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Evaluation of Discriminant functions for differentiating Iron deficiency anemia from Beta Thalassemia trait: A Single Center Study

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Abstract

Objective: To evaluate the effectiveness of discriminant functions (DF) in differentiating iron deficiency anemia (IDA) from beta thalassemia trait (BTT).

Methodology: A comparative cross sectional descriptive study was conducted at The CITI Lab, Rawalpindi. A total of 1538 patients from August 2023 to March 2025 were assessed after taking relevant history. Blood samples were assayed for complete blood count (CBC), Hemoglobin (Hb) electrophoresis and Serum ferritin. CBC was run within 6 hours on automated hematology analyzer Beckman Coulter unicel DxH 800. Hb electrophoresis was done on Sebia Capillary 2 Flex- piercing (Sebia capillary electrophoresis system). Serum ferritin estimation was done on Architect i1000 SR immunoassay analyzer.

Results: The best DF were Hisham and Wongprachum indices with Youden index values of 50.2% and significant differences were observed in all CBC parameters except MCHC between BTT and IDA patients ($p < 0.001$).

Conclusions: The Hisham and Wongprachum indices showed the best performance in differentiating IDA from BTT and thus can be used for screening to improve diagnosis.

Keywords: Microcytic Hypochromic Anemia, Differential Diagnosis, Iron deficiency anemia, Thalassemia Trait

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Introduction

Anemia is identified as a global health problem by WHO especially in developing countries like Pakistan[1].It is a condition in which number of red blood cells (RBCs) or hemoglobin concentration (Hb) is decreased.[2] According to WHO 40 percent children, 37 percent pregnant females and 30 percent females in the world are affected by anemia.[3] In resource limited countries microcytic hypochromic anemia (MHA) have high prevalence. Iron deficiency anemia (IDA) and beta thalassemia trait (BTT) are the main differential of MHA[4]. Thalassemia is a genetic disease due to mutation in HBB gene on chromosome 11 leading to decrease production of beta globin chains. It has autosomal recessive pattern of inheritance[5] whereas IDA is characterized by insufficiency of healthy RBCs due to lack of iron resulting in defective heme synthesis.[6] In the world approximately 1 billion people are suffering from IDA.[7] It causes detrimental effects on human health especially in pregnancy and childhood[8]. The prevalence of IDA in pregnancy in Pakistan is 70–80%[9]. Increased incidence of BTT in World is seen in areas like Mediterranean, Middle East, Indian subcontinent, Southeast Asia and South China. In Pakistan BTT frequency is 5-8 percent and 5000 children are born each year with thalassemia major.[10]

IDA can occur due to reduced iron intake, impaired absorption of iron, increased physiological or pathological requirement and chronic blood loss but in developing countries like Pakistan the most common cause of IDA is dietary insufficiency.[11] Erythropoiesis occurs in the marrow and evidence suggests that the number of cell divisions and RBC size is related to the rate of Hb synthesis. Whenever there is defective heme synthesis (IDA) or globin synthesis (thalassemia) the concentration of Hb will fall and the RBCs will keep dividing. Thus numerous microcytic RBCs are seen in both IDA and thalassemia with decreased Hb level. Hence the RBC count is higher with respect to Hb level in both IDA and thalassemia but thalassemia has an additional hemolytic component so the RBC count of thalassemia is higher than IDA.[12]

Authorship Contribution: [1,2]Conceived and planned the idea of the study, drafting the work or revising it critically for important intellectual content[1, 2] final approval of the version to be published,

