Extended-spectrum β-lactamases
Aspects on human carriage
and distribution in aquatic environments

PhD thesis by Silje Bakken Jørgensen

Department of Laboratory Medicine
Section for Medical Microbiology
Bærum hospital
Vestre Viken Hospital Trust

Faculty of Medicine
University of Oslo

Oslo 2018
© Silje Bakken Jørgensen, 2018

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo


All rights reserved. No part of this publication may be
reproduced or transmitted, in any form or by any means, without permission.

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.
# 1 Table of contents

2 Abstract .......................................................................................................................... 1

3 List of papers .................................................................................................................. 4

4 Abbreviations .................................................................................................................. 5

5 Introduction ..................................................................................................................... 7

5.1 General introduction: Antimicrobial resistance and the challenges we face ............. 7

5.2 Enterobacterales ......................................................................................................... 8

5.2.1 *Klebsiella pneumoniae* .............................................................................................. 9

5.2.2 *Escherichia coli* ..................................................................................................... 9

5.3 Genetic changes and genotyping methods .................................................................. 10

5.3.1 Core genome, pan-genome and genetic changes ..................................................... 10

5.3.2 Epidemiological typing methods and definitions of strains and clones ............... 11

5.3.3 Whole genome sequencing .................................................................................... 14

5.4 Antimicrobial resistance ............................................................................................. 15

5.4.1 Historical overview ................................................................................................. 15

5.4.2 β-lactams and β-lactamases .................................................................................. 16

5.4.3 Colistin and colistin resistance .............................................................................. 20

5.4.4 Plasmids and mobile genetic elements ................................................................ 21

5.4.5 Successful clones .................................................................................................... 23

5.5 Environmental dissemination of ESBLs – the One Health perspective ..................... 24

6 Aim of this doctoral thesis ............................................................................................ 26

7 Summary of materials and methods ............................................................................ 27

7.1 Ethics .......................................................................................................................... 27

7.2 Study population ....................................................................................................... 27

7.3 Sample collection from recreational water and wastewater .................................... 28

7.4 Laboratory methods .................................................................................................. 28

7.4.1 Bacterial culture and identification ........................................................................ 28

7.4.2 Antimicrobial susceptibility testing ....................................................................... 30

7.4.3 Detection of ESBL phenotype ................................................................................ 31

7.4.4 Detection of ESBL genotype .................................................................................. 31

7.4.5 Whole genome sequencing .................................................................................... 32

7.4.6 Multilocus variable number of tandem repeats assay ........................................... 33

7.5 Statistical methods ..................................................................................................... 33
Appendix: Papers I - IV
2 Abstract

Background
Antimicrobial drug resistance is an important problem for global health. Without effective antimicrobial drugs, medical advances such as organ transplants, cancer treatment and neonatal intensive care will suffer. Because antimicrobial drugs are used in animals as well as in humans, because people travel and food products are distributed all over the world, and because bacteria and drugs are spread into natural environments, a One Health perspective is needed to understand and combat the increasing challenge of multidrug resistance. Extended-spectrum β-lactamases (ESBLs) are enzymes which confer resistance to our most frequently used antimicrobial drugs, the β-lactams. ESBL-producing Enterobacterales (ESBL-E) are now frequently found among clinical bacterial isolates in Norway.

Aims
The overarching aim of this thesis was to increase our knowledge regarding the occurrence, dissemination and persistence of ESBL-E in different ecological compartments in Norway.
To achieve this, we specifically aimed to
1) examine the prevalence of ESBL-E faecal carriage in patients with gastroenteritis, and explore the possible association between carriage and foreign travel
2) detect patient and microbe related risk factors for prolonged faecal ESBL-E carriage in patients with urinary tract infection caused by ESBL-E
3) analyse intra-individual strain diversity of ESBL-\textit{Escherichia coli} (ESBL-EC) detected in urine and consecutive faecal samples
4) investigate the occurrence of ESBL-EC in recreational water and wastewater
5) perform a genetic comparison of ESBL-EC strains from recreational water and wastewater with geographically related clinical isolates
6) examine the presence of multidrug resistance and plasmid mediated colistin resistance genes in ESBL-EC strains from patients and aquatic compartments.
Abstract

Materials and methods
The prevalence of ESBL-E faecal carriage and the duration of such carriage were explored through observational studies of two different patient cohorts, patients with gastroenteritis and patients with urinary tract infection caused by ESBL-E. Samples from four recreational water locations and a wastewater plant were retrieved on multiple occasions. ESBL-E isolates were identified and characterised by pheno- and genotypic methods, including whole genome sequencing and multilocus variable number of tandem repeats assays (MLVA).

Results
The overall ESBL-E faecal carriage rate was 15% in patients with gastroenteritis, while the sub-group who had travelled to Asia were carriers in 56% of cases. Among patients who had suffered from urinary tract infections caused by ESBL-E, the carriage rate was 77% at inclusion, 39% at 13 months, 19% after two years, and 15% after three years. Prolonged carriage was associated with previous infection with ESBL producing *E. coli* strains belonging to phylogroup B2 or D. We observed large intra-individual genotype variations in ESBL-E isolates from consecutive faecal samples. ESBL-E were present in eight out of 20 samples from recreational water, and in all samples from the wastewater plant. Multidrug resistance was more common in clinical isolates, but plasmid mediated genes conferring colistin resistance were only detected in strains from recreational water. Eight different ESBL-E sequence types and isolates with identical MLVA-patterns were detected across all compartments.

Conclusion
This work confirms that patients suffering from gastroenteritis after foreign travels to Asia have a very high risk of being ESBL-E carriers. Carriage of specific genetic lineages of ESBL-EC is associated with prolonged colonisation, and carriers are likely to be colonised by several different ESBL-E strains over time. This knowledge may be of importance for clinicians when choosing empirical treatment regimens, and for infection control personnel outlining recommendations for screening, outbreak detection and contact precautions in hospitals.
ESBL-EC that are capable of causing infections are present in wastewater and in recreational water with a potential for human exposure. Multidrug resistance is more frequent in clinical ESBL-EC strains than in environmental strains, and plasmid mediated colistin resistance genes can be found in ESBL-EC in Norwegian recreational water. The demonstration of ESBL-EC presence in wastewater and recreational water suggests that these are potential sources for ESBL-EC dissemination and possible infection in humans. This knowledge may contribute to the development of improved surveillance strategies, and should encourage further research on water as a transmission route for antimicrobial drug resistance.
3 List of papers

This thesis is founded upon the following papers referred to in the text by their Roman numerals. Reprints were made with permission from the publishers.

I. High prevalence of faecal carriage of ESBL-producing Enterobacteriaceae in Norwegian patients with gastroenteritis.
Scandinavian Journal of Infectious Diseases, 2014; 46: 462–465

II. Fecal carriage of extended spectrum β-lactamase producing Escherichia coli and Klebsiella pneumoniae after urinary tract infection - A three year prospective cohort study.
PLOS ONE DOI:10.1371/journal.pone.0173510

III. A comparison of extended spectrum β-lactamase producing Escherichia coli from clinical samples, recreational water and wastewater.
PLOS ONE DOI: 10.137/journal.pone.0186576.

IV. First environmental sample containing plasmid mediated colistin-resistant ESBL-producing Escherichia coli detected in Norway.
## 4 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLP</td>
<td>Amplified fragment length polymorphism</td>
</tr>
<tr>
<td>AMR</td>
<td>Antimicrobial resistance</td>
</tr>
<tr>
<td>AST</td>
<td>Antimicrobial susceptibility testing</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CARD</td>
<td>Comprehensive Antibiotic Resistance Database</td>
</tr>
<tr>
<td>CA-UTI</td>
<td>Community acquired urinary tract infection</td>
</tr>
<tr>
<td>CTX-M</td>
<td>Cefotaximase München</td>
</tr>
<tr>
<td>EAEC</td>
<td>Enteroaggregative <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohaemorrhagic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EIEC</td>
<td>Enteroinvasive <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended-spectrum β-lactamase</td>
</tr>
<tr>
<td>ESBL-E</td>
<td>Extended-spectrum β-lactamase producing Enterobacterales</td>
</tr>
<tr>
<td>ESBL-EC</td>
<td>Extended-spectrum β-lactamase producing <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
</tr>
<tr>
<td>IMP</td>
<td>Imipenem metallo-β-lactamase</td>
</tr>
<tr>
<td>KPC</td>
<td><em>Klebsiella pneumoniae</em> carbapenemase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix assisted laser desorption/ionization time of flight</td>
</tr>
<tr>
<td>mcr</td>
<td>Mobilized colistin resistance gene</td>
</tr>
<tr>
<td>MLEE</td>
<td>Multilocus enzyme electrophoresis</td>
</tr>
<tr>
<td>MLST</td>
<td>Multilocus sequence type</td>
</tr>
<tr>
<td>MLVA</td>
<td>Multilocus variable number of tandem repeats assay</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>NDM</td>
<td>New-Dehli metallo-β-lactamase</td>
</tr>
<tr>
<td>OXA</td>
<td>Oxacillinase</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin-binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>Rep-PCR</td>
<td>Repetitive element palindromic polymerase chain reaction</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td>VIM</td>
<td>Verona integrone-encoded metallo-β-lactamase</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number tandem repeat</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
</tr>
</tbody>
</table>
Introduction

5 Introduction

5.1 General introduction: Antimicrobial resistance and the challenges we face

Antimicrobial resistance (AMR) is an increasing problem that has gained public attention world-wide. Descriptions like “the most important medical challenge for the next 100 years” and “the climate-crisis of medicine” illustrate the great importance of this issue for international public health (1, 2). Gradually, the international society has recognised that a One Health approach is needed to battle the AMR-problems. This implies that we make “…collaborative effort of multiple disciplines – working locally, nationally, and globally – to attain optimal health for people, animals and our environment” (3).

The Scandinavian countries have for a long time been in a fortunate position regarding AMR in both Gram-positive and Gram-negative bacteria (4, 5). Strict regulations of antimicrobial drug use in food production as well as in human medicine have been a key factor (4, 6). Simultaneously, rigorous infection control measures have been established to prevent resistant microbes to spread and become an established part of the microbial flora of hospitals. For example, the Scandinavian countries and the Netherlands have adopted a “search and destroy” policy to combat the international epidemic of methicillin resistant Staphylococcus aureus (MRSA) (7, 8). This approach seems to have been successful so far, although there are increasing reports regarding MRSA in live-stock and human transmission in Denmark (9). Eager tracing of cases and eradication of carriage in patients and health care personnel have been instrumental in preventing the spread of MRSA in health care institutions (8, 10). However, this strategy cannot be adapted to the resistant Gram-negative intestinal bacteria, e.g. Enterobacterales with extended-spectrum β-lactamase (ESBL-E). The spread of ESBL-encoding genes on easily transferrable plasmids, and the lack of efficient eradication measures of virulent strains that thrive in the human intestines, make ESBL-E a far more challenging problem than MRSA (11, 12).
As ESBL-E are frequently retrieved from clinical samples, the drugs that so far have been regarded as our last line of defence, i.e. carbapenems and colistin, is used more often, with increasing resistance rates as the inevitable result (6, 13). Moreover, patients in many parts of the world do not have access to carbapenems or colistin, and third generation cephalosporins are still the only available treatment option, even if they in many cases are no longer effective (14). The consequences for morbidity and mortality are enormous (2, 14). A need for more knowledge regarding human faecal carriage of ESBL-E and possible transmission routes for these bacteria has been an important incentive for the research presented here.

