Thesis

Molecular detection of bacteria and fungi in blood samples: comparison of two molecular assays with conventional blood culture

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Declaration

Herewith I declare, that the thesis has been written by my own without any external unauthorized help. Where other sources of information have been used, they have been acknowledged.

Date: ....

Signature: ....

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First of all, I would like to thank my supervisor Prof. Harald H. Kessler for his encouraging words, supportive criticism, and for providing the opportunity to perform my doctoral thesis at the Research Unit Molecular Diagnostics.

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Furthermore, I would like to thank my family for supporting me all the time.

Abstract

Background: Rapid and accurate diagnostics to identify microorganisms responsible for blood stream infections are essential for accurate antimicrobial therapy. Blood culture is the current gold standard for diagnosis of microorganisms in blood. New molecular tests have been developed which may accelerate diagnosis.

Objectives: The aim of this study was to compare molecular assays, the LightCycler® Septi*Fast* Test M^{GRADE} and SepsiTest^{TM} Blood, with conventional blood culture for detection of microorganisms producing bloodstream infections.

Study design: Seventy-five specimens were collected from patients with or without sepsis and tested with the LightCycler® Septi*Fast* Test M^{GRADE}, the SepsiTest^{TM} Blood, and conventional blood culture. Results obtained were compared.

Results: All tests gave negative results for 49 of 75 (65%) specimens with 15 of 26 (57%) in the sepsis group and 34 of 49 (69%) in the non-sepsis group. Positive results were obtained for 2 of 75 (3%) specimens with all tests in the non-sepsis group. For 24 of 75 (32%) specimens, discrepant results were obtained. When all tests were compared, an overall agreement of 68% was found.

Conclusions: The molecular assays provided a more rapid detection and identification of microorganisms responsible for bloodstream infections when compared to blood culture. The LightCycler® Septi*Fast* Test M^{GRADE} requires fewer hands on time and offers better-defined identification results, whereas the SepsiTest^{TM} Blood is capable of detecting a larger number of microorganisms. However, due to the need of susceptibility testing for appropriate antimicrobial treatment, molecular tests do not have the potential to replace the current gold standard blood culture.

Content

1 Background

Sepsis is still a major challenge in medicine. Huge resources have been invested in developing and evaluating potential therapies, and remarkable effort has been undertaken to understand the systemic inflammation and multiple system organ failure characteristics of severe sepsis. (Levy et al. 2003, Russell 2006, Bodmann 2001) When initiated early, appropriate antimicrobial therapy has been shown to decrease mortality rate and to have a favorable effect on the outcome in critically ill patients. (MacArthur et al. 2004, Kollef et al. 1999, Niederman 2003)

For adequate sepsis therapy, accurate diagnostic tests are essential to identify etiologic agents correctly. Andrade et al. describe the perfect world for diagnosing sepsis as follows: “In a perfect world, there would be a kit named I/NI (infected/noninfected), in which the results of a single finger puncture, obtained from patients initially diagnosed with SIRS, would tell the physician if the patient is infected or not. By testing a second drop of blood, now using the kit BVF (bacteria, virus, or fungus), and –voila!-the clinical laboratory would also inform the physician that the infection is caused by bacteria, virus or fungi.” (Andrade et al. 2008) Despite the fact that these tests do not exist, advances in the diagnosis of infectious disease have been made in the past years (Houpikian et al. 2002).

Recently, molecular methods for direct detection of pathogens in blood in order to detect blood stream infection have been introduced (Andrade et al. 2008, Peters et al. 2004). However, the significance of these new methods for diagnosis of sepsis remains unclear.

2 Introduction

2.1 Sepsis

2.1.1 Definition

In 1897-1980, Louis Pasteur showed for the first time that bacteria were present in blood from patients with puerperal septicemia. The fact that one woman survived puerperal septicaemia, leaded Pasteur to state that “Natura medicatrix won the victory” an opinion which is consistent with the observation that sepsis is a systemic response to fight off pathogens. (Annane et al. 2005)

2.1.1.1 Bacteremia and Fungemia

First of all it is useful to differentiate the terms “bacteremia” “fungemia”, and “sepsis”. Bacteremia and fungemia are terms that identify the presence of bacteria or fungi in blood. Invasion of the blood by microorganisms is generally caused by one of two mechanisms: drainage from the primary focus of infection via the lymphatic system to the vascular system, or contaminated intravascular medical devices such as catheters or graft material. Sepsis is defined as the presence of clinical symptoms of infections in a patient with a positive blood culture. (Reimer et al. 1997)

2.1.1.2 Sepsis, SIRS, sever Sepsis

The term “sepsis”, in popular usage, implies a clinical response emerging from infection. It is evident that a similar or identical response is possible in the absences of infection. Therefore the phrase systemic inflammatory response syndrome (SIRS) is proposed to describe this inflammatory process, independent of its cause (Fig. 1.1). In 1991 the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) convened a “Consensus Conference” with the ambition to provide a conceptual and practical framework to work out definitions of SIRS and Sepsis. (Bone et al. 1992)

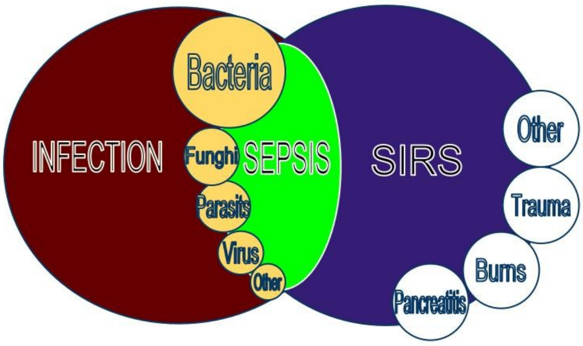


Fig. 1.1: The interrelationship between SIRS, Sepsis, and source of blood borne infection; adapted from (Bone et al. 1992)

The statement of 1992 from the ACCP/SCCM Consensus conference introduced the following criteria:

SIRS is considered to be present when patients have more than one of the following clinical findings:

- Body temperature >38°C or >36°C (hyperthermia or fever)
- Heart rate >90/min (tachycardia)
- Hyperventilation evidenced by
  - respiratory rate >20/min or
  - blood gas P_aCO_2 <32 mmHg
- white blood cell count: >12000 cells/mm^3 or <4000 cells/mm^3 or >10% band forms (leucocytosis, leucopenia, bandemia)

Sepsis is defined as suspected or proven infection plus a systemic inflammatory response syndrome. (Bone et al. 1992)
Severe sepsis is defined as sepsis with organ dysfunction recognized by hypotension, hypoxemia, oliguria, metabolic acidosis, thrombocytopenia, or obtundation. Septic shock is defined as severe sepsis with hypotension, despite adequate fluid resuscitation. Septic shock and multiorgan dysfunction are the most common causes of death in patients with sepsis. (Russell et al. 2000)

These general definitions are widely used in practice and serve as the basis for inclusion criteria of numerous clinical trials. But there are critical views claiming that as concepts, SIRS provide a useful intellectual framework for investigation, but the clinician must treat diseases, not acronyms. (Marshall 2000)
This conflict and the recently observed increase in septic patients raised the need for a review of the 1992 definitions of sepsis and related conditions.

2.1.1.3 PIRO

The 2001 International Sepsis Definitions Conference held in Washington D.C. had the final goal to identify methodologies for increasing accuracy, reliability and/or clinical utility of the diagnosis of sepsis. PIRO (Predisposition, Insult, Response, and Organ dysfunction) a hypothetical model for staging sepsis was presented. The PIRO system (details are shown in Tab. 2.1) is proposed as a template for future investigation and is a work in progress rather than a model to be adopted. (Levy et al. 2003)

Tab. 2.1: The PIRO System for Staging Sepsis.

Domain	**Present**	**Future**	**Rational**
Predisposition	Premorbid illness with reduced Probability of short term survival. Cultural or religios beliefs, age, gender	Genetic polymorphisms in components of inflammatory response (e.g.Toll-like receptor, tumor necrosis factor, interleukin 1, CD 14); Enhanced understanding of specific interactions between pathogens and host diseases	At the present, premorbid factors impact on the potential attributable morbidity and mortality of an acute insult; deleterious consequences of insult heavily dependent of genetic predisposition (future)
Insult (Infection)	Culture and sensitivity of Infecting pathogens; detection of disease amenable to source control	Assay of microbial products (lipopolysaccharide, bacterial DNA); gene transcript profile	Specific therapies directed against inciting insult require demonstration and characterization of that insult
Response	SIRS, other signs of sepsis, Shock, CRP	Nonsepcific markers of activated inflammation (e.g. procalcitonin or interleukin 6) or impaired host responsiveness (e.g. HLA-DR); specific detection of target of therapy (e.g. protein C, tumor necrosis factor, platelet-activating factor)	Both mortality risk and potential to respond to therapy vary with non-specific measures of disease severity (e.g. shock); specific mediator-targeted therapy is predicated on presence and activity of mediator
Organ Dysfunction	Organ dysfunction as number of failing organs or composite score (e.g. MODS[a], SOFA[b], LODS[c] PEMOD[d], PELOD[e])	Dynamic measures of cellular response to insult – apoptosis, cytopathic hypoxia, cell stress	Response to pre-emptive therapy (e.g. targeting micro-organism or early mediator) not possible if damage already present; Therapies targeting the injurious cellular process require that it be present

[a] MODS = multiple-organ dysfunction syndrome, [b] SOFA = sequential organ failure assessment, [c] LODS = logisitc organ dysfunction system, [d] PEMOD = pediatric multiple organ dysfunction, [e] PELOD = pediatric logistic organ dysfunction; adapted from (Levy et al. 2003)

2.1.2 Epidemiology

Although potent antibiotics and refined supportive care are increasingly available the mortality of septic patients remains high, with an estimated overall mortality rate of 30%, increasing to 50% when associated with shock (Parrillo et al. 1990).

Seven hundred fifty one thousand cases (3.0 cases per 1000 population) of sepsis or septic shock occur each year in the United States, being responsible for as many deaths as acute myocardial infarction (Angus et al. 2001a).

Annane et al describes the occurrence of peaks in the epidemiology of septic shock in the sixth decade of life (Annane et al. 2003). Factors which predispose patients to septic shock include cancer, immunodeficiency, chronic organ failure, other underlying diseases, iatrogenic factors, (Annane et al. 2003, Alberti et al. 2002) and genetic factors as being male (Angus et al. 2001b, Hubacek et al. 2001, Martin et al. 2003). Rates of pneumonia, bacteremia, and multiple-site infections as cause of sepsis have increased steadily over time, whereas abdominal infections have remained stable and genitourinary infections have decreased (Annane et al. 2003, Alberti et al. 2002, Kumar et al. 2006).

Gram-positive bacteria with (30-50%) have been reported the leading causative pathogens in patients with septic shock, followed by gram-negative bacteria (25-30%) in patients with septic shock (Tab. 2.2) (Annane et al. 2005). However, multidrug-resistant bacteria and fungi show a steady increase (Annane et al. 2005, Edmond et al. 1999, Harbarth et al. 2003). Viruses and parasites are rarely isolated (2-4%), however, frequency could be underestimated. In about 30% of cases blood cultures stay negative, mainly in patients with community-acquired sepsis who already received antibiotic treatment. (Annane et al. 2005) It is important to consider that the causal micro-organisms differ with the origin and source of infection as e.g. pneumonia, peritonitis or urinary-tract infections. The percentage of gram-positive cocci, *Candida spp*., and fungi are very similar in different categories as community-acquired and nosocomial acquired infections, whereas patients with nosocomial infections show a higher incidence of infections caused by gram-negative bacilli (48-49% versus 35%) due to a higher frequency of other pathogens in community acquired infections. (Alberti et al. 2002)

Tab. 2.2: Main pathogens in septic shock

Pathogens	Frequency
Gram-positive bacteria	**30-50%**
Meticillin-susceptible *S. aureus*	14-24%
Meticillin-resistant *S. aureus*	5-11%
Other *Stpahylococcus spp*.	1-3%
Streptococcus pneumoniae	9-12%
Other *Streptococcus spp.*	6-11%
Enterococcus spp.	3-13%
Anaerobes	1-2%
Other gram-positive bacteria	1-5%
Gram-negative bacteria	**25-30%**
Escherichia coli	9-27%
Pseudomonas aeruginosa	8-15%
Klebsiella pneumonia	2-7%
Other Enterobacteriacaea	6-16%
Haemophilus influenzae	2-10%
Anaerobes	3-7%
Other gram-negative bacteria	3-12%
Fungus	**1-3%**
Candida albicans	1-3%
Other *Candida spp.*	1-2%
Yeast	**1%**
Parasites	**1-3%**
Viruses	**2-4%**

adapted from (Annane et al. 2005)

2.1.3 Antimicrobial Therapy

Intravenous antibiotic therapy should be administered early in the course of infection especially prior to development of severe sepsis and septic shock, after appropriate cultures have been collected. (Kollef et al. 1999, Kumar et al. 2006, Harbarth et al. 2003, Dellinger et al. 2004)

Initial empirical anti-infective therapy should include one or more drugs that have activity against the suspected pathogens (bacteria or fungi) and that penetrate into the presumed source of sepsis. (Bodmann 2001, Dellinger et al. 2004, Calandra et al. 2005) In addition, the choice of drug should be guided according to the susceptibility patterns of the suspected organism. (Bodmann 2001, Dellinger et al. 2004) Clinicians should be aware of the pathogens accounting for community-acquired and nosocomial infections in their ICU as well as within the hospitals at which they practice. (Bodmann 2001, Dellinger et al. 2004) This implies that susceptibility testing should be updated on a regular basis in order to detect changes. (Kollef et al. 1999)

For patients with suspected infection who have received prior antimicrobial therapy directed at gram-negative bacteria, subsequent empiric antimicrobial treatment should include coverage of pathogens that may be potentially resistant to the earlier administered antibiotics, for example by selecting a new class of antimicrobial agents for empiric treatment of gram-negative infections or adjust routine administration of combination antimicrobial therapy with agents to which the patient has not had previous exposure and to which antimicrobial resistance is thought to be unlikely. (Kollef et al. 1999)

The antimicrobial regimen should always be reassessed after 48-72 hours on the basis of microbiological and clinical data with the aim of using a narrow-spectrum antibiotic therapy to avoid development of resistance, to reduce

toxicity, and to reduce costs. When the causative pathogen is identified monotherapy is warrantable depending on the pathogen, because there is no evidence that combination therapy is more effective. (Dellinger et al. 2004)
The main reason for the administration of inadequate antimicrobial therapy is the presence of resistant bacteria not appropriately treated by the prescribed antibiotic regime. (Kollef et al. 1999)

Needless to say, that the antimicrobial therapy is just a part of the therapy for sepsis. Consensus guidelines for the management of sepsis have recently been published. (Dellinger et al. 2004) Additionally, there is literature with special focus on early goal-directed therapy (Rivers et al. 2001), the effect of activated Protein C (Vincent et al. 2005), giving references for ventilation in critical ill patient (Brower et al. 2004), and corticoid therapy (Russell 2006).