Funding Source: none
Conflict of Interest: none

Received: Sept 26, 2025
Accepted: Dec 11, 2025

IDA and thalassemia trait both presents with similar symptoms and clinical features. So it is difficult to differentiate IDA from BTT. Both IDA and BTT patients present with similar RBC indices and morphology. In both cases anisocytosis, pikilocytosis, microcytosis and hypochromia is seen. CBC and microscopic analysis of the blood film alone are not sufficient to make diagnosis of IDA or BTT. Thus additional tests like serum ferritin, serum iron, total iron binding capacity (TIBC), transferrin saturation and Hb electrophoresis are required to differentiate IDA from BTT.[13] In IDA serum ferritin, serum iron, and transferrin saturation is low with high TIBC. Serum ferritin which is the most specific indicator of IDA is an acute phase reactant and is elevated in inflammation[14]. Erythrocytosis with low mean corpuscular volume (MCV) irrespective of the Hb is suggestive of BTT which is then confirmed by Hb electrophoresis and normal iron profile. In BTT Hb A2 level is increased however in some BTT cases Hb A2 is not elevated. Some conditions like IDA and megaloblastic anemia can also affect the Hb A2 levels so molecular analysis of DNA sequence is gold standard for diagnosis of thalassemia.

The differential diagnosis of microcytic anemia is therefore complex, time consuming, expensive and often inaccessible to a great number of people. Thus simple and less costly indexes to screen for microcytic hypochromic anemia would be extremely useful. Hematologists have invented discriminant formulas (DF) which are based on basic and readily available RBC parameters to successfully differentiate between IDA and BTT. Currently more than 45 such simple DFs are available.[4]

The purpose of this study is to apply some of these DF in our population and observe the sensitivity and specificity of these formulas in our population. No additional blood sampling or expenditure is required for these formulas and if successful they can be used as a screening program at the population level. This approach can help us use our health care resources rationally and we can provide early and accurate treatment interventions.

Methodology

A comparative cross sectional descriptive study was conducted at The CITI Lab, Rawalpindi from August 2023 to March 2025 after ethical approval from institutional review board. The WHO sample size calculator was used to calculate sample size with 95 percent confidence interval and 5 percent margin of error. Data of 1538 patients were collected of which 50 were healthy controls, 294 patients of BTT and 1194 patients of IDA. After taking the history and verbal consent 5 ml of blood was taken, 2 ml was taken in ethylene diamine tetra acetic acid (EDTA) for CBC and Hb electrophoresis and 3 ml sample was taken in gel for serum ferritin estimation. CBC was run within 6 hours on automated hematology analyzer Beckman Coulter unicel DxH 800 and Hb, RBC count and red cell indices were recorded in all cases. Peripheral blood smears of all samples were made. Hb electrophoresis was done on Sebia Capillary 2 Flex-piercing (Sebia capillary electrophoresis system) and percentages of Hb A and Hb A2 was recorded. Serum ferritin estimation was done on Architect i1000 SR immunoassay analyzer. The analyzers involved in this study were maintained and calibrated according to manufacturer's instructions.

Inclusion criteria

1. Patient of any age group or either gender presenting at our collection points.
2. Hb < 12 for females, < 13 for males and for children Hb less than normal Hb for that particular age in case of BTT and IDA group whereas in control group Hb was normal.
3. MCV less than 80, MCH less than 27 and in children MCV and MCH were lower than normal for that particular age in case of BTT and IDA group whereas in control group MCV and MCH were normal.
4. Ferritin level less than 15 ng/ml in adults and less than 7ng/ml in children was considered as IDA[15]
5. Hb A2 value of more than 3.5% was taken as BTT[16]

Exclusion criteria

1. Patients with Hb less than 6g /dl .
2. Patient who had received a blood transfusion within last 3 months.

Using the Hb, RBC counts and red cell indices various DF indices like Shine and lal, Mentzer index, England and Fraser, Sirdah, Srivastava index etc were calculated for each patient using the formulae as shown in table 1.[15]

