Quantitative detection of bacterial DNA in whole blood in bloodstream infection
Quantitative detection of bacterial DNA in whole blood in bloodstream infection
Abstract

Ingrid Ziegler (2018): Quantitative detection of bacterial DNA in whole blood in bloodstream infection. Örebro Studies in Medical Science 183

This thesis aims to increase the knowledge on how quantitative PCR can be used in the diagnostics of bloodstream infections, with an emphasis on quantitative elements.

In Papers I and II, we evaluated quantitative data from two commercial PCR tests for pathogen detection directly in blood, Magicplex Sepsis (I) and SeptiFast (II), from patients with suspected sepsis. We found that high quantification cycle (Cq) values, indicating low DNA loads, were associated with findings of pathogens with doubtful clinical relevance, whereas low Cq values, indicating high DNA loads, were correlated with sepsis and septic shock, as well as with positive blood culture results.

In Paper III, we aimed to study the bacterial DNA load during *Staphylococcus aureus* bacteremia, in relation to different clinical factors. For this purpose, we developed a droplet digital PCR (ddPCR) for precise DNA quantification, targeting *S. aureus* specifically. We found that a high initial *S. aureus* DNA load was associated with laboratory markers for immune dysregulation as well as with sepsis, endocarditis, and mortality.

In Paper IV, we aimed to develop a tool for repeated DNA quantification during bloodstream infection. For this purpose, we optimized a ddPCR, targeting the universal bacterial 16S rDNA, and performed a comparison with species-specific ddPCRs on spiked blood, and on clinical samples. The performance of the 16S rDNA ddPCR was adequate, and we found that a high 16S rDNA load was associated with sepsis and mortality.

In conclusion, our results indicate that the pathogen DNA load in blood plays an important role in the clinical picture in BSI. In future research on molecular BSI diagnostics, studies on DNA loads and clearance should be included.

*Keywords*: bloodstream infection, bacteremia, sepsis, DNA load, quantitative PCR, droplet digital PCR, 16S rDNA

Ingrid Ziegler, School of Health and Medical Sciences, Örebro University, SE-701 82, Sweden, ingrid.ziegler@regionorebrolan.se
## TABLE OF CONTENTS

### LIST OF ORIGINAL PAPERS

### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>13</td>
</tr>
<tr>
<td>Sepsis</td>
<td>13</td>
</tr>
<tr>
<td>Sepsis epidemiology</td>
<td>13</td>
</tr>
<tr>
<td>Incidence</td>
<td>13</td>
</tr>
<tr>
<td>Outcome</td>
<td>13</td>
</tr>
<tr>
<td>Risk factors for mortality</td>
<td>14</td>
</tr>
<tr>
<td>Onset and focus of infection</td>
<td>14</td>
</tr>
<tr>
<td>Microbiological etiology</td>
<td>14</td>
</tr>
<tr>
<td>Sepsis definitions</td>
<td>15</td>
</tr>
<tr>
<td>Sepsis-1</td>
<td>15</td>
</tr>
<tr>
<td>Sepsis-2</td>
<td>15</td>
</tr>
<tr>
<td>Sepsis-3</td>
<td>16</td>
</tr>
<tr>
<td>Clinical features of sepsis</td>
<td>17</td>
</tr>
<tr>
<td>Fever</td>
<td>17</td>
</tr>
<tr>
<td>Physiological parameters</td>
<td>18</td>
</tr>
<tr>
<td>Common clinical symptoms</td>
<td>18</td>
</tr>
<tr>
<td>Symptoms of specific organ dysfunction</td>
<td>18</td>
</tr>
<tr>
<td>Organ dysfunction in septic shock</td>
<td>19</td>
</tr>
<tr>
<td>Sepsis management</td>
<td>19</td>
</tr>
<tr>
<td>General aspects</td>
<td>19</td>
</tr>
<tr>
<td>Immuno-modelling therapies</td>
<td>21</td>
</tr>
<tr>
<td>Sepsis pathophysiology</td>
<td>22</td>
</tr>
<tr>
<td>Pathogen factors</td>
<td>22</td>
</tr>
<tr>
<td>Host factors</td>
<td>25</td>
</tr>
<tr>
<td>Persistent inflammation-immunosuppression and catabolism syndrome</td>
<td>29</td>
</tr>
<tr>
<td>Diagnostic methods to find infectious etiology in sepsis</td>
<td>29</td>
</tr>
<tr>
<td>Bloodstream infection</td>
<td>30</td>
</tr>
<tr>
<td>Blood culture</td>
<td>31</td>
</tr>
<tr>
<td>Molecular methods</td>
<td>32</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>32</td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>34</td>
</tr>
</tbody>
</table>
Quantification cycle ................................................................. 35
Digital PCR .............................................................................. 36
Droplet digital PCR .................................................................. 36
Target genes for molecular identification of bacteria and fungi ........ 36
Molecular diagnostic methods in bloodstream infection ............... 40
Blood as a specimen in molecular diagnostics ............................ 40
Molecular methods from positive BC bottles ............................... 42
Molecular methods directly from whole blood ......................... 42
Commercial tests ..................................................................... 43
AIMS ....................................................................................... 47
MATERIALS AND METHODS ...................................................... 48
Patients (I-IV) ....................................................................... 48
  SeptiFast Study (II) ............................................................... 48
  Dynamics of Sepsis Study (I, III, and IV) ............................... 48
Blood culture (I-IV) .................................................................. 49
Serial dilutions of samples with reference strain bacteria (IV) .......... 49
DNA extraction and PCR (I-IV) ................................................ 50
  Paper II ............................................................................... 50
  Papers I, III, and IV ............................................................. 50
Clinical data and definitions (II, III, IV) ...................................... 51
Statistics (I-IV) ...................................................................... 51
Ethics (I-IV) .......................................................................... 51
RESULTS .................................................................................. 52
Study populations (I-IV) ........................................................... 52
Evaluation of quantitative data of commercial molecular methods for pathogen identification in whole blood (I, II) ....................... 52
  Paper I ............................................................................... 52
  Paper II ............................................................................... 54
ddPCR for monitoring bacterial DNAemia in bloodstream infections (III, IV) ................................................................. 56
  Paper III ............................................................................. 56
  Paper IV ............................................................................. 58
DISCUSSION ............................................................................ 63
Study populations (I-IV) ........................................................... 63
Papers I-II ............................................................................... 63
Papers III-IV ........................................................................... 64
CONCLUSIONS ........................................................................................................ 67
FUTURE PERSPECTIVES ..................................................................................... 68
SVENSK SAMMANFATTNING .............................................................................. 72
ACKNOWLEDGEMENTS ..................................................................................... 74
REFERENCES .................................................................................................... 77
LIST OF ORIGINAL PAPERS

This thesis is based on the following papers and manuscripts, which are referred to in the text by their Roman numerals:


Papers I and II are reprinted in accordance with the Creative Commons Attribution (CC BY) license.
ABBREVIATIONS

APC    Antigen presenting cell
ARDS  Adult Respiratory Distress Syndrome
AST   Antibiotic susceptibility test
AUC   Area under the curve
BC    Blood culture
BSI   Bloodstream infection
CD    Cluster of differentiation
CFU   Colony forming unit
CI    Confidence interval
CoNS  Coagulase-negative staphylococci
Cq    Quantification cycle
CRP   C-reactive protein
DAMP  Damage-associated molecular patterns
ddPCR Droplet digital PCR
DIC   Disseminated intravascular coagulation
DNA   Deoxyribonucleic acid
dPCR  Digital PCR
ED    Emergency department
EDTA  Ethylenediaminetetraacetic acid
EGDT  Early goal directed therapy
HIV   Human immunodeficiency virus
HLA   Human leucocyte antigen
HLA-DR HLA-D related
ICU   Intensive care unit
IL    Interleukin
ITS   Internal transcribed spacer
LPS   Lipopolysaccharide
MALDI-TOF Matrix-Assisted Laser Desorption Ionization – Time of Flight
MHC   Major histocompatibility complex
MIC   Minimal inhibitory concentration
MIQE  Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MRSA  Methicillin resistant Staphylococcus aureus
MS    Mass Spectrometry
MST   Magicplex Sepsis Real-time Test
NET   Neutrophil extracellular trap
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PICS</td>
<td>Persistent inflammation-immunosuppression and catabolism syndrome</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>qSOFA</td>
<td>Quick SOFA</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal DNA</td>
</tr>
<tr>
<td>RETTS</td>
<td>Rapid Emergency Triage and Treatment System</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SAB</td>
<td><em>Staphylococcus aureus</em> bacteremia</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SOFA</td>
<td>Sequential Organ Failure Assessment</td>
</tr>
<tr>
<td>SSC</td>
<td>Surviving Sepsis Campaign</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TTP</td>
<td>Time to positivity</td>
</tr>
</tbody>
</table>
INTRODUCTION

Sepsis
Sepsis is one of the oldest syndromes in medicine, known even to the ancient Greeks. The word sepsis is derived from the Greek word sepo (σέπο) with the meaning “I rot”. Hippocrates described sepsis as a dangerous biological decay that could occur in the body and cause autointoxication [1]. Sepsis is now recognized as a syndrome with a dysregulated host response with presence of organ dysfunction, secondary to infection. The infectious pathogen in sepsis, is often, but is not necessarily, spread into the bloodstream, causing a bloodstream infection (BSI).

Sepsis epidemiology

Incidence
Sepsis and septic shock are related to high morbidity and mortality worldwide [2]. The incidence is difficult to determine as there are few data from low- and middle-income countries and, in many studies, incidence data are derived from large retrospective datasets, for which diagnosis definitions can vary [3]. However, in a recent meta-analysis [4] the incidence rate was estimated at 437 cases per 100,000 person-years, with an in-hospital mortality of 17% for sepsis and 26% for severe sepsis. Based on these figures, the authors made a tentative global estimate of 50 million cases of sepsis. In a recent Swedish study [5], a sepsis incidence of 780 cases per 100,000 person-years was noted.

Outcome
Epidemiological data have shown a trend with a rising volume of sepsis hospitalizations in the last decade, but a declining in-hospital mortality [6]. Reasons for better survival in sepsis might be successes in implementation of management recommendations for early supportive care and administration of antibiotics [7-9], as well as an increasing awareness of sepsis among both healthcare professionals and the public. However, in recent studies [10-12], it has been shown that a large proportion of sepsis survivors have a poor long-term outcome, with a high burden of physical and cognitive sequelae, a decreased quality of life, and a long-term risk of death.
Risk factors for mortality
Important risk factors for death are multiple organ failure [13, 14], inappropriate, or delayed antibiotic treatment [15, 16], increasing age and presence of comorbidities [2, 17]. The prognosis also depends on focus of infection and etiology [18], where infections with *Staphylococcus aureus* have been reported to be associated with the highest mortality among Gram-positive bacteria, and *Pseudomonas* spp. and *Acinetobacter* spp. with the highest mortality for infections caused by Gram-negatives [18, 19]. In several studies, infections with multi-resistant bacteria have been associated with an increased risk of death [20-22]. Late-onset, secondary sepsis infections are associated with higher mortality rates than primary ones [23].

Onset and focus of infection
Sepsis diagnosed on admission to hospital, or within 48 hours of admission, is considered to be a community-onset infection, which can be community-acquired, or healthcare associated, depending on whether or not the patient was subjected to significant prior healthcare exposure before becoming ill [24]. Sepsis with start of symptoms later than 48 hours post-admission is considered to be a hospital-onset infection. Healthcare associated sepsis, which is more likely in patients with comorbidities, has a different pathogen distribution, higher rates of antimicrobial resistance, and higher mortality rates [25].

In a large, multi-center, multi-national and prospective study of intensive care unit (ICU)-treated patients with sepsis, the most common site of infection was the lungs, representing 64% of cases, followed by the abdomen (20%), the bloodstream (15%), and the renal tract/genito-urinary system (14%) [21]; a similar distribution of infectious foci has been reported from other studies as well [2, 26].

Microbiological etiology
Traditionally, sepsis is referred to as a condition caused by bacterial or fungal infection, but viral infection, such as influenza [27], and parasitic infection, in particular malaria [28], can also lead to sepsis. In this thesis the primary focus is on bacterial sepsis.

Only about a third of sepsis patients have positive blood cultures (BC) [29], probably in some cases due to a lack in sensitivity in BC, and sometimes due to absence of BSI. Including cultures from sites other than the bloodstream, such as sputum, pleural fluid, endotracheal aspirates, and urine, a microbial etiology is found in 60-70% of ICU-treated sepsis patients.
In microbial isolates from an ICU study population 47% were Gram-positive, 62% Gram-negative, and 19% fungal. The most common Gram-positive organism was *S. aureus*, and the most common Gram-negative organisms were *Pseudomonas* spp. and *Escherichia coli* [21].

**Sepsis definitions**
The definition of sepsis has changed several times since the first descriptions by Hippocrates.

**Sepsis-1**
The first internationally accepted definition was established in 1991, after the North American consensus meeting [31]. This definition (Sepsis-1) described sepsis as the host’s inflammatory response to an infection. For clinical sepsis classification, the systemic inflammatory response syndrome (SIRS) criteria were used. These were:

- Heart rate >90/min
- Respiratory rate >20/min
- Temperature >38° or <36°
- Leukocytes >12 or <4 x 10⁹/mL

For sepsis diagnosis, two or more of these criteria had to be fulfilled and be due to a suspected infection, but microbiological confirmation was not required. When sepsis was complicated with organ failure, the term “severe sepsis” was used; severe sepsis accompanied by hypotension that persisted after adequate fluid resuscitation, was termed septic shock.

