Original Research Article

Comparison of automated flowcytometric reticulocyte analysis with manual reticulocyte count

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ABSTRACT

Background: Reticulocytes are young or immature red blood cells released from bone marrow and that contain remanants of ribonucleic acid (RNA) and ribosomes. Reticulocyte count (RC) is the index of erythropoietic activity within bone marrow. The reticulocyte counting methods at clinical laboratories are currently divided into manual and automated.

Methods: A total of 500 samples of study cases were processed by manual method using New Methylene Blue (NMB) and automated method based on flowcytometry by PENTRA XLR HORIBA hematology analyzer. All quality control parameters were evaluated and values obtained by both methods were compared using various statistical methods.

Results: Automated hematology analyzer provides excellent precision and linearity with no significant carryover. On comparing manual and automated RC method good method correlation was found (correlation coefficient r-0.865), however individual case wise percent deviation between manual and automated RC and CRC varied significantly. In addition within run precision calculated for automated RC differed significantly from manual count. The mean of difference between duplicate readings (150 samples) of manual and automated RC (<5%) were 0.3 and 0.01 respectively while 6.3 and 0.15 respectively for >5% RC. Thus, automated method was found to be more precise than the manual RC.

Conclusions: The manual count method for RC associated with significant imprecision compared to flowcytometric method mostly based on interobserver variation and the smaller number of cell being counted. In contrast, the automated method is rapid, easy to operate, count higher number of cells with precise measurement.

Keywords: Automated method**,** PENTRA XLR(HORIBA) hematology analyzer, Manual method, Reticulocyte count

INTRODUCTION

The reticulocyte count has evolved into one of the basic tests in diagnostic hematology for assessing erythropoietic activity in bone marrow. [1,2] Because of its diagnostic and therapeutic implications, it is commonly used parameter for evaluation of various hematological conditions. [3]

The reticulocyte enumeration is useful in following conditions like [4-6]

- Classifications of various anemias
- For diagnosis and assessment of severity of hemolytic anemia and aplastic crisis in hemolytic anemia

- To monitor the bone marrow function following treatments such as chemotherapy and bone marrow transplant
- To monitor response to the treatment for iron deficiency anemia (IDA), B12 deficiency anemia and folic acid deficiency and in renal transplantation engraftment
- To monitor the hydroxyurea therapy in sickle cell anemia
- To see the effects on erythropoietin abuse in sport athletes

Reticulocyte counting is carried out by manual or automated method. Manual counting of reticulocytes by light microscopy with supravital dyes for RNA was developed in the 1940s and remains the standard method of reticulocyte enumeration.[7] However, automated methods of reticulocyte enumeration developed during the past decade are increasingly being performed in the clinical laboratory.[8-10] In addition to accurate reticulocyte enumeration, by automated flowcytometric reticulocyte analysis, the measured fluorescence intensity is directly proportional to the amount of RNA in the immature erythrocytes, this method has the ability to quantitate reticulocyte maturity.[11-13] In addition, the newer techniques provide a variety of reticulocyte-related parameters such as Absolute reticulocyte count(ARC), Reticulocyte with a low RNA content (RETL), Reticulocyte with a medium RNA content (RETM), Reticulocyte with a high RNA content (RETH), Immature reticulocyte fraction (IRF), Mean reticulocyte volume (MRV), Reticulocyte hemoglobin cellular content (RHCC), Mean fluorescence index (MFI) and Mean reticulocyte hemoglobin content (CHr) which are not available with light microscopy and appear valuable in the clinical diagnosis and monitoring of anemia and other diseases and for evaluation of various hematological disorders.[14-17] Hence, the present study is an attempt to compare both manual and automated method for reticulocyte count.

METHODS

The present study is the cross sectional study conducted at the hematology laboratory, department of pathology at tertiary care hospital affiliated with medical college during period of one year. A total of 500 cases were taken for study, from those whose reticulocyte count was requested by clinician at hematology laboratory. The cases included samples from adult male, female and pediatric patient more than 1 year of age. The cases with <1 year of age and history of recent blood transfusion within 3 months were excluded from the study.

