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Collaborative Study on the First WHO Repository of Red Blood Cell Transfusion-Relevant Bacterial Reference Strains

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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 **27 September 2019** 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 Ivana Knezevic** at email: knezevici@who.int.

Summary

Transmission of bacteria by blood products is still one of the major threats in the transfusion field nowadays. Strategies and methods to cope with this risk had been implemented or are under development. This implies also the use of appropriate bacterial strains that provide reliable test results in combination with the respective blood components.

So far, the WHO Expert Committee on Biological Standardization (ECBS) has adopted 4 bacterial strains as part of the 1st WHO International Reference Repository of Platelet Transfusion Relevant Bacterial Reference Strains in 2010. In 2015, the repository was expanded by 10 additional strains. Two international collaborative studies were carried out with Paul-Ehrlich-Institut as coordinator in cooperation with members of the ISBT Working Party Transfusion Transmitted Infectious Diseases (WP-TTID) Subgroup on Bacteria to prove their suitability. However, due to the different storage conditions of platelets compared to red blood cells (RBC), the majority of the already existing reference strains are not suitable for RBC. Therefore, the current repository of transfusion-relevant reference strains is extended by strains specifically selected for RBC.

The six bacterial candidate strains that succeeded in a previous selection process were tested for their growth ability in RBC worldwide and comprised the strains *Listeria monocytogenes* PEI-A-199, *Serratia liquefaciens* PEI-A-184, *Yersinia enterocolitica* PEI-A-105, *Yersinia enterocolitica* PEI-A-176 and the two strains *Pseudomonas fluorescens* PEI-B-P-77 and *Serratia marcescens* PEI-B-P-56 from the platelet reference panel. Within the study, the strains were intentionally inoculated in RBC units with a low concentration of approximately 10-25 Colony Forming Units (CFU)/platelet bag. Samples were taken during 42 days of RBC storage in weekly intervals and the total number of bacterial counts was determined over time.
In order to include donor variability and the different RBC compositions, the study was conducted by 15 laboratories worldwide.

With the exception of *S. marcescens*, all tested strains showed good or excellent growth in RBC. A distinction can be made with respect to the growth kinetics with *L. monocytogenes* showing a slow but steady growth during the whole testing period of 42 days. The other four strains grew faster reaching stationary phase after 21 to 28 days.

Transfusion Relevant Bacteria Reference Strains are provided as frozen suspensions with a known cell count to be used for the assessment of microbiological methods or testing strategies to improve the blood safety. Five of the six tested strains demonstrated robust and consistent growth in RBC and will be proposed to be adopted as the WHO International Reference Repository of Red Blood Cell Transfusion Relevant Bacterial Reference Strains.

Introduction

Despite significant advances in medicine, blood and its individual components are still one of the most frequently applied medicines worldwide. WHO therefore included blood and blood components in the "Model List of Essential Medicines" [1]. Due to its human origin, blood transfusion is associated with a risk of transmission of infectious diseases. Particularly viral contaminations had been regarded as major threats for recipients. A comprehensive donor screening and improved diagnostics led to a significant reduction of viral transmissions [2]. However, bacterial contaminations of blood components resulting in fatalities of recipients after transfusion have been repeatedly reported [3,4]. As a consequence, several measures like shelf-life reduction, first aliquot diversion, effective skin disinfection or predonation screening were implemented in the last two decades to reduce the risk of bacterial transmission [5,6]. In addition, new methods to detect or eliminate potential contaminants in blood components were developed or are currently under review [7–10].

The validation of these new techniques requires both blood and its components as a matrix and microorganisms that represent typical contaminants. However, due to the antimicrobial activity exerted by blood compounds due to the presence of antibodies, leukocytes or complement factors, respective microorganisms have to meet certain requirements. It is well known that bacterial isolates of a single species can generally exhibit different growth patterns in blood which is additionally affected by donor variability of the blood matrix [11]. Therefore, artificial inoculation of blood components with uncharacterized bacteria can lead to false results dependent on the method that is applied.
Two international studies were organized and coordinated by Paul-Ehrlich-Institut (PEI) under the auspice of the International Society of Blood Transfusion (ISBT) Working Party Transfusion-Transmitted Infectious Diseases, Subgroup on Bacteria to identify bacterial strains that show robust growth in platelets and allow a reliable evaluation of respective methods. The results of both studies were submitted to the Expert Committee on Biological Standardization (ECBS) and were adopted by WHO as a Repository of Platelet Transfusion-Relevant Bacteria Reference Strains [11,12].

In the first study, all four tested strains demonstrated reliable growth independent of donor variability and the single platelet unit's composition. In the second study, two out of twelve strains showed only sporadic growth in platelet concentrates (PC) and were not adopted as official Reference Strains.

Compared to PC, Red Blood Cells (RBC) are less prone for bacterial contamination due to their mandatory cold storage conditions. Nevertheless, fatalities and transfusion reactions caused by contaminated RBC units have been reported [13,14]. The spectrum of bacteria being causative of RBC transfusion septic reactions differs compared to PC. Particularly Gram negative psychrotrophic bacteria play a pivotal role. As a consequence, the majority of the already existing Reference Strains are not able to proliferate in cold stored RBC units and fail to be used as reference material. Therefore, suitable candidate strains have to be tested within a collaborative study to show their growth behavior in cold stored RBC. Candidate material was sent to the participants in order to perform the spiking experiment. The results of each single strain are summarized in the following sections.

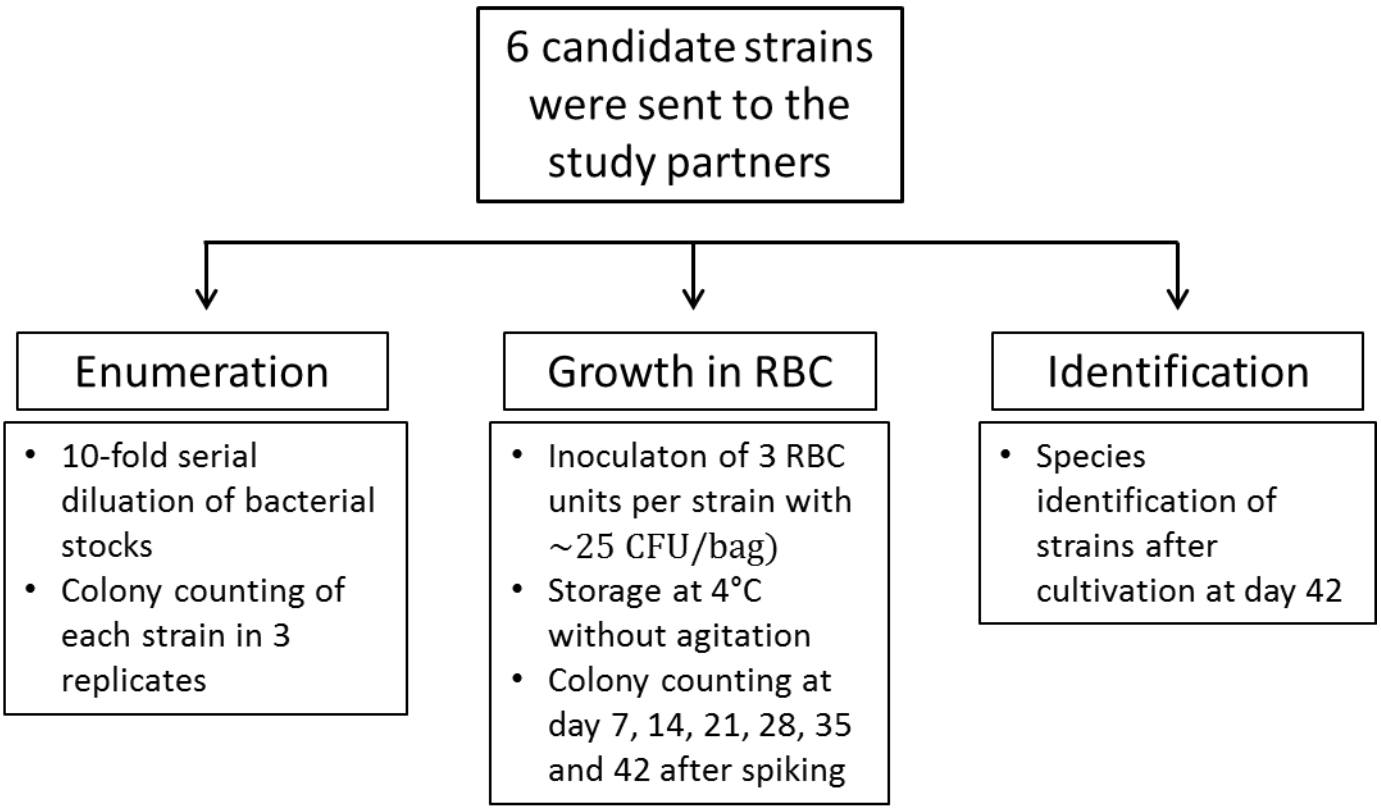
Materials and Methods

Participants and Study Design

Eighteen laboratories worldwide initially agreed on participating in the study, of which fifteen submitted results to be included in the final study report. The participants were recruited from Germany (3), UK (1), Ireland (1), Austria (1), USA (3), Canada (2), Mexico (1), South Africa (1), Hong Kong (1) and Japan (1). Details on participants and laboratories are given in **Appendix 1**.

The study protocol (scheme shown in **Fig. 1**) was discussed and confirmed by the TTID WP subgroup on bacteria and presented at the ISBT Congress in Copenhagen 2017. The results were presented to the TTID WP in several meetings (i.e. ISBT Congress Toronto 2018, ISBT Congress Basel 2019).

Fig 1: Scheme of the international RBC study



Six pre-selected bacterial strains **(Table 1)** were sent to the study partners on dry ice to ensure that liquid samples remain continuously frozen until their use. As shown in **Fig. 1** enumeration was performed for inoculum control and bacterial strain identity was confirmed after growth in RBCs. For each bacterial strain, three RBC units were inoculated with ~25 Colony forming units (CFU) each. Baseline sterility of the RBCs was confirmed before the inoculation step by microbiological control in accordance with the routine standard operating procedure used in each participating laboratory respectively.

For inoculation, the thawed bacterial reference strain solution was serially diluted in sterile saline to achieve a final concentration of ~25 CFU/mL in accordance with a standardized

protocol (see **Appendix 2**). To enumerate the inoculum, 100 µl of the last three dilution steps were plated onto agar plates in triplicates respectively and colonies were counted the following day. Following the inoculation, RBC units were stored under blood bank routine storage conditions. Aseptic sampling was performed on day 7, 14, 21, 27, 35 and 42 of all 3 bags to cover the usual shelf-life of RBC units. A dilution series up to dilution 6 was performed from each sample and 100 µl of each dilution was plated in triplicate onto agar plates. Colonies were counted the next day. Strain identification was performed from day 42 isolates of RBC bags to proof the bacterial identity and to exclude false positive results due to cross-contaminations.

Selection and characterization of bacterial candidate strains

Identification of suitable strains started with the collection of bacterial isolates, most of them being involved in transfusion incidents, from laboratories worldwide. A pre-test for their growth ability in cold stored RBC units was performed. Six out of 32 isolates, two from the official platelet repository and four new strains (**Tab.1**), showed satisfactory growth after low count spiking and were designated as candidate strains.