Table I:DF formulas that will be used to discriminate IDA from BTT			
Rbc index	Formula	Cut off BTT	Cut off IDA
England and Fraser I	MCV- RBC- (5 X HB)	<0.0	>0.0
Srivastava	MCH / RBC	<3.8	>3.8
Mentzer	MCV / RBC	<13.0	>13.0
Shine and Lal	MCV X MCV X (MCH /100)	<1530	>1530
England and Fraser II	MCV- RBC- (5 x Hb) – 3.4	<0.0	>0.0
Telmissani MCHD	MCH / MCV	<0.34	>0.34
Telmissani MDHL	(MCH X RBC)/ MCV	>1.75	<1.75
Kerman I	(MCV X MCH) /RBC	<300	>300
Kerman II	(MCV X MCH x 10) / (RBC X MCHC)	<85	>85
Sirdah	MCV – RBC – (3 X HB)	<27	>27
Ehsani	MCV – (10 X RBC)	<15	>15
Sirachainan	(1.5 X HB) – (0.05 X MCV)	>14	<14
Sehgal	MCV X MCV / RBC	<972	>972
Bordbar	(80 – MCV) X (27 – MCH)	>44.76	<44.76
Matos and Carvalho	(1.91 X RBC) + (0.44 X MCHC)	>23.85	<23.85
RBC count	RBC count	>5	<5
Bessman	RDW	<14	>14
Ricerca	RDW/ RBC	<3.3	>3.3
Green & King	(MCV × MCV × RDW)/ (100 × HB)	<65	>65
Das Gupta	(1.89 × RBC) − (0.33 × RDW) − 3.28	>0.0	<0.0
Hisham	(MCH × RDW) / RBC	<67.0	>67.0
Jayabose RDW	(MCV × RDW) / RBC	<220	>220
Nishad Thal Index	(0.615 × MCV) + (0.518 × MCH) + (0.446 × RDW)	<59	>59
Keikhaei	(HB × RDW × 100) / (RBC × RBC × MCHC)	<21	>21
Wongprachum	(MCV × RDW / RBC) − (10 × HB)	<104	>104
Cruise Jahangiri	MCHC + (0.603 × RBC) + (0.523 × RDW)	>42.63	<42.63

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Using the formulas in table I DFs were calculated in each patient. Sensitivity (Se), specificity (Sp), negative predictive value (NPV), positive predictive value (PPV), construction of receiver operative characteristic (ROC) curves to calculate the area under the curve (AUC) and youden index (YI) were calculated and measured for each index

Calculations were performed as follows:

Se %= (TP) / (TP + FN) x 100,

Sp %= (TN) / (TN+ FP) x 100,

PPV %= (TP) / (TP + FP) x 100,

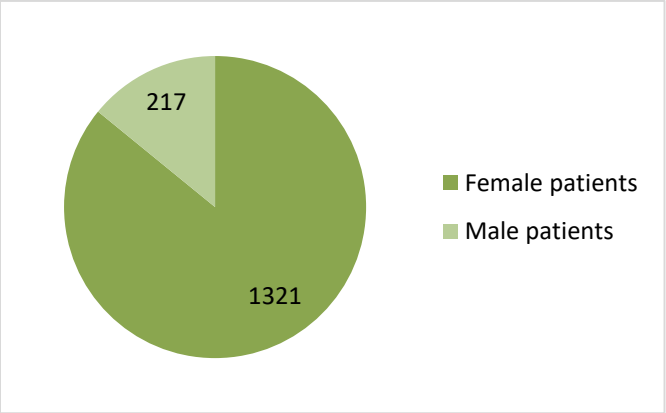
NPV %= (TN) / (TN + FN) x 100,

YI = (Sensitivity + specificity) – 100

Where TP = true positive, FN = false negative, TN = true negative and FP = false positive. Statistical analysis was performed using Statistical Package for the Social Sciences Statistics software (SPSS) version 27.

Results

A total of 1538 patients were enrolled in the study. The distribution of patients is shown in figure 1.



The mean values of characteristics and hematological data of all the groups are summarized in table II. The age and hematological parameters of IDA and TT group were compared using mann whitney u test. A P value of <0.05 were considered significant. In IDA group MCV and MCH were significantly higher whereas Hb and RBC were significantly higher in BTT group. There was no significant difference in terms of age and MCHC.

