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Collateral sensitivity in clinical *Escherichia coli* isolates

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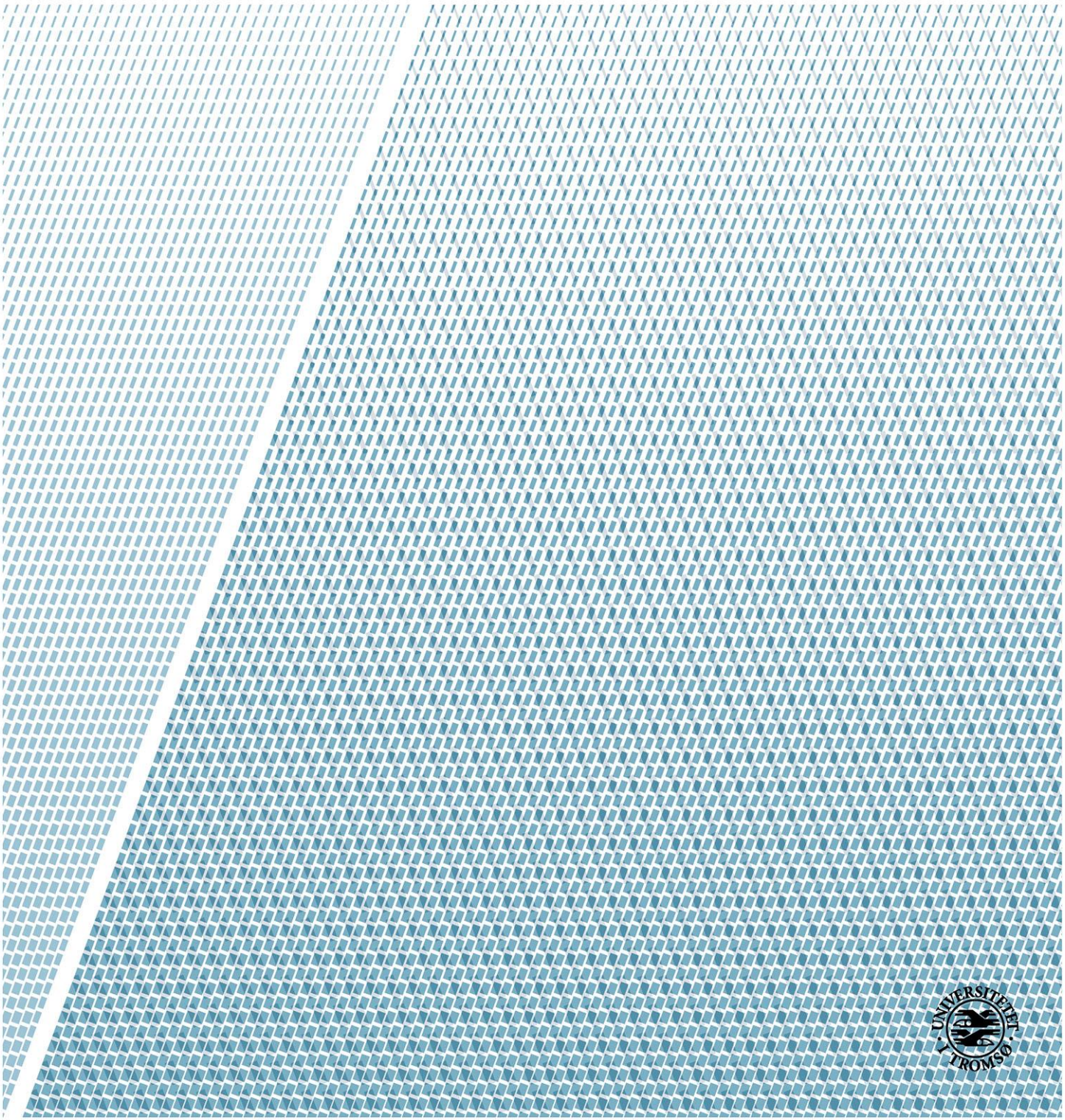

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ABSTRACT

Background

Antimicrobial resistance is a well-known problem and is becoming more dangerous than ever. Antimicrobial resistance threatens the effective prevention and treatment of infectious diseases, and is an increasing threat to global public health. There are still a wide variety of antimicrobial drugs on the market, but the emergence of antimicrobial resistance appears at a higher rate than the development of new antibiotics. An important, but challenging task to combat infections and to slow down the emergence of antimicrobial resistance is to find novel strategies by using existing drugs in an appropriate way.

The continuous increase in resistance development towards antimicrobial agents is a well-known problem, however, insecurities exist about how frequently the emergence of resistance in microbes, at the same time, leads to an increased sensitivity to other antimicrobial agents. This phenomenon is called "collateral sensitivity".

Methods

We evolved resistance in clinical *E. coli* strains towards three antimicrobial drugs used clinically in the treatment of urinary tract infections to determine collateral effects towards seven selected antibiotics. The collateral effects were confirmed by determination of changes in susceptibility by performing E-tests. The resulting collateral sensitivity and resistance profiles were mapped to see if there existed a network or general pattern of collateral sensitivity.

Results

The results predominantly showed a higher level of collateral sensitivity between resistant mutants and drug with an increase in minimum inhibiotiry concentration (MIC) up to 32-fold for the mutant compared to its respective wild type. A low level of collateral resistance was also observed, but collateral sensitivity was observed to a greater extent than collateral resistance. Overall, our project supported a general pattern for the different *E. coli* resistant isolates.

Conclusion

This project provides a proof-of-principle of the phenomenon "collateral sensitivity" and can thus contribute to sustainable use of antimicrobials clinically by slowing down the rate of resistance evolution.

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ABBREVIATIONS

0	No change in susceptibility profile
ddH_2O	Double distilled water
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
E-test	Epsilometer test
ECOFF	The epidemiological cut-off value
ESBL	Extended-spectrum β-lactamase
EtBr	Ethidium bromide
HGT	Horizontal gene transfer
LAF	Laminar Air Flow
LB	Luria Broth Base
LBA	Luria Broth Agar
MHA-plate	Muller-Hinton II agar plate
MIC	Minimal inhibitory concentration
NR	No results
ONC	Overnight culture
PABA	Para-amino-benzoic-acid
PBP	Penicillin binding proteins
PFGE	Pulsed-field gel electrophoresis
R	Collateral resistance
RAPD	Randomly amplified polymorphic DNA analysis
rpm	Rotations per minute
RT	Room temperature
S	Collateral sensitivity
UTI	Urinary tract infections
WT	Wild type

1 INTRODUCTION

1.1 Preface

In 1859, Charles Darwin introduced the term "natural selection" after a voyage around the world studying plants and animals. "Natural selection" describes an evolutionary theory, which acts upon differences in reproductive capability between individuals within a population. Darwin observed several species of finches on the Galápagos Islands and noticed that they differed from each other in beak size and shape. He realized that the differences were depending on the type of environment the species' lived in. Each species' particular variation of beak size and shape apparently gave them an advantage to survive and reproduce in a particular environment. Among the Galápagos finches, as well as bacteria and other organisms, nature selects the best adapted individuals to survive and reproduce (Darwin, 1859).

There are still unanswered questions in relation to bacterial evolution, but the way humans have over- and misused todays existing antimicrobial agents can be seen as an evolutionary experiment.

1.2 Antimicrobial drug resistance

Antimicrobial resistance has become a serious threat to global public health. Inappropriate prescriptions, over- and misuse of antimicrobial drugs has led to a rise of resistance and threatens the effective prevention and treatment of common infectious diseases. The discovery of antibiotic drugs in the 20th century was regarded as the everlasting solution to cure infectious diseases, but bacteria continuously develop mechanisms to detect, evolve and adapt to changing conditions. The WHO reports 25 000 deaths per year in Europe following to the emergence of antimicrobial resistance. This costs our society approximately £1,5 billion per year (WHO, 2014).

A wide variety of antimicrobial agents is available on the market today, but the emergence of antimicrobial resistance appears at a higher rate than the development of new antibiotics. As WHO stated: we may be heading towards a post-antibiotic era.

1.3 Antimicrobial agents

Antimicrobial agents can be broadly divided into those that kill the bacterial cell (bactericides) or those that prevent the growth of bacteria (bacteriostatics) (Pankey and Sabath, 2004). A more accurate and common method to classify the antimicrobial agents is based on their mechanism of action: interference with cell envelope synthesis, inhibition of protein synthesis, interference with nucleic acid metabolism and inhibition of metabolic pathways.

1.3.1 Interference with cell envelope synthesis

This group includes beta lactam antibiotics such as penicillins, cephalosporins, carbapenems and monobactams, but also includes glycopeptides (such as vancomycin) and polymyxins (such as colistin) and more. Beta lactam antibiotics interfere with the synthesis of the peptidoglycan of the bacterial cell wall. Penicillin irreversibly binds to so called penicillin binding proteins (PBPs). These are enzymes (e.g. transpeptidases) that crosslink the peptide chains attached to the backbone of the peptidoglycan. The bactericidal step involves the inactivation of an inhibitor of autolytic enzymes in the cell wall, leading to cell lysis. Glycopeptides also inhibit the synthesis of the bacterial cell wall, but at an earlier stage. Polymycxin antibiotics disrupt the outer cell membrane (Flower et al., 2011).

1.3.2 Inhibition of protein synthesis

Tetracyclines are broad-spectrum antibiotics and are regarded as bacteriostatic. Both, aminoglycosides and tetracyclines inhibit bacterial protein synthesis by binding to the 30S subunit of the bacterial ribosome. Macrolides in contrast, bind to the 50S subunit of the bacterial ribosome and affect protein synthesis by inhibiting translocation of the growing peptide chain (Flower et al., 2011).

1.3.3 Interference with nucleic acid metabolism

Quinolones inhibit bacterial DNA gyrase (topoisomerase II), the enzyme that produces a negative supercoil in DNA and topoisomerase IV. Bacterial transcription or DNA replication will be inhibited. Ciprofloxacin is a broad-spectrum antibiotic and the most commonly used fluoroquinolone (Flower et al., 2011).

1.3.4 Inhibition of metabolic pathways

Para-amino-benzoic-acid (PABA) is an essential precursor in the synthesis of folic acid, which is required for the synthesis of DNA and RNA in bacteria. Sulfonamides are structural analogues of PABA and compete with PABA for the enzyme dihydropteroate synthetase that converts PABA to folate, thus prohibiting the synthesis of precursors for nucleic acid synthesis. While sulfonamides interfere early in the synthesis of DNA, the folate antagonist trimethoprim inhibits the enzyme dihydrofolate reductase (DHFR), one step later in the synthesis of DNA precursors. Clinically, sulfonamides are often combined with trimethoprim (Flower et al., 2011).

1.4 Emergence of resistance towards antimicrobials

"Some men are born great, some achieve greatness, and some have greatness thrust upon them", William Shakespeare wrote in his comedy *Twelfth Night* in 1601. Likewise, bacteria may be naturally antibiotic resistant or resistance can be thrust upon them. Antibiotic resistance may be acquired either by mutations, genetic rearrangements, or acquisition of resistance genes from external sources by horizontal gene transfer (HGT). Non-inheritable resistance in cells called persisters, contributes to treatment failure without resistance traits being genetically encoded (Levin and Rozen, 2006). Persisters are not mutants, but are survivors after an antimicrobial treatment.

1.4.1 Mutations

Mutations can occur spontaneously or they are induced. Spontaneous mutations appear due to a variety of sources, which in the end lead to errors in DNA replication. Subsequently, resistance may emerge because chromosomal genes that do not affect antibiotic susceptibility suddenly code for resistance (Khachatourians, 1998). Mutations can be categorized as deleterious (harmful), neutral or advantageous (beneficial). These categories are based on their effect on fitness, where advantageous mutations increase fitness, deleterious mutations decrease it and neutral mutations do not affect the fitness of bacteria (Loewe and Hill, 2010). Mutations are rare events and occur only at a frequency of $10^{-7} - 10^{-11}$ (Madigan and Martinko, 2006), but nevertheless they contribute to the rise of antibiotic resistance in bacteria (Khachatourians, 1998).

1.4.2 Horizontal gene transfer

HGT includes three mechanisms of transfer of genetic material between bacteria, which are transformation, transduction and conjugation. A fourth mechanism of HGT has recently been proposed and involved outer membrane vesicles (Fulsundar et al., 2014). To understand the mechanisms of HGT, it is important to be able to conceive the evolution of bacterial genomes and also the dissemination of antibiotic resistance genes.

1.5 Resistance mechanisms

Antimicrobial drug resistance, from a microbiological perspective, means the ability of microbes to resist the action of naturally occurring or synthetically produced compounds with bactericidal or bacteriostatic effect (Tapsall et al., 2005). While a wide range of antimicrobial agents were discovered and developed since the 1940s, bacterial have in parallel evolved resistance to many of the available agents (Levin et al., 2014)

Resistance mechanisms can be broadly divided in to three main types:

1.5.1 Altered access to target (altered uptake)

The outer membrane of Gram-negative bacteria makes them less permeable to many antibiotics as compared to Gram-positive bacteria (Kojima and Nikaido, 2013). Another mechanism that decreases the amount of drug that reaches the target is the increased transport of antibiotics out of the cell by bacterial efflux pumps.

