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Report on the International Validation Study on Bacteria Standards (Transfusion-Relevant Bacterial Strain Panel)

AND

Proposal for a validation study for enlargement of the transfusion-relevant bacterial strain panel"

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Abstract

Bacterial contamination of platelet concentrates (PCs) remains a persistent problem in transfusion. No international transfusion-relevant bacterial strain panel currently exists for investigation of methods used to detect or kill bacteria in blood components. Therefore the International Society of Blood Transfusion (ISBT) Working Party Transfusion-Transmitted Infectious Diseases (WP-TTID), Subgroup Bacteria, organised an international study on Bacteria Standards to be used as a tool for development, validation and comparison of both bacterial screening and pathogen reduction methods.

Four blinded Bacteria Standards (A: *Staphylococcus epidermidis*; B: *Streptococcus pyogenes*; C: *Klebsiella pneumoniae*; D: *Escherichia coli*), deep-frozen and defined in identity and count, were prepared and distributed to 14 laboratories in 10 countries. The partner laboratories were asked to identify the bacterial species, estimate the bacterial count and determine their ability to grow in PCs after low spiking (0.3 and 0.03 CFU/mL), to simulate contamination occurring during blood donation.

Data of identification and enumeration were received from 13 laboratories, data of growth ability from 12 laboratories within the fixed time frame. The Bacteria Standards were correctly identified in 98% (1 case reported as *Staphylococcus delphini* instead of the closely related *S. epidermidis*). *S. pyogenes* and *E. coli* grew in PCs in 11 out of 12 laboratories (92.3%), *K. pneumoniae* and *S. epidermidis* replicated in all participating laboratories (100%).

The results of bacteria counting were very consistent between laboratories: the 95% confidence intervals were for *S. epidermidis:* 1.19-1.32 x 10^7 CFU/ml, *S. pyogenes*: 0.58-0.69 x 10^7 CFU/ml, *K. pneumoniae*: 18.71-20.26 x 10^7 CFU/ml and *E. coli*: 1.78-2.10 x 10^7 CFU/ml. This international study demonstrated the stability of the Bacterial Standards and consistency of results in a large number of transfusion laboratories. The Bacteria Standards can be considered as a suitable tool in validation and assessment of methods for improvement of bacterial safety of blood components. This study is a first step in implementation of a relevant bacterial panel as an international standard. The panel will be enlarged in the near future.

1 Introduction

Since the impressive reduction of transfusion-transmitted virus infections, bacterial infections by blood transfusion are representing the most important infection risk. After confusion of blood components respectively recipients, bacterial contamination is considered to be the second most common cause of death from transfusion (for a review, see de Korte *et al.*). The mortality rates for platelet related sepsis range from 1 in 20,000 to 1 in 100,000 donor exposures (de Korte *et al.*), the contamination rate of platelet concentrates reaches from 0.16% up to 0.6% at the end of their shelf life (for a review, see Walter-Wenke *et al.*).

The fundamental difference between contaminations by viruses on the one hand, and bacteria on the other hand is that the latter can replicate strongly in a platelet concentrate during its shelf life. Under the usual storage conditions, (22.5°C), microorganisms contaminating a platelet concentrate can grow up to 10^{10} Colony Forming Units (CFU) and more per bag. In addition to bacterial cells themselves, endotoxines and/or exotoxines are often contained in the bags, depending on bacterial species and strain. Transfusion of a highly contaminated blood component will as a rule lead to immediate septic shock, and, not rarely, to death of the patient. Assigning the contaminated bacterium to pathogenic, non-pathogenic or facultative pathogenic species, as defined in the criteria of clinical microbiology, is only of secondary importance in this context. It has to be pointed out that also usually apathogenic bacteria can cause life-threatening infections in the recipient after transfusion.

Essential instruments suitable for preventing bacterial contamination of blood components include careful donor selection, selection of the punction site, effective skin disinfection, separation of the first volume from the blood donation (pre-donation sampling, also called diversion), and the consistent monitoring of the bag systems including monitoring of additional connections to the bag by the so called sterile connecting device (SCD).

To increase bacterial safety of cellular blood components, there is a choice between two fundamentally different approaches: firstly, so-called pathogen reduction and, secondly, bacteria screening. In order to validate and to assess these methods, it is crucial to apply suitable bacterial strains which are able to proliferate in blood components.

Since those blood relevant bacteria are hitherto not available, bacterial strains have been selected and characterised regarding their capability to multiply in platelet concentrates up to a count of 10^8 to 10^9 CFU/mL applying conditions as used in transfusion medicine routine. This property of the bacterial strains has been confirmed in platelets from at least 100 different donors in order to exclude antimicrobial effects of donor's host defence.

Additionally, a novel procedure has been developed to manufacture these bacterial strains as deep frozen suspensions consisting of living microbial cells only. The bacteria suspensions also referred to as Bacteria Standards are defined in identity and count. They are stable over at least one year and shippable on dry ice. After thawing, the bacteria are ready to use and can be applied immediately for spiking of blood components. The novel manufacturing procedure enables artificial contamination of blood components following "real life" conditions as occurring usually during blood donation, i.e. spiking with 10 CFU per bag corresponding to 0.03 CFU/mL.

Four of those Bacteria Standards have been implemented in the study. It is intended to enlarge the panel in the future, i.e. a meaningful list of representatives of bacteria groups (e.g.

aerobic/anaerobic, Gram-positive/Gram-negative, spore-forming bacteria, slow-growers etc.) should be elaborated.

2 Materials

The panel members are bacterial strains selected for their ability to replicate in PCs under routine storage conditions used in transfusion medicine. The panel members are prepared using a specially developed procedure which guarantees defined bacterial suspensions (deep frozen, ready to use, stable, shippable, defined in count of living cells). The panel is designed to allow objective validation of methods for Bacterial Screening as well as technologies for Pathogen Reduction in PCs under "real life" conditions, i.e. inoculating the PCs with a very low bacteria count (0.03 to 0.3 CFU/mL) followed by growth in the matrix. Until now, there have been no transfusion relevant bacterial reference strains available.

The strains had been isolated from transfusion-transmitted bacterial infections (blood bag and/or recipient). Thereafter, the strains were characterized for their ability to grow in PCs under normal blood bank conditions (original bag volume, storage under agitation, temperature controlled). In order to exclude potential influences of immune system of the blood donors, growth control has been performed in PCs from at least 100 different donors.

2.1 Samples

Sample A
Staphylococcus epidermidis
Code: PEI-B-06-08

Sample B
Streptococcus pyogenes
Code: PEI-B-20-06

Sample C
Klebsiella pneumoniae
Code: PEI-B-08-10

Sample D
Escherichia coli
Code: PEI-B-19-06

2.2 Stability Testing

Enumeration and stability testing was performed 24 hours after production/deep freezing and, additionally, during operating time of the participants at the time points up to 140-181 days post production. For the stability testing three vials of each Bacteria Standard were defrosted and two dilutions serious of each vial were produced. Samples were transferred directly from deep freezer to a dry incubator at 37ºC for 10 minutes. If ice crystals were still evident, the vial was warmed in the hand until the content had melted. The stock suspensions were used immediately after thawing. Plating assays were carried out (n = 6) of one defined dilution of both dilution series (in total 36 plates per Bacteria Standard). Thereafter, mean values were calculated.

Figure 1 shows the results of the stability testing of the four species, performed at different times after production during the operating time of the study. Furthermore, Figure 2 demonstrates the

stability data of other lots of the same bacterial strains (Bacteria Standards) produced in the manner as the preparations used in the validation study. Thus stability could be stated over a longer time period.

2.3. Batch-to-batch consistency

The identity of new lots of the respective Bacteria Standard is guaranteed by a combination of classical and molecular microbiological procedures. Classical characteristics used are growth properties, colony morphology, Gram-staining, and biochemical parameters like metabolising of certain sugars. Additionally, the DNA coding for 16s ribosomal RNA is sequenced in routine. As the final proof of identity, a Random Primed PCR Fingerprinting is performed. For illustration, please see Addendum 1 of this report (Prototype Certificate).

3 Design of study

Four different blinded Bacteria Standards were sent to the participating laboratories as shown in Figure 3. They were asked to identify the bacterial species as well as the count of microbes of the given standard. Additionally, the ability of the bacterial strains to reach high counts in PCs shall be examined when spiked with very low numbers of bacteria (0.03 CFU/mL and 0.3 CFU/mL). Each bacterial strain is provided with the characteristics information.

Blinded vials of the four Bacteria Standards (10 identical vials each of *Staphylococcus epidermidis* (A), *Streptococcus pyogenes* (B), *Klebsiella pneumoniae* (C) and, *Escherichia coli* (D); 7 of them were needed, the residual ones served as reserve) were sent on dry ice to the participating laboratories. They are asked to perform the following experiments as shown in Figure 3:
a) to cultivate the strains and to identify the respective bacterial species.
b) to estimate the bacterial count of each Bacteria Standard in 5 independent replicates.
c) to spike at least 2 platelet bags (if possible: 2 apheresis PCs and 2 whole blood derived PCs): one with ~ 10 CFU per bag corresponding to 0.03 CFU/mL and one with ~ 100 CFU per bag. Thereafter, count after 4 days (96 hours after inoculation) storage of the PCs applying usual blood bank conditions to estimate the microbial count.

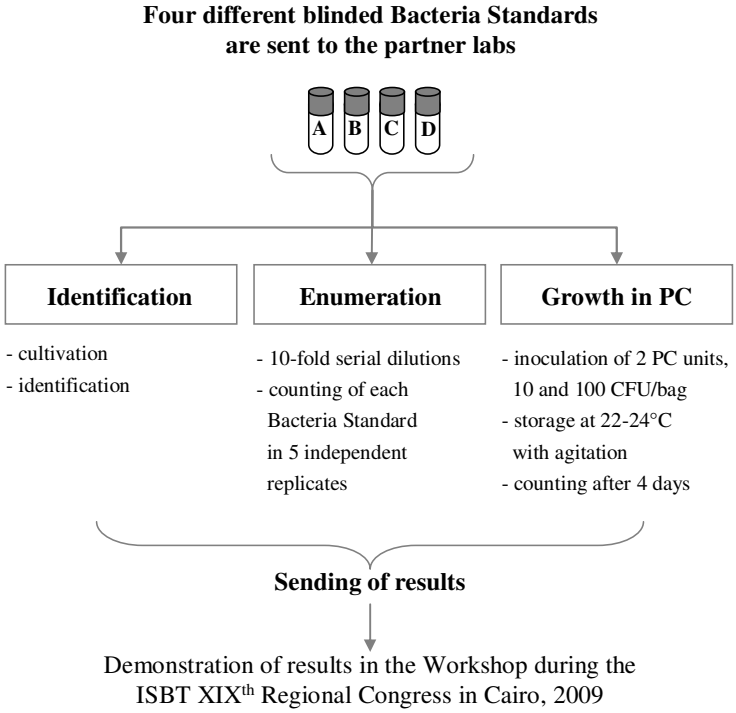

Figure 3 Principle and design of International Validation Study on Bacteria Standards (for more details, see Addendum 2: Protocols)

3.1 Participants

No.	Country	Facility	Partner
1	Austria	Austrian Red Cross, Blutzentrale Linz	Christian Gabriel
2	Canada	Canadian Blood Service, Ottawa	Sandra Ramirez
3	Germany	German Red Cross, Frankfurt/Main	Michael Schmidt
4	Germany	Paul-Ehrlich-Institute, Microbial Safety, Langen	Thomas Montag Melanie Störmer
5	Germany	German Red Cross, Springe	Thomas Mueller Bernd Lambrecht
6	China	Hong Kong Red Cross Blood Transfusion Service	Kong-Yong Lee
7	México	Centro Nacional de la Transfusion Sanguínea	Julieta Rojo Antonio Arroyo
8	The Netherlands	Sanquin Blood Supply Foundation, Amsterdam Academic Medical Center, Amsterdam	Dirk de Korte Jan Marcelis
9	Poland	Regional Centre for Transfusion Medicine, Bialystok	Piotr Radziwon
10	United Kingdom	NHS Blood and Transplant, London	Carl McDonald Siobhan McGuane
11	USA	Walter Reed Army Medical Center, Washington DC	David G. Heath Hector Carrero
12	USA	CaridianBCT Biotechnologies, Denver	Ray Goodrich Shawn Keil
13	USA	Louis Stokes Veterans Administration Medical Center, Ohio Case Western Reserve University, Ohio	Roslyn Yomtovian Michael R. Jacobs
14	South Africa	South African National Blood Service	Tshilidzi Muthivhi

3.2 Assay methods

For all methods used, detailed instructions were given in the "Protocols" (see Addendum 2) which have been provided together with the samples. In the following, the principles of methods will be described in brief.

Identification:

The participants were asked to cultivate and to identify the blinded samples A, B, C and D following their routine protocols as used in the respective microbiological lab (for details see Addendum 2, Protocols).

Enumeration of bacteria:

Enumeration of blinded samples was performed in 5 independent replicates each. For each experiment, tenfold dilutions of the samples were performed. Thereafter, the spread plate method was used, i.e. 100 µl each of each dilution were distributed onto 5 solid agar plates followed by incubation of the agar plates for one to two days at 37°C. Finally, the bacterial colonies were counted and the results were noted in the raw data sheets. Furthermore, the bacteria count in the original sample was calculated using a formula provided by the study board (for details see Addendum 2, Protocols).

Contamination of platelet concentrates

Based on the results obtained in "Enumeration of bacteria" (see above), at least four routine platelet concentrates (either pooled platelet concentrates, PPC, or apheresis platelet concentrates, APC) per Bacteria Standard were artificially contaminated with very low bacterial count. Following a defined procedure, two of the platelet concentrates were spiked with approximately 100 bacteria per unit (corresponding to 0.3 CFU/mL) and the other two platelet concentrates were spiked with approximately 10 bacteria per unit (corresponding to 0.03 CFU/mL). Thereafter, the platelets were stored under routine conditions (22.5°C, agitation) for 96 hours followed by sample drawing and enumeration of the bacterial count (for details see Addendum 2, Protocols).

3.3 Statistical methods

Statistical analysis was performed at PEI based on the raw data sent by the participants. The data were read from the results sheets as recorded by each participant (see Addendum 2, Protocols).

Comparison between laboratories was performed by means of a mixed linear model with fixed factor *laboratory* and random factor *vial*. Confidence intervals for the estimated differences between each participant and PEI as well as p-values were adjusted using the Bonferroni method in order to restrict the overall type I error α (false positive results i.e. false significant differences) to 5% (for description of the Bonferroni method see Abdi).

