Recurrent infection with Extended-Spectrum Beta-Lactamase (ESBL)-producing Enterobacteriaceae

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Cover illustration: ESBL-producing *E. coli*, positive DDT-test

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Il m'a expliqué en souriant que rien n'est blanc ou noir et que le blanc, c'est souvent le noir qui se cache et le noir, c'est parfois le blanc qui s'est fait avoir.

Romain Gary, La vie devant soi, 1975

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ABSTRACT

Infections with Extended-Spectrum Beta-Lactamase (ESBL)-producing Enterobacteriaceae (EPE) are increasing globally. The most common EPE are the gut pathogens *Escherichia coli* (ESBL-*E. coli*) and *Klebsiella pneumoniae* (ESBL-*K. pneumoniae*). The spread of antimicrobial resistance (AMR) in these organisms is due both to the spread of high-risk bacterial clones and to the transfer of AMR-genes via easily transmissible plasmids. This thesis focuses on factors of importance for recurrent EPE- infection. In **paper I,** the frequency of subsequent EPE-positive clinical cultures in an unselected patient group with a fecal screen or clinical culture positive for EPE was investigated. It was uncommon with a following clinical culture in patients with a positive fecal screen, but a new EPE-infection was common (almost 30%) in patients with a previous EPE-positive clinical culture (>90% urine cultures). In **paper II**, the rate of a change of species and possible ESBL-carrying plasmid transfer between clinical ESBL-*E. coli* and ESBL-*K. pneumoniae* isolates in subsequent infections was investigated by a novel plasmid typing technique, Optical DNA mapping (ODM). The rate of a change of species was low (<3%). Possible transfer of plasmids was found in a few cases. ODM in these cases rendered valuable information of plasmid numbers, plasmid sizes and the location of resistance genes. **Paper III** was a retrospective study of bacterial factors of importance for recurrent ESBL-*E. coli* UTI in 123 patients. Almost all isolates causing recurrences were of the same phylogroup as the index isolate. PFGE of a subset of isolates showed strain homology in 98%. Phylogroup B2 dominated, and within this phylogroup, presence of the subclone ST131- O25b-*fimH*30Rx was associated with multiple recurrences. In **paper IV**, ESBL-*E. coli* isolates from recurrent and sporadic UTI were prospectively collected. A comparison of bacterial characteristics with focus on ST131-O25b and its subclones showed an increase in risk of recurrence in patients infected with the virulent subclone. In conclusion, this thesis provides valuable new knowledge about factors influencing recurrences of EPE-infection.

Keywords: ESBL, *E. coli*, recurrent infection, UTI, AMR, phylogroup, *fimH*30Rx
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SAMMANFATTNING PÅ SVENSKA

Antibiotikaresistens är ett snabbt växande globalt hot mot vår hälsa. Tarmbakterier som bär på enzym av typen Extended-Spectrum Beta-Lactamases (ESBL) ökar mest av alla multiresistenta bakterier. ESBLs bryter ner och inaktiverar penicilliner och cefalosporiner, våra viktigaste och mest använda antibiotika. Särskilt oroande är den sorts ESBL som även bryter ner karbapenemer. Ofta har bakterier med ESBL dessutom förvärvat multiresistens, dvs resistens också mot andra antibiotikagrupper.
Andelen patienter som har haft en infektion med eller bär på dessa bakterier ökar även i Sverige. Anledningarna till detta är flera, varav överanvändning av antibiotika, både inom vården och djurhållningen, bristande hygienrutiner och ökat resande är några. Dessutom är vissa av de bakteriekloner som bär ESBL mer spridningsbenägna och sjukdomsframkallande (virulenta) än andra. Bakterierna bär ESBL-genen på plasmider, små ringformade DNA-molekyler som lätt kan överföras mellan bakterier.
Escherichia coli (*E. coli*) och *Klebsiella pneumoniae (K. pneumoniae*) är de vanligaste bakterierna som kan bilda ESBL. De ingår i vår normala tarmflora, men de tillhör också våra vanligaste sjukdomsframkallande bakterier. De kan orsaka ett brett spektra av infektioner såsom blodförgiftning och urinvägsinfektion (UVI). Spridningen av *E. coli* med ESBL-produktion tillskrivs i stor utsträckning en speciell bakterieklon, ST131-O25b-*fimH*30Rx. Klonen bär på ESBL-genen CTX-M-15 på särskilt lättmobila plasmider.
Syftet med avhandlingen var att studera hur vanligt det är med återkommande infektion med ESBL-bildande *E. coli* respektive *K. pneumoniae* hos patienter som tidigare varit positiva i ett odlingsprov, att studera spridning av ESBL-bärande plasmider mellan dessa arter, samt att studera hur bakteriella egenskaper, såsom fylogrupp och klontillhörighet hos ESBL- *E. coli* påverkar risken att få en ny infektion. I det första delarbetet **(I)** studerades över 3000 patienter med tidigare positiva prov taget antingen för att påvisa tarmbärarskap (sk fecesscreenprov) eller kliniska prov avseende efterföljande positiva kliniska prover. De kliniska proverna tas på misstanke om infektion, i dessa fall främst urinvägsinfektion. En mycket liten andel av de med tarmbärarskap visade sig ha ett uppföljande kliniskt prov (<6%) medan det hos patienter med ett tidigare positivt kliniskt prov var vanligt med ytterligare positiva kliniska prov (nästan 30%). I arbete **II** undersöktes hur många patienter med recidiverande infektion som bytte art från ESBL- *E. coli* till ESBL- *K. pneumoniae* eller vice versa mellan infektionsepisoderna. Orsaken till ett sådant byte kan vara att en ESBL-plasmid migrerat mellan bakterierna innan nästa infektion uppstod. Möjlig plasmidmigration studerades med Optical DNA mapping (ODM) i samarbete med Chalmers Tekniska

Högskola. Metoden är nyutvecklad, baseras på nanoteknik och medför en så detaljerad analys av plasmider att man kan fastställa om två bakterier bär på samma plasmider, eller till och med samma delar av plasmider. Byte av art mellan infektionsepisoderna var ovanligt, <3%, och att plasmidmigration föregår en ny recidivinfektion kunde endast beläggas i ett fåtal av dessa fall. I arbete **III** studerades fylogrupp respektive klontillhörighet och stammarna från 123 patienter med recidiverande UVI. I 98% av fallen förekom samma ESBL-*E. coli*-stam vid varje recidiv. Drygt hälften av alla fall med recidiv var infekterade med ESBL- *E. coli* som tillhörde den mer virulenta typen, dvs fylogrupp B2, varav majoriteten tillhörde ST131-O25b-klonen. Patienter infekterade med dess subklon ST131-O25b-*fimH*30Rx hade signifikant fler recidiv än de infekterade med övriga isolat inom fylogrupp B2. I arbete **IV** jämfördes fylogrupper, ST131-O25b och dess subkloner och ESBL-gener hos prospektivt insamlade ESBL-*E. coli*-isolat från 68 patienter med recidiverande och 235 patienter med sporadisk UVI i Västra Götalandsregionen. Fylogrupp B2 dominerade och förekomst av den virulenta subklonen visade sig öka risken för recidiv påtagligt.

Sammantaget ger avhandlingen viktig och ny information om återkommande infektioner hos patienter med tidigare bärarskap eller infektion med ESBL-bildande bakterier och om bakteriella faktorer som påverkar risken att få en ny infektion.

LIST OF PAPERS

I. Lindblom A, Karami N, Magnusson T, Ahrén C. Subsequent infection with extended-spectrum β-lactamase-producing Enterobacteriaceae in patients with prior infection or fecal colonization. Eur J Clin Microbiol Infect Dis. 2018; 37: 1491-1497.

II. Lindblom A, Kk S, Müller V, Öz R, Sandström H, Ahrén C, Westerlund F, Karami N. Interspecies plasmid transfer appears rare in sequential infections with extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae. Diagn Microbiol Infect Dis. 2019; 93: 380-385

III. Karami N, Lindblom A, Yazdanshenas S, Lindén V, Ahrén C. Recurrence of urinary tract infections with ESBL-producing *Escherichia coli* are caused by homologous strains among which clone ST131-O25b is dominant. J Glob Antimicrob Resist. 2020, epub ahead of print

IV. Lindblom A, Karami N, Kristiansson E, Yasdanshenas S, Kiszakiewicz C, Kamenska N, Henning C, Ahrén C. Recurrent urinary tract infections with ESBL-producing *Escherichia coli* are caused by isolates of specific phylotypes and clones. *Manuscript*

CONTENT

ABBREVIATIONS

AMR	Antimicrobial resistance
AST	Antibiotic sensitivity testing
BSI	Bloodstream infection
CPE	Carbapenemase-producing Enterobacteriaceae
CTX-M	CefoTaXimase-Münich
Ears-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EHEC	Enterohemorragic *E. coli*
EPE	ESBL-producing Enterobacteriaceae
ESBL	Extended-Spectrum Beta-Lactamase
$ESBL_A$	Classical ESBL
$ESBL_{CARBA}$	Carbapenemase
$ESBL_M$	Miscellaneous ESBL
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic *E. coli*
HGT	Horizontal gene transfer
IBC	Intracellular bacterial community
ICU	Intensive care unit
IPEC	Intraintestinal pathogenic *E. coli*
IS	Insertion sequence
LPS	Lipopolysaccharide
MDR	Multi-drug resistance
MGE	Mobile genetic element
MRSA	Meticillin-resistant *Staphylococcus aureus*
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PPV	Positive predictive value
RUTI	Recurrent urinary tract infection
SHV	Sulfhydryl-variable, an early beta-lactamase enzyme
TEM	Temoneira, an early beta-lactamase enzyme
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
VF	Virulence factor
WGS	Whole-genome sequencing

1 INTRODUCTION

The discovery of antibiotics remains one of the greatest advances in modern medicine. Without effective antibiotics, treatable infections become fatal again, and advanced healthcare such as organ transplants, cancer treatments and neonatal care become hazardous to perform due to the risk of hard-to-treat complications[1,2]. Consequently, antimicrobial resistance (AMR) is one of the biggest threats to global public health. Among drug-resistant bacteria, Enterobacteriaceae producing Extended-Spectrum Beta-Lactamases (EPE) is of particular concern. The situation was in 2015 highlighted by the World Health Organisation (WHO) with a global program to fight the worrying development, with measures to improve awareness, prevention and diagnostics of AMR[3]. This includes increased knowledge of factors that contribute to recurrent infection with EPE, which is the focus of this thesis.
The Gram-negative gut bacteria *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are the most common EPE. They are involved in a wide range of conditions, from asymptomatic fecal colonization, urinary tract infections (UTI), abdominal infections and life-threatening sepsis[4]. Extended-Spectrum Beta-Lactamases (ESBLs) are enzymes that inactivate the penicillins and the cephalosporins, our most used antibiotics[5]. The proportion of patients with a history of a previous EPE-positive culture is rising. In 2018, ESBL-*E. coli* caused about 60 infections per 100 000 inhabitants in Sweden, more than a doubling compared to 2009[6]. In addition to clinical infections, screening regimens aiming to halter the spread of EPE in the health-care setting results in augmented numbers of patients with a known history of fecal EPE-colonization. In case of a new suspected infection in these patients this can pose a clinical problem. Although it has previously been shown that colonizing strains are less virulent than infecting strains[7], severe infections with EPE are linked to higher morbidity and mortality[8], and warrants prompt treatment with last-resort broad-spectrum antibiotics. Thus it is important to identify patients at risk of a new EPE-infection.

In **paper I**, the frequency of, and the time to, subsequent positive EPE-cultures in patients with a previous positive EPE-culture in a fecal screen or clinical culture in an unselected patient group was outlined. The study formed

the basis for the following studies in the thesis in demonstrating that recurrent EPE-infection occurs in almost 30% of patients and thus is a matter of concern. The spread of resistant *E. coli*- and *K. pneumoniae*- isolates is due to the expansion of successful virulent bacterial clones, belonging to certain sequence types (STs) and to horizontal transfer of resistance genes (HGT) between bacteria. The ESBL-genes are located on easily transmissible plasmids considered epidemic due to their rapid spread[9]. Transfer of resistance genes through conjugation is known to occur in the human gut microbiome. In a clinical context, this could mean that patients with a previous EPE-infection could be at risk of new infections with other ESBL-producing Enterobacteriaceae. In **paper II**, the rate of a change of species between ESBL-*E. coli*- and ESBL-*K. pneumoniae*-isolates in patients with recurrent EPE-infection was investigated. Possible ESBL-carrying plasmid transfer among ESBL-*E. coli* and ESBL-*K. pneumoniae* was investigated with Optical DNA Mapping (ODM), a plasmid typing technique developed in recent years. In non- ESBL *E. coli*, UTI recurrences are most often caused by the same strains and phylogroups[10]. Increased understanding of the differences in microbial pathogenesis among ESBL-*E. coli* isolates causing recurrent and sporadic UTI, and similarities to UTI:s with non-resistant *E. coli*, can help in foreseeing the probability of recurrent ESBL-*E. coli* UTI. The possibility that bacterial properties, such as strain type, phylogroup and/or ESBL-type influence the risk of developing subsequent infections with ESBL-*E. coli* UTI was investigated in **paper III** and **IV**.

1.1 Enterobacteriaceae

Bacteria can be classified as Gram-positive or Gram-negative, depending on the structure and appearance of their cell wall after Gram staining (Figure 1). As opposed to the thick (20-80 nm) peptidoglycan cell wall that characterizes Gram-positive bacteria, the Gram-negative cell wall is thinner (7-8 nm), consisting of an inner cytoplasmic cell membrane, a thin peptidoglycan layer and an outer membrane with lipopolysaccharide (LPS), also called endotoxin, a powerful stimulator of the immune system. LPS consists of lipid A, the core polysaccharide and the O-antigen, long polysaccharides, creating an outer membrane important for the protection of the bacteria against hydrophobic compounds such as antibiotics. Inside the outer membrane is the periplasmic space, also important for bacterial defense as it contains enzymes

that inactivate antibiotics and selective efflux pumps. To transport necessary nutrients through the cell wall, the Gram-negatives have developed porines; specialized protein channels[11].

