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Plasmid mediated antibiotic resistance

- with focus on extended spectrum β-lactamases (ESBL)

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Cover photo: Scanning Electron Microscopy photo of ESBL-producing *E. coli*. The picture was created through a collaboration between the Swedish Institute for Communicable Disease Control and the Unit for Electronmicroscopy at Karolinska Institutet, Huddinge. **Photographer:** Kjell Hultenby. **Colour editing:** Emil Sellström and Alma Brolund.

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To my family ♥

ABSTRACT

Enterobacteriaceae is a family of bacteria including *Escherichia coli* and *Klebsiella pneumoniae* that are common colonizers of the gastrointestinal tract. Extended spectrum β-lactamases (ESBL) are bacterial enzymes that degrade β-lactam antibiotics and thus make the bacteria resistant to β-lactam antibiotics. ESBL-producing *Enterobacteriaceae* (EPE) is an increasing problem constituting a burden on health care systems conferring excess morbidity, prolonged hospital stay and mortality. There are many hundreds of different ESBL-genes described. The most common are the $bla_{\text{CTX-M}}$ group. ESBL-genes are often situated on plasmids. This enables a fast dissemination since they can be spread both vertically and horizontally. Horizontal dissemination also enables them to spread between bacteria of different species. Outbreaks of resistant bacteria, such as EPE, must be rapidly detected and contained. Today the main focus during an outbreak is to conduct epidemiological typing of the bacterial strain. However, a horizontal outbreak of resistance genes in the bacterial population can also occur and go undetected if only the strain is subjected to epidemiological typing. There is a need for better understanding of plasmid dissemination and their role in transferring antibiotic resistance genes.

In this thesis I have evaluated different methods for epidemiological typing of both strains and plasmids by analyzing different nationwide collections of Swedish EPEs from 2007 to 2011.

In Sweden EPE became notifiable according to the communicable disease act in 2007. The number of reported cases has since increased from ~2000 to ~7000 cases per year. In **paper I and II** we found that both bacterial strain types and ESBL-genotypes were stable over the five-year period. However, a decline in median age for contracting an EPE infection in the Swedish population was seen. This suggests that EPE-carriage is increasing among healthy people in the community. The decline of EPE amongst elderly people might also be an effect of increased awareness and improved infection control in hospitals limiting outbreaks amongst elderly and multi-ill individuals.
Paper III describes plasmids of EPE in detail and presents a new plasmid typing approach using next generation sequencing (NGS). The Swedish EPE were found to often carry several plasmids as well as multi-replicon plasmids of IncF-type. Small cryptic plasmids were common in the EPE isolates which raises the question of their role in the bacterial cell. **Paper IV** describes the strain-plasmid-interplay in the gut flora of three EPE carriers over time. The three patients presented different examples of this interplay. In one of the patients isolates with identical epidemiological strain types were found to also have identical plasmids. In a second patient plasmid variation was observed between identical epidemiological strain types. A third patient had a mixed EPE flora with different epidemiological strain types and different species where similar plasmids were seen in divergent strains as well as the opposite. These results most likely reflect the effect of antibiotic usage and EPE risk factors, such as travel abroad, on the gut flora in terms of bacterial communication and dissemination of ESBL-genes.

This thesis has contributed to increased knowledge of the Swedish epidemiology of EPE and increased awareness of the impact of plasmids in the dissemination of common resistance genes.

LIST OF PUBLICATIONS

I. **Brolund A**, Hæggman S, Edquist PJ, Gezelius L, Olsson-Liljequist B, Wisell KT, Giske CG. *The DiversiLab system versus pulsed-field gel electrophoresis: characterisation of extended spectrum β-lactamase producing Escherichia coli and Klebsiella pneumoniae.* J Microbiol Methods. 2010 Nov; 83(2):224-30.

II. **Brolund A**, Edquist PJ, Mäkitalo M, Olsson-Liljequist B, Söderblom T, Tegmark Wisell K, Giske CG. *Swedish epidemiology of ESBL-producing Escherichia coli 2007-2011.* Manuscript accepted for publication in Clinical Microbiology and Infection.

III. **Brolund A**, Franzén O, Melefors Ö, Tegmark Wisell K, Sandegren L. *Plasmidome-analysis of ESBL-producing Escherichia coli Using Conventional Typing and High-throughput Sequencing.* PLoS One. 2013 Jun 13;8(6):e65793.

IV. **Brolund A**, Giske CG, Alm E, Melefors Ö, Tegmark Wisell K, Titelman E, Sandegren L. *Plasmidome sequencing of extended spectrum β-lactamase producing Enterobacteriaceae isolated from the fecal flora of carriers at five timepoints during one year.* Manuscript.

Related work by the author not included in this thesis:

I. Börjesson S, Jernberg C, **Brolund A**, Edquist P, Finn M, Landén A, Olsson-Liljequist B, Tegmark Wisell K, Bengtsson B, Englund S. *Characterization of plasmid-mediated AmpC-producing E. coli from Swedish broilers and association with human clinical isolates.* Clin Microbiol Infect. 2013 Jul;19(7):E309-11.

II. Woksepp H, Jernberg C, Tärnberg M, Ryberg A, **Brolund A**, Nordvall M, Olsson-Liljequist B, Wisell KT, Monstein HJ, Nilsson LE, Schön T. *High-resolution melting-curve analysis of ligation-mediated real-time PCR for rapid evaluation of an epidemiological outbreak of extended-spectrum-β-lactamase-producing Escherichia coli.* J Clin Microbiol. 2011 Dec;49(12):4032-9.

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V. **Brolund A**, Sundqvist M, Kahlmeter G, Grape M. *Molecular characterisation of trimethoprim resistance in Escherichia coli and Klebsiella pneumoniae during a two year intervention on trimethoprim use.* PLoS One. 2010 Feb 16;5(2):e9233

VI. Kitchel B, Rasheed JK, Patel JB, Srinivasan A, Navon-Venezia S, Carmeli Y, **Brolund A**, Giske CG. *Molecular epidemiology of KPC-producing Klebsiella pneumoniae isolates in the United States: clonal expansion of multilocus sequence type 258.*Antimicrob Agents Chemother. 2009 Aug;53(8):3365-70

VII. Källman O, **Motakefi A**, Wretlind B, Kalin M, Olsson-Liljequist B, Giske CG. *Cefuroxime non-susceptibility in multidrug-resistant Klebsiella pneumoniae overexpressing ramA and acrA and expressing ompK35 at reduced levels.* J Antimicrob Chemother. 2008 Nov;62(5):986-90.

VIII. Grape M, **Motakefi A**, Pavuluri S, Kahlmeter G. *Standard and real-time multiplex PCR methods for detection of trimethoprim resistance dfr genes in large collections of bacteria.* Clin Microbiol Infect. 2007 Nov;13(11):1112-8.

CONTENTS

LIST OF ABBREVIATIONS

AmpC	Ampicillinase C (β-lactamase of $ESBL_M$-type)
bla	β-lactamase
BSI	Blood stream infection
CAZ	Ceftazidime
CC	Clonal complex
CI	Confidence interval
CTX	Cefotaxime
CTX-M	Cephotaximase Munich (β-lactamase of $ESBL_A$-type)
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
E. coli	*Escherichia coli*
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EPE	ESBL-producing *Enterobacteriaceae*
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GIT	Gastrointestinal tract
IS*CR*	Insertion sequence common region
IS-element	Insertion sequence element
K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MST	Minimal spanning tree
NAG	N-acetylmuramic acid
NAM	N-acetylglucosamine
NGS	Next generation sequencing
OXA	Oxacillinase (β-lactamase of $ESBL_M$-type and $ESBL_{CARBA}$-type)
PBP	Penicillin binding protein
PBRT	PCR based replicon typing
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PFGE	Pulsed-field gel electrophoresis
pHMM	Profile Hidden Markov Model
PK	Pharmacokinetics
pMLST	Plasmid multilocus sequence typing
pRFLP	Plasmid restriction fragment length polymorphism
RIVM	The Netherland Institute for Public Health and the Environment
SHV	Sulphydryl variable (including β-lactamases of $ESBL_A$-type)
SIR	Sensitive, intermediate or resistant
SMI	Swedish Institute for Communicable Disease Control
ST	Sequence type
TEM	Temoniera (including β-lactamases of $ESBL_A$-type)
UTI	Urinary tract infection

1 INTRODUCTION

1.1 *ENTEROBACTERIACEAE*

Bacteria can be classified as Gram-positive or Gram-negative based on the structure of their cell wall (Figure 1). This can be determined by Gram staining. The Gram-positive or Gram-negative bacteria are colored differently in the staining process and can thus be differentiated in an ordinary light microscope. The main difference is that Gram-negative bacteria have two double layered membranes and a very thin peptidoglycan layer which is located between the two membranes, whereas Gram-positive bacteria only have one double layered membrane and a thick, 20-80nm, peptidoglycan layer facing outwards from the cell. The peptidoglycan layer of Gram negatives is 7-8nm.

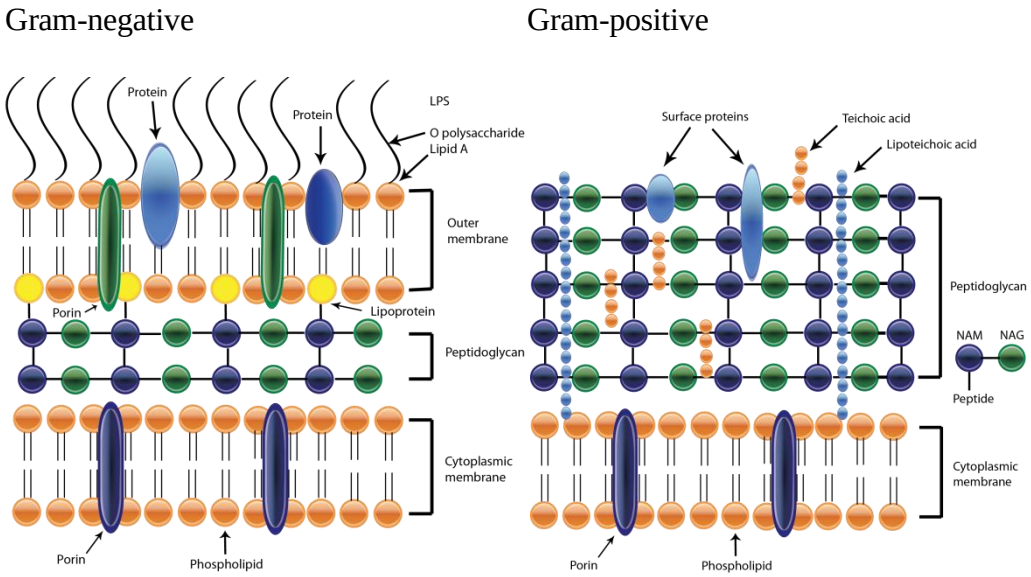


Figure 1. Differences in the cell wall composition between Gram-positive and Gram-negative bacteria.

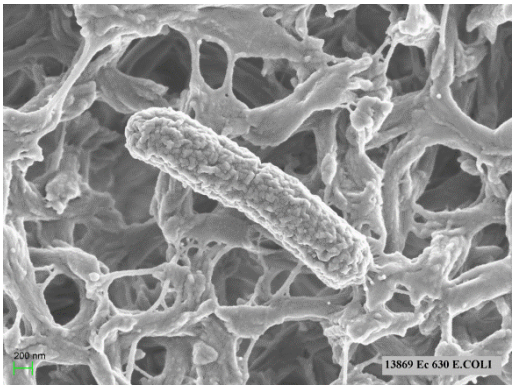
Enterobacteriaceae is a family of Gram-negative bacteria classified within γ-proteobacteria. *Enterobacteriaceae* include many hundreds of species, but only a limited number are of clinical relevance. The most studied bacteria of clinical importance in this family are: *Escherichia coli*, *Klebsiella* spp., *Salmonella enterica*, *Shigella* spp., *Proteus spp.*, *Enterobacter* spp., *Citrobacter* spp., *Morganella morganii* and *Yersinia* spp.

Enterobacteriaceae are rod-shaped, 1-5µm in length, non-sporulating, facultative anaerobic bacteria. They ferment sugars (e.g. glucose and lactose) to a variety of end products and reduce nitrate to nitrite, produce catalase and are oxidase negative. All these criteria help detecting them in the clinical microbiological laboratories.

As the name implies they are common colonizers of the gastrointestinal tract (GIT) and are highly important for the wellbeing of human and most animals as part of the natural bacterial flora. *Enterobacteriaeae* are also widespread in the environment. However,

they can also be pathogenic causing infections in the central nervous system, lower respiratory tract, bloodstream, gastrointestinal and urinary tract. They possess many different characteristics that contribute to their survival, the most common being: endotoxin, capsule, antigenic phase variation, type III-secretion systems, sequestration of growth factors and resistance to serum killing. (Murray et al., 2009, Madigan et al., 2003)

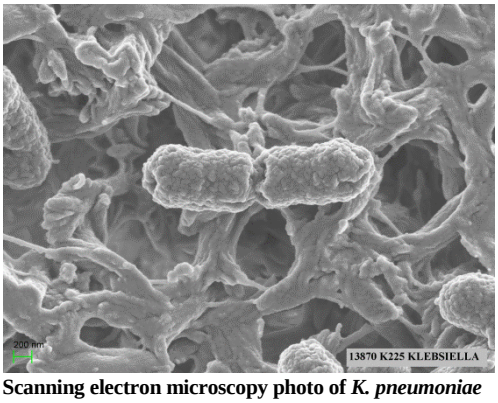
1.1.1 *Escherichia coli*



Scanning electron microscopy photo of *E. coli* cell division

Escherichia coli are part of the normal gut microflora of humans and many animals. They are important to humans since they synthesize vitamins, especially vitamin K which we are unable to produce ourselves. *E. coli* represents one of the most studied bacterial species. It has been frequently used as a model organism, but is also of high clinical relevance. *E. coli* are pathogenic if they invade sites that are normally sterile, such as the urinary tract, cerebrospinal fluid c the blood. *E. coli* is the causative agent of >85% of all community acquired urinary tract infections (UTI). Women are more affected than men due to anatomical differences. There are *E. coli* strains that can produce toxins that cause disease in the GIT, examples are enterohemorrhagic, enterotoxigenic, enteroinvasive, enteroaggregative *E. coli*. (Madigan et al., 2003, Murray et al., 2009)

1.1.2 *Klebsiella pneumoniae*



Scanning electron microscopy photo of *K. pneumoniae* cell division

Klebsiella pneumoniae are most commonly found in soil and water but they can also colonize the GIT. They are often the first bacteria to colonize the gut of newborn children who are completely sterile at birth. But they are less dominating in the microflora of adults. *K. pneumoniae* can cause pneumonia, mostly in immunocompromised humans. They are the second most common agents after *E. coli* to cause UTI and bloodstream infections (BSI).
K. pneumoniae are more commonly acquired in hospital environments than in the community e.g. in patients with urinary catheters or immunocompromised patients. (Madigan et al., 2003)

1.2 ANTIMICROBIALS

Antimicrobials, e.g. antibiotics (or antibacterials), antivirals and antifungals, are agents used to treat infections caused by microorganisms. The ideal antimicrobial acts on targets that are unique for the microorganisms and do not interfere with human processes, which minimizes the risk for adverse effects. Antibiotics affect bacteria and can be either bacteriostatic (inhibits bacterial growth) or bactericidal (kills the bacteria). Antibiotics can be divided into different classes depending on structural composition and what bacterial process they interfere with. Examples are antibiotics targeting cell wall synthesis (e.g. β-lactams), DNA synthesis (e.g. fluoroquinolones) protein synthesis (e.g. tetracyclines) and folic acid synthesis (e.g. trimethoprim). Figure 2 describes the different targets and classes of antibiotics.

