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Characterization of quinolone resistant *Escherichia coli* from broilers with focus on plasmid-mediated quinolone resistance

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Preface

This master thesis is a 30 credits thesis carried out at Norwegian Veterinary Institute (NVI) as a part of QREC-risk project. It is a part of the master of Food Science at the Faculty of Chemistry, Biotechnology and Food Sciences at the Norwegian University of Life Sciences (NMBU).

I would like to thank my supervisors at NVI Jannice Schau Slettemeås and Camilla Sekse for guidance, flexibility, positivity and help during the experimental work and the writing phase. Thank you for going great lengths to give me feedback, even during holidays and on seminars. It has all been of great value.

I would also like to thank my fellow masterstudents at NVI Andrea and Kingsley for providing support, comfort and joy in these last three months. Last, but not least, I would like to thank friends and family for providing support in one way or another.

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Abstract

Widespread use of antimicrobial agents has generated antimicrobial resistance (AMR) among bacteria. The occurrence of AMR in Norwegian broiler production is low in a European and a global perspective and this is probably because of the restricted usage of antimicrobial agents. The World Health Organization has defined fluoroquinolones as critically important for treatment of infections in humans. Plasmid-Mediated Quinolone Resistance (PMQR) comprises a large group of genes conferring low-level resistance to fluoroquinolones and these are *qnr*-genes, *aac(6')-Ib-cr* gene, *qepA*, and *oqxAB* genes. Presence of both PMQR and chromosomally mediated resistance mechanisms to quinolones has shown to give a higher level of resistance in isolates of *Enterobacteriaceae*. In the absence of chromosomally mediated quinolone resistance mechanisms, the acquisition of only a PMQR gene will not display clinical resistance.

The aim of this study was to characterize five *E. coli* strains isolated from poultry encoding PMQR genes and to characterize and circularize one plasmid from one of the strains and compare it to other plasmids. Characterization of strains were carried out by phenotypic methods such as susceptibility testing, conjugation, and transfer frequency, molecular genotyping and identification of resistance mechanisms based on Whole Genome Sequencing (WGS) data. The plasmid characterization was based on WGS data, circularization was done by using PCR and Sanger sequencing. Annotations were carried out by RAST and comparison by BLAST and BRIG.

The five *E. coli* harboured the PMQR genes *qnrS1* or *qnrB19* encoding quinolone resistance. In addition, they harboured a $bla_{\text{TEM-1B}}$–gene encoding a β-lactamase. Four of the five strains carrying a *qnrS1* gene contained a self-transferable IncX1 plasmid. The *E. coli* strain carrying a *qnrB19* were not able to conjugate under the conditions used in this experiment. All five *E. coli* strains displayed MIC to ciprofloxacin above the ECOFF. The four strains carrying *qnrS1* displayed MIC to ciprofloxacin above the clinical breakpoint, thus were clinically resistant to ciprofloxacin. Plasmid pNVI7234 was 47 686 bp in size and was isolated from an *E. coli* O23:H16 and ST-453. pNVI7234 shared close homology with plasmids isolated from two *Shigella flexneri* strains. Annotation of the plasmid revealed a plasmid backbone encoding genes involved in conjugal transfer and partitioning systems, and a toxin-antitoxin system ensuring persistence in bacterial hosts.

Sammendrag

Utstrakt bruk av antimikrobielle midler har forårsaket antimikrobielle resistens i bakterier. I norsk fjørfeproduksjon er forekomsten av antimikrobiell resistens lav både i et europeisk og globalt perspektiv og den lave forekomsten skyldes sannsynligvis begrenset bruk av antimikrobielle midler. Verdens Helseorganisasjon har definert fluorokinoloner som kritisk viktige for behandling av humane infeksjoner. Plasmidmediert kinolonresistens omfatter en stor gruppe gener som gir resistens mot fluorokinoloner ved lave konsentrasjoner og disse genene er *qnr*-gener, *aac(6')-Ib-cr*, *qepA* og *oqxAB* gener. Forekomst av plasmidmediert og kromosomal kinolonresistens har i kliniske *Enterobacteriaceae* isolater gitt høygradig resistens mot kinoloner. Forekomst av kun et enkelt plasmidmediert kinolonresistensgen gir ikke klinisk resistens.

Formålet med studien var å karakterisere og sammenlikne fem *E. coli* stammer isolert fra fjørfe som inneholdt plasmidmedierte kinolonresistensgener. Fra en av stammene skulle et plasmid karakteriseres og lukkes for videre sammenlikning med liknende plasmider. Karakterisering og sammenlikning av stammer ble utført ved fenotypiske metoder som testing av følsomhet mot antimikrobielle midler, konjugeringsforsøk og overføringsfrekvens ved konjugering, og molekylær genotyping og identifisering av resistensmekanismer ved bruk av data fra helgenomsekvensering. Plasmidkarakteriseringen ble utført ved helgenomsekvensbaserte metoder og lukking av plasmid ble utført ved PCR og Sanger sekvensering. Annotering av plasmidet ble utført ved bruk av RAST, og sammenlikning med andre plasmid ble utført ved bruk av BLAST og BRIG.

De fem *E. coli* inneholdt de plasmidmedierte kinolonresistensgenene *qnrS1* eller *qnrB19*. Alle *E. coli* isolatene inneholdt i tillegg et $bla_{\text{TEM-1B}}$-gen som kodet for en β-laktamase. Fire av stammene inneholdt et *qnrS1*-gen på et overførbart IncX1 plasmid. Stammen som inneholdt et *qnrB19* gen lot seg ikke overføre under de eksperimentelle betingelsene. Alle fem *E. coli* uttrykte MIC mot ciprofloxacin høyere enn det epidemiologiske brytningspunktet (ECOFF) og fire av fem stammer var klinisk resistente mot ciprofloxacin. Det sirkulariserte plasmidet pNVI7234 var i størrelsesorden 47 686 basepar og ble isolert fra en *E. coli* O23:H16 og ST-453. pNVI7234 var nært beslektet med plasmider fra to *Shigella flexneri* stammer. Annotering av plasmidet avslørte en konservert del bestående av gener som koder for overføring, oppdeling (partitioning systems), og et toksin-antitoksin system som sikrer plasmidets persistens i vertsbakterien.

Content

1. Introduction

Widespread use of antimicrobial agents has generated resistance to antimircrobials among bacteria (Destoumieux-Garzon et al., 2018). Antimicrobial agent are used in human medicine, veterinary medicine, animal husbandry, plant production and aquaculture, and can be found in lakes, rivers and soil. Resistance genes, resistant bacteria and antimicrobial agents may spread between the listed environments as wastewater, sludge, manure and/or runoff (Andersson & Hughes, 2012). In 2003, the World Health Organization (WHO) concluded that antimicrobial resistance was of health concern in terms of global public health and animal health, and that antimircrobial agents used in animals regardless of purpose were frequently the same or closely related to antimircrobial agents used in human medicine. A list of Critically Important Antimircrobial agents (CIA) was developed in 2005 to ensure prudent use in human and veterinary medicine. Quinolones, 3rd, 4th and 5th generation cephalosporins, glycopeptides, macrolides and polymyxins were assigned the highest priority in the CIA list (World Health Organization, 2017).

Antimircrobial agents are natural or synthetic chemical substances that inhibit microbial growth (Keen & Montforts, 2012; Verraes et al., 2013). There are multiple strategies among microorganisms to counteract the action of antimircrobial agents and these include modification of target site, modification of cell wall (Andersson & Hughes, 2012; Verraes et al., 2013), enzymatic degradation of the antimircrobial agents (Verraes et al., 2013), and efflux pumps that reduce accumulation of antimircrobial agents within the cell (Andersson & Hughes, 2012). These mechanisms grant the bacterium resistance to one or more antimircrobial agents. The term antimicrobial resistance in this thesis refers to a biological definition that is, any increase in minimum inhibitory concentrations (MIC) or conversely, reduction in susceptibility (Robicsek et al., 2006).

Sensitivity to antimicrobial agents is measured by MIC. MIC measures the lowest concentrations of an antimicrobial agent that inhibits the visual growth of a bacterial culture (Andersson & Hughes, 2014). MIC is measured under standardized *in vitro* conditions such as defined growth medium, inoculum size, incubation temperature, and duration (Andersson & Hughes, 2014).

Understanding antimicrobial resistance in the context of this thesis requires elaboration on concepts such as selective pressure, fitness, fitness costs, cross-resistance and co-selection. Selective pressure is the effect on reproductive success (fitness) of a bacterium when exposed to any agent (i.e antimicrobial), resulting in a change in frequency of the bacterium in a population (Keen & Montforts, 2012). Fitness is a term that refers to an organism's survival and reproductive success in an environment (Durao et al., 2018). Fitness costs are the costs of having i.e. a resistance determinant in terms of reduced growth rate on bacterial populations (Andersson & Hughes, 2012). Co-selection is

the process of acquiring mobile genetic elements (i.e plasmids, integrons, transposons) with resistance to multiple antimicrobial agents during exposure to one antimicrobial agent (Keen & Montforts, 2012). The mobile genetic element with multiple resistance to multiple antimicrobial agents keeps being attractive to the bacteria as long as one of the resistance determinants are necessary for growth, even though the resistance determinants are superfluous (Hudson et al., 2017). Cross-resistance is an acquired ability of a microorganism to tolerate other antimicrobials with the same mode of action (Keen & Montforts, 2012).

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines antimicrobial resistance based on threshold values. EUCAST develops threshold values for defining a genera or species of bacteria resistant to one specific antimicrobial agent. Epidemiological cut-off value (ECOFF) is a threshold value that determines if a bacterium is regarded as Wild-type (WT) or Non-Wild-type (NWT) to a specific antimicrobial agent. The WT/NWT distinction defines a bacterium as resistant in a biological sense by indicating if the bacterium has acquired resistance(Ellington et al., 2017; EUCAST). Ellington et al. (2017) define ECOFF as the "highest MIC value for organisms devoid of phenotypically detectable acquired resistance mechanisms". The ECOFF value also has other purposes such as being a cut-off value when screening for low-level resistance. Acquired resistance mechanisms in this context only refer to the ability of a bacterium to acquire resistance mechanisms and do not specify whether it is acquired by vertical- or horizontal gene transfer. A NWT bacterium has acquired higher tolerance to one antimicrobial compared to a WT. Clinical breakpoints are threshold values used to determine whether a genus or a species of bacteria is regarded as clinically resistant or susceptible to one antimicrobial agent. Clinical breakpoints categorize bacteria into resistant (R), intermediate (I) and susceptible (S). A resistant bacterium in clinical settings could resist therapeutic treatment (Ellington et al., 2017; EUCAST; EUCAST, 2018).

1.1 Sublethal antimicrobial resistance

Sublethal levels of antimircrobial agents are concentrations of antimircrobial agents that are below the MIC for a particular bacterial culture when exposed to a specific antimicrobial agent. These conditions are not lethal to the microbiota nor to the pathogens and can therefore persist. Exposure to sublethal levels of antimircrobial agents might result in reduced growth rate of a susceptible strain compared to conditions without exposure (Andersson & Hughes, 2014). Andersson and Hughes (2012) argue that non-lethal concentrations of antimircrobial agents are more problematic because it slows down the growth rate of the bacteria rather than killing it, and acts as a selector for AMR. Sublethal drug concentrations affect the rate of emergence of mutants and the selection of resistance mechanisms (Andersson & Hughes, 2012). A broader range of mutants is selected for when exposed to sublethal concentrations of antimircrobial agents and it favours cheap resistance mechanisms in

terms of fitness costs, over too costly resistance mechanisms that are outcompeted (Andersson & Hughes, 2014).

1.2 Usage of critically important antimicrobial agents in Norway and occurrence of quinolone resistant *E. coli* in Norwegian broiler production

Norway has low usage of veterinary medicinal products for therapeutic use in food-producing animals (NORM/NORM-VET, 2015) and very low sales of critically important antimircrobial agents such as 3rd and 4th generation cephalosporins, fluoroquinolones and macrolides for use in food-producing animals (NORM/NORM-VET, 2016; NORM/NORM-VET, 2017). The usage of macrolides and fluoroquinolones were negligible in 2014, and in addition, cephalosporins are not marketed towards food-producing animals in Norway (NORM/NORM-VET, 2015).

