Ind J Clin Biochem (Jan-Mar 2017) 32(1):61–67
DOI 10.1007/s12291-016-0574-9

ORIGINAL ARTICLE

Using Red Cell Indices and Reticulocyte Parameters for Carrier Screening of Various Thalassemia Syndromes

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Received: 8 February 2016 / Accepted: 28 April 2016 / Published online: 9 May 2016

Abstract Primary screening for thalassemia carriers usually involves an accurate blood count using an automated blood cell analyzer. We analyzed the red cell and reticulocyte parameters from 200 samples of various types of thalassemias and identified the discrimination criteria for differential diagnosis. These were separated into four groups based on genotypes. These groups included α-thalassemia, β-thalassemia, β-thalassemia with Hb E and Hb E trait, which are the important target of thalassemia screening. To compare the effectiveness of the screening strategies, seven selected screening tools were compared, including MCV alone (cutoff <80 fL); MRV alone (cutoff <100 fL); SD-C-NR alone (cutoff >28.5); a combined MCV and MRV; a combined MCV and SD-C-NR; a combined MRV and SD-C-NR; and a combined MCV, MRV and SD-C-NR. The combination of MCV, MRV and SD-C-NR has highest sensitivity for discrimination in all thalassemia, β-thalassemia, α-thalassemia, Homozygous Hb E and Hb E trait groups as 99.5, 100, 98, 100 and 100 %, respectively. The effectiveness of the combination of MCV, MRV and SD-C-NR parameters in the present study was determined as the faster and higher sensitivity screening tool than the other methods, including simple, reliable, cost-effective, and using only one automated hematology analyzer, saving labor costs.

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Keywords Thalassemia syndrome carrier · Red blood cell indices · Reticulocyte parameters · Automated blood cell analyzer

Introduction

Thalassemia syndrome is the most common genetic disorder of microcytic and hemolytic anemias, about 250 million people in the world carry thalassemia genes mainly in the Mediterranean area, the Middle East, the Indian subcontinent, and in Southeast Asia [1]. This genetic disorder results in defective globin synthesis, poorly or absent synthesized of α-globin chains in α-thalassemia and β-globin chains in β-thalassemia. The gene–gene interactions in this population can lead to cause several types of thalassemia syndrome. The three prime targets of prevention and control are homozygous α^0-thalassemia (causing the hemoglobin (Hb) Bart hydrops fetalis), homozygous β-thalassemia, and β-thalassemia/Hb E [2]. This imbalanced synthesis results in a variable degree of anemia which stimulates erythropoietin production, leading to proliferation and expansion of the bone marrow. The β-thalassemias have a considerable phenotypic variation depending on multiple factors, which include the nature of the mutation involved. This leads to a wide range of presentations from profound anemia (requiring lifelong blood transfusions—β-thalassemia major) to extremely mild anemia (β-thalassemia trait) [3].

In Thailand, about 1 % of Thai people (600,000 persons) were thalassemia patients [4] and about 40 % of Thai people have a thalassemia trait [5]. Therefore, in the prevention and control program are concern for the rapid, accurate, and inexpensive screening protocols to identify carriers of α^0-thalassemia, β-thalassemia, and Hb E, especially in a prenatal population at risk for Hb disorders.

The conventional method for primary screening of all forms of thalassemia relies on hematologic index cutoffs analyzed by an accurate blood cell count analyzer. Mean corpuscular volume (MCV) values less than 80 femtoliter (fL) and mean corpuscular hemoglobin (MCH) values less than 27 picogram (pg) are examined as cutoff to confirm or exclude the diagnoses of α-thalassemia and β-thalassemia [6, 7]. However, it has been demonstrated that a single-tube osmotic fragility (OF) test, it might be an attractive alternative to identify carriers of α- and β-thalassemias [8–10]. The used of a combined modified OF test and modified dichlorophenolindophenol (DCIP) test for Hb E [11] has been proposed for screening in rural communities of Southeast Asia [12].

Reticulocytes are juvenile red blood cells. They contain the ribosomal ribonucleic acid remnants which appear in large amounts in the cytoplasm of the nucleated precursor red blood cells. The number of reticulocytes in the peripheral blood is an accurate reflection of erythropoietic activity assuming that reticulocytes are released from the bone marrow after the normal time, and that they remain in circulation for the normal period of time [13]. Now-a-days all automated blood cell analyzers, which were developed under the advance technologies, can be provide erythrocyte and reticulocyte parameters for screening these various types of thalassemias discrimination from the normal individuals. The aim of the present study was to analyze the red cell indices and reticulocyte parameters provided by the Coulter LH 750 automated analyzer for discrimination of thalassemia carriers.

