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Collateral Sensitivity and Resistance Networks in Clinical *Escherichia coli* Isolates

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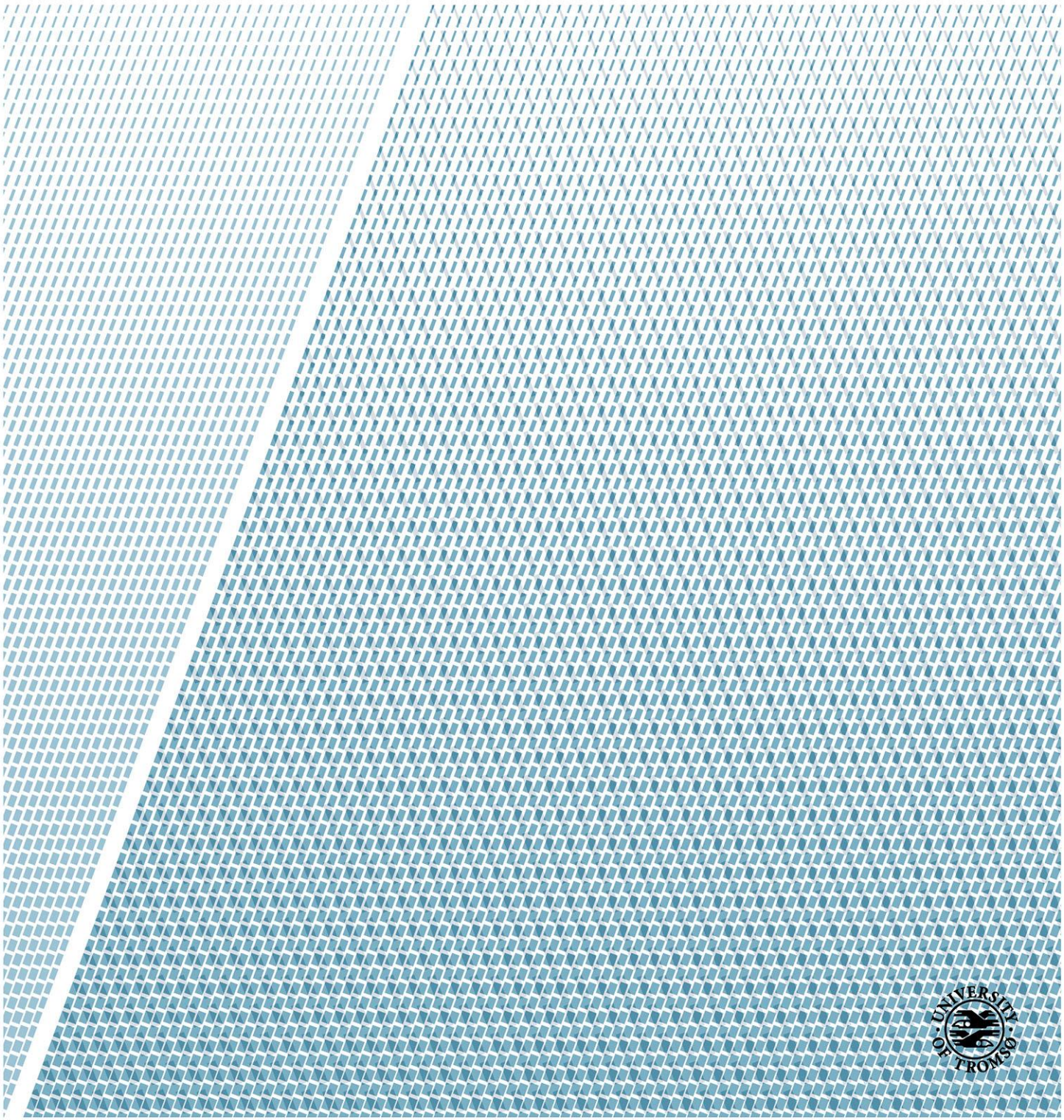

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Mari Norvik,

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Abstract

Because of an increasing development of antimicrobial resistance, treatment options for bacterial infectious diseases are narrowed. A strategy to reduce the evolution of resistance is to take advantage of antimicrobial collateral networks. In this thesis, we explored these collateral networks with a focus on antimicrobials used in Norway for the treatment of cystitis. Cystitis is the most common outcome of urinary tract infection.

Five clinical strains from a collection of pan-susceptible *Escherichia coli* (*E. coli)* isolated from uncomplicated urinary tract infection (ECO•SENS) were used. Spontaneous, single step mutants selected on trimethoprim, ciprofloxacin and nitrofurantoin were generated and their susceptibility determined by E-test. They were confirmed to belong to *E. coli* species by performing random amplified polymorphism DNA polymerase chain reaction (RAPD PCR). The susceptibility of the mutants was thereafter tested towards seven different antimicrobials. These results were compared to the ancestral strains' susceptibility profiles to the same antimicrobials. This method represented a scenario where the susceptibility of an isolate is decreased towards trimethoprim, ciprofloxacin or nitrofurantoin due to a spontaneous mutation, but at the same time, the mutants' susceptibility can increase or decrease for a different antimicrobial. The collateral networks of mutants with decreased susceptibility to three antimicrobials were visualized in heat maps, where blue color displays collateral sensitivity and red color indicates drug combinations that show collateral resistance. The use of drug combinations that show collateral resistance should be avoided and instead, the use of drug combinations that display collateral sensitivity should be applied to hinder the increase of antimicrobial resistance. The presented data suggest that acquisition of trimethoprim resistance simultaneously increases susceptibility to aminoglycosides and mecillinam. Moreover, we provide evidence that the temporal order of antimicrobial consumption can be optimized for resistance to trimethoprim and that the use of nitrofurantoin may be carried out with caution.

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Abbreviations

Bp	Base pair
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
dH_2O	Distillated water
ddH_2O	Double distillated water
E. coli	*Escherichia coli*
ECOFF	Epidemiological cut-off value
HGT	Horizontal gene transfer
Kbp	Kilo base pair
LB	Luria Broth
LBA	Luria Broth Agar
MPC	Mutant prevention concentration
MIC	Minimum inhibitory concentration
MSC	Minimal selective concentration
MSW	Mutant selection window
MHA2	Mueller-Hinton Agar 2
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphism DNA
RPM	Rotations per minute
ST	Sequence type
UTI	Urinary tract infection
WT	Wild type

1 Introduction:

1.1 State of the art

Sir Alexander Fleming discovered the first antibiotic in 1928, and it is clear that penicillin revolutionized medicine. Without antimicrobials, current treatment options would have been much narrower. They enable treatment of patients on an extensive scale. For example, performing organ transplantations without antimicrobials would have been extremely risky, and it probably would not have been feasible to achieve the current survival rates. (Davies and Davies, 2010).

After 1928, humans have underused, overused and misused antimicrobials to the point where resistance to antimicrobials has evolved even further (Davies and Davies, 2010). In Europe alone, the death toll from infections due to antimicrobial resistance bacteria is 25,000 people annually, and the total cost is estimated to be 1.5 billion EUR (ECDC/EMEA, 2009). Moreover, these numbers are estimated to increase in the future (de Kraker et al., 2011). It is not only the clinical treatment of humans that leads to the dissemination of antimicrobial resistance. In 2010, 13 million kilos of antimicrobials were fed to animals in the US to boost their growth rate (Spellberg et al., 2013). Adding to the growing problem, pharmaceutical companies are not inventing and developing new antimicrobials (Conly, 2005). What can be expected for the future?

One out of many clinical relevant diseases that can be difficult to treat because of the evolution of antimicrobial resistance is urinary tract infection (UTI). Recently an increase in resistance to antimicrobials commonly used to treat UTIs has been observed (Winstanley et al., 1997, Garau et al., 1999). Uropathogenic bacteria such as *E. coli* show decreased sensitivity to antimicrobials like ampicillin, amoxicillin, sulphonamides, trimethoprim and quinolones (Winstanley et al., 1997, Garau et al., 1999). A proposed method to deal with the lack of treatment strategies for several bacterial infections is renewing antimicrobial stewardship. Already in 1913, Paul Ehrlich discussed combination therapy as a strategy to outcompete resistant microbes (Ehrlich, 1913). Recent studies support this theory of using drug combinations to surmount antimicrobial resistance (Imamovic and Sommer, 2013, Kim et al., 2014).

1.2 Antibiotics/Antimicrobials

Antibiotics are produced by microorganisms in order to kill, or inhibit the growth of other microorganisms. Since Antimicrobials can be either natural, semisynthetic or synthetic, antibiotics are classified as antimicrobials, but antimicrobials are not necessarily antibiotics (Goering et al., 2013).

There are many ways to categorize antimicrobials. When an antimicrobial manages to affect both Gram-negative and Gram-positive bacteria, it has a broad spectrum of activity. Naturally, an antimicrobial agent with a narrow spectrum of activity affects simply Gram-negative or Gram-positive bacteria or an even smaller subpopulation. An antimicrobial can also be either bactericidal or bacteriostatic. Through a bactericidal effect of an antimicrobial, the organism is killed. An organism's growth can also be inhibited if the activity of the antimicrobial is bacteriostatic. Antimicrobials can also be classified by their mechanism of action. There are five main mechanisms based on the inhibited processes: cell wall synthesis, membrane function, protein synthesis, nucleic acid synthesis or other metabolic processes (Goering et al., 2013). In this thesis, we isolated mutants with reduced susceptibility to trimethoprim, ciprofloxacin, and nitrofurantoin, and therefore I focus mainly on these antimicrobials throughout the thesis.

1.2.1 Mechanism of action: trimethoprim

Trimethoprim has primarily a bacteriostatic effect (Felleskatalogen, 2015b). Mammalian, bacterial and protozoan cells inherently have the enzyme dihydrofolate reductase (DHFR), which is important for folic acid synthesis. By competitive inhibition, trimethoprim binds the enzymatic site of DHFR in bacteria. Human cells are resistant to trimethoprim. Therefore, trimethoprim can be used clinically with minimal side effects (Goering et al., 2013).

1.2.2 Mechanism of action: ciprofloxacin

Ciprofloxacin has a broad spectrum of activity and a bactericidal effect. It inhibits DNA gyrase and topoisomerase II and IV. These enzymes are important for bacterial replication, transcription, repair and recombination (Felleskatalogen, 2015a).

1.2.3 Mechanism of action: nitrofurantoin

The mechanism action of nitrofurantoin is complex. Intermediates of nitrofurantoin are formed through flavoproteins allowing the intermediates to inhibit bacterial ribosomal proteins, which prevents protein synthesis, aerobic energy metabolism, DNA, RNA, and cell wall synthesis. Nitrofurantoin has a bactericidal effect when treating urinary infections and in the presence of acidic urine the antimicrobial effect is increased (Felleskatalogen, 2012, UpToDate, 2015a).

1.3 Antimicrobial Resistance

Antimicrobial resistance is either classified as intrinsic or acquired. Intrinsic resistance is species specific, and the result of bacteria naturally encoding for a distinct resistance mechanism or lacking the structure or metabolic process of which the antimicrobial is targeting. Acquired resistance results from genetic changes to the bacteria that was originally sensitive to the antimicrobial. This kind of changes in the bacteria can occur by mutations in the bacterial chromosome or following the acquisition of new DNA (Hollenbeck and Rice, 2012).

