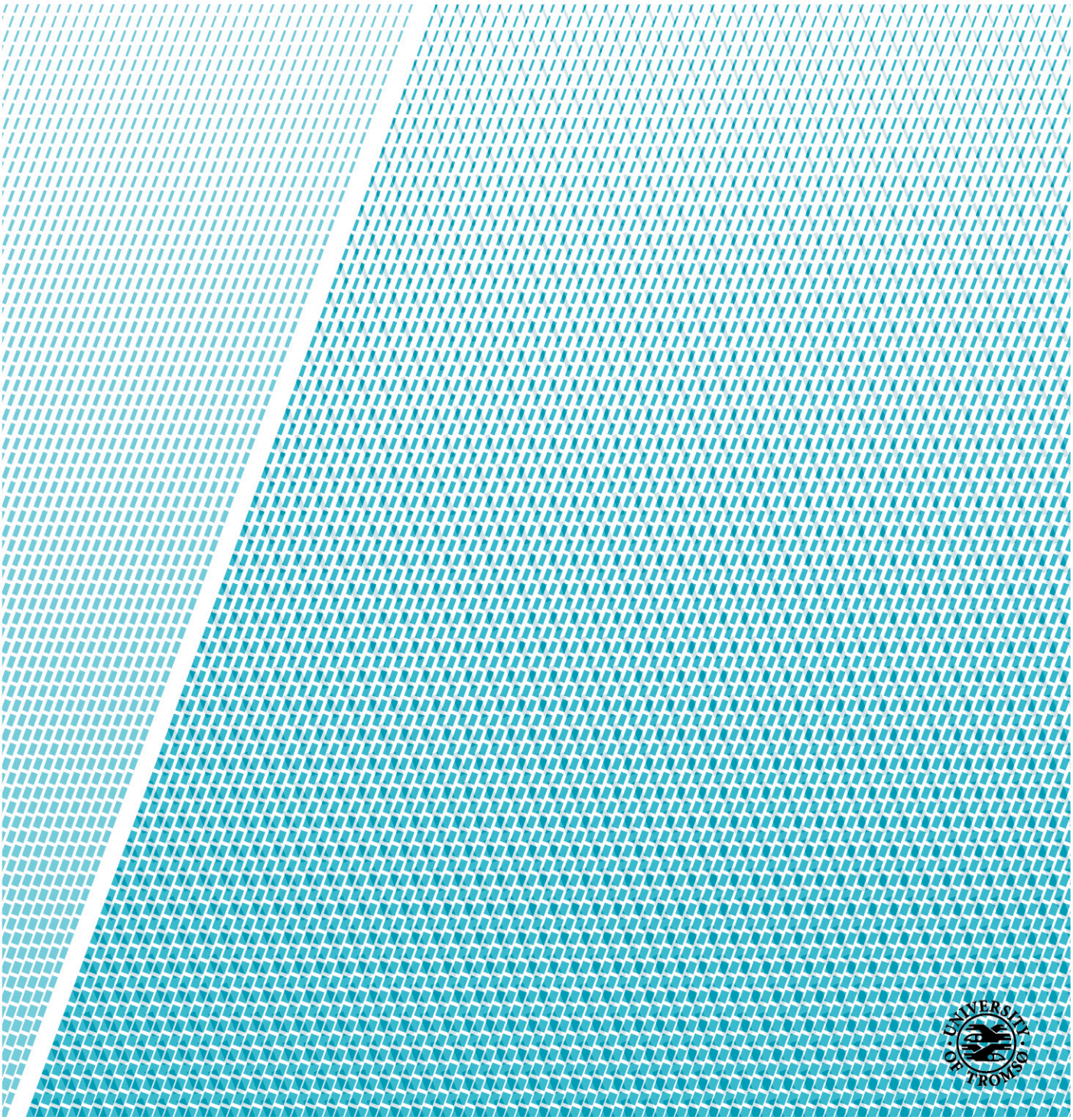
UiT
THE ARCTIC
UNIVERSITY
OF NORWAY

Department of Medical Biology
Faculty of Health Sciences

The population structure of human carriage and clinical isolates of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* in Norway

Lotte L. E. Andreassen

Master's thesis in Masterprogram in Biomedicine MBI-3911 May 2017



Acknowledgements

This master`s thesis is the culmination of two very interesting years of studies. Neither my thesis, nor my additional studies would have been possible for me to have undertaken alone. This is the result of a massive collaboration involving family, friends, co-workers and supervisors, and could not have happened without all these amazing individuals.

I have to start by thanking my brilliant main supervisor, Ørjan Samuelsen for his incredible patience, calmness, availability and for his unshakeable belief in my ability to finish this project. I also have to send a huge thank you to my co-supervisor Bjørg C. Haldorsen for her technical assistance and encouragement. And to my last two supervisors, Arnfinn Sundsfjord and Iren H. Löhr for their inspiration and good advices.

I do not think this project would have been finished in time without the help of K-res engineers Ellen H. Josefsen and Bettina Aasnæs. Thank you both for your technical support and for keeping up with my periodical whining. And a special thanks to bioinformatician, Jessin Janice James Peter for introducing me to the world of bioinformatics.

I have also received expert help from several of my co-workers at the Department of Microbiology and Infection control. Espen W. Bruvold, the master of agars, Marit Wiklund, who helped me configure Analytix, Miriam Nilsen, who assisted with the screening samples, and the rest of the gang at AMS, thank you!

My gratitude also goes to my co-students, and especially fellow office-inhabitants Siri Skarsfjord and Aud Malin Hovd for all their support, understanding and encouragement throughout the last two years.

I also have to thank my family for their encouragement, moral and financial support, and my two four legged children, Kaos and Dixi, who has been paramount for my mental health during stressful periods. And last but definitely not least, Tom Stangnes, for his optimism, understanding and sacrifices during these two years.

Lotte Leonore Eivindsdatter Andreassen, May, 2017

Summary

The increasing emergence of antimicrobial resistant bacteria worldwide is recognized as a severe threat to public health on a global scale. Without effective antimicrobial agents to treat bacterial infections, modern medicine will be set back several decades and deaths caused by bacterial infections will increase. The most widely used class of antimicrobials, is β-lactams, and the increase in resistance against β-lactams due to β-lactamases, and especially extended-spectrum β-lactamases (ESBL) is a great concern. In this study, our aims were to determine the carriage rate of ESBL-producing *E. coli* and *K. pneumoniae* in a random population, and to investigate the population structure of ESBL-producing *E. coli* and *K. pneumoniae* isolates from both carriage- and clinical samples. The carriage isolates were obtained by screening of fecal samples from inhabitants in the Tromsø municipality, collected through the Tromsø-7 population study, and the clinical isolates were obtained from the 2014 NORM collection of ESBL-producing *E. coli* and *K. pneumoniae*, isolated from blood cultures and urine in different hospitals in Norway during 2014. An additional aim was to determine the carriage rate of *K. pneumoniae*, irrespective of resistance, in the Tromsø population.

Screening of fecal samples from inhabitants in the Tromsø municipality, showed the carriage rate of ESBL-producing *E. coli* and *K. pneumoniae* to be 3.2%. We also found the carriage prevalence of *K. pneumoniae*, irrespective of resistance, to be 14.7%.

Whole-genome sequencing (WGS), was used to determine the population structure of the ESBL-producing carrier strains and the ESBL-producing clinical strains. The genotypic characterization of the ESBL-producing *E. coli* isolates showed both the carrier strains and the clinical strains were dominated by ST131, with CTX-M-15 as the most prevalent ESBL. Genotypic characterization of the clinical *K. pneumoniae* strains, showed a dominance by ST307, also with CTX-M-15 as the most prevalent ESBL.

Our results show the carriage rate of ESBL- producing *E. coli* and *K. pneumoniae* in Norway is lower compared to other countries. The populations of carriage strains of both *E. coli* and *K. pneumoniae* is, however, dominated by known high risk clones. We recommend further surveillance of these populations should be performed on a regular basis.

Table of Contents

Abbreviations

AAC	Aminoglycoside-modifying acetyltransferases
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
BLC	Blood culture
CAZ	Ceftazidime
CIP	Ciprofloxacin
CLED	Cysteine Lactose Electrolyte Deficient
CTX	Cefotaxime
CXM	Cefuroxime
EARS-Net	European antimicrobial resistance surveillance network
ES	Extended spectrum
ESBL	Extended spectrum β-lactamase
Eucast	European Committee on Antimicrobial Susceptibility Testing
GEN	Gentamicin
IS	Insertion sequence
K-res	Norwegian National Advisory Unit on Detection of Antimicrobial Resistance
MALDI TOF MS	Matrix assisted laser desorption ionization time of flight mass spectrometer
MBL	Metallo β-lactamase
MDR	Multidrug resistant
MEM	Meropenem
MIC	Minimal inhibitory concentration
MLST	Multi locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NGS	Next generation sequencing
NORM	Norwegian surveillance system for antibiotic resistant microbes
NS	Narrow spectrum
NSBL	Narrow spectrum β-lactamase
PBP	Penicillin-binding protein
PMF	Peptide mass fingerprint
PMQR	Plasmid-mediated quinolone-resistance
SCAI	Simmons citrate agar with inositol
SCC	staphylococcal cassette chromosome
ST	Sequence type
SXT	Trimethoprim-sulfamethoxazole
TZP	Piperacillin-tazobactam
WGS	Whole genome sequencing

1 Introduction

Antimicrobial resistance (AMR) is by the World`s Heath Organization (WHO) considered to be one of the most severe threats to global public health in modern time [1]. Without effective treatment of bacterial infections the treatment situation will be set back several decades, meaning relatively common infections could potentially be fatal [1]. The increasing prevalence of antimicrobial resistant bacteria provides a severe cause for concern on a global scale. As an example, antimicrobial resistant Tuberculosis is emerging as a major cause of death globally, and WHO estimated 170 000 people died from antimicrobial resistant tuberculosis globally in 2012 [2]. The exact numbers are near impossible to obtain as many of these cases are from countries without proper surveillance programs [2].

Because infections with antimicrobial resistant bacteria are much harder to treat, the consequences can be increased mortality, longer hospital stays for the individual patients and therefore also higher costs per patient [1]. Antimicrobial resistant bacteria are often categorized in three different classes, depending on the extent of their resistance [3]. Some isolates are multidrug resistant (MDR), meaning they demonstrate resistance to agents belonging to three different antimicrobial classes [3]. Other isolates are classified as extensively drug resistant, meaning they express resistance to agents belonging to all but two of the different antimicrobial classes [3]. Some isolates even demonstrate resistance to all known antimicrobial agents, these are classified as pan-drug resistant [3].

On February 27th 2017, WHO published a priority list of antimicrobial resistant bacteria [4]. The purpose of this priority list was to set focus on resistant bacteria considered to be the most severe threat to public health, and to help coordinate a global research effort in the fight against antimicrobial resistant bacteria [4]. The resistant bacteria were divided into three categories: priority 1 (critical), priority 2 (high) and priority 3 (medium) [4]. Bacteria belonging to the family *Enterobacteriaceae*, resistant to carbapenems and/or third generation cephalosporins were categorized as critical, along with carbapenem-resistant *Acinetobacter baumannii* and carbapenem-resistant *Pseudomonas aeruginosa* [4].

In May 2016, Review on Antimicrobial Resistance published an extensive report on the prospects on the effects of AMR [5]. The work on this report was led by economist Jim O`Neill

and the report is therefore often referred to as "the O`Neill report" [5]. One of the stipulated alarming scenarios described in this report was the increase in annual deaths caused by AMR from 700 000 people globally in 2014, to 10 million people in 2050, unless action is taken [5]. This report points out certain areas where the authors recommend efforts should be made. Among these areas is increase of public awareness, improved focus on hygiene and sanitation, improved global surveillance of AMR and use of antimicrobial agents, and intensify research on antimicrobial resistant bacteria and the development of new antimicrobial agents [5].

1.1 Antimicrobial agents

The discovery of antimicrobial agents is without a doubt one of the major triumphs in modern medicine [6]. The ability to suddenly treat previously incurable infections, like syphilis, had an enormous impact on both healthcare and social life in the 1940`s [6]. It all started in 1928 with Alexander Fleming`s discovery of the inhibitory effects of the mould *Penicillium notatum* on colonies of *Staphylococci* growing on an agar plate [7, 8]. After some difficulties, the active substance was isolated in 1940 by Howard Florey and Ernest Chain [9]. The drug was called penicillin [7]. This was the starting point of "the antibiotic era", and Fleming, Florey and Chain received the Nobel Prize in 1945 for their work with penicillin [7]. In his acceptance speech, Fleming warned against misuse of the drug, claiming it would cause the emergence of resistant bacteria [10]. Since the discovery and further development of penicillin, a large number of different antimicrobial classes have been discovered [6]. Figure 1 illustrates the timeline of development of the major antimicrobial classes [6].

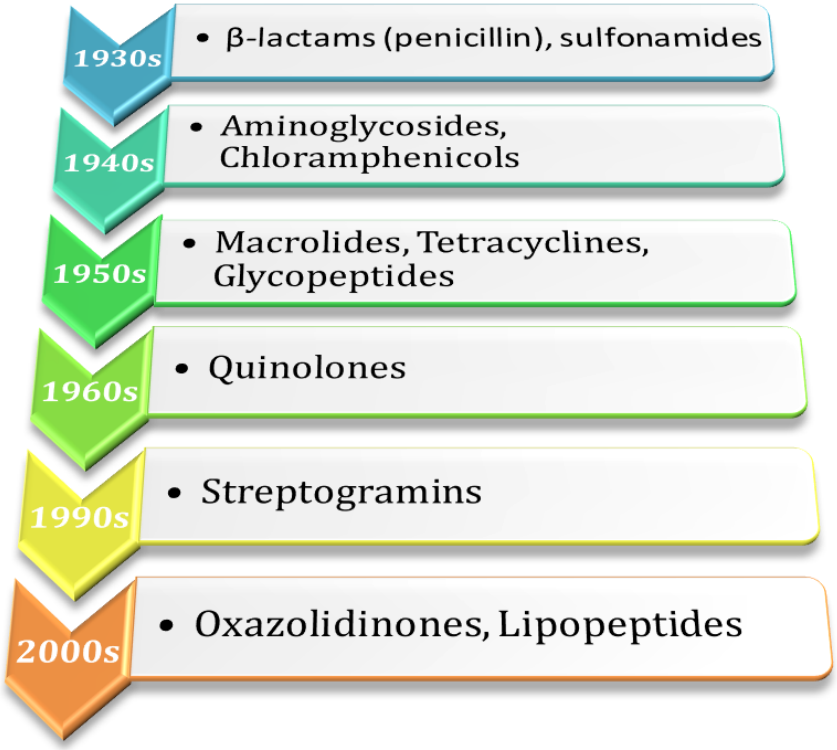


Figure 1: Timeline showing the development of different antimicrobial classes by decade of introduction. (figure modified from [6]).

1.2 Mechanisms of action of antimicrobial agents

Today more than 20 different classes of antimicrobial agents have been discovered, each of these classes comprise of different specific compounds [11]. Some classes are extensively developed with a large number of different specific antimicrobial agents, while some classes only consist of a few different drugs [12]. All antimicrobial agents work by a specific mechanism of action. In general, there are four different main mechanisms by which antimicrobial agents can kill or inhibit growth of bacteria; inhibiting cell-wall synthesis, hindering protein synthesis, interfering with metabolic processes in the bacterial cell, or inhibition of DNA or RNA synthesis [13].

Maintenance and production of the bacterial cell wall is paramount for the survival of the bacterium [13]. An important feature of the cell wall is the peptidoglycan layer which consists of disaccharide subunits (N-acetylglucosamine and N-acetylmuramic acid) crosslinked to one another with peptide bridges [13]. Penicillin-binding proteins (PBPs) are bacterial enzymes

anchored in the cell membrane, which are essential for cell wall synthesis [13]. Antimicrobial agents targeting these enzymes, will inhibit the synthesis of peptidoglycan and the cell will succumb due to instability of the cell wall [13]. There are two main classes of antimicrobial agents targeting the cell wall synthesis, the β-lactams and the glycopeptides [13]. The largest class is the β-lactams, named by the β-lactam ring in the core of their molecular structure [13]. The β-lactams will be discussed in more detail in the next chapter. The other major class of cell wall targeting antimicrobial agents are the glycopeptides [13]. Agents belonging to this class does not bind to PBPs directly, but instead to precursors that are being incorporated into the cell wall by PBPs [13]. This inhibits the assembly of the cell wall and results eventually in cell lysis [13]. Glycopeptides are effective against most Gram-positive bacteria, and the most commonly used agent is vancomycin [12, 13].

Another antimicrobial target is the protein synthesis process of the bacterial cell [13]. The antimicrobial agents bind to either the 30S or the 50S ribosomal subunit, thereby inhibiting crucial steps in the protein synthesis [13]. Antimicrobial agents belonging to the two major classes aminoglycosides and tetracyclines, binds to the 30S subunit, inhibiting the ribosome translating mRNA [12]. Aminoglycosides have especially good antimicrobial activity against Gram-negative bacteria, while tetracyclines have good activity against many Gram-negative and Gram-positive bacteria [12]. Antimicrobial agents belonging to the major classes lincosamides and macrolides, also interfere with the protein synthesis, but these agents bind to the 50S subunit of the ribosome and prevent the elongation of peptide chains during protein synthesis [12]. Lincosamides have good antimicrobial activity against Gram-positive cocci, while macrolides work on most Gram-positive and some Gram-negative bacteria [12].

Some antimicrobial agents interfere with metabolic processes important for the bacterial cell, like the metabolism of folic acid, a metabolic pathway where the end products are precursors necessary for the DNA synthesis [13]. This particular process is inhibited by two different classes of antibiotics; sulphonamides and trimethoprim [13]. Sulphonamides bind to an enzyme involved in the folic acid pathway, dihydropteroate synthase, and consequently disrupt the pathway [13]. Trimethoprim function much in the same way, but binds to another enzyme in the pathway, dihydrofolate reductase (DHFR) [13]. Both these antimicrobial classes have activity against many Gram-positive and Gram-negative bacteria [13]. Trimethoprim-

sulfamethoxazole is a combination drug which consist of both agents, and therefore inhibits the same metabolic process in two different places in the pathway, which makes it effective against a wider range of bacteria [13].

Several antibiotic classes work by inhibition of DNA and RNA synthesis [13]. Fluoroquinolones, for instance, bind to DNA gyrase, an enzyme that is involved in regulation of supercoiling of DNA which is essential for DNA replication, and thus inhibit DNA synthesis [13]. The activity spectrum of fluoroquinolones varies by the specific agent in question [12]. Narrow spectrum agents like nalidixic acid work against a number of Gram-negative bacteria, but are ineffective against Gram-positive bacteria, while broad-spectrum agents like ciprofloxacin have excellent effect against many Gram-negative and Gram-positive bacteria [12]. Rifampicin is another antimicrobial class with a similar mode of action [13]. It binds to the RNA polymerase and interferes with mRNA synthesis [13]. Rifampicin is active against many Gram-positive and Gram-negative bacteria [12]. Metronidazole is another antimicrobial agent inhibiting the DNA synthesis. This agent inflicts direct damage to the bacterial DNA by generating highly cytotoxic compounds or free radicals that ruptures the DNA [12]. Metronidazole displays activity against most anaerobic bacteria [12]

Table 1 gives an overview of some of the most common antimicrobial classes, their target and some examples of specific antibiotic agents belonging to the different classes.

Table 1: Overview of selected common antibiotic classes, presented with bacterial target and examples of specific antibiotic agents [12, 13].

Examples of antibiotics		**Antibiotic class**		**Target**
Penicillin G, penicillin V, ampicillin, amoxicillin, oxacillin, piperacillin		Penicillins	ß-lactams	**Cell wall synthesis**
cefazolin, cefadroxil, cephalexin, cephadrine	1. gen.	Cephalosporins		
cefuroxime, cefamandole, cefoxitin, cefotetan, cefaclor	2. gen.			
ceftriaxone, ceftazidime, cefpodoxime, cefotaxime	3. gen.			
cefepime, cefpirome	4. gen.			
ceftaroline	5. gen.			
Meropenem, ertapenem, doripenem, imipenem		Carbapenems		
Aztreonam		Monobactams		
Sulbactam, tazobactam, clavulanic acid		ß-lactamase inhibitors		
Vancomycin, teicoplanin		Glycopeptides		
Gentamicin, amikacin, tobramycin, streptomycin		Aminoglycosides	S30 subunit	**Protein synthesis**
Doxycycline, tetracycline, tigecycline		Tetracyclines		
Clindamycin, lincomycin		Lincosamides	S50 subunit	
Erythromycin, azithromycin		Macrolides		
Ciprofloxacin, norfloxacin		Fluoroquinolones	**DNA synthesis**	
Sulfamethoxazole		Sulfonamides	**Folic acid synthesis**	
Trimethoprim		DHFR inhibitor		
Metronidazole			**DNA damage**	
Rifampicin			**mRNA synthesis**	

1.2.1 β-lactams

As mentioned in the previous chapter, β-lactams comprise of a β-lactam ring in the core of their molecular structure [13]. β-lactams function as bactericidal drugs by binding to PBPs in the bacterial cell wall [14]. Different species of bacteria have different sets of PBPs, and each cell can have from three to eight different types of PBPs [14]. Different β-lactams have different affinity for different types of PBP [14]. Due to the diversity of PBPs among different bacterial species, the general activity spectrum of β-lactams is also diverse, meaning some β-lactams work best on Gram-positive bacteria, while others work best on Gram-negative [14]. The most important PBPs in Gram-negative bacteria is PBP1a, PBP1b, PBP2 and PBP3 [14].

β-lactams is a vast class of antimicrobial agents, comprised of several subclasses, like penicillins, cephalosporins, carbapenems and monobactams, each with a large number of different derivatives evolved through history [13]. Every discovery of a new class of β-lactams or a specific agent, has been made out of necessity caused by resistant bacteria or a need to produce antimicrobial agents that would work on a broader selection of bacterial species [14].

Penicillin, the first antimicrobial agent on the market, was a β-lactam. The first penicillins (benzylpenicillin and phenoxymethyl penicillin) were active against most Gram-positive bacteria, but had little effect on most Gram-negative bacteria [15]. In the 1950`s penicillin-resistant *Staphylococci* started to emerge due to isolates producing penicillinase (a penicillin hydrolysing enzyme belonging to the β-lactamase family, discussed further in chapter 1.3.3), and in the early 1960`s, penicillinase-stable penicillins; methicillin and cloxacillin, were introduced [15]. Most of these penicillins were less active against Gram-negative bacteria, and there was a necessity to develop more broad-spectrum drugs to treat infections caused by these bacteria [15]. Examples of these broader spectrum penicillins, are ampicillin, amoxicillin and piperacillin [15].

The first cephalosporin (cephalosporin C) was actually discovered in 1948, and exhibited a broad-spectrum of activity, but it`s effect was seemingly very low [15]. However, it was later discovered the molecule was more stable than penicillin, so it could be produced semi-synthetically, which made it easier to manufacture larger amounts of the drug [15]. The first cephalosporin on the market, cephaloridine, was introduced in 1964, and several more

derivatives was developed during the 1960`s and 1970`s, like cephalexin and cephapirin [15]. These were the first generation cephalosporins, and they had effect on a large number of Gram-positive bacteria, including penicillinase-producing *Staphylococci*, and some Gram-negative bacteria, like *Escherichia coli* and *Klebsiella pneumoniae* [15]. The basic molecular structure of cephalosporins was relatively easy to modify, and in the 1970s the second generation of cephalosporins was introduced with cefamandole in 1973 [15]. Furthermore, cefaclor was introduced in 1976 and cefuroxime in 1984 [15]. These second generation drugs had activity against a wider spectrum of Gram-negative bacteria, but less activity against Gram-positive bacteria [15]. The third generation of cephalosporins surfaced in the late 1970s, with the introduction of drugs like cefotaxime and ceftazidime [15]. The third generation cephalosporins were a solution to a growing problem with bacteria resistant to previous generations of cephalosporins, caused by β-lactamase production [15]. These new generation drugs had a limited spectrum of activity against Gram-positive bacteria, but worked better against Gram-negative bacteria compared to the previous generations, including β-lactamase producing strains [15]. In the late 1980s a fourth generation of cephalosporins was developed [15]. This generation consisted of drugs like cefpirome and cefepime, which had even better activity against Gram-negative bacteria, and was considered to have an increased stability against the effect of β-lactamases [15]. A fifth generation cephalosporin, ceftaroline, was introduced in 2010 [16]. This cephalosporin is termed "anti-MRSA cephalosporin" and it was produced as a solution to the rising problem with methicillin-resistant *S. aureus* (MRSA) [15]. This drug had extensive activity against Gram-positive bacteria compared to the previous generations of cephalosporins, including MRSA isolates [15]. Cephamycins, is another group of β-lactams often classified with second generation cephalosporines, as the antimicrobial spectrum of cephamycins are similar to that of second generation cephalosporines [15]. Cephamycins are however, much more stable against β-lactamases, including some of the extended-spectrum β-lactamases [15]. In general cephamycins are stable towards class A extended-spectrum β-lactamases, but less so towards class C β-lactamases (the different classes of extended-spectrum β-lactamases are explained further in chapter 1.3.3) [15]. An example of a common cephamycin, is cefoxitin [15].

In the late 1970s studies into a new class of β-lactams, carbapenems, were executed [15]. These compounds had a wider spectrum of activity compared to all previously known β-lactams and

were additionally very effective as β-lactamase-inhibitors [15]. The first generation of natural carbapenems were rather unstable and only functional when combined with an inhibitor of natural enzymes present in the human body [15]. The first synthetically manufactured carbapenem was imipenem, this drug also had to be combined with an inhibitor not to be decomposed by enzymes in the human body [17]. The first carbapenem not dependent on an inhibitor of human enzyme activity, was meropenem, soon followed by others, like ertapenem and doripenem [15]. Due to the antimicrobial potency of carbapenems, and their effect on β-lactamase producing bacteria, these drugs are considered to be the last-resort treatment of patients with severe infections caused by antimicrobial resistant bacteria [17].

Monobactams is another class of β-lactams, first discovered in the early 1980s, these agents had a limited activity spectrum, with no activity against Gram-positive bacteria, but exhibited good activity against Gram-negative bacteria [15, 18]. In 1983, the agent most widely used monobactam, aztreonam, was introduced as an agent for use against Gram-negative bacteria [15].

The most prevalent reason for AMR to β-lactams, is the production of bacterial β-lactamases, making the drug ineffective [15]. To address this problem, some β-lactams were combined with β-lactamase-inhibitors to form a combination drug with effect against bacteria known to produce β-lactamases [15]. An example of these combination drugs is amoxicillin-clavulanic acid, which had improved effect against β-lactamase producing Gram-positive and Gram-negative bacteria [15]. Tazobactam is another inhibitor of β-lactamases and is combined with piperacillin to form the drug piperacillin-tazobactam [15].

In general, β-lactams are widely used, due to the wide selection of different agents, their bactericidal effect and their low toxicity to humans [13]. According to the NORM (Norwegian surveillance system for antibiotic resistant microbes) report published in 2015, β-lactams is the most frequently used group of antimicrobial agents in Norway [19].

1.3 Antimicrobial resistance

Already before penicillin was introduced as a treatment option, resistance to the drug had been observed among some Staphylococcus strains, and in the late 1940`s, penicillin resistant bacteria were becoming a fast growing problem [20]. Several new β-lactams were introduced as a solution to the problem, but reports of resistance typically followed a few years later [20]. For instance, in 1960 the β-lactam methicillin was introduced, and in 1962 methicillin-resistant Staphylococci emerged [20]. In 1985 imipenem was introduced as a last resort treatment option for infections caused by highly resistant strains, and in 1998 there were reports of imipenem resistant Enterobacteriaceae [20].

The annual report published by the European antimicrobial resistance surveillance network (EARS-Net), reports of high, and increasing, levels of resistance in Gram-negative bacteria in many parts of Europe, and the situation is defined as "especially worrying" [21]. Many of these isolates were also multidrug-resistant, displaying non-susceptibility to third-generation cephalosporins, fluoroquinolones and aminoglycosides [21]. Figure 2 shows an overview of *E. coli* isolates with combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides in Europe in 2015 [21]. As shown in figure 3, the same multidrug-resistance is much higher in *K. pneumoniae* isolates, where some countries report a prevalence of more than 50% [21].

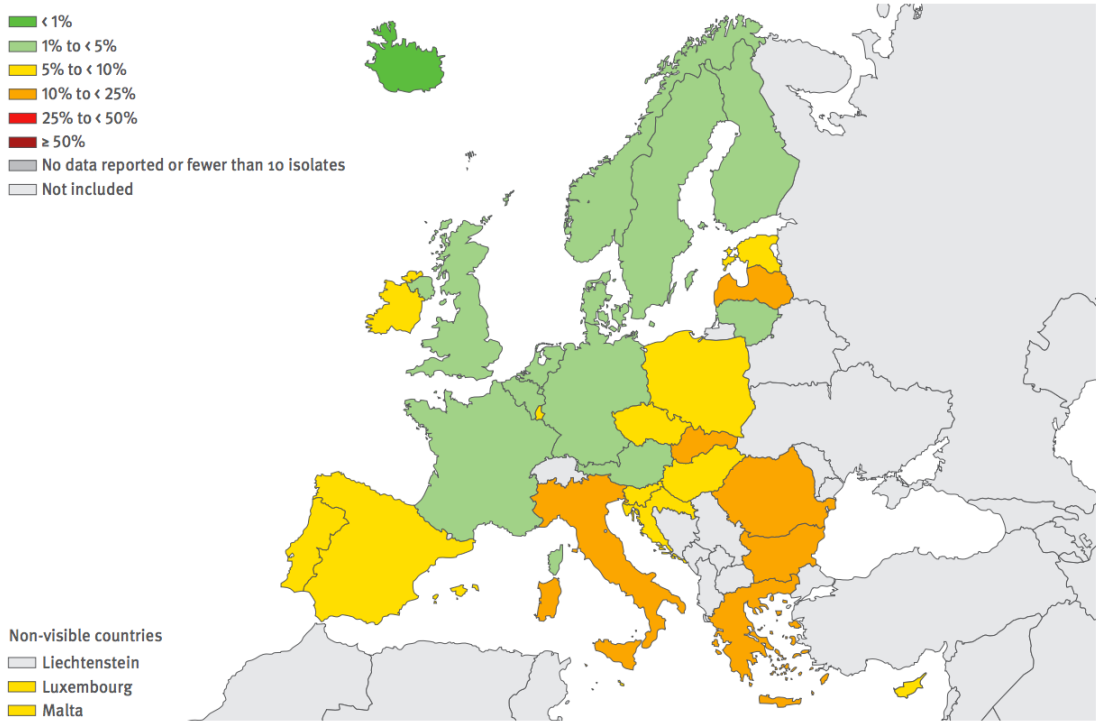


Figure 2: Percentage of invasive E. coli isolates with combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides in European countries in 2015. Figure retrieved from EARS-Net [21].

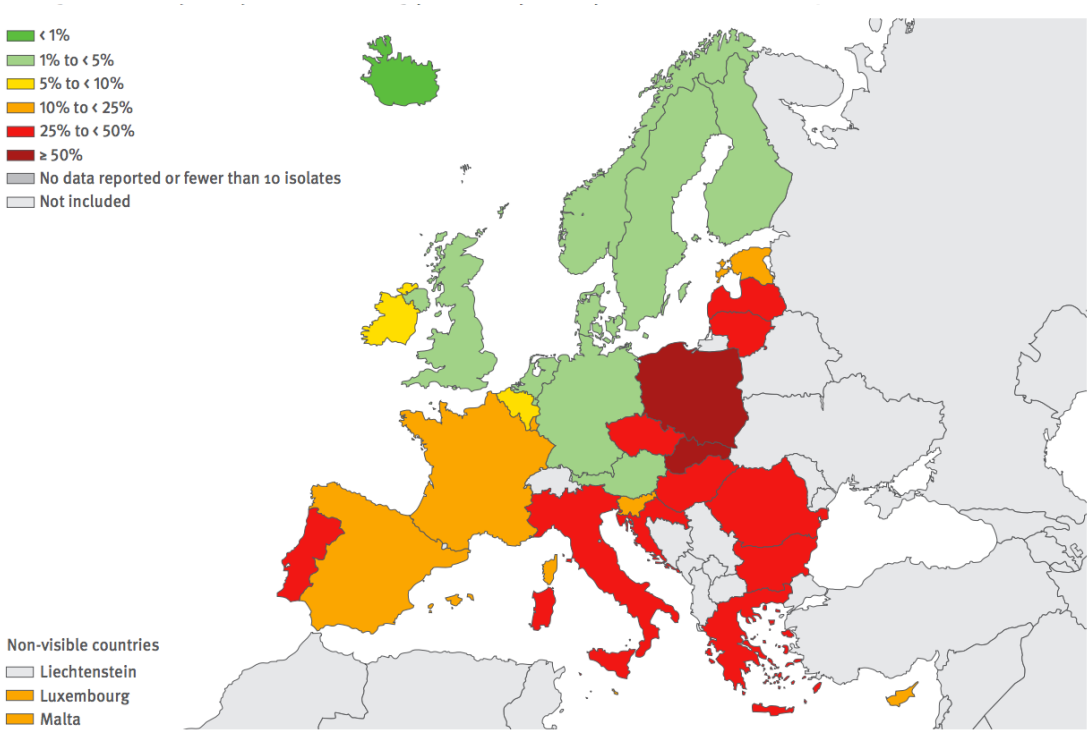


Figure 3: Percentage of invasive K. pneumoniae isolates with combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides in European countries in 2015. Figure retrieved from EARS-Net [20].

There is also an increase in the prevalence of carbapenem-resistant Enterobacteriaceae, especially for *K. pneumoniae*, with a mean of 6.2% in 2012 to 8.1% in 2015 [21]. Looking at individual countries, the prevalence varied from <1% to a staggering 61.9% in Greece in 2015 [21]. Figure 4 shows an overview of carbapenem-resistant *K. pneumoniae* in Europe in 2015 [21].

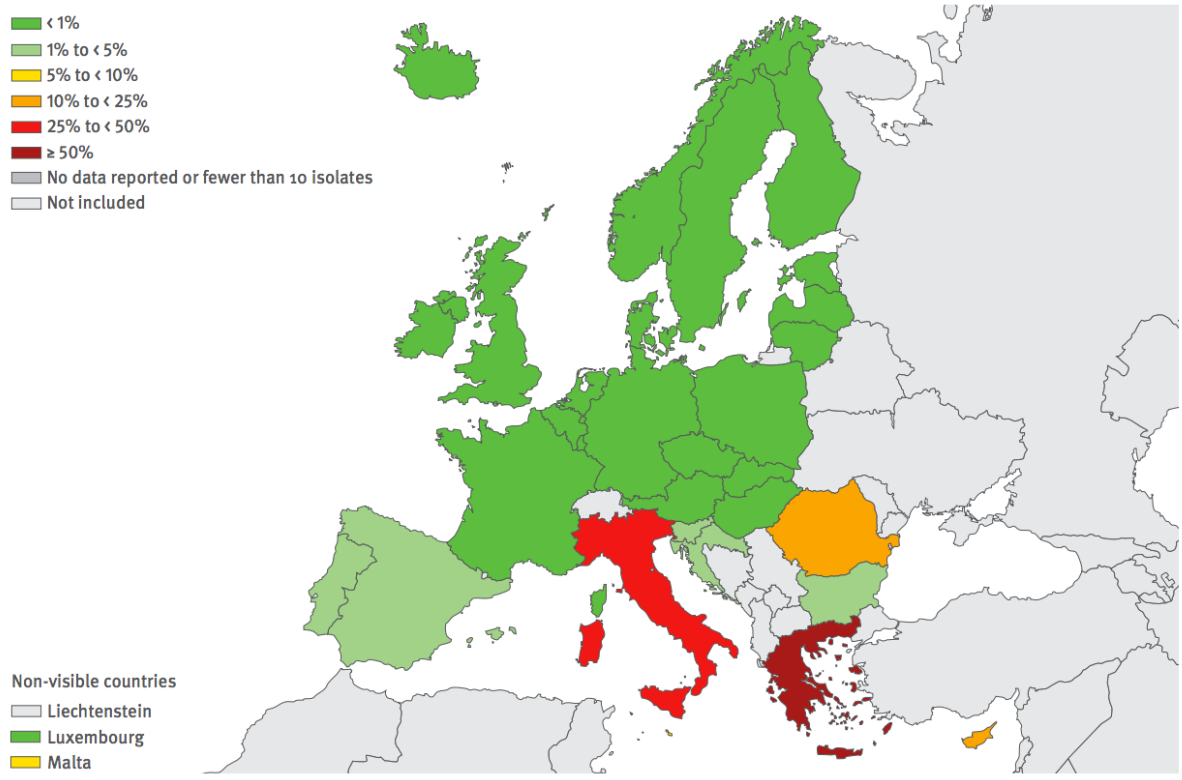


Figure 4: Percentage of invasive K. pneumoniae isolates with resistance to carbapenems in European countries in 2015. Figure retrieved from EARS-Net [20].

