Rapid Bacteria Detection in Blood Components

+ PEI Blood Bacteria Standards for Validation of Screening and Reduction Methods

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What are PEI Blood Bacteria Standards ?

1. PEI Blood Bacteria Standards are able to grow in PCs up to high counts (what is not automatically given in case of reference strains like ATCC strains).
2. Strains grow up in PCs independent on donor properties (tested for multiplication in PCs from at least 100 different donors).
3. The standards are deep frozen, ready to use, stable, and shippable (manufactured by a special procedure).
4. They are defined in count and consist mainly of living cells (as a rule > 95% living cells).
5. The standards allow “real life” spiking of blood components, i.e. artificial contamination with ~ 10 CFU per bag corresponding to 0.03 CFU per millilitre.
6. Thus, PEI Blood Bacteria Standards are a feasible tool for objective validation and assessment of methods for screening and pathogen reduction in blood components.

ISBT Working Party Transfusion-Transmitted Infectious Diseases (WP-TTID),

Bacteria Group (Chair: Thomas Montag)

ISBT Macao, China, 2008

1. European Expert Meeting on "Platelet Screening by Rapid Methods for Bacteria Detection
   September 10 - 11, 2007, Bacharach, Germany
   Reason: Preparation of a Clinical Study on Post-Storage Bacteria Screening using Flow Cytometry

2. **Main work:**
   Preparation of ISBT-WHO International Validation Study on Blood Bacteria Standards as prerequisite for their implementation as WHO Blood Bacteria Standards
   Preparation of Blood Bacteria Standards following the requirements of WHO (Committee of Biological Standardisation; Blood Bacteria Standards represent a new principle in comparison to e.g. Protein or Virus Standards)

   - Development of "Passports" of Blood Bacteria Standards
   - International discussion: Which bacteria species respectively strains are blood relevant? Which properties of the bacteria are required? What International Standards are necessary?
   - Study design

“Passports” of Blood Bacteria Standards

Example

(will be changed into “Certificate”)

Working Party on
Transfusion Transmitted Infectious Diseases

WHO-ISBT International Validation Study

Passport

Blood Bacteria Standard

Species: *Staphylococcus epidermidis*
Code: PEI-B-06-(Lot)
Lot: PEI-B-06-07

store below -70°C

Developed by: Paul Ehrlich Institute
Federal Agency for Sera and Vaccines
Division Microbial Safety
Paul-Ehrlich-Str. 51-59
63225 Langen

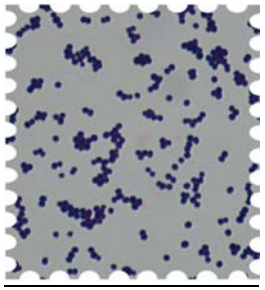
Generated:
Date: 2005/09/28

Approved:
Date: 2008/02/06

Passports Blood Bacteria Standards

(Page 3)

Lot:	PEI-B-06-07
Species:	*Staphylococcus epidermidis*
Isolated from:	platelet concentrate (PC)
Supplied as:	deep frozen in 10% Human Serum Albumin in saline (150 mM NaCl)
Volume:	> 1 mL
Bacterial load:	$2.18 \pm 0.29 \times 10^8$ cfu/mL
Growth in PC:	Blood Bacteria Standard *Staphylococcus epidermidis* PEI-B-06 grows donor independently in PCs.



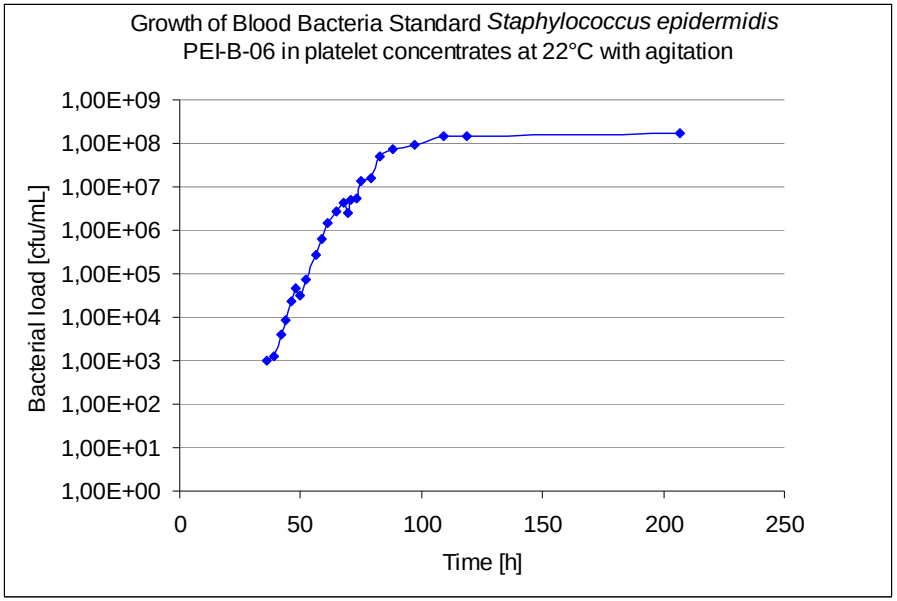


Fig.1 In the in vitro study pooled PCs (n=4) were inoculated with 0.03 cfu/mL of Blood Bacteria Standard PEI-B-06 of isolate *Staphylococcus epidermidis*. Sampling was performed during aerobic storage at 22°C and the presence of bacteria was assessed by plating culture.

Passports Blood Bacteria Standards

(Page 4)

2. Bacterial Strain (Blood Bacteria Standard)

Phylum:	Firmicutes
Class:	Cocci
Order:	Bacillales
Family:	*Staphylococcaceae*
Species:	*Staphylococus epidermidis*
Collection no.:	none (isolate)
Isolated from:	platelet concentrate
Characteristics:	Gram-positive cocci (0.7 - 1.2 µm), colonies are often surrounded by a clear zone of hemolysis (beta hemolysis) due to production of hemolysins tissue invasive, produce purulent (pus-filled) lesions, nonmotile, nonsporeforming, facultatively anaerobic, obligatory pathogenic, grows at 6.5°C to 46°C, grows at pH 4.2 - 9.3.

3. Microbiological identification

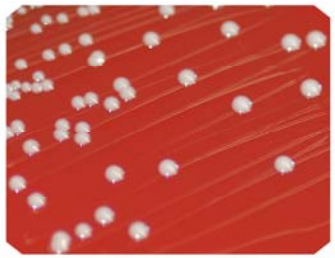


Fig. 2 *St. epidermidis* (PEI-B-06) on sheep blood agar after 24hours incubation at 37°C

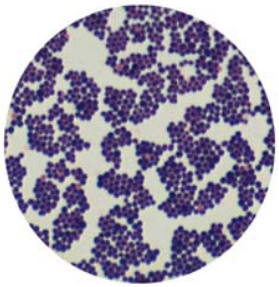


Fig. 3 GRAM-stain of *St. epidermidis* (PEI-B-06)

Colonies: small, white or yellow color, approx. 1-2 mm in diameter after overnight incubation; no hemolysis

GRAM-stain: GRAM-positive

Passports Blood Bacteria Standards

(Page 5)

4. Molecular genetic identification (16S rRNA Sequence)

Automated microbial DNA sequencing was performed by using the MicroSEQ® Microbial Identification System (Applied Biosystems).

Name	Resultat Microseq	% Match	Specimen Score	Consensus Length
PEI-B-06-07	Staph.epi ATCC 12228	100	42	488

Staphylococcus epidermidis partial 16S rRNA gene

GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCC
TCTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGATAA
CTTCGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATGGTTCAATAGTGAAAGA
CGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCT
TACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGG
AGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACA
AATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCT

5. Production

5.1 Production principle

After the bacterial identification process using microbiological, biochemical by using the API Staph multitest identification system (bioMérieux) in accordance with the manufacturer's instructions and molecular genetic methods (16S rRNA sequencing, RAPD-PCR), an impedance-monitoring system is used to characterize bacterial growth kinetics of Blood Bacteria Standard PEI-B-06 under definded conditions (e.g. media, temperature). Following bacteria are removed during the logarithmic phase, enumerated and frozen in 10% Human Serum Albumin in saline (150 mM) at -80 °C. Viabilty control is performed 24 hours after production while stability control is performes quarterly. The bacterial identity of each charge of Blood-Bacteria Standard PEI-B-06 is confirmed by biochemical, molecular genetic methods including 16S rRNA sequencing and DNA fingerprinting (RAPD-PCR).

5.2 Master Bank

Bacteria of Blood Bacteria Standard PEI-B-06 are cultured on appropiate agar media to a sufficient bacterial count. Under aseptic condentions bacteria are transferred to six vials of a Cryobank system to the manufacturers` insructions and stored at -80 °C. Cryobank tubes contain a medium for suspending the bacterial culture and 25 color-coded ceramic beads. The suspending medium comprises tryptone soy broth supplemented with glycerol and sucrose. Cryobank systems offer a reliable, convenient and versatile system for storing and preserving fastidious bacteria over long periods.

Passports Blood Bacteria Standards

(Page 6)

6. Batch Quality Control

6.1 Viability

To affirm the viabilty of the Blood Bacteria Standard PEI-B-06, vials of PEI-B-06 are defrozen 24 hours after production and enumerated as described in the application section. In this regard a standard deviation of < ± XX is strictly adhered.

6.2 Stability

The stability of the Blood Bacteria Standard PEI-B-06 is confirmed quarterly by defreezing and enumerating as described in the application section.

		Production		Last Stability control	
Species	**Charge**	**Date**	**Bacterial load [cfu/mL]**	**Date**	**Bacterial load [cfu/mL]**
Staphylococcus epidermidis	PEI-B-06-07	28.09.2005	2,18 ± 0,29E+08	06.02.2008	1,56 ± 0,33E+08

6.3 Identity (Fingerprint)

Random amplified polymorphic DNA analysis (RAPD) was performed using different single oligonucleotide primers of arbitrary sequence. Products underwent electrophoresis on an agarose gel (2%) and were visualized using ethidium bromide staining.

