

Rapid Detection of Bacterial Contamination of Platelet-Rich Plasma-Derived Platelet Concentrates Using Flow Cytometry

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Abstract. Flow cytometry can be used to detect bacterial contamination of platelet products. In this study, we investigated whether the incubation of a minimal volume of platelet-rich plasma (PRP)-derived platelet concentrates (PCs) with growth medium improved the analytical sensitivity of flow cytometry. Five bacterial strains (Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Klebsiella pneumoniae, and Escherichia coli) were used. Platelets were inoculated with 10, 10^2, and 10^3 CFUs per mL; 0.5 mL, 1.0 mL, and 2.0 mL aliquots of spiked platelets were incubated with growth medium at 37°C for 24 hours. During the incubation period, the numbers of events were analyzed every 4 hours by flow cytometry. We could detect a low concentration (10 CFUs per mL) of bacteria in a small volume (minimum 0.5 mL) of PCs. Irrespective of spiking concentrations and incubation volumes, the detection times of S. aureus and S. epidermidis were 24 hours or less, while those of B. cereus, K. pneumoniae, and E. coli were 16 hours or less. A higher spiking concentration made it possible to shorten the detection time. The numbers of detected bacteria increased during the incubation. However, the graphs corresponding to K. pneumoniae and E. coli showed peak levels and decreasing patterns during the incubation period. The incubation of small volumes of PC with growth medium increased the analytical sensitivity of flow cytometry for bacterial detection. Therefore, flow cytometry can serve as a useful method for sterility testing using PRP-derived PCs with only low levels of consequent platelet loss.

Key words: platelet concentrates, PC, bacterial contamination, flow cytometry

Introduction

Platelet concentrates (PCs) are the most common source of transfusion-associated infection [1] due to their storage temperature. Because PCs are stored at 20 to 24°C with agitation to maintain platelet function, bacteria can multiply in PCs [2]. The frequency of sepsis and fatality due to transfusion-associated bacterial infection is higher for PCs than for other blood products [3]. It has been reported that approximately 1 in 2,000 to 1 in 3,000 units of PCs are contaminated with bacteria [4], and the risk of sepsis is approximately 1 in 15,000 to 1 in 100,000 [5]. Most bacteria identified in PCs are normal skin flora, such as Staphylococcus epidermidis, Staphylococcus aureus, and Bacillus cereus [1, 4, 6]. The introduction of nucleic acid amplification testing (NAAT) for blood products has markedly reduced the risk of viral transmission [2]; however, the risk of bacterial infection is over 100 times higher than the risk of viral transfusion-associated infection [7].
Several methods of detecting bacterial contamination of blood components have been developed. Many countries have introduced sterility tests as a detection method for PCs. Common sterility tests include the automated bacterial detection system using culture methods (BacT/ALERT, bioMérieux, Durham, NC) monitoring CO₂ production and an enhanced bacteria detection system (Pall eBDS, East Hills, NY) monitoring oxygen consumption [8-10], and real-time polymerase chain reaction (PCR) [7, 11]. Recently, flow cytometry was introduced as a sterility test for PCs [12, 13]. Most previous studies using flow cytometry for sterility tests used buffy coat (BC)-derived PCs. However, platelet-rich plasma (PRP)-derived PCs, which have a lower volume than BC-derived PCs, are routinely used in Korea and the United States [14]. Therefore, it is difficult to obtain large sample volumes of PCs for sterility tests.

In the present study, we used a smaller volume of PCs than did previous studies and introduced a pre-incubation step using growth media prior to flow cytometry analysis. We investigated whether the pre-incubation step increased the analytical sensitivity and reduced the sample volume and detection time.

**Materials and Methods**

**Study design.** Among the PRP-derived PCs supplied by the Korean Red Cross Institute, we used PRP-derived PCs (discarded for various reasons) within the term of validity. The following bacteria isolated from patients were used: *S. aureus*, *S. epidermidis*, *B. cereus*, *Klebsiella pneumoniae*, and *Escherichia coli*. Each of the bacterial species was spiked into three tubes containing 25 mL of PCs, and the final concentrations of spiked PCs were adjusted to 10, 10², and 10³ CFUs per mL for each bacterial strain. Subsequently, 0.5 mL, 1.0 mL, and 2.0 mL aliquots from the spiked PCs of each concentration for each bacterium were incubated with 8 mL of bacterial growth medium (BBL Fluid Thioglycollate Medium, BD Diagnostic Systems, Sparks, MD) at 37°C for 24 hours. One control tube of PCs without bacterial spiking was simultaneously incubated with medium. All procedures were performed in duplicate for each bacterium.