Tab. 1: List of candidate strains

Strain	**ID**	**Origin**
Listeria monocytogenes	PEI-A-199	Blood screening isolate, England
Pseudomonas fluorescens	PEI-B-P-77	WHO repository, enlargement panel
Serratia liquefaciens	PEI-A-184	RBC isolate, CDC
Serratia marcescens	PEI-B-P-56	WHO repository, enlargement panel
Yersinia enterocolitica	PEI-A-105	RBC isolate, Japan
Yersinia enterocolitica	PEI-A-176	RBC isolate, CDC

Batches of the respective strains were produced at PEI using an established protocol which guarantees defined bacterial suspensions. All steps described below were performed in a class II MSC under aseptic conditions. In brief, bacteria from the Working Cell Bank were cultivated in rich CASO medium at 37°C (30°C for *P. fluorescens*). Bacteria were cultured until early exponential phase to maintain a maximum amount of viable cells. The bacterial culture was subsequently diluted 1:2 with 20% of cold stored albumin serving as a cryoprotectant (cold stored to avoid bacterial growth during the filling process). The bacterial suspension was stirred constantly during the filling in cryotube vials. The filled vials were sealed via a screw cap and stored immediately at -80°C. The manual filling (1,5 ml in 1,8 ml vials, PEI) was performed on 14th December 2016 for *S. liquefaciens* PEI-A-184, 15th December 2016 for *Y. enterocolitica* PEI-A-176, 28th December 2016 for *L. monocytogenes* PEI-A-199, 3rd January 2017 for *Y. enterocolitica* PEI-A-105, 8th February 2017 for *S. marcescens* PEI-B-P-56 and 8th June 2017 for *P. fluorescens* PEI-B-P-77. The colony count of each batch was determined prior and after the freezing process at PEI to determine the impact of the freezing step itself on the number of viable cells. The integrity of the sealed vials was not further analyzed as the material is not lyophilized and it is expected that a potential ingress of moisture has no impact on the quality and viability of the cells.

Real time stability testing was performed after the production of the frozen bacterial suspensions and within the operating time of the study in an interval of several months in a total period of two years. For this purpose, six vials of each strain stored at -80°C were defrosted and two dilution series of each vial were prepared. Samples of a predefined dilution were plated of each dilution series and the mean colony count/mL was determined the following day. The effect of increased storage temperatures was not analyzed as bacterial survival at temperatures >-80°C usually leads to a sharp decrease in the shelf life of cryopreserved bacteria.
The identity of the bacteria was verified by a combination of classical and molecular microbiological procedures. Classical analysis such as growth properties, colony morphology and Gram-staining was combined with 16S rRNA gene sequencing for identification to species level.

A total of 320 vials of each strain were filled at PEI. Subtracting the vials used in the collaborative study and the stability testing there are 180 vials available for the bacterial RBC repository. In the past, we have experienced for *S. marcescens* higher in-batch inconsistency for batches with more than 160 vials. Therefore, only 20 vials batch#3 are available which will be solely used for the platelet repository due to the failure of *S. marcescens* PEI-B-P-56 in the current study. PEI will act as custodian and batches are stored under temperature controlled conditions at -80°C at PEI (Paul-Ehrlich-Straße 51-59, 63225 Langen, Germany).

Shipment of the strains to participating sites

Vials were stored at -80°C prior the shipment. In order to guarantee the frozen state, the candidate strains were delivered on dry ice to each collaborating site.

Sterility control for baseline sterility of RBC units

All RBC units were tested for contaminants before bacterial inoculation to assure baseline sterility of the original RBC 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 inoculation of RBCs, the concentrated bacteria suspension had to be diluted prior the spiking experiment (**Fig. 2**). The 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.

Fig. 2: Inoculation and sampling procedure

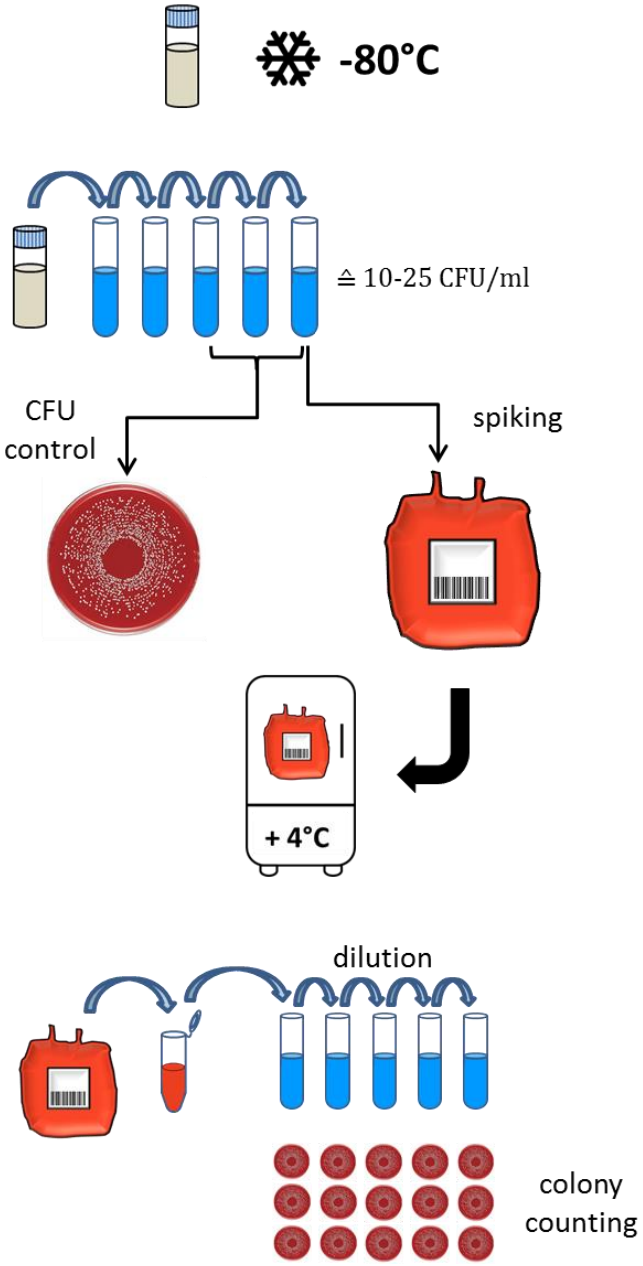
1. Shipment of frozen vials with defined bacterial concentration to study partners

2. Serial dilution of the stock suspension to reach a final concentration of 10-25 CFU/ml.

3. Inoculation of 1 ml of the last dilution into a RBC bag.
Plating and CFU determination of the last three dilutions for control of the inoculum.

4. Incubation of spiked bags under routine cold storage conditions for a total of six weeks (42 days).

5. Weekly sampling and CFU determination.



Sampling, enumeration and documentation

Sampling was performed in a weekly interval on day 7, 14, 21, 28, 35 and 42 after inoculation during RBC storage (stationary storage at 2-6°C). Sampling was performed following the study protocol (**Fig.2**).

Statistical methods

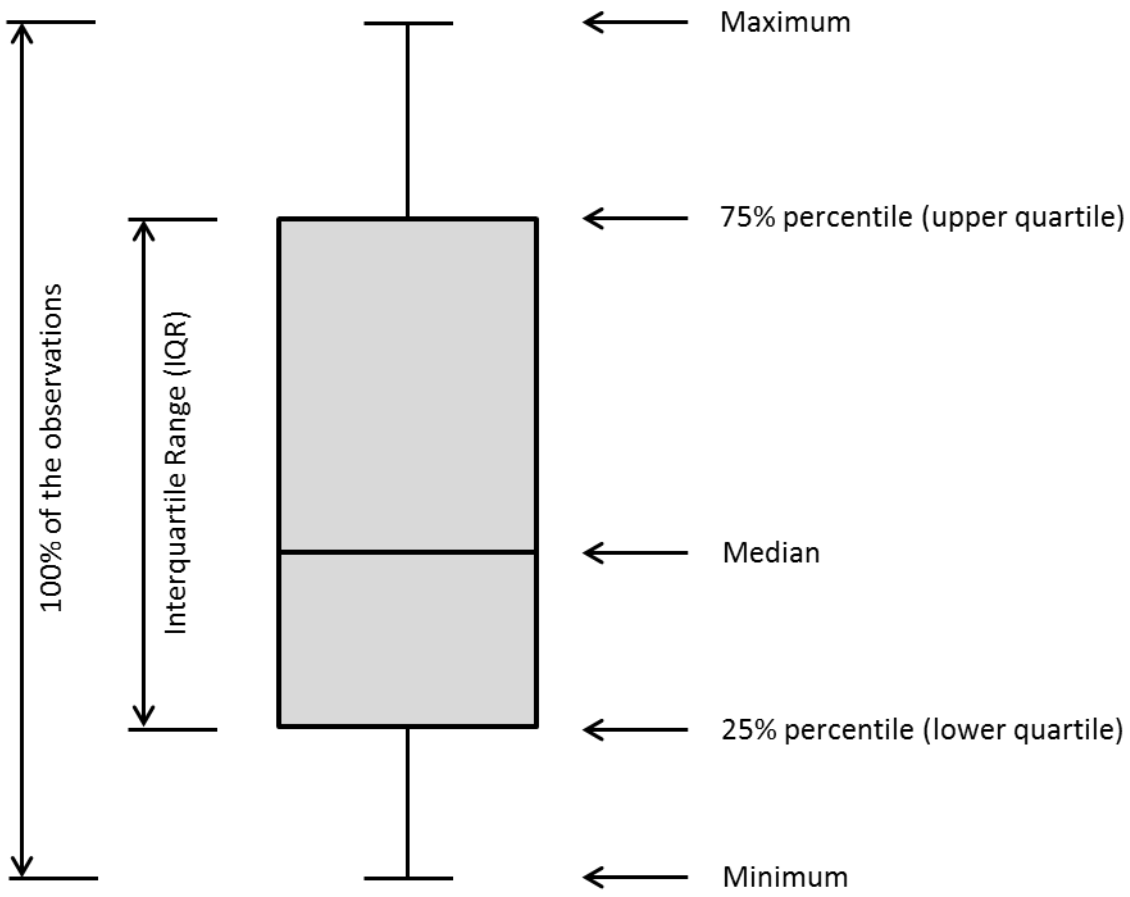
Statistical analysis was performed at PEI based on the raw data sent by the participants. Evaluation was based on log10 CFU/ml; zero CFU/ml concentrations were set to 0.01 before log-transformation. Growth data was analyzed per strain group and day.

Inoculum data was compared between PEI and participants descriptively and graphically (stapled boxes and Box-and-Whisker plot). Overall mean for each strain group were estimated by means of a mixed linear model with log10 CFU as dependent variable and random factor and participant.

Analysis of stability of inoculum data at PEI was done for up to six determinations per test strain and time point 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 9.4, SAS System for Windows. Results for bacterial growth were presented in Box- and-Whisker plots (**Fig. 3**).

