NOVEL DNA MICROARRAY IN SEPSIS DIAGNOSTICS

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Front cover: Image from hybridized microarray by Prove-it™ Advisor, hybridization performed by Heli Keränen. Three spots are modified as music notes.

Abstract

Sepsis is defined as a documented infection with systemic inflammatory response syndrome (SIRS). When pathogens have been detected by blood culturing method, the condition is classified as a bloodstream infection (BSI). The frequency of severe sepsis is approximately 90.4 cases per 100 000 population in Europe and circa 751 000 cases annually in United States. Sepsis is associated with high mortality rates ranging up to 50 % in most severe cases. The presence of immunocompromising conditions, chronic diseases, prosthetic devices such as intravenous lines or urinary catheters and higher age are factors which typically increase the infection risk. Currently, common causative bacteria such as *Staphylococcus aureus,* other staphylococci, *Escherichia coli* and *Klebsiella pneumoniae* are detected using blood culturing method. It is time-consuming, especially in case of fastidious and slow growing bacteria and thus initial empirical therapy typically contains broad-spectrum antimicrobial(s).

Rapid methods for sepsis/BSI diagnostics are needed to improve patient outcomes, decrease length of stay in hospital and related costs. When causative pathogens are identified earlier, also appropriate antimicrobials can be administered earlier. The aim of this study was to develop a polymerase chain reaction (PCR) and microarray-based assay for the detection of main causative pathogens and methicillin resistance marker from patients with suspected sepsis/BSI. The assay, which utilized the Prove-it™ TubeArray platform, was first developed for detection of 12 bacterial species, coagulase negative *Staphylococcus* group and methicillin resistance marker. The performance of this assay was evaluated with blood culture samples. The bacterial panel was further improved for the detection of over 50 causative pathogens in sepsis/BSI. This optimized assay was clinically validated with over 3300 blood culture samples collected from HUSLAB, Finland and UCLH, United Kingdom. The developed assay, named Prove-it™ Sepsis, demonstrated 94.7 % sensitivity and 98.8 % specificity. Based on this validation study, the assay was CE-marked for *in vitro* diagnostics in Europe. This diagnostics assay with the improved target panel was also successfully transferred and optimized to the Prove-it™ StripArray platform, whose capacity of 1-96 simultaneous analyses responds to the need of hospital laboratories dealing with larger sample amounts.

Another aim of this study was to evaluate the PCR and microarray assay's suitability for identification of pathogens directly from whole blood samples without a culturing step. The assay was combined with a selective bacterial deoxyribonucleic acid (DNA) isolation method and the performance of this combination was evaluated with spiked blood samples. Detection limit of 11-600 colony forming units per mL was obtained depending on the target organism. In addition, analytical sensitivity of 1-21 genome equivalents for the PCR and microarray assay was demonstrated. These results showed proof-of-concept for the combination assay and feasibility of the PCR and microarray assay to be used for more sensitive applications after an extensive optimization phase.

Molecular assays have opened a new era in microbiological laboratories and brought a broadened perspective parallel to the conventional culturing and phenotype-based method. Also in this study, genotype-based characterization was utilized to offer more accurate identification than conventional culturing. In future, understanding the clinical relevance of DNAemia may open new strategies to the management of septic patients using nucleic acids-based assays.

Tiivistelmä

Sepsis tarkoittaa vakavaa yleisinfektiota ja tulehdusreaktio-oireyhtymää, johon liitetään usein veriviljelypositiivisuus. Yleisyys Euroopassa on 90.4 tapausta 100 000 ihmistä kohden ja Yhdysvalloissa noin 751 000 tapausta vuosittain. Sepsikseen liitetään korkea kuolleisuus, jopa 50 %. Heikentynyt immuunipuolustus, krooniset sairaudet sekä korkea ikä saattavat lisätä sairastumisriskiä. Yleisimpiä aiheuttajabakteereita ovat muun muassa *Staphylococcus aureus* ja muut stafylokokit, *Escherichia coli* ja *Klebsiella pneumoniae.* Resistentit ja multi-resistentit bakteerikannat ovat yleensä hoidollisesti vaikeimpia, koska tehokkaan mikrobilääkehoidon kohdistaminen saattaa olla vaikeaa. Tällä hetkellä sepsis osoitetaan veriviljelydiagnostiikan avulla, jolloin mikrobi pyritään tunnistamaan potilaan verestä. Viljely on hidas menetelmä vaativissa kasvuolosuhteissa kasvavien mikrobien kohdalla, siksi potilaan empiirinen ensihoito koostuu yleensä laajakirjoisesta mikrobilääkkeestä tai lääkeyhdistelmistä.

Nopeutetun diagnostiikan avulla mikrobi(t) pystyttäisiin tunnistamaan nopeammin ja näin ollen kohdistettu lääkehoito aloittamaan aikaisemmin. Tämän työn tavoitteena oli kehittää PCR-monistus- ja mikrosirutekniikkaan perustuva testi sepsiksen aiheuttajamikrobien tunnistamiseen. Ensin kehitettiin tunnistus 12 bakteerilajille, koagulaasinegatiiviselle stafylokki-ryhmälle sekä metisilliiniresistenssi-geenimarkkerille positiivisesta veriviljelynäytteestä. Testialustaksi optimoitiin Prove-it™ TubeArray -mikrosiru, jolla pystyi analysoimaan 1-24 näytettä kerrallaan. Testin toimivuus arvioitiin kerätyillä veriviljelynäytteillä. Seuraavassa vaiheessa mikrobipaneeli laajennettiin kattamaan yli 50 sepsiksen aiheuttajamikrobia. Tämän parannetun testiversion toimivuus arvioitiin yli 3300 veriviljelynäytteen avulla, jotka oli kerätty HUSLAB:ssa Suomessa ja UCHL:ssä Isossa-Britaniassa. PCR- ja mikrosirutesti nimettiin Prove-it™ Sepsis -testiksi, jolle määritettiin 94.7 %:n herkkyys ja 98.8 %:n tarkkuus, kun testitulosta verrattiin veriviljelyn mikrobilöydöksiin. Tämän arvioinnin perusteella testi CE-merkittiin *in vitro* diagnostiikkaan Euroopassa. Kehitystä jatkettiin Prove-it™ TubeArray -testialustan lisäksi myös Prove-it™ StripArray -testialustalle, jolla saattoi analysoida 1-96 näytettä samanaikaisesti. Useamman näytteen yhtäaikainen analysointi vastaa paremmin tarvetta isoissa laboratorioissa, joissa näytekapasiteetti on suurempi.

Lisäksi tutkittiin PCR- ja mikrosirutestin soveltuvuutta mikrobitunnistukseen suoraan potilaan verinäytteestä ilman rikastusvaihetta. Spesifinen bakteeri-DNA:n eristysmenetelmä potilasverinäytteestä yhdistettiin PCR- ja mikrosirutestin kanssa. Tätä yhdistelmää arvioitiin verinäytteillä, joihin oli lisätty tietty pitoisuus bakteereita. Analysoinnin tuloksena tämän yhdistelmätestin herkkyydeksi määritettiin bakteerilajista riippuen 11-600 pesäkettä muodostavaa yksikköä per mL. Lisäksi PCR- ja mikrosirutestin analyyttiseksi herkkyydeksi määritettiin 1-21 genomiekvivalenttia. Tulokset osoittivat, että PCR- ja mikrosirutesti saattaisi olla kehitettävissä myös herkempiin sovelluksiin kuin rikastettuun näytemateriaaliin, esimerkiksi muokkaamalla testiä yhdessä kuvatun DNA-eristysmenetelmän kanssa.

Molekyylipohjaiset testit ovat jo avanneet uuden aikakauden mikrobiologisissa laboratorioissa. Mikrobien geenipohjainen luokittelu ja karakterisointi tarjoavat sellaisia mahdollisuuksia, joita fenotyyppipohjaisella luokittelulla ei pystytä välttämättä saavuttamaan. Näitä havaintoja tehtiin myös tässä tutkimuksessa, kun PCR- ja mikrosirutesti tunnisti bakteereja potilasnäytteistä, joissa viljely epäonnistui tai ei antanut oikeaa tulosta. Sepsispotilaan verenkierrosta löytyvän bakteeri-DNA:n kliininen merkittävyys infektioissa ei ole vielä täysin selvää. Sen ymmärtämisen myötä voidaan kehittää nopeampia nukleiinihappopohjaisia strategioita sepsispotilaan diagnosointiin.

Contents

List of original publications

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I. Järvinen AK, **Laakso S,** Piiparinen P, Aittakorpi A, Lindfors M, Huopaniemi L, Piiparinen H, Mäki M (2009). Rapid identification of bacterial pathogens using a PCR- and microarray-based assay. *BMC Microbiology* 9:161.

II. Tissari P, Zumla A, Tarkka E, Mero S, Savolainen L, Vaara M, Aittakorpi A, **Laakso S**, Lindfors M, Piiparinen H, Mäki M, Carder C, Huggett J, Gant V (2010). Accurate and rapid identification of bacterial species from positive blood cultures with a DNA-based microarray platform: an observational study. *The Lancet* 375:224-230.

III. **Laakso S**, Kirveskari J, Tissari P, Mäki M (2011). Evaluation of High-Throughput PCR and Microarray-Based Assay in Conjunction with Automated DNA Extraction Instruments for Diagnosis of Sepsis. *PLoS ONE* 6(11):e26655

IV. **Laakso S**, Mäki M. (2013). Assessment of a semi-automated protocol for multiplex analysis of sepsis-causing bacteria with spiked whole blood samples. *MicrobiologyOpen* 2(2):284-292

Contribution of the author to papers I - IV

I. The author performed a part of the experimental work comprising DNA extraction, PCR optimization, sequencing experiments, PCR- and microarray experiments. She participated in result interpretation and writing of the article together with other authors.

II. The author performed a part of the experimental work, including design of oligonucleotide probes for new targets, sequencing experiments and troubleshooting. She also optimized the hybridization protocol on the microarray and performed part of the DNA extractions and Prove-it™ Sepsis analysis experiments in UCLH. The author participated in the interpretation of results and commented on the article together with other authors.

III. The author designed and performed all the DNA-based experimental work, including *e.g.* DNA extractions, PCR and microarray experiments, real-time PCR and sequence homology searches. She interpreted the data and had the main responsibility for writing the article under the supervision of Docent Minna Mäki. All authors jointly commented on the manuscript.

IV. The author designed and performed all the experimental work, interpreted the data and had the main responsibility for writing the article under the supervision of Docent Minna Mäki.

Abbreviations

A	Absorbance
ACCP	American College of Chest Physicians
ATCC	American Type Culture Collection
bp	Base pair
BSI	Bloodstream infection
CA-BSI	Community acquired BSI
CA-MRSA	Community acquired MRSA
ccr	Recombinase gene in SSCmec
CD64	Neutrophil, studied as biomarker
CE	*Conformité Européenne*
CFU	Colony forming units
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CNS	Coagulase negative Staphylococcus
CO_2	Carbon dioxide
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dsDNA	Double-stranded deoxyribonucleic acid
dxs	Chromosomal d-1-deoxyxylulose 5-phosphate synthase gene
EDTA	Ethylenediaminetetraacetic acid
ESKAPE	Group of pathogens including *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* sp.
ESI-MS	Electrospray ionization mass spectrometry
EU	European Union
FDA	US Food and Drug Administration
FISH	Fluorescent *in situ* hybridization
FN	False negative
FP	False positive
GE	Genome equivalent
gyrB	Gene encoding the subunit B protein of DNA gyrase
HA-MRSA	Hospital acquired MRSA
HCA-BSI	Healthcare associated BSI
HPA	Hybridization protection assay
HRP	Horseradish peroxidase
HUSLAB	Helsinki University Hospital Laboratory
ICU	Intensive care unit
IL-18	Interleukin-18, studied as biomarker
ISO	International organization for standardization
ITS	Internal transcribed spacer
IVD	*In vitro* diagnostic

IVDD	*In vitro* medical device directive
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
mecA	Gene included in the SCC*mec* element, encoding penicillin binding protein PBP2a
mmHg	Millimeters of mercury
MRSA	Methicillin-resistant Staphylococcus aureus
MSSA	Methicillin-sensitive Staphylococcus aureus
MS	Mass spectrometry
NA	Nucleic acid
OD	Optical density
parE	Gene encoding the subunit E of topoisomerase IV
PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction
PCT	Procalcitonin
POC	Point-of-care
rRNA	Ribosomal ribonucleic acid
SCCM	Society of Critical Care Medicine
SIRS	Systemic inflammatory response syndrome
SOAP	Sepsis occurrence in acute ill patient
SCC*mec*	Staphylococcal cassette chromosome mec
ssDNA	Single-stranded deoxyribonucleic acid
sp./spp.	Species (singular/plural)
Tm	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine substrate
TN	True negative
TP	True positive
UCLH	University College London Hospital
UK	United Kingdom
US	United States
USA	United States of America

1 Introduction

1.1 Sepsis

1.1.1 Definition

The definitions of sepsis, severe sepsis and septic shock were introduced in the consensus conference of American College of Chest Physicians (ACCP) and Society of Critical Care Medicine (SCCM) at the beginning of the 90s (Bone *et al.*, 1992). Earlier terms like septicemia, bacteremia and sepsis syndrome were used without precise definitions to characterize patients with severe generalized infection. According to the consensus conference, sepsis is defined as a documented infection with systemic inflammatory response syndrome (SIRS) (Bone *et al.*, 1992). Definitions were revised in 2001, but were left practically unchanged. The expanded list of diagnostic criteria for sepsis, including a list of variables related to the general, inflammatory, hemodynamic, organ dysfunction and tissue perfusion symptoms were prepared to help recognition of sepsis, but none of those were specific for sepsis (Levy *et al.*, 2003). Sepsis is defined severe when associated to organ dysfunction, hypoperfusion or hypotension. Manifestations of hypoperfusion may include, but are not limited to, lactic acidosis, oliguria or an acute alteration in mental status. The most complicated condition is septic shock, which is defined as the presence of sepsis and refractory hypotension, *i.e.* systolic blood pressure less than 90 mmHg, mean arterial pressure less than 65 mmHg or a decrease of 40 mmHg in systolic blood pressure compared to baseline unresponsive to a crystalloid fluid challenge of 20 to 40 mL / kg (Bone *et al.*, 1992; Levy *et al.*, 2003; Annane *et al.*, 2005). Definitions of common sepsis-related terms are shortly summarized in Table 1.

