Application of multiplex real-time PCR and Fluorescence Resonance Energy Transfer for the detection and differentiation of the most frequent causative agents of systemic infections from biological fluids

Ph.D. Thesis

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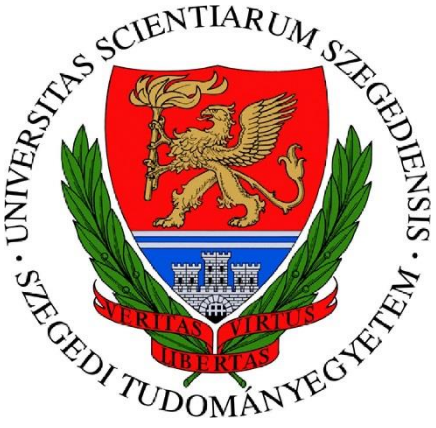

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Introduction

The exogenous and endogenous infections in the surgical departments and intensive care units are important health care challenges. These infections can lead to sepsis and septic shock indicating that this problem demands attention. While progress in prevention of these infections has been made, there are no complete solutions for nosocomial infections.

Bloodstream infections are life-threatening, especially in individuals with serious underlying conditions or an impaired immune system.

The number of reported cases of bloodstream infections in the USA between 1979 and 2002 was 10,319,418 and demonstrated an annualized increase of 8.7%. This trend is expected to continue due to aging of the population, increased use of transplantation, immunosuppressive therapy, chemotherapy, and invasive procedures. The causative microorganism of sepsis is an important determinant of outcome, in addition in critically ill patients, the majority of infections are caused by bacteria .

However, fungal infections account for only 4.6% of all infections, they have a significant impact on public health, furthermore it has been found that the annual number of cases of sepsis caused by fungal organisms in the United States increased by 207% between 1979 and 2000. Mixed fungal/bacterial infections are not uncommon, incidences of combined *Candida* and bacterial bloodstream infections have been reported in as many as 23% of all episodes of candidaemia.

Despite its relatively low frequency, fungal blood stream infections can progress to severe sepsis and septic shock, associated with a drastic rise in mortality; therefore, early and appropriate treatment of such infections is critical. The majority of these mycoses-related deaths were associated with *Candida*, *Aspergillus*, and *Cryptococcus* sp. infection. In vitro studies have indicated that emerging *Candida* species such as *C. glabrata* and *C. krusei* are demonstrating an increased incidence of resistance to antifungal agents, in particular to fluconazole. For this reason, the management of sepsis have great importance and the source of infection should be identified and controlled.

Since molecular diagnosis in sepsis is reliable, and faster than the classical blood-culturing techniques, there has been an increase in interest in methods such as PCR, ligase chain reaction (LCR), nucleic acid sequence based amplification (NASBA), and nested PCR. Nevertheless, these molecular approaches are applied only following the positivity of the blood culture; therefore, they require a substantial amount of elapsed time.

In contrast to conventional blood culture methods and molecular techniques, which are applied following the positivity of the blood culture, there are novel approaches that allow the amplification directly from blood tubes. These techniques have the great advantage that they can detect the microorganisms in 2-5 h, have high sensitivity and the quantification of the bacterial load is possible.

The LightCycler PCR assay is fast, reliable and relatively easy to perform - even in small laboratories. This method is based on a previously-reported FRET technique which has been proved to be an appropriate method for discriminating between the commonly occurring pathogen Gram-positive and Gram-negative bacteria. Differentiation of bacterial pathogens through a simple melting point analysis at 540 nm is impossible since there are no highly variable regions in the bacterial genome. For this reason the use of 16S rRNA coding region for the detection of the prokaryotic pathogens seems to be suitable.

The most effective solution for the differentiation of bloodstream infection related fungal pathogens is the use of ITS (internal transcribed spacer) region (ITS1;ITS2). These non-coding regions are highly variable rRNA coding regions which are adaptable for the identification of clinically relevant fungi over the broadest range. The ITS 2 region is situated between the 5.8S rRNA and 28S rRNA coding fraction and highly variable in different fungal species. Due to the highly variable ITS 2 region is the 7 most frequent *Candida* species can be differentiated by the use of LC capillary real-time PCR, moreover the complete detection of 10 *Candida* and 4 *Aspergillus* species, *Cryptococcus neoformans*, *Fusarium oxysporum* and *Mucor hiemalis* is possible through HRM (High Resolution Melting Analysis) and by the use of Bio-Rad CFX96 real-time PCR machine. The HRM is simply a precise warming of the amplicon DNA from around 50˚C up to around 95˚C. At some point during this process, the melting temperature of the amplicon is reached and the two strands of DNA separate or "melt" apart. The secret of HRM is to monitor this process happening in real-time. This is achieved by using intercalating dyes. The PCR machine which is adaptable for HRM detect this process by measuring the fluorescence. The machine then simply plots this data as a graph known as a melt curve. This curve shows the level of fluorescence vs. the temperature.