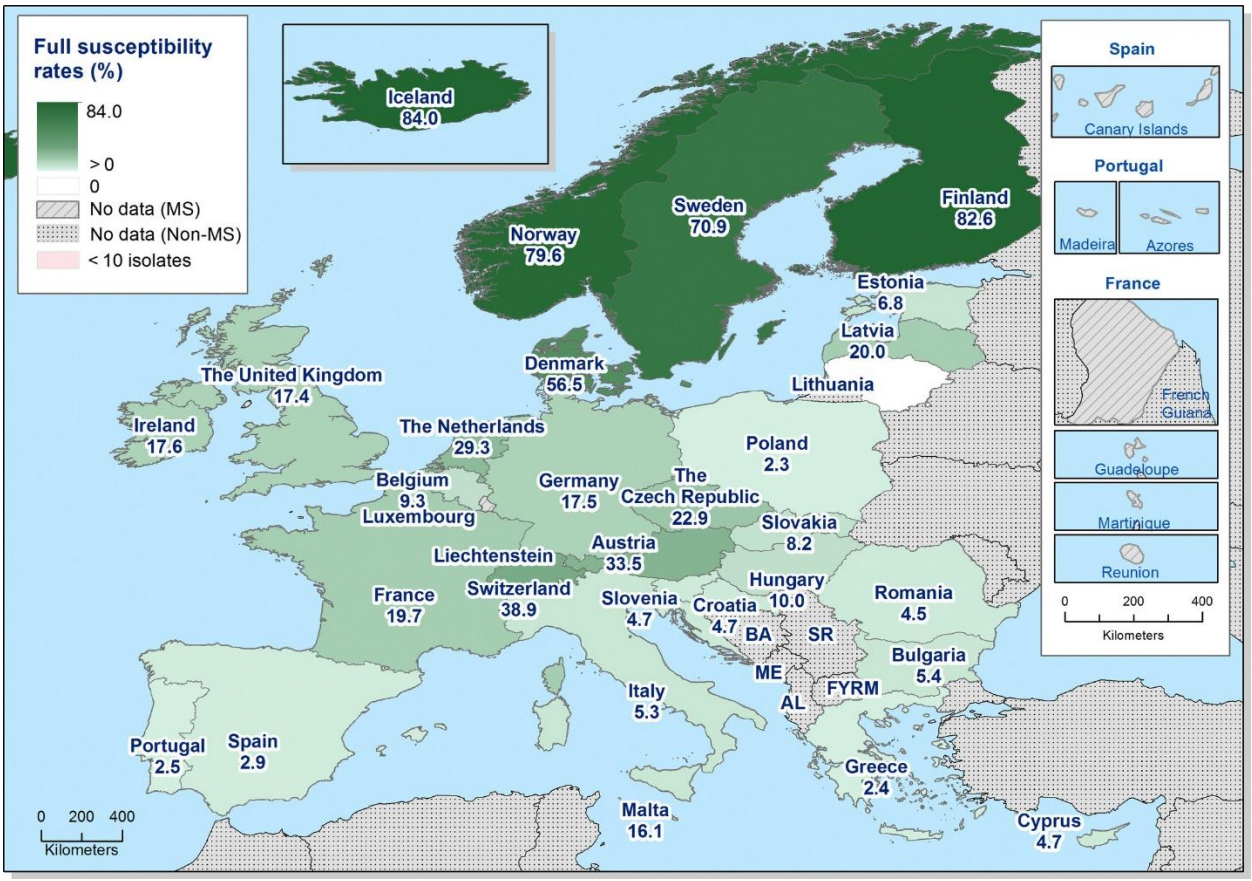

Figure 1: Percentage of indicator E. coli from broilers being susceptible to all antimircrobial agents being tested for in 30 EU/EEA member states in 2016. Reproduced from (EFSA & ECDC, 2018).

The occurrence of AMR in Norwegian broiler production is low (figure 1) in a European (EFSA & ECDC, 2018) and international perspective and this is probably because of the restricted usage of antimircrobial agents. Figure 1 and 2 shows the favourable situation in Norwegian broiler production in terms of complete susceptibility to all antimircrobial agents tested for and in resistance to ciprofloxacin in particular. Although there is no selective pressure from quinolone usage in the

Norwegian broiler production, a relatively high number (3.4 % in 2014, 2% in 2011 and 8% in 2009) of *E. coli* expressing quinolone resistance was detected by non-selective screening of indicator *E. coli* in 2014. This year a selective method for presence of quinolone resistance were introduced using a selective agar containing ciprofloxacin and 210 caecal samples from broiler flocks and 198 samples of broiler meat were screened. Quinolone-resistant *E. coli* (above ECOFF to ciprofloxacin) were identified in 188/210 (89.9 %) caecal samples and 140/198 (70.7%) samples from broiler meat (NORM/NORM-VET, 2015).

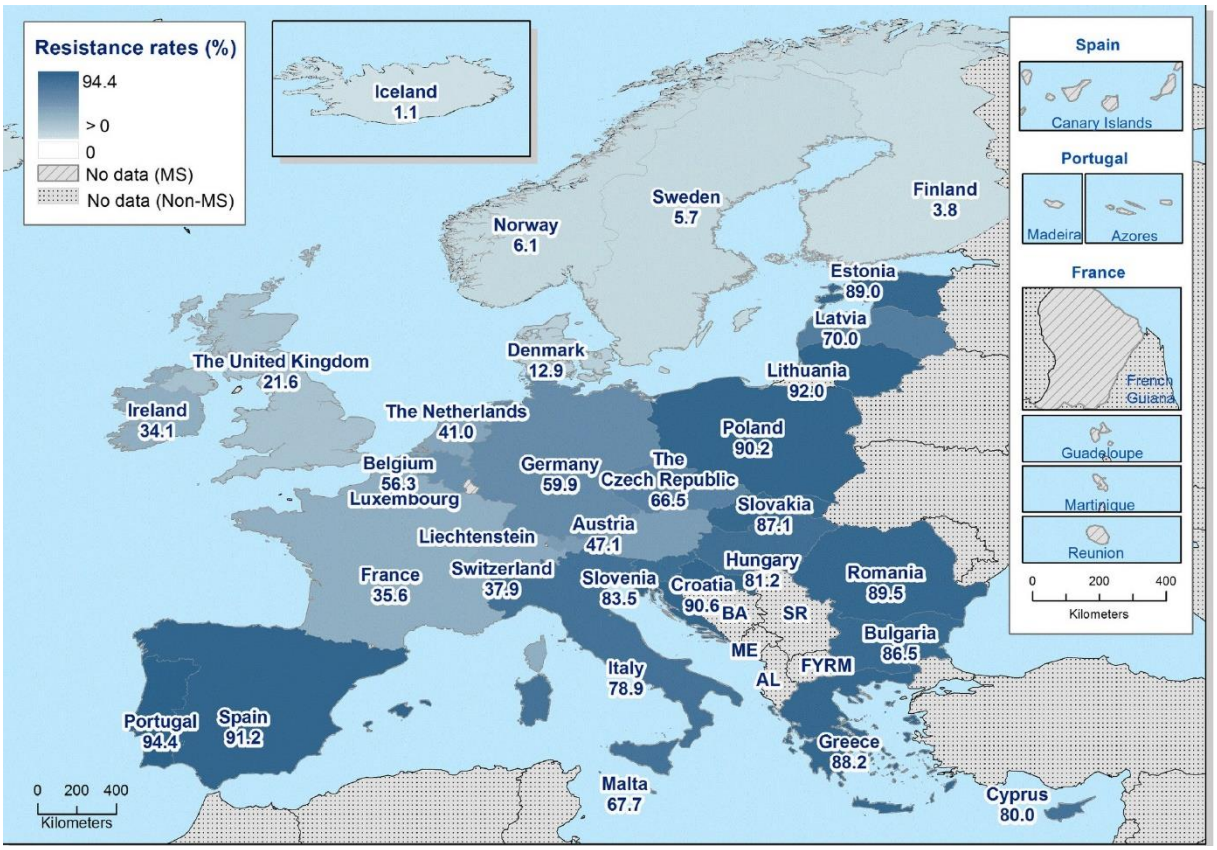

Figure 2: Percentage of indicator E. coli from broilers showing resistance (above ECOFF) to ciprofloxacin in 30 EU/EEA member states. Reproduced from (EFSA & ECDC, 2018).

1.3 Transfer of antimicrobial resistance

Transfer of AMR can occur by vertical and horizontal transfer. Vertical transfer of AMR is transfer of genes by direct inheritance from parent to daughter cells (Keen & Montforts, 2012; Melnyk et al., 2015) Vertical transmissible genes are a part of the host's genome. Resistance transferred by vertical gene transfer is often chromosomal mutations (Keen & Montforts, 2012). Horizontal gene transfer is transfer of genes by mechanisms including transformation, conjugation and transduction (Melnyk et al., 2015), as shown in figure 3. Transformation is the uptake of naked plasmid DNA. Transduction is transfer of DNA from injection by bacteriophages (Hayes, 2003a). Conjugation is an

energy-driven, contact-dependent transmission of plasmids from donor to recipient cells (Carattoli, 2011).

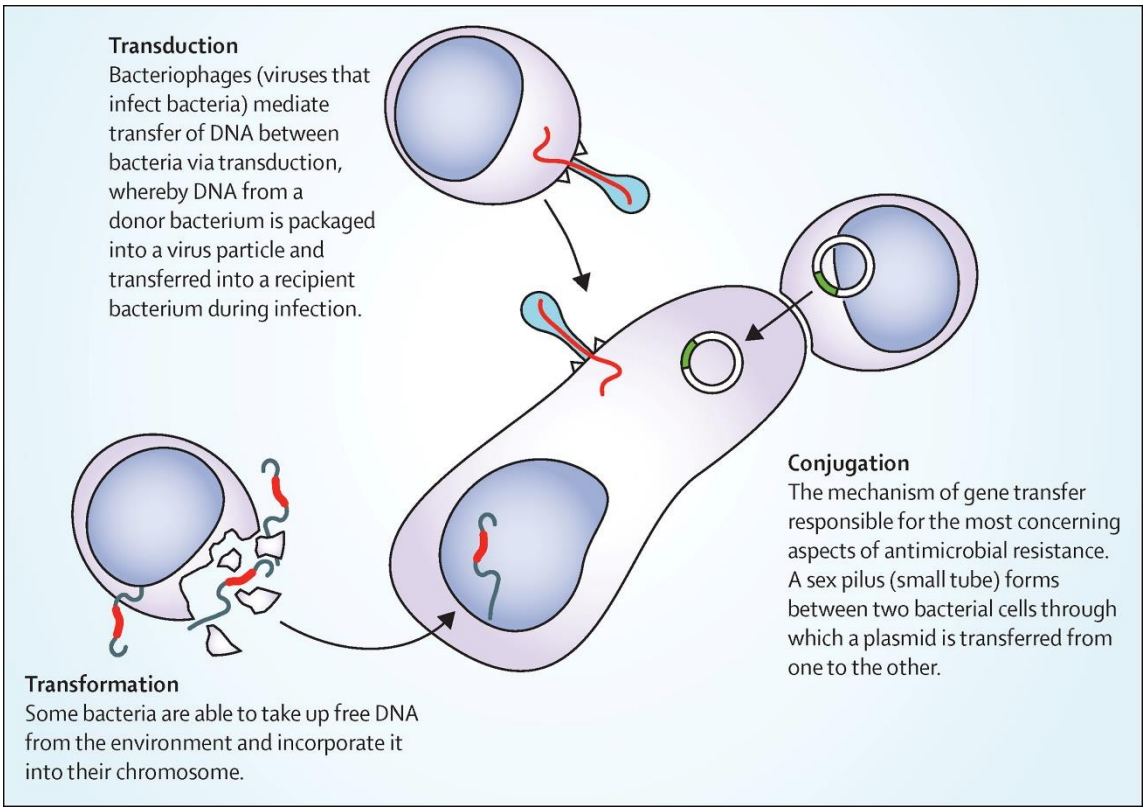

Figure 3: Horizontal gene transfer mechanisms; conjugation, transformation and transduction. Reproduced from Holmes et al. (2016).

1.3.1 Plasmids

Plasmids are by definition a double-stranded, circular or linear DNA molecule capable of autonomous replication (Carattoli, 2011). The topology of plasmids is determined by DNA gyrase and DNA topoisomerase and plasmids may have more than one topological form. Circular, covalently closed, supercoiled topological form is the most common. Plasmids can vary greatly in size, from less than two kilobases (kb) to many hundreds of kb. The smallest plasmids are just capable of replication, while the larger ones may constitute a significant part of the host genome. Large plasmids often consist of a backbone (small plasmid), and a varying number of added mobile genetic elements such as transposons, insertion sequences and bacteriophages. The contribution of plasmids to the host bacterium genome depends on the number of different plasmids in the bacterium, the size of each individual plasmid and their copy number (Hayes, 2003a). Mobile genetic elements such as insertion sequences and transposons with AMR genes can be acquired by plasmids (Carattoli, 2013).

