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**Culture-based bacterial detection systems for platelets: the effect of
time prior to sampling and duration of incubation required for
detection using aerobic culture**

Short title: Time-dependence for sampling and detection of bacteria in platelets

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ABSTRACT (205 words)

BACKGROUND: Bacterial contamination of platelet products (PLTs) occurs at low concentrations requiring a period of incubation for growth to minimize sampling error. Culture-based detection methods also need sufficient incubation time; together these periods may limit the useful life of PLTs. This study characterizes the impact of sampling and detection times using two commercially-available bacteria detection products.

STUDY DESIGN AND METHODS: Apheresis PLTs inoculated with nine bacterial species at low concentrations were sampled immediately, and after 24 hours following inoculation. Test results were analyzed following incubation at 16, 20 and 24 hours after sampling using two bacterial detection systems.

RESULTS: When sampled immediately after inoculation, BacT/ALERT (BioMerieux) and Pall eBDS (Pall Corporation) failed to detect *S. epidermidis*, *S. liquefaciens* or *P. aeruginosa* and *S. epidermidis*, *S. liquefaciens*, *B. cereus* or *P. aeruginosa*, respectively. The BacT/ALERT was better at 20 hours ($p < 0.02$), but not at 16 or 24 hours for time 0 sampling. When sampling occurred 24 hours after inoculation, there were no difference between the two systems.

CONCLUSION: Results suggest that for either bacteria detection system, holding PLTs

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for 24 hrs prior to sampling improves the detection sensitivity for PLTs contaminated with low concentrations of bacteria and longer incubation periods improve detection.

ABBREVIATIONS: PLTs = platelet concentrates; TSA = trypticase soy agar; CFU = colony forming units

INTRODUCTION

Bacterial contamination is a major problem with transfusion of platelet products (PLTs) that can lead to serious morbidity and mortality. The bacterial contamination of PLTs has been estimated to occur at frequencies of 1 in 2000 to 1 in 3000 PLTs transfusions.¹ Several countries have a national surveillance system for collecting and monitoring data on the occurrence of adverse effects of transfusion. The French Blood Agency Haemovigilance Surveillance System has attributed 18 deaths to blood components contaminated by bacteria in the period from 1994 to March 1998.² In the USA, the BaCon Study revealed six fatal transfusion-related platelet transfusions between 1998 and 2000.³ The UK surveillance system SHOT (Serious Hazards of Transfusion) reported 22 incidents, including six fatalities, caused by the bacterial contamination of blood components between 1995 and 2002.⁴

AABB has since March, 2004, required all transfusion services implement methods to limit and detect bacterial contamination in platelet components.⁵ For culture screening systems, there is a choice between the semiautomatic system BacT/ALERT (bioMerieux, Marcy l'Etoile, France)⁶ and the nonautomated system eBDS (Pall Corporation, East Hills, NY, USA).⁷ These two detection methods have been cleared for the quality control of PLTs by the Food and Drug Administration (FDA). An ideal

screening system for detecting bacterial contamination should be rapid and sensitive. The above two methods require, in accordance with the manufacturers' instructions for use, a holding period of 24 hours prior to sampling. The methods of detection are based upon growing the sampled bacteria to sufficient levels during a period of incubation such that surrogate markers of growth will signal the presence of bacteria. The Pall eBDS system detects the reduction of oxygen in air above the incubated sample whereas the BacT/Alert system monitors the carbon dioxide produced by respiring bacteria. Therefore, in addition to the pre-requisite incubation prior to sampling and the duration of incubation of the sample to effect changes in the surrogate marker required for detection, PLTs may be released and used before results are available. On the other hand, if these products are used outside the recommendations of manufacturers as a release criterion, these requisite durations of time may impact upon the shelf life of the products. Recent data are available suggesting further characterization of incubation time prior to sampling and duration of bacteria testing is warranted.^{8,9,10} If either or both of the studied bacterial detection systems are able to detect in a shorter time, effective use of PLTs can be performed. We have evaluated PLTs spiked with common bacteria. The efficacy of detecting bacterial contamination after a reduced holding time and/or shorter bacteria detection incubation period was tested using the two bacterial culture methods.

