Original Research Article

Utility of reticulocyte hemoglobin in diagnosing latent iron deficiency and iron deficiency anemia

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ABSTRACT

Background: Iron deficiency is the most common cause of anemia and studies have shown poor cognition, psychomotor and social/emotional development in children who are deficient in iron, even with normal hemoglobin levels, the so-called Latent phase of Iron deficiency. It is therefore crucial to identify LID, as well as IDA at the earliest stage, in order to initiate treatment. Many tests like serum ferritin and soluble transferring receptor(sTfR) have been described collectively as a panel to detect iron deficiency; however no single test is specific enough to be used independently. Also during treatment it takes weeks to observe changes in Hb, hematocrit or RBC indices, hence the need for a more sensitive and reliable test. Objective was to evaluate effectiveness of CHr in diagnosing LID and IDA.

Methods: Samples were collected from 180 children, clinically suspected to be anemic. Complete hemogram and Iron profile were measured. Three groups were defined, LID (Tfsat <20%, Hb >11g/dL; n=52), IDA (Tfsat <20%, Hb <11g/dL; n=84) and controls (Tfsat >20%, Hb >11g/dL; n=44). The mean values of RBC indices, Iron profile and CHr was compared across the groups. A cut off value of <26 pg CHr was taken to represent Iron deficiency state.

Results: Comparison between anemic group and control found that all RBC indices were found to be significantly lower including Reticulocyte hemoglobin. All of the variables in anemic group were lower compared to latent iron deficient group except MCHC and reticulocyte count. CHr was found to be statistically lower in LID and IDA group in comparison to control group.

Conclusions: CHr can be used as a valuable indicator in diagnosis as well as follow-up of LID and IDA, which is easily available and inexpensive.

Keywords: Diagnosis of iron deficiency, Iron deficiency anemia, Iron deficiency state, Latent iron deficiency, Reticulocyte hemoglobin, Serum ferritin

INTRODUCTION

Iron deficiency is one of the most common nutritional deficiencies and leading cause of anemia especially in growing children. As per WHO report on global prevalence of anemia, 273.2 million (42.6%) people were anemic. In India, 59% of children aged 6-59 months were anemic, 42% (38 to 46%) of which is attributed to iron deficiency, the single most common nutrient deficiency. Iron deficiency exists in a state of continuum ranging from Latent Iron Deficiency (LID) to Iron Deficiency Anemia (IDA). A child maybe in a state of LID utilizing iron stores for a prolonged period maintaining Hemoglobin levels within normal limits hence maybe clinically unrecognized. Both LID and IDA occurring in the first two years of life have been associated with poor cognitive and psychomotor development. Long-term follow-up of infants with moderate iron deficiency by
Lozoff et al demonstrated the lower cognitive functioning even at age 19 despite correction of anemia, emphasizing the irreversible nature of the cognitive impairment. Therefore it is imperative to diagnose LID early, before the development of anemia for timely intervention, in order to prevent long-term sequelae.

Many tests have been described collectively as a panel to detect a state of Iron deficiency however there is a lack of single simple and inexpensive test which can effectively detect both LID and IDA. Bone marrow aspirates for stainable iron is considered the most reliable for diagnosis of iron deficiency; latent and anemic phase, however because of its invasive nature it is less commonly used.

Iron deficiency is diagnosed by a combination of various parameters, such as serum iron, serum ferritin, transferrin saturation (Tfsat), Total Iron Binding Capacity (TIBC), soluble transferrin receptor, zinc protoporphyrin/haem ratio. All these parameters measure different aspects of iron physiology, have individual limitations, are confounded by developmental changes and is cost inefficient especially in developing countries. Serum ferritin is most specific for iron status however as it is also an acute phase reactant hence increases in anemia secondary to systemic disorder despite marked iron deficiency. Transferrin Saturation is known to fluctuate diurnally due to diurnal variation of serum iron, and serum iron levels decrease with infection, inflammation, and malignancy and increase with liver disease.

