ORIGINAL INVESTIGATION

A multiplex real-time PCR assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples

Lutz Eric Lehmann · Klaus-Peter Hunfeld · Thomas Emrich · Gerd Haberhausen · Heimo Wissing · Andreas Hoeft · Frank Stüber

Abstract Early detection of bloodstream infections (BSI) is crucial in the clinical setting. Blood culture remains the gold standard for diagnosing BSI. Molecular diagnostic tools can contribute to a more rapid diagnosis in septic patients. Here, a multiplex real-time PCR-based assay for rapid detection of 25 clinically important pathogens directly from whole blood in <6 h is presented. Minimal analytical sensitivity was determined by hit rate analysis from 20 independent experiments. At a concentration of 3 CFU/ml a hit rate of 50% was obtained for *E. aerogenes* and 100% for *S. marcescens, E. coli*, *P. mirabilis*, *P. aeruginosa*, and *A. fumigatus*. The hit rate for *C. glabrata* was 75% at 30 CFU/ml. Comparing PCR identification results with conventional microbiology for 1,548 clinical isolates yielded an overall specificity of 98.8%. The analytical specificity in 102 healthy blood donors was 100%. Although further evaluation is warranted, our assay holds promise for more rapid pathogen identification in clinical sepsis.

Keywords Bacterial and fungal pathogens · Blood culture · ICU · Multiplex PCR assay · Sepsis · Simultaneous detection

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Introduction

Blood culture, which currently remains the gold standard in the microbiological diagnosis of bacterial or fungal bloodstream infections (BSI), typically becomes positive 8–36 h after sampling, and therapy can then be adapted based on presumptive bacterial identification suggested by Gram-stain characteristics. A more precise pathogen identification and susceptibility profile, however, is not available until up to 24–48 h [1, 2]. For the majority of patients with clinically apparent sepsis, the blood culture is negative, making the optimal antimicrobial therapy empiric [3]. Early detection and adequate treatment of causative pathogens within the first 6–12 h, however, is critical for a favorable outcome in patients with BSI [4–6]. The development of rapid diagnostic methods for BSI has been identified as an important medical need to supplement conventional blood culture diagnostics and molecular techniques have potential to fulfill this need [1]. Nucleic acid based diagnostic systems, including polymerase chain reaction (PCR) methods as well as the application of DNA and RNA probes are well known sensitive techniques for a more rapid detection and the specific identification of pathogens involved in BSI [7–15]. From a theoretical point of view, PCR-based diagnostic techniques, therefore, hold promise for sensitive and specific detection

of target pathogens within much shorter turn around times than usually exercised with conventional blood cultures.

Microbiological and epidemiological data clearly indicate that only a limited number of bacteria and fungi are responsible for the majority of all bloodstream infections on the intensive care unit (ICU). As revealed in recent studies, about 20–25 species account for >90% of all detected nosocomial pathogens in BSIs [16, 17]. To correctly identify a pathogen in a patient with clinical sepsis and to cover the broad spectrum of microorganisms involved in BSI, several parallel or serial specific PCR analyses or a more universal PCR assay followed by specific probe hybridization or sequencing of the genomic target must be performed. Such an approach, however, is technically challenging, laborious, and time-consuming. Consequently, only PCR-based methods designed to specifically detect single pathogens (i.e., PCR for certain viruses) have been widely adopted in the routine clinical laboratory [18]. To date, several attempts have been made to translate the more universal diagnostic approach of blood-cultures into PCR-based assays that provide broad diagnostic coverage for the most frequent nosocomial pathogens involved in BSI [3, 15, 19, 20]. This has been done by the use of universal primers for PCR amplification that target conserved and specific genomic regions of pathogens and subsequent analysis of the PCR amplicon for pathogen identification by melting point analysis, hybridization probes, or direct sequencing [3, 20]. However, most of the broad range PCR assays introduced so far, still suffer from important technical limitations: preparation is done from plasma and not from whole blood, thus, missing bacterial cells or bacterial DNA already engulfed by phagocytic cells such as granulocytes and macrophages. Moreover, fungi are usually not part of such pathogen panels and most assays lack process controls to check for correct DNA preparation and PCR amplification. Finally, such assays commonly lack application of efficiently decontaminated reagents or alternatively, the use of high quality DNA-free reagents and plastic ware, to avoid potential workflow contamination resulting in impaired test sensitivity and specificity. To circumvent current technical limitations and to contribute to a more rapid detection and identification of pathogens in patients with clinical sepsis, here, we describe the development of a new sensitive and specific multiplex real-time PCR-based assay for amplification of bacterial and fungal DNA directly from whole blood samples.

Materials and methods

Assay workflow

The assay follows three steps: (1) specimen preparation by mechanical lysis and purification of DNA, (2) Real-time PCR amplification of target DNA in three parallel reactions (Gram positive, Gram negative, fungi) and subsequent detection of PCR products by specific hybridization probes, and (3) automated identification of species and controls. All specimen handling preparations and the PCR reaction set up were performed in a laminar flow box to minimize the risk of workflow contamination. Moreover sterile powder free gloves were worn. All instruments used were decontaminated from bacterial DNA prior to performance of the assay either by UV radiation or by decontamination reagents (e.g., LTK 008).

