Evaluation of Two Hematologic Indices and Extrapolated HbA2 Values in the Differential Diagnosis of Iron Deficiency Anemia (IDA) and Beta Thalessemia Traits with IDA

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ABSTRACT

We evaluated the combination of two indices of red blood cell count (RBC) and red blood cell distribution with index (RDWI) and extrapolated HbA2 values in differential diagnosis of iron deficiency anemia (IDA) and beta-thalassemia traits (BTT) associated with IDA. A total 129 subjects were included in this study. RBC/RDWI was able to diagnose 32% beta-thalassemia with iron deficiency while 66% had IDA. HbA2 showed beta-thalassemia cases accounted 48% of the total, while IDA accounted 52%, giving RBC/RDWI approach of diagnosing BTT/IDA vs. IDA sensitivity rate of 77% and specificity rate 100% in diagnosing BTT; whereas it had sensitivity rate 76% and specificity rate 77% in diagnosing IDA. HbA2 considered as gold standard, sensitivity of RBC/RDWI approach 100% and specificity 95% in diagnosing BTT while IDA, sensitivity 95% and specificity 100%.

RBC/RDWI in diagnosing BTT and IDA seems to be an effective tool. As hemoglobin electrophoresis still standard in this respect, decisions based on this recommended approach plus extrapolating HbA2 values may circumvent problem of long waiting time, particularly in situations where rendering of the most probable diagnosis at shortest time.

Key Words: Beta Thalassemia, Iron deficiency anemia, RBC index, RDWI, Extrapolated HbA2

ÖZET

Demir Eksikliği Anemisi ve Demir Eksikliği ile Birlikte Görülen Beta Talasemi Olgularında Ekstrapole HbA2 Değerlerinin Ayırıcı Tanısında İki Farklı Hematolojik İndeksin Değerlendirilmesi

Bu çalışmada demir eksikliği anemisi (DEA) ve demir eksikliği ile birlikte görülen beta talasemi (BT) olgularında ekstrapole HbA2 değerlerinin ayırıcı tanısında İki farklı hematolojik İndeksin (eritrosit sayısı ve eritrosit hücre dağılım indeksleri) değerlendirilmiştir. Çalışmada toplam 129 olgu incelenmiştir. RBC/RDWI indeksleri BT olgularının %32'sini ve DEA olgularının %66'sını saptayabilmiştir. HbA2 ise BT olgularının %48'ini DEA olgularının %52'sini saptamıştır. RCB/RDWI indeksleri BT tanısında %77 sensitivite ve %100 spesifisite göstermiştir. DEA tanısında ise sensitivite %76, spesifisitesi %77 olarak bulunmuştur. HbA2, BT tanısında %100 sensitivite ve %95 spesifisite; DEA tanısında %95 sensitivite ve %100 spesifisite göstermiştir.

RBC/RDWI indeksleri, BT ve DEA tanılarında etkin bir araçtır. Her ne kadar hemoglobin elektroforezi standart tanı aracı olsa da, işlemin çok uzun sürmesi, özellikle hızlı tanı konması gereken durumlarda bir sorun oluşturabilir.

Anahtar Kelimeler: Beta Talasemi, demir eksikliği anemisi, RBC indeksi, RDWI indeksi, Ekstrapole HbA2

