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Bloodstream infections with ESBL-producing Enterobacterales

– prediction, rapid diagnosis and molecular epidemiology

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Cover design by Inga Fröding.

*cgMLST minimal spanning tree showing the molecular epidemiology of ESBL-producing Escherichia coli from patients with community-onset bloodstream infection (**Study I and IV**).*
Pink: Patient with a positive Stockholm score but no septic shock. Plum: Patient with a positive Stockholm score and septic shock. Black: Patient with a negative Stockholm score and septic shock. White: Patient with a negative Stockholm score and no septic shock.
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Bloodstream infections with ESBL-producing Enterobacterales – prediction, rapid diagnosis and molecular epidemiology

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Antimicrobial-resistant bacteria are a threat to public health worldwide. Particularly, the increase of extended-spectrum β-lactamase (ESBL)-producing Enterobacterales (EPE) is a significant clinical problem. ESBLs confer resistance to cephalosporins and carbapenemases confer resistance to carbapenems. *Escherichia coli*, the most important member of the Enterobacterales, is the most common cause of bloodstream infection (BSI). First-line treatment is in most cases the cephalosporin cefotaxime, but EPE are resistant to cefotaxime. Thus, patients with EPE BSI are at risk of prolonged hospitalization and increased mortality caused by empirical treatment failure. This thesis aimed to improve care for patients with EPE BSI in three ways: early prediction, rapid diagnosis and increased understanding of resistance transmission and the importance of microbial virulence for the severity of disease.

The aim of **Study I** was to develop a practical prediction-score to identify patients at risk of EPE BSI which could be used to improve the appropriateness of empirical treatment. Risk factors for EPE BSI were assessed and two clinical scores evaluated. The strongest predictors (named the Stockholm score), prior EPE-positive culture (especially recent samples), prostate biopsy and healthcare abroad, were present in 50% of cases. In other patients, prediction was difficult, hence rapid diagnostics and susceptibility testing are necessary.

Therefore, the aim of **Study II** was to evaluate a method for rapid susceptibility testing. EUCAST disk diffusion read after 6 hours was compared to 18 hours on *E. coli* and *Klebsiella pneumoniae*-isolates representing clinically important resistance mechanisms. Results showed that 6-hour reading was accurate if adapted breakpoints were used.

Resistance traits are mainly spread through clonal expansion of bacterial cells and/or horizontal gene transfer trough plasmids. Development of appropriate intervention methods depend on knowledge of the mode of transmission. **Study III** aimed to determine the molecular epidemiology of *K. pneumoniae* harbouring the carbapenemase-gene $bla_{\text{NDM-1}}$. Isolates from India, UK and Sweden were subjected to MLST, plasmid replicon typing and PCR of virulence genes. Diverse sequence types and plasmids, rather than a single high-risk clone, were responsible for the early dissemination of *K. pneumoniae* with $bla_{\text{NDM-1}}$.

BSI is often complicated by sepsis and septic shock, associated with high mortality. With resistance on the rise, the search for new therapies includes anti-virulence agents. Thus, a correct understanding of the influence of microbial virulence on pathogenesis is important. The aim of **Study IV** was to determine the respective contributions of microbial virulence and patient factors to the severity of disease. Whole genome sequencing (WGS) was performed on *E. coli* from patients with EPE BSI. The virulence gene *iss*, increased serum survival, was associated with septic shock, especially in immunocompetent patients.

In conclusion, the treatment of patients with EPE BSI could be improved by these results. Use of the Stockholm score identified many, but not all, patients at risk of EPE BSI, thereby improving the appropriateness of empirical therapy. Rapid disk diffusion with adapted breakpoints gave accurate results and would enable early correction of therapy. $bla_{\text{NDM-1}}$ was found in diverse sequence types of *K. pneumoniae*. The virulence gene *iss* was associated with septic shock in EPE BSI and might have potential as an anti-virulence treatment target.

LIST OF SCIENTIFIC PAPERS

I. **Fröding I**, Karlsson Valik J, Bolinder L, Naucler P, Giske CG. Prediction of bloodstream infection caused by extended-spectrum beta-lactamase-producing Enterobacterales in patients with suspected community-onset sepsis. Int J Antimicrob Agents. 2019;53:820-829.

II. **Fröding I**, Vondracek M, Giske CG. Rapid EUCAST disc diffusion testing of MDR *Escherichia coli* and *Klebsiella pneumoniae*: inhibition zones for extended-spectrum cephalosporins can be reliably read after 6 h of incubation. J Antimicrob Chemother. 2017;72:1094-102.

III. Giske CG, **Fröding I**, Hasan CM, Turlej-Rogacka A, Toleman M, Livermore D, Woodford N, Walsh TR. Diverse sequence types of *Klebsiella pneumoniae* contribute to the dissemination of $bla_{\text{NDM-1}}$ in India, Sweden, and the United Kingdom. Antimicrob Agents Chemother. 2012;56:2735-8.

IV. **Fröding I**, Hasan B, Sylvin I, Nauclér P, Giske CG. ESBL-producing *E. coli* causing community-onset bloodstream infection and the association of bacterial clones and virulence genes with septic shock. Manuscript.

OTHER RELATED PUBLICATIONS

1. Giske CG, Dyrkell F, Arnellos D, Vestberg N, Hermansson Panna S, **Fröding I**, Ullberg M, Fang H. Transmission events and antimicrobial susceptibilities of methicillin-resistant *Staphylococcus argenteus* in Stockholm. Clin Microbiol Infect. 2019;25(10):1289 e5- e8.

2. Enstrom J, **Fröding I**, Giske CG, Ininbergs K, Bai X, Sandh G, Ullberg M, Fang H. USA300 methicillin-resistant *Staphylococcus aureus* in Stockholm, Sweden, from 2008 to 2016. PLoS One. 2018;13(11):e0205761.

3. Fang H, **Fröding I**, Gian B, Haeggman S, Tollstrom UB, Ullberg M, Nord CE. Methicillin-resistant *Staphylococcus aureus* in Stockholm, Sweden: Molecular epidemiology and antimicrobial susceptibilities to ceftaroline, linezolid, mupirocin and vancomycin in 2014. J Glob Antimicrob Resist. 2016;5:31-5.

CONTENTS

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
APEC	Avian pathogenic *E. coli* (ExPEC subtype)
AST	Antimicrobial susceptibility testing
ATU	Area of technical uncertainty
BLAST	Basic local alignment search tool
BMD	Broth microdilution
BSI	Bloodstream infection
CA	Category agreement
CAT-ID	Carbapenemase activity test identification disk
cgMLST	Core genome multilocus sequence typing
CPE	Carbapenemase-producing Enterobacterales
CTX	Cefotaxime
CTX-M	Cefotaxime-Munich β-lactamase. Common group of ESBL-enzymes.
EA	Essential agreement
EARS-Net	European Antimicrobial Resistance Surveillance Network
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPE	ESBL-producing Enterobacterales
EPEC	Enteropathogenic *E. coli*
ESBL	Extended spectrum β-lactamase
ESBL-EC	ESBL-producing *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extra-intestinal pathogenic *E. coli*
FDA	United States of America Food and Drug Administration
GLASS	Global Antimicrobial Resistance Surveillance System
Hier CC	Hierarchical clustering of cgMLST
ICU	Intensive care unit
ISO	International Organization for Standardization
KPC	*K. pneumoniae* carbapenemase
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
ME	Major error
mE	Minor error
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing

MRSA	Methicillin-resistant *Staphylococcus aureus*
NDM	New Delhi metallo-β-lactamase
NGS	Next-generation sequencing
NMEC	Neonatal meningitis-associated *E. coli* (ExPEC subtype)
NPV	Negative predictive value
OR	Odds ratio
OXA	Oxacillinase
OXA-48	Carbapenem-hydrolysing class D enzyme. A group of oxacillinase-related enzymes with carbapenemase-activity.
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PPV	Positive predictive value
rep-PCR	Automated repetitive sequence-based PCR
ROC	Receiver operating characteristic
SIR	Susceptibility categories. EUCAST definition: S= Susceptible, I= Susceptible, Increased exposure, R= Resistant
SNP	Single nucleotide polymorphism
SOFA	Sequential (sepsis-related) organ failure assessment score
ST	Sequence type (MLST-derived)
TLA	Total laboratory automation
UPEC	Uropathogenic *E. coli* (ExPEC subtype)
UTI	Urinary tract infection
VIM	Verona integron-encoded metallo-β-lactamase
VME	Very major error
WCA	Work cell automation
WGS	Whole genome sequencing
3GC	Third generation cephalosporin
3GCR	Third generation cephalosporin-resistant (mainly caused by ESBL-production)

A note on terminology:
In general, the names of genes are composed of a three-four letter code, always italicized. The name of the protein product is capitalized with variants of the genes identified with a serial number based on the order of first observation. Thus bla_{NDM-1} and $bla_{CTX-M-15}$ refers to the β-lactamase genes (*bla*), which encode the enzymes NDM-1 (first NDM-variant observed) and CTX-M-15 (15^{th} CTX-M-variant observed), respectively. Genes mentioned in this thesis are not included in the list above.

1 INTRODUCTION

At the centre of attention of this thesis is the patient with bloodstream infection caused by extended-spectrum β-lactamase-producing Enterobacterales. Enterobacterales are Gram-negative enteric bacilli of which the clinically most important species are *Escherichia coli* and *Klebsiella pneumoniae*. *E. coli* is the most common cause of bloodstream infection (BSI). Extended-spectrum β-lactamase (ESBL) is an enzyme that causes resistance to cefotaxime, which is the first-line empirical treatment in Sweden for patients with suspected bloodstream infection. The rate of ESBL-production in *E. coli* increased rapidly in Sweden between 2000 and 2010 and meant that the first-line treatment more frequently was inadequate leading to treatment failure. Thus, it is important at an early stage to identify which patients have an infection with ESBL-producing Enterobacterales (EPE) to adjust the antibiotic treatment. This can be achieved either by prediction based on risk-factors (**Study I**) or by rapid diagnostic methods (**Study II)**.

Resistance genes are spread either through clonal expansion and transmission of nearly identical bacterial strains, or through horizontal transfer by plasmid-borne resistance genes between different bacteria. Appropriate measures for infection control depend on the route of transmission. Hence, it is important to study the molecular epidemiology of AMR bacteria to discern if a resistance gene is mainly clonal or mainly plasmid-borne (**Study III)**. The severity of disease in patients with BSI caused by ESBL-producing *E. coli* is influenced by patient comorbidity and immune status. Whether also microbial factors have a significant impact on the severity of disease is unclear. Increased understanding of the pathogenesis of disease severity in EPE BSI could possibly identify properties that have a potential utility in future methods to identify patients with higher risk of severe infection or potentially become targets for new adjuvant anti-virulence treatment in sepsis (**Study IV**).

1.1 ANTIMICROBIAL RESISTANCE IN *E. COLI* AND *K. PNEUMONIAE*

1.1.1 Evolution of antimicrobial resistance

The origin of most antibiotic resistance genes can be found in environmental bacteria, in many cases in antibiotic-producing *Actinobacteria* [1, 2]. The function of antibiotics in these bacteria was inter-cellular signalling and protection [3]. Resistance genes evolved in parallel, to protect the antibiotic-producing bacteria from the antibiotic action. There is evidence that resistance genes are ancient, *vanA*-genes have been found in Canadian High North permafrost-samples, dating 30,000 years ago [4]. In a previously completely closed cave in Lechuguilla Cave in New Mexico, which has been isolated from the surface eco-system since at least 4 million years, *Paenibacillus* species was found carrying antibiotic resistance genes to several antibiotics, including aminoglycosides [5]. The environmental resistome is a term that refers to all potential resistance genes, includes genes that confer phenotypic resistance, silent genes that are not expressed and pro-resistance genes which are genes that do not cause resistance in their present form, but in which only minor mutations are needed in order to cause resistance [2].

In the environmental resistome, genes are commonly located on the chromosome but sometimes on plasmids, which are mobile circular DNA-fragments (further described in

Section 1.5.2). The transfer from chromosome to plasmid is a relatively rare event, but when the resistance gene has been mobilied to a plasmid, it has a higher potential to spread to clinically relevant bacteria, termed pathogens.

In the 1920s, before antibiotics were introduced to the clinic, common human pathogens were generally very susceptible to antibiotics. However, within a few years of the first introduction of each new class of antibiotics, pathogens with resistance to that class were observed (**Figure 1**) and rapidly spread [6].

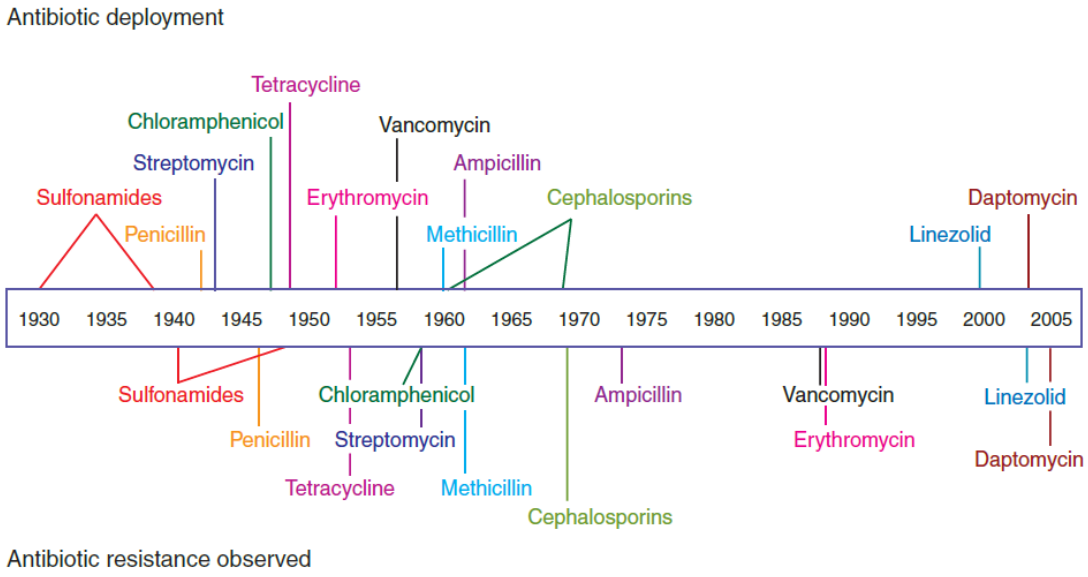


Figure 1. *Timeline of antibiotic deployment and the evolution of antibiotic resistance. The year each antibiotic class was introduced in clinical use is shown above the timeline and the first observation of resistance below. Only a selection of antibiotic classes are shown [6]. Reprinted with permission.*

The global expansion of resistant bacteria that has occurred especially in the past 20 years is considered a serious threat to human health. It is caused by a complex relationship between antimicrobial use in humans and animals, antimicrobials from pharmaceutical industry, genetic events in microorganisms, the environment, international travel and transmission in hospitals and community (**Figure 2**) [7].

The World Health Organization has identified pathogens of critical priority for development of new antibiotics [8]. The most critical pathogen is *Mycobacterium tuberculosis*, followed by carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa* and third generation cephalosporin-resistant (3GCR, mainly due to ESBL) *E. coli.*

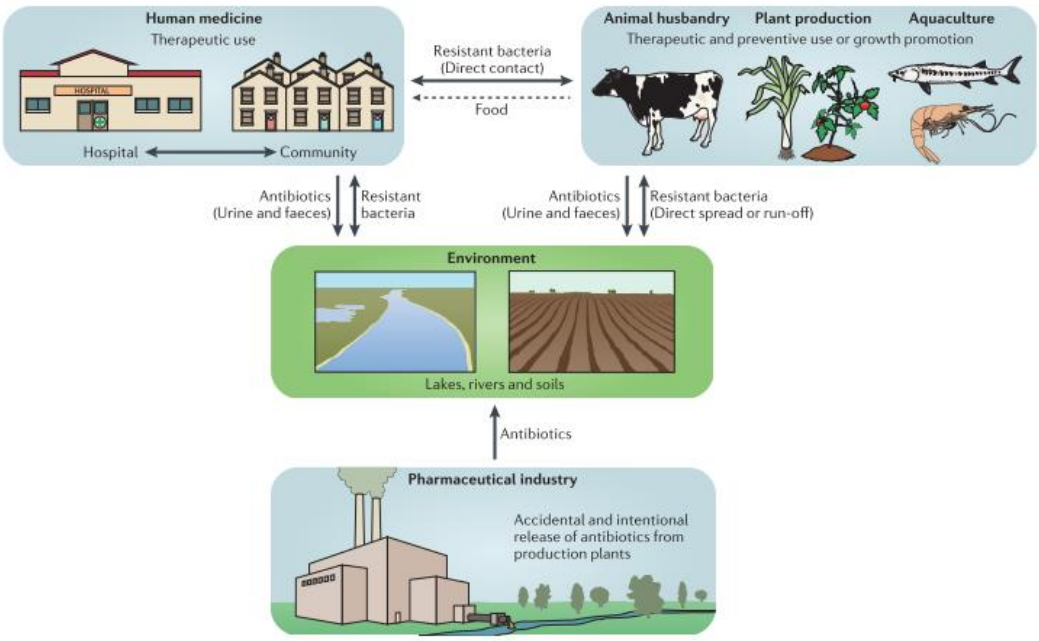


Figure 2. *Ecology of antibiotics and antibiotic resistance. Selection and induction of antibiotic resistance occur in all ecological niches where antibiotics and bacteria co-exist. These include microbiota in humans, livestock/agriculture and the environment. Antibiotics in the environment come from human and hospital waste, food production and pharmaceutical industries. Resistant bacteria are circulated between the different niches [7]. Figure reprinted with permission.*

Cassini *et al.* have estimated the burden of disease of AMR bacteria in the European Union and the European Economic Area in 2015 [9]. The pathogen with the highest burden of disease was 3GCR *E. coli* for which the estimated number of infections were 297,416 and the number of attributable deaths 9,066. It seems like infections with AMR bacteria is added on the burden of infection of susceptible bacteria instead of replacing infections with susceptible strains. The burden is higher in southern European countries, especially Italy and Greece. Additionally, in these countries with high rates of antimicrobial resistance, there has been a shift in the species-distribution in bloodstream infections, with species such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* increasing. These species feature intrinsic resistance to many antibiotics, which further aggravates the burden of AMR [10].

1.1.2 Antibiotics with effect on Gram-negative bacteria

The potential of an antibiotic agent to have effect on bacteria is heavily dependent on the bacterial cell wall. Gram-negative and Gram-positive bacteria have very different cell wall properties. Thus, the effect of an antibiotic agent is often limited to either Gram-positive or Gram-negative bacteria. **Table 1** summarizes the target and major resistance mechanisms (explained in **Figure 3**) of clinically relevant antibiotics with effect on Gram-negative bacilli. The table also displays their activity on Enterobacterales producing the β-lactamases covered in this thesis: ESBL and New Delhi Metallo-β-lactamase (NDM).

Table 1. *Overview of antibiotics with effect on Gram-negative bacteria in clinical use. Table originates from Morar et al. [3]. Modified and reprinted with permission.*

Antibiotic class	Examples	Activity against EPE[A]	Activity against NDM -isolates [11]	Target	Mode of resistance
β-lactams					
Penicillins	Ampicillin	-	-	Cell membrane - peptidoglycan biosynthesis	Hydrolysis Altered target Porin loss Efflux
3G-cephalosporins	Cefotaxime	(-)	-		
	Ceftriaxone	(-)	-		
	Ceftazidime	(-)	-		
Carbapenems	Ertapenem	+	-		
	Imipenem	+	+/-		
	Meropenem	+	+/-		
Monobactams	Aztreonam	-	+/-		
β-lactams with β-lactamase-inhibitors [12]	Piperacillin-tazobactam	+	-		
	Ceftazidime-avibactam	+	-		
	Ceftolozane-tazobactam	+	-		
	Meropenem-vaborbactam	+	+/-		
Other classes					
Aminoglycosides	Amikacin	+	(-)	Translation	Phosphorylation Acetylation Nucleotidylation Efflux Altered target
	Gentamicin	+/-	(-)		
	Tobramycin	+/-	(-)		
Fluoroquinolones	Ciprofloxacin	+/-	(-)	DNA replication	Acetylation Efflux Altered target
	Levofloxacin	+/-	(-)		
Pyrimidines/ Sulfonamides	Trimethoprim/ Sulfamethoxazole	+/-	(-)	Nucleotide synthesis - C1 metabolism	Efflux Altered target
Cationic peptides	Colistin	+	+	Cell membrane - lipid A	Altered target Efflux
Rifamycin	Rifampicin	(+)	(+)	Transcription	Altered target Efflux
Glycylcyclines (Tetracycline-derivates)	Tigecycline	+	(-)	Translation	Monooxygenation Efflux Altered target
	Eravacycline	+	(-)		
Phosphonic acid [13]	Fosfomycin	+	(-)	Cell membrane - peptidoglycan biosynthesis	Decreased permeability Enzymatic modification

[A]Susceptibility according to supplemental material for **Study I**.
Legend for activity columns: + resistance uncommon (<20%); (+) agent should only be used in combination with other active agents; +/- resistance common (>20%); (-) usually resistant, susceptible isolates can occur; - always resistant. 3G: Third generation

1.1.2.1 β-lactam antibiotics – structure and target.

The β-lactam antibiotic group is the most widely used antibiotic class [12]. The main advantages with β-lactams are their low toxicity, high efficacy and that there is a variety of β-lactams. Some have a narrow Gram-positive spectrum (*e.g.* phenoxymethylpenicillin) while other are broad-spectrum, potent antibiotics (*e.g.* meropenem). β-lactam-antibiotics are organic molecules with a β-lactam ring. They cause a bactericidal effect on dividing cells by their covalent binding that inhibits penicillin-binding-proteins (PBP). PBPs are important in stabilizing the cell wall when the cell wall is expanded after cell division. PBPs are transpeptidases that create peptide-cross-linking between the peptide chains when the peptidoglycan layer is built. There are many different PBPs. Each bacterial species has its own set of PBPs, often three to eight different types, of which usually one or two are essential. In Gram-negative bacteria PBP1a, PBP1b, PBP2 and PBP3 are essential [12]. Inhibition of PBP will arrest cell wall building and cause cell death. The different sub-classes

of β-lactam agents have different affinity to different PBPs, which explains the difference in antimicrobial spectrum.

1.1.2.2 β -lactam-β-lactamase inhibitor combinations

Combining a β-lactam with a β-lactamase-inhibitor restores the activity of the β-lactam. The β-lactamase-inhibitor acts as a suicide inhibitor as it is hydrolysed by the β-lactamase during the reaction that causes inactivation of the β-lactamase. Amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftazidime-avibactam, ceftolozane-tazobactam and meropenem-vaborbactam are combinations that have effect on ESBL-producing Enterobacterales (EPE) (**Table 1** and **Table 2**) [14]. Avibactam and vaborbactam are more potent β-lactamase inhibitors than tazobactam and have effect both on EPE and some carbapenemase-producing isolates, but not on metallo-β-lactamases (*e.g.* NDM) [15].

1.1.2.3 Cephalosporins

As penicillins mostly have Gram-positive activity, the cephalosporins were developed for enhanced activity on Gram-negative bacteria. The first cephalosporins were oral, and resistance developed rapidly, with the TEM-1 penicillinase first observed in 1974. Since then, several generations of cephalosporins were developed [12]. The 1st generation cephalosporins are cefaclor and cefalexin.

2nd generation cephalosporins include oral compounds such as cefadroxil, which is used for uncomplicated cystitis and as a screening substance for EPE in some countries. Cefuroxime, which has some effect on Gram-negative bacteria, but more pronounced Gram-positive activity also belongs to this group [12].

3rd generation cephalosporins (called extended-spectrum cephalosporins) including cefotaxime, ceftriaxone, ceftazidime and ceftibuten, were very potent when they were introduced in the clinic in the 1980s and quickly became first-line treatment for Gram-negative infections [12]. Cefotaxime is still the empirical treatment of choice for suspected Gram-negative infection in immunocompetent patients in low resistance-prevalence countries such as Sweden. The increase in resistance to these antibiotics (3GCR), mainly caused by extended-spectrum β-lactamases, ESBLs, is a public health threat [8].

4th generation cephalosporins, *e.g.* cefepime, is not widely used in Sweden. The antimicrobial spectrum is similar to 3rd generation cephalosporins and $ESBL_A$-production also causes resistance to cefepime. However, cefepime has higher hydrolytic stability to AmpC-enzymes compared with the 3rd generation cephalosporins [16].

5th generation cephalosporins include ceftolozane which is combined with tazobactam. It has an improved effect on isolates with porin-loss and has a potent effect on *Pseudomonas aeruginosa*, but also on EPE [12, 14]. However, resistance may occur through hyperproduction of AmpC cephalosporinase, which can develop during therapy [17]. Other agents in this group are ceftaroline and ceftobiprole that have anti-MRSA effect in contrast to all other β-lactam-antibiotics, but the Gram-negative activity is similar to that of cefotaxime.

1.1.2.4 Carbapenems

The carbapenems (ertapenem, imipenem, meropenem) have the broadest spectrum of β-lactam antibiotics and they are stable against hydrolysis by ESBL- and AmpC-cephalosporinases [12]. They are, however, susceptible to hydrolysis by carbapenemases. Some carbapenemase-producing isolates can retain a degree of susceptibility to meropenem and/or imipenem. These agents are thus still useful in combination therapy for carbapenemase-producing isolates. However, NDM-producing isolates often have high-level resistance to meropenem, particularly this is true for *K. pneumoniae* [18].

1.1.3 Resistance mechanisms

1.1.3.1 Overview

There are four principal bacterial resistance mechanisms that inactivate the effect of antibiotics (**Figure 3**) [19]: A) **Impermeability**. In Gram-negative bacteria this means a loss or conformational changes of porins in the cell membrane [20]. This restricts the amount of antibiotics that can enter the periplasmatic space, which in combination with a β-lactamase aggravates the level of resistance. However, impermeability is often associated with decreased fitness, since important nutrients cannot enter [21]. B) **Efflux pumps** in the cell membrane cause active transport of antibiotics out of the cell. C) **Target modifications** can occur through resistance mutations or through enzymatic modification of the target site. D) **Drug inactivation.** Enzymes, either in the cytoplasm or in the periplasmatic space inhibit the activity by binding or through enzymatic degradation.