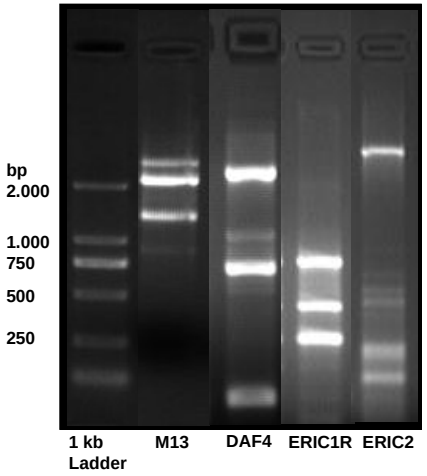


Fig. 4 RAPD-PCR (DNA-Fingerprint) of *St. epidermidis* PEI-B-06 using different single oligonucleotide primers (n=4)

Pre-validation Study of Blood Bacteria Standards between Paul Ehrlich Institute and National Blood Service London (25 samples, 7 replicates each, blinded)

	Paul Ehrlich Institute Langen, Germany			National Bacteriology Laboratory National Blood Service, London, UK	
No.	Strain	Code	cfu/ml	Identification	cfu/ml
1.	Bacillus cereus (isolated spores)	PEI-B-07-13	4.45 E+04	Bacillus cereus	5.5 E+04
2.	Staphylococcus aureus	PEI-B-23-05	4.0 E+07	Staphylococcus aureus	5.9 E+07
3.	Escherichia coli	ATCC 25922	2.0 E+07	Escherichia coli	8.7 E+06
4.	Negative	-	0	Sterile	0
5.	Bacillus cereus	PEI isolate	9.0 E+06	Bacillus cereus	6.5 E+06
6.	Candida albicans	PEI-B-21-02	2.0 E+06	Yeast	3.6 E+06
7.	Bacillus subtilis	ATCC 6633	3.7 E+02	Bacillus subtilis	2.8 E+02
8.	Bacillus cereus (isolated spores)	PEI-B-07-05	3.0 E+03	Bacillus cereus	1.6 E+03
9.	Klebsiella pneumoniae	PEI-B-24-02	1.7 E+08	Klebsiella pneumoniae	1.9 E+08
10.	Staphylococcus aureus	ATCC 6538	1.0 E+04	Staphylococcus aureus	8.2 E+03
11.	Propionibacterium acnes	PEI-B-22-04	2.9 E+07	Propionibacterium acnes	2.0 E+07
12.	Candida albicans	ATCC 10231	1.2 E+04	Yeast	5.0 E+03
13.	Negative	-	0	Sterile	0

Topic: identification of species and cell counting

Partners of WHO-ISBT Validation Study on Blood Bacteria Standards

Institution	Country	Contact	asked	Con-firmed
Blutzentrale Linz	Austria	Christian Gabriel	+	yes
Navigant	USA	Ray Goodrich	+	yes
Partner Lab of Sanquin	The Netherlands	Jan Marcelis	+	yes
National Blood Service London	United Kingdom	Carl McDonald	+	yes
Hong Kong Red Cross Blood Transfusion Service	Hong Kong SAR China	Cheuk-Kwong Lee	+	yes
German Red Cross Blood Transfusion Service Springe, Partner Lab of BD Biosciences	Germany	Thomas Müller	+	yes
South African National Blood Service	South Africa	Tshilidzi Muthivhi	+	yes
Fukushima Medical University School of Medicine	Japan	Hatisho Otho	+	(?)
Canadian Blood Service	Canada	Sandra Ramirez-Arcos	+	yes
German Red Cross Blood Transfusion Service University of Frankfurt/Main	Germany	Michael Schmidt	+	yes
University of Amsterdam	The Netherlands	proposal Henk Reesink	+	+
Regional Centre for Transfusion Medicine in Bialystok	Poland	Piotr Radziwon	+	(+)

Study Start: end of September 2008

Use of PEI Blood Bacteria Standards in Validation of Methods for Pathogen Reduction (example)

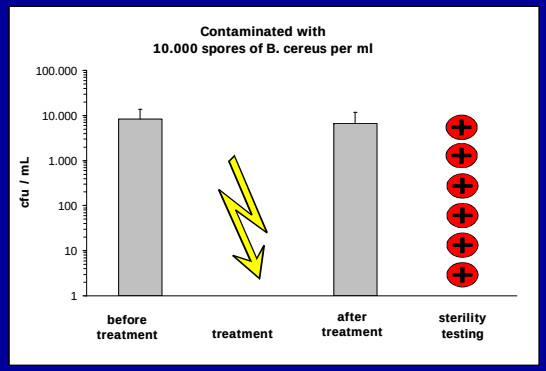
Bacterial spores are not killed by Amotosalen / UVA

Experiment 1:

contamination of 6 PPC with 3 x 10^6 isolated spores of *Bacillus cereus*

treatment with Amotosalen / UVA

-> no inactivation



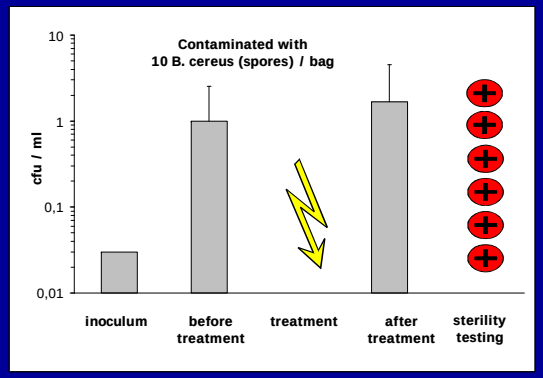
Experiment 2 ("real life"):

contamination of 6 PPC with 10 isolated spores of *Bacillus cereus* (= 0.03 cfu / ml)

storage for 1 day

treatment with Amotosalen / UVA

-> no inactivation



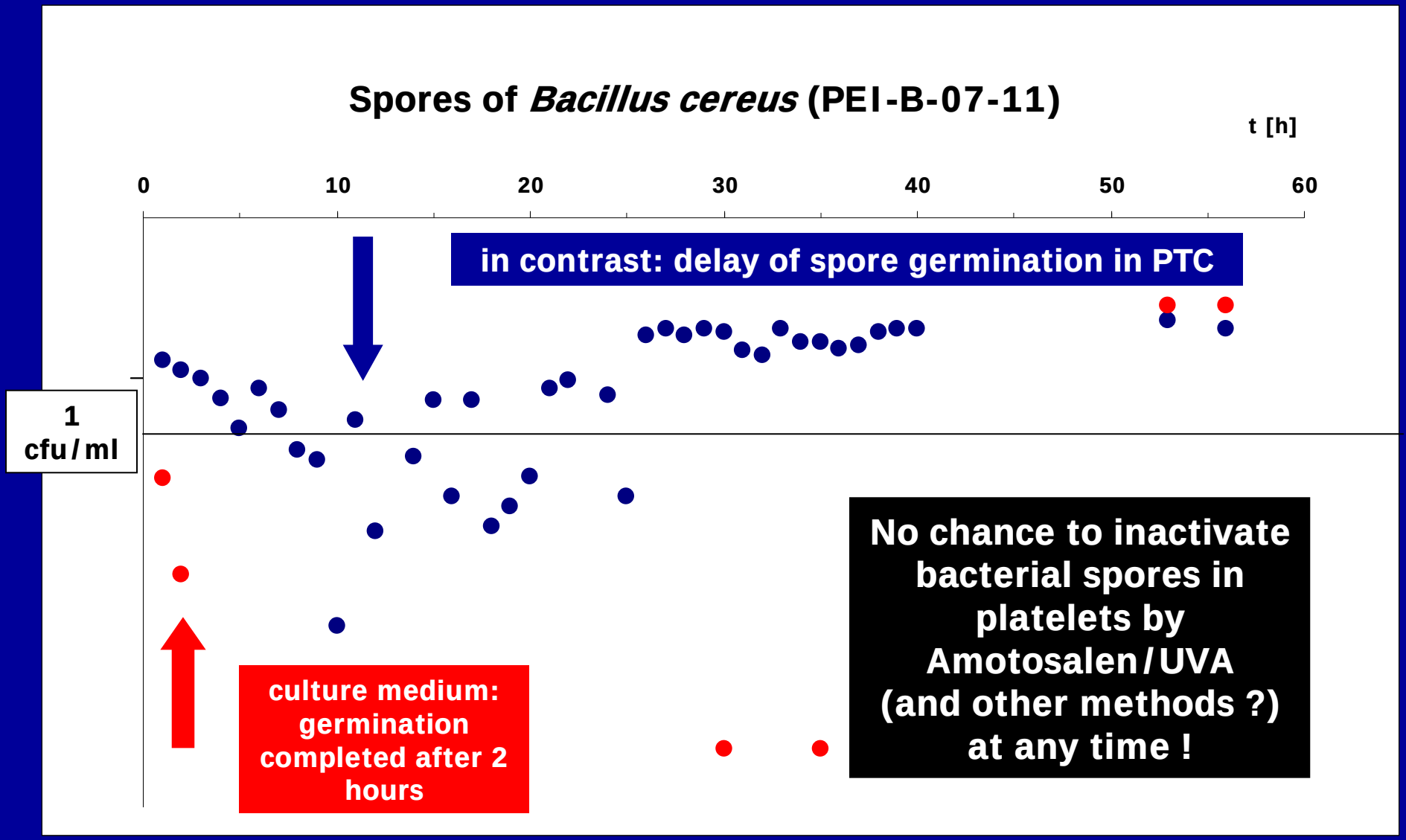
“Life style” of spore forming bacteria in PCs ?

- Contamination with 10 spores of *Bacillus cereus* per PC bag
- Storage for 18 – 24 hours (22.5°C, agitation)
- **Expectancy:**

Spores germinate to **vegetative cells**, which will be **killed** by Amotosalen / UVA.

- Why did *Bacillus cereus* survive ?

Germination of bacterial spores in platelets versus microbiological culture medium



Are spore forming bacteria clinically relevant ?

