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EFFECTIVENESS OF DISCRIMINANT FUNCTIONS AS SCREENING TESTS IN PREDICTING B THALASSEMIA TRAIT AMONG PAEDIATRIC POPULATION WITH MICROCYTIC ANEMIA

Dr. Vara Prasad Bm[1*], Dr. Vamseedhar Annam[2], Dr. Vinitra K[3]

[1*]Assistant Professor: Ramaiah Medical College, Bangalore.
[2]Professor & HOD, Shri Chamundeshwari Medical College, HOs, Bangalore.
[3]Assistant Professor: BGS Global Institute of Medical Sciences , Bangalore.

***Corresponding Author:** Dr. Vara Prasad Bm
*Assistant Professor: Ramaiah Medical College, Bangalore.

Abstract

Aim: The objective of the present study was to study the effectiveness of various discriminant functions as screening tests in predicting BTT among paediatric population with Microcytic anemia, who were later confirmed by increased HbA2 levels on Agarose gel electrophoresis at alkaline pH (8.6).

Methods: The present study was conducted in the Hematology section Department of Pathology Central Laboratory RRMCH Hospital Bangalore during the period from January 2015 to May 2017. A total number of 300 paediatric patients were included in the present study.

Results: The commonest age group affected was 0-1 year accounting to 48% followed by age group 2-3 year with 22%. Males showed higher preponderance compared to female with M:F ratio [1.4:1]. The prevalence of BTT was 28.33% in the present study. Out of 300 cases 107(35.6%) cases were positive for either both or any one of the indices. All these cases were subjected to electrophoresis. Among these, 85cases showed increased HbA2 levels.

Conclusion: The present study demonstrates that a set of cost-effective screening test like Discriminant Indices along with routine hemogram data in Microcytic Anemia cases can effectively discriminate between BTT and non-BTT. The confirmation of BTT can be done by Hb electrophoresis on agarose gel and HbA2 quantification by automated densitometer scanner.

Keywords: Discriminant functions, microcytosis, Microcytic anemia, BTT

INTRODUCTION

Thalassemia is the most common hereditary hemolytic anemia in the world. It is an autosomal recessive disease characterized by varying degrees of anemia. The genetic defects affect the synthesis of proteins called globins which are components of hemoglobin; the oxygen transport pigment in the red cells. Impaired synthesis of globin chains lead to compromised hemoglobin synthesis thus anemia.[1,2] Microcytic hypochromic anemia, especially in the Indian context, usually is due to beta thalassemia trait (BTT) or iron deficiency anemia (IDA). IDA is known to be the most common nutritional disorder in the world.[3] Screening of thalassemia minor is the only method to prevent the occurrence of homozygotes in the society. The definitive diagnosis is made by iron profile for IDA

and high performance liquid chromatography (HPLC) for BTT. These are expensive and require sophisticated analyzers.

The frequency of the β-thalassemia gene is population dependent. It is prevalent in a broad belt extending from the Mediterranean basin to Southeast Asia. It is estimated that 1.5% of the world's population carries β-thalassemia, i.e. at least 80–90 million people with an estimated 60,000 new carriers are born each year.[4] A WHO update on β-thalassemia in India indicated a similar overall carrier frequency of 3–4%, which given the current national population would translate to between 35.6 and 47.5 million carriers of the disorder nation-wide.[5]

Inheritance of one gene from Beta thalassemia results in BTT. Due to high prevalence of BTT in various regions of India correct identification of BTT assumes great importance. BTT presents as Microcytosis and should be differentiated from IDA which is the commonest cause among paediatric population. When the Microcytic cases are inappropriately diagnosed and administered with iron it leads to iron overload and complications in BTT cases. Effective population screening of BTT can dramatically decrease the incidence of birth of a thalassemia major child. Like other recessive illnesses, it can be eliminated if the carriers of the disease are fully detected and treated. Through genetic counselling, birth rate of β-thalassemia major can be reduced by as much as 90%. Screening for BTT therefore assumes importance in preventing the potential of a homozygous offspring.[6]

Applicability of different red cell parameters and red cells indices in determination of potential beta-thalassemia carrier status and their value in predicting BTT had been assessed in many studies.7-10

The objective of the present study was to compare the effectiveness of various discriminant functions as screening tests in predicting BTT in Microcytic anemia, who were later confirmed by increased HbA2 levels on Agarose gel electrophoresis at alkaline pH(8.6).

