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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

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ABSTRACT

Automated counting of reticulocytes has greatly increased the precision and accuracy of this assay compared with traditional manual counts. In addition, reticulocyte maturity can now be assessed based on the staining intensity of the reticulocytes, which is proportional to their RNA content. Newly produced or immature reticulocytes may be defined by a relatively high degree of RNA staining, whereas more mature forms show less staining. This difference in staining makes possible the identification of the youngest highly fluorescent reticulocytes prematurely delivered into the circulation from the bone marrow in conditions of increased erythropoietic stimulation or during regeneration of erythropoietic activity in patients receiving bone marrow or stem cell transplants. We evaluated reticulocyte counting and measurement of the immature reticulocyte fraction (IRF) with the ABX Pentra 120 Retic blood analyzer (Montpellier, France) using over 300 blood samples. Results were compared with those obtained from the Sysmex XE-2100 Automated Hematology analyzer (Sysmex Corporation, Kobe, Japan). Comparison of the 2 methods showed excellent correlation for reticulocyte percentage and absolute count (Pearson product moment [*r*] = 0.95 for both parameters). There was also good correlation between the IRF expressed by the ABX Pentra 120 Retic and the Sysmex XE-2100 (*r* = 0.77), although the ABX Pentra gave significantly higher values than did the XE-2100. This difference in values is a result of methodological differences in deriving the IRF measurements, ie, the influence of both the fluorescent stain used and the software thresholds for the IRF. Because the IRF has now been proven to be a useful clinical parameter, there needs to be some standardization and calibration of IRF methods. *Lab. Hematol.* 2001;7:75-80.

KEY WORDS: Reticulocyte count · Immature reticulocyte fraction · Automated hematology analyzer · Anemia · Standardization

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INTRODUCTION

The reticulocyte count has evolved into one of the basic tests in diagnostic hematology for assessing erythropoietic activity. It is essential for the diagnosis, classification, and treatment monitoring of anemias, for the confirmation of bone marrow regeneration after chemotherapy or transplantation, and in the monitoring of hemopoietic restoration during or after erythropoetin treatment. Traditional visual counting by light microscopy of whole blood with reticulocyte

RNA stained by supravital dyes (such as brilliant creysl blue or new methylene blue) has been the established practice [1]. However, visual counting is labor intensive and imprecise, with interobserver coefficients of variation between 25% and 50% [2,3].

During the last few years the use of a dedicated flow cytometer has become the reference technique for measuring reticulocyte counts and parameters of reticulocyte maturation [4]. Compared to manual counting, these automatic techniques have led to improvement in the accuracy and precision of reticulocyte counts [5] and have provided the ability to reliably measure new parameters of reticulocyte maturation. These new parameters are based on the measurement of the RNA content of red blood cells (RBCs) and were originally called the reticulocyte maturity index (RMI) [4], but more recently "immature reticulocyte fraction (IRF)" has become the internationally accepted term. This reticulocyte grading, rather than just the reticulocyte count, may be a superior parameter in the monitoring of the bone marrow erythropoietic activity [4,6].

Several fully automated hematology analyzers that provide a reticulocyte count and maturity grading based on flow cytometric techniques are now available, and this more complete reticulocyte analysis is increasingly becoming routine laboratory practice. Different analyzers use different reagents and techniques to measure the reticulocytes. The Coulter STKS, MAXM, and GEN-S (Beckman Coulter, Miami, FL) and the Abbott Cell-Dyn 3500 and 3700 (Abbott Diagnostics, Santa Clara, CA) methods employ the supravital stain new methylene blue, and the RNA-containing RBCs are stained and counted by volume, conductivity, and light scatter [7]. Among other automated instruments, various fluorescent or absorbent dyes and lasers are used: Thiazole Orange for ABX instruments, Auramine-O for the Sysmex R series, a polymethine dye for the Sysmex XE-2100, Oxazine 750 for the Bayer Advia 120 (Technicon, Tarrytown, NY) and a cyanine dye, CD4K530, on the Cell-Dyn 4000 (Abbott Diagnostics). Intermethod biases have been well documented for both reticulocyte counts and the IRF measurements, however, most authors have not thought these differences to be clinically significant [8,9].