Clinical relevance of spore forming bacteria

McDonald CP, Hartley S, Orchard K, Hughes G, Brett MM, Hewitt PE, Barbara JAJ
Fatal *Clostridium perfringens* sepsis from a pooled platelet transfusion
Transfusion Medicine 1998; 8: 19 - 22

Blajchman MA, Sarwal SS, Loeb M
A second example of a transfusion-associated septic reaction associated with *Clostridium perfringens*
Transfusion 2001; 41: 427

Morduchowicz G, Pitlik SD, Huminer D, Alkan M, Drucker M, Rosenfeld JB, Block CS
Transfusion Reactions Due to Bacterial Contamination of Blood and Blood Products
Reviews of Infectious Diseases 1991; 13: 307 – 314
Fatality, transfusion-transmitted infection with *Bacillus cereus*

Sazama K
Reports of 355 transfusion-associated deaths: 1976 – 1985
Transfusion 1990; 30: 583 -590
2 fatal cases by *Bacillus species*

Dave J, Brett M, MacLennan S, Shields M
Sepsis associated with blood transfusion
The Lancet 1996; 347: 1773
Bacillus cereus

Marley EF, Saini NK, Vekatraman C, Orenstein JM
Fatal *Bacillus cereus* meningoencephalitis in an adult with acute myelogenous leukaemia
South Med J 1995; 88: 969 -972

Cone LA, Dreisbach L, Potts BE, Comess BE, Burleigh WA
Fatal *Bacillus cereus* endocarditis masquerading as an anthrax-like infection in a patient with acute lymphoblastic leukaemia: case report
J Heart Valve Dis. 2005; 14: 37 -39

Spore forming bacteria in platelets

Frequency of spore formers in platelets

Yomtovian et al., Transfusion 1993; 33: 902 – 909
1 *Bacillus cereus* in 3141 platelet pools
~ 1 in 3,000

Loukhmas L et al., Transfus Clin Biol 2000 ; 7: 171 -176
4 *Bacillus cereus* in 3,458 platelet concentrates
~ 1 in 900

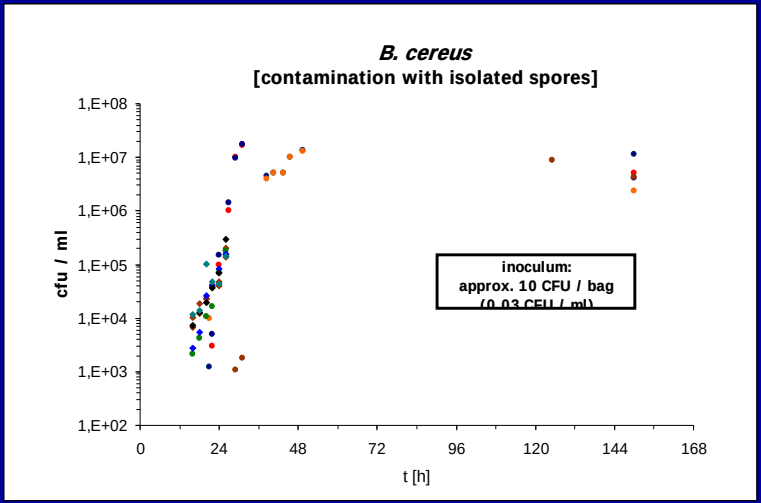
Werch JB et al., Transfusion 2002; 42: 1027 -1031
2 *Bacillus cereus* in 3093 platelet concentrates
~ 1 in 1,500

Rock et al., Transfusion 2004; 44 : 337 – 342
1 *Bacillus species* in 12,062 platelet concentrates
~ 1 in 12,000

Mohammadi et al. Transfusion 2005; 45: 731 – 736
2 *Bacillus cereus* in 2146 platelet concentrates
~ 1 in 1,000

Yomtovian et al., Transfusion 2006; 46: 719 – 730
1 *Bacillus cereus* in 418 platelet pools
~ 1 in 500

Growth of spore formers in platelets



10 experimentally contaminated platelet pools (of 4)

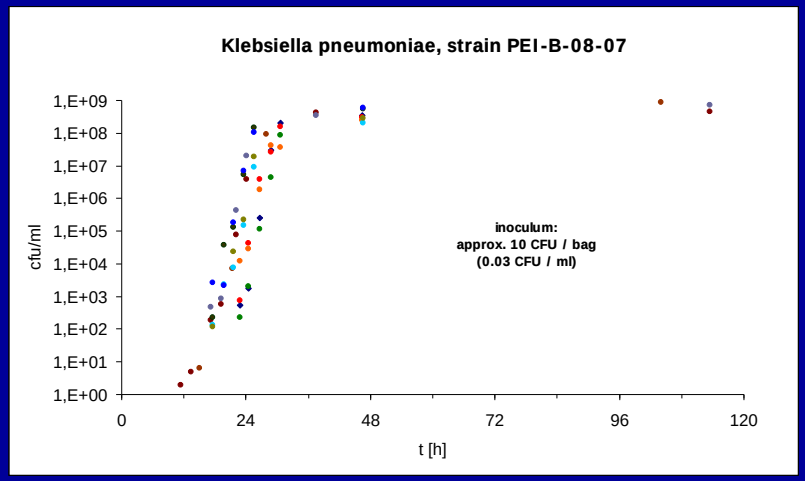
alcoholic disinfectants are not sporicidal !

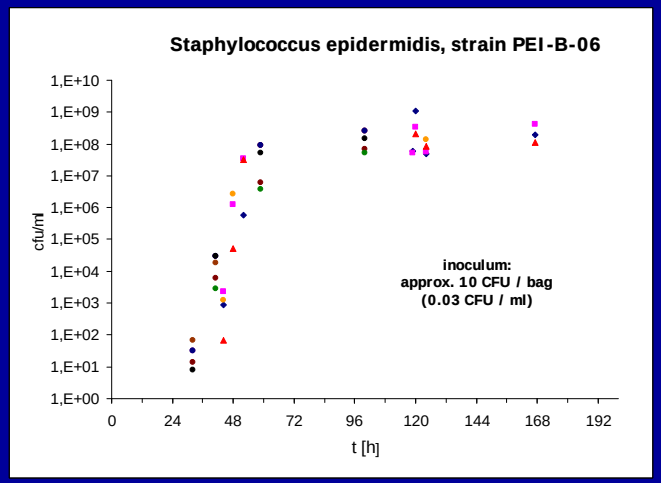
Use of PEI Blood Bacteria Standards in Validation of Screening Methods

Crux in Bacteria Screening of Blood Components

Usually, bacteria are contaminating blood donations in a very low count (10 to 100 CFU per bag corresponding to 0.03 to 0.3 CFU / ml).

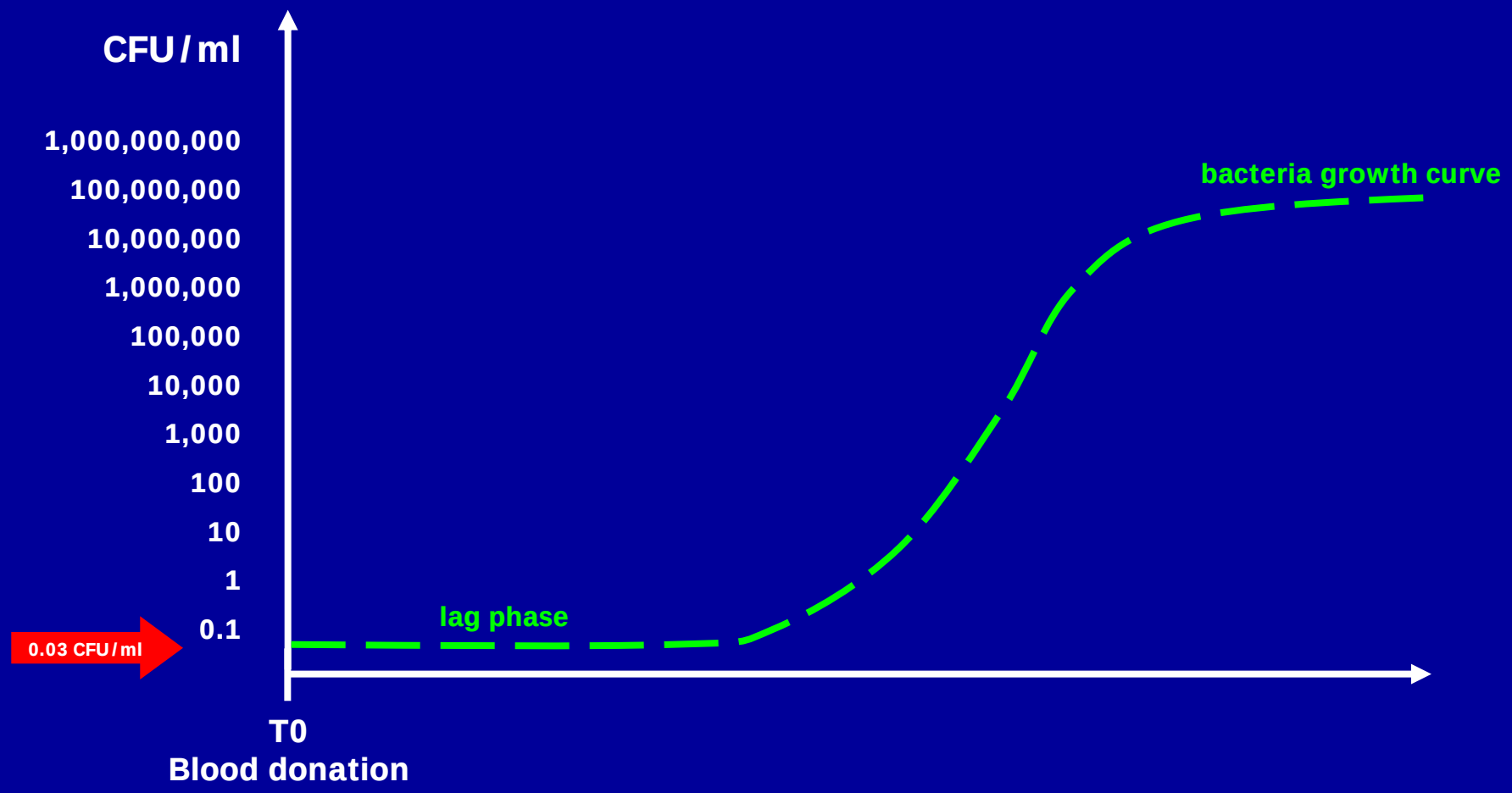
Thereafter, they can grow up in platelets to 10^8 to 10^9 CFU / ml (in dependency on species and strain).