Consensus conference defined also the term bacteremia, which is the presence of viable bacteria in the blood (Bone *et al.*, 1992). When pathogens have been detected from blood culture and clinical symptoms of systemic infection have been obtained, the condition is called a bloodstream infection (BSI). BSIs can be further divided to primary and secondary infections. Shortly, infection is a primary BSI if the pathogen identified from one or more blood culture samples is not related to an infection at another site. Primary infection is often associated with intravascular catheters. Infection is secondary BSI if the pathogen cultured from blood is related to an infection with the same pathogen at another site (Paolucci *et al.*, 2010; Juan-Torres and Harbarth, 2007).

According to the definition, detection of sepsis does not require detection of BSI. A high portion of blood cultures are negative although the majority of patients have sepsis related symptoms such as fever, hypotension or oliguria. Several reasons, such as pathogen-produced pyrogenic agents, may cause clinical signs of sepsis with negative blood culture. This condition is called clinical sepsis. Characterization of clinical sepsis may be

difficult since the patient's condition may not be unambiguous and only one of the main signs (fever, hypotension, or oliguria) is required together with other criteria (Garner *et al.*, 1988; Hugonnet *et al.*, 2004; Soraya *et al.*, 2008).

Table 1. *Definitions of sepsis-related terms.*

Terms	Definition
Bacteremia	Presence of viable bacteria in blood.
Bloodstream infection (BSI)	Presence of clinical symptoms of systemic infection and positive blood culture results.
Systemic imflammatory response syndrome (SIRS)	Presence of two or more of the following: - Body temperature > 38 °C or < 36 °C - Heart rate > 90 beats per min - Respiratory rate > 20 breaths per minute or arterial CO_2 tension < 32 mm Hg or need for mechanical ventilation - White blood cell count > 12 000/mm^3 or < 4000/mm^3 or immature forms > 10 %
Sepsis	The systemic response to a documented infection together with SIRS criteria.
Severe sepsis	Presence of sepsis associated with organ dysfunction, hypoperfusion, or hypotension. The manifestations of hypoperfusion may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status.
Septic shock	Presence of sepsis with hypotension despite adequate fluid resuscitation. It includes perfusion abnormalities such as lactic acidosis, oliguria, or an acute alteration in mental status.
Clinical sepsis	Presence of either fever, hypotension, or oliguria, and all of the following: - Blood not cultured or no microorganism isolated - No apparent infection at another site - Appropriate antimicrobial therapy for sepsis have been directed

(References: Bone *et al.,* 1992; Levy *et al.,* 2003; Annane *et al.,* 2005; Garner *et al.,* 1988).

1.1.2 Incidence and costs

Estimation of incidence of severe sepsis is around 18 million cases worldwide annually and circa 1400 patients die from severe sepsis each day (Angus *et al.*, 2001; Bone *et al.*, 1992 Daniels *et al.*, 2011). In the United States (US), sepsis is defined to be the 10th leading cause of death and septic shock to be the first cause of death in intensive care units (ICU) (Minino *et al.*, 2007). Approximately 751 000 cases of severe sepsis occur annually in the US and an average length of stay in hospital for a patient with severe sepsis is 19.6 days with an associated cost of $22 100. Treatment of these patients involves an economic cost estimated at $16.7 billion annually (Angus *et al.*, 2001). In the European Union (EU), the frequency of severe sepsis is estimated to be 90.4 cases per 100 000 population and the management of patients with severe sepsis bear around €7.6 billion healthcare costs per year in Europe (Daniels, 2011).

High mortality rates are associated with sepsis and BSI. Angus and co-workers (2001) demonstrated that the mortality rate of patients with severe sepsis was 28.6 % in the US. Age has a strong influence on the incidence of severe sepsis and mortality increased from 10 % to 38.4 % when pediatric patients were compared to a group of > 85 year age patients. Similar values were also obtained in Europe in the Sepsis Occurrence in Acute Ill Patient (SOAP) study. The mortality rates in the ICU were 27 % for patients with sepsis, 32 % for patients with severe sepsis and 54 % for patients with septic shock (Vincent *et al.*, 2006).

1.1.3 Infection sites and etiology

Sepsis is associated with community- or hospital-acquired infections, and the classification is typically difficult. Several seemingly harmless conditions may cause sepsis, but often it is caused by a more serious medical primary infection, such as pneumonia or meningitis. The presence of immunocompromising conditions, chronic diseases, prosthetic devices such as intravenous lines or urinary catheters and higher age are factors which typically increase the infection risk (Nguyen *et al.*, 2006). The most frequent causes of infections in septic patients are pneumonia, bloodstream infections (including infective endocarditis), intravascular catheter-related sepsis, intra-abdominal infections, urosepsis and surgical wound infections (Harbarth *et al.*, 2003; Calandra and Cohen, 2005). Many studies have also described the same typical infection sites like lung (50 - 68 % of the patients), abdomen (20 - 25 % of the patients), urinary tract (7 - 14 % of the patients), wounds and blood (Ebrahim, 2011; Vincent *et al.*, 2006; Vincent *et al.*, 2011).

Different microbes can cause sepsis, such as bacteria, fungi and viruses, but diagnosis of bacterial and fungal sepsis is the most studied. Therefore, the present study focused only on bacterial sepsis diagnostics. Both Gram-positive and Gram-negative bacteria cause

sepsis-related infections. Vincent and co-workers (2006) reported the distribution of bacteria in their European SOAP study of 3147 patients with the median age of 64 years. Gram-positive bacteria were identified in 40 % of the positive samples, Gram-negative bacteria in 38 % of the positive samples and fungi in 17 % of the positive samples (Vincent *et al.*, 2006). In another study concerning neonatal sepsis cases in Nepal, the distribution was 44.1 % of Gram-positive and 55.9 % of Gram-negative bacteria (Gyawali and Sanjana, 2012). According to several studies, the most common Gram-positive bacteria detected from blood cultures are *Staphylococcus aureus,* coagulase negative *Staphylococcus* (CNS) (including *Staphylococcus epidermidis*), *Streptococcus pneumoniae* and other streptococci. Common Gram-negative bacteria are *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus* spp. and other members of *Enterobacteriaceae* group (Vincent *et al.*, 2006; Beekmann *et al.*, 2003; Reimer *et al.*, 1997; Harbarth *et al.*, 2003; Nguyen *et al.*, 2006). In addition, especially among neonates *Haemophilus influenzae* and *Neisseria meningitidis* are common findings in infections (Nizet and Klein, 2011).

It is estimated that *S. aureus, E. coli* and other members of the *Enterobacteriaceae* group, *P. aeruginosa, S. pneumoniae* and *Candida albicans* represent typically true causative pathogens in infections when detected from blood culture. Pathogens such as CNS, *Corynebacterium* spp., *Bacillus* spp. and *Propionibacterium acnes* are often classified as contaminations when detected from blood culture. Contaminations are typically originated from skin (Reimer *et al.*, 1997, Hall and Lyman, 2006). However, studies have shown the clinical importance of also these bacteria as causative agents in infections and the interpretation of these bacterial findings needs to be investigated carefully (Otsuka *et al.*, 2005; Adler *et al.*, 2005; Park *et al.*, 2011).

1.1.4 Antimicrobial resistances and MRSA

The number of infections caused by bacteria resistant to one or more of the current antimicrobials has been estimated to increase. The well-studied antimicrobial resistances are methicillin (among *S. aureus* and other *Staphylococcus* ssp.) and vancomycin (among *e.g. Enterococcus* ssp.) resistances. In addition, bacteria including *Enterobacteriaceae* group and generating resistances by producing extended spectrum β-lactamase, metallo-β-lactamase or carbapenemase enzymes are under extensive investigation (Bhattacharya, 2013). Bacterial resistances have a major influence on the outcome of septic patients and it has been assessed that the presence of bacterial resistance approximately doubles the mortality rate associated with sepsis (Turnidge, 2003). A highly resistant group of bacteria which is related to worse patient outcomes is named the ESKAPE group. Pathogens included in this group are *Enterococcus faecium, S. aureus, K. pneumoniae, Acinetobacter baumannii, P. aeruginosa* and *Enterobacter* species (Boucher *et al.*, 2009; Rice, 2008). In addition, methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa, A.*

baumannii, and *Stenotrophomonas maltophilia* constitute another group typically classified as highly multi-resistant bacteria and difficult to treat (Trouillet *et al.*, 1998).

Methicillin-resistant *S. aureus* is the one of the most known and studied resistant bacterial species causing high mortality and associated often with inadequate antimicrobial treatment (Lodise *et al.*, 2003). According to a large European sepsis study, blood cultures were positive in 60 % of the patients with sepsis and MRSA was detected in 14 % of those samples (Vincent *et al.*, 2006). Another study related to BSI showed that *S. aureus* was the causative agent in 24 % of the samples and 31 % of those were resistant to methicillin (Latif *et al.*, 2009). *S. aureus* is the second most common pathogen causing BSIs and the most common causative pathogen in nosocomial BSIs. Shorr and co-workers (2006) reported that *S. aureus* was the causative agent in 25.7 % of healthcare-associated BSIs (HCA-BSI), in 29.7 % of nosocomial BSIs and in 17.8 % of community acquired BSIs (CA-BSI) in the US. The prevalence of MRSA in these groups was 41 %, 52 % and 26 %, respectively. Around 25 % of healthy humans are colonized with *S. aureus* and 1.5 - 3 % with MRSA. It has been estimated that in over 80 % of people who have *S. aureus* BSI, the same isolate can also be isolated from their nares (del Rio *et al.*, 2009).

Methicillin resistance in *Staphylococcus* species is associated with the additional penicillin binding protein PBP2a, which has low affinity for all β-lactam antimicrobials. PBP's role as a transpeptidase is to catalyze the formation of cross-bridges in bacterial cell wall peptidoglycan. Semisynthetic penicillin such as methicillin, nafcillin and oxacillin has been designed for the treatment of infections caused by beta-lactamase-producing staphylococci. Typically these β-lactam antimicrobials bind to the methicillin-sensitive *S. aureus* (MSSA) native PBPs disrupting the synthesis of peptidoglycan cell wall and resulting in bacterial death. PBP2a has low affinity for all β-lactam antimicrobials which leads to no disruption in cell wall peptidoglycan synthesis and resulting in normal bacterial growth (Berger-Bachi and Rohrer, 2002; Hanssen and Ericson Sollid, 2006; IWG-SCC, 2009). The highly mobile element of *Staphylococcu*s species, the staphylococcal cassette chromosome SCC*mec,* carries the *mecA* gene which encodes PBP2a. SCC*mec* elements are classified based on their putative cassette chromosome recombinase genes (*ccr*) and overall genetic composition. Currently, at least 11 types of SCC*mec* (types I-XI) elements and several variants have been reported based on differences in their structure and size (Peng *et al.*, 2010; Shore *et al.*, 2011). SCC*mec* types I-III are usually related to hospital-acquired MRSA (HA-MRSA) and types IV and V to community-acquired MRSA (CA-MRSA) (Berglund and Söderquist, 2008).

1.1.5 Administration of appropriate antimicrobial therapy

According to the Surviving Sepsis Campaign recommendation, intravenous antimicrobial therapy should start within the first hour of recognition of severe sepsis or septic shock and the empirical therapy should contain one or more antimicrobials (Dellinger *et al.*, 2008). Combination therapy is supposed to cover the spectrum of all possible pathogens. In case of multi-microbial infections or resistant bacteria, targeted selection of antimicrobials leads to better patient outcomes (Nguyen *et al.*, 2006). Combination therapy should not be administered longer than 3-5 days and antimicrobial therapy should be revised daily to avoid the development of resistance and to reduce toxicity and costs. When the causative agent and susceptibility profile has been defined, narrowed antimicrobial treatment should be used. There is no evidence that combination therapy would give better response than directed mono-therapy if the causative agent has been identified. The suitable duration of the therapy is typically 7–10 days (Dellinger *et al.*, 2008; Nguyen *et al.*, 2006).

Delayed antimicrobial treatment in patients with severe sepsis or septic shock is known to increase mortality. Inappropriate therapies are often related to the pathogen resistances, such as MRSA, which were noted also in the recommendations of Surviving Sepsis Campaign (Dellinger *et al.*, 2008). One study showed that almost 1/3 of patients received inappropriate antimicrobial treatment and in most of those cases the causative agent was either vancomycin-resistant *Enterococcus*, CNS, *P. aeruginosa* or *C. albicans* (Ibrahim *et al.*, 2000). In another study, inappropriate therapy was associated mainly with multi-resistant bacteria such as *P. aeruginosa, S. maltophilia, Acinetobacter* spp. and MRSA (Harbarth *et al.*, 2003). It has been demonstrated that every additional hour without appropriate antimicrobial treatment increases the risk for death in septic patients by 7.6 % during the first six hours from hypotension onset (Kumar *et al.*, 2006). Harbarth and co-workers (2003) compared 28-day mortality between initially an appropriately treated group and an inappropriately treated group. The mortality rates were 24 % and 39 %, respectively. Similarly, another study demonstrated mortality rates in Gram-negative bacteremia to be 18 % for the group of appropriately treated and 34 % for the group of inappropriately treated patients (Bochud *et al.*, 2004). In addition to the mortality rate, the administration of ineffective therapy correlated also to the length of stay in hospital increasing the related costs. High mortality rate and costs cause pressure to develop faster methods for the identification of causative agents giving guidance to the appropriate antimicrobial therapy earlier (Carrigan *et al.*, 2004; Harbarth *et al.*, 2003; Beekman *et al.*, 2003).

Detection of sepsis is often difficult and therefore adequate treatment may be delayed. Attempts to improve the situation have included finding specific markers indicating the patient's condition and helping in the diagnosis. Several biomarkers for sepsis are under investigation. These may provide information suitable for diagnostics, monitoring and therapeutic decision making (Lever and Mackenzie, 2007). Probably the most investigated diagnostic biomarkers, which could indicate the presence or absence of a

disease state or other clinical condition, are C-reactive protein (CRP) and procalcitonin. CRP is a hepatocyte- produced acute-phase reactant found in the blood, which amount is increasing within 4–6 hours of an inflammatory stimulus. Procalcitonin (PCT) is a precursor for the hormone calcitonin and the level of PCT has been found to increase in children with sepsis and bacterial infection. As a monitoring biomarker, the level of PCT is decreasing quickly when appropriate antimicrobial therapy is initiated. In addition, there are also other biomarkers under investigations, such as CD64, IL-18 and lactate. Clinicians may recognize patient condition faster by screening biomarkers. However, the role of these biomarkers is still under investigation (Standage and Wong, 2011; Lever and Mackenzie, 2007; Schuetz *et al.*, 2011).

