THE AGA KHAN UNIVERSITY

eCommons@AKU

Department of Pathology and Laboratory Medicine | Medical College, Pakistan

November 2010

Is manual reticulocyte count a reliable option for under resourced countries

Afsheen Farzand Ali
Aga Khan University

Bushra Moiz
Aga Khan University

Sadia Omer
Aga Khan University

Follow this and additional works at: http://ecommons.aku.edu/pakistan_fhs_mc_pathol_microbiol

 Part of the Pathology Commons

Recommended Citation

Ali, A., Moiz, B., Omer, S. (2010). Is manual reticulocyte count a reliable option for under resourced countries. *Journal of the Pakistan Medical Association, 60*(11), 892-6.
Available at: http://ecommons.aku.edu/pakistan_fhs_mc_pathol_microbiol/24

Original Article

Is manual reticulocyte count a reliable option for under resourced countries?

Afsheen Farzand Ali, Bushra Moiz, Sadia Omer
Section of Haematology, Department of Pathology and Microbiology, The Aga Khan University, Karachi, Pakistan.

Abstract

Objectives: To establish the credibility of manual reticulocyte counts without compromising the quality of care, and to evaluate the degree of acceptability of manual reticulocyte counts in terms of accuracy and cost effectiveness in comparison with two automated haematology analyzers.

Methods: Visual reticulocyte enumeration was evaluated for comparability, within-batch precision and costing with respect to Coulter® STKS and Gen S haematology analyzers.

Results: The results of reticulocyte estimation for 80 samples as obtained by 3 modes were correlated using Pearson's correlation coefficient (r) which were computed as 0.884 , 0.875, and 0.793 for manual-Gen.S, Gen.S-STKS and manual-STKS respectively thus showing positive association of these results. STKS had the CV of 10.4% and was more precise compared to Coulter® Gen.S (CV=11.6%) while manual counts showed the least precision with a CV of 19.8%. The cost per test was calculated to be $ 0.11 for manual technique in contrast to $0.45 for Gen S and $1.09 for STKS.

Conclusion: Visual counting of reticulocytes can be used as a reliable tool for estimating reticulocytes in resource strained countries as it is not only cost effective but can also efficiently discriminate between high and low reticulocyte ranges which are required for sound clinical judgment (JPMA 60:892; 2010).

Introduction

Reticulocytes are immature red blood cells, which contain intracellular Ribonucleic acid (RNA), Mitochondria and Ribosomes.[1] The significance of reticulocyte count in the diagnosis of anaemia cannot be underestimated as it provides vital information about the classification and pathogenesis of anaemia.[2] Reticulocyte count is the index of erythropoietic activity within bone marrow.[3] Hence, reticulocytosis would depict increased erythropoiesis in response to various clinical scenarios like blood loss, haemolysis or post successful therapy in iron, vitamin B12 or folate deficiency states. Similarly, conditions such as untreated nutritional anaemia or bone marrow failure would suppress red cell production and thus the reticulocyte count.[4] Enumeration of reticulocytes can aid in monitoring the response of erythropoietin therapy in chronic renal failure[5] and may also herald post chemotherapy or transplant marrow recovery in aplastic anaemia or malignant disease.[6] Traditionally, reticulocyte quantification had relied upon microscopic techniques but recently automated reticulocyte analysis has become widely available.

However, in countries like Pakistan, manual technique is still the most common procedure utilizing a trained microscopist and supra-vital stains like methyelene blue. Although relatively simple, it is flawed with 25-50% inter-observer variation.[7-9] The reason may be multi-factorial; like reliance upon the expertise of a technical observer, the use of ocular inset and the homogeneous distribution of reticulocytes in a well spread film and the number of cells counted.[10]