MATERIALS AND METHODS

The present study was conducted in the Hematology section, Department of Pathology & Central Research Laboratory, RRMCH Hospital, Bangalore during the period from January 2015 to May 2017. A total number of 300 paediatric patients were included in the present study.

The inclusion criteria consisted of paediatric age group less than 5 years with MCV < 70fl and Hb<10g/dl (based on SYSMEX XN1000)

The relevant personal history was obtained from either the hospital records or by personal interview with the patient and/or their attendants , whenever possible as per the proforma .

The exclusion criteria were: Age above 5 years, hemolysed and clotted samples were excluded from the study.

Methodology

2ml of venous blood was collected into a tube containing K3EDTA (1mg/1ml). Complete blood counts (CBC) were performed on all the samples as a routine test by SYSMEX XN1000. MCV and RBC count is based on electrical impedence.

The peripheral blood smear examination was carried out on all cases as a routine procedure by manual method. The discriminant functions used were:

1. Sehgal Index =MCV x MCV/RBC
2. Mentzer index (MI) = MCV / RBC

The cases positive for either indices or either one of the indices were subjected to hemoglobin electrophoresis.

BTT was confirmed by agarose gel hemoglobin electrophoresis at 8.6 pH and quantitation of HbA2 by an automated densitometer scanner using HELENA software. HbA2 ≥ 3.5% was considered diagnostic for BTT.

PRINCIPLE: Electrophoresis is the movement of charged particles in an electric field. At pH 8.4 - 8.6, hemoglobin is a negatively charged protein. The hemoglobin therefore migrates towards the anode in an electric field with agarose gel as the support medium. During electrophoresis, various

hemoglobins separate, because of charge differences caused by structural variations. This separation allows preliminary identification of different hemoglobins. After separation the various hemoglobins, can be quantitated by using an automated densitometer scanner and Helena software platinum.

Equipment:

- Horizontal electrophoresis tank and power supply (Helena Laboratories, Beaumount, Texas)
- Densitometer scanner with Helena Platinum 4 Software
- Agarose gel plates. (The SAS-MX alkaline Hb-10 kit)

Contains agarose gel plate in Tris/EDTA/Glycerin buffer with sodium azide a preservative.
Applicators and Glassware

1. Application templates
2. Gel blotter
3. Template blotters
4. Micropipette - 2-10µ, 20-200µ and 1000µl
5. Test tubes (5ml)
6. Bullets

Reagents:

1. Tris/Borate buffer concentrate (contained concentrated Tris/Borate buffer with sodium azide and thiomersal as preservatives -1x100ml) - Contents were diluted with distilled water to make up to one litre.
2. Acid Blue stain concentrate (contained concentrated acid blue stain - 1x75ml)-Contents were diluted with distilled water to make up to 700ml and allowed stand overnight and stored in a tightly stoppered bottle. This stain was filtered and used.
3. Hemoglobin lysing agent (Contained Triton X-100 in purified water with potassium cyanide and thiomersal as preservatives - 1x50ml). The lysing agent was ready for use.
4. De-stain solution concentrate (contained concentrated de-stain solution -1x40ml).

Contents of the bottle were diluted with distilled water, made up to 2 litres and stored in a tightly stoppered bottle.
Fixation Solution: 500ml of methanol, 500ml of distilled water and 100 ml of glacial acetic acid were mixed well and stored in a tightly stoppered bottle. Saline solution: 0.85% NaCl.

