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Transmission, intestinal colonization and host response

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ESBL-producing *Enterobacterales*

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— Transmission, intestinal colonization and host response

Oskar Ljungquist

LUND
UNIVERSITY

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Abstract

The prevalence of colonization and incidence of infections with bacteria producing extended spectrum beta-lactamases (ESBL) has steadily increased during the past decades. Antimicrobial resistance, such as ESBL-producing bacteria, are recognized by the World Health Organization (WHO) as a major health care concern. The acquisition and subsequent colonization of ESBL-producing *Enterobacterales* (EPE) is a prerequisite for developing such infections. Even though ESBL-producing *Enterobacterales* have been extensively studied, there are still several important knowledge gaps; such as questions on the average duration of intestinal EPE colonization and on what factors that affect prolonged colonization in a low-endemic setting. Moreover, knowledge whether colonization can be eradicated by specific drugs or therapy is lacking. In this thesis, I want to explore the epidemiology of EPE further, investigate risk factors for a prolonged colonization of EPE and examine a possible eradication therapy.

In Paper I, we performed a cross sectional study to examine whether, and to what extent, EPE could be transferred between humans and dogs of the same household. The dogs of owners with known EPE colonization/infection were selectively cultured for EPE and compared to a control group consisting of dogs of owners without EPE carriage/infection. For 2 of 22 households studied in Paper I, identical EPE strains with respect to bacterial species, antibiogram, genotype, and multiple locus VNTR analysis (MLVA) type were found in humans and dogs. We have thus shown that transfer of EPE can and does occur, albeit to a limited extent and of unknown directionality, between humans and dogs of the same household.

In Paper II, we performed a prospective cohort study of patients with known EPE colonization/infection, who were selectively cultured for EPE at least three months after the initial positive EPE culture. To identify risk factors for prolonged colonization, characteristics of patients who remained carriers were compared to individuals with a negative EPE culture. The data was analysed using multivariate logistic regression. We found that urological intervention within 6 months and a history of EPE infection were independently associated with prolonged intestinal colonization with EPE. Furthermore, we observed that 50 % of the patients in this study were not adequately informed about their EPE status by their treating physician.

In Paper III, we performed a randomized, placebo-controlled, single-blinded clinical superiority trial. In a parallel group trial design, we compared whether the probiotic Vivomixx® could eradicate EPE colonization in chronic EPE carriers as compared to placebo. There was no statistical difference in EPE eradication with Vivomixx® as compared to placebo, but the study was limited due to missing outcome data and limited power. Although the study failed to demonstrate significant effect on the primary endpoint, Paper III contributes important data to this field of research as studies on possible EPE eradication therapies are urgently needed.

In Paper IV, we investigated the prevalence of specific serum IgG-antibodies directed against CTX-M-15 and CTX-M-27 IgG antibodies in patients following bacteremia with an ESBL-producing *Enterobacterales*. We compared this to individuals that had experienced bacteremia with third generation cephalosporin susceptible *E.coli* (3GCSE). A total of 59 patients were included in the EPE-group and another 42 patients with 3GCSE were included in the control group. In 28% of patients with EPE blood isolates carrying either $bla_{CTX\text{-}M\text{-}15}$ or $bla_{CTX\text{-}M\text{-}27}$, IgG antibodies to the corresponding CTX-M-type were detected. In contrast, only 9.5% of patients in the 3GCSE control group were positive for anti-CTX IgGs (p=0.03). Thus, we demonstrated the presence of specific IgG antibodies targeting CTX-M enzymes although the significance of their presence is still unknown.

Finally, in Paper V we selectively cultured stool samples of international travellers with traveller's diarrhoea for EPE, if the referral stated foreign travel. Out of 304 travellers, 84 (28%) carried EPE after returning home to Sweden. EPE prevalence was highest among travellers returning from Africa (54%), Asia (45%) and North America and the Caribbean (25%). The EPE prevalence of 28% was slightly higher than in an investigation in the same population one decade earlier (24%), but the difference was not statistically significant. Out of 86 strains available for whole genome sequencing (WGS), 47 different sequence types were identified, of which 2 where novel. Only 5 strains belonged to ST131. Out of the 79 *E. coli* isolates, 76% carried at least one type 1-fimbriae gene, 29% carried at least one *pap* (encoding p-fimbriae) gene and 43% extraintestinal pathogenic *E. coli* (ExPEC) or uropathogenic *E. coli* (UPEC). Over half of the *E. coli* strains (57%) were intestinal pathogenic *E. coli*, most commonly Enteroaggregative *E. coli* (EAEC, 33%), and Enteroinvasive *E. Coli* (EIEC, 22%).

Key words ESBL, pAmpC-producing Enterobacterales, Escherichia coli, Gut flora

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— Transmission, intestinal colonization and host response

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MADE IN SWEDEN

“The devil is in the details.”

To Ditte, Ebbe and Olle.

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1. **Ljungquist O**, Ljungquist D, Myrenås M, Rydén C, Finn M, Bengtsson B. Evidence of household transfer of ESBL-/pAmpC-producing Enterobacteriaceae between humans and dogs – a pilot study. *Infection Ecology Epidemiology*. 2016; 6: 10.3402/iee.v6.31514.

2. **Ljungquist O**, Schönbeck M, Riesbeck K, Tham J. Risk factors associated with prolonged intestinal colonization of ESBL-producing *Enterobacteriaceae* – a prospective cohort study. *Infection and Drug Resistance* 2019:12 2637–2648.

3. **Ljungquist O,** Kampmann C, Resman F, Riesbeck K, Tham J. Probiotics for intestinal decolonization of ESBL-producing *Enterobacteriaceae*; a randomized, placebo-controlled clinical trial. *Clinical Microbiology and Infection*, https://doi.org/10.1016/j.cmi.2019.08.019.

4. Sahlström T*, **Ljungquist O***, Su Y-C, Mattsson E, Resman F, Tham J, Riesbeck K. Bacteremia with ESBL-producing *Enterobacterales* is associated with IgG antibodies reacting with CTX-M-15 and/or CTX-M-27. Manuscript, submitted.
   * both authors contributed equally.

5. **Ljungquist O,** Camporeale A, Giske C, Nematzadeh S, Resman F, Riesbeck K, Tham J. ESBL-producing *Enterobacterales* in patients with traveller's diarrhoea- a cross sectional cohort study. Accepted Antimicrobial Agents and Chemotherapy 2020.

Abbreviations

3GCSE	Third generation cephalosporin-susceptible *E. coli*
ABU	Asymptomatic bacteriuria
AmpC	Ampicillinase C
CAD	Catéter à demeure
CFU	Colony-forming unit
CMY	Cephamycinase
CPE	Carbapenemase-producing *Enterobacterales*
CTX-M	Cefotaximase-München
DHA	Dhahran beta-lactamase
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
ECDC	European centre for disease and control
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ESBL	Extended spectrum beta-lactamase
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
IBD	Inflammatory bowel disease
ICU	Intensive care unit
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MIC	Minimal inhibitory concentration
MLST	Multi locus sequence type
MLVA	Multiple-locus variable tandem repeat analysis
MRSA	Methicillin resistant *Staphylococcus aureus*
NDM	New Delhi metallo-beta-lactamase

LPS	Lipopolysaccharide
OR	Odds ratio
OMPs	Outer membrane proteins
OMVs	Outer membrane vesicles
OXA	Oxacillinase
PAIs	Pathogenicity-associated islands
pAmpC	plasmid encoded Ampicillinase C
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
RCT	Randomized controlled trial
SHV	Sulfhydryl variable beta-lactamase
ST131	Sequence type 131
TEM	Temoneira beta-lactamase
UTI	Urinary tract infection
VIM	Verona integron-encoded metallo-beta-lactamase
VRE	Vancomycin-resistant *Enterococci*
WGS	Whole genome sequencing

1 Introduction

> "Boy, that escalated quickly. I mean, that really got out of hand fast."
>
> Ron Burgundy

Antimicrobial resistance is ever-evolving around us. The emergence of *Enterobacterales* producing extended spectrum beta-lactamases (ESBL) has been correlated to the introduction of third generation cephalosporins in healthcare settings (1). Infections due to multidrug-resistant *Enterobacterales* constitute a major health care concern and are associated with increased mortality compared to infections with third generation-susceptible *Enterobacterales* (2-5). Treatment options for such infections are few. Intestinal colonization of *Escherichia coli* and/or *Klebsiella pneumoniae* producing ESBL is a requirement for developing such infections (6). It is vital to learn more about the mechanisms, spread and impact of ESBL-producing *Enterobacterales* (EPE).

The theme of this thesis is intestinal colonization of ESBL-producing *E. coli* and *K. pneumoniae*, the most common bacteria carrying this particular resistance. The primary aim of this thesis is to investigate transmission, risk factors for prolonged colonization, eradication and international travel as a risk factor for intestinal colonization of EPE.

2 The normal gut flora

The microbiota is defined as a population of microbes that inhabits a given ecosystem. The combined genetic material of the microbes in the defined microbiota is called the microbiome. Each individual carries unique sets of bacteria and genes. The intestinal microbiota of people living in different countries is distinctly different, due to many factors of which dietary differences are one (7, 8). Each bacterial strain, and the genomes of viruses, fungi and archaea in the intestine, together carry thousands of genes. This makes the intestinal microbiome far more versatile than our own human genome.

Structure

The human intestines contain an estimated 500-1000 species of bacteria, in total about $10^{10} – 10^{11}$ bacterial cells per gram of faeces (9). The total amount of bacteria in the intestines was previously estimated to 10^{14}. However, a newer assessment of the total amount of bacteria in the body of an average adult of 70 kg weight is closer to trillions of bacteria (10^{12}). The total weight of these bacteria is 0.2 kg, about 0.3 % of the total body weight of a 70 kg adult (10). The total amount of bacteria in the body roughly equals the total number of cells in the body, as opposed to older estimates of 10:1 in favour of bacteria. The rich supply of nutrients in the gut provides an excellent microclimate for the bacteria.

The microbiota is often compared to a human organ and it also includes, e.g., microbes covering skin, teeth and mucous membranes and inhabits the border between the body and the surrounding milieu (11). Most interactions between the body and the surrounding environment involves bacteria to some extent.

Function

The function of the microbiota is versatile, and the full extent of its purposes is presently unknown. One important function of the microbiota is digestion of dietary fibre that are indigestible by human enzymes (12). This has the dual effect of providing the body with energy that would otherwise have been lost; as well as with a vast array of e.g., short chain fatty acids (SCFAs), potent regulatory physiological molecules (13). Furthermore, the microbiota is involved in the development of the immune system, host intestinal endocrine function, provides protection against the adhesion of pathogenic bacteria, synthesis of vitamins and neurotransmitters and

acts as eliminator of toxins (14, 15). The function of inter-species interactions is, however, currently under-explored.

The gastrointestinal tract covers about 30 m^2 of mucous membrane and thus a single stool sample, or a faecal culture, is a poor representative of the entire microbiota. A faecal culture is a poor model for grasping the complexity of the "true" microbiota. Most bacteria of the microbiota are unculturable with standard techniques, and recent technological advances has enabled advanced analyses of the microbiota.

Development

The normal gut microbiota is established during infanthood and is affected by a number of different factors, including mode of delivery, feeding/diet, antibiotic use and consumption of probiotics (7, 12, 15). The supply of breastmilk is the most significant factor of microbiota structure during the first year. After the cessation of breast feeding, the microbiota continues to develop rapidly. It is dominated by phylum *Bacteroidetes* and *Firmicutes*, of which the main genera are *Bacteroides,* and *Bacillus* and *Clostridium*, respectively (16). The most common genus found in the gut is *Bacteroides*. It also contains, in smaller proportions, Actinobacteria, Proteobacteria, Verrucomicrobia, and other microorganisms as Archaea, yeasts and phages (14).

The ability of bacteria to effectively colonize the intestines is in large part determined by the presence of oxygen. The majority of the bacteria in the colon are anaerobe bacteria.

The role in disease

Ordinarily, a stable equilibrium state exists between the individual and the microbiota, a *symbiosis*. In contrast, the term dysbiosis is often used to describe a state where the host-microbial balance is disturbed in a way that is detrimental to the host. This is often associated with a loss of diversity and a low-grade inflammation of the intestinal mucosa. Dysbiosis is context and disease specific. Dysbiosis of the microbiota has been associated with the disease progression of numerous diseases, including diabetes mellitus and IBD (17).

A decrease in diversity of the microbiota is associated with several diseases, including IBD, obesity, diabetes or recurrent infection with *Clostridioides difficile*. Additionally, some strains of bacteria seem to be specific of IBD (18). Microbiota composition have also been demonstrated to affect the response to cancer treatment (19). It is important to note, that these associations have not been established as causative. It is presently unknown if the altered microbiota is a cause or result of the disease in question (15). In mechanistic animal models of human disease, the microbiome is involved in the pathogenesis of autoimmune disease, obesity and neurological diseases. This causality has not been established in humans.

The resilience phenomenon is the ability of the gut flora to return to an equilibrium state following a disturbance. A disturbance of the microbiota can be chemical (e.g. antibiotics), physical (e.g. diarrhoea), microbial (e.g. probiotics). The resilience is associated with the diversity of the microbiota; the more diverse, the more easily will equilibrium be restored (17). In parallel, a more varied gut microbiota is less susceptible to disturbance (17). This could be related to human health; a person with a healthy microbiota with a high level of resilience may have better protection against dysbiosis and infection (11).

EPE and the microbiota

As the topic of this thesis is EPE, this leads to two fundamental questions; what is the role of ESBL-producing *Enterobacterales* in the microbiome? What is the role of the microbiome in protecting against colonization and persistence of ESBL-producing bacteria in relation to other risk factors?

Presently, to my knowledge, studies on this topic are limited.

Studies on travellers returning from areas endemic of EPE show high prevalence of EPE colonization, with or without traveller's diarrhoea or antibiotic consumption during the trip (20-25). Strains of *E. coli* not producing CTX-M types are simultaneously acquired during travel. In colonizing the intestines, EPE are often polyclonal, and they seem to at least temporarily replace strains of commensal *E. coli* (26). How this is done is unknown, one theory is that the EPE could induce a small inflammatory response, which releases by-products that can be used in anaerobic metabolism. The invading EPE strain is able to use these substrates more efficiently than the commensal *E. coli*. This leads to a competitive growth advantage and the EPE strain can displace the commensal *E. coli* (27). Prolonged colonization of EPE enables further dissemination to new hosts.

2.1 Colonization resistance

Colonization resistance is the ability to prevent pathogenic bacteria to adhere and grow in the intestines. The association between the microbiota and host defence was made decades ago when mice with dysbiosis (post antibiotic exposure) were shown to have an increased risk of enteric infection (28). The precise mechanism in which pathogenic bacteria are prevented from colonizing the intestines of a healthy gut is not fully comprehended, but it involves competing for nutrients, production of antimicrobial products, gut barrier integrity and distribution of bacteriophages (29). Examples of antimicrobial products are bile acids, bacteriocins and short chain fatty acids.

A prospective Swedish study found that travellers with a less diverse microbiota were more susceptible to *Campylobacter* infection (30). Antibiotics do not differ

between pathogenic or commensal bacteria and may promptly induce dysbiosis (31). Consequently, this can increase the risk of pathogenic or opportunistic bacteria colonizing and infecting the intestines of which *C. difficile* is a notable and clinically relevant example (32). As a consequence of antimicrobial treatment, overgrowth of toxin-producing *C. difficile* can result in enteritis and disease. Interestingly, colonization with Clostridiales is also associated with increased colonization resistance (12).

Commensal strains of *E. coli* and EHEC compete for signals and nutrients produced by bacteria in the gut. In mice, multiple strains of commensal *E. coli* with similar nutritional requirements as EHEC prevent the latter from colonizing the intestines (32). Another study linked colonization resistance to an acidic environment with production of high concentrations of short fatty acids- this inhibited the expansion and supported clearance of EPE among others (33).

In a study on mice, the antibiotics clindamycin, dicloxacillin and cefotaxime all promoted the overgrowth of and CTX-M-15-producing *E. coli* ST131. Of these, only clindamycin was associated with both the suppression of *Bacteroides* and prolonged colonization of the CTX-M-15-producing *E. coli* ST131 (34).

In addition to antibiotics, many other classes of drugs can impact the microbiota, such as proton pump inhibitors, antidiabetics and antipsychotics (29).

Furthermore, a continuous stress to the microbiota, as in a permanent dietary switch from high protein diet to a carbohydrate rich diet (or vice versa), could permanently alter the microbiota in what is sometimes called a ‘regime shift’ (17).

3 *Escherichia coli*

Escherichia coli was named after the German paediatrician Theodor Escherich (1857-1911), who first described the bacterium in 1886 (35). *E. coli* is a Gram-negative, rod shaped, non-sporulating facultative anaerobe, found in humans and warm-blooded animals. The pathogenic range of *E. coli* is vast; it is a common commensal of the intestines but also a potentially severe pathogen leading to severe sepsis and death. Thus, the within-species diversity of *E. coli* is immense. Profound knowledge of the resistance and pathogenicity of *E. coli* has been gained since it is an exemplary bacterium to work with in the laboratory; it grows easily, fast and its genome has been thoroughly investigated.

In the intestines, the concentration of *E. coli* is 10^7 -10^9 cfu per gram of faeces (36). The intestinal concentration of *E. coli* is highest early in life. In two thirds of vaginally delivered babies, the source of the *E. coli* is maternal, the rest are environmental strains (37). This is true for the entire microbiota of vaginally delivered babies, as it is similar to the vaginal and gut microbiota of the mother (14). The microbiota of babies born with caesarean section is less diverse compared to vaginally delivered babies (15).

At two years of age, the amount of *E. coli* stabilizes around 10^8 cfu per gram of faeces and then decreases gradually with age. Thus, depending on age, between 0.01-10% of all bacteria in the gut are *E. coli*, primarily located in large intestine.

Infections caused by *E. coli* can be both intestinal and extraintestinal, depending on bacterial characteristics. This is in turn based on the extent of virulence factors expressed to overcome the immune system of the host. Strains causing extraintestinal disease are unable to cause intestinal disease, and vice versa (38). During the past decades, detailed knowledge of *E coli* genes, of both chromosomal and plasmid origin, has been acquired through new sequencing methods on strains causing disease in humans. This has increased knowledge regarding the pathogenicity of *E. coli* and led to better categorization of the different types that exist.

Clinically important strains of *E. coli* to humans can be divided into commensal strains, intestinal pathogenic or extraintestinal pathogenic *E. coli* (ExPEC) (38). Infections caused by ExPEC strains can be non-severe, e.g. lower urinary tract infections (i.e. symptomatic, not causing severe disease), or severe life-threatening infections, such as septic shock. The true population-based incidence rate of

worldwide *E. coli* infections is not known, and different models exist predicting this, but an estimated two million people perish every year from *E. coli* infections globally (38-40).

Commensal *E. coli* lack virulence factors that are generally needed to cause disease in hosts without compromised immune systems. By adherence to the intestines, production of bacteriocins and other mechanisms, they protect the host against colonisation of other bacteria with greater pathogenicity (36). In severely immunocompromised hosts, however, commensal *E. coli* can act as opportunists and cause extraintestinal infection (41).

Intestinal pathogenic *E. coli* are commonly divided into five different pathotypes; EAEC, EHEC, EIEC, EPEC and ETEC. Although there is overlap between them, these pathotypes each have a combination of specific virulence factors giving them each individual pathogenic potential (35). When the host is infected with a sufficient number of bacteria, each of these pathotypes can cause gastroenteritis and/or colitis. Thus, clinically, the symptomatology of the different intestinal pathogenic *E. coli* is shared and cannot be used as a basis to differ between them. For this, molecular methods such as PCR must be used. The treatment of patients with infections by intestinal pathogenic *E. coli* is primarily symptomatic.

Inflammation of the gut causes expansion of *E. coli* which in turn can lead to transmission to new hosts through faecal-oral route. Consequently, strains that cause enteritis and disease can spread more successfully and have thus been favoured in the natural selection, outcompeting less virulent strains. All bacteria in the intestines compete for the same nutrients and a limited space of mucous membrane to adhere to.

ExPEC are defined as all non-commensal *E. coli* isolates able to cause extra-intestinal disease, in numerous anatomical sites (41). Common foci include the blood, urinary tract, abdomen and the meninges. ExPEC strains share similar genetic traits, such as a range of virulence factors. Interestingly, ExPEC do not cause gastroenteritis, but colonizes the intestines of the healthy host prior to extra-intestinal infection (42). For extra-intestinal infection to occur, the bacteria must depart the intestines and enter an extra-intestinal site, such as the urinary tract. This ability is what differentiates ExPEC from commensal strains and is reflected in the possession of specific virulence factors. As presented schematically in Figure 1, general *E. coli* virulence factors include toxins (e.g. hemolysin), adhesins (e.g. P- and S-fimbriae), iron-acquisition systems (e.g. aerobactin) and host defence-avoidance mechanisms.