Specimen preparation

Samples of 3 ml whole blood specimens, were subjected to mechanical lysis with ceramic beads in a MagNALyzer® instrument (Roche Diagnostics GmbH, Mannheim, Germany) for 30 s at 6,500 rpm. After lysis, bacterial DNA was prepared using the Septi*Fast* Prep Kit Mgrade (Roche, Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations. Briefly, the lysed specimens were incubated at 56°C for 15 min with gentle agitation with a protease and chaotrophic lysis buffer (containing 50% guanidinium thiocyanate, 20% Triton X-100, 2% Proteinase K) that releases the nucleic acid and protects the released DNA from DNAses in the whole blood. Internal control (IC; 10 µl) was introduced into each specimen along with the lysis buffer. The IC consists of a mixture of synthetic double stranded DNA molecules with primer binding sites identical to those of the target sequences but differing in their hybprobe binding sites, thus allowing differentiation of the amplified IC and the target-specific amplicon. After addition of binding buffer the mixture was transferred to a spin column with a glass fiber insert (1,900g; 3 min). Human genomic DNA and the bacterial/fungal target DNA in the sample were then adsorbed to the surface of the glass fiber. Unbound substances (salts, proteins, cellular fragments) were removed by a washing step (4,200g; 2 min) with 1,800 µl of inhibitor removal buffer (containing 50% guanidinium thiocyanate, 40% ethanol, Tris–HCl buffer) and a subsequent washing step (4,200g, 10 min) with 1,600 µL of wash buffer (containing 0.2% sodium chloride, 80% ethanol, Tris–HCl buffer). Adsorbed nucleic acids were then eluted from the column after incubation (5 min) with 100 µL preheated (70°C) elution buffer and centrifuged for 2 min at 4,200g.

PCR amplification on the LightCycler® 2.0 Instrument

The eluate was subjected to three different PCR reactions using the LightCycler® Septi*Fast* Kit Mgrade (Roche Diagnostics GmbH, Mannheim, Germany) as recommended by the manufacturer. Briefly, three 50 µL aliquots of the

prepared specimen DNA were added to the master mix and loaded onto the LightCycler capillaries (100 µl). The target DNA was amplified in three parallel reactions using the Hot start Taq-Polymerase. Gram positive, Gram negative or fungal sequences were amplified in the corresponding capillaries according to the bacterial and fungal master list (Table 1). The internal transcribed spacer region (ITS) was selected as specific target for the detection of bacterial and fungal pathogens. Sequence information of selected ITS target sequences is disclosed in patent applications WO 2004/052606, WO 2004/053156, WO 2005/075673, and WO 2006/035062. The target sequences are located between the 16S and the 23S ribosomal DNA sequences of Gram negative and Gram positive bacteria and between the 18S and 5.8S ribosomal sequences of fungi. The target sequences of the different species covered by the assay were amplified either with universal or specific primers. The assay principle including applicable primer and hybridization probe sequences of the ITS target regions is disclosed in patent applications WO 2004/053148 and WO 2004/053156. More detailed PCR-amplification conditions are outlined in Table 2.

Real time detection of PCR products by hybridization probes

During PCR reaction, increase of specific products was determined using dye-labeled hybridization probes and continuous automated fluorescence measurement. After completion of amplification, melting curve analysis was performed to further prove specificity of the products. The melting curve profile is given in Table 2. The probes hybridize to internal sequences of the amplified fragments during the annealing phase. Emitted fluorescence was then measured in one of four detection channels of the Light-Cycler® 2.0 Instrument. The melting temperature depended upon fragment length, composition of sequence, and degree of homology between the hybridization probe and the target

Table 2 LightCycler amplification and melting curve profiles

| PCR steps	Temperature (°C)	Time	Temperature change (°C/s)
PCR amplification profile			
Preconditioning			
Decontamination	40	5 min	20
Enzyme activation	95	10 min	20
15 cycles pre-amplification			
Denaturation	95	15 s	3.0
Annealing	58	50 s	20
Elongation	72	40 s	3.0
30 Cycles amplification			
Denaturation	95	15 s	3.0
Annealing	50	50 s	20
Elongation	72	40 s	3.0
Melting curve profile			
Denaturation	95	60 s	20
Annealing	40	60 s	20
Melting	80	0 s	0.1

DNA. All probes were designed to reliably discriminate between the different species as detected in the same detection channel of the instrument by use of specific melting temperatures of the corresponding amplicons. Examples of melting curve registrations and subsequent specific identification of microorganism, and internal controls by melting point analysis of the PCR-products are given in Fig. 1a–c. The corresponding specific melting temperatures for all PCR products and probes as derived from the respective pathogens are given in Fig. 2a–c.

Automated identification of species and controls

Amplicons resulting from PCR reactions in specimens and controls were analyzed by a pathogen identification software (SIS; Septi*Fast* Identification Software, Roche

Table 1 Master list of detectable microorganisms

Gram negative	Gram positive	Fungi
Escherichia coli	*Staphylococcus aureus*	*Candida albicans*
Klebsiella (pneumoniae/oxytoca)	*ConS*	*Candida tropicalis*
Serratia marcescens	*Streptococcus pneumoniae*	*Candida parapsilosis*
Enterobacter (cloacae/aerogenes)	*Streptococcus* spp.	*Candida krusei*
Proteus mirabilis	*Enterococcus faecium*	*Candida glabrata*
Pseudomonas aeruginosa	*Enterococcus faecalis*	*Aspergillus fumigatus*
Acinetobacter baumannii		
Stenotrophomonas maltophilia		

ConS coagulase negative *Staphylococci* (including e.g., *S. epidermidis*, *S. haemolyticus*)