**Sepsis-2**
The Sepsis-1 definition came under criticism as the SIRS criteria were considered unspecific, and the criteria for organ failure, indicating severe sepsis, were vague. To meet these objections, a second International consensus conference was held in 2001 [32]. However, in Sepsis-2 the basic definition of sepsis remained unchanged, only some clinical signs and laboratory markers, indicating organ dysfunction and impaired tissue perfusion, were added. The criticism of the SIRS criteria as being unspecific, as well as insensitive, persisted. Many non-infectious inflammatory conditions fulfilled the SIRS criteria for sepsis, and could be misdiagnosed as sepsis [33], whereas many patients treated for severe infections in the ICU did not fulfill the criteria [34]. Sepsis-2 still did not include clear-cut criteria for organ
dysfunction defining severe sepsis, and different criteria were used in different studies, leading to heterogeneity in classification [35, 36].

**Sepsis-3**
The present definition of sepsis was launched in 2016, by a third international consensus sepsis conference [37]. In the new definition, the SIRS criteria are abandoned, as hyperinflammation is no longer seen as the cornerstone of sepsis. Instead, according to new understandings of sepsis pathophysiology [38], activation of both pro- and anti-inflammatory responses occur, along with major alterations in other pathways, such as cardiovascular, neuronal, autonomic, hormonal, metabolic, and coagulation pathways.

In the current definition, Sepsis-3, sepsis is defined as “life-threatening organ dysfunction caused by a dysregulated host response to infection”. Organ dysfunction is clinically classified using the Sequential Organ Failure Assessment (SOFA) score [39], and an increase of ≥2 points from baseline, due to infection, is the criterion for sepsis (Table 1).

A sepsis screening tool for clinical use called quick SOFA (qSOFA) is proposed in Sepsis-3. qSOFA includes three clinical parameters; altered mental status, systolic blood pressure ≤100 mm Hg and respiratory rate ≥22 breaths/minute. If two of these three parameters are present, sepsis should be suspected. qSOFA has been criticized, mainly due to low sensitivity [40].

Microbiological confirmation is not required to establish a sepsis diagnosis. However, presence of a suspected or verified infection is essential to diagnose sepsis. One major reason for a delayed sepsis diagnosis is probably that infection is not suspected in a patient with organ dysfunction. Thus, there is a need for improved strategies to identify an underlying infection in populations presenting with organ dysfunction.
Table 1. The sequential organ failure assessment (SOFA) score. Adapted from Singer et al [37].

<table>
<thead>
<tr>
<th>Organ system</th>
<th>SOFA Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Respiration: PaO₂/FiO₂, kPa</td>
<td>≥53.3</td>
</tr>
<tr>
<td>Coagulation: thrombocytes x10⁹/L</td>
<td>≥150</td>
</tr>
<tr>
<td>Liver: bilirubin, µmol/L</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Cardiovascular: mean arterial pressure, mm Hg</td>
<td>≥70</td>
</tr>
<tr>
<td>Central nervous system: Glasgow Coma Scale</td>
<td>15</td>
</tr>
<tr>
<td>Renal: Creatinine, µmol/L Diuresis, mL/day</td>
<td>&lt;110</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dopamine, Epinephrine and Norepinephrine doses are given as µg/kg/minute. PaO₂, partial pressure of oxygen; FiO₂, fraction of inspired oxygen.

Clinical features of sepsis
The clinical presentation of sepsis can vary greatly, and early identification of the condition is sometimes difficult. Symptoms depend on site of infection, causative pathogen, comorbidities, the stage of disease in which the patient seeks healthcare, and the pattern of organ dysfunction.

Fever
Fever is considered to be a part of the immune system’s adaptive response and is not a sign of organ failure. A Swedish study has recently shown that fever in sepsis patients treated in ICU is associated with lower mortality [41], regardless of differences in hospital care in the study groups. Sepsis
patients without fever are at higher risk of misdiagnosis, resulting in longer time to antibiotic administration and supportive care [41]. However, almost 40% of septic patients have normal body temperature [34], and fever is frequently present in harmless conditions. Thus, fever has no discriminatory value for sepsis identification.

**Physiological parameters**
At the prehospital setting and the Emergency Department (ED) in our hospital in Örebro, as in most places in Sweden, all patients are screened by the Rapid Emergency Triage and Treatment System (RETTS) including assessment of physiological parameters, i.e. oxygen saturation, respiratory rate, blood pressure, pulse, level of consciousness, and body temperature. The presence of an elevated respiratory rate is particularly important to mention, as it is both an early sign of sepsis and a predictor of severe disease with poor outcome [42].

**Common clinical symptoms**
Two recent Swedish retrospective studies [43, 44] focusing on the clinical presentation of the septic patient, have described several distinct, common symptoms, all with sudden onset. These are: fever, dyspnea, altered mental status, pain, pronounced weakness of muscles, and vomiting or diarrhea. The last three of these symptoms are not routinely considered in sepsis diagnostics. Thus, an increased awareness of them can potentially result in earlier identification of more sepsis patients.

**Symptoms of specific organ dysfunction**
Acute organ dysfunction most commonly affects the respiratory and cardiovascular systems, where respiratory symptoms are dyspnea and hypoxia, and cardiovascular manifestations are hypotension or an elevated serum lactate level. Acute kidney dysfunction is manifested as a decreasing urine output and an increasing serum creatinine level. An acute altered mental status is a sign of cerebral dysfunction, an elevation of serum bilirubin reflects hepatic dysfunction, and low platelets are a sign of failure in the coagulation system. Accordingly, these six organ functions (respiration, circulation, cerebral function, kidney function, liver function, and coagulation) are included in the SOFA score. Figure 1 illustrates those six organ systems, and how they can be monitored.
Organ dysfunction in septic shock
In patients with septic shock, the respiratory failure can progress to Adult Respiratory Distress Syndrome (ARDS) [46] with hypoxia and bilateral infiltrates of non-infectious, non-cardiac origin. The cardiovascular failure in septic shock patients is manifested as a persistent hypotension despite adequate volume expansion, necessitating use of vasopressors, and possibly myocardial dysfunction [39]. The elevated serum lactate seen in patients with septic shock is a sign of both circulatory failure and a persistent cellular and metabolic stress [47].

Sepsis management

General aspects
The Surviving Sepsis Campaign (SSC) was established in 2001 in connection with the sepsis definition conference, with the main goal to reduce mortality in sepsis. Since 2004, the campaign has published, and regularly updated, international guidelines for sepsis and septic shock; the latest guidelines are from 2016 [48] with a recent minor update from 2018 [49]. In the SSC guidelines from 2012 [50] the use of so called early goal directed therapy (EGDT) was emphasized, which included protocolized resuscitation with
quantitative endpoints, based on the results of the study by Rivers et al [51], in which EGDT showed a significant reduction in in-hospital mortality among patients with severe sepsis and septic shock, compared to standard therapy. Since then, three randomized trials [52-54] on patients with sepsis with shock or hypoperfusion, have compared EGDT to standard therapy regarding mortality. Together, these trials suggest that EGDT is safe, but not superior to, standard non-protocolized care. However, standard therapy has evolved over the last decade, including more aggressive fluid resuscitation, and rapid administration of appropriate antibiotics. The last update of the SSC guidelines still contains strong recommendations for standardized therapies, such as antibiotics, initial fluid volume, blood pressure goals and vasopressor choice, but the use of EGDT as a package protocol of care, with specific target endpoints is removed. Instead, the latest SSC guidelines emphasize frequent clinical reassessment, and patient-tailored therapy.

Sepsis management has two major components, managing infection and managing organ failure. Therapies for both these components must be tailored to the specific patient, depending on comorbidities, body weight, immune status, and other factors. In regard to managing the infection, the treatment must, in addition, be tailored to the causative pathogen, and the focus of infection.

The SSC guidelines latest update contain an “hour-1-bunde” with recommendations about the management during the first hour, with the following content:

- Measure lactate level. Re-measure if initial lactate is >2 mmol/L
- Obtain BCs prior to administration of antibiotics
- Administer broad-spectrum antibiotics
- Rapidly administer 30 mL/kg crystalloid for hypotension or lactate ≥4 mmol/L
- Apply vaspressors if patient is hypotensive during or after fluid resuscitation to maintain mean arterial pressure ≥ 65mmHg

There is evidence that each hour of delay in administration of appropriate antimicrobials is associated with an increase in mortality in sepsis and septic shock [9, 15, 48]. The choice of antibiotic agents must be made individually, based on several factors:
• Suspected focus of infection, taking into account the pathogens that are most likely to cause that type of infection, and antibiotic penetration at the site
• Prevalent pathogens and their resistance patterns within the community and hospital
• The patient’s immune status (neutropenia, splenectomy, poorly controlled human immunodeficiency virus [HIV] infection and other immunodeficiency diseases)
• The patient’s age and comorbidities including chronic illness (diabetes, liver or renal failure)

Concerning dosing strategies for optimal use of antibiotics, the SSC recommendations are vague, due to lack of evidence. According to pharmacokinetic/pharmacodynamic principles, the time above the minimal inhibitory concentration (MIC) could be optimized by continuous infusion of β-lactam antibiotics, but so far there is no convincing evidence that this reduces mortality in sepsis patients. A recent meta-analysis [55] showed an association between continuous β–lactam infusion and decreased in-hospital mortality in patients with severe sepsis, but only three studies with a total of 632 patients were included. Looking at patients with a higher level of disease severity and/or infected with less-susceptible pathogens such as Pseudomonas spp. and Acinetobacter spp, the body of evidence arguing for benefit from continuous infusion is more convincing [56].

Altogether, more knowledge is needed on how antibiotic treatment can be optimized to improve sepsis survival. Many studies on sepsis do not take the infectious etiology into account or are not powered for subgroup analyzes of the separate causative pathogens. Hopefully, this will change with the trend for more individually tailored sepsis care.

**Immuno-modelling therapies**

Many studies have been conducted to find therapies that can treat the sepsis patient’s dysregulated immune response. Since the first trial in 1982 [57] on an antiserum to endotoxin, numerous clinical trials have evaluated specific mediators of the immune response and plasmapheresis therapies in sepsis patients [58]. Only one drug, activated protein C, an anti-thrombotic and anti-inflammatory agent, has been licensed for treatment of sepsis [59]. Unfortunately, the drug was later found to be non-efficacious, and was removed from the market [60]. Research on sepsis therapies used to focus on
ways to block the hyperinflammation, but in recent years has endeavored to target the immunosuppression as well [61]. With recent insights into the complexity and heterogeneity of sepsis, ongoing and upcoming clinical trials will hopefully bring us new therapeutic agents. Most probably, these therapies will be tailored for sub-groups of septic patients, and etiology and immune status will be taken into account.

Today, in Sweden, we use two agents in sepsis treatment which target the host’s response, not the pathogen. These are intravenous immunoglobulin, for treatment of necrotizing fasciitis and toxic shock syndrome caused by *Streptococcus pyogenes* [62], and corticosteroids, for treatment of refractory septic shock [63]. They are both examples of agents that might be beneficial for a subset of sepsis patients, even though they have been non- efficacious when tested on unselected sepsis patients.

**Sepsis pathophysiology**
Sepsis pathogenesis is driven by a combination of factors related both to the invading pathogen and the host. The host response involves many concomitant and opposing mechanisms of both pro-inflammatory and anti-inflammatory type.

**Pathogen factors**
The “Germ theory”, stating that many diseases are caused by microorganisms, was presented by Louis Pasteur to the French Academy of Medicine in 1878. He also demonstrated that *Streptococcus* spp. could cause puerperal sepsis [1]. Pasteur, Semmelweiss, Coch and other 19th century scientists described an early pathophysiological theory for sepsis, illustrated by the new term “blood poisoning”. This was described as a condition in which microorganisms had invaded the bloodstream and released toxins that caused systemic symptoms, shock and death [64]. Today we know more about the virulence factors, and their impact on the immune system in sepsis. Many pathogens share common mechanisms in terms of ability to adhere, invade, and cause damage to host cells and tissues, as well as to survive host defenses and establish infection [65].

- **Capsules:** Some bacteria have capsules that protect them from phagocytosis by macrophages and neutrophils. The capsule causes indirect enhancement of the inflammatory response, as the macrophages and neutrophils produce more inflammatory cytokines in an attempt to clear the bacteria [66].
• **Toxins:** Many bacteria produce toxins, substances which purpose is to destroy or damage the host cell. Endotoxins are produced by Gram-negative bacteria; they are also known as lipopolysaccharides (LPS). Exotoxins are enzymes that can be secreted by the pathogen into the surrounding milieu or injected into host cell cytoplasm. Examples of exotoxins are A and B toxins (found in *Pseudomonas aeruginosa*, *E. coli*, *Vibrio cholerae*, *Corynebacterium diphtheriae* and *Bordetella pertussis*) proteolytic exotoxins (found in *Clostridium botulinum* and tetani) and superantigens (found in *S. aureus* and *S. pyogenes*). Superantigens can induce non-specific activation of T-cells and massive cytokine release, which can result in sepsis and toxic shock syndrome [65].

• **Adhesins:** A key step in the host-pathogen interaction is adherence of the pathogen to host surfaces. This is facilitated by adhesion factors, adhesins, which are expressed by all bacteria, and can be made from polypeptides or polysaccharides [65].

• **PAMPs:** The microbes express several genetically conserved signature molecules called “pathogen-associated molecular patterns” (PAMPs) which interact with immune cells and are particularly important in the pathogenesis of sepsis [67]. Examples of PAMPs include LPS, and flagellin in Gram-negative bacteria, and lipoteichoic acid and peptidoglycan in the Gram-positive bacterial cell wall. Bacterial DNA from both Gram-positive and Gram-negative bacteria has been found to interact with immune cells and are considered to be PAMPs [61, 68]. PAMPs can serve the pathogen in different ways, such as toxins or adhesins [69].

