Norwegian University of Life Sciences
Faculty of Veterinary Medicine and Biosciences

Philosophiae Doctor (PhD)
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Quinolone resistant *Escherichia coli* in Norwegian livestock – A comparative genomics study

Håkon Kaspersen

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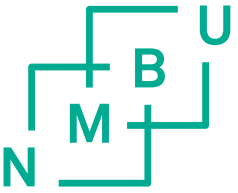
Adamstuen 2019



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"Wisdom begins in wonder"

— Socrates

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— Håkon Kaspersen, Oslo, 2019

Disclosure

The work of this thesis was carried out at the Norwegian Veterinary Institute (NVI), Oslo, Norway, from 2016 to 2019. The PhD project was part of a project named "Quinolone resistant *E. coli* despite low antimicrobial usage: mechanisms and possible preventive measures", in collaboration with the School of Veterinary Medicine, Norwegian University of Life Sciences, and the private industry. This thesis was written in RStudio with R version 3.6.1 (2019-07-05) [1] using the Bookdown package [2]. All code is published on github: github.com/hkaspersen/hkaspersen_thesis.

Funding

This study was funded by the Research Funding for Agriculture and the Food Industry (NFR project 255383).

Contents

1 Summary (English)

The occurrence of antimicrobial resistance in Norwegian livestock is generally low. This is a consequence of good animal health and welfare, which reduces the need for antimicrobial use. Quinolones are important antimicrobial compounds both for human and animal health. An increased occurrence of resistance towards these compounds in livestock have been observed in many European countries. In Norway, the use of quinolones in livestock has been restricted. Prophylactic use of any antimicrobial is prohibited. The Norwegian monitoring programme for antimicrobial resistance in feed, food and animals (NORM-VET) performs surveillance of antimicrobial resistance among commensal *E. coli*. The programme identified a low occurrence of quinolone resistance among several tested animal species. However, quinolone resistant *E. coli* (QREC) has been detected at low levels in a high proportion of samples from both pigs and broilers. Due to the low quinolone usage, this finding was somewhat surprising. Thus, further investigation of the origin of these bacteria was warranted. The aim of this study was to compile existing data on quinolone resistance occurrence. Furthermore, quinolone resistance mechanism characterization was performed. Relationships between the isolates were investigated by using a high resolution phylogenetic approach. The results showed a low occurrence of QREC among the included animal species. A significantly higher occurrence was observed in broilers. *In silico* characterization of quinolone resistance mechanisms identified chromosomal mutations as the major resistance determinant. Phylogenetic analysis of QREC provided evidence for dissemination in the broiler and pig production chains. Possible persistence of QREC was detected in the broiler production environment. Major QREC sequence types were detected among the samples from broilers. Some of these sequence types had previously been reported in other Nordic countries. Furthermore, phylogenetic analysis indicate that commensal *E. coli* rarely develop quinolone resistance in the broiler production environment. These results provide evidence for introduction of QREC to the Norwegian broiler production via imported breeding birds. The results highlight the importance of biosecurity measures at the top of the pyramid, to prevent dissemination of QREC.

2 Summary (Norwegian)

Norge har en av de laveste forekomstene av antibiotikaresistens i verden grunnet god dyrehelse og –velferd. Forbruket av kinoloner, som er svært viktige antibiotika for både dyr og mennesker, er svært lavt i Norge, og profylaktisk bruk av antibiotika er forbudt i husdyrproduksjonen. I mange andre europeiske land er det oppdaget en økende forekomst av kinolonresistente *E. coli* (QREC), som sannsynligvis er koblet til et økt forbruk av dette antibiotikumet. I Norge derimot viser data fra overvåkningsprogrammet for antibiotikaresistens i mikrober fra fôr, dyr og næringsmidler (NORM-VET) en lav totalforekomst av QREC blant norske husdyr. QREC har likevel blitt detektert fra en stor andel prøver fra gris og slaktekylling, men mengden QREC i hver prøve ser ut til å være lav. Disse funnene førte til spørsmål rundt deres opphav. Målet med dette prosjektet var å sammenfatte eksisterende data på forekomst av QREC i ulike dyrearter i Norge, karakterisere kinolonresistensmekanismer i QREC stammer, samt beskrive forholdet mellom stammene ved bruk av dype fylogenetiske metoder. Resultatene viser en overordnet lav forekomst av QREC, men en signifikant høyere relativ forekomst ble observert i slaktekylling. Kromosomale mutasjoner ble identifisert som hovedmekanisme for den observerte kinolonresistensen. Fylogenetiske analyser av sekvensdataene viste en klonal spredning av QREC i både slaktekyllingproduksjonen og slaktegrisproduksjonen, og mulig persistens av QREC i miljøet der slaktekyllingene oppholder seg. Videre fylogenetisk analyse av både villtype *E. coli* og QREC viste at villtype *E. coli* i liten grad utvikler kinolonresistens i slaktekyllingproduksjonen. Resultatene viser at introduksjon av QREC fra importerte foreldredyr er hovedårsaken til den observerte forekomsten av QREC i den norske slaktekyllingproduksjonen. Disse resultatene belyser viktigheten av biosikkerhetstiltak høyere i slaktekyllingpyramiden for å hindre spredning av QREC nedover i produksjonen.