The manual reticulocyte count though inexpensive is tedious and shows low reproducibility. Introduction of automated technologies have greatly increased the accuracy and precision of reticulocyte count with the coefficient of variation (CV) of 3-12.3%.[11] Based on the principle of flowcytometry, reticulocytes are estimated after staining with fluorescent (thiazole orange, auramin-O, cyanene) or non fluorescent (Oxazine 750, new methylene blue) dyes[12] which precipitate residual RNA while an acidic reagent clears haemoglobin. Usually 32,000 red cells are counted and assayed by volume, conductivity and light scatter (VCS). With introduction of maturation indices and volume measurements, automated reticulocyte counting provides a new and meaningful approach to this analysis.[13] The inclusion of these parameters has shown promising results for diagnostic and therapeutic purpose.[14-16] Moreover, the initial expense of automated instruments and their reagents is offset by reduction in time consumption, making them an attractive cost effective option.[17] The presence of Howell jolly bodies, red cells fragments, plasmodia, nucleated red cells, siderotic inclusions, cell debris, large platelets and platelet clumps in the samples, interferes with the proper enumeration of reticulocytes.[18]

In developing and under resourced countries, automation cannot be offered in most laboratories especially in a rural setting. Therefore, the present study was designed with the aim to evaluate the manual reticulocyte counting with two haematology analyzers Coulter® STKS and Gen S in terms of accuracy, precision and cost effectiveness.

Material and Methods

The study analyzed 80 unselected samples (from 50 adults and 30 children) for which reticulocyte count was requested during March 1 - March 31, 2007 at the clinical laboratories of Aga Khan University Hospital. The adults' age range was 15 to 85 years (median 40 years) and paediatric population age range was 0.0 (newborn) to 10 years (median 0.02 years). All samples sent for reticulocyte counting during this study period were included, to ensure sufficient samples in higher values outside the reference range to validate the results. The samples were divided into 2 groups A and B corresponding to reticulocyte range of 0-2, 2.1 and above respectively. The reason for this grouping was our population based reference range which was 0.2-2.0% for adults and children aged 2-12 years.

Five ml of blood was collected in vacutainer containing K3-EDTA (Becton Dickinson) after informed consent and were kept at room temperature throughout testing and analyzed within 6 hours of collection. Reticulocyte analysis of each sample was done through visual technique and by two instruments Coulter® STKS and Coulter® Gen.S (Coulter electronics, Hialeah, Fl, USA).

Briefly, manual counting was performed by mixing 50 µl of new methyelene blue (Sigma-Aldrich®, Germany) with 100 µl of blood sample in a test tube and after 10 minutes of incubation at room temperature, and remixing, a thin smear was prepared. One thousand red cells were counted on each smear of all samples. The percentage of cells containing stained RNA was recorded microscopically through Olympus® BX51 (Japan) by two independent observers who examined all 80 cases individually to minimize subjective variation. The ocular inset was not utilized.

Coulter® STKS is a semi automated while Coulter® Gen.S is a fully automated system. The instruments were used according to manufacturer's instructions and all results were recorded in percentage as our clinicians are more familiar with these values.All instruments were calibrated according to the manufacturer's instructions. Daily start up and shut down procedures were performed as well as all recommended quality control (Coulter® Retic-CTM Cell control, Beckman Coulter, Inc., USA) was run on both instruments on daily basis.

Within batch precision was determined on 5 different routine samples by analyzing each of them, 10 times repeatedly on both instruments. As for the manual method, 10 slides were prepared from each sample and observed by 2 independent observers. The results were expressed as a mean of the two observer readings.

The cost per test for each mode was estimated by adding labour cost to the expense of the reticulocyte reagent used. The latter was calculated by noting the reagent consumption for a period of a month and dividing the volume by the number of tests performed over the period. The cost of equipment was disregarded as these are already established instruments in the clinical lab.