Fig. 3: Box-and-Whisker plots for growth analysis

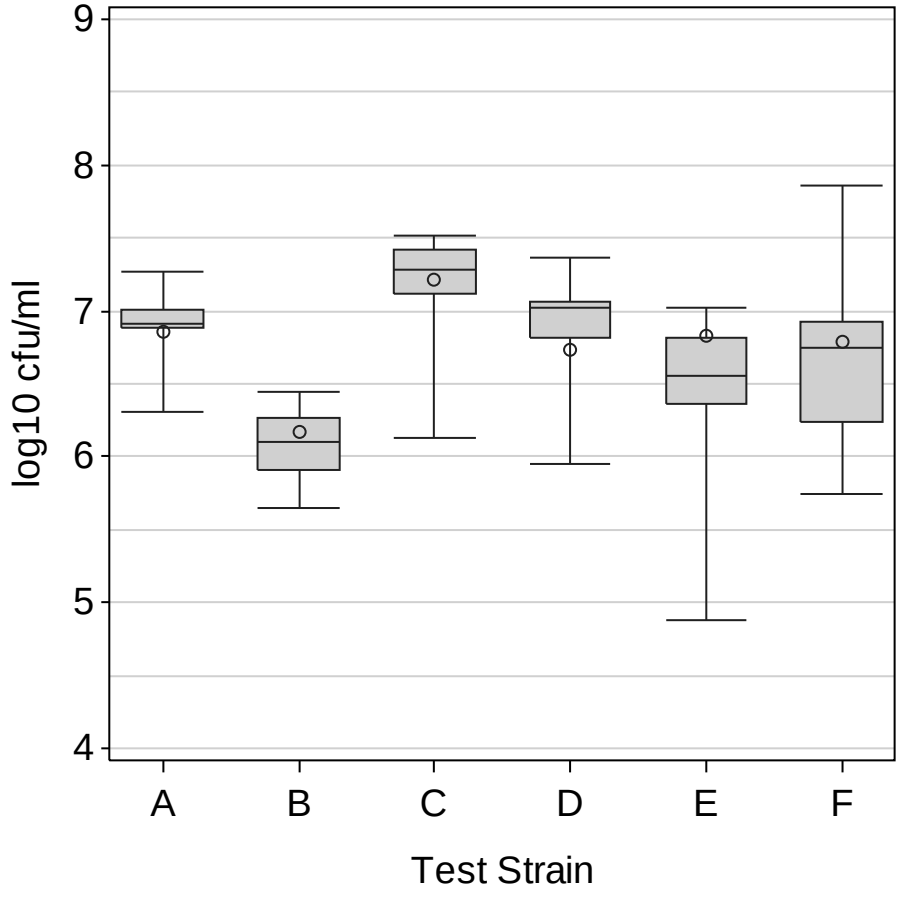


Results

Recovery of inoculum

The bacteria stock suspensions were diluted for low count spiking. The cell count of each bacterial strain was provided in the study protocol and expressed as colony forming units per mL (CFU/mL). The match of inoculum by the participating labs was statistically evaluated and results are shown in **Fig. 4** and **5**.

Fig. 4: Box-and-Whisker-Plot for the recovery of inoculum

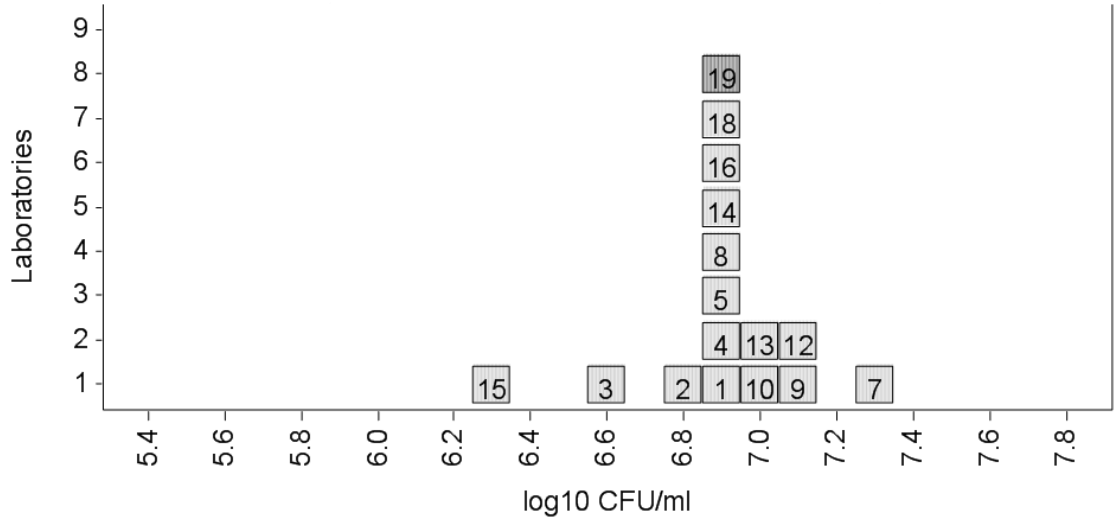


Box-and-Whisker-Plot of recovery of inoculum values by test strains (circle: PEI inoculum; A= *Listeria monocytogenes* PEI-A-199; B= *Pseudomonas fluorescens* PEI-B-P-77; C= *Serratia liquefaciens* PEI-A-184; D= *Serratia marcescens* PEI-B-P-56-01-03; E= *Yersinia enterocolitica* A-105; F= *Yersinia enterocolitica* A-176)

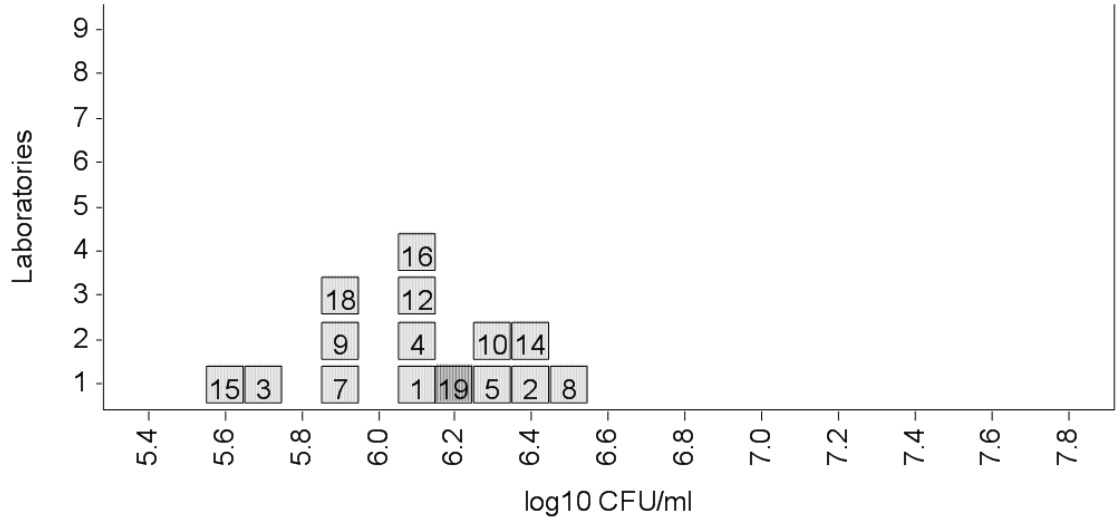
The participating sites generally confirmed the stock concentration previously determined by PEI. For *S. marcescens*, the average inoculum value was slightly higher than the one determined at PEI.

Fig. 5: Recovery of PEI inoculum values by participants, mean log10 CFU/mL plotted as stacked boxes (PEI results dark grey shaded).