Artificial contamination of platelets imitating "real life" conditions:
-> contamination with 0.03 CFU / ml
-> storage at 22.5 °C under agitation

Kinetic of microbial growth in blood components



Platelet Bacteria Screening (“Dutch Approach”)

BacT/Alert, BioMerieux

BACTEC, Becton Dickinson

CFU/ml

1,000,000,000

100,000,000

10,000,000

1,000,000

100,000

10,000

1,000

100

10

1

0.1

0.03 CFU/ml

Early sampling for culture methods

eBDS, Pall

lag phase

bacteria growth curve

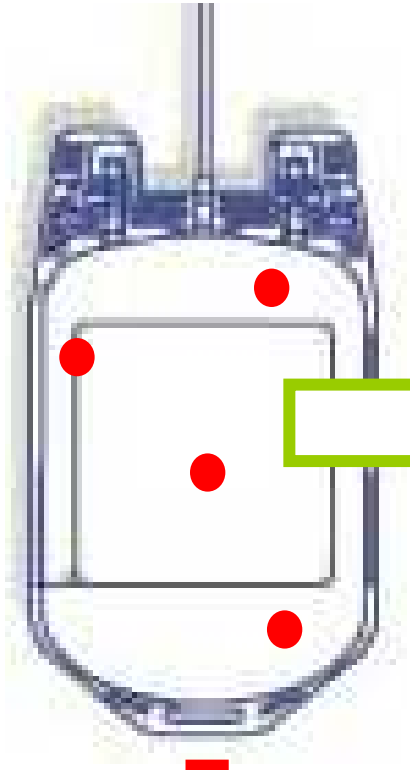
T0

Blood donation

T1

24 h after donation

clinical case: apheresis PTC (2 therapeutic units)



sampling 20 h after donation

incubation in BacT/Alert + Pall eBDS

cultures sterile

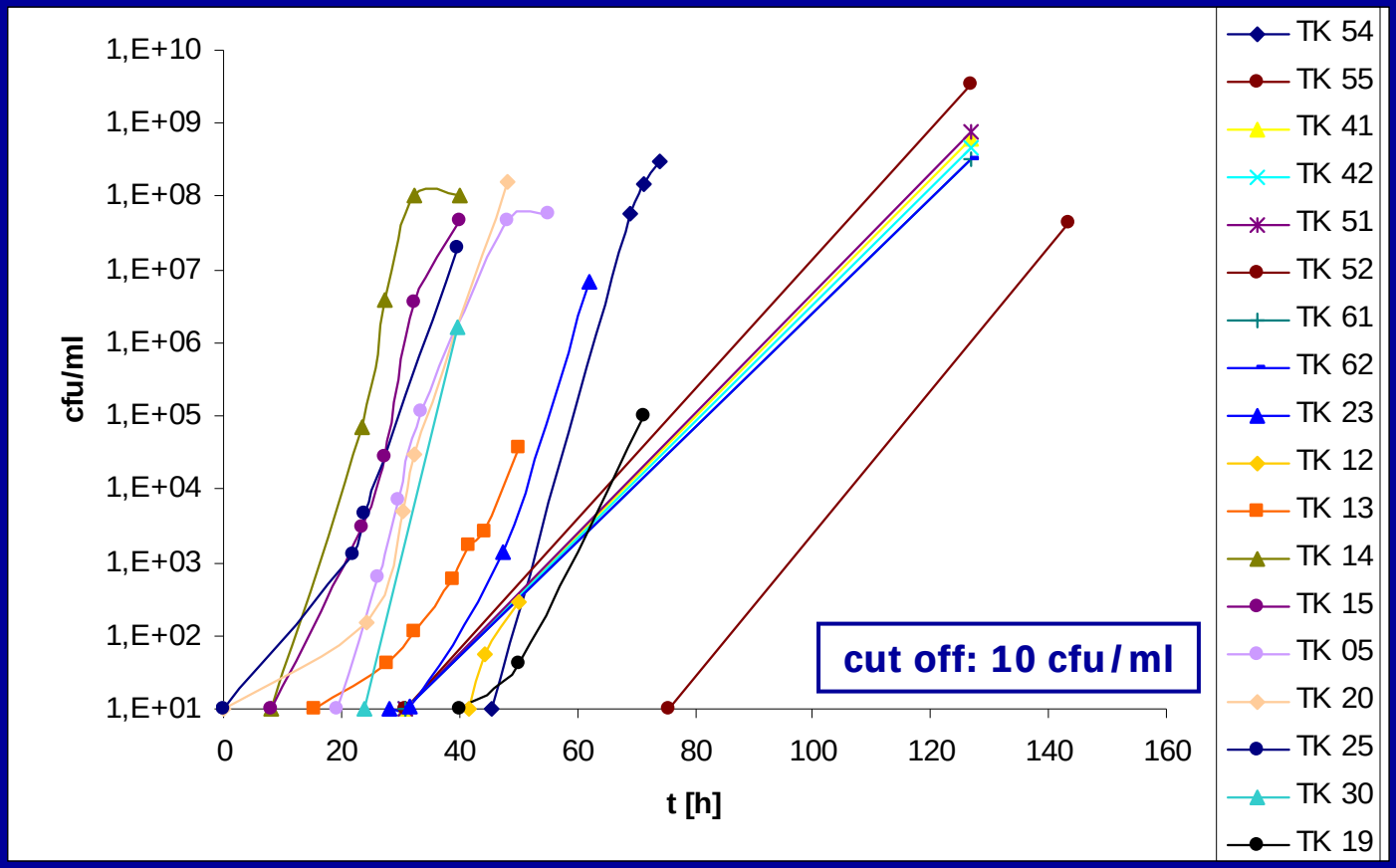
fatal case (s)
by Klebsiella pneumoniae

false negative result

bags: 10^8 – 10^9 CFU/mL

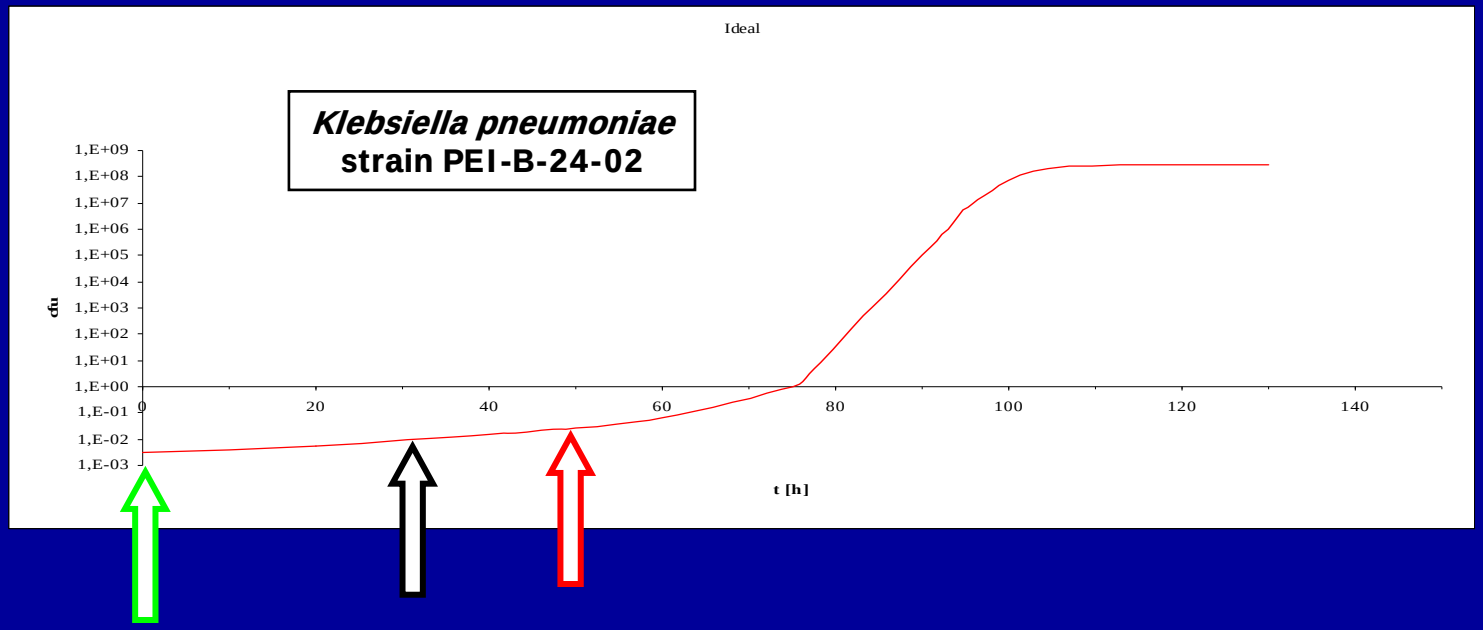
Spiking experiments with *Klebsiella pneumoniae* strain PEI-24-02

(contamination with 10 bacteria per bag corresponding to 0.03 cfu/ml)



delay of multiplication start ("prolonged lag-phase") in dependency on donor properties