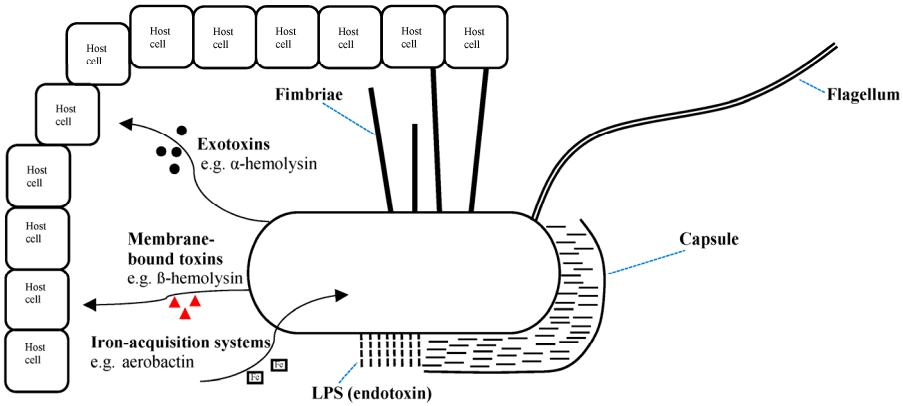


Figure 1. Schematic presentation of *E. coli* virulence factors.

Fimbriae are used to adhere to host epithelial cells of the intestines, kidneys or lower urinary tract. Exotoxins and membrane bound toxins can be secreted to damage host cells or disrupt normal cellular metabolism. Iron-acquisition systems (siderophores) are used to obtain essential iron molecules. The LPS stabilize the membrane structure and can induce a severe reaction from the immune system of the host. The capsule can mimic cells of the host, thereby evading cells of the immune system.The flagellum is mainly used for movement but also serves as a sensory organelle.

For instance, *E. coli* in the blood originating from an pyelonephritis is associated with the expression of P-fimbriae (42). Expression of multiple virulence factors is more common in *E. coli* causing pyelonephritis or urosepsis compared to strains causing lower UTI or ABU (42).

Genes encoding for virulence factors are often concentrated together in pathogenicity-associated islands (PAIs), which are blocks of chromosomal DNA, or alternatively on plasmids (43, 44).

Using molecular methods, ExPEC are defined as *E. coli* carrying two or more of the following VFs in the genome; *papA* and/or *papC*, *sfa/foc*, *afa/draBC*, *kpsM II* and *iutA* (45).

E. coli can be further divided into phylogroups of which there are 8 documented; A, B1, B2, D, E, F, G and Clade 1. Although colonization with *E. coli* of B2 is common, this phylogroup is the one most often associated with virulence and extra-intestinal infections (44). ExPEC strains most often belong to phylogroups B2 or D in non-immunocompromised individuals, while commensal *E. coli* and intestinal pathogenic *E. coli* most often belong to A/B1 or A/B1/D, respectively (44).

3.1 *Klebsiella pneumoniae*

Klebsiella pneumoniae was named after the German pathologist Edwin Klebs (1834-1913). It was first described by Carl Friedlander in 1882, isolated from the lungs of patients who had died from pneumonia (46). *Klebsiella pneumoniae* is a Gram-negative, rod shaped, encapsulated, facultative anaerobic, non-sporulating bacteria. It colonizes the intestines of humans and animals and is also found in the environment, e.g. in the soil and in plants. In humans, it is the most clinically important bacteria of the genus, but *K. oxytoca* can also, rarely, cause disease. *Klebsiella pneumoniae* is classified as an ESKAPE pathogen, along with *Enterococcus faecium, Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species (47). The ESKAPE pathogens are associated with antimicrobial resistance and are recognized as priority pathogens for which new antibiotics are urgently needed. *Klebsiella pneumoniae*, as well as the other ESKAPE pathogens, are associated with nosocomial infections, often seen in immunocompromised patients with multiple comorbidities. It has been estimated that *K. pneumoniae* cause 8 % of all nosocomial bacterial infections in Europe and in the United States (48). Thus, it can be an opportunistic pathogen with nosocomial spread, responsible for one third of all Gram-negative hospital infections in total (49). However, during recent years, *K. pneumoniae* with increased virulence and widespread antimicrobial resistance have emerged, with the ability to cause diseases in immunocompetent hosts. Compared to *E. coli*, *K. pneumoniae* is generally less susceptible to several classes of antibiotics when compared to *E. coli*, especially to cephalosporins and carbapenems. Compared to *E. coli*, invasive infections with *K. pneumoniae* are more often nosocomial, frequently affect patients with multiple comorbidities and are associated with higher mortality (50).

Due to its ability to colonize the environment and spread rapidly, in-hospital transmission and outbreaks of nosocomial infections of *K. pneumoniae* occur worldwide, especially in neonatal units (48). Nosocomial infections are primarily lung and urinary tract infections. In hospitals, the colonization rates of *K. pneumoniae* in patients increases proportionally with the length of stay and is facilitated by antibiotic usage, concordant with other nosocomial pathogens common in hospital flora such as *P. Aeruginosa* (51). Reducing antibiotic consumption in general, and fluoroquinolones and cephalosporins in particular, can reduce the risk of hospital outbreaks, along with improved hygiene measures and infection control (52).

3.2 Dominant clones

The word clone is derived from the ancient Greek word klon, meaning twig. It is defined as a cell (bacteria) that is genetically identical to the organism from which it is derived. In the past decades, virulent clones of *E. coli* have emerged with global dissemination. These clones represent lineages of *E. coli* that can carry virulence and resistance genes which enables further transmission, of which the clone B2 O25:H4 ST131 carrying CTX-M-15 is the most prominent example (43). The ST131 lineage of ESBL-producing *E. coli* was first described in 2008 as a common cause of hospital infections in many countries. Recently, molecular studies have estimated the emergence of this clone in the early 1990s in North America, likely stimulated by the broad usage of extended spectrum cephalosporins and fluoroquinolones (53). Other notable clones are ST410 and ST648 (27).

Unlike *E. coli*, there seem to be geographical limits in the dissemination of different *K. pneumoniae* clones, i.e. not pandemically spread. Also, unlike *E. coli*, dominant clones of *K. pneumoniae* (such as ST11) to a greater extent carry genes conferring resistance to both cephalosporins and carbapenems, sometimes including colistin (27). A common trait of both *E. coli* and *K. pneumoniae* is that the successful spread of dominant clones is facilitated by plasmids.

4 Colonization versus infection

The transmission of EPE is complex and must be viewed from a one-health perspective. The gastrointestinal tract of humans and animals are reservoirs for EPE, and EPE can increasingly be found in the environment (54). EPE can spread between humans both within the community and in hospitals and could be driven by international travel to EPE endemic areas.

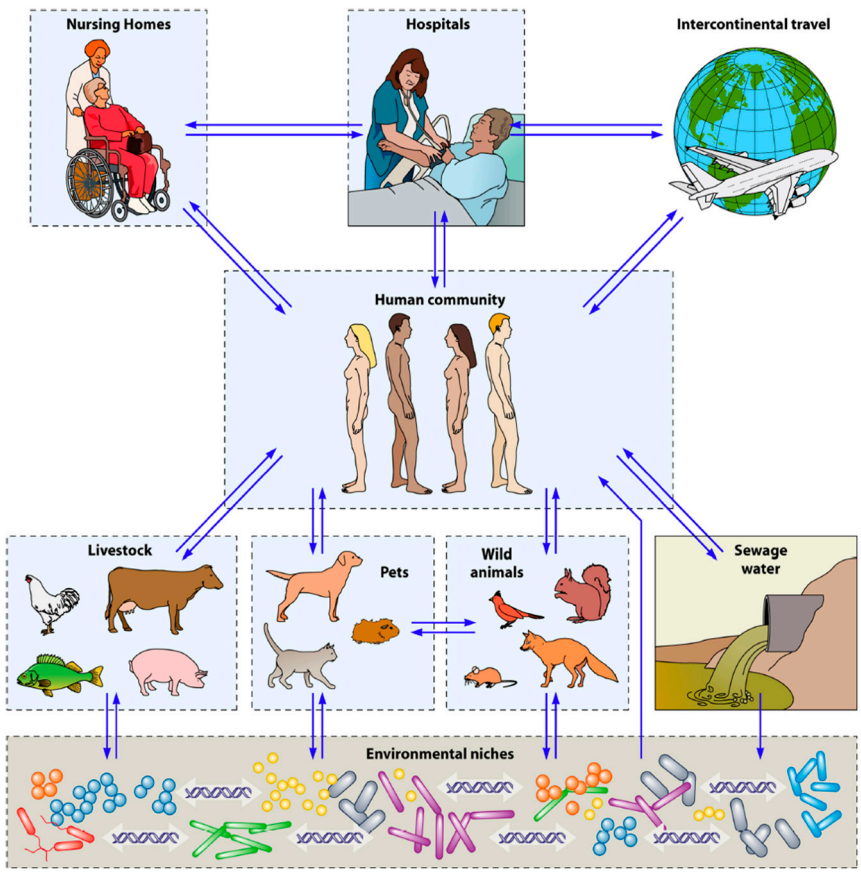


Figure 2. Schematic presentation of important reservoirs of EPE.
Figure reprinted with permission from author Paul-Louis Woerther (55).

It is widely accepted that intestinal colonization with EPE is the *sine qua non* of developing subsequent infections from EPE (6). Once colonized by EPE, the risk of developing serious EPE infections is relatively low, and mainly occurs within a year of detecting EPE (56, 57). A Swedish study showed that the pathogenicity of faecal strains is lower compared to invasive EPE strains (58). However, many studies acknowledge prior EPE colonization as an important risk factor of developing invasive EPE infections (59-61).

Distinguishing EPE infection from colonization is imperative and can be a challenge when seeing patients in the clinic. If EPE is found in cultures from sterile sites, such as the blood, joints or cerebrospinal fluid, a clinician can be certain that the patient has a manifest, clinically relevant, infection which must be promptly treated with antibiotics. However, when EPE are found in other cultures, e.g. from urine, skin or sputum, the situation is much more precarious. It could represent an infection but also mere colonization. Patients are frequently colonized by EPE in other localizations than the gastrointestinal tract. In these cases, the EPE strains are likely commensals and should rarely be treated with antibiotics. It is of uttermost significance to differentiate between these two situations, as antimicrobial treatment of colonization does not permanently eradicate colonization but increases the risk of selection and persistence of resistant, virulent EPE clones (62).

Sometimes quantification of bacteria is used to try to distinguish between colonisation and infection, examples are urine and lower airways of intubated patients. However, there are currently no biomarkers that can differentiate between infection and colonisation. Thus, unless for infection control purposes, cultures should only be taken if there is a clinical suspicion of infection.

5 The beta-lactams

Beta-lactam antibiotics are arguably the most effective and widely used class of antimicrobials. Since sir *Alexander Fleming* discovered the antibacterial properties of penicillin in 1928, beta-lactam antibiotics have continued to dominate the market of antibiotic production and consumption (63). Beta-lactam antibiotics are characterized by the beta-lactam ring, the principal structure of penicillins, cephalosporins, carbapenems and monobactams. Beta-lactams were originally derived from naturally occurring substances; penicillin was derived from the fungi *Penicillium notatum*. Today, the producer organisms of beta-lactams are *Penicillium chrysogenum* and *Acremonium chrysogenum* (63).

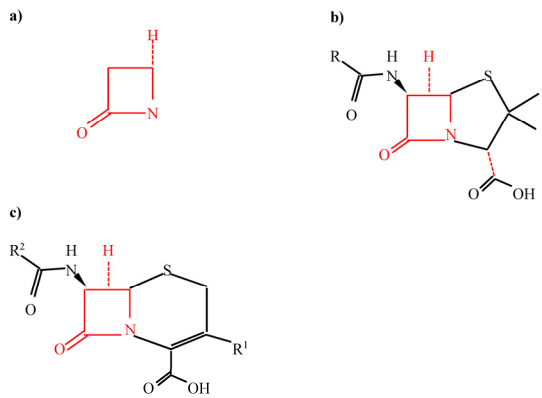


Figure 3. The molecular structure of **a)** the beta-lactam ring, **b)** penicillin and **c)** cephalosporin. Note the fundamental beta-lactam ring in red.

'Beta-lactam antibiotics inhibit the synthesis and the maintenance of the bacterial cell wall, in particular the formation and remodelling of the polymer peptidoglycan. Under normal circumstances, the bacterial peptidoglycan is formed by the cross-linking of beta-linked *N*-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) polysaccharide chains, which produces a rigid three-dimensional structure (64). This process is called the transpeptidation. The enzymes DD-transpeptidase, commonly referred to as penicillin binding proteins (PBP), aid this

process by catalysis. There are several different variants of PBPs, each targeted by different beta-lactam antibiotics.

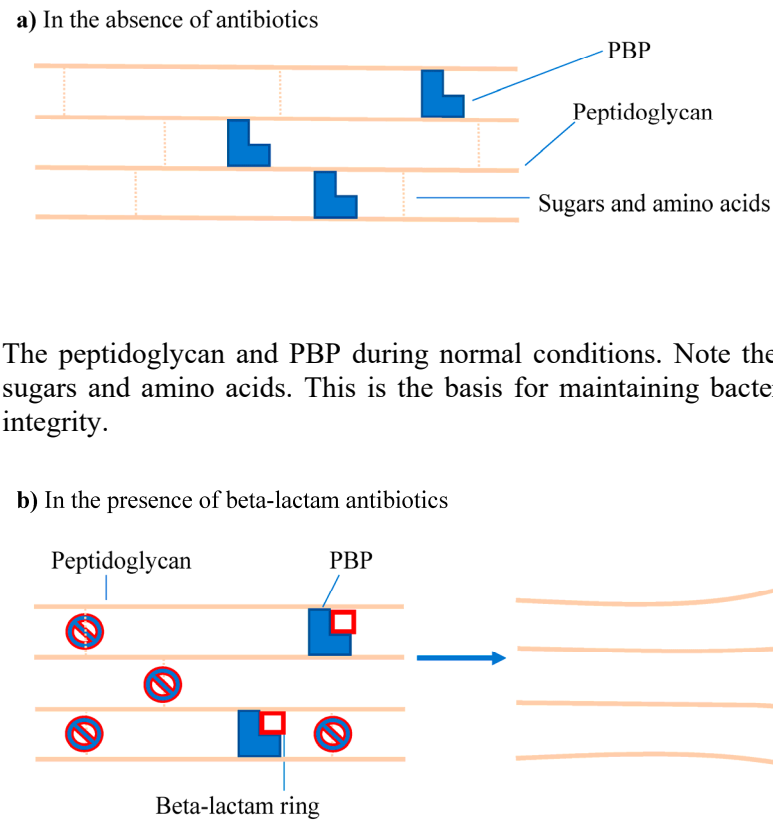


Figure 4. Schematic presentation of mechanism of action of beta-lactam antibiotics.

The PBPs are inhibited by beta-lactam antibiotics, disrupting cross linkage between peptidoglycans. This leads to bacterial lysis.

The beta-lactam ring inhibits the PBP, which prevents transpeptidation. As a result, the peptidoglycan is not properly formed, cell wall integrity cannot be maintained, leading to lysis of the bacterial cell. Thus, beta-lactams are bactericidal and highly effectively in killing bacteria. Side effects and adverse events are few, with no effect on eukaryotic cells. Gastrointestinal symptoms during treatment are common, as the effect is not limited to pathogenic bacteria, but to commensal bacteria of the microbiota as well. Since the introduction of beta-lactam antibiotics, however, they have been pursued by clinically significant resistance.

5.1 A brief history of beta-lactamases

> Brace yourself, I'll take you on a trip down memory lane.
>
> Kendrick Lamar

Since the 1960s, a broad diversity of different beta-lactamases has been discovered. Not surprisingly, they emerged shortly after the introduction of corresponding beta-lactam antibiotics into healthcare . Figure 3 shows the timeline of the most prominent beta-lactamases.

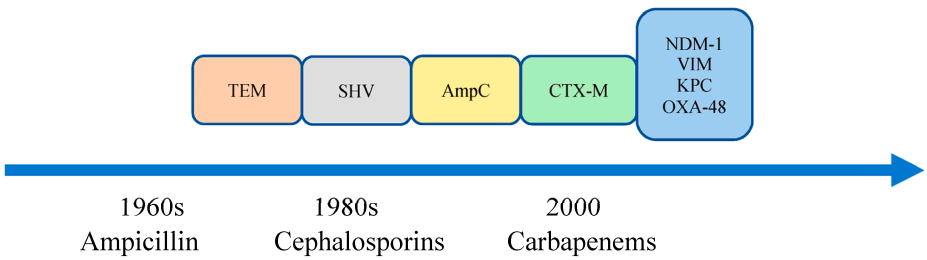


Figure 3. Timeline of the first identification of various beta-lactamases.

5.1.1 TEM

The first plasmid-mediated beta-lactamase of Gram-negative bacteria was discovered during the 1960s in Greece. It was isolated from a strain of *E. coli* found in a blood culture of a patient, Temoneira, thus naming the enzyme TEM (65). Gradually, as antibiotic consumption increased, TEM became more prevalent in health care settings worldwide. TEM can be found in bacteria of the *Enterobacterales,* in *Pseudomonas aeruginosa, Haemophilus influenzae* and in *Neisseria gonorrhoeae.* Not long after that, TEM-2 was found, a closely related beta-lactamase only differing one amino acid from TEM-1 (66). TEM-1 and TEM-2 are active against narrow spectrum cephalosporins, such as cephalothin, but not third generation cephalosporins. Further mutations have led to TEM-type ESBLs, conferring resistance to third generation cephalosporins.

5.1.2 SHV

Sulfhydryl variable beta-lactamase (SHV-1) emerged in the 1970s as a similar but less common beta-lactamase (67). TEM-1, TEM-2 and SHV-1 have no activity against cefotaxime, ceftazidime or aztreonam. During the 1980s antibiotics with

broader spectra were introduced in health care and the use of third generation cephalosporins became widespread in hospitals all over the world. As a direct consequence, new beta-lactamases appeared. The first of these with activity against third generation cephalosporins such as cefotaxime was named SHV-2 (68). It was found in *Klebsiella ozeanae* in Germany.

5.1.3 CTX-M-type beta-lactamases

During the 1980s and 1990s, TEM- and SHV-types of ESBL were the most prevalent beta-lactamases, and particularly *K. pneumoniae* carrying these enzymes caused nosocomial outbreaks. Since the beginning of the new millennium, however, community onset infections with *E. coli* with CTX-M-types have been the dominating EPE worldwide (66, 69). Within two years after the introduction of the extended spectrum cephalosporins cefotaxime and ceftazidime, new ESBLs in *E. coli* and *K. pneumoniae* arose (70). The term extended-spectrum beta-lactamases was first coined in a review article from 1989 (71). Contrasting TEM and SHV, CTX-M-type beta-lactamases do not origin from mutations of plasmid mediated penicillinases. They originate from the chromosomal genes of the non-pathogenic commensal *Kluyvera* spp. and were mobilized by insertion sequences onto plasmids (72). CTX-M-type beta-lactamases were first described in *E. coli* strains isolated from a German patient in 1990, and soon many similar reports followed (73-76). The name CTX-M is an abbreviation of the beta-lactamase cefotaximase and refers to the great hydrolytic activity against cefotaxime, and the M denotes Munich, Germany, where it was first described (77).

CTX-M-type beta-lactamases are transferred by plasmids and there are over 220 different types known. They are characterized into five different clusters or subtypes depending on the amino acid sequences; CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (69). Early geographical hotspots for the emergence of CTX-M beta-lactamases were Central Europe, South America and Japan (78).

The CTX-M-15 enzyme belongs to the subgroup CTX-M-1 and is the most globally spread enzyme, prevalent worldwide. It was first described in 2001, from patients hospitalized in New Delhi, India (73). Since then, the spread of CTX-M-15 has increased both in the community and in hospitals.

5.1.4 Carbapenemases

Soon after carbapenems became commercially available in hospitals in the second half of the 1980's, reports of carbapenemases arose (79, 80). Carbapenem resistance can also arise as a result of ESBL or AmpC in combination with decreased permeability (porin loss) or increased efflux (80). Bacteria carrying such resistance are commonly multidrug-resistant, and few (if any) antibiotics are available for

treatment. Even though carbapenemases are clinically important and was actively sought for in my different projects, they were not the main focus of this thesis.

5.2 The definition of ESBL-producing bacteria

ESBLs are beta-lactamases produced by bacteria that hydrolyse the beta lactam-ring of extended spectrum cephalosporins, and thereby inactivating them (81). Typically, they degrade ceftriaxone, cefotaxime, ceftazidime and oximino-monobactam. In contrast, carbapenems, cephamycins and beta-lactamase inhibitors stay intact. Thus, clinical infections caused by ESBL-producing bacteria cannot be treated with extended-spectrum cephalosporins or monobactams, but carbapenems can be used successfully in most cases.

Furthermore, the gene encoding resistance should according to the definition be transferrable between bacteria of the same or different species. In clinical routine diagnostics, this is not used as a criterion of ESBL as it requires sequencing methods, which is relatively expensive and time consuming. Instead, if a strain is resistant against one or more of the third generation cephalosporins and is positive in phenotypical and/or genetical beta-lactamase tests, it is characterized as ESBL-producing (82).

Internationally, ESBLs are generally characterized depending on which beta-lactamase they exhibit, and usually the term ESBL is accompanied with the enzyme family; e.g. CTX-M-15 ESBL (83). In Sweden and Norway, ESBLs are characterized phenotypically into $ESBL_A$ and $ESBL_M$ based on how they are inhibited as defined by Giske et al. (84). This does not require sequencing of the bacteria, which is primarily used in research settings. In this thesis, the later Swedish nomenclature will be used.

