Rapid Screening Method for Detection of Bacteria in Platelet Concentrates


Hemosystem, 13006 Marseille, France

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Public awareness has long focused on the risks of the transmission of viral agents through blood product transfusion. This risk, however, pales in comparison to the less publicized danger associated with the transfusion of blood products contaminated with bacteria, in particular, platelet concentrates. Up to 1,000 cases of clinical sepsis after the transfusion of platelet concentrates are reported annually in the United States. The condition is characterized by acute reaction symptoms and the rapid onset of septicemia and carries a 20 to 40% mortality rate. The urgent need for a method for the routine screening of platelet concentrates to improve patient safety has long been recognized. We describe the development of a rapid and highly sensitive method for screening for bacteria in platelet concentrates for transfusion. No culture period is required; and the entire procedure, from the time of sampling to the time that the final result is obtained, takes less than 90 min. The method involves three basic stages: the selective removal of platelets by filtration following activation with a monoclonal antibody, DNA-specific fluorescent labeling of bacteria, and concentration of the bacteria on a membrane surface for enumeration by solid-phase cytometry. The method offers a universal means of detection of live, nondividing, or dead gram-negative and gram-positive bacteria in complex cellular blood products. The sensitivity is higher than those of the culture-based methods available at present, with a detection limit of 10 to 10^2 CFU/ml, depending upon the bacterial strain.

In the field of blood transfusion, blood products are now routinely screened by ultrasensitive techniques to minimize the risk of transmitting viruses to recipients. The risk of bacterial contamination remains, however, and is now the most common transfusion-related infectious adverse event (1). Preparations of platelet concentrates (PCs) are particularly at risk, as they must be stored at 20 to 24°C to maintain optimal viability and functional properties. The risk of bacterial contamination in PC transfusion, estimated to be 1 in 1,000 to 1 in 2,000, is 50 to 250 times higher than the combined risk of human immunodeficiency virus, hepatitis B and C virus, and human T-cell leukemia virus type 1 and 2 contamination (4–6, 18, 26). Unlike with the transmission of a viral component, bacterial infections are associated with acute reactions, rapid onset of sepsis, and high rates of mortality in the period immediately following transfusion (24). In many countries the only bacterial screening technique currently routinely used to avoid this is platelet concentrate swirling (29), the detection limit of which is 10^7 to 10^8 bacteria/ml, well above the threshold of clinical significance, which is taken to be approximately 10^5 CFU/ml (21). This value is, however, arbitrary, as it is influenced by several factors, including the bacterial strain, the patient’s existing medical condition, promptness in recognition of a septic reaction, identification of the bacterial strain, and the remedial action taken (6, 11, 24). Another method for screening for bacteria in PCs is that of liquid culture, which, although very sensitive, requires prolonged culture periods (2 to 4 days).

With the recognition of the limitations of these screening methods and the increasing risk of clinically significant levels of bacteria with the time stored prior to transfusion, in vitro storage of platelets has been reduced from 7 days to 5 days and has even been reduced to 3 days in some countries (14).

We have developed a highly sensitive system to screen for bacterial contamination of PCs by solid-phase cytometry. The system has previously been described for the detection of fluorescence-labeled cells (20) and for the detection of bacteria in simple filterable solutions (17, 30) and acellular clinical samples (3), but the screening of cellular biological fluids was not previously feasible. The two main problems were the selective removal of cellular material, which would have interfered with the analysis and masked any bacteria present, and the development of a method capable of labeling bacteria in complex solutions.

In the method described here, to selectively remove platelets, platelet aggregation is induced by a monoclonal antibody (MAb) and the large aggregates are removed by filtration. The gram-positive and gram-negative bacteria present in the eluate are permeabilized, and the DNA is labeled with a DNA-specific fluorescent marker before the sample is filtered through a 0.4-μm-pore-size black membrane, which retains the bacteria on its surface. The membrane is then transferred to a solid-phase laser scanning cytometer (Scansystem; Hemosystem, Marseille, France). Based on the measurement of different parameters (fluorescence, size, and shape), fluorescence-labeled microorganisms can be detected with a high sensitivity and can be discriminated from cellular debris and background material.