Sample Collection and Hemolysate Preparation:

Freshly collected EDTA anticoagulated blood was the specimen of choice.
Sample was stored in refrigerated at 2-6ºc for up to 1 week. For optimal results, saline washed red cells was used to prepare lysate so as to remove possible interference from plasma proteins.

1. 200 µl of well mixed whole blood was mixed with 1000µl of saline solution and centrifuge to sediment the red cells.
2. 1000µl of the supernatant was discarded.
3. Further 1000µl of saline solution was added and mixed well.
4. The above steps (2-4) were repeated for 2 more times.
5. 1000µl of supernatant was removed after final centrifugation and the remaining sample was treated as whole washed blood.
6. Each patient sample/control was diluted with hemoglobin lysing agent and hemoglobin concentration adjusted to 1.0 - 2.0g/dl

Electrophoresis Procedure:

1. Gel was removed from the packing and placed on tissue paper towel. The gel surface was blotted with a gel blotter.

2. The sample application template was aligned with the arrow at the edges of the gel. The template blotter was placed on top of the template and rubbed with a finger across the slits to ensure good contact. The blotter was removed and retained for use in the step 5.
3. 3µl of hemolysate was applied to each slit and allowed to absorb for 5minutes.
4. During absorption time, 30ml of buffer was added into each inner section of the SAS-MX chamber.
5. After 5 minutes of sample absorption, the template was again blotted with the template blotter retained from step 2 and removed both blotter and template.
6. The gel was placed into the chamber in such a way that, the agarose side facing down and aligned the positive (+) and negative (-) sides with the corresponding positions on the chamber.
7. With uninterrupted power supply, the gel electrophoresed at 150volts for 30 minutes.
8. Following electrophoresis the gel was fixed in a fixative solution for 5 minutes and dried the gel at 60-70^{o}c.
9. The dry gel was immersed in stain solution for 10 minutes.
10. The gel was de-stained with de-stain solution for one minute each for two to three times.
11. The gel was dried and stored.

Densitometer Scanning Procedure

Following electrophoresis, (well fixed, stained & de-stained and dried) the gel plate was placed on the automated densitometer scanner. The HbA2 level was estimated in computer using Helena software platinum.

Interpretation Results:

Qualitative Evaluation: The possible identity of the hemoglobin types present in the samples were determined by visual evaluation of the completed gel.

Quantitative Evaluation: The relative percentages of each hemoglobin type on the gel could be determined by densitometry of the completed gel at 595 nm .HbA2 ranging from 3.5-7% is taken as confirmatory for diagnosis of BTT.

Statistical analysis

Chi-square test has been used to test the significance of proportion of lab parameters between BTT and NON BTT diagnosed based on electrophoresis.

RESULTS

The present screening study was designed to pick up possible BTT consisted of 300 children with age group less than 5years having both MCV<70fl and Haemoglobin < 10g/dl. The effectiveness of various RBC parameters and their discriminant functions were compared with Hb electrophoresis [gold standard] at alkaline pH on agarose gel

Table 1: Age wise Distribution with Sex in cases with MCV <70Fl

Age in years	Male	Percentage	Female	Percentage	Grand Total	Percentage
0-1	85	28.3	59	19.66	144	**48**
1-2	40	13.3	27	9	67	**22.33**
2-3	25	8.33	13	4.33	38	12.66
3-4	12	4	13	4.33	25	8.33
4-5	15	5	11	3.66	26	8.66
Grand total	**177**	**59**	123	**41**	300	100

The commonest age group affected was 0-1 year accounting to 48% followed by age group 2-3 year with 22%. Males showed higher preponderance compared to female with M:F ratio [1.4:1]. Table 1.

Table 2: Prevalence of BTT in MCV <70fl

Beta Thalassemia Trait (BTT)	Number	%
Present	85	28.33
Absent	215	71.66
Total	300	100

The prevalence of BTT was 28.33% in the present study.

