ORIGINAL PAPER

Establishment of the first International Repository for Transfusion–Relevant Bacteria Reference Strains
ISBT Working Party Transfusion-Transmitted Infectious Diseases (WP-TTID), Subgroup on Bacteria

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Vox Sanguinis

Background   Bacterial contamination of platelet concentrates (PCs) still remains a significant problem in transfusion with potential important clinical consequences, including death. The International Society of Blood Transfusion Working Party on Transfusion-Transmitted Infectious Diseases, Subgroup on Bacteria, organised an international study on Transfusion-Relevant Bacteria References to be used as a tool for development, validation and comparison of both bacterial screening and pathogen reduction methods.

Material and Methods   Four Bacteria References (*Staphylococcus epidermidis* PEI-B-06, *Streptococcus pyogenes* PEI-B-20, *Klebsiella pneumoniae* PEI-B-08 and *Escherichia coli* PEI-B-19) were selected regarding their ability to proliferate to high counts in PCs and distributed anonymised to 14 laboratories in 10 countries for identification, enumeration and bacterial proliferation in PCs after low spiking (0·3 and 0·03 CFU/ml), to simulate contamination occurring during blood donation.

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Results Bacteria References were correctly identified in 98% of all 52 identifications. *S. pyogenes* and *E. coli* grew in PCs in 11 out of 12 laboratories, and *K. pneumoniae* and *S. epidermidis* replicated in all participating laboratories. The results of bacterial counts were very consistent between laboratories: the 95% confidence intervals were for *S. epidermidis*: $1 \cdot 19–1 \cdot 32 \times 10^7$ CFU/ml, *S. pyogenes*: $0 \cdot 58–0 \cdot 69 \times 10^7$ CFU/ml, *K. pneumoniae*: $18 \cdot 71–20 \cdot 26 \times 10^7$ CFU/ml and *E. coli*: $1 \cdot 78–2 \cdot 10 \times 10^7$ CFU/ml.

Conclusion The study was undertaken as a proof of principle with the aim to demonstrate (i) the quality, stability and suitability of the bacterial strains for low-titre spiking of blood components, (ii) the property of donor-independent proliferation in PCs, and (iii) their suitability for worldwide shipping of deep frozen, blinded pathogenic bacteria. These aims were successfully fulfilled. The WHO Expert Committee Biological Standardisation has approved the adoption of these four bacteria strains as the first Repository for Transfusion-Relevant Bacteria Reference Strains and, additionally, endorsed as a project the addition of six further bacteria strain preparations suitable for control of platelet contamination as the next step of enlargement of the repository.

Key words: bacteria reference strains, bacterial contamination, international reference material, pathogen reduction, platelet screening, repository, validation.

Introduction

Bacterial infections by blood transfusion, particularly of platelet concentrates (PCs), represent the most important residual infection risk in developed countries since the impressive reduction in transfusion-transmitted viral infections in many countries, and the third leading cause of death from transfusion after TRALI and haemolytic transfusion reactions due to blood group incompatibilities [1,2]. The reported prevalence of bacterial contamination of PCs is highly variable and difficult to assess due to differences in surveillance, testing methodologies and case definitions. Reported mortality rates for platelet-related sepsis range from 1 in 20 000 to 1 in 100 000 donor exposures and contamination rate of PCs from 0·16% up to 0·6% at the end of their shelf life [3–6].

A fundamental difference between contaminations by viruses and bacteria is that the latter can continue to replicate in a PC during its shelf life, such that under the usual storage conditions at 22–24°C, even extremely small numbers of bacteria can multiply to vast and clinically dangerous levels during their storage period [3]. In addition to bacterial cells, endotoxins and/or exotoxins may be present in blood products, depending on bacterial species and strain, which can also be very harmful [2]. Transfusion of a highly contaminated blood component typically leads to immediate septic shock and sometimes to death of the patient.

