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Assessment of diagnostic reliability of RBC-Based Indices followed by Molecular Screening of genetic basis in the thalassemia population

Bhowad Shruti R[1], Samant Parineeta M[2], Seth Bageshree[3]

[1]Department of Biochemistry, MGM Institute of Health Sciences, Navi Mumbai, India.

*[*2]Department of Biochemistry, MGM Institute of Health Sciences, Navi Mumbai, India.*

[3]Department of Paediatrics, MGM Medical College & Hospital, Navi Mumbai, India.

Abstract

Thalassemia, an autosomal recessive blood disease, shows a variety of clinical expressions in terms of asymptomatic to severe blood transfusion dependence. Its prevention, compared to treatment, is cost-effective, possible and practical. The study aimed to evaluate the haematological indices followed by molecular analysis in screening of beta-thalassemia in the population. 200 participants were screened for microcytic hypochromic using CBC. After performing a complete blood count (CBC), 152 microcytic hypochromic patients were selected. These cases were further analysed by iron profile, RBC-based indices and high-performance liquid chromatography (HPLC). Further, beta-globin mutations of the suspected cases were analysed using ARMS-PCR. Five common mutations namely IVS I-1 (G-T), IVS I-5 (G-C), Codon 8-9 (+G), Codon 15 (G-A), Codon 41/42 (-TCTT) and 619 bp were analysed. In the study population, 152 out of 200 (76%) patients had microcytic hypochromic. 63.50% (127 cases) were detected using the Shine and Lal Index, 60.5% (121 cases) were detected using the Mentzer Index, iron profiling and HPLC detected 57.5 % (115 cases) of suspected thalassemia. The sensitivity of the Mentzer Index, Shine and Lal Index was 97.44%, and 96.61% respectively and specificity was 100%. And that of HPLC was 100%. Further, in molecular analysis mutations detected were IVS I-5 (G-C), CD-15 (G-A), IVS I-1(G-A) and IVS I-110 (G-A). IVS I-5 (G-C) was observed with the highest frequency of 61.7 % and IVS I-110 (G-A) with the lowest frequency of 7%. As the Mentzer Index and Shine and Lal Index have good sensitivity and specificity, they can be considered reliable screening tools, particularly in prenatal diagnosis. In molecular analysis, the most common mutation in the population was IVS I-5 with a prevalence of 61.7%. These indices may also have the potential to advance genetic diagnostics.

Keywords: Thalassemia Trait, Menzter Index, Shine and Lal Index, HPLC, Reliability

Full length article **Corresponding Author*, e-mail: parineeta.samant13@gmail.com

1. Introduction

The most prevalent inherited diseases in India are hemoglobinopathies, which pose a serious threat to the nation's health. [1] A group of common disorders as hemoglobinopathies are inherited and result in abnormal haemoglobin molecule synthesis or structure. Thalassemia and sickle cell disease are the two main forms [2]. The incidence of β-thalassaemia in India is 3.3% with 1-7% of couples being affected annually [3,4]. Beta-thalassaemia occurs as a result of β-gene mutation in β-chain globin synthesis presenting in autosome 11 [5]. Two alleles (β/β) of the β-gene regulate the synthesis of β-globin. These individuals are categorised into three groups based on haematological indices, including Hb variants: major β-thalassaemia (β0/β0), intermedia β+/β+ or β0/β+, and minor β+/β or β0/β. Blood transfusions are necessary for β-thalassaemia major, although required occasionally for β-thalassaemia intermedia. 'Mild' is the third category [6]. The mutation is passed to the offspring of this thalassemia carrier in an autosomal recessive way. [6] Early detection of heterozygous people bearing beta-globin gene mutations is of relevance due to the rise in occurrences of beta-thalassemia major. The identification of thalassemia carriers holds a vital role in preventing the inheriting of this disease [6]. It is important to know the diagnostic and epidemiology approach regarding the cause of anaemia in thalassemia patients' families since they have a higher risk of inheriting thalassemia from their descendants [7,8,9]. In clinical laboratories, a complete blood count test, which measures Hb, mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), is a standard blood test to detect beta-thalassemia carriers. Several haematological indices are introduced, including the Mentzer index (MCV/RBC), Srivastava index (MCH/RBC), Shine & Lal index (MCV [2]

*MCH/100), and many others [11]. Moreover, the specificity and sensitivity values, as well as the positive and negative predictive values among erythrocyte indices have been documented in many previous studies, yet with different results among populations [12]. Even though haematological indices can be used to identify presumed beta-thalassemia carriers, a recent discovery indicates that Hb analysis, including fractions of HbA2, HbA, or HbF, can either confirm or rule out the beta-thalassemia carrier state [13]. Since thalassemia is a monogenic disease with more than hundreds of different mutations and deletions in α- or β-globin gene, it is necessary to confirm and map the genetic spectrum of the population. Therefore, the goal of the study was to evaluate the haematological indices followed by molecular analysis and screening of beta-thalassemia.

