Journal of Clinical Laboratory Analysis 24 : 252–255 (2010)

Flow Cytometric Reticulocyte Counting: A Comparison Between Two Methods

M. Maconi,[1]* P. Danise,[2] L. Cavalca,[2] and D. Formisano[3]

[1]*Department of Laboratory Medicine, AO Arcispedale Santa Maria Nuova, Reggio Emilia, Italy*

[2]*Department of Oncology Hematology, ASL Salerno1, Salerno, Italy*

[3] *Unit Statistical and Clinical Epidemiology, AO Arcispedale Santa Maria Nuova, Reggio Emilia, Italy*

The peripheral reticulocyte count is commonly used as an indicator of the erythropoietic activity of the bone marrow. Manual counting provides results with a high degree of inaccuracy and imprecision. Automation of counting is therefore needed. The increase in the number of methods available requires however that the results from the various methods agree with one another. The aim of our study was to evaluate the analytic performance of two automated hematology analyzers by a parallel study. We compared the analyzers between them and with manual counting. We enrolled in our study a total of 100 healthy subjects and an additional 80 patients affected by various hematological diseases. Difference between methods is statistically significant: the reference intervals of ADVIA2120 are higher than the Sysmex XE-2100. The correlation between methods and correlation with the microscopic method are excellent and statistically significant. In conclusion, we can affirm that total automation of reticulocyte counts represents a definite improvement over microscopic counts. This study confirms the diversity of the reference intervals still exists in the new automated hematology analyzers. J. Clin. Lab. Anal. 24:252–255, 2010.

Key words: reticulocyte; flow cytometric count; erythropoiesis; reference intervals; blood disease

INTRODUCTION

The peripheral reticulocyte count is commonly used as an indicator of the erythropoietic activity of the bone marrow. It is essential for the diagnosis, classification, and the monitoring of the treatment of anemias, as well as in the follow up of the bone marrow regeneration after intensive chemotherapy, bone marrow transplantation, and in the follow-up hemopoietic restoration during or after erythropoietin therapy (1,2). The ICSH standard (3) proposes a microscopic reference method for reticulocyte counts. The reticulocytes are identified by optical microscopy, using supravital stain (brillant cresyl blue, new methylene blue) that binds to ribosomal RNA. The percentage of reticulocytes among the total erythrocytes and the total reticulocytes count per microliter of blood are calculated. Manual counting of a large number of cells (minimum 1,000 erythrocytes, including reticulocytes) provides results with a high degree of inaccuracy and imprecision and a large inter- and intra-observer variability (4,5).

Automation of counting is therefore needed. Reticulocyte counting by flow cytometry using a large variety of fluorescent dyes (thiazole orange, acridine orange, thioflavin, Pyronin Y, Auramine-O) that bind to ribosomal RNA, yields more reliable results. The degree of fluorescence emission caused by the excitation (at 488 nm with an argon-laser light)of the bound dye is proportional to the amount of RNA in erythrocyte. During the last years, flow cytometry has become the reference technique for measuring absolute reticulocyte counts and parameters of reticulocyte maturation (6–10).

Problems still exist that essentially depend on the differing sensitivities of the dyes used to stain the RNA of reticulocytes, on the technology used to identify positive cells (fluorescence, light scattering, absorbance)

*Correspondence to: M. Maconi, Department of Laboratory Medicine, AO Arcispedale Santa Maria Nuova, Viale Risorgimento, 87, 42100 Reggio Emilia, Italy. E-mail: mariacaterina.maconi@asmn.re.it

Received 11 December 2009; Accepted 24 May 2010

DOI 10.1002/jcla.20394

Published online in Wiley InterScience (www.interscience.wiley.com).

and the software that is more or less capable of separating reticulocytes from erythrocytes (as there is a physiologic continuum between these populations) and from other cells such as platelets or nucleated red blood cells (NRBCs). A further limit is due to the different performances on samples with severe reticulocytopenia (11,12). Differences also have been noted in reticulocytosis (13), but rarely do these have implications for clinical decision making, as in patients with reticulocytopenia.

