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Collateral sensitivity in clinical mecillinam resistant isolates of *Escherichia coli*

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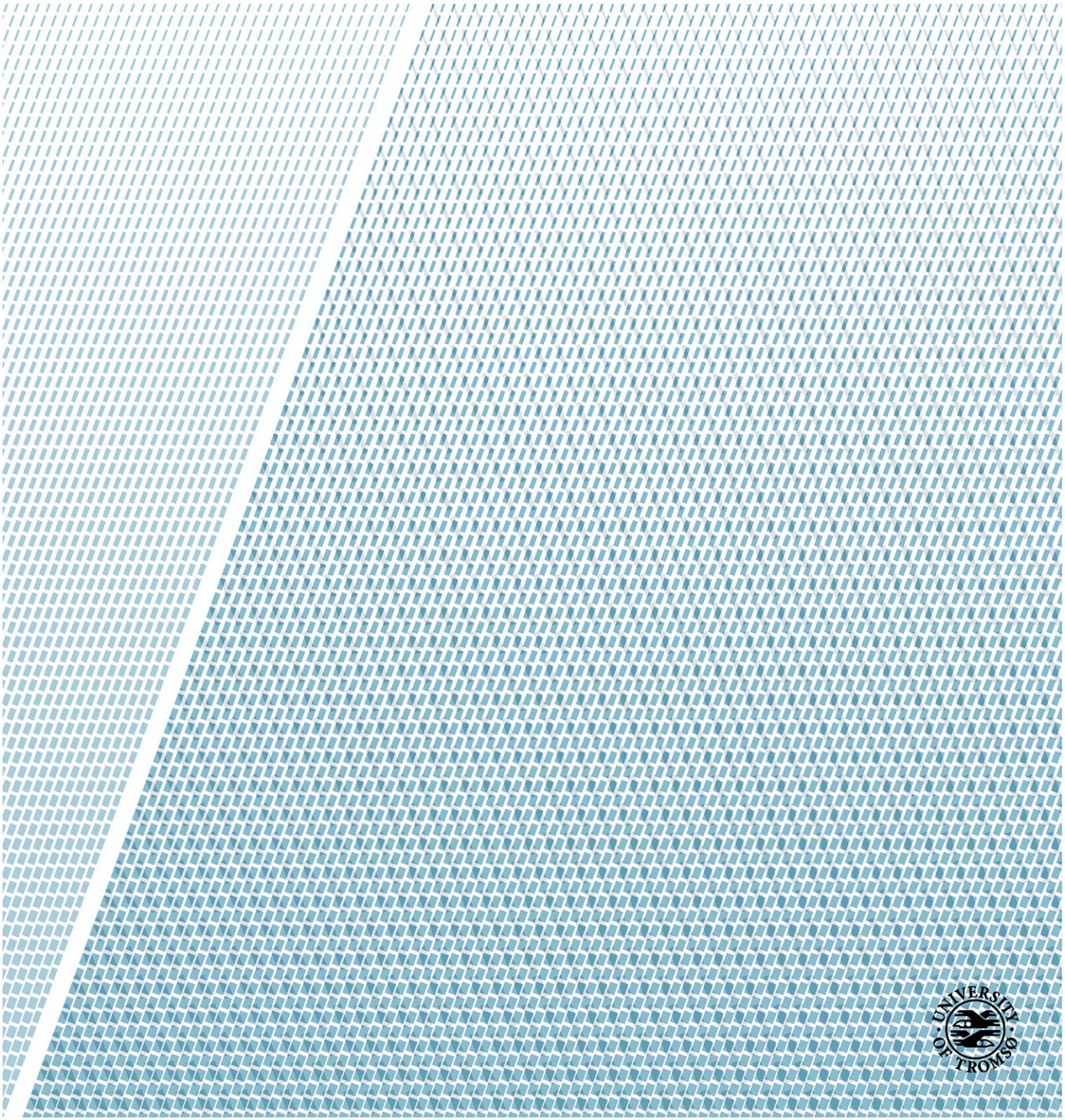

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ABSTRACT

Background

The rapid increase in antimicrobial resistance (AMR) has become a major threat to the successful management of infectious diseases. To counteract this global threat, development of novel treatment strategies is essential. A promising strategy may be exploiting collateral sensitivity; a phenomenon that occurs when a microorganism that has developed resistance to one antimicrobial agent, exhibits increased susceptibility to another antimicrobial agent. In order to develop novel treatment strategies and prevent further resistance development, we aimed to explore the generality of the concept of collateral sensitivity in clinical urinary tract isolates of *E. coli*. Furthermore, we wanted to investigate the underlying mechanisms of collateral sensitivity.

Methods

We evolved resistance to mecillinam in a collection of clinical isolates of *E. coli*. Ten were selected for further determination of possible collateral sensitivity and cross-resistance networks. The IC_{90}-assay with micro broth dilution was used for this purpose, which we tested for eight different antimicrobial agents. The results were displayed in heat maps and graphs showing the distribution of AMR to various agents. PCR and DNA sequencing were performed for the *mrdA* gene to detect mutations that may confer mecillinam resistance.

Results

According to our results both collateral sensitivity and cross-resistance occurred in mecillinam resistant isolates. Chloramphenicol presented the highest tendency of collateral sensitivity, while ciprofloxacin presented the highest tendency of cross-resistance. In general, a substantial tendency for collateral sensitivity frequently appeared compared to cross-resistance. Moreover, 13 synonymous point mutations were observed in the *mrdA* gene, leading to no alteration in the amino acid sequence.

Conclusion

Based on our *in vitro* results, we suggest mecillinam could be a good candidate to be employed as the first drug of choice for UTIs caused by *E. coli*. Mecillinam resistant isolates exhibited a clear tendency for collateral sensitivity, which we believe would occur on the population level as well. Further investigations of the underlying mechanisms of collateral sensitivity are required.

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DEFINITIONS

Antimicrobial resistance (AMR): "resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it" (1).

Cross-resistance (CR): occurs due to a single resistance mechanism which confer resistance to an entire class of antimicrobial agents (2).

Collateral sensitivity (CS): in the context of microbiology, CS occurs when a microorganism that has developed resistance to one antimicrobial agent exhibits increased susceptibility to another antimicrobial agent (3).

Community-acquired infection: infections are acquired in the community, in contrast of hospital-acquired infections (4).

Hospital-acquired infections: infections are acquired in hospitals and other healthcare facilities, in contrast of community-acquired infections. The patient in this case must have been admitted for reasons other than infection, and has shown no signs of active or incubating infection (4).

In vitro: "in glass", studies are performed with microorganisms, cells or biological molecules outside their normal biological context (5).

In vivo: "in the living", studies are performed in living organisms (5).

Isolate: a population of bacterial cells in pure culture derived from a single colony. In the context of clinical microbiology, isolates are usually derived from the primary culture of a clinical specimen obtained from an individual patient (6).

Multidrug-resistance (MDR): defined as acquired non-susceptibility to at least one antimicrobial drug in three or more antimicrobials categories (2).

Nucleotides: building blocks of nucleic acids in DNA and RNA. Nucleotides have three characteristics components; a nitrogenous base (purine or pyrimidine), a pentose and a phosphate (5).

Nucleic acids: biomolecules essential for all known form of life. Nucleic acids, which include DNA and RNA, are made from monomers known as nucleotides (5).

Pathogen: a bacterium, virus or other microorganism that can invade the human body and cause disease (7).

Plasmid: an "extrachromosomal", circular DNA in the bacterial cells that replicates independently of the chromosome and regulate its own replication (8).

Species: the basic category of bacteria; a named group of bacteria which shows a high degree of overall similarity as compared to other, more vaguely related strains. Currently there are no universally accepted species definitions (6).

Strain: the descendants of a single isolation in pure culture, usually derived from a single initial colony. It may be considered as an isolate or a group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic or genotypic characteristics. E.g. Two isolates can be representatives of one strain, however two strains can never be the same isolate (6).

Transposon: "jumping gene", a DNA sequence that can move from one place in the DNA to a different place (8).

1 INTRODUCTION

1.1 Preface

The phrase *"survival of the fittest"* originated from an evolutionary theory that describes the mechanism of natural selection (9). As early as in the 1850s, Charles Darwin discovered the foundation of a scientific theory on adaptation of animals and plants and their incredible biodiversity. Few would expect that this would give rise to human's biggest public health threat in the 21st century; antimicrobial resistance, a global threat driven by the evolution of resistant microorganisms. To combat this threat, novel infectious treatment strategies are essential. A promising strategy may be exploiting collateral sensitivity; a phenomenon brought to light in 1952, which for the past few years has caught the interest of researchers again. Translating collateral sensitivity networks into treatment-guidelines may retard the evolution of antimicrobial resistance by constraining the evolutionary paths towards resistance.

1.2 Antimicrobial agents

The discovery of penicillin by Alexander Fleming in 1928 is considered the beginning of the history of modern medicine, as well as one of the greatest discoveries of the 20th century. An antimicrobial agent is a substance that either kills or inhibits the growth of microorganisms (4). These agents are categorized according to the microorganisms they act primarily against. Antibacterial agents are used to treat bacterial infections, antifungal drugs act against fungi, antiviral drugs are used specifically for treating viral infections, and antiprotozoal drugs act against protozoa (4). In this thesis antimicrobial agents or antimicrobials refer to antibacterial drugs.

1.2.1 Classification of antimicrobial agents

Antimicrobial agents are derived naturally from microorganisms, chemically modified or produced fully synthetic by pharmaceutical chemists (4). They can be classified in several ways, including:

- *spectrum of activity*; either broad- or narrow-spectrum
- *effect on bacteria*; antimicrobials can either kills the microorganisms (bactericidal) or inhibits their growth (bacteriostatic). This distinction is rather blurred since some of the drugs have bactericidal effect on some species, but have bacteriostatic on others
- and *mode of action* (4).

I have chosen to describe antimicrobials agents in the context of their mode of action, which categorize them into five categories based on their site of activity (4):

i) Inhibition of cell wall synthesis

The bacterial cell wall is essential for maintenance of the integrity of bacterial cells (4). The cell synthesis is hence an important target for antimicrobial agents. A unique and main component of the bacterial cell wall is peptidoglycan, which is a mixed polymer of hexose sugars and amino acids (4). In Gram-positive bacteria, peptidoglycan forms a thick layer external to the cell membrane, and may contain other macromolecules (4). While in Gram-negative bacteria, the peptidoglycan layer is thin and is also overlaid by an outer membrane with other components such as lipoproteins and lipopolysaccharides (4). β-lactams target the bacterial cell wall synthesis by specific covalent binding to penicillin-binding proteins (PBPs), such as mecillinam and PBP 2 (10). The mode of action for mecillinam differs from other β-lactams, which bind to other PBPs (10). PBP 2 is responsible for the elongation of rod-shaped cells and generates the mature peptidoglycan molecules. Thus cells treated with mecillinam will have impaired formation of cross-links and become enlarged, non-dividing spheres that ultimately lyse (11, 12).

ii) Inhibition of nucleic acid synthesis

DNA and RNA are the keys to replication of all living forms. Antimicrobials, such as quinolones (e.g. ciprofloxacin), disrupt DNA or RNA synthesis by interfering with either nucleotide or nucleic acid biosynthetic processes in the cell (4). This causes interference of normal cellular processes, for instance bacterial transcription or replication, and thus cell viability as well (4).

iii) Inhibition of protein synthesis

Enzymes and most of cellular structures are primarily made of proteins, which are essential components necessary for bacterial cell growth and replication (4). Many antimicrobials

target bacterial protein synthesis by binding to either the 30S or 50S subunits of the ribosomes (the site of protein synthesis). Consequently, this will lead to cell death of the organism or inhibition of its cell growth or replication (4). Antimicrobial classes that have this mode of action are for instance aminoglycosides (e.g. gentamicin), macrolides, tetracyclines and miscellaneous (e.g. chloramphenicol and nitrofurantoin) (4).

iv) Inhibitor of cell membrane functions

Disruptions or damage to the cell wall structure may result in the loss of important solutes essential for survival of the cell (4). Unfortunately both eukaryotic and prokaryotic cells have this cell wall structure and consequently some of the antimicrobials in this class may often be lead to severe adverse effects for systemic use in humans (4). Therefore most clinical use is restricted to topical applications, such as polymyxin B.

v) Inhibition of other metabolic pathways

Inhibition of other metabolic pathways includes the folic acid pathway, a process which is needed for production of precursors for nucleic acid synthesis (4). For instance trimethoprim acts by preventing tetrahydrofolic acid synthesis. This is required for the synthesis of different nucleotides in the DNA and RNA (4).

1.3 Antimicrobial resistance

The successful discovery of antimicrobial agents was unfortunately compromised by the inevitable emergence of antimicrobial resistance (AMR) from the time they were first used (1). As the quote *"Some men are born great, some achieve greatness, and some have greatness thrust upon them"* by William Shakespeare, some bacteria are born resistant, others have resistant thrust upon them. In other words, enhanced levels of resistance can be achieved by mutations in the bacterial genome (*de novo*) or by acquisition of resistance-conferring genes through horizontal gene transfer (HGT) (4). This will be described in more detail in the section on AMR mechanism (section 1.3.4). I will first address the true cost of AMR, the current status in Europe and give a brief introduction to possible novel strategies to deal with the issue of AMR development both from the scientific/medical communities as well as the action plan suggested by the World Health Organization (WHO).

1.3.1 The true cost of AMR

In the last 70 years, there has been a continual race between the discovery and development of novel antimicrobials and the bacteria that will respond to the selective pressure and novel AMR mechanisms that are developed and selected for (4, 13).

At the present time, AMR is listed as one of the greatest threats to human health according to recent World Economic Forum Global Risks reports (14). It is also recognized as a growing global threat to the successful management of infectious diseases (15), with over 2 million AMR infections per year in the US alone (16). According to WHO, deaths caused by infectious diseases represent more than half of disease-related deaths globally (17). The emergence of bacteria with a diversity of resistance mechanisms has intensified the challenges associated with infection control and treatment strategies.

1.3.2 Current status and possible strategies for improvement of treatment strategies

Though AMR occurs naturally, over- and misuse of antimicrobial agents accelerates this process (18, 19). According to the annual surveillance reports by the European Centre for Disease Prevention and Control (ECDC), there is an association between the consumption levels of antimicrobials and the levels of AMR (20, 21). Figure 1 gives an overview of the consumption of antimicrobials for the majority of European countries in 2014 (20). Greece, Romania and France were the three countries with the highest consumption of antimicrobial agents. Another report of ECDC from the same year gave the latest data on AMR for different bacterial species in Europe (21). For invasive isolates of *E. coli* with resistance to third-generation cephalosporins in 2014, there is a clear correlation of resistance prevalence and antimicrobial agents consumption (Figure 2). Bulgaria, Cyprus, Italy, Romania and Slovakia were countries with highest resistance prevalence in 2014, in which the percentage of resistance were 25% to < 50%.

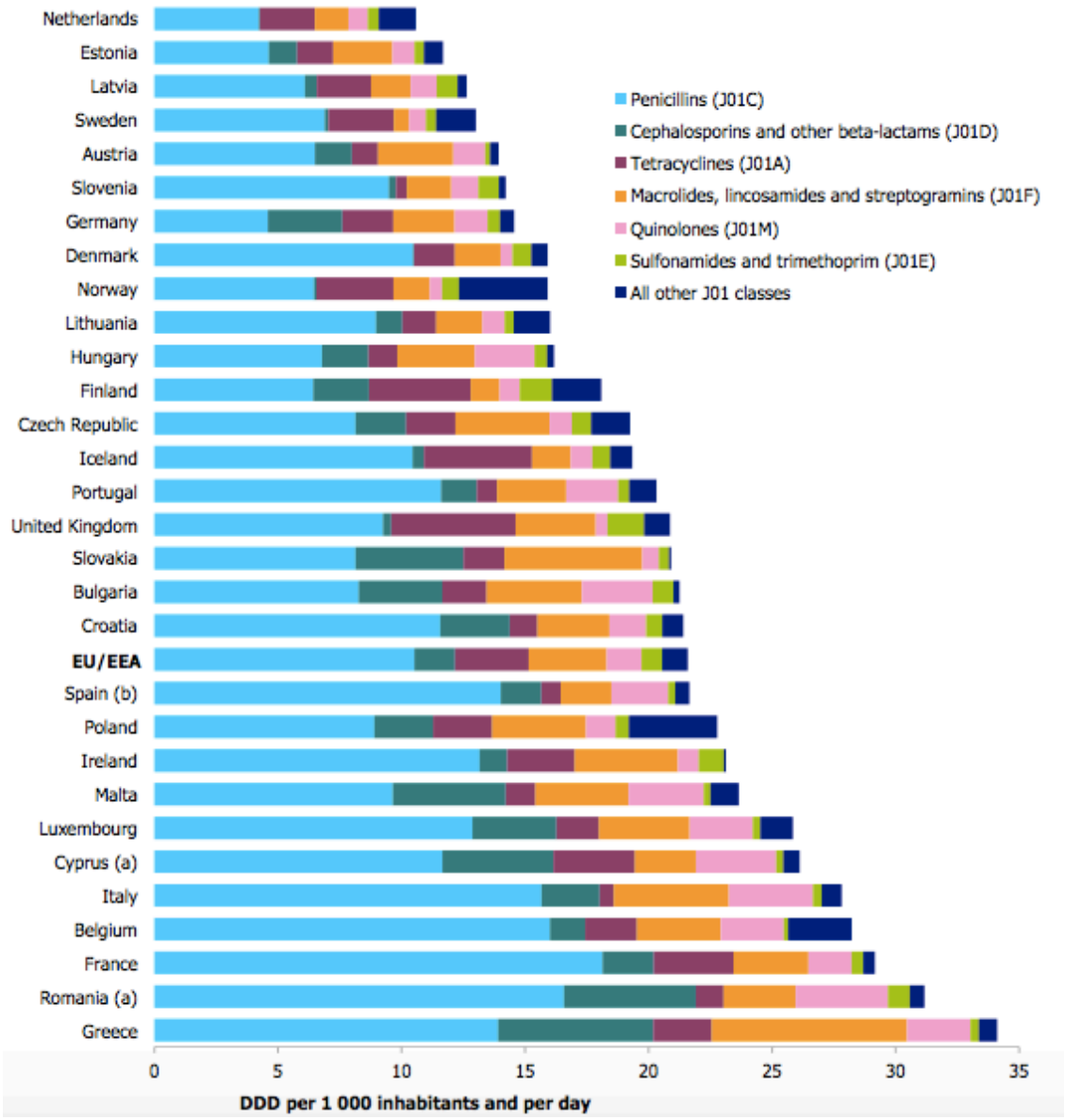


Figure 1: Consumption of antimicrobials for systemic use in the community for different European countries 2014. The different color codes denote various antimicrobial groups. Permission obtained from: (20, 22).

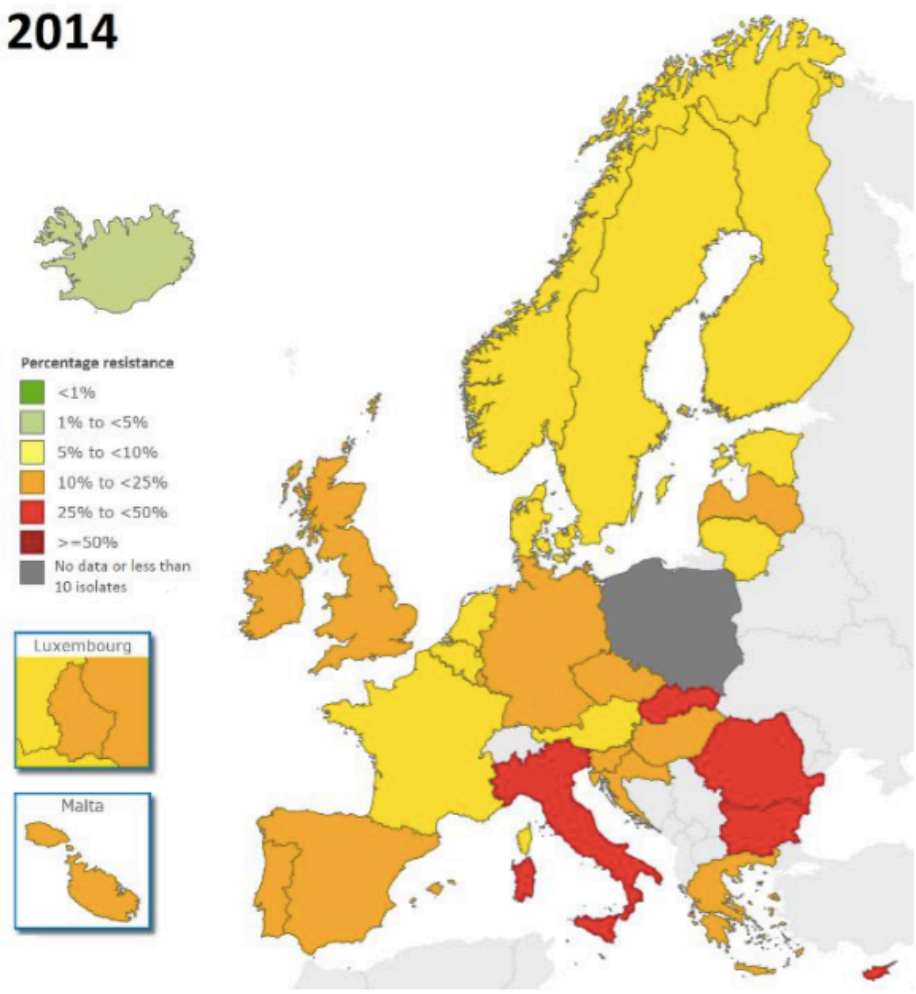


Figure 2: Percentage of invasive isolates of *E. coli* with combined resistance to third-generation cephalosporins in 2014. Permission obtained from: (21, 22).

Microorganisms develop defensive mechanisms, which protect them from antimicrobials and allow them to become less sensitive (4). Switching to another antimicrobial or higher dosage is not always sufficient. In the latter, increased dosage is associated with greater adverse effects and toxicity for the patient. Moreover, use of high doses of antimicrobials has the potential to promote increase of cross-resistance in clinical settings (23).

So why do we not develop novel antimicrobials that can combat resistant bacteria? In order to do this, the developed antimicrobials must be effective against resistant microorganisms (e.g. those with novel antimicrobial mechanism(s)), which is a tremendously challenging and time-consuming task (24). The dramatic decrease in novel antimicrobial agents approved is not only due to scientific but also other several factors, including commercial ones. Pharmaceutical companies consider development of antimicrobial agents a poor economic investment compared to drugs that treat chronic illness (24). An example of this is drugs

treating high blood pressure (hypertension) that are taken daily for the rest of a patient's life while antimicrobials are taken only for a short period of time. This is one of the major reasons why companies have stopped developing antimicrobial agents. In addition to the mentioned factors, use of a novel antimicrobial agent will probably eventually lead to development of AMR as well. Hence changing our antimicrobial treatment strategies may be a better solution.