MATERIALS AND METHODS

Study Design

Following inoculation of PLTs with bacteria, samples were taken for incubation and detection in either bacteria detection system and for durations of incubation including 16, 20 and 24 hrs. The remaining volume of contaminated PLTs was stored at room temperature (20-24 °C) with agitation. Following 24 hrs, sampling from the contaminated PLTs was repeated for incubation at 16, 20 and 24 hrs of incubation with each bacteria detection system.

Post inoculation quantitative culture was performed by [standard streak plate method with incubation on TSA](#) at 37 °C for 20 to 24 hours. The culture bottles and pouch samples after sampling were tested to confirm true positivity or true negativity for the presence of bacteria by plating 0.5 to 1 mL on TSA. The bacterial growth in PLTs, [including extinct bags](#), was quantified [every day](#) up to 5 days.

Apheresis platelets

PLTs have a shelf life of 3 days in Japan. Expired (> 72 hours after collection) apheresis PLTs, [that were collected by Haemonetics \(Braintree, Massachusetts, USA\) machines \(70%\) with polyolefin storage bag, Terumo \(Tokyo, Japan\) machines \(20%\) with](#)

polyolefin bag, or by Gambro (Lakewood, Colorado, USA) machines (10%) with polychloride-vinyl bag, 4 and 5 days old, were aseptically preserved in an appropriate condition until use and shipped to our laboratory from Red Cross Blood Centers in platelet preservation boxes. PLTs sterility was assured by sampling prior to inoculation with bacteria and testing sterility of the PLTs using the standard streak plate method.

Bacteria

Nine bacterial species were obtained from the American Type Culture Collection (KWIK-STIKT™ DuoPak, Kanto Reagents, Tokyo, Japan) (Table 1). They were revived from frozen (-80 °C) vials by growing in trypticase soy agar (TSA; Becton Dickinson, Franklin Lakes, NJ, USA). To quantify bacteria, undiluted samples were serially diluted one in ten with saline up to a dilution 10^{-9} . A 100 µl aliquot from dilution of 10^{-6} to 10^{-9} bacteria were plated, in duplicate, in order to obtain a dilution that gave more than 20 and less than 200 CFU per plate from which calculations of concentration were made. Dilutions (0.5-5mL) were used such that targeted concentrations of 1-10 CFU/mL were made in each of four PLTs for each that bacteria species were studied. One ml of sample from each PLT was drawn and a 100 µl aliquot for each plate was spread onto each of ten TSA plates. The final bag inoculation density

was determined after incubation of plates at 37 °C for 20 to 24 hours.

Bacteria detection systems

BacT/ALERT

This system consists of aerobic and anaerobic culture bottles (BPA and BPN culture bottles, bioMerieux), and the BacT/ALERT 3D system (bioMerieux, Marcy l'Etoile, France). The BacT/ALERT automated microbial detection system uses a colorimetric sensor at the bottom of the culture bottles that changes color in the presence of carbon dioxide produced during bacterial proliferation. The bottles are examined approximately every 10 minutes the computer software monitors the rate of color change produced by sensor. This is nearly a closed system except for the transfer of sample from the PLTs to the sample bottle with a syringe and needle used in a clean workstation. We used an aerobic culture with 4 mL of sample each. Although readings are taken every 10 minutes throughout incubation, we recorded the state of bacteria detection following 16, 20 and 24 hours of incubation.

eBDS

The Pall eBDS system consists of a disposable sample set, in which a pouch contains a readily dissolvable tablet of sodium polyanethol sulfonate (SPS) in trypticase soy broth

(TSB), a flatbed agitator and an incubator (Helmer, Noblesville, IN, USA), and an oxygen analyzer (PBI-Densensor; Ringsted, Denmark). This system is based on the principle that growing aerobic and facultative anaerobic bacteria consume oxygen in plasma that will equilibrate with air in the space within a sample pouch. After collection of PLTs, the pouch is heat-sealed and incubated at 35 °C before measuring oxygen concentration using an oxygen analyzer. The sample set was attached to a segment from a PC unit with a sterile connecting device (TSCD; Terumo Corporation, Tokyo, Japan). Pouches of eBDS were incubated for 24 hours at 35 °C with agitation on a horizontal shaker and evaluated 16, 20 and 24 hours after inoculation. Oxygen in the headspace was measured by the attached oxygen analyzer. A positive result was determined as any O₂ reading less than 9.4 percent.

