World Health Organization

WHO/BS/2015.2269
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 12 to 16 October 2015

Collaborative Study to Enlarge the First WHO Repository of Platelet Transfusion-Relevant Bacterial Reference Strains

Eva Spindler-Raffel, Kay-Martin Hanschmann, Thomas Montag-Lessing†* and the Collaborative Study Group**

**Division of EU cooperation / Microbiology, Head Dr Isabelle Bekeredjian-Ding*
Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, D-63225 Langen, Germany
***ISBT WP Transfusion Transmitted Infectious Diseases Subgroup on Bacteria*
Chairs: Carl P McDonald and Richard J Benjamin
See Appendix 1

NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **14 September 2015** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr M. Nübling** at email: nueblingc@who.int

Summary

Bacterial contamination of platelet concentrates (PCs) remains a persistent problem in transfusion [1, 2, 3, 4 and 8]. For method validation and for assessment of blood safety measures it is crucial to use bacterial strains that are able to proliferate in blood components, e.g. in PCs under usual storage conditions [5, 6].

Four bacterial strains were adopted by ECBS in 2010 as the 1st WHO International Reference Repository of Platelet Transfusion Relevant Bacterial Reference Strains: *Staphylococcus epidermidis* PEI-B-P-06, *Streptococcus pyogenes* PEI-B-P-20, *Escherichia coli* PEI-B-P-19 and *Klebsiella pneumoniae* PEI-B-P-08. [7] The committee requested detailed instructions for use, which were provided by PEI in February 2011. The four bacterial strains have been cultivated and distributed by the Paul-Ehrlich-Institut since 2014 (PEI code number 8483/13).

A proposal for future expansion of the repository was endorsed by WHO ECBS in 2010. To characterize new candidate strains, a second international collaborative study was performed. This study was coordinated by the Paul Ehrlich Institut (PEI) in cooperation with the ISBT Working Party Transfusion Transmitted Infectious Diseases (WP-TTID), Subgroup on Bacteria.
Eleven further bacterial candidate strains were evaluated together with the already established strains in an international study under routine conditions, which means the simulation of bacterial contamination during blood donation by low bacterial cell count spiking (< 1 Colony Forming Unit per milliliter) directly into PC-bags and determination of their ability to proliferate in PC from multiple donors. Bacterial counts were performed at days 2, 4 and 7 after inoculation to assess the bacterial growth kinetics.

The candidate strains included the 2 spore forming bacterial strains (*Bacillus cereus* PEI-B-P-07-S and *Bacillus thuringiensis* PEI-B-P-57-S), Gram-negative species (*Enterobacter cloacae* PEI-B-P-43, *Morganella morganii* PEI-B-P-74, *Proteus mirabilis* PEI-B-P-55, *Pseudomonas fluorescens* PEI-B-P-77, *Salmonella choleraesuis* PEI-B-P-78, *Serratia marcescens* PEI-B-P-56), and Gram-positive species (*Staphylococcus aureus* PEI-B-P-63, *Streptococcus dysgalactiae* PEI-B-P-71, and *Streptococcus bovis* PEI-B-P-61). The study was performed in fourteen centres (ten different countries) that tested each bacterial strain in triplicate. With the exception of the *Morganella morganii* strain, all bacterial strains showed moderate to excellent growth at day 7 after inoculation. The individual growth curves showed variation from slow to fast growth. *Bacillus cereus*, *Bacillus thuringiensis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, *Serratia marescens*, *Staphylococcus aureu*s and *Streptococcus dysgalactiae* showed growth of significantly more than 2 $\log_{10}$ CFU/mL up to 8 $\log_{10}$ CFU/mL by day 2 of storage. For *Enterobacter cloacae*, *Proteus mirabilis*, *Staphylococcus epidermidis*, *Streptococcus bovis* and *Streptococcus pyogenes*, this growth level was reached at day 4. Growth for *Salmonella choleraesuis* was lower than for the other strains and showed a high variability among the results of the different participants. In addition, the study provided information regarding the growth behaviour and kinetics of different bacterial species in PC. Stability testing was performed at PEI to ensure the growth ability at a defined content of living cells in deep frozen suspensions.

Platelet Transfusion Relevant Bacteria Reference Strains which are provided as ready to use deep frozen suspensions in a defined cell count are a feasible tool for validation and assessment of various microbiological methods for improving blood safety. Neither resuspension nor cultivation is needed prior to use. In the collaborative study referenced, 11 candidate strains were characterized. Those that demonstrate growth independent of donor effects under "real life" conditions, will be recommended for inclusion in the WHO International Reference Repository of Platelet Transfusion Relevant Bacterial Reference Strains.

Introduction

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 initial volume from the blood donation (pre-donation sampling, also called diversion). Nevertheless bacterial contamination is considered as one of the most common transfusion-associated causes of death [1, 2, 3, 4, 11].

The fundamental difference between contamination by viruses in comparison to bacteria is that the latter can replicate strongly in a PC during its shelf life [5, 6]. Under the usual storage conditions at 22.5°C with agitation, microorganisms contaminating a PC can grow up to 10 $\log_{10}$ Colony Forming Units (CFU) per bag. In addition to bacterial cells themselves, pyrogenic substances (i.e. endotoxins and/or exotoxins) may accumulate in the PC bags, depending on the bacterial species and strain. Even relatively apathogenic bacterial strains can cause life-threatening infections in the recipient after transfusion [10].

The International Society of Blood Transfusion (ISBT) Working Party Transfusion-Transmitted Infectious Diseases, Subgroup on Bacteria (former chair: Dr Thomas Montag-Lessing) had organized an international validation study on Platelet Transfusion-Relevant Bacteria Reference Strains (PTRBRS) to be used as a tool for development, validation and comparison of the respective methods. Four blinded bacterial strains had been sent in replicates to participating laboratories worldwide for bacterial count calculation, strain identification and evaluation of growth properties in PCs. The results were submitted to the Expert Committee on Biological Standardization (ECBS) and were established as the WHO Repository of Platelet Transfusion-Relevant Bacteria Reference Strains [7].

They are available at PEI as the First WHO Platelet Transfusion Relevant Bacterial Reference Strain Repository.

In the first collaborative study (WHO BS/10.2154, 2010) the four strains were sent to the participating laboratories. The study partners had to identify, enumerate and spike the bacterial strains into the PCs in two different concentrations (10 CFU and 100 CFU per bag). Sampling and enumeration was performed on day 4. In contrast, in the enlargement study, the candidate bacterial strains were not blinded with respect to the strain identity and only one concentration was spiked into PCs (10 to 25 CFU per bag). To get more information of the growth kinetics of the candidate strains sampling and enumeration was carried out on day 2, 4, and 7 after inoculation. To ensure the stability of the bacterial strains (number of living cells in the deep

frozen suspension) during the study period and even beyond, cell counting had been performed routinely at PEI.

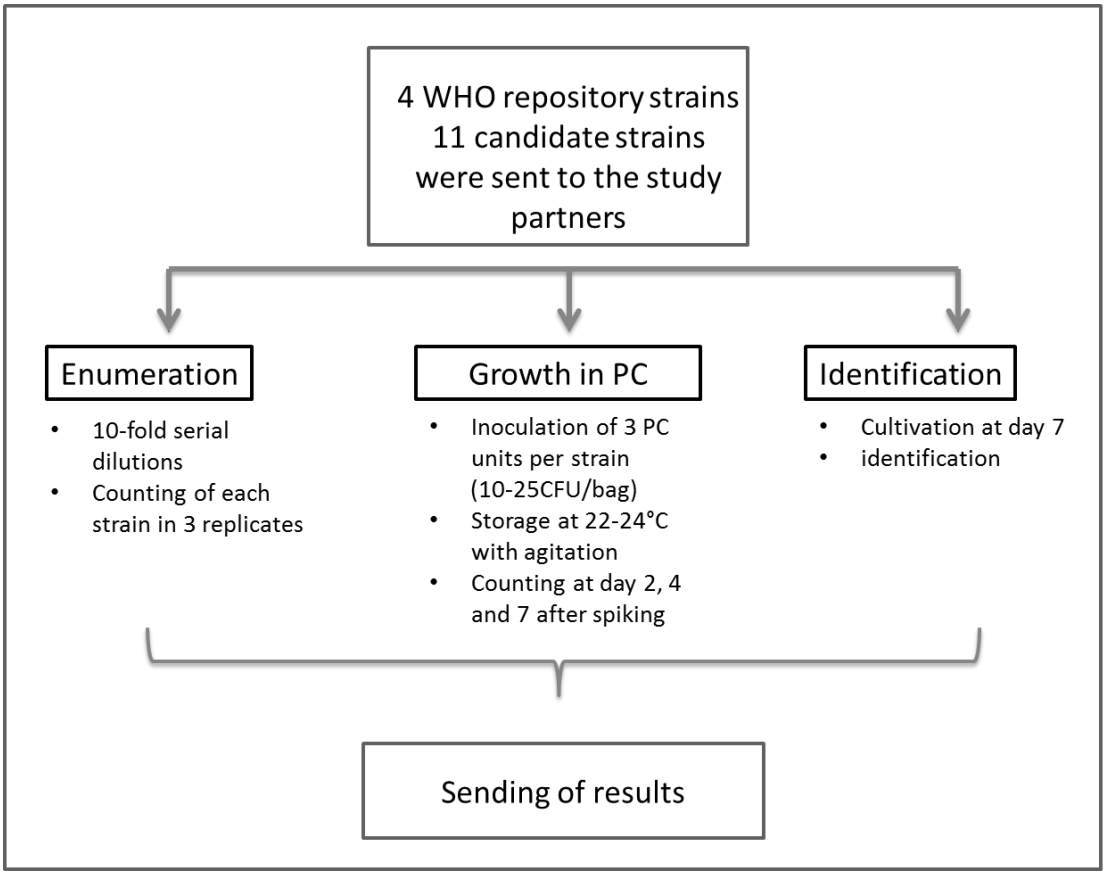
Those 9 strains that demonstrated donor independent growth properties under "real life" conditions and showed stability during the storage time will be recommended for inclusion in the WHO International Reference Repository of Platelet Transfusion Relevant Bacterial Reference Strains. As the tested *Morganella morganii* PEI-B-P-74 strain showed no growth, a second strain was tested in 8 laboratories in accordance with the study protocol. The new strain showed growth in all tested PCs. The data are very consistent as the statistical evaluation shows. The growth potential as well as the match of inoculum is comparable to the already existing WHO bacteria repository. It is recommended to add this strain to the bacteria extension list.

Materials and Methods

Participants and Study Design

Sixteen laboratories world-wide were asked, fourteen which participated in the enlargement study and received the samples. All fourteen study partners finished the tests and sent the results. The participants were from Germany (3), Austria (1), The Netherlands (1), England (1), Canada (1), USA (3), Mexico (1), Pakistan (1), Japan (1), and South Africa (1). Details on participants and laboratories are given in **Appendix 1: Design of the collaborative study**
The study protocol (scheme shown in **Fig.1**) was discussed and confirmed by the TTID WP subgroup on bacteria and presented at the meeting in Amsterdam 2013. The results were presented to the TTID WP in several meetings (i.e. ISBT Congress Seoul 2014, extraordinary meeting TTID WP subgroup on bacteria Philadelphia 2014, ISBT Regional Congress London 2015).

Fig. 1: Scheme of international enlargement study

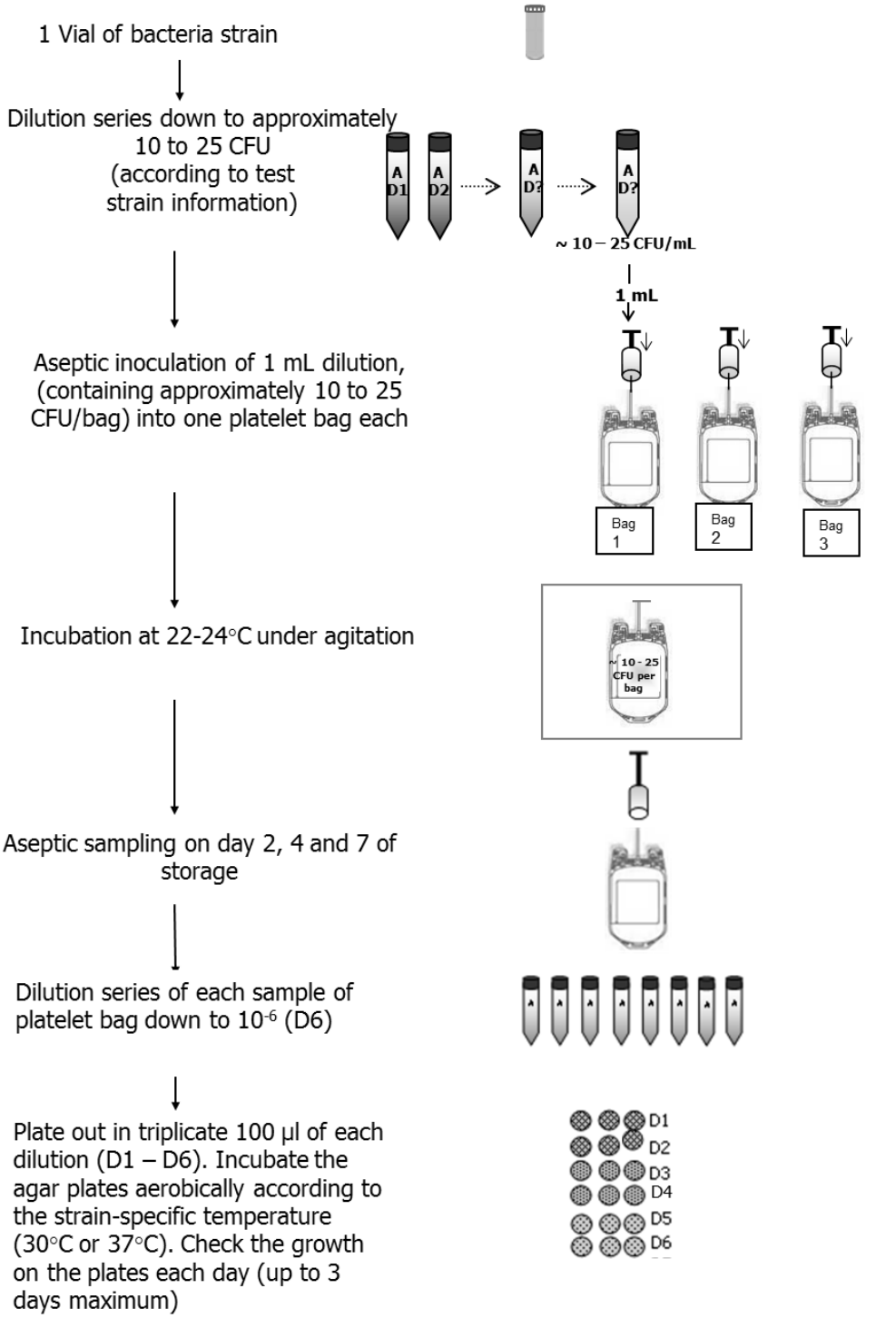


Fifteen selected bacterial strains (Table 1) were sent to the 14 study-partners frozen on dry ice primarily for the evaluation of bacterial strain growth properties in PCs. As shown in Fig. 1 enumeration and bacterial strain identification was performed for inoculums control and bacterial strain identity confirmation after growth in PCs. For each bacterial strain, three PCs (buffy coat derived pooled and/or apheresis PCs) were inoculated with 10-25 CFU/bag. Before inoculation the baseline sterility of the PCs was confirmed by microbiological control in accordance with the routine standard operating procedure used in the participating laboratory.

For inoculation, the bacterial reference strain solution was serially diluted in sterile NaCl to achieve a final concentration of ~10-25 CFU/mL in accordance with a standardized provided protocol (Fig. 2). To enumerate the inocula, 100 µl of the last three dilution steps were plated onto agar plates and colonies were counted the day after incubation. After contamination, the PCs were stored under blood bank routine storage conditions. Aseptic sampling was performed in accordance with the protocol on day 2, 4 and 7 from all 3 PC bags. A dilution series up to dilution 6 was performed from each sample (in total 9 dilution series per strain) and 100 µl of each dilution is plated in triplicate onto agar plates in accordance with the provided protocol. Colonies were counted after they became visible. Strain identification was performed from day 7 isolates of PC bag 1, 2 and 3 to guarantee that the inoculated bacterial strain grew in the PC bag.

The study protocol is attached in **Appendix 2.**

Fig.2: Inoculation and sampling procedure



Selection and characterization of bacterial candidate strains

In total, 15 bacteria strains (4 established repository strains, 11 new candidate strains, 4 vials each strain) were sent to the participants in purpose-built containers on dry ice. Each vial was labelled with the name of the bacterial strain and PEI-identification/lot number (PEI-B-P-XX).
The candidate strains are bacterial strains which were selected for their ability to replicate in PCs under routine storage conditions used in transfusion medicine. The strains are prepared using a specifically developed procedure which guarantees defined bacterial suspensions (deep frozen, ready to use, stable, shippable, defined in count of living cells) [7].

The enlargement strains are either isolates from blood products (blood bag and/or recipient) or tested regarding their growth ability in PCs at PEI. The bacterial strains were characterized for their ability to grow in PCs under current routine blood bank conditions (original bag volume, storage under agitation, temperature controlled).

Enumeration and stability testing was performed at PEI before and after production/deep freezing and, additionally, during the operating time of the study. For the stability testing six vials of each bacterial strain solution were defrosted and two dilution series 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) for one defined dilution of both dilution series. Thereafter, mean values were calculated.

The identity of the bacteria strains was tested by a combination of classical and molecular microbiological procedures. Classical characteristics used are growth properties, colony morphology, Gram-staining, and biochemical parameters like metabolism of certain sugars (API-System). Additionally, part of the 16s ribosomal RNA gene was sequenced.

Tab. 1: List of tested bacteria strains

First WHO Repository Platelet Transfusion-Relevant Bacteria Reference Strain	
Staphylococcus epidermidis	PEI-B-P-06
Streptococcus pyogenes	PEI-B-P-20
Escherichia coli	PEI-B-P-19
Klebsiella pneumoniae	PEI-B-P-08
Candidates for enlargement Study	
Bacillus cereus, spores *Bacillus thuringiensis,* spores	PEI-B-P-57 PEI-B-P-07
Enterobacter cloacae	PEI-B-P-43
Morganella morganii morganii	PEI-B-P-74
Proteus mirabilis	PEI-B-P-55
Pseudomonas fluorescens	PEI-B-P-77
Salmonella choleraesuis (heterotypic synonym: *Salmonella enterica)* Serotyp Heidelberg	PEI-B-P-78
Serratia marcescens	PEI-B-P-56
Staphylococcus aureus	PEI-B-P-63
Streptococcus dysgalactiae	PEI-B-P-71
*Streptococcus bovis (*old name – reclassified as *Streptococcus gallolyticus)*	PEI-B-P-61

Sterility control for baseline sterility of platelet concentrates

All PCs were sampled before bacterial inoculation to assure baseline sterility of the original platelet bags. Sterility testing was performed based on the commonly used methods in the laboratories (e.g. aerobic and anaerobic cultivation in automated systems).

Dilution procedure and artificial contamination

For low count spiking the bacteria reference strain solutions were diluted. The test strains were defrosted and vortexed for 15 seconds. Serial dilution of each vial was performed in sterile saline, as described in the dilution procedure in the protocol.
The first dilution was termed the D1 (10^{-1}) dilution, the following was termed D2, D3 etc. to the final dilution step containing ~10-25 CFU/mL. Inoculation of each PC bag was perfomed in accordance with the study protocol.

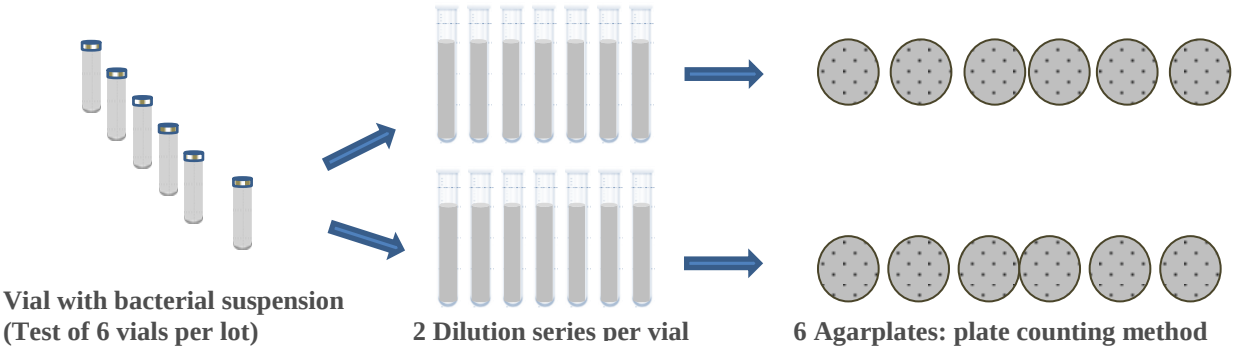
Sampling, enumeration and documentation

Sampling was performed on day 2, 4 and 7 (48, 96, 168 hours) after inoculation during PC storage (storage at 20 - 24°C under agitation). Sample withdrawal was performed following the study protocol (Fig.2).