One approach to counteract drug resistance development in bacteria is combination therapy where two or more drugs are administered simultaneously. The first case showing success with combination therapy was in 1940s, based on a so-called synergistic effect (25). Synergy is the interaction of drugs where the combined effect is greater than the sum of drugs individually. Combination therapy has been considered a promising drug therapy strategy to the rising health threat of AMR until associated severe adverse effects were observed for some combination therapies. For instance the combination of tobramycin and piperacillin has shown renal toxicity, skin rash and ototoxicity (26). Furthermore, several studies have shown no difference in clinical outcomes between combination therapy and monotherapy (26).

However, a more favorable strategy may be alternating therapy, also known as collateral sensitivity cycling. Translating collateral sensitivity networks into treatment-guidelines may retard the evolution of antimicrobial resistance by constraining the evolutionary paths towards resistance (27). This will be described in more detail in the section 1.4. To approach solutions for AMR we need to understand how currently used antimicrobial agents work and how bacteria are able to survive treatment with these antimicrobials.

1.3.3 WHO global action plan to tackle AMR

Several antimicrobial stewardship programs have developed strategies to control the emergence of AMR. In May 2015, The World Health Assembly of WHO endorsed a global action plan to tackle AMR (28). Their goal is to ensure continuity of successful treatment and prevention of infectious diseases with effective and safe medicines, used in a responsible way, and accessible to all who need them. They set out five strategic objectives to achieve this goal:

1) *"to improve awareness and understanding of AMR"*; this can promote behavioral change by raising the issue through effective communication, education and training.

2) *"to strengthen knowledge through surveillance and research"*; integrated programs are needed for surveillance of AMR in all countries, which aim to extend reduction in prevalence of AMR by using the collected data. This should be supported by national governments, professional organizations and industries through the generation of such knowledge and bringing it into practice.
3) *"to reduce the incidence of infection"*; this can be achieved through better hygiene and infection prevention (e.g. better sanitation, hand washing, food and water safety), vaccine programs and sustainable husbandry practices.
4) *"to optimize the use of antimicrobial agents"*; there is need for effective, rapid, low-cost diagnostic tools for guidance on the optimal use of antimicrobials. It is also important to improve regulations of the purchase and compliance of patient and health care provider.
5) *"develop the economic case for sustainable investment that takes account of the needs of all countries, and increase investment in new medicines, diagnostic tools, vaccines and other interventions."*; studies on the economic cost of AMR should be implemented for all countries, not only limited to developed countries. Additionally there is need for affordable tools to inform health personnel of the susceptibility of the pathogens to available antimicrobials (28).

1.3.4 Development and dissemination of antimicrobial resistance

Some bacteria are naturally resistant to specific antimicrobials (intrinsic resistance). **Intrinsic resistance** is the inherent ability of a species to resist the mode of action(s) of a certain antimicrobial agent through its inherent structural or functional characteristics (4). The mechanisms of this could be the lack of drug target. While **acquired resistance** occurs when a particular species, initially susceptible to a certain antimicrobial, obtains the ability to resist the mode of action(s) to the respective antimicrobial agent (4).

This thesis only deal with acquired resistance mechanisms. Definitions and examples of such resistance mechanisms will be described in section 1.3.4.2. But first, I will address how antimicrobial resistance occurs and how it spreads in bacterial populations.

1.3.4.1 Emergence of genes conferring resistance

A mutation is a permanent alteration in the sequence of nucleotides in a DNA molecule. When there are changes in the DNA sequence it can lead to genetic variation and affect the phenotype (8). However, a DNA mutation may or may not affect the phenotype of the organism depending on if the mutation leads to alteration in the amino acid sequence (29). A mutation can be classified by the type of alteration in the DNA, the alteration it causes in the protein, or by whether the mutation is a spontaneous change in the genetic material or induced by a mutagen in the environmet (e.g. plasmids or transposons) (29). The different types of mutation are:

- **Point mutation:** a nucleotide in one chromosomal position is substituted of another nucleotide. This usually takes place during DNA replication. There are different types of point mutations;
  - i) Transition: substitution of a nitrogenous base, such as a purine base (A or G) with another purine, or a substitution of a pyrimidine (T or C) with another pyrimidine (29).
  - ii) Transversion: substitution of a purine with a pyrimidine or vice versa. This is the most common type of mutation, which may or may not alter the functional properties and stability of the protein (4).
  - iii) The formation of a STOP codon in the nucleotide sequence: causing premature termination of a certain protein, which almost always inactivates the protein (8).
- **Deletion or addition of a nucleotide:** occurs during DNA replication. This may be induced by mobile elements such as transposons (29).

When mutations in the nucleotides do not lead to alteration in the amino acid sequence (the protein), they are called **synonymous mutations** (30). This is because the mutated codon still encodes for the same amino acid as the original codon. Whereas mutations that may change the protein sequence are called **non-synonymous mutations** (30).

Mutations in the DNA may also arise through **inversion** (flipping a region so that it lies in reverse orientation), **deletion** (a number of base pairs have been removed) or **insertion** (incorporation of another DNA sequence) (8). An insertion or a deletion of nucleotides other than in groups of three leads to a **frame shift** mutation. The reading frame is changed and this

leads to a change in the protein amino acid sequence, which can lead to alteration in the protein function, reduction of activity or inactivation the protein.

1.3.4.2 Vertical and horizontal gene transfer

AMR mechanisms can be transferred vertically. This is transmission of genes conferring resistance from the bacteria to its daughter cells during DNA replication and cell division (13).

Development of AMR can occur through mutations (as mentioned above) and also through acquisition of new AMR genes by HGT. HGT occurs when the genetic material is contained in mobile genetic elements, such as plasmids, transposons and bacteriophages, and transferred between the same or different species (13). HGT is primarily mediated by conjugation, transduction or transformation (4, 8);

1. **Conjugation:** occurs when the donor cell transfers DNA to the recipient cell by direct contact through a pilus. The genetic material is exchanged between bacterial cells, whereas this process involves transfer of mobile genetic elements such as plasmids or transposons (4, 8).
2. **Transduction:** gene transfer through transduction involves bacteriophage, a virus that infects and replicates within a bacterium. When the bacteriophage infects the recipient cell, it will at the same time donate its own DNA into it. Thus if the DNA of the bacteriophage contains resistant genes, which has been incorporated with its own DNA when being replicated inside a resistant bacterium, the resistant gene will be transferred to other cells (4, 8).
3. **Transformation:** occurs when a bacterium lyses, leading to release of free extracellular DNA, often in the form of plasmid. This may be taken up by the recipient bacterial cell, which will incorporate it into its genome. Hence lysis of a resistant cell will cause release of resistant genes that may be taken up by other recipient cells (4, 8).

Mutations are clearly important with respect to resistance development. However, the main problem in spread of AMR is the mobile genetic elements that harbor resistance determinants (4). This occurs often for the antimicrobial agents of last resort, e.g. vancomycin and carbapenems (31, 32). Another example is colistin, an important antimicrobial agent where

only rare chromosomal mutation resistance was described. Nonetheless, a recent study reports the emergence of colistin resistance on a plasmid (33).

1.3.5 Fitness cost of AMR

AMR is caused by the development of different antimicrobial resistance mechanisms (section 1.3.4). Newly acquired resistance is (often) costly and reversal of resistance in drug-free environments occurs mainly as a function of these costs. As an example, several studies have shown that antimicrobial resistant isolates may have slower growth rates than susceptible isolates (34, 35), hence AMR can lead to harmful adverse effects for the bacteria. This is referred to as the "cost" of resistance (36).

In principle, antimicrobial sensitivity may be renewed by temporarily exclusion the antimicrobials for which resistance has emerged, thus allowing competitive replacement of resistant bacteria with sensitivity ones that have higher fitness (36). However, this disadvantage can be ameliorated by compensatory evolution through for example, mutations that increase or restore the fitness of the resistant isolates (37). This allows resistance to persist even without the presence of antimicrobials (32).

Conventional antimicrobial cycling is based on the assumption that resistance is accompanied by a biological fitness cost. In the absence of selective pressure imposed by drug treatment, the resistance frequency of a population with high fitness cost is expected to be outcompeted by their non-resistant parental WTs and disappear from the bacterial population (32). However, due to the uncanny ability to bacteria to adapt, renewing sensitivity is difficult because of natural selection and the evolutionary adaption of the resistant bacteria (32). Furthermore, the reversal is expected to be slow even if a fitness cost is present (32).

1.4 Collateral sensitivity

As mentioned earlier, a possible favorable strategy to combat AMR is to constrain the mutational paths towards resistance taking advantage of the phenomenon of collateral sensitivity. Collateral sensitivity is a phenomenon which arises when bacteria acquiring

resistance to one antimicrobial agent become more susceptible to others (3, 38). Szybalski and Bryson used this term for the first time in 1952 when they did research on cross-resistance (3). However, it never got much attention until recently. Although there have been several studies on this phenomenon, the underlying mechanisms of collateral sensitivity still remain unclear.

In 2013, Imamovic and Sommer suggested that collateral sensitivity cycling could contribute to the sustainable use of antimicrobial agents in the clinic for controlling resistant bacteria (38). They approached this hypothesis by evolving parallel lineages of *E. coli* resistant to 23 antimicrobial agents. These resistant isolates were derived from a laboratory strain (MG1655) and two clinical isolates of *E. coli*. Collateral susceptibility profiles for the different isolates were determined by using microtiter plates and 2-fold dilutions of the tested antimicrobial.

The theory behind Imamovic and Sommer's suggestions is displayed in Figure 3, and can briefly be explained as follows: a pathogenic wild type (WT) cell population (black circles) being treated with drug A (blue arrow) at time t_0. Over time emergence of resistance for drug A will arise (blue circles) and drug A becomes ineffective (t_1). By switching to drug B (red arrow), which drug A-resistant isolates have become collaterally sensitive to (t_2), leading to susceptibility of drug A-resistant isolates and selection for cells with WT resistance levels. Eventually, resistance to drug B (red circles) will arise (t_3) and treatment will get switched back to drug A to, which drug B-resistant isolates have become collateral sensitive, resulting in susceptibility of drug B-resistant isolates (t_0). Therefore through rational cycling between drugs A and B, they are counter selecting for resistance thus the emergence of antimicrobial resistant populations can be prevented.

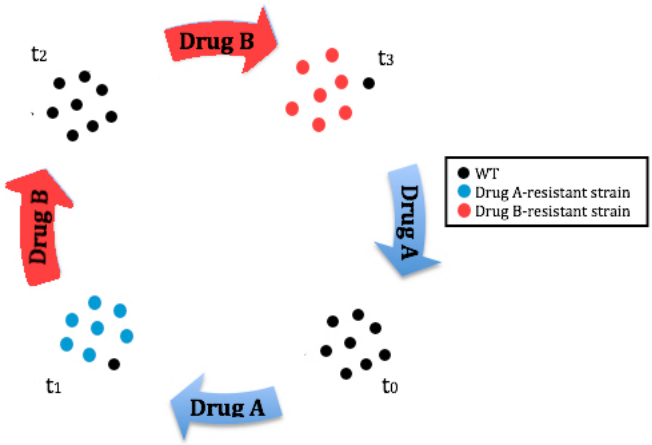


Figure 3: Collateral sensitivity cycling. A general model demonstrating the principle of collateral sensitivity cycling, showing the eradication of resistant strain when antimicrobials with reciprocal collateral sensitivity profiles (Drug A and B) are rotated. Adapted and modified from: (38).

Imamovic et al. also conducted an experiment to provide proof of a principle for collateral sensitivity cycling. According to their heat map, showing antimicrobial susceptibility profiles of drug-resistant strains relative to the WT, gentamicin (GEN) exhibited collateral sensitivity for cefuroxime (CFX). Hence a population of *E. coli* WT was evolved to become resistant toward gentamicin. Once that was achieved, gentamicin-resistant cells were mixed with WT cells, and the mixed population was exposed to cefuroxime. This treatment led to complete killing of the gentamicin-resistant cells. The same was done with the remaining WT cells, but this time they evolved resistance to cefuroxime. Again, cefuroxime-resistant cells were mixed with WT cells and exposed to gentamicin. This resulted in complete killing of cefuroxime-resistant cells and survival of WT cells. Here they demonstrated how collateral cycling could be applied to select against resistance (38).

In recent years, several studies have aimed to extend the knowledge of this phenomenon (27, 39-44). As mentioned earlier, the underlying mechanisms of collateral sensitivity still remain unclear. Many studies offer an insight into this; one among them involved reduction in the activity of efflux pumps (39, 43). This is the best-described mechanism, which was first explained by Lázár et al. (39). In this work they also performed evolutionary experiments to study networks of collateral sensitivity interactions. Three main patterns emerged from their susceptibility map; first, collateral sensitivity interactions occurred frequently. Second, the mode of antimicrobial action has a strong influence on the distribution of interactions. And third, the majority of the collateral sensitivity interactions involved aminoglycosides.

Lázár et al. suggested that resistance to aminoglycosides, caused the reduction in activity of efflux pumps, which altered the susceptibilities to multiple different antimicrobials such as ampicillin, fosfomycin and nitrofurantoin (39). The theory was that uptake of aminoglycosides is affected by changes in the cell membrane potential, and resistance to this antimicrobial group was caused by a reduction in the membrane potential. Simultaneously, efflux pumps that use the proton motive force had reduced function because of the reduced membrane potential. This leads to increase intracellular accumulation of several other antimicrobials whose efflux was dependent on these pumps. This theory was supported through whole-genome sequencing (WGS). It revealed that following adaption to aminoglycosides, mutations that most likely diminish the function of efflux pumps are frequently found (39).

This so-called trade-off, where the organism is losing one quality or aspect of something in return for gaining another quality or aspect, is an important factor of collateral sensitivity (27). In the Lázár-study, bacterial trade-off gives rise to alteration of the cell membrane permeability, that may not only cause decreased intracellular accumulation of one antimicrobial, but also increased intracellular accumulation of others (27, 39).

Earlier studies on collateral susceptibility frequently used *Escherichia coli* (*E. coli*) as the prokaryotic model organism (38, 39, 43). It is an advantageous model organism due to its rapid growth, the vast knowledge humans have about this bacterium and the diversity of molecular tools available. Additionally, it is a very relevant pathogenic bacterium, which will be reviewed below. For these reasons, *E. coli* was chosen as the model of organism for the current study.

1.5 Escherichia coli

E. coli is a rod-shaped, Gram-negative bacterium, which is motile and can be with or without a capsule (4). *E. coli* is also non-fastidous and bile-tolerant facultative anaerobe (4).

This bacterium causes urinary tract infections (UTIs), diarrheal diseases, neonatal meningitis and bloodstream infections (septicemia) (4). However, harmless types of *E. coli* normally inhabit the gut of humans and animals, and may also colonize the lower end of the urethra and vagina (4). It is when *E. coli* spreads to other locations outside the intestinal tract, by contact and ingestion (faecal-oral route), that it primarily becomes a pathogen (4, 45). This may be food-associated or endogenous. The bacterium possesses different antigens, which can be used for diagnostics to characterize strains by serotyping (4).

1.5.1 Pathogenic categorization

E. coli is a diverse group of bacteria and some *E. coli* strains are more pathogenic than others (4). This is due to the carriage of different virulence factors, and makes it suitable to categorize pathogenic *E. coli* into different pathotypes:

- **Enteropathogenic *E. coli* (EPEC):** causes sporadic cases and outbreaks of infection in babies and young children. The virulence factors they possess are bundle-forming pili, intimin and an associated protein (4).
- **Enterotoxigenic *E. coli* (ETEC):** the most important bacterial cause of diarrhea in children. The virulence factors they possess are colonization factors and production of enterotoxins (4).
- **Entereohaemorrhagic *E. coli* (EHEC):** sporadic cases and outbreaks worldwide, which food and unpasteurized milk are important for cause of infection spread. Their production of verotoxins affects tissue cultures resulting in diarrhea (4).
- **Enteroinvasive *E. coli* (EIEC):** the most common bacteria for diarrhea in areas of poor hygiene. By using plasmid-mediated genes they invade the cells by endocytosis (4).
- **Enteroaggregative *E. coli* (EAEC):** causes diarrhea in children in areas where resources are poor. They also have characteristic attachment to tissue culture cells. Their virulence factors are plasmid-mediated fimbrae and heat-labile toxins (4).
- **Diffuse-aggregative *E. coli* (DAEC**): causing diarrhea in children as well but is somewhat controversial. They produce an alpha haemolysin and cytotoxic necrotizing factor 1 (4).

Among the diseases mentioned above, UTIs are one of the most common infections in the world, which this project will be focusing on (10, 46).

1.5.2 *E. coli* – clinical relevance

The current spread of Gram-negative bacteria is a therapeutic challenge. In this project the focus will be on *E. coli*, the most frequently isolated etiological agent from a range of infections such as UTIs and septicemia worldwide (4). Beside UTIs being among the most common infections seen in the community (10), they are also associated with prominent morbidity (4). The annual number of uncomplicated UTIs cases is 130-175 millions worldwide, which usually are caused by *E. coli* (46). Septicemia, originating in the urinary tract, is a common and serious complication of UTIs. To reduce mortality of this illness, appropriate treatment is critical. However the increasing prevalence of AMR *E. coli* limits clinical options and delays suitable therapy (46). Furthermore, *E. coli* isolates producing extended-spectrum β-lactamases (ESBLs), which degrade a wide range of antimicrobials

(specifically β-lactams) through hydrolysis, are becoming more prevalent in the community (10, 47). Carbapenems have been regarded as the drugs of choice to combat multi-resistant ESBL-producers (4). However, the emergence of resistant bacterial species to carbapenems have been reported and is highly associated with numerous healthcare-related risk factors and with high mortality (48). Thus efficient infection-control practices for containment of outbreaks and novel treatment strategies are necessary against the emergence of antimicrobial resistance in *E. coli*.

1.6 Urinary tract infections

The urinary tract includes the kidneys, the ureters (the tubes that carry urine from the kidneys to the bladder), the bladder (which stores urine) and the urethra (the tube that carries the urine from the bladder to the outside) (49).

UTIs occur when bacteria are introduced into the urethra and move up into the bladder. *E. coli* is the most common organism causing UTIs (4). If the infection remains only in the bladder, the infection is called “cystitis”, however if infection travels up past the bladder and into the kidneys, it is called “pyelonephritis” (4).

Cystitis is the most common bacterial infection and causes symptoms such as burning during urination and the need to urinate frequently (7). Pyelonephritis is less common than cystitis and they have similar symptoms (7). However, pyelonephritis may also cause fever, back pain and nausea or vomiting (7). Both infections are more common in women than men due to anatomical reasons. Women usually have uncomplicated cystitis and are easily treated with a short course of antimicrobial treatment (50). In men, cystitis may also affect the prostate gland and therefore is more complicated so treatment for a longer period is necessary (51).

UTIs may be community- or hospital-acquired (4). There are a variety of mechanical factors that can predispose someone to develop a UTI, such as disruption of normal urine flow, incomplete emptying of the bladder or factors that facilitate access of organisms to the bladder (4).

1.6.1 Treatment of UTIs

According to the Norwegian guidelines from the directorate of Health ("Helsedirektoratet"), the antimicrobials recommended for treatment of acute uncomplicated and complicated cystitis are trimethoprim, nitrofurantoin and pivmecillinam. The duration of treatment depends on whether it is an uncomplicated or complicated infection, 1-3 or 5-7 days, respectively (52).

Treatment guidelines for other part of the world vary in their recommendations compared to Norway. For instance, in the United Kingdom (UK), for children of 3 months or older with cystitis or lower UTIs, the drugs of choice for treatment are trimethoprim, nitrofurantoin, cephalosporins, or amoxicillin (53). First-line agents for symptomatic lower UTIs for non-pregnant women are trimethoprim or nitrofurantoin in Scotland (54). And according to the Infectious Diseases Society of America (IDSA), trimethoprim-sulfamethoxazole is one of the traditional first-line drugs in the US (55). With a focus on the situation in Norway and based on previous results from the Microbial Pharmacology and Population Biology Research group (MicroPop) at UiT – The Arctic University of Norway (Tromsø), which will be described below, we have given mecillinam special attention in this study.

1.7 Mecillinam

Mecillinam is a β-lactam antimicrobial, which was discovered in the 1970s with bactericidal effect. Mecillinam, unique among β-lactams, binds selectively to PBP 2 in the Gram-negative cell wall, especially *E. coli* (10). *E. coli* replicate through binary fission. This is a process where cells increase in length and split in two by constricting at the middle of the cell leading to synthesis of new cell poles (Figure 4) (56). Directed by a so-called Z-ring, which functions as a scaffold for other proteins to attach (such as FtzQ, -A and -Z), the formation of a septum is formed and divides the cell into two daughter cells.

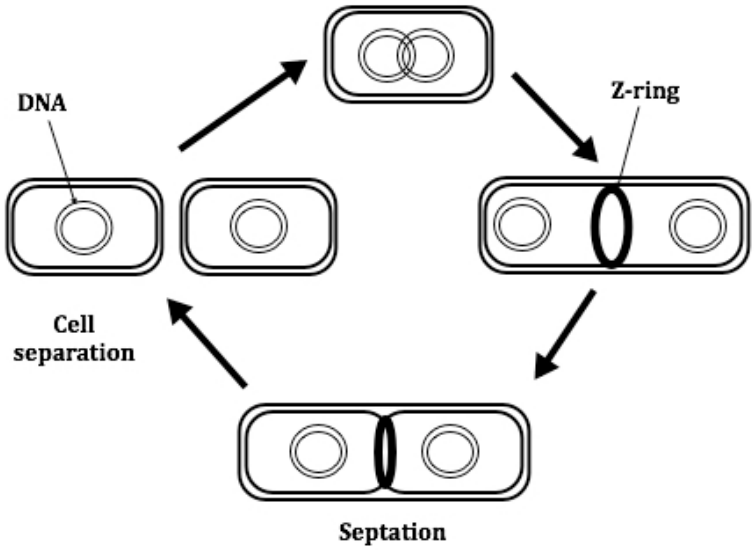


Figure 4: A model of cell division including various cell division proteins.

Mecillinam affects the bacterial cell wall by binding to PBP 2 and inhibits the transpeptidase activity of the enzyme. PBP 2 is responsible for the elongation of rod-shaped cells. Thus cells treated with mecillinam become enlarged, non-dividing spheres that leads to lysis (12).

Pivmecillinam, which is the prodrug of mecillinam, is a synthetic penicillin for oral use (10) (Figure 5). According to the Norwegian guidelines, pivmecillinam dosage is usually 200 mg 3 times daily for treatment of uncomplicated UTIs (52). In contrast of mecillinam, pivmecillinam absorbs readily from the gastrointestinal tract and undergoes enzymatic hydrolysis by esterases that liberate the active mecillinam (10).