INTRODUCTION

Beta-thalassemia is one of the most prevalent hemoglobinopathies worldwide particularly in the Mediterranean countries, the Middle East, and Asia (1). In the absence of an effective, simple and fast screening program for thalassemia and combined with a lack of awareness among frontline medical practitioners this problem remains undetected in up to 46% of men, women and children many of whom are generally asymptomatic to mildly symptomatic (2). Not all demographically affected countries have national screening programs in which all patients who present with suspicious or probable thalassemic red blood cell indices are subjected to hemoglobin electrophoresis or high performance liquid chromatography (HPLC) to confirm a possible hemoglobinopathy. Economic constraints and lack of simple, rapid, and inexpensive tests are major problems to routine screening. Compounding the problem is the pervasive incompetence of practitioners in analyzing electronic hematologic indices in detecting a possible thalassemia associated with iron deficiency anemia versus pure iron deficiency anemia (2), and, when suspected cases do undergo electrophoretic studies, technical problems such as inhibition of HbA2 expression occur due to iron deficiency anemia (IDA). Iron replacement therapy for anemia usually takes up to 20 weeks (average 16 weeks) for significant correction to take place, only after which a recommended follow-up electrophoresis to detect a rise in HbA2 is instituted, an approach that takes up to least half a year to complete, a turn around time that is unacceptably too long, to the dismay of both doctor and patient.

Currently recommended discrimination indices based on hematologic analyzer parameters that attempt to diagnose iron deficiency anemia and beta-thalassemia trait (BTT) associated with IDA generally are not highly sensitive and specific, and are mostly not reliable at certain levels or situations.

In this study we embark on the use of two hematologic indices namely, (a) the red blood cell count (RBC) and (b) the RDWI (red blood cell distribution width index), and do an arbitrary extrapolation of HbA2 values whenever applicable to screen what fraction of a local population of anemic Saudi patients who come to the National Hospital, Riyadh Hematologic laboratory Section, classified as cases of beta-thalassemia trait (BTT) or probable thalassemia associated with iron deficiency anemia (BTT/IDA) without undergoing a 20-week iron replacement therapy and without a post- serum iron correction hemoglobin electrophoretic study.

MATERIALS AND METHODS

All patients who came to the Hematology laboratory section of the National Hospital Riyadh from January 2006 to December 2007 who presented with a complete blood count (CBC) suggestive of anemia associated with a possible thalassemia, including those with a normal hemoglobin and hematocrit but decreased MCV and MCH were registered. Excluded from the program were: pregnant women and patients with a thyroid problem, those who were recently transfused, those with a megaloblastic anemia, those who present with signs and symptoms or a possible history of lead poisoning, or those with a history of a chronic disease. Controls included those with sickle cell disease or trait "abnormal controls" and hematologically "normal" cases for comparison during electrophoresis.

The following patients were considered as having beta- thalassemia trait: (1st) those who had evidence of anemia and show an HbA2 peak on electrophoresis, or, (2nd) those who had anemia and show a Hgb F peak alone or in combination with HbA2, or, (3rd) those who had anemia and show a borderline HbA2 peak with a resultant value of equal to or above 3.3% after arbitrarily adding 0.5% to the actual value, or, (4th) those who had anemia and had a combination of RBC count of > 5 M/μl and RDWI of < 220 in the hematologic indices report. If patients did not demonstrate the aforementioned criteria, they are classified as having a probable "pure" IDA (Table 1).

A follow up electrophoresis was not performed for anemic cases that did not show an HbA2 peak or only showed a "borderline" HbA2 value (defined here as 2.8-3.2%) during the first study; at the same time, oral iron therapy was not carried out for all cases with a borderline HbA2 level; instead, a value of 0.5 % was arbitrarily added to their individual HbA2 values taking into consideration the results of a study (3). If the resulting sum of the latter re-

Table 1. RBC indices used to differentiate IDA from BTT in this study.

Hematologic Index	IDA	BTT
RBC count (M/μl)	< 5	> 5
RDWI (MCV X RDW/RBC)	> 220	< 220

ached the upper limit for HbA2 (3.3%), the patient is counted as a probable case of thalassemia trait.

At initial screening, patients who presented with and fulfilled the following red blood cell indices-based criteria were immediately separated from the program and were considered for future testing: (1st) normal hemoglobin and hematocrit (2nd) decreased MCV and decreased MCH. These two criteria are currently used to screen possible cases of thalassemia trait that do not present with anemia.

