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Collateral sensitivity in clinical *Escherichia coli* isolates resistant to ciprofloxacin

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Abstract

The increase in antimicrobial resistance in bacteria is a serious problem in public heath limiting the efficacy of available antimicrobials for treatment of infections. *E. coli* is the most frequent cause of uncomplicated urinary tract infections. Ciprofloxacin is an important antimicrobial for treatment of uncomplicated urinary tract infections. Unfortunately, resistance to ciprofloxacin is commonly found in the clinics and confers cross-resistance to other antimicrobials. Recent studies on a previously discovered phenomenon known as collateral sensitivity, the opposite of cross-resistance, where resistance to one antimicrobial confers increased sensitivity to others. Recently, some have suggested that using collateral sensitivity to choose the order of antimicrobial used can beneficially slow the progress of antimicrobial resistance during drug cycling. Therefore, we aim to study the collateral sensitivity and cross-resistance profiles across different clinical isolates of *E. coli* from urinary tract infections.

In this study we generated 10 ciprofloxacin resistant mutants and tested their susceptibility to 8 different antimicrobials. Our results show that *E. coli* resistant to ciprofloxacin above clinical breakpoints are cross-resistant to many other clinically-relevant antimicrobials, such as mecillinam, trimethoprim, nitrofurantoin, chloramphenicol, and ceftazidime. Our results also showed that ciprofloxacin resistant mutants are collaterally sensitive to gentamicin. This study provides important data on cross-resistance and collateral sensitivity in a collection of clinical *E. coli* isolates resistant to ciprofloxacin. Since it is important to confirm previous findings on laboratory strains with clinical isolates, hopefully these findings will add to growing data on collateral sensitivity and inform future drug cycling treatment strategies to combat antimicrobial resistance.

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Abbreviations

AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
CAZ	Ceftazidime
CIP	Ciprofloxacin
CIP_x	Ciprofloxacin-containing agar plate at x µg/mL
CFU	Colony forming unit
CHL	Chloramphenicol
CLSI	The Clinical and Laboratory Standards Institute
COL	Colistin
CR	Cross-resistance
CS	Collateral sensitivity
dH_2O	Distilled water
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off values
E. coli	*Escherichia coli*
e.g.	For example
E-test	Epsilometer-test
EtOH	Ethanol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GEN	Gentamicin
HCCA	Cyano-4-hydroxycinnamic acid
IC-90	Inhibitory concentration-90
LB	Luria Broth
LBA	Luria Broth agar
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-Of-Flight
MIC	Minimum inhibitory concentration
MEC	Mecillinam
MH	Mueller-Hinton
MHA2	Mueller-Hinton agar 2

NaCl	Sodium chloride
NC	Negative control
NIT	Nitrofurantoin
OMP	Outer membrane porin
PBP-2	Penicillin binding protein-2
PCR	Polymerase chain reaction
QRDR	Quinolone resistance-determining regions
q.s.	Sufficient quantity
R	Resistant
RNA	Ribonucleic acid
rpm	Rotations per minute
S	Sensitive
SFI	Streak for isolation
SNP	Single-nucleotide polymorphism
TFA	Trifluoroacetic acid
TMP	Trimethoprim
UNN	University Hospital of North Norway
UTI	Urinary tract infection
WHO	The World Health Organization

1. Introduction

1.1 Preface

There is no doubt that the discovery of antibiotics in the late of 19th century contributed to reductions in human morbidity and mortality (Davies et al. 2010). As we believed these antibiotics were perfect to eliminate bacterial infections without any consequences, the inappropriate use of these agents led us to a new problem, the development of antimicrobial resistance (AMR). AMR can either be innate or occur via several mutation-based mechanisms that microorganisms acquire and maintain during antibiotic pressure (Blair et al. 2015). The emergence of multidrug resistance microorganisms is currently classified as a global threat to public health by organizations such as the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC) (Roca et al. 2015).

The 2014 ECDC report shows that the prevalence of AMR in *E. coli* to third-generation cephalosporins, fluoroquinolones, and aminoglycosides is increasing in some countries in Europe, with a mean percentage increase from 3,8% in 2011 to 4,8% in 2014. Infections with multidrug-resistant bacteria cause 25 000 deaths in Europe each year and cost the European Union over €1,5 billion annually (ECDC, 2014). Urinary tract infections (UTIs) are among the most common human infections. They play an important role in global antibiotic use and AMR development due to their high prevalence and the amount of antimicrobials used for treatment (Zalmanovici Trestioreanu et al. 2010) (Zowawi et al. 2015).

The slow progress in development of novel antibiotics to combat the emerging threat of AMR and growing frequency of AMR isolates threatens the efficacy and limits our available treatment options (Silver 2011). A strong focus should be directed to optimal antimicrobial stewardship and re-assessment of clinically abandoned antibiotics, which could prolong the efficacy of remaining antibiotic agents and provide us more time to develop new antibiotics (Spellberg et al. 2013). Antimicrobial stewardship includes strategies used to combat the emergence of antimicrobial resistance defined as " the optimal selection, dosage, and duration of antimicrobial treatment that results in the best clinical outcome for the treatment or prevention of infection, with minimal toxicity to the patient and minimal impact on

subsequent resistance" (Doron et al. 2011). Antimicrobial stewardship interventions are based on recommendations from WHO´s publication in 2001, where some improvements within the hospital settings can be changed, such as formulary restriction, employ general guidelines, improve educations of clinicians, drug cycling etc. (WHO, 2001) (Doron et al. 2011). A novel antimicrobial stewardship idea is adapting drug cycling procedures, so that the order and use of certain drugs will be based on "collateral sensitivity", as proposed by Imamovic and Sommer as a beneficial way to combat AMR (Imamovic et al. 2013) (see **section 1.7**). The collateral sensitivity phenomenon was first discovered in the 1950s (Szybalski et al. 1952). In this study, we focus on examine the collateral sensitivity (CS) and cross-resistance (CR) profiles in ciprofloxacin resistant mutant.

1.2 Antibacterial agents

The antibacterial agents that are used in this study are all used for treatment of bacterial infections in clinical settings (**Table 1**). Antibacterial agents can exhibit either a bactericidal effect that causes death of the bacterium or a bacteriostatic effect that inhibits bacterial growth and assists the host defense. There are four main processes that antibacterial agents commonly interfere with in bacteria; these are cell wall synthesis, protein synthesis, nucleic acid synthesis, and folate synthesis.

Table 1. Antimicrobial agents used in this study.

Antibacterial	Antimicrobial class	Antimicrobial target
Ciprofloxacin	Fluoroquinolone	DNA replication (gyrase), separation of chromosome (topoisomerase IV).
Mecillinam	Broad-spectrum β-lactam	Cell wall synthesis (PBP-2)
Nitrofurantoin	Other class	Poorly understood mechanisms: Inhibition of protein synthesis, aerobic energy metabolism, and nucleic acid and cell wall synthesis.
Trimethoprim	Antifolates	Folic acid synthesis (DHFR)
Ceftazidime	Cephalosporin (3.generation)	Cell wall synthesis (PBPs)
Chloramphenicol	Other class	Protein synthesis (50S ribosome subunit)
Gentamicin	Aminoglycoside	Protein synthesis (50S and 30S ribosome subunits)
Colistin	Polymyxin	Cell wall synthesis (Cytoplasmic membrane)

1.2.1 Drug target - cell wall and cell membrane

Both the Gram-positive and Gram-negative bacterial cell wall contains a peptidoglycan layer in the outer-membrane. This layer is important for bacteria to survive in hypotonic environments. There are various antibacterial agents that damage the cell wall by different mechanisms, such as mecillinam and ceftazidime. Mecillinam and ceftazidime interfere with the final cell wall formation by antagonistic binding of penicillin binding proteins (PBPs), including the transpeptidase enzyme, which is responsible for cross-linking of new peptidoglycan to the preexisting cell wall peptidoglycan. Without active cross-linking of the peptidoglycan the cell wall lyses. Colistin acts by interfering with the lipid layer in the cytoplasmic membranes of bacteria. This results in altered permeability of the cell and leads to cell death (Neu et al. 1996).

1.2.2 Drug target - protein synthesis

Bacterial ribosomes are required to synthesize proteins in bacteria. Bacterial ribosomes contain two subunits; these are the 30S and 50S subunits. Antibacterial agents, such as chloramphenicol and gentamicin, can interfere with bacterial protein synthesis by binding either the 30S or 50S subunits. Chloramphenicol inhibits protein synthesis by binding specifically to the 50S subunit; this binding leads to inhibition of the peptidyltransferanse enzyme that is responsible for peptide bond formation (Neu et al. 1996). Gentamicin binds to both the 30S and 50S subunits. The irreversible binding of the 30S subunit leads to misreading the genetic code (Neu et al. 1996).

1.2.3 Drug target - nucleic acid synthesis

There are several ways that antimicrobial agents can interfere with nucleic acid synthesis, these include inhibition of nucleotide synthesis, the reading of the DNA template, and enzymes involved in the replication and transcription of DNA. Antimicrobials such as ciprofloxacin will bind DNA gyrase and topoisomerase IV and interfere with DNA replication and decatenation of the daughter chromosomes (Neu et al. 1996) (also see **section 1.4**).

1.2.4 Drug target - folate synthesis

Folate is essential for normal synthesis of DNA, RNA, and bacterial cell wall proteins in bacteria. Antimicrobials such as trimethoprim interfere with folate synthesis by

antagonistic binding of the bacterial enzyme dihydrofolate reductase (DHFR) (Neu et al. 1996).

1.3 Survival mechanisms of bacteria: antimicrobial resistance

There are three different ways bacteria can be antimicrobial resistant, by intrinsic resistance characteristics, following adaption, or acquisition of resistance traits (Fernandez et al. 2012). Intrinsic resistances are the result of innate properties of a specific microorganism that make them resistant to certain antibiotics. Intrinsic resistance is often caused by the lack of or altered/insensitive antimicrobial target, presence of drug inactivation enzymes, and/or altered permeability and efflux of antimicrobials (Olivares et al. 2013). Alternatively AMR can result from survival mechanisms, where microorganisms are forced to evolve by selective antimicrobial pressure or occur spontaneously by random chance mutation. Bacteria adaptation can include temporary alterations in genes or protein expression in the bacteria that allow it to survive in the presence of antimicrobials or other stress environments, such as nutrient condition. The evolved bacteria with new abilities, such as altered drug target and changed permeability of a drug through the cell membrane, may be antimicrobial resistant. Genetic changes can be transmitted vertically to succeeding generations of bacteria. Genetic adaptations that cause resistance through mutations within chromosomally located genes often change the outer-membrane porins or the activity of efflux pumps (Fernandez et al. 2012). Finally, susceptible microbes can also acquire resistance by horizontal gene transfer (HGT) of resistance genes, such as multi-drug resistance plasmids.

1.3.1 Acquisition of resistances through DNA mutation

A mutation is an alteration in the deoxyribonucleic acid (DNA). There are several types of genetic mutations including nucleotide substitutions, additions or deletions, tri-nucleotide repeats, and chromosomal rearrangements that were described by (AJF et al. 1999).

Point mutations can occur spontaneously or are induced by a mutagen in the environment. These mutations can have a neutral, beneficial, or harmful effects. When a mutation has taken place, phenotypic variations may be observed between the mutant and non-mutant due to changes in gene expressions or protein function. For

instance, when a mutation occurs in an efflux pump repressor, there is higher expression of the genes that encode for the efflux pump, which makes the bacteria more resistance to some specific antimicrobials than the wild-type population (AJF et al. 1999).

Substitution point mutations are also called single-nucleotide polymorphisms (SNP), since there is variation in a single nucleotide at a specific position in the genome. Within protein-coding regions there two types of SNPs, these are synonymous and non-synonymous. A synonymous substitution is a DNA mutation that maintains the amino acid sequence. When a DNA mutation changes the encoded amino acid with one of similar chemical properties, this is called a silent substitution or conservative mutation that likely has a neutral effect on protein structure and function. Finally, a non-synonymous substitutions, is a mutation that replaces an amino acid with a chemically different amino acid, this mutation is also called missense mutation and can alter the protein structure and its function, leading to beneficial, neutral or harmful effects.

Additions and deletions are mutations where there is adding or deleting of a single or multiple nucleotides in DNA. These changes are called frame shift mutations when the reading frame of protein-coding DNA is changed. Frame shift mutations typically make proteins non-functional. Repeats are similar to additions where the new nucleotides match the previous ones. Addition of a repeat other than in multiples of three will cause frame shift mutations. A trinucleotide repeat is mutation that gives a gene duplicated DNA in triplet nucleotides. This will add amino acids to encoded proteins and may interfere with function of the protein or it may be a conserved mutation, where the protein can still act normally.

Chromosomal rearrangements is an abnormal chromosomal mutation that gives changes in the chromosome structure by involving mechanisms that can cause deletions, translocations, and duplications. Chromosomal rearrangements can also be harmful, neutral or even beneficial depending on where the rearrangement actually occurred (AJF et al. 1999).

1.3.2 Horizontal gene transfer

Horizontal gene transfer (HGT) includes several DNA transfer mechanisms that allow organisms to spread or exchange their gene material between others that are not in a parent-offspring relationship. For a successful integration of transferred genes into a recipient from a donor host, the genes must "do no harm" in the recipient. These processes are summarized below as describe by Soucy et al. (Soucy et al. 2015).

There are three main HGT mechanisms; these are conjugation, transformation and transduction. Conjugation transfers genetic material via physical contact between a donor and a recipient cell through a conjugation pilus. Single stranded DNA and also selfish genetic elements such as plasmids and integrative conjugative elements can be transferred through conjugation. Selfish genetic elements can be carrying antibiotic resistance genes, metabolic genes, virulence factors, etc. that can provide selective advantages for the recipient cell. The DNA transfer in transformation is through uptake of fragments of DNA by the recipient cell from its surrounding environment. Double stranded DNA is degraded into single-stranded during cell entry and recombines into the chromosome. In transduction, the DNA from a donor is loaded into a bacteriophage and is transferred into a recipient cell by binding and insertion of DNA (Soucy et al. 2015).

1.3.3 Common resistance mechanisms

As mentioned above, AMR can either be intrinsic or acquired. There are three main types of resistance mechanisms; drug target alteration, drug modification or degradation by enzymes, and changes in membrane permeability.

Drug target alteration causes changes in the drug target site that the antimicrobial is unable to bind and perform its antimicrobial activity. An example of this is when point mutations to the penicillin binding proteins results in a decrease of affinity of penicillin (Alekshun et al. 2007). Enzymatic modification or degradation of a drug can occur in bacteria that contain enzymes like β-lactamases. β-lactamases are well-known resistance mechanisms that can hydrolyze the β-lactam ring and inactivate some β-lactam antimicrobials (Jacoby et al. 1985). Changes in the membrane permeability to antimicrobials can be caused by either reduced expression or structural changes to outer-membrane porins (OMPs). For Gram-negative bacteria,

passage of some drugs through the outer membrane is controlled by porins, due to low permeability of lipid layer in outer membrane. A point mutation in single gene can lead to reduced expression of OMPs, and a such reduction can influence the uptake of ciprofloxacin (Cohen et al. 1989). Finally, efflux pumps are energy dependent membrane-associated proteins that export substrates like antimicrobials out of the cell and leads to a decrease in drug accumulation within the cell. Efflux pumps are often referred as a multi drug resistance (MDR) mechanisms because they can influence the accumulation of many different antimicrobials (Nikaido 1994). Both reduced permeability and the overexpression of efflux pumps results in lower antimicrobial concentrations inside the bacterial cell, which can make the bacteria resistant.

1.4 Antibiotic of interest, ciprofloxacin

1.4.1 Mode of action

Ciprofloxacin (CIP) is a potent broad-spectrum antimicrobial that exhibits bactericidal activity against a variety of Gram-negative bacteria and many Gram-positive bacteria as well with significant eradiation (Emmerson et al. 2003). CIP belongs to the quinolone antimicrobial agent class, and is further characterized as a fluoroquinolone by inclusion of fluorine in the chemical structure. CIP was synthesized from the first quinolone on the market, nalidixic acid. It was used for the treatment of urinary tract infections caused by Gram-negative bacteria.