Blood services worldwide have implemented interventions to reduce the risk of transmission of bacteria by transfusion. These include (i) primary prevention of the introduction of bacteria during blood donation, through vigorous donor selection, effective skin disinfection, diversion of the first volume from the blood donation and component production process monitoring; (ii) diagnostic detection by component culture and/or rapid detection methods; and (iii) pathogen reduction [3–5,7–12]. To validate and assess the methods for bacterial screening and pathogen reduction in a consistent manner, bacterial strains which are able to proliferate in blood components are needed. Studies by the Paul-Ehrlich-Institute (PEI, Federal Institute for Vaccines and Biomedicines, Germany) as well as several other reports have shown that not all bacteria, including established bacterial reference strains, are suitable for validation studies of transfusion-related bacterial detection and reduction methods, because they are not able to multiply reliably in blood components [13,14]. However, no international references currently exist for investigative purposes concerning methods to improve microbial safety of blood components. For this reason, a procedure has been developed to manufacture deep frozen bacterial suspensions (Transfusion-Relevant Bacteria References, TRBR) of defined species with known count and ability to grow in PCs. After thawing, the TRBR are ready to use and can be applied immediately for low spiking of blood components with 10 CFU per bag, corresponding to approximately 0·03 CFU/ml to represent the bacterial load potentially present after venepuncture during blood donation [8]. To prove the stability and robustness of the TRBR and their growth characteristics under routine conditions in

PCs worldwide, an international validation study was organised by the Subgroup on Bacteria of the Working Party on Transfusion-Transmitted Infectious Diseases of the International Society Blood Transfusion (WP-TTID of ISBT).

Material and methods

Study design

The project was co-ordinated by a small working party (study board) of the Subgroup on Bacteria of the ISBT WP-TTID. Four TRBR (10 identical vials each) were prepared by the PEI in August 2008 and distributed in collaboration with the American Type Culture Collection (ATCC) to 14 participating expert laboratories worldwide (Table 1). The partners were asked to (i) culture and identify the bacterial species, (ii) estimate the bacterial count of each TRBR in 5 independent replicates, and (iii) spike at least two platelet bags [apheresis PCs (APCs) or pooled, whole blood-derived PCs (PPCs)]: one with 10 CFU ($\sim$0·03 CFU/ml) per bag and one with 100 CFU per bag ($\sim$0·3 CFU/ml). Detailed protocols for the identification of species, counting of bacteria, and spiking of PCs as well as data sheets for the results were provided by the study co-ordinators. All experiments had to be carried out in parallel by all participants within a fixed time period (November 2008–February 2009). Results had to be sent up to a defined deadline before de-blinding at the ISBT Regional Meeting in Cairo, Egypt, 2009.

Production of Transfusion-Relevant Bacteria References (TRBR)

Four TRBR that caused infections in transfusion or other biological drug recipients were characterised regarding identity and their ability to grow up to high counts in PCs from at least 100 different donors after low count spiking. Afterwards, they were manufactured as deep frozen suspensions which were stable, shippable and exactly defined in count of living bacterial cells. For this purpose, the growth kinetics of theses bacterial strains were characterised in Trypticase Soy broth (heipha Dr. Mueller GmbH, Eppelheim, Germany) at 37°C in an automated culture system (BacTrac, Sy-Lab, Neupurkersdorf, Germany), to define their specific logarithmic growth period. During the second cultivation, all bacterial species were harvested in their logarithmic growth period and thereafter deep frozen at −80°C. Human serum albumin 20% (Biotest AG, Dreieich, Germany) was added as a stabiliser to the bacterial suspension at a ratio of 1:1 before freezing at −80°C. The bacterial count was enumerated before freezing and 1 day after thawing. Finally, each vial of the TRBR contains 1·5 ml of deep frozen bacteria suspended in Trypticase Soy broth and 10% human serum albumin.

Table 1 Study board and participants of international validation study

Country	Facility	Investigator
Study board		
Germany	Paul-Ehrlich-Institute, Microbial Safety, Langen	Thomas Montag Melanie Störmer
Australia	Australian Red Cross Blood Service, Melbourne	Erica Wood
United Kingdom	NHS Blood and Transplant, London	Carl McDonald
Participating laboratories		
1. Austria	Austrian Red Cross, Blutzentrale Linz	Christian Gabriel
2. Canada	Canadian Blood Service, Ottawa	Sandra Ramirez-Arcos Dana Devine
3. China	Hong Kong Red Cross Blood Transfusion Service	Cheuk-Kwong Lee
4. Germany	German Red Cross, Frankfurt/Main	Michael Schmidt
5. Germany	Paul-Ehrlich-Institute, Microbial Safety, Langen	Thomas Montag Melanie Störmer
6. Germany	German Red Cross, Springe	Thomas Müller Bernd Lambrecht
7. México	Centro Nacional de la Transfusion Sanguínea	Julieta Rojo Antonio Arroyo
8. Poland	Regional Centre for Transfusion Medicine, Bialystok	Piotr Radziwon
9. The Netherlands	Sanquin Blood Supply Foundation, Amsterdam	Dirk de Korte
	VU Medical Centre, Amsterdam	Annika Petterson
10. United Kingdom	NHS Blood and Transplant, London	Carl McDonald Siobhan McGuane
11. USA	CaridianBCT Biotechnologies, Denver	Ray Goodrich Shawn Keil
12. USA	Louis Stokes Veterans Administration Medical Center, Ohio	Roslyn Yomtovian
	Case Western Reserve University, Ohio	Michael R. Jacobs
13. USA	Walter Reed Army Medical Center, Washington DC	David G. Heath Hector Carrero
14. South Africa	South African National Blood Service	Tshilidzi Muthivhi