Stability testing of the bacterial strains

The stability of all strains was tested at PEI continuously during the storage time and the results were statistically evaluated. For this purpose, 6 vials per lot (start, mid, end of production) were diluted and the colony forming units (CFU) were determined by plate counting method on 6 agar-plates in parallel (Fig.3).

Fig. 3: Inoculation and sampling procedure performed for stability testing



Statistical methods

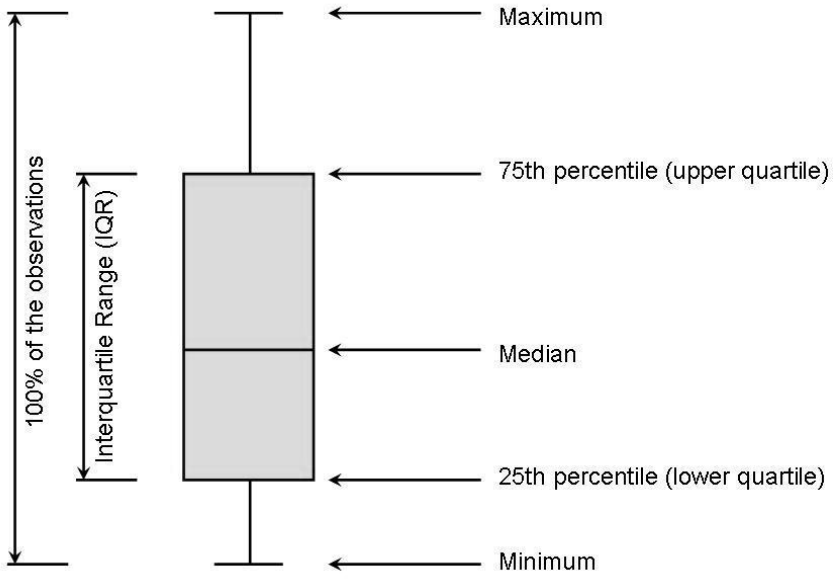
Statistical analysis was performed at PEI based on the raw data sent by the participants. Evaluation was based on $\log_{10}$ CFU/mL; zero CFU/mL concentrations were set to 0.1 before log-transformation. Taking into account an inoculation of 10-25 CFU/bag and bag sizes of 100 to 300 mL, inoculation per milliliter ranged from 0.033 to 0.250 CFU/mL (i.e. -1.48 to -0.60 $\log_{10}$ CFU/mL).

Growth data were analyzed per strain and day. Overall mean for each strain was estimated by means of a mixed linear model with log_{10} CFU as dependent variable and random factors bacterial strain and participant.

Analysis of stability of inoculum data at PEI was performed for up to five determinations per test strain by means of a linear regression model with dependent variable log_{10} CFU and date of determination as explanatory variable.

The statistical analysis was performed with SAS®/STAT software, version 9.3, SAS System for Windows. Results for bacterial growth were presented in Box- and-Whisker plots (Fig. 4.)

Fig. 4: Box-and-Whisker plots for growth

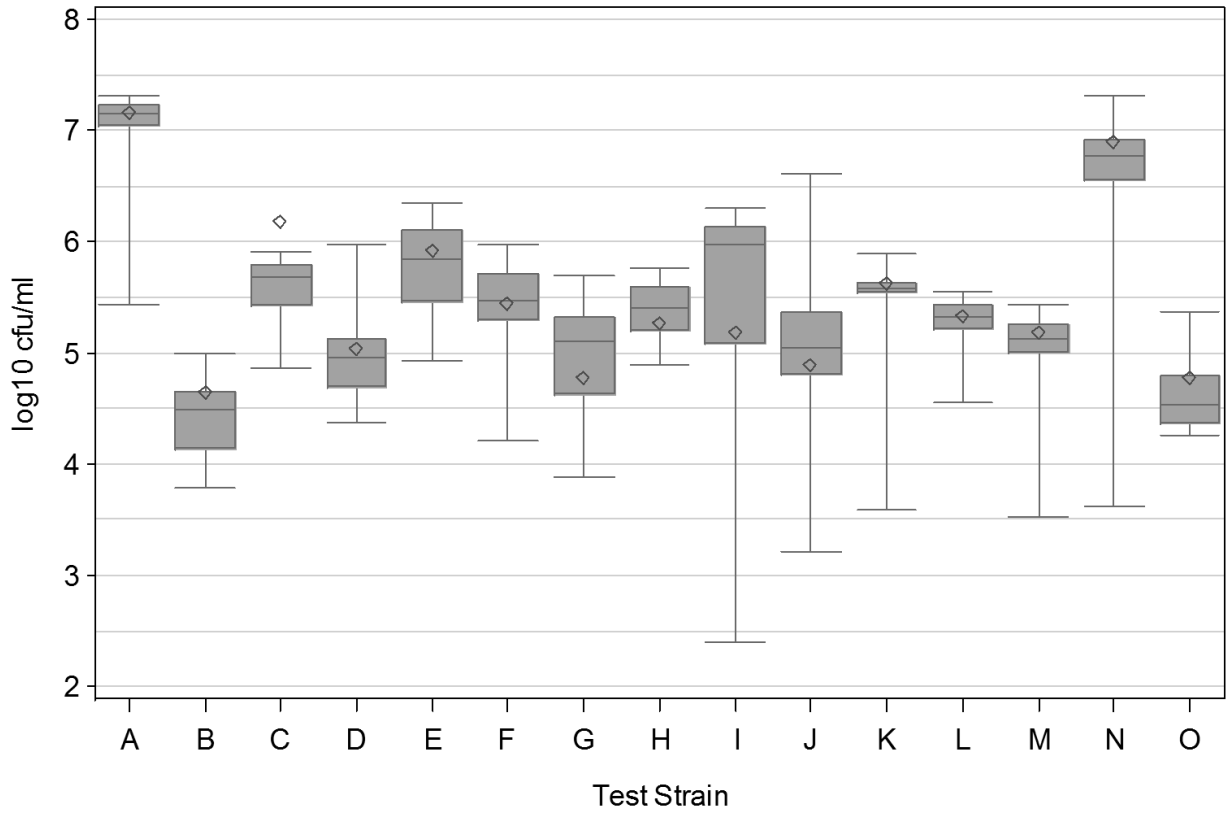


Results

Recovery of inoculum

The bacteria reference strain solutions were diluted for low count spiking. The cell count of each bacterial strain was provided in the study protocol and displayed in colony forming units per mL (CFU/mL). The match of inoculum by the participating labs was statistically evaluated and results are shown in Fig. 5 and 6.

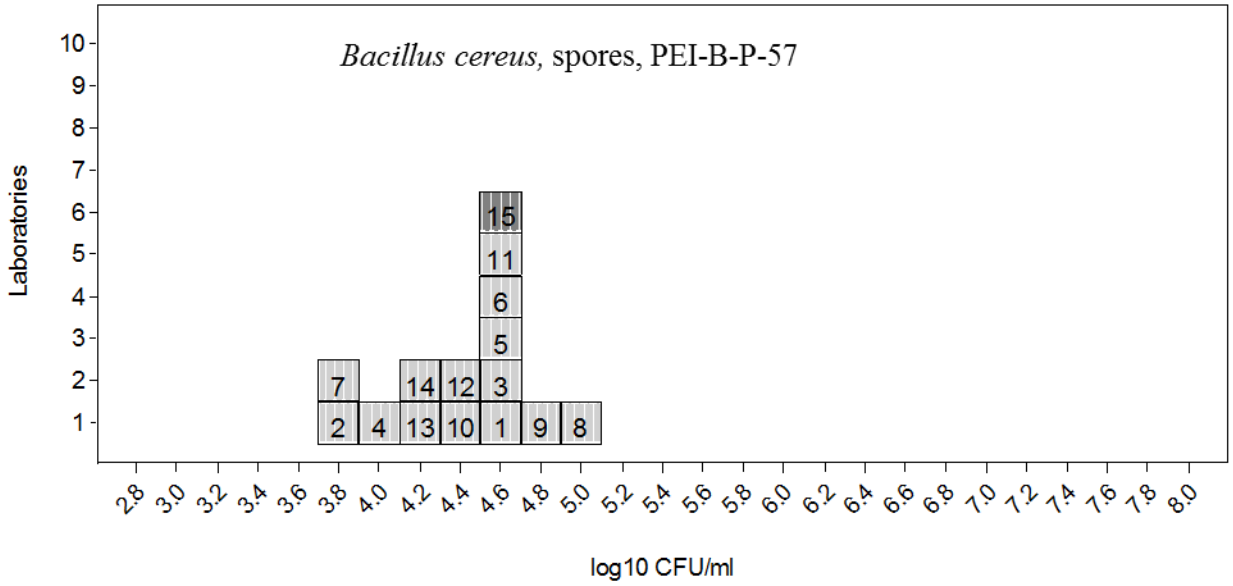
Fig. 5: Box-and-Whisker-Plot for the recovery of inoculum

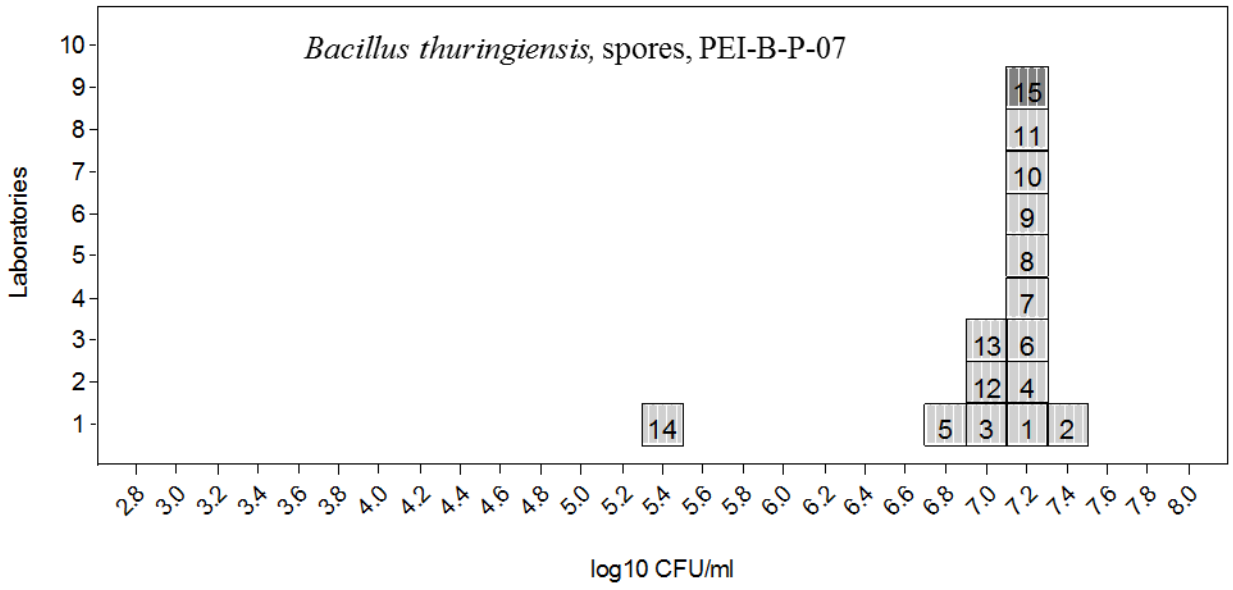


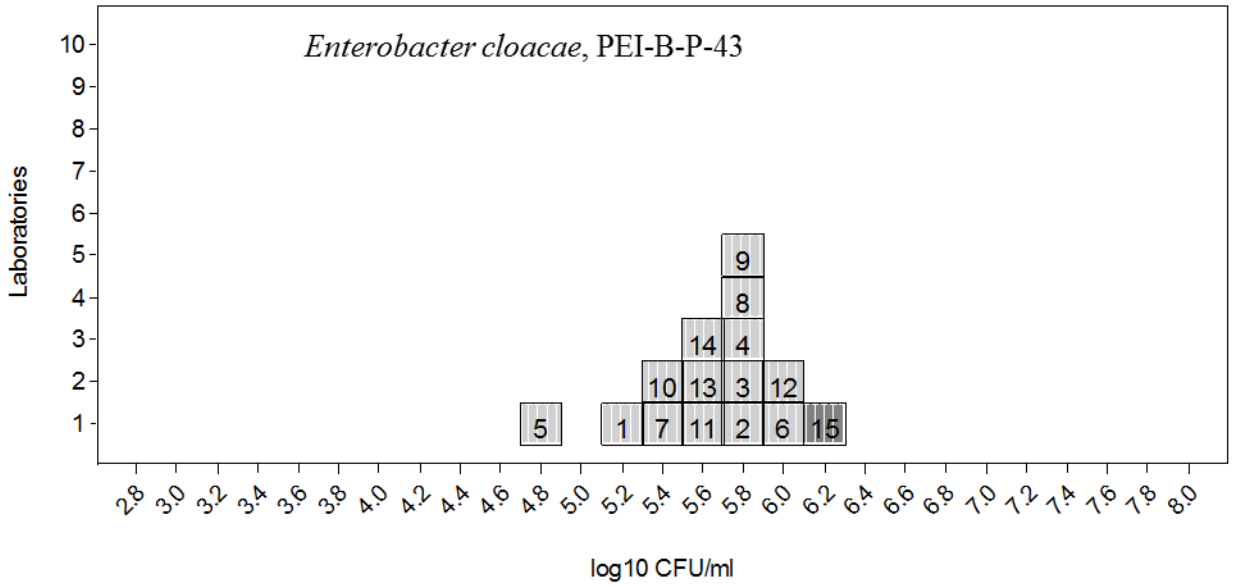
Box-and-Whisker-Plot of recovery of inoculum values by test strains (rhombus: PEI inoculum; A=*Bacillus cereus* spores, B=*Bacillus thuringiensis* spores, C=*Enterobacter cloacae*, D=*Escherichia coli*, E=*Klebsiella pneumoniae*, F=*Morganella morganii*, G=*Proteus mirabilis*, H=*Pseudomonas fluorescens*, I=*Salmonella choleraesuis*, J=*Serratia marcescens*, K=*Staphylococcus aureus*, L=*Staphylococcus epidermidis*, M=*Streptococcus bovis*, N=*Streptococcus dysgalactiae*, O=*Streptococcus pyogenes*)

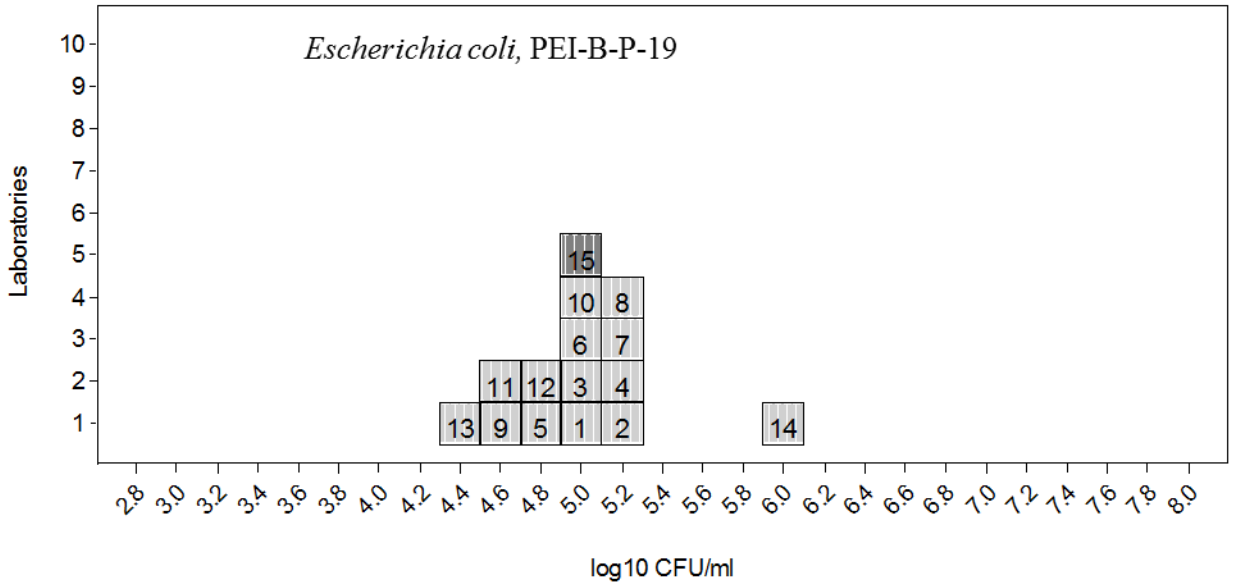
With the exception of *Enterobacter cloacae* (reflected as rhombus ◊ in Figure 5) the participating labs confirmed the inoculum value calculated by PEI. Nevertheless, *Enterobacter cloacae* was also inoculated successfully.

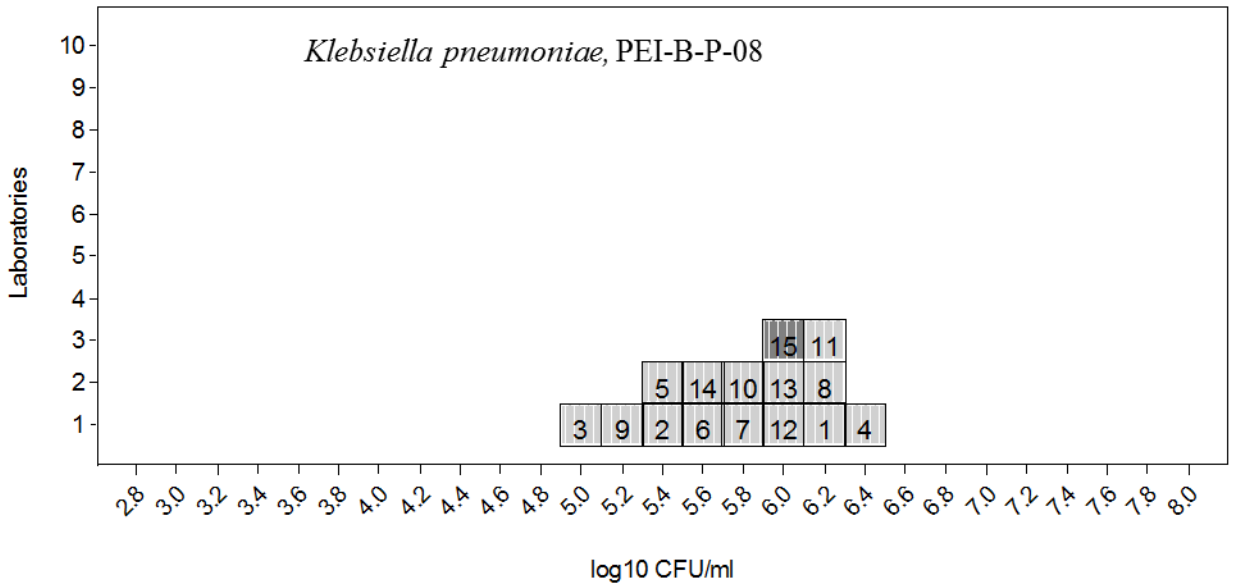
Fig. 6: Recovery of PEI inoculum values by participants, mean log_{10} CFU/mL plotted as stacked boxes (PEI results dark grey shaded).

