RESEARCH ARTICLE

Rapid, sensitive detection of bacteria in platelet samples with Fountain Flow Cytometry

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Background: There is a current need to develop a technique for bacterial screening of platelet donations that is more rapid, sensitive, and economical than alternatives. The objective of this research was to perform a pilot test of the viability of Fountain Flow Cytometry (FFC), for the rapid and sensitive detection of bacteria in platelet donations.

Methods: Platelet samples were inoculated with serial dilutions of five selected bacterial strains. Samples were then centrifuged, reconstituted in buffer, and stained with a live/dead bacterial stain cocktail. The resulting aqueous sample was measured by FFC, in which the sample passed as a stream in front of an LED, which excited the fluorescent labels. Fluorescence was detected with a digital camera as the sample flowed toward it.

Results: Fountain Flow Cytometry enumeration yielded results that were linear with bacterial concentration, having an R^2 of ≥ 0.98 with a detection efficiency of $92\% \pm 3\%$. Measurements of uninoculated samples showed a false-positive detection rate at ~ 400 colony forming units (CFU)/mL. Detection of bacterial concentrations in platelets above this threshold can be made in ~ 15 minutes, including sample preparation time.

Conclusion: This pilot study supports the efficacy of FFC for the rapid and sensitive screening of platelet donations for bacteria.

KEYWORDS

bacteria, biodetection, cytometry, fountain flow, platelets

1 | INTRODUCTION

Approximately three million units of platelets are transfused in the United States annually.¹ Of these, an estimated one in one thousand to one in three thousand units is contaminated with potentially pathogenic bacteria.² The transfusion of contaminated platelets can result in bacterial sepsis in the recipient. Platelet transfusion-associated sepsis remains a significant transfusion-related risk in the United States. In recognition of this danger, the AABB instituted AABB standard 5.1.5.1. This standard requires that all member blood banks and transfusion services employ a method to detect or inactivate platelets for bacteria before they are released for use.

The adoption of AABB Standard 5.1.5.1 has driven the development of several technologies in an attempt to solve the problem of bacterial contamination. Currently, the FDA has cleared for quality control four instruments for use in the United States.³ The two in wide

use are bioMérieux's BacT/ALERT 3D and Pall Corporation's eBDS, both requiring a long (>24 hours) predetection incubation. The PGD by Verax and BacTx by Immunetics are rapid but relatively insensitive ($>10^3$ – 10^4 colony-forming units (CFU)/mL)^{4,5} and are, therefore, typically used for point-of-care measurements shortly before transfusion. All apheresis and whole blood platelets have an FDA-approved shelf life of 5 days from donation to transfusion.

Photochemical pathogen reduction technology (PRT) is commonly used in Europe for platelets, but it has only recently been approved for use in the United States by the FDA.⁶ One of the main drawbacks of PRT is the lack of independent research substantiating bacterial elimination in high-volume samples over the shelf life of stored platelet units.⁷ There is concern that *K. pneumonia* and *B. cereus*, in particular, have grown in PRT-treated pooled platelets.⁷ This leads to the question as whether some spore-forming bacterial strains can regrow after PRT, because their spores are not inactivated by PRT.⁷ In addition, PRT

FIGURE 1 Schematic diagram of an LED-illuminated Fountain Flow Cytometer. (A) A prototype Fountain Flow Cytometer utilized low-level detection of bacteria in platelet donations. (B) The FFC optical configuration illustrating 1-Flow cell with sample inlet (1a) and outlet (1b); 2-Dichroic mirror; 3-LED-focusing lens; and 4-LED (The CCD camera and 35-mm lens are seen in A&C.) (C) A schematic representation of Fountain Flow Cytometric detection. A sample of fluorescently tagged cells flows through the flow cell toward the CCD camera and fore optics. The cells are illuminated in the focal plane by an LED. Labeled bacteria pass through the CCD camera focal plane and are imaged by the camera and lens through the flow cell window, as a bandpass filter isolates the wavelength of fluorescence emission. The fluid in which the cells are suspended then passes by the window and out the flow cell drain tube (reproduced with permission⁹)

is likely to be significantly more costly and more labor intensive than methods now in use.^{7,8} One recent economic analysis for a Spanish hospital showed that the cost of chemical deactivation for platelets is much higher than conventional tests, 93€ per unit for chemical deactivation vs 21€ per unit for conventional bacterial testing.⁹