3 Abbreviations

Term	Abbreviation
Akaike's Information Criteria	AIC
Antimicrobial resistance	AMR
Antimicrobial Resistance Identification By Assembly	ARIBA
Antimicrobial susceptibility testing	AST
Basic Local Alignment Search Tool	BLAST
Bayesian Information Criteria	BIC
Epidemiological cut off	ECOFF
European Committee on Antimicrobial Susceptibility Testing	EUCAST
High Throughput Sequencing	HTS
Maximum likelihood	ML
Minimum inhibitory concentration	MIC
Non-Metric Multidimentional Scaling	NMDS
Open Reading Frame	ORF
Plasmid mediated quinolone resistance	PMQR
Principal Component Analysis	PCA
Quinolone resistance determining region	QRDR
Quinolone resistant E. coli	QREC
Sequence Type	ST
Single Nucleotide Polymophism	SNP
Whole genome sequencing	WGS

4 List of publications

Occurrence of quinolone resistant *E. coli* originating from different animal species in Norway.

Håkon Kaspersen, Anne Margrete Urdahl, Roger Simm, Jannice Schau Slettemeås, Karin Lagesen, Madelaine Norström (2018). *Veterinary Microbiology*, 217(February):25-31. (10.1016/j.vetmic.2018.02.022).

Dissemination of quinolone resistant *Escherichia coli* in the Norwegian broiler and pig production chain, and possible persistence in the broiler production environment.

Håkon Kaspersen, Camilla Sekse, Eve Zeyl Fiskebeck, Jannice Schau Slettemeås, Roger Simm, Anne Margrete Urdahl, Madelaine Norström, Karin Lagesen. Submitted to *Applied and Evironmental Microbiology*.

Comparative genome analyses of wild type- and quinolone resistant *Escherichia coli* indicate dissemination of QREC in the Norwegian broiler breeding pyramid.

Håkon Kaspersen, Eve Zeyl Fiskebeck, Camilla Sekse, Jannice Schau Slettemeås, Anne Margrete Urdahl, Madelaine Norström, Karin Lagesen, Roger Simm. Submitted to *Frontiers in Microbiology*.

5 Introduction

5.1 Antimicrobial resistance

Antimicrobial resistance (AMR) is the ability of microorganisms to survive and grow in the presence of antimicrobials [3]. Quinolone resistance is of concern as quinolones are on the list of critically important antimicrobials [4]. Resistant bacteria pose a threat to both human and animal health, as fewer treatment options exist. For the animal and food industry this means a loss of productivity and livelihood [5]. Antimicrobial usage has been linked to an increased occurrence of resistant bacteria [6]. In Norway, antimicrobial usage is low among livestock, and quinolones are hardly used [7, 8] (Figure 5.1). This is reflected in the good animal welfare situation [9], as good health reduces the need for antimicrobial treatment. In this chapter, the broiler and pig production in Norway is presented. Antimicrobial usage within these populations is briefly described. The chapter then gives an overview of the occurrence of quinolone resistance in Norway and Europe. Finally, quinolone resistance mechanisms are presented.

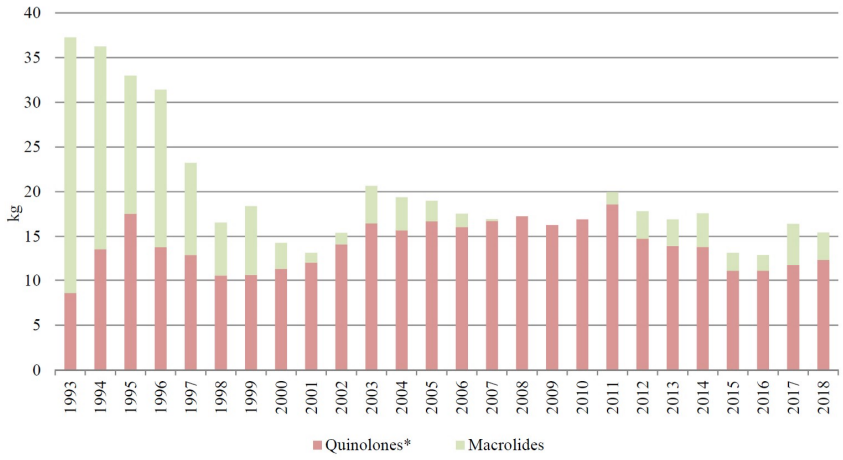

Figure 5.1: Overall sales, in kg active substance, of quinolones and macrolides for therapeutic use in terrestrial livestock animals in Norway in 1993 - 2018, reprinted with permission from NORM/NORM-VET 2018 [7].

Norwegian animal husbandry

Broiler production

The Norwegian broiler production is part of a pyramidal structure with pure-bred animals at the top, breeding animals in the middle, and broilers at the bottom [10]. The pure-bred animals originate from Scotland or Germany. Eggs that are laid by these pure-bred animals are imported to Sweden and become grandparent animals. The grandparent animals lay eggs that are imported to Norway. These eggs are hatched in Norwegian hatcheries and become parent animals. The parent animals are reared for around 18 weeks, and are subsequently used for breeding of broiler chickens until around 60 weeks old. The broiler chickens are hatched in a few hatcheries, and distributed to broiler farms all over the country. Most of the broiler chickens are reared for 28 - 32 days, depending on weight, then slaughtered [11]. The broiler farms have high biosecurity, i.e. measures are taken to prevent exposure to harmful agents.

