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From DEPARTMENT OF LABORATORY MEDICINE, DIVISION OF CLINICAL MICROBIOLOGY

Karolinska Institutet, Stockholm, Sweden

# RAPID DIAGNOSIS OF SEPSIS PATHOGENS

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RAPID DIAGNOSIS OF SEPSIS PATHOGENS

# THESIS FOR DOCTORAL DEGREE (Ph.D.)

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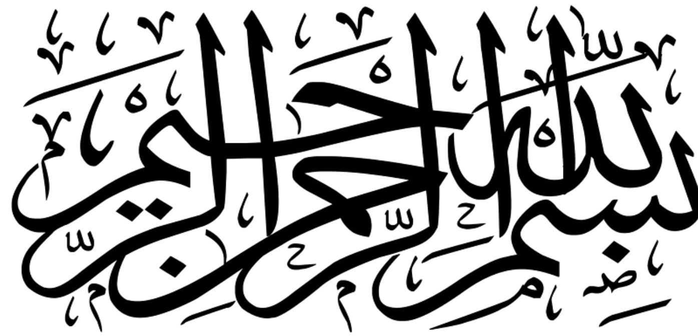

(In the name of Allah the Merciful)

Every challenging work needs self-efforts as well as guidance of other people especially those who were very close to my heart. My humble effort I dedicate it to my loving Mother and Father whose affection, encouragement, love and prayers of day and night make me able to get such success and honor, along with all the love and faithfulness sweet Wife.

# ABSTRACT

Sepsis is a growing global healthcare concern and is related to high morbidity and mortality. Studies have repeatedly demonstrated that the rapid administration of appropriate antimicrobial treatment is crucial for patient survival. Thus, receiving timely and actionable information from the laboratory on identification of the microorganism causing sepsis is crucial for patient management.

In today's routine diagnostics, blood culture is the standard method for diagnosing sepsis and identification of microorganisms is based on sub-culturing the positive blood culture bottles.

The overall aim of this thesis was to evaluate and improve the use of rapid identification methods in identification of microorganisms directly from blood culture bottles.

The thesis focused on two methods, FilmArray and MALDI-TOF MS. The present studies showed that the identification of microorganisms from a blood culture bottle by FilmArray and MALDI-TOF MS were ready in 65 min and 30 minutes respectively.

The most common form of sepsis is caused by a single microorganism. In paper 1 we studied the performance of FilmArray in identification of microorganism's form blood culture bottles. The FilmArray could identify the microorganism in 91.6% of the blood culture bottles.

The anaerobic bacteria are not covered by current rapid identification methods including FilmArray. In study 3, we analyzed the performance of MALDI-TOF MS in identification of anaerobic bacteria from four different blood culture bottle types. MALDI-TOF MS could identify anaerobic bacteria in between 75-79% of the different blood culture bottle types.

The incidence of detection of polymicrobial growth in blood culture bottles is increasing. This is an obvious challenge both for conventional and rapid identification methods. In study 1 and 4 we evaluated the performance of rapid methods in identification of polymicrobial growth directly from blood culture (BC) bottles after positivity. FilmArray correctly identified all microorganisms in 17/24 (71%) and 99/115 (86.1%) of the BC bottles in study 1 and 4 respectively. In contrast, the present MALDI-TOF MS method showed poor performance and could identify both microorganisms in only 2/115 (1.7%) blood culture bottles.

The high analytical performance of the current rapid methods stimulated us to ask the question if we can identify microorganisms from bottles before the blood cultures signals positive. We called this unique approach as semi-culture based identification since the full-term culture is not needed.

In study 2, we analyzed the semi-culture based identification by FilmArray and MALDI-TOF MS. We analyzed both simulated and clinical blood cultures with this approach. MALDI-TOF MS failed to identify the microorganisms prior to positivity even in the simulated blood culture bottles. Interestingly, FilmArray could identify microorganisms from bottles before

the blood cultures signals positive both in simulated and clinical blood culture bottles. In simulated samples, the median time to detection (TTD) of growth for the bottles in the blood culture system was 11.1 h, whereas FilmArray could identify microorganisms after 5 h incubation in the system.

In conclusion, the present thesis shows that the FilmArray is a reliable method for identification of microorganisms from positive blood culture bottles with mono- as well as polymicrobial growth. The data from the studies showed also that it is possible to improve the use of rapid identification methods as in the case of semi-culture based identification.

## LIST OF SCIENTIFIC PAPERS

I. Altun O, **Almuhayawi 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.
II. **Almuhayawi M**, Altun O, Stralin K, Ozenci 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.
III. **Almuhayawi M**, Altun O, Abdulmajeed AD, Ullberg M, Ozenci 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):e0142398.
IV. **Almuhayawi M**, Kynning M, Lüthje P,Ullberg M, Sandström G, Özenci V. Direct identification of microorganisms by FilmArray and MALDI-TOF MS from blood culture bottles with polymicrobial growth (Manuscript).

## LIST OF RELATED SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Altun O, **Almuhayawi M**, Ullberg M, Ozenci V. Rapid identification of microorganisms from sterile body fluids by use of FilmArray. Journal of clinical microbiology. 2015;53(2):710-2.
II. Altun O, Athlin S, **Almuhayawi M**, Stralin K, Ozenci 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 : official publication of the European Society of Clinical Microbiology. 2016;35(4):579-85.
III. Altun O, **Almuhayawi M**, Luthje P, Taha R, Ullberg M, Ozenci 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.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| BSI | Blood stream infection |
| BC | Blood culture |
| SIRS | Systemic inflammatory response syndrome |
| SOFA | Organ failure assessment score |
| EMS | Emergency medical service |
| CFU | Colony-forming unit |
| ICU | Intensive care unit |
| PCR | Polymerase chain reaction |
| PNA-FISH | Fluorescence in situ hybridization using peptide nucleic acid |
| MRSA | Methicillin resistant staphylococcus aureus |
| VAN | Vancomycin resistant Enterococcus |
| KPC | Carbapenem resistant |
| SD | Standard deviation |
| MALDI-TOF MS | Matrix-assisted laser desorption ionization–time of flight |
| MS | Mass spectrometry |
| FilmArray BCID | FilmArray Blood Culture Identification Panel |
| TTD | Time to detection |
| RT | Room temperature |
| RCF | Relative centrifugal forces |
| TAT | Turn-around time |

# 1 INTRODUCTION

Sepsis is a major health concern worldwide since it is related to high morbidity, mortality and huge health care related costs (Goto and Al-Hasan, 2013, Angus et al., 2001). The true incidence of the disease is unknown. However there is accumulating evidence that sepsis is an increasingly common problem affecting both developed and developing countries (Kissoon et al., 2011).

## 1.1 DEFINITION OF SEPSIS

The devastating nature of sepsis has not only stimulated modern clinical researcher but also the scientific community in ancient times.

The complex nature of sepsis has brought complex and interesting questions that needed to be answered by the scientific community. It is possible to state that one of the major problems with sepsis has been to define what sepsis is.

The definition of the disease is obviously decisive in order to describe the etiology, epidemiology, pathophysiology of the disease and to develop effective diagnostic approaches. In addition, the clinical studies aiming to develop and study new treatment approaches is completely dependent on the definition of the disease.

The term “sepsis” was first used by Hippocrates (ca. 460-370 BC). It is originated from the word “sipsi” meaning “make rotten” in Greek. Later, Ibn Sina (979-1037 BC) described the coincidence of fever and blood putrefaction (sepsis).

In 1850s, Semmelweiss (Noakes et al., 2008) described the cause of childbed fever as "decomposed animal matter that entered the blood system". Subsequently, Pasteur and others proposed the theory of sepsis or “blood poisoning” as disseminated infection caused by an invasion of a pathogen (Altemeier, 1982).

The first consensus conference in establishing a set of definitions for sepsis and its sequel was made in 1991 (Sepsis-1). The international recommendations for definition of systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock were established and published in 1992 (Bone et al., 1992).

Improved understanding of the pathophysiology of sepsis during time resulted in the second consensus conference. The definition from the first conference was modified (Singer et al., 2016). The consensus conference defined sepsis to be the clinical syndrome defined by the presence of both infection and a systemic inflammatory response (Sepsis-2) (Fig. 1).

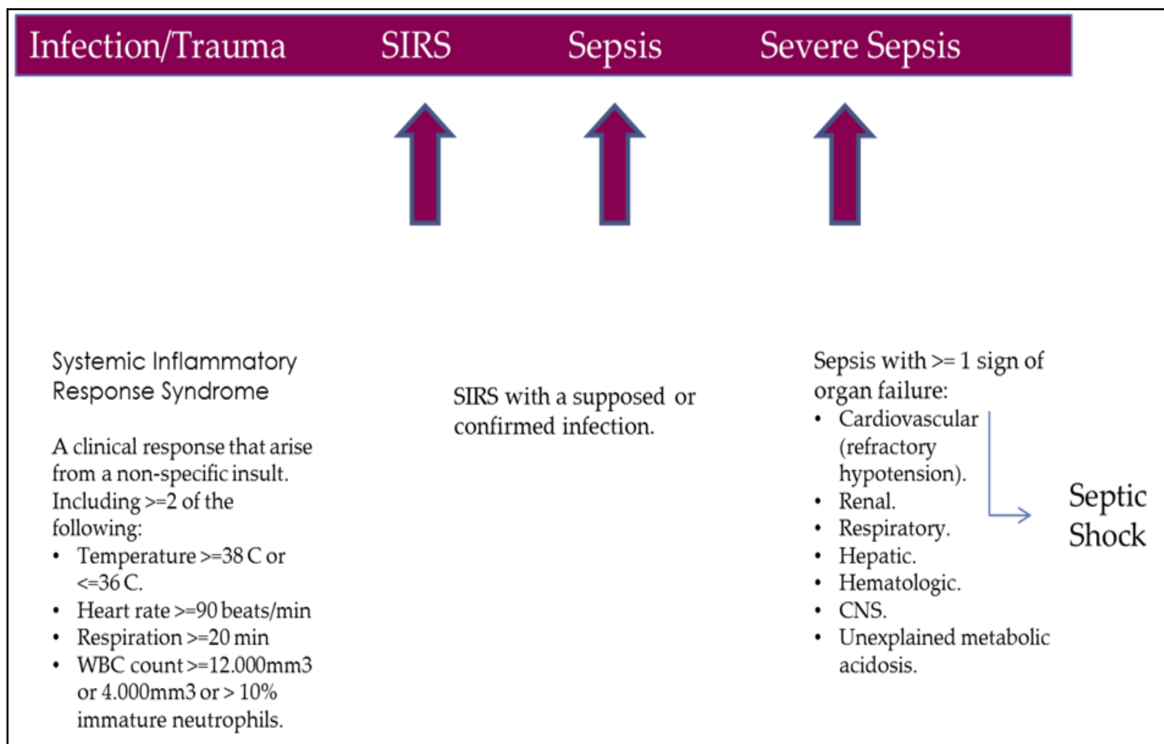


Fig. 1 Definitions of SIRS, Sepsis, Severe Sepsis and Septic Shock.

However, several studies have shown that the use of SIRS criteria as a parameter for sepsis diagnosis resulted in incorrect diagnosis as sepsis even in the absence of infection (Singer et al., 2016). The need for a more specific definition resulted in the third international definitions for sepsis and septic shock (Sepsis-3).

According to the new classification, sepsis is now defined as an evidence of infection with life-threatening organ dysfunction, clinically characterized by 2 points or greater in organ failure assessment score (SOFA) (Singer et al., 2016).

The new criteria for septic shock include fluid-unresponsive hypotension, serum lactate levels more than 2 mmol/L, and the requirement for vasopressors to maintain the arterial pressure of 65 mmHg or more.

Another definition that is probably most related to microbiological diagnosis of sepsis is blood stream infection. This definition is based on detection of microorganisms in the blood stream. For newborns and infants however, the term clinical sepsis is used (Horan et al., 2008).

The present doctoral thesis is focused on microbiological diagnosis of sepsis. Therefore the present work is inevitable challenged by continuously changing definitions of the disease. During the study period the patients were diagnosed in the clinical routine according to Sepsis-2 criteria.

However, the project focused on positive blood cultures. Therefore, in our scientific article we preferred to use the term bloodstream infections because it represents the microbiologically diagnosed patients with sepsis.

However, in writing the thesis the term sepsis is preferred. The underlying reason for this discrepancy and using the term sepsis is to describe the published material on etiology, epidemiology of the disease. The majority of these articles preferred to use the term sepsis.

## 1.2 INCIDENCE OF SEPSIS

As described above the problems in definition of sepsis have resulted in difficulties in measuring the incidence and prevalence of the disease. However, it is important to describe the current data on the incidence of sepsis in different settings and geographical locations regardless of the limitations.

Several studies have shown that sepsis incidence is increasing with the population growth in USA (Martin et al., 2003, Dombrovskiy et al., 2005, Dombrovskiy et al., 2007, Martin, 2012). Other studies existing from Australia, UK, Croatia and Saudi Arabia showing similar figures (Finfer et al., 2004, Harrison et al., 2006, Degoricija et al., 2006, Baharoon et al., 2015, Sudhir et al., 2011, Sundararajan et al., 2005).

At high income countries sever sepsis incidence is reported to be between 50 and 100 cases per 100,000 (Danai and Martin, 2005). Sepsis incidence is shown to be three to four times higher than the percentage of organ dysfunction syndrome which is the most severe form of sepsis (Martin et al., 2003).

At present more than 1,000,000 cases of sepsis of patients admitted to the hospital per year at USA (Martin, 2012).

