Epidemiology of Extended-Spectrum Beta-Lactamase (ESBL)-producing *E. coli* with special reference to outbreak detection

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ABSTRACT

Multidrug resistant bacteria, particularly extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* (EPE), are becoming a major health concern. ESBL-producing *Escherichia coli* (ESBL-*E. coli*) is the most prevalent type. ESBL-genes are carried on plasmids, often by bacteria belonging to clones with properties that facilitate transmission. An example is *E. coli* of sequence type (ST) 131, and its sublineage ST131-O25b. The most prevalent ESBLs world-wide are the CTX-M enzymes, most often belonging to the CTX-M-1 group. In **Paper I** the epidemiology of ESBL-*E. coli* causing urinary tract infection was studied from the first detected cases until these bacteria were established in the greater Gothenburg area. The first cases where seen in women from the community setting in 2003-2005. In 2008-2009, the elderly and men were also affected. The ST131-O25b sublineage became established during the study period, but otherwise the emergence of ESBL-*E. coli* was polyclonal. There was a shift in ESBL types in favor of the CTX-M-1 group enzymes. In **Papers II and III** PFGE, standard method for epidemiological typing at the time, was compared to other methods. For investigation of a polyclonal ESBL-*E. coli* outbreak, MLVA was found comparable to PFGE, whereas MLST-analysis was not useful. For continuous epidemiological surveillance of ESBL-*E. coli*, both MLVA and MLST were inferior to PFGE, especially for typing the ST131-O25b sublineage. This thesis demonstrates how the epidemiology of ESBL-*E. coli* might change over time, emphasising the need of continuous surveillance using optimal typing methods to detect outbreaks at the local level. We propose that an abbreviated MLVA might be useful to preselect isolates for more discriminating typing methods.

Keywords: ESBL, *E. coli*, epidemiology, outbreak investigation, surveillance.

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SAMMANFATTNING PÅ SVENSKA

Antibiotika används för att behandla bakteriella infektioner, som utan behandling kan bli livshotande. Högspecialiserad vård, exempelvis cancerbehandlingar eller stora kirurgiska ingrepp, går inte att genomföra utan antibiotika. Hög antibiotikaförbrukning leder till att bakterier utvecklar resistens mot antibiotika. Enligt WHO utgör antibiotikaresistens ett allvarligt hot mot människors hälsa. Antibiotikaresistens är ett globalt hot. Framför allt ökar resistensmekanismen ESBL (Extended-Spectrum Beta-Lactamase). ESBL-enzymet gör att en viss typ av antibiotika, betalaktamer, förlorar sin verkan. Vissa bakterier i vår tarmflora, framför allt *Escherichia coli* (*E. coli*), kan vara bärare av ESBL. *E. coli* kan även orsaka infektioner, tex urinvägsinfektion.

Syftet med avhandlingen var att studera hur förekomsten av ESBL-*E. coli* hos patienter i Göteborgsregionen förändrats över tid, samt att utvärdera olika typningsmetoden för övervakning av ESBL-bakterier. Typning innebär att bakterier inom en art delas upp i undergrupper genom att de typas till stamnivå. Avsikten med typning är att upptäcka om det pågår smittspridning av en viss bakteriestam.

Avhandlingen innefattar tre arbeten. I det första arbetet undersöktes förekomsten av ESBL-*E. coli* som orsak till urinvägsinfektioner i Göteborgsregionen. Resultaten visar att förekomsten ökade från perioden 2003-2005 till 2008-2009, och att ökningstakten var snabbare hos sjukhusvårdade patienter jämfört med patienter från öppenvården. En särskilt framgångsrik *E. coli*-typ kallad ST131-O25b, som sprids globalt, etablerade sig även i Göteborgsregionen under den studerade perioden. I det andra och tredje arbetet jämfördes typningsmetoden PFGE (Pulsed-field gel electrophoresis), som länge varit den vanligt förekommande typningsmetoden för ESBL-*E. coli*, med en alternativ metod, MLVA (Multiple-Loci VNTR Analysis). I det andra arbetet analyserades *E. coli* med ESBL från ett utbrott på Sahlgrenska Universitetssjukhuset, där flera patienter smittats med samma stam av ESBL-*E. coli*. MLVA visade sig kunna användas vid utredningar av den typen av utbrott, men behövde kompletteras med ytterligare metoder. I det tredje arbetet analyserades alla ESBL-*E. coli* som detekterades i prover från sjukhus och vårdcentraler under tiden för det pågående utbrottet. Syftet var att värdera om MLVA kan användas för att upptäcka utbrott. När isolaten fick olika typningsresultat med MLVA var de med stor sannolik olika stamtyper. Om typningsresultaten däremot blev lika varandra med MLVA krävdes analyser med mer diskriminerande typningsmetoder för att kunna bekräfta ett utbrott. Sammanfattningsvis har förekomsten av ESBL-*E. coli* ökat över tid i Göteborgsregionen, och nya varianter förväntas komma framgent. Det kräver tillgång till effektiva typningsmetoder för övervakning. MLVA har visats vara ett enkelt och snabbt alternativ att använda som initial screeningmetod vid övervakning och utbrottsutredning av dessa bakterier.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Helldal L*, Karami N*, Florén K, Welinder-Olsson C, Moore ER, Ahrén C. **Shift of CTX-M genotypes has determined the increased prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* in south-western Sweden.** *Clin Microbiol Infect.* 2013;19(2):E87-90.
*These authors contributed equally to this work

II. Karami N, Helldal L, Welinder-Olsson C, Ahrén C, Moore ER. **Sub-typing of extended-spectrum-beta-lactamase-producing isolates from a nosocomial outbreak: application of a 10-loci generic *Escherichia coli* multi-locus variable number tandem repeat analysis**. *PLoS One.* 2013;8(12):e83030.

III. Helldal L, Karami N, Welinder-Olsson C, Moore ER, Ahrén C. **Evaluation of MLVA for epidemiological typing and outbreak detection of ESBL-producing *Escherichia coli* in Sweden.** *BMC Microbiol.* 2017;17(1):8.

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ABBREVIATIONS

AST	Antimicrobial Susceptibility Testing
BSI	Blood stream infection
CC	Clonal complex
CPE	Carbapenemase Producing *Enterobacteriaceae*
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic (Shiga toxin-producing) *E. coli*
EPE	ESBL-producing *Enterobacteriaceae*
ESBL	Extended-spectrum beta-lactamases
$ESBL_A$	Classical ESBL
$ESBL_M$	Miscellaneous ESBL
$ESBL_{CARBA}$	Carbapenemases
ExPEC	Extraintestinal Pathogenic *E. coli*
GECM	Generic *E. coli* MLVA
HGT	Horizontal Gene Transfer
IPEC	Intestinal Pathogenic *E. coli*
MALDI-TOF MS	Matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry
MBL	Metallobetalactamases
MDR	Multi-drug Resistance
MGE	Mobile Genetic Element
MLST	Multi-Locus Sequence Typing

MLVA	Multiple-Locus Variable number tandem repeats Analysis
MRB	Multidrug resistant bacteria
MRSA	Methicillin Resistant *Staphylococcus aureus*
NGS	Next generation sequencing
OXA	Oxacillinase-type beta-lactamase
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
ST	Sequence type, as determined by MLST
UTI	Urinary tract infection
VNTR	Variable number tandem repeats
WGS	Whole Genome Sequencing
WHO	World Health Organization

1 INTRODUCTION

The increasing resistance to antibiotics is a severe threat to public health on a global scale [1]. Access to antibiotics is a basic requirement in order to attain safe and cost-effective treatments for many common infections that would otherwise be life threatening. Furthermore, the highly specialized healthcare available in high income countries, such as immunocompromising chemotherapy and major surgical interventions including organ transplants, would not be achievable without antibiotics being available to deal with adverse outcomes.

However, development of antibiotic resistance is an inherent part of bacterial evolution. Indeed, many of the antibiotic substances used originate either from microbial products with the original purpose to kill competing bacteria, or synthetic derivatives thereof [2]. Consequently, bacteria have evolved effective measures to protect themselves against such attacks, which also gives them the ability to develop ways to evade antibiotic treatment [3]. However, misuse of antibiotics, both for humans as well as in veterinary medicine and in the food-production chain, in combination with poor hygiene and sanitation in areas where these bacteria thrive, have accelerated and accentuated this evolutionary process to the point where there today are infections with bacteria harboring resistance mechanisms that render them unable to treat [4, 5].

In the Nordic countries the increase in antibiotic resistance is evident, although not yet as alarming as in many other parts of the world [6, 7]. This advantageous situation is supposedly due to strictly regulated terms for antibiotic usage in food production and veterinary practice, and also because of optimised antibiotic treatment in human medicine, including keeping antibiotics as a prescription drug [8]. Nevertheless, the antibiotic resistance mechanisms are not limited to certain geographical areas, nor accepting nation borders. There will inevitably be an influx of resistant bacteria, not only by human carriers travelling and migrating over the globe, but also with food products and agricultural raw material being traded across borders. On a global scale, there is need to address the problem and to reach common agreements on how to deal with it [9, 10].

When a particular resistance mechanism becomes more common in a bacterial species it will start to spread not only within the hospital setting but also in the community, and there will be a constant influx of these bacteria to the hospital setting. Thus, there will be a continuous and increasing need to prevent transmission of resistant bacteria within the healthcare system, especially those

that are multidrug resistant. To achieve this, Infectious disease control officials need information about local epidemiology, including what strain types that are in circulation in the community. In addition, there must be a continuous surveillance of strains isolated from the hospital setting, enabling early detection of outbreaks [11]. Therefore, there is a need for straightforward and cost-effective typing methods for surveillance of local epidemiology, in order to enable outbreak detection and subsequent contact tracing. These issues will be assessed in this thesis with focus on one of the most prevalent multidrug resistant bacteria today, which is *Escherichia coli* (*E. coli*) producing extended-spectrum beta-lactamase (ESBL), named ESBL-*E. coli*. More precisely, paper I describes the early epidemiology of ESBL-*E. coli* in the greater Gothenburg area over a five-year period from the first detected case in late 2003. In 2008, a polyclonal outbreak with ESBL-producing *Enterobacteriaceae* (EPE) was detected in a neonatal ward. Subsequently, this lead to the assessment of novel typing methods, primarily Multiple-locus variable number tandem repeats analysis (MLVA), for investigation of outbreaks due to ESBL-*E. coli* (paper II), but also for continuous surveillance of these bacteria in a routine clinical setting at the local setting (paper III).

1.1 Enterobacteriaceae

Most bacteria can be classified into one of two large groups; Gram-positive or Gram-negative, depending on the structure and staining properties of their respective cell walls [12]. The cell envelope of a Gram-negative bacteria comprises the inner cytoplasmic cell membrane, followed by a thin peptidoglycan cell wall and then an outer membrane containing lipopolysaccharides (LPS) consisting of lipid A, core polysaccharide, and a unique polysaccharide, referred to as the O-antigen in *Enterobacteriaceae*. In the outer membrane there are porins, allowing passive diffusion. The outer membrane protects the Gram-negative bacteria from several antibiotics [13].

The family *Enterobacteriaceae* belongs to the domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, and order Enterobacteriales, and is a large family of Gram-negative bacteria. They are rod-shaped (typically 1–5 μm in length), facultative anaerobe and include many hundreds of species, both commensal bacteria, as well as pathogens such as *Salmonella* and *E. coli* [14].

1.1.1 Escherichia coli

E. coli is a motile, nonsporulating rod. It is the most abundant facultative anaerobe in the normal gut flora (Figure 1). Most *E. coli* strains live harmlessly

in the intestines and rarely cause disease in healthy individuals. The commensal *E. coli* benefit their host by producing vitamin K and preventing colonization with pathogenic bacteria [15, 16]. Nevertheless, there are certain more pathogenic strains, associated with diarrhoeal disease (IPEC; Intestinal Pathogenic *E. coli*), as well as extra-intestinal infections (ExPEC; Extraintestinal Pathogenic *E coli*) [17]. The pathogenic potential of a particular *E. coli* strain depends on the expression of specific virulence genes [18, 19]. The IPEC strains are regarded as obligate pathogens in humans [20], while the ExPEC strains are facultative pathogens that can be part of the normal gut flora

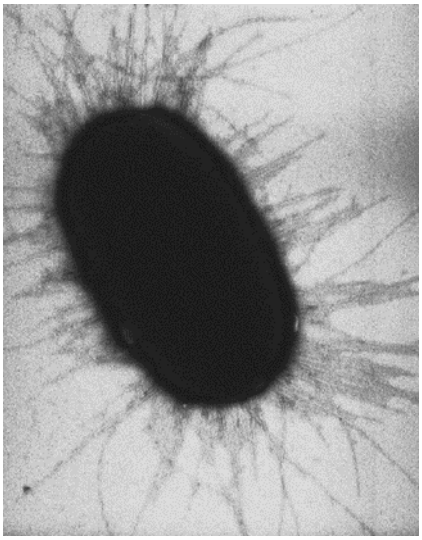


Figure 1. E. coli with fimbriae. Image Creative Commons, licensed under CC BY 2.5: https://creativecommons.org/licenses/by/2.5/deed.en. Couresy of Gross [21].

in healthy individuals, but turn pathogenic if they reach other body sites than their normal habitat, and especially normally sterile sites [22-24].

The *E. coli* genome encodes up to 5000 genes. Only approximately half of these genes constitute the core genome shared by all *E. coli*, while the remaining genes comprise the highly variable accessory genome, which generates a wide genomic diversity within the species [25, 26]. The accessory genome can for example encode for certain virulence factors and other properties that enhance bacterial survival in certain ecological niches. Examples of virulence factors are adhesins, secretion systems, various toxins, iron uptake systems and capsule synthesis [19, 27].

E. coli displays a clonal structure, meaning that isolates typically form different clusters of genetically related strains, referred to as genetic lineages, or, in the

wider sense of the word, as clones [28, 29]. There are several *E. coli* genetic lineages that have acquired specific virulence determinants, thereby increasing their ability to cause disease. Thus, these are demarcated genetic lineages sharing specific virulence factors. Only successful combinations of virulence determinants remain in circulation and become specific "pathotypes" of *E. coli*, such as Enterohemorragic *E. coli* (EHEC) within IPEC [19, 27, 28]. The *E. coli* pathotypes are traditionally sorted into clonal groups originally defined by shared O-antigens, sometimes with the addition of flagellar (H) antigens [30, 31].

Diarrhoeal illnesses caused by different *E. coli* lineages is a major public health problem in low income countries, and contributes significantly to morbidity and mortality, especially in young children [32, 33]. For the diarrhoeagenic *E. coli*, the specific strains that constitute the currently known IPEC pathotypes, are enteropathogenic *E. coli* (EPEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive E. coli (EIEC), and diffusely adherent *E. coli* (DAEC) [19, 20]. Clonal dissemination of IPEC strains, such as *E. coli* O157:H7, is well known [34, 35].

ExPEC is an important human pathogen and is the most common cause of urinary tract infection (UTI) [22], and also the most common Gram-negative bacteria associated with bloodstream infections (BSI) of all bacteria [36, 37]. In addition, it can cause infection in various organs, ranging from the biliary system to the CNS. In the gut they can act as harmless commensals, until leaving the gastrointestinal tract which enables them to cause infections in other parts of the body. ExPEC strains with a tendency to cause UTIs are designated Uropathogenic *E. coli* (UPEC). An important virulence factor of UPEC is an adhesin on the tip of the type 1 fimbriae, FimH. These fimbriae allow the bacteria to adhere to and invade bladder epithelial cells, which facilitates infection [38]. Just as for the IPEC pathotypes, there are also certain ExPEC lineages that represent major pandemic clonal lineages, responsible for human extraintestinal *E. coli* infections [39, 40]. ExPEC strains belonging to phylogenetic groups B2 and D show higher virulence in humans, whilst commensal strains mostly belong to phylogroups B1 and A [16, 41-43].

1.2 Antibiotics

Antibiotics are antimicrobials that are used to treat infections caused by bacteria in human and veterinary medicine. The discovery of antibiotic compounds during the first decades of the 20th century, and the subsequent

wide scale production and sale from the 1940s and onwards, revolutionized human medicine, as previously deadly infections now could be treated.

Antibiotics are either based on naturally occurring compounds or synthetic pharmacological substances produced in laboratories. The compounds found in nature are produced by microorganisms in order to kill neighboring bacteria, thus gaining an advantage when competing for limited nutrients and space [44].

There are several groups of antibiotics, the main ones being:

- Beta-lactam antibiotics
- Fluoroquinolones
- Macrolides and lincosamides
- Tetracycline
- Aminoglycosides
- Glycopeptides

There are also trimethoprim, mecillinam and nitrofurantoin, which are often used to treat UTI in Sweden. Antibiotics that are bactericidal drugs promote cell death, while bacteriostatic agents merely inhibit bacterial growth. Antibiotics can be divided into four principal groups depending on how the particular compound affects the bacterial cell [45, 46]:

- Disruption of cell wall synthesis (e.g. beta-lactam antibiotics and glycopeptides),
- Interference with DNA-synthesis (e.g. fluoroquinolones),
- Inhibiting protein synthesis (e.g. tetracyclines and aminoglycosides)
- Inhibiting folic acid synthesis (e.g. trimethoprim)

1.2.1 Beta-lactam antibiotics

Beta-lactam antibiotics constitute a large group of antibiotics with low toxicity to humans. They are the most frequently used antibiotics for empirical therapy in Sweden and world-wide [6, 47]. The compounds work by inhibiting the synthesis of the peptidoglycan layer of the growing cell wall. The last step in the synthesis of the peptidoglycan layer is facilitated by enzymes called transpeptidases, or penicillin-binding proteins (PBPs). The beta-lactam ring of beta-lactam antibiotics has a structure similar to the terminal amino acid residues on the peptide subunits of the peptidoglycan layer. These structural similarities facilitate the beta-lactams binding to the PBPs. This binding to PBP

is irreversible, and prevents the final crosslinking of the growing peptidoglycan layer, resulting in inhibition of cell wall synthesis [47].

The beta-lactams are divided into penicillins, cephalosporins, monobactams and carbapenems. They all share a common structure; the beta-lactam ring, shown in Figure 2.

The penicillins were the first beta-lactams to be developed, only to soon be followed by emerging resistance through beta-lactamases as outlined below. The development of new compounds meant that the beta-lactam molecules

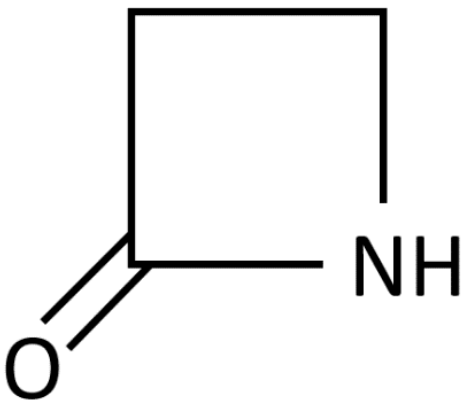


Figure 2. The beta-lactam ring

became more complex and with larger side chains, to prevent the emerging beta-lactamase enzymes from degrading the beta-lactam ring.

With growing resistance, the need for protecting beta-lactam antibiotics from degradation was recognised. This was achieved with co-administrating beta-lactam antibiotics with beta-lactamase inhibitors, for example clavulanic acid or tazobactam, with amoxicillin and piperacillin, respectively. To date, these types of combinations are increasingly used. Beta-lactam inhibitors are derived from beta-lactams, but in themselves have little direct antimicrobial activity. The beta-lactamase inhibitors bind to beta-lactamases and inactivate them [47].

1.2.2 Antibiotic resistance

Already soon after the discovery of penicillin by Alexander Fleming in 1928 there were concerns about resistance development [49], and since then there has been a dramatic increase in antibiotic resistance in bacterial populations. However, one must bear in mind that the concept of antimicrobial resistance is as old as the existence of bacteria. Resistance mechanisms have developed as the result of interaction between microorganisms and their environment. Many antimicrobial compounds in clinical use are derivatives of naturally occurring molecules, because bacteria residing in the same niches have evolved mechanisms to overcome the anti-microbial action of their co-residents in order to survive [50].

