Evaluation of the efficacy of flow cytometry in detecting bacterial contamination in platelets

Introduction: Bacterial contamination of platelet products is a major risk of infections in blood transfusion. Due to their storage conditions at room temperatures (22 to24°C), cases of septicemia and even death caused by platelet injection have been reported. Therefore, use of appropriate diagnostic methods can improve the health of this product. In this study, flow cytometry was used to detect contaminated platelet units.

Methods: This study was a diagnostic interventional type. 15 units of platelet concentrate was prepared at a minimum interval time after production. *Staphylococcus epidermidis* and *Escherichia coli* (E. coli) bacteria were each added to 6 platelet bags, with a concentration of 10 CFU / ml, while 3 bags were used as negative control. Platelets were stored in a shaking incubator at 22 - 24 ° C, for 0, 6, 24 and 48 hour-intervals after inoculation. Samples were then taken at 1 ml volume and evaluated by flow cytometry.

Results: The sensitivity of the flow cytometry method to detect contaminated platelet units in infections with both *Staphylococcus epidermidis* and *E. coli*, in a 1 ml volume in all samples at 0, 6, 22 and 24 hours after inoculation, was 100%, and the number of bacteria increased in 24 hours of incubation, except for *E. coli* that decreased after 24 hours.

Conclusion: This study shows that flow cytometry can be a useful method for detecting bacterial contamination in platelets, and can detect low concentrations (10 CFU / ml) of bacteria in small volumes of sample (ml) in a short time.

Keywords: Flow cytometry, bacterial contamination, platelet units

Introduction

People with thrombocytopenia are vulnerable to bleeding due to bone marrow deficiency and low platelet production. In these people, one of the main therapeutic options is platelet injection. Bacterial contamination is a major risk of infections in the transfusion of blood products such as platelets [1]. In the meantime, platelets are more susceptible to contamination with bacterial agents during the production and preparation process, and despite the advances made in collecting, supplying and storing platelet concentrates, the bacterial infection caused by the transfusion of contaminated platelets is still serious challenge. In some cases the risk of developing bacterial infections is higher than the risk of transmissible viruses such as HIV and hepatitis. The prevalence of this infection in platelets has been reported 1 in 1,000 to 2000[2, 3]. In the United States, bacterial contamination of blood products is considered as the second leading cause of death caused by blood transfusion second only to laboratory errors [4].It is estimated that 2,000 and 4,000 people annually receive bacterial contaminated platelets, of which 200 to 600 will lead to clinical sepsis and 40 to 533 deaths. The risk of death in the United States is estimated to be 1 in 100,000 cases of platelet concentrate transfusion [5]

An appropriate method of detecting bacterial contamination in platelet products should have high diagnostic potency, high and rapid sensitivity, so that it prevents transfusion of infected platelets, as well as false positive results, which could lead to unnecessary removal of healthy products .

The effect of bacterial detection in platelet concentrates depends on factors such as sampling time, sample size, diagnostic sensitivity and bacterial growth rate sensitivity. If sampling for early testing takes place, the probability of detecting any bacterial contamination is low. Because the infected bacteria do not have enough time to reproduce to reach a detectable number. However, the sampling time cannot be delayed due to the short duration of maintenance of the platelets. The larger the sample size obtained from platelets, the more likely it is to detect bacterial contamination. A large sample size, on the other hand, reduces the volume of available platelet units for injection. Therefore, using sensitive and rapid methods, only a small amount of sample is required to identify bacterial contamination [7]

In order to screen for platelet units for bacterial contamination, the Food and Drug Administration (FDA) approved two culture systems of Bact/Alert and eBDs. In the Bact/Alert system, the basis for identifying bacteria in blood products is based on CO_2 gas production. This system makes it possible to continuously monitor the culture on the machine screen by using a dedicated environment for the device [8]. Apart from culture systems, rapid methods for screening bacteria in platelet products include molecular methods such as everse transcription polymerase chain reaction (RT-PCR) and methods such as flow cytometry [9, 10]Flow cytometry with the high sensitivity and specificity and the need for a small sample size allows the identification of bacteria in platelet products in the shortest possible time. Several studies have been conducted to evaluate the efficacy of flow cytometry in diagnosing bacterial infections [7, 9, 11]Vollmer et al. in 2009, in Germany, used BactiFlow flow cytometer to detect and count bacteria based on the activity of esterase in living cells, and compared the results with the Bact/Alert culture system. The results of their study showed that the automatic culturing system Bact/Alert was able to detect all species of aerobic and anaerobic bacteria within 22 hours [9].

Considering the proper characteristics of flow cytometry and the importance of rapid diagnosis of bacterial infections, this study tries to investigate the efficacy of flow cytometry using a dye combination for the diagnosis of bacterial infections.