In the low-income countries incidences of sepsis are less well-described (Adhikari et al., 2010). It has been reported that the incidence of sepsis increases in young individuals in low income counters (Berkley et al., 2005).

In countries where data for sepsis are obtainable the number of sepsis cases has increased (Kaukonen et al., 2014, Shen et al., 2010, Hall et al., 2011).

It was pointed that the number of hospital admissions for sepsis increased up to three-fold in comparison with myocardial infarction and stroke that remained the same over the past few decades (Seymour et al., 2012, Kempker and Martin, 2016) (Fig. 2).

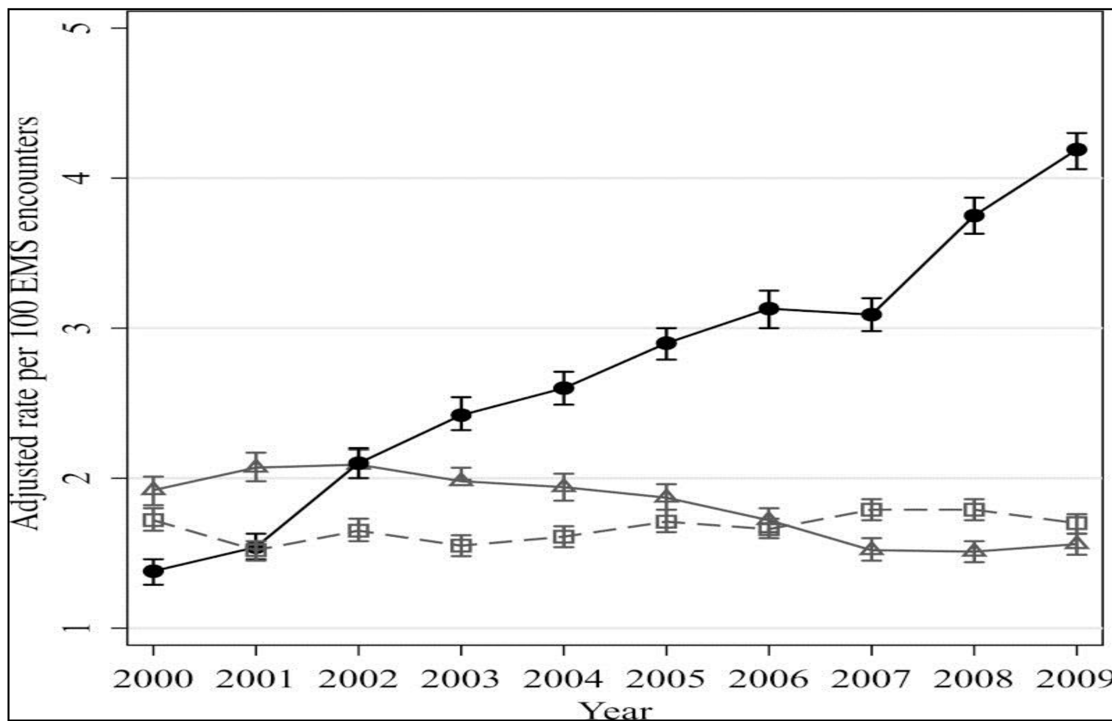


Fig. 2 Hospital admissions of sepsis compared to stroke and myocardial infarction among emergency medical service (EMS). (Circles= severe sepsis; Triangles=AMI; Squares= stroke). This figure was adopted from (Seymour et al., 2012).

Recent studies showed that ethnic background and gender may contribute to the risk for development of sepsis (Martin et al., 2003, Esper et al., 2006, Mayr et al., 2010). Males have higher risk than females in developing sepsis regardless of age (Martin et al., 2003, Dombrovskiy et al., 2007, Esper et al., 2006).

The reasons behind the differences in the incidence of sepsis regarding the ethnicity and gender are not known (Esper et al., 2006, Dombrovskiy et al., 2007, Martin et al., 2003).

## 1.3 PATIENT OUTCOME

Sepsis remains one of the main causes of decreasing life expectancy (Semeraro et al., 2010). The mortality rates differ worldwide due to numerous factors such as age, comorbid disease, access to health care, regional health patterns as well as genomic influences (Martin, 2012).

The patients with sepsis in ICUs have higher average of mortality rates (Vincent et al., 2006). Furthermore, costs to treat patients with sepsis are quite large with an estimate from cost from US$25,000 to $50,000 per episode worldwide (Edbrooke et al., 1999, Schmid et al., 2002, Brun-Buisson et al., 2003, Schmid et al., 2004, Moerer et al., 2007).

From data shown back in 1979 the risk of dying was near 30% in the early years and the year 2000 the risk has been under 20% (Martin, 2012). In USA, each year more than 200,000 patients die because of sepsis (Martin, 2012).

## 1.4 ETIOLOGY OF BLOODSTREAM INFECTION (BSI, SEPSIS)

It is possible to suggest that a wide range of microorganisms have the ability to cause sepsis. These include bacteria, fungi and protozoa. However the most common cause of sepsis is bacteria and yeast.

There are several different types of underlying diseases and conditions which may induce the development of sepsis, including chronic illnesses, or the patients receiving chemotherapy or immunosuppressive drugs (Angus et al., 2001, Barchiesi et al., 2016).

Among the underlying diseases in ICU patients, respiratory infections are the most common source for sepsis, followed by genitourinary and abdominal infections (Martin et al., 2003, Danai et al., 2007).

Table 1 Underlying disease of sepsis. This table was adopted and modified from (Sudhir et al., 2011).

| **Source of infection if sepsis** |
|---|
| 1. Respiratory tract infection |
| 2. Urinary tract infection |
| 3. Gastrointestinal tract infection |
| 4. Malaria |
| 5. Cellulites |
| 6. Dengue |
| 7. Source not found |

The microorganism causing the initial infection naturally dominates as BSI pathogen.

In general, the proportions of Gram-negative and Gram-positive bacteria causing sepsis are similar. The most common Gram-negative bacteria are *Escherichia coli*, *Klebsiella* species, *Pseudomonas aeruginosa*, *Enterobacter* species and *Serratia* species, while *Staphylococcus aureus*, CoNS, *Enterococcus* species, viridans streptococci and *Streptococcus pneumoniae* are reported to be the most common Gram-positive bacteria causing sepsis (Biedenbach et al., 2004, Tosson and Speer, 2011).

Even anaerobic bacteria are important as causative microorganisms of sepsis. The predominant anaerobic bacterial species in sepsis is *Bacteroides fragilis*. In contrast, anaerobic cocci and *Clostridium* species are infrequently isolated from sepsis patients (Nord, 1982).

The incidence of sepsis due to yeast are increasing. Candidemia is currently regarded as the fourth most common BSI in ICUs. *Candida albicans* has been dominating as the major pathogen causing candidemia. The epidemiology of candidemia has however changed rapidly over time resulting in more frequent detection of other *Candida* species, including *Candida glabrata* and *Candida krusei* (Diekema et al., 2012, Pittet and Wenzel, 1995, Trick and Jarvis, 1998).

The extended diversity among the microorganisms causing sepsis and the differences in antimicrobial susceptibility profiles result in difficulties in treating patients. Therefore the microbiological diagnosis of sepsis has the most importance for selection of effective and appropriate antimicrobial treatment of patients with sepsis.

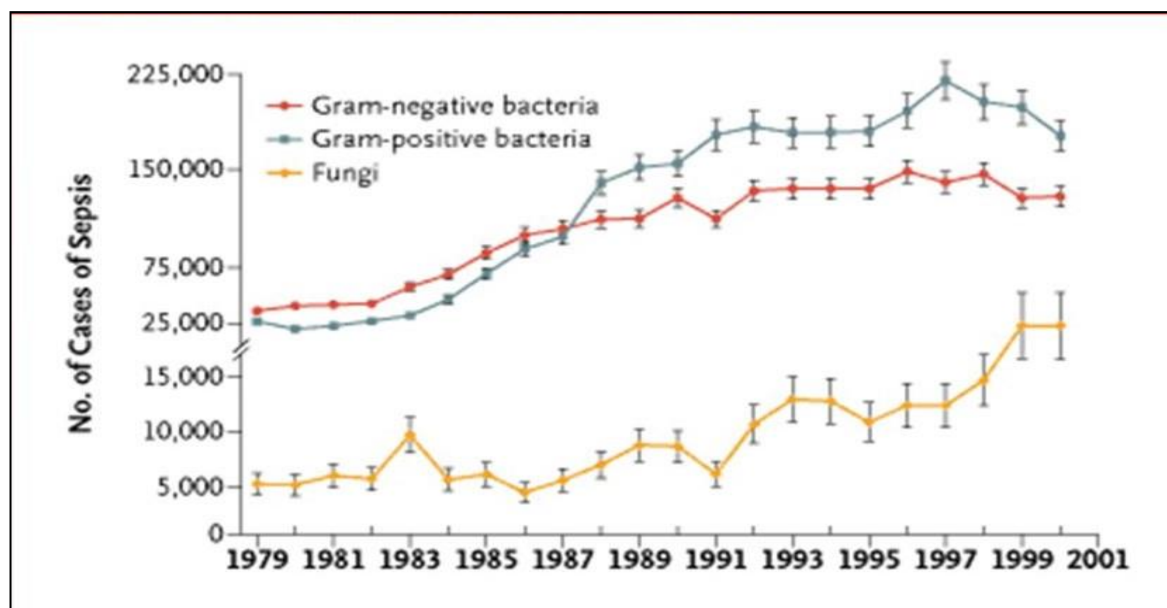


Fig. 3 Shows the microorganisms causing sepsis over time (Martin et al., 2003).

## 1.5 TREATMENT OF SEPSIS

Empiric antibiotic therapy is defined as broad spectrum treatment that covers the most common microorganisms causing sepsis in a patient and is chosen in relation to other clinical signs and the prevalence of antimicrobial resistance in a given geographical area.

The International Committee on Surviving Sepsis (SSC) recommends that patients with suspected sepsis need to be treated with antibiotics within the first hour of diagnosis (Dellinger et al., 2004). It was shown that this early empiric antibiotic therapy reduces mortality rate (Dellinger et al., 2013, Levy et al., 2014, Ferrer et al., 2014). The majority of

these studies were observational and retrospective studies (Ibrahim et al., 2000, Zaragoza et al., 2003, Kollef et al., 1999, Vogelaers et al., 2010).

On the other hand, empiric antibiotic treatment can have several negative effects, such as adverse effects related to the drug itself, alterations of the host microbiome, development of opportunistic infections and probably the increased risk for multidrug-resistant microorganisms (Havey et al., 2013).

Most importantly, empiric antibiotic treatments cannot cover all microorganisms and can thus have only limited effect (Leekha et al., 2011).

Therefore, the microbiological diagnosis is the only way for an effective, safe and economic treatment of patients with sepsis. The SSC recommends blood samples to be drawn before initiating antibiotic therapy so that the initial empirical therapy can be corrected for appropriate, targeted antibiotics when the culture result is available.

## 1.6 LABORATORY DIAGNOSIS OF SEPSIS

The significant changes in a broad range of parameters in patients with sepsis provide the possibility to diagnose sepsis by the help of laboratory analysis of blood. As described above the diagnosis of sepsis and SIRS depends mainly on laboratory results describing biochemical and hematologic parameters.

These results are generally available within a short period of time, not exceeding an hour. The information obtained by these rapid laboratory investigations is invaluable help to start supportive therapy in order to save the lives of patients.

Unfortunately, the information is mainly unspecific and does not reveal the identity of the causing microorganism. Microbiological diagnostic remains therefore crucial for selection of specific antimicrobial therapy.

## 1.7 MICROBIOLOGICAL DIAGNOSIS OF SEPSIS

The microbiological diagnosis of sepsis is a complex process. Today there are two approaches to detect and identify microorganisms from patients with sepsis, culture-independent methods, and culture-dependent methods.

The culture-independent methods have been developing during the recent years and have so far very limited availability in the clinical routine. In contrast, blood cultures have been used in the clinical routine for more than 100 years and are still regarded the gold standard in microbiological diagnostics of sepsis.

Unfortunately, blood culture-based diagnosis is not optimal as the turn-around time for the microbiology results is relatively long.

There are several different aspects of blood culture-based diagnostics, which need to be considered in the development of new methods providing reliable microbiological results within short turn-around time (Banerjee et al., 2016).

They can be assigned to three major parts in this process, namely pre-analytical, analytical and post-analytical procedures. These parts in turn include several different moments as described in Table 2.

The pre-analytical part covers the procedures prior to detection of growth in the blood culture. When the clinical diagnosis of sepsis is made, the first step is to collect blood cultures for microbiological diagnosis of sepsis.

Then the bottles need to be transported to the laboratory. After arrival in the laboratory the bottles should be registered in the laboratory information system and placed onto blood culture instruments.

The analytical part starts with the detection of growth in the blood culture bottles. The bottles are then removed from the system and the process of identification and antimicrobial susceptibility testing of microorganisms is initiated.

The third and the last section is the post-analytical part. In this final step, the microbiological diagnosis is reported to the clinic where the antimicrobial treatment of patients is started or adjusted accordingly.

The main goal of microbiological diagnostics is to obtain reliable and rapid information on the identity and the susceptibility profile of the microorganism causing sepsis. Therefore improvement in all of the three steps is similarly essential for shortening the turn-around time until reliable results can be obtained.

However, the characteristics of these three parts are significantly different from each other. The pre-analytical part is mostly hospital or laboratory specific, e.g. transport times of blood cultures differ significantly among laboratories and the improvements in shortening the transport time are mostly center-specific (Ronnberg et al., 2013).