1.2 Diagnosis of pathogens causing sepsis and BSI

Conventional blood culture including pathogen subculturing on appropriate media and antimicrobial susceptibility evaluation are the gold standard methods for identification of sepsis and BSI causing pathogens. Phenotype-based characterization such as staining as well as microscopy and testing biochemical properties of pathogens are described as traditional microbiological methods in diagnostic laboratories. In order to respond to the need for more rapid diagnostics, new assays with various detection strategies have been developed. One novel approach is to use mass spectrometry for characterization of pathogens based on their proteomic profile. Another strategy is to use nucleic acids (NA) for identification of pathogens from clinical samples. NA-based assays are typically classified as hybridization- or amplification-based assays depending on the used technique. In addition, NA-based mass spectrometry applications have also been developed (Mancini *et al.*, 2010; Weile and Kanbbe, 2009; Peters *et al.*, 2004).

The sample type for these new assays is either positive blood culture or patient blood. In some assays, clinical sample cannot be used as such and thus, an additional culturing step on appropriate agar media from the original sample is required before the final analysis. Recent developments show a trend to provide identification of Gram-positive and Gram-negative bacteria, fungi and resistance markers in the same assay or simultaneous identification with several parallel reactions. However, the coverage of the target panel is linked to the used technique and varies between assays (Afshari *et al.*, 2012; Weile and Kanbbe, 2009; Mancini *et al.*, 2010). Table 2 summarises examples of well-established assays and techniques used in sepsis diagnostics (some also in BSI diagnostics).

Table 2. *Examples of commercially available systems and assays used in sepsis diagnostics (some also in BSI diagnostics).*

Method description	Sample type	Assay (Manufacturer)
Blood culturing (automated systems)		
Blood culturing automates, where increasing levels of CO_2 or headspace gas pressure are continuously monitored with fluorometric or colorimetric sensors, indicating growth of pathogens in the culture media.	Whole blood	BACTEC™ FX/9000 series (Becton Dickinson, USA)
	Whole blood	BacT/ALERT series (bioMérieux, France)
	Whole blood	VersaTREK (Thermo Fisher Scientific, USA)
Phenotypic-based characterization (automated systems)		
Pathogen identification from pure bacterial/fungal culture by screening biochemical properties, including antimicrobial susceptibility evaluation.	Pure bacterial/fungal culture from positive blood culture	VITEK® (BioMérieux, France)
	Pure bacterial/fungal culture from positive blood culture	BD Phoenix™ (Becton Dickinson, USA)
	Pure bacterial/fungal culture from positive blood culture	Microscan WalkAway® (Siemens Healthcare Diagnostics, Germany)
Protein-based characterization (automated systems)		
Pathogen identification from pure bacterial/fungal culture by screening of proteins with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).	Pure bacterial/fungal culture from positive blood culture	Flex™ MALDI-TOF series (Bruker, Germany)
	Pure bacterial/fungal culture from positive blood culture	VITEK® MS (BioMérieux, France)
	Pure bacterial/fungal culture from positive blood culture	AXIMA (Shimadzu Corporation, Japan)

Nucleic acid- and hybridization-based assays		
Pathogen identification by fluorescent *in situ* hybridization (FISH)-based technology, where fluorescently labeled probes are hybridized to conserved rRNA sequences.	Positive blood culture	PNA-FISH (AdvanDX, USA)
	Positive blood culture	HemoFISH assays (miacom, Germany)
Pathogen identification by hybridization protection assay (HPA) technology and analysis with Hologic Gen-Probe's luminometers.	Positive blood culture	AccuProbe (Gen-Probe Inc, USA)
Pathogen identification based on oligonucleotides attached to gold nanoparticles followed by hybridization on microarray.	Positive blood culture	Verigene® assay (Nanosphere Inc, USA)
Nucleic acid- and amplification-based assays		
Pathogen identification by multiplex real-time PCR assay.	Whole blood	LightCycler® SeptiFast Test MGRADE (F. Hoffmann-La Roche, Germany)
	Whole blood	MagicPlex (SeeGene, Korea)
	Positive blood culture	Gene Xpert MRSA/MSSA assay (Cepheid, USA)
Pathogen identification by multiplex PCR followed by gel electrophoresis analysis.	Whole blood	VYOO® (Sirs-Lab, Germany)
Pathogen identification by broad-range PCR followed by sequencing analysis.	Whole blood	SepsiTest® (Molzym GmbH & Co., Germany)
Pathogen identification by multiplex PCR followed by electrospray ionization mass spectrometry (ESI-MS) analysis.	Whole blood and positive blood culture	PLEX-ID (Abbott Ibis Bioscience, USA)

(References: www.bd.com; www.biomerieux.com; www.trekds.com; www.medical.siemens.com; www.bruker.com; www.shimadzu.com; www.advandx.com; www.miacom-diagnostics.com; www.gen-probe.com; www.nanosphere.us; www.roche.com; www.seegene.com; www.cepheid.com; www.sirs-lab.de; www.molzym.com; www.ibisbiosciences.com).

1.2.1 Blood culture as a gold standard method

Blood culturing is the current gold standard method for determination of causative agents in case of sepsis and BSI. Blood sample is taken from the patient and inoculated in aerobic and anaerobic blood culture bottles containing suitable growth media for micro-organisms. According to Clinical and Laboratory Standards Institute (CLSI) guidelines for blood culture (2007), two to three different sets of blood cultures per septic episode are recommended. During the 24 hour period, no more than three sets are typically needed (Ntusi *et al.*, 2010). The blood volume inoculated to the blood culture bottle varies between manufacturers. According to CLSI (2007), suitable sample volume would be 20-30 mL from adults per culture and no more than 1 % of infant's total blood volume. Typical dilution ratio of blood in broth is $\geq$ 1:5 and maximum blood volume varies between bottle types, starting from 10 mL (CLSI, 2007; Reimer *et al.*, 2005; Ntusi *et al.*, 2010). Nowadays most of the laboratories use automated blood culture systems in which fluorometric or colorimetric sensors continuously monitor bottles. The detection of positive reaction is based on either increasing CO_2 production or headspace gas pressure. Examples of well-established automated blood culture systems are BACTEC™ FX/9000 series (Becton Dickinson, USA), BacT/ALERT series (bioMérieux, France) and VersaTREK (Thermo Fisher Scientific, USA) (CLSI, 2007; Weile and Knabbe, 2009). Blood culture bottle incubation time varies, but a large portion of pathogens can be detected after 24 hours incubation. Nearly 100 % of pathogens can be detected after 4-5 days incubation and some recommendations range up to 7 days before blood culture is classified as negative if no growth has been detected (Coccerill III *et al.*, 2004; Ntusi *et al.*, 2010).

After a blood culture has been flagged positive, further investigation of the causative agent is performed with subculturing on appropriate media and investigating morphological features and cell wall characterization by staining and microscopy (*e.g.* Gram stain, Ziehl-Neelsen stain) (CLSI, 2007; CLSI, 2011). In addition, antimicrobial susceptibility evaluation is performed together with subculturing. CLSI (2011) guidelines list the most typical microbes and antimicrobial resistances which should be tested in a routine laboratory. For example MRSA findings are increasing and when *Staphylococcus* spp. has been detected from the sample, oxacillin susceptibility testing is recommended (CLSI, 2011).

1.2.2 Phenotypic-based characterization of microbes

Rough characterization of pathogens by staining and microscopy may already guide clinicians to revise antimicrobial treatment. Typically, species identification is still achieved by culturing on appropriate media followed by pattern of biochemical tests such as catalase production and oxidase reaction. These tests give an overview of bacterial biochemical properties and can be used for identifying bacteria. Biochemical test may be

performed as single manual tests but several automated systems are commercially available allowing high-throughput analysis. Examples of well-established automated systems are VITEK® (BioMérieux, France), BD Phoenix™ (Becton Dickinson, USA) and Microscan WalkAway® (Siemens Healthcare Diagnostics, Germany). Most of the systems perform pathogen identifications by screening biochemical properties and antimicrobial susceptibility testing by serial dilution (Houpikian and Raoult, 2002; Klouche and Schröder, 2008; CLSI, 2007).

Phenotypic methods have been used as a standard microbiological procedure for pathogen identification from positive blood culture. Although it is used as a reference method when new technologies are developed, some limitations can be still identified. Phenotype-based characterization requires pure bacterial culture which prolongs the time to identification especially in case of slow growing and fastidious bacteria. Old cultures may not show typical biochemical patterns as expected, and variation may be found between different strains from the same species, which may affect the accuracy of species-level identification. Ongoing antimicrobial therapy may affect the typical biochemical properties of pathogens. Databases used for interpretation and comparison of pathogens' biochemical properties may also contain limited number of species (Kim *et al.*, 2008, Weile and Knabbe, 2009).

In addition to investigating bacterial biochemical properties, immunoassays allow the detection of antigens or presence of specific antibodies raised in response to pathogen antigens. Several immunoassay formats such as enzyme immunoassays, immunofluorescent assays and latex agglutination assays are available (Weile and Knabbe, 2009; Houpikian and Raoult, 2002). MRSA-Screen latex agglutination test (Denka Seiken Co., Ltd., Japan) is one of the immunoassays used for screening MRSA. The assay uses a monoclonal antibody for the detection of PBP2a and the results are obtained in around 20 minutes. The sensitivity has been shown to be at a sufficient level for accurate detection (Atay and Gülay, 2004).

1.2.3 Protein-based characterization of microbes

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been originally used as a research tool for protein analysis and it's use has recently emerged also in clinical microbiology and sepsis diagnostics. Pathogens from positive blood culture are subcultured and growth colonies can be used for analysis by MALDI-TOF MS. During sample analysis, the device forms a mass-to-charge ratio spectrum with peaks indicating the molecular masses and charge densities of components present in a biological sample. The measured peaks, generated from ionization of highly conserved proteins are compared against the reference spectra of the integrated database provided by the manufacturer. Species and genus identification is based on the comparison of peaks to reference spectra. Scores calculated by comparing the spectra

indicate the confidence of identification (Cherkaoui *et al.*, 2010; Kaleta *et al.*, 2011). Analysis does not require operation with batches and the method is considered to be fast. Reagent costs are low but a device investment is expensive. Subculturing is still required which lengthen the analysis time but development is also ongoing to use blood culture as a sample type. Some limitations have been found with the sensitivity and specificity in case of multi-infection samples, as well as with the coverage of target panel. Also, evaluations of antimicrobial susceptibilities are still limited. Well known MALDI-TOF MS manufacturers are *e.g.* Bruker (Germany), BioMérieux (France) and Shimadzu Corporation (Japan) (Cherkaoui *et al.*, 2010; Kaleta *et al.*, 2011; La Scola and Raoult, 2009).

1.2.4 Pathogen identification by nucleic acid- and hybridization-based assays

NA- and hybridization-based assays require a large number nucleic acids of target cells and therefore are often targeted to ribosomal ribonucleic acids (rRNA) molecules which are present in high copy numbers per cell (Weile and Knabbe, 2009; Klouche and Schröder, 2008). Fluorescence- and chemi-luminescence-based assays are available for pathogen identification from positive blood culture samples. These include PNA-FISH (AdvanDX, USA), HemoFISH assays (miacom, Germany) and AccuProbe (Gen-Probe Inc, USA) (Asfari *et al.*, 2012; Peters *et al.*, 2004; Miacom Diagnostic, 2011). The most commonly used hybridization-based assay utilizes a fluorescent *in situ* hybridization (FISH) technology, which is based on fluorescently labeled species specific probes hybridizing to conserved regions of rRNA in bacterial cells. Different probes are labeled with different fluorochromes and when the hybridized sample is viewed under a fluorescence microscope, pathogens can be distinguished based on the fluorescence signals (Bauerfiend *et al.*, 2012; Miacom Diagnostic, 2011, Harris and Hata, 2013). While studies using PNA-FISH have shown that identification is achieved faster than with conventional methods, some limitations are still identified. Depending on the diagnostic need, a suitable kit detecting a certain number of pathogens can be chosen based on Gram-staining results. Thus, results might be needed simultaneously from several different FISH-based assays since one assay may identify only few targets. There also might be sensitivity problems in case of slow growing and fastidious organism since PNA-FISH requires at least 10^5 colony forming units (CFU) per mL for positive detection (Harris and Hata, 2013).

1.2.5 Pathogen identification by nucleic acid- and amplification-based assays

Polymerase chain reaction (PCR) is maybe the most common amplification method used in molecular assays. The starting material for PCR is deoxyribonucleic acids (DNA) and therefore many complex sample types, such as blood culture and tissue require sample preparation and NAs extraction steps before amplification. Two types of primers are generally used: species specific primers targeted to certain bacterial or fungal gene areas or to genes responsible for resistances, and universal broad-range primers which are typically targeted to conserved gene regions, amplifying a high number of different pathogens using the same set of primers. Both primer strategies can be combined in multiplex PCR, where several gene targets are amplified in the same reaction (Peters *et al.*, 2004; Dark *et al.*, 2009). Typical genes and regions which are generally used for taxonomical characterization, and also in many commercial assays, are 16S rRNA, *gyrB/parE* genes and internal transcribed spacer (ITS) region. These contain highly conserved areas flanking variable areas for accurate distinguishing of bacterial or fungal species (Wellinghaussen *et al.*, 2009; Casalta *et al.*, 2008; Metso *et al.*, 2013). Ribosomal 16S rRNA gene and ITS region are present in high copy numbers in cells. *gyrB/parE* are single-copy genes encoding small subunits of type II and IV topoisomerases, respectively, which regulate the over- or underwinding of DNA during the replication period (Forterre *et al.*, 2006; Soraya *et al.*, 2008).

Two types of PCR assays are available; real-time PCR and conventional end-point PCR assays. Real-time PCR enables detection and simultaneous quantification of targeted DNA molecules during amplification, representing the key advantage of these assays. Amplified products are labeled either with non-specific fluorescent dyes (*e.g.* SYBR green) which binds to the any double stranded DNA (dsDNA) or labeled probes which hybridize to a specific sequence of the target organisms (*e.g.* molecular beacons, Taqman probes). Several probes with different fluorochromes may be used for differentiation of target organisms in the same reaction. Result interpretation is based on fluorescent signal monitored during the amplification. Well-studied multiplex real-time PCR assays directed to the identification of sepsis causing bacteria from whole blood samples are LightCycler® SeptiFast Test MGRADE (F. Hoffmann-La Roche, Germany) and MagicPlex (SeeGene, South-Korea). In addition, one example of multiplex real-time PCR assays using positive blood culture sample type is Gene Xpert MRSA/MSSA assay (Cepheid, USA) (Dark *et al.*, 2009; Josefson *et al.*, 2011; Heid *et al.*, 1996).

