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RAPID MICROBIOLOGICAL DIAGNOSIS OF INFECTIONS IN STERILE BODY FLUIDS INCLUDING BLOOD

Osman Altun

Karolinska Institutet

Stockholm 2020

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ISBN 978-91-7831-787-5

RAPID MICROBIOLOGICAL DIAGNOSIS OF INFECTIONS IN STERILE BODY FLUIDS INCLUDING BLOOD

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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This thesis is dedicated to my family, friends and colleagues whose support made this work possible.

Especially to my parents, whose words of encouragement, push for excellence and to always remain humble continues to guide me in life. To my wife Elsa and daughter Adella, whose presence in my life has filled it with love and laughter. To my friends and colleagues who made this work a great educational experience that has reshaped my scientific perspective and goals.

Osman Altun

ABSTRACT

Infections of normally sterile body fluids (SBF) including blood, cerebrospinal-, pleural-, peritoneal, – pericardial and synovial fluid are critical and require early diagnosis and rapid initiation of appropriate antimicrobial therapy. With the advanced and invasive care of modern medicine, infections of SBF are increasing worldwide. The clinical microbiology laboratory is crucial in facilitating targeted therapy through early microorganism detection, identification and antibiotic susceptibility testing. This actionable information will allow correct antimicrobial therapy early in the infectious process.

Blood culture systems as well as blood culture bottles are the standard diagnostic method in blood cultures and are utilized for SBF cultures. The overall aim of this thesis was to evaluate and improve the microbiological diagnosis of SBF including blood from positive blood culture bottles.

The first study focused on BacT/Alert Virtuo blood culture system introduced into the market to replace the BacT/Alert 3D blood culture system by the same company. The study presented in this thesis showed a significantly shorter time to detection for bottles incubated in the Virtuo system than those incubated in the BacT/Alert 3D system (median 12 h and 15 h, respectively; $p < 0.0001$) in simulated blood cultures.

In Study 2, the performance a unique method of short-term culture followed by MALDI-TOF MS was analyzed prospectively. The study showed a successful identification of 424/515 (82.3%) bacteria with MALDI-TOF MS after 5.5 h subculture on solid media.

In Study 3 and 4, the FilmArray BCID panel was prospectively evaluated for accurate identification and limited antibiotic susceptibility testing. In Study 3, the FilmArray BCID showed accurate identification of microorganisms in 153/167 (91.6%) and 17/24 (71%) culture positive blood culture bottles with monomicrobial- and polymicrobial-growth. In Study 4, the performance of FilmArray BCID was analyzed for SBF other than blood. The assay could identify all microorganisms in 84/84 (100%) and 18/24 (75%) SBF samples with mono- and polymicrobial growth respectively. These studies showed that the FilmArray BCID panel is a reliable and fast method that may be utilized in blood cultures as well as other SBF that are cultured in blood culture bottles.

In Study 5, the performance of four *S. pneumoniae* antigen tests including ImmuLex™, SlideX® pneumo-Kit, Wellcogen™, and BinaxNOW® were evaluated. The study revealed similar sensitivity of 99.6%-100% for all tests when excluding invalid results in the calculation. However, the study showed that the BinaxNow® had the lowest specificity (64.1% 9, p<0.01) among the four methods. The ImmuLex™ test had lower specificity than the Slidex® test (82.6% vs 97.6%, p<0.01) and performed similar to the Wellcogen™ test (84.5%, p=ns).

In conclusion, the present thesis showed that the new Virtuo blood culture system allows faster detection of microorganisms in blood culture bottles. The thesis further presented several methods for rapid identification of microorganisms after detection of growth in blood culture bottles. These tests included FilmArray BCID panel and the latex agglutination tests directly from blood culture bottles while MALDI-TOF MS was performed after sub-culture on solid media. These rapid tests are important complements to the rigorous and extensive conventional culture methods and provide early critical actionable information for appropriate antimicrobial stewardship.

LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. **Altun O**, Almuhayawai M**,** Lüthje P, Taha R, Ullberg M, Özenci V. Controlled Evaluation of the New BacT/Alert Virtuo Blood Culture System for Detection and Time to Detection of Bacteria and Yeasts. Journal of Clinical Microbiology. 2016;54(4):1148-51. PMID: 26842707

II. **Altun O,** Botero-Kleiven S, Carlsson S, Ullberg M, Özenci V. Rapid identification of bacteria from positive blood culture bottles by MALDI-TOF MS following short-term incubation on solid media. Journal of Medical Microbiology. 2015, 00, 1-7. PMID: 26361761

III. **Altun O,** Almuhayawai M, Ullberg M, Ozenci V. Clinical evaluation of the FilmArray blood culture identification panel in identification of bacteria and yeasts from positive blood culture bottles. Journal of Clinical Microbiology. 2013;51(12):4130-6. PMID: 24088863

IV. **Altun O,** Almuhayawai M, Ullberg M, Özenci V. Rapid identification of microorganisms from sterile body fluids by use of FilmArray. Journal of Clinical Microbiology. 2015;53(2):710–2. PMID: 25520440

V. **Altun O,** Athlin S, Almuhayawai M**,** Strålin K, Özenci V. Rapid identification of Streptococcus pneumoniae in blood cultures by using the ImmuLex, Slidex and Wellcogen latex agglutination tests and the BinaxNOW antigen test. European Journal of Clinical Microbiology & Infectious Diseases. 2016;35(4): 579–85. PMID: 26796552

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. **Altun O**, Özenci V. Correction of Previously False-Positive Result by Improved Software. Journal of Clinical Microbiology. 2015 Feb;53(2):750. PIMD: 25502521.

II. Almuhayawai M, **Altun O,** Strålin K, Özenci V. Identification of microorganisms by FilmArray and matrix-assisted laser desorption ionization-time of flight mass spectrometry prior to positivity in the blood culture system. Journal of Clinical Microbiology. 2014;52(9):3230-6. PMID: 24 951 811

III. Almuhayawai M, **Altun O,** Abdulmajeed AD, Ullberg M, Özenci V. The Performance of the Four Anaerobic Blood Culture Bottles BacT/ALERT-FN, -FN Plus, BACTEC-Plus and -Lytic in Detection of Anaerobic Bacteria and Identification by Direct MALDI-TOF MS. PloS one. 2015;10(11):0142398. PMID: 26554930

IV. Athlin S, **Altun O,** Eriksen HB, Özenci V. Strålin K. The Uni-Gold™ Streptococcus pnumoniae urinary antigen test: an interassay comparison with the BinaxNOW Streptococcus pneumoniae test on consecutive urine sample and evalustion on patients with bacteremia. European Journal Clinical Microbiology Infectious Disease. 2015 Aug;34(8):1583-8. PMID: 25926305

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LIST OF ABBREVIATIONS

AST	Antibiotic Susceptibility Testing
BD	Becton Dickinson
CO_2	Carbon Dioxide
CFU	Colony-forming Unit
FDA	Food and Drug Administration
FilmArray BCID	FilmArray Blood Culture Identification Panel
GNB	Gram Negative Bacteria
GPB	Gram Positive Bacteria
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer
MSSA	Methicillin sensitive *Staphylococcus aureus*
MRSA	Methicillin resistant *Staphylococcus aureus*
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
PCR	Polymerase Chain Reaction
SBF	Sterile Body Fluid
TTD	Time to Detection
TTI	Time to Identification

1 INTRODUCTION

Sterile body fluids (SBF), such as blood, cerebrospinal, pleural, peritoneal, pericardial and synovial fluid, are all susceptible for serious invasive bacterial and fungal infections. The advancement of modern medicine including invasive procedures has led to increased infections of normally sterile body fluids including blood[1]. Early detection and rapid identification of the microorganisms is critical for appropriate management and targeted antimicrobial therapy [1–5]. The clinical microbiology laboratory is central in facilitating appropriate antimicrobial therapy in the management of SBF infections by confirming the presence of infectious pathogens, identifying them and providing antimicrobial susceptibility testing that allows targeted antimicrobial therapy. This thesis presents studies aimed at further improving the microbiological diagnosis of SBF infections including detection of microorganisms, identification and antibiotic susceptibility testing (AST) from positive blood culture bottles.

1.1 DEFINITION OF INFECTIONS OF STERILE BODY FLUIDS

The etiology of infections of SBF varies and may include surgery, trauma and burn injuries that introduce infectious pathogens to the normally sterile sites. Infection of each sterile site or SBF is separately defined and may describe the anatomical site, organ involved, etiology, clinical parameters/presentation and pathophysiology (e.g. bacteremia, sepsis, bacterial meningitis, bacterial peritonitis etc.). However, infection of SBF sites is in general associated with significant mortality and morbidity and need to be readily assessed and treated with appropriate antimicrobial therapy[2,4–6]. Initial antimicrobial treatment of suspected infection of SBF is guided by local microorganism susceptibility patterns as well as clinical societal guidelines and often includes using broad spectrum antimicrobials pending microbiological evaluation. In response to recommendations to limit the use of broad-spectrum antimicrobial therapy and the extended time to detection of the infectious pathogens, major research efforts have been placed in improving the microbiological analysis by decreasing time to detection, identification and antibiotic susceptibility testing.

1.2 LABORATORY DIAGNOSIS OF INFECTIONS OF STERILE BODY FLUIDS

1.2.1 Time to detection of microorganisms

The first challenge encountered in the microbiological diagnosis of SBF is the ability to detect microorganisms causing the infection of the SBF. The microorganisms are often very low in number and uneven distributed in the SBF that require highly sensitive methods. The first methods developed for detecting infection in SBF were the manual culture systems. The first manual culture systems involved a broth-based culture method (monophasic) where the specimen was inoculated in the culture bottles with culture medium and manually inspected[7]. The manual culture system had the advantages of simplicity and for the laboratories to be able to produce their own blood culture vials. However, the manual system was labor intensive requiring venting the culture bottles to create an aerobic atmosphere, manual reading for culture growth that took time from doing other diagnostic work and the challenge to inspect them with the frequency required to give early detection of growth. The manual method was then further developed to include a biphasic method where the blood culture bottles contained both solid media as well as a liquid phase described by Castaneda et al., in 1947 (Figure 1)[8]. The biphasic culture method was performed by inoculating the bottles and then processing the culture by modifying the air in the bottles, tilting to allow the fluid over the solid medium, incubate the bottles in upright position and examining the bottles visually on a scheduled time intervals[8]. Several other biphasic methods were developed with the most widely known biphasic method being the Septi-Chek. The Septi-Chek bottles were inoculated with blood and visually inspected twice for the first two days and then once per day until seven days of incubation had been completed[7]. The manual mono- and biphasic culture methods provided the foundation that allowed the development of the automated blood culture system.

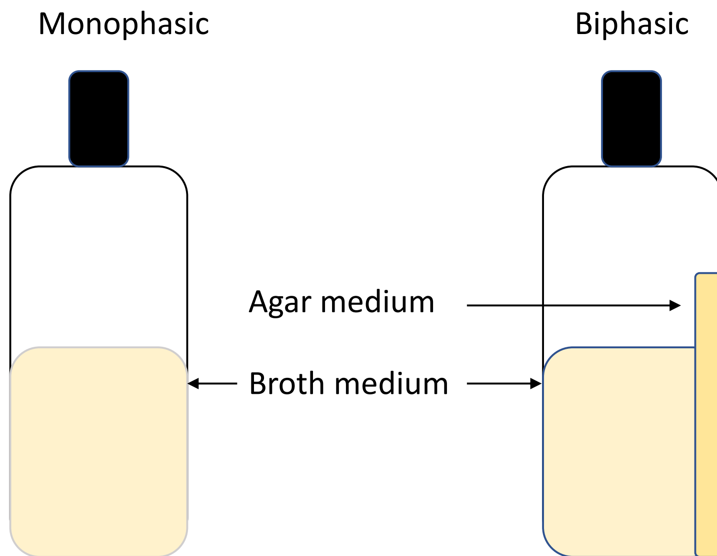
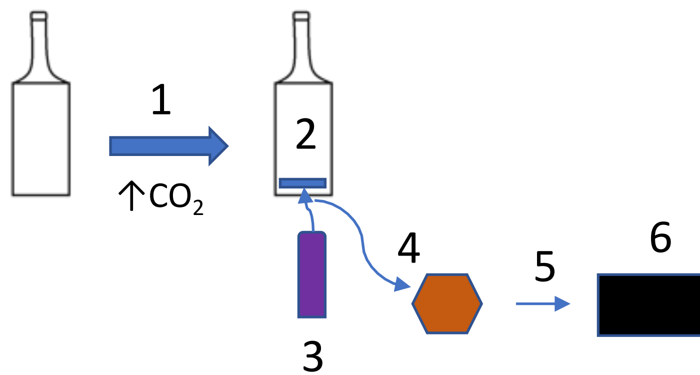


Figure 1. Display of mono- and biphasic blood culture bottles.

Automated blood culture systems were developed in response to the aforementioned challenges in the manual culture systems with the purpose to minimize the technician hands-on time for specimen processing while increasing the detection rate as well as decreasing the time to detection. The first blood culture instruments for automated detection were introduced in the 1970s and were based on the detection of increasing carbon dioxide (CO_2) concentrations seen with microbial growth in culture bottles. One such early blood culture system was developed by Johnston Laboratories (now known as Becton Dickinson Diagnostic Instrument Systems) using broth media that contained radioactive ^{14}C in blood culture bottles. During the microbial metabolism, the radioactive ^{14}C was released as $^{14}CO_2$ into the headspace of the bottles. The bottles were analyzed for growth with a heat-sterilized needle, an aliquot was collected from the headspace and analyzed for radioactivity to assess for microbial growth[7]. Radioactive reagents are hazardous to the health and the environment and is a major disadvantage in any diagnostic method due to the challenges of using them as well as disposing them after use safely. In addition, the bottle heads had to be punctuated for gas analysis to allow detection of growth exposing the technician to radiation.

In the 1980s, a new generation of blood culture systems were introduced with the development of infrared spectrophotometric CO_2 detection. The new infrared based blood culture systems did not have the disadvantage of using radioactive substances. However, the system still required punctuation of the bottle head to collect gas for analysis, replacing removed gas and risking contaminating blood culture bottles[9]. These first automated detection of growth using CO_2 in blood culture systems made manual inspecting of culture growth obsolete, but still required manual monitoring of growth via other methods that exposed the technicians to radioactive substances and/or risked contaminating the culture bottles as well as delaying the detection.

The introduction of continuous automated monitoring gave the automated blood culture system a significant advantage where the instruments could serve as incubators and continuous agitation while under interrogation of growth independent of a technician. The systems reduced handling time, decreased risk for cross contamination and monitored for microbial growth every 10-25 minutes providing opportunity for early detection of growth. The first automated blood culture system that provided continuous monitoring for growth and was Food and Drug Administration (FDA) approved was the BacT/Alert (Organon Teknika, Turnhout, Belgium) system[10]. The BacT/Alert system utilized a sensor internally attached to the bottom of the blood culture bottles. The sensor was separated from the culture broth by a membrane permeable only to CO_2. Like the previously described methods, the microbial metabolism increased the CO_2 production. The CO_2 diffused over the membrane to the sensor causing acidification. The acidification caused a color change to the sensor that was monitored every 10 minutes by light-emitting diodes light on the sensor while photodiodes measure the amount of red light reflected from the sensor. The red light reflected was proportioned to the color sensor and correlates with the CO_2 production (Figure 2). The BacT/Alert system then applied an algorithm to differentiate positive culture bottles from negative culture bottles depending on the rate of the sensor color change [7].



