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Occurrence of quinolone resistant *Escherichia coli* in Norwegian dairy cattle

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Occurrence of quinolone-resistant *Escherichia coli* in Norwegian dairy cattle

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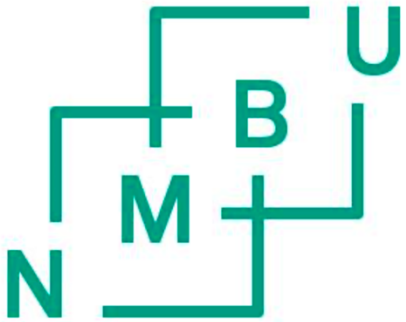

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Ås, February 22
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Abstract

This study aimed to obtain further understanding of the occurrence of quinolone-resistant *Escherichia coli* (QREC*)* from cattle despite negligible quinolone usage in cattle treatment, to identify risk factors for QREC among Norwegian dairy cattle, and to gain more knowledge of QREC dynamics over time.

QREC occurrence was investigated according to the intensity of milk production in a cross-sectional study. In parallel with this, a longitudinal study was conducted in which QREC occurrence in cows and their calves was screened during the first three months postpartum.

QREC occurrence was more abundant in high-intensive farms. Clinically resistant QREC isolates were detected. The most abundant phenotypic multi-resistance pattern was against ciprofloxacin, nalidixic acid, ampicillin, sulfamethaxazole, trimethoprim, and tetracycline. A co-occurrence of QREC isolated from cows, calves, and the environment at the farm level was found. There was a higher within-sample prevalence of QREC in calves compared to postpartum cows. Both studies found a clonal distribution of QREC isolates within the farms. Sequence type 162 that has been isolated from other farm- and wild animals in Norway, was detected in one farms. The within-sample prevalence of QREC seemed low in terms of total *E. coli*. Repeated findings of QREC in bovine faecal material could indicate a widespread occurrence of QREC despite Norway's long-term low usage of quinolones.

Abbreviations

AMEG	*Categorisation of antibiotics in the European Union*
AMR	*Antimicrobial resistance*
AMS	*Automatic milking systems*
ARIBA	*Antimicrobial Resistance Identification by Assembly*
CFU	*Colony-forming unit*
DF	*Dilution factors*
ECOFF	*Epidemiologic cut-off value*
EFSA	*European Food Safety Authority*
EKM	*Energy-correlated milk*
EUCAST	*European Committee on Antimicrobial Susceptibility Testing*
ESVAC	*The European Surveillance of Veterinary Antimicrobial Consumption*
H - farms	*High-intensive farms*
L - farms	*Low-intensive farms*
MALDI–TOF	*Matrix-assisted laser desorption ionisation–time of flight*
MDR	*Multidrug resistant*
MIC	*Minimal inhibitory concentration*
MLVA	*Multiple locus variable–number tandem repeat analysis*
MLST	*Multi-locus sequence typing*
NDHRS	*Norwegian Dairy Herd Recording System*
NOMRS	*Norwegian official milk recording scheme*
NORM/NORM-VET	*Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway*
OIE	*World Organisation for Animal Health*
PCU	*Population correction unit*
PCR	*Polymerase chain reaction*
WHO	*World Health Organization*
QRDR	*Quinolone resistance-determining region*
QREC	*Quinolone-resistant* Escherichia coli

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1. Introduction

1.1 Introduction to the Norwegian dairy industry

Norwegian dairy herds are small in the European context. Nevertheless, the production units have changed to larger ones with the increasing use of automatic milking systems (AMS). From 2000 to 2018, the number of cows in Norwegian dairy herds doubled on average from 14 to 28, and milk per cow per year increased from 6,200 kg to 7,987 kg (Vik et al., 2019, Nørstebø, 2019).

A cattle population spread out over a large geographic area containing challenging topography has been proven advantageous in avoiding infections and handling national eradication programs. Norwegian eradication programmes have been successful against infectious diseases such as bovine tuberculosis, ringworm, brucellosis, and bovine viral diarrhoea. The active approach to controlling infectious diseases and the benefit provided by geographical distance could potentially have contributed to reducing the transmission of antimicrobial-resistant bacteria. There is a restricted sale of live animals and a trend to raise calves and recruit heifers individually at each farm. All entrances to the farms require sluice gates. As mandated by national regulations, there are also separate entrances for farmers, visitors, and deliveries (Dyrehelseforskriften, 2002). Additionally, there is a financial benefit for farmers with an operative contingency plan to prevent infectious diseases (Matmerk, 2018). With the absence of several serious diseases, the Norwegian dairy cattle population is in good health overall.

Norway has some of the lowest therapeutic antimicrobial use in animal food production (ESVAC, 2021), and antimicrobial growth promoters have never been used for cattle production in Norway (Grave et al., 2006). The Norwegian Medicine Agency has published therapy recommendations for the treatment of farm animals as part of a project sponsored by the Ministry of Health and Care Services. The recommendations mainly advise the use of small-spectrum antibiotics, predominantly penicillin. The recommendations correspond well to the yearly published report of antimicrobial usage, which states that penicillin continues to be the most-sold antibacterial class (NORM-VET 2020, Figure 1.1). With its low

antimicrobial use, Norwegian dairy production provides a unique platform for building basic knowledge and gaining insight into alternative mechanisms behind the development and spread of antimicrobial resistance.

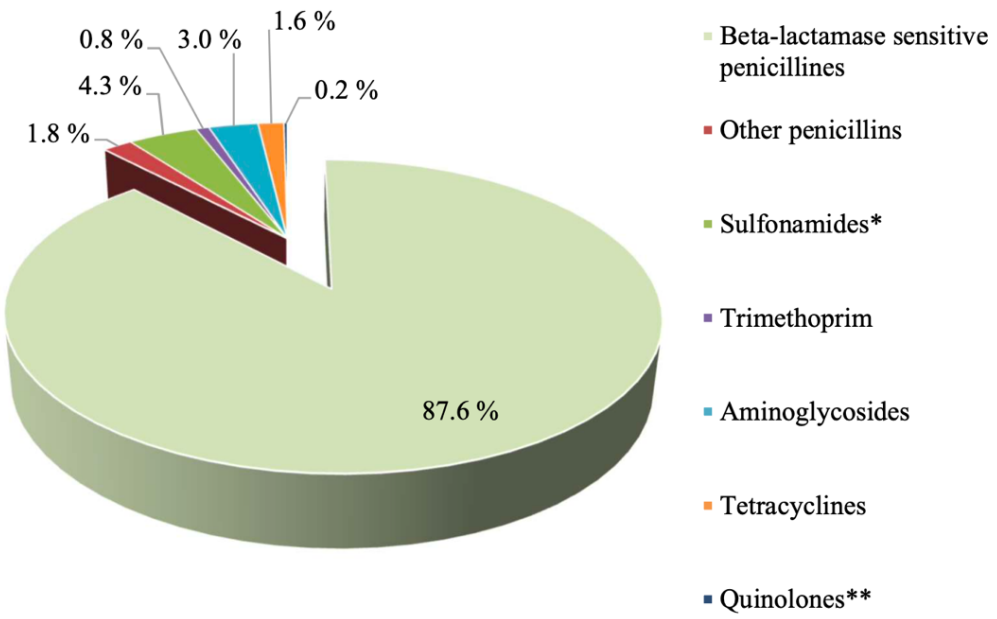


Figure 1.1 Prescribing patterns, in kg active substance, of antibacterial veterinary medicinal products for cattle in Norway in 2020. Penicillin remains the most-sold antibacterial class, whereas quinolone represents 0.2% of the total sales of antimicrobials for cattle. Data were obtained from the Veterinary Prescription Register. *In combination with trimethoprim only; ** Fluoroquinolones only (NORM-VET, 2020)

1.2 Surveillance and occurrence

1.2.1 Norwegian surveillance programme

Surveillance of antibiotic resistance and the use of antimicrobials is documented in the yearly published national report, NORM/ NORM-VET, which includes companion animals and farm animals, as well as human medicine. NORM-VET was established in 2000 as a political initiative and is now an elemental part of the national action plan on antimicrobial resistance.

E. coli and *Enterococcus* spp. are used as indicator bacteria for bovine faecal material in the

surveillance of antibiotic resistance. In addition, selective methods are used for the detection of methicillin-resistant *Staphylococcus aureus* (NORM-VET, 2015, 2018) and the detection of resistance in *Streptococcus agalactiae* (NORM-VET, 2015) and *Salmonella typhimurium* (NORM-VET, 2015, 2018) in bovine faecal samples.

Commensal intestinal *E. coli* is considered a key component for surveillance programmes, and the usage of *E. coli* as an indicator bacterium is in line with the FSA's recommendation to harmonise the monitoring of food-producing animals across Europe (Aerts et al., 2019).

1.2.2 Occurrence of antimicrobial resistance in bacteria isolated from cattle

The NORM-VET report of 2019 states that 93.3% of commensal *E. coli* isolates from cattle caecal samples were susceptible to all antimicrobial classes included in the test panel, indicating low resistance among bovine intestinal *E. coli* (NORM/ NORM-VET, 2019). This is a slight decrease in sensitivity compared to the report of 2015 (95.4%) (NORM/ NORM-VET, 2015). *E. coli* resistant to sulfamethoxazole and tetracycline were most frequently observed, followed by ampicillin-resistant phenotypes (NORM/ NORM-VET, 2019). Sulfamethoxazole and tetracycline are both sold for animal use in Norway. Ampicillin, an extended-spectrum penicillin, is reserved for human use only.

1.2.3 Occurrence of quinolone-resistant bacteria isolated from cattle

Monitoring QREC from cattle has been a part of NORM-VET in 2001, 2003, 2005, 2010, 2015, 2017, and 2019. A single quinolone-resistant isolate was detected in 2001, while subsequent investigations in 2003, 2005, and 2010 did not reveal any QREC from cattle. In 2013, a more selective method for QREC isolation was introduced, which could make comparisons to previous years difficult. However, the occurrence of QREC isolated from cattle after introducing this more selective method was still low. Two isolates of QREC were detected in 2015 (0.8%) and none *E. coli* displayed any resistance to quinolones in 2017 or 2019, indicating a prevalence below 1.2%. Data obtained from the surveillance programme have been used in a study conducted by Kaspersen et al., (2018), “Occurrence of quinolone-resistant *E. coli* originating from different animal species in Norway.” This recent work states that the prevalence of QREC in Norwegian cattle is less than 1% (Figure 1.2; Kaspersen et al.,

2018). From a European perspective, resistance among *E. coli* isolated from cattle in Norway is among the lowest of the countries reporting to EFSA (EFSA and ECDC, 2020).

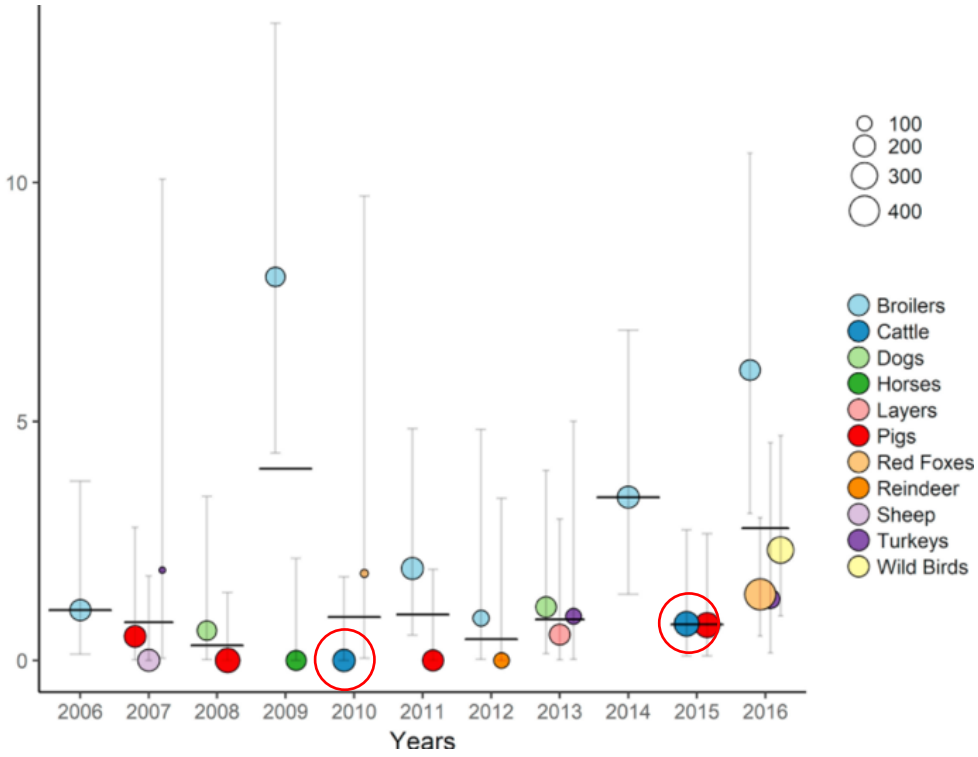


Figure 1.2 Percent occurrence of QREC for each animal species per year. The total mean occurrence per year is represented as a black horizontal line. The size of each point represents the number of isolates for each respective animal species. Cattle are included in 2010 and 2015 and are visualised as dark blue points with a red circle (modified from Kaspersen et al., 2018).

1.3 Quinolones and their usage in cattle production

1.3.1 The antimicrobial class

Quinolones are antibiotics obtained by chemical synthesis. The first agent among the quinolones to be used clinically was nalidixic acid. This is a broad-spectrum antimicrobial class with concentration-dependent bactericidal activity (Riviere, 2018). Quinolones with a fluorine atom at the sixth position and a major ring substituent at position seven are called fluoroquinolones (Figure 1.3, Correia et al., 2017). Fluoroquinolones include well-known antibiotics such as ciprofloxacin, enrofloxacin, ofloxacin, and norfloxacin. Quinolones and

fluoroquinolones inhibit the activity of DNA gyrase and topoisomerases II and IV, enzymes that relax the supercoiling of bacterial DNA and complete cell division (Ruiz, 2003). However, non-fluorinated quinolones should not be used anymore because their antibacterial activity is far inferior to fluorinated quinolones (Naber et al., 1998).

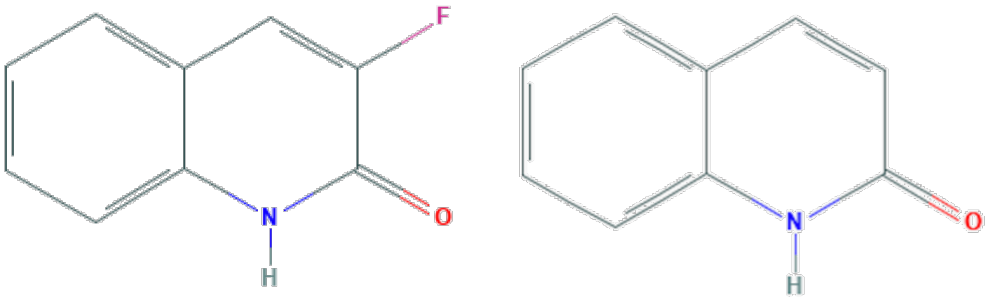


Figure 1.3 Structure of the fluoroquinolone (left) and quinolone (right) molecules (PubChem, 2004).

The World Health Organization (WHO, 2018) deemed fluoroquinolones are critically important and, to preserve their effectiveness, should mainly be reserved for treating the severest human infections. In this thesis, *quinolone* will be used as a common term, including quinolone and fluoroquinolone, unless stated otherwise.

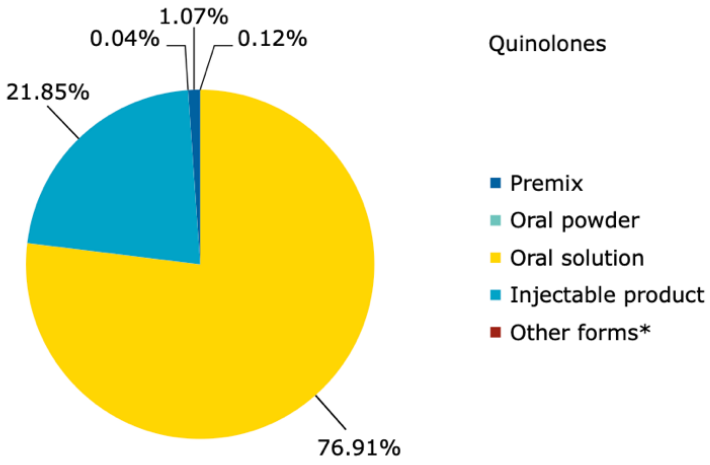
1.3.2 Usage of quinolones for cattle in Norway

Quinolone sales for food-producing animals in Norway are negligible (ESVAC, 2021). Of the total sales of antimicrobials, only 0.2% are quinolones explicitly used for treatment of cattle (Figure 1.1, NORM/NORM-VET, 2020), an increase of 0.1% from the previous year (NORM/NORM-VET, 2019). Baytril®, an enrofloxacin, is the only marketed quinolone for food-producing animals in Norway. Baytril® is indicated for use in cattle only after antimicrobial sensitivity tests and after absent responses to other antibiotic classes are documented (Felleskatalogen, 2020). Quinolones are not recommended for the treatment of any specific cattle diseases by the Norwegian Medicines Agency (Terapianbefalingene, 2012).

1.3.3 Usage of quinolones for food-producing animals in Europe

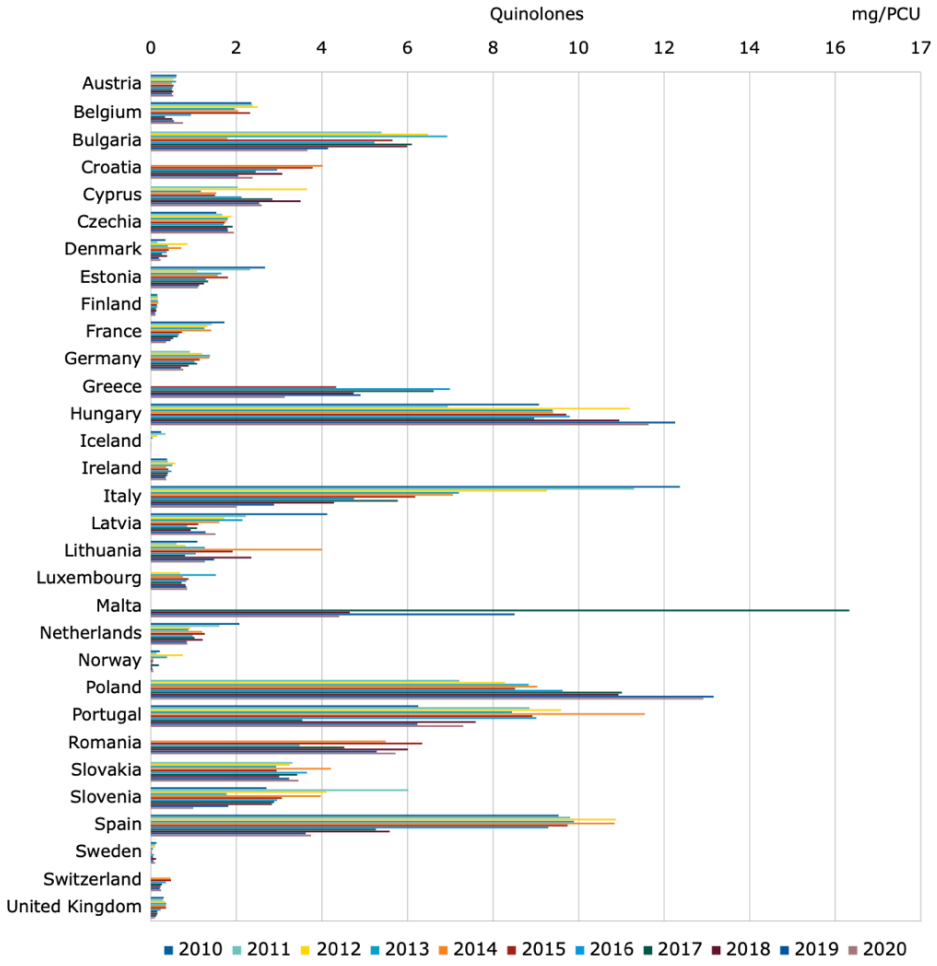
The European Medicines Agency (EMA) is an agency of the European Union (EU) responsible for the scientific evaluation, supervision, and monitoring of the safety of medicines for both human and veterinary medicine. The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project collects antimicrobial drugs used in animals across the EU. The ESVAC report has been published yearly since the project started in 2010. The usage of quinolones for treating food-producing animals in Europe varies highly between countries (Figure 1.5). Norway has one of the lowest usage levels among the European countries, with less than 0.01 mg of quinolones per population correction unit (PCU). The highest PCU among the European countries is found in Malta, Hungary, and Bulgaria, with 15.3 mg/PCU, 8.8 mg/PCU, and 5.7 mg/PCU, respectively. Many countries have reduced the usage of quinolones from 2010 to 2018, a trend found in Italy (from 1.7 to 2.3 mg/PCU), Lithuania (0.7 to 2.3 mg/PCU), and Portugal (5.6 to 7.6 mg/PCU). Fluoroquinolones and quinolones account for 2.5% and 0.3% of the total antimicrobial sales for food-producing animals in Europe where oral solutions are the main pharmaceutical form sold (Figure 1.4).