The AMR situation in Norway is closely monitored by NORM, which publishes an extensive report on the prevalence of AMR and the usage of antimicrobial agents in Norway each year [19]. These reports conclude there have been increases in resistance against many important antimicrobial agents the last decade [19]. For instance, the prevalence of *E. coli* and *K. pneumoniae* isolates resistant to third generation cephalosporins has increased from 3-6% from 2003 to 2015 [19]. The prevalence of clinical *E. coli* isolates non-susceptible to gentamicin (aminoglycoside), have increased severely from a little over one percent in 2000, to 6.4 % in 2015 [19]. Of these isolates, 40.3% were also resistant to third generation cephalosporins,

making them multidrug resistant, as previously presented through the European data from EARS-Net [19, 21, 22]. *Klebsiella spp*. isolates non-susceptible to aminoglycosides in Norway, has increased from 1.0% in 2012, to 2.8% in 2015 [19]. There is also an increase in *E. coli* isolates with resistance to ciprofloxacin, from 7.3% in 2013 to 9.4% in 2014 [19]. The prevalence of *K. pneumoniae* isolates resistant to fluoroquinolones seem to be stable at 3-4% [19].

The prevalence of Enterobacteriaceae with reduced susceptibility to carbapenems are still low in Norway, but the number of clinical isolates has increased from only a few isolates in 2007, to over 30 in 2015 [19]. The most worrying development within this category is the 3-fold increase in isolates from 2014 to 2015 [19]. A similar increase in resistance to carbapenems is seen with clinical strains of *Acinetobacter baumannii* and *Pseudomonas* spp. as well, from a few isolates in 2004 to 22 isolates in 2015 [19]. However, the sudden increase in later years is not seen, instead these isolates seem to have a more steady increase over several years [19].

The increase in AMR in these bacterial species, makes the overall treatment of bacterial infections more difficult due to the limited options of antimicrobial agents left to choose from [1]. When the first-choice drugs are ineffective, more broad-spectrum drugs are administered, which are often more expensive and likely to cause even more resistance [23]. The treatment of infections caused by resistant bacteria is more time consuming, which means the patient needs to be hospitalized for a longer period of time, which again equals higher costs per patient [5]. In addition, the general decrease of effective antimicrobial agents also cripple other medical fields, like cancer-treatment or surgery [5]. Cancer patients often undergo treatment that compromise their immune system, like chemotherapy, and without effective antimicrobial agents, this form of treatment is extremely risky [5].

1.3.1 Mechanisms of AMR

AMR is the direct consequence of different resistance mechanisms expressed by bacterial cells [24]. These mechanisms of resistance can be sorted into three main categories; the minimization of the antimicrobial agent inside the cell (either by decreased uptake through the cell membrane

or active efflux), alterations of the antimicrobial target and degradation or alteration of the drug by microbial enzymes [24].

Decreased uptake of antimicrobials through reduced permeability of the bacterial cell is caused by downregulation or modification of porin proteins in the bacterial cell membrane [24]. Hydrophilic antimicrobial agents (like β-lactams) are dependent on these porins as gateways to the intracellular environment [25]. Hydrophobic drugs (like macrolides and aminoglycosides) on the other hand can diffuse through the lipid bilayer to gain access to the interior of the cell [25]. The major outer-membrane porins of Enterobacteriaceae (OmpF and OmpC) are believed to be non-specific channels, where antibiotic agents pass through to access binding sites inside the bacterial cell [24]. Resistance to these antimicrobial agents can therefore be achieved by the downregulation or modification of these proteins to become more selective, which leads to reduced uptake of the drug [24]. An example of the loss of porins leading to resistance, is the OmpF porin in *E. coli [24]*. This is one of the most abundant porins in the outer membrane of *E. coli* [25]. In *E. coli* micF RNA (a small noncoding RNA), is responsible for the negative regulation of expression of OmpF [26]. This downregulation is triggered by the presence of antimicrobial agents, and can cause resistance to, for instance, β-lactams [26, 27].

Efflux is another way for the bacterial cell to increase the concentration of antimicrobial agents intracellularly [24]. This ejection of the drug is caused by efflux pumps, located in the cell-wall, actively transporting antimicrobial agents out of the cell as soon as it enters through the cell membrane [24]. Many different efflux pumps have been described, with different substrate specificity [24]. Some efflux pumps only exports a very narrow-spectrum of substrates, while others transport a wide range of different substrates, these pumps are known as "multidrug resistance efflux pumps" (MDR) [24]. An example of an MDR efflux pump found in *E. coli*, among others, is the AcrAB pump, belonging to a family of MDR efflux pumps known as the resistance nodulation division (RND) family [24]. This efflux pump consist of three main structures, an inner-membrane protein, an adaptor protein in the periplasmic space and a third protein in the outer membrane [28]. This AcrAB pump has a substrate profile that includes tetracycline, chloramphenicol, β-lactams, novobiocin, fusidic acid, nalidixic acid and fluoroquinolones [29]. Most bacteria have chromosomal genes encoding efflux pumps, and high levels of resistance is linked to an overexpression of these pumps [24]. This overexpression

can be caused by mutations in genes responsible for regulating the gene expression of efflux pumps [24]. Some efflux pump genes have also been mobilized, from the chromosome to plasmids, which can be transferred between bacterial cells [24]. In 2007 a new plasmid-mediated gene for an efflux-pump called QepA was identified in an *E. coli* isolate from Japan [30]. This efflux pump conferred resistance to fluoroquinolones [30].

Another resistance mechanism is alterations of antimicrobial targets, so that the antimicrobial agents cannot bind to the bacterial target [24]. These alterations are caused by mutations in the genes encoding this target [24]. It can be alterations of the target molecule itself, leading to reduced affinity for the antibiotic agent, or the addition of another chemical group that will function as protection for the primary binding site [24]. For instance, the acquisition of a gene belonging to the gene-family *qnr*, confers resistance to fluoroquinolones [24]. The *qnr* genes encode specific proteins (pentapeptide repeat proteins) that will bind to the antimicrobial target of fluoroquinolones (DNA gyrase) and thereby protect the bacteria from the effects of the drug [24]. Another possibility of target alterations, is the acquisition of another target, similar to the original [24]. An example of this target change is methicillin resistant *Staphylococcus aureus* (MRSA), where the original penicillin-binding protein, PBP (important for cell wall synthesis), is supplemented by another version, PBP2a [24]. If a β-lactam antibiotic binds to the original PBP, in order to inhibit its function, the bacterial cell will still have a functioning penicillin-binding protein in PBP2a, and will not be affected by the β-lactam drug [24]. The production of this homologous protein is caused by the acquisition of a new gene, *mecA* or *mecC* [24]. These genes are located on a mobilized gene-element called staphylococcal cassette chromosome *mec* element (SCC*mec*) [24].

The direct inactivation of an antimicrobial agent can be caused by bacterial enzymes modifying the agent by the addition of a chemical group, or inactivating the agent by hydrolysis [24]. As an example, aminoglycoside-modifying acetyltransferases (AAC) is a group of enzymes with the ability to inactivate aminoglycosides by acetylation [31]. There are several types of AACs conferring resistance to different antimicrobials, like AAC(3)-II which confers resistance to aminoglycosides like gentamicin and tobramycin, and AAC(6)-Ib which additionally confers resistance to fluoroquinolones [31]. The first antimicrobial hydrolysing enzyme ever described, was a penicillinase in 1940 [24]. Since then a large number of different enzymes have been

discovered [24]. Today thousands of different enzymes able to degrade antimicrobial agents belonging to most of the major classes of antimicrobial agents, like β-lactams, aminoglycosides and macrolides have been discovered [24]. Enzymatic degradation of antimicrobial agents is within many bacterial species, including *E. coli* and *K. pneumoniae* the main cause of antimicrobial resistance to certain antibiotics [24]. The largest and most diverse class of these hydrolysing enzymes, are the β-lactamases [24]. This enormous class of enzymes can hydrolyse agents belonging to all the different groups of β-lactams [24]. The β-lactamases will be described further in chapter 1.2.3.

Bacteria are also likely to express a combination of several different mechanisms, for instance, genes encoding β-lactamases can be found on the same plasmid as genes encoding AAC enzymes or efflux pumps [24]. This combination of different resistance mechanisms may result in resistance to many different antibiotic classes simultaneously within one bacteria, by definition making it multidrug resistant [24]. The production of several versions of each main mechanism is also common, like the production of different β-lactamases within one bacterial cell [24].

1.3.2 Mechanisms of transfer of AMR

Antibiotic resistance can be intrinsic within a bacterial species, meaning the mechanism is inherent in that particular genus or species and is passed on to the next generation of cells by vertical gene transfer [24]. Intrinsic resistance is typically a result of structural or functional features within this specific genus or species [24].

Acquired resistance on the other hand is when one or several resistance mechanisms are attained by chromosomal mutations or horizontal transfer of resistance-genes originating from another bacterial cell [24]. These genes can be implemented in the bacterial chromosome or in plasmids within the cell, the transfer of entire plasmids are also common [24]. Resistance genes can be acquired through mutations or horizontal gene transfer [24].

A mutation is essentially a change in the nucleotide sequence of a gene [13]. This alteration can be limited to a single nucleotide, parts of a gene, an entire gene or several genes in combination [13]. Mutations can occur spontaneously, by a replication error for instance, they can be the

consequence of exposure to mutagens, like chemical factors, or the product of biological factors, like foreign DNA being introduced to the bacterial cell [13]. Such a mutation can result in a phenotypic change in the bacterial cell, sometimes giving the mutated cells an advantage [32]. For instance, if the mutation results in antimicrobial resistance against penicillins, the mutants will have a greater chance of survival in an environment with high exposure to penicillin [32]. Consequently, the mutants will then multiply and the new gene(s) will be implemented in the population [32]. For a mutation to be considered stable, it must have been passed on from one generation to the next as a part of the bacteria`s genetic makeup [13]. Due to the fact that a majority of all bacteria are haploid for most of their genes, and their short generation-time, mutations may arise very quickly, for instance as a result of antimicrobial treatment [32].

Another way for bacteria to acquire resistance genes is through horizontal gene transfer, meaning transfer of genes from one cell to another, unlike vertical gene transfer of the entire genome from mother- to daughter-cell during replication [13]. There are three main mechanisms of horizontal gene transfer, transformation, transduction and conjugation [13].
Transformation is the uptake of free fragmented DNA originating from another bacterial cell undergone lysis. After uptake, the foreign DNA fragments can be implemented in the genome of the recipient cell [13]. Many bacterial species are capable of uptake and integration of naked extracellular DNA fragments, for example *Streptococcus pneumoniae* and *Haemophilus influenza* [33]. There are indications that being exposed to antimicrobial agents, may induce the bacteria`s ability to execute uptake of DNA through transformation [33].

Transduction is the transfer of genetic material from one bacterial cell to another by bacteriophages [13]. During infection of a bacterial cell the bacteriophage integrate its own DNA into the bacterial chromosome, in order to use the bacterial replication systems to produce viral products [13]. When this process is completed, the bacteriophage cut the viral DNA from the bacterial chromosome and package the DNA in protein cases [13]. These packages of viral DNA may also contain fragments of the infected cell`s DNA [13]. The infected cell lyses and the bacteriophage is released and free to infect another bacterial cell [13]. When it then releases its DNA in the new cell, the bacterial DNA from the first infected cell is also integrated in the

new cell`s chromosome [13]. Mobile genetic elements can also be transferred through transduction [33].

Conjugation is genetic transfer through cell-to-cell contact [13]. This contact is facilitated by a sex pilus originating from the donor cell, or surface adhesins [13]. This sex pilus makes an intercellular bridge between the donor and the recipient cell, enabling the transfer of genetic material [13]. The conjugation machinery is typically encoded by genes located on a mobile genetic element, like plasmids or transposons [33]. The DNA transferred from the donor cell can either be chromosomal or located on the same mobilized genetic elements [13]. To transfer chromosomal DNA through conjugation, the chromosome must first be mobilized [13]. This is done by synthesis of a new DNA strand which is transferred to the recipient cell, which in turn synthesise a complementary DNA strand [13].

Transposons are transposable elements, meaning DNA sequences that have the ability to change location within the chromosome, a plasmid, or move between the two genetic elements [13]. The transposons consist of genes necessary for movement as well as genes encoding qualities like AMR [13].

Plasmids are closed, circular genetic elements, which vary greatly in size from one-two kilo bases to more than one mega base [13]. Plasmids typically contain genes important for replication or transfer of the plasmid, and genes encoding features that can give the bacteria advantages over others, like AMR genes [13]. Some plasmids are transferable by conjugation, this is usually initiated with the replication of the plasmid in the donor cell, and the original plasmid is then transferred [13]. Plasmids are highly associated with transfer of antimicrobial resistance genes between bacteria [33]. There are many known plasmids harbouring specific AMR genes, that have been successfully spread through different bacterial species and genus [34]. These AMR plasmids, can be divided into two main groups, narrow-host-range and broad-host-range plasmids [34]. Narrow-host-range plasmids can usually only be conjugated to other cells within the same species, while broad-host-range plasmids have compatibility to different species of bacteria [34]. Narrow-host-range plasmids are usually belongs to incompatibility group F (IncF), while broad-host-range plasmids often belongs to incompatibility groups A/C, L/M or N [34]. IncF plasmids are highly associated with antimicrobial resistant *E. coli* [34].

Plasmids belonging to this group are generally very diverse and varies in size from 50-200kb, and are known to hold a wide range of resistance genes to multiple classes of antimicrobial agents on the same plasmid [34]. Considering the magnitude of resistance genes that can be found on a single plasmid, and their potential for rapid spread through conjugation, it is obvious that these genetic elements play an immense part in the world-wide distribution of AMR.

1.3.3 β-lactamases and β-lactamase mediated resistance

The first β-lactamase was described in 1940 and derived from an *E. coli* isolate. This β-lactamase was chromosomally encoded and categorized as an penicillinase as it hydrolysed penicillin [35]. In 1965 the first plasmid mediated β-lactamase, from a Gram-negative bacteria, was discovered in Greece, it derived from an *E. coli* isolated from blood culture [35]. This β-lactamase was called TEM-1, and it was soon discovered in other Gram-negative bacteria as well [35]. Another plasmid-mediated β-lactamase from *E. coli* and *Klebsiella pneumoniae* was also discovered around the same time, this one was called SHV-1 [35]. TEM-1 can hydrolyse penicillins and first generation cephalosporins, also referred to as narrow-spectrum (NS) cephalosporins, while SHV-1 confers resistance to broad-spectrum penicillins (for example ampicillin and piperacillin)[36]. As an answer to the emerging problem with β-lactamase producing bacteria, new antibiotic agents that were considered to be β-lactamase stable were developed, like second generation cephalosporins [35]. Subsequently, new variants of the known β-lactamases were identified, like TEM-3 and SHV-2, and these new variants had the ability to hydrolyse the new extended-spectrum (ES) β-lactams [35]. Consequently, these improved enzymes were categorized as "extended-spectrum β-lactamases" or ESBLs [35]. Since the discovery of the TEM and SHV enzyme families, a large number of other β-lactamase families have emerged in Gram-negative bacteria, like CTX-M, PER and OXA [36].

CTX-M is one of the most common families of β-lactamases found in Enterobacteriaceae [37]. CTX-M genes are proven to have been transferred into Enterobacteriaceae from different species of *Kluyvera* [37]. Due to bacterial evolution, this large β-lactamase family today comprise of over hundred different enzymes [37].

The OXA family consists of a large number of extremely diverse enzymes with varying activity spectrum [38]. All OXA enzymes are oxacillinases, meaning they hydrolyse oxacillin, in

addition, some are extended-spectrum OXAs (ES-OXA) and also exhibits activity against cephalosporins, and some are OXA-carbapenemases, hydrolysing carbapenems as well [38].

The emergence of β-lactamases able to hydrolyse carbapenems has been of great concern seeing as carbapenems have been the antibiotic agents of choice when dealing with infections caused by ESBL producing bacteria [39]. The first carbapenemases discovered in the late 1980`s were chromosomally encoded, but soon other plasmid-mediated carbapenems were discovered, like KPC in *K. pneumoniae* [39].

The diversity and complexity of the β-lactamase enzymes call for systemized classification schemes to maintain an overview of the different enzymes. Several classification systems are currently in use, some based on the enzymes molecular properties, some based on more functional and clinically practical properties [38]. The β-lactamases can be divided into two major structural groups of enzymes; serine β-lactamases and metallo β-lactamases [40]. Serine β-lactamases uses serine to hydrolyze the β-lactam antibiotics, while metallo β-lactamases are dependent on hydrolysis by a hydroxide ion, stabilized by two zinc ions [40, 41]. The different enzymes` phenotypic resistance profile varies greatly, both between the specific enzyme-families and the individual enzymes [38, 41]. Another scheme for further classification of β-lactamases based on each enzyme`s molecular structure, is the Ambler classification scheme, which consists of four different classes; A, B, C and D [38]. Class A includes both narrow-spectrum β-lactamases (NSBL), like TEM-1, and extended spectrum β-lactamases (ESBL), like TEM-3, and carbapenemases, like KPC [38]. Class B includes all the metallo β-lactamases (MBL), like NDM [38]. Class C includes AmpC cephalosporinases and class D are the oxacillinases (OXA), which can be NSBL, ESBL and even carbapenemases (Carbap.) [38]. Figure 5 shows this classification with examples of different enzymes/enzyme families and their general activity spectrum against specific ß-lactam classes.

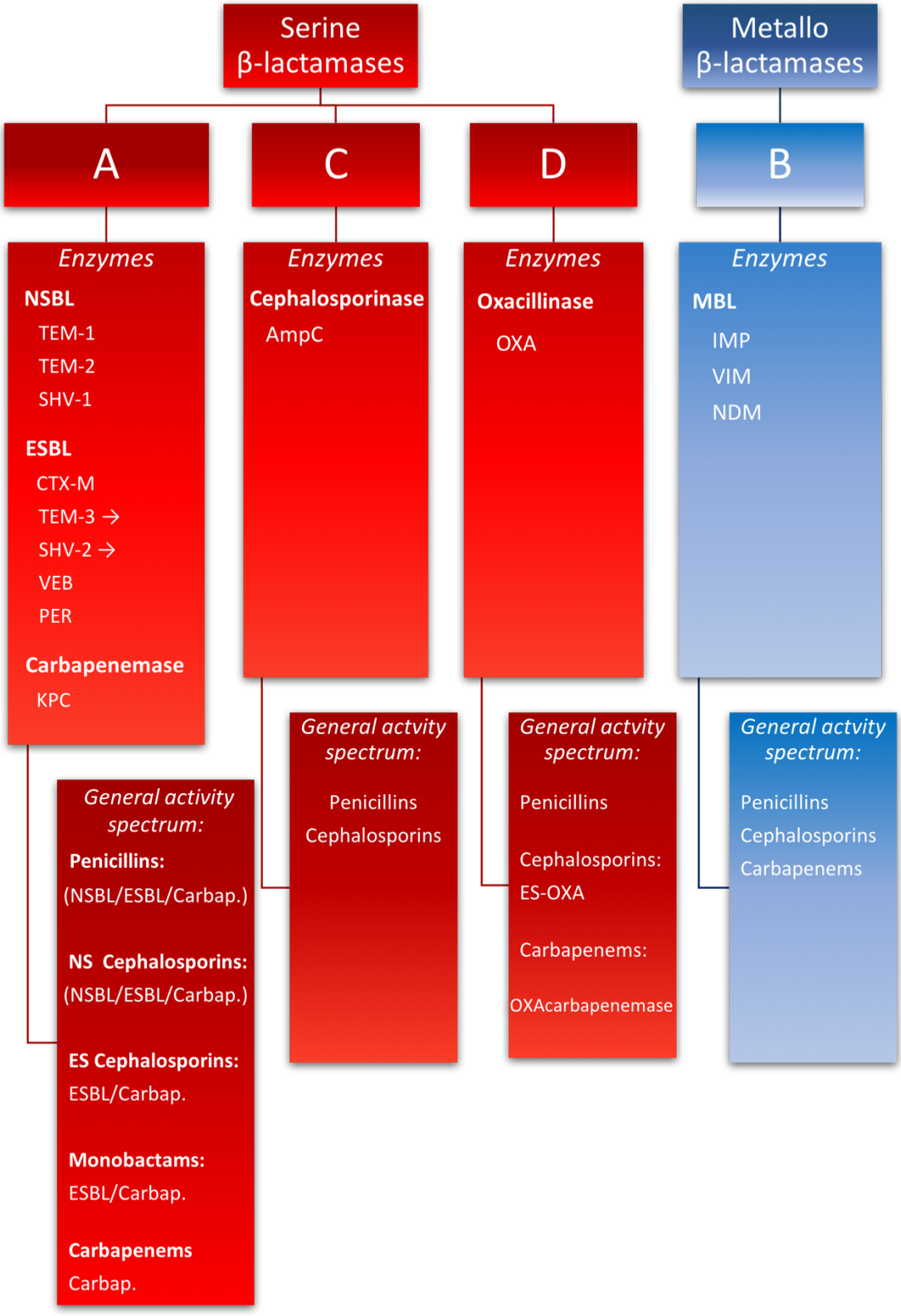


Figure 5: Classification of β-lactamases. Basic classification based on biochemical structures, and further using the Ambler classification system (A, B, C, D) with examples of enzymes in each class and their general activity spectrum. [38]

1.3.4 ESBL

As described in the previous chapter, the first ESBL enzymes were derivatives of previously narrow-spectrum β-lactamases, like the TEM and SHV enzyme family. TEM, SHV and CTX-M all belong to Ambler class A, but the CTX-M enzymes are by far the most successful [37]. In the 1980-90s ESBLs belonging to the TEM and SHV families were the most dominating, but in 2000-2010 the CTX-M enzymes took over as the most prevalent ESBLs in Enterobacteriaceae [37]. The first CTX-M was discovered already in 1989 in Munich, Germany [37]. The enzyme was found in an *E. coli* strain displaying resistance against cefotaxime, isolated from an infant with otitis [37]. The enzyme was called CTX-M-1 (CTX being an abbreviation of cefotaxime, and the M standing for Munich) [37]. Since then, hundreds of different CTX-M enzymes have been discovered, belonging to five main groups, or clusters, of enzymes, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (See table 2 for origins and examples of enzymes) [37]. In general, CTX-M enzymes gives phenotypic resistance to 3rd and 4th generation cephalosporins, and monobactams, they are generally inhibited by β-lactamase inhibitors like clavulanic acid, tazobactam and sulbactam [37]. Many bacteria expressing a CTX-M ESBL, also demonstrates co-resistance, resistance against other antimicrobial agents, like aminoglycosides and fluoroquinolones [37]. This co-resistance is usually caused by the acquisition of plasmids harbouring an assortment of different resistance genes encoding different resistance mechanisms [42]. Plasmid-mediated quinolone-resistance (PMQR) genes are for instance often carried on the same plasmid as CTX-M-genes [42].

The most successful CTX-M enzyme is without a doubt CTX-M-15, belonging to the CTX-M-1 group [37]. This enzyme was first discovered in Enterobacteriaceae isolates in India in 1999, but soon spread to other continents [37, 43]. In 2001 CTX-M-15 emerged in the United Kingdom and during the following five years, CTX-M-15 producing *E. coli* strains were responsible for several outbreaks [37, 44]. The majority of these isolated strains were later proved to belong to sequence type 131 (ST131) [44]. This specific *E. coli* clone will be discussed further in chapter 1.4.1.2. Bacteria producing CTX-M-15 disseminated rapidly, and it is now the most prevalent CTX-M enzyme in the world [37]. Different CTX-M enzymes seem to have arisen in different geographical areas, like CTX-M-10 mainly described in Spain and France, and CTX-M-14 in Korea, but then spread to other regions [37]. As mentioned, CTX-M-14 was first described in Korea in 1995 [37]. It then appeared in China in 1997, Taiwan

in 1998, France 1999 and in Brazil the same year [37]. This shows the enormous success of the CTX-M enzymes, and how fast bacterial populations can evolve, especially subjected to environments containing antimicrobial agents. The $bla_{\text{CTX-M}}$ genes are normally located on plasmids, which can be one explanation for the rapid spread of the CTX-M enzymes through conjugation [37]. The origin of the CTX-M enzymes were genes chromosomally located in different *Kluyvera* species, that became mobilized on mobile genetic elements, like plasmids (for details, see table 2) [37]. Carrying plasmids containing $bla_{\text{CTX-M}}$ became a significant advantage for the bacterial cell, and natural selection in a cephalosporin rich environment resulted in the success of CTX-M producing clones, like ST131 [37]. In addition to the sheer survival of these clones, international travel and immigration, along with the export of food products, are probably all factors in the international dissemination of these clones [37].

Table 2: The different CTX-M groups or clusters, with the origin of each of the groups and examples of specific CTX-M enzymes belonging to each group. (Based on Table 1 in "CTX-M enzymes: origin and diffusion" [37])

CTX-M group (cluster):	**Origin:**	**Examples of CTX-M enzymes:**
CTX-M-1	*Kluyvera cryocrescens* (ancestral gene: *kluC*)	CTX-M-1, CTX-M-3, CTX-M-15
CTX-M-2	*Kluyvera ascorbate* (ancestral gene: *kluA*)	CTX-M-2, CTX-M-44
CTX-M-8	*Kluyvera georgiana* (ancestral gene: *kluG*)	CTX-M-8
CTX-M-9	*Kluyvera georgiana* (ancestral gene: *kluY*)	CTX-M-9, CTX-M-14, CTX-M-24, CTX-M-27
CTX-M-25	*Kluyvera georgiana* (ancestral gene: $bla_{\text{CTX-M-78}}$)	CTX-M-25

1.4 Bacterial species

This project targets two different bacterial species, *E. coli* and *K. pneumoniae*, both Gram-negative bacteria belonging to the large family Enterobacteriaceae. This large and comprehensive bacterial family consists of at least several hundred species, and is steadily expanding due to the advances in technology, especially that of genomic analysis. Species belonging to this vast family range from species considered relatively harmless for humans, such as *Aranicola proteolyticus* and *Buchnera aphidicola*, to species well known to cause severe infections in humans, like *Salmonella typhimurium* and *Shigella sonnei*. [12]

Species belonging to the Enterobacteraceae family are well known for causing hospital acquired infections, like ventilator associated pneumonia, blood stream infections, urinary tract infections and post-operative wound infections [45, 46].

1.4.1 *Escherichia coli*

E. coli is a common commensal species inhabiting the gut of humans and animals, but the species also have pathogenic potential and can therefore be labelled as an opportunistic pathogen [47]. *E. coli* is the main cause of urinary tract infections, but can also cause more severe infections if it gains access to sterile sites, like the bloodstream, and is proven to be the dominating cause of blood stream infections by Gram-negative bacteria [47]. According to the 2015 NORM report, *E. coli* is the leading cause of all blood stream infections in Norway [37]. In 2015 32.4% of blood stream infections were caused by *E. coli*, while the second most common cause was *S. aureus*, which was responsible for 14.4% of the infections, when bacteria considered to be skin flora contaminants had been disregarded [37]. *E. coli* is also known to cause infections like meningitis, wound infections, osteomyelitis, etc. [47]. *E. coli* is also frequently associated with nosocomial infections like ventilator associated pneumonia, hospital acquired blood stream infections, surgical sites infections and nosocomial urinary tract infections [46].

1.4.1.1 Antimicrobial resistance in *E. coli*

As described in chapter 1.3, the prevalence of *E. coli* isolates with a wide selection of different acquired resistance-genes have increased over the last decades. The 2014 WHO report on

antimicrobial resistance shows a high level of antibiotic resistance in *E. coli* isolates worldwide [2]. Five of six world regions report 50% or more of *E. coli* isolates to be antibiotic resistant [2].

β-lactam resistance in *E. coli* is most frequently caused by the production of β-lactamases [48]. The *E. coli* chromosome holds the gene encoding an intrinsic AmpC β-lactamase, however, the gene is usually weakly expressed [48]. This expression of AmpC in *E. coli* is non-inducible, but can still be regulated by alterations in the promoter region and isolates producing high levels of AmpC have been identified in clinical samples [48]. Isolates like this will typically express phenotypic resistance to for example penicillins and some 2nd generation cephalosporins, like cefoxitin [48]. They can also express resistance to expanded-spectrum cephalosporins if the upregulation of the AmpC gene is extensive [48]. In addition to the chromosomally encoded AmpC β-lactamase, a large number of acquired β-lactamases have been identified in *E. coli* worldwide, this subject will be addressed in more details in the next chapter.

In addition to the intrinsic AmpC β-lactamase, *E. coli* may also carry plasmid-mediated AmpC enzymes [49]. The most frequently found plasmid-mediated AmpC in *E. coli*, is CMY-2 [49]. The gene encoding this AmpC β-lactamase, $bla_{\text{CMY-2}}$, is related to a chromosomally encoded AmpC gene in *Citrobacter freundii* [49]. The CMY-2 enzymes confer resistance to 1st, 2nd and 3rd generation cephalosporins, and enhanced resistance to penicillins [49].

The main cause of fluoroquinolone resistance in *E. coli* is mutations in the bacterial targets, DNA gyrase and topoisomerase IV (*gyrA* and *parC)*, but other mechanisms are also represented, like reduced uptake in the cell due to loss or downregulation of porins or efflux mechanisms, like QepA [50]. Fluoroquinolone resistance can also be caused by pentapeptide proteins (Qnr proteins) protecting the bacterial targets, or fluoroquinolone-modifying aminoglycoside acetyltransferases (AAC(6)-Ib) which modify the drug [51].

Aminoglycoside resistance in *E. coli* is mainly caused by the production of aminoglycoside modifying enzymes, like aminoglycoside acetyltransferases (ACC), modifying the aminoglycoside, or 16S rRNA methylases, which alters the binding site of 16S rRNA [52].

Aminoglycoside resistance in *E. coli* can also be conferred by the upregulation of efflux pumps [53].

The *E. coli* genome have in recent years been extensively studied, leading to a greater understanding of the species` genomic diversity [47]. The genome can be divided into two main parts. One consists of a core genome which has been conserved throughout the evolution [47]. The other main part is extremely flexible and consist of mobile genetic elements, like plasmids, bacteriophages, transposons and insertion sequence (IS) elements [47]. These mobile genetic elements may harbour genes encoding pathogenic properties or antibiotic resistance [47].

Analysis of the *E. coli* genome can yield important information which can be used to determine which clonal group the isolate belongs to, and compare it to other isolated strains [47]. These genomic progresses are extremely important epidemiological tools, for instance in outbreak surveillance [47].

1.4.1.2 ESBL-producing *E. coli*

As mentioned in the previous chapter, CTX-M enzymes has arisen as the dominant ESBLs in *E. coli* [54]. Enzymes of the TEM and SHV family is still common, but CTX-M is today the most prevalent ESBL in *E. coli* [54]. CTX-M enzymes have been found in *E. coli* isolated from clinical samples (both nosocomial and community acquired), human carrier strains, animals (both production animals and pets), food products and from environmental samples like sewage [54]. Most prevalent of the CTX-M enzymes in *E. coli*, is CTX-M-15 [54]. The dissemination of this enzyme worldwide can be accredited to the rapid spread of *E. coli* ST131 [54]. This clonal group emerged in several different regions in 2008, more specifically in Canada, a few European countries (France, Spain, Portugal and Switzerland) and in four Asian countries (India, South Korea, Lebanon and Kuwait) [55]. Soon after it was also detected on the African continent and in Oceania, along with numerous other countries in America, Europe and Asia [55]. ST131 is today the predominant *E. coli* sequence type causing infections in humans [55, 56]. *E. coli* ST131 isolates have an extensive repertoire of both virulence and antibiotic resistance genes [55]. As an example; most ST131 isolates possess $bla_{\text{CTX-M-15}}$, encoding the production of the CTX-M-15 enzyme, making the bacteria resistant to penicillins, extended-spectrum cephalosporins and monobactams [55]. In addition the same clonal linage is also

associated with co-resistance to fluoroquinolones [55]. A large number of plasmids of varying sizes, gene-content and incompatibility groups have been identified in different *E. coli* ST131 strains, of these, IncF type plasmids are the most common [55]. Other *E. coli* clonal groups have also been associated with pathogenic significance, like ST648 and ST38 [54]. ST648 is also associated with the carriage of CTX-M-15, while ST38 is associated with CTX-M-9 [54]. Another clonal group associated with clinical samples, is ST405 [57]. Isolates belonging to this sequence type is known to produce CTX-M-15, and a carbapenemase called NDM-1 (New Dehli metallo-β-lactamase) has also been identified in ST405 strains [57].

1.4.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae is known as an environmental bacterium, residing in habitats like soil and surface waters [58]. It is a known colonizer of mucosal surfaces of humans, especially the gastro intestinal tract and the oropharynx [58]. It is an opportunistic pathogen, and colonizing strains can cause infections in an immunocompromised host, like pneumonia, hospital acquired blood stream infections, post-operative wound infections, urinary tract infections and liver abscesses [58]. *K. pneumoniae* also has the ability to colonize artificial surfaces of medical devices, like urinary catheters and ventilator tubes due to the formation of biofilms [59]. Due to the opportunistic nature of *K. pneumoniae* causing infections in hospitalized patients and its ability to endure in hospital environments, it is well established as a nosocomial pathogen [60]. According to the 2015 NORM report, 9,1 % of blood stream infections in the participating hospitals in Norway in 2015, was caused by *Klebsiella* spp. once bacteria considered to be skin flora contaminants had been disregarded [37].

In recent years, the advances in molecular analysis, and in particular the possibilities for whole genome sequencing, have resulted in the definition of three different phylogroups of *K. pneumoniae*; KpI, KpII and KpIII [61]. It has also been proposed that these phylogroups should be redefined as three different species; *K. pneumoniae* (KpI), *K. quasipneumoniae* (KpII) and *K. variicola* (KpIII) [61].

1.4.2.1 Antimicrobial resistance in *K. pneumoniae*

As described in chapter 1.2, the emergence of *K. pneumoniae* clones showing increasing levels of antibiotic resistance has been a severe concern for the last decades [58]. As a result of this increasing resistance, treatment of infections caused by *K. pneumoniae* is consequently getting more difficult [58].

The 2014 WHO report on antimicrobial resistance shows a general higher level of antibiotic resistance in *K. pneumoniae* isolates worldwide, compared to *E. coli* [2]. All six world regions report a resistance prevalence of 50% or more for *K. pneumoniae* isolates [2].