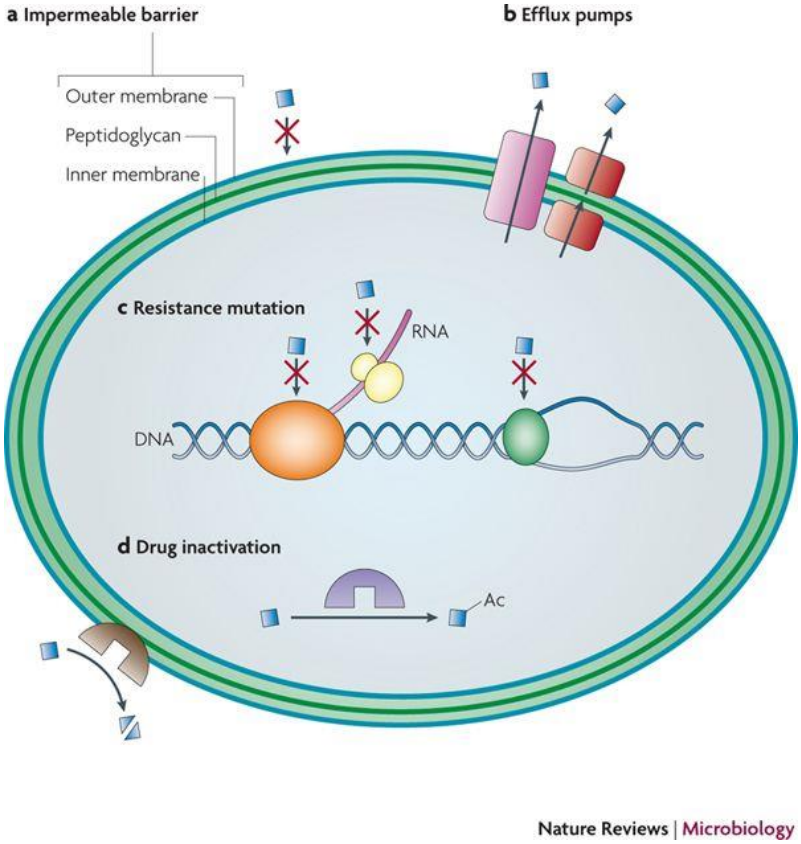


Figure 3. *Principal resistance mechanisms in Gram-negative bacteria [19]. Drug inactivation (d) is the major resistance mechanism for β-lactam antibiotics in Gram-negative bacteria. β-lactamase enzymes hydrolyse β-lactam antibiotics in the periplasmatic space between the inner and outer membrane in the bacterial cell wall. Reprinted with permission.*

For β-lactam agents the two most important resistance mechanisms are enzymatic inactivation by β-lactamases in the periplasmic space and target modifications of PBPs (often due to expression of an alternative PBP). Porin loss can aggravate the effect of β-lactamases and efflux pumps are common causes of carbapenem resistance in *Pseudomonas aeruginosa*. However, in Enterobacterales the most prevalent and damaging resistance mechanism is β-lactamase-production.

1.1.3.2 β-lactamases in Enterobacterales

There were more than 2,800 unique β-lactamases described by 2018 [15]. **Table 2** summarizes various aspects of the most clinically important β-lactamases. β-lactamases are located in the periplasmic space in the cell wall, where they hydrolyse the β-lactam-ring and thus inactivate the agent. The various β-lactamases have different substrate specificity, which means that they can only hydrolyse certain kinds of β-lactam classes. Every time a new class of β-lactam (developed to overcome the resistance caused by current β-lactamases) has been clinically introduced, new β-lactamases have evolved that also hydrolyse that class.

***Table 2**. Overview of β-lactamases.*

Swedish ESBL-Terminology	Common name	Ambler class	Examples	Resistance phenotype[A] Pc./ 1GC	3GC	Carba-penem	Diagnostic inhibitor	Clinical inhibitor
	Penicillinase	A	Staphylococcal enzymes	Pc. only			-	CLA, TAZ, AVI, VAB
-	Oxacillinase	D	OXA-1	Oxacillin			-	AVI
	Broad-spectrum penicillinase	A	TEM-1[B], SHV-1[B]	x			-	CLA, TAZ, AVI, VAB
$ESBL_A$	Extended-spectrum β-lactamase	A	**CTX-M**, TEM[B], SHV[B]	x	x		CLA	CLA, TAZ, AVI, VAB
$ESBL_M$	Plasmid AmpC cephalosporinase	C	pAmpC CIT, CMY, DHA	x	x		Cloxacillin	AVI, VAB
-	Chromosomal AmpC cephalosporinase	C	cAmpC	x	x		Cloxacillin	AVI, VAB
$ESBL_{CARBA}$	Serine carbapenemase	A	KPC, IMI, SME	x	x	x	Boronic acid	AVI, VAB
	Metallo-β-lactamase	B	**NDM**, IMP, VIM	x	x	x	EDTA	-
	Carbapenem-hydrolysing class D enzyme (Oxacillinase-derived)	D	OXA-48	x		x	-	AVI

[A]"x" means that the agent is a substrate for the enzyme. The level of resistance varies, some isolates appear susceptible despite presence of the enzyme.
[B]There are hundreds of different TEM- and SHV- enzymes. Some can hydrolyse 3GC ($ESBL_A$) and some (*e.g.* TEM-1 and SHV-1) only hydrolyse 1GC and are not classified as ESBLs.
1GC: 1st generation cephalosporin; 3GC: 3rd generation cephalosporin; Pc.: penicillin; CLA: clavulanic acid; TAZ: tazobactam; AVI: avibactam, VAB: vaborbactam
Table based on information in several reviews [15, 22-26].

In this thesis, the two terns ESBL and 3rd generation cephalosporin-resistant (3GCR) are widely used. The definition of the term ESBL used is the Swedish reporting definition, which includes both $ESBL_A$ (classic Extended-spectrum β-lactamase, inhibited by clavulanic acid) and $ESBL_M$ (plasmid-mediated AmpC-cephalosporinase, inhibited by cloxacillin). From clinical and infection-control perspectives, the spectrum of resistance (implications for treatment) and the location of the β-lactamase on a plasmid (implications for transmission) are more important than the type of inhibition, which is the rationale for this classification [25]. The term 3GCR denotes all isolates resistant to 3rd generation cephalosporins. In addition to $ESBL_A$ and $ESBL_M$, this term includes isolates with chromosomal AmpC and

isolates with other resistance mechanisms, such as hyperproduction of penicillinase in combination with impermeability. However, in *E. coli* and *K. pneumoniae* most 3GCR is due to $ESBL_A$.

$ESBL_{CARBA}$ is the Swedish term for carbapenemase. The type of carbapenemase produced influences the level of resistance to carbapenems, diagnostic sensitivity of screening- and confirmation-tests and predicts resistance to ceftazidime-avibactam [15].

New Delhi metallo-β-lactamases (NDM) and other metallo-β-lactamases (MBL), generate high-level resistance to carbapenems. They are thus easy to detect with diagnostic tests and are always resistant to ceftazidime-avibactam and meropenem-vaborbactam [27].

The OXA-carbapenemases that occur in Enterobacterales are found in the OXA-48-group, while many other OXA-carbapenemases (OXA-23-group, OXA-51-group, OXA-58-group) are restricted to *Acinetobacter species* [28]. OXA-carbapenemases often have a weaker hydrolysing effect on carbapenems than the other carbapenemases. Because of this, they are more difficult to identify but are still important from an infection control perspective as minor mutations, changes in expression or porin loss can rapidly cause high-level resistance to carbapenems. OXA-carbapenemases do not hydrolyse cephalosporins. Many, but not all, isolates are nevertheless resistant to cephalosporins due to cephalosporinases. They are always resistant to piperacillin-tazobactam and most types confer resistance to temocillin [29]. However, temocillin-resistance is not specific for OXA-carbapenemases and as was noticed in **Study II**, not all OXA-carbapenemases display *in vitro* resistance to temocillin. Temocillin susceptibility seem to be especially common with OXA-244, one of the members of the OXA-48-group (unpublished data). Thus, detection of OXA-carbapenemases is a challenge for laboratories. OXA-carbapenemases are inhibited by avibactam.

The serine carbapenemase *K. pneumoniae* carbapenemase (KPC) is globally the most common carbapenemase and is inhibited by avibactam, vaborbactam and boronic acid [15]. However, resistance for ceftazidime-avibactam is described and can develop during treatment. In some cases, the resistance is caused by increased expression of KPC combined with porin loss [30].

1.1.4 Global epidemiology of ESBL- and carbapenemase-producing *E. coli* and *K. pneumoniae*

In many countries in Asia, where resistance data are available, more than 50% of *E. coli* in bloodstream infections are 3GCR (**Figure 4**). In most parts of northwest Europe, Canada, the USA and Australia the rate is less than 25% [31]. A majority is caused by enzymes in the CTX-M group, especially CTX-M-15 which is carried by the successful clone ST131 (see **Section 1.5.3**).

The most notable rates of carbapenemase-production in *K. pneumoniae* (**Figure 5**) are reported from India (59% reported resistance in 2017, mainly NDM), Greece (66% in 2017, mainly KPC), Italy (32% in 2017, mainly KPC), the Middle East (17-70% in 2016-2017, mainly OXA-48) and Latin America (8-20% in 2013-2017, mainly KPC) [31-34]. In most countries in northwest Europe, USA, Canada and Australia less than 5% of *K. pneumoniae* are CPE. For *E. coli,* no countries report more than 20% carbapenem-resistance [31].

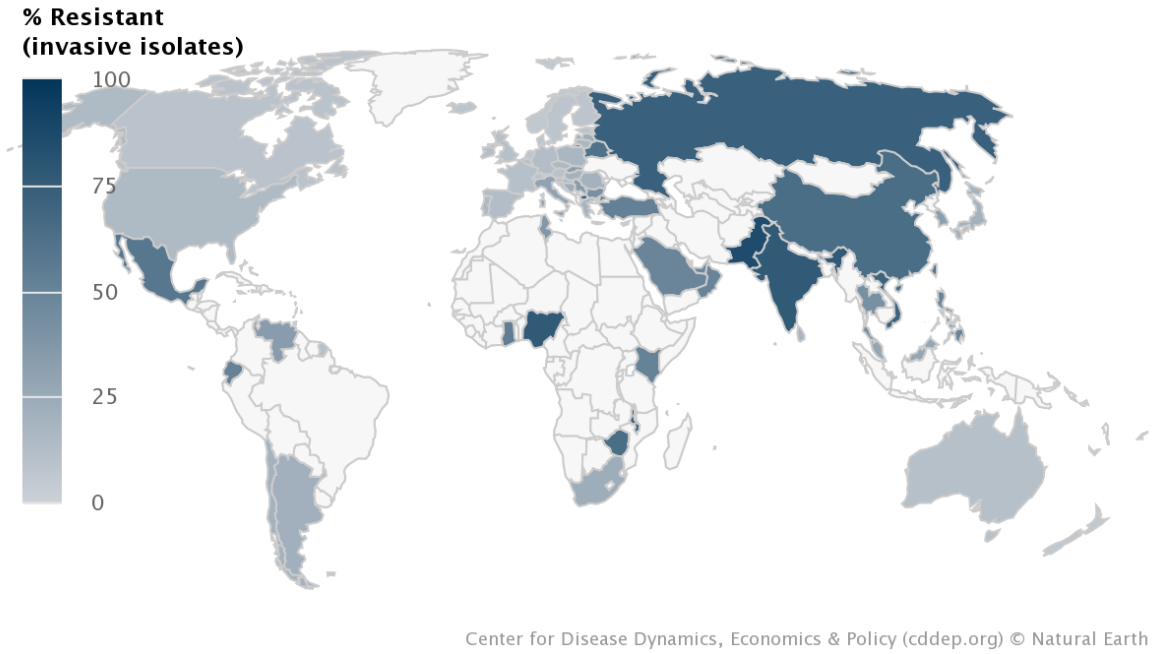


Figure 4. *Resistance of E. coli to 3rd generation cephalosporins 2014-2017. Data from several sources (including EARS-Net and GLASS 2017) compiled by Center for Disease Dynamics, Economics and Policy. https://resistancemap.cddep.org. Data show aggregated resistance rates for isolates from blood and cerebrospinal fluid. Due to differences in sample collection and testing methods, rates should be interpreted with caution. For some countries rates are based on less than 100 isolates. Light grey countries lack data. Note that the colour-scale is different compared with* ***Figure 5*** *[31].*

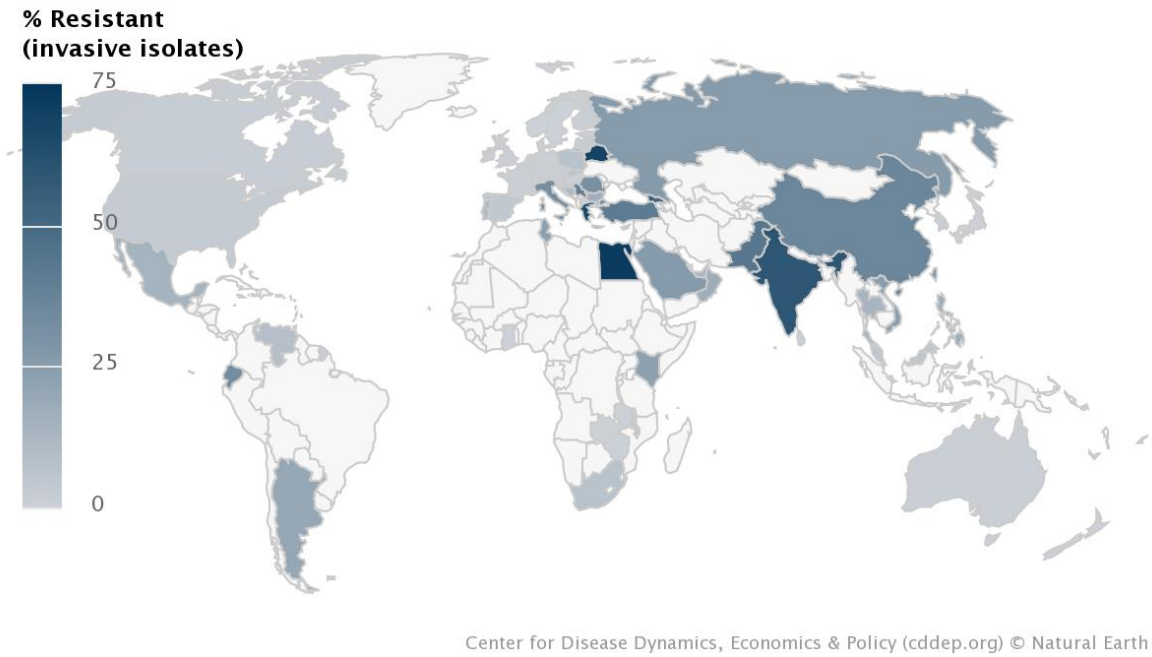


Figure 5*. Resistance of K. pneumoniae to carbapenems 2014-2017. Data from several sources (including EARS-Net and GLASS 2017) compiled by Center for Disease Dynamics, Economics and Policy, https://resistancemap.cddep.org. Data show aggregated resistance rates for isolates from blood and cerebrospinal fluid. Due to differences in sample collection and testing methods, rates should be interpreted with caution. For some countries rates are based on less than 100 isolates. Light grey countries lack data. Note that the colour-scale is different compared with* ***Figure 4*** *[31].*

Information about the situation in Africa is scarce due to lack of structured AMR surveillance and in many cases lack of access to microbiological diagnostics.

1.1.4.1 Local epidemiology of ESBL-producing E. coli and K. pneumoniae in Stockholm

The rate of 3GC-resistance in *E. coli* from blood cultures sampled at Karolinska University Hospital increased rapidly from 2005 to 2012 (**Figure 6**). Since then the rate has stabilized around 10%. For *K. pneumoniae* the rate of ESBL-production was 7.5% in total in four Stockholm hospitals (Karolinska Huddinge, Karolinska Solna, Södersjukhuset and Danderyd Hospital) in 2018. Carbapenem-resistance occurred in less than 1% of both *E. coli* and *K. pneumoniae* in 2018 [35].

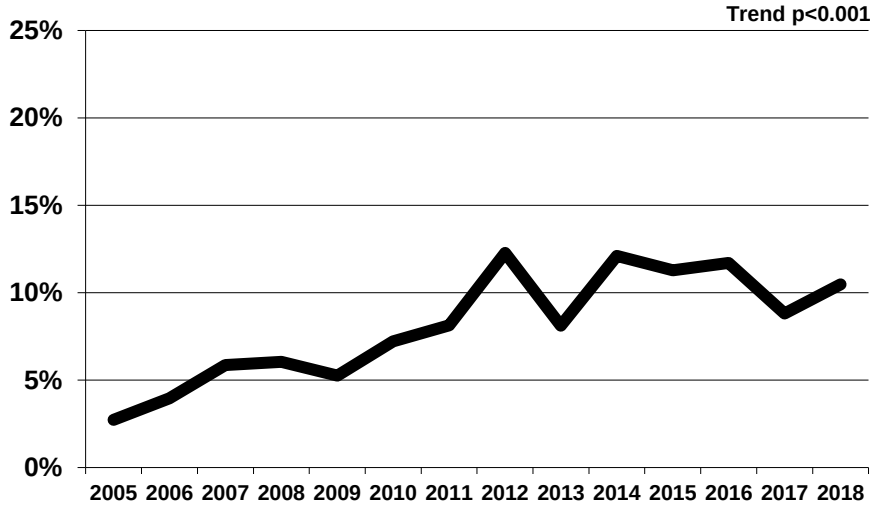


Figure 6*. Proportion of E. coli from blood cultures sampled at Karolinska University Hospital that were cefotaxime-resistant (R) or intermediate (I), per year. Reduced susceptibility to cefotaxime is in most cases caused by ESBL (ESBL$_A$ or ESBL$_M$) and the proportion reflects the increase of ESBL-EC. Local data from Karolinska University Laboratory.*

1.2 BLOODSTREAM INFECTIONS, SEPSIS AND SEPTIC SHOCK

For recognized pathogens, such as *E. coli* and *K. pneumoniae*, a bloodstream infection means that the patient has a positive blood culture with the pathogen and this term is used synonymously with bacteraemia [36]. For skin commensal species that often cause contamination of blood cultures the definition of bloodstream infection/bacteraemia is more complex: the patient needs to have two positive blood cultures with the same species and at least one of the following symptoms: fever, chills or hypotension. Bloodstream infections usually originate in a localized infection in some part of the body, for example urinary tract infection, pneumonia, skin and soft tissue infections or an abdominal infection. Serious infections can cause sepsis, depending on the patient's immune response. The pathophysiology in sepsis is complex and obscure despite intense research on the subject.

Previously sepsis was described mainly as an *overactivation* of the immune response: an excessive activation of the immune response led to hyperinflammation, increased vascular permeability, coagulation and organ failure. However, this model has been revised and sepsis is now expressed as a *dysregulation* of the immune response since both pro- and anti-inflammatory systems are activated at the onset of sepsis which causes a functional inhibition of inflammatory cells [37]. In 2016 the "Sepsis Definitions Task Force" published new

guidelines on the definition of sepsis (Sepsis-3) [38]. The new definitions read:
*"**Sepsis** is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection."*
*"**Septic shock** is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality."*

The Sepsis-3 clinical criteria for sepsis and septic shock emphasise that an infection is a prerequisite for sepsis and they are based on the Sequential (Sepsis-related) Organ Failure Assessment (SOFA) score [38]. The SOFA score grades the level of organ failure based on a set of laboratory variables and clinical interventions. The clinical criteria for sepsis are a suspected or documented infection and an acute increase of ≥2 points of the SOFA score. Clinical criteria for septic shock are sepsis, the need for vasopressor therapy and an increased lactate despite adequate fluid resuscitation [38].

A simplified summary of some of the observations that supports the theory of dysregulation follows. Serum-levels of both pro-inflammatory (IL-1, IL-6 and TNF-α) and anti-inflammatory (IL-10 and IL-1a) are simultaneously increased and then slowly decrease [37]. Neutrophilic granulocytes, a type of leukocytes which are important in the early response to pathogens, are increased in numbers, but are often dysfunctional, do not react appropriately to stimuli and have decreased ability to phagocytose pathogens [39]. Both cytotoxic T-cells and T helper cells are affected by increased apoptosis and fatigue. However, regulatory T-cells are not affected by this, which leads to immunosuppression. In addition, metabolic, endocrine and neuronal signalling pathways are also affected. Combined, the dysregulated immune response leads to organ failure and death in septic shock. If the patient survives it is common with a continued immunosuppression and dysregulation of the immune response, which can lead to secondary infections and increased mortality in a longer perspective.

Further complicating the understanding and the possibility to develop immunotherapies for sepsis is that there is a pronounced heterogenicity between patients in their host response. A recent study analysed genome-wide gene expression in blood leukocytes from patients with sepsis admitted to the ICU [40]. Four endotypes with different expression profiles were identified based on the transcription-profiles. These four profiles differed in mortality and the proportion of patients with septic shock. One group of patients had an endotype characterized by a marked under-expression of genes involved in innate and adaptive immune functions. This group was associated with the highest mortality.

Sepsis is thus an immunologically complex condition characterized by a dysregulated immune response, which in some individuals may lead to death. The level of dysregulation and immune expression varies between patients which probably means that individualized treatment and related diagnostic tests are necessary for success in the development of future immune-modulatory treatments for sepsis. Nevertheless, survival of bloodstream infections is not solely dependent on the host immune response – appropriate antimicrobial treatment and the virulence of the infecting microbe also play important parts.

1.3 THE IMPORTANCE OF APPROPRIATE EMPIRICAL THERAPY

In severe infections the time to appropriate therapy is vital for the survival of the patient [41, 42]. In the absence of ultra-rapid, point-of-care diagnostic tests for detection of sepsis pathogens and resistance markers, to aid in the decision of antimicrobial treatment, empirical treatment needs to be initiated before the identity of the species causing the infection is known. The decision is based on the patient's presentation, history and severity of illness. Invasive infections caused by ESBL-producing Enterobacterales (EPE) are associated with a high risk of receiving inadequate empiric treatment, and these patients seem to be at risk of higher mortality [43].

In a study by Tumbarello *et al.*, on 186 patients with bloodstream infection (BSI) caused by EPE, 48% of the patients received inadequate therapy. Out of these 60% died within 3 weeks, as compared with 18% of patients with adequate initial therapy [43]. However, in a Dutch study, inappropriate therapy within 24 hours was not associated with a higher mortality within 30 days in the multivariable analysis [44]. Other studies have also shown somewhat conflicting results on mortality and according to a review by Trecarichi *et al.*, this is mostly due to limitations in sample sizes [45]. In this review, the authors report four studies with sample sizes larger than 342 patients. In three of these studies ESBL-production was independently associated with mortality. Although it has been difficult in some retrospective studies to show the impact of inappropriate therapy in ESBL-bacteraemia on mortality, a meta-analysis by Schwaber and Carmeli demonstrated a significant increase in mortality associated with ESBL-production [46, 47]. There is also no doubt that inappropriate therapy caused by resistant bacteria increases the length of hospital care and costs [46, 48].

Empiric treatment is given to treat the infecting organism, and when the organism is resistant, a favourable outcome is probably mostly due to the patients' immune system and supportive therapy. Alternatively, the infection might be primarily a urinary tract infection with secondary bacteraemia, and that the high antibiotic concentration in the urine is sufficient despite resistance *in vitro*. The differences between mortality outcomes in different studies might be due to a difference in the severity of the infections included. In severe infections it is important that all treatment is optimized. ESBL-production by the infecting bacterial strain in sepsis was in one study identified as the most important predictor for readmission within 30 days, with an odds ratio (OR) of 4.5 (p=0.01) [49].

1.3.1 Risk factors for invasive infections with ESBL-producing Enterobacterales

It is clear that it is important to identify patients with an increased risk of having an infection with an ESBL-producing strain when empirical treatment is initiated, especially in severe infections. Several studies have been performed to address the risk factors of ESBL bloodstream infection, including one Swedish study [50]. Trecharichi *et al.* reviewed 30 of these studies in 2012 [45]. The following risk factors were in several of these studies associated with ESBL BSI in multivariable analysis: Previous antibiotic treatment, especially third generation cephalosporins and fluoroquinolones; previous hospitalization, residency in long-term care facility and healthcare contacts; presence of indwelling devices, especially urinary tract catheterization; and presence of co-morbidities. Contrarily, in the Swedish study by van Aken *et al.* in 2014, a prior culture positive of ESBL-producing bacteria was the only

significant risk factor for BSI with ESBL in the multivariable analysis [50]. “A prior culture” probably included both screening and clinical cultures, but this is not clearly stated. However, the sample size in this study was quite small; only 70 cases of BSI caused by ESBL-producing *E. coli* and 140 non-ESBL *E. coli* controls were included.

One problem with many of the previous studies is that they include both community- and hospital-acquired infections, without separate analysis. This is unfortunate, since these two patient groups are very different. Rodriguez-Bano *et al.* studied risk factors for ESBL *E. coli* BSI in two separate studies, one on community-acquired BSI and one on nosocomial BSI [51, 52]. They compared the same cases (ESBL-EC BSI) with two separate control groups: non-ESBL *E. coli* BSI and all patients with suspected BSI. They found that somewhat different risk factors were associated with ESBL *E. coli* BSI in nosocomial BSI and community-onset BSI. In the study on nosocomial BSI, the risk factor with the strongest association to ESBL-producing *E. coli* was previous use of third generation cephalosporins [51]. This variable had a significant association regarding both control groups used, patients with nosocomial BSI, and patients with nosocomial BSI caused by non-ESBL *E. coli*. The association was stronger when compared with non-ESBL *E. coli* than when compared to suspected BSI-controls, which supports the theory described above that the importance of previous antibiotic treatment is exaggerated when compared with susceptible strain infections. In the second study, on community-onset BSI, three variables were associated with ESBL-EC in multivariable analysis when compared to both control groups: healthcare associated bacteraemia, urinary catheter use and recent antimicrobial use [52].

At least five additional studies on risk factors for ESBL-production in community-onset BSI have been published in recent years, from Israel, South Korea and China [53-57]. The design of the studies varies, which makes them difficult to compare, and in several of them susceptible strains of the same species have been used as controls. However, the variables that were associated with ESBL in more than two of these studies in multivariable analysis were: 1) admission from long-term care facility/healthcare-associated infection, (four studies) [53-56]; 2) recent use of antibiotics/previous third generation cephalosporin use/admitted on antibiotics (four studies) [54-57]; and 3) severe infection at admittance (two studies) [54, 56].

1.3.2 The importance of local epidemiology

The risk factors for infections with ESBL-producing bacteria vary between different regions and are related to the level of resistance. For example, in the Chinese study of risk factors of ESBL BSI by Quan *et al.*, 55% of *E. coli* recovered from blood cultures were ESBL-positive, [55] while the resistance level was still below 10% in Sweden in 2014 [58]. Therefore, it is not possible to apply a risk-scoring system developed in another epidemiological setting, without local validation and adaptation. However, due to differences in sampling practices, it is difficult to compare resistance rates from different regions. For example, in some countries blood samples are only taken in treatment failures or nosocomial infections, which of course results in a higher proportion of resistant isolates.