STATISTICAL METHODS

All analysis were performed using the Instat (Instat Biostatistics, Graphpad Package USA). Normal distributed data were analyzed using student t-test values calculated. Non-parametric tests were used when data were compared between patients and control subjects. Mann-Whitney-U test was also used. The two-sided p-value was applied and less then 0.05 was considered significant.

RESULTS

A total of 129 subjects were included in the study. Sixty-four (64) were classified into the IDA vs. IDA/BTT category based on results of hematologic indices; 22 were males, while females numbered 42. The Normal Control group consisted of 44 patients all of whom showed a normal hematologic profile and normal hemoglobin electrophoretic pattern. Males composed half of this group. The Sickle Cell group (abnormal control) consisted of 20 patients whose diagnoses were confirmed by electrophoretic results as either having sickle cell disease or trait; however, 4 (25%) patients in this group showed an HbA2 peak, reclassifying them as SS/BTT coexpression. One patient was dropped due to a diagnosis of a probable case of heterozygous lambda-beta-thalassemia (HbF 27%, HbA2 3.0%).

Patients were divided into three groups based on the hematologic analyzer reports using Cell Dyne 1800: Group I (normal, no anemia on CBC), Group II (mild to moderate iron deficiency anemia with a possible thalassemia on CBC, Hb range: 7.6-10.0 mg/dl), Group III (normal hemoglobin and hematocrit but decreased MCV and MCH on CBC). Only patients belonging to Group II were included into the reclassification scheme using extrapolated HbA2 values and RBC/RDWI indices.

Using a combination of two hematologic indices (RBC and RDWI) where an RBC count of > 5 favored BTT while an RBC count of < 5 favored IDA, and an RDWI of > 220 favored IDA while an RDWI of < 220 favored BTT, 66% of anemic patients were reclassified as IDA while 32% were reclassified as BTT with IDA. One was dropped due to conflicting RBC and RDWI results (Table 2).

Table 2. Reclassification of anemic patients after using RBC/RDWI and electrophoresis results.

Approach	IDA	BTT/IDA	Total
RBC/RDWI approach	41 (66%)	22 (32%)	63
HbA2 only	46 (72%)	18 (28%)	64
Extrapolated HbA2	33 (52%)	31 (48%)	64

Table 3A. Sensitivity and specificity of RBC/RDWI approach compared to extrapolated * electrophoretic results.

Disease	Sensitivity	Specificity	Youden's Index
BTT	77%	100%	77
IDA	76%	77%	53

* Actual HbA2 value + 0.5%

Table 3B. Sensitivity and specificity of RBC/RDWI approach compared to actual electrophoretic results.

Disease	Sensitivity	Specificity	Youden's Index
BTT	100%	95%	95
IDA	95%	100%	95

Electrophoresis of all anemic patients showed elevated HbA2 (BTT/IDA) in 28% of the anemic patients which was adjusted to 48% after extrapolation of HbA2 values by 0.5% (cut-off = 33.3%), an increased rate of 20%. This leaves 52% of all anemic cases as "pure" cases of IDA.

Using the extrapolated electrophoretic HbA2 results as the gold standard for comparison, the RBC+RDWI approach showed a sensitivity of 77% in diagnosing BTT and a specificity of 100%; while the sensitivity of the RCBC/RDWI approach in diagnosing IDA alone is 76% with a much lower specificity of 77% compared to BTT. Despite only a moderately high sensitivity in diagnosing BTT, the RBC+RDWI approach has excellent specificity. The sensitivity and specificity of the RBC/RDWI approach for diagnosing IDA are fairly the same and modest (Table 3A). However, the measure of validity of the RBC/RDWI as a tool when diagnosing BTT is much better (Youden's index score = 77) than when it is being used to diagnose IDA (Youden's index score= 53). The Youden's index for RBC/RDWI for diagnosing BTT is significantly higher than those achieved in previous studies (4) while that for IDA falls slightly behind in comparison. In a study by Beyan and co-workers (2007) they found the Youden's index of the RBC count to be 73.3 (77 in our study) when used for diagnosing IDA and BTT while the Youden's index for RDWI in doing the same task was 63.4 (53 in our study). Note that Beyan and coworkers (2007) did not evaluate the ability of these indices to diagnose BTT and IDA separately. When actual (non-extrapolated) HbA2 values are used as the gold standard, the sensitivity of the RBC/RDWI approach is 100% with a specificity of 95% in diagnosing BTT while for that of IDA, the sensitivity is 95% while the specificity is 100% (Table 3B).