Minimal levels of antimicrobials are used in the Norwegian broiler production [7]. During

the last six years, only 0.02 - 0.18% of all broiler flocks have been treated with penicillins [7]. Quinolones are not used in the Norwegian broiler production (personal communication: Høy T., The Norwegian Medicines Agency, October 2017).

Swine production

Swine production in Norway has a pyramidal structure with unidirectional flow of animals, similar to the broiler production [12]. Import of live pigs from other countries to the commercial Norwegian pig population is negligible [13]. The genetic nucleus herds, where the pure-bred grandparent animals are produced, are at the top of the pyramid. Hybrid parent animals are produced in multiplier herds, either by recruiting pure-bred animals from nucleus herds or having a self-recruitment strategy. Gilts from the multiplier herds are distributed to piglet producing herds (either farrow to finish or farrow to grower herds). The piglet producing herds have a mean weaning age of 33 days, followed by a growing stage until the grower pig is approx. 30 kg and 10-11 weeks of age. The grower pig is then transferred to a specialized fattening pig farm or fattening unit at the same farm.

The data reported to the Veterinary Prescription Register on prescription and use of antibiotics for terrestrial food-producing animals are not complete. These represent approximately two thirds of the sales of injectables, oral powder, and oral solution [7]. However, the percentage use of each antimicrobial type, as kilograms of active substance, is known. Of all prescribed antimicrobials to pigs in 2018, only 0.1% were quinolones, compared to 86.8% for penicillins [7]. Quinolones are therefore used at a very low level in Norwegian pigs.

Monitoring AMR and QREC epidemiology

The situation in Europe

Harmonized monitoring of AMR in commensal *E. coli*, i.e. *E. coli* that is naturally present in the gut, from food and food-producing animals has since 2014 been obligatory for all EU/EEA member states. This was implemented by the European Commission (SANCO/11591/2012). The commission describes the sampling frame and analysis of isolates. Commensal *E. coli* is usually isolated from caecal samples. Antimicrobial susceptibility testing (AST) is carried out using broth microdilution. With this method, the minimum inhibitory concentration (MIC) value is determined for multiple antimicrobials. A MIC value is defined as the lowest concentration of the antimicrobial needed to inhibit growth of a micro-organism [14]. Based on the MIC value,

an isolate may be categorized as either susceptible or resistant. This is based on breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). In a clinical setting, the clinical breakpoint is used to determine if the treatment will be successful [15]. However, for monitoring purposes, the epidemiological breakpoint (ECOFF) is frequently used. The ECOFF is the highest MIC value for the susceptible (wild type) population [15, 16], see Figure 5.2. It is important to distinguish between these two breakpoints. Isolates categorized as resistant with the ECOFF value may still respond to treatment in a clinical setting. Using the ECOFF enables detection of resistant isolates before the potential development of clinical resistance. The ECOFF therefore works as a precautionary measure.

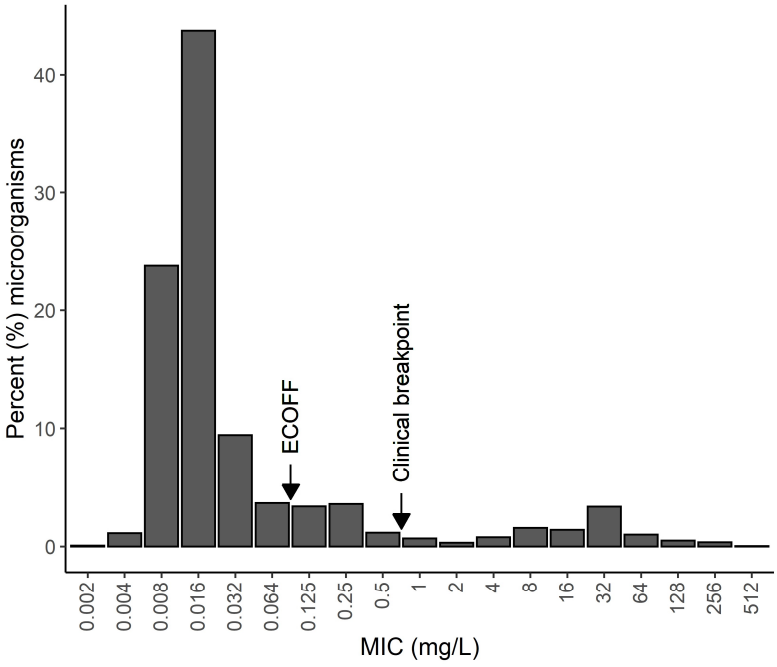

Figure 5.2: Distribution of MIC-values for ciprofloxacin in *E. coli*. Data: www.eucast.org.

Occurrences are often reported as a percentage, but can sometimes be referred to with terms such as “low” and “high”. Guidelines from EFSA and ECDC specify the percent occurrence that correspond to each of these terms [17]. The corresponding terms and percentages are “rare” (< 0.1%), “very low” (0.1 - 1%), “low” (> 1 - 10%), “moderate” (> 10 - 20%), “high” (> 20 - 50%), “very high” (> 50 - 70%), and “extremely high” (> 70%).