The samples for the analysis of reticulocyte count by both manual and automated method were collected in EDTA (ethylene diamine tetra acetate) vaccutainer and processed within 2 hours of collection. The samples of all the study cases were processed by manual method using conventional/traditional NMB and automated method based on flowcytometry by HORIBA PENTRA XLR hematology analyzer.

By manual new methylene blue method, reticulocyte count (% of reticulocytes in RBC population) and corrected reticulocyte count-CRC (% reticulocytes x patient hematocrit/normal hematocrit) were performed and recorded.

For automated method, samples were processed on PENTRA XLR 5 part hematology analyzer by HORIBA. PENTRA XLR is a quantitative multi parameter, automated hematology analyzer for in vitro use in clinical laboratories to identify and enumerate various complete blood count (CBC) parameters as well as following reticulocyte parameters: RET# (Reticulocyte absolute value), Ret % (Reticulocyte percentage), RETL*, RETM*, RETH* (Reticulocyte with low, medium and high RNA content), CRC (Corrected reticulocyte count), MRV (Mean reticulocyte volume), IRF (Immature reticulocyte fraction), RHCC (Reticulocyte hemoglobin cellular content).

The instrument works on use of fluroscent dye such as thiazol orange and laser optical bench, measures the fluorescence of the cells passing through the measuring point into the flow cell and the volume by impedance. A maximum of 32,000 cells are analyzed and the instrument, using customized gating for each sample, separates reticulocytes from mature RBCs, white blood cells (WBCs), and platelets. The instrument was calibrated according to the specification of manufacturer such as during installation, maintenance or service interventions.

The following quality control procedures were conducted for automated reticulocyte method

Repeatability/within batch precision

Repeatability/ within batch precision was analyzed for reticulocyte parameters using samples with low, normal and high values processed in 20 replicates.

Reproducibility/between batch precision

Reproducibility was assessed using low, normal and high levels of single lot of control material with each batch.

Linearity

Linearity was performed on 10 successive dilution of whole blood with high reticulocyte value . Each dilution was analyzed in triplicate and mean value at each dilution was plotted against the expected theoretical value and the linearity graph was prepared.

Carry over

Carry over was done using blood samples with high reticulocyte value which was processed in triplicate (S1,

S2, S3) followed by three cycles of ABX diluents (D1, D2, D3). Carry over was calculated according to the following formula: (D1-D3)/ (S3-D3) X100. S=Sample with high reticulocyte count, D=Diluent

Retained sample stability for reticulocytes

Blood samples were preserved at room temperature and at 4°C. They were analyzed immediately after receiving at the laboratory and subsequently at 2, 4, 6, 8, 24, 48 and 72 hours. The initial reticulocyte count for each sample was considered the reference point. From this value the deviation for each analysis at scheduled processing time was calculated.

Comparision between automated and manual methods

Concordance was evaluated between standard manual method and automated method for reticulocytes.

A. Comparison of mean and standard deviation of all measured values:

The mean and SD of all 500 examined samples for manual and automated RC was calculated.

B. Case wise percentage deviation between manual and automated method

The deviation of values for manual and automated RC and CRC was calculated for each sample.

C. Comparison of within run precision between automated and manual method

Run precision between automated and manual method: Out of total 500 samples examined; 150 samples were run on automated hematology analyzer in duplicate. The same samples were analyzed for manual count by conventional method by two different observers. The mean of difference between the duplicate results of both automated and manual methods was compared and SD was calculated.

D. Correlation and regression analysis of manual and automated reticulocyte count

Using a linear regression analysis, RC obtained by both manual and automated method was compared and both Pearson's product moment coefficient correlation and intraclass correlation coefficient were derived.

RESULTS

Quality Control procedures for automated reticulocyte count

Repeatability/ within batch precision

Repeatability/ within batch precision values for low, normal and high level of reticulocyte concentration for RC %, ARC and IRF are described in Table 1.