1.3.1 Mechanisms of antimicrobial resistance

There are three different mechanisms of antimicrobial resistance. The first one prevents the antimicrobial from reaching the target either by reducing permeability or increasing efflux. The bacteria adapt reduced permeability by downward adjustment or exchanging of pores. Efflux is where the bacteria pump the antimicrobial out of the cell. The second mechanism of resistance results from changes to the antimicrobial targets by spontaneous mutations in the gene that encodes for target molecules. Alternatively, drug targets can be modified by, for instance, the addition of a chemical group. The third and final mechanism is modification of the antimicrobial. This can happen, for example, by hydrolysis of the β-lactam ring by β-lactamase enzymes or by the addition of a chemical group (Blair et al., 2015).

1.3.2 Genetic variation in bacteria

Gene transfer mechanisms are clearly involved in the spread of bacterial resistance determinants. Gene transfer can be vertical or horizontal. Vertical gene transfer is a linear transfer of genes from its ancestor, where it originally evolved, to two daughter cells. Horizontal gene transfer is the transfer of genetic material to another cell that is not it's offspring. Moreover, all horizontally acquired genetic changes can then be transferred vertically and be maintained in the population (Brown, 2003).

1.3.3 Horizontal gene transfer (HGT)

Horizontal gene transfer (HGT) is a well-known mechanism of spreading antimicrobial resistance. This allows bacteria to exchange DNA either through plasmids carrying multiple resistance determinants or bacteriophages. Mechanisms of HGT that are understood best are conjugation, transduction and natural transformation (**Figure 1**).

Conjugation can take place across the same or various species. The transfer occurs from the donor cell to the recipient cell through a pilus as the connector. The plasmid's origin of transfer is pulled through an exporter and into the recipient cell. The plasmid can be transferred further through other conjugation events or inherited through cell division. Transduction occurs when a bacteriophage transfers DNA between two bacteria. The bacterial virus binds to the recipient bacteria and inserts its nucleic acid. In natural transformation, a competent recipient cell takes up a fragment of DNA from the environment. The DNA fragment that was taken up into the recipient cell has to recombine with the recipient chromosome or be stabilized as a plasmid in order to be maintained and eventually expressed. (Holmes RK, 1996).

1.3.4 Point mutation

As reviewed in (Mohammad B. Habibi Najafi, 2013), mutations are changes in the deoxyribonucleic acid (DNA) sequence (**Figure 1**). These changes occur through errors during replication or non-repair of DNA damages. Mutations can be either spontaneous or induced. The consequence of a mutation can be beneficial, neutral, or harmful to the organism.

A point mutation occurs spontaneously through substitution, deletion or insertion of a base. This usually happens during DNA replication. Spontaneous or growth-dependent mutations will, in general, occur in 1 out of 10^6 – 10^8 cells (Giedraitiene et al., 2011).

There are three mechanisms of mutations, a substitution of a nucleotide, deletion, or addition of a nucleotide. A substitution of a base pair to a different base pair can be divided into two categories, transition and transversion. When a transition happens, the base substitution occurs with a base from the same chemical category. Alternatively, a transversion is when the replacement of a base occurs with a base from a different chemical category. Deletion or addition of a nucleotide occurs during DNA replication.

Point mutations can be silent, missense or nonsense mutations, or cause frameshifts. A silent substitution is a change to the amino acid codon that does not alter the amino acid sequence. A missense mutation is the change of a codon that originally belonged to another amino acid so that it now encodes for a different amino acid. When a codon is replaced by a stop codon, it is called a nonsense mutation. Finally, frameshift mutations occur when the total nucleotide number changes and the amino acid codons of the remaining sequence are displaced (Griffiths AJF, 2000, Mohammad B. Habibi Najafi, 2013).

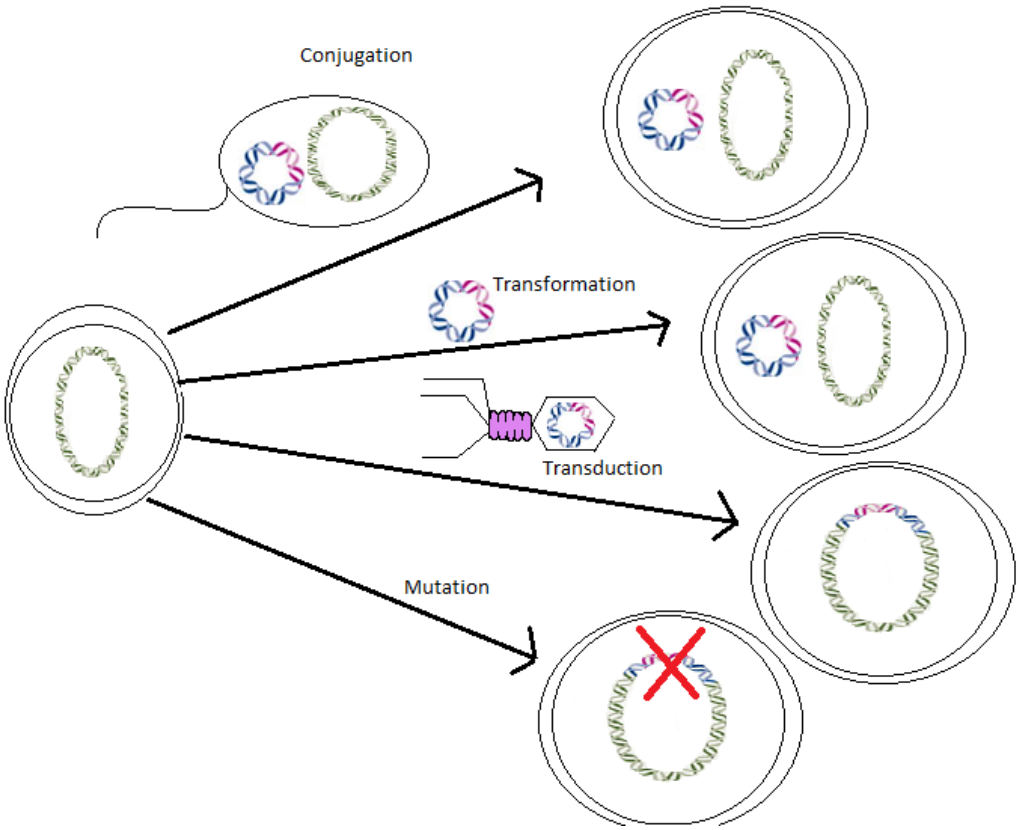

Figure 1: Illustration of conjugation, transformation, transduction and mutations. Adapted from (Andersson and Hughes, 2010).

1.3.4.1 Resistance to ciprofloxacin

Resistance to fluoroquinolones can occur by changes in the target enzymes, DNA gyrase and topoisomerase IV. This affects quinolone binding. A change in bacterial cell wall permeability can also arise and result in decreased uptake or increased the efflux of the drug through the presence of efflux pumps.

The target that is most tolerant of mutations will in a chronological order evolve changes. In *E. coli,* mutations occur first in the DNA gyrase enzyme. Further mutations will arise in several other genes that are also related to resistance (Jacoby, 2005). When *E. coli* evolves resistance to quinolones, a change in seven or more amino acids in the *gyrA* gene or three amino acids in *parC* gene are usually responsible (Giedraitiene et al., 2011) The *qnr* gene is also involved in the generation of ciprofloxacin mutants (Briales et al., 2011).

1.3.4.2 Resistance to trimethoprim

Susceptible strains can develop acquired resistance to trimethoprim. This is frequently caused by a chromosomal mutation that follows the production of DHFR, which may be less vulnerable to trimethoprim inhibition. Resistance rarely occurs through plasmids, but when it does, a high level of resistance takes place. If transposons transfer from one replicon to another resistance can also evolve. A mechanism through efflux pumps is also seen in *Burkholderia* efflux pumps (Podnecky et al., 2015).

1.3.4.3 Resistance to nitrofurantoin

Resistance to nitrofurantoin develops through stepwise mutations, which occur in the *nfsA* and *nfsB* genes. These genes encode for oxygen-insensitive nitroreductases. One-step mutations occur in the *nfsA* gene while two-step mutations are found in the *nfsB* gene. To obtain high-level nitrofurantoin resistance, it is necessary to inactivate these two genes (Sandegren et al., 2008).

1.3.5 Mutant selection window

To study how antimicrobial resistance can emerge in nature, microbiologists in the lab select for resistant isolates by culturing sensitive bacteria in the presence of the antimicrobial of interest. As reviewed in (Courvalin, 2008), an appropriate antimicrobial concentration is crucial to obtain mutants. Concentrations should not be as high as the mutant prevention concentration (MPC), where it is almost impossible to see any bacterial growth. However, they must be higher than the MIC, in order to exclude the growth of the wild type (WT). Somewhere between the MPC and MIC, the mutant selection window (MSW) exists and this is where spontaneous single step mutations usually occur (**Figure 2**) A new phenomenon termed minimal selective concentration (MSC) suggests increased relevance of antimicrobial concentrations below MIC with respect to MSW (Gullberg et al., 2011).

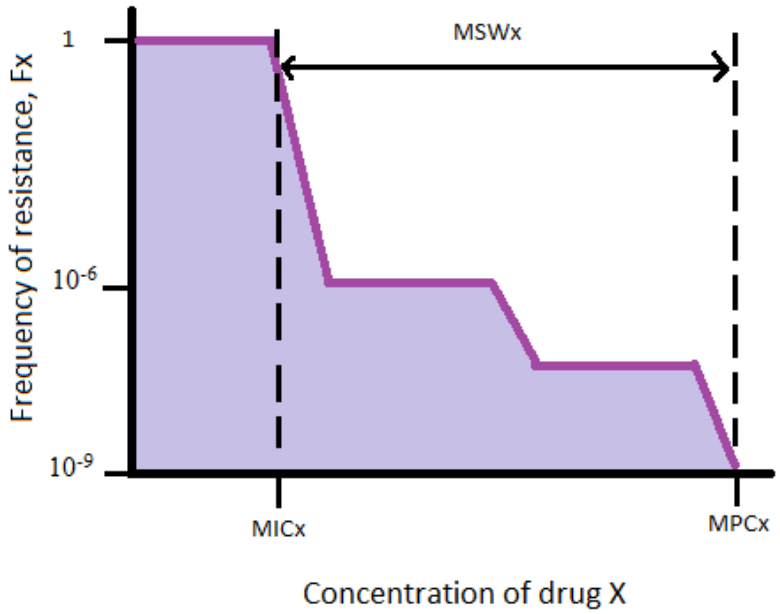

Figure 2: Graph is illustrating how the MSW lies between MIC and MPC. Figure adapted from (Michel et al., 2008)

1.4 Antimicrobial susceptibility testing

The organism of interest's susceptibility to antimicrobials can be tested *in vitro* for determination of its minimal inhibitory concentration (MIC), and is defined as " the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation" (Andrews, 2001). The three most common techniques to determine the MIC are the broth dilution method, gradient method (E-test) and disk diffusion method. The MIC-value indicates a strain's susceptibility to the tested antimicrobial and the strain can thus be categorized as either "susceptible", "intermediate" or "resistant" (Reller et al., 2009). MIC is also used to determine which antimicrobial to use for clinical treatment and to set the appropriate dose regimen (Mouton and Punt, 2001, Senekal, 2010)

An increase in MIC indicates a reduced susceptibility. If the MIC has decreased, the susceptibility has increased. Clinical breakpoints decide whether the bacterial susceptibility change is defined as susceptible, intermediate or resistant. These breakpoints are standardized and interpreted by using MIC (µg/mL) or a disk diffusion test with the zone of inhibition in diameters (mm) (Turnidge and Paterson, 2007). Clinical breakpoints are determined by "dosages, pharmacokinetics, resistance mechanisms, MIC distributions, zone diameter distributions pharmacodynamics and epidemiological cut-off values (ECOFFs)" (EUCAST, 2015). The ECOFF value distinguishes wild type from non-wild type (with resistance mechanism) for a given antimicrobial (EUCAST, 2007).