Statistical Analysis

All statistical analysis was done using SPSS version 14.0 (SPSS, Chicago, IL, USA) and Med Calc® (Med Calc software version 9.6.3.0, Mariakerke, Belgium). Descriptive statistics including mean (± standard deviation) for quantitative variable were used. Linear relationship and the distribution of reticulocyte measurements by three methodologies were studied through scatter-plot and histogram and were found to be normal. Pearson correlation

coefficient (r) was utilized for determining the strength of linear association between various results for groups A (reticulocyte count 0-2%) and B (counts 2.1% and above) separately. The threshold for significance was 0.05 for two-tailed test. Bland and Altman plots[19] were used to calculate mean difference (bias) and agreement between three methodologies; it was considered that 95% of all values lying within ±2SD indicate good agreement. When comparing a test method against reference method, it is important to interpret that the two can be used interchangeably if there is no random, absolute or proportional bias between the two. Random error can be estimated through correlation coefficient (r). The absolute (or constant) and proportional errors are defined respectively as fixed, and percentage change, in results given by test method when compared with reference method. These can be analyzed utilizing linear regression models such as Passing and Bablok.[20] This equation states that there is no statistical difference between reference and test method if respective confidence intervals include a slope of (B) 1 and intercept (A) of zero indicating absence of absolute and proportional bias respectively.

Results

During the study period, 80 samples were collected from 44 (55%) males and 36 (45%) females. The ages of these patients showed considerable heterogeneity ranging from 0.0 (newborn) to 85 years (median 23 years). Manual reticulocyte results ranged from 0.2 to 12.7%, while automated results from Gen.S and STKS ranged from 0.46 to 14.5 and 0.1 to 16.6% respectively.

Reticulocyte counts (mean ± 1.0 SD) were 2.7 ± 2.4% and 2.6 ± 1.88% by STKS and Gen.S respectively. Manual method gave marginally lower results at 2.4% ± 2.07%.

The results of reticulocyte estimation obtained by 3 modes were correlated using Pearson's correlation coefficient (r). These were computed as 0.884 (p=0.000), 0.875, (p=0.000) and 0.793 (p=0.000) for manual-Gen.S, Gen.S-STKS and manual-STKS respectively thus showing positive association of these results. Group A (reticulocyte count <2.0 %) and group B (reticulocyte count >2.1%) also showed similar linear association for the reticulocyte estimations by the three techniques (Table-1).

Table-1: Correlation Coefficient for reticulocyte enumeration (Groups A & B) through visual technique, Coulter Gen.S and Coulter STKS.

Group A (n=40)	Manual-Gen S	Manual- STKS	Gen S-STKS
r	0.698	0.562	0.5770
P-value	<0.0001*	0.0002*	0.0001*
95%CI	0.494-0.829	0.304-0.734	0.324-0.753
Group B (n=40)	**Manual-Gen S**	**Manual- STKS**	**Gen S-STKS**
r	0.841	0.704	0.850
P-value	<0.0001*	<0.0001*	<0.0001*
95%CI	0.717-0.913	0.503-0.833	0.732-0.918

* Statistically significant P-value.
Where r=coefficient of correlation and CI = confidence interval.

Table-2: Comparison of three techniques using Passing and Bablok equation.

Group A	Manual-Gen S	Manual-STKS	GenS-STKS
Intercept A	0.18	0.62	0.57
95%CI	-0.13 to 0.42 (=0)	0.38 to 0.81	0.29 to 0.8
Slope B	0.61	0.43	0.76
95%CI (=1)	0.44 to 0.83	0.28 to 0.63	0.48 to 1.06
Group B	**Manual-Gen S**	**Manual-STKS**	**GenS-STKS**
Intercept A	-0.50	1.12	1.38
95%CI	-1.86 to 0.38 (=0)	0.25 to 1.77	0.66 to 1.86
Slope B	1.25	0.59	0.53
95%CI	0.85 to 1.58 (=1)	0.38 to 0.86	0.39 to 0.71

The remarks (=0) or (=1) are added for where 95% confidence interval for intercept A or slope B equals 0 or 1 respectively for absolute and proportional bias.