Bacteria display several principally different resistance mechanisms in relation to their action [51, 52]. They can be summarized as follows:

- Efflux pumps: Efflux pumps are located in the cytoplasmic membrane. They can expel toxic compounds, including antibiotics, from the bacterial cell. This mechanism of resistance affects a wide range of antimicrobial classes and also other toxic compounds that bacteria might encounter, such as biocides, metals etc., with the risk of co-resistance development.
- Decreased permeability: Many antibiotics have intracellular targets. Therefore, the antibiotic must penetrate the outer and/or cytoplasmic membrane in order to perform its antimicrobial effect. Bacterial resistance mechanisms that modify permeability are either downregulation of membrane porins or altering the porin structure. In both cases the antibiotic is prevented from diffusing into the bacterial cell.
- Production of enzymes: Enzyme production enables the bacteria to inactivate the antibiotic compound. Some enzymes degrade antibiotics, such as the beta-lactamases, while others modify the antibiotic, such as aminoglycoside modifying enzymes.
- Modification of the antibiotic target site: This is one of the most common mechanisms of antibiotic resistance in bacteria, resulting in impaired binding of the antimicrobial molecule to its target. The target changes may consist of either enzymatic alteration of the target site, point mutations in the genes encoding the target site or replacement/bypass of the original target. An example of the latter is the alternative

penicillin binding proteins (PBP) in MRSA, where a *Staphylococcus aureus* has acquired an exogenous PBP (PBP2a) that competes with the binding of the antibiotic.

There is a distinction between so called natural resistance, due to resistance determinants that are intrinsic to a species rendering all isolates of the species resistant, and acquired resistance, implicating that the species was originally susceptible to the antibiotic compound in question, but part of the isolates belonging to the species have acquired resistance mechanisms. Acquired resistance is considered the main upcoming threat [50].

1.2.3 Horizontal gene transfer

There are two genetic strategies for resistance development; mutations in genes that affect the activity of the antibiotic compound, and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT) [2, 3]. HGT facilitated by plasmids has greatly contributed to the emergence and dissemination of multidrug resistance Gram-negative bacteria that we now encounter [53, 54].

HGT can take place through three main strategies [54, 55];

- Conjugation: Genetic material is transferred between bacterial cells by direct cell-to-cell contact, where genetic elements are transferred through a pilus
- Transformation: Naked DNA is picked up and incorporated in the bacterial host DNA, although this is mainly used by a limited number of clinically relevant bacteria
- Transduction: DNA is transferred by bacterial phages

In conjugation the mobile genetic element (MGE) is a conjugative plasmid. The plasmids transfer genetic information between bacteria, often within a species but also, although generally less frequently, between species (Figure 3). If these plasmids carry multiple resistance genes, which often is the case for ESBL-carrying plasmids, the recipient bacteria will immediately become multi-resistant in one step [56].

The ability of MGEs to transfer antibiotic resistance genes between bacterial isolates is well recognized [57]. MGEs are traditionally divided into plasmids, phages or transposons that facilitate the movement within a chromosome or between bacteria. Plasmids are small circular, double-stranded DNA molecules, which replicate independently of the bacterial chromosome. They often carry genes that confer advantageous properties for bacterial survival,

such as a variety of antimicrobial resistance genes or virulence factors [58]. They are traditionally classified into incompatibility groups (Inc groups) based on their plasmid replicon type. With the development of sequencing methods, so called plasmid-MLST is increasingly substituting replicon-typing to classify plasmids [59].

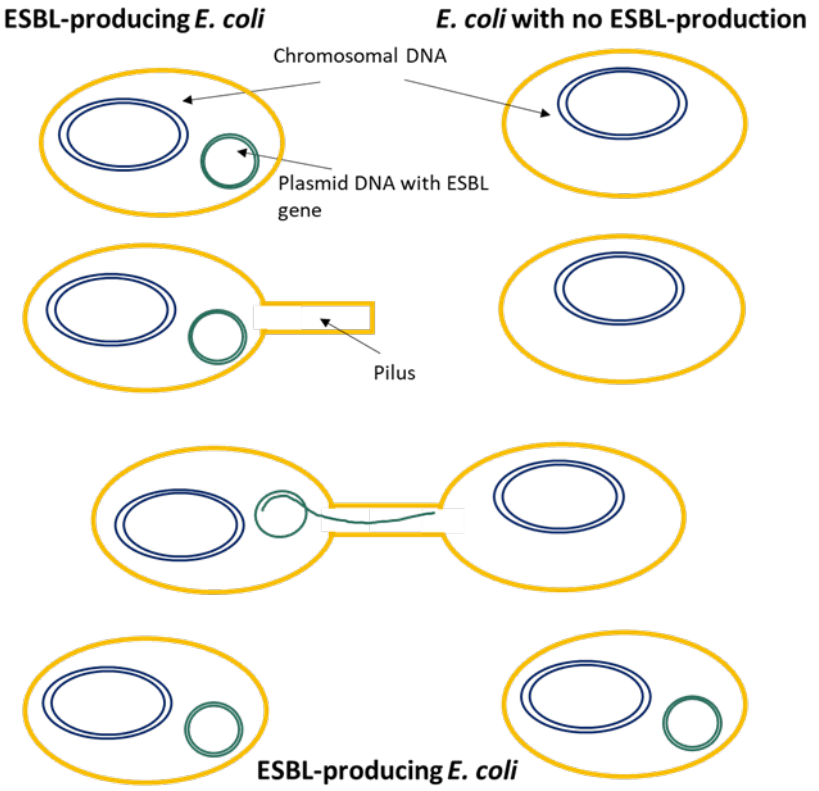


Figure 3. Schematic illustration of conjugation.

1.2.4 The early beta-lactamases

Beta–lactamases are ancient enzymes; their origin is estimated to millions of years ago [60]. The genes encoding beta-lactamases were originally located on the chromosomes of environmental bacteria, where their function was to protect the producing bacteria from naturally occurring beta-lactam compounds. Beta-lactamases have emerged as the major resistance mechanism for beta-lactam antibiotics in Gram-negative bacteria. The first report of an

enzyme with beta-lactamase activity was a penicillinase detected in *E. coli* in the 1940s [61]. Following this, beta-lactam resistance was frequently recognized in Gram-negative bacteria, together with the finding that many Gram-negative bacteria produced inducible chromosomal beta-lactamases.

Beta-lactamases degrade the beta-lactam ring of beta-lactam antibiotics through hydrolysis with varying efficacy [62]. Penicillinases have a propensity for penicillins. TEM-1 is a commonly encountered beta-lactamase in Gram-negative bacteria. It confers resistance to ampicillin in *E. coli* and other *Enterobacteriaceae*, as well as in *Haemophilus influenzae* and *Neisseria gonorrhoeae*. The term TEM was derived from the name of the Greek patient (Temoniera) from whom the first described isolate was recovered in 1963 [63], whereas SHV is an abbreviation for sulf-hydryl variable. SHV-1 also confers ampicillin resistance and shares approximately 70 percent of its amino acids with TEM-1. SHV-1 was identified and is most commonly found in *Klebsiella. pneumoniae* [64].

Third generation cephalosporins (oxyimino cephalosporins), including cefotaxime, ceftriaxone, and ceftazidime, were developed to meet with the emergence of new beta-lactamases. Subsequent resistance was first described in 1983 in a *K. pneumoniae* isolate from Germany, carrying a derivative of SHV-1 designated SHV-2 [65]. This was followed by reports of *Enterobacteriaceae* carrying TEM-derivatives conferring an extended-spectrum resistance. Thus, the enzyme substrate profile had now expanded and now also encompassed the extended spectrum cephalosporins, hence the term Extended-Spectrum Beta-Lactamase (ESBL).

The TEM-, SHV-, and OXA-type ESBL enzymes differed from their precursor enzymes only by a few amino acids clustering around the active site of the enzyme, and thus changing the substrate profile [66]. These early ESBL-enzymes were mainly detected in nosocomial bacteria, such as *K. pneumoniae*, from the hospital setting [66, 67].

1.3 Classification of ESBL

Because of the great plethora of ESBL enzymes in *Enterobacteriaceae*, there is a need for classification. Today there are two main approaches to classification of ESBL beta-lactamases, the Ambler structural (molecular based) classification [68] and Bush-Jacoby-Medeiros functional classification [62, 69]. Assignments to functional groups generally correspond to the molecular classification, although there are exceptions. Initially the ESBL definition included only the clavulanic acid inhibited, molecular class A,

functional class 2be beta-lactamases, capable of hydrolyzing narrow-spectrum cephalosporins, oxyimino-cephalosporins, penicillins and monobactam, although not cephamycins and carbapenems [69].

In Scandinavia, a simplified classification proposed by Giske et al. in 2009 [70] is most commonly used. It includes plasmid-mediated enzymes carried by *Enterobacteriaceae* that are reportable according to Communicable Diseases Act in Sweden. $ESBL_A$ in this scheme represents the classical ESBLs inhibited by clavulanic acid, and generally corresponds to "ESBL" in international literature. The $ESBL_M$ are inhibited by cloxacillin or boronic acid and encompass the plasmid-mediated AmpC enzymes. The $ESBL_{CARBA}$ comprise enzymes with the ability to degrade carbapenems, often referred to as "carbapenemases" in international literature Only bacteria harbouring plasmid-mediated carbapenemases are included in $ESBL_{CARBA}$. Carbapenem-resistance due to other mechanisms, such as production of other beta-lactamases, efflux pumps and/or altered permeability, are not included.

1.4 Further development of ESBL

At the end of the 1980s, there were several reports describing a novel ESBL group; the CTX-M enzymes [71-73]. These enzymes were more active against cefotaxime compared to ceftazidime, and were therefore named CTX-M (CefoTaXimase, first isolated in Munich). Accompanying the emergence of the CTX-Ms from the mid-1990s and onward was a shift in the epidemiology of ESBLs, and since then CTX-Ms have been recognised as the most prevalent ESBLs among *Enterobacteriaceae* [74]. Today there are well over 200 different CTX-M enzymes (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/). The emergence and nomenclature of CTX-M is outlined in more detail in relation to paper I.

The OXA enzymes, yet another type of beta-lactamases, are characterized by hydrolytic activity against oxacillin and cloxacillin, and are poorly inhibited by clavulanic acid. At first the OXA enzymes were considered a minor group of plasmid-encoded beta-lactamases, which mainly affected the penicillins. Since then, several OXA enzymes have been detected with broader substrate profiles, including carbapenems [75].

AmpC beta-lactamases, in contrast to ESBLAs, hydrolyze both broad and extended-spectrum cephalosporins, as well as cephamycins. They are encoded on the chromosome of many *Enterobacteriacea*, including *Citrobacter* and *Enterobacter* species, where the expression is usually inducible. AmpC may also occur on the chromosome of *E. coli* where it is not inducible, although it

can be hyper expressed due to mutations in the promoter and attenuator regions. In addition, plasmid-mediated AmpC (pAmpC) genes are increasingly identified in *E. coli*, of which CMY constitutes the most disseminated type [76]. At the time of the studies in this thesis pAmpC enzymes were rare in our region, and thus were not further explored.

Parallel with the global emergence of ESBLAs, there is an ongoing increase in resistance in *Enterobacteriacea* also to carbapenems, which constitutes an important growing and worrying public health threat [77]. Although known as carbapenemases, these enzymes generally hydrolyze also other beta-lactams.

IMP carbapenemases emerged in *Pseudomonas aeruginosa* and *Enterobacteriaceae* in Japan in the 1990s, and this was the first plasmid-mediated carbapenemases to be recognized [78]. Since then, an increasing number of carbapenemases have been encountered in *Enterobacteriaceae*, often firstly described in *K. pneumonia*, subsequently followed by reports in *E. coli* and increasingly frequent in the community. The most common carbapenemases among clinical *Enterobacteriaceae* globally are the K. pneumoniae carbapenemases (KPCs), the metallo-beta-lactamases, including New Delhi metallobeta-lactamase (NDMs), IMPs and VIMs, and also OXA48–like enzymes [79], although with some geographical variation in their prevalence [80]. In certain areas, like in Southeast Asia, the prevalence is becoming alarmingly high, which also applies to a few countries in southern Europe [81].

1.5 Transmission of antimicrobial resistance

The ability of the bacterial genome to adapt to changes in the environment, in combination with antibiotic-driven selection caused by human activity, are the main driving forces behind the antibiotic resistance problem. In addition, the emergence of highly successful multidrug-resistant genetic lineages in different pathogenic bacteria has made this a global challenge [82].

Antibiotic resistance can be transferred in two ways; either to the bacterial daughter cell in a vertical fashion, or by horizontal transfer to other bacteria. Horizontal transfer of plasmids with antibiotic resistance genes in *Enterobacteriaceae* occurs in the human gut flora, as well as in animals and the environment [83]. In addition, carriage of several resistance genes on the same plasmid confers multi-resistance, whereby one resistance gene may be co-selected for by use of antibiotics other than those to which it confers resistance [84]. The selective effects of pollutants, heavy metals and biocides must also be taken under consideration when antibiotic resistant genes and

MDR bacteria are spread in the environment [85]. They can co-select both for mobile genetic elements carrying multiple resistant gene and MDR bacteria.

The development of antibiotic resistance is a natural phenomenon in bacteria. However, the process is accelerated by the use, and misuse, of antibiotics in humans and animals, leading to selective pressure favoring the resistant bacteria. In veterinary medicine, antibiotics are overused, not only to treat actual infections in the livestock, but also to enhance rapid growth in order to maximize profit. The use of antibiotics in this sector unfortunately is likely to emerge [86]. Antibiotic pressure does not only occur in the gut of a patient or animals treated for a clinical infection. It also takes place on a wider scale in the hospital environment, where many patients are treated with antibiotics enhancing the risk of selection and spread of resistant bacteria in this niche. Additionally, lack of basic hygiene routines in many health care settings world-wide, further facilitates the spread of these organisms [87]. However, there are studies indicating that the spread of ESBL-*E. coli* in the hospital setting is lower than that of *K. pneumoniae* carrying ESBL [88].

In addition to the selective pressure of antibiotics, several additional factors contribute as drivers of antimicrobial resistance and onward transmission of resistant bacteria. Transmission occurs both between people and between people and animals, as well as to and from the environment around us (Figure 4) [87]. International travel and migration of humans and transports of goods further accelerates this process. The role of the food industry and animal farming in the dissemination of MDR bacteria has become obvious in recent years [89]. Apart from the transmission from animals for food production, we appear to share bacteria with domestic animals, and also with family members [90].

High population density, poverty, and insufficient sanitation also facilitate the dissemination of multi-resistant bacteria in the community, not least EPE, as illustrated in Figure 5 [5, 83, 91]. As with any faecal-oral transmitted disease, the role of water pollution cannot be ignored, ESBL-producers can be found in both wastewater and fresh water all over the globe [87, 92].

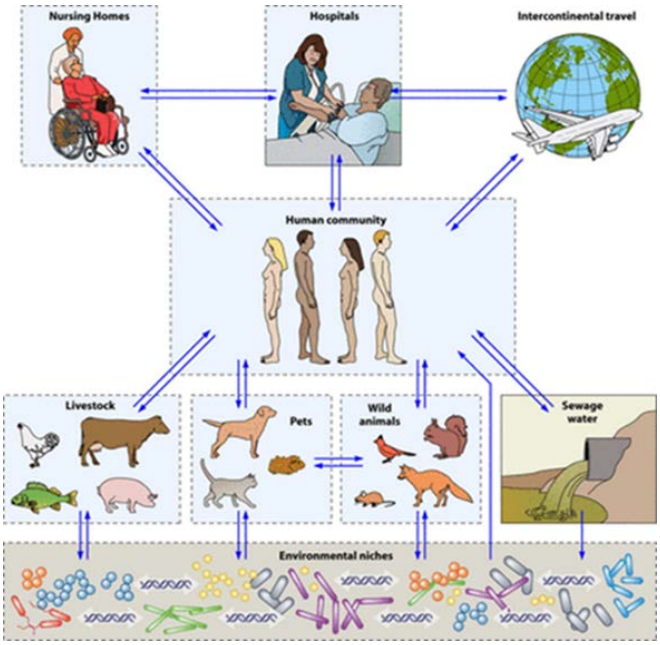


Figure 4. Possible routes of dissemination of MDR Enterobacteriaceae. Reprinted from Woerther et al. [5] with permission from the American Society of Microbiology.

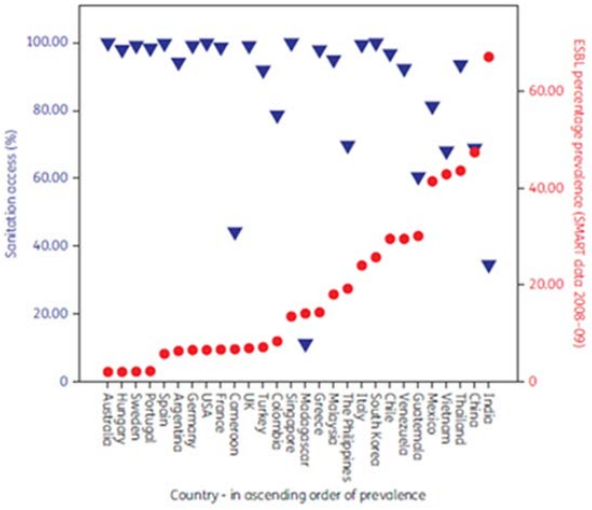


Figure 5. Sanitation access and ESBL prevalence in selected countries worldwide. Reprinted from Bevan et al. [83] with permission from Oxford University Press.

1.5.1 Faecal carriage of ESBL-producing bacteria

The gut flora is the main reservoir from which EPE originate, both for community and hospital acquired infections. In the majority of cases, EPE colonization remains asymptomatic and does not lead to infection [93, 94].

Data on the actual risk of a colonized person developing an infection are scarce, other than for selected patient groups with a high risk of infection development. There are a number of reports on the prevalence rates of faecal ESBL-carriage, demonstrating an increase in asymptomatic faecal carriage of both EPE as well as carbapenemase producing *Enterobacteriaceae* (CPE) among healthy individuals worldwide, especially in less developed regions [5, 95, 96]. In Sweden, asymptomatic EPE carriage has been estimated to almost 5% of the population [97]. This should be compared to developing countries, where carriage rates are considerably higher, ocasionally exceeding 60%. Woerther et al. [5] describes the Western Pacific, Eastern Mediterranean, and Southeast Asia regions as displaying the highest carriage rates, Figure 6, and predicted the most noticeable increases in these countries in the early phase of this epidemic.

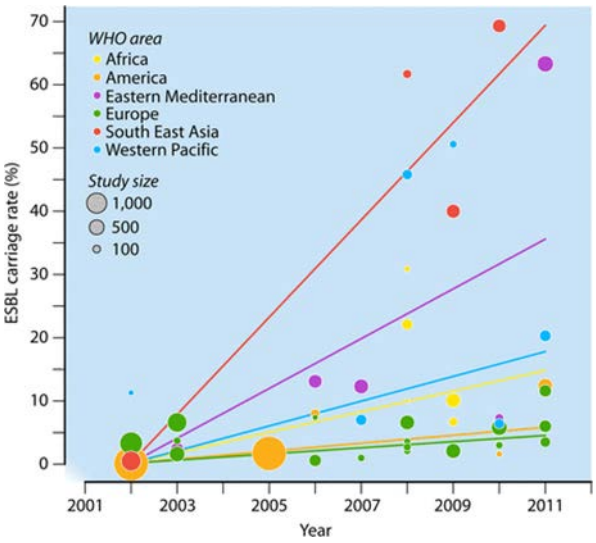


Figure 6. Trend of increasing faecal carriage of EPE in various geographic regions as EPE emerge globally. Reprinted from Woerther et al. [5] with permission from the American Society for Microbiology.

The majority of EPE carriage cases reflect the general global epidemiology of ESBLs and are made up of CTX-M beta-lactamases, especially CTX-M-15 [5]. However, even though the genes are the same as in clinical infections with EPE, more diverse ESBL-producing *E. coli* clones seem to be circulating in the community, and thus as part of the gut flora in colonized individuals. This is in contrast with ESBL-*E. coli* strains causing infection, where *E. coli* of type ST131, has become pandemic. This discrepancy is supported by Ny et al. [97], reporting that the EPE strains found in carriers in Sweden had a lower proportion of EPE belonging to the more virulent types (phylogroup B2), as compared to isolates from blood stream infections (BSIs). Vading et al. [94] also reported that the vast majority of travel-acquired EPE in healthy volunteers lack typical virulence factors of uropathogenic strains.

Travel from countries with low ESBL-EPE carriage rates to places with high ESBL-EPE carriage rates are a source of colonisation, as described in several early studies involving Swedish travelers [98]. There are parts of the world that are considered high-risk areas, such as India, Southeast Asia and the Middle East, and travel to these regions is a major risk factor for acquisition of asymptomatic fecal carriage of EPE, as recently reviewed by Woerther et al. [98]. In addition, there is an enhanced risk of colonization by ESBL-EPE among travelers hospitalized abroad.