1.5.2 Altered target

The antibiotic specifically binds to its target with high affinity, thus preventing the target's normal function. The target may be altered in such way that the affinity of the antibiotic is lowered and the target can function normally. An example is methicillin-resistant staphylococci, which synthesizes an additional PBP to which beta lactams show a much lower affinity (Barber, 1961).

1.5.3 Drug inactivation

Bacteria can resist the action of antibiotics by enzymatic modification or degradation of the antibiotic. Since the discovery of β-lactamase in 1940, thousands of enzymes have been discovered that are also able to modify and inactivate antibiotics (Abraham and Chain, 1940). The antibiotic can be inactivated by modification or the addition of a chemical group causing a steric hindrance for the antibiotic to bind the target site. Different chemical groups can be added to the antibiotic through enzymatic action - including acyl, phosphate and several other groups. The responsible enzymes form a large group of antibiotic-modifying enzymes (Wright, 2005).

1.6 Collateral sensitivity

We are in desperate need of new agents for the treatment of infectious diseases. While we await the development of new antimicrobial agents, novel strategies have been established to maximize efficacy of already existing agents and to reduce the emergence of resistance. The most prominent strategies are drug stewardship programs and combination therapies. For infectious diseases such as tuberculosis, malaria and the human immunodeficiency virus (HIV), combination therapy has been introduced as a golden rule in the treatment of these diseases (Gogtay et al., 2013, Mitchison, 2012, Thompson et al., 2012). In 1953, Garrod discussed the theory of combining one or two chemotherapeutic agents in bacterial infections (Garrod, 1952).

1.6.1 Collateral sensitivity and its usage in drug cycling

Another treatment strategy is a scheduled cycling of different antibiotics, called drug cycling (see Figure 1). It has been demonstrated that an isolate, which develops resistance towards a given antimicrobial agent can show a decreased susceptibility to other antimicrobials (Lázár et al., 2013, Imamovic and Sommer, 2013, Rodriguez de Evgrafov et al., 2015). Nevertheless, it is still unclear how the emergence of resistance for one antimicrobial agent increases susceptibility to other antimicrobials. This phenomenon is called "collateral sensitivity" and may be a solution to slow down the rate of resistance emergence in bacteria.

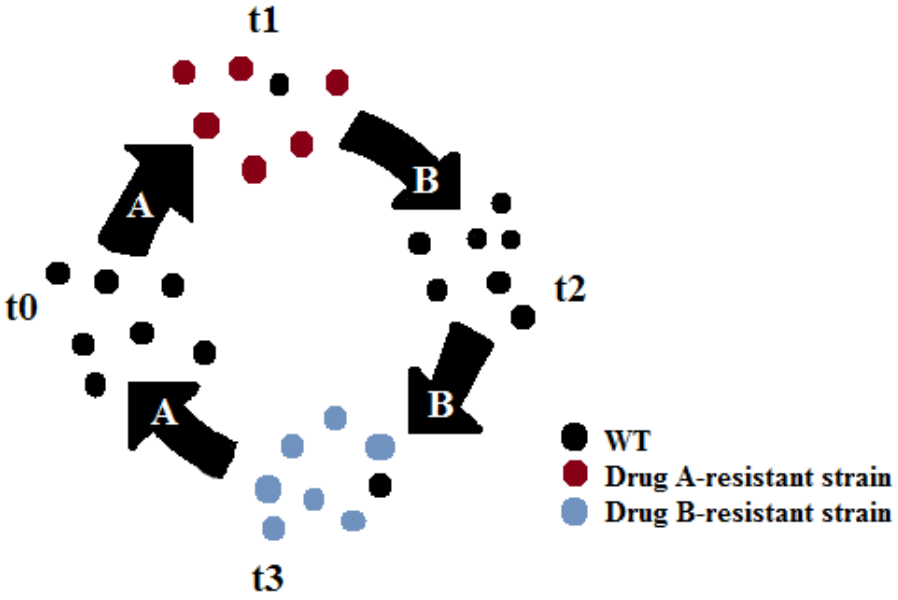

Figure 1: Illustration of drug cycling with drug A and B. At t_0, treatment of the disease-causing WT population (black) starts with drug A. Over time in presence of drug A, resistance to drug A arises (red) and drug A becomes ineffective at t_1. Then, drug A is replaced with drug B, to which drug A-resistant cells show collateral sensitivity at t_2. An elimination of drug A-resistant cells and selection for WT resistance levels occurs. Resistance to drug B (blue) arises at t_3 and treatment is altered back to drug A and the course of the same events continues. Adapted and modified from Imamovic and Sommer 2013.

1.6.2 Previous work on collateral sensitivity

The first work based on this phenomenon was published in 1952. Szybalski and Bryson mapped the occurrence of cross-resistance of *Escherichia coli* to fifteen antibiotics. This work did not get a lot of attention when published, probably because of all the new antimicrobial agents coming onto the market. Unlike today, there was no need for a strategy to counter resistance.

We know that bacteria can adapt to the over-, and misuse of antimicrobial agents and become resistant to these agents. After bacteria become resistant to a specific antibiotic they can develop new phenotypical properties. These resistant strains may show a lower, equal or higher susceptibility than the parental strain to other antibiotics (Szybalski and Bryson, 1952). This is the foundation behind the drug cycling strategy.

Imamovic and Sommer continued to work on the phenomenon of collateral sensitivity in 2013, where they exposed *E. coli* to 23 different antibiotics. As previously, they observed that the bacteria's susceptibility to other antibiotics changed and became more sensitive to other and related drugs. This suggests a new antibiotic stewardship where drug cycling relies on collateral sensitivity effects and hopefully increases the life span of many antimicrobial agents, eventually preventing the emergence of resistant strains (Imamovic and Sommer, 2013). Imamovic and Sommer did also test whether collateral sensitivity affects the kill kinetics of resistant bacteria when drugs were applied at concentrations that were above wild type MIC. They showed that cefuroxime (or gentamycin) resistant *E. coli* strains exposed to a drug to which they were collaterally sensitive were eradicated more quickly than the WT. This finding means that treatment within the collateral sensitivity cycling paradigm could offer more suppression of resistant infections compared to drug cycling without reciprocal collateral sensitivities (Imamovic and Sommer, 2013).

1.6.3 Fitness cost of resistance

In the absence of antibiotics, the expression of antibiotic resistance can influence the growth rate or competitive ability of bacteria, referred to as the fitness cost of resistance. Resistance is often associated with reduced bacterial fitness, which is measured in changes in the relative reproduction rate of the organism. These findings suggest that a reduction in antibiotic use would benefit susceptible bacteria, making them able to outcompete resistant strains over time (Levin et al., 1997, Andersson and Levin, 1999).

1.7 Microbial characterization

In this project, clinical strains from the ECO-SENS project were studied (see 1.9). Compared to laboratory strains, the information about these clinical strains are obscure, except from the information about their susceptibility profile which is pan-susceptible. In microbiology, the identification and characterization of a microbial isolate is essential. There are technologies to detect small variations within species and within individual strains.

1.7.1 Species identification

Sequencing specific sections of ribosomal DNA, called 16S rRNA gene sequencing, can be used to identify bacterial species, as it is the case with random amplified polymorphic (RAPD) PCR amplification analysis (see 0). The results in 16S rRNA sequencing will then be compared to sequences on a database, while the results of RAPD PCR will be compared to a positive control (Janda and Abbott, 2007).

1.7.2 Strain typing

Identifying bacteria at the strain level, called strain typing, can be broadly divided into two categories: phenotypic methods and genotypic methods. Phenotypic methods are used to differentiate strains by characterization of the products of gene expression (Tenover et al., 1997). However, biochemical tests, pathogenicity, killer toxin susceptibility, and antibiotic susceptibility are not variable enough to distinguish closely related strains (Li et al., 2009). Over the last two decades, the phenotypic methods have been replaced by molecular methods.

Genotypic methods, also referred to as DNA fingerprinting, are based on an analysis of the genetic composition of an organism (Tenover et al., 1997). The result of a specific genotyping method can be as unique as a fingerprint. These techniques have become widely used for bacterial strain typing (Li et al., 2009). In our project, the RAPD PCR analysis was used. RAPD PCR is not as widely used as other genotypic methods.

1.8 Antimicrobial susceptibility testing and clinical breakpoints

In clinical microbiology, antimicrobial susceptibility testing of bacterial isolates is important to confirm susceptibility to specific antimicrobial agents or to detect possible drug resistance in bacterial isolates (antibiogram). Antimicrobial susceptibility is important for clinicians to decide the appropriate course of treatment against bacterial infections.

There are several phenotypic methods, but the most widely used method is broth microdilution. Other techniques include disc diffusion and gradient diffusion. In our project, the gradient diffusion method was applied.

1.8.1 Gradient diffusion method

The Epsilometer test (E-test) assay enables quantification of a microorganism's' susceptibility to certain antimicrobial drugs by determining the MIC in μg/mL. The MIC is defined as the lowest concentration of antimicrobial agent required to inhibit growth of the microorganism (Andrews, 2001). The method is described in 0.

The method provides both the numeric MIC value and a qualitative assessment of the bacterial strain using the categories susceptible, intermediate and resistant, as described in the next paragraph.

1.8.2 Clinical breakpoints

Clinical breakpoints are used to predict the clinical outcome of treatment with antimicrobials. According to their MIC, an organism can be categorized as susceptible, intermediate or resistant for a given antibiotic (Reller et al., 2009):

- Susceptible (S): the organism responds to therapy using the recommended dosage of antimicrobial for the specific infection and species
- Intermediate (I): the MIC of the organism approaches or exceeds the threshold for normal dose of antimicrobial, but clinical response is possible with higher doses
- Resistant (R): the organism is not inhibited by concentrations with normal doses

In Europe, the organization EUCAST, (CLSI in the United States) can be considered as the major international contributors to antimicrobial susceptibility testing as

reviewed by Silley and co-workers (Silley, 2012). EUCAST publishes clinical breakpoints based on the antimicrobial's pharmacokinetic and pharmacodynamic properties and on clinical studies.

1.8.3 The epidemiological cut-off value (ECOFF)

The epidemiological cut-off value separates microorganisms without (wild type) and with acquired resistance mechanisms (non-wild type) to a given antibiotic. The values are determined by EUCAST based on the distribution of MICs for one drug in a single bacterial species.

1.9 ECO-SENS study and strains

In our project, clinical *E. coli* isolates from the ECO-SENS projects were studied. The ECO-SENS projects are the first international survey to investigate the prevalence and susceptibility of pathogens causing community-acquired acute uncomplicated urinary tract infections (UTI) (Kahlmeter, 2003, Kahlmeter and Poulsen, 2012). The first ECO-SENS study took place during 1999-2000. Midstream urine samples were taken from 4734 women (not older than 65 years) with symptoms of acute UTI. The samples were collected in 252 community health care centers in 17 countries (16 European countries and Canada).

The urinary tract pathogens found in the ECO-SENS strains, including *E. coli*, were identified and the susceptibility to 12 antimicrobial drugs was determined. In the *E. coli* isolates, 42 % were resistant to one or more of the 12 antimicrobials.

Kahlmeter continued the ECO-SENS project and during 2007-2008, urine samples were taken from 1697 women (aged 18-65 years) in Austria, Greece, Portugal, Sweden and the UK. The chosen countries represented areas of Europe indicated to have more or less problems with resistance. The results from this ECO-SENS II project were then compared to the results in the first ECO-SENS study, and the results showed an increase in resistance to quinolones and trimethoprim from 2.8 % to 5.9 % between the two studies.

The clinical *E. coli* isolates used in this project originate from four countries: Greece, Portugal, Sweden and the United Kingdom, where the isolate from Portugal originate from the first ECO-SENS study while the remaining strains originate from the second study.

1.10 Urinary tract infections

Several of the antibiotics studied here are commonly used antibiotics in Norway, many of them in the treatment of UTIs. According to epidemiological studies the incidence of UTIs is estimated to be 175 million cases worldwide annually (Foxman et al., 2000, Foxman, 2003). UTIs are a frequent reason for the prescription of antibiotics. To avoid an increase in antibiotic resistance, it is important that the decision for a specific antibiotic prescription is carefully considered.

1.10.1 What is UTI?

Urinary tract infections are the most common bacterial infections in women (Foxman, 2003). Acute uncomplicated UTI is the most common and is often caused by *E. coli* (86 %) (Gupta et al., 1999b). Uncomplicated UTI, or cystitis, without known comorbidities or urologic abnormalities is a superficial bacterial infection in the mucosa of the urine bladder and urethra. Symptoms of this condition are frequent urination and dysuria. Uncomplicated acute cystitis can last up to one week. Complicated cystitis is a condition when anatomic or other circumstances can affect the progress. Complicated cystitis includes all cystitis in children, men, pregnant women and elderly people.

1.10.2 Treatment of acute uncomplicated cystitis

According to the 2012 Norwegian guidelines, no single agent is regarded as the best choice to treat acute uncomplicated cystitis. Instead, the following three alternatives are equivalent:

1. Trimethoprim 160 mg x 2 or 300 mg in the evening for 1-3 days
2. Nitrofurantoin 50 mg x 3 for 3 days
3. Pivmecillinam 2++ mg x 3 for 3 days

Fluoroquinolones are consideres as alternative in complicated cases and when resistance to the conventional medication is evident (Health, 2012).