1. Organism metabolic activity in the bottle releases CO_2
2. CO_2 reacts with the dye in the sensor
3. Sensor exposed to light intermittently
4. Photo detector reads light emitted from the sensor
5. Analysis for positivity performed on the light emitted from the sensor
6. Software differentiates positive and negative analysis

Figure 2. Automated blood culture system monitoring of growth of microorganisms based on the CO_2 production.

Becton Dickinson Diagnostic Instruments Systems received FDA approval for the BACTEC automated and continuous monitoring blood culture in 1992. The BACTEC system uses fluorescence to detect CO_2 production changing the membranes instead of the colorimetric method[7]. Extra Sensing Power was the third continuous monitoring method to receive FDA approval monitored for growth by manometric monitoring gas production or consumption by monitoring for pressure changes in the headspace of each bottle. Algorithms were applied to the pressure changes that were monitored to determine for positivity. There are additional automated continuously monitored blood culture systems beyond the scope of this thesis. However, the main blood culture systems in use are the BacT/Alert and the BACTEC system studied in this thesis.

In modern clinical microbiological laboratories, automated blood culture systems are the gold standard for detection of bacteria and fungi in blood as well as other normally sterile body fluids. Blood culture systems as well as the blood culture bottles and supplement to the blood culture bottles have been extensively studied[11–16]. The first two FDA approved automated blood culture systems BacT/Alert (bioMérieux) and BACTEC (Becton Dickinson) are the most widely used in clinical microbiology laboratories worldwide. BioMérieux introduced Virtuo, a new version blood culture system, to the market with automatic loading and unloading, improved detection algorithm and improved temperature stability compared with the BacT/Alert 3D (BioMérieux) system that was in use from the same company. The Virtuo system was evaluated in this thesis in direct comparison with the BacT/Alert 3D system. At the Karolinska University laboratory, more than 200 000 blood culture bottles are analyzed yearly, hence the new Virtuo system had the additional advantage of automatic loading and unloading that would free the technician to do less mechanic work and allow them to focus on diagnostic work. Study 1 is the first evaluation of the new automated blood culture system using simulated blood cultures.

1.2.2 Identification of microorganisms

Following detecting growth in the blood culture bottles, the clinical microbiology laboratory will focus on identifying the microorganisms. Current identification methods have been developed over many decades and will reliably deliver accurate pathogen identification to support the clinicians in guiding antimicrobial therapy. The identification of microorganisms is based on the morphology under microscopic evaluation, Gram stain, biochemical and growth characteristics of the microorganisms. The traditional approach to pathogen identification from positive blood culture bottles at clinical laboratories starts with the Gram stain and microscopic examination of the blood culture broth to examine the Gram stain and pathogen morphology. The Gram stain and morphology will then guide subsequent steps of subculture on relevant agar plates and incubation atmosphere for subculture. Bacterial colonies are collected after 24-72-hour subculture and further analyzed with matrix-associated laser desorption ionization-time-of-flight mass spectrometer (MALDI-TOF MS) (Bruker Daltonik) or the Vitek 2 XL (BioMérieux). MALDI-TOF is a mass spectrophotometry system that performs protein analysis of microorganisms for identification as further described below.

The Vitek 2 XL is a biochemical identification and AST instrument that uses instrument specific cards for microorganism identification and AST. The activity of the bacteria including growth, inhibition of growth, enzymatic activity, acidification, alkalization is used for automated identification and antibiotic susceptibility testing. Each well in the card is monitored by an optical system using different wave lengths every 15 minutes generating raw data. The raw data is then analyzed through a software to match for identification and susceptibility testing. The Vitek 2 XL system uses specific test kits including Gram-negative bacillus, Gram-positive cocci, Gram-negative antibiotic susceptibility and Gram-negative susceptibility. Identification and susceptibility testing take 6-18 hours in total[17].

When the criteria for species identification is not met upon analyzing by the MALDI-TOF MS and/or Vitek 2 XL, identification is performed with additional testing. These may be include morphological testing (repeat Gram stain, growth on specific agar), enzymatic tests (catalase, oxidase, indole spot, DNase and L-pyrrolidonyl-b-naphthylamide) (Remel), agglutination tests (*Streptococcus pneumonia* and Group A-D and G streptococci) (Oxoid) and susceptibility testing (optochin discs on gentian violet blood agar plates are used). Where it is clinically warranted, more advanced techniques may be performed including 16S rRNA gene sequencing. However, despite providing reliable pathogen identification and AST, current culture and identification methods continue to be time consuming, taking 6-72 hours to identify the microorganisms after blood culture positivity. The delay in detection and identification of the pathogens has forced the clinical management of sepsis to use empiric broad spectrum antibiotic therapy pending pathogen identification[18]. Similar guidelines are available for neonatal sepsis[19], meningitis[3] and peritonitis[20]. The microbiological laboratories are now presented with the challenge to provide earlier reliable pathogen identification and AST to aid in the goal of limiting the use of empiric broad spectrum antimicrobials, thereby allowing targeted antimicrobial therapy earlier in the disease course. The studies included in this thesis explored direct identification of microbes from positive blood culture bottles using short-term subculture methods, as well as subculture-independent methods.

MALDI-TOF MS has been described as 'revolutionary' and creating a 'paradigm shift' in clinical microbiology for well-deserved reasons[21]. The method was historically used for protein analysis in both research and in clinical chemistry laboratories for diagnostic purposes. The method was adapted to microbiological analysis and is currently at the center of the workflow at most major clinical microbiology laboratories for pathogen identification. Microorganism analysis is performed by applying sample colonies to the MALDI-TOF MS sample plate from solid culture media and overlay the sample with the MALDI-TOF MS matrix. The plate with the sample is allowed to dry before it is bombarded by the laser system resulting in ionization of the sample proteins and the matrix generating ions. The time-of-flight tube will then allow separation of the ions based on their mass-to-charge. The ions are detected by the MS detector and generate a profile that can be used for spectra analysis and analyzed in the software database for microorganism identification (Figure 3)[21]. The entire process of sample preparation and analysis for identification takes less than six minutes per sample.

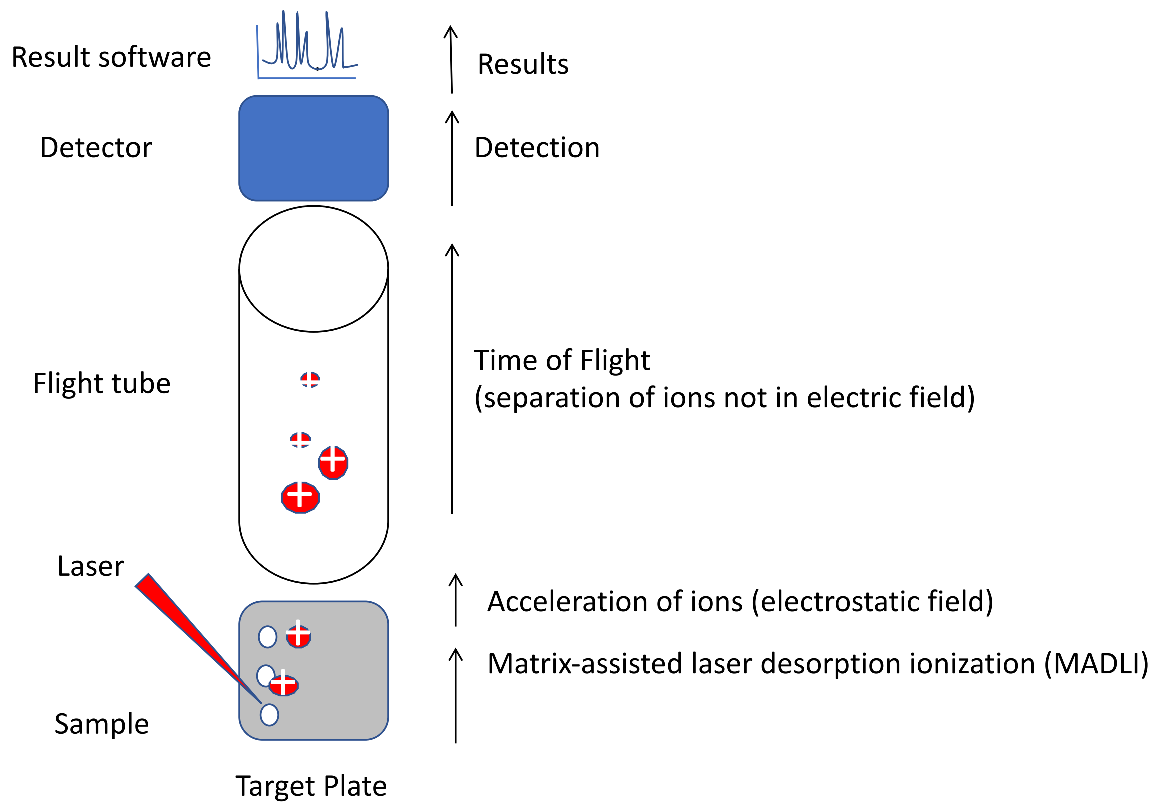


Figure 3. MALDI-TOF MS method for microorganism analysis. Target plate with sample and matrix is bombarded by the laser causing ionization of the proteins in the sample. Acceleration of the ionized proteins occurs in the electrostatic field. The time of flight tube allows separation based on the size of the proteins since all proteins have the same charge. A detector picks up the ions as they reach the detector. The software matches the detection of the ionized proteins with an internal pattern recognition for microorganism identification.

The MALDI-TOF MS became a central diagnostic tool due to the low cost per specimen analysis, the simplicity of using it and the speed in obtaining reliable identification results. The MALDI-TOF MS has been further evaluated for rapid identification of pathogens directly from positive blood cultures without first sub-culturing the culture broth on solid media [22,23]. The protocols of rapid identification of pathogens directly from blood culture bottles is an additional process that may be challenging to integrate into routine diagnostic without significantly consuming dedicated technician time. Interestingly, a novel identification method was published by Özenci et al., in 2008 based on simple routine diagnostic desktop tests following a short incubation time of blood culture broth on solid media[24].

The introduction of MALDI-TOF MS in the laboratory provided the opportunity to apply the MALDI-TOF MS to the novel method presented by Özenci et al., that was already part of the diagnostic process in the laboratory. The study included in the thesis (Study 2) investigated the performance of MALDI-TOF MS in identification of microorganisms after short-term subculture on solid media. The approach would have the potential to identify the microorganism within 2.5 h or 5.5 hours after blood culture positivity, significantly earlier than conventional culture identification methods (Figure 4).

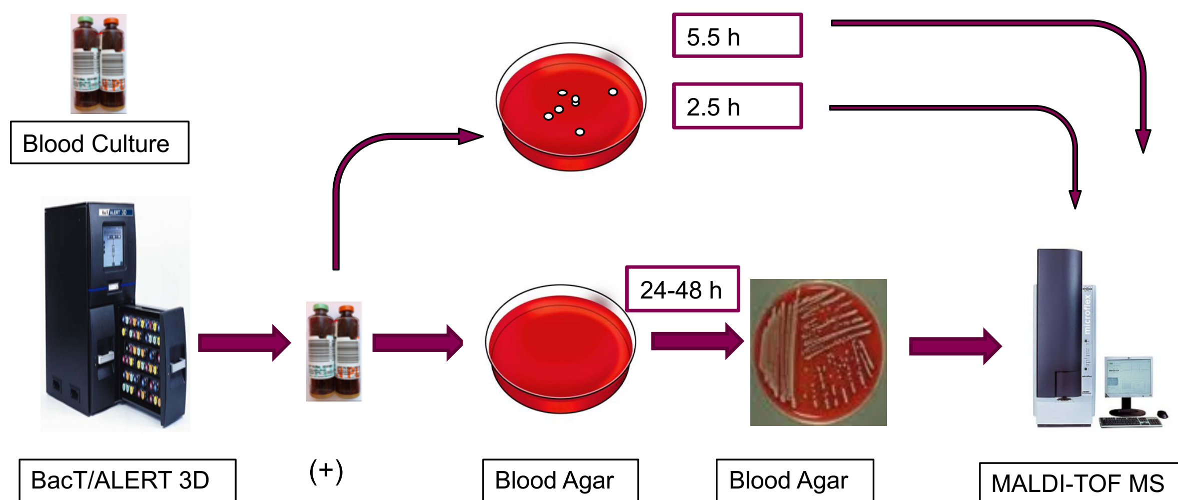


Figure 4. The workflow for traditional- and the short-term culture steps and MALDI-TOF MS analysis. After blood culture positivity, subculture was analyzed with MALDI-TOF MS at 2.5 h, 5.5 h and 24-48 h.

The subculture-independent methods for pathogen identification are characterized through the process of identification microorganisms directly from the blood culture bottles without the need for subculture to solid media with the aim to decrease the turnaround time for pathogen identification. There are currently several different subculture independent methods on the market. These methods may be based on MALDI-TOF MS, polymerase chain reaction (PCR), fluorescent nucleic acid probes, 16S rRNA and antigen/antibody-based methods[25]. The two approaches that were investigated in this thesis were the PCR-based method and the antigen/antibody approach.

The PCR-based methods have dramatically improved over the years with increasing simplicity and a decreased cost per analysis. The FilmArray blood culture identification (BCID) (BioFire, Salt Lake City, UT) panel studied in this thesis (Study 3 and 4) represents an excellent example of these advances. The FilmArray is multiplex PCR-based platform that uses high-order multiplex PCR analysis in a 'Lab-in-Pouch' approach to identify microorganisms and antibiotic susceptibility genes (Figure 5)[26–28]. The 'Lab-in-pouch' method uses a pouch containing the reagents required for sample preparation, PCR reactions, negative/positive control as well as endpoint detection with two-stage nested PCR[27]. In addition to microorganism identification, the method included AST genes as well as the ability to identify multiple microorganisms in the same sample in the setting of a polymicrobial infection. The simplicity and rapid turnaround time of one hour had the potential to decrease the time required by the technician to do the analysis as well decreasing the turnaround time. The FilmArray BCID panel was used in two studies to compare the methods performance with current standard diagnostic methods used for microorganism identification (Study 3 and 4).