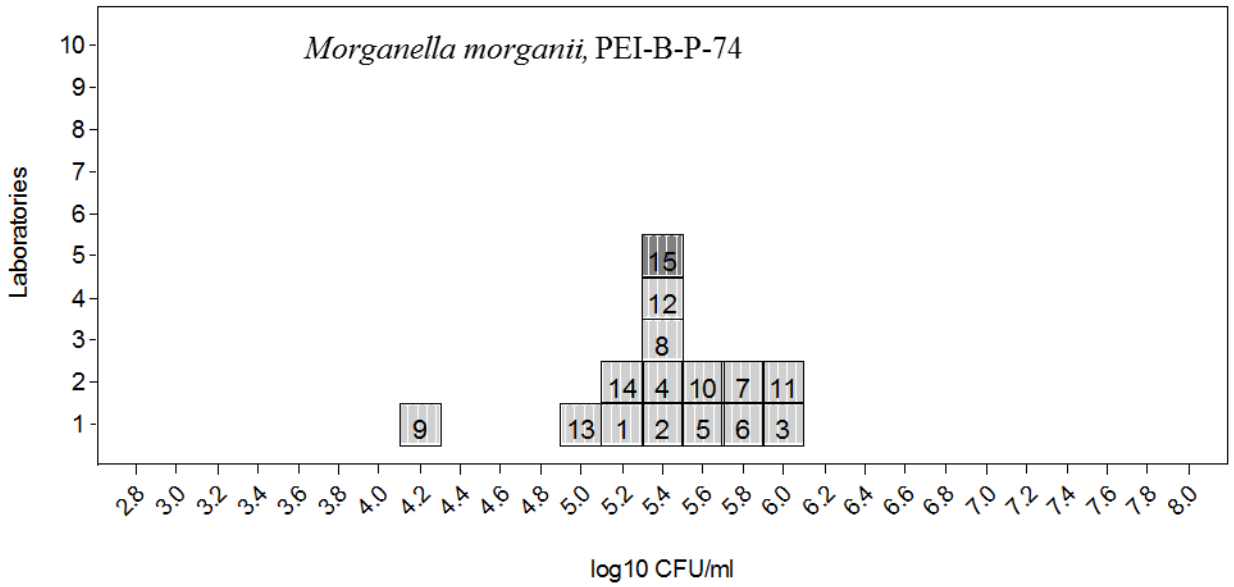


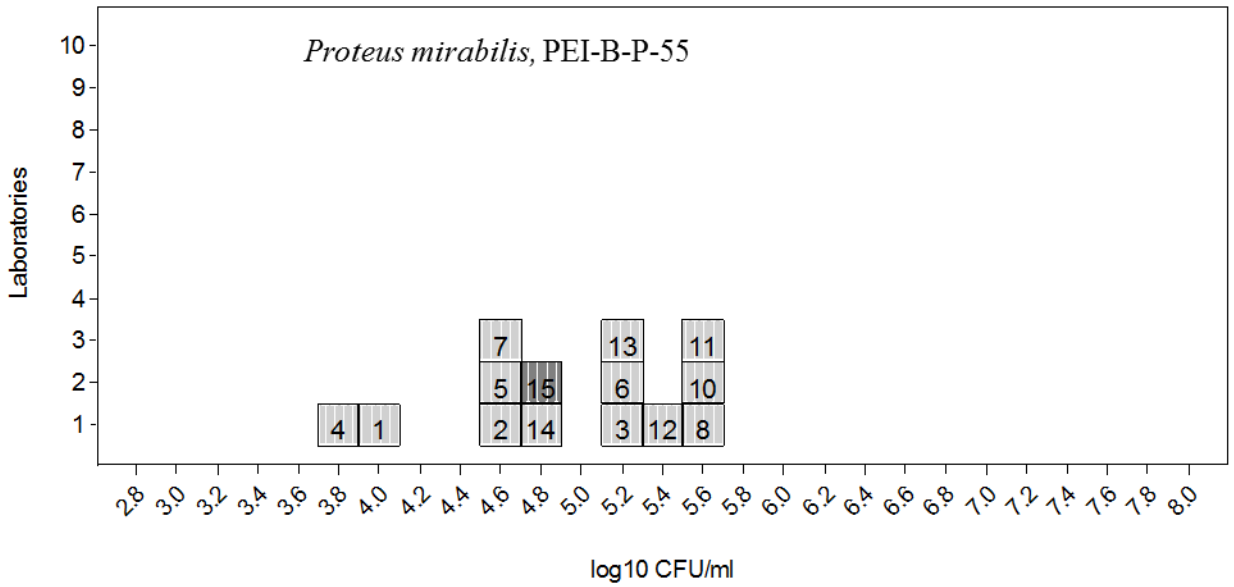


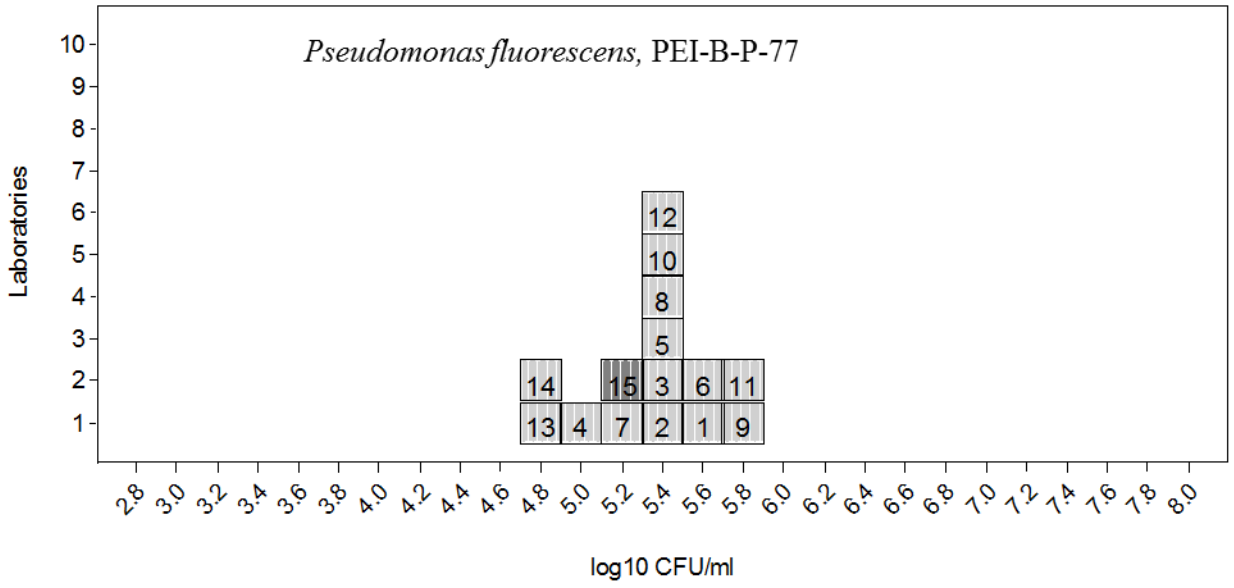

Enterobacter cloacae, PEI-B-P-43
Laboratories
log10 CFU/ml

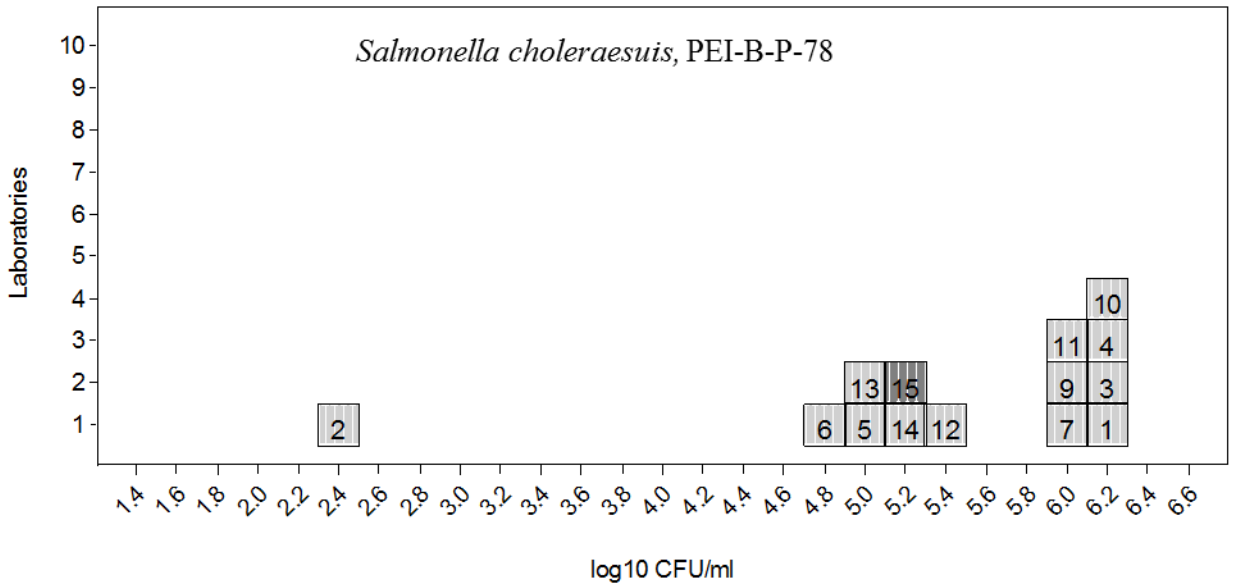

Escherichia coli, PEI-B-P-19
Laboratories
log10 CFU/ml

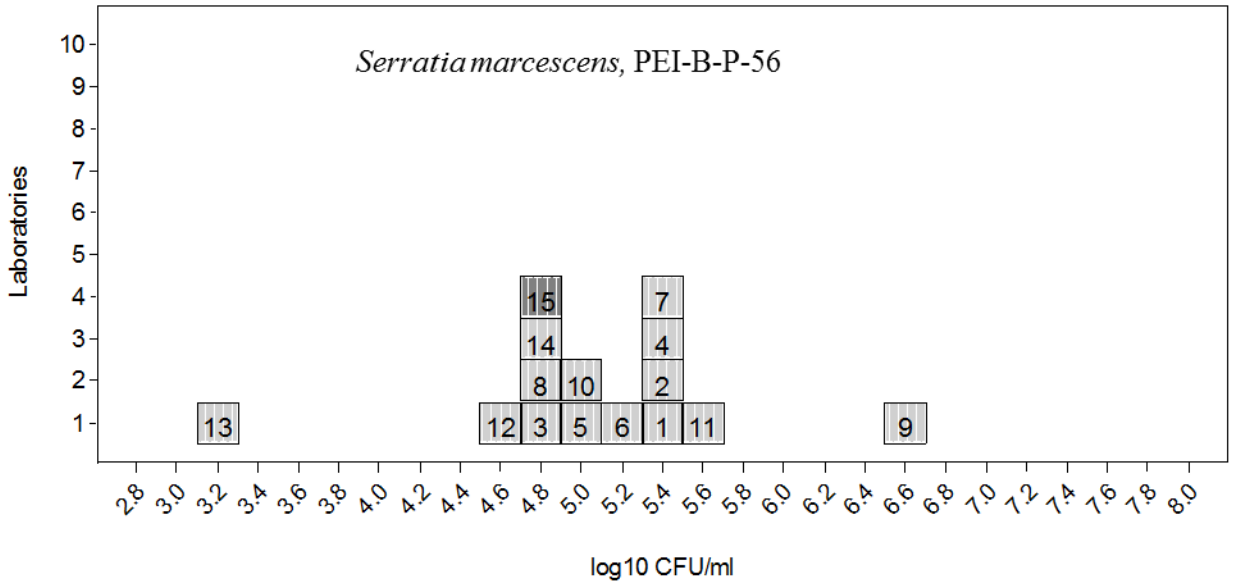

Klebsiella pneumoniae, PEI-B-P-08
Laboratories
log10 CFU/ml
3 9 2 6 7 12 1 4
5 14 10 13 8
15 11

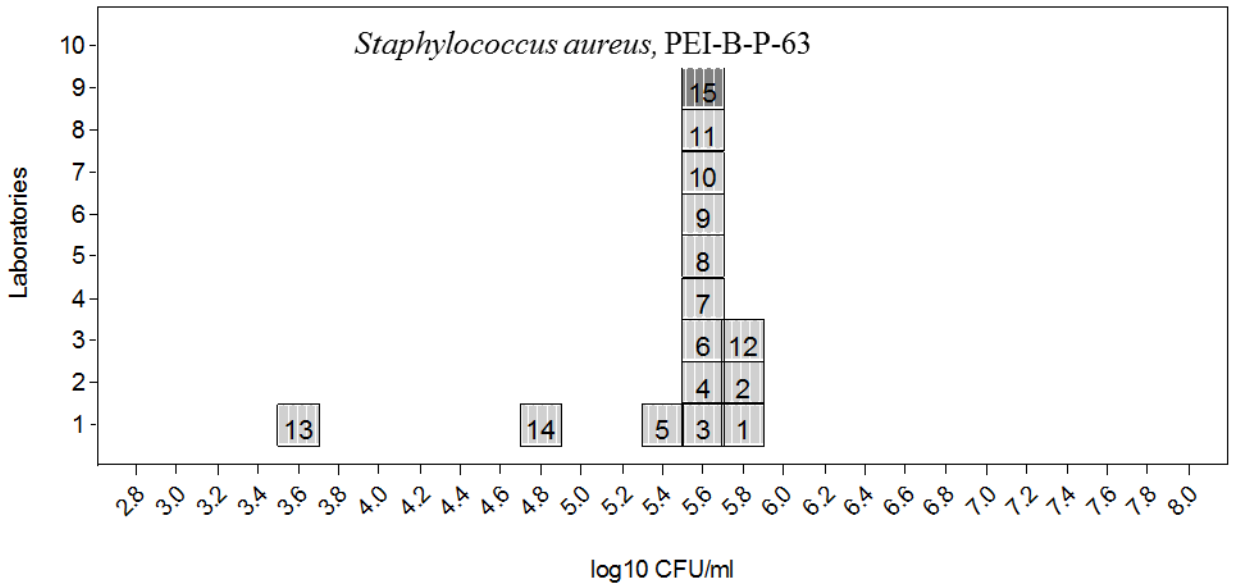

Morganella morganii, PEI-B-P-74
Laboratories
log10 CFU/ml
9
13 1 2 5 6 3
14 4 10 7 11
8
12
15