1.11 Aim, hypotheses and specific objectives

1.11.1 Aim

The aim of this project is to investigate networks of collateral sensitivity and resistance in clinical *E. coli* strains with pre-existing resistance determinants relevant for treatment of urinary tract infections.

1.11.2 Hypotheses

We hypothesize that collateral sensitivity and resistance networks for certain drugs display general patterns in clinical strain collections and that this information can provide the basis for novel evidence based treatment guidelines that delay the evolution of antibiotic resistance.

1.11.3 Specific objectives

1. Generate spontaneous antibiotic resistant isolates of five clinical *E. coli* strains, from the ECO-SENS-collection, and confirm changes in susceptibility by performing E-test for seven different antibiotics
2. Test the collateral effect of a specific set of antimicrobial drugs in the generated isolates of *E .coli*

2 MATERIALS

2.1 Bacterial strains

The bacterial strains studied in this project belonged to the ECO-SENS strain collection. The ECO-SENS studies took place in 1999-2000 and 2007-2008. The clinical *E. coli* isolates of this collection originated from urine samples of women from European countries (Canada was included in the first ECO-SENS study). The countries were chosen to represent areas of Europe where we see increasing numbers of resistant strains in UTIs (Kahlmeter, 2003, Kahlmeter and Poulsen, 2012). In this thesis, five different ECO-SENS isolates from Greece, Portugal, Sweden and the UK were selected and studied as shown in Table 1.

Table 1: ECO-SENS strains used for the generation of spontaneous resistant isolates in this project

Name	**Sequence type**	**Phylogroup**	**Year**	**Country**
K56-12	ST104	B2	2000	Portugal
K56-41	ST420	A	2007-2008	Greece
K56-50	ST100	A	2007-2008	Greece
K56-70	ST550	B2	2007-2008	Sweden
K56-78	ST1235	D	2007-2008	UK

2.2 Growth media

The growth medium used for bacterial cultivation was Difco™ LB Miller Broth (BD, USA). The Luria Broth (LB) base was prepared by dissolving 25 g in 1 liter (L) distilled water. The solution was sterilized by autoclavation at 121 ºC for 20 min (CertoClav Getinge) and stored at room temperature. LB agar (LBA) was prepared by adding 25 g LB powder and 12 g agar (Select Agar, Sigma Aldrich, Germany) in 1 liter distilled water. Autoclavation followed (as above), and the LBA was cooled down to 55 ºC before poured into sterile petri dishes under sterile conditions. For antibiotic containing LBA plates, the appropriate amount of the respective antibiotic was added to the sufficiently cooled (autoclaved) LBA before pouring the plates. The pH-value was adjusted to 7 after the antibiotic was added. The selective plates used in this study included trimethoprim in a concentration of 4 µg/mL, 8 µg/mL and 16 µg/mL, ciprofloxacin in a concentration of 0.1 µg/mL and 0.25 µg/mL and nitrofurantoin in a concentration of 6 µg/mL, 8 µg/mL, 12 µg/mL, 22 µg/mL and 24 µg/mL.

2.3 Antibiotic stock solutions

Antibiotics were stored as stock solutions at -20 ºC and thawed when needed for preparation of antibiotic containing LBA plates. The trimethoprim stock was already prepared by post-doc Nils Hülter in the lab at a concentration of 10 mg/ml in dimethyl sulfoxide (DMSO, Sigma Aldrich, Germany). 100 mg ciprofloxacin (BioChemika, Germany) was suspended in 10 mL distilled water, and < 10 drops of 3.7 % HCl was added to allow the compound to dissolve completely. The pH was adjusted to 7 by adding 3.7 % HCl. The stock solution was then sterile filtered using 0.2 µm Acrodisc® Syringe filters (VWR, USA) and stored at -20 ºC. A nitrofurantoin (Sigma-Aldrich, Germany) stock solution was prepared by dissolving 400 mg nitrofurantoin powder in 10 mL DMSO, resulting in a final concentration of 40 µg/mL. The tube containing the nitrofurantoin stock solution was wrapped in aluminum foil to protect the solution from light, due to the light sensitivity of this antibiotic.

2.4 Freeze stock cultures

For long-term storage of strains, freeze stock cultures of all wild type (WT) strains and mutants were prepared. A loop (10 µl) of colonies (5-6 colonies) of the respective strain was taken from LBA plates and resuspended in 500 µl liquid LB and 500 µl 50 % sterile glycerol, resulting in a final concentration of 25 %-glycerol (Cryo freeze tubes VWR International, USA). The tubes were vortexed and stored at -80 ºC.

2.5 Buffer and other solutions

The table shows an overview of the buffer and other solutions used in this project.

Table 2: Buffer and other solutions

Solution	**Content**	**Preparation**	**Storage temperature**	**Producer/supplier**
1xTAE buffer	Tris Acetate EDTA Distilled water	20 mL of 50xTAE were added to 1000 mL distilled water	RT	Julia Kloos made the 50x buffer
0.9 % NaCl	NaCl Distilled water	9 g of NaCl were added to 1000 mL	RT	Sigma-Aldrich
50 % glycerol	Glycerol Distilled water	57.5 mL of 87 % glycerol were added to 42.5 mL distilled water, 100 mL	RT	Sigma-Aldrich

2.6 PCR reactions

The primer used in this project was M13 (used as a single primer), which binds to random sites in the bacterial genome (see Table 3). See Table 4 for other PCR reagents and paragraph 2.2.3 for more information. A primer dilution with a final concentration of M13 primer stock solution (100 µM) was prepared using double distilled water (ddH_2O).

Table 3: Primer used in the project

Name	**Sequence 5' – 3'**	**Storage temperature**
M13 universal primer, 5 µM	GAGGGTGGCGGTTCT	2-8 ºC

Table 4: Other PCR reagents

Name	**Content**	**Storage temperature**	**Producer**
DreamTaq PCR Master Mix	DreamTaq DNA polymerase 2 2x DreamTaq buffer dNTPs 4mM $MgCl_2$	- 20 ºC	Thermo Scientific
Nuclease-free water	-	- 20 ºC	Thermo Scientific
Loading buffer 6X	Already prepared in the lab	2-8 ºC	-
SmartLadder	-	2-8 ºC	EuroGentec
Agarose 2 % gel	2 g in 100 mL 1xTAE	-	SeaKem, USA
Ethidium bromide 1 mg/mL	Already prepared in the lab	18 ºC - 25 ºC	-

2.7 Epsilometer test (E-test)

Strain susceptibility profiles were determined by Epsilometer tests from various producers as shown in Table 5. MICs were measured as described in paragraph 2.2.5.

Table 5: List of E-tests used in the project

E-test	Antibiotics	Storage temperature	Concentration gradient	Producer
1	Trimethoprim	8 ºC to -20 ºC	0.002 - 32 mg/L	Liofilchem, Italy
2	Ciprofloxacin	8 ºC to -20 ºC	0.002 – 32 mg/L	Liofilchem, Italy
3	Nitrofurantoin	8 ºC to -20 ºC	0.016 – 256 mg/L	Liofilchem, Italy
4	Gentamicin	8 ºC to -20 ºC	0.016 – 256 mg/L	BioMérieux SA, France
5	Kanamycin	8 ºC to -20 ºC	0.016 – 256 mg/L	BioMérieux SA, France
6	Tetracyclin	8 ºC to -20 ºC	0.016 – 256 mg/L	Liofilchem, Italy
7	Colistin	8 ºC to -20 ºC	0.016 – 256 mg/L	Liofilchem, Italy
8	Mecillinam	8 ºC to -20 ºC	0.016 – 256 mg/L	Liofilchem, Italy

3 METHODS

3.1 Bacterial cultivation

Inoculation of a liquid bacterial culture is required to grow a sufficient number of bacteria for experimental use. The selected *E. coli* strains were all frozen down at -80 ºC as a glycerol stock and thawed on ice before they were streaked for single colony growth on LBA plates. To minimize the chance of contamination, this was done every seventh (and a few times, fourteenth) day. The overnight culture was prepared by transferring 35 mL liquid LB to a sterile Erlenmeyer flask. This was done in a Laminar Air Flow (LAF) bench or in a sterile area on the working bench. A single colony from the LBA plate with the *E. coli* WT strains was used to inoculate the liquid media. The flask was then covered with aluminum foil and incubated at 37 ºC for 16-18 hours with 150 rotations per minute (rpm) in a shaking incubator (Infors HT).

To generate spontaneous mutants, the inoculated liquid bacterial culture plated on antibiotic containing LBA plates. The concentration of antibiotic in the plates was chosen according to the results form susceptibility determination using E-test and the Kirby-Bauer method (Disc diffusion), respectively.

Following incubation, 3x10 mL of the overnight culture were pipetted into three separate sterile 50 mL falcon tubes (BDTM Falcon, USA) and centrifuged at 4000 rpm for 10 minutes (Eppendorf swing-bucket rotor centrifuge). The supernatant was removed and the pellets (containing approximately 10^9 cells) were each resuspended with 1 mL of 0.9 % NaCl through and vortexing. Then, 100 µl of the cell suspension was plated on pre-dried antibiotic containing LBA plates.

The antibiotic containing LBA plates were incubated at 37 ºC for 24-72 hours, depending on the growth rate of the colonies. Single colonies of *E. coli* from the antibiotic containing LBA plate were further streaked on plates containing either the same concentration of antibiotic or in some cases on plates with increased drug concentration - to observe if there was any growth.

3.2 Streaking and isolating bacteria on LBA plates

The antibiotic containing LBA plates were marked in sections on the bottom of the petri dish, so several possible mutants could be streaked and isolated on the same plate. A sterile 1 µl loop was used to touch the bacterial colony and gently streak from the outside towards the center of the new antibiotic containing LBA plate. As a negative control, WT *E. coli* was also streaked on the same plate.

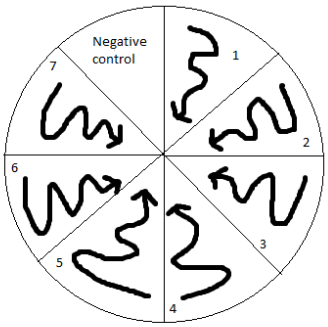

Figure 2: Diagram showing technique of streaking and purifying possible mutants on an antibiotic containing LBA plate

When there was growth on the antibiotic containing LBA plate, freeze stock solutions were prepared and the possible mutant was streaked on a new LBA plate (without antibiotics) for further testing. The possible mutant was gently spread with a sterile 1 µl loop over the plate, as shown in Figure 3, and incubated for 12-18 hours at 37 ºC. It was expected to see single colonies on the plate on the next day. If the bacterial growth was too dense (impossible to separate single colonies), the isolate was re-streaked on a new plate to obtain single colonies.

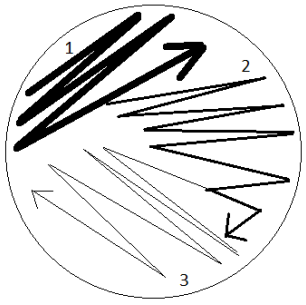

Figure 3: Streaking for isolation

3.3 Randomly amplified polymorphic DNA analysis (RAPD)

The RAPD analysis is a technique to identify genetic variation based on modifications of polymerase chain reaction (PCR) products. A single, short and arbitrary oligonucleotide (ten bases long) primer binds to DNA regions distributed at random sites throughout the genome on opposite strands of the DNA template and the amplification products are random fragments of genomic DNA (Maslow et al., 1993). In a conventional PCR, primers are designed to bind specific regions of interest in the genome. In this case, only that region is amplified, not a random site as in the RAPD-PCR.

Templates for RAPD PCR reaction were prepared by resuspending several bacterial colonies of the possible mutant from the LBA plate in 50 µl ddH_2O. This suspension was boiled for 10 minutes followed by centrifugation at 13000 rpm for 10 minutes (Biofuge Pico, Thermo Scientific, USA). The cellular debris (cell wall, proteins and lipids) was pelleted of and only DNA remained in the supernatant. The template was finally added to the remaining components of the sample for RAPD PCR analysis.

A sample for RAPD PCR analysis consisted of master mix, DNA template, the universal M13 primer and ddH_2O as listed in Table 6. The master mix (DreamTaq PCR master mix: Thermo Scientific, USA) consisted of a heat resistant DNA polymerase together with the four deoxyribonucleoside trisphosphates (dATP, dCTP, dTTP and dGTP) and was used to amplify shorter DNA fragments. See Table 3 and Table 4 for more information.

Table 6: Components for RAPD PCR analysis

Name	**Volume**
DreamTaq Mastermix	15 µl
M13 primer, 5 µM	5 µl
ddH_2O	3 µl
Template (DNA)	2 µl

3.3.1 RAPD-PCR thermocycler program

RAPD analysis involves repeated cycles. These steps are performed in a thermocycler machine, programmed as shown in Table 7. The first and second step involves denaturation, where the DNA becomes single stranded at 94 ºC. The third step is called the annealing step, where the temperature is lowered to 40-60 ºC, and the primer targets complementary sequences on the template. During the fourth and last step, the temperature is set to 72 ºC. This is the temperature at which the activity of the heat stable DNA polymerase is optimal and a new DNA strand is synthesized in 5' → 3' direction. All of these steps were repeated 45 times and as a result random but characteristic segments of the template DNA were amplified and eventually visualized by agarose gel electrophoresis.