**Flow cytometry.** For the flow cytometric measurement of bacteria, we used a BD FACSCalibur system (BD Biosciences, San Jose, CA) equipped with an air-cooled argon ion laser (15 mW and 488 nm) and filter sets for fluorescence (FL) : FL1 (515 to 545 nm), FL2 (564 to 606 nm), and FL3 (> 650 nm). Bacteria were stained with thiazole orange, membrane-permeable dye (BD Biosciences) for five minutes according to the manufacturer’s instructions. The number of fluorescein isothiocyanate-positive cells was increased in the bacterially contaminated sample tubes as compared with the corresponding negative control tubes. At least 100,000 cells were scanned for each sample. The event numbers were analyzed every 4 hours for 24 hours. The flow cytometry result was considered positive when the value obtained by subtracting the event number of the negative control tube from the event number of the spiked PC tube was greater than 100. Data were acquired and analyzed with CellQuest Pro software (BD Biosciences). All procedures were performed in duplicate for each bacterial strain.

**Comparison with the real-time polymerase chain reaction (PCR).** To compare the performance of flow cytometry, we adopted real-time PCR as a reference method. We conducted the comparison experiments for *Klebsiella pneumoniae* and *S. epidermidis*, representative of rapidly-growing and slowly-growing bacteria, respectively. Real-time PCR for 0.5 mL aliquot, the lowest volume, from the spiked PCs of 10, 10², and 10³ CFUs per mL at every 4 hours for 24 hours incubation were performed with flow cytometry. We conducted the real-time PCR according to our previously published real-time PCR protocol for detection of bacterial contamination of platelets [15]. The primers targeted and amplified bacterial 16S rRNA sequences approximately 466 bp in length. The forward primer was 5'- TCC TAC GGG AGG CAG CAG CAG T-3', and the reverse primer was 5'- GGA CTG ATG GGG ATT GCT GGC CAA-3'; both primers were synthesized by Bioneer (Daejeon, Korea). Real-time PCR was performed using the LightCycler 2.0 (Roche, Penzberg, Germany). All procedures were performed in duplicate for each bacterial strain.
In flow cytometry, each sample component is analyzed according to the differences between the side scatter (SSC) and FL1 signals on a logarithmic scale. Figure 1 shows the dot plots of 2.0 mL PCs spiked with high concentrations (10^3 CFUs per mL) of different bacterial species. The spiked PCs were incubated for 24 hours in the growth medium. For all five bacteria, clouds of highly fluorescent dots were located at an identical position distant from the platelet debris, indicating low fluorescence in the dot plots.

Figure 2 shows the dot plots of 0.5 mL of PCs spiked with 10^2 S. aureus per mL. The weakly fluorescent platelets were situated on the left side, and the highly fluorescent bacteria appeared on the right side, after a 12-hour incubation with growth medium. The event numbers in the gated area of high fluorescence increased over the incubation time.

Table 1 presents the detection times and event numbers measured by flow cytometry. The detection time was the earliest time when the value obtained by subtracting the event number of the negative control from the event number of the spiked PCs was greater than 100. S. aureus and S. epidermidis were detected in all experiments after 24-hour incubation, E. coli was detected after 16-hour incubation, and B. cereus and K. pneumoniae were detected after 12-hour incubation. The concentrations of the bacterial inoculate were critical. The higher the spiking concentration, the shorter the time required for detection. However, the effects of incubation volumes varied. In experiments with S. aureus and S. epidermidis, a higher incubation volume resulted in a shorter time required for detection, especially when spiked with a low concentration of bacteria. In the experiments with K. pneumoniae and B. cereus, the detection time did not vary with the incubation volume. In the experiment with E. coli, a larger incubation volume delayed the detection time.
Detection times of flow cytometry were compared with those of real-time PCR for 0.5 mL aliquots from the spiking concentrations of 10, 10^2, and 10^3 CFUs per mL of *Klebsiella pneumoniae* and *S. epidermidis*. In analysis using flow cytometry, *Klebsiella pneumoniae* was initially detected after 8-hour incubation at the spiking concentration of 10^2 and 10^3 CFUs per mL, and it was initially detected after 12-hour incubation at the lowest concentration (10 CFUs per mL). However, in analysis using real-time PCR, it was initially detected after 8-hour incubation at all three spiking concentrations. In analysis using both flow cytometry and real-time PCR, *S. epidermidis* was initially detected after 12-hour, 16-hour, and 24-hour incubation at the spiking concentration of 10, 10^2, and 10^3 CFUs per mL, respectively.