Classification according to $ESBL_A$ is based on phenotypical diagnostics; these strains are inhibited by clavulanic acid. *Enterobacterales* with $ESBL_M$ express resistance against cefoxitin. Cefoxitin is a second generation cephamycin, grouped with second-generation cephalosporins. Many Gram-negative bacteria have naturally occurring chromosomally encoded beta-lactamases. *Klebsiella* spp., *Salmonella* spp. and *Proteus* spp. do not, which is why phenotypical tests are sufficient to establish that they carry pAmpC. For all other species, genetic tests must be performed as well to differentiate between chromosomally or plasmid-encoded AmpC. This is displayed in detail in Figure 18 and 19.

Thus, phenotypically characterizing *E. coli* as $ESBL_M$ does not distinguish between plasmid- or chromosomally encoded AmpC, which could lead to overreporting of ESBL isolates.

Many different bacteria can produce ESBL; clinically relevant ones include species within *Enterobacterales*, such as *Proteus* spp., *Klebsiella* spp., *Salmonella* spp., but also *Acinetobacter* spp. and *Pseudomonas aeruginosa*. Sometimes phenotypic ESBL/pAmpC is be found, but no known gene of ESBL/pAmpC is found when strains are sequenced (85).

5.3 ESBL vs AmpC

The first report of a plasmid-encoded AmpC came from South Korea in 1989, and was found in a strain of *K. pneumoniae* having caused a wound infection (86). From a clinical perspective, the difference between ESBL and AmpC is of academic nature. There is no evidence of difference in potency, virulence or clinical features between strains carrying these two groups of resistant determinants when comparing outcomes of invasive infections (87, 88). When comparing prevalence, ESBL outnumber AmpC by far and it is worth noting that strains can carry genes encoding both ESBL and AmpC resistance simultaneously. In this thesis, ESBL and AmpC will simply be referred to as ESBL.

Generally, AmpC resistance can be characterized into three different groups (89). The historically most common type of AmpC resistance is the inducible resistance, but this only affects cephalosporins of the first and second generation.

Here, two types of AmpC resistance are relevant. First, non-inducible (constitutive) chromosomally encoded resistance due to mutations, causing hyperproduction of AmpC. Secondly, plasmid encoded AmpC (pAmpC). While AmpC resistance in *E. coli* can be encoded both by chromosomes and plasmids, *K. pneumoniae* lacks chromosomal AmpC. There are 29 known genes encoding for pAmpC, divided into 6 different families (90). Common AmpC enzymes include CMY, DHA and ACT. Clinical isolates carrying multiple pAmpC beta-lactamases have not yet been reported.

5.4 Classification of beta-lactamases

Beta-lactamases are commonly classified according to Ambler or Bush-Jacoby-Medeiros (91, 92). Ambler's classification is based on structural homology, whereas Bush-Jacoby-Medeiros is based on hydrolytic properties. In this thesis, Ambler's classification from A through D based on amino acid sequence homology will be discussed in brief. Ambler's classification is displayed in Table 1.

Table 1. Classification of beta-lactamases according to Ambler.

Ambler's Molecular Class	Beta-lactamase	Notable examples
A	Broad-spectrum	TEM-1, TEM-2, SHV-1
	Extended spectrum	TEM-, SHV-, CTX-M-family
	Carbapenemase	KPC-1, KPC-2, KPC-3, IMI, SME
B	Carbapenemase	IMP-, VIM-, NDM- family
C	AmpC	CMY-, ACT-, DHA-families.
D	Broad-spectrum	OXA-family
	Extended spectrum	OXA-family
	Carbapenemase	OXA-23, OXA-24, OXA-25, OXA-26, OXA-27, OXA-40, OXA-48

Ambler class A enzymes is a heterogenous group of enzymes that generally are inhibited by beta-lactamase inhibitors such as clavulanic acid. SHV and TEM are notable examples of extended-spectrum beta-lactamases of this group.

The majority of the class A ESBLs belong to the TEM-, SHV- or CTX-M- groups, and the genes coding for these enzymes are designated bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$, respectively. They are commonly found in, but not restricted to, *E. coli* and *K. pneumoniae*. The CTX-M-group is large and is today the most clinically important family of ESBL enzymes, prevalent all over the world. Other notable ESBLs are BES, GES-1, VEB and PER. Notable class A carbapenemases are KPC, SME and IMI. Class A enzymes are encoded on plasmids, chromosomes and integrons.

Ambler class B enzymes consist of the metallo-beta-lactamases and generally confer resistance to penicillins, cephalosporins, carbapenems but not aztreonams. The bla_{MBL} genes are located on chromosomes, plasmids and integrons. While the beta-lactamases of class A, C and D are serine beta-lactamases, class B beta-lactamases are Zn^{2+}-dependent in the beta-lactam hydrolysis. Therefore, metal-chelating agents like ethylenediaminetetraacetic acid (EDTA) can inhibit this process (93). This is of relevance only in in vitro testing.

Ambler class C AmpC beta-lactamases are usually encoded on the chromosome, but plasmid encoded AmpC (pAmpC) are becoming increasingly prevalent. AmpC beta-lactamases are inhibited by cloxacillin, oxacillin and aztreonam. Bacterial strains with AmpC beta-lactamases are usually resistant to penicillins, beta-lactamase inhibitors, cephalosporins including cefoxitin but not carbapenems.

Ambler class D beta-lactamases are called oxacillinases (OXA). Usually, oxacillinases are encoded chromosomally and confer resistance to penicillins, cephalosporins and carbapenems. Class D beta-lactamases are inhibited in vitro by sodium chloride. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are examples of bacteria that frequently harbour oxacillinases (94).

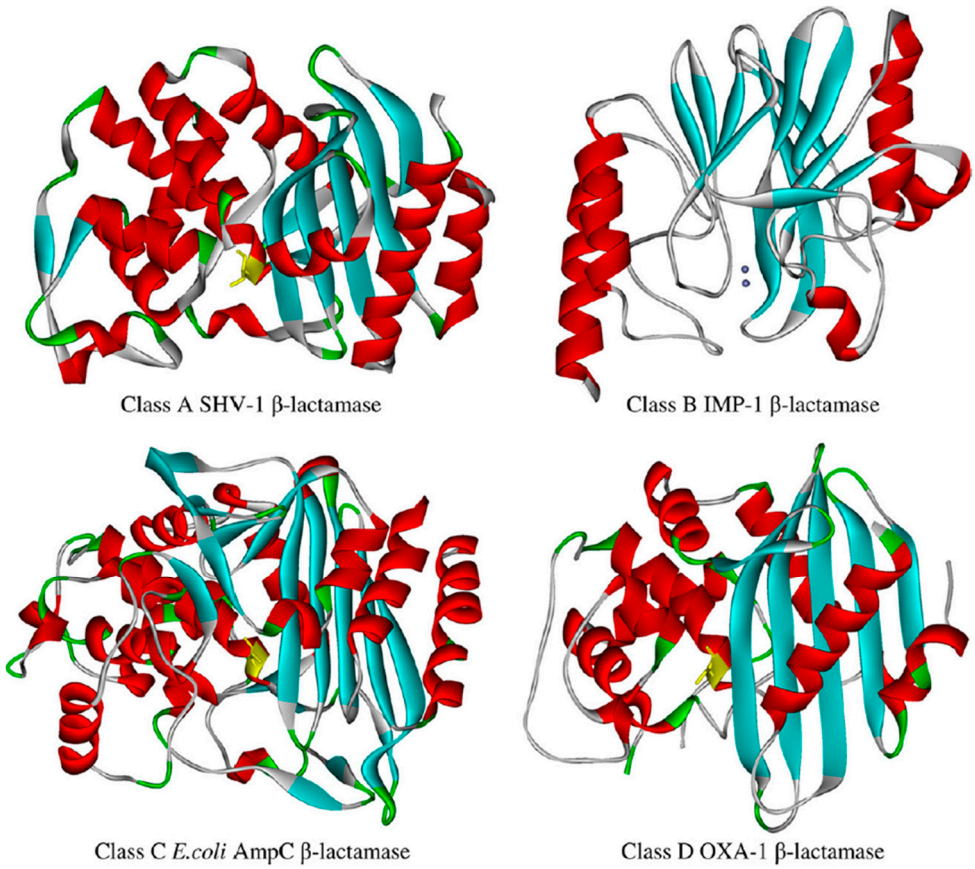


Figure 5. Examples of enzymes from each Ambler class.
Class A SHV-2 beta-lactamase, class B IMP-1 beta-lactamase, class C *E coli* AmpC beta-lactamase, class D OXA-1 beta-lactamase. The serine active sites are displayed in yellow for classes A, C and D. The two Zn^{2+} ions are shown in for class B. Reprinted with permission from author Robert A. Bonomo (93).

5.5 Mechanisms of beta-lactamases

Simplified, the outer part of a Gram-negative bacterium consists of cytoplasmic membrane, periplasmic space and an outer membrane. The peptidoglycan layer is present in the periplasmic space and is relatively thin compared to that of Gram-positive bacteria.

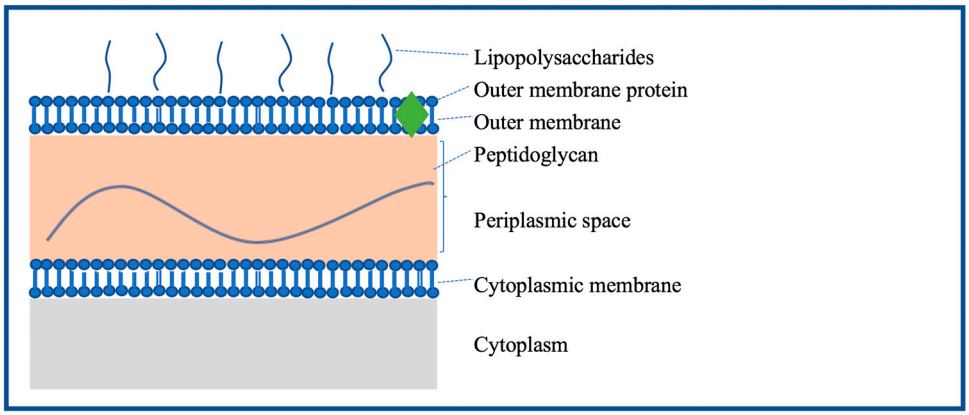


Figure 6. Schematic presentation of a Gram-negative bacterial cell wall.

As previously described, the transpeptidases responsible for peptidoglycan cross-linking are the targets of beta-lactam antibiotics. Both beta-lactams and beta-lactamases are likely evolutionary ancient, and have evolved in parallel during the competition for ecological niches throughout history; some beta-lactamases are estimated to be over 3 billion years old (95). Beta-lactamases are prone to evolve and single-nucleotide mutations can significantly alter the functionality (86).

In *Enterobacterales*, several different mechanisms can inhibit the effect of beta-lactam antibiotics. It is important to note that these mechanisms can occur simultaneously. In the presence of the selective pressure of beta-lactam antibiotics, bacteria with multiple mechanisms of resistance are favoured (95). Below, a brief description of the four main resistance mechanism in *Enterobacterales* are presented.

I. Plasmid-mediated production of ESBLs.

In *Enterobacterales*, this is the most important mechanism of resistance. The antibiotic degrading enzymes produced are often encoded from plasmids of the cytoplasm, and excreted into the periplasm (64). ESBL enzymes hydrolyse the beta-lactam ring, inactivating beta-lactam antibiotics such as penicillins and cephalosporins.

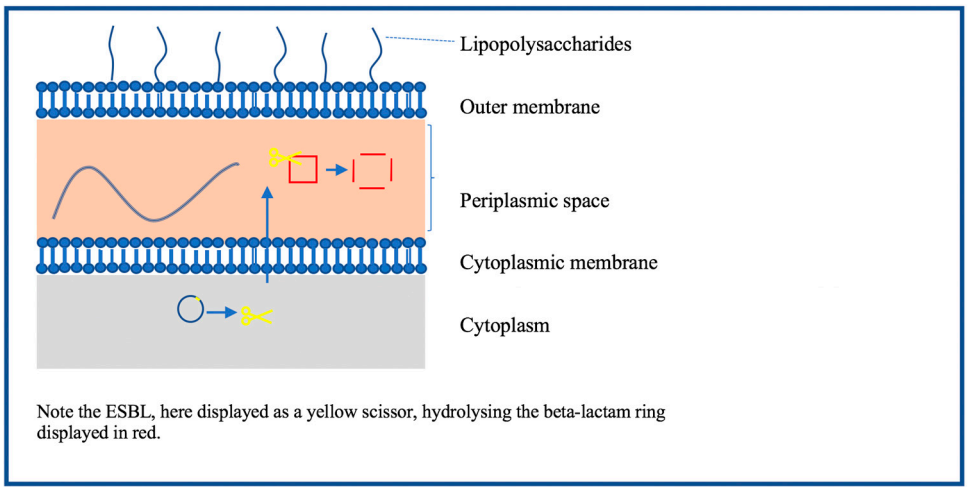


Figure 7. Schematic presentation of antibiotic degrading enzymes.
Note the ESBL, here displayed as a yellow scissor, hydrolysing the beta-lactam ring displayed in red.

Importantly, plasmids carrying ESBL-genes often carry genes encoding resistance to fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole and other antibiotic classes simultaneously. ESBL-producing bacteria are therefore commonly referred to as multidrug-resistant bacteria. Treatment with any of these antimicrobials may facilitate further spread of ESBL. Below, important mechanisms of resistance to beta-lactam antibiotics other than beta-lactamases are discussed in brief.

II. Reduced permeability due to decreased expressions of outer membrane proteins (OMPs).

Beta-lactam antibiotics access the periplasmic space thorough OMPs, and loss of OMPs can lead to reduced permeability. This can be the result of mutations in bacterial genes encoding for OMPs (86). This prevents the beta-lactam antibiotics from entering the bacterium. This resistance mechanism can be effective for several beta-lactams and some Enterobacteriales exhibits resistance to carbapenems in this manner

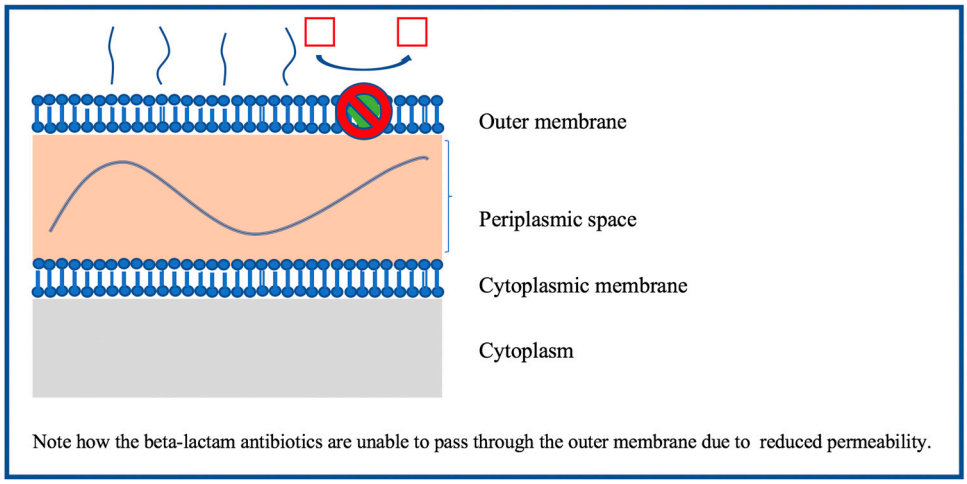


Figure 8. Schematic presentation of reduced permeability.

III. Upregulation of efflux pumps.

Substrates from the cytoplasm and/or the periplasmatic space are actively transported out from the bacteria. This naturally reduces the amount of beta-lactam antibiotics in the periplasmatic space. This is also an important mechanism of the bacteria in conferring resistance to multiple antibiotic classes.

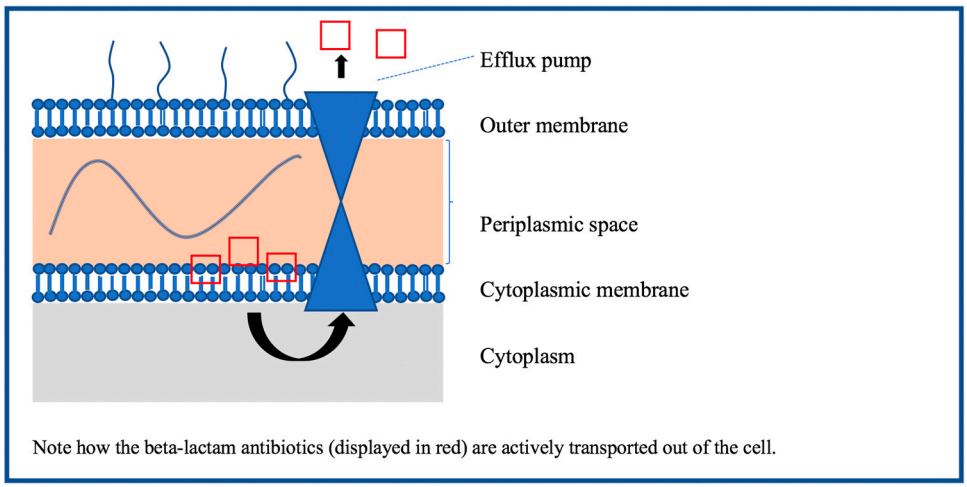


Figure 9. Schematic presentation of upregulated efflux pumps.

IV. Alterations in PBP, that render them with reduced binding. This is an important mechanism of antibiotic resistance in mostly Gram-positive bacteria, of which

methicillin resistance in *Staphylococcus aureus* is a notable example. Interestingly, the PBP seems to be the ancestors to beta-lactamases (96). PBPs are clinically important but will not, however, be further covered in this thesis.

5.6 Horizontal gene transfer

The core of horizontal gene transfer is the acquisition of genetic elements from other bacteria. There are three ways in which DNA can be transferred between cells; transformation, transduction and conjugation (97). In this thesis, conjugation and bacterial plasmids that promotes this process will be discussed in brief.

In the bacterial cell, there are two structures containing genetic material. Most bacterial species have one, circular chromosome in the cytoplasm. Plasmids are circular units of double-stranded DNA, that can be transferred from one bacterium to another of the same or different species (98).

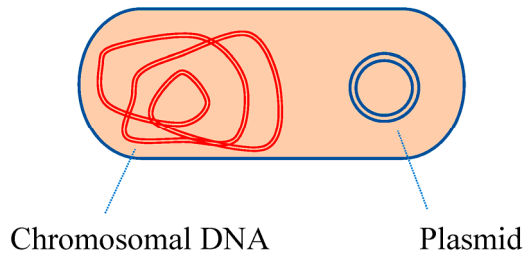


Figure 10. Schematic presentation of the genetic material of bacteria.

Plasmids and chromosomal DNA exist and replicate separately from each other. Plasmids vary in size; some carry 2 or 3 genes, others more than 400. Many plasmids are conjugative plasmids, they carry DNA to enable cell to cell DNA transfer, particularly their own transfer. Plasmids may carry antimicrobial resistance genes, and virulence factors such as aerobactin and other metabolic functions (42).

Examples of clinically relevant plasmids conferring ESBL-resistance include IncI1, IncK, IncF and IncN (99). In *E. coli,* plasmids of the IncF family are almost exclusively responsible for the successful global dissemination of the $bla_{CTX\text{-}M}$-gene (100).

5.7 Conjugation

The process of conjugation needs the involvement of either a plasmid or a transposon. Transposons are mobile genetic elements, carrying resistance genes, able to transfer between bacteria.

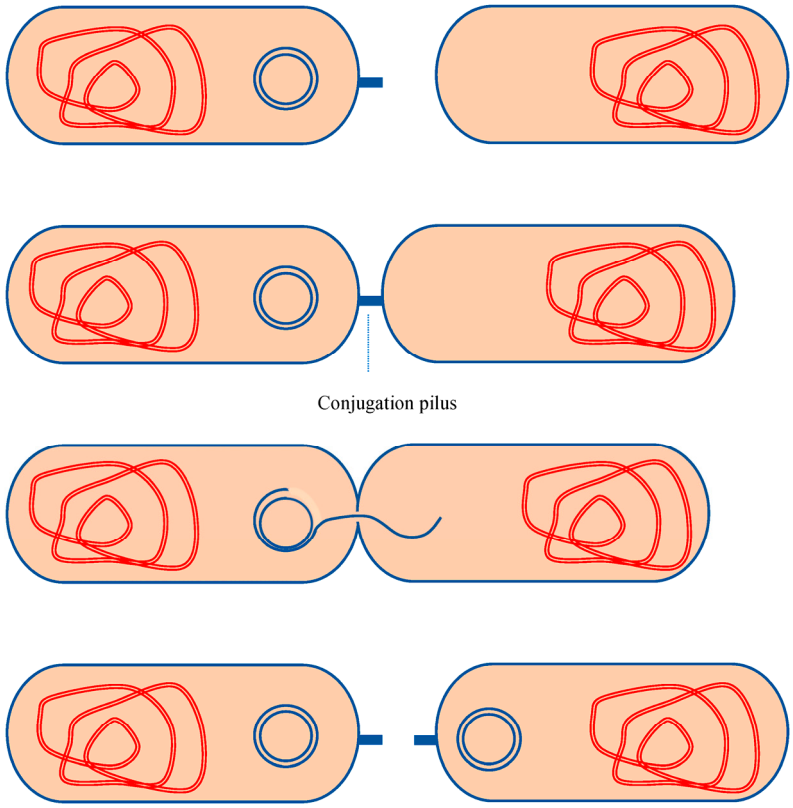


Figure 11. Schematic presentation of conjugation.
The conjugation pilus (plural, pili) draws the two bacterial cells close. The double-stranded DNA of the plasmid is copied into the cytoplasm of the recipient bacterial cell. The recipient bacterial cell will eventually become a donor bacterial cell, with its own plasmid and conjugation pilus.