Table 7: RAPD-PCR thermocycler program

Name	**Step**	**Temperature**	**Time**
RAPD1	1	95 ºC	5 min
	2	95 ºC	1 min
	3	36 ºC	1 min
	4	72 ºC	2 min
	5	Repeat cycle 2-4	45 times
	6	4 ºC	Forever

3.4 Agarose gel electrophoresis

Agarose gel electrophoresis is an effective way to separate DNA fragments of varying sizes ranging from 100 bp to 25 kb (Sambrook and Russel, 2001). The PCR product (10 µl) was mixed with 2 µl loading buffer 6X and loaded into the wells of a 2 % agarose gel. The agarose gel contained ethidium bromide, which contributes to the DNA visualization by intercalating itself between the base pairs and emitting fluorescent light when exited by ultraviolet (UV) light. The 2 % agarose gel was prepared by mixing 2 g agarose (Seakem, USA) with 100 mL 1xTAE-buffer. The solution was heated in a microwave until the agarose was completely dissolved (approximately 1-3 minutes). The solution was cooled down to approximately 60-65 ^{o}C before 20 µl ethidium bromide (EtBr) were added. The still liquid agarose gel was then poured slowly (to avoid bubbles, which can disrupt the gel) into a casting tray and left to solidify for 20-30 minutes at room temperature.

Because the phosphate backbone of the DNA is negatively charged, DNA fragments will migrate to the positively charged end when placed in an electric field. The gel box was then filled with 1xTAE buffer until the gel was covered. To identify the different sizes of the PCR products, 6 µl of a DNA marker (Smart ladder: Eurogentec USA) spanning 200-10000 base pairs was loaded into some of the wells. The marker contained DNA fragments of known sizes. Shorter molecules migrate easier and faster than longer molecules. The rate of migration of the DNA through the gel is determined by:

- Size of the molecule
- Type of agarose and its concentration
- DNA conformation
- Voltage applied
- Presence of ethidium bromide
- Buffers

(Lee et al., 2012)

The electrophoresis was standardized to run for 1 hour at 90 V. This was done to all isolated mutants to obtain characteristic RAPD band patterns. The gels were visualized using a Gel Doc transilluminator (BioRad, USA) and the Quantity One software (BioRad, USA). Using the ladder as a guide, DNA bands from the sample could be compared to a positive control of *E. coli* to confirm if the isolates belonged to *E. coli* species or not.

Possible mutants (at least 5 random isolates from each strain) were screened by RAPD-PCR. The resulting pattern of amplified DNA segments on an agarose gel was compared to the WT strain (positive control) to confirm if the possible mutants were *E. coli* and no contaminations. Other controls which were included in the analysis were *Acinetobacter baylyi* as a negative control and a water control.

3.5 Gradient diffusion method (E-test)

The MIC is defined as the lowest concentration of antimicrobial agent required to inhibit growth of the microorganism and determined by E-test (Andrews, 2001). An E-test is a strip containing a continuous *exponential* gradient of a specific antibiotic. The E-tests were kept at room temperature 30 minutes before use. The inoculum was prepared by suspending 2-4 individual bacterial colonies in a tube containing 0.9 % NaCl and vortexing. The turbidity of the bacterial suspension was measured using a suspension turbidity densitometer and adjusted to 0.5 McFarland standard.

The bacterial suspension was distributed with a sterile cotton swab on a Mueller-Hinton II agar plate (MHA-plate) and streaked equally in three different directions. The E-strip was then applied in the center of the plate. The plate was incubated at 37 ºC over night (16-19 hours).

The MIC was interpreted at the point where the elliptic zone of growth inhibition intersects the E-test strip. The bacteriostatic antibiotics (tetracycline, nitrofurantoin and trimethoprim) showed two zones of inhibition, a 100 % and 80 % inhibition zone. Only the 80 % inhibition zone value was noted (Citron et al., 1991). Some of the bactericidal antibiotics also showed both, a 100 % and an 80 % inhibition zone, but only the 100 % inhibition zone was noted. At the 80 %, there is significant inhibition of growth, but a few colonies are still growing at higher concentrations. The MIC results between the standard two-fold dilution steps were rounded up to the next higher step. This was done for all MIC determinations.

MICs were determined to confirm decreased drug susceptibility in the generated mutants. Comparison of determined MICs to clinical breakpoints (EUCAST) allows classification of the mutant into susceptible, intermediate or resistant to the respective antibiotic. The determined MICs of the generated mutants were also compared to the ECOFF-value (EUCAST).

3.6 Collateral sensitivity profiles

Each strain's susceptibility profile was determined by MIC determination using E-test. For all the five clinical strains, the trimethoprim, ciprofloxacin and nitrofurantoin resistant *E. coli* mutants were further MIC tested with different antibiotic E-tests as listed in Table 5. The mutants were tested to confirm a change (increased, decreased or no change) in MIC for seven different antibiotics. The method was done as described in 3.5. The MIC determined from this MIC testing showed whether the mutant's susceptibility profile had changed compared to the WT towards the selected antibiotics.

Collateral sensitivity was defined as growth-inhibition of the resistant strain at lower concentrations than the WT ($MIC_{\text{Resistant strain}} < MIC_{\text{WT}}$). No change in susceptibility was defined when as growth-inhibition of resistant strain and WT at the same antibiotic concentration ($MIC_{\text{Resistant strain}} = MIC_{\text{WT}}$). Collateral resistance was defined as growth-inhibition of a resistant strain at a higher drug concentration compared to the WT ($MIC_{\text{Resistant strain}} > MIC_{\text{WT}}$).

4 EXPERIMENTAL RESULTS

4.1 Generation of spontaneous mutants

Antibiotic resistant mutants were generated from five clinical strains of *E. coli* originating from the ECO-SENS-study. The aim was to generate at least five mutants for each strain per selected antibiotic. Three antibiotics with different mechanisms of action and varying resistance mechanisms were used in this project: trimethoprim, a dihydrofolate reductase inhibitor, ciprofloxacin, which interferes with the DNA-gyrase preventing bacterial replication and nitrofurantoin which modulates and damages proteins necessary for DNA, RNA, protein and cell wall synthesis. These three antibiotics were chosen because both trimethoprim and nitrofurantoin are often used, either single or in combination with other drugs, to treat UTIs (Health, 2012).

When generating mutants, contaminations could be observed on the LBA plates. Often, it was easy to discover and detect a contaminant on the plate by their different look, color (yellow) and smell. *E. coli* does not have very unique colony morphology on plate, but the sweet smell is easy to recognize. Contaminations could also be detected by RAPD-PCR analysis (see Figure 5).

When generating spontaneous mutants resistant to nitrofurantoin, it became clear how important a negative control was. Since nitrofurantoin is light sensitive, it was possible to observe if our procedure of handling nitrofurantoin was correct. In initial experiments, the WT of *E. coli* was growing well on selective plates, as they have not been protected from light during preparation. The nitrofurantoin was probably degraded and the isolated bacteria were probably not the desired mutants. A negative control was also streaked on these plates when isolating possible mutants. Here, there was also growth of the negative control.

To protect the nitrofurantoin containing LBA plates from light, the plates were wrapped in aluminum foil. Unfortunately, the negative control still showed good growth. This happened several times, and there was no difference in colony appearance. The generation of spontaneous mutants for nitrofurantoin never succeeded. It remained unclear when or if the nitrofurantoin was degraded: in the plates used to plate out the initial ONC, or in the plates used for selection of mutants or in the plates used afterwards for isolation and purification of selected colonies. The negative control was streaked out in both the last two steps during generation of spontaneous mutants, not in the first step when the ONC

was plated out. Some of the colonies that grew were tested further to be able to decide if the generation of nitrofurantoin resistant mutants should be continued or not.

In total, 40 possibly trimethoprim resistant mutants (see Appendix 1), 46 possibly ciprofloxacin resistant mutants (see Appendix 2) and 20 possibly nitrofurantoin resistant mutants (see Appendix 3) were isolated.

4.2 Confirmation of resistant mutants

4.2.1 RAPD-PCR analysis for confirmation of mutants

After bacterial cultivation, colony picking, isolation and further streaking of possible mutants, RAPD fingerprinting was performed to confirm whether the mutants belonged to *E. coli* species. The fingerprint (band pattern) of the possible mutants was compared to a fingerprint of the WT *E. coli* (positive control). *Acinetobacter baumannii* was used as a negative control. The obtained fingerprints typically consisted of several bands in different sizes. In some cases not all bands appeared.

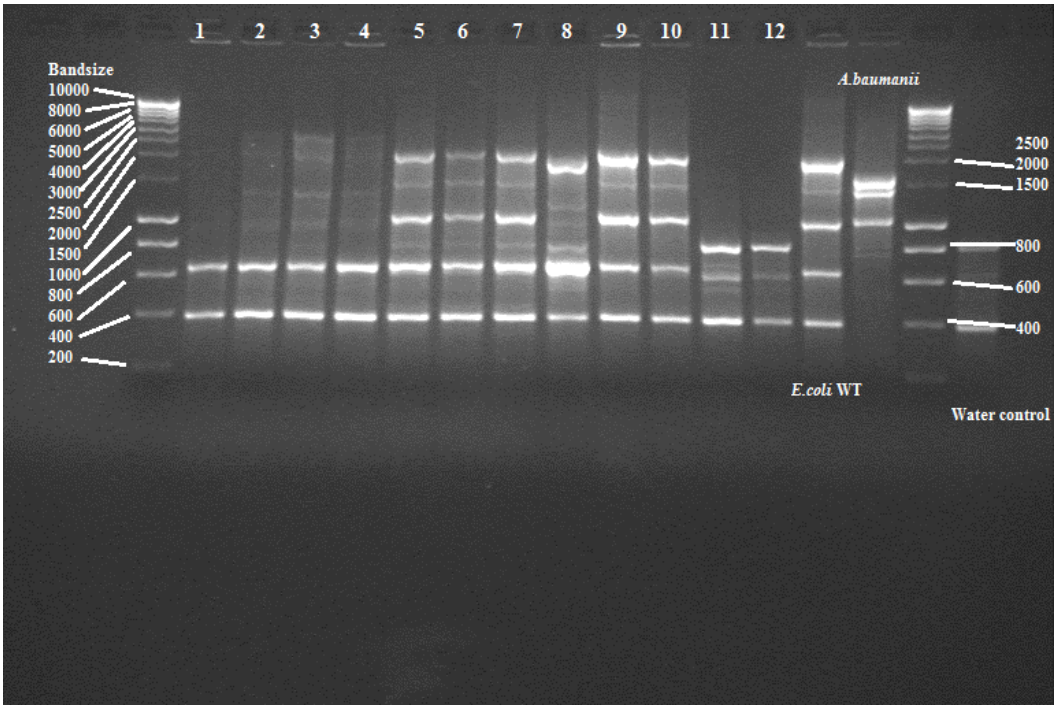

Figure 4: RAPD-PCR analysis for trimethoprim resistant mutants. Positive control: WT E. coli, negative control: A. baumannii. Water control: no template DNA was added.

RAPD-PCR analysis was first performed on the selected trimethoprim resistant mutants. All 12 samples depicted in Figure 4 had similar RAPD-patterns when compared to the ancestor. Samples 1, 11 and 12 only showed few bands. Despite a low number of bands, the fingerprints were still comparable to the ancestor and were therefore confirmed positive.

From 40 generated possible trimethoprim resistant mutants, 34 could be confirmed as *E. coli* by RAPD-PCR analysis. Figure 5 shows an example of an isolate that could be detected as contamination using RAPD-PCR analysis.

Table 8: Detonations of trimethoprim mutants confirmed as E. coli with RAPD-PCR analysis.

Strain	***Mutants***	***Result RAPD-PCR***
K56-12	4TP-12.1	+
	4TP-12.2	+
	4TP-12.4	+
	4TP-12.7	+
	4TP-12.8	+
	8TP-12.2	+
K56-41	4TP-41.1	+
	4TP-41.2	+
	8TP-41.3	+
	8TP-41.7	+
	8TP-41.9	+
	16TP-41.3	+
K56-50	4TP-50.1	+
	4TP-50.2	+
	4TP-50.3	+
	4TP-50.4	+
	4TP-50.5	+
	4TP-50.6	+
	4TP-50.7	+
	4TP-50.8	+
	4TP-50.9	+

Table continues on next page.