The TRBR were encoded with the letters: A (*S. epidermidis* PEI-B-06), B (*S. pyogenes* PEI-B-20), C (*K. pneumoniae* PEI-B-08) and D (*E. coli* PEI-B-19), respectively. The identities of new lots of TRBR were confirmed by the API Identification System (BioMerieux, Durham, NC, USA), microscopic and macroscopic morphology, Gram staining and DNA sequencing (MicroSeq 16S rDNA Bacterial Identification System; Applied Biosystems, Foster City, CA, USA).

To assure the batch-to-batch consistency, DNA fingerprinting [15] was performed. Growth abilities of the new lots were shown in four pool PCs each inoculated with 10 CFU/bag and monitored by plate assay.

Enumeration and stability testing of TRBR

Enumeration was made, and stability testing (ST) were performed, before freezing (ST_0), between 24 and 144 h after production (ST_1) and during storage from August 2008 to January 2009 after 23 ± 7 days (ST_2), 120 ± 5 days (ST_3) and 164 ± 9 days (ST_4) after production (for details see Table 2). For this purpose, a defined number of vials of each TRBR ($n_{ST0} = 1$, $n_{ST1} = 6$, $n_{ST2} = 3$, $n_{ST3} = 6$, $n_{ST4} = 5$) were transferred directly from a freezer ($-80°C$) to a dry incubator at $37°C$ for 10 min. If ice crystals were still evident, the vial was warmed in the hand until the content melted completely. The stock suspensions were used immediately after thawing. With the exception of ST_4, which was performed according to the study protocol, two dilution series per vial were produced. The spread plate method was used, and 100 μl of one defined dilution was distributed onto six solid agar plates followed by aerobic incubation at $37°C$ for 1–2 days. In contrast, 100 μl of each dilution was spread on five agar plates for ST_4 according to the study protocol, incubated for 1–2 days at $37°C$; enumerated and mean values were calculated.

Study protocols

Culture and identification of bacterial species
Participants were asked to culture on Trypticase Soy Agar or Columbia Blood Agar plates and to identify the blinded samples following their routine laboratory protocols and identification panels, including conventional identification by Gram stain, as well as microscopic and macroscopic observations.

Enumeration of bacteria by participants
Blinded samples were enumerated in five independent replicates of each TRBR. For each experiment, tenfold serial dilutions in sterile saline (0·9%) up to dilution step 6 (10^{-6}) of the samples were performed. The spread plate method was used, and 100 μl of each dilution was distributed onto five solid agar plates followed by aerobic incubation at $37°C$ for 1–2 days. Finally, the bacterial colonies were counted. Agar plates containing ≥300 colonies were discarded from the evaluation.

Growth of bacteria in PCs after low spiking
PCs were sampled before bacterial inoculation and tested by sterility testing routinely used in the participating laboratory to assure baseline sterility, using an automated culture system or performing conventional inoculation into liquid media and incubation. Afterwards, at least two PCs [APC or pooled platelet concentrate (PPC)] which are commonly used in the participating laboratory were inoculated with very low bacterial count of each TRBR, based on the results obtained during previous enumeration of bacteria. Following a defined procedure, one of the PCs was spiked via a Luer-lock connection device with approximately 100 bacteria per unit (corresponding to ∼0·3 CFU/ml) and the other PC was spiked with approximately 10 bacteria per unit (corresponding to ∼0·03 CFU/ml). For this purpose, a PC was combined with a Luer-lock connection device, and 5 ml of PC was drawn from each bag using a sterile syringe for final flushing of the connecting device. In the next step, 1 ml of the final dilution containing the recommended