PAMPs can be recognized by the innate immune system’s pattern recognition receptors (PRRs). The recognition of PAMPs by PRRs will cause a series of intracellular signaling cascades, resulting in induction of transcription factors, which will upregulate the expression of pro-inflammatory and anti-inflammatory cytokines [69].

Microbes show great diversity concerning virulence factors, triggering the sepsis syndrome in different ways depending on the microorganism. There is evidence that Gram-positive bacteria affect the host’s response differently to Gram-negative organisms [70, 71]. The most recognized explanations for this are differences in the cell wall structure, where Gram-negatives contain
LPS and Gram-positives a thick layer of peptidoglycan, as well as the production of powerful exotoxins in certain Gram-positive pathogens [23]. While Gram-negative sepsis seems to be associated with higher mortality than Gram-positive sepsis in first-hit (primary) infections, an inverse relationship between Gram-status and mortality has been observed in second-hit infections [23]. The underlying reason for this is multi-factorial, but differences in virulence factors and their influences, both hyperinflammatory and immunosuppressive, on the host’s immune response, are certainly important. Numerous studies performed in vivo on mice, or in vitro, have focused on virulence factors in different bacteria, such as *P. aeruginosa*, *S. aureus* and *S. pneumoniae*. A myriad of virulence expressions have been described, resulting in both hyperinflammation and immunosuppression, often in a dose-dependent manner [72, 73]. However, in most clinical studies performed on sepsis immunology, the etiology has not been taken into account [74].

The higher the pathogen load, the more virulence factors will be excreted from infectious microorganisms, such as toxins and PAMPs, inducing the sepsis immune response [61]. There are convincing data that the microbial load plays an important role for disease severity in sepsis. Studies in this field were performed already in the 1950s, when Hall and Gold found, in a study of 35 patients with septic shock, that all six patients with a bacterial count >100 colony forming units (CFU)/mL blood died, while only 41% of those with lower counts died [75]. Larger studies during the following decades (60s-70s) thereafter confirmed this association [76]. At this time, quantitative BCs were still performed, using the techniques of pour plate (blood and molten agar were mixed) or spread plate (blood was spread over the surface of an agar plate) [76]. As the quantification process was both cumbersome and of limited use, this routine was discarded by most laboratories when automatic BC systems were introduced. In the 90s, BC devices were introduced that measured the time between incubation onset and growth detection - the time to positivity (TTP) [77]. A link between the TTP and the microbial inoculum has been confirmed by measurements of clinical isolates of several microorganisms in different concentrations [77], and can consequently be used as a surrogate marker for the microbial load in blood. Several studies have reported an increasing risk of mortality in sepsis and septic shock in *S. aureus*, *S. pneumoniae*, *E. coli* and *Klebsiella pneumoniae* BSI, with shorter TTP of BCs [78-82]. However, variables other than the pathogen load influence the TTP, such as the volume of cultured blood, the
time between sampling and start of incubation, individual pathogen factors, presence of multiple pathogens and prior administration of antibiotics.

The introduction of molecular methods such as polymerase chain reaction (PCR) has opened up new possibilities in BSI diagnostics, including quantification. Now, several studies have reported an association between the bacterial DNA load in blood and sepsis severity and mortality in BSI caused by several different pathogens, such as *Neisseria meningitidis*, *S. pneumoniae* and *S. aureus* [83-86]. The rate of bacterial clearance appears to be another important factor, but is less studied. Concerning *S. aureus* bacteremia (SAB) it is well known that persistent bacteremia is associated with complicated infection and risk of mortality [87], and therefore follow-up BCs are recommended in international guidelines [88]. There are a number of reports concluding that a sustained short TTP in follow-up BCs is associated with complicated infections and risk of death [89, 90], and one study shows that slow clearance of bacterial DNA in blood is associated with increased mortality in patients with methicillin resistant *S. aureus* (MRSA) bacteremia. Apart from SAB, the bacterial clearance in BSI is poorly studied from a clinical point of view. Two studies, published by Chuang et al [91, 92] have described the bacterial clearance rate in BSI caused by *Acinetobacter baumannii*, a pathogen that is associated with multiresistance and high mortality in sepsis. The studies showed that slow clearance of bacterial DNA was related to poor outcome, and also independently associated to both immunosuppressive treatment and inappropriate antibiotic treatment.

The role of the microbial load at the site of infection, and in the tissues of end organs in sepsis, is only sparsely studied. In studies on mice [93, 94] with induced pneumonia and intestinal infection, high loads of bacteria in lungs, spleen, liver, heart and kidney, as well as high loads of inflammatory cytokines at the same sites, have been related to sepsis severity and death.

**Host factors**

The arrival of modern antibiotics in the 1930s [1] opened up future prospects whereby doctors had a tool that could completely cure their patients of deadly infectious diseases. Indeed, survival dramatically increased [95], but despite the use of effective antibiotics many sepsis patients continued to die. Thus, the hypothesis that host factors were important in sepsis pathogenesis grew and, ever since, much research has concentrated on the host’s immunological response in sepsis.
The first theory was that the sepsis syndrome was caused by a profuse and un-controlled inflammation. This assumption was based on animal studies where infusion of large amounts of bacteria resulted in release of inflammatory mediators, such as tumor necrosis factor (TNF) α and interleukin (IL) 1 [96, 97]. Later, this theory developed to include a compensatoryanti-inflammatory phase after the initial hyperinflammation [98]. Now we know that the host response in sepsis involves both hyperinflammatory and immunosuppressive processes that occur both subsequently and simultaneously [67]. The innate immune system appears to induce most hyperinflammatory responses, while the adaptive immune responses are characterized by immunosuppression.

Upon detection of an invading pathogen, the host innate immune cells are activated. The recognition is made by PRRs, of which four classes have been identified: toll-like receptors (TLR), C-type lectin receptors, retinoic acid inducible gene1-like receptors, and nucleotide-binding oligomerization domain-like receptors. The PRRs recognize PAMPs from the pathogen, but also damage-associated molecular patterns (DAMPs) [69]. DAMPs, or alarmins, are endogenous molecules released from injured or stressed cells, such as human DNA (nuclear DNA and mitochondrial DNA), heat-shock proteins, high-mobility group protein B1, and S100 proteins. DAMPs are also released during sterile cell injury, such as burns or trauma [67, 99]. The PRRs initiate transcription of type I interferons and cytokines such as TNF-α, IL-1, and IL-6 [61]. Some of these PPRs can assemble into molecular complexes termed inflammasomes [100], which are important for the secretion of cytokines IL-1β and IL-18, and which can induce a profuse hyperinflammation and programmed cell death. This inflammatory cytokine response gives rise to the typical signs of infection, such as fever, and triggers processes to achieve infection control. This includes:

- A rapid and abundant release of innate immune cells, such as neutrophils and macrophages, with a prolonged lifespan. Macrophages act as phagocytes and can, after phagocytosis of the pathogen, secrete IL-12, which activates natural killer cells that kill host cells infected with intracellular pathogens [101]. Neutrophils have several functions; they are phagocytes, they can secrete soluble antimicrobials from their granules, and release neutrophil extracellular traps (NET), that trap bacterial DNA [102].
• Induction of hepatic acute phase proteins, such as C-reactive protein (CRP) [101].

• A release of complement factors, in particular C5a, a potent inflammatory peptide, important for reactive oxygen species production and chemotactic responses [61, 67].

• Activation of the coagulation system. This is induced by several factors, such as upregulation of tissue factor expression, release of NETs, and depression of the fibrinolytic system [67].

• A release of acetylcholine, through activation of the vagus nerve, leading to suppression of proinflammatory cytokines [61].

When this immune response exceeds a certain threshold it will cause a systemic dysregulation, with damage of cellular proteins, lipids, and DNA, impaired mitochondrial function, disseminated intravascular coagulation (DIC) and endothelial hyperpermeability, as possible consequences. In Figure 2 the innate immune response to an invading pathogen is illustrated.
The adaptive immune system consists of specialized cells that are antigen specific and can generate a memory. The adaptive response is triggered by the recognition of antigens from phagocytized pathogens, which are presented to CD 4+ T helper (Th) cells by the major histocompatibility complex (MHC) class II molecules (also known as human leucocyte antigen, HLA). MHC class II molecules are present in dendritic cells, B-cells, monocytes and macrophages, in this context termed antigen presenting cells (APC). CD 4+ Th cells control other cells and help them to clear infection, but cannot kill infected cells themselves [103]. After antigen presentation, two types of Th cell responses can be induced to eliminate different types of pathogens,

*Figure 2. Host response to sepsis. Adapted from Van der Poll et al [61] with permission from Elsevier*
Th 1 and Th 2. In general, Th 1 cells act in response to antigens from intra-
cellular bacteria, and Th 2 cells to extracellular or parasite antigens, even
though the responses overlap. The Th 1 response is characterized by the
production of Interferon-gamma, which activates bactericidal activities of
macrophages, and induces B cells to produce antibodies.

The Th 2 response is characterized by the release of IL 4 and 5, which
helps B cells to differentiate to plasma cells and to release eosinophils [103].

In sepsis, the T cell functions are deeply dysregulated, demonstrating cel-

tuar death, exhaustion and hyporesponsiveness [104]. The Th 1 cells and
MHC class II (HLA-DR) molecules on APCs are suppressed by anti-inflam-

matory IL-10, produced by regulatory T cells and Th2 cells which leads to
a suppressed T cell response, with predominance of Th 2. Exhausted T cells
have an upregulation of inhibitory receptors, such as programmed cell death
1 ligand, inducing apoptosis [105]. The result is a marked lymphopenia,
which sometimes can be persistent, and is then associated with a higher in-
cidence of secondary infections and risk of death [106].

**Persistent inflammation-immunosuppression and catabolism syndrome**

A new term has been proposed for patients who survive the initial sepsis but
remain critically ill, “persistent inflammation-immunosuppression and ca-
tabolism syndrome” (PICS) [107, 108]. Possibly this condition is induced
by sustained exposure to PAMPs and DAMPs. In the definition of this syn-
drome, elevated CRP and neutrophil count are used as markers for persis-
tent inflammation, a decreased lymphocyte count is used as a marker for
immunosuppression, and a low albumin level as a marker for catabolism
[109].

**Diagnostic methods to find infectious etiology in sepsis**

Administration of adequate antibiotics is crucial for sepsis survival, and in
order to counteract antibiotic resistance it is important to perform de-esca-
lation, once the etiology has been established. Consequently, diagnostic ef-
forts are of utmost importance, in order to early identify the causative path-
ogen, and the site of infection. Culture is traditionally the diagnostic method
for pathogen identification and should be done before administration of an-
tibiotics. As BSI is common in sepsis, the bloodstream is the most important
culture site, but cultures should be obtained from all suspected sites of in-
fection, as long as the culture process does not substantially delay admin-
istration of antibiotics.
Today, molecular techniques have been developed as a complement to culture. At our hospital in Örebro, Sweden, we use molecular diagnostic tests on cerebrospinal fluid (CSF) in meningitis diagnostics, on feces for diagnosis of gastro-intestinal infections, and on respiratory secretions for detection of viral respiratory tract infections. We do not routinely perform molecular diagnostic tests directly on blood, but we perform molecular identification with Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) on blood from BC bottles that signal positive.

**Bloodstream infection**

Bacteremia is usually defined by growth of bacteria in BC. In Denmark, the overall annual incidence of bacteremia has been found to be 114–166 episodes per 100,000 person-years [110], and this figure has increased considerably in recent years.

Bacteremia can sometimes occur transiently without symptoms of infection. BSI is an infectious disease where bacteria or candida is detected in blood, even though the primary focus can be located elsewhere. Not all patients with BSI have sepsis, and not all sepsis patients have BSI. In addition, because BC lacks sensitivity, especially for slow-growing and fastidious organisms and when antibiotics have been given prior to culture [111, 112], BSI can be falsely BC negative.

*S. aureus*, coagulase-negative staphylococci (CoNS) and *Enterococcus* spp. are reported to be the Gram-positive bacteria most frequently found in BC, and *E. coli*, *K. pneumoniae* and *Pseudomonas* spp. the most frequently found Gram-negatives [26]. Although Gram-negative bacteria used to be the most common finding in BC, Gram-positive BSI outnumbered Gram-negative BSI at the end of the 20th century [113]. Thereafter, there has been another shift, at least among ICU-treated patients, where a large, multi-center study involving 75 countries has shown that Gram-negative bacteria represent a majority of BC findings [21]. This study also revealed a trend with an increasing number of BCs positive for candida.

The most prevalent pathogens in community-onset BSI are *S. aureus*, *S. pneumoniae* and *E. coli* [110, 114, 115].
Blood culture

BC is still the gold standard in BSI diagnostics, despite several limitations such as a long time to result and suboptimal sensitivity and specificity. False-negative results can be due to a low quantity of microbes in the blood, administration of antibiotics prior to culture, or presence of a fastidious, slow-growing or non-culturable pathogen. False-positive results are most often due to contamination from the skin flora.

Adequate volume sampling is the most important parameter for best sensitivity, as the bacterial concentration in blood is very low in most patients with BSI. Several reports, most written by the time quantitative BCs were routinely performed, have stated that 50% of BSI episodes are associated with a bacterial concentration of about only 0.01–1 CFU/mL [116-118]. An association between the volume of blood cultured and the positivity rate has been reported from several studies [119-121]. The current guidelines [116] recommend 4 bottles, divided into 2 sets, with paired aerobic and anaerobic bottles, corresponding to 20-30 mL blood per set. There is no evidence that the timing of specimen collection for BC, depending on body temperature or presence of chills, affects the positivity rate [122]. Even though there is a widespread recommendation to draw the different BC sets from separate puncture sites and at separate timepoints, it is unclear if this practice is better than collecting the total volume of blood in one single draw (105). The arguments for a multi-sampling practice are that bacteremia might be intermittent, and that separate samples may help to discriminate contaminants from pathogens. However, most cases of clinically significant BSI seem to be associated with continuous bacteremia, but with low concentrations of circulating microorganisms [121, 123]. For the interpretation of BC results positive for microbes to commonly represent contamination, in particular CoNS, it is not clear which practice, single or multiple sampling, is preferable [124, 125]. Advantages with a single sampling practice are potentially shorter time to antibiotics, a higher chance that the second BC set actually will be drawn, and less inconvenience for both patient and healthcare workers. One randomized study by Dargère et al [125] has compared the two sampling strategies and found single sampling superior regarding positivity rate and risk of contamination. However, the sample size was limited with 300 BC positive patients, and more studies in this field would be welcome.