Statistical Analysis

A Fisher's exact test was used to compare the measurements. $P < 0.05$ is considered to indicate statistically significant difference.

RESULTS

The average initial bacterial inoculum densities for the PLTs ranged from 1 to 20 CFUs per mL (Table 1). All PLTs inoculated with *S. aureus*, *B. cereus*, *S. marcescens*, *E. coli* and *K. oxytoca* showed increased growth to concentrations exceeding 5 log CFU per mL at 48 hours after inoculation. *S. epidermidis* reached this level at 72 hours (Fig. 1).

These bacteria were extinct in 1 of 4 PLTs inoculated with *P. aeruginosa* at 24 hours, and in 3 of 4 PLTs inoculated with *S. liquefaciens* and *E. cloacae*, at 5 days. The remaining 3 PLTs with *P. aeruginosa* and 1 PC with *S. liquefaciens* reached a density \geq 5 log CFU per mL at 24 hours. *E. cloacae* required 120 hours to reach this level.

Time 0 Study

As shown in Table 2, BacT/ALERT needed mean times of 9.6 to 18.2 hours for detection in when sample were taken immediately after inoculation. None of samples inoculated with *S. epidermidis* or *S. liquefaciens* and only 1 of 4 samples with *P. aeruginosa* were detected at 16 hours of incubation. When incubated for 20 hours or 24 hours, all the negative samples became positive.

The eBDS system, a positive result was determined in all specimens with an oxygen reading less than 9.4 percent. There were no false-positive results. No specimens inoculated with *S. epidermidis*, *S. liquefaciens*, or *P. aeruginosa* were

detected at 16 hours of incubation. Seventy percent of the specimens inoculated with *B. cereus* were detected at 16 hours. When incubated for 20 hours, *B. cereus* and *S. liquefaciens* were detected in 3 of 4 samples. Specimens inoculated with *P. aeruginosa* were not detected at 20 hours of incubation; however, 25 % of those specimens were detected at 24 hours of incubation.

The BacT/ALERT was superior at 20 hours overall ($P < 0.02$), but not at 16 or 24 hours for those inoculations for the time 0 sampling (Table 3A).

Time 24 Study

As shown in Table 2, BacT/ALERT needed mean times of 3.7 to 15.9 hours when samples were taken 24 hrs after inoculation. All the samples, except those inoculated with *S. epidermidis* in which only 1 of 4 PLTs was detected, were detected efficiently at 16 hours of incubation. At 20 and 24 hours, there were no false-negative samples, as confirmed by repeated samples up to 5 days.

In the eBDS system, there were no false-positive results. Although specimens inoculated with *P. aeruginosa* had a 67% detection rate at 16 hours of incubation, the specimens inoculated with the other bacteria had a 100 % detection rate. After a 24-hour hold and subsequent sampling, there were no statistical differences in sensitivity between the two systems (Table 3B).

DISCUSSION

The principal objectives of the detection of bacterially contaminated platelets are the prevention of transfusion-related sepsis and the extension of platelet shelf life. Among various bacterial detection schemes, the FDA of the USA has approved two bacterial culture systems for use in quality-control testing to monitor platelet contamination: BacT/ALERT and Pall eBDS.^{6, 7} At present, bacterial detection in platelets is routinely performed on all PC products in Belgium, the Netherlands, Wales, and Hong Kong and on most PLTs in the USA¹¹ and some European countries.^{12, 13}

In 2005, the FDA, sanctioned the use of leukoreduced apheresis PLTs stored for 7 days in an approved storage container provided that aerobic and anaerobic release cultures are procured from all units 24 to 36 hours after collection with an additional set of study cultures procured from all outdated units after 7 days of storage. Although recent advances in screening systems and their ability to detect bacteria are improving the safety of blood supply,^{6, 7} a perfect screening system does not exist yet. Culture systems are very sensitive but they still require several days to detect a positive unit and case reports of false negatives have appeared.^{8, 9, 10}

Platelets require shaking at room temperature for preservation to maintain their optimum functions.¹⁴ Coincidentally, bacteria can proliferate under these conditions

from low concentrations (< 1 CFU per mL) at the time of collection to very high concentrations (> 8 log CFU per mL) throughout storage.¹⁵ Our results highlight that some bacteria grow rapidly under the conditions of platelet storage and would be captured if sampling occurs at 24 hrs (*B. cereus*, *E. coli*, *S. marcescens*, *P. aeruginosa*, and *K. oxytica*) whereas others grow more slowly and their concentrations are increased at 24 hrs but not greatly (*S. epidermidis*, *S. aureus*, *S. liquefaciens* and *E. cloacae*). These data are applicable only to the ATCC strain tested and should not be generalized to all strains and certainly not to different species of the same genus. More importantly, these data highlight the issue that some bacteria grow slowly and their concentrations at the time of sampling may be too low to capture. Longer storage durations prior to sampling clearly reduce the risk of sampling error.