In our previous work,^{10,11} we introduced an alternative method, Fountain Flow Cytometry (FFC), for the detection of microorganisms at low concentrations in volumes in excess of 100 mL. With FFC, as with conventional flow cytometry, the suspended microorganisms are stained to facilitate detection. The resulting aqueous sample is passed as a stream in front of an LED, which excites the fluorescent dyes (Figure 1). Unlike conventional flow cytometry, the resulting fluorescence is measured with a digital camera, as the sample flows toward the camera.

The contribution of the research described here will be a first step in the development of FFC as an economical, rapid (<1 hour), alternative to established methods that is one to two orders of magnitude more sensitive for the detection of bacterial contamination of platelets. This approach has the potential to decrease the use of contaminated platelet units along with lengthening the time window between end of test and expiration. In addition, adoption of this technique for rapid bacterial detection at low concentrations would allow detection with minimal or no incubation, thus allowing for the use of fresher (<1-day-old) platelets which may provide a larger post-transfusion platelet count to the recipient and may reduce the number of some transfusion reactions (e.g. febrile, non-hemolytic reactions). In this pilot study, we perform research to assess the feasibility of FFC as a method to test for bacterial contamination of platelet units,

immediately post-donation (at a blood center), just prior to shipping, and/or immediately pre-transfusion (at a hospital).

2 | MATERIALS AND METHODS

2.1 | FFC protocol for measuring samples

Fountain Flow Cytometry measurements of apheresis platelet samples inoculated with serial dilutions of bacteria in addition to uninoculated samples were made with a Fountain Flow system, as described elsewhere,¹² with essentially the same measurement and calibration protocols as in previous work.¹³ Ten-milliliter diluted samples were measured with an FFC flow rate of 0.8 mL/min. For each 10-mL (post-dilution) sample enumerated with the FFC, the sample was counted in multiples of 300 video frames (each frame obtained with a 60-msecond exposure) with each set of 300 frames representing a 0.25-mL subsample. There was no significant “dead” time interval between exposures so that the sample flow in the FFC is continuously monitored. A mean and standard deviation of enumerated bacteria were determined from the ensemble of the 300-frame subsamples representing a single sample. Samples were provided by the Garth England Blood Bank of the Poudre Valley Hospital (Fort Collins, Colorado).

All samples (whether inoculated or uninoculated, measured by FFC or hemocytometry) were treated with the protocols given below, using three proprietary (SoftRay, Inc., Laramie, WY, USA) dye cocktails: LSS1 (LightSpeed Solution 1), LSS2, and LSS3. These protocols use a lysis and counterstaining step (with LSS1), followed by centrifugation,

FIGURE 2 Histogram of detections of labeled *E. coli* from a PBS sample inoculated with bacteria at a concentration of 1040 bacteria/mL. Intensities (digital FFC counts) were computed by summing the individual pixel intensities in a 10×10 pixel box

removal of supernatant, resuspension of the pellet in buffer with LSS2, and then by further dilution with LSS3.

All FFC detections in this study were made using SoftRay's Biocount software.^{10,13} Our detections for bacteria were based on a 100 digital count (FFC intensity units) threshold for fluorescence, consistent with the histogram of *E. coli* photometric intensities shown in Figure 2. In addition, fluorescing objects larger than nine pixels in size (with each pixel 4 μm on a side) were eliminated from enumeration as they would be indicative of objects larger than bacteria.

2.2 | Stain selection

For this project, we chose to illustrate the efficacy of detecting viable Gram-positive and Gram-negative bacteria at relatively low concentrations using a viability stain cocktail including the DNA/RNA dye SYBR Green I (Life Sciences, Eugene, OR, USA) with a propidium iodide counterstain to assess bacterial viability. The former stain will penetrate both live and dead cells, while the latter is normally excluded from cells without permeable cell walls and is thus an indicator of cell non-viability.¹⁴ The FFC in this work only counts viable cells which emit in the green.