5.2 Enterobacterales

Enterobacterales (formerly Enterobacteriales) is a bacterial order that previously consisted of only one family with a validly published name, i.e. the Enterobacteriaceae (15). After comprehensive comparative genomic studies, it was recently suggested that the Enterobacterales should be divided into seven different families; Enterobacteriaceae, Erwiniaceae, Pectobacteriaceae, Yersiniaceae, Hafniaceae, Morganellaceae and Budviciaeae (15), [https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=91347 last accessed April 23\textsuperscript{rd} 2018]). The Enterobacterales are non-sporulating, facultative anaerobe, Gram-negative rods. They inhabit a wide spectrum of niches, e.g. soil, water, plants and animal intestines (15, 16). Some members are considered to be overt pathogens, such as Salmonella and Shigella, while others are considered opportunistic pathogens, such as Enterobacter or Citrobacter (16, 17). In clinical samples, Escherichia coli (E. coli) and Klebsiella pneumoniae (K. pneumoniae), both from the Enterobacteriaceae family, are the most frequently encountered Enterobacterales. They primarily cause urinary tract infections, but also blood-stream infections, soft tissue infections and meningitis (4, 16, 17). In the following, after a short introduction to K. pneumoniae, I will focus on E. coli, as this is the major ESBL-producing species found in Norwegian blood and urine cultures (4).
5.2.1 *Klebsiella pneumoniae*
In healthy humans *K. pneumoniae* may colonise the human intestinal tract, the oro- and nasopharynx. On intact skin, these bacteria are considered to be part of the transient flora, and contamination of perineum and the inguinal skin occurs frequently (18, 19). *K. pneumoniae* may colonise wounds, and is a common cause of urinary tract infections, bacteraemia, liver abscesses and nosocomial pneumonia (16). They can also be retrieved from environmental surfaces and water (20). *K. pneumoniae* may spread in hospital environments, and ESBL-producing strains have caused numerous nosocomial outbreaks, also in Norway (11, 21, 22).

5.2.2 *Escherichia coli*
*Escherichia coli* (*E. coli*) have their natural habitat in the intestines of vertebrates, and are also frequently found in soil and water (16, 17, 23). The presence of *E. coli* in aquatic environments is often regarded as a sign of faecal contamination (16, 23). Fimbriae cover the bacterial surface, and these are important for attachment to human cells and the ability to colonise the hosts (17). The bacterium produces a variety of enzymes, which are important for their access to nutrients and survival in various environments, interactions with the surrounding microbiome, and also for pathogenicity (17).

Like many other bacterial species, *E. coli* is an opportunistic pathogen. This means that it is a natural part of the human microbial flora, causing no harm under normal circumstances. These normally occurring *E. coli* strains are referred to as commensals (23). However, when transferred from the intestinal mucosa to other organs such as the urinary tract, the gall bladder or the blood stream, *E. coli* may cause potentially lethal infections. Around one third of all positive blood cultures from Norwegian hospital laboratories contain *E. coli* (4).

*E. coli* bacteria may also cause intestinal disease. The strains which are responsible for diarrhoea contain virulence factors like enterotoxins and adhesins (16, 17). Many of them have names according to their pathogenic properties; enterotoxigenic *E. coli*
(ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC) (16). Virulent strains which do not cause diarrhoea, but rather affect other organs, are referred to as extraintestinal pathogenic *E. coli* (ExPEC) (24). *E. coli* infections are the cause of approximately two million human deaths per year, intraintestinal and extraintestinal diseases combined (23).

### 5.3 Genetic changes and genotyping methods

#### 5.3.1 Core genome, pan-genome and genetic changes

All *E. coli* share a core genome which mostly consists of highly conserved housekeeping genes necessary to fulfil the bacterial life cycle. The average *E. coli* genome contains between 4,000 and 5,000 genes, but only 2,000 genes with high homology are conserved among all strains, thus constituting the core genome (25, 26). Other parts of the *E. coli* genome are variable, as genes are exchanged through horizontal gene transfer, either by direct cell-to-cell contact (conjugation), DNA transfer by a bacteriophage virus (transduction) or uptake of DNA from the surroundings (transformation) (23, 27). Recombination events, including integration events (when new genes are added without any old genes being lost or altered), occur mostly in distinctive hotspots spread along the circular genome (25). Hence, *E. coli* can keep a clonal frame of conserved genes, while at the same time allowing changes in other genes conferring environmental adaptation, virulence or antibiotic resistance (23). Additionally, spontaneous mutations occur during DNA replication, and may also lead to alterations in gene functions or expression.

The pan-genome of *E. coli*, i.e. the collection of all genes that have been described in *E. coli*, is steadily growing as new strains are analysed (28). The plasticity and large diversity in the genome give the bacteria rich opportunities for adapting to environmental changes and various selection pressures including exposure to antibiotics. All *E. coli*, both commensals, ExPEC and diarrheagenic strains, are potential recipients of genes conferring antibiotic resistance. Virulence factors and
antibiotic resistance often occur together in the same strains, creating epidemiologically successful clones with an enhanced ability to colonise, persist in the host and resist a vast range of antimicrobial drugs (29, 30).

5.3.2 Epidemiological typing methods and definitions of strains and clones

Infection epidemiology is the study of the occurrence and spread of infections in defined populations. In molecular epidemiology studies, molecular biology techniques are combined with traditional epidemiological methods to elucidate reservoirs and transmission routes for dissemination of defined bacteria.

“Strain” and “clone” are important terms in bacterial epidemiology, but their meaning is often subject to interpretation. In a paper from 2007, van Belkum et al offer the following definitions (31), which I will use in this thesis:

**Clone:** Bacterial isolates that, although they may have been cultured independently from different sources in different locations and perhaps at different times, still have so many identical phenotypic and genotypic traits that the most likely explanation for this identity is a common origin within a relevant time span. As the word “clone” in other settings is often used to describe isolates that are directly descendent from an original, “clone” is also used as an alternative for “strain” in the literature (32).

**Strain:** The descendants of a single isolation in pure culture, usually derived from a single initial colony on a solid growth medium. A strain may be considered an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic and genotypic characteristics. Cultures of a particular microorganism, isolated at the same time from multiple body sites of a patient and indistinguishable by typing, also represent a single strain.

Several epidemiological typing methods have been used to characterise bacteria, and which typing method to choose depend on the epidemiological setting and the genetic
characteristics of the microbe in question. Studies focusing on global epidemics which develop over a long time period require different typing methods than an outbreak investigation that spans over a restricted area and a short time-frame (31, 32). Moreover, the genetic stability of microbes is largely variable. *Staphylococcus aureus* for instance, has a rather stable genome, which makes it possible to trace distinct clones over large distances over many years (33). *E. coli* on the other hand, frequently exchange large amounts of DNA with its surrounding microbiome as described (23, 25). This may cause large variation in resistance mechanisms, virulence factors and gene expression within a time-span of a few generations only. These changes makes it challenging to choose adequate typing methods in different settings (23).

Different pheno- and genotypic approaches have been used to characterize and type the *E. coli* strains. The list below is not complete, but contains some of the most frequently and historically important epidemiological typing tools:

**Serotyping:** Since the 1940s, serotype analysis of the H- (flagellar, protein), K- (capsular, carbohydrate) and O- (somatic, cell membrane lipopolysaccharide) antigens has been used to divide the species into distinct serotypes (16, 17).

**Multilocus enzyme electrophoresis (MLEE):** In the 1980s, isolates were further characterized by the relative electrophoretic mobility of several water-soluble housekeeping cellular enzymes. Comparison of *E. coli* strains using the MLEE type of 38 different enzymes made it possible to construct a phenogram which identified four major phylogroups (A, B1, B2 and D) and two accessory groups (C and E) in the species (31).

**Multilocus sequence typing:** In the late 1990s, multilocus sequence typing (MLST) emerged as a powerful tool for bacterial population genetics. With this method, the nucleotide sequence of several housekeeping genes is determined for each isolate. Each new genetic sequence described is given a specific number, so that each strain can be described by a sequence of numbers; one number for each housekeeping gene.
There are several MLST-schemes available for *E. coli*, all using different combinations of housekeeping genes (34, 35).

**Phylogrouping**: A triplex PCR which made it possible to assign *E. coli* isolates to one of the four main phylogenetic groups was introduced in 2000 (36). These phylogenetic groups had been identified through MLEE. Later, a modified quadriplex method has been described, which can distinguish isolates from seven separate phylogroups (37). Recent studies have revealed extensive sub-structures in the *E. coli* species (38). The phylogroups are not randomly distributed with respect to their source of isolation, as strains causing extra-intestinal infections are more likely to be of phylogroup B2 or D.

**Pulsed-field gel electrophoresis (PFGE)**: Genomic DNA is cut by restriction endonucleases into a few (usually less than 30) large fragments, which are separated in an agarose gel by periodic alteration in the electric field’s direction. PFGE has remarkable discriminatory power and reproducibility, and has therefore become a widely applicable method for comparative typing of almost all bacterial species (31).

**Rep-PCR**: In the bacterial DNA, we find interspersed repetitive DNA elements that can be amplified by species specific primers. The amplified products can be separated by gel-electrophoresis to create a genetic fingerprint comparable to PFGE (39).

**Amplified fragment length polymorphism (AFLP)**: A PCR-based method relying on selective amplification of DNA fragments generated by one or two restriction enzymes, usually the combination of a frequent and a rare cutter (40). The amplified fragments are separated on agarose gel, or on DNA sequencing instruments with automated data capture.

**Multilocus variable number of tandem repeats assay (MLVA)**: All bacterial genomes contain multiple loci of repetitive DNA, so called variable number tandem repeats. These repetitive units are often incorrectly copied during bacterial replication, resulting in shortening or lengthening of the repeat region (31). In MLVA, several different selected repeat regions are amplified by PCR, and the size of each PCR-
product is measured by capillary electrophoresis. Hence, for each repeat locus, a digit can be assigned, representing the number of repeats implied. MLVA assays are usually very fast, they reduce the amount of labour as compared to traditionally used methods and they offer high-discrimination. Moreover, the data generated can easily be reproduced in different laboratories, and shared in public databases (41). Reduced typing time with high resolution makes this a valuable tool for resolving large and complex outbreaks.

**Single nucleotide polymorphism (SNP) analyses** for genotyping involves comparison of the nucleotide bases in defined positions. By this definition, MLST is a variant of SNP genotyping (31). As whole genome sequencing (WGS, see below) has become more available, it is now possible to search through entire bacterial genomes to determine the complete SNP-variations between bacterial isolates (42). If there are very few SNPs that differ between the isolates in their core genomes, one can assume that the isolates are closely related.

**5.3.3 Whole genome sequencing**

Microbial whole-genome sequencing (WGS) is an emerging technique that allows species identification, genotype determination, detection of antibiotic resistance genes and virulence genes in one single analysis. The method has become increasingly fast and affordable as next generation sequencing techniques and powerful bioinformatics tools have been developed during the last decade. As the new technology develops, several questions arise regarding the use of WGS and the quality assessment of the generated data. When comparing a sequenced genome to genomes in a database, the performance quality and the extent of data in the database, as well as the selected matching thresholds will influence the results (43). Another important challenge for clinical microbiologists is the prediction of antibiotic susceptibility according to WGS, as there is lack of data linking pheno- to genotype and vice versa (44). Phenotypic susceptibility testing will still be necessary in many settings to detect resistance encoded by unknown mechanisms and mechanisms due to alterations in gene
expression levels. Hence, selection of antibiotic treatment should primarily be based on susceptibility tests (43, 44).