The most prevalent cause of resistance against cephalosporins in *K. pneumoniae* is the production of β-lactamases [62]. *K. pneumoniae* (KpI) strains have a chromosomally encoded SHV β-lactamase, while all *K.variicola* and have a chromosomally encoded LEN β-lactamase, and *K. quasipneumoniae* harbours an intrinsic OKP β-lactamase [61]. The production of this β-lactamase usually occurs at low levels, but it still gives phenotypic resistance against penicillins like ampicillin, amoxicillin, ticarcillin and carbenicillin [63]. In addition, numerous acquired β-lactamases have been identified in *K. pneumoniae*, this will be discussed further in chapter 1.4.2.2.

Fluoroquinolone resistance in *K. pneumoniae* can be caused by several different resistance mechanisms, like mutations or protection of the target molecule, downregulation of porins and increase in the production of efflux pumps [64]. Resistance can be adverse by a combination, and sometimes all these mechanisms in cooperation [64]. An example of target protection is the Qnr-proteins which are pentapeptide proteins that protects DNA gyrase and topoisomerase IV from the inhibitory effect of fluoroquinolones [64]. Genes encoding these proteins (*qnr*) are often found on plasmids [64].

The most prevalent cause of aminoglycoside resistance in *K. pneumoniae* is the production of drug-modifying enzymes, for instance aminoglycoside acetyltransferases (ACC), which have been described previously in chapter 1.3.1, the genes for these enzymes are usually found on mobile genetic elements [31]. Another mechanism of resistance to aminoglycosides found in *K. pneumoniae* is the aminoglycoside-modifying enzyme 16S rRNA methyltransferase [65].

This enzyme confers resistance to aminoglycosides by altering the binding site of 16S rRNA [65]. Other resistance mechanisms responsible for aminoglycoside resistance are loss or downregulation of porins and the production of efflux pumps [65].

1.4.2.2 ESBL-producing *K. pneumoniae*

In addition to the intrinsic SHV-1 β-lactamase, a wide range of acquired β-lactamases have been discovered in *K. pneumoniae* isolates worldwide [66]. When ESBLs started to emerge, the most commonly encountered ESBLs in *K. pneumoniae* were mutants of TEM and SHV type enzymes, but as with *E. coli*, the CTX-M enzymes later took over as the dominating ESBLs in *K. pneumoniae* [62]. And as was the case with *E. coli*, CTX-M-15 has had enormous success in *K. pneumoniae* as well [62].

The 2014 WHO global report on antimicrobial resistance shows several world regions have a higher prevalence of *K. pneumoniae* isolates showing resistance to third generation cephalosporins, than *E. coli* isolates [2]. In fact, a large number of countries, belonging to all six WHO world regions, reported more than 50% of clinically isolated *K. pneumoniae*, was resistant to this group of antimicrobials [2].

The advances in genomic methods, makes it easier to determine lineages of highly virulent or antibiotic resistant clones. The most infamous of these clones have, by MLST, been given the designation ST258 [61]. This sequence type was first described in 2009 [67]. Isolates belonging to this sequence type, often produces a KPC β-lactamase (carbapenemase), making the isolate resistant to penicillins, cephalosporins, monobactams and carbapenems [61].There are several other *K. pneumoniae* clones considered to have clinical importance, some of them will be briefly accounted for. *K. pneumoniae* ST17 was the main clone responsible for an outbreak in a neonatal intensive care unit at Stavanger university hospital in Norway in 2008-2009 [68]. This clone produced CTX-M-15 and TEM-1 [68]. *K. pneumoniae* ST11 is another clinically important clone associated with different CTX-M enzymes as well as KPC [69]. This clone is widespread in Asia and is the dominating clone associated with the dispersal of KPC producing *K. pneumoniae* in China [69]. *K. pneumoniae* ST307 is another clone known to produce a CTX-M-15, and some strains even produce a KPC in addition. This clone has replaced ST258 as the most dominant *K. pneumoniae* clone associated with the spread of KPC in Italy [70]. Another

K. pneumoniae sequence type associated with co-production of both CTX-M-15 and KPC, is ST340 [71]. This sequence type belong to the same clonal group (CG258) as ST258 and ST11 [71].

1.5 Carriage of ESBL-producing *E. coli* and *K. pneumoniae*

Both *E. coli* and *K. pneumoniae* is considered parts of the commensal intestinal flora in humans, it is therefore logical that among these commensal strains, there may be some which have acquired genes for ESBL production. Different studies undertaken in different countries and regions, show very diverse results when it comes to the community carriage of ESBL-producing *E. coli* and *K. pneumoniae*. In Norway, a study from Stavanger university hospital, showed a prevalence of fecal carriage of ESBL-producing Enterobacteriaceae to be 2.9% (*n*=26) among pregnant women (*n*=901) [72]. Another Norwegian study describing ESBL-producing Enterobacteriaceae carriage in patients with gastroenteritis (*n*=273) in 2014, showed a prevalence of 15.8% [73]. Another study done in Norway from 2016, determined the carriage rate of ESBL-producing Enterobacteriaceae in "healthy individuals" to 4.9% [74]. A French study from 2012 showed the carriage rate of ESBL-producing *E. coli* in individuals in the Paris area to be 4.9% [75]. The same study showed this prevalence was a 10-fold increase in five years [75]. A Swedish study from 2016 concluded with a community carriage rate of ESBL-producing *E. coli* at 4.7% [76], and a Dutch study from the same year, showed a prevalence of ESBL producing Enterobacteriaceae of 8.6% [77]. An overview of these six studies is presented in table 3.

Table 3: Selected studies investigating carriage rates of ESBL-producing Enterobacteriaceae

Author:	**Year:**	**Country:**	**Population:**	**Prevalence:**
Rettedal *et al.* [72]	2015	Norway	Pregnant women (*n*=901)	2.9%
Jørgensen *et al.* [73]	2014	Norway	Patients w/ gastroenteritis (*n*=273)	15.8%
Ulstad *et al.* [74]	2016	Norway	Healthy individuals (*n*=284)	4.9%
Nicolas-Chanoine *et al.* [75]	2012	France	French inhabitants (*n*=345)	4.9%
Ny *et al.* [76]	2016	Sweden	Swedish inhabitants (*n*=2134)	4.7%
Reuland *et al.* [77]	2016	Holland	Dutch inhabitants (*n*=1695)	8.6%

On a global scale, a large review article by Woerther *et al*. was published in 2013, investigating studies on community fecal carriage of ESBL-producing Enterobacteriacea, from different world regions [78]. This review shows carrier rates in all regions were low (less than 10%) before 2008, but in some regions, increased rapidly after 2008 [78]. In 2008, the first incidence of carrier rates exceeding 60% was reported in Thailand [78]. This review also shows great difference between different world regions [78]. In Europe, the carrier rates were mostly limited to under 10% with little alteration from 2001 to 2011 [78]. The same seems to be the case for America, while for the Eastern Mediterranean, Western Pacific and South East Asia, the carriage rates have increased from under 10% to well over 60% in the same time period [78]. The review stipulates that in 2011 there were 1.1 billion community carriers in South East Asia, 280 million in the Western Pacific region, 180 million in the Eastern Mediterranean, 110 million in Africa, 48 million in America and 35 million carriers in Europe [78]. The same article pointed to international travelling as a risk factor for the acquisition of colonizing ESBL-producing bacteria [78]. The highest acquisition rates seemed to be associated with travel to India, South-East Asia, Thailand and The Middle East [78]. The Norwegian study of ESBL-carriage in patients with gastroenteritis also looked at possible risk factors for acquiring ESBL-producing Enterobacteriaceae, and also concluded that travel to Asia was a significant risk factor [73].

1.6 Carriage of *K. pneumoniae*

Most studies of colonization of *K. pneumoniae* today are focused on isolates with specific resistance markers, like carbapenemase- or ESBL-producing *K. pneumoniae.* There are few studies concentrating on carriage of *K. pneumoniae* as a species, disregarding antimicrobial susceptibility. A few studies are presented in table 4. These four different studies range in prevalence from 21.1% to 62.1% [79-81]. The two oldest studies were carried out in 2012 in China and Korea [79, 80]. In both studies a similar amount of samples (954 and 1174) were taken from adult inhabitants and the prevalence rates were 21.1% for Korea and 62.1% for China [79, 80]. An American study, carried out in 2016, found a prevalence of 23% [81]. This study only included hospitalized patients, and the number of samples collected were 1765 [81]. The most recent of the selected studies was published in January 2017, and also included hospitalized patients admitted to an intensive care unit in an Australian hospital [82]. This study found a prevalence of carriage of 6% among patients admitted from the community, and 19% among patients whom had been recently hospitalized, total carriage rate of both populations was 10.4% [82].

Table 4: Selected studies investigating carriage rates of K. pneumoniae.

Author:	**Year:**	**Country:**	**Population:**	**Prevalence:**
Martin *et al.* [81]	2016	USA	Hospital patients (n=1765)	23%
Lin *et al.* [79]	2012	China	Adult Chinese inhabitants and Chinese adults living in other Asian countries (*n*=954)	62.1%
Chung et al. [80]	2012	Korea	Korean adults and foreign adults who are ethnic Koreans (*n*=1174)	21.1%
Gorrie et al. [82]	2017	Australia	Patients admitted to ICU (n=498)	10.4%

2 Aim of the study

The main aim of this study was to determine the population structure of ESBL-producing *E. coli* and *K. pneumoniae* isolates from both carriage- and clinical samples. In order to achieve this, three separate queries were addressed:

- What is the carriage rate of ESBL-producing *E. coli* and *K. pneumoniae* among individuals in Tromsø?
- What is the population structure of ESBL-producing *E. coli* and *K. pneumoniae* in clinical isolates and in community carriers?

An additional query was also included in the project as it proceeded:

- What is the carriage rate of *K. pneumoniae* among individuals in Tromsø?

3 Materials and methods

3.1 Sample collection

To achieve the aims of this study, several bacterial samples had to be obtained. Fecal samples from individuals in Tromsø was used to determine the carriage rate of *K. pneumoniae,* and the prevalence of ESBL-producing *E. coli* and *K. pneumoniae,* as well as the population structure of the latter. To determine the population structure of ESBL-producing *E. coli* and *K. pneumoniae* in clinical isolates, ESBL producing *E. coli* and *K. pneumoniae* isolated from urine and blood cultures were obtained through NORM.

3.1.1 Fecal samples from Tromsø-7

The Tromsø Study is a series of large population studies carried out in Tromsø over a 40-year time span [83]. The first Tromsø Study, Tromsø-1, was carried out in 1974 [83]. This first study targeted cardiovascular diseases, and compiled data from 6595 men [83]. Since then, six more extensive studies have been completed, gathering data for research of a wide range of medical and social fields of interest [83].

Tromsø-7 is the latest of these population studies, carried out in 2015-2016, and collecting data for a large number of different research projects, such as cancer, obesity, mental health, diabetes and antimicrobial resistance [84]. In total, 33 423 men and women over the age of 40, were invited to participate in the study [84]. The study was composed of two main parts. Part one was a general health survey accompanied by several questionnaires, 21 083 men and women participated in this first part of the study [84]. A selection of 9000 individuals were also invited to participate in the second part of the study, which composed of more extended and specialized surveys [84]. As a part of this survey, 6358 participants were asked to deliver a fecal sample in order to study the carriage of antimicrobial resistant bacteria in the random population [84]. From these individuals, a total of 5015 fecal samples were collected [84]. Further details on the collection and preparation of these samples are given in chapter 3.2.1.

3.1.2 Bacterial isolates from NORM

Norwegian surveillance system for antibiotic resistant microbes (NORM) is a health register for data on resistant microbes in Norway [85]. Every year specific isolates are collected from Norwegian clinical microbiology laboratories and the statistical data from these isolates are used as a basis for monitoring the occurrence and spread of resistant microbes throughout the country [85]. This yearly surveillance serves as an effective tool to assess the development of antimicrobial resistance over time [85].

The data collected through NORM is also reported further internationally to the European Antimicrobial Resistance Surveillance Network (EARS-Net), who summarizes data from 29 European countries [21].

In this project, 126 ESBL-producing *E. coli* and 39 ESBL-producing *K. pneumoniae* isolated from blood cultures in 2014 originated from different hospitals in Norway, were analyzed using whole-genome sequencing to examine the population structure, ESBL-variant and other resistance genes. Results from susceptibility testing of these isolates along with results from 1243 non-ESBL producing *K. pneumoniae* collected in 2014, was also retrieved from NORM for comparison [22]. Table 5 shows a complete overview of the different isolates included in this project.

Table 5: Table showing specifications for all the bacterial isolates included in the project with the source of collection, type of sample and number of isolates. (*) The non-ESBL producing K. pneumoniae from NORM 2014 were not analyzed during this project. Previously registered data was used for comparison purposes.

Collected through:	***Species:***	***No. of isolates:***	***Sample origin:***
Tromsø-7 (Carrier strains)	ESBL-producing *E. coli*	23	Faeces
	ESBL-producing *K. pneumoniae*	2	Faeces
	K. pneumoniae	97	Faeces
NORM 2014 (Clinical strains)	ESBL-producing *E. coli*	126	Blood culture (*n*=90)
			Urine (*n*=36)
	ESBL-producing *K. pneumoniae*	39	Blood culture (*n*=24)
			Urine (*n*=15)
	Non-ESBL producing *K. pneumoniae**	1243	Blood culture (*n*=618)
			Urine (*n*=625)

3.2 Cultivation of bacteria (Tromsø-7 study)

ESBL-producing *E. coli* and *K. pneumoniae* (both ESBL-producing and non-ESBL-producing strains) was isolated from 662 fecal samples from individuals participating in the Tromsø-7 study. The cultivation was performed using different selective and differentiating growth media as described below.

3.2.1 Screening of Tromsø-7 samples

The fecal samples had been prepared in ESwab tubes (Copan) by each of the individuals from whom it came. All the selected participants had received a kit containing an ESwab tube with a sterile swab for sample collection, gloves, a label with a unique identification number, a return envelope and description of how to collect and prepare the sample.

The following procedure was presented to the participants:

1. Open the packaging containing the ESwab tube. Make sure the swab does not come in contact with any surfaces.
2. Rub or roll the end of the swab in toilet paper with faeces. There should be visible faeces on the swab.
3. Open the ESwab tube and place the swab into the tube.
4. Break the swab at the red mark, and put the lid on the ESwab.
5. Place the label on the tube, place the tube in the return envelope and send or deliver the sample to "Tromsøundersøkelsen".

Upon arriving to the laboratory, each of the E-swab tubes had been given an internal laboratory identification number, and 200µl 85% glycerol (Merck) was added before they were frozen at -70°C.

To administrate the screening process of the fecal samples, the laboratory information system (LIS) CGM Analytix, version 5.12, (CGM) was used. The system does not have separate modules designated for research purposes and therefore had to be configured for this purpose. A unique registration code (T7-DYRK-PR) was created and the four different agars used in the project was linked to the registration code. In addition, a new freezing system had to be

implemented to cope with the large number of isolates to be frozen, as well as refreezing the sample itself. This was done by creating five separate storage-lists for the frozen samples: refreeze of the sample, freezing of material from the CLED control agar, freezing of *K. pneumoniae* colonies from the SCAI agar, freezing of *E. coli* and/or *K. pneumoniae* colonies from the CHROMagar ESBL plate, and freezing of *E. coli* and/or *K. pneumoniae* colonies from the CHROMagar mSuperCarba plate.

Before cultivation, each sample had to be reactivated in the Analytix system, re-registered with the new registration code and entered into the storage list for refreezing of the remaining sample material. This had to be done manually for each sample.

The samples were then thawed and 100 µl was cultivated on four different agar plates, each described in more details below. The remaining sample material in the E-swab tube was then frozen at -70°C in a separate Cryovial 2ml cryotube (Simport) for the prospects of future research. The agar plates were incubated at 37°C.

Each sample was examined after ~24 hours incubation. A swipe of the growth on the CLED control plate was frozen, using standard freezing broth (see attachment A) for additional research purposes in the future. Each sample was therefore registered into the CLED agar storage list. Suspicious colonies growing on SCAI, CHROMagar ESBL and/or CHROMagar mSuperCarba (more detailed description in chapter 3.2.2 to 3.2.5.) were identified using a MALDI Microflex LT mass spectrometer by Bruker Daltonics (the procedure is described in details in chapter 3.3). If the result was *E. coli* or *K. pneumoniae*, the sample was registered in the appropriate storage list, and the isolate was frozen at -70°C using a standard freezing broth (see attachment A). When required, the colonies were re-plated on CLED agar for better isolation prior to identification or freezing. This procedure was repeated for all the 662 samples included in the project.

After completion of the general screening of the fecal samples, all the collected (frozen) strains were inoculated on lactose agar, with and without ampicillin (see attachment A) and incubated at 37°C for ~24 hours. Each isolate was then subjected to phenotypic antibiotic susceptibility testing either by disk diffusion (*K. pneumoniae*) or by microbroth dilution (ESBL-producing *K.*

pneumoniae/*E. coli*) (both methods are described in more details in chapter 3.5). DNA extracted from the strains accumulated from the ESBL agar was then sent to whole-genome sequencing at The Genomics Support Center Tromsø at UiT – The Arctic University of Norway (DNA isolation procedure is described in chapter 3.4.1). The sequences were then processed by several different bioinformatics tools as described in chapter 3.4.3. Figure 6 shows the overall workflow for the Tromsø-7 samples.

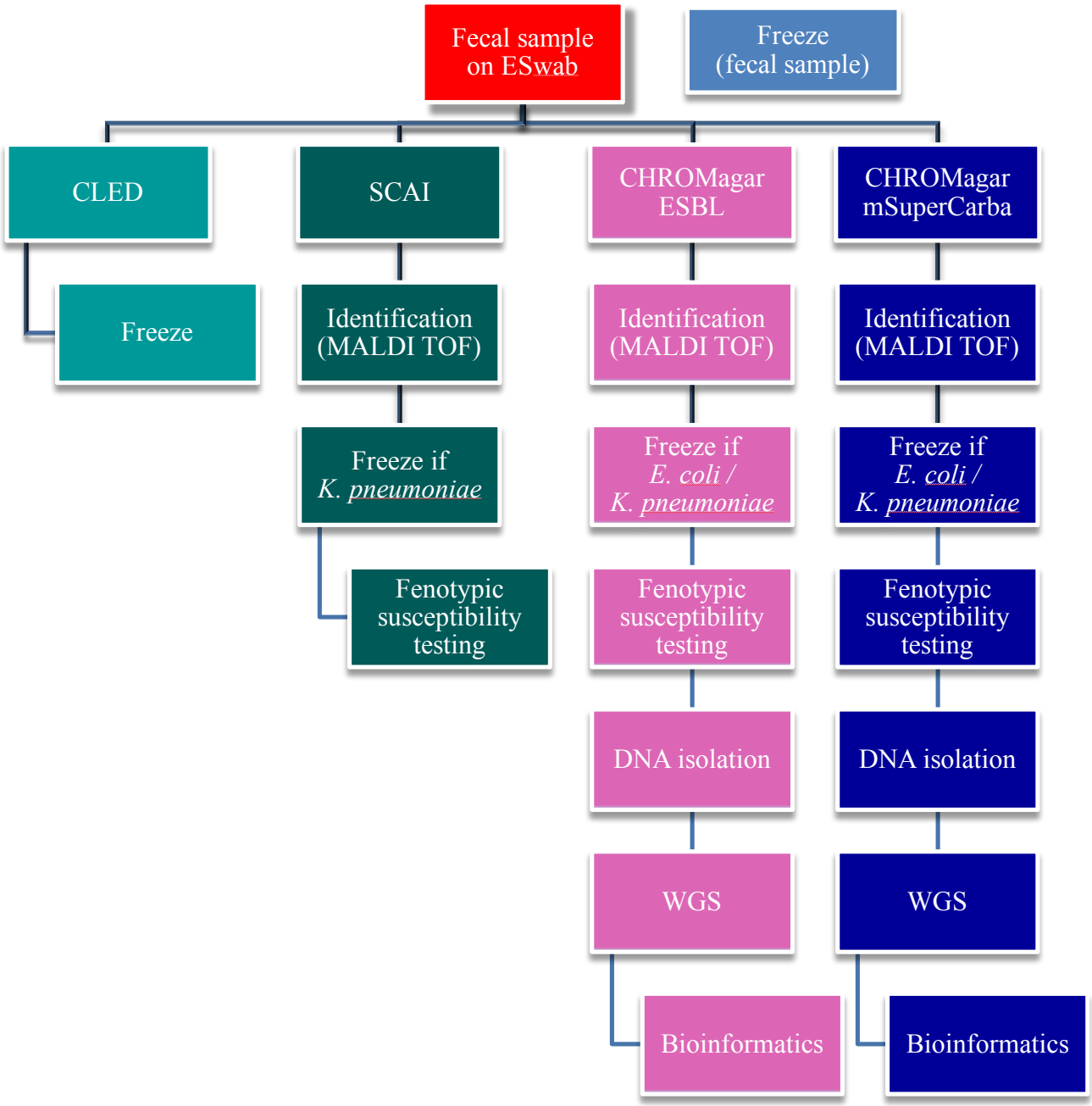


Figure 6: Workflow chart for the screening of fecal samples from the Tromsø-7 study.

3.2.2 Cled agar

Cysteine Lactose Electrolyte Deficient (CLED) agar (see attachment A for details) was used as a growth control, meaning if there was no growth on the control agar the sample was discarded. This agar was chosen due to its inhibitory effect on the swarming of *Proteus* sp., which was expected to be present in some of the fecal samples [86]. The CLED agar was produced according to the manufacturer`s instructions (MAST).

3.2.3 SCAI agar

Simmons citrate agar with inositol (SCAI) is a selective agar for *K. pneumoniae* and *Klebsiella oxytoca* (see attachment A for details on contents and recipe) [87]. Isolating *K. pneumoniae* from fecal samples is challenging due to the abundance of *E. coli* in faeces [87]. *K. pneumoniae* is often represented far less numerous compared to *E. coli* in fecal samples [87]. The SCAI agar contains citrate and inositol as the only carbon sources, both of which can be fermented by *K. pneumoniae* and *K. oxytoca*, but not by *E. coli* [87]. The agar also contains bromthymol blue as a pH indicator which means the desired *Klebsiella* sp. colonies can also be differentiated based on the colour of the colonies [87]. The fermentation of inositol leads to acid production which lowers the pH and the colour changes from blue to yellow [87]. The fermentation of citrate elevates the pH and the colonies becomes blue [87]. Figure 7 shows a *K. pneumoniae* strain growing on a SCAI agar plate. Studies have shown the sensitivity of this agar to *K. pneumoniae* to be 92,5 % [88]. The agar was incubated overnight at 37°C and any yellow or blue large colonies were identified using MALDI TOF. The SCAI agar recipe is presented in attachment A.

Figure 7: K. pneumoniae isolate (Positive control strain ATCC 700603) growing on a SCAI agar plate. (Photo by L. L. E. Andreassen).

3.2.4 CHROMagar ESBL

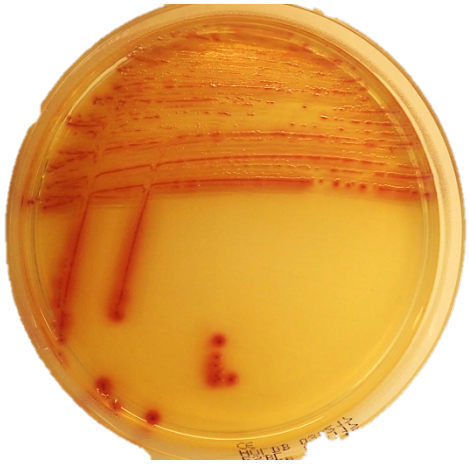


Figure 8: E. coli isolate (Positive control strain EO 499) growing on a CHROMagar ESBL agar plate. (Photo by L. L. E. Andreassen)

CHROMagar ESBL (CHROMagar) is a selective and differentiating agar designed to detect ESBL-producing Gram-negative bacteria (see attachment A for details) [89]. The agar contains antibiotics favoring ESBL-producing strains, while non-ESBL producing strains will be inhibited [89]. CHROMagar ESBL also contains color indicators which allows for differentiation between different species based on color change [89]. *E. coli* will grow on the agar with large pink/reddish colonies, while *K. pneumoniae* will grow with dark blue colonies [89]. Because of false positive results caused by bacteria with intrinsic AmpC expression, the agar is designed to inhibit AmpC-producing bacteria [89]. The agar was produced according to the manufacturer's (CHROMagar) instructions, and consists of a CHROMagar base powder, in addition to a CHROMagar ESBL supplement for selection of ESBL producing bacteria. After seeding the samples, the agar plates were incubated for 18-24 hours in 37°C. The manufacturer specifies the sensitivity of the CHROMagar ESBL to be 99,2 %, and the specificity to be 89 % [89]. Figure 8 shows a CTX-M-15-producing *E. coli* growing on a CHROMagar ESBL agar plate.

3.2.5 CHROMagar mSuperCarba

CHROMagar mSuperCarba (CHROMagar) is a selective and differentiating agar designed to detect carbapenemase-producing Gram-negative bacteria (see attachment A for details on contents) [90]. The agar contains antibiotics favoring strains displaying carbapenemase-production and is designed to detect Gram-negative bacteria producing a wide selection of carbapenemases, including KPC, NDM, VIM, IMP and OXAs [90]. Along with the CHROMagar ESBL, this agar also contains color indicators, which allows for differentiation between different species based on color change [90]. *E. coli* will grow on the agar with large

pink/reddish colonies, while *K. pneumoniae* will grow with dark blue colonies [90]. The agar was produced according to the manufacturer's (CHROMagar) instructions. After seeding the samples, the agar plates were incubated for 18-24 hours in 37°C. The manufacturer does not specify this agar`s sensitivity or specificity [90]. Figure 9 shows a OXA-48 carbapenemase-producing K. variicola.

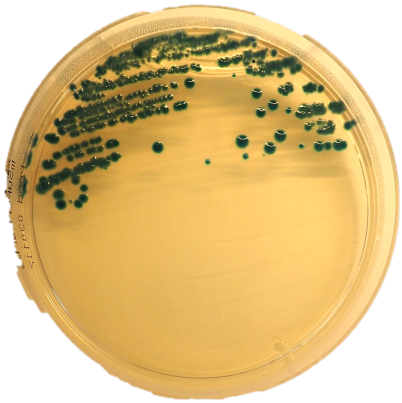


Figure 9: K. variicola isolate (Positive control strain 5087 8013) growing on a CHROMagar mSuperCarba agar plate. (Photo by L. L. E. Andreassen)

3.2.6 Other agar plates

In addition to the four main agar plates included in the screening pipeline, three other agar plates were used in this project, lactose agar, lactose agar with 100mg/L ampicillin and Mueller-Hinton agar (see attachment A for details on contents in each medium). The lactose agar was used to seed frozen isolates, as growth control during susceptibility testing and as additional seeding media when needed. The lactose agar containing ampicillin was used to seed the frozen ESBL-producing isolates from NORM and the isolates from Tromsø-7 suspected of producing ESBLs. It was also used as growth control during susceptibility testing on the same isolates. Mueller-Hinton agar was used to perform susceptibility testing by agar diffusion.

3.2.7 Control strains

All three of the selective and differentiating agars were produced at the media production unit at the Dept. of Microbiology and Infection Control, University Hospital in Tromsø. To ensure the media`s quality different control strains with and without β-lactamase genes was used. Table 6 lists all the control strains used and specifies the expected result on the different agars and also what kind of β-lactamase the positive control strains produce. Some of the control strains are commercially available strains (ATCC), while others are obtained from patient samples at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res).

Table 6: List of control strains for quality control of the different agar plates.

SCAI agar			
ATCC 700603	*Klebsiella pneumoniae*	Pos. control	ESBL- producing (SHV-18)
ATCC 25922	*E. coli*	Neg. control	
50878013	*Klebsiella variicola*	Pos. control	Carbapenemase-producing (OXA-48)
CHROMagar ESBL			
ATCC 25922	*E. coli*	Neg. control	
ATCC 29212	*Enterococcus fecalis*	Neg. control	
ATCC 700603	*Klebsiella pneumoniae*	Pos. control	ESBL-producing (SHV-18)
EO 499	*E. coli*	Pos. control	ESBL-producing (CTX-M-15)
CHROMagar mSuperCarba			
50878013	*Klebsiella variicola*	Pos. control	Carbapenemase-producing (OXA-48)
K71-77	*E. coli*	Pos. control	Carbapenemase-producing (NDM)
EO 499	*E. coli*	Neg. control	ESBL-producing (CTX-M-15)
ATCC 700603	*Klebsiella pneumoniae*	Neg. control	ESBL-producing (SHV-18)

3.3 Identification of isolates using MALDI TOF

Cultivated bacteria was identified using the MALDI Microflex LT mass spectrometer by Bruker Daltonics. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) identifies different bacteria based on protein composition [91]. A single bacterial colony is smeared on a steel target plate , as seen in Figure 10, and covered in an energy-absorbent matrix [91]. The target plate is then loaded into the MALDI TOF MS, where the sample is ionized by a laser beam [91]. The charged particles are then separated in an electrically charged field and proceeds into the "time-of-flight" tube [91]. The time required to travel through this tube to the detector in the other end, is proportional to the protein`s size [91]. The MALDI TOF produces a spectrum visualizing the intensity of a range of proteins of different sizes, called a peptide mass fingerprint (PMF) [91]. This PMF is then compared to known PMFs in the database and a probable identification is set with a score describing the probability of the match [91]. A report is then produced containing a list of the ten best matches for each isolate.

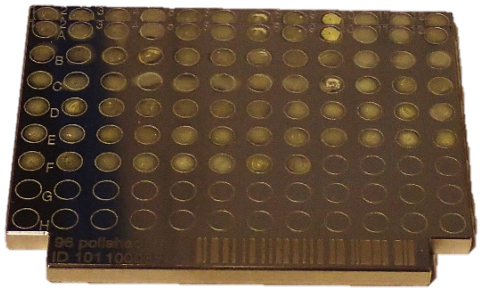


Figure 10: The MALDI TOF steel target plate with isolates applied to specific positions on the plate and covered in Matrix solution. (Photo by L. L. E. Andreassen)

Procedure:

1. A small portion of a colony was applied to a specific position on the MALDI TOF target plate using a wooden toothpick.
2. The position was then covered with 1µl matrix solution (Bruker Daltonics. See attachment A for details).
3. When the matrix solution was visibly dry, the target plate was inserted into the MALDI TOF MS.
4. Each of the samples on the target plate was registered in the MALDI Biotyper Realtime Classification software program, version 3.1. (Bruker Daltonics) and the identification process was started. The identification procedure was run with a standard method,

MBT_AutoX, and each PMF was compared to the Bruker Daltonics database, MBT compass library DB-5989 #1829023 (version v5.0.0.0).

5. The list with the end results were printed out and each result was manually entered into the Analytix LIS program, with designated species and top score.

3.4 Whole-genome sequencing (WGS)

165 clinical strains of ESBL-producing *E. coli* and *K. pneumoniae*, isolated from urine and blood cultures through NORM, were sent to GATC Biotech AG in Germany for WGS. The company also provided isolation of DNA. The NORM isolates were stored at -70°C upon collection in 2014, and was therefore first inoculated on a total of three lactose agar plates, the inoculation pattern was extremely dense to yield the amount of material requested by GATC Biotech AG. After overnight incubation, pellets were made by suspending the colonies in 1ml 0.85% NaCl (see attachment A for details) in a 1.5ml Micro-tube (Sarstedt) and then centrifuging the tube at 15 000 RPM for 10 minutes using a Microfuge 18 centrifuge (Beckman Coulter). This process was then repeated to achieve the goal of a pellet weighing 500mg, which was the amount of material recommended by GATC Biotech AG. Once the pelleting process for each isolate was done, the Eppendorf tube containing the pellet was again frozen at -70°C. When this procedure was completed for all 165 isolates, the Eppendorf tubes were packed in small freezing boxes and sent to Germany in a larger box containing freezing elements.

25 strains (2 *K. pneumoniae* and 23 *E. coli*) suspected of being ESBL-producers, isolated from the fecal samples collected through the Tromsø-7 study were delivered to the Genomics Resource Center Tromsø at UiT for WGS. DNA isolation from these strains was done in-house as described below.

3.4.1 DNA isolation

DNA isolation from the ESBL-producing strains from Tromsø-7 was performed using the NucliSens EasyMAG system (BioMerieux). This system is based on the binding of free DNA to magnetic silica beads [92]. The bacterial cells are first lysed to gain access to the bacterial DNA [92]. During the automatic isolation process in the EasyMag instrument, bacterial DNA is bound to the magnetic silica beads and the beads are then immobilized by a magnetic device

[92]. Several washing steps are performed to purify the DNA in each sample [92]. After the last washing step, the bound DNA is released from the beads by adding an elution buffer. The magnetic device then removes the silica beads from the eluate [92].

Procedure:

1. Pellets of each bacterial isolate was made by making a 4 McF suspension in 0.85% NaCl (see Attachment A for details).
2. 1.4 ml of the suspensions were transferred into 1.5 ml Eppendorf-tubes (Eppendorf).
3. The tubes were then centrifuged at 9000 RPM for two minutes, using a Microfuge 18 centrifuge (Beckman Coulter) and the supernatant was discarded. The procedure was repeated twice to increase the volume of pelleted cells.
4. The bacterial pellets were then suspended in 200 µl lysozyme solution (Sigma-Aldrich) containing 50mg/mL of lysozyme added to NucliSens®easyMAG Lysis Buffer (Biomerieux), and the solution was incubated for 30-40 minutes at 37°C. The lysozyme (Sigma-Aldrich) is an enzyme, extracted from chicken egg white, that breaks down bacterial cell walls [93].
5. After incubation, 20 µl (20 mg/ml) Proteinase K PCR Grade (Roche) was added and the tubes were incubated on a heat block (Grant) at 55°C. Proteinase K is an enzyme used to inactivate endogenous nucleases during DNA isolation [94].
6. 800 µl NucliSens®easyMAG Extraction buffer 3 (Biomerieux) was added to individual wells in the sample cassettes belonging to the easyMAG system,
7. 200 µl of the lysates was then added to each well.
8. The sample cassettes were placed in the instrument and a lysisbuffer was added by the instrument according to a standardized procedure.
9. The automated isolation process was then started. The instrument was set on a program for extracting DNA from whole blood.
10. A solution containing the silica beads (Biomerieux) for binding of DNA was added after 10 minutes.
11. When the instrument had finished, after about an hour, the eluates were transferred into low-bind tubes (Eppendorf) to avoid loss of DNA.

After the isolation process, the purity of DNA was measured along with the amount of DNA in each sample.

The purity of the extracted material was determined using Nanodrop (Thermo Scientific). Nanodrop is based on spectrophotometry, where ultraviolet light is passed through the sample [95]. DNA and RNA will absorb a fixed amount of this light, while the rest passes through to a detector [95]. The detector records the amount of light absorbed by the sample, which is directly correlated to the amount of DNA and RNA in the sample [95]. A wavelength of 260 nm is used to determine the amount of DNA in the sample, while the ratio between measurements at 260 nm and 280 nm is used to determine the purity of DNA in the sample compared to the amount of protein contaminants, as proteins absorbs light at 280 nm [95]. Additionally, the ratio between 260/230 nm is also registered, to assess possible contamination of other organic compounds [95]. Recommended criteria set for purity ratios are generally for 260/280 nm: 1.8 – 2.0, and for 260/230: 2.0 – 2.2 [95].

The amount of DNA present was determined using a Qubit fluorometer (Thermo Fisher Scientific). This method is based on target-specific fluorescence, which is achieved by adding a fluorophore to the eluates that will bind exclusively to the DNA in the sample [96]. The intensity of the fluorescent signal is therefore proportional to the amount of DNA in the sample [96]. The exact amount of DNA is determined using a set of standards of known concentration to create a standard curve, from which the sample is interpreted [96]. For this project, the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) was used, the kit contains assay reagent (fluorophore) and DNA standards [96]. HS means “high sensitivity” and the kit is designed to be used on sample concentrations from 10pg/µl to 100ng/µl [96]. The recommended criteria for satisfactory amount of DNA was > 20µg/µl [96].