4 Results

4.1 Data received

The results of the study have been discussed during the ISBT Regional Meeting in Cairo, Egypt, March 21st, 2009. All data received up to February 28th, 2009, have been considered for calculation (for raw data sheets, see Addendum 2, Protocols).

Unfortunately, Dr. Tshilidzi Muthivhi, South African National Blood Service, Cape Town, South Africa did not receive the samples in time (both permission by the government and transfer through the customs postponed). Therefore, his data could not be considered in the study results.

4.2 Results

4.2.1 Sample A

Identification:
Sample A was identified in 12 of the participating laboratories as *Staphylococcus epidermidis*. One participant identified the sample as *Staphylococcus delphinii* (see discussion below).

Counting [CFU/mL]:
Median of all participants: 1.13 Exp+07
Mean of all participants: 1.25 Exp+07
95% Confidence Interval: 1.19 Exp+07 to 1.32 Exp+07

Growth in platelet concentrates:
Sample A (*Staphylococcus epidermidis*) grew in platelet concentrates in all participating laboratories after spiking with both 0.3 CFU/mL and 0.03 CFU/mL (Figure 4)

4.2.2 Sample B

Identification:
Sample B was identified in all participating laboratories as *Streptococcus pyogenes*.

Counting [CFU/mL]:
Median of all participants: 0.50 Exp+07
Mean of all participants: 0.63 Exp+07
95% Confidence Interval: 0.58 Exp+07 to 0.69 Exp+07

Growth in platelet concentrates:
Sample B (*Streptococcus pyogenes*) grew in platelet concentrates in 12 out of 13 participating laboratories after spiking with 0.3 CFU/mL (Figure 5). Furthermore, the strains grew in platelets in 11 out of 13 participating laboratories after spiking with 0.03 CFU/mL (see discussion below).

4.2.3 Sample C

Identification:
Sample C was identified in all participating laboratories as *Klebsiella pneumoniae*.

Counting [CFU/mL]:
Median of all participants: 19.45 Exp+07
Mean of all participants: 19.49 Exp+07
95% Confidence Interval: 18.71 Exp+07 to 20.26 Exp+07

Growth in platelet concentrates:
Sample C (*Klebsiella pneumoniae*) grew in platelet concentrates in all participating laboratories after spiking with both 0.3 CFU/ml and 0.03 CFU/mL (Figure 6, for details see Addendum 2, Protocols).

4.2.4 Sample D

Identification:
Sample D was identified in all participating laboratories as *Escherichia coli*.

Counting [CFU/mL]:
Median of all participants: 1.71 Exp+07
Mean of all participants: 1.94 Exp+07
95% Confidence Interval: 1.78 Exp+07 to 2.10 Exp+07

Growth in platelet concentrates:
Sample D (*Escherichia coli*) grew in platelet concentrates in 12 out of 13 participating laboratories after spiking with 0.3 CFU/mL. Furthermore, the strains grew in platelets in 11 out of 13 participating laboratories after spiking with 0.03 CFU/mL (Figure 7, see discussion below).

4.3 Discussion

Two of the Bacteria Standards (*Staphylococcus epidermidis and Klebsiella pneumoniae*) replicated in platelet concentrates in all participating laboratories (100%). The samples B (*Streptococcus pyogenes*) and D (*Escherichia coli*) grew in 12 out of 13 participating laboratories (92.3%). The most likely interpretation of these failures is the existence of specific antibodies towards the bacterial strain in the blood donor population. The latter could prevent bacterial growth and/or kill the microorganisms. The same interpretation is offered in the two cases in which only the higher spike (100 bacteria per platelet bag corresponding to 0.3 CFU/mL) led to bacterial growth (for details, see figures 1 to 4 at the end of the report). All in all, the ability of the Bacteria Standards to grow up to high counts in platelet concentrates obtained from donors in different regions of the world should be considered as to be confirmed.

The results of bacteria counting of all participants are well homogenous since a divergence below factor 2 represents an acceptable value in estimation of high particle counts.

Sample A has been identified by one of the participants as *Staphylococcus delphinii* instead of *Staphylococcus epidermidis*, but at least as so called Coagulase-negative Staphylococcus (CNS), the identification failure is most likely due the commercial identification kit used in this lab. Those misinterpretations are not unusual in microbiological routine diagnostic. Sequencing of bacterial 16S rRNA (as had been done prior to the study in the PEI, see Addendum 1, Prototype Certificate) represents the state of the art in determination of bacteria species. Thus, identification of sample A as *Staphylococcus epidermidis* is proven.

5 Conclusions and proposals

The Bacteria Standards for the control of platelet concentrates contamination should be considered as a suitable tool in validation and assessment of methods for improvement of

bacterial safety of blood components. In the study, both homogeneity and stability could be demonstrated. Additionally, the general ability of the chosen strains to grow up to high counts in platelet concentrates under routine conditions in transfusion medicine could be demonstrated as well. It is proposed to implement the blood relevant bacterial panel as WHO Standards.

6 References

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Abdi, H:
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7. Tables

Table 1: Results of participating laboratories (mean and confidence interval)

Participant	BBS A *Staphylococcus epidermidis*			BBS B *Streptococcus pyogenes*			BBS C *Klebsiella pneumoniae*			BBS D *Escherichia coli*		
	Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI	
Amsterdam	1.14	0.90	1.37	1.20	1.03	1.38	17.08	14.46	19.70	1.20	0.52	1.88
Austria	1.17	0.94	1.41	0.71	0.53	0.88	21.71	19.09	24.33	1.13	0.46	1.81
Canada	1.24	1.00	1.47	0.76	0.59	0.94	9.01	6.39	11.63	1.60	0.93	2.28
Germany Frankfurt	0.88	0.65	1.12	0.24	0.07	0.42	9.54	6.92	12.16	1.75	1.07	2.42
Hongkong	1.53	1.29	1.76	0.71	0.54	0.89	22.70	20.08	25.32	0.94	0.26	1.61
UK	1.17	0.93	1.40	0.53	0.36	0.71	25.26	22.64	27.88	1.48	0.80	2.15
Mexico	1.22	0.99	1.46	0.60	0.43	0.78	14.70	12.08	17.32	1.73	1.05	2.40
Germany PEI	1.69	1.46	1.93	0.77	0.60	0.95	20.37	17.75	22.99	2.32	1.64	2.99
Poland	1.74	1.50	1.97	0.84	0.67	1.02	31.01	28.39	33.63	2.41	1.73	3.08
Germany Springe	0.95	0.72	1.19	0.55	0.37	0.72	24.56	21.94	27.18	3.03	2.36	3.71
USA Denver	1.30	1.06	1.53	0.59	0.42	0.77	22.05	19.44	24.67	1.02	0.34	1.69
USA Ohio	0.80	0.57	1.04	0.32	0.15	0.50	18.32	15.71	20.94	2.05	1.38	2.73
USA US Army	1.48	1.25	1.72	0.36	0.19	0.54	17.03	14.41	19.65	4.58	3.90	5.26

Mean bacterial count (E+07 CFU/mL); least square estimators derived from a mixed linear model and 95%-confidence limits (CI).

Table 2: Results of participating laboratories (mean standard deviation and coefficient of variation)

Participant	No. Vials	BBS A *Staphylococcus epidermidis*			BBS B *Streptococcus pyogenes*			BBS C *Klebsiella pneumoniae*			BBS D *Escherichia coli*		
		Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
Amsterdam	5	1.14	0.12	10.4	1.20	0.19	15.5	17.08	1.16	6.8	17.08	1.16	6.8
Austria	5	1.18	0.14	12.2	0.71	0.17	23.3	21.71	1.78	8.2	21.71	1.78	8.2
Canada	5	1.24	0.31	24.7	0.76	0.31	40.6	9.01	1.74	19.3	9.01	1.74	19.3
Germany Frankfurt	5	0.88	0.07	7.7	0.24	0.05	19.1	9.54	2.18	22.8	9.54	2.18	22.8
Hongkong	5	1.53	0.16	10.7	0.71	0.20	28.5	22.70	4.54	20.0	22.70	4.54	20.0
UK	5	1.17	0.19	15.9	0.53	0.08	14.6	25.26	3.18	12.6	25.26	3.18	12.6
Mexico	5	1.22	0.40	32.7	0.60	0.22	36.4	14.70	6.43	43.7	14.70	6.43	43.7
Germany PEI	5	1.69	0.44	26.2	0.77	0.15	19.6	20.37	1.92	9.4	20.37	1.92	9.4
Poland	5	1.74	0.42	24.1	0.84	0.07	8.6	31.01	1.64	5.3	31.01	1.64	5.3
Germany Springe	5	0.95	0.12	12.3	0.55	0.11	20.9	24.56	3.22	13.1	24.56	3.22	13.1
USA Denver	5	1.30	0.12	9.1	0.59	0.21	34.8	22.05	1.48	6.7	22.05	1.48	6.7
USA Ohio	5	0.80	0.07	8.5	0.32	0.07	22.8	18.32	1.39	7.6	18.32	1.39	7.6
USA US Army	5	1.48	0.42	28.1	0.36	0.08	21.2	17.03	3.12	18.3	17.03	3.12	18.3

Mean bacterial count (E+07 CFU/mL), standard deviation and coefficient of variation (CV).

Table 3: Overview: mean values of participants

BBS	Median	Mean	95% Confidence Interval	
A, *Staphylococcus epidermidis*	1.13	1.25	1.19	1.32
B, *Streptococcus pyogenes*	0.50	0.63	0.58	0.69
C, *Klebsiella pneumoniae*	19.45	19.49	18.71	20.26
D, *Escherichia coli*	1.71	1.94	1.78	2.10

Mean bacterial count (E+07 CFU/mL)

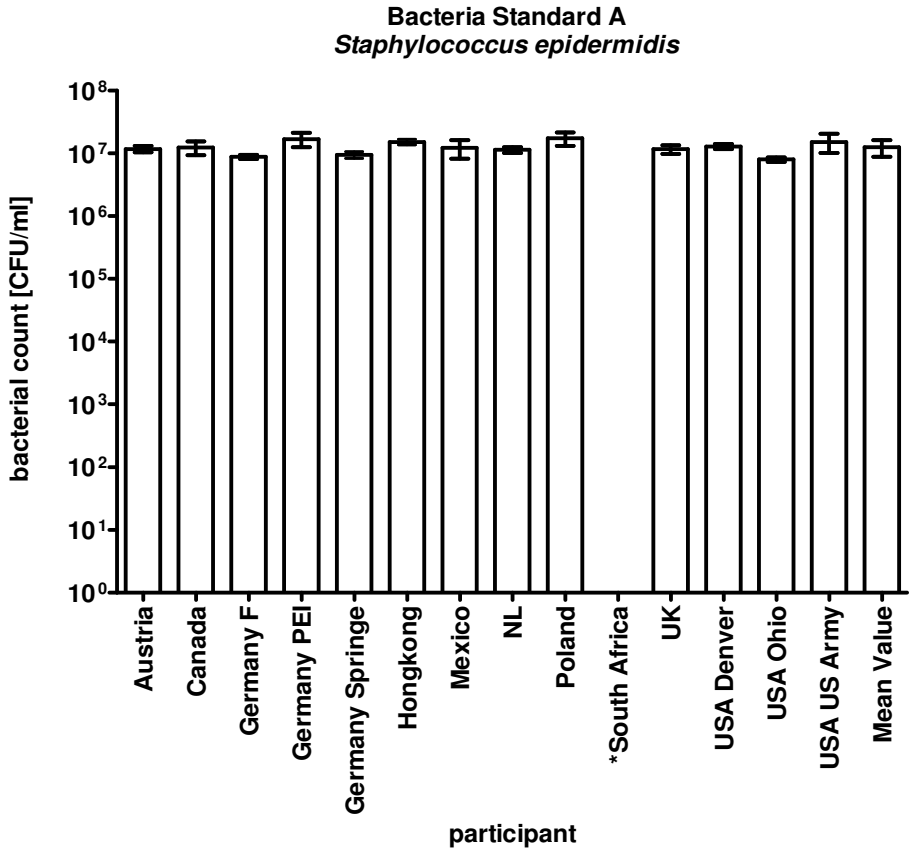


Table 4: Sample A: ***Staphylococcus epidermidis***

Raw data, mean, standard deviation (SD) and coefficient of variation (CV)

Participant	Vial	#1	#2	#3	#4	#5	N	Mean	SD	CV
Amsterdam	501	1.09	1.17	0.80	1.18	1.26	5	1.10	0.18	16.4
	502	0.93	0.80	0.76	1.26	1.16	5	0.98	0.22	22.4
	503	1.02	1.40	1.02	2.17	0.94	5	1.31	0.51	38.9
	504	1.14	1.33	0.78	1.48	1.04	5	1.15	0.27	23.5
	505	0.79	1.10	1.76	0.97	1.07	5	1.14	0.37	32.5
Austria	536	0.98	1.55	1.66	1.68	1.23	5	1.42	0.31	21.8
	537	1.73	0.74	0.70	1.07	1.00	5	1.05	0.41	39.0
	538	0.67	1.19	1.27	1.04	1.43	5	1.12	0.29	25.9
	539	0.83	2.04	0.75	1.33	0.88	5	1.17	0.54	46.2
	540	1.27	0.50	0.66	0.72	2.45	5	1.12	0.80	71.4
Canada	541	0.98	0.97	0.99	1.01	1.29	5	1.05	0.14	13.3
	542	0.98	1.01	1.13	1.51	1.05	5	1.14	0.22	19.3
	543	2.94	1.39	1.65	1.63	1.27	5	1.78	0.67	37.6
	544	1.06	1.13	1.04	1.17	0.96	5	1.07	0.08	7.5
	545	0.69	1.08	1.25	1.62	1.10	5	1.15	0.33	28.7
Germany	506	1.04	1.31	0.96	0.76	0.65	5	0.95	0.26	27.4
Frankfurt	507	0.68	1.44	0.64	0.95	0.75	5	0.89	0.33	37.1
	508	1.04	0.67	0.89	1.03	0.99	5	0.92	0.15	16.3
	509	0.56	1.02	0.54	0.51	1.25	5	0.77	0.34	44.2
	510	0.87	1.42	1.07	0.31	0.71	5	0.88	0.41	46.6