1.3.3 Predicting the probability of infection with ESBL-producing Enterobacterales at admission to hospital

One of the most important reasons for studying risk factors for BSI with ESBL-producing bacteria, is the desire to be able to identify patients at risk for having this type of infection, at the time empiric therapy is initiated. However, the source population in many of the studies described above does not adequately reflect the patient population in which the question of suitable empirical therapy arises, which is further discussed in the methods section (**Section 3.1.1.1**). Additionally, the odds ratios for several of the associated variables are usually quite small, although significant, and the predictive value is thus limited.

As Rottier *et al.* showed in a Dutch study 2015, basing therapy decisions solely on previous carriage with ESBL-producing Enterobacterales (EPE) or antibiotic treatment leads to unnecessary use of broad-spectrum antibiotics [59]. They evaluated the effects of Dutch recommendations to give broad-spectrum antibiotics (third generation cephalosporin + aminoglycoside, or carbapenem) to all patients with suspected sepsis of unknown origin who had previously documented EPE-colonization or previous cephalosporin/fluoroquinolone use. In this study, the positive predictive value (PPV) of prior colonization of with ESBL <3 months was 7.4, while the PPV was only 1.8, when both prior EPE-colonization <3 months and use of cephalosporin/fluoroquinolones <1 month were considered. The authors concluded that strict adherence to the guidelines would, for bacteraemia caused by Enterobacterales, lead to an overtreatment rate of 14%, a rate of carbapenem use of 18% and an inappropriate treatment rate of 3.5%. The corresponding rates for following guidelines, but disregarding previous antibiotic use, were a carbapenem use rate of 7.4%, an overtreatment rate of 4% and an inappropriate treatment rate of 4.2%. Thus, decreasing the inappropriate treatment to a minimal extent from 4.2% to 3.5%, would lead to a considerable amount of overtreatment with carbapenems.

In Italy, Tumbarello *et al.* developed a risk-scoring system in order to identify patients having infections with ESBL-producing bacteria, at admission to hospital [60]. The cohort consisted of patients admitted to hospital and the controls were matched for hospital ward and month of admission. In the risk-scoring system, they included the following variables that were associated with isolation of ESBL-producing Enterobacterales from a clinical sample: recent hospitalization, admission from another healthcare facility, Charlson co-morbidity index ≥4, previous therapy with β-lactams or fluoroquinolones, history of urinary catheterization, and age ≥70 years old. The different variables were assigned a score of 2 or 3. A total score of ≥6 gave a PPV of 82% and NPV of 81% for any infection (*i.e.* growth in clinical sample) caused by ESBL-producing bacteria. In their derivation cohort, only 17% were from blood samples, the other samples were from urinary tract (64%), skin and soft tissues (12%), lower respiratory tract (7%), biliary tract (4%), and surgical wounds (2%). However, patients with a known history of infection with ESBL-producing bacteria were excluded in this study. The Tumbarello-model thus predicts the probability of having any infection with ESBL-producing bacteria in patients without known history of ESBL-infection and admitted to hospital, regardless of clinical diagnosis.

The Tumbarello-model has been validated and adapted to local conditions in North Carolina, USA, by Johnson *et al.* [61]. In this study the cohort also consisted of patients admitted to

hospital, and cases were patients with any clinical sample positive for ESBL-producing bacteria. However, it is unclear whether patients with previous history of infection with ESBL-producing bacteria have been excluded, as they were in the Italian study. The distribution of sample sources was similar to that of the Italian study, as 15% were derived from blood cultures. The authors adapted the Italian model, by exchanging some variables, and changing the score of others. In this model, called the Duke model, the following variables were included: previous hospitalization; transfer from another healthcare facility; previous therapy with β-lactams or fluoroquinolones; recent history of urinary catheterization; and immunosuppression. With the Duke model, a score of ≥8 gives a PPV of 79% and an NPV 85% for having any infection with ESBL-producing bacteria. Given this, and if patients with a score of ≥8 were given empiric therapy covering ESBL-producing bacteria, 85% would have been given appropriate therapy, 3% overtreatment, and 12% inappropriate treatment.

In another study in the Netherlands, Plattel *et al.* tried to establish a system for predicting ESBL-carriage (not infection) at admittance to hospital, but failed with this objective since they could not find any sufficiently discriminatory variables [62].

1.4 METHODS FOR SUSCEPTIBILITY TESTING OF BACTERIA

1.4.1 MIC-determination

The reference method for AST according to ISO20776-1 is Minimal Inhibitory Concentration (MIC)-determination by broth dilution [63]. A standardized inoculum is added to tubes or microtiter-wells with antibiotics in specific concentrations with doubling dilution steps including the concentration 1 mg/L and incubated 16-24 hours depending on species. The MIC is the lowest concentration that prevents visible growth after this time.

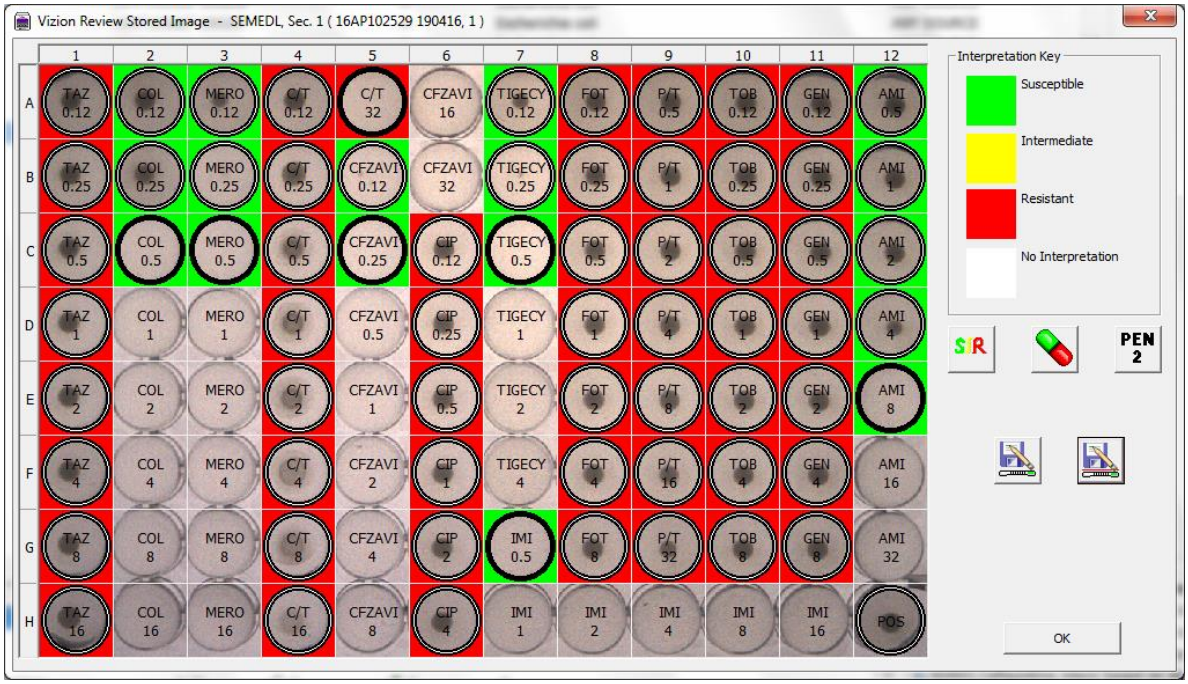


Figure 7. *Broth microdilution with a Sensititre panel read with Vizion software (Thermo Fisher Scientific, Waltham, Massachusetts). ESBL$_A$-producing E. coli from an external quality control programme. Photo from Karolinska University Laboratory.*

Traditionally this has been a labour-intensive method, and it has not been available in most laboratories. A commercially available product for broth microdilution (BMD) susceptibility testing is Sensititre® (Thermo Fisher Scientific, Waltham, Massachusetts), which offers standard panels with pre-prepared antibiotic dilutions in 96-well microtiter plates (**Figure 7**) [64]. The most common method for routine MIC-determination in clinical laboratories is MIC-determination with gradient tests. Plastic or paper strips with a predefined gradient of antibiotic concentrations are placed on Mueller-Hinton agar inoculated with a bacterial suspension [65]. However, this method is unsuitable for certain antibiotics, such as colistin [66] and EUCAST has since 2015 issued several warnings concerning the performance of gradient tests for some antibiotics, including piperacillin-tazobactam [67].

1.4.2 Disk diffusion

Prediction models for identifying patients at risk for bloodstream infection caused by ESBL-producing bacteria at admittance could be an important aid in improving empirical therapy. However, definite therapy must always be guided by identification of the causing species and susceptibility testing. In most cases this is done by bacterial culture in blood culture systems, followed by species identification with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or phenotypic tests. Time to detection in blood culture systems is usually 4-48 hours (for Enterobacterales mostly less than 24 hours) with species identification possible within 1- 4 hours after the blood culture bottle has turned positive.

Culture is followed by susceptibility testing, which traditionally unfortunately requires overnight incubation, further delaying susceptibility results with at least 16 hours. The routine susceptibility testing method recommended by the European committee of antibiotic susceptibility testing (EUCAST), is EUCAST disk diffusion (
Figure 8) [68]. Bacterial colonies are suspended in saline solution to a density of 0.5 McFarland. The suspension is inoculated on Mueller-Hinton agar plates (or Mueller-Hinton Fastidious agar), and standardized antibiotic disks are applied on the inoculated plates. After 16-20 hours of incubation, the inhibition zones are measured with a pair of callipers. The inhibition zones measured are interpreted as "Susceptible, "Susceptible, Increased exposure" , or "Resistant" according to breakpoints provided in EUCAST breakpoint tables [68].

Disk diffusion is an old and manual method, which requires trained staff and quality control for accurate results but has several advantages over new automated methods [69]. Apart from staff costs, the method is cheap, well validated and flexible. The selection of antibiotics that are tested can easily be adapted for local needs, sample types, resistance patterns and new antibiotic agents. The latter is an important point, in the development of new antibiotic agents, the most suitable disk strength for disk diffusion is developed in parallel with clinical trials. This means that disk diffusion disks are often commercially available within a few months of the approval of a new antibiotic, while the process of validating and developing automated AST methods often take more than two years [69].

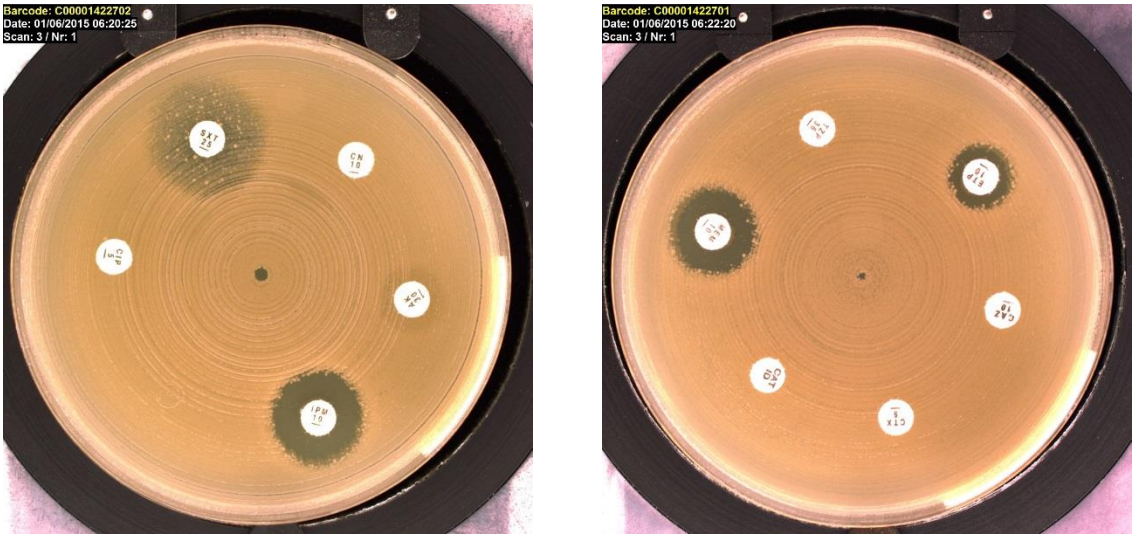


Figure 8. *EUCAST disk diffusion photographed at 18 hours of incubation. NDM-producing K. pneumoniae from* ***Study II****. The isolate was resistant to all tested antibiotics including piperacillin-tazobactam, cephalosporins, carbapenems, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole. Photo taken at Karolinska University Laboratory with BD Kiestra TLA.*

1.4.2.1 Automation of disk diffusion

Although disk diffusion traditionally is manual, there is a huge potential for full automation, including streaking of plates, application of antibiotic disks, incubation, photography and reading of inhibition zones, and some studies have shown improved standardization of disk diffusion with automation [70, 71]. Some fully automated methods for disk diffusion exist, such as WASP™ system (Copan Italia SpA, Brescia, Italy) [71] and there are also specialized instruments for automatic reading of inhibition zones such as SIRscan (i2a diagnostics, Montepellier, France) [70], Scan 4000 (Interscience, Saint Nom, France) [72] and the stand-alone software AntibiogramJ [73]. For the total lab automation systems such as the BD Kiestra™ TLA/WCA [74, 75] in use at Karolinska University Hospital, the important steps in disk diffusion – spreading of plates, application of disks and assigning inhibition zones on digital images – are nevertheless still manually performed by laboratory technicians. A limitation for automation is that specialized software for reading of inhibition zones in many cases is restricted to the software supplied by the automation company, and a competing software that might be better for disk diffusion reading is difficult (almost impossible) to implement. A total lab automation system is an expensive investment for a laboratory and is not easily changed. This means that the laboratory is dependent on the willingness of the company to develop and validate automated reading of disk diffusion.

1.4.3 Phenotypic versus genotypic methods

As was described in **Section 1.1.3** bacteria can have many different resistance mechanisms that can co-act and together confer a higher level of resistance. For some resistance mechanisms, *e.g.* porin loss, there are many different genes. Additionally, changes in the regulation of expression can influence the level of resistance [76].

Phenotypic methods such as BMD and disk diffusion described above can be used both for demonstrating susceptibility and resistance. However, while PCR-based methods are rapid, they can only demonstrate the presence of a resistance gene [77]. For some genes, such as NDM, this reliably predicts phenotypic resistance for β-lactam-antibiotics. But other genes might be present without causing a clinically relevant resistance. With advances in whole genome sequencing techniques, this method could potentially be developed for prediction of resistance and possibly susceptibility but it will require advanced bioinformatic power and continuous development and validation against phenotypic methods to ensure detection of previously unknown resistance mechanisms not included in the database [76].

1.4.4 Rapid methods for susceptibility testing

Species identification is necessary for susceptibility interpretation. While the development of MALDI-TOF MS has revolutionized laboratory work in clinical microbiology, by shortening the time for isolate species identification from 20 hours to 15 minutes, a comparably rapid and reliable method of susceptible testing is still lacking [77].

As discussed above, PCR-based approaches are only able to identify resistance, but not susceptibility. Several PCR-based rapid methods for detection of sepsis pathogens, *e.g.* FilmArray® (BioFire, Salt Lake City, Utah) include selected resistance genes, for example KPC, which can be useful in high-prevalence regions [78, 79].

Currently there are many efforts to find ways to shorten the time for AST, including among others, digital time-lapse cytometry, microfluidic platforms, automated digital microscopy, two-photon excitation fluorometry, and mass-spectrometry based approaches [65, 77, 80-86]. Some of the new methods show promising results, but they are still labour-intensive, expensive and commonly suitable only for single sample tests and a limited number of antimicrobials. In relation to other methods, rapid disk diffusion has a potential as a cost-effective and flexible method if reproducible and accurate results can be produced. At the time of publication of **Study II**, there were some conference abstracts but only a few published studies describing the potential of rapid disk diffusion [87-90].

1.5 MOLECULAR EPIDEMIOLOGY AND VIRULENCE

The study of molecular epidemiology and genetic characteristics of bacterial strains is important for understanding of the transmission and evolution of resistance and virulence of bacteria. A correct understanding of transmission and selection mechanisms are vital for development of appropriate intervention strategies to limit antimicrobial resistance and to be able to identify transmission events and outbreaks in clinical settings. Identification of important virulence factors for infection and severity can also suggest potential targets for future treatment [6]. The methods referred to in this chapter are described in detail in the methods **Section 3.3**.

1.5.1 Evolutionary phylogeny of *E. coli*

The evolution of *E. coli* has been studied in detail because *E. coli* is such a diverse species, with individuals ranging from harmless commensals to highly pathogenic Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC) and *Shigella* species. Molecular studies have shown that *Shigella* is so closely related to *E. coli* that they are phylogenetically the same species, but *Shigella* carries important toxins causing a clinically well-recognized presentation which motivates keeping the name and distinction of *Shigella* [91].

The classic division of *E. coli* into phylogroups, A, B1, B2, D was proposed by Ochman and Selander in 1984 based on results of multilocus enzyme electrophoresis (MLEE) and further developed by Clermont *et al.* in 2013 with addition of the phylogroups C, E and F based on multilocus sequence typing (MLST) [91, 92]. As Wirth *et al.* showed in 2006, the original phylogroups were relevant from an evolutionary perspective, but more detailed insights in the phylogenetic relationship within *E. coli* could be elucidated with Achtman's MLST [91]. Although there was a good concordance between STs and phylogroups, there were also some intermediate groups with a mixture of genetic material from different phylogroups, as evidence of recombination. These intermediate groups were named ABD and AxB1. Their analysis indicated that the current *E. coli* population probably evolved from a common ancestor some 10-30 million years ago, after a bottleneck decrease in the population had occurred. The four ancestral phylogroups had then evolved clonally, but with different degrees of recombination. The amount of recombination is of importance for the study of phylogeny, since the horizontal gene transfers confuse the building and interpretation of phylogenetic trees. The extent to which recombination has occurred during evolution and continuously occurs, differed between the various phylogroups. According to the MLST-based analysis of Wirth *et al.*, the most conserved phylogroup was B2, while ABD was the most recombinant. *Shigella* and EIEC mostly belonged to ABD and B1. Other pathogenic *E. coli* were also associated with a high level of recombination (EIEC belonged to ABD, B1 and AxB1, EPEC to ABD, B1 and B2). The authors suggested that the high recombination in these pathogens possibly could be caused by a reduced function of the methyl-directed mismatch repair (MMR). A deficiency in this post-replicative repair system causes both a high mutation rate and high transfer rate. This gave these strains an advantage to enter and stay in the pathogenic niche, instead of in the commensal niche, since they need to adapt to the continuous selective pressure of the human immune system [91].

Interestingly however, ST131 (see detailed description below), which was responsible for a substantial part of the expansion of ESBL-producing *E. coli* belonged to phylogroup B2 and had a low rate of recombination in the core genome [93]. For ESBL-producing *E. coli* in Sweden, there was also a difference between the phylogroups found in the commensal faecal microbiota of carriers and those found in bloodstream infections [94]. In both groups phylogroup D was common. Phylogroups A and B1 were associated with faecal carriers, while phylogroup B2 (mainly ST131) was associated with bloodstream infection. This indicates that not only the level of recombination is important for pathogenicity and clonal expansion, other variables are also important.

1.5.2 Definition of high-risk clones and successful plasmids

The global increase of AMR in Enterobacterales is mainly driven by two mechanisms: successful high-risk clones and horizontal plasmid transfer [95]. Successful high-risk clones have been shown to have an epidemic potential and are common in all parts of the world. A clone in microbiology refers to closely related strains with a common ancestor. However, all individuals in a clone do not have identical genomes, small differences will always exist caused by mutations, horizontal gene transfers and recombination events [96]. Multidrug resistant (MDR) high-risk clones have been defined as clones that 1) show international distribution; 2) possess several types of clinically important resistance traits (MDR) 3) are able to colonize hosts for a prolonged period of time; 4) are efficiently transmitted between hosts; 5) show extended pathogenicity and fitness; and 6) are able to cause severe and/or recurrent infections [95-97]. The spread of these high-risk clones is due to a combination of acquired genetic properties enhancing the abilities described and the selective pressure of antimicrobial treatment [95].

Horizontal transfer of genes and plasmids is the second mechanism. According to Baquero and Coque, the potential success of these mobile genetic elements depends on four different properties necessary for transfer of a plasmid or transposon from one individual to another: "penetration (ability to reproduce and spread), promiscuity (ability to exchange information), plasticity (variability) and persistence (construction of durable links with its environment)." [98]. One important property is the promiscuity, *i.e.* if the plasmid can only transfer between closely related individuals (within the same group or species or even clone) or if inter-species transfer is easy. Plasmids are divided into groups by the incompatibility group typing (Inc). The DNA-region in the plasmid that the Inc-grouping is based on, is a region that is necessary for replication of DNA. This is a conserved region, in contrast to other coding regions of the plasmid, which often undergo mutational and horizontal gene transfer changes [96]. IncF is an example of a narrow-host range plasmid, while IncA/C, IncL/M and IncN belong to the broad-host range group and can easily be transferred between species [96, 99]. This property of horizontal transfer is important. Since the plasmid may transfer to many different individuals (belonging to the same or a different species) that are found in close vicinity of each other in the microbiota, it is thus not dependent on the fitness of the original clone. Plasmids can thereby sometimes have a greater importance for spread of resistance genes than high-risk clones. One example is the rapid spread of the colistin-resistance gene *mcr-1*, which has disseminated around the world and can be found in many different clonal groups and species, carried by plasmids such as IncI2 and IncX4, among others [100].

Although IncF plasmids are narrow-range plasmids, they are still very important, since they are the type that carries *bla*$_{\text{CTX-M-15}}$ in *E. coli* ST131. Other high-risk clones also often carry important AMR genes on IncF plasmids, which are called epidemic plasmids [96]. One reason for the success of these plasmids is that they are stable and are reliably reproduced in both bacterial daughter cells after a cell division.

It is important to have a correct understanding of transmission and spread of AMR bacteria to develop appropriate strategies for intervention of transmission. For this reason, studies like **Study III** are important to clarify the role of high-risk clones relative to plasmid transfer.

1.5.3 *E. coli* ST131 – a high-risk clone

As described above, *E. coli* is an extremely diverse species, both in virulence and resistance, with members of the species ranging from harmless gut colonizers, to potentially lethal toxin-producing Enterohaemorrhagic *E. coli*. Since the genes encoding ESBL-enzymes are carried on plasmids, ESBL-enzymes can be present in basically any strain-type. However, there is one highly successful clone called ST131, which is responsible for a substantial part of the increase of ESBL-producing bacteria around the world [101, 102]. Although ST131 is the dominant clone, there are also many other *E. coli* lineages which are associated with ESBL-production, for example ST405, ST59, ST10 and ST23 [103].

ST131 is divided into the clades A, B and C. ST131 clade C consists of two subclades, ST131 C1 and ST131 C2. The previous nomenclature "ST131 H30-R" refers to clade C and "ST131 H30-Rx" to clade C2. ST131 clade C is characterized by resistance to fluoroquinolones (FQ) due to mutations in *gyrA* and *parC* [104-106]. Ben Zakour *et al.* studied the evolution of ST131 and concluded that clade C separated from Clade B in the middle of the 1980s. Clade C is characterized by its acquisition of the mentioned FQ-mutations, which occurred at the same time as the clinical introduction of fluoroquinolones. Shortly thereafter clade C2 diverged, concomitant with the acquisition of *bla*$_{\text{CTX-M-15}}$, which is almost uniformly found in this subclade. ST131 C2 expanded exponentially during the 1990s and 2000s and is today the dominating subclade. ST131 C1 has also acquired CTX-M, but several different types, both *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-27}}$ are common [107]. The evolution of ST131 in Sweden followed the same pattern as the international expansion and the current population was shaped by several introductions of ST131 into Sweden in combination with national transmission events [108].

ST131 is not only associated with multidrug-resistance, it has also a high number of virulence genes. With a few exceptions, nearly all ST131 are classified as extra-intestinal pathogenic *E. coli* (ExPEC, see below). ST131 is the dominating clone in bloodstream infections, with reported proportions of 20-60% of ESBL-producing *E. coli* from these infections [101, 109].

As discussed above, recombination seems to be important and frequent in pathogenic *E. coli* but ST131 is an exception. McNally *et al.* showed that the expansion of ST131 coincided with a marked reduction in recombination. One explanation could be that after ST131 had acquired genes associated with virulence and resistance genes, another genetic event decreased the recombination and mutation frequency, leading to a clonal expansion [93].

1.5.4 *K. pneumoniae* ST258 – a high-risk clone

The most successful *K. pneumoniae* clone is the KPC-producing ST258 and closely related ST512. These clones have greatly contributed to the spread of carbapenem-resistant *K. pneumoniae*, from its probable origin in USA where it was first described in 1996, to a large part of the world [103, 110]. KPC is endemic and the predominant carbapenemase-type in North America, South America, Greece, Italy, Israel and China [34, 111]. The clonal expansion is to a great extent caused by nosocomial transmission routes [33]. The bla_{KPC} gene has also been reported in isolates belonging to many other sequence types and clonal complexes [103].

1.5.5 ExPEC virulence factors

E. coli isolated from extra-intestinal infections such as UTI, abdominal infections, pneumonia and meningitis, are termed Extra-intestinal pathogenic *E. coli* (ExPEC) in order to distinguish them from the other intestinal pathogenic *E. coli* such as EIEC, EHEC and EPEC. In discussion of virulence traits, ExPEC is an umbrella term that comprises several subtypes called urinary pathogenic *E. coli* (UPEC), neonatal meningitis pathogenic *E. coli* (NMEC) and avian pathogenic *E. coli* (APEC) [112, 113]. The definition of ExPEC is not straightforward, a common definition is that the isolate is recovered from an extra-intestinal infection. There have been several attempts to molecularly define ExPEC and UPEC respectively. ExPEC has been defined as presence of two or more of the following virulence genes: *pap, sfa/foc, afa/dra, kpsMT* and *iutA* [114]. The definition of UPEC is presence of three or more of the genes *chuA, fyuA, vat* and *yfcV* [115]. However, these definitions do not encompass all the variation in isolates causing extraintestinal infections. Other genes are also associated with ExPEC virulence. The most important are presented in **Figure 9** and **Table 3** and the functions of the main types of virulence factors are briefly described below [116, 117]. Most of these genes have been identified in studies comparing isolates from clinical infections with faecal or environmental samples.

1.5.5.1 Adhesins

The function of adhesins is to facilitate adherence to structures in the urinary tract. Most ExPEC infections with *E. coli* are derived from the faecal microbiota, so the ability to attach to the urinary epithelium is essential for the development of UTI [116, 117]. Fimbrial adhesins, or pili, are anchored in the bacterial cell wall and adheres to their receptors through tip-located adhesins. For Type 1 Fimbriae this tip consists of FimH, which is almost always present in *E. coli* and has been used for subtyping of strains. The classification of ST131 H30 (*i.e. fimH* allele 30) mentioned in **Section 1.5.3** is based on typing of the *fimH*-gene. Pap-fimbriae are associated with pyelonephritis.