Strain	*Mutants*	*Result RAPD-PCR*
K56-50	4TP-50.10	+
	4TP-50.11	+
	8TP-50.1	+
K56-70	4TP-70.6	+
	4TP-70.7	+
	4TP-70.8	+
	4TP-70.9	+
	4TP-70.10	+
	4TP-70.11	+
K56-78	4TP-78.1	+
	4TP-78.2	+

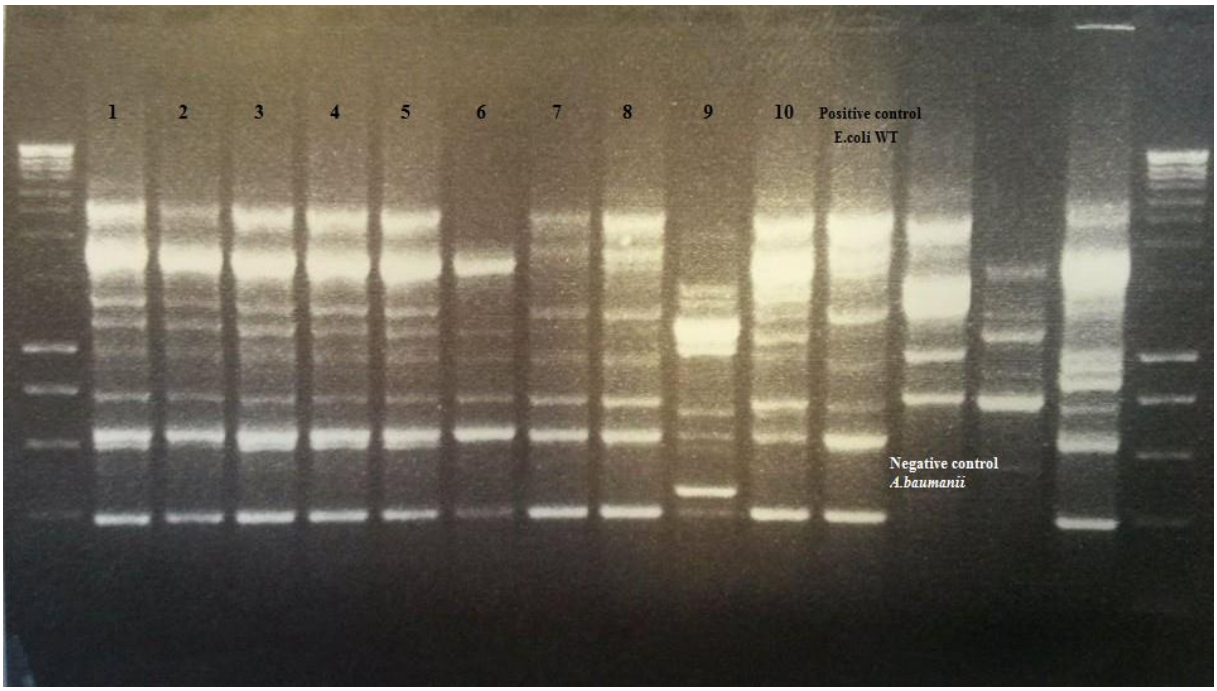

Figure 5: RAPD-PCR analysis for trimethoprim resistant mutants, showing also a contaminant isolate (sample nr. 9 = mutant 16TP-41.1). The contaminant shows a band right above 800 bp. which is not similar to the E. coli positive control. The band patterns from samples nr. 1-8 and 10 are similar to that of the positive control.

In total, 46 ciprofloxacin mutants were generated, of which 28 isolates were screened by RAPD-PCR analysis and 26 could be confirmed as belonging to *E. coli* species.

Table 9: Detonations of trimethoprim mutants confirmed as E. coli with RAPD-PCR analysis.

Strain	*Mutants*	*Result RAPD-PCR*
K56-12	0.1CIP-12.1	+
	0.1CIP-12.2	+
	0.1CIP-12.3	+
	0.1CIP-12.4	+
	0.1CIP-12.5	+
	0.1CIP-12.6	+
	0.1CIP-12.7	+
K56-41	0.1CIP-41.1	+
	0.1CIP-41.2	+
	0.1CIP-41.3	+
	0.1CIP-41.4	+
	0.1CIP-41.5	+
	0.1CIP-41.6	+
K56-50	0.1CIP-50.1	+
	0.1CIP-50.2	+
	0.1CIP-50.3	+
	0.1CIP-50.4	+
	0.1CIP-50.6	+
	0.1CIP-70.2	+
K56-70	0.1CIP-70.5	+
	0.1CIP-70.8	+
K56-78	0.1CIP-78.1	+
	0.1CIP-78.2	+
	0.1CIP-78.5	+
	0.1CIP-78.6	+
	0.1CIP-78.7	+

In total, 20 nitrofurantoin mutants were generated and 15 were screened. These were confirmed to belong to *E. coli* species, except for one.

Table 10: Nitrofurantoin mutants confirmed as E. coli by RAPD-PCR analysis.

Strain	***Mutants***	***Result RAPD-PCR***
K56-78	12NT-78.1	+
	8NT-78.1	+
	8NT-78.2	+
	8NT-78.3	+

The RAPD-PCR only confirmed whether the mutant belonged to *E. coli* species or not. Spontaneous resistance of these mutants indicated a changed susceptibility profile of these isolates to the selected antibiotic, as compared to the WT, and therefore the mutant's susceptibility profile (MIC) was determined.

4.2.2 Gradient diffusion method to confirm mutants

MICs were determined for 30 of the 35 RAPD-PCR-confirmed trimethoprim mutants, for 25 of the 26 RAPD-PCR-confirmed ciprofloxacin mutants and for all the 15 confirmed nitrofurantoin mutants. The determined MICs were compared to the MICs of the respective WT strain to observe whether the susceptibility profile of the mutant had changed (decreased, similar or increased susceptibility).

The MIC values varied a lot among the different strains and the different antimicrobials. The MIC values varied the most among the trimethoprim mutants. A 4- to 48-fold increase in MIC could be observed. Ultimately, for a large number of trimethoprim mutants no inhibition zone could be determined (MIC greater than >32 µg/mL). An overview of the results after the generation and confirmation of the trimethoprim mutants is shown in Appendix 1.

No inhibition zone could be determined for the ciprofloxacin mutants (MIC greater than >32 µg/mL). Appendix 2 shows an overview of the generated ciprofloxacin mutants. The MICs determined for the nitrofurantoin mutants were similar to the WT MICs, without changes in susceptibility. Nitrofurantoin mutants of strain K56-78 however showed a 4-fold increased MIC (see Appendix 3).

In total, 28 trimethoprim mutants, 23 ciprofloxacin mutants and four nitrofurantoin mutants were characterized both by RAPD-PCR and E-test. One mutant from each strain was selected for MIC testing using a larger selection of antimicrobials. The choice of selected antimicrobials was based on the results of Sommers paper. The choice was based on interesting combinations of strains resistant to different antimicrobials showing collateral interactions. If the MICs of the mutants varied a lot within a given strain, several mutants with different MICs were selected for further studies.

Table 11 shows an overview of the MIC testing of selected *E. coli* resistant isolates, and the MIC values of both the WT and mutant isolates.

Table 11: Overview of selected E. coli mutants for MIC testing

Antibiotic	***Strain***	***Mutants***	***MIC WT, µg/ml***	***MIC mutant, µg/ml***
Trimethoprim	K56-12	4TP-12.1	0.25	≥32
	K56-41	4TP-41.1	0.50	≥32
		8TP-41.3	0.50	100 %: 2
		8TP-41.9	0.50	100 %: 2
	K56-50	4TP-50.1	100 %: 0.50 80 %: 0.25	≥32
		4TP-50.2	100 %: 0.50 80 %: 0.25	100 %: 12
		4TP-50.3	100 %: 0.50 80 %: 0.25	100 %: 4
		4TP-50.5	100 %: 0.50 80 %: 0.25	8
		4TP-50.6	100 %: 0.50 80 %: 0.25	4
	K56-70	4TP-70.3	100 %: 1 80 %: 0.25	≥32
	K56-78	4TP-78.1	1	≥32
Ciprofloxacin	K56-12	0.1CIP-12.2	0.008	≥32
	K56-41	0.1CIP-41.1	0.008	≥32
	K56-50	0.1CIP-50.1	0.008	≥32
	K56-70	0.1CIP-70.5	0.004	≥32
	K56-78	0.1CIP-78.1	0.016	≥32
Nitrofurantoin	K56-78	8NT-78.1	4	16

In addition, two double mutants (trimethoprim+ciprofloxacin) from another ongoing project in our laboratory were included in MIC testing using a larger selection of antimicrobials.

Table 12: Overview of selected E. coli double mutants (trimethoprim+ciprofloxacin) for MIC testing

Antibiotic	**Strain**	**Mutants**	**WT MIC (µg/ml)**	**Mutant MIC (µg/ml)**
Trimethoprim + ciprofloxacin	K56-41	CIP-TP 41.7	TP: 0.38 (80 %) CIP: 0.016	TP: 6 (80 %) CIP: 0.50
	K56-78	CIP-TP 78.3	TP: 0.75 (80 %) CIP. 0.016	TP: $\geq$32 CIP: 0.50

4.2.3 EUCAST and clinical relevance of the changed MICs

The MIC values of the generated mutants showed that the mutants' susceptibility had increased. A mutation may have occurred that influenced the isolates susceptibility. But comparing MIC values of the mutant and the WT strain alone is not enough to say whether the mutant can be classified as drug resistant. The MIC values of the generated mutants were therefore compared to the clinical breakpoints and ECOFF values (see Table 13 below).

Within the same strain, determined mutant MIC values differed for a given antibiotic. Isolates with a MIC value greater than >32 μg/mL are, according to EUCAST, categorized as clinically resistant. Four out of 11 selected trimethoprim mutants had a MIC value below the clinical breakpoint, but equal or above the ECOFF value which indicated an acquisition of resistance to the given drug. The nitrofurantoin mutant could not be classified as resistant even though its susceptibility was reduced.

Table 13: Clinical breakpoints and ECOFF values for E. coli WT strains and the antibiotics trimethoprim, ciprofloxacin and nitrofurantoin

	Trimethoprim	**Ciprofloxacin**	**Nitrofurantoin**
Clinical breakpoints	S $\leq$ 2 R > 4	S $\leq$ 0.5 R > 1	S $\leq$ 64 R > 64
ECOFF	2.0	0.064	64

4.3 Collateral sensitivity profiles

E. coli mutants with reduced drug susceptibility to trimethoprim, ciprofloxacin and nitrofurantoin were used to map a network of collateral sensitivity interactions. This was done by measuring the susceptibility of each mutant against seven different antibiotics (see Table 5) using E-tests. The MICs were compared to the WT MIC to see whether the mutant's susceptibility profile had changed.

4.3.1 MIC determination

For most of the antibiotics, the MIC was easy to read and the inhibition zone had clear and sharp, except for the bacteriostatic antibiotics (tetracycline, trimethoprim and nitrofurantoin), where two zones of inhibition were visible (100 % and 80 % inhibition). MIC determination of tetracycline was challenging, because the inhibition zone was fuzzy. A slow gradient of reduced growth and single small colonies made it hard to determine the MIC. In the case of MIC determination with colistin the end points of growth inhibition varied between the two sides of the E-test. Here, the lowest concentration was notated.

The results of MIC determination for trimethoprim mutants are listed in Table 14 including the previous MIC values determined during confirmation of the mutant (MIC mut.). The WT MICs for each of the different antibiotics and ECOFF values for WT *E. coli* and the respective antimicrobial drug are also listed. The results of the ciprofloxacin, nitrofurantoin single mutants and trimethoprim+ciprofloxacin double mutants are also listed in tables below (see Table 15, Table 16, Table 17).

Table 14: MIC values (µg/ml) of E. coli trimethoprim mutants and their respective WT strain

Strain	Mutant	MIC mut.	WT GEN	GEN	WT KAN	KAN	WT CIP	CIP	WT TET	TET	WT COL	COL	WT NIT	NIT	WT MEC	MEC	WT TRI	TRI
K56-12	4TP-12.1	>32	**2**	0.50	**8**	0.50	**0.008**	0.012	**3**	0.50	**0.50**	1	**8**	8	**0.25**	0.125	**0.25**	-
K56-41	4TP-41.1	>32	**1**	0.25	**4**	1	**0.008**	0.008	**2**	0.50	**1**	1	**4**	8	**0.25**	0.125	**0.50**	-
"	8TP-41.3	2	**1**	0.50	**4**	1	**0.008**	0.008	**2**	0.50	**1**	1	**4**	8	**0.25**	0.125	**0.50**	-
"	8TP-41.9	2	**1**	0.25	**4**	0.50	**0.008**	0.004	**2**	0.50	**1**	1	**4**	4	**0.25**	0.125	**0.50**	-
K56-50	4TP-50.1	>32	**1**	0.25	**4**	1	**0.008**	0.008	**0.125**	1	**1**	1	**16**	16	**0.50**	0.25	**0.50**	-
"	4TP-50.2	16	**1**	0.125	**4**	1	**0.008**	0.008	**0.125**	0.25	**1**	1	**16**	16	**0.50**	0.125	**0.50**	-
"	4TP-50.3	4	**1**	0.25	**4**	1	**0.008**	0.008	**0.125**	0.25	**1**	1	**16**	8	**0.50**	0.125	**0.50**	-
"	4TP-50.5	8	**1**	0.25	**4**	1	**0.008**	0.008	**0.125**	0.50	**1**	1	**16**	8	**0.50**	0.125	**0.50**	-
"	8TP-50.6	4	**1**	0.50	**4**	2	**0.008**	0.016	**0.125**	0.50	**1**	1	**16**	8	**0.50**	0.125	**0.50**	-
K56-70	8TP-70.3	>32	**1**	0.25	**8**	0.25	**0.004**	0.002	**2**	0.25	**0.50**	1	**4**	1	**0.125**	0.064	**1**	-
K56-78	4TP-78.1	>32	**1**	0.50	**4**	2	**0.016**	0.016	**2**	0.50	**0.50**	1	**4**	32	**0.25**	0.125	**1**	-
ECOFF			2.0		8.0		0.064		8.0		2.0		64.0		1.0		2.0	

Table 15: MIC values (µg/ml) of E. coli ciprofloxacin mutants and their respective WT strain