Figure 1.4 Distribution of sales of quinolones for food-producing animals, in mg/PCU, by the major pharmaceutical forms sold. Aggregated by the 31 European countries, for 2020 (ESVAC, 2021).



The European Commission requested in 2013 that all antimicrobials should be categorised by AMEG (Categorisation of Antibiotics in the European Union). Quinolones are in Category B, "for restrictive use," together with third- and fourth-generation cephalosporins and polymyxins. Category B is only for use when no alternatives from Categories C or D are clinically effective. The total sales of antimicrobials in Category B have shown a decreasing

trend from 2011 to 2018, with fluoroquinolone sales decreasing by 4.2% and other quinolone sales decreasing by 74.4% (ESVAC, 2020). The variation between countries in the sale of antimicrobials should be interpreted with great care. EFSA now publishes guidelines to harmonise monitoring.



[1] In Austria, Denmark, Finland, Iceland, Ireland, Norway, Sweden, Switzerland and the United Kingdom combined sales of fluoroquinolones and other quinolones were <1 mg/PCU for all years; in Luxembourg, sales were <1 mg/PCU for 2011 and 2013–2020.

Figure 1.5 An overview of quinolone sales for food-producing animals among the European countries. Data are from the eleventh ESVAC report. Numbers are given in mg/PCU for quinolones.

1.3.4 Usage of quinolones for food-producing animals worldwide

Limited data on resistance patterns in animal pathogens or commensal bacteria is available worldwide. In 2015, the World Organisation for Animal Health (OIE) launched its first data collection of antimicrobial agents intended for animal use, which it has published every year

since. A total of 153 countries, including OIE members and non-OIE members, are participating in the fourth round of data collection (OIE, 2020). Many members have reported the total usage from the veterinary sector, including companion animals, whereas 18 member countries have provided quantitative data for antimicrobial agents for food-producing animals. Among these countries, fluoroquinolone is the eighth most common prescribed antimicrobial class (Figure 1.6, OIE, 2020).

Thirty-five countries reported the use of antimicrobial agents for growth promotion, and among these countries, the most frequently listed antimicrobial agent was flavomycin, followed by bacitracin and tylosin (OIE, 2020). The usage of fluoroquinolones as growth promoters has been decreasing over the last few years. In 2017, two countries reported the usage of fluoroquinolones as growth promoters for farm animals. Only one country reported use in 2018 and none in the latest report of 2019 (OIE, 2020).

1.4 Quinolone resistance mechanisms

Bacteria become resistant through mutations in target genes or the horizontal transfer of genes encoding efflux pumps, degradative enzymes, alternative housekeeping enzymes, or ribosomal protection proteins. Quinolone resistance has traditionally been described as mediated by mutations in genes coding for quinolone targets: DNA gyrase and the topoisomerases (Redgrave et al., 2014). Chromosomal resistance is more stable than plasmid-mediated resistance and is likely to persist even if selective pressure is withdrawn (Strahilevitz et al., 2009). Plasmid-mediated fluoroquinolone resistance (PMQR) associated with *qnr* genes was first detected in 1994 (Robicsek et al., 2006). PMQR genes are often located on transferable plasmids and are co-transmitted with other important resistance genes (Wasyl et al., 2013). PMQR genes can be transferred between bacterial species at higher frequencies than chromosomal mutations can, making their spread much faster (Kao et al., 2016). There is a worldwide emergence of PMQR due to plasmid mobility (Robicsek et al., 2006). In recent years, quinolone resistance in Gram-negative bacteria has been on the rise worldwide, particularly in *E. coli* (Robicsek et al., 2006).

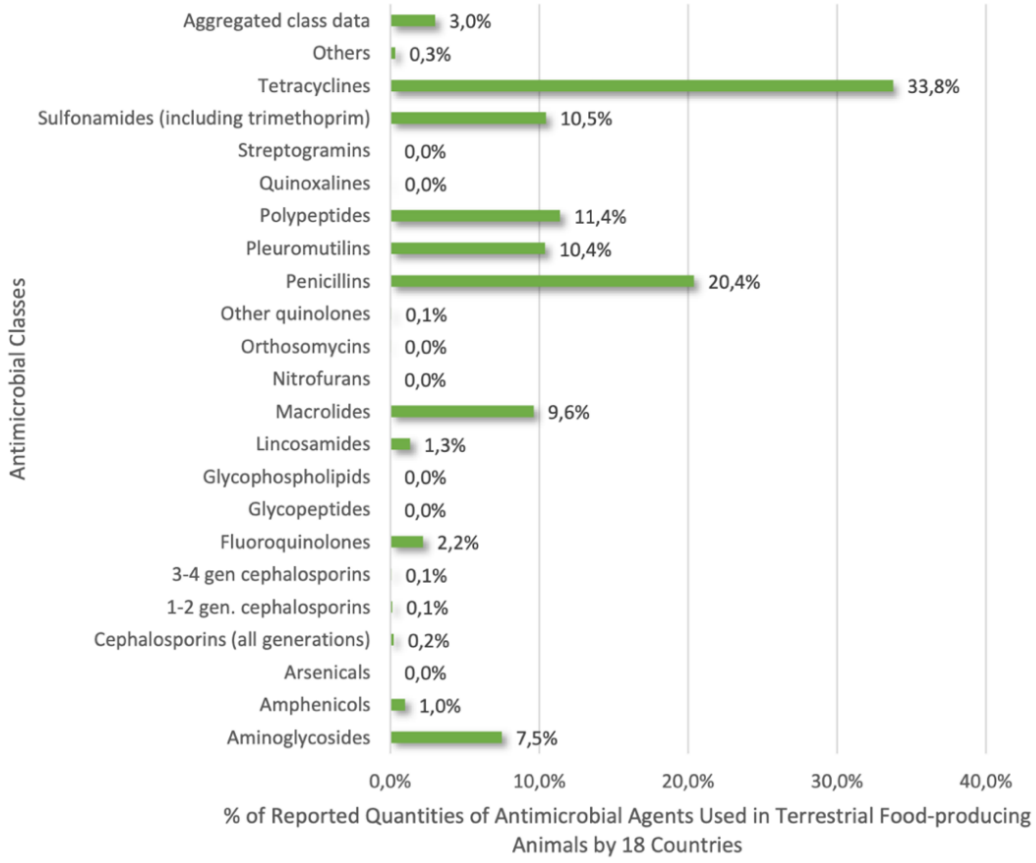


Figure 1.6 Proportion of antimicrobial quantities (by antimicrobial class) reported for use in food-producing animals worldwide (OIE, 2020).

1.4.1 Chromosomal mutations

In Gram-negative bacteria, point mutations located in the quinolone resistance-determining region (QRDR) are the most frequent cause of quinolone resistance (Figure 1.7 (a)). QRDR consists of clustered genes that encode for subunits of the enzymes DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). Mutations make these enzymes less sensitive to inhibition by quinolones. Mainly the *gyrA* gene and its homologous region in the *parC* gene are affected. Mutations in the *gyrB* and *parE* genes appear to be of minor importance and are rare contributors to quinolone resistance (Yang et al., 2004, Feng et al., 2019)

Highly quinolone-resistant *E. coli* are reported to be associated with four mutations in QRDR. Point mutations appear to repeatedly occur in the *gyrA* gene and the *parC* gene, resulting in substitutions of the amino acids Ser83 and Asp87 in the DNA gyrase and Ser80 in the

topoisomerase IV (Jurado et al., 2008, Suk-Kyung et al., 2010).

1.4.2 Plasmid-mediated resistance

Plasmid-mediated quinolone resistance (PMQR) is associated with low-level quinolone resistance that by itself does not exceed the clinical threshold for susceptibility but facilitates the selection of higher-level resistance (Robicsek et al., 2006, Jacoby et al., 2014).

Three groups of PMQR have been found in isolates from cattle globally (Aguilar-Montes de Oca et al., 2015, Awosile et al., 2018, Tamang et al., 2012, Thu Hang et al., 2019, Yang et al., 2018).

Plasmids carrying *Qnr* genes, which code for a pentapeptide repeat family of proteins, protect DNA gyrase from the attachment of quinolones (Figure 1.7 (d)). Additionally, the presence of genes that code for efflux pumps and a protein that modifies and inhibits the activity of ciprofloxacin have been found. The modifying enzyme, AAC(6′)-Ib-cr is a variant of aminoglycoside acetyltransferase (Figure 1.7 (d2)). AAC(6′)-Ib-cr seems to have emerged more recently and might be even more prevalent than the Qnr proteins (Cattoir et al., 2009, Robicsek et al., 2006,).

There has been a worldwide attention to PMQR since the first report of its occurrence in 1994 in the United States. Since then, PMQR has been reported in Asia, Africa, Australia, and several countries in Europe (Ajayi et al., 2012, Kirchner et al., 2011, Robicsek et al., 2006). In the countries close to Norway, PMQR has been identified in Gram-negative bacteria from cattle in England, Wales (Kirchner et al., 2011), and Scotland (Murray et al., 2008). In comparison, the first detection of PMQR in Scandinavia was in two human isolates found in Denmark in 2007 (Cavaco et al., 2007). Further studies have detected PMQR in humans in Norway and Sweden (Samuelsen et al., 2008, Karah et al., 2010). Farm animals other than cattle have been detected as carriers in Norway, including turkey (Slettemeås et al., 2019) and chicken (NORM/NORM-VET, 2013). To the best of the author's knowledge, PMQR has not been detected in cattle in Norway.

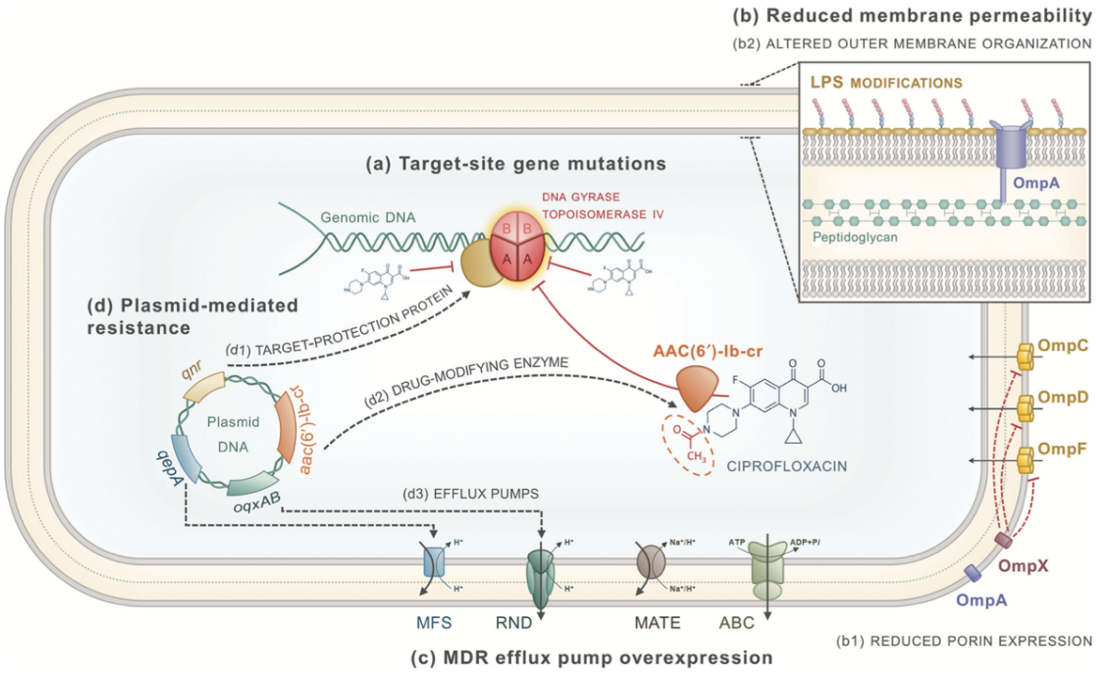


Figure 1.7 Mechanisms of quinolone resistance. (a) Chromosomal mutations within the QRDRs of the genes encoding the A and B subunits of DNA gyrase and topoisomerase IV structurally change the target protein, reducing its drug-binding affinity. (b) Chromosomal mutations leading to reduced outer membrane permeability, by either reduced porin expression (b1) or modifications in the outer membrane organisation (b2), and mutations leading to an increased expression of efflux pumps (c), contribute additively to resistance by decreasing cytoplasmic quinolone accumulation. (d) Plasmid-encoded quinolone resistance genes can produce *Qnr*-target-protection proteins (d1), *AAC(6)-Ib-cr* acetyltransferase variants capable of modifying certain quinolones (d2) or *QepA* and *OqxAB* efflux pumps that actively extrude quinolones (adapted from Correia et al., 2016).

1.5 Occurrence and dissemination of QREC in cattle

1.5.1 Occurrence of quinolones in the farm environment

Although antimicrobial usage exerts a selective force on resistance development, there is evidence that the prevalence of resistance in a bacterial population may not be related only to levels of antimicrobial usage. Resistant organisms sometimes persist after quinolones have been withdrawn (Hoyle et al., 2005). Because quinolones are strongly absorbed pharmaceuticals, they tend to accumulate in soil and sediments (Pico et al., 2007, Xiiong et al., 2015). Accumulation of quinolones in the farm environment could originate from several sources, including human waste and excretion, aquaculture, other companion or farm animals, and residual water from the pharmaceutical industry (Figure 1.8). A seven-year survey of

bovine pathogenic strains from eight European countries found that QREC were present before the marketing of quinolones in 1997 (Meunier et al., 2004).

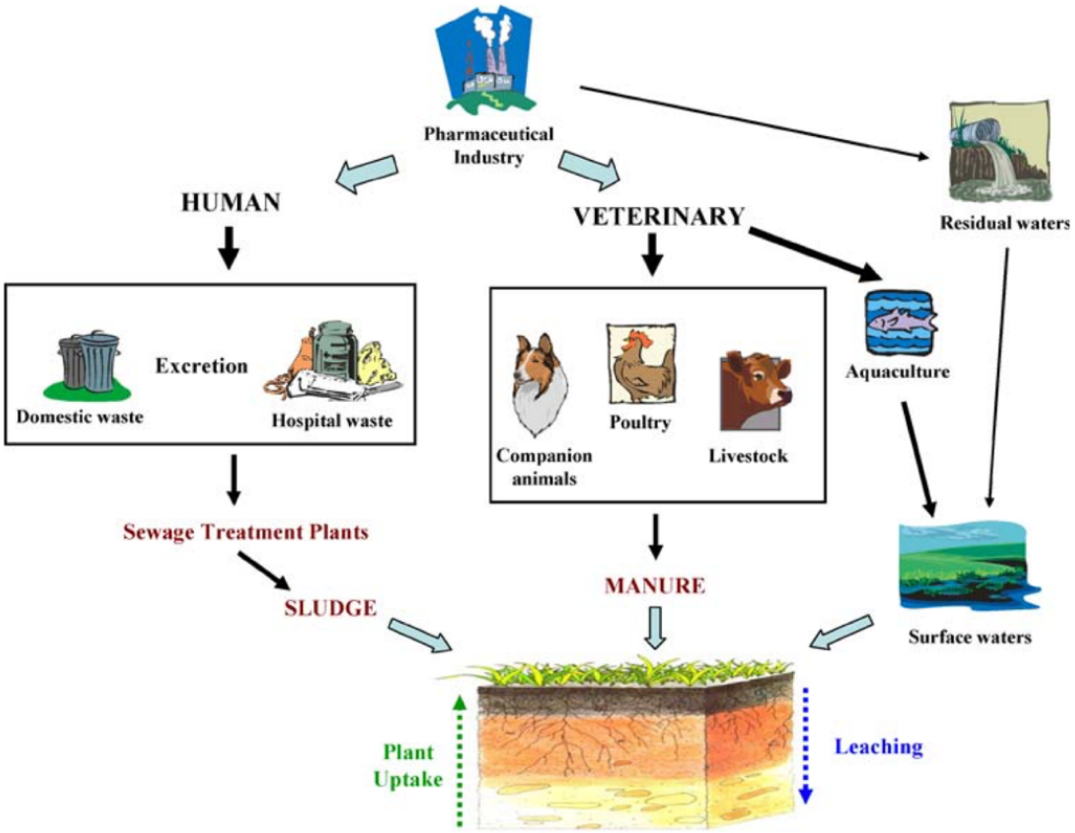


Figure 1.8 Pathways into the environment for fluoroquinolones. Quinolones are strongly absorbed pharmaceuticals and tend to accumulate in soil and sediments (Pico et al., 2007, Xiiong et al., 2015).

1.5.2 Dynamics of quinolone resistance

Several studies have now demonstrated that young bovines show a higher prevalence of resistant faecal *E. coli*, including QREC, than older stock. Thus, the susceptibility of calves to colonisation by resistant bacteria seems to be linked to their age (Hoyle et al., 2004, Edrington et al., 2012, Duse et al., 2015). QREC have been found in calves on the day of birth and then declines significantly with age (Hoyle et al., 2004, Duse et al., 2015). Early detection could be associated with calving in group pens (Duse et al., 2015).

The age-related decline in the shedding of resistant *E. coli* among calves is a well-established phenomenon. Berge et al., (2005) found a decrease in the occurrence of antimicrobial

resistance beginning when the calves were aged 4–6 weeks. In contrast, Donaldson et al., (2006) stated that antimicrobial-resistant strains commonly peak in calves at 14 days of age, and a Swedish paper from Duse et al., (2015) focusing specifically on QREC shedding found a sudden and significant reduction in the relative number of QREC around 18 days of age (Figure 1.9). The same study found age to be the most critical risk factor for faecal shedding of QREC.