Antibiotics are compounds that can be found in the environment produced by other organisms (e.g. penicillin) to resist bacterial attacks. Chemically synthesized compounds are also available and are called xenobiotics (e.g. trimethoprim), but the term is less used today and antibiotics has become more broadly used and usually also includes the chemically synthesized compounds. When xenobiotics were first developed there was a common hope that they would not give rise to bacterial resistance since they possessed new mechanisms of action that were not already present in the environment. This hypothesis was however rapidly proven wrong.

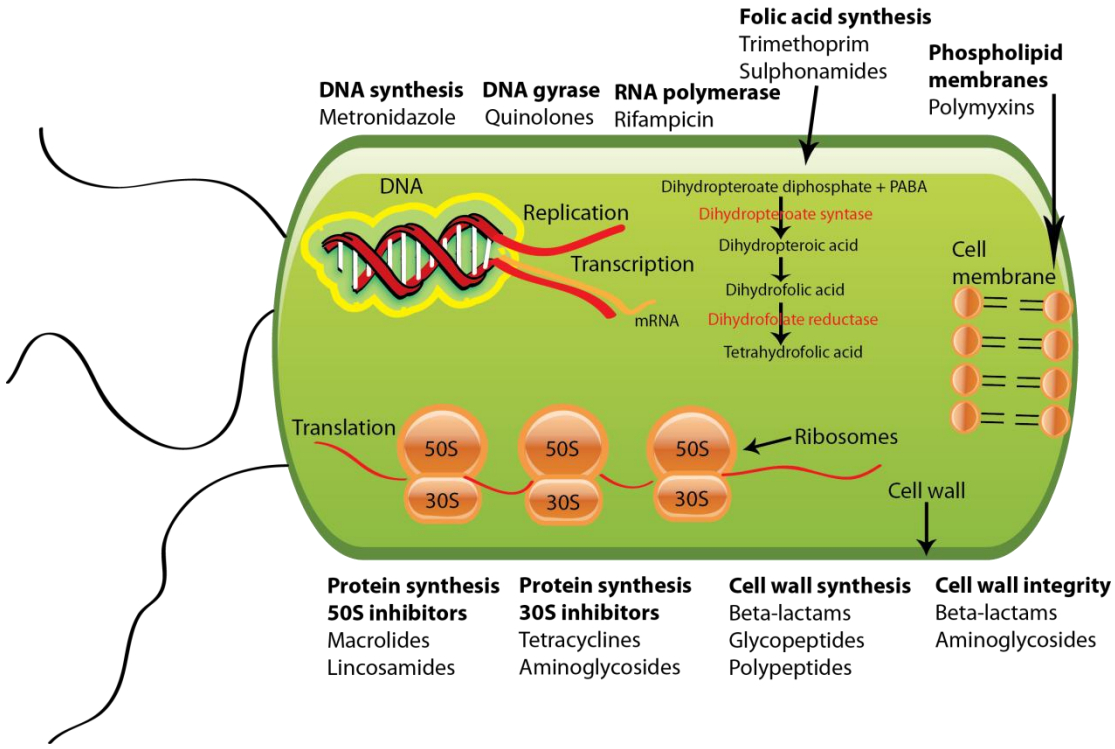


Figure 2. Antibiotic mechanisms of action illustrated for examples of different antibiotic classes.

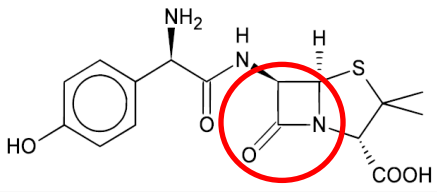
1.2.1 β-lactams

β-lactams represent the most commonly used class of antibiotics. They can be divided into four groups: penicillins, cephalosporins, carbapenems and monobactams. They are extensively used for the treatment of common infections, such as pneumonia and urinary tract infections (UTI), but also for severe and life threatening infections such as bloodstream infections. β-lactams are also frequently used as prophylaxis treatment before surgery.

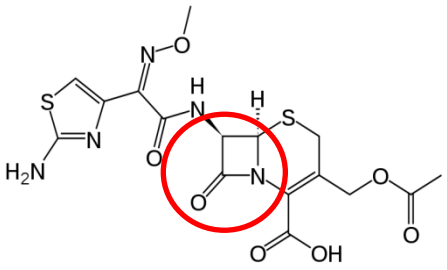
β-lactam compounds all share a β-lactam ring in their structure (illustrated in red, Figure 3).

Penicillin was the first antibiotic to be used in large scale. The antibacterial effect was discovered by Sir Alexander Fleming in 1928 for which he received the Nobel Prize in Medicine and Physiology in1945 together with Howard Florey and Ernst Boris Chain (Sternbach and Varon, 1992). Many variants of penicillin have since been discovered or invented and produced in order to change the antibiotic spectrum of activity or circumvent resistance amongst bacteria. This has led to the different groups of β-lactams and the many different compounds within each group. As the development of new compounds in this class of antibiotics has progressed the molecules have become more complex with larger side chains surrounding the β-lactam ring making them less accessible to degrading β-lactamases.

Amoxicillin (penicillin)



Cefotaxime (cephalosporin)



Meropenem (carbapenem)

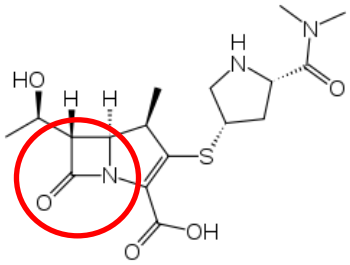


Figure 3. Examples of β-lactam structures. The red circle marks the β-lactam ring.

β-lactams act by disrupting cell wall synthesis. They inhibit the last step in the peptidoglycan synthesis through interference with transpeptidation. β-lactams also affect other inner membrane proteins that are thought to influence peptidoglycan

synthesis. These proteins and transpeptidases are often referred to as penicillin-binding proteins (PBPs). Bacteria in growth phase are killed by β-lactams (Sköld, 2006). Some bacterial species are naturally (intrinsically) resistant to β-lactams. Examples are Mycoplasma which lack a peptidoglycan cell wall and Mycobacteria which produce a chromosomally-encoded class A (Ambler) β-lactamase, BlaC, which detgrade most β-lactams. However, there are exceptions; Mycobacteria have shown to be susceptible to carbapenems in combination with clavulanic acid (Hugonnet et al., 2009).

1.3 ANTIBIOTIC RESISTANCE

Bacteria have a remarkable ability to adapt to changes in the environment. For example, by changing the genome and thereby add on or modify/downregulate functions to be able to survive under different conditions. Changes in the genome can occur either vertically through mutations that are passed on to following generations or horizontally by taking up genes or pieces of DNA from the environment (Harbottle et al., 2006). Antibiotic treatment is an example of a selection pressure that drives these genetic changes. As long as we have known about antibiotics, resistance has been observed. Already Alexander Fleming worried about further driving the resistance evolution if the drugs were to be misused (Sternbach and Varon, 1992).

There are several ways for the bacteria to become resistant to antibiotics as illustrated in Figure 4. (Sköld, 2006)
Efflux pumps in the bacterial cytoplasmic membrane can pump out the antibiotics from the cell and thus lowering the intracellular concentration. These can be either general, pumping out structurally different antibiotics, or antibiotic specific (e.g. the quinolone efflux pump).
The permeability of the cell wall can be modified and hinder the antibiotics to get through. This occurs either by downregulating membrane porins or by changing the porin structure so that the drug is blocked from diffusion.
Degrading enzymes inactivate the antibiotic so that the antibacterial effect is lost, whereas antibiotic altering enzymes modify the antibiotic and lowers the affinity to the binding target.
Bacteria can also present alternative proteins that compete with binding of the antibiotic. One example is alternative penicillin binding proteins (PBPs) as in the case of methicillin resistant *Staphylococcus aureus* (MRSA). Another example is trimethoprim resistance that involves alternative enzymes in the folic acid pathway.
Modifications in the antibiotic targets that lead to decreased affinity of the antibiotic also occur, as in the example of chromosomal quinolone resistance through e.g. mutations in the gyrase genes. (Harbottle et al., 2006, Kohanski et al., 2010)

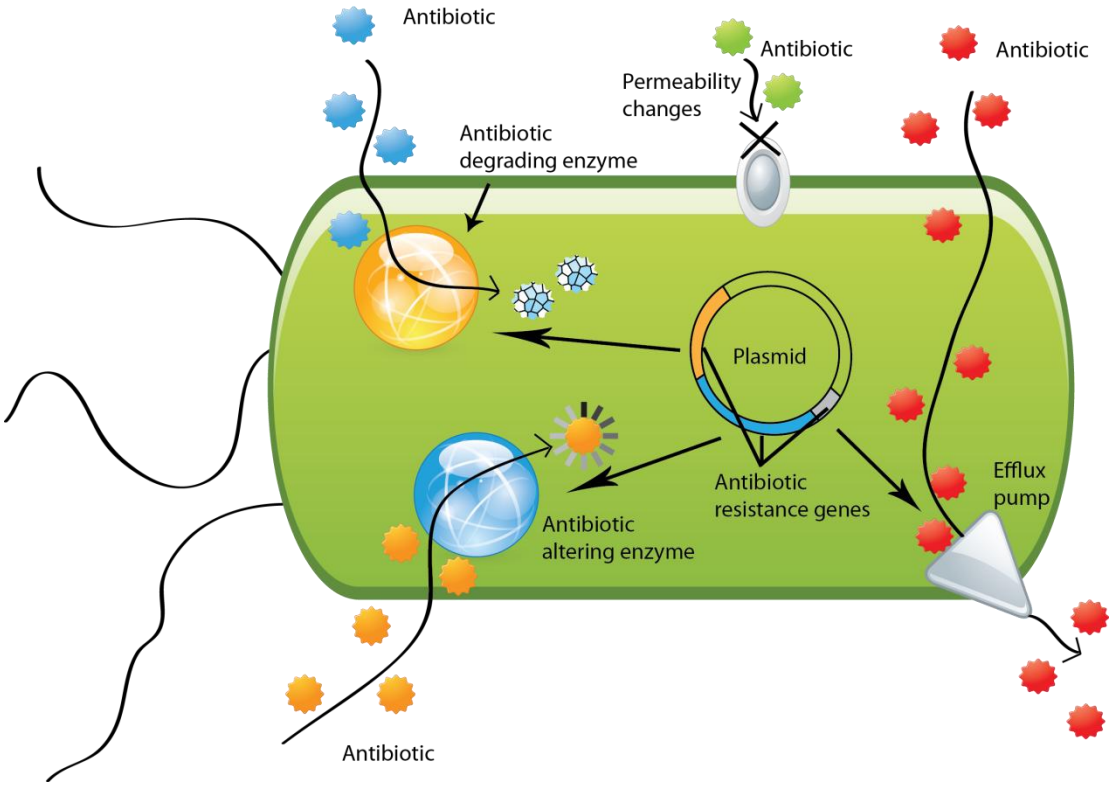


Figure 4. Resistance mechanisms. Chromosomally located genes or mutations also occur and can contribute to antibiotic resistance as discussed in the text. This is not illustrated in the figure.

1.3.1 β-lactamases

β-lactamases are enzymes produced by bacteria that degrade β-lactam antibiotics. Extended spectrum β-lactamases (ESBLs) are enzymes that can degrade later classes of β-lactams (e.g. 3rd generation cephalosporins) (Paterson and Bonomo, 2005). There are many types of β-lactamases and different nomenclatures have been proposed and debated. Also what to include in the definition of ESBLs is being discussed.

Traditionally two different β-lactamase classification systems have been used for the term ESBL: The Ambler classification (Ambler et al., 1991) and the Bush-Jacoby-Medeiros classification (Bush and Jacoby, 2010) (see Table 1).
Common for these two classification systems is that they only include enzymes conferring resistance to third generation cephalosporins that are inhibited by clavulanic acid and are horizontally transferrable.

In 2009 Giske and co-authors proposed a new classification scheme with a more generous definition of ESBL (Table 1). Their main argument for changing the definition was to simplify it and to make it more comprehensible for persons outside the microbiology laboratories, such as clinicians, infection control professionals, hospital management staff and policy makers (Giske et al., 2009). When this publication came out it attracted quite some attention and several comments and

responses have been published by other experts in the field not agreeing with the suggested classification.

Giske et al divided ESBLs into three main groups; $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$.

$ESBL_A$ represent the "classical ESBLs" (according to previous definitions) that confer resistance to penicillins and cephalosporins. They are inhibited by clavulanic acid and the most common ones can be further divided in three main groups of enzymes; CTX-M, SHV and TEM.

$ESBL_M$ are miscellaneous ESBLs that are resistant to penicillins, cephalosporins and sometimes also carbapenems. They are inhibited by cloxacillin or boronic acid and the main group is the acquired AmpC enzymes. Some OXA-ESBLs are also classified in the $ESBL_M$ group.

$ESBL_{CARBA}$ are enzymes that degrade all β-lactam antibiotics. They are inhibited by either EDTA (ethylenediaminetetraacetic acid) or DPA (dipicolinic acid) as in the case of metallo-β-lactamases (MBLs), or boronic acid or avibactam as the KPCs. Some OXA-enzymes are also included in the $ESBL_{CARBA}$ group. These can sometimes be difficult to detect since they usually show quite low levels of resistance.

Table 1. β-lactamase classifications and definitions.

Ambler classification	
Class A	Serine based penicillinases
Class B	Zink based metallo-β-lactamases
Class C	Serine based cephalosporinases
Class D	Serine based oxacillinases
Bush-Jacoby-Medeiros classification	
Group 1	Cephalosporinases not inhibited by clavulanic acid
Group 2:	
Group 2a	Penicillinase
Group 2b	Extended-spectrum penicillinase
Group 2be	ESBLs
Group 2br	Penicillinases not inhibited by clavulanic acid
Group 2c	Hydrolysing carbenicillin
Group 2d	Hydrolysing cloxacillin
Group 2e	Extended spectrum cephalosporinases
Group 2f	Carbapenemases
Group 3	Metallo-β-lactamases inhibited by EDTA and DPA
Group 4	Penicillinases not inhibited by clavulanic acid
Giske classification	
$ESBL_A$	Classical ESBLs inhibited by clavulanic acid
$ESBL_M$	Miscellaneous enzymes including acquired AmpC and OXA-ESBLs
$ESBL_{CARBA}$	Carbapenemases
$ESBL_{CARBA-A}$	Carbapenemases such as KPC, NMC and SME
$ESBL_{CARBA-B}$	Metallo-β-lactamases
$ESBL_{CARBA-D}$	OXA-carbapenemases

In this thesis and in the included studies the definition by Giske et al is applied. The studies are focused on $ESBL_A$ and $ESBL_M$ producing bacteria.

There are many hundreds of different ESBL types described. The most common are the *bla*$_{CTX-M}$ variants. They were first discovered in the 1980's and since 1995 they have spread to all continents and the numbers have increased tremendously. The *bla*$_{CTX-M}$ genes are divided into five phylogroups; CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Birkett et al., 2007). The CTX-M-1 phylogroup includes *bla*$_{CTX-M-15}$ which is the most common type found on all continents. In Sweden phylogroups CTX-M-1 and CTX-M-9 are the most frequently found.
Acquired AmpC enzymes, includes the subgroups CIT, MOX, FOX, DHA, ACC and EBC (Perez-Perez and Hanson, 2002). The most common genotypes found are the *bla*$_{CMYs}$ where *bla*$_{CMY-2}$ is dominating worldwide. The *bla*$_{CMYs}$ belong to the CIT enzyme subgroup.

1.4 DISSEMINATION OF RESISTANCE

Outbreaks involving genes encoding ESBL enzymes can occur either by emerging bacterial clones or by horizontal gene transfer between bacteria. In the latter case, plasmids containing the resistance genes are spread between bacteria of the same and/or different species.