2.1.4 Sepsis and cirrhosis

Infection is an important problem in patients with liver failure. The most common sites of infection are ascites, lungs, urinary tract, and blood. Patients with cirrhosis have a higher risk of developing sepsis, sepsis-induced organ failure, and sepsis-related death. The most frequently pathogens detected in cirrhotic patients with bacteraemia and sepsis are *Escherichia coli*, Staphylococcus *aureus*, Viridans Group Streptococci, *Enterococcus faecalis*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*. Fungal infections are involved in up to 15% of sepsis in cirrhosis. (Fernandez et al. 2002, Foreman et al. 2003, Gustot et al. 2009)

2.2 Microbiological diagnosis of sepsis

2.2.1 Blood cultures

Blood cultures should be considered when there is a reasonable expectation of sepsis. Today, blood cultures are usually performed with automated blood culture systems including the BACTEC® (BD Diagnostic Systems, Franklin Lakes, NJ, USA) and the BacT/Alert® (bioMérieux, Etoile, France) system. Both walk-away systems monitor cultures continuously using similarly composed soybean-casein digest broths.

2.1.1.1 Requirements for reliable blood culture results

Volume

Volume of blood cultured should be according the used blood culture bottle and medium. In adults with bacteremia, there are usually only a few microorganisms in the blood (<1 to 10 colony forming units per milliliter), hence it is necessary to collect an adequate volume of blood for improving microbial recovery. The volume of blood is the most important factor affecting the sensitivity of blood cultures. (Chandrasekar et al. 1994, Shafazand et al. 2002)

Ratio of blood to broth

The volume added should be up to 10 ml blood for an aerobic adult bottle (Plus Aerobic/F) to reach blood-to-broth ratio of 1:5 to 1:10. There is no need to overfill the bottles because the media have been supplemented with anticoagulants, and antimicrobial agent removals like resin materials. (Reimer et al. 1997)

Bottle headspace atmosphere

When blood culture bottles for manual usage where used it was necessary to vent bottles to room air prior to incubation. Nowadays there is no need for venting because blood culture bottles are manufactured with a carefully controlled atmosphere in the bottle headspace (the inner part of the bottle above the broth medium). Aerobic bottles generally contain ambient atmosphere to which different amounts of carbon dioxide have been added. Anaerobic bottles typically contain an atmosphere composed of carbon dioxide and nitrogen. (Reimer et al. 1997)

Number of blood cultures

For clinical suspicion of endocarditis blood culture sets, drawn 30 min to 1 hrs apart, are recommended. For all other suspected cases of bacteremia, up to three blood culture sets are recommended. Blood cultures should be taken prior to empiric antibiotic treatment. Two sets of culture samples drawn from separate venipuncture sites will help the clinician distinguish contaminants from true-positive results. (Shafazand et al. 2002) There are additionally recommendations to obtain blood for 6 bottles (for a total volume of 35-42 ml) at the same time (Lamy et al. 2002). To diagnose catheter-related infections it is suggested to obtain one pair of central-blood, and one pair of peripheral-blood simultaneously to detect the differential time to positivity (Blot et al. 1999).

Incubation of blood cultures

The bottles are incubated at 35°C with rocking agitation for a total of five to seven days. The BACTEC 9240 unit tests each bottle every 10 min. (Nolte et al. 1993) There are still discussion about five versus seven days of Incubation of blood cultures. Huang et al. suggests a period of five days of testing because the recovery of clinically important microorganisms with the system does not require seven day testing, nor does it justify its cost. (Huang et al. 1998)

Avoidance of contamination

There are different possibilities for preventing contamination of blood culture. First, skin infection should be performed carefully with the appropriate time which is required for the antiseptic to have maximal effect. Second, it should be standard praxis to avoid contamination of the sterile tops of the culture bottles before inoculating them with blood.(Hall et al. 2006, Madeo et al. 2005) Third, blood should be obtained percutan instead from vascular catheter. Finally, Phlebotomy Teams are in discussion because well trained clinicians decrease contamination rates. (Hall et al. 2006)

2.1.1.2 Indications for blood cultures

Mandell et al. recommended blood cultures for critically ill patients in intensive care units, patients with cavitary infiltrates, severe chronic liver disease, leucopenia, active alcohol abuse, asplenia, positive pneumococcal urinary antigen test or pleural effusion (Tab. 2.3). (Mandell et al. 2007)

Tab. 2.3: Clinical indications for more extensive diagnostic testing

Indication	**Blood culture**	**Sputum culture**	**Legionella UAT**	**Pneumococcal UAT**	**Other**
Intensive care unit admission	x	x	x	x	x[a]
Failure of outpatient antibiotic therapy		x	x	x	
Cavitary infiltrates	x	x			x[b]
Leukopenia	x			x	
active alcohol abuse	x	x	x	x	
Chonic severe liver disease	x			x	
Severe obstructive/structural lung disease		x			
Asplenia (anatomic or functional)	x			x	
Recent travel (within past 2 weeks)			x		x
Positive Legionella UAT result		x[d]	NA		
Positive pneumococcal UAT result	x	x		NA	
Pleural effusion	x	x	x	x	x[e]

Note: NA, Not applicable; UAT, urinary antigen test; [a] Endotracheal aspirate if intubated, possibly bronchoscopy or nonbronchoscopic bronchoalveolar lavage. [b] Fungal and tuberculosis cultures. [d] Special media for Legionella. [e] Thoracentesis and pleural fluid cultures; adapted from (Mandell et al. 2007)

2.3 Conventional methods for identification of microorganisms out of positive blood cultures

2.3.1 Gram-stain

Gram-stain, named after Hans Christian Gram who developed this method, is most commonly used for direct microscopic examination of specimens. This multistep, sequential staining protocol separates bacteria into four groups on cell morphology and cell wall structure: gram-positive cocci, gram-negative cocci, gram-positive rods, and gram-negative rods. Furthermore it is possible to identify nonbacterial forms like fungi or trichomonads. (Koneman et al. 1997)
When a blood culture is identified as positive by the BACTEC 9240 unit the bottle is immediately analyzed by gram staining and a subculture is done (Fig. 2.2). (Nolte et al. 1993, Hautala et al. 2005) The finding of the gram-stain is directly reported to the clinicians. Hautala et al reports that gram-stain result of positive blood cultures along with the knowledge of where the infection was acquired, allows early accurate targeting of antimicrobial therapy for blood stream infections. (Hautala et al. 2005) Additionally, the gram-stain result is useful for the choice of further test methods like different biochemical tests.

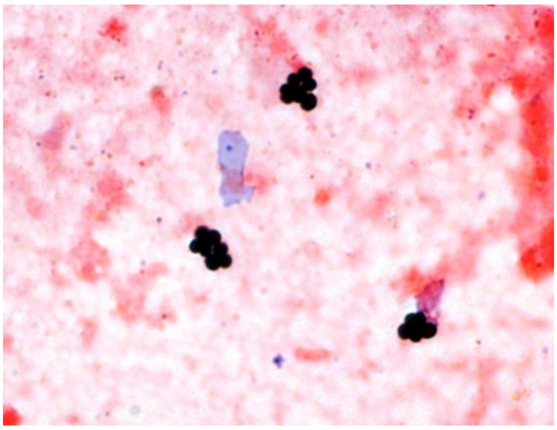


Fig 2.2: Direct gram-stain of *Staphylococcus aureus*.

2.3.2 Biochemical tests for Identification

There are several biochemical tests, different selective agar plates and test systems available like API or Vitek2 (bioMérieux) for Identification of microorganisms. Metabolic characteristics of microorganisms like fermentation of sugar are used for identification shown in Fig. 2.3. (Koneman et al. 1997, Ligozzi et al. 2002, Smith et al. 1972, Ling et al. 2001)

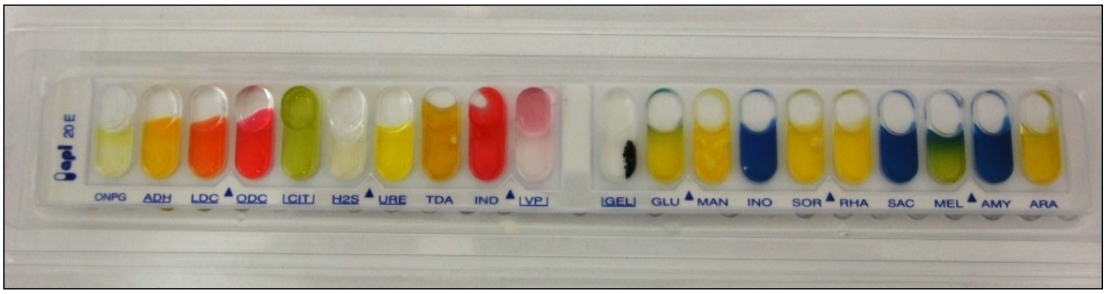


Fig. 2.3: API-20E of E. coli

2.3.3 PNA FISH

Peptide nucleic acid (PNA) molecules are build-on like Deoxyribonucleic acid (DNA). They just differentiate in their backbone. In PNA, the sugar phosphate backbone of DNA is replaced by a polyamide or peptide backbone. Due to the uncharged backbone of PNA, these probes have superior hybridization characteristics such as higher specificity and better hybridization kinetics compared to traditional DNA probes. Bacteria and yeast cells produce a large quantity of ribosomal ribonucleic acids (rRNAs) that contain species specific, highly conserved regions. FISH using PNA probes targeting rRNAs combines the unique performance characteristics of PNA probes with the advantage of using rRNA as a target (Fig. 2.4). (Perry-O'Keefe et al. 2001, Stender 2003)

This method has been recently applied for culture identification of *Stapyhlococcus aureus*, *Candida albians*, and *Enterococcus faecalis* directly from smears of positive blood culture bottles (Fig. 2.5). The first PNA FISH kits for rapid diagnosis of bloodstream infections have been developed and are today commercially available from AdvanDx (USA). (Stender 2003)

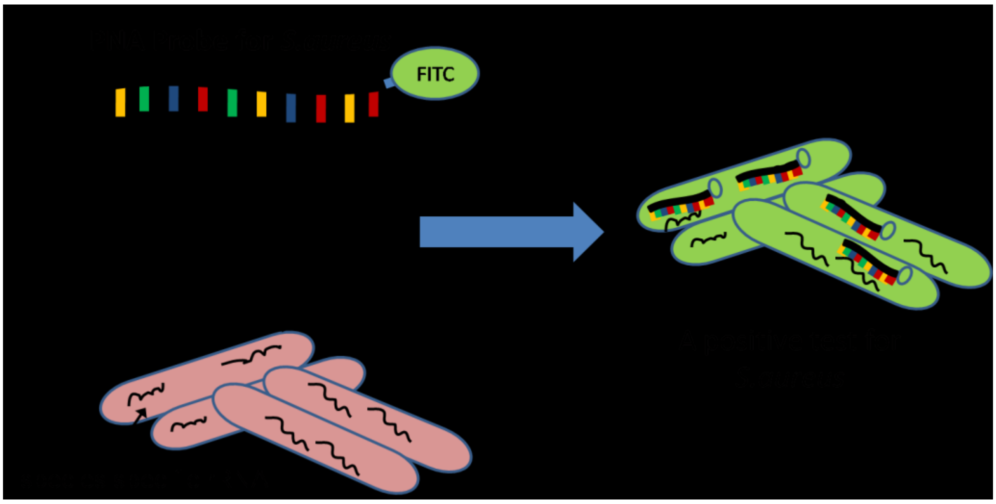


Fig. 2.4: Illustration how a PNA-FISH probe hybridization works.