In order to be active, CIP crosses into the cell through porins into the cytoplasm, where it interacts with two type II topoisomerases, DNA gyrase and topoisomerase IV. In Gram-negative bacteria the DNA gyrase is the primary target, while for Gram-positive topoisomerase IV is the primary target in the bacteria cell (Hooper 1999, Jacoby 2005, Ruiz 2003). DNA gyrase is composed of two *gyrA* subunits and two *gyrB* subunits. It is responsible for maintaining and removing the positive superhelical twist before DNA helicase separation during DNA transcription. Topoisomerase IV consists of two *parC* subunits and two *parE* subunits. The topoisomerase IV enzyme is responsible for decatenation/separation of interlinked daughter chromosomes and removing knots in the bacterial chromosome in the terminal stage of DNA replication (Deibler et al. 2001). Ciprofloxacin binds in the cleavage-ligation active site at the topoisomerase enzymes to form enzyme-DNA-drug complexes. This results in

inhibition of DNA replication, triggering of the SOS response, and other poorly understood mechanisms that ultimately lead to cell death. (Drlica et al. 2008)

1.4.2 Mechanisms of resistance to CIP

CIP resistance is acquired either by chromosomal mutations or plasmid-borne resistance genes (Hooper 1999). High-level resistance to ciprofloxacin by chromosomal mutation arises in a step-wise manner and requires a number of mutation steps to reach stable clinical resistance level (Fernandez et al. 2012). There are three mechanisms of resistance to CIP: target-mediated (drug target alterations), changes in drug permeability and efflux, and enzymatic inactivation (Hooper 2001, Jacoby 2005, Ruiz 2003).

Target-mediated CIP resistance occurs when there are specific mutations in either or both target enzymes, DNA gyrase and topoisomerase IV. For *E. coli* the first step CIP resistance mutation occurs in either *gyrA* or *gyrB* encoding the DNA gyrase enzyme (Ruiz 2003). Mutations are more often observed in *gyrA* than in *gyrB*, in a region called the quinolone resistance-determining region (QRDR). Resistance mutations in topoisomerase IV are found in the *parC* subunit; *ParE* mutations seem to be irrelevant (Ruiz 2003). In general, one mutation in DNA gyrase or topoisomerase IV exhibits a ≤10-fold change in resistance, and mutations in both enzymes can give a higher level of resistance, resulting in 10-100-fold changes in drug susceptibility (Aldred et al. 2014).

Chromosomal mutations that change the entry and efflux of CIP can directly reduce the drug concentration in the cell, which reduces the drug efficacy. In *E. coli*, mutations that cause resistance in this manner can be found in the *marR* gene. This gene is part of the MarRAB regulon, which is responsible for expression of antimicrobial resistance and oxidative stress genes (Ariza et al. 1994). Mutation of *marR* results in decreased expression of *ompF* and an increase in *acrAB* expression. *OmpF* is a porin in the outer membrane that regulates the influx of CIP, while *acrAB* genes encode major components of the AcrAB-TolC efflux pump that export CIP and other antimicrobials out of the bacterial cell. Due to the reduced permeability of porins and efflux of specific drugs, including chloramphenicol and tetracycline, mutations in *marR* can lead to reduced susceptibility to other antimicrobials (Cohen et al. 1989).

Alternatively, CIP resistance can be plasmid-borne, and is encoded on plasmids containing the *qnr*, *aac(6′)-lb-cr* and *qepA* genes. The *qnr* gene encodes for a protein that can prevent CIP from binding to its target enzymes (DNA gyrase or topoisomerase IV). The *aac(6′)-lb-cr* encodes an acetyltransferase that can modify CIP, which decreases its antimicrobial activity. The *qepA* gene encodes for an efflux pump that leads to resistance by export of CIP (Drlica et al. 2009).

1.4.3 Significance of ciprofloxacin in this study

The reason we selected ciprofloxacin for further investigation was because in previous studies reduced susceptibilities were observed in generated *E. coli* CIP resistant mutants from the ECO•SENS collection when changes in susceptibilities to 15 other antimicrobial agents were determined. The previous investigation in the Johnsen lab showed that CIP resistant mutants were also resistant to other antimicrobials such as chloramphenicol, trimethoprim, termocillin, mecillinam, and azithromycin with significant in susceptibility from 2 to as high as 32 fold (Podnecky et al., unpublished data). A Similar pattern was also described in a recent paper by Imamovic and Sommer (Imamovic et al. 2013). But some of the CIP resistant mutants generated in the Johnsen lab showed increased sensitivity to fosfomycin, gentamycin, nitrofurantoin, and ertapenem (**Appendix Figure a1**), which were not observed in Imamovic and Sommer′s investigation.

1.4.4 Other antimicrobials used in this study

Mecillinam (MEC): A broad-spectrum β-lactam antibiotic used for the treatment of uncomplicated UTIs caused by Gram-negative bacteria, such as *E. coli*. MEC has a bactericidal effect through its specific antagonist binding to penicillin binding protein-2 (PBP-2) and prevention of cell wall synthesis (UPTODATE 2016).

Nitrofurantoin (NIT): Is a urinary tract antiseptic to treat UTIs caused by *E. coli*, NIT is converted to a reactive electrophilic intermediate by bacterial nitroreductases. Once in active form, NIT inactivates or alters bacterial ribosomal proteins leading to inhibition of protein synthesis, aerobic energy metabolism, and nucleic acid and cell wall synthesis (UPTODATE 2016).

Trimethoprim (TMP): Is a bacteriostatic antibiotic commonly used against uncomplicated UTIs caused by *E. coli*. TMP inhibits folic acid synthesis by blocking the bacterial enzyme dihydrofolate reductase in bacteria (UPTODATE 2016).

Ceftazidime (CAZ): Used for treatment of complicated and uncomplicated UTIs caused by *E. coli.* It has a bactericidal effect and inhibits cell wall synthesis by binding to one or more of the penicillin-binding proteins (PBPs) (UPTODATE 2016).

Chloramphenicol (CHL): Is a broad-spectrum antimicrobial with bacteriostatic effect that inhibits protein synthesis by reversibly binding the 50S ribosomal subunits in susceptible bacteria, such as *E. coli* (UPTODATE 2016).

Gentamicin (GEN): Is a broad-spectrum antimicrobial with bactericidal activity against Gram-negative bacteria including *E. coli.* GEN interferes with protein synthesis by binding to the 30S and 50S ribosomal subunits (UPTODATE 2016).

Colistin (COL): Used to treat acute or chronic infections caused by Gram-negative bacteria. It has a bactericidal effect. Colistin damages the outer cell membrane, which leads to cell death(UPTODATE 2016).

1.5 Antimicrobial susceptibility testing and interpretation of susceptibility

Antimicrobial susceptibility testing (AST) is used to determine the susceptibility of microorganisms, such as bacteria, to certain antimicrobial agents. This method is useful to detect susceptible microorganisms and to decide which antimicrobial will likely be the most effective for treatment in clinical settings. As described by (Jorgensen et al. 2009), the most commonly used AST methods are broth dilution and antibiotic diffusion assays, such as antimicrobial gradient diffusion and disk diffusion methods.

The broth dilution tests are one of the earliest methods used and is considered the standard for AST. This method is further classified as macro-broth dilution where the final testing volume is 2 ml in test tubes and as micro-broth dilution where the volume is ≤ 500 μl in microtiter plates. The micro-broth dilution tests are often performed in a 96-well microtiter plate, with liquid growth medium containing geometrically

decreasing concentrations of an antimicrobial agent and a consistent number of bacterial cells. The gradient diffusion test, commonly known as Epsilometer test (E-test), is used for ASTs in clinical laboratories. The E-test method uses plastic or paper test strips containing a dried antibiotic concentration gradient on the underside and a concentration scale on upper side, and employ on agar plates with a consistent number of bacterial cells, to determine the susceptibility of microorganisms by observed zone inhibition. Disk diffusion test is an AST testing method employing paper disks with a defined concentration of antibiotic placed on MH agar plates to observe the zone of inhibition, which can be used to interpret the susceptibility (Jorgensen et al. 2009).

These methods are used to determine the susceptibility of microorganisms expressed as the minimal inhibitory concentration (MIC), 90% inhibition level (IC_{90}), or IC_{50}. MIC and IC_{90} are expressed as the minimal drug concentration that is required to inhibit bacterial growth, the IC_{50} is more commonly used for comparing drug effects (Munck et al. 2014). The micro-broth dilution method and IC_{90}s were used to describe drug susceptibility patterns in our project.

1.5.1 Clinical breakpoints and epidemiological cutoff value (ECOFF)

To interpret the MIC results from AST and assess the susceptibility or resistance of microorganisms to an antimicrobial treatment, clinical breakpoints are needed that define a microorganism as resistant, intermediate or susceptible. The Clinical and Laboratory Standards Institute (CLSI) defines a bacterial strain as susceptible when the antimicrobial agent used for infection at the recommended dosage for treatment of an infection inhibits the isolates, intermediate when the MICs of isolates approach or exceed the level of antibiotic used for an ordinary dosage and the clinical response is likely less than it would be with a susceptible strain, and resistant when the isolate is not inhibited by the antimicrobial agent with an ordinary dosage (Turnidge et al. 2007).

The epidemiological cutoff values (ECOFF) are breakpoints that differentiate the wild-type population of a bacterial species from the resistant population with acquired or selected resistant mechanisms. The European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) publishes the ECOFF values and clinical breakpoints that are used throughout Europe, as well as guidelines for AST methods.

1.6 *Escherichia coli*

E. coli belongs to the *Enterobacteriaceae* family. It is characterized as a Gram-negative, rod-shaped bacterium and is a facultative anaerobe with relatively simple growth requirements. This versatile microorganism plays an important role in the normal intestinal flora of humans but also as a pathogen causing infection (Pupo et al. 1997) (Kaper et al. 2004). *E. coli* with specific virulence factors can adapt to other environments and cause disease (Kaper et al. 2004). Three common infections caused by of *E. coli* are urinary tract infections (UTIs), diarrheal disease and sepsis/meningitis (Kaper et al. 2004).

1.6.1 Urinary tract infections

Urinary tract infections (UTIs) occur when microbes, typically bacteria, overcome the immune system and successfully colonize in the urinary tract. This requires specific adhesion factors, adhesins. In a clinical context, UTIs are categorized as either uncomplicated or complicated. Uncomplicated UTIs are among the most reported cases of bacterial infections caused by *E. coli,* and can be further differentiated into infections in the lower urinary tract (cystitis) and in the upper urinary tract (pyelonephritis) (Kaper et al. 2004). Uncomplicated UTIs are frequent; each year there are 6-8 million cases in the United States and 130-175 million cases worldwide in all age groups. Of these infections the most common UTI causative pathogen is *E. coli,* which contributes to 75-95% of all uncomplicated UTIs cases in the United States (Nordstrom et al. 2013).

In Norway antibiotics for the treatment of UTIs make up from just over 10% to over 30% of total antibiotic prescriptions, depending on the age group (**Figure 1**). Uncomplicated UTIs are treatable with numerous antimicrobials with trimethoprim, nitrofurantion, pivmecillinam as the standard first line treatment and fluoroquinolones (ciprofloxacin) when there is a failure of the standard treatment caused by resistance (Helsedirektoratet 2012). According to the recommendations of the Helsedirektorat in Norway, the usage of fluoroquinolones should be limited because of increasing resistance development. In Norway, current resistance rates to fluoroquinolones were at 11% in 2014 (ECDC, 2014).

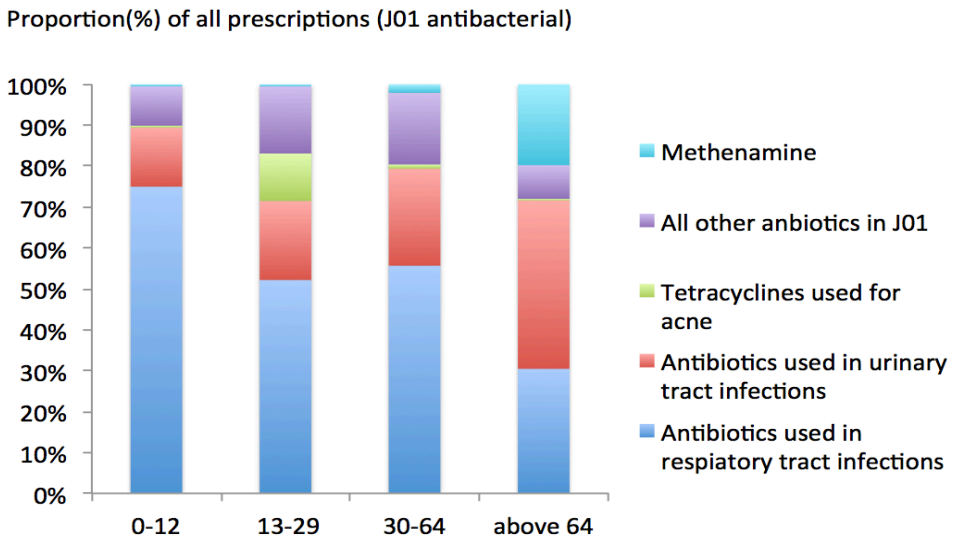


Figure 1. Antimicrobial prescriptions from 2010-2014 in Norway. This figure is showing proportion of prescriptions of antimicrobials for treatment within 4 age groups. Respiratory tract infections (blue) and urinary tract infections (red) contribute to frequent use of antimicrobials across the different age groups. Figure modified from: (Folkehelseinstitutt 2015).

1.6.2 *E. coli* strains from the ECO•SENS collection

The ECO•SENS studies, were based on data generated from antimicrobial susceptibility testing with commonly used antimicrobials to uropathogenic *E. coli* isolates from uncomplicated UTIs in women collected over periods between 1999-2000 and 2007-2008 from 16 European countries and Canada (Kahlmeter 2000) (Kahlmeter et al. 2012). The authors were trying to investigate the differences in antimicrobial resistance prevalence between the European countries. From their final reports in 2003 and 2012, the prevalence of resistance in *E. coli* to ciprofloxacin increased from 1,1% (1999-2000) to 3,9% (2007-2008) (Kahlmeter et al. 2003, Kahlmeter et al. 2012). See **Section 2.1** for more information on which strains were chosen from the ECO•SENS project for this thesis work.

1.7 Collateral sensitivity

Collateral sensitivity (CS) is related to Szybalski and Bryson´s research in 1952, where they discovered this phenomenon during their work on cross-resistance (CR) patterns between different antimicrobial agents. They described the new phenomenon "a strain resistant to one antibiotic may become more sensitive than parent strain to

another drug" and termed it as the reverse phenomenon of CR (Szybalski et al. 1952). Recently this "idea" has been studied further; collateral sensitivity interaction networks have been described and examined in drug-resistant *E. coli* (Imamovic et al. 2013). CS/CR networks (susceptibility profiles) can be visualized as heat-maps (**Figure 3**) and average change in the susceptibility across many strains (**Figure 4**).

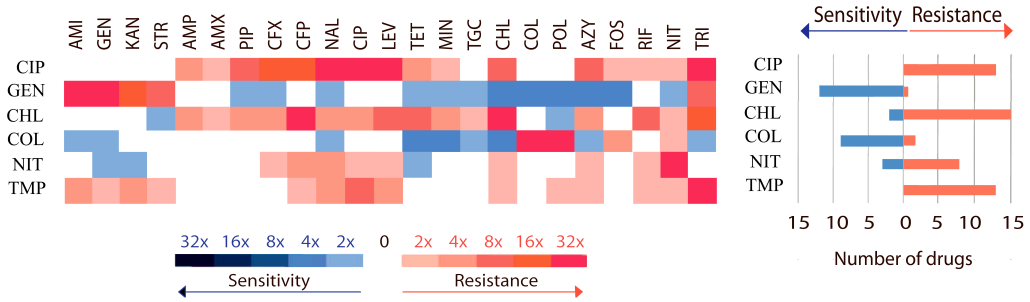


Figure 2. Heat map of CS/CR changes in AMR *E. coli*. (A) The susceptibility profiles of drug-resistant *E. coli* strains to 23 different antibiotics are shown with a color-scale showing the fold-changes from the wild-type strain. (B) This bar chart shows for a specific drug-resistant *E. coli* the number of drugs where CS or CR effects were observed. Figure modified from: (Imamovic et al. 2013).