Table II Mean values of characteristics and hematological parameters in patients with IDA, BTT and control group.				
Mean values	IDA	TT	Control group	P value using mann whitney u test
Age	24.91	25.87	24.2	>0.05
Females	1054	226	41	
Males	140	68	9	
Hb	8.77	9.73	12.9	<0.001
MCV	67.4	61.8	85.4	<0.001
MCH	21.0	19.3	28.8	<0.001
MCHC	31.06	31.09	33.7	>0.05
RBC	4.3	5.1	4.5	<0.001

Table III Efficiency of the DF indices to differentiate between BTT and IDA

Index	AUC	Proposed cut off	Se	Sp	NPV	PPV	YI	Optimum cut off	Se	Sp	NPV	PPV	YI
England and Fraser I	0.831	<0	22.1	99.3	83.8	89.0	21.4	<16.78	82.65	66.86	94	38	49.51
Srivastava	0.786	<3.8	50.7	84.8	87.5	45.2	35.5	<4.2	65.99	77.8	90.3	42.3	43.79
Mentzer	0.812	<13	61.2	83.3	89.7	47.4	44.5	<14.45	76.2	73.3	92.6	41.3	49.5
Shine and Lal	0.714	<1530	99.3	4.9	96.7	20.4	4.2	<1060	90.82	45.06	95.20	28.93	35.88
England and Fraser II	0.831	<0	33.7	98.2	85.8	82.5	31.9	<13.38	82.65	66.83	93.99	38.02	49.48
Telmissani MCHD	0.506	<0.34	93.2	8.3	83.2	20.0	1.5	<0.32	67.3	39.45	83.1	21.5	6.75
Telmissani MDHL	0.183	>1.75	27.6	98.7	84.8	83.5	26.3	>1.4	75.2	72.7	92.2	40.4	47.9
Kerman I	0.778	<300	78.2	67.1	92.6	36.9	45.3	<300	78.2	67.1	92.6	36.9	45.3
Kerman II	0.792	<85	65.6	78.6	90.3	43.0	44.2	<85	65.6	78.6	90.3	43.0	44.2
Sirdah	0.821	<27	51.2	90.4	88.3	56.6	41.6	<32	71.1	76.8	91.52	43	47.9
Ehsani	0.809	<15	63.9	81.2	90.1	45.5	45.1	<18.6	71.8	74.6	91.5	41.1	46.4
Sirachainan	0.279	>14	13.6	99.6	82.4	88.9	13.2	>10	75.9	53.2	89.96	28.5	29.1
Sehgal	0.795	<972	80.6	68.2	93.5	38.4	48.8	<972	80.6	68.2	93.5	38.4	48.8
Bordbar	0.289	>44.76	91.2	41.5	95.0	27.7	32.7	>90	77.6	61.0	91.7	32.9	38.6
Matos and Carvalho	0.208	>23.85	42.5	95.4	87.1	69.4	37.9	>22.5	71.1	73.7	91.2	40.0	44.8
RBC count	0.182	>5.0	52.4	91.2	88.6	59.5	43.6	>4.8	60.9	85.6	89.9	51	46.5
Bessman	0.413	>14	0	98.7	78.5	0	-1.3	>19	65.9	54.6	85.6	28.1	20.5
Ricerca	0.268	<3.3	43.9	98.0	86.6	85.7	41.9	<3.5	48.8	94.7	87.3	71.4	43.5
Green & King	0.186	<65	46.3	98.7	87.2	90.5	45.0	<70	51.2	96.1	88.0	77.8	47.3
Das Gupta	0.757	>0	51.2	93.4	87.7	67.7	44.6	>0	51.2	93.4	87.7	67.7	44.6
Hisham	0.207	<67.0	56.1	94.1	88.8	71.9	50.2	<67	56.1	94.1	88.8	71.9	50.2
Jayabose RDW	0.183	<220	53.7	94.7	88.3	73.3	48.4	<230	58.5	91.4	89.1	64.9	49.9
Nishad Thal index	0.255	<59	90.2	56.6	95.5	35.9	46.8	<58	85.4	63.2	94.1	38.5	48.6
Keikh0aei	0.182	<21	51.2	97.4	88.1	84.0	48.6	<23	58.5	90.8	89.0	63.2	49.3
Wongprachum	0.192	<104	46.3	98.0	87.1	86.4	44.3	<130	56.1	94.5	88.8	71.9	50.2
Cruise Jahangiri	0.622	>42.63	95.1	27.0	95.3	26.0	22.1	>43.3	85.4	42.1	91.4	28.5	27.5