Conventional end-point PCR is a standard PCR reaction, containing either species-specific or broad-range primers for amplification. The end product of the PCR reaction are dsDNA or single stranded DNA (ssDNA) amplicons, which can be further analyzed by a detection method such as gel electrophoresis, sequencing, hybridization on a microarray or electrospray ionization mass spectrometry (ESI-MS). Increasing numbers of alternative detection technologies are being developed with various advantages and result interpretation is dependent on the used method (Afshari *et al.*, 2012; Klouche and

Schröder, 2008; Mancini *et al.*, 2010). Some protocols may be time-consuming and require educated/skilled personnel (Dark *et al.*, 2009). Examples of end-point PCR assays using different detection technologies are VYOO® (Sirs-Lab, Germany), SepsiTest® (Molzym GmbH & Co., Germany) and PLEX-ID (Abbott Ibis Bioscience, USA).

VYOO® is a PCR and gel electrophoresis assay directed to whole blood samples. Gel electrophoresis enables size-based separation of different fragments and indicates the success of amplification step (Fitting *et al.*, 2012). Amplicons can be further analyzed by sequencing and sequence homology searches for identification of the pathogen. SepsiTest® utilizes gel electrophoresis and sequencing technology from whole blood samples (Wellinghausen *et al.*, 2009). Amplicon analysis by PCR-ESI-MS is a new detection method in sepsis diagnostic. PLEX-ID is a PCR-ESI-MS device following the same principle than MALDI-TOF but instead of analyzing proteins, the device uses amplicons for characterization. The mass to charge ratios of PCR amplicons are measured and the obtained spectrum is compared to a reference database for pathogen identification. The system uses both culture and whole blood sample types and it has been also used for epidemiological purposes (Kaleta *et al.*, 2011; Afshari *et al.*, 2012; Soraya *et al.*, 2008).

1.2.5.1 DNA Microarray-based assays

Hybridization on a DNA microarray is one of the detection strategies for analysis after end-point PCR. This approach was also used in this study when molecular assays for sepsis diagnostics were developed. The key advantage of microarrays is the potential of simultaneous identification of a large panel of pathogens and detection of resistance markers (Soraya *et al.*, 2008). DNA microarrays contain DNA fragments or oligonucleotide probes which are immobilized onto a chemically modified solid surface such as a glass or silica slide. Depending on the amount of targets and oligonucleotide probes, arrays can be distinguished into high-density (around 10^4-10^6 probes) or low-density (around 100-1000 probes) arrays. Oligonucleotides are typically short 20-30 base pair (bp) long synthetic ssDNA products which are covalently attached to the surface for example via amino modifications in the 5'-terminus. One oligonucleotide probe may be printed on the microarray as duplicate or triplicate. This printing strategy can improve detection of target DNAs instead of unwanted interfering substances. The amount of replicates is however fully dependent on the detection strategy. Oligonucleotide probes are designed for variable regions of the gene target and several different specific probes may be designed per each target in order to confirm the detection of target organism (Cleven *et al.*, 2006; Ulyashova *et al.*, 2010; Cuzon *et al.*, 2012; Roth *et al.*, 2004; Weile and Knabbe, 2009).

After an amplification of target DNA from a sample, labeled ssDNA amplicons are hybridized with oligonucleotide probes using suitable conditions and reagents. Probes are printed on the microarray in a certain order and the pathogen can be identified when

hybridization is detected with the specific probes. Several studies have been published using microarray with colorimetric or fluorescent detection technology (Roth *et al.*, 2004; Cuzon *et al.*, 2012; Wiesinger-Mayer *et al.*, 2011). Shortly, one colorimetric detection method, which was employed also in this study, is based on biotin labeled DNA fragments which are hybridized with complementary probes on the microarray. During the conjugation step, streptavidin-horseradish peroxidase (HRP) conjugate binds to biotin. In the final precipitation step, HRP catalyzes the oxidation of the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) or its analogue inducing a blue reaction-product. The reaction is visualized by camera with a visible light source. Fluorescent detection is based on fluorochrome-labeled amplicons which are hybridized with probes on the microarray and detected with a fluorescence reader. Both detection technologies contain several carefully optimized steps which provide suitable conditions for hybridization, for example to decrease the interfering background signal level and promote good spot morphology for detection (Sauer *et al.*, 2009; Cuzon *et al.*, 2012).

Signal intensities from each hybridization complexes are calculated and compared to the background signal. Sophisticated analysis software is typically used for analysis of microarray images and interpretation of detected spots facilitated by built-in analysis rules. In optimal cases, identified pathogens or gene markers are reported without result interpretation by user. However, building complex functional analysis algorithms is time-consuming and many published studies report manual microarray result analysis (Wiesinger-Mayer *et al.*, 2011). An example of an assay utilizing hybridization technology for identification of pathogens from positive blood culture is the Verigene® assay (Nanosphere Inc, USA) (Anderson *et al.*, 2012).

1.2.5.2 Challenges in sample preparation in sepsis diagnostics

Sample preparation and NA extraction are critical steps in molecular assays, because efficient extraction is required for further NA analysis. Point-of-care (POC) assays contain sample processing and analysis in one closed system. However, a majority of molecular assays include only downstream analysis steps and a method for sample processing is needed separately (Anderson *et al.*, 2012; Weile and Knabbe, 2009).

Blood culture and whole blood are the main sample types for assays used for sepsis diagnostics. These sample types cause challenges to sample preparation and selection of an appropriate NA extraction method. The ability to disrupt microbial cell walls is important since Gram-positive bacteria as well as fungi contain cell walls which are harder to lyse. Sample material may contain low amounts of causative pathogens such as 1-30 CFU/mL in whole blood, and therefore recovery of microbial DNA in extraction should be high. Removal of inhibitors such as heme, anticoagulants (*e.g.* Ethylenediaminetetraacetic acid (EDTA)) and heparin from blood samples is important for amplification (Ecker *et al.*, 2010; Al-Sould *et al.*, 2000). Blood samples contain also

high amounts of human DNA which may interfere with the amplification of microbial DNA. Extraction methods designed to remove human DNA may increase the sensitivity of amplification. Suitable sample and eluate volumes should be optimized for the downstream application. Several user requirements need to be taken into account in assay design and development such as high reproducibility, required level of automation, throughput requirements, cost-effectiveness, user-friendliness and flexibility of methods (Horz *et al.*, 2009; Regueiro *et al.*, 2010).

Many automated high-throughput and semi-automated extraction devices are available such as NucliSENS®easyMAG® (bioMérieux, France), MagNA Pure LC (F. Hoffmann-La Roche, Germany), EZ1® (Qiagen, Germany) and NorDiag Arrow (NorDiag, Norway). These devices employ different extraction kits for different purposes. Also manual kits for lower sample throughput are available. Extraction methods typically utilize chaotropic agents for lysis and silica particles, magnetic beads or silica columns for binding of released NAs (Wiesinger-Mayer *et al.*, 2011, Bergman *et al.*, 2013; Brownlow *et al.*, 2012). Typically these extraction solutions, evaluated for blood or blood culture sample material, extract total NAs including human and microbial NAs from the clinical sample. Only few methods have concentrated on the separation and extraction of microbial DNA from total NAs. Molzym GmbH & Co. (Germany) is one company offering manual and semi-automated solutions for microbial DNA extraction from whole blood samples. The method first enzymatically degrades human DNA and then extracts microbial DNA from concentrated microbial cells (Wiesinger-Mayer *et al.*, 2011).

1.2.6 Comparison of gold standard method with novel technologies

A high amount of rapid assays have been developed for the detection of causative pathogens from patients with suspected sepsis or BSI. None of those assays have replaced the current gold standard blood culture method but are valuable tools, especially when directed to certain groups of patients under higher risk (Paolucci *et al.*, 2010; Soraya *et al.*, 2008). In situations where the conventional method fails to identify the causative agent, molecular assays may enable faster and more targeted management of patients. Genotype-based characterization has already opened a new era and brought broadened perspective to the conventional culturing method in microbiological laboratories (Mancini *et al.*, 2010; Soraya *et al.*, 2008; Klouche and Schröder, 2008).

The main disadvantages of current blood culturing and further subculturing method are the delay of results and low sensitivity in case of slow growing bacteria and fungi. Occasionally, if the patient does not respond to initial therapy, the clinician may need to change the empirical therapy before the blood culture has turned positive (Paolucci *et al.*, 2010). Around 30-40 % of all blood cultures taken for the diagnosis of BSIs turn positive and large amounts remain negative even if there is a strong clinical suspicion of infection (Klouche and Schröder, 2008). False negative blood cultures may be due to the previous

use of antimicrobial treatment, insufficient volume of blood cultured, fastidious and slow growing pathogens or for example bacterial production of toxins, such as toxic shock syndrome toxin 1 by *S. aureus* or pyrogenic toxins by *S. pyogenes* (Liang *et al.*, 2013; Ross and Onderdonk, 2000; Carrigan *et al.*, 2004; Nguyen *et al.*, 2006; Klouche and Schröder, 2008). Some bacteria are sensitive to growth conditions and inappropriate pre-analytic handling may disturb exponential growth. Studies have also reported problems with skin flora contamination in the blood culture bottles. The fraction of false positives depends on the used system or media and may range from 0.6 % to over 6 % (Klouche and Schröder, 2008; Hall and Lyman, 2006). However, blood culture is still a valuable method in the detection of microbes and after bacteria have been detected from blood culture bottles, susceptibility testing can be started and suitable therapy administered (Klouche and Schröder, 2008; Dark *et al.*, 2009). Lots of effort has been put to improve blood culture method by developing higher level of automation and new growth media with inhibitor-neutralizing agents. Correct timing in taking blood samples, suitable blood volume and sufficient amount of blood samples taken per set may increase sensitivity and detection of real causative agents instead of contaminants (Paolucci *et al.*, 2010; Hall and Lyman, 2006; Coccerill III *et al.*, 2004).

The advantage of molecular assays is the time benefit in the identification of pathogens, which is critical for appropriate antimicrobial therapy. Dependent on the panel of used molecular assays, typically most of the important pathogens and resistance markers can be screened within one analysis. Molecular assays can also detect slow growing and fastidious organism which typically require several days of culturing by the conventional method. NA assay does not necessarily require viable microbes, enabling detection of pathogen DNA after antimicrobial treatment and identification of autolysed pathogens (*e.g. S. pneumoniae*) (Paolucci *et al.*, 2010; Martner *et al.*, 2009). However, analysis with molecular assays is not always unambiguous. Interpretation of findings may be difficult and the significance of pathogen DNA as a marker of infection is under investigation. Complicating the interpretation, there is a lack of reference method especially when assays are performed directly from whole blood (Dark *et al.*, 2009; Paolucci *et al.*, 2010). Furthermore, an adequate sensitivity is difficult to achieve due to the low pathogen concentrations in whole blood. Some assays have difficulties also in differentiating pathogens in the case of multi-infections (La scola and Raoult, 2009).

Molecular assays face additional problems such as environmental contaminants (*e.g.* skin contaminants), bioburden coming from reagents (*e.g.* genomic DNA from host bacteria during the production of polymerases) or manufacturing processes (Ecker *et al.*, 2010; Mühl *et al.*, 2010). Strict rules and guidance for collection and preparation of samples could lower the level of contaminants. Lots of work has also been done to improve industrial processes to develop DNA-free reagents. In addition, clinical relevance of DNAemia/circulating DNA or negative results by molecular assays and their influence on patient condition are under intensive investigation (Dark *et al.*, 2009; Klouche and Schröder, 2008; Peters *et al.*, 2004).

Cost issues are always a question since traditional culturing method is relatively cheap and molecular assays typically bring higher costs. Some new assays for sepsis diagnostics require also expensive initial device investment. However, every additional day in hospital ward causes substantial costs and thus novel methods, bringing earlier results, can decrease the length of stay and related cost in hospital (Agnus *et al.*, 2001). Lodise and co-workers (2003) demonstrated that a delay in appropriate antimicrobials for *S. aureus* bacteremia by over 45 hours increased length of hospital stay from 14 to 20 days. Also Davis and co-workers (2012) presented in their cost-benefit analysis that longer patient treatment in hospital causes higher expenses.

1.3. Validation of new assays for diagnostics: regulation and legislation

New assays for diagnostics are designed and developed according to the *in vitro* diagnostic (IVD) regulations. IVD products offered for sale in EU member countries are required to conform to IVD directive requirements and to be IVD-*Conformité Européenne* (CE) marked. Generally, IVD tests are medical devices, including reagents, instruments *etc.* which are intended to be used for the examination of human specimens (*e.g.* blood, tissue) and producing medically important information. CE mark represents conformity of the IVD product to the *in vitro* diagnostic medical device directive (IVDD) 98/79/EC. Requirements for attaining CE marking include particular verification and validation of technical characteristics. Demonstration of performance must include for example sensitivity and specificity determinations, accuracy, repeatability, reproducibility, including control of known relevant interference, and limit-of-detection determinations. The 98/79/EC directive specifically defines important terms and its purpose is to ensure that only safe and functional products are placed on the market in EU countries. EC declaration of conformity is the procedure whereby the manufacturer who fulfills the requirements of IVDD declares that the product meets all the provisions which apply (Directive 98/79/EC, 1998).

Manufacturers often follow harmonized international standards which help to meet the specified requirements for IVDs, such as International Organization for Standardization (ISO) 9001 and ISO 13485 standards. ISO 9001 is a quality management system standard applicable to many industries. ISO 13485 is a standard specific to medical device quality management systems. Being ISO 13485 certified is the industry standard of ensuring and communicating that the quality management system of the company complies with 98/79/EC directive (SFS-EN-ISO-9001, 2008; SFS-EN-ISO-13485, 2004).

In the US, similar regulations and guidance are implemented by the US Food and Drug Administration (FDA). In contrast to the separate regulations of medical devices and IVD directives in Europe, the same medical device regulations apply to IVD's as any other medical device in the US. FDA classifies IVD's based on medical risk to Class I-III,

where Class I contains low risk devices and Class III contains high risk devices. Standard ISO 13485 is also recognized by FDA and Quality System Regulations (21CFR Part 820) are consistent with ISO 13485. In general, FDA guidance is more prescriptive than the EU IVDD and can be used in the development of an IVD for Europe (FDA, 2013; Kimmelman, 2003). When new IVD assays are developed, both EU IVDD and FDA regulations should be considered carefully with regard to intended market scope.

1.3.1 Estimation of sensitivity and specificity

CLSI is an international organization which develops worldwide recognized consensus standards and guidelines for patient testing and related health care issues. It has produced guidelines for qualitative diagnostic tests in order to standardize the experimental details as well as the data analysis of qualitative information. When new diagnostic assays are developed, validation can proceed according to these CLSI guidelines. These guidelines were also followed in the clinical validation of the molecular assay developed in this study.