In short, transformation is the process where the DNA of a bacterial cell is released, fragmented and taken up and incorporated into the genome of a different bacterial cell. Transduction involves bacterial lysis, the release of fragmented DNA which is incorporated into the genome of other bacteria by bacteriophages (97). Additionally, integrons of gene cassettes are helpful tools for bacteria in acquiring resistance genes.

5.8 Outer membrane vesicles (OMVs)

Another way in which bacteria can interact with the surroundings is to produce OMVs. OMVs could be regarded bacterial escape pods, but the functions of these OMVs are versatile. One of many purposes is to transport resistance and virulence genes out of the cells (101, 102). Recently, it has been demonstrated that EPE can spread the *bla*$_{\text{CTX-M-15}}$ gene by releasing OMVs (103).

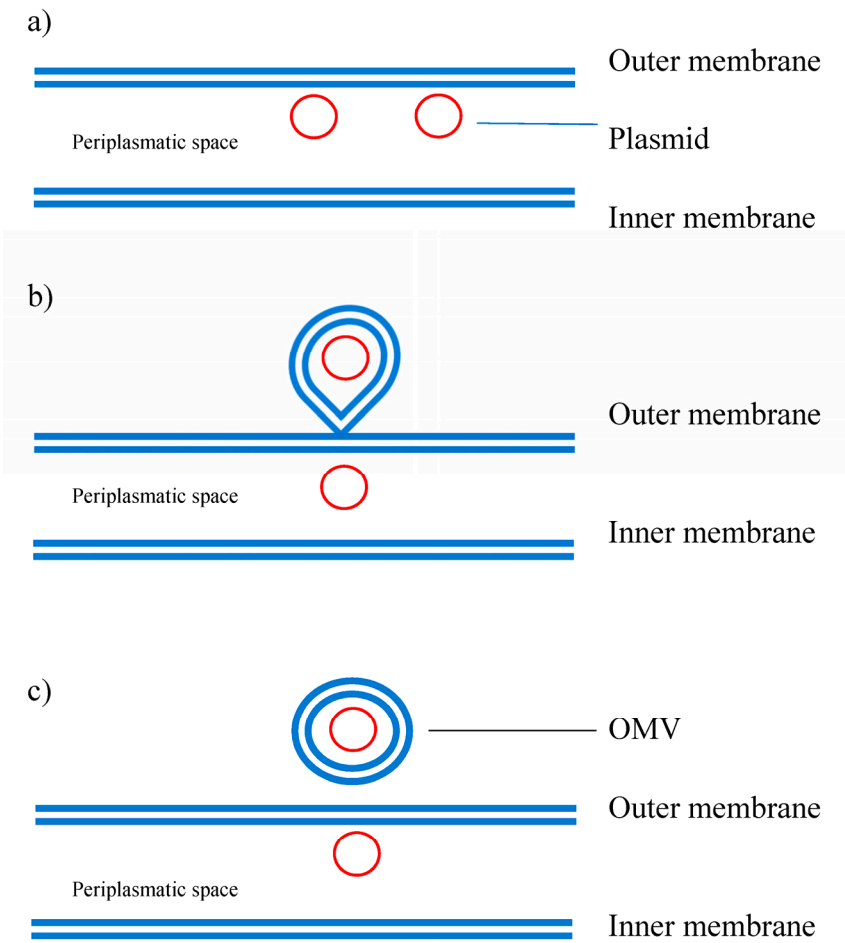


Figure 12. Schematic presentation of the formation of OMVs.
As seen above, plasmids can reside in the periplasmatic space (a). A circular form of the outer membrane is detached to form the outer membrane vesicle (b). It could be regarded as a bacterial “escape pod”, filled with genetic material ensuring further existence and proliferation of bacterial DNA (c).

As a conclusion, using these mechanisms of horizontal gene transfer, the entire global bacterial gene pool is potentially available as a source for bacteria to gather resistance genes. This is a logic outcome of Darwin's theory of evolution by means of natural selection; the more resistance genes, the more likely to survive when exposed to antibiotics. It however also tangles the evolutionary tree, since not all genetic traits in bacteria are inherited from "parent" strains.

5.9 Fitness cost

Is there a downside of carrying resistance genes? If not, why are not all bacteria multidrug resistant? The dominating theory is that with increased carriage of resistance genes comes reduced bacterial fitness. In the absence of antibiotics, in vitro models have shown that the acquisition of resistance genes is associated with fitness costs (104). Once a plasmid is acquired by a bacterial cell, transcription/translation machinery is seized in order to enable plasmid gene expression. This happens on cost of essential chromosome transcription/translation, resulting in changed cell protein expression and overall loss of fitness. Thus, a bacterial cell compensatory mechanism is plasmid degradation, to maintain the essential chromosome transcription/translation (27). However, the issue of bacterial fitness is complex, and in a small number of clones, compensatory mutations can occur, enabling the continued expression of plasmid gene expression without a loss of fitness. Although this mechanism is not fully understood, this is a theory behind the successful dissemination of ESBL-producing *E. coli* ST131, among other genes carrying the ESBL genotype $bla_{CTX\text{-}M\text{-}15}$.

6 Epidemiology

6.1 EPE in Skåne

Compared to the rest of the world, the prevalence of EPE in clinical cultures in Skåne is low and comparable to other parts of Sweden. However, the prevalence of faecal carriage of EPE in individuals in Skåne is unknown, as studies investigating the prevalence in Skåne are rare. One small study investigating the faecal carriage in the community found that in 2008 and 2010, the prevalence (obtained at primary health care units) was 2.1% and 3%, respectively. The rate of faecal carriage of the patients of a University hospital in Skåne in 2008 and 2010 were 1.8% and 6.8%, respectively (105). Another study compared the rate of faecal EPE carriage in elderly people living at nursing homes, 11%, with elderly living at their own homes, 8.7%, and found no statistical difference (106).

Regional epidemiological data from the Clinical Microbiology laboratory of Skåne University hospital is available (107). One should be cautious to draw any conclusions when the actual numbers are low, but the data shows that the trend over these 11 years is a (slowly) rising prevalence of ESBL in clinical cultures.

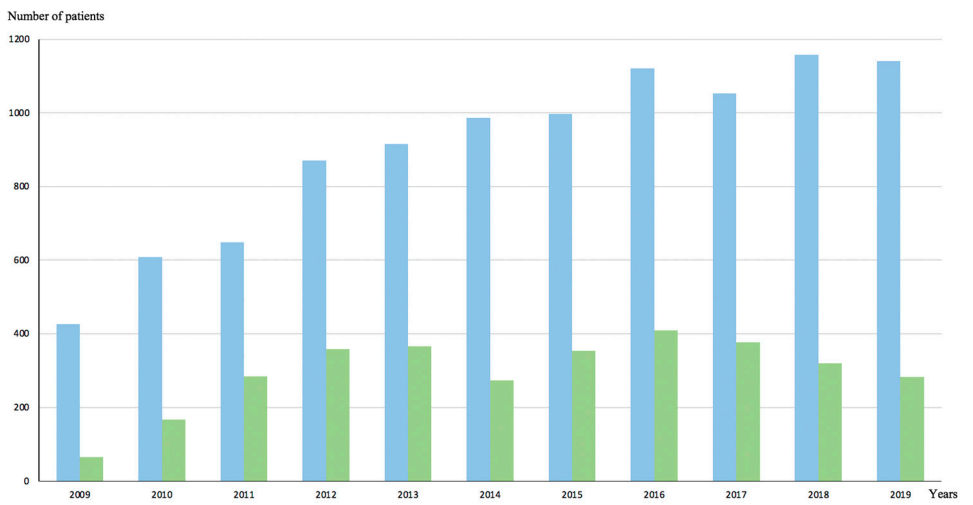


Figure 13. EPE in Skåne, Sweden 2011-2019.
The number of patients with a positive culture for an ESBL-producing bacterium. Green stacks show positive screening samples of ESBL, obtained from faeces or rectum. The blue stacks represent positive clinical samples of ESBL; from urine, blood, skin/soft tissue, airway, cervix or other. It is important to note that all patients with a history of receiving in patient health care in a foreign country during the last 6 months are screening for ESBL-producing bacteria. These patient samples constitute the bulk of the green stacks. Regional epidemiological data from the Clinical Microbiology laboratory of Skåne University hospital (107).

Between the years 2011 and 2019, the incidence of invasive (bacteremia) ESBL-producing *E. coli* and *K. pneumoniae* increased from 4.7% and 2.8% to 7.2 % and 4.7%, respectively.

Table 2. Invasive ESBL-producing *E. coli* and *K. pneumoniae* in Skåne, Sweden 2011-2019.

Year	Invasive *E. coli*	ESBL-producing *E. coli*	ESBL, (%)	Invasive *K. pneumoniae*	ESBL-producing *K. pneumoniae*	ESBL (%)
2011	1045	49	4.7	215	6	2.8
2012	1183	71	6	187	9	4.8
2013	1211	79	6.5	200	5	2.5
2014	1271	85	6.7	209	11	5.3
2015	1365	93	6.8	214	6	2.8
2016	1332	104	7.8	265	14	5.3
2017	1422	97	6.8	242	12	5
2018	1362	104	7.6	271	13	4.8
2019	1470	106	7.2	258	12	4.7

The actual numbers are low, but the trend is a rising prevalence of ESBL-producing strains. Regional epidemiological data from the Clinical Microbiology laboratory of Skåne University hospital (107).

In Skåne, invasive infections with carbapenem-resistant *E. coli or K. pneumoniae* are extremely rare. During the 9 years between 2011-2019, invasive infections with

carbapenem-resistant *E. coli* and *K. pneumoniae* occurred in 5 and 2 patients, respectively. Interestingly, during these years, the invasive carbapenem-resistant *E. coli or K. pneumoniae* cases in Skåne made up 33 % of all cases in Sweden.

Table 3. Invasive carbapenem resistant *E. coli* and *K. pneumoniae* in Skåne, Sweden 2011-2019.

Year	Invasive carbapenem resistant *E. coli*	Invasive carbapenem resistant *K. pneumoniae*
2011	0	0
2012	1	1
2013	1	0
2014	0	0
2015	1	0
2016	1	0
2017	0	0
2018	1	1
2019	0	0

Regional epidemiological data from the Clinical Microbiology laboratory of Skåne University hospital (107).

For both *E. coli* and *K. pneumoniae*, invasive ESBL-producing strains are often resistant to aminoglycosides (data available for gentamicin and tobramycin, ~40-60 %), ciprofloxacin (~60%) and trimethoprim-sulfamethoxazole (~60-85%).

In Skåne, as well as the majority of other counties in Sweden, all patients with a history of receiving health care (admitted to hospital or advance outpatient care) in a country outside of the Nordic countries during the previous 6 months are screened for MRSA, VRE and EPE at admission or prior to admission.

Additionally, patients are screened if they have been in highly endemic areas (Asia, Africa, the Middle East, South or Central America) for more than two months alternatively present with a skin wound and/or soft tissue infection that arose during or adjacent to the trip.

These cultures are obtained from vestibulum nasi, throat, perineum and rectum. When relevant, additional cultures are obtained from urine catheters, central venous catheters, eczema, stomia and from clinical wounds.

6.2 EPE in Sweden

The largest nationwide study investigating the prevalence of community carriage of EPE in Sweden gathered data between November 2012 and December 2013. It found an asymptomatic faecal carriage rate of 4.7% in healthy Swedes (58). When comparing these strains with invasive EPE strains using molecular methods, the authors found less multidrug resistance and lower pathogenicity of the faecal strains.

The rate of community carriage was slightly higher compared to other, smaller studies conducted in Sweden during the same time (23, 105, 108).

The faecal carriage of EPE in toddlers at preschools has been investigated in Uppsala, Sweden, and published in 2011 and 2018. The rate of faecal carriage of EPE increased rapidly between the years 2011 and 2018, from 2.9% to 20.1%, respectively (109, 110).

Epidemiological data on the prevalence of invasive infections of EPE is available from the European Centre for Disease Prevention and Control (ECDC), through the European Antimicrobial Resistance Surveillance System Network (EARS-Net) (111). There is no data from intestinal colonization of EPE. Between the years 2011 and 2018, the incidence of invasive ESBL-producing *E. coli* and *K. pneumoniae* in Sweden more than doubled; it increased from 3.6 and 2.3% to 8.3 and 5.5%, respectively.

Table 4. Invasive ESBL-producing *E. coli* and *K. pneumoniae* in Sweden 2011-2018.

Year	Invasive *E. coli*	ESBL-producing *E. coli*	ESBL (%)	Invasive *K. pneumoniae*	ESBL-producing *K. pneumoniae*	ESBL (%)
2011	5102		3.6	943	22	2.3
2012	5537		4.5	977	28	2.9
2013	7532		5.2	1300	47	3.6
2014	6546		5.6	1000	45	4.5
2015	5995		6.2	1001	33	3.3
2016	6958		8.3	1537	75	4.9
2017	5790		7.4	1034	58	5.6
2018	5390		8.3	1089	60	5.5

Data from European Antimicrobial Resistance Surveillance System Network (EARS-Net) (111). When this thesis was written, the latest data available was for 2018.

During these years, invasive infections caused by carbapenem-resistant *E. coli or K. pneumoniae* were rare events in Sweden.

Table 5. Incidence of invasive carbapenem-resistant *E. coli* and *K. pneumoniae* in Sweden 2011-2018.

Year	Invasive carbapenem resistant *E. coli*	Invasive carbapenem resistant *K. pneumoniae*
2011	0	0
2012	1	1
2013	2	0
2014	0	0
2015	4	0
2016	6	2
2017	1	1
2018	1	2

Data from European Antimicrobial Resistance Surveillance System Network (EARS-Net) (111). When this thesis was written, the latest data available was for 2018.

6.3 EPE in the rest of the world

During the past two decades, numerous studies investigating the prevalence of faecal colonization of EPE in healthcare settings and the community have been published, in both healthy and symptomatic individuals. For many reasons, the results of these should be interpreted with caution. The definition of what constitutes EPE differs between studies. Diverse laboratory methodology of how EPE is detected has been used. Most notably, they vary in study design, size and target population, resulting in questionable external validity. Not one study can be generalized to reflect the EPE prevalence of the inhabitants of the whole country.

By early 2000, the CTX-M-enzymes had replaced the hitherto dominating beta-lactamases TEM and SHV. Since then, the CTX-M-type of beta-lactamases have emerged as the most common ESBLs in both developed and developing countries (112). As previously stated, there are several hundred different CTX-M-types, but the most common are CTX-M-15, CTX-M-14 and CTX-M-27 (100, 112). CTX-M-14 dominates in China, Japan, South Korea, Spain and south-east Asia. In South America and Israel, CTX-M-2-type beta-lactamases are important. In the rest of the world, however, CTX-M-15 is the most prevalent CTX-M-enzyme (72, 78).

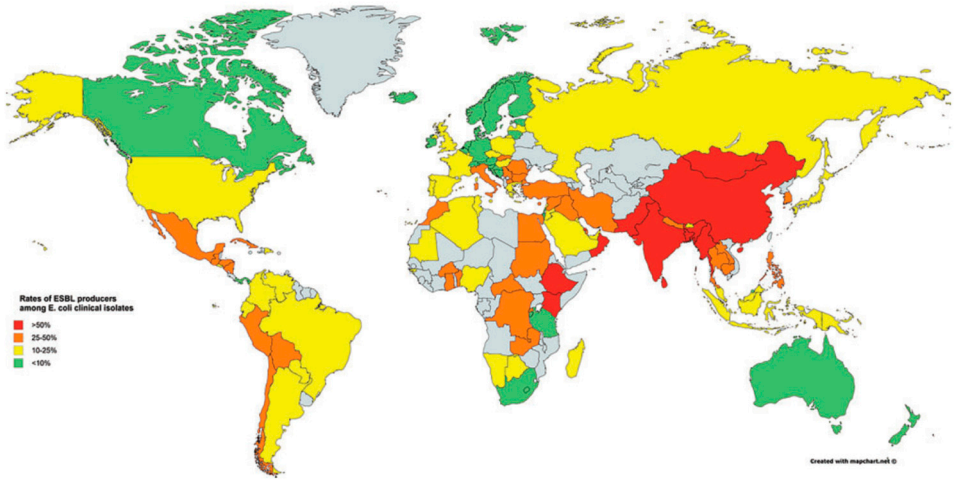


Figure 14. Estimated rates of ESBL producers among *E. coli* clinical isolates.
Data from 2014, published by the World Health Organization. Grey indicate countries with insufficient data. Reprinted with permission from author Yohei Doi (112).

As previously stated, the successful dissemination of CTX-M-15 is partly attributed to the clone ST131, including its subclone H30-Rx (113). The worldwide spread is attributed to the expression of certain virulence factors, resistance genes and the ability to colonize and persist in the intestines. Depending on geographic region, the proportion of ST131 in clinical human *E. coli* isolates range from 12.5 to nearly 30% (114).

In a review article on carriage of class A ESBL in healthy individuals, the pooled prevalence, when accounting for 66 worldwide prevalence studies, was estimated to be 14%, with an annual increasing trend of 5% (115).

6.3.1 Incidence of invasive EPE in Europe.

In Europe, data for 2018 obtained from EARS-Net, show the highest rates of cephalosporin-resistant invasive *E. coli* for Bulgaria (38.7%), Cyprus (37.1%), Slovakia (28.1%) and Italia (28.7). In contrast, Norway (6.8%), Netherlands (7.3%), Finland (7.6%) and Denmark (7.7%) reported the lowest rates. For invasive *K. pneumoniae*, the highest rates of cephalosporin-resistant strains were seen for Bulgaria (77.7), Greece (70.7%), Poland 64.6 (%) and Romania (61.4%). Lowest rates were reported from Finland (4.5%), Sweden (5.5%), Norway (7.5%) and Denmark (6.5%) (111).

The rates of carbapenem-resistant invasive *E. coli* were low in 2018; Cyprus (2%), Greece (1%) and Bulgaria (1.4%) had the highest rates. For carbapenem-resistant invasive *K. pneumonia,* however, the percentages were significantly higher; Greece (63.9%), Romania (29.5%), Italy (26.8%) and, finally, Cyprus (21.8%).

6.3.2 Increased prevalence of EPE faecal carriage in Europe

Community carriage of EPE in healthy individuals was first reported in 2003 from Spain and Poland (116, 117). Since then, European countries have reported rising prevalence of community carriage of primarily CTX-M-15 EPE strains, except for Spain where CTX-M-14 and CTX-M-9 have been the dominating beta-lactamases found in EPE isolates (118). In eastern Europe, the main ESBL beta-lactamase has been CTX-M-3 (119).

In the Netherlands, a country with relatively low EPE resistance detected in clinical isolates, community carriage of EPE in 2011and 2016 were 8.6% and 4.5 %, respectively (120, 121). Most studies conducted in nursing homes in Europe show higher rates. Elderly people living in long-term care facilities found EPE carriage rates of 14.5%, 11.3% and 40.5% in the Netherlands, Belgium and Northern Ireland, respectively (122, 123). The rate of EPE faecal carriage in patients admitted to French hospitals were 17.7% and 17.0% in 2014 and 2016, respectively, and a study on healthy children at French Day care centres found an EPE prevalence of 6,7% (124-126). The prevalence of EPE in healthy individuals was found to be 5.8%, 2.0% and 3.0% in Switzerland, Portugal and Hungary, respectively (127-129). A recent study from hospitalized patients in London reported an EPE prevalence of 9.0 % (130). In Norway, patients with diarrhoea had an EPE prevalence of 15.8% (131). One study identified ESBL-producing *E. coli* in Latvia, Germany, Finland, Sweden, Poland and Russia to be 1.6%, 4.7%, 6.6%, 8.0% and 23.2%, respectively. ESBL-producing *K. pneumoniae* was only detected in Sweden, Finland and Russia in rates of 0.3%, 1.1% and 2.0%, respectively (132). A relatively large study from Netherlands found an EPE colonization of 5% in the general population (133).

The pooled prevalence of class A ESBL in healthy individuals was estimated to 4% in the European population in general (115).