BC devices report TTP for all positive samples, and this information can be used as a surrogate marker for the microbial load [77]. The TTP can help to distinguish CoNS bacteremia from contamination [126], and can be used
in the diagnostics of catheter-related BSI, by calculation of the differential TTP [127].

**Molecular methods**

Molecular methods, including PCR, microarray and nucleic acid sequencing now have an important place in clinical laboratories. Nevertheless, even though this is a field undergoing rapid development, the applications routinely used for BSI diagnostics are still limited.

Molecular methods can shorten the time to result depending on the method and local laboratory routines. In some situations, when BC has low sensitivity, for example when the pathogen is difficult to grow, or when antibiotics have been given prior to culture, molecular identification is a valuable alternative. The low detection limit can potentially make molecular methods more sensitive than BC but, on the other hand, sensitivity is negatively affected by the low sample volume in molecular tests. The possibility of quantification is an important advantage with molecular diagnostics in BSI, and can be useful for several reasons:

- For interpretation of findings of suspected contaminants, as contamination bacteria are generally found in very low amounts.
- For determination of the relative abundance of each pathogen in cases of polymicrobial infections
- In sepsis management for indication of infection severity and response to treatment [84, 86, 92].

Drawbacks with molecular techniques are lack in sensitivity in some cases (depending on method, pathogen and specimen), risk of false-positive results (due to contamination or detection of non-viable or not clinically relevant DNA), no possibility of phenotypic resistance testing, and that they often are expensive and laboratory-intensive. Most molecular diagnostic tests are pathogen-specific or contain a limited multiplex panel, which is a limitation in BSI diagnostics, where the etiologic question most often is broad.

**Polymerase chain reaction**

Among molecular methods developed for amplification of nucleic acids, PCR is the first, and the most ground-breaking technique, now indispensable in laboratories worldwide. The first paper on the PCR technique was published in 1985 by Saiki et al [128], and described a new method for
amplification of beta-globin genomic sequences for diagnosis of sickle cell anemia. Kary Mullis, co-writer in this article, was the chemist who first, in 1983, developed and demonstrated the technique, and in 1993 he received the Nobel Prize in chemistry for the innovation of PCR.

Using PCR, any specific DNA sequence present in a sample can be targeted and amplified in a cyclic process to a large number of identical copies. In brief, the template, that contains the DNA target, is mixed with primers (two short DNA sequences designed to bind to the start and end of the DNA target), free nucleotides, and the DNA polymerase enzyme. Thereafter, the mixture is heated to denature the double-stranded DNA template into single strands. Then, the mixture is cooled so that the primers can anneal to the DNA template. At this point, the DNA polymerase begins to synthesize new strands of DNA, starting from the primers. At the end of this first cycle, each double-stranded DNA molecule consists of one new and one old DNA strand that have hybridized. These newly synthesized DNA sequences serve as templates in later cycles, and the DNA target is thus exponentially amplified. In Figure 3, the principle of PCR is schematically illustrated. There are numerous PCR systems on the market, performing this process in automatic, programmed steps (128).

**Polymerase chain reaction - PCR**

![Polymerase chain reaction diagram](image)

1. Denaturation at 94-96°C
2. Annealing at ~68°C
3. Elongation at ca. 72 °C

*Figure 3. Schematic drawing of the first cycles of a polymerase chain reaction. Source: Enzoclop/Wikimediacommons/public domain*
Quantitative PCR

Quantitative PCR (qPCR), also known as real-time PCR, or second-generation PCR, monitors the amplification of the target DNA in real-time, compared to conventional PCR, where the result cannot be seen until the end of the process. For real-time monitoring, a fluorescent reporter is used, which binds to the product formed and reports its presence by fluorescence. This can be done in two ways using unspecific dyes or specific probes.

Fluorescent dyes will bind to all double-stranded DNA. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity measured for each cycle. The drawback with dyes is that the DNA binding is unspecific [129] and therefore only singleplex testing is possible with the technique. There is also a risk of interference between the intended target sequence and an unwanted non-specific PCR product.

Probes detect only the DNA containing the sequence complementary to the probe, which increases method specificity. In multiplex qPCR, when several target sequences are monitored in the same assays, probes are used as they can be designed with different colored labels.

The most commonly used probes are hydrolysis probes, also called Taq-Man probes. These probes have two fluorescent units, a reporter at one end and a quencher at the opposite end. As long as the reporter and the quencher are close to each other, no fluorescence is emitted, but if they are separated the fluorescence emission starts. During PCR, both probe and primers will anneal to the DNA target. Synthesis of a new DNA strand are initiated from the primers, and when the polymerase reaches the probe, its 5’-3’-exonuclease degrades the probe, separating the fluorescent reporter from the quencher and resulting in an increase in fluorescence. Probes based on a single fluorescent unit, whose fluorescence changes upon binding target DNA are also available [129]. Figure 4 illustrates a qPCR where TaqMan probes are used for real-time monitoring.
Quantification cycle
The detected fluorescence is measured in a real-time PCR machine, and the amount of fluorescence produced will rise exponentially for each PCR cycle. The quantification cycle (Cq) occurs when the PCR amplification curve meets a predefined threshold, which depends on how many cycles were run before the threshold was reached. The Cq indicates the same thing as the cycle threshold (Ct) and the crossing point (Cp), which are expressions used by different manufacturers of real-time PCR machines but, according to guidelines (The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments) [131], the term Cq should be used. The Cq contains quantitative information; the lower the Cq, the more target DNA in the original template. However, to get an absolute quantification, the result must be compared to a standard curve, where the qPCR
has been performed on samples in serial dilutions with known concentration of the target sequence.

**Digital PCR**
The third generation of PCR is digital PCR (dPCR) [132]; a method developed from qPCR, but with the possibility of absolute quantification without the use of standard curves. The principle is the same as in qPCR, but in dPCR the PCR mixture is divided into numerous separate portions and the PCR is run on each of them individually. After PCR, the samples are checked for fluorescence using a binary readout of 0 or 1, and is the reason it is called “digital”. The fraction of fluorescing droplets is recorded, and the absolute target DNA template concentration is calculated by using Poisson distribution statistics for the correction of more than one positive reaction in the same droplet.

**Droplet digital PCR**
Droplet digital PCR (ddPCR) has become the most widespread application of dPCR. ddPCR uses a water-and-oil emulsion technique, where the target template is divided into about 15,000 nanoliter-sized water-and-oil droplets by a droplet-generator [133]. Compared to qPCR, ddPCR has demonstrated a greater quantification precision and a higher reproducibility, with a comparable sensitivity [134].

**Target genes for molecular identification of bacteria and fungi**
Bacteria are prokaryotes, one-celled organisms that lack membrane-bound organelles, such as a nucleus and mitochondria. Instead, bacterial DNA resides inside the bacterial cytoplasm, where the processes of translation, transcription and DNA replication, as well as interaction with ribosomes, occur. Most DNA are organized into one circular chromosome, although some examples of linear DNA exist [135].

Together with the chromosomal DNA, most bacteria contain plasmids, which are extrachromosomal circular DNA structures that can replicate independently [135]. In Figure 5, the structure of the bacterial cell is schematically illustrated.
Genus- or species-specific chromosomal genes are used as targets for the design of suitable, specific primers for detection of different bacteria. Some genes encoding for antibiotic resistance are plasmid-mediated, and is the reason plasmid genes can be targeted for genotypic detection of certain resistance genes, such as many carbapenemase genes [136].

In addition to the chromosome and plasmids, the bacterial cytoplasm contains numerous ribosomes, in which protein synthesis take place. The ribosome consists of ribosomal RNA (rRNA) and ribosomal proteins. All prokaryotes have 70S ribosomes, each consisting of a small (30S) and a large (50S) subunit. The large 50S subunit contains two rRNA molecules, 5S RNA and 23S RNA. The small 30S subunit contains the 16S rRNA molecule [135].

The genes encoding for the 5S, 16S, and 23S rRNAs are located together on the chromosome, organized into an rRNA operon, as illustrated in Figure 6. The 16S rRNA gene, or 16S rDNA, is the section of the rRNA operon with genes encoding for the 16S rRNA molecule. The 16S rDNA is universally present in all bacteria and has conserve regions, which are suitable targets for amplification by PCR, as well as variable regions, which enables subsequent characterization by DNA sequencing. As a result, this gene has
become the most important target in phylogenetic studies in bacterial ecology. In addition, 16S rDNA sequencing plays an important role in medical microbiology in the diagnostics of elusive infections where a broad-range approach is needed [135].

![Diagram of the ribosomal RNA operon](image)

*Figure 6. The ribosomal RNA operon. Sizes of the genes for 16 S, 23 S, 5 S rRNA and the internal transcribed spacer (ITS) region are indicated in nucleotide base pairs (bp). Adapted from Mims’ Medical Microbiology [135] with permission from Elsevier*

The internal transcribed spacer (ITS) region is a spacer DNA situated between the genes encoding for the 23SrRNA and the 16SrDNA in prokaryotes, and is another gene used as a universal target. ITS has both highly conserved sequences, and sequences with a high degree of variation, even between closely related species [135].

In contrast to other chromosomal genes, the rRNA operon appears in multiple copies in each bacterial genome, from 1 to 15. Copy numbers can vary between bacteria from the same family and genus, but are most often conserved at the species level [137].

One explanation for why these genes are multiple, is that their products (rRNA, responsible for the protein synthesis) are needed in higher concentrations than the products of the other single copy genes. In addition, the rRNA operon copy number/bacterial genome has been correlated to growth conditions, where oligotrophic bacteria tend to have a low rDNA copy number [138].

The “rRNA operon copy number database” [139] is based on large data of sequenced genomes, and is a publicly available, searchable tool, for copy number information for specific bacteria and archaeb. Figure 7 illustrates the variability in 16S rDNA copy numbers in bacterial genomes, and Table 2 lists the 16S rDNA copy number/genome in selected bacteria.
Figure 7. Bacterial phylogeny with genomic 16S rDNA copy number indicated with black bars (determined using the rRNA operon copy number database [139], and taxonomic order indicated with color shading of branches. Adapted from Kembel et al [140] and reprinted in accordance with the Creative Commons Attribution (CC-BY) licence.

Table 2. 16S rDNA copy numbers/genome in a selection of bacterial species, according to the rRNA operon copy number database [139]

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Number of 16S rDNA/genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>12-14</td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td>9-11</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>7</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>6</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>6</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>6</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>5-6</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5-6</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>4-6</td>
</tr>
<tr>
<td>Neisseria meningitides</td>
<td>4</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>4</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>3</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>1</td>
</tr>
<tr>
<td>Rickettsia rickettsii</td>
<td>1</td>
</tr>
</tbody>
</table>
Fungi are eukaryotes that have 80S ribosomes, consisting of a large (60S) and a small (40S) subunit, inside which a total of four rRNA molecules are found. The 18S rRNA gene, encoding the 18S RNA in the small subunit, is frequently used as a target for universal fungal-specific primers, as well as the ITS region of the fungal rDNA.

**Molecular diagnostic methods in bloodstream infection**

**Blood as a specimen in molecular diagnostics**
When optimizing molecular techniques for application on blood, several specific factors must be taken into account. The main components in blood that affect molecular approaches are illustrated in Figure 8.

*Figure 8. Blood as a sample for microbial diagnosis during bloodstream infection. The main components of the blood that affect the molecular diagnosis are represented. Adapted from Opota et al [141] with permission from Elsevier.*

**INGRID ZIEGLER**  *Quantitative detection of bacterial DNA in whole blood*
Factors that complicate molecular BSI diagnostic areas:

- The high quantity of human DNA, leading to unspecific primer-binding during PCR
- PCR inhibitors present in blood, such as hemoglobin, lactoferrin and immunoglobulins [142]
- DNA from both dead and viable microorganisms can be detected
- The low number of circulating microorganisms in blood during BSI [117, 118]

To overcome these issues, different DNA extraction strategies are used prior to PCR or other molecular assay. Many different commercial kits are available for this purpose. For degradation and removal of human DNA and PCR inhibitors, the template can be pre-treated with special buffers that lyse the human cells and inhibitors. Reagents in this process may include salts, proteases, and phenol-chloroform. Magnetic bead technology can be used to bind and extract target DNA. Mechanical treatment, such as dilution, centrifugation, filtration and washing is used to produce a highly concentrated and pure final sample [143]. Thereafter, another buffer treatment for lysis of pathogen cells can be added to isolate free microbial DNA.

In order to extract live microorganisms and not dead ones, some DNA extraction kits use buffers that not only degrade human cells, but also lysed bacterial cells and free DNA. Such methods can reduce the DNA from non-viable microbes, but probably only partly [144].

The hardest issue to overcome is the low number of microbes in blood. In regard to BC, the evidence can be summarized in: “the higher the volume of blood cultured, the higher the yield” [116]. Plausibly, this can be applied for molecular diagnostics as well, but the maximal sample volume for DNA extraction devices is limited, most often to 1-2 mL. Recently, several companies have developed DNA extraction methods where an initial volume of 5-10 mL of whole blood can be used for pathogen DNA isolation. Among the commercially launched molecular assays for pathogen identification directly from whole blood, those using an initial blood volume of 5 mL, have shown better sensitivities than methods starting with lower volumes [144-146].

