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KLEBSIELLA PNEUMONIAE AND ESCHERICHIA COLI

MULTIDRUG-RESISTANCE AND DIFFERENT ASPECTS OF INVASIVE INFECTIONS

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Klebsiella pneumoniae and Escherichia coli – multidrug-resistance and different aspects of invasive infections

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

ABSTRACT

Klebsiella pneumoniae and *Escherichia coli* are pathogens belonging to the Enterobacteriaceae family. They can cause infections ranging from uncomplicated urinary tract infection to severe bloodstream infection (BSI). The prevalence of extended spectrum β-lactamase-producing Enterobacteriaceae (EPE) is increasing worldwide and carbapenemases (CPE), a subgroup of EPE where antibiotic treatment is very limited, is a major threat to patients.The aims of this thesis were to get expanded molecular and epidemiological knowledge about *K. pneumoniae*, its association to morbidity and mortality in BSI (**II, III**), to increase sensitivity in detection of carbapenemase-producers (**I**), to determine risk to acquire fecal colonization with EPE during traveling, and to characterize colonizing EPE in terms of virulence factors and phylogroups (**IV**).

In **paper I** methods for antimicrobial susceptibility testing were evaluated for detection of *K. pneumoniae* carbapenemase (KPC)- and Verona integron-encoded metallo-β-lactamase (VIM)-producing *K. pneumoniae* in order to define appropriate screening breakpoints. Strains (n=51) were tested against different carbapenems using disk diffusion, gradient test, and automated susceptibility testing. Results were interpreted with the European (EUCAST) and American (CLSI) antimicrobial susceptibility testing breakpoints. We found that clinical breakpoints cannot be used for carbapenemase screening. Meropenem was the most suited carbapenem to use for screening purposes. A breakpoint of 0.5 mg/L detected all isolates with an at the same time good separation from the wild type population.

In **paper II and III** a cohort of patients with BSI caused by *K. pneumoniae* was evaluated retrospectively and compared with BSI caused by *E. coli*. Data on risk factors, prognostic factors and mortality was retrieved from 1251 medical charts (**III**). The late mortality (within 90 days) was significantly higher among patients with BSI caused by *K. pneumoniae* and could be explained by higher comorbidity. Contrary to European trends our study showed low antibiotic resistance among *K. pneumoniae* isolates supporting the hypothesis of absence of successful multidrug-resistant *K. pneumoniae* clones in the Stockholm area. For a subset of the patients (n=139) molecular analysis was performed on the *K. pneumoniae* isolates (**II**). Based on multilocus sequence typing, the isolates could be separated in three phylogenetic clades: KpI (n=96), KpII (n=9) and KpIII, also known as *K. variicola* (n=34). Patients infected with strains belonging to *K. variicola* had higher 30 days mortality (29.4 %), also when adjusting for age and comorbidity (OR for KpIII = 3.0 (95% CI: 1.1-8.4) compared to KpI). Only three of the isolates causing mortality within 30 days belonged to any of the virulent serotypes, had a mucoid phenotype, or harbored virulence genes. Hence, the increased mortality could not be related to any known strain factor. In general, a high level of comorbidity was observed in the *K. pneumoniae* cohort.

Paper IV was a prospective study. Fecal samples and survey data were collected from 188 Swedes traveling to four regions of high EPE prevalence, and molecular characterization was performed on EPE. Colonization incidence varied by visited region; the Indian subcontinent 49%, northern Africa 44%, Southeast Asia 19% and Turkey 10%. Few strains harbored virulence factors connected to uropathogenicity, and most *E. coli* strains belonged to phylogroup A, rarely associated with extraintestinal infections. No clinical infections were seen in follow-up. No CPE was found, but one strain contained the plasmid-mediated colistin resistance gene, *mcr-1*. Independent risk factors for EPE acquisition were travelers´ diarrhea and use of antibiotics during travel. EPE acquired during travel have seemingly low pathogenicity as indicated by the low frequency of virulence factors and phylogroups associated with extraintestinal infections.

In summary this thesis provides new knowledge about *K. pneumoniae* BSI in a clinical and a molecular perspective. It also adds to the knowledge about molecular features of EPE colonizing the intestine, and appropriate breakpoints to use in detection of CPE.

LIST OF PUBLICATIONS

I. **Vading M**, Samuelsen Ø, Haldorsen B, Sundsfjord A and Giske CG. Comparison of disk diffusion, Etest and VITEK2 for detection of carbapenemase-producing *Klebsiella pneumoniae* with EUCAST and CLSI breakpoint systems Clin Microbiol Infect. 2011; 17(5): 668-74.

II. Matallah M*, **Vading M***, Kabir M, Bakhrouf A, Kalin M, Nauclér P, Brisse S and Giske CG. *Klebsiella variicola* is a frequent cause of bloodstream infection in the Stockholm area, and associated with higher mortality compared to *K. pneumoniae*. PLoS One 2014. 26;9(11).

III. **Vading M**, Nauclér P, Diaz Högberg L, Kalin M and Giske CG. High long-term mortality in Swedish invasive infections caused by *Klebsiella pneumoniae* and low prevalence of resistance compared to European EARS-Net data. Manuscript.

IV. **Vading M**, Kabir MH, Nauclér P, Kalin M, Iversen A, Wiklund S, and Giske CG. Frequent acquisition of low-virulent strains of extended-spectrum beta-lactamase-producing *Escherichia coli* in travelers. Submitted manuscript.

* Both authors contributed equally to the paper

ADDITIONAL RELEVANT PUBLICATIONS

Wiklund S, Fagerberg I, Örtqvist Å, **Vading M**, Giske CG, Broliden K, Tammelin A. Knowledge and understanding of antibiotic resistance and the risk of becoming a carrier when travelling abroad: a qualitative study of Swedish travellers. Scand J Public Health. 2015; 43(3):302-8.

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LIST OF ABBREVIATIONS

Amp C	Ampicillinase C (β-lactamase of $ESBL_M$-type)
AMR	Antimicrobial resistance
bla	Gene encoding β-lactamase
BSI	Bloodstream infection
CI	Confidence Interval
CLSI	Clinical Laboratory Standards Institute
CPE	Carbapenemase-producing Enterobacteriaceae
CTX-M	Cefotaximase Munich, β-lactamase of $ESBL_A$-type
EARS-Net	The European Antimicrobial Resistance Surveillance Network
ECDC	The European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off value
EEA	The European Economic Area
ESBL	Extended-spectrum β-lactamases
$ESBL_A$	Classical ESBL (SHV-, TEM- and CTX-M-variants)
$ESBL_M$	Miscellaneous ESBL (plasmid-mediated AmpC)
$ESBL_{CARBA}$	Carbapenemases (KPC, NDM, VIM and OXA-48-variants)
EPE	ESBL-producing Enterobacteriaceae
EU	The European Union
EUCAST	European Committee of Antimicrobial Susceptibility Testing
ExPEC	Extra-intestinal pathogenic *Escherichia coli*
fim	Genes encoding Type-1 fimbriae (involved in bacterial adhesion)
KPC	*Klebsiella pneumoniae* carbapenemase
mcr-1	Gene encoding plasmid-mediated colistin resistance
MDR	Multidrug-resistance
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
NDM	New Delhi metallo-β-lactamase, a type of carbapenemase
OXA	Oxacillinase-type β-lactamase, a type of carbapenemase
pap	Pili associated with pyelonephritis (involved in bacterial adhesion)
PCR	Polymerase chain reaction
RA	Relative abundance
SHV	Sulfhydryl variable, β-lactamase of $ESBL_A$-type
ST	Sequence type
TEM	Temoneira, β-lactamase of $ESBL_A$-type
UTI	Urinary tract infection
VF	Virulence factor
VIM	Verona integron-encoded metallo-β-lactamase, a type of carbapenemase
WT	Wild type, the “natural” bacterial phenotype

1 INTRODUCTION

Extended spectrum β-lactamase (ESBL) -production among bacteria belonging to the gram-negative family Enterobacteriaceae is the clinically most significant mechanism causing antibiotic resistance among gram-negatives and is rapidly increasing worldwide. Enterobacteriaceae is a large bacterial family comprising several species. Apart from being colonizers of the gut microbiota of both animals and humans and being found in water and soil, some of the members also serve as human pathogens. Infections range from urinary tract infection (UTI), abdominal infection and pneumonia to severe bloodstream infection (BSI) [1]. The pathogens focused on in this thesis are two most common pathogens in the Enterobacteriaceae family - *Escherichia coli* and *Klebsiella pneumoniae*. In most geographic regions *K. pneumoniae* is the pathogen with the highest rate of ESBL-associated resistance [2].

ESBL-producing Enterobacteriaceae (EPE) most often cause infections of similar severity as non-EPE. However, patients with infections caused by EPE are subjected to longer hospitalization and more frequently suffer from complications due to delayed adequate antibiotic treatment. Infections caused by EPE are also associated with higher costs [3-5]. In Sweden the proportion of EPE among *E. coli* and *K. pneumoniae* is, compared to many other countries, still relatively low. In 2014 the Public Health Agency in Sweden published a study on intestinal EPE-colonization among healthy Swedes. The prevalence was 4.8% for samples collected 2012-2013 [6]. However resistance rates are higher in hospital settings and increase in a manner that at this date causes clinical challenges on an everyday basis. At Karolinska University Hospital in Stockholm, the rate of ESBL-producers among invasive *E. coli* isolates was 4 percent in 2006. In 2014 the rate for the first time exceeded 10% [7].

β-lactam antibiotics are a broad class of antibiotics including the penicillins, cephalosporins, monobactams and carbapenems. The group is important and forms the basis of antibiotic treatment of severe infections caused by both gram-negative and gram-positive bacteria. Common to all β-lactams is the molecular ring-shaped structure, the β-lactam ring, forming the mode of action by binding to penicillin-binding proteins in the bacterial cell wall thus inhibiting cell wall synthesis (Fig. 1) [1].

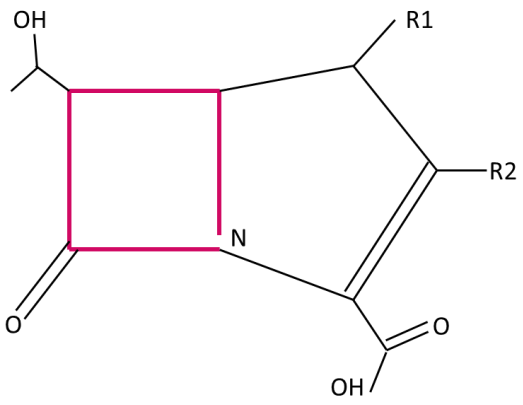


Figure 1. *Structural features of a carbapenem with the β-lactam ring (red)*

Antibiotic resistance among Enterobacteriaceae can occur by several mechanisms (Fig. 2). Efflux pumps in the bacterial cell wall reduce the bacterial accumulation of antibiotics. Due to loss of porins in the outer bacterial membrane, permeability decreases preventing antibiotic influx. However, the most important mechanism is antibiotic degrading enzymes, ESBLs. ESBLs are a group of diverse, mostly plasmid-mediated enzymes that can be produced by all Enterobacteriaceae. The most common and clinically most important species are *E. coli* and *K. pneumoniae*. Through hydrolysis the enzymes cause an opening of the β-lactam ring of penicillins, cephalosporins and monobactams, which in turn leads to an inactive form of the β-lactam antibiotic [2].

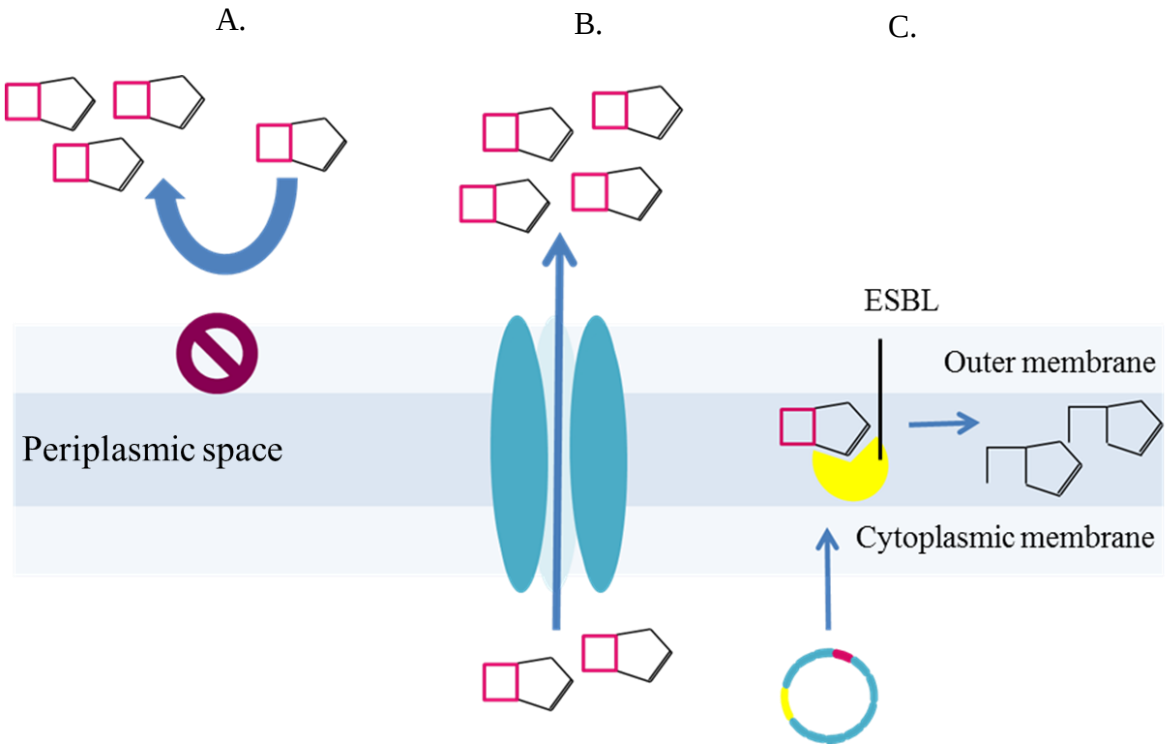


Figure 2. *Mechanisms of antibiotic resistance in Enterobacteriaceae.*
The bacterial cell wall consists of an outer and an inner membrane surrounding the periplasmic space.

- *A. Decreased permeability due to loss of porins*
- *B. Reduced antibiotic accumulation due to active efflux pumps*
- *C. Plasmid-mediated enzyme production inactivating β-lactams through hydrolysis*

Co-resistance to other important antibiotic classes such as fluoroquinolones, trimethoprim-sulfamethoxazole and aminoglycosides is common among EPE challenging antibiotic treatment even more. In severe infections caused by EPE the drugs of choice are the carbapenems [8, 9], a group of β-lactam antibiotics that resist inactivation from classical ESBLs.

The major worldwide threat is carbapenemase producing Enterobacteriaceae (CPE), EPE resistant also to carbapenems. Severe infections caused by CPE have high mortality due to very few, if any, treatment options, and the increasing rate of their occurrence is very worrisome.

