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Performance Evaluation of Fully Automated Reticulocyte Count As A Validated Method In Comparison with Manual Count In Neonates

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ABSTRACT

Objective: To evaluate the performance of fully automated reticulocyte count as a validated method in comparison with manual count in neonates.

Study Design: cross-sectional study.

Place and Duration of Study: Department of Haematology, Armed Forces Institute of Pathology (AFIP), from July 2020 to December 2020.

Methodology: 103 healthy neonates of either gender were selected using non-probability convenience sampling. Venous blood of the participants was collected and reticulocyte count was determined using both manual count as well as Sysmex XN-3000 Automated Haematology Analyzer. The data was recorded, tabulated and processed to ascertain the degree of correlation and agreement between the two methods using Linear Regression Analysis and Bland-Altman plot respectively.

Results: The mean age of participants was 14 days. Out of the total of 103 participants, gender-wise distribution was 55(53%) males and 48(47%) females. The mean manual reticulocyte count was 2.0+1.1% and automated reticulocyte count was 2.0+ 1.0%. The two sets of data showed good correlation with a coefficient of correlation (r) of 0.97. The mean bias of data was 0.002 with good agreement as all recorded data points fell between upper and lower limits of agreement.

Conclusion: Our findings suggest that automated haematology analyzers can offer a high-throughput method of determining reticulocyte count with an adequately close agreement of results to the well-established manual method.

Keywords: Reticulocyte count, Automation, Haematology analyzers.

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INTRODUCTION

Erythrocytes begin their life cycle as Pluripotent Haematopoetic Stem Cells within the bone marrow. These immature cells gradually traverse morphologically distinct stages of development under the influence of a complex and interconnected cascade of growth factors, which are in turn driven by environmental stimuli. During this process, they decrease in size, lose their nuclear material and their cytoplasm becomes progressively orthochromatic. Subsequently, their nucleus is ejected before leaving the bone marrow.

Reticulocytes are juvenile erythrocytes which do not have nuclei but retain residual RNA (Ribonucleic Acid). Although they are able to perform the vital task of oxygen transport to tissues, they lack the characteristic biconcave shape of mature erythrocytes, which renders them mechanically unstable and vulnerable to shear stress. The complete maturation of reticulocytes in peripheral blood lasts 3 days. During this brief time period, they undergo extensive transformation including up to 20% membrane loss, membrane re-modelling and elimination of residual RNA.[1]

It has been estimated that approximately 2 million reticulocytes enter the circulation of an average healthy adult every second.[2] The percentage of reticulocytes in peripheral blood is considered an index of the erythropoietic activity of bone marrow. Reticulocyte count as a percentage of total erythrocytes is a frequently requested laboratory parameter, with applications in diagnosis as well as monitoring treatment response to several hereditary and acquired haematological disorders including haemolytic anaemias, iron deficiency anaemia, megaloblastic anaemia and myelodysplastic syndrome (MDS). At birth, the reference range is reported to be 3.0-7.0% which drops to adult values (0.5-2.5%) by the 7th day of life.[3]

Traditionally, the determination of reticulocyte count has depended upon the microscopic manual

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enumeration of supravitally stained reticulocytes exhibiting a web or "reticulum" of Ribonucleic Acid (RNA) remnants within their cytoplasm. As per the definition of the National Committee for Clinical Laboratory Standards (NCCLS) any non-nucleated erythrocyte containing two or more particles of blue-stained material corresponding to ribosomal RNA is considered a reticulocyte4. Heilmeyer categorizes reticulocytes into four stages, with gradual loss of demonstrable RNA accompanying progressive maturation.[1] In addition to being dependent on the skill and experience of the laboratory technician and pathologist, this relatively simple method is potentially error-prone due to smaller number of erythrocytes counted, misidentification of morphologically similar inclusions, low reproducibility and inter-observer variability.[5]

With the increasing advent of automation in every aspect of Diagnostic Haematology, reticulocyte count can be determined by many modern hematology analyzers including the Sysmex XN-3000 utilizing the principles of fluorescence and flow cytometry. Inside the reticulocyte channel (RET) of the analyzer, cell membranes of erythrocytes are perforated sufficiently by a lysing agent (CELLPACK DFL) to introduce a fluorescence marker (Fluorocell RET), while leaving the cells largely intact. Intracellular nucleic acids are labelled by this marker, with the intensity of the fluorescence signal directly proportional to nucleic acid content. By measuring both Forward Scattered Light (FSC) and the intensity of fluorescence signal, the reticulocyte population is separated from mature erythrocytes as a function of RNA content.[6] The reticulocytes can also be further categorized on the basis of progressively decreasing intensity of fluorescence signal (corresponding to increasing maturity) into high, intermediate and low fluorescence reticulocytes.[7] Researchers have demonstrated that quantification of immature reticulocyte fraction (sum of high and intermediate fluorescence reticulocytes) based on measurement of fluorescence signal intensity by flow cytometric analysis may aid in early diagnosis and monitoring response to treatment of anaemia due to various etiologies including iron deficiency anaemia.[8] and thalassaemia trait.[9]

Since the adoption of any new method of analysis in laboratory settings may have significant potential financial and clinical implications, there remains a need to thoroughly evaluate the technical aspects before widespread adoption and acceptance of any new method of analysis.