Enrichment of bacteria before molecular identification, such as identification from positive BC broth, is another way to bypass the problem of low concentrations of microbes in blood.
Molecular methods from positive BC bottles
There are several molecular methods using positive BC broth for BSI diagnostics [147], whereof the most commonly used is the MALDI-TOF MS. Nowadays, this is integrated in the workflow of many clinical laboratories, including ours. The method can be used for identification of isolated pure colonies, following culture on agar plate, or directly from specimen, such as positive BC broth. The technology includes an ionization method, where excitation by laser catalyzes charge transfer from matrix to analyte, which releases ions. Detection is made by mass analysis of the time of flight of each ion species and, from this data, an output with the spectral profile based on the mass-to-charge ratio of all proteins in the specimen, is produced. Comparing this spectral profile with a reference spectral library its identity can be determined. This approach demonstrates excellent sensitivities, but the reduction in time to result compared to BC is limited. However, the implementation of MALDI-TOF MS systems has significantly shortened the time to identification of BSI pathogens and, recently, this reduction has been described as leading to significantly shorter time to adequate antibiotic treatment [148, 149].

Molecular methods directly from whole blood
Specific singleplex PCRs targeting bacterial pathogens have been developed for use on whole blood, mostly for identification of fastidious organisms causing BC negative endocarditis such as Bartonella spp. and Coxiella spp. [150, 151]. However, in BSI diagnostics the etiologic question is most often broad and is the reason the diagnostic tools need to be designed to detect more than one organism. To meet this demand, several commercial molecular tests for BSI diagnostics have been developed with multiplex design or with broad-range identification after DNA amplification. These technologies have the potential to both shorten the time to result and to improve the possibilities for microbial diagnosis in BSI, but the implementation process has been slow. The diagnostic value in relation to costs has so far been questionable, due to insufficient sensitivities. The bacterial concentration in blood during BSI can be extremely low [116], and even though the pathogen DNA load is reported to be higher [152], it is probably sometimes below the limit of detection for most molecular methods, especially when small samples volumes of 1-2 mL are used.

In addition, it is difficult to evaluate the performance of the tests, when BC, the method used as reference standard, has insufficient sensitivity and
specificity itself. Consequently, the literature evaluating the tests is heterogeneous, and mostly observational [153]. Study populations with varying risks for BSI are used, including neonates, ICU-treated sepsis patients, patients with hematological malignancies and patients in the Emergency Department. In some studies, BC is used as comparison standard for the statistical analyses, but in some studies a combination of culture findings and clinical data is used. Some studies include all culture and molecular findings in the statistical analyses, while others exclude all findings with suspected contamination bacteria. A few studies have been performed on PCR-guided therapy, most of them retrospectively, where changes in antibiotic therapies due to PCR results are registered [154, 155]. Even if PCR results clearly affected the choice of antibiotics and frequently led to changes in administration of antifungal agents, there is no data on outcome after treatment adjustment in these studies. To enable a well-grounded evaluation about the value of PCR diagnostics in whole blood in BSI, randomized intervention studies investigating the clinical impact and outcome would be welcome.

**Commercial tests**

Several molecular tests for detection of BSI-causing pathogens directly on whole blood have been developed, some with a multiplex technology and some using broad-range identification [147]. None of the assays reports quantitative results, even if quantitative data, such as Cq values, are sometimes available.

The Lightcycler SeptiFast (Roche Molecular System, BASEL, Switzerland) is a real-time PCR system that can identify 19 pathogens (eight Gram-negative bacteria, six Gram-positive bacteria and five fungi) and the *mecA* gene (associated with MRSA) present in blood samples, within 5 to 8 hours. SeptiFast was launched in 2004 as the first commercial test in this field, and by now has been well-investigated in clinical studies. The studies have included different pools of patients and gold standards for the statistical analyses, and are the reasons for the wide variation in the results on sensitivity and specificity. In a meta-analysis Dark et al [156] concluded that the SeptiFast test seemed to have better specificity than sensitivity, but with the reservation that the studies included had important deficiencies. The low clinical sensitivity (43-70%), despite high analytic sensitivity, is probably mainly due to the low sample input-volume, 1.5 mL. The SeptiFast identification method targets the ITS region within the rRNA operon with species-
specific probes. SeptiFast performs a quantitative analysis, but the test result is only qualitative, as Cq values are not available to the user.

The Magicplex Sepsis Real-time Test (Seegene, Seoul, Korea) is a PCR method that can identify more than 90 pathogens at the genus level, including 25 pathogens (19 bacteria and 6 fungi) at the species level, as well as three resistance genes (mecA, vanA and vanB). It is a three-step procedure, where pathogen DNA is first amplified by conventional PCR, and thereafter the pathogen identification is performed by qPCR to genus level in a second step, and then to species level in a third step. The input volume is 1 mL and the time to result is 3 to 5 hours. The method has only sparsely been evaluated in clinical studies with sensitivity ranging from 37-65%, and specificity between 77-92% [157, 158]. It is not commercialized as a quantitative method, but in the second and third steps Cq values are available as indirect information about the DNA load.

The Vyoo system (Analytik, Jena, Germany) can detect 34 bacteria and 7 fungi to species level, and 5 resistance genes. The input sample volume is 5 mL and the turnaround time 8 hours. The technique is based on DNA amplification using multiple PCRs targeting 16s rDNA, followed by electrophoretic separation of target-specific amplicons. The performance of the VYOO test has been evaluated in a few studies [159, 160] where the sensitivities to detect culture-positive BSI were 60-70%.

The T2 magnetic resonance (M2) system, (T2 Biosystems, Lexington, MA, USA) including the T2Candida and the T2Bacteria panels, is a fully automated technology for detection of 5 species of candida and 6 species of bacteria, respectively, and the most recent contribution to the BSI diagnostic field. The technology amplifies the DNA by using a thermostable polymerase and primers, targeting the candida-universal ITS region in the T2Candida test, and targeting Enterococcus faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa and E. coli species specifically in the T2Bacteria test. Thereafter, T2MR detection of the amplified product is performed by amplicon-induced agglomeration of super-magnetic particles, through the binding of attached species-specific probes [161]. The input sample volume is 5 mL and the turnaround time is 4-6 hours.

Despite its novelty there are already several reports evaluating the method, in particular the T2Candida, which also is the first and only FDA-cleared sepsis pathogen detection panel for use direct on blood. The diagnostic performance is stated superior to BC and serum β-glucan, and in two
trials the sensitivity and specificity for candidemia were 90% and 98%, respectively [161, 162]. There are also studies evaluating the clinical impact after implementation of T2Candida, showing that the method reduces time to appropriate anti-fungal therapy, shortens courses of empirical therapy, and save costs in antifungals [163, 164].

One study, from a hospital in Rome, has been published evaluating the T2Bacteria panel [165]. It reports sensitivity and specificity of 89% and 98%, respectively, for detection of BSI caused by bacteria included in the panel, confirmed by BC or fulfilling criteria for infection. The pathogens detected by the T2Bacteria panel represent problematic, often multi-resistant bacteria [166] that can cause BSI, why a rapid diagnosis is particularly important.

The performance of the T2MR system is promising, and especially the T2Candida panel seems to find a place in clinical diagnostics. The drawback of the panels is that they detect only a limited number of pathogens, and many common BSI causing microorganisms are not included.

The **SepsitTest** (Molzym, Bremen, Germany) is a qPCR system with universal primers targeting the 16S (bacteria) and 18S (fungi) rDNA. The amplified product is sequenced by Sanger sequencing methodology and analyzed using the free online tool SepsitTest-BLAST. The process takes about 8-12 hours, including several separate steps; non-automated DNA extraction, qPCR, sequencing and online sequence analysis. Originally, the input volume was 1 mL, but the company has now developed an add-on kit for DNA extraction from volumes up to 10 mL, to be used together with SepsitTest. The performance reported for this assay is widely variable, with a sensitivity ranging from 11% to 85% and a specificity ranging from 58% to 96% [158, 167, 168]. These data are based on the earlier version of the SepsitTest, with 1 mL sample volume.

The **IRIDICA BAC BSI Assay** (Abbott Diagnostics, Lake Forest, IL, USA) is a molecular assay for universal detection and identification of bacteria and candida, and can, in addition, detect important resistance genes. The IRIDICA system amplifies DNA using broad-range PCR with 18 primers pairs, targeting rRNA genes and other conserved regions of bacteria and candida genomes, as well as four resistance genes. Electrospray ionization mass spectrometry (ESI-MS) is thereafter used for analysis of base composition (adenine, cytosine, guanine and thymine) of the amplicon, including information about the exact mass and length. The result is compared to a database, which enables identification of the pathogen. Semi-quantitative
information is included in the result, indicating a “level”. The sample volume is 5 mL and the time to result is 6 hours. Several studies have reported sensitivities and specificities of about 90% and 95%, respectively [145, 169]. Despite promising results, the IRIDICA system has formally been withdrawn from the market.
AIMS

PCR-based methods for the diagnosis of BSI directly from whole blood will most probably be implemented as diagnostic tools in a near future. PCR enables DNA quantification, but still we know little about the importance of the bacterial DNA load for disease severity, immune-dysregulation, and treatment results in BSI. The underlying aim with this thesis was to learn more about the role of the bacterial DNA load in BSI.

Aims of this thesis were:

- To investigate if use of quantitative data from a commercial PCR test for BSI diagnostics can improve the diagnostic performance (Paper I)

- To investigate if quantitative data from a commercial PCR test for BSI diagnostics can provide information about disease severity (Paper II)

- To study the bacterial DNA load during S. aureus bacteremia in relation to clinical factors, such as sepsis, immune dysregulation and mortality (Paper III)

- To develop an in-house broad-range 16S rDNA ddPCR for detection and quantification of bacterial DNA in whole blood, and to evaluate its diagnostic and clinical performance (Paper IV)

- To validate ddPCR methods for quantification of bacterial DNA in whole blood (Papers III, IV)
MATERIALS AND METHODS

Patients (I-IV)
The Department of Infectious Diseases, Örebro University Hospital, Sweden, provides services for a population of 275,000 inhabitants in the County of Örebro, and is organized into an outpatient clinic and a ward for 30 adult patients. The study populations in Papers I-IV derive from two studies conducted at the department: the SeptiFast Study conducted from October 2007 to September 2008 (Paper II) and the Dynamics of Sepsis (DOS) Study conducted from February 2011 to June 2014 (Papers I, III and IV).

SeptiFast Study (II)
This was a prospective study, aiming to evaluate the SeptiFast assay in patients subjected to BC at the Emergency Department (158), for which patients were consecutively enrolled over one year. After obtaining written informed consent, an EDTA whole blood sample for PCR analysis was taken at the same time as BC from the included patients. In total, 1,093 patients were included in the study, and 113 positive SeptiFast PCR were found in 107 patients. These 107 patients form the study population in Paper II.

Ten PCR tests positive for CoNS were excluded because they had no microbiological or clinical support for infection with CoNS, and were therefore considered to represent skin contamination. Four PCR tests positive for fungi were also excluded because the study focus was on bacteria. Finally, 99 positive PCR tests from 94 patients were included in the study population in Paper II.

Dynamics of Sepsis Study (I, III, and IV)
This prospective study of patients diagnosed with BSI had several aims, including detection and quantification of bacterial DNA during the course of the BSI. An EDTA whole blood sample was collected from the same venous puncture as BC samples from patients at the Emergency Department. The EDTA blood samples were stored in a Biobank. These blood samples were used in Paper I, without obtaining informed consent from the patients, but after anonymization, and without access to any clinical data except information about gender, age and BC results.

The study populations for Papers III and IV consisted of patients who had given written, informed consent. Enrollment was performed when a BC
bottle signaled positive (Days 1-2). Blood samples and EDTA tubes were then collected on Days 1-2, 3-4, 6-8, 13-15, and 26-30. Figure 9 shows a flow-chart of patients included in Paper I, III and IV.

Exclusion criteria were age under 18 and infection with HIV, Hepatitis B, or Hepatitis C.

Figure 9. Flow-chart of patients included in Study I and in the DOS Study

**Blood culture (I-IV)**

In accordance with routine practice, two sets of duplicate BCs were collected per patient. For each BC a volume of 8-10 ml of venous blood was inoculated in a Bactec Aerobic/F bottle and the same volume in a BactecPlus Anaerobic/F bottle. The BC bottles were then incubated in the BACTEC (Becton Dickinson and Company, Franklin Lakes, NJ, USA) system for up to 7 days.

**Serial dilutions of samples with reference strain bacteria (IV)**

Reference strain bacteria, *S. aureus* (CCUG 35601), *S. pneumoniae* (CCUG 33638), and *E. coli* (CCUG 7620), were cultivated and incubated
overnight, and two to three colonies of each were mixed with 1 mL saline solution. Serial dilutions were then performed, first taking 2.5μL of the aliquot suspension into 2.5 mL of whole blood, for a dilution of 1:10\(^3\), then by diluting another five times down to 1:10\(^8\).

**DNA extraction and PCR (I-IV)**

No later than three days after clinical sample collection EDTA blood was frozen in aliquots at -70°C. Samples from the DOS study were mixed with 20% Glycerol before freezing.

Samples in Paper I and the bacteria-spiked samples in Paper IV, were never frozen or mixed with glycerol, as they were subjected to DNA extractions within three days.

**Paper II**

DNA was extracted manually from 1.5 mL of the EDTA blood, using the SeptiFast Prep KitMGRADE (Roche Diagnostics GmnH). Quantitative PCR was performed using the SeptiFast method, which is described in detail elsewhere [170].