There is a lack of knowledge concerning the duration of EPE carriage, and whether these bacteria can indeed be eliminated. Persistence studies are difficult to perform, because a negative sample does not necessarily mean that colonisation has truly terminated. Reports show that travel-acquired EPE carriage tends to be relatively short, only 5–35% of those with travel-acquired EPE were carriers 6 months later [98]. The mean carriage duration for patients colonized during hospitalization was reported to be six months [5] In patients with a clinical infection due to an EPE isolate prolonged persistence has been reported. In the study of Alsterlund et al. [99] all carriage for several years was detected. According to Titelman et al. [100] 43% of patients with UTI due to EPE were still colonized in stool after one year.

1.6 Problems with antibiotic resistance

Antibiotic resistance affects not only human public health on a global scale but will ultimately affect several sectors within our communities, not the least food production, as recently highlighted by the World Bank [101]. There is a need of a “One health” perspective where humans, animals and environmental factors are seen as one connected entity. This is increasingly realized by leading stakeholders [102], and is summarized in the WHO Global action plan

on antimicrobial resistance [9], launched in 2015. Improving sanitation, increasing access to clean water, and ensuring good governance, as well as increasing public health-care expenditure and better regulating the private health sector are all necessary to reduce global antimicrobial resistance, according to Collignon et al. [103].

Within the human health sector both hospital and community acquired infections are affected. Infections with antibiotic resistant bacteria might be more costly, and could even be impossible, to treat, resulting in increased morbidity and mortality and increased health care costs [104, 105]. Antibiotics are used within all health care areas, both to treat common community acquired infections, such as UTI or pneumonia, as well as serious infections in neonatal and intensive care patients, or in patients that are immunocompromised because of cancer therapy or organ transplants. For a critically ill patient, treatment failure might well become life threatening. Without access to necessary antibiotics to treat infections, specialised medical procedures and treatment regimens will become impossible to accomplish.

According to the World Health Organization (WHO) the estimated costs for antibiotic resistance are still largely unknown [9], even if attempts have been made at estimating the extended costs, both economically and in lost life years. Present estimates of millions of deaths annually and billions in cost are likely to increase dramatically within the upcoming decades [106]. In Sweden, considered a low incidence country with respect to antibiotic resistance, the Public Health Agency estimates the cost for antibiotic resistance due to multidrug resistant bacteria amount to 4.3 billion Swedish crown by the year 2030 [107].

2 AIMS

2.1 General Aim

The overalls aim was to explore the need for continuous surveillance of ESBL-producing *Enterobacteriaceae* at the local level, and to evaluate various epidemiological methods for surveillance enabling simple and rapid detection of outbreaks.

2.2 Specific Aims

I. To describe the epidemiology of ESBL-producing *Enterobacteriaceae* in the greater Gothenburg area in western Sweden, from the first detected case until these bacteria were established five years later, with focus on ESBL-*E. coli* causing urinary tract infection.

II. To evaluate different epidemiological typing methods in comparison to pulsed-field gel electrophoresis (PFGE) for investigation of a polyclonal outbreak caused by ESBL-*E. coli.*

III. To evaluate different epidemiological typing methods in comparison to pulsed-field gel electrophoresis (PFGE) for local surveillance of ESBL-*E. coli*, with focus on multiple-locus variable number tandem repeat analysis (MLVA).

3 PATIENTS AND METHODS

Details on materials and methods in the respective papers will not be repeated, since they can be found in the papers. However, to facilitate understanding of the discussion for papers II-III this Patient and methods section also includes an overview of methods for subtyping of *E. coli*.

3.1 Study designs

Paper I – Retrospective epidemiological study

Paper II - Retrospective descriptive and methodological study

Paper III - Methodological study

3.2 Setting of these studies

During the study period the clinical microbiology laboratory at Sahlgrenska University Hospital provided health care for approximately 750 000 inhabitants in the greater Gothenburg area in the southwest of Sweden, including a 2000-bed university hospital, a 200-bed tertiary hospital, 110 long-term-care facilities, and 75 outpatient clinics. The study period was set to 2004–2009, including the first EPE cases detected in late 2003. During this period, the number of blood cultures increased from 25 000 to 35 000 and urine cultures remained unaltered around 65 000 samples/year.

3.3 Methods for identification *E. coli* and antibiotic susceptibility testing

During the past two decades MALDI-TOF (matrix-assisted laser desorption/ionization) mass spectronomy (MS) has revolutionized modern clinical microbiology. It is now used as a routine method for species identification of clinically relevant microorganisms worldwide. [108-110]. However, biochemical analyses for species detection still exist in parallel, either because it is more rapid than the MALDI-TOF or as an additional method to complement the MALDI-TOF. During the period when isolates for paper I-III were collected, species identification of *E. coli* at our laboratory was performed with standard biochemical analyses, after initial detection according to morphological features on a selective agar plate for Gram-negative bacteria.

Antibiotic susceptibility testing (AST) today is increasingly performed according to the guidelines of EUCAST (European Committee on Antimicrobial Susceptibility Testing) or CLSI (Clinical and Laboratory Standards Institute, US). Routine AST is generally carried out through disc diffusion, or commercially available automated systems using broth cultures in the presence of defined antibiotic concentrations. For disc diffusion, bacteria grow on a specific agar-plate under standardised conditions, in the presence of discs of known antibiotic concentration. Because the antibiotics diffuse into the surrounding agar, an antibiotic concentration gradient is established. Susceptible bacteria will fail to grow where the antibiotic concentration is high enough, hence creating a zone with no growth around the disc (Figure 7). The disc diameter is measured and determined as either S (susceptible), I (intermediate) or R (resistant), according to standardized breakpoint tables set by EUCAST [111]. AST of the isolates in this thesis was performed according to the national guidelines existing at the time from the Swedish National Antimicrobial Susceptibility Testing Committee; SRGA (the Swedish Reference Group for Antibiotics). These guidelines were based on EUCAST methodology.

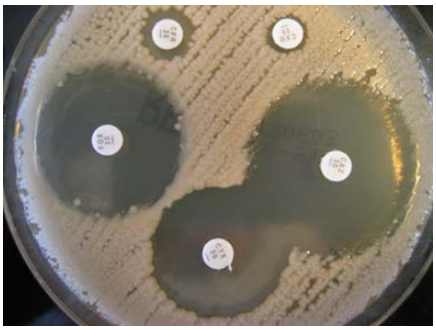


Figure 7. Antibiotic susceptibility testing using disc diffusion at the time of the study. Image courtesy of C. Åhrén.

The disc diffusion method is based on MIC-values; MIC meaning Minimum Inhibitory Concentration, which is determined from a serial dilution of antibiotic concentration, where MIC is the lowest concentration of the antibiotic necessary to inhibit visible growth. An approximation of the MIC value can be accomplished by using a gradient test, Figure 8. However, this method, although widely used in routine clinical microbiology, is affected by methodology problems. Therefore, golden standard today for determining an appropriate MIC value is broth microdilution.

There are several methods for phenotypic detection of EPE in the laboratory. The main principle of detection is based on growth in the presence of a third-generation cephalosporin, sometimes in addition with a chromogenic media or by methods based on inhibition of clavulanic acid, or some other inhibitory substance depending on which beta-lactamase enzyme to be confirmed (Figure 8). When there is need to confirm the finding of an EPE, detecting the beta-lactamase gene with PCR or sequencing methods is needed. For this thesis work, ESBL-*E. coli* were initially detected by resistance to a third-generation cephalosporin in routine AST. Confirmation was performed by a double disk diffusion test (DDT) [112]. The principle of the DDT is that the disc placed in the middle of the plate contains clavulanic acid. Both the clavulanic acid and the antibiotic content from discs containing a third-generation cephalosporin diffuse into the same space in the agar, thereby creating an area where there is an optimal concentration of both substances for growth inhibition to occur. This creates the enlarged, so called "ghost zones", seen in Figure 8.

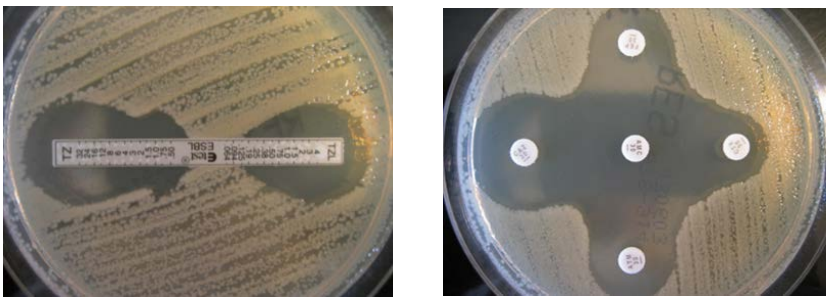


Figure 8. Phenotypic detection of EPE using gradient test (left) or double disc diffusion test, DD (right). Image courtesy of C. Åhrén.

3.4 Overview of methods for subtyping of *E coli*

To determine the source of outbreaks and establish transmission pathways there is a need for typing methods that differentiate *E. coli* strains beyond the species level. A strain typing method should provide enough discriminatory power to distinguish between epidemiologically unrelated isolates, and, in addition, cluster isolates from the same source [113, 114]. So far development of *E. coli* typing methods has been focused mainly on enteropathogenic isolates. However, there is an increased need for typing methods for non-enteropathogenic strains for the purpose of surveillance and outbreak

detection, especially for multidrug resistant (MDR) *E. coli* [115]. There is a plethora of methods available for strain typing of *E. coli*, and it is not in the scope of this thesis to mention them all. Rather, in the subsequent section there will be a summary of commonly used methods. The typing methods used in paper II and III, i.e, MLVA, PFGE and MLST, are more extensively described at the end of this section.

3.4.1 Subtyping based on phenotypic characteristics

Conventional typing methods that assess phenotypic traits, such as staining properties, biochemical properties and antigenic properties, have historically formed the foundation of descriptive bacterial epidemiology. An important limitation of phenotypic methods in general is that they are not variable enough for discriminating between closely related strains [113, 116]. Also, the expression of the phenotypic traits might vary over time.

3.4.2 Serotyping

Serotyping has traditionally been an important typing method from the early days of microbiology [117]. For *E. coli*, Ørskov et al. [30] developed a typing scheme based on the presence of surface antigens, i.e. O-antigens (LPS), H-antigens (flagella), and K-antigens (capsule). Since few laboratories had capabilities to type the K antigen, serotyping based on O- and H-antigens became the gold standard for *E. coli* typing. Major limitations of serotyping are that not all isolates are typeable, and several strains within a serotype cannot be distinguished [115]. However, serotyping is still used for designation of clonal lineages, especially pathogenic strains, such as EHEC O:157 or the pandemic ST131-O:25b genetic lineage.

3.4.3 Antibiogram

Antibiogram typing based on the resistance profile is traditionally used to rapidly differentiate possibly related strains, for instance in outbreak surveillance of MDR bacteria in hospitals. The discrimination is, however, dependent on the diversity, stability, relative prevalence of a particular resistance mechanism and also the mobility of the resistance genes [117]. Isolates referred to the same strain by other typing methods might still display different antibiograms, and for *E. coli* and other *Enterobacteriaceae* that are capable of harboring plasmids with multiple resistance determinants, antibiogram-based typing is becoming less reliable.

3.4.4 PhenePlate (PhP) system

The PhP system results in biochemical fingerprinting. It characterizes bacterial strains by evaluating the kinetics of biochemical reactions. The method is

rapid, non-laborious and a substantial number of isolates can be studied simultaneously. The suggested use by the manufacturer is as an initial screening method (http://www.phplate.se/). However, we found the method both to under- and overestimate differences, which limits its use. Our experiences from analysing the isolates in paper II using the PhP system were discouraging, especially for the ST131 outbreak isolates (unpublished data).

3.4.5 MALDI-TOF

In MALDI-TOF MS the generated spectrum of an unknown microbial isolates is compared with the MS spectra of known isolates in a database for species identification [118]. The technique has also been assessed for subtyping of diverse microorganisms, for instance *Clostridium difficile* and *Staphylococcus aureus* [119, 120]. There are several technical advantages in favor of this method, including speed and cost, but for some species the discriminatory power is not good enough and so far, successful examples for *E. coli* are rare [109, 121].

3.4.6 Subtyping methods based on PCR

Typing with polymerase chain reaction (PCR) provides a rapid inexpensive and unambiguous means for assigning types, where primers directed towards specific sequences or genes constitute the base. These results are thus highly portable between laboratories. The PCR can be aimed at a particular gene, such as resistance genes like the CTX-M genes, or a specific allele at a certain locus, such as the PCR for detection of ST131-O25b. If multiple genes are targeted, generally a multiplex-PCR set up is used.

3.4.7 *E coli* phylogenetic grouping

Phylogenetic grouping has been used for decades to distinguish *E. coli* with the ability to cause extra-intestinal infection from commensals of the human gut [43]. Strains belonging to the different phylogenetic groups of *E. coli* are not randomly distributed, and strains responsible for extra-intestinal infections are more likely to be members of phylogenetic groups B2 or D, rather than A or B1 [42]. By real-time multiplex PCR methods targeting specific genes isolates can be divided into phylogenetic groups. Currently, seven groups are recognized; A, B1, B2, C, D, E and F, for *E. coli* sensu stricto [122]. The discriminatory power of phylotyping is, however, too low to allow for more detailed subtyping of *E. coli*.

3.4.8 ESBL-genotyping

Typing of the ESBL-genes is often part in surveillance of EPE, and numerous methods, including PCR-based assays, have been developed for identification

of the growing number of different ESBL-genes [123, 124]. Some of these ESBL-genes differ by no more than a single amino-acid which makes it almost impossible to design PCR-assays for the individual ESBL-genes, particularly for the very large number of CTX-M genes. Thus, PCR-typing results generally need to be followed by DNA sequence analysis of the PCR amplicons to resolve which type it is.

The CTX-M enzymes can be assigned to one of five groups; CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 or CTX-M-25, based on their amino acid sequence [125]. The group name corresponds to the first identified CTX-M gene within the group, and the number of different genes within each group varies considerably. An alternative way to group the CTX-M genes is based on the clustering of CTX-M genes, i.e. cluster -2, -3, -8, -14, -25, -45 and -64 are chosen as the representative enzymes in each cluster [126]. However, the CTX-M genes within each group are very similar for both ways of assigning CTX-M groups, but different CTX-M genes have been chosen as group-representatives.

Often a step-wise approach, like in paper I-III, is used for identifying the ESBL-genes. Firstly, a multiplex-PCR is used to differentiate between TEM, SHV and CTX-M genes, followed by a second multiplex-PCR to differentiate between CTX-M groups. Lastly DNA sequence analysis is performed if necessary. For the specific PCR used in this thesis, please see the respective papers.

The high prevalence of certain CTX-M genes greatly limits the use of ESBL-gene typing for surveillance but may be of importance in outbreak management.

3.4.9 Detection of isolates belonging to the *E. coli* ST131-O25b genetic lineage

With the emergence of this lineage it has become increasingly important to differentiate MDR *E. coli* isolates of ST131 from those of other linages. A PCR developed by Clermont et al [127] for easy identification of isolates belonging to the ST13-O25b linage has been applied in a large number of studies [128, 129], and also in this thesis work. It is based on nucleotide sequence polymorphisms in the *pabB* gene. Also, there are multiple ST131 subclones, also within the ST131-O25b, of which H30R and Rx have gained most interest. There are several conventional PCRs described to detect fimH30 and its subclones [130-132]. In this thesis-work the H30Rx PCR described by Banerjee et al. was applied [133]. With the emergence of next generation

sequencing an alternative way to differentiate isolates of ST131 has been described for epidemiological studies. It is based on three different clades in relation to the respective fimH allele; A (H41), B (H22) and C (H30) [132, 134]. Clade C includes the subclades C1, C2 and the recently described C1-M27 subclade. Matsumura et al. [134] recently described a multiplex PCR assay that identifies all ST131clades, including the C subclades, in a single PCR-reaction.

3.4.10 High resolution melting analysis

High resolution melting analysis (HRM) is a technique used to determine whether two PCR amplicons of similar size have identical sequences or not. It is used to assess single nucleotide polymorphisms (SNPs) and genetic variation in strains of bacteria [135, 136]. A PCR is performed amplifying the sequence of interest. The amplicon DNA is subsequently gradually heated and the melting pattern of the amplicon, when the two strands of DNA are separated, is monitored in real-time and subsequently compared between isolates. In the study by Woksepp et al. [137] using restriction enzyme cleavage and ligation-mediated quantitative PCR followed by subsequent high-resolution melting-pattern analysis of DNA fragments (LM/HRM) the resolution of the LM/HRM method for the identification of a nosocomial outbreak of O25b-ST131-associated ESBL-positive *E. coli* isolates was comparable to that of PFGE. In another study HRM was used for identification of ST131 from non-ST131 [138].

3.4.11 Subtyping based on DNA fingerprinting techniques

Genotyping, which refers to the discrimination of bacterial strains based on their genetic content, has become widely used for bacterial strain typing due to its high resolution, as compared to phenotypic methods. DNA fingerprinting technique provides indirect access to DNA sequence polymorphisms and consists of DNA fragments separated from each other, traditionally by electrophoresis in an agarose gel. They provide a whole-genome analysis but with much less information than present sequencing methods.

3.4.12 Pulsed Field Gel Electrophoresis

Pulsed Field Gel Electrophoresis (PFGE) has been used as the gold standard for epidemiological typing in this thesis, and will be explored in more detail below.

3.4.13 The Amplified Restriction Fragment Polymorphism technique

The Amplified Restriction Fragment Polymorphism (AFLP) technique is based on the selective PCR amplification of a subset of restriction fragments. The technique is based on presence or absence of restriction fragments, rather than fragment length differences. Following PCR, the reaction products are separated and visualised for comparison to other isolates [139]. AFLP is highly discriminatory and provides a whole-genome analysis, and is often used in the same context as PFGE. However, the final data output is complex, thus interpretation can be difficult and, consequently, also interlaboratory comparison.

3.4.14 Random Amplification of Polymorphic DNA

Random Amplification of Polymorphic DNA (RAPD) typing is based on random amplification of multiple fragments of different lengths. A short primer used at low annealing temperature, which is not directed at any specific sequence in the genome, will hybridize at random sites [140]. The amplified fragments are traditionally visualised by electrophoresis. RAPD has a good discriminatory power and no knowledge of the DNA sequence of the targeted genome is required. However, a limitation is the lack of interlaboratory reproducibility, due to the short random primer sequences and the low PCR annealing temperatures required.

3.4.15 Repetitive sequence-based PCR

Repetitive sequence-based PCR (Rep-PCR) uses specific primers that target noncoding repetitive sequences that are present at many sites in the bacterial genome, and demonstrates higher discrimination power and higher reproducibility than RAPD [141]. The number and sites of these repeat sequences are variable from strain to strain, and therefore lead to a number of different-sized fragments that can be resolved electrophoretically and thus create a DNA fingerprint [142]. Rep-PCR has been commercially adapted to an automated format known as the DiversiLab system using a standardised protocol. A limitation is that DiversiLab often needs to be used in combination with other typing methods to obtain sufficient differentiation, as reported by Brolund et al. [143]. The method can for instance not differentiate ESBL-*E coli* isolates within the ST131-O25b linage.

3.4.16 Multiple-locus variable number tandem repeat analysis

Multiple-locus variable number tandem repeats analysis (MLVA) represents a major part of this thesis and is described in more detail below.

3.4.17 Microarrays

Microbial diagnostic microarrays (MDMs) consist of nucleic acid probe sets, fixed on a solid support [144]. The microarray technology offers simultaneous detection of thousands of targets in a high-throughput environment. Isolates are compared by the pattern obtained by the presence or absence of the genes/alleles included in the assay. Depending on the probes, current MDMs can provide resolution at various taxonomic levels, and even strain level [145]. The bacterial DNA in the sample hybridise to the probes, and hybridisation is generally detected by fluoresence or chemiluminesence [146]. However, the microarray can only identify already described genes and the construction of DNA microarrays is still too expensive for routine application. Future use of arrays in epidemiology depends on the development of more cost-effective protocols. In addition, international consensus would have to be achieved for data interpretation before use for epidemiological purposes.

3.4.18 Subtyping methods based on sequencing

3.4.19 Whole genome sequencing

Whole-genome sequencing (WGS) and genome comparison is the ideal way to illuminate the genetic variability within a bacterial species [147]. The commercial introduction of next-generation sequencing (NGS) technologies has made it possible to perform WGS of bacteria relatively rapidly and at more affordable costs. By applying NGS, thousands of places throughout the genome are sequenced at once via massive parallel sequencing [148].