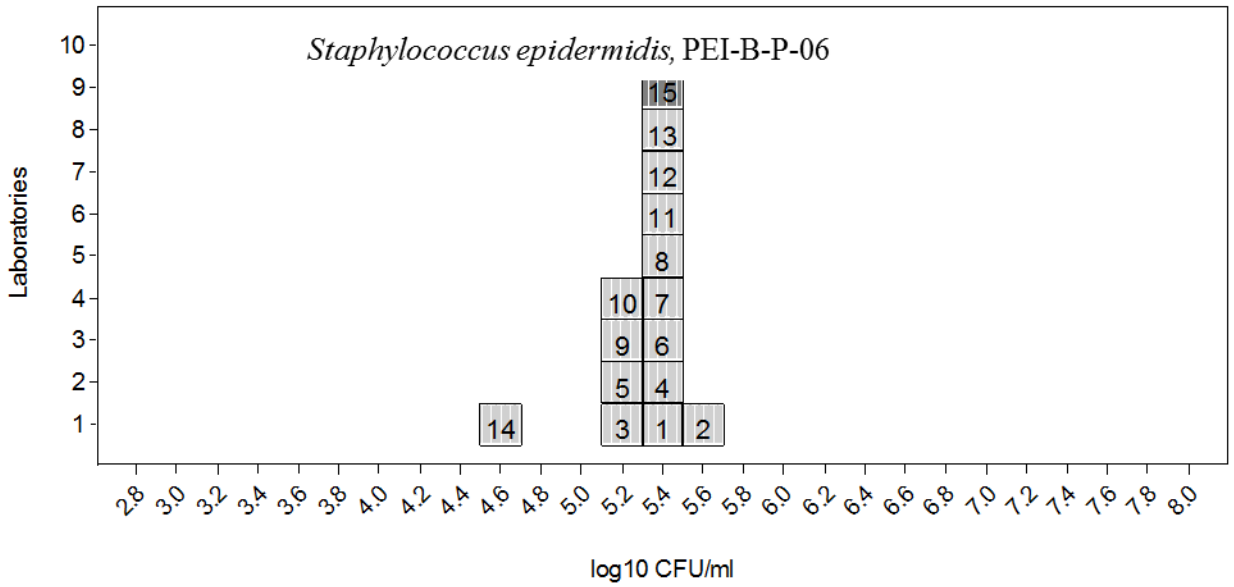

Proteus mirabilis, PEI-B-P-55
Laboratories
log10 CFU/ml

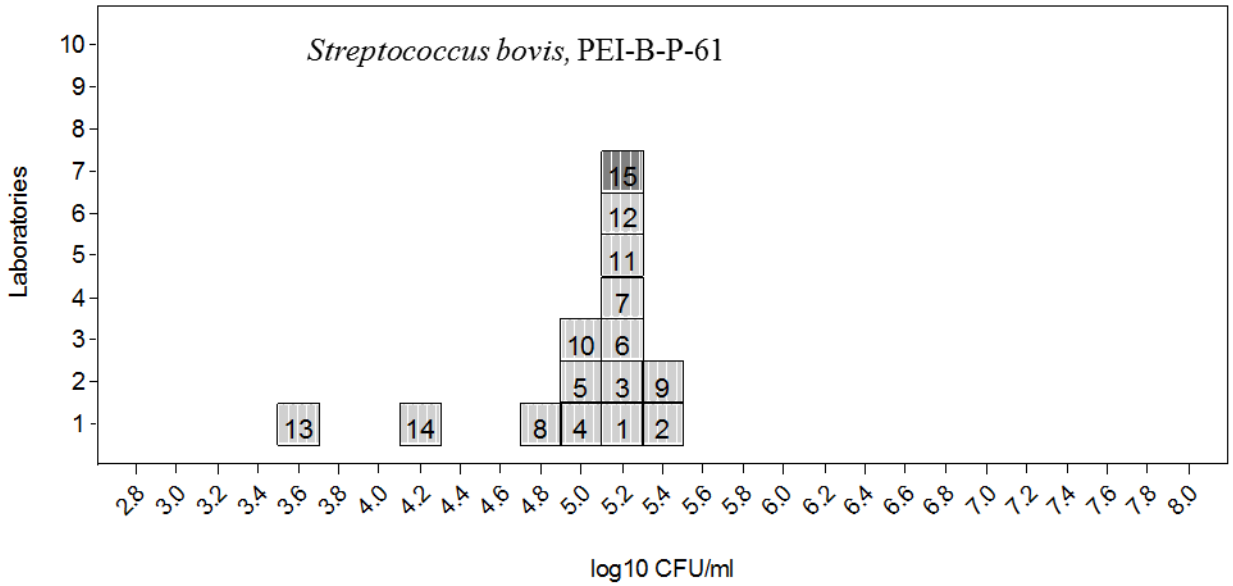

Pseudomonas fluorescens, PEI-B-P-77
Laboratories
log10 CFU/ml

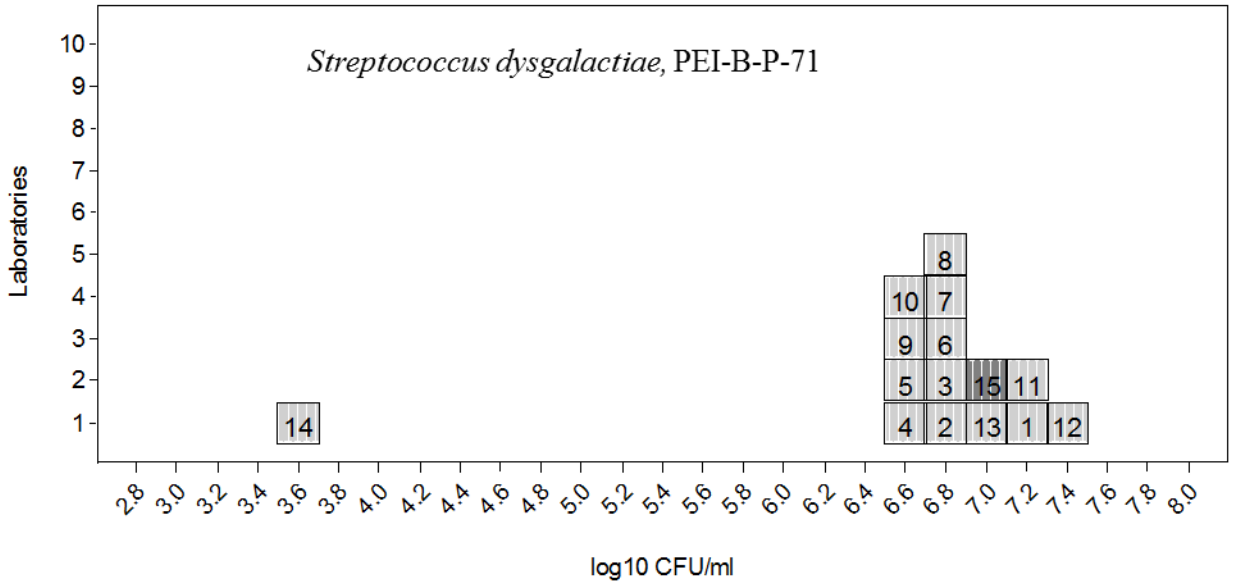

Salmonella choleraesuis, PEI-B-P-78
Laboratories
log10 CFU/ml

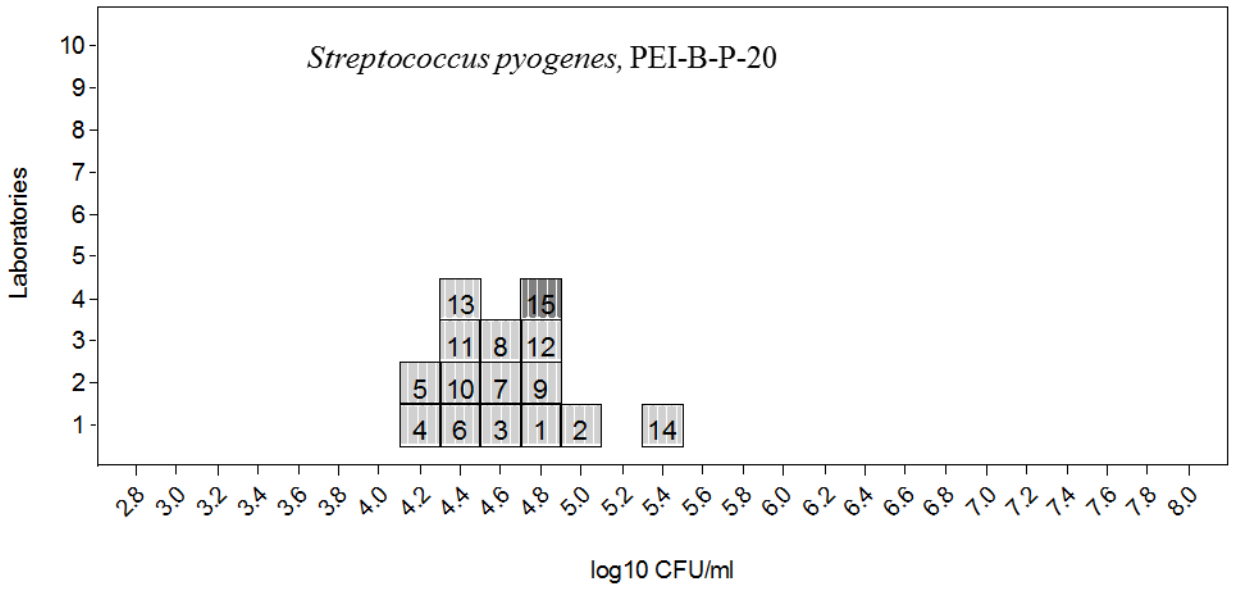

Serratia marcescens, PEI-B-P-56
Laboratories
log10 CFU/ml


Staphylococcus aureus, PEI-B-P-63
Laboratories
log10 CFU/ml


Staphylococcus epidermidis, PEI-B-P-06
Laboratories
log10 CFU/ml


Streptococcus bovis, PEI-B-P-61
Laboratories
log10 CFU/ml


Streptococcus dysgalactiae, PEI-B-P-71
Laboratories
log10 CFU/ml


Streptococcus pyogenes, PEI-B-P-20
Laboratories
log10 CFU/ml
13
15
11
8
12
5
10
7
9
4
6
3
1
2
14

Bacterial Growth in PCs

To gain more information about the growth behaviour of each bacterial strain, bacterial cell counting was performed at day 2, 4 and 7 after spiking. Cell counts are summarized in Table 2 and results are presented as Box- and Whisker plots in Fig. 7 and 8.

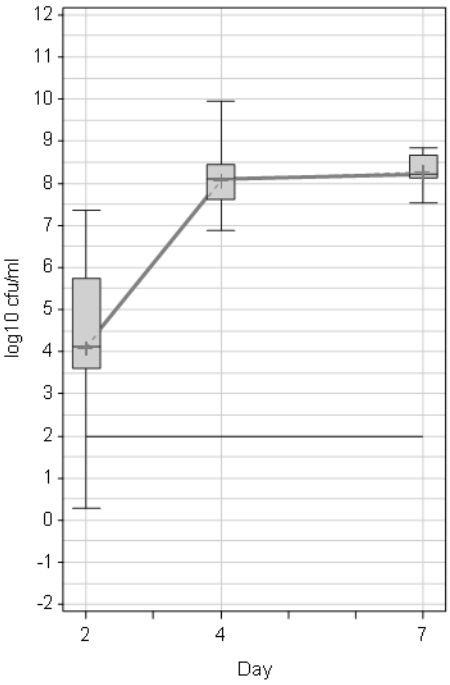
Tab. 2: Statistical evaluation of growth ability for each bacterial strain and day post inoculation

Bacterial Strain	Day	N[1]	Mean[2]	95% CI[3]		Median	Min	Max
Bacillus cereus spores	2	14	7.25	7.02	7.47	7.28	6.33	7.71
PEI-B-P-57	4	14	7.12	6.80	7.44	7.14	6.33	7.97
	7	14	6.79	6.52	7.07	6.82	5.87	7.66
Bacillus thuringiensis spores	2	14	6.21	5.21	7.21	6.71	0.94	7.79
PEI-B-P-07	4	14	5.86	4.87	6.86	6.34	0.79	7.77
	7	14	5.94	4.83	7.04	6.39	0.16	7.81
Enterobacter cloacae	2	14	3.24	0.90	5.58	3.51	-2.00	8.36
PEI-B-P-43	4	14	5.77	3.72	7.83	7.33	-2.00	8.64
	7	14	6.36	4.19	8.53	7.98	-2.00	9.23
Escherichia coli	2	14	4.08	2.89	5.27	4.11	0.28	7.36
PEI-B-P-19	4	14	8.06	7.64	8.49	8.09	6.88	9.95
1st WHO Repository	7	14	8.28	8.04	8.51	8.22	7.53	8.85
Klebsiella pneumoniae	2	14	7.80	6.68	8.92	8.56	1.67	8.96
PEI-B-P-08	4	14	8.44	7.23	9.65	8.98	1.80	11.03
1st WHO Repository	7	14	8.53	7.37	9.68	9.08	1.79	9.70
Morganella morganii	2	14	-0.51	-2.44	1.41	-2.00	-2.00	7.73
PEI-B-P-74	4	14	-0.74	-2.59	1.11	-2.00	-2.00	7.16
	7	14	-0.48	-2.38	1.42	-2.00	-2.00	7.61
Proteus mirabilis	2	14	1.00	-0.62	2.62	-0.16	-2.00	7.66
PEI-B-P-55	4	14	4.95	3.40	6.49	4.77	0.63	9.11
	7	14	7.78	6.31	9.24	8.86	0.77	10.23
Pseudomonas fluorescens	2	14	7.77	7.40	8.14	7.94	6.10	8.46
PEI-B-P-77	4	14	8.48	8.06	8.90	8.72	7.11	9.23
	7	14	9.12	8.71	9.53	9.19	7.90	10.76
Salmonella choleraesuis	2	14	1.81	-0.48	4.10	0.75	-2.00	8.46
PEI-B-P-78	4	14	2.87	0.47	5.27	3.26	-2.00	8.40
(heterotypic synonym: *Salmonella enterica)*	7	14	3.46	0.92	5.99	3.46	-2.00	9.04
Serratia marcescens	2	14	6.89	5.38	8.40	8.49	1.53	9.37
PEI-B-P-56	4	14	7.39	5.71	9.08	8.44	1.42	11.04
	7	14	7.64	5.90	9.38	9.03	0.84	10.03

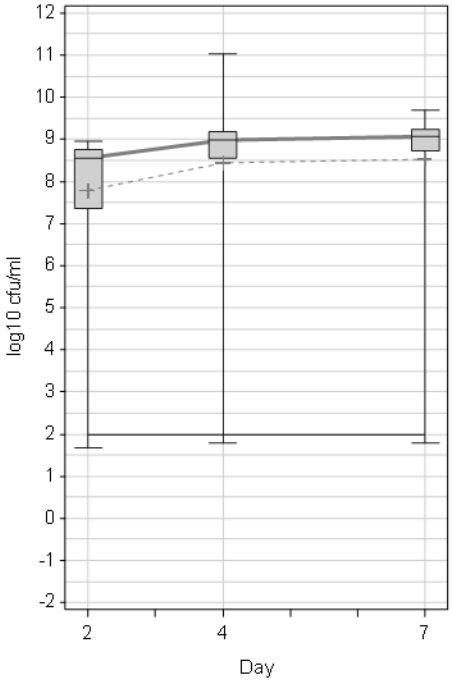
Bacterial Strain	Day	N[1]	Mean[2]	95% CI[3]		Median	Min	Max
Staphylococcus aureus	2	14	5.92	5.12	6.73	5.76	3.01	8.16
PEI-B-P-63	4	14	7.78	7.41	8.16	7.99	6.29	8.45
	7	14	8.08	7.63	8.54	8.36	5.90	9.25
Staphylococcus epidermidis	2	14	2.23	0.57	3.88	2.43	-2.00	6.69
PEI-B-P-06	4	14	6.37	5.22	7.51	7.17	2.76	8.56
1st WHO Repository	7	14	7.38	6.64	8.11	7.77	4.40	8.76
Streptococcus bovis	2	14	1.33	-0.35	3.00	1.07	-2.00	7.65
PEI-B-P-61	4	14	3.80	1.77	5.82	4.84	-2.00	7.45
(old name – reclassified: *Streptococcus gallolyticus)*	7	13	3.59	1.50	5.68	4.13	-2.00	7.10
Streptococcus dysgalactiae	2	14	4.78	3.12	6.44	5.45	-2.00	8.12
PEI-B-P-71	4	14	6.85	5.94	7.75	7.38	3.59	8.43
	7	14	6.50	5.65	7.36	7.02	3.87	8.00
Streptococcus pyogenes	2	14	2.33	0.09	4.58	1.89	-2.00	8.02
PEI-B-P-20	4	14	4.14	2.34	5.94	4.39	-2.00	7.93
1st WHO Repository	7	14	4.15	2.48	5.81	4.00	-2.00	7.86

Fig. 7: Box- and Whisker plots of growth kinetics - WHO-Repository strains

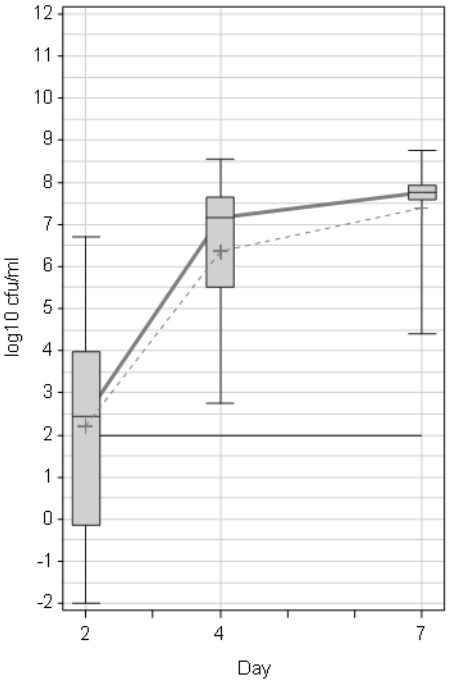
Escherichia coli, PEI-B-P-19



Klebsiella pneumoniae, PEI-B-P-08



Staphylococcus epidermidis, PEI-B-P-06



Streptococcus pyogenes, PEI-B-P-20

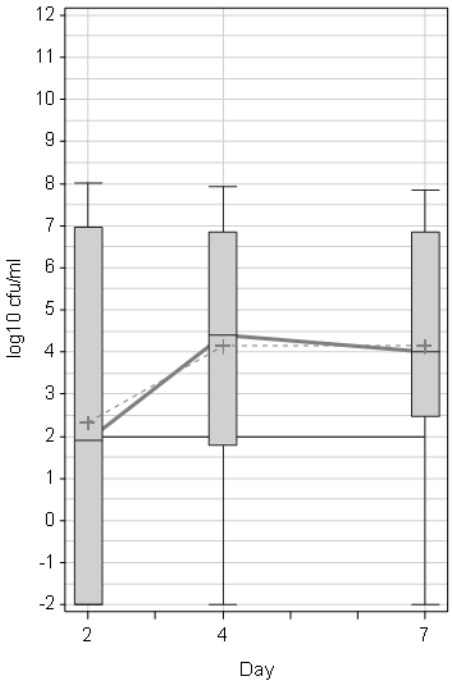
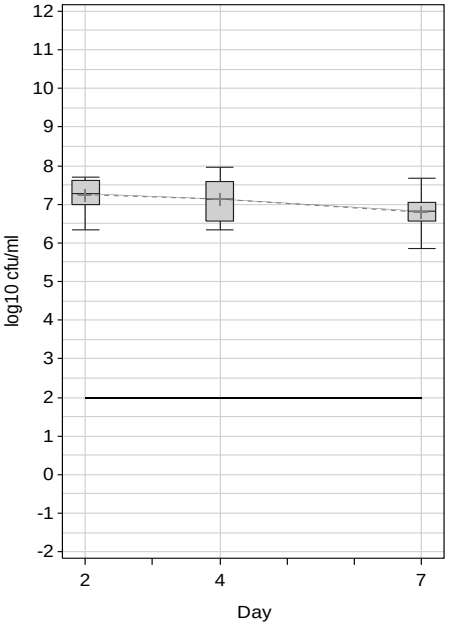
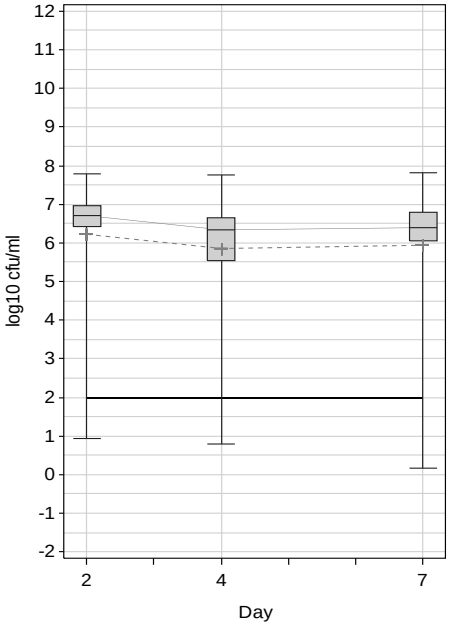


Fig. 8: Box- and Whisker plots of growth kinetics - Candidate strains

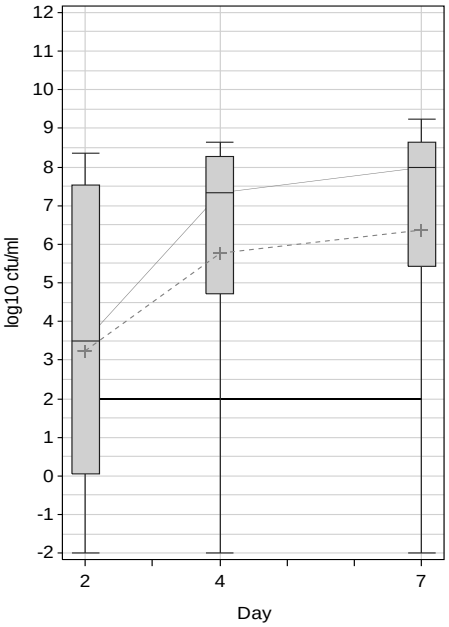
Bacillus cereus, spores, PEI-B-P-57



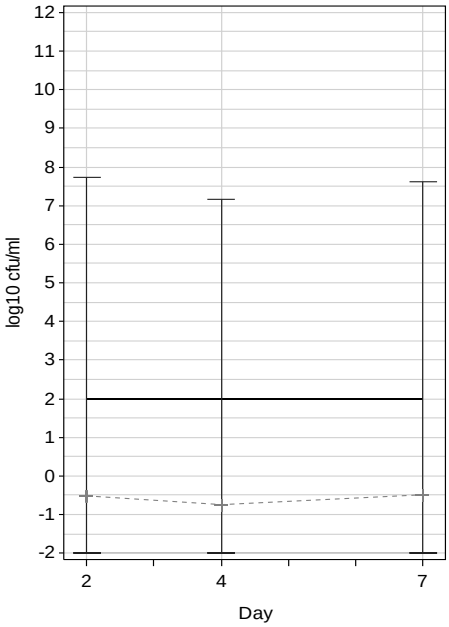
Bacillus thuringiensis, spores, PEI-B-P-07