Methods

Preparation of platelet concentrate bags

One-day platelet concentrates were prepared from the Tehran Blood Transfusion Center. Bags were randomly selected, and there was no freedom of choice regarding the blood types of the bags. The bags were quickly transferred to the incubator at 22 to 24°C temperature, with rotational motion. It should be noted that at each work time, four one-day platelet concentrates were selected simultaneously. The basis for counting the days of platelet bags was considering blood donation day as day zero.

Preparation of microbial strains

The bacteria for this study were Gram Negative ATCC strains of *E. coli* (and Gram-positive *Staphylococcus epidermidis*), which were prepared from clinical samples of patients in the microbiology lab of the Iranian Blood Transfusion Organization. The identification steps for *E. coli* were such that the bacterium on the blotting agar had smooth, circular, gray and glossy colonies, and created a metallic molding colony on the Eosin methylene blue (EMB) (Merk, Germany)agar medium. The identification steps for *Staphylococcus epidermidis* were such that the bacteria on the blood agar environment had smooth, round, convex colonies, and no growth was observed on the EMB agar medium. Then, specific tests were used to confirm each bacterium. For E. coli bacteria, warming and culturing were performed on different environments of Citrate, Urea, Solfid-indole-motility agar (SIM) (Merk, Germany) and Triple sugar iron agar (TSI) (Merk, Germany), respectively. For *Staphylococcus epidermidis*, gram staining and catalase and coagulase tests were performed respectively.

Flow cytometry procedure

At minimum intervals after production of platelet concentrate units, all the bags were sampled and cultured on culture media which produced negative culture results. Of all 15 bags under sterile conditions under the hood sampling was performed using a sterile syringe. A 10 CFU / ml concentration of *E. coli* and *Staphylococcus* were added to 6 bags of platelet each. And 3 platelet bags were considered as negative controls, to which no bacteria were added. At each experimental stage, three bacterial infected platelet concentrates and five negative control platelet concentrates (control) were performed. For negative controls, no growth was observed throughout the study. The sampling time for each bacterium was the same; T = 0 immediately after inoculation, T = 6, T = 24, T = 48 in hours.

To conduct the test by flow cytometry, at first, 5 ml of the consentrates were removed from contaminated platelets with a 5/50 buffering (Separately for each bacterium). 500 µl of the suspension were poured into a separate tube. Then, 5 µl of Thiazole orange dye (TO), (BD

Biosciences, USA) was added to the suspension. Next, 5 µl of Propidium iodide dye (PI), (BD Biosciences, USA) was added to a mixture of dye and suspension. The resulting solution was vortexed and incubated for 1 minute at room temperature. Liquid counting beads (BD Biosciences, USA) were applied to the room temperature before use, and then vortexed slowly for 30 seconds. 50 µl of it was added to each tube containing dyes, buffers and the bacteria-containing suspensions. These beads emit fluorescence and were counted and analyzed in their respective graphs. To measure the flow cytometry of bacteria from a Partec cy-flow space system (Partec GmbH, Münster, Germany) equipped with air-cooled argon ion laser (15 mW and 488 nm) and filter sets for fluorescence (FL): FL1 (515 to 545 nm), and FL3 (> 650 nm), was used.

Statistical analysis

For statistical analysis, SPSS software version 16 was used. One-way ANOVA analysis was used to determine the difference between the mean number of *E. coli* and *Staphylococcus epidermis.*

Results

Incidence of bacterial contamination by flow cytometry

Due to the different growth rates of the bacteria in platelets, the results of tests with *E. coli* and *Staphylococcus epidermidis* were evaluated separately. The number of bacteria in contaminated platelet units for each bacterium was calculated based on the amount of inoculations and the sampling time. Concerning the results of *E. coli* bacteria, the bacterial contamination level according to the sample size taken from the infected platelet units is summarized in Table 1.

Table 1: Counted number of *E. coli* and *Staphylococcus epidermis* bacteria in a volume of 1 ml of platelet bags inoculated with 10 CFU / ml concentration by flow cytometry.

Time	0		6		24		48	
Sample number	Staphylococcus epidermidis	Escherichia coli	Staphylococcus epidermidis	Escherichia coli	Staphylococcus epidermidis	Escherichia coli	Staphylococcus epidermidis	Escherichia coli
1	177	188	237	293	269	191	184	192
2	210	25	213	77	227	27	165	27
3	306	142	313	217	355	87	236	134
4	216	112	229	173	247	89	165	87
5	173	339	192	294	146	278	166	243
6	180	151	200	131	147	131	189	120

As demonstrated in table 1, the number of *E. coli* bacteria increased by 6 hours after inoculation and decreased at 24 and 48 hours. The number of bacteria in *Staphylococcus epidermidis* was increased 24 hours after inoculation and decreased in 24 hours. The sensitivity of the flow cytometry detection method was 100% for both bacteria. One-way ANOVA analysis was used to determine the difference between the mean numbers of *E. coli* bacteria. As shown in Table 2, the average number of *E. coli* bacteria did not show any significant difference at the expected times.