3.4.2 Illumina sequencing

All the isolated strains were sequenced using Illumina Next Generation Sequencing (NGS) technology [97]. This technology is based on sequencing by synthesis, meaning the different nucleotides is detected as they are built into the synthesized chain [97]. This is achieved by labelling the four bases with different fluorescent dyes, and the emitted fluorescence is detected [97].

This process begins with the fragmentation of DNA in the sample and the addition of different adapter sequences that binds to the ends of the DNA fragments [97]. These adapters consist of different primers, unique barcodes and specific fragments complementary to oligos in the flow cell in the Illumina sequencer [97]. The fragmented DNA is then entered into this flow cell with the complementary adapters (oligos) attached to the surface, and one end of the fragments will then bind to these immobilized adapters [97]. A new strand is then synthesised by a polymerase and the original strand is then washed away. The adapter on the other end of each of the synthesized fragments will then bind to another (different) complementary adaptor on the flow cell, forming a bridge [97]. The different strains in the flow cell are then synthesized and one end of the bridge is then detached, forming two copies of the original fragment [97]. This process is done repeatedly to create clusters of synthesized fragments on the surface of the flowcell [97]. When the amplification is done, the reverse strands of all the fragments are cleaved off, this leaves only the forward strands left on the flowcell [97]. The actual sequencing of the fragments now starts by adding single nucleotides to create a new copy of all the fragments [97]. The nucleotides are labelled with a fluorescent dye and the emitted light is detected by a camera as the synthesis progresses, this process is called sequencing by synthesis [97]. After all the forward strands have been synthesized and read, the new copy of the strands are discarded and the process starts again, only this time the forward strains are cleaved off and discarded [97]. The result is millions of reads from each sample [97].

3.4.3 Bioinformatic tools

The sequences provided by Illumina came in the form of a large number of overlapping paired-end sequence reads [98]. To obtain contiguous sequences, assembly of these overlapping reads is required [98]. Once the reads are assembled into contigs, the data can be analysed, for instance by comparing the contigs to known sequences and/or to other obtained sequences. Figure 11 gives an overview of the different bioinformatics tools included in the bioinformatics pipeline for assembly construction and further analysis of the data.

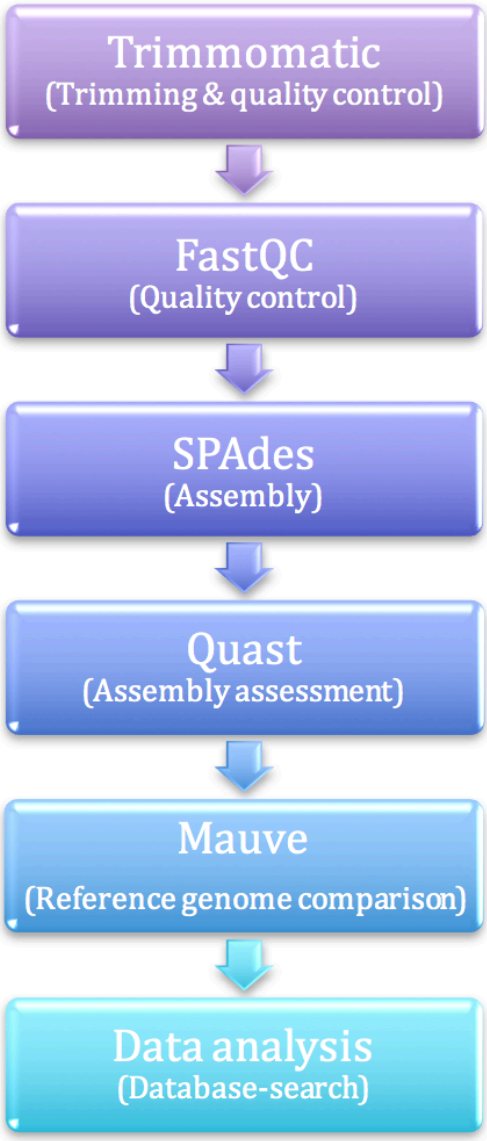


Figure 11: Overview of the different bioinformatics tools involved in the bioinformatics pipeline for assembly construction and data analysis.

3.4.3.1 Trimmomatic

Trimmomatic is a very useful tool that is optimized for trimming the Illumina reads, for example to get rid of adapter sequences added during the sequencing process and poor quality bases that can be problematic during the assembly of the reads [99]. The forward and reverse reads from the WGS were stored in separate files. It was therefore important to use a pre-processing tool that was pair-aware, so that the positioning (connection) of the reads belonging to the same fragment was maintained for later assembly [99]. Trimmomatic was designed to meet the need for an effective, pair-aware preprocessing tool for Illumina data especially. The

main processing steps within the program are identification and removal of adapter sequences, and quality filtering [99].

The following command was used to run Trimmomatic:

```
"java -jar Trimmomatic-0.36/trimmomatic-0.36.jar PE
P19_71_lib119254_4654_5_1.fastq.gz
P19_71_lib119254_4654_5_2.fastq.gz pair_R1.fastq.gz
unpair_R1.fastq.gz pair_R2.fastq.gz unpair_R2.fastq.gz
ILLUMINACLIP:Trimmomatic-0.36/adapters/TruSeq3-
PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15
MINLEN:36"
```

"LEADING:3" and "TRAILING:3" indicate the minimum quality required to keep a base in the start and end of the read, respectively.
"SLIDINGWINDOW:4:15" is the specified threshold for the quality of the read. "4" specifies the window size (number of bases to average across), while "15" is the average quality threshold.
"MINLEN:36" is the minimum length required for the reads, any reads under this length will be discarded.

3.4.3.2 SPAdes

To assemble the reads into contiguous sequences (contigs), the assembly algorithm "St. Petersburg genome assembler" (SPAdes) was used. The SPAdes algorithm is based on using a de Bruijn graph to construct contigs [100]. The short reads are converted into K-mers which are used to build a de Brujin graph [98]. K-mers illustrate all the possible substrings (of for example four bases – 4-mers) of a specific read [98]. The distance between these K-mers are estimated, and the overlapping K-mers are then assembled into contigs [98]. The contigs are now stored as fasta files [98]. This assembly approach where the reads are assembled without the use of a reference genome is called "De novo" assembly [98].

The following command was used to run SPAdes:

```
"SPAdes-3.8.0-Linux/bin/spades.py -1 pair_R1.fastq.gz -2
pair_R2.fastq.gz -s unpair_R1.fastq.gz -s
unpair_R2.fastq.gz --careful -o assembly"
```

The "-1" and "-2" refer to the forward and reverse reads, respectively.

3.4.3.3 Quast

Quality assessment tool for genome assemblies (QUAST) is a very useful tool to evaluate assembled genomes. The program generates reports on the quality of the assembled genome along with an extensive visualization of these data [101]. Some examples of the assessed parameters are the number of contigs in the assembly (the fewer the better), the length of the largest contig, the total number of bases in the assembly and the GC content [101]. For this project, the quality criteria for the assembly was set to <400 contigs in the assembly, 40x coverage, and the GC content and total genome size should not deviate too much from the reference genome for the particular species. Another parameter for assembly evaluation is the N50 value [101]. To determine this value all the contigs are sorted by size, and the N50 value is the size of the median contig, if this is large, there is a likelihood for the rest of the contigs being large too [101]. If the N50 value is small, the majority of the contigs are most likely very short. These parameters can also be visualized in different plots [101].

3.4.3.4 Mauve

Mauve is a tool for aligning two or more genomes with unequal gene content [102]. The program is originally designed for comparing genomes for the identification of genome rearrangements [103]. In this project, it was used to align the sequenced strains with reference genomes as a quality assessment of the assembled genomes. The reference genomes, *E. coli* ATCC 25922 and *K. pneumoniae* MGH 78578, were downloaded from genbank. The assembled genomes from NORM and Tromsø-7 were then compared to the coinciding reference genome. If more than 50% of the contigs did not align with the reference genome, the assembled genome was discarded.

3.4.3.5 Databases

The assemblies of the sequenced *K. pneumoniae* and *E. coli* genomes were submitted to several different databases for detection of specific genes. The Centre for Genomic Epidemiology (CGE) is situated in Denmark and hosts a series of different genomic databases, such as "ResFinder" and "MLST" [104].

In this project ResFinder, version 2.1, was used to identify known acquired antimicrobial resistance genes within the sequenced isolates. The ResFinder database contains thousands of known acquired resistance genes originating from a wide range of bacterial classes [105]. The database is designed to process data from four different sequencing platforms (including Illumina), and the data can be pre-assembled, or short reads or complete (or partially complete) genomes [105]. The antimicrobial configurations selected for this project was an %ID threshold of 90%, selected minimum length of 60%, and the type of reads as "Assembled genome/contigs".

The MLST database was used to determine which sequence type each isolate belonged to. This is determined by identifying and comparing nucleotide sequences in certain housekeeping genes to make up the sequence type [106]. There are different MLST schemes for different bacterial species [106]. In the CGE MLST database, there are two different schemes for *E. coli* [104]. Scheme #1 was chosen for this project, it consists of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) located in the *E. coli* chromosome [107]. Scheme #2 is based on the same number of genes, but the genes are different compared to Scheme #1(*dinB*, *icdA*, *pabB*, *poIB*, *puP*, *trpA*, *trpB*, *uidA*), additionally the number of profiles included in Scheme #1 (6875 profiles) was much higher compared to Scheme #2 (823 profiles) [107, 108]. The *K. pneumoniae* scheme also consisted of seven genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*), and 2644 profiles were included in this scheme [107]. The selected MLST configuration was "*Klebsiella pneumoniae*" and "*Escherichia coli#1*", and the type of reads was "Assembled genome/contigs".

3.4.3.6 Phylogenetic trees

The phylogenetic trees were made from isolates of ESBL-producing *E. coli* from Tromsø-7 and NORM 2014, and ESBL-producing *K. pneumoniae* from NORM. Multiple sequence alignment

was performed on protein level using MAFFT to determine homology of the sequences in each population [109]. The MAFFT alignments were then linked and the phylogenetic trees were constructed using the program RAxML (Randomized Axelerated Maximum Likelihood) [110]. The RAxML command PROTGAMMAAUTO was used to construct a best maximum likelihood tree with 100 rapid bootstrap replicates [110].

3.5 Phenotypic susceptibility testing

For interpretation of zone diameter and MIC values to the SIR system, clinical breakpoints from EUCAST (European Committee on Antimicrobial Susceptibility Testing), version 7.1 (www.eucast.org) was used.

3.5.1 Agar diffusion

Agar diffusion is an effective and easy method for determining a bacterium`s susceptibility to antimicrobial agents [13]. The method is based on creating an antimicrobial gradient on an agar plate and measuring inhibition zones for the different applied antimicrobial agents (for details on the specific agents used see table 7) [13]. When applying paper discs containing antimicrobial agents to an agar plate already inoculated with a bacterial suspension, the antimicrobial agents will diffuse into the agar, creating a concentration gradient [13]. The bacteria will then start growing (dividing) on the plate, except in the areas around the discs where the antimicrobial concentration is high enough to inhibit growth of this particular bacterium [13]. The effect will be inhibition zones with no bacterial growth around the discs. The diameter of these zones (mm) are then measured and compared to set breakpoints and translated into susceptible (S), intermediate (I) or resistant (R) for each antibiotic agent on the plate [13].

Procedure:

1. Bacterial colonies were suspended in 0.85 % NaCl (see attachment A for details) to create a 0.5 McFarland suspension using a DEN-1McFarland Densitometer (Biosan).
2. The 0.5 McFarland suspension was inoculated on Mueller-Hinton agar plates (see attachment A for details) and different paper discs (Oxoid) containing known

concentrations of different antibiotics was placed on the agar plate (for details, see table 7). The suspension was also inoculated on a lactose agar plate for growth control.

3. The plate was then incubated for 18-24 hours in 37°C.
4. The diameter of each inhibition-zone was then registered in Analytix.

Susceptibility testing by agar diffusion was performed for the following antimicrobial agents: piperacillin-tazobactam, cefuroxime, cefotaxime, ceftazidime, meropenem, Gentamicin, aztreonam, ciprofloxacin and trimethoprim-sulfamethoxazole. The concentration of each disc is presented in table 7.

Table 7: Antimicrobial agents (Oxoid) tested by disc diffusion with specific concentrations.

Antimicrobial agent	**Disc concentration**
Piperacillin-tazobactam	36 µg (piperacillin 30 µg, tazobactam 6 µg)
Cefuroxime	30 µg
Cefotaxime	5 µg
Ceftazidime	10 µg
Meropenem	10 µg
Gentamicin	10 µg
Aztreonam	30 µg
Ciprofloxacin	5 µg
Trimethoprim-sulfamethoxazole	25 µg

Agar diffusion was used for *K. pneumoniae* isolated from SCAI agar from Tromsø-7 (*n*=97). Previously registered data from susceptibility testing by agar diffusion was also acquired from the 2014 NORM collection for both *K. pneumoniae* (*n*=1243), ESBL-producing *K. pneumoniae* (*n*=39) and ESBL-producing *E. coli* (*n*=126).

3.5.1.1 Combination discs for detection of ESBL-producers

First indication of the occurrence of ESBL-production in a random isolate is reduced susceptibility to third or fourth generation cephalosporins [111]. Parallel to the standard susceptibility testing by agar diffusion, a phenotypic test for the detection of ESBL-production was also performed. This test is based on the properties of clavulanic acid as a β-lactamase inhibitor [112]. Figure 12 shows the effect of the combination discs on an ESBL-producing *E. coli*. This test was performed on all the strains from Tromsø-7 isolated from the CHROMagar ESBL plate (*n*=25).

Figure 12: Illustration of the effect of the combination discs for detection of ESBL producing bacteria. The photo depicts an ESBL-producing E. coli isolate. (Photo by L. L. E. Andreassen)

.

Procedure:

1. A 0.5 McF suspension of the desired bacteria in 0.85% NaCl (see attachment A for details) was prepared and some of the suspension was inoculated on a lactose agar plate for growth control.
2. The suspension was evenly inoculated on a Muller-Hinton agar plate (see attachment A for details) and two pairs of combination discs were applied. One pair consisted of one disc with 30 µg cefotaxime and a complementary disc with cefotaxime (30 µg) combined with clavulanic acid (10 µg) The other pair consisted of one disc with 30 µg ceftazidime and one with ceftazidime (30 µg) combined with clavulanic acid (10 µg).
3. The agar plates were incubated over night at 37 °C.
4. The next day the diameter of inhibition zones were registered. A difference in zone diameter between the disc containing the cephalosporins alone and its counterparts containing the clavulanic acid of 5 mm or more, was interpreted as positive phenotypic ESBL-production in the isolate [112].

3.5.2 Microbroth dilution system for antibiotic susceptibility testing

Susceptibility testing of suspected ESBL producing bacteria from Tromsø-7, (*n*=25) was performed using the Sensititre system (Thermo Scientific). This system comprises of Sensititre plates with 96 wells containing a selection of antibiotic agents in different concentrations, creating a dilution series. The setup of the two Sensititre plates used (NONAG4 and NONAG5), is shown in attachment A.

Procedure:

1. A 0,5 McFarland suspension in 0.85% NaCl of the isolate in question was prepared and the suspension was also inoculated on a lactose agar plate for growth control.
2. 10 µl of the suspension was added to a Sensititre Cation adjusted Mueller-Hinton broth with TES buffer (Thermo Fisher Scientific), and the tube-cap was then replaced with a special dosage-cap (Thermo Fisher Scientific).
3. 50 µl of this MH broth was then added to the wells in each Sensititre plates using the Sensititre AIM pipetting unit (Thermo Fisher Scientific).
4. A plastic film was added to cover wells and the plates were incubated at 37°C for 18-24 hours.
5. After the incubation, each well was examined for growth using the Vizion Digital MIC Viewing System (Thermo Fisher Scientific). The MIC (minimal inhibitory concentration) value was determined by the first well in the dilution series without visible growth, see figure 13. Each plate also contained a positive-growth control well. If there was no bacterial growth in this well, the plate was discarded.

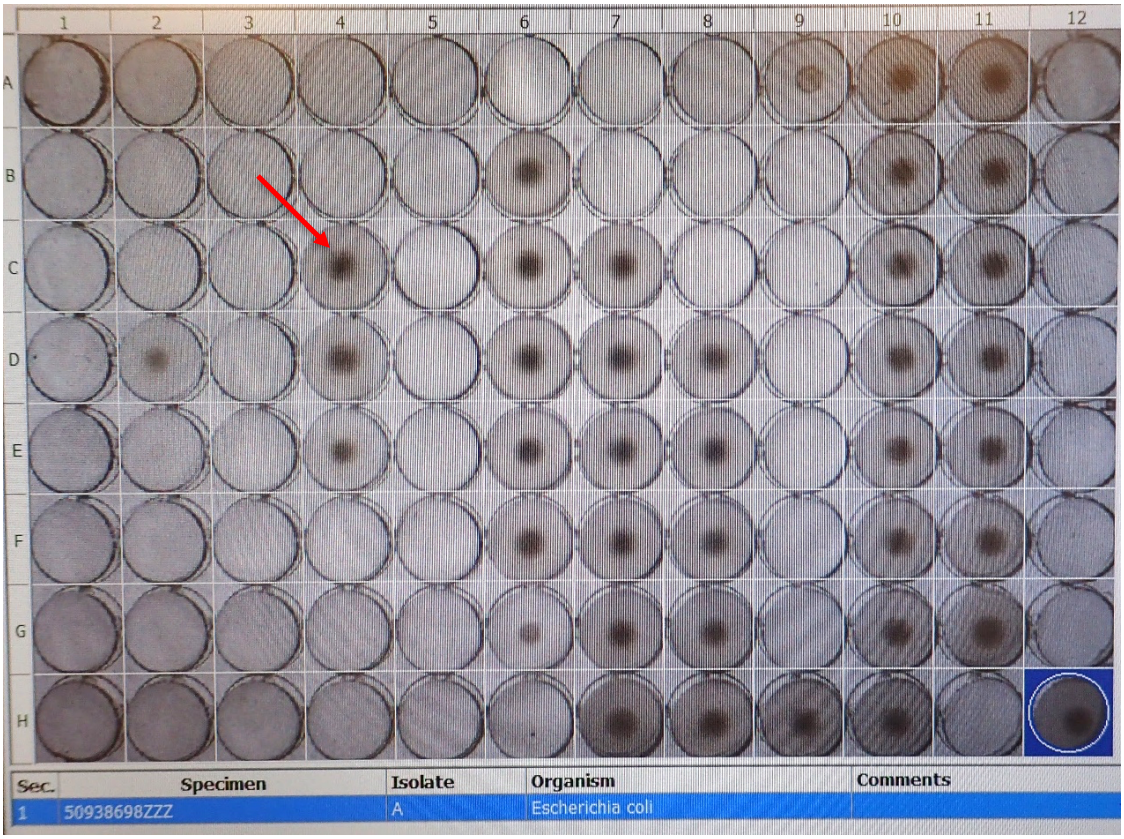


Figure 13: Caption of the examination of a Sensititre plate inoculated with an E. coli isolate. Bacterial growth is seen as dark areas (red arrow) in the wells (Photo by L. L. E. Andreassen)

Susceptibility testing by broth dilution was performed for the following antibiotic agents: amoxicillin-clavulanic acid, piperacillin-tazobactam, cefepime, cefotaxime, cefoxitin, ceftazidime, ceftazidime-avibactam, cefuroxime, doripenem, ertapenem, imipenem, meropenem, aztreonam, ciprofloxacin, amikacin, gentamycin, tobramycin, tigecycline, colistin, fosfomycin, nitrofurantoin, temocillin and trimethoprim-sulfamethoxazole.

4 Results

A total of 662 fecal samples were screened for detection of (i) *K. pneumoniae* irrespective of resistance (SCAI agar), (ii) ESBL-producing *K. pneumoniae* and (iii) ESBL-producing *E. coli*. Only one isolate in each group from each fecal sample with positive growth on the agar plates were selected for subsequent analysis. Ninety-seven isolates of *K. pneumoniae* were obtained from cultivation on SCAI agar. From the CHROMagar ESBL plates, 25 isolates were initially obtained of which 23 were *E. coli* and two were *K. pneumoniae*. No *E. coli* or *K. pneumoniae* were observed on the CHROMagar mSuperCarba agar. The control strains were cultivated on the different agar plates for each new production batch of each of the agars. All the positive control strains grew on the respective agar plates as expected, and none of the negative control strains were able to grow.

4.1 Results *Klebsiella pneumoniae* from Tromsø-7

From the 662 fecal samples, 97 *K. pneumoniae* isolates were acquired from SCAI agar plates, resulting in a carriage prevalence of 14.7 %. All the strains were identified as *K. pneumoniae* using MALDI TOF MS. Results of antimicrobial susceptibility testing performed by agar diffusion of the 97 *K. pneumoniae* isolates are presented in figure 14. All isolates were susceptible to cefuroxime, cefotaxime, meropenem and aztreonam. For piperacillin-tazobactam, 85 of the isolates (87.6%) were sensitive, 11 isolates (11%) were intermediate and one isolate (1%) was resistant. For ceftazidime, 95 isolates (98%) were sensitive, while one isolate (1%) was intermediate and one (1%) was resistant. For gentamicin, 92 isolates (95%) were susceptible and five isolates (5%) were intermediate. For ciprofloxacin, 86 isolates (89%) were susceptible, while 11 isolates (11%) were intermediate. For trimethoprim-sulfamethoxazole, 96 isolates (99%) were susceptible, while 1 isolate (1%) was resistant.

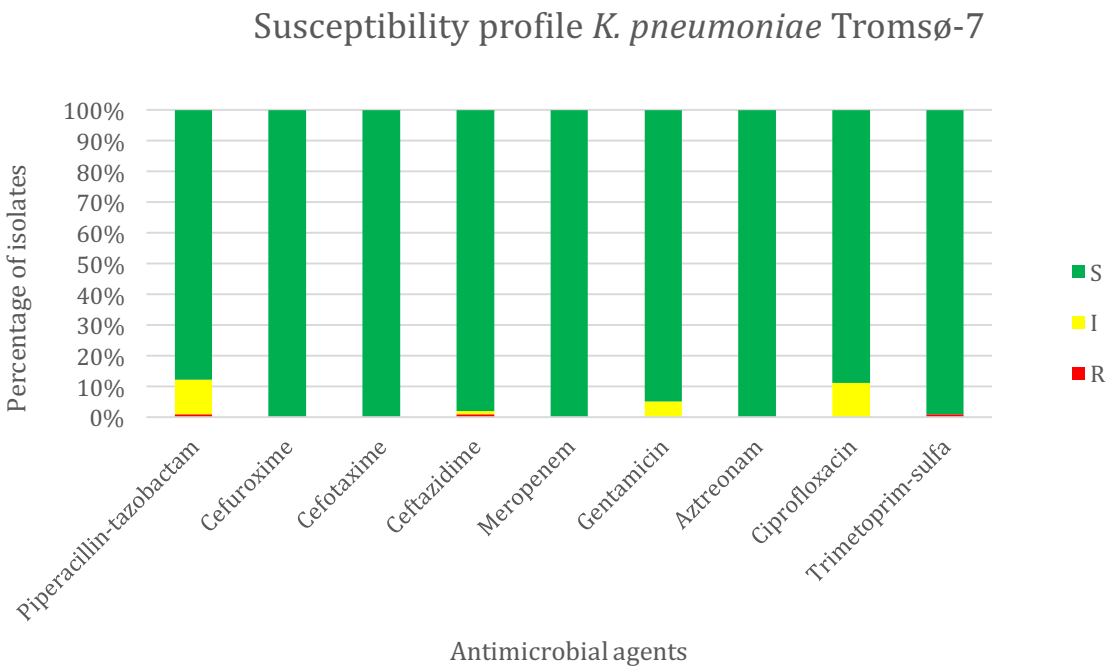


Figure 14: Susceptibility profile of K. pneumoniae isolates obtained from Tromsø-7, presented with percentage of isolates categorized as sensitive (S), intermediate (I) or resistant (R) for each of the antibiotic agents included in the analysis.

To compare the susceptibility profile of *K. pneumoniae* carriage isolates with clinical *K. pneumoniae* isolates, the susceptibility profiles of the 97 *K. pneumoniae* strains obtained from the Tromsø-7 fecal samples were compared to data for 1243 *K. pneumoniae* clinical strains gathered through NORM 2014. With respect to the NORM strains, 625 were isolated from urine samples and 618 were isolated from blood cultures. Data on these strains were obtained from the NORM 2014 report [22]. Figure 15 shows a comparison of the Tromsø-7 carriage isolates to the NORM 2014 clinical isolates. As the diagram shows, there were only minor differences in susceptibility between the clinical strains and the carrier strains for most of the antimicrobial agents included in the analysis. However, there is a general trend of higher levels of non-susceptibility for the tested antimicrobial agents in the clinical isolates compared to the carrier strains. The most prevalent difference in susceptibility seems to be for trimethoprim-sulfamethoxazole, for which 1% of the carrier isolates were resistant, while over 10% of the clinical strains showed reduced susceptibility. Moreover, for cefuroxime (CXM) and cefotaxime (CTX), all the carrier strains were susceptible, while 8% and 3% of the clinical strains were resistant. There are however some exceptions from this general trend, as seen for

piperacillin-tazobactam, ciprofloxacin and gentamicin, where there is a higher level of non-susceptibility in the carriage strains. However, a higher proportion of the clinical strains were fully resistant to these agents while the carrier strains were categorized as intermediate. All isolates in both collections were susceptible to meropenem.

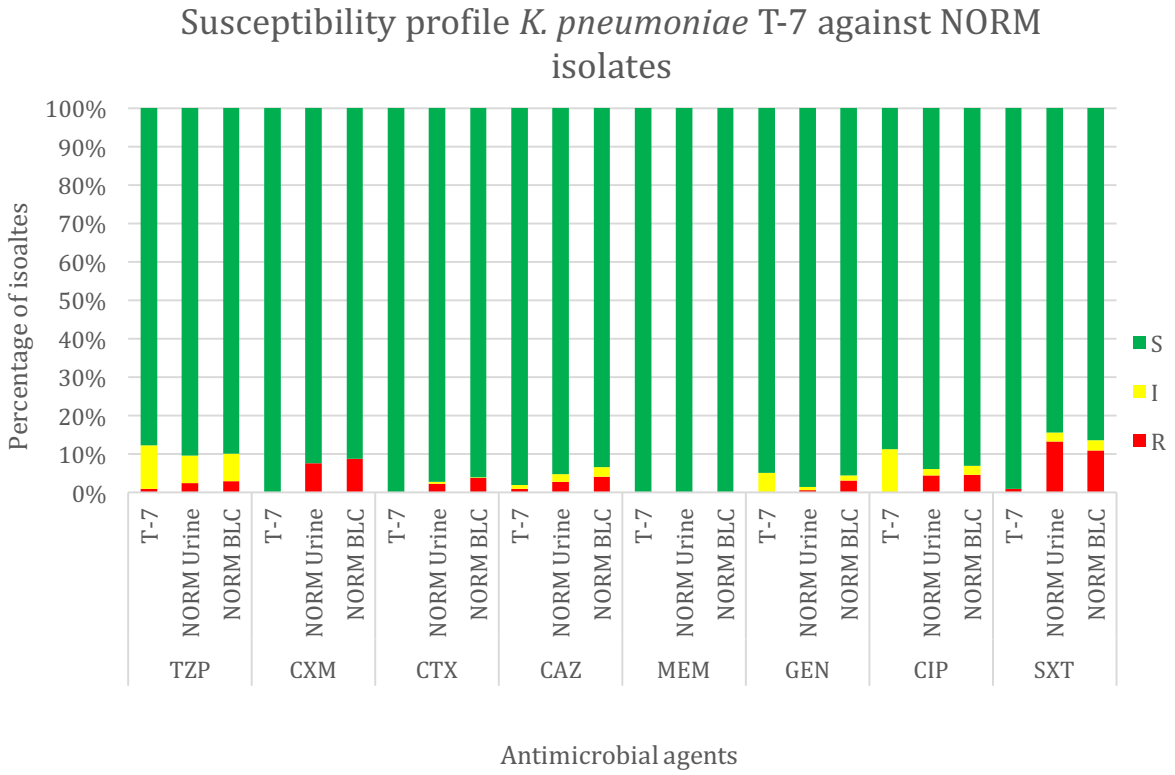


Figure 15: Susceptibility profiles of K. pneumoniae carriage isolates obtained from Tromsø-7 compared to the susceptibility profiles of clinical K. pneumoniae isolates from the 2014 NORM collection. Data obtained from NORM are divided in strains isolated from urine, and strains isolated from blood cultures (BLC). The susceptibility profiles are all presented with percentage of isolates categorized as sensitive (S), intermediate (I) or resistant (R) for each of the antibiotic agents included in the analysis; piperacillin-tazobactam (TZP), cefuroxime (CXM), cefotaxime (CTX), ceftazidime (CAZ), meropenem (MEM), gentamicin (GEN), ciprofloxacin (CIP) and trimethoprim-sulfamethoxazole (SXT).

4.2 Results ESBL-producing isolates from Tromsø-7

From the 662 fecal samples, 25 isolates suspected of being ESBL-producers were obtained using the CHROMagar ESBL agar. Using MALDI TOF, 23 of these isolates were identified as *E. coli* and two isolates as *K. pneumoniae*. One fecal sample yielded both *E. coli* and *K. pneumoniae*.

4.2.1 Phenotypic results ESBL-producing isolates from Tromsø-7

Susceptibility testing of the suspected ESBL-producing isolates from Tromsø-7 was performed by broth microdilution. Figure 16 shows the phenotypic susceptibility profile of the *E. coli* isolates, and figure 17 shows the phenotypic susceptibility profile of the *K. pneumoniae* isolates.

As shown in figure 16, all 23 of the *E. coli* isolates were susceptible to ceftazidime-avibactam, doripenem, ertapenem, imipenem, meropenem, amikacin, colistin, forsfomycin and tigecycline. For amoxicillin-clavulanic acid, 10 of the isolates (43%) were susceptible, while 13 (57%) were resistant. For piperacillin-tazobactam, 21 isolates (91%) were sensitive, one (4%) was intermediate and one (4%) was resistant. For cefepime, three isolates (13%) were susceptible, 12 isolates (52%) were intermediate, and eight isolates (35%) were resistant. For cefotaxime, two isolates (9%) were sensitive, two isolates (9%) were intermediate, and 19 isolates (83%) were resistant. For cefoxitin, 14 isolates (61%) were sensitive (using the screening breakpoint) and nine isolates (39%) were resistant. For ceftazidime, nine isolates (39%) were sensitive, six isolates (26%) were intermediate and eight isolates (35%) were resistant. For cefuroxime, two isolates (9%) were sensitive, while 21 isolates (91%) were resistant. For aztreonam, four isolates (17%) were sensitive, six isolates (26%) were intermediate and 13 isolates (57%) were resistant. For ciprofloxacin, 12 isolates (52%) were susceptible, two isolates (9%) were intermediate and nine isolates (39%) were resistant. For gentamycin, 15 isolates (65%) were sensitive, while eight isolates (35%) were resistant. For tobramycin, 15 isolates (65%) were susceptible, two isolates (9%) were intermediate and six isolates (26%) were resistant. For nitrofurantoin, 22 isolates (96%) were susceptible, while one isolate (4%) was resistant. For

trimethoprim-sulfamethoxazole, nine isolates (39%) were susceptible, while 14 isolates (61%) were resistant.

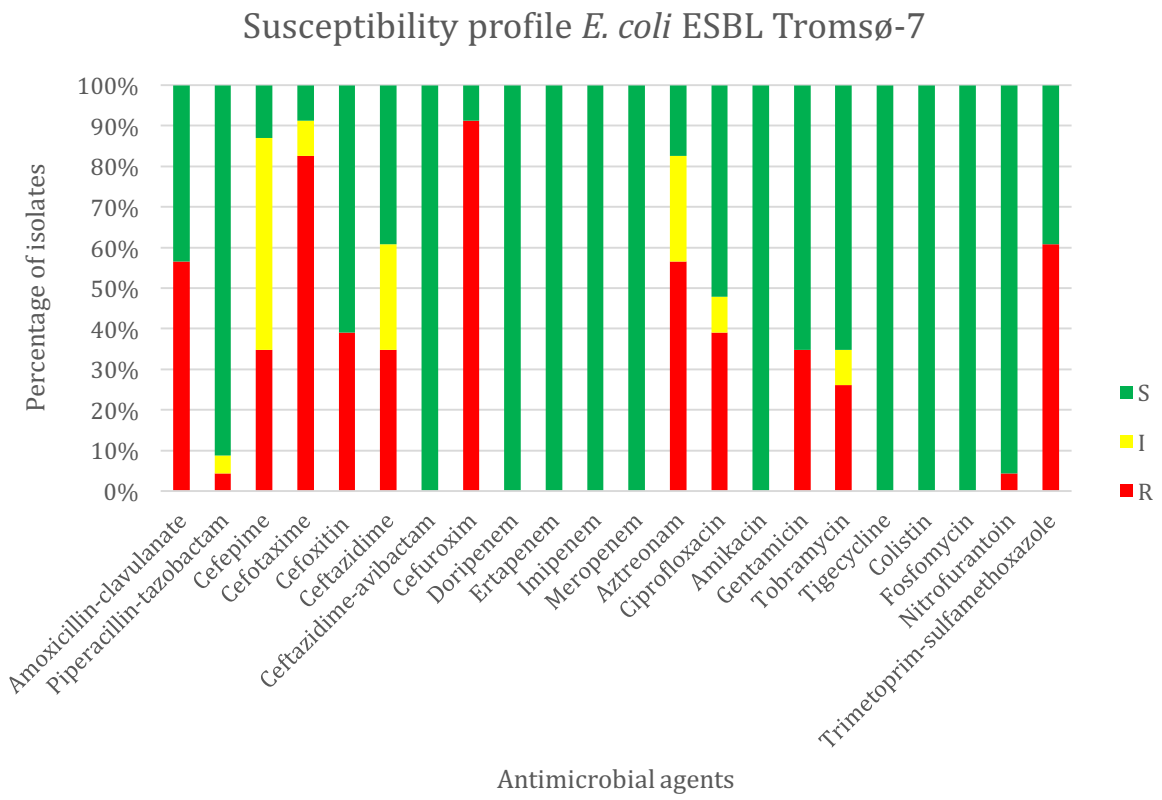


Figure 16: Susceptibility profile of suspected ESBL-producing E. coli isolates obtained from Tromsø-7, presented with percentage of isolates categorized as sensitive (S), intermediate (I) or resistant (R) for each of the antibiotic agents included in the analysis.

As shown in figure 17, both *K. pneumoniae* isolates were susceptible to cefepime, ceftazidime-avibactam, meropenem, imipenem, ertapenem, doripenem, gentamicin, tobramycin, amikacin, colistin and fosfomycin. Both isolates were resistant to cefuroxime. Further, one of the isolates (50934266) was also susceptible to cefoxitin, cefotaxime, ceftazidime, aztreonam, ciprofloxacin and trimethoprim- sulfamethoxazole, resistant to amoxicillin-clavulanic acid, piperacillin-tazobactam and nitrofurantoin, and intermediate for tigecycline. The other isolate (50936472) was susceptible to amoxicillin-clavulanic acid, piperacillin-tazobactam, nitrofurantoin and tigecycline, and resistant to ceftazidime, cefotaxime, aztreonam, ciprofloxacin and trimethoprim- sulfamethoxazole.