**Papers I, III, and IV**

Bacterial DNA was extracted from 1 mL EDTA blood using the Select NA Blood Pathogen Kit (Molzym, Bremen, Germany) on an Arrow instrument (DiaSorin, Solna, Sweden), in accordance with the manufacturer’s instructions.

In Paper I, pathogen detection was performed using the Magicplex Sepsis Real-time Test, including a first conventional PCR step for amplification, and then two qPCR steps for identification to genus and species level.

In Papers III and IV, extracted DNA was subjected to *S. aureus*-, *S. pneumoniae*- and *E. coli*-specific ddPCR protocols targeting *nuc*, *lytA* and *uidA* genes, respectively. In Paper IV, a broad-range ddPCR with primers and a probe targeting the 16S rDNA were used.

The DNA was detected and quantified using the QX100 Droplet Digital PCR system (Bio-Rad Laboratories Inc., Pleasanton, CA, USA) according to the manufacturer’s recommendations. The PCR master mix was mixed with droplet generator oil on a droplet generator cartridge and placed into a droplet generator. The generated droplet emulsion was transferred to a 96-well PCR plate and amplified in a thermal cycler. After amplification the plates were transferred to and read in a droplet reader.
In Paper III, samples were also analyzed using a qPCR protocol with nuc gene primers, with subsequent quantification using a standard curve.

**Clinical data and definitions (II, III, IV)**

Retrospective chart review was performed for evaluation of severity of illness. Collected data included demographic characteristics, comorbidities, focus of infection, antibiotic treatment, length of hospital stay, ICU admission, and mortality. Comorbidity was evaluated using the Charlson Comorbidity Index [171]. The patient’s clinical condition on admission was classified as SIRS, sepsis, severe sepsis or septic shock using the Sepsis-2 criteria [31] in Paper II, and as non-sepsis, sepsis or septic shock according to the Sepsis-3 definition in Papers III and IV [37].

**Statistics (I-IV)**

Descriptive statistics were presented as medians together with minimum and maximum values for continuous variables, and as percentages for categorical variables. Sensitivities, specificities and predictive values were calculated from cross-tabulations. The non-parametric Mann-Whitney U test was used for comparison between two groups, and Fischer’s exact test was used for comparison of proportions. Pearson’s correlation and the non-parametric Spearman’s rho coefficient were used to assess correlation between two variables, depending on data distribution. Receiver-operating characteristic (ROC) curves were constructed to illustrate various cut-off levels. Area-under-curve values (AUC) were reported with 95% confidence interval (CI). A p-value of <0.05 was considered significant, with the exception of Spearman’s test, where significant correlation was set at <0.01 (two-tailed). The SPSS software package (IBM, New York, USA), versions 21 and 22, was used for the statistical analyses. For calculation of DNA template concentration in ddPCR (Papers III and IV), Poisson distribution statistics were performed in the Quantasoft software package (Bio-Rad).

**Ethics (I-IV)**

The studies were performed in accordance with the Declaration of Helsinki and were approved by the Regional Ethical Review Board in Uppsala, Sweden (approval number 2007/071 for Paper II and 2009/024 for Papers I, III and IV). All patients (or a next-of-kin) provided written informed consent in Papers II, III and IV. In Paper I, data were anonymized and thus no written informed consent was required.
RESULTS

Study populations (I-IV)
The study populations in Papers I-IV consisted of patients who sought medical care at the Emergency Department at Örebro University Hospital with suspected bacterial infection. Table 3 shows the distribution of gender and age in the different studies.

Table 3. Gender and age distribution in Papers I-IV

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Female; n (%)</td>
<td>294 (42)</td>
<td>35 (37)</td>
<td>4 (15)</td>
<td>39 (47)</td>
</tr>
<tr>
<td>Age, years, median, range</td>
<td>67 (18-97)</td>
<td>74 (14-96)</td>
<td>77 (24-92)</td>
<td>72 (24-93)</td>
</tr>
</tbody>
</table>

Evaluation of quantitative data of commercial molecular methods for pathogen identification in whole blood (I, II)

For broad routine use of PCR techniques in BSI diagnostics, there is a need for validated commercial assays. There are a limited number of commercial tests available for molecular identification of pathogens directly on blood samples [147] whereof two are evaluated in Papers I and II: Magicplex Sepsis Real-time Test (MST) and SeptiFast.

Paper I
In Paper I, we evaluated the performance of MST by comparing with BC in a large study cohort of patients with suspected sepsis. MST has been described in only a few publications, by Carrara et al [157], Loonen et al [158], and Ljungström et al [172].

Of the 696 study patients, 322 (46%) patients were positive using at least one method; 128 (18%) were BC positive and 268 (38%) were MST positive. Considering BC to be the gold standard, MST had an overall sensitivity of 47%, a specificity of 66%, positive predictive value (PPV) of 23%, and a negative predictive value of 87%.

Of the MST positive samples with a negative BC, a large number were positive for pathogens that rarely cause community-acquired BSI, including CoNS (n=129), Streptococcus agalactiae (n=26), Stenotrophomonas maltophilia (n=24) and A. baumanii (n=10). These results, showing that MST detected not only an important proportion of BSI pathogens but also much bacterial DNA with doubtful clinical significance, were consistent with that
previously reported for MST [157, 158, 172]. In order to explore whether the positive results with doubtful clinical significance were due to very low quantities of DNA, we looked more closely at the Cq values. Among the MST positive cases, those with concordant positive BC had significantly lower Cq values than those with a negative BC. This pattern was noted for the three largest pathogen groups, i.e. *S. aureus*, *S. pneumoniae* and *E. coli*, as illustrated in Figure 10.

![Figure 10. Comparison of median quantification cycle (Cq/Ct) values between blood culture negative and blood culture positive samples for all Magicplex Sepsis Real-time Test (MST) positive samples, and samples with MST positive for Staphylococcus aureus, Streptococcus pneumoniae and Escherichia coli.](image)

With a lower Cq cut-off value, i.e. 6.0 for *Staphylococcus* spp. and 9.0 for all other species, the number of MST positive cases decreased to 83 (12%) and the overall sensitivity decreased to 38%. However, the PPV increased to 59% and the specificity increased to 96%, because many MST positive results for CoNS and bacteria, which rarely cause community-acquired BSI, turned MST negative (Figure 11).
Figure 11. Sensitivities, specificities and positive predictive values of the Magicplex Sepsis Real-time Test (MST) at different quantification cycle (Cq) cut-off values for predicting blood culture positivity.

Even with this lowered Cq cut-off, the sensitivity of the MST was comparable with most other commercial tests (at the time) in the same field of application. However, this gives insufficient diagnostic value, and is certainly the most important reason why molecular methods directly on blood have not yet found a place in clinical use.

Moreover, the fact that MST showed high positivity for microbes that probably had no clinical significance hampers its clinical usefulness. In Paper I, we propose that to overcome this issue Cq values can be used as a tool for evaluating whether a positive MST test represents contamination or is clinically relevant.

**Paper II**

In Paper II we evaluated quantitative data in relation to BC positivity and disease severity in a study population of 94 patients with suspected sepsis and positive SeptiFast test.

The prevalence of severe sepsis/septic shock in the study was 29% according to Sepsis-2 criteria. SeptiFast positive tests from patients with severe sepsis/septic shock had significantly lower Cq values compared with those
from patients with non-severe sepsis, with a median of 16.9 versus 20.9, p<0.001. This association remained significant (p=0.002) within the group of 45 patients with concordant positive BC and Septifast test.

PPVs from the SeptiFast test for identifying severe sepsis/septic shock were 34% at Cq cut-off <25.0, 35% at Cq cut-off <22.5, 50% at Cq cut-off <20.0, and 73% at Cq cut-off <17.5. Figure 12 illustrates the number of samples from patients with severe sepsis/septic shock in relation to PPVs.

![Graph showing PPVs](image)

**Figure 12.** Bars in blue show cumulative numbers of SeptiFast positive samples from patients with severe sepsis/septic shock in the entire study population at different quantification cycle (Cq/Cp) cut-off values. The green line shows positive predictive values for detection of severe sepsis/septic shock at different Cq cut-off values.

Patients with a positive Septifast test with a Cq value <17.5 had significantly more severe sepsis/septic shock (73% versus 15%, p<0.001), were more often admitted to the Intensive Care Unit (23% versus 4%, p=0.016), were more frequently BC positive (100% versus 32%, p<0.001) and had longer hospital stays (median 19.5 versus 5 days, p<0.001).
ddPCR for monitoring bacterial DNAemia in bloodstream infections (III, IV)

In Papers III and IV we studied the bacterial DNA load, measured by in-house ddPCRs in patients with verified BSI, at several timepoints from hospital admission (Day 0, Days 1-2, Days 3-4 and Days 13-15).

Paper III

In paper III we quantified S. aureus-specific nuc DNA in blood at several timepoints during the first month after admission in 27 patients, with culture-proven SAB, included in the DOS Study. Among included patients, 13 (48%) fulfilled the Sepsis-3 criteria, and 6 (22%) died due to the infection.

On Days 0-2, nuc DNA was detected in 22 patients (81%), and on Days 6-8 in 3 patients (all non-survivors). The nuc DNA load on Days 1-2 was significantly elevated in patients with sepsis (median 2.69 vs. 1.32 log10 copies/mL; p=0.014) and in non-survivors (median 2.5 vs. 1.0 log 10 copies/mL; p=0.033). Patients with a high nuc DNA load (>1000 copies/mL) on Days 1-2 had significantly elevated CRP levels at all timepoints, and significantly decreased lymphocyte counts on Days 0, 1-2, 13-15 and 26-30, as illustrated in Figure 13 a-b.
Figure 13 a-b. Box plots presenting C-reactive protein (a) and lymphocyte counts (b) at different timepoints in patients with negative nuc PCR, with nuc DNA load <1000 copies/mL, and with nuc DNA load >1000 copies/mL. In the statistical analysis, the group with negative nuc PCR was combined with the group with nuc DNA load <1000 copies/mL, and then compared with the group with nuc DNA load >1000 copies/mL.
When studying patients with sepsis and/or infectious endocarditis separately, the associations between high initial nuc DNA load and elevated CRP persisted at most timepoints in contrast to the association with low lymphocyte counts which did not persist. Consequently, the association between nuc DNA and CRP elevation appears to be independent of sepsis syndrome and/or IE, while the lymphopenia might be dependent on these clinical conditions.

The proportion of patients with persistent DNAemia one-week post admission was 3/5 (60%) for non-survivors (excluding one patient who died on Day 1) versus 0/19 (0%) for survivors (p=0.004) and 3/11 (27%) for patients with sepsis versus 0/13 for patients without sepsis (p=0.08). In Figure 14, individual nuc DNA load at the different timepoints are demonstrated, with non-survivors marked in red.

![Graph showing nuc DNA load over time for different patient groups.](image)

*Figure 14. Quantitative nuc DNA data in 22 individual patients with Staphylococcus aureus bacteremia and detected nuc DNA. Non-survivors are marked in red.*

**Paper IV**

In Paper IV, we developed an in-house ddPCR targeting the bacteria universal 16S rRNA gene. The performance of the method was evaluated by comparison with species-specific ddPCRs (*nuc* gene for *S. aureus*, *lytA* for *S. pneumoniae*, and *uidA* for *E. coli*) in serial dilutions of whole blood spiked
with *S. aureus*, *S. pneumoniae*, or *E. coli*, the three most prevalent pathogens in community-onset BSI [110, 114]. Because the copy number of the 16S rRNA operon/bacterial cell varies between species, while the species-specific genes are present in one copy/cell, we expected higher copy numbers of 16S rDNA than species-specific DNA. The correlation between 16S rDNA and species-specific DNA for all spiked samples, positive in both methods, is illustrated in Figure 15.

![Figure 15. 16S rDNA in relation to species-specific DNA (nuc for *Staphylococcus aureus*, lytA for *Streptococcus pneumoniae*, and uidA for Escherichia coli), in whole blood samples spiked with bacteria in serial dilutions](image)

For all bacteria, the dilution at lowest concentration with a positive result for 16S rDNA was $1:10^8$, and for species-specific DNA it was $1:10^7$. Because 16S rDNA was still detected in the final dilution, we can assume a limit of detection for the 16S rDNA ddPCR that is at least 10-fold higher than that of the species-specific ddPCRs.

The median ratio between the 16S rDNA and the species-specific load was 6.6 for all samples together, 7.6 for *S. aureus*, 4.3 for *S. pneumoniae*,
and 7.7 for E. coli. These figures do not correspond absolutely to the 16S rDNA copy numbers/bacterial cell found in the literature for S. aureus, S. pneumoniae and E. coli, which have been estimated at 5-6, 4, and 7, respectively [137], and there was no significant difference in median ratios between the three bacteria. However, exact concordance is certainly not to be expected, especially not in samples with low concentrations. Based on the strong correlation between 16S rDNA and species-specific DNA, even without adjusting for species-variation in copy numbers/bacterial cell, we concluded that this in-house 16S rDNA ddPCR appeared to be a useful method for measurement of bacterial load in blood. Figure 16 illustrates the results from the analyses of spiked blood in serial dilutions, with the typical droplet pattern achieved by the ddPCR method.
Figure 16. Illustration of droplet digital PCR results from analyses of samples in serial dilutions, spiked with Staphylococcus aureus (A-B), Streptococcus pneumoniae (C-D) and Escherichia coli (E-F). A,C,E: 16S rDNA, B: nuc DNA, D: lytA DNA and F: uidA DNA. The positive droplets are blue and the negative are black.
Thereafter, we ran both 16S rPCR and species-specific ddPCRs on clinical samples from 83 patients included in the DOS Study, with culture-proven BSI caused by *S. aureus*, *S. pneumoniae*, or *E. coli*. The highest positivity rates were seen in *S. aureus* BSI, where 92% of the patients were 16S rDNA-positive and 85% *nuc*-positive at least at one timepoint. Corresponding figures for *S. pneumoniae* and *E. coli* BSI were 43% (16S rDNA)/40% (*lytA*) and 46% (16S rDNA)/62% (*uidA*), respectively. Among patients with *S. aureus* BSI, both methods had a sensitivity of 100% at admission (Day 0), and bacterial DNA was still detected in blood by both methods in 75% of cases on Days 1–2. Thereafter, positivity rates declined, and on Days 13–15 no patients had detectable *nuc* DNA, while four still had detectable 16S rDNA. Initial sensitivity was lower among patients with *S. pneumoniae* and *E. coli* BSI, and only a few patients had detectable bacterial DNA with any of the methods on Days 3–4.