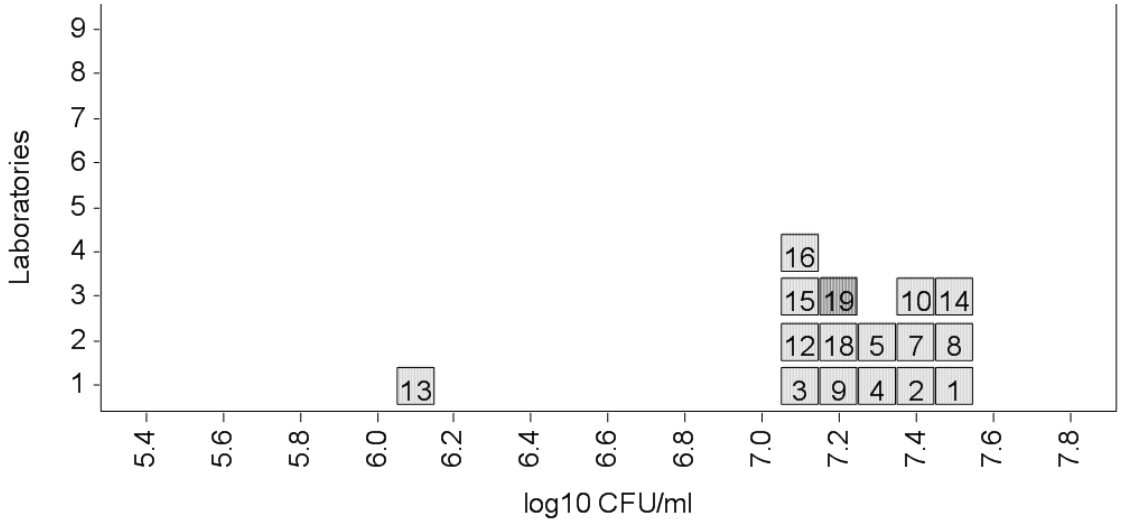
Listeria monocytogenes PEI-A-199



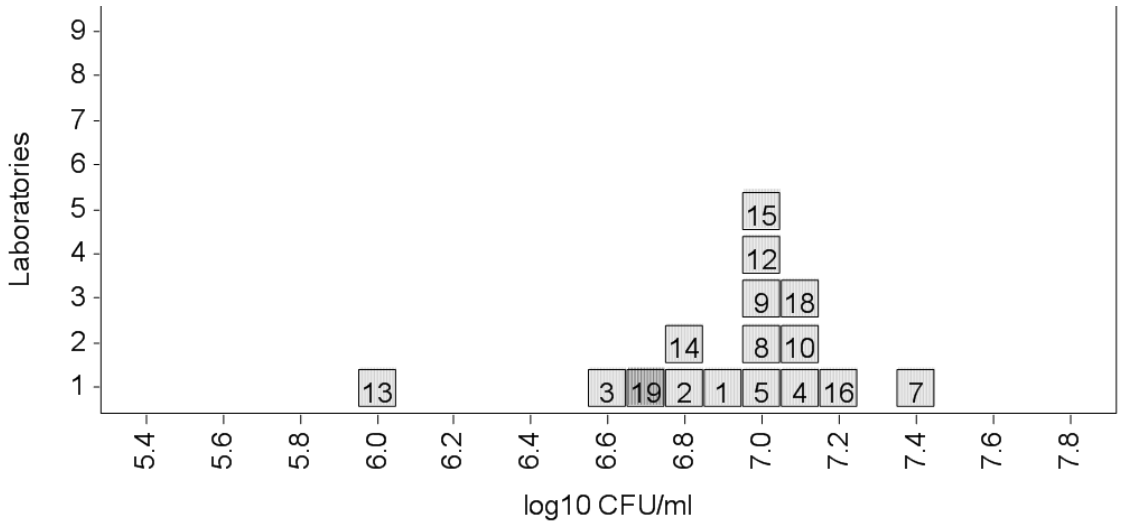
Pseudomonas fluorescens PEI-B-P-77



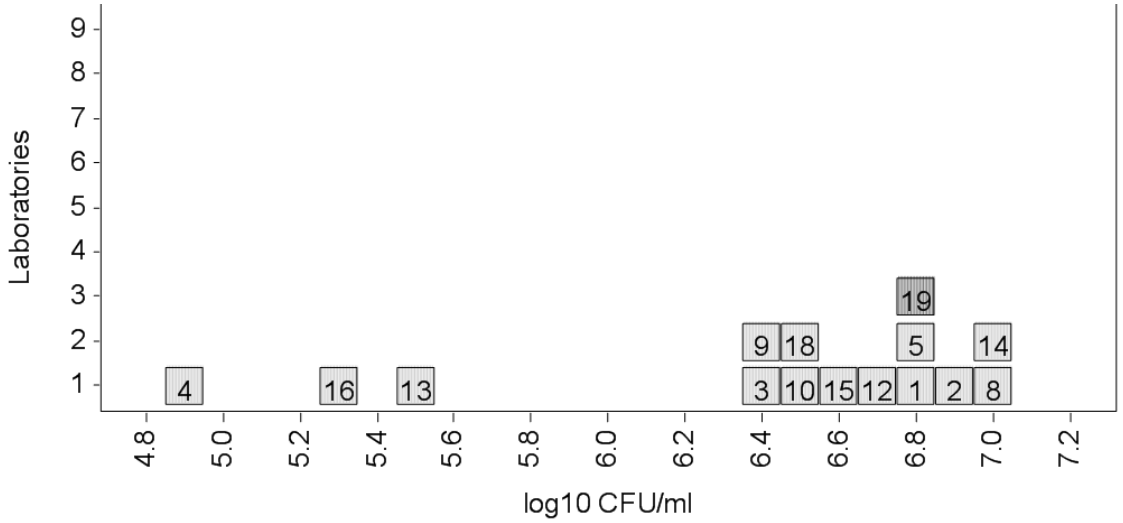
Serratia liquefaciens PEI-A-188



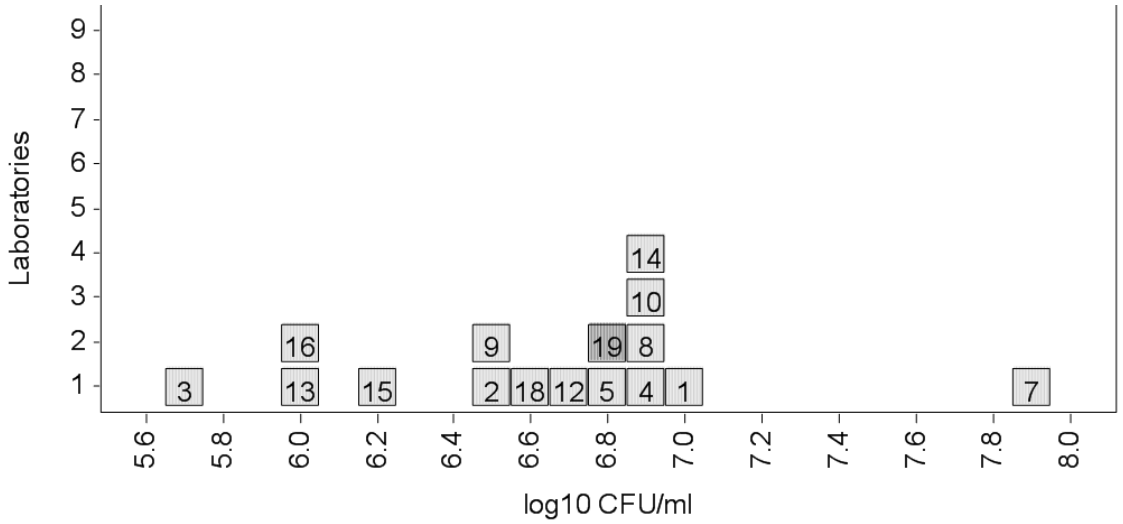
Serratia marcescens PEI-B-P-56



Yersinia enterocolitica PEI-A-105



Yersinia enterocolitica PEI-A-176



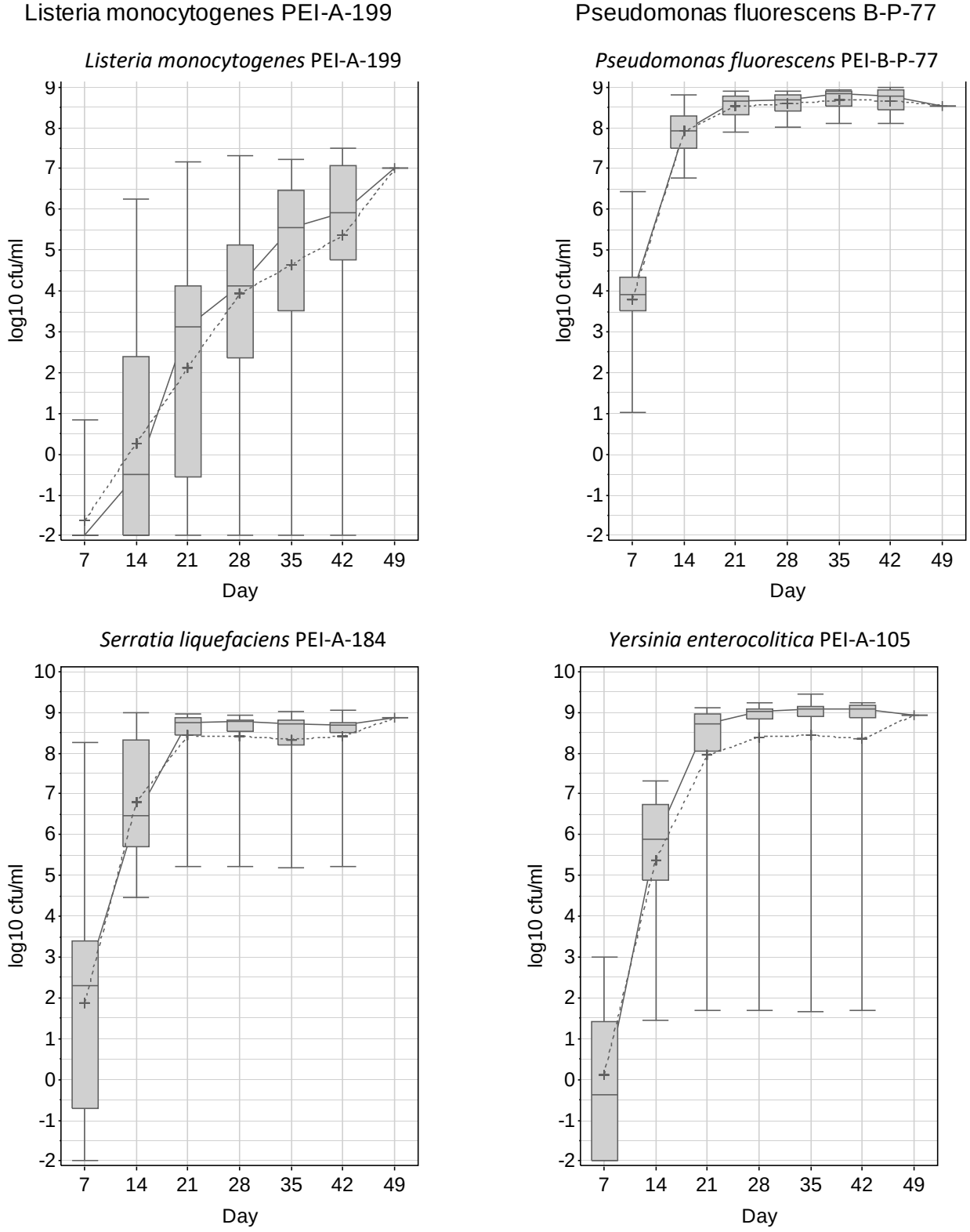
Bacterial Growth in RBCs

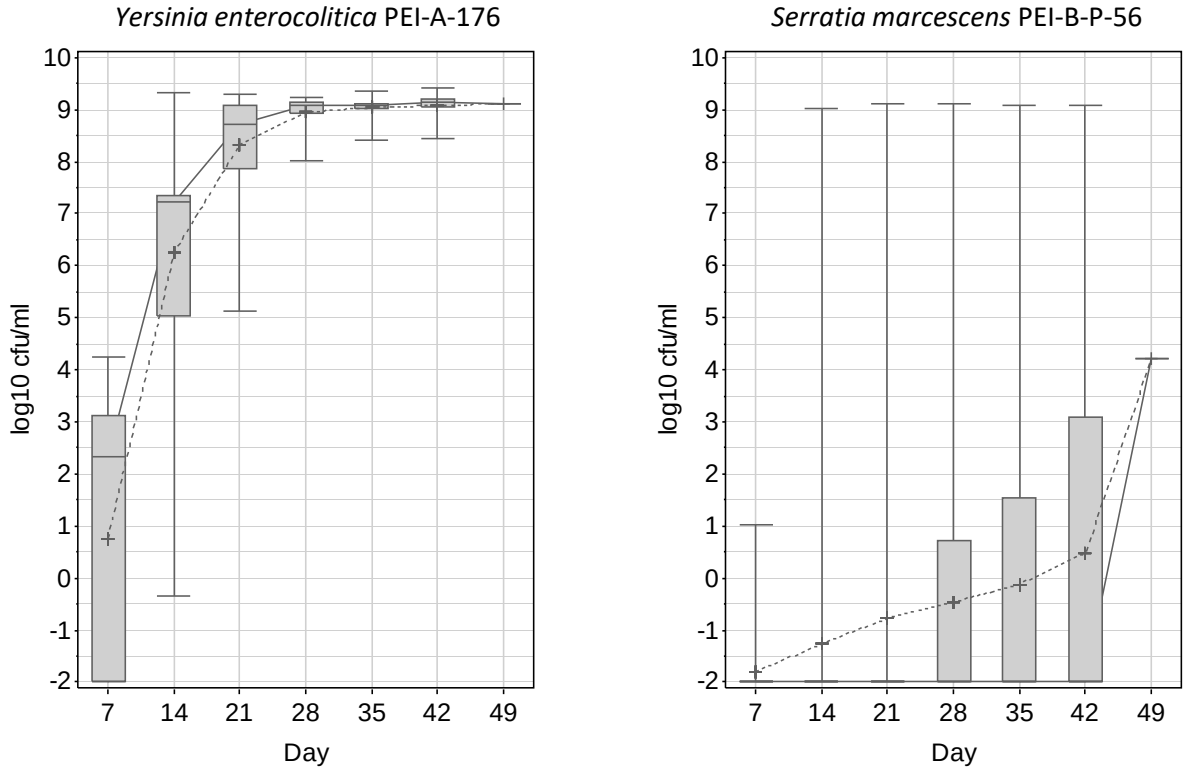
In order to characterize the growth kinetic of each bacterial strain in RBC under cold storage conditions, CFU determination was performed at day 7, 14, 21, 28, 35 and 42 post spiking. Cell counts are summarized in **Table 2** and results are presented as Box- and Whisker plots in **Fig. 6**.