Materials and Methods

Subjects

Study participants included 300 apparently healthy couples, pregnant women and anyone who suspected the hemoglobin disorders. Sample selection was those who consecutively attended for care service between January and December 2014 at Thalassemia clinic at Sawanpracharak Hospital, Nakhonsawan Province, Thailand. Our study was approved by the institutional ethical committee of Naresuan University, Phitsanulok, Thailand. After informed consent was obtained EDTA-anticoagulated blood samples were obtained and transferred on ice within 2 h to the Medical Technology Laboratory Unit of Sawanpracharak Hospital where all laboratory testing was performed.

Screening and Hematologic Analysis

Complete Blood Counts (CBC) and RBC indices were determined using the Coulter LH 750 automated analyzer (Beckman Coulter, Fullerton, CA USA).

Reticulocyte Analysis

Reticulocyte indices were also determined using the Beckman Coulter LH 750 analyzer. The procedure of reticulocyte analysis in this analyzer uses new methylene blue dye (supravital dye) incubation with whole-blood samples, to precipitate the basophilic RNA network within the reticulocytes. The function of the reticulocyte stain is to identify and delineate the reticulocytes from mature red cells. Hemoglobin and unbound stain are removed by using a clearing reagent, then leaving clear spherical mature reticulocytes and darkly stained reticulocytes. Stained reticulocytes are differentiated from mature red cells and other cell populations by light scatter, direct current measurements, and opacity characteristics. Once the cells have been stained and cleared, the volume, conductivity and light scatter (VCS) Technology of reticulocyte analysis is employed, utilizing a unique flow cytometric means of cell interrogation to count and classify reticulocytes. All reticulocytes are identified and classified by simultaneous three-dimensional analysis using Volume, Conductivity, and Light Scatter.

Hemoglobin Analysis

Beta-Thalassemia traits were examined in samples with Hb A_2 levels exceeding 4.0 % using high performance liquid chromatography in our laboratory (HPLC) [18, 19]. Hb Constant Spring (Hb CS) was also examined by using HPLC. We also confirmed the separation and quantitation of Hb A_2 and Hb E by using automated capillaries zone electrophoresis (Sebia, Bio-Rad) [14]. The capillaries system uses the principle of capillary electrophoresis in free solution. Electrophoresis was performed in alkaline buffer (pH 9.4) allowing separation to be directed by pH and endosmosis. All Hb-types concentrations were measured at a wavelength of 415 nm.

DNA Analysis

The polymerase chain reaction (PCR) analysis of genomic DNA extraction from peripheral blood leukocytes was used as gold standards for α-thalassemia. Alpha-thalassemia^0 mutations (SEA type) were identified by allele-specific PCR [15–17].

Statistical Analysis

Descriptive statistics, median and interquatile range in non-parametric data were used to describe hematologic features of all subjects. All effectiveness variables from Coulter LH 750 auto-analyzer based on RBC and reticulocyte indices in each group were compared by using Mann-Whiteney

Table 1 Thalassemia genotypes observed in 200 carriers

Thalassemia genotype	No. of cases
α-Thalassemia	50
α^0-Thalassemia trait (SEA type)	18
Hb H	14
Hb H/CS	7
Hb CS trait	11
β-Thalasemia	50
β-Thalasemia trait	50
β-Thalassemia with Hb E/Homozygous Hb E	52
Homozygous Hb E	40
β-Thalasemia/Hb E	12
Hb E trait	48

U-test. We combined the red cell and reticulocyte parameters for thalassemias and Hb E screening. We also calculated the sensitivity and specificity of the red cell and reticulocyte parameters by using Receiver Operating Characteristic (ROC) analysis. All analysis was performed by SPSS version 13.0 (SPSS, Chicago, IL, USA).