To obtain an insight into the potential clinical value of the measurement of 16S rDNA on BSI patients we related the initial DNA load on Days 1-2 to presence of sepsis (Sepsis-3 definition) on admission, and to mortality. This timepoint was chosen because it was the only occasion when the positivity rate was high enough to enable statistical analyses. The initial 16S rDNA load was higher in BSI patients with sepsis than without sepsis (median 2.38 vs. 0 lg10 copies/mL; p=0.031) and in non-survivors than in survivors (median 2.38 vs. 0 lg10 copies/mL; p=0.006).
DISCUSSION

Study populations (I-IV)

As noted in Table 3, only 15% of patients in Paper III were female. This is a surprisingly low figure, even though men are known to have an increased risk for SAB compared to women, with a relative risk of 1.62, according to a population-based study by Laupland et al [173]. In the S. pneumoniae and E. coli BSI groups in Paper IV, the proportion of women was 21/30 (70%) and 14/26 (54%), respectively. Laupland et al [24, 114] have reported that E. coli BSI is more common in women, while no significant differences were seen between genders in the incidence of S. pneumoniae BSI. The overall incidence of community-onset BSI was comparable in males and females in this study, similarly to the Danish epidemiological study by Sogard et al [110]. The median age among BSI patients was 71 years in the study by Sogard et al [110], not very different from our study populations. The sizes of the study populations in the different studies are noted in Table 3. The study population in Paper I consists of a large number of well-defined, consecutively included patients, which is an important strength of this study. In Papers II and IV, the study populations are large enough for simple statistics, based on the total cohorts, but the sample sizes are too small to perform convincing subgroup analyses, or to make adjustments for confounders. The small sample size in Paper III is, indeed, a major limitation of the study. The results reached significance level despite this, but no firm conclusions can be drawn from this small patient cohort.

Papers I-II

Most commercial tests for detection of bacterial DNA are only qualitative and not quantitative. It would be potentially clinically beneficial to include quantitative information in the test results and, in most cases, when the assays are qPCR-based Cq values are easily available, even if not presented to the user. Quantitative aspects of commercial tests have not been studied previously, even though several studies on various in-house PCR assays have shown that a high DNA load is associated with disease severity and mortality [84-86, 174]. In Papers I and II, we investigated the potential clinical usefulness of quantitative data from two of the commercial tests for pathogen detection in blood. Our results, showing that MST positive samples with high Cq value often represent contamination bacteria, are not surprising as such bacteria are expected only in small quantities. A limitation of
Paper I is the lack of clinical data, because the ethics permission only allowed storage of personal information on age, sex and BC results. Consequently, our estimation of clinical relevance only applies to BC results in combination with general epidemiologic data on the bacteria that normally cause community-acquired BSI. The bacteria we classified as contaminants were most frequently CoNS, representing skin contamination, but there were also cases of S. maltophilia and A. Baumanii, most likely caused by laboratory contamination. Even though these bacteria normally do not cause community-acquired BSI, they can cause serious infections in immune-compromised patients and these findings could have serious implications. Therefore, it is important to utilize all available options for estimating the clinical relevance of ambiguous results; accessibility to Cq values can be valuable for this purpose. The TTP of BCs can provide similar information on CoNS findings, as shown by Kassis et al [126].

The results of Paper II, showing a link between SeptiFast Cq values and disease severity, are in line with that reported previously for in-house methods for specific BSI pathogens. This association was seen in the largest groups of bacteria, but also in the whole study cohort, which is in fact quite remarkable. Different pathogens behave differently in the infected host, and probably the DNA levels also depend on the etiology. In addition, SeptiFast PCR amplification targets the ITS region, which is part of the rRNA operon, a gene complex which exists in varying copy number in different bacteria. Despite this, our results indicate that quantitative data might be useful, even without taking the underlying pathogen into account. As far as we know, this is the first study to show a correlation between bacterial DNA load and disease severity, using a commercial multiplex PCR test.

In conclusion, the results of Papers I and II indicate that quantitative data, such as Cq values, can provide important clinical information, and therefore should be included in commercial PCR tests for BSI diagnostics.

**Papers III-IV**

Our intended focus in Papers III and IV was to study the DNA load in BSI patients during treatment, both in regard to absolute DNA levels and the rate of DNA clearance. This is a field of great interest, yet poorly studied. Elimination of the infectious pathogen, using antibiotic treatment and source control, is a major goal in sepsis management, well supported by evidence. However, currently, treatment result is not normally measured in terms of effect on the microbial load in blood. In virology, in contrast, the measurement of viral load in blood is well established as an important part...
of diagnostics and treatment-response monitoring in HIV and many other infections [175]. Quantification of microbial DNA in BSI assessment has been proposed as a potential prognostic marker [176] and, in a few studies, default DNA clearance has been associated with treatment failure and mortality [86, 91, 92].

In candidemia and SAB, follow-up BCs are recommended [88, 177] as a method to detect microbial persistence in blood, but this method is non-quantitative and has a long time to result. Monitoring of pathogen DNA instead, by specific or broad-range ddPCR, could potentially bring great clinical value to the management of such infections.

The sizes of our study populations were, as noted, limited, and is the reason the results on DNA persistence are mostly descriptive. Only in Paper III was it possible to compare patients, with or without persistent DNA in blood, to clinical data. The small study groups, and the possible impacts of confounding factors, mean that the results of the analyses must be interpreted with caution. Notwithstanding, one interesting finding in Paper III is the association between mortality and persistent DNAemia, which was statistically significant despite the small sample size.

In Paper IV, we found that the absolute majority of cases with 16S rDNA positivity on Days 3-4, and all cases positive on Days 6-8, were S. aureus BSI patients. Probably E. coli and S. pneumoniae DNA persistence are uncommon in BSI, given that the infections are correctly treated. Accordingly, persistent DNA positivity might be an indicator for treatment failure in BSI of these etiologies.

Most of the statistical analyses in Papers III and IV are based on quantitative DNA measurements from Days 1-2. At this timepoint almost all patients had been sampled, and many were PCR positive. In Paper II, we showed associations between high nucleic DNA levels on Days 1-2, and sepsis, mortality, endocarditis, elevated CRP and lymphopenia. Some of these clinical factors are related, and therefore we cannot claim that they are independently associated with high DNA loads. However, our results indicate a potential high clinical usefulness of quantitative DNA measurements. Among bacterial BSI infections, SAB is probably the condition where such monitoring would be most valuable. SAB is a disease with varying presentation, ranging from uncomplicated manifestations to life-threatening infections. Persistent bacteremia has been linked to a complicated disease course [87] and, potentially, DNA measurements could be a simpler, faster, and more informative alternative to BC for identification and monitoring of pa-
tients with complicated SAB. The high DNA positivity rate among SAB patients in our study further indicates that this method is highly suitable for this purpose.

In Paper IV we linked a high 16S rDNA load on Days 1-2 to sepsis and mortality. This is in line with the results of other researchers for BSI of different etiologies [84-86, 91, 174]. The innovation in our study is that we did not measure species-specific DNA, but universal bacterial DNA. Perhaps quantitative 16S rDNA could be used in sepsis management, as a tool to screen for presence of bacterial DNA in blood and to evaluate treatment response. Certainly, high-throughput technologies for broad-range species identification are striding ahead, but currently a 16S rDNA PCR without bacterial identification is considerably more time- and cost-effective.

In Papers III and IV we used ddPCR for DNA amplification. Several previous studies have compared ddPCR with qPCR and reported a smaller effect of inhibitors, greater precision, and higher reproducibility [134, 178]. ddPCR has been used for DNA quantification in various fields, such as quantifying HIV DNA [178]. The method has been used only rarely to quantify bacterial DNA in human samples [179] and no previous reports have evaluated this method in BSI assessment.

In Paper III, the ddPCR results were compared with qPCR results to validate the ddPCR method. The two methods showed high concordance, which indicates that ddPCR is a suitable method for quantifying bacterial DNA in blood.
CONCLUSIONS

- In the Magicplex Sepsis Real-time Test, high Cq values were associated with results with doubtful clinical relevance; thus, adjustment of Cq cut-off could improve diagnostic performance.

- High loads of bacterial DNA in whole blood are associated with disease severity in bloodstream infection.

- In *S. aureus* bacteremia (SAB) patients, persistent DNAemia one week after admission was associated with mortality.

- In SAB patients, a high initial bacterial DNA load was associated with high CRP levels and low lymphocyte counts in the first month after presentation.

- 16S rDNA dynamics were correlated with dynamics of species-specific DNA in patients with BSI caused by *S. aureus*, *S. pneumoniae*, and *E. coli*, and could potentially be a method to monitor bacterial DNA load.

- ddPCR results showed high concordance with qPCR results, and this appears to be a suitable method for quantifying bacterial DNA in blood.
FUTURE PERSPECTIVES

Efforts in improving outcome in sepsis are of the utmost importance because, despite modern critical care, mortality and morbidity remain high. Patients with sepsis belong to an extremely heterogenic group, both regarding host factors (such as variances in genetic factors, comorbidities and immune status) and pathogen factors (such as pathogen etiology, virulence factors, the pathogen’s resistance-pattern and microbial burden). To achieve the best treatment for each patient, the therapies must be individualized. To enable this, it is necessary to identify important host and pathogen factors in the individual patient. There are many promising novel biomarkers for host factors, both for early identification of sepsis, such as micro-RNAs [180] and heparin-binding protein [181], and for identification of immunodysfunction, such as HLA-DR [182]. Our research aims to increase the understanding of the pathogen factors in sepsis. The starting point for the future perspectives emerging from this thesis are the increasing possibilities for rapid pathogen identification with novel solutions utilizing molecular techniques.

The implementation of molecular techniques for rapid diagnostics on signal-positive BC has been successful, in particular the use of MALDI-TOF MS, with its significantly shorter time to adequate antibiotic treatment for many BSI patients [148, 149]. However, the reduction in time to result compared to BC is limited and, in sepsis, correct and timely antibiotic administration is crucial, not only in regard to patient survival but also in regard to the global problem of antibiotic resistance. Accordingly, a breakthrough in molecular methods for direct testing on whole blood has the potential to revolutionize sepsis assessment. For broad, routine use of such techniques, validated, commercial assays are essential that not only have high diagnostic performance, but which are also rapid, easy-to-use and affordable.

Evaluations of molecular tests on whole blood have mostly shown a limited concordance with BC for pathogen identification. Thus, it is unlikely that molecular methods will replace BC in the near future, but they will surely become a complement. The most important challenge for upcoming methods is to achieve better sensitivities, despite the frequently low bacterial concentration in BSI. Even with a limit of detection of 1CFU/ mL, if there is no pathogen DNA in the limited volume of blood tested, nothing will be detected. Methods starting with larger blood volumes, such as 5 mL, before DNA extraction [144-146], have shown better sensitivities, and probably “the higher the volume, the higher the yield” [116], as said about BC, can
be applied to molecular diagnostics as well. Thus, the commercial methods evaluated in Papers I and II, the Magicplex Sepsis Real-time test and SeptiFast test, starting with 1 and 1.5 mL blood, will in the future probably be outcompeted by methods that can process higher volumes.

BSI can be caused by numerous pathogens, and an optimal diagnostic method should be broad-range, without a risk of missing the target. IRIDICA BAC BSI was designed to be such a diagnostic method and it was found to detect more pathogens than BC [183]. Even though the IRIDICA assay is no longer available [184], the experiences with the method illustrate the potential of novel molecular methods for detection of BSI. Modern high-throughput sequencing technologies, such as the Next Generation Sequencing (NGS) platform [185], are promising, but have not yet been clinically evaluated for detection of BSI. Most probably, standardized protocols with open source bioinformatics tools, and user-friendly data-analysis interfaces will soon be developed, and facilitate clinical applications of NGS.

While waiting for fast, cheap, user-friendly, broad-range methods, available for everyone at any time of the day, we should probably start by identifying patient groups who will potentially benefit greatly from robust diagnostic molecular methods. Eligible patients could be patients with septic shock, immunocompromised patients, patients with a high risk of infections caused by multi-resistant bacteria, neonates, and patients with elusive infections, where conventional diagnostics have been unsuccessful. The T2MR system, including the T2Candida and the T2Bacteria panels, have been developed for use on such selected patient groups. The tests target candida and six problematic, often multi-resistant bacteria. There appears to be at least a role for T2candida in clinical assessment, with a diagnostic performance superior to BC [161, 162]. Studies evaluating the clinical and financial impact of T2Candida are underway and already showing benefits [163, 164]. Such studies are very important for the implementation of novel techniques and should be prioritized in future research on molecular BSI diagnostics. To prove the value of new methods, randomized intervention studies, investigating the clinical impact and outcome, are needed.