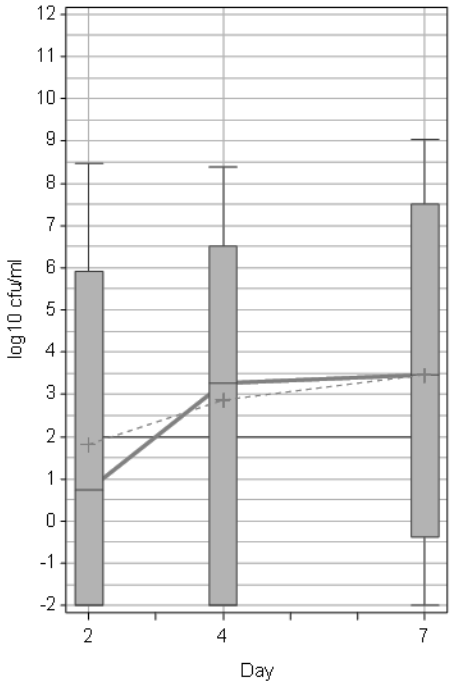
Enterobacter cloacae, PEI-B-P-43



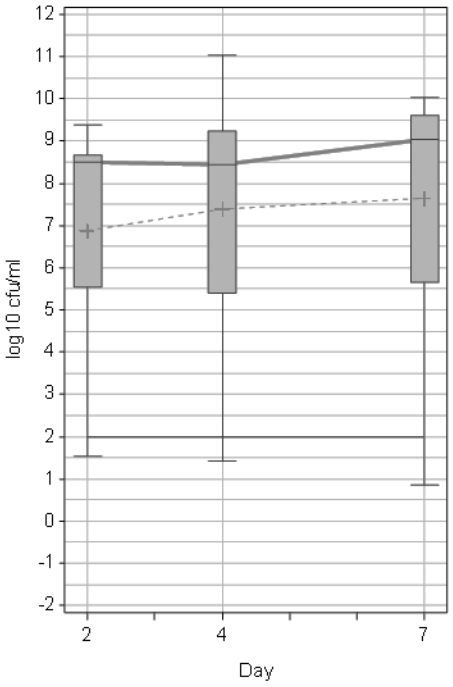
Morganella morganii, PEI-B-P-74



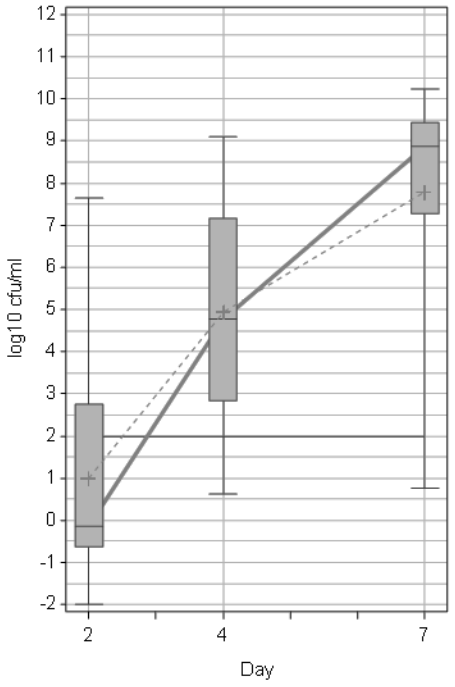
Salmonella choleraesuis, PEI-B-P-78



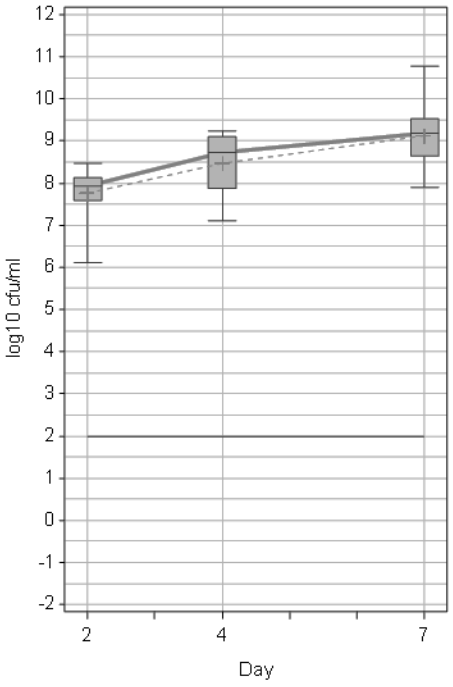
Serratia marcescens, PEI-B-P-56



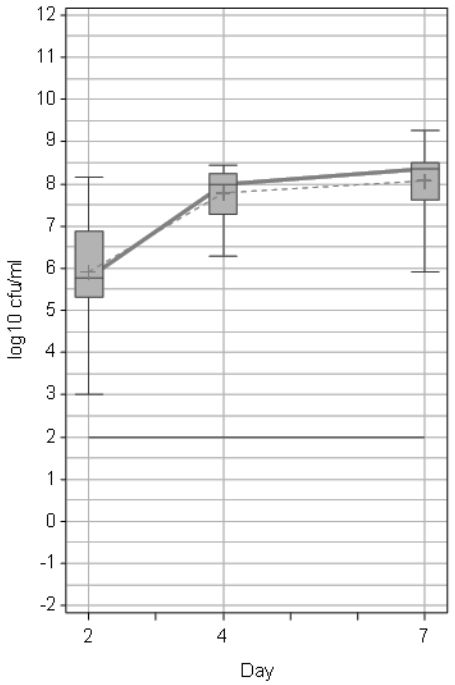
Proteus mirabilis, PEI-B-P-55



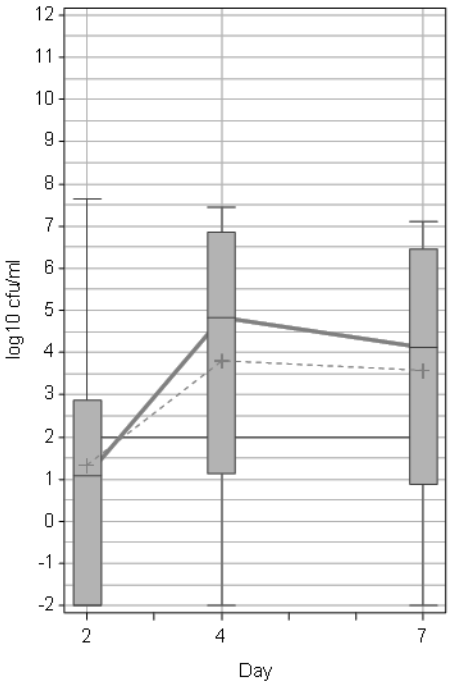
Pseudomonas fluorescens, PEI-B-P-77



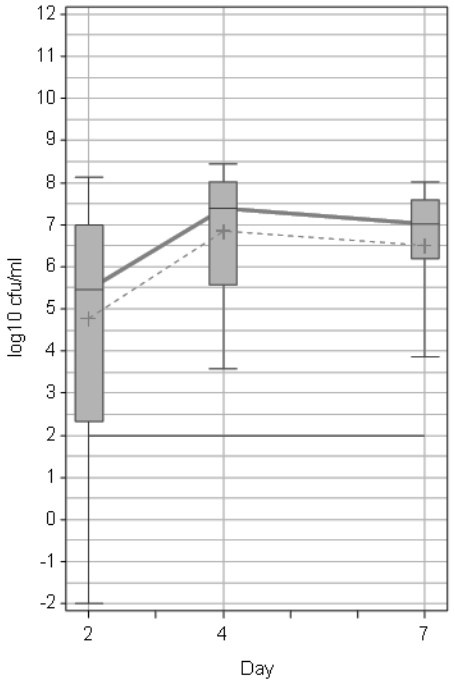
Staphylococcus aureus, PEI-B-P-63



Streptococcus bovis, PEI-B-P-61



Streptococcus dysgalactiae, PEI-B-P-71



Stability testing

Tab. 3: Estimation of stability parameters by means of a Linear Regression (Intercept and Slope=Time) for each test strain

Bacterial Strain	Variable	Estimate	LowerCL	UpperCL
Bacillus cereus spores	Intercept	7.23	7.15	7.32
PEI-B-P-57	Day[1]	-0.00005	-0.00023	0.00013
Bacillus thuringiensis spores	Intercept	4.67	4.64	4.70
PEI-B-P-07	Day[1]	-0.00004	-0.00008	0.00000
Enterobacter cloacae	Intercept	6.02	5.88	6.17
PEI-B-P-43	Day[1]	0.00045	0.00022	0.00068
Escherichia coli	Intercept	5.12	5.02	5.21
PEI-B-P-19, 1st WHO Repository	Day[1]	-0.00022	-0.00045	0.00001
Klebsiella pneumoniae	Intercept	6.00	5.88	6.13
PEI-B-P-08, 1st WHO Repository	Day[1]	-0.00011	-0.00029	0.00006
Morganella morganii	Intercept	5.84	5.44	6.24
PEI-B-P-74	Day[1]	-0.00145	-0.00233	-0.00057
Proteus mirabilis	Intercept	4.88	4.57	5.19
PEI-B-P-55	Day[1]	0.00003	-0.00048	0.00053
Pseudomonas fluorescens	Intercept	5.30	5.14	5.47
PEI-B-P-77	Day[1]	-0.00015	-0.00044	0.00014
Salmonella choleraesuis (heterotypic synonym: *Salmonella enterica)*	Intercept	5.52	5.13	5.91
PEI-B-P-78	Day[1]	-0.00096	-0.00157	-0.00035
Serratia marcescens	Intercept	5.12	4.79	5.45
PEI-B-P-56	Day[1]	-0.00033	-0.00085	0.00019
Staphylococcus aureus	Intercept	5.66	5.63	5.69
PEI-B-P-63	Day[1]	-0.00009	-0.00013	-0.00004
Staphylococcus epidermidis	Intercept	5.33	5.31	5.35
PEI-B-P-06, 1st WHO Repository	Day[1]	0.00000	-0.00004	0.00004
Streptococcus bovis (old name – reclassified: *Streptococcus gallolyticus)*	Intercept	5.18	5.14	5.22
PEI-B-P-61	Day[1]	-0.00001	-0.00008	0.00006
Streptococcus dysgalactiae	Intercept	6.99	6.88	7.11
PEI-B-P-71	Day[1]	-0.00005	-0.00023	0.00014
Streptococcus pyogenes	Intercept	4.81	4.72	4.90
PEI-B-P-20, 1st WHO Repository	Day[1]	0.00000	-0.00017	0.00017
1 – Decrease per day [$\log_{10}$ CFU/mL]				

Fig. 9: Stability WHO-Repository Strains (mean values) performed by PEI

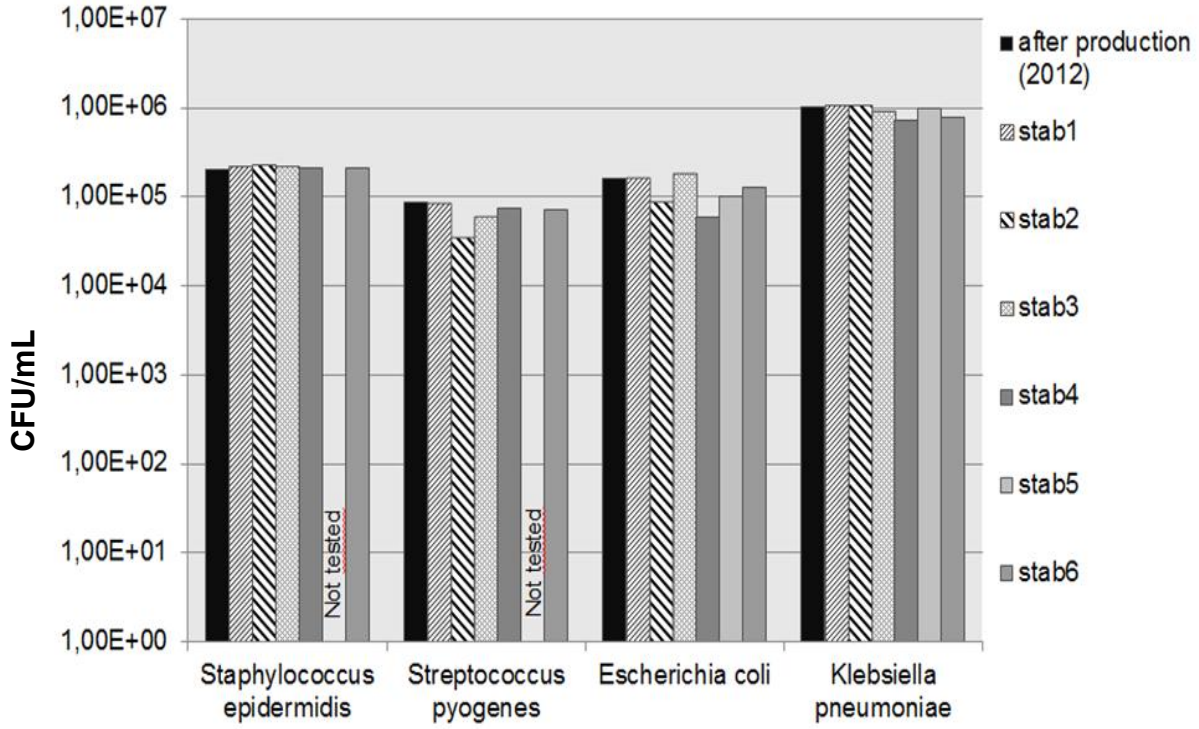
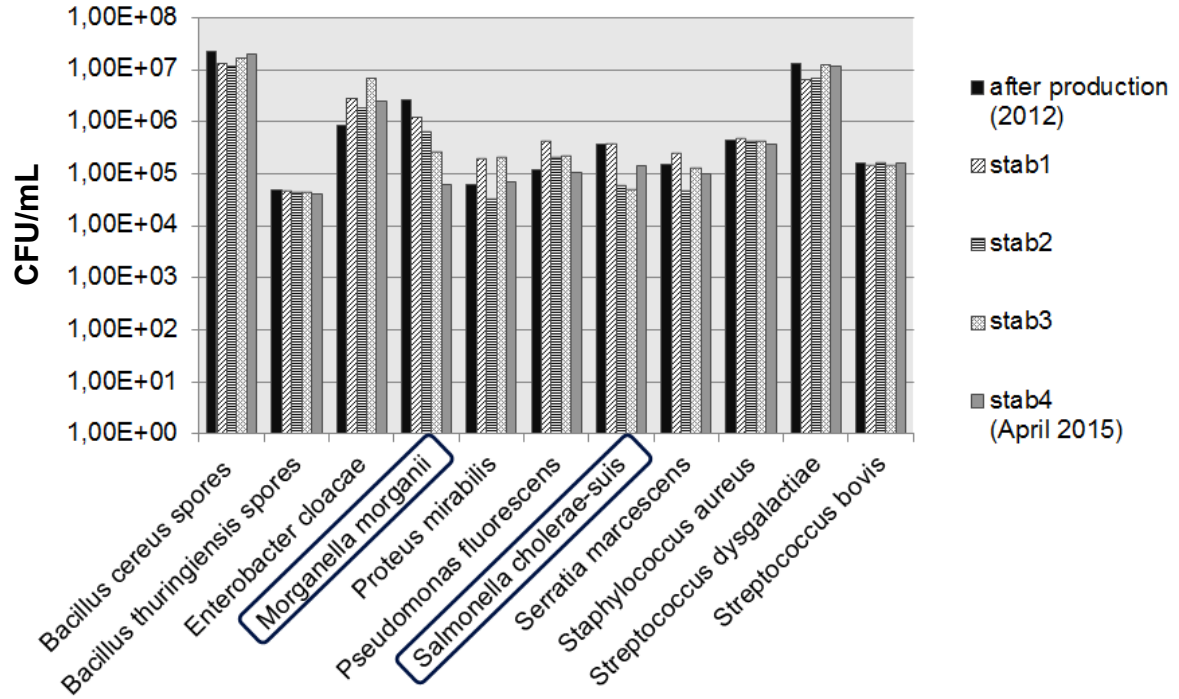


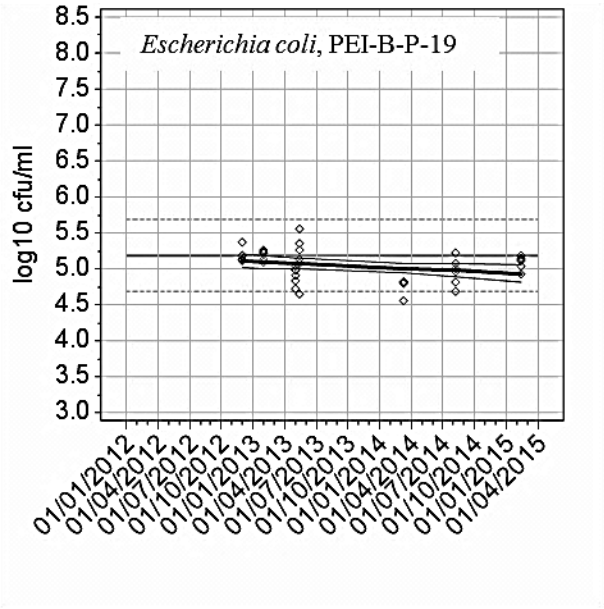
Fig. 10: Stability Candidate Strains (mean values) performed by PEI

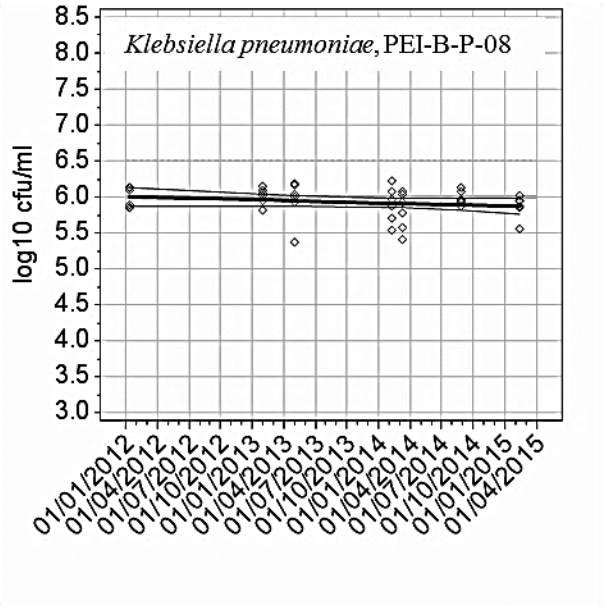


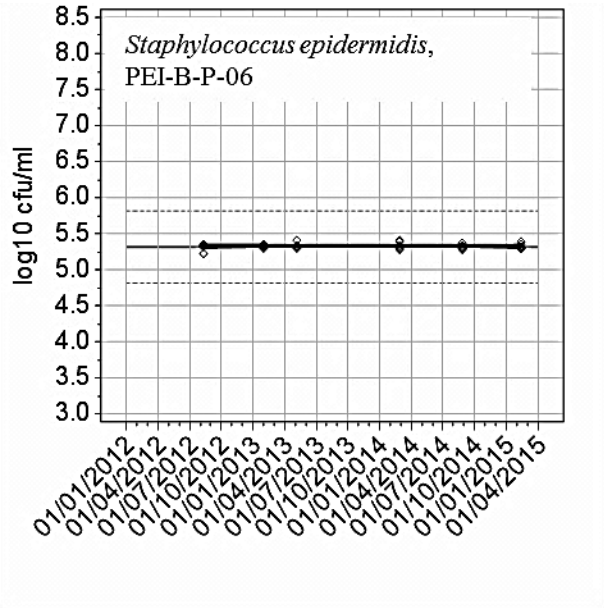
Stability testing – Plots

Specification was set by data from first determination at PEI as mean (bold base line) ± 0.5 $\log_{10}$ CFU/mL (dotted lines). A strain can be regarded as stable until the lower 95% confidence interval for an individual prediction (thin line; including variance of the error as well as variance of the parameter estimates) for the linear trend (thick line) intersects the lower specification limit (dotted line).

Fig 11: Stability WHO-Repository Strains performed by PEI







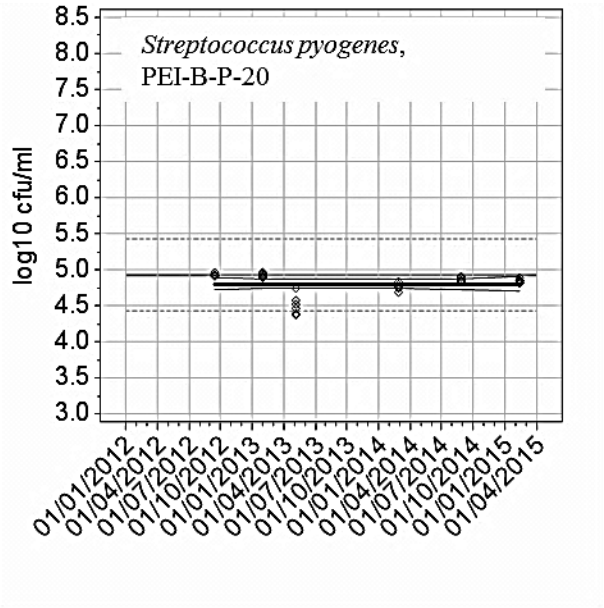
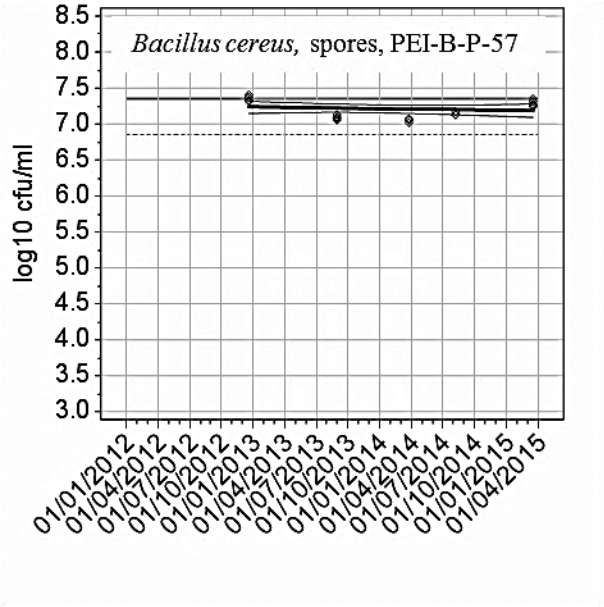
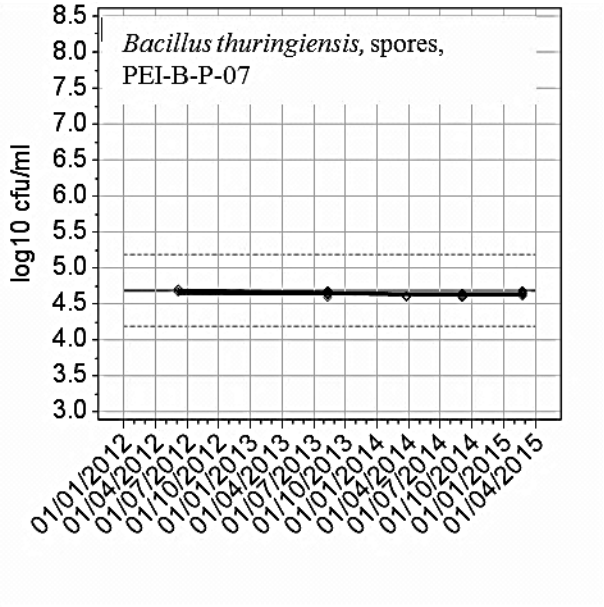
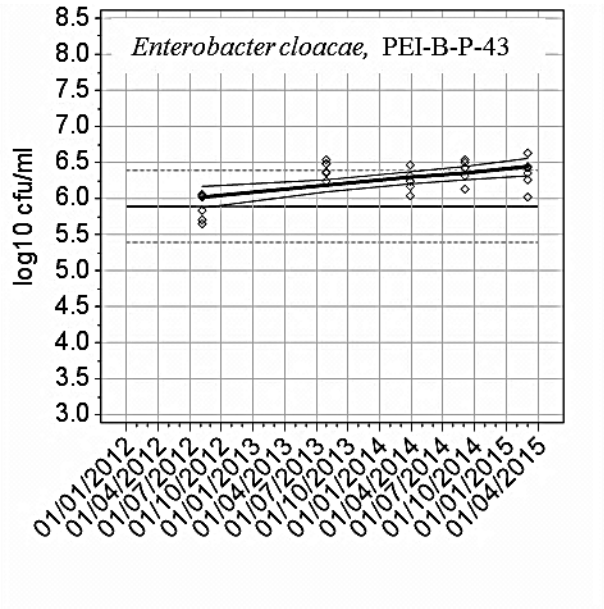
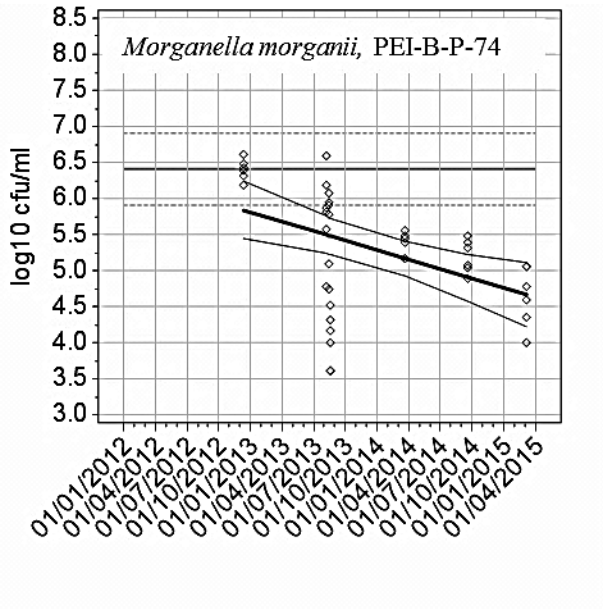