Finally, the use of an epifluorescence microscope linked to the cytometer allows confirmation of the result and preliminary identification of the class of bacteria.
MATERIALS AND METHODS

Bacterial propagation. All bacterial strains used in this study originated from the bacterial collection of the Pasteur Institute, Paris, France: Bacillus cereus (ATCC 7064), Enterobacter aerogenes (ATCC 13048), Escherichia coli (ATCC 25922), Klebsiella pneumoniae (CIP 79.21), Serratia marcescens (ATCC 43862), Staphylococcus aureus (ATCC 6538), Staphylococcus epidermidis (ATCC 12228), Streptococcus agalactiae (ATCC 12403), and Streptococcus bovis type 1 (ATCC 9475). Following overnight culture (16 to 18 h) in Trypticase soy broth (TSB), the turbidity of the culture was adjusted to match a 0.5 McFarland standard and dilutions were prepared with phosphate-buffered saline (PBS; pH 7.4). In all experiments, the concentrations of bacteria spiked were confirmed by quantitative culture on Mueller-Hinton agar plates or esterase labeling (17).

Blood products. Apheresis PCs, fresh frozen plasma (FFP), and pooled PCs (PPCs) were obtained from the French blood transfusion service, Etablissement Français du Sang. PCs were prepared according to established French regulatory guidelines (Journal Officiel Français, Vol. 123, p. 9109, 28 May 2003). All experiments were carried out with platelets between 2 and 4 days old.

Determination of the optimal test sample volume. FFP was spiked with 2-µm-diameter fluorescent beads (Standard-G, Chemunex) at a final concentration of 1, 2, 5, or 10 beads/ml. The resulting sample was mixed for 30 min on a flatbed rocker; and 1, 2, 3, 4, or 5 ml was filtered through a 0.4-µm-pore-size black membrane (Whatman). The number of fluorescent beads per membrane was then determined on a Scansystem solid-phase cytometer.

Removal of platelets and bacterial recovery from PCs. PBS and PCs were inoculated with 10² or 10³ CFU of E. coli or S. epidermidis per ml. Three milliliters of the spiked sample was immediately mixed with 1 ml of purified MAb CD9 6B1 (INSERM U268, Villejuif, France) at 7.5 µg/ml. After incubation for 30 min at 22°C on a flatbed rocker, the samples were passed through a 5-µm-pore-size filter ( Pall Inc.), which retained the platelet aggregates. Purified MAb CD9 SN4 (Ancell) was used as a reference standard for platelet aggregation. Prior to the addition of the CD9 MAb and after filtration, (i) the platelets were enumerated with a cell counter (ABX Micro-60) to calculate the percentage of aggregated platelets; and (ii) aliquots were plated on solid medium for quantitative culture. The bacterial colonies were counted at 24 and 48 h.

Platelet aggregation and labeling of bacteria with BLS1 and BLS2 solutions. Three milliliters of PC was mixed with 1 ml of BLS1 platelet aggregation solution (polyethylenimine [PEI], 60 mg/ml; MAb CD9 6B1, 30 mg/ml; 1:2,000 dilution of Picogreen nucleic acid-binding dye [Molecular Probes]) in distilled H₂O, and the mixture was incubated for 40 min at room temperature on a flatbed rocker. The platelet aggregates, visible as opaque flocculates, were removed by passing the sample through a 5-µm-pore-size filter (Pall Inc.). The resulting platelet-depleted sample was incubated for 20 min at room temperature with 7 ml of permeabilizing and labeling reagent BLS2 (EDTA, 1.86 g/liter; nisin, 8 mg/liter; depleted sample was incubated for 20 min at room temperature with 7 ml of m-pore-size filter (Pall Inc.)). The resulting permeabilized sample was washed with PBS and PCs were inoculated with 10² or 10³ CFU/ml, respectively. Three milliliters of each spiked sample was processed either with 8 ml of BLS1 or BLS2 solution, as described above, or with 7 ml of EDTA reagent (5 mM EDTA, Picogreen [1:2,000]) by the reference method (27). The number of labeled bacteria was determined with a Scansystem analyzer.

