

The Paediatric Iron Deficiency Assessment with Reticulocyte Haemoglobin Equivalent (Ret-He) in Comparison with Biochemical Markers of Serum Ferritin and Transferrin Saturation

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ABSTRAK

Terdapat cabaran dalam diagnosis kekurangan zat besi anemia atau "iron deficiency anaemia" (IDA) pada masa kini. Hal ini kerana jika menggunakan kaedah konvensional, penyakit ini sering dikesan pada peringkat akhir. Kandungan retikulosit hemoglobin berguna untuk mengenal pasti IDA pada peringkat awal. Objektif kajian ini adalah untuk menilai retikulosit hemoglobin setara (Ret-He) dalam mendiagnosis IDA pada kanak-kanak dan membandingkannya dengan kaedah konvensional yang lain. Kajian prospektif ini dijalankan ke atas 120 orang pesakit pediatrik berusia 12 tahun dan ke bawah, yang hadir ke Hospital Sultanah Aminah Johor Bahru, Malaysia dengan kadar hemoglobin <12 g/dL. Ret-He dan serum besi, ferritin dan saturasi transferin telah diukur. Dengan menggunakan titik 'cut-off' 20% untuk saturasi transferin, 81 daripada 120 subjek (67.5%) didapati kekurangan zat besi. Berdasarkan diagnosis IDA, nilai cut-off untuk Ret-He apabila diuji menggunakan analisis keluk "Receiver Operating Characteristics" (ROC) adalah sebanyak 22.65 pg. Ret-He menunjukkan sensitiviti dan spesifisiti yang baik, di mana nilai masing-masing ialah 77.8% dan 66.7%. Jika dibandingkan dengan Ret-He, serum ferritin menunjukkan hanya 18.9% sensitiviti, namun, spesifisiti sebanyak 100% menunjukkan ianya berguna untuk ketetapan penyakit ini tetapi tidak sesuai bagi tujuan ujian saringan. Saturasi transferin menunjukkan sensitiviti dan spesifisiti yang baik, namun secara biologi ianya berubah-ubah dan tidak efektif sebagai alat

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ujian saringan. Kajian korelasi menunjukkan serum zat besi dan saturasi transferin mempunyai hubungan positif, signifikan dengan Ret-He ($r = 0,415$ hingga $0,518$), namun, hubungan di antara Ret-He dan serum feritin tidak signifikan ($r = 0,051$, $p = 0,578$). Kajian ini menunjukkan bahawa Ret-He pada titik cut-off 22.65 pg mempunyai sensitiviti yang lebih baik dan berpotensi di gunakan sebagai alat ujian saringan pada populasi pediatrik.

Kata kunci: kekurangan zat besi anemia, pediatrik, retikulosit hemoglobin setara

ABSTRACT

Diagnosis of iron deficiency anaemia (IDA) is a challenge as the conventional methods often diagnose the disease at the later stage. Haemoglobin content of reticulocytes is useful to identify IDA at earlier stage. The objective of this study was to evaluate reticulocyte-haemoglobin equivalent (Ret-He) in diagnosing IDA in children and to compare it with other conventional methods. This prospective study was conducted on 120 paediatric patients aged 12 years and below, who attended Hospital Sultanah Aminah Johor Bahru, Malaysia with haemoglobin <12 g/dL. Ret-He and serum iron, ferritin and transferrin saturation were measured. Using a cut-off point of 20% for transferrin saturation, 81 out of 120 subjects (67.5%) were found as iron deficient. Based on the diagnosis of IDA, cut-off value for Ret-He using the Receiver Operating Characteristics (ROC) curve analysis was found as 22.65 pg. Ret-He showed a good sensitivity and specificity of 77.8% and 66.7%, respectively. As compared with Ret-He, serum ferritin showed a sensitivity of only 18.9%. However, a good specificity of 100% suggest it is useful for ruling in the disease but not suitable for screening. Transferrin saturation showed a good sensitivity and specificity, but it is biologically variable and not cost effective as a screening tool. Correlation study showed serum iron and transferrin saturation have significant positive correlation with Ret-He ($r=0.415$ to 0.518). However, there was no correlation between Ret-He and serum ferritin ($r=0.051$, $p=0.578$). This study shows that Ret-He at a cut-off point of 22.65 pg has a better sensitivity and potentially be useful as a screening tool in the paediatric population.