Real-time measurements of the fungal ITS 2 region is possible at 540 nm and require a non-specific intercalating dye.

Until now, parallel detection of fungal and bacterial infections in the same reaction tube with real-time system has been an unresolved problem, however, there are several tests in the market with the same purpose.

The differentiation of the bacterial pathogens, *vi*a melting temperature of the overall PCR product (by the use of 16S rRNA gene) and the melting point of the Gram specific probes, allow the creation of subgroups within the Gram-positive and Gram-negative strains. This system requires less than 4 h, inclusive of the time needed for the DNA preparation and the evaluation of the PCR results.

Real-time PCR detection can be performed by using free dyes or labelled sequence-specific probes. The novelty of our prototype system lies in the use of non-specific SYBR Green dye as a donor molecule, instead of a labelled primer or other specific anchor probe. This technique allows to examine pathogenic fungi, Gram-positive and Gram-negative bacteria in a single tube multiplex PCR reaction. Another novelty is the use of HRM analysis for the better discrimination of fungal infections and novel PCR mixes to reduce the time required for the differentiation of the pathogens involved in bloodstream infections. One combination of the two techniques uses unlabeled probes for the amplicon detection and T_m determination. Another parallel application was the combination of TaqMan chemistry and the very new, aspecific dye, BOXTO, as a multiplex PCR.

Materials and methods

Reference strains of the multiplex PCR

Reference strains of 17 clinically relevant bacterial species were collected, as typical of the main causative agents of bloodstream infections. Nine reference strains, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 4701, *Bacteroides fragilis* ATCC 25285, *Pseudomonas aeruginosa* ATCC 27853, *Haemophilus influenzae* ATCC 49247, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were from the American Type Culture Collection [ATCC]. *Streptococcus pyogenes* OKI 80002 provided by the National Centre for Epidemiology, Hungary [OKI] and *Proteus vulgaris* HNCMB 60076 was from the Hungarian National Collection of Medical Bacteria [HNCMB]. Furthermore, to confirm the reliability and reproducibility of the technique, clinical strains of *S. aureus* (n = 4), *S. epidermidis* (n = 6), *S. pyogenes* (n = 2), *E. faecalis* (n = 2), *E. faecium* (n = 3), *L. monocytogenes* (n = 1), *B. fragilis* (n = 2), *P. aeruginosa* (n = 1), *H. influenzae* (n = 1), *E. coli* (n = 5), *K. pneumoniae* (n = 5), *P. vulgaris* (n = 3), *Stenotrophomonas maltophilia* (n = 2), *Serratia marcescens* (n = 2), *Enterobacter aerogenes* (n = 2), *E. cloacae* (n = 2) and *Acinetobacter baumannii* (n = 3) from the Institute of Clinical Microbiology at the University of Szeged, Hungary were also included. The species identities of the clinical isolates were confirmed by conventional biochemical methods.
Ten fungal strains were examined in the same reaction vessel. Five reference strains, *Candida albicans* ATCC 10231 and ATCC 14053, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019 and *C. glabrata* ATCC 39316, were from the [ATCC], *Cryptococcus neoformans* IFM 5844 and IFM 5855 were from IFM Quality Services Pty Ltd [IFM], and *Aspergillus fumigatus* SzMC 2486, *A. flavus* SzMC 2536 and *A. niger* SzMC 2761 were from the Szeged Microbiological Collection [SzMC]. Furthermore, clinical strains of *C. albicans* (n = 14), *C. glabrata* (n = 5), *C. tropicalis* (n = 4), *C. parapsilosis* (n = 5), *C. krusei* (n = 4), *C. quillermondii* (n = 4), *C. lusitaniae* (n = 3), *C. norvegensis* (n = 1), *C. inconspicua* (n = 2), *C. dubliniensis* (n = 2) and *Cryptococcus neoformans* (n = 2) from the Institute of Clinical Microbiology at the University of Szeged, Hungary were also tested.