The occurrence of quinolone resistance among commensal *E. coli* in Europe seems to follow a north - south gradient [17, 18]. The northern countries seem to have a low occurrence while the southern countries a higher occurrence (Figure 5.3). The overall occurrence of QREC from 2006 to 2017 from production animals including pigs, cattle less than one year old, and various poultry animals was 26.1%. More specifically, the EU countries had a mean QREC occurrence of 49.67% in poultry from 2006 to 2016 [16, 17, 19–25]. This level corresponds to a high occurrence. A mean occurrence of 8.73% was observed in pigs within the same time frame [16, 19–22, 24, 26], corresponding to a low occurrence. To the authors’ knowledge, no systematic monitoring of wild animals have been done at a European level.

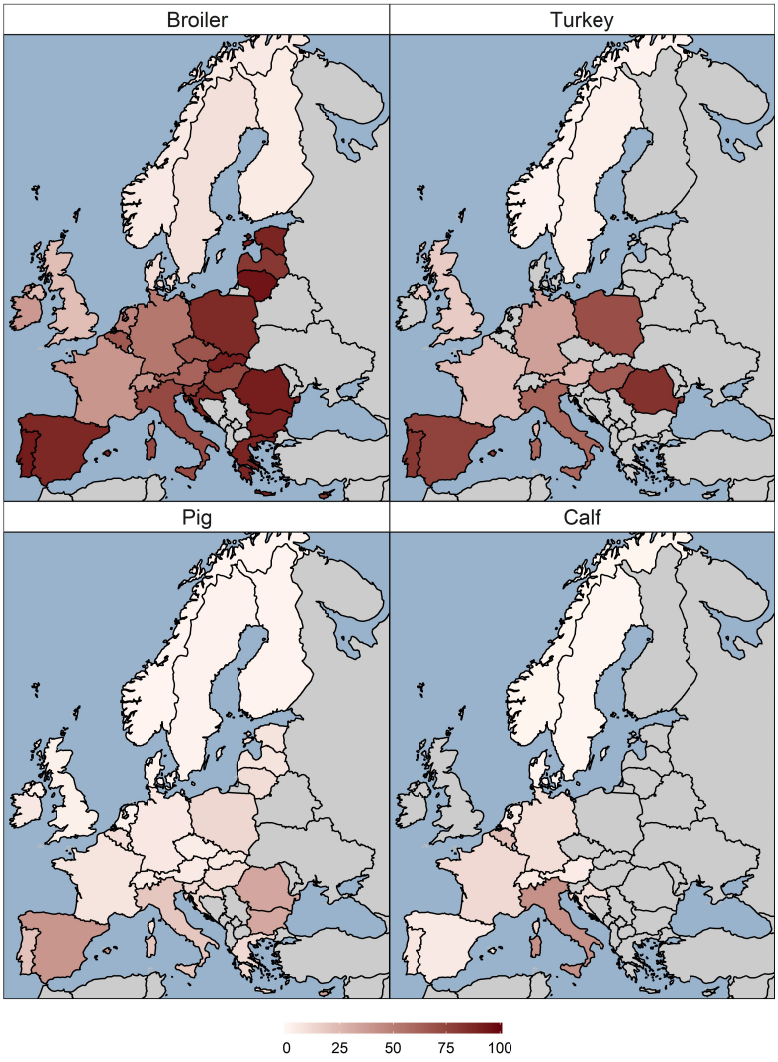

Figure 5.3: Mean occurrence of quinolone resistance in commensal *E. coli* from 2011 - 2017. Mean values from broilers was based on data from 2011, 2012, 2013, 2014, and 2016, for pigs from 2011, 2012, 2013, 2015 and 2017, for calves from 2015 and 2017, and for turkeys from 2014 and 2016. Grey areas represent missing data. Data taken from European Union summary reports on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food 2011 - 2017 [17, 18, 22–24, 24–26].

The situation in Norway

As part of the Norwegian government's strategy plan against antimicrobial resistance, the Norwegian monitoring programme for antimicrobial resistance in feed, food and animals (NORM-VET) was implemented in 2000. The programme has since 2014 followed the recommendations for surveillance as given by the EU (2013/652/EU) in parallel with national surveillance needs. A variety of animal species have been sampled in NORM-VET, but all animals are not sampled each year due to the sampling regime. The sample material is commonly faecal or caecal matter and meat, but boot swab samples are also included some years. In broiler and turkey flocks, 10 caecal samples are collected from each flock and pooled before culturing. For pigs and cattle, one individual is sampled per herd. Wild animals are sampled individually. Standardized culturing and isolation methods are used to identify *E. coli* from the samples collected. These isolates are used as an indicator for the presence of resistance in the given animal population on a national level. Quinolone resistance in commensal *E. coli* has been monitored in NORM-VET since 2000. Here, samples are plated onto lactose-bromothymol blue agar (pre-2013) or MacConkey agar (2013 until today). A single colony of presumptive *E. coli* is randomly selected for susceptibility testing, hereafter referred to as the traditional method.