1.5.5.2 Immune evasion

Bacterial infection induces a strong host immune response. Bacteria in the blood are normally rapidly killed by the complement system or are opsonized and subsequently killed by phagocytes. However, ExPEC isolates have several mechanisms to protect the bacteria from this. Two factors that are associated with serum resistance are capsular polysaccharides,

especially belonging to group 2 capsular antigens (*kpsMT*) and a cell membrane-bound lipoprotein encoded by a gene called *iss*, increased serum survival [112, 117].

1.5.5.3 Invasion

Invasion virulence factors are especially associated with the NMEC subtype of ExPEC, where *e.g. ibeA*, *ompA* and *fdeC* increase the ability of the bacteria to invade through the blood-brain barrier and cause cellular invasion in other infection types [118].

1.5.5.4 Iron uptake-siderophores

Iron is an essential nutrient for bacteria, but in the urinary tract there is a limited amount of iron. Hence, bacteria with increased ability to absorb low amounts of iron have an infectious advantage [116].

1.5.5.5 Toxins

Toxins are secreted into extracellular space where the toxins damage host cells, which contributes to symptoms in infection. The lysis of host cells causes release of nutrients (including iron) that the infecting bacteria can utilize [116].

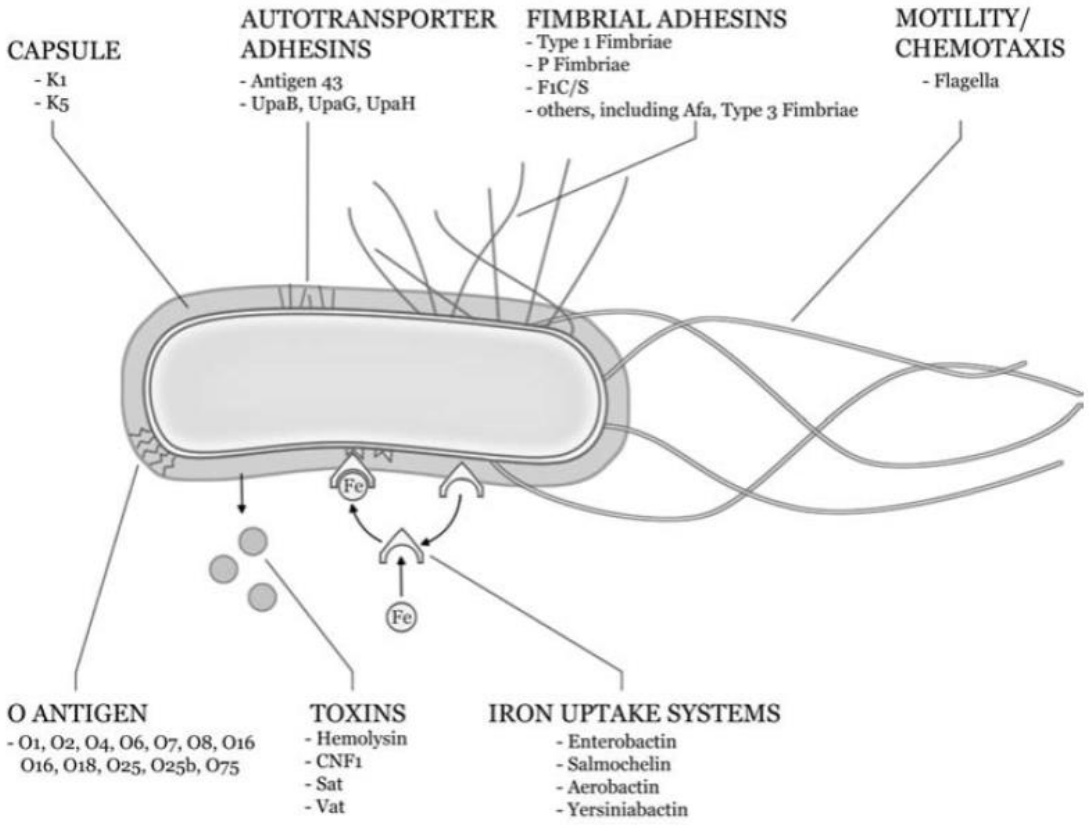


Figure 9. *Schematic figure of principal ExPEC virulence factors [116]. Reprinted with permission.*

Table 3. *Overview of important virulence traits with published association with ExPEC.*

Group	Operon	Name	Included in definition of ExPEC/ UPEC	Reference*	Included in Study IV
Adhesins	*afa/dra*	Dr Adhesins fimbriae	ExPEC	A	**Yes**
	Sfa/foc	F1C fimbriae/S-fimbriae	ExPEC	A	**Yes**
	pap	P-fimbriae	ExPEC	A	**Yes**
	fim	Type 1 fimbriae		A	**Yes**
	bma	M fimbriae		B	No
	clpG	Fimbrial adhesin CS31A		B	No
	F17	F17 fimbriae		B	No
	iha	Adhesin-siderophore		B	No
Immune evasion	K1	K1 Capsular antigen (subset of group 2)		B	No
	K2	K2 Capsular antigen (subset of group 2)		B	No
	kfiC	K5 Capsular antigen (subset of group 2)	ExPEC	C	**Yes**
	kpsMT	Group 2/3 capsule	ExPEC	A	**Yes**
	iss	Increased serum survival		C	**Yes**
	tcpC	Tir domain containing protein tcpC		A	**Yes**
	traT	Serum-resistance associate protein		B	No
Invasion	*aslA*	Putative arylsulfatase ser-type periplasmic non-aryl sulfatase		A	**Yes**
	fdeC	Invasin, adhesin FdeC		A	**Yes**
	ibeA	Invasion protein IbeA pyridine nuclotide-disulfide oxidoreductase		A	**Yes**
	aap	Anti-aggregation protein dispersin		B	No
	ompA	Outer membrane protein A		A	**Yes**
	ompT	Outer membrane protease VII		D	**Yes**
Siderophores - Iron uptake	*iuc*	Aerobactin		A	**Yes**
	iutA	Ferric aerobactin	ExPEC	A	**Yes**
	chu	Chu hemin uptake, outer membrane heme/hemoglobin receptor	UPEC	A	**Yes**
	ent	Ferrienterobactin		A	**Yes**
	iroN	IroN salmochelin receptor		A	**Yes**
	ireA	Siderophore receptor		B	No
	irp2	Yersinia biosynthesis gene		B	No
Toxins	*pic*	Pic serine protease		A	**Yes**
	sat	Sat protease, Secreted auto transporter toxin		A	**Yes**
	vat	Tsh (vat) protease. Temperature sensitive hemagglutinin/vacuolating autotransporter	UPEC	A	**Yes**
	hly	Hemolysin		A	**Yes**
	cnf1	Cytotoxic nectrotizing factor 1		A	**Yes**
Miscellaneous	*malX*	Pathogenicity island marker		C	**Yes**
	hsp	Uropathogen specific protein, type VI secretion system		C	**Yes**
	yfcV	Putative fimbrial-like adhesin protein	UPEC	E	**Yes**
	fyA	Pesticin/yersiniabactin receptor	UPEC	E	**Yes**
	clbB/N	Colibactin synthesis		B	No
	cvaC	Microcin V		B	No
	fliC	H7 flagelin		B	No

*Reference codes: A= VFDB [119]; B= Olesen 2017 [120]; C= Johnson 2019 [121], Hung 2019 [122] and Kanamori 2017 [123]; D= Johnson 2003 [114] and Hung 2019 [122]; E= Spurbeck 2012 [115]

2 AIMS

2.1 OVERALL AIM OF THE THESIS

The overall aim of this thesis is to investigate strategies for improving care of patients with bloodstream infections (BSI) caused by ESBL-producing Enterobacterales (EPE), particularly *E. coli* and *K. pneumoniae* (**Figure 10**).

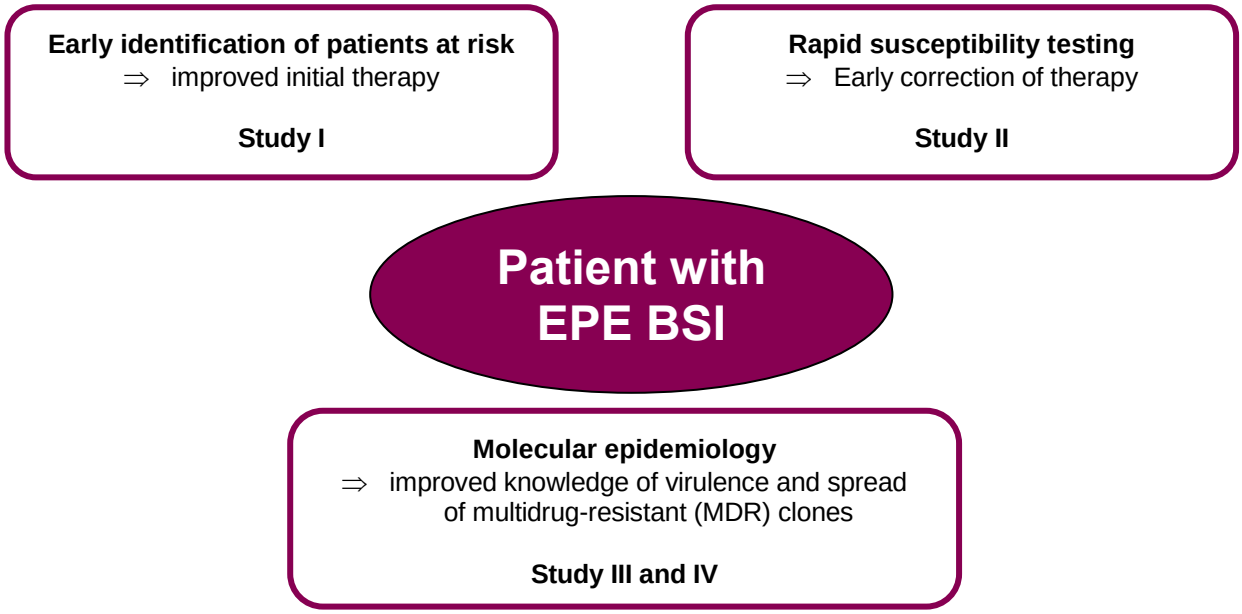


Figure 10. *Overview of thesis aims.*

2.2 SPECIFIC AIMS OF INCLUDED STUDIES

- To identify risk factors for community-onset EPE BSI and from these create a clinically useful, simple screening tool (Stockholm score) as an aid for decisions on empiric therapy (**Study I**).
- To compare the diagnostic performance of the newly developed Stockholm score to that of a previously suggested score (Utrecht score) (**Study I**).
- To evaluate the performance of 6-hour reading of disk diffusion inhibition zones and phenotypic tests for detection of ESBL- and carbapenemase-production for *E. coli* and *K. pneumoniae* (**Study II**).
- To determine the clonal, serotype and plasmid diversity of an international collection of *K. pneumoniae with* production of the carbapenemase NDM-1 (**Study III**).
- To explore the potential of using automated repetitive sequence-based PCR (rep-PCR) as a proxy for MLST to rapidly identify successful clones of *K. pneumoniae* (**Study III**).
- To describe the molecular epidemiology of community-onset BSI caused by ESBL-producing *E. coli* in Stockholm (**Study IV**).
- To examine the association of virulence genes, resistance genes and clonality with severity of disease and source of infection in community-onset BSI caused by ESBL-producing *E. coli* (**Study IV**).

3 METHODOLOGICAL CONSIDERATIONS

For detailed accounts of the material and methods used, see the method section of each publication. Here general considerations for the choice of methods are described. In addition, the different methods for molecular epidemiology used in this thesis are also explained in detail and discussed, since the differences between them might not be clear for all readers.

3.1 EPIDEMIOLOGY AND PREDICTION

3.1.1 Case-control study (I)

Study I was a retrospective case-control study with 277 cases with EPE BSI and 400 matched controls from the source population of patients visiting the emergency department with a suspected severe infection with Gram-negative bacilli in the years 2012-2015 (**Figure 11**). The source population was defined as patients ≥18 years old with a blood culture sampled at one of four emergency departments in Stockholm (Karolinska University Hospital in Solna and Huddinge, Södersjukhuset and Danderyd Hospital), admitted to hospital, and antibiotic treatment with an agent with activity against Gram-negative bacilli started within 24 hours.

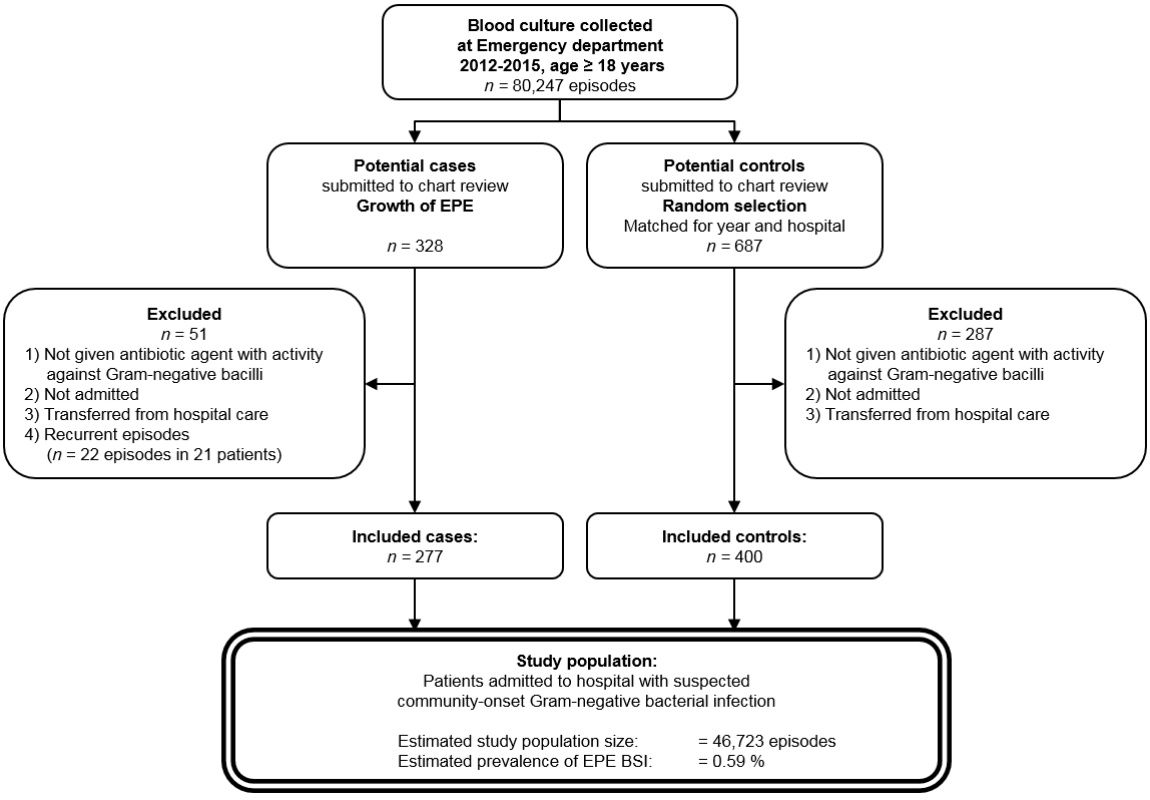


Figure 11. *Flowchart of* ***Study I*** *(illustration from publication).*

The choice of the source population in a case-control study is very important for the generalizability and possibility for application of the obtained results. The results are valid only for the population studied. This is especially important in the study of AMR bacteria.

3.1.1.1 The important choice of source population

Special considerations are needed for study design regarding prediction of infections with AMR bacteria. The majority of prior studies on risk factors for ESBL BSI have compared patients with BSI caused by ESBL-producing bacteria to BSI caused by the same pathogen without ESBL-production [45]. However, this method might bias the results by overestimating the importance of previous antibiotic treatment [124, 125]. Patients who have received active antibiotics are less likely to have a bloodstream infection with a susceptible strain, since it would be inhibited by the treatment. This causes the exposure to antibiotic treatment to be less probable in the control group. On the other hand, if one uses the entire population of patients with BSI as the source population, there is a risk that an association detected is a surrogate marker for BSI with the pathogen in question, *E. coli* or Enterobacterales, depending on how cases are defined. A suggested alternative study-design is to compare cases with both source populations as was done in two studies by Rodriguez-Baño *et al.* [51, 52]. Unfortunately, this approach increases the time and cost necessary for performing the study.

Table 4 summarizes important differences between studies using a source population with patients with *E. coli* BSI versus patients with suspected infection with Gram-negative bacilli. As our aim was to develop a clinically useful risk score to guide empiric therapy, we chose to use all patients presenting at the emergency department with suspected Gram-negative infection as the source population.

Table 4. *Comparison of two alternative study designs of* ***Study I****.*

	Type of source population	
Variable	***E. coli* BSI**	**Suspected BSI with Gram-neg bacilli**
Cases	ESBL-producing *E. coli* in blood culture	ESBL-producing Enterobacterales (EPE) in blood culture
Controls	Non-ESBL-producing *E. coli* in blood culture	Patients with suspected BSI caused by Gram-negative bacilli who are admitted to hospital, without EPE in blood culture
Generalizability	Only patients with a positive blood culture with *E. coli*	Patients with suspected BSI caused by Gram-negative bacilli who are admitted to hospital
Time after blood culture sampling	20 - 48 hours	0 hours
Disadvantages	Overestimates the risk caused by prior antimicrobial treatment Only ESBL-EC, not other EPE studied	Risk for dilution effects Prediction of EPE or Gram-negative BSI?
Advantages	Simple	The population reflects the desired population for application of the results
Approximate size of source population	≈ 5,000 episodes	46,723 episodes
Prevalence in the cohort of study I	≈ 10%	0.59 %

3.1.1.2 Matching variables

In case-control studies controls preferably are randomly selected from the source population. Matching on strong confounders is a method frequently used to improve the efficacy of the study. Otherwise there might be little overlap between cases and controls so that the control

group would have to be excessive in size (*e.g.* if a study on dementia failed to match for age, the number of controls in the relevant age-groups would be too low). The matching variables must be corrected for in the statistical analysis, and the influence of the matching variables in themselves cannot be studied [126]. In **Study I** we matched and adjusted for hospital and year of blood culture sampling, since these factors were strongly related to the proportion of bacterial samples that were EPE.

3.1.2 Cohort study (IV)

In **Study IV** the cohort consisted of a subset of cases from **Study I**, patients with community-onset BSI caused by ESBL-EC attending the emergency departments of Karolinska Hospital, Södersjukhuset and Danderyd Hospital 2012-2015 (**Figure 12**). The cohort design enabled studies of associations of patient characteristics and microbiological determinants with several different outcomes, including severity of disease, mortality, mode of infection, repeated BSI episodes and patients with a low risk for EPE BSI.

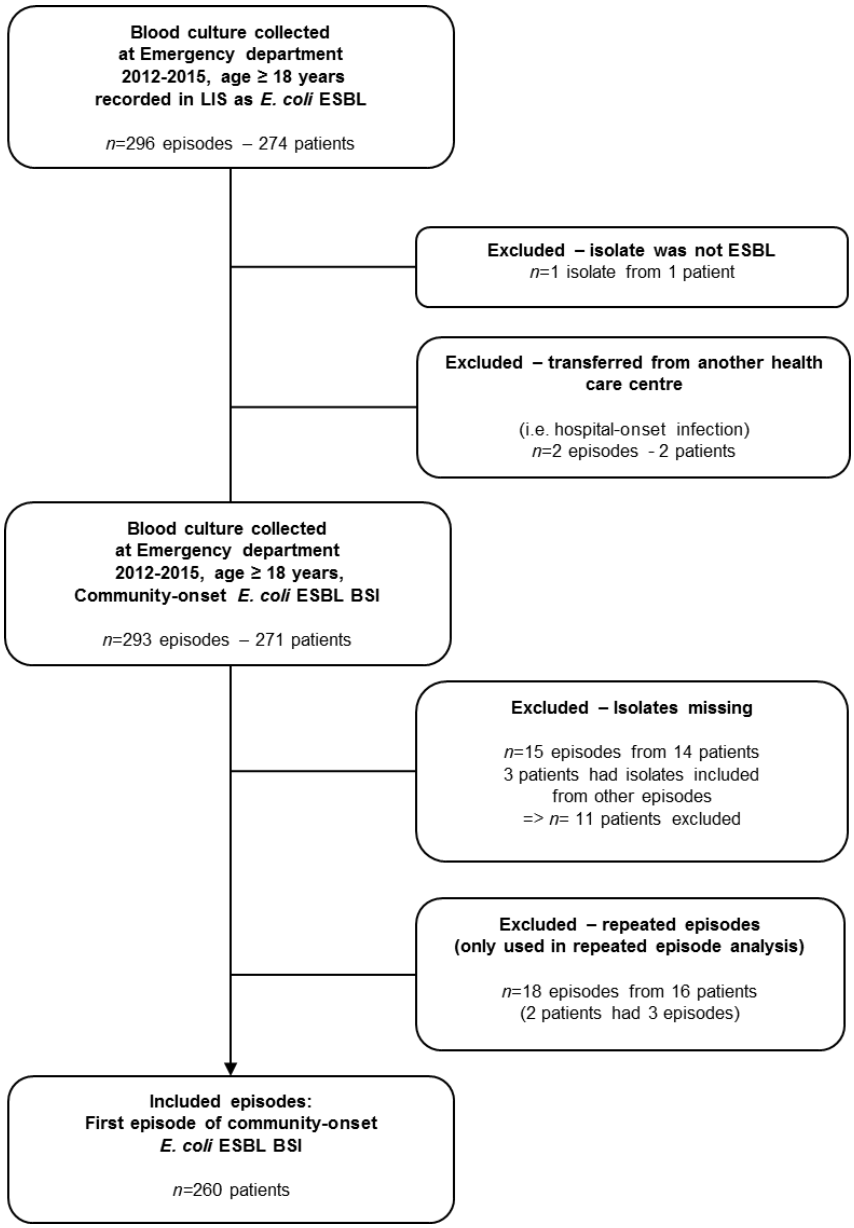


Figure 12. *Flowchart of* ***Study IV*** *(illustration from manuscript). LIS: laboratory information system.*

3.1.3 Multivariable logistic regression (I, IV)

In **Study I** and **IV**, multivariable logistic regression was used as the main statistical method to assess the relationship between the examined variables and the outcomes. In **Study I** randomly selected controls were matched for hospital and year, and the univariate logistic regression was adjusted for these two variables. In **Study IV**, there were no matching variables to consider, since the study was based on all patients in the cohort and there were no selected controls. Purposeful selection of variables to include in multivariable analysis was performed according to the steps described by Hosmer, Lemshaw and Sturdivant [127]. In short, this process included: 1) A careful univariate analysis. 2) Thoughtful selection of variables to include, considering a) clinical relevance b) a univariate/adjusted p-value of <0.20. c) avoiding inclusion of intermediate variables between the exposure and the outcome variable. 3) The model was fitted with all selected variables. The variables with the highest p-value were removed, and a new model was fitted. 4) The new model was compared with the old model by post-estimation with the likelihood ratio test. If the new model was significantly different, the latest removed variable was reintroduced. 5) This process was continued until no further variables could be removed without a significant difference from the previous model.

In **Study IV** there were several occasions with contingency tables with a zero cell, which is not possible to include in normal logistic regression. For some cases this problem was solved by combining groups, in other cases when there was no sensible group to combine with, univariate analysis was performed by Fisher's exact test.

3.2 EVALUATION OF DIAGNOSTIC METHODS

3.2.1 AST-method evaluations (II)

Study II compared the performance of rapid antimicrobial susceptibility testing (AST) with disk diffusion after 6 hours of incubation compared to standard 18 hours of incubation on 128 isolates of *E. coli* and *K. pneumoniae* specially selected for representing a wide variety of resistance mechanisms.

A microorganism is categorized as S, I or R by applying the appropriate breakpoint in a defined phenotypic AST-test system. At the time of the study the EUCAST definitions of the AST categories were S – Susceptible, I – Intermediate (since 1 January 2019 changed to "Susceptible, increased exposure") and R – Resistant [128]. A description of the disk diffusion method is included in the introduction **Section 1.4.2**.

When evaluating AST-methods, the results are reported as minor error (mE), major error (ME), very major error (VME), categorical agreement (CA) and essential agreement (EA), according to the definitions outlined in **Table 5**.

The criteria for acceptable performance for approval of newly developed AST-methods are defined by ISO-standard and FDA [129, 130]. ISO recommends including at least 300 isolates and that the collection should include as many unrelated strains representing different degrees of susceptibility as possible. FDA recommends 300 isolates plus an additional 75 challenge isolates with MICs near breakpoints. When calculating the ME rate, the denominator used is the number of isolates tested susceptible with the reference method, and

for VME rate the denominator is the number of isolates tested resistant, according to both FDA and ISO.

In **Study II** the reported ME and VME rates used the denominator total number of tested isolates, therefore these rates cannot be directly compared with the ISO/FDA criteria.

Table 5. *Definitions of terms in evaluation of AST-methods, according to ISO 20776-2 2007 and FDA 631 2009.*

ISO-abbreviation	Category	Explanation	Criteria for acceptable performance of AST-method	
			ISO	**FDA**
VME	Very Major Error	Report S when reference is R	<3% [a]	<3%[a]
ME	Major Error	Report R when reference is S	<3% [b]	≤3%[b]
mE	Minor Error	All errors which includes I		
CA	Category Agreement	Correct SIR	≥90%	≥90%
EA	Essential Agreement	Disk diffusion +/- 3mm MIC +/- 1 MIC-dilution	- ≥95%	- ≥90%

Notes: ISO and FDA use the term "Discrepancy" instead of the in publications commonly used term "Error".
[a]VME rate based on the number of **resistant** organisms tested. Depends on the number of included resistant isolates.
[b]ME rate based on the number of **susceptible** organisms tested.

Discrepancy rates are very dependent on the level of resistance of included isolates. If all isolates are highly susceptible or resistant, the discrepancy rates are naturally much smaller than if many borderline resistant isolates are included. With increasing levels of resistance, and the spread of new resistance mechanisms, AST methods are constantly being challenged. Methods with good performance when they were FDA approved might not be able to distinguish between borderline-resistant and -susceptible isolates. However, with increasing resistance rates correct grading of susceptibility is also increasingly important. When there are few therapeutic options left in MDR isolates, one might have to resort to using an antimicrobial despite acquired resistance to that agent.

EUCAST changed the definition of the SIR-category on 1 January 2019, which will have some implications for future evaluations of AST-methods, see **Section 5.2.3**.

3.2.2 Receiver operator characteristics (ROC) (I, II)

ROC-analysis is a method for comparing the performance of two different diagnostic tests and identifying the appropriate cut-off of a diagnostic test. This is done by plotting the diagnostic test sensitivity (true positive rate) on the Y-axis, and 1-specificity (false positive rate) for each value of the test [131].

Figure 13 shows a hypothetical ROC-analysis comparing test A and test B. A cut-off set at the sensitivity-level of 0.90 corresponds to a specificity of 0.97 (1-specificity: 0.03) for test A and 0.75 (1-specificity 0.25) for test B. Thus, test A is a better performing test than test B. If only test B is available, the choice of cut-off is dependent on how the test will be used. For screening tests, most often a high sensitivity is sought and positive test results are then confirmed with a more specific test (often more complicated and expensive).