Fungal reference strains of the HRM analysis

The reference strains *Candida albicans* ATCC 10231 and ATCC 14053, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, and *C. glabrata* ATCC 39316 were obtained from the American Type Culture Collection (ATCC, Manassa, VA, USA), *Cryptococcus neoformans* IFM 5844 and IFM 5855 were from IFM Quality Services Pty Ltd (IFM, Ingleburn, NSW, Australia) and *Aspergillus fumigatus* SzMC 2486, *A. flavus* SzMC 2536, *A. niger* SzMC 2761, *A. terreus* SzMC 1932 were from Szeged Microbiological Collection (SzMC, Szeged, Csongrad, Hungary).

To check the liability of the method, nonhuman pathogenic fungal strains *Fusarium oxysporum* SzMC 0609 and *Mucor hiemalis* SzMC 0478 were also examined. Furthermore, to confirm the reliability and reproducibility of the technique, clinical strains of *C. albicans* (n=14), *C. glabrata* (n=5), *C. tropicalis* (n=4), *C. parapsilosis* (n=5), *C. krusei* (n=4), *C. quillermondii* (n=4), *C. lusitaniae* (n=3), *C. norvegensis* (n=1), *C. inconspicua* (n=2), *C. dubliniensis* (n=2) and *Cryptococcus neoformans* (n=2) from the Institute of Clinical Microbiology at the University of Szeged, Hungary were also tested. These strains were maintained on BBL™ Sabouraud–chloramphenicol slant agar (Becton, Dickinson and Company, Sparks, MD, USA)

and periodically sub-cultured. 24-h cultures were used for all subsequent experiments. The species identities of the clinical isolates were confirmed by conventional biochemical methods.

Bacterial DNA purification

The bacterial strains were grown on BBL™ Columbia agar plate (Becton, Dickinson and Company, Sparks, MD, USA) under aerobic conditions, except that *Bacteroides fragilis* was grown under anaerobic conditions. The bacterial DNA was extracted with the QIAamp® DNA Blood Mini Kit (QiaGene Inc, Chatsworth, Calif., USA), following the manufacturer's instructions in "Protocols for Bacteria". One milliliter of log-phase culture suspension, at a concentration of 10^7 CFU/mL, was used for the preparation. For determination of the sensitivity of the reaction, 100 μL of the serially diluted *S. aureus* reference strain was used for DNA extraction. The number of bacterial cells was determined by plating aliquots of serially diluted samples onto BBL™ Columbia agar plate (Becton, Dickinson and Company, Sparks, MD, USA).

For lysis of the rigid multilayered Gram-positive bacterial cell wall, we used a pre-incubation step with 20 mg/mL lysozyme (in 20 mM Tris • HCl, pH 8.0, 2 mM EDTA, 1.2% TritonX100). The spin protocol for "DNA Purification from Tissues" was followed, after incubation at 30°C for 30 min. The final concentration of DNA was 2.0-13.8 ng/μL, with a ratio A260/A280 = 1.6-1.8 after purification.

Fungal DNA purification

All the fungi were grown on BBL™ Sabouraud–chloramphenicol agar (Becton, Dickinson and Company, Sparks, MD, USA) medium. The fungal DNA was extracted from 1 mL of a log-phase culture suspension containing 9.6×10^7 of fungal cells. For determination of the sensitivity of the reaction, 100 μL of the serially diluted *C. albicans* reference strain was used for DNA extraction. The number of fungal cells was determined by plating aliquots of serially diluted samples onto Sabouraud-glucose agar.

The QIAamp® DNA Mini Kit Protocol for Yeasts was followed in the DNA purification. In this case, additional reagents were required for elimination of the complex fungal cell-wall structure: sorbitol buffer (1 M sorbitol, 100 mM EDTA, 14 mM β-mercaptoethanol) was used, and the samples were incubated with lyticase for 30 min at 30°C. Efficient and complete lysis was achieved in 1.5 hour in a shaking water-bath. This purification yielded 2.0–25 μg of DNA in 100 μL of water (2.0–13.8 ng/μL), with A260/A280 = 1.6–1.8.

