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Original Research Article

Comparative Assessment of Manual and Automated Methods of Counting Reticulocytes and the Effect of Sample Storage on Reticulocyte Count: An Analytical Assessment

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Abstract

Aim: The purpose of the study was to assess comparability between manual and automated methods of reticulocyte counting and to understand the variation of reticulocyte count over time.

Methods: The cross-sectional study conducted in the Department of Pathology of a Darbhanga medical college and Hospital, Darbhanga, Bihar, India over a period of six months. In this study, comparison of RC by the manual and automated method was done in 100 patients with anemia and 100 control samples which were matched for age and sex.

Results: Basic demography in study cases showed 46 females and 54 males. Out of 100 cases, 12 cases were infants (up to 1 year), 38 cases were children (>1 year to 14 years) and 60 cases were adults (>14 years). The mean automated RC for males was 4.76 ± 4.20 and it was 4.90 ± 4.36 by the manual method. In the case of females, the mean automated RC was 3.65 ± 3.44 and that by the manual method, it was 3.88 ± 3.66. No statistically significant difference was found between the automated and manual count among the male (P = 0.77) as well as female patients (P = 0.61) by z-test for the difference between the mean automated RC and mean manual RC.

Conclusion: We conclude that there was no significant difference between automated and manual methods for reticulocyte counting in any gender for microcytic, normocytic, or macrocytic patients.

Keywords: Anemia, automated reticulocyte count, manual reticulocyte count, Immature reticulocyte fraction

Introduction

Careful assessment of the blood is the first step in the assessment of hematological function and diagnosis of the related diseases. [1] Reticulocytes are young or immature red blood cells that are released from bone marrow and that contain remnants of ribonucleic acid (RNA) and ribosomes. [2] Reticulocyte count (RC) is the index of erythropoietic activity within the bone marrow and its measurement provides an initial assessment of anemia. [1,2]

The reticulocyte counting methods at clinical laboratories are currently divided into manual and automated. The manual reticulocyte counting by microscopy became traditional and has been considered the standard method since 1940, for its simplicity and low cost. [3,4]

The RC can be done by either by manual or by an automated method. Manual RC by microscopy has been considered as the standard method since 1940 because of its simplicity and low‑cost [3] However, it presents some inconvenience and limitations such as lack of accuracy, more time required for analysis, lack of quality of the stain, and inappropriate blood films. [5] Automated RC, through continued instrument, software, and reagent developments, provides an improved precision for the RC. It is a rapid and simple investigation which assists in providing a sensitive approach to the diagnosis and therapeutic monitoring of the anemic patients. [6]

Automated reticulocyte counting makes the determination fast, specific, and efficient by counting a large number of red blood cells. It can provide information about individual cell characteristics, such as hemoglobin content of reticulocytes, hemoglobin content of mature erythrocytes, percentages of microcytic erythrocytes and hypochromic cells, mRNA content and of cellular indices such as volume, hemoglobin concentration, and content. All these novel parameters are useful in reporting and interpretation in the diagnosis of specific anemia. [7]

Reticulocyte count is considered as less stable at room temperature because of in vitro maturation of reticulocytes to red blood cells and the count begins to fall within 6–8 h of blood collection.8 The Clinical and Laboratory Standards Institute (CLSI) recommends that samples stored at room temperature should be analyzed for reticulocytes within six hours of collection. For storage greater than six hours, refrigerated temperatures (4 °C) should be preferred to prevent deterioration of the sample. [9] The automated count may be performed using a fluorochrome for staining the remnant ribonucleic acid (RNA) present at the reticulocyte. After being stained, the fluorescent cells can be enumerated using a flow cytometer of general use. [8]

The purpose of the study was to assess comparability between manual and automated methods of reticulocyte counting and to understand the variation of reticulocyte count over time.

Methods

The cross-sectional study conducted in the Department of Pathology of a Darbhanga medical college and Hospital, Darbhanga, Bihar, India over a period of six months. In this study, comparison of RC by the manual and automated method was done in 100 patients with anemia and 100 control samples which were matched for age and sex. EDTA anticoagulated blood samples of patients sent to hematology laboratory for routine complete hemogram in the first morning batch where reticulocyte count was indicated were included.

Insufficient or grossly hemolysed samples were excluded. Sample size was calculated using OpenEpi Software version 3 with estimated mean difference between reticulocyte count measured using manual and automated method to be 0.2 [manual-1.81(0.416) vs automated- 1.61(0.236)] from previously published data.10 The sample size was calculated as 100 with 99% confidence interval and 90% power.