5.4 Antimicrobial resistance

5.4.1 Historical overview

The discovery of antimicrobial drugs early in the 20th century has been prerequisite for the development of modern medicine. Without efficient antimicrobial drugs, advanced surgery, intensive neonatal care, organ transplants and chemotherapy would be impossible. With increasing bacterial resistance, we may not be able to support these medical advances. Thus, antimicrobial resistance surveillance and infection prevention and control are important for the welfare of humanity (2).

During millions of years, bacteria have developed resistance mechanisms to diminish the effects of antimicrobial substances released from competing bacteria, plants and fungi. The antibiotic era started in 1928 with Alexander Fleming’s discovery of penicillin, a substance produced by a fungus which had the ability to inhibit bacterial growth. In 1935, Gerhardt Domagk documented the antibiotic effects of sulphonamides. Within few years, sulphonamide, penicillin and an increasing number of other antimicrobial drugs were produced industrially and used to cure and prevent bacterial infections in animals and humans. Soon it also became evident that many bacteria adapted to the new drugs (Fig. 1), e.g. by changing their target proteins, developing efflux pumps to remove drugs from the bacterial cell, changing their metabolic pathway affected by the drug or by producing enzymes inactivating or blocking the drug (45).

As resistance to β-lactams and colistin are the main issues in this thesis, a short introduction to the development of bacterial resistance towards these drugs follows below.
**5.4.2 β-lactams and β-lactamases**

β-lactams represent a broad class of antibiotics characterised by a β-lactam ring in their molecular structure. Penicillin is the best known β-lactam, but a diversity of new members of this group of antibiotics have been discovered and developed during the last 60 years. Even today, ninety years after Fleming’s discovery of penicillin, β-lactams are our most important antibiotic drugs (4).

In the different β-lactam antibiotics, the β-lactam ring is bound to other molecules, and according to these additional molecules we classify the drugs into the following subgroups: penicillins, cephalosporins, carbapenems and monobactams. The drugs bind to penicillin binding proteins (PBPs) in the bacterial cell wall, thereby disrupting the cell-wall synthesis. β-lactams are considered bactericidal antimicrobial agents.

Resistance to β-lactams has developed along several axes (46):

1. Aminoacid alterations in the bacterial PBPs can reduce drug affinity, and bacteria may acquire new PBP-encoding genes with lower drug affinity.
Introduction

Changes in PBP affinity is the mechanism behind methicillin resistance in *Staphylococcus aureus* (MRSA).

2. In Gram-negative bacteria, the β-lactams must penetrate through the bacterial outer membrane porins to reach the PBPs. Changes in these porins can reduce permeability, and cause antibiotic resistance.

3. Another way for the bacteria to defend themselves against β-lactams is by producing β-lactamases. β-lactamases are enzymes that resemble PBPs, and therefore are able to hydrolyse and thereby inactivate β-lactams.

Many genera of Gram-negative bacteria have naturally occurring β-lactamases which belong to the same superfamily of proteins as the PBPs. These enzymes have probably existed for several million years (47). The first β-lactamase, a penicillinase, was discovered in *E. coli* in 1940, even before penicillin was released for clinical use (48). However, the increasing use of penicillin during the post-war years was accompanied by fast emergence of plasmid-mediated penicillinases in *Staphylococcus* species. Today, penicillin is no longer a primary treatment option for staphylococcal infections.

By adding new molecules to the β-lactam ring, new agents could be protected against the penicillinases. However, in the early 1960s, the first transferable broad spectrum β-lactamase in Gram-negatives was discovered in a strain of *E. coli* derived from a patient called Temoniera, and the enzyme was designated TEM-1 (49). The TEMs quickly spread world-wide to other strains of *E. coli* and to other Enterobacterales, as well as to other common pathogens like *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* (50). Another common type of plasmid-encoded broad spectrum β-lactamase is SHV-1 (for sulphhydryl variable). This enzyme is intrinsic in *Klebsiella pneumoniae*, but plasmid mediated in *E. coli* and other Enterobacterales (49).

As the antibiotic race developed, the cephalosporins and carbapenems were introduced in the 1970s and 1980s, and shortly thereafter, new transferable extended-spectrum β-lactamases (ESBLs) as well as carbapenemases appeared. Plasmid encoded mutants of TEM and SHV were the first ESBLs to be detected (50, 51). Today, the dominating
ESBLs on all continents are the CTX-Ms, which is short for cefotaximase München (52), named after the preferred substrate of the first CTX-M to be discovered, and the city where it was found.

Since their appearance in the late 1980s, a vast diversity of CTX-M enzymes have been described, and consecutively numbered. They have also been divided into phylogenetic groups according to their amino acid sequence similarities (52), with the consequence that the different groups comprise CTX-Ms with diverse numbers. E.g. CTX-M group 1 consists of CTX-M 1, 3, 10, 12, 15, 22, 23, 28 and growing, as new CTX-M enzymes are characterized continuously. A multiplex PCR with consensus primers that detects the genes of the most common CTX-M groups has been developed as a valuable screening tool in epidemiological research when determination of the exact nucleotide sequence is unnecessary (53). Information on the diversity of β-lactamases can be retrieved from the National Center for Biotechnology Information (NCBI) which curates a database of antimicrobial resistance genes, including β-lactamase alleles and protein sequences (www.ncbi.nlm.nih.gov/pathogens/submit-beta-lactamase/, last accessed May 19th 2018).

The CTX-M enzymes are thought to originate from the chromosomes of different Kluyvera spp. When these genes were mobilized from chromosome to plasmids, they could rapidly spread to new species (52). Selective pressure from the increasing use of extended spectrum cephalosporins in hospitals during the 1980s and 1990s allowed for the fast dissemination of CTX-M producing bacteria, primarily E. coli and K. pneumoniae (54). From 2000 and onwards, CTX-M has become a global problem, frequently detected in bacterial strains from humans, animals, and the environment (55). This coincides with the release of third generation cephalosporins from patents and their introduction as easily available, oral generic antibiotics. In Europe today, and also in Norway, the most common groups are CTX-M group 1 and CTX-M group 9 (4, 32, 55).

As new types of β-lactamases have been discovered, new nomenclature and different classification systems have been proposed. Initially, the designation extended-spectrum β-lactamase (ESBL) was not used for all β-lactamases with abilities to
hydrolyse the extended-spectrum cephalosporins, but was reserved for enzymes with
distinct biochemical (substrate and inhibitor) profiles, the Bush-Jacoby-Medeiros
classification (56), or distinct amino acid sequences, the Ambler classification (57).
Several transferable β-lactamases with different molecular and chemical properties,
e.g. AmpC β-lactamase, were not ESBLs by these definitions.

In 2009, Giske and co-workers suggested a new ESBL-classification, which aimed to
provide a functional nomenclature accessible to clinicians, infection control
professionals, health care leaders and politicians as well as microbiologists (58). They
suggested calling all transferable β-lactams with the ability to hydrolyse extended-
spectrum cephalosporins and/or carbapenems ESBLs, and to divide this diverse group
into three sub-classes (Figure 2). Enzymes belonging to the functional class 2be after
the Bush-Jacoby-Medeiros definition were grouped together and called ESBLA. These
are predominantly of Ambler class A, and are able to hydrolyse oxyimino-
cephalosporins and monobactams, but not cephamycin or carbapenems. They are also
inhibited by classical β-lactamase inhibitors. Plasmid mediated AmpC (Ambler class
C) and OXA β–lactamases (Ambler class D) were put together in a common group,
ESBLM, where the M stands for miscellaneous. β–lactamases with the ability to
hydrolyse carbapenems were called ESBLCARBA. In this thesis, “ESBL” will refer to
ESBLA unless stated otherwise.

Figure 2  ESBL-classification according to Giske et al.
(Source: Søraas: Extended spectrum beta-lactamase producing Enterobacteriaceae – aspects on
carriage, infection treatment. University of Oslo 2014). Printed with permission from A. Søraas.)
5.4.3 Colistin and colistin resistance

Colistin was discovered in the 1940s, and belongs to a class of antimicrobial drugs called polymyxins. It is produced by the bacterium *Paenibacillus polymyxa subsp. Colistinus*, previously known as *Bacillus polymyxa* (59, 60). The positively-charged polymyxins act by cationic disruption of the negatively-charged lipid A component of lipopolysaccharide (LPS) of the Gram-negative bacterial cell wall. The polymyxins have no effect on anaerobes or Gram-positive bacteria, as they do not have an LPS-containing outer membrane. Several Gram-negative species also have intrinsic resistance to polymyxins, e.g. *Proteus sp.* and *Serratia sp.*, presumably caused by constitutive gene expression resulting in addition of cationic molecules to LPS (59).

Due to potential nephron- and neurotoxicity, colistin use in humans had greatly diminished by the 1980s (61), but the drug was still extensively used in farm animals in many parts of the world (62). As the ESBLs and carbapenemases emerged and challenged cephalosporin and carbapenem treatment for Gram-negative bacteria, colistin gained new credit as a “last line of defence”-drug in human medicine (59, 63).

The plasmid borne *mcr-1* gene conferring colistin resistance was first described in China in 2015 (64). Prior to this discovery, resistance to colistin had only been known to be mediated by chromosomal mutations leading to changes in the LPS by addition of cationic molecules (59). The product of *mcr-1* is a phosphoethanolamine transferase, catalysing the addition of cationic molecules to LPS, similar to the resistance mechanism for chromosomal resistance (63, 64).

During 2016 and 2017, there have been reports from many parts of the world that *mcr-1* frequently occurs in combination with CTX-M type β-lactamases, rendering the isolates resistant to both cephalosporins and colistin (65, 66). Co-resistance to carbapenems has also been found (61). Lately, several new plasmid encoded variants of *mcr-*genes have been discovered (63, 67). As resistance towards cephalosporins and carbapenems increases globally, colistin now is on the WHO list of critically important antimicrobials for human medicine (6). The occurrence of a resistance mechanism
easily transmitted between clones and species by several different plasmids has caused great concern (68).

5.4.4 Plasmids and mobile genetic elements
As mentioned above, the CTX-M enzymes have gradually become the most frequent ESBLs in Enterobacterales during the last 20 years (54). This epidemic spread seems to be the result of several factors. The introduction of \( bla_{CTX-M} \) genes in highly mobile broad-host range genetic platforms (plasmids and conjugative transposons) has allowed the genes to be transferred between many different species and strains. Secondly, the introduction of these elements in particularly successful bacterial clones has favoured their dissemination even more (32, 54).

Plasmids are extra-chromosomal DNA molecules capable of autonomic replication (17, 69), which can be transferred between bacteria of the same species or similar species (narrow host range plasmids), but often also between bacteria of different species, genera and kingdoms (broad host range plasmids) (17, 70). Not all plasmids encode for their own mobilization and transfer, but such plasmids can be transferred along with chromosomal self-transferrable genetic elements (conjugative transposons) or with conjugative plasmids which encode for transfer (70). The structure of plasmids is normally circular, with genetic backbones encoding plasmid replication and maintenance functions. They can acquire mobile genetic elements called transposons, which beside essential genes for plasmid mobilization and transfer, may contain accessory determinants that encode antibiotic resistance or virulence factors (Figure 3) (69, 71). Hence, plasmids represent one of the most difficult challenges for counteracting the spread of antimicrobial resistance (29).
Figure 3 A simplified illustration of a typical IncF plasmid. The backbone/core regions include: replicons (yellow), transfer region (orange) and maintenance region (green). Variable regions (red and pink) are flanked by IS elements (blue). Undefined regions are grey. (Source: Løhr, Extended spectrum β-lactamase producing Klebsiella pneumoniae. A neonatal intensive care unit outbreak, long-term colonization in children and plasmid characteristics. University of Bergen 2014). Printed with permission from I. Løhr.