Streptococcus species (including e.g., *S. pyogenes*, *S. agalactiae*, *S. mitis*)
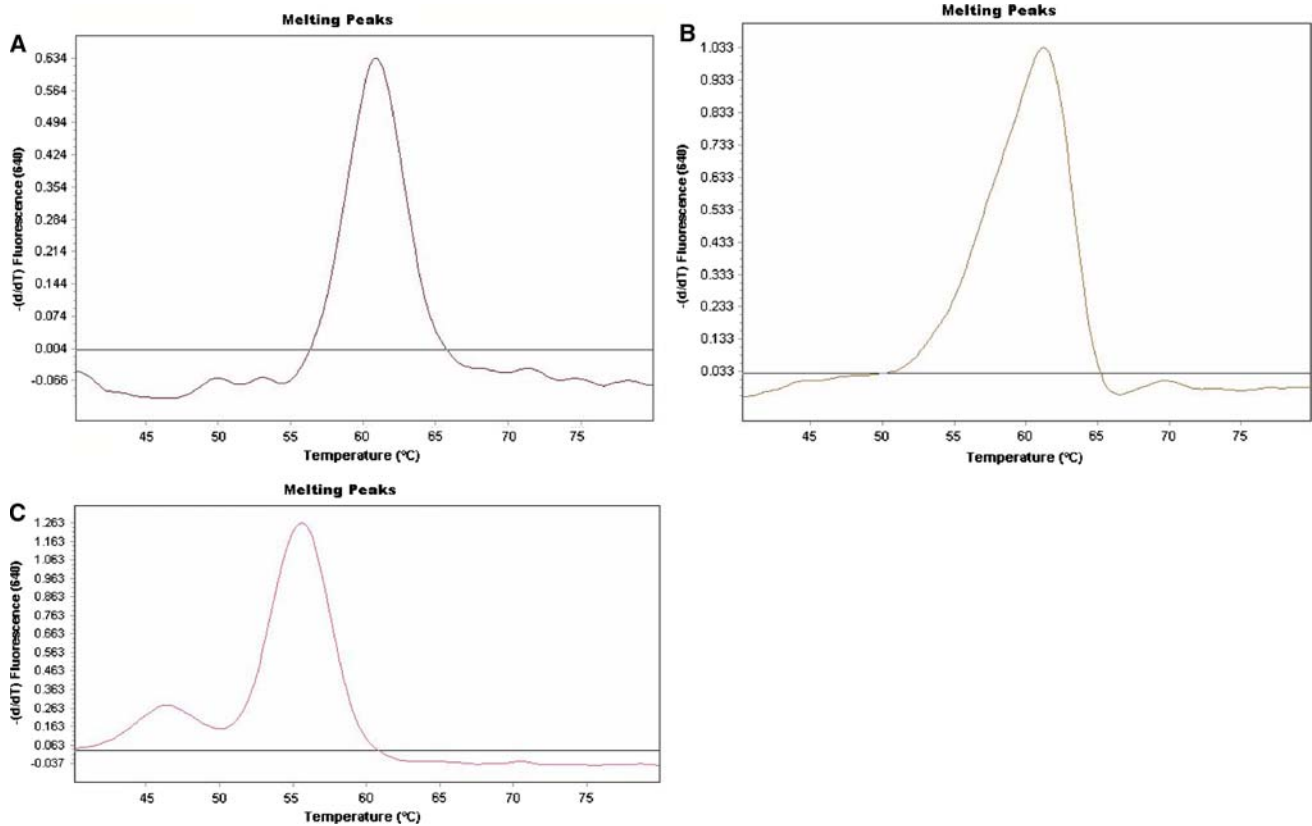
Fig. 1 Examples of characteristic melting curve registrations of spiking experiments and microorganism identification by respective melting temperatures. **a** *K. oxytoca* (T_m = 61°C), **b** *S. aureus* (T_m = 62°C), **c** *C. albicans* (T_m = 55°C), and internal control (T_m = 46°C)

Diagnostics GmbH, Mannheim, Germany) specifically designed for the interpretation of melting temperatures of PCR products. After the completion of each run on the LightCycler® 2.0 Instrument, all melting curves were marked manually with vertical sliders. Once a slider was set, the software automatically calculated the melting point (T_m) value and the corresponding peak height. Specific T_m boundaries had been pre-defined for each melting peak maximum. This procedure was repeated for each of the four detection channels and with all the three assays (Gram+, Gram-, Fungi; Fig. 2a–c). Minimal peak heights for all specified melting peaks were derived from a total of 13,727 data sets obtained with target negative specimen.

Preparation of blood specimen spiked with bacterial and fungal reference strains

Spiked specimen were prepared by spiking target-negative K-EDTA blood from healthy volunteers without clinical and laboratory evidence of infection with quantified cryostock preparations from bacterial or fungal reference strains to a final concentration of 100, 30, and 3 CFU/ml respectively. The list of reference strains used throughout the

evaluation is given in Table 3. Cryostocks were prepared from conventional microbiological cultures of reference organisms. Aliquots of the prepared cryostocks were then stored at –80°C until use. Concentration of microorganisms were determined and adjusted by standard counting of colony forming units (CFU) after plating suspensions on solid media.

Evaluation of assay precision

The assay precision as measured in each detection channel was evaluated using pre-prepared K-EDTA whole blood (see above) spiked with 10^3 and 10^2 CFU/ml of microorganisms representative for each of the four detection channels of the LightCycler® 2.0 Instrument. *S. marcescens* was used for the 610 nm channel, *K. oxytoca* was used for the 640 nm channel, *C. krusei* was used for the 670 nm channel, and *E. faecalis* was used for the 705 nm channel. Specimens were analyzed in replicates of seven for intra-assay precision using six different instruments. For inter-assay precision, six independent experiments were performed. Lot-to-lot variability was investigated by including two different test lots.