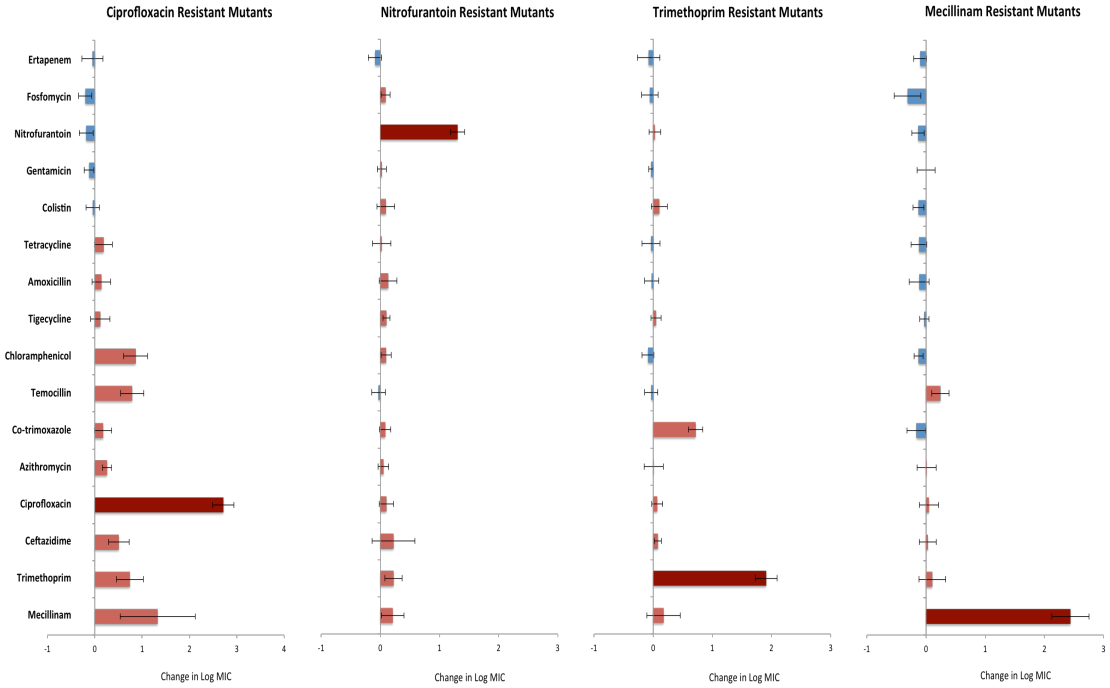


Figure 3. Observed CS/CR changes in AMR *E. coli* clinical isolates. This figure shows the average change in susceptibility of *E. coli* mutants resistant to ciprofloxacin, nitrofurantoin, trimethoprim and mecillinam to 16 drugs. Results from this research represent the average of 10 strains and are expressed in fold-changes of MICs from the wild-type strain. The most interesting finding here is that CIP mutants show CR to many drugs, and MEC mutants show the opposite. Figure kindly provided by Podnecky et al., unpublished data.

Drug cycling programs have been proposed as a beneficial way to slow resistance evolution based on the theory that resistance comes at some fitness cost. AMR isolates with high fitness costs are expected to be outcompeted by their ancestral strains in a mixed population (Andersson et al. 2010, Kim et al. 2014). Drug cycling based on CS is proposed to give the wild-type population an even greater growth advantage in the presence of an antimicrobial (Imamovic et al. 2013).

This principle of drug cycling with two antimicrobials that cause CS to each other is shown in **Figure 5**, where the wild-type population causing disease (t_0) is treated with drug A (violet arrow). Because of survival mechanisms, AMR to drug A will evolve, most of the population will become resistant, and this leads to treatment failure with drug A. At the same time the resistant strain also become more susceptible of Drug B (green arrow), this will lead to a successful eradication of the resistant strain to Drug A. When resistance to Drug B emerges, the treatment can be changed back to Drug A.

These proposed cycles can also have more than 2 drugs, if the antimicrobials are effective and resistant bacteria develop CS to other antimicrobials to complete the cycle (Imamovic et al. 2013).

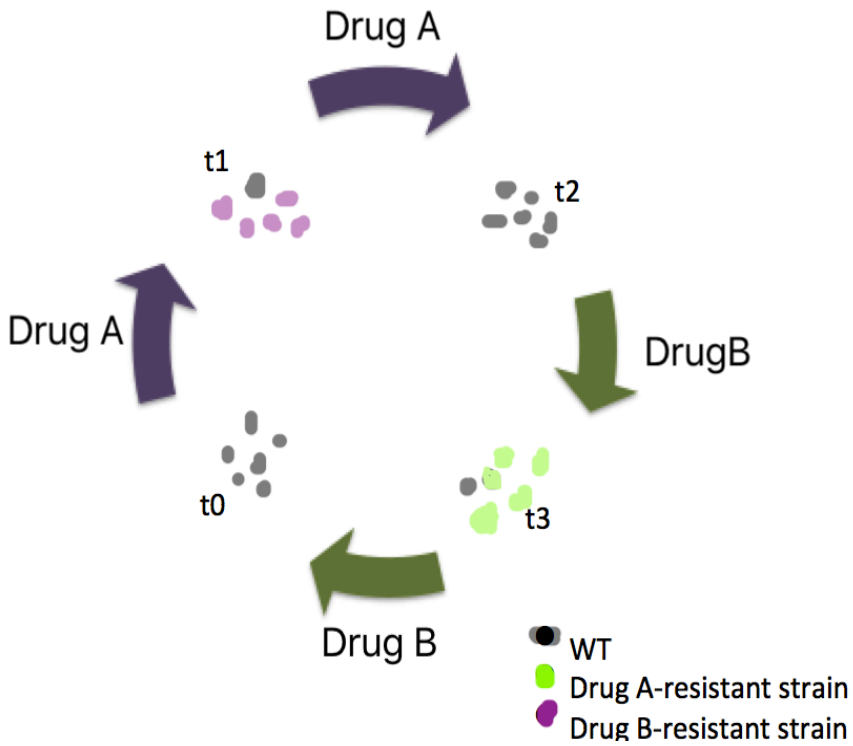


Figure 4. Suggested drug cycling process based on CS. This figure shows the principle how drug cycling would work when we know the collateral sensitivity network. Figure modified from **(Imamovic et al. 2013)**.

1.8 Research aims

Previous work in the Johnsen lab has shown that cross-resistance to many antimicrobials is common in 10 ciprofloxacin resistant mutants selected from pan-susceptible clinical *E. coli* isolates. Our aim in this project is to investigate cross-resistance patterns of ciprofloxacin resistant mutants to other clinically-relevant antimicrobials, and compare our results to observations from earlier publications (Imamovic and Sommer 2013) (Lazar and Pal 2013) and previous work in the Johnsen lab. By doing so we aim to determine if cross-resistance is more common than collateral sensitivity and what factors contribute to variations in the antimicrobial susceptibilities.

1.9 Hypothesis

In this investigation we expected to observe more cross-resistance interactions in ciprofloxacin resistant mutants than collateral sensitivity. We believe that genetic variation among clinical *E. coli* isolates does not contribute to distinct differences in susceptibility patterns. Instead, the resistance mechanisms of the CIP mutants are expected to be a greater contributor to CS patterns than the genetic background.

2. Material and Methods

2.1 Bacterial strains

Bacterial strains used for generating CIP resistant mutants are listed in **Table 2**. These strains are from the ECOSENS collection of pan-susceptible *Escherichia coli*, and were isolated from uncomplicated urinary tract infections (UTIs) (Kahlmeter, 2003) (Kahlmeter and Poulsen, 2012). All strains listed in **Table 2** were found to be plasmid free by S1 nuclease and replicon PCR (Bengtsson, 2012) and are pan-susceptible to commonly used antimicrobials.

Table 2. *E. coli* UTI isolates used in this study.

Strains	Sequence type	Phylogroup	Country of origin	Year
K56-5	ST998	B2	Greece	2000
K56-17	ST73	B2	Portugal	2000
K56-18	ST998	B2	Portugal	2000
K56-20	ST127	B2	Portugal	2000
K56-22	ST73	B2	Sweden	2000
K56-30	ST1161	B2	Sweden	2000
K56-31	ST638	B2	UK	2000
K56-43	ST550	B2	Greece	2007-2008
K56-49	ST127	B2	Greece	2007-2008
K56-61	ST80	B2	Sweden	2007-2008

2.2 Growth media

Growth media is needed for cultivation of bacteria and allows us to get high-density growth for work within this study. Cultivation of bacteria was performed in liquid broth media or on agar plates with growth nutrients incubated overnight at 37 °C.

2.2.1 LB broth

Recipe for 800 ml medium:

- 20 g Difco ^{TM} Miller Luria Broth (Sigma-Aldrich, USA)
- 800 ml dH_2O

This growth media is nutrient rich and is commonly used to cultivation of *E. coli*. The manufacturer instructions specify to reconstitute 25 g powder in 1 L water. To make a

smaller volume, 20 g powder was added to 800 ml dH_2O, stirred and autoclaved for 20 minutes at 121°C (CertoClav, Getinge). Sterilized LB was stored in a cold-room at 4°C.

2.2.2 LB agar

Recipe for 40 LB agar (LBA) plates:

- 20 g Difco ^{TM} Miller Luria Broth (Sigma-Aldrich, USA)
- 12 g Select agar (Sigma-Aldrich, Mexico)
- 800 ml dH_2O

To make 40 LBA plates, 20 g LB powder, 12 g Select agar powder and 800 ml dH_2O was combined, stirred to suspend the mixture, and autoclaved for 20 minutes at 121 °C. Then the suspension was cooled to 50-60 °C before it was poured into sterile polystyrene petri dishes (VWR International, USA). LBA plates were stored at in a cold-room at 4 °C.

2.2.3 Mueller Hinton agar and broth

Mueller Hinton (MH) agar and broth is used for antimicrobial susceptibility testing (AST). What makes MH medium the most suitable for AST is that it has optimized cation concentrations (Mg2+ and Ca2+), thymine and thymidine content, and in-agar medium diffusion properties that can affect the MIC value. MH media satisfies the requirements of the EUCAST and CLSI for AST. MH agar and broth was obtained from the UNN media kitchen for AST or MH agar plates were prepared in laboratory following the manufacturer instructions (**Section 2.5.2**).

2.2.4 Recipes for other solutions and reagents

80% glycerol (sterile) – 100 mL

- Glycerol solution (86-89% purity,T) (Sigma-Aldrich, Germany)
- dH_2O

Measure 54 ml glycerol solutions on a 100 ml graduated cylinder. Fill with dH_2O up to 100 ml in total volume. Transfer the suspension to a bottle and autoclave for 20 minutes at 121 °C.

0,85% saline (sterile) – 80 mL

- Sodium chloride (≥99,5% Fluka, Germany)
- dH_2O

Dissolve 0,65 g sodium chloride in 80 ml dH_2O. Autoclave for 20 minutes at 121 °C with 20 psi pressure.

2.3 Bacteria cultivation

2.3.1 Overnight culture

Bacteria are inoculated into 25 ml of nutrient-rich medium (*i.e.* LB medium) by picking an single isolated colony from an LBA plate or from a frozen glycerol stock with a sterile loop and resuspending it in the media. The sample is incubated at 37 °C with shaking at 150 rpm for 18-20 hours.

2.3.2 Freeze stock culture

This method allows us to keep our bacterial culture viable for years when stored at -80°C. The sample is prepared by adding 750 μl of an overnight culture and 250 μl of 80% glycerol into 1,8 mL Falcon freeze tubes (VWR international, USA).

2.3.3 McFarland standard

A McFarland suspension is a bacterial suspension with an adjusted density that can be used to estimate the number of bacteria in the sample. McFralnd standards are used for AST to be sure that the same amount of bacteria is always used for testing. A 0,5 McFarland standard used for susceptibility testing, and it is correspond to an approximate cell density of 1,5 $x10^8$. To prepare a 0,5 McFarland bacterial suspension, few isolated bacterial colonies are picked and suspended in a tube containing 0,85 % NaCl (see **Section 2.2.4**). The volume of saline can be adjusted to correct the turbidity of the bacterial suspension that is measured in a calibrated densitometer (BIOSAN, DEN-1 McFarland densitometer).

2.4 Common plating techniques

There are several different plating methods to obtain good-isolated single colonies for purity or to obtain confluent growth on agar plates. In this research we used the

following common plating techniques: 3-zone streaking, glass beads plating, and swabbing for confluent growth.

2.4.1 Streak for isolation technique

The 3-zone streaking method shown in **Figure 6** was used to streak bacteria for isolation using a sterile loop to get good isolated colonies. The inoculum is picked either from an LB plate, liquid medium, or frozen glycerol stock. Zone 1 is struck on the plate to obtain a high concentration of bacteria, then a new sterile loop is used to streak a few lines from zone 1 into zone 2. A new loop or opposite edge of the loop is used to streak zone 3 from zone 2. The plate is then incubated for 16-18 hours overnight at 37° C.

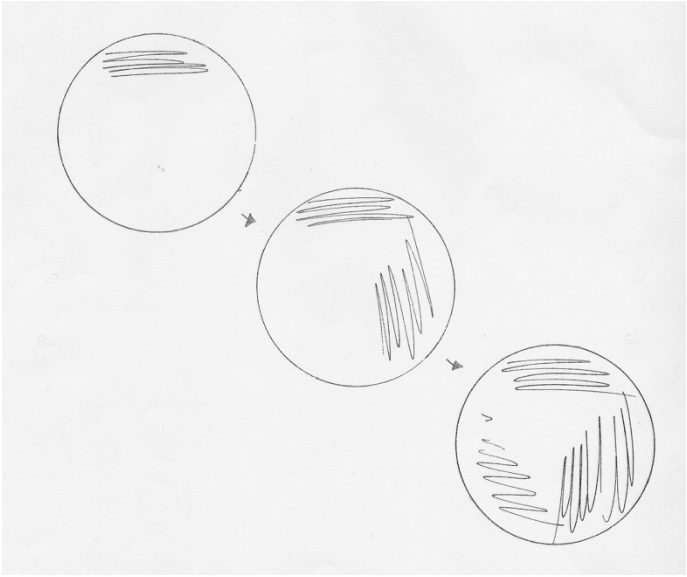


Figure 5. 3 zone streak for isolation technique. This figure shows the steps of 3-zone streak starting from left to right.

2.4.2 Spread plating

Glass beads were used to plate for confluency from liquid bacteria culture. 15-20 sterile glass beads were added to a plate with 50-200 μl of bacterial culture, the plate is then shook back and forth with wrist movement to equally distribute the culture until the moisture on the plate surface has been absorbed. The glass beads are collected from the plate afterwards for decontamination and sterilization.

2.4.3 Swabbing for confluent growth

A sterile cotton swab is dipped into a 0,5 McFarland of bacteria suspension (see **Section 2.3.3**). Two perpendicular lines across the plate are then swabbed on the agar

plate. Then the plate is placed on a plate rotator and the bacteria suspension is spread evenly by moving the cotton swab with gentle pressure from the edge of plate to the center and back for a total of 10 seconds.

2.5 Step-wise static ciprofloxacin resistance selection

A step-wise static resistance selection method was used to generate mutants that have reduced susceptibility to ciprofloxacin. The generated mutants were used to observe AMR emergence and spontaneous mutation rates, identify different resistance mechanisms that may appear in the evolution, and to investigate patterns of CS and CR to different antimicrobial agents. This method is designed for work with susceptible *E. coli.*

2.5.1 Ciprofloxacin stock solution

A ciprofloxacin (CIP) stock solution was used to make MHA CIP plates and overnight cultures with certain concentrations of CIP to maintain the same antibiotic pressure on bacteria. A stock solution of 25 mg/ml was prepared as follows: 50 mg ciprofloxacin (≥ 98% HPLC, Fluka, China) was dissolved in 2 ml of 0,1 N HCl (provided by Nicole Podnecky). The suspension was then sterilized using a 0,2 μM filter unit (Pall Acrodisc, USA). The stock was stored in small single-use aliquots in sterile tubes stored at -20°C.