Imitation of diagnostic in the clinical case cited



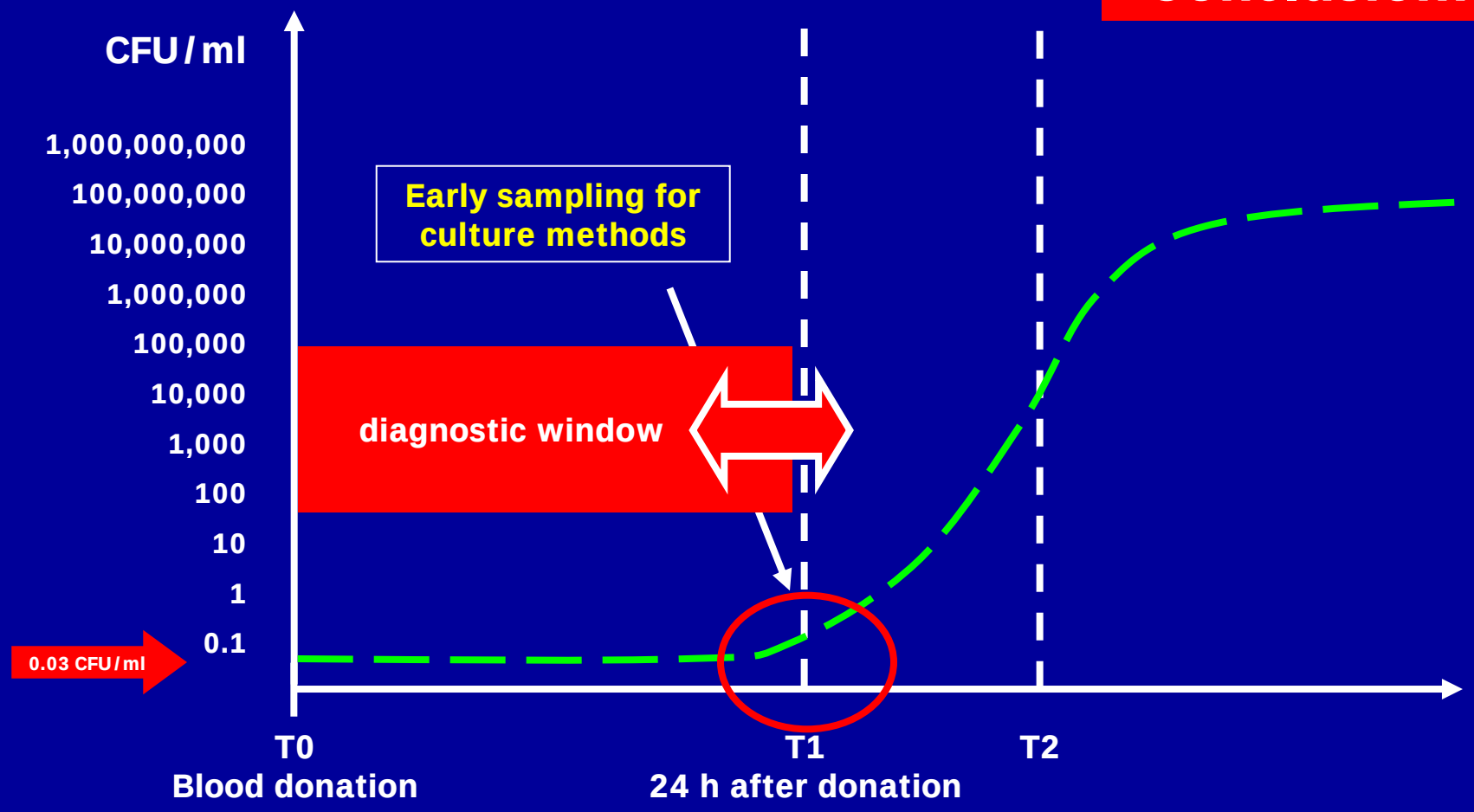
spiking of platelet pools with strain PEI-B-24-02, ~ 0.03 cfu per millilitre

sample drawing after 31 hours and incubation in BacTAlert: **negative**

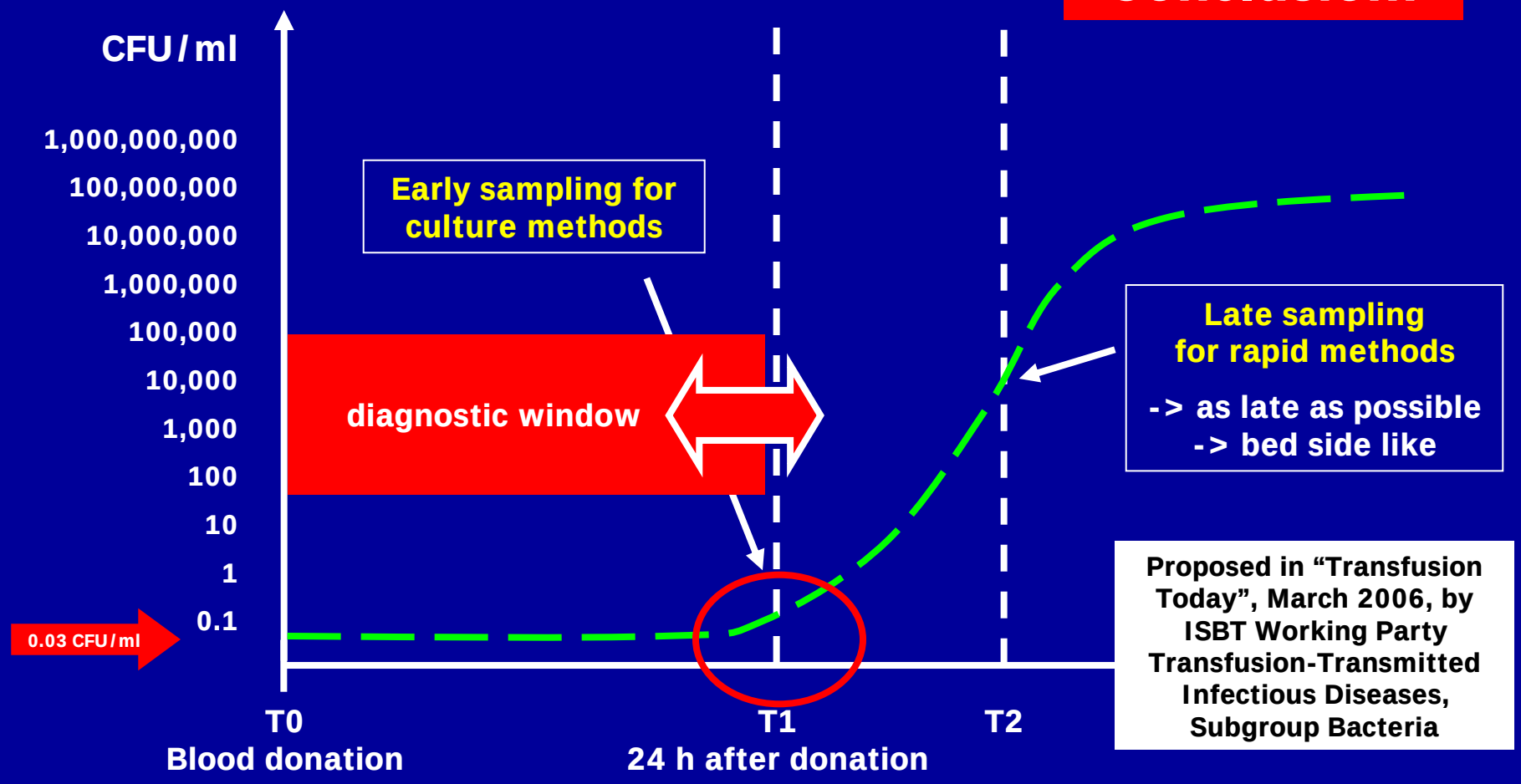
sample drawing after 48 hours and incubation in BacTAlert: **positive**

Two different strategies for platelet bacteria screening

Conclusion:



Two different strategies for platelet bacteria screening



Rapid Methods for bacteria detection in PCs

Flow Cytometry, BD Biosciences Europe

PGD Sytem (Pan Genera Detection)
Verax Biomedical / Abbot

Universal Bacteria PCR / NAT
(still no commercial variant)

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pH kinetic
(feasibility studies)

Rapid Detection of Microbes in PCs using

Flow Cytometry

(bacteria and fungi)

Bacteria screening of platelet concentrates by Flow Cytometry

1. Mixing of 50 µl sample with 450 µl TO-buffer

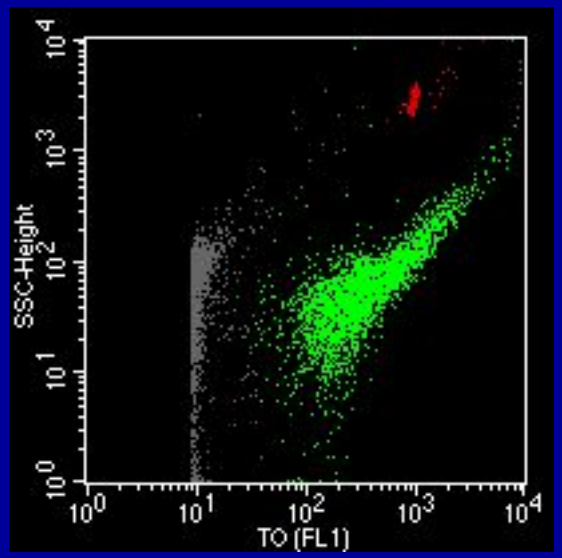
2. Incubation for 5 minutes at room temperature