6.3.3 The prevalence of EPE faecal carriage in Asia is higher compared to Europe

Asia is commonly regarded a hotspot for multidrug resistant bacteria; the highest reported community carriage prevalence of EPE intestinal carriage in the world origin from Asia. In stool samples obtained in China between 2013-2014, 73.9 % of individuals tested carried *Enterobacterales* with a CTX-M-gene (134). Furthermore, studies from Thailand report EPE intestinal colonization rates of

61.7% and 69.3 %, respectively (135, 136). One study from Nepal in 2019 reported a rate of 68.9% of EPE carriage of healthy individuals (137). However, not all countries in Asia report rates of this magnitude. In Taiwan, only 1.9% of asymptomatic outpatients were colonized by EPE in 2017; in Japan 6.4% of healthy volunteers were colonized (138, 139). In Korean patients screened for ESBL-producing *K. pneumoniae* when admitted to an ICU, 6.4% were colonized (140). However, when screened for EPE in general in similar settings in Korea, colonization rates were higher; 28.2% and 42.5%, with a prevalence in healthy individuals of 20.3% (141, 142). A recent study on healthy adults from Taiwan found an EPE prevalence of 41.4% (143).

In Nepal, prevalence of EPE in children with diarrhoea rose from 1.5% in 2001 to 35% in 2016 (144). In Cambodia, the rate of EPE colonization among healthy individuals was 20% in 2011, with a variation between 5-62% when comparing 10 different villages (145). Prevalence studies from China report rates of EPE colonization in healthy individuals between 30.5-73.9%, 46.9% in nursing homes (134, 146-149).

A small study out of Lebanon conducted in 2013 reported an EPE carriage rate of healthy children of nearly 25% (150). In India, reports of EPE colonization rates of healthy individuals are scarce and most reports are on international travellers returning from India. One study, however, report a carriage rate of cephalosporin-resistant *Enterobacterales* of 61.4% in healthy Indian individuals (151). A recently published study from Pakistan reported an EPE prevalence of 43% among healthy children (152).

6.3.4 EPE faecal carriage in North America

Data of EPE colonization in healthy individuals from the USA is surprisingly limited. In a survey of hospitalized patients in 2007-2009, the EPE colonization rate was 2.6% and another report conducted between 2000 and 2005 saw an increase of EPE colonization from 1.3 to 3.2 % (153, 154). In Californian children under 15 years of age, EPE colonization rate was 3.5%, where the highest prevalence (5.9%) was seen in the subgroup of children below two years of age (155). In a study performed in Pennsylvanian long-term care facilities in 2006-2008, 3.4 % of the elderly were colonized with EPE (156). In California, a 17% prevalence of intestinal EPE carriage was found in 2015, and a recent study conducted in the same state between 2016-2017 found a prevalence of 38% in long-term care facilities and 34% and nursing homes (157, 158).

In two studies performed on international travellers in 2012 and 2013, one out of sixty and none of fifty-eight participants, respectively, carried EPE before travelling (159, 160).

A small study from 2010 performed in Canada found an EPE colonization rate of healthy individuals of 9%, however only 67 individuals were included (161).

In Mexico between 2014 and 2015, 17.5 % of cancer patients who were screened prior to elective surgery were EPE positive (162).

6.3.5 EPE faecal carriage in South America

Recent studies from South America investigating EPE colonization in humans are uncommon. An older study investigating EPE colonization in children in Bolivia and Peru showed an increase from 0.1% in 2002 to 1.7% in 2005 (163). Another study in children of Bolivia showed an EPE rate of 12.4 % in 2011 (164). In Argentina, cultures of outpatients with gastrointestinal complaints revealed an EPE colonization rate of 26.8% (165).

6.3.6 EPE faecal carriage in Oceania

In a study conducted between 2008-2009 in Australia on 106 international travellers, 2 % were EPE colonized in the pre-travel stool sample (166). A study on patients admitted to the ICU in Melbourne in 2011 found an EPE prevalence of 13.9% (167). In the same city, the prevalence of EPE in three nursing homes was 12%, and for patients undergoing transrectal prostate biopsy an EPE prevalence of 7 % was reported (168, 169).

6.3.7 EPE faecal carriage in Africa

In northern Africa, intestinal carriage rates of EPE have been reported from Tunisia (7.3 %) and Egypt (63.6%). In sub-Saharan Africa, rates have been described for Senegal (10.0%) and Niger (30.9%) (55). During the last years, community carriage in Africa has been studied more extensively, primarily in children. A study on children in Tanzania, both hospitalized and in the community, showed EPE rates of 50.4% and 11.6%, respectively. Interestingly, *K. pneumoniae* dominated in prevalence rather than *E. coli* (170). Additional studies from Tanzania show EPE faecal carriage of 31.8% in children, and 16.5% and 60% in adults, respectively (171-173). Furthermore, a small study of children in Central African Republic showed a rate or EPE carriage of 59% (174). In children of Guinea-Bissau and Togo, rates of positive EPE in stool samples were 32.6% and 60.5%, respectively (175, 176). In Chad, the prevalence of intestinal EPE carriage was found to be 38% in healthy volunteers and 51 % in hospitalized patients (177).

In south Africa, the prevalence of EPE colonization was 22.7% in one study (178). The pooled prevalence of class A ESBL in healthy individuals was estimated to 22% in Africa in 2016 (115).

7 Devil in a new dress; the carbapenemases.

Carbapenemases are beta-lactamases able to hydrolyze penicillins, cephalosporins, aztreonam and carbapenems. Clinically relevant carbapenem antibiotics are imipenem, meropenem, ertapenem and doripenem. Carbapenemases rose in prevalence in clinical isolates after the introduction of carbapenem antibiotics in health care in the late 1990s. Invasive infections with strains resistant to carbapenems are increasing in prevalence and are associated with high morbidity and mortality, with mortality rates of some carbapenemase-producing bacteria up to 50% (179). Carbapenemases belong to Ambler classification system class A, B and D. In parallel with ESBLs, carbapenemases are hydrolyzing enzymes, but bacteria frequently carry other resistance mechanisms such as production of efflux pumps and porin mutation or loss (180). Bacteria carrying carbapenemases also frequently carry genes encoding for ESBLs, and some isolates carry multiple carbapenemases (86).

Clinically, therapeutic options to treat carbapenem-resistant infections include colistin, fosfomycin, tigecycline and aminoglycosides, but resistance has been reported for all of these antibiotics. Some strains are resistant to all commercially available antibiotics investigated (181). For patients with invasive infections of strains resistant to carbapenems, a combination treatment of at least two susceptible antibiotics is generally recommended (180). Novel promising agents potentially becoming available commercially in the near future are plazomicin, meropenem/vaborbactam, eravacycline and cefiderocol, with activity against many carbapenemases occurring at the present time (180, 182-184).

8 Probiotics

Probiotics are defined as living microbial food supplements that offer a health promoting benefit on the host when ingested in adequate amounts (185). Probiotics have surfaced as an aid to reverse a dysbiosis of the intestines. They are regarded safe, although it involves the intake of an enormous quantity of live bacteria (186, 187). There is a vast number of different probiotics available, many of which are sold over the counter. Some contain only one single probiotic bacterium, some have multiple species. The fungus *Saccharomyces boulardii* is regarded a probiotic. The use of probiotics has been popularized during recent years, and there are data of clinical effects on patients. In clinical randomized controlled trials, probiotics reduced gastrointestinal symptoms in patients with inflammatory bowel disease (188-190).

In a study from India, probiotics given together with fructooligosaccaride reduced the incidence of death and sepsis in new-borns compared to placebo (191). Probiotics have shown effect in treating antibiotic associated diarrhoea, and several RCTs have shown benefit in a variety of gastrointestinal diseases including risk of developing *C. Difficile* infection (192, 193).

Probiotics have been proposed as therapy to reduce carriage of multidrug resistant bacteria, but most existing clinical studies are inconclusive and have conflicting results (191, 194-197). The proposed mechanism is by competitive exclusion, and in vitro experiments of several *Lactobacilli* have shown inhibitory effect on the growth of ESBL-producing *E. coli* (198). In Paper III, we used Vivomixx®, which contain *Lactobacillus plantarum, L. paracasai, L. acidophilus, L. delbrueckii* ssp. *bulgaricus, Bifidobacterium longum, B. infantis, B. breve* and *Streptococcus thermophiles*. Vivomixx® is available over the counter and was previously known as VSL#3.

Probiotics act through four main mechanisms; i) production of acids and toxins which lower the intestinal pH and inhibit pathogenic bacteria; ii) colonization of the gut which prevents adherence of pathogenic bacteria; iii) stimulation of the immune system, and, finally, iv) by strengthening the defence mechanism of the mucosa (199-202).

There are several challenges associated with probiotic therapy. They are generally not inexpensive. Optimal dosage is unknown. Also, there could be a problem with product sustainability; generally probiotic bacteria are unable to survive for longer periods, especially at room temperature. Furthermore, another problem is persistence in the intestines. The perseverance of probiotics in the intestines is only a few days (188). Thus, once the ingestion of probiotics is terminated, resident bacteria of the microbiota may outcompete the probiotics strains.

The dose and duration of administration of Vivomixx® used in Paper III, was discussed with its inventor, professor De Simone. This dose is identical to what had been used safely and tolerably in previous studies (190).

9 A brief and simplified account of the immune response to microbes

The healthy human immune system is an immensely complex system. It is divided into two key components, the innate and the adaptive immune system. The innate immune system is evolutionary older and shared with plants, fungi and other less advanced organisms. It is described as the first line of defence against pathogens; it is rapid and non-specific. It involves physical and chemical barriers, pattern-recognition receptors and cell-mediated components (203).

The cells of the adaptive immune system are capable of immune memory; once stimulated, they can persist for months or years to become reactivated once an antigen reoccurs. It reacts slower than the innate immune system but is highly specific. T- and B-lymphocytes form the backbone of the adaptive immune system.

For a long time, the adaptive and the innate immune system have been regarded as two entirely separate systems. Recently, however, the connection and interplay between the two has been mapped (204). Natural killer (NK) cells, for instance, have long been classified as cells of the innate immune system. Recent research consider them to be a link between the adaptive and the innate immune system, sharing common features of both systems (205).

B-lymphocytes, or B- cells, express B cell receptors on their cell membrane which allow the B-cell to bind to a specific antigen. This activates the B-cells and triggers antibody production. Immunoglobulin G the most prevalent type of antibody in the human body and represent 10-20% of plasma proteins (206).

While it is undetermined if protective and detectable antibodies are produced in response to colonization with *Enterobacterales*, invasive infections in humans, however, give rise to an increased antibody response compared to controls (207, 208).

Peyer's patches and mesenteric lymph nodes are involved in the innate immune response against bacteria entering the host through the intestinal mucosa. Also, the adaptive immune response plays an important role in this defence. Individuals immunized with *Salmonella Typhi* vaccine develop substantial levels of IgG and IgA targeting *S. Typhi* (209). Apart from serum produced IgG and IgM, mucosal

production of IgA (as well as in serum) is a major component of adaptive immune response against bacterial enteritis, including *C. difficile* (210).

Oral therapy with chicken antibodies (IgY), where hens were immunized with epitopes from EPE, has been proposed as a way to achieve eradication of intestinal carriage of EPE (211). Recently, monoclonal antibodies specifically reacting to CTX-M enzymes in Gram-negative bacteria have been described (212).

10 Gaps of knowledge

Although refined clinical and laboratory methods increase our knowledge of EPE, there are several unanswered questions regarding EPE in general and intestinal colonization of EPE in particular. The impact, consequences and host response of EPE colonization and infections needs of further research.

10.1 Risk factors for acquisition of EPE

Several risk factors for EPE acquisition are known, of which travel to high-endemic countries, antibiotic treatment are most prominent (130). For community carriage of EPE, one study associated a gut microbiota composition of the phylum *Proteobacteria* and the family *Enterobacteriaceae*, as well as travel to Southeast Asia in the past year (143) to EPE colonization. Another study identified vegetarians of significantly higher risk of EPE colonization compared to non-vegetarians (213). A relatively large Dutch study identified risk factors related to travel and hygiene, and another Dutch study found seasonal variances in intestinal EPE colonization, with highest prevalence in August and September (133, 214).

Furthermore, risk factors for EPE carriage and infections of hospitalized patients have been studied. Sequencing of EPE isolates found in food and livestock revealed that they differ from invasive EPE strains found in hospitalized humans, suggesting that the food chain may not be a central route of acquisition of EPE in humans (215). The predominant route of EPE transmission seems to be person-to-person contact, and other sources such as contact with farm and companion animals, environmental sources and food are recognized to a lesser extent (216). Within-household transmission of EPE is regarded a plausible source, along with other elements, of the extensively dissemination of EPE in the community (217).

A recent multi-centre study identified antibiotic therapy (hazard ratio 2.38), spring season and old age as independent risk factors of acquiring EPE during hospitalization, and the risk was highest for monotherapy with a cephalosporin (218). This risk could be affected by infection prevention measures and vary in low- and high endemic setting.

Several scores, such as the Stockholm and the Utrecht score, has been proposed to predict the risk of an invasive EPE infection, based on risk factors. The former identified a recent (particularly $\leq$ 3 months) EPE positive faecal culture, recent prostate biopsy and healthcare abroad (219). Another study from the same group identified, other than previous antibiotic therapy, urological and immunological disorders, haematological malignancy, solid tumours and diabetes as risk factors of blood stream infection by EPE in a large, population-based study (220).

The Utrecht score is comprised of two prediction rules for EPE, one for community onset and one for hospital onset suspected invasive EPE infections, which was recently validated in an international multi-centre prospective cohort study (221). It takes into account, among other factors, recent antibiotic used, immune status and suspected source of source of infection.

It has been well established that traveling to areas where EPE colonization is endemic is a risk factor for EPE acquisition. However, the situation is potentially quite dynamic as awareness increases, travel patterns change, endemic situations and clonotypes may fluctuate. Furthermore, in certain areas of the world the prevalence carbapenemase-producing *Enterobacterales* (CPE) is increasing, but the rate of community spread is largely unknown. In Skåne, a study on EPE colonization in individuals with traveller's diarrhoea highlighted the risk of EPE acquisition during international travel and informed on differential risks for different destinations (21). However, these data are now ten years old, and renewed information is needed to assess details on influx of EPE from international travellers, especially regarding what clones dominate and if CPE strains occur. What is the pathogenicity of these strains? What type of *E. coli* are they; commensal strains, intestinal pathogenic or ExPEC? What is the susceptibility of these strain to our available antibiotics?

In conclusion, the current knowledge of transmission routes is incomplete, and more studies are needed regarding EPE and the risk of spillover; zoonotic infections with pandemic potential.

Could EPE spread between companion animals within the same household? Could EPE spread between companion animals and humans of the same household? Is the microbiome protective against EPE- in line with colonization resistance?

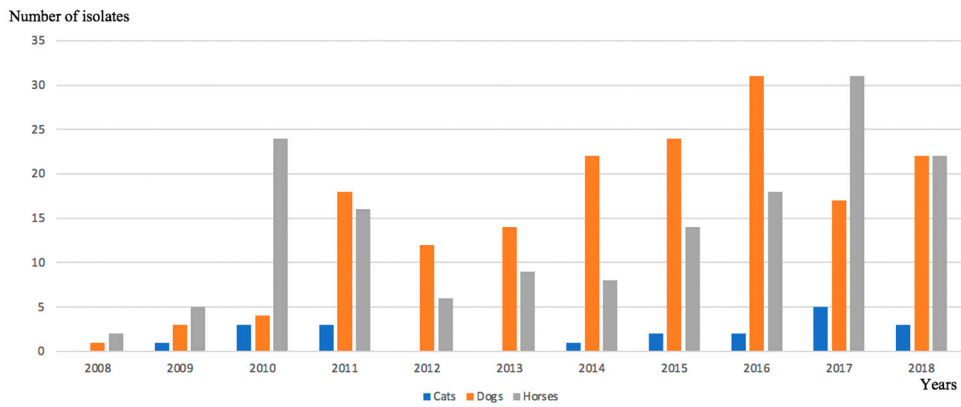
10.2 Prevalence of EPE

The development and dissemination of resistance in *Enterobacterales* is complex and rapidly changing. EPE in clinical cultures are continuously monitored and reported by most countries, although scarce resources, surveillance quality variations and unwillingness to report information make global data incomplete.

Several smaller studies worldwide, as reported above, report the prevalence of intestinal colonization of EPE. However, they are quickly outdated and rely upon the interest and scientist or governmental bodies to perform repeated surveillance studies and share data. Ideally, global updated data of EPE colonization should be gathered and shared in an easily accessible data bank.

Furthermore, the prevalence of EPE in the environment, in foodstuffs and in different animals is underexplored. In Sweden, the detection of EPE in clinical cultures of companion animals such as dogs is rare. However, one must remember the ease of which cutting edge diagnostical methods are available for humans in countries with resources. This is seldom the case for the care of companion animals, even in industrialized countries. At veterinary clinics, in-house microbiological analyses are largely unavailable. Even at the largest veterinary hospitals in Sweden, advanced microbiological analyses such as blood cultures, MAST-test etc are not readily accessible as diagnostic tools. The presence of ESBL is generally determined at the National Veterinary Institute of Sweden, in Uppsala, which gathers and reports data on e.g. EPE prevalence. However, many clinics use other private laboratories and data from the National Veterinary Institute of Sweden is therefore not representable as a measurement of EPE incidence in Sweden. Thus, there is need for data on EPE prevalence in companion animals. Additionally, EPE in farm animals, foodstuffs, sewage plants and in the environment needs to be further explored.

Table 6. Number of clinical isolates of different bacterial species of *Enterobacterales*, producing $ESBL_A$ or $ESBL_M$ from companion animals and horses, 2008-2018.



Reproduced from Swedres-Svarm 2018 (222).

10.3 Duration of colonization and risk factors for prolonged persistence

As of date, it is unknown if successful decolonization of EPE can be achieved. While multiple travel studies report the spontaneous loss of intestinal colonization of EPE, it unknown if this represent perpetual loss of EPE or reflects poor sensitivity of EPE faecal samples (223, 224). Small numbers of EPE in the intestines could be difficult to detect with current tools of diagnostics. Several questions follows. What determines the duration of colonization? Are there specific bacterial characteristics, patient characteristics, environmental factors or a combination of different factors?

If EPE is undetected by available culture methods, but still are colonizing the intestines in small amounts, is there still a risk for infection or person-to person transmission from them?? Could they cause severe disease? Could a patient, once proven colonized with EPE, ever be classified as decolonized of EPE? Should the EPE always be considered when initiating empirical clinical treatment? If the gut flora has returned to an equilibrium state- what happens if it is provoked with antibiotics? Would this lead to selection of EPE resurgence and subsequent increased risk for infection?

In this field of research, there is absence of studies on how to predict prolonged EPE colonization, which could form the basis of eradication strategies.

10.4 Eradication therapies

Several different strategies have been evaluated, such as per oral antibiotic treatments and faecal transplantation. There is, however, currently no therapy or method recommended for decolonization of EPE (225). Can the intestinal colonization with EPE be eradicated with a drug or therapy? Could future vaccines target EPE or selected clones of EPE causing severe infections?

Such eradication therapies are neither possible nor desirable for all patients, as instant recolonization is likely in high endemic countries, and based on the discussion in the section on prolonged colonization, it is still a matter of debate if complete eradication is at all possible.

Targeting and eliminating virulent EPE strains causing repeated or invasive infections, however, is highly desirable particularly in specific populations, e.g. immunocompromised hosts. Since colonization in itself is not harmful, such eradication therapy must have a clinically relevant number needed to treat (NNT), with few or no adverse effects.

10.5 Host immune response to EPE colonisation/infection.

Numerous clinical studies and new laboratory techniques such as whole genome sequencing have provided valuable insights of virulence and resistance genes of EPE during the past decades.

But little is known about the host immune response to EPE colonization and infection. Is there a humoral response to the CTX-M-type beta-lactamase?

Recurrent infections with E. coli in general and ESBL-producing E coli in particular, most notably urinary tract infections, are not uncommon. Such infections have been demonstrated to occur with bacteria carrying identical beta-lactamases suggesting that infections with E. coli do not induce protective immunity (226). But it is not known whether specific antibodies directed against the specific beta-lactamases are generated, neither IgA nor IgG, in what circumstances they may be and what functional role such antibodies may have. If antibodies are produced, could they be used as diagnostic tools or even for adjunctive therapy for EPE infections or colonization?

Why are some individuals only colonized with EPE and others develop infections? Is this due to bacterial characteristics or underlying conditions of the host? Is it a combination of the two? Is the host immune response to EPE colonisation/infection involved? If so, in what way?

11 Overall aim of the thesis

The overall aim of the thesis was to study different aspects of EPE acquisition, colonization, loss of colonization and infection. The included studies specifically examine spread to or from companion animals, risk factors for carriage persistence, an updated assessment of acquisition from international travel, an eradication trial and humoral response following infection.

11.1 Specific aims

- To study potential and frequency of spread of EPE between humans and dogs of the same household (Paper I).
- To identify risk factors associated with prolonged intestinal carriage of EPE (Paper II).
- To identify to what extent patients with EPE infection/colonization is adequately informed about EPE (Paper II).
- To evaluate if a probiotic mixture could eradicate intestinal carriage of EPE (Paper III).
- To investigate if specific IgG antibodies directed against the ESBL enzymes CTX-M-15 and CTX-M-27 can be detected in patients having undergone EPE bacteremia (Paper IV).
- To re-assess the rate of EPE carriage in patients with travellers' diarrhoea ten years after an initial study in the same region was performed (Paper V).
- To identify if patients with travellers' diarrhoeal carry carbapenem-resistant *Enterobacterales* (Paper V).
- To study phenotypic and genotypic traits of EPE identified, and likely acquired during travel, with special regard to antimicrobial resistance and virulence factors (Paper V).