2.5.2 Preparation of ciprofloxacin selective plates

- 15,2 g MH agar 2 (Sigma-Aldrich, Switzerland)
- 400 mL dH_2O
- Various volumes of CIP stock solution (see **Table 3**).

For making 20 MHA plates with CIP, 15,2 g powder of MHA2 is mixed with 400 ml dH_2O and autoclaved at 121°C for 15 minutes. The solution is cooled to ~50°C and varying volumes of CIP 25 mg/ml is added and mixed well. The agar is then poured into petri dishes.

Table 3. Volume of CIP stock solution required for making MHA CIP plates.

MHA CIP plate	$CIP_{0,016}$	$CIP_{0,032}$	$CIP_{0,064}$	$CIP_{0,128}$	$CIP_{0,25}$	$CIP_{0,5}$	CIP_1	CIP_2
Concentration of CIP (μg/ml)	0,016	0,032	0,064	0,128	0,25	0,5	1	2
Volume of CIP added to 400 ml of MH agar (μl)	0,256	0,512	1,024	2,048	4	8	16	32

2.5.3 Generation of CIP resistant mutants

Clinically-resistant CIP resistant mutants were generated by step-wise static selection. The *E. coli* strains had to go through several steps of selection with increasing concentrations of CIP until they are above the clinical breakpoint, 1 μg/ml CIP.

The initial selection started from a single colony of the wild-type strain:

1. Streak the isolate of interest on LB agar and incubate overnight to get isolated colonies.
2. Pick one colony from LB agar and inoculate it into 25 ml LB broth. Incubate the overnight culture with shaking at 150 rpm.

Each CIP mutation selection step included the following:

3. Pellet 10 ml of the overnight culture in a sterile 15 ml centrifuge tube (VWR, USA) and resuspend it with 1 ml MH medium to obtain a concentrated bacteria culture. If the overnight culture was not dense (as observed by the size of the cell pellets), then pellet 10 ml more of the overnight culture and mix it together with the other resuspended pellet.
4. Add 100 μl of the resuspended pellet to MHA plates with CIP and plate for confluency using sterile glass beads (see **Section 2.4.2**). Incubate the plates overnight at 37°C.
    a. For the first round of selection, plates containing 0,032 μg/ml, 0,064 μg/ml, 0,128 μg/ml and 0,25 μg/ml CIP are used. For slow-growing strains we include plates with 0,016 μg/ml CIP.
5. 100 μl of the resuspended pellet is also added to a sterile 96-well plate and serially diluted in 9,10-fold dilution steps. Typically 100 μl of the 7^{th} and 8^{th} dilutions are plated to achieve countable colonies on non-selective LBA

plates. The 9-dilution steps were setup as shown in **Table 4**. These plates are also incubated overnight at 37°C.

6. After overnight incubation, or up to 48 hours, visible colonies on CIP selective plates and non-selective LBA plates are counted to determine the mutation frequency (see **Section 2.5.4**).
7. If growth is not above the clinical breakpoint, a mixture of colonies from the highest concentration plate with growth is used to inoculate a new flask with 25 ml MH medium containing the corresponding CIP concentration.

Steps number 3 to 7 are repeated with increasing concentrations of CIP selection until growth on MHA with CIP at 2 μg/ml is achieved. Then selected mutants are purified by streaking for isolation on MHA CIP 2 μg/ml plates. Single isolated colonies are inoculated into non-selective 3 ml LB medium, incubated overnight, and then stored as freeze stocks at -80°C in 20% glycerol (see **Section 2.3.2**).

Table 4. Dilution series for viable cell count determination.

Dilution Step	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Dilution	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10
Inoculum (µl)	100	100	100	100	100	100	100	100	100
Diluent vol (µl)	900	900	900	900	900	900	900	900	900
Total dilution factor	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9
Expected plate count (CFU/100 µl)	10^9	10^8	10^7	10^6	10^5	10^4	10^3	100	10

2.5.4 Calculation of the mutation frequency

The total amount of bacteria plated from the re-suspended pellet is determined by the dilution factor (**Table 4**) and the number of bacteria colonies counted on the non-selective dilution plate. The following formula can be used:

$$Total\ bacteria\ plated\ (CFU) = dilution\ factor\ 10^x \text{ x counted colonies } (\frac{\text{CFU}}{100\mu\text{l}})$$

The mutation frequency was calculated for each selection step of each strain. It is the ratio of plated bacteria that are able to grow on the selective CIP plate. The number of

bacteria counted on selective plates and the total bacteria concentration can be used to estimate the mutation frequency:

$$Mutation\ Frequency\ = \frac{colony\ count\ on\ MHA\ CIP}{Total\ bacteria\ plated}$$

2.6 Identification of *E. coli* by MALDI-TOF

Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry together with MALDI Biotyper software is a fast and sensitive technique to inspect the proteins of a microorganism. The masses of proteins are calculated by measuring the time from laser pulse to detecting ions as a signal in the mass spectrometer. Looking at the total protein from bacteria will give a specific spectrum, "fingerprint", of the sample. The MALDI Biotyper software will match a fingerprint to a known database of different bacteria and will give a calculated score to indicate the confidence of identification.

2.6.1 MALDI-TOF sample preparation

Materials:

- 70% Ethanol
- 80 % Trifluoroacetic acid (TFA) (#T6508, Sigma-Aldrich GmbH)
- Matrix stock solution from UNN (50% acetonitrile, 47,5% water and 2,5% TFA)
- Cyano-4-hydroxycinnamic acid (HCCA) (Single-use vial, #255344, BRUKER Daltonik)
- Ultra pure water (#39253, Sigma-Aldrich)
- Formic acid (#33015, Sigma-Aldrich GmbH)
- MALDI 96-well plate
- Wooden sticks (sterilized)
- Pipette
- Eppendorf tubes (Brand, Germany)
- Cryotubes (Thermo Fischer scientific, Denmark)
- Petri dishes
- Absorbent tissue from lab

Cleaning the 96-well target plate, the day before testing:

The target plate is cleaned by covering the whole plate with 70% EtOH in a petri dish for 5 min. Then rinse the plate with distilled water and physically clean it with absorbent tissue moistened with 70% EtOH. Then rinse again with distilled water and dry it with absorbent tissue. 100 µl of 80% TFA is pipetted onto the clean target plate and every well on target plate is cleaned with absorbent tissue. Finally rinse the plate once more with distilled water and it is ready for use.

Loading samples on the target plate:

CIP resistant mutants are struck out on LBA plates the night before, incubated at 37°C overnight. The matrix solution is prepared by adding 250 µl prepared stock solution to a single-use vial containing HCCA. Vortex the mixture until the solution is clear. 1 µl of matrix solution is pipetted onto the first two well positions on the target plate as negative controls. Then use sterile toothpicks to pick a single isolated single colony from the overnight LBA plate and spread the bacteria evenly within a well of target plate. The rest of wells on the plate are filled with one strain per well, then 1 µl of matrix solution is added to each well with bacteria. The target plate with samples is ready to send in for analysis by UNN.

2.6.2 Analysis of MALDI-TOF data

The MALDI-TOF analysis is returned with identification of bacterial species and a confidence score from the MALDI Biotyper RTC.

- A score over 2.000: indicates a reliable identification on the species level.
- A score between 1.700-1.999: indicates a non-reliable identification on species level, but can be reliable on the genus level.
- A score between 0.000-1.699: indicates no reliable identification.

2.7 Minimal inhibitory concentration testing

Minimal inhibitory concentration (MIC) gradient diffusion test strips are paper or plastic strips containing a predefined gradient of antibiotic over 15 two-fold dilutions on one side and with a quantitative scale for observation of the MIC printed on the other side. When the strip is placed on an inoculated agar plate with figures scale up, the antibiotic from the strip will diffuse out into the agar. After incubation for 16-20

hours, one will either observe no inhibition or an elliptical inhibition zone. The scale of the strip is used to read the MIC at the point where the edge of the inhibition zone intersects the strip. With a bactericidal antibiotic there will be a sharp edge to the zone of inhibition. While with a bacteriostatic antibiotic there can be a diffuse zone of inhibition, and the MIC is determined where 80% growth inhibition is observed. With any antimicrobial, the growth on one side of the strip growth is higher than the other side; the highest value is used as the MIC.

MIC testing using the gradient strip method was used to confirm that CIP resistant mutants generated by selective plating had MICs above the clinical breakpoint. MIC testing was performed following protocols used at UNN (K-res, unpublished protocol).

1. CIP mutants are struck for isolation onto non-selective LBA plates and incubated at 37°C overnight.
2. MIC test strips (CIP 0,002-32 μg/ml, Liofilchem® MIC, Italy) used for testing are taken out from freezer and allowed to warm to room temperature for 1 hour before opening to prevent condensation.
3. Then 6-8 isolated colonies, or more if the colonies were not very large, are picked with a sterile cotton swab from the LBA plate and suspended in 0,85% sterile saline. The turbidity of the saline solution is checked with a McFarland densitometer and adjusted if necessary to get a 0,5 McFarland density.
4. Within 15 minutes, the 0,5 McFarland bacteria suspension is spread on MHA plates with sterile cotton swabs using the swabbing for confluency method (see **Section 2.4.3**)
5. The MIC strip is then placed on the MHA, softly pressed onto the agar with sterile toothpicks, and without moving it after application.
6. The plate is then incubated for 16-18 hours.
7. MIC results are interpreted following the manufacturer′s guidelines

MICs are interpreted using breakpoints such as the EUCAST clinical breakpoint or the ECOFF value (see Section 1.5.1). The EUCAST breakpoint for clinical resistance and sensitivity and the ECOFF value for interpretation of CIP MICs are shown in

Table 5. EUCAST clinical breakpoints and ECOFF values for ciprofloxacin.

	Ciprofloxacin
EUCAST Clinical breakpoints	Sensitive (S) ≤ 0,5 Resistant (R) > 1
ECOFF	0,064

2.8 IC_{90} antimicrobial susceptibility testing

Micro-broth dilution assays were used in this study to test for changes in the susceptibility (collateral sensitivity) or resistance (cross-resistance) of the CIP resistant mutants to other drugs of interest. We determined the susceptibility of the CIP mutants and wild-types of 10 strains against 8 different single drugs by both 2-fold and 1,5-fold micro-broth dilutiosn in 96 wells. Results were interpreted as the inhibition concentration-90 (IC_{90}). Inhibition was determined by comparing the optical density at 600nm (OD_{600}) of bacteria with and without antimicrobial after 18 hours incubation.

Materials used in this assay:

- 96-well plate (Thermo Fisher Scentific, Denmark)
- Multichannel pipette (Pipetman Neo®, France)
- VWR reagent reservoirs (VWR, USA)
- Microplate reader (VERSAMAX)
- Microplate Shaker (Edmund Buhler)
- MH broth medium

2.8.1 Antimicrobial drug stocks and testing concentrations

The master stocks of each antimicrobial were provided by Nicole Podnecky. Antimicrobial master stocks were prepared following manufacturer, EUCAST or CLSI guidelines, aliquoted into small single-use tubes, and stored at -20°C. The master stock concentrations are shown in **Table 5.** Master stocks were diluted in MH broth to achieve the desired working concentrations for testing. Working stocks were made 2-fold higher than the highest concentrations tested. This is because after adding the bacterial strain inoculum the concentration of antimicrobial is reduced by 2-fold. The highest concentrations for testing the CIP resistant mutants and wild-types (**Table 5)** were based on previous MIC data of CIP resistant mutants and wild-types of 10

similar but different ECO-SENS isolates from the Johnsen Lab (Podnecky, et al., unpublished data). The highest testing concentrations were chosen so that the resulting range of test concentrations would include the expected result.

Table 6. Highest concentration of antimicrobials used for IC_{90} testing.

Antimicrobial	Master Stock Concentration (mg/ml)	Highest Concentration for testing CIP resistant mutants (μg/ml)	Highest concentration for testing of wild-types (μg/ml)
Ciprofloxacin	10, 2 and 0,1	32	2
Mecillinam	4 and 1	4	4
Nitrofurantoin	10	64	64
Trimethoprim	10	32	32
Ceftazidime	10 and 1	32	32
Chloramphenicol	12,5	256	256
Gentamicin	10	32	32
Colistin	10	32	32

2.8.2 AST strategies and quality control

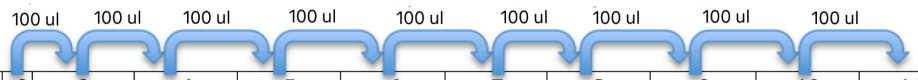
Initially we used 2-fold micro-broth dilution IC_{90} experiments because we could cover a larger range of antimicrobial concentrations. We then switched to 1,5-fold experiments to get more accurate results. The measurement of the IC_{90} is performed using a plate reader to check the optical density at 600nm (OD_{600}) after 18 hours incubation.

An *E. coli* quality control strain (ATCC 25922) and an ECO-SENS derived K56-70 CIP mutant were included in each plate as control strains. These controls allowed us to check that the antimicrobial working stock concentrations were correct in each plate. Also in each plate we had positive and negative growth controls, with no antimicrobial in the media.

2.8.3 IC_{90} 2-fold experimental setup

1. CIP mutants are struck for isolation onto non-selective LBA plates and incubated at 37°C overnight.
2. 96-well plates are prepared by adding MH broth to columns in the plate as shown by the colored cells in **Figure 7**.

3. The working stock of the antimicrobial is made by diluting the master stock in MH broth to 2X the highest tested concentration.
4. 200 µl of the antimicrobial working stock is added in column 2.
5. 100 µl of the antimicrobial working stock is taken from column 2 to column 3 (as the blue arrow shows in **Figure 7**. The contents of column 3 are mixed by pipetting up and down 15-20 times. The 2-fold serial dilutions are carried out as the remaining blue arrows show.



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

Figure 6. 2 fold dilution IC_{90} setup.

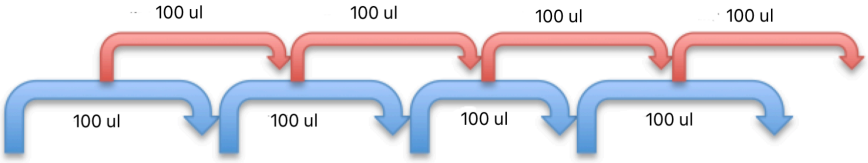
6. After mixing the contents of column 11, 100 µl from column 11 is taken out and discarded.
7. A 0,5 McFarland (~$1x10^8$ to $2x10^8$ cells) from each strain is prepared in 0,85 % sterile saline, as described (see **Section 2.6**).
8. The 0,5 McFarland is diluted further (1:1000) 5 µl into 4,995 mL MHB.
9. 100 µl of the diluted McFarland is added in column 1-11, where each row contains a different strain, including the ATCC 25922 and K56-70 CIP resistant controls.
10. The 96-well plate is incubated for 18 hours at 37 °C shaking at 700 rpm.
11. The OD_{600} is measured after 18 hours incubation.

2.8.4 $ASIC_{90}$ 1,5-fold experimental setup

1. Perform steps #1-3 as described above (**Section 2.8.3**).
2. 200 µl of the antimicrobial working stock is added in column 2, and 150 µl is added to column 3.
3. 100 µl of the antimicrobial working stock is taken from column 2 to column 4 (as the blue arrow shows in **Figure 8**. The contents of column 4 are mixed by

pipetting up and down 15-20 times. The serial dilutions are carried out as the remaining blue arrows show in every other column.

4. 100 µl of the antimicrobial working stock is taken from column 3 to column 5 (as the red arrow shows in **Figure 8**. The contents of column 5 are mixed by pipetting up and down 15-20 times. The serial dilutions are carried out as the remaining red arrows show in every other column.
5. After mixing the contents of columns 10 and 11, 100 µl from column 10 and 11 is taken out and discarded.



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

Figure 7. 1,5 fold dilution IC90 setup.