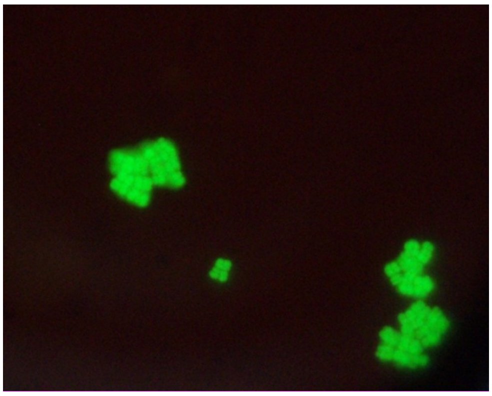


Fig. 2.5: PNA FISH of *S. aureus*

2.3.4 GenoType blood culture

GenoType blood culture (HAIN Lifescience, Nehren, Germany) is a Polymerase chain reaction (PCR) based method combined with hybridization of blotted DNA probes. It is a commercial available system, a DNA strip assay, designed for detection of the most relevant bacterial sepsis pathogens from positive blood cultures. The GenoType assay consist of two different panels, one designed for the identification of gram-positive cocci and one designed to identify gram-negative rods, hence a gram stain result is required (Fig. 2.6). Due to the interest in susceptibility patterns the detection of the *mecA* gene as well as the *van* genes are included for the identification of meticillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE), respectively. (Eigner et al. 2005)

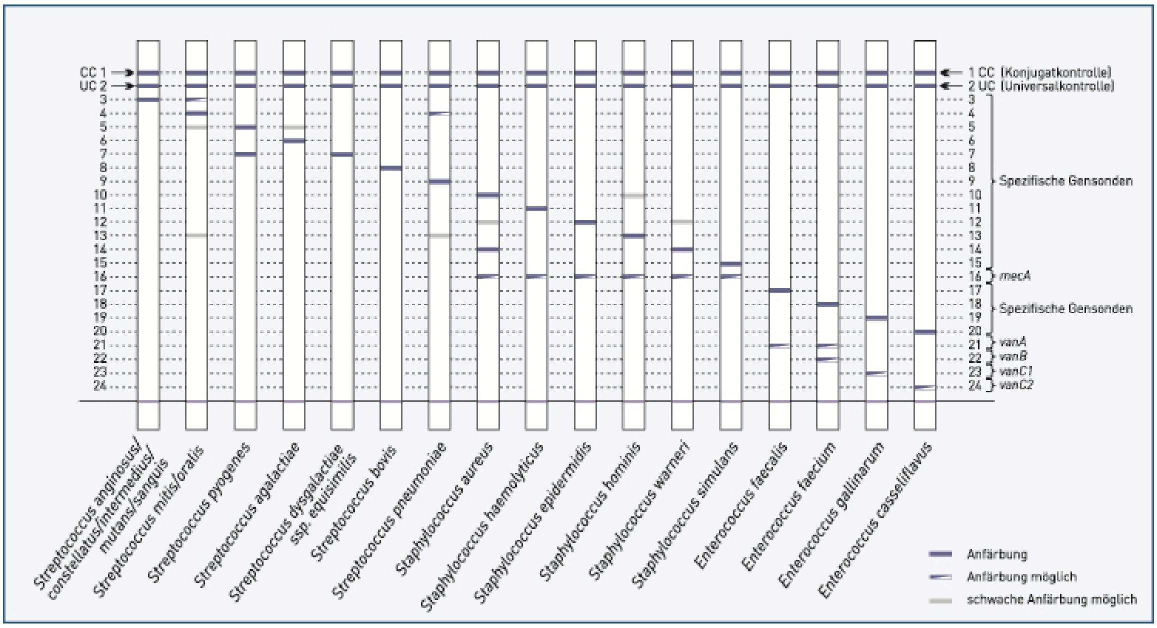
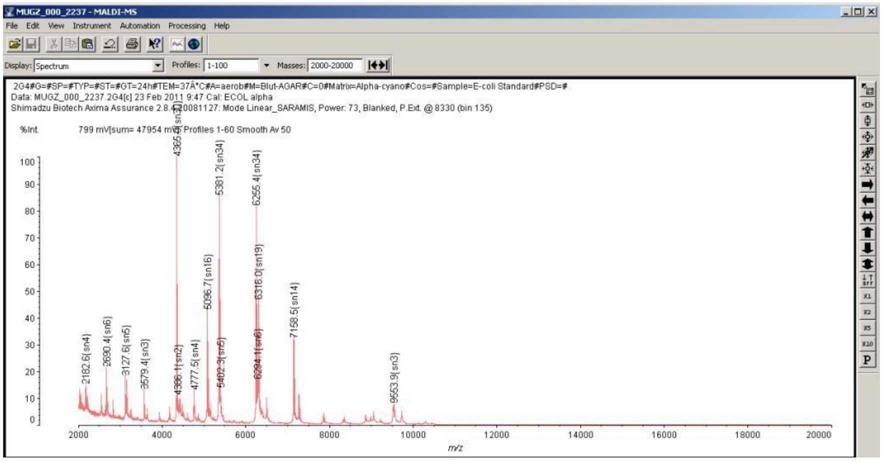


Fig. 2.6: GenoType panel for gram-positive cocci © HAIN Lifescience

2.3.5 MALDI-TOF MS

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI TOF MS) was recently introduced for identification of microorganisms. For this purpose microorganisms are mixed with a matrix, which result in a crystallization of the sample within the matrix. This mixture is loaded into the mass spectrometer, where it is bombarded with a UV laser. The matrix absorbs energy from the laser and the sample becomes vaporized, releasing ions of various sizes. These ions with different size results in different time of flight through the flight tube and strike the detector at different times. The time of flight required to strike the detector is used to calculate the masses of the ion and to create a mass spectrum (Fig. 2.7). The mass spectrum is then matched with a database to get the identification result. (Giebel et al. 2010, Seng et al. 2009)

This enormous fast identification tool can be used for identification direct from the fresh agar subculture. Furthermore, there are protocols available for identification direct from positive blood culture fluid by stepwise centrifugation to remove blood cells and extraction of cell peptides with formic acid. This approach may replace routine biochemical identification procedures and will deliver reliable results in a short time. (Christner et al. 2010, La Scola et al. 2009).



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Data: MUGZ_000_2237.2G4[c] 23 Feb 2011 9:47 Cal: ECOL alpha
Shimadzu Biotech Axima Assurance 2.8.4.20081127: Mode Linear_SARAMIS, Power: 73, Blanked, P.Ext. @ 8330 (bin 135)
799 mV[sum= 47954 mV] Profiles 1-60 Smooth Av 50
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Fig. 2.7: Mass spectrum of *E. coli* obtained from the Shimadzu instrument

2.4 New developments: Direct Diagnosis of Microorganisms from whole blood for diagnosis of bloodstream infections

Results of conventional cultures may take 6 to 48 hours and negative blood culture results may be the consequence of the previous use of antimicrobial agents which can have an inhibitory effect on bacterial growth. Recently, a step forward was done with introducing molecular test systems for direct use for diagnosis of bloodstream infections out of whole blood. The most commonly used systems at present are Real-time PCR formats and PCR plus Sequencing analysis. (Andrade et al. 2008, Peters et al. 2004)

2.4.1 Brief description of commercially available systems used for diagnosis of Sepsis

2.4.1.1 LightCycler® SeptiFast Test MGRADE

The LightCycler® Septi*Fast* Test M^{GRADE} (Roche Applied Systems, Penzberg, Germany) is a combination of mechanical lyses of cells and purification of DNA with a multiplex real-time PCR assay for differentiation of 25 bacterial and fungal pathogens from whole blood. (Lehmann et al. 2008)

2.4.1.2 SepsiTestTM Blood

The SepsiTest^{TM} Blood test (Molzym, Bremen, Germany) is a combination of enzymatic lyses of cells and purification of DNA with a universal 16S rRNA gene amplification and subsequent sequencing analysis of the amplicon. (Wellinghausen et al. 2009) Furthermore 18S rRNA gene amplification and subsequent sequencing for differentiation of fungi is included. (Molzym)

2.4.1.3 YVOO – Sepsis pathogen identification test (SirsLab, Germany)

YVOO- Sespis pathogen identification test (SirsLab, Jena, Germany) is a combination of separation of specific binding prokaryotic DNA from initially extracted whole DNA from blood with multiplex PCR detected on Gel-Electrophoresis. (Horz et al. 2008, Sirs-Lab)

2.4.2 Future prospects

2.4.2.1 PLEX ID

This new strategy, PLEX ID (Abbott, Illinois, USA) for the molecular detection of bloodstream infections combines broad-range PCR amplification with mass spectrometry. First multiple pairs of primers are designed to amplify carefully selected regions of bacterial of fungal genomes. Second, following PCR amplification, a fully automated electrospray ionization/mass spectrometry (PCR/ESI-MS) is performed. The mass spectrometer effectively weigh the PCR amplification products, or the mixture of amplicons, which sufficient mass accuracy that the composition of A, G, C and T can be deduced for each amplicon present. The base compositions are compared with a database of calculated base compositions derived from the sequences of known organism and to signature from reference standards previously determined via PCR/ESI-MS. (Ecker et al. 2010, PLEX ID, Abbott, A Promise For Live)

2.4.2.2 Microarray technologies

This might be e further advance in molecular blood stream diagnostics because with this approach not only the identification of different microorganism is possible but it also permits the detection of their antimicrobial resistance and / or virulence genes. (Andrade et al. 2008) The basic concept is that DNA probes specific to selected targets are spotted on a glass or silicon slide in a known order. Target DNA fragments are labeled with a reporter molecule and hybridized to the array, and duplexes formed with a specific probe are detected in advanced platforms by measuring the fluorescent signals. (Miller et al. 2009)

2.5 Background of Molecular techniques used for this study

2.5.1 DNA extraction

There are different options for lysis of cells. The LightCycler® Septi*Fast* DNA extraction protocol is based on mechanical lysis with the Septi*Fast* Lys Kit M^{GRADE} followed by the purification of DNA with the Septi*Fast* Prep Kit M^{GRADE}.
The Septi*Fast* Lys Kit M^{GRADE} contains vials with ceramic beads for mechanical lysis of both blood cells and potential pathogens in the MagNA Lyser® Instrument. Furthermore by addition of lysis buffer, pathogens are fully broken up and DNAs are released while simultaneously stabilized. (Lehmann et al. 2008)
The SepsiTest™ Blood kit uses enzymes for lysis of cells. In a first step, blood cells are lysed selectively in presence of chaotropic buffer. Human DNA is degraded with MolDNase. Then, pathogens are lysed using BugLysis and Lyticase in presence of chaotropic buffer.
Finally, the pathogen’s DNA is isolated by a quick bind-wash-elute procedure. This procedure starts with binding buffer added, and total nucleic acids bind to the silica surface in presence of chaotropic salt. The mixture is transferred to a spin column with glass fiber insert. Both, human genomic DNA and pathogen DNA in the sample are adsorbed to the surface of the glass fiber. During washing steps, unbound substances (salts, proteins, cellular fragments) are removed. Adsorbed nucleic acid is then eluted from the column using heated elution buffer.

2.5.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) allows amplification of a small amount of nucleic acids. The fragment to be amplified is determined by single stranded oligonucleotides also referred to as primers. Primers are usually 20 to 30 base pairs long and complementary to the beginning or the end of the DNA fragment amplified. They anneal by binding to the DNA template at these starting and ending points, where the DNA polymerase begins the synthesis of the new DNA strand. The *Taq* polymerase used is a thermo stable enzyme that withstands heating up to 96°C, the temperature used to denature double stranded DNA.

The PCR process consists of cycles with three steps (Fig. 2.8):

I. Denaturation of the double stranded DNA by heating up to 96°C in order to separate the strands.
II. Annealing of primers at lower temperature to allow hybridization to the single DNA strands.
III. Elongation by the DNA polymerase to copy the DNA strands. The polymerase starts at the annealed primers and works along the DNA strand.

This cycle is repeated several times until a sufficient amount of amplification products is generated.

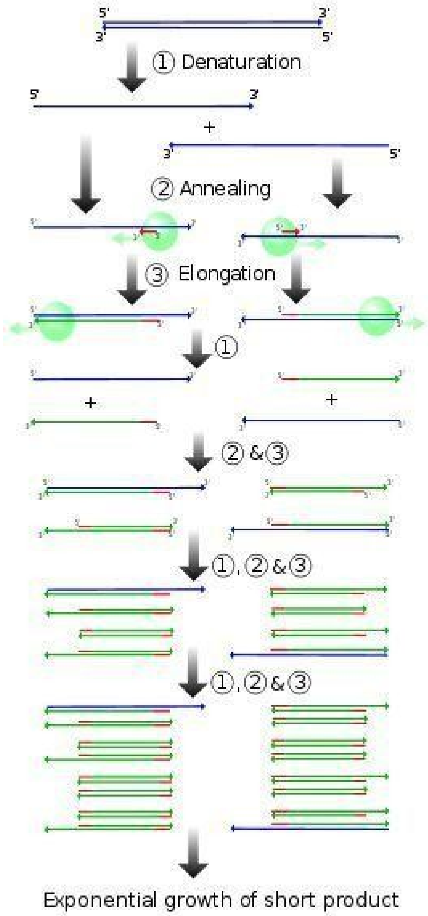


Fig. 2.8: Schematic drawing of the PCR cycle. (1) Denaturing at 94–96 °C. (2) Annealing at ~65 °C (3) Elongation at 72 °C. Four cycles are shown here. The blue lines represent the DNA template to which primers (red arrows) anneal that are extended by the DNA polymerase (light green circles), to give shorter DNA products (green lines), which themselves are used as templates as PCR progresses.

2.5.3 Real-time PCR

The Real-time PCR uses fluorescent dyes to detect PCR amplification. Fluorescent dyes allow continuous monitoring as template DNA is amplified which is the reason for the term real-time PCR. Real-time PCR instruments simultaneously amplify and detect, eliminating the need to open tubes containing PCR amplification products and therefore reducing the risk of contamination. One platform for real-time PCR is the LightCycler® Instrument which combines thermal cycler and fluorimeter. (Lyon et al. 2009)

2.5.4 Hybridization probes

Hybridization probes are included in the LightCycler® Septi*Fast* test. Each hybridization probe has a single dye attached on two adjacent probes. The energy transfer donor is attached to one probe, and a long-wavelength acceptor is placed on the other probe. During PCR, the probes hybridize next to each other at the annealing segment of each cycle (Fig. 2.9). The donor transfers absorbed energy to the acceptor and the acceptor emits a different wavelength. The increased emission of the acceptor is monitored to follow the appearance of target. (Wittwer et al. 2001) Identification of pathogens or resistance genes is provided by melting analysis performed after completion of real-time PCR. (Lyon et al. 2009) The LightCycler® Septi*Fast* test utilizes hybridization probes with different melting temperatures (T_m) labeled with four different dyes allowing the identification of 25 different pathogens simultaneously. (Lehmann et al. 2008)

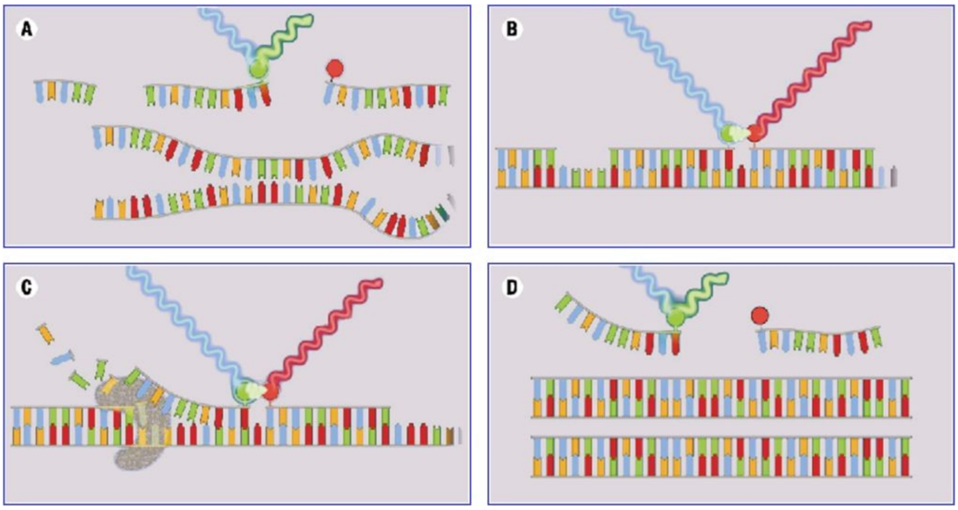


Fig. 2.9: Real-time PCR with hybridisation probes. A: Dentaturation step, B: Annealing step (fluorescence measurement), C: Elongation step, D: End of Elongation step, © Roche