The Enterobacteriaceae (domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria and order Enterobacteriales), is a large family of Gram-negative rods that includes members of the normal gut microbiota as well as pathogenic species. The family contains around 50 genera and over 250 species, including *E. coli* and *K. pneumoniae*[12].

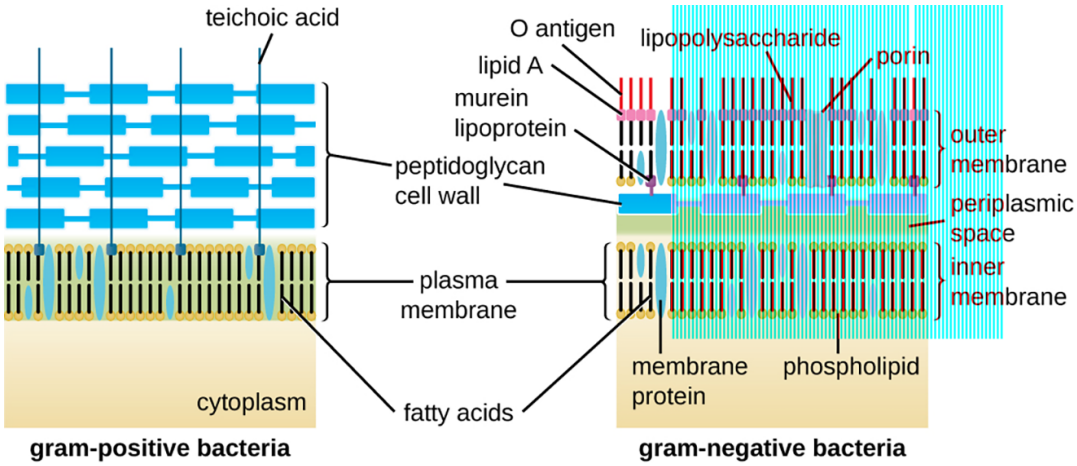

Figure 1. The Gram-positive and Gram-negative bacterial cell wall. Image Creative Commons, licensed under CC BY 4.0: https://openstax.org/books/microbiology/pages/1-introduction

1.1.1 Escherichia coli

E. coli is a non-sporulating, motile, lactose-fermenting, facultative anerobic Gram-negative rod. As a normal inhabitant of the mucus layer of the colon, *E. coli* has important biological functions. By being the most abundant aerobic commensal, *E. coli* prevents colonization by pathogenic strains. *E. coli* is also responsible for the synthesization of vitamin K, which we are unable to produce ourselves[11].

However, expression of virulence factors that make it possible to invade, thrive and persist in their host disturbes the normal physiology and distinguishes commensal from specific pathotypes of *E. coli*[13]. In this respect,

a pathotype classifies *E. coli* into groups that cause similar disease manifestations. Besides commensal *E. coli*, two main pathotypes of *E. coli* exist, the extraintestinal pathogenic *E. coli* (ExPEC) and the intestinal pathogenic *E. coli* (IPEC). ExPEC can be part of the normal gut flora as opposed to IPEC, which are obligate diarrheagenic pathogens that can be further divided into six pathotypes; e.g. EHEC (Enterohemorragic *E. coli*) and ETEC (Enterotoxigenic *E. coli*)[13,14].

ExPEC includes the uropathogenic *E. coli* (UPEC) and pathotypes causing bacteremia, abdominal infections and neonatal meningitis. UPEC is by far the most common cause of UTIs. It causes 75-95% of community-acquired and 65% of hospital-acquired UTIs[15-17].

E. coli can be classified into serotypes and divided into phylogroups. Serotyping is based on the O-antigen, i.e. LPS, and the H-antigen, i.e. flagellar antigens, and occasionally on the capsular K-antigen. There are over 180 serotypes of *E. coli*. The different phylogroups of *E. coli* differ in virulence properties, ecological niche and ability to cause disease. There are presently eight *E. coli* phylogroups (A, B1, B2, D, C, E, F and G). UPEC strains have been shown to belong mainly to B2 and D, whereas commensal, colonizing strains have been associated to phylogenetic groups A and B1[7,18].

For both IPEC and ExPEC certain clonal lineages with colonizing, spreading and persistence properties that facilitate their establishment as a pathogen have emerged. A bacterial clone consists of evolutionary similar isolates sharing the same traits, although they are not genetically indistinguishable. For IPEC, a well-known example is EHEC serotype O157:H7[4]. The emergence of the pandemic antibiotic-resistant ExPEC strains is largely due to a single clonal group, ST131, serotype O25b:H4, belonging to phylogenetic group B2. Presence of ST131 has been associated with recurrent and complex *E. coli* infections[7,19].

1.1.2 Klebsiella pneumoniae

K. pneumoniae is the most common isolate of the *Klebsiella* genus. It is a non-sporulating, lactose-fermenting and non-motile facultative anaerobic Gram-negative rod. *K. pneumoniae* can be found in several environmental niches; in soil, vegetation and wastewater[20]. By phylogenetic analyses seven

phylogroups of *K. pneumoniae sensu latu* have been described[21]. *K. pneumoniae sensu stricto* (KpI, *K. pneumoniae*) is the most common clinically encountered. The protective and antigenic polysaccharide capsule is an important virulence factor of *K. pneumoniae*. LPS, fimbriae, siderophores and efflux pumps are also main virulence factors[22].

Compared to *E. coli*, *K. pneumoniae* is to a greater extent associated with hospital-acquired infections as well as health-care transmission of antibiotic resistance, and patients in intensive care units (ICUs) are particularly at risk. *K. pneumoniae* causes UTI:s, bloodstream infections and pneumonia, often in immunocompromised patients with predisposing conditions such as old age, alcoholism, organ failure, malignancies and in neonates[23,24]. They are a leading cause of ventilator-associated pneumonia in the ICU[25]. Colonization is linked to the length of hospital stay and antibiotic usage[26-28]. During the 1980s and 1990s, *K. pneumoniae* strains carrying TEM- and SHV-ESBLs caused several outbreaks in ICUs[29].

1.2 Mechanisms of action for antibiotics

Antibiotics are either naturally derived, produced by microorganisms (e.g. penicillin, streptomycin) or synthetically produced (e.g. sulfonamide). Since the launch of sulfonamide for therapeutic purposes in the 1930s, followed by penicillin becoming available in the late 1940s, the development and search for new antibiotics has continued[30].

To exert their effect antimicrobials need a target in the bacterial cell to act on, and to avoid toxicity, preferably the target is absent in the human cell. Antibiotics can induce cell death (bactericidal drugs) or inhibit cell growth (bacteriostatic drugs). The beta-lactams and glycopeptides interfere with bacterial cell wall synthesis by blocking steps in the peptidoglycan synthesis, thereby causing cell lysis. The quinolones block DNA-gyrase and topoisomerase IV, inducing DNA-strand breaks and stopping DNA supercoiling leading to cell death. The macrolides, lincosamides, aminoglycosides and tetracyclines block ribosomal protein translation. Sulfonamide and trimethoprim block folic acid synthesis[11,31].

1.2.1 Beta-lactams

Penicillin G was the first beta-lactam to be discovered by Sir Alexander Fleming in 1928[32]. It revolutionized the treatment of streptococcal infections although it took until the mid 1940s until it was approved and distributed worldwide. In 1945, Fleming, together with Howard Florey and Ernst Boris Chain received the Nobel Prize in Medicine and Physiology[33].

The beta-lactam antibiotics are, due to their low toxicity and high efficacy, still the most widely used antibiotic class both for common and severe infections. The beta-lactams are bactericidal agents that all share a common structure, the beta-lactam ring (Figure 2) which inhibits bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs). Thus, only growing cells are affected. The penicillin-binding proteins (PBPs) are enzymes, transpeptidases, involved in the stabilization of the cell wall by crosslinking of peptidoglycan in both Gram-positive and Gram-negative bacteria. Bacteria of different species carry diverse subsets of PBPs which explains the differences in the antimicrobial spectrum of the various beta-lactam classes. In Gram-negative bacteria, PBP 1a, 1b, 2 and 3 are important targets for the beta-lactams[34].

During the decades after their discovery resistance to the beta-lactams gradually emerged. New derivatives stable to staphylococcal beta-lactamases (e.g, methicillin) and with a greater Gram-negative spectrum (e.g, ampicillin) were developed[34]. The beta-lactam ring was modified with larger side chains to protect them from new emerging beta-lactamases (Figure 2). At present, four main classes of beta-lactam antimicrobials; the penicillins, the cephalosporins, the carbapenems and the monobactams are in clinical use.

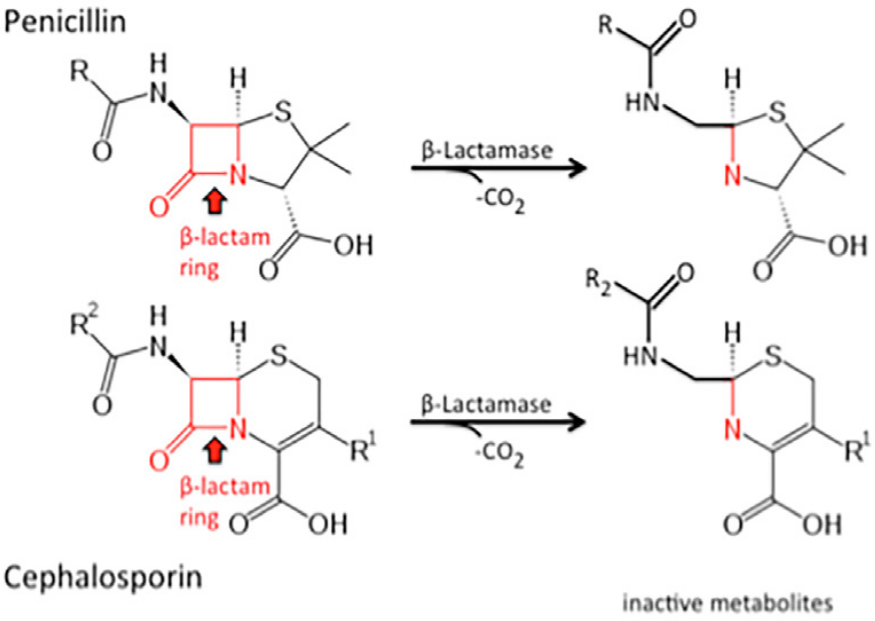

Figure 2. The beta-lactam ring (red) of the penicillins and the cephalosporins and the hydrolytic, inactivating effect of the beta-lactamases. Image Creative Commons, licensed under CC BY-SA 4.0. http://tmedweb.tulane.edu/pharmwiki/doku.php/betalactam_pharm

1.2.1.1 Beta-lactam-beta-lactamase inhibitor combinations

Co-administrating beta-lactam antibiotics with beta-lactamase inhibitors is a way to protect the beta-lactam from degradation. The beta-lactamase inhibitor acts as a substrate for the beta-lactamase by binding irreversibly and inducing chemical reactions in the enzyme's active site. Clavulanic acid, sulbactam and tazobactam are all examples of commercially available beta-lactamase inhibitors, effective against CTX-M, TEM-1, TEM-2 and SHV-1[35]. Amoxicillin-clavulanic acid and piperacillin-tazobactam are examples of combinations extensively used in Sweden. Avibactam (in combination with ceftazidime) and vaborbactam (in combination with meropenem) are more potent and have an additional effect on some carbapenemase-producing isolates, with the exception of metallo-beta-lactamases[36]. The combination ceftazolan-tazobactam has been shown to also have an effect on EPE, but especially on *Pseudomonas aeruginosa* with porin-loss[37].

1.2.1.2 Cephalosporins

The cephalosporins were introduced in the 1980s, rapidly followed by emergence of new beta-lactamases that inactivated them. The cephalosporins

are grouped into generations based on their coverage against Gram-negative and Gram-positive bacteria: 1st generation (e.g. cefadroxil, cefazolin, cephalexin), 2nd generation (cefuroxime, cefoxitin, cefaclor), 3d generation (cefotaxime, ceftriaxone, ceftazidime, ceftibuten), 4th generation (cefepime), 5th generation (ceftaroline, ceftobiprole).
The 1st and 2nd generations are more active against Gram-positive bacteria (streptococci and staphylococci). The 3d generation has a more Gram-negative profile. 3d generation cephalosporins have increased stability to SHV-1 and TEM-1 beta-lactamases and a more potent activity against Gram-negative bacteria. These are still one of our most important antibiotics used for severe infections, but are strongly threatened by the ESBL pandemic. Cefepime has a similar spectrum to 3d generation cephalosporins but is more stable against AmpC-enzymes[34,35].

1.2.1.3 Carbapenems

The carbapenems (meropenem, imipenem, ertapenem) have a potent broad-spectrum activity against Gram-negative, Gram-positive, aerobic and anaerobic bacteria. Due to their binding to PBP1a, 1b and 3, they are stable to most beta-lactamases with the exception of the emerging carbapenemases[34].

1.3 Principles of mechanisms for antibiotic resistance

A natural part of bacterial evolution is adaptation and protection via intrinsic resistance mechanisms. Organisms that produce antibiotics also contain self-resistance mechanisms against their own antibiotics. Production of small amounts of antibiotics is a natural phenomenon and various antibiotic compounds can be found in the environment. The problem arises when resistance genes are spread to pathogenic bacteria and when excessive antibiotic pressure allows resistant strains to outconquer the natural microbial population. An example of this is the mobilization of the CTX-M beta-lactamase-genes from the terrestrial *Kluyvera spp*[38].