An insertion sequence element is in itself the simplest form of transposon, consisting of a transposase encoding gene surrounded by inverted repeated elements (69). The transposase enables mobilization of the surrounding gene segments. A composite transposon contains both an insertion sequence element and additional genes. Such transposons may contain a variety of genes, flanked by insertion sequence elements, which enable mobilization of their surrounding gene segments (69, 71).

Historically, plasmids have been categorized into separate incompatibility types (Inc types), based upon the observations that plasmids with the same replicon type will not stably co-exist in the same bacterial cell. More recently, PCR based replicon typing methods and WGS based methods for plasmid characterisation have been developed (71, 72). ESBLs are often linked to plasmids of the IncF-type, but they are also frequently found on other plasmid subtypes (71, 73, 74).
5.4.5 Successful clones

The spread of antimicrobial resistance through successful clones are effective for several reasons. These clones are characterized by increased ability to effectively colonise new hosts and survive in the environment (75). Collection of resistance mechanisms towards multiple antibiotic classes in a single bacterial clone can be advantageous for the clone’s survival in hospitalized hosts where they are subject to selective pressure from various antibiotics. All their resistance genes are transferred from mother to daughter cells, by virtue of the strain’s spread and increasing prevalence (32). Furthermore, the successful clone has multiple opportunities to act as a donor and transfer its resistance elements horizontally to other strains, species or genera (32, 74). It may also be that a widespread, successful coloniser is picking up genetic resistance elements on many occasions, and from different sources (74).

When the ESBLs were first discovered, *K. pneumoniae* was the most prevalent ESBL host, but today *E. coli* is the most frequently reported ESBL-producer from clinical samples, and the CTX-M-type ESBLs dominate (54). Since their first appearance in the late 1980s, the CTX-M enzymes have spread globally. According to recent publications it seems that there is some consensus in that their rapid dissemination is supported by both horizontal gene transfer and the spread of successful clones (32, 76). Thus, there are three important factors that have supported the CTX-Ms world dominance:

1. The collection of gene cassettes in integrons containing CTX-M-genes and genes conferring resistance to other drugs (co-resistance) and virulence factors that facilitate bacterial survival in different environments (co-selection).
2. The incorporation of such integrons into broad-host range plasmids.
3. The incorporation of these plasmids in a successful clone, such as *E. coli* B2 O25:H4-ST131.

The *E. coli* B2 O25:H4-ST131 clone has received particular attention as it is a virulent, uropathogenic clone that dominates in clinical ESBL-producing isolates from several
Introduction

continents (32, 55). The clone has various PFGE-types and MLVA-types, and hence can be divided into different strains. By investigating historical collections of ExPEC from several countries, it has been shown that the clone has been present both in communities and hospital settings on different continents for decades, but that ESBL acquisition through the adaptation of IncF plasmids probably occurred around the year 2000 (55).

5.5 Environmental dissemination of ESBLs – the One Health perspective

In the recent years, the One Health perspective has become more prominent in national and international action plans against antimicrobial resistance (1, 77). Enterobacterales thrive in various environmental compartments, and it is recognized that ESBL-E is not a problem restricted to samples from health care services, but also occur frequently in healthy humans (78, 79), companion animals (80), live-stock (80), wild life (81-83), soil (84), sewage (85), food (86) and water (23, 85, 87). However, the prevalence of ESBL-E in hospital and community environments varies substantially throughout the world. Historically, surveillance data have been scarce from most countries, and countries with surveillance programs have to a large extent used data from human clinical samples and neglected data collection in other compartments (88).

Transmission of ESBL-E occurs between many ecological niches (89, 90), and several research initiatives have been launched to improve our understanding of transmission routes, and to develop effective means to hinder further dissemination (91). Examples of possible interventions are improvements in sewage plants and drainage systems (85), closed containment of hospital waste (82), and strict control of animal manure and human sewage sludge used as fertilizers (91-93).

The major bacterial clones and ESBLs from human clinical samples do not always dominate in soil, water, food, live-stock and wild life (23, 94, 95), even though the successful ExPEC clones are readily detectable in other ecological niches in many parts of the world (82, 96, 97). An explanation for this may be that the different clones are adapted to survive in different environments, and that ESBL-E dissemination is
due to a combination of clonal spread and plasmid transfer between different clones and species (55).
6 Aim of this doctoral thesis

The overall aim of this thesis was to increase our knowledge regarding the occurrence, dissemination and persistence of ESBL-E in different ecological compartments in Norway.

The specific aims were:

- to examine the prevalence of ESBL-E faecal carriage in patients with gastroenteritis, and explore the possible association between carriage and foreign travel
- to detect patient and microbe related risk factors for prolonged faecal ESBL-E carriage in patients with urinary tract infection caused by ESBL-E
- to analyse intra-individual strain diversity of ESBL-EC detected in urine and consecutive faecal samples
- to investigate the occurrence of ESBL-EC in recreational water and wastewater
- to perform a genetic comparison of ESBL-EC strains from recreational water and wastewater with geographically related clinical isolates
- to examine the presence of multidrug resistance and plasmid mediated colistin-resistance genes in ESBL-EC strains from patients and aquatic compartments
Materials and methods

7 Summary of materials and methods

7.1 Ethics
The project and all its subprojects involving human participants have been approved by the Helse Sør-Øst Regional Committee for Medical and Health Research Ethics, following the Declaration of Helsinki principles (reference number: 2009/2037 BS-08901b). It is registered at ClinicalTrials.gov (Identifier: NCT01838213).

7.2 Study population
In the papers that constitute this work we have included two different human populations (papers I-III). One was a cohort of patients seeking medical help for gastrointestinal symptoms (paper I) and the other was a cohort suffering from urinary tract infection (papers II and III). The latter has previously been included in a case control study regarding risk factors for UTI caused by ESBL-E and effect of mecillinam treatment on UTI caused by ESBL-EC (98, 99). Both cohorts mainly included patients attending primary health care services.

The study population in paper I consisted of 273 patients in the catchment area of Akershus University Hospital (Ahus). The hospital’s catchment area includes parts of the capital city Oslo, but also rural regions to the north and east. The population of 450 000 people has a large immigration population compared to other parts of Norway. The included patients were between 0 and 94 years old at inclusion, median 37 years, 131 women and 142 men.

The study population in the other papers was from the catchment area of Vestre Viken Hospital Trust, which is of approximately the same size as Ahus. The Vestre Viken area is located west of Oslo, and has a similar mixed urban and rural catchment area, not comprising any parts of the capital, but containing the city of Drammen. The investigated cohort consisted of 101 patients, 12 men and 89 women, aged 19 to 95, median 55 years.
7.3 Sample collection from recreational water and wastewater

Recreational water samples were collected on five different dates during the summer season (May-September 2010) at one freshwater and three saltwater beaches located in the Vestre Viken area close to the Norwegian capital Oslo. Sterile containers of 1 liter were rinsed three times at the sampling site before the first sample was collected from a depth of 0.5-1 meter.

Daily flow proportional influent wastewater samples were collected from our local wastewater treatment plant (Vestfjorden Avløpsselskap) on the same days as the environmental samples. The samples were transported in sterile containers.

7.4 Laboratory methods

7.4.1 Bacterial culture and identification

Faecal samples (papers I and II) were cultured on commercial, selective, chromogenic medium (ChromID ESBL, bioMérieux, France) to detect Gram-negative, ESBL-producing strains. The plates contain cephalosporins which suppress growth of antibiotic susceptible strains, and allowed us to isolate antibiotic resistant colonies. Additionally, the medium contains drugs that inhibit AmpC, making it even more selective for ESBL<sub>A</sub> producing strains. Chromogenic media also have the advantage that the colonies change colour according to species, which further simplifies identification.

The faecal samples in paper II were also cultured on a non-selective agar for growth control, and in an enrichment broth (brain-heart-infusion broth with cefotaxime 2.5 mg/liter), which was incubated for 48 hours before sub cultivation on ChromID ESBL plates.

The urine samples (papers II and III) were cultured on a non-selective chromogenic medium (ChromID CPS3) and identification was done by the VITEK system (both from bioMérieux, France). The VITEK is a semi-automatic test method where a
bacterial suspension is added to cards containing small wells, each with specific reagents designed to reveal a biochemical trait by an optical manifestation. The machine reads the biochemical reactions in the wells, and based on patterns interprets the bacterial identification.

The recreational water samples (papers III and IV) were vacuum-filtered in portions of 10-500 mL, and the filters were incubated on both ESBL-selective and non-selective chromogenic agar plates (Brilliance agar, Oxoid, UK, and ChromID ESBL plates). Blue and purple colonies growing on the Brilliance agar plates were counted to estimate the amount of coliform bacteria and \textit{E. coli} in the samples. This method allowed us to calculate a CFU-ratio of ESBL-EC/total of \textit{E. coli}, and a CFU-ratio of ESBL-EC/total of coliform bacteria.

Wastewater samples were filtered, and portions of 1-50 µL of filtered water were subsequently spread on the same types of agar plates as the recreational water samples. Individual purple colonies on ChromeID ESBL plates representing potential ESBL-EC were frozen for later identification, AST and ESBL confirmation as described in the sections below.

The matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) method was used as an extra method for species identification on some of the isolates (all papers). These were either faecal isolates that did not contain any \textit{bla}_{CTX-M}, or isolates from faeces, water or wastewater that did not have MLVA-profiles corresponding to \textit{E. coli}. By this method, we detected a few \textit{Citrobacter}, \textit{Enterobacter} and \textit{Rahnella spp.} which had been misinterpreted as \textit{E. coli} due to their colour on the chromogenic plates.

The MALDI-TOF allows identification of a bacterial species on a molecular basis within minutes (100). A small volume of pure culture bacteria are smeared onto a metal plate and covered by liquid matrix. Then, a pulsed laser irradiates the sample, triggering ablation and desorption of the sample and matrix material. The bacterial molecules are protonated and deprotonated to become ionized, and then accelerated into a time-of-flight mass spectrometer. Finally, the mass spectra generated are
analysed by dedicated software and compared with stored profiles to identify the bacterial species.

### 7.4.2 Antimicrobial susceptibility testing

Three different methods of antimicrobial susceptibility testing (AST) have been used in this work. For testing of faecal samples (paper I), we used the EUCAST disk-diffusion method (101). In this method, a bacterial suspension of predefined density is added evenly to an agar plate. Subsequently, disks containing antimicrobials are put on the agar plate, and the plate is incubated at 35°C in ambient air conditions. After 16-24 hours the plate is examined for growth around the antimicrobial disks, and the zone-diameters of growth inhibition are measured. By comparing the zone diameters to international, standardized and validated species-specific breakpoints, we can determine whether or not the isolate is susceptible to the relevant drug (101).

For AST of clinical urine samples, environmental samples and faecal samples from long-term carriers (papers II and III) we used the VITEK method (bioMérieux, France). This is a semi-automatic test where a bacterial suspension is added to cards containing small wells with different antimicrobials in different concentrations. The machine monitor bacterial growth in the wells and the software interprets the results into an antibiogram related to the bacterial species and predefined antimicrobial susceptibility breakpoints for the relevant species.

Susceptibility testing for colistin was performed at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance by a broth microdilution method using Sensititre plates (ThermoFisher, USA). The wells in the plates contain different concentrations of several antimicrobials including colistin. A standardized bacterial inoculum is added to each well, and after overnight incubation (16 – 20 hours) at 35°C, bacterial growth is detected visually or automatically by a photometer.

