ORIGINAL ARTICLE

A Multicentre Based Observation of a Screening tool to Differentiate Microcytosis and Hypochromia

TF Dipta[1], AL Kabir[2], MH Rahman[3], SS Gazi[4], AZ Hossain[5], N Fatema[6], *S Ferdousi[7]

[1]*Dr. Tashmim Farhana Dipta, Associate Professor, Transfusion Medicine and Haematology, BIRDEM General Hospital and Ibrahim Medical College,*

[2]*Dr. Amin Lutful Kabir, Associate Professor, Department of Haematology, Bangabandhu Sheikh Mujib Medical University, Shahbagh, Dhaka*

[3] *Dr. Md. Hafizur Rahman, Incharge, Haematology unit, ICDDRB, Mohakhali, Dhaka*

[4]*Dr. Gazi Sharmin Sultana, Consultant, General Laboratory (Department of Clinical Pathology, Clinical Biochemistry and Haematology), BIRDEM General Hospital, Shahbag, Dhaka*

[5]*Dr. Ahmed Zahid Hossain, Consultant, Department of Paediatric Surgery and Incharge of Emergency, Square Hospitals Ltd, Panthapath, Dhaka,*

[6]*Dr. Nafisa Fatema, Resident Medical officer, Department of Paediatrics, Institute of Child Health and Shishu Hospital, Sher-E Bangla Nagar, Dhaka*

[7]**Prof. Dr. Shamima Ferdousi, Associate professor and Head, Department of Pathology, Ibrahim Medical College, Shahbag, Dhaka*

Corresponding Author

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ABSTRACT

Back ground: Iron deficiency anemia (IDA) and beta-thalassaemia trait (B-TT) are the most common causes of hypochromic microcytic anemia. Many indices have been defined to quickly discriminate these similar entities via parameters obtained from automated blood count analyzers.

Methodology: The purpose of the study was to evaluate the predictive value of these indices in differential diagnosis of IDA and B-TT in adult cases. In this study we use auto-analyzer based formula of percentages of microcytic and hypochromic red cells (M/H ratio = % of Microcytosis/% of Hypochromia) as a screening tool for thalassaemia trait in Bangladeshi population.

Results: A total of 150 subjects w ere included in this study with 50 known obligate carrier of beta-thalassaemia trait and 100 patients with hypochromia and microcytosis. Confirmatory tests for IDA were performed if serum ferritin level was <12 ng/ml and confirmatory test for Beta-thalassaemia trait (BTT) and Haemoglobin E trait can be considered when $HbA_2 > 3.5\%$, on agarose gel Haemoglobin electrophoresis at an alkaline pH (8.6) where, in addition, MCV <76 fl and/or MCH <27 pg. BTT was selected with $HbA_2 > 3.5\%$, while the non- BTT group were those with $HbA_2 < 3.5\%$.The final analysis of the result revealed that M/H ratio is a very sensitive index for beta-thalassaemia trait. In our study, the sensitivity, predictive value and diagnostic accuracy of the M/H ratio for the beta thalassaemia trait were 96%, 90.4% and 90.4% respectively and also identify all cases of coexistent iron deficiency.

Conclusion: Thus M/H ratio is an easy, reliable and sensitive index which can be used for mass screening of beta-thalassaemia trait, particularly in a population where iron deficiency anemia is also prevalent.

Key Words: M/H ratio, thalassaemia trait

Introduction

It has been estimated that 20% of the world's population is iron deficient. Iron deficiency anemia is the most common type of anaemia throughout the world and dietary deficiency is the commonest cause[1]. Whereas, thalassaemia which is a commonest inherited gene disorder is prevalent worldwide[1,2]. Bangladesh is situated in the thalassaemia belt and Beta-thalassaemia is common here[2,3]. There are a significant number of patients who have concurrent iron deficiency and beta-