Hongkong	516	0.77	1.33	2.17	1.37	2.38	5	1.60	0.66	41.3
	517	0.99	2.95	1.47	1.69	1.64	5	1.75	0.73	41.7
	518	1.37	1.35	0.95	2.89	1.12	5	1.54	0.78	50.6
	519	1.63	1.53	1.65	1.14	1.21	5	1.43	0.24	16.8
	520	1.04	1.12	1.54	1.77	1.13	5	1.32	0.32	24.2
UK	521	1.15	1.51	1.46	0.72	1.17	5	1.20	0.32	26.7
	522	0.86	1.47	1.16	1.03	0.79	5	1.06	0.27	25.5
	523	0.77	1.28	1.12	0.88	1.67	5	1.14	0.35	30.7
	524	1.13	1.51	1.52	1.47	1.66	5	1.46	0.20	13.7
	525	0.83	1.68	0.95	0.77	0.62	5	0.97	0.41	42.3
Mexico	546	0.81	0.58	0.55	1.35	0.84	5	0.82	0.32	39.0
	547	1.92	1.48	3.54	1.07	1.17	5	1.84	1.01	54.9
	548	0.59	0.94	0.80	0.98	1.79	5	1.02	0.46	45.1
	549	1.00	2.56	1.39	1.60	0.35	5	1.38	0.81	58.7
	550	1.23	1.29	0.82	0.99	0.93	5	1.05	0.20	19.0
Germany	110	1.09	0.96	1.43	1.13	1.51	5	1.22	0.23	18.9
PEI	111	2.23	1.84	2.12	3.17	1.50	5	2.17	0.62	28.6
	112	1.65	1.41	1.55	3.88	1.21	5	1.94	1.09	56.2
	113	0.91	3.15	3.06	1.34	1.07	5	1.91	1.10	57.6
	114	1.07	1.06	1.31	1.33	1.34	5	1.22	0.14	11.5
Poland	551	1.58	1.30	1.16	3.60	1.71	5	1.87	0.99	52.9
	552	1.84	1.64	0.92	2.10	1.38	5	1.58	0.45	28.5
	553	1.30	1.88	1.29	1.52	0.97	5	1.39	0.34	24.5
	554	3.91	2.50	1.63	1.86	2.17	5	2.41	0.90	37.3
	555	1.20	1.86	0.98	1.85	1.33	5	1.44	0.40	27.8
Germany	511	1.09	1.51	0.62	1.16	0.63	5	1.00	0.38	38.0
Springe	512	0.65	0.87	1.05	0.76	1.35	5	0.93	0.27	29.0
	513	0.68	0.73	1.12	1.04	0.87	5	0.89	0.19	21.3
	514	0.64	0.88	0.63	0.68	1.22	5	0.81	0.25	30.9
	515	1.11	0.62	0.87	1.41	1.59	5	1.12	0.39	34.8
USA	526	1.32	1.26	1.54	1.03	1.72	5	1.37	0.27	19.7
Denver	527	1.23	1.00	0.76	1.72	0.91	5	1.13	0.38	33.6
	528	1.10	1.16	0.78	1.28	2.15	5	1.29	0.51	39.5
	529	1.88	1.13	0.98	0.77	1.48	5	1.25	0.44	35.2
	530	1.46	1.48	1.81	1.16	1.30	5	1.44	0.24	16.7
USA	531	0.92	0.80	0.88	0.75	0.73	5	0.81	0.08	9.9
Ohio	532	0.74	0.80	0.73	1.05	0.65	5	0.79	0.15	19.0
	533	1.16	0.70	0.69	0.59	0.89	5	0.81	0.23	28.4
	534	0.93	0.73	0.89	0.83	1.10	5	0.89	0.14	15.7
	535	0.70	0.76	0.70	0.68	0.68	5	0.70	0.03	4.3
USA	561	1.42	1.47	1.34	0.74	1.21	5	1.24	0.30	24.2
US Army	562	0.79	3.22	1.39	0.91	1.86	5	1.63	0.98	60.1
	563	2.88	2.53	1.20	1.83	2.33	5	2.15	0.66	30.7
	564	1.55	1.53	1.09	0.93	1.09	5	1.24	0.28	22.6
	565	1.83	0.84	1.14	0.72	1.20	5	1.15	0.43	37.4

Mean bacterial count (E+07 CFU/mL)

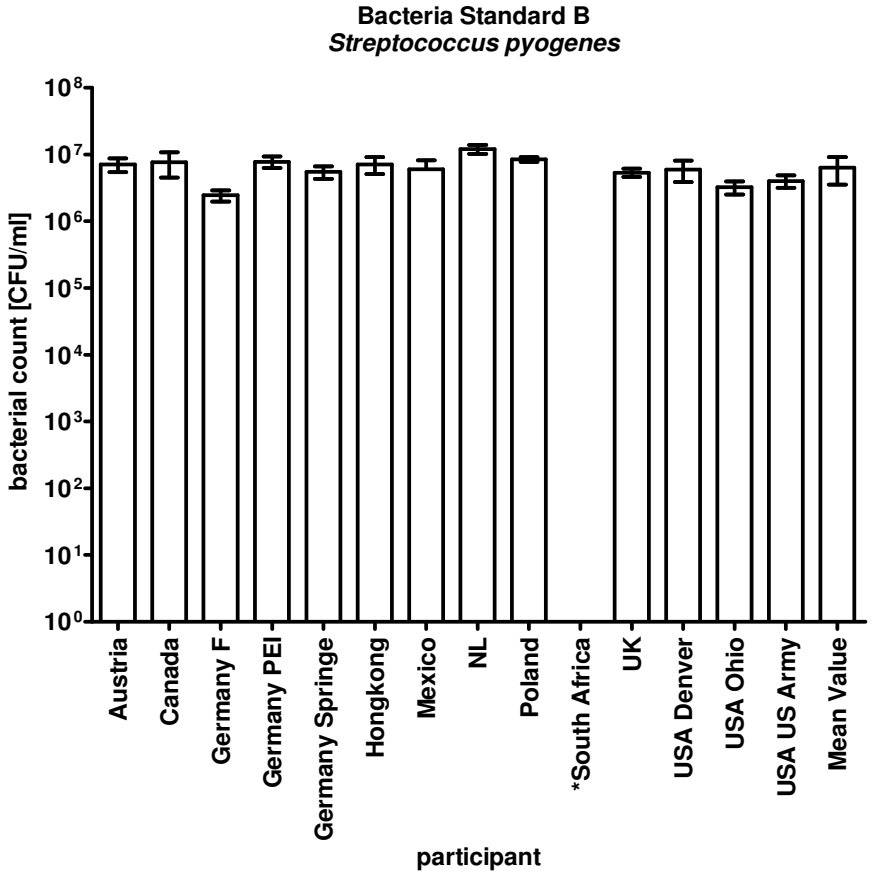


Table 5: Sample B, *Streptococcus pyogenes*

Raw data, mean, standard deviation and coefficient of variation (CV)

Participant	Vial	#1	#2	#3	#4	#5	N	Mean	SD	CV
Amsterdam	501	1.28	0.83	1.25	1.39	1.56	5	1.26	0.27	21.4
	502	0.55	3.68	0.77	1.04	0.82	5	1.37	1.30	94.9
	503	1.06	0.34	1.48	0.68	1.33	5	0.98	0.47	48.0
	504	0.62	0.73	0.36	3.03	0.42	5	1.03	1.13	109.7
	505	0.84	0.93	0.43	3.67	1.00	5	1.37	1.30	94.9
Austria	536	0.35	0.18	0.56	1.25	0.90	5	0.65	0.43	66.2
	537	0.39	0.46	0.41	0.26	0.88	5	0.48	0.23	47.9
	538	0.62	1.94	0.60	0.46	0.29	5	0.78	0.66	84.6
	539	0.37	0.76	1.30	0.61	1.59	5	0.93	0.50	53.8
	540	0.96	0.23	0.65	0.44	1.26	5	0.71	0.41	57.7
Canada	541	0.62	1.00	0.48	0.51	0.46	5	0.61	0.23	37.7
	542	0.50	0.39	0.46	0.54	0.47	5	0.47	0.05	10.6
	543	0.65	0.52	0.75	0.79	0.69	5	0.68	0.10	14.7
	544	1.51	3.15	1.03	0.28	0.43	5	1.28	1.15	89.8
	545	0.35	1.04	1.05	0.73	0.70	5	0.77	0.29	37.7
Germany	506	0.29	0.32	0.31	0.15	0.20	5	0.25	0.08	32.0
Frankfurt	507	0.08	0.17	0.12	0.41	0.13	5	0.18	0.13	72.2
	508	0.15	0.68	0.23	0.12	0.35	5	0.31	0.23	74.2
	509	0.08	0.09	0.28	0.21	0.48	5	0.23	0.17	73.9
	510	0.08	0.42	0.16	0.22	0.36	5	0.25	0.14	56.0

Hongkong	516	0.84	0.70	1.66	0.50	0.50	5	0.84	0.48	57.1
	517	1.32	0.96	0.35	1.79	0.27	5	0.94	0.64	68.1
	518	0.23	0.28	0.59	0.75	0.21	5	0.41	0.24	58.5
	519	0.68	0.93	0.48	0.77	0.78	5	0.73	0.17	23.3
	520	0.32	0.71	0.82	0.77	0.58	5	0.64	0.20	31.3
UK	521	0.46	0.49	0.40	0.44	0.40	5	0.44	0.04	9.1
	522	0.54	0.67	0.30	0.38	0.80	5	0.54	0.20	37.0
	523	0.74	0.33	0.67	0.70	0.39	5	0.57	0.19	33.3
	524	0.64	1.08	0.38	0.53	0.55	5	0.64	0.26	40.6
	525	1.00	0.33	0.26	0.30	0.50	5	0.48	0.30	62.5
Mexico	546	0.37	0.41	0.63	0.34	0.23	5	0.40	0.15	37.5
	547	0.97	0.51	0.56	0.72	0.46	5	0.64	0.21	32.8
	548	0.31	0.14	0.68	0.77	1.29	5	0.64	0.45	70.3
	549	0.27	0.34	0.50	0.51	0.40	5	0.40	0.10	25.0
	550	0.58	0.69	0.40	1.05	1.95	5	0.93	0.62	66.7
Germany	110	0.94	0.29	1.10	0.67	0.88	5	0.78	0.31	39.7
PEI	111	2.34	0.95	0.21	0.75	0.62	5	0.97	0.81	83.5
	112	0.76	0.63	0.90	0.42	0.36	5	0.61	0.23	37.7
	113	0.54	0.40	0.46	0.51	1.27	5	0.64	0.36	56.3
	114	0.59	0.76	0.82	1.42	0.76	5	0.87	0.32	36.8
Poland	551	1.87	0.38	0.83	0.57	0.20	5	0.77	0.66	85.7
	552	0.69	0.61	1.03	2.06	0.38	5	0.96	0.66	68.8
	553	0.50	0.55	0.87	1.44	0.62	5	0.80	0.39	48.8
	554	0.61	1.17	1.14	0.66	0.63	5	0.84	0.29	34.5
	555	0.73	0.76	0.93	1.33	0.47	5	0.85	0.32	37.6
Germany	511	0.70	0.95	0.31	0.35	0.28	5	0.52	0.29	55.8
Springe	512	0.64	0.35	0.71	0.48	0.79	5	0.59	0.18	30.5
	513	0.52	0.20	0.32	0.95	1.43	5	0.68	0.51	75.0
	514	0.39	0.54	0.36	0.91	0.69	5	0.58	0.23	39.7
	515	0.61	0.19	0.41	0.25	0.38	5	0.37	0.16	43.2
USA	526	0.46	0.33	0.57	0.60	0.59	5	0.51	0.12	23.5
Denver	527	0.20	0.26	0.89	2.91	0.40	5	0.93	1.14	122.6
	528	0.40	0.31	0.54	0.25	0.53	5	0.40	0.13	32.5
	529	0.59	0.32	0.39	0.44	0.71	5	0.49	0.16	32.7
	530	0.24	0.58	0.88	0.59	0.90	5	0.64	0.27	42.2
USA	531	0.21	0.26	0.28	0.22	0.21	5	0.24	0.03	12.5
Ohio	532	0.30	0.27	0.23	0.34	0.24	5	0.27	0.05	18.5
	533	0.28	0.37	0.39	0.27	0.26	5	0.31	0.06	19.4
	534	0.43	0.39	0.43	0.49	0.39	5	0.42	0.04	9.5
	535	0.31	0.28	0.40	0.47	0.38	5	0.37	0.08	21.6
USA	561	0.74	0.52	0.27	0.28	0.47	5	0.45	0.19	42.2
US Army	562	0.34	0.67	0.50	0.26	0.31	5	0.42	0.17	40.5
	563	0.43	0.20	0.16	0.42	0.15	5	0.27	0.14	51.9
	564	0.30	0.15	0.19	0.72	0.13	5	0.30	0.25	83.3
	565	0.24	0.33	0.90	0.18	0.27	5	0.38	0.29	76.3

Mean bacterial count (E+07 CFU/mL)

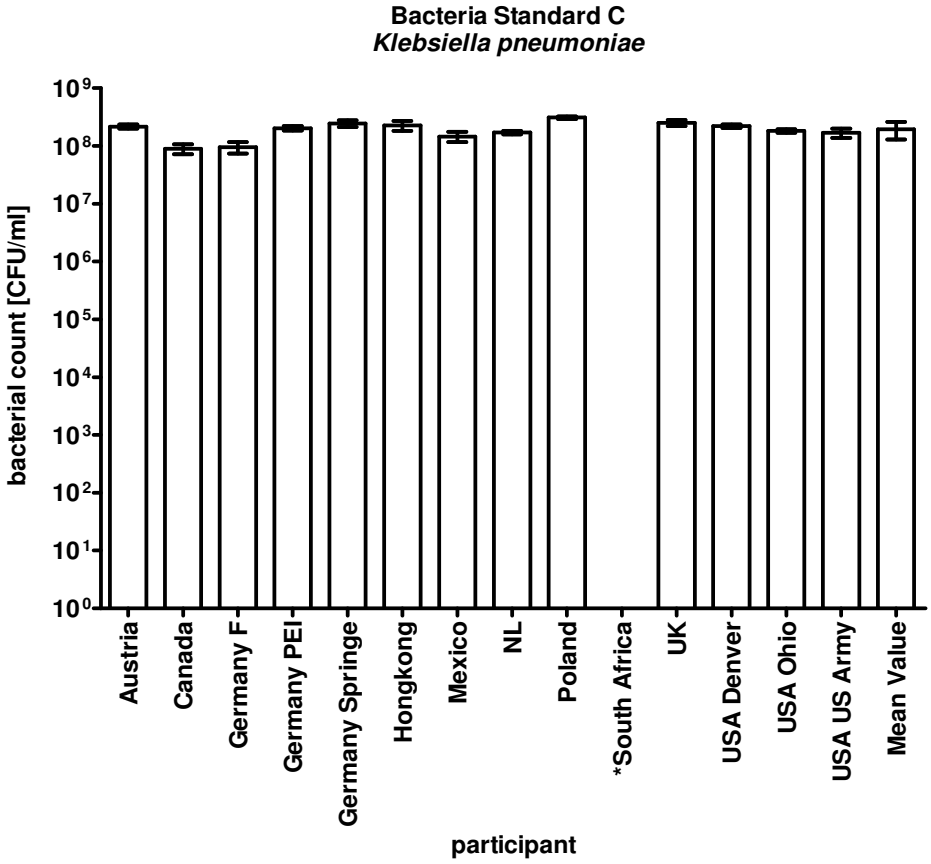


Table 6: Sample C. *Klebsiella pneumoniae*

Raw data, mean, standard deviation and coefficient of variation (CV).