Hemoglobin is most commonly used as screening test for iron deficiency, however it lacks specificity as several factors can affect hemoglobin. Also, Hemoglobin is derived from the entire red blood cell population with a lifespan of approximately 120 days, delaying the detection of Iron deficiency. Since reticulocytes persist in the circulation for only 24-48 h, measurement of the hemoglobin content of the reticulocytes (CHR) can identify early stages of iron deficiency, when other traditional biochemical parameters are non-informative. Modern day analyzers provide this information as part of the hemogram for no additional blood sample or cost. Hence, we studied the effectiveness of using CHR to diagnose latent iron deficiency and iron deficiency anemia in children.

**METHODS**

The study was conducted in a tertiary care hospital between March 2018 and November 2018. A total of 274 subjects were recruited into the study. Children aged between 6 months and 18 years were included in the study, among which those receiving iron supplements or blood transfusion in the preceding six months, known cases of anemia secondary to non iron deficient causes like Thalassemia (MCV>100 fl), those with signs of infection/inflammation in the preceding one month were excluded as per exclusion criteria. After taking informed consent from parents/guardians, detailed history was obtained and physical examination, anthropometry was conducted.

Venous blood samples were obtained for laboratory tests. 2ml of EDTA sample for hematological parameters and 2.5 ml of serum sample for biochemical analysis were collected and processed within 6 hrs of collection. The following laboratory tests were performed: hematological parameters included a complete blood count (CBC) with RBC indices and Reticulocyte Hemoglobin (CHR). Biochemical parameters included serum iron, plasma ferritin, Transferrin Saturation (Tfsat), Total Iron Binding Capacity (TIBC).

Hematological parameters were measured using automated fluorescence differential flow cytometer (Sysmex XE-2100). The CHR is determined from measurements of light scatter at 2 different angles after isovolumetric spining of oxazine 750 stained reticulocytes. From the amount of light scattered at the 2 different angles, the hemoglobin concentration and cellular volume of individual reticulocytes are independently determined. Ferritin was measured in serum by using an Access Immunoanalyzer. The percentage of transferrin saturation was calculated from serum iron, and the serum total iron binding capacity.

Three groups defined were, controls (Tfsat >20%, Hemoglobin>11 g/dl), Latent Iron Deficiency (Tfsat <20%, Hemoglobin >11 g/dl) and iron deficiency anemia (Tfsat <20%, Hemoglobin <11 g/dl). Various hematological and biochemical parameters were compared across these groups.

**Statistical analysis**

For all patients two clinical outcomes were evaluated: Latent Iron Deficiency and Iron Deficiency anemia. Descriptive statistics were reported using Mean and Standard Deviation for Normal Distribution Continuous variables. For Non-normal distribution, Median and IQR analysis of Variance was used to compare the clinical parameters across the three groups.

The Kolmogrov-Smirnov goodness-of-fit revealed no significant departures from normality for any variables. Post hoc analysis was done using Bon-Ferloni correction. Correlational coefficient was calculated to assess the relationship between CHR with other biochemical parameters.

Receiver Operating Characteristic was used to illustrate the diagnostic performance of CHR and to establish a cut-off based on optimal combination of sensitivity and specificity. To validate the CHR cut-off, the patient population was divided into healthy and abnormal subgroups and all the test parameters were compared. All analysis was done using SDSS version 14.2, p value <0.05 was taken as significant.
RESULTS

Table 1: Comparison of the study variables between the three groups.