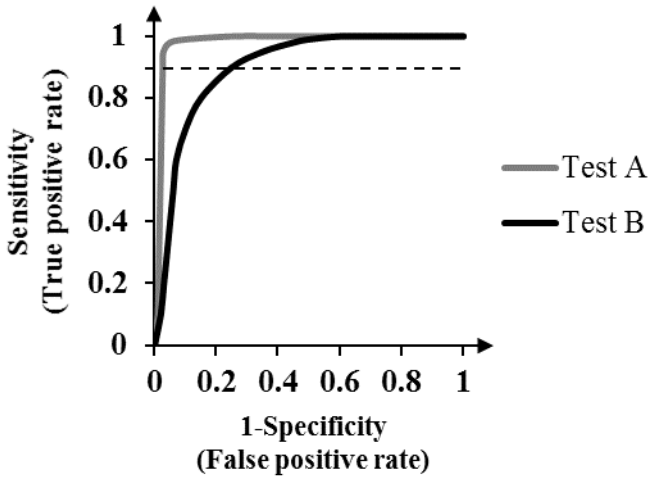


Figure 13. *Example of receiver operator characteristics (ROC) curve used in evaluation of diagnostic tests.*

ROC-analysis was used in **Study I** to calculate and compare the specificity and sensitivity of the Stockholm and Utrecht scores for prediction of ESBL-EPE BSI, and to assess if an alternative cut-off for the Utrecht score would improve the results. In **Study II** the method was used to compare 6-hour disk diffusion screening for CPE with meropenem and faropenem disks.

3.3 METHODS USED FOR MOLECULAR EPIDEMIOLOGY (III, IV)

Identifying different types of organisms within a species, typing, is important for local outbreak investigations and for global surveillance through monitoring of trends, evolution and spread of microorganisms. Typing is frequently applied for both foodborne pathogens and multi-resistant bacteria. Molecular typing methods have been in use for the last 30 years and the restriction-enzyme method pulsed-field gel-electrophoresis (PFGE) has long been considered the gold standard for species typing of *E. coli* and *K. pneumoniae* and many other species [132]. PFGE was especially useful in outbreak investigations since it had excellent discriminatory ability, good intra-laboratory reproducibility and was cost-effective, but the method was less useful for global molecular surveillance since comparisons between PFGE performed in different laboratories was not possible.

Many other molecular methods for bacterial typing have been used but in recent years generally abandoned with the technological advance, increase in availability and decrease of costs of whole genome sequencing (WGS). These prior commonly used methods include amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), automated repetitive sequence-based PCR (rep-PCR), variable-number tandem repeat (VNTR), multilocus VNTR (MLVA), single locus sequence typing (for example *spa*-typing for *S. aureus*) and comparative genomic hybridisation with DNA microarrays. A detailed description of these typing methods is beyond the scope of this thesis and have been described elsewhere [132]. Only the methods used in the included studies are described in detail: multilocus sequence typing (MLST), rep-PCR and WGS-based analyses.

3.3.1 Multilocus sequence typing (MLST) (III, IV)

For regional and global surveillance typing for common bacterial pathogens, the most suitable and widely used method is multilocus sequence typing (MLST) [133]. However, the discriminatory power is too low to be used in outbreak situations and local surveillance.

The first MLST typing scheme was introduced in 1998 for *Neisseria meningitidis*, and the method was rapidly adapted to other species [132, 134]. MLST uses a set number, usually seven to eight, of conserved housekeeping genes that are present in all strains of the species. The genes were chosen based on their optimal intra-species variability for the purpose of following evolution and spread over time and space [135]. In traditional MLST (**Study III**) these genes are amplified with PCR, then the nucleotide base sequence is determined by Sanger sequencing technique. The obtained DNA-sequence of each housekeeping gene is compared by basic local alignment search tool (BLAST) to a database of previously sequenced isolates and assigned to an allelic number, if the sequence is previously known. New sequences are assigned a new allelic number and deposited in the database. The combination of the obtained seven allelic number are compared to previous allele-combinations, and assigned to a present or new, sequence type (ST) serial number. Two isolates with the same ST have identical sequences in the seven housekeeping genes.

For *Klebsiella pneumoniae* the Pasteur MLST database was used in **Study III**, which includes the seven housekeeping genes *gapA, infB, mdh, pgi, phoE, rpoB* and *tonB.* (http://bigsdb.pasteur.fr/ecoli/ecoli.html) [136].

For *E. coli* there are three different MLST-schemes using different housekeeping genes: the University of Warwick (Achtman) MLST, including the seven genes *adk, fumC, gyrB, icd, mdh, purA,* and *recA* (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search) [91], the Pasteur institute MLST database including the eight genes *dinB, icdA, pabB, polB, putB, trpA, trpB,* and *uidA* (http://bigsdb.pasteur.fr/ecoli/ecoli.html) [137] and one specialized for enterotoxigenic *E. coli* at the Michigan State University that includes the genes *aspC, dpX, fadD, icd, lysP, mdh* and *uidA* [138]. STs obtained by the different schemes were compared by Clermont *et al.* [138]. The University of Warwick scheme had the lowest resolution of the three schemes but seemed to be the most robust and had the most congruent results with whole genome sequence methods. The University of Warwick nomenclature is the most widespread and is the one used for *in silico* MLST of WGS-sequences in **Study IV**.

From whole genome sequencing data, information can be extracted *in silico* for genes included in the traditional MLST, which now has become less labour intensive and cheaper than traditional MLST (see below).

3.3.2 Automated repetitive-sequence-based PCR (rep-PCR) (III)

The commercial automated repetitive-sequence-based PCR (rep-PCR) method DiversiLab (bioMérieux, Marcy d'Etoile France) was evaluated for NDM-1-producing *K. pneumoniae* as a part of **Study III.** This rep-PCR method (also called repetitive-element PCR) used PCR with primers for noncoding regions that are present in several separated regions interspersed in the bacterial genome [132, 139]. The regions in between the primers were amplified, which produced multiple PCR-products of different lengths, depending on the placing of the

primer-sequences in the genome. The amplicons were then separated by high-resolution chip-based microfluidic capillary electrophoresis and detected and visualized as trees and virtual gel band-patterns through the product-specific software. The method was a rapid, less labour-intensive method compared to PFGE and was found to be useful for primary identification of hospital outbreaks especially for *Klebsiella species* and *Acinetobacter species*, but for other species the discriminatory power was too low for outbreaks. Results should be confirmed with another method if isolates in an outbreak was assigned to the same type [132]. Production of the DiversiLab instrument and kits have been discontinued by the company bioMérieux and is no longer available [140]. A probable reason for this is the increased availability and reduced costs of microbial typing with whole genome sequencing.

3.3.3 Whole genome sequencing (WGS) (IV)

The classic sequencing method Sanger sequencing introduced in 1977 was based on incorporating fluorescent-labelled terminating base pairs in *in vitro* DNA replication [141]. Sanger sequencing is limited to sequences < 1,000 bases in length and is useful for individual genes, but not for entire organism genomes. An *E. coli*-genome consists of about 4,500,000 base pairs. Sanger sequencing was used for MLST genes in **Study III**. Sequencing methods have been rapidly developed in the last 15 years and the cost of sequencing dropped rapidly between 2007 and 2015, with the introduction of new powerful high-throughput sequencing methods [142]. The cost of sequencing is further reduced by centralizing sequencing instruments and bioinformatic knowledge for both human and microbial genome sequencing in institutions such as Science for Life Laboratory (SciLifeLab, Solna, Stockholm).

There are several different next-generation sequencing (NGS) technologies that can be used for sequencing of entire genomes called whole genome sequencing (WGS). These methods include Ion Torrent, Illumina, Pacific Biosciences (Pac Bio) and Oxford Nanopore [141]. In **Study IV** Illumina HiSeq 2500, placed at the Science for Life laboratory (SciLifeLab), was used for WGS. Illumina uses a sequencing-by-synthesis method which detects release of fluorescent labels from incorporated nucleotides to determine the sequence [141]. The main advantages of Illumina sequencing are a low error rate, massive throughput capacity, with hundreds of microbial genomes sequenced simultaneously and low cost per output. Disadvantages are longer run time and short reads, which limit the possibility for *de novo* assemblies and plasmid detection.

Short reads of DNA sequences generated by Illumina are assembled by mapping to a reference genome (reference-based mapping) or *de novo* assembly, which gives the entire gene sequence of the isolate. Although the obtained sequence is referred to as the "whole genome", it is not exactly a "true" genome, as errors in the sequence can arise in several parts of the process, including during the extraction-process of DNA, contamination, sequencing process errors, and assembly errors. To minimise these errors, several quality control checks are done during the process.

Table 6. *Main steps of WGS and location of work/software used in* ***Study IV.***

No.	Step	Location/Software used
1	Extraction of DNA	Karolinska University Laboratory
2	Library preparation	SciLifeLab
3	Sequencing generating Illumina short reads	SciLifeLab
4	*De novo* assembly with SPAdes v3.1 Assembly polish and quality assurance steps	microSALT pipeline and Enterobase
5	Detection of - Virulence genes - Resistance genes	 microSALT + Selected virulence genes microSALT + Resfinder
6	Phylogenetic analysis - SNP - cgMLST + Hier CC - MLST	Enterobase + GrapeTree + iTOL

3.3.3.1 Quality of the extraction process

The influence of the extraction process on the quality of sequences was illustrated in the work with **Study IV**. The initial extraction of DNA was performed with MagnaPure 96 (MP96, Roche, Basel, Switzerland) which is a standard platform for DNA-extraction before PCR. However, the quality control of the sequencing reads showed unexpected low read depth. The quality of a sequence is measured in sequence read depth, *i.e.* the average depth of overlapping reads at any locus. There are not yet established thresholds for quality control, but an *average* read depth of >30x is generally considered as adequate quality [141], while others recommend a *minimum* read depth of >30x [76]. The initial sequencing after extraction with MP96 yielded many isolates with low quality reads, with 10x coverage less than 90%, and several isolates which failed in MLST allelic calling in Enterobase, mainly for *fumC* but also for other MLST genes. For this reason, 54 isolates were re-sequenced with extraction by EZ1 (Qiagen, Hilden, Germany). Both methods use magnetic bead technology but the EZ1 has protocols developed for the needs of WGS. The quality of the genome-sequences finally used in **Study IV** clearly varied depending on the extraction method used. In total 58 isolates were extracted with EZ1, of these 97% had a 30x coverage rate over 97% (corresponds to minimum read depth of >30x) and 100% had both 30x and 50x >50% (corresponds to average read dept of >30x). For the 220 isolates that were extracted with MP96, only 38% had a 30x coverage rate over 97%, and 97% had 30x >50% while 79% had 50x >50%.

3.3.4 Core genome multilocus sequence typing (cgMLST) (IV)

Core genome multilocus sequence typing (cgMLST) is a method for bacterial typing which is more discriminatory than traditional MLST but based on the same principle. Instead of using 7 housekeeping genes identified by PCR (or *in silico* from WGS), cgMLST uses *in silico* detection of >2,000 core genome genes, which optimally are present in all, or at least most isolates of a species. The Enterobase scheme for cgMLST includes 2,513 genes for *E. coli* [143]. Each gene is assigned an allelic serial number and the 2,513 allelic numbers are used to obtain a serial core genome sequence type (cgST) number. Two isolates with identical cgST have the same gene sequence in all the 2,513 genes. There are several schemes of cgMLST on different platforms for *E. coli* and an international standard is lacking.

The drawback of cgMLST is that not all isolates have all genes. Deletions and insertions can give rise to distortions in the phylogenetic relationships. The more isolates that are tested, the fewer genes all isolates have in common. However, cgMLST is a powerful method, rapid

after the initial designation, needs less bioinformatic power than single nucleotide polymorphism and can easily produce trees with >10,000 genomes [143, 144].

Woksepp *et al.* demonstrated the usefulness of a cgMLST scheme (SeqSphere+, Münster, Germany) in a nosocomial outbreak of ESBL-EC of ST131 in Sweden and obtained comparable results as with PFGE and SNP [145].

The normal MLST nomenclature is useful in communication about high-risk clones and changes in global epidemiology. However, the cgST types are not sensible to use in communicating clones, not even in outbreak situations. Since more than 2,000 genes are included, it is likely that mutations occur in some isolates within the outbreak, and they will then have different cgST numbers. As the cgST numbers are allocated in the order of appearance, the cgST number in itself does not give any information about the relatedness of isolates. Instead the number of allelic differences between the genomes in question, preferably presented as a phylogenetic tree, is more informative.

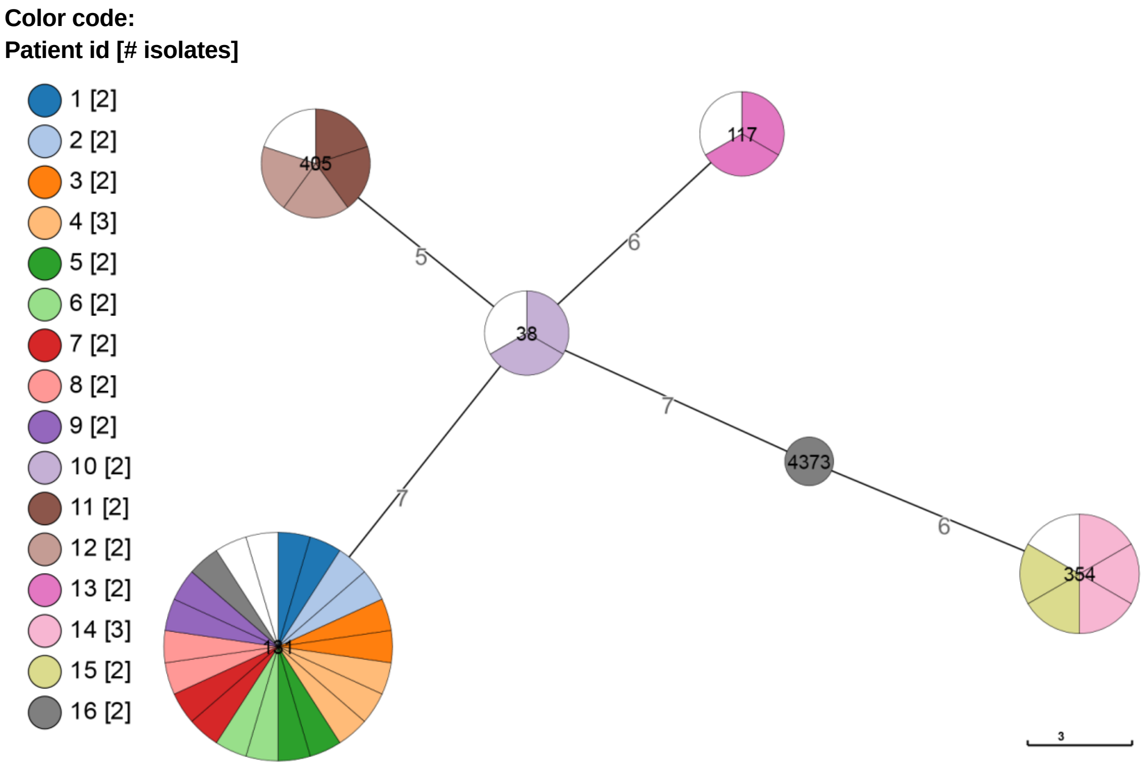
3.3.5 Single nucleotide polymorphism phylogeny (SNP) (IV)

Single nucleotide polymorphism, SNP-analysis of WGS-genomes has become a common and useful method for outbreak and transmission analysis. This is performed through reference-based mapping of either reads or assembled contigs. Repeated regions, regions that are missing in a certain proportion of the genomes (typical threshold 5%) and regions that are the same in all compared genomes are filtered out. The remaining positions contain an SNP in at least one of the compared genomes. The SNP-differences can be displayed as a matrix (**Figure 14 E)** between the compared genomes or as phylogenetic trees. Phylogenetic trees, for both SNP and cgMLST are normally obtained through one of several maximum likelihood tree algorithms, such as the randomized accelerated maximum likelihood program (RAxML) [146].

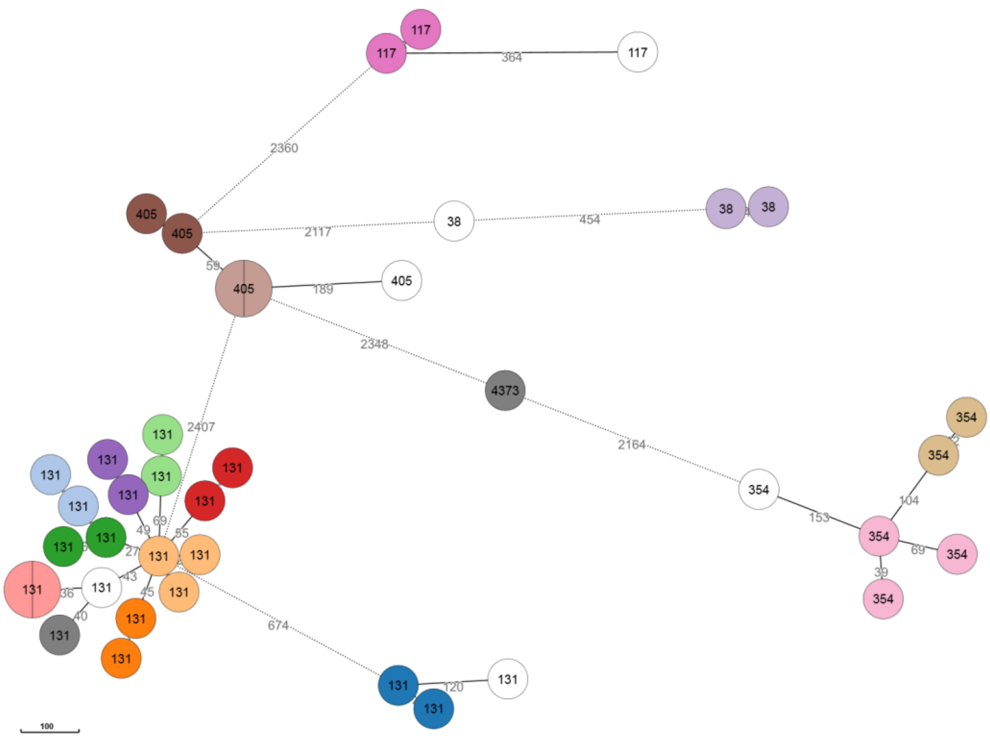
A limitation with SNP-based phylogeny is that the result varies due to the number and types of genomes included. It works best when only including a few, closely related genomes with a closely related reference genome. If more distantly related genomes are included in the analysis, the number of mapped bases decreases, and the risk of mis-mapping increases [147].

3.3.6 Comparison of MLST, cgMLST and SNP

A comparison of the usefulness of MLST, cgMLST and SNP-analysis is provided in **Table 7**. The outputs and results of the different methods have been applied on the same set of genomes in **Figure 14 A-E.** In this comparison the same 40 genomes are included in all analyses. Six are reference genomes, and the rest are from 16 patients with repeated episodes of ESBL-EC BSI from **Study IV**. Repeated isolates from the same patient but sampled >30 days apart are considered to have an epidemiological relationship. Each patient is coloured in the same colour in all figures. For 15 of the 16 patients, both episodes were caused by closely related isolates, and all methods give the same result, but with different resolution. In **Figure A**, Achtman 7-gene MLST assigns repeated episodes to the same ST, but within-ST relationships cannot be determined. With cgMLST in **Figure B** it is clear that isolates from the same patient are more related to one another than to isolates from other patients within the same ST. This is seen for ST131, ST405 and ST354. However, each node has an individual

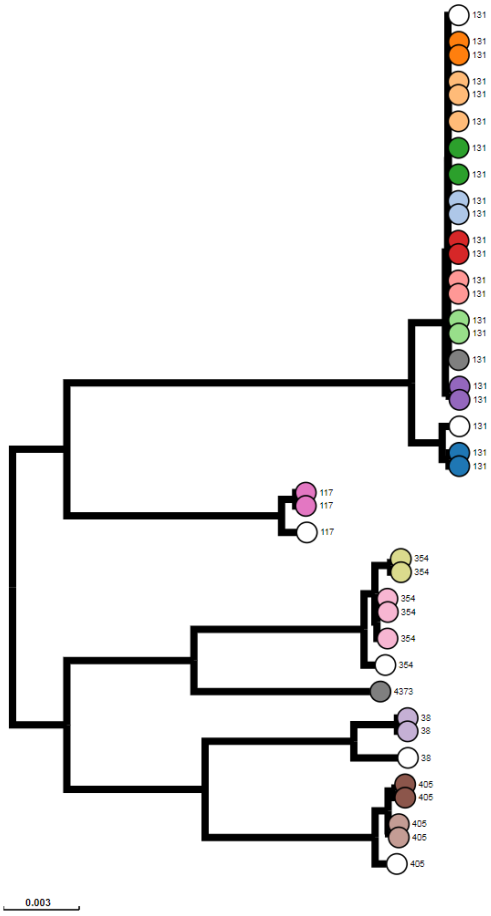


A. *Achtman 7-gene MLST, minimal spanning tree. Distance on branches are number of allelic differences. Node size is relative to number of isolates. Number on node refers to ST.*

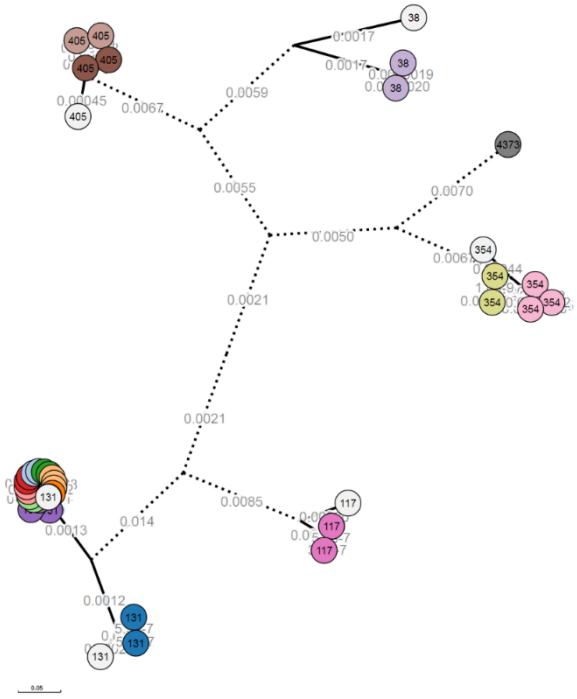


B. *Enterobase cgMLST, minimal spanning tree. Distance on branches are number of allelic differences. Dotted branches (length > 500) are shortened to facilitate visualisation. Node sizes are relative to number of isolates. Number on node refers to ST (Achtman 7-gene MLST).*

Figure 14. A-D. *Comparison between different molecular typing methods. The phylogenetic relationship between the same 40 genomes (34 isolates from 16 patients from study IV and 6 reference genomes) are shown. Isolates from the same patient are the same colour in all figures according to legend. White nodes are reference genomes All trees are made with Enterobase Grape Tree.* ***E.*** *SNP-matrix for the four patients with ST131 clade C1.*



C. Maximum likelihood SNP-dendrogram. Scale of branch length is the number of substitutions per site. Labels refer to ST (7-gene MLST).



D. Minimal spanning tree based on the same SNP-data as in figure C. Number on branch refers to number of substitutions per site. Dotted branches are shortened to facilitate visualisation.

		Patient 3		Patient 2		Patient 5		Patient 4		
		Jul 2015	Dec 2014	Aug 2013	Mar 2013	Dec 2014	Jan 2013	Feb 2014	May 2014	Apr 2012
		EF542	EF479	EF347	EF312	EF481	EF301	EF411	EF435	EF215
Patient 3	EF542	0	4	414	372	368	362	1295	1299	1292
	EF479	4	0	412	370	366	360	1293	1297	1290
Patient 2	EF347	414	412	0	44	58	58	1272	1277	1269
	EF312	372	370	44	0	22	16	1230	1235	1227
Patient 5	EF481	368	366	58	22	0	12	1226	1231	1223
	EF301	362	360	58	16	12	0	1220	1225	1217
Patient 4	EF411	1295	1293	1272	1230	1226	1220	0	15	7
	EF435	1299	1297	1277	1235	1231	1225	15	0	8
	EF215	1292	1290	1269	1227	1223	1217	7	8	0

E. Example of SNP-matrix. Matrix showing the number of SNPs between 9 isolates from four patients with ST131 clade C1. Headings are coloured with the same colour as in figure A-D. Cells in matrix are toned according to number of SNPs (White 0-10 SNPs, Dark brown >500 SNPs).

Table 7. *Comparison of MLST, cgMLST and SNP for epidemiological typing of E. coli. Table compiled from references [141, 144, 145, 147, 148].*

Comparator	MLST	cgMLST (Enterobase)	SNP
Discriminatory power	Low	High	High
Suggested relatedness threshold in outbreak settings			
Related	NA	≤10 allelic differences	≤10 SNPs
Not related	NA	>100 allelic differences	>100 SNPs
Description	7 conserved house-keeping genes	2,513 core genome genes, present in most isolates of a species	Genomes are mapped to reference. Repeat regions, regions that are missing in ≥5% and bases that are the same in all compared isolates are filtered out
Method for dealing with recombination	Only conserved genes are included, recombination in other parts of the genome does not affect the result	Recombination events can occur and somewhat distort phylogenetic relationships. Recombinant regions will be collapsed into a smaller number of allelic changes. Less impact of recombination in cgMLST than wgMLST	Only SNPs in positions present in all compared genomes are compared
Speed	With WGS, high	Very rapid to perform comparisons after initial assignment of alleles	Slow
Capacity	With WGS, high	High, >20,000 genomes have been compared in the same analysis	Works best with few closely related strains (preferably within the same or closely related STs) with a closely related reference genome
Interlaboratory Standardization	High, the same database for MLST are used. Only quality of sequencing varies	High, if the same cgMLST scheme is used. There are several different platforms	Low, somewhat different results depending on included genomes
Standardization and usefulness of nomenclature	High, global lineages have the same ST	Standardisation is high within the same cgMLST scheme. But resolution is too high for cgMLST-names to be useful	No nomenclature
Main area of usefulness	Global epidemiology Nomenclature Sequence types	Outbreaks Global epidemiology Phylogenetic trees	Outbreaks SNP-matrix Phylogenetic trees

cgMLST number (not shown). The number of allelic differences between isolates from the same patient is small, often <10 alleles. The same is clear from the SNP dendrogram in **Figure C** but is less obvious when the SNP is presented as a minimal spanning tree (**Figure D**). The individual number of SNPs that differs between included genomes can conveniently be displayed as a matrix (**Figure E**).