Reproducibility/ between batch precision

Mean and CV % obtained for RC (%) and ARC for low, normal and high level control are described in Table 2.

Table 1: Within batch precision data of low, normal and high RC%, ARC and IRF.

Values	Low level (RC %)	Normal level (RC %)	High level (RC %)	Low level (RET 10^6/mm³)	Normal level (RET 10^6/mm³)	High level (RET 10^6/mm³)	Low level (IRF)	Normal level (IRF)	High level (IRF)
Mean	0.7	1.1	7.2	0.015	0.045	0.149	0.027	0.153	0.418
SD	0.09	0.08	0.26	0.001	0.002	0.005	0.002	0.009	0.010
CV (%)	12.56	7.01	3.61	6.6	4.4	3.3	11.1	5.8	2.39

Table 2: Reproducibility of RC (%) and ARC.

Control	No of samples	Mean (RC%)	CV(%) (RC%)	Mean (ARC)	CV(%) (ARC)
Level 1	31	1.4	3.2	0.070	5.71
Level 2	31	5.2	1.3	0.182	3.2
Level 3	31	10.3	0.9	0.376	2.1

Linearity

Figure 1 shows the linearity graph. correlation coefficient obtained was r=0.998.

Carryover

Carryover (%) was found to be 0, indicating no significant carry over using automatic run for samples.
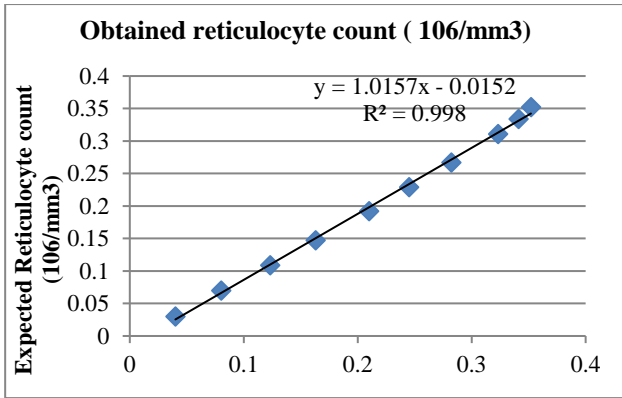
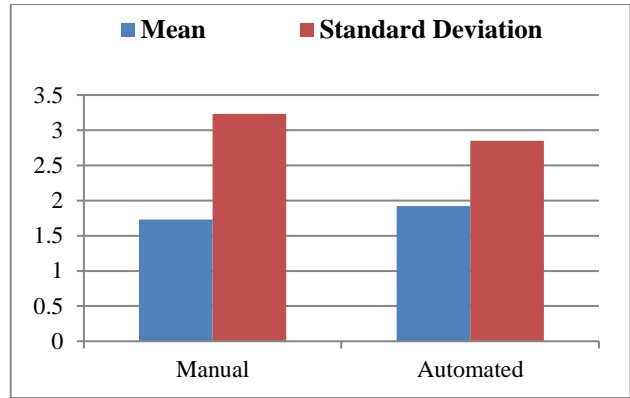**Figure 1: Linearity of reticulocyte counting by PENTRA XLR.**

Figure 2: Mean and standard deviation of automated and manual reticulocyte count.

Table 3: Summary statistics for percentage reticulocyte count obtained with Manual methods and Pentra XLR (<% 5 Rticulocyte count and >% Reticulocyte count).

	All data		<5 % reticulocyte count		>5 % reticulocyte count	
Methods	Manual	Pentra XLR	Manual	Pentra XLR	Manual	Pentra XLR
No of samples	500	500	472	472	28	28
Mean (%)	1.73	1.92	1.23	1.31	10.70	10.22
Standard deviation	3.23	2.85	1.07	0.9	9.27	7.28

Retained sample stability for reticulocytes

A significant decrease in reticulocyte count % noted after 6 hours of storage at room temperature and after 48 hours of storage at 4°C. The significant decrease in IRF noted at 8 hours of storage at room temperature and at 4°C. Thus, cold storage of reticulocyte at 4°C did not prevent modifications of IRF parameters.