2.5.5 Color Compensation

A multiplex real-time PCR assay with differently labeled dyes (e.g. Fluorecein, LightCycler RED 610, LightCycler RED 640, LightCycler RED 670, LightCycler RED 705) needs color compensation to correct the spectral overlap of the fluorescence channels in multi-color applications. (Wittwer et al. 2001)

2.5.6 SYBR Green I

SYBR Green I is included in the SepsiTest^{TM} Blood test. It is a DNA intercalating dye, which allows template analysis during amplification in a real time PCR assay (Fig. 2.11). This dye detects all dsDNA, including primer dimers and other unwanted products. The specificity of detection is dependent on the specificity of amplification with no verification of product identity. Because the melting curve of a product is dependent on GC content, length, and sequence, PCR products can be distinguished through melting curve analysis. (Ririe et al. 1997)

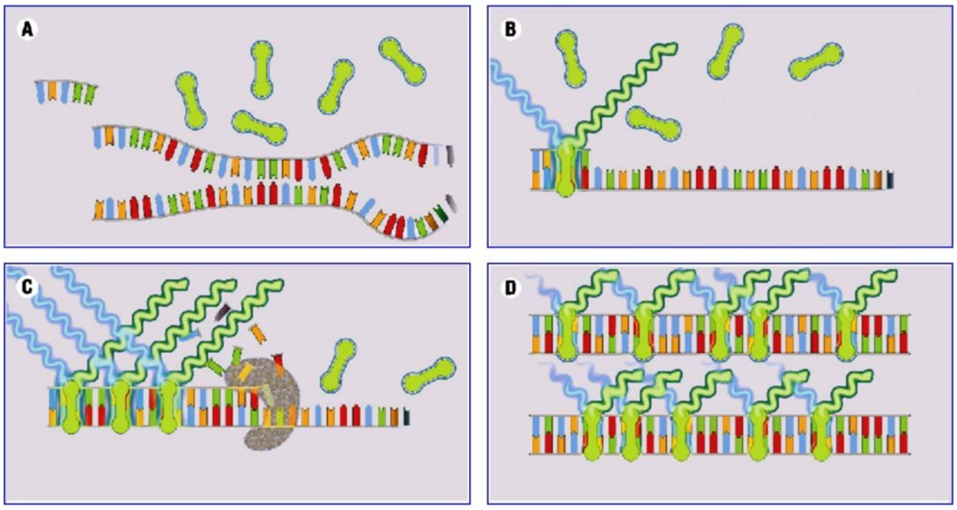


Fig. 2.11: Real-time PCR with SYBR Green I, A: Denaturation step, B: Annealing step, C: Elongation step, D: end of Elongation step (fluorescence measurement), © Roche

2.5.7 Sequencing

DNA sequencing has become a powerful technique in molecular diagnostics affording analysis of genes at the nucleotide level. Sanger´s method, which is also referred to as dideoxy sequencing or chain termination, is based on the use of di-deoxynucleotides (ddNTPs) in addition to the normal nucleotides (dNTPs) found in DNA. Di-deoxynucleotides are essentially identical to nucleotides except of containing a hydrogen group (H) on the 3´carbon instead of a hydroxyl group (OH). These modified nucleotides, when integrated into a sequence, prohibit the addition of further nucleotides: The phosphodiester bond cannot form between the di-deoxynucleotide and the next incoming nucleotide, and thus the DNA chain terminates. For the sequencing reaction, a short oligonucleotide sequence complementary to the template at that region is used. The primer is extended using DNA polymerase for replication of the DNA, dNTPs, and a low concentration of ddNTPs with each base labeled using a special fluorescent dye. Limited incorporation of the chain terminating ddNTPs by DNA polymerase results in a series of related DNA fragments which can be separated in a gel or capillary to obtain a sequence. (Nelson et al. 2001)

2.5.8 PCR targets

For the LightCycler® Septi*Fast* Test M^{GRADE}, the internal transcribed spacer (ITS) region was selected as the target region for bacterial and fungal species differentiation. It is located between the 16S and 23S ribosomal DNA (rDNA) sequences of all gram-positive and gram-negative bacteria and between the 18S and 5.8S rDNA sequences of all fungi.
For the SepsiTest^{TM} Blood, the 16S rDNA for bacteria and the 18S rDNA for fungi were selected as target region.

2.6 Aims of the study

The aim of this study was to compare new molecular methods, the LightCycler® Septi*Fast* and SepsiTest^{TM} Blood kits with conventional culture for detection of pathogens producing bloodstream infections. Both performance of assays and results obtained from patients with and without sepsis were studied.

3 Materials and Methods

3.1 Study design

Results obtained with the LightCycler® Septi*Fast* and SepsiTest^{TM} Blood tests were compared with conventional blood culture. The flowchart is shown in Fig.3.1.

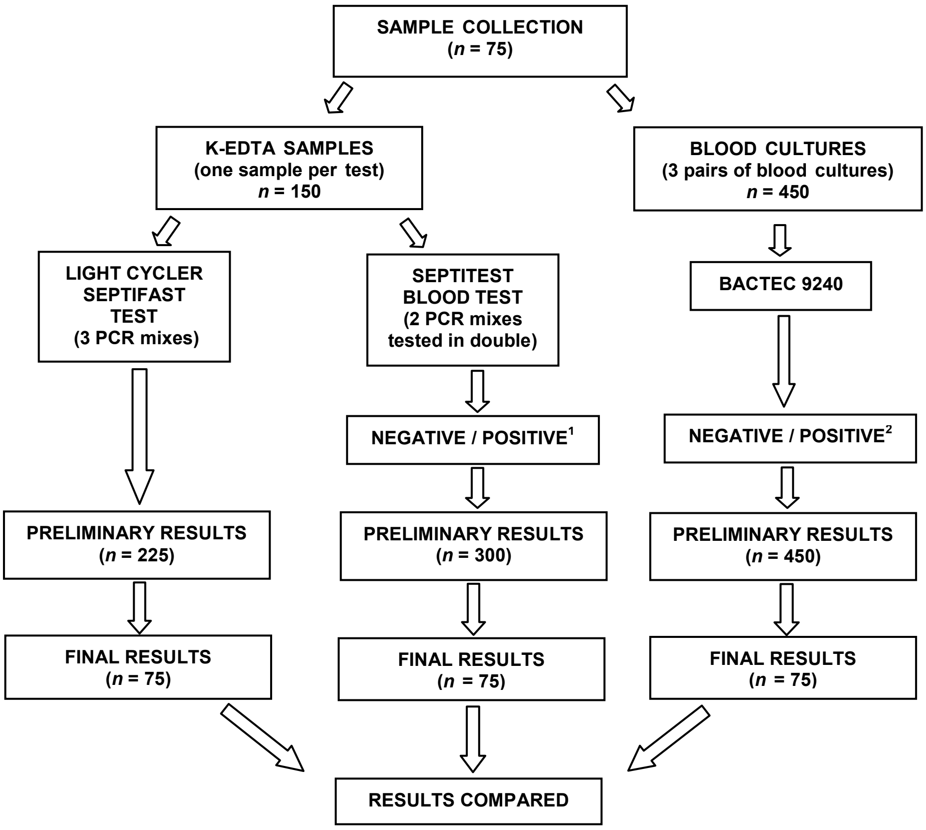


Fig. 3.1: Flowchart of the study.
[1]Positives were sequenced additionally.
[2]Positives were conventionally identified.

3.2 Materials

3.2.1 Patients

Seventy-five samples were collected from 57 patients and tested in parallel with the LightCycler® Septi*Fast* and the SepsiTest™ Blood tests and with conventional blood culture. Patients were classified into two groups: Patients with sepsis (*n*=14) and patients without sepsis (non-sepsis) are suffering from different stages of liver disease (*n*=43). The age range of sepsis patients was 39 to 82 years (male:female, 11:3) and that of non-sepsis patients 27 to 47 years (male:female, 31:12). Twelve patients in the sepsis group and 14 patients in the non-sepsis group received antimicrobial treatment at the time of sample collection. Patients were investigated at baseline and once a week thereafter until discharge or death. Inclusion/exclusion criteria are shown in Tab. 3.1. The study was approved by the ethical committee of the Medical University of Graz, Austria. Informed consent was obtained from each participant.

Tab. 3.1: Inclusion/exclusion criteria of the study.

Inclusion criteria	**Exclusion criteria**
Sepsis: Consecutive patients with sepsis without evidence for cirrhosis or sepsis-associated liver dysfunction admitted to the ICU	Sepsis: underlying hematologic disease
Non-sepsis: Consecutive hospitalized patients with liver cirrhosis or acute-on-chronic liver failure (ACLF)	Pregnancy
Age ≥18 years	Age <18 years

3.2.2 Sample Collection

For blood cultures, blood was collected in three pairs of aerobic/anaerobic blood culture bottles (BD Diagnostic Systems) according to current guidelines. Blood was taken by peripheral venous puncture or a freshly placed indwelling peripheral, central venous or arterial catheter. Immediately after blood sampling for cultures, 2 x 6 ml blood was collected in K-EDTA blood tubes (Greiner Bio-One, Kremsmünster, Austria) from the identical source.

3.2.3 Transport and storage of samples

After collection, blood cultures were immediately placed into the BACTEC 9240 instrument. The K-EDTA blood samples were immediately transferred to the diagnostic laboratory. The sample for the LightCycler® Septi*Fast* Test M^{GRADE} was kept frozen at ms20°C until a minimum of 4 samples were available. The sample for the SepsiTest^{TM} Blood was prepared immediately until step 7 of the extraction protocol and kept frozen until a minimum of 6 samples were available.

3.3 Instruments

3.3.1 BACTEC^{TM} 9240

The BD BACTEC^{TM} 9240 (BD Diagnostic Systems, Franklin Lakes, NJ, USA) is a walk-away system monitoring cultures continuously (Fig. 3.2). It is used with the blood culture bottles Plus Aerobic/F and Plus Anaerobic/F with soybean-casein digest broths (Fig. 3.3). The principle of the system is that metabolizing microorganisms in the culture medium release carbon dioxide into the medium which interacts with a dye. The effect is a measurable fluorescence level which corresponds to the released CO_2 from the organism. Above a certain cutoff, the instrument reports the bottle as positive.

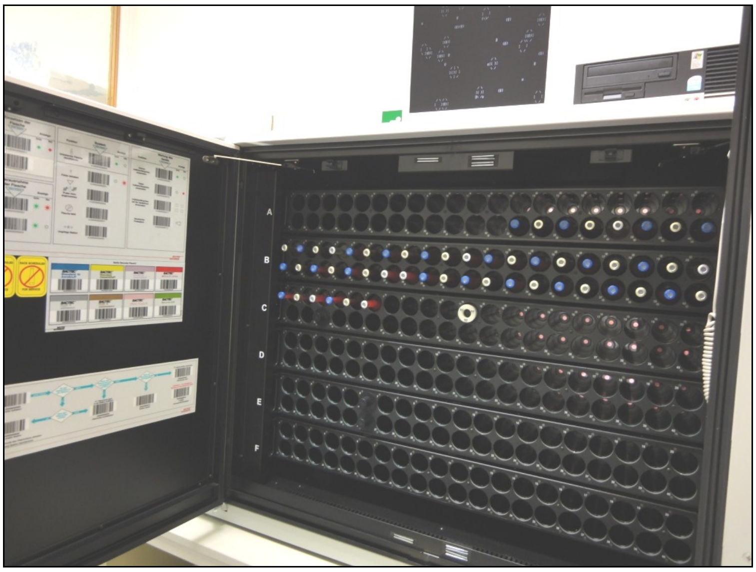


Fig. 3.2: BACTEC 9240: An insight view of the instrument

Fig. 3.3: BACTEC bottles:
Plus Aerobic/F and Plus Anaerobic/F

3.3.2 MagNA Lyser®

The MagNA Lyser® instrument (Roche) is a bench top device for mechanical disruption of cells or other biological materials (Fig. 3.4). It is used together with vials containing ceramic beads.

Fig. 3.4: The MagNA Lyser® instrument.

3.3.3 LightCycler® 2.0

The LightCycler® 2.0 (Roche) is a real-time PCR instrument (Fig. 3.5). For this study, the version which conforms to the European Directive for *in vitro* Diagnostic Medical Devices (98/79/EC) was employed. The LightCycler® consists of an upper and a lower unit. The upper unit contains the heating soil and the lower the thermal chamber, fluorimeter, drive units, electronic boards, and power supply.

Real-time PCR is performed in specially designed glass capillaries with an optimal surface-to-volume ratio to ensure rapid equilibration between the air and the reaction components. To avoid contamination, the capillary is tightly closed during the run. This instrument was used for both assays.

For the LightCycler® Septi*Fast* Test M^{GRADE}, the Septi*Fast* Identification Software Set v2.0 is required to run the test.

Fig.3.5: LightCycler® 2.0 instrument.

3.3.4 Applied Biosystems Sequencer 3130

The Applied Biosystems 3130 Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA, USA) is a four capillary automated sequencer (Fig. 3.6). In this study, it was used for sequencing the amplification product obtained with the SepsiTest™ Blood for identification of either bacteria or fungi.

