

Original article

Diagnostic accuracy of reticulocyte hemoglobin content in Thai patients with microcytic red cells as a test for iron deficiency anemia

Jindaratn Chaipokam, Thanyaphong Na Nakorn, Ponlapat Rojnuckarin

Division of Hematology, Department of Medicine, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand

Background: Reticulocyte hemoglobin content (CHr) is helpful for early diagnosis of iron deficiency anemia (IDA). However, its use is limited in thalassemia carriers.

Objectives: To determine the accuracy of CHr and reticulocyte count as a test for iron deficiency anemia in Thai patients with microcytic red cells given the high prevalence of thalassemia carriers in Thailand.

Methods: Thai patients with adult microcytic anemia (mean corpuscular volume ≤ 80 fL) were prospectively enrolled. We conducted an automated complete blood count, reticulocyte count (with CHr), ferritin, and hemoglobin analysis. IDA was defined by a ferritin reference level < 50 $\mu\text{g/L}$ and ≥ 1 g/dL response within a month to iron dietary supplement. Cutoff points were determined from receiver operating characteristic curves.

Results: We included 168 patients (53 with IDA, 50 with anemia of inflammation (AI), 49 with thalassemia traits, and 16 with thalassemia diseases) and 99 healthy controls. The CHr in patients with IDA and thalassemia disease were significantly less than those in patients with AI and thalassemia trait ($P < 0.001$), who in turn had lower CHr compared with normal controls ($P < 0.001$). Patients with thalassemia were distinguished by their high reticulocyte counts. After excluding thalassemia, a cutoff CHr < 27.0 pg showed a sensitivity of 84.9% and specificity of 77.8% for IDA diagnosis. Additionally, mean corpuscular hemoglobin concentration (MCHC) < 31.6 g/dL showed a sensitivity of 81.1% and specificity of 83.7% for IDA diagnosis.

Conclusions: CHr combined with additional information about reticulocyte count and MCHC is useful for diagnosis of IDA in Thais.

Keywords: Diagnostic test, iron deficiency, microcytic anemia, reticulocyte hemoglobin content

The criterion standard, or index test, for the diagnosis of iron deficiency anemia (IDA) is the absence of stainable bone marrow iron. In addition, serum ferritin is a reliable marker for the diagnosis of IDA and the best marker representing iron storage in bone marrow [1, 2]. In clinical practice, serum ferritin is helpful for diagnosis of IDA with high specificity and has positive predictive value [3, 4]. However, these tests are not readily available in many hospitals in Thailand because they are not routine. Furthermore, they require a longer turnaround time and are more expensive than complete blood counts (CBC) with reticulocyte hemoglobin content (CHr).

One of the first laboratory investigations for anemia is automated reticulocyte counting, which

concurrently yields the reticulocyte hemoglobin content. This variable indicates the hemoglobinization of the youngest red blood cells, which have entered the circulation within the past 1–2 days. CHr values in IDA samples are significantly lower than those of control and hemolytic anemia samples [5, 6]. The CHr measured using an automated blood cell analyzer, for example a Sysmex XE 2100 or Bayer ADVIA120, has been shown to be useful in the diagnosis of iron deficient states in patients undergoing chronic dialysis [7-9]. A CHr < 28 pg shows optimal accuracy for diagnosis of IDA in patients with a mean corpuscular volume (MCV) of < 100 fL. Using Prussian blue staining of bone marrow aspirate to determine the iron status, the sensitivity of CHr is 73.9% and its specificity 73.3%. Therefore, measurement of the CHr in peripheral blood samples is effective for early diagnosis of an iron-depleted state [2, 10-12]. This index is also useful in the early detection and monitoring of erythropoietic function after 3–4 days

Correspondence to: Ponlapat Rojnuckarin, Division of Hematology, Department of Medicine, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand. E-mail: ponlapat.r@chula.ac.th

of erythropoietin therapy in patients undergoing dialysis [13-18]. Furthermore, addition of CHr to the screening CBC improves detection of IDA in apparently healthy adolescents [19].

As CHr can be obtained readily using automated blood cell analyzers, it enables diagnosis and treatment of IDA without the need for a more time-consuming and costly iron study. However, its utility in Thai patients may be limited because of the high prevalence of thalassemia and hemoglobinopathy, which cause hypochromia [20]. The reported CHr cutoff values may not be sufficiently sensitive or specific to differentiate thalassemia/hemoglobinopathy carriers from patients with iron deficiency [6].