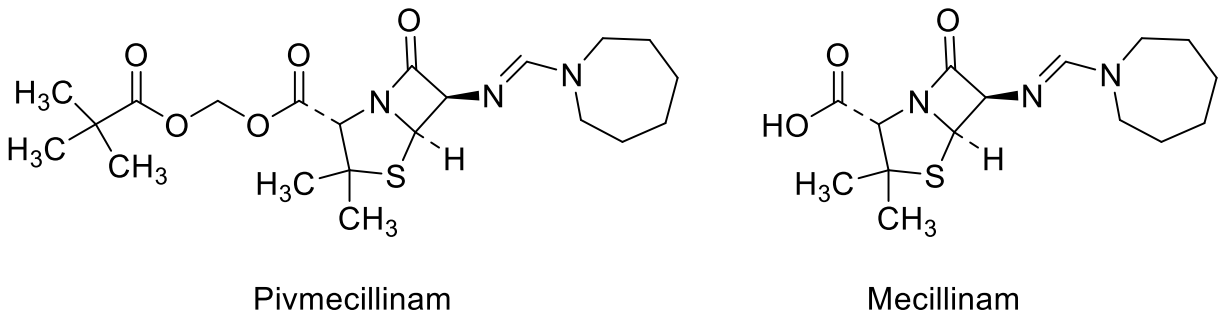


Figure 5: Chemical structures of pivmecillinam (prodrug in ester form) and mecillinam (active form).

Mecillinam has high clinical efficacy for the treatment of UTIs, and is also well tolerated with a low side-effect profile (10, 57). However, MDR bacteria that are resistant to mecillinam have been observed and are threatening the treatment of UTIs (58, 59). According to the NORM/NORM-VET report, the prevalence of mecillinam resistance has slowly increased in UTIs, from 4,2% in 2012, to 5,0% in 2013 and to 6,0% in 2014 (58). Another study that aimed to provide an update of antimicrobial resistance in *E. coli* causing uncomplicated UTIs, also showed similar results (59). This update for 2014 in Spain presented a prominent increase in resistance, including to mecillinam (1% to 6,5%) (59).

In the current project, mecillinam was the drug of choice for our focus in making resistant mutants. This choice is based on the previous results from MicroPop. In their work, they evolved clinical *E. coli* isolates (isolates from the same ECO-SENS collection as in our study) resistant to four different antimicrobial agents, ciprofloxacin, mecillinam, nitrofurantoin and trimethoprim. For each isolate they performed MIC testing to determine the susceptibility profiles for 16 antimicrobial agents. They generated an overview of distribution of collateral sensitivity and cross-resistance as displayed in Figure 6. Based on the results from MicroPop, mecillinam gives a strong collateral sensitivity profile compared to the other three antimicrobial agents.

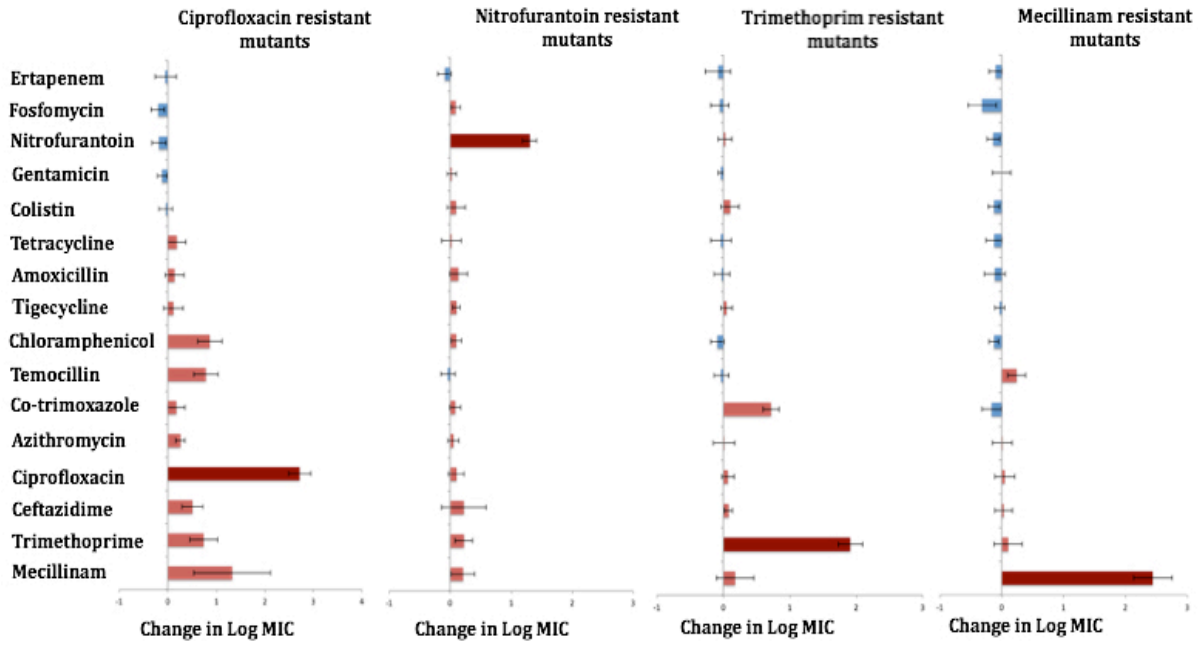


Figure 6: AMR to different antimicrobial agents in various collateral sensitive/cross-resistance phenotypes. The average log MIC changes for each AMR group to 16 antimicrobial are compared. Blue coloring indicates collateral sensitivity, and red coloring indicates cross-resistance. Permission obtained from: MicroPop research group at UiT – The Arctic University of Norway in Tromsø.

1.7.1 Use of and prevalence of resistance to mecillinam in Norway

Mecillinam is one of the first line drugs of choice for uncomplicated UTIs treatment in Norway (52). According to the NORM/NORM-VET report from 2014, pencillins with extended spectrum constitute 41% of penicillins used in Norway (58). This is an increase in use compared to 30% in 2003, which is due to increased use of amoxicillin and pivmecillinam (58). Pivmecillinam is being used more for UTIs, replacing the role of sulfonamides and trimethoprim. Also, the same report shows sales of single antimicrobials, where pivmecillinam is one of the antimicrobials that are most frequently used for outpatients. Together with phenoxymethylpenicillin and doxycycline, these three represent 47% of all prescriptions for outpatients given from primary care when methenamine is excluded.

As mentioned above, the prevalence of resistance has slowly increased for mceillinam (58). However, the report also shows that the susceptibility test results are difficult to reproduce for this antimicrobial and therefore, the observed differences may not reflect real changes in prevalence. Even though resistance rates among urinary tract isolates have remained relatively stable over the last decade, it is trending upwards for most antimicrobials and mecillinam is unfortunately one of them (Figure 7).

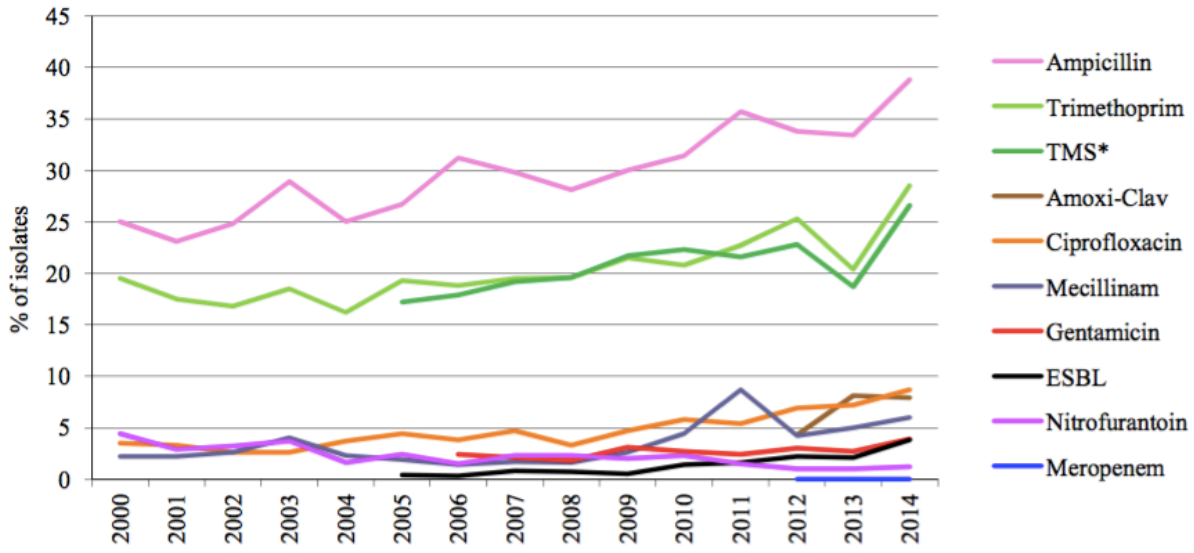


Figure 7: Prevalence of resistance to different antimicrobials in *E. coli* urinary tract isolates from 2000-2014.
Permission obtained from: (58).

1.7.2 Use of and resistance prevalence of mecillinam in the world

Although mecillinam is the most used agent for UTIs in Northern countries (60), the drug is not available everywhere (e.g. it is not used in Canada and the US) (57), or not a drug of choice in the recommendations of UTIs treatment (54, 55). According to IDSA, trimethoprim-sulfamethoxazole is the major first-line agent in the US (55). Studies have shown high susceptibility rates to mecillinam in Europe and in some countries in the Americas (61, 62). For instance according to a surveillance study from 2008 the susceptibility rate of *E. coli* to mecillinam was above 90% for all countries that were included (9 European countries and Brazil) (61). This was the second highest susceptibility rate with 95,8% for all countries in total, right after fosfomycin with 98,1%. Similar results were reported by Kahlmeter and his research group, which the mean level of resistance was less than 2% for all 5 countries (Austria, Greece, Portugal, Sweden and the UK) (62). These countries were selected to represent different geographical areas in Europe.

The vast use of mecillinam in northern Europe raises the question if there are any reasons for the low prevalence of resistance to mecillinam? This will be addressed on the next section on mecillinam resistance mechanisms.

1.7.3 AMR mechanisms to mecillinam in *E. coli*

There are different AMR mechanisms to mecillinam. Resistance may occur due to four major mechanisms:

i) antimicrobial inactivation/modification (63, 64)
ii) alteration in the cell wall composition (e.g. liposaccharide, PBPs) (65-69)
iii) reduced expression of cell wall porins or
iv) over-expression of efflux pumps.

Antimicrobial inactivation/modification may be caused by the production of β-lactamases (70). β-lactamases are a heterogeneous group of enzymes classified according to what subclasses of β-lactams they are degrading through hydrolyzing. As mentioned earlier, ESBLs degrade a wide range of different β-lactams (47, 63, 64). Other groups of β-lactamases that also degrade mecillinam are for instance carbapenemases (31, 71) and metallo-β-lactamases (MBLs) (72, 73).

Alteration in the cell wall composition is another resistance mechanism of mecillinam. An example of this is modification of PBPs, which may be caused by mutations of different resistance-encoding genes that confer resistance. PBPs have a distinct role in cell shape, division and elongation. Even in the absence of PBP 2 activity, high levels of cell division proteins (e.g. FtzQ, -A and -Z, and MrdA and -B) (65, 68, 74) and positive effector for septation (e.g. ppGpp, a signal for the stringent response) (69) are observed, and this effect is suggested to be a compensation mechanism, where cell division is restored (75).

Reduced expression of cell wall porins is the third resistant mechanism of mecillinam. Gram-negative bacteria are more impermeable than Gram-positive due to its double layer of cell membranes. The outer cell membrane of Gram-negative bacteria forms a permeable barrier, thus hydrophilic antimicrobials may cross the outer membrane by diffusing through outer membrane porin proteins. There are three major porin types in *E. coli*: OmpC, OmpF and PhoE (76). The first two are the most important for uptake of β-lactams (77). Thus either the down-regulation of porins or the replacement of porins with more selective channels will result in decreased influx of antimicrobial agents (78).

Over-expression of efflux pumps is the last one of the resistance mechanisms. Diffusion of antimicrobials through the outer cell membrane to reach its target in the periplasm may be stalled by efficient removal by efflux pumps. There are different types of efflux pumps, such as the resistance-nodulation-division (RND) efflux pumps, which include the AcrAB-TolC and AcrAD-TolC. Mutations in one or more efflux expression regulators may cause an increase in their expression and confer resistance (79).

As mentioned, the prevalence of resistance to mecillinam has remained low in the world in general. This is contradictory comparing to in a laboratory settings where the frequency of mutations is high (12). To explore this matter, Thulin et al. displayed an overview of different resistance-encoding genes that confer mecillinam resistance *in vitro* (Table 1) (12).

Table 1: The known mecilliam resistance-encoding genes and their functions. Adapted and modified from: (12).

Genes(s) (alias[es])	Function	References
mrdA (pbpA)	Cell division and elongation	(68, 80)
mrdB (rodA)		(68, 81)
mreB (envB)		(67)
mreC		(67)
ftsQ		(65, 74)
ftsA		(65, 74)
ftsZ		(65, 74)
rpoB	RNA synthesis	(82)
cysB	Cysteine biosynthesis	(83, 84)
cysE		(83, 84)
argS (lov)	tRNA synthetases	(69)
alaS		(69)
slt	Transglycosylation	(84)
lon	Rcs regulatory system	(85)
rcsB		(66, 85)
rcsC		(66, 85)
yrfF (mucM, igaA)		(85)
cyaA	Global regulation	(86, 87)
crp		(86, 87)
spoT	ppGpp degration and synthesis	(12)
rfa, rfb, rfc	Lippolysaccharide	(12)
galE		(12)
aroK	Shikimate kinase	(11)

Their work revealed that the frequency of mutations leading to mecillinam resistance is very high in *in vitro* selection-experiments compared to clinical settings, where resistance development seems to be rather uncommon. They suggested that mutations, which confer resistance in laboratory selections, have higher fitness costs. Hence their growth rates are reduced below the threshold level needed for stable maintenance in the bladder during treatment.

In this project, the following genes were chosen for close scrutiny; *mrdA*, *thrS*, *aspS* and *gtlX*. These genes encode PBP 2, threonyl-tRNA synthetase, aspartate tRNA ligase and glutamyl-tRNA-synthetase respectively (12). The aim is to detect mutations that may confer mecillinam

resistance, hence this work may reveal the resistance mechanism in the mecillinam resistant isolates. The selection of these genes was based on the work by Thulin et al. in which the respective genes showed a high frequency of mutations observed in laboratory settings (12) and on the basis of the gene encoding for the drug target of mecillinam.

1.8 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is central in the current project. Therefore the following is an introduction to different methods, which may be used for determination of antimicrobial susceptibility *in vitro*, with a focus on the methods used in this project.

Microorganisms can be tested for their susceptibility to a certain antimicrobial using various laboratory techniques. Commonly, these are used to determine the minimum inhibitory concentration (MIC) (88). The MIC is defined as the lowest antimicrobial concentration that prevents growth of the microorganism. The MIC is an important result in diagnostic laboratories to confirm antimicrobial resistance, but can also be used to determine the potency of novel antimicrobials (88). There are different methods to perform AST:

Disk diffusion: One of the oldest methods and remains as one of the most widely used AST in routine clinical laboratories (89). The method is based on disks containing antimicrobials, which are applied on MHA plates with bacterial inoculum and are incubated overnight. If an antimicrobial is effective against the bacteria, either bactericidal or bacteriostatic, there will be an area around the disk where bacteria growth is not visible. The size of the clear area (zone of inhibition) is dependent on how effective the antimicrobial is against that bacteria, thus an effective antimicrobial will create a larger zone.

E-test: This method confirms the susceptibility of organisms to a certain antimicrobial agent by measuring the MIC (88). The strips used in the MIC-test have predefined and continuous concentration gradient of different antimicrobial agents. When applying these strips to inoculated agar plated and incubated, an eclipse of inhibition that intersects the strips will occur. The MIC value (μg/ml) can be read using the scale on the strip.

Micro broth dilution: This method is performed by using multiple microtiter plates filled with MH broth (90). Then a serial dilution of different concentrations of the antimicrobial is

made in the plate and followed by adding the bacterial inoculum of interest. The optical density is measured the day after the incubation to observe the visible growth of the bacteria.

For the current project, both MIC-test and micro broth dilution have been used. The MIC-test was to confirm whether the isolated phenotypes were resistant or susceptible to mecillinam. Micro broth dilution with the IC_{90}-assay was used to describe possible collateral sensitivity and collateral resistance networks in mecillinam resistant isolates.

1.8.1 EUCAST

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is a committee that published breakpoints and technical aspects of phenotypic *in vitro* AST in Europe (88). These clinical breakpoints are used in clinical laboratories, providing guidance to clinicians with respect to the potential use of agents for treatment in patients. The organization publishes MIC breakpoint interpretations based on several factors, including pharmacokinetic and pharmacodynamics data, clinical studies, resistance mechanisms, commonly used dosing regimens and the WT MIC distributions for relevant species of organisms (91).

In the latter, the highest MIC within the WT MIC distribution is used as the epidemiological cut-off value (ECOFF) (92). The ECOFF is used as a tool to distinguished isolates without (WT) and with acquired resistance mechanisms (non-WT) to a given antimicrobial agent. Isolates with a MIC above the ECOFF (hence likely to possess resistance mechanisms) are often clinically resistant. Together, ECOFFs and clinical breakpoints are used to determine the rate of resistance development.

The clinical breakpoints are categorized into three groups:

- *Susceptible:* The organism should respond to the therapy using recommended antimicrobial dosage for the given site of infection and species.
- *Intermediate:* The organism's MIC approaches or exceeds the threshold for normal antimicrobial dosing, but clinical response is possible with higher doses or if the antimicrobial concentrates at the site of infection.
- *Resistant:* The organism is not inhibited by the concentrations achieved with normal dosing.

Depending on which type of microorganisms and antimicrobial agents are being tested, the MIC-value will determine the sensitivity profile of the microorganism according to the EUCAST clinical breakpoints (93).

1.9 ECO-SENS projects

The first international survey to investigate the prevalence and susceptibility of pathogens causing community-acquired uncomplicated UTIs is the ECO-SENS Project (94). There have been two ECO-SENS studies (62, 94). The first one took place in 1999-2000 and included 4734 women, not older than 65 years, from 16 European countries and Canada (94). Patients with symptoms of an acute UTI provided a midstream urine sample, which was tested for the presence of bacteria and the susceptibility of the isolated bacteria to 12 antimicrobials was determined. The conclusion of the surveillance study indicates that antimicrobial resistance was lowest in the Nordic countries and Austria and highest in Portugal.

The second ECO-SENS study was in 2008-2009 (62). This time, the surveillance study only included five countries and 1697 women gave urine samples for bacterial isolation and antimicrobial susceptibility testing to 14 antimicrobials. The countries were selected to represent areas of Europe, which were indicated to have more (Greece and Portugal) or less (the UK, Austria and Sweden) problems with resistance. In this project, 11 resistance isolates with ESBLs were identified, compared to the previous ECO-SENS study where there were none.

In the first ECO-SENS study in 2000, AMR of *E. coli* to mecillinam was less than 3%, and similarly low for ciprofloxacin, gentamicin and nitrofurantoin. However, ciprofloxacin resistance was apparent in Portugal (5,8%). The second surveillance study in 2010 also showed low AMR, including mecillinam, gentamicin and nitrofurantoin in *E. coli*, less than 2%. While ciprofloxacin and trimethoprim resistance had increased between the first and the second surveillance study; 1,1% to 3,9% for ciprofloxacin and 13,3% to 16,7% for trimethoprim. In both studies amoxicillin, chloramphenicol and tetracycline were not included.

All of the bacterial strains used in this project belong to the ECO-SENS strain collection.

2 HYPOTHESIS AND AIMS

2.1 Hypothesis

Collateral sensitivity networks have been demonstrated for single isolates of *E. coli*. We hypothesize that such networks also exist on a population level.

2.2 Aims

In this project we aim to explore the generality of the concept of collateral sensitivity in clinical urinary tract isolates of *E. coli* from the ECO-SENS collection. With the emergence of increased multi-drug resistance (MDR), we aim to inform recommendations for novel treatment strategies to improve efficacy of treatment for UTIs and to prevent further resistance development. Furthermore, we want to investigate the underlying mechanisms of the reciprocal collateral sensitivity between antimicrobials agents.

3 MATERIALS

3.1 Bacterial strains

All of the bacterial strains used in this project belonged to the ECO-SENS strain collection described previously (62, 94). In this project 15 different ECO-SENS isolates from Greece, Portugal, Sweden and the UK were used to make mecillinam resistant mutants, as shown in Table 2. Ten of our resistant mutants were selected for further analysis. All isolates used are pan-susceptible, meaning the isolate is susceptible to all antimicrobial agents, and plasmid-free.

Table 2: ECO-SENS isolates employed in this project. Isolates in bold were used for further analysis. Sequence type was determined by multilocus sequence typing and phylogroup was determined by triplex PCR-based method (95).

Name	Sequence type	Phylogroup	Year	Country
K56-5	998	B2	2000	Greece
K56-17	73	B2	2000	Portugal
K56-18	998	B2	2000	Portugal
K56-20	127	B2	2000	Portugal
K56-23	73	B2	2000	Sweden
K56-24	73	B2	2000	Sweden
K56-25	73	B2	2000	Sweden
K56-30	1161	B2	2000	Sweden
K56-31	638	B2	2000	The UK
K56-66	372	B2	2007-2008	Sweden
K56-69	1230	A	2007-2008	Sweden
K56-71	607	A	2007-2008	The UK
K56-76	978	B2	2007-2008	The UK
K56-77	1236	B2	2007-2008	The UK
K56-80	141	B2	2007-2008	The UK

3.2 Buffers, growth media and other chemicals

Table 3: An overview of growth media and other chemicals used in this project.

