Enumeration of CD34+ haemopoietic stem cells: comparative study of the performance of the SYSMEX XN-1000 hematology analyzer in a dual-platform approach versus a single-platform approach

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Abstract

Data regarding the quantification of CD34+ hematopoietic stem cell HSC in dual-platform (DP) approach using a hematological analyzer featuring dedicated nRBC channel are limited if not inexistent. The objective of this study is to compare the performance of the DP approach on quantification of CD34+ HSC using a new generation of hematological analyzer featuring dedicated nRBC channel, Sysmex XN-1000, with the single-plate (SP) form approach using Beckman Coulter's Navios flow cytometer. Using the International Society for Hematotherapy and Transplantation protocol, a total of 86 samples were analyzed in multiple myeloma patients planned for autologous HSC transplantation. The Beckman Coulter's Stem-Kit™ reagents were used to perform both approaches DP and SP. The count of CD34+ cells of the SP deduced directly from the software that manage the Navios flow cytometry was compared to CD34+ cell count obtained by DP. The DP approach was made by combining flow cytometry analysis and the Sysmex XN-1000 hematological cell analyzer data. The analysis of the agreement of the results between these two approaches using the Bland-Altman diagram gave a bias of one cell/μl. As for the equation of the right of Passing-Bablok, it is $Y(\text{SP}) = 0.166667 + 0.969697 X(\text{DP})$. The slight differences in CD34+ stem cell counting between these two approaches did not achieve statistical significance. Both SP and DP approaches were effective and yield similar results. DP seems to be a simple and affordable approach that is well suited to the needs of laboratories where this type of analyzer is available.

Keywords Mobilized peripheral blood stem cells · CD34+ · Flow cytometry · Sysmex XN-1000

Introduction

Since its clinical introduction in 1986, the transplantation of mobilized hematopoietic stem cells into peripheral blood (PBSC) has become the most common transplantation procedure performed in medicine [1]. It is widely used to treat blood disorders, malignant tumors, and genetic abnormalities.

To collect a satisfactory graft, the choice of the best timing to initiate the cytopheresis cycle is a critical factor, and this process depends essentially on the number of CD34+ stem cells mobilized in the peripheral blood [2]. Flow cytometry analysis is currently one of the fastest and most accurate solutions to vigilantly monitor the effectiveness of the entire CD34+ hematopoietic stem cell (HSC) mobilization process.

Several approaches have been proposed to enumerate CD34+ HSCs by flow cytometry. Among these, two are the most commonly used: The first method is called a single platform (SP), which is considered a reference method where CD34+ HSCs are marked in the presence of a known number of beads (fluorospheres) in a tube. The cytometer relates the number of beads counted to the number of CD34+ cell counted, and the results are expressed in percentage and absolute numbers of the CD34+ HSCs by applying a precise formula [3, 4]. The second method called dual platform (DP) combines two instruments, flow cytometry and a hematological

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analyzer. Here, the absolute number of CD34+ HSCs is deduced from the percentage of CD34+ cells evaluated via flow cytometry and the absolute number of nucleated cells and/or the number of white blood cells (WBCs) obtained by a hematological analyzer [4].

Published works comparing these two approaches known as single and dual platforms as regards of enumeration of CD34+ HSCs are limited, and even less those comparing these two approaches through the application of the ISHAGE protocol. In several published works, the DP approach was carried out using hematological analyzer cells whose operating principles are basically working on the impedance principle (Coulter principle). With such analyzers, there is a potential risk of a false increase of the total number of white blood cells due to the integration of nucleated red blood cell (nRBC) levels into white blood cell levels. Leading to a higher risk of error when calculating CD34+ HSCs by DP technique [5–7].

The aim of this study is to evaluate the contributions of the nRBC channel technology in a Sysmex XN-1000 hematological analyzer in the enumeration of CD34+ HSCs by the DP approach and to compare these results to the SP, reference method recommended by ISHAGE.