Fig. 2 Distribution of melting temperatures (T_m) and respective detection channels for all microorganisms and internal controls. **a** Gram negative bacteria, **b** Gram positive bacteria, and **c** fungi
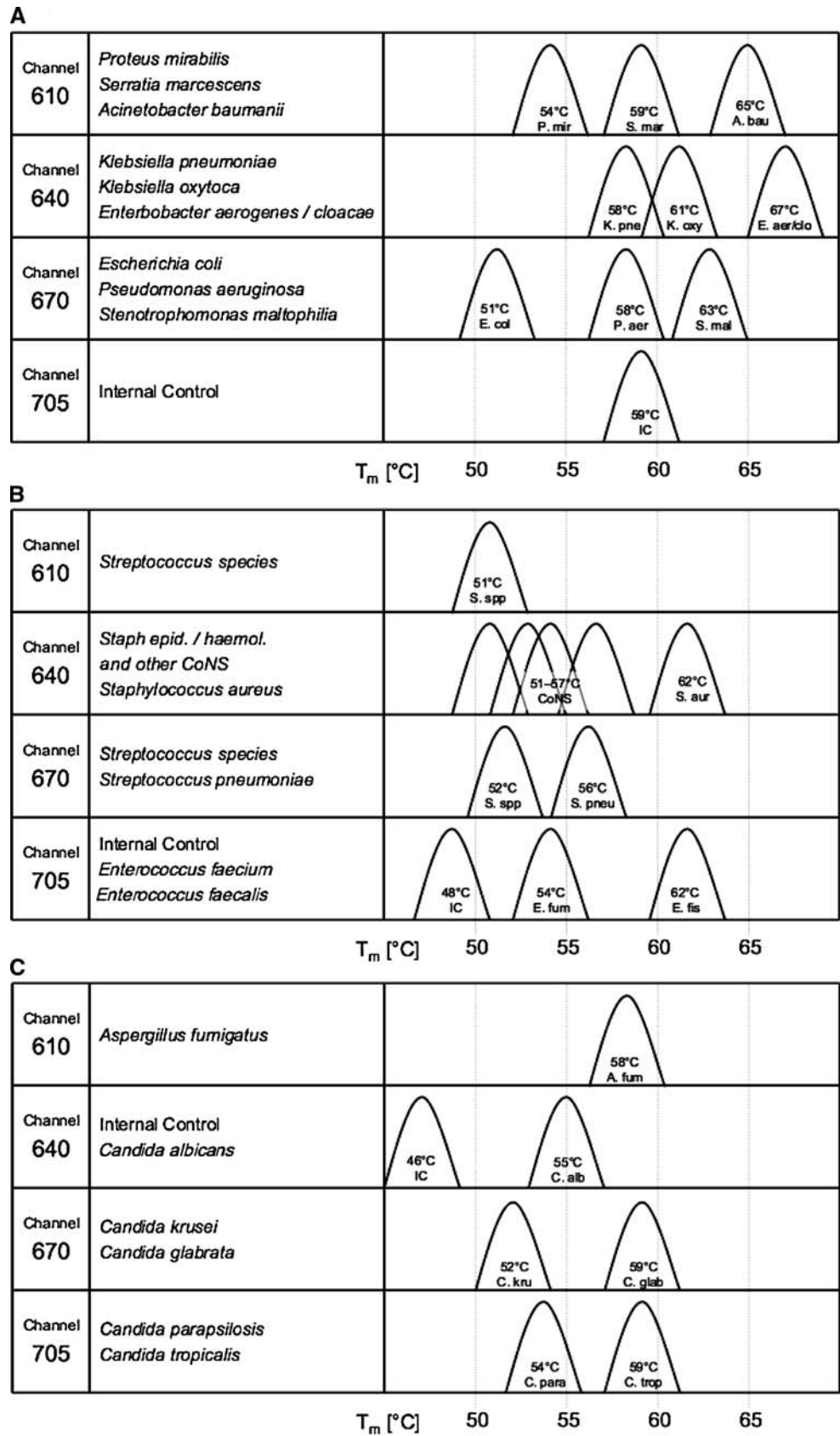
Table 3 Minimal analytical sensitivity (%) of the assay in spiked ETDA-blood samples

Assay	Pathogen	DSMZ/ATCC	100 CFU/ml ($n = 8$) (%)	30 CFU/ml ($n = 12$) (%)	3 CFU/ml ($n = 20$) (%)
Gram negative	*A. baumannii*	30007/19606	100	100	85
	E. aerogenes	30053/13048	100	100	50
	E. cloacae	30054/13047	100	100	75
	S. marcescens	30121/13880	100	100	100
	S. maltophilia	50170/13637	100	100	95
	E. coli	30083/11775	100	100	100
	K. oxytoca	5175/13182	100	100	85
	K. pneumoniae	30104/13883	100	100	80
	P. mirabilis	4479/29906	100	100	100
	P. aeruginosa	50071/10145	100	100	100
Gram positive	*S. aureus*	20231/12600	100	100	75
	S. epidermidis	20044/14990	100	75	0[a]
	S. haemolyticus	–/29970	100	50[a]	0[a]
	S. pneumoniae	20566/33400	100	100[a]	30[a]
	S. agalactiae	2134/13813	100	92[a]	10[a]
	E. faecalis	20478/19433	100	100[a]	85
	E. faecium	20477/19434	100	100[a]	65
	S. pyogenes	20565/12344	100	67	0[a]
	S. mitis	12643/49456	100	100	65
Fungi	*A. fumigatus*	–/790	100	100	100
	C. glabrata	–/2001	100	75	0
	C. albicans	–/18804	100	100	70
	C. krusei	3433/24210	100	100	55
	C. parapsilosis	5784/22019	100	100	85
	C. tropicalis	11953/4563	100	100	70

[a] Results for ConS and *Streptococcus* spp. are given with applied cross-contamination threshold

Analytical sensitivity for all pathogens

DSMZ German collection of microorganisms and cell cultures (http://www.dsmz.de)

ATCC American type culture collection (http://www.atcc.org)

Determination of minimal analytical sensitivity and specificity (inclusivity/exclusivity)

The assay minimal analytical sensitivity for each of the 25 species of the master list was analyzed by hit rate analysis. Target negative K-EDTA blood specimen of healthy donors (see above) serially spiked with bacterial and fungal reference strains (Table 3) to a final concentration of 100, 30, and 3 CFU/ml were then analyzed for the presence of the corresponding microorganisms in replicates of 8, 12, and 20, respectively. To guarantee assay inclusivity for different strains of the pathogens in the clinical setting, the assay was tested on a collection of 1,548 clinical isolates originating from various geographic areas in Europe (northern, central, and southern Europe) and US. All isolates were microbiologically characterized by phenotypical and biochemical methods and analyzed with the PCR test. In addition, 31 clinically common species not related to blood stream infections (Table 4) were tested for exclusivity of the assay by spiking of target negative K-EDTA blood samples and subsequent hit rate analysis.