Likewise the post-analytical steps are generally hospital and laboratory specific, e.g. reporting of positive blood cultures differ significantly between countries and centers.

In contrast, improvements regarding the analytical part may be implemented in different laboratories and geographical regions independently of the pre- and post-analytical processes. Therefore the analytical part has been the focus for scientific and clinical research during the recent years.

Table 2 The major steps in microbiological diagnosis of sepsis.

| Pre analytical | Analytical | Post analytical |
|---|---|---|
| Meeting the patient | Removing positive BC bottles from the system | |
| Clinical diagnosis of sepsis | Gram staining | Report Gram-staining results and start/adjust antimicrobial therapy |
| Collecting BC | Identification  from BC bottles | Report rapid ID result |
| Sending BC bottles to the laboratory | Subculture of bottles onto plates | |
| Place the BC bottles into BC system | Growth of bacteria on agar plats | |
| | Conventional ID and susceptibility testing | Report rapid ID result and adjust antimicrobial therapy |

In comparison to the other two parts, the analytical part of microbiological diagnostics significantly improved during the last decade. The introduction of state of the art commercial methods and development of reliable and rapid in-house methods suitable for clinical routine has opened a new era in clinical microbiology.

However, as all other improvements in clinical medicine, information and know-how regarding the performance of these novel methods is decisive for their implementation into clinical routine. Today, there is still limited information on the analytical and clinical performance of a number of modern microbiological methods.

## 1.8 IDENTIFICATION OF MICROORGANISMS

### 1.8.1 Culture-independent methods

As the name describes, the goal of culture-independent methods is to bypass the long process of blood cultures and subsequent cultures. In this approach blood sample from the patients will be analyzed directly and the results on detection, identification and hopefully

susceptibility profile of the microorganisms causing sepsis will be available in a couple of hours. The clinical effect of this approach in patients with sepsis will probably be very positive since the turn-around time is significantly shorter than for culture-dependent methods.

Recent improvements in molecular microbiology provided methods that have been tested in clinical studies. Hitherto, there are five systems developed for broad range detection of microorganisms directly from blood (Table 3).

However, preliminary experiences showed limited sensitivity and specificity, and in combination with the high cost per sample the clinical usage of these methods in routine microbiological diagnostics has been very restricted.

The major challenge in the approach to detect microorganisms directly from blood samples is the minute amount of microorganisms in combination with high amounts of human DNA and other hematogen molecules (Opota et al., 2015b).

This requires high sensitivity in the presence of multiple substances which may interfere with the reaction and may lead to inconclusive results.

Table 3 The commercially available systems in culture-independent microbiological diagnostics of sepsis.

| **System** | **Manufacturer** | **Method** | **Total Coverage (bacteria and fungi + resistance marker)** | **Turnaround time (hours)** |
|---|---|---|---|---|
| SepsiTest | Molzym, Bremen, Germany | Broad-range PCR + sequencing | >345 | 6 |
| SeptiFast | Roche Molecular System, Basel, Switzerland | Multiple broad-range real-time PCR | 21 + *mecA* | 3.5-5 |
| MagicPlex | Seegene, Seoul, Korea | Multiple PCR + multiplex real-time PCR | 117 + *mecA*, *vanA/B* | 3-5 |
| VYOO | SIRS-Lab, Jena, Germany | Multiplex PCR + electrophoresis | 39 | 8 |

| | | | | |
|---|---|---|---|---|
| IRIDICA (PLEX-ID) | Abbott Molecular, Carlsbad, CA, USA | Multiplex broad-range PCR/electrospray ionization mass spectrometry | >800 + *mecA*, *vanA/B* | 6 |

## 1.8.2 Culture-dependent methods

### *1.8.2.1 Conventional methods*

The gold standard method in the analytical part of microbiological diagnostics of sepsis is to identify microorganisms using phenotypic and biochemical tests. These methods require pure culture (at least isolated single colonies) on agar plates and are summarized as conventional methods.

Obtaining pure cultures with visible singular colonies is a time consuming process that takes around 20 h. It can take up to 72 h for slow-growing microorganisms such as anaerobic bacteria. Next, the identification of microorganisms using conventional biochemical methods takes another 8 - 48 hours.

The introduction of methods such as Matrix-Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) and nucleic acid-based methods made it possible to shorten the time for identification of microorganisms from days to a couple of minutes. Indeed the identification of microorganisms with MALDI-TOF MS takes minutes including the hand-on time.

The next step on improvement of identification of microorganisms is to focus on approaches bypassing the long period of sub-culture period. It is only possible by development and establishment of sub-culture independent microbiological methods.

### *1.8.2.2 Rapid identification methods*

The rapid identification methods that will be described in this section are blood culture-dependent but sub-culture independent.

Currently, there are several different types of methods available for identification of microorganisms directly from blood culture bottles.

These include multiplex PCR, MALDI-TOF MS and microarray and florescence in situ hybridization (Opota et al., 2015a). The characteristics of the commercially available methods are described in Table 4.

Table 4 The commercially available systems in blood culture-dependent microbiological diagnosis of sepsis.

| **System** | **Manufacturer** | **Method** | **Total Coverage**<br>**(bacteria and fungi + resistence marker)** | **Turnaround time (hours)** |
|---|---|---|---|---|
| FilmArray | Idaho Technology, Salt Lake City, UT, USA | Multiplex PCR | 24<br>+ *mecA, vanA/B, KPC* | 1 |
| MALDI-TOF MS | Brucker Daltonics (Bremen, Germany)<br>bioMérieux (Marcy l'Etoile, France) | Mass-spectrometry | >5000 | <1 |
| Prove-it Sepsis | Mobidiag,<br>Esbo, Finland | Microarray | 73<br>+ *mecA* | 5 |
| Verigene (Gram-negative/Gram-postive) | Nanosphere, Northbrook,<br>IL, USA | Microarray | 12/8<br>+ *mecA*, *vanA/B* /CTX-M, *KPC*, NDM, VIM, IMP, OXA | 2.5 |
| QuickFISH<br>(*Staphylococcus*/ *Enterococcus*/ Gram-Negative/ *Candida*) | AdvanDx, Woburn, MA, USA | Fluorescence in situ hybridization | 2/2/3/3<br>+*mecA* | 1-3 |

The turn-around time for non-culture based methods and rapid identification methods are presented in Fig 4.

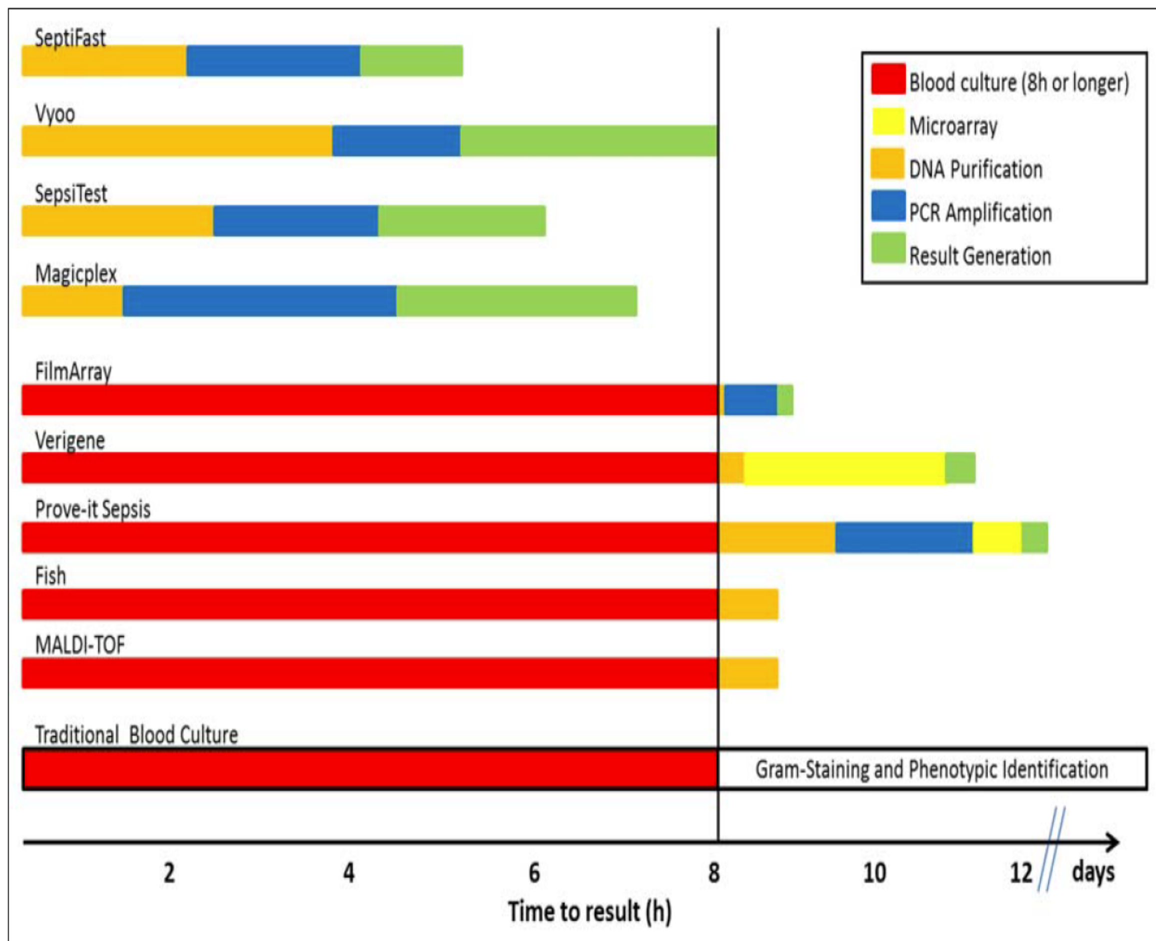


Fig. 4 This figure was adopted from (Liesenfeld et al., 2014).

# 2 AIM OF THE PROJECT

The overall aim of this doctoral project was to improve the microbiological diagnosis of sepsis by evaluating and extending the use of rapid methods in identification of microorganisms from blood cultures.

**Specific aims were to**

**Paper 1**

Evaluate the clinical performance of FilmArray in identification of microorganisms from positive blood cultures collected prospectively.

**Paper 2**

Analyze the performance of FilmArray and MALDI-TOF MS in identification of microorganism's blood cultures before the bottles signal positive in the BC systems.

**Paper 3**

Evaluate the performance of (i) four different blood culture bottles and two different blood culture systems in detection and time to detection of anaerobic bacteria and (ii) the performance of MALDI-TOF MS in identification of anaerobic bacteria from different bottle types.

**Paper 4**

Asses the performance of FilmArray and MALDI-TOF MS for identification of microorganisms from BC bottles with polymicrobial growth.

# 3 MATERIALS AND METHODS

The studies were performed at Karolinska University Laboratory in Huddinge, Sweden, that serves the southern part of the greater Stockholm area and surrounding cities and suburbs. The laboratory receives BC samples from three tertiary-care hospitals: Karolinska University Hospital in Huddinge, Stockholm, Södertälje Hospital in Södertälje and South General Hospital, Stockholm, with a total of 1,569 beds.

## 3.1 BLOOD CULTURE SYSTEMS AND BLOOD CULTURE BOTTLES

The BC systems employed in the studies were the BacT/Alert 3D (bioMérieux, Marcy l'Etoile, France) and the BD Bactec 9240 (Becton Dickinson Instrument Systems, Sparks, MD) BC systems. The BacT/Alert 3D BC system was used with aerobic BacT/Alert FA Plus (resin-based), anaerobic BacT/Alert FN (charcoal-based) and FN Plus (resin-based), and pediatric BacT/Alert PF Plus BC bottles (resin-based), containing non-specific media for the detection of yeasts, aerobic and aerobic bacteria.

The BD Bactec 9240 BC system was used with Bactec Mycosis IC/F BC bottles (resin-based) for selective culture and recovery of yeast, and Bactec Plus (resin-based) and Bactec Lytic BC bottles (no absorbents) for cultivation of anaerobic bacteria (Table 5). As for clinical routine practice, BC bottles were incubated until they signaled positive or for a maximum of five days.

Table 5 BC bottles and BC systems used in the studies.

| Study | **BacT/Alert BC system** | | | | **BD Bactec BC system** | | |
|---|---|---|---|---|---|---|---|
| | FA Plus | FN Plus | FN | PF Plus | Mycosis IC/F | Plus | Lytic |
| **1** | × | × | | × | × | | |
| **2** | × | × | | | | | |
| **3** | | × | × | | | × | × |
| **4** | × | × | | × | | | |

## 3.2 CLINICAL BLOOD CULTURES

All patient samples were obtained from BC samples arriving at the Karolinska University Laboratory for routine microbiological diagnostic.

In Paper 1, a total of 206 BC bottles including Bactec Mycosis IC/F BC bottles, BacT/Alert FA Plus, BacT/Alert FN Plus and BacT/Alert PF Plus BC bottles were investigated. Bottles were collected for analyses when they had signaled positive.

In Paper 2, BacT/Alert FA Plus and BacT/Alert FN Plus BC bottles were analyzed a) prior to BC positivity and b) prior to incubation. a) From a quadruplicate set of BC bottles, the fourth

BC bottle was analyzed before signaling for BC positivity, when the other three bottles had signaled positive. b) From a total of 400 BC bottles, an aliquot was taken prior to incubation and stored frozen. The pre-incubation broth from BC bottles which later on signaled positive was then thawed and analyzed.