Plasmids are extrachromosomal, self-replicating genetic elements (Carattoli, 2011) that can be horizontally transferred by conjugation between different species genera and kingdoms (Carattoli,

2011; Carattoli, 2013). Plasmids can harbour a wide variety of traits including AMR (Carattoli, 2013) as well as traits giving the host an advantage over other bacteria in their environment (Hayes, 2003a). The dissemination of a plasmid in an environment are determined by properties such as the ability to conjugate, conjugation efficiency (Carattoli, 2013) and the capacity to transfer to and replicate in a range of bacterial hosts. A plasmid with a broad host range has the capacity to transfer to and replicate in many bacterial hosts and can with relative ease spread to different niches (Carattoli, 2011). As a consequence, plasmid-encoded traits can be disseminated among bacteria with relative ease compared to chromosomal-encoded traits. Plasmids are important genetic elements in the gene pool since they could provide functions in addition to those encoded by the chromosome (Carattoli, 2011; Hayes, 2003a) and that could aid in survival by helping bacteria persist in a hostile environment or pathogenic properties beneficial to the bacteria (Hayes, 2003a).

Plasmids often encode traits that help the bacterium to endure in environments that otherwise may be lethal, or is limiting to growth (Hayes, 2003a). Such traits can be genes for resistance to antimircrobial agents (Carattoli, 2011), metal ions such as lead, mercuric and sink (Hayes, 2003a), virulence factors such as bacteriocins, siderophores, cytotoxins, or adhesion factors (Carattoli, 2011; Hayes, 2003a), virulence factors that aids in colonization of hosts and surviving hosts defence systems, metabolic functions enabling utilization of different nutrients and biodegradation of toxic substances such as toluene, organic hydrocarbons, herbicides and pesticides. Other survival mechanisms that could be conferred by plasmids are resistance against bacteriophages, restriction of foreign nucleic acids, and antirestriction systems – that are systems protecting the plasmids from degradation by host restriction systems (Hayes, 2003a). Plasmids conferring AMR are often found to contain multiple resistance genes on the same plasmid (Carattoli, 2013). All these traits grant the host bacteria advantages under a variety of selective pressures in a variety of environments.

Plasmids are categorized by their ability to replicate and propagate within the same host cell and this categorization is called Incompatibility (Inc) groups. Plasmid incompatibility is defined by Novick (1987) as “the failure of two plasmids present in the same cell to be stably inherited in the absence of external selection”. Plasmid incompatibility does not arise because of presence of an incompatibility gene, or Inc-gene, but as a consequence of normal activities of certain plasmid maintenance and replication functions (Novick, 1987). Plasmids in different Inc groups can replicate and propagate in the same cell and to their daughter cells because they do not share the same control systems. In contrast, plasmids in the same Inc group are incompatible in the sense that they share the same control systems and therefore cannot be replicated together (Carattoli, 2013; Novick, 1987). There are 27 Inc groups for *Enterobacteriaceae* (Carattoli, 2011; Shintani et al., 2015).

Replication of plasmids is carried out by rolling circle replication (figure 4) in many plasmids (Hayes, 2003a). Replication of plasmids are initiated by the hosts' replication systems and plasmids frequently uses the host replication system for its own replication, thereby minimizing the amount of genetic information needed on the plasmid for its own replication (Carattoli, 2013). In order to guarantee that the plasmid is present in the daughter cells, some plasmids have devised a strategy where they are copied in large quantities and are distributed evenly in the cell. These plasmids are said to replicate by high-copy numbers and they rely on random diffusion. Replication by low-copy numbers uses other strategies to guarantee presence in daughter cells during replication. Active partitioning systems ensure that each daughter cell gets at least one copy of the plasmid by positioning the plasmids appropriately within the cell. In contrast, low-copy number plasmids do not rely on random distribution in the cell and active partitioning systems are widely distributed among low-copy number plasmids. Control of plasmid replication is important because lack thereof would unnecessarily tax the metabolic capacity of the host bacteria and offer a disadvantage to the cell harbouring the plasmid in competition with a plasmid-free counterpart (Hayes, 2003a).

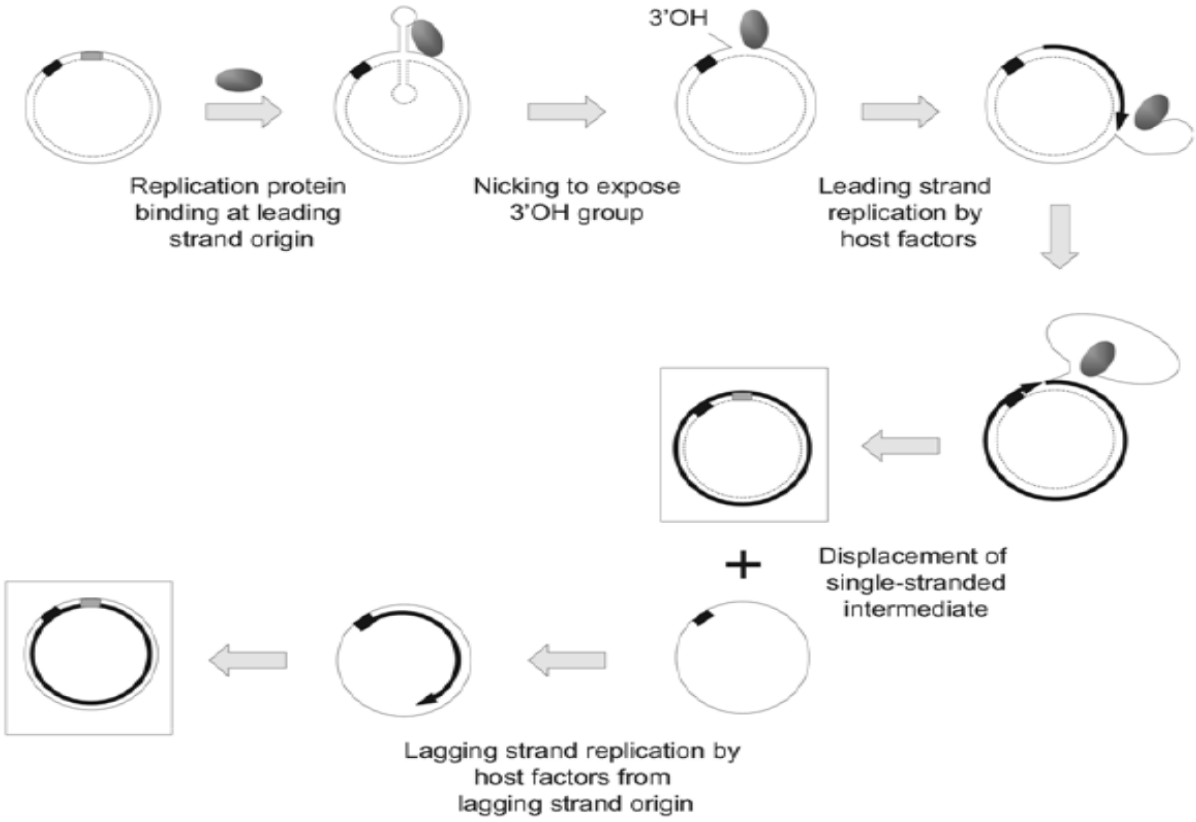

Figure 4: Rolling circle replication in plasmids. Reproduced from Hayes (2003a).

Some plasmids use other strategies to ensure persistence in a population over generations and one such system is toxin-antitoxin system (TA). An illustrative example of a TA system is shown in figure 5. Plasmids use TA systems that impair growth or kills bacteria unable to acquire plasmids after cell

division, and thus favour persistence of plasmids in bacteria. TA systems work by releasing toxins in plasmid-free cells after cell division/cell segregation that impair growth or kills the bacteria. In cells with plasmids, release of antitoxins neutralizes the toxin by acting on the toxin or inhibiting its translation. A variety of different TA systems are widely disseminated on bacterial plasmids, although the intracellular targets for the toxin components of these systems probably differ (Hayes, 2003b).

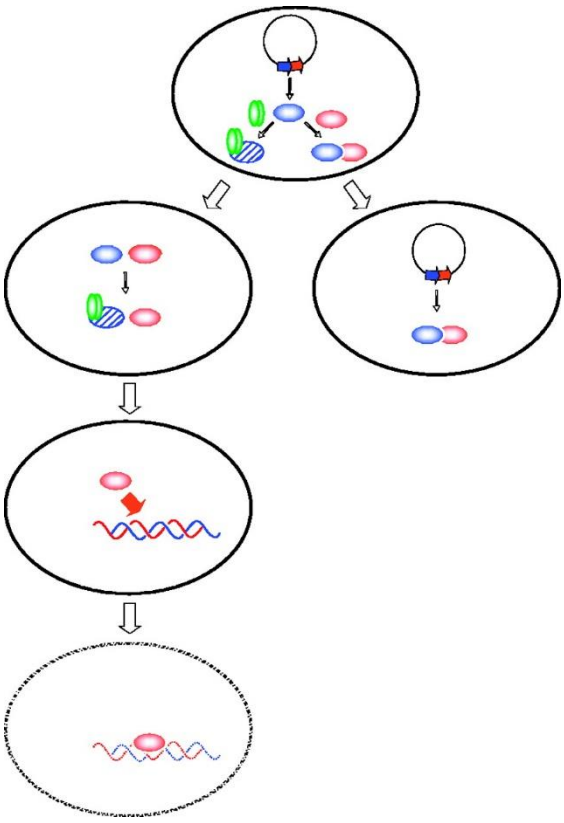

Figure 5: Type II Toxin-antitoxin mechanisms. When the plasmid is present in the daughter cells, the toxin (red) and antitoxin (blue) forms a tight complex that neutralizes the toxin. A protease degrades the antitoxin, preventing formation of the neutralizing complex when the plasmid is not present, and the toxin can act by impairing growth or killing the cell. Reproduced from Hayes (2003b).

1.4 Quinolones and fluoroquinolones

The WHO has defined fluoroquinolones as critically important for treatment of infections in humans (World Health Organization, 2017). Quinolones are a class of fully synthetic antimicrobial agents (Robicsek et al., 2006), developed in the 1960s and 1980s (Jacoby et al., 2015), with bactericidal effects on most *Enterobacteriaceae* (Robicsek et al., 2006). It is useful to distinguish between firstgeneration quinolones developed in the 1960s and second-generation quinolones named fluoroquinolones developed in the 1980s. Nalidixic acid belongs to the first-generation quinolones and was developed to treat urinary tract infections (Jacoby et al., 2015). Ciprofloxacin, norfloxacin, oxofloxacin, pefloxacin and enrofloxacin belongs to the second generation (fluoroquinolones) (Poirel et al., 2012) showing greater potency and broader spectrum (Jacoby et al., 2015; Poirel et al., 2012).

They are particularly effective against Gram-negative bacteria and several Gram-positive and intracellular bacteria. Fluoroquinolones are characterized by an additional fluorine atom at C-6 position (Poirel et al., 2012; Robicsek et al., 2006) and a piperazinyl or related ring at C-7 position on the quinolone molecule (Robicsek et al., 2006). The structure of quinolones and fluoroquinolones (quinolones and fluoroquinolones will hereby collectively be referred to as quinolones) are shown in figure 6. Introduction of quinolones led to an increase in the usage and, subsequently, resistance towards these substances. Soon after the introduction of nalidixic acid, resistance was observed (Jacoby et al., 2015). It seemed unlikely that quinolone resistance would develop because the antimircrobial agents were fully synthetic and thus evolution of quinolone resistance would not occur in nature (Robicsek et al., 2006). However, resistant isolates emerged after only several years of presumably inadequate use of quinolones (Rodriguez-Martinez et al., 2016). Resistance towards quinolones has become widespread among *Enterobacteriaceae* in the decades since they were first introduced (Strahilevitz et al., 2009).