Therefore, we prospectively evaluated the use of CHr in Thai patients with microcytic red cells (MCV \leq 80 fL) for diagnosis of IDA, compared with the criterion standard, or index test, of low serum ferritin levels and hemoglobin responses to iron dietary supplement. The cutoff value, sensitivity, and specificity of the CHr test were explored.

Methods

This was a prospective study of diagnostic accuracy. The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (approval No. 222/2011). Every participant included signed an informed consent form. We included consecutive adult inpatients and outpatients (age \geq 18 years), who had a MCV $<$ 80 fL and were treated at the King Chulalongkorn Memorial Hospital from August 1st, 2010 to January 31st, 2012. Exclusion criteria were pregnancy, creatinine clearance $<$ 30 mL/min (calculated using the Cockcroft–Gault equation), a history of iron supplement use within the previous 3 months, or an unclassifiable type of diagnosis.

Venous blood samples were collected by venipuncture and immediately processed (within 6 h) at the King Chulalongkorn Memorial Hospital laboratory for CBC and reticulocyte count with CHr, using an XE-AlphaN automated blood cell analyzer (Sysmex Corporation, Kobe, Japan). The serum ferritin and serum iron, total iron binding capacity (TIBC) were determined using an Elecsys 2010 immunoassay analyzer (Roche Diagnostics, Mannheim, Germany). Hemoglobin analysis was conducted using isoelectric focusing electrophoresis (IEF) or high performance liquid chromatography (HPLC), or both.

Three diagnostic groups were defined. An IDA group with a ferritin reference level $<$ 50 μ g/L and hemoglobin level that rose \geq 1 g/dL within one month after oral iron therapy. Patients in the thalassemia group had hemoglobin analysis showing thalassemia and/or hemoglobinopathy and/or confirmed α -thalassemia traits using gap-polymerase chain reaction (gap-PCR). We measured serum ferritin in all thalassemia samples to exclude concomitant IDA. The last group of patients had anemia of inflammation (AI), showed a ferritin level \geq 200 g/L and a history of inflammatory diseases. Patients who could not be classified into one of these groups were excluded. The control participants were nonanemic healthy volunteers with normal MCV and normal red cell distribution width (RDW).

Data were analyzed using SPSS statistical software for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA) to identify the cutoff value for optimal sensitivity and specificity using receiver operating characteristic (ROC) curves. Multiple comparisons were made using a one-way analysis of variance (ANOVA) at 95% confidence interval (CI) and the differences were considered significant at the 0.05 level. A Pearson correlation coefficient was used to define relationship between CHr and other variables.

The sample size was calculated at 95% confidence with a 6% acceptable error. At least 70 patients with microcytic anemia, 51 of whom were iron deficient, and at least 70 normocytic control samples were required to provide adequate power for this study.

Results

We recruited 99 healthy volunteers into the control group and screened 181 patients with microcytic red blood cells. Sixteen patients were excluded because 2 were $<$ 18 years old, 2 had creatinine clearance $<$ 30 mL/min, 5 patients were in unclassifiable groups of diagnosis, and 7 patients had suspected -thalassemia trait (microcytic anemia with normal ferritin level), but a gap-PCR had not been performed to confirm α -thalassemia. Ultimately, 168 patients were enrolled in this study; 53 with IDA, 50 with AI, and 65 with thalassemia.

In the thalassemia group, there were 49 patients with thalassemia traits; 15 with hemoglobin E trait, 14 with homozygous hemoglobin E, 9 with β -thalassemia trait, 6 with hemoglobin E with α -thalassemia trait, 4 with α -thalassemia trait (confirmed by gap-PCR), and 1 with a β -hemoglobin variant. Sixteen patients with

thalassemia disease were 4 with β -thalassemia with hemoglobin E, 7 with hemoglobin H diseases, 3 with hemoglobin H with hemoglobin CS, 1 with hemoglobin AE Bart's with hemoglobin CS, and 1 homozygous hemoglobin CS.