Introduction of CP cut-offs and performance evaluation of analytical assay specificity

In first step, serial semi quantitative analysis of 574 blood samples obtained from a fresh veni-puncture site in 259 healthy volunteers according to common guidelines [21] was performed to determine the relative bacterial loads and positivity rates in such blood specimens of coagulase negative *Staphylococci* (ConS) and *Streptococcus* spp. upon application of semi-automated real-time PCR. These experiments aimed at establishing optional semi quantitative crossing point (cp) cut-off values for the LightCycler® 2.0 Instrument to potentially discriminate between significant bacterial loads of such organisms in clinical samples and low amounts of bacterial DNA (background reactivity) resulting from cross-contamination of samples by colonizing skin flora during the initial blood draw.

In second step, the previously established semi quantitative cp cut-off values were then evaluated by processing whole blood samples that had been collected from 102 individuals at two independent study sites under routine conditions of high throughput ICUs after approval of the local

Table 4 Species tested for exclusivity

Species	DSMZ	ATCC
Actinobacillus actinomycetemcomitans	8324	33384
Aeromonas hydrophila	30188	19570
Bacillus cereus	345	10702
Bacteroides fragilis	1396	29762
Bartonella henselae	–	49793
Bordetella pertussis	5571	12743
Borrelia burgdorferi	4680	35211
Burkholderia cepacia	7288	10856
Campylobacter jejuni	4688	35918
Cardiobacterium hominis	8339	14900
Clostridium perfringens	756	10388
Corynebacterium jeikeium	7171	43216
Cryptococcus neoformans	n.a.	n.a.
Gemella haemolysans	–	10379
Haemophilus influenzae	4690	35039
Histoplasma capsulatum	n.a.	n.a.
Legionella pneumophila	7513	43662
Listeria monocytogenes	20600	15313
Moraxella catarrhalis	9143	8193
Morganella morganii	6675	35200
Mycobacterium fortuitum	43074	23010
Mycobacterium tuberculosis	43465	25584
Mycoplasma pneumoniae	–	15293
Neisseria meningitidis	10036	13091
Pantoea agglomerans	3493	53769
Peptostreptococcus magnus	20470	15794
Porphyromonas gingivalis	20709	49417
Prevotella denticola	20614	33184
Proprionibacterium acnes	1897	25746
Proteus vulgaris	13387	12454
Yersinia enterocolitica	4780	29913

n.a. not available (clinical isolate)

ethics committee and acquisition of written informed consent. To simulate blood sampling in ICU patients with indwelling devices undergoing invasive measures in the ICU, the blood draw was performed in individuals scheduled for elective cardiothoracic surgery. No clinical or laboratory signs of infection were obvious in these patients upon inclusion in the study. Blood cultures and EDTA specimens for PCR were collected in parallel directly from central lines or indwelling arterial catheters that had been inserted immediately before the blood draw using sterile precautions and after appropriate skin disinfection. Conventional blood cultures and PCR samples were then analyzed in parallel as described above at both study sites. Moreover, all PCR results were further re-analyzed for potential cross-contaminating skin flora by examining the raw data without using the automatic software threshold.

Statistics

Data was summarized and described by standard descriptive statistical methods (e.g., mean, standard deviation, variance, etc.) using Excel 2003 for Windows. Analysis of differences between melting peak temperatures of different isolates of the same species was done by Mann–Whitney U test using GraphPad Prizm 3.0.

Results

DNA extraction and assay performance

The DNA of all 25 bacterial and fungal species (Table 1) was successfully extracted directly from spiked whole blood samples without any preincubation and samples were then further analyzed by PCR using DNA free reagents. Microorganisms of each group (Gram positive, Gram negative, fungi) as well as the internal controls were identified by their specific melting points (T_m) in the predefined detection channels of dedicated wavelength. Examples of melting curve registrations of typical spiking experiments and consecutive microorganism identification by respective melting temperature analysis are given for *K. oxytoca* ($T_m = 61°C$; Fig. 1a), *S. aureus* ($T_m = 62°C$; Fig. 1b) and *C. albicans* ($T_m = 55°C$; Fig. 1c). Summarized information on detection wavelength and T_m for each pathogen and the internal control is illustrated in Fig. 2a–c. Ambiguous T_m were observed for several ConS species (51–58°C), but these could be clearly distinguished from the clinically important *S. aureus* ($T_m = 62°C$; Fig. 2b). Moreover, *E. aerogenes* and *E. cloacae* yielded identical T_m at 67°C (Fig. 2a) and were reported as *E. aerogenes/cloacae*.

Assay precision

Precision of the T_m determination was tested for all detection channels of the LightCycler® 2.0 Instrument with four suitable reference organisms (*S. marcescens*, ATTC: 13880; *K. oxytoca*, ATTC: 13182; *C. krusei*, ATTC: 24210; *E. faecalis*, ATTC: 19433) at two different sample concentrations (1,000 and 100 CFU/ml) including two different test lots. Using seven replicates per sample on six different instruments a maximal intra-assay variance of 0.22°C (SD: 0.03–0.22°C) was observed. The maximum coefficient of variation calculated for T_m was 0.38% (SD: 0.06–0.38%). More detailed results are given in Fig. 3a. The overall inter-assay precision calculated from six independent experiments varied between 0.19 and 0.54°C (SD: 0.12–0.27°C). The maximum coefficient of variation calculated for T_m was 0.87% (SD: 0.37–0.87%). More detailed results are given in Fig. 3b.
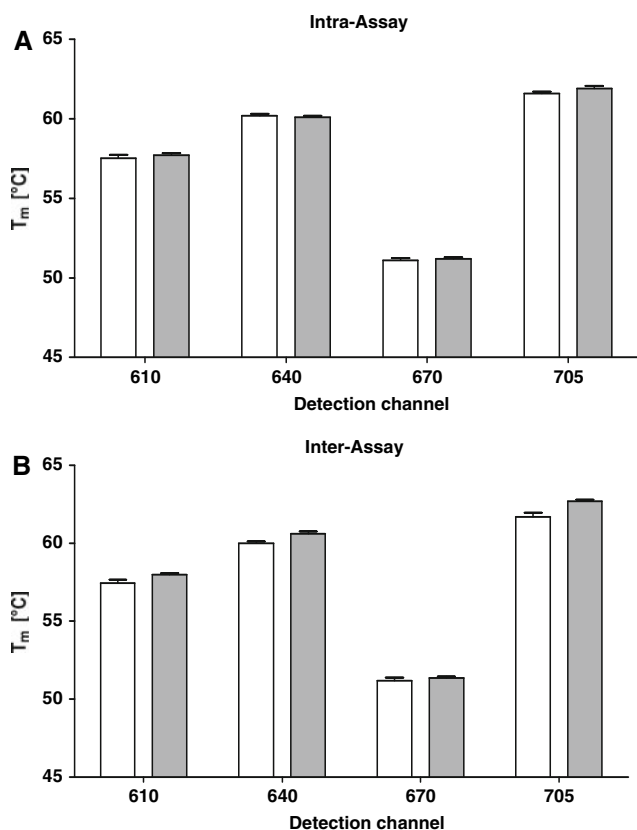
Fig. 3 Assay precision: precision of individual channels was tested with the following pathogens *S. marcescens* (channel 610), *K. oxytoca* (channel 640), *C. krusei* (channel 670), and *E. faecalis* (channel 705). The mean melting temperatures (T_m) (±SD) for all results are presented. **a** Intra-assay precision was tested with 1,000 CFU *open bars* or 100 CFU *grey bars* of pathogen concentration for each detection channel in replicates of seven. **b** Inter-assay precision was tested with two different lots of bacteria *open bars* for lot A and *grey bars* for lot B, for each detection channel in six independent experiments. Data are given as mean ± SD