Compared to the overall occurrence of quinolone resistance among indicator *E. coli* in the EU, the overall occurrence in Norway is low. For instance, the mean occurrence of QREC between 2006 and 2018 was 5.22% in broilers, and 0.3% for pigs [7, 27–35], corresponding to a low and very low occurrence, respectively. However, a peak in QREC occurrence was detected in 2009 at 8% [29], with no apparent explanation at that time. This triggered the introduction of a selective method in 2014 to further investigate this unexplained occurrence. With the selective method, the samples are plated onto MacConkey agar with 0.06 mg/L ciprofloxacin. Analysis with this method revealed that QREC were present in 89.5% of included broiler caecal samples in 2014. Corresponding analyses on samples from other animal species, such as pigs, red foxes, wild birds, breeder flocks and horses were performed in 2015, 2016 and 2017. Here, the occurrences ranged from 2.4% in horses, 5.6% in wild birds, 14.8% in red foxes, 54.3% in pigs to 100% in broiler breeder flocks [33–35]. In comparison, the occurrence of quinolone resistance among commensal *E. coli* identified with the traditional method in the same samples was 0% in horses and breeder flocks, 1.2% in red foxes, 2.3% in wild birds, and 0.7% in pigs. This difference in occurrence between the two methods indicates that the QREC are generally present at low levels.

Quinolones

Quinolones were introduced to the market in the late 1960s [36]. The first quinolone, nalidixic acid, was discovered as a by-product of chloroquine synthesis [37], and was clinically in use in 1967. A few decades later, the synthesis of novel quinolones increased the effectiveness of nalidixic acid towards Gram positive bacteria, and reduced its toxicity by altering its chemical structure. This structural change involved, among others, the addition of a fluoro-group to the molecule, which also increased its potency towards Enterobacteriaceae [36]. This also lead to a change in nomenclature for these compounds, as the molecules that harboured this structural change were then called fluoroquinolones.

The main target for quinolones and fluoroquinolones (hereafter called quinolones) in *E. coli*, is the type II topoisomerases DNA gyrase (also known as topoisomerase II) and topoisomerase IV [38]. The type II topoisomerases regulate DNA topology by cutting DNA strands, passing another DNA strand through the break, and then sealing the break [39]. Both DNA gyrase and Topoisomerase IV are comprised of two subunits, A and B. These are encoded by the genes *gyrA* and *gyrB* for DNA gyrase, and *parC* and *parE* for topoisomerase IV. Quinolones bind to these enzymes through a water-metal ion bridge (Figure 5.4), connecting the quinolone molecule to two specific amino acids; serine (S) 83 and aspartic acid (D) 87 in DNA gyrase, and S 80 and glutamic acid (E) 84 in topoisomerase IV [40]. This binding inhibits the ligase activity of the enzyme, and fragments the bacterial chromosome, leading to cell death.

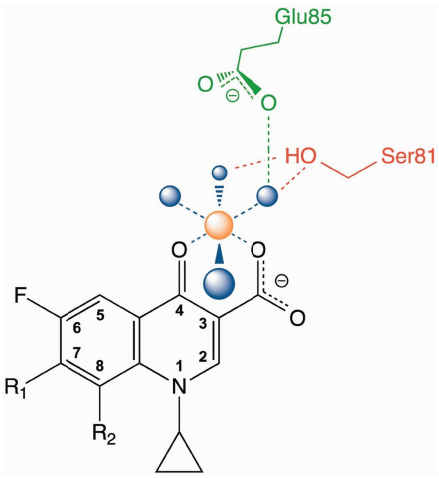

Figure 5.4: Overview of the water-metal ion bridge that mediates the quinolone-topoisomerase IV interaction, reprinted from Aldred et al. 2013 [41] with permission from Nucleic Acids Research.

Quinolone resistance mechanisms in *E. coli*

Resistance mechanisms may be disseminated either by vertical inheritance by cell division or through horizontal transfer of genetic material from one cell to another. Multiple mechanisms that confer resistance towards quinolones have been discovered in *E. coli*, and they are generally divided into four categories: chromosomal mutations, reduced membrane permeability, efflux pump overexpression, and plasmid mediated quinolone resistance (PMQR) [42], see Figure 5.5. Except for PMQR, these resistance mechanisms are mediated by mutations in the chromosome that either lead to structural changes of the encoded proteins or changes in expression through various pathways. The four categories are therefore described as two different categories below: chromosomal mutations and PMQR.

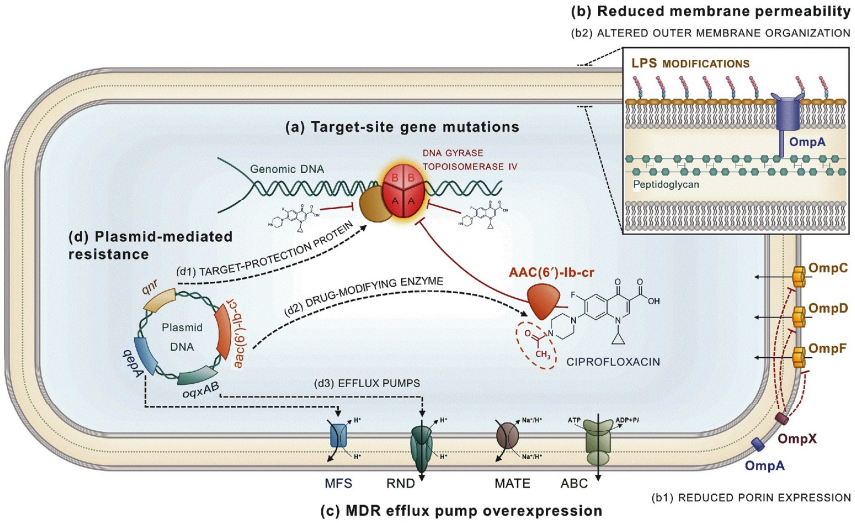

Figure 5.5: Quinolone resistance mechanisms in *E. coli*. Reprinted from Correia et al. 2017 [42] with permission from the Microbiology Society.