Regression equations (calculated by Passing and Bablok) along with the calculated values for groups A and B are summarized in Table-2. Absolute bias was seen in STKS against both manual and Gen.S while proportional bias was seen in manual counting vs. automation in group A. However, in group B, Visual counting and GenS showed good correlation, however both intercepts and slopes were observed in manual vs. STKS and Gen.S vs. STKS. An inter-method bias was observed as reticulocyte percentage was consistently higher on GenS compared to visual

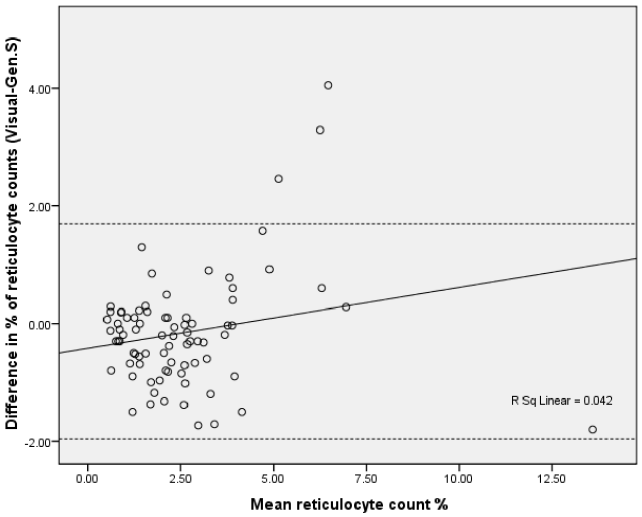

Figure: Difference against average of Visual and Gen.S measurements, with 95% limits of agreement (broken line) and regression line.

counting (Figure) and on STKS than Gen.S. This was particularly evident when percentage of reticulocyte count was < 5. No inter-method bias was observed in visual and STKS results.

In batch precision of investigated methods was tested by serial measurements of 5 routine samples 10 times. We found that STKS had the CV of 10.44% and was more precise compared to Coulter® Gen.S (CV=11.68%). Inter-assay precision of the visual count was determined by preparing 10 separate blood films from one sample. Mean reticulocyte count was 2.6±0.53 and as expected showed least precision in comparison to automation with a CV of 19.85%

The cost per test was calculated to be $ 0.11 for manual technique in contrast to $0.45 for Gen S and $1.09 for STKS.

Discussion

Reticulocyte enumeration is an important indicator of bone marrow erythropoiesis[3] which is required by clinicians in a number of clinical situations.[2] Because of its diagnostic and therapeutic implications; it is usually the most commonly requested test in the evaluation of anaemia.[4] Visual reticulocyte counting is a widely utilized and accepted test in our laboratories, as it is highly cost effective in comparison to automation. However, a number of studies have shown this technique to be time intense, tedious, inaccurate as well as imprecise when compared with automation.[10] The present study was undertaken to compare visual counting with automation in order to assess the feasibility of various methodologies in Pakistan.

The study showed a high degree of correlation between visual counting and automation similar to previously reported results.[21] However, such positive association between manual and automation techniques has been denied by others.[22] It is interesting to note that the results are comparable to those studies that have used similar Coulter®systems as used here. Perhaps the difference in visual counting and automation may be attributable to various haematology analyzers utilized in various studies.

It was observed that the three techniques were positively correlated in lower count as well as at higher reticulocyte counts with statistically significant results. Coulter® STKS gave 5 unreliable high counts compared to other two techniques. Our study did not address specifically the cause of this discrepancy, but it is well known that interference factors like nucleated red cells, Howell Jolly bodies, or other red cell inclusions as well as haemoglobinopathies or high white cell count can result in such erroneous results.[8,23] It has been suggested that such implausible results should be counterchecked through differential counts and or manual reticulocyte counting.[24] The results analyzed through the three techniques showed good agreement as majority of results lie within 95% limits of confidence interval. It was also generally observed that such correlations became weak with the increase in difference in measurements. Also, reticulocyte analysis by STKS was consistently higher compared to results by visual and Gen.S.