Participant	Vial	#1	#2	#3	#4	#5	N	Mean	SD	CV
Amsterdam	501	13.60	16.40	15.30	16.40	17.60	5	15.86	1.50	9.5
	502	18.90	18.95	14.80	14.80	20.95	5	17.68	2.76	15.6
	503	18.10	17.05	13.80	15.40	14.90	5	15.85	1.72	10.9
	504	14.60	16.95	20.05	21.55	18.90	5	18.41	2.71	14.7
	505	22.05	17.70	15.50	14.70	18.05	5	17.60	2.87	16.3
Austria	536	23.05	21.20	22.40	19.70	16.70	5	20.61	2.53	12.3
	537	20.40	19.45	16.50	23.55	19.90	5	19.96	2.52	12.6
	538	18.55	22.00	23.60	17.90	22.95	5	21.00	2.61	12.4
	539	19.30	18.50	28.70	23.35	23.25	5	22.62	4.06	17.9
	540	28.50	20.45	20.55	25.85	26.45	5	24.36	3.66	15.0
Canada	541	9.85	11.70	13.00	11.35	10.75	5	11.33	1.17	10.3
	542	7.40	6.30	10.65	9.25	5.40	5	7.80	2.14	27.4
	543	7.60	5.60	6.00	9.60	5.95	5	6.95	1.67	24.0
	544	12.05	9.20	8.40	9.95	5.15	5	8.95	2.52	28.2
	545	9.10	11.90	7.85	9.20	12.10	5	10.03	1.88	18.7
Germany	506	8.90	7.55	7.25	10.05	10.60	5	8.87	1.48	16.7
Frankfurt	507	8.60	10.95	7.75	9.65	8.35	5	9.06	1.26	13.9
	508	7.55	8.85	6.80	5.35	6.90	5	7.09	1.27	17.9
	509	9.60	8.35	9.25	10.60	10.30	5	9.62	0.89	9.3
	510	17.10	10.45	12.05	11.60	14.00	5	13.04	2.61	20.0

Hongkong	516	32.55	24.10	26.35	21.35	27.95	5	26.46	4.21	15.9
	517	28.45	29.55	30.20	28.95	25.50	5	28.53	1.82	6.4
	518	21.10	20.00	23.10	21.60	19.05	5	20.97	1.55	7.4
	519	17.90	21.80	18.15	15.65	17.75	5	18.25	2.22	12.2
	520	19.15	18.90	14.65	23.35	20.40	5	19.29	3.14	16.3
UK	521	31.40	26.80	29.35	29.90	29.60	5	29.41	1.66	5.6
	522	19.80	22.00	21.50	24.80	20.90	5	21.80	1.87	8.6
	523	25.05	26.35	25.50	23.75	25.70	5	25.27	0.97	3.8
	524	19.85	22.50	24.85	23.90	21.70	5	22.56	1.94	8.6
	525	28.80	27.45	27.00	28.10	25.00	5	27.27	1.44	5.3
Mexico	546	17.60	30.80	26.15	24.00	12.85	5	22.28	7.09	31.8
	547	11.75	25.75	20.05	14.00	11.50	5	16.61	6.16	37.1
	548	11.75	25.75	20.05	14.00	11.50	5	16.61	6.16	37.1
	549	10.35	8.75	14.45	13.35	19.20	5	13.22	4.05	30.6
	550	1.95	3.80	4.55	6.90	6.75	5	4.79	2.09	43.6
Germany	110	16.60	19.40	20.60	17.40	17.35	5	18.27	1.67	9.1
PEI	111	21.90	18.70	24.30	25.15	23.05	5	22.62	2.51	11.1
	112	19.20	22.70	15.65	14.00	23.15	5	18.94	4.10	21.6
	113	20.80	16.60	22.90	20.60	29.60	5	22.10	4.77	21.6
	114	17.35	28.60	18.30	17.40	17.95	5	19.92	4.87	24.4
Poland	551	24.50	24.85	29.85	34.15	30.35	5	28.74	4.07	14.2
	552	31.00	29.30	33.25	36.20	31.75	5	32.30	2.60	8.0
	553	33.35	28.20	32.40	40.15	29.75	5	32.77	4.61	14.1
	554	35.15	32.00	28.50	26.60	28.35	5	30.12	3.43	11.4
	555	31.50	28.05	31.00	34.45	30.50	5	31.10	2.29	7.4
Germany	511	28.85	22.30	24.65	29.05	27.95	5	26.56	2.97	11.2
Springe	512	18.05	23.35	28.50	19.65	22.50	5	22.41	4.02	17.9
	513	22.25	22.00	20.80	18.30	22.60	5	21.19	1.75	8.3
	514	27.95	32.65	28.35	21.00	35.50	5	29.09	5.50	18.9
	515	29.85	21.95	19.05	18.40	28.40	5	23.53	5.30	22.5
USA	526	18.35	22.50	24.45	19.10	20.75	5	21.03	2.49	11.8
Denver	527	22.60	25.35	21.40	23.05	22.95	5	23.07	1.43	6.2
	528	21.10	24.80	25.90	16.75	11.65	5	20.04	5.90	29.4
	529	23.35	21.70	24.90	22.40	20.15	5	22.50	1.78	7.9
	530	22.05	25.00	27.80	22.85	20.45	5	23.63	2.85	12.1
USA	531	15.55	16.25	19.00	17.95	16.75	5	17.10	1.38	8.1
Ohio	532	15.70	17.50	24.00	21.00	17.50	5	19.14	3.33	17.4
	533	18.40	24.75	19.00	21.50	17.50	5	20.23	2.93	14.5
	534	16.05	17.40	17.10	17.60	16.50	5	16.93	0.64	3.8
	535	17.10	15.55	21.80	16.90	19.75	5	18.22	2.51	13.8
USA	561	13.45	12.20	12.70	15.25	10.50	5	12.82	1.74	13.6
US Army	562	15.20	19.60	17.10	12.95	14.50	5	15.87	2.56	16.1
	563	15.30	16.65	18.45	16.45	18.25	5	17.02	1.32	7.8
	564	20.60	18.65	17.60	19.20	14.20	5	18.05	2.41	13.4
	565	19.40	20.20	21.95	25.35	20.00	5	21.38	2.41	11.3

Mean bacterial count (E+07 CFU/mL)

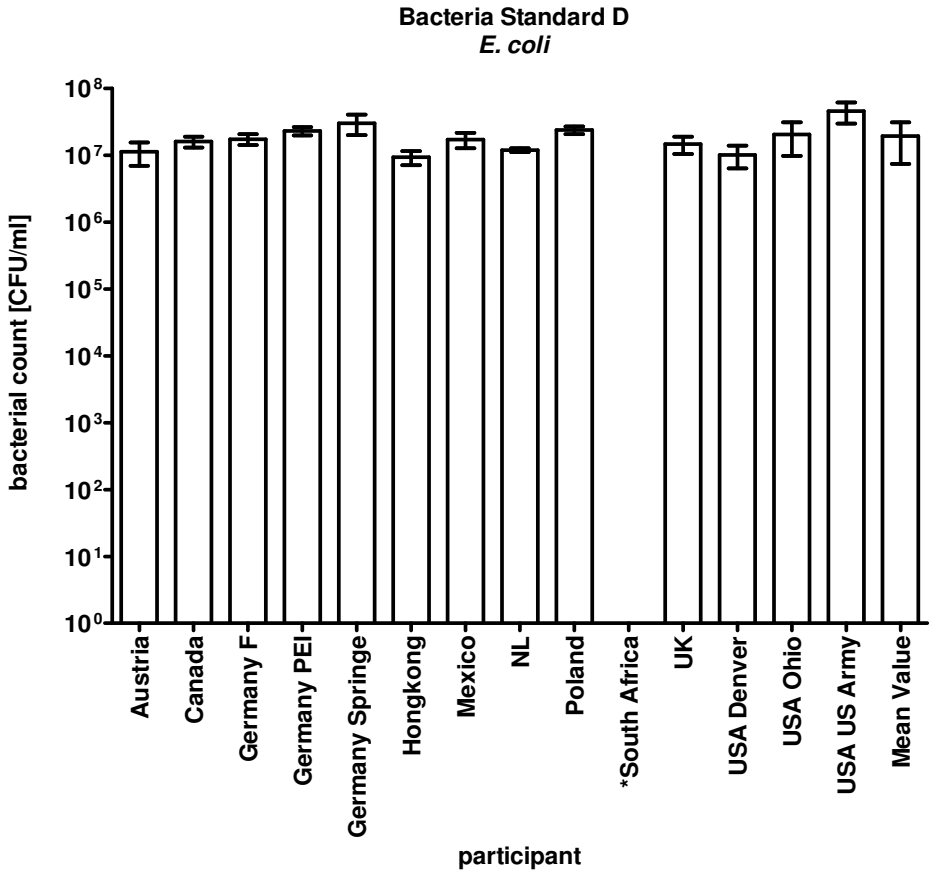


Table 7: Sample D, *Escherichia coli*

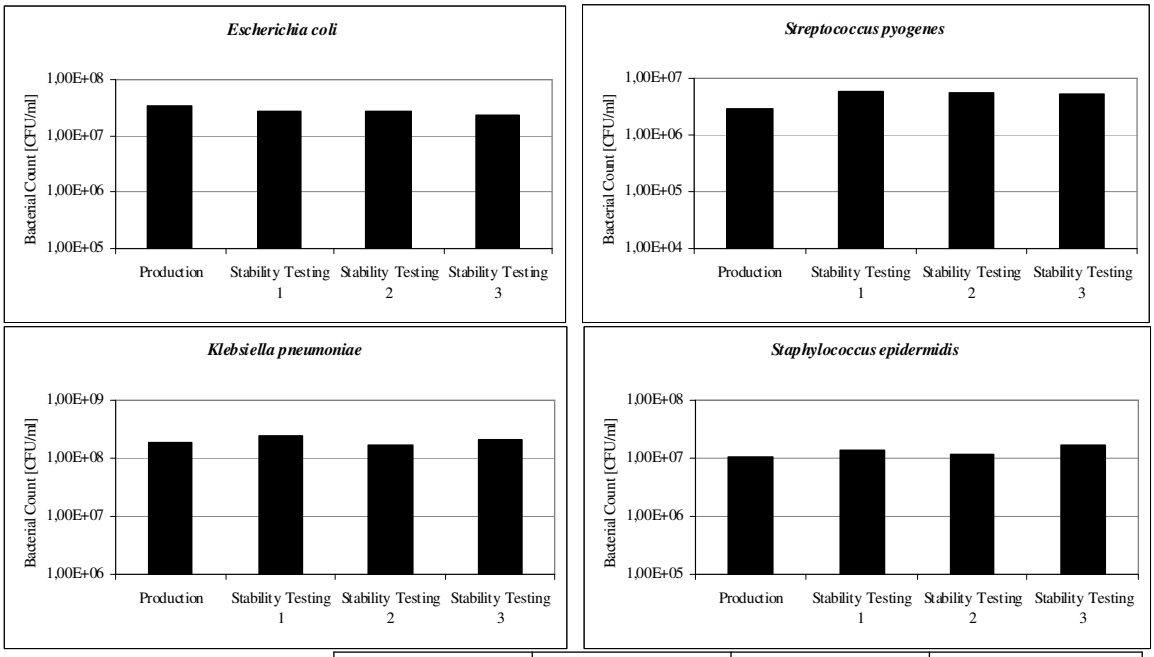
Raw data, mean, standard deviation and coefficient of variation (CV)

Participant	Vial	#1	#2	#3	#4	#5	N	Mean	SD	CV
Amsterdam	501	1.16	1.15	1.08	1.08	1.61	5	1.22	0.22	18.0
	502	1.17	1.00	1.37	1.35	0.74	5	1.13	0.26	23.0
	503	0.66	0.86	1.91	1.36	1.91	5	1.34	0.58	43.3
	504	1.13	1.10	1.04	1.22	1.12	5	1.12	0.07	6.3
	505	1.17	0.71	0.70	2.50	0.90	5	1.20	0.75	62.5
Austria	536	1.89	1.07	1.24	1.93	1.38	5	1.50	0.39	26.0
	537	1.07	2.03	2.18	1.01	1.53	5	1.56	0.53	34.0
	538	0.86	0.92	0.88	0.89	0.87	5	0.88	0.02	2.3
	539	1.12	0.47	0.53	0.33	0.27	5	0.54	0.34	63.0
	540	2.15	0.66	0.86	1.32	0.86	5	1.17	0.60	51.3
Canada	541	2.33	1.09	1.76	1.21	1.41	5	1.56	0.50	32.1
	542	2.06	1.74	1.05	1.22	2.05	5	1.62	0.47	29.0
	543	1.28	2.04	1.19	0.87	0.67	5	1.21	0.52	43.0
	544	1.59	1.60	1.35	1.39	1.90	5	1.57	0.22	14.0
	545	2.32	1.96	2.83	2.38	0.76	5	2.05	0.79	38.5
Germany	506	1.64	2.09	1.94	2.53	1.44	5	1.93	0.42	21.8
Frankfurt	507	1.50	1.77	1.24	1.94	1.88	5	1.67	0.29	17.4
	508	1.24	1.26	1.34	1.31	1.25	5	1.28	0.04	3.1
	509	1.51	2.29	1.58	1.96	1.24	5	1.72	0.41	23.8
	510	1.79	2.61	3.19	1.32	1.77	5	2.14	0.75	35.0