Table 2: One-way variance analysis (ANOVA) to determine the difference between the average numbers of *E. coli* bacteria

	Time(Hour)	MEAN±SD(n=6)	*P value*
The Number of E.coli bacteria	0	159.5±103.68	0.57
	6	197.5±87.58	
	24	132.83±89.08	
	48	133.83±76.28	

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Figures 1 and 2 show the growth of *E. coli* strain at T = 0, T = 6, T = 24, T = 48 after inoculation of 10 CFU/ml in contaminated platelet units, using flow cytometry.

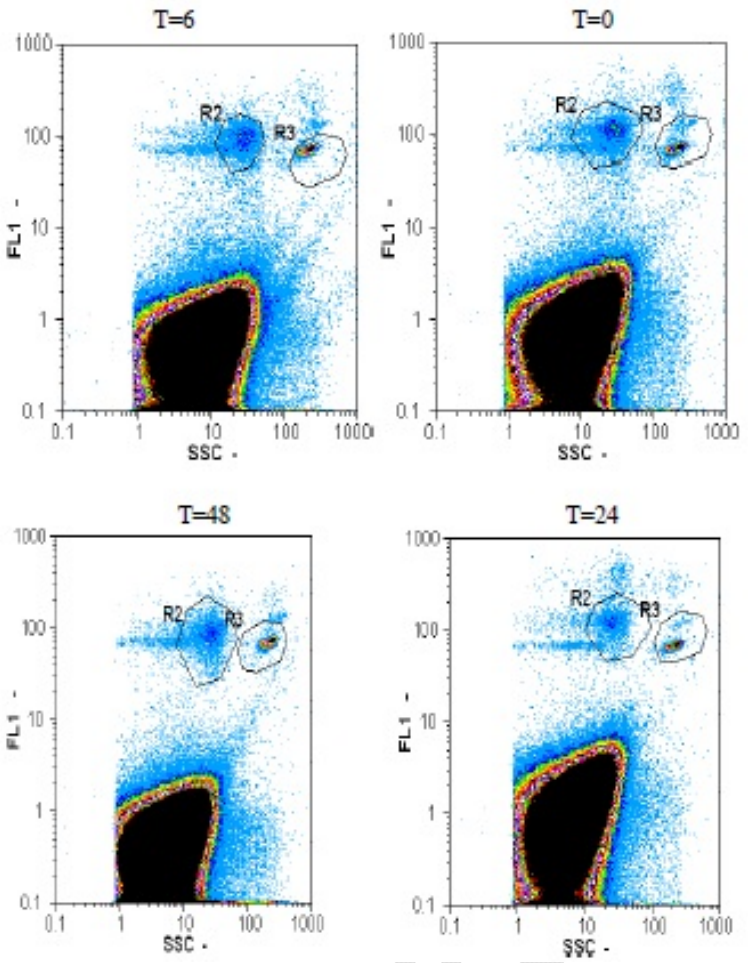

Figure 1: *E. coli* growth at T = 0, T = 6, T = 24, T = 48 after inoculation of 10 CFU / mlin contaminated platelet units. R2: Gates related to the number of bacteria, R3: Gates related to the number of beads

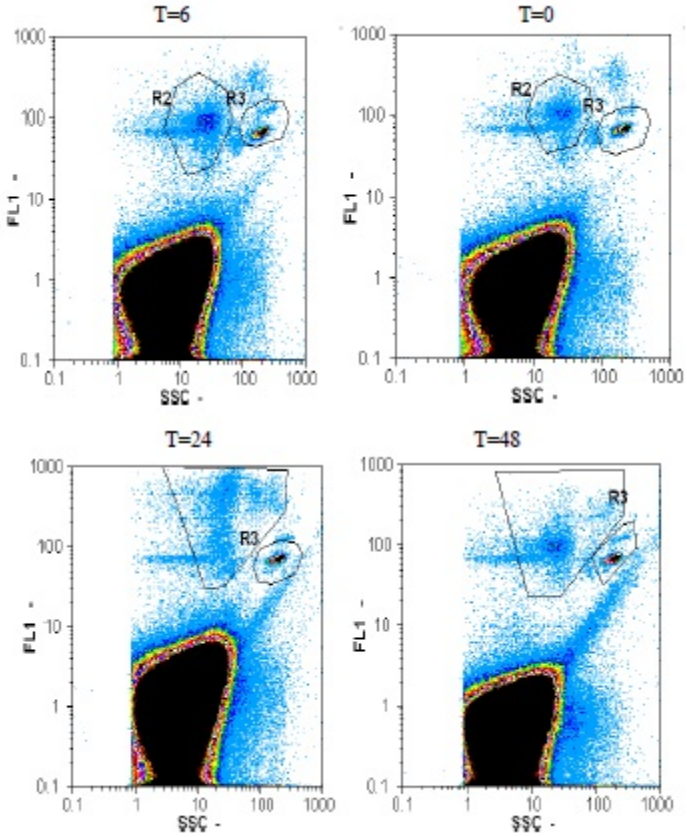

Figure 2: Growth of *Staphylococcus epidermidis* at T = 0, T = 6, T = 24, T = 48 after inoculation of 10 CFU/ml in contaminated platelet units. R2: Gates related to the number of bacteria, R3: Gates related to the number of beads