thalassaemia trait & other haemoglobinopathies[4,5]. World Health Organization (WHO) estimates that at least 6.5% of the world populations are carriers of different inherited disorders of Haemoglobin. A conservative World Health Organization (WHO) report showed about 3.0% of populations are carriers of Beta thalassaemia and 4.0% are carriers of Hb-E in Bangladesh, which means that there are about 3.6 millions carriers of beta thalassaemia and 4.8 millions carriers of Hb-E and affected birth per thousand of Beta thalassaemia is 0.106 & 0.300 of Hb-E/Beta thalassaemia. It is presumed that approximately six thousand thalassaemic children are born each year in Bangladesh[6,7]. Several screening procedures have been devised but these are neither cost effective nor sensitive enough[8-11]. A simple procedure utilizing cell counter introduced recently, for discrimination of beta-thalassaemia trait from iron deficiency anemia, is the ratio between percentage of microcytic and hypochromic red cells (M/H ratio)[10-12]. The quantification of the percentages of microcytic and hypochromic erythrocytes has proved clinically useful in the differential diagnosis between thalassaemic and non-thalassaemic microcytosis[12]. Iron-deficient erythropoiesis is characterized by the production of RBC with a decreased haemoglobin concentration and high hypochromic cells percentage, while microcytes of beta thalassaemia are generally smaller with low haemoglobin content but more preserved haemoglobin concentration. The percentage of hypochromic cells, defined as RBC with low haemoglobin concentration, tends to be higher in patients with IDA than in thalassaemia carriers, while the percentage of microcytes, increased in thalassaemia carriers, shows the opposite trend; the quotient % Micro/% Hypo, M/H ratio amplifies the difference between the two groups[10,12,17]. However, the reason for the discrepancies among the various studies may be also due to the different sample size and the inclusion of patients with severe and mixed cases of thalassaemia and iron deficiency anaemia. Many formulae, based on haemoglobin, RBC count, MCV and RDW are useful to discriminate between these two common causes of microcytic hypochromic anaemia in uncomplicated cases, but in patients who have both IDA and BTT, a situation common in Indian subcontinent, needs a special consideration[4]. In our population there is a high prevalence of iron, vitamin B_{12} and folic acid deficiencies along with high incidence of BTT which affects the RBC indices, so we may also have a large number of atypical cases with concomitant diseases[1]. Objective of our study was to evaluate and quantification of the % microcytic and % hypochromic ratio index (M/H ratio), which was calculated from RBC parameters, using fully automated haematology cell-counter to observe this ratio as a tool to evaluate clinical usefulness in the differential diagnosis between thalassaemic and non-thalassaemic microcytosis.

Materials & Methods

The material comprised of 150 subjects referred to the Department of Pathology, Institute of Child Health and Shishu Hospital, Sher-E Bangla Nagar, Dhaka, Department of Transfusion Medicine and Haematology section of Department of Clinical Pathology, Clinical Biochemistry and Haematology, BIRDEM General Hospital, Dhaka and Department of Haematology, BSMMU, Dhaka from 1st January 2003 to 31st December, 2005, for complete blood counts (CBC) for the various reasons. They comprised of both sexes, all ages with children above 12 years. The total 150 subjects were divided into two groups. Group I comprised of 50 subjects of known obligate carrier of beta-thalassaemia trait diagnosed by HbA_2 (also by family studies if necessary). Serum ferritin was within the normal limits in all of them. Estimation of M/H ratio, repeat haemoglobin electrophoresis, serum ferritin level and $NESTROF_{12}$ (Naked Eye Single Tube Red Cell Osmotic Fragility) were also done. The age of Group I ranged from 18 to 55 years (median 24 years) and male to female ratio was 1.5:1. On the other hand, Group II was composed of 100 patients with hypochromia and microcytosis diagnosed by cell counter, peripheral blood film, haemoglobin electrophoresis and serum ferritin level. This Group II was further divided into four groups by the observation of HbA2 and serum ferritin level. The age of Group II subjects ranged from 12-60 years (median 35 years) and male to female ratio of 1.3:1. The parents of 56% were first cousins and of 11% were related but not first cousins. The parents of 33% had no previous relationship. M/H ratios were calculated from the

blood samples with the help of the autoanalyzer using the calculation below. Study of peripheral blood film after properly staining with Giemsa stain was observed. Hypochromia was defined as central pallor more than 1/3rd in a RBC, where the visible field showed no overlapping and even distribution of RBC. This % Microcytosis / % Hypochromia haematological parameters were obtained from cell counter (fully automated haematology auto-analyzer Advia, USA and Sysmex, Japan). All individuals were subjected to detailed interview, adequate counseling and thorough clinical examination. About 5 to 6 ml o venous blood was collected from each of the subject from both study and control groups through a clean vene-puncture and distributed in the following manner:

1. 1.5 ml blood was properly mixed with potassium EDTA to a final concentration of 2.0 mg/ml and used for blood count by haematology auto-analyzer and a peripheral blood film was also made.