Another aspect to highlight, is the need for rapid antibiotic susceptibility testing (AST). Molecular methods can detect resistance genes, but for multifactorial resistance detection, phenotypic AST must be performed. Semimolecular phenotypic AST from signal-positive BC will certainly be available in the near future. The Swedish company Q-linea has developed an automated method for phenotypic AST, not yet commercialized, where an an-
tibiogram with MIC values is delivered 3-6 hours after signal of BC positivity. Our hospital in Örebro participated in a clinical evaluation of the method, and reported a correlation of 96% compared to the reference method [186].

One of the key issues for successful clinical implementation of molecular methods is that they must be user-friendly. A rapid test will not be as valuable if it requires a laboratory which could be closed for the night, is located far away, or lacks trained staff. Therefore, upcoming point-of-care instruments, such as the recent Septiflo™ device developed for the detection of the Gram status of BSI in under 10 minutes, will be highly appreciated. This device works on the principle of identifying PAMPs, such as LPS and lipoteichoic acid [187]. The method appears promising, but is not yet commercialized or evaluated in clinical studies.

Indeed, rapid molecular tests are of high interest, and many innovative technologies are striding ahead. In contrast, the main subject of this thesis - the role of pathogen DNA load and pathogen DNA persistence/clearance in BSI - is not included in the scope of these technologies. Just recently, the SSC published research priorities for sepsis and septic shock [188]. Herein, rapid molecular tests for pathogen identification are discussed, but there is nothing relating to quantitation possibilities. In particular, the subject of pathogen DNA clearance in BSI is neglected. Surprisingly few studies are published in this field, and those with small study populations of only 20-50 patients [86, 91]. Indeed, this is a promising field of research, with possible direct clinical implications. Diseases such as candidemia and SAB, both known to cause persistent infections, are particularly interesting, and could potentially be ideal for monitoring using PCR instead of BC.

Pathogen DNA monitoring might be a method for evaluation of early antibiotic therapy appropriateness, in situations such as treatment of septic shock when optimal dosage and choice of antibiotics are crucial. The idea behind the 16S rDNA ddPCR we developed in Paper IV, was to find a simple method for detection and quantification of unspecified bacterial DNA during BSI. In a future study at our hospital, we plan to use this assay for frequent, repeated measurement of bacterial DNA in patients with septic shock during the first days after admission to ICU. A disadvantage with this 16S rDNA ddPCR is that it does not provide the bacterial identity of the detected DNA. Thus, my hope is for a future sensitive and quantitative molecular method designed for blood that can both detect bacterial etiology and monitor bacterial load.
Regarding future plans, I have a draft of a new study, with the departure point in Paper III. Even though no firm conclusions can be drawn from Paper III due to the small sample size, I believe that the results of Paper III are highly interesting. Our *nuc* ddPCR showed excellent sensitivity for detection of SAB, and the *nuc* DNA load and persistence were clearly related to important clinical factors. SAB infections are often complicated, but can also be simple, and today almost every SAB patient receives long antibiotic treatment and undergoes complicated investigations. *nuc* DNA monitoring might be a way to discriminate between uncomplicated and complicated infections. In this potential, future study, I would include SAB patients and perform daily monitoring with *nuc* ddPCR, both for measurement of DNA load and time to DNA clearance. The primary aim would be to compare *nuc* DNA monitoring to standard-of-care monitoring with follow-up BCs, for prediction of complicated infections. Possibly, in the near future, follow-up BCs will be something from the past.
SVENSK SAMMANFATTNING

Sepsis är ett livshotande tillstånd där kroppens immunförsvar reagerar på ett okontrollerat sätt till följd av en infektion, i många fall med spridning av bakterier i blodbanan. För att påvisa bakterierna används blododling, en metod som dock har flera nackdelar. Dels tar det flera dagar att få resultatet, och dels är den inte alltid tillförlitlig. Blododlingar kan bli falskt negativa om patienten fått antibiotika före odlingen, eller om infektionen orsakas av svårodlade bakterier.

På senare år har molekylära tekniker utvecklats, såsom PCR, för påvisning av DNA, vilket kan användas för att hitta bakterie-DNA i blodet vid sepsis. Provresultatet från ett PCR-test kan fås efter några få timmar, och i vissa fall kan bakterier, som inte hittas med blododling, påvisas. Sannolikt kommer PCR spela en viktig roll inom området för bakteriepåvisning i blod i framtiden, men än så länge är användningen begränsad.

I tidigare studier har man sett att mängden bakterie-DNA i blodet är kopplat till infektionens svårighetsgrad. Ju högre mängd, desto allvarligare sjukdomsbild och desto högre risk för död. Även den takt med vilken DNA försvinner från blodet, har kunnat kopplas till hur snabbt patienten svarar på antibiotikabehandling.

Min avhandling syftar till att öka kunskapen om hur PCR-teknik kan användas vid diagnostik av infektioner med bakterier i blodbanan, med tonvikt på vilken roll mängden bakteriellt DNA i blodet har för den kliniska bilden.

I avhandlingens två första arbeten utvärderades två kommersiella PCR-metoder för påvisning av bakteriellt DNA i blod. Vi fann att mängden DNA i blodet spelade roll för tolkningen av provresultatet. Låga koncentrationer DNA kunde tyda på att det rörde sig om en förorening, och inte ett sant positivt resultat. Hög DNA-koncentrationer var förknippat med en mer allvarlig sjukdomsbild. I provresultatet anges i nuläget endast vilken bakterie som påvisats, utan någon information om mängden DNA, varför våra resultat skulle kunna bidra till att förbättra användbarheten av dessa tester.

I avhandlingens tredje arbete studerades mängden bakteriellt DNA, mått med en egenutvecklad PCR-metod, hos patienter där bakterien *Staphylococcus aureus* påvisats med blododling. Blodprovtagning för PCR skedde vid upprepade tillfällen under sjukdomsförföljden. Resultaten från studien visade att patienter med höga initiala nivåer eller kvarvarande bakterie-DNA i blodet efter en vecka, hade ett mer påverkat immunförsvar och högre dödlighet än övriga patienter.
Även i det sista arbetet användes en egenutvecklad PCR-metod, utvecklad i syfte att kunna mäta bakteriellt DNA under sjukdomsförloppet hos patienter med sepsis och bakterier i blodbanan. För att kunna använda samma metod oavsett vilken bakterieart som orsakat infektionen, användes en mål-gen, 16S rDNA, som finns hos alla bakterier. Utvärdering av metoden visade att den var lika känslig som PCR-metoder riktade mot enskilda bakterier. Vi fann också att en hög mängd DNA var kopplat till sepsis och ökad dödlighet, hos patienter med bakterier i blodbanan.

Sammanfattningsvis visar våra resultat att mängden bakteriellt DNA i blodet spelar en betydande roll för sjukdomsbilden vid infektioner med bakterier i blodbanan. I framtida forskning om molekylära metoder för påvisning av bakteriellt DNA i blodet, bör man även fokusera på mängden DNA, och takten med vilken DNA-nivån sjunker och försvinner från blodbanan.
ACKNOWLEDGEMENTS

I would like to express my great appreciation to all those who have contributed to this theses, and to all who have helped, encouraged and supported me all along the way. You all made this work possible! In particular, I want to thank:

Kristoffer Strälin, my supervisor. Back in 2010, you invited me into the world of scientific research, by giving me an active role in the start-up of the DOS-study. Since then you have kept on inspiring and challenging me, with endless enthusiasm combined with great knowledge. You have been a brilliant supervisor; present, engaged and accessible.

Paula Mölling, my co-supervisor. You once guided me through all steps and pit-falls in the laboratory work behind the set-up of the nuc PCR used in this thesis. You have generously shared your laboratory skills and impressive knowledge in molecular biology, and continuously contributed to the progress of our research. I appreciate you greatly for your competence, but just as much for your fantastic supportive and inclusive attitude.

My co-authors; Per Olcén, Anna Fagerström, and Per Josefsson, for your contributions in the conception of the studies, data collection and for valuable manuscript reviews. Theresa Ennefors, Sofia Lindström and Magdalena Källgren, for many hours of laboratory work, data collection and review of manuscripts.

Sara Cajander and Gunlög Rasmussen, co-authors and colleagues. Your involvement in data processing and input in manuscript writing, have been a great help. However, most of all I appreciate to share everyday weekdays with you, with discussions and chitchats, sometimes with scientific content, but most often without.

Co-researchers, in related fields, at our clinic, and at the Department of Laboratory Medicine, for creative collaborations and rewarding discussions. Anna Lange, for being my confidant in moments of frustration and ambivalence concerning my research. You always cheer me up with your mix of curiosity, sincerity and humour! Jan Källman, for your efforts in flying the flag for science and research at our Department. Karolina Prytz for fruitful discussions, and great company while we learnt about Molecular Microbiological Diagnostics in Maastricht. Simon Athlin, for our fellowship as PhD students under Kristoffer’s umbrella, and useful exchange of experiences. Martin Sundqvist, for your valuable advice and inspiring attitude to science.
All my work-mates at the Department of Infectious Diseases. You all contribute to a great atmosphere, welcoming and generous, making it possible for me, and others, to grow. Henrik Eliasson, former Head of Department, for your great concern for our clinic, and supportive approach. Martin Widlund, Head of Department. I do appreciate that you prioritize research in your visions for the Department. However, I also appreciate that you make your best to steal some of my research-time, by involving me in these visions. I look very much forward to future collaboration in leadership!

Anders Magnusson, for greatly appreciated statistical assistance. Lars-Göran Jansson, for professional and rapid service, in making posters, photos and figures.

BG Nilensjö, for competent coaching in running; an important piece in my work-life puzzle. Sometimes I have given a running session priority over the work with my research data, which possibly was wrong in a short perspective. However, I believe I made the right choice, not only for my marathon personal record, but also for important insights about my drive forces and capability.

My fellow runners, in particular Ia and Elin. I have spent so many early mornings in your company, in rain, snow, wind and sunshine. Our jogs have given me balance in life and energy to work, and, into the bargain, fantastic friends.

My parents, Kjellrun and Staffan. For love and support, throughout life. My sisters and brothers, with families. You are all important to me.

Our beloved children Albert, Viktor and Julius. You are fantastic and unique, and I feel such a gratitude to call you mine.

Bruno. For always being by my side, with all your love, support and generosity. I love you.

Finally, I wish to acknowledge the financial support provided by Region Örebro County’s Research Committee, Nyckelfonden and ALF research funding.
Figure 17. Finally at the finish line! Medal from Frankfurt marathon, 2016.
REFERENCES


89. Hsu MS, Huang YT, Hsu HS, Liao CH: Sequential time to positivity of blood cultures can be a predictor of prognosis of patients with persistent *Staphylococcus aureus* bacteremia. *Clin Microbiol Infect* 2014, 20(9):892-898.


116. Lamy B, Dargere S, Arendrup MC, Parienti JJ, Tattevin P: How to Optimize the Use of Blood Cultures for the Diagnosis of


149. Vlek AI, Bonten MJ, Boel CH: Direct matrix-assisted laser desorption ionization time-of-flight mass spectrometry improves


Publications in the series
Örebro Studies in Medicine


35. Söderqvist, Fredrik (2009). Health symptoms and potential effects on the blood-brain and blood-cerebrospinal fluid barriers associated with use of wireless telephones.


41. Gustafsson, Sanna Aila (2010). The importance of being thin – Perceived expectations from self and others and the effect on self-evaluation in girls with disordered eating.

42. Johansson, Bengt (2010). Long-term outcome research on PDR brachytherapy with focus on breast, base of tongue and lip cancer.

43. Tina, Elisabet (2010). Biological markers in breast cancer and acute leukaemia with focus on drug resistance.


46. de Leon, Alex (2010). *Effects of Anesthesia on Esophageal Sphincters in Obese Patients*.


52. Loiske, Karin (2011). *Echocardiographic measurements of the heart. With focus on the right ventricle*.


60. Lindgren, Rickard (2011). *Aspects of anastomotic leakage, anorectal function and defunctioning stoma in Low Anterior Resection of the rectum for cancer*.


64. Nordin Olsson, Inger (2012). Rational drug treatment in the elderly: "To treat or not to treat".


67. Thuresson, Marie (2012). The Initial Phase of an Acute Coronary Syndrome. Symptoms, patients’ response to symptoms and opportunity to reduce time to seek care and to increase ambulance use.


75. Gustavsson, Anders (2012): Therapy in Inflammatory Bowel Disease.


83. Lönn, Johanna (2013): The role of periodontitis and hepatocyte growth factor in systemic inflammation.


96. Sundh, Josefin (2013): *Quality of life, mortality and exacerbations in COPD.*


98. Palmetun Ekbäck, Maria (2013): *Hirsutism and Quality of Life with Aspects on Social Support, Anxiety and Depression.*


102. Söderström, Ulf (2014): *Type 1 diabetes in children with non-Swedish background – epidemiology and clinical outcome*

103. Wilhelmsson Göstas, Mona (2014): *Psychotherapy patients in mental health care: Attachment styles, interpersonal problems and therapy experiences*


105. Demirel, Isak (2014): *Uropathogenic Escherichia coli, multidrug-resistance and induction of host defense mechanisms*


109. Törös, Bianca (2014): Genome-based characterization of Neisseria meningitidis with focus on the emergent serogroup Y disease


120. Pelto-Piri, Veikko (2015): Ethical considerations in psychiatric inpatient care. The ethical landscape in everyday practice as described by staff.


139. Elwin Marie (2016): *Description and measurement of sensory symptoms in autism spectrum.*
140. Östlund Lagerström, Lina (2016): "The gut matters" - an interdisciplinary approach to health and gut function in older adults.


157. Olsson, Emma (2017): *Promoting Health in Premature Infants – with special focus on skin-to-skin contact and development of valid pain assessment.*


177. Christos Karefylakis (2018): Vitamin D and its role in obesity and other associated conditions.