2.3 | Filter selection

All FFC data were taken with SEMRock filters (Rochester, NY, USA) designated for fluorescein (an excitation filter at 472-nm, 30-nm bandwidth and an emission filter at 520-nm, 35-nm bandwidth). The excitation filter was used to bandpass limit the emission from a blue LED, a Luminus (Sunnyvale, CA, USA) CBT-90-B-C11-KJ300 with a 3 mm $\times$ 3 mm emitting area, and a 460-nm peak emission wavelength, running at 3.0 A current.

2.4 | Preparation of serial dilutions of platelet samples inoculated with bacteria

Samples of two-fold serial dilutions of bacteria were prepared to be inoculated into platelet samples. A 1-mL stock sample of bacteria was prepared from overnight liquid culture media, centrifuged, and resuspended

in 1 mL of PBS. Ten microliters of stock was then inoculated into 0.5 mL of platelet sample and enumerated as detailed in the following section. The goal was to produce FFC counts at the highest concentration between 1000 and 3000/mL. Based on this initial measurement, for each bacterial strain, subsequent measurements were made with 0, 1.25, 2.5, and 5.0 μL of bacterial stock into 0.5 mL of platelet sample, or 0, 2.5, 5.0, and 20.0 μL of stock, producing a two-fold dilution sequence.

2.5 | Preparation of apheresis platelet samples inoculated with bacteria for FFC

Fountain Flow Cytometry preparation consisted of five steps: (1) Each 0.5 mL platelet sample was inoculated with 0–20 μL of bacterial stock in a microcentrifuge tube prior to FFC enumeration; 0.5 mL of PBS and 110 μL LSS1 staining solution were added and subsequently vortexed. (2) The sample was incubated in a shaking incubator at 37°C and vortexed. (3) The sample was centrifuged for 4 minutes at 16 000 g. Then supernatant was discarded, leaving approximately 0.1 mL of fluid. (4) 0.9 mL of LSS2 was added to the sample prior to 5 minutes of incubation and vortexing to reduce background fluorescence. (5) Finally, the sample was added to 9 mL of LSS3, vortexed, and introduced to the FFC for enumeration. Approximately 0.25 mL was processed by FFC for each individual measurement.

2.6 | FFC enumeration of inoculated platelet samples

Measurements of the two-fold serial dilutions of bacteria are shown in Figure 3. Each bacterial strain was tested with a different platelet sample on a different date. To confirm that the original sample was uncontaminated by bacteria, 100 μL of uninoculated sample was spread plated on tryptic soy agar and incubated for 2 days at 37°C.

2.7 | Hemocytometer calibration

For comparison of FFC-measured cell concentrations in buffer with an independent counting method, we chose hemocytometry, using the protocol described in the following steps: (1) A 0.5 mL volume of

FIGURE 3 Fountain Flow Cytometer enumeration of bacteria inoculated into fresh apheresis platelet samples diluted by 10 $\times$ in PBS. Each data point represents a 0.25-mL sample labeled with SYBR Green I. Diamonds represent FFC counts, equivalent to CFUs in 0.25 mL of the sample. (To obtain the equivalent concentration of bacteria in the original platelet sample, each FFC count must be multiplied by 40.) A square marker represents the FFC counts predicted from hemocytometry calibration. Shown are Fountain Flow Cytometer measurements of platelet samples inoculated with serial dilutions of (A) *E. coli*, (B) *S. epidermidis*, (C) *S. agalactiae*, (D) *S. marcescens*, and (E) *S. aureus*

PBS was inoculated with 10 or 20 μ L of bacterial stock (corresponding to the maximum volume used for the serial dilution series described above). (2) A 15 μ L volume of 10% Triton X-100 and 5 μ L of propidium iodide stock solution (1 mg/mL in PBS) were added to the sample and vortexed. (3) The sample was incubated for 1 minute at 37°C. (4) A 5 μ L volume of Sybr Green I stock solution (30 μ L of 1000 $\times$ Sybr Green I in 900 μ L DMSO) was added to the sample, which was then vortexed. (5) The sample was incubated at RT for 5 minutes and vortexed again. (6) A 50- μ L volume of sample was loaded into a hemocytometer cell mounted on the stage of an Olympus BH-2 epifluorescent microscope and cells were counted with a total magnification of 400 $\times$.