The differences between the methods are presented in **Table 7**. In summary, the sequence types obtained by MLST, which is the least discriminatory method, are most useful as a common nomenclature for global surveillance of high-risk clones and association of different STs with resistance genes, virulome and infection types. cgMLST and SNP-analysis can both be used for outbreak analysis but are more difficult to compare between analyses. Results obtained by different cgMLST schemes cannot be compared. SNP is at its best when applied on a small number of closely related isolates within a suspected transmission event, while cgMLST has potential to elucidate detailed phylogenetic relationships in comparisons that include a high number of genomes.

However, recombination and horizontal gene transfer, is a common cause of distortion of phylogenetic relationships and creates pitfalls for all methods. In order to minimize the impact of recombination, a subset of genes/single nucleotide variants are used, but this problem is necessary remember, and the "true" phylogenesis is impossible to obtain [91].

3.3.7 Detection of virulence and resistance genes (IV)

For identification of virulence genes in **Study IV**, a selection previously described virulence genes with association with ExPEC, was compiled and analysed with Basic Local Alignment Search Tool (BLAST) through the in-house bioinformatic pipeline microSALT (https://github.com/Clinical-Genomics/microSALT), for detection of 108 virulence genes in the 278 genomes. For some genes the web tool NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for further characterisation and trouble-shooting. For resistance gene detection the resistance database of the microSALT pipeline was used, which in turn used the ResFinder database v2.1 [149].

Establishing presence or absence of a gene in a WGS genome has some inherent difficulties. For sequencing methods that generates short-end reads as the method used in **Study IV**, Illumina sequencing, the following assembly of the short reads can cause difficulties. Depending on settings in the assembly method, contigs can be truncated at different sites. If a gene of interest is divided between two contigs, the gene will not be detected. Additionally, where to define the threshold for the similarity of a gene (identity %) needed to predict the presence of a gene is not obvious and thresholds must be adapted to the context. Some genes have conserved sequences and do not show much variation, since a mutation might alter the function completely. Other genes, *e.g.* surface exposed virulence genes, have a high allelic diversity, but still the same function. An example is *fimA* which is under constant pressure to diversify to avoid the immune system [150]. Hence it might be appropriate in some circumstances to have a conservative strict identity threshold and in other cases to have a permissive threshold.

For detection of resistance genes, the cut-off used in microSALT pipeline **Study IV** was 97% identity and 90% gene coverage, the same as is used in clinical samples. For virulence genes,

the cut-off for identity was initially set at 90% identity and 90% gene coverage, but this turned out to be too high, for some genes with high allelic diversity. Hence the cut-off was lowered to 80% identity and 80% gene coverage. There is no international consensus for which cut-off to use for different genes, but the 80/80 cut-off was applied previously for detection of virulence genes in ESBL-producing ExPEC [123].

3.4 SOFTWARE

Microsoft Access database was used to collect and store data for **Study I** and **IV**. Microsoft Excel was used for data storage in **Study II** and **III** and for calculations in all studies. Stata IC statistical software: release 13 (StataCorp LP, College Station, TX) was used for statistical calculations in **Study I**, **II** and **IV**.

In **Study IV** several web resources for analysis of genomic sequences and graphical illustrations was used: Enterobase (https://enterobase.warwick.ac.uk/) [143, 144], the Enterobase embedded function GrapeTree software [151] and the Interactive tree of life (iTOL) (https://itol.embl.de/) [152].

In **Study III**, BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) was used for visualisation of minimal spanning tree.

3.5 ETHICAL CONSIDERATIONS

The collection of clinical data in **Study I** and **IV** was performed by review of medical records of the included patients. This inevitably involves a violation of patient privacy, as sensitive information will be collected and stored. Patient consent was not sought, instead permission was sought and obtained from the Regional Ethical Review Board in Stockholm (Dnr 2014/277-31).

From a utilitarian perspective, the ethical conflict is between the principle of the individual's right to informed consent compared to the medical research value for the society and future patients. However, informed consent was deemed not possible since some of the patients included in the study were deceased at the time of the study. The value of the medical research obtained by the study is significant. By identifying risk factors for community-onset bloodstream infections caused by EPE, the study has resulted in knowledge that will improve empiric therapy for patients with EPE BSI. The new knowledge will also help avoid unnecessary broad-spectrum antibiotic treatment, and thus contribute to minimizing further antibiotic resistance development. Additionally, our understanding of the epidemiology and virulence of ESBL-EC in BSI has increased.

All data were handled strictly confidential and identities were pseudonymized. The key was kept separately in a password protected file, and only the investigators had access to the key.

Study II and **III** were *in vitro* studies with bacterial strains without attached patient metadata. Although the bacterial isolates originated from human infections, what was stored and used were solely bacteria, and no human DNA or tissue remained. Thus, the strains were completely unrelated from their origin. No humans or animals were harmed. As a general rule, bacterial isolates are exempted from ethical permissions.

4 RESULTS

4.1 STUDY I

Prediction of bloodstream infection caused by extended-spectrum beta-lactamase-producing Enterobacterales in patients with suspected community-onset sepsis.

The dilemma at hand when choosing empirical therapy for patients with suspected serious infections at admittance at the emergency department, is the need for prompt appropriate therapy for the causing microorganism, but at the same time avoiding overuse of broad-spectrum antibiotics, which causes selection of even more resistant bacteria and further fuels the evolution of AMR bacteria. The aim of **Study I** was to develop a simple prediction score for EPE BSI to facilitate this choice at the emergency department, and to evaluate a previously published, complex prediction score, which we called the Utrecht score [153].

Study I was performed as retrospective case-control study, set in Stockholm during 2012-2015 (**Figure 11**). The final multivariable logistic regression model (summarized in **Table 8)** identified three major risk factors that seemed promising for use in a score, any prior EPE-positive culture, a recent prostate biopsy and prior healthcare abroad. These were combined to form the Stockholm score which was compared to the Utrecht score when applied on the cases and controls.

Table 8 *Final multivariable prediction model of EPE BSI. Adapted from* ***Study I****.*

Risk factor	Controls n=400 (%)	EPE BSI n=277 (%)	Multi-variate OR	P-value
Any prior EPE-positive culture	3	33	19.1	**<0.001**
Prostate biopsy ≤30 days	1	14	22.2	**<0.001**
Prior healthcare abroad ≤6 months	2	6	3.9	**0.016**
Age category				
<40	11	3	1.0	(Ref.)
40-49	7	7	3.0	**0.047**
50-59	9	15	2.8	**0.045**
60-69	19	32	3.3	**0.011**
70-79	27	21	1.6	0.317
≥80	28	22	1.6	0.352
Suspected diagnosis at admission				
Unknown	33	25	1.0	(Ref.)
Abdominal	9	6	1.0	0.998
LRTI	23	7	0.4	**0.005**
SSTI	5	1	0.1	**0.015**
UTI	26	60	2.6	**<0.001**
Other infection	5	1	0.5	0.350
SOFA-score				
0-1	39	29	1.0	(Ref.)
2-3	38	34	1.5	0.086
4-5	15	21	2.1	**0.014**
≥6	9	17	4.0	**<0.001**

The final source population (estimated size 46,723 episodes) consisted of patients admitted to hospital with a community-onset suspected Gram-negative infection (*i.e.* who were given empirical treatment with an antibiotic agent with activity against Gram-negative bacilli). The estimated prevalence of EPE BSI, which we aimed to predict, was 0.59%. At first thought, this might sound surprisingly low. However, the explanation for the low prevalence in the population in question is that only about 15% of sampled blood cultures yield a positive result. Out of positive bottles 20% grow *E. coli*, an additional 5-10% grow other members of the Enterobacterales group and 10% of the *E. coli* are ESBL-producing.

The consequence of the low prevalence is that the specificity of a score becomes very important for the number needed to treat (NNT). The predictors included in the Stockholm and Utrecht scores are outlined in **Table 9** and their performance in **Table 10**.

Table 9. *Comparison of the included predictors in the two prediction scores.*

Predictor	**Stockholm Score**
Any prior EPE-positive culture	1
Prostate biopsy <30 days	1
Prior healthcare abroad <6 months	1
The Stockholm score is positive if the sum is ≥1	

Predictor	**Utrecht Score**
Prior EPE-positive culture <1 year	100
Suspected UTI	50
Suspected pneumonia	-50
Immunocompromized	25
Any use of antibiotic <2 months	25
Age per year	Age
The Utrecht score is positive if the sum is ≥120 [153]	

The two scores had similar sensitivity, Stockholm score 50% versus 54% for the Utrecht score, but the specificity of the Stockholm score was much higher, 96% compared to 77%. **Figure 15** shows a ROC-comparison of the two scores and the individual predictors in the Stockholm score. The difference in specificity causes a great difference in the positive predictive value, 6.2% for the Stockholm score, and 1.4% for the Utrecht score. However, since the prevalence in the population is only 0.59, the numbers of patients needed to be treated, in order to treat one patient with EPE BSI with appropriate treatment, are quite high.

If one uses a strict application of each score and thus treats all patients with a positive score with meropenem, the number needed to treat (NNT) can be calculated. In the following section, the definition of NNT is the number of patients (with and without EPE BSI) that would need to be treated in order to treat one patient with EPE BSI, with a strict application of each score. This concept is similar to, but not the same as, the NNT usually reported in randomized controlled trials. NNT_{ALL} (refers to all patients in the source population) would be 16 for the Stockholm score (to treat the 139 EPE BSI-cases that were positive for the Stockholm score, 2,103 patients without EPE BSI would have to be treated with meropenem, in total 2,242 patients treated). For the Utrecht score NNT_{ALL} would be 74 (149 EPE BSI cases and 10,863 cases without EPE BSI.).

Two components of the Stockholm score, prior EPE-positive culture and prior healthcare abroad, are already recognized risk factors for EPE infection, which many infectious disease specialists and ED doctors already take into consideration when choosing empirical therapy. This means that the benefit of implementing such a score might be limited. We therefore compared a hypothetical strict use of the score with the empirical therapy the patients actually

received. Improved therapy for cases was defined as appropriate therapy started more than 24 hours earlier with application of the score. For controls the impact of the score was measured as a change of therapy from any other treatment to meropenem. For the Stockholm score, a strict application on the included patients would have meant that 14% of cases would have received improved therapy but 3% of patients without EPE BSI would have been given meropenem instead of another agent, corresponding to a number needed to treat ($NNT_{IMPROVED}$) of 39 for improved therapy. For the Utrecht score, 19% of cases would have received improved therapy, but 21% of the population would have been given meropenem, $NNT_{IMPROVED}$ would be 182 patients.

Table 10. *Summary of the overall performance of the two prediction scores*

	Stockholm score	Utrecht score
Sensitivity	50.2%	53.8%
Specificity	95.5%	76.8%
Positive predictive value (PPV)	6.2%	1.4%
Negative predictive value (NPV)	99.7%	99.6%
Number of cases with a positive score	139	149
Estimated no. of controls with a positive score in the source population	2,103	10,863
Number needed to treat (NNT_{ALL})[A]	16	74

[A]NNT_{ALL}: Total number of patients that need to be treated to treat one patient with EPE BSI, with a strict application of the score, regardless of the empirical treatment that *de facto* was given.

If all patients with a positive score had been given meropenem empirically

	Stockholm score	**Utrecht score**
Cases (EPE BSI) with improved treatment[B]		
Proportion of cases in study	14%	19%
No. of patients in the source population	40	53
Controls (no EPE BSI) with changed treatment		
Proportion of controls in study	3%	21%
Estimated no. of patients in the source population	1,518	9,578
Number needed to treat ($NNT_{IMPROVED}$)[C]	39	182

[B]Improved treatment: appropriate therapy started >24 hours earlier with application of score compared to what *de facto* was given.
[C]$NNT_{IMPROVED}$: NNT to improve therapy compared with what *de facto* was given.

Patients *de facto* treated with meropenem as empirical treatment

	Cases	**Controls**
Proportion (no.) treated with meropenem in study	25% (68)	8% (30)
Estimated number of patients in the source population	68	3,504

The second important finding of the study was that a prior EPE-positive culture is the most important risk factor for EPE BSI in community-onset gram-negative infection, and that the time passed since the latest prior EPE-positive culture is important for the risk (**Figure 16**). The more recently the culture is taken, the higher odds ratio for EPE BSI, but the increased risk remains regardless how long time has passed since the sample was taken. A recent culture (sampled 8 days-3 months before the blood culture) has an odds ratio (OR) of 32.8 compared to having no prior EPE-positive culture, while the OR for a sample taken >2 years before was 8.4. There was no statistically significant difference between a prior EPE-positive culture in a clinical sample or in a faecal screening-sample.

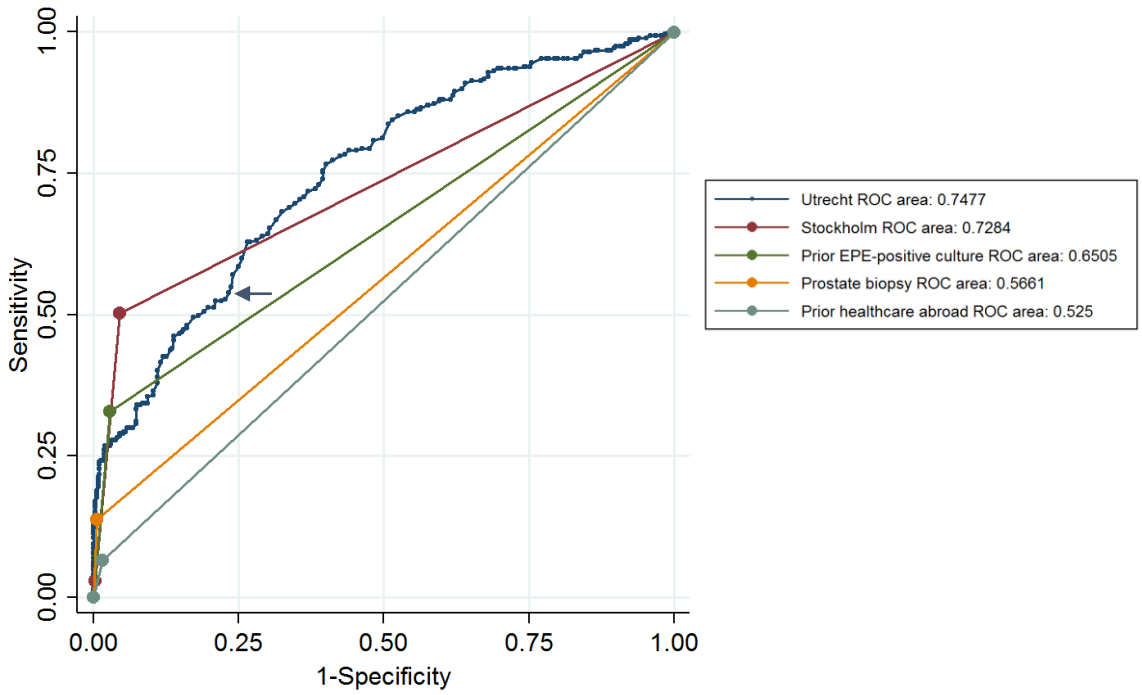


Figure 15. *ROC-comparison between the Utrecht score, the Stockholm score and the three individual predictors included in the Stockholm score. The blue arrow indicates the Utrecht score cut-off at ≥120. First episodes, n=677.*

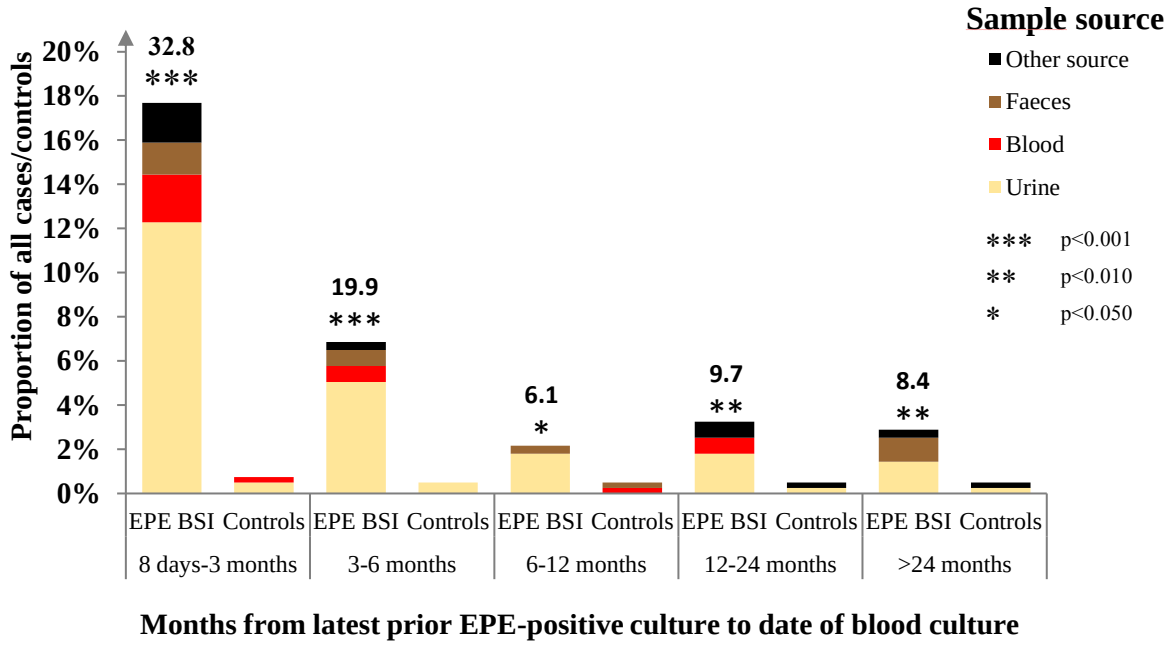


Figure 16. *Time from latest prior EPE-positive culture to date of blood culture. Bars show the proportion of all cases/controls that have a prior EPE-positive culture, coloured by sample type. Numbers over the bars represent ORs from logistic regression for each time-category compared with having no prior EPE-positive culture, adjusted for matching variables. Stars indicates the statistical significance-level of the OR. Graph adapted from* ***Study I****.*

4.2 STUDY II

Rapid EUCAST disk diffusion testing of MDR Escherichia coli and Klebsiella pneumoniae: inhibition zones for extended-spectrum cephalosporins can be reliably read after 6 h of incubation.

4.2.1.1 Development of inhibition zones over time

We programmed the BD Kiestra™ total lab automation system to photograph AST agar plates at repeated times during incubation (at 5, 6, 7, 12 and 18 hours) which made it possible to follow the development of inhibition zones over time. The isolates tested were overnight cultures from frozen isolates of ESBL-producing, carbapenemase-producing and wild-type *E. coli* and *K. pneumoniae*.

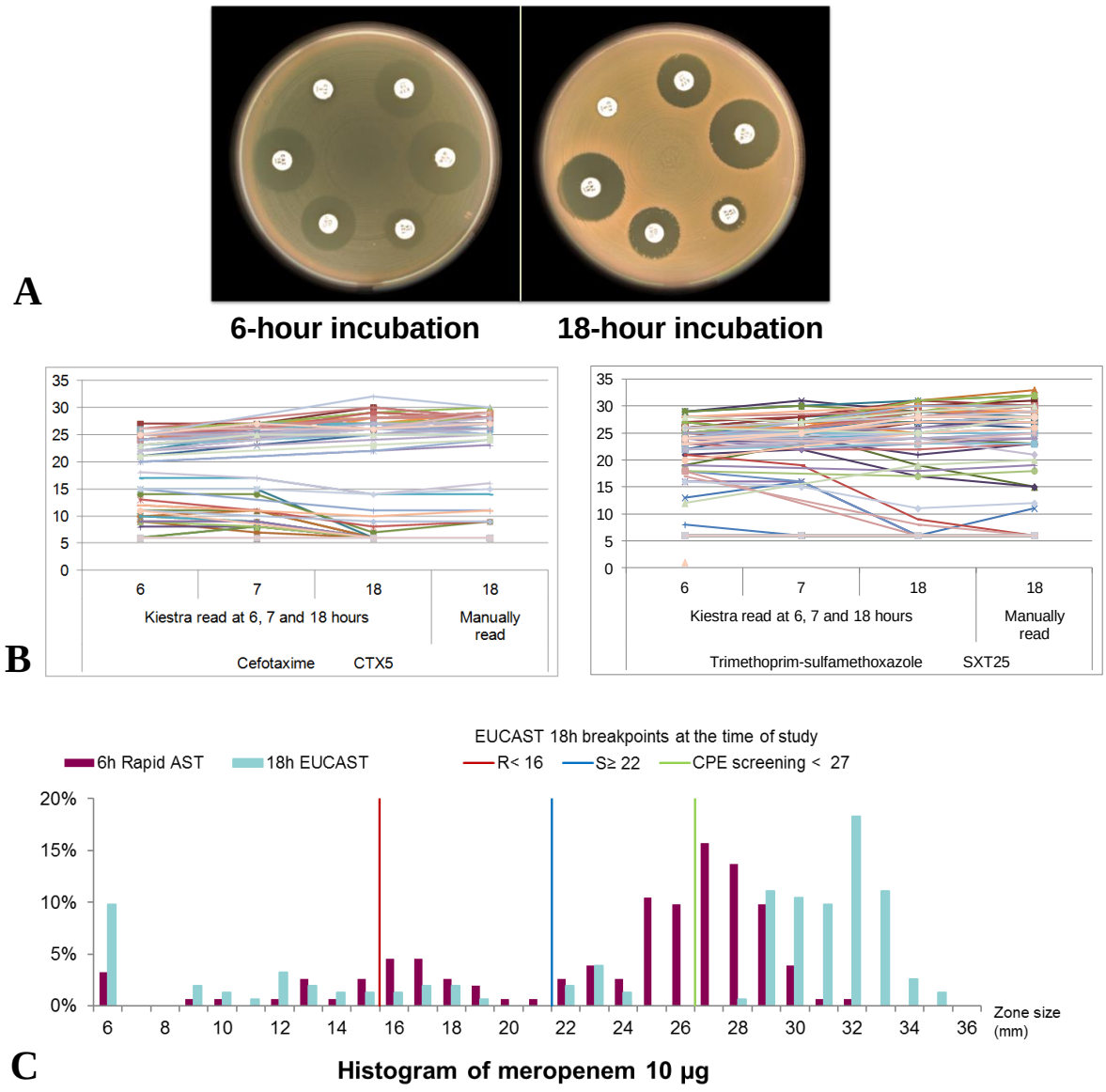


Figure 17. *Development of inhibition zones over time. A) Photograph of the same disk diffusion plate after 6 hours and 18 hours of incubation photographed with BD Kiestra TLA. B) Development of inhibition zones over time for cefotaxime and trimethoprim-sulfamethoxazole. C) Histogram of meropenem with 6-hour reading (plum) compared to 18-hour EUCAST reading (blue).*

At 5 hours of incubation, inhibition zones were difficult to read. In the final publication we compared 6-hour Kiestra-reading to 18-hour standard EUCAST manual reading.

As is shown in **Figure 17**, the inhibition zones for wild-type isolates typically increase in size over time, while the inhibition zones of resistant isolates become smaller. This results in less separation between susceptible and resistant isolates when inhibition zones are read after a short incubation time. This trend was true for all tested antibiotics, although the effect was more pronounced for meropenem and trimethoprim-sulfamethoxazole. The consequence is that adapted breakpoints for each reading time and for each species is necessary to avoid errors in interpretation.

4.2.1.2 Performance of rapid disk diffusion compared to standard disk diffusion

For cefotaxime the category agreement was excellent, 98.7%, even when standard EUCAST breakpoints were applied, with no major errors and no very major errors (**Figure 18**). However, for most other antibiotic agents, there were unacceptably many errors when using standard EUCAST breakpoints (**Table 11**). These errors could to a large extent be eliminated for cefotaxime, ceftazidime, piperacillin/tazobactam, meropenem, ciprofloxacin and gentamicin, by using the adapted tentative breakpoints suggested by Jonasson *et al.* at the time of study [90]. The only error that remained was one isolate with a minor error for meropenem.

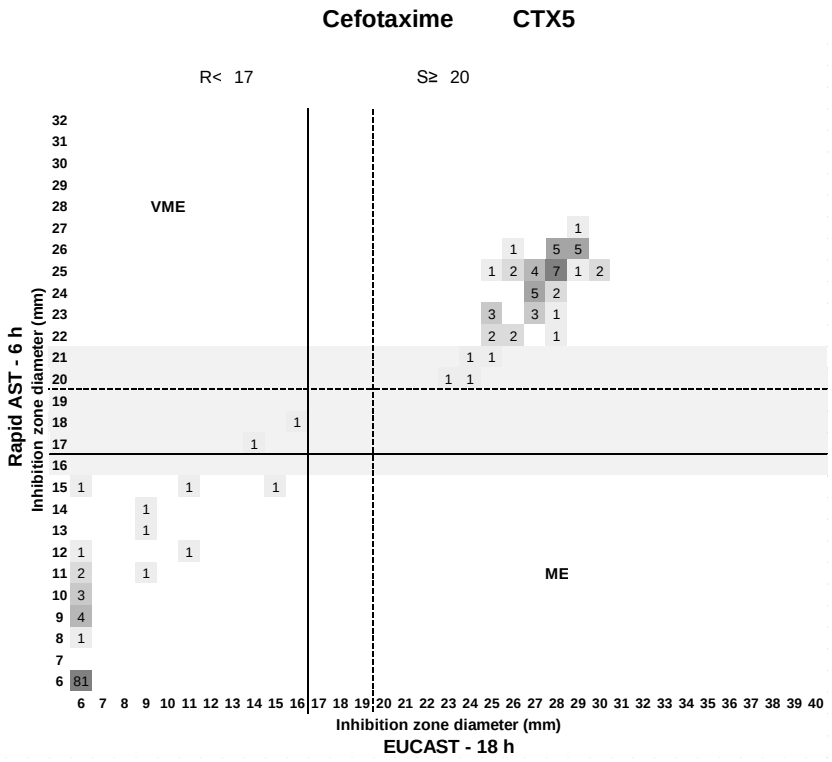


***Figure 18**. Correlation between rapid AST inhibition zone reading at 6 hours with standard EUCAST reading at 8 hours for cefotaxime. EUCAST breakpoints applied to both readings are shown as lines (solid line, R/I breakpoint; dashed line S/I breakpoint.) The numbers refer to the number of observations for each data point. Results within the grey shaded area are classified as uncertain according to the tentative 6-hour reading breakpoints available at the time of study, these results should not be reported. Figure from **Study II**.*

Table 11. *Performance of selected antimicrobials with standard EUCAST breakpoints and breakpoints adapted for 6-hour reading available at the time of study. Table adapted from* ***Study II****.*

	Cefotaxime	Cipro-floxacin	Piperacillin-tazobactam	Meropenem
EUCAST 18h breakpoint ≥S/<R (mm)	22/17	22/19	20/17	16/22
RAST adjusted breakpoint[A] ≥S/<R (mm)	22/16	22/17	21/15	25/17
Errors with EUCAST 18h	**1.3% mE**	**11% mE**	**15% mE** **0.7% ME[B]**	**12% mE** **0.7% VME[B]**
Errors with adjusted bp	**0%**	**0%**	**0%**	**0.7% mE**
Adjusted breakpoints for Rapid AST always have ATU - category – do not report				
Proportion of results that fall into ATU	**4%**	**15%**	**33%**	**20%**

[A]Tentative breakpoints available at the time of study.
[B]All reported errors are proportions of all tested isolates.