Strain	**Mutant**	**MIC mut**	**WT GEN**	**GEN**	**WT KAN**	**KAN**	**WT CIP**	**CIP**	**WT TET**	**TET**	**WT COL**	**COL**	**WT NIT**	**NIT**	**WT MEC**	**MEC**	**WT TRI**	**TRI**
K56-12	0.1CIP-12.2	≥32	**2**	0.50	**8**	2	**0.008**	-	**4**	1	**0.5**	1	**8**	8	**0.25**	0.064	**0.25**	0.50
K56-41	0.1CIP-41.1	≥32	**1**	0.50	**4**	2	**0.008**	-	**2**	0.50	**1**	1	**4**	8	**0.25**	0.125	**0.50**	0.50
K56-50	0.1CIP-50.1	≥32	**1**	0.25	**4**	1	**0.008**	-	**0.125**	0.50	**1**	1	**16**	16	**0.50**	0.125	**0.25**	0.125
K56-70	0.1CIP-70.5	≥32	**1**	0.50	**8**	2	**0.004**	-	**2**	0.125	**0.50**	1	**4**	4	**0.125**	0.064	**0.25**	0.25
K56-78	0.1CIP-78.1	≥32	**1**	0.50	**4**	2	**0.016**	-	**2**	0.25	**0.50**	1	**4**	8	**0.25**	0.125	**1**	0.50
ECOFF			2.0		8.0		0.064		8.0		2.0		64.0		1.0		2.0	

Table 16: MIC values (µg/ml) of E. coli nitrofurantoin mutants and their respective WT strain

Strain	Mutant	MIC mut	WT GEN	GEN	WT KAN	KAN	WT CIP	CIP	WT TET	TET	WT COL	COL	WT NIT	NIT	WT MEC	MEC	WT TRI	TRI
K56-78	8NT-78.1	16	**1**	0.50	**4**	2	**1**	0.008	**2**	0.50	**0.25**	1	**4**	-	**4**	0.064	**0.50**	0.50
ECOFF			2.0		8.0		0.064		8.0		2.0		64.0		1.0		2.0	

Table 17: MIC values (µg/ml) of E. coli trimethoprim-ciprofloxacin double mutants and their respective WT strain

Strain	Mutant	TP mut.	CIP mut.	WT GEN	GEN	WT KAN	KAN	WT CIP	CIP	WT TET	TET	WT COL	COL	WT NIT	NIT	WT MEC	MEC	WT TRI	TRI
K56-41	CIP-TP 41.7	6	0.50	**1**	0.50	**4**	2	**0.008**	0.50	**2**	2	**1**	1	**4**	100 %: 16 80 %: 8	**0.25**	0.125	**0.50**	1
K56-78 ST1235	CIP-TP 78.3	≥32	0.50	**1**	0.50	**4**	4	**0.016**	0.50	**2**	1	**0.50**	1	**4**	100 %: 16 80 %: 8	**0.25**	0.125	**1**	8
ECOFF				2.0		8.0		0.064		8.0		2.0		64.0		1.0		2.0	

4.3.2 Collateral sensitivity profiles of antibiotic resistant E. coli clinical strains

MICs values of the mutants and a selection of different antibiotics were mapped. The heat map shows whether the susceptibility profile of the mutant had increased or decreased compared to the respective WT for a given antimicrobial: red color indicates a decreased susceptibility profile (collateral resistance) whereas blue color indicates an increased susceptibility profile (collateral sensitivity).

An increase in susceptibility (>32-fold) was observed for trimethoprim resistant mutants and kanamycin, but most of the results showed a 2- to 4-fold change in MIC compared to the WT. A high increase in susceptibility (>32-fold) was observed for ciprofloxacin mutants and tetracycline (see Figure 7). This was also observed in nitrofurantoin mutants exposed to ciprofloxacin and mecillinam (see Figure 8). On average, the results suggest a 4-fold increase in sensitivity and the same in resistance, with a few exceptions, which show a higher increase or decrease in susceptibility.

Each of the five strains, K56-12, K56-41, K56-50, K56-70 and K56-78, exhibited collateral sensitivity towards at least four other antibiotics, including aminoglycosides (gentamycin and kanamycin).

Trimethoprim, ciprofloxacin and nitrofurantoin mutants showed a decreased susceptibility profile (collateral resistance) towards colistin and nitrofurantoin. A decrease in susceptibility could be observed (increase in MIC >8-fold). Both, trimethoprim and nitrofurantoin are commonly used in treatment of UTI and the collateral resistance observed could contribute to the failure of drug cycling programs.

The changes in susceptibility (changes in MIC) determined for the spontaneous mutants were compared to the current clinical EUCAST breakpoints (see Appendix 4). When comparing the results from MIC testing of trimethoprim-, ciprofloxacin- and double-mutants that showed an increased susceptibility (collateral sensitivity), with the clinical breakpoints of the specific antibiotic, these mutants could all be classified as susceptible. The results from the MIC testing with nitrofurantoin mutants also showed an increased susceptibility (collateral sensitivity), but these mutants were not classified susceptible according to EUCAST.

The results from MIC testing which showed a decreased susceptibility (collateral resistance), had MIC-values below the clinical breakpoints. This means that these results are not clinical relevant. EUCAST tables do not specify clinical breakpoints (MICs or zone diameters) for *E. coli* towards tetracycline and kanamycin, so interpretation of MICs with these antibiotics was not possible.

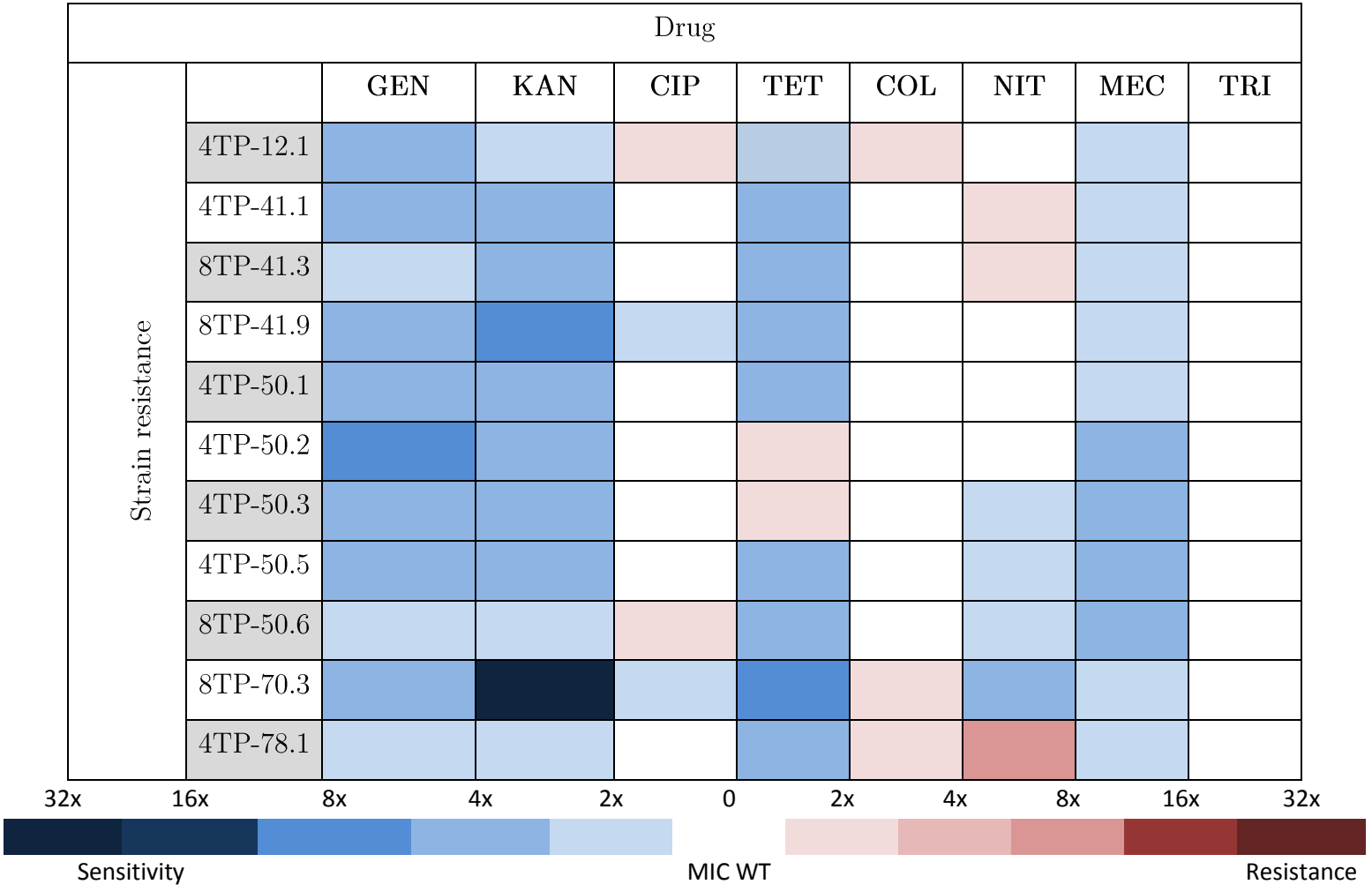

Figure 6: Collateral sensitivity profiles of E. coli trimethoprim mutants relative to the WT

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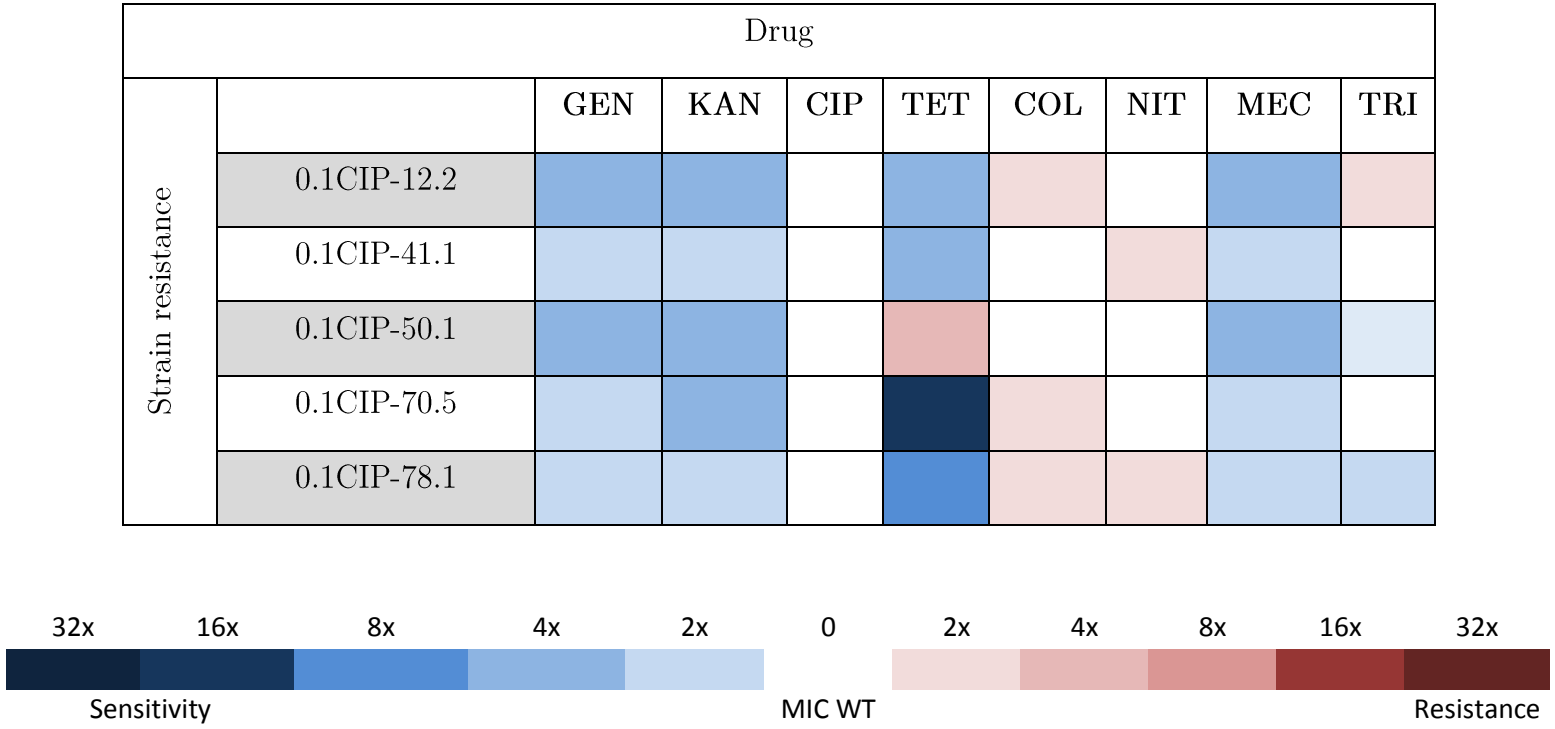

Figure 7: Collateral sensitivity profiles of E. coli ciprofloxacin mutants relative to the WT.