2. 1.5 ml blood was properly mixed with potassium EDTA to a final concentration of 2.0 mg/ml and used for preparation of haemolysate for Haemoglobin electrophoresis and estimation of HbA_2.

Rest of the blood was transferred into a clean glass test tube and allowed to clot. Clear serum was separated into a clean test tube and preserved for estimation of serum ferritin. In our study, M/H ratio has been calculated as:

M/H ratio = % of Microcytosis / % of Hypochromia[12-13, 15].

Finally, sensitivity & specificity, and positive & negative predictive values were calculated as follows:

Sensitivity: True positive / (true positive + false negative)

Specificity: True negative / (true negative + false positive)

Positive predictive value: True positive / (true positive + false positive)

Negative predictive value: True negative/ (true negative + false negative) and

Youden's index14= [(sensitivity + specificity) x 100] - 100.

Here, Youden's index takes into account both sensitivity and specificity and gives an appropriate measure of validity of particular technique. Serum ferritin estimation was performed by Radio-immuno assay (RIA) technique. Calibration was performed by using standards of 0-1000 ng /ml. Quality control was also performed within assay and between assays running of controls. The sensitivity, predictive value and diagnostic accuracy of M/H ratio for Beta-thalassaemia trait was calculated according to the method of Galen and Gambino[1-3, 10-17].

Results

This was a cross sectional analytic study, although we use Group I and Group II, the results were seen in an observatory manner and Group II was divided into four groups by the observation of HbA2 and serum ferritin level . The results of various haematological parameters and diagnostic tests of the Group I and the Group II with hypochromia and microcytosis are presented in table-I. The sensitivity of the M/H ratio of Beta-thalassaemia trait was calculated to be 96% from the above calculation. Table -II showed, on the basis of HbA2 and serum ferritin level study subject (n=100) were divided into four groups: Beta-thalassaemia trait (BTT) was 67%, Beta-thalassaemia trait with iron deficiency (BTT with IDA) in 18%, iron deficiency anaemia in 9% and miscellaneous / unclassified in 6%. The M/H ratios have been implemented to correctly identify all the cases of BTT and BTT with IDA. There were 9 false positive which actually belonged to IDA category. The sensitivity and predictive value of M/H ratio for BTT was 96% and 90.4% respectively found from the above calculation. The diagnostic accuracy was 90.4% using this calculation. Six cases belonged to the miscellaneous (unclassified group) with normal levels HbA2 and serum ferritin. These case could be of beta-thalassaemia trait, -thalassaemia or -thalassaemia trait, but the exact cause could not be identified.

Table-I: Haematological parameters and diagnostic tests of Group I (obligate carrier of beta-thalassaemia trait) and Group II (% Hypocromic and %microcytic)

Parameters & Diagnostic tests	Group I (n=50)	Group II (n=100)
Hb (gm/L)	125 ±18.50	113 ±2.35
TRBC (10^{12}/L)	5.8 ±1.80	5.1 ±0.85
MCV (fl)	65.3±4.30	66.0±7.30
MCH (pg)	20.0±1.50	21.40±3.20
Serum ferritin (ng/ml)	88.0±5.50	85.6±4.80
HbA_2	4.8 ± 0.80	3.7 ± 0.80
%Hypochromia	17.6± 12.0	18.0± 10.0
%Microcytosis	32.0± 15.80	27.5± 14.80

Table-II: On the basis HBA_2 and serum ferritin level Group II (n=100) were divided into four groups. All the Haematological parameters of these groups were shown in this table.