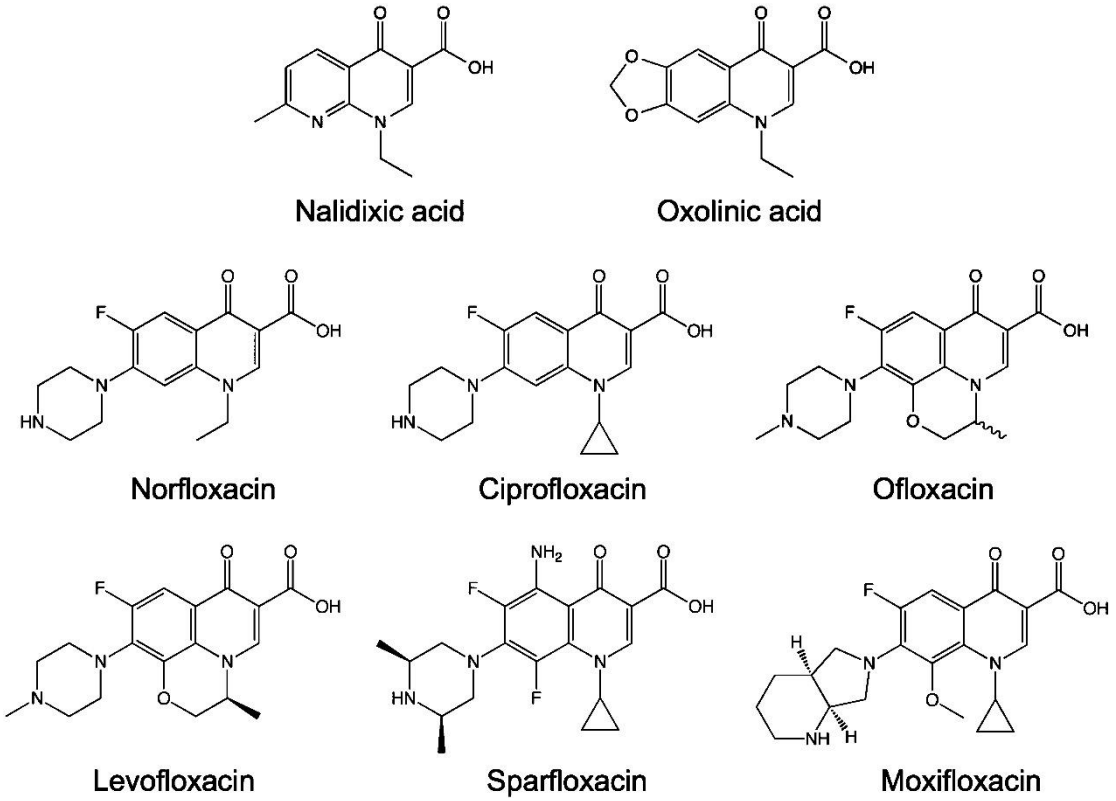

Figure 6: Structures of quinolone and fluoroquinolone. Quinolones; nalidixic acid and oxolinic acid. Fluoroquinolones; norfloxacin, ciprofloxacin, ofloxacin, levofloxacin, sparfloxacin and moxifloxacin. Levofloxacin, sparfloxacin and moxifloxacin are newer-generation fluoroquinolones. Reproduced from Aldred et al. (2014).

The target of quinolones and quinolones are the type II topoisomerases DNA gyrase and DNA topoisomerase IV. These enzymes transiently break and reseal DNA thereby affecting the topology of DNA. DNA topology is essential for DNA replication, transcription, recombination, and DNA repair (Poirel et al., 2012; Robicsek et al., 2006). Regulation of DNA topology by type II topoisomerases are

done during normal cellular growth (Strahilevitz et al., 2009) and are essential for bacterial growth (Poirel et al., 2012). Quinolones inhibit the activity of these enzymes leading to rapid inhibition of DNA synthesis thereby inhibiting growth (Poirel et al., 2012) and accumulation of unrepaired double-stranded DNA breaks which is lethal to the cell (Poirel et al., 2012; Robicsek et al., 2006). Accumulation of double-stranded breaks occurs because quinolones block the religation of cleaved double-stranded DNA by binding to the complex of DNA and topoisomerase (Jacoby et al., 2015).

1.4.1 Quinolone resistance by chromosomal mutations

Resistance towards quinolones may be caused by chromosomal mutations (figure 7). Chromosomal mutations are the most common resistance mechanism of quinolone resistance and these mutations occur in a region on the genes of DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*) termed the quinolone resistance determining region (QRDR) (NORM/NORM-VET, 2015; NORM/NORM-VET, 2016; NORM/NORM-VET, 2017). Mutations in *gyrA* and *parC* are most prevalent in *Enterobacteriaceae*. Mutations in *gyrA* protect DNA gyrase and *parC* protects DNA topoisomerase IV (Poirel et al., 2012) by altering the ability for quinolones to bind to the enzymes (Robicsek et al., 2006). The resistance mechanisms in which mutations occur in the QRDR are encoded on a chromosome and it is mainly inherited from one bacterium to its progeny as vertical transmission (Robicsek et al., 2006).

The most common mutations in *gyrA* in *E. coli* are substitutions in codon 83 and 87 (Poirel et al., 2012; Ruiz, 2003). In codon 83 in *gyrA* the substitution could be from aminoacid serine found in WT *E. coli* to either leucine, tryptophan, alanine or valine, and a substitution from serine to leucine is designated S83L or Ser83Leu mutation in *gyrA* (Ruiz, 2003). In codon 87 in *gyrA* substitution could be from aspartic acid found in WT *E. coli* to either asparagine, glycine, valine, tyrosine and histidine (Ruiz, 2003). Clinical quinolone-resistant *E. coli* usually have the Ser83Leu mutation in *gyrA* (Andersson & Hughes, 2012).

1.4.2 Plasmid-Mediated Quinolone Resistance

PMQR comprises a large group of genes conferring low-level resistance to fluoroquinolones and these are *qnr*-genes, *aac(6')-Ib-cr* gene and the *qepA* and *oqxAB* genes (figure 7). *Enterobacter, Klebsiella, Salmonella and E. coli* are species within genera *Enterobacteriaceae* found to carry PMQR genes (Jacoby et al., 2015). *qnr* encodes pentapeptide repeat proteins conferring reduced susceptibility to fluoroquinolones and nalidixic acid (Strahilevitz et al., 2009). *aac(6')-Ib-cr* encodes an aminoglycoside transferase that confers resistance to fluoroquinolones such as norfloxacin (Jacoby et al., 2015; Robicsek et al., 2006) and ciprofloxacin (Jacoby et al., 2015; Strahilevitz et al., 2009). *qepA* and *oqxAB* encode efflux pumps that protect the host by reducing accumulation of quinolones (Jacoby

et al., 2015). PMQR genes are usually found on plasmids carrying multiple resistance determinants and the type of resistance genes, plasmid size and plasmid incompatibility group vary considerably (Rodriguez-Martinez et al., 2016).

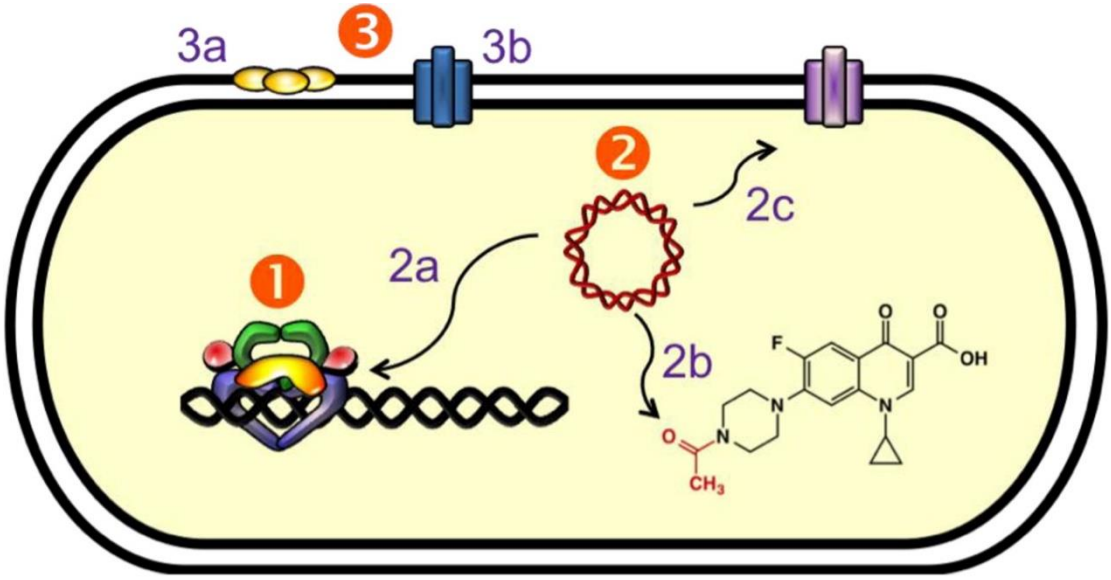

Figure 7: Mechanisms of quinolone resistance. (1) Chromosomal mutations in the genes encoding type II topoisomerases. (2) Plasmid-Mediated Quinolone Resistance - PMQR; (2a) qnr proteins acting on type II topoisomerases by weakening the topoisomerase-DNA interactions, (2b) aac(6')-lb-cr – aminoglycoside acetyltransferase acting on ciprofloxacin and norfloxacin fluoroquinolone molecules, (2c) qepA and oqxAB – plasmid-encoded efflux pump acting on quinolones and other antimicrobial agents. Reproduced from Aldred et al. (2014).

qnr-genes are a large group encoding proteins with comparable resistance activity (Robicsek et al., 2006). In order of prevalence, *qnrB* seems to be more common than *qnrS* and *qnrA*, followed by *qnrD* as the least common. *qnrB1*, *qnrB2*, *qnrB4*, *qnrB6*, *qnrB10* and *qnrB19* are the most frequently detected *qnrB* alleles (Jacoby et al., 2015). The proteins encoded by the *qnr*-genes belong to the pentapeptide repeat family (Strahilevitz et al., 2009). The group of *qnr*-genes are by far the largest in terms of various alleles. As of May 2018, there are 120 different alleles of *qnr*-genes admitted to the database http://www.lahey.org/qnrstudies. There are seven *qnrA* alleles, 94 *qnrB* alleles, one *qnrC* allele, nine *qnrS* alleles and seven *qnrVC* alleles (Clinic; Jacoby et al., 2008). Although the detailed mechanisms of the *qnr*-genes are still unknown, *qnrA* has been shown to bind to DNA gyrase subunits *gyrA* and *gyrB*, as well as the DNA gyrase holoenzyme, suggesting that the *qnr* proteins interact with these enzymes. Additive effects on susceptibility by harbouring more than one *qnr*-gene are unclear, but results of co-occurrence of *qnrB4* and *qnrS1* showed no observed elevated MIC on ciprofloxacin, suggesting that they competed for the same binding to DNA gyrase (Strahilevitz et al., 2009).

qnr-genes are frequently found on multidrug resistance plasmid, co-located with a wide variety of resistance genes conferring resistance to other classes of antimircrobial agents. Plasmids with *qnrA* and *qnrB* often confer resistance to β-lactams, aminoglycosides, chloramphenicol, tetracycline,

sulphonamide, trimethoprim or rifampin. Co-localization of AMR as noted for *qnrA* and *qnrB* is less frequent for plasmids harbouring *qnrS. qnrA* and *qnrB* which are often co-located with β-lactamase resistance genes. *qnrS* are often co-located with the β-lactamase resistance genes *LAP-1*, *TEM-1*, *LAP-2* and *SHV-12*. The association between resistance to extended-spectrum cephalosporins and quinolones have been noted by other researchers (Rodriguez-Martinez et al., 2016).

aac(6')-Ib-cr encodes an aminoglycoside acetyltransferase conferring low-level resistance to norfloxacin (Jacoby et al., 2015; Robicsek et al., 2006) and ciprofloxacin (Jacoby et al., 2015; Strahilevitz et al., 2009). The acetyltransferase encoded by *aac(6')-Ib-cr* acts by drug modification, acetylating norfloxacin and ciprofloxacin. *aac(6')-Ib-cr* are commonly found on multi-resistant plasmids with extended spectrum β-lactamase gene *CTX-M-15*, and also co-localized with other PMQR genes (Jacoby et al., 2015).

The genes *qepA* and *oqxAB* are efflux pumps with activity towards fluoroquinolones. Efflux pumps function by removing unwanted compounds from the cytoplasm and membranes. *qepA* acts on multiple antimicrobial agents such as aminoglycosides, broad-spectrum β-lactams and hydrophilic fluoroquinolones (Rodriguez-Martinez et al., 2016; Strahilevitz et al., 2009); predominantly ciprofloxacin and norfloxacin (Jacoby et al., 2015). *oqxAB* encodes efflux pumps that act on three classes of antimicrobial agents; chloramphenicol (Jacoby et al., 2015; Strahilevitz et al., 2009), trimethoprim, and quinolones such as ciprofloxacin, flumequine, norfloxacin and nalidixic acid (Jacoby et al., 2015).

1.4.3 Clinical relevance

PMQR genes can be clinically important in spite of conferring reduced susceptibility insufficient to survive clinical concentrations of quinolones because they can act by facilitating selection of higher levels of quinolone resistance (Jacoby et al., 2015). Selection for chromosomally-encoded resistance mechanisms to fluoroquinolones is favoured when PMQR genes are present and have been reported to occur since 1988 (Carattoli, 2013). Mutations in the chromosomally located gene *gyrA*, have evolved in isolates harbouring *qnr*-genes in patients treated with quinolones for *E. coli* or *Salmonella enterica* infections (Jacoby et al., 2015). Presence of both PMQR genes and chromosomally-mediated mechanisms to fluoroquinolones have shown to give a higher level of resistance in clinical isolates of *Enterobacteriaceae*. In the absence of chromosomally-mediated quinolone resistance mechanisms, the acquisition of a PMQR gene leaves MIC values for quinolones still in the susceptible category, according to clinical breakpoints from both Clinical & Laboratory Standards Institute (CLSI) and EUCAST (Rodriguez-Martinez et al., 2016).