6. Perform steps #7-11 as described above (**Section 2.8.3**).

2.8.5 Calculating the IC_{90} result

The OD_{600nm} result of the positive and negative controls, as well as the sample with different antimicrobial concentrations is required to calculate the percent inhibition of growth.

$$\%\ Inhibition = \left(1 - \frac{(OD_{600}\ drug\ treated - OD_{600}\ negative\ control)}{(OD_{600}\ positive\ control - OD_{600}\ negative\ control)}\right) x\ 100$$

When the percent inhibition of growth is less than 90% it indicates that the isolate is capable of growth at the tested antimicrobial concentration, but if it is greater than or equal to 90% it indicates that the growth is inhibited. The IC_{90} is the lowest concentration where ≥ 90% growth inhibition was measured, and the result is reported as the drug concentration.

2.9 Isolation of genomic DNA

The GenElute^{TM} Bacterial Genomic DNA Kit (Sigma-aldrich, NA2100/2110/2120) was used for isolation of high quality *E. coli* genomic DNA. DNA isolation using the GenElute^{TM} kit requires the following materials and equipment:

- Lysozyme solution
- Gram-positive lysis solution
- Proteinase K
- RNase A solution
- Lysis solution C
- GenElute Miniprep Binding column/ Wash solution / Wash solution 1
- Ethanol 96%
- Shaking incubator
- Heraeus microfuge pico centrifuge
- Waterbath (37 °C)
- Heatblock (55 °C)

Isolation of genomic DNA was performed following the GenElute bacterial genomic DNA Kit protocol, as described below:

1. The culture is prepared by making an overnight liquid culture of the isolate in 5 ml LB (see **Section 2.3.1**) and incubate with shaking 225 rpm at 37 °C for 18±2 hr.
2. Bacterial cells are harvested by pelleting 1,5 ml of overnight culture by centrifugation for 2 min at 13.000 rpm. Remove the culture medium.
3. Resuspend the pellet in 200 µl of lysozyme solution and incubate for 30 min at 37 °C (water bath).
4. RNase treatment is performed by adding 20 µl of RNase A solution and incubating for 2 min at room temp.
5. The bacterial cells are lysed by adding 20 µl of Proteinase K solution and 200 µl of Lysis Solution C to the sample and vortexing it for about 15 sec then incubating it at 55°C for 10 min (heatblock).
6. The spin column is prepared by adding 500 µl of Column Preparation solution to each pre-assembled GenElute Miniprep Binding Column, which is seated in

a 2 ml collection tube. The assembly is then centrifuged at 13.000 rpm for 1 min and the eluate is discarded.

7. Next 200 µl of ethanol is added to the bacterial lysate and vortexed for 5 to 10 sec.
8. The entire sample is transfered into binding column and centrifuged at 13.000 rpm for 1 min. The eluate in collection tube is discarded.
9. The first wash of the column is performed by adding 500 µl of Wash Solution 1 to the column and centrifuging for 1 min.
10. The column is transferred to a new collection tube.
11. The second wash of the column is performed by adding 500 µl of Wash Solution to the column and centrifuging for 3 min. It is necessary to centrifuge one more minute (or more) if there is still ethanol in the column.
12. The column is placed into new collection tube.
13. Bacterial DNA was eluted by pipetting 100 µl of 10 mM Tris-base onto the center of the column, incubating for 5 min at room temp., and centrifuging for 1 min.
14. The eluate containing isolated DNA was analyzed using a Nanodrop and stored at -80°C. The stored isolated DNA can be used for PCR and targeted DNA sequencing.

2.9.1 Determining the concentration, quantity, and quality of extracted DNA

Determination of DNA concentration and purity is performed using a Nanodrop spectrophotometer. The result from the spectrophotometer shows the concentration of DNA in µg/ml, the 260/280, and 260/230 ratios.

We can use the concentration of DNA to determine the total amount of DNA in our sample. The 260/280 ratios are used to describe purity of isolated DNA. "Pure" DNA should have a ratio around 1.8. If the 260/280 ratio is lower than 1,8, it indicates the presence of protein, phenol, or other contaminants. If the ratio is higher than 1,8, it indicates the presence of RNA. 260/230 ratios are also used to assess the purity of nucleic acids. Measured 260/230 ratios are commonly in the range of 2.0-2.2; if values are lower it indicates the presence of contaminants (ie. EDTA, carbohydrates, and phenol).

2.10 Polymerase chain reaction

Polymerase chain reaction (PCR) is a method used to amplify specific DNA-segments enzymatically *in vitro*. Amplifying genetic material can be a useful tool to manipulate DNA, detect infectious organisms, and detect genetic variations in genes. PCR requires a DNA template to be amplified (for this project it is our high quality chromosomal DNA, see **Section 2.9**), specific primers, dNTPs (nucleotides), and a thermostable DNA polymerase enzyme. **Table 6** shows the primers used in this study. These primers are taken from a published paper (Komp Lindgren et al. 2003), and were designed by the authors to bind and amplify QRDRs where CIP resistance mutations typically occur.

Table 7. PCR primers for amplification of CIP AMR genes.

Gene	Primer name	Oligonucleotide sequence (5′ to 3′)	Melting temperature	Expected band size
gyrA	GyrAR1000 GyrAFQ322	GAGCGCGGATATACACCTT GAGCTCCTATCTGGATTAT	53°C	680
parC	ParCR981 ParCFQ107	GTGGTAGCGAAGAGGTGGTT GACCGTGCGTTGCCGTTTAT	65°C	875
marR	MarORR2011 MarORF1139	GAGTAACCCGAACGCTCTGA GCCAGGCCAAGAAATAACGC	56°C	873
acrR	AcrR9934R AcrR8900F	CTGAACCTGAAGAACGACCTG ACTGTTACTACGCCAACG	57°C	1035

Primers are tested with following temperatures:

1. Initial denaturation: 98°C (1 minutes)
2. Denaturation: 98 °C (10 seconds)
3. Annealing: 45-72 °C (20 seconds)
4. Primer extension: 72 °C (1 minutes)
5. Repeat steps 2-4 for 25-35 cycles
6. Final extension: 72 °C (10 minutes)
7. Hold: 4-10 °C

2.10.1 Phusion® DNA polymerase PCR setup

Unlike ordinary *Taq*-polymerases, the Phusion PCR enzyme provides high fidelity and rapid amplification, and it is especially used for DNA sequencing where the DNA fragment needs to be correct after amplification. The polymerase is stable at

temperatures up to 95°C, which is why the enzyme remains active and stable through all cycles.

PCR includes three steps; denaturation (dsDNA is denatured completely to ssDNA), annealing (primers bind specifically to denatured ssDNA when the mix is cooled to below the melting temperature of the primers), and extension (the heat-stable DNA polymerase enzyme recognizes and binds to the ssDNA with primer, dsDNA is formed by addition of dNTPs. The polymerase is given enough time to synthesize the target DNA (usually 1 min per 1000 bp), The PCR machine (BIO-RAD, PTC-200 Peltier Thermal cycler) that controls the heater and rapidly shifts between temperatures in the following steps:

8. Initial denaturation: 98°C (30 seconds)
9. Denaturation: 98 °C (5-10 seconds)
10. Annealing: 45-72 °C (10-30 seconds)
11. Primer extension: 72 °C (15-30 seconds per kb)
12. Repeat steps 2-4 for 25-35 cycles
13. Final extension: 72 °C (5-10 minutes)
14. Hold: 4-10 °C

PCR samples were setup using the recipe for the Phusion master mix, see **Table 7**. All of the reaction components are combined, but the Phusion DNA polymerase is added to the mastermix in the end, and then the mastermix is aliquoted into separate tubes where the different DNA templates are added.

Table 8. Mastermix recipe for Phusion PCR.

Reagents	20 µl reaction	Final concentration
Milli-Q water	q.s. 20 µl	
5X Phusion HF buffer	4 µl	1X
10 mM dNTPs	0,4 µl	200 µM
10 µM forward primer	1 µl	0,5 µM
10 µM reverse primer	1 µl	0,5 µM
Genomic Template DNA	Variable (50-250 ng)	< 250 ng
Phusion DNA polymerase	0,2 µl	1.0 units/50 µl PCR

2.10.2 Primer optimization by temperature gradient method

Using primer optimization method, we were able to detect the optimum annealing temperature for the primers that we interested in this study. Prepare 12 PCR tubes with 20 µl Phusion PCR reaction (see **Table 7**) using the same genomic DNA template. Place these tubes in order in gradient PCR machine from left to right and setup the temperature as the normal Phusion PCR setup (see **Section 2.9.1**), except the annealing is changed to a range of test temperatures. Visualization on a gel (see **Section 2.10**) is used to compare the PCR band intensities and purity to determine which tube contains amplified PCR product with the optimum annealing temperature, which is read from the gradient PCR machine.

2.11 Agarose gel electrophoresis of PCR products

Gel electrophoresis is an analysis method used to visualize DNA fragments, for example PCR products. DNA moves with an electrical current from the (-) electrode to the (+) electrode, because DNA is (-) charged. DNA fragments are separated from each other according to size by an electric field produced by the gel electrophoresis machine and the resistance of the DNA moving through the agarose. By adding ethidium bromide the DNA fragments be detected visually with UV-light.

2.11.1 Preparation of 1% agarose gel

1 g of agarose (SeaKem® LE Agarose, USA) is dissolved in TAE buffer (provided by the Johnsen lab) by heating the mixture in a microwave oven. The solution is cooled for few minutes, and 20 µl ethidium bromide (10 mg/ml, Sigma-Aldrich, USA) is added to achieve a 0,5 µg/ml final concentration. The agarose solution is then poured into a gel-casting tray with a comb to create the wells and cooled at room temperature for 20-30 min to stiffen.

2.11.2 Agarose gel electrophoresis

The agarose gel and casting tray are placed in the gel electrophoresis chamber and the chamber is filled with enough TAE-buffer to cover the gel. DNA samples are prepared by adding 5 µl loading buffer (6x loading buffer, provided by the Johnsen Lab) to 10 µl of DNA sample. The entire 10µl sample is the loaded into the agarose gel and 3 µl of Smart Ladder is added to the first and last well on gel. The gel is run

typically for 1-hour at 80V. The DNA fragment separation is visualized on the gel by UV-detection.

2.11.3 DNA extraction from an agarose gel

The gel extraction method is used for purifying PCR-products from an agarose gel. In this study, gel extraction method was needed to obtain a PCR product in specific band size visualized on agarose gel, when multiple bands were present. Briefly, the desired DNA band with a specific band size is cut out of the gel and purified using the QIAquick® Gel Extraction Kit protocol, as described below.

Materials used in this method:

- Buffer QG (QlAquick® Gel Extraction Kit, QIAGEN)
- Buffer PE with ethanol (QlAquick® Gel Extraction Kit, QIAGEN)
- Buffer EB (QlAquick® Gel Extraction Kit, QIAGEN)
- Isopropanol 100% (Sigma-Aldrich, Germany)
- 2 ml collection tube (QlAquick® Gel Extraction Kit, QIAGEN)
- QIAquick column (QlAquick® Gel Extraction Kit, QIAGEN)
- 1,5 ml microcentrifuge tube (QlAquick® Gel Extraction Kit, QIAGEN)
- Scalpel
- Water bath (50ºC)

The gel slice with a specific DNA is cut out and weighed in a 2 ml collection tube. 3 volumes of Buffer QG added to 1 volume gel (where 100 mg gel equals 100 µl volume) and incubated in a 50ºC waterbath for 10 min, the gel slice should be completely dissolved. 1 gel volume of isopropanol is added to sample and it is mixed by vortexing. The DNA to the column by applying the whole sample to the QIAquick column and centrifuging for 1 min at 13,000 rpm. The eluate is discarded, then 500 µl buffer QG is added and centrifuged for 1 min, and the eluate is discarded again. To wash the column, 750 µl Buffer PE is added to column with the bound DNA and is centrifuged for 1 min, the eluate is discarded. After this wash the column with bound DNA is place into a clean 1,5 ml microcentrifuge tube. 50 µl of Buffer EB is added to the center of the column to elute the DNA bound in column and is centrifuged for 1 min. Then the purified DNA (eluate) can be analyzed with Nanodrop (see **Section 2.8.1**), and then the purified DNA can be used for DNA sequencing.

2.12 Target DNA sequencing

PCR products that did not have multiple bands were sent for DNA sequencing at the DNA sequencing core facility in University hospital of North Norway (UNN). Prior to analysis of the sequencing, we preformed the sequence reactions on a PTC-200 Thermal Cycler by following the BigDye terminator v3.1 cycle sequencing kit protocol (UNN). The BigDye terminator reaction was carried out as shown in **Table 8**. For each sample, a sequence reaction in the forward direction and a reverse direction were carried out separately.

Table 9. Components of a Big-dye sequencing reaction.

Reagent	Quantity
Big-Dye v3.1	1 μl
Sequencing buffer	3 μl
Template DNA	200 ng
Primer (5 pmol)	1 μl
Milli-Q water (sterile)	q.s., 20 ul

2.12.1 DNA Sequencing Analysis

The UNN core sequencing facility analyzed the samples using Applied Biosystems 3130xI Genetic Analyzers for traditional Sanger sequencing. Sequence data obtained from the UNN sequence lab were visualized using Sequencher® (v. 5.3, Gene Codes Corp. Ann Arbor, MI, USA), a DNA sequence analysis software package for DNA comparisons and analyzing sequences. We used this software to look for CIP resistance mutations by comparing CIP resistant mutants to their wild-type strains and the MG1655 *E. coli* reference strain. SNPs were defined as changes in nucleotides in the CIP resistance mutants compared to wild-type strain. Gene Construction Kit (v. 4.0.3, Textco BioSoftware Inc., W. Lebanon, NH, USA) was used to look at the MG1655 genome, to visualize gene-coding regions, and determine if identified mutations cause amino acid changes.

3. Experimental results

3.1 CIP resistant mutants

3.1.1 Generation of spontaneous mutants

In this study, CIP resistant mutants were generated from 10 clinical strains selected from the ECO•SENS collections. Each strain was evolved to achieve resistance to CIP above the EUCAST clinical breakpoint through the stepwise static antimicrobial resistance selection method (see **Section 2.5.3** and **Table 5**). For each of the 10 strains, 3 CIP resistant mutants were generated from 3 single colonies that grew on CIP_1 or CIP_2 plates. These mutants were purified on new CIP_2 plates. All of the isolates that were purified were characterized by the "naked eye" as colonies that were small (~1mm), opaque, smooth, shiny, and circular on CIP_2 plates, which are typical characteristics of *E. coli*. However, the CIP mutant of strain K56-17, K56-17 CIP (1), was observed to have slower growth on LBA, but faster growth on CIP_2 plates. One isolate from each strain was picked for further investigation (**Appendix Table a2**).

3.1.2 Mutation Frequency

During the selection of CIP resistant mutants, the mutation frequencies (**Table 9**) were calculated based on the colony forming units (CFUs) counted from both the CIP-containing MHA plates and the non-selective LB plates (**Appendix Table a1**). The mutation frequency was no longer determined once the strains grew on CIP_1 plates. The mutation frequencies ranged from as high as $2{,}3 \times 10^{-9}$ to as low as $8{,}8 \times 10^{-25}$, where the average frequency was at 1×10^{-16} for all strains. We observed that in most of our generated CIP resistant mutants, a general trend that strains require 2-3 antimicrobial selection steps to reach a clinical resistance.

Table 10. Mutation frequencies calculated from each mutation step.