DNA preparation from infected blood

Samples of 180 μL healthy donor bloods in EDTA tubes were infected with 20 μL of log-phase culture suspension at a concentration of 10^8 CFU/mL bacterial and/or fungal cultures. Bacterial and fungal cells were quantified in Bürker chamber, by viable counts. For the sensitivity testing of the prototype system, the blood samples were infected with five dilutions of the log-phase culture suspension at a final volume of 20 μL. The first dilution contained 50 copies in 1 μL template DNA ($2.5x10^4$ CFU/mL blood), the second contained 10 copies ($5x10^3$ CFU/mL blood), the third 5 copies ($2.5x10^2$ CFU/mL blood) and the fourth 2 copies ($5x10^2$ CFU/mL blood). The red blood cells were disrupted by lysis buffer, the bacterial and fungal cell wall lysed using the freezing-thawing method. After digestion with Proteinase K, the DNA extraction was carried out as reported previously.

Bacterial and fungal primer design, FRET probes

Two primer pairs were used for multiplex amplification of bacterial and fungal DNA. The bacterial primer pair was PLK1 (TAC GGG AGG CAG CAG) forward and PLK2 (TAT TAC CGC GGC TGC T) reverse, which are highly conserved in different groups of bacteria and amplify the 16S rRNA sequence. The PLK2 reverse primer was modified and used without the inner fluorescence labelling. Originally, the labelled primer excited the Gram specific probes. We applied the non-specific SYBR Green dye for excitation; it also serves for visualization of the fungal amplicons. This primer-pair produces a 187 bp fragment in each species.

Previously, hybridization probes were used for the Gram classification ISN2 (5′-CCG CAG AAT AAG CAC CGG CTA ACT CCG T-3′) labelled with LCRed 640 was specific for Gram-negative, and ISP3 (5′-CCT AAC CAG AAA GCC ACG GCT AAC TAC GTG-3′) labelled with LCRed705. In our experiments ISP2 probe was labelled with Cy5.5 at the 5′ end which was specific for Gram-positive bacteria. The reason why we change the labeling dye because the producers offered Cy5.5 dye instead of LCred705.

The ITS86 forward (GTG AAT CAT CGA ATC TTT GAA C) and the ITS 4 reverse (TCC TCC GCT TAT TGA TAG C) primers were used for detection of the fungi. These primers amplify a 192–494 bp sequence of ITS2 region, which is a highly variable part between the 5.8S and 28S rRNA sequence.

Conditions of the multiplex real-time PCR

Multiplex real-time PCR and FRET was performed using a LightCycler 1.2 real-time PCR instrument (Roche Diagnostics GmbH, Mannheim, Germany). The reaction volume of 10 μL contained 1 μL of DNA (with a final concentration of ~10 ng/μL), 1 μM of each of the primers, 0.7 μM of each of the probes, an appropriate amount of master mix, and 0.2 mM BSA (in the cases of the Fermentas and BioRad master mixes).

The PCR conditions were as follows: initial denaturation at 95 °C for 600 s, followed by 40 cycles of denaturation (95 °C for 0 s, 20 °C/s), annealing (55 °C for 15 s, 20 °C/s), and extension (72 °C for 20 s, 2 °C/s). The emitted fluorescence was measured after the annealing steps. The melting-curve analysis procedure consisted of 1 cycle at 95 °C for 10 s, 40 °C for 120 s, followed by an increase in the temperature to 95 °C at 0.2 °C/s. The fluorescence signal (F) was monitored continuously during the temperature ramp, and plotted against temperature (T).

Conditions of the broad range real-time PCR

Bio Rad CFX96 real-time PCR instrument (Bio-Rad Laboratories, Hercules, CA, USA) was used for the amplification. The reaction volume of 10 μl contained 1 μl of fungal DNA, 1.0 μM of each of the primers and 5 μl of reaction buffer, which includes EvaGreen dye (SsoFast Supermix; (Bio-Rad Laboratries, Inc., Hercules, CA, USA)). The PCR conditions were as follows: initial denaturation at 95 ˚C for 420 s, followed by 40 cycles of denaturation (95 ˚C for 30 s), annealing (55 ˚C for 60 s) and extension (72 ˚C for 60 s). The melting curve analysis at the end of the amplification consisted of one cycle starting at 72 ˚C for 20 s, the temperature subsequently being increased to 95 ˚C in 0.1 ˚C/s increments (HRM analysis).