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

Test Strain	Day	N[1]	Mean[2]	95% CI[3]		Median	Min	Max
PEI-A-199, *Listeria monocytogenes*	7	15	-1.63	-2.17	-1.10	-2.00	-2.00	0.84
PEI-A-199, *Listeria monocytogenes*	14	15	0.27	-1.08	1.62	-0.50	-2.00	6.24
PEI-A-199, *Listeria monocytogenes*	21	15	2.12	0.53	3.72	3.11	-2.00	7.18
PEI-A-199, *Listeria monocytogenes*	28	15	3.94	2.61	5.27	4.12	-2.00	7.31
PEI-A-199, *Listeria monocytogenes*	35	15	4.65	3.30	5.99	5.54	-2.00	7.24
PEI-A-199, *Listeria monocytogenes*	42	15	5.36	4.04	6.68	5.90	-2.00	7.50
PEI-B-P-77, *Pseudomonas fluorescens*	7	14	3.80	2.98	4.62	3.90	1.03	6.44
PEI-B-P-77, *Pseudomonas fluorescens*	14	13	7.92	7.54	8.29	7.94	6.78	8.80
PEI-B-P-77, *Pseudomonas fluorescens*	21	13	8.52	8.32	8.72	8.64	7.90	8.88
PEI-B-P-77, *Pseudomonas fluorescens*	28	13	8.59	8.41	8.77	8.70	8.01	8.89
PEI-B-P-77, *Pseudomonas fluorescens*	35	13	8.68	8.50	8.85	8.85	8.11	8.93
PEI-B-P-77, *Pseudomonas fluorescens*	42	12	8.67	8.47	8.87	8.78	8.09	8.99
PEI-A-184, *Serratia liquefaciens*	7	15	1.87	0.35	3.39	2.31	-2.00	8.26
PEI-A-184, *Serratia liquefaciens*	14	15	6.81	6.07	7.55	6.47	4.47	8.98
PEI-A-184, *Serratia liquefaciens*	21	14	8.43	7.87	8.99	8.75	5.23	8.97
PEI-A-184, *Serratia liquefaciens*	28	13	8.41	7.82	9.01	8.77	5.22	8.93
PEI-A-184, *Serratia liquefaciens*	35	14	8.33	7.77	8.90	8.71	5.18	9.01
PEI-A-184, *Serratia liquefaciens*	42	13	8.42	7.82	9.01	8.69	5.22	9.04
PEI-B-P-56-01-03, *Serratia marcescens*	7	15	-1.80	-2.23	-1.37	-2.00	-2.00	1.03
PEI-B-P-56-01-03, *Serratia marcescens*	14	15	-1.27	-2.84	0.31	-2.00	-2.00	9.02
PEI-B-P-56-01-03, *Serratia marcescens*	21	15	-0.77	-2.46	0.91	-2.00	-2.00	9.11
PEI-B-P-56-01-03, *Serratia marcescens*	28	15	-0.46	-2.22	1.29	-2.00	-2.00	9.11
PEI-B-P-56-01-03, *Serratia marcescens*	35	15	-0.12	-2.02	1.79	-2.00	-2.00	9.09
PEI-B-P-56-01-03, *Serratia marcescens*	42	15	0.47	-1.61	2.56	-2.00	-2.00	9.09
PEI-A-105, *Yersinia enterocolitica*	7	13	0.12	-0.99	1.23	-0.36	-2.00	3.02
PEI-A-105, *Yersinia enterocolitica*	14	12	5.39	4.16	6.61	5.88	1.45	7.32
PEI-A-105, *Yersinia enterocolitica*	21	13	7.95	6.73	9.17	8.72	1.69	9.11
PEI-A-105, *Yersinia enterocolitica*	28	13	8.39	7.17	9.62	9.01	1.70	9.24
PEI-A-105, *Yersinia enterocolitica*	35	13	8.44	7.20	9.68	9.09	1.66	9.44
PEI-A-105, *Yersinia enterocolitica*	42	11	8.35	6.86	9.84	9.08	1.69	9.22
PEI-A-176, *Yersinia enterocolitica*	7	13	0.75	-0.87	2.37	2.35	-2.00	4.24
PEI-A-176, *Yersinia enterocolitica*	14	13	6.25	4.70	7.79	7.22	-0.33	9.33
PEI-A-176, *Yersinia enterocolitica*	21	15	8.33	7.75	8.91	8.71	5.13	9.28
PEI-A-176, *Yersinia enterocolitica*	28	14	8.95	8.75	9.14	9.07	8.01	9.23
PEI-A-176, *Yersinia enterocolitica*	35	14	9.04	8.92	9.17	9.09	8.41	9.36
PEI-A-176, *Yersinia enterocolitica*	42	15	9.08	8.95	9.21	9.13	8.44	9.42

Fig. 6: Box- and Whisker plots of growth kinetics (continuous line connecting the median values per day, denoted by horizontal line within each box; dotted line connecting mean values, denoted by "+").





Listeria monocytogenes shows growth up to 6 $\log_{10}$ cfu/ml, still ongoing at day 42. All other strains show a steep increase with up to 9 $\log_{10}$ cfu/ml almost reached at day 21, with good agreement between most laboratories. *Serratia marcescens* shows only a flat growth until day 42 with different onset of growth between participants.

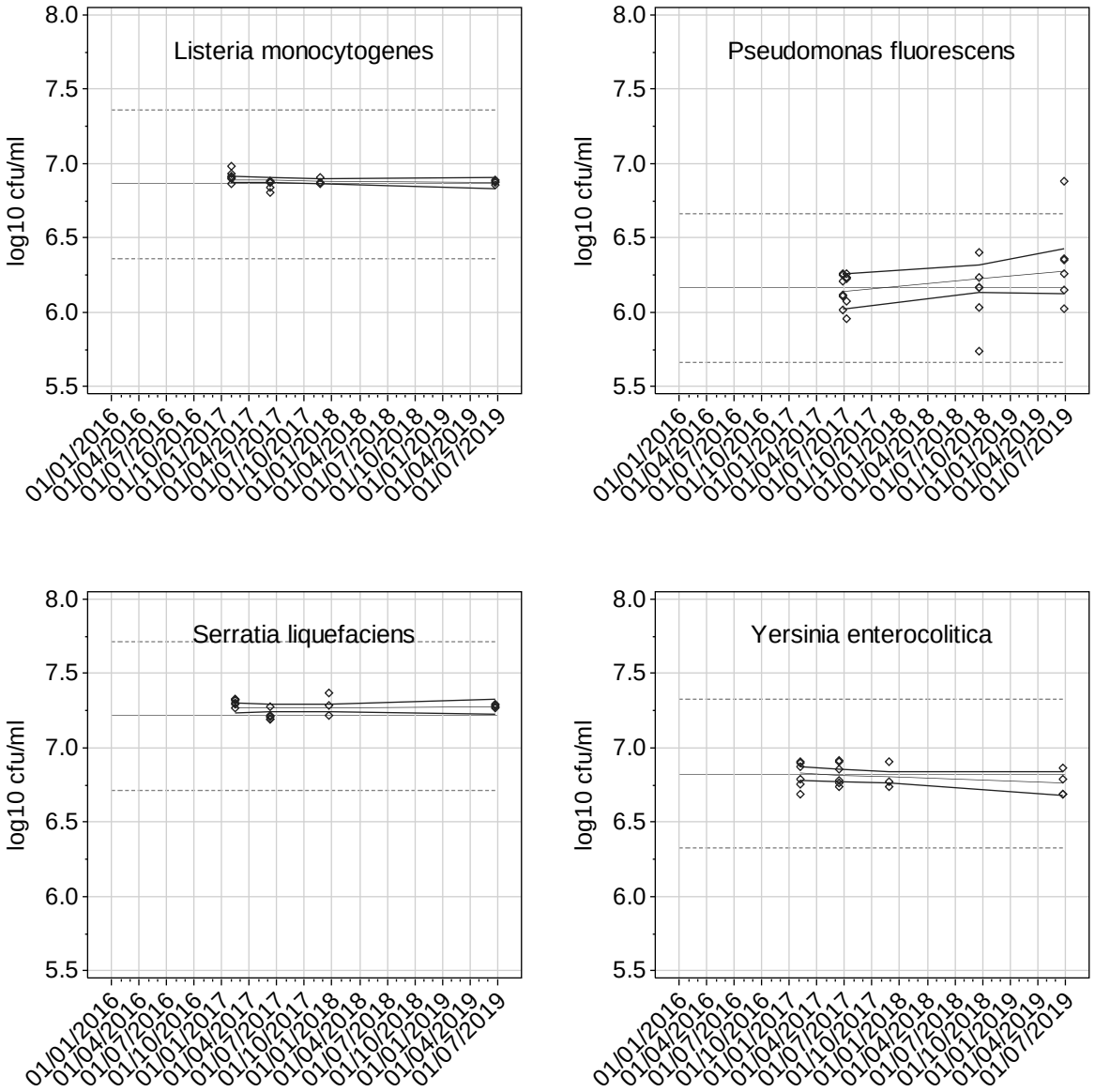
Stability Testing

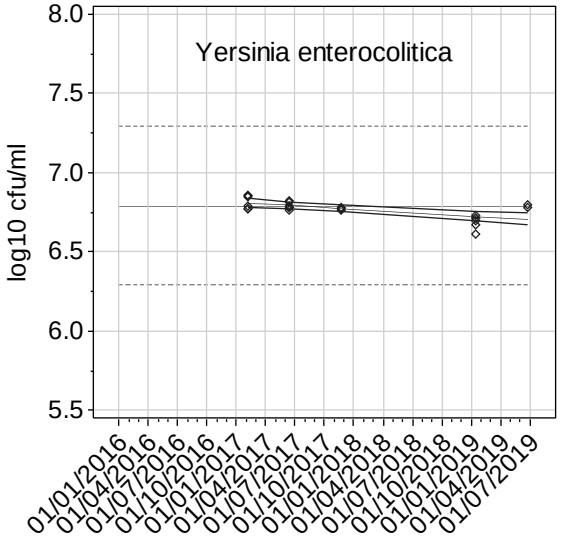
Tab. 3: Estimation of stability parameters by means of a Linear Regression

Test Strain	Variable	Estimate	LowerCL	UpperCL
Listeria monocytogenes, PEI-A-199	Intercept	7.40	6.28	8.51
	Time	-0.00002	-0.00008	0.00003
Pseudomonas fluorescens, PEI-B-P-77	Intercept	2.31	-3.59	8.20
	Time	0.00018	-0.00009	0.00046
Serratia liquefaciens, PEI-A-184	Intercept	7.05	5.42	8.68
	Time	0.00001	-0.00007	0.00009
Serratia marcescens, PEI-B-P-56-02, batch 2	Intercept	0.21	-21.64	22.06
	Time	0.00031	-0.00074	0.00136
Serratia marcescens, PEI-B-P-56-03, batch 3	Intercept	-25.95	-69.65	17.75
	Time	0.00157	-0.00052	0.00366
Yersinia enterocolitica, PEI-A-105	Intercept	8.40	5.92	10.88
	Time	-0.00008	-0.00019	0.00004
Yersinia enterocolitica, PEI-A-176	Intercept	9.25	7.99	10.52
	Time	-0.00012	-0.00018	-0.00006

Fig. 7: Stability of strains

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 (thin black lines above and below linear trend) for the linear trend (thick black line) intersects the lower specification limit (lower dotted line). For *Serratia marcescens*, batch 3, same specifications as for batch 2 were used in the respective figures.