Strain	Step 1		Step 2		Step 3		Total[1]
	CIP[2]	MF1	CIP[2]	MF2	CIP[2]	MF3	MF
K56-5 CIP (1)	0,032	$2,1x10^{-8}$	0,128	$3,2x10^{-7}$	0,5	$6,4x10^{-8}$	$4,3x10^{-22}$
K56-17 CIP (1)	0,032	$2,9x10^{-8}$	0,25	$1,1x10^{-8}$			$3,2x10^{-16}$
K56-18 CIP (1)	0,032	$4,4x10^{-8}$	0,128	$5x10^{-9}$	0,5	$4x10^{-9}$	$8,8x10^{-25}$
K56-20 CIP (1)	0,032	$8x10^{-10}$	0,25	$5,8x10^{-7}$			$4,7x10^{-16}$
K56-22 CIP (1)	0,064	$2,2x10^{-9}$	0,5	$8,5x10^{-8}$			$1,9x10^{-16}$
K56-30 CIP (1)	0,032	$2,3x10^{-9}$					$2,3x10^{-9}$
K56-31 CIP (9)	0,032	$3,8x10^{-10}$	0,25	$2,3x10^{-8}$			$8,8x10^{-18}$
K56-43 CIP (1)	0,016	$3,9\ x10^{-7}$	0,128	$3,2\ x10^{-9}$	0,5	$4,4x10^{-6}$	$5,5x10^{-21}$
K56-49 CIP (1)	0,032	$8,3\ x10^{-10}$	0,25	$1,4\ x10^{-7}$			$1,2x10^{-16}$
K56-61 CIP (1)	0,032	$7,1\ x10^{-9}$	0,128	$2,4\ x10^{-7}$	0,5	$2,5x10^{-9}$	$4,3x10^{-24}$

Abbreviations: MF – mutation.
[1] - ciprofloxacin selection concentration (μg/ml)
[2] - Total mutation frequency = MF1 x MF2 x MF3

Strain K56-30 was remarkable in that after only one selection step (following growth on a $CIP_{0,032}$ plate) it was able to grow on CIP_1 plates, with dense (not-countable) growth on all CIP selective plates and non-selective plates. Strain K56-31 had a slower mutation frequency than the rest of the strains evolved in two steps. Strains K56-5 CIP (1), K56-18 CIP (1), K56-43 CIP (1), and K56-61 CIP (1) required three steps to reach resistance at CIP 1 μg/ml.

3.1.3 Confirmation of bacterial species by MALDI-TOF

The selected CIP resistant mutant from each strain was analyzed with MALDI-TOF mass spectrometry to confirm that the selected mutants are truly *E. coli*. MALDI-TOF analysis showed that all of the CIP resistant mutants tested were identified as *E. coli* and had confidence score values over 2000, which indicates that a reliable identification was made on the species level (see **Appendix, Table a3**).

3.1.4 MIC determination of CIP resistant mutants

The CIP MIC for 10 CIP resistant mutants was determined with E-test to verify that the mutants were truly "resistant" where the MIC is at or above the clinical resistance level and the ECOFF value. Those MICs are shown in **Table 11**. All of the CIP

resistant mutants tested had a CIP MIC over 1 μg/mL, and were classified as clinically resistant using the EUCAST clinical breakpoints (see **Table 5**).

Table 11. Ciprofloxacin MICs of selected mutants.

CIP resistant mutant	CIP MIC (μg/mL)	Phenotypic Variation
K56-5 CIP (1)	4	No
K56-17 CIP (1)	3	No
K56-18 CIP (1)	8	No
K56-20 CIP (1)	4	No
K56-22 CIP (1)	3	Mix
K56-30 CIP (1)	4	No
K56-31 CIP (9)	2	No
K56-43 CIP (1)	2	No
K56-49 CIP (1)	2	No
K56-61 CIP (1)	2	No

During MIC testing the phenotype of the CIP resistance mutants was observed both when the mutants were struck for isolation on LBA and when swabbed for confluency on MHA2. The growth of each mutant looked uniform on non-selective plates, however K56-22 CIP (1) had a mixed phenotype on non-selective plates. Two more additional steps of purification (streak for isolation of a single colony) were performed, however a single phenotypic population was not achieved. The occurrence of mix-phenotypes was not observed when the isolate was plated on selective media (CIP_2 plates).

3.2 Description of CS/CR profiles

3.2.1 IC_{90} results from micro-broth dilution

Each of the 10 generated CIP resistant mutants and their WT parental strains were tested to determine the IC_{90} with 8 different antimicrobials CIP, MEC, NIT, TMP, CAZ, CHL, GEN, and COL. Initially, 2-fold IC_{90} experiment data (see **Appendix Table a4**) were used to determine a testing range for the 1,5-fold IC_{90} experiments. Subsequently, the 1,5-fold IC_{90} assay was used to determine the final IC_{90} result more precisely (**Table 12**).

For the slow-growing K56-17 (1) CIP strain, the OD_{600} was measured both after 18 hours and 42 hours. Because the OD_{600} of the positive control after 18 hour was below 0,2, while for other mutants of the same strain were typically above 1,2, the data for K56-17 (1) is from the 42-hour time point. After the longer incubation the OD_{600} of the positive control was above 1,0 and indicated dense growth of bacteria. The measured OD_{600} values were used to calculate the % inhibition of growth. The reported IC_{90} results are the lowest antimicrobial concentration that exhibited inhibition ≥ 90%.

The expected range for ATCC controls tested to CIP (0,004-0,016), MEC (0,03-0,25), NIT (4-16), TMP (0,5-2), CHL (2-8), GEN (0,25-1), CAZ (0,06-0,5) and COL (0,25-1). The ATCC control strain was within the expected range.

Table 12. IC_{90} results of strains tested with 8 different antimicrobials.

Strain	Isolate	IC_{90} (µg/mL) using 1,5-fold dilution method							
		CIP	MEC	TMP	NIT	GEN	CHL	CAZ	COL
K56-5	WT	0,012	0,125	0,125	6	0,25	3	0,063	0,375
	CIP (1)	4	0,375	0,75	8	0,094	12	0,188	0,375
K56-17	WT	0,016	0,094	0,25	8	0,25	4	0,25	0,375
	CIP (1)[1]	1,5	0,188	3	24	0,063	16	0,75	0,188
K56-18	WT	0,012	0,094	0,125	12	0,25	3	0,094	0,375
	CIP (1)	8	0,375	1	8	0,094	12	0,75	0,5
K56-20	WT	0,008	0,063	0,25	4	0,375	3	0,063	1
	CIP (1)	3	0,188	1	6	0,094	16	0,5	0,75
K56-22	WT	0,012	0,094	0,375	12	0,5	4	0,094	1
	CIP (1)	3	0,188	0,375	8	0,125	16	0,188	0,75
K56-30	WT	0,016	0,094	0,375	12	0,375	4	0,125	0,75
	CIP (1)	3	0,1875	1	6	0,063	24	0,5	0,5
K56-31	WT	0,012	0,094	0,375	8	0,25	4	0,063	0,25
	CIP (9)	1	0,125	0,5	4	0,094	16	0,188	0,188
K56-43	WT	0,008	0,094	0,5	6	0,375	3	0,094	0,375
	CIP (1)	1,5	0,094	0,5	12	0,375	6	0,125	0,375
K56-49	WT	0,008	0,094	0,25	12	0,375	3	0,047	0,25
	CIP (1)	1	0,25	1	12	0,188	6	0,125	0,188
K56-61	WT	0,012	0,094	0,75	4	0,25	3	0,063	0,375
	CIP (1)	3	0,75	1,5	8	0,094	16	0,5	0,375
ATCC 25922		0,008	0,094	0,5	4	0,25	3	0,25	0,375

[1] IC_{90} result of slow-growing strains was determined after 42 hours incubation.

3.2.2 Calculated fold-changes from the IC_{90} results

The final results of the 1,5-fold IC_{90} of the 10 strains were used to compare the susceptibility of the new CIP resistant mutant to the parent WT strain and describe the CS/CR profiles. To compare the IC_{90} data the fold changes were calculated, where the result of a CIP resistant mutant was divided by susceptibility of its WT:

$$\text{Fold change in drug susceptibility} = \frac{IC_{90}\ of CIP\ resistant\ mutant}{IC_{90}\ of\ WT\ strain}$$

The calculated fold changes are shown in the **Appendix, Table a5.** Simply having a fold change above 1 suggests a decrease in susceptibility, and less than 1 suggests increased susceptibility, and a fold change of 1 would have no change. However, when we interpreted the fold changes only fold changes equal to or above 2 and equal

to or below 0.5 were considered true changes in the drug susceptibility, while everything else was considered to have no change.

3.2.3 Interpretation of CS/CR in CIP resistant mutants

Using these fold change results between CIP resistant mutants and their corresponding WTs and definitions of change in susceptibility, a heat-map was generated, (**Figure 8)**. The fold-change in IC_{90} result are shown and each cell was color-coded based on the amount of change, where red colors show decreased susceptibility and blue colors show increased susceptibility.

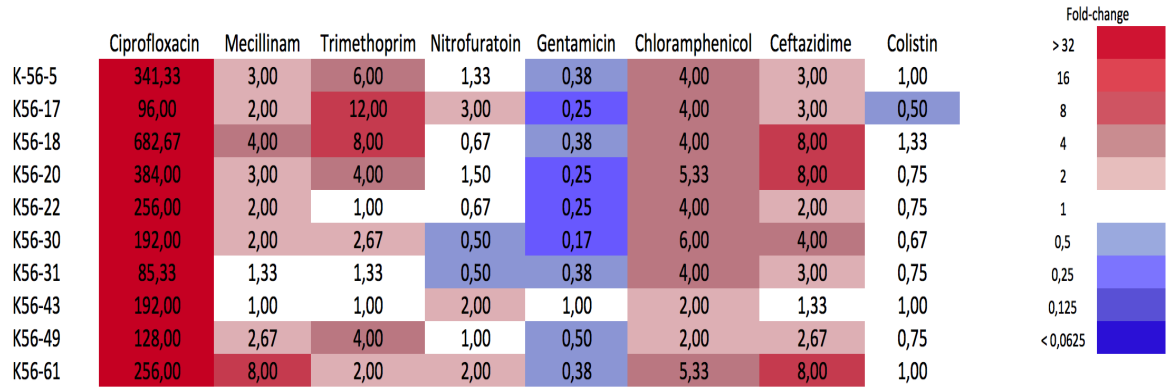


Figure 8. Fold change in drug susceptibility of 10 CIP resistant mutants. The fold-change in IC_{90} results are shown and each cell was color-coded based on the amount of change, where red colors show decreased susceptibility and blue colors show increased susceptibility.

We observed decreases in susceptibility in all CIP resistant mutant strains to ciprofloxacin, which is expected. The fold changes ranged from 85 up to 683. For many of the other drugs we observed decreases in susceptibility, or CR and in some cases also increases in susceptibility, CS. Specifically, we observed CR to MEC ranging from 2 to 8 fold, however in K56-31 CIP (1) and K56-43 CIP (1) there was no change in MEC susceptibility. For most of the strains, there was CR to TMP varying from 2 to 12 fold, while strains K56-22 and K56-43 showed no change for TMP. For CHL, CR was found in all 10 of the strains with fold changes from 2 to 6. Finally for CAZ, CR was found with fold changes varying from 2 to 8, except strain K56-43, which showed no change in susceptibility.

Interestingly, we also observed that there was increased susceptibility (CS) for all the CIP resistant mutants to GEN, which varied from 0,5 to 0,17 fold, except strain K56-

43 that showed no change in susceptibility. Additionally, CIP resistant isolates of K56-30 and K56-31 showed CS to NIT, while the CIP resistant mutant of K56-17 showed CS to COL.

3.2.4 Average CS/CR changes across 10 CIP resistant mutants

In addition to looking at the differences in CS and CR for each of the 10 different strains, an average of the fold-changes was used to represent the general trend of clinical UTI *E. coli* resistant to CIP. The average fold-changes of the 10 strains were calculated and we assessed the variation of the data from the average by calculating the standard deviations. The standard deviations of CIP resistant mutants with each of the 8 antimicrobials were as follows: CIP (0,28), MEC (0,25), TMP (0,38), NIT (0,27), GEN (0,21), CHL (0,16), CAZ (0,27) and COL (0,12). These data represent the spreading of the fold-changes and shows the highest variation between the CIP resistant mutants when tested with TMP, while the change in susceptibility to COL had the lowest variation. Overall the standard deviations are low and indicate the fold-changes are comparable across the 10 CIP resistant mutants tested. The average fold-changes were log-transformed and plotted with the 95% confidence interval (see **Figure 9**).

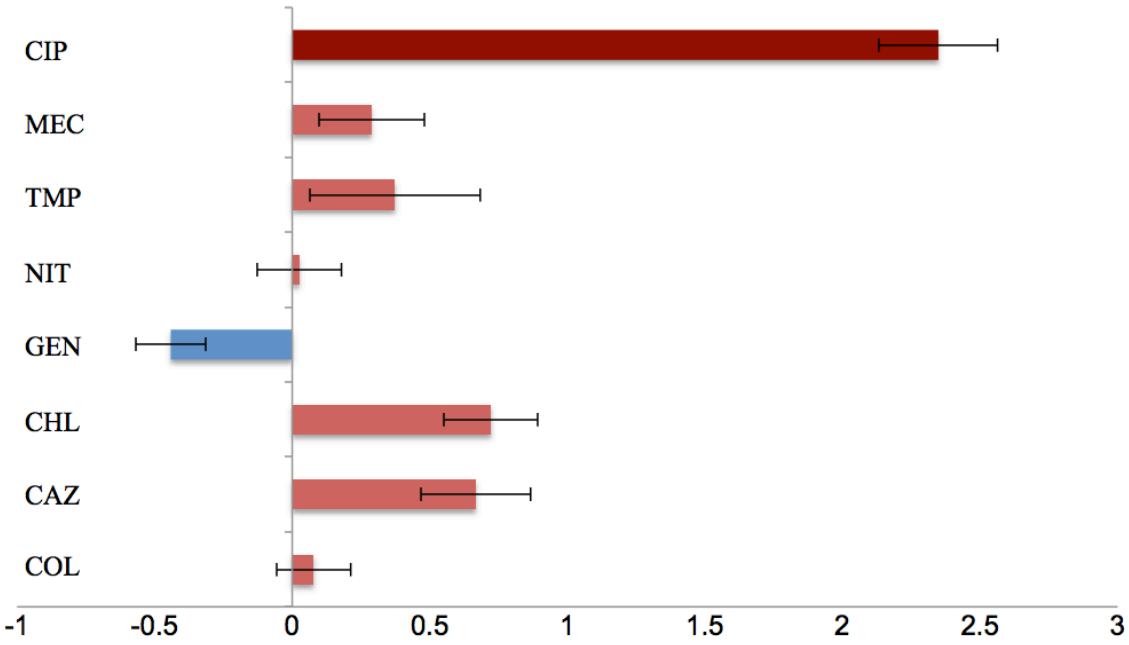


Figure 9. Average CS/CR changes of 10 CIP resistant mutants. This histogram shows the average change in susceptibility, where the dark red color shows resistance to CIP, the light red color shows CR, and blue color shows CS to different drugs. Error bars show the 95% confidence interval.

On average CR of the 10 CIP resistant mutants was found for MEC, TMP, NIT, CHL, CAZ, and COL, and CS was found to GEN. However, with the 95% confidence interval, we only expect true CR for CIP resistant strains to MEC, TMP, CHL and CAZ, and CS to GEN. For the CIP resistant mutants change in susceptibility to NIT and COL can vary between low CR, CS or no change, and accordingly the 95% confidence interval spans between CR and CS.

3.3 Identifying DNA mutations in CIP resistance genes

3.3.1. PCR amplification

PCR was used to amplify genes commonly know to contribute to CIP resistance, *gyrA*, *parC*, *marR* and *acrR*. Each PCR reaction was visualized on an agarose gel to see the DNA band and confirm its size using a DNA ladder for each amplified gene. An example gel image with some of these amplified genes (*gyrA*, *parC* and *marR*) is shown in **Figure 10**.