In batch variability was determined in routine samples by all three methods. The obtained CV values were between 10.4-11.7 % for 2 automations. The fine precision of automated counting has been observed in numerous previous studies evaluating various haematology analyzers.[24,25] In CAP reticulocyte project report, it was recommended that for an automated system r-value should be greater than 0.95 and CV should be 15% or less.[7] The automated reticulocyte count evaluated in our study met this criterion of CV. Visual counting showed less reproducible and more variable results with a CV of 19.85%. Such results were not unexpected as they have been described before.[7,10] The reason for this variability may be due to sample staining, because of less number of cells being counted compared to automation, inaccurate identification of reticulocytes, non uniform distribution on smear, or differences in technologists' experiences in visual counting.[25] It has been observed that inconsistent microscopy rather than sample staining is the single most important factor responsible for this inaccuracy.[21] To overcome this inherent problem associated with visual counting, we utilized 2 microscopists with different experiences and the results were expressed as average of their counts.

When one compares the cost of manual counting vs. automation in our setting, it is usually the former which is least expensive as seen in our study also. The reason may be partly because of low labour cost in our country in comparison to automation which requires expensive imported consumables. This is in sharp contrast to reports from developed countries where instrumentation has been described to be more cost effective.

The present study indicated that manual reticulocyte counting is more cost effective but less precise. A linear relationship with respect to automations was observed at lower as well as higher reticulocyte counts. Hence, visual technique can aid in the interpretation of clinical cases and therefore would facilitate a clinician's judgment which will not be any different from the one made on the basis of automated results.

The present study was evaluated to have certain limitations in terms of lack of clinical details, and not

analyzing the various techniques for linearity, carry over, stability of reagents and between-batch precision. However, we feel that our study in spite of limitations sufficed our purpose.

Hence, automated techniques owing to its higher cost should be limited to clinical laboratories associated with tertiary care hospitals. Small laboratories in urban areas and those in rural setting can perform visual reticulocyte counting with considerable degree of confidence. This will be economical for them as well as cost effective from a patient's point of view. However, in patients where the results do not correlate clinically, reticulocyte analysis should be done by automation. This can easily be achieved by sending them to larger clinical laboratories that can act as a referral centre for them.

Acknowledgement

We would like to thank the Aga Khan University Hospital for utilizing their resources.