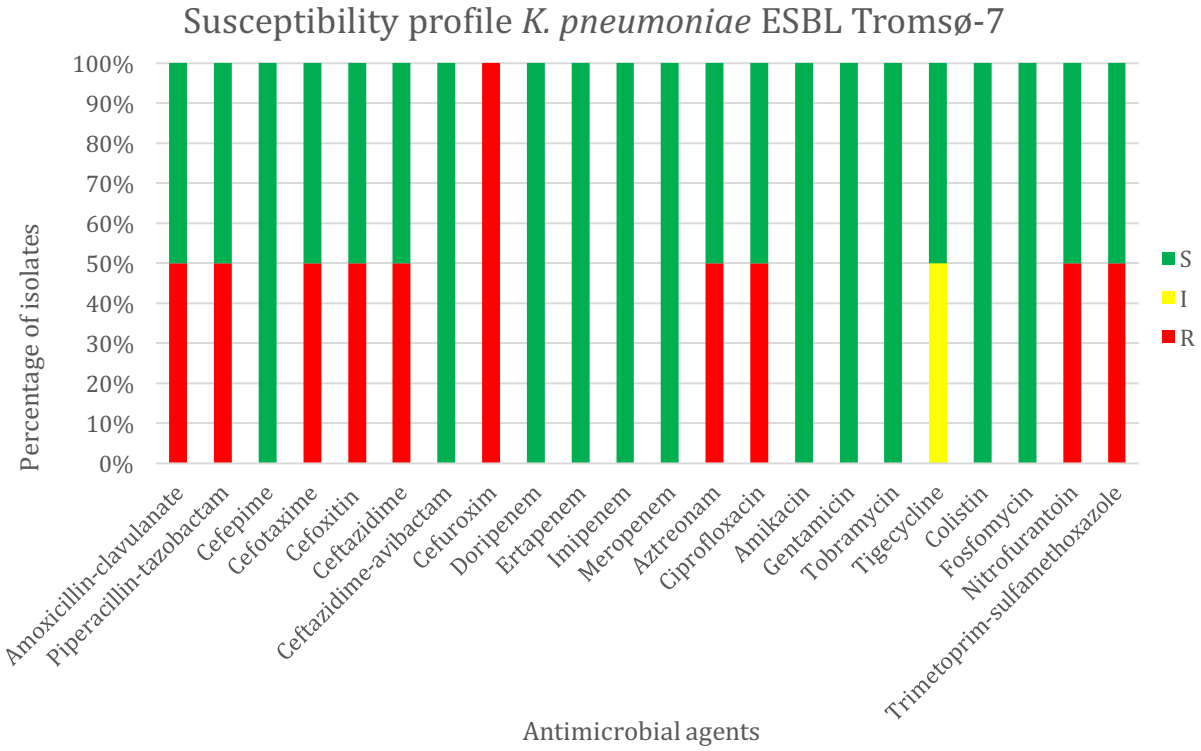


Figure 17: Susceptibility profile of ESBL producing K. pneumoniae isolates obtained from Tromsø-7, presented with percentage of isolates categorized as sensitive (S), intermediate (I) or resistant (R) for each of the antibiotic agents included in the analysis.

Parallel to the phenotypic susceptibility testing, each isolate was phenotypically tested for ESBL-production by a combination disc test. Two *E. coli* isolates (50929671 and 50929669) and one *K. pneumoniae* isolate (50934266) gave a ratio below the set parameters, resulting in a negative test result. One *E. coli* isolate (50922842) had a very low ratio, just over the set parameters. The remaining isolates were phenotypically positive for ESBL-production.

4.2.2 Genotypic results ESBL-producing isolates from Tromsø-7

Al isolates from Tromsø-7 suspected of being ESBL-producers based on growth on CHROMagar ESBL plates, were subjected to WGS, including the isolates with negative results from the phenotypic ESBL-test. Due to a misunderstanding of the labelling of the *E. coli* and the *K. pneumoniae* isolates originating from the same sample (50936472), the DNA from these two isolates were unfortunately mixed during WGS, and the sequences had to be discarded. These two isolates are therefore not included in the genetic analysis.

The genomic data were subsequently processed and used for database searches, to determine STs and presence of known AMR-genes. For the ESBL-producing *E. coli* isolates, these data showed in total 13 different STs, along with two isolates with novel sequence types. The dispersal of the different STs are presented as a pie chart in figure 18. The dominant sequence type was ST131 (32%). The second most dominant sequence type was ST12 (9%). The other represented STs were ST10, ST1290, ST2178, ST38, ST448, ST450, ST617, ST648, ST69, ST88 and ST93, each represented with one isolate. Two isolates had a novel ST-type not previously described. The two isolates that were phenotypically negative were of ST88 and ST131, and the isolate with a low-ratio in the phenotypic test were of a novel ST.

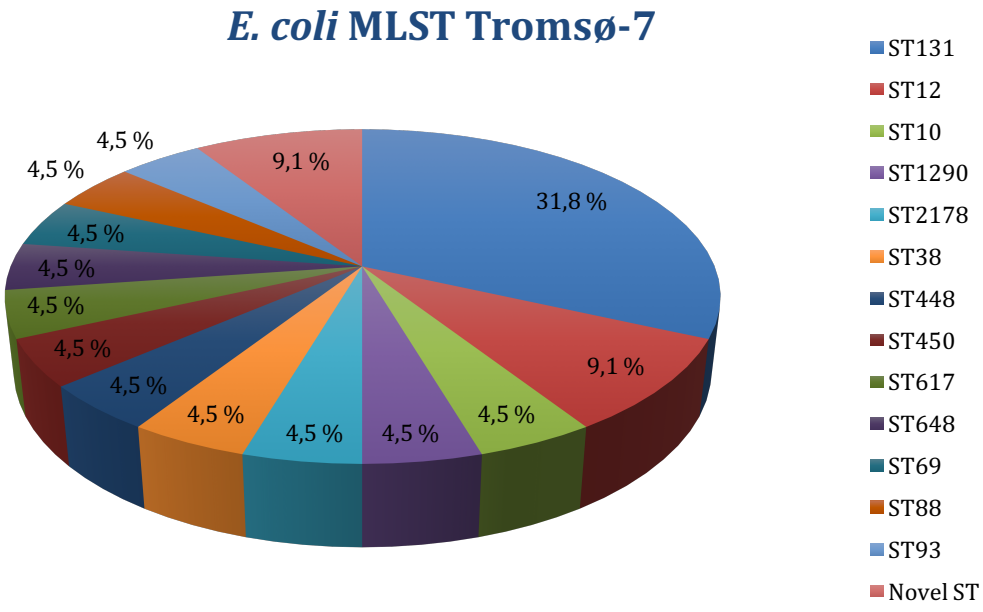


Figure 18: ST dispersal within ESBL-producing E. coli from Tromsø-7, shown in percentage

The WGS data also revealed different known AMR genes, and especially interesting for this project was the ESBL-genes. As shown in figure 19, the dominating ESBL in *E. coli* isolates from Tromsø-7 was CTX-M-15, which was found in nine isolates (47.4%). Closely following was CTX-M-14, which was found in seven isolates (36.8%). CTX-M-8 and CTX-M-27 were also present in one (5.3%) and two (10.5%) isolates, respectively. No other ESBL-genes were found in this population, and no isolate carried more than one ESBL-gene. Three isolates had no known ESBL-gene included in the database, these were the two isolates that were

phenotypically ESBL negative (50929671 and 50929669) as well as the isolate with a low ratio in the phenotypic test (50922842).

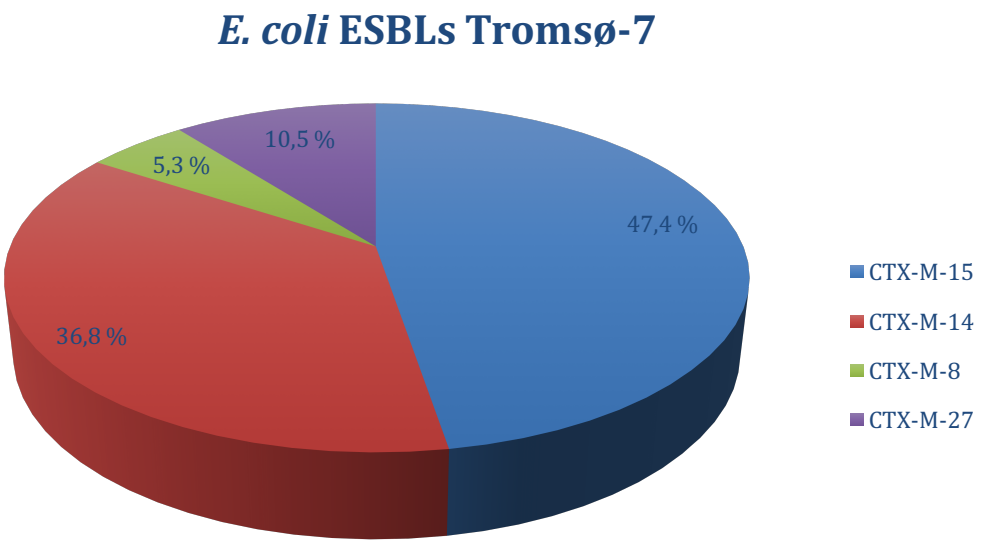


Figure 19: The dispersal of ESBL variants found in E. coli isolates from Tromsø-7, shown in percentages.

The isolates were also analysed with respect to the presence of other selected AMR determinants, including plasmid-mediated AmpC genes, plasmid-mediated quinolone resistance genes, 16S rRNA methylases and carbapenemases.

Three of the *E. coli* isolates, carried the aminoglycoside acetyltransferase-gene, *aac(6`)lb-cr*, which is also shown to affect some fluoroquinolones including ciprofloxacin [51]. All these isolates also carried the ESBL-gene for CTX-M-15. No carbapenemase, plasmid-mediated AmpC or 16S rRNA methylase genes were identified in any of the *E. coli* isolates from Tromsø-7.

As shown in table 8, within the ST131 isolates, there was an evenly dispersal between CTX-M-14, CTX-M-15 and CTX-M-27, with two enzymes of each within the group. The two isolates belonging to ST12, both possessed a CTX-M-14. The rest of the MLSTs seemed to have a random dispersal of CTX-M-14, CTX-M-15 and CTX-M-8. The three isolates with *aac(6`)lb-cr* belonged to three different sequence types, one of them is ST131.

Table 8: An overview of the different sequence types of the ESBL-producing E. coli isolates from Tromsø-7, and the dispersal of selected AMR genes.

ESBL-producing *E. coli* from Tromsø-7							
MLST		**ESBL**					
	n	CTX-M-14	CTX-M-15	CTX-M-8	CTX-M-27	*aac(6')Ib-cr*	Others
ST131	7	2	2	-	2	1	1 with no ESBL gene (50929669)
ST12	2	2	-	-	-		-
ST10	1	-	1	-	-	1	-
ST1290	1	1	-	-	-		-
ST2178	1	-	1	-	-		-
ST38	1	1	-	-	-		-
ST448	1	-	1	-	-		-
ST450	1	1	-	-	-		-
ST617	1	-	1	-	-		-
ST648	1	-	1	-	-	1	-
ST69	1	-	1	-	-		-
ST88	1	-	-	-	-		No ESBL gene (50929671)
ST93	1	-	-	1	-		-
Novel	2	-	1	-	-		1 with no ESBL gene (50922842)
Total:		7	9	1	2	3	

The genealogy and evolution of these isolates are presented in a phylogenetic tree in figure 20. Most of the isolates appeared to be relatively unrelated, with a few exceptions. Two distinct clusters of seven isolates in all (outlined in red) were clonally related. All these isolates belong to sequence type ST131. Although closely related, six of these isolates harboured three different CTX-M ESBLs, while the last isolate did not harbour an ESBL enzyme at all (sample 50929669). Another cluster of two isolates, outlined in green, were also clonally related. These two both belonged to ST12 and they both carried a CTX-M-14 ESBL.

Figure 20: Phylogenetic tree with metadata for ESBL-producing E. coli from Tromsø-7. The complete phylogenetic tree is seen in the top figure, and a magnified version in the red frame. The columns on the left shows selected metadata. The column on the far left is the different sequence types, the middle column depicts the differences in CTX-M genes and the column on the right shows which isolates have the aac(6')-Ib-cr gene. Two clusters are outlined in red, these all belong to ST131. The ST12 cluster is outlined in blue. The phylogenetic tree was generated based on protein coding sequences using RAxML [110]. Association to metadata was done using Phandango [113].

4.2.3 Prevalence of ESBL-carriage in Tromsø-7

Based on both the phenotypic and genotypic results, three *E. coli* and one *K. pneumoniae* isolate which initially grew on the CHROMagar ESBL plate, were found to be ESBL-negative. This resulted in an overall prevalence of carriage of ESBL-producing *E. coli* and *K. pneumoniae* of 3.2%. For *E. coli* and *K. pneumoniae* specifically the prevalence was 3% and 0.2%, respectively.

4.3 Results ESBL-producing isolates NORM 2014

A total of 165 ESBL-producing *E. coli* and *K. pneumoniae* isolates were obtained through the NORM 2014 collection. Susceptibility testing on these isolates had already been performed using agar diffusion and these data were accessed through NORM. In this collection, 126 of the isolates were *E. coli*, 90 of these were isolated from blood cultures, and 36 were isolated from urine samples. Thirty-nine of the isolates in were *K. pneumoniae*, of which 24 were isolated from blood cultures and 15 were isolated from urine samples.

4.3.1 Phenotypic data ESBL-producing *E. coli* NORM 2014

The *E. coli* isolates showed limited differences in susceptibility comparing blood cultures and urine isolates, as shown in figure 21. The majority (75%) of the isolates were resistant to amoxicillin-clavulanic acid. Only a few isolates showed susceptibility to the third generation cephalosporins cefotaxime (1%) and ceftazidime (9%). A majority of the isolates were also resistant to cefotaxime (98%). One blood culture isolate was intermediate for meropenem (1%), the rest were susceptible. For gentamicin 51% of the isolates were resistant, and 83% of the isolates were also resistant to ciprofloxacin. For trimethoprim-sulfamethoxazole, 73 % of the isolates were resistant. Tigecycline, cefepime and piperacillin-tazobactam was only tested on blood culture isolates. All the isolates were susceptible for tigecycline, 95% showed reduced susceptibility to cefepime and 69% the isolates were susceptible to piperacillin-tazobactam.

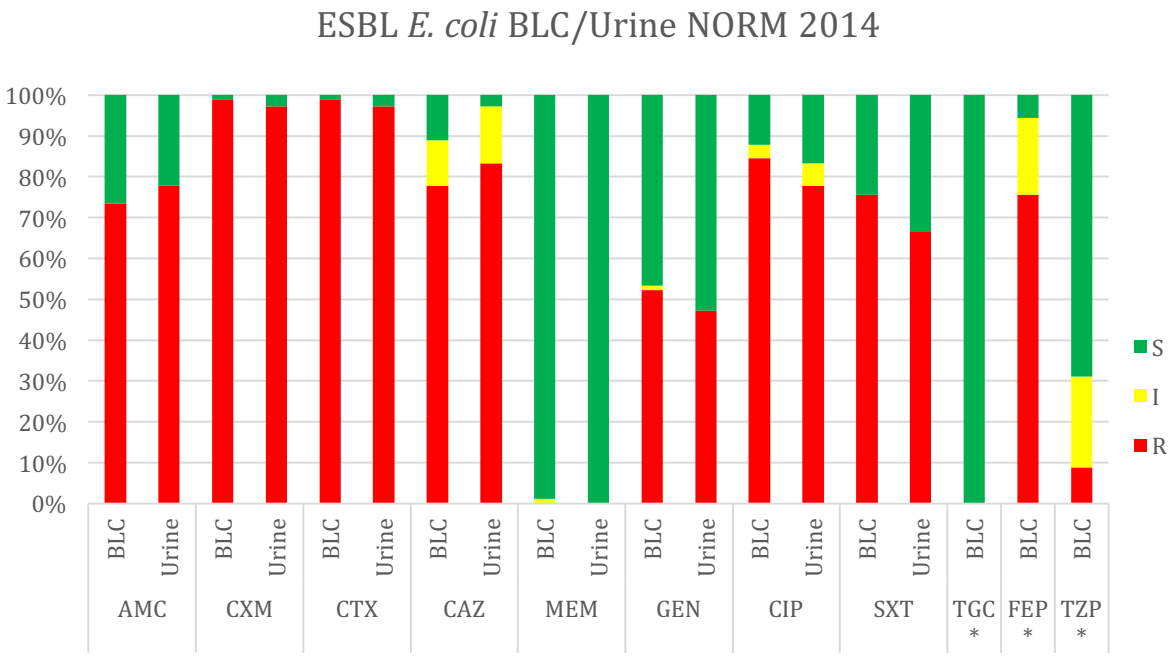


Figure 21: Susceptibility profile of ESBL-producing E. coli isolates from blood cultures (BLC) and urine. The data was obtained from NORM 2014. The susceptibility profile is presented with percentage of isolates categorized as sensitive (S), intermediate (I) or resistant (R) for each of the antibiotic agents included in the analysis; amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), cefotaxime (CTX), ceftazidime (CAZ), meropenem (MEM), gentamicin (GEN), ciprofloxacin (CIP), Trimethoprim-sulfamethoxazole (SXT), tigecycline (TGC), cefepime (FEP) and piperacillin-tazobactam (TZP). (*) Data on tigecycline, cefepime and piperacillin-tazobactam was only available for blood culture isolates.

4.3.2 Phenotypic data ESBL-producing *K. pneumoniae* NORM 2014

The *K. pneumoniae* isolates showed some differences in susceptibility comparing blood cultures and urine samples, as shown in figure 22. The susceptibility profiles will therefore be presented both collectively and separately for the antibiotic agents where there were apparent differences. In general, the blood culture isolates had a higher degree of resistance. For the cephalosporins (cefepime was only tested on blood culture isolates), almost all the isolates were resistant (92%-97%). For amoxicillin-clavulanic acid, there were some differences, all the blood culture isolates were resistant, while 60% of the urine isolates were resistant. The same ratio was seen for piperacillin-tazobactam, 32% of the blood culture isolates, and 20% of the urine isolates were resistant. For meropenem, 95% of the isolates were susceptible, while one blood culture isolate was intermediate and one urine isolate was resistant. For ciprofloxacin, there was a higher resistance rate within the blood culture isolates (92%), than with the urine

isolates (60%). The difference between the two sample groups was most prevalent for gentamicin, where 75% of the blood culture isolates were resistant, to only 19% of the urine isolates. Tigecycline was only tested on blood culture isolates and 75% of the isolates were susceptible. From trimethoprim-sulfamethoxazole, the two groups were very similar with around 82% of the isolates resistant.

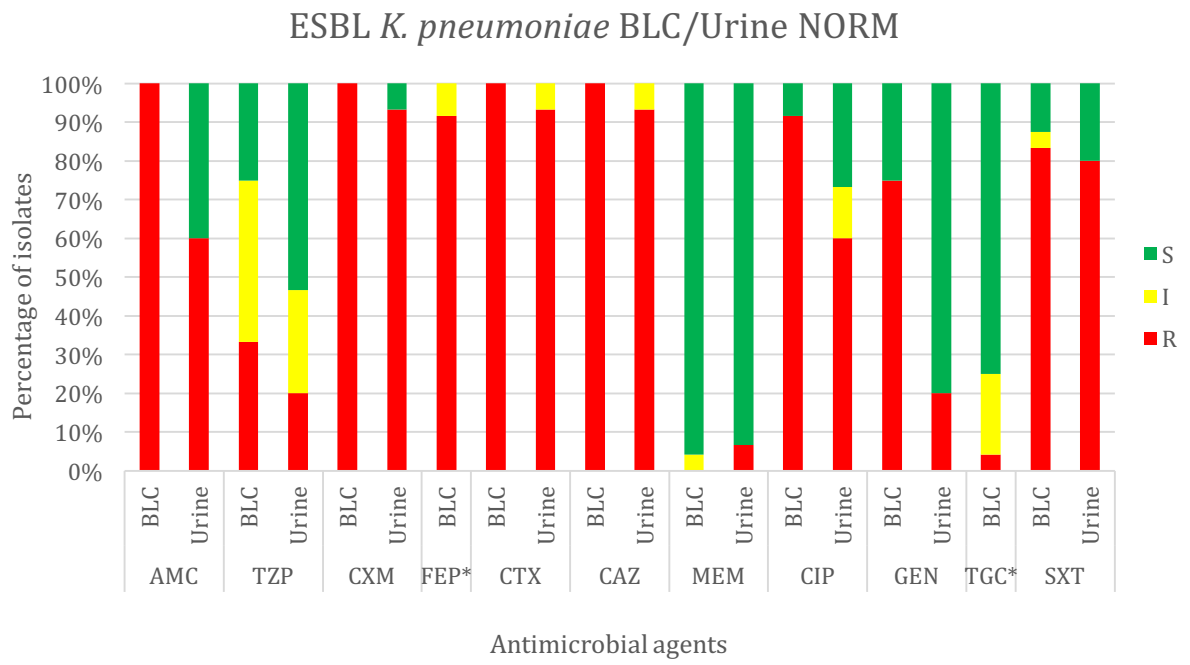


Figure 22: Susceptibility profile of ESBL-producing K. pneumoniae isolates from blood cultures (BLC) and urine. The data was obtained through NORM 2014. The susceptibility profile is presented with percentage of isolates categorized as sensitive (S), intermediate (I) or resistant (R) for each of the antibiotic agents included in the analysis; amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), cefuroxime (CXM), cefepime (FEP), cefotaxime (CTX), ceftazidime (CAZ), meropenem (MEM), ciprofloxacin (CIP), gentamicin (GEN), tigecycline (TGC) and trimethoprim-sulfamethoxazole (SXT) (*) Tigecycline and cefepime was only available for blood culture isolates.

4.3.3 Genotypic results ESBL-producing isolates from NORM 2014

All the NORM 2014 isolates were subjected to WGS. One of the isolates (P19_61) was characterized as a *K. pneumoniae*, but when the sequences was obtained, *E. coli* sequences were identified indicating contamination. This isolate was therefore not included in further genetic analysis.

4.3.3.1 *E. coli*

As illustrated in figure 23, the WGS data showed the NORM *E. coli* isolates to be strongly dominated by ST131, with 56% of the isolates belonging to this sequence type. The second most prevalent sequence type was ST405, represented by 8% of the isolates. ST648 and ST38 both accounted for 5% of the isolates and 3% belonged to ST10. ST617, ST1193, ST372 and ST95 each accounted for two percent of the isolates. Two isolates belonged to novel MLSTs and the rest of the isolates were distributed among 18 other MLSTs, with one isolate belonging to each group.

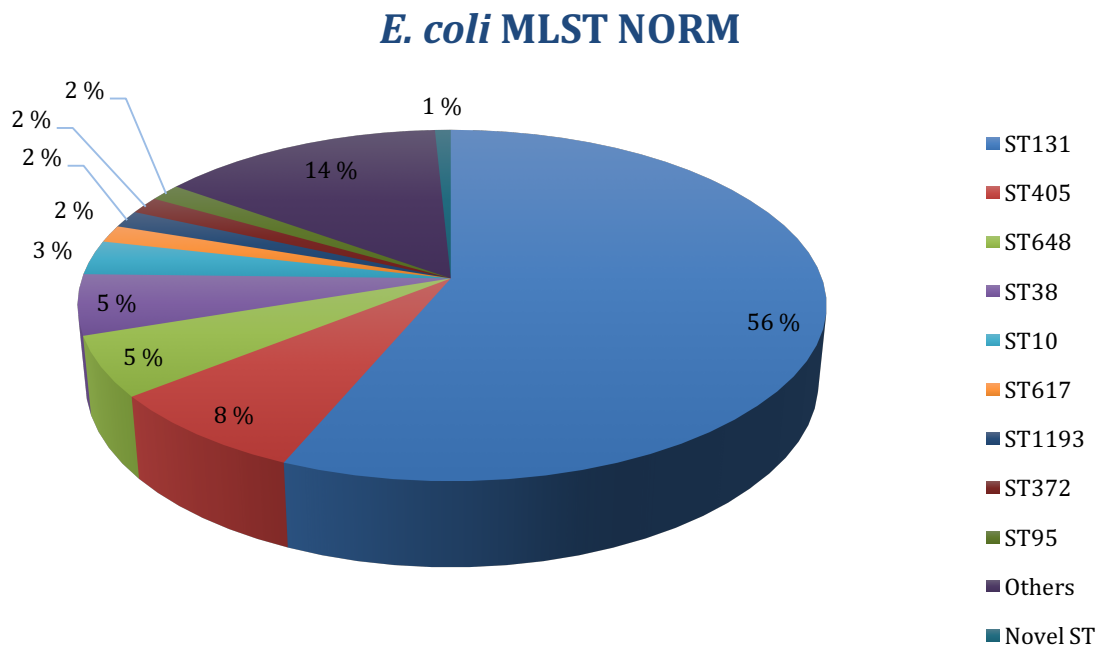


Figure 23: STs in NORM E. coli isolates. Presented in percentages of the total number of isolates.

The distribution of ESBL variants within the NORM *E. coli* population is illustrated as a pie chart in figure 24. The population was greatly dominated by CTX-M-15 which made up 58% of the total number of ESBLs. The second most prevalent ESBL was CTX-M-14 with 16%, and third was CTX-M-27 with 15.5%. Four isolates carried a CTX-M-1 (3%) and 3 isolates carried a CTX-M-55 (2.3%). Two isolates carried a SHV-12, and two isolates had a CTX-M-24 (1.6%). One isolate had a CTX-M-2, and one isolate harboured a TEM-33-like ESBL (0.8%).

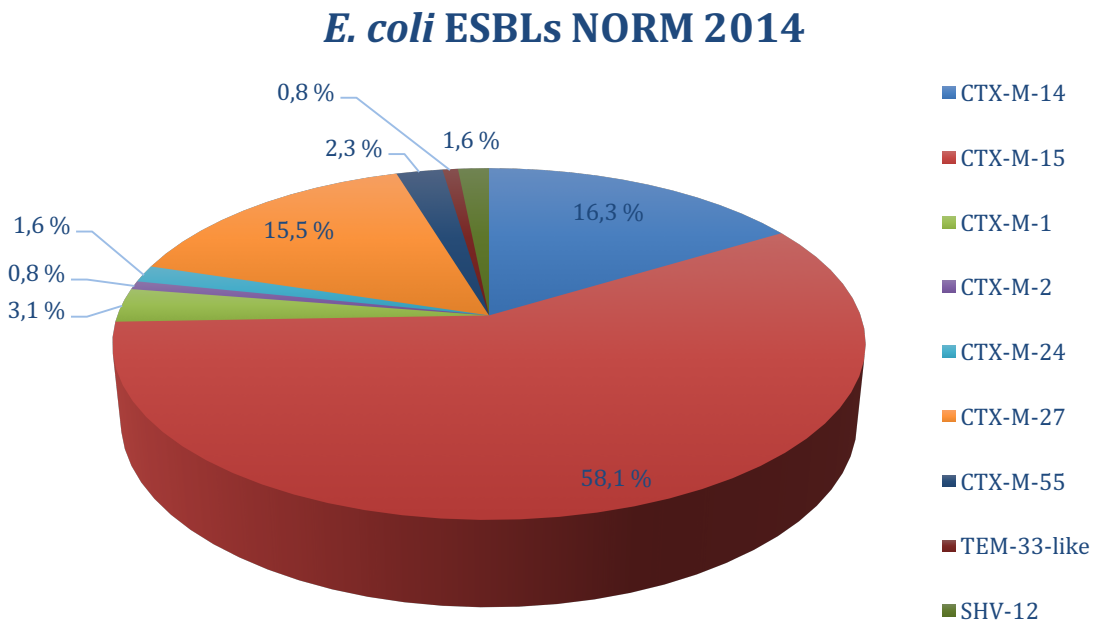


Figure 24: Distribution of ESBL variants in E. coli isolates from NORM 2014. Presented in percentages of the total number of ESBLs.

With respect to other selected AMR determinants, 55 isolates (43.7%) carried the *aac(6`)lb-cr* gene. Eight isolates had a *qnr* gene variant, these were in total two *qnrB19*, two *qnrB66-like*, one *qnrB1-like*, one *qnrB7*, one *qnrS1* and one qnrVC4. One isolate had a *qepA*-gene. Two isolates carried a 16SrRNA methylase, ArmA. One isolate harboured a plasmid-mediated AmpC β-lactamase (CMY-2) and two isolates possessed carbapenemase-genes ($bla_{\text{IMP-26}}$ and $bla_{\text{OXA-181}}$), these two isolates had sample number P20_10 and 20_09.

Table 9 shows the correlation between the different sequence types and selected AMR genes. Within the ST131 population, CTX-M-15 was the dominating ESBL (55%), followed by CTX-M-27 (25%). CTX-M-15 was also the dominating ESBL in ST405 (80%), ST648 (57%), ST38 (71%), ST10 (75%) and both ST617 isolates carried a CTX-M-15. In ST1193, ST372 and ST95, CTX-M-15 constitutes 50%, present in one out of two isolates. Within the group "Other STs", CTX-M-15 was also the dominating ESBL with 56%. The isolate with the novel ST, also carried a CTX-M-15. One of the ST-131 isolates had a TEM-33 β-lactamase, and the same isolate also carried a CTX-M-1. Two isolates had both a CTX-M-14 and a CTX-M-15, both these isolates belonged to ST38. The isolate which possessed a IMP-26 carbapenemase (P20_10), also carried

a CTX-M-15, and belonged to ST95. The isolate carrying an OXA-181 carbapenemase (P20_09), was the same isolate that carried the plasmid-mediated AmpC, it also possessed a CTX-M-15 and it belonged to sequence type ST410. This was the only isolate of this sequence type in the collection.

Table 9: An overview of the different sequence types of the ESBL-producing E. coli isolates from NORM 2014, and the dispersal of selected AMR genes. The selected AMR determinants are ESBLs, plasmid-mediated quinolone resistance (PMQR) and aminoglycoside resistance (16S rRNA methylases).

ESBL-producing *E. coli* NORM 2014															
MLST		**ESBL**								**PMQR**					16SrRNA methylase *armA*
	n	CTX-M-14	CTX-M-15	CTX-M-1	CTX-M-2	CTX-M-24	CTX-M-27	CTX-M-55	Other ESBLs / carbapenamases	*aac(6')Ib-cr* / *aac(6')Ib-cr*-like	QnrB	QnrS1	QnrVC4	qepA	
ST131	71	8	39	4	-	2	18	-	1 TEM-33-like	30	1 QnrB1-like	-	-	-	-
ST405	10	1	8	-	-	-	-	1	-	9	-	-	-	-	1
ST648	7	2	4	-	1	-	-	-	-	3	1 QnrB66-like 1 QnrB19	-	-	-	-
ST38	7	4	5	-	-	-	-	-	-	4	-	-	-	-	-
ST10	4	1	3	-	-	-	-	-	-	1	-	-	-	1	-
ST617	2	-	2	-	-	-	-	-	-	2	-	-	-	-	-
ST1193	2	-	1	-	-	-	1	-	-	1	-	-	-	-	-
ST372	2	1	1	-	-	-	-	-	-	1	-	-	-	-	-
ST95	2	-	1	-	-	-	-	1	1 IMP-26 1 OXA-181	-	-	-	-	-	-
Other ST (n=18)	1 of each	4	10	-		-	1	1	2 SHV-12 1 CMY-2	4	1 QnrB7 1 QnrB19 1 QnrB66-like	1	1		1
Novel ST	1	-	1	-		-	-	-	-	-	-	-	-	-	-
Total		21	75	4	1	2	20	3	5	55	6	1	1	1	2

The phylogenetic analysis of the ESBL-producing isolates from NORM 2014, resulted in a very large phylogenetic tree. The complete tree is presented in figure 25, however, considering the size of the tree, it is divided into two parts (A and B) and presented separately in figure 26 and 27, respectively. As seen in figure 19, part A generally consisted of many different lineages with a series of small clusters, while part B consisted of large clusters all originating from the same lineage.

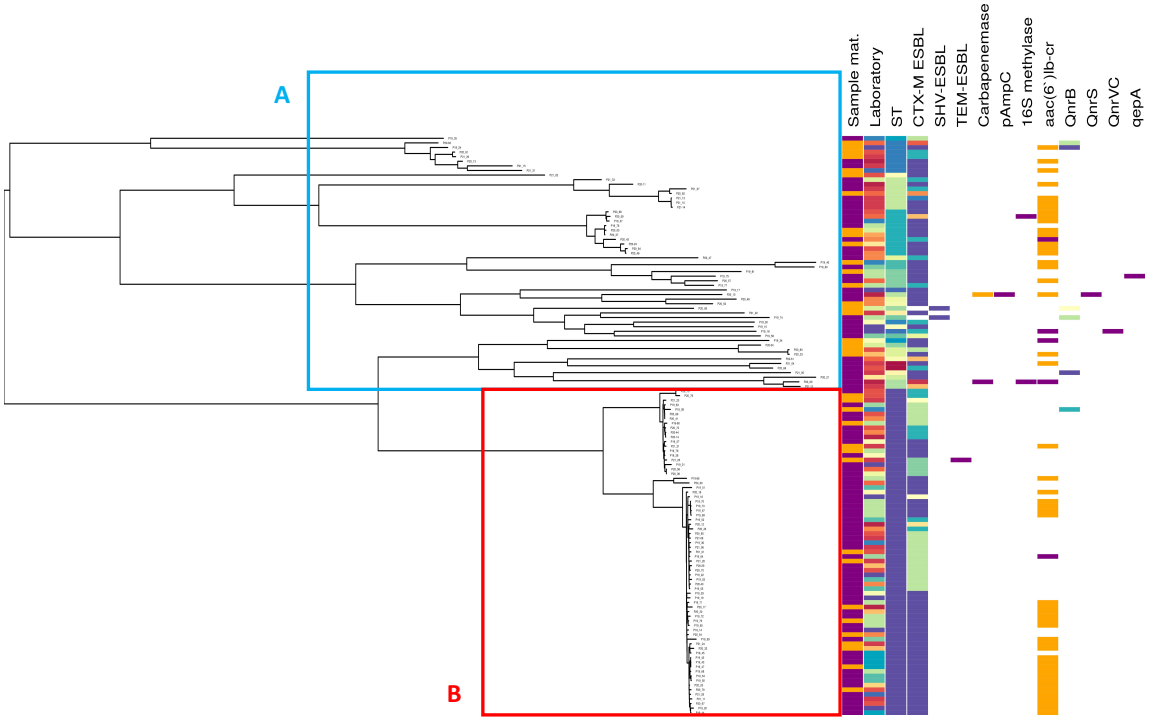


Figure 25: Phylogenetic tree with metadata for ESBL-producing E. coli from NORM. The columns on the right shows selected metadata. Due to the size of the tree, it is divided into two parts. Part A is the general part of the tree, while part B consists of closely clonally related isolates. Part A and B are presented separately in figure 20 and figure 21, respectively. The columns on the tight shows selected metadata. The phylogenetic tree was generated based on protein coding sequences using RAxML [110]. Association to metadata was done using Phandango [113].

As seen in figure 26, part A of the phylogenetic tree for the NORM ESBL *E. coli*, showed four clusters consisting of four to ten isolates, apparently originating from the same linage. Cluster one consisted of seven isolates belonging to ST648, cluster two also comprised of seven isolates, belonging to ST38. Cluster three was the largest, with ten isolates, all belonging to ST405, and cluster four comprised of four isolates belonging to ST10. All these clusters showed diversity in terms of CTX-M ESBL, sample material and laboratory.