K. pneumoniae is second to *E. coli* the most common gram-negative pathogen causing BSI and UTI. The pathogen disseminate more easily than *E. coli* in hospitals probably due to circulating clones hosting specific virulence factors (VFs) [10-12]. The patient population affected by invasive infections caused by *K. pneumoniae* show high comorbidity - diabetes, immunosuppression, malignancies and hospitalization are risk factors for BSI caused by *K. pneumoniae*. Invasive infections caused by this pathogen are associated with high mortality both in settings with high and low antibiotic resistance rates [13-15]. Most recent studies on *K. pneumoniae* focus, due to the escalating proportion of resistance, on β-lactamase producing isolates.

Taxonomic studies on *K. pneumoniae* have led to splitting of the species into three distinct species. Isolates belonging to phylogroup KpI are now called *K. pneumoniae sensu stricto*, KpII *K. quasipneumoniae*, and KpIII *K. variicola* [16, 17]. The knowledge on differences in disease presentation and prognosis in infections caused by the different species is limited due to the novel change of classification and its limited impact on clinical practice thus far.

Antimicrobial resistance among bacteria can be traced several thousand years back and the bacterial inherent ability to adapt to environmental changes and express resistance genes is incredible [18-20]. High use of antibiotics select for resistant strains by suppressing the commensal microbiota and creating a niche for resistant strains to breed. Chromosomally mediated antibiotic resistance occurs by spontaneous mutations in the bacterial genome that are vertically inherited when bacteria divide. The success of EPE has several aspects. One important factor is that genes coding for ESBL not only are inherited on bacterial division, but also are transferable between bacteria horizontally through plasmids [21]. The mechanism is called bacterial conjugation and is further explained in Fig. 3.

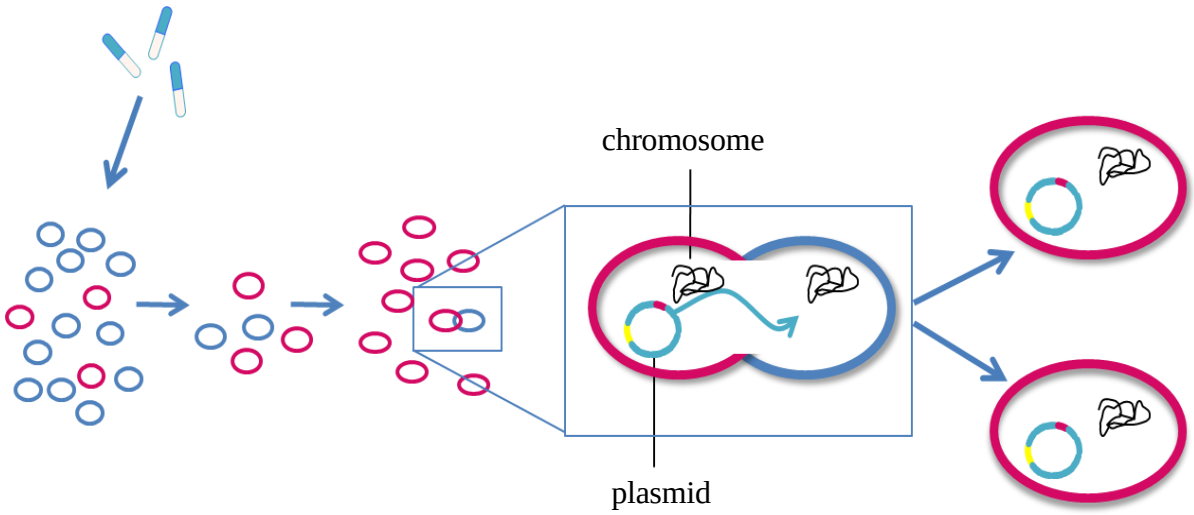


Figure 3. *Schematic illustration of antibiotic resistance selection and bacterial conjugation. Susceptible intestinal bacteria (blue) are suppressed due to antibiotic exposure, favoring resistant bacteria (red) to breed. A pilus (not shown in figure) hooks to join the donor and recipient bacterium. In turn the plasmid, carrying genes encoding for virulence and antibiotic resistance, replicate independently and transfer to susceptible bacteria causing horizontal antibiotic resistance transfer.*

Another factor contributing to the increasing proportion of EPE is the resistant strains´ adaptation as environmental habitants as well as pathogens. High levels of antimicrobial resistance, for example metallo-β-lactamase-production in *Salmonella* Typhimurium [22], is associated with bacterial fitness reduction in terms of decreased growth rate and loss of invasiveness. However, some successful clones of resistant Enterobacteriaceae show both good adaptations as part of the commensal microbiota, as well as low fitness cost. The mechanisms are not yet fully understood. One example is the *E. coli* clonal group, sequence type 131 (ST131), commonly reported to harbor *bla* $_{\text{CTX-M-15}}$, and other co-resistance genes. ST131 is a common intestinal colonizer, but also a frequent uro- and bloodstream pathogen and a strain known for worldwide dissemination. It belongs to phylogroup B2, a phylogroup often associated with extraintestinal infections [23-25]. Some β-lactamase producing *K. pneumoniae* strains have been suggested to possess even higher pathogenic potential than non-producers due to simultaneous expression of plasmid encoded adhesins, VFs facilitating bacterial adherence to host surfaces [26, 27].

The dissemination of EPE around the world has been shown both in prevalence studies and in studies following travelers to endemic areas [28, 29]. Intestinal colonization with EPE is common in many parts of the world, and the duration of fecal carriage varies - colonization seem to persist longer following a clinical infection and among newborn than among healthy individuals without clinical symptoms [30-32]. However, the future risk of developing a clinical infection caused by EPE when colonized is not yet well known.

The overall aim of this thesis was to study the bacterium *K. pneumoniae* from different aspects – risk factors for and prognosis in severe infections in comparison with *E. coli*, bacterial characteristics in terms of virulence and phylogroups in relation to prognosis, diagnostics for multi-resistant isolates, and finally evaluate risk factors for EPE acquisition and molecular features of EPE in intestinal colonization.
More specifically the aims of the studies presented in papers **I-IV** were:

1. To determine screening breakpoints for laboratory detection of *K. pneumoniae* producing $ESBL_{CARBA}$ (**I**)
2. To determine phylogroups and virulence characteristics in *K. pneumoniae* causing BSI in the Stockholm area (**II**)
3. To evaluate differences in prognosis in *K. pnuemoniae* BSI in relation to molecular characteristics (**II**)
4. To define risk factors for and prognosis in BSI caused by *K. pneumoniae* in relation to *Escherichia coli* (**II, III**) in a low resistance setting
5. To study how relative frequencies of BSI caused by *K. pneumoniae* versus *E. coli* vary with the frequency of ESBL-producing isolates in those species (**III**)
6. To study risk factors for acquisition of EPE when traveling in high-prevalence areas (**IV**)
7. To study molecular characteristics of EPE colonizing the intestine (**IV**)

In this thesis frame I will give an introduction to the commensal gut microbiota, its importance, and changes in composition due to antibiotic pressure. I will then focus on the pathogens of importance, and their molecular characteristics, followed by an introduction to the worrying dissemination of EPE and CPE. Lastly I will discuss tools used in the diagnostics of carbapenemase-producers, treatment of CPE, and future aspects of multidrug-resistance (MDR). The findings in the four papers included in this thesis will be integrated within each section. The methods used in the studies are fully described separately in the papers for which reason they are only partly repeated here to facilitate the understanding.

2 THE INTESTINE – THE BODY´S LARGEST BACTERIAL RESERVOIR

The community of microbes living in association with its host is called the microbiome or the commensal microbiota. The intestine contains the main proportion of the commensal microbiota. It is a fascinating ecosystem consisting of 1-2 kg of at least 1000 different bacterial species. The bacterial number has for a long time been estimated to 10:1 (10^{13} bacteria or 10^{11}–10^{12}/g) compared to number of cells in the body, although this ratio has recently been questioned [33][34]. The bacterial establishment starts during the birth process when the child on the way through the birth canal is colonized by the mother's vaginal- and intestinal microbiota. *E. coli* is one of the first bacterial species to colonize the gut during infancy, reaching high density before the anaerobes establish. After the age of two the intestinal composition has stabilized and now, depending on the intestinal conditions, more than 99 % of the bacteria are anaerobic [35]. Of the facultative bacteria, *E. coli* still is the most common, but is now outnumbered by the anaerobic bacteria by between 100:1 to 10000:1 [36]. The intestinal ecosystem in adults is diverse and complex and the relative abundance (RA) of different species is constantly changing due to interaction with present bacteria, nutrition and other host factors such as age, lifestyle factors and diet [37]. As bacterial transmission from mother to newborn occurs at birth, EPE colonization of the mother is a risk factor for colonization also of the newborn [38, 39]. In a study from India carrier rates of EPE were 14.3% day one, and as high as 41.5% day 60, among vaginally delivered babies [40].

2.1 THE NORMAL GUT MICROBIOTA – FUNCTION AND IMPORTANCE

The full complexity of the microbiota is only in the beginning of being understood and currently intensively explored [41]. It contributes with several beneficial functions for the host. Colonization resistance serves as a key defense both against exogenously introduced gastrointestinal infectious pathogens as well as overgrowth of potential pathogens [42, 43]. The mechanisms of action are both direct and indirect (immune-mediated). By occupying the colonization niche the commensal microbiota prevents access to mucosal adherence sites and inhibits colonization of exogenous pathogens, such as *Salmonella Typhimurum* and *Vibrio cholerae* or certain strains of pathogenic *E. coli*. It also prevents overgrowth of indigenous potential pathogens such as *Clostridium difficile* and yeasts. Indirectly commensal bacteria and their products can activate the host's immune response targeting pathogenic bacteria [44]. The specific bacteria of most importance in this defense are not yet fully understood but seem to constitute the majority of the intestinal microbiota including the two dominating commensal bacterial phyla, the Bacteroidetes (including the anaerobic *Bacteroides* spp) and the Firmicutes (including the *Lactobacillus* spp), and also the Actinobacteria (including *Bifidobacterium* spp) [44].

Enzymes produced by the microbiota also facilitate digestion of complex carbohydrates and generates essential nutritional factors, including vitamins [45]. Recently several diseases –

particularly autoimmune - such as type 2 diabetes and inflammatory bowel disease, but also obesity and even neuropsychiatric disorders have been found to be linked to imbalance in the gut microbiota [46-48]. The RA of some members of the intestinal microbiota depend more on host genetics, while others, e.g. the *Bacteroides* spp, are more influenced by environmental factors, for example food and intake of antibiotics [49].

2.1.1 *Escherichia coli*

E. coli are gram-negative, non-sporulating, motile, fermenting, rod-shaped bacteria, with an enormous diversity within the species. Most *E. coli* strains have low pathogenicity and belong to the commensal microbiota where they are the most frequent facultative anaerobes in the mucus layer of the colon, with a quantity of 10^7-10^9 cfu/gram of feces [36]. It is not entirely known why *E. coli* is such a successful competitor in the colon, however, better utilization of available nutrients than other species is speculated [50]. Apart from being a gut-colonizer, *E. coli* is also the most important gram-negative pathogen causing infections ranging from lower to upper UTI, abdominal infection, pneumonia, BSI and meningitis. Certain strains can also cause intestinal infections [51].

The barrier that separates commensialism from infection is a complex balance between host and bacteria. Studies on *E. coli* have revealed that bacterial behaviour differ between phylogroups why attempts to link phylogenetic background with other bacterial traits have been made. *E. coli* can be divided into four major phylogroups; A, B1, B2 and D. Nowadays, using a quadruple PCR, totally eight *E. coli* phylogroups are known; in addition to the major ones also C, E, F and *Escherichia cryptic clade I* [52]. Commensal strains mostly belong to group A and B1 while phylogroup B2 and, to some extent, D, are connected to longer duration of intestinal colonization, more virulent strains, and are more often causing extra-intestinal infections (ExPEC) [53-55]. *E. coli* strains belonging to a non-B2 phylogroup to a greater extent show multiresistance, i.e. non-susceptibility to 3 or more different antibiotic classes. The reason is unknown but one hypothesis is that commensal strains, commonly non-B2, become more intestinally exposed to antibiotics [56, 57].

By multilocus sequence typing (MLST), analyzing DNA-sequences in 7 different housekeeping genes, sequence types (STs) can be determined [58] in order to characterize genetic relationships among bacterial isolates. International databases store information on registered STs, at present almost 5900 different ST:s are registered in the MLST database (http://mlst.ucc.ie).

Several VFs are of importance for *E. coli*. VFs help bacterial attachment to host mucosal surfaces, stimulate inflammation and help the pathogen to overcome immunologic respons. Some virulence genes have been correlated to successful intestinal colonization in human, i.e. certain traits classically regarded as VFs may also be colonization factors [51]. The main VFs associated with ExPEC include adhesins, (e.g. *pap* - pili associated with pyelonephritis - encoded P-fimbriae and *fimH*- encoded type 1 fimbriae), toxins (hemolysin), iron acquisition

systems (aerobactin, siderophore), capsule production (K1/K2/K5) and protectins (colicin) [59, 60].

2.1.2 *Klebsiella pneumoniae*

Klebsiella spp is also a genus of gram-negative, non-sporulating, fermenting, facultative anaerobic, rod-shaped bacteria. They are nonmotile. Like *E. coli*, *K. pneumoniae* have a high degree of plasticity, with gene loss or gain of genomic segments by lateral gene transfer. The bacteria can thrive in a variety of environmental niches due to metabolic versatility. Apart from being colonizers of mucosal surfaces (i.e. intestine, upper respiratory tract) both in human and other mammals, the bacteria can be found both in water, plants, insects and soil [61]. Colonization rates vary with increasing rates among hospitalized patients and with consumption of antibiotics [62, 63].The bacteria also constitute importance as pathogens causing clinical infections such as UTI, pneumonia, abdominal infection, BSI and meningitis, often in immunocompromised patients [64]. In some parts of the world, particularly Southeast Asia, *K. pneumoniae* is a cause of pyogenic liver abscess among previously healthy individuals [65-67]. There are several species within the genus, of which the clinically most important is *K. pneumoniae*.

By genotypic methods *K. pneumoniae sensu latu* can be divided into three different phylogroups; KpI, KpII and KpIII. Taxonomic work has proposed the names *K. variicola*, for phylogroup KpIII and *K. quasipneumoniae* for phylogroup KpII [16, 17]. This phylogenetic separation into three different species is novel and yet little is known about differences in pathogenesis and virulence. In turn, *K. pneumoniae* comprises three subspecies: *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis*. The two latter subspecies are rarely encountered and are associated with specific diseases (rhinoscleroma and ozena, respectively) [68]. *K. quasipneumoniae* also includes two subspecies (Fig. 4).