The purpose of this study was to compare the reticulocyte counting and IRF results from the ABX Pentra 120 Retic blood analyzer with those obtained from the Sysmex XE-2100. Previous studies have included appraisals of reticulocyte precision, linearity, carryover, and stability for both the ABX Pentra 120 Retic [10] and the Sysmex XE-2100 [11], so these studies were not repeated.

MATERIALS AND METHODS

Residual K_3EDTA anticoagulated samples were taken from the routine hematology workload. A total of 302 either normal or pathological samples were analyzed for reticulocyte counts and indices by both methods within 4 hours of specimen collection. The pathological samples were selected to represent a wide range of reticulocyte counts; these were essentially from adult patients with malignant hematological disease, hemoglobinopathies, iron deficiency, or anemia with chronic disease. Normal samples were from healthy volunteer donors.

Recommended quality control was run on both the ABX Pentra 120 Retic and the Sysmex XE-2100 instruments on a daily basis. ABX Minotrol was used to monitor the complete blood count (CBC) values and Minotrol Retic for reticulocyte quality control. Sysmex CBC and Retic Check were used on the XE-2100.

The ABX Pentra 120 Retic analyzes whole blood, giving proportional and absolute reticulocyte counts. A 0.8-μL aliquot of whole blood is mixed and incubated at 35°C for 25 seconds with 2.5 mL of ABX Retix, which contains Thiazole Orange, a patented fluorescent stain specific to nucleic acids (Becton Dickinson, San Jose, CA). The cells are analyzed in the flow cell using a 20-mW argon ion laser producing 3 types of information: the size of the cell measured by resistivity, forward-scattered light, and the fluorescence signal. 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 results are displayed on a reticulocyte matrix with RNA content on the y-axis and cell volume on the x-axis. Reticulocyte maturity is assessed and classified into 3 classes, low RNA content (RetL), medium RNA content (RetM),

TABLE 1. Summary Statistics for Percentage Reticulocyte Counts Obtained With the ABX Pentra 120 Retic and Sysmex XE-2100, Also Broken Down Into Reticulocyte Counts Above and Below 5% (as Measured on the Sysmex XE-2100)

	All Data		≤5% Reticulocytes		>5% Reticulocytes	
	Pentra 120	XE-2100	Pentra 120	XE-2100	Pentra 120	XE 2100
No. of samples	302	302	278	278	24	24
Mean	2.58	2.14	1.8	1.28	11.08	12.00
Median	1.65	1.13	1.54	1.09	10.23	11.33
Standard deviation	2.10	3.40	1.18	0.83	3.96	5.56
Minimum	0.16	0.06	0.16	0.06	3.54	5.90
Maximum	19.10	25.10	8.92	4.99	19.10	25.10

TABLE 2. Summary Statistics for Absolute Reticulocyte Counts ($\times 10^9$/L) Obtained With the ABX Pentra 120 Retic and Sysmex XE-2100, Also Broken Down Into Reticulocyte Counts Above and Below 5% (as Measured on the Sysmex XE-2100)

	All Data		≤5% Reticulocytes		>5% Reticulocytes	
	Pentra 120	XE-2100	Pentra 120	XE-2100	Pentra 120	XE 2100
No. of samples	302	302	278	278	24	24
Mean	9.93	7.72	7.80	5.21	34.56	36.71
Median	7.20	4.91	6.84	4.73	31.70	28.25
Standard deviation	9.56	11.22	4.41	2.90	16.22	23.83
Minimum	0.6	0.2	0.63	0.2	9.59	15.33
Maximum	69.69	97.5	30.00	17.61	69.69	97.50

and high RNA content (RetH). Global reticulocyte RNA content is quantified using a mean fluorescence index (MFI) [10]. In addition, cells in the immature area (IMM) of the matrix, where highly fluorescent elements such as immature reticulocytes and nucleated RBCs are located, are also quantitated. The IRF is calculated using the sum of absolute counts of (RetH + RetM + IMM)/Total Retic Count and is expressed as a fraction (range, 0.00-1.00). Fractions are the favored format, rather than the percentages used by Sysmex analyzers, as decided by the participants of the ISLH-sponsored Immature Reticulocyte Fraction Workshop [12].

