Microcalorimetry: a novel method for detection of microbial contamination in platelet products

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BACKGROUND: Measuring heat from replicating microorganisms in culture may be a rapid, accurate, and simple screening method for platelets (PLTs). Microcalorimetry for detection of microorganisms in in vitro contaminated PLT products was evaluated.

STUDY DESIGN AND METHODS: *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus sanguinis*, *Escherichia coli*, *Propionibacterium acnes,* and *Candida albicans* were inoculated in single-donor apheresis PLTs to achieve target concentrations of 10^5, 10^3, 10, or 1 colony-forming units (CFU) per mL of PLTs. Contaminated PLTs in growth medium were incubated at 37°C for 5 days in a calorimeter. Positivity was defined as heat flow of at least 10 μW above the lowest value of the power–time curve.

RESULTS: With microcalorimetry, inocula of 10 CFUs per mL PLTs could be detected with the following detection times: *S. epidermidis* (31.65 hr), *S. aureus* (24.24 hr), *S. sanguinis* (7.82 hr), *E. coli* (7.53 hr), *P. acnes* (73.57 hr), and *C. albicans* (43.77 hr). The detection time was less than 4 hr at 10^5 CFUs per mL PLTs for *S. aureus*, *S. sanguinis*, and *E. coli*. Noncontaminated PLTs remained negative. The total heat ranged from 2.8 (*S. sanguinis*) to 8.3 J (*E. coli*).The shape of the power–time curve was species-specific and independent from the initial concentration of microorganisms.

CONCLUSION: The detection limit of microcalorimetry was 1 to 10 CFUs per mL PLTs. Microcalorimetry is a promising novel method for detection of contaminated PLTs. Applying this method to all PLT products could reduce the frequency of transfusion-related sepsis and prolong the shelf life of PLTs.

Improvements in blood donor screening procedures for indicators of viral infections have substantially reduced the risk of transfusion-transmitted infections. Bacterial contamination of blood donations, however, remains a serious complication for recipients of transfusion products, particularly platelets (PLTs).[1-4]

Bacterial contamination of PLT products is the most frequent infectious risk from transfusion overall, occurring in approximately 1 of 2,000 to 3,000 units and a frequent cause of death from transfusion overall (after transfusion-related acute lung injury and clerical errors) with mortality rates ranging from 1:20,000 to 1:85,000 donor exposures.[5-8] PLT products are at special risk for bacterial contamination because they are stored at room temperature (22 ± 2°C) to preserve PLT function. The ambient temperature supports the rapid replication of a wide range of bacteria.[9] The reported numbers of transfusion-related sepsis (especially the nonfatal episodes) are probably underestimated because many episodes are not associated with the preceding transfusion or the clinical symptoms are partially masked by concomitant antimicrobial therapy.[10] In response to increasing concern about transfusion-associated bacterial sepsis, the AABB implemented a new standard requiring that blood

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banks and transfusion services shall apply methods to limit or detect bacterial contamination in all PLT components by March 1, 2004.[11,12] A similar requirement has also been promulgated by The College of American Pathologists.

The sterility test should be rapid, sensitive, specific, affordable, and simple to perform.[3] At present, no single laboratory technique meets these criteria.[13-15] Microcalorimetry is a nonspecific analytical tool for measurement of heat produced or consumed over time by chemical reactions or physical changes of state in a specimen, including heat generated by complex biologic processes in cultured cells. Basic microcalorimetry data are recordings of heat flow rates, frequently referred to as heat power–time curves.[16]

All living organisms produce heat as a result of their metabolism. Metabolic heat generated by single cells ranges from about 1 to 80 pW. Normal human connective tissue cells (e.g., fibroblasts, adipocytes) have reported metabolic rates of approximately 25 to 80 pW per cell, and chemical reaction–based theoretical calculations of single-cell metabolism are in the same range. In contrast, most microbes produce significantly smaller amounts of heat (approx. 1-3 pW per cell).[16] Despite the low heat output of microorganisms, their exponential replication in culture allows their detection by microcalorimetry within hours even when the starting numbers are low (e.g., 1-10 colony-forming units [CFUs]/specimen).