DISCUSSION

The need for discriminating IDA from BTT associated with IDA (BTT/IDA) has been around for decades since several studies concluded that IDA directly affects the rates of HbA2 synthesis in the bone marrow (5) and therefore, iron therapy lasting up to 20 weeks should be instituted3 after which a repeat serum iron with a repeat electrophoresis are done to confirm elevated HbA2 levels.

This study did not include repeat electrophoretic studies after a serum iron assay at the end of a 20 week iron replacement therapy regimen, in stark contrast to that of the Egyptian initiative done at the Al Azhar University by El-Agouza (3) where 730

Table 4. Indices used for differentitating IDA from BTT/IDA

• Mentzer Index (MI)	: MCV / RBC
• Shine and Lal Index (S & L)	: MCV x MCH x 0.01
• England and Fraser (EF)	: MCV – RBC –(5 x Hb) – k
(where 'k' is calculated to be 5.19 or as calculated based on the kind of counter used)	
• Srivastava Index (S)	: MCH / RBC
• Green and King index (G&K)	: MCV2 x RDW / 100 x Hb
• RDW Index (RDWI)	: MCV x RDW / RBC

university students were studied for iron deficiency anemia and levels of hemoglobin subtypes (including HbA1C). In this study, they concluded that low iron levels significantly affected HbA2 levels and that correction of the iron deficiency by iron replacement therapy for 20 weeks increased previous HbA2 levels by up to 0.5%. We adopted these findings and applied it directly to extrapolate suspicious or borderline HbA2 values to "projected" levels to circumvent the problem of a 20-week delay and additional electrophoretic studies. We project the actual HbA2 values to the presumed HbA2 values by directly adding 0.5%, using 3.3% as the cutoff. In fact, a study in 1976 by Cartei and co workers (6) showed that superimposed IDA actually decreases HbA2 proportionately more than it does total Hb ($p < 0.001$), further reinforcing our hypothesis for the utilization of this approach for increasing the sensitivity of the electrophoretic technique in diagnosing BTT.

Another study by Harthoorn-Lasthuizen and coworkers done 23 years later in the Netherlands corroborated this finding at the biomolecular level by showing (using 150 patients with IDA and 71 healthy controls) that a linear correlation exists between HbA2 and MCV, and HbA2 and erythrocyte zinc protoporphyrin (ZPP) (5).

Recent studies4 in areas of high prevalence such as Turkey and the Middle East have resulted in the evaluation of several recommended discrimination indices to differentiate IDA from BTT associated with IDA (Table 4).

This culminated in a study by Aysin and coworkers where RBC and RDWI were found to have the highest Youden's indices (82% and 80%, respectively), although still emphasizing the need for a confirmatory electrophoretic study after therapeutic correction of serum iron levels (7).

Thus initially without extrapolation, the actual number of cases with elevated HbA2 was 28% (18/64) but after adjustment, the number went up to 48% (31/64). When considered together with hematologic counter–based discrimination indices such as an RBC > 5 and RDWI < 220, we found a significant concordance between the post–extrapolation number of cases with thalassemia and the number of possible thalassemia cases based on these two discrimination indices.