The Sysmex XE-2100 uses a new patented polymethine fluorescent dye to stain the RNA of reticulated RBCs. In the flow cell, each single cell is passed through the beam of a semiconductor diode laser (more than 30,000 cells are counted for each sample) [11]. Due to their higher fluorescence intensity, leukocytes and nucleated RBCs are separated from the reticulocytes and other RBCs. The XE 2100 reports reticulocytes as percentages of RBCs and gives an absolute count. This analyzer also produces a reticulocyte differential based on the RNA content of the cell. Fluorescence intensity and forward-scatter light intensity of each cell is analyzed, the RNA content and cell size is registered, and the different maturity stages are resolved. The 3 fractions, high fluorescence ratio (HFR), middle fluorescence ratio (MFR), and low fluorescence ratio (LFR) are determined. In addition, the fraction of very early reticulocyte population, the IRF, which is the sum of HFR and MFR, is reported as a percentage of the reticulocytes.

Comparison of reticulocyte counts and indices was performed using the Pearson coefficient of correlation. To allow comparison of the IRF results, the Sysmex IRF, which is reported as a percentage, was divided by 100 to make it equivalent to the ABX Pentra 120 Retic IRF.

RESULTS

The results for the reticulocyte counts obtained from the ABX Pentra 120 Retic and Sysmex XE-2100 are given in Tables 1, 2, and 3. Tables 1 and 2 show summary statistics for the 2 counts, and Table 3 shows correlation statistics. Figures 1 and 2 show the correlation graphs, and Figures 3 and 4 show mean difference plots between the 2 methods.

The Pearson product moment (r) values were 0.95 for both the reticulocyte percentage and absolute counts, therefore there was overall very good correlation between the 2 methods.

There are 6 results that show significantly higher reticulocyte counts on the Sysmex XE-2100 than on the ABX Pentra 120 Retic. These are more easily identified in the plots of the mean reading of each of the 2 methods against the difference in readings of the 2 methods (Figures 3 and 4). These samples were from 2 patients with pyruvate kinase deficiency, 3 patients with β thalassemia major, and 1 with hemoglobin S disease. All of these samples contained varying numbers of nucleated RBCs (range, 1.5-171.5 per 100 WBCs).

There are 2 additional discrepant results, 1 from a patient with β thalassemia major and the other of unknown diagnosis, where the ABX Pentra 120 Retic gives a higher count than the Sysmex XE-2100; these samples did not contain nucleated RBCs or other RBC inclusions. An intermethod bias was observed, the percentage and absolute count values

TABLE 3. Correlation Statistics For Reticulocyte Counts Obtained With the ABX Pentra 120 Retic and Sysmex XE-2100, Also Broken Down Into Reticulocyte Counts Above and Below 5% (as Measured on the Sysmex XE-2100)

	All Data		≤5% Reticulocytes		>5% Reticulocytes	
	% Retics	Reticulocytes ($\times 10^{12}$/L)	% Retics	Reticulocytes ($\times 10^{12}$/L)	% Retics	Reticulocytes ($\times 10^{12}$/L)
No. of samples	302	302	278	278	24	24
r Value	0.953	0.959	0.950	0.937	0.836	0.937
Slope	0.832	1.113	1.348	0.616	0.595	1.376
Intercept	0.807	3.339	0.117	0.402	3.984	10.86