The laboratory data are shown in **Table 1**. Patients with AI were significantly older than those in the other groups (**Table 1**). The mean CHr in this study was 27.3 pg. The laboratory normal range is 25–35 pg. The CHr values of patients in the AI and thalassemia trait groups were significantly less than those in the control group, but significantly higher than those in the groups with thalassemia disease or IDA. Patients in the group with thalassemia disease had

significantly higher reticulocyte counts than those in all the other groups including IDA. Because of the non-normal distributions, reticulocyte and ferritin data were transformed logarithmically. Comparisons of the transformed data had similar outcomes to those shown in **Table 1** (data not shown).

A Pearson correlation analysis showed that CHr was positively correlated with hemoglobin (Hb) or degree of anemia ($r = 0.77, P < 0.001$), MCV ($r = 0.86, P < 0.001$), mean corpuscular hemoglobin (MCH, $r = 0.89, P < 0.001$), and mean corpuscular hemoglobin concentration (MCHC, $r = 0.57, P < 0.001$), but it was not correlated with age or RDW at 95% confidence interval (**Table 2** and **Figure 1**).

Table 1. The means (standard deviations) for age, sex, and red cell indices of the 3 groups of microcytic patients and healthy controls

Diagnosis	Control	AI	Thalassemia		IDA
			Trait	Disease	
N	99	50	49	16	53
Age (years)	37.3 (10.8) ^{a.027}	54.8 (20.6) ^{b.014}	45.9 (18.0) ^{c>0.99}	35.8 (14.7) ^{d.39}	44.8 (16.8) ^{d.05}
Sex (%): male/female	18.2/81.8 ^{a.350}	28.0/72.0 ^{b.051}	30.6/69.4 ^{c.10}	18.8/81.2 ^{d.96}	54.7/45.3 ^{d.22}
MCV (fL)	88.8 (3.5) ^{a<.001}	72.8 (5.4) ^{b.002}	69.0 (8.4) ^{c>0.99}	64.1 (8.1) ^{d.54}	68.5 (9.2) ^{d.03}
Hb (g/dL)	13.5 (1.0) ^{a<.001}	9.4 (1.6) ^{b.11}	10.7 (2.1) ^{c<.001}	8.8 (1.0) ^{d>.99}	8.5 (2.7) ^{d.29}
MCH (pg)	29.3 (1.4) ^{a<.001}	23.3 (2.0) ^{b.006}	22.5 (2.8) ^{c.019}	18.9 (2.5) ^{d.52}	20.4 (3.7) ^{d<.001}
MCHC (g/dL)	33.0 (0.8) ^{a<.001}	32.2 (2.2) ^{b>.99}	32.7 (1.8) ^{c<.001}	29.6 (2.6) ^{d>.99}	29.3 (3.0) ^{d<.001}
RDW (%)	13.5 (0.6) ^{a<.001}	17.9 (3.2) ^{b>.96}	16.7 (2.8) ^{c<.001}	24.3 (4.9) ^{d.06}	20.1 (4.3) ^{d.03}
CHr (pg)	33.1 (2.4) ^{a<.001}	26.6 (3.9) ^{b.009}	25.2 (4.1) ^{c<.001}	20.5 (3.5) ^{d>.99}	21.2 (5.5) ^{d<.001}
Reticulocyte (x10 ⁹ /L)	46.2 (14.7) ^{a>0.99}	52.1 (31.3) ^{b.58}	63.5 (50.1) ^{c.34}	146.1 (101.2) ^{d.014}	46.7 (26.5) ^{d.93}
Ferritin (µg/L)	Not done	1,766.0 (3,772.0) ^{b.15}	386.5 (459.8) ^{c<.001}	974.6 (1,212.3) ^{d.060}	9.5 (8.4) ^{d.011}

Control, volunteers with normocytic red cells; AI, anemia of inflammation; IDA, iron deficiency anemia; MCV, mean corpuscular volume; Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; CHr, reticulocyte hemoglobin content; ^a*P* control vs IDA, ^b*P* AI vs thalassemia, ^c*P* thalassemia trait vs IDA, ^d*P* thalassemia disease vs IDA, ^e*P* IDA vs AI

Table 2. The correlation of age and other variables (95% CI)

Age	Parameters					
	MCV	Hb	MCH	MCHC	RDW	CHr
<i>r</i>	-0.12	-0.25	-0.13	<0.001	0.07	-0.07
<i>P</i>	0.04	<0.001	0.04	0.99	0.25	0.27