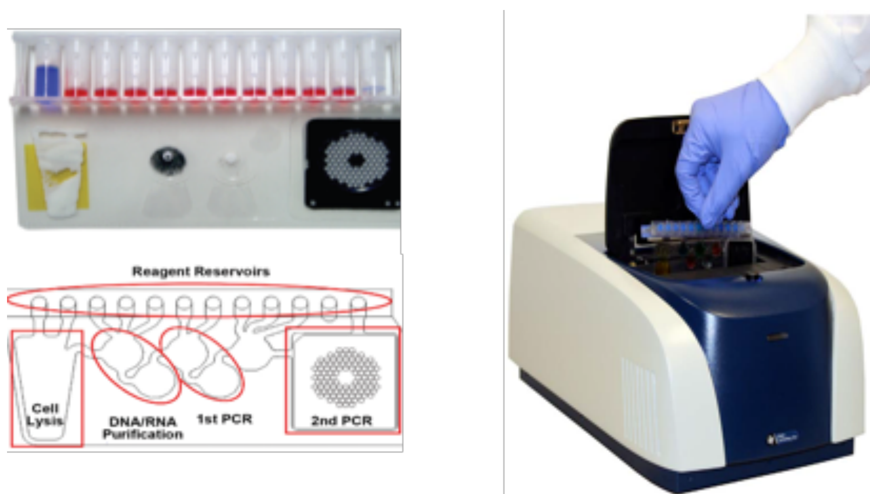


Figure 5. Outline the FilmArray pouch, reactions and the instrument. (Figure modified from Poritz et al.,[28])

Targeted single pathogen identification methods are often developed due to the high detection frequency of specific pathogens in specific cultures. *S. pneumoniae* is a good example of pathogens that are commonly encountered in several different cultures including SBF. *S. pneumoniae* has a unique feature of producing a cell wall enzyme in the stationary growth phase that causes autolysis of the microorganism in the blood culture bottle. Depending on the extent of the autolysis it may prevent *S. pneumoniae* from growing on subculture, hence preventing final identification and AST[29]. Such pathogens provide additional challenges in identification as it is diagnostically difficult to differentiating *S. pneumoniae* from other viridians group streptococci as they share phenotypical and genotypical features[30]. Furthermore, at the time of the study, the MALDI-TOF MS system had made extensive gain in the diagnostic approach to most pathogens but did not reliably distinguish the viridians streptococci from the *S. pneumoniae* due to the similarities in the protein structures of the organisms[23]. Rapid and reliable methods to identify the *S. pneumoniae* continue to be in demand despite the availability of the MALDI-TOF MS system. In the fifth and final study 5, the performance of the ImmuLex™ *S. pneumoniae* Omni (Statens Serum Institut, Denmark) latex agglutination test was compared with the three other tests that included Slidex® pneumo-Kit (bioMérieux, France), the Wellcogen™ *S. pneumoniae* (Remel, UK) and the BinaxNOW (Alere, USA) in the detection of *S. pneumoniae* antigen directly from positive blood culture bottles. The simplicity of the methods provided an opportunity to rapidly identify microorganisms with cost effective and user-friendly agglutination tests.

2 AIM OF THE THESIS

The aim of this doctoral project was to improve the microbiological diagnosis of infections in normally sterile body fluid specimens including blood when utilizing blood culture bottles for pathogen detection.

The specific aim(s) for each study:

2.1 STUDY 1:

To evaluate the performance of new BacT/Alert Virtuo (bioMérieux) blood culture system for detection and time to detection of bacteria and yeasts utilizing simulated blood cultures.

2.2 STUDY 2:

To analyze the performance of short-term culture followed by MALDI-TOF MS (Bruker) in rapid identification of bacteria from positive blood cultures.

2.3 STUDY 3:

To assess the performance of the FilmArray BCID (Biofire) in identification of bacteria and yeasts from positive blood culture bottles.

2.4 STUDY 4:

To evaluate the FilmArray BCID panel (Biofire) in identification of bacteria and yeasts in SBF cultured in blood culture bottles.

2.5 STUDY 5:

To compare ImmuLex™ *S. pneumoniae* Omni (Statens Serum Institut), Slidex® pneumo-Kit (bioMérieux), the Wellcogen™ *S. pneumoniae* (Remel) latex agglutination test and the BinaxNOW® (Alere) test in identification of *S. pneumoniae* directly from positive blood cultures.

3 MATERIALS AND METHODS

All studies were performed at the clinical microbiology laboratory at Karolinska University Hospital, Huddinge, Sweden. The microbiology laboratory serves the three tertiary-care hospitals: Karolinska University Hospital in Huddinge, Södertälje Hospital in Södertälje and South General Hospital in Stockholm serving a total of 1569 beds that include both adult and pediatric beds.

3.1 BLOOD CULTURE SYSTEMS

The blood culture systems used in each study are outlined in Table 1. The blood culture systems include BacT/Alert 3D (bioMérieux, Marcy l'Etoile, France), BacT/Alert Virtuo (bioMérieux, Marcy l'Etoile, France) and Becton Dickinson (BD) BACTEC (Becton Dickinson Instrument Systems, Sparks, MD, USA). BacT/Alert 3D was the main blood culture system used for the clinical specimens during the duration of the thesis period and was included in all studies. BacT/Alert Virto was only utilized in the first study because the system was in the process of validation and clinical evaluation. BD BACTEC blood culture system was only used for the BD Mycosis (Becton Dickinson) blood cultures to evaluate for yeast infections in the clinical routine analysis at the laboratory for the purpose of evaluating for fungemia.

Table 1. Outline of blood culture systems used each study including in the thesis.

Study	BacT/Alert 3D	BacT/Alert Virtuo	BD BACTEC
1	√	√	
2	√		
3	√		√
4	√		
5	√		

3.2 BLOOD CULTURE BOTTLES

The blood culture bottles used in the studies dependent on both instrument limitations and specific clinical utilization. The blood culture bottles used in each study are outlined in Table 2. The first study compared BacT/Alert 3D (bioMérieux, Marcy l'Etoile, France), BacT/Alert Virtuo (bioMérieux, Marcy l'Etoile, France) using aerobic (FA Plus) and anaerobic (FN Plus) BacT/Alert blood culture bottles (bioMérieux, Marcy l'Etoile, France). The second study included all blood cultures used for diagnostic purposes at the three tertiary hospitals. These additional blood culture bottles were FA charcoal, FN charcoal and the pediatric blood culture bottle (PF) Plus (bioMérieux, Marcy l'Etoile, France). Charcoal blood culture bottles were not approved to be used on the FilmArray blood culture identification panel (BCID) (BioFire, Salt Lake City, UT, USA) and were therefore excluded from the third and fourth study. The fourth study excluded the use of charcoal bottles as well as BD Mycosis (Becton Dickinson Instrument Systems, Sparks, MD) because they were not utilized to diagnose infections of sterile body fluids. Finally, the fifth study excluded the BD Mycosis blood culture bottles that contain antibiotics to prevent bacterial growth and are not used to detect bacteremia including *Streptococcus pneumoniae*.

Table 2. Blood culture bottles used in each study included in the thesis.

Study	BacT/Alert FA/FN Plus	BacT/Alert FA/FN	BacT/Alert PF Plus	BACTEC Mycosis
1	√			
2	√	√	√	
3	√		√	√
4	√		√	
5	√	√	√	

3.3 SIMULATED BLOOD CULTURES

Simulate blood cultures were only used in Study 1 to compare the two blood culture systems BacT/Alert 3D (bioMérieux, Marcy l'Etoile, France), BacT/Alert Virtuo (bioMérieux, Marcy l'Etoile, France). In total, 115 frozen clinical bacterial and fungal isolates were used in the study. Originally, the isolates were collected from clinical blood cultures that had been processed in the routine microbiological analysis for clinical diagnostics at Karolinska University Laboratory and stored in a frozen stock. Isolates were recovered from frozen stocks by culture on appropriate agar for 24 h (aerobe bacteria) or 48 h (anaerobe bacteria) in a temperature of 36°C. The bacteria were then suspended in phosphate-buffer saline solution (pH 7.3-7.4) to a McFarland of 0.5 (1.5×10^8 CFU/ml) to allow approximately 15 colony-forming-units (CFU) to be inoculated in the blood culture bottles. The suspension was sub-cultured on agar to assess the CFU for control of the final inoculum. Each blood culture bottle was inoculated with eight milliliter defibrinated horse blood. Finally, blood cultures were incubated in the blood culture system until positivity or for a maximum of five days. Blood culture that were negative until final incubation day were sub-cultured on agar for control of growth.

3.4 CLINICAL CULTURES

The clinical blood cultures and cultures of sterile body fluids were obtained from specimens that were sent to Karolinska University Laboratory for clinical diagnostic purpose. Clinical blood cultures were collected and inoculated by standard collection method to avoid contamination. Blood cultures were incubated in the BacT/Alert 3D blood culture system until positivity for a maximum of five days. The number of positive blood cultures included in Study 2 was 579, Study 3 was 206, and Study 5 was 375. Study 4 included 116 sterile body fluid specimens cultured using blood culture bottles. Becton Dickinson (BD) Mycosis (Becton Dickinson) blood culture bottles were similarly inoculated with blood and incubated in BD BACTEC (Becton Dickinson) until positivity or for a max incubation of 14 days. Briefly, 8-10 ml blood or sterile body fluid specimen were inoculated into BacT/Alert FA, FA Plus, FN and FN plus blood culture bottles (bioMérieux, Marcy l'Etoile, France). The PF bottles (bioMérieux, Marcy l'Etoile, France) were inoculated with 0.5-4 ml blood or other SBF. The blood culture bottles with blood were placed in the blood culture system upon arrival to the laboratory within normal working hours. The blood culture bottles with SBF

were placed in the blood culture system after inoculation of the SBF in blood culture bottles the laboratory.

3.5 THE FILMARRAY BLOOD CULTURE IDENTIFICATION ASSAY

The FilmArray blood culture identification (BCID) assay (BioFire, Salt Lake City, UT, USA) is an integrated reverse-transcription-PCR that utilizes a pouch to dry store all necessary reagents for sample preparation, reverse-transcription-PCR, PCR and for detection of bacterial and fungal pathogens. The FilmArray BCID panel includes 19 bacteria, five *Candida* species and three antimicrobial gene targets (Table 3).

FilmArray analysis was performed by inserting one milliliter hydration buffer into the pouch using the accompanied product syringe and hydration buffer. Then, 100 µl blood culture bottle broth (with blood or sterile body fluid) was diluted in 500 µl accompanied FilmArray dilution buffer. Finally, 300 µl of the diluted sample was injected in the FilmArray pouch before loading the pouch in the FilmArray system for analysis. FilmArray automatically performed the DNA extraction, amplification, melt curve analysis, two internal control (for primary amplification and the analyze-specific detection stages) and target detection within 60 minutes. The automated system only provided results if the internal quality controls were detected and would otherwise result in invalid result.

Table 3. The microorganisms and antimicrobial resistance genes included in the FilmArray BCID Panel.

Category	Target
Gram-negative bacteria	*Enterobacteriaceae*
	Escherichia coli
	Enterobacter cloacae complex
	Klebsiella oxytoca
	Klebsiella pneumoniae
	Serratia marcescens
	Proteus spp.
	Acinetobacter baumannii
	Haemophilus influenzae
	Neisseria meningitidis
	Pseudomonas aeruginosa
Gram-positive bacteria	*Staphylococcus* spp.
	Staphylococcus aureus
	Streptococcus spp.
	Streptococcus agalactiae
	Streptococcus pyogenes
	Streptococcus pneumoniae
	Enterococcus spp.
	Listeria monocytogenes
Fungi	*Candida albicans*
	Candida glabrata
	Candida krusei
	Candida parapsilosis
	Candida tropicalis
Antibiotic resistance markers	*mecA*
	vanA/vanB
	KPC

Reference: Altun O, Almuhayawai M, Ullberg M, Ozenci V. Clinical evaluation of the FilmArray blood culture identification panel in identification of bacteria and yeasts from positive blood culture bottles. Journal of Clinical Microbiology. 2013;51(12):4130-6.

3.6 MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

MALDI-TOF MS (Bruker Daltonik, Hamburg, Germany) analysis was performed directly on bacteria grown on agar plates. In brief, a thin layer from bacterial colonies grown on agar plates was scraped using a one milliliter bacterial loop and smeared on a spot on the 96-spot stainless steel target plate (Bruker Daltonik, Hamburg, Germany). Protein extraction was performed on the plate using 70% formic acid solution (Fluka) and allowing the sample to dry for two minutes. One microliter alpha-cyano-4-hydroxycunnamic acid (CHCA matrix) (Bruker Daltonik) was then applied on the sample and dried before analyzing it with the MALDI-TOF MS system (Bruker Daltonik). Spectra analysis was performed using Bruker Biotype 3.1 software and library (version 4613, Bruker Daltonik). Score cut offs for identification were used according to the MALDI-TOF MS manufacturers guidelines. Scores >1.7 were interpreted as low-confidence identification but considered as successful identification if confirmed by conventional methods obtained overnight. A Score of <1.7 was considered failed identification.

3.7 STREPTOCOCCUS PNEUMONIAE ANTIGEN TESTS

In study 5, the performance of four *S. Pneumoniae* antigen tests was evaluated. These tests were ImmuLex™ *S. pneumoniae* Omni (Statens Serum Institut, Denmark) LA, SlideX® pneumo-Kit (bioMérieux, Marcy l'Etoile, France), Wellcogen™ *S. pneumoniae* (Remel, UK) LA and BinaxNOW® *S. pneumoniae* (Abbot, USA). The tests were done according to the manufacturer's instructions. Briefly, the ImmuLex™ test was performed by mixing one drop of the blood culture drop with one drop of the anti-*S. Pneumoniae* latex suspension on the reaction card. A positive result was obtained if agglutination was observed within five minutes. The ImmuLex™ had a negative control using one drop from the negative control bottle with one drop of the latex suspension. The Slidex® test was performed by centrifuging one milliliter blood culture broth at 2000 rpm for 10 minutes and using one drop of the supernatant to mix with one drop of anti-*S. pneumoniae* latex suspension on a glass slide for two minutes or until agglutination was observed. The negative control for the Slidex® tests was done by applying one drop of the supernatant on a glass slide and mixing it with the accompanied control latex suspension. The Wellcogen™ test was performed by mixing one milliliter drop of the anti-*S. pneumoniae* latex suspension on the reaction card. The negative control was done by mixing one drop of the blood culture broth with one drop of accompanied control latex. The specimens were mixed on the card for two minutes or until agglutination

was observed. For all tests, the presence of agglutination in the tests and the absence of agglutination in the negative control was considered a positive test while agglutination in the negative control was considered an unreliable for identification (invalid test result). An invalid test was repeated at least once. The BinaxNOW® was performed by applying one drop from the positive blood culture broth and visually reading the membrane for positive and control line after 15 minutes. The presence of a positive line on the testing card and the absence of the negative control line was interpreted as a positive test.

3.8 CONVENTIONAL METHODS FOR IDENTIFICATION OF MICROORGANISMS

The conventional methods for identification of microorganisms from positive blood culture bottles were utilized in the laboratory for routine clinical diagnostic purposes. Positive blood cultures broth was first analyzed by Gram-stain. The broth was subsequently cultured on agar plates based on the Gram-stain and incubated for 24-48 hours. Identification of microorganisms was done by analyzing growth on agar plates with MALDI-TOF MS (Bruker Daltonic) or Vitek2 XL (bioMérieux, Marcy l'Etoile, France). Validated desk spot tests, agglutination tests, ozidase, indole spot and L-pyrrolidonyl-β-naphthylamide as indicated. Discrepant results were further analyzed with subcultures, selective media and repeat MALTI-TOF MS and/or Vitek2 XL analysis. Susceptibility testing was performed with the disc diffusion method in accordance with the EUCAST guidelines (http://www.eucast.org). *Staphylococcus* spp. were tested for methicillin resistance with the cefoxitin disc diffusion method. *Klebsiella pneumoniae* carbapenems testing was with the imipenem and meropenem disc diffusion method.