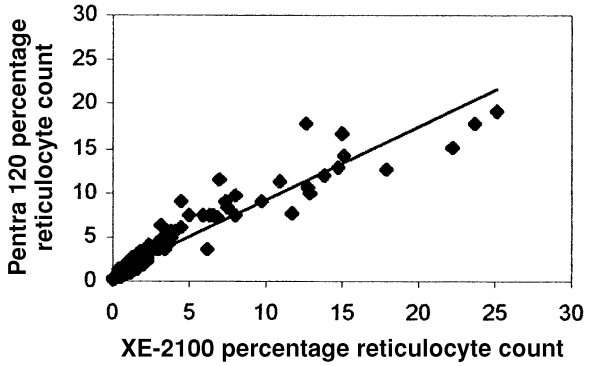

FIGURE 1. Correlation graph of XE-2100 percentage reticulocyte count against Pentra 120 percentage reticulocyte count.

were consistently higher on the ABX Pentra than the Sysmex XE-2100. This bias was particularly evident when the reticulocyte percentage was less than or equal to 5% as measured on the XE-2100 (Figures 3 and 4).

Results for the comparisons of the IRF parameters are shown in the summary statistics (Table 4) and the correlation statistics (Table 5). Figure 5 shows the correlation graph, and Figure 6 shows the plot of the mean IRF of the 2 methods against the difference in readings between the 2 methods. The IRF results show good correlation (r = 0.75) but the ABX Pentra 120 Retic gives consistently significantly higher results than the Sysmex XE-2100 (point of intercept, 0.11). This bias is particularly evident when the reticulocyte count as measured on the XE-2100 is greater than 5% (point of intercept, 0.256). There is less good correlation with these samples (r = 0.639). The ABX Pentra 120 Retic mean IRF with this group is 0.451 and for the XE-2100, 0.187. In addition to the differences in IRF values between the 2 instruments, the ABX Pentra 120 demonstrated a much greater dynamic range in the reported IRF values with a range of 0.00 to 0.87 compared to 0.00 to 0.45 with the XE-2100.

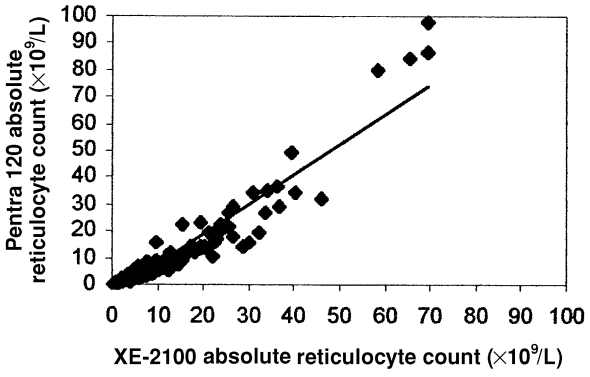

FIGURE 2. Correlation graph of XE-2100 absolute reticulocyte count against Pentra 120 absolute reticulocyte count.

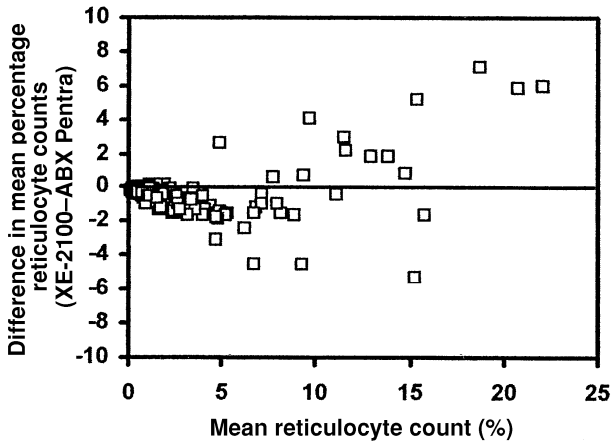

FIGURE 3. The difference in percentage reticulocyte counts between the XE-2100 and Pentra 120 (Altman Bland plot).

DISCUSSION

Both automated systems show good correlation (r = 0.95) for reticulocyte percentage and absolute reticulocyte counts. However, a slight bias was seen when the reticulocyte count was less than 5% with ABX Pentra 120 Retic giving higher results than the Sysmex XE-2100

Six samples with high reticulocyte counts by both methods, showed significantly higher results from the XE-2100 compared to those from the ABX Pentra. Some interferences are well known to affect reticulocyte counting (eg, nucleated RBCs, RBC inclusions, hemoglobinopathies and high WBC count) [13]. Nucleated RBCs were present in all of these samples, however, there were an additional 45 samples used in the study with nucleated RBCs present (range, 0.5-355 per 100 WBC) that did not give discrepant reticulocyte counts.