Results

The results of the thalassemia types are summarized in Table 1. Among 300 participants studied, 200 (66.7 %) were found to carry thalassemias or hemoglobinopathies. No thalassemia was detected in the remaining 100 subjects (33.3 %). The results and comparison of these groups are demonstrated in Table 2. The comparison results of all hematologic characteristics of each group of thalassemia participants are demonstrated in Table 3. As expected, the most common genotype was Hb E heterozygote, which was identified in 100 subjects, included 12 β-thalassemia/Hb E, 40 homozygous Hb E and 48 Hb E trait. In the former group, fifty β-thalassemia traits were detected. Fifty α-Thalassemias, including 18 α^0- thalassemia traits (SEA type), 14 Hb H, 7 Hb H/CS and 11 Hb CS traits were identified and summarized in Table 1.

We used the ROC curve to calculate the area under curve (AUC) of the important of red cell and reticulocyte parameters. We found that MCV, MCH, Mean reticulocyte volume (MRV) and Standard deviation conductivity non-

Table 2 Comparison of all blood cell indices of normal control and β-thalassemia, α-thalassemia and hemoglobin E carrier

Variables	Normal (n = 100)	All thalassemia carrier (n = 200)	*p* value
Age (years)	35.9 (8.5–44.0)*	23.10 (2.85–41.90)*	0.057
WBC ($\times 10^9$/L)	7.30 (5.63–8.60)	8.50 (6.83–10.30)	0.001
RBC ($\times 10^{12}$/L)	3.685 (3.263–4.175)	4.620 (3.538–5.378)	<0.001
Hb (g/dL)	10.65 (9.03–12.10)	9.20 (7.70–10.90)	<0.001
Hct (%)	31.7 (27.4–36.2)	29.45 (25.23–34.28)	0.019
MCV (fL)	88.40 (83.88–91.90)	66.55 (60.63–72.55)	<0.001
MCH (pg)	29.25 (27.53–30.80)	20.70 (18.93–22.48)	<0.001
MCHC (g/dL)	33.15 (32.50–33.80)	31.40 (30.50–32.10)	<0.001
RDW (%)	14.60 (13.80–17.10)	19.55 (17.10–26.55)	<0.001
Plt ($\times 10^9$/L)	248.50 (195.50–345.25)	253.00 (198.00–347.75)	0.783
% Ret count (%)	1.80 (1.29–2.63)	2.41 (1.58–3.09)	0.001
Ab Ret ($\times 10^6$/L)	0.0649 (0.0476–0.0904)	0.1046 (0.0674–0.1445)	<0.001
MRV (fL)	110.05 (102.48–116.35)	95.00 (86.25–99.98)	<0.001
IRF (ratio)	0.370 (0.310–0.420)	0.295 (0.200–0.378)	<0.001
M-V-Ret	54.70 (51.25–57.75)	47.40 (43.00–50.10)	<0.001
M-C-Ret	73.25 (69.90–77.80)	87.05 (82.80–93.18)	<0.001
M-S-Ret	137.85 (128.80–144.53)	140.35 (127.80–151.10)	0.091
SD-V-Ret	14.40 (12.95–16.02)	16.63 (14.69–18.76)	<0.001
SD-C-Ret	25.32 (23.02–27.35)	30.79 (27.55–34.51)	<0.001
SD-S-Ret	18.19 (16.95–19.96)	16.96 (15.25–19.15)	<0.001
M-V-NR	46.40 (44.03–48.48)	35.40 (33.13–38.68)	<0.001
M-C-NR	72.70 (69.95–77.70)	90.00 (85.05–96.88)	<0.001
M-S-NR	78.30 (65.78–84.60)	78.25 (61.83–87.35)	0.454
SD-V-NR	10.91 (10.41–11.50)	11.29 (10.00–13.35)	0.140
SD-C-NR	24.73 (22.88–26.26)	30.58 (30.23–33.17)	<0.001
SD-S-NR	14.04 (13.15–15.43)	16.76 (13.95–20.24)	<0.001

* Median and interquartile range

Table 3 Comparison of all blood cell indices of α-thalassemia, β-thalassemia and hemoglobin E carrier

