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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Publications related to the thesis

I. Horváth Á, Pető Z, Urbán E, Vágvölgyi Cs, Somogyvári F: A novel, multiplex, real-time PCR-based approach for the detection of the commonly occuring pathogenic fungi and bacteria BMC Microbiology 2013;13:300.

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II. Somogyvari F, Horvath A, Serly J, Majoros H, Vagvolgyi Cs, Peto Z: Detection of invasive fungal pathogenes by real-time PCR and high-resolution melting analysis. In vivo 2012;26(6):979-83.

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III. Horvath A, Santha P, Horvath V, Torok N, Nagy I, Jancso G, Vagvolgyi Cs, Somogyvari F: Rapid genotyping of genetically modified laboratory animals from whole blood samples without DNA preparation. Acta Biologica Hungarica 2013;64(2):262-5.

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List of abbreviations

AST	antibiotic susceptibility testing assessment
ATCC	American Type Cell Culture
CFU	colony forming unit
F	fluorescence signal
FISH	fluorescence *in situ* hybridization
FRET	Fluorescence resonance energy transfer
gDNA	genomic DNA
HNCMB	Hungarian National Collection of Medical Bacteria
HRM	High Resolution Melting Analysis
IFM	IFM Quality Services Pty Ltd
ITS	internal transcribed spacer (fungal non coding sequence)
LCR	ligase chain reaction
LC	Roche LightCycler 1.2 PCR machnie
MMX	master mix
MODS	multiple organ dysfunction syndrome
MOF	multiple organ failure
NASBA	nucleic acid sequence based analysis
OKI	National Centre for Epidemiology, Hungary
PCR	polymerase chain reaction
SD	standard deviation
SIRS	systemic inflammatory response system
SzMC	Szeged Microbiological Collection
T_m	melting temperature
T_m A	melting temperature of the amplicon
T_m P	melting temperature of the probe

1. Introduction

1.1 Definitions for bloodstream infections

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 [1].

Sepsis is a potentially fatal whole-body inflammation caused by a severe infection [2]–[4].

Sepsis is induced by the immune system's response to a serious infection most commonly bacteria but also fungi, viruses and parasites in the blood, urinary tract, lungs, skin, or other tissues. Pneumonia is the most common cause of sepsis that accounts for half of all cases, followed by intraabdominal and urinary tract infections [5].

The reaction of the immune system to an infection also known as systemic inflammatory response system (SIRS). SIRS can be a response to a noninfectious offense as well, however, when it is the result of a confirmed infectious process, it is termed sepsis. Another definition for bloodstream infections is the bacteremia which means the presence of viable bacteria in the blood. The definitions viraemia, fungaemia and parasitemia are described in the same manner, respectively. The term septicemia, the presence of microorganisms or their toxins in the blood, is no longer used by the consensus committee [3].

The typical signs and symptoms of the sepsis are fever (>38ºC) or hypothermia (<36ºC), elevated heart rate (>90 beats per minute), rapid breathing (>20 breaths per minute or $PaCO_2$ <32 mm Hg) and the number of white blood cells (>12,000/cu mm, <4,000 cu mm, or > 10% immature (band) forms). The systemic response to an infection is manifested by two or more of these conditions. Further symptoms can be the confusion and the edema [2]. Sepsis is defined as severe when these findings occur in association with signs of organ dysfunction, such as hypoxemia, oliguria, lactic acidosis, elevated liver enzymes, and altered cerebral function [6].

Following a bloodstream infections multiple organ dysfunction syndrome (MODS) than multiple organ failure (MOF) can evolve [7], [8].

Bloodstream infections are life-threatening, especially in individuals with serious underlying conditions or an impaired immune system [9]. Risk factors of sepsis can be divided into two/ three groups: risk factors for infection, contingent upon developing infection and possibility

for organ dysfunction [10], [11]. For example age, black race, male gender, health conditions are more susceptible to bloodstream infections. Moreover there is an inverse relationship between the socioeconomic status and the possibility of sepsis [12]. Severe sepsis most likely occurs in patients with chronic disease such as diabetes, cancer, chronic renal and liver disorders or obstructive pulmonary disease furthermore the residence in long-term care facilities, malnutrition, in addition the abnormalities in the immune response system can be risk factors for the infection [5].

1.2 Incidence of sepsis

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% [13]. 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. The most common Gram-positive isolates are *Staphylococcus aureus* and *Streptococcus pneumonia*, whereas *Escherichia coli*,

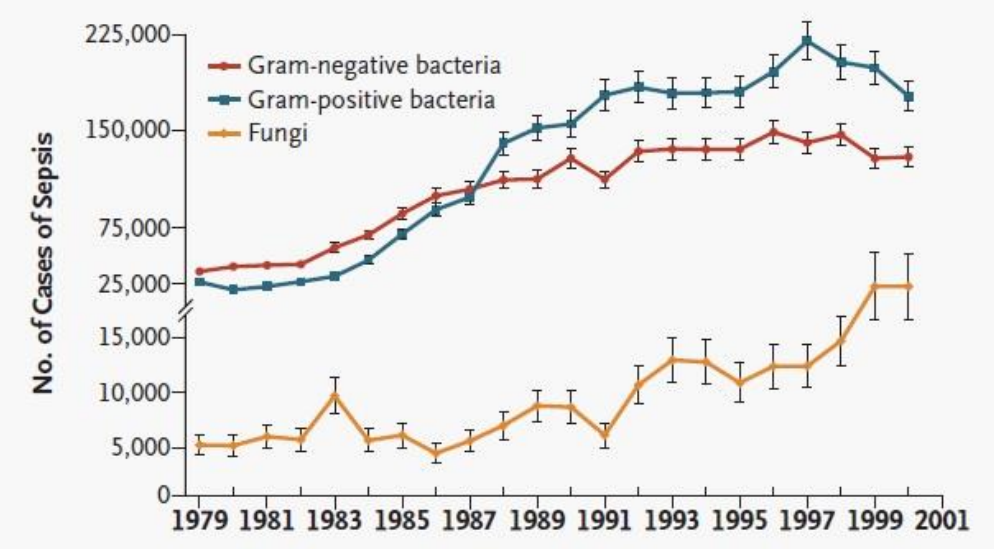

Figure 1.**Numbers of cases of sepsis in the United States according to the causative oranism from 1979 to 2001** (Martin et al. 2003) [13]

Klebsiella species, and *Pseudomonas aeruginosa* predominate among Gram-negative isolates [14].

In the past three decades Gram-positive bacteria have shown increases in frequency of sepsis and are now almost as common as infections caused by Gram-negative bacteria [15], [16].

During a 22 - year period incidence of Gram-positive infections increased by an average of 26.3 percent per year [13].

However infection caused by Gram-negative bacteria have decreased - in a systemic literature review of 510 articles published during the last 30 years was reported that Gram-negative bacteremia was associated with a higher mortality compared with Gram-positive bacteremia [17]. In a more recent study involving 75 countries and 14,000 ICU patients, Gram-negative bacteria were isolated in 62% of patients with severe sepsis who had positive cultures, Gram-positive bacteria in 47 % and fungi in 19% [18].

The most common bloodstream infections caused by *Staphylococcus* and *E. coli* were associated with a relatively low mortality (20% and 19%, respectively) compared with *Candida* (43%) and *Acinetobacter* (40%) species [19].

However, fungal infections account for only 4.6% of all infections, and 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 [13]. 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 [20].

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 [21]. *Candida albicans* is currently the most frequent causative agent of fungal sepsis, but the frequencies of invasive mycoses caused by *C. parapsilosis, C. tropicalis, C. glabrata, C. kefyr, C. inconspicua* and *C. krusei* have been reported to be increasing especially in immuncompromised patients [22], [23]. The majority of these mycoses-related deaths were associated with *Candida*, *Aspergillus*, and *Cryptococcus* sp. infection [13]. 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 [24], [25].

Nowadays, a fourth of patients who develop severe sepsis will die during their hospitalization [26], [27]. In an epidemiological study where the authors analyzed the incidence, outcome and associated cost of care the average length of stay was 19.6 days and cost per case was $22,100 [5]. For this reason, the management of sepsis have great importance and the source of infection should be identified and controlled.

1.3 Current diagnosis of bloodstream infections

According to the protocol used in case of bloodstream infections, bacteriological testing is performed in blood culture bottles. The patient's blood is loaded into automated detection system that examines the production of CO_2. Whenever microbial growth is suspected, blood is withdrawn from the positive bottle for sub culturing in plates, antibiotic susceptibility testing assessment (AST) and gram staining to provide the first identification. The bacterial identification is based on growth and culture characteristics, Gram-staining and further testing of biochemical properties [28].

The detection of systemic fungal infections is difficult because the patient has only a few specific symptoms. In addition, the typical signs of the infection may be not present in the patient because of the immunosuppression of a patient. In case of fungi colonization is difficult to distinguish from invasive disease, blood culture is often negative and fungal identification depends on the microbial growth. Waiting for positive fungal blood culture results and potentially susceptibility testing leads to a significant delay in appropriate therapy and in turn higher mortality [29]–[31].

It can be concluded that the entire procedure of identification the bacterial and/or fungal pathogens can be achieved in days because these methods require grown cultures [32]–[35]. Molecular identification after growth in blood culture media for example fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence based analysis (NASBA) provide faster identification of living bacteria compared to the gold standard culture based methods but it is still time-consuming because these molecular approaches are applied only following the positivity of the blood culture [36]–[39] (Figure 2).

The availability of fast and reliable procedures for the differentiation of *Candida* species is another important issue for clinical laboratories. The diagnosis of candidaemia or haematogenous candidiasis can be problematic because of the low sensitivity of analysis *via* blood cultures [39]. Non-culturing methods, such as galactomannan antigenaemia, Western blotting and PCR based approaches are therefore being developed and evaluated for the detection of mycotic infections [40]–[42].

1.4 Why do we need improved methods?

The main disadvantages of the standard culture techniques are the delay between blood sampling and information returned to the clinician (typically days), the need for bacteriological expertise, the personnel workload and reagent costs, as well as the inaccuracy of the method for detecting microorganisms that are fastidious or have been previously exposed to antibiotics. Moreover, the total hospitalization costs of sepsis are relatively high: there is an increase from $15.4 billion in 2003 to $24.3 billion in 2007 [43].

1.5 New possibilities in the diagnosis

Molecular diagnostic methods for the detection of the pathogens have a continuously growing importance due to their rapidity and sensitivity. Different nucleic acid detection tests have been performed for detection of bloodstream infections such as FISH, NASBA, LCR, and PCR. [44].