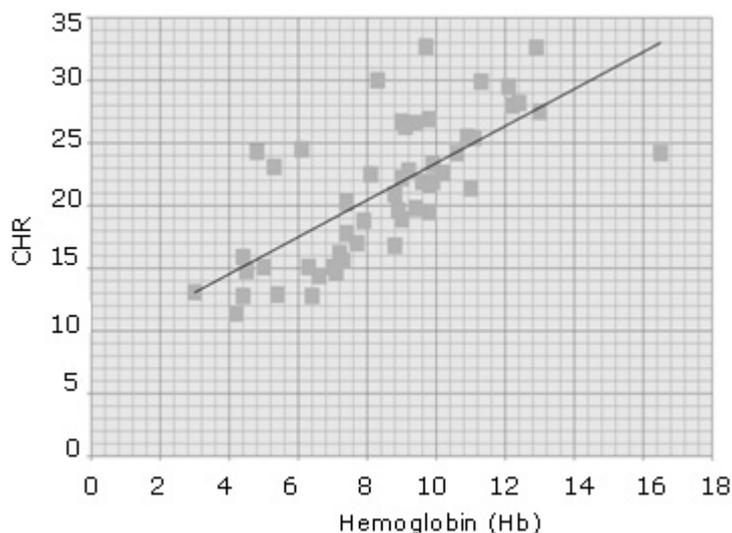


Figure 1. The correlation between the reticulocyte hemoglobin content (CHr) values and hemoglobin levels in patients with microcytic red blood cells ($r=0.77$, $P<0.001$)

Receiver operating characteristic (ROC) curve analysis was performed to determine appropriate cutoff values of CHr as shown in **Figure 2**. Using a CHr cutoff point of 24.6pg, the sensitivity was 73.6%, specificity was 74.8%, positive predictive value (PPV) was 42.4%, and negative predictive value (NPV) was 92.0% for diagnosis IDA for the whole population (**Figure 2A**). Many false positive samples were from thalassemia patients. Normal volunteers were not included in these calculations.

When patients in thalassemia groups (traits and diseases) was excluded, the cutoff point CHr of 27.2 pg showed a sensitivity of 84.9%, specificity of 81.2%, PPV of 60.8%, and NPV of 93.8% (**Figure 2B**). As there was a statistical difference in CHr between the thalassemia trait and thalassemia disease ($P=0.001$), only thalassemia disease was excluded in a reanalysis. The result was similar to that obtained by excluding all thalassemia patients (**Figure 2C**).

In addition, MCHC of the IDA group was similar to thalassemia disease, but significantly lower than those of the other groups (**Table 1**). On ROC analysis using a MCHC of 31.6 g/dL as the cutoff point, the sensitivity was 81.1%, specificity was 83.7%, PPV was 81.5%, and NPV was 81.3% for differentiating IDA from thalassemia trait (**Figure 2D**).

Discussion

Our study demonstrates a caveat in using CHr in diagnosis of IDA in Thais because of the high prevalence of thalassemia and hemoglobinopathy.

Consistent with a previous study [6], the cutoff value of CHr for the diagnosis IDA in Thai patients (24.6 pg) is <28 pg as specified elsewhere [2] because of the higher prevalence of microcytic red cells that are correlated with low CHr. As shown in **Table 1**, the CHr is useful to differentiate IDA from AI and thalassemia trait, but cannot accurately differentiate IDA from thalassemia disease in patients with microcytic anemia. However, thalassemia disease has a distinctive feature of high reticulocyte counts that can differentiate it from all the other causes of microcytic anemia (**Table 1**). Furthermore, the present study shows that a low MCHC is another helpful parameter in differentiating IDA from thalassemia traits.

The differential diagnoses of microcytic anemia include IDA, AI, thalassemia trait, and thalassemia disease. As initial tests, CBC and reticulocyte count should be routinely investigated simultaneously. If absolute reticulocyte count is high ($>100 \times 10^9/L$), thalassemia disease should be considered (**Figure 3**) correlating with clinical history of long-standing anemia since childhood and physical examination of chronic hemolysis, such as splenomegaly and jaundice. If the CHr value is >24.6 pg, a diagnosis of IDA is unlikely (NPV 92.4%). The presence of chronic infection, inflammation or malignancy suggests AI and a positive family history suggests thalassemia trait.