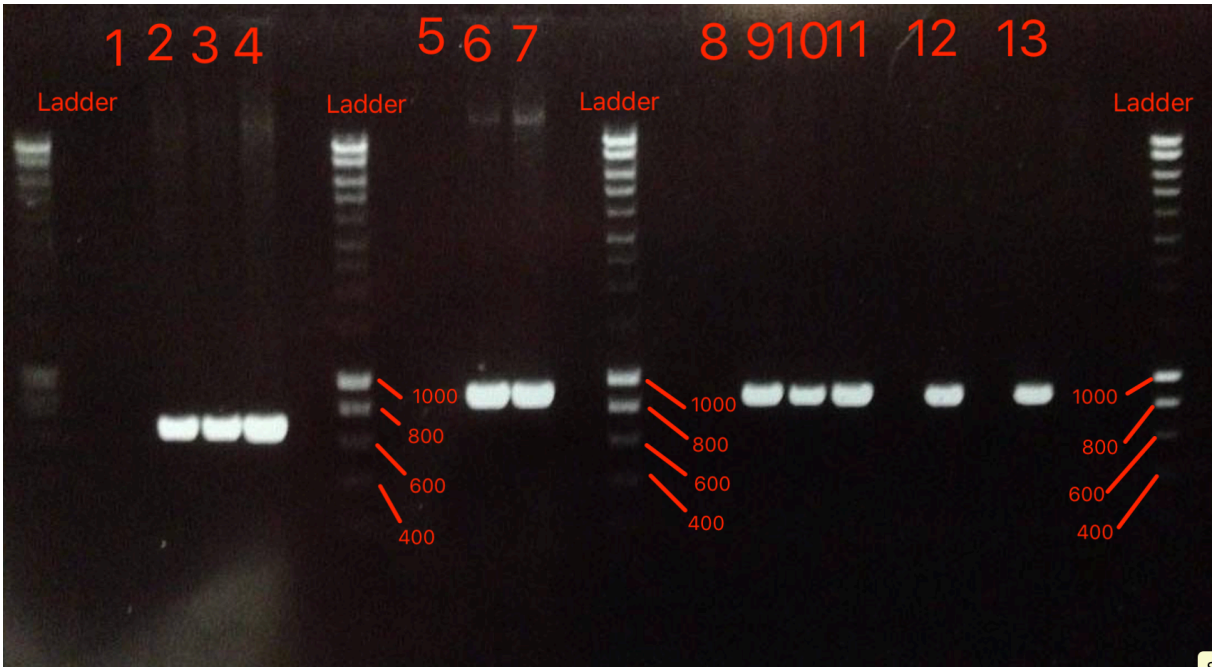


Figure 10. Gel image of PCR products on a 2% agarose gel. Wells numbered 1-4 for amplified *gyrA* (1 negative control (NC) with ddH_2O, 2 K56-5 WT, 3 K56-30 WT and 4 K56-49 (1)), lanes 5-7 for *parC* (5 NC, 6 K56-30 WT and 7 K56-49 CIP) and lanes 8-13 for *marR* (8 NC, 9 K56-5 WT, 10 K56-5 CIP, 11 K56-20 WT, 12 K56-31 CIP and 13 K56-43 CIP). Ladder = SmartLadder (see **Appendix Figure a2**).

During the PCR amplifications occasionally amplification of specific genes failed or we found double (Appendix Table a?) or multiple bands. Amplification of the *acrR* gene yielded multiple bands the majority of the time, see **Figure 11**.

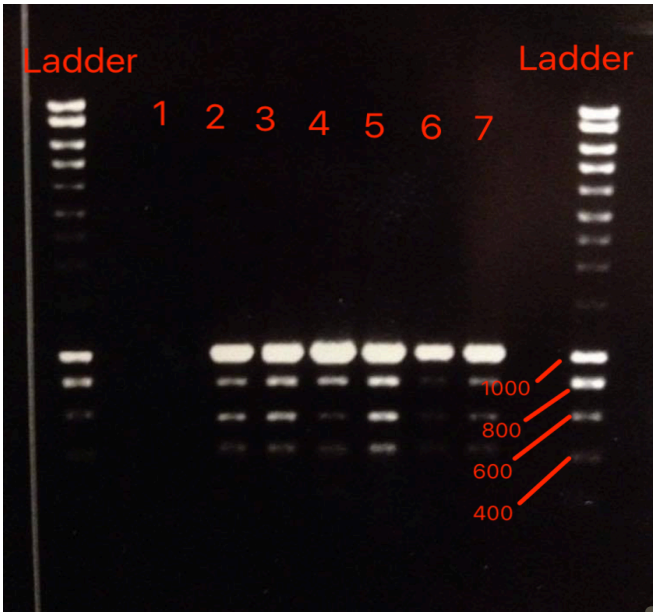


Figure 11. Gel image of the *acrR* gene PCR products Ladder: Smartladder (see **Appendix Figure a2**) 1:NC, 2:K56-17WT, 3:K56-17CIP, 4:K56-18WT, 5:K56-18CIP, 6:K56-61WT and 7:K56-61CIP.

Despite issues with the PCR amplifications, all of the desired PCR products were successfully amplified and confirmed by visualization on gel. Either by repeating the PCR reactions or isolating single bands from the gel, single PCR products were sent for DNA sequencing for all 4 genes with all 10 strains (see **Appendix Table a6**).

3.3.2. Confirmed point mutations from sequenced data

With help from the Sequencher DNA sequence analysis software we analyzed all of the sequence data, and point mutations were identified in *gyrA* gene within the QRDR (see **Table 4**) when the CIP resistant mutants were compared to their WT. No point mutations were identified in *parC* and *marR* genes from the sequence data. Poor quality sequences were obtained with the *acrR* gene, and it was not possible to compare the data using Sequencher.

Table 4. Identified point mutations in *gyrA*.

Isolate	*gyrA* DNA mutation	GyrA amino acid position	WT amino acid	Mutant amino acid
K56-5 (1) CIP	G259T	87	Aspartate (Asp)	Tyrosine (Tyr)
K56-17 (1) CIP	G259A	87	Aspartate (Asp)	Asparagine (Asn)
K56-18 (1) CIP	G259T	87	Aspartate (Asp)	Tyrosine (Tyr)
K56-20 (1) CIP	C248T	83	Serine (Ser)	Leucine (Leu)
K56-22 (1) CIP	C248T	83	Serine (Ser)	Leucine (Leu)
K56-30 (1) CIP	C248T	83	Serine (Ser)	Leucine (Leu)
K56-31 (9) CIP	G259A	87	Aspartate (Asp)	Asparagine (Asn)
K56-43 (1) CIP	G259T	87	Aspartate (Asp)	Tyrosine (Tyr)
K56-49 (1) CIP	C248T	83	Serine (Ser)	Leucine (Leu)
K56-61 (1) CIP	G259T	87	Aspartate (Asp)	Tyrosine (Tyr)

4. Discussion

The growing frequency of antimicrobial resistance is considered to be a global health problem, as this reduces the efficacy of available treatment options. New antimicrobial treatment approaches should be explored. The idea of applying CS in drug cycling programs as proposed by Sommer (Imamovic et al. 2013), can be a possible approach to prevent AMR and maintain availability of effective drugs. But with limited research in this field, focused on only laboratory strains or a few clinical isolates tested *in vitro*, further investigation in this field is required before considering the use of CS to inform drug cycling or other practical uses in clinical settings.

The goal of this research was to investigate CS/CR patterns in pan-susceptible, genetically diverse, clinical *E. coli* strains in order to determine if CR occurs more common in CIP resistant mutants than CS and what factors contribute to these patterns. We also aimed to compare our findings in clinical isolates to those described in previous studies by Imamovic and Sommer and Lázár and Pál in 2013, which primarily focused on laboratory strains of *E. coli*. Finally, very little is know about factors contributing to changes in these patterns, so we used DNA sequencing to compare the resistance mechanism(s) of the generated mutants to test if this factor contributes to changes in the CS/CR patterns.

4.1 Generation of CIP resistant mutants

We were able to use a stepwise static AMR selection method to generate our CIP resistant mutant strains. Using this method we generated 10 CIP resistant mutants of *E. coli* from the ECO•SENS collection with stable clinical resistance and IC_{90} values that were at least 85 times higher than the parent WT isolates. No major difference in the IC_{90} of the mutant which required only one selection step to become CIP resistant, K56-30 CIP (1), with CIP (3 μg/ml), compared to those who required (average of 2,9 μg/ml).

Higher resistance to CIP can occur through multiple mutations, where several point mutations in different target enzymes are required (Hooper 2001). While single mutations in *gyrA* were found in all the isolates, it is unclear if CIP mutants with

higher CIP MICs had more mutations in other genes that could contribute to CIP resistance. However, K56-5 and K56-18 both required 3 selection steps and are resistant to CIP at a high level (4 and 8 µg/ml respectively) and over a 300 fold decrease in susceptibility to CIP. CIP resistant mutant K56-20 CIP (1) also had over 300 fold-change in susceptibility to CIP, and this strain required 2 mutational steps to adapt to clinical resistance. In general our *E. coli* strains required 2 or 3 selection steps to achieve CIP clinical resistance.

4.1.1 Challenges and limitations in generation of CIP resistant mutants

There were some challenges when generating CIP resistant mutants in the laboratory. These include the overgrowth of strains during CIP resistance selection steps, difficulties in the purification of single phenotype bacterial populations, and on occasion contamination was observed. For some strains during selection steps, there was confluent growth on all selective plates with CIP and non-selective plates (LBA). This occurred with K56-5 once following plating from $CIP_{0,032}$, however when the experiment was repeated typical growth and results were found. For K56-30 confluent overgrowth occurred on every selection step from $CIP_{0,032}$ and higher, all the way to CIP_1. There were observations of dense growth of these bacteria on the plates and a strong odor from K56-5 and K56-30.

For isolate K56-22 CIP (1) we were unable to achieve a purified single-phenotype bacterial population. Mixed phenotypes of CIP and MEC resistant mutants on non-selective plates were also observed in the Johnsen lab, during the previous work. This could be due to adaptation of the CIP resistance mechanism, other compensatory mutations, or possibly a change in the expression of different genes related to CIP resistance or other adaptions.

A contamination was also observed during the CIP selection steps for K56-5; yellow and rounded cells that had a very different phenotype than *E. coli* were found on selective plates with CIP and LBA non-selective plates. It is unclear what was the source of the contamination. But by starting the selection process again from the glycerol freeze stock of the WT we were able to avoid the contamination.

4.2 Confirmation of point mutation

In this study, we were able to detect point mutations in the *gyrA* gene, which are nonsynonymous, Ser83 to Leu and Asp87 to Asn and Tyr. These chromosomal mutations are common point mutations also found in previous studies (Hooper 1999); (Komp Lindgren et al. 2003, Ruiz 2003). But we were not able to detect point mutations in the *parC* and *marR* genes that were amplified and sequenced, additionally sequencing attempts of the *acrR* gene failed. AcrR is an important component that regulates the expression of the AcrAB-TolC efflux pump. This efflux pump can confer CR to other drugs. Within our 10 tested CIP resistant isolates, we observed CR to drugs that can be efflux pump substrates, such as CHL and some β-lactams (Nikaido 2001). We speculate that mutations to *acrR* or other components of efflux pumps may exist in our CIP resistant isolates and may contribute to changes in the CS/CR susceptibility patterns. Further DNA sequencing attempts could explain our findings. However, currently we are not able to compare the susceptibility patterns of the 10 generated CIP resistant mutants by their CIP resistance mechanism because of lack of data.

4.2.1 Challenges and limitations in confirmation of point mutation

All primers used to amplify *gyrA*, *parC*, *marR,* and *acrR* genes in this study are taken from a previous study (Komp Lindgren et al. 2003). There is missing information about which melting temperatures were used to amplify *parC*, *marR,* and *acrR* genes. We calculated the theoretical melting temperatures for *parC*, *marR,* and *acrR*, and tested these theoretical melting temperature using gradient PCR (see **Section 2.9.3**). We were able to find an optimal melting temperature and use these melting temperatures (**Table 7**) to amplify these genes.

During the PCR process we observed contamination in the negative controls. A lot of interventions were tried to prevent this contamination, such as new sterile Milli-Q-water, the use of filter-tips for mixing PCR-reagents, frequent changing of tips, increased awareness to prevent cross-contamination from pipette tips between PCR

tubes, and handling of fewer samples at a time. Contamination of the negative controls was eliminated following these interventions.

Double bands were observed for some strains, which suggested that the primers bind at multiple sites. However, when these experiments were repeated the double bands in the amplified PCR-product disappeared. Also in amplified PCR-products of the *acrR* gene we observed multiple bands on an agarose gel. This indicates that these primers bound less efficiently to the specific target site, which yielded lower concentrations of the desired PCR-product. Because of the multiple bands, gel slices with the specific band size for *acrR* were cut out and DNA was extracted with the gel extraction method (see **section 2.13**). The extracted DNA typically had low DNA concentration (< 20 ng/µl) detected by Nanodrop, but was still sent for sequencing. Poor sequence data was obtained, which could not be used to identify mutations. Re-design of the *acrR* primers for PCR and sequencing could allow us to improve the quality of the sequence data and identify mutations in the *acrR* gene.

4.3 Observed CS/CR profiles in this study

Initially we used 2-fold micro-broth dilution IC_{90} experiments to predict a range of antimicrobial concentrations that exhibit inhibition ≥ 90% (see **Appendix Table a4**). Comparing the IC_{90} measurements of the 2-fold dilution and 1,5-fold dilution setups, there are no big changes in the IC_{90} data and the fold-changes are consistent. However, we did observe over 2-fold differences of IC_{90} data for strain K56-18 CIP (1) and K56-30 CIP (1) tested with CHL, K56-20 WT and K56-22 WT tested with COL, and K56-43 WT tested with TMP. These variations could indicate human or random errors in the study since both 1,5-fold and 2-fold dilutions were only performed once. The comparison of 2-fold and 1,5 fold IC_{90} data was not possible with the slow-growing strain K56-17 CIP since the IC_{90} with 2-fold dilution was not determined.

The 1,5-fold IC_{90} assay was performed to determine susceptibility of 10 generated CIP resistant mutant strains to CIP, MEC, TMP, NIT, GEN, CHL, CAZ, and COL. Based on the fold change in the IC_{90} results, CR to MEC, TMP, CHL, CAZ, and NIT (CR to NIT in only 3 strains) was observed, and CS was observed to GEN, COL and NIT. Specifically, we see that all generated CIP resistant mutants had CS to GEN

(except for strain K56-43), two strains (K56-30 and K56-31) had CS to NIT, and one strain, K56-17, had CS to COL while the rest had no change in susceptibility.

4.3.1 Challenges and limitations of IC_{90} assay

In this study, isolate K56-17 CIP (1) grows slower in MH medium than other strains. This can interfere with our IC_{90} results obtained after 18 hours, and it was necessary to incubate for 42 hours to get denser growth and correct calculation of the IC_{90}. The real IC_{90} values for K56-17 CIP (1) to the 8 testing antimicrobials could be inaccurate since the longer incubation time could affect the results. One reason could be that the longer incubation can affect the antimicrobial activity of antimicrobials, however it depends on which drug and its stability (half-life) at 37°C and in MH.

4.4 Comparison with previous studies on CR and CS profiles

In many ways it is challenging to compare CS/CR data from this study to other studies because there is lack of information about the highest concentration of CIP the isolates in other studies were evolved to. In comparison to the results from Imamovic and Sommer in 2013 (Imamovic et al. 2013), where CR/CS profiles were also determined base on IC_{90}s, we can see some similarities in our study specifically in CR patterns to CHL, NIT and TMP. In Imamovic and Sommer´s study CIP resistant mutants showed CR with over 32-fold change in IC_{90} to TMP, over 8-fold change to CHL, and over 2-fold change to NIT. Their results also showed no changes in susceptibility to GEN and COL. In our study, our CIP resistant mutants showed similar patterns of CR with as high as a 12-fold change to TMP, 6-fold change to CHL, and 3-fold change to NIT. We also observed CS for our CIP resistant mutants to GEN, NIT (K56-30 and K56-31) and COL (K56-17), which is not described by Sommer et al. (Imamovic et al. 2013). In studies by Lázár et al. (Lazar et al. 2013), they found CS in a low-level CIP resistant mutant to NIT and GEN, and CR to TMP, but not to CHL (Lazar et al. 2014).