The discriminatory power is superior to other typing methods and WGS can overcome the caveats of conventional outbreak management, which often fails to distinguish genetically closely related strains. A major advantage is that WGS is a universal method that does not require species-specific protocols [149], and that data are comparable regardless of the platform used to generate them. WGS does not only provide information about epidemiological linkage but can also identify a number of important genes such as antibiotic resistance genes, virulence factor genes, plasmids or combinations of genes, such as the housekeeping genes of MLST or multiple core genes as in core MLST. Assembly of plasmids and highly repetitive parts of the DNA has, however, proven to be challenging [150].

3.4.20 Multi-locus sequence typing

The method is described in more detail below.

3.4.21 CH-typing

There are MLST schemes based on fewer genes than the traditional seven-gene approach. For *E. coli*, Weissman et al [132] has developed the CH-typing assay based on the *fumC* housekeeping gene of MLST in combination with an internal fragment of the *fimH* gene. The method is becoming increasingly used and there is a webtool available [151]. It provides good discriminative power and is able to split large STs into subgroups, including ST131, and can also identify the H30 subclone of ST131 [152]. CH-typing is said to correspond to specific STs, or ST complexes, with 95% accuracy [132]. However, it cannot replace standard MLST for definitive characterization of the clonal phylogeny of E. coli [153].

3.4.22 Epidemiological typing methods used in this thesis

3.4.23 Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-Field Gel Electrophoresis (PFGE) is based on analysis of the restriction patterns after degradation of the bacterial chromosome. It is performed by restriction enzymes cleaving the bacterial DNA into fragments of various size [154, 155]. In 1984, Schwartz and co-worker developed a variation on the standard gel electrophoresis protocol by introducing periodic change of field direction, which made the separation and visualisation of very large DNA fragments possible. This technique became known as pulsed-field gel electrophoresis [156]. Every genetic change is not detected, but the sum of the visible fragment sizes represents more than 90% of the total genome. Minor genetic changes, such as gain of resistance genes, will not be detected, nor is DNA from plasmids usually visualised. The method for performing PFGE is similar for many different bacteria, with only the choice of the restriction enzyme and electrophoresis conditions optimized for each species [157].

In PFGE, DNA is embedded in agarose gel. This is followed by digesting the chromosomal DNA with restriction enzymes that cleave infrequently. The result is a limited number, usually fewer than 30, of macro-restriction fragments ranging from approximately 50-70 kb to >1 Mb [158]. Slices of agarose containing the chromosomal DNA fragments are then inserted into the wells of an agarose gel, and the restriction fragments are resolved into a pattern of discrete bands. The applied pulsed field causes the DNA molecules to re-orient in the agarose gel matrix. The technique exploits the fact that smaller molecules require less time to reorient than larger DNA, so a gradual increase in the length of electrophoretic pulse times in different directions allows separation of DNA fragments over a wide range of sizes. Generally, 11 bands of 78kb- 453kb sizes are analyzed for *E. coli*, leaving the smallest and largest

bands aside. A well-characterized control strain should be run along with the unknown isolates being tested, and a molecular size standard should be present on the gel to provide size orientation of the fragments [159]. In PFGE for *E. coli*, *Salmonella* Braenderup H9812 is often used as control strain. The DNA fragments produce a DNA fingerprint with a specific pattern to be further analysed.

Visual analysis of PFGE banding patterns may be very adequate for comparison of a few isolates on the same gel, but its usefulness is limited when there is a distance in time between the patterns being compared, therefore computer assisted programs generally are used. However, they are not always capable of correctly identifying all bands in a PFGE pattern. Even optimised settings cannot replace the detection of subtle pattern differences that the human eye may detect. Therefore, manual confirmation of PFGE patterns, and judgement based on additional information, such as epidemiological data, are necessary [117, 158].

Currently, there is no consensus nomenclature for PFGE patterns, and no common international database available for comparison. According to Tenover [159], isolates are designated “indistinguishable” if the restriction patterns have the same numbers of bands and the corresponding bands are the same size, i.e. the same strain (A). Subsequently, an isolate is considered to be “closely related” i.e. subtypes of the same strain (A1, A2 etc) if their PFGE-pattern differences are consistent with a single genetic event, i.e. two to three band differences. Isolates are considered “possibly related” if their PFGE-pattern differences are consistent with two independent genetic events, which can be explained by simple insertions or deletions of DNA or the gain or loss of restriction sites, i.e. four to six band difference. If the PFGE pattern changes are consistent with three or more independent genetic events, i.e. generally seven or more band difference, it is considered “unrelated” i.e. a different strain (type A, B, C etc).

Tenover et al. [159] suggested that, for healthcare-associated outbreaks of limited duration (usually of half a year or less), isolates differing by one to four bands should be considered as “closely related” and therefore “probably part of the same outbreak”. In short term outbreak typing, even single band differences should be deemed important [117]. However, random genetic events, including point mutations and insertions and deletions of DNA, may change the PFGE patterns during the course of an outbreak. Also, variations of two to three bands have been observed in strains of some species when they are cultured repeatedly over time or isolated multiple times from the same patient [113, 157].

In surveillance studies up to six bands difference is often allowed to signify strain relatedness. Van Belkum et al. [117] has suggested a more limited definition where only isolates differing by a single genetic event (i.e. up to four bands difference) are considered related subtypes of a strain.

When using cluster analysis and similarity index for determining strain relatedness, as outlined below, ≥ 90% is generally considered equivalent to "indistinguishable", ≥ 80% to closely related, ≥ 70% possibly related and less than 70% similarity is used to define different strains (Table 1).

Table 1. Criteria for interpreting PFGE-patterns to determine isolate relatedness. Adopted from Tenover et al. [159].

No. of band differences with PFGE	Similarity index (%) with computer assisted program	Determination of isolates relatedness
0	> 90	Indistinguishable
2-3	80-89	Closely related
4-6	70-79	Possibly related
≥ 7	< 70	Different

For designation of strain types in paper II and III it is important to highlight that the PFGE types were arbitrarily denominated with capital letters following the alphabet. Therefore, it is not possible to deduce strain relationship based on the designated letter, i.e. types AE and AB are not subtypes within strain A, which otherwise is a common way to denote subtypes.

Despite its long history as a typing method, PFGE is still a widely applicable method for comparative typing of many bacterial species, especially in outbreak investigation and for local epidemiological studies. It is a reliable and highly discriminating method and has often been used as gold standard in epidemiological studies, such as in this thesis work [117]. The major limitations of PFGE is that the method is quite labor-intensive and slow. It takes two to four days to obtain results. Bands of same size may not come from the same part of the chromosome, which makes it difficult to resolve bands of similar size. Gels need to be analyzed carefully, the process is partly subjective, and years of experience is needed for interpreting the data. The lack of international standard for nomenclature is a limitation for comparison of and communicating results between laboratories. However, if standardized protocols, equipment, and analysis tools are used, the results are reproducible

and can be shared between different laboratories. Since PFGE remains broadly applicable with an enormous data and user base, it is reasonable to believe that it will continue to be a meaningful approach to molecular typing also for some years to come. However, as with all typing methods, this method is likely to eventually be replaced, likely by sequencing-based technologies.

3.4.24 Multiple-locus variable number of tandem repeat analysis (MLVA)

Simple Sequence Repeats (SSRs), or Short Tandemly Repeated sequences (STRs), are DNA sequences consisting of one to six nucleotides that are tandemly repeated at a locus [160]. These so-called variable number of tandem repeat regions (VNTRs) have been identified in pro- and eukaryotic species [117]. In each locus, the repeat copy number can vary between different strains within a species, which is used for subtyping. When multiple VNTRs are targeted for analysis, the technology is called multiple-locus VNTR analysis, or MLVA.

The first studies of repetitive DNA loci in bacteria were on the *H. influenzae* genome [117]. Later on, MLVA also proved to be a way to address the genetic diversity of highly monomorphic species such as *Bacillus anthracis* and *Yersinia pestis*. Most of the early work was performed with such bioterror-related microorganisms [161]. Subsequently, work has been done to develop the typing method for other human pathogens, mainly enteric pathogens such as *Salmonella* and EHEC/STEC. The first adaptations of MLVA for enteropatogens, i.e. EHEC/STEC *E. coli* O157:H7, were published in late 2003 and since then there are many studies reported in the literature on using MLVA typing for analysing EHEC/STEC isolates [162, 163]. Lindstedt et al. [164] developed a so called generic *E. coli* MLVA in 2007, proposed for typing all *E. coli* and not only enteropathogens. It was subsequently modified by Loebesli et al. [165] in 2011, which was the method used in this thesis. The *E. coli* O157 assay was, however, very specific for the *E. coli* O157, and it is important to recognize that the targeted loci of the EHEC/STEC-MLVA and generic *E. coli*-MLVA using 10 loci (GECM-10) differ.

The MLVA-process is shown in Figure 9. The protocol starts with PCR-amplification. Sequence regions flanking the repeat loci are generally sufficiently well-conserved targets so that PCR-mediated amplification of the respective loci can be performed [164]. The amplificated VNTR fragments are then separated by a high-resolution capillary electrophoresis (automatic sequencer), enabling alleles differing in size only by a single repeat unit to be resolved. In capillary electrophoresis, the fragments are run through a gel

matrix in an electric field. An internal size standard is included in each lane, with a distinct dye color, for size determination. By choosing a different fluorophore for each loci, the PCR amplicons will display different colors, so that they can be run together and still be typed individually. The data is displayed as an electropherogram (Figure 10), and each peak is identified according to color and size by computer software [166].

To our knowledge there is no consensus on how to report *E. coli* data generated by MLVA. At each locus, the number of copies of each repeat, based on the observed fragment size, is assigned an allele number. The resulting information is a code, with a number representing the allele type found in each of the loci, i.e. 03-07-11-05-04-N-02-00, where N means that there was no PCR-product amplified for that particular loci, and 00 means that there was an amplified PCR-product, but no detected repeats [165]. Lindstedt et al, [164] proposed an arbitrary designation of different alleles at a specific locus, although based on the number of repeats. Others have used the actual number of repeats in each locus as the allele number. It has also been proposed that the actual fragment length could be used for comparison [167]. We compared all three types of data interpretation in paper III and found no significant differences for the final typing results.

Since the evolution of the VNTR loci is rapid the MLVA method can provide good discriminatory power among closely related isolates. The analytical power for detecting more distant relationships is limited but can be overcome, to some extent, by adding more VNTR loci with a range of mutation rates [168]. However, MLVA is not considered suitable for performing studies of evolution and phylogeny.

There are several reasons for why MLVA has not become more widely used for international surveillance. Differences in the choice of loci, nomenclature, PCR-primer, capillary electrophoresis -platform and/or chemistry differences, and also interpretation of incomplete or partial repeats, are difficulties in inter-laboratory comparisons [166]. Discrepancy in fragment lengths arises for instance from variation within the laboratory set-up used for fragment analysis, why some form of standardization and calibration would be desirable.

Figure 9. Schematic description of the MLVA process

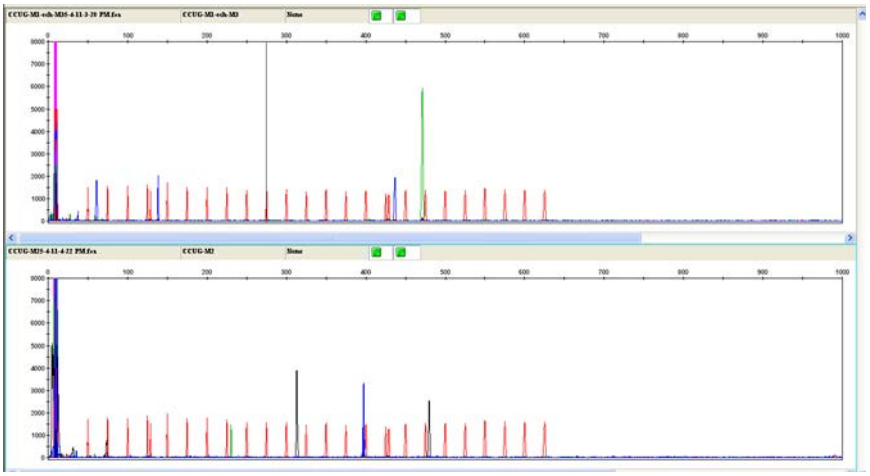


Figure 10. MLVA electropherogram showing the DNA standards of known size in red, and the sizes of the PCR products in blue, green, and black.

Also, there is no international agreement on which nomenclature to use. A common curated database easily accessible on the internet, such as there is for MLST, would greatly aid inter-laboratory communication. Another hampering factor is the independent development of multiple protocols leading to several different schemes for each organism. For example, six protocols have been described for EHEC/STEC O157 [166].

3.4.25 Designation of MLVA strain types in this thesis

In paper II the strain nomenclature was based on MLVA with ten loci, which was designated GECM-10 (generic *E. coli* MLVA with ten loci), Table 2. The type assigned to a particular strain, for example G06-04, means six repeats in

loci CVN001 and the forth type detected within the study with a change in at least one of the remaining loci. In paper III we concluded that the GECM-7 (with seven loci instead of ten), Table 2, was sufficient. The same naming strategy for designation of MLVA types as in paper II was used, i.e. starting with the number of repeats in CVN001, and so on. To facilitate comparison of paper II and paper III, in this thesis the designation G_{10}XX-YY is used when referring to GECM-10 types, and G_7XX-YY when we refer to a GECM-7 type in paper III.

Table 2. The different sets of generic MLVA that are discussed in this thesis, the loci used in each of them are marked with colour.

	CVN 1	CVN 2	CVN 3	CVN 4	CVN 7	CVN 14	CVN 15	CCR 1	CVN 16	CVN 17
GECM-3										
GECM-7										
GECM-10										

The MLVA-types were based on single loci differences as we aimed for as high discriminatory power as possible. For surveillance purposes another approach may be favorable using cluster-analyses and including isolates with up to four loci differences to form MLVA-Type Complexes, as proposed in the study of Naseer et. al. [169].

When proceeding with the MLVA method and adapting it to the routine clinical setting, we suggest a different approach for denomination of strain types. A string of numbers related to the actual number of repeats in a certain locus would be preferable. Indeed, this was the only possible approach when we compared our studies with those of others published later. The number of repeats in the three loci CVN001, CVN004 and CVN014 (Table 2) could form the basis for such a denomination system, i.e. MLVA-type 6-14-6, with the opportunity to add more numbers when a higher resolution is desirable.

A different approach to denomination of types is the system used for the spa-typing method for MRSA. Here, the string of numbers corresponding to the different count of repeats in each locus is converted to an arbitrary number. However, this method requires a curated database to avoid any duplicated strain type numbers. It could also not be extended with additional loci, if need for enhanced resolution is required.

With the denomination procedure suggested above, it is also still possible to deduce from the string of numbers whether two isolates share the same number

of repeats in any of the chosen loci and thus are closer related than isolates that do not share the same alleles.

3.4.26 Multi-locus sequence typing

Multi-locus sequence typing (MLST) was proposed in 1998 as a sequence-based method for identifying clonal relationship among bacteria. MLST determines the genetic relatedness by analyzing the sequences of a variable number, of metabolic, or housekeeping, genes present in all isolates within a species and that are, genetically speaking, relatively stable. The first MLST scheme was developed for *Neisseria meningitidis*, and since then the approach has been applied to a growing number of organisms [170].

The MLST-protocol starts with PCR amplification and sequencing of the panel of targeted housekeeping genes. Sequences of the loci are queried via online submission against an appropriate established database with known allelic sequences for the desired bacterial species [154]. The method usually employs allele fragments, approximately 400 to 600 bp, rather than the whole gene, due to historical and practical reasons [171]. The maintenance of curated, web-accessible databases is an important part of MLST schemes [170].

Currently, three MLST schemes for *E. coli* can be found online; Pasteur (France) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi), Warwick (https://enterobase.warwick.ac.uk/) and at Michigan State University, USA (http://www.shigatox.net/ecmlst/cgi-bin/index). An effort to determine correspondence of these three MLST schemes was reported by Clermont et al [152]. For assigning STs in the papers of this thesis, sequences were submitted to the Warwick MLST database for *E. coli* (at that time to http://mlst.ucc.ie/mlst/dbs/Ecoli).

There is a universal nomenclature scheme for storing and interpreting MLST data. In most MLST schemes, seven loci are indexed, for which each unique sequence for each locus is assigned an arbitrary and unique allele number in order of discovery. The designations for each of the loci are incorporated into an allelic profile (for example, 2-3-4-3-8-4-6) thus summarizing information from thousands of base pairs in a string of digits [172]. The allelic profile represents a sequence type (ST), which is assigned a numerical designation, for example ST11. Clonal complexes (CC) are groups of closely related but not identical isolates that are likely to come from a recent common ancestor [171]. Currently, a clonal complex is designated by defining a central sequence-type and related sequence-types, i.e. those that share up to four identical MLST loci. Naming of the CC is often derived from the ST regarded as the ancestor, or the dominating ST within this CC.

One major advantage of MLST is that it is nucleotide sequence based. Also, the method does not require access to specialized reagents or training and, in addition, the results are highly reproducible, and can easily be exchanged and compared between different laboratories. The web-based databases make international sharing of the results possible, and also enables a worldwide overview of the distribution of strains simply by tracing their STs [155]. Although an ST represents only a tiny percentage of the conserved parts of the genome, the large number of STs in many bacterial populations demonstrates that as few as seven housekeeping loci provide enough discriminatory power to compose a representative sample of the entire genome. In this respect MLST has successfully described population diversity and structure for a wide range of bacteria, thus demonstrating phylogenetic relationships for several bacterial lineages. However, polymorphisms in the slowly evolving housekeeping genes, which are its targets, may not be differentiating enough for useful epidemiological comparisons over a short time period, as in most outbreak investigations. Thus, MLST is mainly suitable for comparing relatedness of strains on a global scale over longer periods of time [170-172].

An important limitation with conventional MLST is that it is time consuming and that the costs have been high because of the need for DNA sequencing [155], which limits its power for genotyping large numbers of samples. This, however, could be overcome by using a high-throughput MLST (HiMLST) method and by employing next-generation sequencing (NGS) technology, to generate large quantities of high-quality MLST data [173]. Furthermore, MLST has been used for analyzing a large number of species, resulting in the identification of major STs and CCs of clinical relevance, for instance *E. coli* ST131 [117]. Therefore, the MLST approach, primarily in combination with NGS protocols, will probably still be considered an important part of epidemiological typing, including also ribosomal MLST (rMLST), and core genome MLST (cgMLST).

3.4.27 Cluster analysis for typing methods used in this thesis

The ability to rapidly and reliably differentiate among related bacterial isolates is essential for epidemiological surveillance [117]. Results from the varying typing techniques for surveillance of microbial variability and also outbreak detection, as described in the previous sections, need to be presented in a comprehensible way to facilitate interpretation. By using cluster analysis, data

can be divided into groups of isolates that are similar or dissimilar from each other.

The two most used analyses are hierarchical clustering and partitional clustering [174].

3.4.28 Hierarchical clustering

In hierarchical clustering, clusters and subclusters are organized as a tree (dendrogram). Each cluster in the tree is the union of its subclusters and the root of the tree in includes all the objects. A dendrogram constructed with UPGMA (unweighted pair-group method) is a common approach to compose a phylogenetic tree from a distance matrix. For this thesis the PFGE band patterns were analysed, using the BioNumerics software version 6.6 with the Dice coefficient for calculating pair-wise similarities, and the UPGMA algorithm for constructing dendrograms of estimated relatedness. Position tolerance and optimization were set at 1.0. Bands within the size range of78-453 kb were included in the analysis as shown in Figure 11.

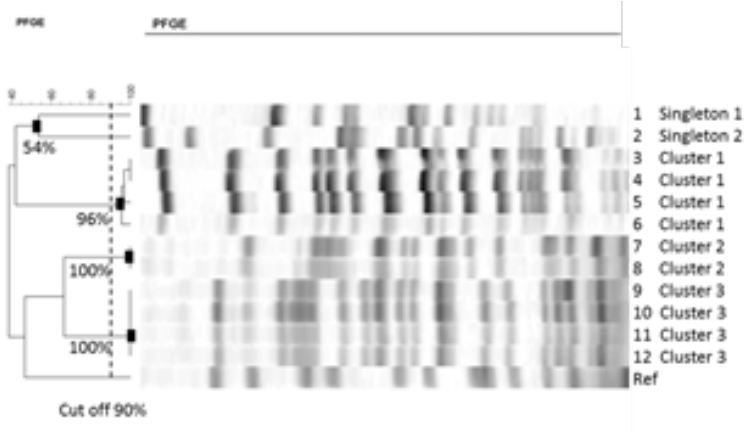


Figure 11. PFGE-fingerprint image and cluster analysis of ESBL-E. coli using BioNumerics software and the UPGMA algorithm and dice coefficient for calculating pair-wise similarities.