**Figure 3** shows the changes in the numbers of events of 0.5 mL PC spiked with various concentrations of five bacteria, according to the incubation time. The numbers of events in *S. aureus*, *S. epidermidis*, and *B. cereus* increased during the 24-hour incubation. However, *K. pneumoniae* and *E. coli* showed peak numbers of events at 12 to 20 hours and at 16 hours of incubation, respectively. After the peak time, the number of events decreased during the incubation. Similar patterns were observed independent of the incubation volume (data not shown).

**Discussion**

The efficiency of bacterial detection in PCs depends on factors such as sampling time, sample volume, the sensitivity of the detection method, and...
bacterial growth kinetics [16]. Because the samples for the sterility test for PCs were obtained early, the chance of detecting any bacterial contamination was low because the contaminating bacteria would not have had enough time to proliferate to a detectable number [16, 17]. However, the sampling time could not be delayed due to the short storage time of the platelets. The larger the sample volume obtained from PCs, the higher the chance of detecting bacterial contamination; an increased sample volume reduced the volume of PCs available for transfusion [18, 19]. PRP-derived PCs are widely used in Korea and the United States. The volume of PRP-derived PCs is approximately 41 mL less than the volume of the BC-derived PCs used in Europe and Canada [20]. Therefore, rapid and sensitive methods using only a small sample volume are needed for the detection of bacterial contamination in Korea and the United States.

A number of methods have been introduced for the detection of bacterial contamination of PCs, such as culture, real-time PCR, and flow cytometry [4]. Culture is the most sensitive method for the detection of bacterial contamination of PCs; however, the requirements of large sample volume and relatively long (more than 24 hours) incubation time are drawbacks to its application to platelet products [1, 4]. Real-time PCR is a rapid and sensitive method for the detection of bacterial contamination of PCs; however, the requirements of large sample volume and relatively long (more than 24 hours) incubation time are drawbacks to its application to platelet products [1, 4].

Table 1. Detection times and event numbers of different bacteria according to the spiking concentrations (10, 10², and 10³ CFUs per mL) and pre-incubation volumes (0.5, 1.0, and 2.0 mL) of the spiked platelets.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Detection Time (hr)</th>
<th>Events (/ul) at Detection Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mL</td>
<td>1.0 mL</td>
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<tr>
<td><em>S. aureus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 CFU/mL</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>10² CFU/mL</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>10³ CFU/mL</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 CFU/mL</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>10² CFU/mL</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>10³ CFU/mL</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 CFU/mL</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>10² CFU/mL</td>
<td>8</td>
<td>8</td>
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<tr>
<td>10³ CFU/mL</td>
<td>8</td>
<td>8</td>
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<tr>
<td><em>K. pneumoniae</em></td>
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<tr>
<td>10 CFU/mL</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>10² CFU/mL</td>
<td>8</td>
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<tr>
<td>10³ CFU/mL</td>
<td>8</td>
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<tr>
<td><em>E. coli</em></td>
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<tr>
<td>10 CFU/mL</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>10² CFU/mL</td>
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<tr>
<td>10³ CFU/mL</td>
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</table>

aThe earliest time when events were greater than 100
bThe value obtained by subtracting the event number of the negative control from the event number of the spiked PCs
Flow cytometry is a rapid, easy procedure to perform, which makes it a good candidate for the detection of bacterial contamination. In flow cytometry for bacterial detection, the bacteria are stained with a fluorescent dye – thiazole orange – and event numbers are measured according to fluorescence intensity. Thiazole orange is an asymmetrical cyanine dye that binds to nucleic acids and enters both viable and dead cells [22]. Previous studies have reported on the suitability of flow cytometry for sterility testing of PCs [12, 13]. In these studies, PC samples spiked with bacteria were incubated at 35 or 37°C for 24 hours. Large volumes of sample (minimum 20 mL) were available because these studies were performed on large pools of four BC-derived PCs. Therefore, the detection time was shorter and the sensitivity higher without growth media. As mentioned above, the sample volume was taken into consideration in the sterility test because of the small volume of PRP-derived PCs. In