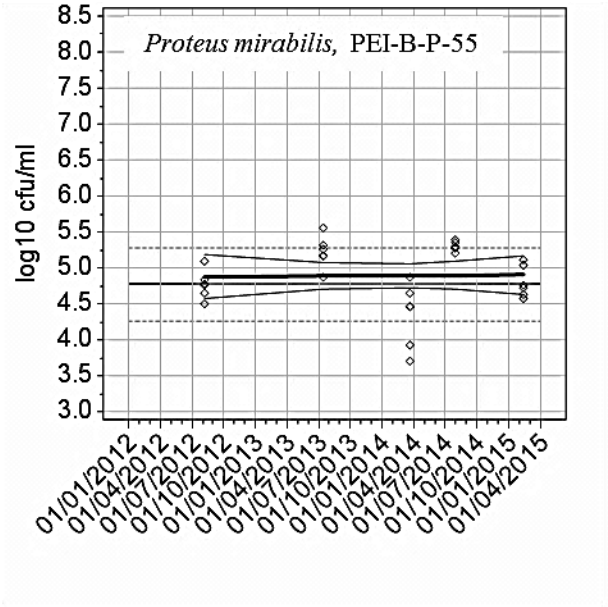
Fig 12: Stability Candidate strains

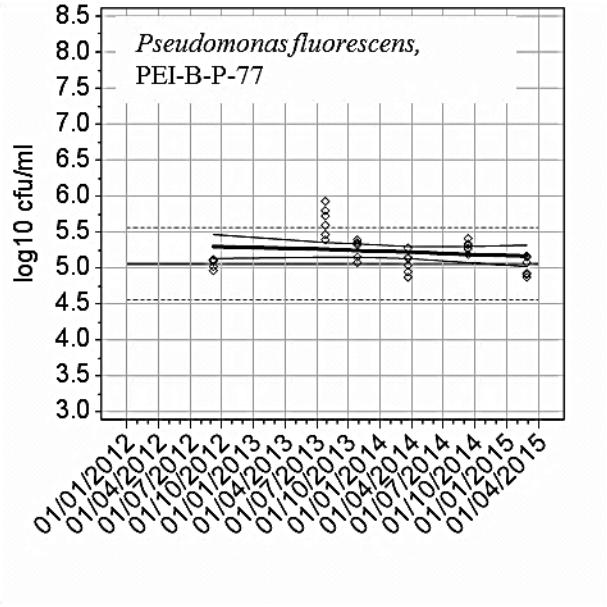


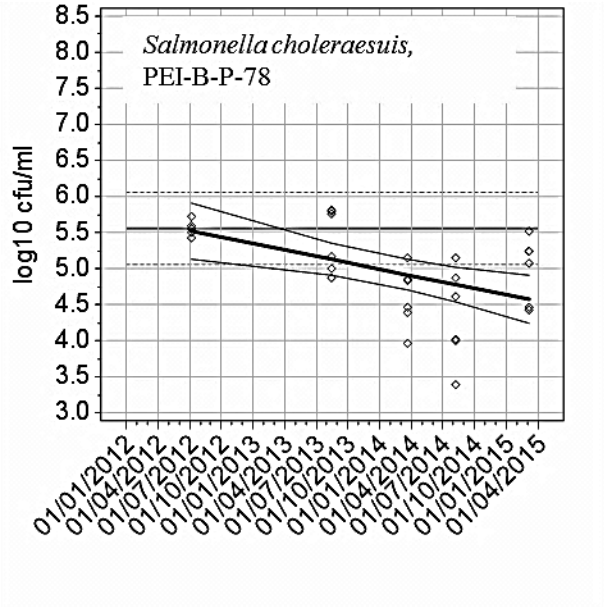


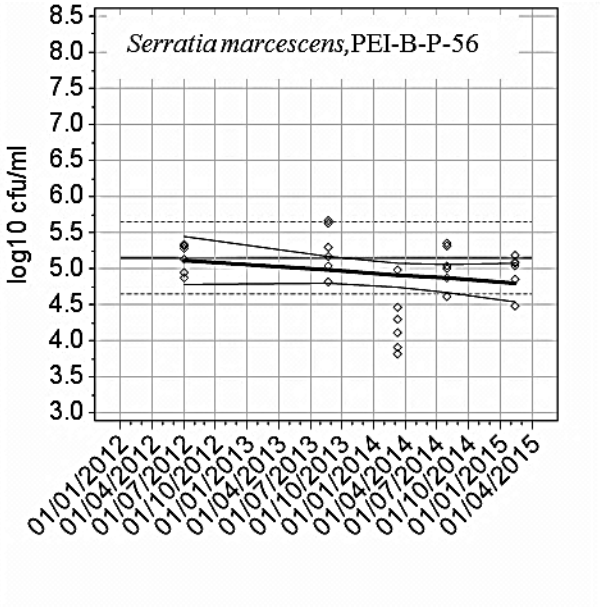


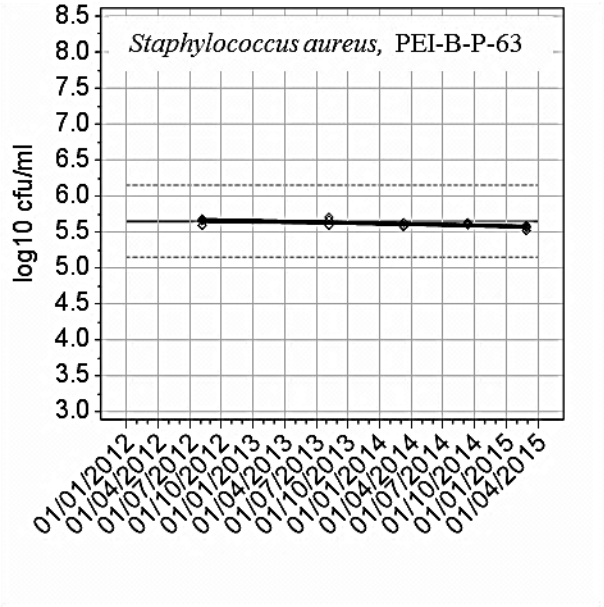


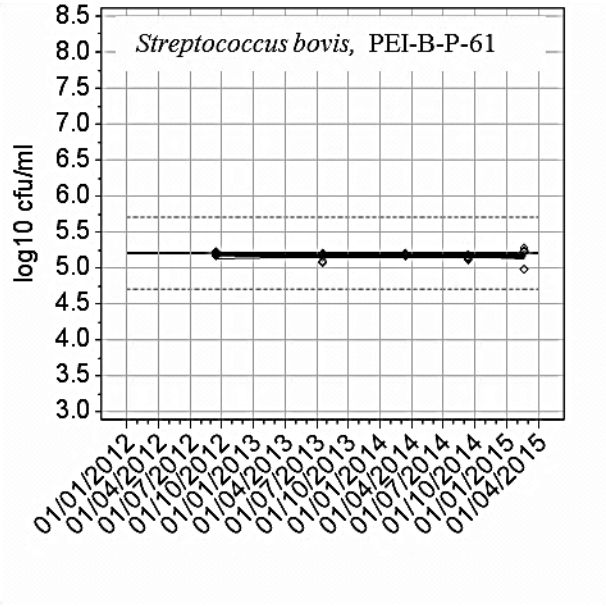

Proteus mirabilis, PEI-B-P-55
log10 cfu/ml
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
01/01/2012
01/04/2012
01/07/2012
01/10/2012
01/01/2013
01/04/2013
01/07/2013
01/10/2013
01/01/2014
01/04/2014
01/07/2014
01/10/2014
01/01/2015
01/04/2015

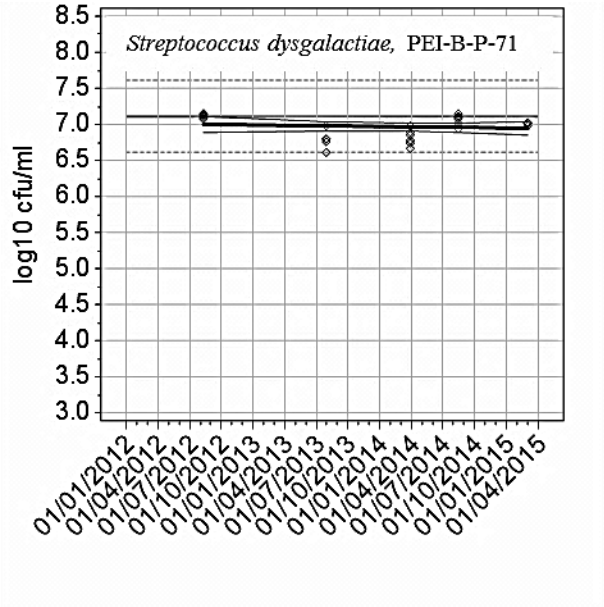

Pseudomonas fluorescens,
PEI-B-P-77
log10 cfu/ml
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
01/01/2012
01/04/2012
01/07/2012
01/10/2012
01/01/2013
01/04/2013
01/07/2013
01/10/2013
01/01/2014
01/04/2014
01/07/2014
01/10/2014
01/01/2015
01/04/2015


Salmonella choleraesuis,
PEI-B-P-78
log10 cfu/ml
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
01/01/2012
01/04/2012
01/07/2012
01/10/2012
01/01/2013
01/04/2013
01/07/2013
01/10/2013
01/01/2014
01/04/2014
01/07/2014
01/10/2014
01/01/2015
01/04/2015


Serratia marcescens, PEI-B-P-56
log10 cfu/ml
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
01/01/2012
01/04/2012
01/07/2012
01/10/2012
01/01/2013
01/04/2013
01/07/2013
01/10/2013
01/01/2014
01/04/2014
01/07/2014
01/10/2014
01/01/2015
01/04/2015


Staphylococcus aureus, PEI-B-P-63
log10 cfu/ml
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
01/01/2012
01/04/2012
01/07/2012
01/10/2012
01/01/2013
01/04/2013
01/07/2013
01/10/2013
01/01/2014
01/04/2014
01/07/2014
01/10/2014
01/01/2015
01/04/2015


Streptococcus bovis, PEI-B-P-61
log10 cfu/ml
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
01/01/2012
01/04/2012
01/07/2012
01/10/2012
01/01/2013
01/04/2013
01/07/2013
01/10/2013
01/01/2014
01/04/2014
01/07/2014
01/10/2014
01/01/2015
01/04/2015


Streptococcus dysgalactiae, PEI-B-P-71
log10 cfu/ml
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
01/01/2012
01/04/2012
01/07/2012
01/10/2012
01/01/2013
01/04/2013
01/07/2013
01/10/2013
01/01/2014
01/04/2014
01/07/2014
01/10/2014
01/01/2015
01/04/2015

Bacillus thuringiensis spores, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus bovis* showed stability with good precision. Another six strains, *Bacillus cereus* spores, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas fluorescens, Streptococcus dysgalactiae*, and *Streptococcus pyogenes*, are stable over the observed period with variability of data. For *Pseudomonas fluorescens* and *Streptococcus pyogenes* this could be due to outliers at the second (third) timepoint. *Enterobacter cloacae* was stable until April 2014 (upper prediction limit intersects the dotted specification line). Here a sampling error at the first stability testing might be responsible for the "increase" in bacteria cells during storage. *Serratia marcescens* showed stability until July 2014 (for 1.5 years). This could be due to outliers at the third timepoint. The two remaining strains (*Morganella morganii* and *Salmonella choleraesuis)* are less stable than the other strains.

Bacterial Identification

After the testing of the growth kinetics of the bacterial strains the participants identified the strains following their routine protocols as used in the respective microbiological lab (Tab. 4).
The results of identification corresponded with the results provided by PEI. According to classification issues *Enterobacter cloacae* is a gene complex containing six genovars. One of them is *Enterobacter hormaechei steigerwaltii*. *Salmonella choleraesuis* (hemolytic synonym and serotype of *S.enterica*) identified as *Salmonella enterica*. *Streptococcus bovis* (biotype I, taxonomy of the group) is currently named *Streptococcus gallolyticus pasteurianus* due to phylogenetic results.

Tab. 4: Identification of the strains by the participating labs

Method of Identification		PEI		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13	Lab 14
	AccuGENX-ID	MALDI-TOF ID	AccuPRO-ID	Gram stain/API	Gram stain/API	Maldi-TOF MS /Vitek MS	BD Phoenix Identification	Vite ID / BBL chystal gram positive 4.0	Mass spectrometry	Gram stain/Colony morphology	Maldi-TOF	Gram stain / Microscan/ API	MALDI mass spectrometry system / Biotog EL4808	MALDI-TOF	16rRNA gene sequence	16S rRNA	Gram stain/ Colony morphology
WHO Repository																	
Staphylococcus epidermidis	PEI-B-P-06	Staphylococcus epidermidis		✓	✓	✓	✓	✓	✓	Gram positive coccus	✓	✓	✓	✓	100%	98%	Gram positive coccus
Streptococcus pyogenes	PEI-B-P-20	Streptococcus pyogenes		✓	✓	✓	✓	✓	yes (Streptococcus type A)	Gram positive coccus	✓	✓	✓	✓	99%	98%	Gram positive coccus
Escherichia coli	PEI-B-P-19	Escherichia coli. **Serotyping**: Escherichia coli, Serovar O6:H16		✓	✓	✓	✓	✓	✓	Gram negative rods	✓	✓	*Escherichia coli / (Stenotropomonas maltophilia in bng 1)*	✓	99%	98%	Gram negative rods
Klebsiella pneumoniae	PEI-B-P-08	Klebsiella pneumoniae		✓	✓	✓	✓	✓	✓	Gram negative rods	✓	✓	✓	✓	99%	99%	Gram negative rods
Candidates for Enlargement study																	
Bacillus cereus spores	PEI-B-P-57	Bacillus cereus	Bacillus spp	✓	Possibility of Bacillus thuringiensis - low discrimination	Bacillus cereus group	✓	✓	✓	Gram positive rods	✓	✓	*Bacillus cereus/ thuringiensis*	✓	Bacillus cereus / Bacillus thuringiensis (2 species had equal scores) 100 %	99%	Gram positive rods
Bacillus thuringiensis spores	PEI-B-P-07	Bacillus thuringiensis		✓													
Enterobacter cloacae	PEI-B-P-43	Enterobacter hormaechei stegerwaltii	**Serotyping:** Enterobacter cloacae	✓	✓	✓	✓	✓	✓	Gram negative rods	✓	✓	*Bacillus cereus/ thuringiensis*	✓	99%	99%	Gram negative rods
Morganella morganii	PEI-B-P-74	Morganella morganii subsp. morganii	Morganella morganii	✓	✓	✓	✓	✓	no growth (identifiation of control)	Gram negative rods	✓	✓	no growth	✓	99%	99%	Gram negative rods
Proteus mirabilis	PEI-B-P-55	Proteus mirabilis		✓	✓	✓	✓	✓	✓	Gram negative rods	✓	Yes- Gram negative rods /API	✓	✓	100%	99%	Gram negative rods
Pseudomonas fluorescens	PEI-B-P-77	Pseudomonas sp	Pseudomonas fluorescens	✓	✓	✓	✓	✓	✓	Gram negative rods	✓	no / Pseudomonas putida	✓	✓	99%	98%	Gram negative rods
Salmonella choleraesuis (heterotypic synonym)	PEI-B-P-78	Salmonella enterica NCTC 6017 Control	Salmonella enterica Serotyping: Salmonella Heidelberg	Salmonella spp/Salmonella choleae	Salmonella spp	Salmonella spp (Serotyp: heidelberg)	Salmonella spp.	yes	Salmonella spp.	Gram negative rods	✓	Antisera typing: Salmonella choleraesuis	Salmonella species (unable to spectate organism)	Maldi-TOF/ Kaufman-white schema: Salmonella choleraesuis	no growth	98%	Gram negative rods
Serratia marcescens	PEI-B-P-56	Serratia marcescens / nematodiphila	Serratia marcescens	✓	✓	✓	✓	✓	✓	Gram negative rods	✓	✓	*Serratia spp.*	✓	99%	100%	Gram negative rods
Staphylococcus aureus	PEI-B-P-63	Staphylococcus aureus / Staphylococcus aureus ATCC 6538 Control	Staphylococcus aureus	✓	✓	✓	✓	✓	✓	Gram positive coccus	✓	✓	Staphylococcus aureus ss aureus	✓	100%	99%	Gram positive coccus
Streptococcus dysgalactiae	PEI-B-P-71	Streptococcus dysgalactiae equisimilis	Streptococcus dysgalactiae	✓	no growth	✓	✓	✓	Streptococcus dysgalactiae ssp. equisimilis	Gram positive coccus	✓	✓	Streptococcus dysgalactiae ssp. equisimilis	✓	100%	99%	Gram positive coccus
Streptococcus bovis (old name - reclassified)	PEI-B-P-61	Streptococcus gallolyticus pasteurianus	Streptococcus gallolyticus	✓	✓	✓	✓	✓	✓	Gram positive coccus	✓	✓	*Streptococcus gallolyticus*	Streptococcus gallolyticus	Streptococcus bovis/ Streptococcus pasteurianis (equal scores) 100 %	99%	no growth

Discussion

All participants received the deep frozen bacteria strains in good condition without complaint. As in the first study, deep frozen, pathogenic bacteria strains could be shipped worldwide without any difficulties. The tested inocula proliferated well and were successfully used for spiking. The bacterial identification performed by the study partners complied with the ID of PEI. The results of bacteria counting of all participants are homogenous since the measured divergence factors represent an acceptable value in the estimation of high bacteria cell counts.

The results of the four strains of the existing WHO Repository are equivalent to the first study. With an initial bacterial count of approx. 0.03 CFU/mL (spiking of 10 to 25 CFU per PC unit) *Escherichia coli* and *Staphylococcus epidermidis* grew up until day 7 in 100%, *Klebsiella pneumoniae* in 95% of the tested PC units. These three strains showed consistent growth at all test sites. *Streptococcus pyogenes* proliferated in 73% of the tested PC units. This strain showed no growth at all at only one site. In the previous collaborative study, this strain did not grow at 3 sites at an inoculum of 10 CFU per bag. The most likely interpretation of these failures are specific or unspecific inhibitory mechanisms directed at the bacterial strain in the donor population [7].

The actual results confirmed the suitability of the strains as 1st WHO Repository.

Nine of the eleven candidate strains showed good (70%) to excellent growth (100%). *Enterobacter cloacae* and *Streptococcus bovis* failed to proliferate at two sites. The tested *Morganella morganii* strain grew only at two sites. *Salmonella choleraesuis* showed no growth at five sites. These results might be explained by antibodies against these strains in the blood donor population. These two strains also exhibited lower stability [Fig.12].