The AST results were interpreted according to EUCAST clinical breakpoints (www.eucast.org).
7.4.3 Detection of ESBL phenotype

In paper I, a disk approximation test (102), which was a modified version of the double-disk synergy test (103) was used for detection of an ESBL-phenotype. In this test, a standardized bacterial suspension is smeared onto a Mueller Hinton agar plate, and a paper-disk containing ampicillin and clavulanate is placed in the centre. Paper disks containing different cephalosporins are placed 2.5 cm from the centre, and the plate is incubated for 16-24 hours. After incubation, the plates are examined for additional inhibition areas between the disks. Asymmetrical inhibition areas indicate synergy between the clavulanate in the middle and the cephalosporins, and therefore potential ESBL activity.

In paper II and III, E-test (AB-Biodisk, bioMérieux, France) was used to confirm ESBL and AmpC phenotypes, by using strips with cefotaxime/clavulanic acid, ceftazidime/clavulanic acid and cefotetan/cloxacillin respectively. Additionally, the combination disk test (Rosco Diagnostica, Denmark) (104) was performed on isolates which were negative for \( \text{bla}_{\text{CTX-M}} \). This test requires six disks, one with cefotaxime, one with ceftazidime, one with a combination of each cephalosporin with clavulanate (ESBL inhibitor) and one containing each cephalosporin in combination with cloxacillin (AmpC inhibitor). A difference in zone diameter >5mm between one or both of the cephalosporins and the combination disks indicates the presence of ESBL or AmpC respectively.

7.4.4 Detection of ESBL genotype

Several methods have been used to detect ESBL genotypes. In paper I, our primary method was a microarray system, the Check-MDR CT101 (Check-Points, the Netherlands). In this method, purified bacterial DNA is subject to a ligation step, followed by PCR amplification and labelling. PCR products are then hybridised to the array and colourimetrically revealed by use of a tube-reader device and software from the manufacturer. The results are visualised by a pattern of easily detectable spots that represent a positive or negative result for each of the included resistance gene targets. The Check-MDR CT101 enables detection of \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{NDM}}, \text{bla}_{\text{KPC}} \), as well as
the following plasmid mediated AmpC variants: CMY, DHA, FOX, ACC, ACT, MIR and MOX.

In paper II, we initially searched for \( \textit{bla}_{\text{CTX-M}} \) by a multiplex PCR that allowed us to detect \( \textit{bla}_{\text{CTX-M}} \) of group 1, group 9 and group 2. This PCR also contained a generic probe for the detection of \( \textit{bla}_{\text{CTX-M}} \) of groups other than group 1 (53). Isolates which were negative in the \( \textit{bla}_{\text{CTX-M}} \) PCR were investigated further by the microarray as described above.

In papers III and IV, WGS was used for the detection of resistance genes (see below), and isolates containing both ESBL\textsubscript{A} and ESBL\textsubscript{M} were included in the statistical analyses.

### 7.4.5 Whole genome sequencing

All \textit{E. coli} isolates from urine and wastewater, and one isolate representing each \textit{E. coli} strain from recreational water were analysed by WGS. This was performed at the Wellcome Trust Centre for Human Genetics, Oxford UK on the HiSeq platform from Illumina, USA, generating 150 base pairs paired-end reads. DNA-extraction and quality assessment was performed at Akershus University Hospital. The bacteria were cultured on lactose agar, suspended in ATL-buffer with proteinase K and lysed in a shaker-incubator. Then we added Rnase before extracting DNA with QIAsymphony DNA Mini Kit (Qiagen, Germany). Quality checks were performed by NanoDrop spectrophotometer, followed by fluorometric measurement of DNA content with Quant-iT (both instruments from ThermoFischer Scientific, USA) prior to dilution and shipment. For assembly, MLST-typing, detection of plasmid replicons, antimicrobial resistance genes and virulence genes we used publicly available tools from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/index.html). We used the threshold default settings in the software tools, which at the time were a threshold of 98% match and a minimum length of 60% for ResFinder and VirulenceFinder, and a selected threshold for ID match of 98% for PlasmidFinder. Phylogroups B2 and D were detected through BLAST-search for the \textit{chuA} gene.
7.4.6 **Multilocus variable number of tandem repeats assay**

As described in the introduction, multilocus variable number of tandem repeats assay is a typing method used for various bacteria. It is a PCR-based method that analyses the variation in number of tandem repeats (VNTRs) in specific DNA loci with variable mutation rates. The MLVA protocol used in this thesis was developed by Lindstedt and co-workers in 2007 (105), and uses 7 different loci to describe the MLVA profiles. PCR-products were subjected to capillary electrophoresis, and each peak was identified according to colour and size by GeneMapper software (Applied Biosystems, USA). Allele numbers were assigned according to fragment sizes as previously described (105). Character values were entered into BioNumerics (Applied Maths, Belgium), and dendrograms were constructed using categorical coefficients and the Ward algorithm. A standard minimum spanning tree was generated using categorical coefficients together with the single and double locus variance priority rules.

7.5 **Statistical methods**

Statistical analyses in all papers were conducted using PASW statistics software, version 21.0 (IBM SPSS, USA). Additionally, we used Stata Statistical Software release 10 (paper I) and 13 (paper II) (StataCorp, USA). Univariate analyses were performed by Fisher’s exact test for categorical data and the Student’s t-test for continuous data. The association between potential risk factors and ESBL-E carriage was quantified by odds ratio (OR) with 95% confidence interval (CI). A p-value ≤0.05 was considered significant. The continuous variables were dichotomized in the presented models, but the main results were robust to alternative operationalizations.

In paper I we defined the age-groups “elderly” as those aged ≥70 years and “children” as those aged ≤10 years for the analysis of risk factors. Due to incomplete data regarding antimicrobial use and previous ESBL status, these risk factors were excluded from the analysis.

In paper II, the planned sample size was calculated from the assumption that the prevalence of ESBL-E carriage after one year would be 50% in the population who
was exposed to any risk variable, with an OR above 2.3 compared to the unexposed population. With alpha set to 0.05 and beta 0.2, about 70 participants would be needed for the study. Point prevalence rates were calculated for each three-month period, and carriage survival was estimated in a Kaplan-Meier curve. Because many patients with negative samples later became positive in new samples, we found it appropriate to define ESBL-E clearance as two consecutive negative samples, and to present an estimate where the event end-point was set to occur at the first negative sample if the following sample also was negative. For further risk factor analysis, we used carriage at 13 months after inclusion as the dependent variable in uni- and multivariable analyses. Multivariable analyses were preceded by estimation of correlation between risk factors in a multiple logistic regression model. To reveal significant risk factors for persisting ESBL-E carriage at 13 months, we conducted backwards elimination of variables, starting by including previously described risk factors related to microbe- and patient characteristics. There was only one variable significantly associated with prolonged carriage. Thus, an exploratory model of hypothetical risk factors was presented.
8 Summary of results

8.1 Prevalence of faecal ESBL-E carriage in patients with gastroenteritis and the association between carriage and travel history (Paper I)

ESBL-E were identified in 16% (43/273) of patients with diarrhoea. In samples submitted for detection of C. difficile, the prevalence of ESBL-E was 10% (5/51), as opposed to 17% (38/222) in the samples submitted for detection of enteropathogenic Enterobacterales, a difference which was not regarded as significant (p=0.29). The prevalence rate of ESBL-E was 9% (19/203) among patients who had not travelled abroad, or had missing travel information. The prevalence rate for those who had travelled abroad was 34% (24/70), while patients who had travelled to Asia were carriers in 56% of cases (18/32). Thus, patients who had travelled to Asia, had a strikingly higher risk of ESBL-E carriage compared to patients who had not travelled abroad or where information on travel history was missing (OR 16, p<0.001). In the small group of ten patients where it was explicitly confirmed that there had been no travel, we did not detect any ESBL-E. There were no differences in carriage risk related to the patients’ sex or age group.

E. coli was the most frequently detected ESBL-producing species (N=44), and two samples contained isolates of atypical EPEC. Other ESBL producing species were Klebsiella pneumoniae (N=4), and Rahnella sp. (N=1). We did not detect any ESBL-producing Salmonella, Shigella or Yersinia. CTX-M group 1 (80%) and CTX-M group 9 (20%) were the only detected ESBLs in E. coli, while one K. pneumoniae isolate produced a SHV-ESBL, and the Rahnella contained a CTX-M β-lactamase of the RAHN- group.
8.2 ESBL-E faecal carriage in patients with urinary tract infection caused by ESBL-E; clonal diversity and risk factors for prolonged carriage (Paper II)

Ninety-three patients with UTI caused by ESBL-producing \textit{E. coli} or \textit{K. pneumoniae} were followed for one year, 43 patients for two years, and 13 patients for three years or more. Ten participants were male, and the median age at inclusion was 55 years (18-95).

The observed faecal ESBL-E carriage rate was 72/93 (77\%) at inclusion, 53/87 (61\%) at 4 months, 48/86 (56\%) at 7 months, 40/84 (48\%) at 10 months, 33/84 (39\%) at 13 months, 8/43 (19\%) at two years, and 2/13 (15\%) after three years or more. 13 of 93 patients (14\%) with at least one negative sample were positive in later samples. The prevalence rates were similar to the results obtained from survival analysis.

There was a significant positive association between phylogroups B2/D in urine samples and faecal carriage at 13 months (OR 3.8, p=0.027). The B2/D phylogroups were represented by 14 different STs, with ST131 constituting the largest group (N=33/64). We compared the patient group who had ESBL-EC belonging to phylogroup B2 or D with the other patients one year after UTI. In paper II we did not investigate further how the ESBL-EC prevalence varied in the two groups earlier in the investigation period. This variation is illustrated in Figure 4, where we see that the two groups separate right from the beginning. The patients with UTI caused by ESBL-EC of phylogroups B2 or D are also more likely to be faecal carriers within the first month after the UTI.
Results

Figure 4 ESBL-EC faecal carriage prevalence at different time points
Patients with ESBL-EC of phylogroup B2 or D (N=64-59) in urine compared to patients with ESBL-EC of other phylogroups (N=25-21). Number of included patients (N) decline with time.

MLVA of faecal isolates from participants who were carriers for more than a year revealed a large diversity among ESBL-producing *E. coli* strains within the same individual. Changes in MLVA-type occurred during protracted faecal carriage in 89% of the patients. In the patients who were carriers for one year or longer, the patients’ faecal MLVA-type differed from the urinary type in 54% of the samples. Only two of 35 long-term carriers (6%) carried the original urinary MLVA-type in all their faecal samples. We also observed that ESBL occurred in several species of *Enterobacteriaceae* within the same patient.

We found no correlation between duration of carriage, sex, age, number of household members, chronic UTI, comorbidity or antimicrobial use. Nor did we observe any association between prolonged carriage and foreign travel.
Results

8.3 Detection and characterization of ESBL-EC in recreational water and wastewater and genetic comparison with human ESBL-EC (Paper III)

In the studies of recreational water and wastewater, both strains containing ESBL<sub>A</sub> and strains containing ESBL<sub>M</sub> were included in the analyses. We detected ESBL-EC in 8/20 (40%) recreational water samples, representing all investigated sites. The ratio of ESBL-EC to total number of *E. coli* colony forming units varied from 0 to 3.8%. ESBL-EC were present in all wastewater samples in ratios of 0.56-0.75%. ST131 phylogroup B2 dominated in urine samples (34/94), was frequent in wastewater samples (16/91), but rare in recreational water samples (5/82). ST38 phylogroup D was well represented in all sources, while ST10 phylogroup A dominated in water samples. Eight STs were detected in all three compartments, and CTX-M 15 was the dominating ESBL regardless of source. Indistinguishable ESBL-EC MLVA-types were detected in all compartments.