1.5 Aim of Study

The main aim of this study was to characterize five *E. coli* strains isolated from poultry encoding plasmid-mediated quinolone resistance genes. The main aim was divided into two secondary objectives:

Secondary objective 1:

Characterize and compare *E. coli* strains with regards to AMR and possible clonal relationship. Characterization and comparison based on phenotypic methods such as susceptibility testing (MIC), conjugation, and transfer frequency, molecular genotyping and identification of resistance mechanisms based on Whole Genome Sequencing (WGS) data.

Secondary objective 2:

Characterize and circularize one plasmid carrying a PMQR gene based on WGS data and closing gaps using PCR and Sanger sequencing. Annotate the plasmid and compare to other closely related plasmids.

2. Materials and methods

2.1 Materials

In this study five *E. coli* from poultry with PMQR genes *qnrS1* and *qnrB19* were examined to determine their plasmids conjugational properties and transfer frequency, their strain susceptibility and an in-depth characterization of one of the plasmids.

The five strains were derived from a project examining samples from poultry ofmeat and caecal material, for the occurrence of *E. coli* resistant to quinolones, using a selective method. All samples were mixed 1:9 in Buffered Peptone Water (Oxoid Ltd, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated overnight at 37°C before plated onto MacConkey (Beckton, Dickinson and Company, Le Pont de Claix, France) agar containing 0.12 mg/L ciprofloxacin (Fluka, Sigma-Aldrich, St. Louis, MO, USA). A selection of *E. coli* isolates from; caecal (n=47) and meat (n=53) samples were sequenced by WGS using Nextera XT on HiSeq 2500 w. Rapid run. Identification of antimicrobial resistance genes was done using the ARIBA program with the CARD database (Slettemeås et al., 2017). WGS revealed five *E. coli* strains that harboured PMQR genes. The occurrence of the *qnrS1* gene was identified in four of the strains and a *qnrB19* gene was identified in the last strain. The strains were labelled 2014-01-5749 (*qnrS1*), 2014-01-5792 (*qnrB19*), 2014-01-6924 (*qnrS1*), 2014-0-7234 (*qnrS1*) and 2014-01-7375 (*qnrS1*).

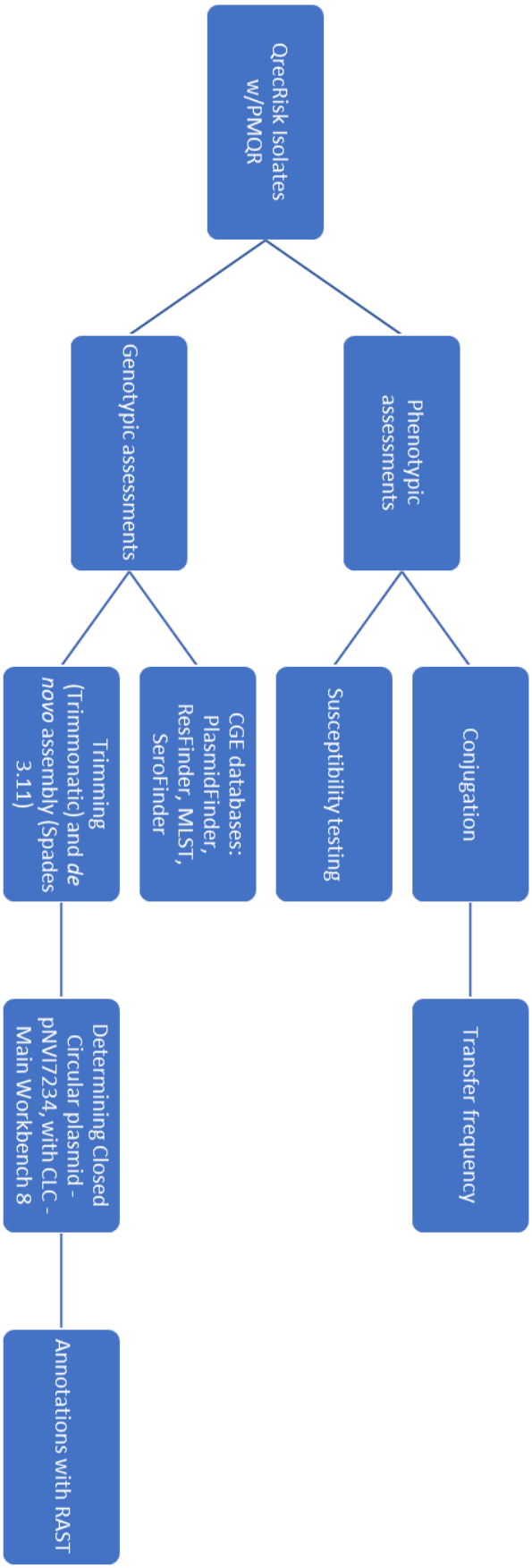

Figure 8: Process chart of the experimental work on the master thesis; phenotypic and genotypic experiments.

2.1.1 DNA extraction

DNA extraction was done by suspending of a loopful of bacteria in 100 µl MilliQ water (MQ). The suspension was boiled at 100°C for 10 minutes on a heat block (Thermo Scientific, Thermo Fischer Scientific Inc., Waltham, MA, USA) and centrifuged at 10 000 rpm for 5 minutes in an Eppendorf Centrifuge 5415D/5424 (Eppendorf AG, Hamburg, Germany). The supernatant was used as a DNA template and stored at -20°C for later use.

2.1.2 Verification of PMQR genes by PCR

Verification of the *qnrS gene* was done by PCR. Template DNA was extracted, as described in 2.1.1, from all donors containing conjugational plasmids; 2014-01-5749, 2014-01-6924, 2014-01-7234, 2014-01-7375, its respective transconjugants and the recipient *E. coli* OneShot™ cells with pCR™ II vector encoding kanamycin resistance (Invitrogen™, LifeTechnologies, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Table 1: Primer pairs used in the PCR to verify the presence of qnrS1 *in the donor strains and transconjugants after conjugation and transfer frequency experiments.*

Target category	Target gene	Primer sequence	Amplicon size (bp)	Annealing temp (°C)	Reference
PMQR gene	*qnrS.F*	5'-CGACGTGCTAACTTGCGTGATA-3'	537	57	(Cavaco et al., 2008)
	qnrS.R	5'-TACCCAGTGCTTCGAGAATCAG-3'			

The reaction volume of 25 µl contained 18.15 µl MQ water, 2.5µl 10x PCR buffer (Qiagen, Hilden, Germany), 0.25 µl 10 mM dNTP mix (Qiagen, Hilden, Germany), 1.5 µl 25mM $MgCl_2$ (Qiagen, Hilden, Germany), 0.25µl 10 µM primer F, 0.25 µl 10µM primer R, 0.1 µl 5U/µl Taq polymerase (Qiagen, Hilden, Germany) and 2 µl template DNA. MilliQ water was used as negative control and *Salmonella enterica* subsp*. enterica* Saintpaul *qnrS1+* (EU-Reference Laboratory - Antimicrobial Resistance, Technical University of Denmark, Denmark) was used as positive control.

The PCR program was carried out on a T100™ Thermal Cycler (BIO RAD Laboratories Inc, Hercules, CA) and contained denaturation for five minutes at 95°C, followed by 30 cycles of denaturation for one minute at 95°C, primer hybridization for one minute at 57°C and elongation for one minute at 72°C. The 30 cycles were followed by a final elongation for one minute at 72°C and infinite time at 8°C.

Gel electrophoresis was carried out to visualize the presence of a *qnrS* gene. The gel electrophoresis was carried out with an O'Generuler 50 bp ladder (Thermo Fischer Scientific Inc.,

Waltham, MA, USA) and 10 µl PCR product was added to each well. PCR product contained 10 µl PCR product and 2 µl 6X Gel Loading Dye. The gel contained 1% agarose and Tris Borat EDTA (TBE) buffer and 10 µl GelRed. The gel electrophoresis ran for 45 minutes at 90 volts.

2.1.3 Verification of species by MALDI-TOF

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF, Bruker Daltonics) was used to verify the presence of *E. coli*.

A toothpick of one representative colony was transferred to a tray compatible with MALDI-TOF. 1 µl of formic acid and 1 µl matrix were added to the sample material on the plate before the tray was loaded into the MALDI-TOF.

2.2 Susceptibility testing – MIC

Susceptibility testing was performed by broth microdilution following recommendations by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) on five *E. coli* strains from poultry, four *E. coli* transconjugants and *E. coli* OneShot™ recipient to a standard panel of antimicrobials Sensititre™ TREK EUVSEC panel (Thermo Fischer Scientific Inc., Waltham, MA, USA). *E. coli* ATCC 25922 was used as a quality control.

Colony material was diluted in Sterile Distilled Water (SDW) to McFarland 0.5. Two large loopfuls of the dilution (approx. 20 µl) were transferred to 11 ml Cation Assisted Mueller-Hinton Broth HT Broth with TES buffer (CAMHBT) (Oxoid Ltd, Thermo Fisher Scientific, Waltham, Massachusetts, USA). 50 µl CAMHBT were inoculated on Sensititre™ YEVUSEC plates and incubated for 18-24 hours at 35°C. The purity of the CAMHBT dilution was assessed by inoculation on blood agar and incubating overnight at 37°C. MIC was determined as the concentration of the antimicrobial substance where there is no presence of visual growth of bacteria on the Sensititre™ EVUSEC plates.

The Sensititre™ EVUSEC plates were read using a mirror adapted for the reading of microtiter plates.

2.3 Whole Genome Sequencing and databases

Sequences of the *E. coli* strains with *qnrS1* (four) and *qnrB19* (one) were trimmed using Trimmomatics (Bolger et al., 2014) and *de novo* assembled using Spades 3.11 (Bankevich et al., 2012). The processed (trimmed and *de novo* assembled*)* sequence data were analyzed *in silico* with regard to multi-locus sequence type (MLST), serotype, acquired resistance genes, chromosomal point mutations and plasmid replicon types by the use of MLST 1.8, SeroypeFinder 1.1, ResFinder 3.0 and PlasmidFinder 1.3 available online at www.genomicepidemiology.org from Center for Genomic Epidemiology (CGE), DTU, Denmark (Carattoli et al., 2014; Joensen et al., 2015; Larsen et al., 2012; Zankari et al., 2012).

2.4 Conjugation

Transferability of plasmids containing *qnrS1* and *qnrB19* were done by conjugational experiments. Strains with reduced susceptibility to quinolones were subjected to conjugation with *E. coli* OneShot™ cells with pCR II vector encoding kanamycin resistance (Invitrogen™, LifeTechnologies, Thermo Fischer Scientific Inc).

Conjugation experiments were carried out essentially by the same procedure but differed in the medium used during the mating phase of the experiment. The mating phase of the experiments was carried out in Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) and on Trypton soy agar (Oxoid Ltd, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for four hours and 24 hours at 37°C. Prior to initiating matings, overnight cultures of all donors and *E. coli* OneShot (Invitrogen™, LifeTechnologies, Thermo Fischer Scientific Inc.) were made in LB broth for the conjugation in LB broth and on blood agar (Oxoid Ltd, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for conjugation in agar. Mating in LB broth was initiated by inoculating 500 µl *E. coli* OneShot™ (Invitrogen™, LifeTechnologies, Thermo Fischer Scientific Inc.) recipient cells with a loopful of donor cells from one donor. Mating on Trypton soy agar was initiated by mixing a large loopful (approx. 10 µl) *E. coli* OneShot™ with a small loopful (approx. 1 µl) donor cells from one donor. Matings were spread onto Mueller-Hinton agar (Difco, Beckton Dickinson and Company, Sparks, MD, USA) with 0.06 mg/l ciprofloxacin (Fluka, Sigma-Aldrich, St. Louis, MO, USA) and 50 mg/l kanamycin (Sigma-Aldrich, St. Louis, MO, USA) after four and 24 hours of mating, and incubated at 37°C overnight. Mueller-Hinton agar plates with growth of presumptive transconjugants were transferred onto blood agar plates and bromthymol blue agar plates and incubated overnight. Phenotypic assessments (colony size, shape and lactose fermentation) were assessed on blood agar and bromthymol blue agar. *E. coli* was verified using MALDI-TOF as described in 2.1.3. Verification of PMQR genes was carried out by PCR as described in 2.1.2.