Microcalorimetry has been widely used in life sciences, pharmacology, biotechnology, and ecology because of its high sensitivity, accuracy, and simplicity.[17-28] The clinical use of calorimetry, however, was previously hindered by insufficiently sensitive instrumentation, insufficient capacity, and the lack of appropriate software.[29,30] These problems have been overcome in microcalorimetry instrument designs of the type used in this study. In this exploratory study, we evaluated the potential of isothermal microcalorimetry for rapid, accurate, and simple detection of bacterial contamination with in vitro contaminated PLT products.

MATERIALS AND METHODS

Test organisms and inoculation culture

We used six strains of American Type Culture Collection (ATCC)-annotated microorganisms: *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 29213), *Streptococcus sanguinis* (ATCC 10556, deposited as *Streptococcus sanguinis*), *Escherichia coli* (ATCC 25922), *Propionibacterium acnes* (ATCC 11827), and *Candida albicans* (ATCC 14053). Strains were stored at –70°C with the cryovial bead preservation system (Microbank, Pro-Laboratory Diagnostics, Richmond Hill, Ontario, Canada) before the experiment. Test organisms were grown overnight on 5 percent sheep blood agar plates (Becton Dickinson, Heidelberg, Germany) at 37°C in 5 percent CO_2. Individual colonies were aseptically removed from the agar plate, inoculated into 2 mL of sterile saline (0.85% NaCl), and adjusted to a turbidity corresponding to a McFarland value of 0.5 with a Densimat (bioMérieux, Marcy-l'Etoile, France). From this suspension, serial 10-fold dilutions were prepared in sterile saline to attain final concentrations targeted at 10^5, 10^3, 10^1, and 1 CFU per mL of PLTs. Experiments were performed in triplicate in different runs. Sterile saline was used as control. The accuracy of the inoculum size was confirmed by quantitative cultures on blood agar plates. Colonies were counted from plates with 10 to 200 colonies, and the CFUs per mL was calculated.

Source and preparation of PLTs

Leukoreduced single-donor apheresis PLTs were provided from the Transfusion Service of Basel (Blutspendezentrum beider Basel, Switzerland) when they were 6 to 7 days old. Outdated PLTs were used in this study to preserve younger PLT products for clinical use. Units of PLTs were routinely obtained from healthy, volunteer blood donors with the continuous-flow cell separator (Amicus, Baxter Healthcare, Deerfield, IL). After standard processing, PLT products were stored at room temperature and continuously shaken on a flatbed agitator before usage. Samples were drawn to ensure baseline sterility of the units, before the PLTs were artificially contaminated. Aliquots of 1 mL of PLTs were transferred with a needle through sterile connection of the unit bag into reaction tubes and mixed with 0.1 mL of inoculation culture at different concentrations as described above to simulate contaminated PLTs.

Microcalorimetry instrumentation

A microcalorimetry thermostat (Thermal Activity Monitor, Model 3102 TAM III, TA Instruments, New Castle, DE) equipped with 48 channels was used to measure the heat flow (power–time) curves. In isothermal mode, the liquid in the thermostat was maintained at the set temperature 37.0°C with an absolute accuracy of 0.02°C. The microcalorimeters had installed aluminum metal reference specimens having a heat capacity approximately equal to that of a 4-mL glass ampoule containing 3 mL of water. This matching reduces equilibration time and improves the stability of the heat flow rate measurements.

Any heat generated or absorbed by the sample is measured continuously over time (Fig. 1). After the initial equilibration time of 15 minutes, heat flow rates were recorded over 5 days at 10-second intervals. Each calorimeter was calibrated weekly by placing a sealed 4-mL ampoule containing 3 mL of water in its measurement position and with an electrical substitution method which employs calibration heaters built into each microcalorim-