3.4.29 Partitional clustering

Partitioning clustering means dividing a set of strains into non-overlapping clusters, so that each data object is found in only one cluster. The data needs to be of categorical character, i.e. data sets such as MLST, spa-typing or MLVA. An example of partitioning clustering is eBURST. In the eBURST

algorithm there is an anticipated founder within the clonal group, and related strains sharing five or six loci [171, 175]. For *E. coli* Minimum Spanning Tree (MST) is commonly used for partitional clustering today. The MST method uses the distances between STs based on the number of differences in their allelic profiles. A tree is constructed that minimizes the total distance between all STs included in the analysis. MST was also used to form MLVA clonal complexes with up to 4 locus variation by Naseer et al [169].

We have used the MST algorithm on the website (http://enterobase.warwick.ac.uk/) to form an MST tree from the MLST data of the isolates in paper III, and the interrelationship is presented graphically in Figure 12. The digits between STs show the number of allelic differences between neighboring STs. Clonal complex sharing at least six loci of their allelic profiles with at least one other member are encircled, i.e. the four *E. coli* Clonal complexes (Cplx) 10, 12, 155 and 354, of which the major cluster, Cplx 10, includes the following STs; 10, 43, 48, 167, 239,617,694 and 1284.

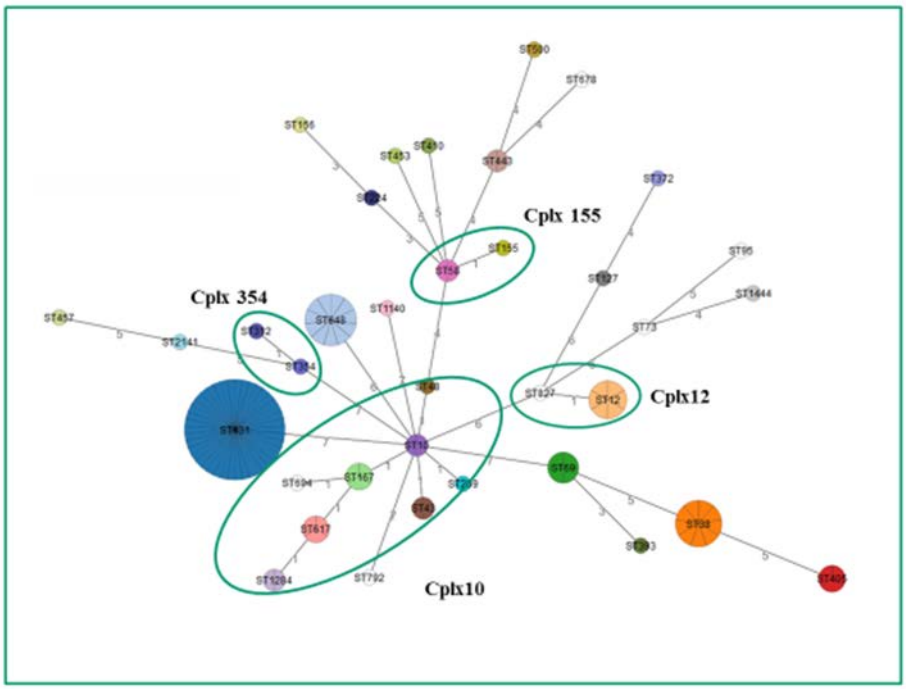


Figure 12. Minimum spanning tree (MST) and E. coli Clonal complexes (Cplx) using MLST data of the E. coli isolates in paper III.

4 RESULTS AND DISCUSSION

4.1 The emergence of ESBL-*E coli* in the Gothenburg area (paper I)

A brief introduction of the ESBL-*E. coli* and CTX-M epidemiology globally and in Sweden will introduce this section as a background before presenting the findings in paper I.

4.1.1 Introduction to the epidemiology of ESBL-*E. coli*

Since the emergence of EPE in the 1990s there has been a steady increase worldwide, and ESBL-*E. coli* has become the major player within the group. Data on colonisation rates in the healthy population has been outlined in the Introduction section. Proper surveillance data on clinical cases are missing in many countries, which is highlighted by WHO in the global action plan against antimicrobial resistance [9]. Data are especially scarce from low-income countries, where rates may be high. Therefore, a proper estimate of the EPE global prevalence can not be made. The highest rates are presently seen in India, China, South-Asia, Mexico and a few African countries that provide data, according to estimates made by Center for Disease Dynamics Economics & Policy [176]. The SENTRY Program recently launched a new report on the resistance in *Enterobacteriaceae* isolated in 199 hospitals from 42 countries worldwide over 20 years [177] and detected the same trend in all countries: Resistance to third generation cephalosporins had increased from 10.3% to 24.0% during these 20 years, and ESBL-*E. coli* rates increased from 3.3% to 15.8%. The increase was also accompanied by an increase in MDR rates, as well as resistance to carbapenems (Figure 13).

In Europe the EARS-Net/EARSS (European antimicrobial resistance surveillance network/ system) surveys resistance rates in invasive isolates since the beginning of the 2000s and for the last decade these are summarised in yearly reports. Rates reported from 2009 and 2017, respectively, are shown in Figure 14 [7, 178].

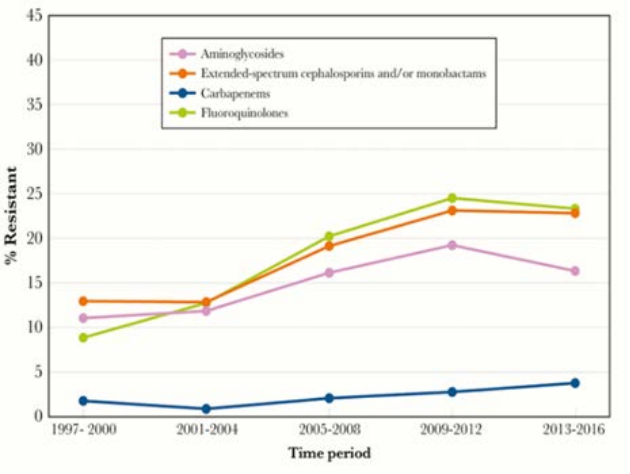


Figure 13. Antimicrobial resistance trends for selected antimicrobial classes in all Enterobacteriaceae. Reprinted from Castanheira et al.[177] with permission from Oxford University Press.

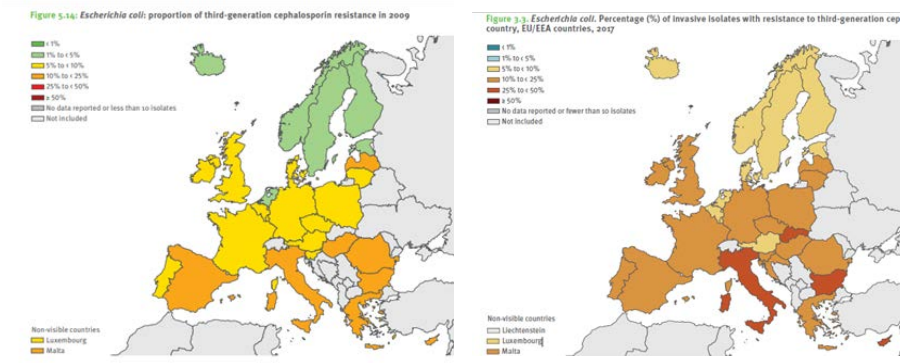


Figure 14. Resistance to third-generation cefalosporins in invasive E. coli in 2009 (left) and 2017 (right) in EU/EEA countries according to the EARS-Net/EARSS network [7, 178].

4.1.2 The global epidemiology of CTX-M

Since the turn of the millennium the CTX-M enzymes have emerged globally [179]. The origin of CTX-Ms is mobilization of chromosomal beta-lactamase genes from *Kluyvera* species, by incorporation onto plasmids [180]. The first CTX-M was in 1989 [72], as outlined in the general introduction. The most common ESBL globally, i.e. CTX-M-15, was first detected in India in 1999 [181], but it was not until the first decade of the 2000s that the CTX-M beta-

lactamases became predominant globally [179]. In Sweden, the first CTX-M beta-lactamases were detected at the beginning of the 2000's [123]. In our region the first clinical cases occurred in 2003 (paper I).

The CTX-M enzymes can be assigned to one of five (six) groups. Each group is named after the first described member. The number of different genes within each group varies considerably, and with the detection of new CTX-M enzymes new types have been added to the groups. There are two classification systems, as described in the method section, but that of Bonnet et al [125] is mostly used. The most prevalent CTX-M genes within the respective group is outlined in Figure 15.

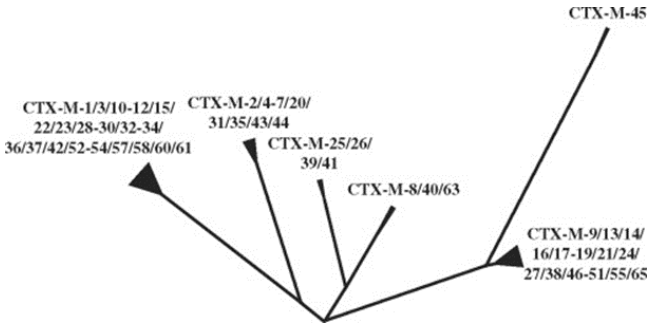


Figure 15. The five (six) known groups of CTX-M-type beta-lactamases. Reprinted from Rossolini et al. [182] with permission from Science Direct.

Though CTX-M-15 are found globally and dominate among ESBLs there are geographical differences in the distribution of these enzymes, both within Europe, as well as on a worldwide scale. However, an increase for CTX-M-15 is reported for most countries, and is currently dominant in most regions [83]. In Europe, the majority of the enzymes belong to the CTX-M-1-group (CTX-M-3 and -15) and the CTX-M-9-group (CTX-M-9 and -14). In China, Japan and South East Asia CTX-M group 9 variants, especially CTX-M-14, are found, while reports from India indicate that CTX-M-15 is common [183]. Until 2007, reports of isolates producing CTX-Ms were rare in the USA, rather, TEM and SHV types dominated. However, from 2007 CTX-M-15 and -14 have been reported [184]. In Canada, CTX-M-15 is also the most frequent CTX-M. In South America there is a particular domination of CTX-M-2, as has been the case since the start of the CTX-M pandemic [83].

In Figure 16 the CTX-M distributions at the time of paper I are demonstrated. Since then the picture has non-surprisingly become more diverse, as described

by Bevan et al [83]. In the early reports of CTX-M epidemiology the studies were often small and there are only limited data from under-resourced countries.

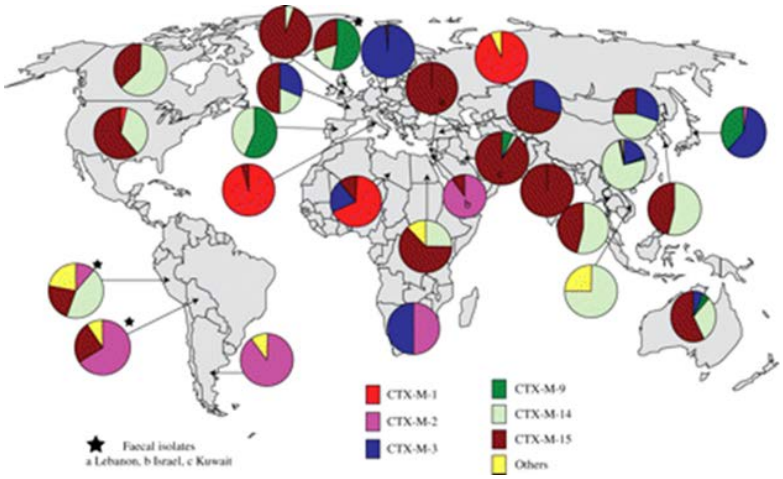


Figure 16. Global distribution of CTX-M. Reprinted from Hawkey et al [184] with permission from Oxford University Press.

CTX-Ms are traditionally considered to be plasmid-mediated but can nowadays also be seen incorporated into the chromosome [131]. These beta-lactamases can be found on a range of plasmids, mostly those belonging to replicon types IncFII, IncN and IncI1 [58]. CTX-M-15 is often carried on highly transmissible plasmids that have become epidemic, which has contributed to the successful global emergence of CTX-M-15 as compared to other CTX-Ms. In addition, the CTX-M-15 genes frequently co-exist on the plasmids with genes conferring resistance to other classes of antibiotics, like fluoroquinolones and aminoglycosides, which leads to co-selection [185], further favoring the emergence of CTX-M-15 in addition to its link to the successful ST131 linage.

4.1.3 The epidemiology of *E. coli* of sequence type 131 and its multi-resistant sublineages

The clonal spread of virulent strains is important for global dissemination of antibiotic resistance. Mathers et al. [29] have summarized important requirements for an international multidrug-resistant high-risk clone:

- It has a global distribution
- It harbours antimicrobial resistance mechanisms
- It is adapted to colonising a human host
- It can be transmitted among human hosts
- It has enhanced pathogenicity
- It is capable of causing severe infection

E. *coli* of sequence type 131*(E. coli* ST131) fulfill all these requirements. The ST131 lineage was originally identified in 2008, although retrospective studies suggest that it may have been of importance as early as 2003 [186-188]. Today, ST131 is the predominant *E. coli* lineage among ExPEC isolates worldwide [130]. It is recognized as a worldwide pandemic clone, causing predominantly community onset infections, mostly associated with urinary tract and bloodstream infections [189].

It has been proposed that the ST131 acquired virulence factors during the 1960s to 1980s, and this was followed by its global success in the 1990s to 2000s. It has been suggested that the dramatic transmission of this linage was the result of acquisition of fluoroquinolone resistance and, subsequently, ESBL production, at the same time as these antibiotic substances were widely adopted for clinical use and therefore became the driving forces for selective pressure [53].

The majority of the ST131 strains belong to serotype O25:H4, with a specific O25b type. This sublineage, often named ST131-O25b, has obtained considerable attention over the years due both to its virulence potential, as it belongs to phylogroup B2, as well as its increasing resistance. Today, most interest is focused on the resistant sublineages of ST131-O25b, that is, the subclones H30R and H30Rx, associated with fluoroquinolone resistance and the latter also with CTX-Ms, especially CTX-M15 [53, 131]. These sublineages have been delineated in recent years with use of WGS. Clade C (H30) of ST131 is currently the most dominant clade globally and includes the sublineages C1 and C2. C2 corresponds to H30Rx while C1 (H30R) is often negative for ESBLs. Other CTX-M genes also occur in ST131 [189], particularly blaCTX-M-14/14-like variants, for example, in Canada, China,

and Spain [130] In Japan, where CTX-M 15 has been rare, ST131 with CTX-M 27 has expanded within a novel global C1 subclade named C1-M27 due to its associated with CTX-M-27 [190]. The C1-M27 subclade was recently reported also to emerge in Europe by Merion et al [191], reflecting that the ESBL-pandemic is continuously changing.

However, ST131 *E. coli* isolates with ESBLs of serotype O16:H5 have also been identified. [53]. These isolates are generally less resistant to fluoroquinolones but often resistant to gentamycin and trimethoprim-sulfamethoxazole [192].

There are also other ExPEC clones, such as ST405, ST695, ST405, ST354, and ST28, reported to carry CTX-M including CTX-M-15 [186]. However, these clones have not been transmitted as successfully as ST131 subclade C. The reasons for this remains unclear, but it has been suggested that coevolution of the C1 and C2 subclades with the plasmids harbouring multiple resistance determinants has played an important role for the spread of this pandemic clone [53].

4.1.4 The epidemiology of ESBL-*E. coli* in Sweden

Historically, surveillance data has indicated that Sweden, just as our Nordic neighboring countries, have a low background prevalence of resistant organisms [178]. There is no data on the prevalence of ESBL-*E. coli* in Sweden for the first study period of paper I (2003). The SWEDRES-report from 2004 [193], containing antibiotic resistance surveillance data from Sweden, shows that in 2004 EPE were rare, only 0.5% of invasive *E. coli* isolates reported to the EARS-Net/EARSS were resistant to third generation cephalosporins. In 2008 this had increased to 1.9%, and in the last available report for 2017 the frequency was 7% [6, 194]. There has been a continuous national surveillance also for urinary *E. coli* isolates in Sweden for many years summarized in the yearly SWEDRES-reports. In 2004 only very, few isolates were resistant to cefadroxil, which is used as an indicator of ESBL presence. In 2008 the frequency increased to 2.4%, and by 2017 it was 6.1 %. Since February 2007, it has been mandatory for clinical laboratories in Sweden to report EPE according to the Communicable Diseases Act. The national incidence in 2017 was 100 cases per 100 000 inhabitants, as compared to 32 cases per 100,000 inhabitants in 2008 (Figure 17).

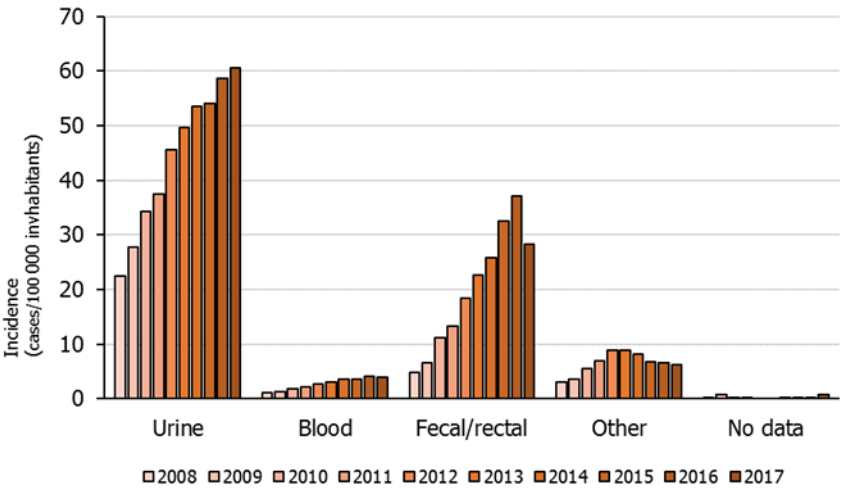


Figure 17. Incidence (cases/100 000 inhabitants) per sample type of notified human cases of ESBL-producing Enterobacteriaceae (EPE) in Sweden 2008-20017. Adopted from data in Swedres [6].

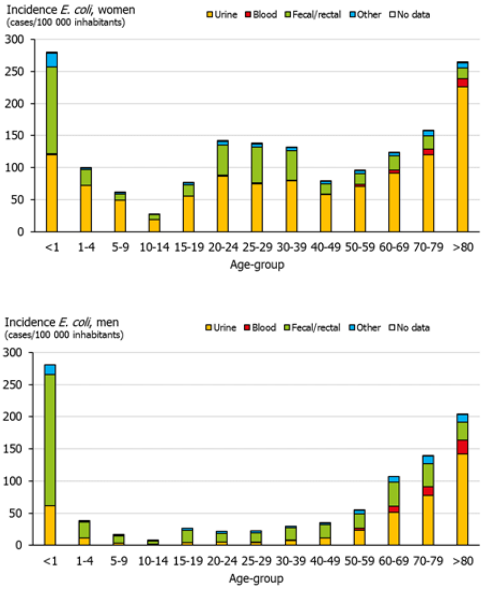


Figure 18. Incidence (cases/100 000 inhabitants) in women and men per sample type of notified human cases of ESBL-E.coli in Sweden 2008-20017. Adopted from data in Swedres [6].

The gender distribution has not changed significantly since surveillance started, with a ratio of 1:2 in favor of women (Figure 18). From the start of the mandatory reporting, *E. coli* has been the most commonly reported species (86% in 2017), and most often detected in urine samples (61% in 2017).

The distribution of genotypes in Sweden and the other Nordic countries is uniform. Genes from the CTX-M-1 group and CTX-M-9 group have been in majority since the first national report [195]. The CTX-M-15 beta-lactamase, belonging to the CTX-M-1 group, dominates in Sweden, with 50–60% of ESBL-producing *E. coli* harboring CTX-M-15. This is followed by blaCTX-M-14, belonging to the CTX-M-9 group.