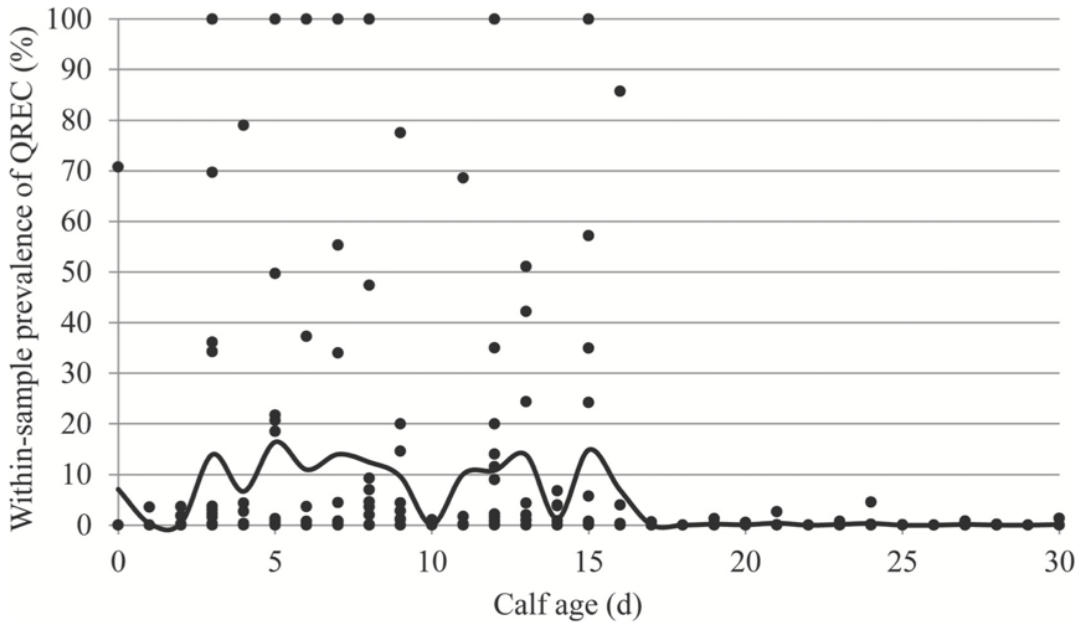


Figure 1.9 The within-sample prevalence of quinolone-resistant *E. coli* from calves (circles for individuals and solid line for the age mean) as a function of calf age. Sampling was performed once per animal. A sudden and significant reduction in the relative number of QREC was found around 18 days of age (Duse et al., 2015). This figure visualises an age-related decline in the shedding of resistant *E. coli* among calves as found in other comparable studies (Berge et al., 2005, Donaldson et al., 2006, Hoyle et al., 2004).

Jurado et al., (2008) have suggested that the decline in resistance prevalence could be artefactual, reflecting an overall reduction in total *E. coli*, with the number of resistant bacteria falling below the detection limits of the assay even though the proportion of resistant bacteria remains unchanged. Another hypothesis is that multidrug-resistant *E. coli* effectively compete only when significant competition is lacking. As the animal ages and the gut matures, the resistance becomes a burden, and the multidrug-resistant *E. coli* is excluded from the system (Edrington et al., 2012). Nevertheless, research on the dynamics of antibiotic resistance expression in the normal faecal microflora of cattle is limited.

1.5.3 Geographical distribution of QREC occurrence

The genetic diversity of QREC demonstrates the spread of distinct clones within and between farms. A Swedish paper stated that cattle on farms located close together were more likely to share the same genotype than cattle on farms located far apart (Duse et al., 2016). Clonal dissemination of QREC from dairy calves has been described, and the identical clones of QREC have been found throughout herds over time (Duse et al., 2016, Hoyle et al., 2005).

Regional distribution of QREC has been documented, with certain regions having higher occurrences. Within-farm diversity was greater for farms that had purchased cattle over the years than farms with a more closed cattle population. On-farm biosecurity was associated with QREC shedding (Duse et al., 2016). Such data could indicate that calves were colonised by resistant strains, with a probable environmental source that could have been transferred within the local community.

Wild mammals and birds are implicated as potential reservoirs for food-borne antimicrobial-resistant bacteria. Wild birds that often visit cattle farms have a great potential to disseminate resistant bacteria among the cattle across a region (Medhanie et al., 2016). Recent work in the United States found a strong positive association between the total number of birds and increased MDR *E. coli* shedding in cattle (Figure 1.10, Carlson et al., 2020 and Chandler et al., 2020). All regions included had a population of birds in which ciprofloxacin-resistant *E. coli* were detected. Birds carry resistant strains over large distances and interact with livestock and human populations. The prevalence and resistance mechanisms of bacteria in migratory birds have not yet been identified in detail, but it appears probable that QREC shedding could be associated with birds.

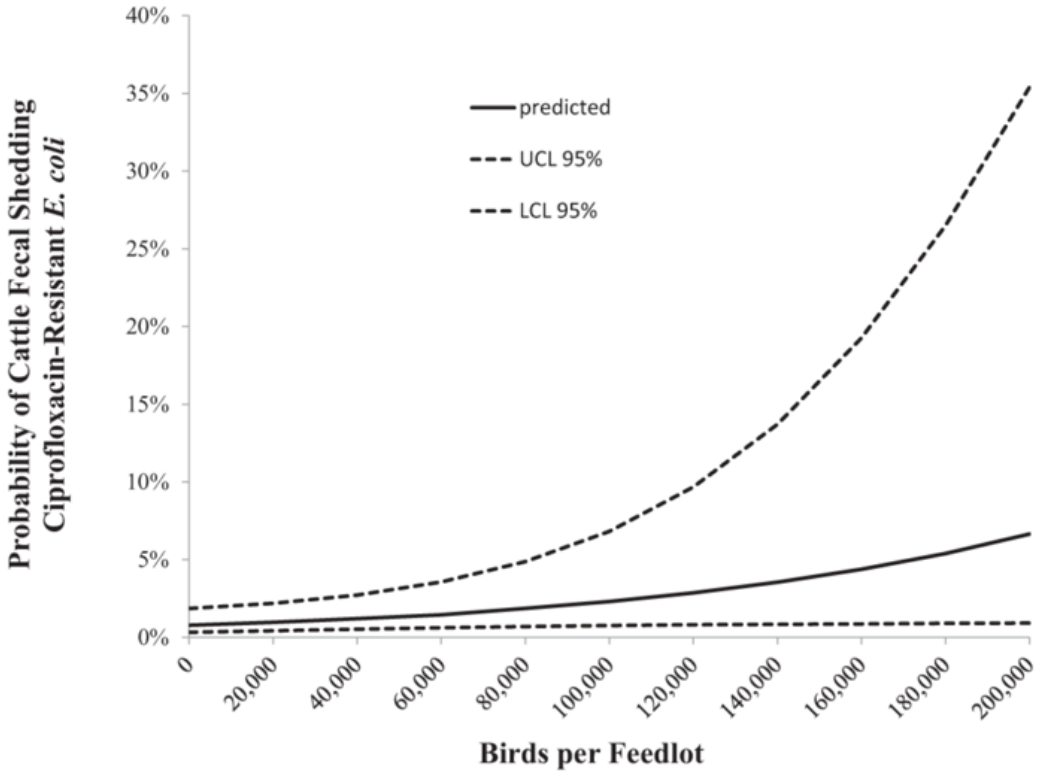


Figure 1.10 A strong positive association has been found between the total number of birds and an increased occurrence of QREC shedding in cattle (Carlson et al., 2020).

1.5.4 Distribution of QREC shedding in other farm animals

A Norwegian study that included farm-, companion-, and wild animal species identified the lowest occurrence of QREC among cattle, horses, reindeer, and sheep and the highest occurrence among broilers and wild birds (Kaspersen et al., 2018). As compared to other farm animals, the lower occurrence among cattle has been observed globally (Kaesbohrer et al., 2012, Ping et al., 2019., Wasyl et al., 2013). Avian species have been found to have the highest occurrence of QREC shedding (Figure 1.11). However, high levels in cattle do occur. A study found an alarmingly high level of quinolone resistance in bacteria isolated from cattle, reaching 77% of tested feedlot cattle in the United States (Tang et al., 2017). Duse et al., (2016) found at least one QREC-positive sample from pre-weaned calves in all but one of 23 sampled farms. An et al., (2017) have stated that unique sets of selective pressures appear to be associated with different livestock environments since identical resistant genotypes are found within the same farm animal species (i.e., avian, bovine, and porcine). The data available to date could indicate that QREC are widespread in the global livestock population,

with great variation in occurrence between species and countries. The variation could arise from unidentified risk factors for QREC appearance.

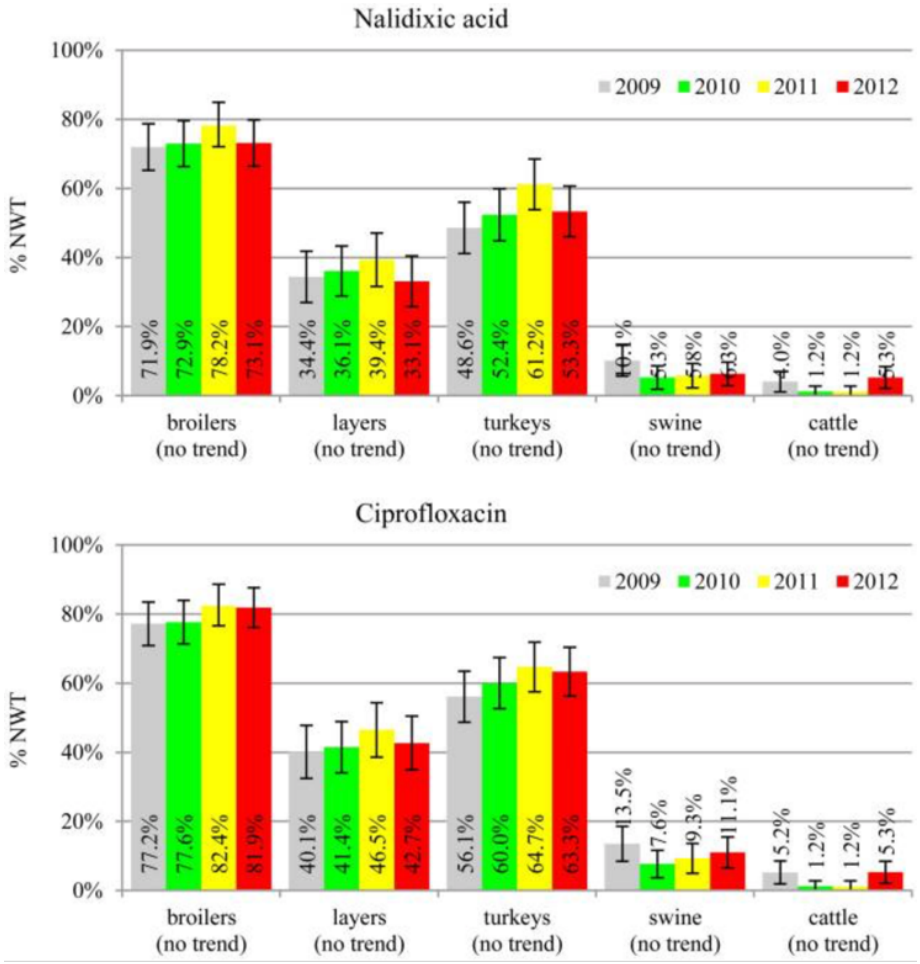


Figure 1.11 Fluoroquinolone resistance in *E. coli* isolated from animals at slaughter in Poland (Wasyl et al., 2013). In cattle isolates, nalidixic acid and ciprofloxacin resistance were observed in 3.0 and 3.3%, respectively

1.6 Weak mutators and farm management factors

1.6.1 Fitness cost

The distribution of fitness effects is a fundamental entity in genetics that describes the proportion of new mutations that are advantageous, neutral, or harmful. Quinolone resistance has been reported to have a deleterious impact on one or more bacterial growth parameters, suggesting that mutations causing quinolone resistance are generally costly for bacteria (Bhatnagar et al., 2019).

On the other hand, a study of *Salmonella typhi* found that a combination of mutations in the *gyrA* and *parC* genes results in higher fitness than in a scenario of independent fitness effects, suggesting that there is a synergistic interaction between the two mutations (Baker et al., 2013, Redgrave et al., 2014). Baker et al. and Redgrave et al., concluded that mutations associated with quinolone resistance induce significant fitness benefits in the absence of antimicrobial pressure. These results highlight that reducing the occurrence of quinolone resistance is more complicated than simply restricting the use of antimicrobial agents.

1.6.2 Weak mutators and SOS response

Weak mutations, where no single mutation has been found to confer clinical resistance by itself, could be of particular interest for quinolone resistance (Baquero et al., 2001, Örlén et al., 2006). Clinically resistant QREC usually have multiple mutations, implying those resistant lineages have undergone several mutation and selection cycles. The occurrence of weak hypermutator bacteria may reflect their enhanced ability to evolve resistance and persist longer than strong mutators without incurring major fitness costs (Örlén et al., 2006).

Prolonged survival under stress conditions may increase the mutation rate, allowing the emergence of more favourable mutations. Stressors well-established in the *E. coli* literature include nutrient starvation, pH downshifting, reactive oxygen and nitrogen species, membrane damage, hyperosmolarity, and non-optimal high and low temperatures (Poole et al., 2012). Additionally, quinolones are known stressors that increase the mutation rate and activate the SOS response (Qin et al., 2015, Baquero et al., 2001, Philips et al., 1987). Specific bacterial genes associated with the increased horizontal transfer, mutation rate, and DNA damage repair are categorised as taking part in the SOS response, a bacterial cell survival strategy. Activation of the SOS response is particularly important for ciprofloxacin resistance development in *E. coli* (Poole et al., 2012). Regardless, much remains unknown about the role of the SOS response in colonising the intestinal gut (Samuels et al., 2019).

In environments that change rapidly, variants that increase their mutation rates due to the activated SOS response could be selected, since they have an increased probability of acquiring beneficial mutations. Conversely, if the environment is constant, the mutation rate

tends to decrease as the organism becomes maximally adapted because of the costs associated with deleterious mutations (Baquero et al., 2004).

1.6.3 Management variables

Various management variables could interfere with the gastrointestinal microflora and potentially affect QREC shedding. Previous studies have linked environmental factors, including diet, to quinolone resistance in commensal *E. coli* (Boseman et al., 2014, Edrington et al., 2012). Various management factors which could affect *E. coli* as potential stressors are highlighted in the following subsections.

1.6.3.1 Diet

Diet composition is one of the most important factors influencing the structure and function of the gut microbiome (Turnbaugh et al., 2009). Diets change during the different stages of the cow's production cycle, from the maturation of young calves with shifts between milk-based, forage-based, and high-energy rations into cows with marked shifts in diet between the dry period and lactation. Within a few weeks, the forage-to-concentrate ratio can change markedly, resulting in a disturbed microflora vulnerable to competition from new bacterial species (Edrington et al., 2012).

Previous studies of bovine diet and MDR bacteria have focused on calves and the presence of factors related to milk feeding. The pasteurisation of waste milk significantly reduces the occurrence of MDR *E. coli* in calves (Edrington et al., 2012). Supporting literature from Sweden found that calves fed with waste milk were more likely to shed *E. coli* resistant to different antibiotics, including QREC (Duse et al., 2015). Also, the risk of QREC shedding increased when waste milk contained benzylpenicillin. This is of special interest in Norway, where penicillin is the most-selling antibacterial class for cattle (NORM/NORM-VET, 2020).

The primary part of a ruminant diet is plants. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Cowan et al., 1999). Flavonoids are bioactive and exhibit antimicrobial activity against Gram-negative bacteria (Omosa et al., 2013). Isoflavone, a component of flavonoids, targets topoisomerases I and II, one of the same targets as

quinolones, causing antimicrobial effects. Isoflavones are components typically found in plants used for cattle farming, such as peas, soy, and alfalfa (Aarnes et al., 2000). Plants' antimicrobial effects are well established. In contrast, cattle diets in regard to the risk for increased quinolone resistance are not well established.

Formic acid-based additives are an option for preserving silage material with minimal nutrient loss and are a common option for preserving feed for ruminants. Formic acid as a silage additive has an antibacterial effect on many bacterial species, decreasing bacterial counts and thus modifying the balance of bacterial communities in the intestine (Garrido et al., 2004). Garrido et al., (2004) found that organic acids modify the normal intestinal microbiota in monogastric animals like chickens. The effects of long-term exposure to organic acids on the normal microbiota are not well studied, and little is known about their effects on the ruminant microbiome.

Feed could also be a source of contamination by microorganisms, including enteric bacteria such as *E. coli* and *Salmonella*, which can carry and transmit AMR. Post-processing contamination may occur in transport and storage facilities, through wildlife access, or through the contamination of feeders (Taylor et al., 2009, Hoyle et al., 2005).

1.6.3.2 Farm intensity

Farm intensity does not have a final definition but rather is made up of complex assumptions about individual countries' politics and regional natural resources. Farm intensity could be broadly defined as farming with a high level of force to maximise yield in a given farming area (Almås, 2015). Wallenbeck et al., (2019) defined farm intensity as the level of milk yield and the feeding ratio. Milk yield and expected feed intake have a strong positive correlation (Kristensen et al., 1985). The forage-to-concentrate ratio is another important element of milk yield, and this ratio additionally impacts the occurrence of bloat and diarrhoea (Olsson et al., 1997). Changes in the digestive tract microbiome have been identified in cattle exhibiting diarrhoea (Zeineldin et al., 2018). Based on these results, it is tempting to hypothesise that intensive production systems could affect the cattle resistome. Idel et al., (2013) have reported another aspect: intensive production systems are closely associated with frequent veterinary treatments. More intensive management is believed to increase the usage of antimicrobials and heighten the risk of disseminating antimicrobial-resistant bacteria (Idel et al., 2013).

Evidence suggests that QREC can persist and spread within the cattle population despite a negligible usage of quinolone. However, the mechanisms and risk factors are still not fully understood. The negligible usage of fluoroquinolones in the Norwegian cattle production chain provides a unique platform for gaining insight into the risk factors and spread of quinolone resistance. The complex composition of farm management factors is of additional interest as a driver for quinolone resistance development. To the author's knowledge, farm intensity in relation to quinolone resistance has not been studied as a risk factor. It is of interest to study transmission in depth at the farm level. While the clonal dissemination of QREC from dairy calves has been studied, transmission from cows to their calves has not been described. This thesis will address some of these aspects.

2. Aims of the Study

Main objective

The primary objective of this study was to increase the understanding of the occurrence of quinolone-resistant *Escherichia coli* (QREC) in Norwegian cattle despite the negligible quinolone usage in cattle treatment, focusing on farm management risk factors and transmission within the farm and from cows to their calves.

Sub-objectives

1. To investigate differences in QREC occurrence between high- and low-intensive dairy farms

   Achieved by identifying the levels of QREC at different levels of farm intensities (Study I)

2. To investigate the dynamics of QREC occurrence and dissemination between cow and calf

   Achieved by sampling frequently for QREC over the first three months postpartum in both cows and calves (Study II)

3. To obtain knowledge about risk factors for QREC occurrence

   Achieved by comparing the prevalence of risk factors between QREC-negative and QREC-positive farms (Study I)

4. To gain insight into the antibiotic resistance phenotypes of QREC and the distributions of minimum inhibitory concentrations (MICs) related to quinolone resistance

   Achieved by MIC testing of isolated QREC (Study I and Study II)

5. To assess the genetic diversity and relatedness of QREC within and between dairy farms in Norway

   Achieved by phenotypic and genotypic analysis (Study I and Study II)

3. Materials and methods

3.1 Study design and populations

3.1.1 Study I

The study was conducted as a cross-sectional study with faecal sampling of calves from two study groups. Figure 3.1 summarises the selection process.