Yersinia enterocolitica
log10 cfu/ml
8.0
7.5
7.0
6.5
6.0
5.5
01/01/2016
01/04/2016
01/07/2016
01/10/2016
01/01/2017
01/04/2017
01/07/2017
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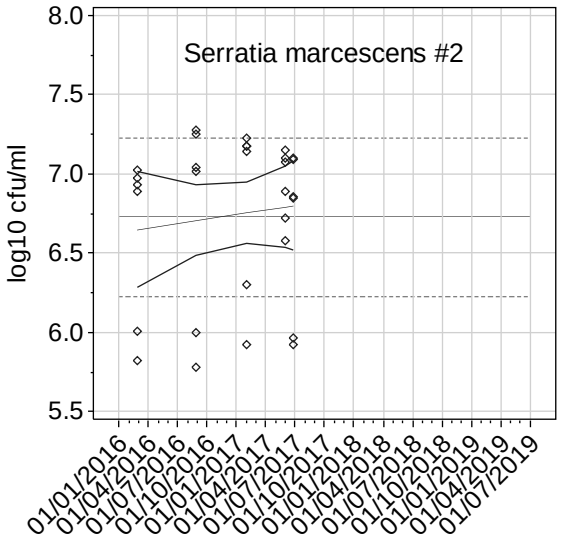
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6.5
6.0
5.5
01/01/2016
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01/01/2017
01/04/2017
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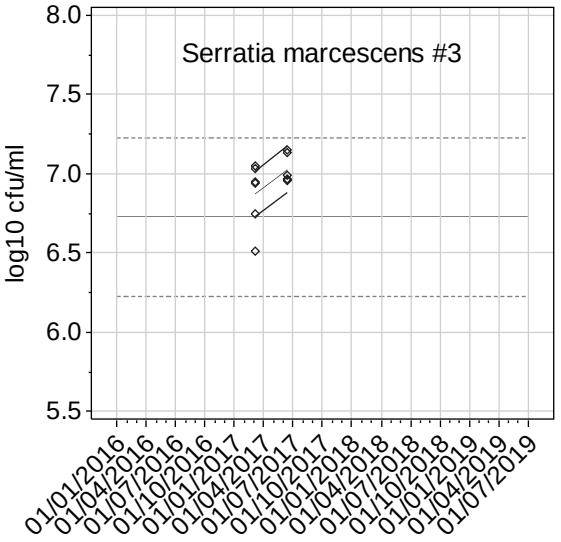
01/07/2019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7.5
7.0
6.5
6.0
5.5
01/01/2016
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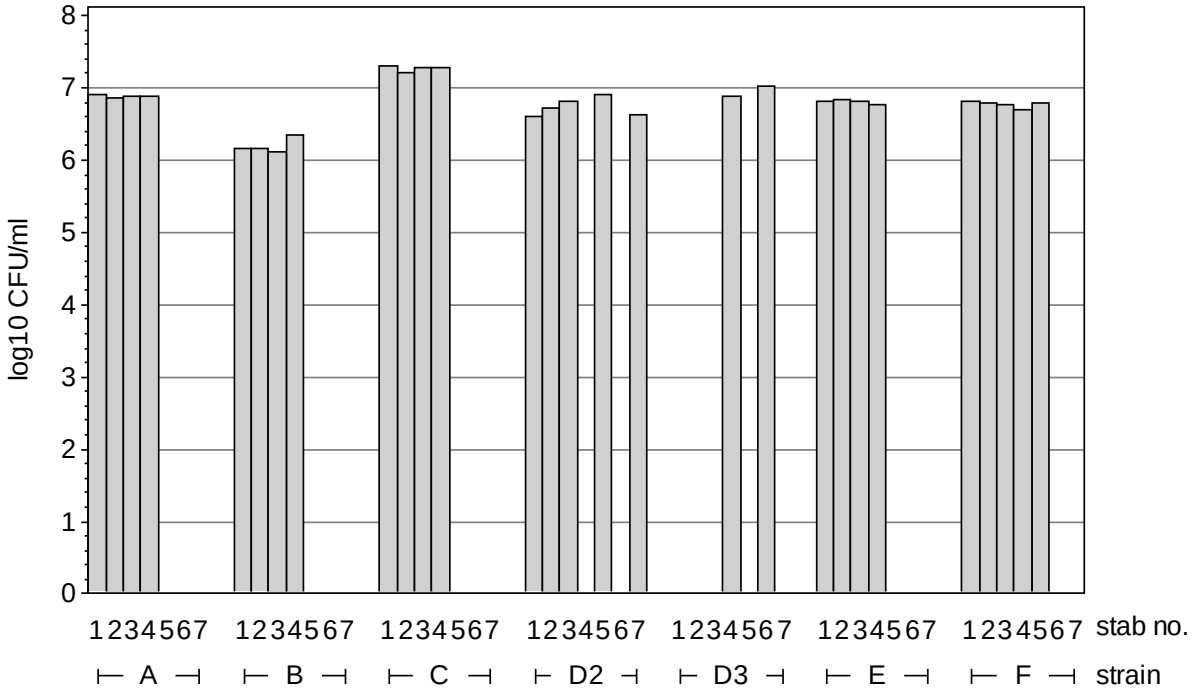
01/07/2016
01/10/2016
01/01/2017
01/04/2017
01/07/2017
01/10/2017
01/01/2018
01/04/2018
01/07/2018
01/10/2018
01/01/2019
01/04/2019
01/07/2019

Fig. 8: Stability of strains over time



Stability Plot of inoculum values by test strains (A= *Listeria monocytogenes* PEI-A-199, B= *Pseudomonas fluorescens* PEI-B-P-77, C= *Serratia liquefaciens* PEI-A-184, D2= *Serratia marcescens* PEI-B-P-56-02 (batch 2), D3= *Serratia marcescens* PEI-B-P-56-03 (batch 3), E= *Yersinia enterocolitica* A-105, F= *Yersinia enterocolitica* A-176)

Five of the six candidate strains show a good stability with respect to the number of CFU over time. *Listeria monocytogenes*, *Serratia liquefaciens* and the two *Yersinia enterocolitica* strains are very stable during the tested period with good precision. For *Pseudomonas fluorescens*, a slight increase of the CFU is observed which could be explained by errors during the sampling and enumeration procedure reflected by a higher variability of the data. Batch #2 of *Serratia marcescens* was replaced by batch#3 during the stability studies due to a high variability within batch#2.

Bacterial Identification

Following 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.

Tab. 4: Strain identification results grouped for each participating laboratory

	Lab1	Lab2	Lab3	Lab4	Lab5	Lab7	Lab8	Lab9	Lab10	Lab12	Lab13	Lab14	Lab15	Lab16	Lab 18
Method of Identification	BBL Crystal	VITEK 2	VITEK 2	VITEK MS	VITEK MS	VITEK 2	Bruker MS	16S	VITEK 2 /MS	16S	API	MS	Maldi	16S / FAME	Maldi/ VITEK2
L. monocytogenes PEI-A-199	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
P. fluorescens PEI-B-P-77	yes	yes	yes	yes	yes	yes	*P. fluorescens/ synxantha*	*P. fluorescens/ synxantha*	*P. fluorescens* complex	yes	yes	*P. fluorescens/ veronii*	yes	*P. cedrina/ fluorescens*	yes
S. liquefaciens PEI-A-184	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	*S. proteamaculans*	yes
S. marcescens PEI-B-P-56	yes	n.a.	n.a.	yes *1	yes	yes	yes	yes	yes	n.a.	yes	n.a.	n.a.	yes	n.a.
Y. enterocolitica PEI-A-105	*Y. enterocolitica* group	yes	yes	yes	yes	yes	Yes	yes	yes	yes	yes	yes	yes	yes	yes
Y. enterocolitica PEI-A-176	*Y. enterocolitica* group	*Y. enterocolitica /frederiksenii*	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes

n.a. not available

BBL Crystal: biochemical analysis
VITEK 2: biochemical analysis
VITEK MS; Bruker-MS; MS; Maldi: mass spectrometry
16S: 16S rRNA gene sequencing
API: biochemical analysis
FAME: fatty acid methyl ester analysis

*1: mixed culture

Discussion

The main objective of this collaborative study was the identification of bacterial strains which are able to proliferate reliably in cold stored RBC units. For this purpose, vials of pre-selected candidate strains were shipped to study partners worldwide. Except for one site, all participants received the bacterial strains in good and frozen conditions. In one case, shipment was delayed and the bacterial samples were already thawed upon arrival. The shipment was successful in a second attempt.

The serial dilution of the bacterial stock concentrations to approximately 25 CFU/bag for low-count spiking of RBCs worked for almost all participating laboratories. Due to the manual dilution, the starting inoculum differed between the sites to a certain degree with a min/max range between 1 and 215 CFU/bag. However, even extreme low concentrations of 3 or less CFU/bag resulted in a detectable growth after several days post inoculation. The varying starting inocula also might explain the different growth kinetics of the single strains within the first two weeks among the laboratories. In addition, the composition of the RBC units, particularly the residual content of leukocytes and plasma as well as the donor variability itself can influence bacterial growth due to growth promoting or inhibiting effect. Except for *S. marcescens* PEI-B-P-56, all strains show robust and reliable growth in 100% for *Y. enterocolitica* 176, 98% for *P. fluorescens* and *S. liquefaciens*, 96% for *Y. enterocolitica* 105 and 90% for *L. monocytogenes* of all tested RBC units. The growth success is comparable with results of the two previous studies for establishment of platelet-relevant reference strains with rates between 70-100% (WHO/BS/2015.2269). Even though *S. marcescens* PEI-B-P-56 is part of the platelet reference panel, it did not grow in nine out of 13 laboratories at all whereas four labs including PEI reported 100% growth in all three tested RBC units respectively.

A detailed analysis of the growth rate showed that the strains *P. fluorescens, S. liquefaciens* and the two *Y. enterocolitica* strains grow to more than 5 $\log_{10}$ CFU/ml by day 14. In comparison, *L. monocytogenes* reached this concentration only to the end of the 42 day testing period.