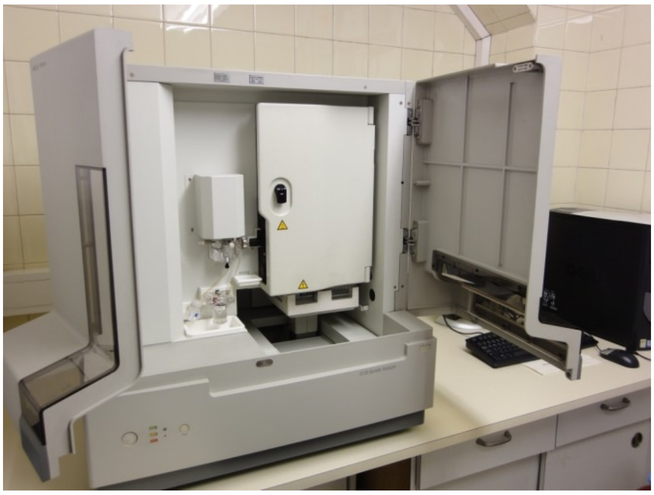


Fig. 3.6: Applied Biosystems 3130 Genetic Analyzer

3.4 Methods

3.4.1 Blood Culture detection

3.4.1.1 BACTEC^{TM} 9240

For conventional blood culture analysis, the BACTEC^{TM} 9240 was used together with the appropriate bottles Plus Aerobic/F and Plus Anaerobic/F for detection of aerobic and anaerobic bacteria and fungi. The Plus Aerobic/F and Plus Anaerobic/F were placed into the instrument immediately after blood collection and incubated at 37°C for 7 days. If a bottle was reported positive by the BACTEC^{TM} 9240, it was removed from the instrument in order to confirm the result through conventional isolation and identification of the microorganism(s).

3.4.1.2 Tests for identification of bacteria and fungi out of blood culture bottles

For identification of (the) microorganism(s) out of a blood culture bottle, a gram stain was carried out. In parallel, an aliquot of the positive blood culture was distributed on the appropriate conventional culture plates (bioMérieux, Etoile, France) for the growth of aerobic and anaerobic bacteria and fungi. If *Streptococcus pneumonia* was suspected after gram staining, the BinaxNOW *Streptococcus pneumonia* Test (inverness medical, Princeton, NJ, USA) was employed additionally. After growth, the result was confirmed with the Slidex® pneumo-Kit (bioMérieux, Etoile, France). For Staphylococci, a STAPHYLASE TEST KIT (Oxoid, Cambridge, UK) was used to distinguish between *Staphylococcus aureus* and coagulase negative staphylococci. For all other microorganisms, the biochemical species identification was done with API® strips manufactured by bioMérieux.

3.4.1.3 Previous blood culture results

To verify positive test results of molecular tests, reports of previously taken blood cultures were screened for positive results.

3.4.2 Molecular assays and performance

While all kits manufactured by Roche and used in this study have been IVD/CE-labeled, those manufactured by Molzym received this label after the completion of this study.

3.4.2.1 Septi*Fast* Lys Kit M^{GRADE}

The Septi*Fast* Lys Kit M^{GRADE} (Roche) consists of vials containing ceramic beads for mechanical lysis of both blood cells and pathogens in the MagNA Lyser® instrument (Fig. 3.7).

Fig. 3.7: Vial containing ceramic beads.

For the Septi*Fast* Lys Kit M^{GRADE}, 1500 µl EDTA whole blood was transferred into the vial containing ceramic beads for lysis (70 seconds at 7000 rpm) in the MagNALyser® instrument. The lysed sample was stored for 10 min at room temperature to allow settling of ceramic beads and cell debris. For each run, one of the negative controls included in the LightCycler® Septi*Fast* Test M^{GRADE} test package was treated in the same way.

3.4.2.2 Septi*Fast* Prep Kit M^{GRADE}

The Septi*Fast* Prep Kit M^{GRADE} (Roche) includes reagents and materials (e.g. proteinase K, buffers, spin columns) required for bacterial and fungal DNA isolation after mechanical lysis (Fig. 3.8).

Fig. 3.8: The Septi*Fast* Prep Kit M^{GRADE}.

One hundred and fifty µl of proteinase K were placed into the 15-ml extraction tube. Then, 1000 µl of the supernatant were added. After this, 10 µl of the internal control and the volume of lysis buffer required were added. After vortexing, the mixture was incubated for 15 minutes at 56°C with gentle mixing. After adding the binding buffer, the mixture was transferred to filter columns and the DNAs were adsorbed to the surface of the glass fibers. After washing steps, nucleic acids were eluted from the column using 300 µl of heated elution buffer.

3.4.2.3 LightCycler® Septi*Fast* Test M^{GRADE}

This assay manufactured by Roche is an *in vitro* nucleic acid amplification test for the detection and identification of bacterial and fungal DNA from microorganisms specified in the Septi*Fast* Test Master List (SML) using the LightCycler 2.0 instrument (Tab. 3.2, Tab. 3.3). The LightCycler® Septi*Fast* Test M^{GRADE} utilizes hybridization probes labeled with four different dyes. The test includes all reagents necessary to prepare the three PCR mixes for gram-positive bacteria, gram-negative bacteria, and fungi as well as the negative and the internal controls. The analytical Sensitivity of the LightCycler Septi*Fast* Test was analyzed by the manufacturer with hit-rate analysis of each analyte of a series of dilution of 100, 30, and 3 CFU/ml in EDTA blood from healthy donors. A minimum sensitivity of 30 CFU/ml was obtained for all species, except for *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus pneumonia*e, and *Streptococcus mitis* (100 CFU/ml).

Tab. 3.2 : Microorganisms specified in the Septi*Fast* Test Master List.

Gram (-)	**Gram (+)**	**Fungi**
Escherichia coli	*Staphylococcus aureus*	*Candida albicans*
Klebsiella (pneumoniae/oxytoca)	CoNS[1]	*Candida tropicalis*
Serratia marcescens	*Streptococcus pneumoniae*	*Candida parapsilosis*
Enterobacter (cloacae/aerogenes)	*Streptococcus spp.*[2]	*Candida glabrata*
Proteus mirabilis	*Enterococcus faecium*	*Candida kruesi*
Pseudomonas aeruginosa	*Enterococcus faecalis*	*Aspergillus fumigatus*
Acinetobacter baumanii[3]		
Stenotrophomonas maltophilia		

[1]Coagulase negative Staphylococci (species which represents the group CoNS are listed in Tab. 3.2); [2]Species which represent the group Streptococcus spp. are listed in Table 3.2); [3]Sepcies often referred as A. calcoaceticus-A. baumanii (Acb compelx) are not detected

Tab. 3.3: Species which represents the group CoNS and the group Streptococcus spp.

Coagulase negative Staphylococci (CoNS)	**Streptococcus spp.**
S. hominis subsp. novobiosepticus	*S. agalactiae*
S. pasteuri	*S. anginosus*
S. warneri	*S. bovis*
S. cohnii subsp. urealyticum	*S. constellatus*
S. hominis subsp. hominis	*S. cristatus*
S. lugdunensis	*S. gordonii*
S. cohnii subsp. cohnii	*S. intermdius*
S. captitis subsp. ureolyticus	*S. milleri*
S. captitis subsp. captisi	*S. mitis*
S. caprae	*S. oralis*
S. saprophyticus	*S. parasanguinis*
S. saprophyticus subsp. sapropyticus	*S. pneumoniae*
S. xylosus	*S. pyogenes*
S. epidermidis	*S. salivarisus*
S. haemolyticus	*S. sanguinis*
	S. thermophilus
	S. vestibularis
	S. viridans

For the preparation of the PCR mixes, the Septi*Fast* Cooling Block was used which simplifies the pipetting procedure (Fig 3.9). For each eluted sample, three PCR mixes for gram-positive bacteria, gram-negative bacteria, and fungi were prepared. Each PCR mix consisted of 50 µl master mix and 50 µl eluted sample. The real-time PCR was run in 100 µl LightCycler capillaries.

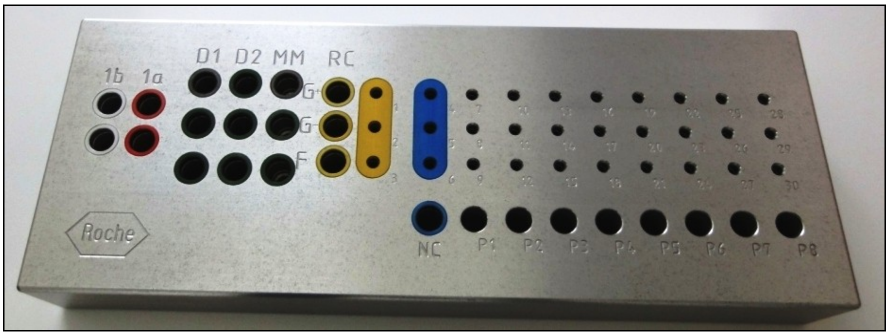


Fig. 3.9: Septi*Fast* Cooling Block

The temperature profile for the real-time PCR run on the LightCycler 2.0 IVD/CE instrument is shown in Fig. 3.10. After amplification, manual T_m calling was required placing the $T_m$bars correctly in the defined $T_m$peaks (Fig. 3.11). After the end of the run, a report was generated automatically and printed. The run file was exported to the Septi*Fast* Identification Software (SIS) and the interpretation report was generated (Fig. 3.12). It was assessed valid if no flags or other massages were shown. The SIS includes a cut-off value for the gram-positive bacteria to avoid false positive signals for coagulase negative staphylococci potentially present in skin flora.

Programs

Program Name	UNG Incubation		
Cycles	1	Analysis Mode	None

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
40	00:05:00	20	0	0	0	None

Program Name	Denaturation		
Cycles	1	Analysis Mode	None

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:10:00	20	0	0	0	None

Program Name	Amplification I		
Cycles	15	Analysis Mode	None

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:00:15	3	0	0	0	None
58	00:00:50	20	0	0	0	None
72	00:00:40	3	0	0	0	None

Program Name	Amplification II		
Cycles	30	Analysis Mode	Quantification

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:00:15	3	0	0	0	None
50	00:00:50	20	0	0	0	Single
72	00:00:40	3	0	0	0	None

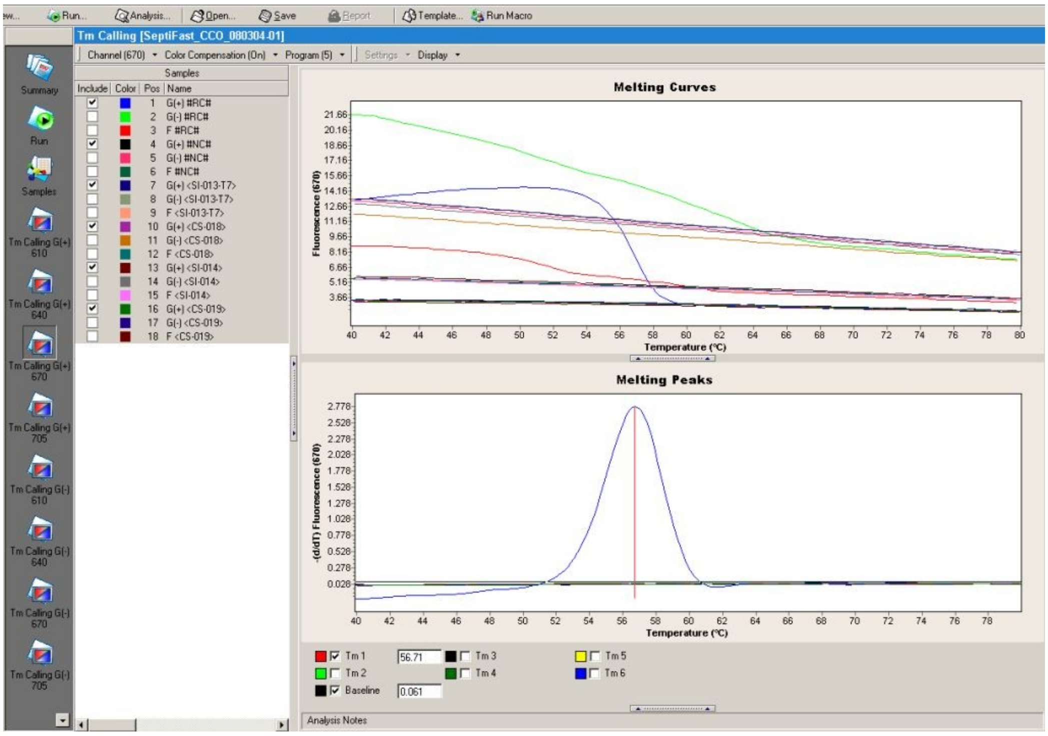
Program Name	Melting Curve		
Cycles	1	Analysis Mode	Melting Curves

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:01:00	20	0	0	0	None
40	00:01:00	20	0	0	0	None
80	00:00:00	0.1	0	0	0	Continuous

Program Name	Cooling		
Cycles	1	Analysis Mode	None

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
40	00:00:30	20	0	0	0	None

Fig. 3.10: Real-time PCR protocol for the Septi*Fast* test



Tm Calling [SeptiFast_CCO_080304-01]

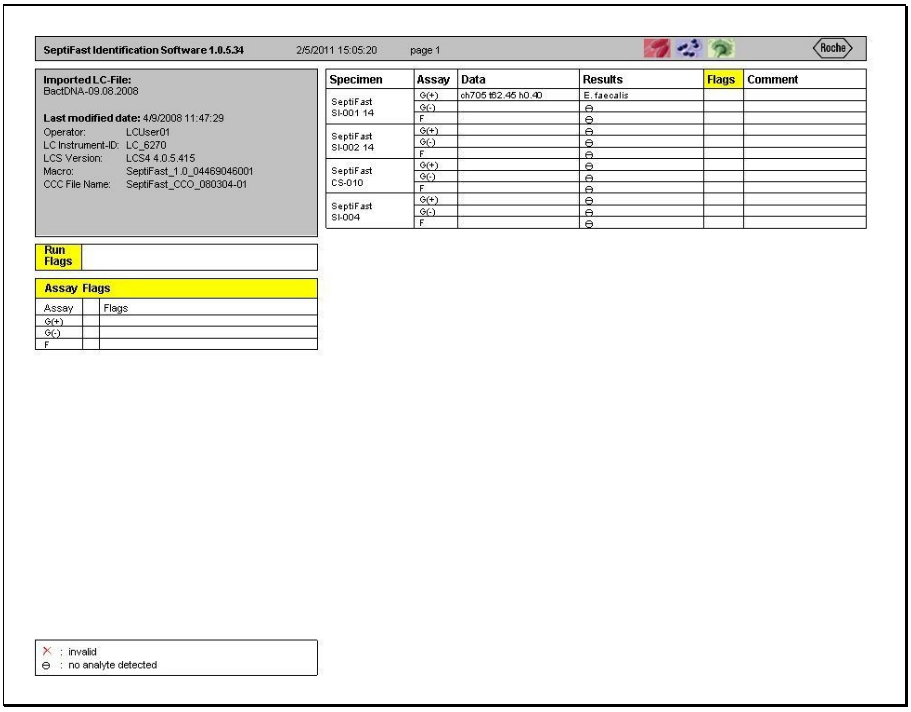
Channel (670) Color Compensation (On) Program (5)