The eBDS has been reported to be ineffective in detecting 2 of 9 (22.2 %) samples inoculated with *B. cereus* at 18 hours of incubation by the 24-hour-holding sampling.¹³ All four samples inoculate with *B. cereus* were detected at 16 hours of incubation by the 24 hour-holding sampling: however, only 3 of 4 of the same samples were detected at 16 hours of incubation by immediate sampling. Only 2 of 3 samples inoculated with *P. aeruginosa* were detected at 16 hours of incubation by the 24-hour-holding sampling. By using BacT/ALERT, none of the four samples inoculated with *S. epidermidis* was

detected in at 16 hours of incubation by the immediate sampling, 3 of 4 of the same samples were detected at 16 hours of incubation by the 24-hour-holding sampling. Therefore, both systems are less sensitive when specimens are sampled immediately after inoculation with *S. epidermidis*, *S. liquefaciens*, *P. aeruginosa*, *E. cloacae* and *B. cereus*.

Some bacteria find it difficult to grow when the inoculum densities are very low (below 1 CFU per mL). It seems that some bacteria have the property of being susceptible to self-sterilization or so-called autosterilization in PLTs. This could be due to killing by preformed antibodies, complement proteins, lysozymes or some lipoproteins in plasma. No live bacteria were observed in the PLTs after 24 hours of incubation in one or more PLTs inoculated with three bacterial species (i.e., *P. aeruginosa*, *S. liquefaciens* and *E. cloacae*) at room temperature. A contamination level less than 5 CFU per mL of contamination has shown a high frequency of autosterilization during 24-hour storage at room temperature.⁷ On the other hand, previous studies have suggested that slow-growing organism with lower inoculums may escape detection with decreased chance of detection.^{16,17} We believe that self-sterilization in this study was involved in negative detection in some PLTs inoculated with *P. aeruginosa*, *S. liquefaciens* or *E. cloacae*, post-inoculation bags hold

for up to 5 days were negative for bacteria.

Two systems can detect sample inoculated with *B. cereus* and *S. liquefaciens* at a sensitivity of 1 CFU per mL. In the Time 0 sampling, the two systems detected specimens inoculated with three bacteria (i.e., *P. aeruginosa*, *S. liquefaciens* and *E. cloacae*). Bacterial screening technology is useful for blocking PLTs that contain high levels of bacteria contamination, but cannot reliably detect low levels of bacterial contamination at time 0. To reduce sampling error (below 1 CFU per mL), in accordance with the manufacture's recommendations for eBDS that PC sampling should be carried out with a 24-hour holding after collection,⁷ BacT/ALERT specifies the same sampling time in the USA. In European countries, BacT/ALERT sampling is performed 2-18 hours after the collection of apheresis platelets and immediately after the production of a buffy coat derived from PLTs, which means 22 hours after the collection of whole blood units.¹⁸ To improve detection sensitivity, the sampling volumes used are 7 to 10 mL (the aerobic and anaerobic bottles used had sampling volumes of 14 to 20 mL). In the USA, 4 to 6-mL samples are generally used.¹⁸ Moreover, bacteria that grow after 24 hours may be overlooked. There have been three published reports on false-negative results when using BacT/ALERT.^{19, 20, 21} Yomtovian and others²² revealed that active surveillance by culture at time of issue detected 38 of 39 contaminated

platelet units with high sensitivity (0.97) and good positive predictive value (0.83).

In conclusion, our results have shown the reduced sensitivity of early sampling for culture-based bacterial screening systems to detect bacteria that grow in PLTs and cause sepsis. Therefore, the sampling errors (i.e., false negative) of the two systems mentioned above are major constraints to the early release of PLTs using any culture-based technique. Ideally, at-issue screening methods with required sensitivity and specificity are hoped for and under development. Alternatively, a point-of-use bacteria detection system may be of value. Results of this study indicate that a 24-hour holding and a 20-hour incubation are required to allow the low level of bacterial contamination to increase sufficiently to be able to detect bacteria in PLTs.