The aim of our study was, through the use of the aforementioned guidelines, to evaluate the analytic performance of two automated hematology analyzers by a parallel study in a single institution. We compared the two analyzers between them and with manual counting in blood samples from healthy individuals and patients with blood disease.

MATERIALS AND METHODS

Specimens

We enrolled in our study a total of 100 healthy subjects selected for calculation of the reference intervals for the two analyzers. The subjects were males and females 4–58 years old. From these subjects only 50 samples were used to compare manual reference method vs. automated method. An additional 80 patients affected by various diseases or conditions (acute and chronic inflammations, neoplastic diseases, patients who had undergone intensive chemotherapy, nutritional anemia, hematological disorders) were examined.

Automated Reticulocyte Count

K_3EDTA blood specimens were analyzed within 3 hr after collection using the two automated hematology analyzers: Sysmex XE-2100 (Sysmex Corporation, Kobe, Japan) and ADVIA 2120 (Siemens Healthcare Diagnostics Inc., Illinois, USA).

These instruments analyze tens of thousands of cells per sample and, with stains that selectively bind to the RNA of reticulocytes, they can separate stained cells (reticulocytes) from nonstained (erytrocytes) and from those whose size or level at staining place them outside the reticulocyte gate (platelets, NRBCs, leukocytes). The XE-2100 reticulocyte count was based on the staining of intracellular RNA with the fluorescent basic dye auramine-O. The fluorescent intensity produced by an argon-laser light source is plotted against the forward light scatter, which reflects cell size. Reticulocytes are separated from mature cells on the basis of the fluorescence they emit. The ADVIA 2120 reticulocyte count was based on the staining of reticulocyte RNA using the dye oxazine 750 and reticulocyte enumeration is determined by helium-laser light absorption and light scatter at low angle (2°–3°) and high angle (5°–15°). An absorption threshold is used to separate stained reticulocytes from unstained RBCs. The stains and general principle used are reported in Table 1.

TABLE 1. Analytic Method of Reticulocyte Counters: Stains and General Principle Used by the ADVIA 2120 and XE-2100

Analyzer	Dye	Technology
ADVIA2120	Oxacine750	Absorbance
XE-2100	AuramineO	Fluorescence

The instruments were calibrated according to the specifications of the manufacturers by specialists from Italian representatives of companies at the time of installation. During evaluation they were checked three times a day using a multilevel control supplied by each manufacturer.

The percentage of reticulocytes in total erythrocytes (Ret%) and the total reticulocyte count per microliter (Ret♯) are given by the instrument.

Manual Reticulocytes Count

The wedge films were prepared within 2 hr after collection for microscopic assessment. The new blue methylene method was used (Sigma diagnostics, St Louis, MO), and reticulocytes were counted according to the indications of the ICSH (3). Two blood smears of each sample were prepared and all the erythrocytes and reticulocytes were counted for each film until a count of at least 1,000 RBCs was reached by two qualified senior technologists. The mean value obtained was used for comparison with the automated methods.

Statistical Analysis

Statistical analysis was made with the use of Analyse-it software, version 1–71, Clinical Laboratory & General module (Analyse-it Software Ltd., Leeds, England, UK) add-in for Microsoft Excel. Summary distribution of hematological and biochemical parameters were evaluated using the nonparametric statistic (median, 2.5° and 97.5° percentile). Coefficients of correlation were calculated by the Pearson's method. Agreement between methods was evaluated by Altman–Bland analysis.

RESULTS

Reticulocyte reference intervals in percentage and absolute values of the two tested methods in healthy subjects are given in Table 2. As the distribution is approximately log-normal, the intervals were calculated with a nonparametric method. Difference between the two methods are statistically significant (Wilcoxon test

TABLE 2. Reticulocyte Reference Intervals in Healthy Subjects ($N = 100$)

		ADVIA 2120	XE 2100
Reticulocyte count (%)	Median	1.41	1.22
	2.5–97.5° percentile	0.84–2.56	0.75–2.13
Reticulocyte count ($\times 10^3\mu l$)	Median	68.1	57.25
	2.5–97.5° percentile	32.79–118.87	32.79–93.52

Reference intervals in percentage and absolute values of the 2 tested methods.