Materials and methods

Sample collection

In order to choose the timing for harvesting mobilized stem cells in multiple myeloma patients programmed for autologous stem cell transplantation, mobilized peripheral blood hematopoietic stem cell (HSPC) samples were obtained in EDTA tubes and cytopheresis bags under the approval of the Research Ethics Board. Primary analysis of the received samples was performed using the Sysmex XN-1000 hematological analyzer which is maintained calibrated and regularly controlled using 3-level quality controls (Hematology Control XN Check™). The WBC count of each sample was adjusted to 15,000 WBC/ μ l by dilution with a phosphate-buffered serum (PBS). All samples were processed simultaneously by both SP and DP methods within 2 h of sampling.

Sample preparation

Using Beckman Coulter's Stem-Kit Reagents (Beckman Coulter Life Sciences, Indianapolis, USA), three tubes were prepared for the analysis of each peripheral blood tube (46 samples) and each cytopheresis tube (40 samples). The first 2 tubes labeled "tube 1" and "tube 2" were prepared identically by staining 100 μ l of the sample initially deposited by reverse pipetting with 20 μ l of CD 45-FITC clone J33 and CD34-PE clone 581 and 20 μ l of viability marker 7-AAD

viability dye. The third tube labeled "control" was prepared identically to tubes "1" and "2"; the only difference is that the sample is stained with isoclonic marker (CD 45-FITC clone j33/IsoClonic Controle-PE clone 581) instead of CD 45-FITC clone j33/CD34-PE clone 581. The three tubes 1, 2, and control were gently vortexed for 5 s and incubated under obscurity for 20 min at room temperature. Erythrocyte lysis was performed by adding 2 ml of the lysis solution (NH₄Cl lysing solution) diluted to 1/10th by distilled water into each of the three tubes. The whole unit was vortexed for 5 s then incubated at 18–25 °C for 10 min under obscurity. A total of 100 μ l of previously homogenized stem-count fluorosphere was added using a calibrated pipette into each of the three tubes, then one last vortex passage to all tubes for 5 s. A positive control was prepared using Stem-Trol control cells (Beckman Coulter Life Sciences, Indianapolis, USA), according to the manufacturer's recommendations and treated under the same conditions as the patients' samples. The acquisition was made immediately on the Beckman Coulter Navios flow cytometer. The control is validated if the obtained Stem-Trol count is within $\pm$ 15% of the Stem-Trol control cell concentration stated on the vial label.

Calculations

Enumeration of viable CD34+ HSCs by these two approaches (SP and DP) was carried out by applying the recommendations of the ISHAGE (International Society of Hematotherapy and Graft Engineering) protocol [9] designated for the enumeration of CD34+ HSCs by flow cytometry.

The results of the CD34+ HSC count by SP technique were derived directly from the software that manages the Navios cytometer version 1.3. A multiplication by the dilution factors is required if the sample was initially diluted with PBS. Since the SP technique is very sensitive to pipetting errors, WBC count and lymphocyte count obtained by cytometry were compared with those obtained with the Sysmex XN-1000 hematological analyzer. They can be used as an internal control on each sample, and have allowed us, in addition to a positive control, to ensure the correct preparation of dilutions and the acquisition of our stem count.

The results of the CD34+ HSC count by DP technique were obtained by combining the percentage of CD34+ HSCs in the analyzed sample deduced from the flow cytometer and the absolute WBC count obtained by the XN-1000 hematological analyzer. The absolute number of CD34+ HSCs was the result of a WBC calculation * % CD34+ HSCs.

Statistical analysis

A study of the concordance between these two methods (SP and DP) was performed using the 2016 EXCEL software and MedCalc version 14.8.1.0 by calculating the correlation concordance coefficients of LIN (ρ) [10] and Pearson (ρ) and

analyzing the Bland and Altman graph [11] to quantify the agreement between the two techniques. A regression study was conducted using the Passing-Bablok model [12], which does not require specific assumptions about sample distribution and measurement errors.