4.1.5 Results and discussion (paper I)

To be able to follow resistance trends and predict potential outbreaks there is a need for local surveillance data. In paper I we describe the emergence of EPE in the greater Gothenburg area on the southwest coast of Sweden over a five-year period, starting from the first detected clinical cases in the autumn of 2003. Since then ESBL-*E. coli* has become the most frequent MDR bacteria detected in our area, predominately in *E. coli* causing UTI. Therefore, the focus of paper I is on the epidemiology of UTI caused by ESBL-*E. coli*.

All consecutive isolates from clinical ESBL-*E. coli* isolates referred to Sahlgrenska University hospital during two time-periods, for 20 months 2003-2005 (n=41) and 12 months 2008-2009 (n=221), were compared. The isolates were characterized for CTX-M, TEM, OXA and SHV genotypes, resistance profile, and selected representative isolates were typed with PFGE and MLST. For further details se paper I.

4.1.6 The emergence of ESBL-*E coli* in BSI cases

The first clinical BSI case was not detected until 2007, and cases continued to be rare during the entire study period. In a recent study [93] from our region approximately 160 bacteremia cases were detected during 2004-2104, primarily in older patients and with no gender differences. In analogy with national and global data, the rate has continuously increased, and at present the frequency of ESBL-*E. coli* among *E. coli* bacteremia cases is 8% at the Clinical Microbiology Laboratory serving the Sahlgrenska University hospital.

In Canada the emergence of EPE bacteremia coincided with the increase of community acquired bacteremia cases caused by the ST131-O25b linage

[188]. We have no continuous data for this clone causing bacteremia in our region at present.

4.1.7 The emergence of ESBL-*E coli* and ST131-O25b linage in UTI cases

For the first study-period the majority of cases were detected in women from the community setting (Table 1, paper I). For the latter period there was an increase in cases, particularly among women in the community, similar to the national trend. Men were few even in the latter period, approximately 20% of all cases and equally distributed between hospital and community settings. However, there was a trend towards a higher increase for men and the elderly over time, although this was not significant. In a recent study from our region it was also reported that men in the age-group 18-65 years are affected by recurrent UTI due to ESBL-*E. coli* more often than women of the same age-group [93].

During the later period we identified an emergence of cases from the hospital setting constituting over a third of the cases (Figure 19 below, and Table 1, paper I). This trend has continued since then according to our local data (unpublished). In the hospital setting, the gender distribution in paper I was approximately equal, and in the later period almost half of the patients belonged to the age group >65 years. The number of cases in this study are not enough to generate significant conclusions. Nevertheless, this finding is in line with more recent studies reporting that clade C of the ST131-O25b clone is predominantly detected in elderly patients in the hospital setting [196].

The emergence of ESBL-*E. coli* in urinary isolates has been accompanied with the emergence of ST131-O25b *E. coli* with ESBL in our region. For 2003-2005, the detected ST131-O25b isolates were very few and only found among women in the community setting. In the later period there was an increase to 24% of all ESBL-*E. coli*, which is lower than other studies from the same period, for instance that of Pitout et al. [188], reporting a frequency of approximately 40% in Canada in 2004-2007. However, these isolates were detected from patients with BSIs. However, the number of isolates in our study is small and numbers in early studies may be difficult to compare due to

inconsistency in typing and nomenclature of ST131-O25b as outlined in paper III.

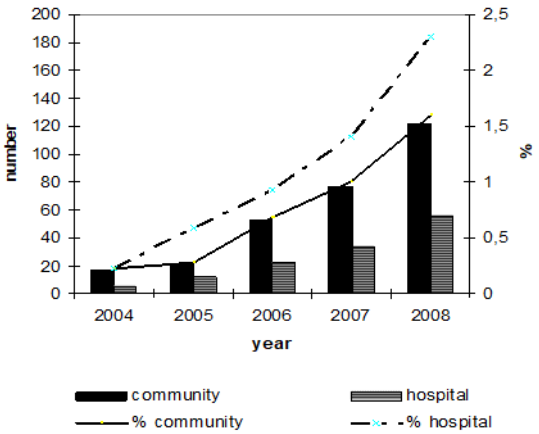


Figure 19. The emergence of ESBL-E. coli among urinary isolates from patients in the community and the hospital setting in the greater Gothenburg area from 2004-2008.

In the later period, 26% (39/148) of the isolates from the community setting belonged to the ST131-O25b clone, the corresponding number for the hospital setting was 20% (13/64), with no noticeable gender differences (Table 1, paper I). At the time of the study the various ST131-O25b clades described above had not been described. However, considering the resistance profiles of the isolates there is reason to believe that several of these isolates might belong to clade C, i.e. H30R and especially H30Rx.

However, the emergence of this clone did not account for the whole increase of ESBL-*E. coli* in our area. For ESBL-*E. coli* that were not part of this clone 26 and 59 different PFGE-types were detected among isolates selected for typing in the early and in the late period, respectively, most were singeltons. If all isolates had been tested the number of detected PFGE-types is likely to have increased. In addition, those isolates analysed by MLST also displayed variety, further supporting the suggestion that the increase for ESBL-*E. coli* was polyclonal. More detailed data on circulating strains during 2008-2009 is described in paper III.

4.1.8 The emergence of CTX-M among urinary ESBL-*E. coli*

In early reports from other countries it was ESBLs of TEM and SHV types that were initially detected. Contrary to this, the CTX-M enzymes dominated already from the start of the ESBL transmission in our region, 88% (36/41) of

the detected ESBL-*E. coli* in the early period carried CTX-M type genes. This applied also to the later period, when the corresponding number was 98% (216/221). These findings are in agreement with studies from Sweden and other Nordic countries at the time [197].

One of the most interesting findings in paper I was the shift in CTX-M profiles during the study period. In the early period, there was an equal distribution between the CTX-M-1 and CTX-M-9 group, but in 2008-2009 we detected a significant shift ($p<0.02$) in favor of the CTX-M-1 group, from 49% (20/41) in the early period to 75% (166/221) in the late period. This latter prevalence is in line with other contemporary studies in Sweden [195, 198, 199]. In the hospital setting the dominance of the CTX-M-1 group was even more prominent, increasing from occasional cases to 86% of all ESBL-*E. coli* with CTX-Ms. Although CTX-M group 1 enzymes, and particularly CTX-M-15, dominate worldwide, there are clearly local variations in CTX-M-types, as a result of locally circulating ESBL-carrying plasmids and establishment of various bacterial clones. Shifts in predominating ESBL-genes has also been seen in other regions, such as Canada and Spain [179, 200]. Also, presently the CTX-M-27 beta-lactamase is emerging in Japan and has also reached Europe, as outlined above.

When CTX-Ms first started to emerge in our region, the CTX-M-1-group was the only detected type for isolates of the ST131-O25b linage. These enzymes still dominated in this clone by 2008-2009, (90%, 47/52), but also CTX-M-9 group enzymes were detected. Interestingly, the CTX-M-27, now emerging globally, was detected in the ST131 clone during the later study period in our region, although we do not know to which clade these isolates belong.

All isolates in our study were typed to the CTX-M group. Partial sequencing to assign specific CTX-M genes within the respective group was performed for selected isolates, for instance all ST131-O25b isolates in our study were found to carry CTX-M-15. Considering the ongoing CTX-M epidemiology at the time, there is reason to believe that CTX-M-15 enzymes would be frequently found also in non-ST131-O25b isolates in our collection carrying CTX-M-1 group genes, but this remains to be clarified.

4.1.9 The emergence of co-resistance in ESBL-*E. coli*

The antibiotic resistance profile for the studied isolates is shown in Table 3. In 2008-2009, 79% of the ESBL-*E. coli* isolates were not sensitive to ciprofloxacin, 50% not to tobramycin and 80 % not to trimethoprim. The most prevalent resistance combinations were those of ciprofloxacin + trimethoprim, and ciprofloxacin + trimethoprim + tobramycin. These resistance levels are in line with what has been

reported on a national level for the same time-period [193, 194]. The resistance levels in ESBL-*E. coli* have remained fairly unaltered over the years, although increasing resistance to tobramycin is being noticed, especially in MDR ESBL-*E. coli* according to our local surveillance of resistance rates.

For the ST131-O25b clone, resistance to fluoroquinolones, such as ciprofloxacin, is well known. In paper I, the highest resistance rates for ciprofloxacin was seen in isolates belonging to the ST131-O25b genetic lineage, 93% (50/54, later period, Table 3). This was not surprising, since this clone is known to be ciprofloxacin resistant, especially the H30R sublineage, comprising clone C. When resistance rates were compared between isolates with ESBL of different CTX-M groups, those with CTX-M-1 group ESBLs were ciprofloxacin resistant more often than those with CTX-M-9 group enzymes, 85% and 70%, respectively. They were also significantly more often MDR than those with CTX-M-9 group enzymes, 66% (77/116) as compared to 22% (10/46), p<0,004).

Table 3. Resistance profiles for ciprofloxacin (CIP), tobramycin (TOB) and trimethoprim (TMP,) in in relation to type of ESBL for urinary ESBL-E. coli isolates from the respective study periods.

	2003-2005			2008-2009		
not sensitive (I/R to	CTX-M-1 group n=20	CTX-M-9 group n=16	Other ESBL n=5	CTX-M-1 group n=166	CTX-M-9 group n=46	Other ESBL n=9
CIP only	1 (1) [a]	2	-	16 (4)	5 (1)	1
TMP only	1	2	-	13 (1)	10	4 (2)
TOB only	-	1	-	-	-	-
CIP+TMP	4	6	4	41 (8)	17 (4)	-
CIP+TOB	4 (1)	1	-	8 (5)	-	-
TOB+TMP	-	-	-	3 (1)	-	-
CIP+TMP+TOB	10 (2)	2	-	77 (28)	10	2
None of CIP+TMP+TOB	-	2	1	8	4	2

[a] Numbers within parenthesis correspond to the ST131-O25b sublineage

4.1.10 In conclusion

This study describes the emergence of ESBL-*E coli* in our region, firstly in the community setting and later on in the hospital setting. The distribution of cases

according to gender and age was in accordance with the distribution of UTIs in the population, which was to be expected as most EPE-cases originate from UTIs caused by *E. coli*. There was a trend towards a higher increase for men and in the elderly, although this was not significant. The ST131-O25 ESBL-*E coli* linage was established in our region during this period and constituted almost one quarter of all urinary cases by the end of the study. For the remaining isolates the emergence of ESBL-*E coli* was polyclonal. We saw a clear shift in the type of ESBLs during the study in favor of the CTX-M-1 group, and with increasing co-multidrug resistance in isolates with this gene. The study clearly demonstrates how the epidemiology for ESBL-*E. coli* can change over time, and that important shifts of both ESBL-*E. coli* clones and ESBL genes might occur. Thus, this warrants continuous surveillance of EPE epidemiology at the local level.

4.2 Evaluation of various typing methods for ESBL- *E. coli* to track possible routes of transmissions in an outbreak situation (paper II)

The polyclonal EPE-outbreak that was part of paper II and III has not been published in its full extent, therefore it will be outlined briefly in this thesis.

4.2.1 The polyclonal EPE-outbreak in a neonatal unit

"Outbreak" is a term used in epidemiology to describe an occurrence of disease greater than would otherwise be expected and has the following definition according to WHO:

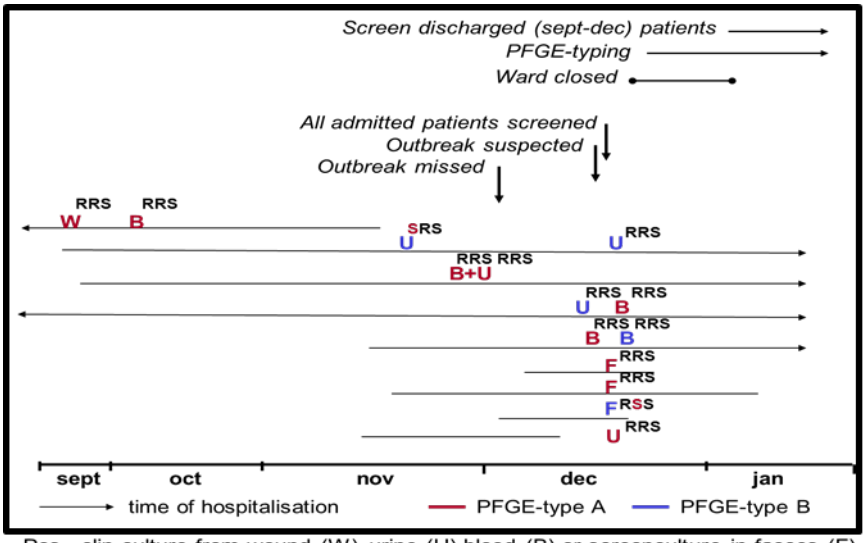
"A disease outbreak is the occurrence of cases of disease in excess of what would normally be expected in a defined community, geographical area or season. An outbreak may occur in a restricted geographical area or may extend over several countries. It may last for a few days or weeks, or for several years".

Investigation of outbreaks due to MDR bacteria, especially within the hospital setting, differ from the general concept of outbreak investigation when an external contaminated source, such as food-products, water sources etc., is suspected to cause accumulation of diseased persons. In these cases, a case-control study is often initiated to solve the problem [201]. This is rarely the case for MDR bacteria outbreaks within health care institutions. Here the source is most often human carriers, patient(s) or, occasionally, staff. External

sources of EPE-outbreaks have been described, for example from poorly disinfected endoscopes [202], but are generally rare. Importantly, asymptomatic carriage must also be taken under consideration in these investigations. There are numerous studies describing substantial carriage in co-patients once an outbreak is revealed [203, 204], similar to the findings in the outbreak described in paper II.

In hospitals, outbreaks due to MDR bacteria are suspected when a deviation is observed in the baseline endemic isolation frequency of these bacteria. The awareness of clinical, laboratory or infection control staff is often crucial to bring attention to this deviation from the expected isolation frequency. With rare pathogens, only a single isolate might be enough to start an outbreak investigation, since none of these pathogens is expected. For more common pathogens, such as EPE, a higher isolation frequency is generally needed before a deviation is noted, and accumulation within a restricted unit or in time may thus be important. High community prevalence of a pathogen, with influx of cases to the hospital setting, poses a further challenge in order to evaluate if a pathogen was acquired in the hospital setting or not.

The outbreak described in paper II took place in 2008 in a neonatal ward at Sahlgrenska University hospital. At the time of the outbreak, descriptions of EPE-outbreaks were relatively rare in the literature, and the polyclonal nature of these types of outbreaks was mostly unknown. The outbreak was finally revealed in late December 2008, and from retrospective analyses of cases in the ward the index case was diagnosed already in early September (Figure 20). In late-November, two clinical *E. coli*-ESBL cases were noted within the same week from this ward. This opened for a suspicion of ongoing transmission, but since these isolates deviated in antibiotic resistance profile no outbreak investigation was initiated until additional cases occurred in late December. As demonstrated in Figure 20, several of the infected children had been hospitalised for many months during the autumn preceding the detection of the outbreak, which probably facilitated the ongoing silent transmission.



Pos clin culture from wound (W), urine (U) blood (B) or screenculture in faeces (F)
RRS , SRS etc : resistans pattern for in turn tobramycin, trim-sulfa, ciprofloxacin

Figure 20. Time of hospitalisation and culture results in infected hospitalised children when the outbreak was revealed.

PFGE		resistance pattern	Index	Case I	Case II	Case III	Case IV	Case XI	Case XVIII
A	E.coli	RRS	W,B	F	U,B	B.F,W	B		
A		SRS		F					F
A		RRS		St		Nose			
B		SRS		U					
B		RRS		U			B		
B		SSS							F
B		RSS				U		F	
D		RSS				F			
	K.pn.	SRS		St,F	F,U			F	

Pos clin culture from urine (U) blood (B) or screen culture in faeces (F) or stoma (St) First found isolate in colour
RRS , SRS etc : resistans pattern for in turn tobramycin, trim-sulfa, ciprofloxacin

Figure 21. Patients part of the EPE-outbreak infected with multiple ESBL-producing E. coli and Klebsiella pneumoniae (K.pn.) of various PFGE-types and resistance profiles.

Once the outbreak was revealed, the ward was closed to new admissions, and in addition other measures were taken. No new cases infected with the outbreak strains occurred after this point. Altogether 125/169 patients hospitalized from September to December were screened for carriage of EPE, revealing 23 cases. The ward reopened in January, and an extensive screening program of admitted and hospitalised patients was initiated, for as long as infected children were still cared for. This screening included admission screening for all admitted children and twice weekly screening of children during their entire hospitalisation period unless they already were known carriers of an EPE-strain. The screening continued until June 2009, and by then more than 900 screening samples were obtained.

Altogether 21 children were considered part of the outbreak. Several children were repeatedly positive and six of them were found to carry different EPE-strains, even strains of the same PFGE-type but with different resistance profiles (Figure 21). This was indeed challenging during the outbreak investigation but was also an important knowledge for the future. From an outbreak investigation perspective, we can conclude that;

- Outbreaks due to EPE may be polyclonal, thus, there is a need to react also on resistance type and not only on bacteria.
- All clinical infections, including reinfections, must be monitored on a ward and all, even repeated, isolates must be saved to enable future analysis if needed.
- Resistance pattern is not a reliable tool for predicting possible transmission.
- If transmission has been ongoing for an extended period of time several types of EPE-strains may be detected, also in the individual patient.

4.2.2 Results and discussion (paper II)

The ability to differentiate isolates with high resolution methods is essential in order to define the strains that are transmitted in a local outbreak from those strains occurring as sporadic infections or through asymptomatic carriage in hospitals and the community setting. This is particularly important for hyperendemic strains, such as ST131-O25b. In addition, when investigating an already established outbreak, and during the subsequent follow-up period, enhanced resolution is important to confirm isolate identity or nonidentity, to be able to ascertain that the outbreak has ceased.

In paper II we evaluated both the GECM-7 and the GECM-10 protocol for typing ESBL-*E. coli*, with the aim to evaluate if MLVA could replace the PFGE method for outbreak investigation in the routine clinical laboratory setting. More precisely, we wanted to establish if the generic *E. coli* MLVA was able to detect ESBL-*E. coli* isolates that were epidemiologically related, and also separate those isolates from sporadic ESBL-*E. coli* detected in children hospitalised during the follow-up period, when infected children still were cared for in the ward and there was a possibility that transmission still might have occurred. Simultaneously, we also evaluated MLST, ESBL-gene typing and antibiotic resistance profile for detection of ESBL-*E. coli* transmission. The outbreak was particularly suitable for these purposes, considering the well characterised epidemiology and the rarity of other sources of ESBL-*E. coli* exposure for these children. Also, it included a substantial number of *E. coli* outbreak isolates.