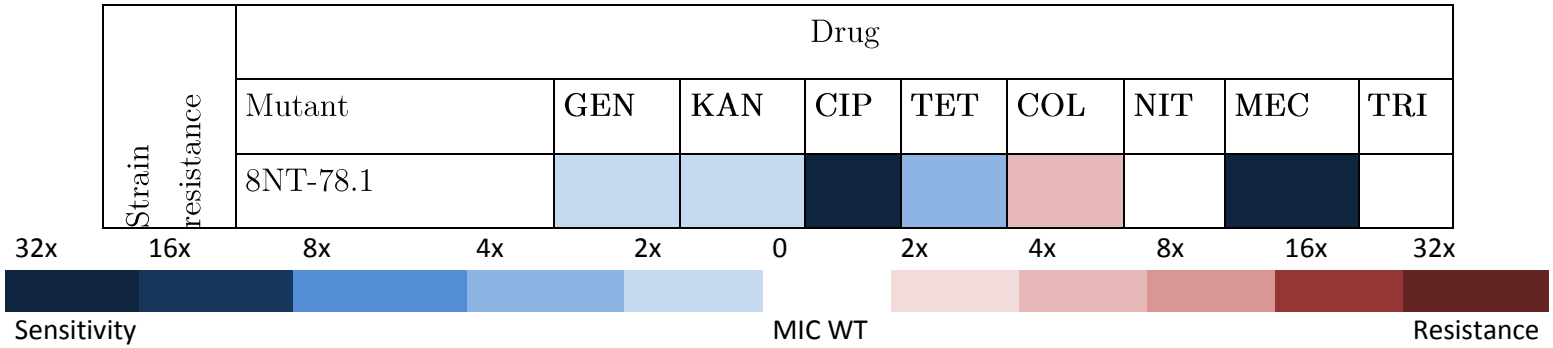

Figure 8: Collateral sensitivity profiles of E. coli nitrofurantoin mutants relative to the WT

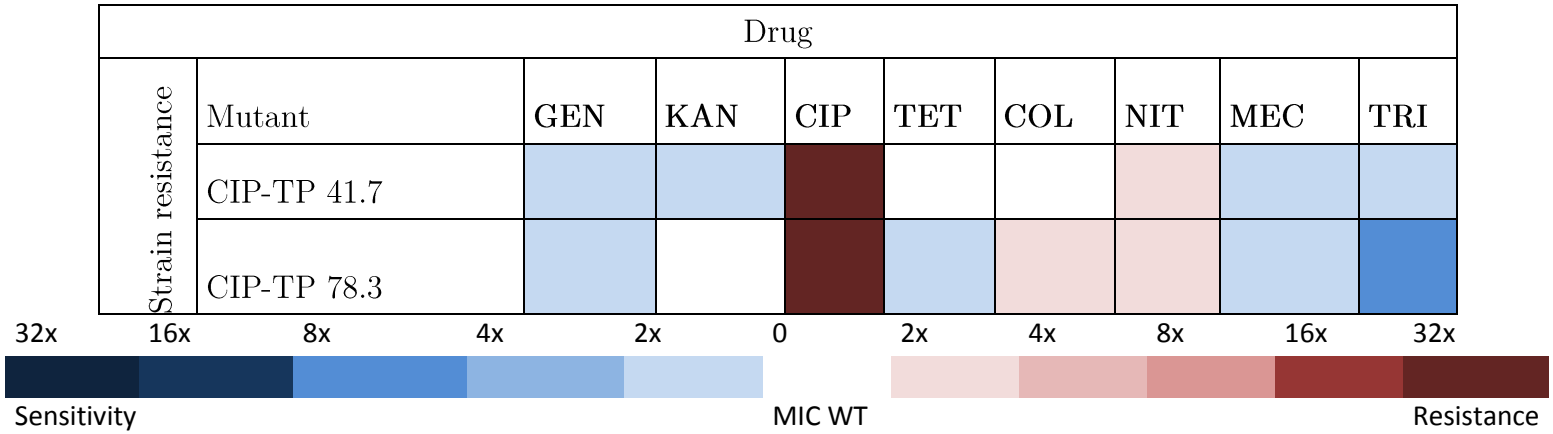

Figure 9: Collateral sensitivity profiles of E. coli trimethoprim-ciprofloxacin mutants relative to the WT

5 DISCUSSION

In this project, I have determined collateral sensitivity/resistance effects in spontaneous antibiotic resistant isolates of clinical strains of *E. coli* strains. These results were summarized in heat maps depicting changed susceptibility profiles of the isolates to other antimicrobial agents. The results show that among resistant isolates of *E. coli* clinical strains both, collateral sensitivity and resistance occurs. To sum up the results, the phenomenon of collateral sensitivity was observed more often compared to collateral resistance, but to the same extend (up to 32x decrease/increase in susceptibility).

Szybalski and Bryson performed the first pioneer experiments on the phenomenon of collateral sensitivity and resistance in 1952. In 2014, Imamovic and Sommer continued to work on this concept and our project provides support for the concept of collateral sensitivity/resistance using heat maps, which is similar to what was done by Sommer et al. Our observations can lead to reduced drug resistance evolution when selected combinations of antibiotics are used successively in drug cycling.

Some of the generated mutants had MIC values below the clinical EUCAST breakpoints for resistance, meaning they were not resistant according to clinical breakpoints. Nevertheless, they showed decreased susceptibility to a given drug when compared to the respective WT MIC. A comparison of the results from the MIC testing with these mutants to Sommers' results is not adequate, because the resistance levels acquired during adaption for *E. coli* strains (measured by MIC) in their study were equal or above the current clinical breakpoints. Generated mutants with MIC values equal or above the current clinical breakpoints, similar to Sommer et al., were comparable to Sommers results.

Those mutant isolates that showed a collateral effect to a given agent in MIC testing, were all equal or below the clinical breakpoints and were not comparable to Sommers' results. Those mutants that showed an increased susceptibility profile (collateral sensitivity) to a given agent in MIC testing, were equal with, if not above, the clinical breakpoints and therefore comparable to Sommer et al.

5.1 From a laboratory perspective

5.1.1 Comparison to previous work on collateral sensitivity/resistance

A few similarities can be found between Sommers' results and this project, but most results have to be regarded contradictory. Compared to Sommer et al., effects of collateral sensitivity were observed between resistant mutants and specific drug combinations in our work for which collateral resistance was observed in their project. One example is collateral resistance between ciprofloxacin mutant and tetracycline (up to 4-fold increase in MIC) seen in Sommers study, but sensitivity in ciprofloxacin mutant 0.1CIP-70.5 and tetracycline (up to a 32-fold decrease in MIC) seen in our project.

Similarities between our project and Sommers' can be observed in MIC testing of trimethoprim mutants. In both studies, collateral resistance is observed between trimethoprim mutants and nitrofurantoin drug (up to a 2-fold increase in MIC), ciprofloxacin drug (up to a 8-fold increase in MIC) and colistin drug (no changes in MIC).

In general, collateral sensitivity was found to a greater extent than collateral resistance, which was the opposite situation in Sommers' findings. On the other hand, Sommer evolved resistance in few clinical strains to 23 drugs, whereas we only evolved resistance in laboratort strains to three antibiotics. All in all, our project supported a general pattern, where collateral sensitivity to aminoglycosides and mecillinam was observed in strains resistant to trimethoprim, ciprofloxacin and nitrofurantoin. No general pattern was observed in Sommers project.

According to another study on collateral sensitivity, 74 % of the evolved resistant lines showed increased sensitivity to one or several drugs (Lázár et al., 2013). Similar to our results, a high rate of collateral sensitivity interactions was also observed. In our project, 55 % (n=83) of the resistant mutants showed increased collateral sensitivity, only 14 % (n=21) showed increased collateral resistance and finally 24 % (n=48) showed no alteration in their susceptibility profile.

5.1.2 Challenges and limitations in this project

5.1.2.1 Contaminations in laboratory

When generating mutants in this project, contaminations were often observed on the plates or detected through RAPD-PCR analysis. Environmental contaminants within microbiological laboratories can create false results and may be a health risk. Good laboratory routines are necessary to prevent this, for example autoclavation of media, sterilization of work bench, 70 % alcohol for disinfection and use of gloves. Sources for contaminations can be found in many steps during preparation and performance of experiments in this project. Preparation of plates was done in a horizontal airflow-bench, where the sterile airflow is supposed to protects the product from contaminations. The experiments (plating out ONC on a large amount of plates) was done on the bench. Here, contaminations may have emerged due to circulation of unsterile air.

Contaminations may have occurred also when preparing the overnight cultures (ONC). The liquid LB medium may have been contaminated even after autoclavation, but the contamination was not yet visible. The contamination may have occurred when LB was pipetted out and transferred into sterile falcon tubes. This procedure was routinely done nearby a bunsen burner, but sometimes this was turned off before the process was completed. There are also other factors that may have caused the contaminations - skin contaminants and other materials from our own or other bodies.

A clean and organized laboratory where good routines are implemented may prevent contaminants in the future. Several techniques can be improved, such as discarding pipette tips often and avoid touching the pipette to non-sterile media and surfaces. Surfaces where dust and other potential contaminants can be collected, such as tops of freezers and incubators, should be cleaned. Use gentle arm movements to reduce disturbances in the LAF-bench while working.

5.1.3 RAPD-PCR analysis

RAPD-PCR analysis was performed to confirm if selected mutants were *E. coli* isolates (see 0). RAPD fingerprinting is a time-efficient and inexpensive method to differentiate between different bacterial species. By comparing the isolates with the controls, which also was RAPD-fingerprinted, contaminations were possible to detect. The RAPD-PCR is not that widely used as other genotypic methods, and not considered as gold standard for bacterial strain typing in the genomic era (Tynkkynen et al., 1999), whereas Pulsed-field gel electrophoresis (PFGE) is considered the gold standard for subtyping bacteria because it is a high-resolution, macro-restriction analysis (Tenover et al., 1995, Gerner-Smidt et al., 2006). PFGE gives a higher level of strain discrimination than RAPD-PCR. PFGE is widely used to separate large DNA molecules in typing of human bacterial pathogens. However, the method is costly and not as time-efficient as RAPD-PCR (Li et al., 2009).The aim in this project was to confirm the possible mutants including an ancestor strain as the positive control, and doing RAPD-PCR was therefore an appropriate method to perform.

5.1.3.1 Agarose gel electrophoresis

A water control was used as a no-template control in the RAPD-PCR. The water control contained reaction components, but no DNA template. The water control showed bands in the gel after electrophoresis, when it was expected not to. The primer used for RAPD-PCR analysis was M13. Primer-derived, non-specific amplification products observed in the negative control of RAPD-PCR analysis is apparently a well-known problem as reviewed in (Pan et al., 1997). Non-specific products were also observed in the negative control reactions with different pairs of universal rDNA primers (Hughes et al., 1994).

5.2 From a clinical perspective

5.2.1 The Norwegian guidelines for treating acute uncomplicated UTI

Collateral sensitivity was observed in trimethoprim resistant isolates to antibiotics belonging to the aminoglycoside class of antimicrobial agents. The same was the case for ciprofloxacin mutants to aminoglycosides. Our results were similar to a study by Lazar, where a dense network of collateral sensitivity-interactions involving aminoglycosides was observed (Lázár et al., 2013). Aminoglycosides are frequently used for treatment of hospital-acquired infections with multidrug-resistant Gram-negative pathogens (Peleg and Hooper, 2010), and therefore is the observed collateral sensitivity-interactions to aminoglycosides a good indicator to improve treatment options.

The general pattern of our results suggests a collateral sensitivity effect on aminoglycosides for strains that are already resistant to trimethoprim, ciprofloxacin and nitrofurantoin. Collateral resistance was seen in ciprofloxacin resistant mutants when susceptibility tested against trimethoprim or nitrofurantoin. In full awareness of the preliminary nature of the presented results, these findings are potentially disturbing since both trimethoprim and nitrofurantoin are alternatives in first line-treatment of acute, uncomplicated UTI according to Norwegian guidelines.

In contrast, mecillinam exposure to trimethoprim, ciprofloxacin and nitrofurantoin resistant mutants showed to be a good alternative, which is also one of the first-choice agents suggested in the Norwegian guidelines.

These results may be a food for thought considering the Norwegian guidelines. Since mecillinam showed an increase in collateral sensitivity when trimethoprim, ciprofloxacin and nitrofurantoin resistance has evolved, mecillinam should not be an equal alternative. Mecillinam should be the third alternative when resistance arises. On the other hand, mecillinam alone as primary treatment showed a high rate of treatment failure in a study investigating community-acquired UTI caused by extended-spectrum β-lactamase (ESBL) producing *E. coli* (Søraas et al., 2014).

5.2.2 Collateral drug cycling versus combination therapy

Combination therapy regimes have long been used to treat viral infections such as HIV (Richman, 2001) and are also a standard therapy regime in treatment of many cancer

(Lane, 2006) and other conditions. A study compared the efficacy of azlocillin alone versus azlocillin in combination with gentamicin or tobramycin for the treatment of children with cystic fibrosis. The study showed that combination therapy was more successful and resistance occurred more frequently during monotherapy as during combination therapy (Michalsen and Bergan, 1981).

Combination therapies have also been practiced by clinicians for the treatment of bacterial infections, especially *Mycobacterium tuberculosis* infections (Mitchison and Davies, 2012). The three advantages of combination therapy are: (1) the possibility that the infecting pathogen will be susceptible to at least one of the agents of the applied combination therapy, (2) prevention of emergence of resistance and (3) the potential of synergistic interactions between chosen agents may reduce mortality as reviewed in (Safdar et al., 2004).