Table 2 Stability testing (ST) of TRBR during validation study

Stability Testing Number of agar plates		ST0 n = 8	ST1 n = 72	ST2 n = 36	ST3 n = 72	ST4 n = 150
Staphylococcus epidermidis	Date	15·08·2008	18·08·2008	02·09·2008	10·12·2008	21·01·2009
	Age	Production	3 days	18 days	117 days	159 days
	Count[a]	1·29 ± 0·31	1·06 ± 0·05	1·37 ± 0·05	1·15 ± 0·13	1·69 ± 0·44
Streptococcus pyogenes	Date	14·08·2008	20·08·2008	02·09·2008	10·12·2008	21·01·2009
	Age	Production	6 days	19 days	118 days	160 days
	Count[a]	0·21 ± 0·05	0·31 ± 0·05	0·59 ± 0·09	0·84 ± 0·15	0·52 ± 0·12
Klebsiella pneumoniae	Date	31·07·2008	01·08·2008	03·09·2008	09·12·2008	28·01·2009
	Age	Production	1 day	34 days	127 days	177 days
	Count[a]	16·8 ± 0·56	18·5 ± 2·24	24·4 ± 0·41	16·7 ± 1·21	20·4 ± 1·92
Escherichia coli	Date	12·08·2008	18·08·2008	03·09·2008	10·12·2008	22·01·2009
	Age	Production	6 days	22 days	116 days	159 days
	Count[a]	2·85 ± 0·61	3·48 ± 0·50	2·77 ± 0·09	2·71 ± 0·34	2·32 ± 0·56

SD, standard deviation; *n*, number of counted agar plates; TRBR, transfusion-relevant bacterial reference(s).
[a]Mean ± SD ($\times 10^7$ CFU/ml)

bacterial count was inoculated via the Luer-lock connection device under aseptic conditions. Finally, the previously removed 5 ml was added to flush the tube segment of the bag. Additionally, 100 µl of the pre-last dilution was spread onto three agar plates for inoculum control. Thereafter, the contaminated PC units were stored under routine conditions at 22–24°C with agitation for 96 h, followed by sampling and determination of the bacterial count by colony counting.

Statistical analysis

Statistical analysis was performed at PEI based on the raw data sent by the participants up to the study deadline. The data were read from the result sheets as recorded by each participant. Comparison between the laboratories was made by means of a mixed linear model with fixed (laboratory) and random (vial) factors. Confidence intervals for the estimated differences between each participant and PEI as well as for P-values were adjusted using the Bonferroni method [16]. For this evaluation, no values were removed from the analysis. The statistical analysis was performed with SAS®/STAT software, version 9.2; SAS System for Windows.

Results

Stability testing of Transfusion–Relevant Bacteria References during study period at PEI

Stability testing was performed during storage from August 2008 to January 2009 at days 23 ± 7 (ST_2), 120 ± 5 (ST_3) and 164 ± 9 (ST_4) after production. As can be seen from the calculated mean values and standard deviations, stability was proven for the regarded period of 6 month which included the production, the shipping and participants enumeration procedures. All details regarding the date of ST, age of TRBR and enumeration results are displayed in Table 2.

Culture and identification by participants

All data received included the identification and enumeration results of 13 of 14 laboratories including PEI. Unfortunately, one participant did not receive the samples in time because of administrative and logistical delays, with the consequence that the results could not be submitted before the fixed deadline.

In the participating laboratories, at least one of the following procedures were performed for bacterial identification: standard biochemical tests, API and VITEK Identification Systems (BioMerieux), Biolog Identification System (Biolog, Hayward, CA, USA), MicroScan Walk-

Away System (Dade Behring, West Sacramento, CA, USA), BD Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD, USA) and DNA sequencing using the MicroSeq 16S rDNA Bacterial Identification System (Applied Biosystems). TRBR A was described as Gram-positive coccus that grew in round, small and white colonies on the agar plate. It was identified in 12 (92%) laboratories as *S. epidermidis*. One participant identified the sample as *S. delphini* using the Biolog Identification System. TRBR B included Gram-positive cocci in chains that grew in white, small and flat colonies with beta-haemolysis on blood agar plates. TRBR B was identified in all 13 (100%) laboratories as *S. pyogenes*. TRBR C was identified in all 13 (100%) laboratories as *K. pneumoniae*, characterised as Gram-negative rods and mucoid, big colonies, and TRBR D as *E. coli*, specified as Gram-negative short rods and shiny round colonies.