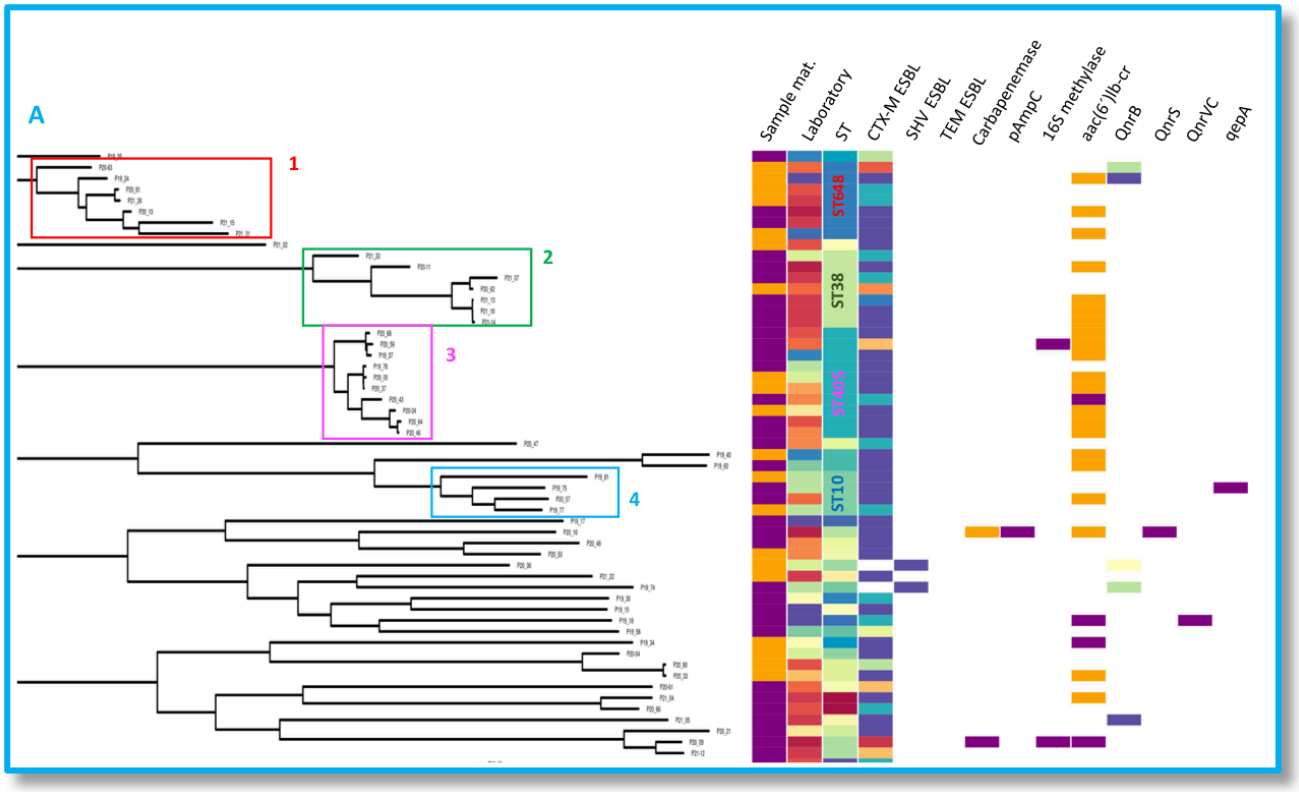


Figure 26: Phylogenetic tree with metadata for ESBL-producing E. coli from NORM, part A. Four different clusters are outlined. Cluster 1 is ST648, cluster 2 is ST38, cluster 3 is ST405 and cluster 4 is ST10.

As seen in figure 27, part B of the phylogenetic tree for the NORM ESBL *E. coli*, showed two large clusters all originating from one lineage. All the isolates in this part of the phylogenetic tree belonged to ST131. The most dominating ESBLs were CTX-M-15 and CTX-M-27. There was much diversity both concerning sample material and originating laboratory.

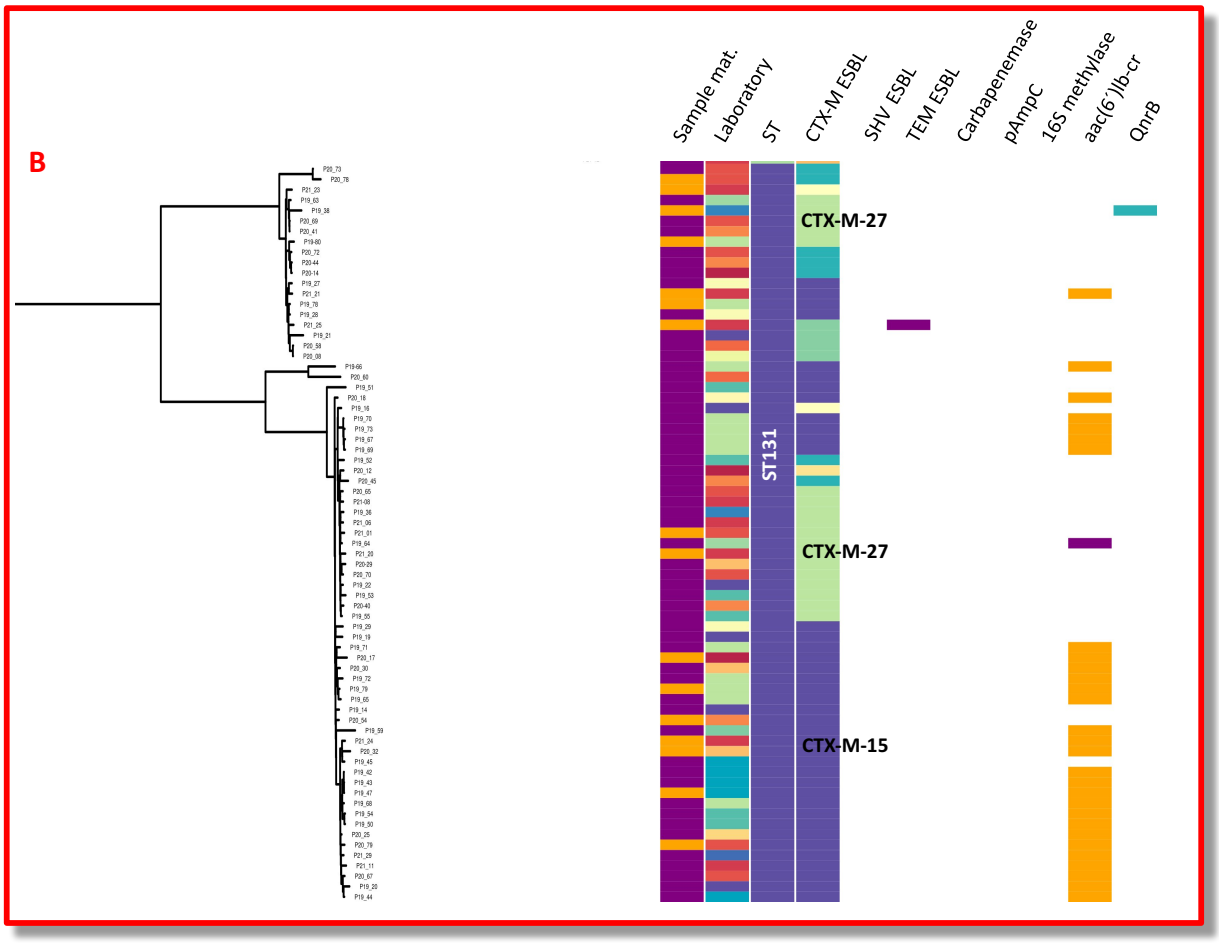


Figure 27: Phylogenetic tree with metadata for ESBL-producing E. coli from NORM, part B. All isolates in this part of the phylogenetic tree belong to ST131.

4.3.3.2 *K. pneumoniae*

The WGS data showed the *K. pneumoniae* isolates from NORM 2014, to be dominated by ST307 (23%), as illustrated in figure 28. The second most dominating sequence types were ST15 and ST340 (both 8%), followed by ST231 and ST45 (both 5%). There was one novel ST in the population, and the rest of the isolates belonged to 18 different sequence types, with one representative of each. In total, this last group consisted of 49% of the population.

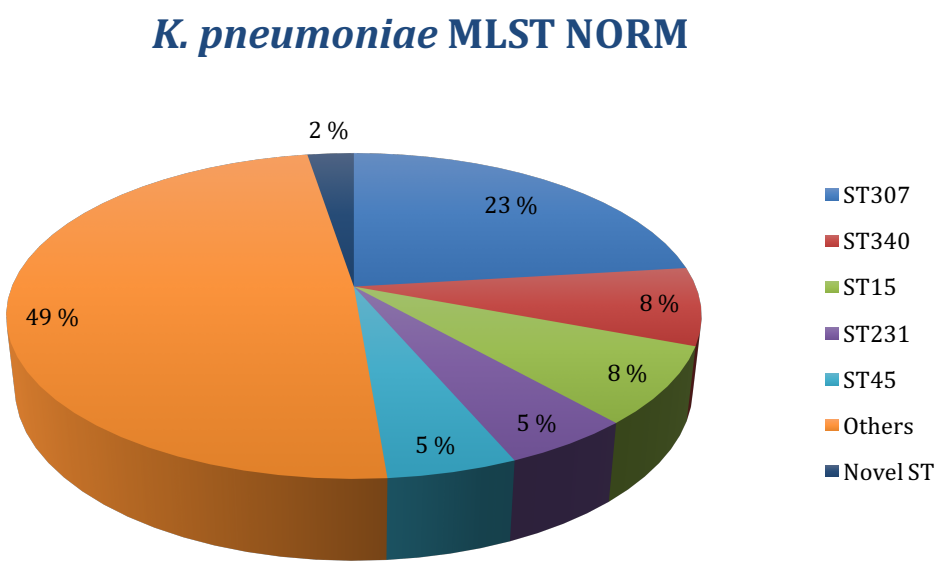


Figure 28: STs in NORM K. pneumoniae isolates. Presented in percentages of the total number of isolates.

Figure 29 shows the distribution of ESBLs within the NORM *K. pneumoniae* population. The population was dominated by CTX-M-15 which makes up 50%. The second most prevalent ESBL in the population was SHV-28 with 19%. Four different ESBLs were each found in two isolates (3%), these were CTX-M-27, SHV-41, SHV-12 and SHV-2-like. Eleven other ESBLs were also only found in one isolate each (1.6%), these were CTX-M-14, SHV-2, SHV-36-like, SHV-14-like, 1 SHV-27, SHV-27-like, SHV-75, SHV-76-like, SHV-99-like, TEM-33 and SHV-129.

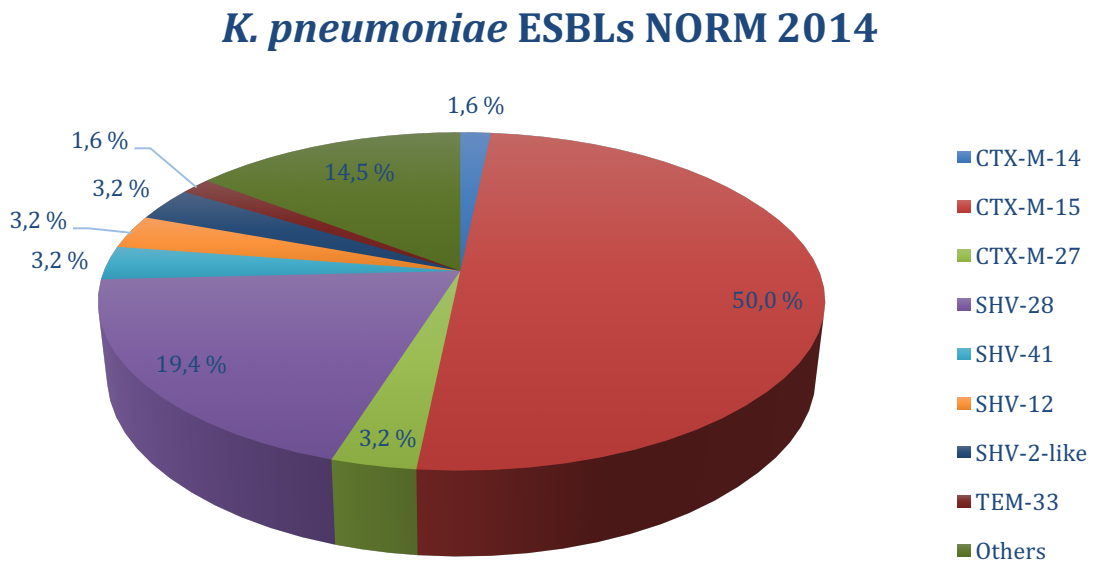


Figure 29: Distribution of ESBLs in K. pneumoniae isolates from NORM 2014. Presented in percentages of the total number of ESBLs.

Concerning other selected AMR determinants, 25 isolates (66%) carried the *aac(6`)lb-cr* gene. Twentyone isolates had a variant of *qnr*-gene, these variants were 17 *qnrB66*-like, two *qnrB1*-like and one *qnrB4*. One isolates carried a 16SrRNA methylase, *armA*. None of the *K. pneumoniae* isolates carried carbapenemase-genes.

Table 10 shows the correlation between the different sequence types and AMR genes characterized in the *K. pneumoniae* population. All the isolates belonging to ST307 carried a CTX-M-15, an SHV-28, and a *aac(6`)lb-cr* gene. In addition, seven of these isolates carried a *qnr*-gene. All the isolates belonging to ST340 harboured CTX-M-15, and carried the *aac(6`)lb-cr* gene. In addition, one of these isolates also carried $bla_{\text{TEM-33}}$, *armA* and *qnrB66*-like. All the ST15 isolates carried CTX-M-15 and a *aac(6`)lb-cr* gene. In addition, two isolates had a SHV-28, one had SHV-129 and one had a *qnrB66*-like-gene. Among the ST45 isolates, one carried a CTX-M-15, a SHV-27, a *aac(6`)lb-cr* gene and a *qnr*-gene (*qnrB1*-like). The other carried an SHV-12. The rest of the isolates represented 18 different STs, and displayed great diversity in AMR markers. The most prevalent ESBL among these isolates was CTX-M-15. Eight of these isolates had a *aac(6`)lb-cr* gene, and 11 carried *qnr*-genes. There was also one isolate of a novel ST, which had a CTX-M-27 and an SHV-41.

Table 10: An overview of the different sequence types of the ESBL-producing K. pneumoniae isolates from NORM 2014, and the dispersal of selected AMR genes. The selected AMR determinants are ESBLs, plasmid-mediated quinolone resistance (PMQR) and aminoglycoside resistance (16SrRNA methylases).

ESBL-producing *K. pneumoniae* NORM 2014													
MLST		**ESBL**								**PMQR**			16SrRNA methylase *armA*
	n	CTX-M-14	CTX-M-15	CTX-M-27	SHV-28	SHV-41	SHV-12	SHV-2-like	Others	*aac(6')Ib-cr* / *aac(6')Ib-cr*-like	QnrB	QnrS1	
ST307	9	-	9	-	9	-	-	-	-	9	7 QnrB66-like		
ST340	3	-	3	-	-	-	-	-	1 TEM-33	3	1 QnrB66-like		1
ST15	3	-	3	-	2	-	-	-	1 SHV-129	3	1 QnrB66-like		
ST231	2	-	2	-	-	-	-	-	-	1			
ST45	2	-	1	-	-	-	1	-	1 SHV-27	1	1 QnrB1-like		
Other ST (18 ulike)	1 of each	1	13	1	1	1	1	2	1 SHV-2 1 SHV-36-like 1 SHV-14-like 1 SHV-27-like 1 SHV-75 1 SHV-76-like 1 SHV-99-like	8	8 QnrB66-like 1 QnrB4 1 QnrB1	1	
Novel ST	1	-	-	1	-	1	-	-	-				
Total		1	31	2	12	2	2	2	10	25	20	1	1

The genetic sequences of the *K. pneumoniae* isolates were used to construct a phylogenetic tree, shown in figure 30, to determine their genealogy and evolution. The phylogenetic tree showed one large cluster, outlined in red, consisting of nine isolates. All these isolates were from blood cultures, they all belonged to ST307 and they all carried the same CTX-M (CTX-M-15) and SHV β-lactamase (SHV-28). They came from several different laboratories, but seemed to be closely related descending from the same *K. pneumoniae* strain. The tree also showed a cluster of three isolates, outlined in green. These three isolates came from blood cultures, but from three different laboratories. They all belonged to ST15, they all had a CTX-M-15 and a *aac(6`)lb-cr* gene, and had ascended from the same strain. The third cluster, outlined in blue, were all ST340 and they all had the same CTX-M (CTX-M-15) and a *aac(6`)lb-cr* gene. Two of them came from the same laboratory while the third came from a different one. They also seemed to be descendants of the same strain.

One isolate, P20_15, was highly unrelated to the rest of the population. This isolate belonged to ST-334 and harboured a SHV-12 ESBL, and in addition possessed an OKP-B-4-like β-lactamase. This isolate is most likely a *K. quasipneumoniae* (KpII), because it is unrelated to the rest of the strains and harbours bla_{OKP}, which is associated with this subspecies [114].

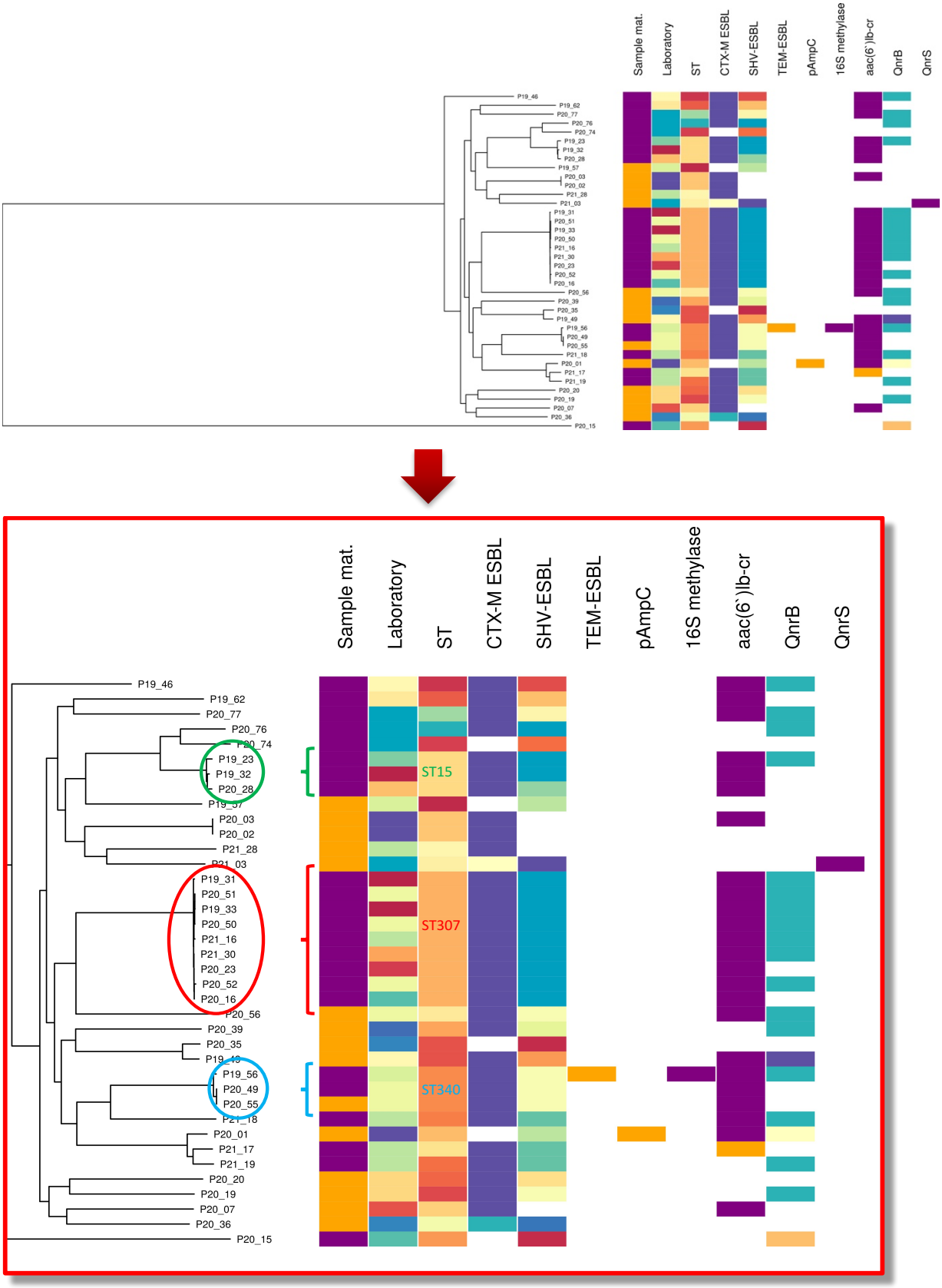


Figure 30: Phylogenetic tree with metadata for ESBL-producing K. pneumoniae from NORM. The complete phylogenetic tree is seen in the top figure, and a magnified version in the red frame. The columns on the right shows selected metadata. The column on the far left is the sample material (purple: BLC, yellow: urine), the next column

depicts the different laboratories which initially handled the sample. The third column shows the differences in STs, and the rest of the columns show differences in AMR-genes. Three clusters are outlined in red, green and blue. The red cluster consists of ST307, the green is ST15 and the blue is ST340.

4.4 Phylogenetic comparison of ESBL-producing *E. coli* from Tromsø-7 and NORM

The sequenced ESBL-producing *E. coli* isolates from both Tromsø-7 and NORM, were also collectively submitted for phylogenetic analysis, resulting in one phylogenetic tree with all the isolates represented. The complete tree is presented in figure 31, however, considering the size of this combination tree, it is divided into two parts (A and B) and presented separately in figure 32 and 33. As seen in figure 31, part A generally consisted of many different lineages with a series of small clusters, while part B consisted of large clusters all originating from the same lineage.

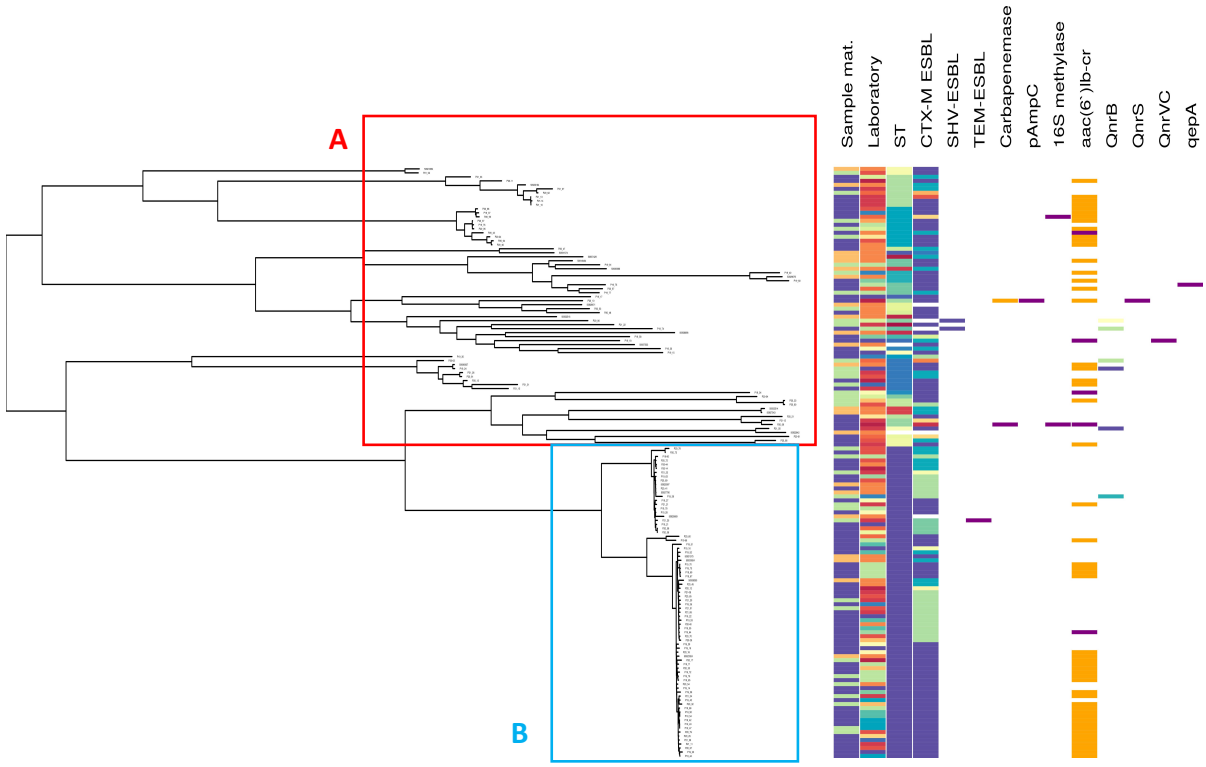


Figure 31: Phylogenetic tree with metadata for ESBL-producing E. coli from both clinical (NORM) and carrier (Tromsø-7) isolates. The columns on the right shows selected metadata. Due to the size of the tree, it is divided into two parts. Part A is the general part of the tree, while part B consists of closely clonally related isolates. Part A and B are presented separately in figure 26 and figure 27, respectively. The columns on the right shows selected metadata. The phylogenetic tree was generated based on protein coding sequences using RAxML [110]. Association to metadata was done using Phandango [113].

As seen in figure 32, part A of the joint phylogenetic tree for the ESBL-producing *E. coli* isolates, showed four clusters consisting of isolates with the same sequence types (as seen in figure 26 in chapter 4.3.3.1). The carrier isolates from Tromsø-7 is marked by red arrows, and as the figure shows, these were mainly distributed along the tree. However, some of the carrier isolates were clonally related to clinical isolates.

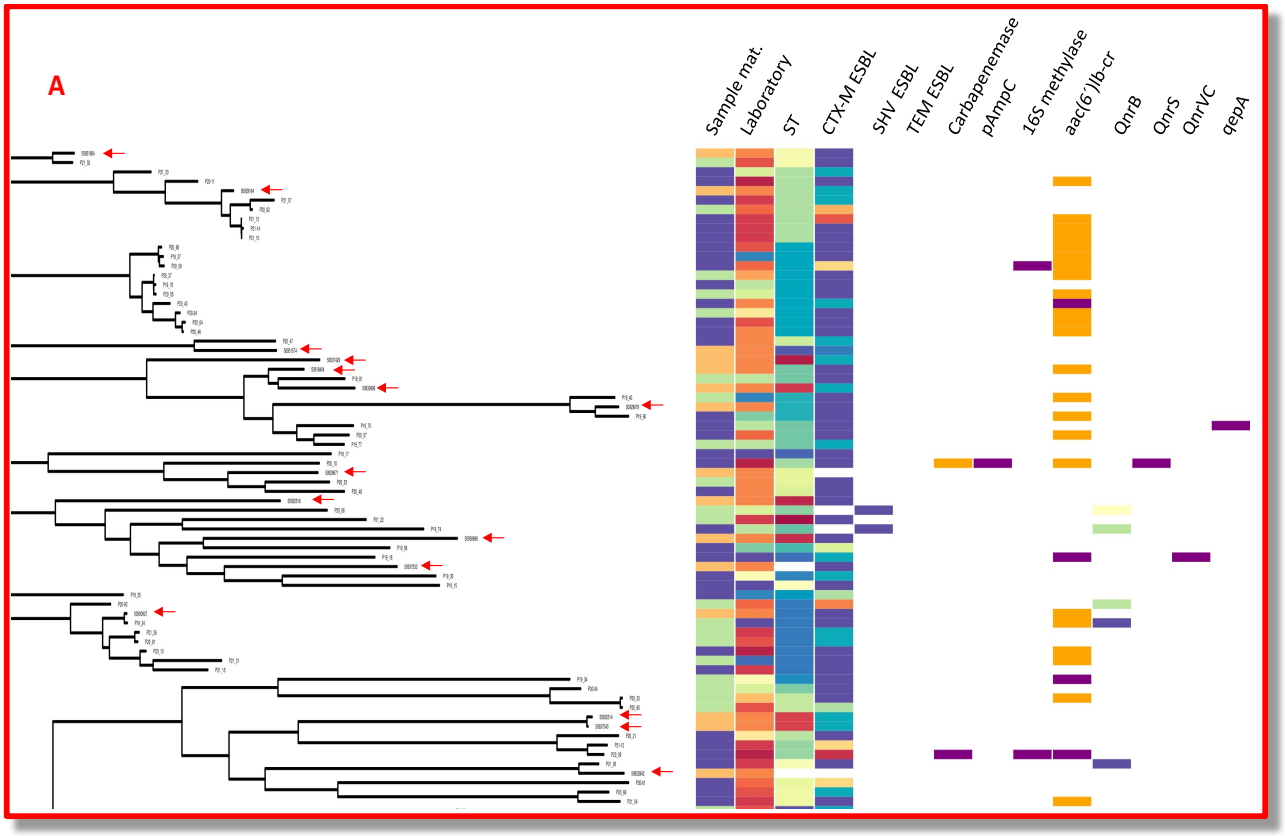


Figure 32: Part A of the phylogenetic tree with metadata for ESBL-producing E. coli from both clinical (NORM) and carrier (Tromsø-7) isolates. The columns on the right shows selected metadata. The carrier isolates from Tromsø-7 are marked by the red arrows.

Part B of the joint phylogenetic tree for the ESBL-producing *E. coli* isolates is presented in figure 33 and showed two large clusters, all originating from one lineage (as seen in figure 27 in chapter 4.3.3.1). This part comprised only of isolates belonging to ST131. The carrier isolates from Tromsø-7 is marked by red arrows, and were distributed in the ST131 lineage.

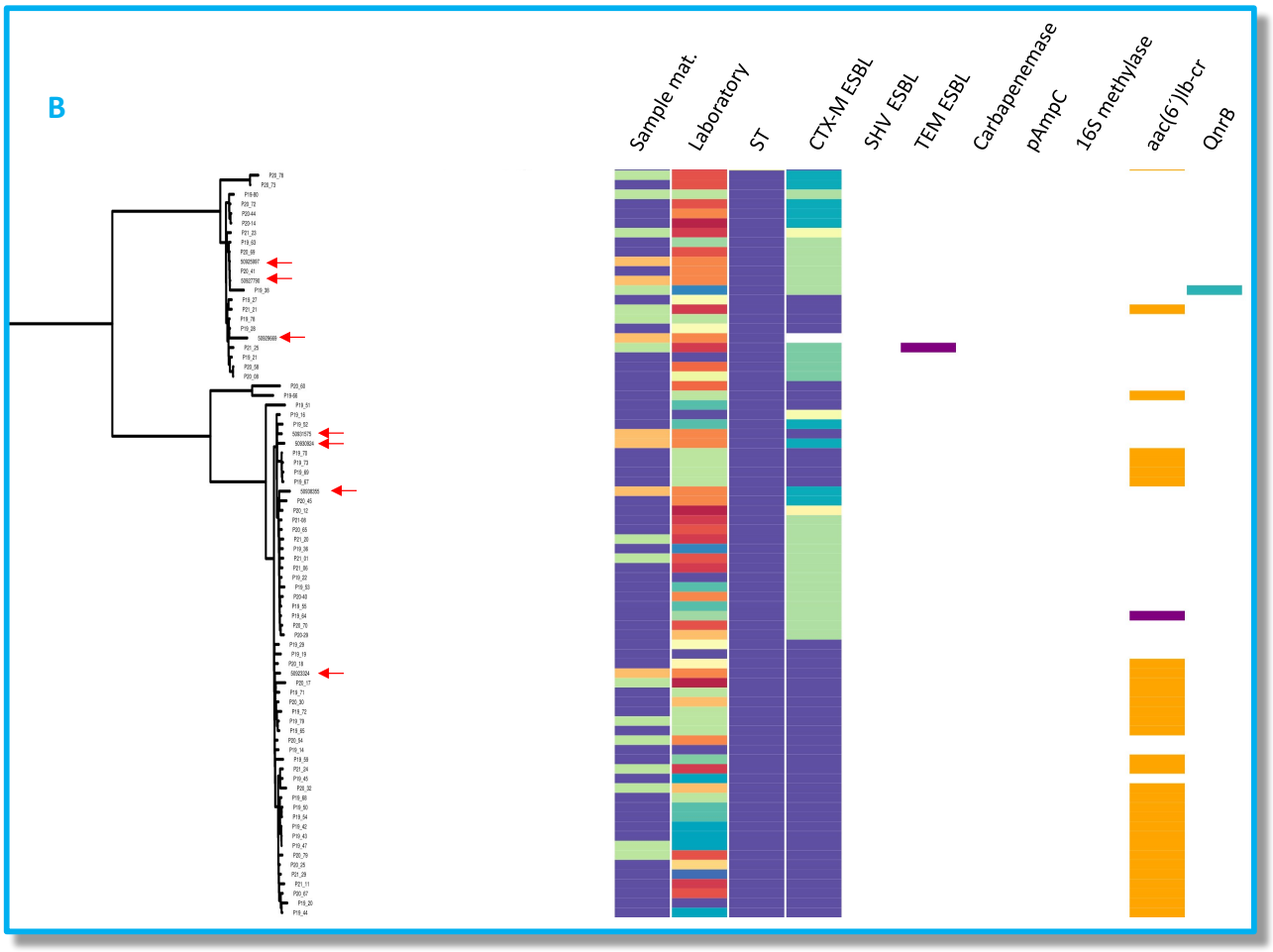


Figure 33: Part B of the phylogenetic tree with metadata for ESBL-producing E. coli from both clinical (NORM) and carrier (Tromsø-7) isolates. The columns on the right shows selected metadata. The carrier isolates from Tromsø-7 are marked by the red arrows.

5 Discussion

This study investigated the prevalence of carriage of ESBL-producing *E. coli* and *K. pneumoniae* in inhabitants in Tromsø. Further, the phenotypic susceptibility of these isolates was investigated, and the population structure was determined using WGS. The WGS data was also compared to WGS data of ESBL-producing isolates from the NORM 2014 collection, which was also sequenced as part of this project.

The Tromsø-7 fecal samples were also used to determine the prevalence of *K. pneumoniae* carriage irrespective of resistance in inhabitants in Tromsø. Additionally, the phenotypic susceptibility profiles for these isolates was determined and compared to phenotypic susceptibility profiles from clinical isolates collected through NORM 2014.

5.1 Fecal carriage of *K. pneumoniae* in Tromsø-7

A total of 97 *K. pneumoniae* isolates irrespective of resistance, were found in 662 fecal samples from the Tromsø-7 study, resulting in a prevalence of 14.7 %. This carriage prevalence is considerably lower than a Chinese study (62%), an American study (23%) and a Korean study (21%) [79-81]. However, the carriage rate in the Tromsø-7 population was higher than the prevalence in an Australian study (10%) [82]. Some of these differences could be due to difference in methodology in the respective studies. The study with the highest prevalence (Chinese study) analysed stool samples from the participants, which were inoculated on MacConkey agar and a selective medium for *K. pneumoniae* (the article does not specify which selective medium) [79]. The Korean study also analysed stool samples inoculated on MacConkey agar, but these samples were also inoculated in an enrichment broth to increase the chance of finding *K. pneumoniae* [80]. The American and the Australian study both analysed rectal swabs, which can give a false negative result as the selection of bacteria in the samples is likely to be narrower compared to a stool sample [81, 82]. The Australian study however, used an enrichment broth in addition to the MacConkey agar, which will increase the chances of finding any *K. pneumoniae* represented in low numbers in the samples [82]. The fecal samples from Tromsø-7 were not inoculated in enrichment broth, so there is likely a false negative rate. In addition, the investigation of bacterial growth on the SCAI agar plates was challenging as bacterial colonies of other species was often represented in great numbers, which could repress the growth of *K. pneumoniae*. In addition, the Tromsø-7 samples were frozen for

a considerably amount of time before inoculation, although adding glycerol to the fecal samples prior to freezing, this may have led to a loss of bacterial diversity on the agar plates, and possibly false negative results. The Australian study also found a higher prevalence of carriage in the previously hospitalized patients (19%) compared to the community associated group of patients, this shows the carriage of *K. pneumoniae* is likely to increase in hospitalized patients, and may account for the higher carrier rates in the American study [82].

A majority of the *K. pneumoniae* isolates were susceptible to all antimicrobial agents included in the analysis. There were limited differences in susceptibility between carrier isolates and clinical isolates, however the clinical isolates showed higher levels of non-susceptibility for the tested antimicrobial agents compared to the carrier strains. This could indicate that the clinical strains have more AMR markers than the carrier strains. The clinical strains were isolated from patients suffering from urinary tract infections and blood culture infections, and it is likely a large portion of these patients have undergone some sort of antimicrobial treatment, which in turn may have selected for more resistant strains of *K. pneumoniae*. However, to accurately compare these populations, more studies are needed. WGS data would give a more extensive base for comparison of these populations.