In Paper 4, a total of 48 BC bottles BacT/Alert FA Plus and BacT/Alert FN Plus with polymicrobial growth as judged by Gram stain were included. Another 134 BC bottles were used to prepare 67 simulated polymicrobial samples by mixing equal volumes of broth from each bottle. In total, 115 polymicrobial samples were analyzed.

## 3.3 SIMULATED BLOOD CULTURES

Simulated BC bottles were prepared by inoculation of a defined number of bacteria into BC bottles together with defibrinated horse blood. Bacteria from frozen stocks were cultured on blood agar plates in appropriate atmosphere at 36°C for 24 h (aerobe bacteria) or 48 h (anaerobe bacteria). Colonies from agar plates were suspended in 0.01 M phosphate buffered saline (pH 7.3-7.4) to 0.5 McFarland ($1.5\times10^8$ CFU/ml), diluted and added to a BC bottle. Aliquots of the suspension were cultured on blood agar plates to control the bacterial density in the final inoculum.

In Paper 2, four clinical isolates of each *S. aureus* and *E. coli*, and two reference strains, *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were analyzed in BacT/Alert FA Plus BC bottles. Each bottle was inoculated with 1000 CFU in 100 µl buffer and 10 ml horse blood.

In Paper 3, 100 isolates of anaerobe bacteria were analyzed in BacT/Alert FN and FN Plus BC bottles incubated in the BacT/Alert 3D BC system and in Bactec Plus and Bactec Lytic BC bottles incubated in the Bactec 9240 BC system. Each bottle was inoculated with 750 CFU in 50 µl buffer and 5 ml horse blood.

## 3.4 THE FILMARRAY BCID ASSAY

The FilmArray Assay (BioFire, Salt Lake City, UT) is a closed *in vitro* diagnostic system for the detection of bacterial and fungal pathogens. The FilmArray blood culture ID (BCID) panel covers 19 bacterial targets and 5 *Candida* species as well as three antimicrobial resistance genes (Table 6).

The analysis was performed following the manufacturer’s recommendations. Briefly, 1 ml hydration buffer was injected into the pouch using the FilmArray product syringe. Then, 100 µl of the BC broth was diluted in 500 µl FilmArray dilution buffer, and 300 µl of the diluted sample was injected into the FilmArray pouch before loading on the FilmArray system for analysis. DNA extraction, amplification, detection of the target and melt curve analysis of the amplification product are automated within the system and results are provided by the software automatically within 60 min.

Each pouch includes two internal run controls for both the primary amplification and the analyze-specific detection stages.

Results of the assay are provided by the software only if the quality control reactions are appropriately detected. When either of the two controls fails, the result is listed as invalid.

Table 6 The FilmArray BCID panel.

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

### 3.5 DIRECT MALDI-TOF MS

MALDI-TOF MS was performed directly from BC bottles, omitting subculture on solid media. An in-house protocol with slight modifications between the studies was used. In general, 1.5-5 ml BC broth was first centrifuged for 5-10 min at low speed (110-180 rcf) to remove larger particles.

The supernatant was then transferred to an Eppendorf tube and centrifuged for 2 min at high speed (20,800 rcf) to collect the microorganisms. The pellet was washed with molecular-grade water; in Paper 4, additional washing steps with saponin (0.01% in water) and ammonium chloride were performed for more efficient removal of interfering blood components. Proteins were extracted by exposing the pellet to organic acid (50% acetonitrile, 47.5% water, 2.5% trifluoracetic acid) and 70% formic acid.

After centrifugation at high speed, the supernatant was spotted on a steel 96-spot MALDI target plate (Bruker Daltonics, Bremen, Germany). In some cases, extraction was performed directly on the MALDI target plate using 70% formic acid. The sample was allowed to dry before application of 1 µl alpha-cyano-4-hydroxycinnamic acid matrix and analysis with the MALDI-TOF MS (Bruker Daltonics, Bremen, Germany).

The Bruker Biotyper 3.0 software and library (Bruker Daltonics) were used for spectra analysis. Scores of >2.0 were considered identification at the species level and scores of >1.7 were considered identification at the genus level, according to the manufacturer's recommendation.

### 3.6 CONVENTIONAL METHODS FOR IDENTIFICATION OF MICROORGANIMS

Gram stains were done directly from positive BC bottles. According to the results of the staining, the broth was cultured on relevant agar plates. Microorganisms grown on the agar plates were identified by MALDI-TOF MS (Bruker Daltonik) or Vitek2 XL (bioMérieux, France). Validated desktop spot tests, including catalase, oxidase, indole spot and L-pyrrolidonyl-β-naphthylamide, were performed as indicated. In case of discrepant results between the study methods and conventional methods, additional subcultures were performed on appropriate selective media.

### 3.7 PARTIAL 16S rRNA GENE SEQUENCING

In Paper 3, partial 16S rRNA sequencing, including the hypervariable regions V3 and V4, was performed in cases of discrepant results from direct MALDI-TOF MS and previous bacterial identification by conventional methods. Bacterial DNA was extracted using the automated Biorobot M48 system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification was performed by adding 3 µl of the extract to a master mix containing 10 µM of each primer (5´-CGGCCCAGACTCCTACGGGAGGCAGCA-3´ and 5´-GCGTGGACTACCAGGGTATCTAATCC-3´) together with 25 µl HotStarTaq master

mix (Qiagen) to give a final volume of 50 µl. After initial denaturation, the cycling parameters were 32 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 7 min.

The PCR products were purified using chemical purification (PE Applied Biosystems) according to the manufacturer's instructions. Sequencing of both strands was carried out using an ABI Prism Big Dye Terminator v3.1 cycle sequencing kit (Applied Bio systems, Foster City, CA) with a Gene Amp 9700 thermocycler (Applied Biosystems). Sequencing primers used in the two reactions were 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-GWATTACCGCGGCKGCTG-3', 1µM each.

The sequence cycling products were analyzed by capillary electrophoresis and fluorescence detection with an Applied Biosystems ABI 3100 genetic analyzer. The fluorescence data were analyzed with the SeqScape Software program (version 4.5; Gene Codes Corporation, Ann Arbor, MI). A BLAST search was run on the obtained sequences to determine bacterial identity.

## 3.8 STUDY PROTOCOL

### 3.8.1 Paper 1

The study was performed prospectively on BC bottles received between April 2013 and June 2013 for routine microbiological diagnostic at the Karolinska University Laboratory. Only one sample per patient was used. Due to interference with downstream applications, only resin-based BC bottles were included. BC bottles were analyzed after yielding a positive signal in the BC system. Microorganisms were identified using the FilmArray Assay BCID panel. Conventional identification methods served as reference method in this study.

### 3.8.2 Paper 2

The performance of the FilmArray Assay and direct MALDI-TOF MS was analyzed in identification of bacteria and yeast from BC bottles prior to positivity in the BC system in different settings. Simulated BC bottles were used to investigate the performance of the two methods in relation to incubation time in the BC system (after 2.5 h, 5 h and 7.5 h), with and without a simulated transport time (2.5 h RT).

The influence on assay performance by transport time without further incubation in the BC system was investigated by incubation of BC bottles at RT (2.5 h, 8 h and 24 h). To investigate the assay performance on clinical BC bottles prior to incubation in the BC system, a 2-ml aliquot was removed from BC bottles upon arrival in the laboratory and stored frozen until the corresponding bottle yielded a positive signal. Alternatively, one BC bottle from a quadruplicate set of bottles was analyzed after partial incubation (prior to positivity). The time point for analysis of the fourth bottle was determined as the time when the third bottle of the set had reached positivity.

### 3.8.3 Paper 3

A collection of 100 anaerobic bacteria isolated from positive BC bottles at Karolinska University Hospital, Huddinge, Sweden and stored at -70°C was used in this study. From each isolate, simulated BC bottles were prepared to compare four different types of BC bottles for the recovery of anaerobic bacterial species in two different types of BC systems. Detection rates, time to detection and the performance of direct MALDI-TOF MS for bacterial identification was determined and compared between the BC bottle types.

### 3.8.4 Paper 4

The study was performed prospectively on BC bottles received between September 2015 and December 2015 for routine microbiological diagnostic at the Karolinska University Laboratory. Positive BC bottles with polymicrobial growth as judged by Gram stain were included. In addition, simulated polymicrobial BC samples were prepared from two positive clinical BC bottles. All polymicrobial BC samples were analyzed by FilmArray with the BCID panel and direct MALDI-TOF MS. Conventional culture-based isolation and identification methods were used as reference method for comparison.

## 3.9 STATISTICAL ANALYSIS

The Fisher's exact test and the Chi-square test was used to compare detection rates between two or more different BC bottle types, respectively (Paper 1 and Paper 3). The Wilcoxon matched-pairs signed-rank test was used to compare time to detection for different BC bottles (Paper 3). Differences with p-values $<0.05$ were considered statistically significant.

## 3.10 ETHICAL PERMISSION

An ethical permission was not required because no patients' data were included in all studies.

# 4 RESULTS

## 4.1 MICROORGANISMS AND BLOOD CULTURES

In **Paper 1,** a total of 206 blood culture bottles were studied. Only one blood culture per patient was included in the prospective evaluation of the FilmArray BCID panel. There were 167 BC with monomicrobial growth and 24 positive BC with polymicrobial growth. In 12 samples, Gram staining and subcultures were negative although the bottles signaled positive in the BC system. The FilmArray results were invalid in 3/206 (1.5%) bottles; these samples were excluded from further evaluation. Fig 5 depicts the study design.

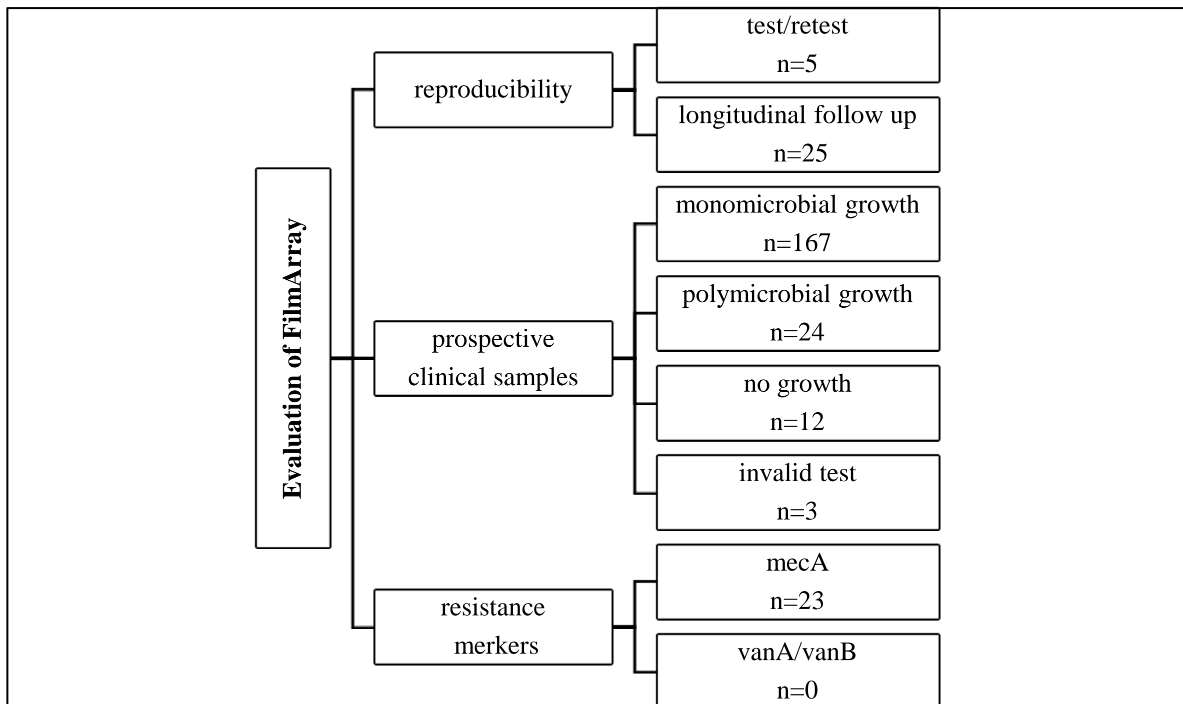


Fig. 5 FilmArray BCID evaluation schemes (N represents number of BC bottles tested). Conventional microbiology methods were used as reference methods.

During the study period 35 different species were identified by conventional methods, 24/35 (69%) covered by the FilmArray BCID panel. The three *Enterococcus* species; *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus avium* were identified at the genus level.

Among the 191 positive BC bottles with growth, 175 (91.6%) contained microorganisms that were included in the FilmArray BCID panel.

**In Paper 2,** both clinical and simulated BC bottles were investigated. In total, 30 simulated BC bottles and 800 clinical samples were included in the study. Samples from 400 clinical BC bottles were collected in order to evaluate the performance of the FilmArray BCID

panel for identification of microorganisms prior to incubation in the BC system. Sixteen of these 400 BC bottles signaled positive for growth and were analyzed.

In addition, 400 BC bottles from quadruplicate BC samples were investigated in order to analyze the performance of the FilmArray assay after incubation but prior to signaling positive in the BC system. Twenty-three of these bottles were analyzed in this study, as soon as the other three bottles taken from the same patient signaled positive. Fifteen of 23 (65%) bottles later became positive within 5-day incubation period, and 8/23 (35%) remained BC-negative. All of the thirteen different species that were identified from clinical BC bottles were included in the FilmArray BCID panel.

In **Paper 3,** a total of 100 anaerobic bacteria that were previously isolated from positive blood cultures were included in the study. The isolates are presented in Fig 6.

All of the isolates were included in the MALDI-TOF MS database applied in the study. These isolates were used to prepare 400 simulated BC samples.