1.4.1 Broth dilution test, antimicrobial gradient method and disk diffusion test

In the broth dilution test, it is necessary to create a two-fold dilution of antimicrobials. An amount of bacteria is incubated in liquid growth medium (2.2.1) at 35 °C for 16-24 hours. After incubation, the MIC is determined as the lowest antimicrobial concentration where no bacterial growth is visible (Reller et al., 2009).

The methods on how to use antimicrobial gradient method (E-test) are supplied in (3.6,3.7).

The disk diffusion test utilizes a disk with a set antimicrobial concentration. The disk is placed on a pre-streaked LBA plate with the organism of interest and incubated overnight at 35 °C. The MIC is interpreted by reading the diameter in millimeters where the bacterial growth stops (Reller et al., 2009).

1.5 How antimicrobials can work together

Collateral sensitivity, also known as negative cross-resistance (Kim et al., 2014), is a term used for many years and in several contexts. It is mostly spoken of in cancer multidrug resistance (Pluchino et al., 2012), but it recently emerged as a hot topic in antimicrobial resistance research (Imamovic and Sommer, 2013).

As Imamovic and Sommer present it; "On the basis of these data, we propose a new treatment framework – collateral sensitivity cycling – in which drugs with compatible collateral sensitivity profiles are used sequentially to treat infection and select against drug resistance development" (Imamovic and Sommer, 2013). The theory proposes that when bacteria evolve resistance to one antimicrobial, the sensitivity may be improved to another antimicrobial. Collateral sensitivity networks can lead to more efficient killing if drugs are used in the same order. This could eliminate resistant bacteria to a greater extent as compared to antimicrobials not having this sensitivity network (Imamovic and Sommer, 2013).

It is envisioned that a form of cycling or rotation with two or more antimicrobials can delay the evolution of antimicrobial resistance. One criterion is that after ending the rotation, the same order of antimicrobials needs to be repeated (**Figure 3**). On the other hand, collateral resistance is a term used when a drug combination evolves a greater resistance than expected. This unfortunate outcome is not desired (Kim et al., 2014, Pluchino et al., 2012, Imamovic and Sommer, 2013).

The collateral sensitivity cycling is competition based. It is argued that collateral sensitivity will lead to a competitive replacement if two antimicrobials with reciprocal collateral sensitivity are cycled. This competition between the two resistant and susceptible strains are based on the idea that susceptibility to the next drug to be higher in the accumulated resistant (previous drug) strain than in the WT. Consequently, cycling will lead to a continuous selection against resistance(Imamovic and Sommer, 2013).

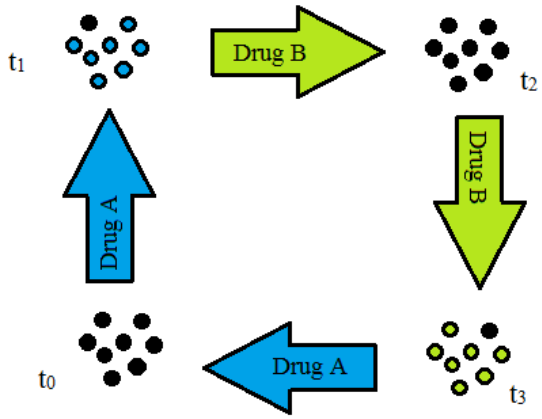

Figure 3: This illustration shows how collateral sensitivity networks can eradicate antimicrobial resistance. When treating a susceptible pathogen with Drug A for a while, resistance will develop (blue pathogens, t_1). When switching to Drug B an eradication of resistant bacteria from Drug A will take place (t_2). After treating with Drug B, the pathogen will become resistant (t_3) and a switch back to Drug A is necessary to outcompete resistance (t_0) (Imamovic and Sommer, 2013).

There are two different ways of combining antimicrobials. The alternating method is described as periodic switching between antimicrobials (as above). The second way is to combine antimicrobials as a multidrug, usually consisting of two antimicrobials, called "combination therapy" (Kim et al., 2014).

As reviewed in (Bell, 2005), antimicrobials can cooperate by inhibiting resistant bacteria in a more effective way. Drugs possessing this capacity have a "synergistic" quality. Conversely, a drug combination can also carry out an "antagonistic" effect, by killing fewer bacteria than anticipated. When drugs do not interact at all, they are additive (

Figure 4).

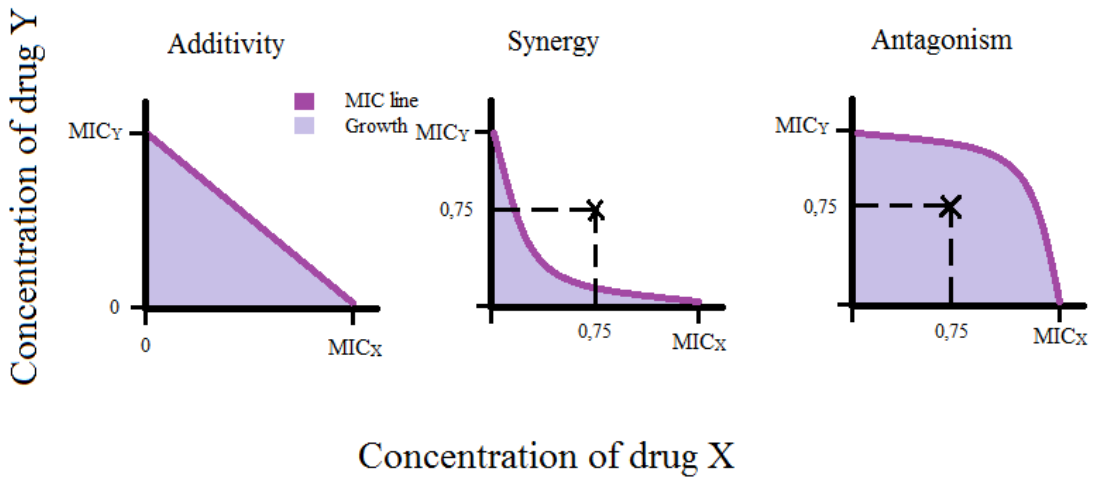

Figure 4: Difference between drug additivity, synergy and antagonism. Adapted from (Michel et al., 2008)

1.6 Organism of interest

E. coli is a mammalian, intestinal, Gram-negative bacterium, which the German pediatrician, Theodor Escherich, described for the first time in 1885 (Escherich, 1885). *E. coli* is a member of the family *Enterobacteriaceae* (Starr, 1986). A Short time after birth, *E. coli* is present in the human intestine. In a few cases, *E. coli* can be pathogenic and cause disease. When *E. coli* travel from their source to another area of the body, *E. coli* can often outcompete resident members of the normal flora and cause disease. The most common diseases caused by *E. coli* are diarrhea, UTIs and meningitis (Kaper et al., 2004).

1.6.1 ECO•SENS isolates

The ECO•SENS I and II studies mapped the susceptibility profiles of uropathogenic *E. coli* isolates originating from uncomplicated UTIs in women and included samples from 16 European countries and Canada. Identified isolates were susceptibility tested against antimicrobials commonly used for the treatment of UTIs. The time difference between the two studies, ECO•SENS I and II, was twelve years. The authors tested how the susceptibility had evolved between study I and II and found there were increases of resistance to nalidixic acid (4.3% to 10.2%) ciprofloxacin (1.1% to 3.9%) and trimethoprim (13.3% to 16.7%) (Kahlmeter, 2000, Kahlmeter and Poulsen, 2012). In this thesis, we work on several isolates from this strain collection that are “pan-susceptible” (Kahlmeter, 2000, Kahlmeter and Poulsen, 2012).

1.6.2 UTI

As many as 150 million incidents of urinary tract infection occur worldwide each year. The estimated financial costs are in the range of 6 billion dollars. *E. coli* is present in 70-95% of community-acquired UTIs (Kucheria et al., 2005). UTI is a collective term for cystitis, pyelonephritis and asymptomatic bacteriuria. Cystitis can be complicated or uncomplicated. An uncomplicated cystitis affects healthy, non-pregnant women and is the most frequent form of UTI. Complicated cystitis is caused by anatomic conditions often affecting pregnant women, men, children and elderly (Helsedirektoratet, 2012). 25-30% of all women with uncomplicated cystitis will have recurrences of cystitis. Uncomplicated cystitis causes significant morbidity especially when recurring (Kucheria et al., 2005). Treatment options for uncomplicated cystitis are narrowed because of antimicrobial resistance development (Grigoryan et al., 2014).

The current Norwegian guidelines for treatment of uncomplicated cystitis (**Figure 5**) include trimethoprim, nitrofurantoin and mecillinam as a first choice and ciprofloxacin as a backup if resistance has developed. The guidelines are similar to the treatment of complicated UTI except ciprofloxacin can also be used as a second choice treatment, regardless of resistance (Helsedirektoratet, 2012).

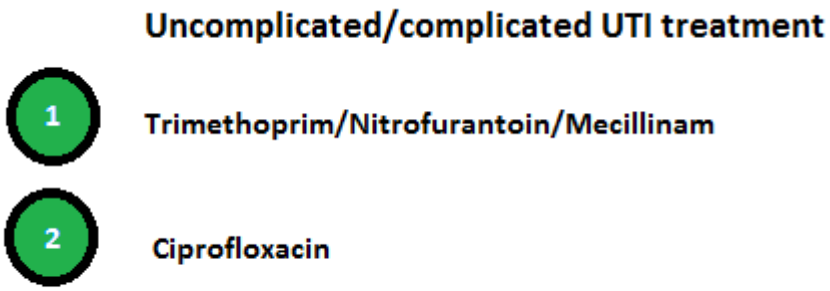

Figure 5: Norwegian treatment guidelines for uncomplicated and complicated UTI.

1.7 Aim

The aim of this study is to investigate networks of collateral resistance and collateral sensitivity in clinical *E. coli* strains with pre-existing resistance determinants relevant for the treatment of UTIs.

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

2 Materials:

In addition, Sambrook, Russell, Molecular Cloning - A laboratory manual, 2001 was used for materials and methods.

2.1 Bacterial strains

The bacterial strains used in this work are listed in **Table 1**. This is a collection of pan-susceptible *E. coli* clinical isolates, which were isolated from UTIs.

Table 1: *E. coli* isolates used in this study.

Name	***Sero Type***	***Phylogroup***	***Country***	***Year***
K56-2	ST73	B2	Greece	2000
K56-75	ST69	D	UK	2007-08
K56-68	ST95	B2	Sweden	2007-08
K56-16	ST127	B2	Portugal	2000
K56-44	ST12	B2	Greece	2007-08

2.2 Growth media

2.2.1 Luria Bertani (liquid) medium (LB medium) – 200 mL

In order to be able to do multiple experiments, a growth media was needed for the bacteria. Luria Broth (LB) is a nutrient often used to culture bacteria. The solution consisted of 5 g Difco™ Luria Broth, Miller (USA) powder and 200 mL distillated water (dH_2O). One liter LB medium consisted of 10 g tryptone, 5 g yeast extract and 10 g sodium chloride. The pH was measured at 7 with a TriTest (Mecherey – Nagel, Germany). A pH adjustment was therefore not needed. The LB media was autoclaved for 20 minutes at 121 °C (CertoClav, Getinge).