| Parameters         | Control                     | Iron deficiency            | Latent Iron Deficiency (LID) | Iron Deficiency Anemia (IDA) |
|--------------------|-----------------------------|-----------------------------|------------------------------|-----------------------------|
|                    | Mean (SD)                   | Mean (SD)                   | Mean (SD)                   | Mean (SD)                   | p value | p value | p value | p value |
| PCV*               | 39.2(3.8)                   | <0.0001                     | 34.2(7.4)                   | <0.0001                     | 0.173   | 28.3(3.6) | <0.0001 | 0.070 |
| MCV*               | 82.5(3.4)                   | <0.0001                     | 77.8(3.1)                   | <0.0001                     | 69.7(2.8) | <0.0001 | 65.1(17.9) | 0.149 |
| MCH*               | 30.7(2.6)                   | <0.0001                     | 26.8(1.6)                   | <0.0001                     | 21.4(2.6) | <0.0001 | 19.6(7.9) | 0.112 |
| RDW*               | 12.3(1.5)                   | <0.0001                     | 13.4(1.1)                   | <0.0001                     | 15.2(1.1) | <0.0001 | 19.0(4.3) | <0.0001 |
| Retic%             | 1.25(0.53)                  | <0.0001                     | 1.29(1.08)                  | <0.0001                     | 1.19(0.43) | <0.0001 | 1.19(0.43) | <0.0001 |
| Iron*              | 114.1(36.6)                 | 0.070                       | 65.1(17.9)                  | 0.149                       | 19.6(7.9) | 0.112 |
| TIBC*              | 332.4(55.9)                 | <0.0001                     | 388.9(49.6)                 | 0.547                       | 449.4(56.4) | 0.348 |
| TSat*              | 27.7(5.0)                   | <0.0001                     | 13.3(4.7)                   | 0.385                       | 14.3(1.4) | <0.0001 |
| Ferritin*          | 177.8(108.5)                | <0.0001                     | 34.2(2.8)                   | 0.517                       | 21.0(7.4) | 0.985 |
| CHr*               | 30.4(2.49)                  | <0.0001                     | 25.9(1.42)                  | <0.0001                     | 23.5(1.58) | <0.0001 |

*significant difference between the groups in post hoc analysis
#There was no significant difference between iron deficiency and iron deficiency anemia

Table 2: Comparison haematological and biochemical parameters across CHr.

| CHr     | CHr<26.3pg | CHr<26.3pg | p value |
|---------|------------|------------|---------|
|         | Mean       | SD         | Mean    | SD     |         |
| Hemoglobin* | 9.46        | 1.55       | 12.33   | 0.81   | <0.0001 |
| PCV*    | 29.77      | 5.34       | 38.6    | 4.76   | <0.0001 |
| MCV*    | 71.58      | 4.28       | 82.17   | 3.47   | <0.0001 |
| MCH*    | 22.65      | 3.3        | 30.37   | 2.61   | <0.0001 |
| RDW*    | 14.81      | 1.29       | 12.3    | 1.52   | <0.0001 |
| RETIC   | 1.27       | 0.75       | 1.21    | 0.51   | 0.4274  |
| IRON*   | 22.00      | (16.00,42.00) | 112.50 | (85.00,139.00) | <0.0001 |
| TIBC*   | 433.76     | 62.74      | 339.04  | 56.89  | <0.0001 |
| TST*    | 14.30      | 3.06       | 25.94   | 6.90   | <0.0001 |
| Ferritin* | 25.25       | (16.50,31.25) | 112.40 | (67.00,249.00) | <0.0001 |

* statistically significant difference between the hematological parameters and CHr.
# median and inter quartile range.

Figure 1: Receiver operating characteristic for reticulocyte hemoglobin.

Table 1 represents the comparison of study variables between the three groups. Hematological parameters like PCV, MCV, MCH, reticulocyte percentage and RDW showed significant difference between the three groups. Biochemical parameters showed varying results between the groups. Mean serum iron level was much higher in control group compared to the LID and IDA, however there was no statistically significant variation between the groups (Table 2).

Total Iron Binding Capacity was significantly lower in the control group than the iron deficiency group, however it showed no significant variation between the LID and the IDA groups. Transferrin Saturation and serum ferritin levels showed no significant difference between Latent Iron Deficiency and Iron Deficiency Anemia group, although they were successful in differentiating iron deficiency from control group. CHr showed variation...
between all three groups which was statistically significant.

Through the ROC analysis we arrived at an optimal cutoff point for CHr, as 26.3 pg with a sensitivity of 89.05% and specificity of 99.22%, with an area under the curve of 0.978 and a maximal Youden index of 0.8828 (Figure 1).

