% sensitivity, 97.34% specificity, PPV was 84.85% and NPV was 98.39%, hence a high NPV. Therefore it can be used as a mass screening test. Multivariate logistic regression of NESTROFT and red cell parameters showed NESTROFT, RBC > 5 x 1012 / l and mildly decreased Hb were best combination for prediction of BTT with adjusted odds ratio of 749, 14.43 & 14.04 respectively. Mean HBA2 was found to be 4.53±0.65% on Cellulose Acetate Electrophoresis and HBA2 elution. Hence we conclude that the differentiation of BTT from Non BTT has important clinical implication in hematology and medicine. The present study demonstrates that set of cost effective screening tests like NESTROFT, DF2 & DF3 along with routine haemogram data (RBC & Hb) in microcytic cases can effectively discriminate between BTT and Non BTT, and diagnosis of BTT can be reliably done by CAE electrophoresis and HbA2 quantitation by elution with HbA2 > 3.5%.

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