Hongkong	516	0.49	0.45	0.93	0.54	0.78	5	0.64	0.21	32.8
	517	0.66	0.21	0.10	0.19	0.12	5	0.26	0.23	88.5
	518	1.35	0.77	0.39	1.77	0.70	5	1.00	0.55	55.0
	519	1.91	1.39	1.01	1.03	1.05	5	1.28	0.39	30.5
	520	1.71	1.58	1.30	1.64	1.31	5	1.51	0.19	12.6
UK	521	1.24	0.68	1.46	1.77	1.49	5	1.33	0.41	30.8
	522	1.88	1.28	1.24	1.74	2.37	5	1.70	0.47	27.6
	523	2.64	2.40	2.97	4.11	2.82	5	2.99	0.66	22.1
	524	0.63	0.09	0.18	0.41	1.00	5	0.46	0.37	80.4
	525	1.01	1.28	0.95	0.69	0.57	5	0.90	0.28	31.1
Mexico	546	0.78	0.50	0.41	0.46	0.89	5	0.61	0.21	34.4
	547	1.08	2.09	1.01	1.42	1.83	5	1.48	0.47	31.8
	548	1.21	0.71	1.66	0.65	1.05	5	1.06	0.41	38.7
	549	2.50	2.66	3.57	4.73	1.80	5	3.05	1.13	37.0
	550	2.80	1.90	3.90	1.86	1.71	5	2.43	0.92	37.9
Germany	110	2.72	1.46	1.80	2.29	1.51	5	1.96	0.54	27.6
PEI	111	1.58	2.38	1.99	3.35	2.53	5	2.36	0.66	28.0
	112	0.90	2.66	3.08	2.72	2.36	5	2.34	0.85	36.3
	113	2.20	2.26	2.44	3.62	3.53	5	2.81	0.71	25.3
	114	1.19	1.60	1.64	4.17	1.97	5	2.11	1.18	55.9
Poland	551	2.98	2.17	2.66	2.08	3.14	5	2.61	0.47	18.0
	552	2.86	2.31	1.81	2.01	1.74	5	2.15	0.46	21.4
	553	2.20	2.06	2.73	4.56	2.67	5	2.84	1.00	35.2
	554	1.80	2.01	2.24	1.82	2.53	5	2.08	0.31	14.9
	555	2.17	2.47	2.27	2.26	2.65	5	2.36	0.19	8.1
Germany	511	3.48	3.23	3.40	3.93	3.30	5	3.47	0.28	8.1
Springe	512	2.20	2.73	2.55	3.38	2.08	5	2.59	0.52	20.1
	513	4.84	4.77	4.32	4.13	5.07	5	4.63	0.39	8.4
	514	1.37	2.19	2.69	2.06	2.71	5	2.20	0.55	25.0
	515	3.08	2.42	2.08	2.48	1.35	5	2.28	0.63	27.6
USA	526	1.66	1.71	1.81	1.72	1.24	5	1.63	0.22	13.5
Denver	527	0.45	0.79	1.30	0.80	0.87	5	0.84	0.30	35.7
	528	1.18	1.85	0.85	1.03	0.67	5	1.11	0.45	40.5
	529	0.39	1.30	0.50	0.86	0.46	5	0.70	0.38	54.3
	530	0.74	1.57	0.74	0.43	0.51	5	0.80	0.45	56.3
USA	531	3.03	2.55	2.45	2.57	3.00	5	2.72	0.27	9.9
Ohio	532	1.49	1.35	0.93	0.65	1.39	5	1.16	0.36	31.0
	533	1.01	0.92	0.93	0.89	1.41	5	1.03	0.21	20.4
	534	1.95	0.93	1.99	1.73	2.41	5	1.80	0.54	30.0
	535	3.47	3.35	3.87	4.17	2.90	5	3.55	0.49	13.8
USA	561	1.59	3.82	2.91	2.64	1.96	5	2.59	0.87	33.6
US Army	562	3.88	2.59	2.62	6.50	3.72	5	3.86	1.59	41.2
	563	3.07	3.25	4.90	2.20	9.35	5	4.55	2.85	62.6
	564	2.33	6.07	7.08	12.75	6.50	5	6.95	3.74	53.8
	565	4.16	4.03	3.50	2.28	10.78	5	4.95	3.34	67.5

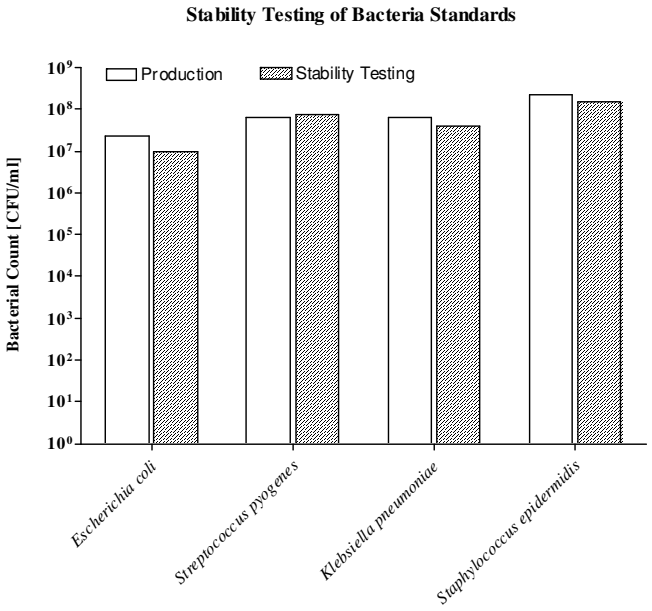
Mean bacterial count (E+07 CFU/mL)

8. Figures



		Production		Stability Testing 1		Stability Testing 2		Stability Testing 3	
Species	**Standard BTBS**	**Date**	**Count [CFU/ml]**	**Date**	**Count [CFU/ml]**	**Date**	**Count [CFU/ml]**	**Date**	**Count [CFU/ml]**
Escherichia coli	PEI-B-19-06	12.08.2008	**3,48E+07**	03.09.2008	**2,77E+07**	10.12.2008	**2,71E+07**	22.01.2009	**2,32E+07**
Streptococccus pyogenes	PEI-B-20-06	20.08.2008	**3,08E+06**	02.09.2008	**5,85E+06**	10.12.2008	**5,74E+06**	21.01.2009	**5,20E+06**
Klebsiella pneumoniae	PEI-B-08-10	31.07.2008	**1,85E+08**	03.09.2008	**2,44E+08**	09.12.2008	**1,67E+08**	28.01.2009	**2,04E+08**
Staphylococcus epidermidis	PEI-B-06-08	15.08.2008	**1,06E+07**	02.09.2008	**1,37E+07**	10.12.2008	**1,15E+07**	21.01.2009	**1,69E+07**

Figure 1: Stability of Bacteria Standards during operation time of Validation Study



Stability Testing after 2 years (*E.coli*), 1 year (*St. pyogenes*), 5 years (*K. pneumoniae)*, 3 years (*St. epidermidis*).

		Production		Stability Testing	
Species	**Standard**	**Date**	**Count [CFU/ml]**	**Date**	**Count [CFU/ml]**
Escherichia coli	PEI-B-19	19.10.2006	2,35E+07	20.02.2008	9,98E+06
Streptococcus pyogenes	PEI-B-20	17.04.2007	6,49E+07	18.02.2008	7,55E+07
Klebsiella pneumoniae	PEI-B-08	17.04.2003	6,61E+07	20.02.2008	4,01E+07
Staphylococcus epidermidis	PEI-B-06	28.09.2005	2,18E+08	06.02.2008	1,56E+08

Figure 2: Stability of Bacteria Standards during storage over years.

Figure 3: please see page 6

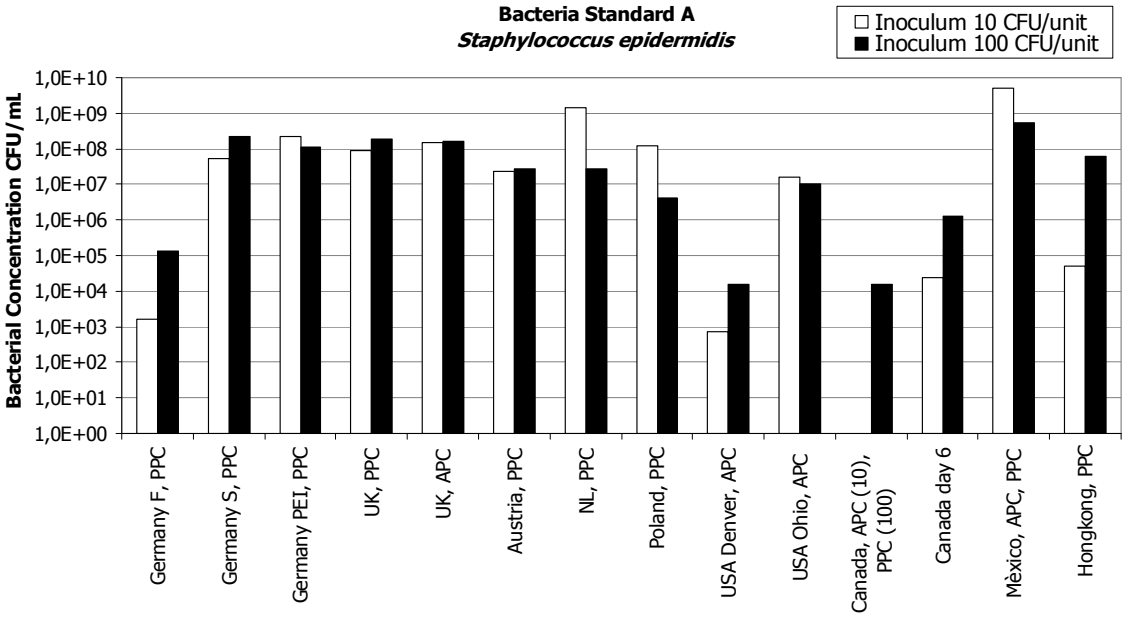


Figure 4: Growth of *Staphylococcus epidermidis* (Sample A) in platelet concentrates.

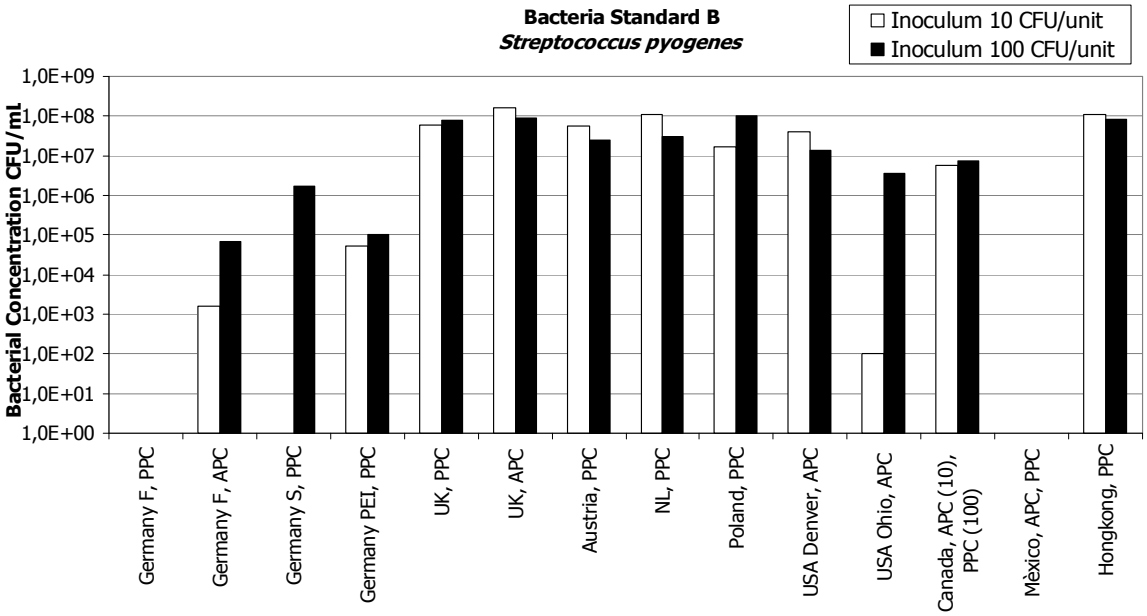


Figure 5: Growth of *Streptococcus pyogenes* (Sample B) in platelet concentrates.

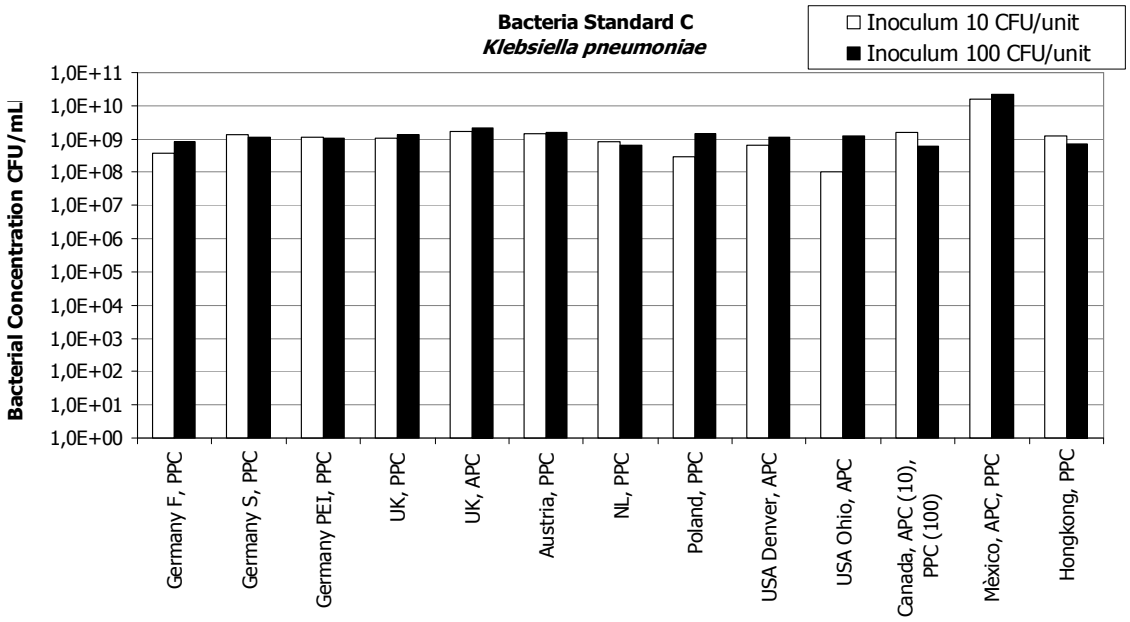


Figure 6: Growth of *Klebsiella pneumoniae* (Sample C) in platelet concentrates.