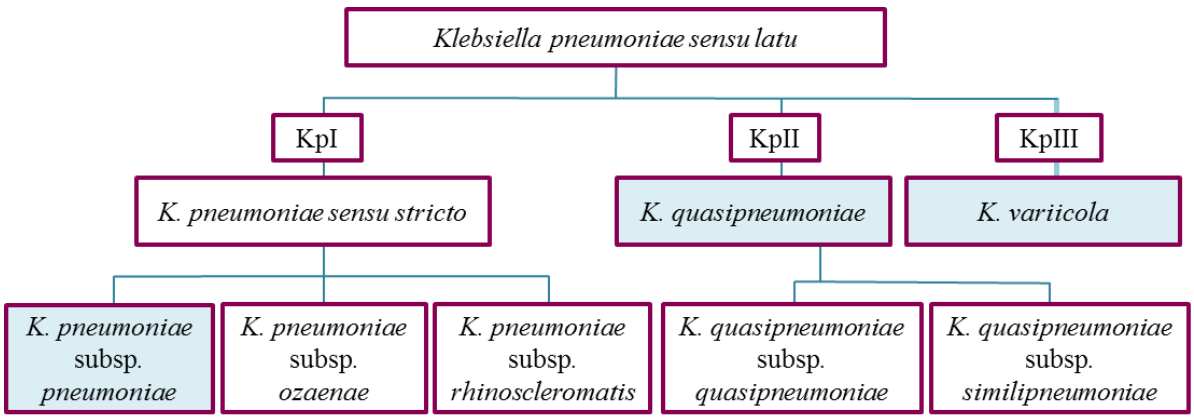


Figure 4. *K. pneumoniae in a taxonomical perspective*

The bacterial capsule is considered an important VF, 78 different capsular (K antigen) serotypes are known [64, 69]. It is composed of polysaccharides forming thick bundles of

fibrillous structures covering the bacteria thus protecting it from macrophage-mediated phagocytosis and bactericidal serum factors. Different K antigens have been associated with invasive disease (K1, K2, K5, K20, K54 and K57) [70, 71] as well as pyogenic liver abscess (K1, K2) [72, 73]. Lipopolysaccharide (LPS) protects from complement-mediated killing activity and mediate adhesion to uroepithelial cells *in vitro* [74, 75]. Strains of *K. pneumoniae* forming hypermucoviscous colonies (Fig.5), often serotype K1 and K2, are associated with virulence due to the extra layer of polysaccharide, and *rmpA* (regulator of the mucoid phenotype) and *magA* (mucoviscosity associated gene A) are genes encoding factors connected to hyper-virulent *K. pneumoniae* [76-78]. Other VFs include adherence factors (pili, type 1 fimbriae and other adhesins) facilitating host mucosa adhesion, and siderophore (enterochelin, aerobactin) activity [64].

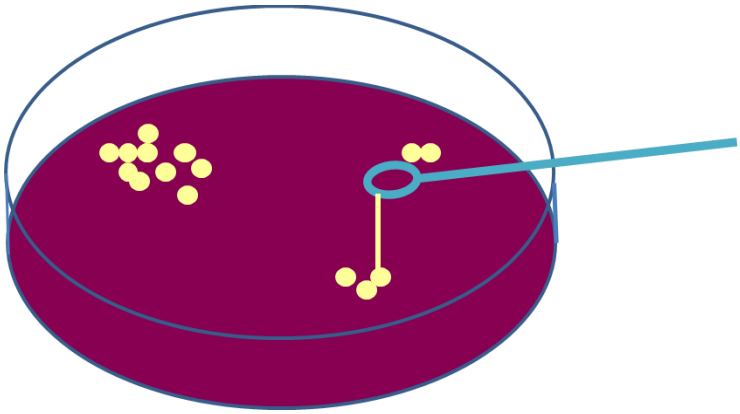


Figure 5. *K. pneumoniae, hypermucoviscous phenotype, forming viscous strings larger than 5 mm in length when pulling with inoculation loop.*

As for *E. coli*, sequence types (STs) can be determined with MLST. At present over 2000 different STs are registered in the Klebsiella Sequence Typing database (http://bigsdb.web.pasteur.fr/klebsiella/). Isolates differing in only one allele in MLST form a clonal complex. Examples of successful sequence types are ST258, a KPC-producer, which rapidly have disseminated worldwide, and ST15 and ST147, often hosting CTX-M-15 [11, 24, 79, 80].

2.1.2.1 *Klebsiella pneumoniae sensu stricto*

K. pneumoniae is clinically the most significant of the *Klebsiella spp*, causing a wide range of infections. It is second to *E. coli* the most frequent gram-negative pathogen.

2.1.2.2 *Klebsiella variicola*

The bacterium was for the first time in 2004 described as a separate *Klebsiella*-genus difficult to distinguish from *K. pneumoniae* by biochemical tests but more reliably differentiated by genotypic methods. [16, 81] It has frequently been isolated from various plants, and in a few studies also been described from clinical samples causing infections among human. Since

around 20% of clinical isolates of *K. pneumoniae sensu latu* in fact are either *K. variicola* or *K. quasipneumoniae*, the clinical impact of these pathogens is not yet well known [81].

2.1.2.3 *Klebsiella quasipneumoniae*

The pathogen has recently been described as a novel species closely related to *K. pneumoniae* [17]. It has been isolated from persons with hospital-acquired infections or gut colonization, but there are also a few case reports describing liver abscess and a hypermucoviscous isolate of *K. quasipneumoniae* causing BSI [82, 83].

2.2 ANTIBIOTICS AND IMPACT ON THE INTESTINAL ECOSYSTEM

Antibiotics change gut microbiota composition by suppressing species sensitive to the specific antibiotic. By killing commensal micro-organisms, both direct bacterial inhibition and microbiota-mediated innate immune defenses decrease enabling remaining, antibiotic-resistant, species to proliferate. When anaerobic bacteria, such as the beneficial *Bacteroides* spp, are suppressed, *Clostridium difficile*, a species commonly isolated in low numbers in healthy individuals, can increase and cause clinical symptoms ranging from mild diarrhea to pseudomembranous colitis [84, 85]. Substantial relative changes in microbial abundances are seen on exposure to antimicrobials, changes are inter individual and are seen up to several months after termination of antibiotic treatment [86]. Apart from selecting for more resistant and non-beneficial microbes, the use of antibiotics has also been suggested to be correlated to alterations in glucose metabolism, bodyweight regulation and as a consequence development of diabetes type 2 [87, 88]. Thus in several ways antibiotic treatment, when indication is lacking, can be of harm both on an individual basis and in a global perspective.

3 INVASIVE INFECTIONS CAUSED BY *K. PNEUMONIAE* AND *E. COLI*

3.1 COLONIZATION VERSUS INFECTION

Gram-negative infections in the urinary tract and invasive infections are often preceded by intestinal colonization and/or colonization of the vaginal introitus and the periurethral area. All mammals are colonized with *E. coli*. Colonization per se is not harmful, but strains expressing VFs can easier invade the urinary tract and other host mucosa and cause infection. UTIs caused by *E. coli* are common, with an annual incidence as high as 10-14% among sexually active women [89, 90]. *E. coli* is also the most common species in gram-negative invasive infections, a population-based Canadian study showed an annual incidence of 30/100 000 [91].

K. pneumoniae is in a healthy individual most often outcompeted by other species in the microbiota. However colonization rates vary depending on environmental factors and antibiotic pressure. Patients in intensive care units (ICU) show high colonization rates both in the stool and in the respiratory tract, increasing the risk for future infections [62-64].

The pathogenic potential among Enterobacteriaceae is variable between and within the bacterial species. Bacteria capable of causing infections in healthy individuals are more dependent on VFs for attachment to the infecting site, to facilitate invasion, and to overcome the host's immune-system, than opportunistic pathogens in immunocompromised hosts [92]. With increasing resistance rates in Enterobacteriaceae the prevalence of EPE-carriers increase. EPE-colonization in patients with chemotherapy induced neutropenia as well as in patients at the ICU pose an increased risk for BSI caused by EPE compared non-carriers [93-95]. Both patient groups are vulnerable with lacking functional endogenous barriers, hence presence of VFs is of less importance for the bacteria to overcome host defenses and cause infection. Also, these patients are to a higher extent colonized by more virulent strains and species due to high antibiotic pressure. However, the risk among healthy asymptomatic EPE-carriers to develop invasive infections due to the colonizing strains is not yet well known. In a retrospective study by Rottier et al [96] prior colonization with EPE had a positive predictive value for the risk of a suspected gram-negative BSI to be caused by EPE of 7.5%.

3.2 CLINICAL CHARACTERISTICS

In total 652 adult patients were admitted to Karolinska University Hospital between 2006 and 2012 due to BSI caused by *K. pneumoniae*. To determine risk factors for, and prognosis in, invasive infection caused by *K. pneumoniae* we retrospectively reviewed this cohort of patients (paper **II** and **III**). Patients with bacteria in the cerebrospinal fluid were also included, and the terms invasive infection and BSI are here used synonymously.

To evaluate differences in invasive infections caused by *K. pneumoniae* and *E. coli*, patients not co-infected with *E. coli* (n=599) were in paper **III** matched 1:1 on sex, age and year of

disease, with patients having invasive infection caused by *E. coli*. Data on clinical characteristics were retrieved from the medical charts.

The median age was 68 years, and 58% of the patients were male. Patients with invasive infections caused by *K. pneumoniae* showed in general a higher level of comorbidity (higher Charlson index) than the *E. coli* cohort. The independent risk factors for invasive infection caused by *K. pneumoniae* as compared to *E. coli* are presented in table 1.

Table 1. *Clinical characteristics of patients with invasive infection caused by K. pneumoniae versus E. coli. Factors significant in multivariable analysis* ***(III)****, using conditional logistic regression, are displayed.*

Patients factors	**Adjusted odds ratio (95% CI)** ***K. pneumoniae* vs *E. coli***
Arrhythmia	0.64 (0.41-1.00)
Peripheral vascular disease	4.37 (1.89-10.10)
COPD	2.01 (1.18-3.45)
Kidney disease	2.00 (1.34-2.98)
Bile disease	3.31 (1.57-6.98)
Hematological malignancy	1.91 (1.16-3.15)
Colorectal malignancy	3.09 (1.57-6.11)
Bile/liver/pancreas malignancy	3.34 (1.73-6.46)
Urinary catheter	2.50 (1.73-3.61)
Central catheter	2.29 (1.49-3.53)
Hospital-acquired infection [a)]	0.54 (0.38-0.78)
Healthcare- associated community-onset[a)]	3.14 (2.07-4.76)

a) in relation to community-acquired infection

In concordance with previous studies, patients with an impaired host defense due to either immunocompromising comorbidity, or to indwelling catheters disrupting mucosal membranes, are at risk for invasive infections caused by *K. pneumoniae* [97, 98], also in comparison with *E. coli*. Malignancy was the most common comorbidity in the *K. pneumoniae* cohort; 318 patients (53%) versus 226 (38%) in the *E. coli* cohort ($p<0.001$). The 7-day and 30-day mortality showed no differences between the cohorts, 6 % versus 7%, $p=0.55$, and 15% versus 12%, $p=0.15$ respectively. Higher 90-day mortality was observed in the *K. pneumoniae* cohort, 26%, versus the *E. coli* cohort, 17% ($p<0.001$). Multivariable analysis revealed that the difference could be attributed to higher comorbidity (i.e. malignancies and severe liver disease), non-community acquired infections, more often polymicrobial infections and other source of infections than the urinary tract (**III**).

Prognostic factors were evaluated among all *K. pneumoniae* BSI episodes (n=652). High age, lung malignancy and infections emanating from the lungs were factors associated with both early (within seven days) and late (up to 90 days) mortality. As expected, polymicrobial

infections were associated with fatal outcome within 7 days, while high level of comorbidity (i.e. high Charlson index) and other host factors were of major importance for fatal outcome within 90 days.

3.3 BACTERIAL CHARACTERISTICS

In addition to retrieving clinical information from the medical charts, the isolates from the patients admitted to Karolinska University Hospital Solna with BSI caused by *K. pneumoniae* 2007-2009 (n=139), were in paper **II** investigated with molecular methods to look for links between bacterial characteristics, and prognosis.

PCR was used targeting virulence genes and serotypes associated with invasive disease. Phylogenetic relatedness was determined with MLST. By analyzing DNA-sequences in 7 different housekeeping genes, the method can separate isolates into distinct STs; isolates with identical allelic profiles, and into clonal complexes, here defined as groups of isolates sharing six loci of their allelic profiles with at least one other member of the group. The method is less discriminatory than pulsed-field gel electrophoresis due to the high evolutionary stability in the housekeeping genes, but is useful due to its internationally standardized system and the possibility of interlaboratory comparison. [58, 59]. By using a computerized calculation tool Neighbor-joining trees could be drawn from MLST data and the relatedness is presented graphically in Fig.6.

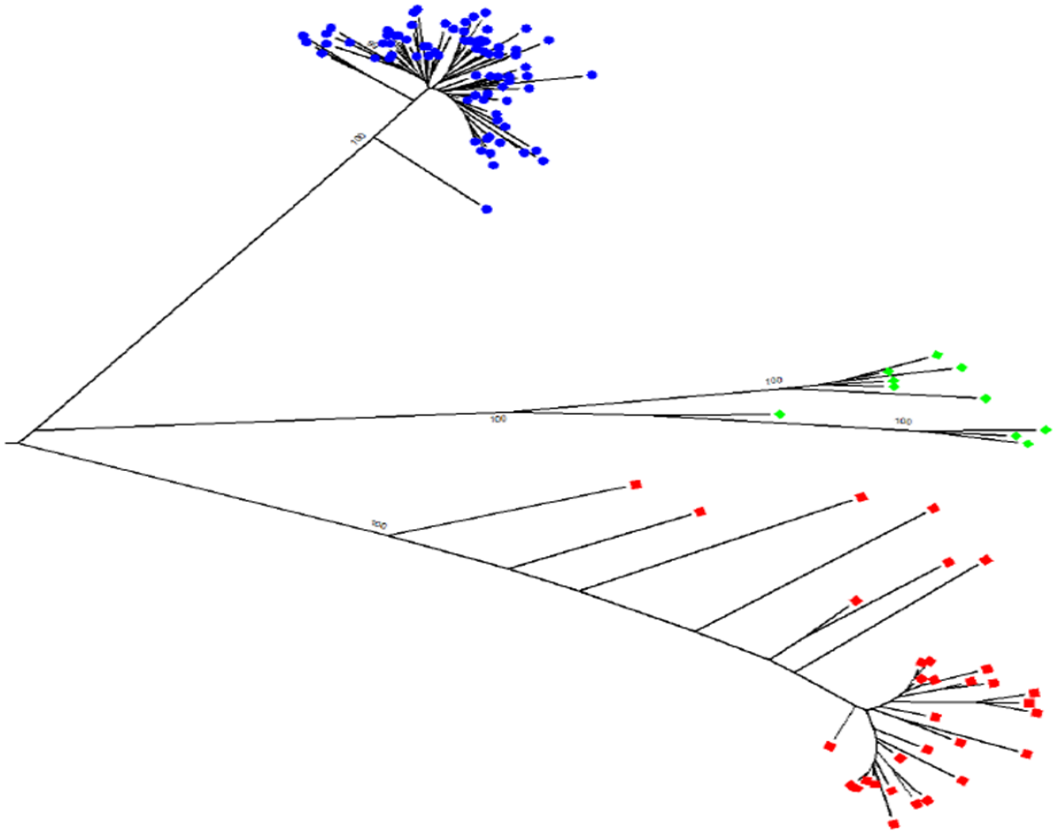


Figure 6A. *Phylogenetic representation of isolates. Phylogenetic group KpI (n=96): blue, KpII (n=9): green and KpIII: red (n=34). From paper* ***II****.*