To our knowledge, the degree of agreement between manual and automated reticulocyte count using this particular instrument has not been subjected to rigorous statistical analysis in any regional or national study. Few studies have attempted to undertake the task without considering that correlation between the two methods does not necessarily translate into agreement, and the appropriate method of evaluating agreement is by determining mean of bias after constructing a Bland-Altman plot.[10]

METHODOLOGY

This cross-sectional study was conducted from 1stJuly 2020 to 31st December 2020 at the Department of Haematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi. Approval from Institutional Review Board (FC-HEM18-8/READ-IRB/20/463) was obtained prior to commencement of the study.

Inclusion Criteria: Healthy neonates were selected for the study as even under physiological conditions, they have a relatively high normal reticulocyte count which could potentially aid us in validation of our results over a relatively wide range of values. All participants were aged <28 days.

Exclusion Criteria: Neonates with non-consenting parents / guardians were excluded from the study. The presence of erythrocyte inclusion bodies resulting from various haematological and non-haematological conditions may interfere with the results of manual as well as automated reticulocyte count.[1] In order to address this potential confounding factor, neonates who were undergoing diagnosis or monitoring of diseases were excluded from the study.

The intent and purposes of the study were explained in detail and informed written consent was obtained from parents / guardians of all participants prior to specimen collection. 3 mL of venous whole blood specimens from all participants was drawn in Ethylenediaminetetraacetic acid (K3EDTA) containing evacuated blood collection containers (Atlas Labovac Italiano). Specimens were appropriately labelled using barcodes and promptly processed within 6 hours of collection.[11,12] Reticulocyte stain was prepared by dissolving 1.0 g of New Methylene Blue in 100 mL of citrate saline solution (0.049 g of trisodium citrate dissolved in 100 mL of normal saline). Equal volumes of reticulocyte stain and specimen were taken in a test

tube. The mixture was incubated at 37°C for 20 minutes before gentle re-suspension and slide preparation. In order to minimize inter-observer variability and possibility of unconscious bias, oil immersion microscopy at 1000x magnification was performed independently by multiple experienced observers in a double-blind manner using Olympus CX21 microscope. The percentage of reticulocytes in 1000 erythrocytes was recorded as the value of manual reticulocyte count. Simultaneously, the specimens were subjected to analysis using Sysmex XN-3000 Automated Haematology Analyzer and value of automated reticulocyte count was recorded.

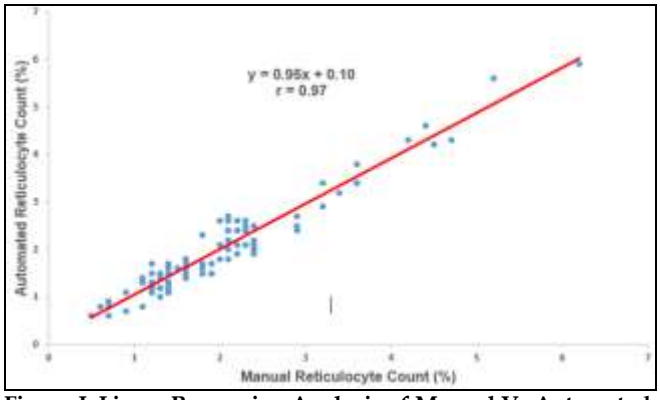

Figure-I: Linear Regression Analysis of Manual Vs Automated Reticulocyte Count

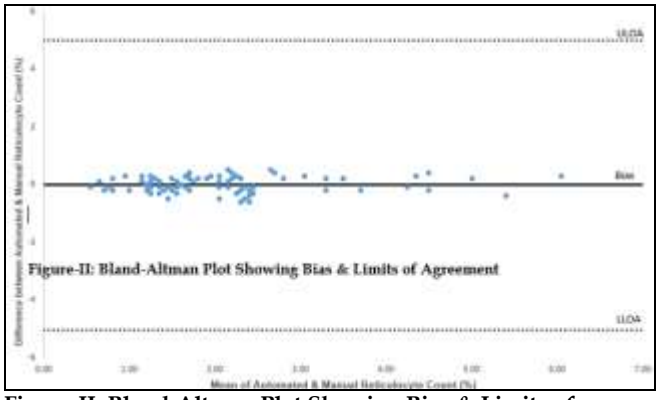

Figure-II: Bland-Altman Plot Showing Bias & Limits of Agreement

The two sets of results of automated and manual reticulocyte count thus obtained were tabulated and the raw data was processed through Microsoft (MS) Excel 2016. The Mean and Standard Deviation (SD) of each group was calculated. Correlation between automated and manual methods was evaluated using Linear Regression Analysis and determining values of coefficient of correlation (R2), slope (X) and intercept (Y). Bland-Altman Plot was constructed to ascertain the degree of agreement between the two methods using mean differences, bias between mean differences, upper limit of agreement (ULOA) and lower limit of agreement (LLOA).