Application of real-time PCR has revolutionized the way to diagnose human pathogens. Novel testing methods can combine PCR chemistry with fluorescent probe detection of amplified product in the same reaction vessel. The use of intercalating dyes allows the reaction kinetics (of the elongation step) and the amplicon melting point (T_m) to be determined easily. The intensity of the fluorescence emitted during the reaction correlates to the amount of DNA product formed. In general, both PCR and amplified product detection are completed in an hour or less, which is considerably faster than the conventional detection methods [45]–[50]. By now due to the innovation of the PCR machines, methods, polymerases, specific master mixes - detection of pathogens in different diseases is faster, reliable and more specific.

1.5.1 Intercalating dyes, probes

Intercalating dyes such as SYBR Green and Eva Green detect the accumulation of any double stranded DNA product. The use of intercalating dyes with appropriate instruments can perform a melting curve analysis to determine the melting temperature (T_m) which is sensitive but not always specific. The T_m is determined by the length of the amplicon and guanine cytosine content [51].

Sensitive and specific detection is possible with real-time PCR by using novel fluorescent probes. In the clinical microbiology, the most frequently used probes are the Fluorescence

resonance energy transfer (FRET), TaqMan and Molecular Beacons. These detection methods all rely on the transfer of light energy between two adjacent dye molecules [52]. FRET technique is a distance dependent interaction between the electronic excited states of two dye molecules [53]. The excitation is transferred from a donor (anchor) molecule to an acceptor (quencher) molecule, without emission of a photon and has been proved to be an appropriate method for discriminating between the commonly occurring pathogen bacteria.

1.5.2 Instruments

The work load and work flow issues may determine which system could be the best for different-sized laboratories and test volumes. Thermocycling in lower capacity instruments such as LightCycler 1.2 (LC) (Roche Diagnostics GmbH, Mannheim, Germany) is faster than in instruments which use solid phase material for heat conductance (heating blocks) [54]. Selection of a real-time PCR instrument and real-time detection format requires consideration of test volume, probe detection conditions, turnaround time for results, personnel requirements, and software.

The LightCycler machine demand special reaction vessels (20 μL sample capillary) and supports fluorescence resonance energy transfer hybridization probe detection with melting curve analysis. Our experiments are based on the use of LightCycler 1.2 because it is fast due to the air heating and cooling for the rapid temperature ramping and susceptible for FRET. These properties are let to analyze the most frequent causative agents of systemic infections, bacteria and fungi respectively.

Real-time detection of a broad range fungal pathogens is possible by the use of Bio-Rad CFX96 (Bio-Rad Laboratories, Hercules, CA, USA) instrument which uses solid phase material for heat conductance and adaptable for High Resolution Melting analysis (HRM).

1.5.3 New generation mixes

Application of hot start DNA polymerases is popular and most often recommended for high-throughput applications. The polymerases employed in Hot Start PCR are unreactive at ambient temperatures. Polymerase activity can be inhibited at these temperatures through different mechanisms, including antibody interaction, chemical modification and aptamer technology [55]. Usually activation of the polymerase is a 10 minutes step before the cycles.

These hot start polymerases are popular because of the specificity and grown sensitivity. In addition, some of our experiments were carried out without microbial nucleic acid purification due to the high temperature activation which is the same step as the cell wall disruption in high temperature and alkaline circumstances. After a 10 minutes digestion step the hot start polymerase can find free nucleic acid strands and starts the amplification [56].
Another novelty is the commercially available "direct PCR" DNA polymerase which enables PCR amplification without any prior DNA purification from blood samples due to the resistance of the enzyme to inhibitors present in blood components. Application of master mixes with these polymerases can reduce the time requirement of a genetic analysis of a certain gene polymorphism. In order to facilitate the work flow and time requirements we have developed a rapid, simple and accurate procedure of genotyping from a single drop of animal blood without DNA preparation [57].

1.6 Existing nucleic acid based detection methods for the determination of the pathogens

Molecular techniques have been developed in order to improve the sensitivity and to detect bloodstream infection earlier [58], [59]. The ideal molecular method would analyze the patient's blood sample and provide all the information required to immediately for direct optimal antimicrobial therapy in case of bacterial or fungal infections. Furthermore, it would provide data to assess the effectiveness of the therapy by measuring the clearance of microbial nucleic acids from the blood over time. None of the currently available molecular methods is sufficiently rapid, accurate or informative to fulfil this requirement.
Several commercially available methods have been introduced, such as SeptiTest; (Molzym, Bremen, Germany), MolYsis (Molzym, Bremen, Germany), SeptiFast (Roche; Basel, Switzerland), Prove-it (Mobidiag, Helsinki, Finland), VYOO (Analytik Jena, Jena Germany) which can reduce the time required for the diagnosis [45]–[50]. Most of them use PCR for the identification of the pathogen. These techniques are similar to our system in some aspects, but they are usually based on previous culturing, they detect the causative microorganism after growth in blood culture bottles.

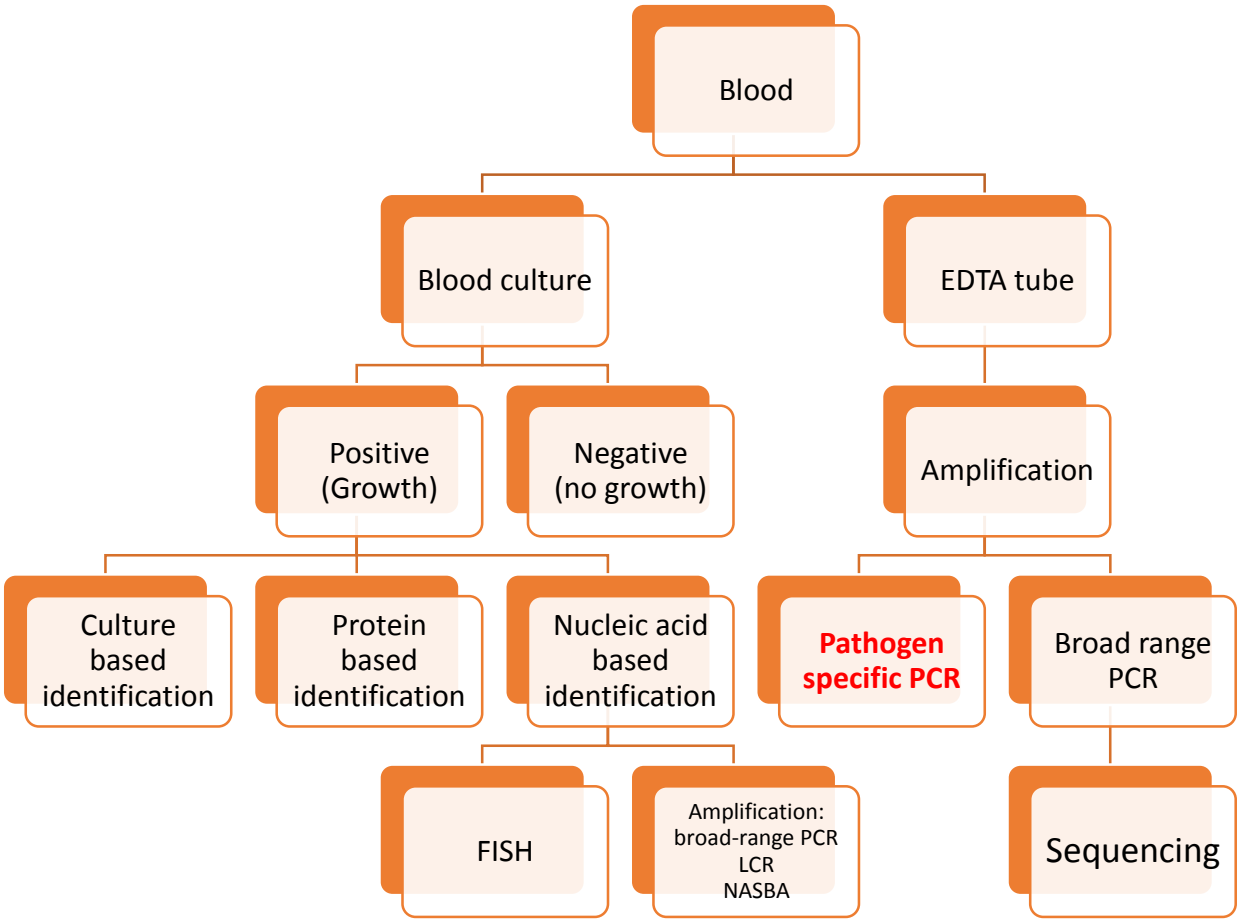

Figure 2. **Possibilities for the detection of the microorganisms causing bloodstream infections.** Compared to the gold-standard culture based method the pathogen specific PCR amplify the PCR product directly from blood without culturing the microorganisms.

1.7 Novel molecular approach to detect the most frequent causative agents in sepsis

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 [60]–[62].

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 [63]. 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 [64].

One approach for identification of the most common clinically relevant fungi is to find species or genus-specific genomic sequences such as heat shock protein 90 [65], aspartic

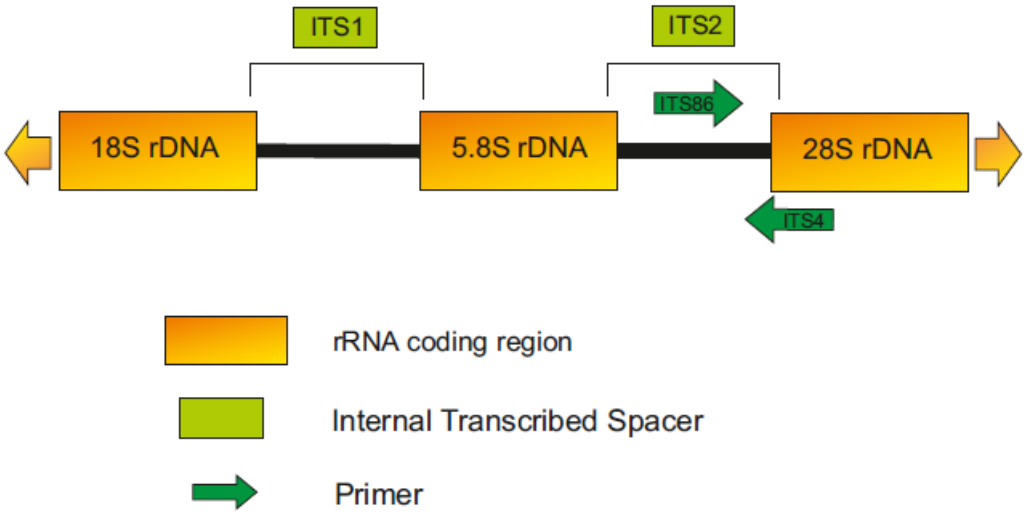

Figure 3. **Internal Trascribed Spacer (ITS) – non coding sequences between rRNA coding regions** (White et al. 1990, Somogyvári et al. 2007) [76], [80]

proteinase [66], chitin synthase [67] or lanostherol synthase [68]. The use of RNA targets for the identification is rare [69]. However, it seems that the most effective solution for the differentiation of bloodstream infection related pathogens is the use of ITS (internal transcribed spacer) region (ITS1;ITS2) [70] [71]. 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 [72]. 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 [73]. 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. Intercalating dye has unique property. When it binds specifically to double-stranded DNA it fluoresces brightly. In the absence of double stranded DNA intercalating dye has nothing to bind to and it only fluoresces at a low level. At the beginning of the HRM analysis there is a high level of fluorescence in the sample because of the billions of copies of the amplicon. But as the sample is heated up and the two strands of the DNA melt apart, presence of double stranded DNA decreases and thus fluorescence is reduced. 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 [74]. HRM is substantially used for genotyping.