Solution	Content	Preparation	Storage temperature	Producer/ manufacture
0,85% saline	Sodiumchloride (≥99,5%) Double distilled water (ddH_2O)	680 mg sodiumchloride were added to 80 mL ddH_2O.	RT	Fluka
80% glycerol	86-89 % glycerol ddH_2O	54 mL glycerol were added to a container and adjusted to 100 mL with ddH_2O.	RT	Sigma-Aldrich
LB broth	LB broth ddH_2O	5 g LB broth were added to 200 mL ddH_2O.	RT	BD Difco™, Miller
LB agar (LBA)	LB broth Select agar ddH_2O	12 g Select agar and 20 g LB broth were added to 800 mL ddH_2O.	-4°C	BD Difco™, Miller Sigma-Aldrich
MH II agar (MHA)	MH II agar ddH_2O	7,6 g MH II agar were added to 200 mL ddH_2O.	-4°C	Sigma-Aldrich
Mecillinam 1 mg/mL	Mecillinam 100 mg Ultra Purewater	100 mg mecillinam were dissolved in 100 mL Ultra Purewater.	-20°C	Sigma-Aldrich
MHA-Mec_{16}	MH II agar Mecillinam 1 mg/mL	3,2 mL mecillinam 1 mg/mL were added to 200 mL MHA.	-4°C	Sigma-Aldrich
50X TAE buffer	Tris-base Glacial acid (100%) 0,5 M EDTA (pH 8,0) ddH_2O	242 g Tris-base were added to 600 mL of ddH_2O. Then 57,1 mL glacial acetic acid and 100 mL of 0,5 M EDTA were added in the mix. The mix was brought to a finale volume to 1 L with ddH_2O.	RT	Sigma-Aldrich Invitrogen™, Gibco®
Cyano-4-hydroxy-cinnamic-acid (HCCA) Portioned	-	-	8°C	Bruker Daltonik GmbH
Triflouracetic acid (TFA)	-	-	RT	Sigma-Aldrich
70% ethanol (EtOH)	96% EtOH ddH_2O	70 mL of 96% EtOH were added to 30 mL ddH_2O.	RT	Sigma-Aldrich

3.3 Polymerase chain reactions and DNA sequencing

Some of the primers used in this project have been published previously (12) and others were designed for this study. The following genes were chosen for close scrutiny; *mrdA*, *thrS*, *aspS*

and *gtlX*. These genes encode PBP 2, threonyl-tRNA synthetase, aspartate tRNA ligase and glutamyl-tRNA-synthetase respectively.

Each gene required 2-3 primer sets for complete coverage of the gene of interest (Table 4). Primers were diluted with nuclease-free water (Thermo Fisher Scientific), first to a stock concentration of 100 μM and then to a final working stock solution of 10 μM. The same primers were used for PCR and sequencing.

Table 4: The primers used in this project.

Primer name	Sequence 5' – 3'	Storage temperature	Amplicon length	Gene	Reference(s)
mrdA-F1	GTGCTGGTCGCAGAGAGTC	-20°C	719 bp	*mrdA*	This study
mrdA-R1	TTACCGATATCATGCGTTGC	-20°C			This study
mrdA-F2	CACGTCATCGGCTATGTGTC	-20°C	767 bp		This study
mrdA-R2	ATGTTGCCGGAACGTTCTTC	-20°C			This study
mrdA-F3	CCGAATGGATGGGTAAATTC	-20°C	750 bp		This study
mrdA-R3	ATTGTGGGATCGAGATGGAC	-20°C			This study
thrS-F1	CTCCGGCTTCTTCTGCTT	-20°C	633 bp	*thrS*	(12)
thrS-R1	CAGCTGGACTTCTCTTTGCC	-20°C			This study
thrS-F2	TTGCGCGGTGAATCATTACC	-20°C	834 bp		This study
thrS-R2	CTGGAAGAAGCCGCGAAAC	-20°C			This study
thrS-F3	ACATTTTGTTGTTGCTGTCGC	-20°C	796 bp		This study
thrS-R3	TAACATCGCTCAACCGGG	-20°C			(12)
aspS-F1	AATTTCCAGTATAATAGCCGCC	-20°C	733 bp	*aspS*	(12)
aspS-R1	AAACCGGACATCATCAGCAG	-20°C			This study
aspS-F2	GACTACCTGGTGCCTTCTCG	-20°C	733 bp		This study
aspS-R2	GACCAAGGTCTTTACCCACTTTC	-20°C			This study
aspS-F3	GGTGCCGACAACAAGAAAAT	-20°C	704 bp		This study
aspS-R3	CCTCTTCGTTGACTGCCTTC	-20°C			This study
gltX-F1	GAATCAGGCGGGAGTGATAG	-20 °C	862 bp	*gltX*	This study
gltX-R1	TTCTGGCAAATAACCGTCATC	-20 °C			This study
gltX-F2	TTTACGCGCACGTTTCTATG	-20 °C	828 bp		This study
gltX-R2	CCGTCTCGATATTGACGAATC	-20 °C			This study

Table 5: Chemicals and enzymes for the PCR mastermix and sequencing reaction.

Name of component	Storage temperature	Producer
Nuclease-free water	RT	Thermo Fisher Scientific
5X Phusion HF buffer	-20°C	New England BioLabs®
10 mM dNTPs	-20°C	New England BioLabs®
Template DNA	-80°C	-
Phusion DNA Polymerase	-20°C	New England BioLabs®
Big Dye[a]	4°C	-
5X Sequencing buffer[a]	4°C	-

[a] Provided by UNN.

3.4 Agarose gel electrophoresis

Listed in Table 6 are the different reagents for agarose gel electrophoresis that were used in this project.

Table 6: Different reagents for agarose gel electrophoresis.

Name of reagent	Content	Storage temperature	Producer
1X loading buffer	Dilute 6X loading buffer 1:6 with sterilized ddH_2O.	RT	-
SmartLadder	-	2-8°C	EuroGentec
Agarose 1% gel	1 g dissolved in 100 mL 1X TAE buffer.	2-8°C	SeaKem, USA
EtBr 0,5 μg/mL	50 μL added to 100 mL agarose 1% gel.	RT	-

3.5 Antimicrobial agents for MIC and IC_{90} determination

Micro broth dilution with IC_{90}-assay was performed to determine collateral sensitivity profiles of mecillinam resistant isolates. The list over different antimicrobial agents employed and what kind of solution was used to dissolve them in are displayed in Table 7.

Table 7: List over antimicrobial agents used in IC_{90}-assay.

Antimicrobial agent	Producer	Catalog no.	Resuspend in
Amoxicillin	Sigma-Aldrich	A0800000	Phosphate buffer, pH 6.0, 0.1 mol/L
Chloramphenicol	Sigma-Aldrich	C0378	95% EtOH
Ciprofloxacin	Biochemika	17850	0,1N hydrochloride (HCl)
Gentamicin	Sigma-Aldrich	G3632-1G	MilliQ-water
Mecillinam	Sigma-Aldrich	33447-100MG	ddH20
Nitrofurantoin	Sigma-Aldrich	N7878-25g	DMSO[a]
Trimethoprim	Sigma-Aldrich	T7883-25g	DMSO[a]
Tetracycline	Sigma-Aldrich	T3383-25g	MilliQ-water

[a] **Dimethyl sulfoxide**

MIC-testing was performed to determine the antimicrobial susceptibility of different isolates in static selection. The MIC-strips of mecillinam used in this study are displayed in Table 8.

Table 8: MIC-strips of mecillinam used for MIC-testing.

Antimicrobial agent	Storage temperature	Concentration gradient (μg/mL)	Producer
Mecillinam	-20°C	0,016-256	Liofilchem, Italy

3.6 Various kits used in this project

Different kits were used for DNA isolation of the strains and isolation of DNA from agarose gels as displayed in Table 9.

Table 9: List over different kits used in this project.

Kit	Catalog No.	Storage temperature	Lot No.	Producer
QIAquick Gel Extraction Kit	28706	RT	127128966	QIAGEN
GenElute Bacterial Genomic DNA Kit	1002047701	RT	SLBN5542V	Sigma-Aldrich
BigDye® Terminator v3.1 Cycle Sequencing Kit	4337455	-20°C		Thermo Fisher Scientific

3.7 Equipment employed in this project

Following are a list over different equipment used in our project for IC_{90}-assay, DNA isolation, isolation of DNA from agarose gel and Nanodrop spectrophotometer, displayed in Table 10.

Table 10: List over various equipment employed in this project.

Name of equipment	Producer	Order/Part No.
Microplater Shaker TiMix 5 control	Edmund Bühler GmbH	6167700
VersaMax ELISA Microplate Reader	Molecular Devices	VERSAMAX
Heraeus Biofuge Pico	Kendro®	75003235
Nanodrop ND-1000 Spectrophotometer	NanoDrop	-

4 METHODS

4.1 Bacterial cultivation

Bacterial isolates used in this project were cultured on appropriate agar plates or in liquid medium. Both techniques are described below.

4.1.1 Streaking and isolating bacteria on solid medium (agar)

For the streaking on agar plates, an inoculation loop was used to i) dip into a bacterial culture, ii) scrape a colony of an agar plate or iii) scrape from the surface of a freeze culture. Then the loop was dragged across the surface of the agar horizontally back and forth until ¼ of the plate had been covered; zone 1. With the same loop it was dragged across the same section, but from another angle this time to get complete coverage of the area/zone 1. The plate was then turned 90 degrees. Starting from zone 1, a new loop was dragged zigzag across the plate until covering 1/3 of the plate (the loop only crossed zone 1 3-4 times); zone 2. This step was repeated once more but now from zone 2, only 2-3 times from that zone, and with bigger space between the zigzag motions. Finally the plate(s) was incubated over night (12-18 hours) at the appropriate temperature (usually 37°C). See Figure 8 for division of the various zones.

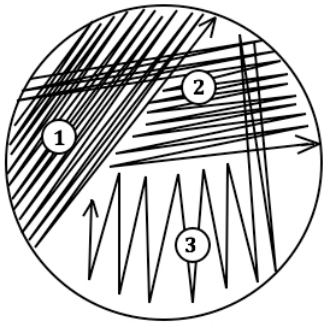


Figure 8: An agar plate showing its different streak zones.

4.1.2 Liquid cultures

Bacteria can be grown in liquid cultures. The desired bacteria were transferred and suspended in a tube containing 4 mL LB broth using an inoculation loop. The tube(s) was incubated overnight with shaking at 225 rpm.

4.2 Storage of the bacterial isolates – Freeze stock cultures

For long term, stable storage of the bacterial isolates used and the mutants generated in this project, freeze stock cultures were used.

Freeze stock cultures were prepared for WT strains and mecillinam resistant mutants for long time storage of bacteria strains. Overnight culture(s) of the respective strain(s) was incubated over night at the appropriate temperature (37°C), 225 rpm. A tube (Cryo freeze tubes VWR International, USA) was added 250 μL of the inoculate and 750 μL of 80% glycerol, resulting in a finale concentration of 20% glycerol. The tube was then stored at -80°C.

4.3 Preparation of bacterial growth media

Bacteria were cultivated in either liquid or solid media. Most of the media were prepared in the lab. MH broth used for the IC_{90} assays were bought from the University Hospital of Northern Norway (UNN) in Tromsø to ensure stable quality throughout the experiments. Antimicrobial stock solutions were prepared in the lab by the technical staff for the IC_{90} assays for common use.

4.3.1 Liquid medium

The liquid media were prepared by adding MH II or LB powder in ddH_2O as specified by the manufacturer (Table 3), and then autoclaved at 121°C with 100 kPa for 20 minutes. The liquid media were then cooled down to the appropriate temperature (55°C) before use.

4.3.2 Agar plates with and without antimicrobial drugs

MHA and LBA were prepared as specified by the manufacturer adding appropriate amounts of agar. After autoclaving of the liquid medium, the solution was cooled down to below 55°C and the appropriate volume of thawed mecillinam stock solution was added to desired finale concentration, e.g. for 16 μg/mL mecillinam 3,2 mL of a 1 mg/mL solution were added to 200

mL MHA. Afterwards the solution was poured in empty sterile petri dishes and cooled/dried overnight. The MHA-Mec plates were stored at 4°C for up to one week.

4.3.3 Antimicrobial stock solution

Mecillinam powder was dissolved in ddH_2O to a finale concentration of 1 mg/ml. Thereafter a 0,2 μM filter unit was used for sterile filtration. Small volumes were aliquoted of the stock solution into sterile eppendorf tubes (850 μL) or Nalgene cryovials (3,5 ml). Single use vials were stored at -20°C.

Other antimicrobial stock solutions prepared were dissolved and diluted in appropriate media according to Clinical and Laboratory Standards Institute (CLSI) or manufacturer's guidelines. More details are given in the materials chapter.

4.4 Static antimicrobial resistance selection and mutation frequency

To acquire spontaneous antimicrobial resistant isolates, in this case of *E. coli*, static selection under antimicrobial pressure was used for this project. Clinically resistant mutants will henceforth be mentioned as resistant mutants in this project.

4.4.1 Inoculum for selection of mecillinam resistant mutants

A streak for isolation of the bacterial isolate(s) of interest was prepared from freeze stock cultures by scraping a small (5-10 μL) sample of the frozen stock and struck on LBA plate(s). The frozen stock was restored immediately to -75°C, and the plate(s) was then incubated at appropriate temperature (37°C) overnight.

4.4.2 Making mecillinam resistant mutants with selective plates

10-20 sterile glass beads were added to an MHA-Mec_{16} plate, which was pre-warmed to RT. 100 µL of undiluted overnight culture (after 18±2 hours) were added on the MHA-Mec_{16} selective plate(s) and was shaken in horizontal plane until inoculum had been completely absorbed. The plates were incubated (24-48 hours) until visible growth was present.

4.4.3 Determination of the initial inoculum with non-selective LBA plates

A 96-well plate was used to fill 6 wells per sample with 900 µL of sterile 0,85% saline. 100 µL of the overnight culture were pipetted into the first well of the dilution series. The well was mixed by pipetting 10-20 times and then the pipet tip was discarded. With a new tip, 100 µL was moved from well 1 to well 2. Again the well was mixed by pipetting 10-20 times and the pipet tip was discarded. These steps were repeated until the entire series had been completed to a finale dilution factor of 10^{-6}. LBA plates were pre-warmed to RT (2 plates for each strain) and 10-20 sterile glass beads were added. 100 µL dilutions, 10^{-5} and 10^{-6}, were added onto each plate and shaken horizontally until the inoculum had been completely absorbed. These dilutions were chosen to give countable amounts of colonies on the plates. Then the glass beads were removed. The plates were incubated at the appropriate temperature (37°C) until visible growth was present (for 24-48 hours) (Figure 9).

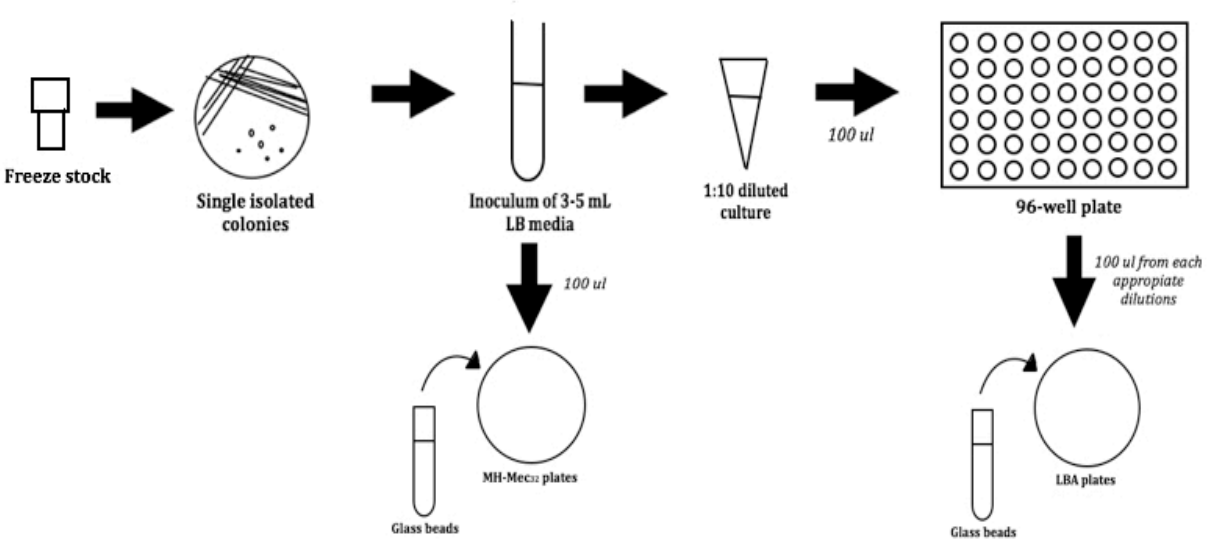


Figure 9: A schematic figure of different steps in the static resistance selection protocol.

4.4.4 Determination of estimated mutation frequency

After day 1, colony-forming units (CFUs) was counted for each dilution and each sample on LBA plates. The plates were thereafter incubated additionally until visible growth was present on the selective plates, and colonies were counted once more. CFUs of mecillinam resistant mutants were counted on day 1 or 2, and the mutation frequency was calculated. The inoculum was calculated from CFU counts from plates incubated for the same amount of time as the selective plates. The following formula was used for the calculation of mutation frequency:

$$\text{Mutation frequency} = \frac{\#\text{mutants on MHA-Mec16 plate}}{\text{Total \# of bacteria plated on selective plate}}$$

4.4.5 Purification and storage of mecillinam resistant isolates

Single isolated colonies were picked from MHA-Mec_{16} plates (either on day 1 or day 2) and struck for isolation on new MHA-Mec_{16} plates (one plate per single isolated colony, and at least 3 colonies were purified). When possible, different phenotypes where chosen and observations of the colony morphologies were noted. After incubation of the plates at appropriate temperature (37°C for 24-48 hours depending on growth), a well-isolated single colony from each streak for isolation plate was picked with a sterile loop and inoculated into 4 mL LB broth. If the colonies were not uniform/homogenous, streaks for isolation were repeated. 3 isolates per parental WT isolate were picked from the plates, inoculated in LB medium and incubated overnight at 37°C with shaking at 225 rpm. Incubation continued until visible growth was present, typically 18-24 hours. This culture was used to prepare freeze stocks.

4.4.6 Confirmation of species

Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) is a method combining mass spectrometry and an identification system based on molecular weight (96). The method is based on the samples being mixed with a matrix. This was accomplished by using a wooden toothpick to pick a bacterial colony from an agar plate and spread it on the

marked positions on the target plate. 1 μL matrix was added in every position when all the samples were applied (recommended within 10 min). Thereafter the target plate was allowed to air dry completely before analyzing. The matrix would absorb the energy from ionizing lasers and lead to ionization and the release of matrix/sample-crystals. An electrical field would migrate the ionized proteins through the "flight-tube". Different mass would get to the detector with different times; hence the time was proportional with the mass. This specificity was utilized to compare the time, which functioned as a "fingerprint", with a database and the identification system would give the sample an ID of a species. A score indicated the probability for the right identification. Scores over 2,0 indicates reliable identification on a species level (Appendix B).

4.5 Antimicrobial susceptibility testing

Microorganisms can be tested for their susceptibility to a certain antimicrobial with measured MIC (88). For the current project both MIC-strip testing and IC_{90}-assay with micro broth dilution were used for determining the antimicrobial susceptibility of the bacteria. IC_{90} is the lowest inhibitory concentration of the antimicrobial that inhibited 90% of the growth of the tested isolate (97). Breakpoints of the tested microorganisms are being compared to EUCAST, which will determine the susceptibility profile of the microorganism (88).

4.5.1 MIC-strip testing

MIC-test is utilized to check the organism´s susceptibility to an antimicrobial agent, in this context the antimicrobial agent is mecillinam. The strips used in the test assay have a predefined and continuous concentration gradient of mecillinam (0,016-256 (μg/ml) . When applying these strips to inoculated agar plated and incubated, an ellipse of inhibition intersecting the strips will occur. The MIC-value (μg/mL) can be read on the strip scale.

The bacterial colonies on the LBA plate(s) were selected using a sterile cotton swab. The cotton swab with bacteria was swabbed in sterile saline 0,85% to make a 0,5 McFarland medium. A cross was made on a new MHA plate with a new cotton pad, which was dipped in the bacterial medium. The plate was placed on a spinning machine, and then with the same

cotton swab it was moved from the edge of the plate to the middle with slow motion. The tip was turned and moved slowly backwards to the initial point (approximately 10 seconds each way). A MIC-strip of mecillinam was placed on the plate with sterile tweezers, and incubated at 37°C with ambient air (18 hours), see Figure 10. The observed MIC-value was compared to the clinical breakpoints defined by EUCAST to determine whether the respective bacterial isolate was resistant to mecillinam or not (Appendix D).

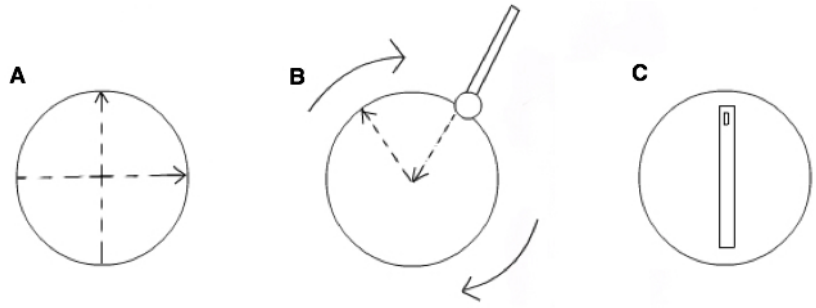


Figure 10: Swabbing the MHA plate with bacteria medium and place the MIC-strip.

4.5.2 IC_{90} determination

As displayed in Table 11, the respective eight antimicrobial agents were chosen for the IC_{90}-assays; amoxicillin, chloramphenicol, ciprofloxacin, gentamicin, mecillinam, nitrofurantoin, tetracycline and trimethoprim.

Table 11: List of antimicrobial agents employed in this project.

Antimicrobial agent	Abbreviation	Class	Target
Amoxicillin	AMX	β-lactam	Cell wall
Mecillinam	MEC	β-lactam	Cell wall
Ciprofloxacin	CIP	Quinolone	Nucleic acid synthesis, DNA gyrase
Gentamicin	GEN	Aminoglycoside	Protein synthesis, 30S
Tetracycline	TET	Tetracyline	Protein synthesis, 30S
Chloramphenicol	CHL	Miscellaneous	Protein synthesis, 50S
Nitrofurantoin	NIT	Miscellaneous	Multiple
Trimethoprim	TMP	Dihydrofolate reductase inhibitor	Metabolic pathways, folic acid biosynthesis

Initially, bacterial isolate(s) of interest, as well as the control isolates ATCC 25922 and K56-44 (mecillinam resistant mutant), from freeze stocks were streaked on LBA plates and incubated overnight. Mecillinam resistant mutants and the parental WTs were always tested together in the same assay. There were performed two replicates of each isolate, with 2-fold or 1,5-fold antimicrobial dilutions. A 96-well microtiter plate was loaded with MH broth as shown in Table 12 and Table 13, depending on which fold change that was being used.

Table 12: 96-well plate filled with MH broth for 2-fold CS/CR.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
B	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
C	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
D	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
E	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
F	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
G	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
H	100 µL		100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL

Table 13: 96-well plate filled with MH broth for 1,5-fold CS/CR.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
B	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
C	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
D	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
E	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
F	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
G	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL
H	100 µL		50 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	200 µL

A working stock of the antimicrobial to be tested at 2x the highest concentration was prepared. The specific MIC testing guidelines and/or manufacturer's guidelines were followed to ensure correct antimicrobial stock solution was made. Dilutions of the stock to the 2x highest concentration were done in MH broth. For 2-fold, 200 µL of the antimicrobial working stock was added in column 2 (Table 14), either with different antimicrobials or different antimicrobial concentrations. For 1,5 fold 150 µL of the same working stock was added into the wells of column 3 as well (Table 15). ATCC strain and K56-44 were included with each antimicrobial being tested.