Chromosomal mutations

Perhaps the most well-studied resistance mechanism towards quinolones is through mutations in the genes that code for the target molecules, *gyrA*, *gyrB*, *parC*, and *parE*. Missense mutations in these genes that lead to a substitution of critical amino acids needed for the binding of quinolones to the target molecules may lead to an increased tolerance towards these compounds. In *E. coli*, substitutions in a stretch of amino acids known as the quinolone resistance determining region (QRDR) in these four genes is highly correlated with quinolone resistance. In GyrA, this region is between amino acid (AA) 67 to 106 [43], in GyrB between AA 333 and 481, ParC between AA 51 and 170, and ParE between AA 366 and 523. Specific substitutions within these areas are often identified in QREC, e.g. the S 83 and D 87 positions in GyrA [44], or the S 80 position in ParC.

Porin channels in Gram negative bacterial membranes are essential for the entry of quinolones into the cell [42]. Specific chromosomal regulons are involved in the expression of these membrane proteins, exemplified by the *mar*, *soxRS* and *rob* regulons [42]. Mutations in these genes may decrease the expression of the Omp-type porins [45, 46].

In *E. coli*, the AcrAB-TolC efflux pump has been identified as a major facilitator of quinolone efflux [47, 48]. Mutations in specific regulatory genes such as *marR* may activate *acrAB* and *tolC*, thus increasing their expression [49]. This will in turn reduce the drug concentration in the cell. Certain mutations in RNA polymerase subunit B (*rpoB*) have been shown to increase the expression of MdtK, which is a multidrug efflux transporter that can reduce the intracellular quinolone concentration [50].

Plasmid mediated quinolone resistance

Some proteins that confer resistance to quinolones are transcribed from plasmids, such as the quinolone resistance proteins (Qnr), the efflux pumps OqxAB and QepA, and the acetyltransferase aac(6')-*Ib-cr*.

Qnr proteins are pentapeptide proteins that are capable of protecting DNA gyrase and/or topoisomerase IV from quinolone action. Qnr proteins bind to topoisomerases and prevent the quinolone from stabilizing the lethal gyrase-DNA-quinolone cleavage complex [51–53], effectively reducing the possible targets for the quinolones. Multiple subtypes of Qnr proteins have been identified, namely QnrA, QnrB, QnrC, QnrD, QnrS, and QnrVC [54]. Other PMQR genes have been identified, such as *mcbG*, which code for pentapeptide proteins thought to have similar effect as Qnr proteins [54–56].

The efflux pump OqxAB has been shown to increase resistance towards chloramphenicol and quinolones, and is dependent on the host TolC outer membrane protein in *E. coli* [57, 58]. Similarly, QepA expression has been found to increase resistance towards ciprofloxacin, norfloxacin and enrofloxacin due to efflux [59].

The acetyltransferase aac(6')-*Ib-cr* infers resistance towards quinolones by altering the quinolone molecule itself, which reduces the activity of the quinolone [60].

5.2 *Escherichia coli*

E. coli is the most studied micro-organism on Earth [61], and is often used as a model organism in various microbiology studies and as an indicator for the presence of resistance in the intestine. *E. coli* is a Gram negative, rod-shaped, facultative anaerobic bacterium often found in the gastrointestinal tract of warm-blooded animals and humans. There, it may be present as a commensal or as a pathogen.

Genetics of *E. coli*

E. coli is a highly diverse species with over 7000 defined sequence types [62]. Several phylogenetic groups have been identified, representing major lineages within the *E. coli* species (Figure 5.6). A huge difference in gene content have been detected among *E. coli* isolates. The pan-genome, i.e. the total amount of genes identified among isolates in a population, can for example range from 15.000 - 40.000 genes [63, 64], depending on the number of included isolates [65]. The genes that are present among at least 99% of the included isolates, i.e. the core genes, seem to converge on approximately 2000 genes [65, 66]. With this apparent genome plasticity, recombination, i.e. the incorporation of horizontally transferred genes or the rearrangement of chromosomal segments, has definitely been important in the evolution of *E. coli* [67]. Early studies on the population structure of *E. coli* presented evidence for a clonal evolution based on electrophoretic movement of enzymes [68, 69], later supported in studies using sequencing methods [66]. Most studies investigating the population structure and evolution of *E. coli* seem to conclude that the species seem to evolve in a clonal manner, regardless of it's relatively high rate of recombination [67]. Recombination is further discussed in Section 5.3.