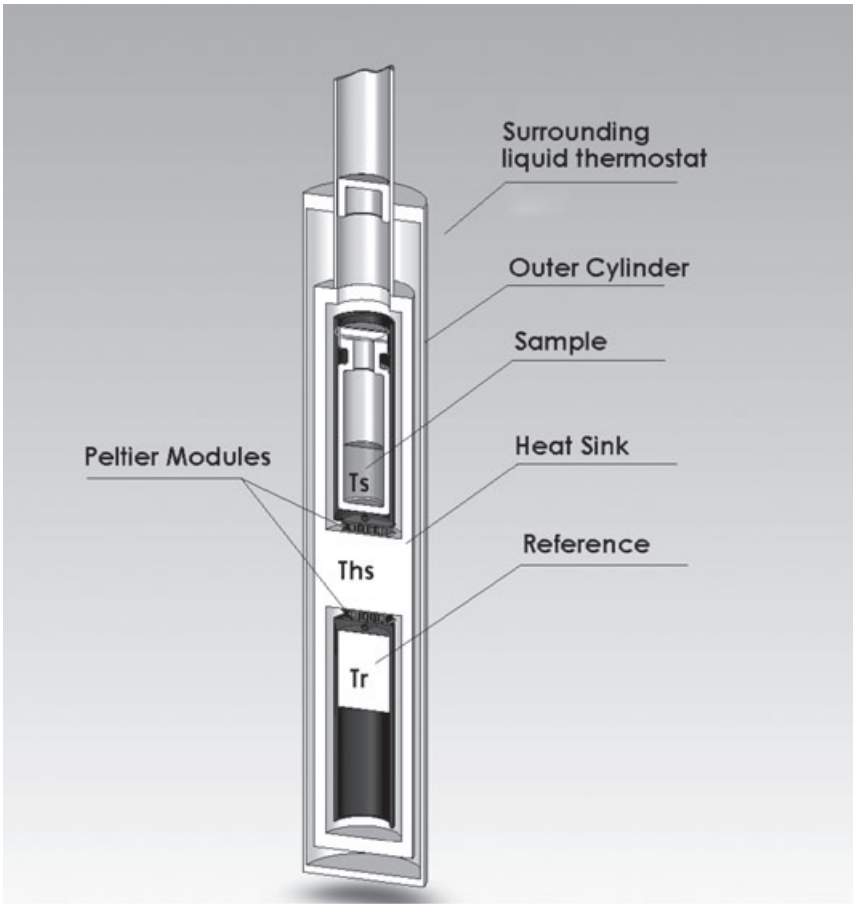


Fig. 1. Temperature differences between the sample (Ts) and a thermally inert reference (Tr) is continuously measured in a heat sink (Ths). The detection limit of the instrument expressed by the manufacturer is ±0.225 μW. Figure courtesy of Thermometric/TA Instruments, Jarfalla, Sweden.

eter and a software module supplied by the instrument manufacturer. The sensitivity of each microcalorimeter expressed by the manufacturer was ±0.225 μW.

Preparation of calorimetry ampoules

Four-milliliter glass ampoules and cap seals were washed with an alkaline detergent, rinsed with deionized water (85°C), and dried at 90°C for 90 minutes in a drying cabinet, followed by autoclaving at 121°C for 20 minutes. The experimenters donned nonpowdered vinyl gloves to prevent deposition of chemical substances on the ampoules that might produce heat flow signals by oxidation or other processes. Each calorimetry ampoule was aseptically filled with 1.9 mL of sterile tryptic soy broth (bioMérieux) and 1.1 mL of contaminated PLTs; the gas phase consisted of 1 mL air. All inoculations were performed in a laminar airflow biosafety cabinet. Ampoules were sealed with a crimped metal lid with silicon rubber seal and sequentially introduced into the instrument previously stabilized at 37°C. Ampoules were first lowered into the thermal equilibration position and left there for 15 minutes before lowering into the measurement position. After the measuring phase, the content of each ampoule was checked quantitatively and qualitatively for presence of microorganisms. Sterility of the negative controls was a basic requirement for the validity of an experiment.