In study 5, amplification and sequencing of 16S rRNA and/or recA was utilized according to the study by Mellmann et al., to differentiate α-haemolytic streptococci species and *S. pneumoniae*[31].

3.9 PROTOCOL DESCRIPTION FOR EACH STUDY

3.9.1 Study 1: Controlled evaluation of the new BacT/Alert Virtuo blood culture system for detection and time to detection of bacteria and yeast

The performance of detection and time of detection of the new BacT/Alert Virtuo (bioMérieux, Marcy l'Etoile, France) blood culture system was compared with the BacT/Alert 3D (bioMérieux, Marcy l'Etoile, France) blood culture system by parallel incubation of 115 clinical bacterial and fungal isolates. Isolates were recovered from frozen stock of isolates that were collected from positive clinical blood cultures at the Karolinska University Laboratory. The isolates were suspended and inoculated in aerobic (FA and FA Plus (bioMérieux, Marcy l'Etoile, France)) and anaerobic (FN and FN Plus (bioMérieux, Marcy l'Etoile, France)) blood culture bottles to simulated blood cultures and use them to compare the performance of the two blood culture systems in detection and time to detection (Figure 6). The aerobic microorganisms *Candida* spp. and *Acinetobacter* spp. were only cultured in aerobic blood cultures. The CE-approved version of the BacT/Alert Virtuo system was intended to operate on the new resin-based blood culture bottles (FA Plus and FN Plus). The charcoal-based blood culture bottles (FA and FN) were included in the investigational-use-only version of the Virtuo employed in this study. Blood cultures were sub-cultured at the end of the incubation period to exclude contamination and confirm true positive/negative results.

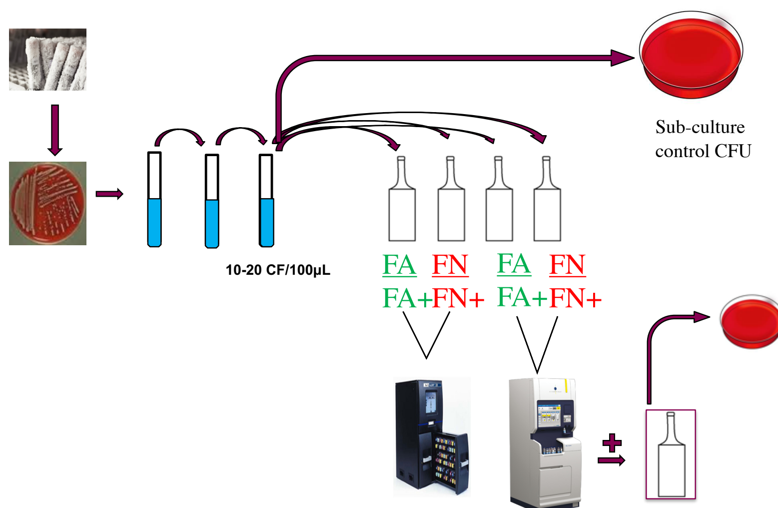


Figure 6. Outlines protocol for study 1. Bacteria from frozen stock sub-cultured, then resuspended. Each blood culture bottle was inoculated with the resuspended bacteria. The resuspension was cultured for control. All positive and negative blood cultures were sub-cultured after final incubation time.

3.9.2 Study 2. Rapid identification of bacteria from positive blood culture bottles by MALDI-TOF MS following short-term incubation on solid media.

The performance of MALDI-TOF MS (Bruker Daltonik, Hamburg, Germany) in identifying microorganisms from blood culture bottles after a short-term incubation on solid media was evaluated in comparison with conventional culture and identification methods. The MALDI-TOF MS analysis was performed after 2.5- and 5.5 h incubation time of positive blood culture broth cultured on solid media. Positive blood cultures were sub-cultured on blood/CLED and chocolate agar in 5% CO_2 atmosphere. When microorganisms were suspected to be anaerobes by Gram stain and morphological evaluation, the sub-culture was done on blood agar plates and incubated in 35°C in anaerobic environment. The MALDI-TOF MS microorganism identification results after short-term incubation was compared with conventional culture and identification methods.

3.9.3 Study 3. Clinical evaluation of the FilmArray BCID in identification of bacteria and yeast from positive blood culture bottles.

This prospective clinical study was performed to evaluate the performance of the new FilmArray BCID panel. The study included only one blood culture per patient and the results of the FilmArray BCID panel were compared with conventional culture and identification as well as AST.

3.9.4 Study 4. Rapid identification of microorganisms from sterile body fluids by use of FilmArray BCID

This prospective clinical study evaluated the performance of the FilmArray BCID in identifying microorganisms in positive blood culture bottles inoculated with sterile body fluid for bacterial and yeast analysis. Reproducibility testing as well as longitudinal testing of the FilmArray BCID panel was performed as well.

3.9.5 Study 5. Rapid identification of *Streptococcus pneumoniae* in blood cultures by using the ImmuLex^{TM}, Slidex$^{®}$ and Wellcogen^{TM} latex agglutination tests and the BinaxNOW$^{®}$ antigen test.

The performance of the new latex agglutination test ImmuLex^{TM} *S. pneumoniae* Omni was compared with Slidex$^{®}$ pneumo-Kit, Wellcogen^{TM} *S. pneumoniae* and BinaxNOW$^{®}$ *S. pneumoniae* antigen test in detecting *S. pneumoniae* in positive blood cultures. This was a prospective clinical study where blood culture bottles with growth for gram positive cocci in pairs suspected to be *S. pneumoniae* were included and analyzed by all tests for comparison.

3.10 STATISTICAL ANALYSIS FOR EACH STUDY

In Study 1, GraphPad Prism 6 was used to perform the statistical analysis. The time to detection (TTD) between the two blood culture systems were compared using the 2-way matched analysis of variance (ANOVA) or Wilcoxon matched-pair signed-rank test. The Mann-Whitney U test was used to compare TTD between the groups of microorganisms. The Spearman correlation was used to assess TTD values by each of the two blood culture systems. The p-value of <0.05 was used to measure the statistical difference.

In Study 2, statistical analysis was not performed as the purpose of the study was not directed at finding a statistical difference between the MALDI-TOF MS short term incubation and the conventional method. Fisher's exact test may be used to compare the two diagnostic methods. However, the rapid test is a complement to the conventional diagnostic methods and a direct percent comparison is sufficient for clinical comparing the diagnostic utility of the rapid test.

In Study 3, Fisher's exact test was used to compare the identification performance of the FilmArray BICD with the conventional culture and identification methods.

In Study 4, a statistical analysis was not applied due to the limited number of each organism detected in the study. Overall statistical analysis would not be clinically valuable for the same reason.

In Study 5, McNamara's test for paired samples was used to compare the proportions. A two-tailed p-value <0.01 was used to evaluate for statistical significance.

3.11 ETHICAL PERMISSION

Ethical permission was not required for the present five studies. In accordance with the Swedish ethical law for research in human subjects or biological material derived from human subjects, the specimens were coded and cannot be traced back to the humans that provided the specimens. The biological material was collected for the diagnostic purposes for clinical management. No additional clinical sample was collected from patients. The human subjects were therefore not exposed to a physical procedure to collect specimens for the studies in the thesis. The study results were not used during the study period to direct patient care and no patient data was included in the results.

4 RESULTS

4.1 MICROORGANISMS

The studies included a total of 1387 isolates, which constituted of 374 Gram-negative (Table 4), 964 Gram-positive (Table 5), and 49 yeast (Table 6) isolates. Table 4, 5 and 6 outlines the number of each Gram negative, Gram positive and yeast isolates respectively in each study. Study 2 excluded cultures with yeasts because of their slow growth. Study 5 did not include any cultures with yeast.

Table 4. Gram-negative bacteria included in the thesis.

		No. of Gram-negative Isolates in Each Study				
Organism	**Total**	**Study 1**	**Study 2**	**Study 3**	**Study 4**	**Study 5**
Escherichia coli	190	10	129	39	12	
Enterobacter asburiae	2		2			
Enterobacter aerogenes	4		3	1		
Enterobacter cloacae	6		2	2	1	1
Enterobacter kobei	8		8			
Klebsiella pneumoniae	50	7	29	9	4	1
Klebsiella oxytoca	19	3	10	3	3	
Raoultella planticola	1		1			
Citrobacter freundii	7		6		1	
Citrobacter koseri	2		2			
Citrobacter farmeri	1				1	
Morganella morganii	1		1			
Proteus vulgaris	1				1	
Proteus mirabilis	10		8	2		
Salmonella spp.	6		4	2		
Serratia marcescens	18	12	4	2		
Nisseria meningitidis	1			1		
Aeromonas spp.	2		1		1	
Acinetobacter spp.	7	7				
Acinetobacter ursingii	1		1			
Pseudomonas aeruginosa	12		2	4	5	1
Brucella spp.	1		1			
Haemophilus influenzae	6		1	2	1	2
Bacteroides fragilis	9		7	2		
Bacteroides vulgatus	1		1			
Bacteroides thetaiotaomicron	1		1			
Parabacteroides distasonis	1		1			
Prevotella spp.	3		1		2	
Veillonella parvula	1		1			
Fusobacterium necrophorum	1		1			
Anaerobic rodd non-speciated	1		1			
Total	**374**	**39**	**229**	**69**	**32**	**5**

Table 5. Gram-positive bacteria included in the thesis.

		No. of Gram-positive Isolates in Each Study				
Organism	**Total**	**Study 1**	**Study 2**	**Study 3**	**Study 4**	**Study 5**
Staphylococcus aureus	130	10	61	22	35	2
Staphylococcus capitis	13		12		1	
Staphylococcus epidermidis	88		84		4	
Staphylococcus haemolyticus	2		2			
Staphylococcus hominis	29		29			
Staphylococcus pettenkoferi	2		2			
Staphylococcus saprophyticus	1		1			
Coagulase negative staphylococcus non-speciated	66	5	2	44	14	1
Enterococcus faecalis	43	5	28		10	
Enterococcus faecium	18	5	7		6	
Enterococcus casseliflavus	1		1			
Enterococcus spp.	53			21	9	23
Streptococcus agalactiae (Group B streptococci)	13		5	5	3	
Streptococcus dysgalactiae (Group G streptococci)	4	2	2			
Streptococcus pyogenes (Group A Streptocci)	11	3	3	3	1	1
Streptococcus anginosus	7		4		2	1
Streptococcus gallolyticus	2		2			
Streptococcus parasanguinis	3		2			1
Streptococcus pneumoniae	306	13	8	14	5	266
Streptococcus salivarius	2		2			
Alpha-streptococci spp.	102	6	9	11	9	67
Streptococcus spp.	6				6	
Gemella sanguinis	2		1	1		
Rothia mucilaginosa	1		1			
Micrococcus spp.	10		3	4	3	
Listeria monocytogenes	5		2	2	1	
Bacillus thuringiensis	1		1			
Corynebacterium spp.	7		2	2	3	
Lactobacillus spp.	16			15	1	
Pediococcus acidilactici	1				1	
Clostridium halophilum	1		1			
Clostridium perfringens	2		1	1		
Clostridium spp.	1		1			
Propionibacterium acnes	4		3	1		
Propionibacterium avidum	1		1			
Eggerthella lentad	2		1	1		
Gram-positive anaerobic rod unspeciated	2		2			
Peptoniphilus spp.	2			2		
Parvimonas spp.	1			1		
Bacillus spp.	2			1	1	
Actinomyces spp.	1				1	
Total	964	49	286	151	116	362

Table 6. The yeasts included in the thesis.

		No. of Yeast Isolates in Each Study				
Organism	**Total**	**Study 1**	**Study 2***	**Study 3**	**Study 4**	**Study 5***
Candida albicans	25	12		7	6	
Candida glabrata	22	15		4	3	
Candida crusei	1				1	
Candida parapsilosis	0					
Saccharomyces cerevisiae	1				1	
Total	**49**	**27**	**0**	**11**	**11**	**0**

**Study two and study five did not include any yeast pathogens.*

4.2 EVALUATION OF BACT/ALERT VIRTUO BLOOD CULTURE SYSTEM.

In Study 1, in total, 115 clinical isolates (Figure 7) were used to inoculate a total of 392 blood culture bottle pairs (total 784 blood culture bottles) to compare the BacT/Alert Virtuo with the BacT/Alert 3D blood culture system. The majority (760/784 (97%)) signaled positive within the five-day incubation period. The number of negative blood culture bottle was 24 (12 bottles in each blood culture system) with no difference of negative blood culture bottle in each system. The negative blood cultures were inoculated with *Acinetobacter* spp. (n=1 pair), coagulase-negative staphylococci (CoNS) (n=3 pairs), alpha-hemolytic streptococci (n=7 pairs), one alpha-hemolytic streptococcus isolate that grew in the Virtuo and one *Acinetobacter* spp. that grew only in BacT/Alert 3D blood culture system. Subculture of the negative blood cultures confirmed the absence of growth and confirmed true negative culture.

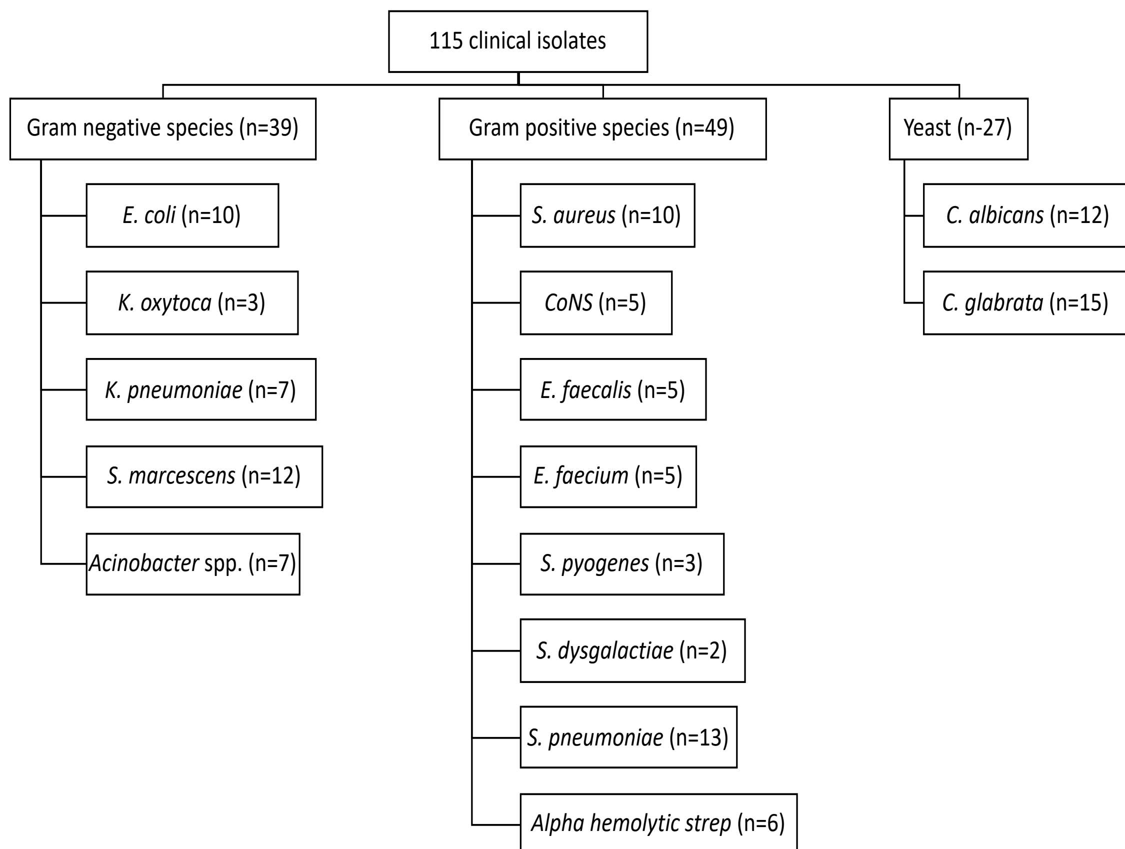


Figure 7. Outline of the clinical isolates used in Study 1.