Determination of semi quantitative analytical cp cut-off values

Statistical analysis of experimental data as obtained from 574 blood samples that were drawn from a fresh veni-puncture site of 259 healthy volunteers after serial semi quantitative analysis by real time PCR revealed significant cross-contamination of the clinical samples in 32.7% for ConS and in 6.3% for *Streptococcus* spp. by colonizing skin flora. To reduce this positivity rate due to potential cross-contamination, application of a semi-quantitative analytical cp cut-off value was evaluated for ConS and *Streptococcus* spp. A cut-off at 25 cycles reduced the positivity rate to 22.4% for ConS and to 4.2% for *Streptococcus* spp.; a cut-off at 20 cycles resulted in a positivity rate of <0.5% for both pathogens. According to these experimental results a semi quantitative analytical cp cut-off value at 20 cycles for ConS and *Streptococcus* spp., seemed suitable to potentially discrimi-

nate between significant bacterial loads of such organisms in clinical samples and low amounts of bacterial DNA (background reactivity) resulting from cross-contamination by colonizing skin flora during the initial blood draw.

Determination of minimal analytical sensitivity and specificity

At concentrations of 100 CFU/ml the minimal analytical sensitivity of the assay, as revealed from analysis of EDTA-blood samples ($n = 8$/reference organism), was 100% for all reference strains tested (Table 3). In EDTA-blood samples ($n = 12$/reference organism) spiked with bacterial and fungal reference strains at a concentration of 30 CFU/ml hit rates were achieved between 70 and 100%, at concentration of 3 CFU/ml recorded hit rates were between 50 and 100% (Table 3). Due to the application of our predefined cross-contamination threshold the detection limit for ConS and *Streptococcus* spp. throughout our experiments was limited for several species and generally ranged between 50% for *S. haemolyticus* and 100% for *S. pneumoniae* (Table 3; *Streptococcus* spp. are denoted by an asterisk) at a concentration of 30 CFU/ml in the spiked samples. The hit rate rate for fungal agents varied substantially and ranged between 0% for *C. glabrata* and 100% for *A. fumigatus* in spiked samples at 3 CFU/ml (Table 3). Differentiation of microorganisms by the PCR test is based on different melting peak temperatures of the target-specific hybridization probes and the specific detection channel used. Evaluation of the melting peak temperatures of all 25 target microorganisms with bacterial and fungal reference strains revealed that the species differentiation specificity (ΔT_m between target organisms detected in the same channel) was 3.37°C for *P. mirabilis* and *S. marcesens* and >5°C for all other target microorganisms, except for *P. mirabilis* and *S. marcesens* ($\Delta T_m = 3$, 37°C; Fig. 2a). No false positive samples were registered when assay specificity was tested. No significant differences in the melting peak temperatures were observed between different isolates of the same species (inclusivity; $p > 0.05$).

Uniformity and homogeneity of the ITS target region

For each of the 25 microorganisms covered by the test, 13–158 different clinical isolates (1,548 isolates in total) were analyzed and compared to the result obtained by phenotypical and biochemical methods (Fig. 4). The overall sensitivity (number of detected isolates/number of isolates tested) is 98.8%, with an overall accuracy of 98.8% (number of correctly detected isolates/number of isolates detected). Discrepant results were found in 5 out of 128 *Enterobacter* strains which were found to have identical ITS regions to *Klebsiella* (*pneumoniae/oxytoca*), therefore, were reported

Fig. 4 Analytical inclusivity: 13–158 different clinical isolates were analyzed. Discrepant results were found in 5 out of 128 *Enterobacter (cloacae/aerogenes)* strains that were interpreted as *Klebsiella (pneumoniae/oxytoca)* due to sequence homologies. *Streptococcus viridans* (14 of 158) strains were interpreted as *S. pneumoniae* due to sequence homologies. In 14 samples no PCR amplification was possible. In total, 19 of 1,548 clinically isolates (1.2%) were found discrepant when comparing traditional phenotypic microbiological characterization and PCR identification

Identification of clinical isolates by SeptiFast Test (n)

Microbiologically characterized clinical isolates (n)