2.5 Transfer frequency

Transfer frequency experiment was carried out by initiating mating by addition of 500 µl *E. coli* OneShot™ (Invitrogen™, LifeTechnologies, Thermo Fischer Scientific Inc.) recipient cells with a loopful of one donor in a 4 ml LB broth (Merck) and incubated for four hours at 37 °C. Overnight cultures in LB broth were made with donor cells able to transfer quinolone resistance by conjugation and *E. coli* OneShot (Invitrogen™, LifeTechnologies, Thermo Fischer Scientific Inc.) recipient cells prior to initiating matings. The matings were diluted ten-fold until a dilution of 10^{-6}. 100 µl of each of three dilutions (10^{0}, 10^{-1} and 10^{2}) were plated on Mueller-Hinton agar (Difco, Beckton Dickinson Company, Sparks, MD) with 0.06 mg/l ciprofloxacin (Fluka, Sigma-Aldrich, St. Louis, MO, USA) and 50 mg/l

kanamycin (Sigma-Aldrich, St. Louis, MO, USA). 100 µl of each of three dilutions (10^{-4}, 10^{-5} and 10^{-6}) were added to Mueller-Hinton agar with 0.06 mg/l ciprofloxacin and Mueller-Hinton agar with 50 mg/l kanamycin. The Mueller-Hinton agar plates were incubated overnight at 37°C. A representative colony from each MH agar; MH with ciprofloxacin, MH with kanamycin and MH with ciprofloxacin and kanamycin were transferred to blood agar and blue agar for phenotypic assessment of colony size, shape and lactose fermentation. *E. coli* was verified using MALDI-TOF as described in 2.1.3. Verification of PMQR genes was carried out by PCR as described in 2.1.2.

Transfer frequency was calculated by dividing number of transconjugants per ml on number of recipients per ml (Kruse & Sorum, 1994; Phornphisutthimas et al., 2007).

2.6 Characterization of a *qnrS*-bearing plasmid

E. coli strain 2014-01-7234 was used for further plasmid analysis using CLC Main Workbench 8 (CLC BIO, Qiagen, Aarhus, Denmark). FASTA sequences from WGS of *E. coli* 2014-01-7234 was uploaded into CLC Main Workbench. Contigs containing *qnrS1* (6834 bp) and $bla_{\text{TEM-1B}}$ (38 802 bp) were aligned with an assembled plasmid (47 674 bp) in a *Shigella flexneri 1a* strain (accession no: CP020088) (Benson et al., 2005). The alignment in the assembly revealed two regions without alignment to the assembled plasmid (CP020088). Primer pairs were designed to investigate if the contigs could be circularized. Three sets of primers were designed in CLC Main Workbench. Substantial overlap in each set of primers indicated that the sequence could be made into a closed circular plasmid.

2.6.1 Primer design and Sanger sequencing

Primers were designed in CLC Main Workbench, ordered from Invitrogen (Thermo Fisher Scientific), amplified using gradient PCR on T100™ Thermal Cycler (BIO RAD Laboratories Inc, Hercules, CA, USA) and sent to Sanger sequencing at the Norwegian Veterinary Institute, Section for Molecular Biology. Primers were designed to investigate two gaps designated gap 1 and gap 2.

Table 2: Overview of primers designed for closing gaps in the qnrS plasmid. Amplicon size was predicted using Primer Blast (Ye et al., 2012). Actual amplicon size was the length of the PCR products sequenced using Sanger sequencing.

Target site	Target sequence	Primer sequence	Predicted Amplicon size (bp)	Lengthof product from Sanger sequencing)
Sequence between contigs	gap 1 (PMQR_7375(=7234)_ende)	CGAAGCCCGGAAAGCAATGGCA	1581[1]	1116
		CGACAGACCCGCAGGAAGCAAT		1098
	gap 2, pair 1 (PMQR_7375(=7234)_midt)	GCACCGCTTCCTGCCCTTTA	2224[1]	1139
		CGGTTGTGGCGTTGGTGAGT		1115
	gap 2, pair 3 (PMQR_7375(=7234)_midt_V3)	TGCACTGTACTGGAGGTTAT	Approx. 1700	1104
		CGGTTGTGGCGTTGGTGAGT		1115

[1] Predicted amplicon size using accession nr: CP020088 in PrimerBlast (Ye et al., 2012). CP020088 revealed close homology with pNVI7234.

The reaction volume was 25 µl with 18.9µl MQ water, 2,5µl 10x buffer (Qiagen), 0.5 µl 10 mM dNTP mix (Qiagen), 1µl 0.4µM primer F, 1 µl 0.4µM primer R, 0.1 µl 5U/µ Taq polymerase (Qiagen) and 1µl template DNA. The PCRs were run with two DNA templates; DNA extracted and purified using either QiaSymphony DSP DNA Mini Kit (Qiagen) or QIAamp DNA Mini Kit (Qiagen) of *E. coli* 2014-01-7234 and DNA extracted from *E. coli* OneShot transconjugants mated with donor *E. coli* 2014-01-7234 using the extraction method described in 2.1.1. PCR products were amplified using primers in table 2 on template DNA purified using QiaSymphony.

Gradient PCR program was carried out on T100™ Thermal Cycler (BIO RAD Laboratories Inc) contained five minutes at 95°C, followed by 30 seconds at 95°C, 30 seconds at temperature gradient 50 – 62°C and 10 minutes at 72°C. The program ran 25 cycles. Temperatures in the gradient PCR were 50°C, 50.8°C, 52.3°C, 54.6°C 57.3°C, 59.6°C, 61.1°C and 62°C.

The three primer pairs (table 2) were subjected to gradient PCR and the PCR products annealed at 50 °C (gap 1), 50°C (gap 2, pair 1) and 54.6°C were sent to Sanger sequencing.

Gel electrophoresis was carried out to assess the size of the PCR products and detect unspecific binding. Unspecific binding was indicated by occurrence of several visual bands of amplified DNA in the same well. The gel electrophoresis was carried out with a 1% agarose gel with GelRed, an O'Generuler 1 kb ladder (Thermo Scientific, Thermo Fischer Scientific Inc., Waltham, MA, USA) and 10 µl DNA sample (12 µl; 10 µl amplified DNA and 2µl 6X Gel loading dye) were added to each well. The gel ran 90 minutes at 90 volts.

2.6.2 Annotations and comparison of plasmids

Gene annotation was done by Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008; Brettin et al., 2015; Overbeek et al., 2014). The plasmid was designated pNVI7234.

Comparison of plasmids with close homology to pNVI7234 was carried out using BLAST ring image generator (BRIG) (Alikhan et al., 2011).

3. Results

Five QREC strains; 2014-01-5792 (*qnrS1*), 2014-01-5792 (*qnrB19*), 2014-01-6924 (*qnrS1*), 2014-01-7234 (*qnrS1)* and 2014-01-7375 (*qnrS1)* were tested for conjugational properties of the plasmids with their respective PMQR genes (*qnrS* & *qnrB19*), transfer frequency of the PMQR genes and susceptibility testing against a number of antimircrobial agents including ciprofloxacin and nalidixic acid as well as genotypic characterization based on WGS data. QREC 2014-01-7234 was used for analysis in order to characterize the *qnr*-bearing plasmid.

3.1 Susceptibility (MIC) of QREC strains

Table 3 shows the sensitivity of donor strains to a predetermined set of antimircrobial agents. The ECOFF values are given for *E. coli*. Of the selected antimircrobial agents in the sensitivity test, ciprofloxacin and nalidixic are of most interest and are therefore highlighted in table 3.

Table 3: overview of susceptibility of the E. coli strains to a selection of antimircrobial agents. Numbers highlighted in bold indicate MIC values above the epidemiological cut-off values.

	Sample (mg/l)					
	ECOFF[1]	2014-01-5749	2014-01-6924	2014-01-7234	2014-01-7375	2014-01-5792
Antibiotics		*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Sulfamethoxazole	64	**>1024**	**>1024**	<= 8	<= 8	<= 8
Trimethoprim	2	**>32**	**>32**	<= 0.25	<= 0.25	<= 0.25
Ciprofloxacin	0.064	**2**	**2**	**2**	**2**	**0.5**
Tetracycline	8	<= 2	<= 2	<= 2	<= 2	<= 2
Meropenem	0.125	<= 0.03	<= 0.03	<= 0.03	<= 0.03	<= 0.03
Azithromycin	ND*	4	4	4	4	8
Nalidixic acid	16	**128**	**>128**	**64**	**64**	**128**
Ceftotaxime	0.25	<= 0.25	<= 0.25	<= 0.25	<= 0.25	<= 0.25
Chloramphenicol	16	<= 8	<= 8	<= 8	<= 8	<= 8
Tigecycline	ND	<= 0.25	<= 0.25	<= 0.25	<= 0.25	<= 0.25
Ceftadizime	0.5	<= 0.5	<= 0.5	<= 0.5	<= 0.5	<= 0.5
Colistin	2	<= 1	<= 1	<= 1	<= 1	<= 1
Ampicillin	8	**>64**	**>64**	**>64**	**>64**	**>64**
Gentamicin	2	<= 0.5	<= 0.5	<= 0.5	<= 0.5	<= 0.5

[1] ECOFF = Epidemiological cut-off values defined by EUCAST. *ND = Not defined by EUCAST.

All *E. coli* strains harbouring *qnrS1;* 2014-01-5749, 2014-01-6924, 2014-01-7234 and 2014-01-7375 yielded a fourfold increase in MIC to ciprofloxacin compared to 2014-01-5792, which harboured *qnrB19*. The MIC values for the strains with *qnrS1* were 2 mg/l to ciprofloxacin, whereas the *qnrB19* carried by 2014-01-5792 had a MIC of 0.5 mg/l to ciprofloxacin. All strains showed sensitivity below the ECOFF for ciprofloxacin (0.064 mg/l).

All strains were above the ECOFF for nalidixic acid (16 mg/l). The MIC to nalidixic acid varied between strains, from 64 mg/l in 2014-01-7234 and 2014-01-7375 to >128 mg/l in 2014-01-6924.

All *E. coli*s were susceptible to tetracycline, meropenem, cefotaxime, chloramphenicol, tigecycline, ceftazidime, colistin and gentamicin and resistant to ampicillin. Two strains; 2014-01-5749 and 2014-01-6924 were resistant to sulfamethoxazole (sulphonamide) and trimethoprim.

3.2 WGS data

Sequence data obtained from WGS of the *E. coli* strains were run through the following web tools from CGE prior to characterization of the plasmids: MLST 1.8, SeroypeFinder 1.1, ResFinder 3.0 and PlasmidFinder 1.3. Sequence type (ST), serotype, resistance genes (acquired and chromosomal point mutations) and plasmid incompatibility groups were predicted and are shown in table 4.

Table 4: Overview of predicted sequence type (ST), serotype, plasmids, acquired resistance genes and chromosomal point mutations in *E. coli* strains.

Strains	Sequence type (ST)	Serotype	Plasmids	Acquired resistance genes	Chromosomal point mutations
2014-01-5749	ST-453	O23:H16	IncX1, IncFII, IncFIB(AP001918)	*aph(6)-Id, aph(3'')-Ib,* $bla_{\text{TEM-1B}}$*, qnrS1, sul2, tet(A), dfrA14*	gyrA S83L
2014-01-6924	ST-453	O23:H16	IncX1,IncFII, IncFIB(AP001918)	*aph(6)-Id, aph(3'')-Ib,* $bla_{\text{TEM-1B}}$*, qnrS1, sul2, tet(A), dfrA14*	gyrA S83L
2014-01-7234	ST-453	O23:H16	IncX1,IncFII, IncFIB(AP001918)	$bla_{\text{TEM-1B}}$*, qnrS1*	gyrA S83L
2014-01-7375	ST-453	O23:H16	IncX1, IncFII, IncFIB(AP001918), IncI1	$bla_{\text{TEM-1B}}$*, qnrS1*	gyrA S83L
2014-01-5792	ST-349	ONT[1]:H15	IncFII, IncFIB(AP001918), IncI1,Incl2, Col(KPHS6), Col156	$bla_{\text{TEM-1B}}$*, qnrB19*	gyrA S83L

[1]O-antigen non-typable.

All strains, except 2014-01-5792, were identified as serotype O23:H16 and sequence type ST-453. For strain 2014-01-5792 the O-group was not possible to identify, but H-type was identified as H15 and sequence type was ST-349.

IncFII and IncFIB(AP001918) plasmids were present in all strains while four out of five strains also harboured an IncX1 plasmid. 2014-01-5792 harboured plasmids that were not present in the other strains; Incl2, Col(KPHS6) and Col156.

qnrS1 gene was present in all strains except 2014-01-5792. The β-lactam resistance gene $bla_{\text{TEM-1B}}$ was present in all strains. The two strains 2014-01-5749 and 2014-01-6924, and the strains 2014-01-7245 and 2014-01-7375 share similar characteristics in presence of acquired AMR genes,

where the first pair also has additional resistance genes that confer resistance towards a wider variety of antimircrobial agents compared to the last pair.

The S83L point mutation in the *gyrA* gene conferring reduced tolerance towards quinolones was found in all *E. coli* strains.