In comparing the time to detection (TTD) among the 379 blood culture bottle pairs that signaled positive in the two systems, the TTD was significantly shorter in the Virtuo blood culture system then BacT/Alert 3D (median TTD 12 h and 15 hours respectively; P<0.0001) which is a 20% reduction in TTD. Further stratification of the positive blood culture pairs showed that 90% of the positive blood culture bottles were positive in 16 h in the Virtuo compared with 21 h in the BacT/Alert 3D system. There was no difference among bacterial species or type of blood culture bottle used. The blood culture bottles inoculated with *Candida* spp. signaled positive within three days (71 h) in the Virtuo compared with four days (81 hours) in the BacT/Alert 3D blood culture system.

4.3 RAPID IDENTIFICATION BY MATRIX-ASSOCIATED LASER DESORPTION IONIZATION-TIME OF FLIGHT MASS SPECTROMETER (MALDI-TOF MS) AFTER SUBCULTURE ON SOLID MEDIA

In study 2, a total of 579 positive blood cultures were studied. For the study, 12 (12/579, 2.1%) blood cultures were excluded due to polymicrobial growth and 52 (52/579, 9%) due to growth of yeast. The remaining 515 blood cultures were 286 (55.5 %) Gram-positive bacteria (GPB) and bottles and 229 (44.5 %) Gram-negative bacteria (GNB) (Figure 8). Conventional methods identified 267/286 (93.4 %) GPB and 223/229 (97.4 %) GNB to the species level after overnight culture. Accurate identification of by MALDI-TOF MS after 2.5 h was 300/515 (58.3 %) and 390/515 (76%) after 5.5 h culture on solid media.

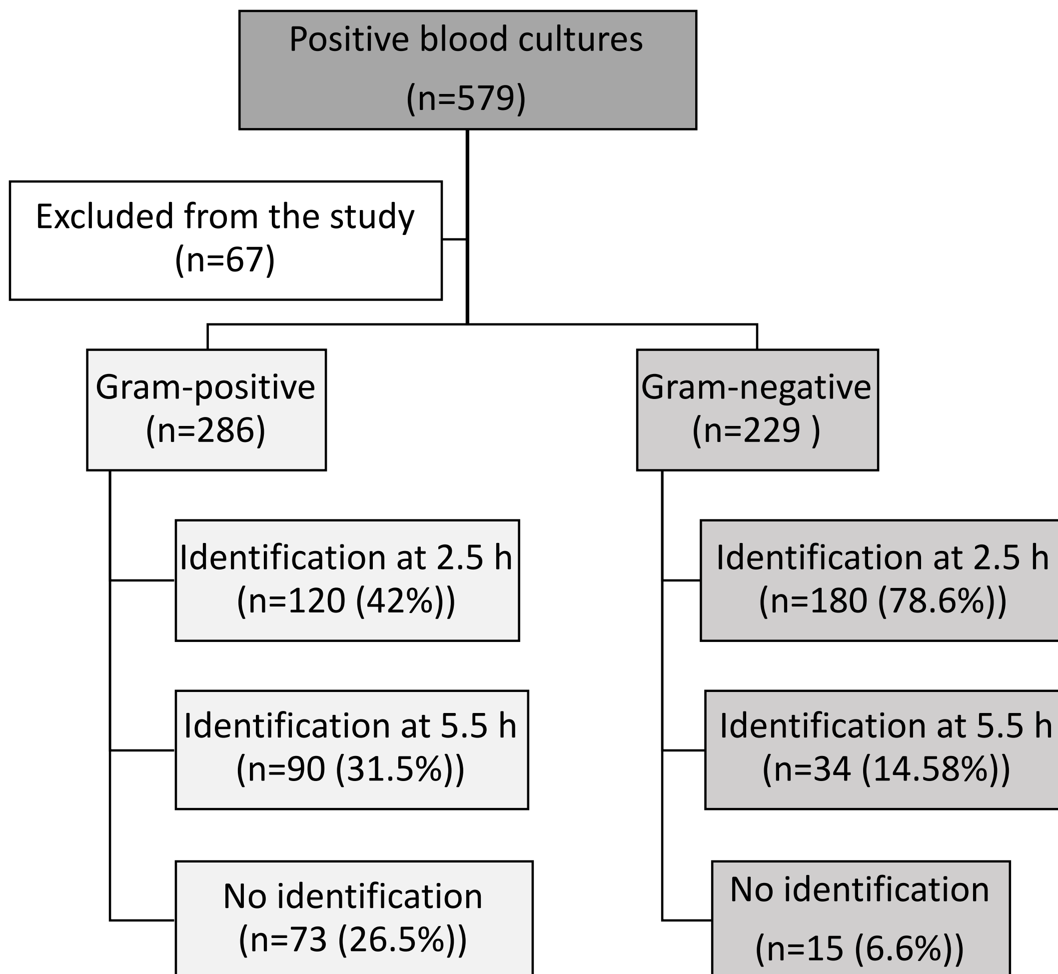


Figure 8. Overview of MALDI-TOF MS identification of Gram-negative and Gram-positive bacteria at 2.5 and 5 hours after subculture on solid media.

As outlined in Figure 8, there is a marked difference in the identification of GNB compared with GPB after 2.5 h subculture,180/229 (78.6 %) and 120/286 (42.0 %), respectively. Further studying the specific pathogens in the GNB (Table 8) and GPB (Table 7) reveals that the majority of Enterobacteriaceae and *S. aureus* were identified in 2.5 h, 173/209 (82.8 %) and 44/61 (72.1 %), respectively.

With the additional 90 GBP and 34 GNB identified after 5.5 h culture on solid media, a total of 424/515 (82.3 %) of all isolates included in the study were identified generating a cumulative identification rate of 73.4 % for GPB and 93.4 % for GNB at this time point. Further analysis of Table 7 reveals that most of the *S. aureus* (56/61, 91.8 %), *Enterococci* spp. (33/36, 91.7 %) and beta-haemolytic streptococci group A, B and G (8/10, 80 %) were correctly identified at the end (5.5 h) of the short-term culture period. In contrast, Major limitations were seen in the identification of *Streptococcus pneumoniae* (2/8), other alpha-hemolytic streptococci (3/19) and anaerobic GPB (2/10).

Table 8 presents the identification results of GNB including the identification of 9/14 anaerobic GNB *Bacteroides* spp. after 5.5 h culture on solid media.

Table 7. Gram-positive bacteria identified during the study period in Study 2.

Organism	Rapid culture followed by MALDI-TOF			Conventional methods
	2.5 h	5.5 h	No ID	>24 h
Staphylococcus **spp.**	96 (49.7 %)	61 (31.6 %)	36 (18.7 %)	193 (100 %)
Staphylococcus aureus	44	12	5	61
Staphylococcus capitis	7	3	2	12
Staphylococcus epidermidis	30	30	24	84
Staphylococcus haemolyticus	1	1	0	2
Staphylococcus hominis	13	13	3	29
Staphylococcus pettenkoferi	1	1	0	2
Staphylococcus saprophyticus	0	1	0	1
Other CoNS*	0	0	2	2
Enterococcus **spp.**	16 (44.4 %)	17 (47.3 %)	3 (8.3 %)	36 (100 %)
Enterococcus faecalis	14	12	2	28
Enterococcus faecium	1	5	1	7
Enterococcus casseliflavus	1	0	0	1
Streptococcus **spp.**	5 (13.5 %)	8 (21.6 %)	24 (64.9 %)	37 (100 %)
Streptococcus agalactiae	3	2	0	5
Streptococcus dysgalactiae	0	0	2	2
Streptococcus pyogenes	1	2	0	3
Streptococcus anginosus	0	0	4	4
Streptococcus gallolyticus	0	0	2	2
Streptococcus parasanguinis	1	0	1	2
Streptococcus pneumoniae	0	2	6	8
Streptococcus salivarius	0	2	0	2
Other α-streptococci†	0	0	9	9
Other GPB	3	2	5	10
Gemella sanguinis	1	0	0	1
Rothia mucilaginosa	0	1	0	1
Micrococcus spp.	0	0	3	3
Listeria monocytogenes	2	0	0	2
Bacillus thuringiensis	0	1	0	1
Corynebacterium spp.	0	0	2	2
Anaerobic GPB	0	2	8	10
Clostridium halophilum	0	1	0	1
Clostridium perfringens	0	1	0	1
Clostridium spp.	0	0	1	1
Propionibacterium acnes	0	0	3	3
Propionibacterium avidum	0	0	1	1
Eggerthella lenta‡	0	0	1	1
Gram-positive anaerobic rod	0	0	2	2
Total	**120 (42 %)**	**90 (31.5 %)**	**76 (26.5 %)**	**286 (100 %)**

*Could not be identified by MALDI-TOF MS or Vitek 2; both isolates were DNase negative.

†These isolates could not be identified by MALDI-TOF MS; they were classified as α-streptococci based on optochin resistance and negative agglutination for group A, B, C, D, G streptococci and *Streptococcus pneumoniae.* Vitek 2 was not performed in these cases.

‡Identified by Vitek 2, with a high identification score of 99 %.

Reference: Altun O, Botero-Kleiven S, Carlsson S, Ullberg M, Özenci V. Rapid identification of bacteria from positive blood culture bottles by MALDI-TOF MS following short-term incubation on solid media. Journal of Medical Microbiology. 2015, 00, 1-7. PMID: 263

Table 8. Gram-negative bacteria identified during the study period in Study 2.

Organism	Rapid culture followed by MALDI-TOF			Conventional methods
	2.5 h	5.5 h	No ID	>24 h
Enterobacteriaceae	173 (82.8 %)	28 (13.4 %)	8 (3.8 %)	209 (100 %)
Escherichia coli	107	17	5	129
Enterobacter asburiae	2	0	0	2
Enterobacter aerogenes	3	0	0	3
Enterobacter cloacae	2	0	0	2
Enterobacter kobei	8	0	0	8
Klebsiella pneumoniae	25	4	0	29
Klebsiella oxytoca	6	2	2	10
Raoultella planticola	1	0	0	1
Citrobacter freundii	4	1	1	6
Citrobacter koseri	2	0	0	2
Morganella morganii	1	0	0	1
Proteus mirabilis	5	3	0	8
Salmonella spp.*	3	1	0	4
Serratia marcescens	4	0	0	4
Other GNB	3	1	2	6
Aeromonas hydrophila	1	0	0	1
Acinetobacter ursingii	1	0	0	1
Pseudomonas aeruginosa	1	1	0	2
Brucella spp.†	0	0	1	1
Haemophilus influenzae	0	0	1	1
Anaerobic GNB	4	5	5	14
Bacteroides fragilis	2	4	1	7
Bacteroides vulgatus	1	0	0	1
Bacteroides thetaiotaomicron	0	1	0	1
Parabacteroides distasonis	1	0	0	1
Prevotella oralis	0	0	1	1
Veillonella parvula	0	0	1	1
Fusobacterium necrophorum	0	0	1	1
Gram-negative anaerobic rod‡	0	0	1	1
Total	**180 (78.6 %)**	**34 (14.8 %)**	**15 (6.6 %)**	**229 (100 %)**

*As all *Salmonella* spp. were identified only at the genus level by conventional methods at ≥24 h, MALDI-TOF MS identification at the genus level was accepted as a successful identification.

†For biosafety reasons, the isolate was sent for typing by PCR to a reference laboratory.

‡Could not be identified by MALDI-TOF MS or Vitek 2 because of insufficient bacterial growth even after >24 h.

Reference; Altun O, Botero-Kleiven S, Carlsson S, Ullberg M, Özenci V. Rapid identification of bacteria from positive blood culture bottles by MALDI-TOF MS following short-term incubation on solid media. Journal of Medical Microbiology. 2015, 00, 1-7. PMID: 263

4.4 RAPID PATHOGEN IDENTIFICATION BY FILMARRAY BCID PANEL DIRECTLY FROM BLOOD CULTURE BOTTLES

The study included a total of 206 positive blood cultures, including 167 (Table 9) and 24 (Table 10) positive blood cultures with mono- and polymicrobial growth respectively and 12 blood cultures that signaled positive but were negative on Gram stain and subculture. Longitudinal performance was assessed by repeatedly analyzing the same five positive blood cultures over four weeks by a total of 29 tests. Reproducibility was assessed by running another set of five positive blood cultures twice during the same day. During the study period, 236 FilmArray tests were performed with 3/236 (1.3%) pouch failures encountered secondary to negative/positive control in the pouch.

Thirty-five different species were identified by conventional identification methods from the included specimens. The FilmArray BCID panel included targets to detect 24/35 (69%) of the isolates (*Enterococcus* spp. were identified on the genus level). The FilmArray BCID panel identified 153/167 (91.6%) pathogens in the monomicrobial growth blood cultures (Table 9). Among the monomicrobial growth blood cultures there was 13/167 (7.8%) microorganisms that were not included in the FilmArray BCID panel including three *Micrococcus* spp., two of each *Corynebacterium* spp., and *Peptoniphilus* spp., and one of each *Parvimonas micra, Bacteroides fragilis*, *Eggerthella lenta*, *Capnocytophaga canimorsus*, *Gemella* spp., and *Lactobacillus* spp. There was 1/167 (0.6%) false negative result and included a CoNS. The FilmArray detected an additional microorganism in 6/167 (3.6%) samples. These six specimens included four blood culture bottles with CoNS, one with *S. pneumoniae* with FilmArray simultaneously detected *Enterococcus* spp., and in one with *C. glabrata* where FilmArray detected also *C. albicans*. In five specimens, FilmArray detected *Enterococcus* spp. that did not grow on subculture.

Tabell 9. Identification of bacteria, yeast and antimicrobial resistance genes from the 167 blood culture bottles with monomicrobial growth included in Study 3.