According to the guidelines (CLSI, 2002; CLSI, 2006; CLSI, 2008), comparison of results should be made against a reference (gold standard) method and optionally also other methods which are in routine use in the laboratory. When results are compared with patient samples whose clinical diagnosis is known, the results should be treated separately from the comparison results with the reference method. Comparison of the results from test method and reference procedure allows categorizing the data into true positive (TP), true negative (TN), false positive (FP) and false negative (FN) results. The result of the tested method is TP if test result gives the same positive identification as the reference test result. Similarly, the result is TN if both the test result and the reference result are negative. The result of the tested method is FP if the test result is positive and the reference result is negative. Similarly, the test method result is FN if the test result is negative while the reference result is positive. If the clinical diagnosis for the patient specimen is known, the obtained test results may be separately compared with the clinical diagnosis and separate values calculated thereby. These values are used for the estimation of parameters such as specificity and sensitivity for the assay. Formulas for calculating sensitivity and specificity are presented below (CLSI, 2002; CLSI, 2006; CLSI, 2008).

Estimated sensitivity = TP / (TP + FN) 100 %

Estimated specificity = TN / (TN + FP) 100 %

During the evaluation of the test method, samples are typically a selection of the specimens and it may cause variability to the measures. This variability can be estimate quantifying confidence limits. CLSI guidelines (2002; 2006; 2008) recommend using score confidence limits for calculation and the formula for 95% score confidence interval (CI) for sensitivity and specificity is described below. Number 1.96 is the quantile from standard normal distribution that corresponds to 95% confidence.

95% score confidence interval = 100 % ($Q_1 - Q_2$)/ Q_3 and 100 % ($Q_1 + Q_2$)/ Q_3

For sensitivity calculations

$Q_1 = 2TP + 1.96^2$

$Q_2 = 1.96\sqrt{1.96^2 + \frac{4\,TP * FN}{TP + FN}}$

$Q_3 = 2\,(TP + FN + 1.96^2)$

For specificity calculations

$Q_1 = 2TN + 1.96^2$

$Q_2 = 1.96\sqrt{1.96^2 + \frac{4\,FP * TN}{FP + TN}}$

$Q_3 = 2\,(FP + TN + 1.96^2)$

2 Aims of the study

Bacterial sepsis is a life-threatening disease with high rates of morbidity and mortality. At the moment, blood culturing is the gold standard method for determining the causative bacteria for sepsis. The procedure is highly manual and bacterial identification together with antimicrobial sensitivity profiling requires typically 2-5 days. To improve diagnostics and start species specific therapy earlier, new approaches are needed for rapid identification of sepsis causing bacteria. The main goal of this study was to develop and optimize a PCR- and microarray-based assay for *in vitro* diagnostic use for identification of sepsis and/or BSI causing bacteria. The study focused also to review DNA extraction methods suitable for the developed diagnostic assay. Another aim was to evaluate the PCR and microarray assay's suitability for identification of pathogens directly from whole blood samples without a culturing step.

The specific aims of this study were to:

- ✓ Develop a PCR- and microarray-based assay for the detection of sepsis causing bacteria using clinical isolates. Evaluate the performance of the assay with blood culture samples and demonstrate proof-of-concept (Publication I).

- ✓ Improve the bacterial panel of the assay to cover the important sepsis causing bacteria and clinically validate the assay's suitability to sepsis diagnostics using blood culture samples (Publication II).

- ✓ Improve the assay's ability to rapidly analyze large sample set by optimizing the assay to a higher-throughput platform allowing a run of 1- 96 samples at a time (Publication III).

- ✓ Investigate the efficiency of sample preparation methods for extraction of bacterial DNA from blood culture samples (Publication III).

- ✓ Evaluate the suitability of the developed PCR and microarray assay to detect sepsis causing bacteria directly from whole blood (Publication IV).

3 Materials and methods

Blood culture samples and clinical isolates used in this study are described in detail in the original publications. The division of these samples is presented in Table 3. A total of 3595 blood culture samples were freshly collected and analyzed. 2716 samples were collected from Helsinki University Hospital Laboratory (HUSLAB), Helsinki, Finland (Publications I, II and III), and 879 samples from University College London Hospital (UCLH), London, UK (Publication II). Blood samples were obtained from patients with suspected sepsis and were further cultured in aerobic or anaerobic blood culture bottles according to the standard laboratory protocols in those laboratories. 2344 of these samples were blood culture positive and 1251 were blood culture negative.

Clinical isolates were collected from American Type Culture Collection (ATCC, US), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany), HUSLAB (Finland) or from the culture collection of Mobidiag Oy, (Finland). A total of 156 clinical isolates were analyzed during the study. Tables 2 and 3 from original publication I and Tables 2 and 3 from original publication IV present the clinical isolates used in those papers. All the methods used in this study are described in detail in the indicated articles and are summarized in Table 4.

Table 3. *Blood culture samples and clinical isolates used in this study.*

Publications	Sample type	Number of samples
I	**Blood cultures**	**186**
	Positive blood cultures	146
	Negative blood cultures	40
	Clinical isolates	**129**
II	**Blood cultures**	**3318**
	Positive blood cultures	2107
	Negative blood cultures	1211
	Clinical isolates	**-**
III	**Blood cultures**	**91**
	Positive blood cultures	91
	Negative blood cultures	0
	Clinical isolates	**1**
IV	**Blood cultures**	-
	Positive blood cultures	-
	Negative blood cultures	-
	Clinical isolates	**26**

Table 4. *Methods used in the study. The Roman numerals refer to the original articles in which the methods were applied.*

Methods	Publications
Bacterial culturing	
Culturing on agar plates	I, III, IV
Blood culturing	I, II, III
DNA extraction	
Manual boiling method	I, IV
Extraction by NucliSENS® easyMAG® device	I, II, III, IV
Extraction by NorDiag Arrow device	
- Viral NA kit	III
- Blood pathogen DNA isolation kit	IV
Spectrophotometer measurements	
Optical density (OD)	III, IV
DNA concentration and purity	III, IV
Amplification	
Primer design	I
PCR amplification	I, II, III, IV
Real-time PCR for *E. coli*	III
Microarray experiments	
Capture oligonucleotide design	I, II
Manufacturing of microarray and spotting of capture probes §	I, II
Hybridization onto TubeArray	I, II
Hybridization onto StripArray	III, IV
Design of target identification rules	I, II
Other DNA analysis methods	
Sequencing	I, II, III
Image analysis by agarose gels	I
Analysis by reference gold standard method	
Blood culturing and phenotypic based characterization	I, II, III
Antimicrobial susceptibility evaluation	I, II, III
Assay sensitivity / specificity calculations	I, II, IV

§ Construction of TubeArray and StripArray microarrays and spotting of oligonucleotides were performed by Clondiag GmbH (Germany) using their standard protocol for microarray manufacturing. Oligonucleotides were 5'-amino-modified prior to spotting on the microarray.

4 Results and discussion

4.1 Development of multiplex-PCR and microarray assay for sepsis diagnostics

4.1.1 Set up of PCR and microarray assay (I)

In this study, the assay which consists of an end-point PCR followed by hybridization and colorimetric detection on a low-density microarray for identification of bacteria was designed. Broad-range bacterial primers targeted to conserved regions of the *gyrB* and *parE* genes were modified using inosines from the primers introduced originally by Roth and co-workers (2004). In addition, species specific primers targeted to *S. aureus gyrB/parE* genes and specific primers targeted to the *mecA* gene were designed and added to the PCR reaction. The optimized multiplex-PCR protocol for these primers produced ssDNA during the amplification step. dsDNA was exponentially produced in the first PCR phase followed by ssDNA production in the second PCR phase. A slightly similar type of PCR method was introduced by Zhu and co-workers (2007). The performance of primers described by Roth and co-workers (2004) and re-designed *gyrB/parE* primers was compared by gel electrophoresis method and sensitivity was shown to be at least on the same level for the bacteria included in the assay panel. Also the production of ssDNA by the re-designed *gyrB/parE* primers was demonstrated (Publication I: Fig. 1).

Oligonucleotide probes were printed as duplicates onto a microarray placed at the bottom of a 2 mL tube (Prove-it™ TubeArray, Figure 1). Since ssDNA was produced during the PCR reaction, hybridization could be performed straight after the PCR step and common methods of ssDNA production such as alkaline, heat treatment or asymmetric PCR were avoided (Gao *et al.*, 2003; Gyllensten and Erlich, 1988). The colorimetric hybridization protocol was optimized carefully for efficient hybridization on the microarray. Positive hybridization spots were detected by a reader instrument including camera and light source (Prove-it™ TubeArray reader, Figure 1). Target identification rules based on spot detection were tested and implemented to the analysis software Prove-it™ Advisor (Figure 3). These identification rules and spot detection threshold values were also evaluated and improved during the optimization of the assay.

Approximately 20 bp long probes were targeted to the *gyrB/parE* or *mecA* genes. The cross-hybridization with sequences of non-targeted bacteria were attempted to avoid using various *in silico*- parameters during the design of the probes after which the accuracy of the probes were evaluated empirically. Species specific oligonucleotides were successfully designed for the detection of *A. baumannii, E. faecium, Enterococcus faecalis, H. influenzae, K. pneumoniae, Listeria monocytogenes, N. meningitidis, S. aureus, S. epidermidis, Streptococcus agalactiae, S. pneumoniae and Streptococcus*

pyogenes. Genus-specific probes were designed for selected CNS species and in addition, *mecA* specific probes were included for detection of methicillin-resistance (Publication I: Table 1). The chosen target panel contained pathogens causing severe infections such as sepsis and also common resistant bacteria (Vincent *et al.*, 2006; Trouillet *et al.*, 1998).

In order to evaluate the specificity of the designed oligonucleotide probes and hybridization protocol, 102 clinical isolates of 70 untargeted bacteria comprising clinically relevant bacterial species, closely related species of targeted bacteria and also bacteria of human normal flora were tested (Publication I: Table 3). Amplicons were hybridized to the microarray and the reported identifications by Prove-it™ Advisor were compared to the known identification data of the samples. The cross-reaction study reported only one discrepant result when *K. pneumoniae* subsp. *ozenae* was reported as *K. pneumoniae* subsp. *pneumoniae*. In conclusion, the results showed high specificity for the PCR and microarray assay.

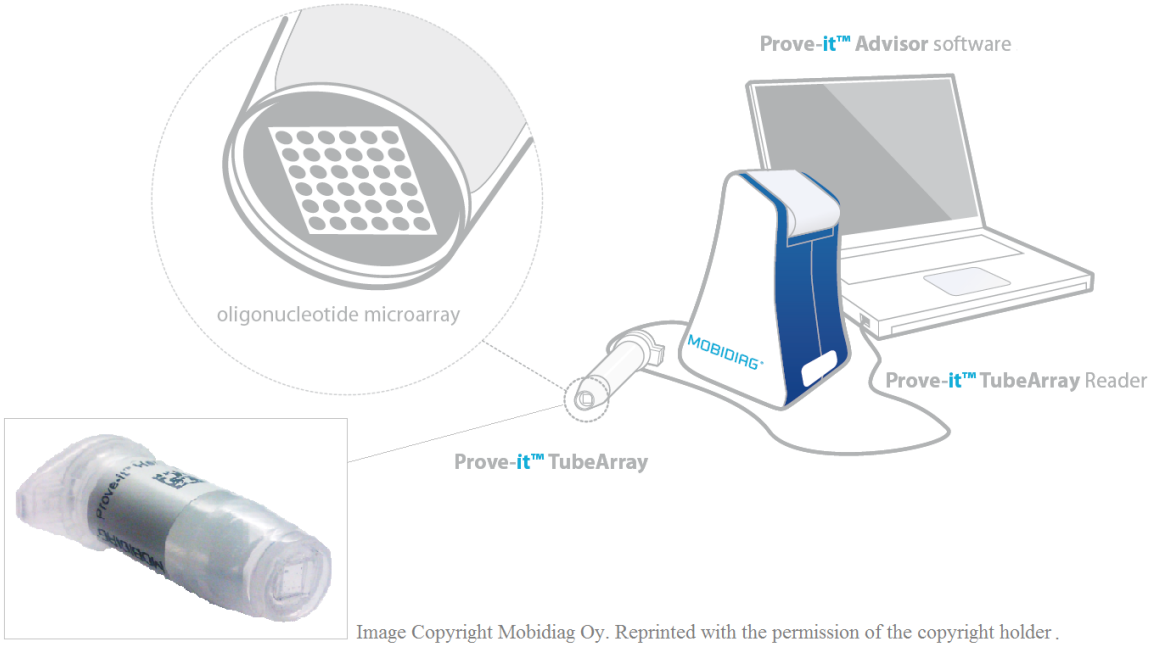

Figure 1 *The microarray platform including a low-density microarray with oligonucleotide probes at the bottom of a 2 mL tube (Prove-it™ TubeArray) and specific colorimetric reader for detection of hybridization reactions from the microarray. Identification of target(s) is based on built-in analysis rules and is reported by the Prove-it™ Advisor software.*

4.1.2 Performance evaluation and proof-of-concept of the assay (I)

In order to evaluate the proof-of-concept and performance of the designed end-point PCR and microarray assay for the detection of sepsis causing bacteria, a set of 186 blood culture samples, including 146 positive and 40 negative blood cultures from HUSLAB

(Finland) were blindly tested. DNA from the samples was first extracted by the NucliSENS®easyMAG® device followed by the PCR and microarray analysis.

Bacteria were identified originally from 69 positive blood cultures by the PCR and microarray assay. The most frequently isolated bacteria were *S. aureus, E. faecalis, S. epidermidis* and *K. pneumoniae*. These microbes are common causative agents in sepsis (Beekman *et al.*, 2003; Schmitz and Lehmann, 2011). The assay reported negative results from 117 blood cultures, of which 40 were blood culture negative samples (Publication I: Table 4). The negative result was correctly reported from species such *Bacillus* sp., *E. coli,* and *E. cloacae* which were not included in the target panel of the PCR and microarray assay.

Results were compared to culturing and 17 discrepant results were observed (Publication I: Table 4). In the two false positive samples, the microarray assay detected the *mecA* gene marker with the atypical species *S. pneumoniae* and *E. faecalis* for an undetermined reason. However, comparison of the results with the reference method showed that the bacterial identifications (*S. pneumoniae* and *E. faecalis*) were similar with the blood culture results. Six samples reported as false negative contained CNS species not included in the CNS panel of the microarray assay and thus were determined to be true negatives. In three samples, the assay did not detect *S. pyogenes, S. aureus* or *S. epidermidis* for undetermined reason. Those samples were thus calculated as false negatives. DNAs from these three samples were also amplified by 16s rDNA PCR, but no amplifications were detected indicating possible PCR inhibitors or degraded DNA in the samples.