Real-time measurements of the fungal ITS 2 region is possible at 540 nm and require a non-specific intercalating dye [75].

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, *via* 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 [76].

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 [77]. Another parallel application was the combination of TaqMan chemistry and the very new, aspecific dye, BOXTO, as a multiplex PCR [78].

2. Aims of the study

The purpose of this study was:

- To find a rapid molecular biological tool for the detection of the bacterial and fungal pathogens which are currently the most frequent causative agents of bloodstream infections.

- Optimization of the parameters of the reactions which enable to detect mixed bacterial and fungal infections in the same reaction tube.

- Application of real-time PCR and FRET in order to differentiate of the pathogens in the same reaction vessel.

- To establish a database with the tested microorganisms and with the appropriate T_m –s for the further analysis of unknown samples.

- To set a broad range PCR for the better discrimination of the fungal pathogens by the use of HRM.

- To detect the pathogens from EDTA blood or serum without gDNA purification.

3. Materials and methods

3.1 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 [79]. 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.

3.2 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.

3.3 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.

3.4 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) [80] 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.

3.5 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 ($2.5\text{x}10^4$ CFU/mL blood), the second contained 10 copies ($5\text{x}10^3$ CFU/mL blood), the third 5 copies ($2.5\text{x}10^2$ CFU/mL blood) and the fourth 2 copies ($5\text{x}10^2$ CFU/mL blood). The red blood cells were disrupted by lysis buffer [81], 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 [33].

3.6 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 [63] 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 [76] 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 [82].

Primer/Probe	**Sequence (5’ – 3’)**
PLK 1 bacterial forward	TAC GGG AGG CAG CAG
PLK 2 bacterial reverse	TAT TAC CGC GGC TGC T
ITS 86 fungal forward	GTG AAT CAT CGA ATC TTT GAA C
ITS 4 fungal reverse	TCC TCC GCT TAT TGA TAG C
ISN 2 Gram-negative FRET probe	LCred CCG CAG AAT AAG CAC CGG CTA ACT CCG T
ISP 3 Gram-positive FRET probe	Cy5.5 CCT AAC CAG AAA GCC ACG GCT AAC TAC GTG

Table 1. **Sequences of the used primers and FRET hybridization probes.** The probes were labelled on the 5’ end.

3.7 Master mixes/excitation dyes

Different, non-specific intercalating dyes are used for real-time PCR investigations. Most of these are accessible in ready-to-use, master mix formulae. Our goal was to choose the best dye for excitation of the labelled probes. The tested dyes were LCGreen “LightCycler® 480 High Resolution Melting Master” (Roche Diagnostic GmbH, Mannheim, Germany); SYBR Green “LightCycler® 480 DNA Master SYBR Green I”, (Roche Diagnostics GmbH, Mannheim, Germany); “IQ™ SYBR® Green Supermix” (Bio-Rad Laboratries, Inc., Hercules, CA, USA) ; “Maxima™ SYBR Green qPCR Master Mix no ROX” (Fermentas, Vilnius, Lithuania); and “LC-FastStart DNA Master Hybridization Probes” (Roche Diagnostics GmbH, Mannheim, Germany) combined with EvaGreen dye (Biotium Inc., Hayward, CA, USA) and “Sso Fast™ EvaGreen® Supermix” (Bio-Rad Laboratries, Inc., Hercules, CA, USA). All master mixes were used according to the manufacturer’s instructions.

3.8 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).

3.9 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 [75].

3.10 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).

4. Results

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

DNA samples from all species involved in the study were prepared successfully. The appropriate annealing temperature could be determined for the amplification of the bacterial and fungal template DNAs in the same reaction vessel. The pathogen templates amplified successfully the FRET probes of Gram-positives and – negatives could be excited by SYBR Green in LightCycler instrument. Species-specific T_{m}-s were obtained by melting-point analysis on three detection channels and all pathogens were identified succesfully as fungi or Gram negative or Gram positive bacteria (Table 1).

At 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. At 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 at the F2 and F3 channels.

Species specific human pathogenic fungal differentiation was obtained by High Resolution Melting (HRM) analysis. However, it requires another instrument and special software for the analysis, therefore the inchoation of prompt antifungal therapy is possible in a shorter diagnostic term.

Microbial strains	T_m P (°C)		T_m A (°C)	
Gram positive (G+)	mean	SD	mean	SD
Enterococcus faecalis	67.94	0.07	84.14	0.36
Enterococcus faecium	67.84	0.21	84.59	0.78
Listeria monocytogenes	67.80	0.19	86.01	0.36
Staphyloccus aureus	64.85	0.21	83.91	0.54
Staphyloccus epidermidis	64.50	0.30	83.60	0.36
Streptococcus pyogenes	46.54	0.56	84.38	0.78
Gram negative (G-)				
Acinetobacter baumannii	66.09	0.15	82.90	0.16
Bacteroides fragilis	48.65	0.18	84.47	0.84
Enterobacter aerogenes	63.95	0.34	83.47	0.48
Enterobacter cloacae	64.98	0.09	84.38	0.24
Escherichia coli	64.69	0.44	84.74	0.54
Haemophilus influenzae	61.99	0.35	84.28	0.30
Klebsiella pneumoniae	65.13	0.23	84.57	0.20
Proteus vulgaris	64.58	0.18	82.87	0.24
Pseudomonas aeruginosa	53.32	0.33	83.00	0.34
Serratia marcescens	64.01	0.30	84.17	0.30
Stenotrophomonas maltophilia	58.10	0.07	84.42	0.15
Fungi				
Candida albicans	-	-	87.1	0.33
Candida dubliniensis	-	-	85.5	0.50
Candida quillermondii	-	-	85.1	0.70
Candida krusei	-	-	89.8	0.02
Candida parapsilosis	-	-	85.4	0.88
Candida tropicalis	-	-	84.5	0.75
Aspergillus fumigatus	-	-	91.0	0.38

Table 2. **Melting points of bacterial and fungal amplicons and probes.** All the amplicons T_m (T_m A) were measured at the F1 channel (540 nm). The signal was generated by aspecific SYBR Green dye. The Gram -positive specific probes produced a signal at the F2 channel (640 nm) the Gram-negative probes at the F3 channel (705 nm) (T_m P). The signals were induced with the help of a special FRET technique.

4.2 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 (Figure 4).

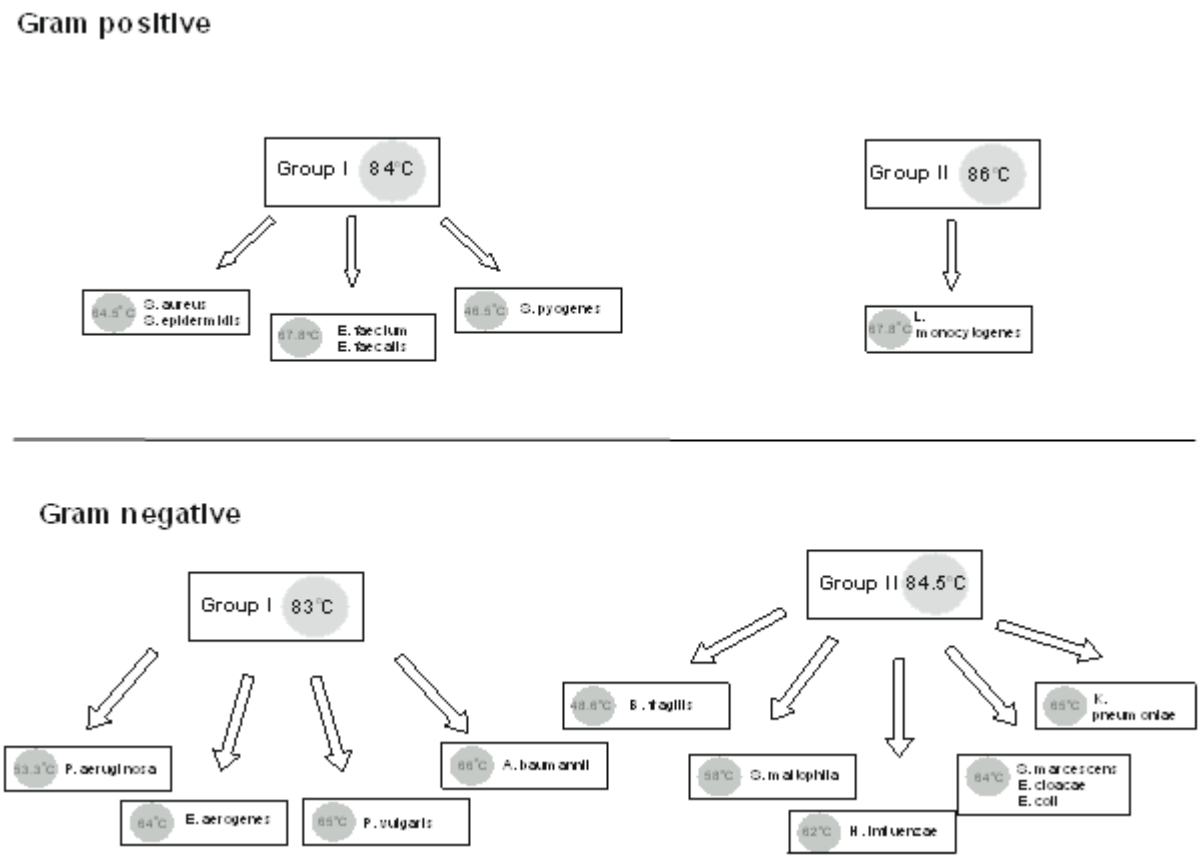

Figure 4. **Differentiation of the bacterial pathogens.** The group temperatures indicate the entire T_m of the pathogens. The subgroup temperatures are the melting temperatures of the hybridization probes.