2.8 | Flow rate calibration

In order to compare FFC enumeration with hemocytometry, it is necessary to determine the volume of sample being analyzed by the FFC in a fixed number of frames, equivalent to a fixed amount of time (in this case 300 frames in 18 seconds). This necessitates a determination of the flow rate of sample through the FFC. Peristaltic pump rates for the pump used in our experiments (Reglo, Ismatech, Glattbrugg, Switzerland) were calibrated each day by weighing the FFC effluent on a digital scale. Variations in pump rate between such calibrations were within approximately $\pm 7\%$.

TABLE 1 Enumeration of bacteria in null (uninoculated), fresh, apheresis samples^a

Sample #	Date drawn	Date FFC	Δt	Counts	CFU/100 μ L
1	3/25/2014	3/29/2014	4	12.6	3
2	3/25/2014	3/29/2014	4	15.0	2
3	3/25/2014	3/29/2014	4	17.6	0
4	3/26/2014	3/29/2014	3	20.2	4
5	3/26/2014	3/29/2014	3	15.2	0
6	3/26/2014	3/29/2014	3	12.8	1
7	3/27/2014	3/29/2014	2	9.2	2
8	3/27/2014	3/29/2014	2	17.0	2
9	3/27/2014	3/29/2014	2	8.6	0
10	3/27/2014	3/29/2014	2	8.4	34
11	3/28/2014	3/29/2014	1	7.0	2
12	4/8/2014	4/9/2014	1	9.4	3
13	4/7/2014	4/9/2014	2	10.4	2
14	4/7/2014	4/9/2014	2	2.6	0
15	4/7/2014	4/9/2014	2	13.0	3
16	4/7/2014	4/9/2014	2	4.8	0
17	4/9/2014	4/12/2014	3	9.4	7
18	4/9/2014	4/12/2014	3	6.2	32
19	4/9/2014	4/12/2014	3	16.4	0
20	4/9/2014	4/12/2014	3	8.2	0
21	4/10/2014	4/12/2014	2	2.2	1
22	4/10/2014	4/12/2014	2	11.2	0
23	4/10/2014	4/12/2014	2	7.8	0
24	4/11/2014	4/12/2014	1	4.2	0
25	4/11/2014	4/12/2014	1	9.8	1
26	4/12/2014	4/12/2014	0	3.8	1
Mean				10.1	
Standard deviation				6.3	

^aFive subsamples were run from each sample. Shown are the date each sample was drawn, the date it was measured with Fountain Flow Cytometry, and the number of days elapsed between the two. In the last column, plate counts (for 100 μ L of sample incubated for 2 days) are given for comparison. Two of the plate counts were at the 1- σ detection threshold for FFC (~400 CFU/mL).

2.9 | FFC measurements to determine the rate of false-positive detections in uninoculated platelet samples

Fountain Flow Cytometry measurements of uninoculated samples of platelets were made in addition to the sets taken with serial dilutions, using the protocol given previously for inoculated samples, with results shown in Table 1.

3 | RESULTS

3.1 | Measurements of serial dilutions of bacteria inoculated into platelet samples by FFC and hemocytometry

Comparisons of FFC measurements of serial dilutions of SYBR Green I/propidium iodide labeled bacteria subsequent to inoculation into

platelet samples against hemocytometry counts of the mother bacterial sample are shown in Figure 3. The data show a linear relationship within the 1- σ error bars (calculated empirically from the five sub-samples comprising each sample). In addition, we measured uninoculated samples with the FFC, all of which show 20.2 counts or less. Best-fit lines were computed for the data from each of the five bacterial strains shown in Figure 3. The R^2 for all of the fits are at or above 0.98.