Calculation of bacterial count of TRBR

In 13 different laboratories worldwide, the bacterial count was estimated for each vial (in total 65 vials of each TRBR resulting from five independent replicates of each TRBR per participant), according to the protocol for the calculation and comparison of the bacterial count of TRBR under distribution conditions. Following, these single values were used for the calculation of the mean value of bacterial count, standard deviation and 95% confidence interval for the collective value of each TRBR. The confidence intervals for the bacterial count analysed from data of all participants were for TRBR A: (*S. epidermidis*) $1·19–1·32 \times 10^7$ CFU/ml, TRBR B (*S. pyogenes*): $0·58–0·69 \times 10^7$ CFU/ml, TRBR C (*K. pneumoniae*): $18·71–20·26 \times 10^7$ CFU/ml and TRBR D (*E. coli*): $1·78–2·10 \times 10^7$ CFU/ml. All results are shown in Table 3.

Growth of bacteria in PCs after low spiking

All data received included the growth results of 12 of 14 laboratories, including PEI. Growth properties of all four TRBR were evaluated by contamination of PCs with very low bacterial count, by spiking at least two routine APCs or PPCs per TRBR. Five laboratories used only PPCs derived from buffy coat from 4 to 5 donors, one used single donor PCs (SPPC) derived from buffy coat, two partners used only APCs, three participating laboratories used APCs or PPCs, and participant number 10 did the whole procedure in both APCs and PPCs. The results are shown in Table 4. *S. epidermidis* and *K. pneumoniae* grew in PCs in all participating laboratories after spiking with both target counts of ∼0·3 and ∼0·03 CFU/ml. The actual bacterial counts for the inocula were dependent on the PC volume in the bag and were in the range of 0·01–0·23 CFU/ml for the low

Table 3 Results of enumeration of all participating laboratories including mean and confidence interval (×10^7 CFU/ml)

Participant		TRBR A *Staphylococcus epidermidis*			TRBR B *Streptococcus pyogenes*			TRBR C *Klebsiella pneumoniae*			TRBR D *Escherichia coli*		
No.	Country	Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI	
1	Austria	1·17	0·94	1·41	0·71	0·53	0·88	21·71	19·09	24·33	1·13	0·46	1·81
2	Canada	1·24	1·00	1·47	0·76	0·59	0·94	9·01	6·39	11·63	1·60	0·93	2·28
3	China	1·53	1·29	1·76	0·71	0·54	0·89	22·70	20·08	25·32	0·94	0·26	1·61
4	Germany, Frankfurt	0·88	0·65	1·12	0·24	0·07	0·42	9·54	6·92	12·16	1·75	1·07	2·42
5	Germany, PEI	1·69	1·46	1·93	0·77	0·60	0·95	20·37	17·75	22·99	2·32	1·64	2·99
6	Germany, Springe	0·95	0·72	1·19	0·55	0·37	0·72	24·56	21·94	27·18	3·03	2·36	3·71
7	Mexico	1·22	0·99	1·46	0·60	0·43	0·78	14·70	12·08	17·32	1·73	1·05	2·40
8	Poland	1·74	1·50	1·97	0·84	0·67	1·02	31·01	28·39	33·63	2·41	1·73	3·08
9	The Netherlands	1·14	0·90	1·37	1·20	1·03	1·38	17·08	14·46	19·70	1·20	0·52	1·88
10	United Kingdom	1·17	0·93	1·40	0·53	0·36	0·71	25·26	22·64	27·88	1·48	0·80	2·15
11	USA, Denver	1·30	1·06	1·53	0·59	0·42	0·77	22·05	19·44	24·67	1·02	0·34	1·69
12	USA, Ohio	0·80	0·57	1·04	0·32	0·15	0·50	18·32	15·71	20·94	2·05	1·38	2·73
13	USA, US Army	1·48	1·25	1·72	0·36	0·19	0·54	17·03	14·41	19·65	4·58	3·90	5·26
All participants		1·25	1·19	1·32	0·63	0·58	0·69	19·49	18·71	20·26	1·94	1·78	2·10

Mean bacterial count (×10^7 CFU/ml); least square estimators derived from a mixed linear model and 95%-confidence limits (CI).