The development of antibiotics during the 20th century has been followed by subsequent discoveries of resistance mechanisms to all antibiotic classes[38]. Bacteria can escape the effects of antibiotics in the following ways, also illustrated in Figure 3:

- Permeability changes: Porine mutations and alterations preventing the antibiotic from diffusing into the bacterial cell
- Efflux mechanisms: Efflux pumps, located in the cytoplasmic membrane, that can expel antibiotics and toxic compounds such as biocides and metals
- Resistance mutations resulting in target modification: Modification of the ribosome, enzymatic alteration in the target site, point mutations in the genes encoding the target site, alterations in penicillin-binding proteins (PBPs)
- Drug inactivation: Degradation or modification of antibiotics by enzymes

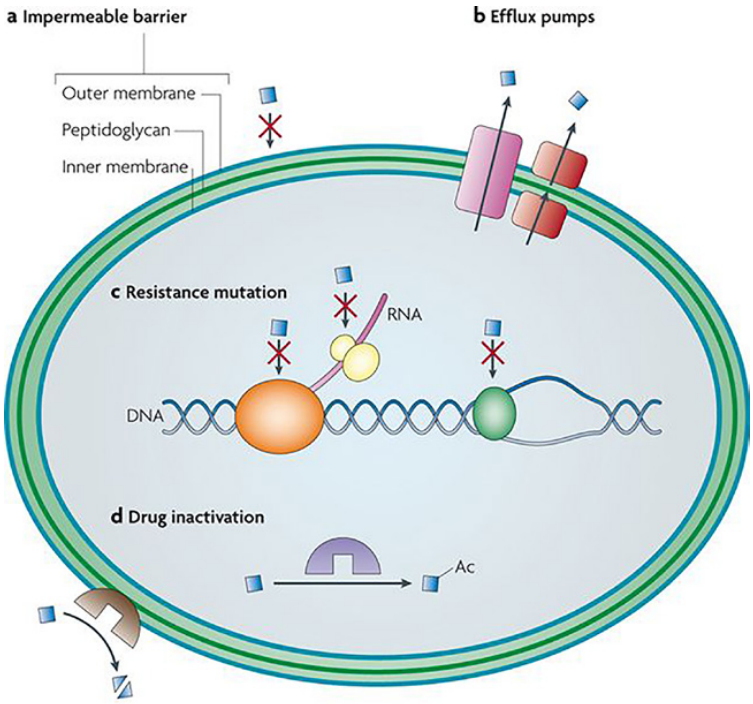

Figure 3. Principal resistance mechanisms in bacteria. For beta-lactam antibiotics, the major resistance mechanism in Gram-negative bacteria is drug inactivation by beta-lactamases in the periplasmatic space (d). Reprinted from Allen et al. [39] *with permission from Springer Nature.*

1.3.1 Extended-Spectrum Beta-Lactamases

Enzymes that inactivate the beta-lactam antibiotics have been known since the 1940s and is the most important resistance mechanism in Gram-negative gut bacteria. The beta-lactamases are present in the bacterial periplasmic space where they inactivate the beta-lactam-ring by hydrolysis. They include the penicillinases (degrading penicillins), the cephalosporinases (degrading penicillins and cephalosporins) and the carbapenemases (which may degrade all beta-lactam antibiotics)[11].

Most Enterobacteriaceae harbor chromosomally-encoded beta-lactamases. The first plasmid-borne β-lactamase (a penicillinase hydrolyzing ampicillin) was identified in 1963. It was named TEM after the patient Temoneira in which it was identified[40]. Some years later the SHV (Sufhydryl-variable)-variants, derived from the chromosome of *K. pneumoniae* and mobilized onto plasmids, were discovered. The TEM and SHV-variants were the most commonly encountered until the end of the 1990s. The development of broad-spectrum cephalosporins revolutionized the treatment of Gram-negative infections during the 1980s, and at the same time, their widespread use meant increased selection pressure and expansion of the substrate specificity of TEM and SHV. By point mutations, TEM and SHV-enzymes became able to inactivate the third-generation cephalosporins such as ceftazidime and cefotaxime. The first isolate with this new cephalosporinase was a *K .pneumoniae*- isolate carrying a TEM-1 derivative named TEM-2[41]. The spread of the new TEM- and SHV-variants were predominantly associated to epidemic clones and *K. pneumoniae* hospital outbreaks[42].

The rate of ESBL-production in Enterobacteriaceae varies worldwide. According to data from 2018 reported by the European Antimicrobial Resistance Surveillance Network the resistance rates against 3rd generation cephalosporins for *E. coli* and *K. pneumoniae* (invasive isolates) in the Nordic countries were on average 8% and 5% respectively. The proportions in Europe ranged from 7% to almost 40% in *E. coli* (Figure 4) and from 0 to almost 80% in *K. pneumoniae*[43].

1.3.1.1 The CTX-M-enzymes

The first reports of the new CTX-M enzymes came in the mid 1980's, and the first clinical isolate was an *E.coli* isolated in Munich in 1989[44]. It was named CTX-M; CefoTaXimase- first isolated in Munich[5,45]. The CTX-M genes have been mobilized from the chromosome of members of the *Kluveyra* genus, a member of the Enterobacteriaceae family and an opportunist that seldom causes disease[46].

The new enzymes were shown to hydrolyse cefotaxime to a greater extent than ceftazidime and were unrelated to the previously known TEM- and SHV-variants. As the CTX-Ms were established, the epidemiology of ESBLs changed from being related to *K. pneumoniae* nosocomial outbreaks to *E. coli*- infections, mostly UTIs, in the community. There was now an entry of CTX-M-genes from the community in to the hospitals[47]. In Western Sweden, the CTX-M-types dominated from the start of ESBL-transmission which was also true for other Nordic countries at the time[48].

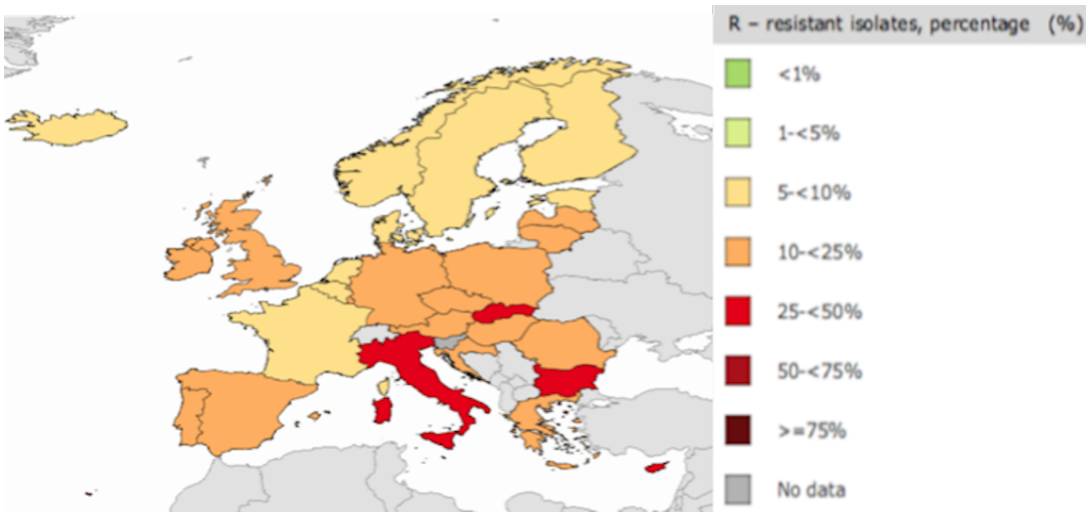

Figure 4. Resistance to third-generation cephalosporins in invasive E. coli 2018 in the EU/EEA countries according to EARS-Net[43].

CTX-M-type beta-lactamases are the most prevalent type of ESBLs worldwide, and often co-exist with resistance to other classes of antibiotics, especially fluoroquinolones, aminoglycosides and co-trimoxazole[49]. They can be found in several Gram-negative bacteria but the largest clinical impact consists of CTX-M-carrying *E. coli* and *K. pneumoniae*[5]. The CTX-M-genes

are very diverse, and there are now over 200 CTX-M-enzymes that can be divided into five groups depending on their amino acid sequences; CTX-M group 1, 2, 8, 9 and 25[50] (Figure 5). These have subgroups where the difference can be as small as a base pair difference (https:/www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/).

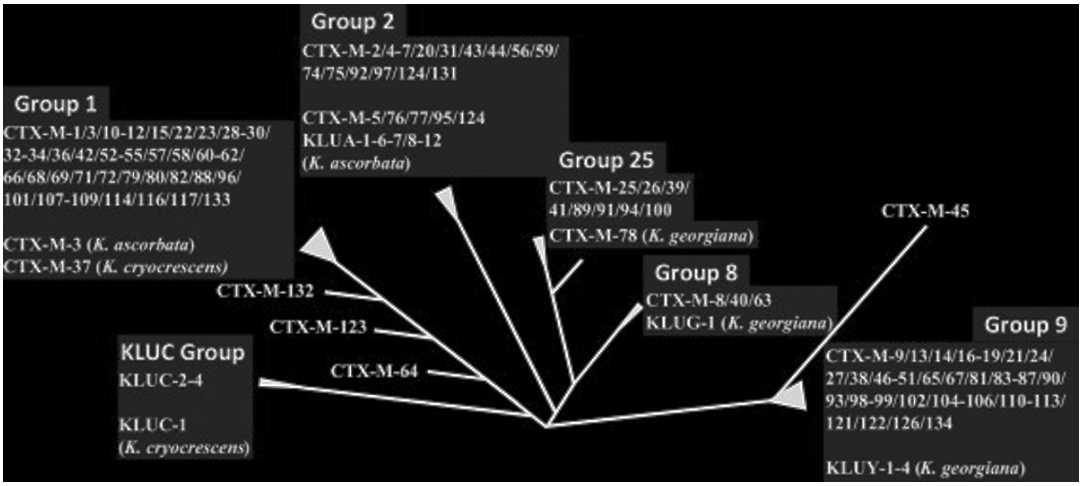

Figure 5. The five groups of CTX-M-type beta-lactamases based on amino acid alignment and putative precursors of Kluyvera spp. Reprinted from d'Andrea et al.[50] with permission from Elsevier.

The most common CTX-Ms are CTX-M-1, CTX-M-3, and CTX-M-15 of group 1, and CTX-M-9 and CTX-M-14 of group 9[45]. CTX-M-15 is the most dominant gene found globally and an increase of this enzyme is reported for most countries[5,51]. Some CTX-Ms are more prominent in certain parts of the world, such as CTX-M-14 in Spain, Portugal and France, CTX-M-3 in Eastern Europe and CTX-M-2 in South America and Japan. However, CTX-M-14 and CTX-M-15 has started to outconquer CTX-M-2. CTX-M-27, a variant of CTX-M-14 (and part of the CTX-M-9 group) is presently on the rise, recently found in Japan, China, South-East Asia, North America and Europe. This CTX-M variant is associated to a sublineage of the successful ST131-clone[46].

1.3.2 Classification of the ESBLs

Three different classifications of the beta-lactamases exist, a structural classification, based on amino acid sequences (the Ambler classification), a

functional classification (the Bush-Jacoby-Meideros classification) and the simplified Giske classification, which is the most used in the Scandinavian countries[52]. The Giske classification is based on treatment options (resistance spectrum) and transmission risks and includes plasmid-mediated enzymes carried by Enterobacteriaceae. It includes three classes of ESBLs. ESBL-A, inhibited diagnostically by clavulanic acid, is the most frequently found in *E. coli* and *K. pneumoniae* (90% of cases), and in the focus of this thesis. ESBL-M is a miscellaneous category including plasmid-mediated AmpC-cephalosporinases and some OXA-ESBLs, inhibited by cloxacillin.

ESBL-CARBA can be divided into class A (serineproteases; mainly KPC), class B (metallobetalactamases; VIM, IMP and NDM-1) and class D (OXA-48). KPC is the most common carbapenemase globally, prevalent in the Americas, China and the Mediterranean countries. The metallo-betalactamases are highly resistant to the carbapenems, and more prevalent in the Indian subcontinent and Eastern Europe. The OXA-carbapenemases are weak carbapenemases that do not hydrolyse cephalosporins in vitro, and also have a weaker carbapenemase-hydrolyzing effect[53].

Table 1 shows the Giske classification and the Ambler classification with the inhibitory substances used for phenotypical detection in the laboratory and the antibiotical classes affected.

Class ESBL	Ambler class	Enzymes	Phenotypic test	Resistance phenotype
$ESBL_A$	A	CTX-M, TEM, SHV	Inhibited by clavulanic acid	Penicillins Cephalosporins
$ESBL_M$	C	pAMPC mostly CMY	Inhibited by cloxacillin	Penicillins Cephalosporins
$ESBL_{CARBA}$	A	KPC	Synergy with boronic acid	Penicillins Cephalosporins, Carbapenems
	B	NDM, IMP, VIM	Synergy with EDTA	Penicillins Cephalosporins, Carbapenems
	D	OXA-48	No available	Penicillins Carbapenems

Table 1.*Classification of the most common ESBL-enzymes. Modified from Giske et al.*[52]

1.3.3 Transmission of antimicrobial resistance

As previously described, selective antibiotic pressure can result in disturbance of the microbial ecology leading to an increased risk of transfer of resistance genes to new hosts. The human gut with kilograms of bacteria is a favourable environment for this[54]. Also, pollutants, biocides and heavy metals in the environment have an important role for the spread of AMR genes and multi-drug resistant (MDR) bacteria[55].

Resistance genes spread among isolates either by transfer and incorporation of new genetic material via horizontal gene transfer (HGT), or vertically, through mutations in the bacterial genome. HGT can occur by three principal ways: ***transformation,*** where bacteria, often in response to changed environmental conditions, can take up extracellular DNA from a deteriorated cell from the environment and incorporate it, ***transduction*****,** where DNA, while replicating in the donor cell, is incorporated into bacterial phages, viruses that package segments of DNA in their capsid and then inject it into a new host, and ***conjugation*****,** where DNA, usually in the form of a plasmid, is exchanged between bacteria through cell-to-cell interaction via a pilus[56] (Figure 6).