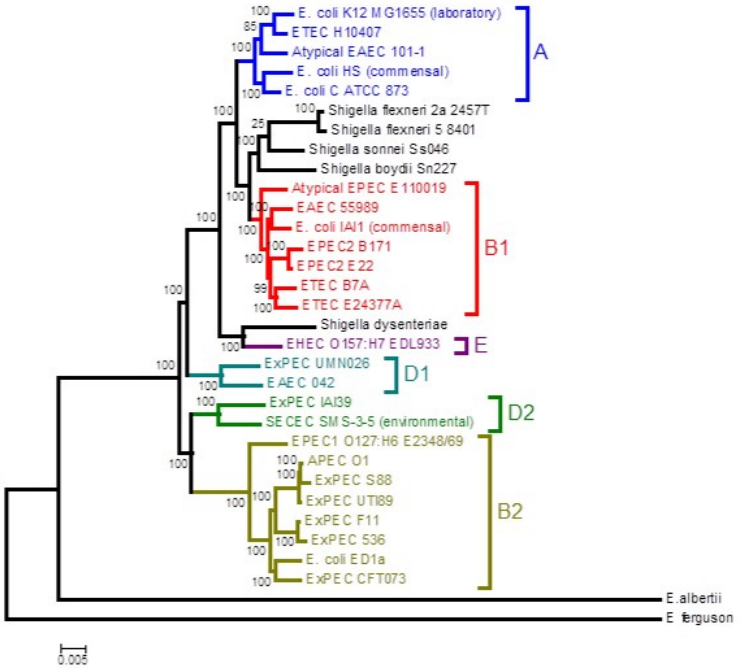

Figure 5.6: Phylogenetic groups of *E. coli*, based on maximum likelihood analysis of a concatenated alignment of 2173 genes, reprinted from Chaudhuri et al. 2012 [67] with permission from Infection, Genetics and Evolution.

5.3 Sequencing and bioinformatic analyses

In this project, we wanted to investigate the evolutionary relationship between isolates in addition to identifying resistance genes and mutations. For these purposes, we used high-throughput sequencing (HTS) to sequence the genomes of the included isolates and subsequently utilised the bioinformatic methods described below. Bioinformatics can be defined as the use of informatics techniques, mathematics, statistics and computer science to understand biological data on a large scale [70]. In this chapter, the concept of high throughput sequencing (HTS) and gene identification is presented, followed by a description of the analysis pathway used in this thesis to go from reads to assembly. Then, various subsequent analyses are described, such as pan-genome analysis and phylogenetics.

High throughput sequencing

The process of Illumina HTS builds upon that of Sanger sequencing. First, genomic DNA is extracted and purified. The gDNA is then fragmented and amplified through a process called library preparation. In this process, the gDNA is fragmented and adapters are placed on each end of each DNA fragment. Then, the fragments are amplified, usually by PCR. Then, the prepared fragments are placed on a flow-cell and the sequencing begins. The machine detects and registers each nucleotide through their unique fluorescent signal. The fragments are polymerized to a given length, usually between 100 - 300 base pairs [71]. Following sequencing, the reads can be used in a multitude of analyses, briefly summarised in Figure 5.7.

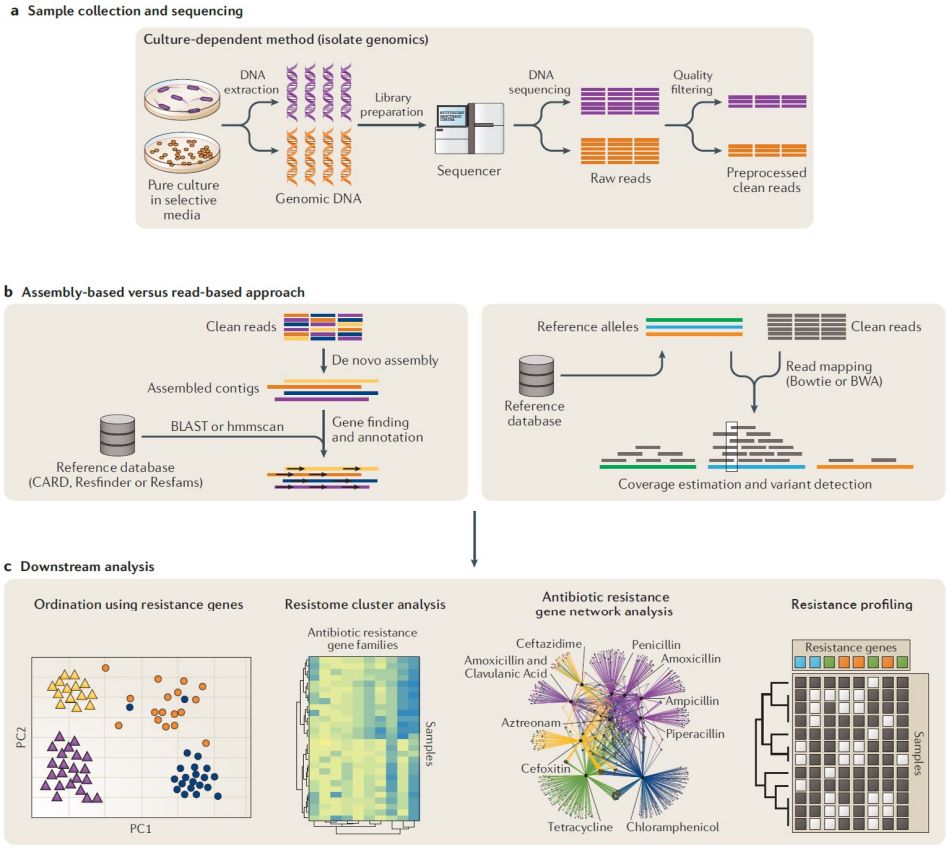

Figure 5.7: Main analysis pathway for culture-based genomic data, modified from Boolchandani et al. 2019 [72] with permission from Springer Nature.