2. Materials and methods

In the present study, 200 participants who visited the MGM Hospitals outpatient clinics between August 2020 and June 2021 were enrolled. Ages ranged from 1 year to 55 years. Based on the CBC (Complete blood count) profile, 152 patients with microcytic hypochromia were selected, from which 51.32% (78) were males and 48.68% (74) were females. These cases were further analysed with iron profiling by estimating serum ferritin levels, haemoglobin analysis by high-performance liquid chromatography (HPLC) and haematological indices were calculated using the following formulas:

Mentzer Index = MCV/RBC
Shine and Lal Index = MCV2 x MCH/100
Srivastava Index = MCH/RBC
Sirdah Index = MCV-RBC-3xHb
Ehsani Index = MCV-10 x RBC
England and Fraser = MCV – RBC-5xHb - 3.4

Further, the suspected cases were analysed for molecular analysis by ARMS-PCR. Five common mutations namely IVS I-5 (G-C), IVS I-1(G-T), Codon 8-9 (+G), Codon 15 (G-A), Codon 41/42 (-TCTT) and 619 bp were analysed. Excel spreadsheets and SPSS version 25 were used to analyse the data.

3. Results and discussion

In the study population, 200 participants based on CBC profiling were screened for microcytic hypochromia. 152 out of 200 were included in the study with 51.32% males and 48.68% females. As represented in Table no. 1. the Average Red blood cells, haemoglobin and Mean corpuscular volume level were found to be 4.47 ± 1.53 million/mm3, 8.28 ± 1.75 g/dl and 70.66 ± 10.00 fl respectively. The blood samples of these microcytic anaemic population were further analysed for serum ferritin levels, haemoglobin electrophoresis and haematological indices such as Mentzer Index, Shine and Lal Index, Srivastava Index, Sirdah Index, Ehsani Index and England and Fraser Index were calculated. The cut-off values for these indices are mentioned in the table no. 2. Serum ferritin levels were categorized into < 30 ng/ml and >30 ng/ml. As represented in Table no. 3, 115 patients were considered to be beta-thalassemia trait suspects. Interpretation of serum ferritin levels acts as an indicator of the relative extent of depletion of iron stores. As per WHO the generally accepted cut-off level for serum ferritin, below which iron stores are considered to be depleted, is <30ng/ml [14]. Further, samples were subjected to Hemoglobin electrophoresis, and 115 patients were found with < 3.5 % HbA2 levels can be considered a thalassemia trait. Cation exchange HPLC is emerging as an excellent diagnostic tool in the quantification of major and minor, normal and abnormal Hb fractions [15]. As represented in Table no. 3, the lowest positive cases which is 57.50% of study subjects by the parameter were by iron profiling, and HPLC. HPLC is a sensitive, accurate and simple technique. It can be applied for screening and diagnosis of hemoglobinopathies [15]. The study from Majeed (2013) in Pakistan shows the BTT case as much as 52% based on haemoglobin electrophoresis [16]. Various mathematical formulas based on erythrocyte indices have been used to determine the BTT. However, there are no single indices that are 100% specific and sensitive enough to filter such conditions. In our study, the frequency of beta-thalassemia trait based on the Mentzer Index was found to be 60.50% and the Shine and Lal index was found to be 63.50% of study subjects. Therefore, to compare the diagnostic reliability of the parameters in screening beta-thalassemia trait, we calculated sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the test. As represented in Table No. 4 the sensitivity, specificity and accuracy of HPLC were found to be 100%. Followed by the Menzter Index which showed 97.44% sensitivity, 100% specificity and 97.96% accuracy and the Shine and Lal index with 96.61% sensitivity, 100% specificity and 97.30% accuracy. In their study, Ehsani MA et al. and Aysel V et al. found that the Mentzer index had the highest percentage of accurately diagnosed patients (94.71%), followed by their new index (92.96%) [17,18]. According to a study by Demir et al, the Shine and Lal index had the highest percentage of accurately diagnosed patients (92%) [19].