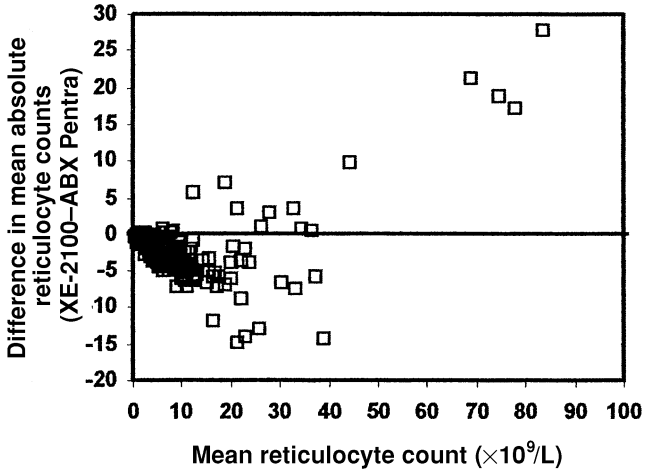

FIGURE 4. The difference in absolute reticulocyte counts between the XE-2100 and Pentra 120 (Altman Bland plot).

TABLE 4. Summary Statistics for the Immature Reticulocyte Fractions Obtained With the ABX Pentra 120 Retic and Sysmex XE-2100, Also Broken Down Into Reticulocyte Counts Above and Below 5% (as Measured on the Sysmex XE-2100)

	All Data		≤5% Reticulocytes		>5% Reticulocytes	
	Pentra 120	XE-2100	Pentra 120	XE-2100	Pentra 120	XE 2100
No. of samples	302	302	278	278	24	24
Mean	0.0257	0.099	0.240	0.090	0.451	0.187
Median	0.229	0.075	0.211	0.071	0.445	0.174
Standard deviation	0.154	0.079	0.142	0.072	0.141	0.086
Minimum	0.008	0.0	0.008	0.0	0.262	0.058
Maximum	0.871	0.452	0.871	0.452	0.765	0.352

It has previously been reported that the ABX Pentra Thiazole Orange reticulocyte count is not affected by the presence of nucleated RBCs [10].

The introduction of automated counting of reticulocytes has markedly increased the precision and accuracy of this assay compared with traditional manual counts.

In the last year the UK National External Quality assurance scheme has distributed 9 samples with varying reticulocyte counts to over 400 registered laboratories. The average geometric coefficient of variance (GCV) for 4 samples with reticulocyte counts in the range of 31×10^9/L to 36×10^9/L was 91.5% for manual counts, 32.8% for 6 different automated hematology analyzers, and 34.2% for dedicated flow cytometers using various dyes. On 3 samples with counts between 118×10^9/L to 152×10^9/L, the average GCV was 59.7% for manual methods, 14.5% for hematology analyzers, and 21.6% for dedicated flow cytometers. On 2 samples with counts of 231×10^9/L and 285×10^9/L, the average GCV was 57.3% for manual methods, 17.5% for hematology analyzers, and 23.2% for dedicated flow cytometers. Interlaboratory GCVs are extremely high for the manual methods but, suprisingly, the hematology analyzers give better GCVs than the dedicated flow cytometers. Similar results are also seen in the College of American Pathologists surveys. Previous studies have shown that with the introduction of flow cytometric techniques for reticulocyte counting interlaboratory variability has not improved as much as expected [14]. The National Committee for Clinical Laboratory Standards proposes a guideline for reticulocyte counting by flow cytometry [13] which involves a calibration protocol using the manual microscopic/new methylene blue method as the suggested calibration procedure. This calibration could also be applied to hematology analyzers.

The reagents used for the automated reticulocyte counting are moderately expensive; however, when labor costs for the manual counting are considered, the automated reticulocyte count is often the least expensive of the 2 methods of counting.