Table 14: 96-well filled with antimicrobial working stock for 2-fold CS/CR.

	1	2	3	4	5	6	7	8	9	10	11	12
A		200 µL										
B		200 µL										
C		200 µL										
D		200 µL										
E		200 µL										
F		200 µL										
G		200 µL										
H		200 µL										

Table 15: 96-well filled with antimicrobial working stock for 1,5-fold CS/CR.

	1	2	3	4	5	6	7	8	9	10	11	12
A		200 µL	150 µL									
B		200 µL	150 µL									
C		200 µL	150 µL									
D		200 µL	150 µL									
E		200 µL	150 µL									
F		200 µL	150 µL									
G		200 µL	150 µL									
H		200 µL	150 µL									

For 2-fold, 100 µL was taken from column 2 and mixed with the wells of column 3. It was pipetted 10-15 times to ensure thorough mixing. The content was completely expelled of the tips into column 3 and 100 µL was taken from column 3 to column 4. The mixing and serial dilutions of the antimicrobial were continued, and 100 µL of the mixture was discarded at the end from column 11.

For 1,5-fold, 100 µL was taken from column 2 and mixed with the wells of column 4. It was pipetted 10-15 times to ensure thorough mixing. The content was completely expelled of the tips into column 4 and 100 µL was taken from column 4 to column 6. The mixing and serial dilutions of the antimicrobial were continued, and 100 µL of the mixture was discarded at the end from column 10. The same mixing and serially dilution process were repeated, but starting from column 3 column to 5 until reached column 11. Here 100 µL was discarded as well.

Preparation of a 0,5 McFarland in 0,85% sterile saline with a few isolated colonies from LBA plates was done. The 0,5 McFarland was diluted 1/1000 into MH broth by adding 5 µL into 4,995 mL MH broth. Lastly, 100 µL of the bacterial isolate inoculum was added to the dilution wells and the positive control wells (Table 16).

Table 16: A schematic illustration of added diluted experimental isolates, each row containing the same type of isolate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	
B	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	
C	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	
D	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	
E	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	
F	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	
G	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	
H	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	

The 96-well plate was incubated at 37°C with continuous shaking at 700 rpm for 18 hours. For the slow growing isolates incubation continued up to 42 hours. By using a plate reader, the A_{600nm} read (using SoftMax® Pro Software v5.4.1) was taken after 18 hours (additional after 24 and 42 hours for some strains). The measurements were used to calculate the IC_{90}, and background-subtracted. The concentration at which %inhibition was ≥90 was calculated with the following formula:

$$\%\ inhibition = 1 - \left(\frac{A600\ drug\ treated}{A600\ positive\ control}\right)\ x\ 100$$

Based on the calculations of the IC_{90}-values, the MIC-value for each isolate was determined. To categorize whether an isolate was resistant or susceptible for the respective antimicrobial agent, the MIC-values of the parental WTs and mecillinam resistant mutants were compared to the clinical breakpoints defined by EUCAST. The control strains were also compared for whether they were in range or not (Appendix D).

4.6 Preparation of genomic DNA for PCR

Isolation of bacterial DNA was prepared to obtain pure extraction of DNA from a variety of cultured bacteria. This was used for further genomic investigation with PCR.

4.6.1 DNA extraction using the GenElute Kit (Sigma)

DNA isolation with GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, NA2100/2110/2120) is a method, which gives high quality DNA with high degree of purity providing genomic bacterial DNA suitable for PCR and WGS. DNA isolation was conducted primarily according to the description of the manufacturer.

The bacterial isolate(s) of interest was inoculated in 5 mL LB media and incubated overnight at the appropriate temperature (37°C) with shaking at 225 rpm (an LB control was always included from each batch of medium). 1,5 mL of overnight culture was pelleted by centrifuging for 2 minutes at 13000 rpm in Heraeus microfuge pico. The supernatant was poured off afterwards. Resuspension of the pellets was done thoroughly in 200 µL of 100 mg/mL of lysozyme in the solution Gram positive lysis solution, which was provided in the kit (prepared fresh), and incubated for 30 minutes at 37°C. 20 µL RNase A solution was added to each sample, and they were incubated for 2 minutes at RT. Thereafter 20 µL of 20 mg/mL Protein K solution were added to the mix, followed by 200 µL of Lysis solution C. The mix was vortexed thoroughly and incubated at 55°C for 10 minutes. For the column preparation, 500 µL Column Preparation Solution was added to each pre-assembled GenElute Miniprep Binding Column and centrifuged at 13000 rpm for 1 minute. The eluate was discarded. The binding was prepared by adding 200 µL EtOH 96% to the lysate and mixed thoroughly by vortexing. Afterwards the entire content of the tube was transferred to the column and centrifuged at 13000 rpm for 1 minute. The collection tube containing the eluate was then discarded. In the washing process, 500 µL Wash Solution was added to the column and centrifuged for 1 minute at 13000 rpm. The collection tube was discarded and the column was placed in a new 2 mL-collection tube. In the second wash 500 µL Wash I Solution was added to the column and centrifuged for 3 minutes at 13000 rpm. Additional 1 minute of centrifuging was performed to remove residual EtOH. The collection tube containing the eluate was then discarded and the column was again placed in a new 2 mL collection tube. Lastly, DNA was eluated with 100 µL of 10 mM Tris-base directly onto the center of the column and incubated for 5 minutes at RT, and then centrifuged for 1 minute at 13000 rpm. DNA purity and concentration was determined on Nanodrop (ND-1000 v3.8.1), and DNA was stored at -80°C.

4.6.2 Determination of DNA concentration and purity with Nanodrop

Nanodrop spectrophotometer (Nanodrop®, ND-1000 V3.8.1) was used to determine the concentration and purity of the DNA. The spectrophotometer was cleaned with ddH_2O and blanked with the appropriate solution, and each sample was measured. The result from the spectrophotometer shows concentration of DNA in µg/ml, 260/280- and 260/230 ratios. The concentration of DNA was used to determine the amount of DNA for e.g. BigDye® Terminator v3.1 Cycle Sequencing Kit. 260/280 ratios described the purity of the isolated DNA. "Pure" DNA should have a ratio around 1.8. If the values were lower than 1.8, it indicated the presence of protein, phenol or other contaminants. While 260/230 ratios were to measure the purity of the nucleic acids. 260/230 ratios were commonly in the range of 2.0-2.2. If the values were lower, it indicated the presence of contamination.

4.7 Polymerase chain reaction using Phusion® High-Fidelity DNA Polymerase

Polymerase chain reaction (PCR) was used to amplify a specific gene of interest, in this case *mrdA* which encodes PBP 2, the drug target for mecillinam. This is to find genomic mutations in mecillinam resistant mutants of *E. coli*, which may confer resistance.

PCR is an amplification technique of small amounts of genetic material. The principle is based on amplification of a sequence of DNA, generating enormous amounts of copies of that particular DNA sequence. There are three main steps in PCR based on repeated amplification cycles. The first involves denaturation of the double stranded DNA (dsDNA) where it is melted and becomes single stranded DNA (ssDNA). In the second step we have annealing of the primers to the complementary regions of the ssDNA. The temperature in this step varies from each primer and has to be lower than the melting temperature of the primers (T_m) hence they can bind properly to the template. The last step involves polymerase extension of the ssDNA, which completes the amplifications of the wanted DNA segments. For this project Phusion® High-Fidelity DNA Polymerase was chosen for DNA synthesis.

Phusion® High-Fidelity DNA Polymerase was the polymerase which was chosen for the amplification. This enzyme offered high fidelity and robust performance. Templates for

Phusion® High Fidelity DNA Polymerase reaction were prepared by using DNA isolation with GenElute Kit (section 4.9).

4.7.1 Preparation of mastermix for phusion PCR

The mastermix recipe is given in Table 17. All the ingredients were stored at -20°C, and the mastermix was prepared fresh for each PCR. The ingredients were thawed on ice, and everything was kept on ice during the procedure.

Table 17: Names and the quantity of each component per 20 µL reaction.

Component	20 µL reaction
Nuclease-free water	to 20 µL
5X Phusion HF buffer	4 µL
10 mM dNTPs[a]	0,4 µL
10 µM Forward primer	1 µL
10 µM Reverse primer	1 µL
Template DNA	2 µL
Phusion DNA Polymerase	0,2 µL

[a] Containing the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP).

4.7.2 Phusion® High-Fidelity DNA Polymerase PCR

The Phusion® High-Fidelity DNA Polymerase thermocycler program was set up as listed in Table 18. See Table 19 for the specific annealing temperature for each primer set.

Table 18: Set up for the thermocycler program with Phusion® High-Fidelity DNA Polymerase.

Step	Temperature/description	Time
1	98°C	5 min
2	98°C	10 sec
3	See Table 19	20 sec
4	72°C	1 min
5	Go to step 2	Repeat cycle 29 times
6	72°C	10 min
7	10°C	Forever

Table 19: Annealing temperatures for different sets of mrdA primers.

Name of primer set	Initial annealing temperature	Annealing temperature after performance of gradient PCR
mrdA 1	58°C	59°C[a]
mrdA 2	58°C	-
mrdA 3	58°C	50°C[a]

[a] See section 6.2.1 for more information.

4.8 Agarose gel electrophoresis

Agarose gel electrophoresis is a method for DNA separation and analysis based on their size and charge (98). By using an electric field the negatively-charged DNA will migrate to the positive electrode through a matrix of agarose. Ethidium bromide (EtBr) is added in agarose gel which functions as a marker by interacting itself between base pairs and emitting fluorescent light when exited by UV light. When placed in an electric field the DNA fragments will migrate to the positively charge end since the phosphate backbone of the DNA is negatively charged. A standard molecule marker, containing DNA molecules of different known sizes (200-10000 base pairs) can be compared to samples to determine the size of the PCR amplicons. Smaller fragments will migrate farther and faster than bigger fragments. The rate of migration of the DNA through the gel is determined by i) size of the molecule, ii) type of agarose and its concentration, iii) DNA conformation, iv) voltage applied, v) presence of EtBr and vi) buffers (98).

Agarose gel was prepared by dissolving agarose (SeaKem®, USA) in 1X TAE buffer to the finale concentration of 1% agarose. The mix was heated in a microwave (approximately 2 minutes) until the agarose was completely dissolved. 50 μL of 1 mg/mL EtBr was added to the solution when the solution was cooled down to approximately 65°C, and poured on an appropriate gel chamber and left to solidify (approximately 20 minutes) at RT. The solidified gel was then placed in an electrophoresis chamber filled with 1X TAE buffer until complete coverage of the gel. 2 μL PCR product was mixed with 10 μL 1X loading buffer and pipetted into the wells of 1% agarose gel. 3 μL of a DNA marker (Smartladder, Eurogentec USA) was loaded in at least one lane on each gel. Gels were run at 80 V for 1 hour. Gel Doc transilluminator (BioRad, USA) and the Quantity One software (BioRad, USA) was used for visualization of the gels and DNA bands were compared to the known DNA markers. A negative control of ddH_2O was included for each PCR set up.

4.9 QIAquick Gel Extraction Kit

For some PCRs, multiple PCR products were observed of different sizes. To try to obtain pure samples for sequencing, bands from the agarose gel at the size expected PCR product was cut

out and DNA extracted using the QIAquick Gel Extraction Kit, primarily according to the QIAGEN's description (99). QIAGEN is the leading provider of innovative samples and technologies for purpose of isolation and detection of contents in any biological sample.

First the DNA fragment was cut out from the agarose gel and the slice was weighed. 3:1 of Buffer QG was added to the gel and incubated at 50°C for 10 minutes (or until the gel slice had completely dissolved). The tube was being vortex every 2-3 minutes during incubation to help dissolving. After incubation the solution was checked if it had the right yellow color which indicating the right pH for optimal DNA binding. 1 gel volume of isopropanol was thereafter added to the sample. QIAquick spin column was placed in a 2 mL collection tube and the sample was applied to the column and centrifuge for 1 minute. The content in the collection tube was discarded and QIAquick column was placed back in the same tube. 0,5 mL Buffer QG were added to the QIAquick column and centrifuged for 1 minute. To wash, 0,75 mL Buffer PE was added and centrifuged for 1 minute (let it stand for 2-5 minutes after application of Buffer PE before centrifuging). The content in the collection tube was discarded and the QIAquick column was centrifuged for an additional 1 minute at 13000 rpm. Then it was placed into a clean 1,5 mL microcentrifuge tube. To eluate DNA, 50 μL Buffer EB was added to the center of the QIAquick membrane, let it stand for 1 minute and centrifuged for 1 minute. To increase DNA concentration, 30 μL elution buffer was added, let it stand for 1 minute and centrifuged for 1 minute (after adding of Buffer EB, increased incubation time to up to 4 minutes may increase the yield of purified DNA.

4.10 DNA sequencing

Sanger sequencing is an automated "cycle" DNA sequencing reaction (100). This method is taken advantage of chemically altered nucleotides; dideoxynucleotides tagged with fluorescents. While making a copy of the DNA template, the enzyme incorporates both nucleotides and the fluorescently tagged dideoxynucleotides. These special dideoxynucleotides will cause the copying process to terminate each time they are incorporated into the growing DNA chain. This process is repeated many times, which will give an enormous number of DNA copies with different lengths. The fragments with

fluorescents attached will be detected by a data analysis software to reveal the sequence of the original piece of DNA (100).

4.10.1 Detection of genetic mutations on mecillinam resistance-encoding genes

BigDye® Terminator v3.1 Cycle Sequencing Kit provided the required reagent components for the sequencing reaction in a pre-mixed format (101). The mix of BigDye v3.1 was added to sequencing buffer, DNA template from PCR, primer (either forward or revers) and brought to a finale volume of 20 μL with deionized water. 200 ng of DNA was added to each sample, which was calculated from the DNA concentration measured from Nanodrop.

See Table 20 for overview of the reagents and the needed quantity of each reagent, and Table 21 for how to perform cycle sequencing on thermal cycler. The samples were sent to the DNA sequencing facility at UNN for sequencing.

Table 20: Reagents and quantity for BigDye® terminator v3.1.

Name of reagent	Quantity
Big-Dye v3.1	1 μL
Sequencing buffer	3 μL
Template	200 ng[a]
Primer 5 mM	1 μL
Deionized water	q.s.
Total volume	20 μL

[a]Nanodrop was performed for quantification of the needed volume of template.

Table 21: Set up for the thermocycler program with BigDye® terminator v3.1.

Step	Temperature/description	Time
1	96°C	5 min
2	96°C	10 sec
3	50°C	5 sec
4	60°C	4 min
5	Go to step 2	Repeat cycle 24 times
6	4°C	Forever

The Sequencher® version 4.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA) was used to assemble sequence reads and detect regions with sequence variation. The genom of the mutants was compared to the parental WTs when possible, or compared to *E. coli* MG1655.

4.11 Gram staining

Gram staining was performed to see if the morphology of the mecillinam resistant mutants were different than that of the WT isolates. Morphology and growth of the cells were compared in pairs where mutants were compared to their parental WT.

This is a common technique used to differentiate Gram-positive and Gram-negative groups based on their different wall constituents by coloring these cells red or violet (102). Gram-positive bacteria stain violet due to the thick layer of peptidoglycan in their cell walls, which retains the crystal violet. While Gram-negative bacteria stain red because of the their thinner peptidoglycan wall. In the decoloring process the crystal violet will not retain in their cell walls.

Overnight culture(s) of the isolate(s) of interest was prepared before staining (37°C with slow shaking, just enough for some motion in the media). The bacterial culture was picked up with a Pasteur pipette and placed onto the middle of a glass slide. The media were spread out, but not too thin. Thereafter the smear was allowed to air dry completely, and the samples were heat fixed to the slide by passing the slide, with smear side up, through the flame rapidly two-three times. The bacterial smear was then completely covered with the primary stain, Crystal violet, and stained for 1 minute. Gently, the dye was washed off with ddH_2O. The slide was then covered with the mordant, Gram's iodine, for one minute. Again the slide was gently washed with ddH_2O. The smear was cautiously rinsed with decolorizer, 96% alcohol, until the purple color no longer came off the smear in the alcohol, and then gently washed off with ddH_2O. The slide was counterstained with safranin for about two minutes, and then gently washed with ddH_2O. Finally the slide was carefully blotted dry with a paper towel. The slides were then investigated in a light microscope at 60X and 100X.

5 EXPERIMENTAL RESULTS

5.1 Isolation of isolates clinically resistant to mecillinam

Clinical *E. coli* isolates with different genetic backgrounds were evolved to become resistant to mecillinam. The results from the optimization of the static selection protocol and the isolation of resistant mutants are described and displayed below.

5.1.1 Optimization of static selection for mecillinam resistant mutants

From the static selection of mecillinam resistant mutants, we aimed to isolate mutants with MICs above 8 μg/mL, the clinical breakpoint. Initially, a concentration of 32 μg/mL of mecillinam in LBA was chosen for the selection (MIC >8 μg/mL). However, this frequently resulted in non-resistant mutants (false positives), as determined by MIC-testing (Table 22). Several reasons for this were considered, and for pinpointing the problem, some factors were investigated: 1) human error; tested by having an observer present during the experiments, 2) comparing MH II and LB with multiple concentrations of mecillinam (8, 16 and 32 μg/mL), 3) retesting a strain that had previously given resistant mutants at 32 μg/mL and 4) rechecking drug concentration calculations and comparing two drug stocks prepared by different members of the lab.

Table 22: Mecillinam resistant mutants in different growth media.

Selection medium	Strain	Mecillinam concentration (μg/mL)	Mecillinam stock (1 mg/mL)[a]	Dilution (1:10) or undiluted[b]	CFU of Mec^R mutants[c]
LBA	K56-5	32	M1	1:10	5
				UD	119
	K56-16	32	M1	1:10	1
				UD	14
MHA	K56-5	8	M1	1:10	5
				UD	133
		16	M1	1:10	0
				UD	50
		32	M2	1:10	0
				UD	1
	K56-16	8	M1	1:10	2
				UD	2
		16	M1	1:10	0
				UD	1
		32	M2	1:10	0
				UD	1
		32	M1	1:10	0
				UD	0

[a] Mecillinam stock prepared by different lab members; M1=member 1, M2=member2.
[b] Diluted (1:10) or undiluted (UD) bacterial overnight cultures which were plated on the selective plates with different media.
[c] Mec^R=mecillinam resistant. CFU observed on the respective selective plates.

Undiluted overnight cultures yielded more CFUs compared to diluted overnight cultures (1:10). This was shown for both LBA- and MHA plates, although for LBA plates they showed more CFUs than for MHA plates. The results showed prominent differences in bacterial growth between the two tested media, but no prominent differences were exhibited for the different mecillinam stocks. Based on these results, the frequency of resistant mutants varies with the type of growth media.

5.2 Mutation frequency and MIC of mecillinam resistant mutants

Mecillinam resistant isolates were generated from ten clinical strains of *E. coli*, originating from the ECO-SENS collection. Three different phenotypes of each strain were isolated, and at least one phenotype isolated for each strain was chosen for MIC-testing. Ten of the 15 mecillinam resistant isolates obtained were selected for further analysis (Appendix A). The results are displayed in Table 23.

Table 23: Mutation frequency and MIC-values for mecillinam resistant isolates. The isolated mecillinam resistant mutants in bold were the ones selected for further analysis. See Appendix A for the complete results.

Parental isolate	Mutation frequency	Isolated mutants	MIC (μg/mL)
K56-5	$1,44x10^{-5}$	**I**	≥256
		II	ND
		III	ND
K56-17	$2,04x10^{-7}$	I	32
		II	48
		III	24
K56-18	$9,54x10^{-6}$	I	8
		II	24
		III	12
K56-20	$1,33x10^{-7}$	I	ND
		II	32
		III	0,5
K56-23	$9,18x10^{-6}$	**I**	32
		II	ND
		III	ND
K56-24	$3,35x10^{-7}$	**I**	64
		II	ND
		III	ND
K56-31	$1,48x10^{-6}$	**I**	24
		II	ND
		III	ND
K56-66	$1,58x10^{-7}$	**I**	≥256
		II	ND
		III	ND
K56-69	$1,02x10^{-6}$	**I**	≥256
		II	48
		III	128
K56-71	$2,35x10^{-6}$	**I**	48
		II	ND
		III	ND

ND = MIC not determined.

The lowest mutation frequency was for strain K56-80 ($7,28x10^{-8}$) and the highest was for strain K56-30 ($6,91x10^{-5}$). The highest MIC-values above the clinical breakpoint were for strains K56-5, -30, -66 and -69 (≥256 μg/mL), and the lowest was strain K56-18 (12 μg/mL) (Table 23).

All isolates were confirmed by MALDI-TOF as *E. coli*. The results showed scores over 2,0 which indicated reliable identification on the species level (Appendix B).

5.3 Collateral sensitivity/cross-resistance networks

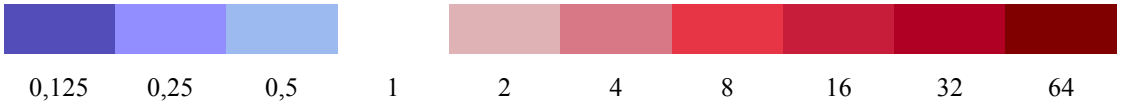
Ten out of 15 strains were selected for further testing and determination of possible collateral sensitivity and collateral resistance networks. The antimicrobial concentration resulting in 90% inhibition of growth (IC_{90}) was determined for the selected isolates for eight antimicrobial agents; amoxicillin, chloramphenicol, ciprofloxacin, gentamicin, mecillinam, nitrofurantoin, tetracycline and trimethoprim.

The IC_{90}-values for the mutants were compared to the parental WTs. The results are displayed in Table 24 as a heat map, presenting the fold-changes in the IC_{90}-values. The fold changes have been color coded according to the description below in the respective table. In this project, only 2-fold changes in collateral sensitivity were considered as relevant changes, thus only ≥2-fold changes have been assigned color codes. Red tones denote cross-resistance and blue tones denote collateral susceptibility. The largest decrease in IC_{90} between the parental WTs and the mutants, showing the highest tendency towards collateral sensitivity, was a 0,125-fold change in amoxicillin susceptibility for isolates K56-23 and K56-66. Three-fold increases in the IC_{90} values to gentamicin and trimethoprim were observed for single isolates, demonstrating cross-resistance.

The average fold changes across all ten isolates were calculated. On average, our mecllinam resistant mutants exhibited the highest tendency to cross-resistance with ciprofloxacin (1,307-fold change), while the highest tendency for collateral sensitivity was to chloramphenicol (0,642-fold change).

Table 24: Collateral sensitivity profiles of mecillinam resistant mutants. A heat map displaying the fold changes for collateral sensitivity(CS)/cross-resistance(CR) for all ten strains that were compared to the parental WTs, and the average of the fold change values (Mecillinam Avg) were calculated for each antimicrobial. The blue coloring denotes CS, red coloring denotes CR and white coloring denotes 1-fold change, alias no fold changes, in CS/CR. For abbreviations see **Table 11**.