Keyword: iron deficiency anaemia, paediatric, reticulocyte haemoglobin equivalent

INTRODUCTION

Iron deficiency anaemia (IDA) is one of the most common forms of nutritional anaemia, affecting around one-fourth of the world's women and children

with a global prevalence of 47% among children under 5 years of age. In Asia, the prevalence of anaemia in children under 5 is 47.7% with the highest prevalence in Africa at 64.6% and lowest in Europe at 16.7% (WHO

2008). In Malaysia, IDA has been reported as one of the most important micronutrient deficiencies for the past several decades. Nutritional anaemia surveys in the early 1990s highlighted the widespread problem of IDA among young children aged less than 7 years, ranged from 12% to 56% in Peninsular Malaysia (Tee et al. 1998). A survey by the Ministry of Health Malaysia supported by UNICEF in 1999-2000 reported that 18.3% of boys and 20.8% of girls below 5 years were anaemic with a haemoglobin concentration of less than 11 g/dl (Khor 2002). A recent survey on 544 Malaysian primary school children aged 7-12 years showed the prevalence of anaemia and iron deficiency were 4.0% and 5.2%, respectively (Nik Shanita et al. 2018).

Iron is an important micronutrient in the human body, constitutes many cellular proteins, enzymes and required for many metabolic processes, such as oxygen transport, electron transport, energy production, DNA synthesis, etc. The majority (95%) of iron comes from the breakdown of old red blood cells (RBC). During the intrauterine period, iron transportation from mother to baby takes place through the placenta. Healthy infants during first 5-6 months of life have enough body iron. However, reduction of iron stores at birth or during infancy can occur due to maternal iron deficiency, fetal-maternal hemorrhage or other perinatal hemorrhages, twin-twin transfusion syndrome, prematurity, and insufficient dietary intake during early infancy (Roganovi & Starinac 2018).

The negative impact of IDA in infancy on psychomotor development has been well documented (Walter 2004). Infants with IDA are reported to have poor neuromotor, behavioral and socio-emotional development (Lozoff 2011). During infancy, there is rapid brain development which needs iron-containing enzymes and hemoproteins for its proper development. Therefore, iron deficiency adversely affects the maturation of the brain disrupting neurophysiological mechanisms resulting in disturbance of regulation of emotion and attention (Lozoff 2011; East et al. 2018).

Taking into consideration that iron is the world's most common single-nutrient deficiency and its deleterious consequences on the developing child, it is important to minimise iron deficiency among children. Developing an effective approach for the diagnosis of iron deficiency in children is pertinent for the improvement of overall child health, in particular the neuro-developmental aspects. Iron deficiency anaemia is often diagnosed by complete blood count, which includes haemoglobin, haematocrit, mean corpuscular volume (MCV), red blood cell distribution width (RDW), and traditional biochemical markers of iron metabolism such as serum iron, transferrin saturation, and ferritin. Typically, these findings may not be evident enough until the iron-deficient erythropoiesis is advanced (Brugnara et al. 1999). Diagnosis of IDA by direct measurement of the mean hemoglobin content (pg) of reticulocytes in early stages of iron deficiency has been studied and found effective when

other traditional parameters are non-informative (Brugnara 2003; Brugnara et al. 2006). The reticulocytes are the immature red blood cells demonstrating the output of red blood cell proliferation recognisable in peripheral blood for just a few days of 1-1.5 before developing into mature RBC (Urrechaga et al. 2011). The mean cellular haemoglobin content of reticulocytes (CHR) is an important parameter for the detection of early iron-restricted erythropoiesis (Garzia et al. 2007). An equivalent parameter to CHR is the reticulocyte hemoglobin equivalent (Ret-He) that measures the amount of iron contained in the reticulocytes (Thomas et al. 2005). The Ret-He represents the measurement of the functional iron available for red cell production over the previous 2-3 days (Urrechaga et al. 2011). The use of these markers helps in the diagnosis of iron deficiency in infants and children, pregnant women, adult blood donors, geriatric patients and patients with chronic kidney disease undergoing hemodialysis as well as for monitoring patients' response to iron replacement therapy and detecting iron restricted erythropoiesis in patients receiving erythropoietin therapy (Peerschke et al. 2014). Thus, instead of using the biochemical parameters and measures of mature erythrocyte indices which are often detected at the later stage of the disease, haemoglobin content of reticulocytes can be used to identify the recent functional availability of iron in red cells. The objective of this present study was to evaluate the Ret-He marker reported by the Sysmex XE 5000, as a tool to rapidly diagnosing iron

deficiency in children and comparing it to other conventional parameters such as haemoglobin, serum iron, serum ferritin, and transferrin saturation.