England and Fraser Index showed the lowest sensitivity, specificity and accuracy with 35.19%, 63.83% and 43.87% respectively. According to the accuracy, the ranking of the diagnostic performance was HPLC > MI > S and L Index > Ehsani I > Srivastava Index > Sirdah Index> E and F Index. As the Mentzer index and Shine and Lal index showed the highest specificity and sensitivity, we also supported their diagnostic performance with the Receiver operating characteristic curve (ROC). The area under the curve determined was 0.642 by the Mentzer Index and 0.617 by the Shine and Lal Index. Therefore, it can be considered as a diagnostic marker. Further, beta-thalassemia traits were confirmed by molecular analysis. The common mutations found were Heterozygous- IVS 1-5 (G-C), CD 15 (G-A), IVS I-1 (G>A) and IVS I-110 (G-A). As represented in Figure no.3 the mutation with the highest frequency was IVS I-5 (G-C) and with the lowest frequency was IVS I-110 (G-A). The β-globin gene is located in a cluster with the other beta-like genes on the short arm of chromosome 11. The cause of β-thalassemia is over 200 mutations. The majority of β-thalassemia cases are caused by point mutations in the gene or its immediate flanking regions, except a few deletions. [20] The pathophysiologic characteristics of thalassemia syndromes are due to the degree of globin chain imbalance. When β-thalassemia major occurs, there is a maximum globin chain imbalance, whereas in silent β-thalassemia, there is a minor imbalance [21] β-thalassemia's heterozygous condition exhibits a remarkable range of phenotypes [22].

Table 1: Shows Mean of Hematological parameters

Haematological parameters	Mean ± SD
RBCs (million/mm3)	4.47 ± 1.53
Pre-hemoglobin (g/dl)	8.28 ± 1.75
MCV (fl)	70.66 ± 10.00
MCH (pg/cell)	24.17 ± 3.54
MCHC (g/dl)	30.77 ± 4.25

Table 2: Threshold values of the Indices used to discriminate between Beta-Thalassemia Trait and Iron Deficiency Anemia

Hematological Indices	Beta-thalassemia Trait
Mentzer Index (MI), 1973	≤13
Shine and Lal Index (S and L), 1977	≤1530
Srivastava Index (SRI), 1973	≤3.8
Sirdah Index (SI), 2007	≤27
Ehsani Index (EI), 2005	≤15
England and Fraser Index (E and F), 1973	≤0

Table 3: Iron profiling in subjects with microcytic anemia

	Serum ferritin (ng/ml)	
	< 30 ng/ml	**> 30 ng/ml**
n (152)	37	115

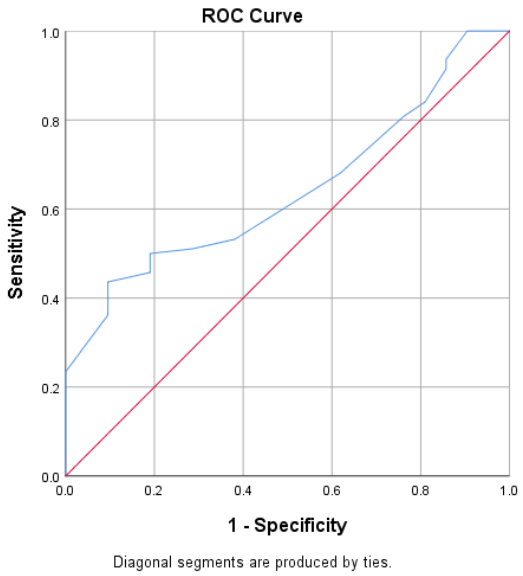

Figure 1: Represents the Receiver Operating Characteristics (ROC) curve for the Mentzer Index

Table 4: Percentage of cases positively identified by different parameters

Parameters	Total No. of positive cases (n=200)	Percentage of positive cases (%)
Complete blood count (CBC)	152	76.00
Mentzer Index (MI)	121	60.50
Shine and Lal Index (S and L)	127	63.50
Srivastava Index (SRI)	135	67.50
Sirdah Index (SI)	142	71.00
Ehsani Index (EI)	130	65.00
England and Fraser Index (E and F)	149	74.50
Ferritin	115	57.50
HPLC	115	57.50