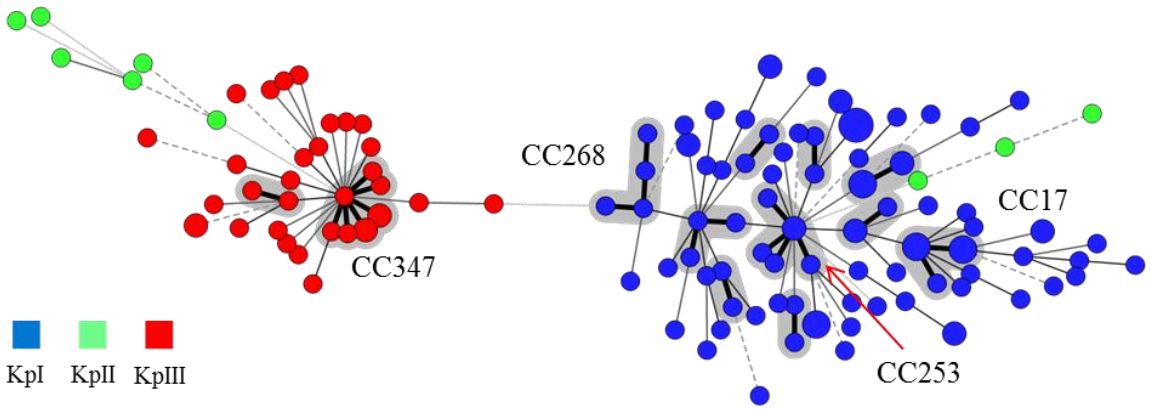


Figure 6B. *Minimal Spanning Tree (MST) based on MLST allelic profiles. The area of each circle corresponds to numbers of isolates. Black lines connect pairs of STs differing in one allele (thick line), two or three alleles (thin line) or four to seven alleles (dashed). Grey zones indicate a clonal complex. CC347, CC268, CC17 and CC253 were the most common clonal complexes (paper **II**).*

Most isolates belonged to phylogroup KpI (n=96), *K. pneumoniae*, followed by KpIII (n=34), *K. variicola*, and KpII (n=9), *K. quasipneumoniae* (Fig. 6). Hence the proportion of *K. variicola* was 24% in the study, relatively more common than reported in other studies [81, 99]. There was a high diversity among the isolates. 116 different STs were found, and the majority did not belong to any clonal complex (Fig. 6B).

Patients' characteristics showed no differences between the phylogroups. Mortality within 30 days was significantly higher among patients with BSI caused by *K. variicola* compared to *K. pneumoniae* , 29,4% versus 13,5% respectively (p=0.004, OR 3.0 (95% CI 1.1-8.4) adjusting for age and Charlson comorbidity index). One limitation was the small number of events (in total 24 out of 139 patients).

Few isolates contained any of the serotypes associated with invasive disease or harbored VFs. Presence of VFs did not differ between patients with a fatal or non-fatal outcome. Over all there were low levels of antibiotic resistance. Resistance to one or several classes of antibiotics used in treatment for gram-negative infections, was 19% in *K. pneumoniae* compared to 6% in *K. variicola* (p=0.08). Previous studies support higher rates of antimicrobial resistance in *K. pneumoniae* isolates compared to *K. variicola* [81, 99]. Five ESBL-producers were found, all belonged to phylogroup KpI, *K, pneumoniae*. This was too few isolates to draw any strong conclusion, but it was in accordance with a study by Valverde et al. determining phylogenetic structure of ESBL-producing *K. pneumoniae* where most isolates belonged to KpI [100]. Thus, none of the investigated strain factors could explain the higher mortality among patients infected with *K. variicola*. To determine the frequency of *K. variicola* in laboratories is still complicated on an everyday basis, since distinction is most often made with molecular methods. Hence *K. variicola* remains underreported and instead reported as *K. pneumoniae*. The poorer prognosis when infected by *K. variicola* (**II**) has not yet been confirmed by other and larger studies.

4 ENTEROBACTERIACEAE AND ANTIMICROBIAL RESISTANCE

"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

The discovery of penicillin by Alexander Fleming, first described in a publication in the late 1920s [101], later in the 1940s leading to production of penicillin G, was revolutionary. Highly mortal diseases as pneumococcal pneumonia and war-associated wounds were now possible to cure. Fleming was awarded with the Nobel Prize in 1945 for his discovery. Already in his speech (above) he warned about microbes developing antimicrobial resistance. Years of consumption and overuse of antibiotics, both among humans, in animal production and agriculture, have now led to a situation threatening modern health care.

Antimicrobial resistance (AMR), the ability of micro-organisms (bacteria, viruses, fungi and parasites) to withstand antimicrobial treatment, is considered by the World Health Organization (WHO) as one of the major human threats (www.who.int/drugresistance/en/). Fig.7 display the estimated annually deaths in 2050 attributable to AMR [102] according to the United Kingdom review on antimicrobial resistance 2014.

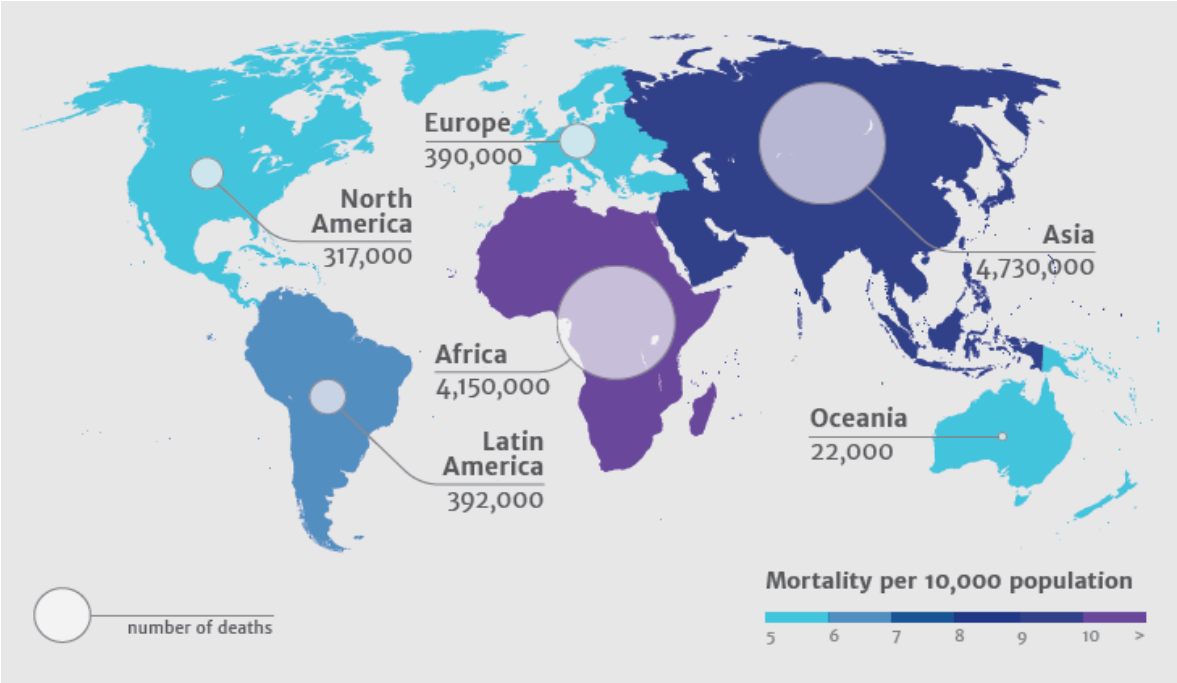


Figure 7. *Estimated number of annual deaths in year 2050 attributable to AMR (per continent) if development continues as now. From Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, 2014.*

Already to this date thousands of persons annually die from complications due to AMR. Increasing resistance not only jeopardizes treatment against infections caused by MDR, but also situations where effective antibiotics are required, such as surgical procedures, advanced cancer treatment and the care of neonates [103].

Plasmids serve as vectors for transmission of resistance genes, and facilitate the dissemination of ESBLs. They acquire mobile genetic elements such as insertion sequences and transposons that mobilize the antimicrobial resistance genes. Plasmids are circular DNA molecules that replicate independently of the bacterial chromosome. A plasmid can contain between one and several hundred of genes encoding proteins for their own transfer, and also for bacterial virulence, antibiotic resistance and metabolic functions. They are not essential for bacterial survival, but can be advantageous for the bacteria. As plasmids often contain genes encoding for resistance to several antibiotic classes, multiresistance is common among EPE. The most frequent antimicrobial groups showing co-resistance to EPE include the fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole [104, 105]. Replicons control the plasmid replication. The classification in incompatibility groups (Inc) is based on the principle that plasmids sharing the same replicon cannot be propagated stably in the same cell. IncFII, IncN and IncI are examples of plasmids often harboring genes conferring resistance in Enterobacteriaceae [106]. Replicon typing was not performed within this thesis.

4.1 PLASMID-MEDIATED B-LACTAM RESISTANCE

4.1.1 ESBL-producing Enterobacteriaceae – molecular features and dissemination

After the first description of ESBLs in Germany in 1983 [107], a large number of plasmid-mediated β-lactamases has been detected. Several different schemes have been used for the classification of β-lactamases. The classification proposed by Giske et al. in 2009 [108] is the most frequently used in Sweden and Norway (table 2).

Table 2. *Classification of ESBL enzymes and areas of endemicity*

Class of ESBL	**Ambler class**	**Enzymes**	**Phenotypic test**	**Areas of endemicity** [109-111]
$ESBL_A$	A	CTX-M, TEM/SHV-ESBL	Inhibited by clavulanic acid	Worldwide
$ESBL_M$	C	Plasmid-AmpC, mostly CMY	Inhibited by cloxacillin	
$ESBL_{CARBA}$	A	KPC	Synergy with boronic acid	Greece, Italy, Israel, Northern America, China
	B	NDM, IMP	Synergy with EDTA	Indian subcontinent, the Balkans, the Middle East
		VIM		Greece
	D	OXA-48	None available, temocillin R	Northern Africa, the Middle East

In initial reports the TEM- (Temoneira) and SHV- (Sulfhydryl variable) enzymes dominated, mainly in *K. pneumoniae*. The number of TEM and SHV variants is now more than 220 and 190 respectively (www.lahey.org/Studies/). CTX-M- (Cefotaximase-) enzymes were rare until the end of the 1980s. The CTX-M β-lactamases now exceed 170 different types (www.lahey.org/Studies/), and can be divided into five groups based on their amino acid identities: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. CTX-M-15 belongs to the CTX-M-1 group, and is the most common enzyme both globally and in Sweden [112] At first most dissemination was associated with *K. pneumoniae*, but with the CTX-M-enzymes, ESBL-producing enzymes were more commonly introduced in *E. coli*. This promoted rapid dissemination in the community and also made the EPE become an increasing problem in common community-acquired infections such as UTIs [104].
In 2007, the year that mandatory laboratory reporting of EPE was introduced in Sweden, almost 2100 new EPE-cases were detected. Since then the rate has increased every year, with over 9500 new cases during 2015 [113].

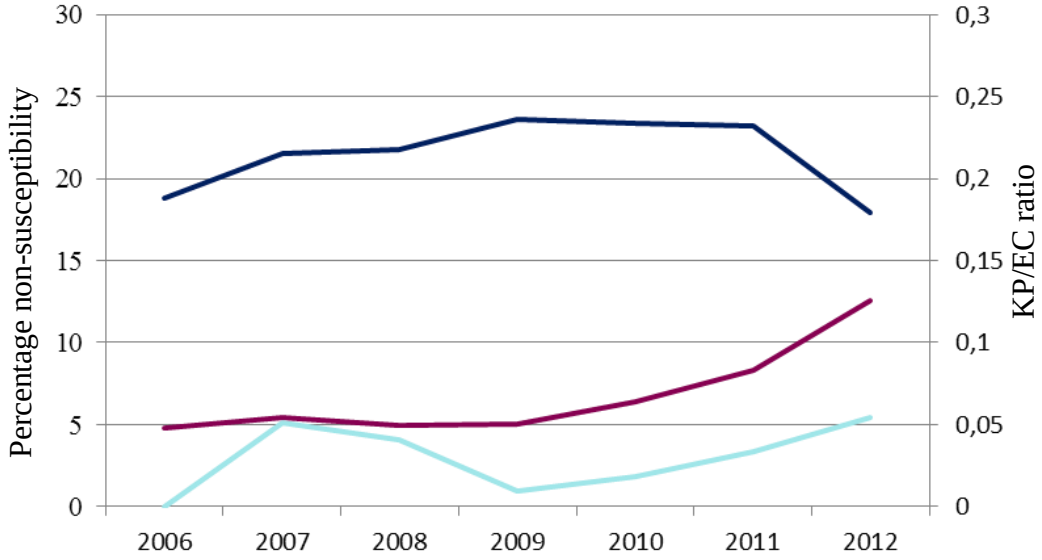
4.1.1.1 *Resistance in the Stockholm area versus Europe*

The European Antimicrobial Resistance Surveillance Network (EARS-Net) is an international network that collects clinical antibiotic susceptibility data from all 28 European Union (EU) member states and two European Economic Area (EEA) countries, Iceland and Norway. The network is coordinated by the European Centre for Disease Prevention and Control (ECDC).

To compare resistance rates in the *K. pneumoniae* and *E. coli* cohort from Karolinska University Hospital with data from European countries, data on invasive *K. pneumoniae* and *E. coli* isolates non-susceptible to third generation-cephalosporins were extracted from the ECDC database for the period 2006-2012 (paper **III**). To avoid sampling bias, countries reporting few isolates (a median of <300 *E. coli* isolates per year) were excluded, leaving 20 countries, including Sweden. Data from EARS-net were population-weighted due to adjust for imbalances in reporting propensity and population coverage.

The *E. coli* population among invasive infections at Karolinska University Hospital shows an increase of non-susceptibility to third generation cephalosporins during 2006-2012, from five to almost 13%, and the rate is comparable with the mean situation among the 20 included EARS-Net countries. In contrast, the *K. pneumoniae* population in the Stockholm area shows stable low rates of non-susceptibility between zero and five percent. This differs from the EARS-Net countries where more than 25% of the isolates were non-susceptible in 2012 (Fig 8, from paper **III**). The few countries reporting higher third-generation cephalosporin non-susceptibility in *E. coli* compared to *K. pneumoniae* all had generally low resistance frequencies, just as the cohort from Karolinska University Hospital [114]. The *K. pneumoniae / E. coli* (KP/EC) ratio was stable in the cohort from Karolinska University Hospital, while the KP/EC-ratio increased significantly (trend test $p<0.001$) over time in the EARS-Net data (Fig 8).