The study population in Study I was recruited from producers delivering milk to the dairy cooperative TINE according to the Norwegian Dairy Herd Recording System (NDHRS). The location was limited to six councils in Norway to avoid a transportation time of more than four hours from farm to laboratory. The study excluded farms with milk production between 5,001 litres and 8,499 litres to get a distinct difference between the low- and high-intensive farms.

The farms remained anonymous throughout the selection process. Contact information and names were first collected after the selection was complete. The selection continued until a total of 21 farmers from 11 high-yielding and 10 low-yielding farms responded to participate. The primary goal was to include 5 to 15 calves below three months from each farm in the study. Initial conversations with the farmers revealed the number of calves and their ages during the sampling period. These data were used to adjust the inclusion criteria to maintain the number of samples. Thus, a minimum level of three calves at each farm was determined, although only two farms had less than five calves at the sampling time. Also, the final inclusion criteria comprised calves younger than four months, although calves up to nine months old were included in three out of 20 farms.

All farmers received an informational letter by email before the visit. Eleven low-intensive and ten high-intensive herds were visited once during the study period. One low-intensive farm was excluded after sampling because it changed its production from dairy to beef during the sampling period.

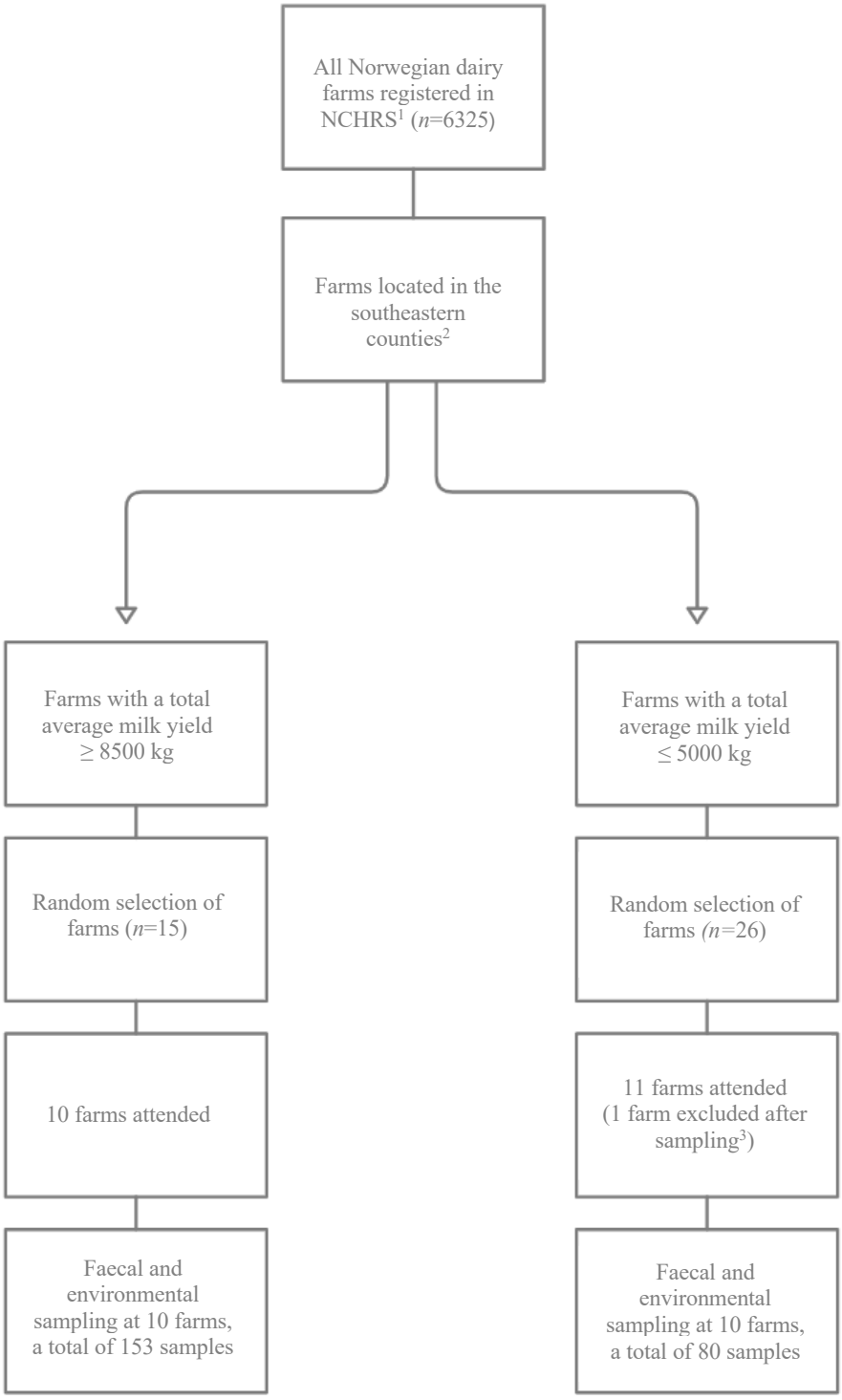


Figure 3.1 Flow diagram of the selection process of farms enrolled in Study I. Notes: [1] Norwegian Cattle Health Recording System (NCHRS). [2] Organisation of counties before 2020 Oslo, Akershus, Buskerud, Østfold, Hedmark, and Oppland were included. [3] One farm was excluded due to changing its production from dairy to beef during sampling.

3.1.2 Study II

The study was conducted as a cohort study, sampling cows and calves postpartum. Figure 3.2 summarises the selection process.

The study population of Study II was recruited from two dairy farms connected to the Faculty's Veterinary Out-Patient Clinic. The farms were located within a one-hour drive of the Faculty of Veterinary Medicine in Oslo to make frequent sampling feasible for one person. The participants were asked for the amount of calving expected during the second week of January 2018. The farmers permitted sampling every second week for three months. Pre-sampling was conducted in ten calves from both farms before the study was initiated to confirm QREC occurrence at the farm. The faecal samples collected for the study were taken from three cows and their respective three calves (Figure 3.2)

3.2 Contributions by the author and others

S.M.F. and A.M.B. conceived and planned the studies. A.M.B. and H.K. supervised the project. S.M.F. collected samples and data and performed the laboratory work. S.M.F., A.M.B., and H.K. contributed to the interpretation of the results. S.M.F., A.M.B., and H.K. analysed the data. H.K. performed the bioinformatics. S.M.F wrote this thesis under the supervision of A.M.B.

3.3 Sampling procedure

In Study I, calf faecal and environmental samples were collected to investigate the occurrence of QREC. In Study II, faecal samples from calves and their respective mother cows were sampled for the occurrence of QREC over time. The sampling for Study I was conducted between October 2017 and April 2018. The sampling for Study II started in January 2018 and lasted until April of the same year.

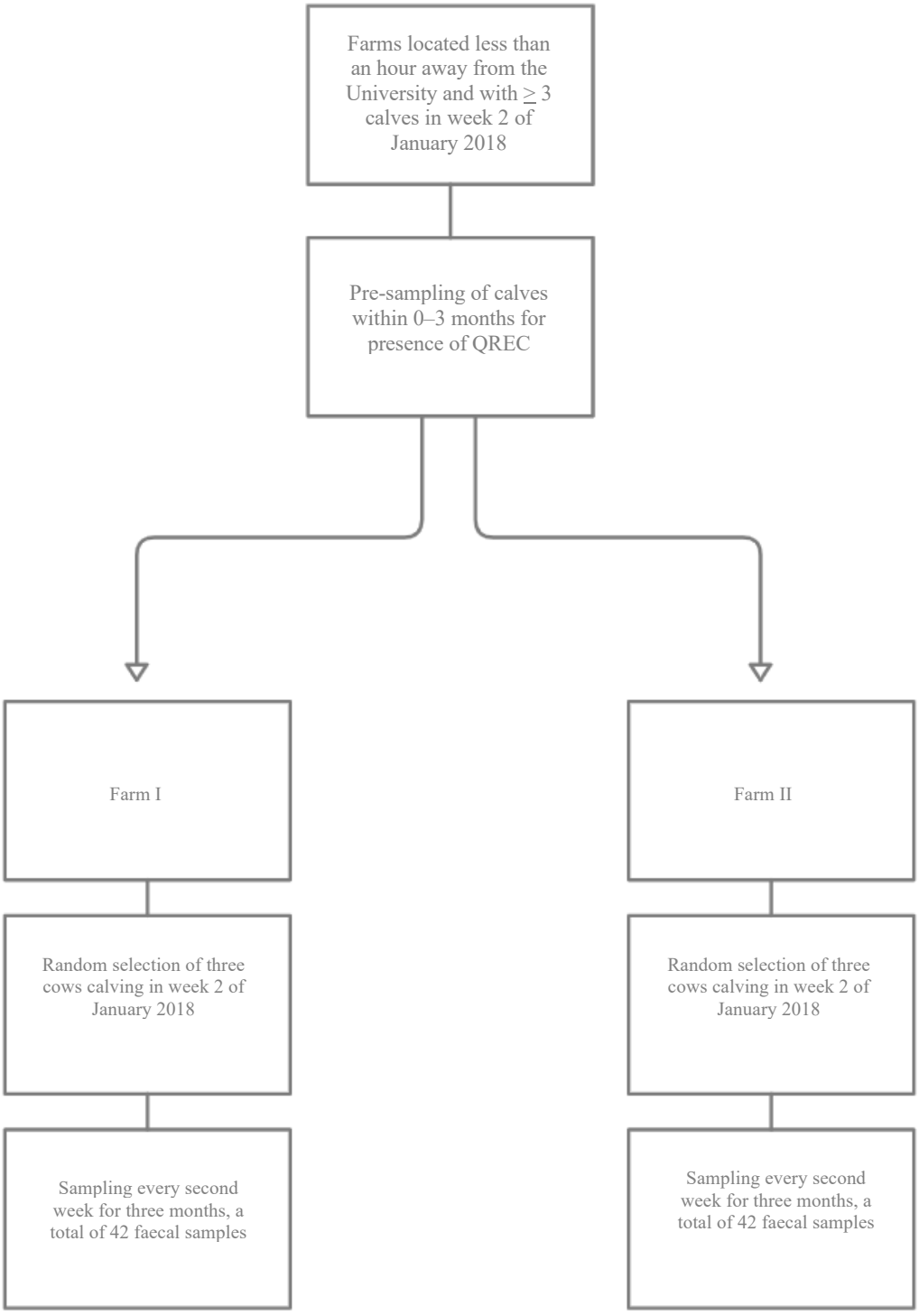


Figure 3.2 Flow diagram of the selection process for cattle enrolled in Study II.

3.3.1 Faecal samples

Faecal samples from individual calves were collected *per rectum*, within 2–5 cm of the rectal opening, and collected in plastic tubes containing Amies Charcoal Medium (VWR International, part of Avantor, Radnor U.S.). The samples were transported to the university by car and stored at 4°C until the analysis. Analysis was performed within 48 hours of sampling, in line with the provider's guidelines.

3.3.2 Environmental samples

A pre-moistened sterile cloth kept in a stomacher bag was used per sampling site for environmental sampling in Study I (Sodibox, Labolytic, Trondheim, Norway). Five specific targeted sites were swabbed at each farm, measured at approximately 0.25 m^2 for each spot. The given locations were (1) calving areas, (2) calf pens, (3) calf water buckets, (4) calf feed buckets, milk buckets, or automatic milk feeders, and (5) floors close to the sampled calves. The transport conditions were the same as with the faecal samples.

3.3.3 Determination of farm hygiene

The general hygiene at the farms was taken into consideration for Study I. The degree of faecal contamination at the environmental sample sites was scored with a three-point user-defined scale: (1) *clean* surfaces, (2) *intermediate* faecal contamination, and (3) *heavy* faecal contamination. Furthermore, the cleaning detergent used for cleaning the farm environment was registered.

The quality of the sluice gate was another aspect of farm hygiene. The functionality was rated on a four-point scale where the presence of physical separation between *clean* and *dirty* zones and access to water, clean wellies, and farm clothing were the basis for the assessment. Each farm was assigned a score: (1) *absent*, (2) *excellent*, (3) *good*, or (4) *insufficient*. The hygiene scores were used as descriptive data only (Appendix I).

3.4 Laboratory methods

3.4.1 Preparation, screening, and identification of QREC

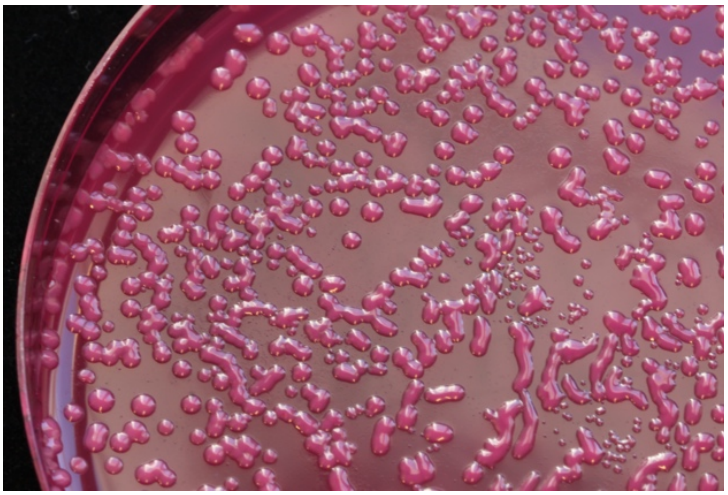
A total of 317 samples (213 faecal samples for Study I, 20 environmental samples for Study I, and 84 faecal samples for Study II) were incubated on MacConkey agar with 0.06 mg/L ciprofloxacin (The Norwegian Veterinary Institute, Oslo, Norway) for selective screening of QREC (Figure 3.3). Bromothymol blue agar (in-house) was used as a QREC non-selective agar. Faecal swabs were transferred to Falcon tubes (Sarstedt 15 ml, Thermo Fisher Scientific Co. L.L.C, USA) with 4.5 ml buffered peptone water (PBW) and mixed using a vortexer (Reax top, Heidolph, Germany) to generate the 10^{-1} dilution. For environmental samples, 100 ml of BPW were added to each sample kept in stomacher bags before mixing it in a stomacher (Star-Blender^{TM} LB 400, VWR, France) for 30 seconds. Then, 1 ml was transferred to a collecting tube to generate the 10^{-1} dilution. Serial dilutions of faecal and environmental samples in isotonic saline were made, and 100 µl were transferred for the final dilution of 10^{-5}. Thereafter, 100 µl were spread on the agar with a sterile, bent plastic rod. Dilution factors (DFs) 10^{-2} and 10^{-3} were used for QREC detection on selective agars. A higher DF was used to quantify commensal *E. coli* on a non-selective plate, 10^{-2} and 10^{-5}. The dilutions were performed in duplicate, and the stock solution was incubated overnight at 37°C. Additionally, the 10^{-1} dilution was incubated overnight at 37°C and then spread on MacConkey agar with 0.06 mg/L ciprofloxacin to detect QREC after enrichment.

Colony-forming units (CFUs) of lactose-positive colonies with typical *E. coli* morphology were counted on QREC-selective and QREC non-selective agar plates. Mean within-sample prevalence of QREC was calculated by dividing the total number of counted CFUs on QREC-selective agar by the total number of counted CFUs on QREC non-selective agar and correcting for dilution factor. An *E. coli* colony was defined as resistant if it grew on selective media containing 0.06 mg/L ciprofloxacin. A total of 55 isolates of QREC were isolated from the agars based upon morphological selection. Resistance was further verified by microdilution with SensiTitre by utilising the ECOFF values defined by EUCAST.

Presumptive *E. coli* colonies were randomly chosen and sub-cultured on blood agar at 37°C overnight. Then, presumptive *E. coli* isolates were tested for production of tryptophanase (indole test) and citrate for first-step species identification. Lactose- and indole-positive and citrate-negative isolates with typical colony morphology (bright pink on MacConkey agar, yellow in Bromothymol blue agar, and a medium-sized [2–3 mm in diameter] opaque morphology) were considered to be *E. coli*. API-20E kits (bioMérieux, Inc., North Carolina, USA) were used in a few situations where colony morphology and biochemical tests were inconsistent. Random and confirmed isolates of *E. coli* were transferred to a 2-mL microtube containing 0.8 mL 50% glycerol and stored at -80°C. For further accuracy of the assumed *E. coli* isolates, matrix-assisted laser desorption ionisation–time of flight (MALDI–TOF, Microflex, Bruker100 Daltonik GmbH) was performed.

Thirty and 25 possible *E. coli* isolates were stored from Study I and Study II after macromorphological and biochemical tests, respectively. All 55 isolates were identified as *E. coli* by MALDI–TOF; however, two isolates from Study II were lost and thus not included in MIC testing. Twenty-one isolates from Study I and all 23 isolates from Study II had MIC values above the epidemiological cut-off and were identified as QREC isolates. All 30 isolates (including isolates above and below cut-off) were whole-genome sequenced, while a random selection of six isolates from Study II was selected for WGS due to cost limitations. The six isolates from Study II provided a restricted overview of genotype variation in these isolates. The isolates were chosen to provide further understanding of genetic diversity in different isolates from calves and cows at the same farm, as well as changes in isolates over time in a single calf. Then, a random selection from the other farm was made. Twenty-one *E. coli* isolates from Study I had MICs for ciprofloxacin above the cut-off, whereas 19 isolates from Study II had MICs above the epidemiological cut-off and were identified as QREC. To compensate for a low amount of WGS, all isolates from Study II were *gyrA* sequenced. In three isolates, *gyrA* was not found by conventional PCR.

Selection for WGS was intended to provide further insight into within-farm genomic variation given a limited selection of isolates. Isolates with similar phenotypic resistance patterns were chosen. In addition, an isolate with a MIC just below the epidemiological cut-off from the same farm, and a random isolate from the other farm, were included.



QREC-selective MacConkey agar with the growth of *E. coli* after enrichment overnight at 37°C. Picture: Silje Mogstad Finstad.

Bromothymol blue agar non-selective for QREC with a mixed culture. Suspected lactose-positive *E. coli* are yellow. Picture: Silje Mogstad Finstad.

3.4.2 Antimicrobial susceptibility testing

The *E. coli* isolates that grew on quinolone-selective agar were tested for a panel of 14 antimicrobial agents to obtain accurate MICs. Sensititre (Sensititre™ TREK Diagnostics, LTD) based on Mueller Hinton broth dilution was used, with each plate pre-dosed with antimicrobial agents at appropriate dilutions. The panel included the following antimicrobial agents: sulfamethoxazole (8–1024 mg/L), trimethoprim (0.25–32 mg/L), ciprofloxacin (0.015–8 mg/L), tetracycline (2–64 mg/L), meropenem (0.03–16 mg/L), azithromycin (2–64 mg/L), nalidixic acid (4–128 mg/L), cefotaxime (0.25–1 mg/L), chloramphenicol (1–16 mg/L), tigecycline (0.25–8 mg/L), ceftazidime (0.5–8 mg/L), colistin (1–16 mg/L), ampicillin (1–64 mg/L), and gentamicin (0.5–32 mg/L). The procedure was conducted according to the manufacturer's protocol, and MIC values were determined manually by visual reading of growth.