Calorimetric detection of microorganisms

Detection time was defined as the time from insertion of an ampoule into a microcalorimeter until exponential growth produced a rising heat flow rate signal and a heat flow 10 μW (i.e., 40 times the effective sensitivity of the calorimeters) above the lowest point of the power–time curve was observed. The time to peak heat flow rate was the time from insertion of an ampoule into the calorimeter until the highest value of the power–time curve was reached. Total heat was determined by integration of the area between the heat flow–time curve and a line tangent to the baseline at two places—at the inception of exponential growth and after cessation of growth and return to baseline. Data analysis was accomplished with the manufacturer's software (TAM Assistant) and Origin 7.5 (Microcal, Northampton, MA).

RESULTS

Detection of microbial contamination

The detection limit was 1 to 10 CFUs per mL of contaminated PLTs (Table 1). The detection time increased with decreasing initial concentration of microorganisms. The mean detection time was shorter for rapidly replicating and highly virulent organisms (*S. sanguinis*, *E. coli,* and *S. aureus*) than for slower replicating and less virulent organisms (*S. epidermidis*, *P. acnes,* and *C. albicans*). *E. coli* and *S. sanguinis*, typically causing severe and potentially fatal transfusion-associated sepsis, were detected by calorimetry within 8 hours at concentrations of 10 CFUs per ml of PLTs. At these inocula, even less virulent pathogens such as *S. epidermidis* were detected within 44 hours (except *P. acnes*, which required 74 hr for detection). The heat flow curves from noncontaminated

TABLE 1. Calorimetry variables of contaminated PLT products

Organism	Targeted CFUs/mL of PLTs†	Calorimetry variables (mean value ± SD)*			
		Detection time (hr)	Time to peak (hr)	Peak heat flow (μW)	Total heat (J)
S. epidermidis	10^5	10.16 ± 0.99	17.62 ± 0.35	154 ± 22	5.53 ± 0.38
S. epidermidis	10^3	21.28 ± 2.69	32.21 ± 8.13	61 ± 26	4.27 ± 2.08
S. epidermidis	10	31.65	40.37	37	3.40
S. epidermidis	1	Negative	Negative	Negative	Negative
S. aureus	10^5	3.54 ± 0.56	9.18 ± 1.10	349 ± 100	7.13 ± 0.80
S. aureus	10^3	6.83 ± 1.63	14.93 ± 1.52	285 ± 83	6.87 ± 0.90
S. aureus	10	24.24 ± 9.51	28.95 ± 8.58	165 ± 226	3.07 ± 3.84
S. aureus	1	26.62	35.43	99	2.89
S. sanguinis	10^5	2.72 ± 0.81	5.51 ± 0.38	311 ± 45	3.37 ± 0.85
S. sanguinis	10^3	5.48 ± 0.45	8.96 ± 1.57	271 ± 50	2.83 ± 0.32
S. sanguinis	10	7.82	10.95	269	2.80
S. sanguinis	1	8.64	11.28	282	2.71
E. coli	10^5	3.22 ± 0.93	6.24 ± 1.60	686 ± 30	8.23 ± 0.76
E. coli	10^3	5.50 ± 1.26	8.39 ± 1.70	660 ± 24	8.10 ± 0.85
E. coli	10	7.53 ± 0.97	10.44 ± 1.46	620 ± 12	7.83 ± 0.75
E. coli	1	Negative	Negative	Negative	Negative
P. acnes	10^5	24.79 ± 18.30	64.51 ± 22.55	59 ± 6	6.73 ± 0.93
P. acnes	10^3	46.42	67.92	47	5.00
P. acnes	10	73.57	94.97	56	5.40
P. acnes	1	Negative	Negative	Negative	Negative
C. albicans	10^5	24.48 ± 36.04	35.31 ± 44.10	88 ± 28	5.03 ± 1.31
C. albicans	10^3	54.90	76.23	40	2.80
C. albicans	10	43.77	52.77	62	3.30
C. albicans	1	Negative	Negative	Negative	Negative

* Experiments were performed in triplicate and data are expressed as means ± SD. Where only one value is shown, one or two replicates were negative. Negative = all three replicates remained negative (i.e., below the threshold of 10 μW above lowest value).