The result of the each DF showing sensitivity, specificity, NPV, PPV, AUC and YI with proposed cut off and optimum cut off are mentioned below in Table III. As shown in table III with proposed cut off the highest sensitivity was shown by Shine and Lal (99.3%) and Cruise Jahangiri (95.1%) but these parameters showed decreased specificity and YI. The highest specificity was showed by Sirachainan (99.6%) and England and Fraser I (99.3%). Youden's index is high in Hisham (50.2%) followed by Sehgal (48.8%) and Keikhaei (48.6%). The lowest YI was shown by bessman. In case of optimum cut off value the highest sensitivity was showed by Shine and Lal (90.82%) followed by Nishad Thal Index (85.4%) and Cruise Jahangiri index (85.4%) whereas highest specificity was showed by Green and king (96.1%) and Ricerca (94.7%). Youden's index was highest in case of Hisham and Wongprachum (50.2%). The lowest YI was shown by MCHD.

ROC curve analysis of all parameters based on their AUC are shown in figure 2 and 3.

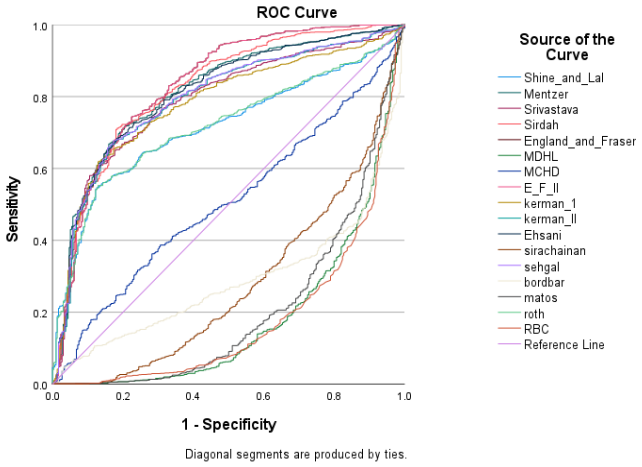


Figure 1:

