Comparative Study for Measurement of Residual Leucocytes in Leucodepleted Red Blood Cells by Two Different Methods

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Abstract Estimation of residual leukocytes in blood components after leukodepletion is crucial for assessment of quality. Flow cytometry (FC) and Nageotte hemocytometer are the most widely accepted methods for counting residual white blood cells (rWBCs) in leukocyte-depleted (LD) blood components. The objective of this study was to compare use of Nageotte counting chamber and FC for quality control of leukodepleted red cell units. A prospective, observational study was conducted in the department of Transfusion Medicine. A total of 80 whole blood donations from healthy donors were subjected to testing by FC and Nageotte hemocytometer for estimation of rWBC in duplicate. Additionally, ten personnel attempted a survey for ease of use of FC. Number of rWBC detected by flow cytometer were between 1 WBC/ μ L and 28 WBCs/ μ L whereas that detected by Nageotte's chamber were between 0 WBC/ μ L (lowest) and 5 WBCs/ μ L. Coefficient of variation was found to be 87.36% by Nageotte hemocytometer method and 43.26% by FC. Linear regression analysis

did not show any correlation (R -squared = 0.01, p = 0.13) between the two methods which signifies that the two methods cannot be used interchangeably. Pearson's correlation coefficient showed a weak relation between results obtained by the two methods. Inter-observer variation was found to be significant with use of Nageotte hemocytometry. Survey for ease of use of FC indicated acceptability of FC with favorable scores. Flow cytometric technique provides a reproducible and objective tool for counting rWBC in leukodepleted blood components compared with the Nageotte hemocytometer.

Keywords Leukoreduction · Leukodepletion · Filtration · Flowcytometry · True count beads · Nageotte

Introduction

Blood transfusion is a very common medical procedure and it carries an inherent risk of infectious and noninfectious adverse events [1, 2]. To enhance safety of blood transfusion, many policies and procedures exist in any patient care setting. One of them is leukodepletion (LD) of blood components, which is used to remove the leukocytes that are known to cause non hemolytic febrile transfusion reaction (NHFT), alloimmunization and refractoriness, CMV transmission and immunomodulation among others [3, 4]. It is now established that reducing leukocytes from blood components would result in reducing these adverse reactions [5, 6]. Methods for LD can be classified based on the log reduction into three categories (1) low performance (< 90%, 1 log reduction), (2) intermediate performance (90–99.9%, 1–3 log reduction), (3) high performance (> 99.99% 4 log reduction). Low-performance methods include saline

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wash, buffy coat removal, and spin-cool filtration. Intermediate-performance methods, such as glycerolization–freeze–thaw–deglycerolization (a method to remove leukocytes by repeated washings), differential centrifugation, and the early adhesion-based filters (modified cotton wool or cellulose acetate). High-performance methods include the so-called third-generation or fourth-generation filters that combine size retention, electrostatic attachment, and receptor-ligand interactions [7, 8]. When used properly, these filters contain less than 5×10^6 leukocytes per unit which is the quality control (QC) criteria for LD in India and USA. However, according to the Council of Europe leukodepleted red blood units (RBC) should not contain $\geq 1 \times 10^6$ cells residual leukocytes [9–11]. To achieve the expected clinical benefits of providing leukodepleted blood and components, the transfusion centers need to control the overall process so that the prepared product has $< 5 \times 10^6$ residual white blood cells (rWBCs) with loss of not more than 10% RBCs. Cell counters do not give accurate results at leukocyte concentrates less than 100 WBCs/ μL . Traditional techniques are cumbersome and operator-dependent. Flow cytometric (FC) estimation of residual leukocytes was applied to assess the quality of leukodepleted blood components in search for a sensitive method. It has been shown that this technique is efficient. However, it finds limited use due the high cost. This study was planned to compare Nageotte hemocytometry and FC for rWBC estimation in leukodepleted components to assess feasibility of both the methods for QC of leukodepleted blood components.

Materials and Methods

Settings and Design

This was a prospective, observational study conducted between December 2017 and January 2018 in the department of Transfusion Medicine of a large tertiary healthcare setup in India. A total of 80 components were assessed for LD by both FC and Nageotte hemocytometer.

Whole Blood Collection

The transfusion service uses 450 mL top and bottom penta bag system with integral filter for LD (Fresenius Kabi, Homburg, Germany). All components were prepared as per standard operating procedure of the department.

Collection of Samples and Complete Blood Count

Pre-LD, 2 mL whole blood samples were aseptically collected from the sample pouch of whole blood donations in EDTA vacutainers (BD Sciences, Franklin Lakes, NJ USA), and post-LD samples were aseptically collected from segments of blood components within 48 h of donation. A complete blood count for all samples was performed by XP-100 analyzer (Sysmex Transasia, Mumbai, India).