A. *Karolinska University Hospital*



B. *EU/EEA, 20 countries reporting to EARS-Net. Population-weighted data*

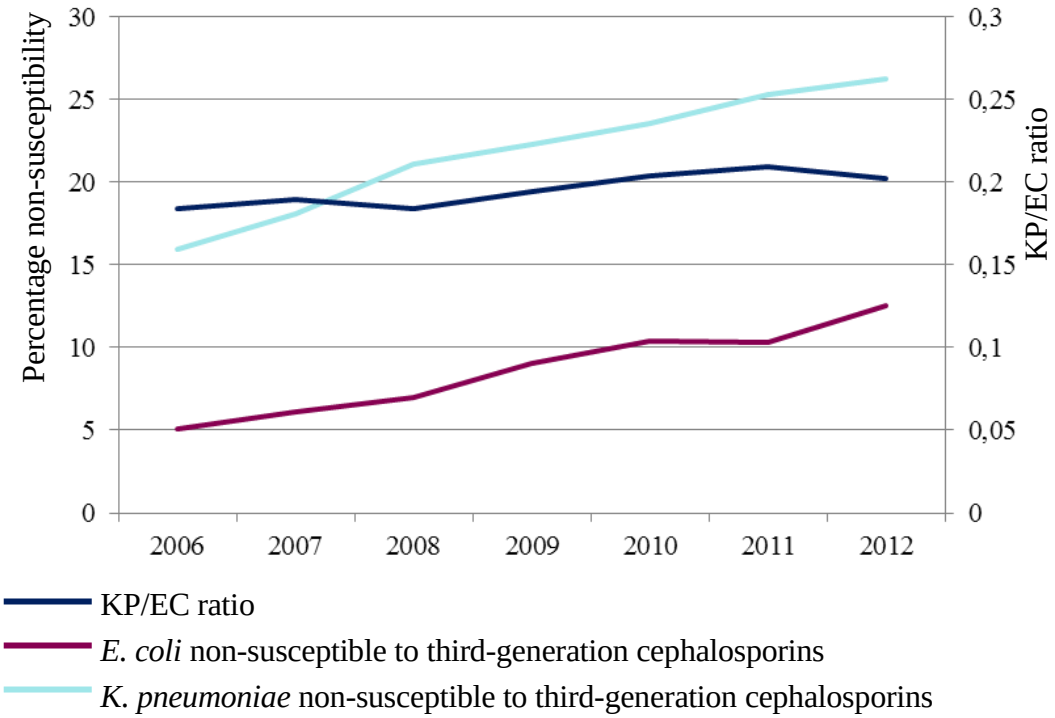


Figure 8. *Rates of invasive isolates non-susceptible to third generation cephalosporins among K. pneumoniae and E. coli 2006-2012, and KP/EC-ratio*

One explanation could be that there are no circulating MDR "high-risk clones" of *K. pneumoniae* in the Stockholm area. The isolates causing invasive infections between 2007 and 2009 (paper **II**) showed a high diversity and internationally disseminating successful clones, such as CC258, could not be detected. The data in Fig. 8 support the absence of high-

risk clones in the Stockholm area until 2012, as these would be expected to clonally expand and confer higher levels of resistance.

4.1.1.2 *Factors important for transmission of resistance*

Dissemination of EPE within a country and worldwide is multifactorial and includes humans, animals and environmental factors. Dissemination between household contacts has been shown in several studies [115, 116]. In a Norwegian study on infants colonized by EPE during an outbreak at the neonatal intensive care unit, transmission within households was observed in 9/28, 32% [32]. EPE has been detected as well in pets [117, 118], poultry and cattle [119] as in wild animals, for example birds [120]. In countries with high prevalence of intestinal EPE-colonization, dissemination occurs easily in the community. Except for the intestine in mammals and birds, several other reservoirs for EPE exist, for example sewage water and the surface of fruits and vegetables [121, 122]. ESBLs and the carbapenemase gene New Delhi metallo-β-lactamase (NDM-1) has even been reported in drinking water from New Delhi [123].

Prevalence of asymptomatic EPE carriership varies within different regions of the world. In Sweden the reported prevalence was 4.8% for samples collected 2012-2013 [6]. In Thailand a prevalence as high as 65.7% was reported from rural areas [124]. The carriership was 19% in a tribal area in India in a study from 2015 [125], while surveillance rectal swabs in outpatients presenting to pediatric oncology unit revealed high EPE carriership, 58.4%, with a rate of CPE carriers of 20.2% [126]. Altogether resistance rates over 50% against extended-spectrum cephalosporins have now been described in all WHO regions, albeit with considerable variation within the regions [127].

International travel has been associated with high frequency of EPE acquisition in several prospective studies. Traveling to southern Asia, including India, poses a particularly high risk. Diarrhea and use of antibiotics during the trip have been considered independent risk factors for acquisition. [29, 31, 128-132]. Paper **IV** is a prospective study on 188 Swedish travelers to four geographic areas with an expected high prevalence of EPE; Southeast Asia, the Indian subcontinent, Northern Africa and the Middle East (all travelers to this region went to Turkey). The study aimed to investigate the molecular features of EPE colonizing the gut after travel, and to determine risk factors for colonization. The travelers submitted one fecal sample before the trip and one upon return, and answered two questionnaires. EPE strains were characterized with molecular methods.

Our results support previous findings with in total 56 out of 175 (32%) pre-travel negative participants colonized with EPE upon return. Colonization rates varied with visited geographic region, highest after traveling to the Indian subcontinent (49%) and northern Africa (44%), followed by Southeast Asia (19%) and Turkey (10%). In accordance with previous studies antibiotic treatment and diarrhea during the trip were independent risk factors for colonization (OR 5.92 (**IV**), table 3. [29, 128, 129, 131, 133].

Table 3. *Risk factors for intestinal acquisition of EPE during travel. Multivariable analysis (paper **IV**).*

Characteristic	Odds ratio (95% CI)
Male sex	2.11 (0.96-4.65)
Age	1.00 (0.98-1.03)
Travel destination	
Southeast Asia	N/A
Indian subcontinent [a)]	5.62 (2.27-13.89)
Northern Africa [a)]	5.50 (1.78-16.94)
Turkey	0.81 (0.15-4.32)
Travelers' diarrhea	2.50 (1.04-6.03)
Antibiotics during trip	5.92 (1.27-27.20)
Chronic disease	0.27 (0.10-0.76)

Bold face = P≤0.05

[a)]In relation to Southeast Asia

The distribution of β-lactamases in relation to geographic region is displayed in Fig. 9 (**IV**). The enzyme distribution correspond to other studies on EPE in these regions with domination of CTX-M-1, and CTX-M-9 being frequently reported in Thailand [124, 132, 134]. CTX-M-9 has only rarely been described in India [135], but was aqcuired by two persons after traveling here (**IV**). The majority of strains were *E. coli* (n=65), the two remaining were *K. pneumoniae* (n=1) and *Citerobacter freundii* (n=1).

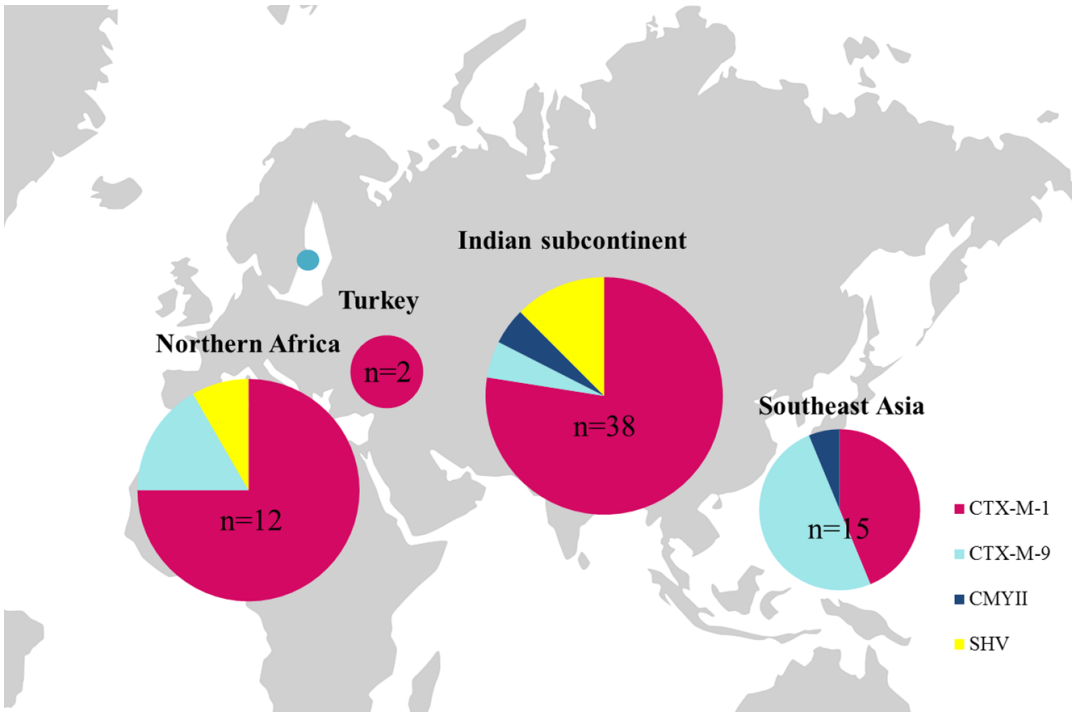


Figure 9. *Geographic distribution of ESBL-producing enzymes and total number of strains. Circle sizes correspond to acquisition rate (from paper **IV**).*

Bacterial characteristics of the ESBL-producing *E. coli* found in the travelers study are displayed in table 4. Strains of phylogroup B2 and D more often showed multidrug-resistance (MDR) than strains belonging to phylogroup A and B1.This is in contrast to other studies reporting higher resistance rates among non-B2-strains [57, 136]. However, these studies included not only ESBL-producing isolates. The dominating resistance gene conferring ESBL in clinical isolates detected in Sweden is $bla_{\text{CTX-M-15}}$, frequently carried by ST131 of phylogroup B2, known for epidemic dissemination and MDR. The distribution of phylogroups between the geografical regions differed – no B2-strains were detected in travelers to Northern Africa or Turkey. However, the small total number (n=8) needs to be considered.

Table 4. *Bacterial characteristics of ESBL-producing E. coli in relation to phylogroup. Modified from paper **IV**.*

	Phylogroup A (n=28)	**Phylogroup B1 (n=12)**	**Phylogroup B2 (n=8)**	**Phylogroup D (n=17)**
MDR *E. coli* (n=32)	10 (36.0)	4 (33.3)	7 (87.5)	11 (64.7)
Several (3 or more) VFs (n=5)	0 (0.0)	0 (0.0)	4 (50.0)	1 (5.9)
Destination				
Southeast Asia (n=15)	7 (25.0)	2 (16.7)	2 (25.0)	4 (23.5)
Indian subcontinent (n=36)	10 (35.7)	10 (83.3)	6 (75.0)	10 (58.8)
Northern Africa (n=12)	9 (32.1)	0 (0.0)	0 (0.0)	3 (17.6)
Turkey (n=2)	2 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)

4.1.1.3 *Duration of intestinal EPE colonization*

Duration of intestinal EPE colonization is individual and affected by both environmental- and host factors. Difficulties in detection of low bacterial numbers in a fecal sample makes data on elimination uncertain, as re-detection of the original strain can occur after several negative samples [137]. One study following colonization among newborns and their parents showed longer persistence among infants (median 12.5 months) than among parents (median 2.5 months) [32]. Colonization seems to persist longer after a clinical infection caused by EPE than among healthy gut-colonized individuals showing no symptoms of clinical infection. Persistence of carriership has been seen in up to 44% one year after infection. [30, 32] Only a limited amount of studies have followed asymptomatic EPE carriers. A recent French study from Ruppé et al [31] investigated the RA of multidrug-resistant Enterobacteriaceae in travelers to tropical regions, and followed the persistence of colonization. The overall acquisition rate was 51% upon return, but only 4.7% remained carriers after three months. Carriage lasted longer in travelers returning from Asia and in travelers with a high RA on return. In our study (**IV)** we did not evaluate duration of carriership. However we found that among 13 travelers with EPE colonization before traveling, EPE was found in only six travelers upon return. Identification of strains with repetitive sequence-based PCR (rep-PCR, DiversiLab) revealed that only four of the initial strains were recovered upon return, all of

them belonged to phylogroup B2. This phylogroup has also previously been connected with prolonged carriership [30]. The CTX-M-1 producing strain ST 131 belongs to this phylogroup, and its adaptation as a commensal as well as a pathogen is well known [23]. In paper **IV** 65 out of 67 EPE strains among the travelers were *E. coli*. Phylogrouping revealed that most strains (57/65, 88%) were non-B2, and that the non-B2 strains carried few VFs. Possibly this could indicate both low risk of future clinical infection and shorter colonization time during these asymptomatic travelers. No clinical infections were observed during 10-26 months of follow-up (**IV**). To my knowledge no studies evaluating RA in relation to bacterial characteristics has been performed, but it would be of interest to study if phylogroup B2 is connected to high RA.

4.2 CARBAPENEMASE-PRODUCING ENTEROBACTERIACEAE

1993 was the first time carbapenemase production was detected in Enterobacteriaceae. It was in a clinical isolate of *Enterobacter cloacae*, a chromosomally encoded *NmcA* [138]. Since then a large variety of plasmid-mediated CPEs have been identified. The carbapenemases belong to the Ambler class A, B and D, and the main ones are listed in table 2.

4.3 CPE IN SWEDEN

Although still rare among clinical infections in Sweden, carbapenemase-production among Enterobacteriaceae is rapidly increasing. In 2012, in addition to mandatory laboratory reporting, $ESBL_{CARBA}$ also became mandatory for clinicians to report. In 2015 there were totally 115 new reports of $ESBL_{CARBA}$, 43 of which from the Stockholm area (Fig. 10). Most cases were found in fecal screening samples. Only 24 out of the 94 cases in Sweden between 2007 and 2012 were isolates from clinical infections [139]. Most cases had recently had a health care contact in an endemic country. As in previous years, the proportion of the isolates acquired abroad during 2015 was almost 80%. During the last months of 2015 there was an increase in reports. Syria (n=19), followed by Turkey (n=11), India (n=7) and Greece (n=5) were the most frequently reported countries in 2015 [140].