5.2 Fecal carriage of ESBL-producing *E. coli* and *K. pneumoniae* in Tromsø-7

Initially 25 isolates suspected of being ESBL-producers were found in the 662 fecal samples analysed. However, three *E. coli* and one *K. pneumoniae* isolate that grew on the CHROMagar ESBL agar (16%), were phenotypically and genotypically ESBL-negative. This was to be expected considering the manufacturer of the CHROMagar ESBL agar reports a sensitivity of 99.2% [89]. A sensitivity this high will inevitably lead to some false positive results, and the specificity is consequently reported to be 89% [89]. The number of ESBL-producing isolates therefore had to be adjusted to 21 isolates, after the results of the phenotypic analysis and whole genome sequencing were obtained. This resulted in a prevalence of 3.2%. This prevalence is slightly lower compared to a similar Norwegian study by Ulstad *et al*. from 2016, which showed a carriage rate of 4.9% [74]. In this study, rectal swabs were inoculated on a different selective agar (MacConkey agar plates supplemented with either cefotaxime, ceftazidime or ciprofloxacin) compared to the Tromsø-7 study [74]. Additionally, a MacConkey broth

supplemented with cefotaxime was used, which gives an advantage in detecting ESBL-producing isolates in samples with a low number of bacteria [74]. The difference in carriage rates between this study and Tromsø-7 may have been caused by the difference in screening media, or as previously discussed, the freezing process of the Tromsø-7 samples, may have led to a lower carriage rate. It is also possible that the difference in prevalence is simply a reflection of different communities in different regions of the country. The Tromsø-7 study only included inhabitants in and around Tromsø, while the Ulstad *et al*. study was limited to inhabitants in the eastern part of Norway [74]. There is also an age difference in the populations between the studies. The study by Ulstad *et al*. inlcuded participants from 18 to 84 years, while in Tromsø-7 all partcicipants were over 40 years old [74]. Similar community carriage rates have been found through studies in both France and Sweden. A Swedish study by Ny *et al*. from 2016, showed a prevalence of *E. coli* ESBL-carriage of 4.7%. This prevalence was based on screening of community fecal samples using a locally manufactured agar (CHROMoriental-agar with cefpodoxime). The French study, by Nicolas-Chanoine *et al.* showed a prevalence of 4.9% [75]. However, this is an older study from 2012, and it concluded with a 10-fold increase in prevalence from 2006-2011, so it is likely the prevalence in France today is higher [75]. Another Norwegian study from 2012, was based on screening of pregnant women in the south of Norway, and found a prevalence of 2.9%, which is in concordance with the Tromsø-7 study [72]. However, this study is also several years old, and considering the trends in other countries this prevalence is likely to be higher today [75, 78]. Another Norwegian study by Jørgensen *et al*., investigated the ESBL carriage rate in patients with gastroenteritis, and found a considerably higher prevalence of 15.8% [73]. Many of these patients had been travelling, and there was a correlation between carriage rate of ESBL and travel to Asia, and especially India [73]. The study showed the ESBL carriage rate among travellers to India (32 travellers), was 56.3%, which most likely accounts for the high overall carriage rate in this study [73]. In Tromsø-7 all participants have responded to a questionnaire including questions about travel. Our future aim is to link our data with the metadata from Tromsø-7 to identify possible risk factors for ESBL-carriage. Compared to other world regions, Norway has a very low prevalence of ESBL-producing *E. coli* and *K. pneumoniae* [19]. As mentioned in the introduction, studies show some countries in Asia to have a prevalence of ESBL-producing Enterobacteriaceae of over 50%, this was however also in older studies (2008-2010), so there is a high possibility this carriage rate is much higher at the present moment [78].

5.3 Comparison of ESBL *E. coli* population structure in Tromsø-7 and NORM 2014

Results from the genotypic analysis, showed the ESBL-producing *E. coli* isolates from Tromsø-7 to be a very diverse group, belonging to many different sequence types, although clearly dominated by ST131 (32%). As mentioned in the introduction, this sequence type is highly associated with outbreaks and is known to have a very efficient dispersal world-wide [55]. Other ESBL carriage studies also support this observation, in a Dutch study, Reuland *et al*. found ST131 to be dominating (15.9%) in the ESBL-producing *E. coli* carriage isolates [77]. Several of the other similar studies discussed in the previous chapter, did not investigate MLSTs among the isolated ESBL strains. The other 12 STs identified were only represented by one or two isolates and appears as random clones in the population. However, the small number of isolates included in the analysis, makes it difficult to make any clear assumptions regarding the population structure of ESBL-producing *E. coli* in the population of Tromsø. However, the identification of the ST131 clone is worrying.

Results from the genotypic analysis, showed less diversity within the NORM *E. coli* isolates, compared to the *E. coli* isolates from Tromsø-7. Although 28 different sequence types were represented within the population, it was clearly dominated by ST131, with 56% of the isolates belonging to this group. Studies have shown ST131 to have extensive pathogenic potential as numerous virulence markers have been investigated, among these are studies of this ST`s ability to establish colonization the intestines, and maintain this colonization over time [55]. Vimont *et al*. proved that ST131 outcompeted non-ST131 commensal strains in mice [115]. Dautzenberg *et al*. investigated the pathogenic potential of ST131, and found that it is 3.2 times more pathogenic compared to non-ST131 [116]. Considering these isolates came from clinical samples, it was expected to find a majority of the isolates belonging to this well known, highly virulent sequence type. The prevalence of ST131 among the NORM ESBL-producing *E. coli* is higher compared to previous Scandinavian studies. A similar study from Denmark in 2013 showed a 38% prevalence of ST131 among ESBL-producing *E. coli* in clinical isolates [117]. Another Norwegian study from 2009, showed 20% of the clinical ESBL-producing *E. coli* isolates included in the study was ST131 [118]. A Swedish study from 2013, investigated clinical ESBL-producing *E. coli* from 2007-2011, and found 34-38% of the isolates belonged

to ST131 [119]. This difference in prevalence of ST131 among clinical strains, most likely depict the dissemination success of this clonal group over the years, as described in other parts of the world [55]. The high prevalence of ST131 among both the Tromsø-7 and the NORM ESBL-producing *E. coli*, suggests this clonal line has established itself as the dominating ST in both clinical and carrier ESBL isolates. This illustrates studies done on this sequence type`s advances in the competition for habitats, especially in terms of its ability to colonize, and its extensive repertoire of resistance genes [55, 116].

One of the Tromsø-7 *E. coli* isolates belonging to ST131 (50929669), did not produce an ESBL. This was the only representative of ST131 from both collections which did not have an ESBL-gene. Considering ST131 is closely associated with several CTX-M ESBLs, this is a divergent observation. It is possible this isolate initially harboured an ESBL, but that it was lost during re-plating and/or freezing of the isolated strain.

The second most prevalent sequence type among the NORM ESBL isolates was ST405 (8%). This sequence type has previously been described in clinical isolates in different parts of the world, and often carrying a NDM carbapenemase [57, 75]. This sequence type was not found in any of the isolates from Tromsø-7, suggesting it has not disseminated in the community with the same success as ST131. However, the ESBL-producing *E. coli* from Tromsø-7 is a very small population compared to the NORM collection, so it is difficult to draw any definite conclusions regarding the less represented sequence types present in the population.

When it comes to AMR genes in the Tromsø-7 ESBL-producing *E. coli* isolates, CTX-M enzymes were clearly dominating, and among these, CTX-M-15 was the most profuse (47%), followed by CTX-M-14 (36.8%). Linking the different STs and the dispersal of ESBL-genes, it is apparent isolates belonging to ST-131 is not dominated by a single ESBL, but rather three different ESBLs (CTX-M-14, CTX-M-15 and CTX-M-27), evenly spread among the isolates (29% of each). Within the NORM collection, CTX-M-15 is the dominating ESBL, carried by 58% of the *E. coli* strains. CTX-M-14 was the second most prevalent ESBL in the NORM collection followed by CTX-M-27, but the prevalence rate (~16%) was lower compared to CTX-M-15. CTX-M-15 was also the dominating ESBL found among the ST131 *E. coli*, with a prevalence of 55%. From other studies it is well known ST-131 is highly associated with

especially CTX-M-15, but also CTX-M-14 and CTX-M-27 [55]. Considering ST131 is the dominating sequence type among both *E. coli* populations, it is therefore to be expected these three ESBLs is well represented in the populations. Among the ST405 from the NORM collection, CTX-M-15 was also the dominating ESBL, in concordance with other studies [57, 75]. No NDM carbapenemase was found among the ST405 isolates.

In addition, two of the NORM *E. coli* isolates possessed the genes for carbapenemases (OXA-181 and IMP-26), however, these two isolates did not display phenotypic resistance to carbapenems. This can be explained by studies showing that the presence of a carbapenemase do not result in phenotypic carbapenem-resistance according to clinical breakpoints unless there are additional AMR markers present, like mutations causing decreased permeability of the cell membrane [23]. In addition, one of the NORM *E. coli* isolates (P21_14) was phenotypically intermediate to meropenem, but did not produce a carbapenemase, only a CTX-M-15. This reduced susceptibility for meropenem could be caused by other AMR mechanisms, like mutation in PBP or downregulation of porins, both mechanisms which have been described in *E. coli* [17].

Three of the *E. coli* isolates carried a gene for aminoglycoside and fluoroquinolone resistance, *aac(6`)lb-cr*, encoding an acetyltransferase. All three isolates also carried a $bla_{\text{CTX-M-15}}$, which can suggest co-localisation on the same plasmid, but this remains to be determined. All three of these isolates were phenotypically resistant to gentamicin and tobramycin, and two were resistant to ciprofloxacin, while the third was susceptible. There were no carbapenemase-genes present in any of the *E. coli* isolates from Tromsø-7, which is supported by the lack of phenotypic resistance for carbapenems within the population and that no isolates were found on CHROMagar mSuperCarba agar plates.

Several plasmid-mediated quinolone resistance genes were found in both *E. coli* populations. The most prevalent of these genes was the *aac(6`)lb-cr*, encoding a fluoroquinolone modifying acetyltransferase, which gives reduced susceptibility to both quinolones and aminoglycosides. This gene was present in 44% of the NORM *E. coli* isolates, and in 13% of the Tromsø-7 isolates. All these isolates also carried one or more ESBL-genes, most prevalent was the combination of *aac(6`)lb-cr* and CTX-M-15, which again was most prevalent in ST131. This

co-resistance is supported by studies showing ESBL-production combined with fluoroquinolone-resistance is common in this sequence type [55]. No other PMQR genes were identified in the Tromsø-7 isolates, but in the NORM isolates, eight *qnr*-genes were found. I addition, a *qepA*-gene, encoding a fluoroquinolone efflux-pump, was found in one of the *E. coli* isolates, and the aminoglycoside resistance gene *armA* was also found in two of the NORM isolates. These findings show there is less co-resistance among the carrier strains, compared to the clinical isolates. The clinical isolates are more likely to have been subjected to selective pressure from antimicrobial agents, which can explain this phenomenon. The study did not investigate for the presence of chromosomal mutations in *gyrA* and *parC* which is the most common mechanism of fluoroquinolone resistance [120].

The phylogenetic tree for the *E. coli* isolates from Tromsø-7, shows most of the isolates are not closely related, but there are also two clusters containing seven isolates in all which are clonally related. These isolates represent the entire group of sequence type ST-131 within this population. This indicates these isolates all originates from one specific strain that has established itself in the community. As mentioned above, even though these isolates are closely related, six of them produce three different ESBLs, CTX-M-14, CTX-M-15 and CTX-M-27. There is also a small cluster consisting of two isolates, 50932514 and 50937543, which both belong to sequence type ST-12, and produces a CTX-M-14.

The phylogenetic tree for the NORM *E. coli* isolates also shows four minor clusters of related strains belonging to sequence types ST648, ST38, ST405 and ST10. However, the isolates belonging to each cluster, come from different laboratories and produce different CTX-M ESBLs, so there is no apparent pattern except the clonal relation. The most interesting element of the NORM phylogenetic tree, however, is the two massive clusters of ST131 isolates. As seen with the Tromsø-7 isolates, these two clusters represent the entire population of ST131 within the NORM isolates. This suggests, as with the Tromsø-7 *E. coli*, these isolates all share a common ancestor. Most likely an ST131 strain has been introduced in Norway and spread throughout the community, and has now become the most dominating ESBL-producing clone in Norway. The combination of these two phylogenetic trees (Tromsø-7 and NORM) show the ST131 carrier isolates are also clonally related to the clinical strains, showing there is no fundamental difference between the ST131 population from clinical samples and carrier strains.

To more accurately determine the connection of these two populations, an analysis of plasmids carried by the different isolates should be performed. Due to time-limitations this analysis unfortunately has not been done within the framework of this project.

5.4 ESBL *K. pneumoniae* population structure in NORM 2014

The population structure of the NORM *K. pneumoniae* isolates, is highly diverse, representing 24 sequence types distributed in 35 isolates. The most prevalent sequence type within the *K. pneumoniae* population was ST307 (23%), this sequence type is highly associated with CTX-M-15 [70], which was found in all the NORM isolates as well. ST307 has in recent years emerged in several different countries, and is considered a rising threat, with special emphasis on KPC-producing strains. The sequence type was first defined in 2008, and has since been identified in high numbers in several countries (Italy, Pakistan, Morocco, Korea and Japan), both associated with clinical outbreaks and carriage strains [121]. A surveillance study in Italy in 2014 revealed that 28% of KPC-producing isolates belonged to ST307, which is very close to the prevalence of ST307 in the NORM *K. pneumoniae* population [121]. Fortunately, none of the NORM ST307 isolates carried a KPC-gene. ST307 was also found to possess traits that might give these strains advantages in a hospital environment, including the production of capsules, as well as harbouring AMR determinants [121]. This is a probable explanation of the dominance of this sequence type in the NORM collection. As there are no ESBL-producing *K. pneumoniae* carrier strains available for comparison, the prevalence rate of this sequence type among carrier isolates is unknown. The second most prevalent sequence types were ST15 and ST340, although in much lower numbers (8%). ST340 is also associated with CTX-M-15 [71], and all three NORM isolates did indeed produce a CTX-M-15, along with all three ST15 isolates. Both ST340 and ST15 have also been associated with KPC and NDM-carbapenemases, and especially ST15 have been associated with outbreaks of carbapenemase-producing *K. pneumoniae* [122]. No carbapenemase gene was found in the *K. pneumoniae* isolates, although two isolates phenotypically showed reduced susceptibility to meropenem.
Both isolates harboured CTX-M-15, but no carbapenemase was identified. The reduced susceptibility to meropenem displayed by these isolates is most likely caused by another AMR mechanism. As an example, the loss of two major porins (OmpK35 and OmpK36) have been known to cause carbapenem resistance in isolates of *K. pneumoniae* [123].

Several plasmid-mediated quinolone resistance genes were found in the *K. pneumoniae* populations. Like the NORM *E. coli* population, the most prevalent of these genes was *aac(6`)lb-cr*, present in 66% of the isolates. This was reflected in the phenotypic susceptibility profiles seen for gentamicin, where about 70% of the *K. pneumoniae* (BLC) were resistant. In addition, one isolate carried an aminoglycoside resistance gene (*armA*).

The second most dominating PMQR-genes in the population were qnr-genes, of which five different variants were identified. Combined, these genes show that the majority of the isolates possessed some sort of PMQR gene, which is reflected in the high rates of phenotypic resistance against ciprofloxacin for the population.

In the *K. pneumoniae* population, all isolates belonging to the three most dominant MLSTs (ST307, ST340 and ST15) carried genes for aminoglycoside- and quinolone-resistance in addition to several different ESBL enzymes, suggesting these genes may have been transferred collectively.

The phylogenetic tree, constructed from the *K. pneumoniae* isolates, showed one large cluster of nine isolates. All these isolates were closely related. They all came from blood cultures, they were all ST307 and they all produced a CTX-M-15, an SHV-28 and carried the *aac(6')-Ib-cr* gene. These isolates came from five different laboratories from different parts of the country, indicating that they do not represent an outbreak. The domination of this clone and the close clonal relation between the different isolates, suggest, like the ST131, that this clone is likely to have successfully established itself in the *K. pneumoniae* population and is now the dominating sequence type in clinical isolates in Norway. But as discussed previously in this chapter, we know nothing of its dissemination among carrier isolates, genomic analysis of carriage isolates is needed to address this issue. There were also two smaller clusters, each consisting of three isolates. One of them (the green cluster) consisted of isolates that all came from blood cultures, but from three different laboratories. These all belonged to ST15, they all had a CTX-M-15 and the *aac(6')-Ib-cr* gene, and they seemed to ascend from the same strain. The third (blue) cluster, were all ST340 and they all produced a CTX-M-15 and the *aac(6')-Ib-cr* gene. Two of these isolates came from the same laboratory (Tromsø) while the third came

from Fredrikstad. These two clusters also indicate that isolates belonging to both these sequence types are descendants from two distinct strains. However, these sequence types are only represented by three isolates each, which is a very limited basis for determining the population structure of these sequence types.

Among the *K. pneumoniae* isolates, there was one isolate highly unrelated to the rest of the population. This isolate belonged to ST334 and it harboured an SHV-12 ESBL, and in addition possessed an OKP-B-4-like β-lactamase. This isolate is most likely a *K. quasipneumoniae* (KpII), as it is unrelted to the rest of the isolates and carries a bla_{OKP}, which is chromosomally encoded in this species [114]. Because these three subspecies of *K. pneuomoniae* are so similar, conventional identification systems like MALDI TOF MS, are not able to differentiate between the different species. Isolates belonging to any of these species will therefore only be identified as *K. pneumoniae*.

6 Conclusion

The results of this study showed a prevalence of fecal carriage of *K. pneumoniae* among adults in Tromsø, to be 14.7 %, which is lower than other similar studies. However, there are some differences in study design, and especially the choice of screening-media could account for some of the differences.

The study further showed a prevalence of fecal carriage of ESBL-producing *E. coli* and *K. pneumoniae* among the same population to be 3.2%. This rate is lower than most other investigated studies, which means the carriage rate of ESBL-producing *E. coli* and *K. pneumoniae* is lower in Norway, than in many other European countries.

The genotypic characterization of the ESBL-producing *E. coli* isolates from Tromsø-7, revealed a dominance of ST131, with CTX-M-14, CTX-M-15 and CTX-M-27 production, among the isolates. The same sequence type was seen to dominate the clinical ESBL-producing *E. coli* isolates from NORM 2014, except among these isolates CTX-M-15 alone was by far the most dominating ESBL. Phylogenetic analysis also revealed the carrier strains and the clinical strains to be closely relaSTted, likely disseminated from one common strain. This suggests ST131 has established itself as the dominating ST in *both* clinical and carrier ESBL-producing *E. coli*.

The genotypic characterization of the ESBL-producing *K. pneumoniae* isolates from NORM-2014, showed ST307 to be the dominating sequence type. The phylogenetic analysis also revealed these isolates were clonally related as well. It is in this study not possible to determine if these findings are reflected in the community carriage of ESBL-producing *K. pneumoniae* as we did not perform WGS analysis of the carriage isolates.

These results show there is still a low carriage rate of ESBL-producing *K. pneumoniae* and *E. coli* in Norway. However, high virulence strains are dominating in carriage of both species, which is a concerning development. This study further shows that surveillance of not only the rate of ESBL-producers, but also the population structure of these isolates should be performed regularly, on both carrier- and clinical strains.

Citations

1. WHO, *Global action plan on antimicrobial resistance*. 2015: Geneva, Switzerland. p. 20.
2. WHO. *Antimicrobial resistance: global report on surveillance*. 2014 15.02.2017]; Available from: http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf.
3. Magiorakos, A.P., et al., *Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance.* Clinical Microbiology and Infection, 2012. **18**(3): p. 268-281.
4. WHO, *Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics*. 2017. p. 7.
5. Review on antimicrobial resistance, *Tackling drug-resistant infections globally: Final report and recommendations*, J. O`Neill, Editor. 2016. p. 81.
6. Lewis, K., *Platforms for antibiotic discovery.* Nat Rev Drug Discov, 2013. **12**(5): p. 371-387.
7. Ligon, B.L., *Penicillin: its discovery and early development.* Seminars in Pediatric Infectious Diseases, 2004. **15**(1): p. 52-57.
8. Fleming, A., *On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzæ.* British journal of experimental pathology, 1929. **10**(3): p. 226-236.
9. Aminov, R.I., *A brief history of the antibiotic era: Lessons learned and challenges for the future.* Frontiers in Microbiology, 2010. **1**: p. 134.
10. Spinler, J.K., C.L. Ross, and T.C. Savidge, *Probiotics as adjunctive therapy for preventing Clostridium difficile infection – What are we waiting for?* Anaerobe, 2016. **41**: p. 51-57.
11. Coates, A.R.M., G. Halls, and Y. Hu, *Novel classes of antibiotics or more of the same?* British Journal of Pharmacology, 2011. **163**(1): p. 184-194.
12. Murray, P.R., Baron, Ellen J., Jorgensen, James H., Pfaller, Michael A., Yolken, Robert H. , *Manual of Clinical Microbiology*. 8th ed, ed. P.R. Murray. Vol. 1. 2003, Washington, DC: American Society for Microbiology.
13. Forbes B.A., S.D.F.a.W.A.S., *Bailey & Scott`s diagnostic microbiology*. 12th ed. 2007, St. Louis, Missouri: Mosby Elsevier.
14. Bush, K. and P.A. Bradford, *β-Lactams and β-Lactamase Inhibitors: An Overview.* Cold Spring Harbor Perspectives in Medicine, 2016. **6**(8).
15. Page, M.G.P., *Beta-Lactam antibiotics*, in *Antibiotic discovery and development*, T.J. Dougherty and M.J. Pucci, Editors. 2012, Springer US: Boston, MA. p. 79-117.
16. Laudano, J.B., *Ceftaroline fosamil: a new broad-spectrum cephalosporin.* Journal of Antimicrobial Chemotherapy, 2011. **66**(suppl_3): p. iii11-iii18.
17. Papp-Wallace, K.M., et al., *Carbapenems: Past, present, and future.* Antimicrobial Agents and Chemotherapy, 2011. **55**(11): p. 4943-4960.
18. Sykes, R.B. and D.P. Bonner, *Discovery and development of the monobactams.* Reviews of Infectious Diseases, 1985. **7**: p. S579-S593.
19. NORM/NORM-VET 2015, *Usage of antimicrobial agents and occurence of antimicrobial resistance in Norway.*, G.S. Simonsen, Urdahl A. M. , Editor. 2016: Tromsø/Oslo.
20. Ventola, C.L., *The antibiotic resistance crisis: Part 1: Causes and threats.* Pharmacy and Therapeutics, 2015. **40**(4): p. 277-283.

21. EARS-Net (European Antimicrobial Resistance Surveillance Network). *Surveillance report: Antimicrobial resistance surveillance in Europe 2015*. 2017 [cited 2017 09.02.2017]; Available from: http://ecdc.europa.eu/en/healthtopics/antimicrobial-resistance-and-consumption/antimicrobial_resistance/EARS-Net/Pages/EARS-Net.aspx.

22. NORM/NORM-VET 2014, *Usage of antimicrobial agents and occurence of antimicrobial resistance in Norway.* 2015: Tromsø / Oslo.

23. Tangden, T. and C.G. Giske, *Global dissemination of extensively drug-resistant carbapenemase-producing Enterobacteriaceae: clinical perspectives on detection, treatment and infection control.* J Intern Med, 2015. **277**(5): p. 501-12.

24. Blair, J.M.A., et al., *Molecular mechanisms of antibiotic resistance.* Nat Rev Micro, 2015. **13**(1): p. 42-51.

25. Delcour, A.H., *Outer membrane permeability and antibiotic resistance.* Biochimica et biophysica acta, 2009. **1794**(5): p. 808-816.

26. Jaktaji, R.P. and F. Heidari, *Study the expression of ompf gene in Esherichia coli mutants.* Indian Journal of Pharmaceutical Sciences, 2013. **75**(5): p. 540-544.

27. Fernández, L. and R.E.W. Hancock, *Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance.* Clinical Microbiology Reviews, 2012. **25**(4): p. 661-681.

28. Du, D., et al., *Structure of the AcrAB-TolC multidrug efflux pump.* Nature, 2014. **509**(7501): p. 512-515.

29. Yu, E.W., J.R. Aires, and H. Nikaido, *AcrB multidrug efflux pump of Escherichia coli: Composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity.* Journal of Bacteriology, 2003. **185**(19): p. 5657-5664.

30. Yamane, K., et al., *New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an Escherichia coli clinical isolate.* Antimicrobial Agents and Chemotherapy, 2007. **51**(9): p. 3354-3360.

31. Ramirez, M.S. and M.E. Tolmasky, *Aminoglycoside modifying enzymes.* Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy, 2010. **13**(6): p. 151-171.

32. Woodford, N. and M.J. Ellington, *The emergence of antibiotic resistance by mutation.* Clinical Microbiology and Infection, 2007. **13**(1): p. 5-18.

33. von Wintersdorff, C.J.H., et al., *Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer.* Frontiers in Microbiology, 2016. **7**(173).

34. Yang, Q.-E., et al., *IncF plasmid diversity in multi-drug resistant Escherichia coli strains from animals in China.* Frontiers in Microbiology, 2015. **6**: p. 964.

35. Turner, P.J., *Extended-spectrum β-lactamases.* Clinical Infectious Diseases, 2005. **41**(Supplement_4): p. S273-S275.

36. Shaikh, S., et al., *Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment.* Saudi Journal of Biological Sciences, 2015. **22**(1): p. 90-101.

37. Cantón, R., J.M. González-Alba, and J.C. Galán, *CTX-M Enzymes: Origin and diffusion.* Frontiers in Microbiology, 2012. **3**: p. 110.

38. Bush, K. and G.A. Jacoby, *Updated functional classification of β-Lactamases.* Antimicrobial Agents and Chemotherapy, 2010. **54**(3): p. 969-976.

39. Queenan, A.M. and K. Bush, *Carbapenemases: the versatile β-lactamases.* Clinical Microbiology Reviews, 2007. **20**(3): p. 440-458.

40. Hall, B.G. and M. Barlow, *Evolution of the serine β-lactamases: past, present and future.* Drug Resistance Updates, 2004. **7**(2): p. 111-123.

41. Palzkill, T., *Metallo-β-lactamase structure and function.* Annals of the New York Academy of Sciences, 2013. **1277**: p. 91-104.

42. Carattoli, A., *Resistance plasmid families in Enterobacteriaceae.* Antimicrobial Agents and Chemotherapy, 2009. **53**(6): p. 2227-2238.

43. Karim, A., et al., *Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1.* FEMS microbiology letters, 2001. **201**(2): p. 237-241.

44. Lau, S.H., et al., *UK epidemic Escherichia coli strains A–E, with CTX-M-15 β-lactamase, all belong to the international O25:H4-ST131 clone.* Journal of Antimicrobial Chemotherapy, 2008. **62**(6): p. 1241-1244.

45. Peleg, A.Y. and D.C. Hooper, *Hospital-acquired infections due to Gram-negative bacteria.* The New England journal of medicine, 2010. **362**(19): p. 1804-1813.

46. Weinstein, R.A., R. Gaynes, and J.R. Edwards, *Overview of nosocomial infections caused by Gram-negative bacilli.* Clinical Infectious Diseases, 2005. **41**(6): p. 848-854.

47. Vila, J., et al., *Escherichia coli: an old friend with new tidings.* FEMS Microbiology Reviews, 2016. **40**(4): p. 437-463.

48. Jacoby, G.A., *AmpC β-lactamases.* Clinical Microbiology Reviews, 2009. **22**(1): p. 161-182.

49. Heider, L.C., et al., *Genetic and phenotypic characterization of the bla(CMY) gene from Escherichia coli and Salmonella enterica isolated from food-producing animals, humans, the environment, and retail meat.* Foodborne Pathog Dis, 2009. **6**(10): p. 1235-40.

50. Karczmarczyk, M., et al., *Mechanisms of fluoroquinolone resistance in Escherichia coli isolates from food-producing animals.* Applied and Environmental Microbiology, 2011. **77**(20): p. 7113-7120.

51. Jacoby, G.A., J. Strahilevitz, and D.C. Hooper, *Plasmid-mediated quinolone resistance.* Microbiology spectrum, 2014. **2**(2): p. 10.1128/microbiolspec.PLAS-0006-2013.

52. Haldorsen, B.C., et al., *Increased prevalence of aminoglycoside resistance in clinical isolates of Escherichia coli and Klebsiella spp. in Norway is associated with the acquisition of AAC(3)-II and AAC(6′)-Ib.* Diagnostic Microbiology and Infectious Disease, 2014. **78**(1): p. 66-69.

53. Tsukamoto, N., et al., *High prevalence of cross-resistance to aminoglycosides in fluoroquinolone-resistant Escherichia coli clinical isolates.* Chemotherapy, 2013. **59**(5): p. 379-384.

54. Bajaj, P., N.S. Singh, and J.S. Virdi, *Escherichia coli β-Lactamases: What really matters.* Frontiers in Microbiology, 2016. **7**(417).

55. Nicolas-Chanoine, M.-H., X. Bertrand, and J.-Y. Madec, *Escherichia coli ST131, an intriguing clonal group.* Clinical Microbiology Reviews, 2014. **27**(3): p. 543-574.

56. Nicolas-Chanoine, M.H., et al., *Intercontinental emergence of Escherichia coli clone O25:H4-ST131 producing CTX-M-15.* J Antimicrob Chemother, 2008. **61**(2): p. 273-81.

57. D'Andrea, M.M., et al., *Persistent carriage and infection by multidrug-resistant Escherichia coli ST405 producing NDM-1 carbapenemase: Report on the first Italian cases.* Journal of Clinical Microbiology, 2011. **49**(7): p. 2755-2758.

58. Paczosa, M.K. and J. Mecsas, *Klebsiella pneumoniae: Going on the offense with a strong defense.* Microbiology and Molecular Biology Reviews, 2016. **80**(3): p. 629-661.

59. Vuotto, C., et al., *Antibiotic resistance related to biofilm formation in Klebsiella pneumoniae.* Pathogens, 2014. **3**(3): p. 743-758.

60. Podschun, R. and U. Ullmann, *Klebsiella spp. as nosocomial pathogens: Epidemiology, taxonomy, typing methods, and pathogenicity factors.* Clinical Microbiology Reviews, 1998. **11**(4): p. 589-603.

61. Holt, K.E., et al., *Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in Klebsiella pneumoniae, an urgent threat to public health.* Proceedings of the National Academy of Sciences of the United States of America, 2015. **112**(27): p. E3574-E3581.

62. Jesus, M.B.D., et al., *Review - Understanding β-lactamase producing Klebsiella pneumoniae*, in *antimicrobial resistance - an open challenge*, M.C. Ossiprandi, Editor. 2015, InTech: Rijeka. p. Ch. 04.

63. Livermore, D.M., *beta-Lactamases in laboratory and clinical resistance.* Clin Microbiol Rev, 1995. **8**(4): p. 557-84.

64. Eftekhar, F. and S.M. Seyedpour, *Prevalence of qnr and aac(6')-Ib-cr genes in clinical isolates of Klebsiella pneumoniae from Imam Hussein hospital in Tehran.* Iranian Journal of Medical Sciences, 2015. **40**(6): p. 515-521.

65. Liang, C., et al., *Molecular epidemiology of aminoglycosides resistance on Klebsiella pneumonia in a hospital in China.* International Journal of Clinical and Experimental Medicine, 2015. **8**(1): p. 1381-1385.

66. Paterson, D.L., et al., *Extended-spectrum β-lactamases in Klebsiella pneumoniae bloodstream isolates from seven countries: Dominance and widespread prevalence of SHV- and CTX-M-type β-lactamases.* Antimicrobial Agents and Chemotherapy, 2003. **47**(11): p. 3554-3560.

67. Samuelsen, O., et al., *Emergence of clonally related Klebsiella pneumoniae isolates of sequence type 258 producing plasmid-mediated KPC carbapenemase in Norway and Sweden.* J Antimicrob Chemother, 2009. **63**(4): p. 654-8.

68. Rettedal, S., et al., *First outbreak of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in a Norwegian neonatal intensive care unit; associated with contaminated breast milk and resolved by strict cohorting.* Apmis, 2012. **120**(8): p. 612-21.

69. Hu, L., et al., *Outbreak by Ventilator-associated ST11 K. pneumoniae with co-production of CTX-M-24 and KPC-2 in a SICU of a tertiary teaching hospital in central China.* Frontiers in Microbiology, 2016. **7**: p. 1190.

70. Villa, L., et al., *Complete genome sequence of KPC-3- and CTX-M-15-producing Klebsiella pneumoniae sequence type 307.* Genome Announcements, 2016. **4**(2): p. e00213-16.

71. Cerdeira, L., et al., *Draft genome sequence of a hospital-associated clone of Klebsiella pneumoniae ST340/CC258 coproducing RmtG and KPC-2 isolated from a pediatric patient.* Genome Announcements, 2016. **4**(6): p. e01130-16.

72. Rettedal, S., et al., *Extended-spectrum [beta]-lactamase-producing Enterobacteriaceae among pregnant women in Norway: prevalence and maternal-neonatal transmission.* J Perinatol, 2015. **35**(11): p. 907-912.

73. Jørgensen, S.B., et al., *High prevalence of faecal carriage of ESBL-producing Enterobacteriaceae in Norwegian patients with gastroenteritis.* Scandinavian Journal of Infectious Diseases, 2014. **46**(6): p. 462-465.

74. Ulstad, C.R., et al., *Carriage of ESBL/AmpC-producing or ciprofloxacin non-susceptible Escherichia coli and Klebsiella spp. in healthy people in Norway.* Antimicrobial Resistance and Infection Control, 2016. **5**: p. 57.

75. Nicolas-Chanoine, M.H., et al., *10-Fold increase (2006-11) in the rate of healthy subjects with extended-spectrum beta-lactamase-producing Escherichia coli faecal carriage in a Parisian check-up centre.* J Antimicrob Chemother, 2013. **68**(3): p. 562-8.

76. Ny, S., et al., *Community carriage of ESBL-producing Escherichia coli is associated with strains of low pathogenicity: a Swedish nationwide study.* Journal of Antimicrobial Chemotherapy, 2017. **72**(2): p. 582-588.

77. Reuland, E.A., et al., *Prevalence and risk factors for carriage of ESBL-producing Enterobacteriaceae in Amsterdam.* Journal of Antimicrobial Chemotherapy, 2016. **71**(4): p. 1076-1082.

78. Woerther, P.-L., et al., *Trends in human fecal carriage of extended-spectrum β-lactamases in the community: Toward the Globalization of CTX-M.* Clinical Microbiology Reviews, 2013. **26**(4): p. 744-758.

79. Lin, Y.-T., et al., *Seroepidemiology of Klebsiella pneumoniae colonizing the intestinal tract of healthy chinese and overseas chinese adults in Asian countries.* BMC Microbiology, 2012. **12**(1): p. 13.

80. Chung, D.R., et al., *Fecal carriage of serotype K1 Klebsiella pneumoniae ST23 strains closely related to liver abscess isolates in Koreans living in Korea.* European Journal of Clinical Microbiology & Infectious Diseases, 2012. **31**(4): p. 481-486.

81. Martin, R.M., et al., *Molecular epidemiology of colonizing and infecting isolates of Klebsiella pneumoniae.* mSphere, 2016. **1**(5): p. e00261-16.

82. Gorrie, C.L., et al., *Gastrointestinal carriage is a major reservoir of K. pneumoniae infection in intensive care patients.* Clin Infect Dis, 2017.

83. UiT. *The Tromsø Study*. 2015 [cited 2017 08.02.2017]; Available from: https://en.uit.no/forskning/forskningsgrupper/gruppe?p_document_id=453582.