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Legends for figures

Fig. 1 Growth curves for nine bacterial species in PLTs. a) *S. aureus*, b) *S. epidermidis*, c) *B. cereus*, d) *E. coli*, e) *S. marcescens*, f) *S. liquefaciens*, g) *P. aeruginosa*, h) *E. cloacae* and i) *K. oxytoca*. Each species was inoculated into four platelet bags at 1.25 to 20.13 CFU per mL on day 0. Data are shown as means \pm standard deviation (n=4). *P. aeruginosa* became extinct after a 24-hour holding in 1 of 4 PLTs, and *S. liquefaciens* and *E. cloacae* in 3 of 4 PLTs.

Log
CFU/mL

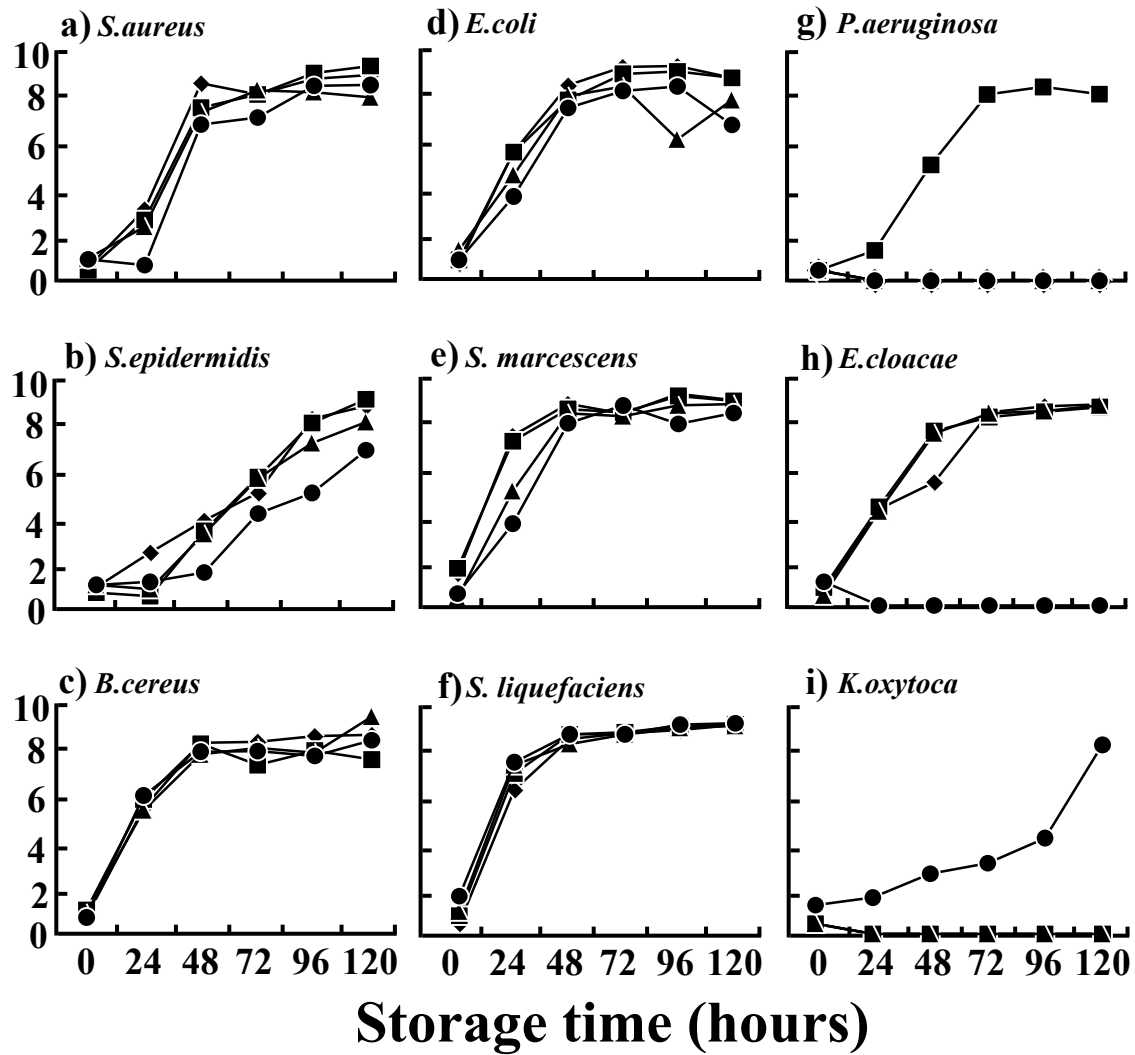


Table 1. Bacterial species used bacterial inoculum density

Species	Bacterial inoculum level (CFU/mL) [†]	ATCC order
<u>Gram positive</u>		
<i>Staphylococcus aureus</i>	6.5 (3-10) (n = 4)	29213
<i>Staphylococcus epidermidis</i>	12.11 (7-15) (n = 4)	49134
<i>Bacillus cereus</i>	1.25 (1-2) (n = 4)	10876
<u>Gram negative</u>		
<i>Escherichia coli</i>	3.25 (1-9) (n = 4)	25922
<i>Serratia marcescens</i>	20.13 (2-48) (n = 4)	43862
<i>Serratia liquefaciens</i>	1.25 (1-2) (n = 4)	27592
<i>Pseudomonas aeruginosa</i>	6 (1-13) (n = 4)	27853
<i>Enterobacter cloacae</i>	6.25 (1-22) (n = 4)	13047
<i>Klebsiella oxytoca</i>	18.75 (1-56) (n = 4)	43086

[†]Data are denoted as mean (range).

The actual number of bacterial cells inoculated was determined by the standard culture method.

Table 2. Results of two systems for inoculum detection in PLTs

Species	BacT/ALERT									eBDS						
	Time 0				Time 24					Time 0			Time 24			