Samples

Include	Color	Pos	Name
☑	🟦	1	G(+) #RC#
☐	🟩	2	G(-) #RC#
☐	🟥	3	F #RC#
☑	⬛	4	G(+) #NC#
☐	🟥	5	G(-) #NC#
☐	🟩	6	F #NC#
☑	🟦	7	G(+) <SI-013-T7>
☐	🟩	8	G(-) <SI-013-T7>
☐	🟧	9	F <SI-013-T7>
☑	🟪	10	G(+) <CS-018>
☐	🟧	11	G(-) <CS-018>
☐	🟩	12	F <CS-018>
☑	🟫	13	G(+) <SI-014>
☐	⬛	14	G(-) <SI-014>
☐	🟪	15	F <SI-014>
☑	🟩	16	G(+) <CS-019>
☐	🟦	17	G(-) <CS-019>
☐	🟫	18	F <CS-019>

🟥 ☑ Tm 1 56.71
🟩 ☐ Tm 2
⬛ ☑ Baseline 0.061
⬛ ☐ Tm 3
🟩 ☐ Tm 4
🟨 ☐ Tm 5
🟦 ☐ Tm 6

Fig. 3.11: T$_m$bar placed correctly in the defined peak



SeptiFast Identification Software 1.0.5.34 2/5/2011 15:05:20 page 1

Imported LC-File:
BactDNA-09.08.2008

Last modified date: 4/9/2008 11:47:29
Operator: LCUser01
LC Instrument-ID: LC_6270
LCS Version: LCS4 4.0.5.415
Macro: SeptiFast_1.0_04469046001
CCC File Name: SeptiFast_CCO_080304-01

Specimen	Assay	Data	Results	Flags	Comment
SeptiFast SI-001 14	G(+)	ch705 t62.45 h0.40	E. faecalis		
	G(-)		⊖		
	F		⊖		
SeptiFast SI-002 14	G(+)		⊖		
	G(-)		⊖		
	F		⊖		
SeptiFast CS-010	G(+)		⊖		
	G(-)		⊖		
	F		⊖		
SeptiFast SI-004	G(+)		⊖		
	G(-)		⊖		
	F		⊖		

Run Flags

Assay Flags

Assay		Flags
G(+)		
G(-)		
F		

✕ : invalid
⊖ : no analyte detected

Fig. 3.12: One example of a SIS report with the Septi*Fast* test

3.4.2.4 LightCycler® Multicolor Compensation Set

The LightCycler® Multicolor Compensation Set (Roche) includes five dyes (Fluorecein, LightCycler RED 610, LightCycler RED 640, LightCycler RED 670, LightCycler RED 705) and a blank solution. The color compensation run is recommended every half year to compensate spectral overlap of analysis. The LightCycler® Multicolor Compensation Set was used for the calibration run performed every six months.

3.4.2.5 SepsiTest™ Blood

The SepsiTest™ Blood (Molzym) consists of a DNA isolation kit and a detection assay (Fig. 3.13). The DNA isolation kit includes buffers, spins, and enzymes for enzymatic lysis of cells and extraction of bacterial and fungal DNA. The detection assay includes the PCR mixes (for bacteria, yeasts, external control), polymerase, DNA staining solution, and sequencing primers for bacteria and fungi. The analytical sensitivity of the test detected by the manufacturer is shown in Tab. 3.4.

Tab. 3.4: Analytical sensitivity of the SepsiTest™ Blood

Microorganism	CFU/ml
Gram-negative bacteria	
Escherichia coli	150
Klebsiella pneumoniae	110
Enterobacter aerogenes	210
Pseudomonas aeruginosa	460
Gram-positive bacteria	
Staphylococcus aureus	40
Staphylococcus epidermidis	20
Streptococcus pneumoniae	40
Enterococcus faecalis	120
Yeast	
Candida albicans	400

The test version used in this study did not contain positive controls for amplification and sequencing. Three EDTA blood samples obtained by a healthy individual were thus spiked, one with strain *Escherichia coli* American Type Culture Collection (ATCC) 25922, one with strain *Staphylococcus aureus* ATCC 29213, and one with strain *Candida albicans* ATCC 90028 with a final concentration of 75000 CFU/ml. After extraction of DNA, a 10 fold dilution series was prepared to check the performance of the sequencing protocol.

Fig. 3.13: The SepsiTest^{TM} Blood.

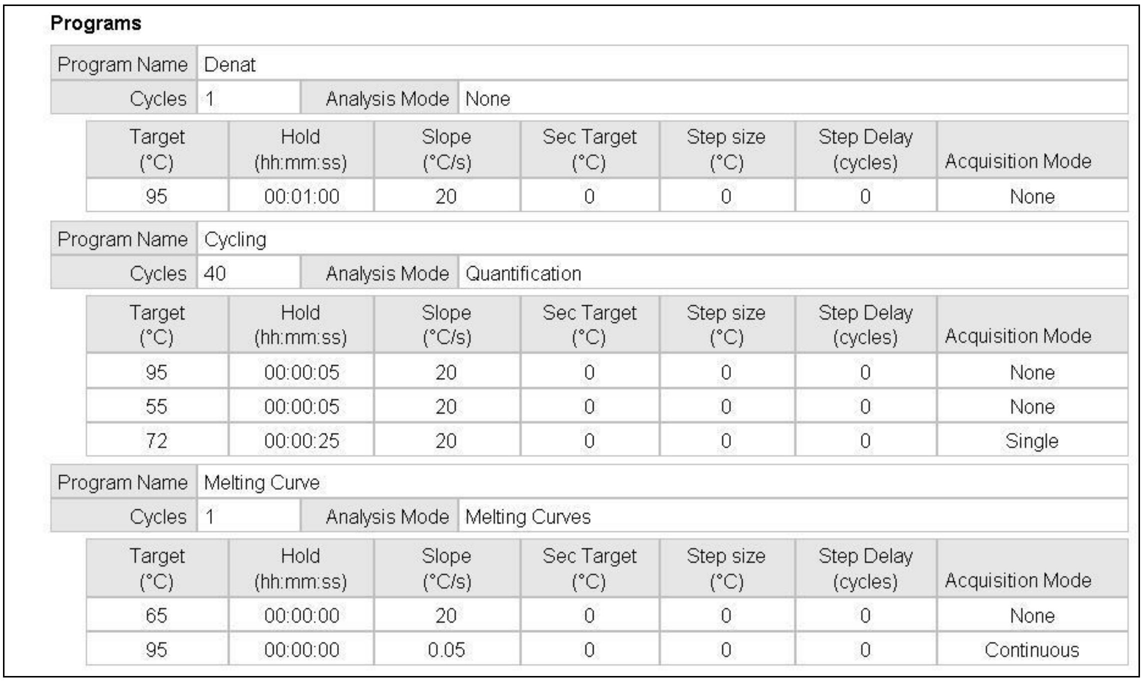
For the manual DNA extraction with the SepsiTest^{TM} Blood, 1000 µl of EDTA whole blood was used. After addition of reagents for the lysis of blood cells, human DNA was degraded with MolDNase and the extraction protocol was carried out until completion of the human cell lysis (step seven). The procedure was interrupted by freezing the sample at ms20°C to allow processing samples in panels of six or eight further on. After thawing the sample, pathogens were lysed using BugLysis and Lyticase in presence of chaotropic buffer. Finally, the pathogen’s DNA was isolated by a bind-wash-elute procedure using Molzym´s CCT$^{®}$ technology. This technology is a nucleic acid purification technology based on PrestoSpinD$^{®}$ columns with a special matrix for binding genomic or plasmid DNA. The DNA binding occurs in the presence of multivalent cations and enables the recovery of femtogram to pictogram amounts of bacterial DNA. The elution volume was 100 µl.

For real-time PCR, the SepsiTest^{TM} Blood DNA Detection assay was employed. Three master mixes for bacteria, fungi, and the external control were prepared. Each PCR mix consisted of 15.8 µl master mix and 5 µl eluted sample. The real-time PCR was run in 20 µl LightCycler capillaries (Tab. 3.5).

Tab 3.5: Bacteria, fungi and external control PCR mix for one reaction.

	Bacteria (16S)	Yeasts (18S)	External control
DNA-free water	5 µl	5 µl	5 µl
2,5 x mastermix	8 µl	8 µl	8 µl
10 x DNA staining solution	2 µl	2 µl	2 µl
MolTaq	0.8 µl	0.8 µl	0.8 µl
Eluate /Negative control	5 µl	5 µl	5 µl
Total volume	20.8 µl	20.8 µl	20.8 µl

Real-time PCR was done according to the manufacturer’s recommendation on the LightCycler 2.0 IVD/CE instrument Fig. 3.14. The PCR analysis was performed using the Absolute Quantification mode and the T_m Calling mode in the fluorimeter channel 530 with color compensation to evaluate the Crossing Points (Cp) and the T_m peak, respectively. A true positive result is defined as follows: External Control appears at the expected value at a Cp 18 ± 2, the sample shows a positive result in the melting curve analysis, and the negative control does not show any signal (except of primer dimers at T_m between 78 and 82°C). A low Cp value of the sample indicates a high pathogen titer in blood and vice versa.



Programs

Program Name	Denat		
Cycles	1	Analysis Mode	None

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:01:00	20	0	0	0	None

Program Name	Cycling		
Cycles	40	Analysis Mode	Quantification

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:00:05	20	0	0	0	None
55	00:00:05	20	0	0	0	None
72	00:00:25	20	0	0	0	Single

Program Name	Melting Curve		
Cycles	1	Analysis Mode	Melting Curves

Target (°C)	Hold (hh:mm:ss)	Slope (°C/s)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	Acquisition Mode
65	00:00:00	20	0	0	0	None
95	00:00:00	0.05	0	0	0	Continuous

Fig 3.14: Real-time PCR protocol for the SepsiTest^{TM} Blood.

3.4.2.6 QIAquick® PCR Purification Kit

The QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany) is used for direct purification of double stranded and single stranded PCR products (100 bp – 10 kb) from amplification reactions. The kit contains buffers and spin columns required for cleanup PCR products from primers, nucleotides, polymerase, salt, and dyes. The kit was used after the PCR run with the detection assay of the SepsiTest^{TM} Blood to prepare amplification products for sequencing analysis.

After real-time PCR, the amplification products were transferred into a 1.5 ml tube by inverted centrifugation of the capillary in a microcentrifuge. Subsequently, amplification products were purified with the QIAquick® PCR Purification Kit according to the manufacturer's package instructions.

3.4.2.7 BigDye® Terminator v3.1 Cycle Sequencing Kit

The BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation) includes reagent components for the chain terminator reaction in a ready, premixed format. The only manual step is to provide the template and the template specific primers in order to perform a fluorescent-based chain terminator reaction.

For the chain terminator reaction, the BigDye Terminator v3.1 and the sequencing primers from the SepsiTest™ Blood Kit were applied. The kit includes forward sequencing primers for gram-positive bacteria, gram-negative bacteria, and fungi. Tab. 3.6 shows the PCR mix for the chain terminator reaction. The PCR program for generation of DNA fragments consisted of an initial denaturation step at 96°C for 1 min, 30 cycles of 15 s at 96°C, 30 s at 53°C, 1.5 min at 60°C, and cooling down and a hold step at 4°C finally. For purification after the chain terminator reaction, ethanol precipitation was done. After addition of Hi-Di™ Formamide, the sample was sequenced with the Applied Biosystems Sequencer 3130 Genetic Analyzer.

Tab. 3.6: PCR mix for the chain terminator reaction

BigDye v3.1	1.8 µl
5X Sequencing Buffer	2 µl
Primer (10 pmol)	1 µl
DNA template	3 µl
ddH2O	2.2 µl
Total volume	10 µl

Subsequently, sequences obtained were analyzed with the Sequencing Analysis Software v5.2. and a BLAST (Basic Local Alignment Search Tool) search was done using the Molzym SepsiTest™ database (http://www.sepsitest-blast.de/de/index.html) and the taxonomy browser of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), GenBank database for identification of bacteria and fungi.

4 Results

4.1 Blood cultures

In this study, 75 clinical blood samples were collected in three pairs of aerobic/anaerobic blood culture bottles each, contributing to 450 blood culture bottles (156 in the sepsis group and 294 in the non-sepsis group). In the sepsis group, 151 (97%) cultures remained negative and five (3%) were detected positive; in the non-sepsis group, 272 (92%) cultures remained negative and 22 (8%) were detected positive. Microorganisms detected are shown in Tab. 4.1.

Tab. 4.1: Microorganisms detected in three pairs of aerobic/anaerobic blood culture bottles.

Prot. no.	Microorganism	No. positive aerobic bottle	No. positive anaerobic bottle
Bottles obtained from sepsis group (*n* = 156)			
SI - 009	CoNS[1]	0	1
SI – 011 T7	CoNS	1	1
	Staphylococcus aureus	1	1
Bottles obtained from non-sepsis group (*n* = 294)			
CS - 007	*Gemella haemolysans*	3	0
CS - 016	*Staphylococcus aureus*	2	0
CS - 024	*Micrococcus sp.*	0	1
CS - 025	*Bacteroides fragilis*	0	1
CA - 008	CoNS	1	0
CI – 002 T7	CoNS[3]	0	3
CI – 002 T21	CoNS	0	1
CI – 003	*E. coli* (ESBL[2] producing)	2	2
CI – 004	*Staphylococcus aureus*	3	3

[1] CoNS; coagulase-negative staphylococci
[2] ESBL; Extended-spectrum beta-lactamase
[3] Two different species of CoNS/bottle detected in all 3 bottles

4.2 LightCycler® Septi*Fast* Test M^{GRADE}

Seventy-five clinical blood samples were studied with three PCR mixes. Results were analyzed with manual T_m bar placing and with the Septi*Fast* Identification Software (SIS) using cut-off values suggested by the manufacturer.