Strain	AMX	CHL	CIP	GEN	MEC	NIT	TET	TMP
K56-5	2	1	1	3	21	1	1	2
K56-17	0,5	0,333	0,75	0,5	21	0,75	1	0,5
K56-18	1	0,5	1	0,375	21	0,667	1	0,667
K56-20	0,5	0,5	1,5	0,5	21	1,5	1	0,658
K56-23	0,125	0,667	1,95	0,376	507	0,5	1,5	3
K56-24	1	0,75	2	1	24	1	1,5	0,752
K56-31	1	0,75	1,333	1	21	1	1	1
K56-66	0,125	0,25	0,75	0,75	64	0,667	0,5	1
K56-69	1	1	2,04	0,665	128	1,5	1	1,5
K56-71	1	0,667	0,75	0,75	85	1	1	1
Mecillinam Avg.	0,825	0,642	1,307	0,892	91,3	0,958	1,050	1,208



In order to graph the average susceptibility changes on the same axis it was necessary to log transform the fold changes as shown in Figure 11, the 95% confidence interval (CI 95%) is also shown. This figure allows for more general description of the collateral sensitivity profile in the collection of ten isolates.

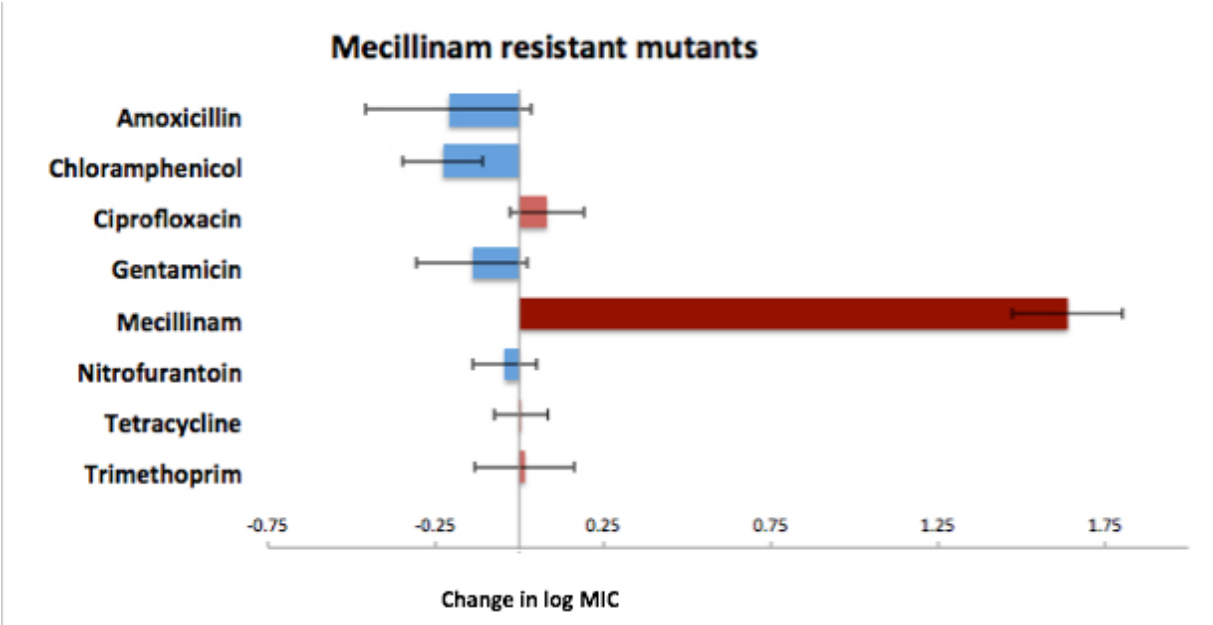


Figure 11: Distribution of collateral sensitivity(CS)/cross-resistance(CR). Showing the average fold change for mecillinam resistant mutants to eight different antimicrobial agents with CI 95%. Blue coloring denotes CS, and red coloring denotes CR.

In general, the mecillinam resistant mutants frequently displayed low-level collateral sensitivity (0,983-fold change in net result). During the experiments, we observed a couple of mecillinam resistant mutants from K56-23 and K56-24 that made small colonies and generally grew slower than their parental WTs (these observations were not performed systematically).

5.4 Optimization of PCR

In this project, we wanted to amplify and sequence certain of genes that are involved in mecillinam resistance. Initially, we struggled with contamination of the PCR reactions, which was evident by having bands in the negative control (mastermix and water). Finally, after switching to aerosol resistant tips and exchanging all buffers and nuclease-free water, the contamination was absent. DNA isolations were also repeated to obtain samples without possible EtOH and protein contaminants, which interfered with the PCR reactions.

Several of the primers that were ordered or designed for this study showed unspecific binding, which caused multiple bands on the agarose gel, and others gave no amplified product at the annealing temperature indicated by the calculations. Gradient PCRs (50-60°C) were performed in an attempt to optimize the running conditions. An example of a gel from a gradient PCR setup is shown in Figure 12. Furthermore, problems with contaminated DNA templates were encountered.

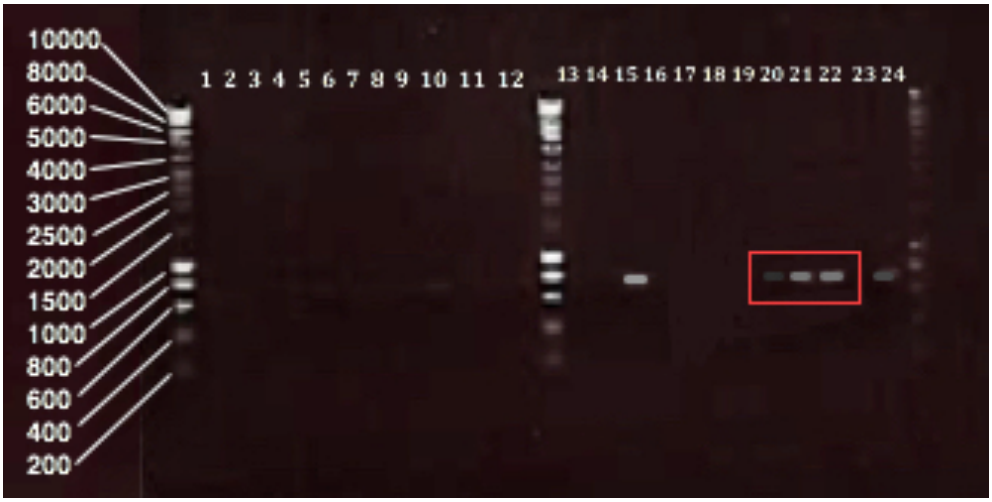


Figure 12: Gradient PCR for mrdA1. The whole gradient spanning from 50°C to 60°C. The area marked in red gave an annealing temperature approximately at 59°C.

The results from the gradient PCR performed for mrdA1 (wells from 1-12 contain samples of negative controls of ddH_2O and wells from 13-24 contain samples of WT K56-24) showed amplified PCR products (bands) at approximately 50- and 59°C. Due two several bands at approximately 59°C, marked in red, a new annealing temperature at 59°C for mrdA1 was chosen.

Finally, investigations for potential mutations were focused on *mrdA* gene, as it is the mecillinam drug target, due to time constraints. To amplify the whole gene, three primer sets were required. Two of these (mrdA1 and mrdA3) gave several bands even with optimization of the annealing temperature, and the amplicons were in the end primarily isolated from the agarose gel to obtain templates for sequencing. An example of such gel is shown in Figure 13.

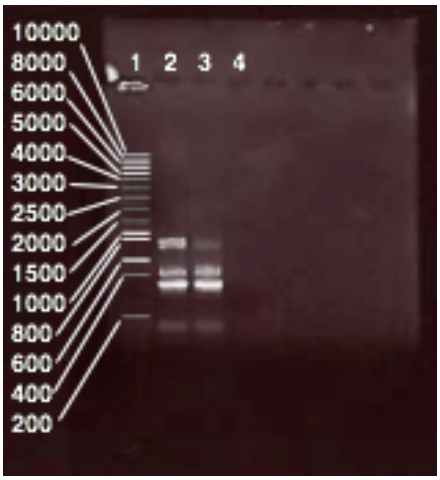


Figure 13: Multiple bands of PCR-products. Displayed is PCR with mrdA1 at 50°C for K56-23 WT (well 2), mutant (well 3) and negative control with ddH_2O (well 4).

5.5 Genetic mutations in the *mrdA* gene

To identify potential mutations in the *mrdA* gene, which may confer mecillinam resistance, the PCR-products were sequenced. The isolates with good quality DNA sequences were aligned to the *E. coli* MG1655 genome. The respective isolates were mutants of strain K56-5, -18, -23 and -66. Only parts of the *mrdA* gene were compared. The results are displayed in Table 25 and Figure 14.

Table 25: The different mutations detected for mecillinam resistant isolates compared to *E. coli* MG1655. The arrow indicates what kind of alteration in the amino acid sequence the mutation caused.

Detected mutations	Amino acid alteration	Detected mutations	Amino acid alteration
CAG498CAA	Gln → Gln	GGC427GGT	Gly → Gly
CCT481CCG	Pro → Pro	CCT430CCA	Pro → Pro
TAC533TAT	Tyr → Tyr	CAG436CAA	Gln → Gln
GCT535GCC	Ala → Ala	ACA459ACG	Thr → Thr
GGT594GGC	Gly → Gly	CCA462CCT	Pro → Pro
GGT595GGC	Gly → Gly	TTG483TTA	Leu → Leu
ACA602ACG	Thr → Thr		

Ala=Alanine, Gln=Glutamine, Gly=Glycine, Leu=Leucine, Pro=Proline, Thr=Threonine and Tyr=Tyrosine.

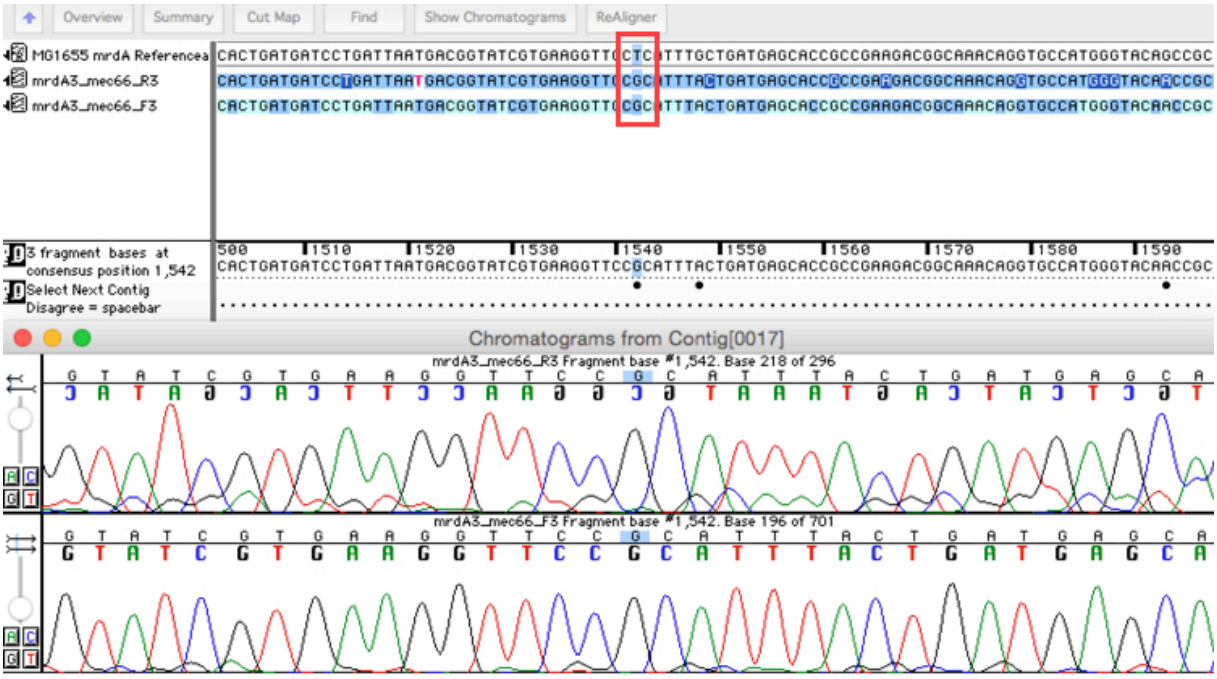


Figure 14: DNA alignment of the mutant and its parental WT. The red marked area is an example of a point mutations observed in the mecillinam resistant mutant (K56-66) compared to MG1655 of *E. coli*.

The results showed 13 synonymous point mutations, in which the mutation in the respective codon still encodes for the same amino acid. Thus leading to no alteration in the amino acid sequence.

5.6 Chain growth effect of *E. coli* cells

All parental WT isolates and their mecillinam resistant mutants were investigated under a light microscope after Gram staining.

In general, observations showed that mutants were more spherical and smaller in size compared to their parental WTs. There were some color intensity differences as well, which in two out of ten cases the mutants showed higher color intensity than their parental WTs (isolates of K56-18 and -23). Observations through the light microscope also showed five out of ten mutants (isolates of K56-5, -18, -20, -24 and -69) grew more in pairs or slightly more in chains than their parental WTs. However, in general, the results showed no prominent differences between the mutants and their parental WTs (Figure 15).

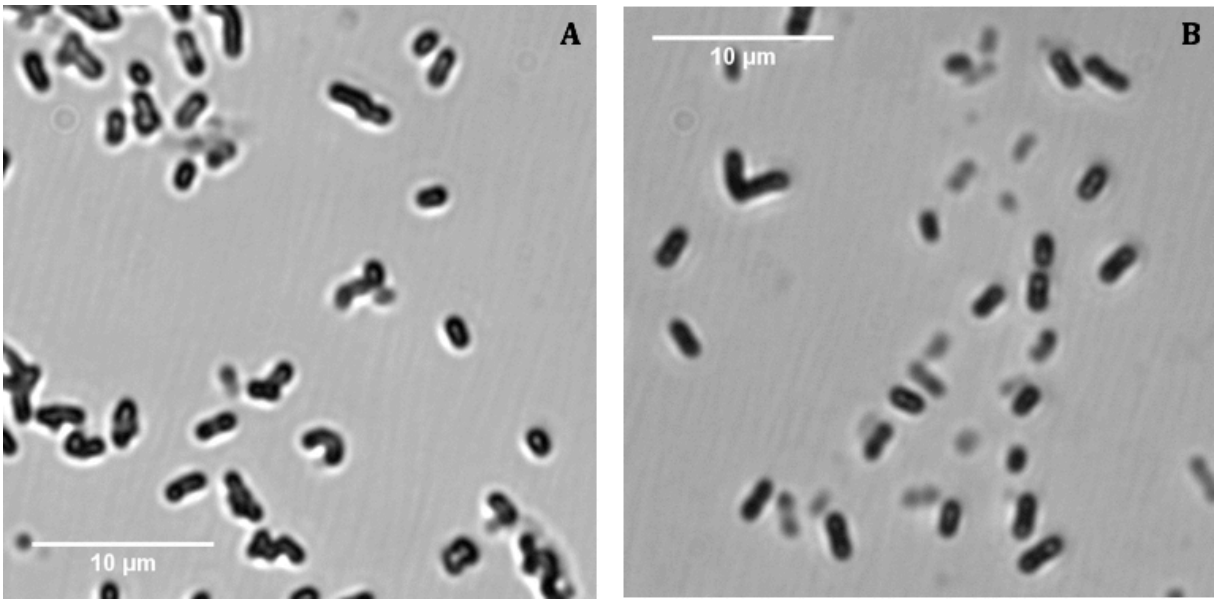


Figure 15: Cells of clinical isolates of E. coli. Comparison of mecillinam resistant isolate (A) of strain K56-5 and its parental WT (B). The observations show no prominent differences in chain growth tendency.

6 DISCUSSION

The successful discovery of antimicrobial agents is unfortunately comprised by the inevitable emergence of AMR (1, 4). Since the first cases of AMR in the 1940s, it has been threatening the success, and modern management of infectious diseases (15), and worse leading to widespread fatalities and economic disruption globally (14). To tackle the bacterial development of AMR mechanisms novel treatment strategies are required to ensure effective treatment of infections. Translating collateral sensitivity networks into treatment-guidelines may retard the evolution of antimicrobial resistance by constraining the evolutionary paths towards resistance. The main focus in this project has been to expand the knowledge of this phenomenon through generating collateral sensitivity/cross-resistance networks. Hence may lay the scientific foundation to establish a novel treatment strategy for UTIs caused by *E. coli* and prevent further resistance development. We approached this by evolving resistant to mecillinam, a relevant antimicrobial in UTI treatment, in clinical strains of *E. coli* from UTIs. We further proceeded to explore the collateral sensitivity/cross-resistance networks of the resistant isolates and took a closer look at some of the mutants in an attempt to find the mechanisms behind our observations.

6.1 Collateral sensitivity/cross-resistance networks

The resulting collateral sensitivity/cross-resistance networks in our study are displayed in heat maps and a graph showing the distribution of antimicrobial susceptibilites to various agents. In this context, we are not using the terms “collateral sensitivity” and “cross-resistance” from a clinical point of view, but rather as changes in the antimicrobial susceptibility that we might be able to employ in order to control AMR development. Our results showed that both collateral sensitivity and cross-resistance occurred for mecillinam resistant isolates. However, a substantial tendency for collateral sensitivity frequently appeared compared to cross-resistance (0,983-fold change in net result). This is in concurrence with previous unpublished preliminary results from our research group for other isolates from the ECO-SENS collection.

In Imamovic et al.'s study, CS drug cycling was demonstrated for various antimicrobial agents (38). However, mecillinam was not included in that study. Our results showed that across all antimicrobial agents tested the overall average fold change was below 1,0. Though, for amoxicillin, chloramphenicol and gentamicin collateral sensitivity was demonstrated for four out of ten strains. Hence at this point our data does not support that the collateral sensitivity patterns are general enough to employ mecillinam in CS drug cycling. However, there are several aspects of the study that should be highlighted. Ten diverse clinical isolates of *E. coli* from the ECO-SENS collection from patients with UTIs were used. Compared to previous studies, this is a substantial number of strains, giving a better description of what is to be expected on the population level (38, 39). Our IC_{90} results were very tight, with the two replicates giving very similar results in most cases. The statistical power of IC_{90}-assay provides high validity and resilience in our data. Additionally, the most interesting finding in our results was the rare tendency of cross-resistance observed for mecillinam resistant mutants. Thus mecillinam might be a good candidate to be employed as the first drug of choice for UTIs. Our findings provide proof of principle for collateral sensitivity, which may be an important key to interfere with resistance development. The study also contributes scientific evidence to other previous works to which our comparisons show high consistency (38, 39). However, these assumptions are based on *in vitro* results. Thus further investigations should be tested *in vivo* and possibly in clinical trials as well.

6.1.1 Comparison to previous works on collateral sensitivity

Imamovic and Sommer, 2013

Imamovic and Sommer demonstrated collateral sensitivity in *E. coli* MG1655 and suggested to use these CS-networks to inform drug cycling strategies (38). In their study, tendency of collateral sensitivity was frequent, where 17 of the 23 resistant *E. coli* isolates exhibit collateral sensitivity to at least one other microbial agent (38). Mecillinam was not included among the antimicrobial agents to which the bacterial isolates were evolved to become resistance to. Hence our comparison of the results is based on the data from the same chemical class as mecillinam (β-lactams).

Similar to this study, a heat map was used to display the collateral sensitivity profiles of antimicrobial resistant *E. coli* strains. According to our results, mecillinam resistant mutants displayed collateral sensitivity towards amoxicillin and chloramphenicol contrasting to their study where cross-resistance was more frequently observed (up to 32-fold change). Their data also show more cross-resistance interactions towards tetracycline (2-fold change increase for all β-lactam-resistant isolates except for one), and towards nitrofurantoin it shows both collateral sensitivity and cross-resistance to a similar extend, while our results exhibited almost no change in the antimicrobial susceptibility towards both agents. Nitrofurantoin affects several targets in the bacterial cell wall, which may partly explain the variation observed for collateral sensitivity and cross-resistance. Resemblances seen in both studies are towards ciprofloxacin and trimethoprim to which prominent cross-resistance is observed for all β-lactam-resistant isolates (up to 8-fold change increase).

In general, we observed more collateral sensitivity for mecillinam-/β-lactam-resistant isolates in our study, especially towards chloramphenicol and amoxicillin in contrast to their study. The limited number of clinical strains they were using may cause the dissimilarities as displayed in our comparison.

MG1655 strain of *E. coli* was selected for resistance to 23 different antimicrobial agents. Two clinical isolates were selected for resistance to a subset of eight of the original 23 antimicrobials. Thus the resistant isolates have a limited variation in their genetic background. We used ten clinical isolates of *E. coli*, giving us more solid data to test the generality of the networks on the population level. As demonstrated in our experiments, both collateral sensitivity and cross-resistance can occur within one antimicrobial agent tested across various strains. Thus it is important to include isolates with various genetic backgrounds to observe if collateral sensitivity networks also exist on the population level.

Lázár et al., 2013

Lázár and co-workers also aimed to determine how frequently collateral sensitivity occurred as well as the underlying mechanisms (39). They noticed populations exposed to the same antimicrobial agent showed very similar antimicrobial susceptibility patterns. The results were charted to a network of collateral sensitivity interactions between several antimicrobial agents, which were grouped according to their mode of action(s). Our comparison is based on the data of cell wall-inhibitors; the same antimicrobial class as mecillinam (mecillinam and amoxicillin were excluded in their study).

Similarities seen in both studies are for instance the same tendency of collateral sensitivity towards gentamicin and nitrofurantoin when strains of *E. coli* develop resistance to antimicrobials with cell wall as their drug target. Based on their results, several major patterns were observed. One of them was that the distribution of interactions was strongly influenced by the mode of action(s) of the antimicrobial agents. The results from both studies may not be comparable since mecillinam was excluded in their study. However, based on this respective pattern, mecillinam resistant mutants might have exhibited the same collateral sensitivity and cross-resistance interactions as the other mutants with adaption to cell wall-inhibitor agents.

The authors reported no collateral sensitivity interactions towards chloramphenicol. The results presented here suggest that in a broader collection of clinical isolates, more CS-networks exist. In their study, the isolates were from the same one ancestral clone that was propagated to ten independent populations. As discussed earlier, this might not be representative on the population level. Furthermore, they were using another method to estimate collateral sensitivity. The sensitivity of each resistant isolate was tested against different antimicrobial agents and the growth in the liquid cultures was measured at half-maximal effective concentration (EC_{50}). EC_{50} and IC_{90} are both units that measure the drug's potency to the isolate of interest. Though, different units of measurement might cause the dissimilarities in our comparisons.

