EFFICACY OF CENTRIFUGATION TO REDUCE THE INTERFERENCES THAT OCCUR DUE TO HIGH LEUKOCYTE COUNT IN HAEMOGLOBIN ESTIMATION BY CYANMETHAEMOGLOBIN METHOD

A.K.L.M. Perera [*] ***and K.G. Jayasekara***
Department of Medical Laboratory Science, University of Ruhuna, Sri Lanka

Haemoglobin (Hb) level in full blood count (FBC) test is a crucial parameter to assess iron status and prevalence of anaemia in a population. Cyanmethaemoglobin method is the gold standard method, utilizing photometry for accurate Hb estimation. This method involves haemolysis of red blood cells (RBC) forming Hb complexes measured by spectrophotometer. Leukocytes which remain unlyzed in this process may cause turbidity leading to falsely elevated Hb levels. The primary goal of this study was to assess the effectiveness of centrifugation in minimizing interferences from high leukocyte counts in Hb estimation by the cyanmethaemoglobin method. Specific objectives included evaluating the relationship between leukocyte count and falsely elevated Hb values and determining a cut-off value for the lowest leukocyte number that causes falsely high Hb value.

The samples were mixed with Drabkin's reagent (DAYTONA private limited, Sri Lanka) and allowed to convert Hb into cyanmethaemoglobin. Absorbance of the reaction mixture was determined with and without centrifugation. Hb values were read using a standard curve. Differences in Hb values between centrifuged and non-centrifuged samples (delta Hb) were compared and statistically analysed using the Statistical Package for the Social Sciences version 23 using paired t-test, correlation and coefficient tests. Leukocyte counts of the samples were determined using automated haematology analyser (Sysmex corporation, Japan). A total of 84 samples were analysed in this study.

Mean delta Hb values varied across leukocyte count groups. Mean delta Hb (g/dL) values were 0.698± 0.28, 0.71± 0.35, 1.04± 0.22, 1.13± 0.30, 1.14± 0.32, 1.21± 0.3, 1.52± 0.28, 1.81± 0.17 with an increasing number of leukocytes (cells/µL) in a sequential group 0 – 4,000, >4,000 -10,000, >10,000 -30,000, >30,000 – 50,000, >50,000 – 70,000, >70,000 – 90,000, >90,000 – 110,000, >110,000. When the leukocyte number increased, mean delta Hb increased. The lowest number of leukocytes causing falsely high Hb was 37,547 cells/µL. Overall, there was a discernible positive correlation (0.677) between delta Hb and leukocyte count.

Centrifugation could be used in cyanmethaemoglobin method to reduce the interferences of high leukocyte count in Hb measurement.

Keywords: centrifugation, cyanmethaemoglobin method, high leukocyte count interferences

[*]*Corresponding Author: amarasinghelakshani@gmail.com*

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A.K.L.M. Perera and K.G. Jayasekara***
Department of Medical Laboratory Science, University of Ruhuna, Sri Lanka

INTRODUCTION

Haemoglobin (Hb) level is one of the important parameters included in the full blood count (FBC) test that is used to evaluate the iron status and anaemia prevalence in a population. The main function of Hb is to transport oxygen from lungs to the tissue where oxygen is utilized for metabolism mainly to facilitate oxidative phosphorylation in mitochondria (Jain, 2020). Low levels of Hb and red blood cells (RBC) cause anaemic conditions in humans.
In order to detect any abnormal level of Hb in the blood, there is a requirement of regular blood test. There are various World Health Organization (WHO)-approved clinical methods for the estimation of Hb levels in the blood in pathology laboratory (Kharkar & Ratnaparkhe, 2017). Among these methods, the direct cyanmethaemoglobin method is the gold standard method for determining the Hb concentration of blood (Srivastava et al., 2014). Photometric cyanmethaemoglobin method is based on the principle that drabkin's reagent reacts with Hb in blood to form cyanmethaemoglobin and the developed colour is measured by photometry at 540nm (Jain, 2020).

Since, this is a spectrophotometric method, turbidity that occurs due to high leukocyte counts, it can affect the actual Hb value measurements (Henry et al., 2022). In the cyanmethaemoglobin method, high leukocyte counts can disrupt accurate Hb level readings due to their interference with light absorption (Rahman, 2004). While the normal leukocyte level does not significantly affect optical density, very high counts can cause turbidity, scattering or blocking of light beams leading to falsely elevated Hb readings (Sulamit et al., 2017). This interference occurs because leukocytes are not lysed or removed by chemicals in this method, affecting the accuracy of Hb examination.