S. aureus and *S. epidermidis* have very close-lying melting temperatures and their species-specific differentiation is not possible via this 16S rRNA coding sequence (Figure 5). 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 were 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 [75]. Based on our experiments we could conclude that the two *Enterococcus* species show similar characteristics [73]. At the same time, *S. pyogenes* and *L. monocytogenes* are clearly differentiable.

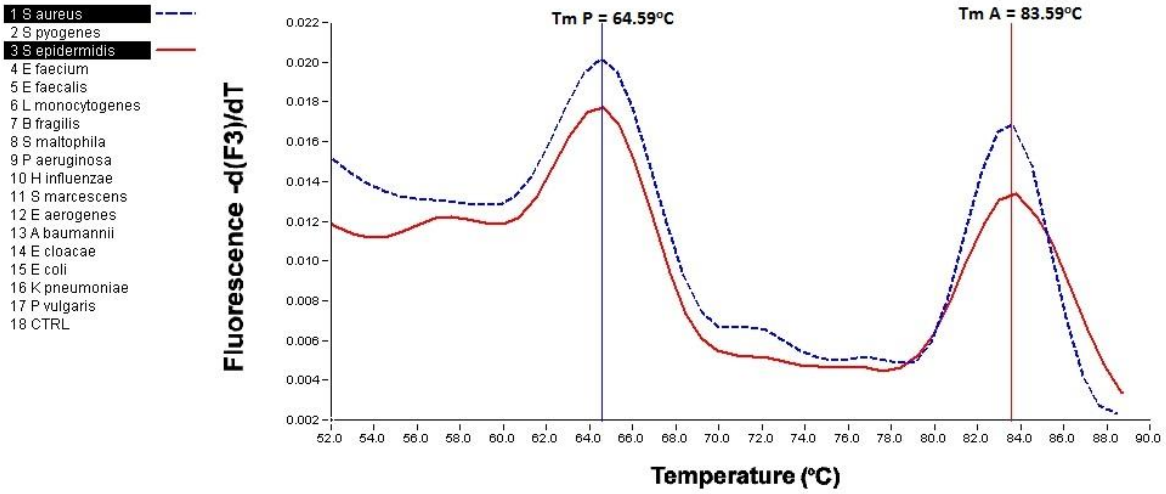

Figure 5. **Melting peaks of *Staphylococcus aureus* and *Staphylococcus epidermidis*** revealing that it is impossible to differentiate these Staphylococcus species via the T_m data of the amplicons or probes.

Among the Gram-negative bacteria, *E. coli* is one of the most common causative agents of bloodstream infections [83]. Unfortunately, it has almost the same T_m as those of *E. cloacae* and *S. marcescens*. Other bacterial strains, such as *H. influenza*, can be differentiated through the melting temperature of the probe (Figure 6) or amplicon. The sensitivity of the reaction was five colony-forming units (CFU) per reaction.

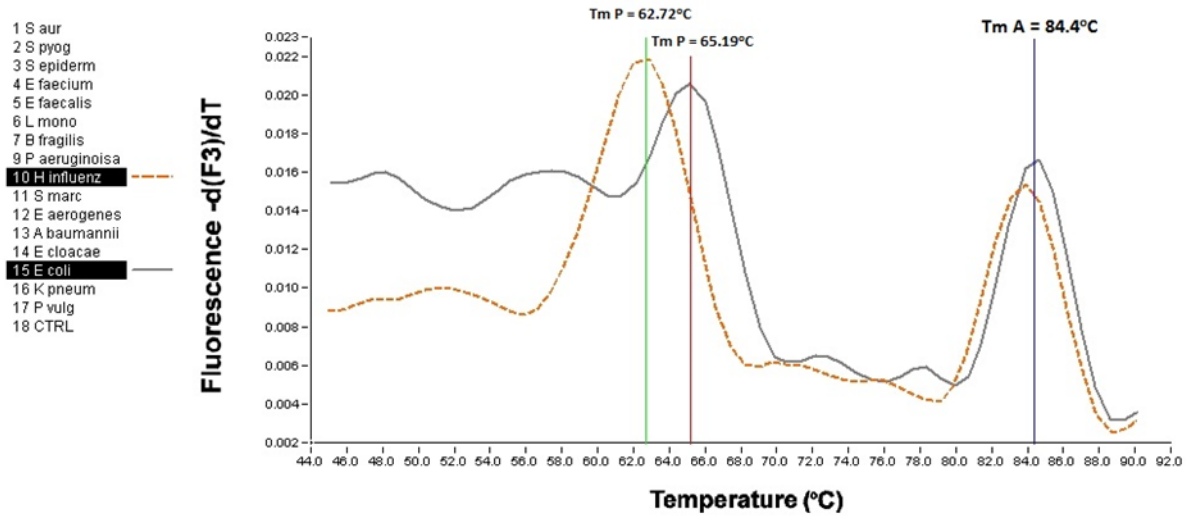

Figure 6. **Differentiation of *Escherichia coli* from *Haemophilus influenzae*.** Although these pathogens have a very similar T_m in the 16S rRNA region, the T_m of the probes are clearly different.

4.3 Determination of fungal pathogens

Based on our results seven frequently-encountered fungal pathogens could be distinguished with LightCycler instrument. The highly variable ITS 2 target sequence allowed correct identification of all of the clinically relevant fungal strains, through the T_m determination on the F1 channel [84]. 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 between 192–494 bp, bacterial amplicons 187 bp).

4.4 Determination of pathogens in 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.

4.5 Calibration of the multiplex PCR

All three non-specific dyes (LCGreen, EvaGreen and SYBR Green) excited all of the labelled probes (LCRed640, LCRed705 and Cy5.5). The most significant results were obtained by the use of SYBR Green intercalating dye.

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

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

The data obtained in this work by the use of multiplex real-time PCR were determined with the use of "Fermentas Maxima SYBR Green, no ROX" in five-eight parallel experiments. No false positive samples were found during the test phase of this method. 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 for all 21 references and 93 clinical isolates included bacterial and fungal strains as well was between 0.08 and 0.88, as listed in Table 1. These data are in concordance with our previous results [85], [86].

4.6 Sensitivity and reproducibility of the multiplex real-time PCR

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 blood containing bacterial suspensions were analyzed. Out of 8 reactions for each species, all of the reactions 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 (Table 3). All the reactions were carried out within the same parameters as described in the PCR conditions section.

Microbial strains	No. (%) of positive PCRs*				
Gram positive (G+)	50 copies	10 copies	5 copies	2 copies	1 copy
Enterococcus faecalis	8 (100)	8 (100)	5 (62.5)	2 (25)	0 (0)
Staphylococcus aureus	8 (100)	8 (100)	7 (87.5)	3 (37.5)	0 (0)
Streptococcus pyogenes	8 (100)	8 (100)	5 (62.5)	5 (62.5)	0 (0)
Gram negative (G-)					
Enterobacter aerogenes	8 (100)	8 (100)	5 (62.5)	2 (25)	0 (0)
Escherichia coli	8 (100)	8 (100)	6 (75)	1 (12.5)	0 (0)
Haemphilus influenzae	8 (100)	7 (87.5)	4 (50)	0 (0)	0 (0)
Fungi					
Candida albicans	8 (100)	8 (100)	5 (62.5)	0 (0)	0 (0)
Candida tropicalis	8 (100)	8 (100)	6 (75)	1 (12.5)	0 (0)

*Out of 8 samples.

Table 3. **Diagnostic sensitivity of the PCR.** Three Gram-positive, three Gram-negative and two fungal strains were used for the infection of healthy donor bloods. All the experiments were carried out eight times using 5 dilutions of the pathogens.

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4.7 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). The T_m data for the fungal strains are listed in Table 4. 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) [75] . 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 (data not shown). 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.

Fungal species	Melting temperature (°C)					
	HRM analysis		Somogyvari et al. [75]		Gutzmer et al. [82]	
	Mean	SD	Mean	SD	Mean	SD
Candida albicans	84.5	0.11	87.1	0.33	87.5	0.04
C. glabrata	83.2	0.09	84.9	0.69	86.3	0.23
C. guilliermondii	82.8	0.12	85.1	0.70	85.4	0.76
C. parapsilosis	82.6	0.12	85.4	0.88	84.9	0.79
C. tropicalis	81.8	0.05	84.5	0.75	84.7	0.77
C. krusei	88.6	0.12	90.8	0.02	91.5	0.34
C. lusitaniae	85.4	0.09	86.1*	0.30	ND	
C. norvegensis	86.2	0.22	86.1*	0.85	ND	
C. inconspicua	86.4	0.24	88.0	1.04	ND	
C. dubliniensis	83.5	0.12	85.5*	0.50	ND	
Cryptococcus neoformans	84.1	0.17	85.6*	0.96	ND	
Aspergillus niger	89.6	0.01	90.3*	0.95	93.9	0.10
Fusarium oxysporum	85.4	0.16	ND		88.3	0.37
Aspergillus fumigatus	90.9	0.14	91.0*	0.38	ND	
A. terreus	ND		92.0*	0.53	ND	
A. flavus	90.4	0.15	90.4*	0.06	ND	
Mucor hiemalis	80.7	0.15	ND		ND	

ND, Not determined; *data not published in the original article

Table 4. **Comparison of melting points of different fungal species.** The previous studies used the LightCycler real-time PCR instrument, SYBR Green dye and melting analysis software. The present data were obtained by means of Bio Rad CFX Polymerase chain reaction machine, EvaGreen dye and High Resolution Melting analysis (HRM). The data were compared with data presented in previous articles [75], [82].

5. Discussions

Sepsis and severe sepsis are the most common cause of death among critically ill patients in non-coronary intensive care units. Currently, real-time PCR is one of the fastest diagnostic method. Several commercially available PCR based method have been introduced, which can reduce the time necessary for the diagnosis. Most of them use PCR for the identification of the pathogen. These techniques are similar to our system in some aspects, but they are usually based on previous culturing, they detect the causative microorganisms after growth in blood culture bottles.

In the following section, I would like to highlight the advantages and disadvantages of the commercially available detection kits:

1.) SeptiTest; (Molzym, Bremen, Germany) can detect bacteria after DNA extraction, PCR amplification of target 16S rDNA, sequencing using sequencing primers. The last step is the evaluation of the data by using BLAST tool [87]. Compared to our method, this technique is not able to detect fungal pathogens.