In addition, six samples containing either *S. agalactiae, S. epidermidis, S. pneumoniae, E. faecalis, E. faecium,* or *S. aureus* were originally reported as false negative caused by either one fully negative probe hybridization signal or one weaker probe hybridization signal. Other duplicates and probes were correctly detected by the Prove-it™ Advisor analysis software, but the results remain under the positive identification criteria and thus, samples were reported as negative. When evaluating less precise identification rules, all of these samples were identified successfully by the microarray assay. These results were also in line with blood culturing results and thus were determined to be true positives when calculating the final specificity and sensitivity values. Evaluating the suitable spot detection threshold and building optimal detection rules for accurate identification of all pathogens by microarray is very challenging and requires several revise cycles (Imbeaud and Auffray, 2005). Such adjustments after the analysis would not be appropriate in a diagnostic assay and thus accurate pathogen identification should be improved by designing new oligonucleotide probes, experimentally setting the best-functioning detection threshold values and formulating efficient identification rules.

Based on the results the initial assay sensitivity was calculated to be 82 %. However, when the described adjustments in the identification of the six samples were done, the final sensitivity of the assay was determined to be 96 % (95 % CI 89.0-98.6 %). Specificity was calculated to be 98 % (95 % CI 93.6-99.5 %) (Table 6). These values

indicated good performance for the designed PCR and microarray assay. Similar results were introduced also by Wiesinger-Mayr and co-workers (2007) with a comparable method, achieving 100 % specificity on the genus level and 97 % on the species level using reference strains and clinical isolates.

In conclusion, proof-of-concept was successfully demonstrated for the PCR and microarray assay. However, the bacterial panel of the assay was somewhat limited and did not cover all important sepsis causing organisms such as *P. aeruginosa* and relevant species from the *Enterobacteriaceae* group including *E. coli* (Beekman *et al.*, 2003; Schmitz and Lehmann, 2011) Thus, an optimal assay with an optimized oligonucleotide probe panel and carefully selected analysis algorithms would increase suitability of the assay for diagnostic use and obviate any manual result interpretation or *post hoc* adjustment.

4.1.3 Extension of target panel and clinical validation of the assay (II)

In order to improve the target panel of the evaluated end-point PCR and microarray assay, new oligonucleotide probes were designed for the detection of over 50 relevant Gram-negative and Gram-positive bacterial species, 24 on species and 26 on taxon level (Table 5). The hybridization protocol was also optimized to be more rapid, easier to use and suitable for the new probe content. The improved assay was clinically validated in two hospitals in Europe, in HUSLAB, Finland and UCLH, London. A set of 3318 blood culture samples were collected in these two laboratories during 2008. Samples were analyzed simultaneously by the improved PCR and microarray assay (named Prove-it™ Sepsis) and conventional culturing method in both laboratories. The validation study is presented in detail in the original publication II. Five samples were excluded from the sample set due to sampling error and 29 samples due to operator or technical error. In total, 3284 samples were included in the analysis, of which 2107 were blood culture positive and 1211 blood culture negative samples. Of all the blood culture positive samples, 86 % contained a pathogen covered by the target panel of the Prove-it™ Sepsis assay. The most frequently identified bacteria were *E. coli, S. aureus, S. epidermidis, S. pneumoniae* and *K. pneumoniae* all of which are also reported as causative organisms in many sepsis related studies (Vincent *et al.*, 2006; Harbarth *et al.*, 2003). The assay panel did not cover organisms such as *Streptococcus viridans* or *Candida* spp. and 1,1 % of *Staphylococcus* sp. found in this study was not covered by the CNS panel of the assay (Publication II: Table 3). These represent obvious areas of improvement to be included in the assay later. Especially, rapid identification of different *Candida* spp. would allow earlier administration of antifungal therapy since for example in the UK the prevalence of fungemia is three to five cases per 100 000 populations (Odds *et al.*, 2007).

Table 5. *Target panel of the improved PCR and microarray assay (Prove-it™ Sepsis assay)*

Gram -	Gram +
Acinetobacter baumannii	*mecA* methicillin resistance marker
Enterobacter aerogenes	
Enterobacter cloacae	*Clostridium perfringens*
Escherichia coli	*Enterococcus faecalis*
Haemophilus inuenzae	*Enterococcus faecium*
Klebsiella oxytoca	*Listeria monocytogenes*
Klebsiella pneumoniae	*Staphylococcus aureus*
Neisseria meningitidis	*Staphylococcus epidermidis*
Proteus mirabilis	*Streptococcus agalactiae*
Proteus vulgaris	*Streptococcus dysgalactiae*
Pseudomonas aeruginosa	ssp. *equisimilis*
Salmonella enterica ssp. *enterica*	*Streptococcus pneumoniae*
Serratia marcescens	*Streptococcus pyogenes*
Stenotrophomonas maltophilia	
Bacteroides fragilis group	Coagulase negative *Staphylococcus*
Campylobacter jejuni/coli	
Enterobacteriaceae	
Neisseria sp. non-*meningitidis*	
Bacteroides fragilis detects at least the following species: *B. fragilis, B. vulgatus, B. thetaiotaomicron.*	
Coagulase negative *Staphylococcus* detects at least the following species: *S. haemolyticus, S. hominis, S. lugdunensis, S. saprophyticus, S. warneri, S. xylosus.*	
Enterobacteriaceae detects at least the following species: *Citrobacter amalonaticus, Citrobacter braakii, Citrobacter freundii, Citrobacter koseri, Enterobacter hormaechei, Enterobacter sakazakii, Kluyvera intermedia, Morganella morganii, Pantoea agglomerans, Providencia rettgeri, Providencia stuartii, Yersinia enterocolitica, Yersinia pseudotuberculosis.*	
Neisseria sp., non-*meningitidis* covers at least the following species: *N. gonorrhoeae, N. subflava, N. sicca, N. cinerea, N. elongata* subspecies *nitroreducens, N. flavescens, N. lactamica.*	
Salmonella enterica subspecies *enterica* detects at least the following serovars: Enteritidis, Oranienburg, Othmarschen, Panama, Paratyphi, Stanley, Typhi, Typhimurium, Virchow, group A,B,C,D.	

Identification results by the Prove-it™ Sepsis assay were compared to the culturing. Based on these results, 18 false positive and 94 false negative samples were reported by PCR and microarray assay (Publication II: Table 5). Over half of the false positive findings (11 out of 18) related to polybacterial finding such as identification of an additional CNS from the seven samples, most probably due to skin contamination in the sample (Richter *et al.*, 2002; Hall and Lyman, 2006). Other false positive findings were cross-hybridizations or caused by undetermined reasons. Most of the false negative findings related to limitations in the sensitivity of the assay (23 samples) or incorrectness in a polybacterial identifications (60 samples). In addition, six false negative samples (five *E. coli* and one *K. pneumoniae*) were identified only on the taxon level instead of species level and only *mecA* was detected from five samples without *Staphylococcus* species identification (Publication II: Table 5). However, the amount of false positive and false negative samples was relatively small (112 samples) from the set of 3284 samples. Based on these results, 94.7 % (95 % CI 93.6-95.7 %) sensitivity and 98.8 % (95 % CI 98.1-99.2 %) specificity was obtained according to the CLSI guidelines (2002) for the assay. These values were similar to the values of the performance evaluation study demonstrating that the extension of the target panel did not negatively affect the sensitivity or specificity of the assay (Table 6). The results were also comparable to published values of other assays for pathogen identification from positive blood culture samples in case of sepsis and BSI. Tormo and co-workers (2012) demonstrated 98.5 % sensitivity and 100 % specificity for Verigene® Gram-positive assay (Nanosphere Inc, USA) with 65 samples. Comparing the concordance of results with reference methods (Publication II: Table 2), Kaleta and co-workers (2011) published similar species level and genus level concordances; 95.6 % and 96.7 %, respectively, for PCR-ESI MS (Abbott Ibis Bioscience, USA) and 94.9 % and 97.1 %, respectively, for MALDI-TOF MS (Bruker, Germany).

Table 6. *Sensitivity and specificity of the improved PCR and microarray assay calculated based on the performance evaluation study (Publication I) and clinical validation study (Publication II) according to CLSI guidelines (2002), when conventional blood culturing was used as the reference method.*

	Positive identification by the PCR and microarray assay	Negative identification by the PCR and microarray assay	Sensitivity (95% CI)	Specificity (95% CI)
Performance evaluation study (n = 186) (Publication I)				
Positive by reference method	73 true positive	3 false negative	**96 %** [a] (89.0-98.6)	
Positive/negative by reference method [b]	2 false positive	108 true negative		**98 %** (93.6-99.5)
Clinical validation study (n = 3284) (Publication II)				
Positive by reference method	1696 true positive	94 false negative	**94,7 %** (93.6-95.7)	
Positive/negative by reference method [b]	18 false positive	1476 true negative		**98,8 %** (98.1-99.2)

[a] Initial sensitivity of 82 % was calculated without described result interpretation adjustment

[b] Blood culture positive samples, including pathogens not covered by the assay

CI = confidential intervals

The validation study showed that the developed Prove-it™ Sepsis assay can be used for sepsis diagnostics and the assay achieved CE-IVD approval. The target identification result could be obtained during the same working day when the blood culture has turned positive. These results indicate that the assay meets the requirements of rapid diagnostics which could improve patient outcome by offering early identification results for targeted antimicrobial therapy (Harbarth *et al*., 2003).

4.1.4 PCR and microarray assay: transfer from tube to strip platform (III)

The amounts of blood culture samples in hospital laboratories are dependent on the size of the hospital. A diagnostic assay directed to sepsis and BSI should cover the functionality and throughput needs of both small and large hospital laboratories. In order to meet these requirements, the end-point PCR and microarray assay was first developed using a single tube array platform (Prove-it™ TubeArray) allowing batching of 1-24 samples (Publications I and II). The performance of this assay was successfully demonstrated (Table 6) with patient samples. The same oligonucleotide content was then transferred onto a higher-throughput platform (Prove-it™ StripArray, Publications III and

IV). The strip array contains eight reaction wells on a strip with a low-density microarray at the bottom of each well and allows batching of 1-96 samples. A specific reader was used for automatic imaging and analysis of strip arrays without manual steps (Figure 2 and 3). The reader consists of an integrated camera, a visible light source and the Prove-it™ Advisor analysis software. Similar built-in analysis rules were used for species and taxon level identification of target organisms than for analysis of tube arrays. The end-point PCR producing ssDNA was the same for both used microarray platforms, but the colorimetric hybridization protocol was optimized for these microarray platforms separately.

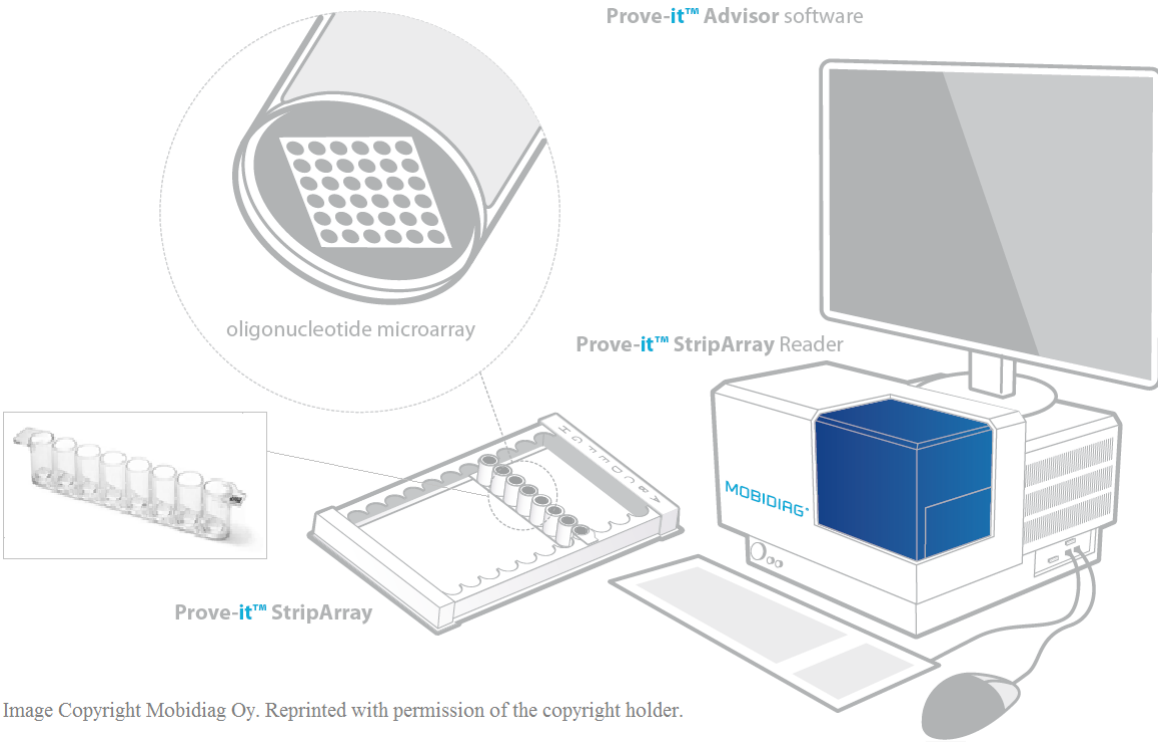

Figure 2 *Microarray platform including low-density microarrays with oligonucleotide probes at the bottom of eight-well strips (Prove-it™ StripArray) and a specific colorimetric reader for detection of hybridization reactions from the microarray. Identification of targets is based on built-in analysis rules and results are reported by the analysis software (Prove-it™ Advisor).*

91 positive blood cultures were freshly collected in HUSLAB, Finland (Publication III) for performance evaluation of the Prove-it™ Sepsis assays in strip array platform. DNA of the samples was extracted as duplicates with NucliSENS® easyMAG® (bioMérieux, France) and NorDiag Arrow (NorDiag, Norway) devices and analyzed with the microarray assay. Simultaneously, conventional blood culturing was performed in HUSLAB according to the CLSI guidelines (2007; 2011). Results showed that 96.7 % (88/91 samples) of the samples were correctly identified by the developed assay. Identified bacteria (*E. coli, S. aureus* and other *Staphylococcus* spp., *K. pneumoniae* and *E. faecalis*) represented typical pathogens found in sepsis and BSI cases (Vincent *et al.,*

2006). When the bacterial identification results were initially compared to the results of conventional method, 12 discrepant results were found (Publication III: Table 2) of which nine were later confirmed to be correct identifications by the PCR and microarray assay. From those nine samples, the microarray assay correctly identified for example *S. pneumoniae* in two samples which remained negative by culturing, *Streptococcus dysgalactiae* subsp. *equisimilis* in two samples which were identified as *S. pyogenes* by culturing and *S. epidermidis* in two samples when only taxon level identification was achieved by culturing. The PCR and microarray assay provided incorrect results in three cases: one multi-infection sample was marked as false negative due to missing identification of *E. faecium*, one sample was false positive due to a contamination of *E. faecalis* in that test series and one sample resulted in identification of *S. aureus* with CNS, while culturing reported only *Staphylococcus capitis*.