LightCycler DNA master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) master mix (MMX), Maxima qPCR SYBR Green no ROX (Fermentas Inc., Vilnius, Lithuania) MMX and the IQ SYBR Green Supermix (Bio Rad) MMX were also used for melting point investigations according to the manufacturer's instructions. For comparison, the SYBR Green melting peak data were measured with the LightCycler real-time instrument (Roche Diagnostics GmbH), as described previously.

Data analysis

In case of multiplex real-time PCR and FRET melting peaks were evaluated using the LightCycler Software V 3.5. (Roche Diagnostic GmbH, Mannheim, Germany). The melting peaks were determined through the manual T_m option on the three detection channels (F1, F2 and F3).
The standard deviation (SD) of the melting-points was calculated from five parallel experiments.

The sensitivity of the multiplex PCR calculated from five dilutions of the bacterial suspension monitored by the use of LightCycler Software V 3.5.

The correct differentiation between bacteria and fungi was verified by means of gel electrophoresis, by the the amplicon length (fungal amplicons 192–494 bp, bacterial 187 bp). For the gel electrophoresis 1.5% agarose gel (AppliChem, Darmstadt, Germany) containing GelRed (10.000x in water) (Biotium Inc., Hayward, CA, USA) was used with HyperLadder™ DNA ladder 100-1013 kb (Bioline, London, UK).

For the evaluation of the broad range PCR data Bio Rad CFX Manager Software version 1.6 and Precision Melt Analysis Software 1.1 (Bio Rad) were used.

Products of the direct PCR were loaded on to 1.5% agarose gel containing GelRed (10.000x in water) (Biotium Inc., Hayward, CA, USA), and data were evaluated with HyperLadder™ DNA ladder 100-1013 kb (Bioline, London, UK).

Results and discussion

Discrimination of the fungal, Gram-positive and Gram-negative bacterial pathogens

DNA samples from all species studied were prepared and amplified successfully with the SYBR Green dye-based method in the LightCycler instrument. Species-specific T_m-s were obtained by melting-point analysis on three detection channels and all pathogens were identified correctly as fungi or Gram-negative or Gram positive bacteria. On the F1 channel (540 nm), the melting points of all the amplicons (T_m A) were visible, due to the fluorescent signal of the SYBR Green non-specific intercalating dye. On the F2 (640 nm) and F3 (705 nm) channels, the Gram-negative and the Gram-positive probes (T_m P), respectively, gave fluorescence signals. After the discrimination of the Gram-negative and Gram-positive strains, the fungal pathogens could be screened, because the fungal strains gave no signal on the F2; F3 channels.

Determination of the bacterial pathogens

Four Gram-positive and nine Gram-negative bacterial subgroups could be distinguished through a joint consideration of the melting points of the probes and the melting point of the overall PCR product. *S. aureus* and *S. epidermidis* have very close-lying melting temperatures and their species-specific differentiation is not possible *via* this 16S rRNA sequence. A comparison of the Gene Bank sequences (*S. aureus* and *S. epidermidis* NCBI Taxonomy ID: NC_009782.1 and JF_799903.1) of these species revealed a variance of only three base-pairs, none of them in the region where the probe is associated with the DNA. Thus, determination of the clinically relevant *Staphylococcus* species requires other gene sequences in which the antibiotic resistance can be detected. The situation is the same for the two *Enterococcus* species. At the same time, *S. pyogenes* and *L. monocytogenes* are clearly differentiable.

Among the Gram-negative bacteria, *E. coli* is one of the most common causative agents of bloodstream infections. Unfortunately, it has almost the same T_m as those of *E. cloacae* and *S. marcescens*. Other bacterial strains, such as *H. influenza*, are clearly differentiable through the melting temperature of the probe or amplicon. The sensitivity of the reaction was five colony-forming units (CFU) per reaction.

Determination of fungal pathogens

Fourteen frequently-encountered fungal pathogens could be distinguished. The highly variable ITS 2 target sequence allowed correct identification of all of the clinically relevant fungal strains, through the T_m points on the F1 channel. There was no signal on the F2 or F3 channel. The sensitivity of the reaction was 5 CFU per reaction.

The correct differentiation between bacteria and fungi was verified by means of gel electrophoresis, with the help of the amplicon length (fungal amplicons 192–494 bp, bacterial 187 bp).

Determination of the co-infection model

In case of co-infections, there are some limitations in the detection. If the ratios of the different agents are higher than 1:10, the system does not detect the infectious agent which is in lower quantities.