Agreement between measurements with FFC and hemocytometry was measured by the ratio of FFC/hemocytometry counts at an FFC sample concentration of ~1000/mL (seen in Figure 3 and Table 2). The variance weighted mean for this ratio was 0.92 ± 0.03 (1- σ) and indicates a good detection efficiency for this FFC application. Concentrations higher than this were not used for calibration as systematic undercounting at higher concentrations becomes an issue because two bacteria seen close together in an image can be counted as a single bacterium.¹¹

TABLE 2 Fountain Flow Cytometry counting efficiency^a

Species	Counting efficiency
<i>E. coli</i>	0.92 ± 0.09
<i>S. epidermidis</i>	0.72 ± 0.17
<i>S. agalactiae</i>	0.79 ± 0.16
<i>S. marcescens</i>	0.93 ± 0.09
<i>S. aureus</i>	1.6 ± 0.23
Weighted mean	0.92 ± 0.03

^aThe counting efficiency is determined from the measured ratio of FFC counts to hemocytometry counts, taken from the data plotted in Figure 3. An efficiency of 1.0 would mean that each bacterial cell in a sample is counted once. The dominant source of error in computed efficiencies is due to counting statistics in the hemocytometry.

3.2 | FFC measurements of the rate of false-positive detections

In addition to the serial dilutions used to measure the linearity and efficiency of detection, 26 samples from the Garth Englund Blood Center were used to assess the rate of false-positive detections using the same protocol and the same set of instrument settings used for the experiments with inoculated samples. The result of these measurements is a mean false-positive detection rate of 10.1±6.3 viable bacteria per sample, corresponding to ~400 CFU/mL (Table 1). (The factor of 40 difference between these numbers reflects a platelet dilution into buffer of 10× and a sample measurement size of 0.25 mL.) The time between donation and FFC ranged from 1 to 4 days, with no obvious correlation between elapsed time and false-positive detection rate.

4 | DISCUSSION

4.1 | Rate of false-positive detections

One of the primary questions addressed in this study is whether or not the selection of dyes and filters used can produce an acceptable rate of FFC false detections of bacteria in platelets. As noted above, data shown in Table 1 address this question and indicate the viability of using FFC to detect bacterial contamination down to a level approaching 400 CFU/mL.

4.2 | Comparison of FFC with hemocytometry

Comparisons of FFC and hemocytometry are similar to those published previously.¹⁰ Comparisons were made of hemocytometry of washed bacteria stained and suspended in PBS buffer, with FFC counts of bacteria from the same mother sample inoculated into platelet samples (as shown in Figure 3). As mentioned previously, the ratio of counts made in this manner (FFC/hemocytometry) was 0.92±0.03. FFC counts of the bacteria are consistent with the technique being nearly 100% efficient. Hemocytometry provides a direct comparison with FFC, because both methods count individual bacteria using a

viability stain/counterstain. Both methods are measuring cell viability based on cell wall integrity, as does conventional flow cytometry using a viability stain/counterstain combination.

4.3 | Performance comparison of FFC with other FDA-approved methods

Of the three methods approved for point-of-issue testing by the FDA, the performance of FFC is easiest to compare with Hemosystem's Scansystem (no longer being sold) which uses a method of fluorescence detection based on laser scanning of filters onto which stained bacteria have been separated from platelet samples. Scansystem has a threshold for detection of ~100 CFU/mL and an efficiency for bacterial recovery onto filters of ~50%.¹⁵ More relevant comparisons for FFC are with the more widely adopted culture-based systems, BacT/Alert and eBDS. The most relevant criteria for comparison are time from donation to result and the threshold bacterial concentration for a positive detection. FFC does not need the incubation period of up to 72 hours required for BacT/Alert and eBDS culture assays. Post-inoculation levels at which most samples inoculated with *E. coli* and *S. epidermidis* rise above the detection threshold for the BacT/Alert have been reported to be as high as 10⁶-10⁷ CFU/mL.¹⁶ Finally, FFC is an order of magnitude more sensitive than other rapid detection methods used for point-of-use detection, such as the Verax PGD.

The simplicity of the FFC platform allows for an inexpensive, portable, platform with a low cost per test, attractive for use in countries with a thinly distributed population (e.g. Australia). Moreover, the sensitivity, speed of detection, and low cost of FFC make it viable for use immediately after donation as well as the same day as transfusion.

4.4 | Future work

Further work is being done to expand the false count rate verification to a much larger number of samples as well as to expand the suite of tested bacterial species.

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