Results

The results of the CD34+ HSC counting on 86 samples collected from patients with multiple myeloma utilizing the DP and SP techniques are included in Table 1. The WBC count of all samples was adjusted around 15.10^3 GB/ μ l before being analyzed by both SP and DP techniques. The median of WBC count in our series is 15.015×10^3 WBC/ μ l. The means and medians obtained by these approaches (SP and DP) are almost identical for stem cells mobilized in peripheral blood (PB) and cytapheeresis samples. The comparison of means of the CD34+ HSC counts measured by both methods reveals a difference of 0.2174 CD34+ stem cells/ μ l stem cells; 95% CI 9428 to 3.3776; $P = 0.8916$ for PB and a difference of 1.8250 CD34+ stem cells/ μ l, 95% CI -4.4977 to 8.1477; and $P = 0.5672$ for PBSC. These results show that the differences between means of CD34+ HSCs measured by the SP and DP method in PB and PBSC are not statistically significant. On the other hand, the nRBC count by the Sysmex XN-1000 hematological analyzer for each sample type shows the presence of a significant number of nucleated red blood cells. The medians are 20 nRBC/ μ l and 40 nRBC/ μ l for peripheral blood and cytapheeresis samples respectively for the same amount of WBC (15.015×10^3 GB/ μ l in our series). They represent between 0.2 and 0.3% depending on the sampling nature, and the range is 80 nRBC/ μ l for both types of samples (Table 2).

Table 1 CD34+ mobilized stem cell count results

Sample	PB		PBSC	
	DP_1_CD_34_I ¹	SP_1_CD_34_I ²	DP_1_CD_34_I ¹	SP_1_CD_34_I ²
Sample size	46	46	40	40
Median	10	10	29.5	28.5
Arithmetic mean	11.63	11.42	32.5000	30.6750
95% CI for the mean	9.343 to 13.917	9.169 to 13.656	27.690 to 37.309	26.416 to 34.933
Standard deviation	7.7010	7.55	15.0384	13.3155
Range	36	35	62	53
<i>T</i> test* (assuming equal variances)				
Difference	0.2174		1.8250	
Two-tailed probability	$P = 0.8916$		$P = 0.5672$	

PB peripheral blood, PBSC mobilized peripheral blood stem cells (in cytapheeresis samples)

*Significance level (α) = 5%

¹ Dual platform: obtained by combining flow cytometry and the XN-1000 hematological analyzer

² Single platform: obtained by flow cytometry (Beckman Coulter's Navios)

Table 2 Nucleated red blood cell (nRBC) count results by Sysmex XN-1000 hematological analyzer

Sample	Average	Median	Range	% of WBC count
PB	31.1	20	80	0.2
PBSC	37	40	80	0.3

The statistical analysis of the CD34+ HSC numeration results shows a very good correlation between these two techniques (Table 3). Lin's concordance correlation coefficient (ρ_c) = 0.9864, Pearson's ρ (precision) = 0.9924, and a bias correction factor Cb (precision) = 0.9939, indicating an excellent agreement between two sets of measurements according to the interpretation of Partik et al. [13]. The analysis of the Bland and Altman graph (Fig. 1) reveals that the bias is 1 cell, indicating that the DP method overestimates the number of CD34+ HSCs by an average of one cell compared to the method (SP) which is the reference method. The Bland-Altman plotted as percentage of differences (Fig. 2) demonstrates that the bias is -2.1% and that the four points to the right of the Bland-Altman plot (Fig. 1) are well within a standard deviation of the overall distribution of differences. The equation of the Passing-Bablok line (Table 4 and Fig. 3) is Y (SP) = $0.166667 + 0.969697 X$ (DP) (the 95% confidence interval of this slope is 0.9130 to 1.0000). The Cusum test did not reveal a significant deviation from linearity ($P = 0.78$).