PEI, Paul Ehrlich Institute; TRBR, transfusion-relevant bacteria reference(s).

inoculum of approximately 10 CFU/bag (~0·03 CFU/ml) and 0·07–1·53 CFU/ml for the higher inoculum of approximately 100 CFU/bag (~0·3 CFU/ml). *S. pyogenes* failed to grow in one participating laboratory (No. 6), after spiking with an actual count of 0·05 CFU/ml, and in one laboratory (No. 7), after spiking with an actual count of 0·5 and 0·05 CFU/ml. *E. coli* did not grow in one PC product inoculated with an actual count of 0·5 CFU/ml (laboratory No. 9).

Discussion

Methods for bacterial detection or bacterial reduction are important tools for improving the safety of blood components [3]. To validate and to assess the analytical qualities of these methods, it is crucial to use bacterial strains which are able to proliferate in blood components because bacterial contamination of blood components will not always result in bacterial multiplication [8,13,14,17]. For example, autosterilisation due to natural bactericidal agents present in the blood component may result in the death of the organisms, or in other instances, the bacteria may survive in the unit in low numbers but not multiply [18,19]. Therefore, the behaviour of bacteria in the setting of transfusion able components must be taken into account, as replication may behave differently than in other microbiological culture media [2].

No international Transfusion-Relevant Bacteria Reference currently exists for the investigation into methods used to detect or kill bacteria in blood components. Therefore, an international validation study was conducted by the Subgroup on Bacteria of the ISBT Working Party on Transfusion-Transmitted Infectious Diseases, responsible for evaluation and advocacy of approaches to increase blood safety throughout the world [20]. The validation study was organised to determine whether these candidate references could be used as a tool for development, validation and comparison of methods for both bacterial screening and pathogen reduction. For this reason, the PEI developed a procedure to manufacture TRBR as deep frozen suspensions. The TRBR are ready to use, defined in identity and count and shippable on dry ice. After thawing, the bacteria can be applied immediately for spiking of blood components with a very low bacterial count [21]. Four TRBR that caused infections in transfusion or other biological drug recipients, and are commonly implicated in bacterial contamination of blood products in the literature, were prepared by PEI and distributed in collaboration with the ATCC to 14 participating laboratories worldwide to identify, enumerate and demonstrate growth abilities in PCs of the bacterial strains [3,22–24].

All blinded TRBR were identified correctly with the exception of one (TRBR A) which was identified by one of the participants as *S. delphini* using the Biolog Identification System. Both *S. epidermidis* and *S. delphini* are the members of the coagulase-negative staphylococci (CNS) group. The identification failure is most likely due to the commercial identification kit used, which measures cellular