In retrospect, several studies in the past decade has accumulated some evidence of predictability of hematologic counter-based discrimination indices (7). However most of these were shown to have actually low sensitivities and specificities when analyzed using the Youden's index. The Youden's index is an epidemiologic tool that provides an appropriate measure of validity of a particular technique or question by taking into account both sensitivity and specificity [(sensitivity + specificity) - 100] and not just either of the two parameters. Thus in a study in Turkey by Cengiz and co-workers in 2007, they found that RBC count (< 5 in favor of IDA and > 5 in favor of TT) and RDWI (MCV x RDW/ RBC) where >220 favors IDA and >220 favors TT have the highest Youden's index after considering the sensitivity and specificity of both indices together. At the bottom of this performance rating is the S & L index (Youden's index = 0) (7).

However, in a small number of patients (up to 10%) it would still be necessary to study body iron status or the HbA2 for a more accurate diagnosis. None of the discrimination indices showed 100% sensitivity and specificity. Our study shows that a combination of RBC count (< or > 5) and RDWI (< or > 220) shows fairly concordant results with other studies done by other workers when BTT alone is being considered (7,4) but the use of this approach for diagnosing IDA seems to fall behind slightly in comparison (Table 3); it is tempting to postulate that the improvement of almost 4 index points (77 vs. 73.7) over the previous study by Beyan and co-workers may be due to a tandem-use of both parameters instead of being utilized separately. Also, it seems that the use in the future of other recently discovered hematologic parameters like the reticulocyte-hemoglobin (Ret-He, Sysmex Corp.) may be of help in increasing the validity of this approach8. IDA does not only complicate the differential diagnosis between IDA and beta–thalassemia trait but also complicates the differential diagnosis between it and anemia of chronic disease (ACD), a task which could prove rather daunting in some patients. Canals and co-workers, using the Sysmex- XE 2=100 hematologic analyzer, recommends the use of the new parameter (Ret-He) whereby a cut-off point of 25 pg has a moderately high sensitivity and specificity in differentiating between IDA and ACD. Future studies may find the possibility of applying this new approach in increasing the sensitivity of the RBC/RDWI approach in further differentiating IDA from IDA/BTT (8).

The current algorithm for the differential diagnosis of IDA and BTT used in some medical centers in Saudi Arabia poses some inherent problems. When discriminating between IDA and BTT/IDA, the algorithm proposes the initial evaluation of hematologic counter parameters after which presumed BTT/IDA cases are subjected to hemoglobin electrophoresis, cases with equivocal or indeterminate electrophoresis results (usually borderline HbA2 values) are allowed a period of between 15-20 weeks for oral iron replacement therapy. Cases presenting with an anemia of < 8 mg/dl are automatically prescribed a 20-week iron replacement program (9). This approach actually poses a risk of iron overload for some cases while in others, an improvement in the serum iron levels results in a proportional increase in HbA2 values. This may also cause a significant delay in diagnosis, preventing doctors to decide on whether or not to provide counseling for the affected person or family members. In other cases, patients lose interest due to the long waiting time involved. Genetic counselors would consider this a nightmare in a region of the world where genetic diseases are at alarmingly high rates due to frequent in-breeding, albeit preventable if only the right preventive measures were taken (1).

A few published studies in Saudi Arabia are sketchy and mainly demographic in nature. Probable reasons for this include the high cost of electrophoretic studies, HPLC, genotyping, globin gene synthesis studies and the unpopularity of hematologic indices (Table 4) as tools for thalassemia screening. Another possible reason is that the long waiting time involving iron replacement therapy could just be too much for many patients to muster. This study may find a way of circumventing this by dramatically shortening the waiting period, avoiding complications of prolonged iron therapy and significant savings in cost of diagnosis and management.

The strengths of this study include the simplicity and ease of this approach in IDA versus IDA/TT discrimination and very high concordance rates between hematological criteria and results of extrapolated and actual HbA2 values after electrophoresis. The approach saves time and is cost effective. Ideally, reflex repeat serum iron assay, repeat hemoglobin electrophoresis testing, genotyping, HPLC and globin gene synthesis studies or PCR (for special cases) are done after 20 weeks of iron replacement therapy but most if not all of these tests are not available in many health centers.