Comparison between automated and manual methods

A total 500 samples were randomly selected for the study. All the samples were processed by both manual method and automated method.

Mean and standard deviation of all measured values

Out of total 500 samples, cases were categorized into two groups (<5% RC and >5% RC). Mean and SD of all values were derived as mentioned in Table 3 and plotted in graph (Figure 2). Mean and standard deviation of values in both the groups differs slightly.

Case wise percentage deviation between manual and automated method

From all 500 cases, percentage deviation between each manual RC and automated RC and between each manual

CRC and automated CRC were calculated as described in Table 4.

Table 4: Percentage deviation of individual RC and CRC values between manual and automated methods.

	Deviation range	Mean	SD	p value
Manual vs. automated RC	-66 to 92	15.5	29.9	
Manual vs. automated CRC	-75 to 91	20.2	34.6	0.001

The difference was not skewed in one direction rather it was on either side i.e. it varied from positive to negative both side significantly.

Comparison of within run precision between automated and manual method

Duplicate readings of both automated and manual RC of 144 samples with <5% RC and 6 samples with >5% RC were recorded. Difference between two readings was calculated for both automated and manual method. The mean and standard deviation of difference were obtained and compared.

The mean of difference between two readings of manual and automated RC (<5%) were 0.3 and 0.01 respectively. The SD of difference between two readings of manual

and automated RC (<5%) were 0.55 and 0.13 respectively (p value <0.05).

The mean of difference between two readings of manual and automated RC (>5%) were 6.3 and 0.15 respectively. The SD of difference between two readings of manual and automated RC (>5%) were 1.87 and 0.42 respectively (P value <0.05).

Correlation and regression analysis of manual and automated reticulocyte count

The linear regression analysis of all 500 cases for reticulocytes measured on PENTRA XLR compared with the manual counting gave the Pearson's coefficient of correlation(r) 0.865 and slope was 0.753. (Figure 3) Pearson's coefficient of correlation was 0.865, which showed good correlation (p value <0.001). To see the agreements between method, intraclass correlation coefficient calculated which was 0.944.
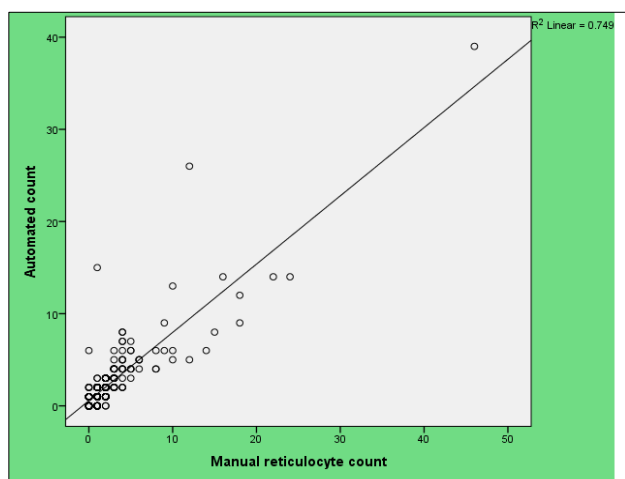

Figure 3: Correlation and regression analysis between manual RC and automated RC.

DISCUSSION

In the present study evaluation of RC by automated flowcytometric method was carried out on 500 samples. The samples were also processed for manual RC by traditional NMB method and light microscopy. The results of both automated and manual reticulocyte count were compared.

Quality control procedures: performance analysis of automated hematology analyzer

The samples were processed on five part hematology analyzer PENTRA XLR by HORIBA on RET mode. The analyzer was calibrated as per manufacturer's guidelines.[18] Various quality control procedures were performed to determine operating characteristics of instrument.

Repeatability/within batch precision

Repeatability was evaluated by 20 consecutive run of samples with low, normal and high level of RC (%), ARC and IRF.