84. UiT. *The Tromsø Study - The seventh survey of the Tromsø Study*. 2016 [cited 2017 08.02.2017]; Available from: https://en.uit.no/forskning/forskningsgrupper/sub?p_document_id=453582&sub_id=453680.

85. NORM. *NORM - Norsk overvåkningssystem for antibiotikaresistens hos mikrober*. 2016 [cited 2017 09.02.2017]; Available from: https://unn.no/fag-og-forskning/norm-norsk-overvakingssystem-for-antibiotikaresistens-hos-mikrober.

86. Fallon, D., et al., *A comparison of the performance of cystine lactose electrolyte deficient (CLED) agar with Oxoid chromogenic urinary tract infection (CUTI) medium for the isolation and presumptive identification of organisms from urine.* Journal of Clinical Pathology, 2002. **55**(7): p. 524-529.

87. Van Kregten, E., N.A. Westerdaal, and J.M. Willers, *New, simple medium for selective recovery of Klebsiella pneumoniae and Klebsiella oxytoca from human feces.* Journal of Clinical Microbiology, 1984. **20**(5): p. 936-941.

88. Passet, V. and S. Brisse, *Association of tellurite resistance with hypervirulent clonal groups of Klebsiella pneumoniae.* Journal of Clinical Microbiology, 2015. **53**(4): p. 1380-1382.

89. CHROMagar. *Clinical Microbiology - CHROMagar ESBL*. 2017 [cited 2017 02.04.2017]; Available from: http://www.chromagar.com/clinical-microbiology-chromagar-esbl-focus-on-esbl-resistance-31.html - .WOEMv_nyjIU

90. CHROMagar. *Clinical Microbiology - CHROMagar mSuperCARBA*. 2017 [cited 2017 02.04.2017]; Available from: http://www.chromagar.com/clinical-microbiology-chromagar-msupercarba-cre-epidemiologic-issues-76.html - .WOEQw_mLTIU

91. Singhal, N., et al., *MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis.* Frontiers in Microbiology, 2015. **6**: p. 791.

92. Biomerieux Inc. *NucliSens easyMAG* 2017 [cited 2017 31.03.2017]; Available from: http://www.biomerieux-diagnostics.com/nuclisensr-easymagr.

93. Sigma-Aldrich. *Lysozyme from chicken egg white*. 2017 [cited 2017 31.03.2017]; Available from: http://www.sigmaaldrich.com/catalog/product/sigma/l6876?lang=en&region=NO.

94. Sigma-Aldrich. *Proteinase K, recombinant, PCR Grade*. 2017 [cited 2017 31.03.2017]; Available from: http://www.sigmaaldrich.com/catalog/product/roche/rprotkro?lang=en&region=NO.

95. Desjardins, P. and D. Conklin, *NanoDrop microvolume quantitation of nucleic acids.* Journal of Visualized Experiments : JoVE, 2010(45): p. 2565.

96. Thermo Fisher Scientific Inc., *Qubit dsDNA HS Assay Kits - user manual* 2015, Thermo Fisher Scientific.

97. Ansorge, W.J., *Next-generation DNA sequencing techniques.* N Biotechnol, 2009. **25**(4): p. 195-203.

98. Edwards, D.J. and K.E. Holt, *Beginner's guide to comparative bacterial genome analysis using next-generation sequence data.* Microbial Informatics and Experimentation, 2013. **3**(1): p. 2.

99. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics, 2014. **30**(15): p. 2114-2120.

100. Bankevich, A., et al., *SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing.* Journal of Computational Biology, 2012. **19**(5): p. 455-477.

101. Gurevich, A., et al., *QUAST: quality assessment tool for genome assemblies.* Bioinformatics, 2013. **29**(8): p. 1072-1075.

102. Darling, A.E., B. Mau, and N.T. Perna, *progressiveMauve: Multiple genome alignment with gene gain, loss and rearrangement.* PLOS ONE, 2010. **5**(6): p. e11147.

103. the Darling lab, c.m.g. *Mauve, multiple genome alignment*. 2017 [cited 2017 01.04.2017]; Available from: http://darlinglab.org/mauve/mauve.html

104. CGE. *Center for Genomic Epidemiology* 2011 [cited 2016 11.11.2016]; Available from: http://www.genomicepidemiology.org/.

105. Zankari, E., et al., *Identification of acquired antimicrobial resistance genes.* Journal of Antimicrobial Chemotherapy, 2012. **67**(11): p. 2640-2644.

106. Larsen, M.V., et al., *Multilocus sequence typing of total-genome-sequenced bacteria.* Journal of Clinical Microbiology, 2012. **50**(4): p. 1355-1361.

107. pubMLST. *MLST allelic profiles and sequences*. 2017 22.04.2017]; Available from: https://pubmlst.org/data/

108. Wirth, T., et al., *Sex and virulence in Escherichia coli: an evolutionary perspective.* Molecular Microbiology, 2006. **60**(5): p. 1136-1151.

109. Katoh, K., et al., *MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform.* Nucleic Acids Res, 2002. **30**(14): p. 3059-66.

110. Stamatakis, A., *RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies.* Bioinformatics, 2014. **30**(9): p. 1312-1313.

111. Tsering, D.C., et al., *Extended spectrum beta-lactamase detection in Gram-negative bacilli of nosocomial Origin.* Journal of Global Infectious Diseases, 2009. **1**(2): p. 87-92.

112. Legese, M.H., G.M. Weldearegay, and D. Asrat, *Extended-spectrum beta-lactamase- and carbapenemase-producing Enterobacteriaceae among Ethiopian children.* Infection and Drug Resistance, 2017. **10**: p. 27-34.

113. Hadfield, J., et al., *Phandango: an interactive viewer for bacterial population genomics.* bioRxiv, 2017.

114. Fevre, C., et al., *Variants of the Klebsiella pneumoniae OKP chromosomal beta-lactamase are divided into two main groups, OKP-A and OKP-B.* Antimicrobial Agents and Chemotherapy, 2005. **49**(12): p. 5149-5152.

115. Vimont, S., et al., *The CTX-M-15-producing Escherichia coli clone O25b: H4-ST131 has high intestine colonization and urinary tract infection abilities.* PLoS ONE, 2012. **7**(9): p. e46547.

116. Dautzenberg, M.J.D., et al., *Epidemic potential of Escherichia coli ST131 and Klebsiella pneumoniae ST258: a systematic review and meta-analysis.* BMJ Open, 2016. **6**(3): p. e009971.

117. Olesen, B., et al., *Prevalence and characteristics of the epidemic multiresistant Escherichia coli ST131 clonal group among extended-spectrum beta-lactamase-producing E. coli isolates in Copenhagen, Denmark.* Journal of Clinical Microbiology, 2013. **51**(6): p. 1779-1785.

118. Naseer, U., et al., *Molecular characterization of CTX-M-15-producing clinical isolates of Escherichia coli reveals the spread of multidrug-resistant ST131 (O25:H4) and ST964 (O102:H6) strains in Norway.* Apmis, 2009. **117**(7): p. 526-36.

119. Brolund, A., et al., *Epidemiology of extended-spectrum beta-lactamase-producing Escherichia coli in Sweden 2007-2011.* Clin Microbiol Infect, 2014. **20**(6): p. O344-52.

120. Johnning, A., et al., *Resistance mutations in gyrA and parC are common in Escherichia communities of both fluoroquinolone-polluted and uncontaminated aquatic environments.* Frontiers in Microbiology, 2015. **6**(1355).

121. Villa, L., et al., *Diversity, virulence, and antimicrobial resistance of the KPC-producing Klebsiella pneumoniae ST307 clone.* Microbial Genomics, 2017. **3**(4).

122. Lee, C.-R., et al., *Global dissemination of carbapenemase-producing Klebsiella pneumoniae: Epidemiology, genetic context, treatment options, and detection methods.* Frontiers in Microbiology, 2016. **7**(895).

123. Ruiz, E., et al., *Acquisition of carbapenem resistance in multiresistant Klebsiella pneumoniae strains harbouring blaCTX-M-15, qnrS1 and aac(6′)-Ib-cr genes.* Journal of Medical Microbiology, 2012. **61**(5): p. 672-677.

Supplementary data

Attachment A

Table 1-A: Media, reagents and solutions used in this project.

0.85 % NaCl: 8,5 g Natrium chloride (Merck) ddH_2O to 1 litre	**SCAI agar plates:** Myo-inositol (Sigma-Aldrich) Simmons Citrate agar (Sigma-Aldrich) ddH_2O
Freeze broth: Brain Heart Infusion (Oxoid) Glycerol 85% (Merck) ddH_2O	**CHROMagar ESBL plates:** CHROMagar Orientation (CHROMagar) CHROMgar ESBL supplement (CHROMagar) ddH_2O
Lactose agar plates: Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Bromthymol blue solution 0,2 % (Merck) ddH_2O HCl (1M and 5M) (Sigma-Aldrich)	**CHROMagar mSuperCarba plates:** CHROMagar mSuperCarba base CHROMagar mSuperCarba supplement S1 CHROMagar mSuperCarba supplement S2 ddH_2O
Lactose agar plates with 100 mg/ml ampicillin: Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Bromthymol blue solution 0,2 % (Merck) ddH_2O HCl (1M and 5M) (Sigma-Aldrich) Ampicillin 100mg/ml (Bristol-Myers Squibb)	**CLED agar plates:** CLED medium (MAST) ddH_2O NaOH (1 M) (Merck)
Mueller-Hinton agar: Mueller-Hinton agar (Oxoid) ddH_2O	**MALDI TOF matrix:** HCC, portioned, matrix for MALDI TOF MS (Bruker Daltonics)
Proteinase K: Proteinase K, recombinant, PCR grade (Roche). Consist of lyophilizate from *Pichia pastoris* which contains serine protease *(*~2 units/mg of protein)	**Lysozyme:** Lyophilizate of lysozyme from chicken egg white (Sigma-Aldrich), ≥40 000 units/mg protein

EasyMAG extraction buffers: NucliSens easyMAG extraction buffer 1 NucliSens easyMAG extraction buffer 2 NucliSens easyMAG extraction buffer 3 (Biomerieux)	**EasyMAG lysis buffer:** NucliSens easyMAG lysis buffer (Biomerieux)
EasyMAG silica beads: NucliSens easyMAG Magnetic Silica (Biomerieux)	**dsDNA HS Assay Kit for Qubit:** Concentrated assay reagent Dilution buffer Pre-diluted DNA standards (Thermo Fisher Scientific)

Recipe for the production of SCAI agar plates:

1. Make a 10% solution of myo-inositol by dissolving 10g myo-inositol powder in 100ml purified water.
2. Make the simmons citrate agar by suspending 21g simmons citrate agar powder to 1L purified water. Mix thoroughly
3. Heat the mixture and boil for 1 minute to completely dissolve the powder.
4. Dispense and autoclave at 121°C for 20 minutes.
5. Cool to 45-55°C
6. Add 100ml of the myo-inositol suspension and add it to 900 ml of the simmons citrate agar, this will give the desired concentration of 1%.
7. Pour into sterile petri dishes and store at 4°C

Setup of the two Sensititre broth dilution plates (NONAG4 and NONAG5):

SENSITITRE CUSTOM PLATE FORMAT

2011140914

Plate Code: **NONAG4** Date: **14-Nov-16**

	1	2	3	4	5	6	7	8	9	10	11	12
A	MERO 0.015	MERO 4	IMI 0.06	IMI 16	ETP 0.015	ETP 4	TAZ 16	FEP 4	P/T4 1/4	FOT 0.12	AZT 0.12	TRM 4
B	MERO 0.03	MERO 8	IMI 0.12	IMI 32	ETP 0.03	TAZ 0.12	TAZ 32	FEP 8	P/T4 2/4	FOT 0.25	AZT 0.25	TRM 8
C	MERO 0.06	MERO 16	IMI 0.25	AUGC 4/2	ETP 0.06	TAZ 0.25	FEP 0.06	FEP 16	P/T4 4/4	FOT 0.5	AZT 0.5	TRM 16
D	MERO 0.12	FOX 2	IMI 0.5	AUGC 8/2	ETP 0.12	TAZ 0.5	FEP 0.12	FUR 1	P/T4 8/4	FOT 1	AZT 1	TRM 32
E	MERO 0.25	FOX 4	IMI 1	AUGC 16/2	ETP 0.25	TAZ 1	FEP 0.25	FUR 2	P/T4 16/4	FOT 2	AZT 2	TRM 64
F	MERO 0.5	FOX 8	IMI 2	AUGC 32/2	ETP 0.5	TAZ 2	FEP 0.5	FUR 4	P/T4 32/4	FOT 4	AZT 4	TRM 128
G	MERO 1	FOX 16	IMI 4	AUGC 64/2	ETP 1	TAZ 4	FEP 1	FUR 8	P/T4 64/4	FOT 8	AZT 8	TRM 256
H	MERO 2	FOX 32	IMI 8	AUGC 128/2	ETP 2	TAZ 8	FEP 2	FUR 16	FOT 0.06	FOT 16	AZT 16	POS

ANTIMICROBICS

MERO	Meropenem
FOX	Cefoxitin
IMI	Imipenem
AUGC	Amoxicillin / clavulanic acid constant 2
ETP	Ertapenem
TAZ	Ceftazidime
FEP	Cefepime
FUR	Cefuroxime
P/T4	Piperacillin / tazobactam constant 4
FOT	Cefotaxime
AZT	Aztreonam
TRM	Temocillin
POS	Positive Control

Figure 34: Specifications for the NONAG4 Sensititre plate with antimicrobial agents and concentrations for each well.

SENSITITRE CUSTOM PLATE FORMAT

2011140914

Plate Code: **NONAG5** Date: **22-Nov-16**

	1	2	3	4	5	6	7	8	9	10	11	12
A	CIP 0.015	CIP 4	CZA 0.5/4	AMI 2	TOB 0.5	TOB 128	GEN 16	NIT 128	FOS+ 2	DOR 0.03	SXT 0.12/2.38	TGC 0.12
B	CIP 0.03	CIP 8	CZA 1/4	AMI 4	TOB 1	TOB 256	GEN 32	NIT 256	FOS+ 4	DOR 0.06	SXT 0.25/4.75	TGC 0.25
C	CIP 0.06	CIP 16	CZA 2/4	AMI 8	TOB 2	GEN 0.25	GEN 64	COL 0.25	FOS+ 8	DOR 0.12	SXT 0.5/9.5	TGC 0.5
D	CIP 0.12	CIP 32	CZA 4/4	AMI 16	TOB 4	GEN 0.5	GEN 128	COL 0.5	FOS+ 16	DOR 0.25	SXT 1/19	TGC 1
E	CIP 0.25	CZA 0.03/4	CZA 8/4	AMI 32	TOB 8	GEN 1	GEN 256	COL 1	FOS+ 32	DOR 0.5	SXT 2/38	TGC 2
F	CIP 0.5	CZA 0.06/4	CZA 16/4	AMI 64	TOB 16	GEN 2	NIT 16	COL 2	FOS+ 64	DOR 1	SXT 4/76	TGC 4
G	CIP 1	CZA 0.12/4	CZA 32/4	AMI 128	TOB 32	GEN 4	NIT 32	COL 4	FOS+ 128	DOR 2	SXT 8/152	TGC 8
H	CIP 2	CZA 0.25/4	AMI 1	AMI 256	TOB 64	GEN 8	NIT 64	COL 8	DOR 0.015	DOR 4	SXT 16/304	POS

ANTIMICROBICS

CIP	Ciprofloxacin
CZA	Ceftazidime/avibactam
AMI	Amikacin
TOB	Tobramycin
GEN	Gentamicin
NIT	Nitrofurantoin
COL	Colistin
FOS+	Fosfomycin+glucose-6-phosphate
DOR	Doripenem
SXT	Trimethoprim / sulfamethoxazole
TGC	Tigecycline
POS	Positive Control

Figure 35: Specifications for the NONAG5 Sensititre plate with antimicrobial agents and concentrations for each wel

Attachment B

Table 1-B: WGS data on ESBL-producing E. coli and K. pneumoniae from NORM 2014.

WGS results: ESBL-producing *E. coli* and *K. pneumoniae* – NORM 2014																
ID	**Species**	**Mater.**	**Laboratory**	**ST**	**CTX-M ESBL**	**SHV-ESBL**	**TEM-ESBL**	**Carbapenemase**	**pAmpC**	**Other β-lactamases**	**16S methylase**	**aac(6`)Ib-cr**	**QnrB**	**QnrS**	**QnrVC**	**qepA**
P19_75	E. coli	BLC	Haukeland	ST-10	blaCTX-M-15											qepA-like
P19_77	E. coli	Urine	Haukeland	ST-10	blaCTX-M-14					blaTEM-1B						
P19_81	E. coli	Urine	Haukeland	ST-10	blaCTX-M-15											
P20_57	E. coli	BLC	Trondheim	ST-10	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_33	E. coli	Urine	Stavanger	ST-1193	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_80	E. coli	Urine	Tønsberg	ST-1193	blaCTX-M-27											
P20_10	E. coli	BLC	Lillehammer	ST-410	blaCTX-M-15			blaOXA-181	blaCMY-2	blaOXA-1, blaTEM-1B		aac(6')Ib-cr		QnrS1		
P19_21	E. coli	BLC	AHUS	ST-131	blaCTX-M-1					blaTEM-1B						
P20_08	E. coli	BLC	Levanger	ST-131	blaCTX-M-1					blaTEM-1B						
P20_58	E. coli	BLC	Trondheim	ST-131	blaCTX-M-1					blaTEM-1B						
P21_25	E. coli	Urine	Ullevål	ST-131	blaCTX-M-1		blaTEM-33-like									
P19_52	E. coli	BLC	Fredrikstad	ST-131	blaCTX-M-14					blaTEM-1B						
P20_14	E. coli	BLC	Lillehammer	ST-131	blaCTX-M-14					blaTEM-1B						
P20_44	E. coli	BLC	Tromsø	ST-131	blaCTX-M-14					blaTEM-1B						
P20_45	E. coli	BLC	Tromsø	ST-131	blaCTX-M-14											
P20_72	E. coli	BLC	Tønsberg	ST-131	blaCTX-M-14					blaTEM-1B						
P20_73	E. coli	BLC	Tønsberg	ST-131	blaCTX-M-14											
P20_78	E. coli	Urine	Tønsberg	ST-131	blaCTX-M-14											

P20_12	E. coli	BLC	Lillehammer	ST-131	blaCTX-M-14-like					blaTEM-1B						
P19_14	E. coli	BLC	AHUS	ST-131	blaCTX-M-15					blaTEM-1B						
P19_19	E. coli	BLC	AHUS	ST-131	blaCTX-M-15											
P19_20	E. coli	BLC	AHUS	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_27	E. coli	BLC	Bodø	ST-131	blaCTX-M-15					blaTEM-1B						
P19_28	E. coli	BLC	Bodø	ST-131	blaCTX-M-15					blaTEM-1B						
P19_29	E. coli	BLC	Bodø	ST-131	blaCTX-M-15					blaTEM-1B						
P19_42	E. coli	BLC	Drammen	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_43	E. coli	BLC	Drammen	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_44	E. coli	BLC	Drammen	ST-131	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P19_45	E. coli	BLC	Drammen	ST-131	blaCTX-M-15											
P19_47	E. coli	Urine	Drammen	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_50	E. coli	BLC	Fredrikstad	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_51	E. coli	BLC	Fredrikstad	ST-131	blaCTX-M-15					blaTEM-1B						
P19_54	E. coli	BLC	Fredrikstad	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_59	E. coli	BLC	Førde	ST-131	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P19_65	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_66	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P19_67	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_68	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_69	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_70	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_71	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_72	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				

P19_73	E. coli	BLC	Haukeland	ST-131	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P19_78	E. coli	Urine	Haukeland	ST-131	blaCTX-M-15					blaTEM-1B						
P19_79	E. coli	Urine	Haukeland	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_17	E. coli	Urine	Lillehammer	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_18	E. coli	BLC	Molde	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_25	E. coli	BLC	Rikshospitalet	ST-131	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P20_30	E. coli	BLC	Stavanger	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_32	E. coli	Urine	Stavanger	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_54	E. coli	Urine	Tromsø	ST-131	blaCTX-M-15											
P20_60	E. coli	BLC	Trondheim	ST-131	blaCTX-M-15					blaTEM-1B						
P20_67	E. coli	BLC	Tønsberg	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_79	E. coli	Urine	Tønsberg	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P21_11	E. coli	BLC	Ullevål	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P21_21	E. coli	Urine	Ullevål	ST-131	blaCTX-M-15					blaOXA-1,blaTEM-1B-like		aac(6')Ib-cr				
P21_24	E. coli	Urine	Ullevål	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P21_29	E. coli	BLC	Ålesund	ST-131	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_16	E. coli	BLC	AHUS	ST-131	blaCTX-M-24					blaTEM-1B						
P21_23	E. coli	Urine	Ullevål	ST-131	blaCTX-M-24					blaTEM-1B						
P19_22	E. coli	BLC	AHUS	ST-131	blaCTX-M-27											
P19_36	E. coli	BLC	Bærum	ST-131	blaCTX-M-27											
P19_38	E. coli	Urine	Bærum	ST-131	blaCTX-M-27					blaTEM-1B			QnrB1-like			
P19_53	E. coli	BLC	Fredrikstad	ST-131	blaCTX-M-27											
P19_55	E. coli	BLC	Fredrikstad	ST-131	blaCTX-M-27											
P19_63	E. coli	BLC	Haugesund	ST-131	blaCTX-M-27					blaTEM-1B						

P19_64	E. coli	BLC	Haugesund	ST-131	blaCTX-M-27							aac(6')Ib-cr-like				
P19_80	E. coli	Urine	Haukeland	ST-131	blaCTX-M-27											
P20_29	E. coli	BLC	Stavanger	ST-131	blaCTX-M-27											
P20_41	E. coli	BLC	Tromsø	ST-131	blaCTX-M-27					blaTEM-1B						
P20_65	E. coli	BLC	Tønsberg	ST-131	blaCTX-M-27											
P20_69	E. coli	BLC	Tønsberg	ST-131	blaCTX-M-27					blaTEM-1B						
P20_70	E. coli	BLC	Tønsberg	ST-131	blaCTX-M-27											
P21_01	E. coli	Urine	Tønsberg	ST-131	blaCTX-M-27											
P21_06	E. coli	BLC	Ullevål	ST-131	blaCTX-M-27											
P21_08	E. coli	BLC	Ullevål	ST-131	blaCTX-M-27											
P21_20	E. coli	Urine	Ullevål	ST-131	blaCTX-M-27											
P20_40	E. coli	BLC	Tromsø	ST-131	blaCTX-M-27											
P19_18	E. coli	BLC	AHUS	ST-156	blaCTX-M-14					blaOXA-10		aac(6')Ib-cr-like			QnrVC4	
P19_30	E. coli	BLC	Bodø	ST-1611	blaCTX-M-14					blaTEM-1B						
P20_47	E. coli	BLC	Tromsø	ST-773	blaCTX-M-14					blaTEM-1B						
P19_58	E. coli	BLC	Førde	ST-345	blaCTX-M-14b-like					blaTEM-1B						
P20_04	E. coli	Urine	Kristiansand	ST-14	blaCTX-M-15					blaTEM-1B						
P21_22	E. coli	Urine	Ullevål	ST-1431	blaCTX-M-15					blaTEM-1B						
P19_15	E. coli	BLC	AHUS	ST-192	blaCTX-M-15					blaTEM-1B						
P20_21	E. coli	BLC	Radiumhospitalet	ST-421	blaCTX-M-15											
P20_66	E. coli	BLC	Tønsberg	ST-372	blaCTX-M-14											
P21_04	E. coli	BLC	Ullevål	ST-372	blaCTX-M-15					blaOXA-1,blaTEM-1A		aac(6')Ib-cr				
P20_11	E. coli	BLC	Lillehammer	ST-38	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				

P20_62	E. coli	Urine	Trondheim	ST-38	blaCTX-M-14,blaCTX-M-15											
P21_07	E. coli	BLC	Ullevål	ST-38	blaCTX-M-14											
P21_10	E. coli	BLC	Ullevål	ST-38	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P21_13	E. coli	BLC	Ullevål	ST-38	blaCTX-M-14-like,blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P21_14	E. coli	BLC	Ullevål	ST-38	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P21_33	E. coli	BLC	Kristiansand	ST-38	blaCTX-M-14											
P19_37	E. coli	BLC	Bærum	ST-405	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_76	E. coli	BLC	Haukeland	ST-405	blaCTX-M-15											
P20_05	E. coli	Urine	Kristiansand	ST-405	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_24	E. coli	Urine	Radiumhospitalet	ST-405	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_37	E. coli	Urine	Telelab	ST-405	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_43	E. coli	BLC	Tromsø	ST-405	blaCTX-M-14							aac(6')Ib-cr-like				
P20_46	E. coli	BLC	Tromsø	ST-405	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_59	E. coli	BLC	Trondheim	ST-405	blaCTX-M-55					blaOXA-1	armA	aac(6')Ib-cr				
P20_64	E. coli	BLC	Tønsberg	ST-405	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P20_68	E. coli	BLC	Tønsberg	ST-405	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P19_34	E. coli	Urine	Bodø	ST-636	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr-like				
P21_02	E. coli	Urine	Tønsberg	ST-69	blaCTX-M-15					blaTEM-1B-like						
P21_05	E. coli	BLC	Ullevål	ST-73	blaCTX-M-15					blaTEM-1B			QnrB66-like			
P19_40	E. coli	Urine	Bærum	ST-617	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr				
P19_60	E. coli	BLC	Førde	ST-617	blaCTX-M-15					blaOXA-1,blaTEM-1C		aac(6')Ib-cr				

P20_53	E. coli	Urine	Tromsø	ST-88	blaCTX-M-15					blaTEM-1B						
P19_24	E. coli	Urine	AHUS	ST-648	blaCTX-M-15					blaOXA-1		aac(6')Ib-cr	QnrB66-like			
P20_13	E. coli	BLC	Lillehammer	ST-648	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P20_81	E. coli	Urine	Tønsberg	ST-648	blaCTX-M-14					blaTEM-1B						
P21_15	E. coli	BLC	Ullevål	ST-648	blaCTX-M-15											
P21_26	E. coli	Urine	Ullevål	ST-648	blaCTX-M-14											
P21_31	E. coli	Urine	Ålesund	ST-648	blaCTX-M-15					blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P20_63	E. coli	Urine	Trondheim	ST-648	blaCTX-M-2								QnrB19			
P19_17	E. coli	BLC	AHUS	ST-977	blaCTX-M-15					blaTEM-1B						
P20_48	E. coli	BLC	Tromsø	Unknown ST	blaCTX-M-15					blaOXA-1						
P19_35	E. coli	BLC	Bærum	ST-1722	blaCTX-M-27											
P20_61	E. coli	BLC	Trondheim	ST-127	blaCTX-M-55					blaTEM-1B						
P20_09	E. coli	BLC	Lillehammer	ST-95	blaCTX-M-15,			blaIMP-26		blaTEM-1B-like	armA	aac(6')Ib-cr-like				
P21_12	E. coli	BLC	Ullevål	ST-95	blaCTX-M-55					blaTEM-1B						
P21_28	K. pneumoniae	Urine	Ullevål	ST-101	blaCTX-M-15					blaSHV-1						
P21_03	K. pneumoniae	Urine	Tønsberg	ST-107	blaCTX-M-14	blaSHV-36-like				blaTEM-1B				QnrS1		
P20_56	K. pneumoniae	Urine	Tromsø	ST-1296	blaCTX-M-15	blaSHV-11				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P20_76	K. pneumoniae	BLC	Tønsberg	ST-14	blaCTX-M-15	blaSHV-28				blaTEM-1B			QnrB66-like			
P21_17	K. pneumoniae	BLC	Ullevål	ST-147	blaCTX-M-15	blaSHV-11-like				blaOXA-1,blaTEM-1B,blaOXA-9-like		aac(6')Ib-cr-like				
P19_23	K. pneumoniae	BLC	AHUS	ST-15	blaCTX-M-15	blaSHV-28				blaTEM-1B, blaOXA-1-like		aac(6')Ib-cr	QnrB66-like			

P19_32	K. pneumoniae	BLC	Bodø	ST-15	blaCTX-M-15	blaSHV-28				blaOXA-1,blaOXA-10-like,blaTEM-1B		aac(6')Ib-cr				
P20_28	K. pneumoniae	BLC	Rikshospitalet	ST-15	blaCTX-M-15	blaSHV-129				blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P20_07	K. pneumoniae	Urine	Kristiansand	ST-16	blaCTX-M-15					blaOXA-1,blaSHV-1,blaTEM-1B		aac(6')Ib-cr				
P20_02	K. pneumoniae	Urine	Haukeland	ST-231	blaCTX-M-15					blaSHV-1,blaTEM-1B-like						
P20_03	K. pneumoniae	Urine	Haukeland	ST-231	blaCTX-M-15					blaOXA-1,blaSHV-1,blaTEM-1B-like		aac(6')Ib-cr				
P20_01	K. pneumoniae	Urine	Haukeland	ST-273		blaSHV-2-like			blaDHA-1	blaOXA-1		aac(6')Ib-cr	QnrB4			
P19_31	K. pneumoniae	BLC	Bodø	ST-307	blaCTX-M-15	blaSHV-28				blaTEM-1B,blaOXA-1		aac(6')Ib-cr	QnrB66-like			
P19_33	K. pneumoniae	BLC	Bodø	ST-307	blaCTX-M-15	blaSHV-28				blaTEM-1B,blaOXA-1		aac(6')Ib-cr	QnrB66-like			
P20_16	K. pneumoniae	BLC	Lillehammer	ST-307	blaCTX-M-15	blaSHV-28				blaOXA-1, blaTEM-1B		aac(6')Ib-cr				
P20_23	K. pneumoniae	BLC	Radiumhospitalet	ST-307	blaCTX-M-15	blaSHV-28				blaOXA-1,blaTEM-1B		aac(6')Ib-cr				
P20_50	K. pneumoniae	BLC	Tromsø	ST-307	blaCTX-M-15	blaSHV-28				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P20_51	K. pneumoniae	BLC	Tromsø	ST-307	blaCTX-M-15	blaSHV-28				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P20_52	K. pneumoniae	BLC	Tromsø	ST-307	blaCTX-M-15	blaSHV-28				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P21_16	K. pneumoniae	BLC	Ullevål	ST-307	blaCTX-M-15	blaSHV-28				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P21_30	K. pneumoniae	BLC	Ålesund	ST-307	blaCTX-M-15	blaSHV-28				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P20_39	K. pneumoniae	Urine	Telelab	ST-323	blaCTX-M-15	blaSHV-99-like				blaTEM-1B			QnrB66-like			

P20_15	K. pneumoniae	BLC	Lillehammer	ST-334		blaSHV-12				blaOKP-B-4-like			QnrB1			
P19_56	K. pneumoniae	BLC	Fredrikstad	ST-340	blaCTX-M-15	blaSHV-11	blaTEM-33			blaOXA-1	armA	aac(6')Ib-cr	QnrB66-like			
P20_49	K. pneumoniae	BLC	Tromsø	ST-340	blaCTX-M-15	blaSHV-11				blaOXA-1		aac(6')Ib-cr				
P20_55	K. pneumoniae	Urine	Tromsø	ST-340	blaCTX-M-15	blaSHV-11				blaOXA-1,blaOXA-10		aac(6')Ib-cr				
P21_18	K. pneumoniae	BLC	Ullevål	ST-37	blaCTX-M-15	blaSHV-11-like				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P21_19	K. pneumoniae	BLC	Ullevål	ST-392	blaCTX-M-15	blaSHV-11-like				blaOXA-1,blaTEM-1B			QnrB66-like			
P20_77	K. pneumoniae	BLC	Tønsberg	ST-405	blaCTX-M-15	blaSHV-76-like				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P20_20	K. pneumoniae	Urine	Molde	ST-416	blaCTX-M-15	blaSHV-14-like										
P19_62	K. pneumoniae	BLC	Førde	ST-420	blaCTX-M-15	blaSHV-75				blaOXA-1		aac(6')Ib-cr				
P19_49	K. pneumoniae	Urine	Drammen	ST-45	blaCTX-M-15	blaSHV-27				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB1-like			
P20_35	K. pneumoniae	Urine	Stavanger	ST-45		blaSHV-12										
P20_19	K. pneumoniae	Urine	Molde	ST-48	blaCTX-M-15	blaSHV-11				blaTEM-1B			QnrB66-like			
P20_74	K. pneumoniae	BLC	Tønsberg	ST-627		blaSHV-2										
P19_46	K. pneumoniae	BLC	Drammen	ST-70	blaCTX-M-15	blaSHV-27-like				blaOXA-1,blaTEM-1B		aac(6')Ib-cr	QnrB66-like			
P19_57	K. pneumoniae	Urine	Fredrikstad	ST-904		blaSHV-2-like										
P20_06	E. coli	Urine	Kristiansand	ST-155		blaSHV-12							QnrB7			

P19_74	E. coli	BLC	Haukeland	ST-4985		blaSHV-12							QnrB19			
P20_36	K. pneumoniae	Urine	Stavanger	Unknown ST	blaCTX-M-27	blaSHV-41										

Table 2-B: WGS data on ESBL-producing E. coli and K. pneumoniae from Tromsø-7.

WGS results: ESBL-producing *E. coli* and *K. pneumoniae* – Tromsø-7											
ID	**Species**	**Sample mat.**	**Laboratory**	**ST**	**CTX-M ESBL**	**SHV-ESBL**	**Carbapenemase**	**pAmpC**	**Other β-lactamases**	**16S methylase**	**aac(6')Ib-cr**
50918404	E. coli	Feces	Tromsø	ST-10	blaCTX-M-15				blaOXA-1		aac(6')Ib-cr
50932514	E. coli	Feces	Tromsø	ST-12	blaCTX-M-14						
50937543	E. coli	Feces	Tromsø	ST-12	blaCTX-M-14						
50930986	E. coli	Feces	Tromsø	ST-1290	blaCTX-M-14				blaTEM-1A		
50923324	E. coli	Feces	Tromsø	ST-131	blaCTX-M-15				blaOXA-1		aac(6')Ib-cr
50925997	E. coli	Feces	Tromsø	ST-131	blaCTX-M-27				blaTEM-1B		
50927790	E. coli	Feces	Tromsø	ST-131	blaCTX-M-27				blaTEM-1B		
50930924	E. coli	Feces	Tromsø	ST-131	blaCTX-M-14						
50929669	E. coli	Feces	Tromsø	ST-131					blaTEM-1B-like		
50931575	E. coli	Feces	Tromsø	ST-131	blaCTX-M-15				blaTEM-1B		
50938355	E. coli	Feces	Tromsø	ST-131	blaCTX-M-14				blaTEM-1B		
50938698	E. coli	Feces	Tromsø	ST-2178	blaCTX-M-15				blaTEM-1B		
50929164	E. coli	Feces	Tromsø	ST-38	blaCTX-M-14				blaTEM-1B		
50932516	E. coli	Feces	Tromsø	ST-448	blaCTX-M-15				blaTEM-1B		
50937429	E. coli	Feces	Tromsø	ST-450	blaCTX-M-14						
50929679	E. coli	Feces	Tromsø	ST-617	blaCTX-M-15						
50930927	E. coli	Feces	Tromsø	ST-648	blaCTX-M-15				blaOXA-1,blaTEM-1B		aac(6')Ib-cr
50921884	E. coli	Feces	Tromsø	ST-69	blaCTX-M-15				blaTEM-1B		
50929671	E. coli	Feces	Tromsø	ST-88					blaOXA-1		
50931574	E. coli	Feces	Tromsø	ST-93	blaCTX-M-8						
50922842	E. coli	Feces	Tromsø	Unknown					blaTEM-1B-like		
50937533	E. coli	Feces	Tromsø	Unknown	blaCTX-M-15				blaTEM-1B		
50934266	K.pneumoniae	Feces	Tromsø	ST-46					blaSHV-1		