The main objective of this research was to determine the efficacy of centrifugation in reducing the interferences of high leukocyte count on Hb estimation by the cyanmethaemoglobin method. Specific objectives were included to determine the association between leukocyte count and the error that occur in Hb values and to determine a cut off value for the lowest leukocyte number that cause falsely high Hb value in blood with a high leukocyte count.

METHODOLOGY

The study was carried out on the 84 retained Tri potassium ethylenediaminetetraacetic acid (K_3EDTA) blood samples received at the Haematology laboratory at Teaching Hospital, Karapitiya (THK) for FBC testing including Hb estimation from September 2023 to October 2023. To meet the inclusion criteria, K_3EDTA anticoagulated blood samples, encompassing a range of leukocyte counts were selected. Specifically, the samples included those with low leukocyte counts (ranging from 0 to 4,000 cells/µl), normal leukocyte counts (ranging from >4,000 to 10,000 cells/µl) and leukocyte counts exceeding the upper reference value (more than 10,000 cells/µl). Additionally, the samples covered variations in Hb values, including normal, low and high values as well as variations in platelet counts ranging from low to normal.

The automated haematology analyzers, Mindray BC 6800 plus (Shenzhen Mindray Biomedical Co.Ltd, China) and Sysmex XN- 1000 (Sysmex corporation, Japan) were used to

obtain the leukocyte count and Hb levels for each selected samples. Before examining the blood samples, those were thoroughly mixed using the inversion technique. Hb analysis was carried out as followed. Briefly, one in 250 dilution of the blood was created by adding 20 µL of the well-mixed blood to 5 mL of Drabkin's solution. The tube containing the solution was mixed well by vortex mixture and the samples were allowed to stand at room temperature for at least 10 minutes to ensure the conversion of Hb to haemoglobincyanide. The entire volume of blood was then divided into two equal parts and placed in separate test tubes. One tube was subjected to centrifugation at 3000 rpm for 15 minutes (cells and plasma separation speed recommended by WHO, 2002). The supernatant was extracted by the micropipette. The Drabkin's solution was used as a blank. The absorbance of the supernatant post-centrifugation was measured in comparison to the blank solution. Finally, the absorbance of the non- centrifuged sample was determined. All the absorbances were measured in both the non-centrifuged and centrifuged samples' supernatants simultaneously at wavelength of 540 nm. Subsequently, the Hb value was read via Hb standard curve. Finally, the Delta Hb values were calculated for each sample and recorded.

Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) software (version 23, IBM, 2021). A paired t-test was used to assess the significance of differences between two related groups. In this study, the observations were paired or matched before and after a centrifugation. The paired t-test evaluates whether the mean difference between paired observations is statistically different from zero. Correlation and coefficient statistical analysis was used to determine the association between leukocyte count and the delta Hb values and to determine the cut off value for the lowest leukocyte number that cause falsely high Hb value in blood with high leukocyte count.

RESULTS AND DISCUSSION

Mean delta Hb values for different leukocyte ranges were calculated by using Hb values before and after centrifugation. Calculated standard deviations were concluded in Table1.

Table 1: Leukocyte count ranges, mean delta Hb values and Standard deviations

Leukocyte count range (cells/µL)	Mean Delta Hb (g/dL)	Standard deviation
0 – 4,000	0.698	0.28
>4,000 -10,000	0.71	0.35
>10,000 -30,000	1.04	0.22
>30,000 – 50,000	1.13	0.30
>50,000 – 70,000	1.14	0.32
>70,000 – 90,000	1.21	0.3
>90,000 – 110,000	1.52	0.28
>110,000	1.81	0.17

The Hb values from selected samples were measured using the cyanmethaemoglobin method both before and after centrifugation, and the paired t-test was applied to each leukocyte range individually. The analysis sought to identify significant differences in Hb values before and after centrifugation. The null hypothesis (H0) posited that there was no difference in Hb values before and after centrifugation ($\mu=0$), while the alternative hypothesis (Ha) suggested that a difference exists ($\mu\neq0$). Paired t test was used to analyze the data. The results are shown

below.

Table 2: Calculated p value with their leukocyte count ranges

Leukocyte range (cells/µL)	**Significant level (p value)**
0 -4,000	0.00 (0.000003)
>4,000 – 10,000	0.00 (0.000008)
>10,000- 30,000	0.00 (2.5602E- 13)
>30,000 – 50,000	0.00 (8.3942E- 10)
>50,000 – 70,000	0.00 (0.000314)
>70,000– 90,000	0.00 (9.9772E- 8)
>90,000 – 110,000	0.00 (0.001587)
More than 110,000	0.000018

The p-value for all leukocyte ranges was found to be less than the significant value of 0.05 (in 95% confidence interval). Consequently, it can confidently reject the null hypothesis and accept the alternative hypothesis. This analysis indicated that centrifugation enhances the accuracy of Hb estimation in both samples with high leukocyte counts and those without high leukocyte counts.