Variables	α-Thalasemia (n = 50)	β-Thalasemia (n = 50)	β-Thal with Hb E/Homo Hb E (n = 52)	Hb E trait (n = 48)	*p* value
Age (years)	24.5 (2.1–50.9)*	18.30 (1.0–40.9)*	33.5 (1.2–62.9)*	33.3 (3.1–58.9)*	0.09
WBC ($\times 10^9$/L)	8.7 (6.4–10.50)	8.2 (6.9–9.9)	8.7 (6.9–10.2)	8.1 (6.8–9.8)	0.719
RBC ($\times 10^{12}$/L)	4.71 (3.46–5.44)	4.61 (3.97–5.71)	4.30 (3.26–5.130)	4.18 (3.78–4.80)	0.263
Hb (g/dL)	9.2 (7.5–11.2)	9.69 (8.0–11.1)	9.2 (7.6–10.3)	9.4 (8.0–10.9)	0.580
Hct (%)	29.2 (25.0–35.5)	31.9 (26.0–37.6)	28.7 (24.1–32.9)	29.8 (25.5–33.5)	0.160
MCV (fL)	66.1 (60.4–70.4)	70.7 (62.9–78.90	65.2 (60.3–69.60	71.2 (66.1–75.5)	0.003
MCH (pg)	20.7 (19.2–22.30	21.0 (18.3–23.0)	20.4 (19.2–22.7)	22.9 (20.2–24.3)	0.019
MCHC (g/dL)	31.7 (31.1–32.1)	30.5 (27.78–32.10)	31.6 (30.8–32.2)	31.9 (30.8–32.7)	0.001
RDW (%)	18.4 (16.5–27.8)	21.55 (15.68–26.10)	19.4 (17.7–26.70)	17.1 (15.2–21.4)	0.015
% Ret count (%)	2.40 (1.38–2.97)	2.68 (1.83–4.05)	2.25 (1.55–2.85)	1.52 (1.11–2.13)	<0.001
Ab Ret ($\times 10^6$/μL)	0.098 (0.065–0.141)	0.117 (0.085–0.1660)	0.103 (0.062–0.139)	0.066 (0.046–0.085)	<0.001
MRV (fL)	93.80 (85.35–102.60)	98.65 (86.00–107.88)	94.85 (86.78–98.00)	91.3 (87.28–96.95)	0.061
IRF (ratio)	0.28 (0.22–0.350)	0.35 (0.23–0.403)	0.27 (0.19–0.380)	0.28 (0.23–0.34)	0.162
M-V-Ret	46.309 (41.48–50.55)	49.35 (42.98–53.830)	47.40 (43.38–49.28)	47.30 (44.15–49.08)	0.392
M-C-Ret	89.85 (84.23–95.150	84.95 (78.43–91.30)	86.90 (84.53–92.03)	81.75 (74.85–88.3)	<0.001
M-S-Ret	141.55 (128.18–151.43)	143.75 (129.30–154.13)	136.70 (121.20–149.23)	115.20 (109.73–126.13)	<0.001
SD-V-Ret	16.13 (14.60–18.49)	16.36 (14.17–18.200)	17.29 (15.23–20.01)	15.23 (12.84–18.00)	0.002
SD-C-Ret	31.23 (27.54–34.29)	30.95 (27.14–34.84)	30.53 (27.62–34.77)	27.79 (24.66–31.67)	0.019
SD-S-Ret	16.52 (15.23–17.62)	15.93 (13.88–19.29)	17.64 (16.53–20.64)	17.77 (15.72–20.10)	0.002
M-V-NR	35.00 (33.25–38.88)	36.10 (32.63–40.15)	35.25 (33.25–37.38)	38.45 (36.23–40.10)	0.010
M-C-NR	91.70 (83.78–100.30)	88.45 (84.00–95.45)	90.00 (87.83–95.98)	80.10 (75.18–86.03)	<0.001
M-S-NR	81.60 (73.05–88.18)	81.40 (65.60–94.40)	69.50 (57.88–80.23)	54.10 (49.15–63.30)	<0.001
SD-V-NR	10.90 (9.47–13.32)	11.65 (9.76–13.57)	11.59 (10.29–12.88)	10.09 (9.56–11.14)	0.001
SD-C-NR	31.77 (28.95–35.80)	31.47 (27.31–34.02)	30.58 (30.58–30.58)	30.58 (30.58–30.58)	0.019
SD-S-NR	15.33 (13.23–19.27)	17.65 (14.08–21.06)	17.15 (15.24–20.43)	13.95 (12.74–15.73)	<0.001