Horizontal transfer of genes can occur in three ways: transformation, transduction or conjugation (Figure 5). Transformation is when bacteria take up free DNA from the environment, it can for example be DNA shredded by dead bacteria. Transduction refers to bacteria being infected by bacterial viruses, so called phages, which can inject DNA in to the bacterial cell. The process where bacteria physically interact with each other and share DNA using plasmids as a vector is called conjugation. Bacteria connect to each other by special pili. (Murray et al., 2009)

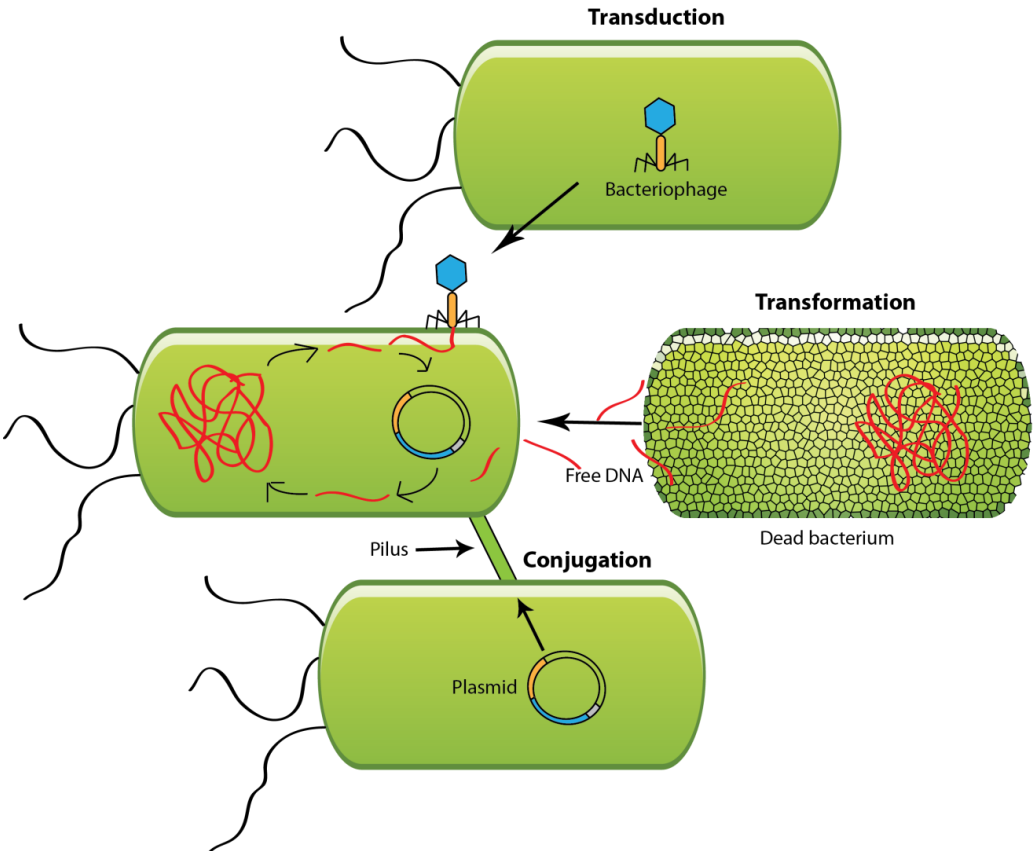


Figure 5. Horizontal gene transfer.

The gut flora is an ideal reservoir for antibiotic resistance genes, where kilograms of bacteria of different species can interact, most often without causing disease and hence to much interference with the immune system. When antibiotics are used the resistant strains are selected and genes important for survival are thought to be shared among species at a higher rate. Plasmids can contain resistance genes to several classes of antibiotics (Bennett, 2008). This can explain the multi-resistance often found amongst ESBL-producing bacteria. The cover of this thesis beautifully illustrates the massive communication going on between *E. coli* bacteria through connection of their pili.

1.4.1 Clonal expansion

Clonal expansion refers to a particular bacterial cell line multiplying and disseminating in a community or causing an outbreak in a hospital for example. The bacteria are then spread vertically – multiplying through cell division. In order to detect clonal expansions molecular epidemiological strain typing tools are important. They allow microbiologists to investigate when and how a particular strain is spreading. This is highly important in order to be able to stop or diminish the source of dissemination.
There are several methods for epidemiological typing of bacterial strains. The most important are described in detail in the materials and methods section. The similarities between strains can e.g. be visualized as dendrograms or minimal spanning trees (MSTs) as illustrated in Figure 6.

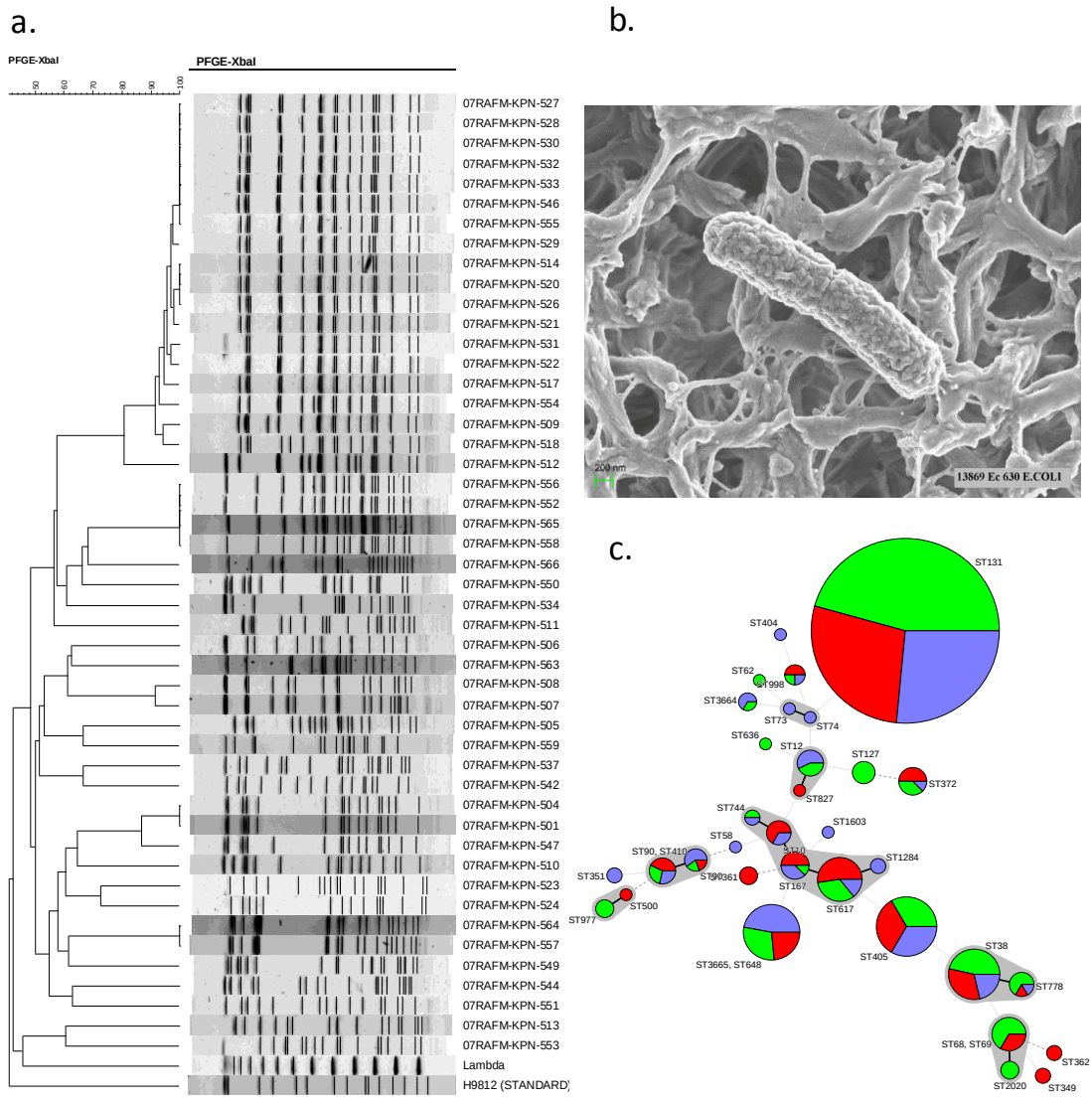


Figure 6. Pulsed-field gel electrophoresis (PFGE) dendrogram (a), electron microscope picture of *E. coli* cell division (illustrating vertical dissemination) (b) and Multilocus sequence type minimal spanning tree (MLST MST) (c).

1.4.2 Mobile genetic elements

Plasmids are double-stranded extra-chromosomal elements that replicate independently of the chromosome in the bacterial cell hosting it. Plasmids can be classified according to incompatibility (Inc) groups, which are based on the principle that plasmids with the same replicon cannot be propagated stably in the same cell (Novick, 1987). The explanation for this is that similar plasmids compete over common cellular functions involved in e.g. plasmid replication control (Couturier et al., 1988). Traditional plasmid typing has been performed through competition assays, where a plasmid is incorporated into an isolate already containing a known plasmid. The daughter cell presents both or just one plasmid depending on incompatibility (Couturier et al., 1988).

A replicon is a highly conserved part of the plasmid where genes encoding replication initiation, control and copy number etc. are situated (illustrated as the plasmid core genome in Figure 7). Nowadays incompatibility grouping have been replaced by

replicon typing which is simpler experimentally and also allows for a subdivision of some of the Inc groups. One example is the IncF1 plasmids that include repFIA, FIB and FIC of which repFIA and FIB plasmids are commonly found to harbor ESBL genes.
The host range of plasmids is largely determined by the Inc-type. IncF plasmids are narrow host range plasmids, meaning that they are found in a restricted number of bacterial species. They are the most common Inc type associated to CTX-M ESBLs. IncA/C plasmids, found associated to $ESBL_M$ and $ESBL_{CARBA}$, are contrarily broad host range plasmids that are found in a wide variety of bacterial species. Some Inc groups are considered as endemic resistance plasmids in *Enterobacteriaceae*. These are IncFII, IncA/C, IncL/M, IncN and IncI (Carattoli, 2011).
Among fully sequenced *E. coli* plasmids harboring $bla_{CTX\text{-}M}$-genes in GenBank (a genetic sequence database) the following plasmid families are found: IncF, IncN, IncN2, IncI1, IncHI2, IncL/M, IncA/C, IncK, IncX4, IncU and RCR (Carattoli, 2013).

Besides the replicon, plasmids are highly diverse. The plasmid genome is often scattered with mobile genetic elements that can move genes around within the plasmid as well as between chromosome and plasmid/s. Plasmids can contain genes that are beneficial for the survival of the bacteria e.g. virulence genes. Plasmids have also developed ways to secure their persistence in the bacterial cell, e.g. through toxin-antitoxin systems. Such systems act by eliminating cells that have lost the plasmid after cell division. (Bennett, 2008)
One of the key elements responsible for the mobilization of genes are the IS elements (insertion sequences). IS elements are short sequences (~700-2500 bp) which only encode genes involved in transposition flanked by inverted repeat sequences. These structures can be inserted or excised by transposases. (Murray et al., 2009)
Two IS elements surrounding a sequence (e.g. an antibiotic resistance gene) compose a transposon. Transposons cannot replicate on their own as plasmids do but they compose an effective way to transfer genes between e.g. plasmids and chromosomes (Bennett, 2008, Harbottle et al., 2006).
Another example of genetic elements that contribute to the spread of antibiotic resistance genes are integrons. Integrons are not mobile themselves but can be incorporated into transposons and thereby become mobilized. However, genes in the form of gene cassettes can be incorporated or excised independently within the integron structure. This is due to resistance gene cassettes being able to exist in two forms, either situated in the integron structure or as a free circular entity (that cannot replicate). They contain an *attC* site in their structure that can recognize an *attI* site in the integron structure and thus be incorporated through site specific recombination (Harbottle et al., 2006). Many gene cassettes can be incorporated after each other in the integron which is also equipped with a strong promoter that can transcribe all the resistance genes. In some bacterial species e.g. *Vibrio cholerae* the presence of so called superintegrons have been found. Superintegrons have been found containing as many as 179 cassettes. Over 60 different antibiotic resistance genes have been found as cassette structures in integrons, covering most of the antibiotics in use (Rowe-Magnus et al., 2001).
A more recently found mobile genetic structure is the IS*CR* elements (insertion sequence common regions), also called common regions or Orf513. IS*CR* elements can move genes through rolling-circle transposition. They are often found close to the

conserved 3'-end of class 1 integrons and have been found to mobilize various ESBL encoding genes (Toleman et al., 2006).

Compared to strain typing relatively little is known on the epidemiology of plasmids. We know that large plasmids, between 50 to 200 kb, of the IncFII, IncA/C, IncL/M, IncN and IncI1 incompatibility groups are found associated to ESBL carriage (Carattoli, 2011). Plasmids within these incompatibility groups can be very different in their variable region. Thus, additional plasmid typing methods have to be combined in order to get a better idea of the similarities between plasmids.

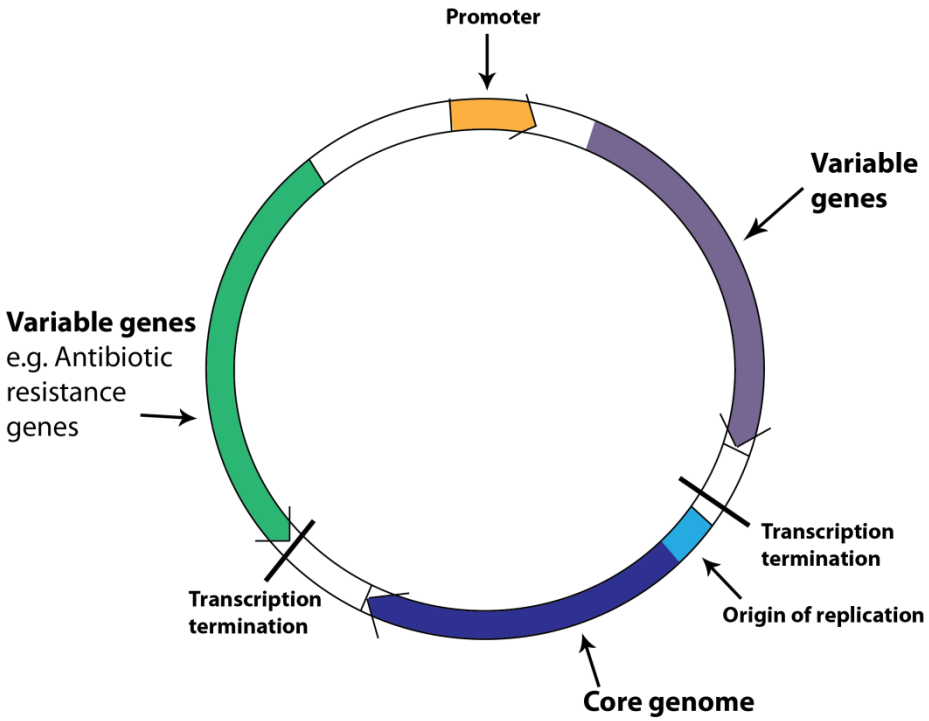


Figure 7. A schematic illustration of a plasmid.