The two dominating replicon types were IncF and IncI1, present in 90% and 31% of the strains, respectively. No significant differences in IncF or IncI1 prevalence were observed between the different compartments. A search for virulence factors associated with gastrointestinal *E. coli* infections (*eae*, *eaeA*, *hfp*, *stx1/vtx1*, *stx2/vtx2* and *ipaH*) revealed two strains with *eae*. These isolates were both retrieved from wastewater. One of them was ST3 and contained *bla<sub>CTX-M-15</sub>* while the other was ST381 and contained *bla<sub>CMY-58</sub>* (ESBL<sub>M</sub>).

8.4 Multidrug resistance and plasmid mediated resistance genes in ESBL-EC strains from patients and aquatic compartments (Papers III and IV)

ESBL-EC isolates from urinary tract infections were more likely to be multidrug-resistant as determined by phenotypic AST compared to isolates from recreational water and wastewater (p<0.001).
Results

Regardless of sample source, CTX-M-15 which belongs to CTX-M group 1 was the dominating ESBL, followed by CTX-M-14 from CTX-M group 9. Several strains contained multiple ESBLs, the most common combination being CTX-M and TEM. There were also strains with combinations of ESBLs and other β-lactamases, predominantly CTX-M combined with OXA-1. Acquired resistance genes conferring resistance towards trimethoprim, macrolides, phenicols, quinolones and aminoglycosides were less frequent in recreational water strains compared to urinary strains.

One in twenty samples from recreational water contained ESBL-producing *E. coli* isolates of ST10 with the plasmid borne *mcr-1* gene conferring colistin resistance. This sample was collected in September 2010, and was investigated by whole genome sequencing in 2016. No acquired colistin resistance genes were detected in strains from clinical urinary samples or wastewater.
9 General discussion

9.1 Prevalence of faecal ESBL-E carriage

Paper I received international attention by being mentioned in the review session “The year in infection control”, at the European Conference on Clinical Microbiology and Infectious Diseases (ECCMID) in Barcelona in 2014. Such a high prevalence rate (15%) of faecal ESBL-carriage in patients with gastroenteritis, even in Norway where antimicrobial consumption is limited, is alarming.

In 2011 when this study was conducted, little was known regarding the prevalence of ESBL-E faecal carriage in the Norwegian population. A large, randomized trial to assess this question was beyond our means, and instead we decided to start by examining the samples we already had access to, i.e. the stool samples submitted to the laboratory for diagnosis of potential gastrointestinal pathogens. Reports from other countries had already indicated high levels of ESBL-E faecal carriage in patients returning from the sub-Indian continent, the Middle-East and Northern Africa, and the prevalence seemed to be higher in patients who had suffered from gastroenteritis during or shortly after their journey (83, 106, 107). More recently, there have been published studies on ESBL-EC carriage in healthy volunteers from Norway (108, 109) and Sweden (110), and they all reveal prevalence rates of less than 5%.

Our samples were from a selected cohort from the north-eastern parts of the counties Oslo and Akershus, and consequently the findings do not necessarily reflect the general level of ESBL-E carriage in patients with gastroenteritis. However, the prevalence rate we describe is very close to the findings of Peirano et al. who found 14% carriage in diarrhoeal stool samples in Canada (107). In their study, like ours, travellers were considerably more likely to be ESBL-E carriers compared to non-travellers. Both studies show that those who have visited Asia are at highly increased risk of faecal ESBL-E carriage. Other studies from Scandinavia describe gastroenteritis as a separate risk factor for post-travel ESBL-E carriage, and based on
those studies, there is reason to believe that travellers who do not develop diarrhoea are also less likely to be colonised by ESBL-E (106, 111).

Identification of patients with increased risk of being ESBL-E carriers is important, as faecal carriage is assumed to be a risk factor for ESBL-E infection. According to Norwegian National Guidelines, empiric antimicrobial treatment for severe infections should be adjusted according to the patient’s risk of being infected with multidrug resistant bacteria (https://helsedirektoratet.no/retningslinjer/antibiotika-i-sykehus/sekjon?tittel=om-multiresiste-mikrober-forekomst-10498, last accessed March 13th 2018).

9.2 Duration of faecal ESBL-E carriage and risk factors for prolonged carriage

To investigate ESBL-E faecal carriage duration, we instigated a longitudinal observational study (paper II), where we relied on the participants to deliver samples and questionnaires regularly. Of a total number of 172, 101 patients agreed to participate, and the epidemiological characteristics of the patients included and those who declined to participate, were quite similar. Only 10% of those included were men. This is as expected, because urinary tract infection is much more frequent in women. Among the included patients, 87% complied with the study protocol, a number judged as satisfactory. Ninety-three patients were followed for one year, 43 were followed for two years, and 13 patients were followed for three years or more.

We used two different measures to estimate the ESBL-E clearance rate 13 months after inclusion; 1) survival analysis with a Kaplan Meier plot and 2) simple point prevalence calculation. The results for the two methods gave quite similar results for carriage at 13 months, 44% versus 39%. The difference is due to the fact that some participants had negative samples at one point, but delivered positive samples later. As clearance was defined as two consecutive negative samples in the survival analysis, some patients were not counted as “cleared” at 13 months, although they had one single negative sample at that time. Other authors have used three consecutive
negative samples as end-point in survival analysis for similar carriage studies (22), while some operate with point prevalence only, and consider patients with one single negative sample as cleared (112, 113).

The carriage clearance rates we observed were comparable to studies from Sweden (112) and Slovenia (113). We also confirmed the findings from the Swedish study that phylogroup B2, and in our case also phylogroup D, were associated with prolonged carriage.

In accordance with the carriage studies mentioned above (112, 113), we did not find antimicrobial consumption to be correlated with prolonged colonisation. However, there are several studies observing association between previous antimicrobial use and infection with ESBL-E (98, 114), and a recent study from the Netherlands also found such a correlation in ESBL-E carriers recruited from a primary health care setting (115). Lack of significant correlation between prolonged carriage and the use of antimicrobials in our study may be a type II error due to our limited sample size. It is also possible that the majority of antimicrobials consumed by our study population (mecillinam and nitrofurantoin) are drugs with limited effect on carriage duration. Other presumed, but rarely occurring risk factors included in the model, such as more than three household members, male sex and high co-morbidity score, may also be a subject to type II errors. These issues are discussed further in chapter 9.7.

Surprisingly, there was no association between prolonged carriage and travel to high-prevalence countries. Our hypothesis was that travel to high-endemic areas would be a risk factor for acquiring new ESBL-E strains, thereby increasing the risk of being ESBL-E positive in subsequent samples.
9.3 Intra-patient variation in ESBL-EC genotypes and implications for infection control and outbreak investigations

We observed that many participants in the carriage-duration study (paper II) delivered a negative sample, but became positive again in later samples. To investigate whether or not this was due to re-colonisation by new strains, we intended to use MLVA-typing of all ESBL-EC. However, because we found considerable variations in MLVA-types in the majority of patients, we could not conclude whether the occurrence of a new MLVA-type after a negative sample was indicative of re-colonisation or not. If we had had WGS-data on all the faecal isolates and not only the urine strains, this could probably have clarified if acquisition of new strains was the likely cause of a genotype shift. However, at the time of publication, only the urine strains had been subject to WGS. In our study, the patients’ faecal MLVA-type differed from the urinary type in 54% of cases. We observed less concordance between urine strains and faecal strains than shown in previous studies of patients with non-ESBL *E. coli* UTI (116), but the differences may be due to different typing methods. Other studies have shown that the MLVA protocol we have used gives a slightly lower discriminatory power than PFGE (117), which in many settings so far has been considered the gold-standard method for outbreak investigations. We expected that the variety of strains would be greater in the travellers than in others. However, the variation in faecal genotypes was large regardless of travel.

Our observations regarding intra-individual *E. coli* strain diversity is supported by a paper published in March 2018. Dixit and co-workers used REP-PCR, WGS of selected strains and SNP-analyses to investigate the diversity in *E. coli* strains in biopsies from patients who had undergone colonoscopy (118). They confirmed that each host is likely to harbour different *E. coli* strains, and found that 83% of the strains detected were the consequence of independent immigration/establishment events. Furthermore they found that when multiple strains belonging to the same phylogroup were present in the same host, independent immigration/establishment events were the cause in 46% of cases, and that within-host evolution was the cause in 54%.
Discussion

In hospitals, we often use tracing of genetically closely related strains to assess whether cases are included in an outbreak or not (119-121). Accurate case definition is essential in all outbreak reports. Most Norwegian infection control personnel in hospitals have experienced MRSA-outbreaks, where we often use spa-typing, and also MLVA or microarray analyses as tools to categorize MRSA-isolates as identical or different (122). Our findings, along with others’, indicate that it is common to carry several different \( E. \ coli \) variants, as there is great plasticity in the \( E. \ coli \) pangenome and high recombination rates. Therefore, substantial genomic changes may occur within few generations (26). Hence, our experience and way of thinking in resolving outbreaks of \( S. \ aureus \) cannot be directly transferred to outbreaks of ESBL-EC. \( E. \ coli \) contamination from a common source should not be ruled out, even if the microbe fingerprints from randomly selected faecal isolates differ between patients. In Switzerland, Tschudin-Sutter and co-workers conducted an interesting study to assess the frequency of ESBL-E transmission between hospitalized patients sharing a room (119). In this study, transmission between room-mates was considered unlikely if the patients had ESBL-EC of different pulsotypes (PFGE-types). The study found that cross-transfer between patients sharing a room for 24 hours or more, occurred in only 1.5% of cases, but the overall occurrence of ESBL-E in the contact patients was 5.3%. As our study has shown that ESBL-EC carriers frequently are colonised with ESBL-EC of different MLVA-types, this Swiss study may have underestimated the rate of transmission between patients.

9.4 ESBL-EC dissemination in recreational water and wastewater

The data analysis in paper III was interesting, because we had no previous knowledge regarding ESBL-EC in recreational water and wastewater in our area. The methods we used to detect \( E. \ coli \) and other coliform bacteria were standard methods recommended by the Norwegian Institute of Public Health to assess the amount of faecal pollution of recreational water. As expected, the amounts of coliforms varied considerably throughout the summer season. Both temperature and recent rainfall are factors that may influence the bacterial load (85, 123), but these variables were not recorded in our study.
We found that ESBL-EC were readily detected in wastewater, and were frequently present in recreational water. However, they constituted only a relatively small portion of total *E. coli* in both environments (3.8% or less). Detection of ESBL-EC was associated with high amounts of total *E. coli*, as previously described by others (85). If the total amount of *E. coli* is very low, and the proportion of ESBL-EC is small, our methods are probably not sensitive enough for ESBL-EC to exceed the detection limits. Although studies on ESBL detection in various habitats are steadily increasing, there are still few studies describing not only the prevalence of ESBL-EC, but also the proportion of ESBL-EC compared to total *E. coli* in recreational water. In a similar study conducted in the Netherlands in 2011/2012, ESBL-EC represented 0-1% of total *E. coli*, and ESBL-EC were present in 62% of recreational water samples (85). In a study from Croatia in 2009 to 2013, Maravic and coworkers conducted a study of recreational seawater. Here they found considerably higher proportions of ESBL-EC (7.7%), even though they used non-selective culture methods (20). Some of the isolates from recreational water were clonally related to clinical isolates as shown by PFGE. As ESBLs in clinical *E. coli* isolates are more common in Croatia than in Norway and the Netherlands (5), it is not very surprising that they are also more prevalent in Croatian environmental samples. However, it is also important to note that the general levels of *E. coli* were much higher in the Croatian samples. Other *Enterobacteriaceae* than *E. coli*, i.e. *Enterobacter* sp. and *Klebsiella* sp., were also frequently detected. In summary, all three studies support our hypothesis that recreational water is a possible source of human exposure to ESBL-EC, and they illustrate that the levels of ESBL-EC vary in different locations.