3.4.3 Preparations for *gyrA* sequencing

DNA extraction and conventional PCR were used to amplify the *gyrA* gene before Sanger sequencing. Twenty QREC isolates sampled in Study II were included in this analysis. DNA was extracted by transferring a few colonies to 500 µl of PCR-grade water in an Eppendorf tube. The suspension was then mixed well and boiled at 100°C for 10 min, followed by

centrifugation at 13,000 rpm (MiniSpin®, Eppendorf, Germany) for 2 minutes. The eluted DNA was stored at -20°C until further processing.

All isolates were screened for the presence of the *gyrA* gene by conventional PCR, using PCR primers as described by Oram et al., (1991). PCR reagents are listed in Table 3.1, and PCR conditions are described in Table 3.2. Subsequent gel electrophoresis was performed using a 1.5% agarose gel in 1X TBE buffer at 90 V for 60 minutes. The PCR products were stained using SYBR® Safe dye added during the agarose gel preparation and visualised using a GelDoc imager. Positive *gyrA* products were cut out and cleaned using QIAquick PCR Clean-up Kit according to the manufacturer's protocol and stored at -20°C until transport for Sanger sequencing (Eurofins GATC Biotech, Ebersberg, Germany). The DNA sequences of amplified *gyrA* genes were used to determine the mutations encoding quinolone resistance in the quinolone resistance-determining region (QRDR).

Table 3.1 PCR reagents

Reagent	**Volume (per sample) in µl**
PCR-buffer	5
MgCl2	1,5
dNTP-mix	1
Taq	0,4
Primer F (TACACCGGTCAACATTGAGG)	2,5
Primer R (TTAATGATTGCCGCCGTCGG)	2,5
H_2O	32,1
Template	5
Total	50

Table 3.2 PCR conditions

Cycles	**Temperature (°C)**	**Time**
	94	5 m
	92	25 s
30	64	1 m
	74	2.5 m
	74	5 m

3.4.4 DNA extraction for whole-genome sequencing (WGS)

All 30 isolates from Study I and six selected isolates from Study II were sub-cultured from MacConkey to blood agar before extraction with the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. The DNA concentration was determined using the broad-range DNA Qubit assay (Qubit™, Thermo Fisher Scientific, USA), and DNA quality was assessed using the NanoDrop (NanoDrop™ One/OneC, Thermo Scientific, USA). A Fragment Analyzer automated capillary electrophoresis system instrument (catalogue number FSV2-DE2-100; Advanced Analytical) and gel electrophoresis were used to determine DNA integrity.

3.4.5 Library preparation and sequencing

Library preparation and sequencing were done in two sets, with the sequencing service first provided (26 isolates) by the Norwegian Sequencing Centre (Oslo, Norway) and thereafter (10 isolates) by the Norwegian Veterinary Institute (Ås, Norway) for practical purposes.

The Norwegian Sequencing Centre performed a Nextera Flex library preparation using the quality-controlled DNA, spiked with PhiX for quality control, followed by sequencing on a HiSeq 3000 instrument (Illumina). The sequencing resulted in paired-end reads of 150 bp. Quality-controlled DNA was used by the Norwegian Sequencing Centre for Nextra Flex (Illumina) library preparation and sequencing in a HiSeq 3000 instrument (HiSeq3/4000 NexteraFlex) and was spiked with PhiX for sequencing quality control, resulting in paired-end reads of 150 bp. The remaining isolates were sequenced at the Norwegian Veterinary Institute using Nextera Flex library preparation, spiking with PhiX, and a MiSeq instrument, resulting in paired-end reads of 300 bp.

3.4.6 Quality control and contaminant screening

Sequences were quality controlled using FastQC (https://www.bioinformatics. babraham.ac. uk/projects/fastqc/) version 0.11.9. Mash (DOI:10.1186/s13059-019-1841-x) version 2.2.2 was used to screen for potential contaminants. A minimum identity value was set at 0.95. Bacterial species other than *E. coli* at levels above this threshold were deemed contaminants, and their samples were excluded from further analysis.

3.4.7 Antimicrobial resistance gene identification and MLST

Antimicrobial Resistance Identification by Assembly (ARIBA) (DOI: 10.1099/mgen.0.000131) version 2.12.1 was used to determine the presence of mutations, acquired resistance genes, and STs from raw reads. The QRDR of *gyrA, gyrB, parC*, and *parE* were investigated for mutations using the MegaRes database (DOI:10.1093/nar/gkw1009). Additionally, the presence of PMQR genes such as *qnr*, *oqxAB,* and *qepA* were determined using the ResFinder database (DOI:10.1093/jac/dks261). STs were determined using the multi-locus sequence typing (MLST) scheme hosted by EnteroBase (DOI:10.1111/j.1365-2958.2006.05172.x)

3.4.8 Genome assembly

Quality-controlled reads were trimmed and adapters removed using Trimmomatic (DOI:10.1093/bioinformatics/btu170) version 0.39, followed by PhiX removal using BBDuk (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/) version 38.76. The trimmed and filtered reads were assembled using SPAdes (DOI: 10.1089/cmb.2012.0021) version 3.14.0, using the “careful” option. The quality of assembly was determined using QUAST (DOI: 10.1093/bioinformatics/btt086) version 5.0.2.

3.4.9 Phylogenetic analysis

A core-gene phylogenetic reconstruction was performed to determine the evolutionary relationships between the isolates. First, the assembled genomes were annotated using Prokka (10.1093/bioinformatics/btu153) version 1.14.6. Then, the pangenome was determined with Panaroo (DOI: 10.1186/s13059-020-02090-4) version 1.2.2. The predicted core genes for

instance, genes that were present in at least 99% of the genomes were aligned and concatenated with MAFFT (DOI: 10.1093/nar/gkf436) version 7.464. IQ-Tree (DOI: 10.1093/molbev/msu300) version 1.6.12 was used to generate a maximum-likelihood phylogenetic tree from the core-gene alignment using ModelFinder plus (DOI: 10.1038/nmeth.4285) and ultrafast bootstrapping (DOI:10.1093/molbev/msx281). Pairwise single nucleotide polymorphism (SNP) distances were calculated with snp-dists (https://github.com/tseemann/snp-dists) version 0.6.3. Phylogenetic trees were visualised using R (R Core Team) version 3.6.2 and the ggtree package (DOI 10.1002/cpbi.96, 10.1093/molbev/msy194, 10.1111/2041-210X.12628.).

A phylogenetic analysis with a higher resolution was used in Study II. Here, the core genome was determined using ParSNP (DOI: 10.1186/s13059-014-0524-x) version 1.5.3. Then, recombinant areas were identified and masked from the alignment using Gubbins (10.1093/nar/gku1196) version 2.4.1 and maskrc-svg (https://github.com/ kwongj/maskrc-svg) version 0.5, respectively. IQ-Tree was subsequently used to reconstruct the phylogeny from the masked alignment similarly described above. Pairwise SNP distances were calculated with snp-dists.

3.5 Statistical methods

Data were entered in a Microsoft Excel spreadsheet (Microsoft Corporation) and transferred to R software (R 3.6.2, R Core Team, Austria). High- and low-intensive farms were compared for the general occurrence of QREC, which was analysed using Fisher's exact test. A *p*-value of less than or equal to 0.05 was considered significant, and the study was designed with a statistical power of 0.8 (Table 3.3). A correlation matrix for changes in QREC occurrence over time was analysed with Spearman's rank correlation coefficient.

Table 3.3 Power analysis for Study I

	Farms	**Sample size**
Sample size 1 (n1)	10	5-15 calves
Sample size 2 (n2)	10	5-15 calves
Total sample size (both groups)	20	100- 300 samples
Proportion 1	0,6	

Proportion 2	0,3
Confidence levels	0,95
Power	0,8
Ratio of sample sizes (n2/n1)	1
Tails	2
Sample size estimated to	107 samples
level of statistical significance	< 0.05

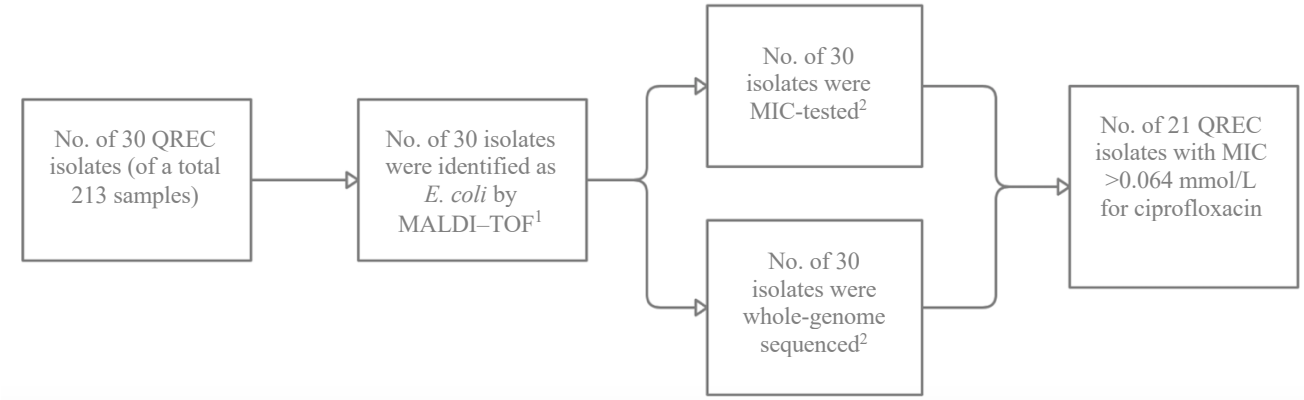


Figure 3.3 Flowchart presenting the number of isolates in different methodology stages included in Study I.

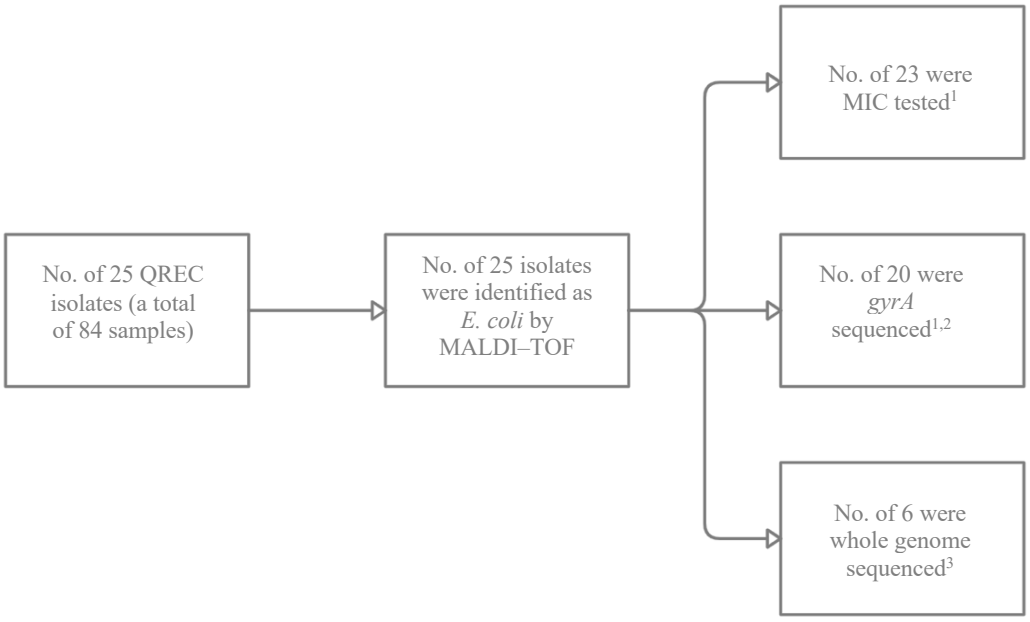


Figure 3.4 Flowchart presenting the number of isolates for different methodology stages included in Study II. [1] Two isolates were lost and thus not stored for further analysis. [2] In three isolates, the *gyrA* sequence was not found by conventional PCR. [3]Only six isolates were whole-genome sequenced due to cost limits.

4. Results

4.1 Study I

4.1.1 Prevalence of QREC

QREC were identified in 9.8% of all faecal samples (21/213) from calves irrespective of production intensity (defined by milk yield at a farm level). Out of 30 faecal samples with growth on selective agar, nine samples were found to have MIC values for ciprofloxacin below the ECOFF values defined by EUCAST (0.064 mmol/L). Interestingly, QREC were more abundant in high-intensive farms where 13.3% (19/143) of all calves sampled carried QREC compared to 2.8% (2/70) of the calves from low-intensive farms. A farm was defined as QREC positive if QREC were identified from one or more calves. Within the high-intensive group, QREC were identified in seven out of 10 farms (70%). In contrast, in the low-intensive group, QREC were isolated from two out of 10 farms (20%). The higher occurrence of QREC identified in high-intensive farms compared to low-intensive farms was significantly different ($p = 0.0322$). Contingency tables are visualised in Table 4.1. QREC were isolated from two out of 20 environmental samples, and both samples were from high-intensive farms in which calves were also found to be QREC positive.

Table 4.1 Contingency table was used to summarise the relationship between the occurrence of QREC and farm intensity. For the analysis, a Fisher`s exact test was conducted. The higher occurrence of QREC identified in high-intensive farms compared to low-intensive farms was significantly different ($p = 0.0322$).

	QREC negative	QREC positive
High-intensive farms	3	7
Low-intensive farms	8	2

The prevalence of calves shedding QREC differed from 7 to 53% at a farm level. The three farms with the highest within-sample prevalence of QREC had the highest prevalence of QREC-shedding calves and were all within the high-intensive group (Table 4.2). The average within-sample prevalence for these three farms was 5.53% QREC among the total number of *E. coli.* Except for these three farms, QREC were only found after enrichment and incubation

overnight, indicating a low proportion of QREC in the intestinal microflora. Generally, low-level quinolone resistance was found in *E. coli* isolated from the calf faecal samples, with an MIC value for ciprofloxacin between 0.12 mg/L and 0.5 mg/L. High MIC values of 8 mg/L for ciprofloxacin and 128 mg/L for nalidixic acid were found only at one farm in Study I, which was among the high-intensive farms. Surprisingly, ciprofloxacin-sensitive bacteria (MIC $\leq$ 0.06 mg/L) in samples from both groups grew on the selective agar. This false-positive QREC phenomenon was more predominant within the low-intensive group. Of the faecal samples with growth on selective agar, 77% were later found to be sensitive to quinolones compared to 4.8% within the high-intensive group.

Table 4.2 Descriptive statistics of studied farms: the proportion of calves shedding quinolone-resistant *Escherichia coli* (QREC) in faeces, the mean within-sample prevalence of QREC among calves, and the median MIC distribution. Each row represents a farm. Only isolates of clinically resistant QREC with MIC for ciprofloxacin above 0.064mg/L are included in this table.

Farm category	Farm prevalence (QREC- positive samples/total no. of samples tested) %	No. of QREC positive/no. tested	Mean within – sample prevalence of QREC (no. QREC / total *E. coli*) %	Median MIC values (mg/L) for ciprofloxacin	[Q1-Q3]	Median MIC values (mg/L) for nalidixic acid	[Q1-Q3]
H	53	8/15	2.4	8	[8-8]	129	[8-129]
	7	1/15	<0.01[1]	0.25	[0.25-0.25]	8	[8-8]
	7	1/14	<0.01[1]	0.25	[0.25-0.25]	64	[64-64]
	27	4/15	11.5	0.25	[0.25-0.37]	64	[64-96]
	21	3/14	2.7	0.25	[0.25-0.25]	128	[64-128]
	0	0/13	-	-	-	-	-
	0	0/15	-	-	-	-	-
	0	0/14	-	-	-	-	-
	7	1/14	<0.01[1]	0.12	[0.12-0.12]	32	[32-32]
	8	1/13	<0.01[1]	0.12	[0.12-0.12]	64	[64-64]
L	0	0/3	-	-	-	-	-
	0	0/9	-	-	-	-	-
	0	0/9	-	-	-	-	-
	0	0/8	-	-	-	-	-
	0	0/6	-	-	-	-	-
	0	0/4	-	-	-	-	-
	0	0/6	-	-	-	-	-
	0	0/5	-	-	-	-	-
	8	1/12	<0.01[1]	0.5	[0.5-0.5]	128	[128-128]
	11	1/9	<0.01[1]	0.12	[0.12-0.12]	64	[64-64]

Abbreviation: H=high-intensive farms. L=low-intensive farms. Q=quantiles 1: 25% and 3: 75%. A hyphen (-) is used for farms where no QREC were found. [1] QREC detected only after incubation in enriched media overnight, classified with a relative abundance of <0.01% in the faecal material.

4.1.2 Antimicrobial susceptibility testing

Of all QREC isolates identified, 14.3% showed only resistance against ciprofloxacin and nalidixic acid (CiNal). Among the low-intensive farms, resistance against CiNal was the only phenotypic resistance profile found among the QREC isolates. The CiNal profile was also found in QREC isolated from one high-intensive farm. This study's most common phenotypic co-resistance pattern was resistance to ciprofloxacin, nalidixic acid, ampicillin, sulfamethoxazole, tetracycline, and trimethoprim (CiNalAmpSuTcTm), which represented 42.9% of the QREC isolates (Table 4.4).

Table 4.3 Distribution of MICs of different antimicrobial classes for *E. coli* isolated from selective agar plate with 0.06mg/l ciprofloxacin. This table include all isolates isolated from the selective agar plates, which involve isolates below the epidemiological cut-off for ciprofloxacin. The distribution visualizes a low level of ciprofloxacin resistance *E. coli* isolated within the low intensive (L) farms, 22.2% of the isolated *E. coli* were found resistant within the L-group. Whereas 95.2% of the isolates within the high-intensive (H) farms were found resistant to ciprofloxacin. The L-farms had a lower occurrence of resistance to other antimicrobial classes than the H-farms. Resistance to a greater verity of antimicrobial classes were found among the isolates from the H-farms, where resistance against six other antimicrobial classes were detected (ampicillin, chloramphenicol, gentamicin, sulfamethoxole, tetracycline, trimethoprim), compare to three classes detected among the isolates for from the L-farms (chloramphenicol, sulfamethaxazole and tetracycline).

Farm cat.	Substance	Total nr. of sampels	Resistance (%)	Distribution (%) of MIC values (mg/L)*																
				0.0075	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	2048
H	Ampicillin	21	81									4.8	9.5	4.8				81		
H	Azithromycin	21	0										85.7	4.8	9.5					
H	Chloramphenicol	21	14.3										81		4.8				14.3	
H	Ciprofloxacin	21	95.2	4.8				9.5	38.1	4.8				42.9						
H	Colitstin	21	0							95.2		4.8								
H	Cefotaxime	21	0					100												
H	Gentamicin	21	23.8						61.9		9.5	4.8				14.3	9.5			
H	Meropenem	21	0		100															
H	Nalidixic acid	21	90.5									4.8		4.8		4.8	33.3	9.5	42.9	
H	Sulfamethoxazole	21	81										9.5			9.5				81
H	Ceftazidime	21	0						100											
H	Tetracycline	21	81								19						47.6	33.3		
H	Tigecycline	21	0				95.2		4.8											
H	Trimpethoprim	21	61.9					19		19							61.9			
L	Ampicillin	9	0									33.3	33.3	33.3						
L	Azithromycin	9	0										44.4	33.3	22.2					
L	Chloramphenicol	9	22.2										33.3		33.3	22.2				
L	Ciprofloxacin	9	22.2	22.2		11.1	44.4	11.1		11.1										
L	Colitstin	9	0							88.9		11.1								
L	Cefotaxime	9	0					100												
L	Gentamicin	9	0						100											
L	Meropenem	9	0		100															
L	Nalidixic acid	9	33.3									55.6		11.1		11.1	11.1	11.1		
L	Sulfamethoxazole	9	11.1										44.4		22.2	22.2				11.1
L	Ceftazidime	9	0						100											
L	Tetracycline	9	11.1								44.4		44.4					11.1		
L	Tigecycline	9	0					100												
L	Trimpethoprim	9	0					11.1		88.9										

*Bold vertical lines denote epidemiological cut-off values for resistance. White fields denote range of dilutions tested for each antimicrobial agent. MIC values higher than highest concentration testes are given as the lowest MIC value above the range

Table 4.4 MICs for quinolones and phenotypic resistance patterns for QREC isolates from calves within high- and low-intensive farms. The nine isolates with the highest MIC values for quinolones were found within the same high-intensive farm. The phenotypic resistance pattern indicates mainly multidrug-resistant isolates from high-intensive farms, while the isolates from the low-yielding farms were only quinolone resistant.