Removal of platelets and bacterial recovery. Purified MAb CD9 6B1 was used at a final concentration of 7.5 µg/ml to induce platelet aggregation in PCs. The mean proportion of residual platelets in the filtrate was 5.5% (standard deviation, 3.4% [n = 36]). The maximal platelet aggregation was achieved by 15 min, with no loss of aggregate quality for up to 40 min. To determine the effects of platelet aggregates on the recovery of bacteria, PBS and PCs were inoculated with E. coli...
after were counted 24 to 48 h later. The mean numbers of CFU counted before and the relative percentages of recovery are reported.

The number of bacteria recovered from spiked PBS for high (10³ CFU/ml) and low (10² CFU/ml) PCs was equivalent to and lower than the number recovered to and after a dilution of 1:2,000, it gave good bacterial DNA labeling, with ing and detection of the bacteria. When it was used at a dilution of 1:200, it showed promise for the labeling of gram-positive and gram-negative bacteria. Although gram-positive bacteria are very sensitive to lysis, they were found to tolerate PEI up to a concentration of 15 mg/liter. This PEI concentration enhanced the labeling of gram-negative bacteria when the incubation period was beyond the 20 min required for the optimal efficiencies of the other reagents. By incorporating PEI into the aggregation solution and extending the incubation period to 40 min, good labeling of gram-negative bacteria was achieved without compromising the labeling of gram-positive bacteria (data not shown).

Bacterial DNA labeling solutions. Picogreen was chosen from among several other nucleic acid-binding dyes for labeling and detection of the bacteria. Although gram-positive bacteria are very sensitive to lysis, they were found to tolerate PEI up to a concentration of 15 mg/liter. This PEI concentration enhanced the labeling of gram-negative bacteria when the incubation period was beyond the 20 min required for the optimal efficiencies of the other reagents. By incorporating PEI into the aggregation solution and extending the incubation period to 40 min, good labeling of gram-negative bacteria was achieved without compromising the labeling of gram-positive bacteria (data not shown).

The BLS1 and BLS2 solutions used in the Scansystem method were compared to the standard EDTA permeabilizing solution (27) for the labeling of gram-positive and gram-negative bacteria present in different solutions (Fig. 2a and b, respectively). Detection of *S. epidermidis* was enhanced 65-, 42-, 16-, and 728-fold in water, PBS, TSB, and plasma, respectively, when the BLS1 and BLS2 solutions were used. The gram-negative bacterium *E. coli* was better detected after labeling with the BLS1 and BLS2 solutions than after preparation with the EDTA solution. The levels of detection were improved 35- and 274-fold for bacterial suspensions in water and PBS, respectively. TSB and plasma did not interfere with the labeling of *E. coli* by the BLS1 and BLS2 solutions, while detection of the bacteria was totally impaired when the EDTA solution was used.

**Validation of the kit for sensitivity and linearity of detection of bacteria.** To validate the method with the Scansystem kit, a broad spectrum of bacteria associated with blood product contamination (23), gram-positive aerobic cocci (*S. aureus, Streptococcus pyogenes, S. epidermidis, S. agalactiae*), rods (*B. cereus*), and gram-negative aerobic rods (*E. coli, S. marcescens, Pseudomonas aeruginosa, K. pneumoniae*) were inoculated into PCs and tested by the Scansystem method. Bacteria were detected in all spiked platelet samples down to concentrations between 10 and 10³ CFU/ml, depending on the strain (Table 2).

For all experiments, PC units were preliminarily checked for bacterial contamination (by the Scansystem method and on culture plates) and were shown to be negative (n = 20).

To determine the linearity of the number of bacteria de-
tected by the bacterial screening system, PCs were inoculated with four different bacterial strains at concentrations ranging from 10 to $10^4$ CFU/ml (Fig. 3). There was a close linear correlation with the bacterial spiking concentration and bacterial detection, with sensitivities down to 100 CFU/ml for E. coli, B. cereus, and S. marcescens and 10 CFU/ml for S. epidermidis.

### DISCUSSION

Different approaches have been taken in an attempt to reduce the risk of transfusion-associated bacterial sepsis, including stricter donor selection criteria, improved puncture site disinfection (13), use of apheresis PCs from a single donor instead of pooled random PCs prepared from several donors (22), reduction of the platelet shelf life from 7 to 5 days (8), in vitro treatment with bactericidal products (12, 19), and the use of bacterial screening systems. The screening methods used by different centers are visual inspection, microscopic examination of stained smears, pH and glucose dipstick tests (29), and tests with automated culture systems (7, 28). No screening method, however, satisfies the main requirements of high sensitivity, high specificity, and rapidity.