Two-way variance analysis (ANOVA) was used to evaluate the mean difference between *Staphylococcus epidermidis* and *E. coli* in different time groups. The results showed that the average number of *Staphylococcus epidermidis* bacteria was 167/156 and the mean number of E. coli bacteria was 214.45, which is significant ($p < 0.05$).

Discussion

Despite all the measures taken in the platelet preparation process to prevent bacterial contamination, pooled platelet is prepared from several donors that may have had asymptomatic bacteremia. On the other hand, platelets should be maintained in room temperature. Therefore, there is a high potential for bacterial contamination in the production process and subsequent

proliferation of bacteria in room temperature. Annually a considerable number of patients who receive platelets, develop bacteremia or bacterial septicemia leading to their death [12, 13]. Previous studies reported the usefulness of flow cytometry in detection of bacterial contamination in platelets. In one of these studies in Korea, platelet samples inoculated with 1 species of bacteria with 3 concentrations of 10, 100 and 1000 CFU / ml in volumes of 0.5, 1, and 2 ml, were incubated in culture media at 35°C for 24 hours. The analysis showed an increase in the number of bacteria at 4 hours, except in the case of *Klebsiella pneumonia*???/ and *E. coli*, whose numbers decreased after 16 hours. Incubation in culture media allowed bacteria to multiply and easily detection by flow cytometry. Moreover, detection time was decreased with increased inoculation concentrations [14]The results of our study revealed that flow cytometry has a high performance in the detection of bacterial infections even at zero time and with minimal bacterial growth that was in line with Vollmer et al. study. whose results indicated that use of the flow cytometry method is very suitable for screening of bacterial infections in the platelets, which can be used even independently of the incubation time and screening strategy [7]In this study, the number of bacteria increased during 24-hour incubation period. However, *E. coli* cases showed peak levels and decreasing patterns during incubation time. For both organisms, an increase in the number of bacteria was observed within 6 hours after inoculation. For E. coli, as a rapidly growing bacterium, the number of bacteria decreased by 24 hours. It seems that this observations can be due to the different growth rate of the bacteria or the size of the sample intended to increase the growth of the bacterium. In the case of *Staphylococcus epidermidis*, as a slowly growing bacterium, decrease of the number was observed within 24 hours. In the present study, flow cytometry allowed early detection of both types of bacteria (zero time) at the lowest inoculation concentration of 10 CFU / ml and at the lowest sample size.

Flow cytometry is a technique for counting and evaluating various properties of particles (including cells) by the flow cytometer, which not only can analyze several parameters simultaneously and rapidly, but also sort and collects various cell populations in a heterogeneous mixture with high efficacy .It can be used to identify cells and examine their characteristics, including size and granularity, surface or internal markers analysis for identification, examining or isolating cells in a heterozygous cell population, examining the function or intracellular signaling, analyzing DNA, examining cell cycle, cell proliferation and apoptosis. Currently, three commonly-evaluated rapid methods are available: BactiFlow, nucleic acid amplification techniques (NAT), and pan genera detection (PGD). These methods are significantly different at detection time. The time to reach the PGD test and the BactiFlow test is about 1.5 hours and for NAT is about 3-4 hours [14-16] Sireis et al also suggested that BacT/Alert can be used as a rapid method for screening on day 3 or 4 after blood donation due to a maximum incubation time of <12 hours for bacterial strains [17]. Flow cytometry Therefore, flow cytometry is of the most common methods of detection and differentiation of cells in various tissues and considerably useful in hematology immunology, cancer biology, clinical oncology, toxicology, pharmacology, botany and microbiology. It is also used to diagnose diseases, determine prognosis as well as detecting malignancies [18]

Conclusion

Flow cytometry method has high speed and precision in the detection of bacterial contamination and its use can promote the health of platelets. The results of this study showed that the sampling time of 0 and 6 hours of contaminated platelet units of at least 5 ml was suitable to ensure the appearance of *E. coli* and *Staphylococcus epidermidis* bacteria contamination using flow cytometry. This method can be used in patients with high risk, generally those who need more platelet units and have a suppressed immune system, especially those with chemotherapy. In fact, using a flow cytometry method is highly recommended for point of issue screening.

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