The CV (%) for low, normal and high level (RC %) were 12.56, 7.01 and 3.61 respectively, CV (%) for low level, normal and high level (ARC-106/mm3) were 6.6, 4.4 and 3.3 respectively and the CV (%) for low, normal and high level (IRF) were 11.1, 5.8 and 2.39 respectively.

The obtained precision data for RC (%), ARC and IRF were close to the claimed manufacturer's precision at all three levels.[18] The PENTRA XLR showed excellent precision for reticulocyte count and its parameter.

Reproducibility/between batch precision

Mean value of RC (%) for level 1,2, 3 controls were 1.4, 5.2 and 10.3 respectively. CV (%) of RC (%) for level 1,2,3 controls were 3.2, 1.3 and 0.9 respectively.

Mean value of ARC (106/mm3) for level 1,2 and 3 controls were 0.070, 0.182 and 0.376 respectively. CV (%) of ARC (106/mm3) for level 1, 2 and 3 controls were 5.71, 3.2 and 2.1 respectively. The values obtained for all the three level of control for RC % and ARC were within the range of manufacturer's specifications which indicated excellent reproducibility for RC.

Linearity

In the present study we found excellent linearity with r value of 0.998.

Carry over

The carry over for ARC found to be 0 in the study, which proved the instrument to be excellent implying no significant carry over for reticulocytes.

Retained sample stability for reticulocytes

In the present study, there was a significant decrease in ARC and RC% after storage at room temperature compared to storage at 4°C. RC% started to fall after 6 hours of storage at room temperature and significantly reduced after 24 hours of storage at room temperature. At 4°C RC started to fall at 48 hours and was reduced significantly thereafter. In the present study, significant decrease in IRF after 8 hours was found after storage at room temperature and at 4°C. Lacombe et al checked reticulocyte % stability after storage at various time periods at room temperature and at 4°C and observed that a significant decrease in reticulocyte percentage appeared after 48 hours of storage at room temperature and no significant difference was noted even after 48 hours at 4°C.[19] In their study IRF parameter was significantly

reduced after 8 hour of storage for both at room temperature and at 4°C. [19]

The in vitro stability of the reticulocyte was checked by Cavill et al; in their study, no significant decrease of reticulocyte count was found at room temperature or at 4°C.[20] However, RT samples with high reticulocyte counts decreased during the first 24 hours but not at 4°C.

Comparison between automated and manual methods

Comparison of mean and standard deviation of all measured values

In the present study, all the study cases (n=500) were categorized on the basis of obtained RC % on automated analyzer in two groups: one with <5% RC and other with >5 % RC values.

Mean value of RC % (<5%) of 472 cases were 1.23% and 1.31% for manual and automated method respectively. Standard deviations were 1.07 and 0.9 for manual and automated method respectively.

Mean value of RC % (>5%) of 28 cases were 10.7% and 10.22% for manual and automated method respectively. Standard deviations were 9.27 and 7.28 for manual and automated method respectively.

Mean and deviation of values in both groups by both manual and automated method differs slightly.

Case wise percentage deviation between manual and automated method

In the present study, there was significant difference in the values of manual RC and CRC Vs. automated RC and CRC. The variation was huge and it varied from -66 to 92 % for automated and manual RC and -75 to 91 % for automated and manual CRC.

The mean value and standard deviation of percentage deviation for manual and automated RC was 15.5% and 29.9% respectively. Mean value and standard deviation of percentage deviation for manual and automated CRC was 20.2% and 34.6 respectively with significant p value (0.001).

Arvind at el carried out similar study with significant deviation in values of manual and automated RC (-26% to 74.9%) and also in values of manual and automated CRC (2.2% to 211%).[3] The difference for both was found to be significant (p value was <0.1).[1,3]

In present study significant difference was found between individual case wise automated and manual RC and CRC; explained by p value (0.001) indicated that the RC and CRC values obtained by both manual and automated methods varies significantly.