Discussion

Peripheral blood stem cell transplant has progressively become the most common transplant procedure performed in medicine, replacing bone marrow transplantation as a source

Table 3 Concordance correlation results of CD34+ mobilized hematopoietic stem cell counts performed by both SP and DP approaches

Sample size	86
Concordance correlation coefficient	0.9864
95% confidence interval	0.9806 to 0.9904
Pearson ρ (precision)	0.9924
Bias correction factor C_b (accuracy)	.9939

of stem cells in nearly 100% of autologous patients and approximately 75% of allogeneic transplant patients [1]. The success of this procedure is mainly based on the number of transfused CD34+ HSCs. Following the integration of flow cytometry as a promising tool for CD34+ HSC counting in 1980, several methods and protocols have since been described to provide a reliable and reproducible number of CD34+ HSCs. Currently, the ISHAGE protocol is the most widely used protocol [14]. The quantification of CD34+ HSCs according to the ISHAGE protocol by flow cytometry could be performed using two approaches, the so-called SP and DP approaches. These two approaches have both benefits and drawbacks. The SP technique is precise but fairly rigorous to be maintained in routine due to the specific properties of the beads. An abnormal bead count can be encountered with this method, in particular during processing of protein-free or low-protein cell samples. This abnormality can occur as a direct consequence of sedimentation or sticking of the beads against the walls of the polystyrene tubes in various situations: During vortexes, electrostatic charges caused by the friction of the polystyrene tubes with the rubber housing of the vortex tube lead to a noticeable change in the concentration of the beads in the tube causing a false CD34+ HSC enumeration. This observed phenomenon can also occur spontaneously over the

Fig. 1 Bland-Altman diagram of differences comparing CD34+ hematopoietic stem cell count results obtained with both SP and DP approaches **Fig. 2** Bland-Altman plot of percent difference comparing CD34+ hematopoietic stem cell count results obtained with both SP and DP approaches

time and called “vanishing counting bead phenomenon” [15]. Furthermore, this method necessitates an average of two counts to provide a final report of CD34+ HSC count. If the variation in values exceeds 10% between two counts, it becomes imperative to repeat the test, which increases the required time and cost to perform the CD34+ cell count using this approach [16].

The DP technique presents a critical problem when processing samples rich in nRBCs. The latter was found in nearly all onco-hematological diseases. And they have also been frequently encountered in mobilized peripheral blood hematopoietic stem cells [20]. In this case, we are experiencing a false evaluation of CD34+ HSCs due to the integration of nRBC levels in the total number of WBC [18, 19]. This increase is in the order of 1% for the treatment of mobilized peripheral blood, 3.08% for the treatment of cytopheresis products and 6.09% for the treatment of umbilical cord blood [20].

Published works comparing these two approaches known as SP and DP for CD34+ HSC count are limited, and even fewer are those comparing these two approaches through the application of the ISHAGE protocol. According to D Barnett et al. inquiry, SP approach is the best in terms of reducing the inter-laboratory variability [21]. This is due to the determining problem that presents the DP approach of reporting the percentage of CD34+ HSCs in relation to the proportion of leukocytes or total nucleated cells including nRBC [4].

Through this, it is clear that the performance of the DP approach is related to the characteristics of the hematological analyzer, i.e., whether it is designed to give the exact WBC count in the case of analyzing erythroblast-rich samples or these results require microscope review to correct the WBC count. In which case, the DP technique would suffer from the same common weaknesses known to be present in all manual

Table 4 Results of the regression analysis

$$Y = 0.166667 + 0.969697 X$$

Systematic differences	
Intercept A	0.1667
95% CI	0.0000 to 0.5870
Proportional differences	
Slope B	0.9697
95% CI	0.9130 to 1.0000
Random differences	
Residual standard deviation (RSD)	1.4947
± 1.96 RSD interval	– 2.9296 to 2.9296
Linear model validity	
Cusum test for linearity	No significant deviation from linearity ($P = 0.78$)

assays, such as intra- and inter-observer reproducibility and sampling errors [22].

Most of the published studies in this regard were based on hematological analyzers that operate on the Coulter principle to perform the DP technique, as Sysmex KX-21 for Ngoma A.M et al. [23], a Coulter counter STKS model for Piedras-Ross J et al. [24], and Sysmex k-1000, Technicon H3 for Gratama JW et al. [4]. All these hematological analyzers, which operation is based on the impedance principle, were reported to have limitations against the presence of nRBC. In addition, most of these studies are limited to correlation analysis and regression analysis to compare these two approaches, although these two parameters do not provide sufficient resolution. According to Keeney M et al., the use of Bland and Altman's presentation is strongly recommended for the analysis of such a situation [3].