Farm category	No. of isolates	CIP mg/ml	NAL mg/ml	Phenotypic resistance pattern by Sensititre ®
H	9	8	>128	CipNalAmpSuTcTm
H	3	0.25	64	CipNalAmpGenSuTcTm
H	1	0.5	128	CipNalAmpGenSuTcTm
H	1	0.25	128	CipNalAmpChlGenSuTc
H	1	0.25	64	CipNalAmpChlGenSuTc
H	1	0.25	128	CipNalAmpSuTc
H	1	0.25	64	CipNalSuTc
H	1	0.12	64	CipNalAmpChl
H	1	0.12	32	CipNal
L	1	0.5	4	CipNal
L	1	0.12	64	CipNal

Three QREC isolates (14.3%) were found to be resistant to ciprofloxacin, nalidixic acid, ampicillin, sulfamethoxazole, trimethoprim, tetracycline, and gentamycin (CiNalAmpGenSuTcTm). Two QREC isolates (9.5%) were identified with a resistance pattern for ciprofloxacin, nalidixic acid, ampicillin, chloramphenicol, gentamycin, sulfamethoxazole, and tetracycline (CiNalAmpChlGenSuTc). Four phenotypic resistance patterns were only found once: CiNalSuTc, CiNalAmpGenSuTcTm, CiNalAmpSuTc, and CiNalAmpChl.

4.1.3 Dissemination and genetic diversity of QREC

Similar phenotypic resistance profiles were identified between QREC isolates from the same farm; however, the profiles diverged between farms. Several phenotypic resistance patterns were identified at two high-intensive farms, and in both cases, a dominant phenotype was represented. QREC were isolated from the farm environment in two farms from the high-intensive group. The faecal and environmental QREC isolated from the same farm showed similar phenotypic resistance profiles and STs, ST 162 and 69 (Figure 4.1)

In all QREC isolates, point mutations were located only in the QRDR, and we did not detect PMQR genes in Study I (Figure 4.1). All 21 QREC isolates were found to have a mutation in *gyrA,* either a single (57.1%) or a double mutation (42.9%). Additionally, a single point mutation in *parC* was found in 66.7% of the isolates. Single point mutations in *gyrA* were located at either S83L (90.5%), D87N (42.9%), D87Y (4.8%), or D87G (4.8%). In five QREC

isolates (23.8%), a single mutation in *gyrA* at S83L was detected together with a mutation in *parC* at S57T. Double point mutations in *gyrA* were found in nine isolates (42.9%) at S83L and D87N, all accompanied with a single mutation in *parC* at S57T. QREC isolates carrying both the double *gyrA* and the single *parC* mutations demonstrated a higher MIC value for ciprofloxacin and nalidixic acid than QREC isolates with only a single mutation detected. All nine QREC isolates with a double mutation in *gyrA* exhibited MIC values for ciprofloxacin of 8 mg/L and nalidixic acid of above 128 mg/L.

Eight unique sequence types (STs) were identified by the core-gene single nucleotide polymorphism (SNP) alignment. All STs were unique to their farm except for ST69, which was identified at two farms. The most abundant ST was ST162 (42.9%, mean distance 15.7 SNPs), which was from the farm with the highest farm prevalence of QREC (Table 4.2). The following most common isolates were ST69 (19%, mean distance 4,425 SNPs) and ST925 (14.3%, mean distance 0 SNPs). Other STs included ST718, ST655, ST300, ST329, and ST301. Thus, the results of the SNP alignment indicate a clonal distribution within the farm.

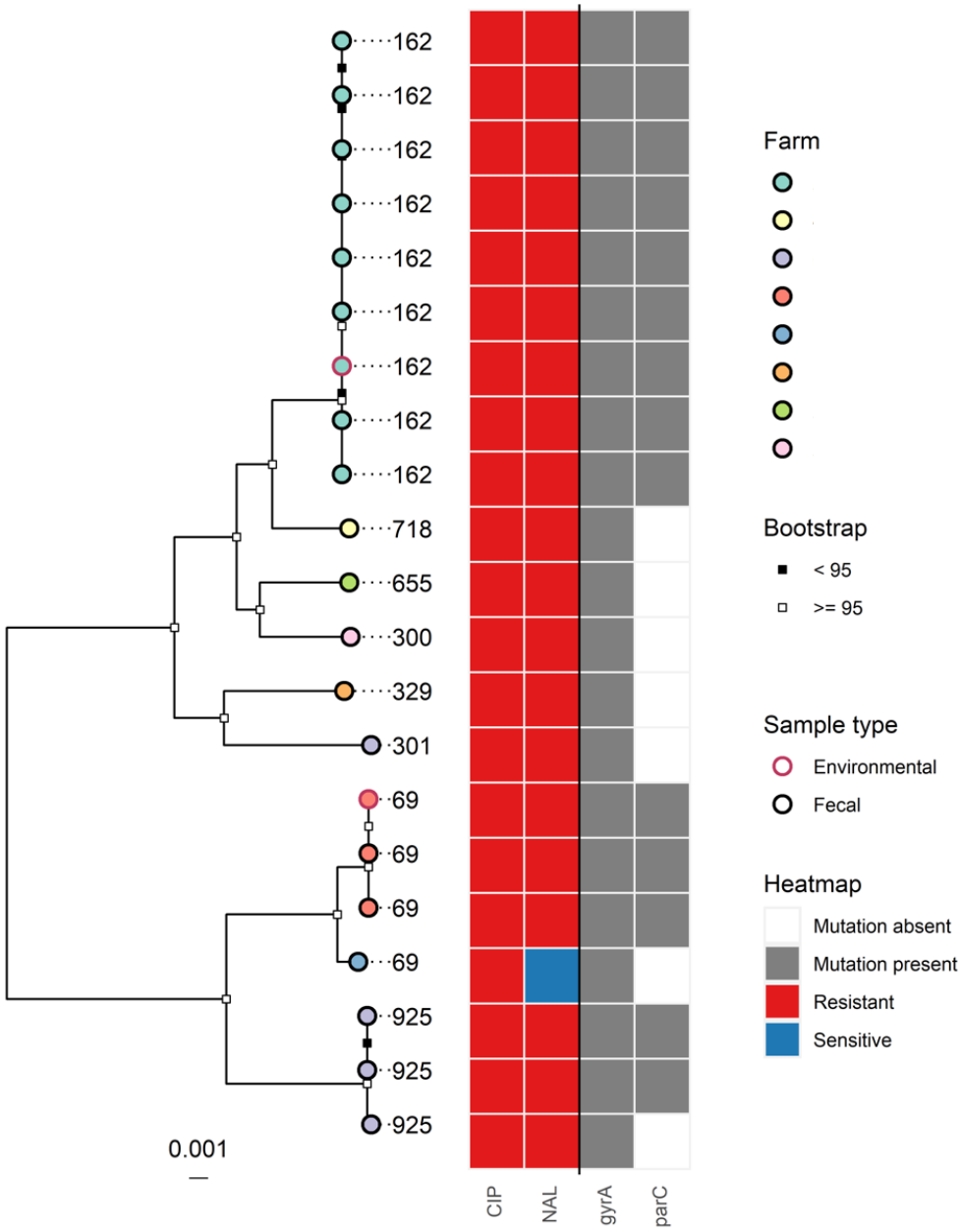


Figure 4.1 Phylogenetic analysis and genetic characterization of QREC isolates are displayed in a maximum-likelihood core-gene SNP tree generated with IQ-Tree (ModelFinder). The sequence type and the coloured circles represent a QREC isolate from a given calf, where the colour depicts the farm origin. The cluster heat map combines the level of resistance to ciprofloxacin and nalidixic acid with the occurrence of mutations in the quinolone resistance-determining region (QRDR). Mutations within the QRDR are highlighted with a grey square if present and a white square if not present. No mutations were found in *gyrB* or *parE*, and no plasmid-mediated quinolone-resistant genes were detected (*qnr, opxAB,* or *qepA*). QREC were found in the environmental samples at two farms, and these environmental isolates are encircled with red. The branch nodes represent the confidence level greater or less than 0.95, coloured in white and black, respectively.

4.1.4 Farm-level variables

Farm variables collected by the questionnaire (Appendix I) are listed in Table 4.5. Questions related to concentrate level and type of product were excluded from the list due to unconcise answers, as well as infrequent usage of concentrate among the calves and changes in types. All responses from the questionnaire are available from the author upon request. Statistical analyses were not performed on the risk factor data obtained from the questionnaire due to the small study population size and a wide range of potential confounding variables.

4.1.4.1 Herd size

A large difference in herd size was found between the high- and low-intensive farms (Figure 4.2). High-intensive farms had between 17.5 and 69 productive cows per year, compared to low-intensive farms with 8 to 20 productive cows per year. Because of the smaller herd size, a lower number of calves was available for sampling within the group of low-intensive farms.

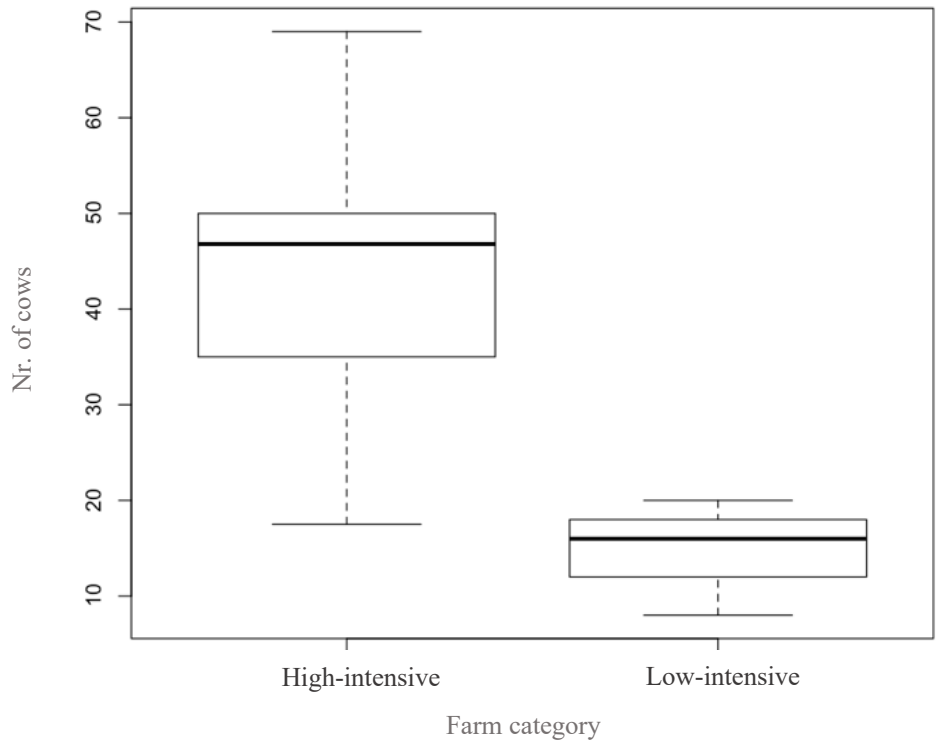


Figure 4.2 Boxplot of the two studies categories high- and low-intensive farms by herd size in number of cows (*y*-axis), visualising the difference in herd size within these two study groups. The boxplot demonstrates the variation in herd size between these two study groups. Whereas low-intensive farms represent small farms below the national average herd size, high-intensive farms have an average herd size considerably larger than the average.

4.1.4.2 Age

At sampling, the calves were on average 56.4 days old. Calves with QREC-positive samples were all less than 75 days of age (on average 30.1 days old). Calves with QREC-negative samples were on average 96.3 days old (Figure 4.3). Calves from high-intensive farms were on average 44.3 days old, whereas calves from low-intensive farms were on average 67.2 days old.

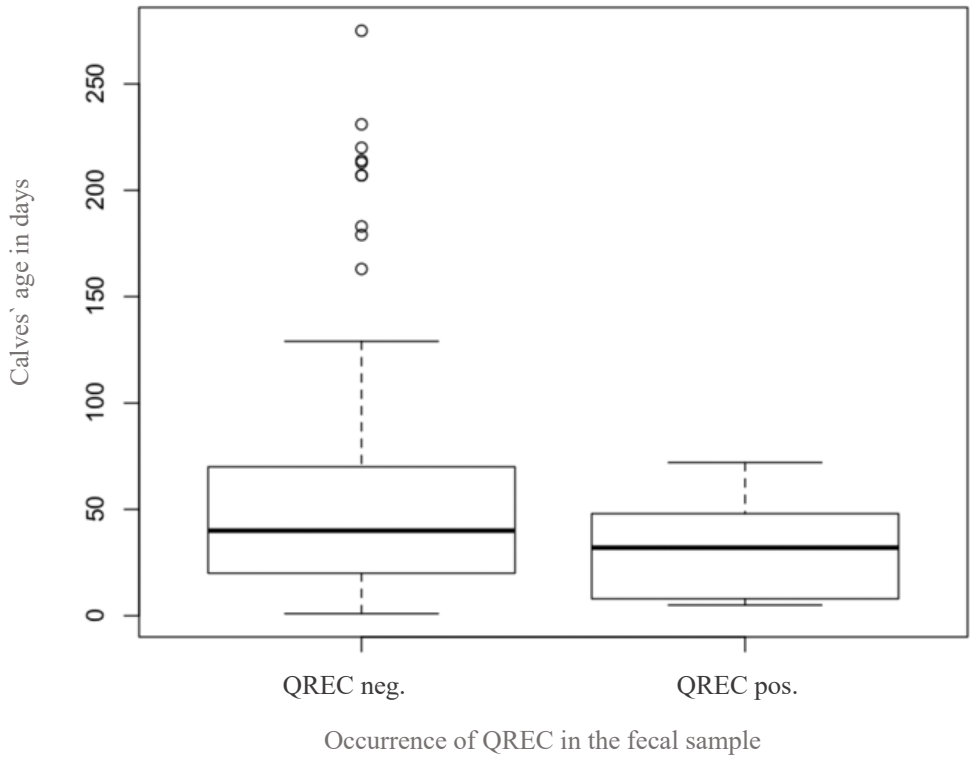


Figure 4.3 Boxplot with the level of detection (*x*-axis) related to the individual calves' age in days (*y*-axis). This boxplot indicates that QREC-positive samples were found in calves younger than 100 days, while QREC-negative calves were found at all ages.

4.1.4.3 Forage conservation

Descriptive information about forage conservation was included in the questionnaire, including forage additives and organic/non-organic status. Most QREC-positive farms (77.8%) used additives for forage conservation. Formic acid was most frequently used (66.7%), followed by the bacterial additive Sill-All® (44.4%). Two of these nine QREC-positive farms used both Sill-All® and formic acid. In contrast, no forage conservation was predominant in farms where no QREC were detected. Of these QREC-negative farms, a proportion used formic acid or Sill-All®, 36.3% and 18.2%, respectively. Non-organic farms

were overrepresented within both study groups (in total, 70% non-organic farms were included). Organic farms were only found among the low-intensive group, and QREC were not detected among the these farms (Table 4.5).

4.1.4.4 Housing and hygiene

This study included ten farms with loose housing systems and ten with tie-stall systems. Loose housing dominated within the high-intensive group (90%), while the low-intensive group mainly had tie-stall housing (90%). QREC-positive calves were housed within both systems, with 66.7% of the QREC-positive farms having loose housing and 33.3% having tie-stall housing. A contradictory phenomenon was found for the QREC-negative farms, where 63.7% had tie-stall and 28.2% loose housing. Nevertheless, the farm with the highest prevalence of QREC had a tie-stall system.

Quantitative hygiene scoring based on the farm environment and the visitors' entrance (disinfection sluice) was done on the sampling day. Interestingly, QREC were isolated from farms with hygiene scores of *clean* (44.4%) or *intermediate* (55.5%), while no QREC were isolated from the three farms scored as *dirty*. QREC were found regardless of the washing detergent used for routine cleaning. The most common cleaning method was only to use water without any detergent, and this method was used both at QREC-positive (77.7%) and QREC-negative farms (72.8%).

The quality of the visitor's entrance as a disinfection sluice was mainly found to be *good* or *excellent* (65%), and this quality was dominant within both the QREC-positive (66.7%) and the QREC-negative (63.6%) farms. Seven farms had disinfection sluices that were either absent or insufficient. Of these farms, 42.9% were QREC positive and 57.1% were QREC negative, respectively (Figure 4.5).

Table 4.5 Farm-level variables. Each row represents a farm and includes its responses to the questionnaire (Appendix I). Abbreviation: farm category; H =high-intensive farm, and L= low-intensive farm. An underlined letter represents a farm in which QREC were isolated from at least one calf. [1]Breeds; STN: Blacksided Trønder and Nordland Cattle STN, NRF: Norwegian Red [2] Hygiene score; 1: clean 2: intermediate 3: dirty [3] Sluice; 1: absent 2: excellent 3: good 4: insufficient. [e] QREC isolated from the environmental samples.

Farm cat.	Type of production	Milking system	Mean milk yield (kg of ECM/cow-yr)	Heard size	Predominant breed[1]	Hygiene score[2]	Sluice [3]	Cleaning detergent	Methods of ensilation
H[e]	Nonorganic	Tie stalls	11552	50	NRFxHolstein	2	4	Water only	Sil-All®
H	Nonorganic	AMS	10700	17,5	NRF	2	3	Water only	Sil-All®
H	Nonorganic	AMS	10761	43,6	NRF	1	3	Water only	Formic acid
H	Nonorganic	AMS	11147	65	Holstein	2	4	Soap	Sil-All® and formic acid
H[e]	Nonorganic	AMS	11552	69	NRF	2	2	Water only	Sil-All® and formic acid
H	Nonorganic	Loose housing	11661	34	NRFxHolstein	3	3	Water only	Formic acid
H	Nonorganic	Loose housing	11416	40	NRFxHolstein	1	3	Water only	Sil-All®
H	Nonorganic	AMS	10929	35	NRFxHolstein	1	2	Water only	No additives
H	Nonorganic	AMS	11091	50	Holstein	1	2	Water only	Formic acid
H	Nonorganic	AMS	10866	50	Mixed breeds	1	2	Water only	Formic acid
L	Organic	Tie stalls	4471	11	STN	2	1	Hydrated lime	No additives
L	Organic	Tie stalls	3486	15	Mixed breeds	1	3	Water only	No additives
L	Nonorganic	Tie stalls	4637	19	NRF	3	4	Virkon S	No additives
L	Nonorganic	Tie stalls	4562	20	Mixed breeds	2	1	Soap	No additives
L	Organic	Loose housing	4173	8	STN	2	2	Water only	Sil-All®
L	Nonorganic	Tie stalls	3702	18	Mixed breeds	2	3	Water only	Formic acid
L	Nonorganic	Tie stalls	4328	18	NRF	3	3	Water only	Formic acid
L	Nonorganic	Tie stalls	4652	15	NRF	2	1	Water only	Formic acid
L	Nonorganic	Tie stalls	4417	12	NRF	2	1	Water only	No additives
L	Nonorganic	Tie stalls	4515	17	NRF	1	2	Virkon S	Formic acid

4.2 Study II

4.2.1 Prevalence of QREC

During Study II, faecal samples were taken from 12 animals (six cows and their calves) from two different farms at seven different time points. Of 84 samples, 22.6% (19/84) were QREC positive. Of the faecal samples from calves, 31% (13/42) were QREC positive, whereas 14.3% (6/42) of the samples from cows were QREC positive. QREC were isolated at least once from every calf and cow during the study period, except from one adult cow. The occurrences of QREC in calves and cows at a farm level coincided. The prevalence of QREC

at each time point changed over time from 0 to 66.7% of sampled animals at Farm I and 0 to 83.3% of sampled animals at Farm II. Faecal samples from cohabiting calves were found to be simultaneously QREC positive before returning to QREC-negative status. This contrasted with the cows, where only one cow was found to shed QREC at each time point (without one sampling at one farm) (Figure 4.4). Within-sample prevalence was less than 0.01% for all QREC-positive cows, whereas the prevalence in calves ranged from < 0.01% to 13.1% (median 0.061%).