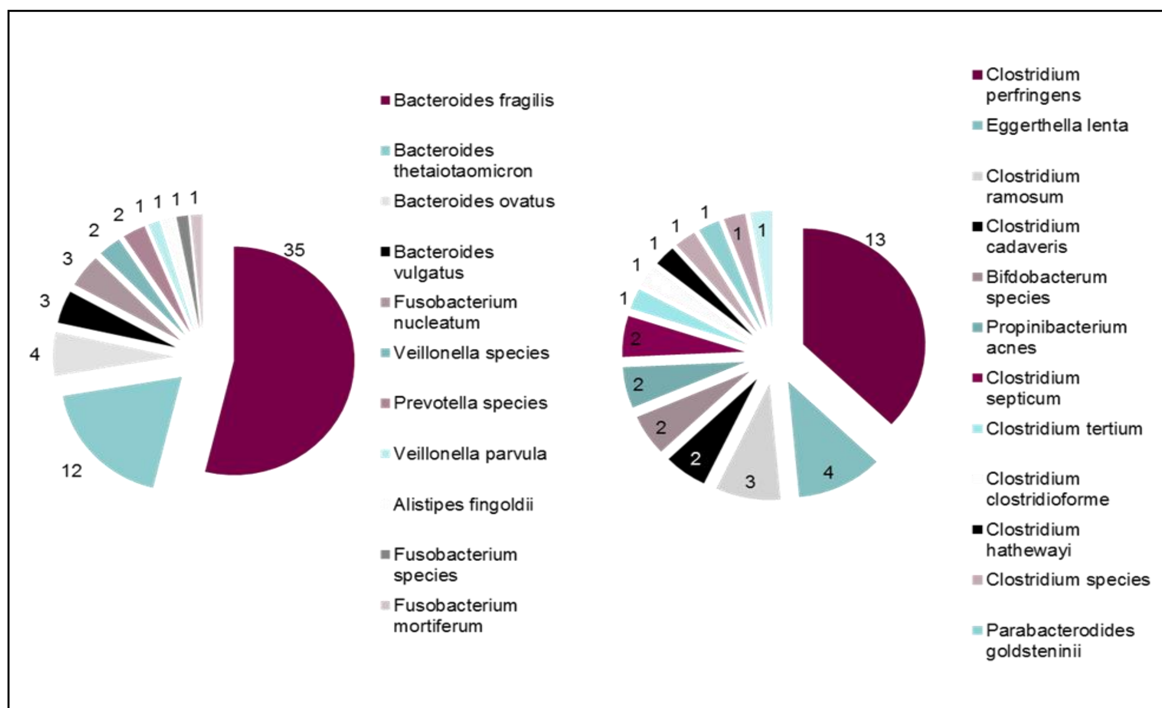


Fig. 6 Anaerobic blood culture isolates diversity.

**In Paper 4,** 32 different types of microorganisms were identified during the study period (Fig 7). The total coverage rate of all microorganisms in the sample material by the FilmArray BCID panel was 96.0% (243/253).

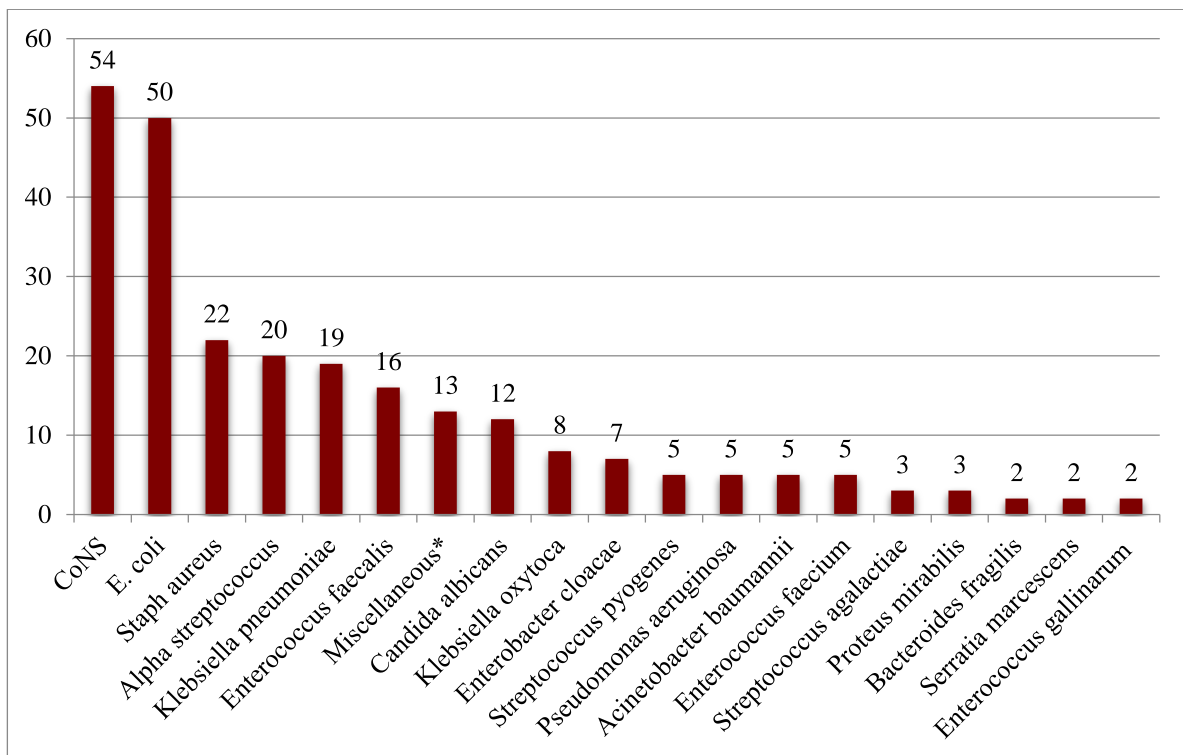


Fig. 7 Clinical isolates diversity. Miscellaneous species* include one of each *Salmonella typhi, Streptococcus pneumoniae, Micrococcus luteus, Enterococcus avium, Bacillus cereus, Acinetobacter pitii, Clostridium ramosum*, Gram-negative anaerobes, *Clostridium* species, *Bacillus* species, Group G streptococcus, Gram-positive cocci and *Candida glabrata.*

## 4.2 IDENTIFICATION BY FILMARRAY FROM BC BOTTLES

In **Paper 1,** monomicrobial growth was observed in 167/191 (87.4%) BC bottles. Identification of the microorganism by FilmArray was successful in 153/167 (91.6%) BC bottles. Importantly, the detection rate for microorganisms covered by the FilmArray BCID panel was 153/154 (99.4%). In the remaining sample, a coagulase-negative staphylococcus (CoNS) could not be identified.

Interestingly, FilmArray identified an additional microorganism in 6/167 (3.6%) BC bottles that was not detected by conventional methods.

In four BC bottles with CoNS and in one bottle with *S. pneumoniae*, the FilmArray assay simultaneously detected an *Enterococcus* isolate. In another BC bottle with *Candida glabrata*, FilmArray detected also *Candida albicans* (Table 7).

Table 7 Identification of bacteria and yeasts from monomicrobial BC by FilmArray.

| Identification | BC and FA positive | BC positive and FA negative | BC negative and FA positive |
|---|---|---|---|
| **Microorganisms included in FA BCID** | | | |
| **Gram-negative** | | | |
| *Escherichia coli* | 34 | | |
| *Klebsiella pneumoniae* | 5 | | |
| *Klebsiella oxytoca* | 2 | | |
| *Proteus mirabilis* | 2 | | |
| *Pseudomonas aeruginosa* | 2 | | |
| *Haemophilus influenza* | 2 | | |
| *Enterobacter cloacae* | 1 | | |
| *Enterobacter aerogenes* | 1 | | |
| *Salmonella* species | 1 | | |
| *Serratia marcescens* | 1 | | |
| *Neisseria meningitides* | 1 | | |
| **Gram-positive** | | | |
| Coagulase negative staphylococci | 37 | 1 | |
| *Staphylococcus aureus* | 19 | | |
| *Streptococcus pneumoniae* | 13 | | |
| *Enterococcus* species | 9 | | 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** | | | |
| *Micrococcus* species | 3 | 3 | |
| *Corynebacterium* species | 2 | 2 | |
| *Peptoniphilus* species | 2 | 2 | |
| *Capnocytophaga canimorsus* | 1 | 1 | |
| *Bacteroides fragilis* | 1 | 1 | |
| *Eggerthella lenta* | 1 | 1 | |
| *Gemella* species | 1 | 1 | |
| *Lactobacillus* species | 1 | 1 | |
| *Parvimonas micra* | 1 | 1 | |
| **Antibiotic resistance markers** | | | |
| MecA | 15 | 1 | 3 |
| VanA/VanB | 0 | | 0 |

In 24/191 (12.6%) positive BC, growth of more than one microorganism was detected by conventional methods. FilmArray could identify all microorganisms in 17/24 (71%) samples. In 6/24 (25%) polymicrobial cultures, FilmArray could not detect any of the microorganisms detected by conventional methods.

In contrast, in one BC bottle with CoNS and alpha-hemolytic streptococci, FilmArray detected an *Enterococcus* isolate in addition (Table 8).

It was not possible to detect the isolates that were only identified by FilmArray even after repeated sub-cultures from these bottles. These results were therefore interpreted as false positive results.

Table 8 Identification 24 polymicrobial blood cultures by FilmArray.

| **Identification** | **ID by Culture** | **ID by FA** |
|---|---|---|
| *E. faecium* + (CoNS) | 1/1 | 1/1 |
| *E. coli* + *K. pneumonia* | 1/1 | 1/1 |
| *C. albicans* + *E. faecalis* | 1/1 | 1/1 |
| *E. cloacae*+*E. faecium* | 1/1 | 1/1 |
| *E. faecalis*+*P.aeruginosa* | 1/1 | 1/1 |
| *E. faecium* + alpha streptococci | 1/1 | 1/1 |
| *E. faecium* + CoNS | 1/1 | 1/1 |
| *E.coli* + alpha streptococci | 1/1 | 1/1 |
| *E.coli* + *K. pneumoniae* | 1/1 | 1/1 |
| *E. coli* + alpha streptococci | 1/1 | 1/1 |
| *K. oxytoca* + *E.faecium* | 1/1 | 1/1 |
| *S.* species + alpha streptococci | 1/1 | 1/1 |
| *Staphylococcus aureus* + CoNS | 1/1 | 1/1 |
| *S. aureus* + alpha streptococci | 1/1 | 1/1 |
| *S. aureus*+*P. aeruginosa* | 1/1 | 1/1 |
| CoNS + alpha streptococci | 1/1 | 1/1 |
| *S. pyogenes* + *E. faecalis* | 1/1 | 1/0 |
| CoNS + *E. faecalis* | 1/1 | 1/0 |
| *E.coli* + *B. fragilis* | 1/1 | 0/X |
| *P. acnes* + *Micrococcus* species | 1/1 | X/X |
| *E.coli*+ *K. pneumoniae* + *E. avium* | 1/1/1 | 1/1/1 |
| CoNS + alpha streptococci + *Enterococcus* | 1/1/0 | 1/1/1 |
| *K. pneumoniae* + *C. perfringens* + alpha streptococci | 1/1/1 | 1/X/0 |
| *S. pneumoniae* + CoNS + *Bacillus* species | 1/1/1 | 1/1/X |
| **Antibiotic resistance markers** | | |
| MecA | 5 | 5 |
| VanA/VanB | 0 | 0 |

Overall, FilmArray could identify all microorganisms in 170/175 (97.1%) BC positive for microorganisms those were included in the FilmArray BCID panel.

In **Paper 2,** in simulated BC bottles, the FilmArray identified 9/10 isolates after 5 h, and the remaining one *E. coli* isolate after 7.5 h of incubation in the blood culture system (Table 9). Interestingly, the median TTD of growth for the BC bottles in the automated BC system was 11.1 h (range, 9.12 to 25 h).

Thus, FilmArray was able to detect bacteria just before samples had been incubated for approximately half of the time to signal positivity in BC system. In order to measure the effect of transport time on FilmArray sensitivity, the bottles were kept at RT for 2.5 h before loading in the BC system. Interestingly, microorganisms in 6/10 (60%) simulated bottles could be identified by the FilmArray already after 2.5 h of incubation in the blood culture system (Table 9).

The remaining four samples (two each of *S. aureus* and *E. coli*) were identified after 5 h of incubation in the BC system. The median TTD in the BC system was 9.1 h (range, 8.2 to 10.8 h). The positive results with the first two sets of experiments encouraged us to test the possibility of identification of microorganisms from bottles prior to incubation in the blood culture system but with simulated transport time only. Interestingly FilmArray could identify microorganisms in 9/10 (90%) bottles 8 h of incubation at RT.

Table 9 In vitro study results from BC bottles inoculated with *Escherichia coli* (n=5) and *Staphylococcus aureus* (n=5). At specified time points in the BC system, BC broth was aspirated and subjected to testing with FilmArray and MALDI-TOF MS. Once a positive result was obtained, no additional testing with the same method was performed.

| Time in blood culture system | Direct incubation in the blood culture system | | | | Blood culture system after incubation at RT for 2.5 h | |
|---|---|---|---|---|---|---|
| | No of bottles with *E. coli* | | No of bottles with *S. aureus* | | No of bottles with *E. coli* | No of bottles with *S. aureus* |
| | FilmArray | MALDI-TOF | FilmArray | MALDI-TOF | FilmArray | FilmArray |
| 2.5 h | 0 | 0 | 0 | 0 | 3 | 3 |
| 5 h | 4 | 0 | 5 | 0 | 2 | 2 |
| 7.5 h | 1 | 0 | NA | 0 | NA | NA |
| Until signaling positive | NA | 5 | NA | 5 | NA | NA |

Similarly, the clinical samples were tested by FilmArray prior to culture positivity. In total, 23 blood culture bottles were analyzed. Fifteen of 23 (65%) bottles signaled positive in the culture system while 8/23 (35%) remained negative after 5 days of incubation.