2.) MolYsis (Molzym, Bremen, Germany) has the great advantage in the buffer system which can eliminate the human DNA background before the purification of the pathogen nucleic acid. This test is specific for *Enterobactericaceae*, *Pseudomonas aeruginosa*, *Staphylococcus Streptococcus* and *Enterococcus*. The test performs more than one PCR for the correct detection of the bacteria moreover, the fungal identification is requires a parallel PCR. This fact increases the time of the identification [88], [89].

3.) SeptiFast (Roche; Basel, Switzerland) is the most commonly used PCR kit in case of whole blood analysis. It is very similar to our system but applies three parallel reaction vessels and requires a special software for the melting analysis and automated identification [90], [91].

4.) Prove-it (Mobidiag, Helsinki, Finland) is a combination of broad range PCR with amplification of resistance genes, because it detects the gyrB and pare topoisomerase genes and the mecA gene – to differentiate methicillin-susceptible from methicillin- resistance *Staphylococcus aureus*. It is a microarray based - technology after the PCR application [33].

5.) VYOO (Analytik Jena, Jena Germany) is also combined microarray and resistance gene amplification method, because it applies a microarray technology and provides information about resistance within in 6-8 h. The great advantage of this technique is the elimination of the human DNA background in the first step [92].

The introduced prototype system uses two primer pairs and Gram specific hybridization probes for the multiplex real-time PCR. The use of rRNA genes for the detection of bacteria that can cause sepsis 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 confirm the results, it is possible to differentiate between fungi and bacteria, or between fungal species by electrophoresis [45], [93] or melting-point analysis [50]. The LightCycler PCR instrument was specially developed to amplify amplicons under 500 bp. The regions amplified by PLK 1/ PLK 2 comprised 187 bp, while the fungal amplicons amplified by ITS 86 /ITS 4 primer pair varied between 192 bp (*Geotrichum candidum*) and 494 bp (*Malassezia furfur*), values which are perfectly suited to this instrument profile.

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. According to the literature the sensitivity of PCR in sepsis to detect the pathogens is generally between 3 and 100 CFU/mL [94]. In contrast the sensitivity of our prototype system was five CFU per reaction, which in combination with an efficient preparation is suitable for the detection of pathogens in bloodstream infections. If commercially available “Midi” preparation kits (i.e.: NucleoSpin Blood L, Macherey-Nagel, Düren, Germany) were used, the sample material 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 in the blood sample. The sample/eluent ratio was equivalent in case of midi and maxi preparation kits which means that increased sample volume does not enhance the sensitivity [48]. The sensitivity of the “gold standard” conventional blood culture technique is one CFU per 10 mL blood sample [95]. We can conclude that 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 cultured bacteria and fungi. The melting points (T_m A and T_m P) were the same as described in Table 3. using "Fermentas Maxima SYBR Green, 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 [96]. 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 concerning 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.

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 [32], [97]–[99]. Molecular diagnostic systems allow species identification in less than 24 hours - which is a drastic improvement relative to the gold-standard, blood culture-based method and Gram staining-based identification methods that yield results in 24 to 72 hours [100], [101].

With the novel method described above, namely the 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 great advantage over traditional FRET-based assays by detecting the T_m of both the probes and the amplicons accurately.

As regards the broad range PCR, the same primers were used in HRM examinations because the shortest amplicons are more sensitive to the sequence alterations than the longer ones. High-resolution software is used for the detection and evaluation of mutations and polymorphisms in the short amplicons. In this context, users apply positive controls to

compare unknown PCR amplicons with chosen genotypes. This method can readily be applied to distinguish certain genetic alterations. The differentiation of the investigated fungal strains with the HRM software is more difficult. The software determines the relative ratios between the fluorescence curves, and thus all of the expected fungal species have to be used as positive controls in every PCR run. The melting peaks offer absolute values for the differentiation. The studies in the literatures indicate that in the case of yeasts, 99% of fungal septic infections are caused by eight species: *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae* and *Cryptococcus neoformans* [102], [103]. These most common fungal pathogens can be differentiated with the help of melting peak differences. Furthermore, *Aspergillus* species can also be detected with this primer pair. The T_m values of the common fungal pathogens such as *A. fumigatus*, *A. flavus* and *A. niger* are different and their discrimination from *Candida* and from each other is possible (Table 4.).

Additionally, we determined the T_m values of filamentous fungi which are non-pathogenic (*M. hiemalis*) or very rarely pathogenic (*F. oxisporum*) to humans. *M. hiemalis* can be easily distinguished, but *F. oxysporum* has same the T_m as that of *C. lusitaniae*. This illustrates that although this method is able to differentiate the most common fungal pathogens, it is not suitable as a general method of identification of fungal species.

The fungal load in fungal sepsis is generally below 10 CFU/mL [104]. As the sensitivity of this PCR is 5 CFU per reaction, in combination with a correct preparation method, it is suitable for the detection of invasive *Candida* infections.

The incidence of fungaemia has increased continuously in recent decades. The early detection of fungal pathogens has a great impact on the clinical outcome of the infection. We have improved the panfungal PCR by means of the HRM investigation, thereby achieving the distinction of 99% of fungal pathogens in sepsis. Thus, the protocol furnishes an opportunity for the rapid detection and reliable differentiation of the *Candida* and *Aspergillus* species most frequently isolated from clinical samples in bloodstream infections.

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Summary

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. Since the gold standard blood culture based technique is time consuming and in case of fungi often yield false negative results due to the low sensitivity, molecular diagnostic methods have been introduced for the detection of pathogens related to sepsis.

Polymerase chain reaction (PCR)-based techniques are widely used to identify fungal and bacterial pathogens causing serious infections. There have been numerous reports of different, new, real-time PCR-based pathogen identification methods although the clinical practicability of such techniques is not yet fully clarified.

The thesis focuses on a novel, multiplex, real-time PCR-based pathogen identification system developed for rapid differentiation of the commonly occurring bacterial and fungal causative pathogens causing bloodstream infections and a real-time PCR detection method for the fast and reliable identification of fungal pathogens.

A multiplex, real-time PCR approach was introduced for the detection and differentiation of fungi, Gram-positive and Gram-negative bacteria. The Gram classification is performed with the specific fluorescence resonance energy transfer (FRET) probes recommended for LightCycler capillary real-time PCR. The novelty of our system is the parallel amplification of bacterial and fungal pathogens in the same reaction vessel and the use of the non-specific SYBR Green dye instead of labelled anchor probes or primers, to excite the acceptor dyes on the FRET probes. In addition, the use of an intercalating dye allows the detection of fungal amplicons. For the better discrimination of the fungal pathogens we investigated a High Resolution Melting (HRM) analysis was applied that is used normally for allele discrimination.

With the novel pathogen detection system, fungi, Gram-positive and Gram-negative bacteria in the same reaction tube can be differentiated within an hour after the DNA preparation *via* the melting temperatures of the amplicons and probes in the same tube. Furthermore, broad range detection of human pathogenic fungi could be possible with HRM technique.

The modified FRET technique and HRM are specific and more rapid than the gold-standard blood culture-based methods. Taking into account that the newly developed system is able to identify Gram-negative and – positive bacteria and fungi, this technique permit rapid and early evidence-based management of bloodstream infections in clinical practice.

Összefoglalás

A műtéti osztályokon, de különösképp az intenzív terápiás részlegeken az endogén és exogén eredetű infekcióknak egyre nagyobb jelentőséget tulajdoníthatunk. Ezek a fertőzések szepszishez és szeptikus shock szindrómához vezethetnek. A diagnosztikai jellegű kutatásokban elért kielégítő eredmények ellenére sem sikerült teljesen megoldani a kórházi infekciók okozta problémákat.

A polimeráz láncreakció alapú technikák széles körűen alkalmazhatók gombás és bakteriális fertőzések kimutatásához. Ez idáig rengeteg új real-time PCR alapú patogén detektálási módszert dolgoztak ki, habár a klinikai hasznosítása ezeknek a technikáknak még nem széleskörű.

Jelen disszertáció egy új multiplex real-time PCR alapú identifikációs rendszert mutat be, mely speciálisan a vérárambeli bakteriális és gomba fertőzések gyors kimutatására alkalmazható továbbá egy még pontosabb gomba detektálásra alkalmas real-time PCR alapú technikát.

Munkánk célja egy multiplex real-time PCR alapú technika kidolgozása volt, mely a gombák, Gram-negatív és Gram-pozitív baktériumok kimutatására szolgál. A Gram specifikus azonosítás FRET technikával történt, melynek kivitelezésére a LightCyler kapilláris real-time PCR készülék bizonyult a legalkalmasabbnak. Munkánk újdonsága a gombák és a baktériumok párhuzamos detektálásának lehetősége egy csőben. A kórokozók párhuzamos detektálásának sikeressége abban rejlik, hogy az interkaláló SYBR Green festék molekulával sikerült gerjeszteni a FRET próbákat, az interkaláló festék segítségével pedig a gomba törzsek is kimutathatók. A pontosabb elkülönítés érdekében High Resolution Melting analysis (HRM) alapú technikát dolgoztunk ki, mely alapvetően a genetikában használnak allél diszkriminációs vizsgálatokra.

Az általunk kidolgozott patogén detektáló rendszerrel a gombák, Gram-pozitív és Gram-negatív baktériumok párhuzamosan kimutathatók a DNS preparálást követő egy órán belül, olvadási hőmérséklet analízis segítségével: az amplikonok és a próba olvadási hőmérsékleteinek kombinációjából. Az általunk kidolgozott rendszer továbbá széles specificitású gomba detektálásra alkalmas real-time PCR és HRM technika segítségével.

A hagyományos tenyésztéses eljárások időigényesek és gombák érzékenységét tekintve érzékenységük nem megfelelő. Jelen módosított FRET technika és HRM analízis sikeressége a gyorsaságban és a specificitásában rejlik. A gombák és baktériumok egy csőben való azonosításának lehetősége gyors kimutatási technikát eredményez a jövőben.

References

[1] P. Varga, Sepsis: az infekció, sepsis, septikus shock, többszervi elégtelenség korszerű szemlélete és ellátása. Budapest: Melania, 1999.

[2] M. M. Levy, M. P. Fink, J. C. Marshall, E. Abraham, D. Angus, D. Cook, J. Cohen, S. M. Opal, J.-L. Vincent, G. Ramsay, and SCCM/ESICM/ACCP/ATS/SIS, "2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference," Crit. Care Med., vol. 31, no. 4, pp. 1250–1256, Apr. 2003.

[3] R. C. Bone, R. A. Balk, F. B. Cerra, R. P. Dellinger, A. M. Fein, W. A. Knaus, R. M. Schein, and W. J. Sibbald, "Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine," Chest, vol. 101, no. 6, pp. 1644–1655, Jun. 1992.