Conclusion and Proposals

A main feature of Transfusion-Relevant Bacteria Reference Strains is their reliable growth in their respective matrix under routine storage conditions. In this way, they can serve as a practical tool for the validation and assessment of different safety measures applied or developed in the blood field worldwide. Five of the six candidate strains meet the criteria to become part of a repository for RBC. After low count spiking of the RBC units, they grew up to high counts under routine conditions independently from individual donors properties. In addition, the panel contains both fast and slow growing organisms thereby providing strains with different properties that can be used depending on the application requirements. It is proposed to designate bacterial strains *Listeria monocytogenes* PEI-A-199, *Pseudomonas fluorescens* PEI-B-P-77, *Serratia liquefaciens* PEI-A-184, *Yersinia enterocolitica* PEI-A-105, and *Yersinia enterocolitica* PEI A-176 as WHO Red Blood Cell Transfusion-Relevant Bacteria Reference Strains.

Comments from participants

As the study report was sent to the participating partners only briefly before the submission of the report, comments from the participants are not yet included. Remarks of the study partners will be supplemented in the final report.

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Carl McDonald, Jennifer Allen, Danuta Sawicka, Victoria Maddox; NHS Blood and Transplant, London, UK

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Cheuk-Kwong Lee, Lily Tang, Rita Lo, Cindy Chan, Wai-Chiu Tsoi; Hong Kong Red Cross Blood Transfusion Service, Kowloon, Hong Kong

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Ute Jentsch, Truscha Niekerk, Xoliswa Mpumlwana, Pontsho Simelane, Hellen Malema, Zanele Dlamini, Refilwe Legodu, Kgomotso Tlhoaele; South African National Blood Service, Roodeport, South Africa

Eva Spindler-Raffel, Anja Schneider, Marie Anders-Maurer, Philipp Windecker, Björn Becker, Jacqueline Mauritz, Sigrid Hanitsch; Paul-Ehrlich-Institut, Langen, Germany

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Appendix 1

Study partners

Country	Facility	Partner
England	NHS Blood and Transplant	Carl McDonald
Canada	Canadian Blood Service	Sandra Ramirez-Arcos
Austria	Blutzentrale Linz	Susanne Süßner
Mexico	Centro Nacional de la Transfusión Sanguínea	Julieta Rojo Medina
Germany	German Red Cross Blood Service NSTOB	Axel Seltsam
Canada	Héma-Québec	Marc Cloutier
USA	Terumo BCT	Susanne Marschner
Japan	Japanese Red Cross	Masahiro Satake
Germany	Transfusion Medicine, University Hospital Cologne, Cologne	Birgit Gathof
USA	Case Western Reserve University	Michael Jacobs
Ireland	Irish Blood Transfusion Service	Niamh O'Flaherty
USA	Cerus	Peter Bringmann
Hong Kong	Hong Kong Red Cross Blood Transfusion Service	Cheuk-Kwong Lee
USA	American Red Cross	Stephen Wagner
South Africa	South African National Blood Service	Ute Jentsch
Germany	Institute for Laboratory and Transfusion Medicine at Ruhr University	Tanja Vollmer

Appendix 2

Study Protocol

ISBT Working Party
Transfusion-Transmitted Infectious Diseases
Subgroup on Bacteria

WHO – Repository
International Validation Study
on Red Blood Cell Concentrates – Transfusion -Relevant
Bacterial Reference Strains

Study Protocol

Test of growth ability
of selected transfusion-relevant bacteria strains in
Red Blood Cell Concentrates

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CONTENTS

1. Background

Bacterial reference strains are a suitable tool for objective validation and assessment of various microbiological methods for blood safety and development of new techniques. Reference strains allow regulatory agencies, blood manufacturers, and companies who are developing novel screening methods and pathogen reduction technologies, to make informed decisions in a standardized manner. As a first milestone, four Platelet Transfusion-Relevant Bacterial Strains were validated in an international study in cooperation with the ISBT WP TTID Bacterial subgroup. These bacterial strains were established in 2010 as the 1st WHO Repository of Platelet Transfusion-Relevant Bacterial Reference Strains (Störmer et al, 2012). After a second collaborative study in cooperation with the ISBT WP TTID Bacterial subgroup, 10 strains were added to the WHO Repository of Platelet Transfusion Relevant Bacterial Reference Strains in October 2015 for a total of 14 reference strains (Spindler-Raffel E et al., 2017, paper submitted).
Most bacteria isolated from platelet components, which are stored at room temperature, are unable to grow or even survive in Red Blood Cell Concentrates (RBCs) under mandatory cold storage conditions from 1 to 6°C. Bacterial strains which are reported to proliferate to clinically significant levels in RBCs are psychrophilic bacteria, primarily Gram-negative species such as *Serratia marcescens* and *Yersinia enterocolitica* (Ramirez-Arcos et al., 2013). Therefore, most of the WHO Repository of Platelet Transfusion Relevant Bacterial Reference Strains are not suitable to be used as bacterial reference strains for RBCs.

Statistically, the prevalence of bacterial contamination in RBC is 1 in 30,000 with septic reactions of 1 in 500,000 and projected fatality rates of approximately 1 in 10 million (Chen et al., 2008). Funk et al. (2011) reported four fatalities between 1997 and 2010 caused by transfusion of bacterially-contaminated RBCs in Germany. The causative bacteria were *Staphylococcus aureus*, *Serratia marcescens* and *Yersinia enterocolitica*. From 2010 to 2014, the FDA reported 1 fatality caused by a RBC unit contaminated with *Pseudomonas fluorescens*. Similarly, there was one case of a fatal transfusion reaction involving RBCs contaminated with *Pseudomonas koreensis* documented in the SHOT report of 2009. *Klebsiella pneumoniae* has also been implicated in a septic transfusion event involving contaminated RBC. (Funk et al, 2011, Niu et al 2006, Perez et al 2001). Frati et al, (2015) published a case report of a RBC transfusion transmitted septic reaction with a fatal outcome in Italy caused by *Yersinia enterocolitica*. In December 2015, there was a case of a fatal reaction with RBCs contaminated with *Aeromonas hydrophila* documented by Héma-Québec (Germain M et al, AABB 2016).

Bacterial transmissions from RBCs are most frequently caused by *Y. enterocolitica*, followed by *Pseudomonas* spp. and *Serratia* spp. In approximately 80 % of the reported cases, the organisms are capable of growing at refrigerated temperature (Wagner 2004). Interestingly, Damgaard et al. (2015) published a list of bacteria that segregated to the RBC fraction after whole blood processing. Similar observations were recently published by Taha et al. (2016).

For a standardized validation of specific parameters (e.g., storage conditions), bacterial screening methods or pathogen reduction systems, it is crucial to use reference strains which are proven to be viable and able to proliferate in the respective blood component.
In line with the strategy to establish Transfusion-Relevant Bacterial Reference Strains for all blood components, the ISBT TTID bacterial subgroup intends to establish a bacterial panel for RBCs. This issue was discussed at the meeting of the ISBT TTID bacterial subgroup in London, June 2015. A proposal was presented to the WHO Expert Committee of Biological Substances (WHO ECBS), who endorsed the establishment of a WHO repository of Red Blood Cell Transfusion-relevant Bacterial Reverence strains during their meeting in Geneva in October 2015.

The list of bacteria with relevance in RBC transfusion, presented to the WHO ECBS in October 2015

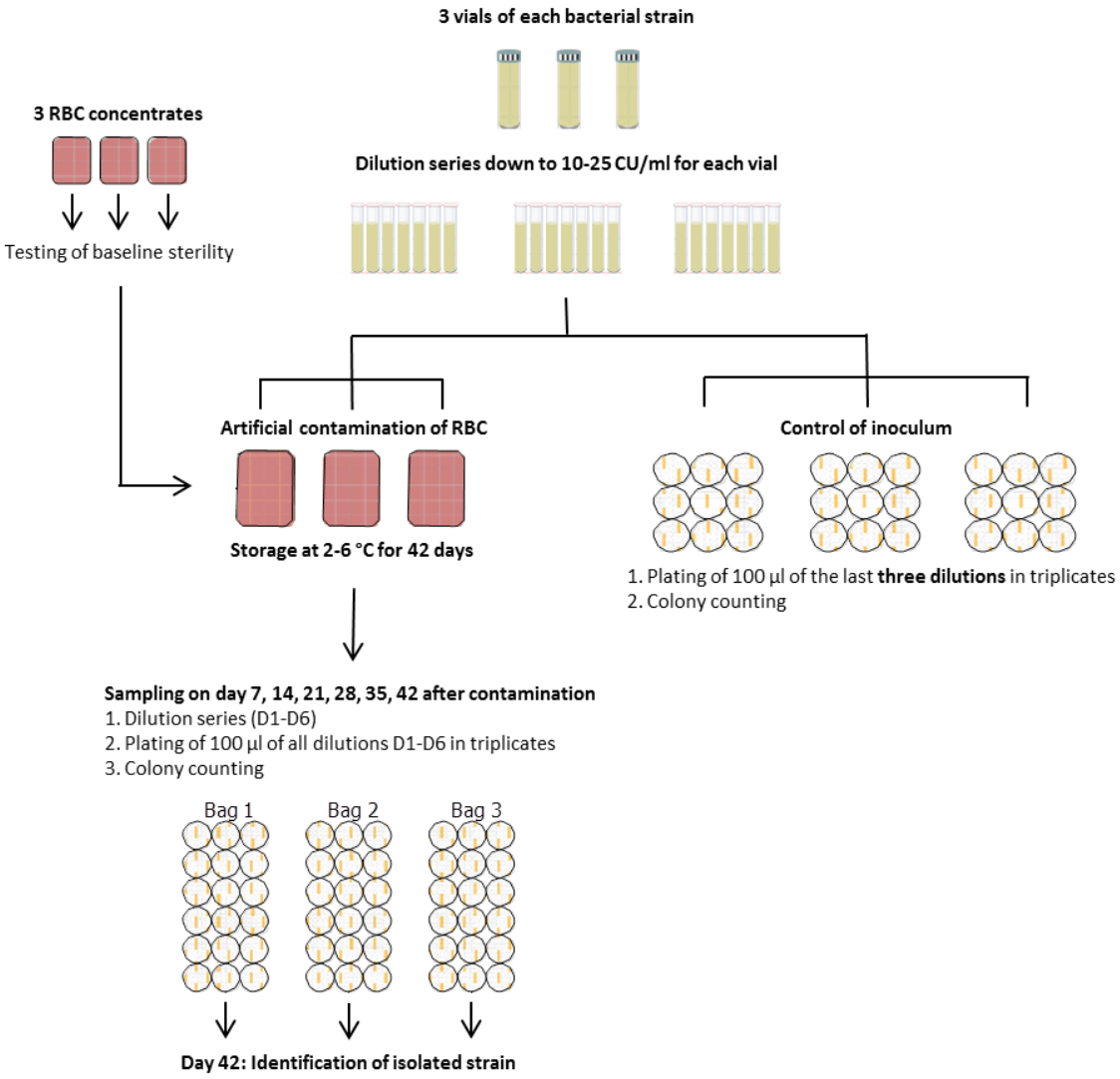
includes:
Gram-positive species: *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pyogenes*, *Listeria monocytogenes*
Gram-negative species: *Pseudomonas aeruginosa, Yersinia enterocolitica, Serratia marcescens, Serratia liquefaciens*
Preliminary work was conducted at PEI with these strains including the analysis of growth characteristics in 2 media (CSB and Thioglycolat) and RBC units with 3 repetitions. Based on this work it was decided that 6 strains will be used for the subsequent studies with international partners. Due to the fact that growth ability may vary among the bacterial species and even at the strain level, it is important to validate the candidate strains in an international collaborative study (cooperation with the ISBT WP TTID Bacterial subgroup).