The FilmArray could identify 14/15 (93%) microorganisms in positive bottles prior to detection in the BC system (Table 10). The only FilmArray-negative and BC-positive sample was one *S. pneumoniae* isolate that signaled positive after 74 h in the BC system. Eight BC bottles that did not signal positive until the end of the incubation time of 5 days in the BC system were also negative in the FilmArray analysis.

Then, the performance of the FilmArray assay in identification of microorganisms from BC bottles before incubation in the BC system was studied. Sixteen positive BC bottles were analyzed. The microorganisms in five samples were directly identified by the FilmArray. These were one each of *E. coli*, *Streptococcus pyogenes*, *S. pneumoniae*, and *Candida albicans* and one polymicrobial BC containing *Klebsiella oxytoca* and *Citrobacter freundii*. Broth from the remaining 11 samples was centrifuged, and the pellet was analyzed with the FilmArray. The FilmArray then identified microorganisms in an additional three samples, each containing *S. aureus*. In total, 8/16 (50%) samples were correctly identified by the FilmArray (Table 11).

Four blood culture bottles that did not signal positive until the end of the incubation time of 5 days in the BC system were also negative on the FilmArray analysis.

Table 10 The identification of microorganisms by FilmArray during incubation in the BC system before detection.

| | No | FilmArray results | Standard method results |
|---|---|---|---|
| **FilmArray positive/ Culture positive** | 14 | | |
| | 4/4 | *Staphylococcus aureus* | *Staphylococcus aureus* |
| | 4/4 | *Staphylococcus* spp. + *MecA* | *Staphylococcus epidermidis* |
| | 1/1 | *Staphylococcus* spp. + *MecA* | Coagulase negative staphylococci |
| | 1/1 | *Enterococcus* spp. | *Enterococcus faecium* |
| | 1/1 | *Enterococcus* spp. | *Enterococcus faecalis* |
| | 2/2 | *Eschericha coli* | *Eschericha coli* |
| | 1/1 | *Klebsiella pneumoniae* | *Klebsiella pneumoniae* |
| **FilmArray negative/ Culture positive** | 1/1 | No detection | *Streptococcus pneumoniae* |
| **FilmArray negative/ Culture negative** | 8/8 | No detection | No growth (5 days) |
| **Total** | 23 | | |

**Table 11** Identification of microorganisms by FilmArray before incubating the bottles in the blood culture system**.**

| | No | FilmArray | Standard methods |
|---|---|---|---|
| **FilmArray positive/Culture positive** | 8/16 | | |
| | | *Klebsiella oxytoca + Enterobacteriacea* | *Klebsiella oxytoca + Citrobacter freundii* |
| | | *Streptococcus pneumoniae* | *Streptococcus pneumoniae* |
| | | *Staphylococcus aureus* | *Staphylococcus aureus* |
| | | *Staphylococcus aureus* | *Staphylococcus aureus* |
| | | *Staphylococcus aureus* | *Staphylococcus aureus* |
| | | *Streptococcus pyogenes* | *Streptococcus pyogenes* |
| | | *Escherichia coli* | *Eschericha coli* |
| | | *Candida albicans* | *Candida albicans* |
| **FilmArray negative/ Culture positive** | 8/16 | | |
| | | Not detected | *Eschericha coli + Streptococcus* spp. |
| | | Not detected | *Staphylococcus epidermidis* |
| | | Not detected | *Staphylococcus aureus* |
| | | Not detected | *Enterococcus faecalis* |
| | | Not detected | *Klebsiella pneumoniae* |
| | | Not detected | *Klebsiella pneumoniae* |
| | | Not detected | *Escherichia coli* |
| | | Not detected | *Escherichia coli* |
| **FilmArray negative/Culture negative** | 4/4 | Not detected | No growth (5 days) |

In **Paper 4,** the FilmArray assay could identify 230/253 (90.9%) microorganisms from BC bottles with polymicrobial growth.

Considering the microorganisms included in the FilmArray BCID panel, the assay could detect and identify 230/243 (94.7%) of the isolates (Fig. 8). If a BC bottle is interpreted as an episode, the FilmArray could correctly identify all microorganisms in 99/115 (86.1%) of episodes (Table 12).

In clinical polymicrobial samples, FilmArray identified both microorganisms in 29/36 (81%) BC bottles with two microorganisms and one of two in seven BC bottles. All microorganisms were identified in 9/12 (75%) BC bottles with growth of three microorganisms and in 3/12 (25%) BC bottles, FilmArray could not identify any of the microorganisms detected by conventional methods.

Among species covered by the BCID panel, the assay missed 10 CoNS (five *Staphylococcus epidermidis*, two *Staphylococcus hominis* and one of each *Staphylococcus sciuri*, *Staphylococcus capitis*, *Staphylococcus warner*) two alpha-streptococci (one of each *Streptococcus anginosus* and *Streptococcus oralis*), and *Enterococcus faecalis* compared to conventional culture-based methods (Fig. 9).

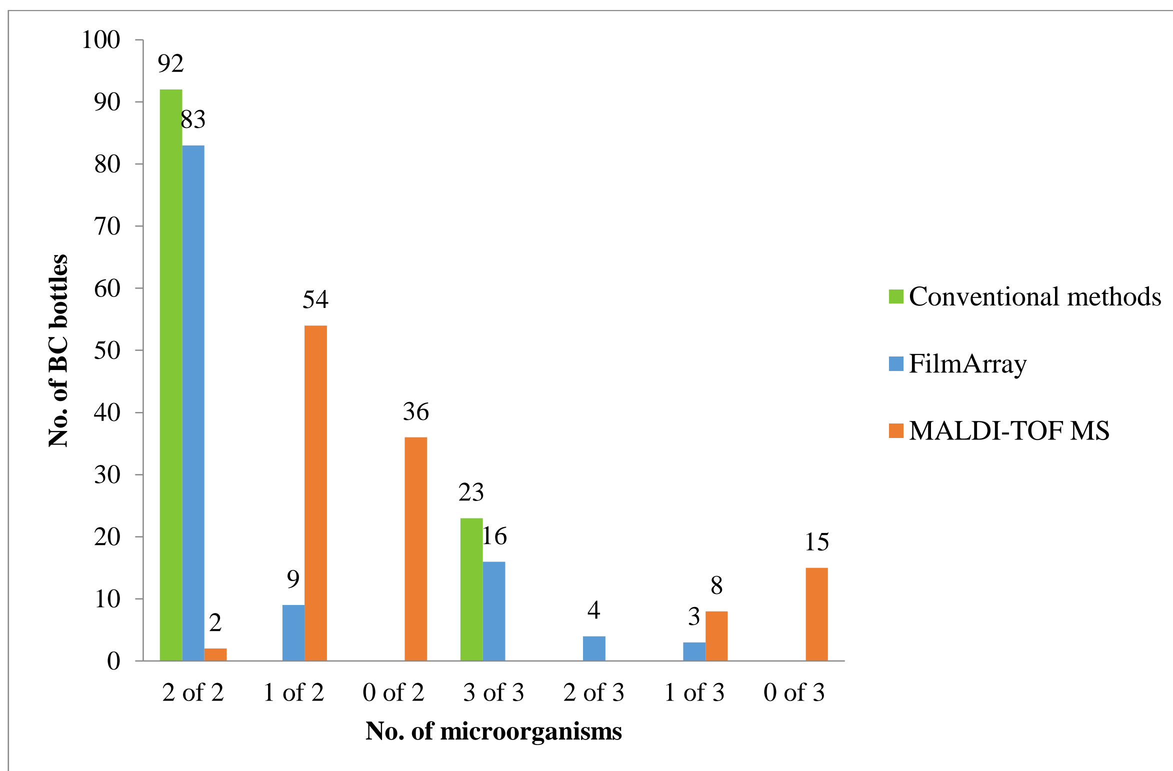


Fig. 8 Identification of microorganisms from blood culture bottles with polymicrobial growth using FilmArray, MALDI-TOF MS and conventional laboratory methods.

Table 12 Detection of microorganisms using FilmArray, direct MALDI-TOF MS with conventional laboratory methods.

| **No.** | **isolate** | **Microorganism** | **Culture results (no.)** | **FA BCID results (no.)** | **MALDI-TOF results (no.)** |
|---|---|---|---|---|---|
| | | **Gram positive** | | | |
| 1 | 54 | CoNS* | 54 | 44 [g] | 11 |
| 2 | 20 | Alpha streptococcus* | 20 | 18 [g] | No ID |
| 3 | 16 | *Enterococcus faecalis* | 16 | 15 [g] | 5 |
| 4 | 22 | *Staph aureus* | 22 | 22 | 16 |
| 5 | 7 | *Enterobacter cloacae* | 7 | 7 | 1 |
| 6 | 5 | *Streptococcus pyogenes* | 5 | 5 | 1 |
| 7 | 5 | *Enterococcus faecium* | 5 | 5 [g] | 2 |
| 8 | 5 | *Acinetobacter baumannii* | 5 | 5 | No ID |
| 9 | 3 | *Streptococcus agalactiae* | 3 | 3 | 1 |
| 10 | 2 | *Enterococcus gallinarum* | 2 | 2 [g] | No ID |

| 11 | 1 | Bacillus species | 1 | $x^{*}$ | No ID |
|---|---|---|---|---|---|
| 12 | 1 | *M. Luteus* | 1 | $x^{*}$ | No ID |
| 13 | 1 | *S. Typhi* | 1 | $1^{g}$ | No ID |
| 14 | 1 | *Streptococcus pneumoniae* | 1 | 1 | 1 |
| 15 | 1 | *Enterococcus avium* | 1 | $1^{g}$ | No ID |
| 16 | 1 | *Bacillus cereus* | 1 | $x^{*}$ | No ID |
| 17 | 1 | *Acinetobacter pitii* | 1 | $x^{*}$ | No ID |
| 18 | 1 | Gram positive cocci | 1 | $x^{*}$ | No ID |
| 19 | 1 | Group G streptococci | 1 | $1^{g}$ | No ID |
| | | **Gram negative** | | | |
| 20 | 50 | *E. coli* | 50 | 50 | 16 |
| 21 | 19 | *Klebsiella pneumoniae* | 19 | 19 | 10 |
| 22 | 8 | *Klebsiella oxytoca* | 8 | 8 | 2 |
| 23 | 5 | *Pseudomonas aeruginosa* | 5 | 5 | No ID |
| 24 | 2 | *Serratia marcescens* | 2 | $2^{g}$ | No ID |
| 25 | 3 | *Proteus mirabilis* | 3 | $3^{g}$ | No ID |
| | | **Yeast** | | | |
| 26 | 12 | *Candida albicans* | 12 | 12 | No ID |
| 27 | 1 | *Candida glabrata* | 1 | 1 | No ID |
| | | Anaerobic | | | |
| 28 | 2 | *Bacteroides fragilis* | 2 | $x^{*}$ | No ID |
| 29 | 1 | *Clostridium ramosum* | 1 | $x^{*}$ | No ID |
| 30 | 1 | Gram negative anaerobe | 1 | $x^{*}$ | No ID |
| 31 | 1 | Clostridium species | 1 | $x^{*}$ | No ID |
| | **253** | **Total** | **253** | **230** | **66** |

N, detection of microorganism to species level; $N^{g}$, detection of microorganisms to genus level; 0, failure to detect microorganisms; X, the microorganism that are not included in the panel. CoNS* includes (37 *S. epidermidis*, 9 *S. hominis*, three of each *S. capitis* and *S. warneri* and one of each *S. haemolyticus* and *S. sciuri)*. Alpha streptococcus* includes (7 *S. anginosus*, 3 *S. salivarius*, 3 *S. mitis,* 2 *S. Sanguinis* and one of each *S. gallolyticus*, *S. oralis*, *S. pyogenes*, *S. parasanguinis*, *S. cristatus*).

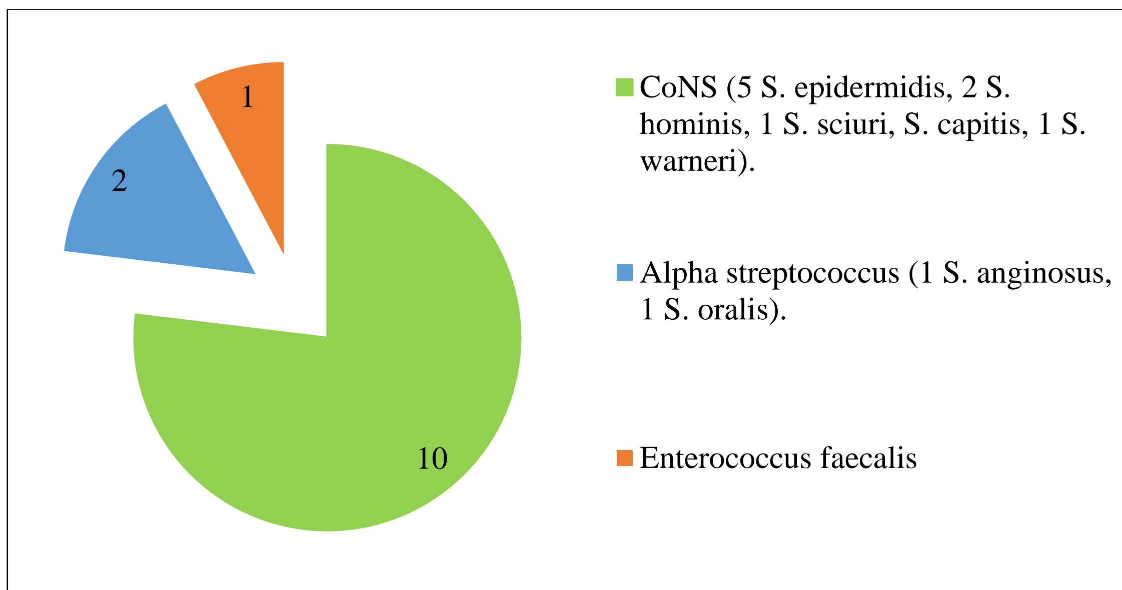


Fig. 9 Microorganisms not identified by the FilmArray *(n*=13).