Identification	No. of samples Blood culture and FA positive	Blood culture positive and FA negative	Blood culture negative and FA positive
Microorganisms included in FA BCID panel			
Gram-negative bacteria			
Escherichia coli	34		
Klebsiella pneumoniae	5		
Klebsiella oxytoca	2		
Proteus mirabilis	2		
Pseudomonas aeruginosa	2		
Haemophilus influenzae	2		
Enterobacter cloacae	1		
Enterobacter aerogenes	1		
Salmonella spp.	1		
Serratia marcescens	1		
Neisseria meningitidis	1		
Gram-positive bacteria			
Coagulase-negative staphylococci	37	1	
Staphylococcus aureus	19		
Streptococcus pneumoniae	13		
Enterococcus spp.	9[a]		4
Streptococcus agalactiae	5		
Alpha-hemolytic streptococci	4		
Streptococcus pyogenes	2		
Listeria monocytogenes	2		
Fungi			
Candida albicans	6		1
Candida glabrata	4		
Microorganisms not included in FA BCID panel			
Micrococcus spp.		3	
Corynebacterium spp.		2	
Peptoniphilus spp.		2	
Capnocytophaga canimorsus		1	
Bacteroides fragilis		1	
Eggerthella lenta		1	
Gemella spp.		1	
Lactobacillus spp.		1	
Parvimonas micra		1	
Antibiotic resistance markers			
mecA	15[b]	1	3[c]
vanA/*vanB*	0		0

[a] Six *E. faecalis* and 3 *E. faecium* isolates.
[b] One MRSA isolate and 14 methicillin-resistant CoNS.
[c] PCR showed that 1/3 CoNS was *mecA* positive, as shown by FilmArray, whereas 2/3 were *mecA* negative, as in the disc diffusion test.

Reference; Altun O, Almuhayawai M, Ullberg M, Ozenci V. Clinical evaluation of the FilmArray blood culture identification panel in identification of bacteria and yeasts from positive blood culture bottles. Journal of Clinical Microbiology. 2013;51(12):41

In blood cultures with polymicrobial growth, the FilmArray BCID accurately detected all microorganisms in 17/24 (71%) cultures (Table 10). FilmArray did not detect one or more organisms in 6/24 (25%) blood cultures with polymicrobial growth. The microorganisms that were not detected in 4/6 of these blood cultures were not included in the panel leaving 2/6 as failure to identify the microorganism

Table 10. Identification of bacteria, yeasts and detection of antibiotic resistance markers in 24 polymicrobial blood cultures by FilmArray BCID panel in Study 3.

Identification	Detection by[a]: Blood culture	FA
Organisms		
Enterococcus faecium, CoNS	1, 1	1, 1
Escherichia coli, *Klebsiella pneumonia*	1, 1	1, 1
Candida albicans, *Enterococcus faecalis*	1, 1	1, 1
Enterobacter cloacae, *Enterococcus faecium*	1, 1	1, 1
Enterococcus faecalis, *Pseudomonas aeruginosa*	1, 1	1, 1
Enterococcus faecium, alpha-hemolytic streptococci	1, 1	1, 1
Enterococcus faecium, CoNS	1, 1	1, 1
Escherichia coli, alpha-hemolytic streptococci	1, 1	1, 1
Escherichia coli, *Klebsiella pneumoniae*	1, 1	1, 1
Escherichia coli, alpha-hemolytic streptococci	1, 1	1, 1
Klebsiella oxytoca, *Enterococcus faecium*	1, 1	1, 1
Serratia sp., alpha-hemolytic streptococci	1, 1	1, 1
Staphylococcus aureus, CoNS	1, 1	1, 1
Staphylococcus aureus, alpha-hemolytic streptococci	1, 1	1, 1
Staphylococcus aureus, *Pseudomonas aeruginosa*	1, 1	1, 1
CoNS, alpha-hemolytic streptococci	1, 1	1, 1
Streptococcus pyogenes, *Enterococcus faecalis*	1, 1	1, 0
CoNS, *Enterococcus faecalis*	1, 1	1, 0
Escherichia coli, *Bacteroides fragilis*	1, 1	0, X
Propionibacterium acnes, *Micrococcus* sp.	1, 1	X, X
Escherichia coli, *Klebsiella pneumoniae*, *Enterococcus avium*	1, 1, 1	1, 1, 1
CoNS, alpha-hemolytic streptococci, *Enterococcus*	1, 1, 0	1, 1, 1
Klebsiella pneumoniae, *Clostridium perfringens*, alpha-hemolytic streptococci	1, 1, 1	1, X, 0
Streptococcus pneumoniae, CoNS, *Bacillus* sp.	1, 1, 1	1, 1, X
Antibiotic resistance markers		
mecA	5	5
vanA/*vanB*	0	0

[a] 1, detection of a microorganism or antibiotic resistance marker in the BCID panel; 0, failure to detect; X, the microorganism was not included in the panel.

Reference; Altun O, Almuhayawai M, Ullberg M, Ozenci V. Clinical evaluation of the FilmArray blood culture identification panel in identification of bacteria and yeasts from positive blood culture bottles. Journal of Clinical Microbiology. 2013;51(12):4130-6.

The antibiotic resistance marker *mecA*, *vanA/vanB* and *bla*KPC was simultaneously tested in all microorganisms. The FilmArray analysis revealed a total of 22 *mecA* (17 in the monomicrobial- and five in the polymicrobial samples, respectively).

In the monomicrobial specimens, three coagulase-negative staphylococcus that were methicillin sensitive with the disc diffusion method were identified as *mecA* positive with the FilmArray. Conventional *mecA* PCR showed that 1/3 CoNS was true *mecA* positive, while 2/3 were *mecA* negative, as determined by disc diffusion. In addition, the only CoNS that could not be detected in the study by the FilmArray was methicillin resistant.

In the polymicrobial specimens, all samples (5/5) that were methicillin resistant according to the disc diffusion method were *mecA* positive on the FilmArray analysis. In 4/5 methicillin-susceptible *Staphylococci,* the FilmArray result were also *mecA* negative. One of these samples contained *S. aureus* plus CoNS, and the FilmArray detected *mecA* without being able to distinguish if the *S. aureus* was a methicillin resistant *S. aureus* (MRSA) or if the CoNS was the pathogens positive for the *mecA* gene making the *S. aureus* a methicillin sensitive *S. aureus* (MSSA) in the sample. Subsequent phenotypical tests were used to determined that the *S. aureus* isolate was MSSA and the CoNS was methicillin resistant.

Conventional culture and susceptibility methods and FilmArray did not identify any *bla*KPC or *vanA/vanB* during the study period.

Overall, the FilmArray identified all microorganisms covered by the FilmArray BCID panel in 170/191 (89.5%) blood culture bottles included in the study.

4.5 FILMARRAY MICROORGANISM IDENTIFICATION IN POSITIVE BLOOD CULTURES INOCULATED WITH STERILE BODY FLUID SPECIMENS

Culture positive SBF specimens (n=116) from 106 patients were included in the study. The specimens were pleural (n=51), synovial (n=38), abscess (n=10), dialysis (n=9), cerebrospinal (n=7) and bile (n=1) fluid. Monomicrobial growth was identified in 92/116 (79%) and polymicrobial growth in 24/116 (21%) (Table 11 and 12). The pouch failure rate was 1/116 (1%) and due to invalid internal run controls. Thirty different species were isolated from the specimens and 25/30 (83%) were covered by the FA BCID panel. FilmArray accurately identified 84/92 (91%) bottles with monomicrobial growth. Eight of the 92 (9%) bottles with monomicrobial growth contained microorganisms that were not included in the the FilmArray BCID panel.

All microorganisms included in the FilmArray BCID panel were detected in 18/24 (75%) blood culture bottles with polymicrobial growth. There were conflicting results in the remaining 6/24 (25%) specimens for FilmArray and the reference method (Table 12).

The results for antibiotic susceptibility testing were in concordance between the FilmArray and the disc diffusion method (Table 11 and 12). There were no *vanA, vanB* or *bla*KPC positive organisms in the study.

Tabell 11. Identification of bacteria, yeast and antibiotic markers in the 92 SBF specimens with monomicrobial growth.

Identification	No. of specimens that were:		
	Culture positive and FA positive	Culture positive and FA negative	Culture negative and FA positive
Microorganism or marker included in FA BCID panel			
Gram-positive bacteria			
Staphylococcus aureus	32		
Coagulase-negative staphylococcus	13		1[a]
Streptococcus pneumoniae	3		
Enterococcus spp.	7		
Streptococcus spp.	6		1[b]
Streptococcus agalactiae	3		
Alpha-hemolytic streptococcus	7		
Streptococcus pyogenes	2		
Listeria monocytogenes	1		
Gram-negative bacteria			
Escherichia coli	3		
Klebsiella pneumoniae	3		
Pseudomonas aeruginosa	1		
Fungi			
Candida albicans	2		
Candida glabrata	1		
Antibiotic resistance marker *mecA*	2		
Microorganisms not included in FA BCID panel			
Corynebacterium spp.		3	
Micrococcus spp.		2	
Aeromonas spp.		1	
Bacillus cereus		1	
Actinomyces spp.		1	

[a] The FilmArray identified *Staphylococcus* spp., while the reference method as well as additional culturing identified *B. cereus*.
[b] The FilmArray identified *Enterococcus* spp. and *Streptococcus* spp., while the reference method and additional cultures only identified *Enterococcus faecalis*.

Reference. Altun O, Almuhayawai M, Ullberg M, Özenci V. Rapid identification of microorganisms from sterile body fluids by use of FilmArray. Journal of Clinical Microbiology. 2015;53(2):710–2. PMID: 25520440Table 9. Detection rate for specimens with polymicrobial growth in sterile body fluids other than blood in Study 4.

Tabell 12. The bacteria, yeast and antibiotic markers detected in the 24 specimens with polymicrobial growth.

Specimen (*n* = 24)	Microorganisms identified by FilmArray and the reference methods[a]
Pleural fluid	***Enterococcus faecalis***, ***E. coli***, ***P. aeruginosa***
Pleural fluid	***E. faecalis***, ***P. aeruginosa***, ***C. albicans***, **coagulase-negative staphylococcus**, **Fox**^{r}[b]
Pleural fluid	***E. coli***, ***Staphylococcus epidermidis***, **Fox**^{r}
Pleural fluid	***Enterococcus avium***, ***E. coli***
CSF	***S. epidermidis***, **Fox**^{s}, ***E. coli***
Pleural fluid	***E. faecalis***, ***P. aeruginosa***
Pleural fluid	***Enterococcus faecium***, ***Citrobacter farmeri***, ***S. epidermidis***, **Fox**^{r}
Pleural fluid	***E. faecium***, ***E. faecalis***, ***E. coli***, ***C. albicans***
Pleural fluid	***E. faecalis***, ***E. faecium***, ***S. aureus***
Pleural fluid	***E. faecalis***, ***E. faecium***, ***C. albicans***
Pleural fluid	***E. faecalis*** (*Staphylococcus capitis*, Fox^{r})
Pleural fluid	***E. faecalis***, ***Proteus vulgaris***, ***Citrobacter freundii*** (*S. aureus*)
Abscess	***E. faecium***, ***E. coli*** (*K. pneumoniae*)
CSF	***E. coli***, Micrococcus spp.
Bile	***E. faecalis***, ***Enterobacter cloacae***, ***Klebsiella oxytoca***, Prevotella spp.
Pleural fluid	***E. faecium***, **alpha-hemolytic streptococcus**, ***Candida krusei***, ***Candida parapsilosis***, Saccharomyces cerevisiae
Pleural fluid	***S. pneumoniae***, Prevotella spp.
Pleural fluid	***C. glabrata***, Pediococcus acidilactici, Lactobacillus spp.
Pleural fluid	***C. glabrata***, ***C. albicans***, ***S. epidermidis***, **Fox**^{r}, *E. faecium*, (*Streptococcus salivarius*)
Abscess	***E. faecalis***, ***E. avium***, ***E. coli***, ***K. oxytoca***, anaerobic flora (*Enterococcus* spp., *E. coli*, *K. oxytoca*, *Streptococcus oralis*, *P. aeruginosa*, *H. influenzae*[c])
Abscess	***E. coli*** (*Streptococcus anginosus*, *K. oxytoca*)
Pleural fluid	***C. albicans*** (*S. pneumoniae*[c]; culture broth was Wellcogen positive)
Pleural fluid	**Alpha-hemolytic streptococcus**, *E. faecalis* (no growth on culture)
Pleural fluid	***S. aureus*** (*S. anginosus*[d])

[a] The reference methods were conventional culture methods. Boldface shows names or markers identified by both FilmArray and the reference method, lightface shows identification only by the reference method, underlined names were not included in the FA BCID panel, and names or markers in parentheses were identified first only by FA BCID and were later confirmed by the reference method after additional subcultures of the bottle culture broth.

[b] FilmArray detected *mecA*, while conventional methods used the disc diffusion method. Fox^{r}, cefoxitin resistant; Fox^{s}, cefoxitin susceptible.

[c] We were unable to confirm *H. influenzae* and *S. pneumoniae* on repeated cultures.

[d] On repeated culturing, *S. anginosus* was found instead of the *Enterococcus* spp. on FilmArray analyses.

Reference: Altun O, Almuhayawai M, Ullberg M, Özenci V. Rapid identification of microorganisms from sterile body fluids by use of FilmArray. Journal of Clinical Microbiology. 2015;53(2):710–2. PMID: 25520440

4.6 RAPID IDENTIFICATION OF *STREPTOCOCCUS PNEUMONIAE* IN POSITIVE BLOOD CULTURES WITH ANTIGEN TESTS

The study included 373 (358 BC bottles with Gram-positive cocci in pairs or chains and 15 bottles negative by Gram stain) blood cultures from 159 patients. *S. pneumoniae* was identified by conventional culture methods in 266/358 (74.3 %) bottles and 92/358 (25.7 %) bottles were positive for non-pneumococcal pathogens. These were α-haemolytic streptococci (n = 67) and other species, *Enterococcus* spp., (n=23), *Klebsiella pneumoniae* (n =1) and *Enterobacter cloacae* (n=1). To differentiate between the α-hemolytic streptococci sequencing of 16S rRNA and/or *recA* genes was performed in 53/67 (79.1 %) isolates. The 15 negative blood cultures included in the study were 12/15 (80%) companion bottles from patients with at least one bottle positive for *S. pneumoniae*. Three bottles were collected from patients with only negative bottles.

Sensitivity analysis (Table 13) revealed similar sensitivity of 99.6–100% in all tests after excluding invalid results in the calculation. When including invalid tests in the performance analysis, the positivity rates of the Slidex and Wellcogen tests were 86.5 % (CI, 81.8–90.1 %) and 92.5 % (CI, 88.7–95.1 %), respectively. The sensitivity was significantly lower than the ImmueLex 99.6% (CI, 97.9-99.9) and the BinaxNow 100% (CI, 98.6–100) ($p < 0.01$).