Table 4 Bacterial counts of TRBR in PCs at time of inoculation and after storage

Partner No.	Target 10 CFU/bag				Target 100 CFU/bag			
	PC	PC Volume[a]	Inoculum[b]	4 day storage[c]	PC	PC Volume[a]	Inoculum[b]	4 day storage[c]
TRBR A: *Staphylococcus epidermidis*								
1	PPC	274	14	2·33E+07	PPC	264	163	2·73E+07
2	APC	215	11	2·39E+04[d]	PPC	309	138	1·25E+06[d]
3	SPPC	48	1	5·15E+04	SPPC	49	18	6·00E+07
4	PPC	293	3	1·62E+03	PPC	280	103	1·35E+05
5	PPC	301	13	2·23E+08	PPC	283	156	1·16E+08
6	PPC	229	11	5·53E+07	PPC	229	115	2·19E+08
7	APC	110	25	5·11E+09	PPC	195	114	5·34E+08
8	PPC	250	8	1·23E+08	PPC	250	80	4·20E+06
9	PPC	350	14	1·40E+09	PPC	350	177	2·64E+07
10	PPC	231	10	8·70E+07	PPC	239	121	1·91E+08
10	APC	158	10	1·53E+08	APC	107	121	1·68E+08
12	APC	240	10	7·00E+02	APC	241	103	1·56E+04
13	APC	240	17	1·68E+07	APC	239	136	1·01E+07
TRBR B: *Streptococcus pyogenes*								
1	PPC	284	6	5·57E+07	PPC	277	82	2·58E+07
2	APC	212	8	5·67E+06	PPC	337	86	7·28E+06
3	SPPC	47	1	1·06E+08	SPPC	52	13	8·48E+07
4	APC	256	13	1·57E+03	APC	287	100	7·03E+04
5	PPC	293	26	5·40E+04	PPC	295	128	1·03E+05
6	PPC	240	11	no growth	PPC	224	86	1·67E+06
7	PPC	300	16	no growth	APC	125	59	no growth
8	PPC	250	8	1·71E+07	PPC	250	84	1·02E+08
9	PPC	350	10	1·12E+08	PPC	350	109	3·00E+07
10	PPC	245	11	5·98E+07	PPC	242	69	7·75E+07
10	APC	177	11	1·62E+08	APC	160	69	9·00E+07
12	APC	239	11	3·93E+07	APC	241	109	1·41E+07
13	APC	193	9	1·00E+02	APC	249	91	3·58E+06
TRBR C: *Klebsiella pneumoniae*								
1	PPC	271	20	1·42E+09	PPC	263	188	1·62E+09
2	APC	182	19	1·53E+09	PPC	318	163	6·23E+08
3	SPPC	47	3	1·19E+09	SPPC	50	26	7·02E+08
4	PPC	302	30	3·60E+08	PPC	285	163	8·36E+08
5	PPC	286	9	1·12E+09	PPC	289	172	1·07E+09
6	PPC	231	11	1·35E+09	PPC	232	130	1·17E+09
7	PPC	202	9	1·64E+10	APC	103	51	2·18E+10
8	PPC	250	10	2·90E+08	PPC	250	100	1·47E+09
9	PPC	350	9	8·43E+08	PPC	350	81	6·27E+08
10	PPC	282	10	1·07E+09	PPC	257	24	1·37E+09
10	APC	176	10	1·77E+09	APC	170	24	2·14E+09
12	APC	239	8	6·73E+08	APC	240	84	1·17E+09
13	APC	150	12	1·05E+08	APC	255	95	1·28E+09
TRBR D: *Escherichia coli*								
1	PPC	257	19	1·21E+08	PPC	258	173	5·05E+08
2	APC	193	15	9·98E+05	PPC	305	207	9·07E+07
3	SPPC	52	1	1·74E+07	SPPC	46	3	2·20E+08
4	PPC	237	16	3·50E+06	PPC	162	133	1·27E+09
5	PPC	298	9	4·75E+08	PPC	280	172	6·20E+08
6	PPC	215	16	2·62E+08	PPC	251	137	4·43E+08
7	PPC	309	6	4·38E+09	APC	150	230	4·61E+09
8	PPC	250	12	5·72E+07	PPC	250	120	2·54E+08

Table 4 (Continued)

Partner No.	Target 10 CFU/bag				Target 100 CFU/bag			
	PC	PC Volume[a]	Inoculum[b]	4 day storage[c]	PC	PC Volume[a]	Inoculum[b]	4 day storage[c]
9	PPC	350	16	no growth	PPC	350	164	1·13E+08
10	PPC	205	14	8·63E+09	PPC	254	140	7·90E+10
10	APC	177	14	1·92E+10	APC	173	140	1·79E+10
12	APC	239	12	3·50E+07	APC	239	124	2·68E+08
13	APC	150	16	1·12E+08	APC	257	157	1·88E+07

PPC, pool platelet concentrate of 4–5 donors; SPPC, single donor buffy coat derived platelet concentrate; APC, apheresis platelet concentrate; PC, platelet concentrate; TRBR, transfusion-relevant bacteria reference(s).
[a]PC volume in ml.
[b]bacterial inoculum in CFU/bag.
[c]bacterial count after storage for 4 days at 22–24°C in CFU/ml.
[d]bacterial count after 6 days of storage.

metabolism. Such misinterpretations are not unusual in microbiological routine diagnostics [25]. These false identifications had at least no transfusion relevance. Genotypic methods have been shown to be more accurate and precise than traditional biochemical and phenotypic techniques [26,27]. Therefore, 16S rRNA sequencing was performed at PEI prior to the study to confirm the identity of sample A as *S. epidermidis*, as supported by 92·3% of the participating laboratories. The results of bacterial counting of all participants were consistently of the same order. These results as well as the stability data confirm the stability of TRBR during the study period of 6 month, including TRBR production, shipping, participant's experiments and sending of results. Moreover, ST is performed by PEI biyearly during storage. The stability depends on the bacterial strain and a lot. Available data indicate stability between 2 and 5 years. Thus, the stability should be considered for at least 1 year (data not shown).