	A. baumannii	A. fumigatus	C. albicans	C. glabrata	C. krusei	C. parapsilosis	C. tropicalis	E. coli	Enterobacter	Enterobacter + Klebsiella	Klebsiella	P. mirabilis	P. aeruginosa	S. maltophilia	S. marcescens	E. faecalis	E. faecium	S. aureus	CoNS	S. pneumoniae	S. pneumoniae + Strep spp.	Streptococcus spp.	negative	Grand Total
Acinetobacter baumannii	67																							67
Aspergillus fumigatus		37																						37
Candida albicans			62																					62
Candida glabrata				49																				49
Candida krusei					37																			37
Candida parapsilosis						49																		49
Candida tropicalis							45																	45
Escherichia coli								86															1	87
Enterobacter aerogenes									56															56
Enterobacter cloacae									67	1	4													72
Klebsiella oxytoca											64												1	65
Klebsiella pneumoniae											84												5	89
Proteus mirabilis												63											1	64
Pseudomonas aeruginosa													53											53
Stenotrophomonas maltophilia														106										106
Serratia marcescens															56									56
Enterococcus faecalis																49							1	50
Enterococcus faecium																	47							47
Staphylococcus aureus																		61						61
Staphylococcus epidermidis																			60					60
Staphylococcus haemolyticus																			13					13
Streptococcus pneumoniae																				50				50
Streptococcus agalactiae																						47		47
Streptococcus pyogenes																						48		48
Streptococcus viridans group																				11	3	139	5	158
Grand Total	67	37	62	49	37	49	45	86	123	1	152	63	53	106	56	49	47	61	73	61	3	234	14	1528
CoNS																			14				6	20

as *Klebsiella* (*pneumoniae/oxytoca*). In addition, some *Citrobacter freundii* and *Salmonella enterica* isolates showed identical ITS regions to *Klebsiella* (*pneumoniae/oxytoca*) and *E. coli*, and therefore, were reported as *Klebsiella* (*pneumoniae/oxytoca*) and *E. coli* by the SIS, respectively. Moreover, due to sequence homologies 14 of 158 *Streptococcus viridans* strains were found identical to *Streptococcus pneumoniae* within the ITS region and were interpreted as *S. pneumoniae*. However, only 19 of 1,548 clinical isolates (1.2%) were found discrepant when PCR results and microbiological characterization of strains were compared. Finally, exclusivity was tested with ATCC strains of 31 clinically common microorganisms in triplicate in two different experiments (Table 4). No species tested for exclusivity was found positive by the assay (total of 186 negative screened samples).

Performance test for diagnostic specificity in healthy volunteers

Whole blood samples of 102 patients scheduled for cardiothoracic surgery but without evidence of infection were analyzed by parallel examinations of conventional blood cultures and PCR samples at two independent study sites. ConS were detected in 20 patients by conventional blood cultures obtained directly from central lines or indwelling arterial catheters that had been inserted immediately before the blood draw using sterile precautions and after appropriate skin disinfection. All of the positive blood cultures (19.6%) yielded positive results 4–6 days after the blood draw, clearly pointing to cross contamination of the cultures with small amounts of residual skin flora. Interestingly, no pathogens were detected using the PCR system combined with subsequent SIS software analysis and activated threshold interpretation for ConS contamination again pointing to low levels of contaminating ConS in the corresponding blood culture positive samples. Re-evaluation of PCR datasets after disabling the established optional semi quantitative cp cut-off value for the LightCycler® 2.0 Instrument, however, showed that 33 (32.3%) ConS positive results could be generated from our sample set. In all these cases, the observed cp related to very low concentrations of pathogens as did the prolonged cultivation time in the positive blood cultures.

Discussion

The ultimate goal of using molecular technology for diagnosis of infection in the clinical setting is for the early detection of pathogens. Here, we introduce a novel molecular biological assay for parallel detection and identification of 25 clinically important BSI microorganisms. Most importantly, the total time-to-result including DNA prepa-

ration, PCR reaction, and data analysis is <6 h starting from a 3 ml K-EDTA whole blood specimen. The novel PCR assay does not require time-consuming pre-incubation of blood cultures prior to running the assay to increase analytical sensitivity. Compared to other PCR based diagnosis tools described previously, e.g., [20, 22] this system, includes external reaction controls to monitor proper function of test ingredients like primers and hybridization probes. Additionally, an internal process control is added to each individual clinical sample prior to DNA preparation. After amplification, the presence of specific amplicons is verified by use of specific dye-marked probes and semi-automated melting point analysis followed by software-assisted analysis to ensure high quality semi-automated species identification.

It has been previously reported, that in non-decontaminated PCR assays using universal primers, background levels of up to 1,000 copies of contaminating *E. coli* DNA can be present, thereby impairing the analytical sensitivity and specificity of such assays [23]. Such non-specific signals may arise from contaminations environmental microorganisms or by bacterial contamination of PCR ingredients. To avoid non-specific signals, and to increase analytical sensitivity, high quality PCR reagents, free of bacterial or fungal DNA contamination, were used according to suggestions recently discussed in the scientific literature [24, 25]. This represents a major improvement as application of conventional non-decontaminated PCR reagents usually show a background threshold of between 500 and 1,000 copies of bacterial target DNA/reaction [23, 25]. To increase analytical sensitivity even further, the internal transcribed spacer (ITS) region was selected as the target region for bacterial (16S–23S) and fungal (18S–5.8S) species identification. The ITS region offers higher analytical sensitivity compared to single copy targets since it is present in multiple operons in the genomes of bacteria and fungi [26]. Moreover, the ITS region is more species specific compared to conserved rDNA genomic region and therefore is more suitable for species differentiation [27].

In serial experiments performed on EDTA-blood samples spiked with different concentrations of bacterial and fungal reference organisms, hit rates of 70–100% were achieved for 23 out of 25 organisms at 30 CFU/ml and for 15 out of 25 organisms at 3 CFU/ml. The detection limit for ConS and *Streptococcus* spp. throughout our experiments was limited for several species due to the application of our predefined cross-contamination threshold and generally ranged between 50% for *S. haemolyticus* and 100% for *S. pneumoniae*. Similarly, the recovery rate for fungal pathogens varied substantially at concentrations of 3 CFU/ml and ranged between 0% for *C. glabrata* and 100% for *A. fumigatus* in the spiked samples (Table 3). The reason for the lower analytical sensitivity in *C. glabrata* positive samples

clearly requires further investigations but may be caused by a reduced efficiency of the amplification reaction owing to the larger genomic target flanked by the primers designed for the ITS region in this specific organism.