† Measured microbial concentrations at targeted concentrations of 10^5 CFUs per mL of PLTs were: for *S. epidermidis* 0.7×10^5, *S. aureus* 1.2×10^5, *S. sanguinis* 1.4×10^5, *E. coli* 0.8×10^5, *P. acnes* 0.7×10^5, and *C. albicans* 5.1×10^4 CFUs per mL.

PLTs showed a steadily declining heat flow signal and remained detection-negative, that is, did not exhibit any exponential rise during the observation time.

Characteristics of heat power–time curves

Figure 2A shows a representative power–time curve for uncontaminated PLTs. There is an initial thermal disturbance as the microcalorimeter heat flow measurement system adjusts to the change in heat capacity caused by introduction of the ampoule into the measuring position. This disturbance is followed by an initial exothermic heat flow of approximately 20 μW, caused by an increase in PLT degradation rate induced by increasing temperature from 22° to 37°C in the microcalorimeter.

As shown in Figs. 2B through 2G, when there are a sufficient number of microorganisms present due to replication in culture, the metabolic heat they produce is added to that produced by the degrading PLTs, and the heat flow curves go through an inflection point and begin to increase. Experiments were highly reproducible (replicate curves are not shown), especially at high microbial concentrations.

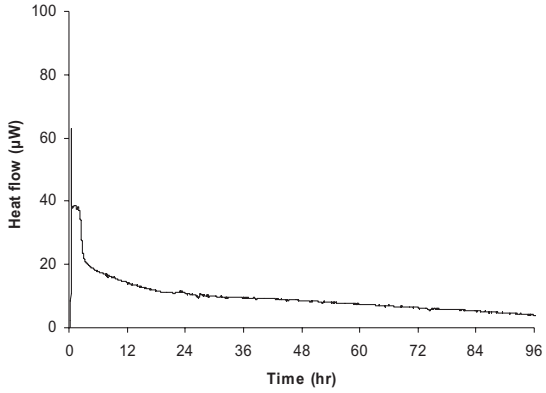
The shape of the heat flow curve appeared to be characteristic for the individual species independent of its initial concentration. Interestingly, the peak heat flow of *S. epidermidis*, *S. aureus,* and *C. albicans* decreased with lower microbial concentration, which was also reflected by lower total heat, whereas the remaining organisms (*S. sanguinis*, *E. coli,* and *P. acnes*) exhibited peak heat flow rates and total heats that were independent of their initial concentration. The total heat was the highest in *E. coli* ranging from 7.83 to 8.23 J, whereas *S. sanguinis* produced the lowest total heat ranging from 2.80 to 3.37 J.

DISCUSSION

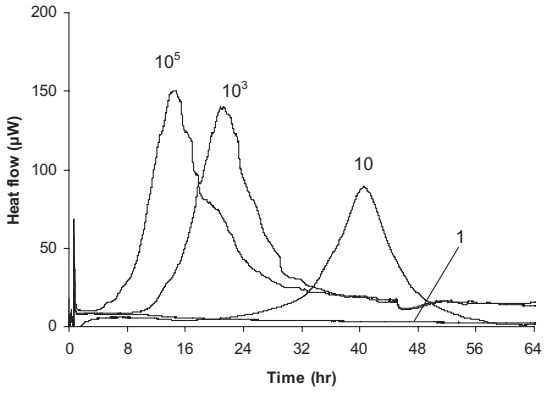
Bacterial contamination of PLT products remains one of the most important causes of transfusion-related morbidity and mortality, causing a continuing risk to patient

Fig. 2. PLTs inoculated with sterile saline were used as control (A). Representative heat power–time curves for PLTs contaminated with test organisms at concentrations 10^5, 10^3, 10, or 1 CFUs per mL PLTs (B-G). The time (hr) on x-axes and the power (μW) on y-axes were plotted on different scales to better demonstrate characteristics of individual heat power–time curves. Heat flow signal at the beginning of each measurement represent disturbances due to the insertion of the microcalorimetry ampoule.