The specificity (Table 14) calculation utilized the non-pneumococcal pathogens in the assessment. The ImmuLex was significantly less specific than the Slidex test (82.6% vs 97.6%, $p<0.01$), and similar to the Wellcogen test (84.5%, p=ns). In comparison, the ImmuLex was more specific than the BinaxNOW test (64.1%, $p<0.01$). The BinaxNow had the lowest specificity (64.1%, $p<0.01$). A positive test result was seen when analyzing alpha-hemolytic streptococci with BinaxNow in 25/67 (37.3%), ImmuLex in 12/67(17.9%), Wellcogen 11/64 (17.2) and Slidex in 2/59 (3.4%).

Table 13. Sensitivity of the four tests in Study 5.

	ImmuLex	Slidex	Wellcogen	BinaxNOW
Positive test result, *n*	265	230	246	266
Negative test result, *n*	1	1	1	0
Invalid test result, *n*	0	35	19	0
Sensitivity, invalid results not included, % (95 % CI)[a]	99.6 (97.9–99.9)	99.6 (97.6–99.9)	99.6 (97.8–99.9)	100 (98.6–100)
Sensitivity, invalid results included, % (95 % CI)[b]	99.6 (97.9–99.9)	86.5 (81.8–90.1)	92.5 (88.7–95.1)	100 (98.6–100)

LA latex agglutination; *BC* blood culture; *CI* confidence interval

[a] Sensitivity was calculated on BC bottles positive for *S. pneumoniae* ($n = 266$) by dividing the sum of positive test results by the number of valid results

[b] In a sensitivity analysis of the study result, sensitivity was calculated on BC bottles positive for *S. pneumoniae* ($n = 266$) by dividing the sum of positive test results by the total number of results including the invalid results

Reference; Altun O, Athlin S, Almuhayawai M, Strålin K, Özenci V. Rapid identification of Streptococcus pneumoniae in blood cultures by using the ImmuLex, Slidex and Wellcogen latex agglutination tests and the BinaxNOW antigen test. European Journal of Clinical Microbiology & Infectious Diseases. 2016;35(4): 579–85. PMID: 26796552

Table 14. Specificity results for the four tests in Study 5.

	n	ImmuLex	Slidex	Wellcogen	BinaxNOW
α-Haemolytic streptococci[a]					
Streptococcus oralis	11	5	1	1	10
Streptococcus mitis	7	1	0	0	4
Streptococcus anginosus	6	0	0	1	0
Streptococcus sanguinis	5	0	1	1	1
Streptococcus tigurinus	5	2	0	3	1
Streptococcus dentisani	4	1	0	1	1
Streptococcus salivarius	3	0	0	0	0
Streptococcus gallolyticus	2	1	0	0	1
Streptococcus constellatus	1	1	0	0	0
Streptococcus intermedius	1	0	0	0	0
Streptococcus lutetiensis	1	0	0	0	1
α-Haemolytic streptococci	10	1	0	2	5
α-Haemolytic streptococci[a] + other species					
S. mitis + CoNS	2	0	0	1	0
S. mitis + *Pseudomonas aeruginosa*	2	0	0	1	0
Streptococcus parasanguinis + CoNS	1	0	0	0	0
S. salivarius + *Streptococcus pyogenes*	1	0	0	0	0
S. sanguinis + CoNS	1	0	0	0	1
α-Haemolytic streptococci + *Staphylococcus aureus*	2	0	0	0	0
α-Haemolytic streptococci + *Haemophilus influenzae*	2	0	0	0	0
Other Gram-positive cocci					
Enterococcus species	23	2	0	2	8
Gram-negative pathogens[b]					
Enterobacter cloacae	1	1	0	0	0
Klebsiella pneumoniae	1	1	0	0	0
Total	92	16	2	13	33
Invalid test results		0	8[e]	3[f]	0
Specificity, invalid results not included, % (95 % CI)[c]		82.6 (73.6–89.0)	97.6 (91.7–99.4)	85.4 (76.6–91.3)	64.1 (53.9–73.2)
Specificity, invalid results included, % (95 % CI)[d]		82.6 (73.6–89.0)	89.1 (81.1–94.0)	82.6 (73.6–89.0)	64.1 (53.9–73.2)

LA latex agglutination; *BC* blood culture; *CoNS* coagulase-negative staphylococci; *CI* confidence interval

[a] A total of 53/67 (79.1 %) α-haemolytic streptococci isolates were analysed by sequencing of 16S rRNA and/or recA genes in order to differentiate between species

[b] Identified by MALDI-TOF MS

[c] Specificity was calculated on BC bottles positive for non-pneumococcal pathogens ($n = 92$) by dividing the sum of negative test results by the number of valid results

[d] In a sensitivity analysis of the study result, specificity was calculated on BC bottles positive for non-pneumococcal pathogens ($n = 92$) by dividing the sum of negative test results by the total number of results including invalid results

[e] Eight BC bottles positive for *S. mitis* ($n = 2$), *S. tigurinus* ($n = 2$), *S. oralis* ($n = 1$), *S. sanguinis* ($n = 1$), *S. dentisani* ($n = 1$) and *S. mitis* + CoNS ($n = 1$) not shown in the table elsewhere

[f] Three BC bottles positive for *S. oralis* ($n = 1$), *S. anginosus* ($n = 1$) and *S. mitis* ($n = 1$) not shown in the table elsewhere

Reference: Altun O, Athlin S, Almuhayawai M, Strålin K, Özenci V. Rapid identification of Streptococcus pneumoniae in blood cultures by using the ImmuLex, Slidex and Wellcogen latex agglutination tests and the BinaxNOW antigen test. European Journal of Clinical Microbiology & Infectious Diseases. 2016;35(4): 579–85. PMID: 26796552

4.7 TIME-TO-DETECTION

Study utilized clinical specimens and simulated blood cultures to evaluate the two blood culture systems. The study showed that the overall TTD was significantly shorter by 18% for bottles incubated in BacT/Alert Virtuo blood culture system than in BacT/Alert 3D (median 12 h and 15 h, respectively, $p<0.0001$).

4.8 TURN-AROUND-TIME

In Study 2, the turn-around-time for identification after subculture was the culture time (2.5 h and 5.5 h) in addition to 30 minutes hands on time considering the time for sub-culture, and MALDI-TOF MS analysis.

In study 3 and 4, the turn-around time for the FilmArray BCID instrument was 65 minutes (5 minutes hands-on followed by 60-minute system analysis).

In study 5, the turn-around-time for all *S. pneumoniae* tests was less than 10 minutes.

5 DISCUSSION

Infections of normally SBFs including blood are associated with high morbidity and mortality [2–4,6,19]. The overall aim of this thesis was to evaluate and improve the process of microbiological analysis of SBF infections from blood cultures including the detection, identification of pathogens and antibiotic susceptibility testing. The standard of microbiology analysis provides the base for clinical management and has implications on mortality, morbidity, length of hospital stays, health care costs and the use of broad-spectrum antibiotics. The infections are commonly encountered in the clinical settings and demand early clinical detection, rapid collection of the SBF specimen for microbiological analysis and initiation of empiric antibiotic therapy as early as possible after clinically suspecting the infection.

Blood cultures are a highly sensitive in detection of microorganisms causing infections of SBF including blood[25]. The automated blood culture systems have further improved the detection and the time to detection through continuous monitoring for growth in the blood culture bottles[32]. The most widely used blood culture systems are BacT/Alert 3D (bioMérieux), VersaTREK (Thermo Scientific) and the Becton Dickinson BACTEC (Becton Dickinson). BioMérieux introduced the new blood culture system, Virtuo, with automated loading and unloading of blood culture bottles in addition to a closed stable incubation temperature as well as a new enhanced colorimetric technology for detection of growth in the blood culture bottles. The established blood culture system in use at the Karolinska University laboratory was the BacT/Alert 3D system. In Study 1, the Virtuo system was compared with the BacT/Alert 3D system in detection and time to detection by parallel incubation of a total of 115 clinical isolates in simulated blood cultures. Simulated blood cultures were used in this study to identify a difference in performance of detection and time to detection in the setting of 'if all things were equal' prior to launching a clinical study. The study showed that the two systems had a similar detection rate of growth suggesting that 'if all things were equal' the new blood culture system was not inferior in detecting growth in the blood culture bottles to the established blood culture system. However, among the 379 bottle pairs that signaled positive in the two blood culture systems the time to detection was significantly shorter for bottles incubated in the Virtuo compare with the BacT/Alert 3D system (median 12 h and 15 h, respectively; $p<0.001$) without significant difference in bacterial species or blood culture bottle type.

Interestingly, the investigation showed marked differences in TTD for two *Candida* species, with 24 h for *C. albicans* and 66 h for *C. glabrata* ($p < 0.0001$).

The significance of these findings is best appreciated when taking in the context of the clinical practice. Overall, the TTD is closely tied with initiating and/or modifying the antimicrobial therapy as well as further processing for pathogen identification and susceptibility testing. The shorter TTD may have major impact on samples processed during normal office hours where a 2-3 hours shorter TTD may be translated to Gram stain, identification and antibiotic susceptibility results being available 12-24-hours earlier. The shorter TTD seen with the yeast isolates may impact the time to appropriate antifungal therapy. *C. glabrata* had shorter TTD by 9 hours in the Virtuo and has a different susceptibility to antifungal agents than *C. albicans*[33].

There are several limitations with Study 1. While the utilization of simulated blood culture bottles even outs the field “if all things were equal” it also cannot rule out if other factors clinically encountered may have an impact on the TTD. These factors include the presence of antibiotics, total parenteral nutrition, transport time, polymicrobial infections and other inhibitory or stimulatory factors may impact the TTD results. Further clinical studies are warranted to evaluate for the factors encountered in clinical specimens and in the processing of clinical specimens including transportation time.

In Study 2, the MALDI-TOF MS was evaluated for rapid identification of microorganisms from positive blood cultures after short term sub-culture on solid media. The sub-culture of positive blood culture was already an integrated part of the diagnostic process in the lab according the to the study presented by Özenci et al., 2008[24]. The method of short term subculture was therefore further explored with the application of the MALDI-TOF MS. MALDI-TOF MS has revolutionized the process of microorganism identification by decreasing the turnaround time, low cost per isolate analyzed, extensive list of organisms that it can identify and simple sample processing[21]. The study included 515 monomicrobial blood cultures and accurately identified 300/515 (58.3%) after 2.5 h and 424/515 (82.3%) bacteria after 5.5 h subculture on the solid media. The overall bacterial identification in the study obtained at 5.5 h are comparable to the identification rates obtained by direct MALDI-TOF MS seen in several studies[22,34,35]. Direct MALDI-TOF MS method is based on several steps of centrifugation to isolate the microorganisms from the blood culture bottle broth and allow the MALDI-TOF MS spectrum analysis.

The challenges with the direct MALDI-TOF MS is the obstacles to integrate it as an additional method alongside the standard methods for identification. In contrast, the short-term subculture approach was modified with the addition of utilizing the MALDI-TOF MS rapid and cost-effective identification capabilities alongside the established conventional identification methods. The additional benefit of short-term subculture is the opportunity to initiate the antibiotic susceptibility testing as described by Idelevich et al., 2014 [35]. The shortcoming of MALDI-TOF MS in identifying *S. pneumoniae* from other alpha-hemolytic streptococci were encountered in the study as previously described[36]. The limitation of the short-term culture method was also seen in slow growing organisms such as the alpha-hemolytic streptococci where the identification rate was poor.

The results of study two showed that a user-friendly method could be utilized alongside the conventional identification methods to identify 82% of the bacteria in monomicrobial blood cultures. It may provide an addition to the opportunity to be able to initiate the antimicrobial susceptibility testing earlier. However, the method was limited by slow growing organisms, polymicrobial blood cultures and yeast organisms.

In Study 3, the performance of the FilmArray BCID system was analyzed for identifying microorganisms directly from positive blood culture bottles. The study included 206 blood culture bottles and accurate identification was made in 153/167(91.6%) with monomicrobial- and 17/24 (71%) with polymicrobial growth and 91.6% of the microorganisms encountered in the study were covered in the BCID panel. The results of identification of bacteria in bottles with monomicrobial growth was higher when compared with the MALDI-TOF MS identification subsequent to short term culture on solid media. The FilmArray detected 9/9 *Candida* spp. detected during the study period. However, similar results can be achieved with direct MALDI-TOF MS for both monomicrobial growth as well as yeast infections[37–39]. The major disadvantage to the direct MALDI TOF MS is the additional preparation steps that is required and hence its performance is user dependent. In contrast, the FilmArray is user-friendly and only requires five minutes for sample preparation and initiation of analysis. The major advantage with the direct MALDI-TOF MS as well as performing MALDI-TOF MS after short-term incubation on solid media is that most major diagnostic laboratories already have an in-house MALDI-TOF MS. The FilmArray was clearly superior to the MALDI-TOF MS in detection of *S. pneumonia* where 14/14 were accurately detected, and that none of the 12 alpha-streptococci were misidentified as *S. pneumonia*.

The FilmArray detected the *Enterococci* on species level only. This is a limitation because the antibiotic treatment for *E. faecium* and *E. faecalis* are different (Vancomycin and Ampicillin, respectively). The FilmArray stood out when looking at the performance in detecting all microorganism in blood cultures with polymicrobial growth with accurate identification of 17/24 (74%). In comparison, direct MALDI-TOF MS cannot reliably identify all microorganism in blood cultures with polymicrobial growth[22].

In Study 3, the FilmArray detected *Enterococcus* spp. in five specimens that were culture negative and thus representing false positive results. After the publication of the paper, the result analysis software in the FilmArray was further optimized by the manufacturer to mitigate false positive results. The software version used in the original paper was 1.3.0. When the five previous false positive samples were analyzed with the new software 1.4.1, no *Enterococcus* were detected. Thus further improved the sensitivity and specificity for *Enteroccus* in Study 3 from 88.9% and 97.3, to 88.9 and 10% respectively[40].

The third study was a prospective clinical study that had some limitations. The number of samples with less common microorganisms such as *Serratia* spp., and *Salmonella* spp., were limited. Similarly, there were no *bla*KPC or *vanA/vanB* detected during the study period. Further clinical studies are therefore warranted to fully explore the performance of the FilmArray.

In Study four, the performance of the FilmArray BCID panel was evaluated in blood culture bottles inoculated with SBF. During the study period, 116 SBF specimens (51 pleural, 38 synovial, 10 abscesses, 9 dialysis, 7 cerebrospinal, and 1 bile fluid) from 106 patients were included. Study four showed a similar performance as study three, where the FilmArray identified all microorganisms in 84/92 (91%) bottles with monomicrobial growth, and 18/24 (75%) specimens with polymicrobial growth when compared with conventional identification methods. Among the monomicrobial growth cultures, 8 of the 92 (9%) contained microorganisms that were not covered by the FilmArray BCID panel. The clinical relevance of these eight microorganisms was not further studied. These microorganisms were *Bacillus cereus Micrococcus* species, and *Corynebacterium* spo., that are usually considered to be contaminants as they are part of normal skin microbiota. In contrast, *Actinomyces* species and *Aeromonas* species were among the pathogens not included in the panel and may be clinically significant findings in certain patient populations, including the immunosuppressed.