The pattern of QREC shedding from calves and cows was markedly different between the two farms in Study II. At Farm I, QREC were only found at two out of seven time points, compared to five out of seven occasions at Farm II. At Farm I, a generally high within-sample prevalence of clinically resistant QREC was found in faecal samples from all three calves and one of the cows within the first week postpartum. After this time, QREC were only detected once in faeces sampled from a cow in its thirteenth week postpartum. In contrast, most individuals at Farm II were found to carry QREC for the first time during the seventh week postpartum. After this time point, a gradual decline of QREC-positive animals was identified in Farm II.

No significant decline in the occurrence of QREC over time was detected for these two farms (Spearman's rank correlation coefficient 0.109, *p*-value = 0.8159). Thus, for the two farms in Study II, QREC shedding did not correlate with the age of the calf.

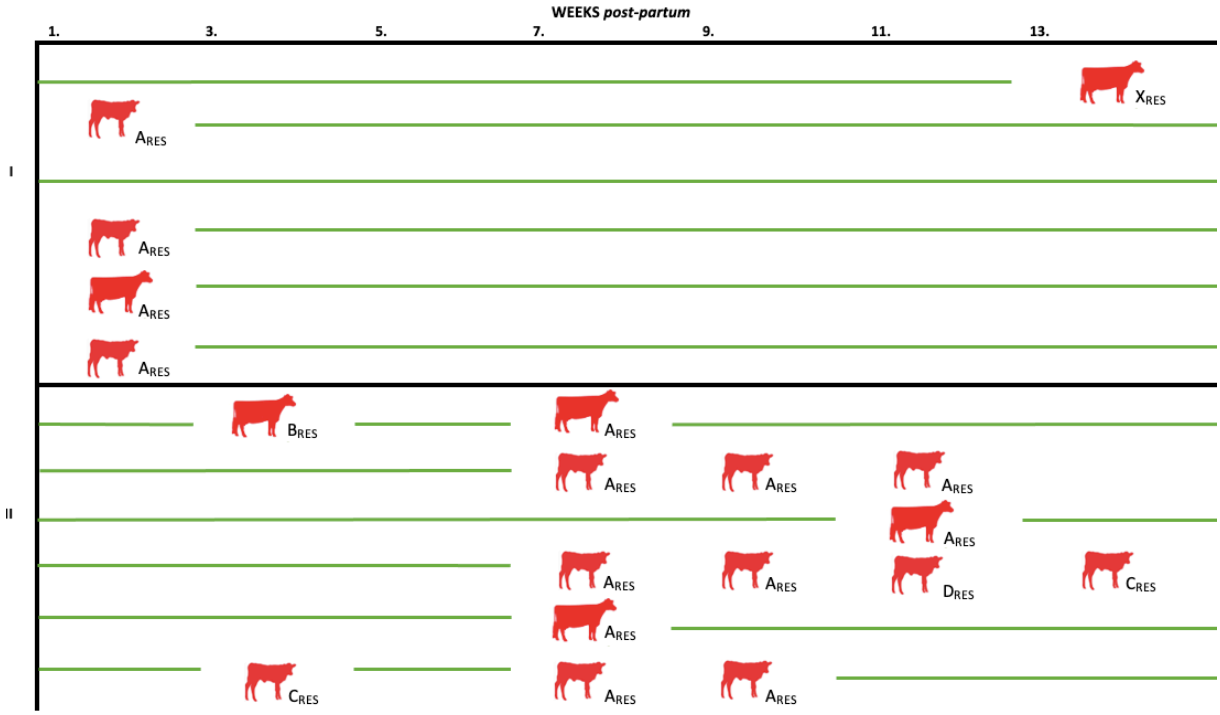


Figure 4.4 QREC occurrence in faecal samples from calves and cows postpartum. The two farms are visualised separately, Farm I above and Farm II below the broad line. Red-coloured calves/cows symbolise QREC isolates detected in a faecal sample from the individual. A green line is used at the time points when QREC were not detected. Abbreviation: ARES: CiNalAmpSuTcTm, BRES: CiNalAmpTc CRES: CiNalAmpChlSuTc, DRES: CipNalAmpChlSuTc, XRES: isolate not MIC-tested.

4.2.2 Antimicrobial susceptibility testing

All QREC isolates had MIC values for ciprofloxacin between 0.25 mg/L and >8 mg/L, with a median of 8 mg/L. *E. coli* isolates conferring a high-level of clinical resistance against quinolones (MIC for ciprofloxacin of 8 mg/L) were found at both farms.

The same phenotypic resistance profile dominated at both farms. At Farm I, this phenotypic resistance pattern was found between cows and calves on the same day of sampling (week 1 postpartum). Antibiotic resistance was found against ciprofloxacin, nalidixic acid, ampicillin, sulfamethoxazole, trimethoprim, and tetracycline, (CiNalAmpSuTcTm) and all isolates had an MIC for ciprofloxacin of 8 mg/L and nalidixic acid above 128 mg/L. No additional QREC were isolated from these calves during the sampling period; however, QREC were found in samples from one cow on week thirteen postpartum.

CipNalAmpSuTcTm was also the major resistance pattern identified in QREC isolated from faecal samples at Farm II. As at Farm I, the MIC values of these isolates were 8 mg/L for ciprofloxacin and over 128 mg/L for nalidixic acid. This dominant phenotypic pattern was isolated multiple times from both cows and calves at Farm II. In addition to this dominant phenotype, another phenotype was identified in samples from two calves at the third and thirteenth weeks postpartum, respectively. This phenotype showed the resistance pattern CipNalAmpChlSuTc and demonstrated MIC values for ciprofloxacin and nalidixic acid of 0.25 mg/L and 128 mg/L, respectively. Finally, a QREC isolate showing a narrower phenotypic resistance pattern, CipNalAmpTc, and with MIC values for ciprofloxacin and nalidixic acid of 0.25 mg/L and 8 mg/L, respectively, was found in a faecal sample from a cow from Farm II on week 3.

4.2.3 Dissemination and genetic diversity of QREC

Mutations in the *gyrA* gene were identified in QREC isolates from Study II by Sanger sequencing. In total, 88.9% of the isolates were found to carry double mutations at positions S83L and D87N. All isolates with this double mutation had MIC values of 8 mg/L for ciprofloxacin and >128 mg/L for nalidixic acid. 11.1% had a single mutation in S83L and were found to have a lower MIC (0.25 mg/L) for ciprofloxacin while their MICs for nalidixic acid varied (8 mg/L and 128 mg/L). Double mutations were found at both farms, while isolates with only single mutations were detected at Farm II at the third and thirteen weeks of sampling, both in calves and in a cow.

The six isolates selected for WGS could demonstrate the within-farm transmission of a successful genotype (Figure 4.5). In Farm I, the same sequence type (ST744) carrying multiple identical mutations in the *gyrA* gene (S83L and D87N) and the *parC* gene (S581) were isolated from all positive calves and a cow in the first week of sampling. However, a different sequence type (ST1609) with only a single mutation in the *gyrA* gene (D87G) was identified from the positive calf on week five postpartum. This isolate showed a MIC value for quinolones below the cut-off for QREC and is, in consequence, not included in Figure 4.4. The sequence type detected at Farm II (ST58) was different from the sequence types detected at Farm I (ST744, ST1609). This QREC isolate originated from a cow with lower MIC values for quinolones compared to other QREC isolates from the same farm. In this isolate, point mutations were identified within the *qnrS1* and *marR* genes, and no mutations were found in

the *gyrA* gene by MLST. The same isolate was found with a mutation in gyrA (S83L) by Sanger sequencing.

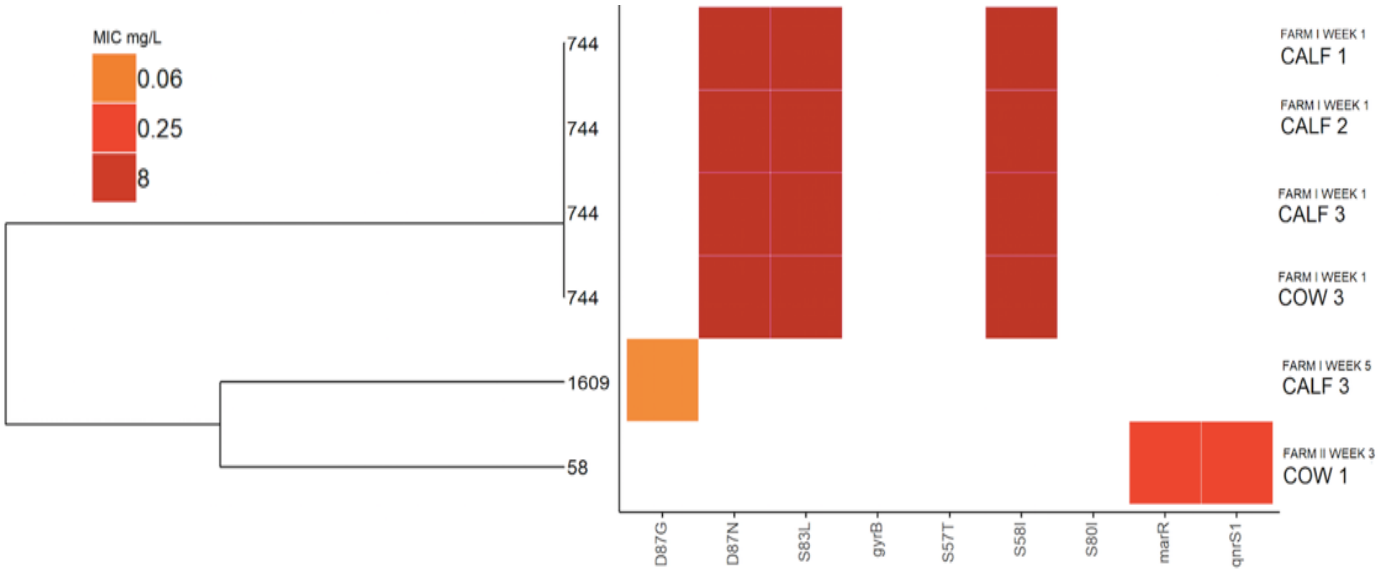


Figure 4.5 Mutations in QRDR-associated genes were identified by whole-genome sequencing of six QREC isolates. The figure specifies the point mutations within *gyrA* (D87G, D87N, and S82L) and *parC* (S57T, S58I, S80I); otherwise, mutations are visualised at a gene level for *gyrB, marR,* and *qnrS1*. The figure demonstrates within-farm transmission of a successful genotype isolated from both cow and calves, with changes in sequence type over time. Plasmid-mediated quinolone resistance was detected in an isolate (*qnrS1*); this isolate was found with a mutation in *gyrA* (S83L) by Sanger sequencing but no mutation in QRDR by MLST. The colours correlate with the MIC value for ciprofloxacin. The relatedness of isolates is displayed as a minimum spanning tree based on multi-locus sequence typing (MLST).

5. Discussion

The primary objective of this study was to increase the understanding of the occurrence of quinolone-resistant *Escherichia coli* (QREC) from cattle despite negligible quinolone usage for cattle treatment and, further, to investigate whether farm management with a focus on intensity influences the occurrence of QREC. In addition, this study aimed to gain a further understanding of QREC dynamics within the farm by studying transmission from cow to calf and general QREC occurrences over a period of time. The results demonstrate the occurrence of QREC in Norwegian dairy farms despite a long history of low quinolone usage in the veterinary treatment of cattle.

5.1 Prevalence of QREC

In Norway, the occurrence of QREC in cattle is less than 1% (Kaspersen et al., 2018). In the present two studies, QREC were identified in 9.8% of all faecal samples. However, in most samples, QREC were only found in a low proportion compared to the total number of *E. coli* isolated. This result could indicate a general low count of QREC within the bovine intestinal microflora. Yet some individuals showed a markedly higher prevalence. The farm with the highest within-sample prevalence of QREC had an average of 11.5% QREC among the total *E. coli* isolated from the faecal samples. This could indicate that QREC in the microbiome poses a real threat to animal and human health should the selective pressure increase.

5.2 Farm intensity

Farm intensity in relation to QREC occurrence was investigated in Study I. Farm intensity was measured as the average milk yield at the farm level. There was a significantly higher occurrence of QREC among the group classified as high-intensive farms. Herd size was found to diverge between the low- and high-intensive farms. Increasing herd size has been described as related to AMR occurrence (Bosman et al., 2014, de Verdier et al., 2012, Rebelo, 2014).

Thus, the differences in herd sizes observed in this study might impact the validity of the results, and they highlight the importance of interpreting the results with care.

QREC isolated from high-intensive farms were mainly (except for a single isolate) multidrug-resistant, compared to QREC detected in low-intensive farms that only showed reduced susceptibility to quinolones. Mutations in *gyrA* and *parC* were detected in isolates from both high- and low-intensive farms. The presence of double mutations in *gyrA,* together with a mutation in *parC,* conferred high-level resistance against quinolones (MIC for ciprofloxacin 8 mg/L). Such high-level resistance was, however, only detected in one farm categorised as a high-intensive farm. To date, the QRDR mutations in *gyrA* and *parC* have only been shown to be acquired by gradual stepwise clonal evolution (Tchesnokova et al., 2019). It could therefore be hypothesised that the farm with high-level quinolone resistance was exposed to higher selection pressure.

Interestingly, in antimicrobial sensitivity testing, *E. coli* colonies grown on selective ciprofloxacin-containing agar were found to be sensitive to ciprofloxacin. This phenomenon was dominating among multiple low-intensive farms. None of the *E. coli* isolates from these farms had mutations in QRDR (data not shown). Sub-inhibitory fluoroquinolone exposure has been found to facilitate low-level quinolone resistance with no causative changes in QRDR, which could explain the present finding (Bai et al., 2012, Boos et al., 2001., Davies et al., 1999, Grkovic et al., 2002). However, if ciprofloxacin-containing agar exposes *E. coli* to sub-inhibitory fluoroquinolone levels, then similar results should have been present in both study groups. It is also possible that organic material from the faecal samples could promote bacterial growth despite the selective pressure. Nevertheless, the detection of multiple QRDR mutations at both low- and high-intensive farms implies the existence of unknown drivers of a stepwise mutation in both study groups.

5.3 Management factors

Feed composition and forage-to-concentrate ratio are known to be the most important factors in increasing milk yield (Volden, 2020). A high concentrate level is associated with high milk yield, defined as high farm intensity in this study. There is no exact definition for the degree

of farm intensity, although milk yield is an easily categorised variable that correlates with input factors such as concentrate. The concentrate is mainly based on starch, which contains easily fermentable carbohydrates. The rumen microbiome produces a high level of short fatty acids from carbohydrates, which results in low rumen pH, in turn inhibiting the growth of some acid-sensitive rumen bacteria (Wang et al., 2020). QREC strains are found to be more resistant to acids and better than quinolone-susceptible strains at using the gut's primary nutrient source (Lastours et al., 2014). Therefore, it is tempting to speculate that the higher prevalence of QREC identified in the high-intensive group is driven by the high concentrate level causing acidic conditions in the rumen that promote the selection of QREC.

Another potential hypothesis could involve the amount of soy added to the feed, which increases in parallel with concentrate levels (Felleskjøpet, 2020). The soy plant has been used for medicinal purposes for millennia and is known for its high levels of secondary plant metabolites, including isoflavones and saponins. The isoflavones are known to have bioactivity that exhibits antimicrobial activity against Gram-negative bacteria (Cowan et al., 1999, Omosa et al., 2013, Sugiyama et al., 2019) by targeting topoisomerases I and II. In addition, isoflavones are found to mimic the effect of ciprofloxacin against bacterial strains (Abreu et al., 2017). Their antibacterial activity has been suggested to originate from a molecular structure comparable to quinolones; however, the exact relationship between chemical structure and isoflavonoid activity is not fully understood (Wu et al., 2013).

As previously discussed, sub-MIC levels of ciprofloxacin are found to give rise to mutations in QRDR (Wang et al., 2010). It could be hypothesised that a molecule with a similar structure, such as isoflavones, could trigger the same SOS response and induce mutations in QRDR. In both high- and low-intensive farms in Study I, soy was included as an ingredient in the concentrate (data not shown). The amount of soy was likely higher in the high-intensive farms since the forage-to-concentrate ratio is the single most crucial factor to increase milk yield. This could be an explanatory factor for the increased occurrence among high-intensive farms.

5.4 Periodical and coinciding occurrences of QREC

A generally high within-sample prevalence of clinically resistant QREC was found in faecal

samples from calves compared to their postpartum cows. Also, postpartum cows were less likely to carry QREC than their calves. Given that calves and cows are exposed to the same environmental microbiome on the farm, the generally lower prevalence of QREC in cows could probably be due to age-dependent colonisation factors. Neonatal ruminates function as monogastric animals. Digestive enzymes transition the calves from being monogastric to being ruminant animals, which could hypothetically impact the ability of QREC to adhere and colonise the gut.

The results indicate co-occurrence of QREC in cattle at a farm level, regardless of age, within this three-month sampling period. While Farm I had a high prevalence of QREC shortly after birth, most individuals at Farm II were QREC positive for the first time during the seventh week postpartum. The co-occurrence may indicate transmission of QREC at the farm level and could suggest that factors other than age alone affect QREC occurrence in the first three months postpartum. This contrasts with comparable studies which have found a significant decline in QREC shedding with age (Berge et al., 2005, Brunton et al., 2014, Donaldson et al., 2006, Duse et al., 2015, Hinton et al., 1985, Hoyle et al., 2004, Watson et al., 2012). Thus, our results strengthen the theory that environmental and management factors affect QREC shedding from ruminants. However, considering our study population size, this result should be interpreted with care.

The pattern of QREC occurrence varied greatly between these two farms. It could be hypothesised that the calves' microbial colonisation conditions varied greatly. Farm I is a conventional farm where calves and cows are separated shortly after birth and placed in separate rooms (data not shown). This diverges from Farm II, an organic farm (data not shown) where calves and cows are kept together for at least three days as required by national regulations for organic farming (Økologiforskriften, 2017). Calves that are separated from their mothers immediately after birth develop less-diverse microbiomes and are thus more susceptible to invasion by unwanted bacteria, such as pathogenic bacteria and QREC.