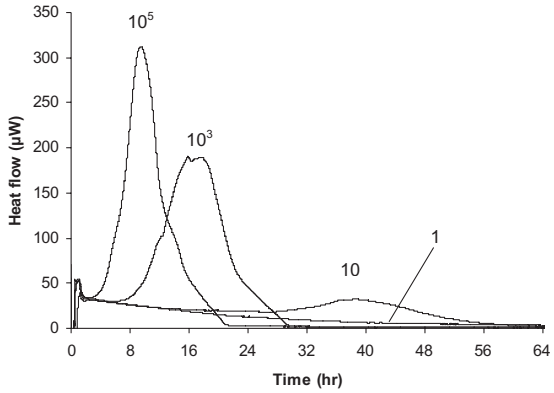
A (Sterile saline)



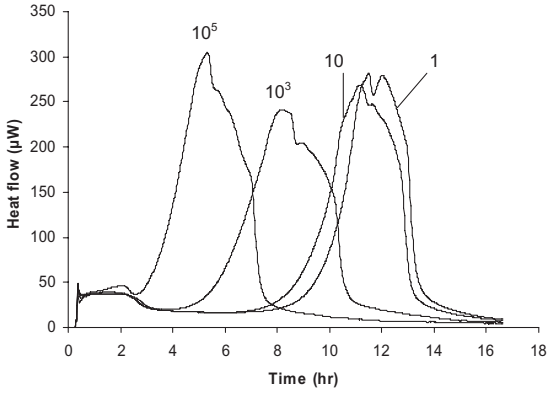
B (*S. epidermidis*)



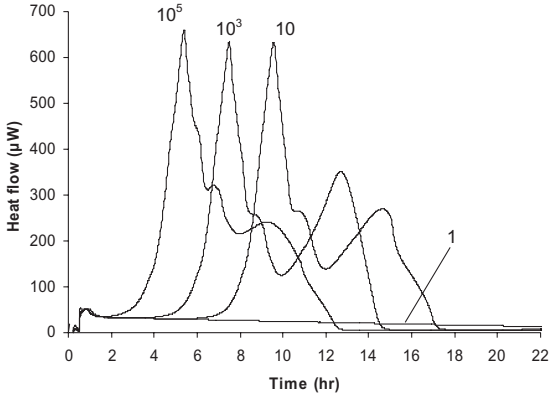
C (*S. aureus*)



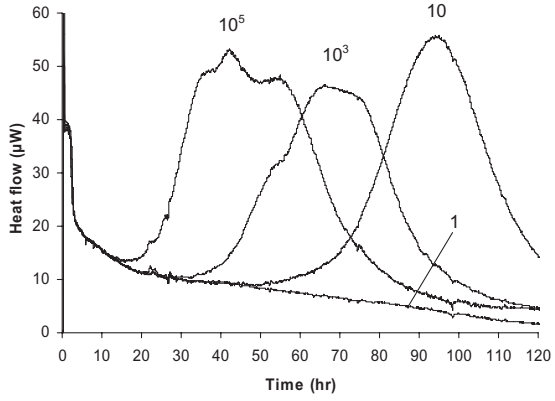
D (*S. sanguinis*)



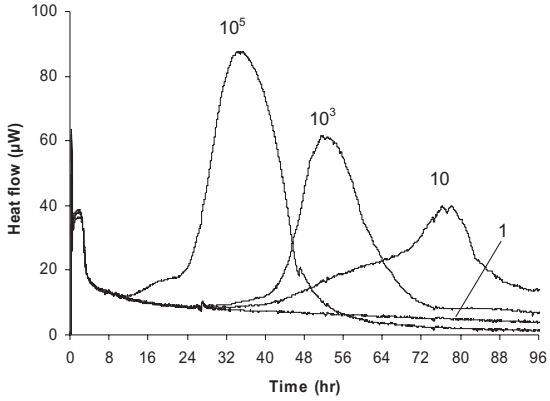
E (*E. coli*)



F (*P. acnes*)



G (*C. albicans*)



safety.[31] At present, no ideal screening method for detection of microbial contamination of donated PLT concentrates exists.[13-15] Therefore, we evaluated isothermal microcalorimetry for detection of microbial contamination with artificially contaminated PLTs. Our findings suggest that microcalorimetry has the potential for simple and sensitive detection of microorganisms in PLTs.