Manual microscopic counting was done on new methylene blue stained smears. Standard technique of mixing equal volumes of whole blood and of dye solution (100 ll) followed by incubation at 37 °C for 15 min and blood film preparation on glass slide using wedge method was used. Number of reticulocytes was counted in a maximum of 10 fields to

a total of 1000 RBC and reticulocyte percentage was calculated as number of reticulocytes counted/1000 9 100.

The same sample was analysed by automated hematology analyser Sysmex XT-2000i (Sysmex corporation, Kobe, Japan). CBC? RETIC mode was used for analysis of sample using RET search II reagent. In the analyser after a predefined response time the stained sample is introduced into the detector, where forward light scatter and side fluorescence emission are measured. The parameters noted were reticulocyte percentage, absolute reticulocyte count, immature reticulocyte fraction (IRF), low fluorescence ratio (LFR), medium fluorescence ratio (MFR) and high fluorescence ratio (HFR).

The samples were analyzed repeatedly by both manual and automated methods at different time intervals (2, 6, 24 and 48 h). The storage temperature was at room temperature (RT) which is usually maintained around 25 °C for 2 and 6 h and at 2–80C for 24 and 48 h. Repeat manual counting was done both on freshly prepared slide as well as stored initial slide (prepared within 2 h). Manual count mentioned as such in the text by default indicates counts done on freshly prepared slide.

Data was analysed in SPSS version 20. Outcome continuous variables like reticulocyte count based on manual and automated methods were first summarized as mean with SD and compared using independent t-test. As the distribution of data was found to be non-normally distributed by Shapiro–Wilk test, so non-parametric equivalent Mann–Whitney test was used to compare medians which is presented. Association between manual and automated methods of counting reticulocytes was done by Spearman's correlation. For normally distributed data of reticulocyte parameters, effect of storage was done using repeated measures ANOVA.

Results

Table 1: Comparison of mean reticulocyte count between male and female by automated and manual method and comparison according to age

Sex (n=100)	Mean automated reticulocyte count (±SD)	Mean manual reticulocyte count (±SD)	Z-test (P)
Male (n =54)	4.76±4.20	4.90±4.36	0.32 (0.77)
Female (n=46)	3.65±3.44	3.88±3.66	0.49 (0.61)
Comparison of mean RC according to age			
Infants (upto 1 year) (n=12)	2.36±1.47	2.18±1.72	0.37 (0.71)
Children (>1–14 years) (n=38)	3.90±2.89	4.18±3.02	0.52 (0.59)
Adults (>14 years) (n=60)	4.46±4.22	4.68±4.40	0.43 (0.66)

Basic demography in study cases showed 46 females and 54 males. Out of 100 cases, 12 cases were infants (up to 1 year), 38 cases were children (>1 year to 14 years) and 60 cases were adults (>14 years). The mean automated RC for males was 4.76 ± 4.20 and it was 4.90 ± 4.36 by the manual method. In the case of females, the mean automated RC was 3.65 ± 3.44 and that by the manual method, it was 3.88 ± 3.66. No statistically significant difference was found between the automated and manual count among the male (P = 0.77) as well as female patients (P = 0.61) by z-test for the difference between the mean automated RC and mean manual RC. Table 1

The mean automated RC among infants (up to 1 year) was 2.36 ± 1.47 and it was 2.18 ± 1.72 by manual method. In the case of children (>1 year‑14 years), the mean automated RC was 3.90 ± 2.89 and 4.18 ± 3.02 by manual method. For adults (>14 years), the mean automated RC was 4.46 ± 4.22 and 4.68 ± 4.40 by manual method. Z ‑test for difference between two means was applied. No statistically significant difference was found in mean RC among the infants (P = 0.71), children (P = 0.59), and adults (P = 0.66) between automated and manual count. Table 1

Table 2: Mean reticulocyte count according to morphologic classification of anaemia in study cases

Sex (n=100)	Automated reticulocyte count (±SD)	Manual reticulocyte count (±SD)	Z-test (P)
Microcytic anaemia (MCV <80) (n=60)			
Male n=32	4.70±4.20	4.75±4.44	0.1 (0.86)
Female n=28	3.25±2.65	3.62±2.80	0.70 (0.39)
Normocytic anaemia (MCV 80–100) (n=30)			
Male n=16	4.50±3.63	4.65±3.71)	0.30 (0.80)
Female n=14	3.70±3.23	3.72±3.43	0.03 (0.97)
Macrocytic anaemia (MCV >100) (n=10)			
Male n=7	5.95±5.45	60.5±5.42	0.09 (0.092)
Female n=3	5.45±6.75	5.50±7.32	0.02 (0.97)

When we divided cases as per morphologic classification of anemia in both sex, the difference between mean manual and automated RC was statistically significant only in the case of males in the macrocytic anemia group (P = 0.092) while it was insignificant in rest cases and sex groups in other groups [Table 2].