12 Patients and methods

Below is a summary of the ethics, patients, setting, laboratory methods and research methodology used in this thesis. For detailed description of the methodology used for all papers, please see the Materials and Methods sections of each paper.

12.1 Ethics

All included studies in this thesis were approved by a local ethics committee (Regional Ethical review Board Lund District Court; DNR2013/713, DNR2016/304, 2016/803, 2018/143 and 2016-740).

12.2 Setting

All five studies were conducted in the Skåne County, located in the south of Sweden with nearly 1.4 million inhabitants in 2019 (227). In Skåne, there is only one laboratory for clinical microbiology, serving the whole county, and no private alternatives exist. In Paper I, additional individuals were recruited to the control group (dog owners with a faecal culture negative of EPE) in Uppsala, Sweden. The majority of the control group was, however, recruited in Skåne.

12.3 Paper I

Paper I was a cross sectional case control study. Patients were recruited from the Department of Clinical Microbiology, Skåne University hospital. In an 18-month period between 2014 and 2015, 498 patients were positive for EPE in at least one faecal culture. Case records were reviewed; new-borns receiving neonatal care and patients with therapeutic/prophylactic antibiotic treatment were excluded. Furthermore, a permanent address and a telephone number was needed in order to contact the patient and to be able to send material for EPE cultivation of the patient

and the household dog. Many of the 498 patients positive for EPE during this time were recently arrived refugees, unable to be included in the study. One cannot be certain but bringing or acquiring a companion animal were likely not a priority for these refugees.

Patients were contacted by telephone and asked about dog ownership or if a dog was present in the household. The control group, individuals with a negative EPE faecal culture, was recruited from the vicinity of the research group. Unfortunately, we did not register the exact number of patients excluded after case records were reviewed, neither how many patients that were contacted by phone. This is regretful and should have been recorded and disclosed in the manuscript. However, not one dog owner declined participation.

12.4 Paper II

Paper II was a prospective cohort study. Inclusion of patients was conducted between 2016-2017, during which 2 148 adult patients with a verified culture of EPE in urine, blood, faeces or any other location were eligible for inclusion. Case records were reviewed, exclusion criteria were alcohol or substance abuse, severe psychiatric disorder, immunosuppressed patients (e.g., immunodeficiency, ongoing cancer treatment, neutropenia/leukopenia, treatment with TNF-α-inhibitors), inpatient care, patients with chronic venous catheters, dementia or inability to provide informed consent, and newly arrived refugees. In all, 820 adult patients with at least 1 documented positive ESBL-culture from blood, wound, cervix, urine or faeces were contacted by mail and asked to submit one faecal sample for microbiological analysis. We defined prolonged EPE colonization as the prevalence of EPE in the faecal culture obtained at least 3 months from the first positive EPE-culture. Of the 820 adult patients invited to take part in the study, 677 declined to submit a faecal sample and 143 accepted.

12.5 Paper III

Paper III was a randomized, single blinded clinical trial of a probiotic mixture. The patients of paper III consisted of the 81 adult patients with no evidence of immunosuppression of paper II who were positive for EPE in the follow up faecal culture, with the exception of one patient who declined further participation after the (EPE positive) faecal culture had been obtained. Thus, eighty patients were in total randomized to placebo or probiotic treatment.

All patients were asked to submit faecal samples for selective ESBL-culturing 1, 3, 6 and 12 months after initiation of probiotics or placebo. Hence, the first follow-up faecal culture was taken during the treatment and the second one 4 weeks after the intervention was completed.

The primary endpoint was successful decolonization defined as three consecutive ESBL-negative faecal samples after completed intervention (at time-points 3, 6 and 12 months).

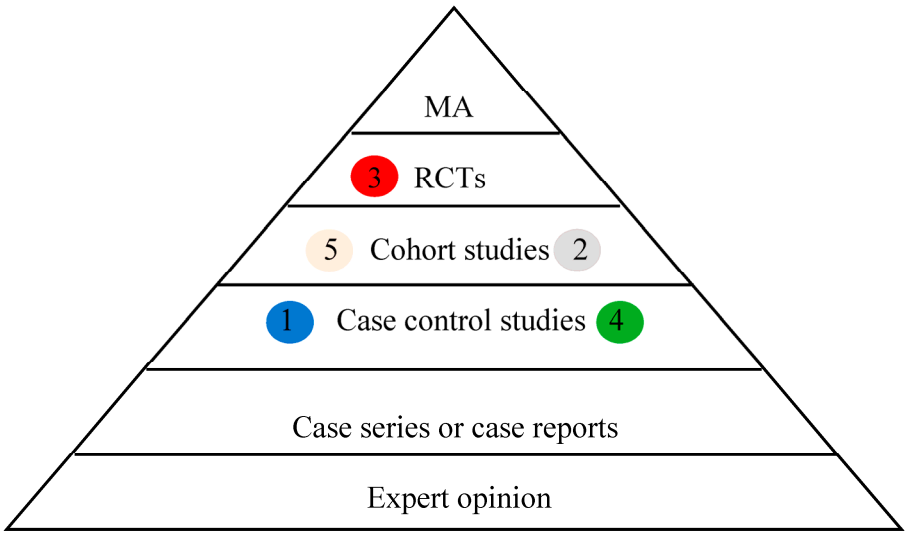
12.6 Paper IV

Paper IV was a cross sectional observational study with a control group. Eligible patients for inclusion in the study were adult patients who had been diagnosed and survived an ESBL-producing *E. coli* or *Klebsiella* spp. bacteremia during the years 2016 and 2017, $n = 147$. The exclusion criteria of this study were primary residency outside Skåne County, non-Swedish citizenship, cognitive impairment, and previous bacteremia with EPE. The control group consisted of patients with 3GCSE bacteremia during the same period. After case records had been reviewed, 121 patients were eligible in the EPE group and invited to participate, of which 62 patients declined or did not reply.

In the 3GCSE group, 135 patients were assessed for inclusion. At this point in time, this was the number of patients in the EPE group thought to be eligible for inclusion. Out of these, 93 patients declined or did not reply. Thus, 42 patients were included in the 3GCSE group.

12.7 Paper V

Paper V was a cross sectional cohort study. Clinical samples sent to the Department of Clinical Microbiology, Skåne University Hospital between February 2017 and January 2018 for culture of *Campylobacter, Salmonella, Yersinia* or *Shigella*, for which the referral stated foreign travel, were included in the study according to staff availability. In total, 314 cultures were included and selectively cultivated for EPE according to the hospital routines. Out of these 314 cultures, the referral stated 'asymptomatic' for 11 patients (restaurant students/employees) and where thus excluded from the study. WGS was performed on all available EPE strains. Countries were categorized according to the United Nations Statistics Divisions (228).



MA = meta analyses, RCTs = randomized controlled trials.
1 = paper I, 2 = paper II, 3 = paper III; 4 = paper IV, 5 = paper V.

Figure 15. Schematic view over study design for each study.

13 Laboratory methods for detecting EPE

When screening for ESBL-producing bacteria, commonly referred to as selective cultures, chromogenic culture media are used. The medium contains a chromogenic substrate which yields a specific colour to each species of microorganisms. Ceftazidime and meropenem are used as markers for ESBL and carbapenem resistance, respectively. After 16-18 hours of incubation, the plates are inspected. Colonies of *E. coli* are pink/burgundy whereas *Klebsiella/Enterobacter/Serratia/Citrobacter* are green/blue to brown/green and *Proteus/Providencea/Morganella* are dark to light brown. Classification according to species is done with MALDI-TOF. Putative ESBL strains are then further analysed using the MAST test. Selective culture for EPE with susceptibility testing according to the EUCAST disk diffusion test methodology was performed in paper I, II, III and V.

13.1 The MAST-test

The MAST-test (Mast group Ltd, Liverpool, England) is an easy to use, phenotypic test for detecting EPE. As previously stated, classic plasmid mediated ESBL, in Sweden referred to as $ESBL_A$, is inhibited by clavulanic acid. AmpC, in Sweden referred to as $ESBL_M$, is inhibited by cloxacillin. This is used in the MAST-test at the clinical microbiology unit to differentia between ESBL and AmpC. If AmpC/ESBL is suspected, the isolate is incubated (35-37°C) overnight. The third-generation cephalosporin cefpodoxime is used as base on the plate.

A = cefpodoxime 10µg
B = cefpodoxime 10µg + clavulanic acid
C = cefpodoxime 10µg + cloxacillin
D = cefpodoxime 10µg + clavulanic acid+ cloxacillin

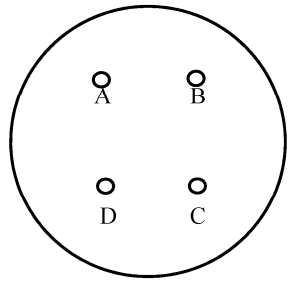


Figure 16. Schematic presentation of the premise of the MAST test.

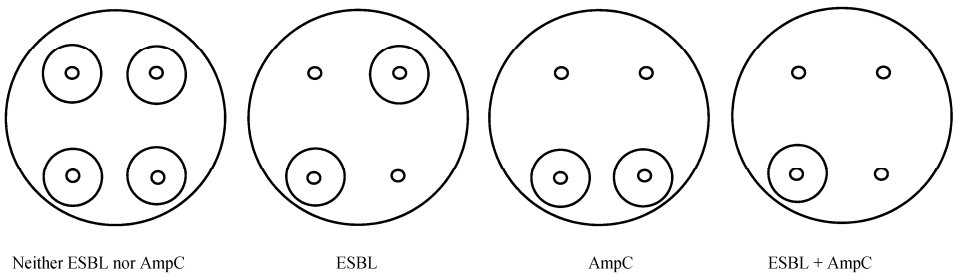


Figure 17. Interpretation of MAST-test

When zones are measured, sizes ≥ 5 mm are used to determine inhibition. Consequently, in zone sizes between 0 - 4 mm, no inhibition has been seen. If the result is negative, or when the result is not assessable, further analysis using polymerase chain reaction (PCR) is performed on the strain.

Multiple beta-lactamases within one organism (e.g. combined ESBL and AmpC) can make phenotypic tests difficult to interpret. Furthermore, phenotypic tests like the MAST-test are not able to differentiate between chromosomal or plasmid-mediated AmpC (pAmpC). For *Klebsiella*, this does not constitute a problem, as *Klebsiella* bacteria do not possess chromosomal AmpC. The detection of pAmpC in klebsiella is therefore straightforward. For *E. coli*, however, the situation is a bit more complicated, as it has both chromosomal and plasmid mediated AmpC. Genotypic verification is needed to conclude that the strain is in fact a pAmpC. MAST-tests cannot differentiate between the different types of pAmpC. For this, PCR is needed. The NordicAST flowchart to detect $ESBL_A$ and $ESBL_M$ in Enterobacterales is displayed in Figure 18 and 19, respectively.

13.2 PCR

PCR is a widely used technique within diagnostics of infectious disease for detecting the DNA of pathogens. In medical microbiology, PCR followed by DNA sequencing is used to identify and characterize genes and genetic sequences that may be related to phenotypical traits, such as resistance and virulence.

It involves the exponential amplification of small fragments of DNA through multiple cycles of denaturation, primer annealing and amplification.

The result is millions of copies of DNA which enables detailed studies on molecular level. The PCR technique was developed during 1980s by Karey Mullis, who later was awarded the Nobel prize in chemistry for this achievement. It has become invaluable for determining and analysing the worldwide spread of ESBL. In particular, PCR based methods for determining beta-lactamase according to CTX-M or pAmpC-type are standard use today (90, 229). PCR was used in paper I, IV and V.

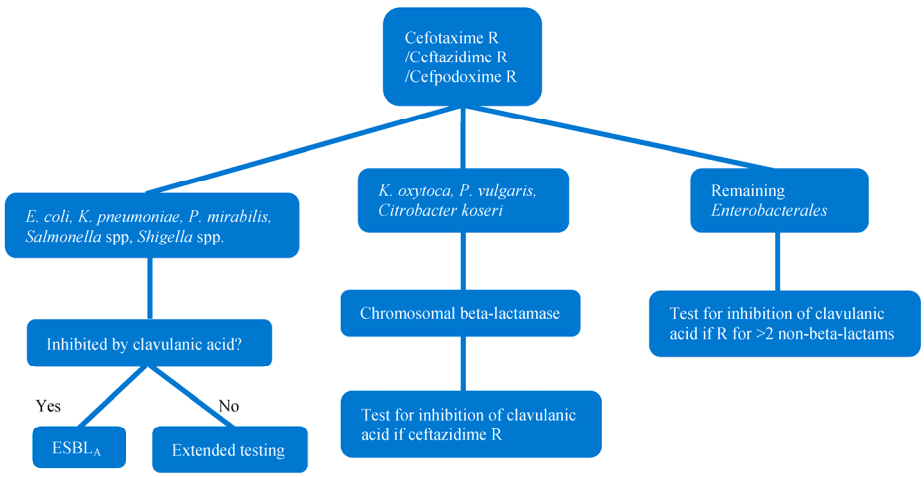


Figure 18. The NordicAST flowchart to detect $ESBL_A$ in *Enterobacterales*.

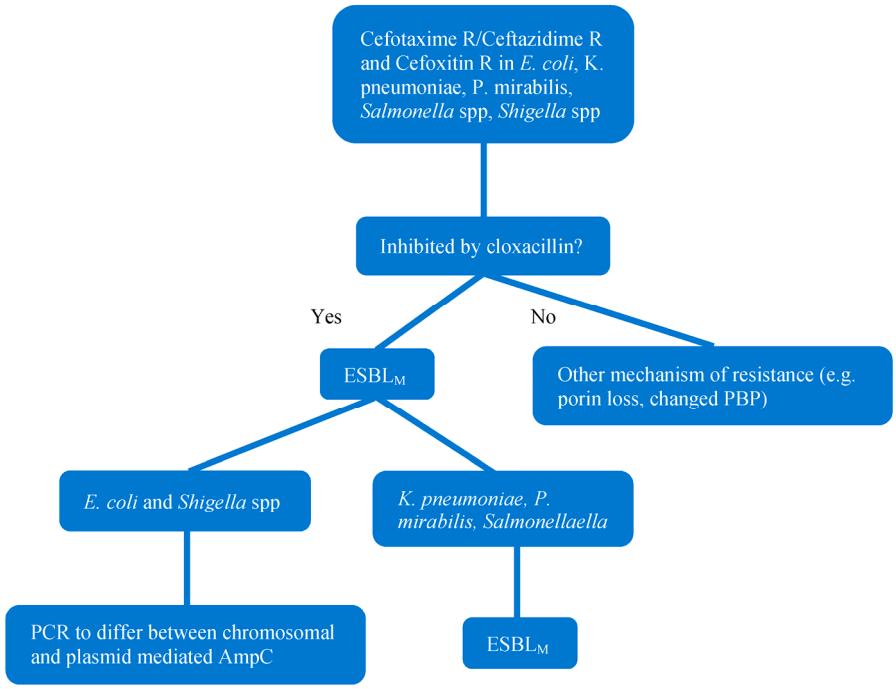


Figure 19. The NordicAST flowchart to detect $ESBL_M$ in *Enterobacterales*.

13.3 Multiple locus VNTR analysis (MLVA)

MLVA a method for epidemiologic typing and can be used in prokaryotes as well as eukaryotes. In bacteriology, it is a useful tool to determine phylogenetic relationships of different isolates, which can be used for research purposes but also for uncovering outbreaks of, for instance, drug resistant bacteria in health care. In the genes of different bacterial isolates, specific sequences of nucleic acids are often identical. However, these sequences are repeated throughout the genes, and the number of repetitions is unique for each organism. The repeats are known as variable number of tandem repeats, VNTR. For *E. coli*, primers targeting ten different pairs of loci of the genome were selected for paper I in this thesis (CVN001, CVN002, CVN003, CVN004, CVN007, CVN0014, CVN0015, CCR001CVN0016 and CVN0017, respectively) (230). By using PCR the number of repeats were analysed. The number of repeats is found by assessing the weight of the entire block of VNTR divided by the weight of the sequence. In identical isolates, the number of repeats is identical whereas in different isolates the number of repeats differs. MLVA was used in paper I.

13.4 MLST

Using PCR and DNA sequencing, a set of housekeeping genes (typically seven different loci) are analysed for nucleotide differences. MLST can be used to differentiate between strains of bacteria of the same species, e.g. in situations of outbreaks and for phylogenetic analysis if the sequences are concatenated. Even though WGS is becoming more available and in many instances replacing MLST, the latter has been used for a long time and classification of *E. coli* strains according to sequence type is still highly relevant. Although MLST was not used specifically for any of the papers in this thesis, sequence types were determined in Paper IV, using data from WGS.

13.5 ELISA

Simplified, ELISA is used to detect the presence of a ligand, commonly a protein in a liquid. In this thesis, the proteins used are antibodies: anti-CTX-M-15 and anti-CTX-M-27. They bind to the walls/bottom of the microplate. The optical density, i.e. the measure of absorbance, of the liquid is compared to standard curve and a cut-off is applied. ELISA was used in paper IV.

13.6 WGS

Due to its small genome size, *Haemophilus influenzae* was the first organism to have its entire genome sequenced in 1995 (231). Today a whole genome shotgun method is used, where many overlapping DNA fragments are sequenced in parallel. A computer software assembles these fragments into larger contigs and (sometimes) eventually chromosomes. Before WGS, subtyping of E. coli was done by measuring antibody levels against O- and H-antigens, and MLST. WGS can more accurately subtype different strains of *E. coli*, as well as provide useful information of resistance and virulence genes (232).

13.7 Statistics

Numerical data was summarized as median and categorical data as proportions (%). For comparison between groups different tests were used for parametric/nonparametric, continuous or categorical data. A two-sided exact significance of ≤ 0.05 was considered statistically significant. In Paper II and IV,

univariate logistic regressions were performed. Odds ratios (OR) with 95% confidence interval (CI) were reported. In Paper II, a stepwise forward multivariate logistic regression model was used. Additionally, the statistical method of weighting was used to account for unequal response rates. For paper III, imputation and a sensitivity analysis was performed. The statistical analyses were performed using IBM SPSS Statistics for Macintosh, Version 25.0. Armonk, NY: IBM Corp.

14 Results

14.1.1.1 Paper I

In the EPE group, twenty-two individuals from twenty-two different households were confirmed EPE carriers as determined by faecal cultures. The age of these individuals ranged from 1 to 82 years. Of these, twenty carried ESBL-producing *E. coli*, one individual also carried an ESBL-producing *K. pneumoniae*. One person carried a pAmpC-producing *E. coli* and another an ESBL-producing *K. pneumonia*.

Of the twenty-two households with individuals carrying EPE, dogs of 2 households (9%) were EPE positive. In each of these households, two additional persons were screened for EPE, but all four were negative. In the control group, none of the 29 humans nor dogs were EPE positive.

In household one, the strains of *E. coli* found in the human and one of the dogs were determined to be identical using MLVA. The other family members, both adults, and the second dog had negative cultures.

In household two, one human and two dogs were determined to carry pAmpC-producing *E. coli*. The pAmpC-producing *E. coli* isolated from the child and from one of the dogs was determined to be identical by MLVA. The other dog had a similar but not identical pAmpC-producing *E. coli* as determined by MLVA. The other family members cultured for EPE were negative.

14.1.1.2 Paper II

In paper two, we included a total number of 143 patients in the study. They carried a total of 147 EPE-strains, of which 134 (91%) were *E. coli*, 12 (8%) were *K. pneumoniae* and 1(1%) was *Proteus* spp. Out of the 147 strains of EPE, 134 (91%) were inhibited by clavulanic acid ($ESBL_A$) 10 (7%) were inhibited by cloxacillin (AmpC or $ESBL_M$) and 3 (2%) were inhibited by both. In the follow up faecal culture after a minimum of three months, 81 patients (57%) remained positive for EPE out of the initial 143 patients. Thus, 62 patients were negative in the follow up faecal culture.

Data was obtained from medical charts and questionnaire and analysed for risk factors of prolonged EPE colonization. In the univariate model, urological intervention within 6 months (p=0.000), an original EPE-positive culture in urine

(p=0.009), a history of EPE infection (p=0.007) and travel to Asia and/or Africa within two years (p=0.020) were associated with a follow-up faecal sample positive for EPE.

In the stepwise forward multiple regression model, urological intervention within 6 months (p=0.007, odds ratio (OR) 4.32, 95% confidence interval (CI): 1.50–12.42) and history of EPE infection (p=0.028, OR 2.82, 95% CI: 1.12–7.10) remained significant risk factors for prolonged EPE carriage. Travel to Africa and/or Asia within the last 2 years (p=0.04, OR 0.42, 95% CI: 0.18–0.96) was associated with a reduced risk of prolonged EPE carriage.

Table 8. Outcome of the stepwise forward multiple regression model.