The statistical evaluation of the growth ability showed for *Bacillus cereus* spores, *Bacillus thuringiensis* spores, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Staphylococcus aureus* and *Streptococcus dysgalactiae* a growth to significantly more than 2 $\log_{10}$ CFU/mL by day 2.

For *Enterobacter cloacae*, *Proteus mirabilis*, *Staphylococcus epidermidis*, *Streptococcus bovis* and *Streptococcus pyogenes* this growth milestone was reached at day 4.

Growth for *Salmonella choleraesuis* was lower than for other strains and showed a high variability among participants which might be explained by the low storage temperature or pre-existing antibodies.

Morganella morganii failed to grow beyond that amount of bacteria in the initial inoculation.
As *Morganella morganii* caused transfusion incidents in several countries it was decided to qualify another strain of this species. In an additional study 8 partners tested a second strain in accordance with the study protocol as a proposal for replacing the *Morganella morganii* strain from the main study. The results are documented in **Appendix** 3 in Fig. 12 and 13.

Conclusion and Proposals

Platelet Transfusion-Relevant Bacteria Reference Strains are a practical tool for the objective validation and assessment of methods for screening and pathogen reduction in blood components. The collaborative study confirmed the growth ability, stability and consistency of the already approved four WHO strains. Nine of the candidate strains fulfilled the predefined requirements for addition to the repository. After spiking with low bacteria counts they grew up to high counts in platelet concentrates under routine conditions independently from individual donors properties. As the candidates cover very fast growing bacteria (high bacterial counts already after day 2) and intermediate growers (to high bacterial counts after day 4 to day 7) the bacterial panel well reflects the different needs both of validation of pathogen inactivation technologies and of bacterial detection systems. It is proposed to add these bacterial strains to the existing bacteria repository as WHO Platelet Transfusion-Relevant Bacteria Reference Strains.
As the tested *Morganella morganii* PEI-B-P-74 strain showed no growth, a second strain was tested in 8 laboratories in accordance with the study protocol. The new strain showed growth in all tested PCs. The data are very consistent. It is recommended to add this strain to the WHO bacteria list as well.

Acknowledgements

Christian Gabriel, Susanne Süssner, Claudia Renke, Ingrid Lindlbauer, Austrian Red Cross, Blutzentrale Linz

Dana Devine, Sandra Ramirez-Arcos, Heather Perkins, Yuntong Kou, Adriana Zapata, Canadian Blood Service, Ottawa Blood Service, Ottawa

Axel Seltsam, Bernd Lambrecht, Birgit Jarck, German Red Cross Blood Service NSTOB, Springe, Germany

Birgit Gathof, Melanie Störmer, Transfusion Medicine, University Hospital Cologne, Germany

Erhard Seifried, Kai Hourfar, Simone Schwientek, German Red Cross, Frankfurt, Germany

Carl McDonald, Kate Aplin, Anjana Roy, NHS Blood and Transplant, London, England

Dirk de Korte, Willy Karssing, Herbert Korsten, Sanquin Blood Supply Foundation, Jan Marcelis, Jaap van Meeteren, Eveline Thijssen, Elisabeth Hospital, Tilburg, The Netherlands

Richard J. Benjamin, Stephen J. Wagner, Anne Hapip, American Red Cross, Blood Component Dep, Rockville, USA

Roslyn Yomtovian†, Michael R. Jacobs, Caryn Good, Case Western Reserve University, Cleveland, USA

Truscha Niekerk, Katlego Moagi, Xoliswa Mpumlwana, Nokuthula Chilwane, Nolwazi Nkambule, South African National Blood Service, Weltevreden Park, South Africa

Susanne Marschner, Shawn D. Keil, Denise Gilmour, Meghan Hudziec, Therumo, BCT, Lakewood, USA

Julieta Rojo, Gabriela Ibañez- Cervantes, Juan Manuel Bello-López, Centro Nacional de la Transfusión Sanguínea, Mexico

Masahiro Satake, Hideto Nagumo, Mami Matsumoto, Kumiko Shinozaki, Kumi Kinno, Moe Kaneko, Japanese Red Cross, Tokyo, Japan

Zainab Mukhtar, Shaheen Sharafat, Institute of Biological, Biochemical and Pharmaceutical Sciences, Dow Medical College, DUHS Karachi, Pakistan

Julia Brachert, Rekia Beshir, Anna-Maria Scheder, Annemarie Mück, Anja Schneider, Sigrid Hanitsch, Utta Schurig, Ute Sicker, Jan-Oliver Karo, Ingo Spreitzer, Paul-Ehrlich-Institut, Langen, Germany

References

1 Brecher ME, Blajchman MA, Yomotovian R, et al: Adressing the risk of bacterial contamination of platelets within the United States: A history to help illuminate the futuere. Transfusion 2013; 53:221-231

2 Lohmann A, Halbauer j, Witzenhausen C, et al: Hemovigilance Report oft he Paul-Ehrlich-Institut, 2012

3 Funk M, Günay S, Lohmann A: Hämovigilanzbericht des Paul-Ehrlich-Instituts, 2014

4 Fatalities Reported to FDA Following Blood Collection and Transfusion – Annual Summary for Fiscal Year 2013

5 Montag T: Perspectives and limitations in the bacterial screening of platelet concentrate. J Lab Med 2006; 30: 60-65

6 Palaveino EL, Yomotovian RA, Jacobs MR: Bacterial contamination of platelets. Transfusion Apheres Sci 2010; 42:71-82

7 Störmer M, Arroyo A, Brachert J, Montag T. et al: Establishment of the first international repository for transfusion-relevant bacteria reference strains: ISBT working party transfusion-transmitted infectious diseases (WP-TTID), subgroup on bacteria. Vox Sang. 2012; 102:22-31.

8 Müller B, Walter-Wenke G, Kalus M, Alt T, Bux j, Zeiler T, Schottstedt V: Routine bacterial screening of platelet concentrates by flow cytometry and is impact on product safety and supply. Vox Sang. 2015, 108 209-218.

9 Schrezemeier H, Walter-Wenke G, Müller TH. Et al: Bacterial contamination of platelet concentrates: results of a prospective multicenter study comparing pooled whole blood-derived platelets and apheresis platelets. Transfusion 2007; 47:644-652

10 Montag T: Strategies of bacteria screening in cellular blood components. Clin Chem Lab Med. 2008; 46:926-32.

11 Störmer M, Vollmer T. Diagnostic Methods for Platelet Bacteria Screening: Current Status and Developments. Transfus Med Hemother 2014; 41:19–27.

Appendix 1

Study partners

No.	Country	Facility	Partner
1	Austria	Austrian Red Cross, Blutzentrale Linz	Christian Gabriel, Susanne Süssner
2	Canada	Canadian Blood Service, Ottawa	Dana Devine, Sandra Ramirez
3	Germany	German Red Cross Blood Service NSTOB, Springe, Germany	Axel Seltsam, Bernd Lambrecht
4	England	NHS Blood and Transplant, London	Carl McDonald, Kate Aplin
5	The Netherlands	Sanquin Blood Supply Foundation; Elisabeth Hospital, Tilburg	Dirk deKorte, Jan Marcelis
6	Germany	German Red Cross, Frankfurt	Erhard Seifried, Kai Hourfar
7	USA	American Red Cross, Blood Component Dep, Rockville	Richard Benjamin, Stephen J. Wagner
8	USA	Case Western Reserve University, University Hospitals Case Medical Center, Cleveland	Michael R. Jacobs, Roslyn Yomtovian†
9	South Africa	South African National Blood Service, Weltevreden Park	Charlotte Ingram, Truscha Niekerk
10	USA	Therumo, BCT, Lakewood	Susanne Marschner Shawn Keil
11	Germany	Transfusion Medicine, University Hospital of Cologne	Birgit Gathof, Melanie Störmer
12	Japan	Japanese Red Cross Kanto-Koshinnetsu Block Blood Center, Tokyo	Masahiro Satake, Hideto Nagumo
13	Mexico	Centro Nacional de la Transfusion Sanguínea	Julieta Rojo, Gabriela Ibañez - Cervantes
14	Pakistan	Institute of Biological, Biochemical and Pharmaceutical Sciences, Dow Medical College, DUHS Karachi	Rafiq Khanani, Zainab Mukhtar

Appendix 2

ISBT Working Party
Transfusion-Transmitted Infectious Diseases
Subgroup on Bacteria

WHO-ISBT WP-TTID International Validation Study on Platelet Transfusion-Relevant Bacteria Reference Strains

Phase 2

Enlargement of Repository of Platelet Transfusion - Relevant Bacterial Reference Strains

Study Protocol

Test of growth ability
of selected transfusion-relevant bacteria strains
in platelet concentrates

Eva Spindler-Raffel
Thomas Montag (†)
Paul Ehrlich Institute
Federal Agency for Sera and Vaccines
Division Microbial Safety
Paul-Ehrlich-Strasse 51-59
63225 Langen
Germany

Project Team:

Co-Chair ISBT WP-TTID, Subgroup on Bacteria
Dr. Carl P. McDonald
Head of Bacteriology
National Bacteriology Laboratory
NHS Blood and Transplant
Charcot Avenue
Colindale
London
NW9 5BG
ENGLAND

Co-Chair ISBT WP-TTID, Subgroup on Bacteria
Dr. Richard J. Benjamin MD PhD FRCPath
Chief Medical Officer
American Red Cross Holland Laboratories
15601 Crabbs Branch Way
Rockville, MD 20855-2743
USA

Dr. Melanie Störmer
Transfusion Medicine
University Hospital Cologne
Kerpener Str. 62
50937 Cologne
Germany

Dr. Eva Spindler-Raffel (Corresponding address)
FG Bakteriologische Sicherheit / Microbial Safety
Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 51-59
63225 Langen
Germany

CONTENTS

1. Background

Bacterial contamination of platelet concentrates (PCs) still remains a persistent problem in transfusion [1]. To mitigate the risk of bacterial contamination of blood components, blood centres have implemented donor screening along with bacterial detection systems or pathogen reduction technologies (PRT). In order to validate and to compare these methods, it is crucial to use bacterial strains which are able to proliferate in blood components, e.g. in PCs during storage [2].

The International Society of Blood Transfusion (ISBT) Working Party Transfusion-Transmitted Infectious Diseases, Subgroup on Bacteria (former chair: Dr Thomas Montag-Lessing) had organized an international validation study on Platelet Transfusion-Relevant Bacteria Reference Strains (PTRBRS) to be used as a tool for development, validation and comparison of the respective methods. Four blinded bacterial strains were sent in replicate to participating laboratories worldwide for bacterial count calculation, strain identification and evaluation of growth properties in PCs. The results were submitted to the Expert Committee on Biological Standardization (ECBS) and were established as the WHO Repository of Platelet Transfusion-Relevant Bacteria Reference Strains [3].

Platelet Bacteria Reference strains (PBRS) are a feasible tool for objective validation and assessment of methods for screening and pathogen reduction in blood components. For this purpose, four PBRS were tested and approved. They are available as the WHO Platelet Transfusion Relevant Bacterial Reference Strains Repository.

The next step will be to enlarge this bacterial panel to approve more strains as PBRS.

In contrast to the previous validation study where both the bacterial count and the growth properties of several replicates in PCs was evaluated, only the ability to proliferate in PCs under "real life"/routine conditions after low spiking (< 1CFU/mL) requires evaluation in this WHO – ISBT enlargement study. For this reason 11 selected bacterial strains that were discussed during the meetings of ISBT WP-TTID Subgroup on Bacteria will be sent to the participating laboratories to evaluate their ability to proliferate in PCs in different regions of the world.

In addition to these 11 new strains, the 4 already approved repository strains will be tested again as a reference.

For statistical reasons each bacteria strain has to be tested in 3 platelet bags for 7 days. Enumeration will be performed on day 2, 4 and 7. Whether tested in parallel or on 3 different days is up to the participating lab, but must be documented.

References

1. Palavecino EL, Yomtovian RA, Jacobs MR. Bacterial contamination of platelets. Transfus Apher Sci. 2010; 42:71-82.

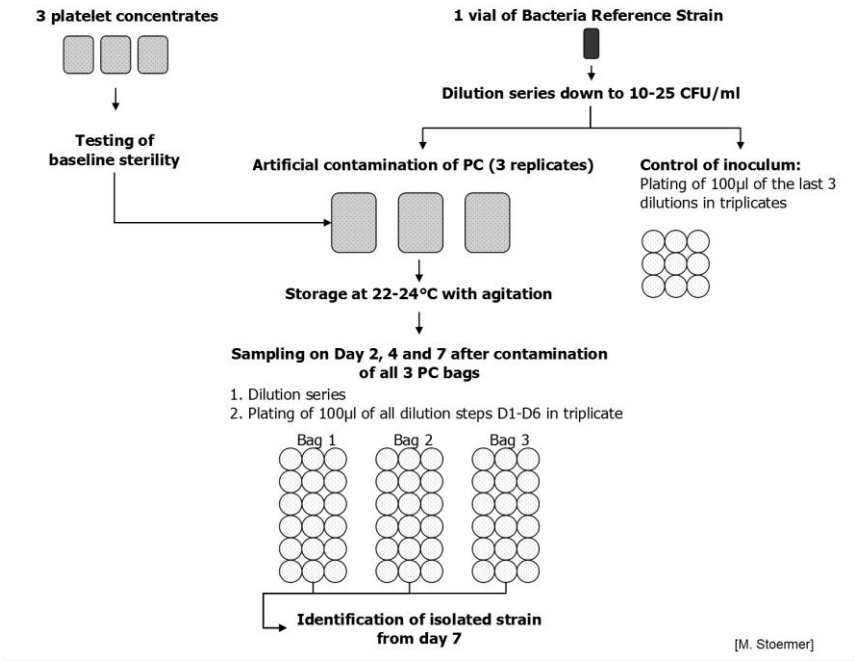
2. Montag T, Strategies of bacteria screening in cellular blood components. Clin Chem Lab Med. 2008; 46:926-32.

3. Störmer M, Arroyo A, Brachert J, Carrero H, Devine D, Epstein JS, Gabriel C, Gelber C, Goodrich R, Hanschmann KM, Heath DG, Jacobs MR, Keil S, de Korte D, Lambrecht B, Lee CK, Marcelis J, Marschner S, McDonald C, McGuane S, McKee M, Müller TH, Muthivhi T, Pettersson A, Radziwon P, Ramirez-Arcos S, Reesink HW, Rojo J, Rood I, Schmidt M, Schneider CK, Seifried E, Sicker U, Wendel S, Wood EM, Yomtovian RA, Montag T. Establishment of the first international repository for transfusion-relevant bacteria reference strains: ISBT working party transfusion-transmitted infectious diseases (WP-TTID), subgroup on bacteria. Vox Sang. 2012; 102:22-31.

2. Study Design

15 different bacterial reference strains will be sent to the partners. For each bacterial strain, three PCs (pooled and/or apheresis PCs) have to be artificially contaminated with 10-25 CFU/bag. Before contamination the baseline sterility of the PCs needs to be proven by microbiological control in accordance with the routine standard operating procedure used in the participating laboratory.

For contamination, the reference strain solution needs to be diluted in sterile NaCl to achieve a final concentration of 10-25 CFU/mL in the tube. To enumerate the inocula, 100 µl of the last three dilution steps are plated onto agar plates and colonies are counted the following day after incubation. After contamination the PCs are stored under routine conditions. Sampling is performed according to the protocol on days 2, 4 and 7 from all 3 PC bags. A dilution series up to dilution 6 is performed from each sample (in total 9 dilution series per strain) and 100 µl of each dilution is plated in triplicate onto agar plates. Colonies are counted. Strain identification has to be performed from day 7 isolates of PC bag 1, 2 and 3 to guarantee that the inoculated bacterial strain grew in the PC bag.



3. Shipping and Storage

The 15 bacterial strains will be sent in purpose-built containers with dry ice. Please check the containers immediately after receiving. To assure the stability of the bacterial load of the Bacteria Strain, the cold chain must not be interrupted and the strains must be used immediately after thawing.

Note: Check the vials immediately after arrival. If the samples show any signs of thawing, they must be discarded!
In this case please inform the study coordinating team immediately.

3.1. *Labelling of Bacterial Reference Strains*

15 different bacteria strains are contained in vials in 4-replicates of 4.
Each vial is labelled with the name of the bacterial strain and PEI-identification/lot number (PEI-B-P-XX).

List of Blood Bacteria Reference Strains

WHO Repository

Staphylococcus epidermidis	PEI-B-P-06
Streptococcus pyogenes	PEI-B-P-20
Escherichia coli	PEI-B-P-19
Klebsiella pneumoniae	PEI-B-P-08

Candidates for Enlargement Study

Bacillus cereus, spores	PEI-B-P-57
Bacillus thuringiensis, spores	PEI-B-P-07
Enterobacter cloacae	PEI-B-P-43
Morganella morganii	PEI-B-P-74
Proteus mirabilis	PEI-B-P-55
Pseudomonas fluorescens	PEI-B-P-77
Salmonella cholerae-suis	PEI-B-P-78
Serratia marcescens	PEI-B-P-56
Staphylococcus aureus	PEI-B-P-63
Streptococcus dysgalactiae	PEI-B-P-71
Streptococcus bovis	PEI-B-P-61

3.2. Storage of Bacterial Reference Strains

Store the vials immediately after arrival in a deep freezer at -80°C without secondary packaging. Test of growth of selected transfusion-relevant bacterial reference strains in platelet concentrates (PCs)

4.1. Materials

- 1 vial of each bacteria test strain.

(The below mentioned materials are calculated for one strain and dilution series for 3 PC bags in parallel. If the spiking is done on different dates you will need more NaCl aqueous solution.)

- 3 platelet units for each strain (apheresis or whole blood derived PCs. Preferably use fresh PCs, if not available use outdated ones)
- Usual platelet storage device (agitation, temperature controlled, 22± 2°C)
- Dry incubator, 37°C and 30°C
- Sterile welding equipment (e.g. sterile connecting device)
- Sterile NaCl aqueous solution (0.85 %) in sterile tubes with caps
- Trypticase Soy Agar plates (alternatively Columbia Blood Agar)
- Sterile applicators (spattles / spreaders)
- Sterile syringes
- Luer Lock connection device/ sterile Coupler spike

Before starting the experiments please ensure sufficient supplies are available i.e. agar plates and NaCl.

4.2. 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 in automated systems) and describe the procedure in the result section.

4.3. Thawing / Defrosting Bacterial Reference Strains

- 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 completely.

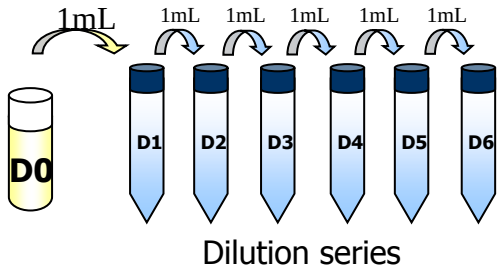
Note: The bacterial strains (stock suspensions) must be used immediately after thawing.

4.4. Dilution Procedure

For low count spiking the bacteria reference strain solutions need to be diluted. For this reason the cell count of each bacterial strain is provided by the organizing committee and displayed in colony forming units per mL (CFU/mL). For each strain the numbers of dilution steps are provided in the appendices.

For all test strains, defrost the vials as described above (Section 4.3.) and vortex for 15 seconds at the highest speed. Perform a series of dilutions of each vial in sterile saline, as described in the dilution procedure (see Section 7 Appendices for each bacterial strain).

The undiluted (stock) suspension is termed the D0 (10^0) dilution. Unless otherwise specified in the dilution procedure, prepare 1:10 serial dilutions using 9 mL of sterile saline (NaCl) each and 1 mL of the stock or 1 mL dilution from previous dilution step. Consequently, each dilution is $1/10^{th}$ the concentration of the previous dilution. The first 10-fold dilution is termed the D1 (10^{-1}) dilution, the following is termed D2, D3 etc. to the final dilution step (containing 10-25 CfU/mL) that is needed.



Dilution series

Dilute each bacterial strain down to approximately 10 to 25 CFU/mL in sterile NaCl.

Note: The final dilution step is dependent on the bacterial count stated in the strain dilution specification (see Section 7 Appendices).

Make sure…

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

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

… that tips are changed after each step!

4.5. *Artificial contamination of platelets /platelet spiking*

If possible: All work mentioned below should be done in a Laminar Flow & Biosafety Cabinet to avoid contaminations.

- Connect each platelet bag with a luer-lock connection device (e.g. a short tube using Sterile Connecting Device), or insert a sterile Coupler spike (with luer-lock Safesite valve) through a port into the pack.