The most common pathogens isolated from PCs are skin flora, including S. epidermidis and Propionibacterium acnes (5, 26). The majority of cases of bacterial contamination of PCs will therefore have occurred at the time of donation due to inadequate skin disinfection. The initial levels of bacteria in the PC unit are usually exceedingly low, below the detection limits of current screening systems and below the level considered clinically significant ($10^5$ CFU/ml) (21). With longer times between sampling and testing, there is a higher probability that all contaminated units will be identified. The short shelf life of platelets, however, means that a delay of testing for 3 to 4 days is not an option. Another factor that influences the probability that units infected with bacteria will be detected is the lag phase before the bacteria start to actively proliferate. Some bacteria remain in a nondividing state for up to 6 days (15).

Liquid culture systems are suitable for most cellular samples, such as whole blood and blood products. They are automatable and have a high level of sensitivity (2), with the presence of a single microorganism theoretically being sufficient to obtain a positive result. Long incubation periods, however, are necessary, in particular for PCs with low bacterial concentrations and slowly growing strains. The detection of P. acnes by the automated blood culture method requires more than 4 days (7, 9), and the detection of Mycobacterium is reported to require 20 days (25).

Solid-phase scanning cytometry has long been recognized as a highly sensitive bacterial screening system, as very low levels of bacteria can be concentrated from a large volume by filtration. This, however, limits the method to use with simple filterable fluids. PCs contain, on average, $5 \times 10^9$ platelets/ml, with the platelets having an average size of 2 to 3 μm. The similarity of the sizes of platelets and bacteria exclude the possibility of preremoval of bacteria by size-selective filtration, and the sheer mass of platelets would overwhelm the membrane retaining the bacteria. To overcome this problem, aggregation of the platelets was induced by using an MAb to the glycoprotein CD9, which is expressed at high levels on the surfaces of platelets. Although its physiological role is not yet known, binding of MAbs to different CD9 antigen epitopes has been shown to induce platelet aggregation through at least one platelet activation pathway (10, 31). The aggregates range in size from 20 to greater than 200 μm, which enables their selective removal by filtration. Filtration of the aggregated platelet samples was shown to influence bacterial retention in the presence of a low concentration ($10^5$ CFU/ml) of bacteria. However, the bacterial recovery rate remains acceptable for the detection of bacteria in PCs by the Scansystem method.

Although the permeabilizing method with EDTA solution has long been accepted as adequate for detection of bacterial contamination in simple solutions (27), it is less suitable when

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**TABLE 2. Sensitivity of Scansystem screening method**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Total</th>
<th>$10^9$</th>
<th>$10^3$</th>
<th>$10^4$</th>
<th>$10^5$</th>
</tr>
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<tbody>
<tr>
<td>Gram-positive bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>25</td>
<td>16</td>
<td>21</td>
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<td>25</td>
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<td>1</td>
<td>2</td>
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<tr>
<td>S. pyogenes</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<td>S. agalactiae</td>
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<td>2</td>
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</tr>
<tr>
<td>Gram-negative bacteria</td>
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<td></td>
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</tr>
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<td>E. coli</td>
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<td>25</td>
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<tr>
<td>S. marcescens</td>
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<td>2</td>
</tr>
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<td>ND</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>P. aeruginosa</td>
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<td>ND</td>
<td>ND</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>41</td>
<td>54</td>
<td>65</td>
<td>65</td>
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<tr>
<td>% Positive</td>
<td>67</td>
<td>89</td>
<td>100</td>
<td>100</td>
<td></td>
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</tbody>
</table>

* Nine different strains, including five gram-positive strains and four gram-negative strains, at each spiking concentration, were spiked in PCs at concentrations ranging from 10 to $10^4$ CFU/ml. Three samples of 3 ml were processed with the kit and analyzed with the Scansystem solid-phase cytometer. The number of samples in which bacteria were detected is reported for each strain at each spiking concentration.