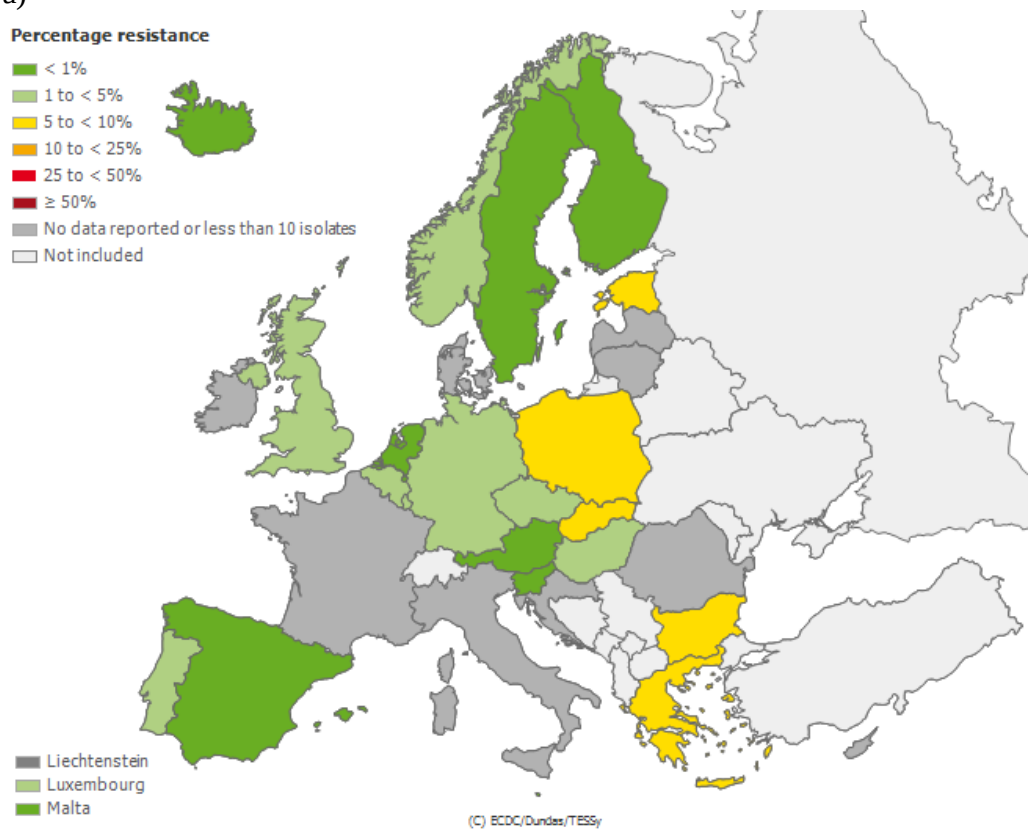
1.5 EPIDEMIOLOGY OF ESBL-PRODUCING *ENTEROBACTERIACEAE*

ESBL-producing *Enterobacteriaceae* (EPE) are spreading fast. In Sweden the number of reported cases have increased from 2099 in 2007 (Feb-Dec) to 7225 in 2012.
There are several studies describing the local frequency in different parts of Sweden; Helldal et al described the prevalence of ESBL-producing *E. coli* in South Western Sweden during 2004-2008 with a higher increase in nosocomially acquired isolates in comparison to community-acquired, 0.2 to 2.5% and 0.2 to 1.6% respectively (Helldal et al., 2013). A study from the county of Östergötland indicated an increased prevalence of EPE from 2002 to 2007, yet the numbers were very low (<1%) (Ostholm-Balkhed et al., 2010). An investigation of the faecal carriage of ESBL-producing bacteria in both Primary Health Care Units and University Hospital setting in Southern Sweden was conducted in 2008-2010. The only species found was *E. coli* and the authors described the prevalence as higher than expected in both groups; 2.1 - 3% and 1.8 - 6.8% respectively (Stromdahl et al., 2011). A recent study from Uppsala investigated the prevalence of EPE in healthy preschool children compared to patients

in the same age group. The authors found a carrier rate of 2.9% in healthy children compared to 8.4% in patients of the corresponding age group (Kaarme et al., 2013).

In Europe the prevalence of bacteria resistant to extended-spectrum cephalosporins differs a lot between countries. They are less frequent in the north and more common in the southern and eastern parts of Europe. Levels of invasive infections caused by *E. coli* resistant to 3rd generation cephalosporins over time are illustrated in Figure 8.
The European network of national surveillance systems of antimicrobial resistance performs on-going surveillance of invasive infections of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, and *Enterococcus faecalis/faecium*, and monitors variations in antimicrobial resistance over time and place. From 2005 invasive isolates of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are also part of the scheme. Since 2012 *Acinetobacter* spp. have also been included. During 2009 a transition of the EARSS management from RIVM in the Netherlands to ECDC in Stockholm was prepared, and from 1st January 2010 the network, renamed as EARS-Net, is coordinated from ECDC. Data collected by EARS-Net is routinely generated quantitative data (MICs or inhibition zones), but the data presented is in the format of susceptibility categories (SIR). The results are summarized in a yearly report available on the ECDC homepage: http://ecdc.europa.eu/. The resistance in the European countries is illustrated on color-coded maps. However, one must keep in mind that the results are biased by differences in reporting, national monitoring and sampling.

a)



b)

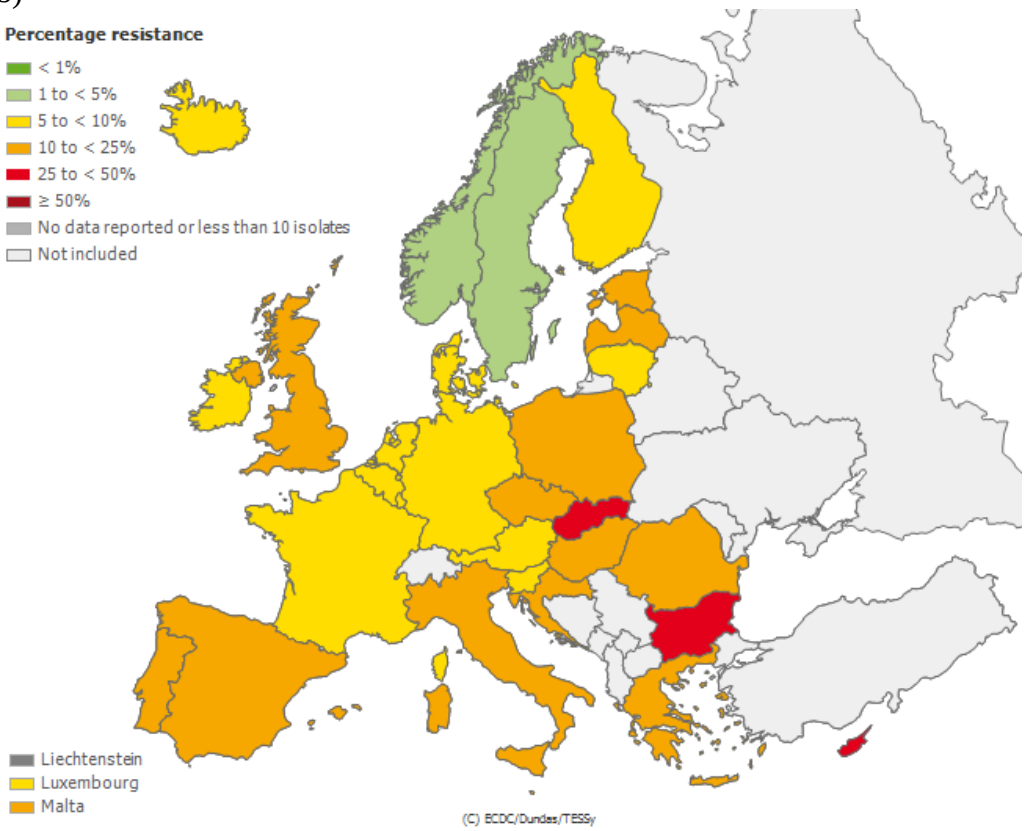


Figure 8. Maps generated by EARS-Net illustrating the proportion of invasive *E. coli* isolates resistant or intermediately resistant to 3rd generation cephalosporins from reporting countries in a) 2001 and b) 2011.
Figure 8 is reprinted with permission from ECDC. Source: ECDC / EARS-Net 2001 and 2011.

Looking at countries outside Europe the ESBL prevalence also varies a lot. A recent study from the USA presents results from the SENTRY surveillance program including 26 hospitals from 20 states. They report the resistance levels to cephalosporins and/or aztreonam in invasive *Enterobacteriaceae* of 6.4% (Castanheira et al., 2013).
A Canadian study has reported ESBL-producing bacteria to make up almost 5% of the *E. coli* population - numbers similar to northern Europe (Simner et al., 2011).
The level of resistant bacteria is truly troublesome in parts of the world. In Thailand carriage of ESBL-producing bacteria, in as high numbers as 52% of healthy volunteers, has been reported (Oteo et al., 2010). In India the reports are also alarming with high numbers seen among patients. A study from two hospitals in Ujjain, India collected consecutive isolates from incoming patients with abscesses, UTI, BSI etc and found ESBL-production in 69% of *E. coli*. These were also highly resistant to fluoroquinolones (Pathak et al., 2012).
High prevalence of ESBL-producers has also been reported from the environment in India. Effluent from drug manufacturers has particularly been reported as a source of polluting the aquatic environment near Hyderabad. Levels exceeded bacterial toxic concentrations 1000-fold (Larsson et al., 2007).

Even if Sweden has relatively low levels of ESBL producing strains compared to other parts of the world, we have many reasons to be worried. The world is getting smaller in

the sense that people travel a lot and the country borders are of less importance. In 2010, Tängden et al studied the acquisition of resistance genes as a result of travel outside northern Europe. Healthy travelers voluntarily participated in taking samples before and after travel. The study resulted in 24% returning as new carriers of EPE after travel. (Tangden et al., 2010).

There are several explanations for the geographical differences. Many countries (e.g. the member states of the European Union) have antibiotic prescribing policies saying that antibiotics have to be prescribed in order to be purchased. Other countries lack such regulations and the implementation of the policies differs between countries. Acquisition "over the counter" in pharmacies or equivalents still occurs in many places. There are also differences in prescribing tradition and culture between countries. Differences in climate affect the frequency of infections and probably the ease of dissemination. Economical status, household conditions and population density also clearly play a role. Infection control routines and programs have a large impact on dissemination of bacteria. Differences in infection control routines are substantial between different countries, also between European countries. Furthermore the microbiological diagnostic traditions and setup of surveillance programs differs. If awareness of the regional situation is low preventive measures will also be. More and more studies show that there is dissemination of bacteria between food, animal and humans, consequently antibiotic usage and inadequate infection control in veterinary medicine, fish farming, irrigation etc also contributes to the increased spread of antibiotic resistance.

1.6 IMPACT OF ANTIBIOTIC RESISTANCE

As the ESBL-carrier rate in the population increases and nosocomial outbreaks become more common the risk of being infected increases. Due to the common multidrug-resistant phenotype of ESBL-producing bacteria therapeutic options for these bacteria are limited. Antibiotic classes to which co-resistance with ESBL are common are fluoroquinolones, aminoglycosides and trimethoprim. Since antibiotics from these groups are common empirical treatment options for severely ill patients, high resistance rates can consequently result in increased morbidity and mortality.
ESBLs also constitute a burden on health care systems conferring excess mortality and prolonged hospital stay. Several studies have confirmed this. For example bloodstream infections in Europe caused by *E. coli* isolates resistant to extended spectrum cephalosporins were estimated to cost >2,700 excess deaths and 18.1 million EUR, represented by >120,000 excess days of hospital stay (de Kraker et al., 2011).
Another example is UTIs that are often uncomplicated and where patients usually get an oral antibiotic prescribed for outpatient use. Such "uncomplicated" infections caused by resistant bacteria, like EPE, can lead to hospitalization and the need for intravenous drug administration because of lack of effective orally administrated drugs. For UTI in men this is increasingly becoming a problem. However, due to high sensitivity rates to nitrofurantoin and pivmecillinam amongst EPE there is no need to deviate from recommended treatment options for such infections in women (André et al., 2007, Sandberg et al., 2011-2012).

1.7 IMPORTANCE OF ADEQUATE TREATMENT

Patients with severe infections are highly dependent on receiving adequate treatment early. Many times the diagnostic procedures available are too slow and treatment has to be given before the causative agent is identified. This is called empirical treatment and is based on epidemiological grounds. Knowledge of the local epidemiology is therefore very important for empirical treatment to be successful. For a patient with a BSI caused by resistant bacteria, such as an ESBL-producer, it can lead to death if an ineffective antibiotic is administered. Changing to an effective regimen after treatment failure might be too late. On the other hand "playing safe" and choosing a non-first line antibiotic, such as colistin or tigecycline, is not always preferable due to less efficacy and more side effects for the patients. Peralta and coworkers studied the rate of adequate empirical treatment in bloodstream infections caused by *Enterobacteriaceae* in 19 Spanish hospitals during four years. They found the empirical treatment to be inadequate in 48.8% of the cases. 24 of the 387 patients included in the study died during the first three days of inadequate therapy (Peralta et al., 2012).

National and global epidemiology knowledge is very important since it provides ways to detect emerging clones or new types of resistant bacteria on the rise. Besides knowing the local epidemiology, this can bring valuable information to the practitioners if the patient has connections to other parts of the country or the world. This is where institutes like the SMI, ECDC and similar governmental institutions and agencies play an important role gathering data through different surveillance tools.

This reasoning also highlights the importance of developing better and not least faster diagnostic tools, to be able to minimize the need or shorten the time for empirical treatment. Adequate treatment and infection control are important tools for controlling and reducing the dissemination of resistant bacteria.

1.8 PERSISTANCE OF ANTIBIOTIC RESISTANCE

Little is known about the persistence of ESBL-producing bacteria in patients. This is mainly due to the difficulties in studying persistence. Fecal cultures represent a snapshot of the flora at the time of study, and low numbers of bacteria can be difficult to detect. It has been shown that a person can present several negative samples and still later in time again come up with a positive one (Alsterlund et al., 2012). Another difficulty is that negative samples prior to colonization or confirmed infection seldom exist.

There are however some studies on persistence. Most are conducted after detecting nosocomial outbreaks. One example is Löhr et al who have followed children and their family members for three years after an outbreak in the neonatal intensive care unit in Stavanger, Norway. They observed carrier length of up to two years in some of the children (Lohr et al., 2013). Persistence in neonates is proposed to be longer than in older children and adults since they have not yet established a naturally protective microbial flora. They also have an undeveloped immune system and are more often treated with antibiotics (Lohr et al., 2013).

Alsterlund et al found some individuals in their study to be colonized with ESBL-producing bacteria in their fecal flora five years after a hospital outbreak in southern Sweden (Alsterlund et al., 2012). Another Swedish study by Tham et al describes

persistence of ESBL in 10% of the patients after three years. They also found two new ESBL-producing strains in one of the patients indicating transfer of the resistance phenotype (Tham et al., 2012). An alternative interpretation is that the patient was infected with different strains, although given the low frequency of EPE in Sweden the probability is low.

Dissemination and persistence of ESBL-producing bacteria have also been studied within households. A Spanish study reported that 16.7% of people living together with known persons with community-acquired infections also were colonized (Valverde et al., 2008). Another Spanish study found fecal carriage in 27.4% of household members of known carriers compared to 15.6% in non-household relatives and 7.4% in a control group of unrelated persons (Rodriguez-Bano et al., 2008). A Chinese study investigated ESBL carriage in children 0-5 years of age admitted to a pediatric clinic in Hong Kong and found CTX-M producing bacteria in 43.5%. Among 53 different households, carriage of an ESBL-producing strain was found in 83% in at least one household member (Lo et al., 2010). A French study found intra family transmission from adoptive children to their new family members at a rate of 23% (Tande et al., 2010).

Cottell et al have studied persistence from a different perspective. They investigated a plasmid, pCT, harboring $bla_{\text{CTX-M-14}}$ (the only resistance gene on that plasmid). They transformed the plasmid into an *E. coli* strain and found it provided little or no additional fitness to the bacteria, but still the plasmid persisted successfully in the absence of selective antibiotic pressure. This suggests that persistence of the plasmid is not always dependent on the resistance gene. (Cottell et al., 2012)

2 AIMS

2.1 GENERAL AIM

The overall aim was to study the epidemiology of Swedish EPE over time and to identify genetic factors important for epidemiological typing as well as dissemination of plasmids harboring extended spectrum β-lactamases.