Determination of rWBC Count by Nageotte Hemocytometer and Microscopy

Nageotte chamber allows manual counting of rWBCs in samples with low leukocyte concentration which is below the threshold of a standard hemocytometer. It is not suitable for samples with normal WBC counts. For this purpose, 400 μL of Turk's solution was added to a test tube. Each sample was diluted to reach a final dilution of 1: 5 by adding 100 μL of blood sample to appropriately labeled test tube containing 400 μL of Turk's solution and mixing thoroughly using vortex. The test tube was left at room temperature for 10 min to lyse red cells. A cover slip was placed over the center of the chamber. The diluted sample was carefully loaded on the chamber without disturbing the cover slip till the chamber was completely charged. The chamber was placed in a moist petridish for 15 min to allow the leucocytes to settle. The leucocytes were counted within 30 min by scanning back and forth under the microscope across the gridded area using 20 X (or 10X) objective lens. Counting was performed in all 40 rectangles and in both the upper and lower grids (for duplicate counting) Results were obtained using the following formula:

$$\text{Leukocytes}/\mu\text{L} = \frac{\text{Cells counted} \times \text{Dilution}}{\text{Gridded area volume (50 } \mu\text{l)}}$$

Determination of rWBC Counts by Flow Cytometry: bead-Based Method

Absolute WBC counts were determined by flow cytometry using no-wash procedure, with the help of Trucount tubes (BD Trucount™ Tubes, San Jose, USA). For this purpose, 200–400 μL of well-mixed red cell sample was dispensed into a clean 12 $\times$ 75-mm polypropylene tube. The BD Trucount tubes were labelled and samples were prepared by adding 400 μL of BD Leucocount reagent and 100 μL of well-mixed sample to each tube. The tubes were capped and gently vortexed for 15 s after which they were incubated for 5 min in the dark at room temperature till they were ready for acquisition. BD Leucocount control cells

(BD, San Jose, USA) with high and low WBC concentrations were used in each run as controls and treated in the same manner in parallel. Results were analyzed using the following formula:

$$\text{Leukocytes}/\mu\text{L} = \frac{\text{Total Beads} \times \text{True Leukocyte Events}}{\text{Total Beads Acquired} \times \text{Total Sample Volume}}$$

Calculation of Log Reduction

Log reduction in the number of leucocytes was calculated using the formula given below.

$$\text{Log reduction} = \frac{(\text{Pre LD Absolute WBC Number} - \text{Post LD Absolute WBC Number})}{\text{Pre LD Absolute WBC Number}} \times 100$$

Inter-observer Variation

Each sample was split into two halves and given a random odd and n even number. These two halves were processed in duplicate by two different individuals, so that the cumulative number of times a test being performed by each method was four. Each observer reported mean result of the duplicate tests rounded off to a whole number. The results were recorded on a case reporting form and submitted. A data analyst kept the details of these sample numbers and the results submitted.

Survey for “Ease of Use” of the Two Methods

A survey was conducted for 10 individuals who perform QC for leukoreduced components on a regular basis to assess the acceptance of FC over Nageotte hemocytometry. The survey had four statements with a 4-point Likert scale. All participants were well versed in both the techniques and were asked to perform the same on unknown samples. All four statements included the routine problems faced with Nageotte hemocytometry and whether FC was a better alternative in terms of the drawbacks of using Nageotte hemocytometry. The survey was attempted by only those personnel who perform QC for LD components on a regular basis and who were well versed in both the techniques and ten such personnel participated in evaluation of ease of use. After performing the test, they were asked to answer the survey individually, without mentioning their identifiers and all forms were filed and submitted for analysis.

Statistical Analysis

All counts and log reduction data was entered in an MS excel sheet. All odd numbered samples were placed in group 1 and all even numbered samples were placed in group 2. Mean values for both these groups were calculated and student t test was applied. To assess the precision of each method, intra assay coefficients of variation (CV) were calculated. For all tests, a probability < 0.05 indicates statistical significance. Linear regression analysis, Pearson's correlation coefficient and coefficient of variance were calculated using SPSS for windows, version 20.0 (SPSS Inc. Chicago, USA).

Ethical Committee Approval

Institutional Review Board (IRB) approval was obtained for the study. Since both Nageotte hemocytometry and flowcytometric enumeration are acceptable methods for quality control of leucodepletion, the Ethical Committee approval was waived off by the institution.

Results

A total of 80 whole blood donations were included in the study with a mean volume of 292.9 ± 20.9 mL for the leukodepleted red cells in additive solution.

Demographic Data

Amongst the 80 donations included in the study, seven were from female donors and the rest were from male donors. The mean age of donors who donated these units was found to be 38.15 ± 14.27 years. Forty-five of the 80 collections were donated by repeat donors, out of which 6 were regular, repeat voluntary donors.

Hematologic Data

The mean hemoglobin of these donors was 15.06 ± 1.25 g/dL and mean leucocyte count was found to be 6654.2 ± 1888.31 per μL .

Results Obtained by Nageotte Chamber Based Estimation

The mean number of rWBC in leukodepleted RBCs was found to be 0.95 ± 0.83 and 1.175 ± 0.78 per μL in group 1 and group 2 respectively. Results have been summarized in Table 1.