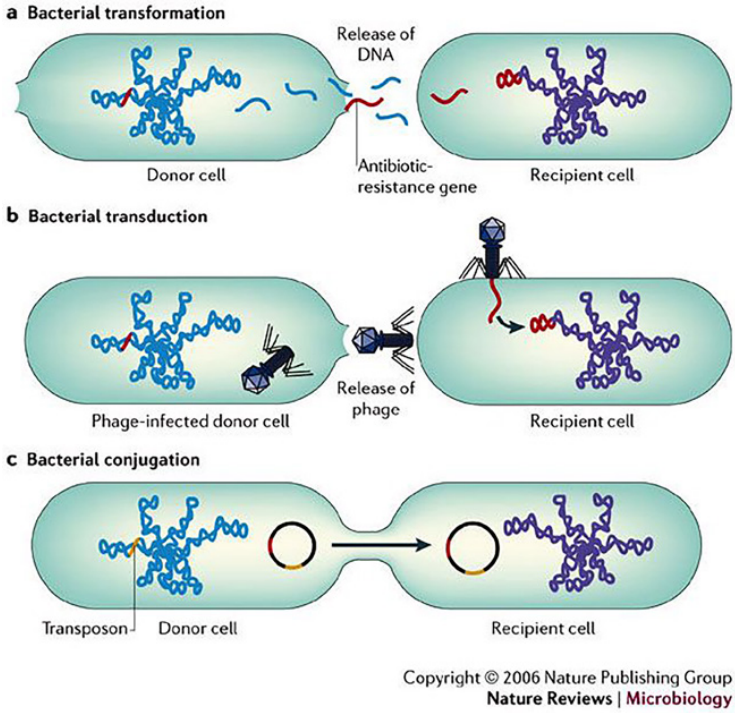

Figure 6. Bacterial transformation (a), transduction (b) and conjugation (c). Reprinted from Furuya et al.[57] with permission from Springer Nature.

Besides the long-term overuse of antibiotics in human healthcare, the dissemination of antibiotic resistance is caused by complex interactions involving several ecological niches[58]. High antibiotic consumption in combination with lack of basic hygiene routines facilitate transmission of AMR in human health care settings. Underdeveloped infrastructure with poor sanitation, overcrowding and limited access to clean water facilitate fecal-oral transmission of resistant gut bacteria (Figure 7). Insufficient sewage treatment contribute to the influx of AMR genes into natural bacterial ecosystems. CTX-M genes have been found in freshwater as well as urban and hospital wastewater allover the world[46,59]. Migration and travelling also strongly contribute to the dissemination of MDR bacteria. Fecal colonization rates up to 75% have been shown in returning travelers from countries with high community carriage rates of EPE[60].

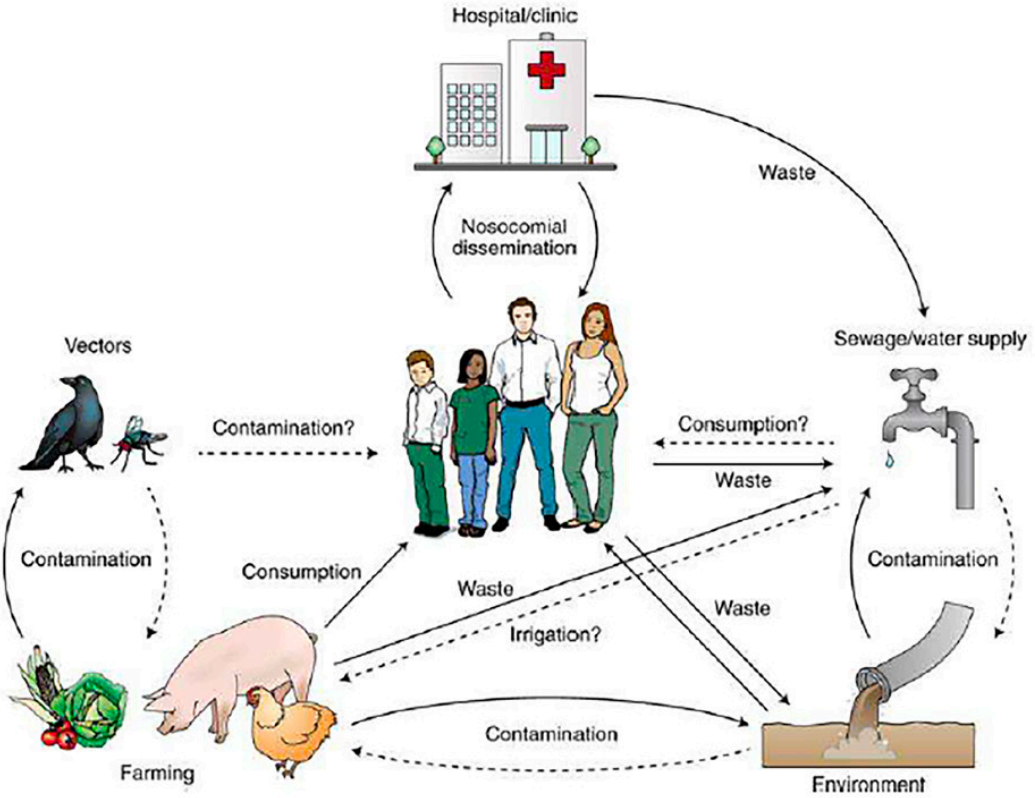

Figure 7. Routes of dissemination of MDR Enterobacteriaceae. Reprinted from Walsh et al.[61] with permission from Springer Nature.

In addition to misuse in human health care, antibiotics are still widely used for growth-promoting purposes in the animal and the agricultural industry[58]. The transmission of EPE via food has been reported[62]. Although antibiotics for growth promotion has been forbidden in Sweden and the EU since many years, globally more antibiotics are used for healthy animals than for sick people. It is estimated that the consumption of antibiotics in livestock only will increase over the next decade, particularly in low- and middle-income countries[63]. During 2018 the EU has updated its legislation on how antibiotics may be used for animals, including a ban on the preventive use of antibiotics in groups of animals and obligations to report antibiotic use to veterinarians[64].

1.3.4 Problems associated with antibiotic resistance

In the WHO Global Action Plan of 2015, the importance of a multisectoral One Health approach recognizing the interconnection between human health, animal health and the environment is emphazised, and this is in agreement with other national and international strategies[65-68]. Poor governance (e.g. corruption and political instability) has been shown to be associated with increased antimicrobial resistance as well as a low healthcare expenditure[69].
The spread of antibiotic-resistant organisms is related to increased healthcare costs[70]. Based on surveillance data Cassini et al. have estimated the burden of disease of infections with antibiotic-resistant bacteria in the EU and European Economic Area in 2015. *E. coli* resistant to third-generation cephalosporins was estimated to cause almost 300 000 infections and 10 000 attributable deaths in 2015[71]. Compared to southern Europe, Sweden still has a favourable resistance situation, but The Public Health Agency of Sweden estimates the direct health-care-associated and indirect costs for society of antibiotic resistance to 400 million SEK in 2030 and 600 million SEK in 2050[72]. From Europe, the British O'Neill report from 2014 estimates that in 2050, 10 million people will die from severe infections from antibiotic-resistant organisms if the development continues[73]. Moreover, AMR heavily affects international trade, especially livestock production in low-income countries[74].

2 AIMS

2.1 General aim

The overall aim of this thesis was to study the epidemiology of subsequent infection in patients positive in a fecal screen culture or a clinical culture with EPE, and to investigate bacterial strain factors, including the transmission of ESBL-carrying plasmids, influencing recurrences of EPE infection.

2.2 Specific aims of included studies

Paper I: To investigate the frequency of, and time to, subsequent infection with ESBL-*E. coli*/*K. pneumoniae* in an unselected cohort of patients previously positive for EPE in a fecal screen or clinical culture in Western Sweden

Paper II: To investigate the frequency of a change of species, and possible transfer of ESBL-carrying plasmids, between subsequent isolates in patients with recurrent infection with ESBL-*E. coli*/*K. pneumoniae* using Optical DNA Mapping

Paper III: To determine phylogroups, ESBL-genes and strain types of ESBL-producing *E. coli* in recurrent UTI with focus on the clone ST131-O25b and its subclone *fimH*30Rx

Paper IV: To compare phylogroups, ESBL-genes and the frequency of the clone ST131-O25b and its subclone *fimH*30Rx in sporadic and recurrent UTI with ESBL-*E. coli*

3 STUDY POPULATION

3.1 Setting of the studies

The studies were carried out in the region of Västra Götaland, Western Sweden. The population augmented from ~1,5 million inhabitants in 2004 to ~1,7 million inhabitants in 2019 of whom just over 1 million presently live in the greater Gothenburg area. The region comprises one 2000-bed university hospital (Sahlgrenska University Hospital), 3 tertiary hospitals (with altogether approximately 2000 beds), 13 minor hospitals and ~200 outpatient clinics. In the region there are four clinical microbiological laboratories, i.e. Sahlgrenska University Hospital (SU), Skaraborg Hospital (SkAS), Södra Älvsborgs Hospital (SÄS) and the NU hospital group (NU).

3.2 Patients and bacterial isolates

In **paper I** the study population consisted of all patients (n=3272) from the in- and outpatient setting in the greater Gothenburg area positive for EPE in a fecal screen culture or a clinical culture between 2004-2014. The number of fecal screen cultures, urine cultures and blood cultures analyzed at SU augmented from respectively 3000-20000, 65000-76000 and 25000-44000 during this study period. In **paper II**, the study population was patients with recurrent EPE- infection (n=513) from the cohort in **paper I**. **Paper III** included all patients with recurrent ESBL-*E. coli* UTI and available urinary isolates (n=123) in the greater Gothenburg area 2004-2014. In **paper IV**, urinary isolates from patients with recurrent (n=68) and sporadic ESBL-*E. coli* UTI (n=235) were prospectively collected from the region's four clinical microbiology laboratories (altogether analyzing approximately 180000 urinary samples/year) between 1/10 2017 to 1/10 2018.

All studies have been approved by the Regional Ethical Review Board in Gothenburg, Sweden (recordals 170-17 and 673-11).

3.3 Inclusion criteria

Patients with a positive fecal screen (n=1436) or clinical culture (blood or urine, n=1836) for ESBL-*E. coli* or ESBL-*K. pneumoniae* were included in

paper I. All subsequent clinical cultures (blood, urine) were identified with focus on the first and last subsequent culture for each patient. Multiple cultures within ≤ 7 days were considered the same infection episode. The follow-up time was at least one year. For **paper II** patients with clinical cultures (blood, urine) indicating reinfection were included. Patients with a shift of species between the episodes (from ESBL-*E. coli* to ESBL-*K. pneumoniae* or vice versa, n=14) were selected and their isolates were further analyzed.

Paper III included patients with multiple ESBL-*E. coli* urinary cultures indicating recurrent UTI. For this study ≥30 days between the episodes was required. The clinical definition of recurrent UTI, ≥2 UTIs during the past 6 months or ≥3 UTIs during the past year was used. A follow-up period of at least one year was required.

In **paper IV** patients with a new positive ESBL-*E. coli* urine culture within 6 months (≥30 days between the episodes) of the index culture were considered having a RUTI and were compared to patients with sporadic UTI. Patients in the sporadic group had no additional positive urinary EPE- culture during a follow-up time of one year after the index culture.

For all studies in the thesis a clinical urine culture was considered a culture with EPE ≥100 000 cfu/mL. Patients of all age groups were included in **paper I, II** and **III**. **In paper IV**, only patients ≥15 years were included. In all four papers only patients with no previous history of EPE-positive cultures (screen or clinical) were included.

4 METHODS

In the below section an overview of the methods used in the thesis is provided. Details of the methods, and the statistical analyses, can be found in the respective papers.

4.1 Databases

Data for **paper I-III** was extracted from the database at Clinical Microbiology, SU. Data for **paper IV** was extracted from the databases of all four laboratories in the region. The databases all contain information about age, gender, sample material and date, culture results including detected bacteria and antibiotic susceptibility (S/I/R) and voluntary referral data. The information was further processed and analyzed in Microsoft Excel.

4.2 Detection of EPE

Clinical samples were cultured and isolates were identified according to routine practice in each laboratory. In **paper I-III** standard biochemical analyses were generally used for species identification. In recent years MALDI-TOF (matrix-associated laser desorption/ionization) has complemented and often replaced traditional biochemical methods as the routine species identification method in all laboratories of the Region Västra Götaland. Antibiotic Sensitivity Testing (AST) was in the beginning of 2000 performed using disk diffusion and/or gradient tests according to the recommendations of the Swedish Reference Group for Antibiotics (SRGA) and later on to those of EUCAST[75].

To detect EPE, the phenotypic double-disc diffusion test (DDT) was used for cephalosporin-resistant isolates. The DDT test (front page) is based on growth inhibition of cephalosporin-resistant isolates by clavulanic acid in the presence of different cephalosporins[76]. The test is considered positive when the inhibition zone around any of the cephalosporin discs are enlarged towards the disc containing clavulanic acid.
For fecal screen samples bacteria were grown on selective media in the presence of a cefuroxime (30 ug) and a ertapenem disc (10 ug). Colonies within, or on the edge of the inhibition zone of different morphological

appearance were tested for EPE with DDT. Species identification was carried out as for clinical samples.

4.3 PCR-analyses and sequencing

PCR (polymerase-chain reaction)-analyses are widely used methods for detection of known bacterial genes and DNA sequences. In this thesis PCR-analyses were used for detection of genes determining phylogroups, ESBL-enzymes and presence of the ST131-O25b clone and its subclone *fimH*30Rx (**paper II- IV**) and for plasmid replicon typing (**paper II**).

To determine *E. coli* phylogroups, the quadruplex-PCR described by Clermont et al.[77], dividing *E. coli* into seven phylogroups (A, B1, B2, C, D, E and F), and *E. coli* cryptic clade I was used. A duplex PCR, detecting polymorphisms in the *pabB* gene was used to detect the ST131-O25b clone[78]. The subclone *fimH*30Rx was detected with the *H*30Rx-PCR described by Banarjee et al[79].

ESBL-enzymes were detected in a stepwise fashion, initially the CTX-M groups (CTX-M group 1, CTX-M group 2 and CTX-M group 9) were assessed by the multiplex-PCR described by Birkett et al.[80]. PCR-typing of CTX-M groups were followed by sequence analysis to assess the exact CTX-M gene in **paper II**. CTX-M genes were amplified by PCR, using primers and protocols published previously[81,82]. For CTX-M negative isolates, a multiplex-PCR differentiating between TEM, SHV and OXA enzymes was used [83].

4.4 PFGE (Pulse-Field Electrophoresis)

PFGE was used for strain typing in **paper II** and **III**. PFGE is a highly discriminating method and has, until the emerge of WGS, been the gold standard for epidemiological typing of strain relatedness. The technique is based on enzymatic cleaving of the bacterial DNA and separating them in an agarose gel matrix by pulse- field electrophoresis. This renders a PFGE pattern that can be compared with that of other strains and that can be analyzed both manually and by computer programs. By a similarity index,

based on band differences, the bacterial isolates' relatedness can then be determined[84].