Comparison of within run precision between automated and manual method

In present study we processed 150 samples in duplicate on PENTRA XLR hematology analyzer. The mean and standard deviation of the difference of duplicate results were derived. The samples were also examined by two different observers by manual methods. Mean and standard deviation of the difference between two readings were derived and compared to the derived values for automated methods. The results were divided into two groups based on RC obtained on automated hematology analyzer taking the cut off value as 5%. There were 144 samples with RC <5% and 6 samples with RC >5%.

The mean of difference between two readings of manual method and automated RC (<5%) were 0.3 and 0.01 respectively. The standard deviation of difference between two readings of manual method and automated RC (<5%) were 0.55 and 0.13 respectively. P value difference was 0.00001, which was significant.

The mean of difference between two readings of manual method and automated RC (>5%) were 6.3 and 0.15 respectively. The standard deviation of difference between two readings of manual method and automated RC (>5%) were 1.87 and 0.42 respectively. P value of difference was 0.005, which was significant.

The mean of difference and standard deviation of paired results were more in manual method compared to automated method in both the groups. The difference was significant indicating relative imprecision for manual count compared to automated method.

Correlation and regression analysis of manual and automated reticulocyte count

In the present study, comparison of automated and manual methods for reticulocyte counting was carried out by regression analysis with the classic pearson's product moment correlation with r value of 0.865 which indicated good correlation between two methods.

Lacombe et al performed comparison of 3 automated and manual methods for reticulocyte counting. With the classic Pearson product-moment correlation (r), they found excellent agreement between all methods.[19] In their study r vaule for manual and automated count performed by ABX PENTRA 120 Analyzer was 0.945, r value for manual and SYSMEX R-2000 was 0.937 and r value for manual and Coulter XL was 0.906; indicating both methods having similar trends.[19]

Brugnara et al. studied regression analysis between manual reticulocyte % and automated reticulocyte % by Miles H*3 analyzer and derived r value of 0.940.[21]

Tichelli et al studied linear regression between SYSMEX R-1000 and manual method and derived r value of

0.966.[22] However, this kind of analysis only indicates a linear trend for both variables to change in same directions and does not permit determination of agreement between any of two methods.

With intraclass correlation coefficient, the level of agreement can be estimated; a satisfactory level is achieved when the lower limit of the 95% confidence interval is at least 0.75. In the present study intraclass correlation coefficient of 0.944 was derived which indicated excellent agreements between two methods. Lacombe et al carried out the comparision of three automated methods with manual method and studied agreements between all methods by intraclass correlation coefficients. In their study intra class correlation coefficients between Manual/SYSMEX R-2000, Manual/Flowcytometry and Manual/PENTRA 120 were 0.935, 0.896, and 0.932 respectively and thus found excellent agreements between all methods. [19]

Brugnara et al, carried out comparison of three automated methods with manual method and studied agreements between all methods by intraclass correlation coefficients. In their study intra class correlation coefficients between Manual/SYSMEX R-2000, Manual/ Flowcytometry and Manual/H*3 analyzer were 0.538, 0.755 and 0.610 respectively. Using these criteria, in their study manual counting of reticulocytes cannot be considered interchangeable with the three automated methods.[21]

However, in the present study, agreement was found to be excellent similar to the findings of study carried out by Lacombe et al.

This result variability noted in different study for intraclass correlation might be due to difference in automated method principles and reagents, inter observer variation of manual count defining a reticulocyte and due to small number of reticulocye being counted by manual method.

CONCLUSION

The present cross sectional study for comparison of automated and manual methods of reticulocyte count proved that automated hematology analyzer based of flowcytometric analysis of reticulocyte count provides excellent precision and linearity with no significant carryover.

Though, the manual count method is significantly cheaper than automated one, it is associated with significant imprecision mostly based on interobserver variation and the smaller number of cell being counted. In contrast, the automated method is rapid, easy to operate without any prior preparation required and thus reducing the labor cost. In addition, they count higher number of cells with precise measurement through specific staining and flowcytometry, so it is way ahead superior than manual method. it would be a desirable option for the hematology laboratory to have the analyzer which performs complete blood count and reticulocyte count on the same instrument.

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