Parameters & Diagnostic tests	BTT (n=67)	BTT + IDA (n=18)	IDA (n=09)	Misc. (n=06)
Hb (gm/L)	11.70 ± 1.45	9.25 ± 2.86	7.90 ± 1.48	12.30±2.35
RBC (10^{12}/L)	5.10 ± 0.90	4.90 ± 0.45	4.10 ± 0.50	5.0 ± 0.90
MCV (fl)	66.5 ± 3.90	63.20 ± 7.50	66.0 ± 4.35	83.0 ± 7.50
MCH (pg)	22.0 ± 2.40	18.5 ± 3.40	20.8 ± 2.90	28.85 ± 2.90
Serum Ferritin (ng/ml)	117.0 ± 10.90	6.10 ± 2.95	2.60 ± 3.90	85.0 ± 7.50
HbA_2	5.10 ± 0.90	4.0 ± 0.40	2.25 ± 0.60	2.90 ± 0.40
%Microcytosis	28.60± 16.80	29.80± 14.80	28.10± 6.60	7.80± 5.90
%Hypochromia	17.6±9.80	19.20±12.60	30.3± 7.10	8.40± 5.90

Discussion

There are only few studies reported previously on the M/H ratio[10-12]. The reference ranges for the new RBC extended parameters and their values in anaemic patients with red cells abnormalities have been recently established[5,17]. Study showed, the % Microcytosis was more increased in thalassaemia than IDA5. Study with the Advia series Siemens 2120 analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) showed, that auto analyzer based M/ H ratio provided by this series has been proven to be a reliable index[13,15]. Mean % microcytosis was much more increased in thalassaemia (36.5%) than in IDA (28.8%; p-0.05), while mean % hypochromic demonstrated an opposite trend (thalassaemia 3.4%, IDA 20.4%; p-0.001) with Advia. On the other hand, mean M/H ratio was higher in thalassaemia (15.1) than IDA (4.6; p-0.001). Here, the hypochromic red cells, measured by the Advia 2120 analyzer, defined by their Hb concentration[13,15]. In iron deficient erythropoiesis, synthesis of haemoglobin molecules are severely impaired leading to the production of RBC with low haemoglobin concentration19. Microcytes of beta-thalassaemia have small volume because of impaired globin synthesis, but almost normal Haemoglobin concentration. Only when these patients have an iron deficiency the percentage of hypochromic cells increases[14,15]. Although IDA and thalassaemia are both microcytic anemia, hypochromia and microcytosis exhibit opposite trends in both diseases. Iron deficient erythropoiesis is characterized by the production of RBCs with a decreased haemoglobin concentration and a high hypochromic cells percentage; while microcytes of beta thalassaemia are generally smaller, with more preserved haemoglobin concentration. In iron deficient erythropoiesis, synthesis of haemoglobin molecules is severely impaired leading to the production of RBCs with low haemoglobin concentration (hypochromic cells), whereas, beta thalassaemic patients have a high rate of microcytosis as a result of the chronic increase of erythropoiesis[7]. In addition, microcytes of beta thalassaemia have a small volume due to impaired globin synthesis, but almost normal haemoglobin concentration. According to a study, the Advia 2120 analyzer M/H ratio index is reliable. This M/H ratio ranked second, compared to the published indices evaluated, with a Youden index of 76.3% and a sensitivity of 99.2%. According to the results of this study, an MH ratio value higher than 3.7 is a highly suspicious feature of beta thalassaemia, so HbA_2 must be quantified to confirm the presence of the disease. Although the specificity is 77.1%, due to its high sensitivity and less false negatives, when patients have a mild anemia and the differential diagnosis is more difficult, this M/H ratio is a reliable index. Whereas, when using the Sysmex XE 5000 analyzer (Sysmex Corporation, Kobe, Japan), studies showed that the percentages of erythrocyte subsets can be calculated and the new parameters, such percentage of microcytic red cells (% Micro