4.5 MLST (Multi-Locus Sequence Typing)

MLST is based on sequencing 450-500 base pairs from seven known "housekeeping genes". These sequences constitute different alleles, giving an isolate an allelic profile. By MLST schemes based on known allelic sequences the isolate can be assigned to a specific sequence type (ST). The Warwick scheme for *E. coli* typing (https://enterobase.warwick.ac.uk/) and the Pasteur scheme for *K. pneumoniae* typing (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) was used in **paper II**.

4.6 Plasmid typing methods

In **paper II**, CTX-M carrying plasmids were studied with different techniques as outlined in section 5.2.

5 RESULTS AND DISCUSSION

5.1 Fecal carriage of EPE

5.1.1 The human gastrointestinal microbiota

The large intestine harbors a population of over 1000 bacterial species, part of the commensal microbiota, established rapidly during and following birth[85]. The first colonizers are facultative aerobe bacteria gradually being replaced by anaerobes (e.g. *Bacteroides spp., Bifidobacterium spp., Peptostreptococcus spp.)* with smaller numbers of aerobic bacteria and potential pathogens like *Clostridioides difficile*, *Enterococcus spp.* and Enterobacteriaceae. The anaerobic bacteria protect against colonization and overgrowth of potential pathogens in several ways; by a direct barrier function; by competing for nutrients; by changing pH and by activating the immune system. The microbiota also takes part in the metabolism of carbohydrates and proteins[86,87]. Disruption of the normal microbiota, the ”colonization resistance” can result in overgrowth of pathogens[88]. An example of this is diarrhea caused by *Clostridioides difficile* under antibiotic treatment.

5.1.2 Prevalence of EPE-carriage

Fecal carriage of EPE is prevalent worldwide (Figure 8). Since fecal carriage is not generally surveilled on a global basis available prevalence rates vary according to the epidemiological setting, time period and geographical region from which data are reported. A systematic review from 2016 estimated the worldwide pooled prevalence of EPE colonization among healthy individuals to 14% with an annual increase of 5,4% between 1991 and 2014[89]. The highest colonization rates have been reported from countries with underdeveloped infrastructure, healthcare and sanitation. In some parts of the world (Asia and Africa) the prevalence can be up to 60%[90-92]. In Europe the rate of fecal carriage is lower[89,93]. Ny et al. studied the prevalence in six European countries including Russia 2015-2017 and found a large variation in prevalence, from 1,6% in Latvia to 23% in Russia[94]. A large cross-sectional population study in Germany found 6,3% to be carriers[95]. In the Netherlands, a prevalence of EPE-carriage of 4,5% in a live-stock dense area,

a prevalence of 8,6% in the general population in Amsterdam and 10% in patients seeking medical care for gastrointestinal symptoms was reported[96-98]. Sweden is, similar to the Netherlands, considered a low-endemic setting. The prevalence of EPE-carriage in healthy patients is however not well-studied. A study of Swedish patients seeking primary health care, published in 2011, reported a colonization rate of 2-3%[99]. In the study of Ny et al., the prevalence in Sweden was 6,6% 2015-2017, an augmentation compared to a study from the same author of 2134 healthy persons 2012-2013 reporting a carriage rate of 4,7%[7,94].

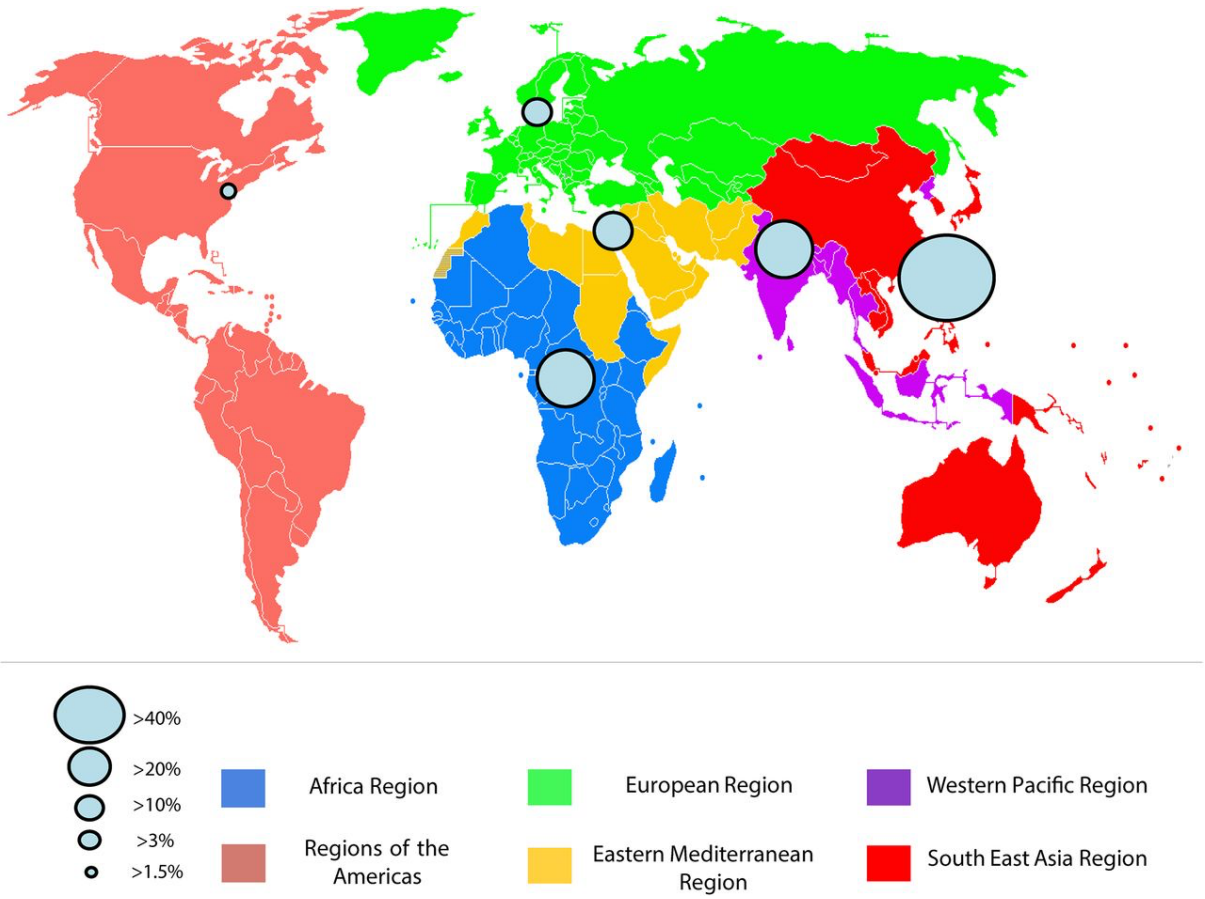

Figure 8. Pooled prevalence of fecal colonization with ESBL-producing organisms. Circle represents the colonization rates. Reprinted from Karanika et al.[89] with permission from Oxford University Press.

5.1.3 Risk factors and duration of EPE-carriage

Humans become EPE carriers through ingestion of contaminated food and water as well as through insufficient hygiene measures both in health-care settings and the community. In most cases, the carrier remains asymptomatic and unaware of the carriership, but EPE carriers could potentially contribute to the spread of EPE by being a natural reservoir for ESBL genes in the community[62,93,100].

Several studies confirm that travel to endemic areas is a risk factor for EPE-colonization, at least in the short-time perspective, with high rates in travelers returning from Southeast Asia, Africa and the Indian subcontinent[60,101].The highest rates were found in patients with travelers diarrhea and patients that had been taking antibiotics during the trip. On the other hand, the duration of the carriership in travelers is generally short, colonizing strains seem less virulent, and follow-up studies have shown a low risk of subsequent infection[102,103]. In Ostholm-Balkheds study from 2018[104] only 11% of travellers who acquired ESBL-PE during their travel abroad had sustained colonisation 12 months after return which is a rate similar to that reported by Arcilla et al. in a large prospective study of 2000 travelers[60].

The role of the commensal gut flora in the risk of prolonged colonization is evident as antimicrobial use also is a well-defined risk factor in many studies[89]. Since the commensal microbiota is first established via transmission from the mother's vaginal and intestinal flora, newborns to mothers that are EPE-carriers are at particularly high risk becoming EPE-colonized[105]. Infants colonized after a nosocomial outbreaks seem to be at risk of longer colonization[106,107].

It is currently difficult to declare a patient free from colonization and patients can after culture-negative fecal samples suddenly present a positive culture[108,109]. After hospital colonization the mean carriage duration has been reported to be 6 months, but is dependent of the patients comorbidity[93]. Immobilisation has been linked to prolonged carriage with EPE[109]. The risk of prolonged colonization is also higher in patients with a history of EPE-infection[108,110]. Titelman et al studied the duration of ESBL-carriage in 61 patients at 1, 3, 6 and 12 months after EPE infection. They found that negative samples did not mean eliminated carriage and that prolonged carriage was associated to bacterial strain factors[108].

5.1.4 Measures to prevent the spread of EPE in the in- and outpatient setting

From the infection control perspective, there is a broad consensus on screening patients referred from hospitalization abroad for MDR carriage such as meticillin-resistant *Staphylococcus aureus* (MRSA) and carbapenem-resistant Enterobacteriaceae (CPE) [111,112]. The benefit of contact precautions for specific patient populations, settings and pathogens is high and screening programs are of importance in the hospital setting, although guidelines vary between countries[100,113,114]. The Swedish recommendations state that patients who have received health care abroad (or in settings with ongoing outbreaks) during the past 6 months are screened for MDR bacteria including EPE upon hospital admission[115]. This particular recommendation does not consider patient-related risk factors for spread in the hospital setting (e.g. diarrhea, presence of drains or catheters or open wounds) or patients at risk of developing infections.

Screening recommendations are generally based on experiences from outbreak situations, and in non-outbreak situations the cost-effectiveness of such measures have been questioned. High-quality evidence is often lacking and contact precautions must be weighed against increased costs and patient safety[113]. Screening large cohorts of previously healthy individuals with a recent visit abroad that are unlikely to develop disease, even when hospitalized, may cause unnecessary anxiety both from the patient's and doctor's perspective. Secondly, today little effort is made to detect patients with no history of recent travel or hospitalization abroad, that are indeed likely to develop disease with EPE, for instance patients with urological risk factors or a history of RUTI. With increasing spread of EPE in the community, in particular ESBL- *E. coli*, this group of patients is likely to increase. Community reservoirs are now the main reservoir for ESBL-*E. coli*, and transmission in households seem to outweigh nosocomial transmission. It is well known that dissemination occurs among relatives and household members[116-118]. Transmission between humans, domestic animals and live-stock is also reported[62,119]. Thus, the present screening regimens can be questioned on clinical and ethical grounds and must be weighed against increased costs and patient safety in non-outbreak situations.

5.1.5 The link between EPE carriage and infection (**paper I**)

As infections with EPE are linked to potentially lethal treatment failures and increased mortality[8,120], it is important to identify reliable risk factors to predict these infections. Presence of indwelling devices (especially urinary tract catheters), previous hospitalization and recidency in long-term facilities, as well as comorbidities and old age are recognized risk factors for EPE-bloodstream infections (BSI)[121]. Recent antibiotic therapy, especially fluoroquinolones and beta-lactams are also linked to EPE-BSI, but in this sense, comparing EPE-BSI with a certain species to a control group with non-EPE-BSI with the same species which many studies have done, might lead to an overestimation of previous antimicrobial use[121].

Studies of the impact of fecal colonization on subsequent infection are often case-control studies focusing on special patient groups like patients with bloodstream infection (BSI) or ICU-patients. In a study of 126 patients with newly diagnosed hematological malignancy, Cornero-Juarez found that being ESBL-positive in feces increased the risk for a subsequent ESBL-bacteremia with the same strain nearly 3,5 times[122]. Translocation of colonizing bacteria from the gastrointestinal tract is indeed a source of infection in this patient group. However, results are divergent, and in a similar patient group (neutropenic patients with acute myeloic leukemia or undergoing stem cell transplantation), Arnan et al did not show any association between ESBL-*E. coli* carriage and risk of ESBL-*E. coli* bacteremia[123]. In a study from Southern Sweden, Van Aken et al. studied patients with EPE-bacteremia. A prior positive EPE-culture was an independent predictor for EPE-bacteremia, but the source of the positive culture and the time to the bacteremia was not described. Also, patients were old, median 71 years, with high comorbidity[124].

In intensive care units (ICUs), colonization with EPE is associated with high frequencies of EPE-infection, occuring in up to 25% of EPE carriers in this vulnerable patient population[125,126]. Another well-known group at risk for EPE-infection are patients undergoing prostate biopsy. Due to the widespread use of fluoroquinolones for prophylactic purposes the risk of sepsis with EPE following this diagnostic intervention is increasing. In this patient group fecal screening could be valuable to target antibiotic prophylaxis[127].

The impact of the carrier state on subsequent infection in a general patient population is less studied. Swedish guidelines state that empiric antibiotic treatment of patients with severe infections and a previous positive fecal screen should include coverage of the colonizing EPE strain (https://strama.se/). This means that the carrier state potentially could predispose for an overuse of the carbapenems with no clinical benefit.

In **paper I,** 1436 patients positive for EPE in a fecal screen between 2004-2014 in the Gothenburg area, with no history of EPE in a previous screen or clinical culture were followed for at least one year (median follow-up time 3,7 years). Laboratory data for these patients showed that 94% of the patients had no subsequent positive clinical culture at all (Figure 9). These results clearly indicate that subsequent infection with ESBL-*E. coli/ K. pneumoniae* is low, even for this cohort of patients, screened at hospital admission. Only 10 patients (0,7%) had a following positive blood culture.