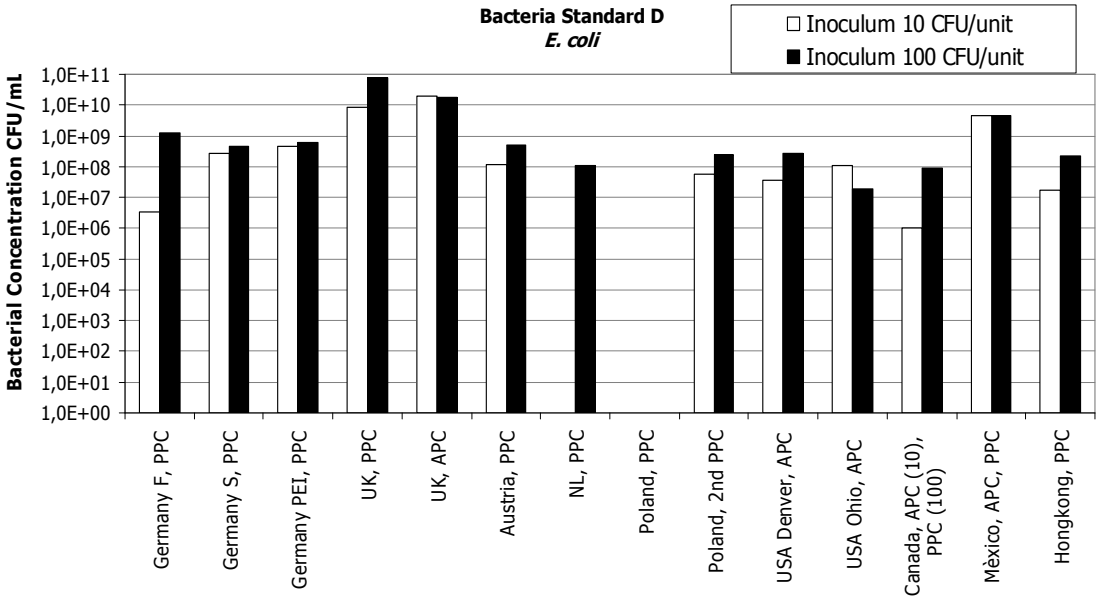


Figure 7: Growth of *Escherichia coli* (Sample D) in platelet concentrates.

Working Party on

Transfusion-Transmitted Infectious Diseases

International Validation Study

Certificate

Blood Bacteria Standard

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

store below -70°C

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

Generated:
Date: 2005/09/28

Approved:
Date: 2008/02/06

CONTENT

1. Certificate

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: 1.5 mL

Bacterial load: 2.18 ± 0.29 x 10^8 CFU/mL

Growth in PC: Blood Bacteria Standard *Staphylococcus epidermidis* PEI-B-06 grows donor independently in PCs.

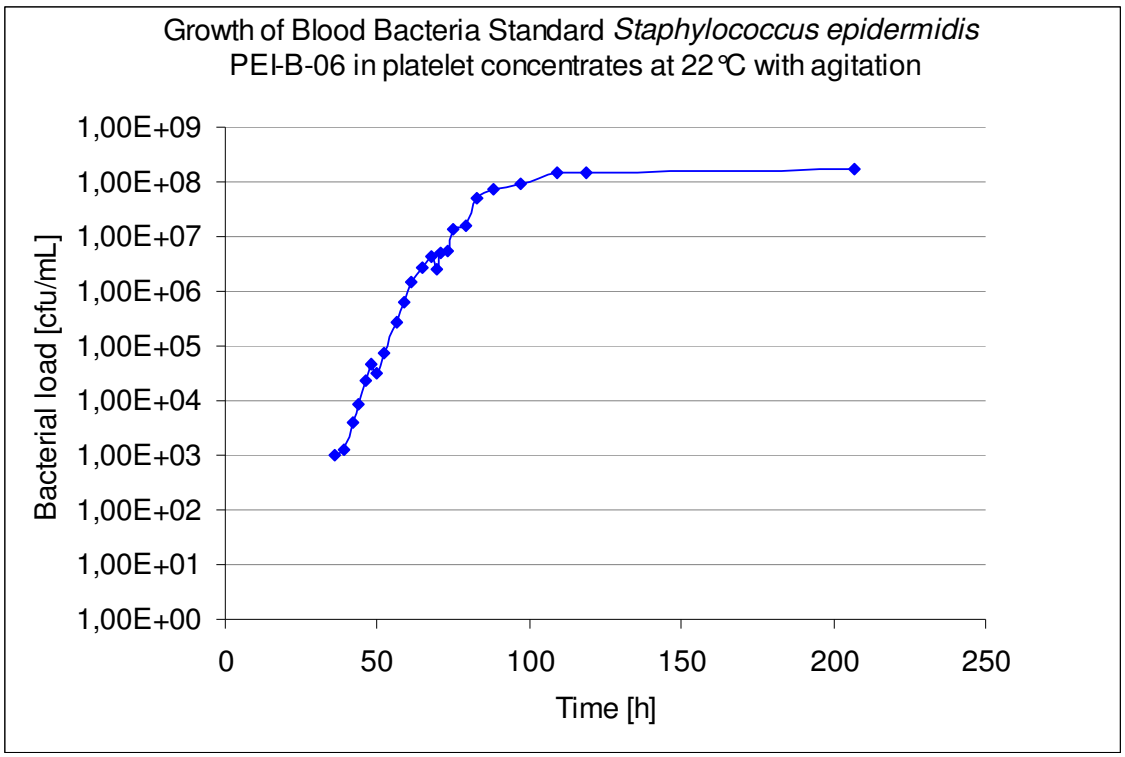


Fig.1 In the in vitro study pooled PCs (n=4) were inoculated with 0.03 CFU/mL of Blood Bacteria Standard PEI-B-06 of isolate *Staphylococcus epidermidis*. Sampling was performed during aerobic storage at 22°C and the presence of bacteria was assessed by plating culture.

2. Bacterial Strain (Blood Bacteria Standard)

Phylum:	Firmicutes
Class:	Cocci
Order:	Bacillales
Family:	*Staphylococcaceae*
Species:	*Staphylococcus 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 haemolysis (beta haemolysis) due to production of haemolysins tissue invasive, produce purulent (pus-filled) lesions, nonsporeforming, facultative anaerobic, obligatory pathogenic, grows at 6.5°C to 46°C at pH 4.2 - 9.3.

3. Microbiological identification

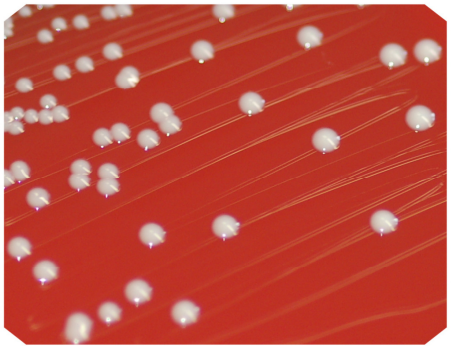


Fig. 2 *St. epidermidis* (PEI-B-06) on sheep blood
agar after 24 hours incubation at 37°C

approx. 1-2 mm in diameter
after overnight incubation;
no haemolysis

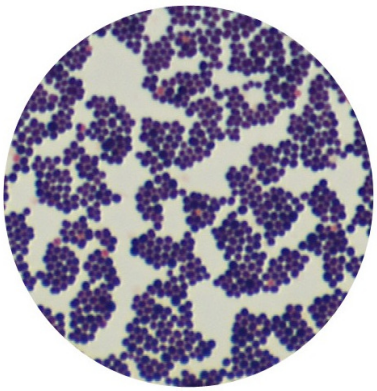


Fig. 3 GRAM-stain of *St. epidermidis* (PEI-B-06)

GRAM-stain: GRAM-positive

4. Molecular genetic identification (16S rDNA 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	*St. epidermidis* ATCC 12228	100 %	46	1469

Staphylococcus epidermidis 16S rDNA sequence

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCC
TCTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGGAT
AACTTCGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATGGTTCAATAGTGA
AAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAGCTAGTTGGTAAGGTA
ACGGCTTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAA
CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCG
AAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTT
ATTAGGGAAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGAAA
GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGG
AATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGC
TCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAAT
TCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACT
TTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTG
CTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
AAGAACCTTACCAAATCTTGACATCCTCTGACCCCTCTAGAGATAGAGTTTTCCCCTTCGG
GGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAG
TTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA
TGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGYAGCGAAACCGCGAGGT
CAAGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTATATGA
AGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTG
TACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATT
TGGAGCTAGCCGTCGAAGGTGGGACAAATGATTGGGGT

5. Production

5.1 Production principle

After the bacterial identification process using microbiological, biochemical (using the API Staph multitest identification system, bioMérieux) and molecular genetic methods (16S rDNA sequencing, RAPD-PCR), an impedance-monitoring system is used to characterize bacterial growth kinetics of Blood Bacteria Standard PEI-B-06 under
defined 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. Viability control is performed 24 hours after production while stability control is performed quarterly. The bacterial identity of each charge of Blood Bacteria Standard PEI-B-06 is confirmed by biochemical

and molecular genetic methods, including 16S rDNA sequencing and DNA fingerprinting (RAPD-PCR).

5.2 Master Bank

Bacteria of Blood Bacteria Standard PEI-B-06 are cultured on appropriate agar media to a sufficient bacterial count. Under aseptic conditions bacteria are transferred to six vials of a Cryobank system to the manufacturer`s instructions and stored at -80 °C. Cryobank tubes contain a medium for suspending the bacterial culture and 25 colour-coded ceramic beads. The suspending medium comprises trypticase 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.

6. Batch Quality Control

6.1 Viability

To affirm the viability of the Blood Bacteria Standard PEI-B-06, vials of PEI-B-06 are thawed 24 hours after production and enumerated as described in the application section.

6.2 Stability

The stability of the Blood Bacteria Standard PEI-B-06 is confirmed quarterly by thawing 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.
PCR products underwent electrophoresis on an agarose gel (2%) and were visualized using ethidium bromide staining.

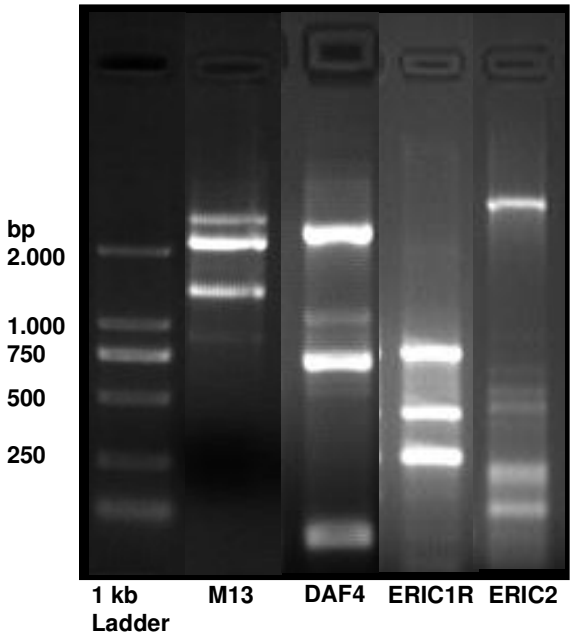


Fig. 4 RAPD-PCR (DNA-Fingerprint) of *St. epidermidis* PEI-B-06 using different single oligonucleotide primers (n=4).

7. Application

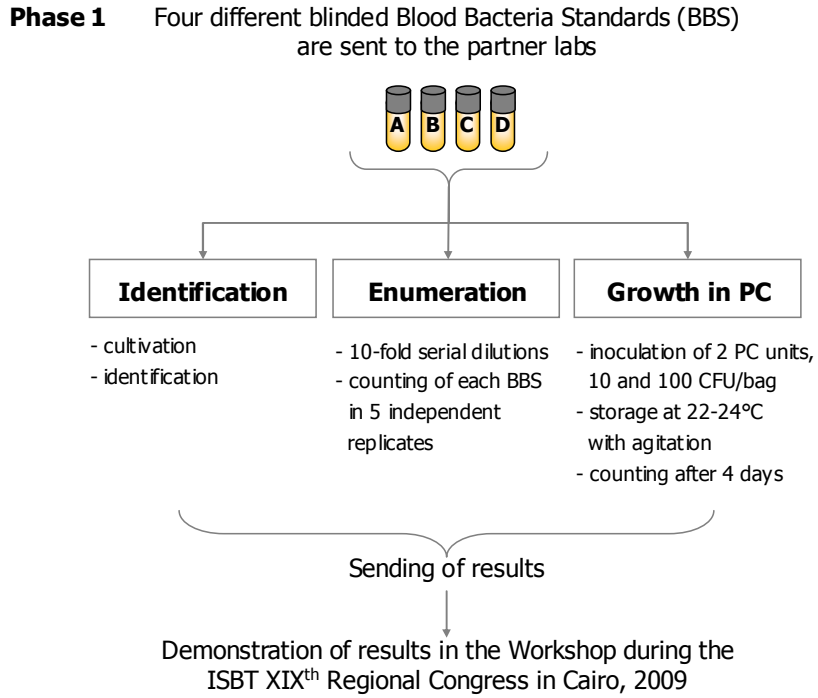
7.1 Storage

The vials of the Blood Bacteria Standard PEI-B-06 have to be stored immediately below 70°C after arrival. To assure the viability of bacteria of the Blood Bacteria Standard PEI-B-06 the cold chain must not be interrupted.

7.2 Utilization

Before use, transfer the vials of the Blood Bacteria Standard PEI-B-06 directly from the deep freezer to a dry incubator and defrost the vials at 37ºC for 10 minutes. If ice crystals are still evident, the vial should be warmed in the hand until the crystals have melted. Vortex the vial for 15 seconds to be sure that all bacteria are evenly spread. Dilution steps and determination of the bacterial count have to be performed as described in the study design protocol.

CONTENT



1. Shipping, Storage and Unfreezing

To assure the stability of the bacterial load of the Blood Bacteria Standards (BBS), the cold chain must not be interrupted and the standards must be used immediately after thawing.
Note: Check the vials immediately after arrival. If the samples show any sign of thawing, they must be discarded! In this case the study coordinating group will provide new samples.

1.1 Labelling of Blood Bacteria Standards

Four different blood bacteria standards of undisclosed identity are contained in vials labelled A,B,C,D (10 vials of each). All vials labelled with the same letter (A,B,C or D) contain the same microorganism and same bacterial count (CFU/mL).

1.2 Storage of Blood Bacteria Standards

- Store the vials immediately after arrival in a deep freezer at -80°C.

1.3 Unfreezing of Blood Bacteria Standards

- Transfer the vial directly from deep freezer to a dry incubator and defrost the vial at 37ºC for 10 minutes.
- If ice crystals are still evident, warm the vial in the hand until the content has melted.

Note: The Blood Bacteria Standards (stock suspensions) must be used immediately after thawing.