## 4.3 IDENTIFICATION BY MALDI-TOF MS FROM BLOOD CULTURE BOTTLES

In **Paper 2,** MALDI-TOF MS could not identify any of the 10 strains (five *S. aureus* and four *E. coli*) from the bottles after 8 h of incubation in the blood culture system. In contrast, all 10 microorganisms could be identified by the method after the BC bottles signaled positive in the system. The method was not tested further for identification of microorganisms from bottles prior to BC positivity.

In **Paper 3,** the influence of different anaerobic BC bottles on the performance of bacterial identification by direct MALDI-TOF MS was investigated. MALDI-TOF MS could accurately identify anaerobic bacteria in 51/67 (76%) from BacT/ALERT FN, 51/67 (76%) from BacT/ALERT FN Plus, 53/67 (79%) from BACTEC Plus and 50/67 (75%) from BACTEC Lytic bottles (Fig. 10). There was no difference in identification of anaerobic bacteria by MALDI-TOF MS among the four BC bottles included in the study.

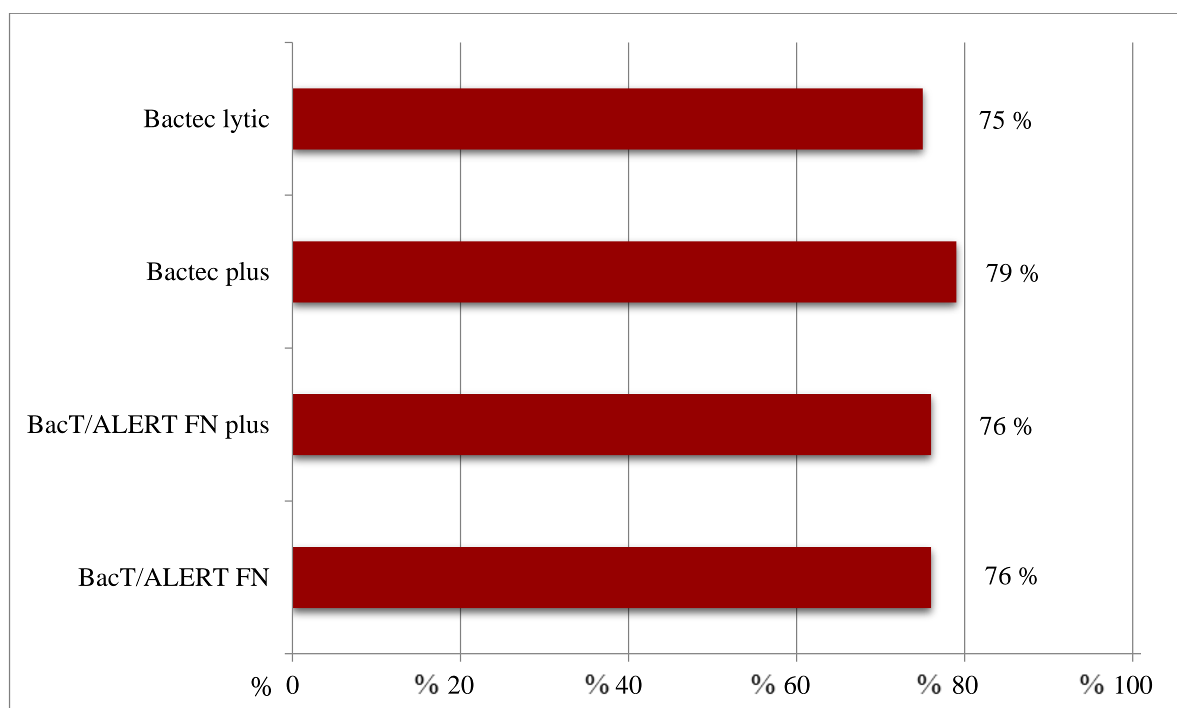


Fig. 10 Identification of anaerobic bacteria directly from blood culture bottles by MALDI-TOF MS.

In **Paper 4,** MALDI-TOF MS could identify all microorganisms in only 2/115 (1.7%) BC bottles with polymicrobial growth. The method could identify one of two or more microorganisms in 66/115 (57%) blood culture bottles.

## 4.4 DETECTION OF MEC A, VAN A AND VAN B AND blaKPC

In **Paper 1,** in BC with monomicrobial growth, consistent results between conventional methods and FilmArray were obtained for one MRSA isolate and 14 methicillin-resistant CoNS. In contrast, three methicillin-susceptible CoNS were tested *mecA*-positive in the FilmArray. In order to evaluate the *mecA* result, conventional *mecA* PCR was performed. The PCR result confirmed the presence of *mecA* in 1/3 CoNS, whereas 2/3 were *mecA* negative, in line with the methicillin-susceptible phenotype. Regarding the BC bottles with polymicrobial growth, there were 5 methicillin-resistant *Staphylococcus* species that were detected both by disc diffusion and FilmArray.

In addition, in one sample with *S. aureus* and CoNS, the FilmArray detected *mecA* without being able to distinguish MRSA or MSSA in the sample. Subsequent phenotypical tests later determined that the *S. aureus* isolate was MSSA and the CoNS were methicillin resistant. There was no microorganism that was *vanA* or *vanB*-positive. Similarly, no carbapenem-resistant or *blaKPC*-positive isolate was detected in the studied material.

In **Paper 4,** the FilmArray detected *mecA* in 23 BC bottles (2 MRSA and 21 methicillin-resistant CoNS) which was confirmed by conventional methods.

## 4.5 TURN-AROUND-TIME

**During the period of study 1, 2 and 4**, the same FilmArray BCID instrument was used. The FilmArray is a fully automated system requiring a maximum of five min hands-on time. The total time from start to the identification result is 65 min for each sample.

In **study 2, 3 and 4,** the turnaround time for direct MALDI-TOF MS was around 25 min with 10 min hands-on time per one sample.

## 4.6 TIME-TO-DETECTION

For **Paper 3,** the shortest median TTD was 18 h in BACTEC Lytic followed by BacT/ALERT FN (23.5 h), BACTEC Plus (27 h) and BacT/ALERT FN Plus (38 h) bottles. There was a significant difference in the average TTD between the four bottle types included in the study ($p<0.0001$).

The TTD was significantly shorter in BACTEC Lytic compared to BacT/ALERT FN, BacT/ALERT FN Plus and BACTEC Plus bottles ($p<0.0001$), similarly BacT/ALERT FN had shorter TTD than BACTEC Plus and BacT/ALERT FN Plus bottles ($p<0.001$ and $p< 0.0001$ respectively). BACTEC Plus had shorter TTD than BacT/ALERT FN Plus bottles ($p<0.0001$) (Table 13).

Table 13 TTD for all four types of anaerobic BC bottles.

| Time to detection | BacT/Alert | | BACTEC | |
|---|---|---|---|---|
| | FN | FN Plus | Plus | Lytic |
| **Mean (h) (SD)** | 25.1(12.8) | 38.5 (14) | 30.9 (17.8) | 19.9 (12.7) |
| **Median (h)** | 23.5 | 38 | 27 | 18 |
| **Range (h)** | 10.6-61 | 14.9-61 | 9.1-101 | 7.3-46 |

## 4.7 DETECTION OF GROWTH (PAPER 3)

During the 5-day incubation, growth of anaerobic bacteria was detected in 89/100 (89%) BacT/ALERT FN, 80/100 (80%) BacT/ALERT FN Plus, 85/100 (85%) BACTEC Plus and 94/100 (94%) BACTEC Lytic BC bottles. There was significant difference in the detection of anaerobic bacteria among the four bottle types studied ($p<0.05$).

BACTEC Lytic had a significantly higher detection rate than BacT/ALERT FN Plus ($p<0.01$). There was no significant difference in detection rates of anaerobic bacteria among the remaining bottle types (Fig. 11).

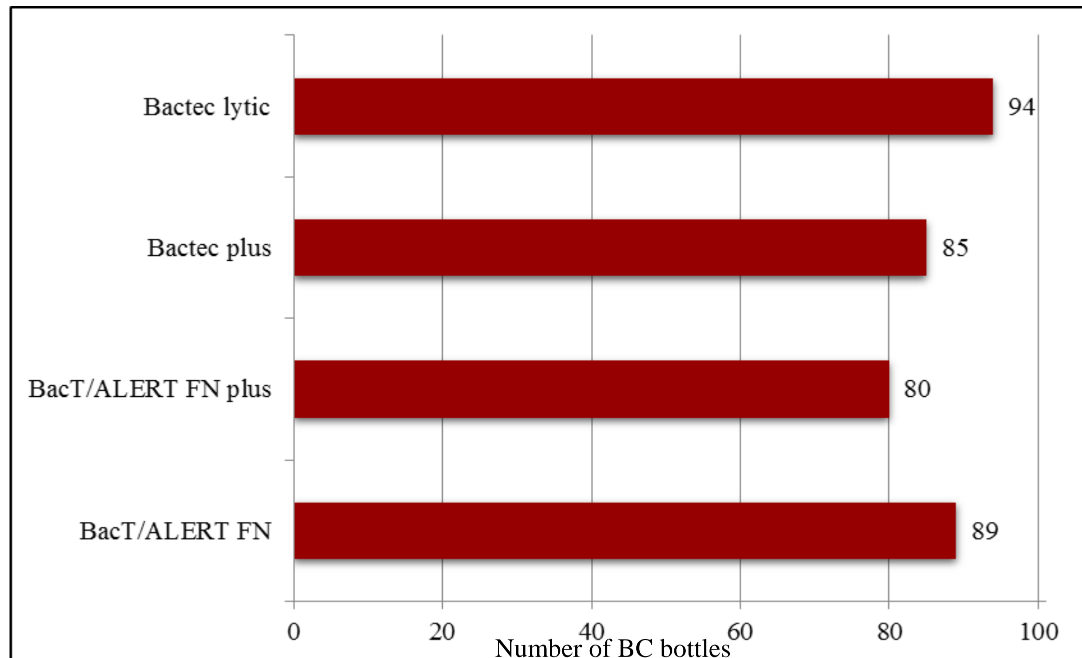


Fig. 11 Overall growth of anaerobic bacteria in four different blood culture bottles.

## 4.8 REPRODUCIBILITY TESTS (PAPER 1)

Five positive BC, one of each with *S. aureus*, MRSA, CoNS, *E. coli* + alpha hemolytic streptococci, and *E. coli* + *K. pneumonia*, were tested with FilmArray BCID panel in duplicate. There was no difference between the two results showing that the FilmArray BCID method is reproducible.

## 4.9 LONGITUDINAL FOLLOW UP WITH FILMARRAY (PAPER 1)

When longitudinal follow-up of positive BC was considered, the FilmArray was positive for the correct pathogen at all time points, i.e., for *S. aureus*, *E. coli*, and CoNS (with *mecA*) on days 1, 7, 14, 21, and 28 and for *C. glabrata* and *K. oxytoca* on days 1, 2, 3, 4, 11, 17, and 21.

## 4.10 PARTIAL 16S R RNA GENE SEQUENCING (PAPER 3)

For **paper 3,** BLAST search showed 100% nucleotide identity to previously registered sequences of the 16S rRNA gene of *Alistipes fingoldii* (330/330 bases) and *Parabacteroides goldsteinii* (330/330 bases). Both species have been described as rare cause of sepsis, which were previously identified by the direct MALDI-TOF MS method.

# 5 DISCUSSION

Sepsis is a devastating disease with global healthcare concerns and associated with both adverse clinical and economic outcomes. The mortality of sepsis is unacceptably high, even in today's modern healthcare environments (Goto and Al-Hasan, 2013, Angus et al., 2001). Several studies have demonstrated that rapid administration of correct antimicrobial treatment is crucial for the survival of the patient (Kumar et al., 2006, Ferrer et al., 2014).

Thus, receiving timely and actionable information from the laboratory on identification and antimicrobial susceptibility of the microorganism causing sepsis is crucial for patient management.

Several studies have shown that the clinical implementation of rapid microbiological diagnostics of sepsis results in decrease in mortality, length of stay and health care costs and shorter time to appropriate antimicrobial therapy (Bauer et al., 2010, Huang et al., 2013).

Currently, blood culture is the gold standard method for diagnosing sepsis. Blood cultures are sensitive, easy to perform and allow detection of viable microorganisms (Opota et al., 2015a). Until recently the identification of microorganisms from sub-cultures of the positive blood culture usually took between 24-72 hours.

The introduction of MALDI-TOF MS in the clinical routine shortened this period to less than 10 minutes. The next step in improvement of microbiological diagnostic of sepsis is therefore to bring solutions to bypass the subcultures that generally take between 20-72 h depending on the growth characteristics of the microorganism detected in blood cultures.