Step	Variable	OR	CI 95%		*p*-value
			Lower	Upper	
Step 1	Urological intervention within 6 months	5.72	2.05	15.92	0.001
Step 2	Urological intervention within 6 months	4.69	1.65	13.33	0.004
	History of EPE infection	2.77	1.13	6.83	0.027
Step 3	Urological intervention within 6 months	4.32	1.50	12.42	0.007
	History of EPE infection	2.82	1.12	7.1	0.028
	Travel to Africa and/or Asia within 2 years	0.42	0.18	0.96	0.040

Out of 820 patients approached in this study, only 143 (17%) were willing to submit a faecal sample. Thus, 677 (83%) individuals were unwilling to participate. An 83% loss could risk the external validity of our results. Therefore, statistical weighting was performed on the variables that were associated with prolonged carriage of EPE (before weighting), as well as age, sex and Charlson comorbidity score.

Table 9. Comparison of baseline characteristics between the patients included in the study and patients that did not provide a follow-up sample.

Baseline characteristics	Patients included (n=143)	Patients not included (n=677)	*P*-value
Age, median (range)	67 (19-87)	60	
Female sex (%)	92 (64)	456 (67)	0.4858
Charlson comorbidity score, median (range)	0 (0-7)	0 (0-8)	
History of EPE infection (%)	116 (81)	458 (68)	0.001
Urological intervention within 6 months (%)	36 (25)	129 (19)	0.097
Resistance to ciprofloxacin + trimethoprim/sulfamethoxazole (%)	71 (50)	250 (37)	0.01

Most of the variables compared did not differ significantly between the patients included and not included.

There was, however, a difference in the percentage of patients with resistance against both ciprofloxacin and trimethoprim/sulfamethoxazole (p=0.01) and infected compared to colonized (p=0.001) in the two groups. In order to adjust for this, statistical weights were computed with regard to resistance and history of EPE infection.

14.1.1.3 The process of weighting

When using weighting based on two different variables, two different 2x2 contingency table are used. One cross tables that shows how the variables are distributed within the patients included in the study, and one cross table for all patients.

Table 10. Cross table for all patients

		History of EPE infection		
		No	Yes	Total
Resistance to ciprofloxacin + trimethoprim/sulfamethoxazole	No (%)	141 (17.2)	358 (43.7)	499 (60.9)
	Yes (%)	105 (12.8)	216 (26.3)	321 (39.1)
	Total (%)	246 (30.0)	574 (70.0)	820 (100)

Table 11. Cross table for included patients

		History of EPE infection		
		No	Yes	Total
Resistance to ciprofloxacin + trimethoprim/sulfamethoxazole	No (%)	10 (7.0)	62 (43.4)	72 (50.3)
	Yes (%)	17 (11.9)	54 (37.8)	71 (49.7)
	Total (%)	27 (18.9)	116 (81.1)	143 (100)

As seen from table 10 and 11, there was a discrepancy in the proportion of patients with no history of EPE infection and at the same time no resistance against ciprofloxacin + trimethoprim/sulfamethoxazole between all patients (17.2%, underlined) and included patients (7.0%, underlined).

The aim of weighing is to level out this difference. The weights are displayed in Table 12.

Table 12. Computed statistical weights.

Variable	Weight
History of EPE-infection and Bacteria resistant to both ciprofloxacin and Trimethoprim/Sulfamethoxazole	0.6976
History of EPE-infection and Bacteria not resistant to both ciprofloxacin and Trimethoprim/Sulfamethoxazole	1.0070
No history of EPE-infection and Bacteria resistant to both ciprofloxacin and Trimethoprim/Sulfamethoxazole	1.0771
No history of EPE-infection and Bacteria not resistant to both ciprofloxacin and Trimethoprim/Sulfamethoxazole	2.4589

In brief, the weight is equal to what the number highlighted in red in table 10 (7.0) has to be multiplied with to equal the number highlighted in green in table 10 (17.2). In this case ≈ 2.46. Corresponding calculations are then done for results of the other variables, as seen in Table 12. Using these weights, a new cross table is constructed for the patients included in the study.

Table 13. New cross table for included patients using weights.

		History of EPE infection		
		No	Yes	Total
Resistance against ciprofloxacin + trimethoprim/sulfamethoxazole	No (%)	25 (17.5)	62 (43.4)	87 (60.8)
	Yes (%)	18 (12.6)	38 (26.6)	56 (39.2)
	Total (%)	43 (30.1)	100 (69.9)	143 (100)

As seen in Table 13, the new, weighted results for the included patients are similar to the results of all included patients, compared to table 10. Thus, the new, computed results better represent the results of all patients eligible for the study.

When the values are used in the multiple regression model, they either have a "boosting" or "reducing" effect. The answers from the underrepresented group (no history of infection, bacteria not resistant to ciprofloxacin + trimethoprim/sulfamethoxazole) will have greater impact, and the overrepresented (history of infection, bacteria resistant to ciprofloxacin + trimethoprim/sulfamethoxazole) less impact.

When patients were asked if they were aware of their EPE infection/colonization before being contacted by the study group, sixty-one patients (50%) stated that they were unaware of this.

14.1.1.4 Paper III

A total of 80 patients were included in this placebo-controlled, single blinded, randomized controlled trial. It was designed as a superiority trial, investigating the effectiveness of a probiotic mixture compared to placebo in eradicating chronic (defined as at least three months) intestinal EPE-carriage. Patients were randomized to placebo (n = 40) or probiotics (n = 40). The median age was 68 years in both groups. The number of females in the probiotics group was 23 (58%) and in the placebo group 28 (70%).

At the end of the trial, 12.5% (5 out of 40) of the patients in the probiotic group had achieved successful eradication of EPE, as defined by the primary outcome, in the intention to treat analysis. In the placebo group, 5% (2 out of 40) of the patients had achieved successful eradication of EPE (odds ratio 2.71; 95% CI 0.49–14.9; p = 0.24).

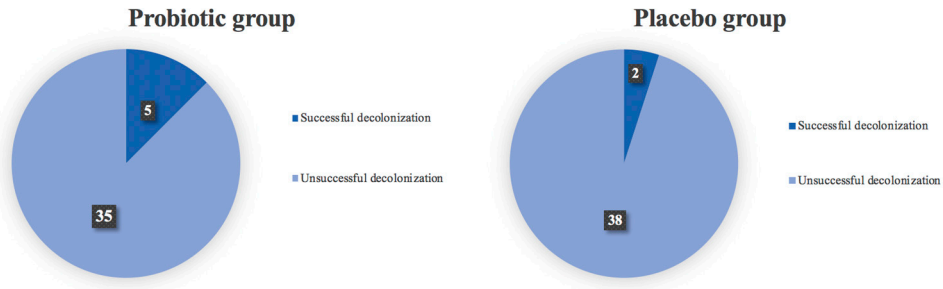
When individuals with fully evaluable outcomes where included only, successful intestinal decolonization was achieved in five out of 33 study persons (15%), compared with two out of 33 (6%) in the control group (p = 0.23).

Of great importance, though, is the fact that two patients in the probiotic group (of the 5 patients with successful EPE eradication) that did meet study criteria of eradication, had clinical cultures positive for EPE during the follow up time. One had multiple urinary cultures and the other one had a faecal culture, positive for EPE. These cultures were all taken in a clinical setting, beyond the study protocol, with identical antibiograms compared to the cultures of the study protocol. The two patients both achieved successful eradication of EPE as defined by the primary outcome but were clearly not EPE negative. This reflects the limited sensitivity of selective faecal cultures even when several samples are obtained as in this study.

Thus, of the 5 patients with successful eradication of EPE, as defined by the primary outcome used in our study, 2 patients evidently did not have successful eradication. Using this information, 7.5% (3 out of 40) of the patients in the probiotic group had

achieved successful eradication of EPE, compared to 5% (2 out of 40) in the placebo group (OR 1.54, 95% CI 0.24-9.75; p 0.64).

a)



b)

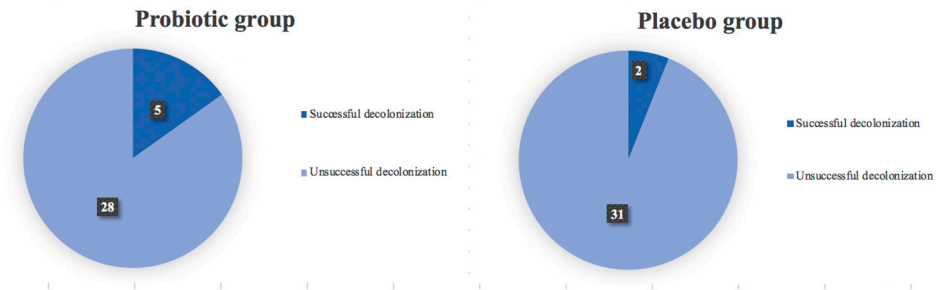


Figure 20. Results of the intervention, a) intention to treat analysis, b) per protocol analysis.

14.1.1.5 Paper IV

A total of 59 patients were included in the EPE-group, and out of these 47 patients had blood isolates carrying either *bla*$_{\text{CTX-M-15}}$ or *bla*$_{\text{CTX-M-27}}$. Of these 47 patients, 13 patients (28%) carried IgG antibodies to the corresponding CTX-M-type. In contrast, the control group consisted of 42 patients with 3GCSE bacteremia of which only 9.5% of patients (4 out of 42) were positive for anti-CTX IgGs (p=0.03).

IgG directed against CTX-M-27 were found in 5 patients with EPE bacteremia, but antibody specificity did not correspond to the genotype of bacteria isolated from

blood; 4 patients were infected by *E. coli* expressing the $bla_{\text{CTX-M-15}}$ gene and 1 patient had an EPE blood isolate carrying $bla_{\text{CTX-M-14}}$.

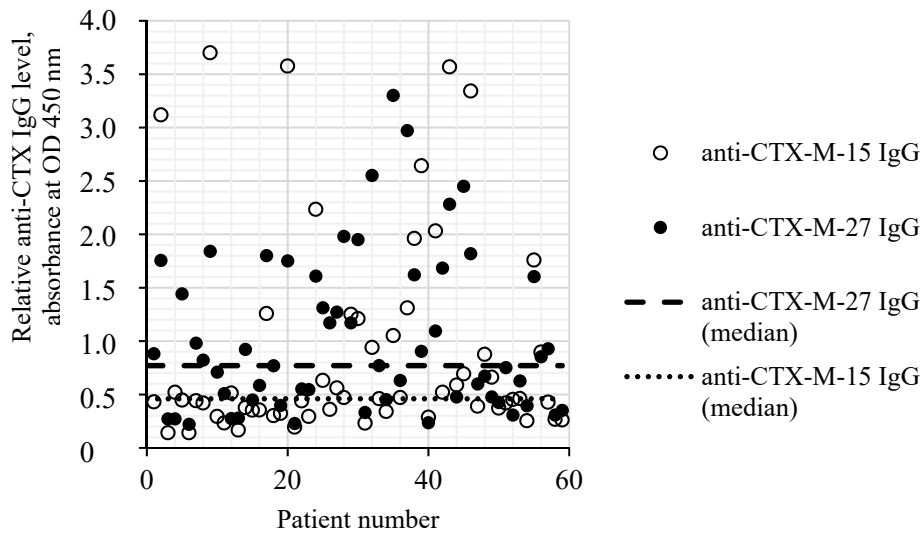
Table 14. Genetic profile of EPE blood isolates and prevalence of antibodies in the EPE and 3GCSE group.

CTX-M beta-lactamase[a]	Only anti-CTX-M-15 IgG (*n*)	Only anti-CTX-M-27 IgG (*n*)	Both anti-CTX-M-15 and anti-CTX-M-27 IgG (*n*)
EPE group (*n*=53)	2	7	9
$bla_{\text{CTX-M-15}}$ (*n*=41)	2	4[b]	8
$bla_{\text{CTX-M-27}}$ (*n*=6)	0	2	1
$bla_{\text{CTX-M-14}}$ (*n*=6)	0	1[b]	0
3GCSE group (*n*=42)	2	2	0

[a] The following $bla_{\text{CTX-M}}$ genes were also identified in 5 clinical isolates, but patients did not develop cross-reacting anti-CTX-M15 or anti-CTX-M-27 IgGs; $bla_{\text{CTX-M-138}}$ (*n*=2), $bla_{\text{CTX-M-174}}$ (*n*=2), and $bla_{\text{CTX-M-79}}$ (*n*=1). The $bla_{\text{CTX-M}}$ gene of one blood isolate could not be determined.

[b] False positive, *i.e.*, antibody prevalence but not to the corresponding genotype.

(a) EPE group



(b) 3GCSE group

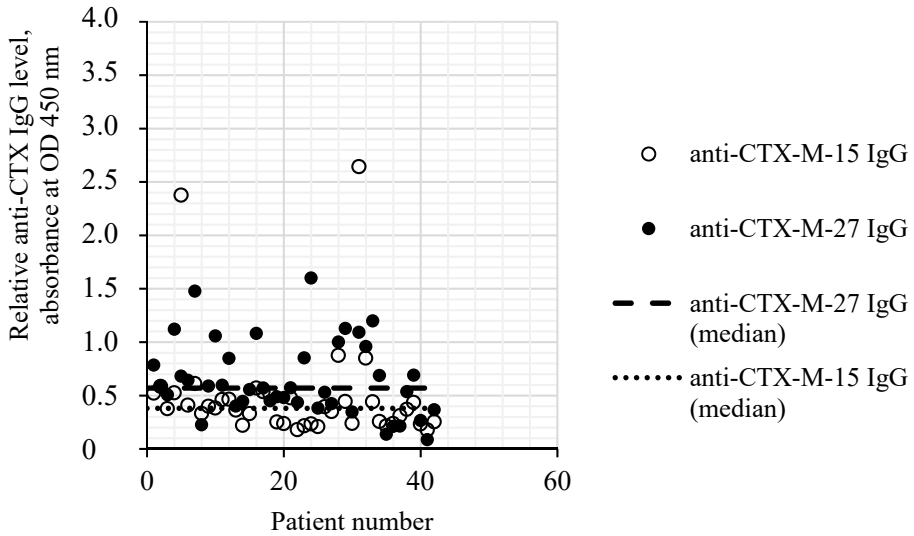


Figure 20. Distribution of antibody levels of anti-CTX-M-15 and anti-CTX-M-27 IgG for the EPE (a) and the 3GCSE group (b).

ELISAs were performed to determine the presence of anti-CTX-M-15/CTX-M-27 IgG antibodies. Sera were analysed in duplicates and mean values of two measurements for each patient were used in the final data analysis. OD = optical density.

14.1.1.6 Paper V

Out of 303 patients with travellers' diarrhoea, eighty-four patients (28%) carried a total of 92 strains of EPE. The majority (92%) were *E. coli* and the remaining 8% were *K. pneumoniae.* In total, 85 strains were inhibited solely by clavulanic acid (ESBL), 9 strains were AmpC-producing, of which 4 strains expressed phenotypical inhibition both for cloxacillin and clavulanic acid

The EPE prevalence of 28% is slightly higher than in an investigation in the same population one decade earlier (24%), but the difference was not statistically significant(21).

When comparing EPE colonization between different continents, highest rates were seen from Africa (54%), Asia (45%) and North America and the Caribbean (22%).

For specific countries, highest EPE colonization rates were seen for India (100%), Egypt (71%), Tanzania/Zanzibar/Kenya (80%), Cuba (57%) and South Africa (50%).

No strains resistant to imipenem, meropenem, ceftazidime-avibactam or amikacin were found in the study.

All 92 EPE strains in the study were susceptible to meropenem, imipenem and ceftazidime-avibactam. Susceptible increased exposure was found for piperacillin-tazobactam (12%), ceftazidime (17%), tobramycin(%), amikacin (2%), trimethoprim-sulfamethoxazole (1%), ciprofloxacin (7%) and temocillin (92%). Resistance was found for piperacillin-tazobactam (9%), ceftazidime (78%), ceftolozane-tazobactam (17%), gentamicin (24%), tobramycin (23%), trimethoprim-sulfamethoxazole (67%), ciprofloxacin (47%) and temocillin (8%).

There was no difference in proportions of patients positive for *Campylobacter, Salmonella, Yersinia* or *Shigella* in the EPE colonized (6.0%) compared to the EPE negative group (5.9%) (p=1).

Out of 86 strains available for WGS, 47 different sequence types were identified, of which 3 where novel. The most common were ST38 (n=9, 11%) and ST10 (n=7, 9%). Only 5 strains belonged to the widely distributed clone ST131.

Most *E. coli* isolates belonged to phylogroup A (n=32, 41%) or D (n=25, 32%). Less prevalent were B2 (n=8, 10%), B1 (n=4, 5%) and F (n=2, 3%).

For more than half of the *E. coli* strains (57%), an intestinal pathogenic pathotype was found, with EAEC (33%), and EIEC (22%) most commonly prevalent. One ETEC, one EPEC and no EHEC strains were found. For 27 isolates, no enteric pathotype could be determined.

Furthermore, 43% were either ExPEC or UPEC, and 30% carried genes shared by ExPEC/UPEC and EAEC or EIEC. There was no statistical difference in the risk of carrying an ExPEC/UPEC strain and travel destination (region).

Out of the 79 *E. coli* isolates, 76% of the strains carried at least one type 1-fimbriae gene and 75% carried at least two. Furthermore, 30% of the strains carried at least one pap (p-fimbriae) gene and 27% carried at least two.

Out of 86 strains available for WGS, 138 different beta-lactam resistance genes were identified in total. ESBL beta-lactamases found were $bla_{\text{CTX-M-15}}$ (n=62), $bla_{\text{CTX-M-27}}$ (n=9), $bla_{\text{CTX-M-14}}$ (n=7) and $bla_{\text{CTX-M-55}}$ (n=3). pAmpC genes found were $bla_{\text{DHA-1}}$ (n=4), $bla_{\text{CMY-2}}$ (n=3), $bla_{\text{CMY-42}}$ (n=2). Other beta-lactamases found were $bla_{\text{TEM-1B}}$ (n=29), $bla_{\text{OXA-1}}$ (n=11), $bla_{\text{TEM-35}}$ (n=4), $bla_{\text{SHV-199}}$ (n=2), $bla_{\text{SHV-27}}$ (n=2), $bla_{\text{SHV-106}}$ (n=1), $bla_{\text{SHV-60}}$ (n=1), $bla_{\text{TEM-1C}}$ (n=1), $bla_{\text{SHV-159}}$ (n=1), $bla_{\text{OKP-B-12}}$ (n=1), $bla_{\text{OKP-B-14}}$ (n=1), $bla_{\text{OKP-B-3}}$ (n=1).

Regarding non-beta-lactam antibiotics, carriage of antimicrobial resistance genes were common for these strains, but corresponding phenotypical resistance was rare.

Of the 86 genotyped EPE strains, 79% carried resistance genes for resistance against aminoglycosides, 51% to fluoroquinolones, 77% to sulphonamides, 78% to trimethoprim, 7% to fosfomycin, 24% to chloramphenicol, 12% to aminoglycosides and fluoroquinolones (combined), respectively. Genotypic and phenotypic expression of AmpC correlated well (n=9).

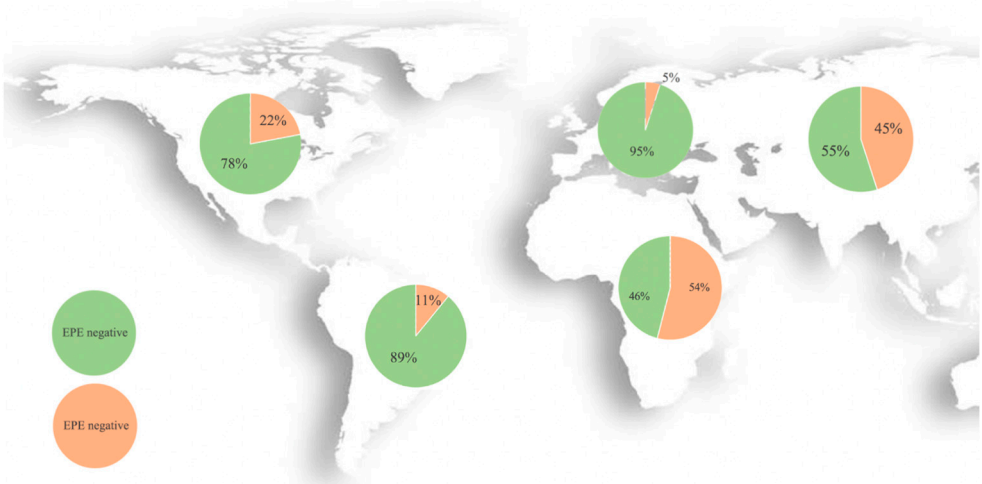


Figure 20. Prevalence of EPE with respect to regions.

15 Discussion

What information does my thesis add to this field of research? How do the findings relate to other studies in the field? What are, if any, the clinical implications?

This thesis sheds some new light on the research area of ESBL-producing bacteria. The strengths lie in its diversity. It includes five studies that are either prospective or cross sectional. On the one hand, it includes studies where the main share of work was done in the laboratory and on the other hand a randomized clinical trial on patients. Since I am principally a medical doctor working in a hospital, I have approached the theme of EPE with a clinician's eyes.