Lázár et al. observed that collateral sensitivity to other antimicrobial classes is uncommon for most of the classes. However, as same as for Imamovic and Sommer, they observed several major patterns in their study (one of them already mentioned above), including exhibition of high tendency of collateral sensitivity towards other classes when strains of *E. coli* develop resistance to aminoglycosides (38, 39).

MicroPop, 2015

In general, our comparison with MicroPop's preliminary data displays high consistency (Figure 16).

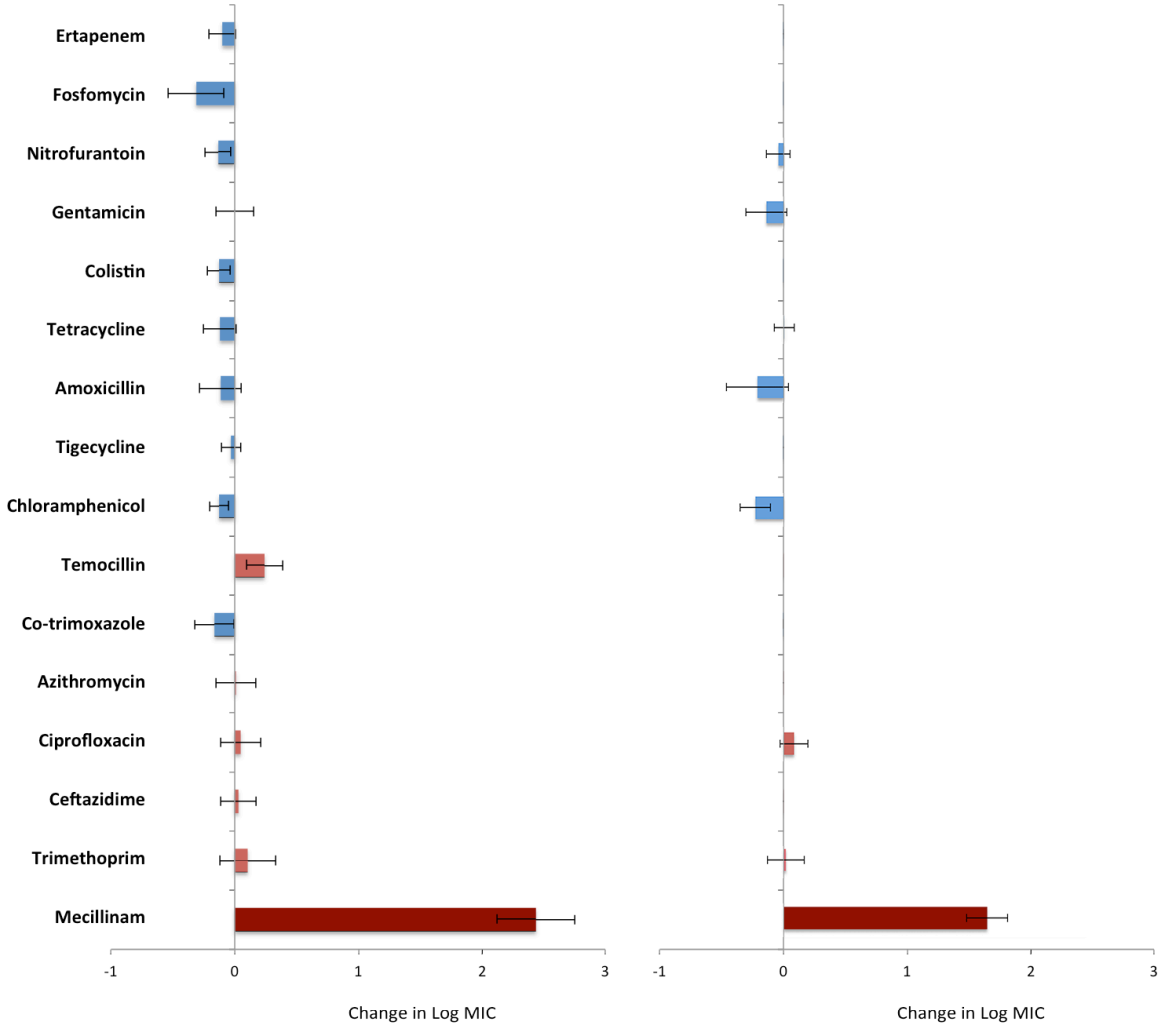


Figure 16: Distribution of collateral sensitivity/cross-resistance. Showing the average log MIC for mecillinam resistant mutants with CI 95%, generated by MicroPop (to the left) and our project (to the right). Blue coloring indicates collateral sensitivity, and red coloring indicates cross-resistance. Permission obtained from: MicroPop research group at UiT – The Arctic University of Norway in Tromsø, unpublished preliminary data.

The same tendency of collateral sensitivity/cross-resistance networks was observed for both studies. As described earlier, MicroPop performed gradient strip diffusion assays for MIC testing to investigate the antimicrobial susceptibility patterns, while we used IC_{90}-assays. Despite using different methods, similar results for collateral sensitivity/cross-resistance for both studies provided comparable data even though only one replicate was performed in their study. In their work, mecillinam resistant mutants displayed collateral sensitivity towards nitrofurantoin, gentamicin, amoxicillin and chloramphenicol. In our study, mecillinam resistant mutants revealed prominently stronger collateral sensitivity towards gentamicin. Similar tendencies for cross-resistance were shown towards ciprofloxacin and trimethoprim.

Tetracycline was the agent that showed contradictory results. In our study, mecillinam resistant mutants showed weak cross-resistance interactions towards tetracycline. While the opposite is demonstrated for MicroPop's study showing clear collateral sensitivity interactions. However, the CI 95%-values between the studies do overlap. CI is used to describe the amount of uncertainty associated with a sample method that includes an unknown population parameter. In other words, the calculated CI 95% shows a range of values where there is a likelihood of 95% that the next replicate performed will fall within the respective interval (103). Thus when the CI 95%-values for both studies include "0" on the x-axis, the "next" mecillinam resistant isolate tested against tetracycline might show no change. Hence the differences for both studies are not considered significant. Ongoing replicate experiments will improve the statistical power in these analyses.

6.1.2 Different strategies for CS cycling

As mentioned in the section 1.3.5, the hypothesis behind conventional drug cycling is based on the assumption that resistance is accompanied by biological fitness cost, thus leading to eradication of resistant bacteria when absence of the selective pressure imposed by drug treatment (32, 43). However, bypass mechanisms may allow genetic adaption and maintenance of the resistance phenotype despite exclusion of antimicrobial agents deployment (32, 37). Furthermore, in clinical settings, eradication of all resistance pathogens is required so that reversibility can occur, even though the rate of reversibility is expected to be slow at the community level (32).

Several studies have suggested CS cycling as a novel treatment strategy, which constrains evolutionary paths to AMR (38, 40).

A study proposed a different approach to the conventional drug cycling (38). This was based on the findings that the development of resistance to one antimicrobial agent might alter the antimicrobial susceptibility profile for that respective bacterial cell. Hence resulting in collateral sensitivity and cross-resistance toward other antimicrobials. They called this novel treatment strategy for CS drug cycling (section 1.4) (38). However, in their experiments, not all antimicrobials could be used in two-drug cycles because use of agents with cross-resistance will provide a selective advantage to the resistant strain over the WT leading to amplification of resistance. Therefore they suggested antimicrobials with collateral sensitivity

profiles could be included in the cycle leading to a change in antimicrobial susceptibility toward collateral sensitivity. However, this means that deployment of four to five drugs in one CS drug cycle would be required, making treatment more complicated. Additional, this strategy is dependent on complete eradication of all resistant strains, which may be challenging in real-life settings due to unknown evolutionary stability of collateral sensitivity/cross-resistance and other factors.

Another study by Gonzales and co-workers suggests a combination therapy of three different β-lactams, all targeting cell wall synthesis (40). This synergistic, collateral sensitive β-lactam combination uses elements from three strategies; targeting multiple nodes (connection points) in the same cellular system, synergistic effect and collateral sensitivity. This combination therapy was suggested to combat methicillin-resistant *Staphylococcus aureus* (MRSA), a multidrug-resistant pathogen. Nevertheless, in their results, high resistance to one of the two β-lactams slightly reduces effectiveness of the synergistic, collateral sensitive β-lactam combination. Furthermore, HGT was not taken into account in their analysis, which can break the synergetic relationship between the β-lactams.

6.1.3 CS cycling versus other antimicrobial treatment strategies

A promising treatment strategy that may improve pathogen eradication and curb the evolution and spread of AMR, is combination therapy. As mentioned above, combination therapy has shown its success by taking advantage of synergistic effects of some drug combinations (section 1.3.2) (25). However, toxicity has been observed and the issue on whether combination therapy reduces mortality in infections with Gram-negative bacteria has been brought to light in a meta-analysis (26). According to the analysis, adverse effects are more common for patients receiving combination therapy. They also suggest that the survival benefit of combination therapy may be none. Moreover, other potential disadvantages including increased financial cost and increase in resistance prevalence associated with combination therapy. Hence collateral sensitivity cycling may be a more favorable solution to the rapidly growing crisis in AMR development. Other treatment strategy includes conventional antimicrobial cycling that was mentioned in previous section (section 1.3.5).

6.1.4 Potential impacts and challenges of CS/CR in clinical settings

Mecillinam is primarily used in Northen Europe for treatment of UTIs (60). Furthermore, it is not available everywhere (e.g. Canada or the US) or not a drug of choice in the recommendations of UTIs treatment (section 1.7.2) (54, 55, 57). For instance, trimethoprim and nitrofurantoin are the recommended antimicrobials for treatment of acute lower UTI in Scotland (54).

Our work may contribute to developing novel treatment guidelines for the treatment of infectious diseases (UTIs). Based on our results, mecillinam might be a good candidate to be employed as the first drug of choice for UTIs. Comparison of the results from the MicroPop group and our study has shown a clear tendency for collateral sensitivity for mecillinam resistant mutants. And, perhaps even more important, the collateral sensitivity profiles indicate that mecillinam resistance gives a fair probability of developing of collateral sensitivity and low probability of cross-resistance (Figure 16).

CS/CR networks could be used as tools to inform the choice of primary and secondary therapies to ensure effective treatment options, based on drug cycling. Furthermore, several studies have anticipated that collateral sensitivity will contribute to the sustainable use of drugs in hospital setting by reducing the rate of resistance evolution (27, 40, 41).

The results from MicroPop and our study are promising. However, more strains need to be explored to verify this. Nonetheless, the results from our study and others are from *in vitro* experiments performed in laboratory settings (38, 40). In Thulin et al.'s paper, their results show that the mutation frequency of mecillinam resistant mutants is much higher *in vitro* than in the clinical settings (12). Mutations were found for various resistance-conferring genes for mecillinam resistant laboratory-selected mutants and for -clinical isolates. However, one remarkable thing was that mutations in the *cysB* gene were found in all of the clinical isolates. Furthermore, mecillinam resistant mutants evolved in laboratory settings had severe fitness costs with lower growth rates compared to *E. coli* MG1655. This tendency has been observed in other studies, as well as in ours (34, 80). Thulin et al. implies that only a small subset of the mutants found *in vitro* are fit enough to become fixed in the bladder (12).

These observations demonstrate the possibility that we might see a different result in real-life settings. In this study, mecillinam resistant mutants were evolved from isolates of *E. coli* from

clinical patients with UTIs. Though our observations might be limited to *in vitro* settings, the mecillinam resistant isolates are clinically relevant.

Also, detection of pathogen species before choice of antimicrobial is not always possible in a clinical setting, such as when a patient is admitted for acute infections. Thus using CS in this context may be challenging if the first line drugs of choice is dependent on the bacterial species. Moreover, the influence of different factors on collateral sensitivity still remains unknown. Such factors are, for instance, acquisition of resistance genes through HGT and pathogens with MDR.

6.1.5 Management of various deviations in our results

The results from our collateral sensitivity/cross-resistance networks were displayed with fold changes. We performed two replicates for all twenty isolates, both mutants and WTs (section 4.5.2). The first replicate was performed with 2-fold dilutions, covering a wide range of MIC-values, to provide us an indication for in what range the IC_{90}-value will be for the respective antimicrobial. The second replicate was performed with 1,5-fold dilutions. The results from the second replicate were used for the final interpretation due to its higher resolution. Furthermore, the results from both replicates were generally consistent. Regarding the more slow growing isolates (the mutants based on K56-23 and-24), which were incubated at 18-, 24 - and 42 hours, the results from 18 hour incubations were used for the interpretation of the overall results. Although with 42 hours we achieved higher density, thus higher accuracy and confidence in our data, this caused difficulties for our interpretation due to the ATCC-values occasionally being out of range after 42 hours and the incubation time not being standardized. However, measurements from the various time points showed high similarities suggesting the antimicrobial potency was still the same.

For the replicates to which the values of the ATCC control and K56-44 resistant isolates (internal quality control strains) were out of range, another replicate was performed. In our final results, ATCC- (for trimethoprim) and K56-44 resistant isolates (for nitrofurantoin) were slightly out of range once (Appendix C). We decided to accept the deviation of K56-44 resistant isolate since the range can vary for mutants, additionally that occurred in our first replicate (2-fold change which MIC-value achieved was 4 μg/mL while the range was 6-24 μg/mL). The range is stricter for the ATCC (0,5-2 μg/mL) since it is defined by EUCAST (104). Therefore

two more replicates were performed for the concerning strains (K56-20 and K56-24). However, we continuously got MIC 0,38 μg/mL which equaled to a 1,5-fold lower than expected. This may be due to the bacteriostatic effect of trimethoprim, which caused difficulties for the range of the ATCC. Furthermore, the range of ATCC-values defined by EUCAST are based on standard microbroth dilution testing hence may cause the deviation (this matter will be discussed in the next section, 6.1.6).

Despite these challenges, these defined values were still used in our study due to the lack of equally well-established values for IC_{90}-assays. Therefore, in order to achieve a MIC-value for ATCC to be in range, the IC_{90}-values calculated for the respective isolates were adjusted 1,5-fold higher. E.g. initially, the WT of strain K56-20 got MIC 0,25 μg/mL which was adjusted to MIC 0,38 μg/mL.

6.1.6 IC_{90}-assessment vs. MIC-testing

Micro broth dilution with IC_{90}-assessment was chosen for the collateral sensitivity profiling. Although IC_{90}-assessment is more time-consuming, there are many advantages regarding this method. For instance it is more cost-effective in which the consumables are cheaper compared to MIC-testing using the gradient strip method. However, the main reason for why micro broth dilution is the preferred method for us is due to the reproducibility. Thus providing us high validity and resilience in our results. We also compared our results from the IC_{90}-assessment with earlier MIC-testing results, which showed high consistency. Furthermore, reproducibility of MIC-testing has shown to be challenging, particularly for mecillinam. Determination of 100% inhibition zone of bacterial growth was difficult, since spontaneous mutants frequently appear. Also, the interpretations were done visually which may affect the results due to inter-variability of different interpreters. This manually readings were not a problem for IC_{90}-assays since the reads were done automatically with a plate reader.

Results from AST for mecillinam have shown to be difficult to reproduce in general (section 1.8) (58). This is due to the high frequency of spontaneous mutations for mecillinam resistant isolates (80). The high mutation rate may be explained by in either of two ways; there are one or a few genes with high spontaneous mutation frequency, or there are many genes with a normal/low spontaneous mutation frequency (80). As mentioned earlier, the frequency of mutation to mecillinam resistance isolates is high *in vitro* due to the large amount of targets

for mutations that result in mecillinam resistance (Table 1) (12). Thus these isolates can easily be mutated further and make the IC_{90}-assay/MIC-test interpreted level to be artificially high.

6.2 Mecillinam resistance-encoding genes

The second aim in our study was to investigate the underlying mechanisms of the reciprocal collateral sensitivity between antimicrobials agents. We approached this by investigating the potential mutations in mecillinam resistance-conferring genes. By exploring the resistant mechanism, we might find some answers in the underlying mechanisms of collateral sensitivity. Potential mutations in the *mrdA* gene were identified through PCR and DNA sequencing. DNA alignments of four mecillinam mutants (K56-5, -18, -23, and -66) were compared to the *E. coli* MG1655 genome. The results displayed 13 synonymous point mutations, meaning these point mutations leads to no alteration in the amino acid sequence. Thus there are no potential mutations in the respective amplicons that may confer resistance and alter the bacterial phenotype. However, our comparison was not optimal. Only part of the *mrdA* gene was compared and the genome of the parental WTs was not the reference genome. *mrdA* gene is one of the known genes that confer mecillinam resistance and shows high frequency of mutations in mecillinam resistant mutants (12, 68, 80). Based on our results, we can not conclude on the mechanism of the mecillinam resistance, and hence not the mechanism of collateral sensitivity observed.

6.2.1 Challenges concerning PCR

Initially, several genes were chosen for close scrutiny for potential mutations in the genomes of mecillinam resistant isolates (1.7.2). However, we experienced challenges regarding PCR, additional to the time limitation, which lead to the decision to focus on the *mrdA* gene only.

Three primer sets were designed to cover the whole *mrdA* gene and DNA sequencing was supposed to be performed for the entire gene. However, we experienced various difficulties during our attempts to amplify the wanted sequence of the gene with PCR. Originally, an annealing temperature at 58°C was chosen for all three primer sets after analyzing the primers at the National Center for Biotechnology Information (NCBI) website with Basic Local

Alignment Search Tool (BLAST) and the OligoAnalyzer 3.1 software. The purpose was to detect whether the primer binding to the DNA was specific enough and to find the optimal annealing temperature for them respectively.

The results from agarose gel electrophoresis frequently showed either no amplifications or multiple bands with the chosen annealing temperatures. Gradient PCR with 50-60°C were therefore performed for mrdA1- and mrdA3-primer sets, which gave us a specific band at 59°C (for mrdA1) and 50°C (for mrdA3).

Multiple bands still occurred after performing the agarose gel electrophoresis for mrdA2- and mrdA3 amplicons. These bands may be caused by unspecific binding of the primers to the genome of *E. coli*. To investigate this issue, analysis of all three primer sets were done through BLAST again to observe whether the primers bind to other microorganisms with higher matches than *E. coli*. The results for all three primer sets showed 100% match for the whole sequence of the primers (all 20 nucleotides) only for PBP 2 in *E. coli*. However, other binding-sites in the *E. coli* genome with partial similarity to the primers may have caused unspecific bands.

Based on the gradient PCRs we adjusted the annealing temperature for the mrdA1 and mrdA3 primer sets. Time limitation restricted us from further exploring the optimal running conditions for these PCRs. Other parts of the running conditions we could have adjusted would be to optimize enzyme concentration, lengthen extension time or increase cycle number (105). Designing of new primers could also be an option.

6.3 Frequency of resistant mutants is depending on type of growth media

To streamline the protocol of static selection of mecillinam resistant mutants to others used in the lab, LBA was exchanged with MHA. According to EUCAST, MH II using for MHA is the recommended medium for supplementation of antimicrobial agents (106). This is due to its defined contents of cation (calcium and magnesium ions) and thymidine, which are known to affect the activity of several agents, such as trimethoprim and trimethoprim-sulfamethoxazole.

The results from the change of medium demonstrated that the two different growth media showed a clear difference in mutation frequency and the number of true resistant mutants (Table 22). Moreover a decrease in mecillinam concentration in MHA gave a clear increase in the mutation frequency. Based on these results, a concentration of 16 μg/mL mecillinam in MHA was chosen for the static selection of mecillinam resistant mutants.

6.4 Patterns of cell growth for mecillinam resistant mutants

Studies have shown that mecillinam resistant mutants are more spherical in shape as a consequence of PBP 2 inhibition (11, 12) and also have slower growth rate (34). The current thinking is that these phenomena and observations may lead and be due to the chain growth effect of the cells. According to our results from Gram staining experiment, there were no great differences between mecillinam mutants and their parental strains regarding the chain growth of the cells. Due to these findings we presume that the incomplete septation during cell division may not lead to chain growth effect. The slower growth of the mutants might be due to other fitness costs in the cell function. However, for our mecillinam resistant mutants the resistant mechanism is still unknown. Therefore further investigations are required to know if these phenomena have an association to the chain growth effect.

6.5 Strengths and limitations in the project

In our project, we included ten clinical *E. coli* isolates providing us strains with different genetic background, which increased our ability to generalize on the population level. Furthermore, the eight antimicrobial agents employed in our experiments have high clinical relevance since they are frequently used in treatment of UTIs. They also belong to distinct chemical classes and have different mode of actions (Table 11).

Nonetheless, our project has only focused on a single species, meaning our understanding on the bacterial evolution globally remains unknown. The medium chosen for bacterial growth LB and MH II broth are very dissimilar to *in vivo* system. Hence, we can not confer our result

in living organisms. For instance in a clinical setting, we will probably get a urine sample from a patient which can vary in its constituents, e.g. level of proteins, hormones and metabolites. Also the anamnesis to a patient and whether the patient is using medicals are important factors to take into account. Furthermore, the immune system to a host plays a crucial part of the medical response, reflecting the state of health to a host.

6.5.1 Improvements

To see how conserved collateral sensitivity is across different species, various types of pathogens causing UTIs may also be included in our project. Also, in order to create a laboratory setting more biologically similar to a clinical setting, we could use urine as medium instead.

Regarding PCR, it has been challenging to investigate the locations of the potential mutations in the *mrdA* gene that may confer resistance. PCR is generally sufficient as a method to identify potential mutations in a resistance-encoding gene. However, besides being a relative large gene (1902 bp), it is also a housekeeping gene (which the bacteria require for the maintenance of basic cellular functions). Thus *mrdA* gene is relatively conserved (68, 107). Hence on the population level the bacteria may allow low degree of mutations in the gene. A better way to investigate potential mutations is to perform WGS for all 20 clinical isolates of *E. coli*. Simultaneously, we can also search for mutations in other regions when knowing that there are at least 38 genes involved in mecillinam resistance (12). This is of course more costly and time-consuming, not to mention the knowledge we have to attain within bioinformatics for comparison of the amino acid variability. With WGS we have the opportunity to do DNA alignment for all known mecillinam resistance-encoding genes and accomplish more information about the mutational patterns. Ideally, we should also use the parental WTs as the reference genome for the DNA alignment regarding the differences in the genetic backgrounds for the clinical strains. This will provide us a more valid result.

Finally, to increase the probability for finding mutations in the genome, genes with same functions could be included e.g. *mrdA* and *mrdB* genes (68). This is based on the assumption that mutations in one gene may also cause mutations in another gene with the same cellular functions. Furthermore, other resistant-conferring genes, which have shown high frequency of mutations found in mecillinam resistant mutants, should be investigated. Examples of such

genes are *thrS*-, *aspS*- and *gtlX* genes, encoding threonyl-tRNA synthetase, aspartate tRNA ligase and glutamyl-tRNA-synthetase respectively (12). Nonetheless, according to Thulin et al.'s paper, mutations in the *cysB* gene are remarkably common for all clinical resistant isolates of *E. coli*. The CysB protein, encoded by the *cysB* gene, is the main positive regulator of cysteine biosynthesis. The mechanism behind this gene conferring resistance still remains unclear, but some suggest that this is partly due to the increased intracellular levels of ppGpp (84). Hence the *cysB* gene may be an interesting gene for further research.