3. Measuring for approximately 30 seconds

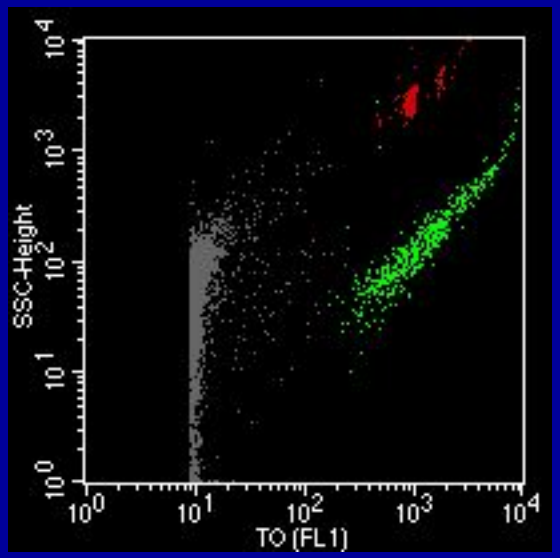
result within 10 minutes

Bacteria grown in Platelet Bags

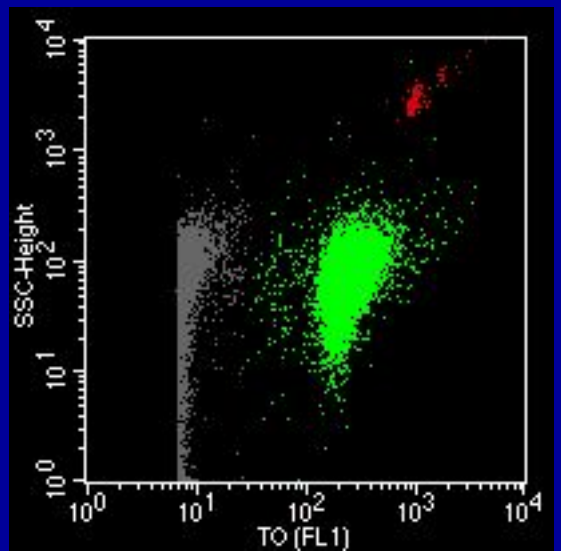
St. aureus



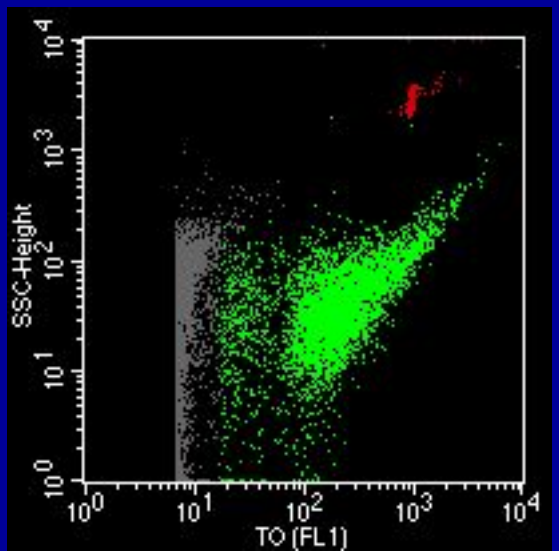
St. epidermidis



B. cereus



K. pneumoniae



Legend

Red:

Absolute Count Beads

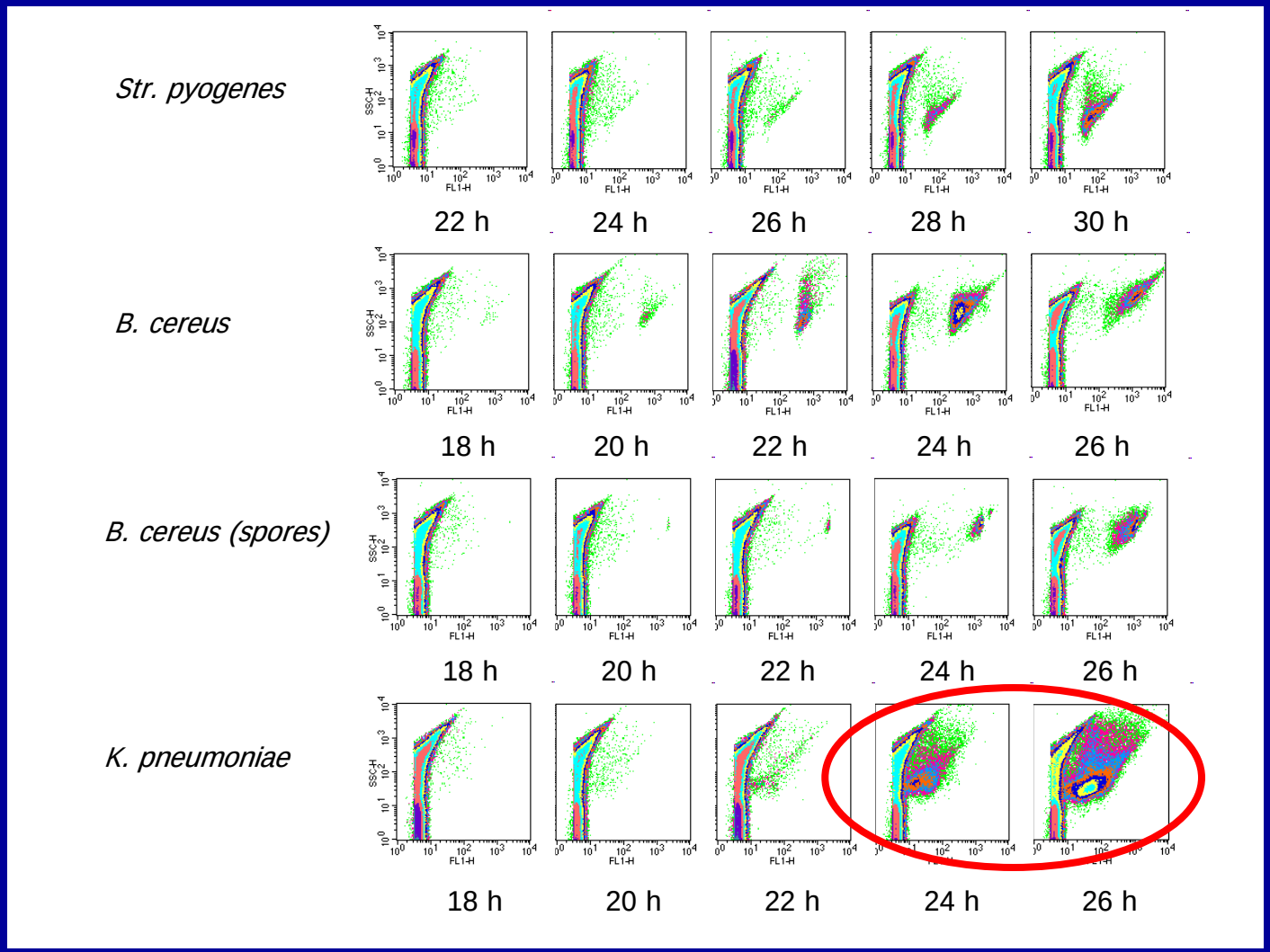
Green:

Stained Bacteria

Grey:

Platelet Debris

Bacteria detection by flow cytometry –
kinetics of different bacteria species
(spiking with 0.03 CFU/mL)



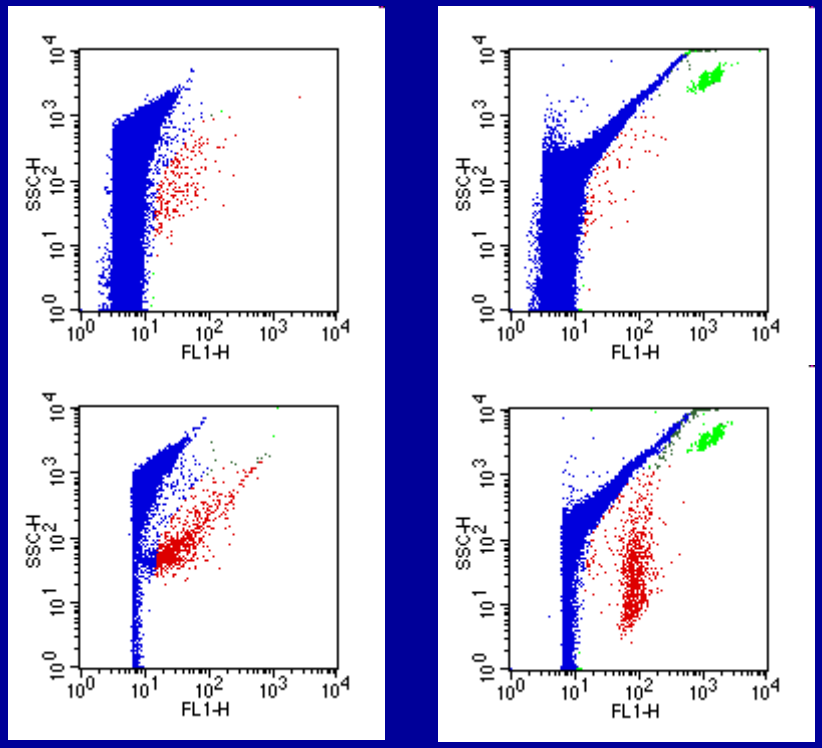
Karo et al. Clinical Chemistry Laboratory Medicine 2008; 46(7): 947-953

Bacteria detection by flow cytometry –
comparison between one-step and two-step method