Calibration of the multiplex PCR

All three non-specific dyes (LCGreen, EvaGreen and SybrGreen) excited all of the labelled probes (LCRed640, LCRed705 and Cy5.5). The best results were obtained with the SybrGreen dye.

The determination of T_m is very sensitive to the composition of the PCR reaction mixture, and especially to the ionic strength. To avoid T_m bias due to pipetting errors between PCR runs, the application of mastermixes is recommended. Limitation of the method is that the various mastermixes offered by different suppliers differ in reagent composition, which may influence the T_m values.

Repeated runs with a certain master mix yield reproducible data. In case of different alteration of master mixes from different suppliers, however, calibration is necessary to establish the new T_m data on the fungal strains.

The data determined in the present work were obtained with the use of "Fermentas Maxima SybrGreen, no ROX" and five-eight parallel experiments. No false positive samples were found when this method was tested. No significant differences in the melting peak temperatures were observed between different isolates of the same species. The standard deviation of the melting peak temperatures of all 21 references and 93 clinical isolates with bacterial and fungal strains was between 0.08 and 0.88. These data are in concordance with our previous results.

Sensitivity and reproducibility

For sensitivity testing of the prototype system, six bacterial and two fungal gDNA preparations were made from artificially infected blood. Eight species, and eight parallel investigations of

five dilutions of the bacterial suspensions in blood were, analysed. Of 8 reactions for each species, all were positive with 50 DNA copies, 98.5% were positive with 10 copies, 67.2% were positive with 5 copies and 21.9% were positive with 2 copies. All the reactions were carried out within the same parameters described in the section PCR conditions.

Determination of fungal pathogens by HRM analysis

DNA samples from all the fungal species involved in this study were prepared and amplified successfully using the EvaGreen dye-based method in the Bio Rad CFX instrument. Species specific melting peaks (T_m) were obtained via HRM analysis, allowing the differentiation of all investigated fungal species. Thus, it was possible to distinguish among the 15 most common fungal pathogens. The clinical strains had the same T_m as the references. Due to the highly saturating EvaGreen dye and the HRM analysis, the accuracy of the resolution was ±0.01–0.24 ˚C. The sensitivity of the reaction was 5 CFU per reaction using the *C. albicans* reference strain. For comparison, all the fungal strains were investigated in the presence of the SYBR Green dye using the Roche LightCycler instrument. The sensitivity of the reaction was similar, but the SD was higher (±0.02-1.04 ˚C). As mentioned before the determination of T_m is very sensitive to the composition of the PCR reaction mixture, and especially to the ionic strength. For example, the difference in T_m between *C. albicans* and *C. dubliniensis* was previously determined with the LightCycler DNA master SYBR Green I MMX to be 1.2 ˚C (87.4 and 86.2 ˚C) . In case of Maxima qPCR SYBR Green no ROX MMX, the difference was 2.0 ˚C (83.5 and 81.5 ˚C), using the IQ SYBR Green Supermix it was 1.5˚C (85.6 and 84.1 ˚C) and with SsoFast Supermix it was 1.0 ˚C (84.5 and 83.5 ˚C). The situation is similar for other fungi. Repeated runs with a certain master mix yield reproducible data. In case of alteration of master mix, calibration is necessary to establish the new T_m data on the fungal strains, this is applied to bacterial investigations as well. The data determined with the broad range PCR were obtained by the use of SsoFast Supermix.