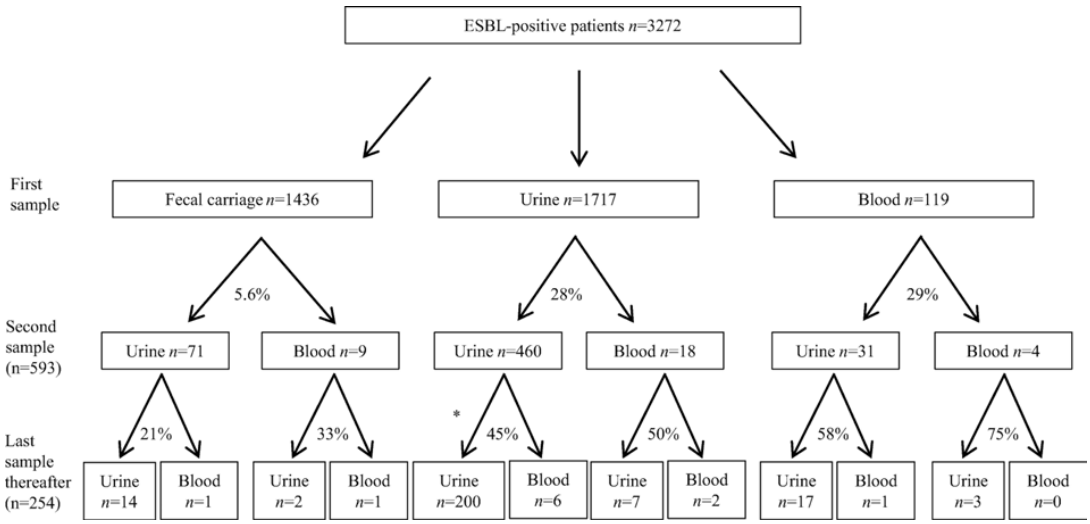

*Figure 9. Flow chart depicting subsequent clinical samples in 3272 patients with initial positive EPE fecal colonization (n=1436), an EPE- positive urine culture (n=1717) or a positive blood culture (n=119). Reprinted from **paper I** with permission from Springer Nature.*

However, subsequent positive cultures were more common in elderly patients than in young patients and in patients previously positive in a fecal screen for

ESBL-*K. pneumoniae* as compared to ESBL-*E. coli* (Table 1, **paper I**). This result could possibly reflect a higher comorbidity in this patient group. In the 80 patients with a clinical culture following a positive fecal carriage, 23% (18 patients) had additional clinical cultures. This frequency approached the frequencies of subsequent samples in patients with initial clinical samples (28%) (Figure 9). Compared to the group with initial positive clinical samples, patients in the group with fecal carriage were younger.

In a recent study from Stockholm of patients with community-onset EPE-BSI aiming to identify predictors for EPE-BSI, the strongest predictor was an EPE-positive urine culture <3 months earlier[128]. A Stockholm score, based on three variables (any positive EPE-positive culture, prostate biopsy ≤ 30 days and prior healthcare abroad) was proposed, with a sensitivity of 50%, a specificity of 96% and a positive predicitive value of 6,2%. In that study, only 4% of the EPE-BSI cases were colonized in feces. Similar scoring systems have been proposed, often with high specificity but similar low sensitivity[129,130], reflecting that the present screening regimens primarily screening patients hospitalized abroad without considering risk factors for EPE-infection do not safely identify patients at risk.

In conclusion, the results in **paper I**, in accordance with previous studies in low-endemic settings[131,132], show a low risk of subsequent infection in the general EPE-colonised patient. These findings indicate that a previous positive fecal screen should not be considered the only reason to choose a certain empirical therapy. The high frequency of subsequent infection in patients with a previous clinical culture (**I**), as well as in certain risk populations in previous publications, indicate that screening regimens in the future should be designed to target patient groups at risk for EPE-infection.

5.2 Transfer of ESBL-carrying plasmids in sequential EPE infections

Bacteria carry their genetic material- their DNA- either in their chromosome or in the form of plasmids. Plasmids are extrachromosomal, small circular double-stranded DNA-molecules, capable of independent replication, that often carry genes that provide a selective advantage for bacteria, including AMR genes, genes that protect them from heavy metals and genes that can

modify nutritional needs. In exchange they take advantage of the host cells replication machinery[133]. Plasmids have a tremendous capability of changing their composition by amplifying advantageous genes and deleting disadvantegous genes. They have developed ways to secure their persistence with addiction systems, e.g. toxin-anti-toxin systems that eliminate cells that have lost their plasmid after cell division[9,134].

5.2.1 Horizontal gene transfer of plasmids and their resistance genes

Horizontal gene transfer (HGT) of resistance genes located on plasmids by conjugation is the most important way by which ESBL-encoding resistance genes are spread among Gram-negative bacteria[9]. As previously described, resistance genes can be found in environmental bacteria, and under favorable circumstances they can be mobilized to plasmids. Conjugation, the transfer of genes from one bacterial cell to another by mechanical contact, occurs via pili. Pili are rod-shaped polymeric proteins creating a channel, connecting the cytoplasms through which a copy of the plasmid is transferred (Figure 6). When resistance genes are transmitted into virulent clones in combination with antibiotic selection pressure the conditions for dissemination of antibiotic resistance are optimal[9,135].

Flanking resistance genes in the plasmid's genome are other mobile genetic elements (MGEs) that efficiently can move genes within the plasmid and from the plasmid to the chromosome[136]. There are three principal recombination systems; insertion sequence elements (IS-elements), transposons, and integrons. IS-elements are DNA segments containing a single open reading frame and a transposase gene (encoding the enzyme responsible for transposition). The IS-elements move themselves to a new location by cut-copy and paste mechanisms. IS*Ecp1* is the IS-element mostly associated with the capture and mobilization of CTX-M genotypes[45]. When two IS elements capture a resistance gene inbetween them, they form a transposon. Integrons use site-specific recombination to move resistance genes and capture mobile gene cassettes consisting of a gene and a recombination site. This process is initiated by an integrase, and the integron also provides a promoter for the expression of the captured cassette genes. It is mainly the class 1 integrons that are associated with the acquisition and mobilization of resistance genes in Gram-negative bacteria[136].

Conjugative plasmids can be divided into narrow-range plasmids and broad-range plasmids. This reflects the plasmid's specialization. Narrow-range plasmids prefer certain species or even certain clones within a species, while broad-host range plasmids can be transferred between different species. Plasmids of different incompatibility groups (Inc-groups) differ in their bacterial host ranges. The narrow-host range plasmids IncF are the most common encountered in *E. coli*. Examples of broad-host range plasmids that can be found in a variety of bacterial species are are IncA/C, Inc L/M and IncN-groups[134].

5.2.2 CTX-M carrying plasmids in *E. coli*

A variety of plasmids of different Inc-groups can carry CTX-M-genes, but the most common plasmid type carrying CTX-M-genes in ESBL-producing *E. coli* is the narrow-range plasmid IncF. IncF can carry multiple replicons alone or in combination, for instance FIA, FIB and FII. By sequencing, the FII plasmids can be further divided into subgroups (e.g., F1 and F2). IncF-plasmids with FIA-FII or FII is strongly associated with the spread of CTX-M-15 in *E. coli* ST131. The IncF-plasmids have also been shown to carry CTX-M-27 and carbapenemase genes. They are low copy-plasmids, >100kb in size that have high conjugative ability and use toxin-antitoxinsystems to firmly secure their persistence[9,137]. Other plasmidtypes that can carry CTX-M-genes are IncI1 (CTX-M-1), IncN (CTX-M-1), IncK (CTX-M-14) and IncL/M (CTX-M-3)[9].

5.2.3 Methods for plasmid detection

There are several methods for plasmid detection all with their own advantages and limitations. Several older methods are laborious and time-consuming, as for instance plasmid-PFGE[138] and are nowadays being exchanged by whole-genome sequencing (WGS) with next-generation (NGS)-techniques[139]. Using WGS it is possible to map the whole nucleotide sequence of the plasmid. Plasmid detection via WGS is complicated because of the large copy numbers compared to the chromosomal sequences of bacteria and the dynamics and frequent recombination events. Plasmids are typically scattered with repetitive elements, such as IS-elements, which make short-read approaches (<1000 bp) less suitable[137]. Long read technologies are becoming increasingly available, but the assembly of reads is still time-

consuming and requires bioinformatic skills and experience. Often a combination of short and long read technologies gives the best results. Plasmid MLST (pMLST) is a simplified sequence technique based on differences in a few gene segments that can be used for subtyping of plasmids. Plasmid genes are compared to pMLST protocols, available for the most common Inc-groups, including Inc I1, IncN and IncF-groups[140].
The methods used for plasmidtyping in **paper II** are described in more detail below.

5.2.3.1 Replicontyping

A replicon is a highly conserved part of the plasmid where genes controlling the plasmids replication are situated. By PCR-based replicon typing, plasmids can be classified into incompatibility groups (Inc-groups). The nomenclature is based on the fact that two plasmids using the same replication mechanisms are incompatible, i.e. unable to persist in the same bacteria[9,56]. Although plasmids can trespass this rule, and become compatible by small nucleotide substitutions, the nomenclature is still used. It is also important to remember that a plasmid can have several replicons, thus the number of replicons is not always proportional to the number of plasmids. The PCR- technique developed by Carattolli et al detects 18 replicons[141]. The method has been extensively used in studies exploring ESBL-plasmids and was used in **paper II**. There are now commercial kits that can detect 25 replicons, and also allows for subdivision of some of the Inc groups like IncF1[142].

5.2.3.2 Optical DNA Mapping

Optical DNA mapping, a plasmid typing technique developed at Chalmers University of Technology, Gothenburg, in recent years, is based on stretching plasmids in nanofluidic channels and visualising them in a fluoroscence microscope. By combining ODM with CRISPR/Cas9-assisted gene identification, the position of the CTX-M gene on the plasmid can be identified[143].

The method is illustrated in Figure 10. The guide RNA is designed to lead Cas9 to the location where the resistance gene is located and cleave it there. Plasmids are then stained with one fluorescent and one non-fluorescent dye. The two molecules- the fluorescent YOYO-1 and the non-fluorescent AT-

selective netropsin- competitively bind to different sites of the DNA. This results in an emission variation along the DNA that reflects the underlying sequence. In the fluoroscence microscopy, each plasmid renders an image with a barcode depicting the DNA sequence, which can be compared to barcodes in a reference database (NCBI). The data is further analyzed to detect the number of plasmids in each sample, their corresponding sizes and the potential presence of a resistance gene, enabling comparison of plasmids and plasmid content[144].

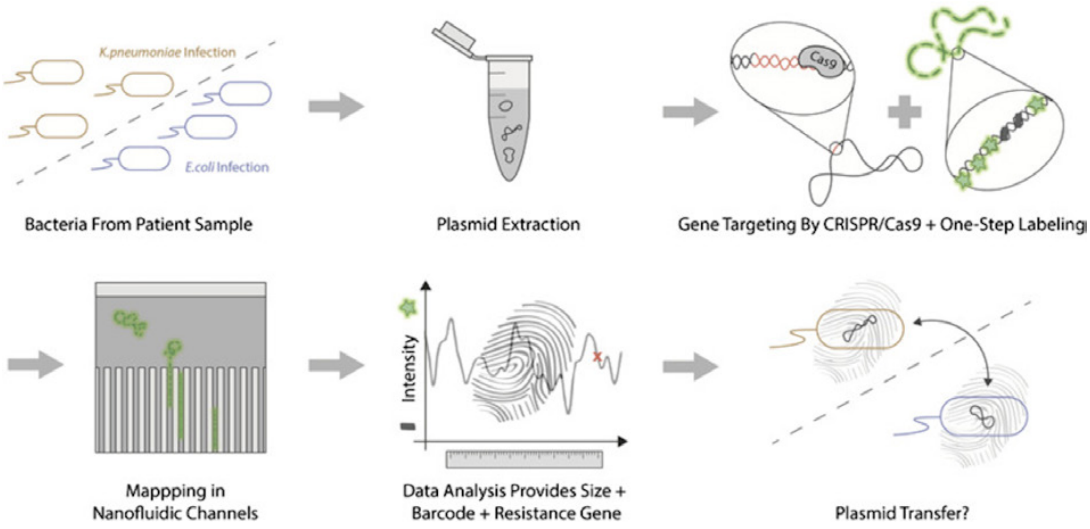

Figure 10. Overview of the Optical DNA Mapping (ODM)-technique. After plasmid extraction, plasmids and the ESBL-gene are targeted by Cas9. Plasmids are stained, stretched in nanofluidic channels and imaged using a fluoroscence microscope. This renders a fingerprint (barcode) depicting the DNA sequence and the position of the ESBL-gene. From the measurements the size of the plasmid can be determined. Reprinted from ***paper II*** *with permission from Elsevier.*

5.2.4 Transfer of resistance genes in patients with recurrent EPE-infection (**paper II**)

As the transfer of plasmids carrying resistance genes is a dynamic and frequent event ongoing in the gut microbiota[145], plasmid transfer of resistance genes between pathogenic bacteria might be involved in recurrent infection with EPE of different species. A worrying scenario in this perspective is when AMR genes move into bacterial clones with high transmissibility associated with more common infections[135]. Plasmid transfer of ESBL-genes among different species of Enterobacteriaceae has previously been described in vitro and in vivo[146-150] and has been described in several outbreak situations, not at least for carbapenem-producing isolates[151-153].

However, the importance of ESBL-plasmid transfer from a clinical perspective is less explored. Is it important to characterize the ESBL-carrying plasmid in EPE-isolates from a previous infection, thereby assessing the risk of a new EPE-infection? **Paper II** was a study attempting to investigate this question. We began by investigating how common it is with a shift of species in subsequent EPE-infections. The study was based on the 513 patients with recurrent EPE-positive samples from the cohort in **paper I**. In 14 of these patients a change of species between ESBL- *E. coli* and *K. pneumoniae* or *vice versa* in sequential infections was noted. Isolates from five of these patients (2-3 sequential isolates/patient) were available for more detailed studies of possible plasmid transfer. The isolates represented both common and rare STs (table 1, **paper II**). We also evaluated a sixth patient with an initial fecal screen positive for *K. pneumoniae* followed by an urinary *E. coli* isolate.

We used ODM in comparison with PCR-based replicon typing for comparison of plasmids in sequential isolates. The ODM-results allowed us to compare the number of plasmids in each isolate, sizes and barcodes of each plasmid and the location of the CTX-M gene on the plasmid. An example of the results is provided in Figure 11.