### 9.5 Genetic comparison of human ESBL-EC strains with strains from recreational water and wastewater samples

We detected a large variation in ESBL-EC multilocus sequence types (STs) in the different compartments examined in paper III. Eight STs were found in all compartments. The pandemic, pathogenic clone ST131 phylogroup B2 dominated in clinical samples, was frequent in the wastewater samples, and rare in the recreational
Discussion

water samples. ST38 phylogroup D was present in all sources, while ST10 phylogroup A dominated in water, but was only found in urine on one occasion. ST10 has previously been described as a successful ESBL-EC occurring in both livestock and humans (80). It has also been detected in surface water and wastewater in Portugal (124), the Netherlands (85), the UK (90), from the river Danub (87), and in migrating penguins (125). The presence of ESBL-EC ST10 in different water and wastewater sources on different dates, calls for further investigation of the dissemination of this strain in other ecological niches in Norway, such as farm animals, manure, birds and wild animals. The other strains that we retrieved from all our sources (ST69, ST405 and ST410) are also well-known *E. coli* hosts of ESBLs, frequently found in both humans and animals (80).

We know from previous reports that recreational water can be a source for outbreaks of pathogenic *E. coli* that cause gastroenteritis. As we have shown in paper II that phylogroup B2 and D are associated with prolonged ESBL-E faecal carriage, one can speculate that these strains have properties that make it easier for them to colonise the human gut, e.g. after exposure to recreational water, through potable water and food or by indirect contact with contaminated surfaces. This is perhaps also reflected by the findings in paper II, where patients with UTI caused by B2/D strains had a higher likelihood of faecal ESBL-EC carriage at inclusion as well as after 13 months. It has been described that the pandemic ST131 clone has properties that favor attachment to human urinary epithelium, and this makes them important pathogens. However, little seems to be known regarding the infectious doses necessary for a ST131 ExPec strain to colonise the gut. The likelihood for an ESBL-colonised person to develop UTI caused by an ESBL-E is probably dependent on a large variety of patient related risk factors, and to investigate this, a comprehensive prospective study would be needed.

9.6 Multidrug resistance and plasmid mediated colistin resistance

Multidrug resistance was more frequent in clinical isolates than in recreational water and wastewater. We used publicly available on-line tools from the Center of Genomic Epidemiology (www.genomicepidemiology.org) in Denmark to search for plasmid
replicons (PlasmidFinder), virulence factors (VirulenceFinder) and acquired resistance genes (ResFinder). We made comparisons between AST and detection of acquired resistance genes for trimethoprim, gentamicin and ciprofloxacin, and found poor concordance between phenotypes and genotypes as detected by ResFinder. This is not surprising, as changes in chromosomal genes (altered expression of intrinsic genes) leading to resistance are common (126), and such mechanisms would not be detected by the ResFinder version 2.1 that was available for us at the time of the analysis (127). One could screen genome sequences for insertion sequences interrupting or modifying the expression of resistance-associated genes, but this is sometimes problematic due to constraints inherent in using short reads (44). Online databases are available for such investigations, i.e. the CARD database at www.card.mcmaster.ca. The applied criteria for gene coverage and gene similarity will also potentially influence the number of hits and the correlation with phenotype. Also, detection of potential resistance genes that are not expressed, may lead to potential over-estimation of resistance if only genetic methods are used. Moreover, many of the genes conferring resistance to aminoglycosides are substrate specific, and thus detection of one or more genes from this category does not imply that the isolate is resistant to all members of this antimicrobial class (128).

An advantage when using ResFinder was the easy detection of multiple β-lactamase genes within the same isolate. With our initial approach of first using PCR to look for bla<sub>CTX-M</sub> and then use microarray only if no bla<sub>CTX-M</sub> was detected, we missed several isolates with combinations of ESBL<sub>A</sub> and ESBL<sub>M</sub>, and some with a combination of several ESBL<sub>A</sub>.

Reports of mcr-1 in <i>E. coli</i> isolates from humans and farm animals are plentiful, as many old and recent strain collections have been examined during the last years. There are also reports on mcr-findings in wild birds and in the environment (61, 65, 66). New variants of plasmid mediated colistin resistance genes, mcr-2 – mcr-7 have been described recently (63), but were not included in the ResFinder database when we did our analyses. As the WGS-data are safely stored, it will be possible to look for new resistance determinants as soon as they are published, by using online tools as BLAST
Discussion

(blast.ncbi.nlm.nih.gov), ResFinder, or the CARD database. Previously, the only report of \textit{mcr-1} from Norway was in a patient presumably infected abroad (129). The discovery of \textit{mcr-1} on a public beach in Norway illustrates that antimicrobial resistance knows no boarders, and that a restrictive antimicrobial policy is not sufficient to keep us safe from being exposed even in our corner of the world.

9.7 Limitations and strengths regarding sample size and study design

In paper I, where we studied travel as a risk factor for ESBL-E colonisation, our only available information regarding travel and clinical symptoms was from the laboratory referral forms. It was not within the frames of the study to collect more detailed clinical or anamnestic information. Hence, the information we got was not standardized, and for the majority of patients (193/273, 71\%) there was no information at all regarding travel history. The study also included samples of two different diagnostic categories; 222 samples were submitted for detection of pathogenic Enterobacterales, and 51 samples were submitted for detection of \textit{C. difficile}. There may have been systematic differences between these two groups, e.g. regarding antimicrobial use prior to sampling, but because information was sparse we did not include antimicrobial consumption as a risk factor in the analyses. ESBL-E were detected in both sample categories, but in unequal amounts (10\% in the \textit{C. difficile} samples vs. 17\% in the others, not a statistically significant difference).

In paper II, information was collected through questionnaires. When asking study participants to fill out a questionnaire, we assume that the information given is reliable, i.e. that the participants remember correctly. However, the interval between the 13-months questionnaire and the three-year follow up questionnaire was long, and thus, the information from the last questionnaire may be less reliable than information from the earlier ones. The participants were also asked to report their travel history, whether they had housed visitors from foreign countries, all antimicrobial treatments that they had received, and whether the prescription came from a hospital or from primary care. It turned out to be essential that this information was supplemented by
data from the National prescription database, as many patients did not remember all prescriptions. On the other hand, whether the patient took their medication or not is not reflected by the data from the registry.

Not all samples and questionnaires were delivered exactly on time, and this influences the number of patients included in the prevalence rates we report. E.g., a patient may have delivered sample number two a month later than planned, and is therefore excluded from the prevalence at four months. However, by reporting the carriage rates using survival analysis, we can still use the information from sample number two, even if we received it later than planned. Moreover, the prevalence rates do not reflect the fact that several participants who delivered a negative sample were positive again in their next sample. Hence, we found it necessary to define ESBL-E clearance as two negative samples in the survival analysis. We discussed whether to define the clearance point at the first negative sample of the two consecutive negative samples, in between these two samples, or at the time of the second, confirmative negative sample. To give a conservative estimate of the carriage duration we chose to define the clearance time as early as possible. As a consequence, the latest possible clearance time in the survival analysis as reported in the paper, was at 13 months.

Our sample size is also a question for discussion. The carriage rates for the first 13 months are fairly reliable, with acceptable confidence intervals. A carriage rate of 39% (point prevalence) and 44% (Kaplan-Meier) after 13 months is in accordance with previous findings from similar studies (112, 113). For follow-up samples taken more than 13 months after the initial urine sample, fewer patients are available due to the study design. Thus, the confidence interval for carriage rate estimates widens. If we had continued faecal samples every three months even after the first year we would have been able to draw a more precise Kaplan-Meier plot of the survival estimate.

Regarding the risk factor analyses, the relatively small number of participants is an important limitation to our study. It is possible that we could have identified more risk factors for prolonged carriage, had the number of participants been higher. In statistical terms, one would say that we may have rejected the null-hypothesis even if
the null-hypothesis is true. This problem is often referred to as a “type II error”. The problem with type II errors diminishes if you have a larger sample size. To find the appropriate inclusion number for a study, researchers do power estimations and sample size calculations before starting their research. In practical life, the sample size is also dependant on limitations in time and resources. To make a sensible sample-size calculation for a prospective observational study, you need to anticipate 1) how frequent the risk factors occur in the study population, and 2) the effect of the risk factors. If a risk factor causes a marginally increased risk (low effect), you need a larger sample size to identify a significant difference between groups. In our case, it was difficult to make accurate anticipations, as we had little previous knowledge regarding effects of the risk factors, and how many in our study population who would experience one or more risk factors during observation. Based on the sparse available literature, sample size calculations were made with the conclusion that at least 70 patients would be needed in order to explore carriage duration and potentially confirm our hypothesis that patients who had received antimicrobials, or had travelled abroad were more likely to be ESBL-E carriers 12 months after UTI caused by ESBL-E.

In conclusion, the study from paper II has more participants than previous studies with similar design, and has to a large extent fulfilled its aim by illustrating the long duration of faecal ESBL-E carriage and identifying infection with ESBL-EC of phylogroup B2 and D as a risk factor for prolonged carriage. The study has also given us useful knowledge regarding antimicrobial consumption, comorbidity, travel habits and ESBL-E prevalence in the patient groups we have studied. Hence we now have the means to make more accurate sample-size calculations for future studies.

9.8 Limitations in sample handling and laboratory methods

An important issue to consider regarding duration of ESBL-carriage (paper II) is why some participants with a negative sample turned positive again later. Is this because the concentration of ESBL-E in the intestines or in the stool varies over time, is it because the sample was poorly taken, or because the bacteria died during transport? Or maybe the patient was indeed cleared of ESBL-E, but became re-infected later?
To rule out the transport issue, all samples were cultured on non-selective plates in addition to the selective ones, to assess that there were bacteria present in all samples. There was only one case where this growth-control failed, in which case a new sample was collected. The average time from sampling to culture was 4.98 days for positive samples and 5.12 days for negative samples, i.e. practically the same.

The selective, chromogenic agar that was used in this study, the ChromID ESBL (bioMérieux, France), have been tested for sensitivity and specificity in several settings earlier and the results have recently been reviewed by Perry et al (130). The agar contains a third generation cephalosporin as the ESBL-selecting agent, but is not designed to detect AmpC producing strains, as it contains AmpC-inhibitors. It will detect class A and B ESBL-CARBA, but generally not OXA-48-like enzymes, because those are weak cephalosporinases. The ChromID ESBL agar sensitivity for detection of ESBL-A is reported to be 75-100% at 48 hours (131-136). The results are dependent on what method you compare to (there is no universal gold-standard or reference method), which ESBL-genes are present in the tested samples, and the composition of the tested material. One must also keep in mind when testing culture media sensitivity, that the sample materials will affect the sensitivity, e.g. the general microbial concentration and biological nutrients and inhibitors (130). Because of this, you would not expect the same detection limits for certain selected bacteria when culturing faecal samples compared to nasopharyngeal swabs, environmental samples, or sterile water spiked with test-strains.