When we compare our generated data on the average CS/CR changes in 10 CIP resistant mutants to previous work in the Johnsen lab with the same antimicrobials, a similar pattern in the data is observed. CIP resistant mutants share a similar CR

pattern to MEC, TMP, CHL, and CAZ, which suggests that a mutant will frequently have CR to those antimicrobials when the CIP resistant mutant is generated *in vitro*. For CIP resistant mutants tested with COL and NIT in our study, there were variable results but a trend towards CR. However, the previous work showed that a CIP resistant mutant was more likely to have CS to COL and NIT. These variations could be due to methodological differences, since in this study we used the IC_{90} method and the previous work in the Johnsen lab was with the E-test method. When looking at the average change in susceptibilities to COL and NIT in both studies, the 95% confidence interval ranges across the border of CS and CR. Surprisingly, CIP resistant mutants in our study show fold-changes suggesting increased susceptibility to GEN than the previous work, which had little change in susceptibility. With statistical methods (see **Section 3.2.3**) we are able to see the bigger picture for the 10 generated CIP resistant mutants instead of one CIP resistant mutant; this allows us to expand the current data to better understand general CS/CR patterns caused by CIP resistance.

4.5 Conclusions and future aspects

With our findings in this study, we are able to observe both CR and CS profiles within 10 generated CIP resistant mutants. While CIP resistance seemed slow to develop in the lab, requiring 2 to 3 selection steps, CIP resistance is commonly found in the clinic (Fasugba et al. 2015). Once *E. coli* adapts clinical resistance to CIP, we found that they can show CR to many other antimicrobials, and this could reduce available treatment options. Unlike previous studies by Imamovic and Sommer (Imamovic et al. 2013), we also observed CS. There is still little known about the mechanisms behind CS, the best understood mechanism was proposed by Lázár and Pál (Lazar et al. 2013), where resistance to aminoglycosides caused reduced efflux function. While we were able to find *gyrA* drug target mutants in all of the strains, but no mutations in *parC* and *marR*, we were not able to determine what factors contribute to CS/CR patterns due to incomplete sequencing data of *acrR* and other unknown mutations in these CIP resistant mutants. The concept of CS still needs further investigation to determine the causal mechanisms.

Further investigation to identify mutations in the *acrR* gene could be a possible way see if efflux with AcrAB-TolC may cause CIP resistance, as it would suggest possible

overexpression of this efflux pump. We suspect that efflux may contribute to changes in our CS/CR-patterns.

The CS of our CIP resistant strains to GEN is an interesting result, where one could consider GEN as a treatment for *E. coli* resistant to CIP as a part of a drug cycling program. However, we discussed the uncertainty in the stability of CS profiles, including GEN, in our CIP resistant mutants. It is possible that these profiles may vary over time as the bacteria adapt to reduce the cost of mutations and changes in their environment (e.g. host defense system, pH). This is something that should also be considered in the future work to better understand factors that affect stability of CS profiles.

Description of CS/CR profiles is important, for example to be aware of the potential of frequent CR when employing CIP for treatment. This suggests that ciprofloxacin is not the best choice for first-line treatment of uncomplicated UTIs. However, CIP resistant mutants in this study often had low CS or very little change in susceptibility to GEN. While GEN is not commonly used for treatment of UTIs, it is used for the treatment of sepsis in Norway and as treatment for many other infections including uncomplicated UTIs in other countries (UPTODATE 2016). I hope that our study can provide some helpful data on the collateral effects of antimicrobial susceptibility caused by resistance to ciprofloxacin.

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6. Appendix

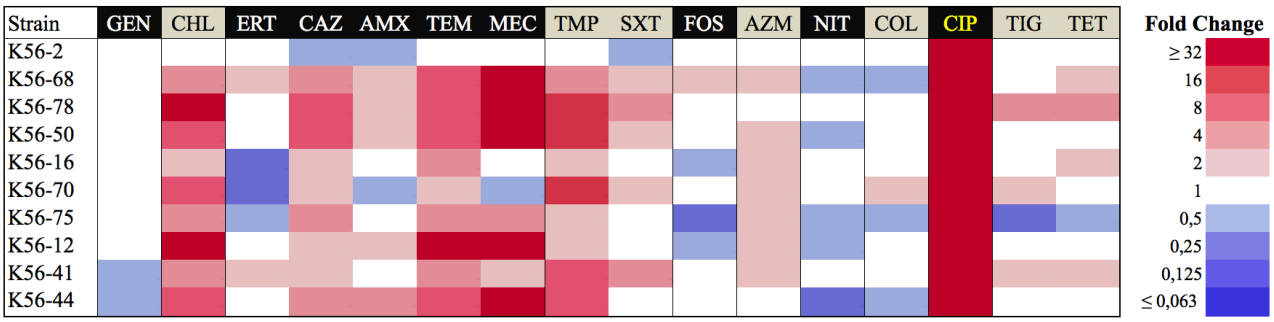

Figure a1. Heat map of CS/CR changes in 10 clinical ECO-SENS *E. coli* isolates resistant to CIP.

Table a1. Total counted CFUs on selective and non-selective plates for each CIP selection step.

Strain	Selection Step 1 (CFU/100µl)		Selection Step 1 (CFU/100µl)		Selection Step 1 (CFU/100µl)	
	LBA	**CIP_x**	**LBA**	**CIP_x**	**LBA**	**CIP_x**
K56-5	D_8: 26	$Cip_{0,032}$: 54	D_8: 31	$Cip_{0,128}$: 1000	D_8: 19	$Cip_{0,5}$: 1208
K56-17	D_8: 8	$Cip_{0,032}$: 23	D_8: 7	$Cip_{0,25}$: 8		
K56-18	D_8: 8	$Cip_{0,032}$: 35	D_8: 12	$Cip_{0,128}$: 6	D_8: 5	$Cip_{0,5}$: 2
K56-20	D_8: 25	$Cip_{0,032}$: 2	D_8: 15	$Cip_{0,25}$: 872		
K56-22	D_8: 9	$Cip_{0,032}$: 2	D_8: 6	$Cip_{0,5}$: 51		
K56-30	D_8: 13	$CIP_{0,032}$: 3				
K56-31	D_8: 26	$Cip_{0,032}$: 1	D_8: 31	$Cip_{0,25}$: 71		
K56-43	D_8: 5	$CIP_{0,016}$: 196	D_8: 22	$CIP_{0,128}$: 7	D_7: 20	$CIP_{0,5}$: 877
K56-49	D_8: 12	$CIP_{0,032}$: 1	D_8: 24	$CIP_{0,25}$: 334		
K56-61	D_8: 7	$Cip_{0,032}$: 5	D_8: 15	$Cip_{0,128}$: 358	D_8: 12	$CIP_{0,5}$: 3

Table a2. Generated CIP resistant mutants for each strain. CIP resistant mutants in bold text were selected for further study.

Strain	CIP resistant mutants
K56-5	**(1)**, (2), (3)
K56-17	**(1)**, (2), (3)
K56-18	**(1)**, (2), (3)
K56-20	**(1)**, (2), (3)
K56-22	**(1)**, (2), (3)
K56-30	**(1)**, (2), (3)
K56-31	(7), (8), **(9)**
K56-43	**(1)**, (2), (3)
K56-49	**(1)**, (2), (3)
K56-61	**(1)**, (2), (3)

Table a3. MALDI-TOF results and score values for confirmation of *E. coli* mutants.

Isolate	Score value	Confirmed *E. coli*
K56-5 CIP (1)	> 2000	+
K56-17 CIP (1)	> 2000	+
K56-18 CIP (1)	> 2000	+
K56-20 CIP (1)	> 2000	+
K56-22 CIP (1)	> 2000	+
K56-30 CIP (1)	> 2000	+
K56-31 CIP (9)	> 2000	+
K56-43 CIP (1)	> 2000	+
K56-49 CIP (1)	> 2000	+
K56-61 CIP (1)	> 2000	+

Table a4. 2-fold IC_{90} results.

		IC_{90} (µg/mL) using 2-fold dilution method							
Strain	**Isolate**	**CIP**	**MEC**	**TMP**	**NIT**	**GEN**	**CHL**	**CAZ**	**COL**
K56-5	WT	0,016	0,125	0,125	8	0,125	4	0,063	0,25
	CIP (1)	4	0,5	1	8	0,063	16	0,25	0,25
K56-17	WT	0,016	0,125	0,25	8	0,5	8	0,125	0,5
	CIP (1)[1]								
K56-18	WT	0,008	0,125	0,25	16	0,25	4	0,063	0,25
	CIP (1)	8	0,5	2	16	0,063	32	1	0,25
K56-20	WT	0,008	0,063	0,25	8	0,25	4	0,063	0,25
	CIP (1)	4	0,25	1	8	0,063	32	1	0,125
K56-22	WT	0,016	0,125	0,5	16	0,5	8	0,25	0,25
	CIP (1)	4	0,125	0,5	16	0,25	32	0,25	0,25
K56-30	WT	0,016	0,125	0,25	16	0,25	4	0,125	0,25
	CIP (1)	4	0,25	1	8	0,125	64	1	0,25
K56-31	WT	0,08	0,063	0,25	16	0,25	8	0,063	0,25
	CIP (9)	1	0,125	1	4	0,125	32	0,25	0,25
K56-43	WT	0,016	0,125	0,125	8	0,25	8	0,125	0,25
	CIP (1)	2	0,125	1	16	0,5	8	0,125	0,25
K56-49	WT	0,008	0,063	1	16	0,25	4	0,063	0,25
	CIP (1)	2	0,25	2	16	0,125	8	0,25	0,25
K56-61	WT	0,08	0,125	0,125	16	0,25	4	0,063	0,25
	CIP (1)	2	1	2	4	0,125	32	1	0,25

[1] K56-17 is a slow growing strain and initial 2-fold IC_{90} were not measured at 42 hours incubation due to time constraints.

Table a5. Calculated fold changes in 1,5 fold dilution IC_{90} results.

	Fold change in drug susceptibility							
Isolate	**CIP**	**MEC**	**TMP**	**NIT**	**GEN**	**CHL**	**CAZ**	**COL**
K56-5 CIP (1)	341,33	3,00	6,00	1,33	0,38	4,00	3,00	1,00
K56-17 CIP (1)	96,00	2,00	12,00	3,00	0,25	4,00	3,00	0,50
K56-18 CIP (1)	682,67	4,00	8,00	0,67	0,38	4,00	8,00	1,33
K56-20 CIP (1)	384,00	3,00	4,00	1,50	0,25	5,33	8,00	0,75
K56-22 CIP (1)	256,00	2,00	1,00	0,67	0,25	4,00	2,00	0,75
K56-30 CIP (1)	192,00	2,00	2,67	0,50	0,17	6,00	4,00	0,67
K56-31 CIP (1)	85,33	1,33	1,33	0,50	0,38	4,00	3,00	0,75
K56-43 CIP (1)	192,00	1,00	1,00	2,00	1,00	2,00	1,33	1,00
K56-49 CIP (1)	128,00	2,67	4,00	1,00	0,50	2,00	2,67	0,75
K56-61 CIP (1)	256,00	8,00	2,00	2,00	0,38	5,33	8,00	1,00

Table a6. PCR amplification of CIP resistance genes using Phusion polymerase.

Strain	Isolate	*gyrA* GyrAR1000 GyrAFQ322	*parC* ParCR981 ParCFQ107	*marR* MarORR2011 MarORF1139	*acrR* AcrR9934R AcrR8900F
K56-5	WT	+	+	+	+
	CIP (1)	+	+	+	+
K56-17	WT	+	+	+	+
	CIP (1)	+	+	+	+
K56-18	WT	+	+	+	+
	CIP (1)	+	+	+	+
K56-20	WT	+	+	+	+
	CIP (1)	+	+	+	+
K56-22	WT	+	+	+	+
	CIP (1)	+	+	+	+
K56-30	WT	+	+	+	+
	CIP (1)	+	+	+	+
K56-31	WT	+	+	+	+
	CIP (9)	+	+	+	+
K56-43	WT	+	+	+	+
	CIP (1)	+	+	+	+
K56-49	WT	+	+	+	+
	CIP (1)	+	+	+	+
K56-61	WT	+	+	+	+
	CIP (1)	+	+	+	+

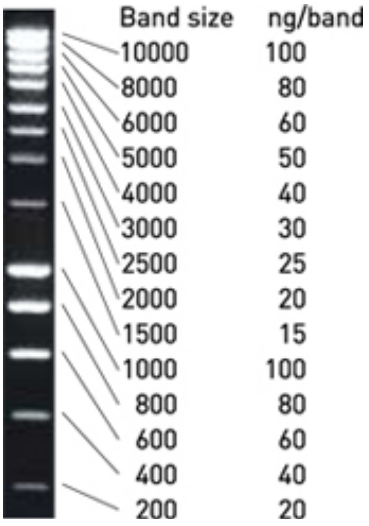

Figure a2. SmartLadder DNA molecular marker.

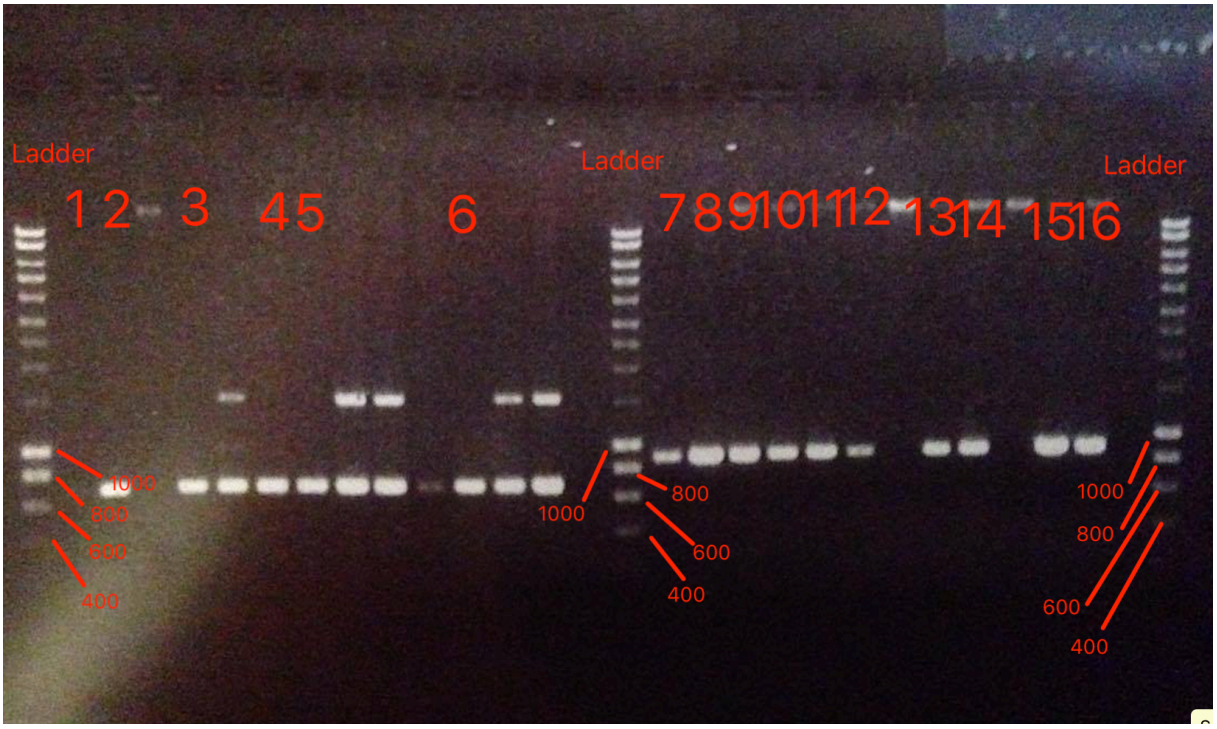

Figure a 3. Gel image of PCR products with double bands on an agarose gel. Gel picture of PCR-product observed on 2 % agarose gel. Ladder: Smartladder (See Appendix). 1-6 for amplified gyrA: 1:NC, 2: K56-22 WT, 3:K56-30WT, 4:K56-31 CIP, 5:K56-43 WT and 6: K56-61 WT. 7-16 for amplified parC: 7:NC, 8:K56-5 WT, 9: K56-5 CIP, 10:K56-17 WT, 11:K56-17 CIP, 12:K56-22 WT, 13:K56-30 CIP, 14: K56-43 WT, 15: K56-20 WT and 16:K56-20 CIP.