TABLE 3. Comparison With Microscopic Methods

Method	*r*-value	*P*-value
ADVIA2120	0.992	0.000
XE-2100	0.992	0.000

Comparison of the two tested instruments (percentage count) with the microscopic reference method. Significance was assumed at $P < 0.01$.

TABLE 4. Comparison of the Reticulocyte Count (Percentage and Absolute Count) Between the Two Methods

	Reticulocyte count (%)		Reticulocyte count ($\times 10^3\mu l$)	
	r-value	*P*-value	*r*-value	*P*-value
ADVIA 2120 vs. XE 2100	0.95	<0.0001	0.93	<0.0001

Significance was assumed at $P < 0.001$.

$P < 0.05$): the reference intervals of ADVIA2120 are higher than the Sysmex XE-2100. The results agree with previous published results obtained for the SE 9500RET and ADVIA 120 (7,11,12,14).

The comparison between the manual reference method and tests according to the NCCLS H44-A recommendations (7) shows a statistically significant correlation with the microscopic method (Table 3).

Pearson correlation between ADVIA 2120 and XE 2100 are shown in Table 4.

The correlation between the two methods is excellent: $r = 0.95$ (95% CI = 0.92 to 0.97) for the percentage of reticulocytes in total erythrocytes and $r = 0.93$ (95% CI = 0.89 to 0.96) for the total reticulocyte count per microliter.

The correlation between the two methods is also statistically significant: $P < 0.0001$.

Bias plot analysis shows a statistically significant difference between the two methods: ADVIA 2120 data are higher than XE-2100 ones, as found also for the reference intervals in healthy subjects (Table 5).

TABLE 5. Bias Plot Analysis of the Two Methods

		Bias	95% IC
Reticulocyte count (%)	ADVIA2120 vs XE-2100	0.285	0.177–0.392
Reticulocyte count ($\times 10^3\mu l$)	ADVIA2120 vs XE-2100	13.362	8.759–17.966

Other studies already published have shown similar results (14).

DISCUSSION

The enumeration of peripheral blood reticulocytes ("reticulocyte counting") is often performed to obtain information about the functional integrity of the bone marrow as the reticulocyte count reflects the erythropoietic activity of the bone marrow, the rate of reticulocyte delivery from the bone marrow into the peripheral blood, and the rate of reticulocyte maturation. Accurate reticulocyte enumeration is critical for the diagnosis of many hematologic diseases, for the classification of patients with anemia, in monitoring bone marrow regenerative activity after chemotherapy or bone marrow transplantation (18). A simple, rapid, and reliable method that replace manual vision counts, which is subjective and highly imprecise, for counting reticulocyte could be useful in clinical practice.

An automated reticulocyte count is now considered as a necessary blood parameter. Comparing with manual reticulocyte counting, the automatic techniques have led to a substantial improvement in the accuracy and precision of reticulocyte counts and in the acquisition of new parameters for measuring reticulocyte maturation. Some of these other reticulocyte parameters, such as the immature reticulocyte fraction or the reticulocyte indices, such as the mean reticulocyte volume or the mean hemoglobin content, have been shown to be useful in several clinical conditions (15–16).

The increase in the number of methods available requires however that the results from the various methods agree with one another: reticulocyte enumeration is the subject of several reviews.

In this study, we perfomed a comparison between two methods for reticulocyte counting which to our knowledge has not yet been compared. We obtained reference intervals in healthy subjects with the two different methods: we found that reference intervals obtained with ADVIA 2120 are higher than obtained with XE-2100. The comparison (percentage values) of the two methods with the microscopic reference method showed generally satisfactory behavior.

These data were confirmed by data from previous studies, even when the comparison was based on different methods for microscopic counting (11,12,17).

We compared the two methods also in patients affected by various diseases to evaluate the performance of these two automated methods in reticulocytopenic and reticulocytosis cases: the need for low imprecision in tests used in these reticulocyte disorders can be justified in certain clinical applications such as monitoring the early erythropoietic response. We found an excellent statistically significant correlation between the two methods, better for the elevated concentrations than for low concentrations. As we found in healthy subjects, also in the patients ADVIA 2120 data are higher than XE-2100.