[4] N. V. Beloborodova, "[Sepsis: a new look at the problem]," Ter. Arkhiv, vol. 85, no. 11, pp. 82–90, 2013.

[5] D. C. Angus, W. T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, and M. R. Pinsky, "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care," Crit. Care Med., vol. 29, no. 7, pp. 1303–1310, Jul. 2001.

[6] The Surviving Sepsis Campaign Guidelines Committee including The Pediatric Subgroup*, R. P. Dellinger, M. M. Levy, A. Rhodes, D. Annane, H. Gerlach, S. M. Opal, J. E. Sevransky, C. L. Sprung, I. S. Douglas, R. Jaeschke, T. M. Osborn, M. E. Nunnally, S. R. Townsend, K. Reinhart, R. M. Kleinpell, D. C. Angus, C. S. Deutschman, F. R. Machado, G. D. Rubenfeld, S. Webb, R. J. Beale, J.-L. Vincent, and R. Moreno, "Surviving Sepsis Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock, 2012," Intensive Care Med., vol. 39, no. 2, pp. 165–228, Feb. 2013.

[7] D. Annane, E. Bellissant, and J.-M. Cavaillon, "Septic shock," Lancet, vol. 365, no. 9453, pp. 63–78, Jan. 2005.

[8] R. Brunner, W. Rinner, C. Haberler, R. Kitzberger, T. Sycha, H. Herkner, J. Warszawska, C. Madl, and U. Holzinger, "Early treatment with IgM-enriched intravenous immunoglobulin does not mitigate critical illness polyneuropathy and/or myopathy in patients with multiple organ failure and SIRS/sepsis: a prospective, randomized, placebo-controlled, double-blinded trial," Crit. Care Lond. Engl., vol. 17, no. 5, p. R213, Oct. 2013.

[9] P. A. Danai, M. Moss, D. M. Mannino, and G. S. Martin, "The epidemiology of sepsis in patients with malignancy," Chest, vol. 129, no. 6, pp. 1432–1440, Jun. 2006.

[10] F. B. Mayr, S. Yende, W. T. Linde-Zwirble, O. M. Peck-Palmer, A. E. Barnato, L. A. Weissfeld, and D. C. Angus, "Infection rate and acute organ dysfunction risk as

explanations for racial differences in severe sepsis," JAMA J. Am. Med. Assoc., vol. 303, no. 24, pp. 2495–2503, Jun. 2010.

[11] D. D. Leedahl, H. A. Personett, O. Gajic, R. Kashyap, and G. E. Schramm, "Predictors of mortality among bacteremic patients with septic shock receiving appropriate antimicrobial therapy," BMC Anesthesiol., vol. 14, no. 1, p. 21, Mar. 2014.

[12] M. L. Mendu, S. Zager, F. K. Gibbons, and K. B. Christopher, "Relationship between neighborhood poverty rate and bloodstream infections in the critically ill," Crit. Care Med., vol. 40, no. 5, pp. 1427–1436, May 2012.

[13] G. S. Martin, D. M. Mannino, S. Eaton, and M. Moss, "The epidemiology of sepsis in the United States from 1979 through 2000," N. Engl. J. Med., vol. 348, no. 16, pp. 1546–1554, Apr. 2003.

[14] S. M. Opal, G. E. Garber, S. P. LaRosa, D. G. Maki, R. C. Freebairn, G. T. Kinasewitz, J.-F. Dhainaut, S. B. Yan, M. D. Williams, D. E. Graham, D. R. Nelson, H. Levy, and G. R. Bernard, "Systemic host responses in severe sepsis analyzed by causative microorganism and treatment effects of drotrecogin alfa (activated)," Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am., vol. 37, no. 1, pp. 50–58, Jul. 2003.

[15] S. Finfer, R. Bellomo, J. Lipman, C. French, G. Dobb, and J. Myburgh, "Adult-population incidence of severe sepsis in Australian and New Zealand intensive care units," Intensive Care Med., vol. 30, no. 4, pp. 589–596, Apr. 2004.

[16] C. Brun-Buisson, F. Doyon, and J. Carlet, "Bacteremia and severe sepsis in adults: a multicenter prospective survey in ICUs and wards of 24 hospitals. French Bacteremia-Sepsis Study Group," Am. J. Respir. Crit. Care Med., vol. 154, no. 3 Pt 1, pp. 617–624, Sep. 1996.

[17] J. Cohen, P. Cristofaro, J. Carlet, and S. Opal, "New method of classifying infections in critically ill patients," Crit. Care Med., vol. 32, no. 7, pp. 1510–1526, Jul. 2004.

[18] J.-L. Vincent, J. Rello, J. Marshall, E. Silva, A. Anzueto, C. D. Martin, R. Moreno, J. Lipman, C. Gomersall, Y. Sakr, K. Reinhart, and EPIC II Group of Investigators, "International study of the prevalence and outcomes of infection in intensive care units," JAMA J. Am. Med. Assoc., vol. 302, no. 21, pp. 2323–2329, Dec. 2009.

[19] M. S. Rangel-Frausto, "The epidemiology of bacterial sepsis," Infect. Dis. Clin. North Am., vol. 13, no. 2, pp. 299–312, vii, Jun. 1999.

[20] S. A. Klotz, B. S. Chasin, B. Powell, N. K. Gaur, and P. N. Lipke, "Polymicrobial bloodstream infections involving Candida species: analysis of patients and review of the literature," Diagn. Microbiol. Infect. Dis., vol. 59, no. 4, pp. 401–406, Dec. 2007.

[21] R. Iatta, C. Cafarchia, T. Cuna, O. Montagna, N. Laforgia, O. Gentile, A. Rizzo, T. Boekhout, D. Otranto, and M. T. Montagna, "Bloodstream infections by Malassezia and Candida species in critical care patients," Med. Mycol., Dec. 2013.

[22] J. M. Klatte, J. G. Newland, and M. A. Jackson, “Incidence, classification, and risk stratification for Candida central line-associated bloodstream infections in pediatric patients at a tertiary care children’s hospital, 2000-2010,” Infect. Control Hosp. Epidemiol. Off. J. Soc. Hosp. Epidemiol. Am., vol. 34, no. 12, pp. 1266–1271, Dec. 2013.

[23] K. Shigemura, K. Osawa, T. Jikimoto, H. Yoshida, B. Hayama, G. Ohji, K. Iwata, M. Fujisawa, and S. Arakawa, “Comparison of the clinical risk factors between Candida albicans and Candida non-albicans species for bloodstream infection,” J. Antibiot. (Tokyo), Jan. 2014.

[24] T. R. Rogers, “Antifungal drug resistance: limited data, dramatic impact?,” Int. J. Antimicrob. Agents, vol. 27 Suppl 1, pp. 7–11, Jun. 2006.

[25] M. A. Pfaller, “Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment,” Am. J. Med., vol. 125, no. 1 Suppl, pp. S3–13, Jan. 2012.

[26] J. Vallés, J. Rello, A. Ochagavía, J. Garnacho, and M. A. Alcalá, “Community-acquired bloodstream infection in critically ill adult patients: impact of shock and inappropriate antibiotic therapy on survival,” Chest, vol. 123, no. 5, pp. 1615–1624, May 2003.

[27] A. Kumar, P. Ellis, Y. Arabi, D. Roberts, B. Light, J. E. Parrillo, P. Dodek, G. Wood, A. Kumar, D. Simon, C. Peters, M. Ahsan, D. Chateau, and Cooperative Antimicrobial Therapy of Septic Shock Database Research Group, “Initiation of inappropriate antimicrobial therapy results in a fivefold reduction of survival in human septic shock,” Chest, vol. 136, no. 5, pp. 1237–1248, Nov. 2009.

[28] American Society for Microbiology, Manual of clinical microbiology, 7th ed. Washington, D.C: ASM Press, 1999.

[29] Z. Salih, J. Cavet, M. Dennis, T. Somervaille, A. Bloor, and S. Kulkarni, “Prognostic factors for mortality with fungal blood stream infections in patients with hematological and non-hematological malignancies,” South Asian J. Cancer, vol. 2, no. 4, pp. 220–224, Oct. 2013.

[30] D. A. Stevens, “Diagnosis of fungal infections: current status,” J. Antimicrob. Chemother., vol. 49 Suppl 1, pp. 11–19, Feb. 2002.

[31] J. Chander, N. Singla, S. K. Sidhu, and S. Gombar, “Epidemiology of Candida blood stream infections: experience of a tertiary care centre in North India,” J. Infect. Dev. Ctries., vol. 7, no. 9, pp. 670–675, 2013.

[32] B. E. E. Cleven, M. Palka-Santini, J. Gielen, S. Meembor, M. Krönke, and O. Krut, “Identification and characterization of bacterial pathogens causing bloodstream infections by DNA microarray,” J. Clin. Microbiol., vol. 44, no. 7, pp. 2389–2397, Jul. 2006.

[33] P. Tissari, A. Zumla, E. Tarkka, S. Mero, L. Savolainen, M. Vaara, A. Aittakorpi, S. Laakso, M. Lindfors, H. Piiparinen, M. Mäki, C. Carder, J. Huggett, and V. Gant, “Accurate and rapid identification of bacterial species from positive blood cultures with a

DNA-based microarray platform: an observational study," Lancet, vol. 375, no. 9710, pp. 224–230, Jan. 2010.

[34] I. Wiegand, K. Hilpert, and R. E. W. Hancock, "Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances," Nat. Protoc., vol. 3, no. 2, pp. 163–175, 2008.

[35] S. X. Zhang, P. Rawte, S. Brown, S. Lo, H. Siebert, S. Pong-Porter, D. E. Low, and F. B. Jamieson, "Evaluation of CLSI agar dilution method and Trek Sensititre broth microdilution panel for determining antimicrobial susceptibility of Streptococcus pneumoniae," J. Clin. Microbiol., vol. 49, no. 2, pp. 704–706, Feb. 2011.

[36] H. Frickmann, A. Hänle, A. Essig, D. Dekker, K. Boahen, S. Acquah, N. Sarpong, Y. Adu-Sarkodie, N. G. Schwarz, J. May, F. Marks, R. M. Hagen, and S. Poppert, "Fluorescence in situ hybridization (FISH) for rapid identification of Salmonella spp. from agar and blood culture broth--an option for the tropics?," Int. J. Med. Microbiol. IJMM, vol. 303, no. 5, pp. 277–284, Jul. 2013.

[37] Y. Choi, H.-Y. Wang, G. Lee, S.-D. Park, B.-Y. Jeon, Y. Uh, J. B. Kim, and H. Lee, "PCR-reverse blot hybridization assay for screening and identification of pathogens in sepsis," J. Clin. Microbiol., vol. 51, no. 5, pp. 1451–1457, May 2013.