Table 3: Comparison between mean immature reticulocyte fraction, corrected reticulocyte count, mean reticulocyte volume and reticulocyte haemoglobin cellular content for normocytic, macrocytic and microcytic cases by automated method in study cases

	Microcytic (±SD), n=60	Normocytic (±SD), n=30	Macrocytic (±SD), n=10	F-test (P)
Mean IRF	0.15±0.08	0.16±0.11	0.25±0.17 7	0.78 (0.001)
Mean CRC	1.65±3.14	1.52±1.14	1.49±1.25	0.10 (0.89)
Mean MRV	87.73±21.63	111.16±24.44	128.26±26.78	58.11 (0.0001)
Mean RHCC	24.64±6.58	32.25±7.90	38.58±8.37	67.35 (0.0001)

When compared for reticulocyte indices, mean IRF, Mean MRV, and mean RHCC was found to be statistically significant among all types of anemia (P = 0.001, 00001, and 0.0001, respectively) while it was insignificant in the case of mean CRC (P = 0.89) [Table 3].

Discussion

Both manual and automated methods showed a strong positive correlation in the detection of reticulocytes at different time intervals. The value which was found to be high by one method was found to be high by other method also. This data was similar with the data from previous studies which also showed an excellent correlation between the methods. [11,12]

Our study showed no statistically significant difference between the mean automated RC and mean manual RC amongst males as well as females (P = 0.77 and P = 0.61). The literature shows

studies with varied results which compared RC between males and females, [13,14] but not the methods. Thus, both methods are suitable for the determination of mean RC, but the manual method can be more preferred as it is cost‑effective.

The comparison of mean RC value by both manual and automated method showed varied results in the literature. Osgood et al. [15] found no significant difference in average RC percentage in the age group of 4–13 years while; Jain P et al. [16] found a significant decrease in the elderly group. Bukhari and Zafar [17] found a significant difference in RC in (<27 days) age group in their study on infants ($P < 0.05$). Tarallo P et al found no statistical difference between boys and girls aged 4–19 years. However, it was significantly higher in men than in women over 20 years of age. [13]

The highest mean RC was observed in macrocytic anemia by both automated and manual methods followed by microcytic anemia in males in our study. The lowest mean RC was seen in microcytic anemia in female patients [Table 2]. The literature did not show such an association between the morphological type of anemia and mean RC. This study was mainly focused on finding out the difference between the manual and automated methods of reticulocyte counting. No statistically significant difference was observed between these two methods in microcytic, normocytic and macrocytic anemia. However, considering that most cases of macrocytic anemia will be having megaloblastic or hemolytic etiology, [18,19] it is expected that there will be reticulocytosis because of hemolysis and subsequent erythroid production.

Our findings are consistent with Lacombe et al., [20] Sindhu et al. [21] and Sunkara and Kotta, [22] all of which found a highly significant difference in IRF values. Rastogi et al. [23] found that a significant difference in the values of CRC obtained by manual versus automated method. The findings of our study showed that there was not much difference in manual and automated counts in both controls and cases, and the difference was also not statistically significant ($P = 0.89$). Our findings are consistent with Butthep et al. [24] who found a highly significant decrease in MRV in iron deficiency anemia patients versus normal (MRV = 95.89 ± 8.57 FL, $P \leq 0.0001$). Hence, it adds to the peripheral smear observation that the size of the reticulocytes observed in iron deficiency anemia is smaller than macrocytic anemia. [25]

Conclusion

We conclude that there was no significant difference between automated and manual methods for reticulocyte counting in any gender for microcytic, normocytic, or macrocytic patients. However, the manual method may be preferred as it is cost‑effective; yet, it is laborious, time‑consuming, need efficient technique, not suitable for heavy loaded laboratories and may be suitable for under‑resourced laboratories. However, the automated method is preferred as it is fast, highly precise and it is mandatory for certain diseases where reticulocyte parameters are required as a statistically significant difference was found among the different parameters such as IRF, MRV, and RHCC.

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