4.2.1.3 Performance of rapid tests for detection of ESBL and carbapenemase-production

The ROSCO Diagnostica ESBL confirm kit 98011 (ceftazidime/ceftazidime+clavulanic acid and cefotaxime/cefotaxime+clavulanic acid), ROSCO KPC/MBL, MAST CAT-ID disk and the EUCAST disk diffusion meropenem-screening breakpoint for detection of CPE were also evaluated. These tests were more difficult to read at 6 hours, than the normal AST. For the ROSCO Diagnostica ESBL confirm kit, 6-hour reading had an overall sensitivity of 87% and a specificity of 98% (negative tests were inconclusive due to cefoxitin-resistance since this indicates the presence of AmpC for which an alternative test is needed). For 6-hour reading of the carbapenemase detection tests the overall sensitivity was low, especially for OXA-48-producing isolates, and some misidentifications occurred.

The conclusion was that ROSCO Diagnostica ESBL confirm kit can be read at 6 hours in isolates lacking cefoxitin-resistance, but that for accurate detection of carbapenemase-production the full 18 hour of incubation is needed.

4.3 STUDY III

Diverse sequence types of Klebsiella pneumoniae contribute to the dissemination of bla_{NDM-1} in India, Sweden, and the United Kingdom.

A total of 39 *K. pneumoniae* isolates carrying the metallo-β-lactamase- (MBL) gene bla_{NDM-1} were characterized in **Study III**, with the aim to identify if there was a domination of a single, or a few, successful clones or if the gene was widespread within different sequence types, and to evaluate if the DiversiLab typing method could be used as a rapid proxy method for MLST.

At the time of the study, this carbapenemase-gene was recently identified, the first description occurred only three years earlier [154]. Isolates carrying this gene are often extremely multi-drug resistant, in many cases only susceptible to last-resort antibiotics such as colistin.

The included isolates originated from various sample types from India (two cities, Chennai and Haryana), the United Kingdom and Sweden. They belonged to 16 different sequence types and ST14 (13 isolates) was the most common and was found in samples from all three countries. Out of these, 10 were from the same hospital in Chennai and might partly reflect a hospital transmission event. All ST14 isolates carried the K2 serotype, which is associated with invasive disease. Other sequence types with more than two isolates were ST11, ST149, ST231 and ST625. None of the virulence genes *allS*, *rmpA* or *wcaG* were detected in any of the isolates.

The bla_{NDM-1} gene was found on several different broad-range plasmids that can easily be transferred between species (IncA/C, IncL/M, IncN, IncHI1) and on the narrow-range plasmid IncF (IncFrepB). In 9 of the ST14 isolates bla_{NDM-1} was found on more than one plasmid type in the same isolate. The plasmid replicon type was untypable in 11 isolates.

Typing by the DiversiLab automated repetitive-sequence-based PCR method (described in the methods section) generated results consistent with sequence types obtained by MLST. However, for hospital transmission investigations, within the same ST/DiversiLab-type a more discriminatory method such as PFGE or WGS might be needed.

4.4 STUDY IV

ESBL-producing E. coli causing community-onset bloodstream infection and the association of bacterial clones and virulence genes with septic shock.

From the cases with community-onset bloodstream infection studied in **Study I**, the subset of patients with growth of an ESBL-producing *E. coli* in the blood culture were selected for further analysis in **Study IV**. A total of 278 isolates from these 260 patients with ESBL-EC BSI were submitted to whole genome sequencing. Association of selected ExPEC virulence genes, resistance genes and sequence types with severity of disease and mode of infection was sought in multivariable analysis taking patient characteristics into account on the 260 first-episode isolates. Isolates from repeated episodes were analysed separately.

The globally successful high risk-clone ST131 was the dominant clone (47% of isolates), as expected (**Figure 19**). The fluoroquinolone-resistant and *bla*$_{\text{CTX-M-15}}$-associated ST131 subclade C2 comprised 30% and subclade C1 11%. Other common sequence types were ST38 (7%), ST648 (6%), ST10 (4%) and ST405 (4%).

The overall all-cause 30-day mortality was low (6.5%), compared to other similar studies. Septic shock or death within 3 days was used as the primary outcome variable as a measure of severe initial disease and affected 27 patients (10%). In multivariable logistic regression, the factors most strongly associated with septic shock were a patient history of hematologic cancer or prior transplantation and detection of the virulence gene *iss* in the *E. coli* (**Table 12** and **Figure 19**). The product of the gene *iss*, increased serum survival, is an outer membrane-bound lipoprotein which causes complement resistance [112]. *iss* was detected in 73% of isolates in **Study IV**. Three patients with a history of hematologic cancer or transplantation had septic shock despite a lack of *iss* in the causing *E. coli*. But all immunocompetent patients that presented with septic shock had a strain in which *iss* was detected, which suggests that *iss* might be of importance for development of septic shock in immunocompetent patients.

The adhesins, especially *pap*, were associated with UTI as the mode of infection, while isolates from patients with post-prostate biopsy bloodstream infection were low in adhesins, had low overall virulence, suggesting that they are normally low-pathogenic isolates and a part of the rectal microbiota but cause bloodstream infection when translocated to the bloodstream through the biopsy procedure. The prophylactic treatment with ciprofloxacin that is commonly administered is unsuccessful due to ciprofloxacin resistance in these strains. Sequence types that were associated with post-prostate biopsy BSI were ST131 subclade C1, ST131 subclade A, ST648 and ST1193.

In **Study I** we saw that prediction of EPE BSI was difficult, and that 20% of cases with EPE BSI lacked the three major risk factors and had not been hospitalized in the last year before the current episode. Characteristics associated with this group of patients were previously healthy females with UTI, caused by an *E. coli* lacking FQ-resistance and MDR but carrying the adhesin *pap* and the protease *pic*. Repeated episodes in the same patient was caused by a closely related strain in 15 out of the 16 patients for whom several isolates were available for WGS. These are described in the methods section, in a comparison of different typing methods that can be applied on WGS data.

***Table 12**. Final multivariable model for association of patient and microbial characteristics with septic shock. Table simplified from **Study IV**.*

	No septic shock or death ≤3 days (*n*=233)	Septic shock or death ≤3 days (*n*=27)	Multivariable OR (95%CI)	P-value
Hematologic cancer or transplantation	17 (7)	9 (33)	10.8 (3.2-36.6)	**<0.001**
iss	167 (72)	24 (89)	5.5 (1.3-22.8)	**0.019**
Daily living activity ≥2	64 (27)	14 (52)	3.8 (1.4-9.9)	**0.007**
UTI or Prostate biopsy	192 (82)	14 (52)	0.3 (0.1-0.9)	**0.026**
Multidrug-resistant	150 (64)	10 (37)	0.3 (0.1-0.7)	**0.010**
papACGH	101 (43)	4 (15)	0.2 (0.1-0.6)	**0.004**

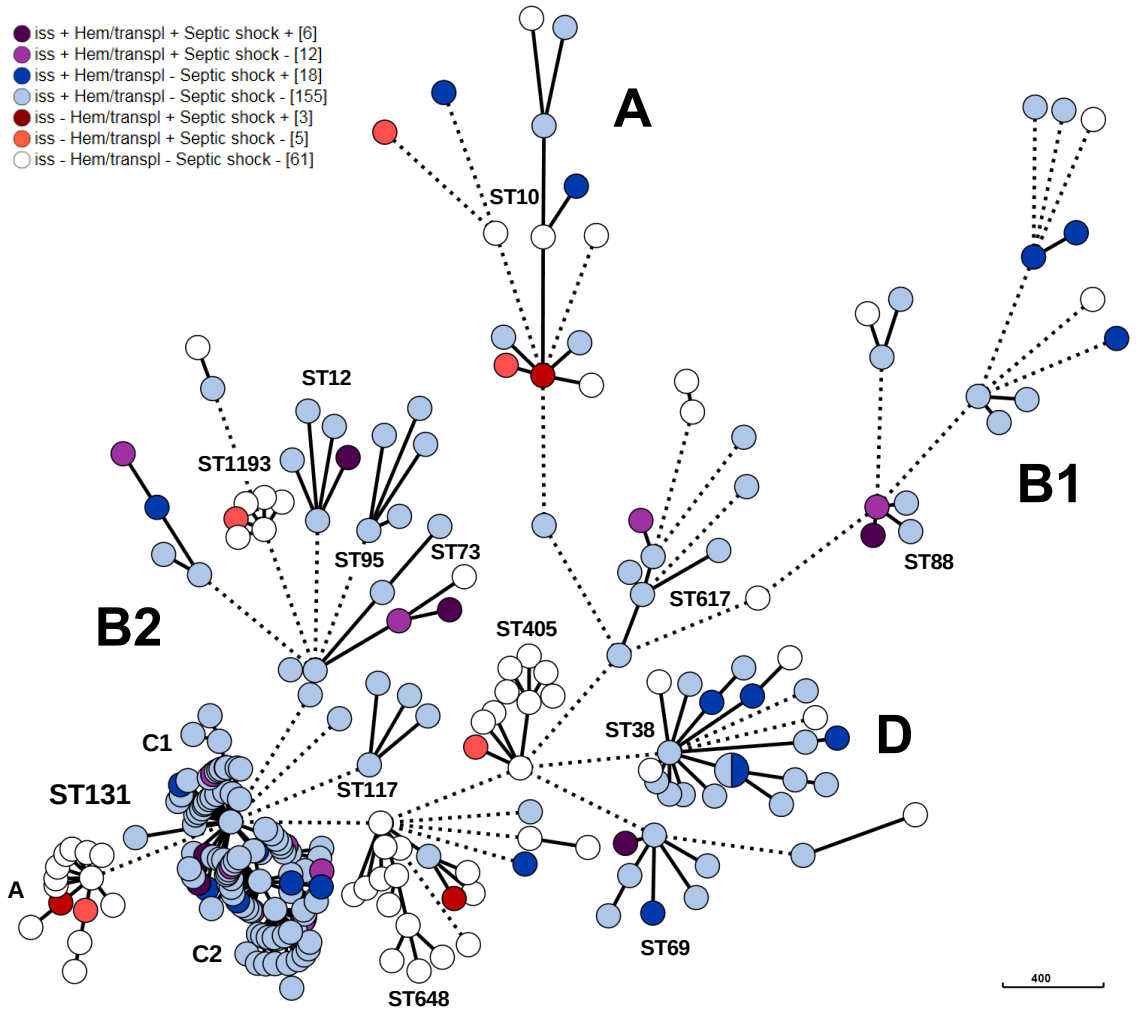


***Figure 19.** cgMLST minimal spanning tree of ESBL-EC from patients with community-onset BSI (n=260). Blue nodes are E. coli with iss; Red nodes are E. coli from patients with haematologic malignancy or transplantation; Purple are E. coli with iss from patients with haematologic malignancy or transplantation. Dark tone for each colour denotes patients with septic shock or death within three days. Phylogroups, STs and ST131 clades with more than three isolates are noted. Tree scale corresponds to number of allelic differences. Dashed lines are shortened to facilitate visualization.*
Iss is clearly differentially distributed among STs: ST131 clade A, ST1193, ST405 and ST648 generally lack iss (one exception for ST648). An E. coli without iss was never recovered from a patient with septic shock if the patient was immunocompetent (i.e. did not have hematologic cancer or transplantation.)
Illustration created with Enterobase GrapeTree. Note: the tree is the same as the thesis front picture but coloured differently.

5 DISCUSSION

5.1 EMPIRICAL THERAPY FOR SUSPECTED COMMUNITY-ONSET GRAM-NEGATIVE INFECTION

Whenever there is a suspicion of a serious bacterial infection in a patient at the emergency department, the physician in charge makes an educated guess of the probable nature of the causing organism and chooses appropriate empirical treatment to cover those that are necessary to cover initially but without causing overuse of broad-spectrum antibiotics. This choice is based on a many different facts, the history of the patient, comorbidities, presentation, severity of disease, suspected source of infection, travel history, the local resistance epidemiology etc. Is it possible to reduce this choice to a simple score and is that desirable?

Scoring systems, such as CRB-65 for pneumonia can be a help for physicians to triage and decide the level of care and standardized use of CRB-65 has been shown to improve care [155-157]. qSOFA was suggested to identify patients at risk for sepsis, but the sensitivity is unfortunately quite low and a better screening tool for sepsis is needed [38, 158, 159]. Scores should never be implemented and used singularly, it is always important to remember the limitations of the used score and to consider additional factors not included in the score that might be important. For example, misuse and misinterpretations of the Centor-criteria for tonsillitis have had adverse consequences, despite its great usefulness when applied in a correct fashion [160, 161]. However, used cautiously with knowledge and care, application of a prediction score for EPE BSI could improve the appropriateness of empirical therapy and be of benefit to these patients.

5.1.1 Development of a prediction score - which predictors to include?

In **Study I** we compared the simple Stockholm score with the more complex Utrecht score. There have been several other attempts to develop risk scores for EPE, but most of these were developed for other patient populations and were not appropriate for the population of patients with community-onset suspected gram-negative infection, which we wanted to target [60, 61]. The Utrecht score was developed in the Netherlands, which has an epidemiological setting concerning ESBL-prevalence similar to Stockholm [59, 153]. In the Rottier study, the estimated prevalence of third generation cephalosporin-resistant (3GCR) Enterobacterales was 0.4% in the source population and the rate of 3GCR Enterobacterales was 8.3% in BSI. In Stockholm, we had 0.59% estimated prevalence in the source population and an ESBL-production rate in *E. coli* from BSI of 7-9% during the years of the study.

We found that for the Swedish setting, the Stockholm score had a higher specificity than the Utrecht score. But interestingly, the aim of the authors of the Utrecht score, was to increase the specificity and minimize over-treatment compared to the Dutch empirical treatment guidelines that were in place at the time. The Dutch guidelines recommended broad-spectrum treatment for patients with a prior EPE-positive culture or prior exposure to fluoroquinolone or third generation cephalosporin, which gave a sensitivity of 53.9%, a specificity of 79.5% and a PPV of 1.0%. With the Utrecht score they obtained 54.3%, 87.2% and 1.7% respectively [153].

The Stockholm and Utrecht scores are similar and several of the risk factors included in the Utrecht score had an OR with a significant association with EPE BSI in univariate analysis also in **Study I.** A prior EPE-positive culture was the most important contributor in both scores, but for the Utrecht score, only prior EPE-positive cultures within one year was included. However, in **Study I**, we saw that the risk of EPE BSI is increased also for patients whose latest EPE-positive culture was sampled more than one year previously. The suspected source of infection is important for the risk of infection. In both **Study I** and the Dutch study, a suspected UTI is associated with an increased risk and pneumonia with a decreased risk. Unfortunately, UTI is so common that including this in an independent way in the score severely diminishes the specificity of the Utrecht score, as any patient >70 years of age, with UTI and prior antibiotic treatment in the last 2 months will become positive. In **Study I**, age was not linearly associated with risk of EPE BSI. Although antibiotic treatment with agents with an impact on the microbiota had a statistically significant association in univariate analysis, it was not significant in the multivariable analysis. This caused the low specificity of the Utrecht score, especially in the subgroup with suspected UTI, where the specificity of the Utrecht score was only 31% compared to 94% for the Stockholm score.

Prior antibiotic treatment lacked a statistically significant association with an increased risk of EPE BSI in the multivariable analysis in **Study I** and was thus not included in the score This contradicts results of many previous studies in which recent antibiotic treatment (variably defined as any antibiotic, fluoroquinolone or cephalosporin, or a composite group of antibiotics with selective activity against Gram-negative bacilli) is associated with an increased risk of EPE BSI in various populations [52, 59, 162, 163]. It is well known that use of antibiotics selects for AMR bacteria, and that sublethal concentrations can induce development of resistance [7]. There were several coactive reasons for this result in **Study I**: a prior EPE-positive culture was a very strong predictor, and prior treatment >3 months with a microbiota impact agent was strongly related to a prior EPE-positive culture. For 49% of the EPE BSI-cases with any prior EPE-positive culture, this culture was a clinical sample collected < 3 months (**Figure 16**). These patients certainly would have been treated with a microbiota impact agent at that time and likely also at previous occasions. Recurrent infections are common in patients with EPE in clinical samples, which is also clear from a recent study by Lindblom *et al.* [164]. In the group of patients that lacked the three major predictors in **Study I**, prior treatment with a microbiota impacting agent was indeed statistically significantly associated with EPE BSI (Multivariable OR 1.9 (95% CI 1.2-3.0) p=0.008) but was in this subset of patients too common in both cases (45%) and controls (32%) to be useful as a predictor in a score. In conclusion, there is an association between prior antibiotic treatment < 3 months and EPE BSI, but this variable should not be used for empirical treatment decisions.

When translating results of risk factor associations into a prediction score, there are several pitfalls. First, the balance between sensitivity and specificity is important - what loss in specificity is acceptable to increase the sensitivity? Inclusion of predictors with a moderate OR but with a high prevalence in both cases and controls decreases specificity. Second, although there might be a statistically significant association in the analysis, how robust is this result? The result could be caused by for example study information bias and random effects. Third, is the predictor a piece of information that is readily available at the time of

decision? Fourth, how complex should the score be? A simple score is easy to use (and to create), but with a more complex score with weighting of the included predictors it might be possible to increase prediction performance.

The strategy of creating a simple score and aiming at a high specificity, meant that we ended up including the two risk factors for EPE which were already well known, a prior positive EPE-culture and prior healthcare abroad. These factors many clinicians already consider when choosing empirical therapy, but our results strengthen the reasons to follow this practice. The third part, prior prostate biopsy, is related to the practice of giving ciprofloxacin-prophylaxis during the procedure. The ciprofloxacin-prophylaxis probably is efficient for limiting BSI of ciprofloxacin-susceptible isolates but has probably no or very limited effect on development of BSI of ciprofloxacin-resistant isolates, which are often ESBL-producing. The procedure including prophylaxis is common in many parts of the country, but still the extent of the risk increase with prior prostate biopsy might be enhanced due to local traditions.

The complexity of the score could be less of a problem, if sufficient programming skills and resources were applied to further development computerized medical records. Scores such as this should not be difficult to integrate into the medical records, most included variables could be automatically retrieved from the medical records and automatically calculated and presented.

The Utrecht score is currently under prospective validation in a worldwide study (10 European countries and Japan) called ESBL-PREDICT, according to preliminary results presented at the 29th ECCMID conference in 2019 [165]. The present validation cohort includes 4,148 community-onset episodes of suspected Gram-negative infection, of which 87 were cases with third generation cephalosporin-resistant gram-negative bacteria (3GCR-GNB) BSI (2.1%). The proportion of patients treated with meropenem was 8.6%. Interestingly, this is very similar to the proportion in **Study I,** in which 8% were treated with meropenem. The authors report that with application of the score 6.7% of patients would be identified as at high risk for 3GCR-GNB BSI and thus require meropenem and they conclude that carbapenem treatment could be reduced. However, this conclusion does not consider the severity of disease or other indications for carbapenem treatment that might be present. The degree of improvement of appropriateness of treatment is not clear from the conference abstract either. The results of the future publication of this project will be interesting to follow.

5.1.2 How should the Stockholm score be used in practice?

Use of the Stockholm score has not been validated prospectively in clinical practice, which should be done in a separate population before application of the score is implemented on a broad scale. The epidemiology changes over time, and the composition of the population also changes. Nevertheless, the results of the studies included in this thesis increase our knowledge of important predictors for an increased risk of EPE BSI, which could be of use when choosing empirical treatment.

A strict application of the Stockholm score was evaluated in **Study I**, where all patients with a positive score were given either meropenem or piperacillin-tazobactam and the result was

that giving all patients with a positive score meropenem would improve the time to appropriate treatment the most. But do all patients with suspected Gram-negative infection and a positive score really need carbapenem treatment as empirical therapy? Calculation of the score should be performed on all patients with a suspected Gram-negative infection and the physician should take the resulting risk for EPE BSI into consideration when choosing empirical treatment. However, individualized medicine with differential prescription depending on the patient, and which of the predictor in the score that is positive, is strongly recommended.

For which patients is the time to appropriate therapy vital? Clearly, there is evidence of improved survival if appropriate therapy is given within a few hours for patients with septic shock [166-168]. Thus, for all patients with septic shock, meropenem treatment is appropriate, regardless of the result of the score. This is partly to cover EPE, as was seen in **Study I**, the risk for EPE BSI is increased in patients with septic shock, but also to have a broad coverage of other causing organisms. In this context it is important to remember that the sensitivity of the Stockholm score is only 50%.

For patients with a milder presentation, sepsis or just bloodstream infection, there is evidence that ESBL-production increases healthcare costs and hospitalization duration [48]. An effect on survival is less evident, although lack of early targeted therapy (< 4 days) was independently associated with 30-day mortality in one study [169]. For these patients the indication of meropenem treatment is more relative and the benefit of the patient needs to be compared to the risk of selecting for further resistance and a future situation with even higher prevalence of carbapenem-resistant bacteria.

Meropenem should probably be given empirically to patients with any prior EPE BSI, a prior EPE-positive clinical culture within 6 months and patients with a positive score and immunosuppression. Prior EPE BSI and other recent clinical samples are associated with a high risk of EPE BSI and it is thus a recurrent infection. Early appropriate therapy in these cases might reduce the time to improvement, development of septic shock and risk of future recurring events.

Patients with a post-prostate biopsy BSI have a high risk of having EPE BSI, but the risk for septic shock is low, the patient is often healthy with unaffected daily living activity, and they are often caused by low-virulent strains as was seen in **Study IV**. For these patients, and for other patients with a positive Stockholm score that have a mild initial presentation and low risk for septic shock, a carbapenem-sparing regime with piperacillin-tazobactam in the high dose 4g every 6 hours might be the best choice.

However, when treating a suspected or verified EPE BSI with piperacillin-tazobactam there are several aspects to consider. Studies comparing the outcome of treatment of EPE BSI with different antibiotics have come to diverging conclusions. Three studies concluded that piperacillin-tazobactam as empirical or definite therapy is a useful alternative to a carbapenem [170-172], while the Merino trial by Harris *et al.* concluded that definite treatment of EPE BSI with piperacillin-tazobactam was inferior, and the study was discontinued in advance [173]. This study has been criticized for the inferior AST method used for determining susceptibility for piperacillin-tazobactam and that almost all mortality

was in the group with terminal cancer [174]. AST of piperacillin-tazobactam generally, but especially in ESBL-producing isolates is notoriously difficult with all methods. The proportion of EPE resistant to piperacillin-tazobactam in **Study I** was 14.8%**.** As was seen in **Study II**, rapid disk diffusion is difficult with piperacillin-tazobactam. If one uses piperacillin-tazobactam empirically one cannot generally expect to have an AST result for this agent until the final AST is performed. Even then, a substantial additional proportion of EPE would end up in the area of technical uncertainty (ATU), where there is uncertainty if the isolate should be classified as resistant (R) or susceptible with or without increased exposure (I/S), regardless if disk diffusion or an MIC-method was used. In conclusion, if one decides to use high-dosage of piperacillin-tazobactam as empirical therapy in a patient with a positive Stockholm score, the patient's clinical response should be closely monitored and if there is any sign of failure of improvement, the therapy should be changed.

Cefotaxime in combination with an aminoglycoside, especially amikacin, has been suggested as an alternative carbapenem-sparing treatment [171]. However, in the event of an EPE this would mean monotherapy with an aminoglycoside, which might result in inadequate concentrations at the infection site and is not recommended in infections other than urinary tract infections [175-178]. In both **Study I** and **Study IV** the rate of resistance to gentamicin was 37%. The resistance to amikacin was lower, but data on amikacin-resistance was incomplete. WGS-data in **Study IV** showed that 96% of the gentamicin-resistant isolates had at least one gene for an aminoglycoside modifying enzyme with effect on gentamicin: 94% carried an *aac(3')-II* (aminoglycoside acetyltransferase gene) and 5% carried *ant(2')* (aminoglycoside adenylyltransferase gene). No phenotypically gentamicin-susceptible isolates carried the genes encoding these two enzymes.

5.2 RAPID DISK DIFFUSION

5.2.1 Why do inhibition zones change over time?

The inhibition zones of wild-type isolates increase over time, but the zones of resistant isolates decrease. The explanation is that disk diffusion is completely dependent on the passive diffusion of the molecules, in contrast to gradient tests, where application of a gradient test rapidly creates an antibiotic gradient in the agar. For wild-type isolates the time of diffusion of the antibiotic from the paper disk through the agar is most important. During the early phases when rapid reading is applied at 4-8 hours, the concentration in the agar at the border of growth is still slowly increasing, and the diffusion does not reach an equilibrium until after about 12-16 hours [179, 180]. The diffusion rate of an antibiotic depends on various molecular properties which explains that some antibiotics have a bigger difference for wild-type bacteria between 6 and 18 hour-readings than others [181]. Resistant isolates, on the other hand, sometimes have a slower growth due to the burden of resistance that decreases fitness [182]. Additionally, in some cases (in **Study II** mostly seen with trimethoprim-sulfamethoxazole and carbapenems) resistant sub-populations and differential expression can cause only individual colonies to appear resistant which might be explained by a lag-phase so that they initially behave as susceptible isolates and only later start to express resistance [77]. These individual colonies are considered when reading 18-hour EUCAST disk diffusion but are difficult to see at early reading.

5.2.2 Clinical implementation of rapid disk diffusion

Rapid disk diffusion, which was evaluated in **Study II,** is a cost-effective way of obtaining rapid AST. Other rapid methods are available, of which some are promising, but they are comparatively expensive, can only be performed on a few positive blood cultures per day, and a limited number of antibiotics [77, 183, 184].

When performed directly from positive blood cultures, inhibition zones for *E. coli* and *K. pneumoniae* are often readable as early as after 4 hours, the bacteria grow faster since they are already in growth mode in the blood cultures. To ensure reproducibility and minimize errors of interpretation, as standardized a method as possible is necessary. When performed directly from positive blood culture bottles, complete standardization of the inoculum is not possible since the concentration of bacteria in the bottle varies between $9.1x10^6$ and $3.4x10^9$ [185]. EUCAST published a validated method with species- and reading time-specific breakpoints in November 2018 and added breakpoints for *Acinetobacter baumannii* in May 2019 [186]. Although EUCAST suggests that the early reading can be considered a final result, if not in the ATU, a reasonable measure to ensure correct AST in bloodstream infection is to also perform a standard disk diffusion. This is needed anyway if any result is in the ATU and for antibiotics such as trimethoprim-sulfamethoxazole for which there is not yet any rapid reading breakpoints established. Considering the results in **Study II** it is doubtful if it will be possible to create useful early breakpoints for this agent. Additionally, in the case of a polymicrobial infection that was not recognized at the time of the early reading, errors in interpretation can occur due to a mixture of bacteria. It is therefore a sensible precaution to warn the clinician that the results are preliminary and will be confirmed.