[38] A. Borst, J. Verhoef, E. Boel, and A. C. Fluit, "Clinical evaluation of a NASBA-based assay for detection of Candida spp. in blood and blood cultures," Clin. Lab., vol. 48, no. 9–10, pp. 487–492, 2002.

[39] R. P. H. Peters, M. A. van Agtmael, S. A. Danner, P. H. M. Savelkoul, and C. M. J. E. Vandenbroucke-Grauls, "New developments in the diagnosis of bloodstream infections," Lancet Infect. Dis., vol. 4, no. 12, pp. 751–760, Dec. 2004.

[40] S. Koo, J. M. Bryar, L. R. Baden, and F. M. Marty, "Prognostic features of galactomannan antigenemia in galactomannan-positive invasive aspergillosis," J. Clin. Microbiol., vol. 48, no. 4, pp. 1255–1260, Apr. 2010.

[41] D. A. Coleman, S.-H. Oh, X. Zhao, H. Zhao, J. T. Hutchins, J. H. Vernachio, J. M. Patti, and L. L. Hoyer, "Monoclonal antibodies specific for Candida albicans Als3 that immunolabel fungal cells in vitro and in vivo and block adhesion to host surfaces," J. Microbiol. Methods, vol. 78, no. 1, pp. 71–78, Jul. 2009.

[42] Z. Erjavec, H. Kluin-Nelemans, and P. E. Verweij, "Trends in invasive fungal infections, with emphasis on invasive aspergillosis," Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis., vol. 15, no. 7, pp. 625–633, Jul. 2009.

[43] T. Lagu, M. B. Rothberg, M.-S. Shieh, P. S. Pekow, J. S. Steingrub, and P. K. Lindenauer, "Hospitalizations, costs, and outcomes of severe sepsis in the United States 2003 to 2007:," Crit. Care Med., vol. 40, no. 3, pp. 754–761, Mar. 2012.

[44] M. W. Pletz, N. Wellinghausen, and T. Welte, "Will polymerase chain reaction (PCR)-based diagnostics improve outcome in septic patients? A clinical view," Intensive Care Med., vol. 37, no. 7, pp. 1069–1076, Jul. 2011.

[45] M. von Lilienfeld-Toal, L. E. Lehmann, A. D. Raadts, C. Hahn-Ast, K. S. Orlopp, G. Marklein, I. Purr, G. Cook, A. Hoeft, A. Glasmacher, and F. Stüber, "Utility of a commercially available multiplex real-time PCR assay to detect bacterial and fungal pathogens in febrile neutropenia," J. Clin. Microbiol., vol. 47, no. 8, pp. 2405–2410, Aug. 2009.

[46] K. Yanagihara, Y. Kitagawa, M. Tomonaga, K. Tsukasaki, S. Kohno, M. Seki, H. Sugimoto, T. Shimazu, O. Tasaki, A. Matsushima, Y. Ikeda, S. Okamoto, N. Aikawa, S. Hori, H. Obara, A. Ishizaka, N. Hasegawa, J. Takeda, S. Kamihira, K. Sugahara, S. Asari, M. Murata, Y. Kobayashi, H. Ginba, Y. Sumiyama, and M. Kitajima, "Evaluation of pathogen detection from clinical samples by real-time polymerase chain reaction using a sepsis pathogen DNA detection kit," Crit. Care Lond. Engl., vol. 14, no. 4, p. R159, 2010.

[47] T. Reier-Nilsen, T. Farstad, B. Nakstad, V. Lauvrak, and M. Steinbakk, "Comparison of broad range 16S rDNA PCR and conventional blood culture for diagnosis of sepsis in the newborn: a case control study," BMC Pediatr., vol. 9, p. 5, 2009.

[48] M. Bauer and K. Reinhart, "Molecular diagnostics of sepsis--where are we today?," Int. J. Med. Microbiol. IJMM, vol. 300, no. 6, pp. 411–413, Aug. 2010.

[49] M. Avolio, P. Diamante, S. Zamparo, M. L. Modolo, S. Grosso, P. Zigante, N. Tosoni, R. De Rosa, P. Stano, and A. Camporese, "Molecular identification of bloodstream pathogens in patients presenting to the emergency department with suspected sepsis," Shock Augusta Ga, vol. 34, no. 1, pp. 27–30, Jul. 2010.

[50] A. Vince, S. Z. Lepej, B. Barsić, D. Dusek, Z. Mitrović, R. Serventi-Seiwerth, and B. Labar, "LightCycler SeptiFast assay as a tool for the rapid diagnosis of sepsis in patients during antimicrobial therapy," J. Med. Microbiol., vol. 57, no. Pt 10, pp. 1306–1307, Oct. 2008.

[51] G. Pals, K. Pindolia, and M. J. Worsham, "A rapid and sensitive approach to mutation detection using real-time polymerase chain reaction and melting curve analyses, using BRCA1 as an example," Mol. Diagn. J. Devoted Underst. Hum. Dis. Clin. Appl. Mol. Biol., vol. 4, no. 3, pp. 241–246, Sep. 1999.

[52] P. Jothikumar, V. Hill, and J. Narayanan, "Design of FRET-TaqMan probes for multiplex real-time PCR using an internal positive control," BioTechniques, vol. 46, no. 7, pp. 519–524, Jun. 2009.

[53] V. V. Didenko, "DNA probes using fluorescence resonance energy transfer (FRET): designs and applications," BioTechniques, vol. 31, no. 5, pp. 1106–1116, 1118, 1120–1121, Nov. 2001.

[54] M. J. Espy, J. R. Uhl, L. M. Sloan, S. P. Buckwalter, M. F. Jones, E. A. Vetter, J. D. C. Yao, N. L. Wengenack, J. E. Rosenblatt, F. R. Cockerill 3rd, and T. F. Smith, "Real-time PCR in clinical microbiology: applications for routine laboratory testing," Clin. Microbiol. Rev., vol. 19, no. 1, pp. 165–256, Jan. 2006.

[55] N. Paul, J. Shum, and T. Le, “Hot start PCR,” Methods Mol. Biol. Clifton NJ, vol. 630, pp. 301–318, 2010.

[56] R. Wahyuningsih, H. J. Freisleben, H. G. Sonntag, and P. Schnitzler, “Simple and rapid detection of Candida albicans DNA in serum by PCR for diagnosis of invasive candidiasis,” J. Clin. Microbiol., vol. 38, no. 8, pp. 3016–3021, Aug. 2000.

[57] A. Horváth, P. Sántha, V. Horváth, N. Török, I. Nagy, G. Jancsó, C. Vágvölgyi, and F. Somogyvári, “Rapid genotyping of genetically modified laboratory animals from whole blood samples without DNA preparation,” Acta Biol. Hung., vol. 64, no. 2, pp. 262–265, Jun. 2013.

[58] M. Paolucci, M. P. Landini, and V. Sambri, “Conventional and molecular techniques for the early diagnosis of bacteraemia,” Int. J. Antimicrob. Agents, vol. 36 Suppl 2, pp. S6–16, Dec. 2010.

[59] T. P. Chung, J. M. Laramie, D. J. Meyer, T. Downey, L. H. Y. Tam, H. Ding, T. G. Buchman, I. Karl, G. D. Stormo, R. S. Hotchkiss, and J. P. Cobb, “Molecular Diagnostics in Sepsis: From Bedside to Bench,” J. Am. Coll. Surg., vol. 203, no. 5, pp. 585–598.e1, Nov. 2006.

[60] H.-Y. Wang, S. Kim, H. Kim, J. Kim, Y. Kim, S.-D. Park, H. Jin, Y. Choi, Y. Uh, and H. Lee, “Real-time PCR TaqMan assay for rapid screening of bloodstream infection,” Ann. Clin. Microbiol. Antimicrob., vol. 13, no. 1, p. 3, 2014.

[61] Á. Horváth, Z. Pető, E. Urbán, C. Vágvölgyi, and F. Somogyvári, “A novel, multiplex, real-time PCR-based approach for the detection of the commonly occurring pathogenic fungi and bacteria,” BMC Microbiol., vol. 13, p. 300, 2013.

[62] M. Book, L. E. Lehmann, X. Zhang, and F. Stüber, “Monitoring infection: from blood culture to polymerase chain reaction (PCR),” Best Pract. Res. Clin. Anaesthesiol., vol. 27, no. 2, pp. 279–288, Jun. 2013.

[63] S. Klaschik, L. E. Lehmann, A. Raadts, M. Book, A. Hoeft, and F. Stuber, “Real-time PCR for detection and differentiation of gram-positive and gram-negative bacteria,” J. Clin. Microbiol., vol. 40, no. 11, pp. 4304–4307, Nov. 2002.

[64] K. H. Wilson, R. B. Blitchington, and R. C. Greene, “Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction,” J. Clin. Microbiol., vol. 28, no. 9, pp. 1942–1946, Sep. 1990.

[65] A. C. Crampin and R. C. Matthews, “Application of the polymerase chain reaction to the diagnosis of candidosis by amplification of an HSP 90 gene fragment,” J. Med. Microbiol., vol. 39, no. 3, pp. 233–238, Sep. 1993.

[66] M. Flahaut, D. Sanglard, M. Monod, J. Bille, and M. Rossier, “Rapid detection of Candida albicans in clinical samples by DNA amplification of common regions from C. albicans-secreted aspartic proteinase genes,” J. Clin. Microbiol., vol. 36, no. 2, pp. 395–401, Feb. 1998.

[67] J. A. Jordan, “PCR identification of four medically important Candida species by using a single primer pair,” J. Clin. Microbiol., vol. 32, no. 12, pp. 2962–2967, Dec. 1994.

[68] T. G. Buchman, M. Rossier, W. G. Merz, and P. Charache, “Detection of surgical pathogens by in vitro DNA amplification. Part I. Rapid identification of Candida albicans by in vitro amplification of a fungus-specific gene,” Surgery, vol. 108, no. 2, pp. 338–346; discussion 346–347, Aug. 1990.

[69] T. Kanbe, T. Arishima, T. Horii, and A. Kikuchi, “Improvements of PCR-based identification targeting the DNA topoisomerase II gene to determine major species of the opportunistic fungi Candida and Aspergillus fumigatus,” Microbiol. Immunol., vol. 47, no. 9, pp. 631–638, 2003.

[70] “PCR Protocols: A guide to methods and applications, edited by Michael A. Innis et al., Academic Press, 1990, 482 pp, $39.95,” Mol. Reprod. Dev., vol. 28, no. 4, pp. 419–419, Apr. 1991.