The hematology analyzers used to perform the DP approach in our study are Sysmex XN-1000. This analyzer contains a specific channel (WNR) to differentiate between WBC, basophils, and erythroblasts [25]. Based on the manufacturer's

specs, Sysmex XN-1000 model is able to detect and correct automatically the blood count when nRBCs are detected in the analyzed sample with a sensitivity up to 1 nRBC/1000 WBC [8].

Statistical analysis of CD34+ HSC count results, by both SP approach using Beckman Coulter's Navios cytometer and DP coupled with the XN-1000 automat, shows a Lin's concordance correlation coefficient (ρ_c): 0.9864, Pearson's ρ (precision) = 0.9924 and a bias correction factor Cb (precision) = 0.9939. These results reflect an excellent agreement between the two sets of measure. The part of the imprecision in our series is $(1 - 0.9924)/((1 - 0.9924) + (1 - 0.9939)) = 55.47\%$ and the part of the inaccuracy is $(1 - 0.9939)/((1 - 0.9939) + (1 - 0.9924)) = 44.53\%$. The difference observed between the two approaches in our series is due to a lack of precision (55.47%) more than to a lack of accuracy (44.53%). To improve further the consistency between these two approaches, random errors between the two sets of measurement must be reduced.

To confirm the efficiency of this specific channel (WNR) on Sysmex XN-1000 in the CD34+ HSC enumeration by DP approach, we can calculate the accuracy without "this correction" by adding absolute nRBC counts which are detected on Sysmex XN-1000 to the WBC counts initially obtained, then recalculate the CD34+ HSCs according to the DP's guidelines. By doing so, the accuracy without this correction is $C_b = 0.9892$ (compared to $C_b = 0.9939$ found previously with "corrected" WBC count). Based on these results, we can conclude that the accuracy always is better with "corrected" WBC count.

On the other hand, the bias value is one cell, and this means that the "DP" method overestimates the number of CD34+ HSCs on average by one cell/ μl compared to the SP method. This difference can be explained by the fact that the hematological analyzer used in DP is basically designed to process whole blood samples drawn in EDTA and to a lesser degree cytopheresis samples drawn in citrate solutions. This

Fig. 3 Correlation diagram according to Passing and Bablok

hypothesis is supported by comparing the differences between means (Table 1) of CD34+ HSC count measured by SP and DP for each type of samples separately (0.2 cells for peripheral blood samples versus a bias of 1.8 for cytapheeresis samples). The Passing-Bablok regression did not indicate a significant difference between these two approaches. The obtained results are similar to those reported from Chapple P.H. et al. using an Abbott Cell dyn 4000 hematological analyzer that integrates cytometry technology in DP. Chapple P.H found a bias of 1.16 and a linear regression $R^2 = 0.9325$ [26]. These results show that the integration of cytometric technology alongside impedance in medium/high-range hematological analyzers makes this latter in DP approach much more reliable as SP for the quantification of CD34+ HSC count.

Umbilical cord blood is known to be one of the richest sources of HSCs with the highest erythroblast concentrations [17], making them as the most vulnerable element of the DP. In comparison of the umbilical cord blood erythroblast count generated by Sysmex XN-1000 with the gold method which is microscopic count, Kim H et al. demonstrated the presence of a strong correlation between these two techniques ($r = 0.865$) [25], which makes us expect that DP paired with Sysmex XN-1000 hematological analyzer remains an extremely promising approach in term of processing HSC sources assumed to contain high levels of erythroblasts. However, a verification of the performance of DP coupled with Sysmex XN-1000 on CD34+ HSC counting on umbilical cord blood remains highly recommended. Also, a prospective and multicenter study is encouraged to complete this study.

Conclusion

The results of the CD34+ HSC count using the DP approach that combines cytometry with the Sysmex XN-1000 hematological analyzer were significantly correlated with the SP approach recommended by ISHAGE. DP seems to be a simple, affordable, and well-adapted approach that suits the needs of laboratories where this type of analyzer is available either as a dedicated method to quantify CD34+ HSCs or as a monitoring procedure to assess the correct execution of the SP technique.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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