Considering the group with low CHr (≤ 24.6 pg) and a normal reticulocyte count, a MCHC <31.6 g/dL suggests the probability of IDA over thalassemia

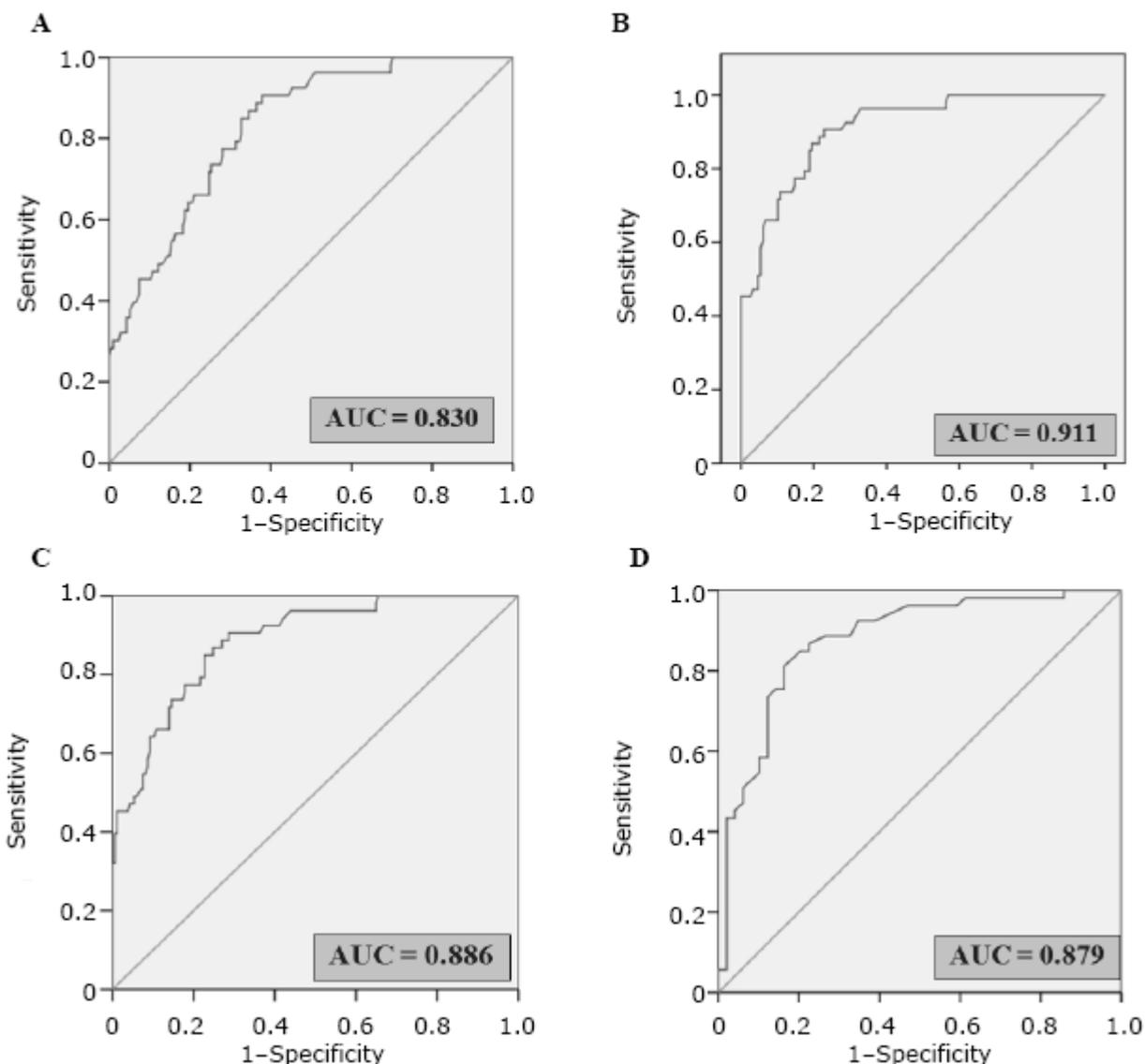


Figure 2. Receiver operating characteristic (ROC) analysis, the areas under the curve (AUC) and the performance of reticulocyte hemoglobin content (CHr) and mean corpuscular hemoglobin concentration (MCHC) in diagnosis of iron deficiency anemia (IDA). **A:** For the entire study population, the CHr cutoff of 24.6 pg showed 73.6% sensitivity, 74.8% specificity, 42.4% positive predictive value (PPV), 92.0% negative predictive value (NPV), and 74.9% accuracy. **B:** When the thalassemia group was excluded, the CHr cutoff of 27.2 pg showed 84.9% sensitivity, 81.2% specificity, 60.8% PPV, 93.4% NPV, and 81.7% accuracy. **C:** When patients with thalassemia disease was excluded, the CHr cutoff of 27.0 pg showed 84.9% sensitivity, 77.8% specificity, 52.3% PPV, 94.7% NPV, and 79.4% accuracy. **D:** ROC curve of the MCHC for differentiation of IDA and thalassemia trait: the MCHC cutoff of 31.6 g/dL showed 81.1% sensitivity, 83.7% specificity, 81.5% PPV, 81.3% NPV, and 81.4% accuracy.