2.2 SPECIFIC AIMS

I. To evaluate different methods for molecular epidemiological typing of ESBL-producing *E. coli* and *K. pneumoniae* and describe the epidemiology in a large collection of Swedish clinical isolates collected in 2007.

II. To describe the epidemiology of Swedish *E. coli* isolates over five years. The results from national surveillance systems are described as well as extensive characterization of isolates from point-prevalence studies conducted in 2007, 2009 and 2011.

III. To evaluate different methods for typing of plasmids harboring ESBLs and describe an alternative approach to plasmid typing as well as to analyze plasmidomes of ten ESBL-producing *E. coli* from 2007.

IV. To study the plasmid evolution and persistence over time in the gut flora of EPE carriers.

3 METHODOLOGY

3.1 CLINICAL ISOLATES

National point-prevalence studies of consecutively collected ESBL-producing *E. coli* have been carried out in Sweden in 2007, 2009 and 2011. Of the 21 Swedish counties 20 participated all three years. During 2007 isolates from all clinical sources (with a large dominance of urinary isolates) were collected during a three month period. Since the number of ESBL-producing *E. coli* has been increasing rapidly the 2009 year collection period was adjusted to one month only and from all clinical sources. During 2011the collection was further limited to contain only urinary isolates collected during one month.

Paper I included ESBL-producing isolates from the point-prevalence study in 2007. Both *E. coli* isolates and *K. pneumoniae* isolates were collected. The inclusion criterion was clavulanic acid-reversible resistance to oxyiminocephalosporins ($ESBL_A$ according to Giske et al (Giske et al., 2009). This resulted in 273 *E. coli* and 48 *K. pneumoniae* isolates.

In paper II ESBL-producing *E. coli* from all three point-prevalence studies were included, which resulted in 273 isolates in 2007, 304 isolates in 2009 and 463 isolates in 2011. The inclusion criteria for the isolates were urinary *E. coli* resistant to cefadroxil (used in Sweden as a screening marker for EPE), and resistance or intermediate susceptibility to cefotaxime and/or ceftazidime.

In paper III ten *E. coli* isolates were selected from the 2007 point-prevalence study collection for in depth plasmid characterization. These isolates were selected to be as diverse as possible. They differed regarding antibiotic susceptibility, real-time PCR for $bla_{CTX\text{-}M}$ phylogrouping, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and were isolated in geographically different parts of Sweden.

Paper IV is based on a different collection of strains that are part of a large study conducted by Emilia Titelman and co-workers at the Karolinska University Hospital in Stockholm. Patients seeking medical advice for different types of infections, (urinary tract infection being the most common) and where the causative agent was found to be EPE, were followed during one year. Samples were taken at five time points; at time zero (when the first clinical culture was taken), after 1 month, 3 months, 6 months and 1 year. In paper IV we studied plasmids in isolates from three of these patients that were positive for EPE throughout all five time points. In total this represents 19 isolates of three different species (17 *E. coli*, two *K. pneumoniae*). A more detailed description of the strains is given in Table 2.

Table 2.

Patient/sample	Species	PFGE-type
2/0	*E. coli*	E-2-1
2/1	*E. coli*	E-2-1
2/2	*E. coli*	E-2-1
2/3	*E. coli*	E-2-1
2/4	*E. coli*	E-2-1
4/0	*E. coli*	E-4-1
4/1-1	*E. coli*	E-4-1
4/1-2	*K. pneumoniae*	K-4-1
4/2-1	*E. coli*	E-4-2
4/2-2	*E. coli*	E-4-3
4/2-3	*K. pneumoniae*	K-4-1
4/3-1	*E. coli*	E-4-2
4/3-2	*E. coli*	E-4-1
4/4	*E. coli*	E-4-3
7/0	*E. coli*	E-7-1
7/1	*E. coli*	E-7-1
7/2	*E. coli*	E-7-1
7/3	*E. coli*	E-7-1
7/4	*E. coli*	E-7-1

3.2 ANTIBIOTIC SUSCEPTIBILITY TESTING

3.2.1 Phenotypic tests

3.2.1.1 Disc diffusion tests and Etests

Disc diffusion tests were performed using antibiotic discs that are plated on agar with inoculated bacteria and incubated. The zone diameter surrounding the antibiotic disc, lacking bacterial growth, is measured and translated to sensitive, intermediate or resistant (Olsson-Liljequist et al., 1997). Breakpoints and methodology valid at the time of testing were used according to the Swedish Reference Group for Antibiotic Resistance Methodology group (SRGA-M) and later to EUCAST. The agents tested were: cefadroxil, mecillinam, trimethoprim, nitrofurantoin, gentamicin, tobramycin, amikacin, meropenem and tigecycline. Etests i.e. antibiotic strips with a fixed concentration gradient of antibiotics were also used on agar with inoculated bacteria. Inhibition of growth presents as a spherical formation. The antibiotic concentration is read where the growth intersects the gradient strip. This enables a direct MIC-determination (Olsson-Liljequist et al., 1997). Gradient tests (Etests) were performed for cefotaxime, ceftazidime, cefepime, piperacillin-tazobactam, ciprofloxacin, fosfomycin and colistin. Breakpoints for SIR-categorization of the Etests were applied according to EUCAST (www.eucast.org). Historical breakpoints were obtained through personal communication with Gunnar Kahlmeter, Centrallasarettet, Växjö, Sweden.

3.2.1.2 Phenotypic ESBL-tests

The activity of β-lactamases produced by bacteria can also be detected with variants of the Disc diffusion method. With this method the susceptibility to different cephalosporins can be compared with or without an enzyme inhibitor. The difference in zone diameter is then compared and a ≥ 5 millimeter change or more indicates ESBL-activity (www.eucast.org).
$ESBL_A$ tests in these studies were performed using combination discs from Becton-Dickinson (Cockeysville, MD, USA) using cefotaxime and ceftazidime with and without clavulanic acid.
$ESBL_M$ tests were performed using combination tablets from Rosco Diagnostica A/S (Taastrup, Denmark) using cefoxitin with and without cloxacillin.

3.2.2 Genotypic tests

3.2.2.1 PCR

PCR is a rather fast and simple method ideal for screening of known resistance genes of interest. In the projects presented in this thesis, real-time multiplex PCR has been used for screening of $bla_{CTX\text{-}M}$ (Birkett et al., 2007) and acquired bla_{AmpC} (Brolund et al., 2010). Due to different chemistry and connection to a detector and a computer real-time PCR enables detection in real-time during the DNA amplification which saves both labor and time. The method is however more expensive and not ideal for amplification of longer DNA fragments (approximately >300 bp). Fast PCR has been used for screening of SHV and TEM genes as well as for genotyping $bla_{CTX\text{-}M}$ and acquired bla_{AmpC} genes prior to sequencing (Rasheed et al., 1997). Fast PCR uses a faster DNA polymerase which enables the time of the amplification to be shortened substantially. However separate detection is still needed.
In the plasmid typing procedures of paper III, a series of multiplex PCRs was performed in the plasmid replicon typing (Carattoli et al., 2005).

3.2.2.2 Sequencing

Sequencing is a method which can be used to define the exact nucleotide sequence of a DNA strand. Different types of sequencing techniques have been used in the studies presented in this thesis.

3.2.2.2.1 Sanger sequencing

Sanger sequencing (dideoxy sequencing) has been used since the 1970s. The technique has become much faster and user friendly since then and the sequencing quality has improved a lot. Today traditional Sanger sequencing can be used to sequence up to 1000 bp. Color-labeled bases are added on to the complementary strand of the sequence using chain-terminating inhibitors. The detection, usually by capillary electrophoresis, and interpretation of the results, determining the order of the nucleotides is performed by a computer. (Hartl and Jones, 2002)

The genotyping performed in paper I and II was conducted by Sanger sequencing, either in-house at SMI or by GATC, Germany.

3.2.2.2.2 Next generation sequencing

The sequencing technology has gone through a very fast development. New platforms have been developed that can sequence whole genomes in hours or a few days depending on the choice of platform. The development is continuously fast and new platforms and chemistries are introduced that lower the price substantially. Now even bench top versions of these next generation sequencing platforms are introduced. In paper III we have used the 454-platform and in paper IV Illumina sequencing was performed.

454-sequencing is based on a sequencing technique called pyrosequencing, which is a technique originally developed by researchers at the Royal Institute of Technology, KTH, in Stockholm in 1996. In 454-sequencing the procedure starts by fragmenting the DNA that is to be sequenced. The fragments are then sorted by size and fragments of a homogenous length are kept for further procedure. An emulsion PCR is performed inside small droplets of water for each of the fragments separately. The droplets are coated onto a pico-titer plate where sequencing reactions are performed on each PCR amplified sequence. In the pyrosequencing procedure bases are added to the sequence one kind at a time (A, T, G or C). Once the correct base is added pyrophosphate release is emitted which is registered. The reagents are washed away after each cycle and the sequence can be challenged with a different base type, and so on. The read-length of 454-sequencing is constantly increasing and has now reached around 700 bp (Liu et al., 2012). A longer read length is beneficial since it facilitates the sequence data analysis and increases the probability of correct predicting of the sequence order. At the time we used 454-sequencing in paper III the read length was about 200 bp which illustrates the high rate of improvement going on in this area. (Novais and Thorstenson, 2011)

Illumina sequencing is performed on a flat surface instead of in oil-emulsions. The size sorted sequence fragments are attached to a surface and amplified into local PCR amplified clones. Fluorescent reversible terminator bases are added to the samples and the bases are added one by one. When light is added to the labeled sequence a fluorescent signal is sent to a camera interpreting the base order. The reagents are then washed away and the next cycle can start. Using this technique nearly unlimited amounts of sequences can be performed simultaneously. However, the read length is so far limited to approximately 250 bp. (Liu et al., 2012)

3.3 EPIDEMIOLOGICAL TYPING

As discussed also in the introduction, epidemiological strain typing is very important in order to detect outbreaks, identify sources of infection and surveillance.

3.3.1 Epidemiological strain typing

There are many different epidemiological strain typing methods available. When choosing an adequate epidemiological typing method it is important to make sure the method answers the questions asked. For local outbreak investigations a method is needed that has a high discriminatory ability and can detect events within a short evolutionary time to avoid overestimating the relatedness between strains. In local outbreak investigations clinical epidemiological data is crucial and the epidemiological typing should be seen as a complement to those. If epidemiological typing results show that strains are related, also a potential epidemiological connection between the patients or medical staff etc, need to be shown in order to draw conclusions regarding possible dissemination from one individual to another. Epidemiological investigations aiming for studies on regional, national or global dissemination of particular epidemiological strain types need to be less discriminative and designed to detect larger evolutionary events that are accumulated over a longer time period. It is also important for such typing results to be easily be compared with those from other laboratories. In order to correctly interpret typing results one will also have to be aware of the prevalence of certain types of strains. If a particular epidemiological strain type is very common in the population, detecting several strains of that type might not be an indication of an outbreak.
Below is a description of the epidemiological typing methods included in the papers of this thesis.

3.3.1.1 PFGE

Pulsed-field gel electrophoresis is a method that can be used for epidemiological strain typing of various bacteria. For *E. coli* and *K. pneumoniae* a restriction enzyme called *Xba*I is normally used to fragment the genome. These fragments are then separated on a pulsed-field gel and the resulting banding pattern is compared between isolates. PFGE is a highly discriminatory method that is considered the gold standard in detecting outbreaks or studying the local epidemiology. The method is however quite time consuming and difficult to perform without previous experience. Experience is also needed when interpreting the data, which makes standardization of the method difficult. This affects interlaboratory comparisons to be made adequately.
Efforts have been made to create a universal PFGE nomenclature (http://www.cdc.gov/pulsenet/index.html) but due to the difficulties mentioned above it has not been used much. (Maslow JN, 1993, Persing, 1993)

3.3.1.2 DiversiLab

DiversiLab (DL) is a semi-automated method based on repetitive sequence PCR that amplifies parts of the bacterial genome. It is commercially available and sold by BioMérieux, France. The PCR fragments are separated using a Bioanalyzer, Agilent Technologies, and a chromatogram of the fragment sizes is generated. This chromatogram can be translated into a virtual gel-image and compared between isolates. This method uses more standardized methodology which facilitates data

interpretation, but there is no universal DL type nomenclature meaning that raw data needs to be compared in order to share results between laboratories. (Healy et al., 2005)

3.3.1.3 MLST

Multilocus sequence typing (MLST) is a method where seven housekeeping genes are amplified and sequenced. Variations in these genes give rise to different allele numbers and the combination of seven numbers make up a sequence type (ST). The method which targets housekeeping genes that are highly stable in evolutionary terms is less discriminatory than the above mentioned, but it has the advantage of the results being very easy to communicate and compare between laboratories. This method is good for comparing relatedness of strains globally and/or over longer time periods. (Wirth et al., 2006)

3.3.1.4 Phene plate (PhP)

PhP is an epidemiological strain typing method developed by researchers at Karolinska Institutet. It is based on the kinetics of biochemical reactions of bacteria. The method is available in different formats with different numbers of biochemicals measured. This commercially available method is performed in micro-titer plates pre-filled with substrates. A bacterial suspension is added to the wells and the plate is incubated at 37°C. The kinetics of the reaction in each well is measured through the absorbance calculated by a computer connected reader after 7, 24 and 48h respectively. The mean value of the readings is calculated and compared between isolates. Different micro-titer reaction plates are available for different species. (Tullus et al., 1999)

3.3.1.5 Phylogenetic grouping of E. coli

In phylogenetic grouping three sets of primers are combined in a multiplex PCR. The three genes amplified are *chuA*, which is a known gene required for heme transport in enterohemorrhagic O157:H7 *E. coli*. The others, *yjaA* and the DNA fragment TSPE4.C2, are of unknown function (Clermont et al., 2000). The combination of PCR products amplified or the absence of PCR products divides the isolates into one of four groups; A, B1, B2 or D. Since there are only four types present the discriminatory ability is of course limited. The method has more been used to explain and define functionality of certain strains such as virulence. Virulent extra-intestinal strains mainly belong to group B2 or D. Commensal strains more commonly belong to group A. (Clermont et al., 2000)

3.3.2 Epidemiological plasmid typing

Plasmid typing is even more complex than strain typing, mostly due to the plastic nature of plasmids and their remarkable ability to change the composition of their variable genetic regions. Plasmid typing has therefore focused a lot on the conserved core parts of the plasmids. Historically plasmid typing was determined through incompatibility assays. Plasmids were then isolated and introduced into strains with an

already known plasmid. If the two plasmids were able to stably coexist in the new cell generation, they were classified as compatible and therefore different. Plasmids of the same incompatibility type are incompatible because they rely on the same cell functions for their maintenance. This results in one of the plasmids randomly being excluded in the following cell generations. Even if the nomenclature from the incompatibility testing is still used, more is known now and the reality turned out to be more complex. Single nucleotide substitutions can cause incompatible plasmids to regain compatibility. Plasmids can also acquire multiple replicons that are useful for them in incompatibility situations. The mosaic plasmids can switch to one of the other replication machineries and maintain its compatibility. The different plasmid typing methods used in paper III and IV are described below.

3.3.2.1 S1/PFGE

There are ways to count the number of plasmids in a bacterial cell. One way is to linearize the plasmids and subsequently separate them on a pulsed-field electrophoresis (PFGE) gel. This also allows for an estimation of the plasmid sizes. The linearization is necessary since plasmids can be present in three different conformations in a cell. They can be either linear, supercoiled circular or open circular. These different conformations migrate differently in agar and can be misinterpreted as having different sizes. By adding S1 nuclease, plasmids are linearized. Supercoiled plasmids are nicked at a random position and turned to open circular. S1 nuclease then cuts a second time in the single stranded gap of the plasmids resulting in a double stranded linear plasmid. By adding a molecular marker to the PFGE experiment the plasmid sizes can be estimated. (Barton et al., 1995)

The PFGE gels with linearized plasmids can be further processed to find out which genes are present on the plasmids using Southern blot. This is however a challenging and labor intensive method for many laboratories to perform with consistent results. As an alternative to the Southern blotting procedure plasmid bands can be cut out from the PFGE-gel, DNA extracted and used as a template for directed PCR. (Barton et al., 1995)

3.3.2.2 Replicon typing

The mostly used plasmid typing tool for replicon typing is a PCR based method (PBRT) (Carattoli et al., 2005). The original protocol detects 18 replicon types. Commercially available kits are now present that can detect 25 replicon types. In *Enterobacteriaceae* there are 27 known replicon types.