* ND, not determined.
more complex solutions, in particular, biological fluids, are tested. To develop a permeabilizing solution more suitable for use in the screening of blood products, a variety of different reagents reported to have effects on membrane permeability and stability were tested. These include enzymes (lysozyme, nisin), chelating agents (EDTA, EGTA), fixatives (formaldehyde, glutaraldehyde, ethanol), permeabilizing components (PEI, digitonin, chlorhexidine diacetate, monesin, NOG, sodium hexametaphosphate, benzalkonium chloride, cetrimide), antibiotics (polymyxin B, rifampin), salts (NaCl, KCl), and other agents (potassium citrate, streptolysin O, urea, sucrose) (16, 27). The reagent composition was chosen by focusing on achieving a universal method for the labeling of bacteria that was sensitive for the detection of a broad spectrum of bacterial strains and that was not inhibited by biologically complex fluids.

As described in the literature (16), PEI was found to be essential for permeabilization of gram-negative strains. At the concentrations optimal for labeling of gram-negative bacteria, all gram-positive bacteria were lost due to lysis. This problem was overcome by reducing the PEI concentration and incorporating it into an extended platelet aggregation stage solution in order to increase the incubation time.

Since PCs do not contain any nucleated cells, other than a low number of residual white blood cells, we chose to use a nucleic acid-binding dye to label the bacteria. Picogreen (Molecular Probes) is a DNA-specific dye commonly used for cellular labeling. Picogreen was compared to several other fluorescent dyes, SYTO13, YOPRO1, and SYBR Green I (Molecular Probes), and was selected for the establishment of the Scansystem screening method. SYBR Green I (dilution, 1:10,000) allowed efficient labeling of the bacteria, with a mean peak intensity of 1,400 ADC counts, whereas Picogreen (diluted 1:2,000) provided a mean peak intensity of 600 ADC counts; however, SYBR Green I induced too many nonspecific fluorescent particles on the membrane surface to be considered further. As Picogreen was found to be unstable when it was stored for long periods in combination with the other reagents in the BLS2 solution (lysis solution), it was incorporated into the BLS1 solution (aggregation solution), in which its stability is maintained at 4°C for long periods (>9 months).

Our method allows labeling and detection of bacteria in complex biological fluids with a high degree of sensitivity. The detection limit of the Scansystem is 10 to 10² CFU/ml for gram-negative and gram-positive bacteria, with a linearity of detection in the range of 10 to 10⁶ CFU/ml. When spiked PCs were stored under usual conditions for several hours (up to 72 h) prior to analysis, the sensitivities of bacterial detection were similar, with 100% of samples containing 10³ CFU/ml being positive (P. Morel, M. Dechaseaux, X. Bertrand, C. Naegele, and D. Talon, Abstr. Ann. Transfusion, vol. 43, abstr. SP13, 2003). When PCs without bacteria (n = 20) were tested by the Scansystem method, they were always confirmed to be negative by the culture plate method. PCs found to be positive by testing by the Scansystem method were always confirmed to be positive as well, emphasizing the high degree of specificity of the method. The method does not depend on bacterial growth and does not require any culture phase. The entire analysis, from the time of sampling to the time that the final result is obtained, actually requires less than 90 min, while culture methods require incubation periods of 24 to 48 h and give results representative of the levels of bacteria in the samples 1 or 2 days earlier. Furthermore, our screening method allows the detection of not only living bacteria but also moribund or nondividing microorganisms, preventing the recipient from being potentially exposed to the effects of bacterial endotoxins.

As the cellular structures of the microorganisms are conserved through the process, microscopic confirmation and preliminary bacterial class identification (rods or cocci) are also possible. At this development stage, microscopic confirmation is necessary to validate the presence of bacteria detected by the Scansystem analyzer software. Software discrimination parameters will be set up precisely after positive PC units are tested in routine studies. This will allow the use of the microscope as an option.

In conclusion, Scansystem is a rapid, specific, and sensitive bacterial screening system that can be used to ensure the increased safety of PCs destined for transfusion. It will certainly permit the possible extension of the shelf life of PCs stored in vitro to their full functional potential of 7 days (8), alleviating shortage problems and reducing wastage. Progress is now being made to extend this screening system to other blood products and for use of this system in clinical situations to test the blood of patients with suspected septicemia. Early diagnosis would improve patient prognosis and reduce the risk of the more serious and debilitating outcomes of this disease.

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REFERENCES