2.2.2 Luria Broth and Agar (LBA) – 40 plates

To make LB agar plates, agar was added to stiffen the solution. For making 40 dishes of LBA, 20 g of LB-broth and 12 g Select agar (Sigma-Aldrich, Germany) were mixed with 800 mL distilled water. The solution was autoclaved for 20 minutes at 121 °C. Following autoclaving, the mixture was cooled to 55 °C and poured into polystyrene petri dishes (VWR International, 90x16.2mm) (Addgene, 2014).

2.2.3 Mueller-Hinton Agar 2 (MHA2) – 40 plates

These plates were used for almost all E-tests. 38 g Mueller Hinton Agar 2 (Sigma-Aldrich, Switzerland) per liter ddH_2O was boiled for 1 minute and autoclaved at 121°C for 15 minutes and cooled to 50°C and poured into polystyrene petri dishes (VWR International, 90x16.2mm).

2.3 Antimicrobial stock solutions

2.3.1 Trimethoprim:

A stock solution (provided by Nils Hülter) at a final concentration of 100 mg/mL was used. The plates were made at concentrations of 3 μg/mL and 4 μg/mL trimethoprim. The volume of the bottle with LB Agar was 800 mL.

2.3.2 Ciprofloxacin:

To make a ciprofloxacin stock of 10 mg/mL, 100 mg ciprofloxacin ≥ 98% (HPLC, Fluka, China) was dissolved in 10 mL of distilled water. The ciprofloxacin dissolved with the addition of 10 drops of 3.7% HCl. The solution was sterile filtrated with a syringe and syringe filters (Acrodisc®, 25 mm) The LBA ciprofloxacin petri dishes were made at a concentration of 0.1 μg/mL ciprofloxacin.

2.3.3 Nitrofurantoin:

The final concentration of the nitrofurantoin stock solution was 40 µg/mL. 4 g Nitrofurantoin (crystalline, Sigma-Aldrich, Germany) was dissolved in 100 mL DMSO. The plates contained nitrofurantoin at a concentration of 6 µg/mL and 12 µg/mL (= 2×MIC). The MIC was 3 µg/mL and 6 µg/mL (Sandegren et al., 2008).

Of the 800 mL liquid LB medium (not yet autoclaved and the agar was not added yet), 20 mL were taken out, and 240 µL of the 40 µg/mL nitrofurantoin stock solution were added. 12 g agar powder was added to the remaining liquid LB and the media was autoclaved. The liquid LB containing nitrofurantoin was sterile filtered. After autoclavation, the solution with nitrofurantoin was added when the agar solution reached a temperature of 50 °C.

2.4 Buffer, reagents and other solutions

50% glycerol solution: For making 100 mL 50% glycerol solution, 57.5 mL 86-89% glycerol (Sigma-Aldrich, Germany) solution were mixed with 42.5 mL distilled water. The solution was autoclaved for 20 minutes at 121 °C.

0.9% NaCl : 9 g NaCl (Sigma-Aldrich, Germany) were dissolved in 1 L distilled water. The solution was autoclaved for 20 minutes at 121 °C.

TAE buffer (50X): TAE (Tris-acetate and EDTA) buffer was used for gel electrophoresis. For making 1 liter of the buffer, 242 g Trizma® Base (Sigma-Aldrich, Germany) was dissolved in 800 mL of distilled water. Additionally, 57.1 mL glacial acetic acid (Merck, Germany) and 100 mL of 0.5 M EDTA (GIBCO® Invitrogen) pH 8.0 were added. The volume was then brought up to 1 L with distilled water. The buffer was diluted to a concentration of 1×TAE before use.

Gel- loading buffer (6X): 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water + 6 mM EDTA, pH 8.

Smart ladder: Eurogentec, 200-10,000 bp, 5 µl/lane.

Mastermix: DreamTaq PCR master mix (2x) (Thermo Scientific, Germany), DreamTaq DNA Polymerase, optimized DreamTaq, $MgCl_2$, and dNTPs.

Primers: M13 primer for RAPD – PCR analysis, 5 µM working solution. With the sequence: 5' – GAGGGTGGCGGTTCT – 3'

Ethidium bromide: concentration of stock solution was 1 mg/mL, working concentration was 0.5 µg/mL.

3 Methods:

3.1 Overnight culture

For inoculation of an overnight culture, a single colony was taken from the bacterial isolate and resuspended in 30 mL of liquid LB medium. Cultures were incubated at 37°C with shaking at 150 rotations per minute (rpm) for 12-18 hours. The total volume of the overnight culture was distributed in three sterile falcon tubes. The tubes were centrifuged at 4000 rpm for 10 minutes, forming a pellet. The pellet was resuspended in 1 mL of fresh liquid LB medium (Addgene).

3.2 Generation of spontaneous mutants

To obtain mutants with reduced susceptibility to trimethoprim, ciprofloxacin and nitrofurantoin individually, the *E. coli* strains of interest were plated on LBA with varying concentrations of the antimicrobial.

100 µL (10^9 cells) overnight culture (see above) was plated using sterile glass beads onto a pre-dried LBA plate, containing the appropriate antimicrobial. Plates were incubated at 37°C for 12-18 hours. Single isolated colonies were chosen and struck for isolation on a new petri dish containing the same antimicrobial concentration to confirm re-growth. Possible mutant colonies were further characterized by confirmation using PCR (3.4) and antimicrobial susceptibility testing (3.6).

3.3 Freeze stock culture

Bacterial strains were stored at -80 °C. To prepare the samples, 1.8 mL Falcon freeze tubes (VWR International, USA) were filled with 500 µL LB and 500 µL 50% glycerol. A sterile 10 µL loop full of bacteria was mixed with the solution and the sample was put in a -80 °C freezer (Addgene).

3.4 Polymerase Chain Reaction (PCR)

A PCR is a reaction where specific portions of DNA are amplified by polymerases.
It switches between different temperatures at different times **Table 2**.

During the first and second step, double-stranded DNA (dsDNA) denatures to single-stranded DNA (ssDNA). In the third step, the primer binds to complementary segments of the ssDNA. DNA polymerase builds the new dsDNA strand, in the fourth step, using the complementary ssDNA as a template. Afterward, steps 2-4 are repeated 45 times.

The PCR technique applied in this project is called random amplified polymorphism DNA (RAPD) analysis **Table 2**.

This process comprises of the "PCR amplification of random fragments of genomic DNA with an arbitrary sequence (Maslow et al., 1993). This method does not require information about the DNA sequence (NCBI, 2015)**.** The primer used in this technique is called M13. M13 is a single primer that binds to arbitrary sequences.

Mutant samples, a positive control and negative controls, (*Acinetobacter*) and water, were prepared for PCR. From the colonies that re-grew on selective media, a single colony was once more picked, but this time was plated on an LBA plate for DNA extraction. The test samples were compared with the positive *E. coli* control. A negative control was included to compare against the positive. Bacterial colonies or isolated DNA (test samples, positive and negative controls) were resuspended in 100 µL double distilled water (ddH_2O). The samples were boiled for 10 minutes and then centrifuged at 13,000 rpm for 10 minutes. The supernatant was used as the PCR template. Each PCR-tube contained DreamTaq mastermix, M13 primer, ddH_2O and template (**Table 3**). All of the preparations were done on ice (Addgene).

Table 2: RAPD PCR program used in this thesis. The temperature and duration for each step are showed.

Step	***Temperature***	***Time***
1	95 ˚C	5 min
2	95 ˚C	1 min
3	36 ˚C	1 min
4	72 ˚C	2 min
5	Repeat 2-4	45 times
6	4 ˚C	∞

Table 3: Proportion of ingredients used for the PCR

Reagent	***Volume***
Mastermix	15 µL
M13 primer	5 µL
dd H_2O	3 µL
Template	2 µL
Total:	25 µL

3.5 Agarose gel electrophoresis

Gel electrophoresis separates DNA according to size. The flow of electricity, electrophoresis, moves a negatively charged DNA molecule towards a positive electrode. The shortest DNA fragments migrate the fastest. The mutant samples were compared to the length of the bands of the positive control. Using this technique, the samples were identified as either a desired *E. coli* mutant or a contaminant. The first step was to make the agarose gel. The concentration of the agarose gel was 2%, for the best separation of the bands. 2 g of agarose (SeaKem® LE Agarose, USA) were dissolved in 100 mL of 1×TAE buffer. The solution was put in the microwave oven for 1-3 minutes. After 5 minutes of cooling, 20 µL ethidium bromide (stock solution: 1 mg/mL) was added to the liquid agarose solution. Then the agarose solution was poured into the gel casting tray and left to stiffen (20-30 minutes). 2 µL loading buffer were added to 10 µL of the RAPD-PCR samples. When the agarose gel was put in the electrophoresis chamber, it was filled with 1×TAE buffer so that all of the gel was covered. 10 µL smart ladder was loaded into the first and the last lane and 10 µL of each sample was loaded in the lanes between the ladders. Then the gel was run at 90 volts for approximately 1 hour. Finally, the DNA fragments were visualized and analyzed under UV light (Addgene). The patterns of the amplified segments from the gel were interpreted by comparing the test samples bands with the bands of the positive control.

3.6 Antimicrobial susceptibility testing: E-test

An E-test is a premade strip of an antimicrobial with a gradient concentration. The MIC is read at the point where the growth stops. Trimethoprim and tetracycline are bacteriostatic antimicrobials, and the inhibition zone needs, therefore, to be read at 80% inhibition of growth. The other antimicrobials are bactericidal and the MICs were read at 100% inhibition (**Table 4**) (AbBiodisk, 2007). A bacterial suspension adjusted to 0.5 McFarland (turbidity standard for bacterial suspensions) was prepared and spread on a LBA/MHA2 dish with a sterile cotton tip in three different directions. The E-test was placed in the middle of the dish with sterile tweezers. The petri dish was incubated at 37°C for 12-18 hours (Hendriksen, 2003).

Table 4: Antimicrobials and their properties (UpToDate, 2015b).

Antimicrobial name	***Abbreviation***	***Mechanism of action***	***Bactericidal/ Bacteriostatic***	***Used for treating UTI:***
Trimethoprim	TRI	Folic acid biosynthesis	Bacteriostatic	Yes
Ciprofloxacin	CIP	Gyrase	Bactericidal	Yes
Nitrofurantoin	NIT	Multiple mechanisms	Bactericidal	Yes
Gentamicin	GEN	Protein synthesis	Bactericidal	Yes
Kanamycin	KAN	Protein synthesis	Bactericidal	Yes
Tetracycline	TET	Protein synthesis	Bacteriostatic	No
Colistin	COL	Lipopolysaccharide	Bactericidal	No
Mecillinam	MEC	Cell wall	Bactericidal	Yes

3.7 MIC testing with E-tests

After all of the mutants were harvested and stored at -80°C, the main experiment was up next. In the last section, testing with different antimicrobial E-tests was going to be carried out. The mutants were tested against seven different antimicrobials: gentamycin, kanamycin, tetracycline, colistin, mecillinam and nitrofurantoin, ciprofloxacin or trimethoprim (**Table 4**). To determine if there was a significant difference between the WT MIC and the mutants' final concentration, the E-test values needed to be read in a specific way. First, the concentration where the bacteria stopped growing was noted down. Second, if the basis value was between a two-fold-dilution, the result was rounded up to the nearest two-fold concentration. Third, the result was significant if there was a two-fold-dilution difference between the values being compared (this was done for all E-tests). The results were visualized in a heat map. Double mutants provided from an ongoing project were added to the experiment. A double mutant consisted of an isolate with decreased susceptibility to both trimethoprim and ciprofloxacin.