3.3 Conjugation

The initial conjugation experiment was performed to examine if strains harbouring plasmids encoding PMQR genes were transferable. All strains were known to harbour one PMQR gene namely *qnrS* and *qnrB*, but not whether the genes were located on transferable plasmids. Table 5 shows which donor strains were able to conjugate after 4 and 24 hours of mating, the efficiency of transfer and verification of *qnrS* in transconjugants.

Table 5: Overview of conjugation experiments on agar and broth, transfer efficiency of conjugation and presence of qnrS.

Mating pair (donor - recipient)	Mating time (h)	Conjugation 37° C		Transfer Efficiency Transconjugants/ recipients		Presence of *qnrS*
		Agar	Broth			
2014-01-5749 → *E.coli OneShot*	4	+	+	3.96E-05	0.000040	+
	24	+	+			+
2014-01-5792 → *E.coli* OneShot	4	-	-	ND	ND	ND
	24	-	-			ND
2014-01-6924 → *E.coli* OneShot	4	+	+	1.51E-04	0.000151	+
	24	+	+			+
2014-01-7234 → *E.coli* OneShot	4	+	+	7.39E-05	0.000074	+
	24	+	+			+
2014-01-7375 → *E.coli* OneShot	4	+	+	1.53E-05	0.000015	+
	24	+	+			+

ND = Not done.

All strains except 2014-01-5792 were able to transfer the gene for quinolone resistance by conjugation (table 5). Transfer of plasmids harbouring the quinolone resistance gene was successful after 4 and 24 hours in both liquid and solid medium. Presence of *qnrS* was verified in all transconjugants.

2014-01-6924 was tenfold (1.53E-05) more efficient in conjugating its plasmid conferring resistance to quinolones compared to the least efficient strain 2014-01-7375 (1.53E-05). 2014-01-7234

was the second most efficient (7.39E-05). The transfer efficiency experiment was only conducted once and the results needs to be confirmed by repeating the experiment.

3.4 Susceptibility testing (MIC) of transconjugants

All strains containing plasmids with a transferable PMQR gene were successfully mated with *E. coli* OneShot. The recipient and the transconjugants were subjected to susceptibility testing against the same selection of antimicrobial agents as the donors. Table 6 shows the MIC values of the recipient and the transconjugants. Ciprofloxacin (a fluoroquinolone) and nalidixic acid (a quinolone) are highlighted because they are the target antimicrobial agents of PMQR.

Table 6: Overview of the results of the susceptibility testing of the recipient and the tranconjugants to a selection of antimicrobial agents.

	MIC (mg/l)					
	ECOFF	2014-01-5749	2014-01-6924	2014-01-7234	2014-01-7375	*E. coli* OneShot
Antibiotics		TC	TC	TC	TC	Recipient
Sulfamethoxazole	64	<= 8	<= 8	<= 8	<= 8	<= 8
Trimethoprim	2	<= 0.25	<= 0.25	<= 0.25	<= 0.25	<= 0.25
Ciprofloxacin	0.064	**0.5**	**0.5**	**0.5**	**0.5**	<= 0.015
Tetracycline	8	4	4	4	8	4
Meropenem	0.125	<= 0.03	<= 0.03	<= 0.03	<= 0.03	<= 0.03
Azithromycin	ND	8	8	8	8	8
Nalidixic acid	16	8	8	8	8	<= 4
Ceftotaxime	0.25	<= 0.25	<= 0.25	<= 0.25	<= 0.25	<= 0.25
Chloramphenicol	16	<= 8	<= 8	<= 8	<= 8	<= 8
Tigecycline	ND	<= 0.25	<= 0.25	<= 0.25	<= 0.25	<= 0.25
Ceftadizime	0.5	1	2*	2*	1	1
Colistin	2	<= 1	<= 1	<= 1	<= 1	<= 1
Ampicillin	8	**>64**	**>64**	**>64**	**>64**	**>64**
Gentamicin	2	<= 0.5	<= 0.5	<= 0.5	<= 0.5	<= 0.5

[1] ECOFF = Epidemiological cut-off values defined by EUCAST. ND = Not defined by EUCAST. *MIC values differed between transconjugants on broth and agar.

All transconjugants; 2014-01-5749, 2014-01-6924, 2014-01-7234 and 2014-01-7375 yielded an increase in the MIC values towards ciprofloxacin and nalidixic acid. The recipient cell (*E. coli* OneShot)

was susceptible to both ciprofloxacin and nalidixic acid, meaning that the increase in MIC could be attributable to the PMQR gene on the conjugational plasmid.

The MIC value of 0.5 mg/l for ciprofloxacin in all transconjugants were above the ECOFF (0.064 mg/l). The MIC value of 8 mg/l for nalidixic acid were below the ECOFF value (16 mg/l).

E. coli OneShot and the donor strains were resistant to ampicillin (>64 mg/l).

3.5 Characterization of an *qnrS1*-bearing plasmid

An IncX1 plasmid, 47 686 bp in size, carrying the resistance genes *qnrS1* and bla_{TEM-1B} was characterized and designated pNVI7234. Figure 9 shows pNVI7234 with annotations using RAST and Figure 11 shows pNVI7234 in comparison to two plasmids showing close homology using BLAST from *Shigella flexneri* 4c (accession number: KJ201886) and *Shigella flexneri* 1a (accession number: CP020088), using BRIG.

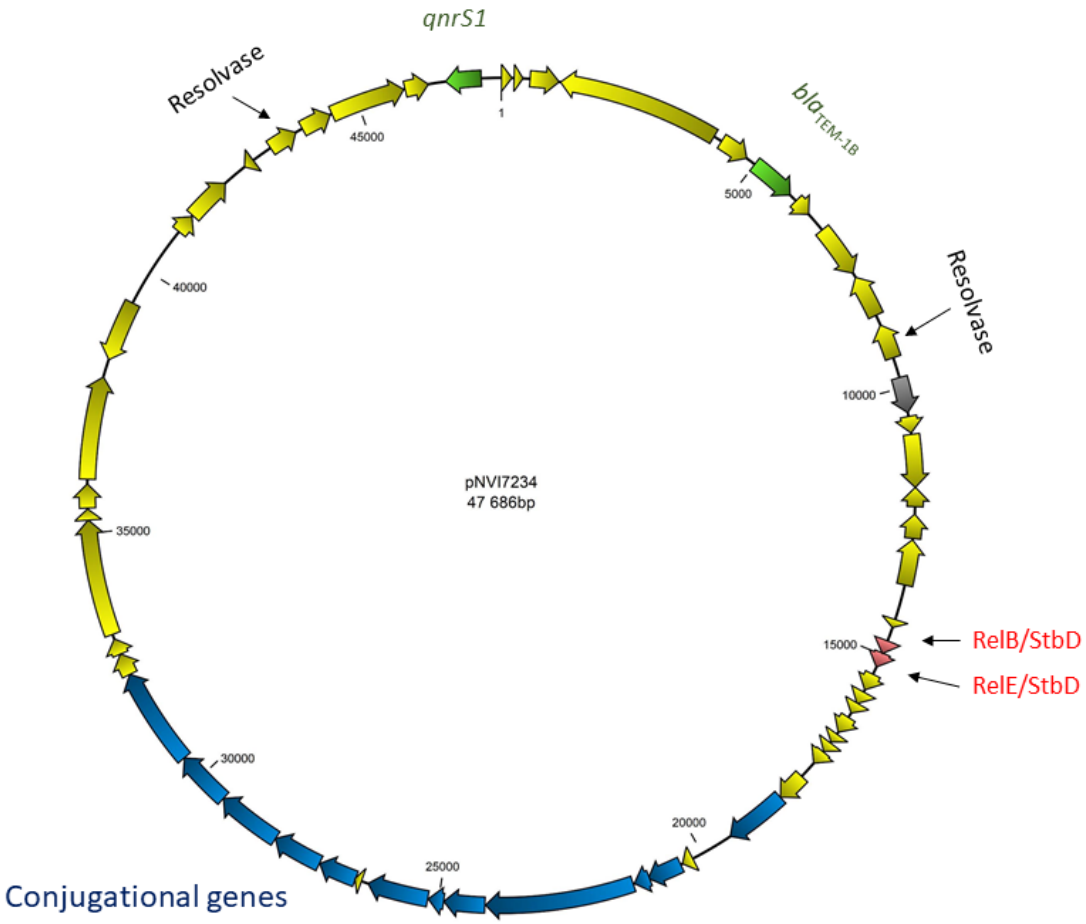

Figure 9: Illustration of plasmid pNVI7234. Blue colour indicates genes responsible for conjugation and transfer activities. Red colour indicates Toxin-Antitoxin systems. Grey colour indicates plasmid stability systems. Green colour indicates resistance genes. Yellow colour indicates mobile genetic elements, hypothetical proteins and remaining genes that do not fit into the other categories based on functionality.

Annotations by RAST revealed genes encoding transfer of plasmid associated with type IV secretion system (T4SS) (fig 7, blue), other transfer associated genes (fig 7, blue), a TA system (RelE/StbD and RelB/StbD) (fig 7, red) and a chromosome partitioning system (ParA) (fig 7, grey). The *qnrS1* and *bla*$_{\text{TEM-1B}}$ were found in a region flanked by two resolvases.

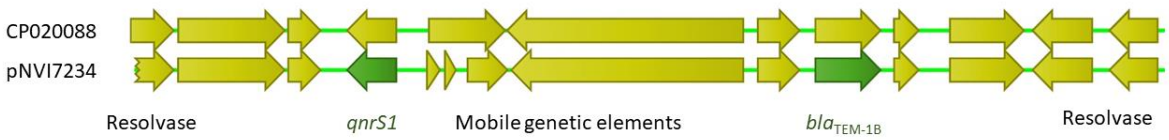

Figure 10: Genetic load region of pNVI7234 with qnrS1 *and* bla$_{\text{TEM-1B}}$*, flanked by a resolvase.*

The region flanked by two resolvases are called the genetic load region in the pNVI7234. The genetic load region shown in figure 10 in pNVI7234 are almost identical to that of the CP020088, except for minor differences in the genes between the *qnrS1* and bla_{TEM-1B} annotated as mobile genetic elements in RAST. The mobile genetic element closest to *qnrS1* showed some differences compared to the CP020088. The bla_{TEM-1B} and *qnrS1* genes were equal in pNVI7234 and CP020088.

Figure 11: A model of pNVI7234 comparing the two plasmids sharing close homology; Shigella flexneri 4c strain (accession number: KJ201886) and Shigella flexneri 1a strain (accession number: CP020088).

The plasmid pNVI7234 showed close homology with two plasmids in GenBank; pSF07202 from a *Shigella flexneri* 4c strain (accession number: KJ201886) and Unnamed2 from a *Shigella flexneri* 1a (accession number: CP020088). The annotations in figure 11 are based on the plasmid Unnamed2 in *Shigella flexneri 1a*, and therefore differ slightly by name from figure 9, however, they do share close

homology. The size of the plasmids is 47 674 bp in *Shigella flexneri* 1a, 47 669 bp in *Shigella flexneri* 4c and 47 686 bp in pNVI7234.

4. Discussion

4.1 Susceptibility of the *E. coli* strains

All *E. coli* strains carried a *qnr*-gene and an S83L mutation in *gyrA* and were considered NWT to ciprofloxacin because they displayed an MIC above ECOFF of 0.064 mg/l. The four *E. coli* strains carrying *qnrS1* and the S83L mutation in *gyrA* displayed a ciprofloxacin MIC value of 2 mg/l. The *E. coli* strain carrying *qnrB19* and the S83L mutation in *gyrA* displayed a ciprofloxacin MIC value of 0.5 mg/l. ECOFF values are given for most relevant genera and species and indicate whether the microorganism displays an MIC to an antimicrobial agent that is expected to be found among the WT of that particular strain. All the *E. coli* strains investigated displayed MIC to ciprofloxacin above the ECOFF indicating that these strains have acquired resistance that would not be present in WT. *Enterobacteriaceae* showing full susceptibility (WT) to ciprofloxacin displays an MIC to ciprofloxacin of 0.004 mg/l (*E. coli* ATCC 25922) (EUCAST).

All *E. coli* strains displayed low tolerance to nalidixic acid above the ECOFF of 16 mg/l. The potency to nalidixic acid varied from MIC >128 mg/l in 2014-01-6924 to MIC 64 mg/l in 2014-01-5749 and 2014-01-5792. Robicsek et al. (2006) report that all fluoroquinolone-resistant bacteria are also resistant to nalidixic acid. Veldman et al. (2011) and Ruiz (2003) reports that mutations in *gyrA* mainly acts upon quinolones such as nalidixic acid and PMQR-genes mainly acts on fluoroquinolones such as ciprofloxacin.