These results with only few discrepant identifications demonstrated that the Prove-it™ Sepsis assay on the strip array platform offered similar performance as the tube array platform. Many studies report that high-throughput; labor-efficient automated analysis could be standardized and could offer faster results (Reguiro *et al.,* 2010). Simultaneous analysis of 1 - 96 microarrays with the automated reader and software offers rapid results without result interpretations by user.

4.2 Development of identification of *Staphylococcus* spp. on microarray

4.2.1 Design and validation of *Staphylococcus aureus* and *mecA* detection (I,II,IV)

S. aureus is responsible for a substantial amount of bloodstream infections and therefore emphasis was put on sensitive identification of the target with the PCR and microarray assay. In addition to the broad-range primers, the multiplex PCR used in the assay contained specific primers for *mecA* gene and *S. aureus* for enhancing the amplification of the target. A set of oligonucleotide probes targeted to *S. aureus* and *mecA* were designed and printed on the microarray. The performance of detection of *S. aureus* or *S.aureus* with *mecA* in case of MRSA was evaluated with clinical isolates and the blood culture samples presented in publication I. Comparison of the assay results with culturing results showed correct identification of *S. aureus* from MSSA strains and *S. aureus* with *mecA* from MRSA strains with two discrepancies. In total, *S. aureus* was correctly detected from 24 blood culture samples (Publication I: Table 4). One sample remained false negative due to an unverified reason, most probably due to degraded DNA or PCR inhibitors present in the sample. The other false negative sample was marked as positive after *post hoc* adjustment of identification parameters.

The performance of identification of *S. aureus* and *mecA* was further improved by design revision after publication I and clinically validated with blood culture samples during the clinical validation of the Prove-it™ Sepsis assay presented in publication II. *S. aureus* was detected from 201 of 209 monobacterial samples (96.2 %) and *S. aureus* with *mecA* indicating MRSA (Figure 3) was detected from 16 samples showing 100 % concordance with reference results (Publication II: Table 2). Of the eight discrepancies, one sample was false negative due to inadequate sensitivity of *S. aureus* detection by the assay. Seven samples were reported as false positive because CNS was reported together with *S. aureus* instead of *S. aureus* alone. These results were most probably due to skin contamination (Hall and Lyman, 2006). The PCR and microarray assay also failed to identify *S. aureus* from nine multibacterial samples. The performance of detection of *S. aureus* and *mecA* by the PCR and microarray assay was comparable to other commercial assays developed for detection of *S. aureus* and MRSA from blood cultures. Stamper and co-workers (2007) evaluated BD GeneOhm (Franklin Lakes, USA) test for positive blood culture samples and obtained 98.9 % sensitivity and 96.7 % specificity for MRSA.

MRSA contains a highly mobile element SCC*mec* which carries the *mecA* gene, the causal factor of methicillin-resistance (Peng *et al.,* 2010; Shore *et al.,* 2011). Different SCC*mec* elements have been identified and therefore the PCR and microarray assay on the strip array platform was tested with important epidemic MRSA clones (Publication IV: Table 1). 18 MRSA clones carrying SSC*mec* types I, II, IV, V or a non-typable SSC*mec* (containing *ccrA1, ccrA2* and class B type of *mec*) were tested with in concentrations (10^5 and 10^3 GE) as duplicates. The results showed that the PCR and microarray assay successfully detected *S. aureus* and *mecA* from each MRSA clone.

The results demonstrated that the developed PCR and microarray assay detected one of the most prevalent sepsis and BSI causing bacterium *S. aureus* from patient samples with high accuracy. The detection of multibacterial samples could still improve. However, 100 % identification of MRSA from blood culture samples and detection of clinically relevant MRSA clones showed that assay is usable for accurate identification of *S. aureus* with *mecA* resistance marker. Simultaneous detection of resistance markers together with causative bacterial species may shorten the time to effective antimicrobial therapy, especially in the case of MRSA which is associated with high mortality and morbidity (Louie *et al.,* 2002; Harbarth *et al.,* 2003).

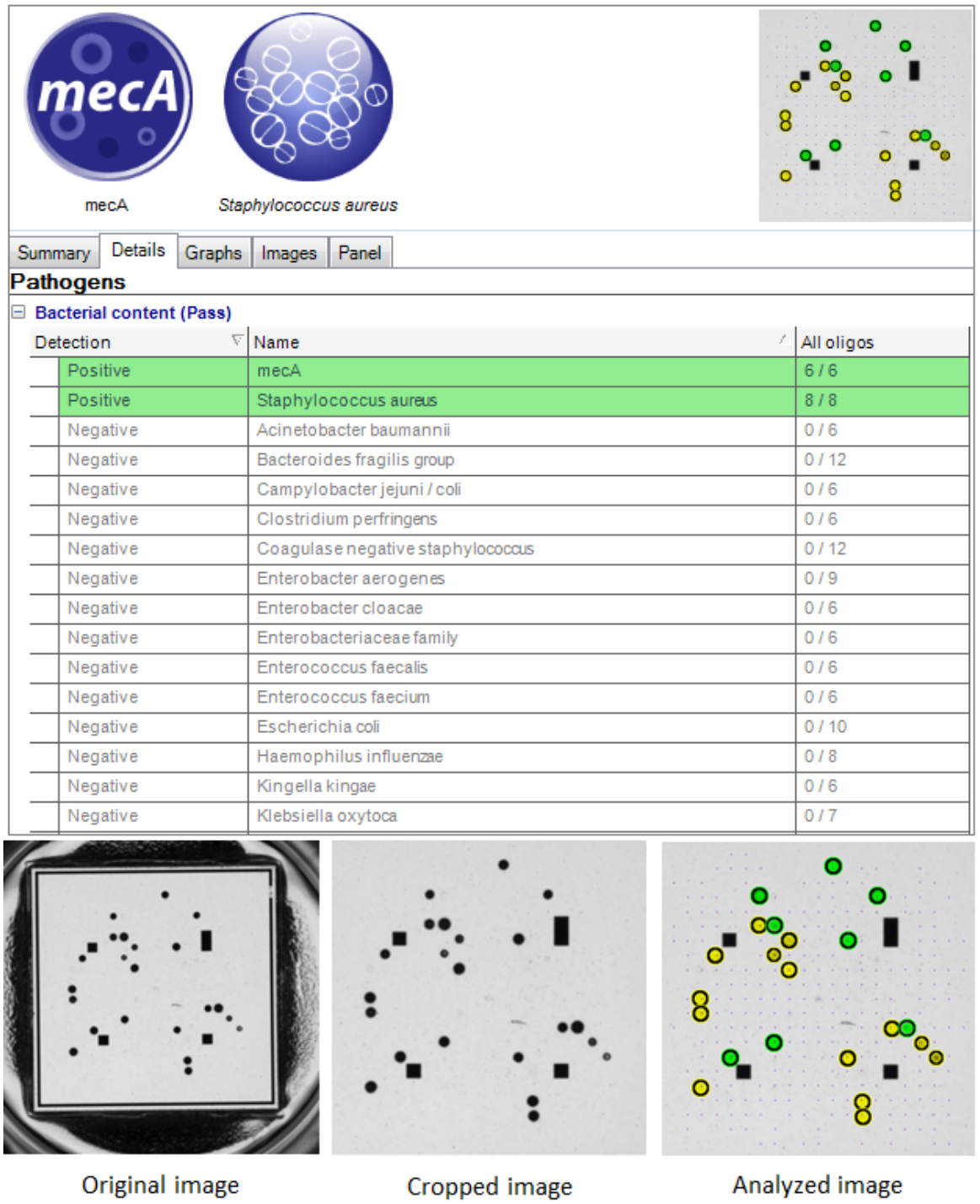

Figure 3 *Analysis report generated by the Prove-it™ Advisor analysis software. The result table indicates the amount of printed and detected oligonucleotides and highlights successful identification of S.aureus and mecA from the sample. The software takes an image from the bottom of the tube, crops the microarray image and analyses it, indicating different detected oligos by colors. Colorimetric positive hybridization is visualized by black spots on the microarray. Yellow marked spots are target specific oligos and green marked spots are control oligos.*

4.2.2 Design and validation of Coagulase-negative *Staphylococcus* detection (I, II)

The PCR and microarray assay was developed to identify *S. epidermidis* on species level and other relevant CNS species on taxon level. The performance of *S. epidermidis* and CNS identification by the PCR and microarray assay was evaluated with clinical isolates and blood culture samples, presented in publication I. *S. epidermidis* was successfully detected from eight blood culture samples. One sample was left false negative, but also 16S rDNA confirmatory PCR was negative indicating the presence of PCR inhibitors or degraded DNA in that sample. One sample was negative due to low quality of the probes and that sample was marked to be positive after adjustment of the built-in analysis rules of the analysis software.

The performance of *S. epidermidis* detection was further improved by design revision after publication I and clinically validated in the Prove-it™ Sepsis assay with blood culture samples presented in publication II. 246 monobacterial *S. epidermidis* findings were obtained by culturing and 240 (97.6 %) of those were detected by the Prove-it™ Sepsis assay. The PCR and microarray assay failed to identify *S. epidermidis* from ten multibacterial samples (Publication II: Table 5). According to Piette and co-workers (2006), over 50 % of the CNS isolates found from clinical samples are *S. epidermidis*. These bacteria are also reported to cause neonatal sepsis infections posing significant burden to public healthcare especially when carrying resistance genes (Cheung and Otto, 2010). The results demonstrated successful identification of *S. epidermidis* especially from monobacterial samples by the designed PCR and microarray assay.

The CNS panel of the PCR and microarray assay was proven to detect prevalent *Staphylococcus haemolyticus, Staphylococcus saprophyticus* and *Staphylococcus xylosus,* and also the highly virulent *Staphylococcus lugdunensis* (Publication I: Table. 2) clinical isolates. When blood culture samples were tested, *S. haemolyticus* was identified as CNS from two samples, while six other samples remained negative containing CNS species such as *Staphylococcus pasteuri, S. capitis* and *Staphylococcus hominis* not included in the target panel of the microarray assay. Therefore the assay coverage was extended to detect also *S. capitis, S. hominis* and *Staphylococcus warneri* in the CNS group. The functionality of this extension was clinically validated with blood culture samples of publication II. CNS was detected from 165 samples by culturing and the CNS panel of the PCR and microarray assay covered 133 of those. Correct identification was achieved from 123 monobacterial samples yielding 92.5 % concordance for CNS detection by the Prove-it™ Sepsis assay when compared to culturing results. The role of CNS as pathogens and their increasing incidence as causative bacteria in infections has been recognized (Piette and Verschraegen, 2009). The results demonstrated accurate identification of important CNS species at the taxon level by the PCR and microarray assay.

4.3 Accurate DNA-based detection compared to culturing and phenotype-based characterization of bacteria (II, III)

DNA-based characterization of bacteria has opened a new era in bacterial taxonomy and opportunities to detect non-viable pathogens from samples where conventional methods may fail (Mancini *et al.,* 2010; Soraya *et al.,* 2008; Klouche and Schröder, 2008). During the presented PCR and microarray development and evaluation study, results revealed examples where DNA-based identification offered more accurate bacterial identification than phenotypic characterization from positive blood culture samples. *S. pneumoniae* was identified from five samples by the PCR and microarray assay when culturing reported negative results most probably because of the autolysis of microbes or the slow growth of bacteria (Publications II and III). *S. pneumoniae* may experience autolysis when stationary growth section is achieved and therefore conventional culturing may fail (Martner *et al.,* 2009). Gram-staining supported *S. pneumoniae* findings and they were also confirmed by sequencing the *gyrB* gene region. Correct identification is important since considerable morbidity and mortality are associated especially to neonatal sepsis when *S. pneumoniae* is the causative agent (Hoffman *et al.,* 2003).

Culturing method reported *S. pyogenes* from two samples when the PCR and microarray assay identified *S. dysgalactiae* subsp. *equisimilis* (Publication III: Table 2). The bacterium was confirmed to be *S. dysgalactiae* subsp. *equisimilis* by sequencing the *gyrB* gene region in both cases. Streptococci are classified based on their Lancefield group antigens and species identification generally utilizes serotyping of streptococci. *S. dysgalactiae* subsp. *equisimilis* may express Lancefield's serogroup C or G, and *S. pyogenes* almost exclusively expresses serogroup A. Brandt and co-workers (1999) investigated blood culture isolates and found similar results where Lancefield's serogroup A was detected from *S. dysgalactiae* subsp. *equisimilis*. They concluded that this kind of *S. dysgalactiae* subsp. *equisimilis* is probably as pathogenic as those exhibiting Lancefield's serogroup C or G antigen and other methods than serotyping should be used for species identification (Brandt *et al.,* 1999).

The PCR and microarray assay identified *Enterobacteriaceae* group from seven blood culture samples while culturing reported *E. coli* (Publications II and III). The sequencing of the *gyrB* gene region showed nucleotide variations and only 96 – 98 % homology to that of *E. coli* sequence. *gyrB* gene regions from *Escherichia fergusonii* and *Escherichia hermannii* were also sequenced since they are closely related species of *E. coli* and can be misidentified as *E. coli* (York *et al.,* 2000). The sequence comparison showed that the obtained sequences were not 100 % similar to the *gyrB* gene regions of *E. fergusonii* and *E. hermannii* sequences or other sequences found from public sequence databases. In conclusion, the isolates could belong to *Escherichia* genus but were not *E. coli*. These results were taxonomically interesting but the clinical significance in these cases would be low.

4.4 Comparison of DNA extraction methods (III)

The developed Prove-it™ Sepsis PCR and microarray assay was aimed at the identification of sepsis causing bacteria from blood culture samples. Blood culture is a complicated sample matrix, and therefore effort must focus on examining the cell wall disruption of microbes and recovery of microbial DNA without putative PCR inhibitors from different DNA extraction methods. NucliSENS®easyMAG® (bioMérieux, France) extraction device was used as the extraction system when the developed PCR and microarray assay was validated with patient samples (Publication II). This extraction system is typically classified as the gold standard method, when different extraction kits are compared. The device has been demonstrated to be efficient, but the reagent costs are not cheap and the original device investment may be high (Dundas *et al.,* 2008). Therefore the performance of the alternative extraction device NorDiag Arrow (Nordiag, Norway) and Viral NA kit was evaluated. The DNA from a set of 91 positive blood cultures was extracted simultaneously with both devices and samples were analyzed by the Prove-it™ Sepsis assay. The bacterial identification results from DNA extracts isolated with both devices showed 100 % concordance (Publication III: Table 1). Both systems successfully lysed and extracted Gram-negative and Gram-positive bacteria from blood culture samples. The amount of Gram-positive bacteria was 35.2 % of all the tested samples and contained species such as *Staphylococcus* spp. and *Streptococcus* spp. whose rigid cell wall is typically difficult to lyse (Sohail, 1998).