3.3.2.3 pMLST

Plasmid multilocus sequence typing (pMLST) is a rather new method that was developed for subtyping of commonly occurring replicon types. Today there are pMLST protocols available for five replicon types, IncI1, IncHI1, IncHI2, IncN and IncF. In pMLST between two and six conserved plasmid genes are sequenced

depending on which Inc-group you are subtyping. Just like with the normal MLST on chromosomes, mutations give rise to new allele types which are given a specific number. The allele number-combination composes a pMLST-type (http://pubmlst.org/plasmid/).

3.3.2.4 Plasmid sequencing

Because of the high variability seen in plasmids of the same replicon type, total plasmid sequencing is basically the only tool available to paint the whole picture. However, more knowledge is needed in order to better understand how fast plasmids are evolving in order to interpret the results in a more correct and meaningful way over time.
Massive amounts of sequencing data can also be challenging and requires bioinformatic knowledge to sort out the sequences.

4 RESULTS AND DISCUSSION

4.1 EPIDEMIOLOGICAL TYPING OF ESBL-PRODUCING *ENTEROBACTERIACEAE*

Epidemiological typing is needed to monitor infectious agents in the surroundings and to follow their transmission. Crucial for epidemiological typing is that the choice of typing method must meet the questions asked. If the global epidemiology is studied, the method of choice must be able to measure relatively distant evolutionary events and it is of outmost importance that the results given are easily interpreted with a nomenclature that can be communicated between laboratories regardless of geographical location. Simplicity and price for an analysis are also important aspects if the method is to be used on a global scale. If the goal of the typing instead is to detect a local outbreak, the requirements are different. The typing method of choice must instead have a high discriminatory ability so that it is able to differentiate between isolates commonly circulating in the community and particular isolates disseminating in e.g. a hospital department. Inter-laboratory communication is always important, but less so when the analysis is to be used for local purposes. A method with too high discriminatory power is however not desirable, since it could contribute to underestimation of dissemination.

In paper I different epidemiological *E. coli* and *K. pneumoniae* typing methods were compared. The methods evaluated were PFGE, DiversiLab and MLST. Additional evaluation of the phene plate method (PhP), 24 reagent test, was also performed but was not included in the publication. ESBL-producing *E. coli* (n=258) and *K. pneumoniae* (n=48) from the Swedish point-prevalence study from 2007 were tested. For outbreak investigation of *E. coli* and *K. pneumoniae* PFGE is generally considered the gold standard method, therefore the evaluation was based on comparison to PFGE-results. The DiversiLab system is a semi-automated commercially available repetitive sequence PCR method. Thereby it has the potential of being more standardized and is delivered with analyzing software, which would facilitate inter-laboratory comparison. We wanted to evaluate if the DiversiLab system could meet the discriminatory power of PFGE. We also tested the reproducibility of the DiversiLab system and the turnaround time for analysis.
PhP is a method that is used for outbreak investigations, e.g. in some Swedish microbiological laboratories. PhP is a method where the kinetics of various biochemical reactions are measured. The system is commercially available. The ESBL isolates were further characterized with $bla_{\text{CTX-M}}$-phylogrouping PCR.

Using a routine protocol for PFGE the typeability (number of isolates that could be typed with the method) of *E. coli* isolates was higher with the DiversiLab system compared to PFGE, 100% vs. 88%. Some of the difficult isolates could potentially have been PFGE typeable under changed conditions such as change of PFGE buffer etc, this was however not performed. Due to the lower typeability of PFGE 226 *E. coli* were subjected to further method comparisons. For *K. pneumoniae* the typeability was 100%

for both methods. The DiversiLab system was stable, reproducible and less time consuming compared to PFGE. Under optimal conditions the DiversiLab system can generate results within one working day. There are different PFGE protocols available, but the one we use in our routine laboratory takes about three days to generate results. Using a similarity cut-off of 90% PFGE identified 27 *E. coli* clusters comprising two or more isolates and 125 singletons. Four *E. coli* PFGE-types included ≥5 isolates. In the *K. pneumoniae* collection five clusters were found. Twenty isolates were classified as singletons and only one large cluster was detected. The DiversiLab system identified 24 clusters and 65 singletons amongst *E. coli* and five clusters and 20 singletons amongst the *K. pneumoniae* isolates when using an indistinguishable cut-off as recommended by the distributors. Seven *E. coli* clusters included more than five isolates. In the *K. pneumoniae* collection only one such large cluster was identified.
All isolates were typeable with PhP. The hands-on time using this method is not so extensive, but the time between measurements still delays the results to >48h. When interpreting the results a 96% similarity cut-off was used for PhP. For *E. coli* PhP identified 34 clusters (>2 isolates/cluster) and 65 singletons. Nine clusters were found to comprise ≥5 isolates/cluster. PhP identified 9 clusters containing more than two isolates and 14 singletons in the *K. pneumoniae* collection. Only one cluster contained ≥5 isolates.

In order to compare typing methods statistical parameters were used. Simpson's index of diversity was used to measure the discriminatory ability of the methods at a 95% confidence interval (CI). Adjusted rand is a statistical tool that identifies the likelihood of agreement between two methods, taken the influence of chance into consideration. Wallace coefficient was used to determine the likelihood of one method typing an isolate the same way as the compared method at a 95% CI. Both Simpson's diversity index and the Wallace coefficients were determined using three different PFGE cut-offs; 80, 85 and 90% similarity.

Comparing DiversiLab and PFGE resulted in a Simpson's diversity index of 98.4 using a 90% similarity cut-off compared to 92.6 for the DiversiLab system in the *E. coli* collection. This difference was statistically significant at a 95% CI. Adjusted rand was 93.9%. The Wallace coefficients showed that when PFGE typed two isolates as similar, there was 90.4% probability that the DiversiLab system would also type them as similar. However, PFGE would only type two isolates the same way as the DiversiLab system in 19.8% of the cases. This means that, when using PFGE as gold standard method, the DiversiLab system overestimates the similarity of *E. coli* strains. Identical types would therefore have to be confirmed by PFGE. These results are in line with other *E. coli* typing comparisons published (Lau et al., 2010, Pitout et al., 2009).
In the *K. pneumoniae* collection the results were different. The DiversiLab system showed higher discriminatory ability than PFGE, Simpson's diversity index was 88.6 compared to 85.6. However, this was not statistically significant at a 95% CI. Adjusted rand showed 97% congruence. According to the Wallace coefficient The DiversiLab system could predict PFGE similarity in 100% of the cases. On the other hand, in 9.6 % of the cases the DiversiLab system overestimated the similarity between isolates using PFGE as Gold standard method.
From these results we conclude that the DiversiLab system is a good method for initial screening of similarity between *E. coli* isolates, but that the isolates identified as similar

would have to be confirmed by PFGE. For *K. pneumoniae* the DiversiLab system might risk missing strains that are related according to PFGE due to the higher discriminatory ability, but in order to fully understand what this means information about the patients and their potential connection is crucial. A more recent study comparing PFGE and the DiversiLab system for typing of several multidrug-resistant pathogens including ESBL-producing *E. coli* and *K. pneumoniae* was published by Deplano et al (Deplano et al., 2011). This study adds an additional dimension to the interpretation of results since they had access to clinical epidemiological data concerning the patients. They conclude that the DiversiLab system showed excellent concordance with PFGE for *K. pneumoniae* typing but lower concordance when typing *E. coli*. They could also see that PFGE is more discriminatory and that the DiversiLab system could identify isolates as identical when there was a true epidemiological link between them (Deplano et al., 2011).

For *E. coli* PhP in comparison with PFGE using a 90% similarity cut-off showed a Simpson's diversity index of 94.8, compared to 98.4 (difference not statistically significant). Adjusted rand was 16.4%. The Wallace coefficient showed that PFGE using a 90% similarity cut-off identified PhP-types in 10.6% of the cases and PhP identified PFGE-types in 35.8% of the cases. This confirms what we saw when comparing type-designations - that the two methods did not correlate well at all. Decreasing the similarity cut-off for PFGE did not change the results, PFGE at 80% similarity cut-off identified 17% of the PhP-types and PhP identified 26.3% of the PFGE-types. The only published study I have found describing a comparison between PhP (PhP-RE using 11 reagents) and PFGE is a poster presented on the 14th ECCMID 2004 (Landgren et al., 2004). Most studies describes the biochemical stability of *E. coli* measured by PhP (Katouli et al., 1990, Kuhn and Mollby, 1986) as well as comparing PhP-typing with other biochemical typing systems (Kuhn et al., 1990). Landgren and co-workers describe a high discriminatory power for both methods. The adjusted rand was high, 92%, but both over- and underestimation of clonal relatedness was observed (Landgren et al., 2004). For *K. pneumoniae* the Simpson's diversity index for PhP was 90.4% compared to 85.6% for PFGE 90% (not statistically significant). Adjusted rand showed 66.5% congruence. The Wallace coefficient showed that PFGE could identify PhP-types in 54.3% of the cases and PhP could identify PFGE-types in 85.9% of the cases. The correlation decreased when lowering the PFGE similarity cut-off. In conclusion PhP had a higher discriminatory ability and was better suited for *K. pneumoniae* than for *E. coli*. Compared to PFGE, PhP however risk missing related strain-types as well as overestimating relatedness between strains for both species when using high resolution 24-well PhP typing. Tullus et al compares high resolution 48-well PhP-typing of *K. pneumoniae* with PFGE which displayed similar results to ours (Tullus et al., 1999). However, it has to be considered that both studies include a limited number (n=33) of isolates.

MLST is a method that is widely used and that generates data that is easily comparable. A web site is publically available where type-designations are found and where anyone can report the finding of new types (http://mlst.ucc.ie/mlst/dbs/Ecoli and http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). An *E. coli* ST found to be widely dispersed internationally is ST131. ST131 is also highly associated to $bla_{\text{CTX-M}}$ and elevated virulence. ST131 is genetically stable and represents 30-40%

of the Swedish ESBL-producing *E. coli* which makes them important to be able to differentiate further with methods more discriminatory than MLST. The 2007 collection was subjected to a simplified PCR-based method for identifying ST131 (detecting *pabB*), and the positive results, 42% of the *E. coli* isolates, were confirmed using the full MLST protocol. Both PFGE and the DiversiLab system were evaluated regarding whether they could identify ST131 as well as differentiate between ST131 isolates. Using PFGE all ST131 isolates clustered at 60% similarity cut-off. For the DiversiLab system ST131 were clustered as similar compared to indistinguishable otherwise used for cluster definition. The DiversiLab system divided the ST131 isolates into five types whereas PFGE with a higher discriminatory ability divided the group in 41 types. Hence, PFGE was clearly better at differentiating between ST131 isolates which is a shortcoming of the DiversiLab system, in accordance with previous studies (Lau et al., 2010, Pitout et al., 2009). Divergent from our results Deplano et al did not see a good correlation between MLST and DiversiLab for *E. coli* (Deplano et al., 2011). However, one must keep in mind that we only studied the concordance to ST131 and no other STs.
PhP subdivided the ST131-isolates into 13 types (7 clusters and 6 singletons), but PhP-types were sometimes disagreeing with the MLST-typing displaying ST-131 positive and negative isolates within a PhP-type.

The epidemiological typing data was also compared with ESBL resistance phylogroups in the samples. Two *bla*$_{\text{CTX-M}}$-phylogroups were identified in the *E. coli* and *K. pneumoniae* collections. CTX-M-1 was found in 74% of the *E. coli* and 69% of the *K. pneumoniae* isolates. CTX-M-9 was found in 22% of the *E. coli* and 8% of *K. pneumoniae*. Genes of both CTX-M-phylogroups were found in two *E. coli* isolates. No CTX-M gene was detected in 4% and 23% of the isolates respectively.
Among the 27 PFGE clusters the CTX-M phylogroups were congruent in 24 which can be compared to the DiversiLab system where the CTX-M phylogroups differed in 14/24 clusters. These results suggest that the PFGE results would be closer to the true epidemiological linkage.
In the *K. pneumoniae* collection both PFGE and the DiversiLab system had mixed CTX-M phylogroups in one of the identified clusters. PhP-typing showed different CTX-M phylogroups in 19/34 *E. coli* clusters and in 6/9 *K. pneumoniae* clusters indicating low probability of epidemiological linkage between the strains.

In conclusion the DiversiLab system works well for excluding relatedness between *E. coli* isolates, but the related isolates needs to be confirmed by PFGE. PFGE showed a high concordance with *bla*$_{\text{CTX-M}}$-phylogroup, whereas the DiversiLab system showed higher discordance. For *K. pneumoniae* one must bear in mind that there was only a limited number of isolates tested. The DiversiLab system was found to have a higher discriminatory ability than PFGE in this collection of isolates. When PFGE is considered the Gold standard this could represent a challenge when using the DiversiLab system as a primary screening method for clonal relatedness, there is a risk that related strains could be typed as different.
Furthermore, PhP-typing is a standardized method that is easy to perform with high reproducibility (Landgren et al., 2005). Using the high resolution 24-well method showed low agreement with PFGE. However, one must take into consideration the

limitations of this study. The number of *K. pneumoniae* isolates was limited and the lack of clinical data makes the interpretation difficult.

Epidemiological strain typing of *Enterobacteriaceae* is difficult. There are no perfect universal methods that suit all purposes of epidemiological strain typing available that are fast, easy to perform and interpret the results from. The bacteria, especially *E. coli*, have a fast evolutionary rate mostly due to the large influence of transposable elements and horizontal gene transfer and also having a short life cycle compared to many other bacterial species. Even if whole genome sequencing were to be more easily available for outbreak investigations one must know what the natural diversity in the population is. In order to interpret similarities and differences correctly, knowledge on the rate of divergence over time is crucial.

4.2 EPIDEMIOLOGY OF ESBL-PRODUCING *ENTEROBACTERIACEAE* IN SWEDEN

Paper II presents the first comprehensive description of the national epidemiology of ESBL-producing *E. coli* in Sweden. Data was combined from different national surveillance systems and from EARS-Net (totally including 10,338 ESBL producing *E. coli* cases). Point-prevalence studies from 2007, 2009 and 2011, including 1,040 isolates, were included and analyzed in detail regarding resistance mechanisms and molecular epidemiology. The *E. coli* collection from 2007 was also included in paper I.

Based on the results described in 4.1 we decided to continue using PFGE for typing of the additional national point-prevalence collections from 2009 and 2011 in paper II. MLST and phylogenetic grouping were applied on the three collections so that the results could be compared to similar studies from other laboratories and countries. Detailed antibiotic susceptibility testing and genotypic analysis of the ESBL mechanisms were also performed.

The PFGE results were very similar in the three point-prevalence collections. About 50% of the isolates are unrelated (singletons) and the most prevalent PFGE-types were recurring in the strain collections from 2007, 2009 and 2011. New PFGE-types from later isolation years were only present with <5 isolates per type, which suggests that the strain types circulating in Sweden are stable over time. The typeability of PFGE for all isolates included in paper II was calculated to 94%.