* Median and interquartile range

reticulocyte (SD-C-NR) are the important parameters for this discrimination and the others as demonstrated in Fig. 1. To compare the effectiveness of the screening strategies, the data were collected as shown in Table 3 were separated into 4 groups based on genotypes. These groups included α-thalassemia, β-thalassemia, β-thalassemia with Hb E/Homozygous Hb E and Hb E trait, which are the important targets of thalassemia screening. Seven selected screening tools were compared, including MCV alone (cutoff <80 fL); MRV alone (cutoff <100 fL); SD-C-NR alone (cutoff >28.5); a combined MCV and MRV; a combined MCV and SD-C-NR; a combined MRV and SD-C-NR; and a combined MCV, MRV and SD-C-NR. As shown in Table 4, the combination of MCV, MRV and SD-C-NR has highest sensitivity for discrimination in all thalassemia, α-thalassemia, β-thalassemia, β-thalassemia with Hb E/Homozygous Hb E and Hb E trait groups as 99.5, 100, 98, 100 and 100 %, respectively. Based on these results, the effectiveness of the combination of MCV, MRV and SD-C-NR parameters in the present study was determined as the faster and higher sensitivity screening tool than the other methods.

Discussion

The aim of screening for thalassemia and hemoglobinopathies in Southeast Asia is to offer carrier testing to the population before they have child [2, 20]. In the present study, we demonstrated the effectiveness combination parameters for thalassemia carrier screening in 200 various thalassemia syndromes. The identification of various types of thalassemia in this group of Thai participants (Table 1) confirms a high prevalence and a diverse heterogeneity of this genetic disorder in Southeast Asia and underlines the need for an appropriate and highly sensitive screening strategy. Hb E was the most common hemoglobinopathy among Southeast Asian populations [21] and subsequently β-thalassemia and α-thalassemias were detected at different frequencies.