4.2.1 Manual analysis of the LightCycler® Septi*Fast* Test M^{GRADE}

Results obtained for sepsis and non-sepsis groups are shown in Tab. 4.2.

Tab. 4.2: Positives obtained from three PCR mixes analyzed manually.

Group	Gram-positive PCR mix	Gram-negative PCR mix	Fungi PCR mix
	No. pos. (%)		
Sepsis (*n* = 26)	11 (42)	1 (4)	0
Non-sepsis (*n* = 49)	5 (10)	1 (2)	0

4.2.2 SIS analysis of the LightCycler® Septi*Fast* Test M^{GRADE}

Results obtained for sepsis and non-sepsis groups are shown in Tab. 4.3.

Tab. 4.3: Positives obtained from three PCR mixes analyzed with the Septi*Fast* Identification Software (SIS) using cut-off values suggested by the manufacturer.

Group	Gram-positive PCR mix	Gram-negative PCR mix	Fungi PCR mix
	No. pos. (%)		
Sepsis (*n* = 26)	7 (27)	1 (4)	0
Non-sepsis (*n* = 49)	2 (5)	1 (2)	0

4.2.3 Comparison of analysis methods

Microorganisms detected with different methods of analysis are shown in Tab.4.4.

Tab. 4.4: Microorganisms detected with manual and SIS analyses.

Prot. no.	Microorganism detected with manual analysis	Microorganism detected with SIS analysis
	Sepsis group (*n* = 26)	
SI - 001	*Streptococcus pneumoniae*	
SI - 002	*Staphylococcus aureus*	*Staphylococcus aureus*
SI - 001 T7	*Enterococcus faecalis*	*Enterococcus faecalis*
SI - 005	CoNS[1]	
SI - 006	*Streptococcus spp.*	*Streptococcus spp.*
SI - 007	*Enterococcus faecalis*	*Enterococcus faecalis*
SI - 008	*Enterobacter aerogenes* + CoNS	*Enterobacter aerogenes*
SI - 009	*Streptococcus spp.*	*Streptococcus spp.*
SI - 011	*Staphylococcus aureus*	*Staphylococcus aureus*
SI - 011 T7	*Staphylococcus aureus*	*Staphylococcus aureus*
SI - 012	*Streptococcus pneumoniae*	
	Non-sepsis group (*n* = 49)	
CS - 020	CoNS	
CS - 021	CoNS	CoNS
SI - 002 T7	CoNS	
SI - 003	*Escherichia coli*	*Escherichia coli*
SI - 004	*Staphylococcus aureus*	*Staphylococcus aureus*
CI - 005	CoNS	

[1] CoNS; coagulase-negative staphylococci

4.3 SepsiTest^{TM} Blood

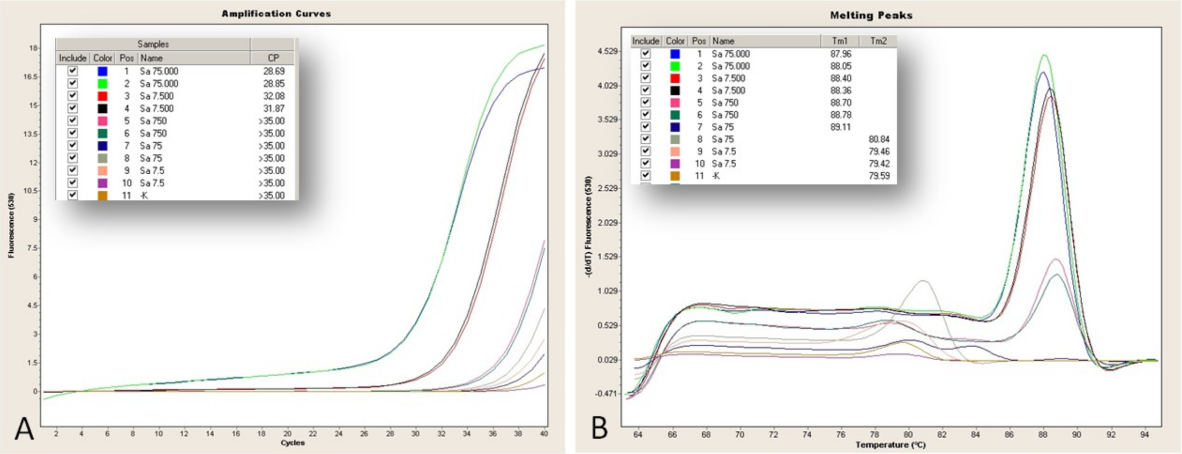
4.3.1 Results obtained from spiked samples

EDTA blood samples obtained by a healthy individual were spiked with 75000 CFU/ml of *Staphylococcus aureus*, *E. coli*, or *Candida albicans*. Ten-fold dilution series were prepared. Each dilution was amplified and analyzed in duplicate.

Fig. 4.1 shows results obtained from the *Staphylococcus aureus* dilution series. The sample containing a concentration of 75000 CFU/ml showed Cp values of 28.69 and 28.85, the dilution containing a concentration of 7500 CFU/ml 32.08 and 31.87. Dilutions containing less than 7500 CFU/ml of *Staphylococcus aureus* showed Cp values >35.00. The specific T_m peak (88 ± 2°C) was always detected at concentrations of 750 CFU/ml and higher. For the sample containing 75 CFU/ml, the peak was detectable only once, whereas for the sample containing 7.5 CFU/ml, no peak could be observed.

The sample containing a concentration of 75000 CFU/ml of *E. coli* showed Cp values of 27.56 and 27.46, the sample containing a concentration of 7500 CFU/ml 31.20 and 31.31. Dilutions containing less than 7500 CFU/ml of *E. coli* showed Cp values >35.00. The specific T_m peak (88 ± 2°C) was always detected at concentrations of 75 CFU/ml and higher. For the sample containing 7.5 CFU/ml, one specific peak could be observed.

The sample containing a concentration of 75000 CFU/ml of *Candida albicans* showed Cp values of 28.54 and 28.55, the sample containing a concentration of 7500 CFU/ml 33.29 and 31.37. Dilutions containing less than 7500 CFU/ml of *Candida albicans* showed Cp values >35.00. The specific T_m peak (88 ± 2°C) was always detected at concentrations of 750 CFU/ml and higher. For the samples containing 75 CFU/ml or 7.5 CFU/ml, the specific peak could be observed only once.



Include	Color	Pos	Name	CP
☑	🟦	1	Sa 75.000	28.69
☑	🟩	2	Sa 75.000	28.85
☑	🟥	3	Sa 7.500	32.08
☑	⬛	4	Sa 7.500	31.87
☑	■	5	Sa 750	>35.00
☑	■	6	Sa 750	>35.00
☑	■	7	Sa 75	>35.00
☑	■	8	Sa 75	>35.00
☑	■	9	Sa 7.5	>35.00
☑	🟪	10	Sa 7.5	>35.00
☑	🟧	11	-K	>35.00

Include	Color	Pos	Name	Tm1	Tm2
☑	🟦	1	Sa 75.000	87.96	
☑	🟩	2	Sa 75.000	88.05	
☑	🟥	3	Sa 7.500	88.40	
☑	⬛	4	Sa 7.500	88.36	
☑	■	5	Sa 750	88.70	
☑	■	6	Sa 750	88.78	
☑	■	7	Sa 75	89.11	
☑	■	8	Sa 75		80.84
☑	■	9	Sa 7.5		79.46
☑	🟪	10	Sa 7.5		79.42
☑	🟧	11	-K		79.59

Fig. 4.1: Results obtained for the dilution series of *Staphylococcus aureus*. A, analysis with the Absolute Quantification mode; B, analysis with the T_m Calling mode.

4.3.2 Sequencing results of spiked samples

For positive samples, sequencing is required to identify microorganisms through BLAST search. Results were generated employing SeptiTest^{TM} and GenBank databases. Results obtained from samples with concentrations of 75000, 7500, and 750 CFU/ml were found to be identical (Tab. 4.5).

For all three pathogens, samples with the lowest positive concentration (7.5 CFU/ml) gave no adequate sequence for the BLAST search; hence, identification was not possible.

Tab. 4.5: Sequencing of *Staphylococcus aureus*, *E. coli*, and *Candida albicans*. Microorganisms showing 100% homology with BLAST search when using SeptiTest^{TM} and GenBank databases.

Staphylococcus aureus	***E. coli***	***Candida albicans***
	Results obtained with SepsiTest^{TM} database	
Staphylococcus simiae	*Shigella sonnei*	*Candida albicans*
Staphylococcus aureus subsp. aureus	*Shigella flexneri*	
Staphylococcus aureus subsp. anaerobius	*E. fergusonii*	
	E. coli	
	Results obtained with GenBank database	
Staphylococcus aureus	*Aeromonas hydrophila*	*Candida albicans*
	E. coli	

4.3.3 Results obtained from clinical samples

Seventy-five clinical blood samples were studied by testing in double with two PCR mixes. Results obtained are shown in Tab. 4.6 and 4.7.

Tab. 4.6. Positives obtained from clinical blood samples when using the SepsiTest^{TM} Blood.

Group	Bacteria PCR mix		Fungi PCR mix	
	First test	**Second test**	**First test**	**Second test**
	No. pos. (%)			
Sepsis (*n* = 26)	10 (39)	3 (12)	8 (31)	6 (23)
Non-sepsis (*n* = 49)	18 (37)	16 (33)	8 (16)	11 (23)

Tab. 4.7: Positives with Cp value >35 obtained from clinical blood samples

Group	Bacteria PCR mix		Fungi PCR mix	
	First test	**Second test**	**First test**	**Second test**
	No. pos. cp>35			
Sepsis	7	3	8	6
Non-sepsis	13	10	8	11

4.3.4 Sequencing of positive samples

All positive samples were sequenced additionally. In case of a positive reaction for bacteria, gram-positive and gram-negative sequencing primers were employed; in case of a positive reaction for fungi, the fungi sequencing primer was employed.

In the sepsis group, for bacteria, all “Cp<35” results (*n*=3) gave an identifiable sequence, whereas for 8 of 10 “Cp>35” results, no analyzable sequence was obtained. For fungi, 8 of 12 “Cp>35” results gave an unidentifiable sequence. (there were no “Cp<35” results for fungi.)

In the non-sepsis group, for bacteria, all “Cp<35” results (*n*=7) gave an identifiable sequence, whereas for 23 of 27 “Cp>35” results, no analyzable sequence was obtained. For fungi, 17 of 19 “Cp>35” results gave an unidentifiable sequence. (There were no “Cp<35” results for fungi.)

Microorganisms identified through analysis with both of the databases are shown in (Tab. 4.8)

Tab. 4.8: Microorganisms identified through sequencing.

	SepsiTest$^{\mathrm{TM}}$ database		**GenBank database**	
Prot. no.	**Homology (%)**	**Microorganism**	**Homology (%)**	**Microorganism**
		Sepsis group		
SI-001				
Bacteria	99,7	*Staphylococcus saprophyticus* *Staphylococcus cohnii*	99	*Staphylococcus saprophyticus* *Staphylococcus cohnii* *Staphylococcus xylosus* *Staphylococcus haemlyticus*
SI-002				
Bacteria	100	*Alkalibacterium olivapovliticus*	99	*Alkalibacterium olivapovliticus* *Alkalibacterium putridalgicola* *Alkalibacterium pelagium* *Alkalibacterium thalassium* *Alkalibacterium indicireducens*
SI-005				
Bacteria	97	*Methylovorus mays*	98	*Methylovorus glucosetrophus*
SI-006				
Bacteria	100	*Streptococcus agalactiae*	100	*Streptococcus agalactiae*
SI-009				
Bacteria	Sequence too short		97	*Streptococcus anginosus* *Streptococcus intermedius* *Streptococcus constellatus*
SI-009				
T7 Bacteria	99	*Streptococcus anginosus*	99	*Streptococcus anginosus* *Streptococcus intermdius*
Fungi	Sequence too short		99	*Malassezia restricta* *Malassezia globosa*
SI-011				
Bacteria	Sequence too short		99	*Staphylococcus aureus*
		Non-sepsis group		
CS-003				
Bacteria	98.1	*Thiobacillus thiparus* *Thiobacillus denitrificans*	99	*Thiobacillus thioparus* *Thiobacillus sayanicus* *Thiobacillus dentitrificans* *Thiobacillus sajanensis*
CS-004				
Bacteria	100	*Staphylococcus warneri* *Staphylococcus simiae* *Staphylococcus saccharolyticus* *Staphylococcus pasteuri* *Staphylococcus lugdunensis* *Staphylococcus hominis Staphylococcus epidermidis* *Staphylococcus caprae* *Staphylococcus capitis* *Staphylococcus aureus*	100	*Staphylococcus aureus* *Staphylococcus haemolyticus* *Staphylococcus warneri* *Staphylococcus pasteuri* *Staphylococcus epidermidis*
CS-005				
Bacteria	99.5	*Pseudomonas flavescens*	100	*Pseudomonas putida*
CS-012				
Fungi	Sequence too short		100	*Fomitopsis pinicola* *Antrodia albida* *Antrodia heteromorpha* *Neolentiporus maculatissimus*
CA-008				
Bacteria	99.6	*Staphylococcus warneri*	99	*Staphylococcus pasteuri* *Staphylococcus warneri* *Staphylococcus haemolyticus*
CI-003				
Bacteria	100	*Shigella sonnei* *Shigella flexneri* *Escherichia fergusonii* *Escherichia coli*	100	*Aeromonas hydrophila* *Escherichia coli*
CI-004				
Bacteria	100	*Staphylococcus simiae* *Staphylococcus aureus*	100	*Staphylococcus aureus*
CI-005				
Bacteria	Sequence too short		100	*Staphylococcus capitis* *Staphylococcus warneri* *Staphylococcus pasteuri* *Staphylococcus epidermidis*

4.4. Comparison study

4.4.1 Comparison of results obtained by molecular tests with blood culture

For the LightCycler® Septi*Fast* Test M^{GRADE}, results obtained from the SIS (as recommended by the manufacturer) and for the SepsiTest^{TM} Blood, results retrieved from the SepsiTest^{TM} database (as recommended by the manufacturer) were used. Comparison of all positive results obtained with different tests studied is shown in Fig. 4.2. All tests gave negative results for 49 of 75 (65%) specimens with 15 of 26 (57%) in the sepsis group and 34 of 49 (69%) in the non-sepsis group. Positive results were obtained for 2 of 75 (3%) samples with all tests. In 24 of 75 (32%) specimens, discrepant results were obtained (Table 4.9).