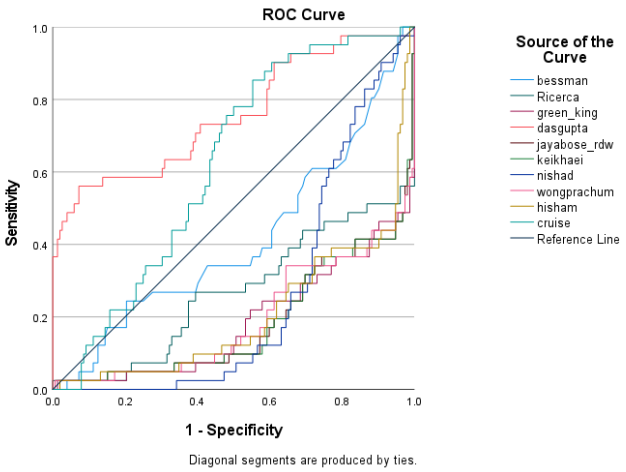


Figure 2

Discussion

Our study showed that the most sensitive DF to differentiate BTT from IDA is Shine and Lal (99.3%). This Observation was similar to studies done by Xiao H et al[18](97.9%), Reis FM et al [19](96%), Jabeen M et al [20] (100%), Wickramaratne KA et al [21] (100%) and Balcázar-Villarroel M et al [17] (100%) however studies done by hoffmann JJ et al [4] showed Jayabose RDW (90.2%) to be most sensitive DF. Singh N et al [15] and Das R et al [22] showed Ricerca to be most sensitive DF (100% and 90.5% respectively) whereas Nerune SM et al [3] showed green and king (90%) and Jayabose RDW (90%) to be most sensitive DF. The specificity of sirachainan and England and Fraser I was high in our study (99%). EFI also showed high specificity in study done by Wickramaratne KA et al [21] and Das R et al [22] (99.7%). Balcázar-Villarroel M et al [17] showed EFI had a specificity of 99.2%. With the optimum cut off our study showed green and king had highest specificity (96.1%) similar to study done by Nerune SM et al [3] which showed green and king had 82% specificity whereas studies done by Singh N et al [15], Xiao H et al[18], FM et al [19] and Jabeen M et al [20] all showed England and Fraser II as the most specific DF. Jayabose RDW (90%) was the most specific DF in study done by hoffmann JJ et al [4]. The highest YI in our study was showed by Hisham and Wongprachum (50.2%). Study by Balcázar-Villarroel M et al [17] showed highest YI by green and king (92.3%) and Wongprachum (90.8%). Sensitivities and YI of different DF in different studies are shown in table IV and V respectively.

Two main causes of microcytic hypochromic anemia are IDA and BTT. It is very important to differentiate IDA from BTT because of difference in prognosis and treatment in both these conditions. Inability to correctly diagnose and treat will lead to iron overload in BTT patients and delay of iron therapy in IDA patients. More over BTT patients can transmit their defective gene to the next generation. To differentiate IDA from BTT we need CBC, Hb electrophoresis, Genetic analysis (in few Cases) and tests for analysis of iron status. These tests are expensive and time consuming for mass screening. Moreover they are not available everywhere. In low resource countries where thalassemia is endemic like Pakistan we need to develop inexpensive and easily accessible methods for mass screening. Various DF derived from RBC parameters have been proposed to distinguish IDA from BTT. By using DF to differentiate between IDA and BTT we can minimize the use of scarce resources and maximize the percentage of population screened

In countries like Pakistan, India, Bangladesh etc where prevalence of thalassemia is high screening programs should be initiated to detect BTT. Full blood count (FBC), red cell indices and DFs can be used to screen BTT. As we can see that none of the DF is strong enough to make a final diagnosis but a combination of DFs can make a probable diagnosis and thus we can effectively select patients that need confirmatory diagnostic testing. It will reduce burden on our healthcare resources. Some DFs performance is good in some population but in other population that DF might not be that effective. It may be due to the fact that some DFs are developed based on small sample size or due to fact that genetic makeup of one population is different than other. The disorder is present worldwide but its prevalence is different in different regions of the world and even within a country the prevalence of this disease is different. Although IDA and BTT are both most common etiologies of MHA but both of them can coexist together thus complicating the given scenario. The parameters of RBC are also show heterogeneity regionally and ethically and thus they also affect DFs. Therefore, independent validation of DFs needs to be done to see the strength of each DF.

LIMITATIONS: There was sample bias as our study was done on patients that presented to us for antenatal screening or as a part of anemia work up. It was a single centre study and further validation in multicentre studies was required.

Conclusion

Due to the above mentioned facts finding DFs which are better at discriminating IDA from BTT may vary from one region to another. Our aim should be to find DFs and the cut offs that are better suited for our population so that we can provide a method to differentiate between BTT and IDA which is both cost effective and efficient. Even we can develop new DFs which are suited to our population.