The choice of antimicrobials used in combination therapy can be divided into three categories (Fischbach, 2011):

1. Inhibition of different targets in the same pathway, for example the combination of two nucleoside reverse transcriptase inhibitors in treatment of HIV (Richman, 2001)
2. Inhibition of different targets in the same pathway, for example the combination of trimethoprim and sulfamethoxazole (Fischbach, 2011)
3. Inhibition of the same target in different ways (streptogramins)

The use of combination therapy may delay and possibly prevent the emergence of resistance. Unfortunately, advantages are followed by disadvantages, especially in the case of aminoglycosides. Increased toxicity may be a problem in combination therapy. A study determined whether antibiotic combination therapy affected mortality and nephrotoxicity in pediatric patients with Gram-negative bacteremia (Tamma et al., 2013). Patients who received combination therapy had a higher incidence of nephrotoxicity compared with those receiving monotherapy.

The development of collateral sensitivity drug cycling as a treatment regime is still at an early stage. Comparing an already existing and practiced therapy regime to a proposed novel antimicrobial stewardship strategy is inadequate. On the other hand, toxicity is associated with combination therapy. In collateral sensitivity drug cycling one alternate several agents after resistance towards a given antimicrobial drug has

developed, and the chance of toxicity may be lower because one does not use several antimicrobials at the same time, but successively.

5.2.3 Combination therapy and collateral responses

Drugs in combination need requirements to be effective in combination therapies. Two drugs in combination that show synergistic effects are believed to maximize killing efficiency (Lehar et al., 2009). Drug combinations which are expected to delay the emergence of resistance are also considered important to affect the long-term efficiency of combination therapies. Munck and his co-workers explored how collateral sensitivity affects the emergence of resistance in combination therapies. They showed that collateral sensitivity-interactions between two antibiotics in combination was a predictor of decreased resistance development during simultaneous exposure to two drugs (Munck et al., 2014).

5.2.4 Collateral sensitivity drug cycling and its efficacy of treatment

Two studies have shown that drug cycling of different antibiotics exhibiting collateral sensitivity slow the rate of resistance development (Imamovic and Sommer, 2013, Kim et al., 2014). The experimental map of cross-resistance and collateral sensitivity resulting from these studies could be used as a tool in therapy. Drug pairs used in drug cycling vary in their ability. The map can provide the selection of right drug pairs. This strategy may minimize inappropriate and unnecessary use of antibiotics by optimizing the selection, dosing, route and duration of administration.

A pre-requisite for successive cycling is reciprocal collateral sensitivity as demonstrated between gentamcin and cefuroxime in Imamovic and Sommer et al. However, a more simplistic approach would be to directly exploit collateral sensitivity in sequential treatment regimes where second line therapies are chosen based on increased killing of secondary options, as demonstrated in time-kill experiments between WT and a mutant with gentamicin resistance exposed to 16x MIC of cefuroxime (Imamovic and Sommer, 2013). More information about CS-networks in clinical strains with pre-existing antibiotic resistance towards single and multiple antibiotics are needed to incorporate such strategies in treatment guidelines.

5.2.5 Challenges and limitations of collateral sensitivity drug cycling

Inappropriate prescriptions and the over- and misuse of antimicrobial drugs has led to a rise of resistance. An important strategy to prevent and delay resistance is to use the antimicrobials in a more appropriate way. That involves consideration of their pharmacokinetic and pharmacodynamics properties, the use of drug combinations and a more elaborated choice of antimicrobials.

Laboratory studies as a proof-of-concept provide no explicit guidelines for clinical practice, rather they serve as a pointer towards potential future therapeutic interventions. Collateral sensitivity may lead to a better usage of existing antibiotics as reviewed in (Hancock, 2014). Although the concept and findings provided for collateral sensitivity are exciting, there are challenges and limitations that must be further investigated.

Infections can be caused by one or several pathogens. What we have seen in previous and ongoing studies that have observed collateral sensitivity in *E. coli*, may not work for other species of pathogens. In UTIs, *E. coli* is the most predominant bacterium, as previous studies have shown (Gupta et al., 1999a, Kahlmeter, 2003, Kamenski et al., 2012), but UTI can also be caused by other bacterial species. Not to mention other, more complicated infections where several bacterial species with different resistance profiles can cause the infection. In addition, the mechanistic understanding of processes leading to collateral sensitivity is still at an early stage. We don't know how frequently interactions in collateral sensitivity occur during the evolution of antibiotic resistance and to observe a general pattern seems challenging.

Collateral sensitivity drug cycling also brings along practical challenges. Usually when a patient gets an acute infection, the next stop is to the hospital where they have the opportunity to identify and determine susceptibility before treatment start. This is not always the case. A patient with an acute infection that requires immediate action cannot always be left on hold until the pathogen and respective resistance profiles have been determined. Collateral sensitivity drug cycling may be a challenge for general practitioners, where you often get antimicrobials for the most common pathogens that may have caused the infection.

In spite of these challenges, collateral sensitivity may be a valuable tool for limiting resistance development if studies on this phenomenon extent to a greater range of strains and species of bacterial pathogens.

5.3 Conclusion remarks and future directions

Work to understand the collateral sensitivity phenomenon is still in progress and there are still many unanswered questions. Findings of this project that show collateral sensitivity-interactions should be transferred to the clinical management of Gram-negative bacterial infections using already approved antibiotics, and may eventually contribute to establishment of drug cycling. Other studies done on collateral sensitivity have also focused on single species, and the evolutionary development and preservation of collateral sensitivity remains mysterious. More species should be investigated and the evolutionary development of these networks should be determined.

This project focused only on a limited amount of strains. The strains had three different genetic backgrounds. Future experiments on this project will be investigating other strains of ECO-SENS collection, and from that get an idea of which genetic factors could contribute to a pattern of collateral effects. Experiments on whether collateral sensitivity affects the kill kinetics of the resistant bacteria in this project should also be performed.

Understanding the mechanisms behind collateral sensitivity is still at an early stage. Most study done on evolution of this phenomenon just focused on acquisition of resistance through genomic mutations, but not by HGT. More work should look at HGT and its impact on collateral sensitivity. Studying and exploiting the fitness cost of antibiotic resistance mechanisms are also highly relevant.

Further experiments looking at the variations of collateral sensitivity networks across a greater strain collection of clinical isolates would help to ensure drug cycling choices appropriate for the majority of pathogens. When, or if, we get that far, in vivo confirmations of collateral sensitivity networks should be involved prior to the last step; introducing drug cycling in human patients .

Summa summarum, even though the phenomenon and the mechanisms behind is not yet fully understood, collateral sensitivity may contribute to the sustainable use of antimicrobials clinically by slowing down the rate of resistance evolution.

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7 APPENDICES

Appendix 1: Summary of results for generated trimethoprim resistant E. coli isolates

Strain	Mutants	RAPD PCR result	WT MIC (µg/mL)	Mutant MIC (µg/ml)	Conclusion
K56-12	4TP-12.1	+	80 %: 0.25	≥32	+
	4TP-12.2	+	"	≥32	+
	4TP-12.4	+	"	≥32	+
	4TP-12.7	+	"	≥32	+
	4TP-12.8	+	"	≥32	+
	8TP-12.2	+	"	80 %: 0.5	+
K56-41	4TP-41.1	+	80 %: 0.50	≥32	+
	4TP-41.2	+	"	≥32	+
	8TP-41.3	+*	"	80 %: 2	+
	8TP-41.7	+	"	80 %: 2	+
	8TP-41.9	+	"	80 %: 2	+
	16TP-41.1	-	"		+
	16TP-41.3	+	"	≥32	+
K56-50	4TP-50.1	+	80 %: 0.50	≥32	+
	4TP-50.2	+	"	80 %: 16	+
	4TP-50.3	+	"	80 %: 4	+
	4TP-50.4	+	"	≥32	+
	4TP-50.5	+	"	80 %: 8	+
	4TP-50.6	+	"	80 %: 4	+

***Few bands were visualized by RAPD PCR**

Table continues on next page

Strain	Mutants	RAPD PCR result	WT MIC (µg/mL)	Mutant MIC (µg/ml)	Conclusion
	4TP-50.7	+	“	80 %: 4	+
	4TP-50.8	+	“	80 %: 8	+
	4TP-50.9	+	“		+
	4TP-50.10	+	“		+
	4TP-50.11	+	“		+
	8TP-50.1	+	“		+
K56-70	4TP-70.3	+	1	$\geq$32	+
	4TP-70.5	+	“	$\geq$32	+
	4TP-70.6	+	“	$\geq$32	+
	4TP-70.7	+	“	$\geq$32	+
	8TP-70.1	-	“	80 %: 1	-
	4TP-70.8	+	“	$\geq$32	+
	4TP-70.9	+	“	$\geq$32	+
	4TP-70.10	+	“		
	4TP-70.11	+	“		
	4TP-70.12	NR	“		
	4TP-70.14		“		
K56-78	4TP-78.1	+	1	80 %: 1	+
	4TP-78.2	+	“	80 %: 1	+
	4TP-78.3	-	“	80 %: 1	-

Appendix 2: Summary of results for generated ciprofloxacin resistant E. coli isolates

Strain	**Mutants**	**RAPD PCR result**	**WT MIC (µg/mL)**	**Mutant MIC (µg/ml)**	**Conclusion**
K56-12	0.1CIP-12.1	+*	0.008	≥32	+
	0.1CIP-12.2	+	"	≥32	+
	0.1CIP-12.3	+*	"	≥32	+
	0.1CIP-12.4	+	"	≥32	+
	0.1CIP-12.5	+	"	≥32	+
	0.1CIP-12.6	+*	"	≥32	+
	0.1CIP-12.7	+*	"		
	0.1CIP-12.8		"		
	0.1CIP-12.10		"		
K56-41	0.1CIP-41.1	+	0.008	≥32	+
	0.1CIP-41.2	+	"	≥32	+
	0.1CIP-41.3	+	"		
	0.1CIP-41.4	+	"	≥32	+
	0.1CIP-41.5	+	"	≥32	+
	0.1CIP-41.6	+	"	≥32	+
	0.1CIP-41.7	-	"		-
	0.1CIP-41.8		"		
	0.1CIP-41.9		"		
	0.1CIP-41.10		"		

***Few bands were visualized by RAPD PCR**

Table continues on next page

Strain	Mutants	RAPD PCR result	WT MIC (µg/mL)	Mutant MIC (µg/ml)	Conclusion
K56-50	0.1CIP-50.1	+	0.008	≥32	+
	0.1CIP-50.2	+	“	≥32	+
	0.1CIP-50.3	+	“	≥32	+
	0.1CIP-50.4	+	“	≥32	+
	01CIP-50.5		“		
	0.1CIP-50.6	+	“	≥32	+
K56-70	0.1CIP-70.1	-	0.004		-
	0.1CIP-70.2	+	“		
	0.1CIP-70.1(1)		“		
	0.1CIP-70.2(1)		“		
	0.1CIP-70.5	+	“	≥32	+
	0.1CIP-70.6		“	≥32	
	0.1CIP-70.7		“	≥32	
	0.1CIP-70.8	+	0.004	≥32	+
	0.1CIP-70.9		“		
	0.1CIP-70.10		“		
K56-78	0.1CIP-78.1	+	0.016	≥32	+
	0.1CIP-78.2	+	“	≥32	+
	0.1CIP-78.3		“		
	0.1CIP-78.4		“		
	0.1CIP-78.5	+	“	≥32	+
	0.1CIP-78.6	+	“	≥32	+
	0.1CIP-78.7	+	“	≥32	+
	0.1CIP-78.8		“		
	0.1CIP-78.9		“		
	0.1CIP-78.10		“		
	0.1CIP-78.11		“		

Appendix 3: Summary of results for generated nitrofurantoin resistant E. coli isolates

Strain	**Mutants**	**RAPD PCR result**	**WT MIC (µg/mL)**	**Mutant MIC (µg/ml)**	**Conclusion**
K56-12	12NT-12.1	+	80 %: 8	80 %: 8	-
K56-41	8NT-41.1	+	80 %: 4	80 %: 4	-
	8NT-41.2		"		
	8NT-41.2(1)	+	"	80 %: 4	-
	8NT-41.3	+	"	80 %: 4	-
	8NT-41.4		"		
	8NT-41.4(1)	+	"	80 %: 2	-
K56-50	24NT-50.1	+	80 %: 16	80 %: 4	-
	22NT-50.1	+	"	80 %: 16	-
	22NT-50.2	NR	"	80 %: 8	-
	22NT-50.3	+	"	80 %: 16	-
	22NT-50.4		"		
K56-70	6NT-70.3	+	80 %: 4	80 %: 2	-
	6NT-70.4	+	"	80 %: 4	-
K56-78	12NT-78.1	+	80 %: 4	80 %: 16	+
	12NT-78.3		"		
	12NT-78.5		"		
	8NT-78.1	+	"	80 %: 16	+
	8NT-78.2	+	"	80 %: 16	+
	8NT-78.3	+	"	80 %: 16	+

Appendix 4: EUCAST clinical breakpoint v.5.0 for Enterobacteriaceae (EUCAST)

Antibiotics	***MIC breakpoint (mg/L)***	
	S <	R >
Gentamycin	2	4
Kanamycin	-	-
Ciprofloxacin	0.5	1
Tetracycline	-	-
Colistin	2	2
Nitrofurantoin	64	64
Mecillinam	8	8
Trimethoprim	2	4