In conclusion, we can affirm that total automation of reticulocyte counts represents a definite improvement over microscopic counts. This study confirms the diversity of the reference intervals. All these results in counts that sometimes disagree with another depending on the method with the consequent need for reference intervals that are method specific (11). In fact, the use of different automated methods creates the problem of incomplete agreement of the counts with the consequent necessity of method-specific reference intervals.

REFERENCES

1. Lesesve JF, Lacombe F, Bernard PH. Les reticulocytes. Rev Fr Lab 1993;52:57–63.
2. Lesesve JF, Lacombe F, Bernard PH, Belloc F, Boisseau MR, Reiffers J. Apport du compteur de reticulocytes Sysmex R-100 pour le suivi de patients en aplasie post-therapeutique. Rev Fr Lab 1993;252:65–69.
3. International Council for Standardisation in Hematology Expert Panel on Cytometry. Proposed reference method for reticulocyte counting based on the determination of the reticulocyte to red cell ratio. Clin Lab Haematol 1998;20:77–79.
4. Goguel A. omptage des reticulocytes sur frottis: Limite de confiance et utilization des resultats. Rev Fr Lab 1991;225:20–22.
5. 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;1:97–112.
6. Tanke HJ, Rothbarth PH, Vossen JMJJ, et al. Flow cytometry of reticulocytes applied to clinical hematology. Blood 1983;61:1091–1097.
7. National Committee for Clinical laboratory Standards. Methods for reticulocyte counting: Proposed standards. Villanova PA: National Committee for Clinical Laboratory Standards, 1985. NCCLS document H16-P.
8. Davis BH, Bigelow NC. Flow cytometric reticulocyte quantification using thiazole orange provides clinically useful reticulocyte maturity index. Arch Pathol Lab Med 1989;113:684–689.
9. Fergusson DJ, Lee SF, Gordon PA. Evaluation of reticulocyte counts by flow cytometry in a routine laboratory. Am J Hematol 1990;33:13–17.
10. National Committee for Clinical laboratory Standards. Methods for reticulocyte counting (flow cytometry and supravital dyes); approved guidelines. Villanova PA: National Committee for Clinical Laboratory Standards, 1997. NCCLS document H44-A.
11. Buttarello M, Bulian P, De Pra' M, et al. Reticulocyte quantification by Coulter MAXM VCS (volume, conductivity, light scatter) technology. Clin Chem 1996;42:1930–1937.
12. Buttarello M, Bulian P, Venudo A, et al. Laboratory evaluation of the Miles H3 automated reticulocyte counter: A comparative study with manual reference method and Sysmex R-1000.Arch Pathos Lab Med 1995;119:1141–1148.
13. Davis BH, Spier CM, Kachin J, et al. College of American Pathologists' reticulocyte proficiency testing program using surrogate blood: Insights into contemporary clinical practice. Lab Hematol 1997;3:84–91.
14. Buttarello M, Bulian P, Farina G, Temporin V, et al. Flow Cytometric reticulocyte counting. Parallel evaluation of five fully automated analyzers: An NCCLS-ICSH approach. Am J Clin Pathol 2001;115:100–111.
15. Davis BH. Immature reticulocyte fraction(IRF): By any name, a useful clinical parameter of erythropoietic activity. Lab Hematol 1996;2:2–8.
16. Brugnara C, Zurakawski D, Di Canzio J, et al. Reticulocyte hemoglobin content diagnose iron deficiency in children. J Am Med Assoc 1999;281:2225–2230.
17. Rudensky B. Comparison of semi-automated new Coulter methylene blue method with fluorescence flow cytometry in reticulocyte counting. Scand J Clin Lab Invest 1997;57:291–296.
18. Lazarus HM, Chahine A, Lacerna K, et al. Kinetics of erythrogenesis after bone marrow transplantation. Am J Clin Pathol 1992;97:574–583.