Comparisons of the IRF results generated by these 2 analyzers demonstrated good correlation but the difference in the values reported was significant. The ABX Pentra IRF was always higher than the Sysmex XE-2100, particularly when the reticulocyte count was greater than 5%. The IRF reference ranges differ significantly. This is a result of differences in reticulocyte reagents and data analysis algorithms deriving the IRF measurements. A similar bias exists between the ABX Pentra 120 Retic blood analyzer and the Sysmex R-2000 [10], which uses a different fluorescent dye, Auramine-O, than that used by the Sysmex XE-2100. The American College of Pathologists surveys IRF values from participating laboratories and the results show a vast difference in IRF values for the same sample when analyzed on different automated

TABLE 5. Correlation Statistics for the Immature Reticulocyte Fractions Obtained With the ABX Pentra 120 Retic and Sysmex XE-2100, Also Broken Down Into Reticulocyte Counts Above and Below 5% (as Measured on the Sysmex XE-2100)

	Immature Reticulocyte Fraction		
	All Data	≤5% Reticulocytes	>5% Reticulocytes
No. of samples	302	278	24
r Value	0.750	0.749	0.639
Slope	1.500	1.474	1.044
Intercept	0.110	0.105	0.256

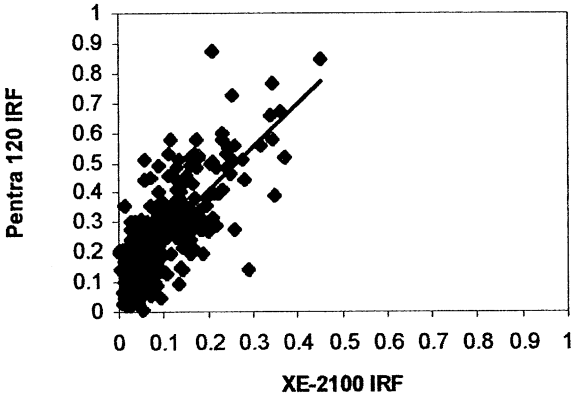

FIGURE 5. Correlation graph of the XE-2100 immature reticulocyte fraction (IRF) against Pentra 120 IRF.

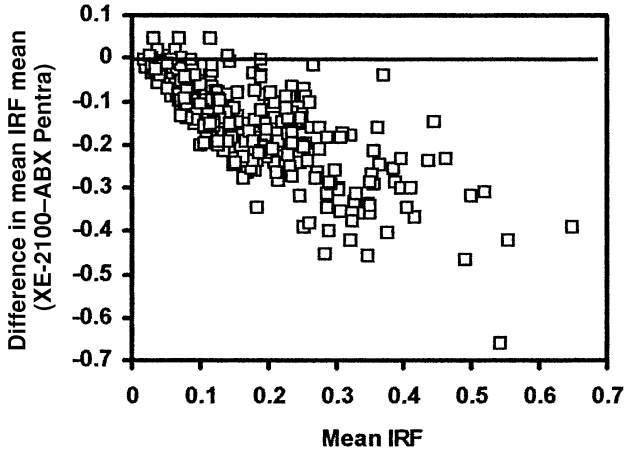

FIGURE 6. The difference in the immature reticulocyte fraction (IRF) between the XE-2100 and Pentra 120 (Altman Bland plot).

instruments. For one sample, the results reported for the IRF ranged from 0.13 to 0.79.

The ABX Pentra 120 Retic and Sysmex XE-2100 appear to exhibit differing dynamic ranges in the IRF values with the ABX Pentra 120 Retic giving a wider range of results for our patient population. This wider dynamic range on the ABX Pentra 120 could provide some greater clinical utility, although additional studies would be required to confirm this supposition.

The clinical utility of the IRF has now been well demonstrated in the diagnostic and serial therapeutic monitoring of neonates, various anemias, bone marrow or stem cell transplantations, and renal failure and transplantation patients [15,16]. However for greater confidence in the use of this measurement, there must be method-specific reference intervals established, calibrators developed, and international standardization before the full diagnostic potential of the IRF can be established.

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