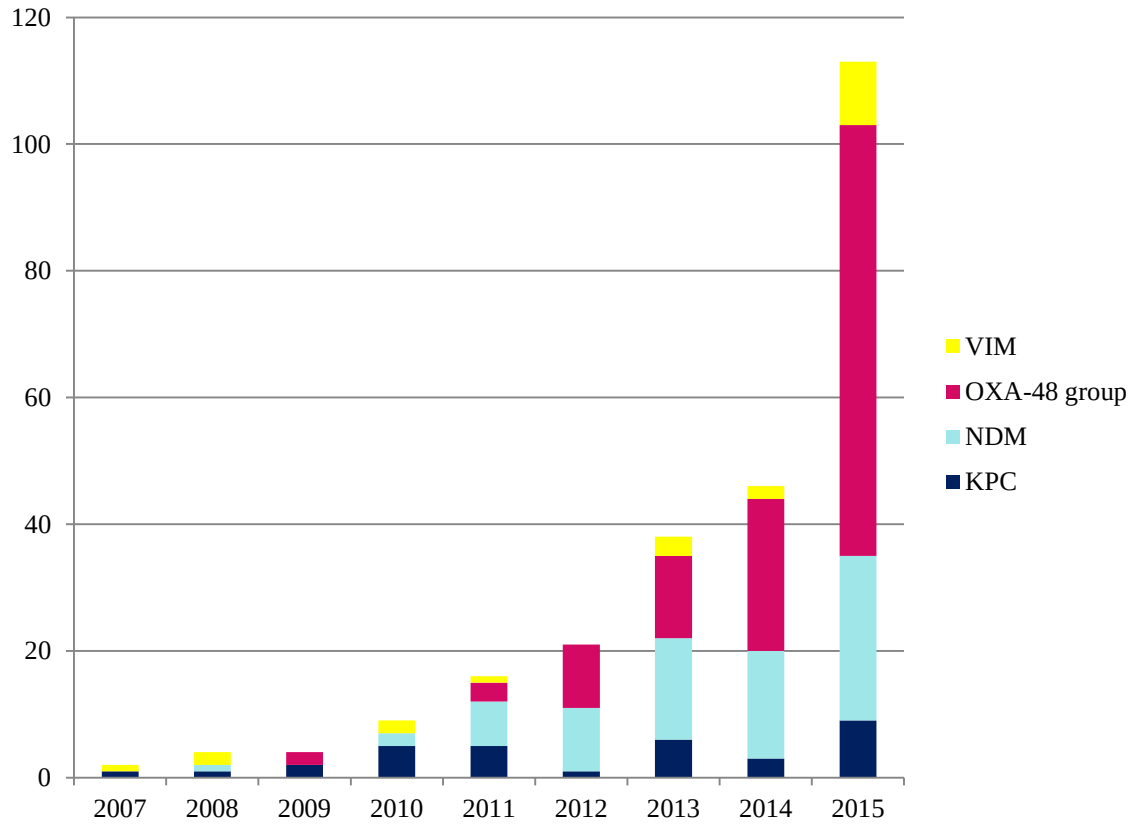


Figure 10. *Number of cases of carbapenemase-producing Enterobacteriaceae in Sweden and type of enzyme, 2007-2015 (SWEDRES 2014, 2015* www.folkhalsomyndigheten.se, *unpublished data). Due to some cases harboring several resistance genes, the figure does not display the exact number but the proportion within each enzyme group.*

4.3.1.1 *Global dissemination of CPE*

KPC enzymes were first detected in a *K. pneumoniae* isolate in the United States in 1996, showing resistance to all β-lactams. In the early 2000s epidemic outbreaks of CPE were reported from Greece, the USA, and later from Israel. KPC is now endemic in Greece, and the most common carbapenemase among Enterobacteriaceae in Europe, but have also disseminated in South America and China. [109, 141]. In data from EARS-net 62% of invasive *K. pneumoniae* isolates reported from Greece in 2014 were non-susceptible to carbapenems. In Italy, an outbreak of KPC among *K. pneumoniae* resulted in an increase of carbapenem resistance in invasive *K. pneumoniae* from one to 27 % between 2009 and 2011. In 2014 the rate had increased further up to 33 % (http://ecdc.europa.eu). KPC is most common in *K. pneumoniae* but can also be expressed by other Enterobacteriaceae as well as by *Pseudomonas aeruginosa*. KPC is associated with the worldwide disseminated ST258 [80].

The metallo-β-lactamases include IMP, VIM (Verona integron-encoded metallo-β-lactamase), and NDM (New Delhi metallo-β-lactamases) enzymes. IMP was first recognized in Japan in the end of the 1980s in *Pseudomonas aeruginosa* [142]. A VIM-positive isolate was first observed in *P. aeruginosa* in Verona, Italy, in late 1997 [143], and later

disseminated to other Enterobacteriaceae, especially *K. pneumoniae*. VIM is still common in Greece, but has been reported also from many other parts of the world [144].NDM-1 was described for the first time in 2010 [145], originating from the Indian subcontinent. NDMs are highly prevalent on the Indian subcontinent and in the Middle East with prevalence rates of NDM-producers among Enterobacteriaceae ranging from 5 to 18.5% in Indian and Pakistani hospitals. It has also been detected in drinking water and seepage samples in New Delhi [123]. NDM has been imported into European countries on several occasions, mainly detected in hospitalized patients transferred from endemic areas. As NDM is being reported not only in *K. pneumoniae*, but also in *E coli* they entail a high risk both for dissemination in the community, and to cause community-acquired infections. NDMs are also seen in *Acinetobacter* spp and, more rarely, in *P. aeruginosa* [146, 147].

Oxacillinase-type β-lactamase (OXA-48) enzyme was for the first time identified in a carbapenem-resistant *K. pneumoniae* in Turkey in 2001 [148]. After that OXA-48-like enzymes have emerged and disseminated in mainly Northern Africa and the Middle East, but also caused outbreaks in several European countries such as the UK, France, Germany, Belgium and the Netherlands [149]. They are still mostly detected among *K. pneumoniae*, but have also been reported in other species. Strains containing OXA-48-like enzymes often express only discrete elevation of carbapenem minimal inhibitory concentration (MIC) [149], challenging detection.

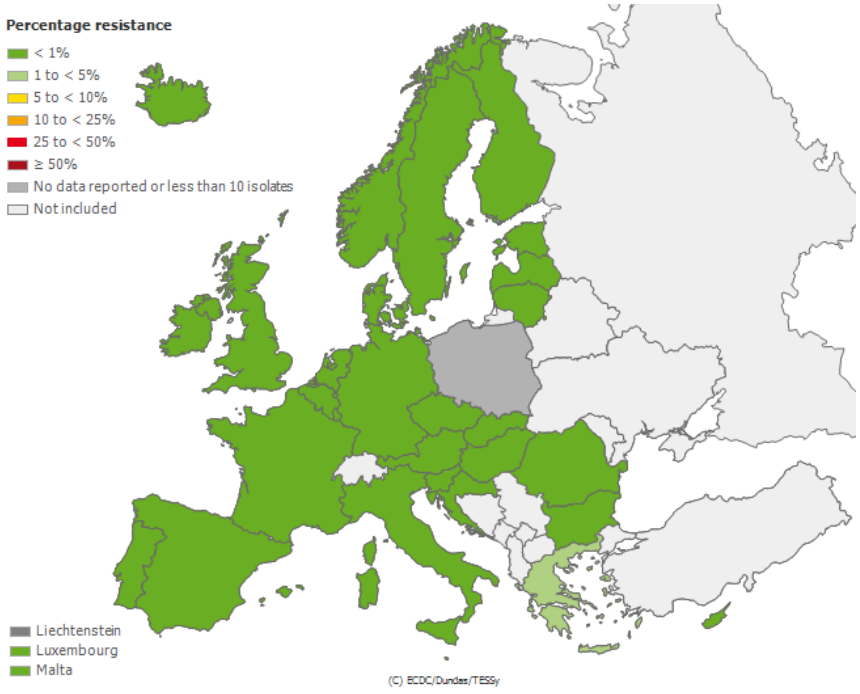


Figure 11A. *Invasive E.coli isolates with non-susceptibility (intermediate or resistant) to carbapenems, EU/EEA countries, 2014, percentage. (EARS-Net database 2014)*

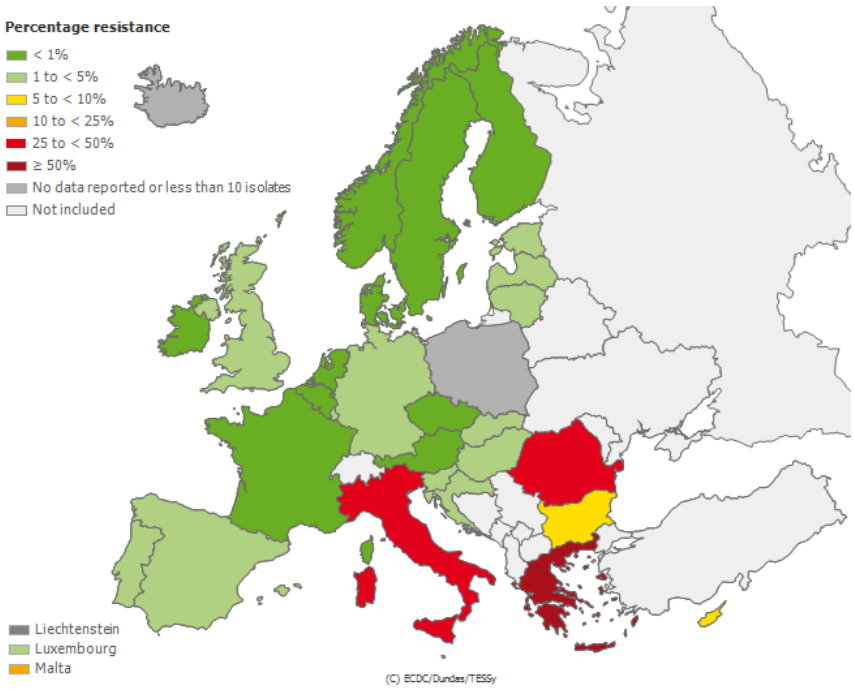


Figure 11B. *Invasive K. pneumoniae isolates with non-susceptibility (intermediate or resistant) to carbapenems, EU/EEA countries, 2014, percentage. (EARS-Net database 2014).*

Carbapenemase production is so far more common in *K. pneumoniae* than in *E. coli* isolates in most European countries (Fig. 11) as well as in other geographical regions. Although high rates of CPE are reported from the regions visited in paper **IV** [150], none of the travelers aqcuired CPE. To this date there are only few reports of healthy travelers acquiring carbapenemase-producers [151] . One possible explanation is that CPE still is more common among *K. pneumoniae* compared to *E. coli*, which is the bacteria most commonly aqcuired in the community. If carbapenem resistance rates increases in the *E. coli* population in the future, the dissemination in the community will probably speed up. Lack of effective therapies against common community-acquired infections caused by *E. coli* will also increase with such scenario.

4.4 PLASMID-MEDIATED NON B-LACTAM RESISTANCE

Plasmid-medicated co-resistance to other antibiotic groups often occurs in EPE. Some of the clinically most important are presented below. The modes of transmission of resistance genes between bacterial strains are similar to those of β-lactam resistance.

4.4.1 Plasmid-mediated aminoglycoside resistance

The most common mechanism is production of aminoglycoside modifying enzymes (AMEs). Several AMEs exist, the most common plasmid-mediated in Enterobacteriaceae is aminoglycoside acetyl-transferase, encoded by *Aac(6´)*. Expression of *Aac(6´)* can confer resistance both to amikacin and gentamicin, although not compulsory [152]. No isolates in our study harbored *Aac(6´)-Ib*, but 10 strains harbored *Aac(6´)-Ib-cr*, encoding a variant of aminoglycoside acetyl-transferase capable of inactivating aminoglycosides as well as

reducing ciprofloxacin activity. Only two out of these 10 isolates showed non-susceptibility against amikacin, while five were resistant against gentamicin (**IV**). The plasmid-mediated 16S rRNA methylase provides high-level resistance to aminoglycoside antibiotics including not only gentamicin, but also amikacin and tobramycin. There are several 16S methylase genes, including *armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD* [153]. The two isolates in our study (**IV**) resistant both to amikacin and gentamycin harbored *rmtB*.

4.4.2 Plasmid-mediated quinolone resistance

In 1998 *qnrA*, a plasmid-mediated quinolone resistance gene, was found for the first time in a urine specimen of a *K. pneumoniae* isolate [154].Up to now, three plasmid-mediated mechanisms of quinolone resistance have been described. The *Qnr* gene group encodes proteins that bind to DNA-gyrase or to topoisomerase IV inhibiting the mechanisms of quinolones. *Aac(6´)-Ib-cr* encoded aminoglycoside acetyl-transferase can reduce ciprofloxacin activity through acetylation, as well as inactivate aminoglycosides. *QepA* and *QqxAB* are plasmid- mediated genes upreglating efflux pumps. All of the 10 isolates harboring *aac (6')-Ib-cr* in our study (**IV**) were non-susceptible to ciprofloxacin. Only 10 out of 24 *qnr*-positive isolates were non-susceptible to ciprofloxacin, 2 of these strains also harbored *aac (6')-Ib-cr*. Thus presence of *qnr* does not always confer clinical resistance, which has also been shown previously [155].

4.4.3 Plasmid-mediated colistin resistance

Chromosomally mediated colistin resistance has been reported both in EPE and non EPE. In November 2015, a plasmid-mediated colistin resistance gene, *mcr-1*, was described in a Chinese study [156]. Expression of *mcr-1* contributes to modification of lipid A, resulting in reduced polymyxin affinity. The gene was detected in *E. coli* isolates both from animals, food and patients. Only a month after the first report, Hasman et al also reported a *mcr-1* positive *E. coli* isolate coproducing also CTX-M-55 and an AmpC (CMY-2). This isolate was detected in blood in a patient in Denmark. *Mcr-1* was also found in EPE from imported chicken meat [157]. Although recently detected, the *mcr-1* gene has been present for a long time. Positive isolates dating back to the 1980s have been found from chicken in China. The prevalence of *mcr-1* is still uncertain since most studies have only investigated *mcr-1* in colistin resistant isolates [158]. We reported the first case of *mcr-1* expression in Sweden (**IV**) from a traveler after a trip to Thailand. The traveler reported neither gastrointestinal symptoms nor antibiotic intake during the trip or on return. As the trip took place during late 2013, more cases of *mcr-1* gut colonization among Swedes most probably exist. Within the Public Health Agency's surveillance program for enterohaemorragic *E. coli* (EHEC), another *E. coli* strain harboring *mcr-1* have been detected in an asymptomatic Swede after traveling in Asia [159]. The future impact of *mcr-1* is too early to predict, but the gene is capable of transfer into rapidly disseminating epidemic EPE producing strains such as *E. coli* ST131. It has also recently been detected in a KPC-producer isolated from an infected wound [160]. The clinical consequences of dissemination of CPE harboring *mcr-1* could be devastating due to the risk of total lack of effective antibiotic treatment against such infections.

5 DETECTION OF CPE

Detection of CPE is complicated as paradoxically some CPE show only low levels of carbapenem resistance. Carbapenem resistance in Enterobacteriaceae can also be caused by other mechanisms than production of carbapenemases. Isolates producing $ESBL_A$ and AmpC can confer also carbapenem resistance when combined with chromosomal porin mutations that prevent influx of β-lactam antibiotics in the bacteria; they are not classified as carbapenemases. Reduced permeability is most often associated with higher cost of fitness than carbapenemase-production, hence EPE resistant also to carbapenems due to loss of porins can be challenging in a therapeutic way but poses less risk of further dissemination. Increased expression of efflux systems in bacteria under antibiotic pressure can also contribute to resistance, also a mechanism separated from carbapenemase production.