	Mean detection time (hrs)	16 hr	20 hr	24 hr	Mean detection time (hrs)	16 hr	20 hr	24 hr	16 hr	20 hr	24 hr	16 hr	20 hr	24 hr		
<i>S. aureus</i>	12.1	4/4	4/4	4/4	7.4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4		
<i>S. epidermidis</i>	17.9	0/4	4/4	4/4	15.9	3/4	4/4	4/4	0/4	4/4	4/4	4/4	4/4	4/4		
<i>B. cereus</i>	9.6	4/4	4/4	4/4	4.8	4/4	4/4	4/4	3/4	3/4	4/4	4/4	4/4	4/4		
<i>E. coli</i>	11.7	4/4	4/4	4/4	6.4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4		
<i>S. marcescens</i>	11.2	4/4	4/4	4/4	5.2	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4		
<i>S. liquefaciens</i>	18.2	0/4	4/4	4/4	12.4	1/1	1/1	1/1	*	0/4	3/4	4/4	1/1	1/1	1/1	*
<i>P. aeruginosa</i>	16.9	1/4	4/4	4/4	9.4	3/3	3/3	3/3	**	0/4	0/4	1/4	2/3	3/3	3/3	**
<i>E. cloacae</i>	12.3	4/4	4/4	4/4	13.2	1/1	1/1	1/1	*	4/4	4/4	4/4	1/1	1/1	1/1	*
<i>K. oxytoca</i>	11.8	4/4	4/4	4/4	3.7	4/4	4/4	4/4		4/4	4/4	4/4	4/4	4/4	4/4	

Spiked PLTs were sampled immediately after inoculation (Time 0) and after a 24 hour room temperature holding (Time 24).

* *S. liquefaciens* and *E. cloacae* became extinct in 3 of 4 PLTs after 24 hours of holding.

** *P. aeruginosa* became extinct in 1 of 4 PLTs after 24 hours of holding.

Table 3. Bacterial detection total positive

A. Time 0 sampling

	<u>at 16 hr</u>	<u>at 20 hr</u>	<u>at 24 hr</u>
BacT/ALERT	25/36	36/36	36/36
eBDS	23/36	30/36	33/36
P value#	0.8	0.02	0.24

B. Time 24 sampling

	<u>at 16 hr</u>	<u>at 20 hr</u>	<u>at 24 hr</u>
BacT/ALERT	28/29	29/29	29/29
eBDS	28/29	29/29	29/29
P value#	1.00	1.00	1.00

Fisher's exact test