4 Results

In this thesis, spontaneous mutants with reduced susceptibilities to trimethoprim, ciprofloxacin, and nitrofurantoin were isolated by selection on the respective antimicrobial agent. Three double mutants with reduced susceptibility to both trimethoprim and ciprofloxacin were provided from an ongoing laboratory project. Selected representative mutants were verified by E-tests and RAPD-PCR and frozen down for further analyzes. Several strains displayed variable abilities to form mutants with reduced susceptibilities to the drugs applied here. This may be a reflection of intrinsic differences between the clinical strains. In some cases, the MIC value varied for the mutations that originated from the same strain. When this occurred, more mutants from that strain were selected for further MIC testing. The susceptibility profiles of mutants with reduced susceptibility to one or two antimicrobials were determined for seven different antimicrobials and heat maps were constructed, as presented in (Imamovic and Sommer, 2013). In these heat maps, blue color indicates increased susceptibility (collateral sensitivity) and red indicates a decreased susceptibility (collateral resistance). The different shades of each color display the fold susceptibility increase or decrease compared to the WT for each of the antimicrobials. A 32-fold increase/decrease has the darkest shades and the 2-fold increase/decrease has the lightest shades.

In general, single and double mutants of ciprofloxacin and trimethoprim tend to show increased MICs to both colistin and nitrofurantoin. The MIC increased as well when the nitrofurantoin mutant was susceptibility tested to colistin, mecillinam and ciprofloxacin.

4.1 Mutant generation and confirmation

A total of 76 mutants were collected with reduced susceptibilities to trimethoprim, ciprofloxacin and nitrofurantoin. Of those 76 isolates, 39 were selected on trimethoprim, 36 on ciprofloxacin, and 1 was found on nitrofurantoin. Altogether, 66 mutants were confirmed by RAPD-PCR and E-tests. The 10 remaining mutants could not be confirmed due to lack of DNA bands in the agarose gel electrophoresis picture. When the gel did not confirm the mutant as *E. coli*, an E-test was not performed (**Table 5**).

It was possible to obtain only one mutant from the selection on nitrofurantoin. The ancestral strain was K56-2, and it was given the isolate name 2.3 NIT. The concentration of the nitrofurantoin plates used for selection were double the MIC of the ancestral strains. Since MIC WT was 8 µg/mL, the plates had a concentration at 16 µg/mL. The MIC of the resulting mutant was 16 µg/mL. The final MIC susceptibility testing results can be seen in **Table 11** and **Table 18**.

The trimethoprim mutants were generated at three different drug concentrations, 3 and 4 µg/mL (**Table 14**). When the MIC was tested for the WT, most of the strains had a MIC of 1 µg/mL, but strain K56-75 had a value as low as 0.25 µg/mL. The 75.3 mutant had a MIC of 32 µg/mL, which was the same for most of the mutants, except for 44.8. Isolate 44.8 had a MIC of 2 µg/mL, which indicates only a 2-fold decrease in susceptibility. The mutant with the highest change in susceptibility was 75.3, with a 128-fold change (**Table 6**).

Ciprofloxacin selection occurred at only one plate concentration, 0.1 µg/mL (**Table 15**). All of the WT strains had a MIC of 0.008 µg/mL, and the MIC of the mutants were either 0.25 or 0.5 µg/mL. The susceptibility decreased, therefore, by 31 or 63 fold (**Table 7**).

The ancestral MICs are listed in **Table 8**. The antimicrobials that had the greatest variation of MIC between the selected mutants and the WTs are mecillinam, trimethoprim, and gentamycin. A total MIC difference of 4 fold can be seen for the gentamycin, mecillinam, and trimethoprim values.

Table 5: Numbers of spontaneous *E. coli* mutants isolated on different selective plates.

Strain	***Total isolates***			***Confirmed***			***Unconfirmed***		
	TRI	CIP	NIT	TRI	CIP	NIT	TRI	CIP	NIT
K56-2	4	8	1	4	8	1	0	0	0
K56-16	12	13	0	12	9	0	0	4	0
K56-44	7	7	0	7	7	0	0	0	0
K56-68	5	4	0	2	2	0	3	2	0
K56-75	11	4	0	11	3	0	0	1	0
Total	39	36	1	36	29	1	3	7	0

Abbreviations: TRI – trimethoprim; CIP – ciprofloxacin; NIT – nitrofurantoin.

Table 6: Trimethoprim mutants used in the thesis with decreased susceptibility.

	Trimethoprim Concentration (µg/mL)			
Mutant	***Selection***	***WT MIC***	***MIC mutant***	***Fold Change***
2.1 TRI	4	1	≥ 32	≥ 32
16.5 TRI	4	1	≥ 32	≥ 32
44.7 TRI	4	1	≥ 32	≥ 32
44.8 TRI	4	1	2	2
44.14 TRI	4	1	16	16
68.13 TRI	4	0.5	≥ 32	≥ 64
75.3 TRI	3	0.25	≥ 32	≥ 128

Abbreviations: TRI – trimethoprim

Table 7: Ciprofloxacin mutants used in the thesis with decreased susceptibility.

	Ciprofloxacin Concentration (µg/mL)			
Mutant	***Selection***	***MIC WT***	***MIC Mutant***	***Fold change***
2.3 CIP	0.1	0.008	0.5	63
2.5 CIP	0.1	0.008	0.5	63
2.7 CIP	0.1	0.008	0.25	31
16.1 CIP	0.1	0.008	0.25	31
16.3 CIP	0.1	0.008	0.25	31
16.11 CIP	0.1	0.008	0.5	63
44.4 CIP	0.1	0.008	0.5	63
44.5 CIP	0.1	0.008	0.5	63
68.1 CIP	0.1	0.008	0.5	63
75.1 CIP	0.1	0.008	0.25	31

Abbreviations: CIP – ciprofloxacin

Table 8: Antimicrobial susceptibility testing of WT *E. coli* strains used in this thesis.

Strain	***Minimal inhibitory concentration (µg/mL)***							
	GEN	***KAN***	***TET***	***COL***	***NIT***	***MEC***	***TRI***	***CIP***
K56-2	1	4	4	1	8	0.25	1	0.008
K56-16	2	4	4	1	4	0.25	1	0.008
K56-44	2	8	2	0.5	8	1	1	0.008
K56-68	4	8	2	1	8	0.5	0.5	0.008
K56-75	2	8	2	0.5	4	0.25	0.25	0.008

Abbreviations: GEN – Gentamycin, KAN – Kanamycin, TET – Tetracycline, COL – Colistin, NIT – Nitrofurantoin, MEC – Mecillinam, TRI – Trimethoprim, CIP – Ciprofloxacin.

4.2 RAPD-PCR and agarose gel electrophoresis

As shown in (**Table 5**) not all of the mutants were confirmed as *E. coli* by agarose gel electrophoresis because of a lack of bands. When DNA bands were displayed as showed in **Figure 6**, *E. coli* had bands at ≈ 400 and 700 base pairs (bp) and at 1.5 and 2 kilo base pairs (kbp).

Figure 6 shows an example of a gel picture. The positive control did not have as bright bands as the rest of the samples, and missed a band at 2 kbp as in numbers 2-8. A band at 1.5 kbp was present in the positive control, 2, 5, 6, and water. Bands at 700-800 bp were present in 1-8, water, and positive control.

Samples 1-8 display similar patterns as observed in the positive control. The results support that the isolated mutants with reduced susceptibility to ciprofloxacin originate from the WT *E. coli* ECO•SENS strain K56-44.

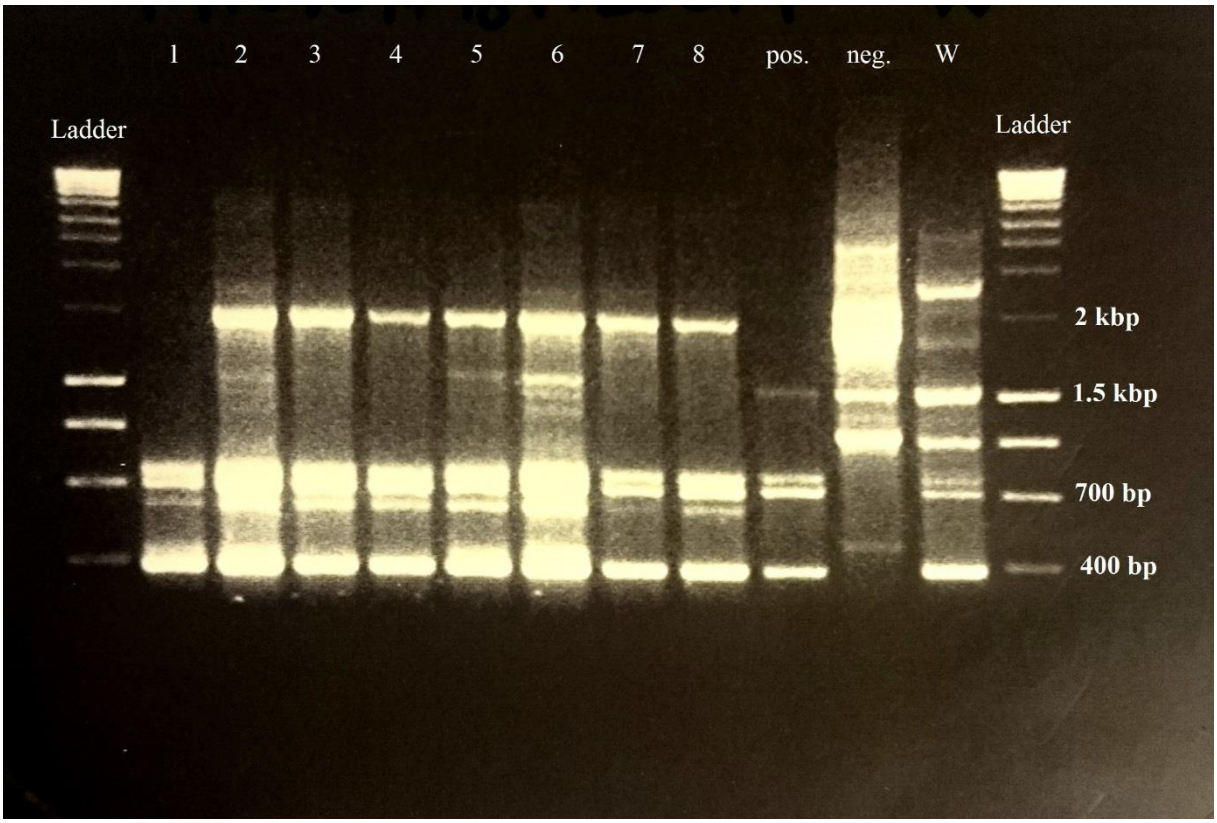

Figure 6: Agarose gel electrophoresis result of K56-44 ciprofloxacin mutants. Number 4 is 44.4 CIP, and number 5 is 44.5 CIP that are used to test the collateral networks.