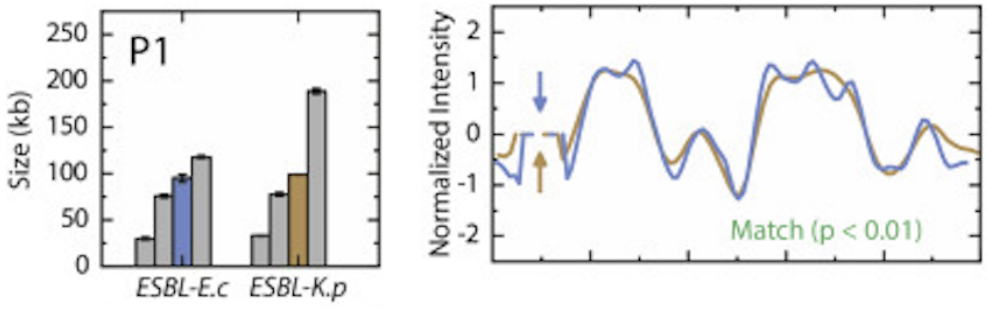

*Figure 11. Comparison of plasmids from a patient (P1) with an initial urinary ESBL-E. coli-isolate and a subsequent urinary ESBL-K. pneumoniae-isolate with Optical DNA mapping (ODM). ODM shows that two plasmids have the same size and position of the CTX-M gene. Reprinted from **paper II** with permission from Elsevier.*

According to sequence data, four of five patients with sequential clinical isolates harbored the same CTX-M-gene. Replicon typing showed similar replicon types in three out of these five patients. In three patients our ODM results showed possible plasmid transfer as the subsequent isolates carried identical or partly identical plasmids by barcode analyses. The results discussed below are illustrated in figure 2, **paper II**.

In patient 1 (P1) the replicon types differed, but ODM identified two plasmids with similar sizes, overlapping barcodes and the same location of the CTX-M gene indicating possible plasmid transfer (Figure 11). In patient 4 and 5 (P4 and P5) the ODM analyses showed overlapping barcodes despite differing plasmid sizes. In these two patients the sequential plasmids were larger, indicating gain of DNA segments. The sequential isolates had the same CTX-M genes, location of CTX-M gene and replicon types. In P5 two sequential isolates were available. In this case ODM indicated duplicate plasmid transfer demonstrating the methods ability to study plasmid dynamics over time. In patient 2 and 3 (P2 and P3) no overlapping barcodes according to ODM were found, although in P2 the same CTX-M-gene, replicon type and similar plasmid sizes were found. P3 differed in replicon types, CTX-M genes and plasmid sizes. In P6 (the patient with an initial fecal screen), ODM showed identical plasmid content and location of the CTX-M-15 gene, despite different plasmid sizes.

In total, a change of species was noted in only 2,7% of the patients with recurrent infection and plasmid transfer could be verified in three out of five cases. From a clinical point of view knowledge of previous ESBL-plasmids thus seems of less importance. Although this study does not evaluate plasmid transfer among the same EPE species, data in **paper III** demonstrates high strain homology in ESBL-*E. coli* suggesting that plasmid transfer in these cases is rare. The findings are in line with the findings of Thingholm et al. demonstrating that CTX-M-15 rarely is transferred between *E. coli* isolates of differing strains and those of Ny et al. demonstrating highly conserved plasmids among *E. coli* ST131[154,155].

The study highlights the need of more advanced methods when studying plasmids, especially in the case of CTX-M carrying plasmids that are generally limited to a few replicon types, and the frequent recombination events of plasmids. ODM rendered valuable information of plasmid transfer that was easy to interpret. It has proved to be a valuable tool for tracing plasmids in previous studies of hospital outbreaks of EPE[106,156]. Also, previous studies have shown a good correlation with sequence data indicating that ODM is an easy alternative to WGS of plasmids[143].

5.3 Bacterial factors influencing recurrences in UTI caused by ESBL-*E. coli*

5.3.1 ExPEC virulence and pathogenesis

The *E. coli* genome consists of around 5000 genes of which about two-thirds is shared by all *E. coli* i.e; the core genome[157]. The remaining accessory genome is highly variable due to frequent acquisition and recombination events helping *E. coli* to adapt to new environments. As previously described, virulence genes can be present on plasmids, but they can also be located on so-called pathogenicity islands (PAIs). These are large unstable genomic regions inserted in the chromosome with clusters of virulence factor genes and MGEs[158].

Bacteria causing UTI originate from the normal fecal flora. Infection occurs when colonizing flora ascend from the periurethral area or the vagina up the urethra to the bladder, ureters and sometimes to the kidneys[18,159,160]. The

pathogenesis involves both host and bacterial factors. There is a great diversity in the repertoire of virulence factors (VFs) in ExPEC and no exact distinguishing subset of VFs has been associated with disease[10,161]. The pathogenesis of ExPEC has been attributed to two theories; the special pathogenicity theory, based on the assumption that ExPEC isolates are more virulent than fecal isolates, and the prevalence definition; based on the observation that ExPEC-isolates dominate over colonizing strains in the fecal flora prior to infection. The two theories are probably not mutually exclusive[18,162].

Examples of important and well-characterized VFs in *E. coli*, according to their functional category, are (also illustrated in Figure 12):

Adhesins: Adhesins facilitate colonization, invasion and induce cellular internalization. The most studied adhesion molecules are the type 1 fimbriae (with the tip-located adhesin FimH) adhering to uroplakins and integrins present on the superficial facet cells of the bladder, and the P-fimbriae (pyelonephritis-associated fimbriae), adhering to kidney epithelial cells. Isolates causing cystitis thus express type 1-fimbriae to a higher extent than isolates causing pyelonephritis that have a higher expression of P-fimbriae.

Toxins: Toxins expressed by ExPEC lyse and damage host cells, e.g. α-hemolysin and cytotoxic necrotizing factor, promoting cell invasion and release of iron and other nutrients.

Protectins: The synthesis of polysaccharide capsules help *E. coli* evade the immune response. Outer membrane proteins *TraT* and *Iss* interfere with complement.

Iron aquisition systems: Siderophore and heme-receptors allow bacterial survival in the iron-limited environment of the urinary tract.

Biofilm formation: Once internalized in the uroepithelium, UPEC can form intracellular bacterial communities, IBCs, with biofilm-like properties. Ag43 is an outer membrane protein that has been shown to be associated with cell aggregation and biofilm formation. In these IBCs, UPEC is hidden from the immune system and protected from antimicrobial treatment. In urine from women with cystitis, intracellular biofilm-like collections of bacteria have been found in exfoliated cells [13,163-166].

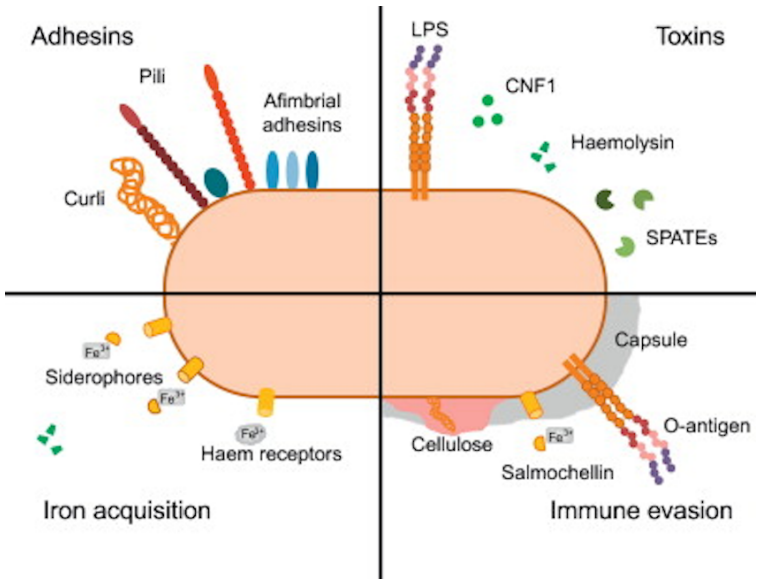

Figure 12. Virulence factors in UPEC. Reprinted from Lüthje et al.[167] with permission from Elsevier.

5.3.2 *E. coli* phylogroups

In 2000 Clermont et al described a phylogenetic approach to divide *E. coli* into phylogroups, i.e. genetically related groups sharing the same ecological niche and virulence properties[168]. From the beginning phylogenetic relationships were based on the multilocus enzyme electrophoresis (MLEE) method, later replaced by a PCR based on three genetic markers, *chuA*, *yjaA* and *TspE4*, initially dividing *E. coli* into four major groups; A1, B1, B2 and D[169].

With the growing knowledge of the *E. coli* genome by MLST it was subsequently expanded with an extra gene, *arpA*. This quadruplex PCR now subdivides *E. coli* into seven phylogroups: A, B1, B2, C, D, E, F and clade I (which is phenotypically similar to *E. coli*, but with small nucleotide differences)[77]. With WGS an additional phylogroup has lately been recognized, that is G. It is a group intermediate between phylogroups F and B2, rare in humans and linked to antimicrobial resistance[170]. Within a phylogroup there may be a number of different strains by MLST, whereas isolates within a ST often are of the same phylogroup and therefore correspondence schedules between MLST schemes and phylogroups have been elaborated [171].

Phylogenetic classification has been extensively compared to virulence, distribution among hosts and antibiotic resistance. Generally, ExPEC clinical isolates have previously been found to predominantly belong to phylogroup B2 and to a lesser extent to D and commensal, colonizing strains to phylogroup A and B1[172-174]. Also, in women with UTI, group B2 has been shown to outcompete other clones in the fecal flora[18]. Studies suggest that a majority of ESBL-*E. coli* B2 isolates are part of ST131[175]. Group D often includes antibiotic-resistant strains which is also true for A, albeit to a lesser extent. Group B1 have been considered non-pathogenic and non-resistant[173,176-178]. It is not unlikely that ESBL- *E. coli* show the same phylogroup distribution as non-resistant *E. coli.* In a study comparing fecal screen isolates from healthy individuals to isolates from patients with BSI, the fecal screen isolates to a greater extent belonged to phylogroup A and B1. The study showed that 53% of BSI-isolates were of phylogroup B2 (45% ST131), compared to 25% of carriers[7]. In a study of fecal colonization in healthy travelers by Vading et al. phylogroup A dominated[102].

5.3.3 ExPEC clones

A bacterial clone implicates bacterial isolates that share similar traits genotypically or phenotypically, belong to the same lineage and have a common ancestor. This definition is however not always straightforward and is dependent on the biochemical or molecular typing method used[134]. The most accurate way to study bacterial clones is nowadays by WGS, but MLST is traditionally a highly reproducible and comparable method when comparing *E. coli* on a global scale. A high-risk MDR clone, in addition to its pathogenic properties, has an international distribution, extended fitness, transmission, colonizing and pathogenic properties, as defined by Baquero et al in 2013[179]. MDR *E. coli* of ST131 fulfill all these criteria.

ExPEC is comprised of many lineages, but only a subset of these cause the majority of infections. One must keep in mind that studies of ST-lineages can be biased according to their preselection strategy. For instance, MDR strains are more likely to be tested and stored in clinical laboratories, and several studies include data on BSI-associated *E. coli* only. In a systematic review of 217 studies by Manges et al. from 2019 ST131 was detected in 91% of the studies. The other major STs were, listed by frequency, ST69, ST10, ST405,

ST38, ST95, ST648, ST73, ST410, ST393, ST354, ST12, ST127, ST167, ST58, ST617, ST88, ST23, ST117, and ST1193 [180].

E.coli ST131, described in the following section, is the dominant clone associated with ESBL-production[181]. Other *E.coli* lineages associated with ESBL are ST405, ST59, ST10 and ST23. ST410 with CTX-M-15 and ST1193 with CTX-M-14 and CTX-M-15 are emerging among ESBL-producing E. coli[182,183].

5.3.4 *E. coli* of sequence type 131 and its MDR subclones

In 2008 the CTX-M-15-producing *E. coli* clone ST131 was discovered. The clone emerged rapidly worldwide and is now one of the most successful MDR Gram-negative clones, today recognized as pandemic, spreading both in the community and health-care setting[184,185]. ST131 belongs to phylogroup B2, mostly to serotype O25:H4. It has a a high capability of causing extraintestinal infections, particularly UTI:s, and has shown to be overrepresented in sepsis[7,186]. It is estimated that 30% of all ExPEC, 60-90% of fluoroquinolone-resistant ExPEC, and 40-80% of ESBL-ExPEC belongs to ST131[186,187]. The successful emergence of ST131 has been attributed to several factors, including person to person transmission, traveling, high prevalence of carriage in livestock and domestic animals and the acquisition of resistance genes on easily transmissible plasmids[19,186,188]. ST131 has been shown to possess a great number of virulence genes and colonization abilities although the composition of virulence genes varies[79,189-191].

Longitudinal studies of ST131 with NGS-techniques have shown that ST131 belongs to three different clades; clade A, clade B and clade C. The three clades are associated to different FimH-alleles. Since the 2000s clade C has emerged and currently up to 80% of ST131 belong to clade C, which has three different subclades: C1 (*H*30R), C1-M27, and C2 (*H*30Rx). WGS studies have shown that clade C evolved from clade B in a stepwise fashion[192,193]. The reason for its success has been attributed to the acquisition of the type-1 fimbrie adhesion molecule *fimH*30 allele, important in colonization, and chromosomal point mutations in *gyr A* and *par C* conferring fluoroquinolone-resistance (Figure 13).