The effect on the strains of carrying an S83L mutation in *gyrA* toghether with a *qnrS1*-gene was a ciprofloxacin MIC of 2 mg/l compared to an MIC of 0.5 mg/l in the transconjugants carrying only *qnrS1*. Similar additive effects of having a mutation in *gyrA* and in addition to a *qnr*-gene have been reported in reviews by Rodriguez-Martinez et al. (2016), Strahilevitz et al. (2009) and Hooper and Jacoby (2015). Rodriguez-Martinez et al. (2016) report that the *E. coli* ATCC 25922 strain shows a 256-fold increase in MIC when carrying an S83L mutation in *gyrA* and *qnrS1*, compared to 32-fold increase when carrying only *qnrS1*. The 256-fold increase in MIC when having both the S83L mutation in *gyrA* and *qnrS1* compared with 32-fold increase with only *qnrS1* demonstrates an additive effect in terms of MIC. The effect of carrying other *qnr*-variants was not demonstrated as strongly with only a 64-fold increase when carrying the combination of an S83L mutation in *gyrA* and *qnrB1*, and a 128-fold increase when carrying the combination of an S83L mutation in *gyrA* and *qnrA1*. (Rodriguez-Martinez et al., 2016). Strahilevitz et al. (2009) report *E. coli* strain J53 and HB101 carrying only *qnrS1* displaying an MIC to ciprofloxacin of 0.25 mg/l and *E. coli* KF130 with mutation in *gyrA* displaying the same MIC to ciprofloxacin. Hooper and Jacoby (2015) supported the findings of equal sensitivity to ciprofloxacin when carrying either mutation S83L in *gyrA* or *qnr*- genes (*qnrA1*, *qnrB1* or *qnrS1*). *E. coli* J53 WT

without PMQR or chromosomal mutations displayed a ciprofloxacin MIC of 0.008 mg/l, *E. coli* J53 carrying *qnrS1* an MIC of 0.25 mg/l and *E. coli* J53 with *S83L mutation in gyrA* displayed an MIC of 0.25 mg/l. The referred papers above reveal that there is an additive effect in *E. coli* when having both a mutation in *gyrA* and carrying either *qnrA1*, *qnrB1,* or *qnrS1 genes*, but the potency of the effect differs.

The strain 2014-01-5792 carrying *qnrB19* displayed a ciprofloxacin MIC of 0.5 mg/l compared with the *qnrS1* containing *E. coli* strains (MIC of 2 mg/l). The lower tolerance to ciprofloxacin cannot be explained by the presence of a chromosomal S83L mutation in *gyrA* since all *E. coli*s carried the same mutation. Rodriguez-Martinez et al. (2016) reported a weaker additive effect of S83L mutations in *gyrA* and *qnrB1* (64-fold increase in ciprofloxacin MIC) compared with an S83L mutation in *gyrA* and *qnrS1* (256-fold increase in ciprofloxacin MIC) in *E. coli* ATCC 25922. Strahilevitz et al. (2009) and Jacoby et al. (2015) reported that the presence of only a *qnrB1* gene in the *E. coli* strains J53 and HB101 displayed an MIC to ciprofloxacin of 0.25 mg/l. Based on these reports there might be a small additive effect of carrying a mutation in *gyrA* and the presence of *qnrB19* which is in concordance with the results in this study.

A strain in the genera *Enterobacteriaceae* is considered clinically resistant to ciprofloxacin if the MIC exceeds >0.5 mg/l and all the *E. coli* strains carrying the *qnrS1* gene together with an S83L mutation in *gyrA* are in this context considered clinically resistant to ciprofloxacin (MIC 2 mg/l). The strain carrying *qnrB19* and an S83L mutation in *gyrA* (MIC 0.5 mg/l to ciprofloxacin) are considered in the intermediate category and are therefore not considered clinical resistant.

4.2 WGS data

The $bla_{\text{TEM-1B}}$–gene encoding a β-lactamase was found in all five strains. The $bla_{\text{TEM-1B}}$-β-lactamase only acts upon penicillins and therefore is not regarded as an extended spectrum β-lactamase (ESBL) (Smet et al., 2010). The five *E. coli* strains were resistant to ampicillin (penicillin) (>64 mg/l) and fully susceptible to the 3rd generation cephalosporins cefotaxime and ceftazidime.

In addition to harbour the resistance genes *qnrS1* and $bla_{\text{TEM-1B}}$, two of the *E. coli* strains; 2014-01-5749 and 2014-01-6924, carried acquired resistance genes to aminoglycosides (*aph(6)-Id* and *aph(3'')-Id*), sulphonamide (*sul2*), tetracycline (*tet(A)*) and trimethoprim (*dfrA14*). Susceptibility testing displayed an MIC >1024 mg/l to sulfamethoxazole, an MIC of >32 mg/l to trimethoprim, an MIC of <=2 mg/l to tetracycline, and an MIC of <= 0.5 mg/l to gentamicin. These results shows that sulfamethoxazole and trimethoprim is above the ECOFF and tetracycline and gentamicin are below. The strains are clinically resistant to sulphonamide (sulfamethoxazole) and trimethoprim, but not to tetracycline or gentamicin (an aminoglycoside).

Characterization of the complete IncX1 plasmid pNVI7234 from *E. coli* strain 2014-01-7234, shows that the *qnrS1* gene and bla_{TEM-1B} are co-located on the same plasmid. Rodriguez-Martinez et al. (2016) report that *qnr-genes* are frequently found on multidrug resistance plasmids. Furthermore, both *qnrB* and *qnrS* are often found co-located with bla_{TEM-1B} (Rodriguez-Martinez et al., 2016). This supports the notion that all the strains might have *qnr*-genes co-located with bla_{TEM-1B}. All *E. coli* strains carrying *qnrS1* also carried an IncX1 plasmid and was identified as serotype O23:H16 and sequence type ST-463.

The *E. coli* strain 2014-01-5792 carried additional plasmids belonging to incompatibility group; IncI2, Col(KPHS6) and Col156, but did not carry an IncX1 plasmid, however, this strain carried a different *qnr*-gene compared to the four other strains. No further work was done to determine which plasmid that was associated with *qnrB19*.

4.3 Conjugational experiments

The four *E. coli* strains; 2014-01-5749, 2014-01-6924, 2014-01-7234 and 2014-01-7375 were able to conjugate the plasmid carrying the *qnrS1* resistance gene into *E. coli* OneShot. These four strains were multi-locus sequence type 453 and serotype O23:H16 in addition to sharing the same resistance gene and an IncX1 plasmid. Based on the similiarity, isolation from the same animal species and their shared characteristics these strains might orginiate from the same source.

The *E. coli* strain 2014-01-5792 carrying *qnrB19* was not able to conjugate under the conditions used in this experiment. However, that does not necessarily imply that the plasmid harbouring *qnrB19* cannot be conjugated. According to Jacoby et al. (2015), *qnrB19* is among the most frequently detected *qnrB* alleles, and *qnrB* seems to be the most prevalent *qnr*-variant, identified in 94 variants as of May 2018. A successful transfer of plasmid from one donor to a new host rely on; the conjugational properties of the plasmid (Carattoli, 2011), the Inc group that the plasmid belongs to (Carattoli, 2011) and the host range that determines the ability of the plasmid to replicate in hosts (Carattoli, 2013) and the presence of essential genes from conjugation on the plasmid.

4.4 Susceptibility (MIC) of the transconjugants

The four transconjugants carrying *qnrS1* displayed an MIC to ciprofloxacin of 0.5 mg/l compared to an MIC to ciprofloxacin of 2 mg/l in the donor strains. The lower tolerance to ciprofloxacin in the transconjugants compared to the donor strains is because only the *qnrS1*-bearing plasmid is transferred by conjugation. The S83L mutation in *gyrA* in the donorstrains is located on the chromosome.

The transconjugants were susceptible to nalidixic acid with an MIC of 8 mg/l and this was a reduction by a factor of eight and 16 respectively, compared to the donor strains. The sensitivity to nalidixic acid in the transconjugants suggests mutations in *gyrA* to play a role in the susceptibility to this antimicrobial agent. This suggestion is supported by Veldman et al. (2011) and Ruiz (2003) in that mutation in *gyrA* primarily act on nalidixic acid whereas PMQR-genes primarily act on fluoroquinolones.

4.5 Characterization of *qnr*-bearing plasmids

The IncX1 plasmid pNVI7234 was found to carry both the *qnrS1* and the $bla_{\text{TEM-1B}}$ gene. IncX1 plasmids have been found in *Enterobacteriaceae* and are thought to have a narrow host range (Johnson et al., 2012). Johnson et al. (2012) report that both *qnrS1* and $bla_{\text{TEM-1B}}$ have been localized on IncX plasmids. However, these genes have also been co-located and identified on an IncX2 plasmid. Dobiasova and Dolejska (2016) report similar findings of isolates harbouring IncX1 plasmids ranging in 40-60 kb in size carrying both *qnrS1,* $bla_{\text{TEM-1B}}$ and $bla_{\text{TEM-135}}$. They also identified the *qnrS1* gene encoded on IncX2 plasmids (Dobiasova & Dolejska, 2016). These reports support our findings of an IncX1 plasmid carrying both *qnrS1* and $bla_{\text{TEM-1B}}$.

Plasmid pNVI7234 is 47 686 bp in size and are isolated from *E. coli* 2014-01-7324, serotype O23:H16 and ST-453. pNVI7234 share close homology with two plasmids isolated from a *Shigella flexneri* 1a (accession number: CP020088) and a *Shigella flexneri* 4c strain (accession number: KJ201886). The size of the two plasmids is 47 674 bp in *Shigella flexneri* 1a and 47 669 bp in *Shigella flexneri* 4c.

The pNVI7234 plasmid has a genetic load region flanked by two resolvases, equivalent to the genetic load region in pOLA52 (Norman et al., 2008). The genetic load region is the region in the plasmid containing resistance genes and mobile genetic elements such as transposons. The genetic load region in pNVI7234 contained both *qnrS1* and $bla_{\text{TEM-1B}}$. Norman et al. (2008) sequenced an IncX1 plasmid named pOLA52 and revealed a “genetic load region”, a backbone region containing genes involved in replication, partitioning system, conjugal transfer and additional genes. The pOLA52 plasmid also carried TA genes. The plasmid backbone contained genes involved in conjugal transfer and partitioning systems. In addition, it contained a type II (Type II; proteins, type I; RNA (Hayes, 2003b)) TA systems of the *RelB*/*StbD* and *RelE*/*StbE* type. TA systems ensure persistence of plasmids in bacteria after cell division by inhibiting growth or killing bacteria without plasmids (Hayes, 2003a). *RelE* is the toxin and *RelB* is the antitoxin. The *RelB* antitoxin is located upstream of the *RelE* toxin. *RelE* toxin acts by impairing growth, by inhibiting translation (Hayes, 2003b). Bustamante and Iredell (2017) report that the *RelB/RelE*-like type II TA system seems to be more prevalent among IncX1 plasmids in an *in silico* analysis of 153 IncX plasmids readily available in databases.

5. Conclusion

Five *E. coli* from poultry harbouring the PMQR genes *qnrS1* or *qnrB19* were examined to determine the conjugational properties and transfer frequency of their plasmid, and the strain susceptibility. In addition to the presence of a *qnr* gene encoding quinolone resistance, all strains harboured a $bla_{\text{TEM-1B}}$–gene encoding a β-lactamase. Four strains carrying a *qnrS1* gene contained a self-transferable IncX1 plasmid. The strains were multi-locus sequence type 453 and serotype O23:H16. Based on their strain similarity, isolation from the same animal species and their shared characteristics, these strains might originate from the same source. The *E. coli* strain carrying a *qnrB19* were not able to conjugate under the conditions used in this experiment.

All five *E. coli* strains displayed MIC to ciprofloxacin above the ECOFF and the four strains carrying *qnrS1* displayed MIC to ciprofloxacin above the clinical breakpoint, thus were clinically resistant to ciprofloxacin. In addition, two *E. coli* strains carried additional resistance to sulphonamide (sulfamethoxazole) and trimethoprim.

An in-depth characterization of one IncX1 plasmid carrying *qnrS1* and $bla_{\text{TEM-1B}}$ were carried out and the plasmid was circularized. Plasmid pNVI7234 was 47 686 bp in size and was isolated from *E. coli* 2014-01-7324, serotype O23:H16 and ST-453. pNVI7234 shared close homology with two plasmids isolated from a *Shigella flexneri* strain 1a and a *Shigella flexneri* strain 4c . Annotation of the plasmid revealed a plasmid backbone encoding genes involved in conjugal transfer and partitioning systems, and a TA system ensuring persistence in bacterial hosts. The genetic load region in pNVI7234 contained both *qnrS1* and $bla_{\text{TEM-1B}}$ and was flanked by two resolvases. The three other *qnrS1* positive *E. coli* were believed to have a similar plasmid, however, more in-depth studies need to be conducted.

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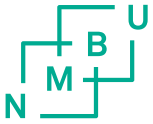

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