4.3 Collateral-sensitivity and –resistance networks

4.3.1 Trimethoprim

In **Table 14** total generated trimethoprim mutants are displayed. Seven trimethoprim mutants were chosen for a closer look at putative collateral networks. Three were originating from the K56-44 strain since the MIC of the mutants varied greatly within the same strain (2-32 μg/mL). For the remaining strains, only one mutant was used because the MIC values of the mutants were more consistent. Trimethoprim resistance is defined by a MIC above the clinical breakpoint of 4 μg/mL, while sensitivity is defined as a MIC at or below 2 μg/mL Of the mutants tested, 6 had MICs above the clinical breakpoint for resistance. Mutant 44.8 is defined as sensitive because the MIC is 2 μg/mL, but the mutant is still less susceptible to trimethoprim than the WT (1 μg/mL) (**Table 9**).

MIC testing was used to determine the susceptibility of these trimethoprim mutants to kanamycin and mecillinam compared to the WT strain. For these antimicrobials, there was a 4-8 fold decrease in MIC of the mutants compared to the WT. This means that the acquisition of trimethoprim resistance led simultaneously to increased susceptibility to kanamycin and mecillinam. In the case of gentamicin and tetracycline, there were 2-8 fold reductions in MIC. No change in the MIC was observed when trimethoprim mutants were tested to ciprofloxacin. Some of the trimethoprim mutants also had no change in susceptibility to colistin and nitrofurantoin (**Table 9**).

A few of the genetic backgrounds of the trimethoprim mutants displayed increased MICs when tested to nitrofurantoin and colistin compared to the WT when tested to these antimicrobials. This indicates that some of the trimethoprim mutants have a decreased susceptibility to nitrofurantoin and colistin compared to the WT's susceptibility to those antimicrobials (**Table 9**).

The MIC values based on the heat map is shown in appendix **Table 16**

Table 9: Fold change in MIC values for trimethoprim-selected mutants compared to the WT. The ECOFF value is 0.064 -2µg/mL, and clinical breakpoints are defined as S ≤ 2, R > 4. The heat map is an inspiration from (Imamovic and Sommer, 2013).

Strain	Mutant nr	MIC WT	MIC mut.	GEN	KAN	TET	COL	NIT	MEC	CIP
K56-2	2.1 TRI	1	32							
K56-16	16.5 TRI	1	32							
K56-44	44.7 TRI	1	32							
K56-44	44.8 TRI	1	2							
K56-44	44.14 TRI	1	16							
K56-68	68.13 TRI	0.5	32							
K56-75	75.3 TRI	0.25	32							

4.3.2 Ciprofloxacin

In **Table 15** the total generated ciprofloxacin mutants are displayed. The ciprofloxacin mutants used for examinating collateral networks are shown in **Table 10**. A total of 10 mutants were used. The mutants were chosen based on how much the MIC varied within the same strain. Three isolates from the K56-2 strain (MIC = 0.25-0.5 µg/mL), 3 from K56-16 (0.19-0.5 µg/mL), 2 from K56-44 (0.38-0.5 µg/mL) and 1 each from K56-68 and K56-75 were selected for further testing.

Ciprofloxacin resistance is defined by having a MIC above the clinical breakpoint of 1 µg/mL, while sensitivity is defined as 0.5 µg/mL or below. All of the mutants are defined as sensitive, but still the susceptibility is lowered compared to the WT.

The mutants with reduced susceptibility to ciprofloxacin were subjected to MIC testing using antimicrobial as in **Table 10.** Strikingly, a near universal pattern of collateral sensitivity to gentamicin, kanamycin, tetracycline, mecillinam and collateral resistance to nitrofurantoin and colistin, as demonstrated for the trimethoprim mutants, was also observed in the mutants with reduced susceptibility to ciprofloxacin (**Table 10**).

The MIC values based on the heat map is shown in appendix **Table 17**.

Table 10: Fold change in MIC values for ciprofloxacin-selected mutants compared to the WT. The ECOFF value is 0.004-0.064 and the clinical breakpoints are defined as S ≤ 0.5, R > 1. The heat map is an inspiration from (Imamovic and Sommer, 2013).

Strain	Mutant Nr	MIC WT	MIC mut.	GEN	KAN	TET	COL	NIT	MEC	TRI
K56-2	2.3 CIP	0.008	0.5							
K56-2	2.5 CIP	0.008	0.5							
K56-2	2.7 CIP	0.008	0.25							
K56-16	16.1 CIP	0.008	0.25							
K56-16	16.3 CIP	0.008	0.25							
K56-16	16.11 CIP	0.008	0.5							
K56-44	44.4 CIP	0.008	0.5							
K56-44	44.5 CIP	0.008	0.5							
K56-68	68.1 CIP	0.008	0.5							
K56-75	75.1 CIP	0.008	0.25							

4.3.3 Nitrofurantoin

Only one nitrofurantoin mutant was available for testing collateral networks **Table 11.** This mutant originated from the K56-2 strain.

Nitrofurantoin resistance is defined by having a MIC about the clinical breakpoint of 64 µg/mL, while sensitivity is defined as a MIC of 64 µg/mL or below. The mutant is therefore not resistant, but the mutant is still less sensitive to nitrofurantoin than the WT (**Table 11**).

The mutant with reduced susceptibility to nitrofurantoin was subjected to MIC testing using the same antimicrobial agents as in **Table 11.** For this nitrofurantoin mutant it was observed collateral sensitivity to gentamicin, kanamycin, and tetracycline, as was present in the trimethoprim and ciprofloxacin mutants. The mutant also displayed a collateral sensitivity towards trimethoprim (**Table 11**).

The nitrofurantoin mutant showed a two-fold increase in MIC when tested to colistin, mecillinam, and ciprofloxacin compared to the WT MIC when tested to these same antimicrobials. This indicates that the nitrofurantoin mutant has a decreased susceptibility to colistin, mecillinam, and ciprofloxacin compared to the WT (**Table 11**).

The MIC values based on the heat map is shown in appendix **Table 18**.

Table 11: Fold change in MIC values for nitrofurantoin-selected mutants compared to the WT. The ECOFF value is 4-64 µg/mL and the clinical breakpoints are defined as S ≥ 64 < R. The heat map is an inspiration from (Imamovic and Sommer, 2013).

Strain	Mutant	MIC WT	MIC Mut.	GEN	KAN	TET	COL	TRI	MEC	CIP
K56-2 ST73	NIT2.3	6	16							

4.3.4 Double mutants

The double mutants used for the collateral network assay are listed in **Table 12.** A total of 3 mutants were used, one double mutant from each of the K56-2, K56-68 and K56-75 strains. All of these mutants had MICs above 4 μg/mL for trimethoprim and are defined as resistant. However for ciprofloxacin the mutants had a MIC below 1 μg/mL, which indicates that they are not resistant, but they are still less susceptible than the WT.

Interestingly, the double mutants with reduced sensitivity to both trimethoprim and ciprofloxacin tend displayed collateral sensitivity and resistance network similar to the trimethoprim and ciprofloxacin single mutants (**Table 13**).

The MIC values based on the heat map is shown in appendix **Table 19**.

Table 12: Overview of the MIC for the strains and mutants and clinical breakpoints and ECOFF value.

	Minimal inhibitory concentration (µg/mL)				
Strain	***Double Mutant***	***WT TRI***	***WT CIP***	***MUT TRI[1]***	***MUT CIP[2]***
K56-2	2.1 TRICIP	1	0.008	32	0.75
K56-68	68.1 TRICIP	0.5	0.008	32	0.5
K56-75	75.1 TRICIP	0.25	0.008	32	0.5

Abbreviations: TRI – trimethoprim, CIP – ciprofloxacin.

[1] – Clinical breakpoints for trimethoprim (S ≤ 2, R > 4), ECOFF values 0.064 – 2

[2] – Clinical breakpoints for ciprofloxacin (S ≤ 0.5, R > 1), ECOFF values 0.004 – 0.064

Table 13: Fold change in MIC values for double-selected mutants compared to the WT. The heat map is an inspiration from (Imamovic and Sommer, 2013).

Strain	**Double Mutant nr**	**GEN**	**KAN**	**TET**	**COL**	**NIT**	**MEC**
K56-2	2.1 TRICIP						
K56-68	68.1 TRICIP						
K56-75	75.1 TRICIP						

- >32 - 16 - 8 -4 - 2 0 + 2 + 4 + 8 + 16 + >32

5 Discussion

In the recent years, antimicrobial resistance has developed, bringing along increased fatality rates than earlier. If this problem continues to increase and is not controlled, the risk of not having any options for the treatment of a bacterial infection is a future possible scenario (ECDC/EMEA, 2009).

In this thesis, a possible way of solving this increasing problem is investigated. The basis of this project is a procedure in which antimicrobials are alternated according to increased susceptibility of otherwise resistant strains, a phenomenon, which is based on a so-called collateral sensitivity effect (Imamovic and Sommer, 2013)

Five clinical strains from the ECO•SENS study (Kahlmeter, 2000) were used to generate mutants with decreased susceptibility to trimethoprim, ciprofloxacin and nitrofurantoin under selective pressure (plating on antimicrobial containing medium). Double mutants were also generated, combining trimethoprim and ciprofloxacin resistance in the same genetic background (representing multi-drug resistance). The results of MIC determination were presented in heat maps inspired by (Imamovic and Sommer, 2013).

These heat maps indicated that mutants of trimethoprim and ciprofloxacin, both single and double mutants, tend to display collateral increased sensitivity to kanamycin, gentamycin, tetracycline, mecillinam, trimethoprim, and ciprofloxacin. On the other hand, the same mutants tend to display collateral reduced susceptibility to colistin and nitrofurantoin. The nitrofurantoin mutant displayed collateral increased sensitivity to kanamycin, gentamycin, tetracycline, and trimethoprim and collateral reduced sensitivity to ciprofloxacin and mecillinam and colistin. It will be interesting to see if these results are general in a larger strain collection.

5.1 Collateral networks

5.1.1 What do the results tell us?

The findings presented in this thesis, if underscored by additional work *in vitro* and *in vivo* can suggest a change to the national and international guidelines of treating UTIs. Today the Norwegian guidelines for treating uncomplicated UTIs include trimethoprim, nitrofurantoin, and mecillinam as the agents of first choice and ciprofloxacin as a backup agent if resistance has developed indicated by treatment failure or susceptibility testing. The guidelines are similar for treating complicated UTIs, with the only difference that ciprofloxacin can also be used as a second choice treatment, regardless of resistance (Helsedirektoratet, 2012) **Figure 5.**

In full awareness that the presented results are preliminary, a possible procedure for alternating antimicrobials is suggested (**Figure 7**). This sequential "combination-treatments" will, following resistance development to first line treatments (**Figure 7**, left panel), ultimately lead to increased treatment efficacy and possibly reduced rates of resistance evolution. The results of this thesis may also suggest which antimicrobials should not be used in a two-way alternation of antimicrobials (**Figure 8**). The pattern of the results in this thesis may also look like it is beneficial to exercise extra caution when using nitrofurantoin either as the first or second line drug. Figure 7 and 8 displays result-tendencies of the isolates from the heat maps (**Table 9**, **Table 10** and **Table 11**), not all of the isolates show similar collateral networks. For example, three out of seven trimethoprim mutants exhibited collateral resistance towards nitrofurantoin, where the rest displayed a no-change in susceptibility.