R), hypochromic red cells (% Hypo He) and percentage of macrocytic red cells (% Macro R) can be obtained[13,14,17,18]. Red blood cells extended parameters including the microcytic (% Micro R) and percentages of hypochromic red cells haemoglobin (% Hypo He) reported by this analyzer revealed that, % Micro R indicates the percentage of microcytic red cells with a volume <60 fl and % Hypochromia indicates the percentage of hypochromic red cells with a Haemoglobin content <17 pg13. There are also various red cell counter based formulae to differentiate BTT from IDA, e.g.: RDWI formula9 (MCV x RDW/RBC, where BTT<220 and IDA>220), Mentzer formula 4, 10(MCV/RBC count) in which BTT <13.0 and in IDA>13.0.(Here, RBC: red blood cells; Hb: haemoglobin; MCV: mean cell volume; MCH: mean cell haemoglobin; RDW: red cell distribution width)[9-17]. Beside this M/H ratio, Red blood cell count (RBC) is an index having both sensitivity and specificity more than 80% when, RBC value more than 5·1012/L in favour of BTT and a value <5·1012/L in favour of IDA, as a powerful indices for adult cases. According to Demir, the Youden indices of RBC count and RDWI were the highest, viz, 82% and 80% respectively[14]. They concluded that RBC count and RDWI are the most reliable discrimination indices in differentiation between BTT and IDA; Mentzer, Ehsani's study showed the best discrimination index with sensitivity 89% and specificity of 81%. Similar sensitivity 90.9% and specificity 80.3% were found by Ghafouri[4,16,18]. However, none of the indices allowed complete discrimination to distinguish BTT from IDA in a better way. None of the formulas are 100% sensitive and specific, therefore are of importance only for screening in areas of high prevalence of BTT1. Automated measurement of the red cells (RBC) microcytosis and hypochromia has proven clinical usefulness for the differential diagnosis. The present study was undertaken with the main objective of evaluating efficiency of M/H ratios in screening for beta thalassaemia only, which is proved to be a reliable index according to the other studies[10,12,13,17-21]. Our MH ratio showed similarity to the published indices evaluated by Urrechaga E study, with a Youden index of 76.3%[13,17-21]. The sensitivity, predictive value and diagnostic accuracy of this index for Beta-thalassaemia trait was found to be 96 %, 90.4% and 90.4% respectively which almost match the results of study carried out by d'Onfrio: 100, 92.3, 93.3; Khattaak: 94.2, 84.4, 89.5 and M. Saleem: 100, 90.4 and 90.4 respectively[1,2,12-20]. Our % Micro R/ % Hypo He (MH ratio), similar to the index provided by Advia instruments and also similar with %Micro R) % Hypo He (M-H) by Sysmex XE 5000 analyzer where, new M-H index proved to be a reliable index; a sensitivity of 98% (2% false negatives) and a specificity of 95.9% (4.1% false positives) are reached when a cut -off threshold of 11.5 is applied[13,21]. M/H ratio also picked up all cases of Beta-thalassaemia trait with iron deficiency anaemia. Findings of our study indicate that M/H ratio is specific and sensitive to be utilized as a population screening tool for detection of thalassaemia traits or carriers. It is superior to all so far described indices. There are only few studies reported previously on the M/H ratio[10-12]. Moreover, cell counter-based formula has been used in the differential diagnosis of microcytic anemia[10-20]. The reference ranges for these new RBC extended parameters and their values in anaemic patients with red cells abnormalities have been recently established as a good indicator by showing the % microcytosis was more increased in thalassaemia than IDA[5,17].

Conclusion

Thalassaemia is an important health problem. Effort should be made to reduce the thalassaemic child-birth by developing a population screening programme. Our study showed that, automated cell counters provide a rapid, technically reliable method for screening of Beta-thalassaemia trait from concomitant Iron deficiency anaemia cases. M/H ratio is one of the good indices but required a specific haematology laboratory where auto-analyzer is also available. Haemoglobin electrophoresis and RIA/ELISA for the estimation of serum ferritin is also expensive and not available throughout the country and only a few centers and institutions have got these facilities. Mass awareness about the danger of thalassaemia, carrier detection and adequate counseling are the prerequisite to prevent silent spread.

Conflict of Interest

The authors report no conflicts of interest.

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