Table 3: Number of cases subjected to Electrophoresis

Electrophoresis	Number	%
Positive	107	35.66
Negative	193	64.34
Total	300	100

Hemoglobin Electrophoresis

Out of 300 cases 107(35.6%) cases were positive for either both or any one of the indices. All these cases were subjected to electrophoresis. Among these, 85cases showed increased HbA2 levels.

Table 4: Association of Sehgal Index with Electrophoresis

		Electrophoresis		
		Positive	Negative	Total
Sehgal Index	Positive	82	23	105
	Negative	3	192	195
	Total	85	215	300

Discriminatory functions i.e Sehgal index and Mentzers index were conducted on 300 children and were co related with electrophoresis.

$\chi 2$ =196.99: DF =5; P < 0.001{by conventional criteria this difference is considered to be extremely stastically significant}.

Hence there is strong association between Microcytic hypochromic anemia and Sehgal Index.

Sensitivity and Specificity 96.44% and 89.30%.

Table 5: Association of Mentzers Index with Electrophoresis

	Electrophoresis			
		Positive	Negative	Total
Mentzers Index	Positive	76	22	98
	Negative	9	193	202
	Total	85	215	300

There is strong association between Microcytic hypochromic anemia and Mentzers Index.

- $\chi 2$ =173.62; DF=5:P<0.001{ by conventional criteria this difference is considered to be extremely stastically significant }.
- Hence there is strong association between Microcytic hypochromic anemia and Mentzers Index.
- The sensitivity and specificity 89.44% and 89.76%

Table 6: Association of Sehgal index, Mentzers Index with BTT

	BTT	Non BTT	Total
Sehgal Index <972	82	23	105
Sehgal Index>972	3	192	195
Mentzers Index<14	76	22	98
Mentzers Index >14	9	193	202

Table 7: Indices and Electrophoresis showing positive for BTT

	Positive	**Negative**
Sehgal Index	82	23
Mentzers Index	76	22
Electrophoresis	85	22

DISCUSSION

Accordingly, use of Hb concentration to discriminate BTT is inappropriate and misleading as it can dangerously exclude a significant proportion of individuals with BTT. As this was a retrospective study, nutritional status of the study subjects could not be assessed by means of dietetic history or by biochemical methods although it is known to influence the interpretation of red cell parameters. Beta thalassemia Minor is also known as β thalassemia trait or carrier state, in which the affected person carries one normal and one mutated thalassemia β globin chain. Thalassemia minor may be present in any person without knowing that they are carriers.

In this study, we used discriminatory indices to segregate BTT and Non BTT cases which is simple, cost-effective screening tests before subjecting to hemoglobin electrophoresis which is expensive technique. The basis of this algorithm was to screen cases of Microcytosis.

Thalassemia affects approximately every 4% of 10,000 live births throughout the world. According to WHO 3.3% is the average frequency of Thalassemia carriers in India. Approximately 15 million people are estimated to suffer from thalassemia disorders world-wide. Reportedly, there are about 240 million carriers of β-Thalassemia worldwide, i.e. 1.5% of world population, and in India alone, the number is approximately 30 million .[11]

The distribution of the Thalassemia gene is not uniform in India and the prevalence is very high among certain communities in India, such as Sindhis and Punjabis from Northern India, Bhanushali's, Kutchis, Lohana's from Gujarat, Mahar's, Neobuddhist's, Koli's and Agri's from Maharashtra, & Gowda's and Lingayat's from Karnataka etc. have a higher carrier rate .[12,13]

Dermir A et al studied 63 children with Microcytosis between 2 – 16 years of which 31 were girls and 32 were boys.[9] The present study included 300 paediatric cases with age <5years of which 59% were males and 41% females [M:F ratio was 1.4:1] . The common affected age group was 0-1 yr(48%) , followed by 22.33% in the age group of 1-2yrs , 12.66% in 2-3yrs, 8.33% in 3-4yrs and 8.66% in 4-5 years.