We compared our study subjects by classifying them into two groups: Group 1 with CHr <26.3 pg and Group 2, with CHr ≥26.3 pg. There was significant difference noted among hematological parameters and biochemical parameters between the two groups. Our ROC analysis of CHr, Serum Ferritin and Tfsat showed that all three investigations had comparable sensitivity and specificity in identifying iron deficiency.

**DISCUSSION**

The relatively newer parameter was evaluated, reticulocyte hemoglobin (CHr) for diagnosis of Latent as well apparent iron deficiency anemia. Given the long lifespan of RBCs, it takes considerable time to reflect factors affecting erythropoiesis in the peripheral blood. Reticulocytes which are immature RBCs circulating in the peripheral blood have a short lifespan of 1-2 days and therefore are a more reliable indicator reflecting the bone marrow activity. Reticulocyte hemoglobin (CHr) is a measurement of hemoglobin inside the reticulocyte. It correlates directly with the functional availability of iron in the marrow, especially in the preceding 48 hours.

The diagnosis of iron deficiency anemia lacks a simple, single, reliable and cost-effective test and usually involves a panel of various hematological and biochemical parameters. These parameters have their own limitations, and therefore their interpretation is ideally done as a whole rather than individual test. Also it is imperative to diagnose iron deficiency status before the development of anemia especially in pediatric age, as it can irreversibly affect cognition and psychomotor development.

Authors evaluated the utility of various hematological and biochemical parameters in the diagnosis of Iron Deficiency State. Hematological parameters like PCV, MCV, MCH, Reticulocyte percentage and RDW showed significant variation between all three groups. Transferrin Saturation and serum ferritin levels, which are believed to be most reliable of the biochemical parameters were only able to differentiate iron deficiency group from healthy individuals and failed to differentiate Latent Iron deficiency from Iron deficiency anemia. Overall CHr correlated well with all the hematological and biochemical parameters. We arrived at a cut-off point of 26.3 pg for CHr which was highly sensitive and specific, making CHr the most valuable screening tool for identifying iron deficiency with or without anaemia. Brugnara et al, studied various hematological and biochemical parameters including Transferrin receptor (Tfr) and Zinc Prottoporphyrin (ZPP), and established that CHr was the strongest predictor among all and they used a cutoff point of 26 pg with a sensitivity and specificity of 83% and 75% respectively. Bakr et al, found that CHr performed in conjunction with complete blood evaluation provided an alternative for diagnosis of Iron deficiency state and used the cutoff point of 26 pg based on Brugnara et al. other studies in adults like Cai et al, used a cutoff point of 27.2 pg based on their data, with a sensitivity of 87.5% and a specificity of 92.9%; Mast et al, reported that CHr of <28 pg had an optimal sensitivity (74%) and specificity (73%) for diagnosis of iron deficiency with reference to the gold standard bone marrow aspirate. They also found that CHr has a high sensitivity and specificity in the diagnosis of IDA, and its comprehensive diagnostic efficacy is better than other traditional indicators such as Transferrin Saturation and sTfR. CHr is much more cost efficient compared to doing current diagnostic panel; as it can be obtained as part of the complete blood count in most of the modern-day analysers, at no extra cost or additional blood sample. CHr can also be used to evaluate response to Iron therapy at the earliest in comparison to other hematological parameters.

Like any other test CHr also has few limitations like ability to differentiate among other microcytic hypochromic conditions like thalassemia. These conditions can be distinguished by using a combination of CHr and the ratio of microcytic to hypochromic red blood cells. The diagnostic value of CHr in the setting of anemia secondary to chronic disorders needs to be established. Further studies are needed to validate an optimal cut-off point with respect to different age groups and gender.

**CONCLUSION**

CHr is a valuable screening tool in identifying Latent Iron Deficiency phase which is simple, cost effective and requires no additional sample.

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**Conflict of interest:** None declared

**Ethical approval:** The study was approved by the Institutional Ethics Committee

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