Table 1 Results of estimation of rWBC in leukodepleted red blood cells by flow cytometry method and Nageotte method—inter-observer variation

Number of samples	Group	Mean WBC (μL) Flowcytometry observations	Mean WBC (μL) Nageotte observations
80	Group 1	13.15 ± 5.69	0.95 ± 0.83
80	Group 2	13.08 ± 5.84	1.175 ± 0.78

Results Obtained by Flowcytometry Based Estimation

All control results were as expected. The mean number of rWBC in leukodepleted RBCs was found to be 13.15 ± 5.69 and 13.08 ± 5.84 per μL in group 1 and group 2 respectively. Results have been summarized in Table 1.

Comparison of Both Methods

Linear regression analysis showed correlation (R -squared = 0.17, $p = < 0.00001$) between the two methods. However, coefficient of variation was found 150.58% in Nageotte hemocytometer method and 55.51% in FC method. Pearson's correlation coefficient was calculated for the two methods. The value of R was found to be -0.1114 , signifying a weak relation between the results obtained by the two methods.

Inter-observer Variation

On comparing inter-observer results, for FC the p value was found to be 0.47; which was not significant and for Nageotte hemocytometry, the p -value was found to be 0.04; which was found to be significant. Therefore, there is significant inter-observer variation with the use of Nageotte hemocytometry and microscopy.

Survey for Ease of Use

Ten individuals participated in the study. On the basis of the responses obtained from the questionnaires, the mean score for each question was calculated. Favorable scores indicate the acceptability of FC for estimation of rWBC after LD in a component (Table 2).

Table 2 Results of survey for ease of use of flowcytometry

S. no.	Question	Mean score (n = 10)
1	Flowcytometry results have better traceability than Nageotte hemocytometry and microscopy	3.2
2	Flowcytometry is less subjective than Nageotte hemocytometry and microscopy	3.6
3	Flowcytometry requires less time than Nageotte hemocytometry and microscopy	2.9
4	Flowcytometry is less cumbersome than Nageotte hemocytometry and microscopy	3.5

Discussion

LD involves the removal of leucocytes from cellular components to reduce the risk of HLA alloimmunization, CMV transmission, and NHFTR primarily. Other useful benefits of LD which have been advocated include reduced mortality in cardiothoracic vascular surgeries, reduced transfusion related immunomodulation, reduced risk of transfusion transmitted bacterial infections amongst many others [5, 6]. Being such a useful technique to healthcare providers, different timings and different methods of leukodepletion were explored and it was found that where possible, pre-storage leukodepletion with the help of leucofiltration is an effective method of leukodepletion. However, to assess the adequacy of this process, rWBC in the component should be measured. Nageotte hemocytometry and FC based enumeration both have been recommended for this purpose. The Nageotte hemocytometry was the first practical method for the enumeration of rWBCs in LD blood components and had been considered suitable for routine QC testing. However, it is quite labor intensive and inter-observer variability is high which was also seen in the present study [8]. The inter-observer variability was found to be significant for results obtained by Nageotte hemocytometry. This leads to difficulties in standardization of quality control procedure. In order to overcome these difficulties, alternative counting methods were sought for. Several studies have compared WBC counts obtained by automated methods to results obtained by the Nageotte hemocytometry [8].

In this study, we compared the performance of Nageotte hemocytometry and FC in terms of counting low leucocyte concentrations in LD red cell units. In our study, the Nageotte counting yielded significantly lower rWBC results than the FC which is in concordance with published

literature concluding that results obtained by Nageotte are lower than those obtained by FC because the latter method is more sensitive. The present study also highlights the fact that results obtained by both the methods do not yield good correlation. When talking about quality control of leukodepleted components, transfusion services look for a test which has good sensitivity. Nageotte hemocytometry gave a negative result in 38 of the 160 observations (24.38%) which includes roughly one-fourth of the observations.

However, there are studies which found that WBC counts in LD RBCs were higher when measured by Nageotte's method than by FC [12, 13]. This could be because of artifacts as the same Nageotte's chamber, and slide cover was repeatedly used and also because the flow cytometry gating was setup to encompass only intact WBCs. Any component which has an increased pre-storage leukoreduction time has more WBC fragments and cell-free DNA in the LD products, which may interfere with rWBC counts. Accurate values of rWBC could be achieved by minimizing the time between collection, component preparation, LD, sample recovery. In the present study appropriate steps were taken to ensure that all products were processed and analyzed on the day of preparation.

The manual method failed to find leukocytes in one-fourth of the samples, due to its high lower detection limit compared to the flow cytometric method. The correlation between the two methods was low in all the samples that were quantified by both. The results demonstrate that FC method is suitable for use in samples with low leukocyte numbers. Automated methods for counting rWBC in LD cellular components offer advantages of improved precision and greater accuracy than are seen with the Nageotte hemocytometer method. Automated methods are less labor-intensive but more expensive than microscopic methods. However, manual method is more cumbersome and time-consuming.

Conclusion

Flow cytometric techniques provide a reproducible and objective tool for counting rWBCs in LD blood components compared with the Nageotte counter.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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