The results of quantitative blood culture studies have shown that most episodes of clinically significant bacteraemia in adults are characterized by low numbers of circulating bacteria. In children, however, the magnitude of bacteraemia is usually much higher than in adults and in general it is inversely related to the child's age. Werner et al. [28] found that 54% of blood cultures from adult patients with *staphylococcal* and *streptococcal* endocarditis contained between 1 and 30 CFU/ml of blood. Moreover, Kreger et al. [29] described that 73% of 77 patients with Gram negative bacteremia had blood cultures that contained <10 CFU/ ml of blood. An analytical assay sensitivity of 3–30 CFU/ml, however, clearly limits the diagnostic capabilities of our assay in patients with paucibacillary bacteremia when compared to a theoretical sensitivity of 1 CFU/culture bottle in conventional blood culture after inoculating ∼10 ml of whole blood. On the other hand, the clinical value of classical blood culture techniques is clearly impaired by the prolonged time-to-result interval, as it usually takes 1–3 days to provide the clinician with Gram-stain results and species identification. At present, our novel PCR based assay system, therefore, cannot fully substitute conventional blood culture due to its potentially lower sensitivity. However, it may provide valuable additional information to the treating physicians very early in the course of disease thereby potentially improving antibiotic coverage in septic patients.

Our system, in principle, permits rapid simultaneous detection of 25 different microorganisms using wide signal-to-signal melting peak differences ($\Delta T_\mathrm{m} = 3.36$–$5.0°C$). Such specific and simultaneous identification of multiple target organisms requires the use of various specific primer and probe systems combined with multiple detection capabilities. In addition, the detection and differentiation of multiple organisms in a blood sample is strongly dependent on the amount of sequence diversity of the target regions in the individual microorganisms to then allow unambiguous differentiation by melting curve analysis after amplification. The overall assay precision for T_m measurements of melting peak temperatures as determined for the representative microorganisms in all four detection channels of the Light-Cycler® 2.0 Instrument, varied between 0.19 and 0.54°C only. The differences in the melting peak temperatures was 3.37°C for *P. mirabilis* and *S. marcescens* and >5°C for all other 23 target organisms. Given the maximal temperature variance of 0.54°C, the observed signal-to-signal difference allowed unambiguous differentiation of all organisms of the master list as detected by the new real-time PCR-based assay system (Fig. 1a–c). In summary, this is a clear

improvement compared to recently published PCR-based pathogen detection methods allowing detection and differentiation of up to 17 organisms only while using narrow signal-to-signal melting peak differences ($\Delta T_m = 0.5$–$1.0°C$) [20, 22].

In another evaluation step, the diagnostic specificity of our assay was assessed by testing the performance in the clinical setting at two independent study sites. To simulate blood sampling in individuals with indwelling devices on the ICU, blood cultures and EDTA-blood specimens for PCR were collected from 102 patients scheduled for cardiothoracic surgery. Samples were taken in parallel directly from central lines or indwelling arterial catheters that had been inserted to the patients immediately before the blood draw under sterile precautions. In 20 out of 102 individuals, blood cultures (19.6%) yielded a ConS positive result after 4–6 days as pointing to cross contamination of cultures with small amounts of residual skin flora. Correspondingly, re-evaluation of PCR data sets after disabling the established optional semi quantitative crossing point cut-off value for the LightCycler® 2.0 Instrument showed that 33 (32.3%) positive results for ConS could be generated from our sample set. In all these cases, the observed cps related to very low concentrations of pathogens, as did the prolonged cultivation time in the positive blood cultures. Interestingly, no ConS positive results, however, were detected by our PCR assay when our semi quantitative crossing point cut-off was applied for software-assisted evaluation of the PCR results, suggesting superior specificity of the assay compared to conventional blood culture under the conditions of our study.

Novel real-time PCR-based molecular biological assays for rapid detection of important pathogens causing BSI can potentially overcome some of the limitations of conventional culture based microbiological techniques. As revealed by many clinical and microbiological studies [4, 30, 31] patient outcome is greatly improved with the early administration of antibiotic therapy. Moreover, microbiological and epidemiological data clearly indicate that a limited number of bacteria and fungi are responsible for the majority of all bloodstream infections on the ICU. About 25 species account for more than 90% of all detected nosocomial pathogens in BSIs [16, 17]. For our assay the number of detectable target microorganisms was thus limited to the 25 most relevant microorganisms thereby covering around 90% of the most common pathogens causing BSI. Our assay at present does not include molecular detection of resistance genes and therefore, cannot substitute conventional blood culture with subsequent susceptibility testing. Detection and species differentiation of microorganisms from the blood of sepsis patients within 6 h after the blood draw however, can provide the treating physician with important complementary information to better tailor initial empiric broad spectrum antimicrobial therapy and to avoid potential gaps in antibiotic coverage of such patients. Moreover, in a second diagnostic step additional PCRs can be performed to detect resistance genes such as *vanA*, *vanB*, or *mecA*, if infection with more resistant pathogens is suspected from the result of the initial PCR [3, 20].

In conclusion, our newly developed real-time PCR assay may be a valuable tool for more rapid diagnosis of pathogens involved in BSI from whole blood without requiring pre-incubation of cultures. Analytical performance data obtained throughout our methodological study indicate good to excellent laboratory detection and differentiation capabilities for 25 bacterial and fungal microorganisms that are known to be most relevant in patients with BSI´s. Furthermore, the assay demonstrated excellent analytical specificity under the conditions of a high throughput ICU in patients with indwelling devices. At this point, our assay is designed to supplement conventional blood cultures due to its higher diagnostic rapidity. Whether the assay will indeed provide higher clinical sensitivity and specificity as well as more reliable identification of microorganisms early on in the course of disease compared to classical blood culture technique and whether it may significantly contribute to a more rapid initiation of better-tailored antimicrobial therapy and improved cost effective patient management in conjunction with other laboratory markers, however, awaits further evaluation of the test in clinical studies on sepsis patients.

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