4.2.3 Overall typing results of ESBL-*E. coli* isolates detected in the outbreak investigation

The 50 isolates studied originated from 28 children and were sampled during the outbreak and the subsequent follow-up period. At the time, 41 *E. coli* isolates from 21 children were considered part of the actual outbreak. The remaining nine isolates from nine children detected in screen samples at admission were regarded as sporadic cases carried by the children prior to submission. Two cluster of isolates containing 35 and 6 isolates, respectively, and with distinct PFGE types (A and B), corresponding respectively to G_{10}06-04 and G_{10}07-02, and ST 131 and 1444, were considered to be part of the outbreak. Thus, this outbreak harbored ESBL-*E. coli* of at least two types, of which the PFGE type A belonged to the ST131-O25b lineage. For summery of typing results for the 50 isolates see Table 4 below including results from table 2 and figure 1 and 2 in paper II and data from reference [205]

*Table 4. Overall typing results for the 50 ESBL-E. coli isolates detected during the investigation and subsequent follow-up period of the polyclonal EPE outbreak. *) only selected isolates within the group were tested*

PFGE-type	isolates (patients)	GEM-10 code (repeats)											ESBL-gene			shared plasmid*	resistance pattern		
		CVN '001	CVN '002	CVN '003	CVN '004	CVN '007	CVN '0014	CVN '0015	CCR '001	CVN '0016	CVN '0017	G10type	MLST ST	ST131-O25b*	H30Rx	CTX-M	OXA	TEM		TOB	TMP	CIP
A	25(15)	6	N	N	14	3	6	4	2	N	N	G$_{10}$06-04	131	+	-	CTX-M-15	OXA-1	TEM-1b	+	R	R	S
A	8(5)	6	N	N	14	3	6	4	2	N	N	G$_{10}$06-04	131	+	-	CTX-M-15		TEM-1b		S	R	S
A	2(2)	6	N	N	14	3	6	4	2	N	N	G$_{10}$06-04	131	+	-	CTX-M-15		TEM-1b	+	R	R	S
B	1	7	N	N	11	3	6	4	2	0	N	G$_{10}$07-02	1444			CTX-M-15	OXA-1		+	R	S	S
B	1	7	N	N	11	3	6	4	2	0	N	G$_{10}$07-02	1444			CTX-M-15	OXA-1	TEM-1b		R	R	S
B	1	7	N	N	11	3	6	4	2	0	N	G$_{10}$07-02	1444			CTX-M-15		TEM-1b		R	R	S
B	2(1)	7	N	N	11	3	6	4	2	0	N	G$_{10}$07-02	1444			CTX-M-15		TEM-1b		S	R	S
B	1	7	N	N	11	3	6	4	2	0	N	G$_{10}$07-02	1444			CTX-M-15				S	S	S
C	2(1)	6	N	N	14	3	11	4	2	N	N	G$_{10}$06-01	131	-	ND	CTX-M-14		TEM-1b		S	R	S
D	1	6	N	N	9	3	5	4	2	N	N	G$_{10}$06-05	73			CTX-M-15	OXA-1		+	R	S	S
E	1	5	0	N	13	3	6	4	2	0	N	G$_{10}$05-01	405			CTX-M-15	OXA-1			R	R	R
F	1	5	12	N	11	3	8	4	2	N	N	G$_{10}$05-02	43			CTX-M-15	OXA-1	TEM-1b		R	R	R
G	1	7	N	N	11	3	10	4	N	N	N	G$_{10}$07-01	69			CTX-M-15				S	R	R
H	1	5	2	N	13	3	6	4	2	9	N	G$_{10}$05-03	1177			CTX-M-15		TEM-1b		S	S	R
I	1	6	N	N	14	3	6	4	2	0	N	G$_{10}$06-03	131	+	ND	CTX-M-15	OXA-1			R	S	R
J	1	6	N	N	14	3	5	4	2	0	N	G$_{10}$06-02	131	+	ND	CTX-M-15	OXA-1			R	R	R

4.2.4 Detection of transmission of ESBL-*E. coli* isolates by comparing PFGE-patterns

In this outbreak investigation we used PFGE for strain-typing, since it was considered the gold standard at the time. Today we most likely would have used WGS. However, at present there are very few reports that compared PFGE and WGS why it is not easy to determine whether PFGE was adequate or not as gold standard. Considering the well-defined epidemiological data that were available we believe PFGE was suitable to use for comparing methods at the time. Interestingly, PFGE is still used in many outbreak investigations (171).

The PFGE type A isolates, comprising 35 isolates from 20 patients, exhibited 93% profile similarity, i.e. very high identity. The isolates were collected only from children hospitalized during the outbreak period, suggesting that PFGE type A was the likely predominant outbreak strain. Isolates of PFGE type B included six isolates from four children, also hospitalised during the outbreak period. Their isolates also showed high level of identity, equal to or more than 90%.

The PFGE type I and J isolates were part of the ST131-O25b linage (Figure 1, paper II), but the PFGE similarity index was only 79% to that of type A, indicating relatedness but not identity, or even partial identity, with type A. Also, the I and J isolates were resistant to ciprofloxacin indicating that they could have been part of the ST131-H30Rx subclone. However, this was not tested at the time. The epidemiological data also aided, since the type I and J-isolates were collected during the follow-up period.

For type C, which also was part of ST131, the similarity index with type A isolates was only 61%, which is below strain similarity.

When assessing the number of band differences instead of similarity indexes, the same results were obtained. Isolates of PFGE types C, D and E were obtained as single isolates in faecal samples from children that were hospitalised during the outbreak period Thus, all three strains were unique and at the time were considered sporadic cases and not part of the outbreak. However, when strain D was analysed at a later stage with a plasmid typing protocol it was revealed that strain D shared the same plasmid as the outbreak strains (170). This child had also been found to previously carry both the A and B outbreak strains (Figure 21) Therefore, in hindsight strain D could be considered part of the outbreak. Strain C had a different ESBL gene; CTXM-14, further implicating non-identity on the plasmid level. The plasmid of strain

E has not been analyzed but it is not unlikely that this strain harbored another plasmid often encountered in ESBL-*E. coli* with CTX-M15 and co-resistance to ciprofloxacin.

4.2.5 Detection of transmission of ESBL-*E. coli* isolates by MLVA using 10 loci (GECM-10)

Ten distinct GECM-10 types were identified among the 50 ESBL-*E. coli* isolates. Each unique GECM-10 type corresponded to a unique PFGE type and vice versa. All isolates in PFGE cluster A shared the same GECM-10 profile, as did all isolates in PFGE cluster B, and they could be separated from all other unrelated isolates. Thus, for this set of isolates GECM-10 was as efficient as PFGE for outbreak investigation. However, considering the findings in paper III, the ability to distinguish the ST131-O25b-isolates in the outbreak might have been sheer luck. Firstly, the ST131-O25b outbreak isolates did not belong to the H30Rx subclone, thus, they were separated from most of the other ST131-O25b isolates circulating at the time, detected in paper III. Lastly, there were five ST131-O25b-isolates in paper III of two distinct PFGE types (V and Z), that had a similar GECM-7 type to that of the PFGE type A outbreak strain (Table 1, paper III; i.e. G_7-06-04). The isolates that were separated by PFGE but not with GECM-7, could not be separated by applying the GECM-10 either (Figure 1, paper III).

The ST131-O25b isolates of PFGE type I could not be separated from the PFGE type A strains by the first seven MLVA loci, GECM-7 (Table 4 above, and Table 1 paper II). However, when adding loci to create the GECM-10 protocol, as suggested by Lobersli et al. [165], the CVN016 locus could be amplified in the PFGE type I isolate, which discriminated it from isolates belonging to PFGE type A. A PCR-product of 475 bp was obtained, but the repeat number was not able to be determined. However, it was only the CVN016 loci that was needed for the enhanced discriminatory power of the GECM-10 as compared to GECM-7. Had we applied a GECM-3 and added the CVN016 locus the results would have been the same. CVN016 has been reported to exhibit variation among ESBL-*E. coli* isolates in other studies, including ST131 isolates [169]. Thus, addition of all three loci in creating the GECM-10 proved to be unnecessary. Also, to separate the ST131-O25b isolate belonging to PFGE type J from the outbreak isolates of PFGE type A, only the first seven loci were needed, and even GECM-3 would have been sufficient, since the type J isolates lacked one repeat in locus CVN014, compared to the type A isolates.

However, in a confirmed outbreak situation, the GECM-10 approach could be of value. If the GECM-10 code of an outbreak isolate is known it could aid in choosing a limited number of loci with unique repeats for the particular outbreak strain (if present), to aid in investigating subsequent transmission. This approach is similar to a study by Fladberg et al. [206], who used a MLVA code to indicate ST131 isolates. However, this procedure requires knowledge about the local MLVA epidemiology through continuous surveillance, to reveal if a particular repeat indeed is unique or not.

From an outbreak investigation perspective, a locus where not all, or even just few, of the tested isolates will display an allele, or where almost all isolates display the same allele, might not prove so useful. The probability that two isolates display either no PCR-product or that all tested isolates display the same allele is simply too high, resulting in inaccurate clustering of isolates that do not share an epidemiological relationship. However, such a non-variable locus could also prove helpful if an outbreak strain happens to display that particular unusual allele outcome, which could thus be used as an outbreak marker to distinguish it from other isolates. For instance, seven repeats in CVN0015, was only seen in the ST1444 outbreak isolates in paper III.

4.2.6 Detection of transmission of ESBL-*E. coli* isolates using MLST

Representative isolates (n= 23) from each GECM-10 and PFGE type, as well as selected isolates carrying each of the different beta-lactamase gene combinations, antibiotic resistance profiles and different sampling sources, were analysed by *E. coli* MLST. Considering the close epidemiological relationship between non-tested and tested isolates we find it unlikely that if all isolates had been tested isolates of additional ST would have been detected, although identical PFGE- and MLVA-types occasionally are described within different STs, as outlined in relation to paper III.

By MLST seven different sequence types were detected, and all but the ST131 isolates could be accurately separated from the outbreak isolates. Similar findings were seen in paper III and there are several reports of ST131 being heterogenous, even within the ST131-O25b clone and its subclones [131, 187](141, 138). This demonstrates the limited value for MLST as a reliable sub-typing method for ESBL-*E. coli* outbreak investigations if the outbreak strain belongs to a widespread ST, such as the ST131 linage, or other locally more prominent STs, such as types 12, 38, 69 and 405 frequently reported from the Scandinavian countries [207]. Isolates of ST 69, 43 and 405, which were detected in this outbreak investigation (Table 4) were also found circulating in

our area in paper III. However, if an outbreak strain belongs to a more uncommon ST, such as the outbreak strain belonging to ST1444, MLST might well be able to distinguish it from sporadic cases. In these cases, probably not all loci need to be tested, if the sequence type is known, similar to the approach of the CH-typing scheme using only the fumC–gene of MLST together with fimH-typing, as developed by Weissman et al. in 2012 [132].

If we were to use MLST today, considering the increase in predominating clones circulating in the community, as well as the number of individuals that nowadays would be asymptomatic carriers of community acquired EPE-strains in an outbreak situation, MLST is likely to be even more unsuitable. Taken together, we found the discriminatory power of MLST too low for outbreak investigation, but there are other values to consider, as discussed above. Also, the ST is easy to extract from WGS-data, contrary to the other methods employed in this study.

4.2.7 Detection of transmission of ESBL-*E. coli* isolates by ST131-O25b-PCR

The simplicity of the method is of course beneficial, but it could not differentiate outbreak isolates from non-outbreak community acquired isolates part of this linage. The global predominance of ST131-O25b further limits its value. However, in case of an outbreak caused by EPE not part of the ST131-O25b linage it could of course be advantageous, at least for initial screening, by ruling out all patients carrying EPE-isolates part of this linage and thus is not part of the outbreak.

4.2.8 Detection of transmission of ESBL-*E. coli* isolates by comparing antibiograms

Relying on the resistance profiles to routinely tested antibiotics for surveillance and outbreak detection is a commonly used approach and has been extensively used for surveilling transmission, for instance for MRSA. It proved to be misleading in this particular investigation. Isolates of the same PFGE type exhibited different resistance patterns, even for repeat isolates from the same patient (Figure 20 and 21 above). The outbreak isolates of type A and B each exhibited three and four different resistance profiles, respectively, but 27 of these isolates shared the most common type, i.e. resistance to tobramycin and trimethoprim (Table 2, paper II).

Interestingly, all isolates part of the outbreak were sensitive to ciprofloxacin (including type D), whereas the others, with exception of the type C isolates, were resistant. Sensitivity to ciprofloxacin was quite rare in ESBL-isolates in

general, especially in isolates that also are resistant to trimethoprim and tobramycin, as described in Paper I (Table 3). This resistance profile could possibly, have been used as a marker for outbreak isolates, especially considering that isolates belonging to the ST131-O25b clone generally are resistant to ciprofloxacin, at least its most prevalent H30R and H30Rx subclones [196] which also was the case of most isolates part of this clone in paper III. Such a resistance pattern deviating from the expected is the kind of marker that could be used to track isolates related to an outbreak, before initiating more distinguishing typing methods. However, with the increasing number of ESBL-isolates being co-resistant to a number of antibiotics the possibility of varying resistance profiles will decrease over time and thus be of less value.

4.2.9 Detection of transmission of ESBL-*E. coli* isolates by anlysing beta-lactamase genes

Strain identity could not be predicted by the presence of certain beta-lactamase genes or combination of these genes, particularly not CTX-M-15, despite it being the gene behind this outbreak [205] The most predominant beta-lactamase gene combination (CTX-M-15, OXA-1, TEM-1b) was represented in 27 isolates (16 children) of three different PFGE strain types (A, B and F). Thus, missing children part of the outbreak. In case of the type F isolate detected after the outbreak had stopped, we falsely could have suspected ongoing transmission by relying on this gene combination for inclusion in the outbreak. Also, since these genes can be found on plasmids the picture may be rather confusing if plasmid migration is involved in an outbreak situation as actually was the case in this outbreak.

The low discriminatory value of typing beta-lactamase genes is evident when a given gene, such as CTX-M-15, has become so predominant in a certain region. On the other hand, if an uncommon CTX-M-gene is involved, gene-typing might be beneficial. Also, ESBL-gene typing could possibly be used for nonidentity; detection of a non-CTX-M 15 isolate would most likely have been considered as different from the outbreak strains, just as for the type C strain carrying CTX-M-14.

4.2.10 Plasmid typing in relation in relation to strain typing when evaluating transmission

In most reported hospital-associated outbreaks due to MDR Gram-negative bacteria it is primarily one strain that is transmitted and is responsible for the accumulation of cases, especially when it comes to *Acinteobacter spp*., *Klebsiella spp.* etc., also when the resistance mechanism is plasmid-mediated

[208]. However, with the emergence of resistance genes on highly mobile plasmids an increasing number of polyclonal outbreaks are reported [209], indicative of plasmid transfer having occurred somewhere on the line between isolates part of the outbreak. It can be debated that plasmid typing should also be performed in an outbreak investigation, at least for polyclonal outbreaks. To our knowledge this has rarely been done, and plasmid analyses are still quite cumbersome despite using sequenced data, primarily due to problems when assembling data. At the time of the outbreak in paper II the methodology was not available to us, although we suspected that plasmid transfer might have occurred since all isolates carried CTX-M-15.

If plasmid analyses had been performed, we would have solved one more case (the type D strain), as outlined above. In the future this might be valuable not the least for outbreaks due to CPE. We detected such a polyclonal CPE outbreak due to OXA-48 at another neonatal unit at Sahlgrenska University Hospital in 2015 (unpublished data). However, to confirm that a common plasmid is involved in an outbreak requires knowledge of the local prevalence of circulating plasmids, both in the community and the hospital setting, in order not to overestimate their importance [48]. At present we have no knowledge of the ESBL-plasmid epidemiology in our region.

4.2.11 Epidemiological data in relation to typing results to evaluate transmission

Isolates from nine patients (PFGE-types C-J) found during the outbreak investigation were considered to be community acquired strains and not part of the outbreak. This clearly demonstrates the importance of epidemiological data being available in the assessment of outbreaks. At the time, ESBL-E. coli isolates were still rare in Sweden. Influx from the community to hospital settings had to be taken into consideration but was considered low. Today, in 2019, when there is an increase in ESBL-*E. coli*, with a prevalence of faecal carriage in Sweden of approximately five percent [97], it has to be taken into consideration that ESBL-*E. coli* isolates detected during an outbreak investigation might be part of the outbreak but might also be sporadic cases of faecal colonization.

4.2.12 In conclusion

The present EPE-outbreak was very well defined epidemiologically and included a comparatively large number of patients. A further strength of this study was that the outbreak was polyclonal and included transmission of ESBL-*E. coli* which has been rarely reported in the literature. To our knowledge, this is one of few reported studies comparing PFGE, MLVA and

MLST for outbreak investigation of ESBL-*E. coli*. A good correlation between PFGE and MLVA was seen and the resolution was better for MLVA than for MLST in relation to PFGE-results. The MLVA performed well in discriminating the E. coli outbreak isolates from non-outbreak isolates even those belonging to the ST131-O25b linage. Only a limited number of isolates of other STs were included in this study, therefore there is a further need to evaluate MLVA for strain-typing. The use of ten loci is probably not needed, and by reducing the number of loci the method will also be easier to use. This is addressed in paper III.

Analyzes with regard to ESBL-gene content and antibiotic resistance profile resulted in inconclusive results, partly due to the polyclonal nature of the outbreak and the fact that there were patients that were coinfected with several different isolates.

For discerning an outbreak strain with low prevalence in the surrounding community, such as the ST1444 isolates in this outbreak, all applied methods performed well. This emphasizes that an outbreak caused by a strain with specific features or a marker, such as specific antibiotic resistance profile or an unusual strain type, will be much easier to both detect in the first place, and also to distinguish from strains that actually are part of an outbreak. Plasmid analyses performed after this study was completed, demonstrated that plasmid analyses may aid in outbreak investigations and with the emergence of resistance genes on highly mobile plasmids, plasmid analyses might also become a significant part of outbreak detection and investigation in the future.

4.3 Comparison of methods for local surveillance of ESBL-*E coli* with the aim to detect outbreaks (paper III)

The results in paper II showed that MLVA could replace PFGE for outbreak investigation, even for isolates belonging to the ST131-O25b clone. However, there was still a need to evaluate how well MLVA would perform in detecting a potential outbreak. Firstly, when typing consecutive ESBL-*E. coli* isolates, as in continuous surveillance, the ability of the typing method to distinguish outbreak strains from sporadically occurring ESBL-*E. coli* strains must be evaluated. This requires that the epidemiology of the strains circulating at the time is known. Secondly, a diverse collection of isolates is needed, including both rarer strain types as well as several subtypes of the more prevalent types. The second requirement is often fulfilled when typing methods are evaluated,

because strain collections are often used, whereas the first requirement tends to be missing and, thus, is quite unique for paper III.

In paper III we investigated all consecutive clinical isolates detected adjacent to the outbreak described in paper II, starting from September 2008 until March 2009, covering all isolates from the greater Gothenburg area. We compared all isolates regarding resistance profile, ESBL-gene profiles, MLST- and MLVA-types with that of PFGE, with a focus on MLVA typing results. Since the comparison in this paper did not aim to detect complete identity but rather strain identity, we chose PFGE using ≥80% similarity index for designation of types for the comparison.

Only one isolate/patient was included, and only clinical samples to mimic the setting in a clinical microbiology laboratory. Altogether 116 isolates were included, 104 sporadic and the twelve clinical isolates detected in the outbreak investigation and during the follow-up period. Out of the 104 sporadically occurring isolates 63 were sampled in the hospital setting, and 41 in the community. We chose not to use the general definition for hospital acquisition, knowing that isolates detected in the hospital may very well be community acquired and vice versa. Instead, we focused on where the patient was sampled, as suggested more adequate for epidemiological studies of MDR bacteria by Skov et al. [210].

4.3.1 Results and discussion (paper III)

From the results of all three papers in this thesis (I-III) it becomes obvious that comparison of antibiotic resistance or ESBL-gene profiles will have a low discriminatory power for outbreak surveillance, and will therefore not be further discussed. The genotyping results for all 116 isolates are gathered in Table 5 below and data are corrected regarding errors detected in paper III.

The distribution of STs displayed a wide variety; altogether 36 STs for the 104 sporadically occurring isolates. As expected, ST131 dominated, followed by ST648, ST38, ST12 and ST69, which is in line with findings of other studies from the Nordic countries [206, 207, 211]. There were 23 MLST singletons, i.e. only one isolate belonging to the particular ST.

As expected, PFGE was the most discriminating method; 82 different types were detected including 65 singletons, out of which four were non-typeable isolates (Table 5 above, and Table 2, paper III). Non typeability by PFGE has been reported previously [167, 195], but appears to be comparatively rare. As reported by others[131, 212], we found several PFGE- types (n=14) within the ST131-O25b genetic lineage. In contrast to PFGE, all isolates were typeable

by MLVA, but the discriminatory power was lower. By applying GECM-7, 56 different types were detected, out of which 39 were singletons.

Table 5. Overall genotyping results in relation to MLST for 104 clinical and 12 outbreak ESBL-E. coli isolates detected in the greater Gothenburg area in the months close to the polyclonal ESBL-E. coli outbreak.

		Number of types per method			
MLST -types	no. of isolates	GEM-3	GEM-7	GEM-10	PFGE-(80%)[b,c]
(n=37 /23)[a]	(n=116)	(n=39/22)[a]	(n=56/39)	(n=60/44)	(n=82/65)
ST 131	42 (9[d])	6		7	18
O25b	34 (9[d])	3		3	14[b]
non-O25 b	8	3	3	4	5[b]
ST 648	10	4	4	5	8
ST 38	9	5	5	8	8
ST 12	6	3	3	3	5
ST 69	4	4	4	4	4
ST 405	3	1	2	2	3
ST 617	3	1	2	2	2
ST 1444	3[d]	1	1	1	1
ST 167	3	1	1	1	1
ST 10, 58, 443	2,2,2	2	2	2	2
ST 43	2	1	1	1	2
ST 1284	2	1	1	1	1
		1	1	1	1
ST singeltons (n=23)	1/ST				

[a] total number of types/numbers of singleton types, for each method; [b] One PFGE type (JB) part of ST131 is found both within and outside the ST131-O25b clone; [c] Non-typeable isolates by PFGE (n=4) are each represented as a unique PFGE-type in this table; [d] Isolates belonging to the polyclonal outbreak

A limitation in our study is the low number of isolates within globally prevalent STs, for instance ST10, 23 and 405. However, our study had a somewhat different aim and is more likely to reflect the use of MLVA for surveillance for the clinical microbiology laboratory routine surveillance protocol. Also, in the local setting variation in types may be less prominent. Brolund et al. [195] surveying isolates in Sweden collected every second year (2007-2011) actually found rather low variation, less than 5% of the detected PFGE-types were new between collection periods.