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Table IV Sensitivities of the DF indices to differentiate between BTT and IDA

	EF1	Srivastava	Ment	S and L	EF2	MCHD	MDHL	Kerman 1	Kerman 2	sirdah	Ehsani	sirachainan	Sehgal	Bordbar	Matos	rbc	bessman	Ri	GK	DG	HI	JR	NT	KE	WO	CJ
Nerune r et al (3)		70	75	62	85												85		90			90				
Hoffman n et al (4)_		90.1	89.8		87.0			88.1	88.8	88.3	86.4				81.1			88.0	89.3	84.7	88.9	90.2				84.9
Singh et al (15)		40	50	80	30					40								100	50			60				
Balcázar et al (17)	51.0	78.4%	90.2%	100	72.5%	98.0%	66.7%	94.1%	84.3	82.4%	90.2%	60.8%	98.0	98.0%	90.2%		74.5	100	96.1	100	100	100	78.4	98	100	52.9
Xiao et al (18)		8.3	16.7	97.9	6.2												81.25	97.9	18.8			43.7				
Reis et al (19)		78	82	96	75					83	91						62.0	93.0	79.0			83.0				
Jabeen et al (20)		84.7	96	100	60.2														83.1			82.0				
Wickra maratne et al (21)	32.6	51.7	67.4	100			58.4			61.8	67.4					62.9		75.7	63.9			69.6				
Das et al (22)	26.2	42	54.1				21.4	38.8		37.1	53.8	16.7		42.8		58	2.1	90.5	39.1	70.7		54.7	58.9		45.4	
Our study proposed cut off	22.1	50.7	61.2	99.3	33.7	93.2	27.6	78.2	65.6	51.2	63.9	13.6	80.6	91.2	42.5	52.4	0	43.9	46.3	51.2	56.1	53.7	90.2	51.2	46.3	95.1
Optimum cut off	82.65	65.99	76.2	90.82	82.65	67.3	75.2	78.2	65.6	71.1	71.8	75.9	80.6	77.6	71.1	60.9	76.2	48.8	51.2	51.2	56.1	58.5	85.4	58.5	56.1	85.4

Table V YI of the DF indices to differentiate between BTT and IDA																									
	EF1	Srivastava	Ment	S and L	EF2	MCHD	MDHL	Kerman 1	Kerman 2	sirdah	Ehsani	sirachainan	Sehgal	Bordbar	Matos	rbc	bessman	Ri	GK	DG	HI	JR	NT	KE	WO	CJ
Nerune r et al (3)		12	45	-33	65												45		72			67				
Hoffmann et al (4)		71.8	76.0		73.6			71.8	75.1	77.4	75.5				68.0			70.8	79.2	73.4	77.1	80.3				17.2
Singh et al (15)		38	49	65	30					39								08	49			57				
Balcázar et al (17)	50.2	70	78	46.6	71.0	-2.0	65.1	72.7	73.6	80.8	79.5	56.2	81.2	68.3	87.1		25.7	47.3	92.3	85.1	82.4	84	70	89.6	90.8	36.9
Xiao et al (18)		8.0	10.2	18.5	5.6												31.54	7.6	5.0			16.3				
Reis et al (19)		59	67	37	67					73	73						28	45	68			68				
Jabeen et al (20)		56.2	85	11.8	44.2														56.6			60.5				
Wickramaratne et al (21)	19	38	49	45			26			48	49					29		1.2	34			42				
Das et al (22)	25.9	40.7	53.1				20.8	35.7		37.0	52.8	-15.4		22.4		53.1	-32.0	55.2	68.0	66.7		74.6	77.8		68.6	
Our study proposed cut off	21.4	35.5	44.5	4.2	31.9	1..5	26.3	45.3	44.2	41.6	45.1	13.2	48.8	32.7	37.9	43.6	-1.3	41.9	45	44.6	50.2	48.4	46.8	48.6	44.3	22.1
Optimum cut off	49.51	43.79	49.5	35.88	49.48	6.75	47.9	45.3	44.2	47.9	46.4	29.1	48.8	38.6	44.8	46.5	28.8	43.5	47.3	44.6	50.2	49.9	48.6	49.3	50.2	27.5