5.1 ANTIMICROBIAL SUSCEPTIBILITY TESTING

The goal of antimicrobial susceptibility testing is to predict the *in vivo* success or failure of antibiotic therapy. Either the minimal inhibitory concentration (MIC-value, mg/L), or zone size (mm) is measured. Bacterial susceptibility for a certain antibiotic is defined as:

S= susceptible. Isolates are inhibited by the achievable concentration of the antimicrobial agent when adequately dosed, clinical efficacy is likely.
I = intermediate. Uncertain effect of the certain antibiotic, response rates may be lower than for susceptible isolates and possibly low-grade resistance against the antibiotic. Clinical efficacy in body sites where the antimicrobials are physiologically concentrated or if the drug is given in higher dosage.
R= resistant. Isolates are not inhibited by the antimicrobial agent in achievable concentrations, clinical efficacy is unlikely.

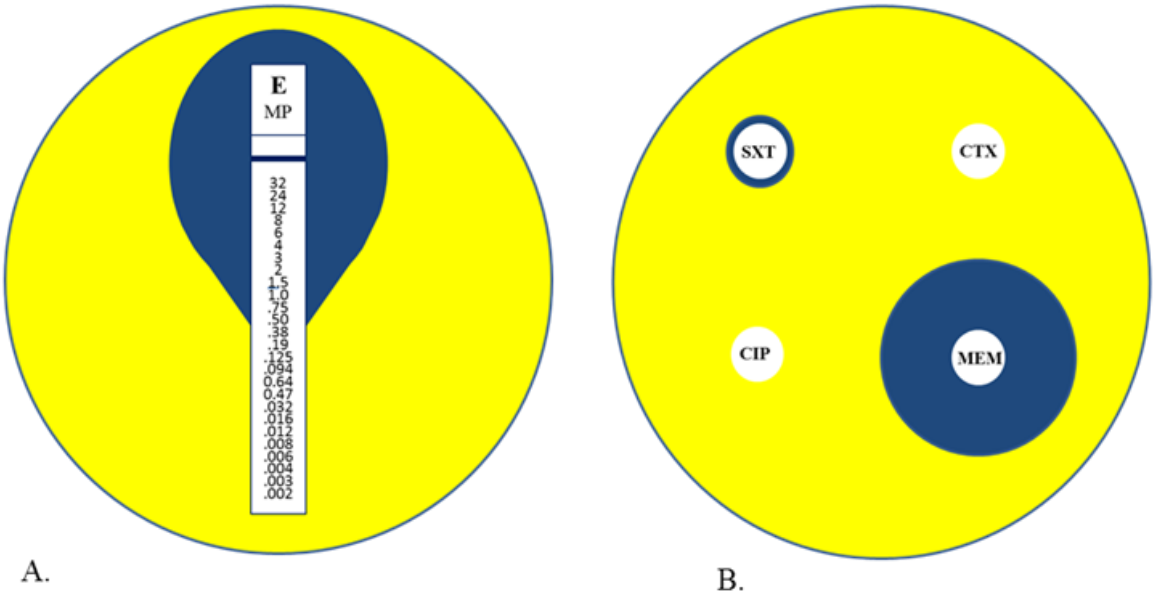
The MIC for a species varies depending on resistance mechanisms, but also within bacterial populations lacking resistance mechanisms against the specific antibiotic, the wild type (WT). The epidemiological cutoff values (ECOFFs) represent in most cases the upper MIC values of the WT distribution [161].

The MIC breakpoints are both used to predict clinical success in antimicrobial treatment against bacterial infections and for surveillance of antimicrobial resistance. When determining the MIC breakpoints several variables are taken into account – e.g. pharmacokinetics, pharmacodynamics, dosages, resistance mechanisms, MIC- and zone diameter distributions and ECOFFs [161].

5.1.1 Determination of the MIC-value

Different methods used to determine antibiotic susceptibility are presented in Fig. 12. Broth microdilution is the reference method according to the ISO standard 20776-1 (2006). A tray containing several wells is prepared with antibiotics diluted in broth in different concentrations before adding samples. After incubation MICs are determined by using manual or automated viewing device for inspection of each well. The MIC is similar to the

lowest antibiotic concentration where no bacterial growth is seen. In the gradient test, a strip containing antibiotic in a gradient concentration, is applied on inoculated plates. After incubation the MIC value is determined. Disk diffusion is widely used at the microbiological laboratories. Antibiotic discs are placed on plates inoculated with bacteria. After incubation the inhibiting zone is measured in mm. This method does not determine the actual MIC but small zones correspond to high a MIC-value, and it thus serves as a surrogate method [162].



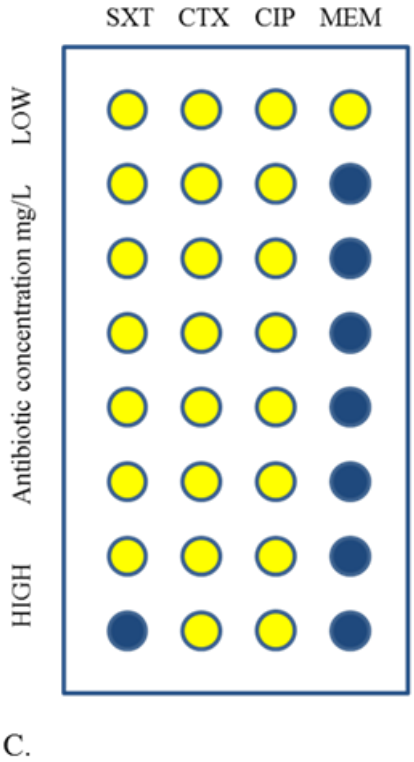


Figure 12. *Antimicrobial susceptibility testing using gradient test, disk diffusion and broth microdilution for a strain susceptible only to meropenem.*

A. The MIC-value is interpreted where the bacterial growth is inhibited, here at a value of 0.5 mg/L.

B. Higher antibiotic concentration close to the antibiotic disks inhibit bacterial growth and the inhibited bacterial zone (dark blue) diameter is measured in mm.

C. Broth microdilution showing bacterial growth (yellow), the lowest antibiotic concentration in a well showing non-growth (dark blue) is the MIC-value.

Different Automated Susceptibility Testing systems (ASTs) have been developed the last years. VITEK2 (bioMérieux), Phoenix, and MicroScan (Siemens) are systems using panels prepared with antibiotics in different gradients. With automated software results can be obtained within a few hours [162].

5.1.2 Challenges in detection of CPE

Rapid detection of carbapenem-resistance in Enterobacteriaceae is of importance. Most empiric antibiotic treatment against severe infections suspected to be caused by gram-negative bacteria, include the use of β-lactams. Since most CPE-isolates are highly resistant to β-lactams including carbapenems, empiric antibiotic therapy is often delayed. Early adequate treatment is crucial for prognosis in severe infections [163].

For epidemiological reasons, and to guide infection control interventions preventing further dissemination, reliable detection of carbapenemases is of major importance. When initializing the first project included in this thesis, it was known that CPE can express carbapenem resistance in different degrees, ranging from resistant to susceptible using the European Committee of Antimicrobial Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI) clinical breakpoints. As carbapenemase expression vary, breakpoints used for treatment of clinical infections were not adequate to use in epidemiological screening. In order to find breakpoints for epidemiological screening we studied 51 isolates of *K. pneumoniae* with a known production of either KPC (n=31) or VIM (n=20) in paper **I**. At the time of the study KPC and VIM were the clinically most important carbapenemases. Antibiotic susceptibility testing was performed with disk diffusion, gradient test (Etest) and automated susceptibility testing (VITEK2) against the carbapenems imipenem, meropenem and ertapenem. The results were interpreted with the, at that time used, clinical EUCAST and CLSI breakpoints for carbapenems as well as EUCASTs ECOFF values (Fig. 13).

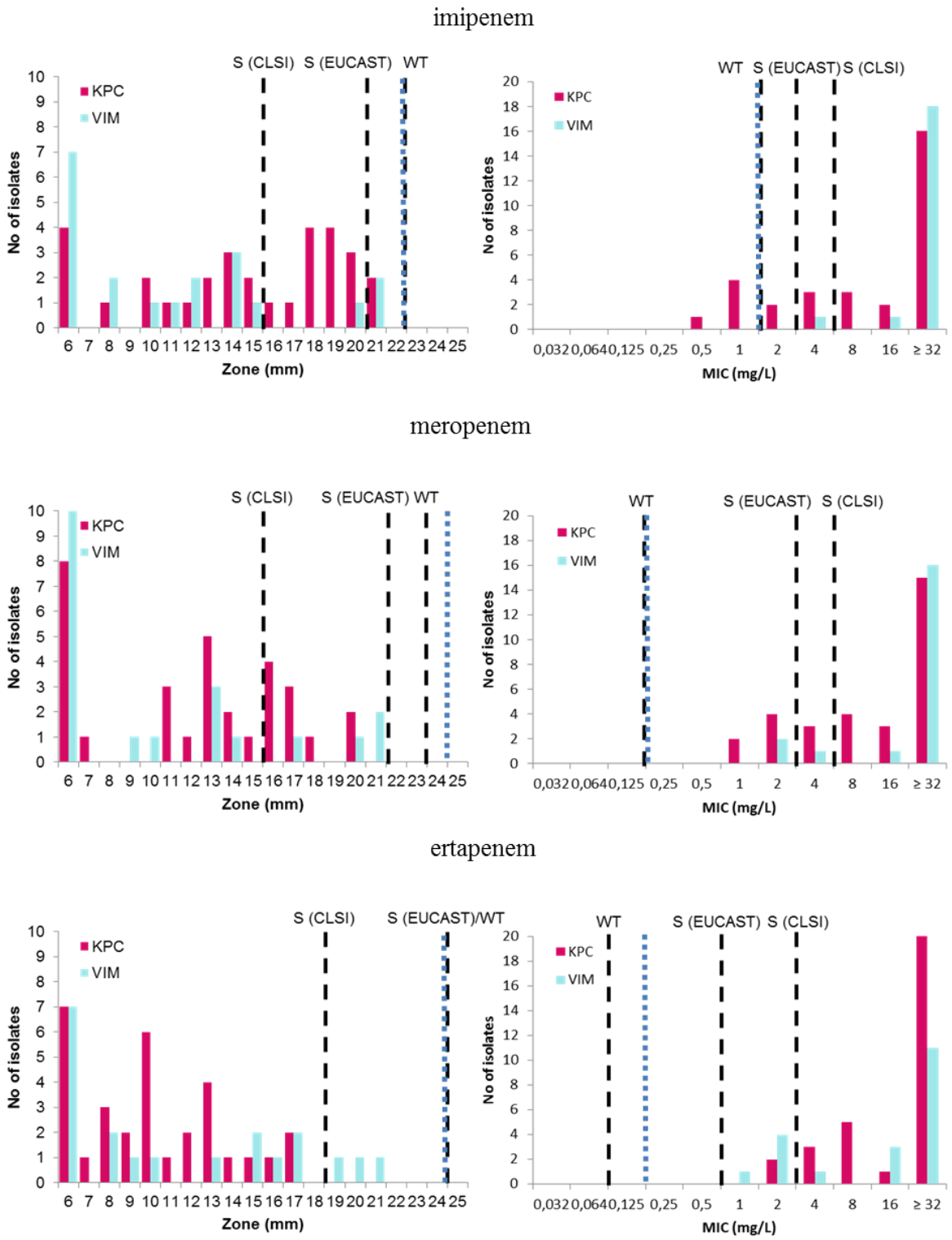


Figure 13. *Susceptibility testing using disk diffusion and gradient test for the carbapenems imipenem, meropenem and ertapenem. The dashed black lines indicate the ECOFF (WT), EUCAST, and CLSIs clinical breakpoints before 2011 (paper **I**). The dashed blue lines indicate the current EUCAST screening cut-off.*

As seen in Fig. 13, the clinical breakpoints used at the time the study was performed are not suitable for epidemiological screening. Meropenem was the best suited carbapenem to use for

screening purposes. A breakpoint of 0.5 mg/L found all isolates with an at the same time good separation from the WT.

After this study was performed another carbapenemase genotype, OXA-48, has emerged. Strains containing OXA-48-like enzymes often express only discrete elevation of carbapenem MICs [149]. Carbapenemase-production has also been introduced in *E. coli*, where MICs usually are below the clinical cut-off values [164]. The diversity of enzymes conferring carbapenemase-resistance in different species has made detection even more challenging.

Previous studies show that ertapenem has high sensitivity but poor specificity, due to impermeability caused by loss of porins, and simultaneous β-lactamase production other than carbapenemases [165-167]. As seen in Fig. 13 imipenem shows a poor separation between the WT and the cut-off value. The best performing carbapenem used in screening is therefore meropenem.

CLSI published new (lowered) MIC breakpoints in January 2011 to capture mainly KPC-producers with the clinical breakpoints. EUCASTs breakpoints are not set for detection of carbapenemases but to be used as clinical breakpoints to predict treatment response. Due to the large span of MIC among CPE, EUCAST has defined a specific cut-off used for screening purposes (table 5).

Table 5. *CLSI and EUCAST current breakpoints for Enterobacteriaceae and carbapenems (http://em100.edaptivedocs.com, http://eucast.org/clinical_breakpoints/)*

	CLSI		**EUCAST**		**ECOFF**	**Screening cut-off (EUCAST)**
Gradient test (Etest)	S MIC (mg/L)	R MIC (mg/L)	S MIC (mg/L)	R MIC (mg/L)	WT MIC (mg/L)	mg/L
Imipenem	≤1	≥4	≤2	>8	≤0.5*≤1**	>1
Meropenem	≤1	≥4	≤2	>8	≤.0.125	>0.125
Ertapenem	≤0.5	≥2	≤0.5	>1	≤0.064	>0.125
Disk diffusion	S Zone diameter (mm)	R Zone diameter (mm)	S Zone diameter (mm)	R Zone diameter (mm)	WT Zone diameter (mm)	
Imipenem	≥ 23	≤ 19	≥22	<16	≥24*≥23**	<23
Meropenem	≥ 23	≤ 19	≥22	<16	≥25	<25***
Ertapenem	≥ 23	≤ 19	≥25	<22	≥29*≥25**	<25

**E. coli*

***K. pneumoniae*

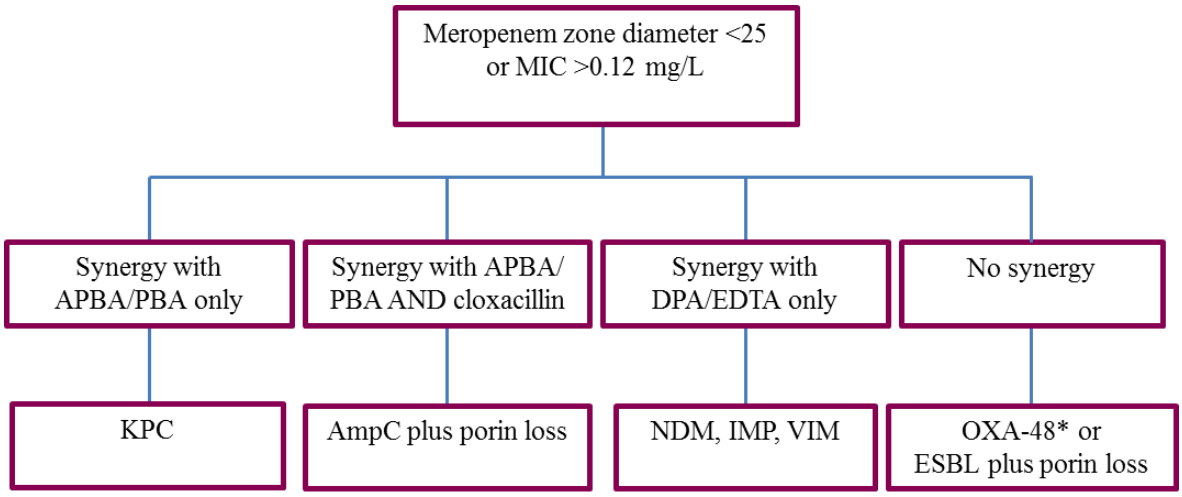
***During outbreaks of OXA-48-producers elevated screening zone diameter can be proposed as OXA-48 in rare cases have zone diameters of 24-26 mm

Using VITEK2 four KPC-producers were not detected when using a card containing imipenem as the only carbapenem (AST N027) (**I**). Woodford et al. compared how well different commercial systems (BD Phoenix, Microscan and VITEK2) inferred the presence of a carbapenemase. Their study showed poor detection of OXA-48-producers but more reliable detection with KPC and metallo-carbapenemases [168]. Automated systems can be useful tools for CPE detection but results must be compared with the carbapenem MIC correlates, and appropriate phenotypic and/or genotypic methods should be used to exclude CPE if carbapenem MIC correlates are above the lower detection limit in the card.