MATERIALS AND METHODS

This prospective cross-sectional study was approved by the Research Ethics Committee of University Kebangsaan Malaysia (UKM) Medical Centre. The study was conducted at the Haematology Unit, Chemical Pathology Unit, and Paediatric Department of Hospital Sultanah Aminah Johor Bahru (HSAJB), Malaysia. This study included the paediatric patients aged 12 years old and below, who attend the paediatric specialist clinic and paediatric ward in HSAJB with the hypochromic microcytic anaemia. In this present study, criteria for diagnosing anaemia were defined as haemoglobin level of less than 12 g/dL (WHO 2008). The exclusion criteria included neonates, children having a recent blood transfusion in the past 3 months, and children with known haematological malignancies as well as those receiving myelosuppressive treatments. In this present study, a total of 120 patients were included throughout one year after considering the inclusion and exclusion criteria. For sample collection, parents of the participants were counseled, and written informed consent was obtained before blood taking. They were interviewed using a questionnaire form to ensure they fulfilled the inclusion and exclusion criteria. A total of 7 ml of peripheral blood was collected where 1 ml was taken in an ethylene diamine

Table 1: The demographic data of the participants (n=120)

Variables		Number (%)	Mean \pm s.d.
Gender	Male	74 (61.7)	
	Female	46 (38.3)	
Ethnicity	Malay	104 (86.7)	
	Chinese	7 (5.8)	
	Indian	1 (0.8)	
	Other	8 (6.7)	
Age (months)			25 (\pm 34.28).

tetra-acetic acid (EDTA) tube and 3 ml each was taken in two plain tubes.

The EDTA tube was used for haematological tests of full blood count (FBC) including haemoglobin, mean cell volume (MCV), mean cell haemoglobin (MCH) and Ret-He marker by Sysmex XE 5000 analyser. Ret-He was analysed by fluorescent flow cytometry technique, using a polymethine dye and using forward light scatter intensity derived from mature erythrocytes and reticulocytes. The plain tubes were used for biochemical tests of serum iron, and transferrin saturation using a chemistry analyser, Roche/Hitachi Modular System COBAS and for serum ferritin by Roche/Hitachi Modular analytics E2170. In this present study, IDA was defined by transferrin saturation of less than 20% and/or serum ferritin less than 12 g/L.

The results were collected and analysed using the SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). The demographic data of the study population was obtained. The values for Ret-He and the other biochemical markers were expressed as a mean and a range. The optimal cut-off value for Ret-He was derived using

the Receiver Operating Characteristics (ROC) curve analysis, based on the diagnosis of IDA (transferrin saturation <20% and/or serum ferritin <12 g/L). Pearson correlation test was carried out to detect the correlation between Ret-He value with the conventional biochemical test of serum iron, serum ferritin, and transferrin saturation in detecting iron-deficiency anaemia. The diagnostic performance of Ret-He, transferrin saturation, and serum ferritin in predicting iron deficiency based on the above-mentioned definition was estimated by their sensitivity, specificity, negative predictive value, and positive predictive value. A p-value of <0.05 (95% confidence interval) was considered statistically significant.

RESULTS

Table 1 showed the demographic profile of the participants. Among the total 120 paediatric patients, male patients predominated compared to female patients (61.7% versus 38.3%). The ethnicity and gender distribution of patients showed Malays and male participants predominate, which may be explained by the Malaysian population statistics. The age

Table 2: Results of the bio-chemical parameters including Ret He (n= 120)

Parameters	Mean \pm s.d.
Haemoglobin, (g/l)	9.08 \pm 1.42
MCV (fl)	67.18 \pm 8.58
MCH (pg)	21.38 \pm 3.61
Ret-He (pg)	21.01 \pm 4.88
Serum ferritin (microg/L)	208.50 \pm 629.95
Iron, (micromol/L)	9.00 \pm 6.99
Transferrin saturation (%)	18.00 \pm 15.89

distribution of the children ranges from 1 month up to 12 years of age, with a mean age of 25 months (\pm 34.28). Table 2 summarises the mean results of the Ret-He and other biochemical parameters of the study subjects.