RESULTS

The total number of participants in the study was 103, with a mean age of 14 days. Gender-wise distribution was 55(53%) males and 48(47%) females. The mean value of manual reticulocyte count was 2.0+1.1% whereas that of automated reticulocyte count was 2.0+1.0%. The relationship between the two methods was expressed as AC = 0.94 MC+0.10, where AC is the automated reticulocyte count shown on y-axis while MC is the manual reticulocyte count shown on x-axis. An r value of 0.97 was ascertained.

Analysis also demonstrated that the mean bias of data was 0.002 with lower limit of agreement (LLOA) of -0.505 and upper limit of agreement (ULOA) of 0.501

DISCUSSION

Technological advancements in methods of analysis of laboratory parameters have been nothing less than a boon for healthcare services and patients alike. It would not be an exaggeration to state that the inherent advantages of automation have revolutionized every aspect of diagnostic services. However, incorporation of new technology within the existing system also has significant potential drawbacks. High cost of maintenance and consumables, specific space and infrastructure requirements, disruption of staff trained in certain methods, generation of potential bottlenecks and increased risk of downtime resulting from malfunction and transition towards a manufacturer-driven laboratory are some of the aspects which need be deliberated upon.[13] Manufacturers' claims regarding performance characteristics of new analytical methods must also be substantiated in real world scenarios before contemplating their addition to the existing test menu. At present, induction of potentially superior technologies into our diagnostic facilities is hampered by the aforementioned considerations. However, with laboratory facilities facing challenges including an ever-escalating workload and rising cost of training and employment of skilled labour, automation is becoming a viable option for an increasing number of laboratories even in relatively resource-constrained settings.

The purpose of our study was to evaluate the performance characteristics of a new method of reticulocyte count, compare it to the well-established and widely-used manual method and to ascertain whether agreement between the two methods was sufficiently adequate for sound clinical decision making.

The recommended method evaluating agreement is by plotting the difference between values obtained by the two methods against their mean. Although no analytical method can be claimed to be error-free, 95% limits of agreement between the proposed method and the reference method are considered a valid measure of agreement. Other methods of statistical analysis including linear regression analysis and paired t-test have been inappropriately applied by many researchers to claim good degree of agreement between a reference method and a proposed new method. Since even a strong correlation does not automatically translate into agreement between two methods, mere employment of linear regression analysis and determination of Pearson's co-efficient may be misleading and does not adequately suffice for furnishing the recommendation of replacement of one method of analysis with another10. Some researchers have incorrectly compared the mean achieved by two methods for the same purpose. It must be noted that paired t-test assesses whether two methods agree on average but gives no information about how well they agree for individual readings.[14]

Utilizing varying methods of statistical analysis, certain aspects of the relationship between the values of reticulocyte count determined by automated and manual methods have previously been studied by many researchers. Simionatto et al reported a systematic error of 0.4%, random error of 3.9% and good correlation (R2 = 0.961) between manual and automated reticulocyte count after conducting a study on 341 patients at Parana´, Brazil in 200915. 80 participants were included by Ali et al in a study conducted at Karachi, Pakistan in 2010. A detailed comparison between manual reticulocyte count and automated count determined by 3 different haematology analyzers was performed and good agreement was demonstrated with 95% values between ULOA and LLOA using Bland-Altman plot, which is in accordance with the results of our study16. Patel et al studied the performance characteristics of the two methods of reticulocyte count including repeatability, reproducibility, linearity and correlation at Gujarat, India in 2019. After evaluating the results from 500 participants, they were able to demonstrate good correlation (coefficient of correlation = 0.9888) and concluded that automated count was rapid, easy to perform and counted high number of cells with precise measurement17. In a 2020 study involving 600 participants at Maharashtra, India, Gangane *et al* opined that there was no significant difference between the two methods of analysis after demonstrating a Pearson's co-efficient of 0.98518. George *et al* studied 86 participants for the comparison between manual and automated reticulocyte count with particular emphasis on effects of specimen storage on results. They concluded that there was strong positive correlation between both manual and automated methods and the differences between the results were not significant at 2, 6 and 24 hours (p = 0.975, 0.967 and 0.227 respectively).[19]

RECOMMENDATIONS

Where resources permit, automated haematology analyzers should be utilized for determining reticulocyte count. Additional studies on automation and precision analysis of haematology analyzers are recommended utilizing the recommended statistical method of construction of Bland-Altman plot before induction into routine laboratory test repertoire.

CONCLUSION

Our findings suggest that automated haematology analyzers can be adopted for determining reticulocyte count with acceptably close results to the widely-used manual method, with added potential benefits of high throughput, rapid turnaround time, low labour-intensiveness and minimization of technical errors.

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Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

UTS & AM: Data acquisition, data analysis, critical review, approval of the final version to be published.

SZ & MB: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

SA & TA: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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