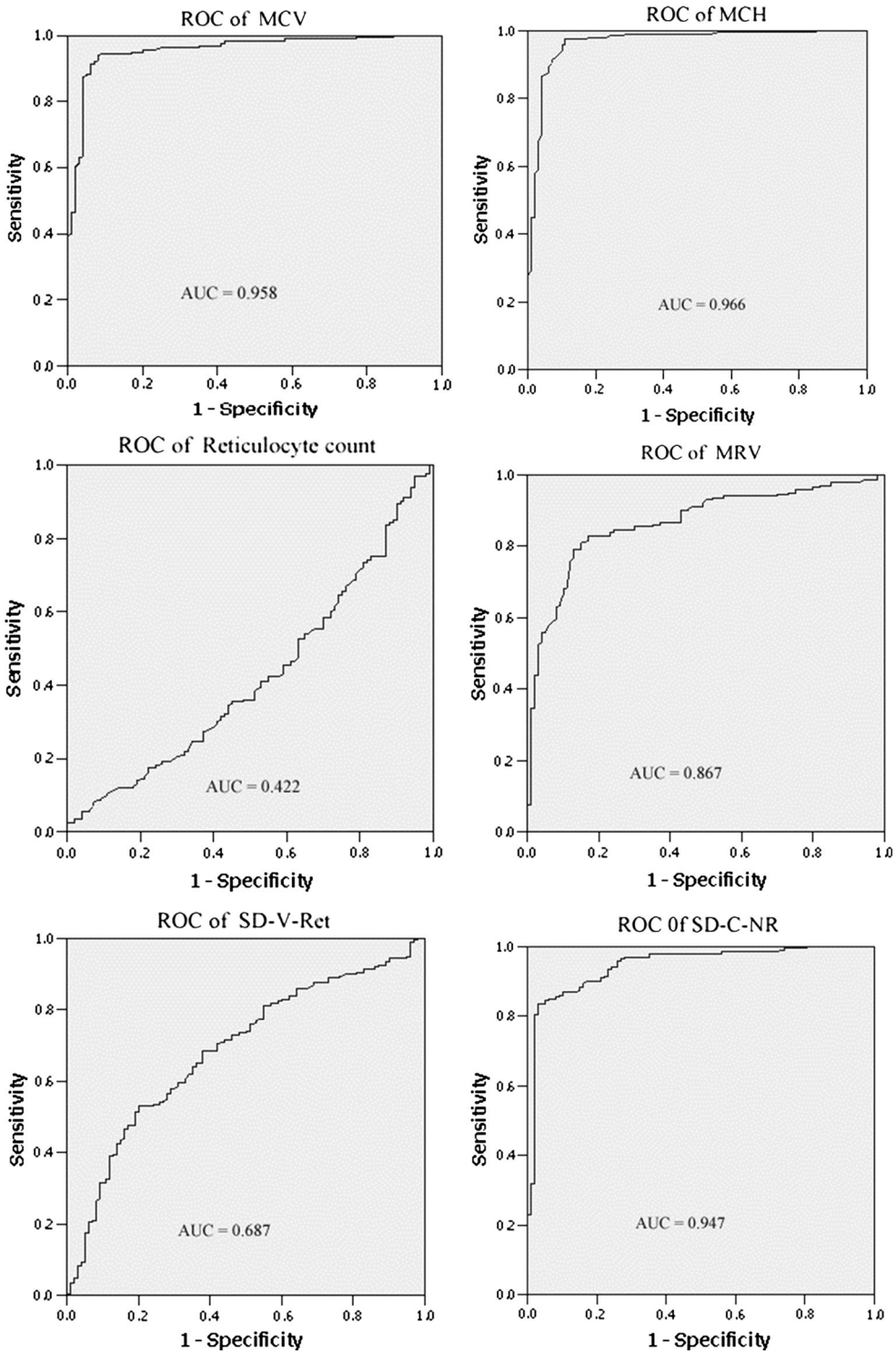

Fig. 1 Demonstration of the AUC of each parameter was used for the thalassemia carrier screening

A flow cytometry-based as an advanced technology in automated hematology analyzer has enabled us to study the heterogeneity of red cell parameters in α-and β-thalassemias [22–24]. The reduction of red blood cell indices such as MCV, MCH, and mean corpuscular hemoglobin concentration (MCHC) are the significant indicators for abnormal red cells in thalassemias [25–27]. MCV and MCH are used as the first screening tools for thalassemia trait since MCV and MCH are decreased in most thalassemias. It is noteworthy that the combined MCV and MCH method provided better sensitivity than that obtained using the MCV or MCH alone. For Hb E heterozygotes, however, many had normal RBC indices and, therefore, had negative results in MCV and MCH screening. The

Table 4 Demonstration of the effectiveness of Thalassemia and hemoglobinopathy screening among 200 carriers using MCV and the combination of MRV and SD-C-NR

Parameters	Sensitivity (%)					Specificity (%)
	All Thallassemia	α-Thalasemia	β-Thalasemia	β-Thal with Hb E/Homo Hb E	Hb E trait	
MCV <80 fLor	94.0	94.0	88.0	98.1	94.0	92.0
MRV <100	81.5	68.0	58.0	100	81.5	85.0
SD-C-NR >28.5	87.5	80.0	70.0	100	87.5	88.0
MCV + MRV	97.0	94.0	94.0	100	97.0	81.0
MCV + SD-C-NR	98.5	100	94.0	100	98.5	82.0
MRV + SD-C-NR	96.0	93.0	94.0	100	96.0	74.0
MCV + MRV + SD-C-NR	99.5	100	98.0	100	99.5	82.0

false negative result with MCV and MCH screening for Hb E carrier is unacceptable, especially when a population screened is known to have a high prevalence of Hb E, as is true for Southeast Asian populations. A reduction in MCV and MCH values also was noted in women with other mild forms of thalassemia, including α^{+}-thalassemia, Hb CS, and Hb PS. Other investigators found similar results in a Chinese population [28, 29].

The results of the present study indicate the sensitivity of the MCV screening protocol was improved greatly (to 100.0 %) when it was used in combination with MRV and SD-C-NR. Based on the results of our study, we strongly recommend the use of the electronic red blood cell and reticulocyte counting for screening Southeast Asian populations to be able to identify and give proper advice to thalassemia carriers. It is interesting that the best screening result with 100.0 % sensitivity and 72.0 % specificity, were obtained with a combined MCV, MRV and SD-C-NR parameters (Table 4).

Conclusion

According to the present study, our screening method consisting of a combination of MCV, MRV and SD-C-NR parameters, is an attractive screening procedure. It is simple, reliable, cost-effective, and used only in one automated hematology analyzer, therefore requiring no extra labor. We propose this study protocol for the first screening to use for prevention and control of severe thalassemia in the provincial hospitals in Thailand. Using this method might provide earlier flagging of the thalassemia carriers, which through genetic counseling, could help reduce the incidence of thalassemia diseases in Southeast Asian communities.

Acknowledgments We sincerely thank Naresuan University for financial support and also thank all co-workers for their blood collection and technical assistance at Medical Technology laboratory Unit, Sawanpracharak Hospital. We sincerely thank who the patients participated in this study and Asst. Prof. Dr. Ronald A. Markwardt, Burapha University, for his critical reading and correcting of the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to report.

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