Table 3 which shows based on serum ferritin cut-off point of <12 g/L, only 14.2% of patients were diagnosed as having IDA while based on transferrin saturation cut of point of <20%, 67.5% of patients were diagnosed as having IDA. Table 4 show the correlation between Ret-He value with serum iron, ferritin and transferrin saturation. Based on the Pearson correlation test, it was found that haemoglobin level, serum iron and transferrin saturation had a significant positive correlation ($p=0.001$, r =range from 0.415 to 0.518)

Table 4: Correlation between Ret-He value with conventional biochemical test of serum iron, serum ferritin, and transferrin saturation

Biochemical test	Ret-He	
	r	p-value
Serum iron	0.415	0.001*
Ferritin	0.051	0.578
Transferrin saturation	0.518	0.001*

r: Pearson correlation coefficient

Table 3: IDA diagnosis based on serum ferritin and transferrin saturation (n=120)

	IDA		Non IDA	
	Number	%	Number	%
Serum ferritin	17	14.2	103	85.8
Transferrin saturation	81	67.5	39	32.5

with Ret-He. There was no correlation between Ret-He and serum ferritin ($r=0.051$, $p=0.578$).

Figure 1 showed the ROC analysis results, which is a method to evaluate the usefulness of the test by calculating sensitivity and specificity. The results of this analysis can be shown as the curve in that the sensitivities were plotted on the y-axis against the value of 1-specificity on the x-axis. If the area under the ROC curve (AUC) is almost 1, the accuracy of the test should be considered excellent. In this study, AUC was 0.802, reflecting that Ret-He facilitates the diagnosis of ID with high

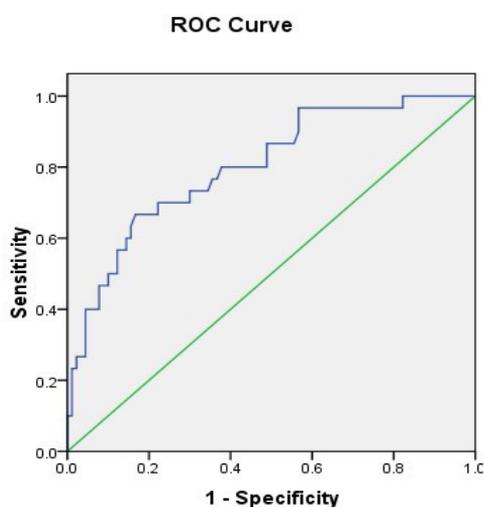


Figure 1: Receiver-operating characteristic curve (ROC) analysis to assess reticulocyte haemoglobin equivalent (RET-He) in the diagnosis of iron deficiency.

Table 5: Sensitivity, specificity, positive predictive value and negative predictive value of Ret-He, serum ferritin and transferrin saturation towards diagnosis of IDA

Markers with	Cut off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Ret-He	22.65 pg	77.8	66.7	87.5	50.0
Serum ferritin	12 g/L	18.9	100.0	100.0	29.1
Transferrin saturation	20%	90.0	100.0	100.0	76.9

PPV: Positive Predictive Value; NPV: Negative Predictive Value

accuracy. The optimal Ret-He cut-off point in detection of iron deficiency was established as 22.65 pg, 95% Confidence Interval: 0.711-0.892, p-value: 0.001*.

Table 5 showed the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Ret-He, serum ferritin and transferrin saturation in diagnosing IDA. The Ret-He with the suggested cut-off point (22.65 pg) has a sensitivity of 77.8% and specificity of 66.7% in the diagnosis of IDA. Transferrin saturation has the highest sensitivity and specificity while serum ferritin has the lowest sensitivity with 100% specificity.