2. Cultivation

Material:

- 1 vial of each Blood Bacteria Standards A, B, C, D
- 8 standard **Trypticase Soy Agar** plates (e.g. according to EP resp. USP, exemplarily see the receipt below) or **alternatively use Columbia Blood Agar**
- Dry incubator 37°C
- Inoculating loops

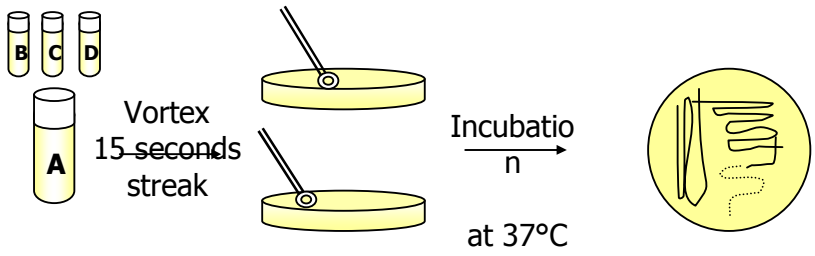
Exemplary example: Composition Trypticase Soy Agar/TSA (1 L):

Peptone from meat	7.8 g
Peptone from casein	7.8 g
Yeast extract	2.8 g
NaCl	5.6 g
D(+)-Glucose	1.0 g
Distilled water	1000 mL
pH	7.5

Procedure:

- Defrost one vial of each Blood Bacteria Standard as described above (see 1.3.) and vortex for 15 seconds at the highest speed.
- Use an inoculating loop to streak the microorganisms in duplicate on agar plates in order to achieve separated single colonies.
- Incubate the agar plates aerobically at 37°C.
- Check the growth on the plates each day (maximally 3 days).

Example for Blood Bacteria Standard A



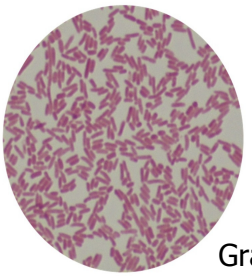
3. Identification

Material:

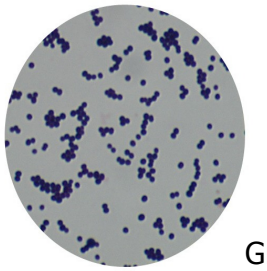
- Freshly grown separated colonies of each Blood Bacteria Standards A, B, C, and D
- Gram-stain equipment
- Gram-positive identification test panel (e.g. API, bioMérieux)
- Gram-negative identification test panel (e.g. API, bioMérieux)

3.1 Gram-Stain

- Perform a Gram-stain for each Blood Bacteria Standard using the freshly grown separated colonies as described in the cultivation section 2. Follow the standard procedure in your laboratory.



Gram-negative bacteria



Gram-positive bacteria

3.2 Identification Test Panel

- Perform an identification test panel, commonly used in your laboratory, for each Blood Bacterial Standard using the freshly grown separated colonies as described in "Cultivation" (see section 2).
- If necessary, perform other characterisation tests such as catalase, oxidase and indole splitting depending on the results of the Gram-stain and colony morphology.

3.3 Documentation

- Please document all methods used and, additionally, the results in the Addendum 1 (6.1.).

4. Enumeration of Blood Bacteria Standards

Material:

- 5 vials of each Blood Bacteria Standard A, B, C, and D
- Dry incubator 37°C, vortexer
- Sterile NaCl aqueous solution (saline, 0.9%)
- Sterile tubes with caps (15 mL; n=120)
- Trypticase Soy Agar plates (alternatively Columbia Blood Agar), n=600 (see "Cultivation" section 2.)
- Sterile applicator (spattle)

4.1 Unfreezing the Frozen Vials for Bacteria Count

- Defrost 5 vials of each Blood Bacteria Standard for 10 minutes in a dry incubator at 37ºC as described in the section "Unfreezing" (1.3). If ice crystals are still evident, warm the vial in the hand until the content has melted.
- Vortex each vial for 15 seconds to ensure that all bacteria are evenly spread.

4.2 Dilution of the Stock Tubes

An undiluted (stock) suspension is termed the 10^0 dilution. This corresponds to the Blood Bacteria Standard as sent. Prepare 10-fold serial dilutions using 9 mL of sterile saline (NaCl) each and 1 mL of the stock resp. of dilution from previous dilution step. In consequence, each dilution is 1/10th the concentration of the previous dilution. The first 10-fold dilution is termed the 10^{-1} (D1) dilution, the following is termed D2 etc.

Note: Make sure…

A: … that the dilution series of the stock tubes is prepared immediately after thawing the stock suspension.

B: … that the stock suspension as well as each dilution is intensively vortexed (highest speed) for 15 seconds.

C: … that tips are changed after each step.

Illustration: Series of 10-fold dilutions:

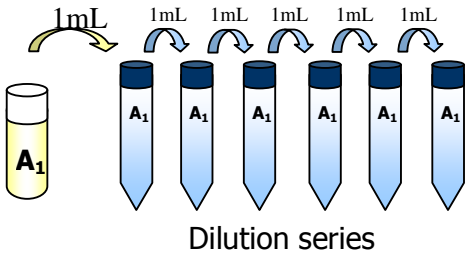
Vortex stock for 15 seconds at highest speed

Start: 1 mL of 10^0 (stock)
Add: + 9 mL NaCl
} Yield: 10 mL → 10^{-1} (D1)

Vortex dilution for 15 seconds at highest speed

Start: 1 mL of 10^{-1} (D1)
Add: + 9 mL NaCl
} Yield: 10 mL → 10^{-2} (D2)

continue



Dilution series

Procedure:

1. Label 6 dilution tubes for each Blood Bacteria Standard vial (e.g. A1/D1, A1/D2, A1/D3….; A2/D1, A2/D2, A2/D3…. etc.; in total 120 tubes).
2. Prepare the dilution tubes with 9 mL each of sterile NaCl solution (0.9%) as diluent.
3. Vortex the thawed vial of the Blood Bacteria Standard (stock) at the highest speed, 15 seconds immediately after unfreezing.
4. Transfer 1 mL of the stock (vial of Blood Bacteria Standard) into the first dilution tube (D1: dilution 10^{-1}).
5. Discard the tip; cap the tube and vortex for 15 seconds at highest speed.
6. Take a new tip and transfer 1 mL out of the first dilution (D1) into the second dilution tube (D2: dilution 10^{-2}).
7. Vortex the dilution D2 for 15 seconds at highest speed.
8. Continue this procedure for dilutions D3, D4, D5 and D6.

4.3 Enumeration

1. Use the spread plate method to distribute 100 µL of each dilution onto 5 solid agar plates (Trypticase Soy Agar, alternatively Columbia Blood Agar). Use a sterile applicator to spread the bacteria on plates.
2. Incubate at 37°C for 1-2 days under aerobic conditions in a dry incubator until colonies are clearly recognizable.

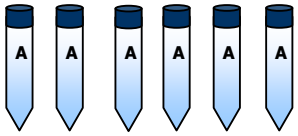
3. Count the colonies per agar plate.

Attention: Discard agar plates containing ≥ 300 colonies from the evaluation and list those as "not countable (n.c.)" in the handwritten document (Addendum 6.2) and as 0 (zero) in the calculation form (Excel-file).

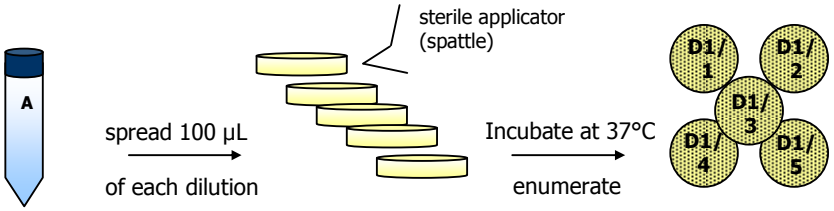
4. Please, document all results for each dilution step in the forms of Addendum 2.

Example for Blood Bacteria Standard A Vial 1

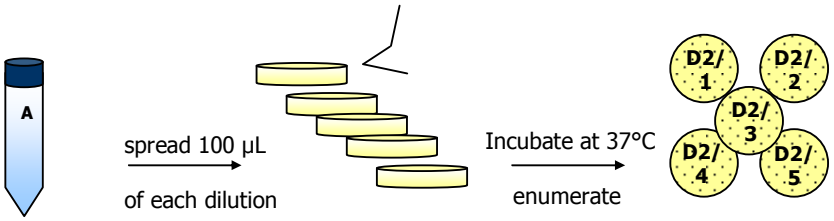
Series of 10-fold dilutions of Vial 1



Procedure example shown for dilution D1



Procedure example shown for dilution D2



→ Repeat procedure for dilutions D3 to D6

4.4 Calculation and Documentation

Document all data in the attached forms in Addendum 2 (handwritten) and, additonally, for automatic calculation in the "Calculation of Bacterial Count-Form" (attached Excel-file). In the Excel-file, please fill in 0 (zero) for all plates that were not countable (≥ 300) because all segments of the tables have to be filled in for mathematical reasons.

Note: All calculations (i.e. estimation of CFU/mL obtained, statistics etc.) will be performed by the study coordinationg group. The participating laboratories are asked only to complete the forms in the Addenda. The calculation procedure below is included for preparation of chapter 5 (see below).

Calculation of bacteria counts [CFU/mL]:

$$\bar{x}_{\text{arithm}} = \frac{1}{n}\sum_{i=1}^{n} x_i = \frac{x_1 + x_2 + \cdots + x_n}{n}$$

$\bar{x}$: mean value
n : number of counted agar plates
x_1 : colony count on plate 1
x_2 : colony count on plate 2

Example

Blood Bacteria Standard A vial 1

Calculation dilution step

	100 µL of…					
Plate	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
1	0	0	170	18	2	0
2	0	0	180	22	3	0
3	0	0	200	20	2	0
4	0	0	220	24	3	0
5	0	0	190	21	1	0
Mean value	0	0	192	21	2.2	0

Calculation vial A1

$$\bar{x}_{A1} = \frac{(\text{MeanD3} \times 10^4) + (\text{MeanD4} \times 10^5) + (\text{MeanD5} \times 10^6)}{3}$$

$$\bar{x}_{A1} = \frac{(192 \times 10^4) + (21 \times 10^5) + (2.2 \times 10^6)}{3} = 2.07 \times 10^6 \text{ CFU/mL}$$

Calculation of bacteria count (CFU/mL) in stock solution A

$$\bar{x}_S = \frac{\bar{x}_{A1} + \bar{x}_{A2} + \bar{x}_{A3} + \bar{x}_{A4} + \bar{x}_{A5}}{5}$$

5. Growth of Blood Bacteria Standards in Platelet Concentrates (PCs)

To determine the growth kinetics of the Blood Bacteria Standards during usual PC storage conditions, PC units are inoculated with approximately 10 CFU per bag and 100 CFU per bag and monitored after 4 days (96 hours after inoculation) of storage at 22-24°C for the bacteria count.

Material:

- 1 vial of each Blood Bacteria Standard A, B, C, and D
- 2 platelet units for each Blood Bacteria Standard A, B, C, and D (total n=8)
  (if possible: 2 apheresis PC and 2 whole blood derived PCs for each BBS, i.e. use preferably fresh PCs, if not available, use outdated ones)
- Usual platelet storage device (agitation, temperature controlled)
- Dry incubator, 37°C
- Sterile welding equipment (e.g. Sterile Connecting Device)
- Sterile NaCl aqueous solution (0.9%)
- Sterile tubes with caps (15 mL)
- Trypticase Soy Agar plates (alternatively Columbia Blood Agar), (in total: 128 for counting and 24 for inoculation control, see point 2 above)
- Sterile applicator 8 (spattle)
- Sterile syringes

5.1 Sterility control for baseline sterility of platelet concentrates

All PCs have to be sampled before bacterial inoculation to assure baseline sterility of the original platelet bags. Perform sterility testing, commonly used in your laboratory (e.g. aerobic and anaerobic cultivation).

5.2 Unfreezing the standards and dilution down to ~100 and ~ 10 CFU/mL

- Thaw 1 vial of each Blood Bacteria Standard (A, B, C, and D) as described in 1.3.
- Dilute each Blood Bacteria Standard down to approximately 100 CFU/mL and 10 CFU/mL in NaCl (e.g. between 50 to 150 CFU/mL, and 5 to 15 CFU/mL) as generally described in 4.2.

Note: The final dilution step (in order to reach approximately 100 or 10 CFU/mL in the final dilution tube) depends on the bacterial count previously calculated in chapter "Enumeration" (see point 4).

5.3 Artificial contamination of platelets ...

- Combine each platelet bag with a luer-lock connection device (e.g. a short tube using Sterile Connecting Device).

5.3.1 ...with approximately 10 CFU per bag

- For each Blood Bacterial Standard, inoculate 1 mL each of the final dilution that contains approximately 10 CFU/mL (i.e. final concentration approximately 0.03 CFU/mL) into one apheresis or whole blood derived platelet concentrate unit (if possible one apheresis and one whole blood derived PC) applying aseptic conditions.
- From the dilution, prior to that of the inoculation dilution, plate 100 µL each onto 3 agar plates and enumerate the colonies after incubation as described in 4.3. (control of bacteria count of inoculum).

5.3.2 ...with approximately 100 CFU per bag

- For each Blood Bacterial Standard, inoculate 1 mL each of the final dilution that contains approximately 100 CFU/mL (i.e. final concentration approximately 0.3 CFU/mL) into one apheresis or whole blood derived platelet concentrate unit (if possible one apheresis and one whole blood derived PC) applying aseptic conditions.
- From the dilution, prior to that of the inoculation dilution, plate 100 µL each onto 3 agar plates and enumerate the colonies after incubation as described in 4.3. (control of bacteria count of inoculum).

Inoculation process:

1) Draw **5 mL** out of the platelet bag using a sterile syringe, but do not discard it (see below).
2) Using a second sterile syringe, inoculate 1 mL of the final dilution (~ 10 CFU/ mL or ~ 100 CFU/mL) through the same port into the platelet bag.
3) Add the previously removed **5 mL** PC sample to flush the tube segment of the bag.

4) Close the luer-lock port.

5) Incubate the contaminated PC units at 22-24°C under agitation for 4 days (96 hours after inoculation).

Note: a) Close the tube by clamp in any case of opening the luer-lock device (e.g. before connection with a syringe, change of syringes etc.)

b) The procedure described is used to overcome the "dead-volume" of the tube, i.e. to bring the inoculum directly into PCs main volume. Additionally, bacteria attached to the inner surface of the tube shall be detached.

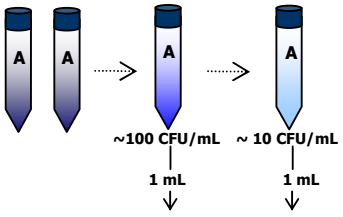
c) Avoid any entry of air into the platelet unit during the inoculation process!