MLST was performed on a subset of isolates. Representative isolates from each PFGE cluster were subjected to MLST. The MLST typeability was 100%. MLST typing showed between 34% - 38% of the isolates in the three collections to be ST131. The other STs found representing 2-6% of the isolates were ST38, ST69, ST405, ST617 and ST648. These STs have all been reported as prevalent from other parts of the world (Aibinu et al., 2012, Peirano et al., 2012, Smet et al., 2010). There are not many studies describing the distribution of STs in Sweden, but Naseer et al have investigated CTX-M- producing *E. coli* from Sweden, Norway and Spain and found that most prevalent STs amongst the Swedish isolates were ST131, ST648 and ST38 (Naseer et al., 2012).

The prevalence and distribution of STs were also very similar over the course of these five years studied. Two new MLST types were found and assigned to the MLST-database, ST3664 and ST3665. ST3664 was found in three isolates; two from 2007 and one from 2011. These are single locus variants of MLST types of the clonal complex (CC) ST95. ST3665 was found in two isolates; one from 2009 and the other from 2011. These are related to STs not assigned to any CC.

All isolates were additionally typeable with phylogenetic grouping. In the entire point-prevalence collection the B2 type was found in 41-47% of the isolates. Phylogroup D was the second most common, present in 27-29% of the isolates. Phylogenetic group A was represented by 15-22% of the isolates and B1 by 9-10%. The distribution of phylogenetic groups followed the ST distribution quite well. The B2 type was the most common all three years and was clearly dominating the ST131 isolates as well as the STs most closely related to ST131. These results also correlate with previous studies, where B2 and D phylogroups have been associated to more virulent *E. coli* (Clermont 2000) and B2, D and A phylogroups are associated to antibiotic resistance such as ESBL-production (Dahmen et al., 2013, Hu et al., 2013, Matsumura et al., 2012b).

Antibiotic susceptibility testing was performed for clinically relevant antibiotics. The antibiotic agents that showed the highest susceptibility among the circulating ESBL-producing *E. coli* isolates were mecillinam, nitrofurantoin and amikacin. Nitrofurantoin and mecillinam are both recommended treatment alternatives for uncomplicated urinary tract infections in Sweden (André et al., 2007, Sandberg et al., 2011-2012). Amikacin should be the aminoglycoside of choice for empirical treatment when targeting a spectrum also covering ESBL-producing *E. coli* isolates. The Swedish Reference Group for Antibiotics recently published new treatment recommendations in this matter (Hanberger et al., 2013). High resistance rates were found for trimethoprim (~70%) and ciprofloxacin (~60%) although the rate varied among different ESBL-genotypes. Co-resistance to these agents is reported from several other studies, both Swedish and international (Fennell et al., 2012, Kaarme et al., 2013, Ostholm-Balkhed et al., 2010, Simner et al., 2011). We also found that the susceptibility to ceftazidime among non-$bla_{\text{CTX-M-15}}$ ESBLs were quite high, 50-70% (data shown in paper II). The susceptibility testing results for bacteria producing all ESBL-genotypes is illustrated in Figure 9. The susceptibility data divided for different ESBL-genotypes are found in paper II.

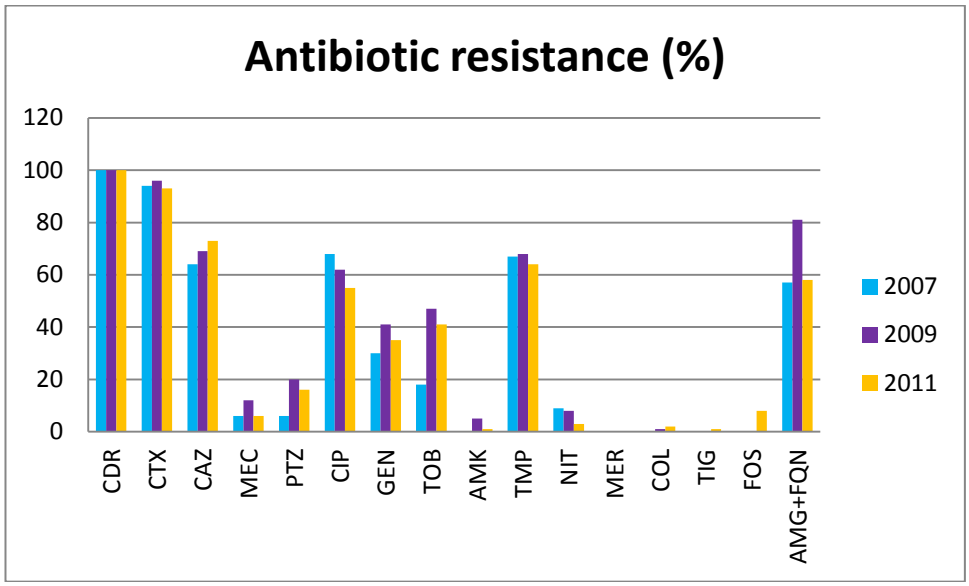


Figure 9. The proportion of isolates from all three point-prevalence studies resistant to the antibiotics tested.
CDR=cefadroxil, CTX=cefotaxime, CAZ=ceftazidime, MEC=mecillinam, PTZ=piperacillin/tazobactam, CIP=ciprofloxacin, GEN=gentamicin, TOB=tobramycin, AMK=amikacin, TMP=trimethoprim, NIT=nitrofurantoin, MER=meropenem, COL=colistin, TIG=tigecycline, FOS=fosfomycin, AMG+FQN=aminoglycoside + fluoroquinolone.

The genes encoding ESBL-production were also investigated. We found very little variation among the *bla*$_{\text{CTX-M}}$ genes as well over time. Just like in all other parts of the world *bla*$_{\text{CTX-M-15}}$ is dominating (54-58%) followed by *bla*$_{\text{CTX-M-14}}$ (11-14%) (Canton and Coque, 2006). Among the ESBL$_{\text{M}}$-genes *bla*$_{\text{CMY-2}}$ is the dominating type (4-5% of all ESBL-producers), this is in concordance with most other countries (Denisuik et al., 2013, Kiiru et al., 2012, Matsumura et al., 2012a, Voets et al., 2012). The distribution of resistance genes is presented in Figure 10.

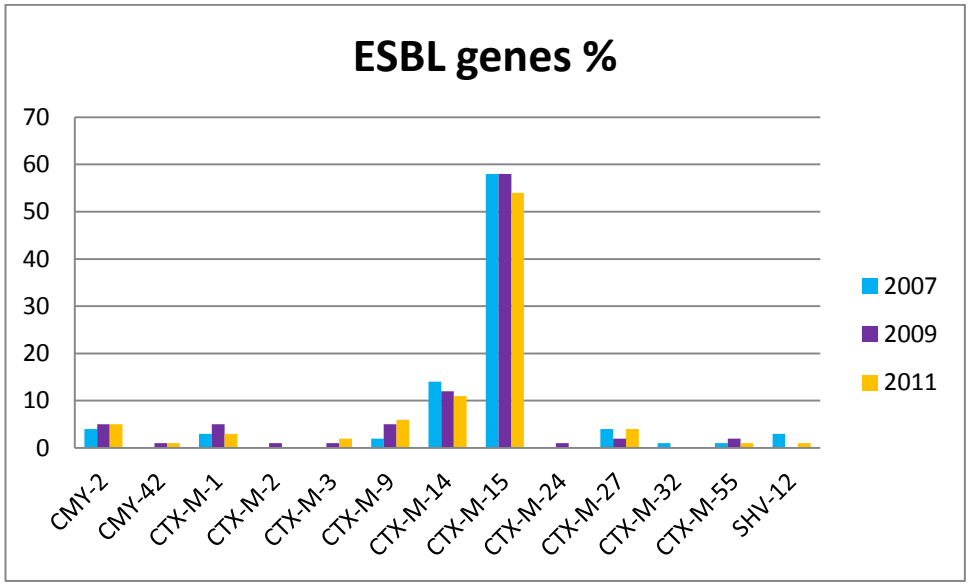


Figure 10. Distribution of ESBL-genes among the three point-prevalence studies.

Analyzing additional surveillance systems monitoring ESBL-producing *E. coli* that are available at the SMI over the same time period, the total reported number of ESBL-producing *E. coli* is steadily increasing in Sweden. The number of reported cases was 1,693 in 2007 compared with 5,068 cases in 2011. Most ESBL-producing *E. coli* are isolated from urine. Women are more commonly affected and are also on average younger than men when contracting an ESBL-producing *E. coli* infection. These results also apply for non-ESBL-producing *E. coli*. One reason for the higher prevalence of ESBL-producing *E. coli* among women is that they are more likely to contract UTI. The anatomy of the female urinary tract favors UTI compared to men who have a much longer urethra. Thereby they are more frequently treated with antibiotics for the UTI – which is a risk factor for ESBL.
We found that there was a decline in the median age for acquiring an EBSL-producing strain in both men and women ($p<0.0001$). This might be a result of EPE increasing in the community (Pitout et al., 2005). In combination with better infection control in the hospitals EPE are to a larger fraction found in uncomplicated infections in persons with no prior co-morbidities. Foreign travel also contributes to acquisition of EPE in healthy people (Tangden et al., 2010, Tham et al., 2010). To make sure these results were not biased by the age distribution in the total Swedish population demographic age data was also analyzed. The demographic data did not display a similar increase in the general population which further suggests that the decline in age is in fact valid.

4.3 EPIDEMIOLOGICAL TYPING OF ESBL-PLASMIDS

Plasmids are important vectors for the spread of ESBL-encoding resistance genes. The plastic genetic structures and high prevalence of repetitive sequences of plasmids however represent a challenge for genetic studies. Many plasmid-typing methods have to be combined in order to get the information needed and sufficient discriminatory power. One of the challenging factors is that bacteria of the *Enterobacteriaeceae* family amongst others often have multiple plasmids. Another complicating aspect of replicon typing is that plasmids can have multiple replicons, thereby a combination of different replicon types found in a bacterial cell do not always correlate to the number of plasmids present. Commonly used plasmid typing methods, such as replicon typing will therefore give confusing results when applied on extracted whole cell DNA. In order to sort out the number of plasmids in a bacterium and determine their replicon types correctly there are several options available. One way is transformation or conjugation of an individual plasmid of interest into a known bacterial background on which further plasmid typing can be applied. Conjugation of plasmids necessitates the plasmid to be conjugative (i.e. harbor all the genes required for transfer through a rolling circle replication type of mechanism and a pilus to connect to the recipient bacteria through which the plasmid can be transferred). If the plasmid is mobilizable it relies on co-transfer of an additional plasmid that is conjugative. Hence, several plasmids are transferred. If electroporation is used, which is forcing transfer of a plasmid by changing the permeability of the cell wall through applying an electrical field that enables passage of a plasmid, plasmid changes in composition cannot be ruled out. Plasmid sequencing with the aim to determine the exact plasmid composition of a

particular plasmid is complicated. Not the least since plasmid DNA is often scattered with identical IS-elements that makes it impossible for sequence analyzing tools to know which contigs to fit together. Further laboratory work is needed through different long range PCRs etc to fill the sequence gaps and connect contigs to each other. Another option is to linearize plasmids in order to calculate the plasmid number and sizes and thereafter use Southern blot or PCR to sort out which plasmid is of what replicon type and perhaps which of the plasmids that is responsible for dissemination of a particular gene of interest.

In paper III we wanted to type plasmids of ten well characterized ESBL-producing *E. coli* from the 2007 point-prevalence study. We were interested in studying the number of plasmid species present in each isolate, to sort out which of the plasmids that carries the ESBL resistance gene and further to study relatedness between the plasmids. Multiple plasmid species are common in *E. coli*, with an average of 2.5 plasmid species per cell (Sherley et al., 2003). We were interested in studying the total plasmid composition (the plasmidome) in the bacterial isolates and with what genes the plasmidome contributes to the isolates. We applied the most commonly used plasmid typing methods and shot-gun sequenced the plasmidomes of the strains with massive parallel sequencing using the Roche 454 platform. This resulted in a proposal of a new sequence based plasmid typing approach.

The classical plasmid typing methods applied were replicon typing, S1 nuclease linearization and Southern blot. These methods provided a good platform for comparison and analysis of the plasmidome sequencing results.

NGS often produces an overwhelming amount of data. Therefore simplified tools to extract information of interest are needed. We wanted to be able to extract information regarding resistance genes present in the plasmid gene pool in a simple and fast way. The sequences were translated to protein in all six reading frames. For this a profile hidden Markov model (pHMM) database was created with reference protein sequences conferring resistance to β-lactams, fluoroquinolones, trimethoprim, sulphonamides, macrolides, aminoglycosides, tetracycline, and chloramphenicol. The output is presented as a heat-map with matches illustrated as filled squares.

Using the same approach a new plasmid typing tool based on NGS was set up. A heat-map analysis was performed using Pfam conserved protein domains of translated sequencing reads without sequence assembly. This gave us insight of the protein-coding content of the *E. coli* plasmidomes. The method also creates fingerprints with a dendrogram that illustrates the relatedness between the plasmidomes which could be used for epidemiological typing of plasmidomes.

One problem that we came across when analysing the NGS data was that one of the ten plasmid DNA samples had been degraded by a phage. Hence, much of the genes confirmed in that isolate using the classical microbiological methods were missing including the $bla_{\text{CTX-M}}$-gene. Presence of phage DNA was also found in two additional plasmidomes which matched the *E. coli* P1-phage to a large extent. P1 phages also replicate as free entities in *E. coli* and could be mistaken for plasmids in S1 nuclease linearization of plasmids.

In paper IV we used paired end Illumina sequencing for the plasmidome analysis. The problems we came across were mostly related to the extremely high sequencing depth obtained (sometimes exceeding 1,000,000x of small plasmids), which hampered the computer-based analysis. Due to the high coverage, we also experienced a much higher background of chromosomal sequences compared to the 454-sequencing. However, this enabled us to extract information from chromosomal sequences in addition to the plasmids. But it also made the interpretation of some genes difficult where the sequence coverage was inconsistent with both plasmid and chromosomal sequences. Heat map analysis of plasmid content was also complicated due to difficulties predicting whether some genes were present because of contamination from chromosomal sequences or possibly other samples.

4.4 CHARACTERIZATION OF PLASMIDS HARBORING ESBL IN SWEDISH ISOLATES

ESBL-producing *E. coli* are known to harbor IncF-plasmids to a large extent. Replicon typing of the entire point-prevalence study of Swedish *E. coli* from 2007 confirmed this. 213 of the 226 isolates carried at least one IncF-plasmid (data not shown). IncF-plasmids are narrow host range plasmids limited to dissemination between *Enterobacteriaceae* (Villa et al., 2010). They are usually large with low copy numbers and often encoding resistance genes (highly associated to $bla_{\text{CTX-M-15}}$) and a number of virulence genes (Villa et al., 2010). IncF-plasmids differ from other plasmid types since they display even higher diversity in terms of plasmid size, number of replicons, ability to conjugate due to extensive recombination (Partridge et al., 2011). The success of the ST131 *E. coli* clone has also been suggested to be favored by the high association to IncFII-plasmids (Novais et al., 2012).
In paper III IncF plasmids were identified in all ten isolates. IncF-plasmids were also identified in most strains from the three patients described in paper IV (Titelman et al., submitted manuscript).

The plasmidomes studied in paper III and IV were found to consist of between two and eight distinct plasmids per isolate. The plasmids were variants of the previously sequenced plasmids pUTI89, pKF3-70, pEK499, pKF3-140, pKF3-70, p1ESCUM, pEK204, pHK17a, p083CORR, R64, pLF82, pSFO157, and R721 available in GenBank. Most of these plasmids have been associated to antibiotic resistance in *E. coli*. In addition, small cryptic high copy-number plasmids were frequent, containing one to seven open reading frames per plasmid. They were found in all but two samples and had a size range of 1.5 to 7.8 kb. The samples contained one to seven different small cryptic plasmids. Small plasmids are not very well characterized and their function or way of persistence is not fully understood. Most cryptic plasmids only contain genes for replication and plasmid mobility. In paper III, three clustered groups of such small cryptic plasmids could be distinguished based on sequence similarity amongst the ten studied isolates. This is interesting since the *E. coli* strains were selected to be as diverse as possible. It is also interesting that we did not find any small plasmid in two of the isolates.