Focusing on the microorganisms included in the BCID panel, FilmArray accurately identified all 84/84 (100%) microorganisms with monomicrobial growth and 18/24 (75%) polymicrobial growth. The study found 100% sensitivity, 100% specificity, and 100% negative and 98% positive predictive values for organisms included on the FA BCID panel in bottles with monomicrobial growth.

The results from study three and four showed that the FilmArray system has a high sensitivity and specificity in detecting microorganisms in both mono-and polymicrobial growth blood culture bottles inoculated with SBF including blood. There were samples with microorganisms that were not included in the panel. This a common limitation with panel based rapid methods. The panel-based rapid methods focus on the most common organisms to limit the costs for each pouch used for the analysis. In addition, by limiting the number of microorganisms, it is feasible to construct user-friendly systems with a low cost per analysis. The major challenge in rapid identification is the ability to identify all microorganisms in cultures with polymicrobial growth. Interestingly, this study demonstrated that standard culture and identification methods used in clinical microbiology may initially fail to identify microorganisms in bottles with polymicrobial growth. The reasons to explain this include the overgrowth of rapidly growing microorganisms in relation to slow-growing organisms in the same bottles and limited selective agar plates used in routine culture. The combined number of polymicrobial growth cultures in both studies were 48. The FilmArray accurately identified all microorganisms in 35/48 (72.9%) included in the panel. Similar high performance was seen in identification of yeast where the FilmArray could identify 3/3 and 9/9 yeasts in mono- and polymicrobial growth, respectively. The distinct advantage seen in identification of yeast infection is the simultaneous identification of bacteria and antibiotic resistance genes more often encountered in immunosuppressed.

Study three and four demonstrates that the FA BCID provides reliable, rapid identification results from positive blood culture bottles with SBF including blood. However, the entire panel was incompletely assessed due to the limited number of organisms encountered in the clinical studies. The FilmArray BCID system should be further evaluated in larger clinical studies to fully assess the performance of the panel on all organisms included. The clinical impact of the method may be further explored in combining the identification capabilities of the FilmArray with antibiotic stewardship programs to assess the hospital costs as well as the clinical outcomes.

Despite the impressive results, the FilmArray can only complement conventional culture, identification and antibiotic susceptibility testing due to the limited antibiotic susceptibility testing and limited number of microorganisms on the BCID panel.

In Study 5, the performance of the ImmuLex *S. pneumoniae* Omni test was compared with two other latex agglutination tests and the BinaxNOW antigen test in blood cultures with Gram stain showing Gram positive diplococci. The study showed a high sensitivity of the ImmuLex test (99.6 %) that was similar to the Slidex and the Wellcogen tests (99.6 and 100 %, respectively). When comparing the specificity of the test, the ImmuLex tests had inferior specificity to the Slidex test (82.6 % vs. 97.6 %, $p < 0.01$) had higher but similar to the Wellcogen test (85.4 %; p = ns). This study contradicted the previous study performed by the manufacturer where the ImmuLex had a specificity of 95%[41].

In summary, the study demonstrated that the new ImmuLex test was sensitive but lacked the specificity with high rate of false-positive test results in blood cultures with alpha-hemolytic streptococci. The number of specimens was sufficient to represent the tests performance. Future studies on the clinical impact of these tests would further elucidate the value of the rapid tests in clinical management of patients with invasive *S. pneumoniae* infections.

6 CONCLUSION

The present thesis showed a shorter TTD in the new Virtuo blood culture system that would allow an earlier initiation of microorganism identification. The rapid identification methods including FilmArray, MALDI-TOF MS after sub-culture on solid media and the latex agglutination tests are all important complements to the conventional culture methods and provide early critical information for appropriate antibiotic stewardship. This thesis presented an approach to focus on each step in the diagnostic process to decrease the turnaround time while maintaining the reliability of the results. The cost of implementing new and improved diagnostic system should be weighed against the final goal - patient outcome including morbidity and mortality as well as limiting the use of broad-spectrum antimicrobials. A close collaboration between the diagnostic microbiology laboratory and the clinical providers is encouraged in the management of SBF including blood for rapid diagnosis and implementation of appropriate antimicrobials early in the disease phase.

7 ACKNOWLEDGEMENT

I am in debt to the medical and scientific community for their generosity and know only how to give back by supporting other individuals in their ambitions in science and medicine to benefit us all in their discoveries. The kind and generous support of many individuals made this thesis possible.

I would like to express my gratitude towards my family for encouraging me to pursue my passion for science and medicine leading to taking on Ph.D. studies at Karolinska and supporting me in my undertaking.

I am highly indebted to the staff at clinical microbiology at Karolinska University hospital in Huddinge, Stockholm, Sweden for their tremendous support in doing this work.

I would like to express a special gratitude and thanks to my adviser, main supervisor, friend and mentor Dr. Volkan Özenci for sharing his expertise in the field of clinical microbiology and generously dedicating his time to mentor and guide me in my career in medicine and research. I will forever remember and value the time and work he sacrificed in making this work possible.

My sincere gratitude to my co-supervisor and friend Dr. Måns Ullberg, for his support, always being available and guidance throughout this work. Dr. Ullberg shared a significant part with Dr. Özenci in making this work possible as well as making the Ph.D. experience enjoyable and fun experience.

To my co-supervisors Kristoffer Strålin and Andrej Weintraub for supporting me and guiding me through this work. To Kristoffer Strålin for his expertise in infectious disease that elucidated the importance of our studies and facilitated additional collaborations. To Andrej Weintraub for his guidance and support in making this work possible and academic mentorship.

I am grateful and extend my sincere gratitude to my opponent Prof. Dr. Evgeny Idelevich, moderator Dr. Peter Bergman and the members of the examination committee Prof. Mikael Rhen, Dr. Anders Ternhag and Dr. Lena Serrander for participating in the final and critical stage of the thesis.

8 REFERENCES

1. Goto, M. & Al-Hasan, M. N. Overall burden of bloodstream infection and nosocomial bloodstream infection in North America and Europe. *Clin. Microbiol. Infect.* **19**, 501–509 (2013).
2. Wiest, R., Krag, A. & Gerbes, A. Spontaneous bacterial peritonitis: Recent guidelines and beyond. *Gut* **61**, 297–310 (2012).
3. van de Beek, D. *et al.* ESCMID guideline: Diagnosis and treatment of acute bacterial meningitis. *Clin. Microbiol. Infect.* **22**, S37–S62 (2016).
4. Angus, D. C. *et al.* Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* **29**, 1303–1310 (2001).
5. Proulx, N., Fréchette, D., Toye, B., Chan, J. & Kravcik, S. Delays in the administration of antibiotics are associated with mortality from adult acute bacterial meningitis. *QJM - Mon. J. Assoc. Physicians* **98**, 291–298 (2005).
6. Van De Beek, D., Brouwer, M. C., Thwaites, G. E. & Tunkel, A. R. Advances in treatment of bacterial meningitis. *Lancet* **380**, 1693–1702 (2012).
7. Rohner, P. & Auckenthaler, R. Review on evaluations of currently available blood-culture systems. *Clin. Microbiol. Infect.* **5**, 513–529 (1999).
8. Castaneda, M. R. A Practical Method for Routine Blood Cultures in Brucellosis. *Exp. Biol. Med.* **64**, 114–115 (1947).
9. Thorpe, T. C. *et al.* BacT/Alert: An automated colorimetric microbial detection system. *J. Clin. Microbiol.* **28**, 1608–1612 (1990).
10. Thorpe, T. C. *et al. BacT/Alert: an Automated Colorimetric Microbial Detection System Downloaded from*. *JOURNAL OF CLINICAL MICROBIOLOGY* vol. 28 http://jcm.asm.org/ (1990).
11. Ericson, E. L., Klingspor, L., Ullberg, M. & Özenci, V. Clinical comparison of the Bactec Mycosis IC/F, BacT/Alert FA, and BacT/Alert FN blood culture vials for the detection of candidemia. *Diagn. Microbiol. Infect. Dis.* **73**, 153–156 (2012).
12. Jekarl, D. W. *et al.* Comparison of the Bactec Fx Plus, Mycosis IC/F, Mycosis/F Lytic Blood Culture Media and the BacT/Alert 3D FA Media for Detection of Candida species in Seeded Blood Culture Specimens Containing Therapeutic Peak Levels of Fluconazole. *J. Clin. Lab. Anal.* **26**, 412–419 (2012).
13. Nylén, T., Saeedi, B., Borg, C., Ullberg, M. & Özenci, V. The performance of 4 different supplements and 5 blood culture bottles types in detection of bacteria and Candida spp. in simulated sterile body fluid cultures. *Diagn. Microbiol. Infect. Dis.* **77**, 1–4 (2013).
14. Kirn, T. J., Mirrett, S., Reller, L. B. & Weinstein, M. P. Controlled clinical comparison of BacT/Alert FA Plus and FN Plus blood culture media with BacT/Alert FA and FN blood culture media. *J. Clin. Microbiol.* **52**, 839–843 (2014).
15. Mirrett, S. *et al.* Controlled clinical comparison of the BacT/ALERT FN and the standard anaerobic SN blood culture medium. *J. Clin. Microbiol.* **42**, 4581–4585 (2004).
16. Rogers, M. S. & Oppenheim, B. A. The use of continuous monitoring blood culture systems in the diagnosis of catheter related sepsis. *J. Clin. Pathol.* **51**, 635–637 (1998).
17. Wallet, F., Loïez, C., Renaux, E., Lemaitre, N. & Courcol, R. J. Performances of VITEK 2 colorimetric cards for identification of gram-positive and gram-negative bacteria. *J. Clin. Microbiol.* **43**, 4402–4406 (2005).
18. Ferrer, R. *et al.* Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: Results from a guideline-based performance improvement program. *Crit. Care Med.* **42**, 1749–1755 (2014).

19. Polin, R. A. *et al.* Management of Neonates with Suspected or Proven Early-Onset Bacterial Sepsis. *Pediatrics* **129**, 1006–1015 (2012).
20. Szeto, C. C. The new ISPD peritonitis guideline. *Ren. Replace. Ther.* **4**, 2–6 (2018).
21. Clark, A. E., Kaleta, E. J., Arora, A. & Wolk, D. M. Matrix-Assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. *Clin. Microbiol. Rev.* **26**, 547–603 (2013).
22. Wüppenhorst, N., Consoir, C., Lörch, D. & Schneider, C. Direct identification of bacteria from charcoal-containing blood culture bottles using matrix-assisted laser desorption/ ionisation time-of-flight mass spectrometry. *Eur. J. Clin. Microbiol. Infect. Dis.* **31**, 2843–2850 (2012).
23. Fothergill, A. *et al.* Rapid identification of bacteria and yeasts from positive-blood-culture bottles by using a lysis-filtration method and matrix- assisted laser desorption ionization-time of flight mass spectrum analysis with the SARAMIS database. *J. Clin. Microbiol.* **51**, 805–809 (2013).
24. Özenci, V., Tegmark-Wisell, K., Lundberg, C. & Wretlind, B. Rapid culture and identification: A practical method for early preliminary laboratory diagnosis of sepsis. *Clin. Microbiol. Infect.* **14**, 177–180 (2008).
25. Opota, O., Croxatto, A., Prod'hom, G. & Greub, G. Blood culture-based diagnosis of bacteraemia: State of the art. *Clin. Microbiol. Infect.* **21**, 313–322 (2015).
26. Doern, C. D., Lacey, D., Huang, R. & Haag, C. Evaluation and implementation of FilmArray Version 1.7 for improved detection of adenovirus respiratory tract infection. *J. Clin. Microbiol.* **51**, 4036–4039 (2013).
27. Seiner, D. R. *et al.* Evaluation of the FilmArray® system for detection of Bacillus anthracis, Francisella tularensis and Yersinia pestis. *J. Appl. Microbiol.* **114**, 992–1000 (2013).
28. Poritz, M. A. *et al.* Filmarray, an automated nested multiplex PCR system for multi-pathogen detection: Development and application to respiratory tract infection. *PLoS One* **6**, (2011).
29. Petti, C. A., Woods, C. W. & Reller, L. B. Streptococcus pneumoniae antigen test using positive blood culture bottles as an alternative method to diagnose pneumococcal bacteremia. *J. Clin. Microbiol.* **43**, 2510–2512 (2005).
30. Arbique, J. C. *et al.* Accuracy of phenotypic and genotypic testing for identification of Streptococcus pneumoniae and description of Streptococcus pseudopneumoniae sp. nov. *J. Clin. Microbiol.* **42**, 4686–4696 (2004).
31. Mellmann, A. *et al.* Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *J. Clin. Microbiol.* **46**, 1946–1954 (2008).
32. Weinstein, M. P. Current blood culture methods and systems: Clinical concepts, technology, and interpretation of results. *Clin. Infect. Dis.* **23**, 40–46 (1996).
33. HM. A. PFALLER, 1, D. J. DIEKEMA, 1, 2, R. N. JONES, H. S. SADER, A. C. FLUIT, R. J. HOLLIS, S. A. MESSER, A. T. S. P. G. E. S. & Group, T. S. P. G. International Surveillance of Bloodstream Infections Due to Candida Species: Frequency of Occurrence and In Vitro Susceptibilities to Fluconazole, Ravuconazole, and Voriconazole of Isolates Collected from 1997 through 1999 in the SENTRY Antimicrobial Surv. *Microbiology* **39**, 3254–3259 (2001).
34. Vlek, A. L. M., Bonten, M. J. M. & Boel, C. H. E. Direct matrix-assisted laser desorption ionization time-of-flight mass spectrometry improves appropriateness of antibiotic treatment of bacteremia. *PLoS One* **7**, (2012).
35. Idelevich, E. A. *et al.* Rapid identification of microorganisms from positive blood cultures by MALDI-TOF mass spectrometry subsequent to very short-term incubation on solid medium. *Clin. Microbiol. Infect.* **20**, 1001–1006 (2014).
36. Idelevich, E. A. *et al.* Acceleration of antimicrobial susceptibility testing of positive

blood cultures by inoculation of Vitek 2 cards with briefly incubated solid medium cultures. *J. Clin. Microbiol.* **52**, 4058–4062 (2014).
37. Rychert, J. *et al.* Multicenter evaluation of the vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for identification of gram-positive aerobic bacteria. *J. Clin. Microbiol.* **51**, 2225–2231 (2013).
38. Ferroni, A. *et al.* Real-time identification of bacteria and Candida species in positive blood culture broths by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* **48**, 1542–1548 (2010).
39. Kok, J., Thomas, L. C., Olma, T., Chen, S. C. A. & Iredell, J. R. Identification of bacteria in blood culture broths using matrix-assisted laser desorption-ionization sepsityper^{TM} and time of flight mass spectrometry. *PLoS One* **6**, 2–8 (2011).
40. Altun, O. & Özenci, V. FilmArray: Correction of previously false-positive results by improved software. *Journal of Clinical Microbiology* vol. 53 750 (2015).
41. Otte, S., Haugan, M., Eriksen, H., Kerrn, M. & Elverdal, P. ImmuLex S. pneumoniae omni - a new latex agglutination test for rapid detection of S. pneumoniae in blood cultures and plate cultures. *SSI Diagnostica, Statens Serum Institut, Copenhagen, Denmark.* 60 (2014).