The most important task for the rapid AST is to detect resistance to the empirical antibiotic treatment the patient received and, in these cases, to report susceptibility for alternative treatments so that therapy correction can be performed as early as possible. Treatment de-escalation for antibiotic stewardship purposes are also an important task for AST, but it is probably in many cases more convenient to wait one more day until the AST-results are confirmed before de-escalation.

The many different breakpoints that are assigned for the three reading times 4, 6 and 8 hours, and each species are as was seen in **Study II**, are completely necessary, but this is at the same time a challenge for routine laboratories to implement. The EUCAST method requires reading of early inhibition zones +/- 5 minutes of the specific incubation time, otherwise to re-incubate and read at the next reading time. This time frame is almost impossible to keep in a busy routine laboratory. Local adaptations such as a wider ATU that encompasses all the ATUs for 4-8 hours is one way to simplify the process and enable breakpoints to be easily programmed in the laboratory information systems. The drawback of this approach is that a higher proportion of isolates will be in the ATU and not possible to report. In special cases, a manual interpretation according to the time- (and species-) specific EUCAST breakpoint tables can then be performed.

For rapid ESBL-detection in third generation cephalosporin-resistant isolates and suspected CPE, lateral flow tests for the most common CTX-M and CPE types [29, 187, 188], respectively, are probably better options than 6-hour reading of ROSCO ESBL and CPE confirmation kits, although further clinical validation of these tests would be valuable,

especially if they are to be applied on 4-6 hour cultures. In **Study IV**, 92% of the isolates carried CTX-M-genes which encode enzymes that, according to the manufacturer, are included in the lateral flow CTX-M Multi-test (NGbiotech, Guipry, France) [189].

5.2.3 Implication of EUCAST new SIR-definitions for future AST-method evaluations

EUCAST changed the definition of the S/I/R-categories on 1 January 2019. The most significant change was that of the definition of the "I"-category [68, 190, 191].

The previous definition (used 2002-2018) was:

"I - Clinically Intermediate, a microorganism is defined as intermediate by a level of antimicrobial agent activity associated with uncertain therapeutic effect. It implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used; ***it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations."*** [192]

The new definition reads:

"I – Susceptible, Increased exposure. A microorganism is categorised as "Susceptible, Increased exposure" when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or its concentration at the site of infection….*
**Exposure is a function of how the mode of administration, dose, dosing interval, infusion time, as well as distribution and excretion of the antimicrobial agent will influence the infecting organisms at the site of infection."* [68]

This change emphasises the importance of exposure and dosing and is a necessary change to accommodate the increasing need to guide antibiotic therapy, and to indicate which agents are treatment alternatives when treatment success is probable when high exposure is achieved despite the presence of resistance mechanisms. At the same time the introduction of a new concept of ATU, area of technical uncertainty, poses greater demands on the laboratories for quality assurance of the AST methods used, and EUCAST recommends individualized interpretations on results in the ATU. However, the removal of the buffer zone interpretation of the I-category and the new concept ATU, complicates comparisons of AST methods. Previously any result that included an I-result was considered a minor error. What comprises a minor error with the new SIR-definitions? When there is no I-category, all discrepancies are either VMEs or MEs. Should discrepancies that include any result that fall into ATU be considered minor errors? Since the I-category now is considered Susceptible, increased exposure – should a discrepancy of I with new method and R with reference method be considered a VME instead of a minor error? This is especially important for agent-microbe combinations where the entire wildtype now is categorized as "I". There is a need for EUCAST to acknowledge these consequences of the change and for the regulatory agency European Medicines Agency to clarify how to handle this issue. It has hitherto not been addressed in the currently available documents about the SIR-definition change on the EUCAST web page, or by ISO [192]. The ISO Standard 20776-2:2007 is currently under revision [193].

5.3 IS THE GLOBAL NDM-1 EXPANSION DRIVEN BY SUCCESSFUL CLONES OR PROMISCIOUS PLASMIDS?

NDM-1 has spread rapidly since the first observation in 2008 [154] and the early years of the expansion when **Study III** was published in 2012. NDM has become the second most common carbapenemase in Sweden, after OXA-48, although the total number of CPE is still low, 144 reported cases in 2018 out of which 31% were clinical infections. Approximately 40% of isolates carried NDM and 50% carried OXA-48 [194]. According to the SMART global surveillance study conducted in 2008-2014, *bla*$_{\text{NDM}}$ was the third most common carbapenemase-gene comprising 19% of carbapenemase-producing isolates, compared to 53% for KPC and 20% of OXA-48 [18, 195]. The study showed large regional differences, with KPC being most prevalent in North America and Latin America, OXA-48 in Africa and the Middle East, and NDM in Asia and the South Pacific. Europe has a mixture of mainly KPC, and OXA-48, but also VIM and NDM [195]. As most (73%) of reported cases of CPE in Sweden are identified in targeted screening at admission to hospital of patients with a history of hospitalization abroad [194], the epidemiology of CPE in Sweden mainly reflects the international travel and healthcare patterns of Swedish patients.

In a thorough review of NDM-carbapenemase epidemiology published in April 2019, Wu *et al.* summarizes the current knowledge which has greatly expanded since **Study III** was published in 2012 [18]. Although the origin of NDM is still not completely defined, there are indications that *bla*$_{\text{NDM-1}}$ originated in *Acinetobacter* species and is a chimera of the aminoglycoside resistance gene *aphA6* and a pre-existing MBL-gene [196]. *bla*$_{\text{NDM}}$ genes have been found in a wide range of Gram-negative bacilli, both mainly environmental species and common human pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and many different species of the Enterobacterales, but *K. pneumoniae* and *E. coli* are the most common. In support of our results from **Study III,** there is still no clearly predominant clone of NDM-positive *K. pneumoniae* on a global scale and NDM is found in many different sequence types. The most common are ST14, ST11 and ST147, which were also observed in **Study III**. Other frequently reported types are ST15 and ST23 (mainly China). The situation is the same for *E. coli*, where NDM is found in diverse sequence types. The genes are usually located on plasmids and 20 different plasmid replicon types have been associated with *bla*$_{\text{NDM}}$ in *Enterobacteriaceae*, including broad-range plasmids IncA/C and IncL/M and the narrow-host-range plasmids IncX3 (hitherto only seen in the *Enterobacteriaceae*), IncFIA, IncFIB and IncFII [18]. This indicates that horizontal transfer is the most important way for the spread of *bla*$_{\text{NDM}}$ and that it is mediated by multiple plasmids with different properties. Nevertheless, local clonal spread of NDM-producing *K. pneumoniae* in hospital settings is a concern [18, 33, 197], which means that intervention strategies must be directed both at minimizing direct transmission and horizontal transfer.

5.4 POSSIBLE TREATMENT TARGETS IN SEPSIS - HOST RESPONSE AND MICROBE VIRULENCE

With increasing levels of resistance that threaten to make effective antibiotics useless, the search for new classes of traditional antibiotics for Gram-negative bacteria has intensified in the last decades but have unfortunately largely failed (with a few exceptions, *e.g.* the promising new class of siderophore cephalosporins (cefiderocol) [27]). There is thus a great need to search for alternative treatment strategies. Clearly, both the host response and microbial virulence influences infection and severity of disease, and thus are potential targets for future sepsis treatment.

The pathogenesis in sepsis is complex and characterized by a dysregulated immune response [38]. Several studies show that the type of dysregulation differs between individuals and the risk of dying of sepsis is associated with human genetic predisposition. As was seen in **Study IV**, it is well known that patients with immunosuppression due to hematologic malignancy and transplantation are at higher risk of infection, septic shock and mortality. However, also in patients without acquired immune suppression, there are important differences regarding immune response between individuals. A sepsis transcriptomics study identified two types of patients with distinct sepsis response signatures (SRS1 and SRS2) based on global genetic expression, the SRS1 group that was characterized by immunosuppression was associated with higher early mortality than SRS2 [198]. Further studies showed that patients could be classified into four groups based on different gene expression patterns [40]. A phase 1b clinical trial testing safety issues for an immunomodulatory therapy in patients with the immunosuppressed SRS1 phenotype was published in 2019. This therapy targets an immune checkpoint pathway, the programmed cell death protein-1 (PD-1) / programmed cell death-ligand 1 (PD-L1) pathway [199]. The pathway regulates the immune response under normal conditions. In certain cancer cells and in sepsis-induced dysregulated immunosuppression an upregulation of this pathway is seen. The upregulation causes T-cell inhibition. Monoclonal antibodies that block PD1 and PD-L1 is an effective cancer treatment and could also be effective in reducing the immunosuppression in sepsis and is a promising new treatment in the group of patients with sepsis characterized by immunosuppression. The Phase 1b study showed promising results, with no sign of a cytokine storm, an overstimulation of the immune response, which is one of the concerns with such therapies.

Virulence factors have been proposed as promising targets for new adjuvant infection treatments [6, 200]. The potential advantages of targeting virulence factors are several, including low impact on host commensal flora, possibly less evolutionary pressure for development of resistance with traditional antibiotics, rapid inactivation of targets [200]. Disadvantages include that they may require combination therapy for multiple virulence factors, empirical treatment is difficult due to species or strains-specific virulence factors and this requires rapid diagnostic methods, the microbes might not be killed and may persist and cause damage when the treatment is stopped. They may be less efficient compared to antibiotics, but adjuvant effects could possibly be achieved. Development of anti-virulence drugs for sepsis requires deep understanding of the sepsis pathogenesis and which roles different virulence factors play for the course of the infection [200]. Ideally blocking of the target would not affect the survival so that the overall selective pressure would not direct

bacterial evolution. Good targets are excreted or exposed on the surface of the microbe. Studies like **Study IV** is a first step for identification of potential important targets.

In **Study IV** we found a clear association of adhesion factors with a urinary tract infection source in patients with community-onset BSI caused by ESBL-EC, which was expected since adhesion factors are of well-known importance for UTI and pyelonephritis [116]. The tip of the *E. coli* adhesin type 1 fimbriae, FimH, is the target of two compounds in preclinical drug development phase, mAb926 and Compound 22 [200]. These agents were effective in mouse UTI models. The monoclonal antibody mAb926 prevented ExPEC attachment to epithelial cells and detached biofilms, while the biarylmannoside receptor analogue Compound 22 prevented UTIs and potentiated the effect of prophylactic antibiotic treatment. Despite their possible benefit in or preventing UTIs or treatment at an early stage of infection, since type 1 fimbriae are important for adhesion to the urinary epithelium, anti-virulence drugs targeting this virulence factor are not likely useful in sepsis since the *E. coli* are already in the blood and other virulence factors are more important for the course of sepsis.

An association with septic shock was found for the virulence factor *iss*, increased serum survival in **Study IV**. This virulence factor has some properties that are favourable for an anti-virulence target. Iss, the product of *iss*, is a lipoprotein located on the outer membrane of *E. coli* [201]. *iss* has primarily been studied as a virulence factor associated with avian colibacillosis, which is a systemic infection with *E. coli* in birds caused by APEC, a subset of ExPEC [201-205]. *iss* was associated with a 20-fold increase in complement resistance and a 100-fold increase in virulence toward 1-day old chicks [112]. Monoclonal antibodies to Iss were developed and used to ascertain the location of Iss on the outer membrane. Iss was also evaluated as an antigen for immunization for colibacillosis in poultry and showed a clear protective effect [204]. One concern about the usefulness of monoclonal antibodies or immunization based on Iss is its similarity, which causes cross-reactivity, with the product of the λ phage derived gene *bor*, which has a 90% similarity to *iss*. The *bor* gene encodes the lipoprotein Bor that is found on the outer membrane of these isolates and is common both in avian commensals and pathogens [201]. However, this similarity does not necessarily diminish the potential of Iss as a target for anti-virulence treatment in human sepsis. Bor was mainly found in *E. coli* that were of bovine or avian origin and less than 10% of human isolates carried Bor [112]. Bor was also uncommon in the phylogroup B2 [112], which is responsible for 50% of bloodstream infections in Sweden [94]. Additionally, Bor also confers serum-resistance, albeit at a lower level [112], so this cross-reactivity would probably not interfere negatively with a potential anti-virulence agent. Combined, these prior studies support that the cell-membrane bound Iss has a potential as a target for a monoclonal antibody that could be used as an adjuvant in sepsis-treatment to limit the severity of sepsis. Further studies on the importance of *iss* and effect of blockade with monoclonal antibodies in various virulence and sepsis models should be performed.

In conclusion, both immunomodulatory pathways and microbe virulence genes are promising new targets for adjuvant treatment in sepsis. However, since there is a great interindividual variation and variation in microbial virulence factors, individualized treatment requires new diagnostic methods that can determine the immune response pattern and determine which microbial virulence factors that should be targeted in each patient.

6 CONCLUSIONS

- Patients with a prior EPE-positive culture, prior healthcare abroad or a prior prostate biopsy with suspected community-onset infection are at risk of having EPE BSI and empiric therapy covering EPE should be considered for these patients at admission.
- Any finding of a prior EPE-positive culture is associated with an increased risk for EPE BSI, but for a recent culture taken within 6 months the increased risk is more pronounced.
- Risk-scoring systems for AMR bacteria are dependent on local epidemiology and must be locally validated before implementation.
- Prediction of EPE BSI is difficult, 50% of patients with EPE BSI lack the three principal predictors and rapid diagnostic methods that include antimicrobial susceptibility testing are important.
- Rapid disk diffusion gives reliable results for the most important agents when adapted breakpoints are used.
- For rapid confirmation of ESBL- and carbapenemase-production rapid disk diffusion is less useful and other methods should be considered.
- *bla*$_{\text{NDM-1}}$ is found in diverse sequence types in *K. pneumoniae*. The global spread of *bla*$_{\text{NDM-1}}$ is to a large part caused by horisontal transfer of plasmids and there are no clearly dominant clones on a global scale. Nevertheless, both intra- and inter-hospital transmission occur and implementation of appropriate infection control measures are necessary to limit further expansion of these strains for which there are very few treatment options.
- The fluoroquinolone-resistant high-risk clone ST131 subclade C2 dominates in community-onset BSI caused by ESBL-producing *E. coli* in Stockholm.
- The *E. coli* virulence factor increased serum survival, *iss*, which causes complement resistance, is associated with septic shock and might be important for the severity of disease in immunocompetent patients.
- Post-prostate biopsy EPE BSI is often caused by low-virulent strains and septic shock is uncommon. Carbapenem-sparing treatment with coverage of EPE might be considered in these patients.
- Repeated episodes of EPE BSI in the same patient is often caused by a closely related strain.

7 FUTURE PERSPECTIVES

Prediction and individualized sepsis-treatment

The Stockholm score has the potential to improve the appropriateness of empirical treatment without a great increase in unnecessary treatments. However, implementation of the score should be accompanied by prospective validation to ensure the usefulness in clinical practice. With current medical journals it is time-consuming work for doctors at emergency departments to look for prior cultures and other important information. Time is limited in busy emergency departments. Improved modernized medical records where data can be easily extracted by computerized algorithms and automatically give the risk score and predicted susceptibility patterns based on relevant prior cultures could further increase the benefit of the score and facilitate validation [206].

This could favourably be combined with immunological tests, to determine patient's type of sepsis immune response, and deep learning (see below) to obtain a truly individualized sepsis treatment with both immunotherapy and appropriate antibiotic treatment.

Implementation of EUCAST direct RAST

Rapid phenotypic testing is important. The EUCAST Rapid AST disk diffusion method directly from positive blood cultures is a cost-effective, flexible method for rapid AST that will be implemented at Karolinska University Laboratory within the coming year. In addition to this, for selected, severely ill patients, ultra-rapid methods for species identification and AST with results within a few hours would be beneficial. There are various methods in development, based on nanotechnology, microfluidic methods, time-lapse microscopy, single cell imaging and flow cytometry [77, 86].

Oxford Nanopore in diagnostics

The new method Oxford Nanopore is still too expensive to be useful for routine diagnostics, but prices fall quickly and there is a high potential for clinical application of this method. Oxford Nanopore has been suggested for prediction of AST through a combination of sequence types and resistance genes [207]. Additionally, this method could shorten the time for infection control and enable early detection of nosocomial transmissions within a few days, compared to the several weeks it takes today. This would be especially useful if software that integrate the results of phylogenetic analysis such as cgMLST and information on patient movement and wards, were used. Combining these data in one software would produce a potent tool for early detection of hospital transmission events and enable early intervention. To accomplish this, high competence in computer software programming and bioinformatics combined with knowledge on infection control needs are necessary.

Studies on complement resistance and iss

The *iss*-gene was associated with septic shock, especially with immunocompetent patients. This warrants further investigations to see if the finding can be repeated in a larger patient material and to find out if the increased severity is mediated through the *iss*-gene, or if it is mediated through an unknown associated mechanism. It would be interesting to study complement-resistance of the isolates in **Study IV**, see if there is an association of

complement resistance both to septic shock and to *iss* in this collection and if this complement resistance could be inhibited with a monoclonal antibody.

Potential applications of deep learning in microbiology

Deep learning, a subset of artificial intelligence and machine learning, is a process of exposing multi-layered artificial neural networks to vast amounts of data, thereby training the networks to perform certain tasks [208]. The computational power of these kinds of neural networks has increased rapidly in the last decade. Deep learning is especially useful for finding patterns in images and other complex datasets and has already been widely used for analyses in radiology and pathology. However, the methods are hitherto not widely applied for microbiology despite the high potential. A PubMed search in October 2019 for deep learning AND radiology gave 1,138 hits; deep learning AND pathology 751 hits but deep learning AND (Microbiology OR antimicrobial OR antibiotic) only 132 hits. Training of artificial neural networks involves exposing them for a high number of annotated images/datasets (labelled with the "true" result as defined by a reference method), with this the neural networks find patterns and improve performance the more datasets they are provided with.

There are several potential applications of deep learning in microbiology related to the studies described in this thesis. As deep learning is especially suitable for image pattern recognition, it could be used on the digital images obtained by the Kiestra BD system, for facilitating the manual digital reading performed today. With proper training it would be useful for identifying possible pathogens and typical normal microbiota findings. In antimicrobial susceptibility testing with disk diffusion it could provide a highly standardized, rapid and accurate reading of inhibition zones.

Deep learning could be applied to clinical prediction models in combination with modern medical records where relevant data could be automatically extracted. Then the risk score and suggested therapy could be presented. The deep learning system could continuously adapt and improve the prediction model over time, although this would require repeated validation. However, proper consideration for patient integrity and data protection must be ensured if these methods are implemented.

As a continuation of the study of the influence of microbiological virulence for septic shock, deep learning in combination with Genome Wide Association Studies (GWAS) could be applied. In **Study IV** 108 genes were studied but with this method the neural networks could look for patterns of association with septic shock in the entire genome, combined with a high number of clinical parameters and results from immunological tests and expression data.

8 SAMMANFATTNING PÅ SVENSKA

Antibiotikaresistens är ett ökande problem, och särskilt resistensförekomst hos tarmbakterier, eftersom de blir mycket svåra att behandla. Utveckling av antibiotikaresistens är en naturlig evolutionär process där antibiotikaproducerande miljöbakterier som funnits i miljontals år och har utvecklat resistensgener för att skydda sig själva, nu överför dessa resistensgener till de bakterier som orsakar infektioner hos människor. Antibiotika utsätter bakterierna för ett evolutionärt tryck, vilket ger de resistenta bakterierna en fördel varmed dessa ökar i antal. Antibiotikaresistenta bakterier är ett hot mot global folkhälsa. I de flesta länder i världen är resistensfrekvensen mycket högre än i Sverige.

Escherichia coli, som tillhör tarmbakteriegruppen Enterobacterales, är en av de viktigaste sjukdomsframkallande bakterierna. Det finns många olika sorters *E. coli*. För det mesta utgör de en viktig del av en frisk tarmflora men vissa är diarré-framkallande och orsakar bl.a. turist-diarré. Andra typer av *E. coli* kan orsaka urinvägsinfektion och blodförgiftning. Dessa bakterier kallas ExPEC, extra-intestinala patogena *E. coli*, vilka förekommer i tarmfloran och orsakar infektion när de hamnar i andra organ. ExPEC bär många olika virulensegenskaper som bl.a. underlättar för bakterien att fastna i urinvägarna. Det kallas bakteriemi när bakterierna från en infektionshärd når blodet, och detta kan leda till blodförgiftning. *E coli* är den vanligaste orsaken till bakteriemi och urinvägsinfektion är för *E. coli* den vanligaste ursprungshärden. Vid blodförgiftning är det viktigt med snabb behandlingsstart med effektiva antibiotika. Antibiotikabehandling som startas innan bakterieodlings-svar finns tillgängliga kallas empirisk behandling. Den vanligaste empiriska behandlingen i Sverige är cefotaxim.

Alla typer av *E. coli* kan förvärva resistensmekanismer mot antibiotika. Den vanligaste resistensmekanismen hos *E. coli* är att de bildar ett enzym som hydrolyserar (bryter ner) antibiotika. Det enzym som ger resistens mot cefotaxim kallas extended-spectrum beta-lactamase (ESBL). Vissa enzym påverkar också karbapenemer och kallas $ESBL_{CARBA}$. Genen som kodar för dessa enzym sitter på en plasmid, ett cirkulärt DNA-fragment, som bakterier lätt kan överföra mellan två bakterier av samma eller olika art, vilket underlättar spridning. Under början av 2000-talet ökade andelen med cefotaxim-resistens bland fynd av *E. coli* i blododlingar provtagna på Karolinska mycket snabbt, från 3% år 2005 till 10% år 2012.

Denna bok handlar om patienter som drabbas av bakteriemi och blodförgiftning med ESBL-bildande bakterier. På grund av resistensen fungerar inte cefotaxim, den vanliga empiriska antibiotikabehandlingen. Fel behandling innebär risk för längre vårdtider och ökad dödlighet. Målsättningen har varit att förbättra behandlingen av patienter med blodförgiftning orsakad av ESBL-bildande bakterier på olika sätt: tidig identifiering av högriskpatienter på akutmottagningen, snabb resistensbestämning samt ökad förståelse av resistensspridning och virulensfaktorers påverkan på svårighetsgraden av sjukdomen.

I **Studie I** var målsättningen att skapa ett enkelt system för risk-klassificering för att identifiera vilka patienter som behöver anpassad empirisk antibiotikabehandling med effekt även mot ESBL-bildande bakterier. Riskfaktorer för bakteriemi med dessa bakterier undersöktes därför i en retrospektiv fall-kontroll studie med 277 fall och 400 kontroller. Två olika risk-klassificeringssystem jämfördes. Studien visade att de tre starkaste riskfaktorerna var fynd av ESBL-bildande bakterier i en tidigare odling (särskilt fynd inom de senaste 6

månaderna), prostatabiopsi inom en månad och sjukvård utomlands senaste halvåret. Av patienterna med bakteriemi orsakad av ESBL-bildande bakterier hade 50% någon av dessa tre riskfaktorer, medan det var svårt att förutspå risken hos övriga patienter. Resultatet understryker vikten av snabb diagnostik och resistensbestämning.

Målsättningen med **Studie II** var därför att utvärdera en snabb resistensbestämningsmetod. Diskdiffusion är en vanlig metod för resistensbestämning. En bakteriesuspension stryks på en agaryta varefter antibiotikalappar läggs på och antibiotikan diffunderar passivt i agarn. Hämningszonens storlek avgör om bakterien är känslig eller resistent. Vanligtvis avläses zonerna nästkommande dag. I studien undersöktes zonavläsning efter kortare tid (6 timmars inkubation) på 128 *E. coli* och *K. pneumoniae* med resistens för viktiga antibiotika och jämfördes med normal (18 timmars) inkubation. Studien visade att zonstorlekarna var annorlunda vid tidig avläsning, samt att skillnaden mellan resistenta och känsliga isolat var mindre. Men 6-timmarsavläsning var ändå en tillförlitlig metod om man anpassade gränserna för vad som rapporterades som känsligt respektive resistent. Detta innebär att cefotaxim-resistens kan påvisas en dag tidigare, och den empiriska behandlingen kan vid behov ändras.

En god förståelse av resistensgeners spridningsvägar är viktigt för utveckling av effektiva strategier för att motverka spridning. I **Studie III** undersöktes 39 *K. pneumoniae* av typen $ESBL_{CARBA}$ från Indien, Storbritannien och Sverige. Dessa bildade ett karbapenemas vid namn NDM, New Dehli metallo-beta-laktamas, som är kopplad till multiresistens och få behandlingsalternativ finns. Studien visade att NDM fanns hos många olika s.k. sekvenstyper av *K. pneumoniae* och på många olika typer av plasmider. Slutsatsen var att resistensmekanismen främst spreds genom plasmidöverföring, men samtidigt förekom lokala utbrott.

Bakteriemi kompliceras ofta av blodförgiftning (sepsis) och septisk chock vilket medför hög risk för död. Med ökande resistensförekomst som gör vanliga antibiotika obrukbara behöver efterforskningar göras för att hitta nya angreppspunkter för läkemedel, t.ex. virulensfaktorer. För att identifiera lämpliga mål krävs en korrekt förståelse av virulensfaktorers betydelse för sjukdomsuppkomst och förlopp vid blodförgiftning. Målsättningen för **Studie IV** var att undersöka den relativa betydelsen av patientfaktorer och bakteriefaktorer för uppkomst av septisk chock hos patienter med blodförgiftning orsakad av ESBL-bildande *E. coli.* Totalt 278 ESBL-bildande *E. coli* från 260 patienter med blodförgiftning (vilka ingick i **Studie I**) undersöktes med helgenomsekvensering för att kartlägga bakteriernas arvsmassa, DNA-sekvens. Resultaten visade att virulensgenen *iss* (increased serum survival), som medför resistens mot immunförsvarets komplementsystem, var associerad med septisk chock, särskilt hos patienter utan hematologisk cancer eller transplantation.

Sammantaget har studierna i denna avhandling visat att behandling av patienter med bakteriemi orsakad av ESBL-bildande bakterier kan förbättras på flera olika sätt. Många patienter med hög risk för detta kan identifieras redan på akutmottagningen, och få anpassad empirisk behandling. Inte alla patienter hittas på så vis, men med snabb diskdiffusion kan tiden till resistensbeskedet förkortas avsevärt och terapin kan tidigt korrigeras. NDM produceras av olika sekvenstyper av *K. pneumoniae* och horisontell plasmidöverföring är en viktig förklaring till den oroande spridningen av dessa multiresistenta bakterier. Virulensfaktorn *iss* var associerad med septisk chock hos patienter med blodförgiftning med *E. coli* ESBL och denna gen kan kanske användas som ett mål för framtida anti-virulens-behandling.

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