trait. The laboratory normal range of MCHC is 33–37 g/dL. By contrast, a MCHC >31.6 g/dL suggests a diagnosis of thalassemia trait. The combination of CHr and MCHC can differentiate IDA from thalassemia better than using CHr alone (sensitivity 69.8%, specificity 88.1%, PPV 59.7%, and NPV 92.0%). In patients with microcytic anemia with normal reticulocytes, CHr ≤24.6 pg and MCHC ≤31.6

g/dL, IDA is very likely (specificity over 90%) as shown in **Figure 3**. In this group, therapeutic diagnosis with oral iron supplement, which should be considered especially for premenopausal women. If hemoglobin rises >1 g/dL in a month, IDA is suggested. If hemoglobin does not improve, iron study and hemoglobin analysis should be performed for a definite diagnosis.

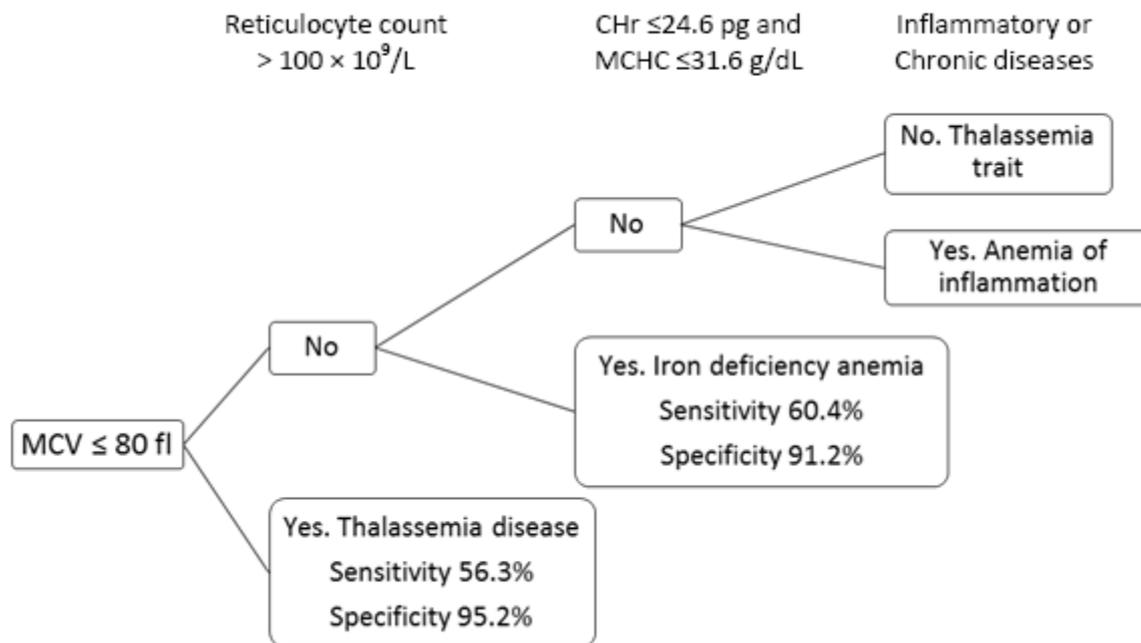


Figure 3. Algorithm proposed for diagnosis of microcytic anemia using complete blood and reticulocyte counts

In conclusion, a CBC and reticulocyte count with CHR are useful investigations for Thai patients with microcytic red blood cells. However, CHR cannot clearly differentiate IDA from thalassemia, unless combined with information about reticulocyte count.

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Conflict of interest statement

The authors have no conflicts of interest to declare. There was no commercial involvement in this project.

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