The overall aim of this thesis was to evaluate and improve the use of rapid identification methods in identification of microorganisms directly from blood culture bottles. The rapid identification methods described in the present thesis refer to identification of microorganisms directly from blood culture bottles bypassing long sub-culture period of 20-72h.

There are several aspects of rapid microbiological identification methods that needs to be evaluated in order to implement the methods in the clinical routine and possibly to improve the use of these methods.

The first prerequisite for rapid microbiological methods is to have a short turn-around time (TAT). It is reasonable to suggest that the results are ready in maximum 3-4 h after the blood culture bottle signals positive.

Two methods, FilmArray and MALDI-TOF MS were investigated. The present studies showed that the identification of microorganisms from a single blood culture bottle by FilmArray and MALDI-TOF MS were ready in 65 min and 30 minutes respectively. The

results from PNA-FISH (AdvanDx, Woburn, MA, USA) and Verigene (Nanosphere, Northbrook, IL, USA) are ready in 2-3 h whereas the TAT for Prove-it (Mobidiag, Esbo, Finland) is around 5 h.

The concept of blood culture based rapid identification of microorganisms relies on blood culture positivity. However, the high analytical performance of the current rapid methods stimulated us to ask the question if we can identify microorganisms from bottles before the blood cultures signals positive i.e. semi-culture based identification. In study 2, we analyzed the semi-culture based identification by FilmArray.

In simulated samples, the median TTD of growth for the bottles in the blood culture system was 11.1 h, whereas FilmArray could identify microorganisms after 5 h incubation in the system. Similar results were obtained when the simulated bottles were incubated for 2.5 h at RT before incubation in the blood culture system. When clinical blood culture bottles were tested, FilmArray could identify the microorganisms from bottles before culture positivity as in the case of simulated samples.

The TAT of FilmArray with semi-culture based approach can even be compared to TAT of non-culture-based molecular diagnostic methods that are performed directly on blood samples. The commercial molecular assays including LightCycler SeptiFast test, the Magicplex real-time PCR test, the VYOO test, and IRIDICA are currently used as non-culture based methods. The TAT of these assays is between 6 to 8 h.

The total time to identification using the semi-culture based approach with the FilmArray is around 6 h. The present data indicate that the semi-culture based identification from bottles after 5 h of incubation in the blood culture system might be a useful approach in a selected number of patients where there is an urgent need for microbiological diagnosis of sepsis. The capacity of the methods is another important parameter in TAT of the rapid identification of microorganisms.

Each FilmArray instrument can analyze one sample at a time in 60 min. This is an important limitation with the assay. Therefore, the manufacturer has recently presented the next generation system with high throughput. The system will be able to analyze up to 12 samples simultaneously. In contrast, MALDI-TOF MS can analyze up to 48 samples simultaneously. Moreover, the method takes less than 10 minutes to analyze the sample.

The microorganism coverage is crucial for the performance of rapid identification methods. There are several methods that can identify only one microorganism including Xpert MRSA/SA Blood Culture (Cepheid, Sunnyvale, CA) or a small number of microorganisms such as PNA FISH (AdvanDx, Inc., Woburn, MA). The limited coverage is a significant challenge to implement these methods since the method requires information from other tests including Gram-staining. Neither FilmArray nor MALDI-TOF MS require prior Gram-staining in clinical practice.

In the first study we showed that the coverage rate of the FilmArray BCID panel is more than 90% in the studied material. Interestingly, six of thirteen of the microorganisms not included in the BCID panel were *Micrococcus* species, *Bacillus* species and *Corynebacterium* species that were detected in only one of the bottles of the blood culture sets indicating that the microorganisms were probably contaminants from the skin flora.

Therefore it is plausible to suggest that the FilmArray BCID panel covers the majority of the clinically relevant microorganisms. Regarding MALDI-TOF MS, the method has one of the largest microorganism coverage among the identification methods in clinical microbiology.

The MALDI-TOF MS that was used in study 2, 3 and 4 has more than 5000 microorganisms in its database. PNA-FISH (AdvanDx, Woburn, MA, USA) includes only 2 to 3 different microorganisms in each test kit. Verigene (Nanosphere, Northbrook, IL, USA) includes two different test kits which are chosen according to Gram-staining result. The Gram-positive kit covers 11 microorganisms and three resistance markers whereas the Gram-negative kit includes 8 microorganisms and 5 resistance markers.

Finally Prove-it (Mobidiag, Esbo, Finland) has the broadest coverage among the panel based molecular methods with more than 60 microorganisms and 3 resistance markers in the panel.

One of the most striking examples of the significance of microorganism coverage was observed in study 3. Two anaerobic bacteria that were previously identified from subcultures by the conventional method Vitek2 XL (bioMérieux, France) as *B. fragilis* and *Prevotella* species identified by MALDI-TOF MS from bottles as *A. finegoldii* and *P. goldsteinii* respectively.

The MALDI-TOF MS results were confirmed later by sequencing. The analytical performance is crucial in implementation of rapid microbiological methods in the clinical routine. It is important to note that the characteristics of the clinical samples play a major role in the measuring the performance of the methods. Karolinska University Laboratory receives blood culture samples from a wide range of clinical wards including several ICUs in Stockholm area.

Therefore we believe that the clinical samples used in evaluation of the methods represent a sufficient diversity in terms of microorganisms and patients in order to draw conclusions for the performance of the methods analyzed in our studies.

The most common form of sepsis is caused by a single microorganism. In the first study we analyzed the performance of a multiplex PCR method, FilmArray, in identification of microorganisms in a prospective clinical material (Altun et al., 2013).

We showed that FilmArray could identify all microorganisms in 91.6% of the blood culture bottles with monomicrobial growth. Several previous studies have described the performance of other rapid identification methods including PNA-FISH (AdvanDx, Woburn, MA, USA),

Verigene (Nanosphere, Northbrook, IL, USA) and Prove-it (Mobidiag, Esbo, Finland) and MALDI-TOF MS (Brucker Daltonics, Bremen, Germany). The analytical performances of these methods were high and comparable to our findings with sensitivity rates between 76-100 % (Hartmann et al., 2005, Bhatti et al., 2014, Liesenfeld et al., 2014, Patel, 2013).

It is increasingly common to isolate microorganisms that are fastidious, slow growing and difficult to culture. One of the most common types of such microorganisms is anaerobic bacteria. The subculture process for anaerobic bacteria is extremely long and is up to several days. Therefore, identification of anaerobic bacteria directly from blood culture bottles is very clinically relevant.

The broad spectrum of anaerobic microorganism present in MALDI-TOF MS database inspired us to test the method for identification of anaerobic bacteria. Previous studies analyzing the performance of MALDI-TOF MS in identification of microorganisms from bottles included very limited numbers of bottles with anaerobic bacteria and therefore showed promising results but were inconclusive (Leli et al., 2013, Moussaoui et al., 2010).

In study 3, we analyzed the performance of MALDI-TOF MS in identification of anaerobic bacteria from four different blood culture bottle types. MALDI-TOF MS could identify anaerobic bacteria in between 75-79% of the different blood culture bottle types. The study with more than 65 anaerobic bacteria showed that the MALDI-TOF MS can be used in identification of anaerobic bacteria from bottles in the clinical routine (Almuhayawi et al., 2015).

As in the case of rare isolates, the incidence of detection of polymicrobial growth in blood culture bottles is increasing. This is an obvious challenge both for conventional and rapid identification methods (Lin et al., 2010, Dodemont et al., 2014).

In study 1 and 4 we evaluated the performance of rapid methods in identification of polymicrobial growth directly from blood culture bottles after positivity. FilmArray correctly identified all microorganisms in 17/24 (71%) and 99/115 (86.1%) of the BC bottles in study 1 and 4 respectively.

It is important to note that 4/24 (17%) and 16/115 (14%) of the bottles with polymicrobial growth included three different microorganisms. The results showed that FilmArray is a reliable method for rapid identification of polymicrobial samples from positive blood culture bottles.

In contrast to promising FilmArray results, the present MALDI-TOF MS method showed poor performance in identification of microorganisms from blood culture bottles with polymicrobial growth. The method could identify both microorganisms in only 2/115 (1.7%) blood culture bottles.

The present results obtained by MALDI-TOF MS are in line with the previously published studies (Kok et al., 2011, Martinez et al., 2014). The difference between the performance of

FilmArray and MALDI-TOF MS in identification of microorganisms from bottles with polymicrobial growth is probably due to the target molecules in the two methods. FilmArray can detect genes and discriminate microorganisms.

In contrast MALDI-TOF MS compare an unknown isolate's protein profile to those of a reference database, more than one protein profile in the sample result in difficulties in identification of different microorganisms.

An additional advantage with the current rapid methods is the possibility to detect a number of resistance markers. FilmArray includes three antibiotic resistance markers: *mecA*, *vanA*/*vanB*, and the *KPC*. Among the positive blood culture bottles in study 1 and 4, there were only 3 MRSA in 306 (0.98%) blood cultures. FilmArray could correctly detect *mecA* gene in all three samples.

The complex work flow and limited resources in today's clinical microbiological diagnosis of sepsis require that the rapid methods to be user friendly. The FilmArray is easy to perform and requires less than 5 minutes hands-on time. Blood culture broth from the bottle is directly applied without a preparation step. In contrast, the MALDI-TOF MS method requires preferable pure cultures of microorganisms.

The blood cells, cell debris and proteins present in the culture media interact with MALDI-TOF MS analysis and may affect spectral peak analysis from organisms cultured in these matrices (Ferroni et al., 2010). Therefore the blood culture broth should be prepared and the microorganisms need to be purified prior to MALDI-TOF MS analysis. This is a time consuming process and takes between 20-30 min per sample with 10-15 min hands on time.

The goal with the development of rapid identification methods is to implement these methods in the clinical routine.

The data from present studies indicate that FilmArray is a reliable method that can be implemented in the clinical routine. In addition the data from study 3 showed that MALDI-TOF MS can be used for microorganisms including anaerobic bacteria that are not present in FilmArray BCID panel.

However, there are several other factors that play important role in choosing the optimal rapid identification method including the size of the laboratory, numbers of positive blood cultures per day, laboratory staffing, patient and provider types and local resistance rates. These factors should be taken into consideration when implementing rapid identification methods in clinical practice.

The present studies have several limitations including lack of clinical data. It is therefore not possible to draw conclusions on possible contaminants detected in clinical samples during the study. However, we analyzed the data from other blood culture samples registered in the

laboratory information system in order to estimate the blood cultures with contaminants as described in study 1.

Similarly, the information from the rapid identification methods obtained during the studies was not used in the clinical routine. Therefore it is not possible to measure the clinical impact of the present methods in our studies. However, the clinical impact of rapid blood culture diagnostics on clinical and economic outcomes has been difficult to analyze. There are several studies with contradictory results (Sango et al., 2013, Forrest et al., 2006, Holtzman et al., 2011).

The underlying reason for different results might depend on multiple factors, including the level of antimicrobial resistance, patient profile, treatment policies and the clinical staff implementing the information from microbiology laboratory in patient treatment. Another limitation would be the use of simulated blood culture bottles in study 2, 3 and 4.

The clinical samples might include parameters that differ from the simulated cultures. These may include variable composition of blood cells, antimicrobial agents and transport time of blood culture bottles that are normally observed in clinical blood cultures.

However, appropriate studies in detection of rare microorganisms such as anaerobic bacteria and rare clinical conditions such as polymicrobial sepsis in prospective clinical studies would be tremendously difficult. The information obtained from simulated samples might probably reflect the analytical performance of the methods in the clinical routine.

# 6 CONCLUSION

The present thesis shows that the FilmArray is a reliable method for identification of microorganisms from positive blood culture bottles with mono- as well as polymicrobial growth. The data from the studies showed also that it is possible to improve the use of rapid identification methods as in the case of semi-culture based identification.

This approach describes the identification of microorganisms from blood culture bottles using FilmArray before the bottles signal positive in the system.

This unique approach may be used in a selective group of patients as a reliable alternative candidate to non-culture based methods. In addition we showed that rapid identification methods with broad coverage such as MALDI-TOF MS can be used in identification of rare microorganisms including anaerobic bacteria.

# 7 A TRANSLATION OF THE THESIS TITLE IN ARABIC (THESIS AND PAPERS)

**الورقه البحثيه الاولى**

التقييم السريري عن طريق الFilmArray BCID في التعرف على البكتيريا والفطريات من زجاجات الدم الإيجابية

**الورقه البحثيه الثانيه**

تحديد الكائنات الدقيقة عن طريق الFilmArray BCID و

MALDI-TOF MS قبل الإشارة الإيجابية في الانظمه الاليه لزجاجات الدم

**الورقه البحثيه الثالثه**

أداء أربع زجاجات الدم اللاهوائية في الكشف عن البكتيريا اللاهوائية مباشره وتحديد الهوية للكائنات الدقيقه عن طريق ال MALDI-TOF MS

**الورقه البحثيه الرابعه**

التعرف المباشر على الكائنات الحية الدقيقةعن طريق الBCID FilmArray وMALDI-TOF MS من زجاجات الدم الايجابيه متعدده المكروبات

اطروحه مقدمه لنيل درجه الدكتوراه

بعنوان

التشخيص السريع للكائنات الحيه الدقيقه المسببه لتسمم الدم

إعداد الدكتور

**محمد بن سعد بن محمد بن صلاح المحياوي**

بكالوريوس الطب والجراحه

## إشراف

**الدكتور** فولكن اوزنجي

**الأستاذ الدكتور** جونر سندستروم

**الدكتور** أمير محمد سعيد

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