15.1.1.1 Paper I

Paper I gives novel insight into the spread of EPE, as it was the first study to report the sharing of identical EPE strains between humans and dogs of the same household. This indicates that within a household transmission of EPE between humans and dogs can take place. The study demonstrates proof of principle, but a statistically significant difference compared to the control group could not be shown, most likely due to the small sample size. The directionality of transfer was unknown.

The possibility of an outside source of these EPE-strains, such as water, foodstuff or an environmental source, spreading to humans and dogs of household 1 and 2, was not, however, evaluated. This seemed less likely, taken into consideration that no other humans were positive for EPE in household 1 and 2. Still, looking back, perhaps the word "evidence" used in the title should have been replaced with "suggestion".

Yet, since our study was published in 2016, additional studies have confirmed our results of co-carriage of identical EPE in humans and dogs of the same household. This has been interpreted by the authors as household transfer.

One case report from Finland in 2018 reported the co-carriage of an identical *E. coli* strain carrying carbapenemase NDM-5 and CTX-M-9 between humans and dogs (233). Although the transfer route was not investigated, they presumed the humans transmitted the strains to the dogs of the household.

A Dutch cross-sectional study investigated the prevalence of EPE in humans, dogs or cats of the same household (234). EPE was detected in 10.6% of the dogs

(59/555), 1.4% of the cats (4/285) and 3.8% of human participants. No co-carriage was seen for humans and cats, but in seven households both humans and dogs carried EPE. Out of these, five carried the same ESBL gene, as determined by WGS; three of these were regarded identical based on analysis of core-genome and plasmid content. The authors concluded a 5% risk of co-carriage between humans and dogs in household where at least one dog is EPE positive.

Thus, the sharing of identical EPE strains between humans and dogs of the same household does occur. The route is unknown.

15.1.1.2 Paper II

As a clinician, it can be frustrating to decide which empirical treatment is suitable for the patient in the emergency room with any previous EPE positive culture. Today, if a patient has had such a positive EPE culture, even if this was many years ago, that patient is considered to be a lifelong carrier of EPE and at risk of such infections. Even though the work of Paper II has not studied prolonged carriage of EPE in relation to a risk of infection, continued EPE colonization could be associated to an increased risk of developing EPE infections.

In these cases, giving empirical antibiotic treatment not covering for the EPE, may risk the health, and in worst case, the life of the patient. On the other hand, a broader antimicrobial treatment increases the risk of developing resistant bacteria. Furthermore, it is an inferior option considering the global ecological situation of antibiotic resistance. At present, it is not known for how long a positive EPE culture is relevant and clinically important (if such a general question has a clear answer at all). A recent study proposed a carbapenem-sparing regime as empirical treatment in non-critically ill patients colonized with EPE, unless a urinary tract infection is suspected (235).

Paper II gives some new information on this matter. It examines risk factors for persistent EPE positivity if the culture is repeated at least three months after an EPE infection/colonization. It identifies urological intervention and a history of EPE infection as variables independently associated with prolonged intestinal colonization with EPE.

Travelling to Africa and/or Asia within 2 years was associated with a decreased risk of prolonged intestinal colonization with EPE. This could reflect transient changes in the microbiota related to travel exposure, and possibly relate to the resilience phenomenon as discussed in the section of microbiota. That is, the ability of the gut flora to return to an equilibrium state following a disturbance. With high level of resilience comes faster restoration to equilibrium, which could theoretically be associated with clearance of acquired strains of EPE. Thus, if the EPE strains are acquired during travel to Africa and/or Asia restoration to equilibrium could mean the clearance of EPE. However, more research is needed as these are mere theories.

Studies of risk factors concerning prolonged intestinal carriage of EPE are few, and epidemiological studies investigating this are urgently needed. Since our study was published, a recent study found prolonged EPE carriage in 59.4% (one month) and 36.5% (six months) (236) Interestingly enough, in prolonged EPE colonization, 82% of strains were identical. The remaining were considered plasmid transferred to other bacteria. Contrary to our study, this study identified travel to Asia as a risk factor for prolonged (six months) EPE carriage. Furthermore, additional risk factors were related to hygiene (not changing the kitchen towel daily) and to swimming in the sea.

The results of regression models are dependent on what variables you choose. In total, eight variables were included in the multivariate regression model in our study.

Statistical methods other than weighting could have been used, such as matching or imputation. However, these methods are in this case inferior to weighting.

15.1.1.3 Paper III

Can EPE be selectively cleared from the intestines with some type of therapy? Paper III explores this in a clinical trial on patients using probiotics, which has been proposed as a method of EPE eradication (237). We performed a superiority study comparing probiotics to placebo. The study did not show a statistically significant difference between probiotics and placebo in eradicating EPE in chronic intestinal carriers. This is, nevertheless, an important result as other alternatives should be pursued. Each individual has its own unique microbiota, and perhaps there is not a single probiotic mixture that works for everyone.

The study has a number of limitations that should be noted. The design did not allow full control of adherence to the treatment protocol, and compliance with the culture protocol was used as a proxy for treatment adherence. The editor and reviewers of this paper wanted to highlight the risk of a type II-error in the conclusion. Looking back, we fully agree that the study is underpowered and carries a risk of a type-II error. The power calculation on which the study was based was very optimistic, and a small or moderate, but clinically meaningful, treatment effect cannot be ruled out; an absence of evidence of an effect does not equal evidence of an absence of effect.

Another limitation of the manuscript was the risk of bias accompanying the proportionally large number of missing outcome data. In the result, individuals with missing outcomes were included in the ITT analysis based on a BOCF (baseline outcome carried forward) assumption/imputation. This is a point of controversy, and many authors are inclined only to include evaluable outcome data in the analysis. It can thus be argued that the analysis noted as per-protocol in our study may be viewed as an ITT analysis based on individuals with evaluable outcomes. Such an approach would not have changed the results of the study but would have increased the risk of a type II error even further.

There are numbers of alternative ways of performing imputation of missing outcomes in order to minimize bias, including best/worst case imputation, latest observation carried forward (LOCF) and various multiple imputation techniques. In the manuscript, only the first of these was included as a sensitivity analysis but implied rather extreme assumptions based on the observed data.

In conclusion, the result of this study found no statistical difference between probiotics and placebo in this cohort. If this study were to be used as the basis for a new RCT including a larger material, the minimum number of subjects needed in order to have sufficient statistical power to detect the same dichotomous primary endpoint could be calculated. With an anticipated difference in incidence of 7.5 %, an alpha of 5%, and a power of 80%, a minimum of 444 patients would need to be included. This is without adjustment for any loss to follow up.

Similar to MRSA, where successful eradication of carriage can be achieved, decolonization therapies for EPE are urgently needed, in particular in the virulent clones with high impact on mortality and morbidity. This has to take into account a clinical meaningful difference of the therapy and spontaneous loss of EPE.

15.1.1.4 Paper IV

Paper IV breaks new ground. It is believed that antibodies will develop following an invasive infection, to protect the host from additional infections of that specific serotype. To the best of our knowledge, it has never before been shown that antibodies targeting the CTX-M-type beta-lactamase itself are involved in the humoral host response to EPE blood stream infections. Sometimes studies that investigate a novel field, as in Paper IV, can provide answers as well as raise questions.
Some answers are provided. It seems clear that individuals having experienced EPE bacteremia more often have detectable IgG antibodies directed against the respective beta-lactamase than individuals having recently experience bacteremia with susceptible *E. coli*. It is also evident that far from all individuals with experienced EPE bacteremia have detectable CTX-M antibodies.

Beta-lactamases are produced in the cytosol and, after being transported across the cytoplasmatic membrane, are principally located in the periplasmatic space. For an immune reaction to occur with antibody production, beta-lactamases must exit bacteria. It is less likely that the reverse is possible, i.e. antibodies can access ESBL-type beta-lactamases in the periplasmic space. This needs to be further investigated.

In what situations are the ESBL presented as an antigen to the immune system? When presented, is the ESBL always considered foreign, leading to formation of antibodies? Are IgA antibodies against ESBLs present in the mucosa of some patients? Can antibodies, in its natural form, or more likely linked to some kind of chaperone molecule, enter the periplasm? Could the antibodies inactivate beta-lactamases, rendering more effective treatment options?

A relatively high proportion of cross-reactivity was seen between the different antibodies anti-CTX-M-15 and anti-CTX-M-27. The different CTX-M types are structurally similar, sometimes only differing as to one or more amino acids. The five different clusters of CTX-M share >94% genetic identity(86). This could perhaps explain the cross reactivity but needs to be furthered examined.

As a diagnostical tool, the implications of our findings are limited. We may not be able to differ between certain types of CTX-M beta-lactamases in patients with previous EPE bacteremia. This could, however, be an advantage if these antibodies could be used as a potential adjuvant therapy. Structural similarity could potentially mean that an infection with a certain CTX-M type of beta-lactamase and IgG therapy does not have to fully correspond, for the adjuvant treatment to be successful.

15.1.1.5 Paper V

Previous studies of returning travellers have e.g. given us insight that travellers' diarrhoea (TD) is associated with EPE acquisition. However, repeated studies of international travellers are important, also serving as sentinel studies for tracking import of carbapenem resistant *Enterobacterales*.

Furthermore, studies investigating the EPE prevalence exclusively in TD patients are rare, and a further strength is that our cohort of travellers is unselected. Similar studies include travellers seeking medical advice pre-travel, which may narrow the geographical distribution down to countries where travellers need malaria prophylaxis, vaccine against yellow fever etc (22, 23). Additionally, sequencing of the strains has given interesting information about their genotypical resistance and pathogenicity.

It is precarious to study global trends in antimicrobial resistance given differences in study samples, methods, travel pattern etc. Owing to increasing international travel and a rising prevalence of EPE in clinical cultures, this study was planned as a follow-up study one decade after the original study in the same low endemic setting. A similar methodology was used, and this allowed us for instance to investigate and compare the EPE prevalence from different countries as well as the prevalence of CPE. It is reassuring that the EPE prevalence in TD patients has not increased during the last decade.

An apparent limitation of our approach is the lack of pre-travel cultures, and thus a lack of proof that the identified isolate is actually acquired during travel.

16 Limitations

All studies have strengths and weaknesses. Below, the main limitations of my five papers are discussed.

16.1 Potential limitations of internal validity.

Firstly, small sample sizes and non-randomization of cohorts risk selection bias. All studies could have benefitted from larger sample sizes. In Paper I the same methodology in the laboratory of diagnosing EPE should have been used for humans and dogs. A larger sample size should have been used, and cases and controls should have been recruited from the same geographical area.

In Paper II and IV, important variables for investigating risk factors could have been overseen in the statistical analysis. Variables included in the regression analyses were putative risk factors, and other, unknown, important variables could have been excluded. While this is not a matter of internal validity, it is a risk of underfitting,

Furthermore, there are methodological concerns. The dichotomization into EPE- or no EPE-colonization is determined by a single selective faecal culture. The sensitivity of the faecal culture in determining EPE is not 100%, which reduces the negative predictive value. This theoretically affects all studies but Paper IV, where no faecal cultures were used. Preferably, several cultures should have been obtained in Paper II over a greater period of time. A control group of non-EPE patients could have been included, to investigate the amount of new EPE carriers during the same period of time.

In Paper III, whether patients received probiotics or placebo should have been blinded also to the primary investigator. In fact, the manufacturer in Italy prepared for a double blinded study.

Also, the randomization with lots from a secured box in a 1:1 style was likely too simplistic. This should have been electronic, perhaps randomized in blocks of eight, for instance.

However, the major limitation was the power calculation which was optimistic and thus the population studied should have been larger. This would have reduced the risk of a type II error.

In Paper IV, the control group was too small. If the same study was to be repeated, it should have been twice the size of the case group, to increase the statistical precision. All patients included in the study, cases and control patients, should have been selectively cultured for EPE in faeces to investigate carriage of EPE in the control group.

In Paper V, not all clinical samples from TD patients sent to the Department of Clinical Microbiology for culture of *Campylobacter, Salmonella, Yersinia* or *Shigella* were included. They were included according to staff availability, and the amount of cultures missed because of this was not registered. However, we believe that the risk of a systematic bias because of this is negligible. Furthermore, we relied on the information on the referral to the Department of Clinical Microbiology, Skåne University Hospital. This data was in no way validated and did not contain information on patient characteristics nor specific travel details.

16.2 External validity (or lack thereof)

One should be cautious to generalize one's results. Particularly for Paper II, the selection and methodology could risk the external validity of our results. For Paper III, the results found specifically to Vivomixx® should not be generalized for all probiotics.

17 Future research

There is still a knowledge gap in the area of intestinal colonization of EPE. There are several major questions that remain to be answered.

First, can the once EPE colonized intestines ever be selectively cleared of EPE? In which patients does EPE disappear from the intestines naturally, without intervention? Is this depending on bacterial or patient characteristics, or both? Could an intervention, a method or therapy be used to reach this goal in chronic carriers? Currently, based on lack of beneficial evidence, routine decolonization of EPE carriage is not recommended (225). Oral antibiotics have been tried with no long-term effect on intestinal EPE carriage (238). The overwhelming risk of increased resistance with this method could be potentially harmful, aggravating the problem. Furthermore, antibiotics combined with faecal transplantation did not eradicate intestinal EPE colonization compared to a control group (239).

In EPE endemic regions, intestinal decolonization strategies could be futile, as new EPE strains would likely be introduced after completed intervention. However, in low endemic areas eradication of EPE is desirable. Also, particularly in virulent strains or in patients with recurrent invasive EPE infections, decolonization strategies would be desirable.

Secondly, more research is needed why some people carry EPE strains in the intestines, but others become ill from infections of the same strain. If this is a matter of virulence traits only, all patients colonized with certain strains would develop infection. This is not an enigma reserved for scientists preoccupied with EPE strains but has puzzled infectious disease-doctors for ages; why do some people only carry certain bacterial strains, without getting ill, while the same strain can be lethal to other patients in close contact?

Prospective, longitudinal studies are needed to investigate the natural course of EPE colonization. To avoid unnecessary usage of carbapenems, a clinical algorithm with high discriminatory accuracy would be very useful to determine if or when a prior EPE culture (of any localization) should be covered in the empirical treatment of severe infections. After how long is an old faecal EPE culture irrelevant and can be discarded when starting empirical antibiotics?

18 Clinical implications.

Why is this thesis interesting for an infectious diseases clinician? What are the clinical implications from the present thesis?

From Paper I we have learnt that humans should be cautious in interacting with a known EPE carrier. EPE transmission between humans and dogs do occur.

Paper II taught us that patients with EPE in clinical cultures are largely uninformed that they carry an EPE. We need to improve our information to patients regarding what EPE is, clinical implications, hygiene aspects etc. Patients should obtain both oral and written information. Secondly, Paper II provided information about factors that are associated with increased (and decreased) risk of prolonged EPE colonization in patients. Theoretically, this could be useful when choosing empirical antibiotics for a patient with prior EPE in a culture. If this patient has had a clinical infection with an EPE and/or a urological intervention within the last six months, he has more likely a faecal culture with EPE. Furthermore, this could be of help when considering infection prevention measures.

Considering the risk of a type-II error, Paper III showed that use of the probiotic Vivomixx was not associated with improved chance of EPE eradication compared with placebo. Other eradication therapies must be explored.

Paper IV has brought us new insights on the host response to invasive EPE infections. A proportion of patients having experienced EPE bacteremia produce specific IgG antibodies against corresponding beta-lactamases. The clinical implications of this are potentially promising but warrant further investigation.

Paper V emphasises the risk for patients with TD of carrying EPE after international travel to endemic areas, most notably south and east Asia as well as Africa. Most of the *E. coli* strains were intestinal pathogenic *E. coli* and a comparatively high proportion of the strains were ExPEC/UPEC, many expressing virulence genes *pap* and/or *fim*. Clinicians should provide antibiotics with EPE coverage for patients with severe infections after international travel, especially if there is a history of TD.

19 Conclusions

Identical EPE strains with respect to bacterial species, antibiogram, genotype, and MLVA can, to a limited extent, be found in humans and dogs of the same household.

Risk factors independently associated with prolonged intestinal colonization with EPE (more than three months) were urological intervention within 6 months and a history of EPE infection.

Travel to Africa and/or Asia within the last 2 years was significantly associated with a decreased risk of becoming a long-term EPE carrier.

Half of the patients with infection/colonization of EPE in Skåne were not adequately informed regarding their EPE status.

The probiotic mixture Vivomixx® was not superior to placebo for intestinal decolonization of adult patients with chronic colonization of EPE.

There is evidence that EPE bacteremia can trigger a host response with the production of IgG antibodies targeting the common ESBL-type beta-lactamases CTX-M-15 and CTX-M-27.

A relatively high proportion of patients with travellers' diarrhoea carry EPE when they return to Sweden, (28%). Highest colonization rates were seen from Africa, Asia and North America and the Caribbean.

Despite increased travel and increased prevalence of EPE in clinical cultures, the rate of EPE carriage in patients with travellers' diarrhoea did not significantly increase compared to the previous study 10 years ago conducted in the same setting.

No imipenem, meropenem, ceftazidime-avibactam or amikacin-resistant strains were found in the study.

Many different sequence types were found, and surprisingly only 5 strains belonged to the widely distributed clone ST131. In our cohort, 3 patients carried strains with STs that had previously never been reported.

Most *E. coli* strains were intestinal pathogenic *E. coli.* A comparatively high proportion of the strains were ExPEC/UPEC, many expressing virulence genes *pap* and/or *fim*.

20 Populärvetenskaplig sammanfattning

Kunskapsläget om bärarskap av antibiotikaresistenta tarmbakterier av typen ESBL (extended-spectrum beta-lactamases) är idag bristfälligt. Det är oklart hur länge bärarskapet fortgår, om och när bärarskapet upphör, hos vilka detta sker och om det går att påverka bärarskapets längd med farmakologisk behandling eller någon slags terapi. Mer kunskap behövs kring riskfaktorer för, och implikationer av, bärarskap av ESBL-bildande tarmbakterier (EPE).

Den här avhandlingen syftar till att öka denna kunskap på flera sätt. Den undersöker nya smittvägar för EPE och riskfaktorer för att bli långvarig bärare av dem. Avhandlingen utvärderar också om probiotika kan ha effekt på detta bärarskap. Vidare undersöks kroppens immunrespons mot infektion med EPE och slutligen efterforskas i hur hög grad man idag ser bärarskap av ESBL-bildande tarmbakterier hos skåningar som har diarré efter utlandsresa.

20.1.1.1 Delarbete I

Delarbete I var en studie som inkluderade 22 hundägare med konstaterat bärarskap av ESBL och en kontrollgrupp på 29 hundägare utan ESBL. I två av 22 hushåll var både hundägare och hund positiva med identisk ESBL-bildande stam.

20.1.1.2 Delarbete II

Delarbete II var en prospektiv kohortstudie som undersöker riskfaktorer för långvarigt bärarskap (mer än 3 månader) av ESBL-bildande bakterier. Urologisk intervention och genomgången infektion med ESBL-bildande bakterier var associerat med förlängt bärarskap, och resa till Afrika/Asien senaste 2 åren var kopplad till minskad risk för förlängt bärarskap.

Dessutom var 50% av deltagarna i studien omedvetna om att de var koloniserade eller hade haft en infektion med ESBL-bildande bakterier. Behandlande läkare behöver bli bättre på att ge muntlig och skriftlig information till patienter som bär ESBL-bildande bakterier.

20.1.1.3 Delarbete III

Delarbete III var en prospektiv, randomiserad, placebokontrollerad studie som undersökte om probiotikan Vivomixx® kunde eliminera bärarskap av ESBL-bildande bakterier jämfört med en kontrollgrupp som fick placebo. Ingen statistisk signifikant skillnad i behandlingsutfall mellan grupperna kunde påvisas.

20.1.1.4 Delarbete IV

Delarbete IV var en studie som undersökte förekomst av antikroppar mot ESBL-enzymet CTX-M-15 och CTX-M-27. Patienter med antibiotikaresistenta tarmbakterier i blodet jämfördes med en kontrollgrupp av patienter med *E. coli*-bakterier i blodet utan sådan särskild antibiotikaresistens. Vi fann en statistisk signifikant skillnad som tyder på att antikroppar mot ESBL-enzymet har en roll i immunförsvarets reaktion mot blodinfektioner med ESBL-bildande bakterier.

20.1.1.5 Delarbete V

Delarbete V var en kohortstudie av tvärsnittskaraktär som undersökte i vilken utsträckning utlandsresenärer som kom hem med turistdiarré hade bärarskap av ESBL-bildande tarmbakterier. Med molekylära metoder undersöktes bakterierna på DNA-nivå för förekomst av resistens- och virulensgener. Av 303 utlandsresenärer hade 84 patienter påvisad ESBL i avföringsprov (28%) Jämfört med en studie som gjordes på liknande sätt för 10 år sedan så var det en liten men icke statistiskt signifikant ökning. Det är betryggande att trots ökad förekomst av ESBL-bildande tarmbakterier i kliniska prover, samt ökat globalt resande, så ökade inte förekomsten av ESBL i avföringsprov hos resenärer med turistdiarré.

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“Don’t act like you’re not impressed”

Ron Burgundy, *Anchorman.*

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About the author

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He has a weak spot for music in general and hip hop/RnB in particular. After six years of mixing bacterial genetics and his favorite music genre, he now has trouble differentiating RNA from RnB. According to his wife the former is always better.