There is a possibility that the ESBL-E detection rate and the relative distributions of detected β-lactamase-genes in our studies could have been different if we had used other culture media. However, in paper II, we used a brain-heart infusion broth containing cefotaxime 2.5 mg/liter (produced in-house) in addition to the direct plating on chromogenic agar, yielding only the additional finding of two isolates of *E. coli* producing plasmid mediated AmpC. In a study from the Netherlands, researchers reported significantly higher yield of ESBL-E by use of selective tryptic soy broth containing cefotaxime 0.25 mg/liter and vancomycin 8 mg/liter (TSB-VS, Cepheid Benelux, the Netherlands) (137) compared to direct culture of rectal swabs. Both the
sampling method, the composition of the broth and the medium to which the broth is compared, may be of relevance for the sensitivity and specificity performance. The broth used in the Dutch study is likely to be more sensitive than ours, as our broth did not contain vancomycin to inhibit Gram-positive bacteria, and the cefotaxime concentration we used was higher. The Dutch study also used different plates for direct culture as comparison to the broth. The EbSA plates (Cepheid Benelux, the Netherlands) which contain MacConkey agar with cefotaxime 1 mg/liter in one section and ceftazidime 1 mg/liter in another section, as well as inhibitors of AmpC and Gram-positive growth, was found to be somewhat less sensitive than ChromID ESBL for direct culture from clinical material according to one previous study (131). It is possible that inoculating the swab with faeces from toilet paper, as we did in the carriage study, is a more sensitive method than rectal swabbing, making enrichment less important. Some sampling procedures for rectal swabbing specifically states that the swab should be visibly contaminated by faeces, but if not, the toilet paper method is perhaps safer for securing sufficient amounts of sample material. In conclusion, the agar and sampling methods we have used for the studies seems adequate for our purpose, but it is important to note that our results reflect the prevalence of ESBL_A and not ESBL_M or ESBL_CARBA.
10 Conclusions

This thesis has fulfilled its overall aim by providing new knowledge regarding the occurrence, dissemination and persistence of ESBL-E in a One Health perspective. This knowledge is useful for clinicians, diagnostic laboratories, infection control personnel and decision makers in several ways. The data obtained support the following conclusions:

1. Faecal ESBL-E carriage was frequent in patients with gastroenteritis. The prevalence rate was substantially higher than those recently reported in healthy Norwegian volunteers.

2. Travellers returning from Asia with gastroenteritis had a substantially increased risk of being ESBL-E carriers, compared to similar patients who had not visited Asia.

3. We observed no significant correlation between ESBL-E faecal carriage duration and patient characteristics such as age, sex, antimicrobial use, comorbidity or travel history. However, the strength in some of these observations is limited due to low numbers in subgroup analyses.

4. ESBL-EC phylogroups B2 and D were associated with prolonged carriage.

5. ESBL-E carriage during more than one year was associated with a large intra-individual variability in faecal ESBL-E species, ESBLs and ESBL-EC MLVA-types.

6. ESBL-EC were detectable in recreational water in varying amounts, and detection of ESBL-EC was associated with increased concentration of *E. coli* in recreational water samples.

7. Known ESBL-EC pathogenic STs were present in recreational water which is a potential reservoir for human exposure, colonisation and infection.

8. ESBL-EC was detected in all the investigated wastewater samples. Identical ESBL-EC STs and MLVA-types were detected in wastewater, recreational water and geographically related clinical samples. This implies that wastewater
Conclusions

may promote dissemination of potentially harmful strains and plasmids through contamination of other environmental sources, e.g. recreational water.

9. Multidrug resistance was more frequent in ESBL-EC strains from clinical urinary samples, compared to those obtained from recreational water and wastewater samples.

10. The plasmid mediated colistin resistance gene \textit{mcr-1} was not detected in clinical urinary samples or wastewater samples, but was present in ESBL-EC ST10 strains from recreational water. Hence, surveillance of transferrable colistin resistance in environment, animals and humans is relevant also in countries where this drug is in minimal use.

Our findings regarding prevalence rates of faecal carriage, duration of carriage, risk factors for prolonged carriage and intra-individual variation in ESBL-E species and genotypes are all relevant for implementation of adequate infection control measures in health care institutions. It can help us to identify patients who should be under contact precautions, help us choose relevant screening algorithms and perform adequate outbreak investigations. Knowledge that helps us to identify patients with increased risk of ESBL-E carriage is also very important when choosing empirical antimicrobial treatment for Gram-negative infections. In addition, paper II of the thesis also demonstrated that one negative faecal sample from a person previously colonised by ESBL-E is not sufficient to prove that the person is no longer an ESBL-E carrier. We recommend at least two negative samples for ESBL-E clearance. Furthermore, we concluded that the enrichment broth we used had few advantages compared to swab samples from faeces on toilet paper.

The detection of ESBL-EC in recreational water suggests that this is a potential source for ESBL-E dissemination and possible infection in humans. This information may contribute to the development of improved surveillance strategies, and should encourage further research on water as a source of ESBL dissemination. The frequent detection in wastewater is also important, and should be investigated further. If the
wastewater can contaminate other environmental compartments, there is a risk of dissemination of potentially harmful strains and plasmids.
11 Future perspectives

The One Health perspective is essential to understand the dissemination of antimicrobial drug resistance and find solutions to how we can preserve both old and new antimicrobial drugs. To achieve this, there is need for detailed knowledge regarding optimal methods for sampling and laboratory analyses, as well as investigations regarding transmission routes and surveillance of antimicrobial drug resistance in humans and animals. The research presented in this thesis raises new questions that need to be addressed:

Based on our findings, we conclude that multiple control samples are necessary to assess clearance of faecal ESBL-carriage. A remaining question is which factors that may lead to ESBL-resurgence in these patients. The optimal interval between control samples should also be investigated further.

Others have shown shorter duration of ESBL-E faecal carriage in returning travellers than what we found in our patients with ESBL-E urinary tract infection (79, 138). This could be due to differences in ESBL-EC phylogroups in the different cohorts. Between the cohorts there may also be systematic variations in the amount of intestinal ESBL-EC, and this variation could be related to phylogroup, or to other factors, such as previous antimicrobial consumption. It would be interesting to determine ESBL-EC STs, phylogroups and ESBL-EC amounts in returning travellers, and relate this information to carriage duration. As we have shown that prolonged carriage is associated with phylogroups B2 and D which frequently cause extraintestinal infections, it may be that commensal non-uropathogenic strains acquired in Asia are easier to clear from the intestine. One should also explore further which properties in B2 and D strains that facilitate acquisition and long-term carriage. These issues are relevant when establishing ESBL-E screening procedures for patients entering health care institutions.

After the publication of paper II we have been able to sequence many of the faecal isolates from the study. Hence, it will be possible to investigate the variation in E. coli
strains during faecal carriage further by comparing WGS data and phylogeny analyses with the previous MLVA-findings. This will give us more knowledge regarding MLVA as a typing-tool, and provide more accurate insight regarding intra-individual clonal changes during protracted ESBL-EC carriage. We have also collected faecal swabs from the patients’ household members, to investigate the occurrence of ESBL-E in the households and look for genomic differences in ESBL-EC within each household.

In a recent commentary, Robinson and colleagues conclude that “an important gap in knowledge relates to the possible extent and mechanisms of transmission of antimicrobial resistance genes between the normal gut flora of animals and that of humans” (3), a notion supported by Huijbers et al in a review from 2015 (94). More knowledge in this field could perhaps reveal the most important contributors of ESBL-EC detected in recreational water.

Affluent wastewater samples frequently contain ESBL-EC. Enterobacterial load is reduced during processing in the plants, e.g. by physical and chemical filtering that separates water from sludge, and biologic rinsing with bacteria which remove nitrogen from effluent water. Even so, to get more knowledge on the potential impact of wastewater as a transmission pathway for ESBL-E, effluent samples should be examined in future studies. Sludge and biosolids increase in use as fertilizers (92), and there are regulations regarding processing to reduce microbial content and acceptable bacterial load in these products (92, 93). However, further investigation of sludge and biosolids as potential sources for transmission of clinically relevant resistance determinants should be encouraged.
Acknowledgements

12 Acknowledgements

This thesis is based on work performed at Vestre Viken Hospital Trust, Akershus University hospital (Ahus), the Norwegian University for Life Sciences and the University Hospital of North-Norway, with research fellowship grants to the Department of Medical Microbiology at Vestre Viken Hospital Trust from the South-Eastern Norway Regional Health Authority.

I wish to express my gratitude to my supervisors, professors Pål Arne Jenum, Arnfinn Sundsfjord and Truls Michael Leegaard for their scientific advice, continuous support and encouragement during the project. My co-PhD fellow and later supervisor Arne Søraas deserves great thanks for his ever-lasting enthusiasm, good mood, and enormous labour to include patients and collect samples from various sources.

Vestfjorden Avløpsselskap was most helpful with the collection of wastewater samples. Lotte Stenfors Arnesen at the Norwegian University of Life Sciences helped to collected and culture water samples, and professor Knut Liestøl at the Department for Informatics at the University of Oslo gave me valuable advice regarding statistical analyses.

I thank my colleagues at Bærum and Drammen hospitals for their support and help, in particular Ola Bjørang, and Inger Marie Brend Johansen for their special participation in laboratory analyses. Bjørn Arne Lindstedt and Inger Løbersli from the National Institute of Public Health provided necessary guidance when we established the MLVA analyses at Ahus. I also wish to thank Bettina Aasnes and Ørjan Samuelsen at the Norwegian National Advisory Unit on Detection of Antibiotic Resistance for valuable help with the microarray analyses, and for giving me a warm welcome and pleasant week at their laboratory in Tromsø.

At Ahus, Karin Helmersen’s guidance and help in the laboratory of the Department of Clinical Molecular Biology was essential for the MLVA, DNA-extraction and quality
control procedures. I have also deeply appreciated the scientific advice and support I have received from researchers Anita Blomfeldt and Hege Vangstein Aamot. Merete Holth, my patient leader, deserves a million thanks for her willingness to facilitate research activities, and for granting me time and flexibility to instigate and complete this work. The goodwill of the other doctors and the infection control personnel at the Department of Clinical Microbiology and Infection Control at Ahus has also been a prerogative for the project, and I am deeply thankful to you all.

Finally I wish to express deep gratitude to my supporting husband Kjetil Telle, to my wise parents and extended family, and to my two sons, Jonas and Even, for their valuable distraction and love.

Silje Bakken Jørgensen, Oslo, May 2018
13 References


41. Løbersli I, Haugum K, Lindstedt BA. Rapid and high resolution genotyping 
of all Escherichia coli serotypes using 10 genomic repeat-containing loci. 

42. Maiden MCJ, van Rensburg MJJ, Bray JE, Earle SG, Ford SA, Jolley KA, et 

43. Rossen JWA, Friedrich AW, Moran-Gilad J. Practical issues in 
implementing whole-genome-sequencing in routine diagnostic microbiology. 

al. The role of whole genome sequencing in antimicrobial susceptibility 
testing of bacteria: report from the EUCAST Subcommitteee. Clinical 
Microbiology and infection. 2017;23(1):2-22.

45. Tenover FC. Development and spread of bacterial resistance to antimicrobial 

1993;21 Suppl 1:S4-9.

47. Hall BG, Barlow M. Evolution of the serine beta-lactamas: past, present 
and future. Drug resistance updates : reviews and commentaries in 

48. Abraham EP, Chain E. An enzyme from bacteria able to destroy penicillin. 

49. Bradford PA. Extended-spectrum beta-lactamas in the 21st century: 
characterization, epidemiology, and detection of this important resistance 


References


104. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. European Comittee on Antibiotic Susceptibility Testing; 2012.


108. Ulstad CR, Solheim M, Berg S, Lindbaek M, Dahle UR, Wester AL. Carriage of ESBL/AmpC-producing or ciprofloxacin non-susceptible
References


References


References

Appendix

Papers I-IV