Luer-Lock

- If using the luer-lock connection device - draw 5 mL out of the platelet bag using a sterile syringe but do not discard it (see below). **Ensure aseptic technique is followed!**
- Using a second sterile syringe, inoculate 1 mL of the final dilution through the same port into the platelet bag. Afterwards the final bacterial load will be 10 – 25 CFU per bag.
- Add the previously removed 5 mL PC sample back into the bag to flush the tube segment of the bag if using the luer-lock connection device.
- Close the luer-lock port.

Coupler-Spike

- If using the sterile Coupler-Spike - inoculate 1 mL of the final dilution (~ 10 CFU/mL to 25 CFU/mL) through the port into the platelet bag.
- Rinse the syringe 3 times with PC.
- Incubate the contaminated PC units at 22-24°C under agitation for 7 days (168 hours after inoculation).

Note:

Close the tube by clamp in case of any opening of the luer-lock device (e.g. before connection with a syringe, change of syringes etc.). The procedure described is used to overcome the "dead-volume" of the tube, i.e. to bring the inoculum directly into PCs main bag.

Additionally, bacteria attached to the inner surface of the tube will be detached.

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

4.6. Control (Enumeration) of inoculum

- Plate 100 µl of the last three dilution steps from abstract 4.4 in triplicate onto agar plates and incubate strain specific at 30°C or 37°C for 24-48h.
- Count the colonies and document the results in the lab protocol (Section 6, "dilution of stock").

4.7. Sampling, Enumeration and Documentation

Following inoculation with approximately 10 to 25 CFU per bag, growth kinetics of the test strains during usual PC storage conditions (storage at 20 - 24°C), are monitored as described below*:*

If possible, all work mentioned below should be done in a Laminar Flow & Biosafety cabinet to avoid contaminations.

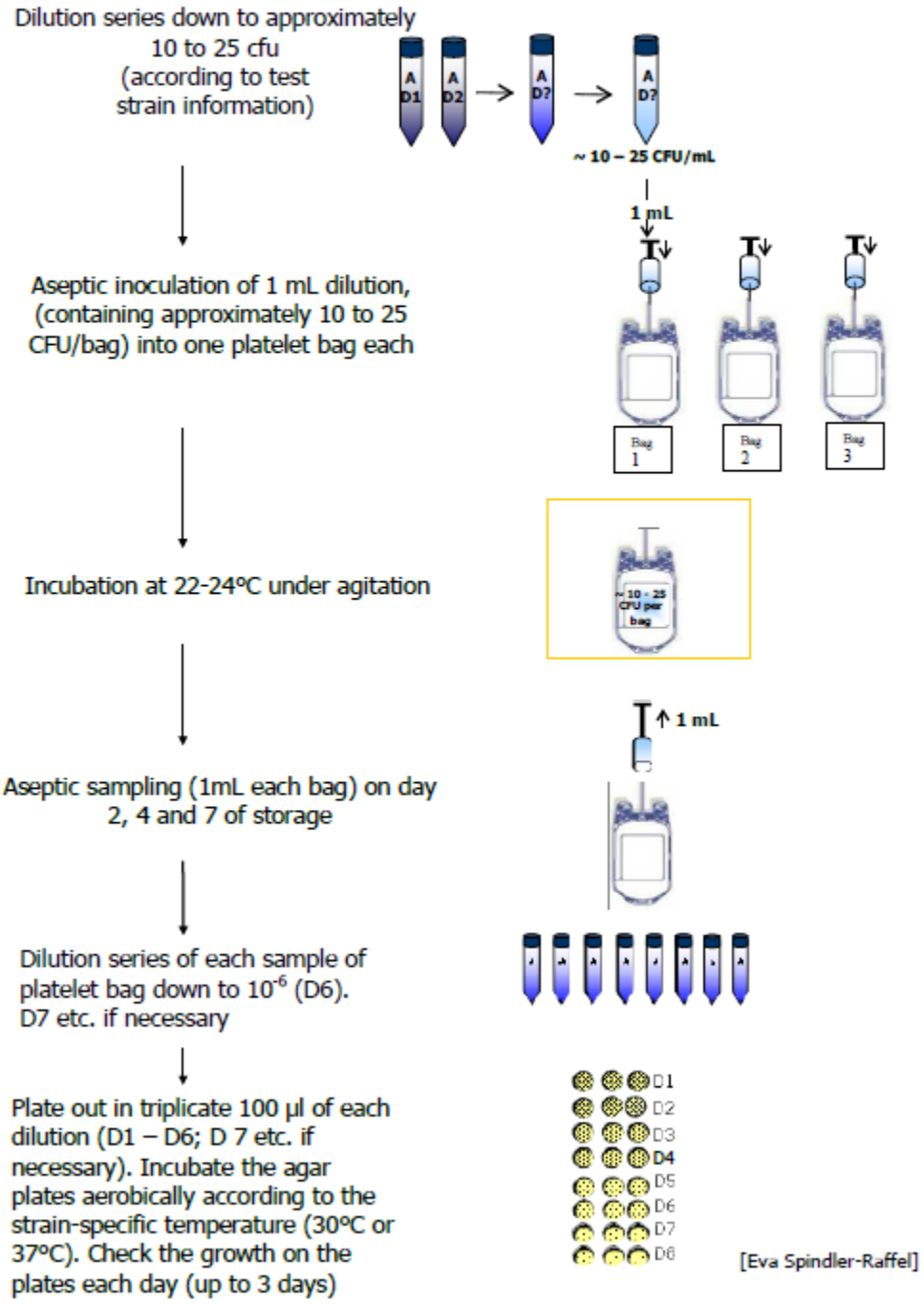
- Sampling will be performed on days 2, 4 and 7 (48, 96, 168 hours).
- Sample drawing shall be performed following the principles described in Section 4.5. (Artificial contamination of platelets /platelet spiking).
- If using the **Luer-Lock** connection device - remove the first **5 mL** of the PC using a sterile syringe but do not discard it, use a second sterile syringe to take a sample (1 mL) of each platelet bag and then add back the previously removed **5 mL** PC sample in order to enable a repetition if necessary.

- If using the sterile **Coupling-Spike** device – rinse the syringe with PC from the pack 3 times to ensure the sample is from the bag, remove a 1 mL sample of each platelet bag and close the luer-lock port.
- Enumerate the bacterial count by diluting the 1 mL sample up to 10^{-6} (D6) and plating out (see Section 4.8).
- Complete documentation in Section 5.

4.8. Summary – Spiking and enumeration scheme

Spiking (10 to 25 CFU/bag) and enumeration of test strains during storage in PC.

Please find the dilution series for the strains in the appendices (7).



[Eva Spindler-Raffel]

Documentation

5.1. *Enumeration*

Please copy for each bacterial strain!

5.1.1. Enumeration 1: after 2 days

Bacterial strain: _____

Pool or apheresis PC:_____

Inocula:_____

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **PC bag 1** **date**	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **PC bag 2** **Date**	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **PC bag 3** **Date**	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

Please copy for each bacterial strain!

5.1.2. Enumeration 2: after 4 days

Bacterial strain: _____

Pool or apheresis PC:_____

Inocula:_____

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **PC bag 1** **Date**	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **PC bag 2** **Date**	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **PC bag 3** **Date**	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

Please copy for each bacterial strain!

5.1.3. Enumeration 3: after 7 days

Bacterial strain: _____

Pool or apheresis PC:_____

Inocula:_____

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
PC bag 1	Dilution 5				
	Dilution 6				
Date					

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
PC bag 2	Dilution 5				
	Dilution 6				
Date					

PC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain xy	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
PC bag 3	Dilution 5				
	Dilution 6				
Date					

Please copy for each bacterial strain!

5.2. *Identification of grown microorganism*

Strain:

Identification (number) of sample:

Growth after day:

Macroscopic view
Colony morphology:

Microscopic view:
(shape: rod, coccus)

Result of Gram-staining: Gram-negative ☐
Gram-positive ☐

Description of identification method
(down to species level, i.e. API) :
(Identification panel)

6. Lab Protocol

Please copy for each bacterial strain!

Test strain_____

Platelet Concentrates : ☐ apheresis-PC ☐ pool-PC (donors n=)
Volume PC:
Shelf life data:

Control inoculum (dilution of stock):_____CFU/mL (mean value)

(CFU plate 1: _____ CFU plate 2:_____ CFU plate 3: _____)

Result of enumeration of inoculum control: _____CFU/ml

Storage conditions:

Bacterial proliferation:

Bacterial growth after storage	Sampling after	Yes (growth)	no
	2 days (48 h)		
	4 days (96 h)		
	7 days (168 h)		

Results of Identification:

Method (please add details like reactions)

Microorganism identified:

Match of inoculated strain (name) _____ yes:☐ no: ☐

Notes

Laboratory:
Responsibility:

Appendices

To obtain comparable results from the participants the strains are put together in groups. Please perform the working steps specified below and send the results of every group to the corresponding address after finishing the tests of each group.

Group 1:

Staphylococcus epidermidis	PEI-B-P-06
Bacillus cereus spores	PEI-B-P-57
Enterobacter cloacae	PEI-B-P-43
Morganella morganii	PEI-B-P-74

Group 2:

Streptococcus pyogenes	PEI-B-P-20
Bacillus thuringingiensis spores	PEI-B-P-57
Proteus mirabilis	PEI-B-P-55
Pseudomonas fluorescens	PEI-B-P-77

Group 3:

Escherichia coli	PEI-B-P-19
Serratia marcescens	PEI-B-P-56
Staphylococcus aureus	PEI-B-P-63
Streptococcus dysgalactiae	PEI-B-P-71

Group 4:

Klebsiella pneumoniae	PEI-B-P-08
Streptococcus bovis	PEI-B-P-61
Salmonella cholerae-suis	PEI-B-P-78

Dilution steps WHO Repository

WHO Repository											
	PEI-Label (strain)	cfu/ml (vial) D0	Dilution (to 10 - 25 cfu/ml for spiking)							Spiking in each PC bag	Incubation Temp. °C
Staphylococcus epidermidis	PEI-B-P-06	1,92E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D3 in 9 ml NaCl)				1 ml D4	37
Streptococcus pyogenes	PEI-B-P-20	6,58E+04	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (3 ml D3 in 7 ml NaCl)				1 ml D4	37
Escherichia coli	PEI-B-P-19	1,69E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D3 in 9 ml NaCl)				1 ml D4	37
Klebsiella pneumoniae	PEI-B-P-08	1,47E+06	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D3 in 9 ml NaCl)	D5 (1 ml D4 in 9 ml NaCl)			1 ml D5	37

Dilution steps, enlargement strains

Candidates Enlargement study												
	PEI-Label (strain)	**cfu/ml (vial) D0**	**Dilution (to 10 - 25 cfu/ml for spiking)**						**Spiking in each PC bag**	**Incubation temp. °C**
Bacillus thuringiensis spores	PEI-B-P-07	4,88E+04	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (3 ml D3 in 7 ml NaCl)			1 ml D4	37
Bacillus cereus spores	PEI-B-P-57	2,19E+07	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D3 in 9 ml NaCl)	D5 (1 ml D4 in 9 ml NaCl)	D6 (1 ml D5 in 9 ml NaCl)	1 ml D6	37
Enterobacter cloacae	PEI-B-P-43	6,75E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D3 in 9 ml NaCl)	D5 (3 ml D4 in 7 ml NaCl)		1 ml D5	37
Morganella morganii	PEI-B-P-74	2,90E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D3 in 9 ml NaCl)	D (5 ml D4 in 5 ml NaCl)		1 ml D4	37
Proteus mirabilis	PEI-B-P-55	4,50E+04	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (3 ml D3 in 7 ml NaCl)			1 ml D4	37
Pseudomonas fluorescens	PEI-B-P-77	2,82E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D2 in 9 ml NaCl)	D5 (5 ml D4 in 5 ml NaCl)		1 ml D5	30
Salmonella choleraе-suis	PEI-B-P-78	3,96E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D2 in 9 ml NaCl)	D5 (5 ml D4 in 5 ml NaCl)		1 ml D5	37
Serratia marcescens	PEI-B-P-56	2,02E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D2 in 9 ml NaCl)			1 ml D4	30
Staphylococcus aureus	PEI-B-P-63	4,14E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D2 in 9 ml NaCl)	D5 (3 ml D3 in 7 ml NaCl)		1 ml D5	37
Streptococcus dysgalactiae	PEI-B-P-71	1,97E+07	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D3 in 9 ml NaCl)	D5 (1 ml D4 in 9 ml NaCl)	D6 (1 ml D5 in 9 ml NaCl)	1 ml D6	37
Streptococcus bovis	PEI-B-P-61	1,93E+05	D1 (1 ml D0 in 9 ml NaCl)	D2 (1 ml D1 in 9 ml NaCl)	D3 (1 ml D2 in 9 ml NaCl)	D4 (1 ml D3 in 9 ml NaCl)			1 ml D4	37

7. Questionnaire

Please complete this questionnaire and return with the first set of completed results to allow accurate assessment.

Study partner:

Contact name:

Contact details:
(Postal address, fax, phone, email)

Were you a participant of the first WHO-ISBT International Validation Study on Blood Bacteria Standards? **Yes / No**

Lab equipment used:

Microbiological Safety Cabinet (Class II) / Laminar flow hood: **Yes / No**
If **yes,** please give details:
(Make, model)

If **no**, please give other details (e.g. performed on bench, with bunsen burner):

37°C incubator: **Yes / No**
If **no**, please give details of temperature used:

30°C incubator: **Yes / No**
If **no**, please give details of temperature used:

22-24°C incubator: **Yes / No**
If **no**, please give details of temperature used:

Blood culture system (automated system for sterility testing):
(Mark, model)

Deep freezer (-80°C): **Yes / No**
If **no**, please give details of alternative used:

Deviation to the protocol:
If any deviations to the protocol have been used please describe:
(e.g. different method of inoculation / sampling)

Microbiological procedures:

Established identification system:
(e.g. grams staining, biochemical methods, automated identification systems)

Established cultivation methods:
(TSA / Columbia Blood Agar / other)

Established enumeration methods:
(Manual counting / automated plate counter)

Many thanks for taking the time to complete this questionnaire.

Appendix 3

Test of *Morganella morganii* PEI-A-91

Morganella morganii caused transfusion incidents in several countries. It would be very necessary to have this strain on the list for enlargement of the WHO repository. As the tested *Morganella morganii* PEI-B-P-74 strain showed no growth, a second strain was tested in 8 laboratories according the study protocol. The new strain showed growth in all tested PCs. The data are very consistent as the statistical evaluation shows. The growth potential as well as the match of inoculum is comparable to the already existing WHO bacteria repository. As the strain is very new the testing of stability is still under progress but very promising. It is recommended to add this strain to the WHO bacteria list.

Fig. 12: Box and whisker plots of *Morganella morganii* PEI-A-91

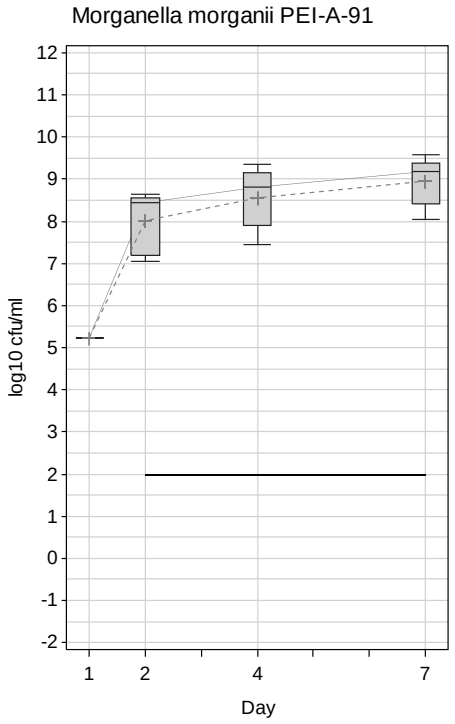
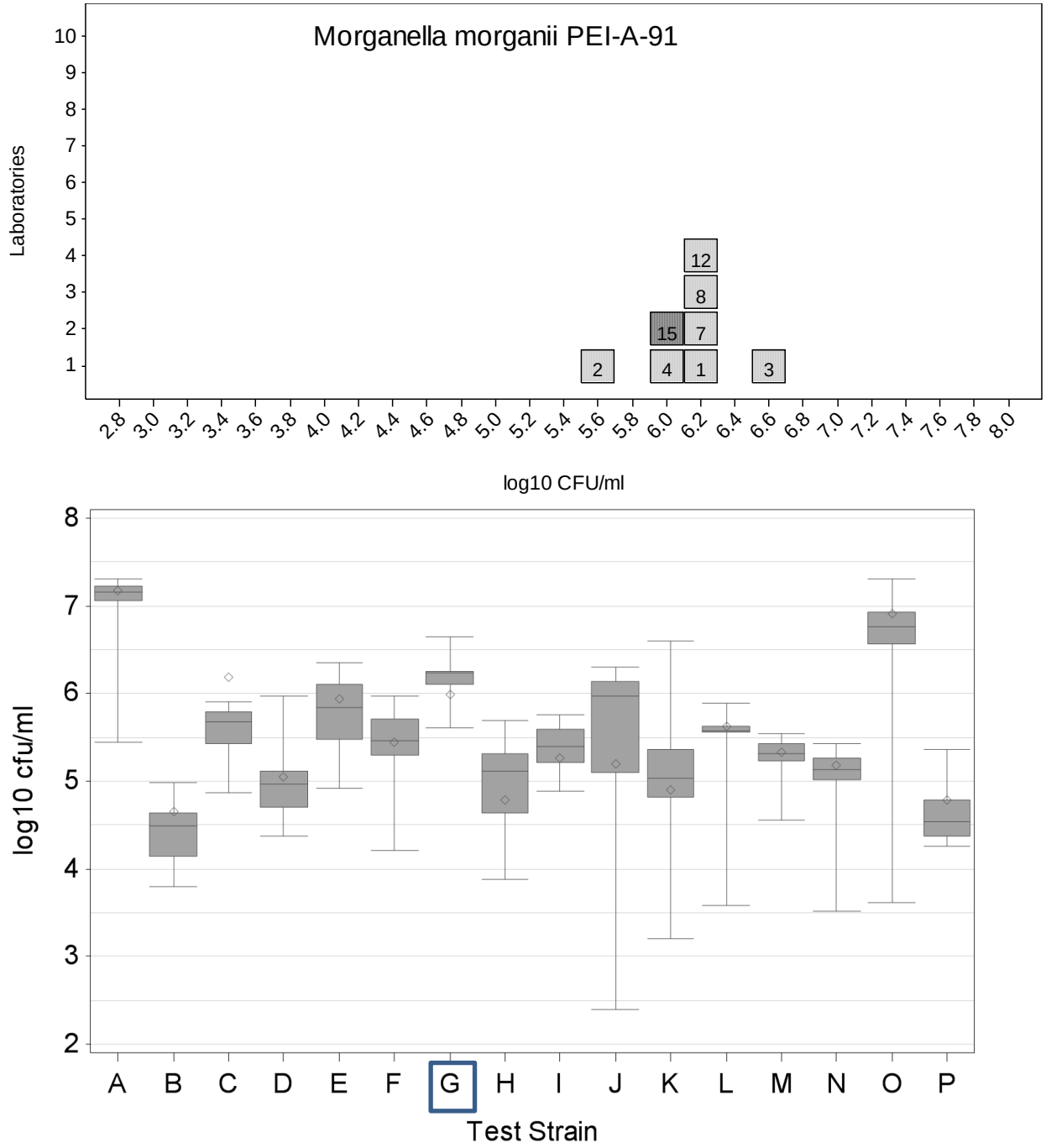


Fig. 13: Match of inoculum *Morganella morganii* PEI-A-91



Box-and-Whisker-Plot of recovery of inoculum values by test strains (rhombus: PEI inoculum; A=*Bacillus cereus* spores, B=*Bacillus thuringiensis* spores, C=*Enterobacter cloacae*, D=*Escherichia coli*, E=*Klebsiella pneumoniae*, F=*Morganella morganii*, G= *Morganella morganii* PEI-A-91, H=*Proteus mirabilis*, I=*Pseudomonas fluorescens*, J=*Salmonella choleraesuis*, K=*Serratia marcescens*, L=*Staphylococcus aureus*, M=*Staphylococcus epidermidis*, N=*Streptococcus bovis*, O=*Streptococcus dysgalactiae*, P=*Streptococcus pyogenes)*.