7 CONCLUSION

We suggest mecillinam could be a potential candidate for the first drug of choice in treatment of UTIs caused by *E. coli*. This suggestion is based on our findings as well as previous unpublished results from our group (MicroPop at UiT – The Arctic University of Norway in Tromsø) showing that collateral sensitivity interaction with other antimicrobial agents frequently occurs for mecillinam resistant mutants. Most strikingly, cross-resistance is rarely seen for mecillinam resistant mutants. We believe that these collateral sensitivity/cross-resistance interactions will occur on the population level as well. Unfortunately, the underlying mechanism(s) of the reciprocal collateral sensitivity between antimicrobial agents still remains unclear.

Furthermore, we insinuate reduction in growth rate for mecillinam resistant mutants found in laboratory selections may have an impact in clinical settings. Thulin et al. have also indicated this, based on reduced fitness cost, these mutants may have difficulties to maintain stable in a clinical setting (12).

8 FUTURE ASPECTS

Since the introduction by the first pioneers, much progress in our knowledge of collateral sensitivity has been explored. However, there are several knowledge gaps that should be studied further concerning collateral sensitivity/cross-resistance patterns (CS/CR).

- How conserved are the CS/CR patterns; regarding the diversity on species level, and even globally. Furthermore, will MDR lead to alteration in the CS/CR patterns?
- What is the underlying mechanism(s) of collateral sensitivity; so far suggestions of different underlying mechanisms and factors may contribute to this matter; such as reduced activity of the efflux pumps and fitness cost. Although, there are many unanswered questions.
- How stable are the CS/CR patterns; will the bacterial evolutionary adaption of antimicrobial agents also occur for CS as for CR, and will these patterns remain stable in *in vivo* settings.

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10 APPENDIX

Appendix A: Complete results from static selection of mecillinam resistant mutants. The isolated mecillinam mutants in bold were the ones selected for further analysis.

Parental isolate	CFU/mL inoculum	CFU/mL mutants	Mutation frequency	Isolated mutants	MIC (μg/mL)	Species confirmation MALDI-TOF
K56-5	$3{,}20\text{x}10^{7}$	460	$1{,}44\text{x}10^{-5}$	**I**	≥256	X
				II		
				III		
K56-17	$5{,}40\text{x}10^{8}$	110	$2{,}04\text{x}10^{-7}$	I	32	
				II	48	X
				III	24	
K56-18	$8{,}70\text{x}10^{7}$	830	$9{,}54\text{x}10^{-6}$	I	8	
				II	24	X
				III	12	
K56-20	$4{,}50\text{x}10^{8}$	60	$1{,}33\text{x}10^{-7}$	I		
				II	32	X
				III	0,5	
K56-23	$1{,}46\text{x}10^{8}$	1340	$9{,}18\text{x}10^{-6}$	**I**	32	X
				II		
				III		
K56-24	$1{,}79\text{x}10^{8}$	60	$3{,}35\text{x}10^{-7}$	**I**	64	X
				II		
				III		
K56-25	$3{,}90\text{x}10^{8}$	840	$2{,}15\text{x}10^{-6}$	I		
				II	24	X
				III		
K56-30	$1{,}10\text{x}10^{7}$	760	$6{,}91\text{x}10^{-5}$	I	≥256	
				II		
				III		
K56-31	$7{,}50\text{x}10^{8}$	1110	$1{,}48\text{x}10^{-6}$	**I**	24	X
				II		
				III		
K56-66	$5{,}70\text{x}10^{8}$	90	$1{,}58\text{x}10^{-7}$	**I**	≥256	X
				II		X
				III		
K56-69	$1{,}66\text{x}10^{8}$	170	$1{,}02\text{x}10^{-6}$	**I**	≥256	X
				II	48	X
				III	128	
K56-71	$2{,}43\text{x}10^{8}$	570	$2{,}35\text{x}10^{-6}$	**I**	48	X
				II		
				III		

The table continues on the next page.

Parental isolate	CFU/mL inoculum	CFU/mL mutants	Mutation frequency	Isolated mutants	MIC (μg/mL)	Species confirmation MALDI-TOF
K56-76	$1{,}20x10^{9}$	760	$6{,}33x10^{-7}$	I	24	X
				II		
				III		
K56-77	$6{,}00x10^{8}$	190	$3{,}17x10^{-7}$	I	48	X
				II		X
				III		
K56-80	$4{,}12x10^{8}$	30	$7{,}28x10^{-8}$	I	48	X
				II		X
				III		

Appendix B: An example of MALDI-TOF result for confirmation of species.

Analyte name	Analyte ID	Organism (best match)	Score value	Organism (second best match)	Score value
A6	1	*Escherichia coli*	2,439	*Escherichia coli*	2,317
A7	1	*Escherichia coli*	2,322	*Escherichia coli*	2,306
A8	1	*Escherichia coli*	2,341	*Escherichia coli*	2,335
A9	1	*Escherichia coli*	2,44	*Escherichia coli*	2,421

Appendix C: IC_{90}-determination. Tested for eight different antimicrobial agents on ten parental WTs and their mecillinam resistant mutants. The table displays fold changes and MIC-values for the respective isolates.

Amoxicillin

Strains:	2-fold highest tested concentration	1,5-fold highest tested concentration	MIC value (µg/mL)		Fold change	
			IC90 2-fold	IC90 1,5-fold	IC90 2-fold	IC90 1,5-fold
K56-5 WT	64 µg/mL	8 µg/mL	2	2	2	2
K56-5 MEC	64 µg/mL	8 µg/mL	4	4		
K56-17 WT	64 µg/mL	8 µg/mL	2	2	0,5	0,5
K56-17 MEC	64 µg/mL	8 µg/mL	1	1		
K56-18 WT	64 µg/mL	8 µg/mL	2	1,5	1	1
K56-18 MEC	64 µg/mL	8 µg/mL	2	1,5		
K56-20 WT	64 µg/mL	8 µg/mL	2	2	1	0,5
K56-20 MEC	64 µg/mL	8 µg/mL	2	1		
K56-23 WT	64 µg/mL	8 µg/mL	4	3	0,25	≤0,125
K56-23 MEC	64 µg/mL	8 µg/mL	1	≤0,375		
K56-24 WT	64 µg/mL	8 µg/mL	2	1,5	1	1
K56-24 MEC	64 µg/mL	8 µg/mL	2	1,5		
K56-31 WT	64 µg/mL	8 µg/mL	2	3	2	1
K56-31 MEC	64 µg/mL	8 µg/mL	4	3		
K56-66 WT	64 µg/mL	8 µg/mL	4	4	0,125	0,125
K56-66 MEC	64 µg/mL	8 µg/mL	0,5	0,5		
K56-69 WT	64 µg/mL	8 µg/mL	8	6	1	1
K56-69 MEC	64 µg/mL	8 µg/mL	8	6		
K56-71 WT	64 µg/mL	8 µg/mL	4	4	1	1
K56-71 MEC	64 µg/mL	8 µg/mL	4	4		
ATCC	64 µg/mL	8 µg/mL	8 or 4	6 or 4		
K56-44 MEC	64 µg/mL	8 µg/mL	8 or 4	4 or 3		

Chloramphenicol

Strain:	2-fold highest tested concentration	1,5-fold highest tested concentration	MIC value (µg/mL)		Fold change	
			IC90 2-fold	IC90 1,5-fold	IC90 2-fold	IC90 1,5-fold
K56-5 WT	32 µg/mL	32 µg/mL	4	3	1	1
K56-5 MEC	32 µg/mL	32 µg/mL	4	3		
K56-17 WT	32 µg/mL	32 µg/mL	4	6	0,5	0,333
K56-17 MEC	32 µg/mL	32 µg/mL	2	2		
K56-18 WT	32 µg/mL	32 µg/mL	4	4	1	0,5
K56-18 MEC	32 µg/mL	32 µg/mL	4	2		
K56-20 WT	32 µg/mL	32 µg/mL	4	4	1	0,5
K56-20 MEC	32 µg/mL	32 µg/mL	4	2		
K56-23 WT	32 µg/mL	32 µg/mL	8	4	0,5	0,5
K56-23 MEC	32 µg/mL	32 µg/mL	4	2		
K56-24 WT	32 µg/mL	32 µg/mL	2	2	1	≤0,75
K56-24 MEC	32 µg/mL	32 µg/mL	2	≤1,5		
K56-31 WT	32 µg/mL	32 µg/mL	4	8	2	0,75
K56-31 MEC	32 µg/mL	32 µg/mL	8	6		
K56-66 WT	32 µg/mL	32 µg/mL	8	6	1	0,25
K56-66 MEC	32 µg/mL	32 µg/mL	8	≤1,5		
K56-69 WT	32 µg/mL	32 µg/mL	8	12	1	1
K56-69 MEC	32 µg/mL	32 µg/mL	8	12		
K56-71 WT	32 µg/mL	32 µg/mL	8	6	1	0,667
K56-71 MEC	32 µg/mL	32 µg/mL	8	4		
ATCC	32 µg/mL	32 µg/mL	4	3 or 4		
K56-44 MEC	32 µg/mL	32 µg/mL	4	3		

Ciprofloxacin

Strain:	2-fold highest tested concentration	1,5-fold highest tested concentration	MIC value (µg/mL)		Fold change	
			IC90 2-fold	IC90 1,5-fold	IC90 2-fold	IC90 1,5-fold
K56-5 WT	2 µg/mL	0,125 µg/mL	0,008	0,012	2	1
K56-5 MEC	2 µg/mL	0,125 µg/mL	0,016	0,012		
K56-17 WT	2 µg/mL	0,125 µg/mL	0,016	0,016	0,5	0,75
K56-17 MEC	2 µg/mL	0,125 µg/mL	0,008	0,012		
K56-18 WT	2 µg/mL	0,125 µg/mL	0,008	0,012	2	1
K56-18 MEC	2 µg/mL	0,125 µg/mL	0,016	0,012		
K56-20 WT	2 µg/mL	0,125 µg/mL	0,008	0,008	1	1,5
K56-20 MEC	2 µg/mL	0,125 µg/mL	0,008	0,012		
K56-23 WT	2 µg/mL	0,125 µg/mL	0,016	0,012	1,98	2,58
K56-23 MEC	2 µg/mL	0,125 µg/mL	0,031	0,031		
K56-24 WT	2 µg/mL	0,125 µg/mL	0,008	≤0,006	1	≥2
K56-24 MEC	2 µg/mL	0,125 µg/mL	0,008	0,012		
K56-31 WT	2 µg/mL	0,125 µg/mL	0,008	0,012	2	1,333
K56-31 MEC	2 µg/mL	0,125 µg/mL	0,016	0,016		
K56-66 WT	2 µg/mL	0,125 µg/mL	0,016	0,016	1	0,75
K56-66 MEC	2 µg/mL	0,125 µg/mL	0,016	0,012		
K56-69 WT	2 µg/mL	0,125 µg/mL	0,016	0,023	2	2,04
K56-69 MEC	2 µg/mL	0,125 µg/mL	0,031	0,047		
K56-71 WT	2 µg/mL	0,125 µg/mL	0,008	0,016	2	0,75
K56-71 MEC	2 µg/mL	0,125 µg/mL	0,016	0,012		
ATCC	2 µg/mL	0,125 µg/mL	0,008	0,008		
K56-44 MEC	2 µg/mL	0,125 µg/mL	0,016	0,016		

Gentamicin

Strain:	2-fold highest tested concentration	1,5-fold highest tested concentration	MIC value (µg/mL)		Fold change	
			IC90 2-fold	IC90 1,5-fold	IC90 2-fold	IC90 1,5-fold
K56-5 WT	32 µg/mL	1 µg/mL	0,125	0,125	2	3
K56-5 MEC	32 µg/mL	1 µg/mL	0,25	0,375		
K56-17 WT	32 µg/mL	1 µg/mL	0,125	0,188	1	0,5
K56-17 MEC	32 µg/mL	1 µg/mL	0,125	0,094		
K56-18 WT	32 µg/mL	1 µg/mL	0,5	0,25	0,25	0,375
K56-18 MEC	32 µg/mL	1 µg/mL	0,125	0,094		
K56-20 WT	32 µg/mL	1 µg/mL	0,25	0,25	0,5	0,5
K56-20 MEC	32 µg/mL	1 µg/mL	0,125	0,125		
K56-23 WT	32 µg/mL	1 µg/mL	1	0,375	0,25	≤0,125
K56-23 MEC	32 µg/mL	1 µg/mL	0,25	≤0,047		
K56-24 WT	32 µg/mL	1 µg/mL	0,5	0,188	0,5	1
K56-24 MEC	32 µg/mL	1 µg/mL	0,25	0,188		
K56-31 WT	32 µg/mL	1 µg/mL	0,25	0,25	1	1
K56-31 MEC	32 µg/mL	1 µg/mL	0,25	0,25		
K56-66 WT	32 µg/mL	1 µg/mL	0,5	0,25	0,5	0,75
K56-66 MEC	32 µg/mL	1 µg/mL	0,25	0,188		
K56-69 WT	32 µg/mL	1 µg/mL	0,25	0,188	0,5	0,665
K56-69 MEC	32 µg/mL	1 µg/mL	0,125	0,125		
K56-71 WT	32 µg/mL	1 µg/mL	0,25	0,25	2	0,75
K56-71 MEC	32 µg/mL	1 µg/mL	0,5	0,188		
ATCC	32 µg/mL	1 µg/mL	0,25 or 0,5	0,25 or 0,75		
K56-44 MEC	32 µg/mL	32 µg/mL	1 or 2	16		

Mecillinam

Strain:	2-fold highest tested concentration	1,5-fold highest tested concentration	MIC value (µg/mL)		Fold change	
			IC90 2-fold	IC90 1,5-fold	IC90 2-fold	IC90 1,5-fold
K56-5 WT	4 µg/mL	4 µg/mL	0,125	≤0,188	≥32	≥21
K56-5 MEC	4 µg/mL	4 µg/mL	≥4	≥4		
K56-17 WT	4 µg/mL	4 µg/mL	0,125	≤0,188	≥32	≥21
K56-17 MEC	4 µg/mL	4 µg/mL	≥4	≥4		
K56-18 WT	4 µg/mL	4 µg/mL	0,25	≤0,188	≥16	≥21
K56-18 MEC	4 µg/mL	4 µg/mL	≥4	≥4		
K56-20 WT	4 µg/mL	4 µg/mL	0,125	≤0,188	32	21
K56-20 MEC	4 µg/mL	4 µg/mL	4	4		
K56-23 WT	4 µg/mL	1 µg/mL	0,125	0,063	≥32	≥507
K56-23 MEC	4 µg/mL	32 µg/mL	≥4	≥32		
K56-24 WT	4 µg/mL	1 µg/mL	0,063	0,063	≥64	≥24
K56-24 MEC	4 µg/mL	32 µg/mL	≥4	24		
K56-31 WT	4 µg/mL	4 µg/mL	0,125	≤0,188	≥32	≥21
K56-31 MEC	4 µg/mL	4 µg/mL	≥4	≥4		
K56-66 WT	4 µg/mL	1 µg/mL	0,125	0,094	16	≥64
K56-66 MEC	4 µg/mL	16 µg/mL	2	2		
K56-69 WT	4 µg/mL	1 µg/mL	0,25	0,125	≥16	≥128
K56-69 MEC	4 µg/mL	16 µg/mL	≥4	≥16		
K56-71 WT	4 µg/mL	1 µg/mL	0,25	0,188	≥16	85
K56-71 MEC	4 µg/mL	16 µg/mL	≥4	16		
ATCC	4 µg/mL	1 µg/mL	0,125	0,094		
K56-44 MEC	4 µg/mL	16 µg/mL	≥4	≥12		

Nitrofurantoin

Strain:	2-fold highest tested concentration	1,5-fold highest tested concentration	MIC value (µg/mL)		Fold change	
			IC90 2-fold	IC90 1,5-fold	IC90 2-fold	IC90 1,5-fold
K56-5 WT	64 µg/mL	32 µg/mL	8	6	1	1
K56-5 MEC	64 µg/mL	32 µg/mL	8	6		
K56-17 WT	64 µg/mL	32 µg/mL	8	8	1	0,75
K56-17 MEC	64 µg/mL	32 µg/mL	8	6		
K56-18 WT	64 µg/mL	32 µg/mL	16	12	0,5	0,667
K56-18 MEC	64 µg/mL	32 µg/mL	8	8		
K56-20 WT	64 µg/mL	32 µg/mL	4	4	2	1,5
K56-20 MEC	64 µg/mL	32 µg/mL	8	6		
K56-23 WT	64 µg/mL	32 µg/mL	8	8	0,5	0,375
K56-23 MEC	64 µg/mL	32 µg/mL	4	3		
K56-24 WT	64 µg/mL	32 µg/mL	8	3	1	1
K56-24 MEC	64 µg/mL	32 µg/mL	8	3		
K56-31 WT	64 µg/mL	32 µg/mL	16	12	1	1
K56-31 MEC	64 µg/mL	32 µg/mL	16	12		
K56-66 WT	64 µg/mL	32 µg/mL	8	6	1	0,667
K56-66 MEC	64 µg/mL	32 µg/mL	8	4		
K56-69 WT	64 µg/mL	32 µg/mL	16	8	1	1,5
K56-69 MEC	64 µg/mL	32 µg/mL	16	12		
K56-71 WT	64 µg/mL	32 µg/mL	4	8	1	1
K56-71 MEC	64 µg/mL	32 µg/mL	4	8		
ATCC	64 µg/mL	32 µg/mL	4 or 8	4 or 8		
K56-44 MEC	64 µg/mL	32 µg/mL	4 or 8	6, 8 or 12		

Tetracycline

Strain:	2-fold highest tested concentration	1,5-fold highest tested concentration	MIC value (µg/mL)		Fold change	
			IC90 2-fold	IC90 1,5-fold	IC90 2-fold	IC90 1,5-fold
K56-5 WT	16 µg/mL	4 µg/mL	1	0,75	1	1
K56-5 MEC	16 µg/mL	4 µg/mL	1	0,75		
K56-17 WT	16 µg/mL	4 µg/mL	1	0,75	1	1
K56-17 MEC	16 µg/mL	4 µg/mL	1	0,75		
K56-18 WT	16 µg/mL	4 µg/mL	1	0,75	1	1
K56-18 MEC	16 µg/mL	4 µg/mL	1	0,75		
K56-20 WT	16 µg/mL	4 µg/mL	1	0,75	2	1
K56-20 MEC	16 µg/mL	4 µg/mL	2	0,75		
K56-23 WT	16 µg/mL	4 µg/mL	1	0,75	2	1,333
K56-23 MEC	16 µg/mL	4 µg/mL	2	1		
K56-24 WT	16 µg/mL	4 µg/mL	0,5	0,25	1	1,5
K56-24 MEC	16 µg/mL	4 µg/mL	0,5	0,375		
K56-31 WT	16 µg/mL	4 µg/mL	1	0,75	2	1
K56-31 MEC	16 µg/mL	4 µg/mL	2	0,75		
K56-66 WT	16 µg/mL	4 µg/mL	2	1	0,25	0,5
K56-66 MEC	16 µg/mL	4 µg/mL	0,5	0,5		
K56-69 WT	16 µg/mL	4 µg/mL	2	1	1	1
K56-69 MEC	16 µg/mL	4 µg/mL	2	1		
K56-71 WT	16 µg/mL	4 µg/mL	1	0,75	1	1
K56-71 MEC	16 µg/mL	4 µg/mL	1	0,75		
ATCC	16 µg/mL	4 µg/mL	1	0,5 or 0,75		
K56-44 MEC	16 µg/mL	4 µg/mL	1	0,75 or 0,5		

Trimethoprim

Strain:	2-fold highest tested concentration	1,5-fold highest tested concentration	MIC value (µg/mL)		Fold change	
			IC90 2-fold	IC90 1,5-fold	IC90 2-fold	IC90 1,5-fold
K56-5 WT	32 µg/mL	2 µg/mL	0,25	0,19	1	2
K56-5 MEC	32 µg/mL	2 µg/mL	0,25	0,38		
K56-17 WT	32 µg/mL	2 µg/mL	0,5	0,38	0,5	0,5
K56-17 MEC	32 µg/mL	2 µg/mL	0,25	0,19		
K56-18 WT	32 µg/mL	2 µg/mL	0,25	0,19	0,5	0,667
K56-18 MEC	32 µg/mL	2 µg/mL	0,13	0,13		
K56-20 WT	32 µg/mL	2 µg/mL	0,25	0,38	1	0,658
K56-20 MEC	32 µg/mL	2 µg/mL	0,25	0,25		
K56-23 WT	32 µg/mL	2 µg/mL	0,5	0,38	2	2
K56-23 MEC	32 µg/mL	2 µg/mL	1	0,75		
K56-24 WT	32 µg/mL	2 µg/mL	0,25	0,25	1	0,752
K56-24 MEC	32 µg/mL	2 µg/mL	0,25	0,188		
K56-31 WT	32 µg/mL	2 µg/mL	0,5	0,38	1	1
K56-31 MEC	32 µg/mL	2 µg/mL	0,5	0,38		
K56-66 WT	32 µg/mL	2 µg/mL	1,0	0,38	0,5	1
K56-66 MEC	32 µg/mL	2 µg/mL	0,5	0,38		
K56-69 WT	32 µg/mL	2 µg/mL	0,5	0,5	2	1,5
K56-69 MEC	32 µg/mL	2 µg/mL	1,0	0,75		
K56-71 WT	32 µg/mL	2 µg/mL	0,25	0,375	1	1
K56-71 MEC	32 µg/mL	2 µg/mL	0,25	0,375		
ATCC	32 µg/mL	2 µg/mL	0,5	0,38 or 0,5		
K56-44 MEC	32 µg/mL	2 µg/mL	0,25 or 0,5	0,25		

Appendix D: Clinical breakpoints for various antimicrobial agents regarding Enterobacteriaceae. The values are defined by EUCAST (93).

Drug	Enterobacteriacea acceptable limits of MIC (μg/mL)			
	EUCAST MIC		ATCC 25922	
	Susceptible	Resistant	QC Limits	K56-44 MEC
Amoxicillin	≤8	>8	2-8	4-16
Chloramphenicol	≤8	>8	2-8	1,5-6
Ciprofloxacin	≤0,5	>1	0,004-0,016	0,012-0,048
Gentamicin	≤2	>4	0,25-1	1,5-6
Mecillinam	≤8	>8	0,03-0,25	16-64
Nitrofurantoin	≤64	>64	4-16	6-24
Tetracycline	≤4	≥16	0,5-2	0,38-1,5
Trimethoprim	≤2	>4	0,5-2	0,25-1