DISCUSSION

The blood concentration of reticulocytes represents a quantitative measure of erythropoiesis and reticulocyte haemoglobin parameters provide real-time information about the amount of iron contained in the red cell precursors haemoglobin. Thus, it gives a direct estimation of the functional availability of the iron in the red cell and the quality of the erythropoiesis (Brugnara et al. 2006). Serum ferritin, although the most commonly used indicator for iron status, has its own

recognised limitations associated with concomitant inflammation and other acute conditions (Daru et al. 2017). A study on children found that ferritin had little or no diagnostic value in children. They revealed that children with a mean age of 2 years showed no significant difference in plasma ferritin levels between non-iron deficient and iron-deficient individuals (Brugnara et al. 1999). In this present study, based on a cut-off value of <12 g/L for serum ferritin, only 14.2% of the population was identified as iron deficient; while based on the cut-off value of 20% for transferrin saturation, 67.5% of the study population was found iron deficient. This is because serum ferritin is an acute phase reactant. Therefore, presence of inflammation or any other acute condition such as malignancy, liver disease, metabolic conditions, or rarely haematological conditions can cause a higher serum ferritin level even though the iron stores are low and there is no raised transferrin saturation. Thus, in patients exposed to such conditions, it is difficult to interpret the true concentration of ferritin (Thurnham et al. 2010; Cullis et al. 2018). As such, it is unreliable to measure serum ferritin as a marker of iron deficiency in children.

The optimal Ret-He threshold

established in this study for the detection of IDA in children based on the ROC curve was 22.65 pg with an AUC of 0.802. This AUC level reflects that Ret-He facilitates the diagnosis of ID with high accuracy. The optimal cut-off value for Ret-He obtained in this study was found to be lower but not far from the manufacturer-established cut-off point of 25 pg with the sensitivity of 76% and specificity of 81%. The manufacturer established a cut-off point of 25 pg based on a study conducted on 504 individuals with a mix of healthy individuals as well as patients of iron deficiency in less than one-third of the population (Canals et al. 2005). However, the age group of the study subjects was not mentioned and thereby inclusion of paediatric population cannot confirm (Canals et al. 2005). To the best of our knowledge, there is no study establishing the reference range of Ret-He for children in Malaysia to date. One large-scale study performed on healthy adults in Malaysia established a reference range of 30.7-38.9 pg (Ambayya et al. 2014). Another, recent study on adult patients and control groups determines the Ret-He value as 16.50 ± 4.90 pg and 34.80 ± 1.97 pg, respectively (Bakri et al. 2020). A study in Indonesia among healthy children showed the Ret-He cut-off point of ≤ 27.8 pg/L (Rungngu et al. 2016). The lower cut-off obtained in our study may be associated with the inclusion criteria whereby we took only children with the hypochromic microcytic anaemia. The Ret-He is found to be lower in IDA patients compared to the non-IDA group and the normal individuals (Uçar et al.

2019).

The established cut-off point of 22.65 for Ret-He used in the present study showed good sensitivity and specificity. Transferrin saturation, although having good sensitivity and specificity, is biologically variable and is not cost-effective as a screening tool. Serum ferritin in contrast was found to have a sensitivity of only 18.9% with a poor NPV of 29.1%. Ferritin however had a good specificity of 100%. Ferritin is useful for ruling in the disease, but not as a screening tool due to its poor sensitivity.

This study shows that Ret-He has a better sensitivity than the traditional biomarker serum ferritin and could potentially be useful as a screening tool. With a good PPV of 87.5%, Ret-He is a potential screening tool in asymptomatic children as well. However, as it has a low NPV of 50.0%, we are unable to rule out that all children above the cut-off point do not have iron deficiency. A correlation study showed Ret-He has a significant correlation with serum iron, haemoglobin level and transferrin saturation. The lack of correlation between Ret-He and serum ferritin could be due to the inclusion of patients who did not reflect the true ferritin level due to underlying inflammation or other acute condition.

As Ret-He uses a method that is based solely on haematological parameters obtained by an FBC and reticulocyte analysis, it is advantageous due to its direct assessment of iron metabolism as well as its potential cost savings. Besides that, Ret-He can be easily incorporated into the FBC panel

and no extra blood volume is needed, which is welcome in the paediatric setting. The measurement time of less than 2 minutes was very minimal, with automatic processing along with complete blood count, and does not require any additional reagents (Toki et al. 2017). This is potentially a very useful tool for early detection and treatment of iron deficiency in children since IDA has far-reaching implications in a child's development.

CONCLUSION

Ret-He is found to be useful in determining an iron deficiency in paediatric patients. In this study, Ret-He showed a cut-off value of 22.65 pg with a good sensitivity of 77.8% and a specificity of 66.7%. We suggest Ret-He which has good sensitivity, is cost-effective while using an automated method with minimal blood sample requirement may be utilised as a screening tool for the diagnosis of IDA in the paediatric population.

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