4.3.2 Typing-results by MLVA in relation to the various loci

It became obvious that the variability in the analysed loci differed considerably (Table 3, paper III), especially for isolates that did not belong to the ST131-O25b linage. Several loci displayed one or two favorable numbers of repeats

(e.g. CCR001, CVN007 and CVN015) whereas for some loci the variation was greater, which applied especially to CVN014. For two loci, CVN003 and CVN017, most of the tested isolates had no detectable PCR-product at all, and for CVN002 and CVN016 this was the case in approximately half of the tested isolates (Table 3, paper III). In fact, the isolates in this study that were part of globally prevalent STs such as ST131, ST12 and ST10 had no detectable products in these four loci. A varying number of repeats was however, seen within CVN002 in the studies of Naseer et al. [169] and Fladberg et al. [206].

Contrary to the considerable increase in number of types reported by Lobersli et al. [165], when adding the three additional loci of GECM-10 to the GECM-7, the discriminatory power was only slightly enhanced for the isolates from paper III; only four more types were noted (Table 5). However, Lobersli et al. tested a different set of strains, including primarily enteropathogenic *E. coli*. In fact, the variability for the last three loci in GECM-10 was rather low (Table 3, paper III), and it was almost only the addition of CVN016 that increased the discriminatory power. Naseer at al. [169] also analyzed CCR001 and CVN016 and found additional variation within these loci, especially for ST38 isolates, which was in comparison with our findings. However, their strain collection was much more diverse than ours. Their isolates were collected over a more extended period, originated from four countries and included *E. coli* with a more diverse set of beta-lactamase genes.

The greatest variability was seen within CVN014 followed by CVN004 and CVN001, which was also the case in two other studies using six and seven loci respectively [167, 206]. We evaluated the outcome of using these three most variable loci, here named GECM-3 (Table 2). The discriminatory power decreased as compared to GECM-7, i.e. reducing the number of detected types by 30% (17/56), similar to what subsequently was reported by Fladberg et al. [206].

4.3.3 The MLVA results for typing the ST131-O25b linage

The results were rather disappointing and not in accordance with other studies reporting an ability to type ST131 and the O25b-ST131 lineage with MLVA [162, 213]. We could only detect three MLVA subtypes within this clone and its multi-resistant H30Rx subclone. Similarly, low diversity was detected among ESBL-*E. coli* ST131 urinary isolates in the study of Hertz et al [211]. Adding the other ST131 isolates not part of the ST131-O25b linage from this study, we detected three additional GECM-7 types, reaching a diversity similar to that of Jorgensen et al. [214]. Fladberg et al. [206] found an increasing number of MLVA-types over time and reported altogether nine types within the ST-O25b-clone for *E. coli* in blood, using GECM-3 or -7. However, they

studied a longer time-period and a somewhat different set of strains than in our study, including also non-ESBL isolates, which may have increased the diversity of the strains. The literature is scarce and rather confusing due to inconsistences in loci used and naming of MLVA types. Also, in many studies the O25b-isolates are not separated from the non-O25b isolates within ST131 and in earlier studies the O25b-PCR was often used to designate isolates to ST131, thus missing ST131-isolates not part of the ST131-O25b clone.

The isolates belonging to the ST131-O25b lineage and, thus, also its H30Rx subclone could be clearly distinguished from the other isolates in paper III, but they could not be separated from each other and the discriminatory power was low and did not increase by adding loci other than CVN004 and CVN0014 (Table 1 and 3, paper III). However, in paper II a PCR-product was also seen for CVN016 in two ST131-O25b isolates.

Nor could the ST131-O25b lineage be distinguished from other isolates based on the presence of a specific number of repeats within a certain locus, although nine or eleven repeats within CVN014 were generally more common among ST131-O25b isolates as compared to five or six repeats observed for the other isolates within ST131. However, using the code for GECM-7 types (6-N-N-14-3-X-5), as suggested by Fladberg at al. [206], a majority of these isolates would be designated to the O25b-ST131 strain type, in accordance with our findings (Figures 1 and 2, Table 3, paper III) and that of Naseer et al. [169], if allowing for additional variation also within CVN004.

One must bear in mind that discrepancies for the repeat numbers in a particular locus when comparing different studies might be due to interpretation issues in calculating the number of repeats. Different platforms for generating data may explain some of the minor differences noted, such as three or four repeats within loci CVN015. These discrepancies also highlight that in cases were the differences in fragment length are very small, i.e. only one repeat, or in cases with an obtained PCR-product but no resolution of number of repeats sequencing of the fragment might help to resolve these issues.

Taken together, the results from paper III and other studies indicate that additional methods to that of MLVA are necessary to verify or dismiss strain relatedness within the ST131-O25b genetic lineage. Other methods, for instance typing by rep-PCR (Diversilab system) have encountered similar and even greater problems in trying to subtype this clone [215]. On the other hand, a low discriminatory capacity may be a valuable property. The emergence of the ST131-O25b linage was initially missed in Scotland due to national surveillance using solely PFGE [216] and not gathering MLST data.

4.3.4 Comparison of MLVA with MLST for epidemiological typing

When comparing MLVA with MLST it becomes clear from Table 5 above that MLVA has a higher discriminatory power compared to MLST, and that all GECM-assays (-3, -7 and -10) are able to break up clusters within a sequence type, as reported also by others [169, 206]. In this respect all the three GECM-assays were almost equally efficient, depending on ST evaluated. Most likely, we underestimated the general discriminatory power of MLVA as compared to MLST due to the low number of isolates within most STs and the short time-span studied, as reasoned above. In other studies, several of the STs that were less prevalent in our study have been shown to contain multiple MLVA-types [169, 206].

Naseer et al. [169] suggested that MLVA using seven loci (GECM-7) might replace MLST, and in most cases a MLVA type corresponded to one ST in our study, especially for more rare STs. However, there are several studies demonstrating the presence of the same MLVA-type divided among different STs [206, 214] in accordance with our results. Not surprisingly, GECM-3 frequently clustered isolates of different STs together, of which a few examples are seen in Figure 22.

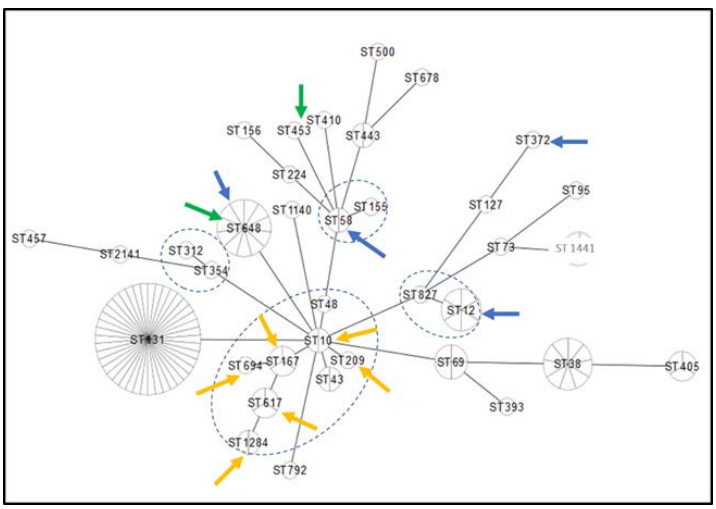


Figure 22. Minimum spanning tree of MLST sequence types (ST) of the ESBL-E. coli isolates in paper III, in relation to the three of the most common GECM-3 types indicated by colored arrows for GECM-3 code 5-11-2 (n=11, yellow), 6-11-9 (n=8, green)and 6-11-6 (n=8,blue). E. coli Clonal complexes (Cplx) are encircled.

4.3.5 Comparison of MLVA with PFGE for epidemiological typing

It becomes clear from Table 5 above and Table 2, paper III, that when studying a more diverse subset of isolates than in paper II, MLVA has a lower discriminatory power than PFGE. The PFGE similarity index within a GECM-7-type was 41-67% within a given GECM-7 type, except for those cases with complete concordance, which was applicable to isolates within PFGE type BF and B (Table 1, paper III). Most GEMC-7 types with more than one isolate/type included several PFGE-types, especially for the ST131-O25b clone, where 3-12 PFGE-types/GECM-7 type were detected.

The opposite to overestimating strain relatedness was also noted, i.e. six PFGE-types included two GECM-7 types (Table 1, paper III). When looking more closely into the cases where MLVA broke up PFGE -clusters, all but one instance involved the ST131-O25b linage. This finding is quite interesting, considering the otherwise low discriminatory power of MLVA for this clone. When applying the PFGE cutoff of ≥90% similarity, the isolates of both PFGE types JB, DA, GA and A were separated into PFGE subtypes with 80-89% identity, which we interpreted as the result of the GECM-7 protocol indeed having a higher discriminatory power than PFGE. That is, MLVA could identify PFGE subtypes within these PFGE (≥80% similarity) clusters. In the example of PFGE-type JB this was further confirmed by the fact that that GECM-7 was able to distinguish O25b-positive from O25b-negative isolates within ST131. More precisely, the two isolates being $G_7$06-08 did not belong to the ST131-O25b genetic lineage, while the one belonging to $G_7$06-11 did.

One important aim of paper III was to evaluate if MLVA could be used for continuous surveillance to detect outbreaks. Thus, we evaluated if the present outbreak strains would have been identified if we had tested all clinical ESBL-*E. coli* isolates at the time of the outbreak-period with MLVA. The PFGE-type B outbreak isolates were separated from the other isolates even with GECM-3, which was not surprising since they belonged to a rather rare ESBL-*E. coli*, i.e. ST1444. Regarding outbreak isolates of the PFGE type A (ST131-O25b), GECM-7 could discriminate these isolates from unrelated PFGE-type A isolates. Nevertheless, five additional isolates belonging to two other PFGE types (Z and V), clustered with isolates of the GECM-7 outbreak type, even when enhancing the resolution by using GECM-10. The four isolates of PFGE type V were, contrary to the outbreak isolates, also part of the H30Rx subclone, further indicating the lack of proficiency for MLVA in typing this clone.

Also, the here described outbreak strain displayed a somewhat atypical type regarding the ST131-O25b linage. If an outbreak is caused by a ST131-O25b H30Rx strain, the likelihood of separating outbreak strains from strains being part of the general influx of community strains probably would prove to be even lower than in this study, and would likely require more discriminatory methods than MLVA, at least if using the loci reported in paper III.

4.3.6 Future surveillance of ESBL- *E. coli*

Considering the increasing number of ESBL-*E. coli* and the high influx from the community setting a simple and rapid pre-selection method is needed for surveillance before applying more advanced methods to dismiss or confirm a possible outbreak. Both identity as well as non-identity will be important to rule out.

A possible future approach could be to firstly identify ST131-O25b isolates by a single PCR, and then apply MLVA to the remaining isolates, preferably the GECM-3 as a start. A recent report [162] suggested a similar approach, that is, to use MLVA as a pre-selection method. We tested this approach on the 116 isolates from paper III, more precisely evaluating the GECM-3 results for the 82 isolates not belonging to the ST131-O25b linage. As shown in Table 2, paper III, 14 clusters containing at least two isolates, and only three larger clusters, were formed, which is outlined in more detail in Table 6. Also, errors from Table 2, paper III are corrected.

Table 6. Number of clusters with two or more ESBL-E. coli isolates and singeltons (n=82) for isolates not part of the ST131-O25b linage detected by the respective genotyping method.

	Typing method			
isolates/cluster	GEM-3	GEM-7	MLST	PFGE
2	3 (2)[a]	7	5	5
3	5 (1)	2	4	4
4	3 (2)	3	1	
5	0	1		
6	0	1	1	
8	2		1	
9			1	
10			1	
11	1			
Cluster types	14	14	14	9
Singelton types	22	39	23	60

[a] Unaltered clusters as compared to GECM-7

Comparing the clustering results of GECM-3 with the other genotyping methods the following results were obtained. Five of the eleven 2-4 isolate clusters by GECM-3 remained unaltered i.e. 15 isolates were correctly clustered as compared to GECM-7. For the remaining clusters, GECM-3 formed falsely large clusters. Altogether 22 isolates were falsely clustered as compared to GECM-7. Compared to PFGE, additional singeltons could be distinguished within the above GECM-3 clusters. This resulted in 39 isolates that were falsely clustered by GECM-3 as compared to PFGE. However, when comparing GECM-7 or MLST with PFGE, there were almost as many isolates, that were falsely clustered together as there was for GECM-3, demonstrating that all these typing methods are rather complementary than congruent. This means that by applying GECM-3 by following the suggested algorithm for this set of isolates a substantial number of isolates would have had to be retested to confirm or dismiss possible relatedness (as defined by PFGE). If isolates are differentiated by GECM-3 they are, however, most likely not related. The 22 GECM-3 singletons were all singletons also by PFGE and GEM-7.

4.3.7 In conclusion

A true advantage of this study was that it mimicked future possible surveillance to detect ESBL-*E. coli* outbreaks for the local clinical microbiology laboratory in that epidemiological data were available and that the ESBL-*E. coli* strains tested included both clinical outbreak isolates, as well as consecutive clinical isolates circulating at the time in our area. When comparing methods for epidemiological typing MLVA proved to overestimate strain relatedness when compared to PFGE, especially for the ST131-O25b clone and its H30Rx subclone. MLVA could not differentiate the ST131-O25b outbreak isolates from other isolates of this linage, despite that these outbreak isolates belonged to a relatively rare variant of ST131-O25b linage. The rare ST1444 type outbreak isolates were well separated.

However, for the ESBL-*E. coli* not part of the ST131-O25b linage the performance of the MLVA method was better, and it could at several occasions separate isolates within a MLST sequence type into different MLVA types. The gain in discriminatory power by using ten loci instead of seven, or even the three most variable loci in the MLVA protocol, was minor. As expected, there were discrepancies between what isolates clustered when applying different typing methods and MLVA, especially the GECM-3 protocol occasionally clustered isolates of different sequence types. On the other hand, MLVA was able to discern subclones within some PFGE ≥80% similarity clusters, and by this added complementary information to the PFGE typing results.

Thus, the MLVA-methods should be considered complementary rather than completely congruent to the other typing methods. The simplicity of the GECM-3 is an advantage, and MLVA might prove to be a suitable preselection method for ESBL-*E. coli* isolates not part of the ST131-O25b linage, in an algorithm for surveillance on the local level before initiating more complex genotyping methods in order to confirm or dismiss strain identity. In case of identical GECM-3-types detected, additional typing methods are necessary to determine strain relatedness. Non-identity results, however, are likely to be confirmatory. If a MLVA protocol should be used routinely, especially the GECM-3 that is based on the most variable loci, it would be important to ascertain that the selected loci could indeed discriminate between the strain types in circulation at the time.

5 CONCLUSION AND FUTURE PERSPECTIVES

Multidrug resistant bacteria, particularly ESBL-producing *Enterobacteriaceae,* are becoming a major health concern, and ESBL-*E. coli* is the most prevalent type. In this thesis the early epidemiology of these bacteria in the greater Gothenburg area is described. More precisely, it focuses on the development from the first case in 2003 detected in the community setting until five years later when EPE were established also in the hospital setting. Possibly the spread of ESBL-*E. coli* in our region originated from transmission in the community from asymptomatic carriers, and subsequently appeared in the hospital setting where the increase that followed was more marked. The distribution of cases according to gender and age was in accordance with the distribution of UTIs caused by antibiotic susceptible *E. coli* in the population, and resembled the national epidemiology of EPE at the time. The MDR ST131-O25b clone became established (0-24%) during this period, but otherwise the emergence of ESBL-*E. coli* was polyclonal. There was a significant shift in ESBL-types in favor of the CTX-M-1 group enzymes, so that by 2009 they were the clearly dominating CTX-M-types. In paper I we could clearly document a shift in the epidemiology of ESBL-*E. coli* over time.

In 2008 one of the first outbreaks due to ESBL-*E coli* in Sweden occurred in a neonatal unit at Sahlgrenska University hospital. From this outbreak investigation we learnt that outbreaks due to EPE may be polyclonal, and that there is a need to react also on resistance type and not only on bacterial species. We also discovered that resistance pattern is not a reliable tool for predicting possible transmission. If transmission has been ongoing for an extended period of time, several types of EPE-strains may be detected, also in the individual patient, possibly as a result of migration of plasmids with ESBL. A need for simple and rapid methods both for outbreak investigation and surveillance of EPE, and especially ESBL-*E. coli*, was therefore requested. Potential methods were evaluated in the subsequent papers in comparison with the golden standard at the time, which was PFGE.

For investigation of this outbreak, MLVA using ten VNTR loci was found to be comparable to PFGE, which did not apply to MLST. The gain by using ten loci instead of seven, or even the three most variable loci, in the MLVA protocol was minor, especially for continuous surveillance of ESBL-*E. coli*. For surveillance, MLVA was inferior to PFGE especially when it came to typing the ST131-O25b sublinage (paper III). However, an abbreviated MLVA using the three most variable loci, might be useful as a screening method to preselect isolates for more discriminating typing methods, especially if isolates

of the ST131-O25b sublinage are sorted out beforehand. This thesis demonstrates how the epidemiology of ESBL-*E. coli* might change over time, emphasizing the need of continuous surveillance to detect outbreaks at the local level. MLVA might serve as a simple and fast typing method on a daily basis in the general clinical microbiology laboratory. With the upcoming threat of *Enterobacteriaceae* with plasmid-mediated carbapenemases that are now also seen in the prevalent *E. coli* clones carrying ESBL, by this thesis work important knowledge has been achieved that might be applicable in the future also when dealing with these extremely resistant bacteria.

From an outbreak and surveillance perspective it is important to follow the local ongoing epidemiology of EPE, with focus on ESBL-*E. coli* as it is the dominant and most important pathogen of the EPE. Firstly, it would be of interest to continue comparing the epidemiology in the community with the hospital setting, and to assess whether the increase for EPE persists in the elderly and men in the latter setting. There might also be certain selected patient groups that need more surveillance attention, for instance men suffering from urology conditions that are frequently prescribed fluoroquinolones.

From the hospital perspective, surveillance of EPE-cases must continue, but typing of all detected cases will not be possible, since there are already too many cases. Thus, a more selective screening and typing strategy will probably have to be the way forward. For this purpose, there is a need to identify the patient categories and healthcare units where more continues typing of EPE is advantageous for reducing transmission and facilitating early detection of outbreaks.

In Sweden today, apart from admission screening of patients previously hospitalised abroad, patients from certain high-risk departments, such as the neonatology, haematology and organ transplant surgery units, are screened for EPE on a regular basis. However, there may be other important units to add to this list, where detection of EPE-cases are generally high. Thus, there is a need to evaluate the effectiveness of the present screening strategies. In this context it is also important to determine whether the EPE-cases detected through screening really reflect those patients that are at risk of developing a clinical infection with EPE. This appears not to be the case, as outlined previously in this thesis. It is important to detect the patients that are colonised with EPE, and in addition also are at risk for developing clinical infections, in order to prevent nosocomial transmission, since these patients are more likely than otherwise healthy faecal carriers to spread the EPE strain.

However, even if more selective screening strategies and more extensive strain typing are implemented, there is still a need to know what EPE-strains and ESBL-carrying plasmids are circulating, in order not to over- or underestimate possible epidemiological relationship in an outbreak situation. Considering the world-wide EPE-epidemiology it is not unlikely that our local epidemiology will change. When it comes to circulating plasmids, this is largely an underexplored field, which applies also to our setting. Also, with the emergence of carbapenem resistant *Enterobacteriaceae* we need to be prepared for new clones and plasmids with $ESBL_{CARBA}$ emerging in our region, in addition to the present ones associated with ESBL-*E. coli*. Therefore it would be of interest to investigate if the ESBL-*E. coli* epidemiology in our region has changed during the last decade as compared to what is described in paper I. More precisely, to investigate if new important clones or ESBL-genes are detected, like the C-M27 clade of ST131, and if the emerging clones, such as ST131-O25b, are becoming even more frequent in the general population or in selected settings or patient groups. It is also important to further evaluate if the suggested algorithm for preselection screening using the three most variable loci in MLVA will be applicable over time, or if a change of loci used may be necessary.

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