[71] C. Gil-Lamaignere, E. Roilides, J. Hacker, and F.-M. C. Müller, “Molecular typing for fungi--a critical review of the possibilities and limitations of currently and future methods,” Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis., vol. 9, no. 3, pp. 172–185, Mar. 2003.

[72] C. L. Schoch, K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen, Fungal Barcoding Consortium, and Fungal Barcoding Consortium Author List, “Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi,” Proc. Natl. Acad. Sci. U. S. A., vol. 109, no. 16, pp. 6241–6246, Apr. 2012.

[73] F. Somogyvari, A. Horvath, J. Serly, H. Majoros, C. Vagvolgyi, and Z. Peto, “Detection of invasive fungal pathogens by real-time PCR and high-resolution melting analysis,” Vivo Athens Greece, vol. 26, no. 6, pp. 979–983, Dec. 2012.

[74] C. Pasay, L. Arlian, M. Morgan, D. Vyszenski-Moher, A. Rose, D. Holt, S. Walton, and J. McCarthy, “High-resolution melt analysis for the detection of a mutation associated with permethrin resistance in a population of scabies mites,” Med. Vet. Entomol., vol. 22, no. 1, pp. 82–88, Mar. 2008.

[75] F. Somogyvari, I. Doczi, J. Serly, S. Ahmad, and E. Nagy, “Rapid discrimination between Candida albicans and Candida dubliniensis by using real-time polymerase chain reaction,” Diagn. Microbiol. Infect. Dis., vol. 58, no. 3, pp. 367–369, Jul. 2007.

[76] S. Klaschik, L. E. Lehmann, A. Raadts, M. Book, J. Gebel, A. Hoeft, and F. Stuber, “Detection and differentiation of in vitro-spiked bacteria by real-time PCR and melting-curve analysis,” J. Clin. Microbiol., vol. 42, no. 2, pp. 512–517, Feb. 2004.

[77] L. Zhou, A. N. Myers, J. G. Vandersteen, L. Wang, and C. T. Wittwer, “Closed-tube genotyping with unlabeled oligonucleotide probes and a saturating DNA dye,” Clin. Chem., vol. 50, no. 8, pp. 1328–1335, Aug. 2004.

[78] K. Lind, A. Ståhlberg, N. Zoric, and M. Kubista, “Combining sequence-specific probes and DNA binding dyes in real-time PCR for specific nucleic acid quantification and melting curve analysis,” BioTechniques, vol. 40, no. 3, pp. 315–319, Mar. 2006.

[79] A. Kilic and A. C. Basustaoglu, “Double triplex real-time PCR assay for simultaneous detection of Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hominis, and Staphylococcus haemolyticus and determination of their methicillin resistance directly from positive blood culture bottles,” Res. Microbiol., vol. 162, no. 10, pp. 1060–1066, Dec. 2011.

[80] A. F. Maheux, L. Bissonnette, M. Boissinot, J.-L. T. Bernier, V. Huppé, E. Bérubé, D. K. Boudreau, F. J. Picard, A. Huletsky, and M. G. Bergeron, “Method for rapid and sensitive detection of Enterococcus sp. and Enterococcus faecalis/faecium cells in potable water samples,” Water Res., vol. 45, no. 6, pp. 2342–2354, Mar. 2011.

[81] S.-N. Bo, J. Bo, Y.-Z. Ning, Y. Zhao, X.-L. Lu, J.-Y. Yang, X. Zhu, and G.-Q. Yao, “Relationship between time to positivity of blood culture with clinical characteristics and hospital mortality in patients with Escherichia coli bacteremia,” Chin. Med. J. (Engl.), vol. 124, no. 3, pp. 330–334, Feb. 2011.

[82] R. Gutzmer, S. Mommert, U. Küttler, T. Werfel, and A. Kapp, “Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR,” J. Med. Microbiol., vol. 53, no. Pt 12, pp. 1207–1214, Dec. 2004.

[83] C. Ferrer, F. Colom, S. Frasés, E. Mulet, J. L. Abad, and J. L. Alió, “Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections,” J. Clin. Microbiol., vol. 39, no. 8, pp. 2873–2879, Aug. 2001.

[84] Z. Khan, A. S. Mustafa, and F. F. Alam, “Real-time LightCycler polymerase chain reaction and melting temperature analysis for identification of clinically important Candida spp,” J. Microbiol. Immunol. Infect. Wei Mian Yu Gan Ran Za Zhi, vol. 42, no. 4, pp. 290–295, Aug. 2009.

[85] E. Bouza, D. Sousa, M. Rodríguez-Créixems, J. G. Lechuz, and P. Muñoz, “Is the volume of blood cultured still a significant factor in the diagnosis of bloodstream infections?,” J. Clin. Microbiol., vol. 45, no. 9, pp. 2765–2769, Sep. 2007.

[86] L. E. Lehmann, J. Alvarez, K.-P. Hunfeld, A. Goglio, G. J. Kost, R. F. Louie, A. Raglio, B. J. Regueiro, H. Wissing, and F. Stüber, “Potential clinical utility of polymerase chain reaction in microbiological testing for sepsis,” Crit. Care Med., vol. 37, no. 12, pp. 3085–3090, Dec. 2009.

[87] N. Wellinghausen, A.-J. Kochem, C. Disque, H. Muhl, S. Gebert, J. Winter, J. Matten, and S. G. Sakka, “Diagnosis of Bacteremia in Whole-Blood Samples by Use of a Commercial Universal 16S rRNA Gene-Based PCR and Sequence Analysis,” J. Clin. Microbiol., vol. 47, no. 9, pp. 2759–2765, Sep. 2009.

[88] S. Gebert, D. Siegel, and N. Wellinghausen, “Rapid detection of pathogens in blood culture bottles by real-time PCR in conjunction with the pre-analytic tool MolYsis,” J. Infect., vol. 57, no. 4, pp. 307–316, Oct. 2008.

[89] A. Benítez-Páez, M. Álvarez, P. Belda-Ferre, S. Rubido, A. Mira, and I. Tomás, "Detection of Transient Bacteraemia following Dental Extractions by 16S rDNA Pyrosequencing: A Pilot Study," PLoS ONE, vol. 8, no. 3, p. e57782, Mar. 2013.

[90] S. Sancho-Tello, D. Bravo, R. Borras, E. Costa, B. Munoz-Cobo, and D. Navarro, "Performance of the LightCycler SeptiFast Test Mgrade in Detecting Microbial Pathogens in Purulent Fluids," J. Clin. Microbiol., vol. 49, no. 8, pp. 2988–2991, Aug. 2011.

[91] P. Josefson, K. Strålin, A. Ohlin, T. Ennefors, B. Dragsten, L. Andersson, H. Fredlund, P. Mölling, and P. Olcén, "Evaluation of a commercial multiplex PCR test (SeptiFast) in the etiological diagnosis of community-onset bloodstream infections," Eur. J. Clin. Microbiol. Infect. Dis., vol. 30, no. 9, pp. 1127–1134, Sep. 2011.

[92] C. Fitting, M. Parlato, M. Adib-Conquy, N. Memain, F. Philippart, B. Misset, M. Monchi, J.-M. Cavaillon, and C. Adrie, "DNAemia Detection by Multiplex PCR and Biomarkers for Infection in Systemic Inflammatory Response Syndrome Patients," PLoS ONE, vol. 7, no. 6, p. e38916, Jun. 2012.

[93] J. R. Waldeisen, T. Wang, D. Mitra, and L. P. Lee, "A real-time PCR antibiogram for drug-resistant sepsis," PloS One, vol. 6, no. 12, p. e28528, 2011.

[94] F. Wallet, S. Nseir, L. Baumann, S. Herwegh, B. Sendid, M. Boulo, M. Roussel-Delvallez, A. V. Durocher, and R. J. Courcol, "Preliminary clinical study using a multiplex real-time PCR test for the detection of bacterial and fungal DNA directly in blood," Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis., vol. 16, no. 6, pp. 774–779, Jun. 2010.

[95] M. P. Weinstein, "Current Blood Culture Methods and Systems: Clinical Concepts, Technology, and Interpretation of Results," Clin. Infect. Dis., vol. 23, no. 1, pp. 40–46, Jul. 1996.

[96] C. Y. Turenne, S. E. Sanche, D. J. Hoban, J. A. Karlowsky, and A. M. Kabani, "Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system," J. Clin. Microbiol., vol. 37, no. 6, pp. 1846–1851, Jun. 1999.

[97] B. Lucignano, S. Ranno, O. Liesenfeld, B. Pizzorno, L. Putignani, P. Bernaschi, and D. Menichella, "Multiplex PCR allows rapid and accurate diagnosis of bloodstream infections in newborns and children with suspected sepsis," J. Clin. Microbiol., vol. 49, no. 6, pp. 2252–2258, Jun. 2011.

[98] C. S. Y. Lim, C. H. Tung, R. Rosli, and P. P. Chong, "An alternative Candida spp. cell wall disruption method using a basic sorbitol lysis buffer and glass beads," J. Microbiol. Methods, vol. 75, no. 3, pp. 576–578, Dec. 2008.

[99] S. A. Miller, D. D. Dykes, and H. F. Polesky, "A simple salting out procedure for extracting DNA from human nucleated cells," Nucleic Acids Res., vol. 16, no. 3, p. 1215, Feb. 1988.

[100] D. Liu, S. Coloe, R. Baird, and J. Pederson, “Rapid mini-preparation of fungal DNA for PCR,” J. Clin. Microbiol., vol. 38, no. 1, p. 471, Jan. 2000.

[101] T. J. Lott, R. J. Kuykendall, and E. Reiss, “Nucleotide sequence analysis of the 5.8S rDNA and adjacent ITS2 region of Candida albicans and related species,” Yeast Chichester Engl., vol. 9, no. 11, pp. 1199–1206, Nov. 1993.

[102] Y. L. Li, S. N. Leaw, J.-H. Chen, H. C. Chang, and T. C. Chang, “Rapid identification of yeasts commonly found in positive blood cultures by amplification of the internal transcribed spacer regions 1 and 2,” Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol., vol. 22, no. 11, pp. 693–696, Nov. 2003.

[103] M. A. Pfaller and D. J. Diekema, “Epidemiology of invasive candidiasis: a persistent public health problem,” Clin. Microbiol. Rev., vol. 20, no. 1, pp. 133–163, Jan. 2007.

[104] J. Loeffler, N. Henke, H. Hebart, D. Schmidt, L. Hagmeyer, U. Schumacher, and H. Einsele, “Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system,” J. Clin. Microbiol., vol. 38, no. 2, pp. 586–590, Feb. 2000.

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.

Appendix

I.

II.

III.