Pearson correlation coefficient was applied to assess the relationship between leukocyte count and the occurrence of falsely elevated Hb levels. Hence, there were two variables, a bivariate 2-tailed approach was adopted, utilizing the Pearson correlation coefficient to analyze the association between them. The correlation coefficient denoted by "r". It quantifies the degree to which changes in one variable are associated with changes in another. In this analysis, the sign of the ‘r’ value was positive and the magnitude of the ‘r’ value was 0.677. Scatter plot of the data was created as follows to visually assess the relationship.

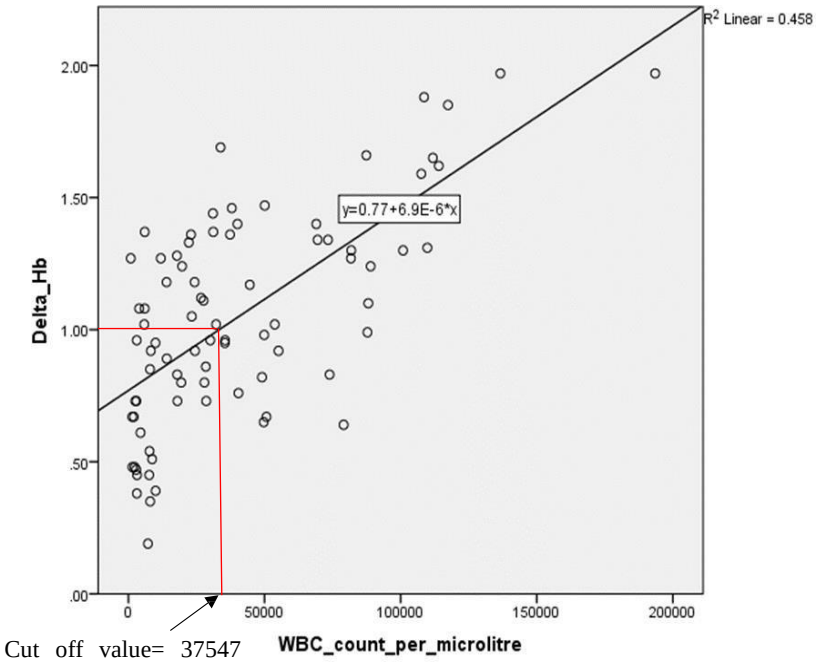

Figure 1: Scatter plot of delta Hb and WBC count per microlitre

The magnitude of 0.677 falls within the range of 0.5 to 0.8, indicating a moderate positive correlation. A scatter plot visually illustrated this positive correlation, emphasizing that higher leukocyte counts are associated with higher mean delta Hb levels. The cutoff value was determined to be 37,547 cells/µl, representing the minimum leukocyte count that leads to falsely high Hb levels.

The results revealed a clear trend in the mean delta Hb values across different leukocyte count ranges. As the leukocyte count increased, there was a corresponding increase in the mean delta Hb. For instance, samples with leukocyte counts between >10,000 and 30,000 cells/µL exhibited a mean delta Hb of 1.038 g/dL, while those with counts exceeding 110,000 cells/µL showed a mean delta Hb of 1.812 g/dL. The study also explored the impact of centrifugation on Hb measurement in samples with low leukocyte counts (0-4,000 cells/µL) and normal leukocyte counts (>4,000-10,000 cells/µL). The mean delta Hb values for these ranges were 0.6975 g/dL and 0.71 g/dL, respectively, suggesting that even within the normal reference range, centrifugation may influence Hb measurement but in very high leukocyte count contained samples shows much more Hb measurement interferences than the normal leukocyte count contained samples. This finding has practical implications for laboratory procedures, indicating that centrifugation can be a valuable step in improving the precision of Hb measurements, particularly in scenarios where high leukocyte counts might introduce interference.

CONCLUSIONS/RECOMMENDATIONS

In conclusion, the centrifugation process proves to be an effective method for minimizing interferences caused by elevated leukocyte counts in Hb estimation through the cyanmethemoglobin method. This approach yields more reliable test results compared to the standard process of Hb estimation, particularly in samples with leukocyte counts exceeding 37,547 cells/µl.

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