The bacterial strains of candidate TRBR were selected regarding their capability to multiply in PCs under routine storage conditions. The growth abilities of these species were proven by spiking studies in PCs from at least 100 different donors with a very low bacteria count of 10 bacteria per platelet bag corresponding to ∼0·03 CFU/ml; this mimics the predicted bacterial load at the time of donation [8]. The growth capability of the bacterial strains in platelets has been confirmed to exclude antimicrobial effects of donor's host defence. In this study, *S. epidermidis* and *K. pneumoniae* replicated in all participating laboratories (100%) using both inocula. In one participating laboratory (8·33%), *S. epidermidis* grew slowly so that a sample was taken again on day six of both inoculated PC bags because no growth was observed after 4 days of storage. *E. coli* grew with the exception of one PC unit contaminated with 10 CFU per bag, while *S. pyogenes* grew in 11 out of 12

participating laboratories (92·3%). One laboratory carried out the contamination of *S. pyogenes* twice because using PPCs, the organisms did not grow. Therefore, they repeated the experiment using APCs for spiking and growth was observed. The most likely interpretations of these failures may be the existence of specific antibodies towards the bacterial strain in the blood population which may prevent bacterial growth and/or kill the microorganisms or the inaccurate handling during contamination or sampling. Another explanation would be the influences of plastic compositions of the platelet bag (e.g. plasticisers). This may also explain the case in which only the higher spike of 100 bacteria per platelet bag led to bacterial growth, i.e. low concentrations of antibodies in the platelet bag could kill the low number of bacteria.

The importance of growth ability and spiking studies using defined low levels of bacteria to blood components provides a basis to evaluate and compare the efficiency of methods for bacterial detection and reduction in the transfusion setting. No direct conclusions can be drawn from experience elsewhere in medical microbiology as to the conditions in blood components [2]. Bacteria cultivated in microbiological media potentially express other biological properties to that grown up in the complex matrix of PCs, i.e. they express another phenotype. That phenotype is achieved applying the concept of low titre spiking of PCs, followed by proliferation of the given strain. In contrast, prior cultivation in microbiological media and high titre spiking afterwards may lead to unreliable results. Several validation studies of detection and reduction methods were carried out without showing the growth ability of the strains studied before treatment or without using untreated control bags as positive controls from the same donation to avoid false negative results [28–30]. In more recent studies, this new thinking is followed to demonstrate the growth

ability of the strains in parallel using low inoculated control PC bags and estimation of the bacterial count during storage [31–35]. Especially, in the case of pathogen reduction methods, reliable results are crucial because one surviving organism may be able to proliferate again up to high counts and endanger the patient.

This study was the result of an international co-operation of members of different areas belonging to the ISBT WP-TTID and can now be considered as a first step in implementation of a relevant bacterial reference material. It should be seen as a pilot experiment with the aim to demonstrate (i) the quality, stability and suitability of the TRBR for defined low titre spiking of blood components, (ii) the property of the respective bacterial strain to grow up to high counts in platelets obtained from donors in different regions of the world, and (iii) to train the logistics of worldwide shipping of deep frozen, blinded pathogenic bacteria. All three of these issues were successfully solved. The 2nd Meeting of the WHO Collaborating Centers on WHO Biological Reference Preparations for blood products and *in vitro* diagnostic devices in 2009 recommended submitting the study results to the WHO Expert Committee Biological Standardisation (ECBS) for consideration. The advancements in this field aiming to establish a recognised reference for worldwide use were discussed during the annual meetings of ECBS in 2009 and 2010. The Committee approved the adoption of four bacteria strains which were included in the international validation study [*Staphylococcus epidermidis* (PEI-B-06), *Klebsiella pneumoniae* (PEI-B-08), *Streptococcus pyogenes* (PEI-B-20) and *Escherichia coli* (PEI-B-19)] as the first WHO Repository for Transfusion-Relevant Bacteria Reference Strains to be held and distributed by the Paul Ehrlich Institute and also endorsed a proposal for addition, within the same Repository, of six further bacteria strain preparations suitable for control of PC contamination as the next step of enlargement of the repository.

Acknowledgements

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