2. Study Design

The six selected bacterial reference strains will be sent to the partners. For each bacterial strain, three RBCs, not older than day 7 after collection, have to be artificially inoculated with 10-25 CFU/bag according to the diagram in Figure 1. Before bacteria inoculation, the baseline sterility of the RBC units needs to be proven according to the routine standard operating procedures used in each participating laboratory.

For bacterial inoculation, 3 vials (one per RBC bag) of one reference strain needs to be 10-fold diluted in sterile NaCl to achieve a final bacterial cell count 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, which will be incubated at the strain specific temperature provided with the dilution protocol. Colonies will be counted the following day after incubation. After bacterial spiking, the RBC units will be stored under routine conditions. Sampling is to be performed according to the protocol on days 7, 14, 21, 28, 35 and 42 of storage from all 3 RBC bags. A dilution series up to dilution step 6 in the dilution protocol is performed from each sample (in total 3 dilution series per strain) and 100 µl of each dilution is plated in triplicate onto agar plates. After plate incubation, bacterial concentration will be determined by colony counting. Bacteria identification will be performed from day 42 isolates of the three RBC bags to confirm the identity of the inoculated bacterium.

Figure 1



3. Shipping and Storage

The six 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 tested 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 please inform the study coordinating team immediately.

3.1. Labelling of Bacterial Reference Strains

Six different bacteria strains are contained in vials in 6-replicates (3 vials serve as a reserve).

Each vial is labelled with the name of the bacterial strain and PEI-identification/lot number.

List of RBC Test Strains

	PEI ID	Strain	Origin
Listeria spp	PEI-A- 199	*Listeria monocytogenes*	Isolate Blood screening, NHS Blood and Transplant, England
Serratia spp	PEI-B-P-56	*Serratia marcescens*	1st WHO repository, enlargement
	PEI-A-184	*Serratia liquefaciens*	Isolate RB; C92-13-01, Roth V, et al Transfusion 2002;40(8):931-5, CDC,
Pseudomonas spp	PEI-B-P-77	*Pseudomonas fluorescens*	1st WHO repository, enlargement
Yersinia spp	PEI-A-105	*Yersinia enterocolitica*	Isolate RBC, Japan
	PEI-A-176	*Yersinia enterocolitica*	Isolate RBC, CDC

3.2. Storage of RBC Test Strains

Store the vials immediately after arrival in a deep freezer at -80°C without secondary packaging.

4. Test of growth of selected transfusion-relevant bacterial reference strains in RBCs

4.1. Materials

- 3 vials of each bacteria test strain. (3 additional vials serve as reserves)

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

- 3 RBC units for each strain
- Preferably use fresh RBC (up to day 7 after collection)
- RBC storage device (temperature controlled, 1-6°C or 2-6°C, according to local procedures)
- Dry incubator at 37°C and 30°C
- Sterile welding equipment (e.g. Sterile Connecting Device) (if applicable)
- 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 RBCs

Prior to bacterial inoculation all RBCs have to be tested for sterility according to local procedures.

The procedure used for sterility should be described in the results section.

4.3. Thawing / Defrosting Bacterial Reference Strains

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

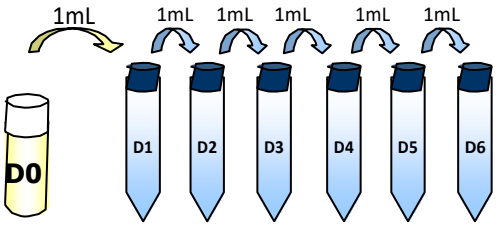
Note: The bacterial strains (stock suspensions) must be used immediately after thawing.

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 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 Red Blood Cell Concentrate / spiking

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

- Connect each RBC 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 RBC 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 RBC bag. Afterwards the final bacterial load will be 10 – 25 CFU per bag.
- Add the previously removed 5 mL RBC 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 RBC bag.
- Rinse the syringe 3 times with RBC.
- Incubate the contaminated RBC units at or 2-6°C for 42 days.

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 RBC main bag.
Additionally, bacteria attached to the inner surface of the tube will be detached. Avoid any entry of air into the RBC 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 (until growth is visible).
- 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 RBC storage conditions (storage at 2-6°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 7, 14, 21, 28, 35 and 42.
- Sample drawing shall be performed following the principles described in section 4.5. (Artificial contamination / spiking of RBC concentrate).

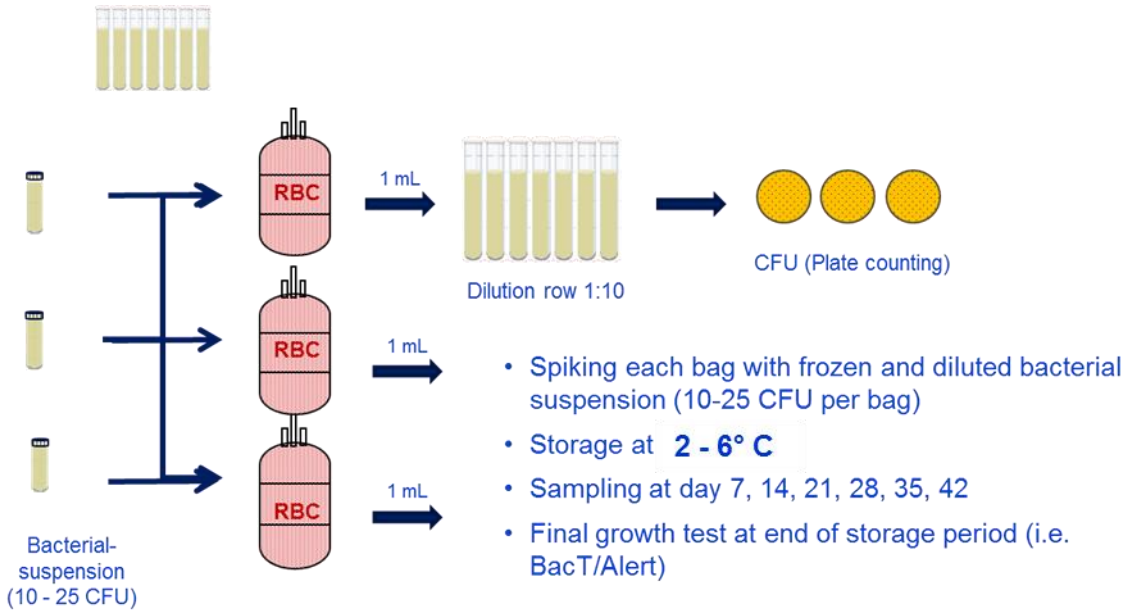
- **Immediately before sampling, mix the content by kneading the bag to reduce sampling errors.**
- If using the **Luer-Lock** connection device - remove the first **5 mL** of the RBC using a sterile syringe but do not discard it, use a second sterile syringe to take a sample (1 mL) of each RBC bag and then add back the previously removed **5 mL** RBC sample in order to enable a repetition if necessary.
- If using the sterile **Coupling-Spike** device – rinse the syringe with RBC from the pack 3 times to ensure the sample is from the bag, remove a 1 mL sample of each RBC 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 RBC.

Please find the dilution series for the strains in the appendices.



5. Documentation

5.1. Enumeration

Please copy for each bacterial strain! (For calculation excel sheets will be sent by mail)

5.1.1. Enumeration 1: after 7 days

Bacterial strain: _____

RBC:_____

Inocula:_____

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 1	Dilution 4				
	Dilution 5				
date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 2	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 3	Dilution 4				
	Dilution 5				
Date	Dilution 6				

5.1.2. Enumeration 2: after 14 days

Bacterial strain: _____

RBC: _____

Inocula: _____

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **RBC bag 1** **Date**	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **RBC bag 2** **Date**	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain **RBC bag 3** **Date**	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
	Dilution 4				
	Dilution 5				
	Dilution 6				

Please copy for each bacterial strain!

5.1.3. Enumeration 3: after 21 days

Bacterial strain: _____

RBC:_____

Inocula:_____

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 1	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 2	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 3	Dilution 4				
	Dilution 5				
Date	Dilution 6				

5.1.4. Enumeration 4: after 28 days

Bacterial strain: _____

RBC:_____

Inocula:_____

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 1	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 2	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 3	Dilution 4				
	Dilution 5				
Date	Dilution 6				

5.1.5. Enumeration 5: after 35 days

Bacterial strain: _____

RBC:_____

Inocula:_____

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 1	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 2	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 3	Dilution 4				
	Dilution 5				
Date	Dilution 6				

5.1.6. Enumeration 6: after 42 days

Bacterial strain: _____

RBC:_____

Inocula:_____

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 1	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 2	Dilution 4				
	Dilution 5				
Date	Dilution 6				

RBC Unit	Dilution 100µl of…	Plate 1	Plate 2	Plate 3	Mean value
Strain	Dilution 0				
	Dilution 1				
	Dilution 2				
	Dilution 3				
RBC bag 3	Dilution 4				
	Dilution 5				
Date	Dilution 6				

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

6. Lab Protocol

Please copy for each bacterial strain!

Test strain:

RBC Concentrates: Exp. Day:

Volume

Blood type /Rh

Result of base line sterility:

Control Inoculum (Dilution of stock): _____CFU/ml (mean value) at dilution D_____

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

Result of enumeration of the stock concentration:_____CFU/ml (mean value)

Bacterial growth after storage	Sampling after	Yes (Growth)	no
	7 days		
	14 days		
	21 days		
	28 days		
	35 days		
	42 days		

Results of Identification:

Method (please add details like reactions)

Microorganism identified:

Match of inoculated strain (name) _____yes: no:

Notes:

Laboratory:

Responsibility:

Dilution steps RBC Test Strains

Strain	PEI-label	CFU/ml (vial) D0	Dilution (to 10 – 25 CFU/ml for spiking)						Spiking in each RBC bag	Incubation temp. °C
Listeria monocytogenes	PEI-A-199	7,29E+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)	D6 (**3 ml** D5 in 7 ml NaCl)	1 ml D6	37
Serratia marcescens	PEI-B-P-56	1,07E+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 (**2 ml** D5 in 8 ml NaCl)	1 ml D6	37
Serratia liquefaciens	PEI-A-184	1,65E+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
Pseudomonas fluorescens	PEI-B-P-77	1,52E+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	30
Yersinia enterocolitica	PEI-A-105	6,79E+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)	D6 (**3 ml** D5 in 7 ml NaCl)	1 ml D6	37
Yersinia enterocolitica	PEI-A-176	6,20E+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)	D6 (**3 ml** D5 in 7 ml NaCl)	1 ml D6	37

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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, e-mail)

Were you a participant of one of the previous WHO-ISBT International Validation Studies 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:

2-6°C Refrigerator: **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. Gram 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.