Table 5: Diagnostic performance of HbA2 and CBC-based Indices in beta-thalassemia trait

Parameters	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HbA2	100	100	100	100	100
Mentzer Index	97.44	100	100	90.91	97.96
Shine and Lal Index	96.61	100	100	88.24	97.3
Srivastava Index	84.26	88.24	95.79	63.83	85.21
Sirdah Index	65.74	63.83	80.68	44.78	65.16
Ehsani Index	91.53	85.71	95.58	75	90.2
England and Fraser	35.19	63.83	69.09	30	43.87

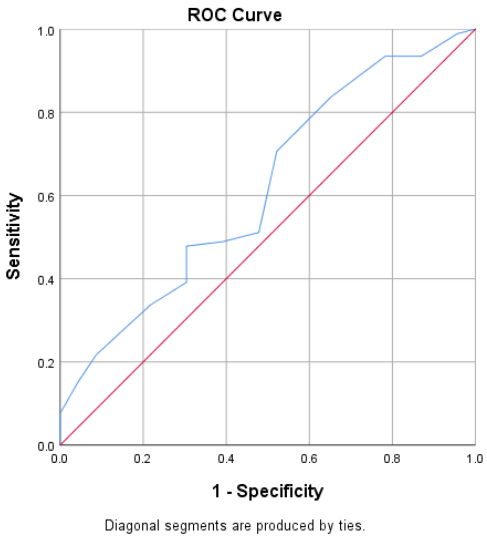

Figure 2: Represents the Receiver Operating Characteristics (ROC) curve for Shine and lal Index

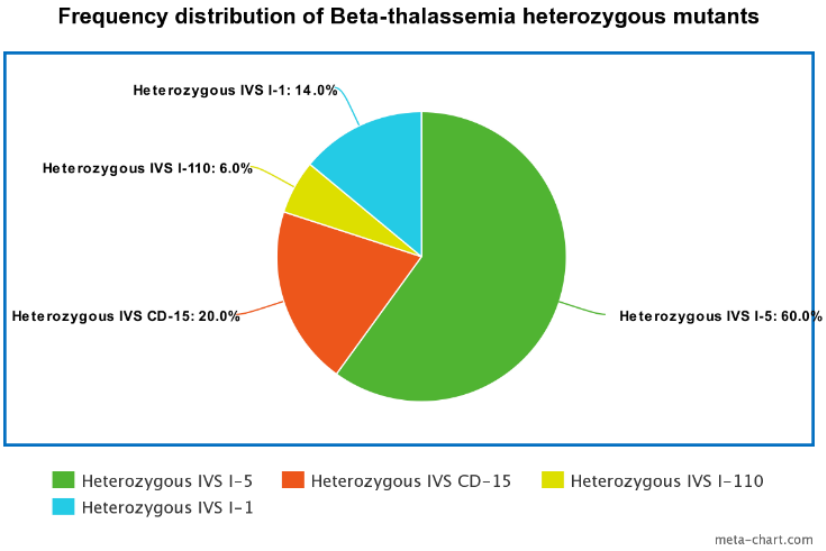

Figure 3: Represents the frequency distribution of beta-thalassemia Trait

4. Conclusion

The development of efficient and cost-effective thalassemia screening procedures has become crucial, particularly in nations where a substantial proportion of the population suffers from such disorders, since screening will remain the main strategy for controlling beta-thalassemia. It is important to distinguish between the beta-thalassemia trait and iron-deficiency anaemia to avoid unnecessary iron therapy and to offer genetic counselling to individuals with the beta-thalassemia trait. In the current study, microcytic hypochromic children and adolescents 76% were suspected to have beta-thalassemia trait by CBC, 63.50% by Shine and Lal Index, 60.50% by Mentzer Index and 57.50% by HPLC. RBC-based Index with high sensitivity and specificity for diagnosing the beta-thalassemia trait would be very helpful. In this investigation, the sensitivity, specificity and accuracy of HPLC were found to be 100% followed by the Menzter Index with 97.44%, 100%, and 97.96% and Shine and Lal Index with 96.61%, 100% and 97.30% respectively. The ROC curve obtained was also found to be reliable. In conclusion, we can state that our research has demonstrated that cell-counter-based parameters like Mentzer Index and Shine lal Index may be useful as early-stage predictors for common mutation observed. This approach based on the determination of red cell indices can be suitable for screening programmes as it is cost-effective.

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Conflict of Interest

None.

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