In addition, dilution series from *E. coli* clinical isolate was used for DNA extraction simultaneously with both devices to examine DNA yields and purity with a spectrophotometer and real-time PCR using the *dxs* gene region for amplification. Three parallel samples were isolated per each concentration in the dilution series with both devices and in general, the performance had only little variation. Standard deviation was high with the NorDiag Arrow device when concentration and purity from parallel DNA samples was compared with NucliSENS®easyMAG®. Variation was not obtained in standard deviation from parallel DNA samples extracted with NucliSENS®easyMAG®, indicating more consistent extraction quality and reproducibility (Publication III: Fig. 1 and Fig. 2). However, none of the isolated samples achieved the eligible purity value of 1.8 $A_{260/280}$. It is noteworthy that the ability of the spectrophotometer to measure low concentrations is a limitation of the method. The measurements out of optimal purity range of DNA (*i.e.* $A_{260/280}$ -ratios of 1.5-2.0) may also cause some variation in the results (Kim *et al.,* 2008). The results showed some variation in the quality and reproducibility between NucliSENS®easyMAG® and NorDiag Arrow and if these devices are used for other purposes, more precise investigation would be recommended. The results of this study demonstrated that both devices can be used for extraction of DNA from blood culture samples when the Prove-it™ Sepsis assay is used for analysis of the DNA extracts.

4.5 Application for whole blood sample type (IV)

Assays for sepsis diagnostics usually use blood culture or whole blood as sample type. The developed PCR and microarray assay (Prove-it™ Sepsis) was demonstrated to be a sensitive detection method of bacteria from blood culture (Publications I-III). Whole blood is a more complicated sample material since the amount of infecting bacteria in clinically significant bacteremia may be low and levels of substances potentially interfering with analysis are high (Al-Sould *et al.,* 2000; Ecker *et al.,* 2010). The suitability of the PCR and microarray assay for identification of pathogens without a blood culturing step was investigated by combining a specific DNA extraction method with analysis by the PCR and microarray assay.

An almost similar PCR and microarray assay using the strip array platform as described in original publication III was slightly modified for analysis of whole blood samples. The assay (named Prove-it™ Bone & Joint) was originally aimed at the detection of bone and joint infection causing pathogens from clinical specimens including osteoarticular fluids and bone biopsies (Metso *et al.,* 2013). These sample types contain low amounts of bacteria and high levels of inhibitors similar to the whole blood sample type. The sample volume per PCR reaction and amount of amplicons for hybridization was increased to enhance assay sensitivity. The hybridization protocol was also optimized to allow a highly sensitive detection with these modifications. The assay was combined together with the commercially available Blood pathogen DNA isolation kit (Molzym, Germany), which is used on the automated NorDiag Arrow (NorDiag, Norway) extraction device (Publication IV: Fig. 1). This specific bacterial DNA extraction method selectively concentrates bacterial cells and degraded human DNA followed by the extraction of bacterial DNA from one mL of whole blood sample.

The combination assay was evaluated by spiking whole blood with a dilution series of bacteria prevalent in sepsis. DNA of spiked samples was extracted as duplicates with the described method and DNA extracts were analyzed by the described PCR and microarray assay. The detection limits for the combined solution were determined to be the lowest amount of CFUs in the blood sample yielding to a correct bacterial identification by the PCR and microarray assay from one or both duplicates. The obtained limit-of-detection (LOD) values were 11 CFU/mL for *E. coli*, 13 CFU/mL for *E. faecalis,* 68 CFU/mL for *K. pneumoniae*, 86 CFU/mL for MRSA (combined detection of *S. aureus* and *mecA*), 250 CFU/mL for *L. monocytogenes*, and 600 CFU/mL for *S. agalactiae* (Publication IV: Table 3). Lehmann and co-workers (2007) introduced detection limits of 3 CFU/mL - 100 CFU/mL depending on the bacterial species with the LightCycler® SeptiFast Test MGRADE assay, which is the best-established solution for whole blood. The results with spiked whole blood samples demonstrated that the sensitivity of the combined assay was on the same level for most of the tested bacteria. However, with some targets (*i.e. L. monocytogenes* and *S. agalactiae*) the assay did not achieve the same sensitivity levels.

To evaluate the dissipation of bacterial DNA during sample preparation steps, DNA from similar clinical isolates which were used for blood spiking, were diluted and tested as duplicates by the modified PCR and microarray assay. Analytical sensitivity was determined to be the lowest amount of GE in the PCR reaction which led to a successful bacterial identification from at least one of the tested duplicates. Detection limits for the PCR and microarray assay were 1 GE for *E. coli*, 8 GE for *K. pneumoniae*, 11 GE for *S. aureus*, 15 GE for *E. faecalis*, 16 GE for *L. monocytogenes*, 17 GE for MRSA (combined detection of *S. aureus* and *mecA*), and 21 GE for *S. agalactiae* (Publication IV: Table 2). These results showed that only few genome copies were sufficient for production of an adequate amount of amplicons for detection on the microarray. Analytical sensitivity values were compared to the detection limits obtained with the combined assay with spiked whole blood samples, although one must note that CFU/mL and GE are not totally comparable units. The results indicated possible PCR inhibitor residues from blood and/or losses of bacterial DNA during the sample preparation step.

In conclusion, sensitivities for some of the bacteria reached a suitable level of 1-30 CFU/mL, values which are typically obtained from whole blood in sepsis (Ecker *et al.,* 2010). Mühl and co-workers (2011) indicated with a similar extraction method that the total amount of positively detected samples by PCR method raised from 50 % to 79 % when sample volume was increased from 1 mL to 5 mL. Similar improvement could also positively affect the sensitivity values obtained in this study. The results demonstrated proof-of-concept for this combination of a specific DNA extraction method with the PCR and microarray analysis assay. Full understanding of the sensitivity and reproducibility of the assay should still be further investigated with a broader bacterial panel as well as with real patient samples.

4.6 User requirements for a diagnostic assay of sepsis (II, III, IV)

Afshari and co-workers (2012) listed several commercially available assays with different detection strategies which have been developed for faster sepsis and BSI diagnostics. New assays need to follow the territorial regulations before they can be offered for diagnostic use. Introducing an assay to the EU market requires CE marking for *in vitro* diagnostic (IVD). Also FDA sets similar requirements for products introduced in US markets (Directive 98/79/EC, 1998; Kimmelman, 2003). In this study, the Prove-it™ Sepsis PCR and microarray assay with tube and strip platform were developed in compliance with the Directive 98/79/EC and therefore attained the CE-IVD marking. They are commercially available in Europe.

High mortality and the reported six hour time window during which the risk for death in septic patient's increases substantially sets high requirements for rapid identification of causative pathogens. Inappropriate patient treatment has been shown to result in poor patient outcomes and an increase hospital costs (Harbarth *et al.,* 2003; Kumar *et al.,*

2006). During the study presented in the original publication II, the turnaround times for identification of bacterial species from blood culture by the Prove-it™ Sepsis assay were compared with conventional culturing using a randomly chosen set of 39 samples. The result showed that the median time difference was 18 hours 19 minutes and in general, the results with the Prove-it™ Sepsis assay were achieved during the same day when blood culture turned positive. This indicated faster results for clinicians for review of patient management. The PCR and microarray assay was also combined with a specific DNA extraction method and the sensitivity of this solution was demonstrated to be on a suitable level for pathogen identification from spiked whole blood samples. This combination offers faster identification results than analysis from positive blood culture samples. However, the study was only a proof-of-concept and the solution needs further development and evaluation.

Many studies conclude that faster results can be achieved by automating previously manual steps. Automation could offer labor-efficient, standardize results between different users and can reduce human errors (Reguiro *et al.,* 2010). Depending on samples and size of the laboratory, a high-throughput platform can speed up diagnostics, when several samples from the same patient as well as increased number of patients can be tested simultaneously. These user requirements were also taken into consideration when the diagnostic PCR and microarray assays were developed in this study. The Prove-it™ Sepsis assay was developed for two platforms, the tube and strip array format. The tube array format allowed analysis of 1-24 samples and the higher-throughput strip array format allowed analysis of 1-96 samples simultaneously. The hybridization protocols for both tube and strip format are still manual and require manual pipetting. The analysis software (Prove-it™ Advisor) was developed to be instinctive and produce analysis results automatically without the need for result interpretation by user (Figure 3). Analysis of results on the strip array format was a wizard-guided automated protocol capable of providing identification results from 96 samples in one run without user intervention after each sample. The level of automation was also increased by using DNA extraction devices together with the developed PCR and microarray assay. Automated NucliSENS®easyMAG® and NorDiag Arrow devices were evaluated with blood culture samples and the semi-automated specific extraction kit combined with the NorDiag Arrow device was tested with spiked whole blood samples.

5 Conclusion and Future prospects

Sepsis, severe sepsis and septic shock are associated with high mortality rates and it is estimated that around 1400 patients die for severe sepsis every day (Ebrahim, 2011; Daniels, 2011). Patients with severe sepsis stay approximately 19.6 days in hospital in the US and related costs are estimated to rise to $16.7 billion annually (Angus *et al.,* 2001). These numbers illustrate the difficulty of treating seriously ill septic patients. Kumar and co-workers (2006) demonstrated that every additional hour during the first six hours from hypotension onset without appropriate antimicrobial treatment increases the risk of death by 7.6 % in septic patients. Other studies have also demonstrated lower mortality rates among adequately treated patients (Harbarth *et al.,* 2003; Bochud *et al.,* 2004).

Sepsis diagnostics needs faster assays, which can offer reliable pathogen identification and resistance screening. The aim of this study was to develop a PCR and microarray assay for rapid detection of pathogens and resistance markers from blood culture samples. The proof-of-concept of the assay was established in publication I. The bacterial panel of the assay was extended to cover over 50 relevant or resistant species which may change antimicrobial treatment when detected from the sample. Targeted patient management based on the identified causative pathogen may result in better patient outcomes and save lives. This improved assay was successfully validated in publication II. The study focused also on modifying the assay to be suitable and easily implementable to small as well as large hospital laboratories. The amount of samples varies significantly between laboratories and thus the developed PCR and microarray assay was optimized for both the tube array platform and the higher-throughput strip array platform capable of identifying 1 - 96 samples simultaneously (Publication III).

The amount of NA-based methods for the identification of causative pathogens from patients with suspected sepsis has increased substantially during the past ten years. In 1997, Reimer and co-workers (1997) concluded that no NA-based identification methods are available for routine use, but lots of research is ongoing in the area. Nowadays, several publications are introducing tens of commercially available NA-based assays, which aim are to improve and offer tools for rapid and accurate diagnostics and decrease the mortality rate of septic patients (Afshari *et al.,* 2012; Paolucci *et al.,* 2010; Dark *et al.,* 2009). The developed PCR and microarray assay (Prove-it™ Sepsis) with both tube and strip array platform received CE-IVD marks and are among those NA-based commercially available assays in Europe. The broad-range primer design and microarray platform of this PCR and microarray assay offers robust and flexible combination where identification of new targets can be easily implemented. Thus, the scope of the Prove-it™ Sepsis assay has already improved after this study with the addition of twelve *Candida* species together with additional bacterial targets (Aittakorpi *et al.,* 2012).

Simultaneous evaluation of bacterial resistances provides earlier information than conventional susceptibility testing and may decrease the time to targeted treatment. Validation of the developed PCR and microarray assay in publication II demonstrated 100

% sensitivity and specificity for identification of *S. aureus* and *mecA* from MRSA samples. In addition, the capacity of this assay to detect epidemiologically important MRSA clones was successfully shown in publication IV. The antimicrobial resistance panel of the Prove-it™ Sepsis assay has already been extended after the present study to detect also vancomycin resistance markers and the addition of other gene markers such as fluoroquinolone resistance markers or other markers related to Gram-negative bacteria can be considered in the future.

Novel assays developed for rapid sepsis diagnostics follow two different strategies based on the sample type. Assays which use blood culture as the sample type offer comparable identification results with gold standard methods. Results are typically achieved faster than with the conventional method, but culturing of pathogens in blood culture bottles is still time-consuming. Whole blood as the sample type offers time advantage when culturing is avoided. However, the achieved results and the significance of pathogen DNA as a marker in infection are still under discussion, since viable bacteria are not required for positive detection. Also the lack of reference methods from whole blood at the moment increases problems to interpret the results.

In this study, a PCR and microarray assay was developed for blood culture sample type since it can be easily implemented in routine laboratories (Publications I-III). In addition, the suitability of the assay to achieve adequate sensitivities required for whole blood was also tested (Publication IV). Since sample preparation is a highly critical step, a specific bacterial DNA extraction method was used together with the PCR and microarray assay. The results demonstrated good proof-of-concept and also the potential of the assay to be used directly for other non-enriched sample types such as tissue and different body fluids. The PCR and microarray assay is already used as a research method for pathogen identification from laryngitis and nasal lavage samples not related to sepsis and can be consider also for other applications (Kinnari *et al.,* 2012, Allen *et al.*, 2013).

In conclusion, sepsis is a serious infection and its diagnosis is not unambiguous. Initial empirical treatment of a septic patient is typically a combination therapy. Targeted antimicrobial therapy can be started immediately after the causative pathogen has been identified. Many molecular and protein-based assays have been developed next to the current culturing-based gold standard method in an attempt to reduce mortality, evolution and spread of bacterial resistance, toxicity and costs. None of those assays have yet replaced the current culturing method but are valuable tools, especially when used for certain groups of patients under higher risk. New assays have faced criticism especially for their high costs, since traditional culturing is relatively cheap and some of the new tests may require expensive device and reagent investments. However, every additional day in hospital causes major costs and thus faster methods which can produce pathogen identification and susceptibility results earlier, can decrease the length of stay in hospital.

NA-based assays have already opened a new era and brought broadened perspective to microbiological laboratories beside conventional culturing methods. Understanding the

clinical relevance of DNAemia/circulating DNA may open new strategies for septic patient management. New attempts for development of point-of-care tests for sepsis diagnostics are increasingly reported. Their protocols of sample in – results out allow fast analysis without manual steps and special skills of the user. This progressing development of new techniques and assays is a trend, which is predicted to spread to all fields in clinical microbiology in the future. Hopefully, these assays can be used for improved patient management worldwide including developing countries.

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