The various laboratory parameters of use in BTT according to various studies are Hb%, RBC counts, MCV and MCH. M B Agarwal et al (1982) studied 143 cases of BTT in which mean Hb was 9.66±2.19 g/dl in symptomatic BTT.[14] Sika et al (1999) studied 337 cases of BTT for which red cell indices and Discriminant functions were studied, they found mean Hb concentration was 11.6±1.6g/dl.[15] Nishi Madan et al (2010) studied 449 cases of BTT , found mean Hb of 10.9±1.1 g/dl.[16]

Mohammed M et al (1999) studied 382 patients of BTT found mean RBC count of 5.45±0.71 million/cumm in BTT patients.[17] Sikka M et al (1999) studied 337 BTT cases and 40 normal controls, they found that mean RBC count was 5.67±0.76million/cumm in BTT cases.[15] In the present study mean RBC count was 4.8±0.57 million/cumm when compared to NON BTT cases which was 3.89 million/cumm, so RBC count was significantly higher in BTT.

Ghosh et al (1985)[18] Pearson et al (1973)[19] and Lafferty et al (1996)[20]derived cut-offs of <75 fL, <80 fL, and <72 fL, respectively, for other populations. Milunsky et al (1998)[21] observed a very high probability of BTT showing MCV <60 fL.MCV had the lowest sensitivity (81.3%) among all the RBC indices in detecting BTT.

The various RBC based indices have been proposed to differentiate BTT from IDA and to screen BTT in patients with Microcytosis. In the present study 300 cases with Microcytosis (MCV<70fl) were screened with various discriminant functions like Sehgal and Mentzers index. The cases were subjected to electrophoresis when Indices shows positivity. Mohammed et al (1999) studied 382 cases

and found sensitivity of Mentzers Index as 70.6%.[17] The Study conducted by Ujwala Maheshwari et al (2017) showed Sehgal index had a sensitivity of 86.6% and specificity of 89.4%.
The study conducted by Ujwala Maheshwari et al (2017) showed Sehgal index had a sensitivity of 86.6% and specificity of 89.4% , while Mentzers Index <14 had a sensitivity of 73.3% and specificity of 91.22% respectively.[22]
In our study we found sensitivity Sehgal Index had a sensitivity of 96.44% and Specificity of 89.30% with P value <0.001. Mentzers Index had a sensitivity of 89.44% and specificity of 89.76% with P value <0.001. The results observed in our study is similar to that observed by Ujwala maheshwari et al.[22]

Electrophoresis is the gold standard for the diagnosis of BTT with estimation of HbA2 levels . A raised level of HbA2 levels ≥3.5% is the criteria for the diagnosis .Electrophoresis was performed on 107 cases after screening for 300 Microcytic cases by Discriminant functions in the present study. Electrophoresis was conducted on cases with alkaline buffered agarose gel and later quantified the various bands using densitometer. Qazi R. A et al (2014) conducted study on 521 of which 26 were diagnosed as BTT based on Hb electrophoresis with mean HbA2 level of 5.8%.
None of the Indices show 100% sensitivity and specificity. The detection of BTT relies mainly on estimation of HbA2 levels by Electrophoresis or HPLC which stands as a gold standard. The main idea of using different indices in Microcytic hypochromic anemia discrimination is to screen the patients having a high probability of requiring appropriate follow-up to reduce unnecessary investigations and costs.

CONCLUSION

The present study demonstrates that a set of cost-effective screening test like Discriminant Indices along with routine hemogram data in Microcytic cases can effectively discriminate between BTT and non-BTT. The confirmation of BTT can be done by Hb electrophoresis on agarose gel and HbA2 quantification by automated densitometer scanner. Effort should be made to reduce the thalassaemic childbirth by developing a population screening programme. M/H ratio is one of the good indices but required a specific haematology laboratory where auto-analyzer is also available. Mass awareness about the danger of thalassaemia, carrier detection and adequate counseling are the prerequisite to prevent silent spread.

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