Figure 3. Changes of event numbers per µL of bacteria during the incubation time. A total of 0.5 mL of PCs spiked with 10 CFUs per mL (●), 10² CFUs per mL (□), or 10³ CFUs per mL (▲) was incubated with 8 mL bacterial growth medium for 24 hours at 37°C.
Korea, single-donor platelet products with volumes of 200 to 250 mL account for only 12% of total platelet products being transfused [23]; therefore, PRP-derived PCs are used for most platelet transfusions. The mean volumes of PCs derived from 320 and 400 mL of whole blood are approximately 41 and 46 mL, respectively [24]. Combined with the lower sensitivity of flow cytometry, especially compared to the PCR method [7], these small volumes are a major obstacle to the introduction of flow cytometry for sterility tests in PRP-derived PCs, because of the loss of platelets in sterility tests. Therefore, some modifications are required, and the incubation of a small volume of PCs in bacterial growth media prior to flow cytometry makes an increase in the analytical sensitivity of the test possible [25].

We showed that incubation with growth media reduced the loss of platelets for sterility testing and increased the sensitivity of the detection of bacterial contamination. We could detect a low concentration (10 CFUs per mL) of bacteria in a small volume (minimum 0.5 mL) of PCs using flow cytometry (i.e., five CFU of bacteria). The initial bacterial concentration, if present, in blood components is thought to be extremely low (below 10 CFUs per mL) [3]; therefore, it is important to be able to detect such a small number of bacteria.

All five of the studied bacterial strains were detected within 24 hours, and there was little wasting of platelets for sterility testing. Moreover, the rapidly-growing bacteria, namely B. cereus, K. pneumoniae, and E. coli, were detected in 16 hours or less. If sampling is performed immediately after platelet production, and the sample is subsequently incubated for 24 hours, the bacteria in contaminated PCs could proliferate and be detected by flow cytometry. Therefore, we believe that a 24-hour delay is reasonable to allow for the investigation of possible contamination of PCs and thus reduce the risk of transfusion-transmitted infections.

A comparison of flow cytometry and real-time PCR indicated no difference in detection time for various concentrations (10, 10², and 10³ CFUs per mL) of bacteria in a small volume (0.5 mL) of PCs, except that the initial detection time for the lowest concentration (10 CFUs per mL) of Klebsiella pneumoniae varied between the two methods. Considering the application of a 24-hour incubation period in the flow cytometry method for detection of bacterial contamination of PCs, we thought that this presented no logistical problem in early 4-hour delay of detection time during 24 hours.

In a previous study of K. pneumoniae, the dot plot of bacteria overlapped with that of platelet debris, reflecting poor discrimination between bacteria and platelet debris [12]. Our study showed that the five bacteria were identically positioned in the dot plots (Figure 1). This difference is probably due to the logarithmic scale used for gating analysis, which may have contributed to the power of discrimination in the low fluorescence range. Decreasing numbers of events were observed in some rapidly-growing bacteria, such as K. pneumoniae, during the incubation. Because growth kinetics vary greatly according to bacterial species [16], and the volume of growth media is limited, rapidly-growing bacteria show early peaks. In this study, the number of events did not decrease below the detection threshold during a 24-hour incubation. Therefore, it is feasible to use flow cytometry for sterility testing of PCs after a 24-hour incubation with growth media.

Early studies of sterility testing of PCs using flow cytometry were performed with pooled BC-derived PCs [7, 12, 13, 25]. In this study, we demonstrated that flow cytometry is valuable for the sterility testing of PRP-derived PCs. In low-volume PC samples with small numbers of bacteria, a 24-hour incubation with growth media can increase the analytical sensitivity of flow cytometry for bacterial detection. Therefore, flow cytometry can serve as a useful method of sterility testing of PRP-derived PCs with only low levels of consequent platelet loss.

References
Flow cytometry for bacterial contamination detection