Because the number of bacteria in contaminated PLT products increases over time of storage, low-sensitivity nonculture methods have been proposed to screen PLTs before transfusion, including visual inspection of the blood products, direct staining of bacteria, or indirect observation of bacterial metabolism by measuring pH, glucose concentration, oxygen consumption, or carbon dioxide production. Suboptimal sensitivity and specificity of these methods, however, may not prevent all transfusion-associated septic episodes.[32] For example, pH can increase, decrease, or even remain unchanged in contaminated PLTs, depending on the type of organism.[7,33,34]

Automated continuous-monitoring blood culture systems are the most accurate standard method and represent the current "gold standard" for detection of low initial concentrations of organisms (1-10 CFUs per mL). Analogous to the microcalorimetry method we describe, this is accomplished by placing specimens possibly containing microorganisms in sealed ampoules containing culture media in which the microorganisms can replicate.[35-37] Detection depends on sensing CO_2 produced by bacteria in the measured sample when sufficient replication has occurred to activate the sensing system. In many countries and blood transfusion centers, PLT samples are randomly checked for microbial contamination with such blood culture systems. This method, however, has several drawbacks, including a large sample volume required (5-10 mL), prolonged culture incubation (24-72 hr), and high cost.[38] In addition, some blood culture systems have failed to detect bacterial contamination, especially when slow-growing bacteria, such as *P. acnes,* were present. In such cases the detection time exceeded the shelf life of PLTs and therefore the transfusion often had already taken place before the detection of the contamination. Most cases of false-negative results from slow growing organisms are caused by sampling error, where no organisms are present in the sample volume but the unit is contaminated with low levels of organisms that grow during storage.

The volume of the test sample should be as small as possible to preserve the majority of PLTs for transfusion, but high enough to ensure adequate sensitivity. For microcalorimetry we used only 1 mL of PLT product, which appeared to have a detection limit similar to the blood culture systems (1-10 CFUs per mL), which use up to 10 mL of blood (data not shown).

Because of prolonged detection time, a incubation period of 24 to 48 hours after culturing and before transfusing PLT units would be appropriate, but is rarely practiced due to high demand and limited durability of PLT products. Unlike viral contamination, which can be detected at the time of donation, bacterial contamination of blood components requires time for the organisms to proliferate before being detectable.[39] Te Boekhorst and colleagues[40] evaluated bacteriologic screening of PLTs with BacT/ALERT and found that 113 of 203 (56%) pooled PLT concentrates had already been transfused at the time of contamination detection.

Molecular techniques for bacterial nucleic acid detection are emerging, capable to detect small numbers of bacteria.[41-43] The potentially high costs, contamination risk, lack of standardization, and the complexity of this method, however, might render it inappropriate for routine testing.[44,45] Also, nucleic acid detection does not indicate whether or not the bacteria are viable. Another method uses antibiotics labeled with fluorescent markers, but it can currently only detect concentrations of 10^5 CFUs per mL or greater. In addition, no antibiotic which binds to all bacteria and fungi has been found.[2]

Pathogen reduction technologies, such as amotosalen plus ultraviolet A light, have been shown to be effective against transfusion-transmitted bacteria, viruses, and parasites in PLTs.[46-48] The downside of this approach is lack of activity against spore-forming bacteria and some non-enveloped viruses, as well as reduced therapeutic efficacy of blood components, necessitating the transfusion of greater quantities and exposing patients to blood from more donors.

By use of the microcalorimetry method, highly virulent organisms, which typically cause severe and potentially fatal transfusion-transmitted sepsis, were detected within 2 days after incubation. In addition, the method is easy to perform and needs only a minimum of manual effort and technical expertise.

In conclusion, this exploratory study suggests that microcalorimetry is a promising novel screening method for microbial contamination of PLTs, potentially allowing the detection of at least 10 CFU of tested organisms per mL. This culture method detects heat, resulting from microbial metabolism and growth. Applying this detection method to screen all donated PLTs could reduce the risk of transfusion-related infectious complications and prolong the shelf life of PLT-enriched plasma.

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