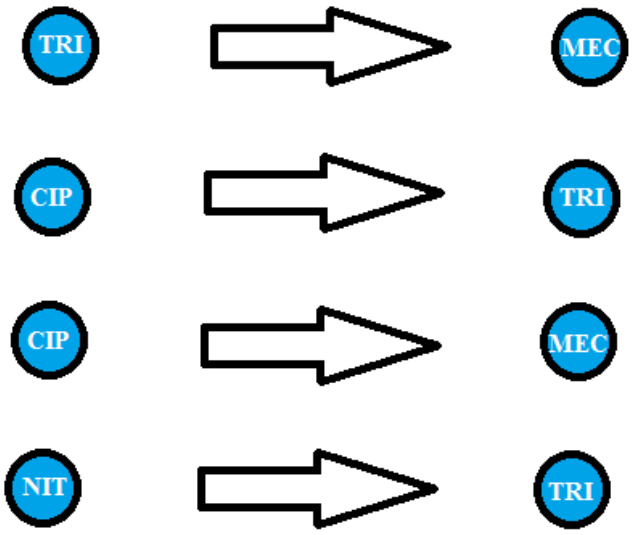

Figure 7: Illustrates tendencies of collateral sensitivity networks in antimicrobials used treating uncomplicated cystitis. This displays possible procedure for alternating antimicrobials. The circles to the left are generated mutants from different antimicrobials and the circles to the right displays what antimicrobial the mutants tend to have collateral sensitivity towards. Based on heat maps (**Table 9**, **Table 10**, **Table 11**). Adapted and modified (Imamovic and Sommer, 2013).

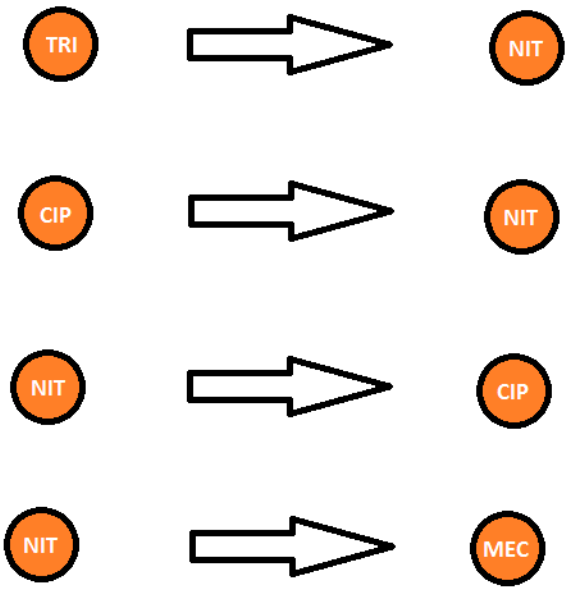

Figure 8: Illustrates tendencies of collateral resistance networks for antimicrobials used treating uncomplicated cystitis. The circles to the left are generated mutants from different antimicrobials and the circles to the right displays what antimicrobial the mutants tend to have collateral resistance networks towards. Based on heat maps (**Table 9**, **Table 10**, **Table 11**). Adapted and modified (Imamovic and Sommer, 2013).

Gentamycin and kanamycin can be used for treating UTIs, but tetracycline and colistin are not used for this indication. Tetracycline and colistin were included in this thesis to show that guidelines for a variety of diseases treated with antimicrobials are necessary to avoid collateral resistance development.

The double mutants used in this thesis exhibited reduced susceptibility to multiple drugs similar to what was demonstrated for single mutants of trimethoprim and ciprofloxacin. Surprisingly, this suggests that the acquisition of two determinants with decreased susceptibility does not seem to affect the collateral sensitivity profiles in *E. coli*. This needs further exploration and investigations on the effects of mobile genetic elements should also be included. If such general patterns exist, it is promising for the future applications of these and similar findings as collateral sensitivity based guidelines can be applied to multi-drug resistant strains.

The collateral networks demonstrated here lay within the range of +/- 8-fold susceptibility change. The isolates generated from the selection on the same antimicrobial, tend to show generally similar results for the different antimicrobial susceptibility testing. Though some showed different fold collateral sensitivity, others displayed, for example, collateral resistance and no fold-change. When trimethoprim mutants were susceptibility tested against colistin, the largest range of fold change was observed (-4 to +2) **Table 9**. As seen in the heat maps, mutants do not always display similarities, even if they are generated by the same selection concentration, the MIC differences between the WT are mutant similar, or if they are originated from the same parental strain. The network differences are likely the result of variation in the genetic background (Imamovic and Sommer, 2013).

All of the trimethoprim-selected mutants are defined as resistant, except for 44.8TRI, which had a MIC of 2 μg/mL. Interestingly, this isolate in within the ECOFF value range of 0.064μg/mL to 2μg/mL, which defines it still as sensitive. Despite these properties, 44.8 TRI tends to show the same collateral networks as the six other trimethoprim mutants that are defined as resistant. Even though 44.8TRI is defined as a sensitive, the MIC has increased compared to its ancestral strain (MIC = 1 μg/mL) and thus the susceptibility towards trimethoprim is decreased. This indicates that the isolate is mutated even though it is still defined as sensitive, and displaying similar networks as resistant mutants. As seen in **Table 6**, the MIC of 75.3 displays a ≥ 128-fold change meanwhile 44.8 shows a 2-fold change compared to MIC WT. The reason for the big range in fold change may indicate different mutation affecting trimethoprim MIC.

5.1.2 How do the results compare to existing literature.

Most of the trimethoprim mutants in this thesis had MICs above the clinical breakpoint. Ciprofloxacin and nitrofurantoin mutants display MICs below the clinical breakpoint, and are not defined as resistant. All isolates in the thesis were selected at just one concentration, and are therefore most likely single-step mutants. Imamovic and Sommer (Imamovic and Sommer, 2013) isolated all mutants above the clinical breakpoint and in several steps suggesting that their isolates acquired several mutations after being tested

to stronger selection pressure than applied in this study. Interestingly, my findings of almost identical collateral sensitivity networks between trimethoprim resistance mutants in different *E. coli* strains resulting in increased sensitivity towards aminoglycosides and mecillinam contrasts the findings in (Imamovic and Sommer, 2013). These authors reported trimethoprim resistant mutants that displayed 2-4 fold MIC increases in susceptibility to gentamycin, kanamycin, ciprofloxacin, and nitrofurantoin. No change in susceptibility was seen towards tetracycline and colistin. The only similarity to the results presented here was a 2-fold increase of MIC when trimethoprim isolates were susceptibility tested with nitrofurantoin. Since the selection pressure applied in Imamovic and Sommers paper differed from this thesis, the number of mutations in an isolate can vary and the collateral networks thereby as well. Moreover, another difference of great importance was between the strains used to generate mutants. One of the strains Imamovic and Sommer used was *E. coli* MG1655. This is a lab strain originally derived from the K-12 strain in 1920s from a stool sample of a diphtheria patient (Madison, 2002-2015). The antimicrobial sensitivity can vary between different strains of *E. coli*. Since we used clinical UTI strains in this thesis, the differing results from Imamovic and Sommer could be expected.

Another recent study (Oz et al., 2014) selected mutants from *E. coli* MG1655 as well, but in two groups. Following “mild” and “strong” antimicrobial selection pressure. The group with strongly selected mutants tended to have a higher number of mutations, but the level of resistance was equal to both groups. Increased collateral sensitivity and resistance networks were also seen in this group compared to the mild selection pressure-group. Oz et. al. (Oz et al., 2014) displayed no change in collateral networks when trimethoprim isolates were susceptibility tested with ciprofloxacin. On the background of the discussion above, the resemblance might be accidental. Further studies with larger and more genetically diverse strains are necessary to determine more general collateral sensitivity networks.

5.2 Generation of mutants

The degree of difficulty to obtain spontaneous mutants varied according to the antimicrobial used. Differences in resistance development are primarily linked to the number of mutations needed to achieve a given MIC. A factor that also may affect the level of difficulty to generate mutants is whether the antimicrobial is bactericidal or bacteriostatic. Ciprofloxacin and nitrofurantoin are bactericidal drugs while trimethoprim is a bacteriostatic drug. It is therefore more easy to evolve mutants for trimethoprim (Stratton, 2003). This might be due to the fact that trimethoprim simply prevents bacteria from growing and depends on the action of the immune system to contribute to the removal of the pathogen. Since no immune system is present on an agar plate, the development of spontaneous mutants may occur even easier. In addition, a note to consider is that most of the trimethoprim selected mutants had MICs above 32 µg/mL, which indicates a high-level of resistance. None of the ciprofloxacin and nitrofurantoin mutants was defined as high-level mutations.

It was most difficult to generate nitrofurantoin mutations mainly because of nitrofurantoin's light sensitivity. Even though the nitrofurantoin plates were exposed to a minimum of light, we had problems with degradation of the antimicrobial. This allowed even the WT to grow on LBA plates with nitrofurantoin, and therefore only one mutant was confirmed.

Mutated isolates can also be generated in LB liquid media (Munck et al., 2014, Rodriguez de Evgrafov et al., 2015). In this thesis, solid agar plates (LBA) were used. The advantage of using liquid media is not the need of an overnight culture, on the other hand when using solid agar plates contaminations may be detected by the eye or the smell. This advantage applies as well for gradient plating, but the procedure can be cumbersome.

Five strains were used in this study, and this number is high compared to other studies. Similar studies used one or two strains for studying collateral networks (Imamovic and Sommer, 2013, Oz et al., 2014, Szybalski and Bryson, 1952, Lazar et al., 2013, Munck et al., 2014, Rodriguez de Evgrafov et al., 2015, Kim et al., 2014). When using several strains, the results become more reliable and it is easier to draw general conclusions.

Using LB media or LBA plates, allows the bacteria to grow under perfect conditions and temperature. These *in vitro* conditions do not create the most realistic picture of how bacteria will act when present in the human body. An immune system will, in addition to an antimicrobial treatment, help to overcome the pathogen. If future studies are performed *in vivo*, the collateral networks may differ from the findings *in vitro*.

5.3 Species confirmation

In this study, the aim was to verify that mutants of *E. coli* were isolated, and not contaminants. RAPD PCR was well suited for this task the technique is fast, simple and inexpensive. Additionally, it does not require DNA sequence knowledge and the same primer can be used for a number of bacteria. The evolutionary development and diversification of the mutants can also be visualized. Some of the disadvantages of using RAPD PCR are that slight alterations in, for example, temperature, pH and time interval can cause changes in the binding sites for the primer and thereby which sequences are amplified. Just variations of common laboratory equipment can cause this result (Anal, 2011).

Some of the samples that were present in the gel electrophoresis pictures showed weak bands that might imply that amplification of the sample was reduced during the PCR reaction. The water control often showed bands on the gel pictures. As explained in (Pan et al., 1997), this is normal because of the M13 primer's ability of easily binding to any DNA present in the water (**Figure 6**). Thus, a trace amount of DNA in polymerase and/or primer solutions would show banding patterns as previously described.

Other methods of strain typing like DNA sequencing and Pulsed-Field gel electrophoresis (PFGE) are more often used. PFGE is often referred as the "gold standard" because of its extent of strain discrimination and its accuracy. When using DNA sequencing, you can determine all the way down to the genotype. And by using PFGE you can determine the bacterial strain.

A study tested how appropriate RAPD PCR is as a technique. They suggest a minimum of two primers when using RAPD and always running a PFGE as a security check (Larrasa et al., 2004). Studies with same purpose as this thesis used genome sequencing to identify mutations or WT (Lazar et al., 2013, Oz et al., 2014, Kim et al., 2014, Rodriguez de Evgrafov

et al., 2015). For later use, I would prefer the use of PFGE or genome sequencing when detecting mutations.

5.4 Antimicrobial susceptibility testing

Normally, when doing E-tests, it is done by three replicates. In this thesis, only one parallel was carried out due to lack of equipment. By doing plural replicates the result becomes more reliable.