one-step method | two-step method

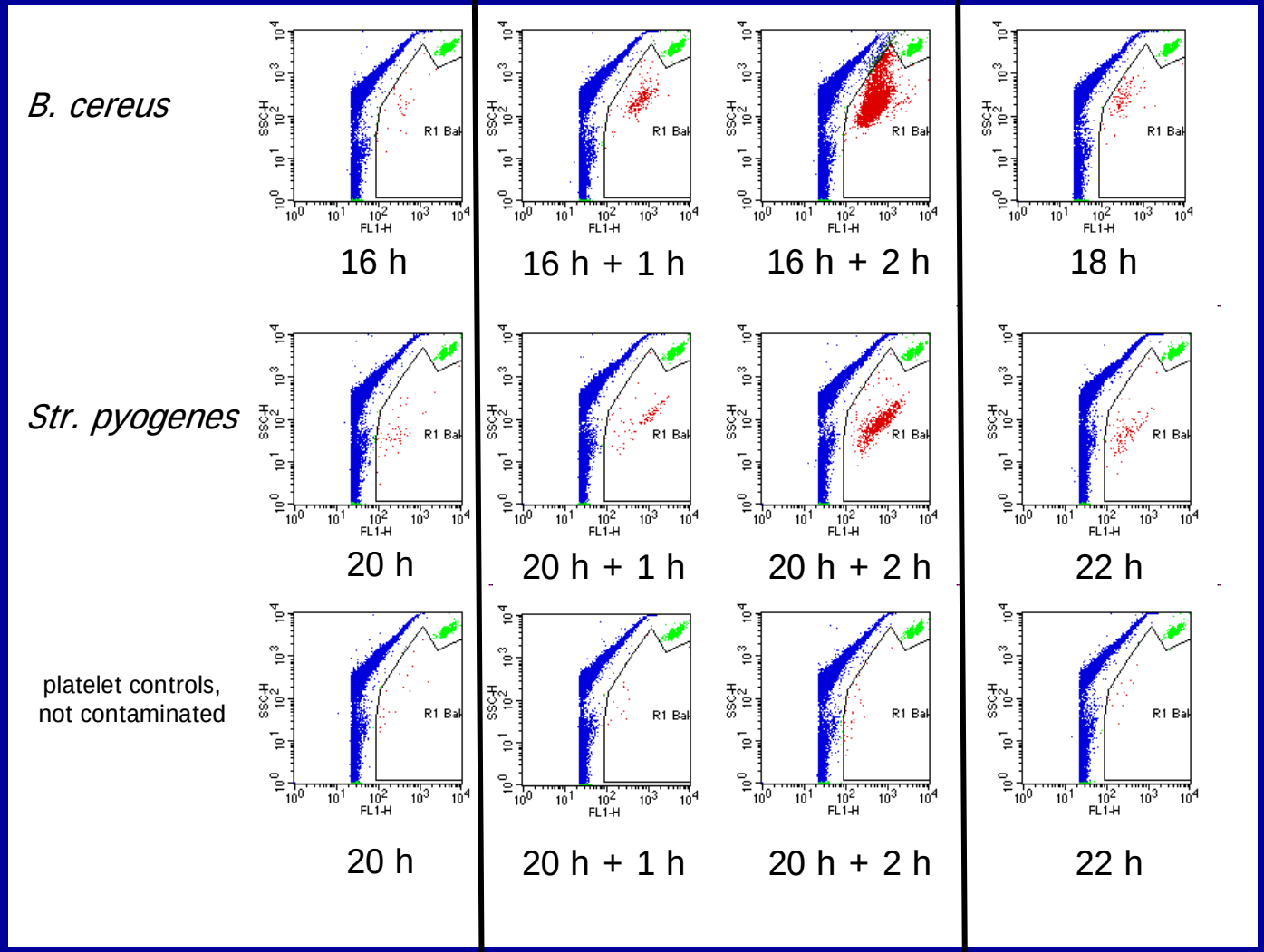
platelet control

Klebsiella pneumoniae



Two-step method: result within 20 minutes

Bacteria detection by flow cytometry – improvement of sensitivity by pre-incubation



Increase in sensitivity: ~ 1 log step per hour of incubation at 37°C

Rapid Bacteria Detection using Flow Cytometry

-> detects bacteria and fungi

-> feasible approach

-> sensitivity 10^3 to 10^4 CFU per mL
(can be improved by pre-incubation)

-> result after 20 minutes

Rapid Bacteria Detection using Pan Genera Detection System

(no own experiences, study in preparation)

-> detects a broad spectre of bacteria

-> feasible approach

-> sensitivity 10^3 to 10^5 CFU per mL

-> result after ~ 1 hour

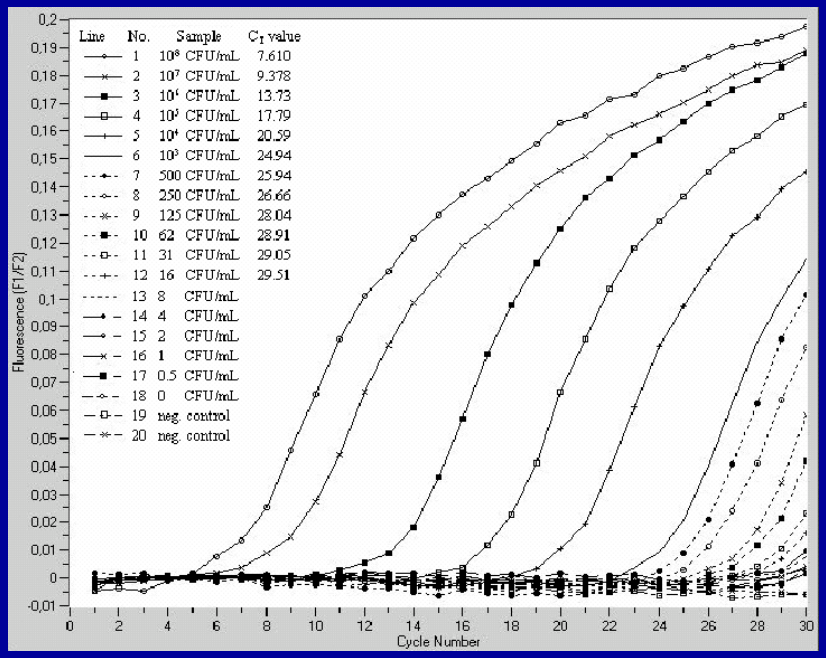
Rapid Detection of Microbes using Real Time Universal Bacteria (Fungi) PCR/NAT

Bacteria detection in platelets by RT-PCR (23S rRNA)

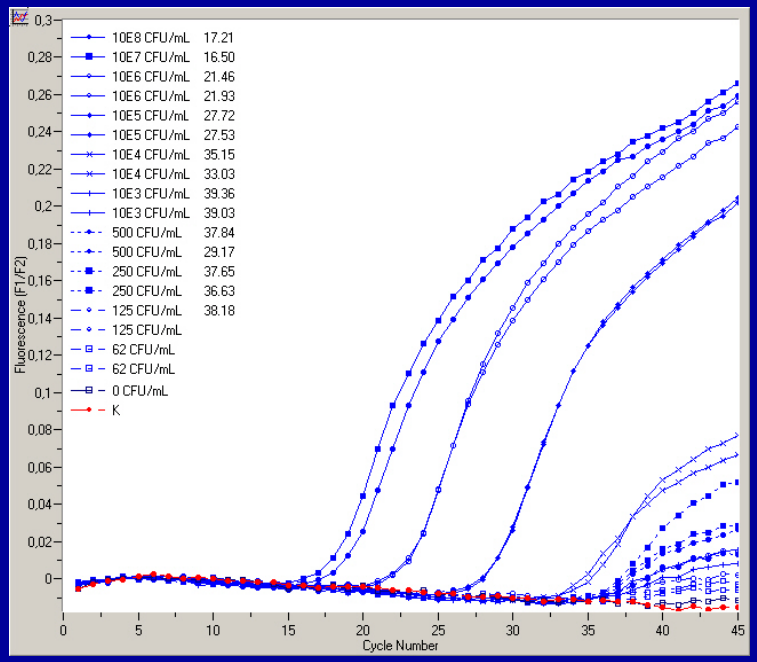
Sensitivity in PCs ~ 10^2 to 10^3 CFU / mL

	One Enzyme System		Two Enzyme System	
Target	CFU/mL	pg RNA/mL	CFU/mL	pg RNA/mL
23S rRNA	500	2	16	0,6
***gro*EL mRNA**	$1 * 10^4$	20	125	2,5

23S rRNA (two enzyme system)



***gro*EL mRNA (two enzyme system**



Data of Melanie Störmer, PEI

Rapid Detection of Microbes using Real Time Universal Bacteria (Fungi) PCR/NAT

-> still no commercial variant
(several in-house methods)

-> feasible approach
(approved in several groups, e.g. Sanquin/The Netherlands, University of Bochum/Germany, University of Frankfurt/Germany, PEI, Linz/Austria ...)

-> sensitivity 10^1 to 10^3 CFU/mL

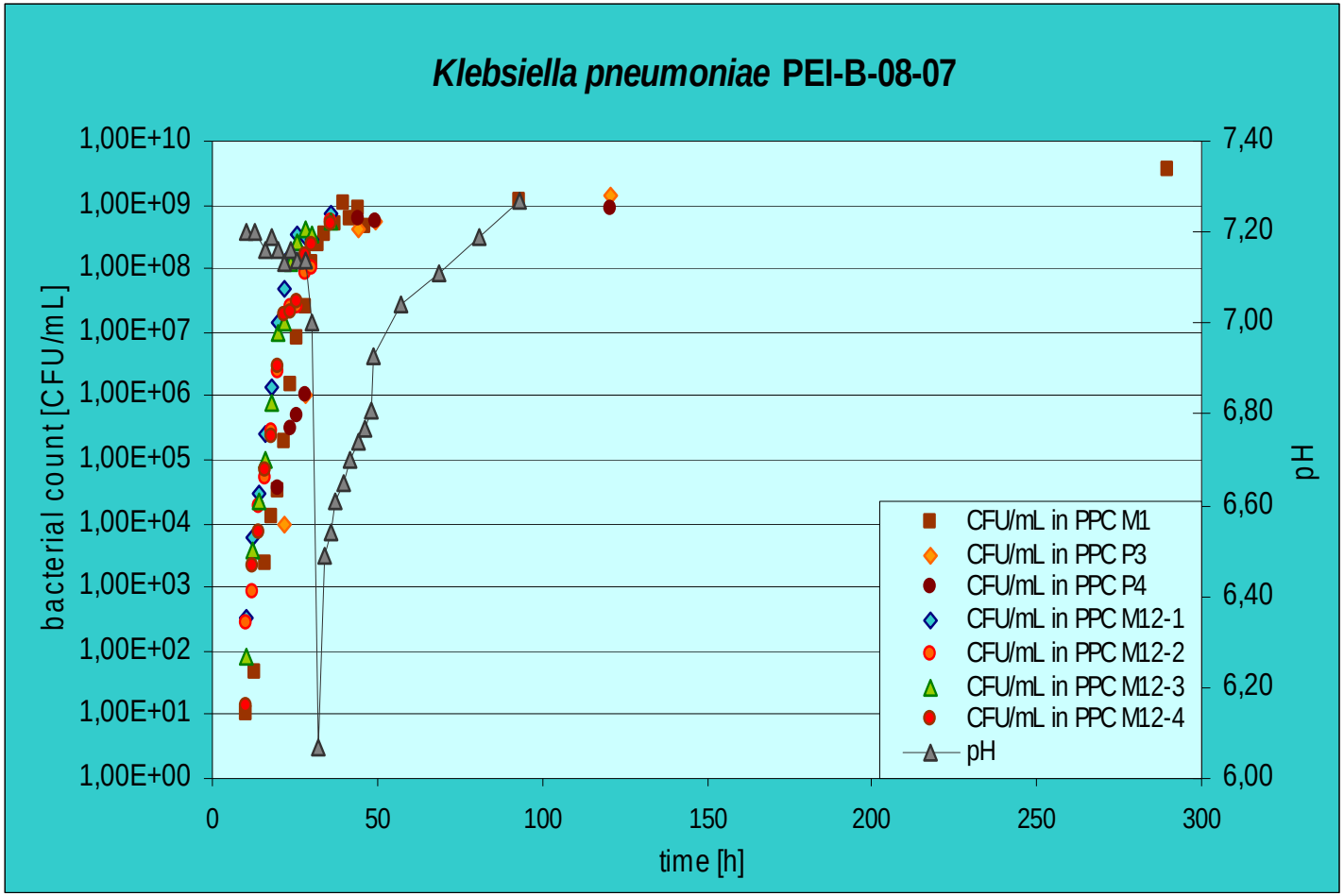
-> results after ~ 4 hours, no real rapid method
(as Real Time RT-PCR, chip technology?)