References

1. Bain BJ. Performing a blood count. In: Blood Cells. A Practical. Guide. 3rd ed. Oxford, England: Blackwell Science; 2002:16-51.
2. Houwen B. Reticulocyte maturation. Blood cells 1992,18: 167-86.
3. Ryan DH. Examination of the blood. In: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. Williams Hematology. 7th ed. New York, NY: McGraw-Hill; 2005: 11-20.
4. Fourcade CH, Belaouri LJH. Reticulocyte analysis provided by the Coulter GEN.S: significance and interpretation in regenerative and non-regenerative hematological conditions. Lab Haematol 1999; 5: 153-8.
5. Goodnough LT, Skikne B, Brugnara C. Erythropoietin, iron, and erythropoiesis. Blood 2000; 96: 823-33.
6. Davies SV, Cavill I, Bentley N, Fegan CD, Poynton CH, Whittaker JA. Evaluation of erythropoiesis after bone marrow transplantation: quantitative reticulocyte counting. Br J Haematol 1992; 81: 12-7.
7. Savage RA, Skoog DP, Rabinovitch A. Analytic inaccuracy and imprecision in reticulocyte counting: a preliminary report from the College of American Pathologists Reticulocyte Project. Blood Cells 1985; 11: 97-112.
8. Davies JI, Smyth MS, Martin JHJ. Automated reticulocyte counting: evaluation of the Coulter® STKS hematology analyzer reticulocyte counting function. Clin Lab Haem 1997; 19: 89-92.
9. Peng L, Yang H, Jiang H, Su J, Peng Z. Automated reticulocyte counting using the Sysmex RAM-1. Clin Lab Haem 2001; 23: 97-102.
10. Peebles DA, Hochberg A, Clarke TD. Analysis of manual reticulocyte counting. Am J Clin Pathol 1981; 76: 713-7.
11. Yu PH, So CC, Wong KF, Lee KC, Chow CS, Yip LK et al. Automated reticulocyte counting--an evaluation of GEN-S, Cell-Dyn 3500 and Cell-Dyn 4000. Clin Lab Haematol 1999; 21: 145-7.
12. Davis BH, Bigelow NC, Koepke JA, Borowitz MJ, Houwen B, Jacobberger JW et al. Flow cytometric reticulocyte analysis. Multi institutional inter-lab correlation study. Am J Clin Pathol 1994; 102: 468-77.
13. Briggs C, Grant D, Machin SJ. Comparison of the automated reticulocyte counts and immature reticulocyte fraction measurements obtained with the ABX Pentra 120 retic blood analyzer and the Sysmex XE-2100 automated hematology analyzer. Lab Hematol 2001; 7: 75-80.
14. Rodriques A, Ortega C, Santos L, Teixeira A, Dinis MJ, Vasconceros I et al. Clinical Utility Of Beckman-Coulter Gen's Reticulocyte Analysis in the Study of Anemia of Chronic Disease (ACD). Lab Haem 2007; 13: 85-92.
15. Kim MH. Clinical significance of reticulocyte maturation parameters in differential diagnosis of macrocytic anemias. Korean J Lab Med 2007; 27: 13-18.
16. Noronha JF, Grotto HZ. Measurement of reticulocyte and red blood cell indices in patients with iron deficiency anemia and beta-thalassemia minor. Clin Chem Lab Med 2005; 43: 195-97.
17. Bowen D, Bentley N, Hoy T, Cavill I. Comparison of a modified thiazole orange technique with fully automated analyzer for reticulocyte counting. J of Clin Pathol 1991; 44: 130-3.
18. National Committee for Clinical laboratory standards. Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes); Approved Guideline-Second Edition, National Committee for Clinical laboratory Standards. NCCLS document H44-A; 1997. (Online) 2004. (Cited 2009, September 3). Available from URL: http://www.clsi.org/source/orders/free/H44-A2.pdf.
19. Bland JM, Altman DG. Comparing methods of measurement: why plotting difference against standard method is misleading. Lancet 1995; 346: 1085-7.
20. Passing H, Bablok W. A new biomedical procedure for testing the equality of measurements from two different analytic methods. Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. J Clin Chem Clin Biochem 1983; 21: 709-20.
21. Brugnara C, Hipp MJ, Irving PJ, Lathrop H, Lee PA, Minchelo EM, et al. Automated reticulocyte counting and measurement of reticulocyte cellular indices. Evaluation of the Miles H*3 blood analyzer. Am J Clin Pathol 1994; 102: 623-32.
22. Lofness KG, Kohnke MI, Geier NA. Evaluation of automated reticulocyte counts and their reliability in the presence of Howell-Jolly bodies. Am J Clin Pathol 1994; 101: 85-90.
23. Lamchiagdhase P, Pattanapanyasat K, Muangsup W. Reticulocytes counting in thalassemia using different automated technologies. Lab Hematol 2000; 6: 73-8.
24. Lohmann RC, Crawford LN, Wood DE. Proficiency testing in reticulocyte counting. Clin Lab Haematol 1994; 16: 57-64.
25. Bossche JVD, Devreese K, Malfait R, Vyvere MVD, Schouwer PDe. Comparison of the reticulocyte mode of the Abx Pentra 120 Retic, Coulter General-S, Sysmex SE 9500, Abbott CD 4000 and Bayer Advia 120 haematology analysers in a simultaneous evaluation.. Clin Lab Haematol 2001; 23: 355-60.