Determination of MIC can be done by dilution in 96-well plates. This is a more demanding procedure due to the quantity of dilutions that is necessary. The E-test is a much easier method, but is far more expensive.

When carrying out E-tests, different types of agar were applied. To measure MIC for ancestral strains and mutants, regular LB Agar was used. When examination of collateral networks, MHA2 was adopt. This agar is more suited for susceptibility testing. When this agar was not chosen consequently for all susceptibility tests, this could have affected the results. This is especially true for trimethoprim as extra thymine in the LB media reduces the effect of trimethoprim. Therefore, MICs on LB can be higher than they would be on MHA2.

5.5 Outlook

Future perspectives include extensive determination of collateral sensitivity networks in a clinical strain collection with and without pre-existing resistance determination. When expanding the study it is easier to make broader conclusions. A proper statistic analyze will allow validated conclusions.

The insertion of resistant plasmids is also interesting and clinically relevant, when studying different gene variations and their effect on collateral networks.

When this theory has enough studies as a support, the project may be carried out as a clinical trial in mice and eventually in humans.

6 Conclusion

The results in this thesis suggest the presence of collateral networks between antimicrobials used to treat UTIs as well as other diseases. These findings may be the starting point for evidence-based antimicrobial stewardship that may in the future affect the order of antimicrobial treatment.

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7 References

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8 Appendix

8.1 Isolated mutants

Table 14: Generated trimethoprim mutants. Raw material from MIC readings (with no two-fold dilution adjustment).

Strain or isolate	***Selection (µg/mL TRI)***	***PCR result***	***MIC (µg/mL TRI)***
Strain K56-2			**0.75**
Nr. 2.1	4	+	≥ 32
Nr. 2.2	4	+	≥ 32
Nr. 2.3	4	+	≥ 32
Nr. 2.4	4	+	≥ 32
Strain K56-16	**-**		**0.75**
Nr. 16.5	4	+	≥ 32
Nr. 16.8	4	+	≥ 32
Nr. 16. 9	4	+	≥ 32
Nr 16.10	4	+	≥ 32
Nr. 16.11	4	+	≥ 32
Nr. 16.14	4	+	≥ 32
Nr. 16.15	4	+	≥ 32
Nr. 16.16	4	+	≥ 32
Nr. 16.17	4	+	≥ 32
Nr. 16.18	4	+	≥ 32
Nr. 16.20	4	+	≥ 32
Nr. 16.22	4	+	≥ 32

Strain or isolate	***Selection (µg/mL TRI)***	***PCR result***	***MIC (µg/mL TRI)***
Strain K56-44			**0.75**
Nr. 44.7	4	+	≥ 32
Nr. 44.8	4	+	1.5
Nr. 44.9	4	+	1.5
Nr. 44.13	4	+	6
Nr. 44.14	4	+	16
Nr. 44.18	4	+	3
Nr. 44.20	4	+	≥ 32
Strain K56-68			0.38
Nr. 68.1	3	ID	ND
Nr. 68.2	3	ID	ND
Nr. 68.3	3	+	≥ 32
Nr. 68.4	3	ID	ND
Nr. 68.13	4	+	≥ 32
Strain K56-75			**0.25**
Nr. 75.1	4	+	4
Nr. 75.2	3	+	≥ 32
Nr. 75.3	3	+	≥ 32
Nr. 75.4	3	+	≥ 32
Nr. 75.5	3	+	≥ 32
Nr. 75.6	3	+	≥ 32
Nr. 75.7	3	+	≥ 32
Nr. 75.8	3	+	≥ 32
Nr. 75.9	3	+	≥ 32
Nr. 75.10	3	+	≥ 32
Nr. 75.11	3	+	≥ 32

Abbreviations and symbols: ND – no data ID – indeterminant + PCR confirmed *E. coli*

Table 15: Generated ciprofloxacin mutants. Raw material from MIC readings (with no two-fold dilution adjustment)

Strain or isolate	***Selection (µg/mL CIP)***	***PCR result***	***MIC (µg/mL CIP)***
Strain K56-2			**0.75**
Nr. 2.1	0.1	+	0.5
Nr. 2.2	0.1	+	0.38
Nr. 2.3	0.1	+	0.38
Nr. 2.5	0.1	+	0.5
Nr. 2.7	0.1	+	0.25
Nr. 2.8	0.1	+	0.38
Nr. 2.9	0.1	+	0.38
Nr. 2.10	0.1	+	0.5
Strain K56-16			**0.006**
Nr. 16.1	0.1	+	0.25
Nr. 16.2	0.1	+	0.25
Nr. 16.3	0.1	+	0.19
Nr. 16.4	0.1	ID	ND
Nr. 16.5	0.1	ID	ND
Nr. 16.6	0.1	ID	ND
Nr. 16.9	0.1	+	0.25
Nr. 16.10	0.1	+	0.19
Nr. 16.11	0.1	+	0.38
Nr. 16.13	0.1	+	0.19
Nr. 16.14	0.1	+	0.25
Nr. 16.15	0.1	+	0.25
Nr. 16.16	0.1	ID	ND

Strain	***Selection (ug/mL CIP)***	***PCR result***	***MIC (µg/mL CIP)***
Strain K56-44			0.008
Nr. 44.1	0.1	+	0.5
Nr. 44.2	0.1	+	0.75
Nr. 44.4	0.1	+	0.38
Nr. 44.5	0.1	+	0.5
Nr. 44.6	0.1	+	0.5
Nr. 44.7	0.1	+	0.5
Nr. 44.8	0.1	+	0.5
Strain K56-68			0.008
Nr. 68.1	0.1	+	0.38
Nr. 68.2	0.1	ID	0.38
Nr. 68.3	0.1	+	0.38
Nr. 68.5	0.1	ID	0.38
K56-75			0.008
Nr. 75.1	0.1	+	0.25
Nr. 75.2	0.1	+	0.25
Nr. 75.3	0.1	ID	ND
Nr. 75.7	0.1	+	0.19

Abbreviations and symbols:

ND – no data ID – indeterminant + PCR confirmed *E. coli*

8.2 MIC values used in "trimethoprim heat map"

Table 16: Overview of MIC values of the different strain WTs and trimethoprim mutants when tested to seven different antimicrobials; gentamycin, kanamycin, tetracycline, colistin, nitrofurantoin, mecillinam and ciprofloxacin.

		Minimal Inhibitory Concentration (µg/mL)						
Strain	***Mutant***	***GEN***	***KAN***	***TET***	***COL***	***NIT***	***MEC***	***CIP***
K56-2	WT	1	4	4	1	8	0.25	0.008
	2.1 TRI	0.25	2	0.5	1	16	0.125	0.008
K56-16	WT	2	4	4	1	4	1	0.008
	16.5 TRI	0.25	2	1	1	8	0.25	0.008
K56-44	WT	2	8	2	0.5	8	1	0.008
	44.7 TRI	0.19	2	0.5	1	8	0.25	0.008
	44.8 TRI	0.25	2	0.5	1	8	0.125	0.008
	44.14 TRI	0.25	1	0.25	1	8	0.25	0.008
K56-68	WT	4	8	2	1	8	0.5	0.008
	68.13 TRI	0.5	2	0.5	1	8	0.25	0.008
K56-75	WT	2	8	2	0.5	4	0.25	0.008
	75.3 TRI	0.25	2	0.5	0.125	8	0.125	0.008

Abbreviations: GEN – Gentamycin, KAN – Kanamycin, TET – Tetracycline, COL – Colistin, NIT – Nitrofurantoin, MEC – Mecillinam, TRI – Trimethoprim, CIP – Ciprofloxacin.

8.3 MIC values used in "ciprofloxacin heat map"

Table 17: Overview of MIC values of the different strains' WT and ciprofloxacin mutants, when tested to seven different antimicrobials; gentamycin, kanamycin, tetracycline, colistin, nitrofurantoin, mecillinam and trimethoprim.

		Minimal Inhibitory Concentration (µg/mL)						
Strain	***Mutant***	***GEN***	***KAN***	***TET***	***COL***	***NIT***	***MEC***	***TRI***
K56-2	WT	1	4	4	1	8	0.25	1
	2.3 CIP	0.5	2	0.5	1	16	0.25	0.5
	WT	1	4	4	1	8	0.25	1
	2.5 CIP	0.5	2	0.5	1	16	0.125	0.5
	WT	1	4	4	1	8	0.25	1
	2.7 CIP	0.25	2	0.5	1	16	0.125	0.5
K56-16	WT	2	4	4	1	4	1	1
	16.1 CIP	0.25	2	0.5	1	4	0.125	0.5
	WT	2	4	4	1	4	1	1
	16.3 CIP	0.25	2	0.5	1	8	0.25	0.25
	WT	2	4	4	1	4	1	1
	16.11 CIP	0.25	2	0.5	1	8	0.125	0.25
K56-44	WT	2	8	2	0.5	8	1	1
	44.4 CIP	0.5	1	0.5	1	8	0.25	0.25
	WT	2	8	2	0.5	8	1	1
	44.5 CIP	0.5	2	0.5	1	8	0.125	0.25
K56-68	WT	4	8	2	1	8	0.5	0.5
	68.1 CIP	0.5	2	0.5	1	8	0.25	0.25
K56-75	WT	2	8	2	0.5	4	0.25	0.25
	75.1CIP	0.5	2	0.5	1	8	0.25	0.25

Abbreviations: GEN – Gentamycin, KAN – Kanamycin, TET – Tetracycline, COL – Colistin, NIT – Nitrofurantoin, MEC – Mecillinam, TRI – Trimethoprim, CIP – Ciprofloxacin.

8.4 MIC values used in "nitrofurantoin heat map"

Table 18: Overview of MIC values of the WT and nitrofurantoin mutant originating from the K56-2 strain, when tested to seven different antimicrobials; gentamycin, kanamycin, tetracycline, colistin, trimethoprim, mecillinam, and ciprofloxacin.

Strain	***Minimal Inhibitory Concentration (µg/mL)***							
	Mutant	***GEN***	***KAN***	***TET***	***COL***	***TRI***	***MEC***	***CIP***
K56-2	WT	1	4	4	1	1	0.25	0.008
	2.3 NIT	0.5	2	2	2	0.5	0.5	0.016

Abbreviations: GEN – Gentamycin, KAN – Kanamycin, TET – Tetracycline, COL – Colistin, NIT – Nitrofurantoin, MEC – Mecillinam, TRI – Trimethoprim, CIP – Ciprofloxacin.

8.5 MIC values used in "double mutant heat map"

Table 19: Overview of MIC values of the different strain WT and the double mutants originating from the respective strains, when tested to six different antimicrobials; gentamycin, kanamycin, tetracycline, colistin, nitrofurantoin, and mecillinam

	Minimal Inhibitory Concentration (µg/mL)					
Double Mutant	***GEN***	***KAN***	***TET***	***COL***	***NIT***	***MEC***
WT	1	4	4	1	8	0.25
2.1 CIPTRI	0.5	2	2	1	16	0.25
WT	4	8	2	1	8	0.5
68.1 CIPTRI	0.5	2	0.5	1	16	0.25
WT	2	8	2	0.5	4	0.25
75.1 CIPTRI	1	4	2	1	16	0.125

Abbreviations: GEN – Gentamycin, KAN – Kanamycin, TET – Tetracycline, COL – Colistin, NIT – Nitrofurantoin, MEC – Mecillinam, TRI – Trimethoprim, CIP – Ciprofloxacin.