In paper III 19 of the 35 resistance gene families included in the pHMM database were identified. The majority of resistance genes were encoded on large plasmids. Only one of the small cryptic plasmids contained resistance genes, a 6.2 kb p62-like plasmid that contained *sul2* and *strAB* genes. In paper IV 24 of the 35 gene families included in the pHMM database were identified.

4.5 EVOLUTION AND PERSISTANCE OF ESBL-PLASMIDS OVER TIME

In paper IV we describe the EPE and their plasmids isolated from three carriers (named 2, 4 and 7) over the course of one year. Samples were taken at five time-points; the initial clinical sample, and fecal samples after 1, 3, 6 and 12 moths. The results demonstrated how the bacteria act in the gut flora and the potential this reservoir creates for genetic exchange.

The three patients represent three different scenarios. In patient 2 *E. coli* isolates with identical epidemiological strain types defined by PFGE were isolated at all time points. The isolates all had the same $bla_{\text{CTX-M}}$ phylogroup and belonged to the internationally dispersed clone ST131. The plasmid content of the strains was identical in the four first samples. The fifth sample contained the same plasmid with the exception of a 22.5 kb deletion between two IS26 elements. The same four small cryptic plasmids were identified in all samples from this patient.
Patient 4 pictures a truly complex situation with several different strain types and species harboring different CTX-M-types. The plasmid content in these strains was just as complex where similar plasmids show up in divergent strains and identical strains containing different plasmids, both large and small.
Patient 7 had identical *E. coli* ST405 isolates over the course of the study. On the contrary the plasmid composition differed between the sampling points. It seems like this is an effect of a dynamic pool of plasmids that merges and diverges constantly and where some plasmids come and go.

It would be interesting to know how these large differences arise. In a study focused on persistence of EPE in the gut flora of carriers from which these patients were chosen, the antibiotic consumption and ESBL risk factors were discussed (Titelman et al., submitted manuscript).
All three patients were initially colonized by an EPE in the urinary tract. Patient 2 was treated with ciprofloxacin and presented no risk factors except for travelling to Gran Canaria within six month before contracting UTI. This patient was not treated with antibiotics during the following year of sampling. Quinolone resistance is most often a result of mutation in the chromosomally located DNA gyrase and topoisomerase IV genes (Ruiz, 2003). Low level quinolone resistance genes can be found on plasmids, however not in the plasmidomes of patient 2. The stability of the ESBL strains and their plasmids can be explained by the lack of risk factors and antibiotic treatment in this patient. The plasmid deletion seen in the fifth sample is not surprising given the lack of selection pressure. The deleted sequence amongst others includes a tetracyklin resistance gene, *tetA*(B). Experiments have shown tetracycline resistance genes (*tet*) to

confer a high fitness cost which could have contributed to this deletion (Sandegren et al., 2012).

Patient 4 was treated with erthapenem and had been hospitalized in Sweden before contracting the UTI. The patient had also been travelling to Nepal and was treated with both tetracycline and ciprofloxacin within 6 months prior to the UTI. This could indeed affect the results of patient 4. He has been treated with several broad spectrum antibiotics that we know affect the gut flora to a large extent. This could have been the driver of the extended plasmid dissemination observed. Genes conferring resistance to both tetracycline and ciprofloxacin were found in the sequenced plasmidomes. The travel to Nepal is also a factor that could respond to acquisition of several ESBL-producing strains and the presence of different ESBL-genotypes (Baral et al., 2012). It cannot be excluded that several strains could have been acquired in Sweden, but the estimated prevalence is much lower (<5% in the Swedish population) and therefore seems more unlikely.

Patient 7 was treated with pivmecillinam for the UTI diagnosed when the first sample was taken. This patient had not been travelling but was admitted to a hospital in Sweden prior to the UTI infection and had a suprapubic catheter and had been treated with ciprofloxacin. During the course of the study this patients had relapsing infections and was treated with both pivmecillinam and nitrofurantoin several times. The effect of continued treatment with antibiotics could respond to the fluctuations in the plasmidome seen for patient 7.

Bias of the results cannot be rule out due to the number of isolates chosen at the time of isolation. We did e.g. not study if there were variations between identical isolates taken at the same point of isolation.
Even if we only studied three patients, the results clearly show that the impact of antibiotic treatment on driving resistance dissemination cannot be underestimated.

5 CONCLUDING REMARKS

This thesis describes several perspectives of EPE in Sweden. The epidemiology in Sweden was followed in detail over five years. Even if the antibiotic policy is rather strict in Sweden compared to many other countries, we still see a steady increase (3-fold in five years) of EPE. The bacterial types and ESBL mechanisms were stable during the course of this time. The dissemination of EPE is shifting from affecting hospitalized individuals with underlying disease to increasingly affecting younger otherwise healthy persons in the community. This trend is also seen in other parts of the world. It may be explained by the increased levels of EPE globally and that these bacteria are imported to a higher extent due to increased travelling. The decreasing number of EPE in hospitals is probably an effect of increased awareness and better diagnostics as well as improved infection control programs and routines.

Different methods for epidemiological typing of bacteria and plasmids have been tested, evaluated and discussed. No perfect universal method fitting the different requirements of local, regional and national epidemiological surveillance is yet available and further research and development on this topic is needed.
We found *E. coli* ST131 to be common among Swedish EPE, representing about 40% of the isolates. About 50% of the isolates were on the other hand unrelated picturing a rather high diversity of strain types circulating. The plasmid typing work of this thesis has shown that IncF-plasmids are highly associated to the dissemination of $bla_{\text{CTX-M}}$ genes in *E. coli*. Although belonging to the same replicon type there was a rather high variation between the plasmids regarding sizes and associated genes. It is however interesting to find that the plasmids we sequenced all resembled previously sequenced plasmids deposited in GenBank. This indicates that although there are large variations among the plasmids there are conserved sequences that reoccur in clinical ESBL-plasmids circulating globally given the still rather limited number of fully sequenced plasmids. This work also points to the small cryptic plasmids in EPE that haven't been studied much and are sparsely described in the literature.
The plasmid typing presented in this thesis also resulted in a suggested new approach for typing of plasmidomes using pHMM analysis of NGS data. New insight is also gained in the difficulties faced and obstacles that have to be overcome with this more and more common sequencing platform.

The persistence and evolution of plasmids was studied in the gut flora of EPE carriers over a period of one a year. This work sheds light on the complexity of bacterial interaction in the gut flora of carriers and the potential for massive genetic exchange.

The impact of antibiotic treatment cannot be underestimated. In order to use these extremely important drugs rationally we need to gain full understanding of how resistance arises and all the factors that influence dissemination. Much research is still needed in this field. If we want to have effective treatment also in the future, new antibiotic agents are needed on the market, as well as optimizing the usage of the already available antibiotics, that can buy us time for solving this important issue that constitutes a major public health problem.

6 FUTURE PERSPECTIVES

There is a need for faster and simpler methods both for epidemiological strain and plasmid typing. Common nomenclature is preferable so that results can be communicated and compared between researchers. International surveillance is increasingly important since the influence of travel is substantial on dissemination of infectious agents, not the least antibiotic resistant bacteria.

National surveillance of EPE is also important. The number of cases and demography need to be monitored carefully. The results of this thesis however suggest that extensive characterization of EPE at a national level might not be needed in as short time intervals as every two years given the stability of the epidemiological strain types and ESBL resistance mechanisms. The increasing prevalence of EPE seen in the community is worrying and will affect the number of cases in seriously ill hospitalized patients in the future. Infection control, effective diagnostic methods and strict treatment guidelines will be increasingly important.

For the near future our research will focus on evaluating the plasmid typing approach presented in paper III. The pfam heat-map will be applied on the plasmidomes of paper IV. This will enable us to see if the dissemination of individual plasmids can be detected in plasmidomes and if this tool could be useful in outbreak situations. Since these plasmids are well characterized and the relation between the strains is known these plasmidomes will make a good collection to evaluate if the heat-map is suitable for detecting plasmid relatedness.

The role of small cryptic plasmids is another interesting field where much is yet to be discovered. Why are they so common? Are they completely selfish elements or is their presence beneficial for the bacteria carrying them?

Strain and plasmid interaction and the contribution to resistance dissemination it confers needs further studies in order to be fully understood. The persistence of EPE in the healthy population is also important to learn more about. Such knowledge would contribute to important information regarding how to handle patients colonized with EPE in hospitals and for how long persistence is to be expected. In the long run we could also get insight in how to optimize antibiotic treatment to be as effective as possible for patients and yet minimize further antibiotic resistance from arising and disseminating.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Enterobacteriaceae är en familj bakterier normalt förekommande i tarmfloran, där bakterier som *Escherichia coli* och *Klebsiella pneumoniae* ingår. β-laktamas med utvidgat spektrum (ESBL) är enzymer som bakterierna kan producera för att bryta ner β-laktamantibiotika, den mest använda gruppen av antibiotika där bl.a. penicillin ingår. β-laktamantibiotika används vid en rad infektioner såsom urinvägs-, blod- och sårinfektioner, men används även som förebyggande behandling efter kirurgiska ingrepp eller ges till patienter med nedsatt immunförsvar t.ex. för tidigt födda barn och cancerpatienter.

Enterobacteriaceae som producerar β-laktamas med utvidgat spektrum (EPE) orsakar stora problem inom sjukvården. I arbete I och II i denna avhandling har vi bl.a. kartlagt EPE i Sverige. Dessa bakterier blev anmälningspliktiga år 2007 och först därefter har vi kunnat övervaka förekomsten på ett utförligt sätt. På SMI har vi vartannat år sedan 2007 samlat in EPE från landets mikrobiologiska laboratorier och karaktäriserat dessa. Vi har även genom våra olika databaserade övervakningssystem kunnat samla in information från laboratorierna löpande. I de två första arbetena i denna avhandling beskrivs förekomsten av EPE i Sverige samt kartläggs de genetiska mekanismer hos bakterierna som orsakar resistensen.

I avhandlingens första arbete utvärderade vi olika metoder för att i detalj karaktärisera ”fingeravtryck” av de olika förekommande bakterieisolaten. Det vill säga metoder för att studera likheter och olikheter mellan bakterier av samma art. Detta för att se vilken metod som lämpar sig bäst för fortsatt karaktärisering av EPE. Vi använde därmed en rad olika metoder för att i detalj studera stora nationella kollektioner av EPE över tid. Resultaten visar att EPE ökar väldigt fort i Sverige på samma sätt som sker i övriga världen. Från 2007-2011 har förekomsten ökat trefaldigt. Men vi ser också att det inte är någon enstaka variant av EPE som dominerar i Sverige vilket pekar på att vi inte har någon stor spridning av en och samma variant utan att det snarare rör sig om olika stammar som t.ex. förvärvats via import från utlandet. Vi ser dock släktskap hos bakterierna på längre håll, vilket är i enlighet med vad som observerats i övriga världen. Cirka 30-40 % av ESBL-producerande *E. coli* i Sverige tillhör den internationellt mycket utbredda klonen ST131 och den vanligast förekommande resistensgenen som kodar för ESBL är $bla_{\text{CTX-M-15}}$. En trend som identifierades är att förekomsten av EPE blir vanligare i lägre åldrar. Det tror vi kan bero på att vi har en ökad förekomst av EPE i samhället hos friska bärare samt att vi blivit bättre på att uppmärksamma EPE inom sjukvården och har jobbat med förbättrade rutiner kring vårdhygien så att den sjukvårdassocierade spridningen avtagit.

Generna som kodar för ESBL enzym sitter ofta på plasmider. Plasmider är cirkulära DNA-molekyler som kan finnas i bakteriecellen i tillägg till kromosomen. Plasmider kan dela sig och spridas på egen hand mellan bakterier. De kan till och med spridas mellan olika arter av bakterier. De utgör ett mycket effektivt verktyg för bakterierna att kunna anpassa sig till nya förutsättningar i deras närmiljö eftersom de ofta innehåller gener som bidrar till att bakterierna blir mer motståndskraftiga (t.ex. mot antibiotika)

och bättre på att orsaka sjukdom. Ju mer antibiotika vi använder desto större fördel blir det för bakterien att förvärva denna typ av motståndskraft. Och ju större andel bakterier som uppvisar resistens desto mer problematiskt blir det att behandla olika sjukdomstillstånd orsakade av bakterierna. Inte minst eftersom en annan effekt av plasmider är att de kan innehålla ett helt batteri av överlevnadsegenskaper, till exempel resistensgener mot flera olika klasser av antibiotika. Man talar om co-selektion när användningen av ett antibiotikum bidrar till att bakterier med resistens mot det medlet anrikas men samtidigt även är resistent mot flera andra antibiotikaklasser, till exempel genom att den har en multiresistensplasmid. Detta fenomen är vanligt förekommande hos EPE vilket ofta gör dem mycket svårbehandlade.

I delarbete III och IV har vi detaljstuderat ESBL-plasmider. I arbete III valde vi ut tio olika ESBL-producerande *E. coli* från vår nationella kollektion från 2007 och jämförde olika plasmidtypningsmetoder. Plasmidtypning är problematiskt, inte minst för att bakterier som till exempel *E. coli* ofta har flera plasmider. Plasmider ändrar också ofta sin struktur, en egenskap som gör att de lätt kan plocka upp för bakterien värdefulla gener. Vi presenterade ett nytt sätt att karaktärisera plasmider genom att bestämma DNA-sekvensen för bakteriens hela plasmidinnehåll. Bakterierna vars plasmider vi studerade i arbete III var utvalda att vara så olika som möjligt. Vi kunde ändå se att en plasmidtyp (IncF) förekom i samtliga bakterier, vilket även rapporterats från internationella studier.

I arbete IV studerade vi hur plasmiderna förändrades under ett år hos tre olika patienter med EPE i tarmfloran. Alla patienterna hade ursprungligen sökt vård för urinvägsinfektion som visat sig vara orsakad av en EPE. Därefter togs avföringsprover efter 1 mån, 3 mån, 6 mån och 12 månader från vilka EPE odlats fram. När vi bestämde DNA-sekvensen hos plasmiderna upptäckte vi tre ganska olika scenarier. Hos två av patienterna identifierades samma bakteriestam vid alla odlingstidpunkter. I den första patienten var även plasmiderna identiska, förutom att en del av en stor plasmid försvunnit vid sista provtillfället. Hos den andra patienten med identiska bakterieisolat såg vi stora förändringar i plasmidinnehållet, där plasmider slagits ihop, delats eller kom och gick under de olika provtillfällena. En tredje patient hade en mycket komplex flora av EPE innehållandes två olika arter av EPE och dessutom olika stamtyper av samma art. Samma komplexitet noterades hos plasmidpopulationen. Plasmiderna kunde se helt olika ut i identiska bakteriestammar och identiska plasmider dök upp i olika bakterier. Skillnaden mellan de tre patienterna kunde till viss del förklaras av sjukdomshistoria och antibiotikaanvändning. Till exempel hade en av patienterna rest till ett högriskområde i världen där denna typ av resistens är mycket vanlig vilket kan förklara att flera olika ESBL-typer fanns i bakteriefloran.

Sammanfattningsvis blir det tydligt att vi behöver ta krafttag mot antibiotikaresistensens utbredning. Mer forskning behövs för att vi ska kunna förstå alla aspekter av hur resistens uppstår och sprids. Vi behöver även lära oss mer om hur antibiotikaanvändning påverkar resistensspridningen om vi ska ha en chans att stoppa den eller åtminstone få den snabba ökningen att klinga av. Vi behöver även tillskott av nya effektiva antibiotika eller vacciner som kan köpa oss mer tid till forskning om vi vill upprätthålla den goda överlevnad i infektionssjukdomar vi har haft sedan antibiotikans inträde på 1940-talet.

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