5.4 Sampling, Enumeration and Documentation

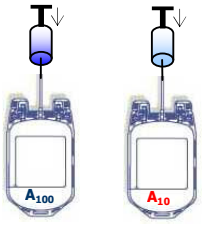
- Sampling is performed 4 days (96 hours after inoculation) after inoculation.
- Sample drawing shall be performed following the principles described in section 5.2., "Inoculation process", i.e. remove the first **5 mL** of the PC using a sterile syringe but do not discard it, use a second sterile syringe to take a sample (1 mL) of each platelet bag, add the previously removed **5 mL** PC sample. This procedure is recommended in order to enable a repetition if necessary.
- Close the luer-lock port.
- Enumerate the bacterial count as described previously (4.3), **but dilute up to 10^{-8} (D8)** (duplicates are sufficient, i.e. two agar plates per dilution).
- Complete documentation in Addendum 3 (6.3).

Example: **Inoculation (100 and 10 CFU/bag) and enumeration of Blood Bacteria Standard A after growth in PC.**

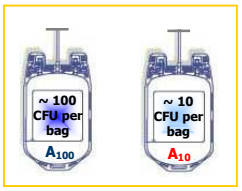
Dilution series down to approximately 100 CFU/mL and 10 CFU/mL (D?). Please calculate the dilution steps from your enumeration in section 4.



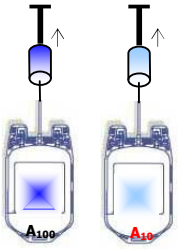
Aseptic inoculation of 1 mL dilution, containing approximately 100 CFU/mL or 10 CFU/mL (10 CFU/bag corresponding to 0.03 CFU/mL) into one platelet bag each



Incubation at 22-24°C under agitation

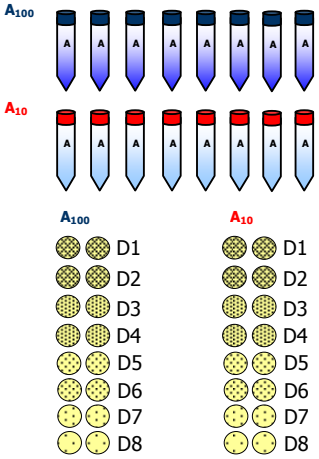


Aseptic sampling after 4 days of storage



Dilution series of each platelet bag (dilution series A_{100} and A_{10}) down to 10^{-8} (D8)

Colony-forming-assay in duplicates of each dilution (D1 – D8) of dilution series A_{100} and A_{10}



6. Addendum

6.1 Addendum 1 - Documentation of Identification

6.1.1 Blood Bacteria Standard A

Colony morphology:

Microscopic view (shape: rod, coccus…) :

Gram-stain : ☐ Gram-negative ☐ Gram-positive

Identification Method (Identification panel) :

Results of Identification Method (please add details like reactions...) :

Microorganism identified in Blood Bacteria Standard A: _____

Notes:

6.1.2 Blood Bacteria Standard B

Cultivation conditions:
(incubation)

Colony morphology:

Microscopic view :
(shape: rod, coccus…)

Gram-stain : ☐ Gram-negative ☐ Gram-positive

Identification Method :
(Identification panel)

Results of Identification :
Method (please add
details like reactions...)

Microorganism identified in Blood Bacteria Standard B:

_____

Notes:

6.1.3 Blood Bacteria Standard C

Cultivation conditions:
(incubation)

Colony morphology:

Microscopic view :
(shape: rod, coccus…)

Gram-stain : ☐ Gram-negative ☐ Gram-positive

Identification Method :
(Identification panel)

Results of Identification :
Method (please add
details like reactions...)

Microorganism identified in Blood Bacteria Standard C: _____

Notes:

6.1.4 Blood Bacteria Standard D

Cultivation conditions:
(incubation)

Colony morphology:

Microscopic view :
(shape: rod, coccus…)

Gram-stain : ☐ Gram-negative ☐ Gram-positive

Identification Method :
(Identification panel)

Results of Identification :
Method (please add
details like reactions...)

Microorganism identified in Blood Bacteria Standard D: _____

Notes:

6.2 Addendum 2 - Documentation of Enumeration (see also Excel-Form)

Blood Bacteria Standard A

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial A_1
A_1	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial A_2
A_2	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial A_3
A_3	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial A_4
A_4	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial A_5
A_5	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Blood Bacteria Standard B

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial B_1
B_1	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial B_2
B_2	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial B_3
B_3	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial B_4
B_4	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial B_5
B_5	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Blood Bacteria Standard C

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial C_1
C_1	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial C_2
A_2	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial C_3
C_3	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial C_4
C_4	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial C_5
C_5	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Blood Bacteria Standard D

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial D_1
D_1	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial D_2
D_2	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial D_3
D_3	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial D_4
D_4	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

Vial	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Mean value of Vial D_5
D_5	Dilution 1						
	Dilution 2						
	Dilution 3						
	Dilution 4						
	Dilution 5						
	Dilution 6						

6.3 Addendum 3 - Documentation of Growth in Platelet Concentrates

6.3.1 Enumeration

Blood Bacteria Standard A

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Mean value of A_{10}
A_{10}	Dilution 1			
	Dilution 2			
	Dilution 3			
	Dilution 4			
	Dilution 5			
	Dilution 6			
	Dilution 7			
	Dilution 8			

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Mean value of A_{100}
A_{100}	Dilution 1			
	Dilution 2			
	Dilution 3			
	Dilution 4			
	Dilution 5			
	Dilution 6			
	Dilution 7			
	Dilution 8			

Blood Bacteria Standard B

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Mean value of B_{10}
B_{10}	Dilution 1			
	Dilution 2			
	Dilution 3			
	Dilution 4			
	Dilution 5			
	Dilution 6			
	Dilution 7			
	Dilution 8			

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Mean value of B_{100}
B_{100}	Dilution 1			
	Dilution 2			
	Dilution 3			
	Dilution 4			
	Dilution 5			
	Dilution 6			
	Dilution 7			
	Dilution 8			

Blood Bacteria Standard C

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Mean value of C_{10}
C_{10}	Dilution 1			
	Dilution 2			
	Dilution 3			
	Dilution 4			
	Dilution 5			
	Dilution 6			
	Dilution 7			
	Dilution 8			

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Mean value of C_{100}
C_{100}	Dilution 1			
	Dilution 2			
	Dilution 3			
	Dilution 4			
	Dilution 5			
	Dilution 6			
	Dilution 7			
	Dilution 8			

Blood Bacteria Standard D

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Mean value of D_{10}
D_{10}	Dilution 1			
	Dilution 2			
	Dilution 3			
	Dilution 4			
	Dilution 5			
	Dilution 6			
	Dilution 7			
	Dilution 8			

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Mean value of D_{100}
D_{100}	Dilution 1			
	Dilution 2			
	Dilution 3			
	Dilution 4			
	Dilution 5			
	Dilution 6			
	Dilution 7			
	Dilution 8			

6.3.2 Summary of Growth in Platelet Concentrates

Blood Bacteria Standard A

Platelet Concentrates : ☐ apheresis-PC ☐ pool-PC (donors n=)

Volume PC A_{10}:

Volume PC A_{100}:

Inoculum A_{10} (Dilution of stock):

Inoculum A_{100} (Dilution of stock):

Result of enumeration of inoculum control A_{10} (pre-last dilution):

Result of enumeration of inoculum control A_{100} (pre-last dilution):

Storage conditions:

Sampling time point after inoculation:

Bacterial proliferation A_{10} : ☐ yes ☐ no

Bacterial proliferation A_{100} : ☐ yes ☐ no

Bacterial count after storage : PC A_{10} =

PC A_{100} =

Notes :

Blood Bacteria Standard B

Platelet Concentrates : ☐ apheresis-PC ☐ pool-PC (donors n=)

Volume PC B_{10}:

Volume PC B_{100}:

Inoculum B_{10} (Dilution of stock):

Inoculum B_{100} (Dilution of stock):

Result of enumeration of inoculum control B_{10} (pre-last dilution):

Result of enumeration of inoculum control B_{100} (pre-last dilution):

Storage conditions:

Sampling time point after inoculation:

Bacterial proliferation B_{10} : ☐ yes ☐ no

Bacterial proliferation B_{100} : ☐ yes ☐ no

Bacterial count after: PC B_{10} =
storage
PC B_{100} =

Notes :

Blood Bacteria Standard C

Platelet Concentrates : ☐ apheresis-PC ☐ pool-PC (donors n=)

Volume PC C_{10}:

Volume PC C_{100}:

Inoculum C_{10} (Dilution of stock):

Inoculum C_{100} (Dilution of stock):

Result of enumeration of inoculum control C_{10} (pre-last dilution):

Result of enumeration of inoculum control C_{100} (pre-last dilution):

Storage conditions:

Sampling time point after inoculation:

Bacterial proliferation C_{10} : ☐ yes ☐ no

Bacterial proliferation C_{100} : ☐ yes ☐ no

Bacterial count after storage : PC C_{10} =

PC C_{100} =

Notes :

Blood Bacteria Standard D

Platelet Concentrates : ☐ apheresis-PC ☐ pool-PC (donors n=)

Volume PC D_{10}:

Volume PC D_{100}:

Inoculum D_{10} (Dilution of stock):

Inoculum D_{100} (Dilution of stock):

Result of enumeration of inoculum control D_{10} (pre-last dilution):

Result of enumeration of inoculum control D_{100} (pre-last dilution):

Storage conditions:

Sampling time point after inoculation:

Bacterial proliferation D_{10} : ☐ yes ☐ no

Bacterial proliferation D_{100} : ☐ yes ☐ no

Bacterial count after storage : PC D_{10} =

PC D_{100} =

Notes :

Please send all results by January 31st 2009

If you have any queries please don't hesitate to contact us.

Thank you for participation!

Prototype Certificate

Working Party on
Transfusion-Transmitted Infectious Diseases

WHO-ISBT International Validation Study

Certificate

Blood Bacteria Standard

Species: *Staphylococcus epidermidis*
Code: PEI-B-06-Charge
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-Strasse 51-59
63225 Langen

Generated: Date: 2005/09/28

Approved: Date: 2008/02/06

Blood Bacteria Standard *Staphylococcus epidermidis* PEI-B-06-07

CONTENT

1

Example: Certificate of Blood Bacteria Standard

Thomas Montag(-Lessing), MD, PhD

Melanie Stoermer, PhD

Paul-Ehrlich-Institute
Federal Agency for Sera and Vaccines
Section 1/3 Microbial Safety
Paul-Ehrlich-Strasse 51-59
D-63225 Langen
Germany

Phone: +49-6103-77-8028, -8020, -8026
Mail to: month@pei.de and stome@pei.de

Proposal	Validation study for enlargement of Transfusion-relevant Bacteria Strain Panel		
Proposer (name of Institution)	PEI	**Principal contact**	Thomas Montag-Lessing Melanie Stoermer
Rationale	It is aimed to enlarge the transfusion-relevant bacterial strain panel intended for the use in standardization of validation and assessment of methods for improvement of microbial safety of blood components (Validation Study Phase 2). As a first milestone, four bacterial strains were validated in an international study and this panel has been submitted to the ECBS (2009 for endorsement, 2010 for adoption/ establishment). This collaborative effort is understood as the first stage in the development of a more comprehensive panel, and moreover the proof of principle which is an excellent basis for ensuring: A) Quality, stability and suitability of bacterial strains for defined low titre spiking of platelet concentrates. B) The property of selected strains to grow up to high counts in blood components obtained from donors in different regions of the world (donor-independent proliferation). C) Training on the logistics of worldwide shipping of deep frozen blinded pathogenic bacteria. In line with the strategy cited above, an enlargement of the panel as well as the inclusion of further blood components (e.g. red cell concentrates) is crucial. For practical reasons, a step wise enlargement of transfusion-relevant bacterial strain panel is necessary. The proposed list includes strains of the following bacterial species: *Bacillus cereus, Bacillus cereus spores, Bacillus subtilis, Bacillus subtilis spores, Clostridium perfringens, Clostridium perfringens spores, Enterobacter cloacae, Klebsiella oxytoca, Morganella morganii, Propionibacterium acnes, Proteus mirabilis, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella cholerae-suis, Serratia marcescens, Staphylococcus aureus, Staphylococcus capitis, Staphylococcus lugdunensis, Staphyloccus warneri, Streptococcus warneri, Streptoccus agalactiae, Streptococcus viridans, Streptococcus bovis and Yersinia enterocolitica*.		
Anticipated uses and users	Control laboratories Blood banks Manufacturers of methods for Bacteria Screening of blood components Manufacturer of approaches for Pathogen Reduction in blood components		
Source/type of materials	Strains of the species listed under “Rationale” above, preferably isolates from transfusion-transmitted bacteria infections, shall be collected by the ISBT Working Party Transfusion-Transmitted Infectious Diseases (ISBT WP-TTID), Subgroup Bacteria. Thereafter, the strains will be processed following the PEI protocol for manufacturing of defined deep-frozen suspensions (ready to use, stable, shippable on dry ice, defined in count of living cells).		
Outline of proposed collaborative study	Bacteria standards will be shipped to the 13 partners worldwide who participated in the establishment of the first panel cited under “Rationale”. The study partners shall check them regarding their ability to grow up in blood components under defined routine storage conditions. As in the first study had been done, the protocol includes very low count spiking (0.03 to		

	0.3 CFU/ml) and, thereafter, monitoring of replication kinetics of the bacteria in the blood component.		
Issues raised by the proposal	None		
Action required	ECBS to endorse proposal		
Proposer's project reference		**Date proposed:**	
CONSIDERATIONS FOR ASSIGNMENT OF PRIORITIES (TRS932)			
Approval status of medicine or in vitro diagnostic method	Approaches for improvement of bacterial safety of blood components (approaches for pathogen reduction in cellular blood components as well methods for bacteria screening of blood components) undergo different levels of regulatory controls in different regions. Regulatory interference reaches from medium to high.		
Number of products or methods	Around 10 different approaches for pathogen inactivation of cellular blood components having different levels of maturity (concrete numerous unknown); one of which on the market; three of which in clinical trial status. More than 10 different approaches for bacteria screening in blood components described having different levels of development (concrete number unknown); 2 automated culture systems on the market involving millions of tests per year, 2 methods for rapid bacteria detection on the market (in start phase).		
Public health importance	No established transfusion-relevant bacterial strain panel existing. Therefore, no principle for standardization of validation and assessment of methods for improvement of microbial safety of blood components (pathogen reduction as well as screening) available		
Global importance	High		
Global need from regulatory & scientific considerations	High		
ECBS outcome			

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