Conclusions

Real-time PCR is one of the fastest diagnostic methods currently available. The use of rRNA genes for the detection is based on the conserved 16S rRNA sequences of the bacteria. As regards fungi, the ITS sequence refers to a segment of non-functional RNA, situated between 5.8S and 28S rRNAs. To reproduce the results, it is possible to differentiate between fungi and bacteria, or between fungal species by electrophoresis or melting-point analysis. The Roche LightCycler PCR was specially developed to amplify amplicons under 500 bp. The amplicons amplified by PLK1/PLK2 comprised 187 bp, while the fungal amplicons amplified by ITS86/ITS4 primer pair varied between 192 bp (*Geotrichum candidum*) and 494 bp (*Malassezia furfur*), values which are perfectly suited to this instrument profile. In this study, the advantage of the LC system was utilised when FRET technique was used to detect and differentiate the bacterial pathogens. As a novel element, excitation of the fluorescent probes was carried out with the help of a non-specific intercalating dye, this is an uncommon procedure in real-time investigations. It allows parallel detection of fungal pathogens and with bacteria in the same tube. As the result of the use of the multiplex PCR in combination with FRET probes and melting point-analysis, the broad-range identification of many frequent causative agents of bloodstream infections becomes possible within four hours. Sensitivity of pathogen PCR in sepsis is generally between 3 and 100 CFU/mL according to the literature. The sensitivity of our prototype system was five CFU per reaction, which in combination with an efficient preparation is suitable for the detection of bloodstream infections. If commercially available "Midi" preparation kits (i.e.: NucleoSpin Blood L, Macherey-Nagel, Düren, Germany) were used, the sample mateial was 2 mL of blood, the elution volume was 100 µL and finally 5 µL of eluate were used for subsequent PCR. The calculated sensitivity was 50 CFU/mL blood. The sample/eluent ratio was the same in case of midi and maxi preparation kits which means that increased sample volume is not enhancing the sensitivity. The sensitivity of the "gold standard" conventional blood culture technique is one CFU per 10 mL blood sample. Our method is less sensitive. The blood culture technique is not replaceable with molecular techniques so far but the time delay until the adequate therapy can be reduce.

To determine the diagnostic sensitivity and reproducibility of the method, experiments with artificially infected blood were performed. The sensitivity of the PCR was 2 to 10 copies per reaction, which was the same as with cultivated cells. The melting points (T_mA and T_mP) were the same as we described before with "Fermentas Maxima SybrGreen, no ROX"; therefore, human gDNA does not inhibit the reaction and does not modify the melting peaks.

With this method, neither the Gram-positive *S. aureus* and *S. epidermidis* nor the Gram-negative *E. coli*, *E. cloacae* and *S. marcescens* can be distinguished, and additional species-specific probes or primers are necessary for the further differentiation of these species.

Antibiotic resistance cannot be determined directly with this prototype system. The susceptibility testing of resistant *E. coli* strains can be performed using a PCR-based technique with other 16S rRNA specific primers. Unfortunately, these investigations require a PCR analysis after the identification of the bacteria.

In spite of its limitations, the prompt and reliable information provided by this new diagnostic method on the most common pathogenic bacteria might permit targeted therapy with narrow-spectrum antibiotics, instead of empirically-administered broad-spectrum antibiotics. To

confirm these findings in clinical practice, a prospective study is now being designed and engineered.

The incidence of sepsis has been continuously increasing over recent decades, and the early detection of the pathogens can have a great impact on the clinical outcome of infections. Molecular diagnostic systems allow species identification in less than 24 hours - which is a drastic improvement relative to the gold-standard, culture-based method and Gram staining-based identification methods that yield results in 24 to 72 hours.

With the novel method described above (multiplex PCR with the new combination of aspecific dyes and labelled probes), the most common causative agents of bloodstream infections can be detected in two hours, without DNA preparation; therefore, this method offers a huge advantage over traditional FRET-based assays by accurately detecting the T_m of both the probes and the amplicons.

New scientific results related to the thesis

The newly developed LightCycler PCR assay is fast, reliable and relatively easy to perform - even in small laboratories. This method is based on a previously-reported FRET technique which has been proved to be an appropriate method for discriminating between the commonly occurring pathogen Gram-positive and Gram-negative bacteria.

Bacterial pathogens, have been differentiated via melting temperature of the overall PCR product by the use of 16S rRNA gene and the melting point of the Gram specific probes, which allow the creation of subgroups within the Gram-positive and Gram-negative strains.

The newly developed prototype system is based on the use of non-specific SYBR Green dye as a donor molecule, instead of a labelled primer or other specific anchor probe.

Due to the highly variable ITS 2 region we 7 most frequent Candida species causing blood stream infections were differentiated by the use of LightCycler capillary real-time PCR.

The complete detection of 10 Candida and 4 Aspergillus species, Cryptococcus neoformans, Fusarium oxysporum and Mucor hiemalis could be introduced by HRM (High Resolution Melting analysis) and by the use of Bio-Rad CFX96 real-time PCR machine.

A novel method was estabilished to determine pathogenic fungi, Gram-positive and Gram-negative bacteria in a single tube multiplex PCR reaction.

The techniques described in this thesis provide a better discrimination of fungal infections and due to the novel PCR mixes the time required for the differentiation of the pathogens involved in bloodstream infections was reduced.

The new system can provide a rapid detection method and can promote the adequate antibiotic therapy in bloodstream infections.

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