The primary limitation of this longitudinal observational study is the low number of individuals included, which has made it challenging to apply the results to a larger population. The sampling was performed at two farms, with a total of 12 animals sampled every second week for three months. The limited number of farms made it possible for one person to conduct the sampling, increasing the reproducibility. Similar longitudinal studies screening

for QREC occurrence have been performed in other countries (Berge et al., 2005, Brunton et al., 2014, Duse et al., 2015, Hinton et al., 1985, Hoyle et al., 2004, Watson et al., 2012), where higher occurrences of QREC were found in young animals as compared to adults, which affirms our results even with a small study population.

5.5 Within-farm transmission

The results of the phenotypic antibiotic sensitivity testing, sequence typing, and phylogenetic analysis of the isolated QREC indicate a strong correlation between the QREC isolated within the farms in both studies. Thus, these results indicate possible clonal transmission at the farm level and could strengthen the theory that management factors at the farm level are triggers for quinolone resistance.

The majority of identified STs were unique for each farm. The high diversity between farms could indicate that drivers for quinolone resistance are found at the farm level. Similar phenotypic resistance patterns were mainly observed in QREC isolated from the same individual at different time points in Study II, although diversity was observed. The changes in QREC genotypes in an individual over time may indicate a persistent driving factor affecting the intestinal commensal bacteria. Another explanation could be a naturally occurring diversity of QREC within the farm, and the individual animals, which the isolation of a single bacterial colony from each sample taken would not reflect. A single QREC isolate was selected from each individual at each time point, which does not capture the microbial diversity of the faecal content. The *E. coli* population in the GI tract is numerous, and only a small proportion of the total *E. coli* population is present in the faecal sample (Hinton et al., 1985). This is a significant challenge when drawing conclusions based on the susceptibility of a single, random *E. coli* sample from a calf or a cow. Nevertheless, other studies also have described one major QREC genotype as dominating in a herd (Duse et al., 2016, Hoyle et al., 2005).

5.6 Farm environment

QREC were isolated from environmental samples in two out of twenty farms, with both farms belonging to the high-intensive group. The STs were similar for QREC isolated from the environment and calves, which could indicate an environmental source and contamination of the farm environment. Previous publications have found contaminated environments to increase the dissemination rate of QREC and have established clonal dissemination of QREC strains within farms to be facilitated by poor farm hygiene (Bosman et al., 2014, Duse et al., 2015). However, in this study, QREC were isolated from farms with *clean* or *intermediate* hygiene scores, while no QREC were identified in the farms scored as *dirty*. Our results could indicate that management factors other than farm hygiene could be drivers of QREC transmission within the farm. The study population size could also have affected the results. The environmental samples represent a limited area of the total farm environment, which could have led to under-estimating the occurrence of QREC in the farm environment. Loose housing compared to tie-stall housing increases the intermingling of animals, the dissemination of one strain of *E. coli* to another place in the farm, and the contact between donors and potential recipients. Nevertheless, it is worth mentioning that the farm with the highest within-farm and within-sample prevalence of QREC had a tie-stall system. This could indicate that farm housing is inferior to other factors impacting QREC occurrence in a specific farm.

The farm environment, including fields and grazing areas, could be exposed to wild mammals, birds, or human wastewater that could be potential sources of quinolones. Low levels of ciprofloxacin in the environment have been found to select for resistance (Gullberg et al., 2011). Research conducted in Norway within the last few years isolated QREC through the environmental sampling of wastewater (Paulshus et al., 2019). Additionally, the usage of quinolones for the treatment of both animals and humans could constitute a risk for QREC occurrence within the cattle production chain.

The STs found in our study indicate similarities with isolates from other species in Norway and suggest a possible interaction between wildlife and anthropogenic sources. Birds as a vector in QREC shedding have been a focus globally (Carlson et al., 2020, Chandler et al., 2020, Medhanie et al., 2016), and wild birds have been included in recent Norwegian studies (Kaspersen et al., 2018, Mjelde and Kallbekken 2020). The main QREC resistance phenotype

found in the present study demonstrates resistance to ciprofloxacin, nalidixic acid, ampicillin sulfamethoxazole, tetracycline, and trimethoprim (CiNalAmpSuTcTm). This is the same phenotypic resistance pattern found in wild geese in Norway and could indicate a possible successful clone/resistance pattern. Mjelde and Kallbekken (2020) found ST162 to be the most phenotypically resistant ST among wild geese in Norway. ST162 has also been found in broiler chickens and red foxes in Norway (Kaspersen et al., 2018). This agrees with isolates from our study, among which a total of eight calves and one environmental sample were found to have ST162. Wild mammals, particularly birds, could act as transporters or as reservoirs for QREC and therefore have important epidemiological roles in its transmission. Apparently, wild mammals account for some QREC transmission. Still, the diverging genotypes between farms could support the theory that farm management factors are important explanatory factors for QREC occurrence in the dairy population.

5.7 Clinical resistance and a *One Health* perspective

In both studies, high-level quinolone resistance was detected in *E. coli* from farm environments, calves, and cows. Additionally, all but three QREC isolates were found to be multidrug-resistant. Plasmid-mediated quinolone resistance was detected in one sample. To the author's knowledge, PMQR has not been previously isolated from cattle in Norway. However, the transfer of a coliform bacteria with a multidrug-resistant plasmid of bovine origin to a human in a farm environment has been documented in Norway (Oppegaard et al., 2001).

While a low-level resistance to ciprofloxacin demonstrating a MIC below clinical cut-off (0.5 mg/L) was found in Study I, most QREC isolates found in this research project showed MIC values above the clinical breakpoint for ciprofloxacin resistance. The diverging level of farm prevalence of QREC and the occurrence of high-level quinolone resistance highlights the importance of increasing our knowledge about quinolone resistance in livestock. Cattle, as carriers of zoonotic bacterial strains and non-pathogenic strains carrying resistance genes, pose a risk for the horizontal transfer of quinolone resistance from cattle to humans (Orden et al., 1999, Marshall et al., 2011, Todorović et al., 2018). Dairy cattle are unique because they may be sources of QREC contamination through milk, meat, direct contact with humans, and manure-fertilised plants. It can be assumed that higher proportions of antimicrobial-resistant

E. coli on a farm increase the risk of their spreading to humans.

5.8 Methodological considerations

5.8.1 Study design

Study I was a cross-sectional study, whereas Study II was a longitudinal observational study. The study population for Study I originated from the general dairy cattle population based on farms that participated in NDHRS, which includes 97.5% of all dairy farms in Norway. The farms in Study II were chosen by their proximity to the laboratory facilities. Inclusion criteria were based on the number of calves and the milk yield. Only farms within five counties were included in Study I. A minimum exclusion criteria increases the external validity (Pannucci et al., 2011). While the external validity for Study I is believed to be strong, a lower validity was estimated for Study II. Nevertheless, Study II's inclusion criteria made the targeted population small and, as a result, increased its internal validity. For Study II, the geographical area was limited, and only farms with three or more calvings were included within the second week of January. The high amount of calving likely excluded small herds.

A cross-sectional study is a good option for monitoring and detecting prevalence, but the study design will be highly affected by the incidence of the diseases (Setia et al., 2016). A low occurrence of QREC could have a biasing effect. With a low incidence, the estimated sample size should be larger to obtain sufficient power for the statistical analysis. For our cross-sectional study, we collected 233 faecal samples from 20 farms. The sample size required was estimated based on a power analysis to give an adequate statistical power of 0.8 (Table 3.3).

There was a notable difference in response rate between high- and low-intensive farms. The lower response rate among low-intensive farms could have produced sampling bias. An important aspect worth mentioning is that the small herd sizes found among the low-intensive farms reduced the number of calves available for sampling below the original exclusion criterion. Calves from a wider age range were included from the low-intensive group, which could have affected the results.

5.8.2 Sampling procedure and susceptibility testing

A pilot study was run during autumn 2017 to gain valuable calibrations and knowledge for the main study. To avoid possible bias, only one person conducted the sampling. Despite only having one person sampling and collecting all data, subjective bias could have occurred, mainly when qualitative data were obtained. Qualitative data were collected using the questionnaire, in which hygiene and sluice gate quality could have been particularly affected by subjective bias.

EFSA has recommended monitoring the prevalence of resistant bacteria using a "single sample per farm" approach. This approach assumes that isolates from the same farm show similar resistance patterns due to a common environmental microbiota. The dynamics of QREC occurrence in Norwegian dairy herds is a novel research area, and we aimed to understand within-sample prevalence better. Based on this aim, we sampled up to 15 calves at each farm, which reduced the total number of farms we could include in our study. Since only one QREC colony was isolated from each sample and further genotyped, the potential diversity of QREC STs in each individual's microbiota was likely overlooked. However, our results showed that a lower number of calves is probably sufficient to determine the farm-level status of QREC, in line with the recommendation from EFSA (EFSA, 2014).

We used ciprofloxacin-selective plates where multiple isolates were tested simultaneously, with an antimicrobial concentration based upon the epidemiological cut-off for resistance issued by EUCAST. The repeated-measure design of Study II allowed us to demonstrate a general trend present within the cohort. We observed the growth of isolates on selective agar, which were found quinolone sensitive after MIC testing. This phenomenon raises questions about the method used, even though we adopted the same standard for sampling and susceptibility testing described by the Norwegian Surveillance Programme for Antimicrobial Resistance (The Norwegian Veterinary Institute, 2017).

The inclusion criteria of the present studies included milk yield, location, and the number of calves during the sampling period. However, the history of quinolones at the farm level was not obtained. Quinolones are still available for selling and treating food-producing animals in Norway. Although the total sales of quinolones in Norway are low, a veterinarian can freely prescribe unlimited amounts of the drugs for use in their practice, making it possible to have

hotspots of quinolone usage in specific areas. Hence, regional differences in quinolone usage in Norway could have impacted our results.

5.9 Generalizability

This research provides information about QREC occurrence in Norway but may also be of global interest. Our study population is too small to determine the occurrence for the entire Norwegian dairy population. The higher occurrence of QREC among highly intensive farms as compared to low-intensive farms should be interpreted with care. However, our findings indicate clonal transmission of QREC and a high diversity of QREC genotypes within the population. Our results are strengthened by being comparable to other studies. In contrast to similar studies, our results indicate factors other than age as important drivers for QREC occurrence, but these conclusions require further research before a conclusion can be made. A general interpretation of our results could be made in the context of current research conducted in Norway over the last few years.

6. Conclusions

- QREC occurrence was more abundant in farms defined as high-intensive
- At the farm level, QREC co-occurred in cattle regardless of age, suggesting dissemination between animals
- Calves carried a higher within-sample prevalence of QREC in faecal samples compared to their postpartum cows.
- The results suggest that loose housing systems and using formic acid as a feed conservator could be potential risk factors for QREC occurrence.
- QREC isolates were mainly multidrug-resistant; the most common phenotype showed resistance against ciprofloxacin, nalidixic acid, ampicillin, sulfamethoxazole, trimethoprim and tetracycline.
- High levels of clinically resistant QREC isolates with a ciprofloxacin MIC of 8 mg/L were identified at multiple farms. Generally, a low-level quinolone resistance was dominating.
- Mutations in QRDR were identified in *gyrA* (S83L, D87N, D87Y, D87G), *parC* (S57T, S58I), and in the regulatory gene *marR*. No mutations were detected in *gyrB* and *parE.*
- Plasmid-mediated quinolone resistance, with a mutation in *qnrS1*, was detected in one isolate from an adult cow. The same isolate was found with a low level of quinolone resistance.
- A clonal distribution of QREC isolated from calves, cows, and the farm environment was identified. The results do not indicate a clonal distribution between farms, suggest dissemination mainly at a farm level.

7. Further perspectives

Quinolone resistance found in the animal production chain is essential in the *One Health* perspective. It needs attention, as quinolones are one of the four highly prioritised, critically important antimicrobials for global human medicine. The antimicrobial class of quinolones is classified as Category B by the European Medicine Agency (EMA); restricting the use of antibiotics in this category is critically important for human medicine, and their application in veterinary medicine should be restricted to mitigate the risk to public health. A veterinarian may freely prescribe an unlimited amount of the drug in their practice, which makes it possible to generate hotspots of quinolone use in specific areas. In Norway, the National Service publishes antimicrobial usage in the human health service at a regional level (https://www.antibiotika.no/kas/). A similar regional reporting of antimicrobial use among farm-animal veterinarians could make room for discussion and improve antimicrobial prescription in the veterinary sector.

It will be of future interest to screen for the occurrence of co-resistance. Multiple studies have analysed the occurrence of co-resistance between fluoroquinolones and extended-spectrum ß-lactamases (ESBLs) in *Enterobacteriaceae* from humans. ESBLs are enzymes that cause resistance to some of the most used antibiotics, including penicillin, cephalosporins, and monobactams (Hu et al., 2019). Co-occurrence with ESBL has also been detected in bacteria isolated from cattle (Aguilar-Montes et al., 2015). Although it is documented to have a relatively low frequency in cattle compared to other animal species, it could be of interest for public health to know more about the presence of co-occurrence with quinolone resistance.

To dive deeper into the occurrence of QREC in cattle, it could be relevant to use a retrospective study to examine a population of farms where QREC demonstrating high clinical resistance have been detected. Antibiotic prescription practices at the farm level should then be acquired. By tracking QREC dynamics over a more extended period, for example, a year, one could potentially highlight different management variables as potential drivers for quinolone resistance, including the bird migration period.

More research is needed to understand the dynamics of antibiotic resistance in the normal faecal microbiota of cattle. Metagenomic next-generation sequencing is now easily available

and could give further insight into the diversity of the resistome, potentially connecting feeding regimes or other potential drivers of resistance development to QREC occurrence.

8. Populærvitenskapelig oppsummering

Utfordringene med økt forekomst av antibiotikaresistens har nådd alarmerende høye nivåer. Antibiotika benyttes for å behandle bakterieinfeksjoner hos mennesker og dyr. Bakteriene kan bli motstandsdyktige, utvikle resistens, mot antibiotika. Motstandsdyktige bakterier kan gi store utfordringer ved behandling av infeksjoner. Antibiotikaresistens er en naturlig forsvarsmekanisme hos bakteriene som beskytter de mot andre bakterier og også andre komponenter som finnes i for eksempel planter eller sopp.

Antibiotikaresistente bakterier hos dyr kan overføres til mennesker via direkte kontakt eller matvarer som melk og kjøtt. Kolibakterier er en naturlig del av tarmfloraen hos dyr og mennesker, og utgjør vanligvis ingen skade, men de kan gi sykdom hos svake individer. *Escherichia coli* (*E. coli*) er en av kolibakteriene. Kolibakteriene kan være resistente mot mange typer antibiotika.

Verdens helseorganisasjon (WHO) har kategorisert hvilke antibiotika som er aller viktigst å spare til behandling av kritisk syke mennesker, og kinoloner er en av de fire viktigste antibiotikum for behandling av menneske. Bruken av kinoloner til behandling av dyr i Norge anses som ubetydelig lav på et nasjonalt nivå. Likevel har det blitt funnet kinolonresistente *E. coli* (QREC) i avføring til flere av produksjonsdyrene i Norge, deriblant storfe. Årsaken til dette er ikke kjent. Målet med denne studien var derfor å se nærmere på forekomst av kinolonresistente bakterier i norske melkekubesetninger, se nærmere på hvordan bakteriene spres i miljøet og undersøke mulige risikofaktorer som kan øke denne forekomsten. Samtidig studerte vi forekomst av kinolonresistens fra kalv og melkekyr de første tre månedene etter kalving. Unge kalver er nemlig kjent for å ha mer resistente bakterier i tarm enn eldre storfe, selv uten at de har vært behandlet med antibiotika. Vi fulgte opp de samme kalvene over tid for å danne en bedre forståelse av når QREC først oppdages i avføring hos kalvene, og om det var et gitt tidspunkt de reduserte utskillelsen.

I dette studiet ble det funnet høyere forekomst av kinolonresistens i besetninger med høy produksjonsintensitet. QREC ble funnet i avføringsprøvene fra 13.3% av kalvene fra gårder som drev en intensiv melkeproduksjon, i motsetning ble QREC funnet blant 2.8% av kalvene

fra lav-intensive gårder. Det ble funnet kinolonresistens i både båsfjøs og løsdriftsfjøs. Renhold så ikke ut til å ha en direkte sammenheng med forekomst. Kalvene som ble fulgt opp over tid hadde høyere forekomst av kinolonresistente *E. coli* enn mødrene og det var en stor sammenheng i forekomst hos kalvene innad på den samme gården. Innenfor besetningene hadde stort sett både voksne kyr, kalver og prøvene fra fjøsmiljøet de samme typene av resistente bakterier. Imellom gårdene var det stort sett forskjellige typer resistente bakterier som ble funnet. Det betyr at smitten skjer innad på gården og i mindre grad mellom gårdene. Bakteriene som ble funnet på en av gårdene kan ligne på de samme som er funnet fra ville fugler, rev og kylling i andre studier gjort i Norge. Ville dyr og fugler, spesielt trekkfugler som reservoar og smitteutskillere av QREC er beskrevet av andre som en mulig risikofaktor for smitte til husdyr.

Det ble funnet bakterier i flere besetninger med en høy grad av kinolonresistens, som betyr at det må høye doser antibiotika til for å drepe bakterien ved en eventuell behandling. De fleste bakteriene hvor kinolonresistens ble oppdaget var multiresistente, altså var bakterien resistent mot flere enn tre forskjellige antibiotika. Disse funnene var aktuelle både for høy-intensive gårder og individene vi fulgte opp over tid. I lave-intensive gårder ble ingen multiresistente bakterier funnet, kun bakterier som var resistente mot kinoloner.

Forekomsten av multiresistente bakterier i fjøsmiljøet kan utgjøre en utfordring for folkehelsen. Videre innsamling av data og forskning på emnet blir viktig i tiden fremover. Resistens mot kinoloner er meldepliktig i Norge i dag. Det nasjonale overvåkningssystemet for bruk av antibiotika og forekomst av resistente bakterier hos både mennesker og dyr er et viktig verktøy i arbeidet mot mer målretta bruk av antibiotika og bekjempelse av antibiotikaresistens for fremtiden. Det er et søkelys på riktig bruk av antibiotika til produksjonsdyr og handlingsplaner er etablert for å redusere bruken ytterligere. Ved å tillate regionale oversikter over antibiotikaforbruk i produksjonsdyrpraksis på lik linje med forbruket til menneske kan eventuelle overforbruk i enkelte området identifiseres. Dette kan gi grunnlag for en diskusjonsplattform med mål om ytterligere forbedret forskrivingsrutiner.

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Appendix I - Questionnaire

Spørreskjema til besetningsbesøk ved prøvetaking

Besetningsnr. _____ Navn: _____Dato: _____

Generelt om gården		
	Besetningsstørrelse, antall årskyr	
	Økologisk / konvensjonell	
	Rase	
	Fjøssystem: båsfjøs, løsdrift med/uten robot	
Hygiene		
	Hygiene score (1-3)	
	Kvalitet på sluse for besøkende (1-4)	
	Vaskemidler	
	Fjøsvaskrutiner	
	Rengjøring av kalvebingene	
Fôring		
	Kraftfortype voksne	
	Kraftfortype kalver	
	Mineral/vitamintilskudd ja/nei	
	Ensileringsmiddel	

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