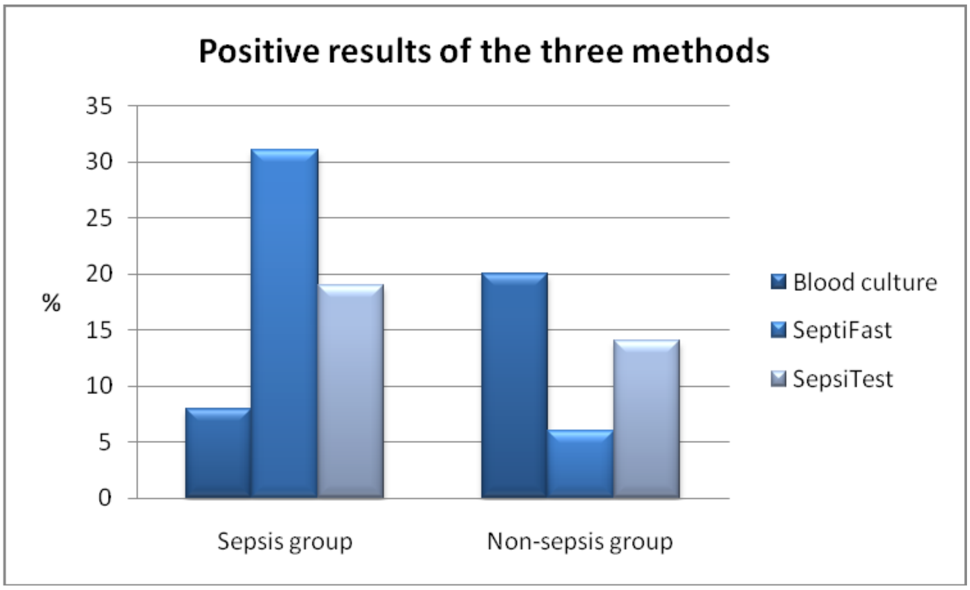


Fig. 4.2.: Comparison of all positive results obtained with the three tests studied.

Tab. 4.9: Comparison of microorganisms identified in the 24 samples showing discrepant results.

Prot. no.	Blood culture	Septi*Fast*	SepsiTest
		Sepsis group	
SI-009	CoNS[1]	*Streptococcus. spp*	neg.
SI-001 T14	neg	*E.faecalis*	neg
SI-007	neg	*E.faecalis*	neg
SI-008	neg	*Enterob. aerog/cloacae*	neg.
SI-005	neg	neg	*Methylovorus mays*
SI-001	neg	neg	*S. saprophyticus/ S. cohnii*
SI-009 T7	neg	neg	*Streptococcus anginosus*
SI-002	neg	*S.aureus*	*Alkalibacterium olivapovliticus*
SI-011	neg	*S.aureus*	neg.
SI-006	neg	S.spp	*Streptococcus agalactiae*
SI-011 T7	*S. aureus* / CoNS	*S.aureus*	neg.
		Non-sepsis group	
CS-025	Bacteroides fragilis	neg	neg
CS-007	Gemella haemolysans	neg	neg
CS-024	Micrococcus sp.	neg	neg
CS-021	neg	CoNS	neg
CS-005	neg	neg	*Pseudomonas flavescens*
CS-004	neg	neg	*Staphylococcus warneri/aureus*
CS-003	neg	neg	*Thiobacillus thioparus/denitrificans*
CS-016	*S. auresus*	neg	neg
CA-005	CoNS	neg	neg
CA-008	CoNS	neg	*S. warneri*
CI-002 T7	CoNS	neg	neg.
CI-002 T21	CoNS	neg	neg.
CI-005	neg	neg	*S. epidermidis*

[1] CoNS: coagulase-negative staphylococci

4.4.2 Previous blood culture results

Screening for positive results in blood cultures taken before specimen collection for this study resulted in detection of four positive results in the sepsis group and none in the non-sepsis group (Table 4.10). When including these results in the comparison study, the consistence improved from 15 of 26 (57%) to 16 of 26 (62%) in the sepsis group.

Tab. 4.10: Microorganisms found additionally in previously taken blood cultures and comparison with results obtained from the study samples.

	Results obtained from			
Prot. no.	**Previous blood cultures**	**Study blood cultures**	**Septi*Fast***	**SepsiTest**
SI-006	*Streptococcus agalactiae*	neg	*Streptococcus spp.*	*Streptococcus agalactiae*
SI-008	*Enterobacter amnigenus*	neg	*Enterobbacter aerog/cloacae*	neg
SI-009	*Streptococcus constellatus*	CoNS[1]	*Streptococcus spp.*	neg
SI-011	*S. aureus*	neg	*S.aureus*	neg

[1] CoNS; coagulase-negative staphylococci

4.4.3 Overall agreement of the three tests

When all tests were compared, an overall agreement of 68% was found. The overall agreement for blood culture versus SeptiFast was 79%, for blood culture versus SepsiTest 77%, and for SetiFast versus SepsiTest 79%.

5 Discussion

The aim of this study was to compare new molecular assays, the LightCycler® Septi*Fast* Test M^{GRADE} and SepsiTest^{TM} Blood with conventional blood culture for detection of pathogens producing bloodstream infections.

Blood culture is the gold standard for the diagnosis of blood stream infections; recommendations for best praxis using this system exist. (Reimer et al. 1997, Riedel et al. 2010) In this study, a blood culture set of six bottles (three aerobic and three anaerobic) from one source were collected together with EDTA blood for molecular tests. Considering the growth characteristics of organisms isolated, three blood culture sets showed a positive result in all bottles. Moreover, two positive results in two aerobic and anaerobic bottles were obtained additionally. Inconsistent results were found mainly with coagulase-negative staphylococci (CoNS). These findings support the opinion that a higher blood volume may lead to improved sensitivity when using blood cultures for diagnosis of blood stream infections and may be helpful in differentiation of contaminants. (Chandrasekar et al. 1994, Shafazand et al. 2002, Lamy et al. 2002, Hall et al. 2006, Hall et al. 2006, Horan et al. 2008) However, regarding CoNS, the number of positive culture bottles cannot predict the clinical significance of CoNS reliably. (Mirrett et al. 2001)

The limitation of blood culture is that only viable microorganisms are detectable. It must be considered that after start of appropriate antimicrobial therapy, the sensitivity decreases substantially. The advantage of blood culture is that viable microorganisms may be tested easily for antibiotic susceptibility of the microorganism in order to optimize antimicrobial therapy. In this study, one extended-spectrum beta-lactamase (ESBL) producing *E. coli* isolate was detected showing this advantage. (Jones 2001, Kollef 2000, Reacher et al. 2000)

The LightCycler® Septi*Fast* (IVD/CE) is a multiplex real-time PCR-based test using hybridization probes for specific detection of 25 clinically important pathogens directly from whole blood in about 6 hours. It includes a simplified DNA extraction protocol employing tubes with defined volumes that can be decanted avoiding time-consuming pipetting steps. Moreover, PCR mix preparation is simplified employing a specially designed cooling block. All necessary controls for qualitative PCR analysis are defined (positive, negative, and internal control). The negative control monitors the whole extraction process and PCR reaction. (Burkardt 2000)

One aspect worth mentioning is the elution volume of 300 µl. In each PCR run (for gram-positive and gram-negative bacteria and fungi), 50 µl of the eluate are added which leads to higher sensitivity. Considering the pipetting error, the remaining volume is less than 150 µl, which does not allow re-analysis in case of a technical problem.

To avoid contamination, strong efforts have been made to develop M^{GRADE} products for the working procedures. M^{GRADE} means special purity of both, reagents and disposables labeled with this brand. Products are designed for highly sensitive bacterial and fungal nucleic acids detection while ruling out DNA contaminations. M^{GRADE} is a trademark of Roche Diagnostics. In this study, these products were used for both molecular tests.

According the instruction manual, the SIS must be used to analyze the results obtained by real-time PCR. When analyzing only those peaks seen distinctly, less positive results were obtained when compared with manual analysis. This difference may be explained by the increased cut-off value given by the manufacturer to reduce false positive results with potential contaminants such as CoNS. (Lehmann et al. 2008). In this study, two results for *Streptococcus pneumoniae* were obtained with manual analysis only, confirmed by previously positive blood cultures reflecting that DNA was still present after appropriate therapy.

The only limitation of this test is that only those microorganisms defined in the SeptiFast Test Master List are detectable.

The SepsiTest^{TM} Blood is a real-time PCR-based test that requires subsequent sequencing of positive results obtained to define the microorganism. DNA extraction and real-time PCR take 4 hours with an additional 3 to 4 hours for subsequent sequencing. The necessity of the sequencing equipment is a limiting factor for the use of this test. There is the possibility to send the sample for sequencing to an external laboratory; however, this would lead to another time delay.

To check the performance of the sequencing protocol which was not specified by the manufacturer, a dilution series of spiked samples was tested. Results obtained showed that several samples gave a Cp value of >35 indicating that low positive results may not be identifiable with sequencing.

The negative control (DNA-free water) included in the test monitors the PCR run only. Several results obtained by the SepsiTest^{TM} Blood (e.g. *Alkalibacterium olivapovliticus, Methylovorus mayus, Thiobacillus spp.*) indicate possible contamination with environmental bacteria. Inclusion of a negative control to be added at the beginning of the procedure monitoring the whole test procedure is thus strongly recommended.

For the BLAST search, two databases were used, the SepsiTest^{TM} database and the GenBank database. GenBank is the largest database of nucleotide sequences but no quality-controlled database; therefore, the SespiTest^{TM} database is provided. However, SepsiTest^{TM} does not provide results in case of a sequence of <200 basepairs (bp) which corresponds to the Clinical and Laboratory Standards Institute (CLSI) guidelines. (CLSI et al. 2008) To improve the quality of a sequence not only a forward primer but also a reverse primer should be included in the SepsiTest Blood sequencing procedure. Another limitation of SepsiTest Blood sequencing is that 16S rRNA gene sequencing does not provide sufficient discrimination for all bacteria. (CLSI et al. 2008) This may influence antibiotic treatment. Furthermore, polymicrobial infection with bacteria or fungi may not be detectable because of the limited ability of conventional sequencing to detect sequences with low abundance (<20% prevalence). Finally, in contrast to the 18S rRNA gene utilized for sequencing of fungi with the SepsiTest Blood, the ITS region has been proposed as appropriate target for genus/species identification of fungi. (CLSI et al. 2008, Iwen et al. 2002)

When comparing test investigated in this study, in the sepsis group, the Septi*Fast* assay gave the highest number of positive results followed by the SepsiTest and blood culture. In contrast, in the non-sepsis group, blood culture gave the highest number of positive results followed by the SepsiTest and the Septi*Fast*. These results correspond to the fact that 86% of patients with sepsis received antimicrobial treatment at the time of sample collection, whereas in the non-sepsis group only 33% received antimicrobial treatment. The Septi*Fast* gave the highest number of positive results in the sepsis group which corresponds to the fact that this test contains the most relevant pathogens found in septic patients. The low positivity of the SepsiTest cannot be explained, as it should detect these microorganisms, too. The overall consistence of all test systems was 68%; the consistence of molecular tests investigated was 79%. When the Septi*Fast* was compared with blood culture, the consistence was 79%, similar to the percentage obtained with comparison of the SepsiTest (77%). These percentages are comparable with other studies. (Wellinghausen et al. 2009, Dierkes et al. 2009, Mancini et al. 2008)

In this study, environmental microorganisms were detected with SepsiTest indicating an increased risk of laboratory contamination when employing this test. Similar to culture, contamination must always be suspected if unexpected microorganisms are identified. Especially, CoNS which are commensals of the skin are highly associated with contamination due to the possibility of contamination during sample collection and test procedure. At least, it is still a challenge to differentiate between contamination and actual infection when using blood culture and molecular tests. (Shafazand et al. 2002, Blot et al. 1999, Lehmann et al. 2008, Wellinghausen et al. 2009, Mirrett et al. 2001)

In this study, a few results could be clarified by means of results of previous blood cultures reflecting the effect of appropriate antibiotic treatment. There is still an ongoing discussion concerning the clinical interpretation of a positive molecular test result. It must be considered that such a result reflects only presence of DNA ("DNAemia") in the sample. Either the DNA detected may originate from microoganisms already killed by antibiotics or represent fragments of unknown origin. (Peters et al. 2004, Paolucci et al. 2010)

A general disadvantage of molecular tests for detection of microorganisms involved in bloodstream infections is the lack to provide information about antimicrobial susceptibility of the organism identified. This lack of information may lead to inadequate antimicrobial therapy. To overcome this problem, conventional blood culture must continue to be part of the current sepsis workflow. (Andrade et al. 2008, Jones 2001, Kollef 2000)

For conventional blood culture, the time to result may range from one to five days. Molecular tests provide significantly shorter times to result (six hours when using the Septi*Fast* assay, nine hours when using the SepsiTest). In Europe, the daily working hours are usually limited by eight hours; thus it might be a challenge for laboratory organization to complete these tests within one working day. (Ecker et al. 2010, Paolucci et al. 2010) Furthermore, the cost of these molecular tests must be evaluated carefully. Special equipment and specially trained laboratory staff is necessary to perform these tests. Cost and cost-effectiveness of these tests for different scenarios in the clinical workday life has not been investigated yet. (Dierkes et al. 2009) To shorten the time to result of molecular tests, automation of DNA extraction should be introduced; however, may be complicated through contamination issues.

In comparison to conventional blood culture, the molecular assays investigated in this study provide a more rapid detection and identification of microorganisms responsible for bloodstream infections. The LightCycler® Septi*Fast* Test M^{GRADE} requires fewer hands on time and offers better-defined identification results whereas the SepsiTest^{TM} Blood is able to detect a larger number of microorganisms. Automation of DNA extraction must be included in these tests to make them useful in the routine diagnostic laboratory. However, due to the need of susceptibility testing for appropriate antimicrobial treatment they do not have the potential to replace the current gold standard blood culture.

6 List of Figures

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8 References

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