Determining the cut-off value for screening for CPE is a balance striving for high sensitivity in combination with highest possible specificity. However, since carbapenemases even with low carbapenem MICs are important to detect for infection control purposes, sensitivity is of greater importance than specificity. In a recent study [164] application of CLSI screening recommendations captured only 86% of CPE isolates, whereas using the EUCAST recommendations showed a high sensitivity, 98.4%. In our study (**I**) the EUCAST screening cut-of for meropenem would detect all isolates, while the current CLSI MIC breakpoints for meropenem would interpret 49 out of 51 (96%) of the isolates as non-susceptible (I or R). A high sensitivity, however, our study contained only KPC and VIM-producing *K. pneumoniae* isolates.

5.2 PHENOTYPING AND GENOTYPING

For detection of carbapenemases, phenotypic methods are applied on isolates with reduced susceptibility to carbapenems. As different enzymes conferring carbapenem-resistance show different phenotypes, EUCAST has proposed an algorithm for detection of carbapenemases as displayed in Fig. 13.



*OXA-48, temocillin R

Figure 13. *Algorithm proposed by EUCAST for detection of CPE.*

The presence of OXA-48-like enzymes is confirmed with genotypic methods as there are no available inhibitors at present. Isolates carrying OXA-48-like enzymes show high-level resistance to temocillin (MIC >32 mg/L), however temocillin has low specificity as other mechanism can confer resistance against the antibiotic [169]. To confirm genotype among CPE, most often PCR is performed targeting common enzymes.

5.3 FUTURE DIAGNOSTICS

Direct molecular detection of carbapenemases is appealing due to the time gain in detection. There are some commercial systems for direct molecular detection in feces available, most suitable for high-prevalence areas or during outbreaks [170-172]. One limitation of these methods is that they can only detect the genotypes included in the analysis for which reason novel or rare genotypes will remain undetected.

Whole genome sequencing (WGS), the most comprehensive method for analyzing bacterial genomics, can be performed with different methods. Next-generation sequencing is a rapid method based on pyrosequencing. In the future WGS will most likely be more frequently used in routine diagnostic settings, for surveillance of antimicrobial resistance, contract tracing and analysis of outbreaks [173, 174]. The advantages of WGS (and publicly available sequence databases) are exemplified in the case of *mcr-1*, where knowledge has rapidly expanded within just a few months after the first description.

6 TREATMENT OF INVASIVE INFECTIONS CAUSED BY CPE

Implications on antibiotic treatment of CPE were not included in this thesis, but will be described in short. Invasive infections caused by CPE are associated with high mortality, ranging from 20 to 70% [175] and the death attributable to carbapenem-resistance has been calculated to 26-44 % [5]. The lack of therapeutic options in infections caused by MDR Enterobacteriaceae has led to the reintroduction of old antibiotic agents. New antibiotic classes with novel mechanisms of action against gram-negative infections are not to be expected in the near future. Treatment regimens against CPE are debated and the literature consists mostly of small retrospective studies or case-reports, where either monotherapy or different combination therapies have been used. In summary most studies favor combination treatment always including a carbapenem if MIC≤ 4 (-8) mg/L plus at least one of the antibiotics listed below depending on susceptibility [176-179].

6.1 CARBAPENEMS

The carbapenems (e.g. imipenem, meropenem, ertapenem and doripenem) are β-lactam antibiotics stable to $ESBL_A$ and $-_M$. They possess the widest spectrum of antibacterial activity covering most gram-positive and gram-negative aerobic and anaerobic bacteria and play an important role in the treatment against severe infections caused by EPE. Reduced membrane permeability due to loss of porins and efflux pumps in combination with ESBL-production can however cause a carbapenem resistant phenotype. CPE hydrolyze also carbapenems, but not all CPE express high-level resistance. The use of a carbapenem in combination therapy against severe infections caused by CPE has shown improved outcome when carbapenem MIC-values are ≤ 4 and even up to 8 mg/L [176, 178].

6.2 COLISTIN

Colistin is a polypeptide antibiotic discovered in 1947, but rarely used until lately, primarily due to kidney toxicity. Now though, due to MDR, it has been revived as the drug of choice in treatment against infections caused by CPE. The target of action is the lipopolysaccharide, where destabilization of the outer membrane of gram-negative bacteria causes increased permeability and cell death. However, resistance rates are increasing in areas where the agent is extensively used. From Italy colistin resistance rates of >40% have been reported in KPC producers [180]. Used in monotherapy, high mortality rates of 50% are reported [177]. Combination therapies with either meropenem, tigecycline or an aminoglycoside, or combination of several of these agents, have been more successful [178, 179].

6.3 FOSFOMYCIN

Fosfomycin acts on bacterial cell wall synthesis, conferring bactericidal effect on a broad spectrum of gram-positive and gram-negative bacteria. It shows effect against most CPE-producers. Resistance due to plasmid-mediated enzymatic modification of the antibiotic is now emerging, and resistance rates are increasing, especially in areas where the agent is

widely used. However, development of resistance in clinical studies appears lower than rates expected from *in vitro* data [181, 182]. Clinical studies on fosfomycin are scarce in invasive infections caused by CPE, but one study show effect when adding fosfomycin in severe CPE infections [183]. Fosfomycin can be both orally and intravenously administrated. Intravenous fosfomycin is presently not available in Sweden.

6.4 TIGECYCLINE

Tigecycline is a tetracycline derivative. Low renal excretion limits the usefulness in infections originating from the urinary tract. Despite the high susceptibility rates among ESBLs including carbapenemase-producing *E. coli*, tigecycline has limitations in the treatment of severe infections due to its bacteriostatic effect. Breakthrough bacteremia has been reported during treatment against VIM-producing *K. pneumoniae* susceptible to the agent, as well as increased mortality in clinical studies. Higher dosing regimens have been proposed as low serum concentrations occur with standard dosing. Tigecycline is not recommended as monotherapy unless other options are lacking [184, 185].

6.5 AMINOGLYCOSIDES

Aminoglycosides are bactericidal agents, inhibiting bacterial protein synthesis. They are used for treatment of infections in the urinary tract, where the antibiotic concentration is high, but their efficacy in severe infections of other source than the urinary tract is more limited. Some CPE are susceptible to gentamycin, and a few to amikacin. In case of susceptibility an aminoglycoside can be used in combination therapy against infections caused by CPE [186, 187].

6.6 NEW AGENTS

New antibiotic classes acting against CPE are not to be expected in the near future. However, some new compounds within already existing classes are recently registered or under investigation in clinical trials.

Plazmomicin is a semisynthetic derivate of sisomicin, an aminoglycoside with improved activity against amikacin- or gentamicin-resistant strains. It has shown high in vitro effect against CPE, however, strains harboring ArmA or RmtC 16S RNA methyltransferase (mainly NDM-producers) are resistant. A phase III trial is currently ongoing comparing the agent with colistin when combined with a second antibiotic (either meropenem or tigecycline) in the treatment of patients with BSI, hospital acquired bacterial pneumonia, or ventilator-associated bacterial pneumonia due to CPE [188][186, 189].

Avibactam is a β-lactamase-inhibitor that, in combination with ceftazidime, has been clinically available in the USA since February 2015. The indications are complicated intra-abdominal infections (in combination with metronidazole), and complicated urinary tract infections (including pyelonephritis), with limited or no alternative treatment options. *In vitro* avibactam can inhibit the activity of Ambler class A, C and D, but not class B metallo-β-

lactamases (NDM, VIM or IMP). Addition of avibactam to ceftazidime greatly reduces the MIC value for most Enterobacteriaceae isolates compared with ceftazidime alone [179, 190].

Eravacycline is a tetracycline derivate that inhibits protein synthesis. It shows activity against KPCs. Phase III studies comparing efficacy versus carbapenems in intraabdominal infections and versus levofloxacin in UTIs are ongoing [191].

7 FUTURE

Antibiotics reduce mortality in severe infections. Efficient antibiotics are a prerequisite for modern health care. New antibiotic classes against gram-negative infections are not to be expected in the near future, why the currently available must be used in a rational way, considering the growing challenge from antibiotic resistance.

Prescribing patterns vary worldwide and mirror the level of antimicrobial resistance. In Europe high-prescribing countries have higher prevalence of antimicrobial resistance and vice versa. Greece showed the highest prescription rates of antibiotics in the primary care sector during 2014, 34.0 daily delivered doses (DDD) per 1000 inhabitants and per day. This is more than three times as much as the lowest prescribing country, the Netherlands, with a prescription rate of 10.6 DDD per 1000 inhabitants and per day. In Sweden the rate was 13.0 DDD per 1000 inhabitants and per day in 2014 (http://ecdc.europa.eu). Increased use of broad-spectrum antibiotics due to high prevalence of MDR doubtlessly leads to further selection of resistant strains. The regional differences in prescribing patterns are substantial even within Sweden, where Stockholm County has one of the country's highest prescriptions rates of antibiotics. However, between 2009 and 2015 the number of prescriptions decreased with 18%, from 430 to 352 per year and 1000 inhabitants (Concise, e-health Authority, www.ehalsomyndigheten.se), still over-prescription can be further reduced.

To limit the use of antibiotics therapeutic alternatives are sought in the treatment of uncomplicated infections. One example is in sporadic urinary tract infections, where antibiotics currently mainly are offered as symptomatic treatment in healthy individuals. A German study on 80 patients with cystitis showed no inferiority in relief of symptoms using ibuprofen compared to ciprofloxacin [192].

The intestine serves as a huge reservoir for resistant Enterobacteriaceae. Different attempts to affect the intestinal microbiota have been studied in order to eliminate colonization. Selective digestive or oral decontamination (SDD, SOD) - administration of topical and/or oral antibiotics - has been used in ICUs to prevent from invasive infections. A recent meta-analysis supports its use [193]. Colistin and aminoglycosides are common components in SDD due to their gram-negative profile, their sparse effect on anaerobes, and their minimal systemic uptake when orally administered. However, several studies report increased colistin and aminoglycoside resistance during or after the use of SDD [194-196]. The recent finding of *mcr-1* has again put focus to the question in order not to jeopardize possible treatment alternatives against multidrug-resistant gram-negative infections. In studies on decolonization of intestinal EPE carriers where SDD has shown effect, it has not been persistent [197, 198].

Another approach is fecal transplantation, a treatment proven efficient against severe clostridial infections. Fecal microbiota transplantation is reconstitution of normal microbiota from a healthy donor [85, 199]. The mode of administration has prevented large-scale use why new packages are under development. In American pilot study 20 patients with recurrent diarrhea caused by *C. difficile* received capsulized frozen fecal microbiota. The cure rate was

70 and 95% with one and two treatment courses and no adverse events occurred [200]. Combining SDD and fecal transplantation is a theoretically interesting approach, but to my knowledge, no such study has yet been published. If SDD is to be used in order to eliminate EPE carriership, patients must be selected carefully, as the choice of antimicrobial agent. A carrier of a more pathogenic strain is probably at higher risk for a future infection caused by that strain than a carrier of a strain without factors of pathogenesis. However, as plasmids share genetic information both within the species to bacteria with other phylogenetic origin, and to other species, there is a great complexity. No studies evaluating RA and phylogenetic origin of EPE have, to my knowledge, yet been performed, but it would be of interest to see if there is a link, as it is easier to determine phylogroup than RA.

Further studies on risk assessment of infections caused by EPE when colonized are needed in order to prevent the overuse of broad-spectrum antibiotics ”just in case”. Studies on how often a gram-negative BSI is caused by EPE versus non EPE when a patient is colonized with ESBL-producing bacteria could be one approach.

Lastly, the recently discovered (but not new) plasmid-mediated colistin resistance gene *mcr-1* exemplifies the difficulties and challenges that are to be expected in the future in the combat against the explosive dissemination of antimicrobial resistance.

8 CONCLUSIONS

Overall this thesis provides new insights in invasive infections related to *K. pneumoniae* and sister species, increased knowledge on detection of CPE and on molecular characteristics of EPE colonizing the intestine after travel. More specifically:

- Meropenem is the most suitable carbapenem to use in epidemiological screening for KPC- and VIM-producing *K. pneumoniae*.
- There are currently no signs of dissemination of high virulent *K. pneumoniae* clones in the Stockholm area.
- *K. variicola* (*K. pneumoniae* phylogroup KpIII), is a frequent pathogen among invasive infections diagnosed as *K. pneumoniae,* and associated with higher mortality than phylogroup KpI (*K. pneumoniae sensu stricto*).
- Invasive infection caused by *K. pneumoniae* affects patients with high comorbidity and mortality within 90 days is high due to patient factors.
- The risk for intestinal acquisition of EPE is high when traveling to the Indian subcontinent and Northern Africa, and also of importance when traveling to Southeast Asia and Turkey.
- The risk for acquisition of CPE is still low even when traveling to areas with an estimated high prevalence of CPE.
- Traveler's diarrhea and antimicrobial treatment are both independent risk factors for intestinal EPE acquisition.
- Phylogroup A and B1, normally commensal strains, are in majority among gut-acquired EPE during travel; strains host few virulence factors and are less co-resistant than clinical isolates.
- Plasmid-mediated colistin resistance gene, *mcr-1*, has now been detected in Sweden, after acquisition in Thailand.

Hopefully the studies in this thesis contribute to reliable detection of CPE, a deeper understanding of the molecular features of EPE as a gut colonizer, and new insights in *K. variicola* as a pathogen with substantial mortality in invasive infections.

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