Detection of microbial contamination in PCs by continuous pH monitoring ?

In-line-pH-measuring by BCSI Device

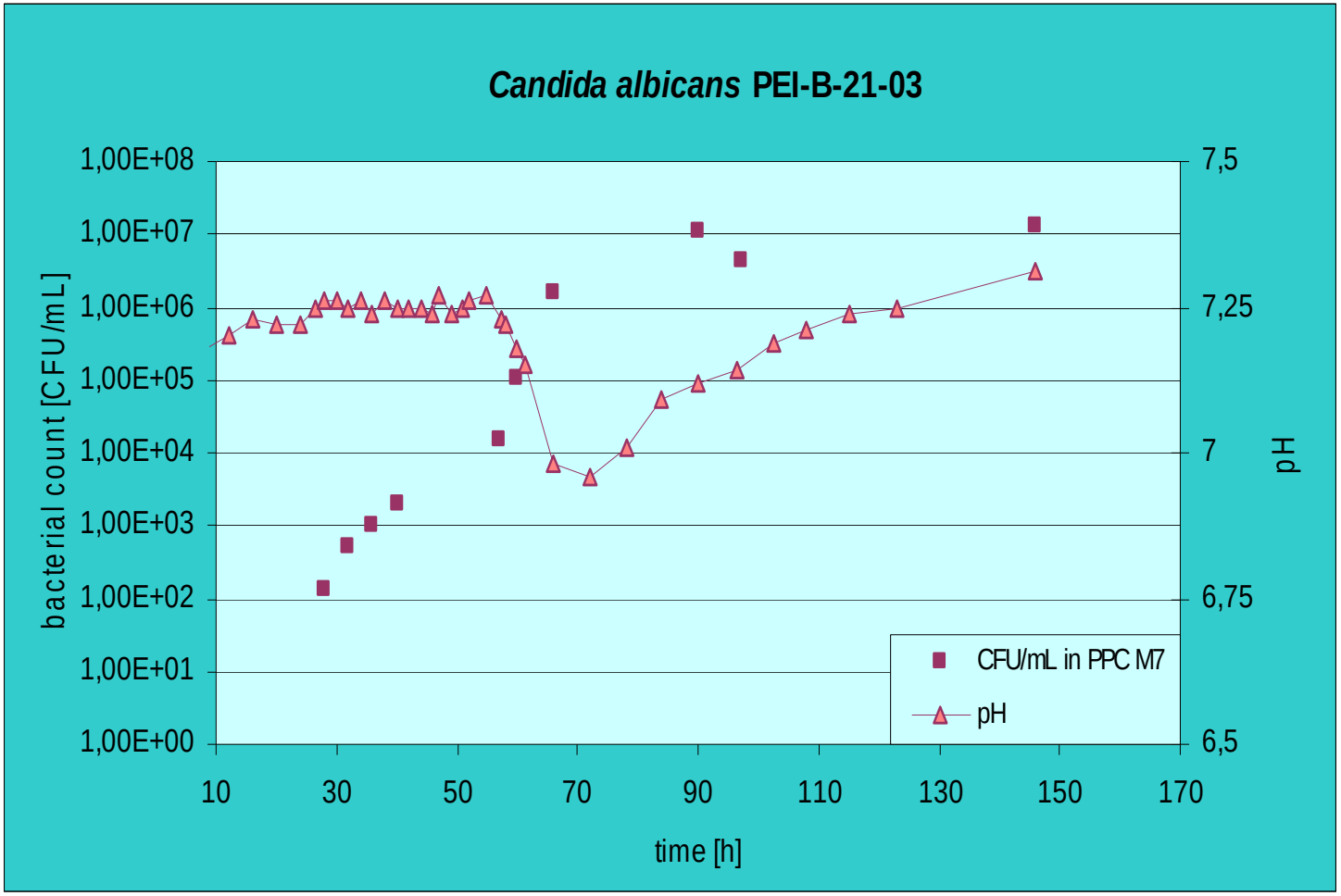
Kinetic of bacteria growth and pH after artificial contamination of PPC

(~ 10 bacteria per bag corresponding to 0.03 CFU/mL)



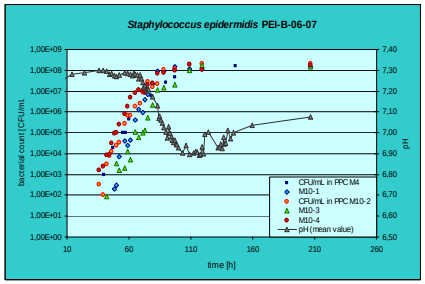
Kinetic of fungal growth and pH after artificial contamination of PPC

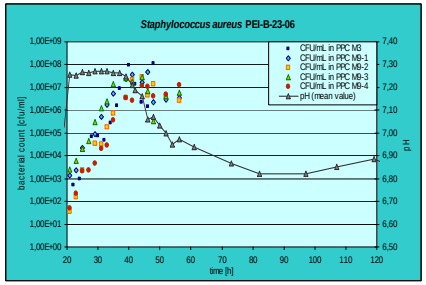
(~ 10 cells per bag corresponding to 0.03 CFU/mL)

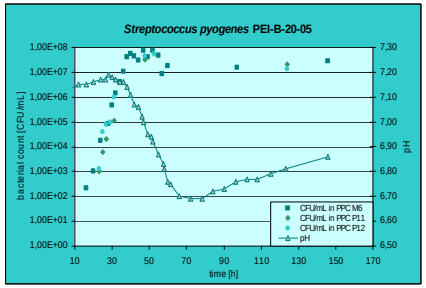


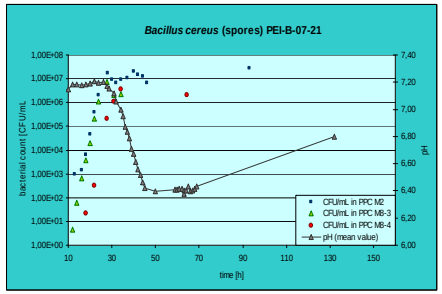
Kinetic of bacteria growth and pH after artificial contamination of PPC

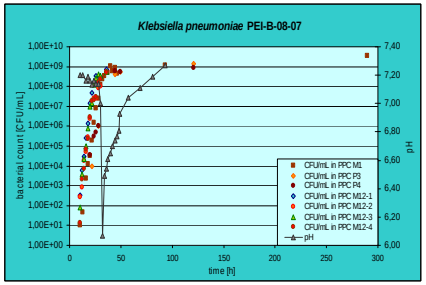
(~ 10 bacteria per bag corresponding to 0.03 CFU/mL)

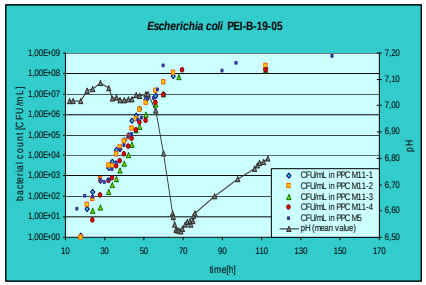


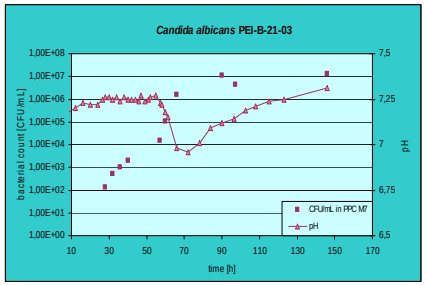


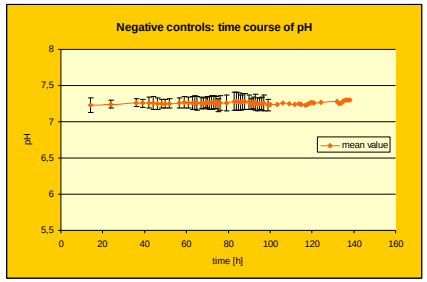












Conclusions

(pH monitoring)

Generally, pH measurement in PCs is not acceptable to be a solitary approach for Platelet Bacteria Screening because of its low sensitivity.

On the other hand, there are time points at which the pH provides a warning signal to prevent transfusion of bacterially contaminated PCs.

Additionally, the BCSI pH 1000 System allows pH measuring immediately before transfusion without opening the bag which gives a clear effort in comparison with pH estimation by strips or dip sticks (as recommended, for instance, by AABB in 2004).

There is a need for further studies (and further development).

Need for clinical studies on rapid bacteria detection methods

BLOOD PRODUCTS ADVISORY COMMITTEE
92nd Meeting, September 10 – 11, 2008
Rockville, MD

FDA Proposal for Conditions of Use of 7 Day Platelets

....

Rapid bacterial detection devices have not been validated in clinical setting and thus use of this device during the clinical trial would validate its clinical sensitivity and possibly justify its use as stand-alone quality control or release test.

.....

Meeting in Amsterdam, The Netherlands, Sanquin (Dirk de Korte) September 4th, 2008:

Sanquin is going to organize a study

-> comparing early and post-storage sampling

-> including rapid bacteria detection methods (Flow Cytometry, PGD, Universal Bacteria PCR/NAT)

Post-storage Rapid Bacteria Detection

→ cannot guarantee sterility of tested PCs and will not prevent transfusion-transmitted bacterial infections

BUT

→ can identify highly contaminated PCs.

→ The latter are leading to septic shock, multi organ failure up to death.

Post-storage Rapid Bacteria Detection in PCs is capable to prevent fatal cases in transfusion medicine.

Thank you !

to my co-workers

to the collaborators

to you for your attention