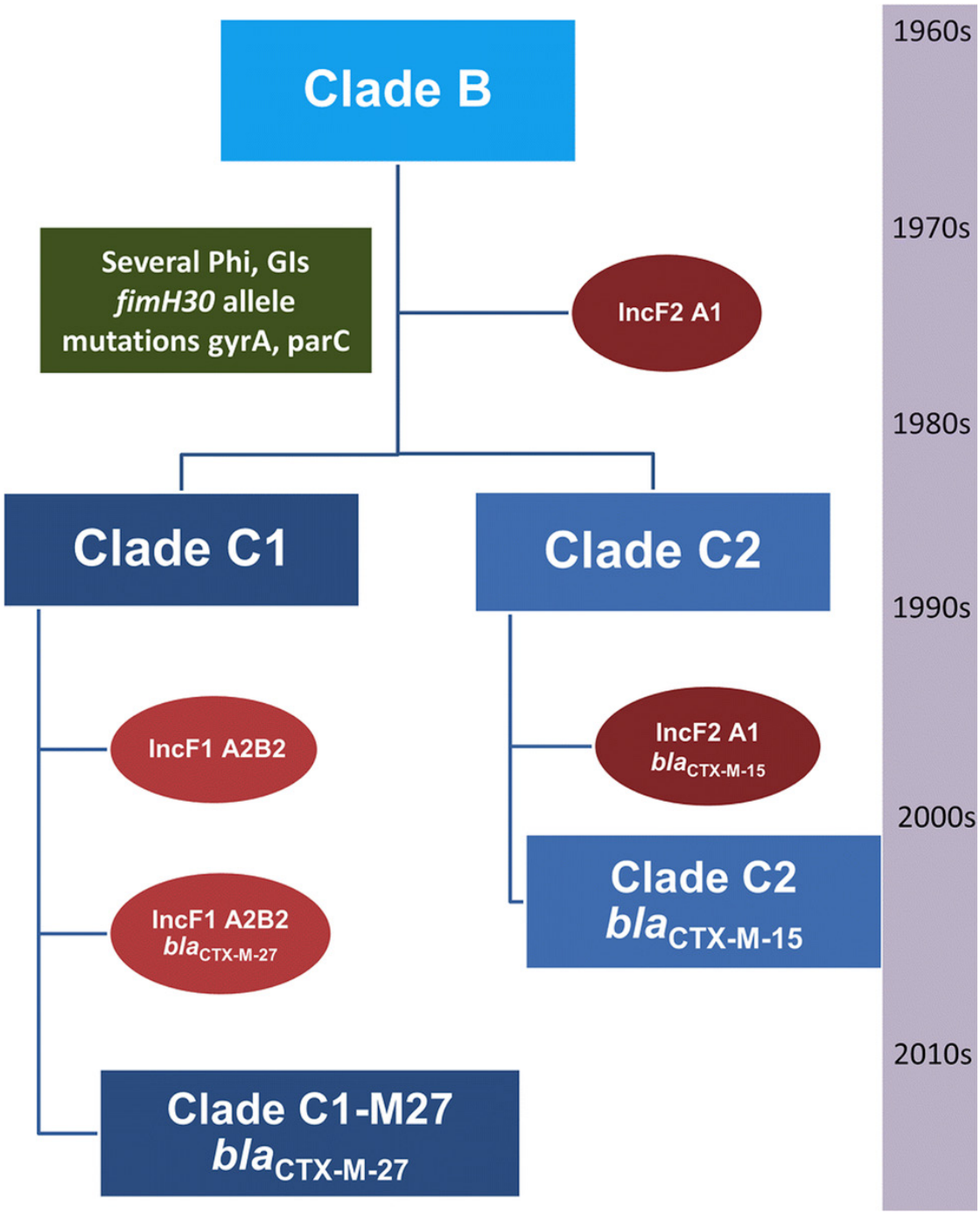

Figure 13. Evolution of E. coli subclades C1/H30R, C1-M27 and C2/H30Rx from clade B through the aquisition of prophages (Phis), genomic islands (GIs), mutations within gyrA and parC, the fimH30 allele and plasmids carrying CTX-M-genes. Reprinted from Peirano et al.[134] with permission from Springer Nature.

Within the C subclade different IncF plasmids were introduced. Clade C2 subsequently retained its IncFII plasmid, picked up CTX-M-15 and evolved into the subclone C2 (*H*30Rx), outcompeting other lineages[192]. Clade C1 later acquired CTX-M-14 and CTX-M-27. These ST131-H30R-isolates have also played a role in the clonal dissemination of CTX-M genes. In Japan, the subclade C1-M27, carrying CTX-M-27, has shown to be responsible for the increase in ESBL-producing *E. coli*, and it has since 2010 emerged also in Germany, France and other European countries[194-196].

5.3.5 Recurrent urinary tract infection

Recurrent UTI (RUTI) refers to a repeated occurrence of UTI within a certain timeframe. Glover at al concludes that various publications since the year 2000 define the condition as "at least three episodes during the previous 12 months or "at least two episodes during the previous 6 months", which is the definition used in Swedish guidelines[197,198]. RUTI is common. In an American study of college women, 27 % had a new UTI within the following 6 months. In a Danish study similar frequences were reported with the highest rate during the first two months[199]. In two Finnish studies, 40-44% of women had a recurrence within one year [200,201].

The terms *reinfection*, *persistence* and *relapse* are often used in parallel. The terminology can be somewhat confusing and inconsistent, and may vary depending on study objectives. The Swedish guidelines refer reinfection to a new infection with a different bacterial strain, or the same strain in case of a negative culture inbetween the episodes and >2 weeks since treatment. A relapse is considered a recurrence within two weeks caused by the same strain. In comparison, in the study by Erjnaes et al. from 2006 reinfection referred to a new infection with a new strain, relapse to an infection with the same strain when there was a negative urine culture or a culture with a strain different from the primary infecting strain separating the two urine cultures, and persistence was used for UTI with the same strain as the primary infecting strain[202].

In the present studies we have refrained from using this terminology and have instead focused on recurrences of UTI within a certain timeframe.

5.3.6 Strain types and *E. coli*-clones in recurrent and sporadic UTI (**paper III and IV**)

In **paper III** we characterized 319 urinary isolates from 123 patients with 2-7 RUTI with ESBL-producing *E. coli* from the Gothenburg area. The patients were retrospectively identified in the laboratory database and selected to meet the criteria of the Swedish guidelines for RUTI. Almost all, 96%, of the patients had their first recurrence within 6 months. As the sequential isolates were of the same phylogroup as the index isolate in 119/123 cases we could conclude that RUTI in the majority of cases is caused by the same infecting strain by phylogroup. By performing PFGE of selected cases we then could conclude strain homology in 43/44 cases. Similar observations have earlier been made of *E. coli* not producing ESBL. In a randomized controlled Danish study of 156 women with relapse of community-aquired *E.coli* 77% were caused by *E. coli* of the initial infecting strain, investigated by PFGE[202]. Köljalg et al found persistent *E. coli* clones in children with RUTI[203]. Our results demonstrating strain homology in RUTI indicate that the initial infecting strain and its properties are of importance, and support the theory of persistent strains establishing biofilm-like bacterial communities in the bladder epithelium[166]. Other studies have shown that the infecting UTI-clones are also the dominant fecal and vaginal clones, and that these clones possess comparatively higher virulence scores[18,162]. Furthermore, there are studies demonstrating that certain clones persist longer in the gut flora. For instance, the study of Overdevest et al. clearly demonstrated prolonged colonisation with ESBL-*E. coli* ST131 as compared to ESBL-*E. coli* not belonging to ST131[204].

Paper III is large study of strain characteristics- nevertheless, it is a descriptive study of available RUTI-isolates from a retrospective collection which inevitably implies bias. **Paper IV** presents comparative data on ESBL-*E. coli* isolates causing sporadic (n=235) and recurrent (n=68) UTIs, prospectively collected from the entire region Västra Götaland. Considering the distribution of RUTI in **paper I** and **III**, with few patients with RUTI after 6 months, RUTI in **paper IV** was defined as a new UTI within 6 months. As in **paper III** isolates ≤30 days from the first were not included. The median age was similar in **paper III** (68 years for women and 72 for men) and for patients with RUTI in **paper IV** (75 years for women and 71

for men) and women dominated. The median time to the first recurrence also corresponded (73 days (**III**) and 55 days (**IV**)). Thus, despite the differences in study design and time period, the two papers are discussed together.

The most common phylogroup in RUTI both in **paper III** (Figure 14) and **IV** was B2 (56% vs 66%) followed by D (19% vs 13%) which was an expected finding considering the known high prevalences of B2 and D in ExPEC infections[172]. The prevalence of phylogroup B2 and D was however also common in sporadic UTI and no significant differences in the frequencies were found between sporadic and recurrent UTI in relation to phylogroup **(IV)**. There was furthermore no differences in the time to the first recurrence or the number of recurrences in relation to phylogroup (**III** and **IV).**

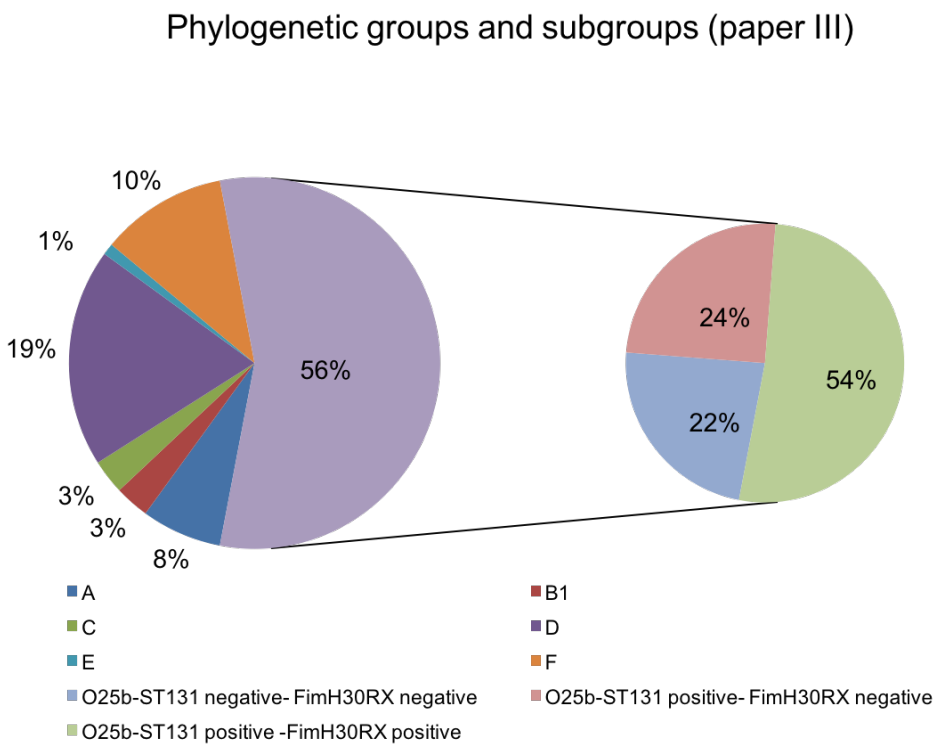

*Figure 14. Distribution of phylogroups and the subclones of phylogroup B (non-ST131-O25b, ST131-O25b-fimH30Rx-negative and ST131-O25b-fimH30Rx-positive isolates) in 119 patients with RUTI (**paper III**).*

Accordingly, the results from **paper III** and **paper IV** suggest that knowledge of only the phylogroup is not enough for prediciting recurrences in ESBL-*E. coli* RUTI. Of B2 isolates causing RUTI (n=67) in **paper III** most belonged to the ST131-O25b clone (n=52) and of these, 36 belonged to the subclone *fimH*30Rx. Even if there are other ST131 lineages producing ESBL, this is the most common subclone. Likewise, in **paper IV**, a high frequency of ST131-O25b (n=28) and *fimH*30Rx (n=20) among B2-isolates was found. The impact of *fimH*30Rx on recurrences was evident in both studies. In **paper III**, *fimH*30Rx was shown to be associated with a high number (≥3) of recurrences and this observation was significant as compared to other isolates in the B2 group (Table 2, **paper III**). In **paper IV**, the presence of *fimH*30Rx clearly increased the risk of recurrences.

Phylogroup D was the second most common phylogroup in both **paper III** (19%) and **paper IV** (13% of RUTIs and 21% of sporadic UTIs). In **paper IV**, 85% of isolates of D were from patients with sporadic UTI and 88 % were women. Compared to B2- isolates, a substantial part of D- isolates carried group CTX-M 9 both in **paper III** and **paper IV** (48% vs 56%).

As expected, almost all *fimH*30Rx positive isolates in **paper III** and **IV** carried CTX-M group 1-enzymes, but merely the knowledge of the CTX-M group could not predict recurrences. Interestingly enough, although the *fimH*30Rx- isolates in **paper III** did display a significantly higher degree of multi-drug resistance, this did not influence the number of recurrences.
To summarize, we found that ESBL-*E. coli*-RUTI most often is caused by the same infecting *E.coli* strain and that the virulent subclone *fimH*30Rx is of major importance for recurrences.

6 CONCLUSION AND FUTURE PERSPECTIVES

Overall, this thesis provides new knowledge of how the fecal carrierstate, previous EPE-infection and bacterial ESBL-*E. coli* properties influence recurrent EPE-infection. More specifically:

It is uncommon with a subsequent EPE-infection in the general patient with a history of fecal EPE-carriage. A better understanding of the predictive value of EPE fecal colonization for subsequent infection is necessary. When it comes to screening regimens it is important to target the actual patients at risk. One way of studying this would be to prospectively screen patients in the Västra Götaland region known to be at risk of Gram-negative infections, for instance patients with urological comorbidity and patients with immune deficiencies, and compare strain types of colonizing and subsequent infecting strains to a control population.

Almost one third of patients with a previous positive clinical sample had a following clinical sample. Patients infected with the ST131-O25b-*fimH*30Rx-subclone were more likely to develop RUTI, and also had an increased number of recurrences. We need a deeper understanding of why, and how, certain bacterial clones so readily outconquer others, and in the future develop rapid tests to detect them in patients. Not all *E. coli* are the same- but it seems to be the same and fittest strain types that cause reinfection by persisting in their host. Other virulent clones may also be on the rise, and it would, for instance, be of interest to characterize the isolates of phylogroup D in **paper IV** more carefully. Also, a retrospective study of medical records of the patients in **paper IV** to assess the patient-related risk factors associated with ESBL-*E. coli* RUTI would be valuable. Furthermore, since we cannot extrapolate our results to BSIs, it would be of interest to study if bacterial factors are of importance also for subsequent and recurrent EPE BSI.

Transfer of ESBL-carrying genes to new bacterial species through plasmid migration was a rare event. Even if our results strenghten the theories of persistence outlined above, it would be interesting to study possible plasmid transfer among bacteria of the same species. Our study also clearly demonstrates the need for advanced methods for plasmid characterization. ODM proved to be a valuable, easy and fast tool in this respect.

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