This study may serve as an impetus for establishing an integrated antenatal hemoglobinopathy screening program in Saudi Arabia and neighboring Arab countries. Patients in need of an urgent necessary surgical operation who may be subjected to a pre-anesthetic sickle screening may also benefit from this approach keeping in mind numerous undiagnosed variants of thalassemia that show coexpression with sickle cell disease or trait. Anemic pregnant patients with a hemoglobin of < 8 mg/dl may need correction of the iron deficiency before confirmation of HbA2 levels but there may be no ti-

me for this during pregnancy and therefore may be more appropriate to make immediate decisions when the female is historically at risk of being a carrier, in which situation the partner will also be required to undergo the same screening.

The formalization and implementation of a simple and easy algorithmic approach to thalassemia screening is therefore important because with over 800 mutations responsible for the production of variant hemoglobins and over 300 mutations responsible for the thalassemia syndromes it is usually not possible to identify the precise protein structure or mutation without detailed protein or DNA analysis. The diagnosis of alpha thalassemia in particular usually is made by exclusion of IDA and beta thalassemia in a person with hypochromic microcytic red cell indices. In some circumstances DNA analysis will still be required (9).

At present, not all laboratories observe the same reference ranges for HbA2 (3.3-3-5%) as they do for blood counts due to a lack of national, or regional standards. A possible action to take is for individual laboratories to make minor changes to the "normal", "borderline, and "raised" cut – off limits for HbA2 quantitation (9). More importantly, further studies are needed to determine how much and to what extent are iron levels or hemoglobin levels directly related to HbA2 expression, such as for example, if extrapolations can be 'gradated' (e.g. + 0.1%, 0.2%, 0.3%, 0.4%, 0.5%) according to the degree of severity of the anemia and if other forms of hemoglobin (e.g. HbA1c) can be used as a form of internal quality control or surrogate test. Also, we suggest that the RBC/RDWI approach should be made part of the initial steps of the algorithm used in the screening process for beta- thalassemia trait.

Although it is a well known fact that hypochromic, microcytic red cell indices are compatible with carriers for alpha or beta thalassemia or IDA or any combination of these, care must be taken regarding the validity of MCV values since stored blood will cause considerable increase in MCV values with a storage of a blood sample or when the length of time and storage temperature between venesection and analysis is not known. Acquired defects of HbA2 may also occur occasionally and should be considered when faced with patients with a history of thyrotoxicosis, megaloblastic anemia, lead poisoning, anemia of chronic disease, sideroblastic anemia, hypothyroidism, acquired HbH disease (9). A meticulous history-taking and physical examination with a high index of suspicion for these conditions should always be borne in mind. In practice it is reasonable to consider IDA and or BTT if the MCH is < 27 pg and the HbA2 is not raised, but when the MCH goes below 25 pg, alpha thalassemia is more possible especially when the individual in question is of southeast Asian, Greek, Turkish or Cypriot origin (10,11).

Finally, recommendations regarding reporting of results are important and should include the following in the written report: (1) whether the patient has sickle cell trait or disease (2) whether bands are present on HPLC or electrophoresis and whether they are normal or not in the patient's situation (e.g. HbF is normal during pregnancy) (3) statement as to whether the patient is a thalassemia carrier or not (4) and a statement on whether partner testing is recommended or not.

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Acknowledgement:

I wish to express my sincere thanks to Dr. Amani Sayed Emara, the head of department laboratory National Hospital, and many thanks to Annolie Bulan, Nenita Manares, Debbie Hope Denate, and Lisa de Luna for help me on the sitting the methods. Also special thanks to Dr. Cezar Valentino N Cepe for for their encouragement and helpful comments during the preparation of research manuscript.

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