Computational identification of genes

Computational identification of genes is mainly done in two ways, either from assemblies or from raw reads [72]. In both cases, a database containing the genes of interest is needed. Only the genes that are represented in the database can be detected. In assembly based methods, the references in the database are compared to the assemblies. A specific threshold of similarity is used to define the two sequences as the same gene. A popular method for gene identification with assemblies is the Basic Local Alignment Search Tool (BLAST) [73]. In read based methods, the raw reads are mapped to the references, either directly as whole reads or broken down as k-mers of

length *k*. Here, the mapped reads or k-mers are locally assembled and compared to the reference. Identifying genes and mutations by using whole assemblies is computationally demanding while read-based methods using k-mers are faster [72].

From reads to assembly

Reconstructing genomes is an important part of comparative genomics. Having a plausible reconstruction of each bacterial genome opens up the possibility of comparing genomic elements between isolates, and therefore makes it possible to deduce the relationship between them. This section briefly describes the analysis process from quality control of reads to the final assemblies.

Quality control of nucleotides in sequencing reads is important to make sure that the underlying data is of adequate quality for assembly. Quality parameters in fastq files, which is the common format for Illumina data, can be checked with software like fastQC [74]. The fastq files include quality information for each nucleotide in each read. Reads are trimmed to ensure that only high-quality nucleotides are included, and that residual adapter sequences from the sequencing reaction are removed. After trimming, the reads are ready to be assembled.

Genome assembly is the process where sequence reads are put together into longer, contiguous sequences called contigs, based on overlapping sequences in the reads. Most assemblers in use for Illumina data today are based on de Bruijn graph algorithms [75]. De Bruijn graph algorithms are based on separating the reads into *k*-mers of a specific length, then creating a graph by identifying overlaps. Then, the algorithm "walks" through the graph, and identifies the optimal path where each edge in the graph is visited only once, also known as an Eulerian path [76], see Figure 5.8. This optimal path then becomes the assembly sequence. It is important to note that genome assemblies are only plausible reconstructions of the original genomes in the cells from which the DNA was extracted, and is therefore only a computed approximation of the "real" genome.

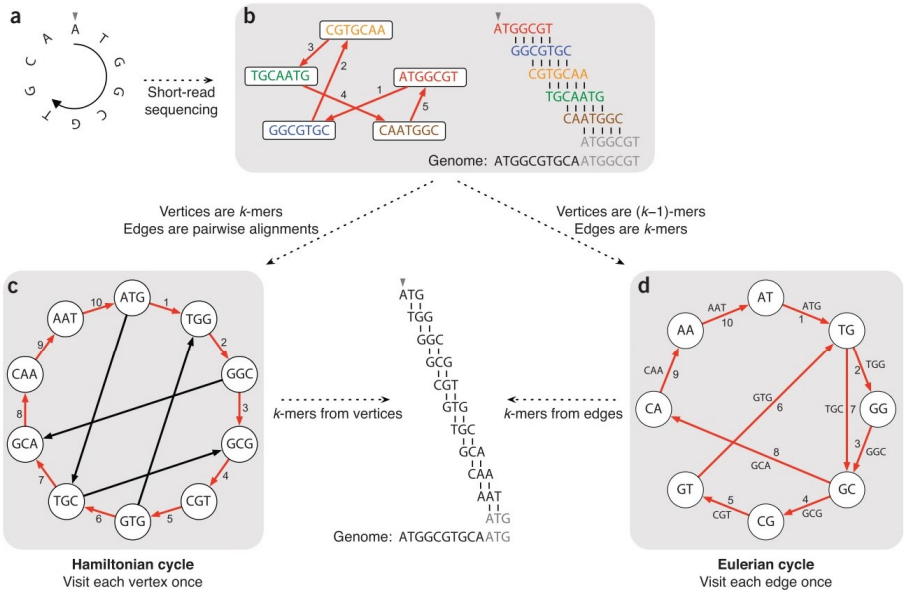

Figure 5.8: The two de Bruijn graph based assembly methods for genome assembly: Hamiltonian cycles and Eulerian cycles. Reprinted from Compeau et al. 2011 [77] with permission from Nature Biotechnology.

Post-assembly analyses

Genome annotation is the process where relevant features in the assembled genome is identified and labelled [78]. A commonly used procedure of annotating the genome is to first predict genes from the assemblies using software that identifies coding regions, such as open reading frames (ORFs). Then, the predicted genes are compared to a database of known genes and the most significant match is identified by alignment methods. The annotation of this match is then transferred to the predicted gene [78, 79]. Annotating the genome enables the identification of core genes [80]. This is done with a pan-genome analysis, which is a method of grouping genes based on their presence among the included genomes. For example, core genes are present in 99% of all included genomes. Identifying core genes is useful, as it highlights which genes are highly conserved among the included genomes.

An alignment of core genes can subsequently be used to analyse the genetic relationship between the isolates. In an alignment, the included sequences are assumed to be homologous. The

alignment contains blocks of highly similar sequences, and gaps where sequences are either missing or highly divergent. To be able to determine patterns of change between sequences, homologous sequences need to be aligned to be able to compare differences between them correctly [81]. Therefore, aligning sequences is the first step in a phylogenetic analysis, i.e. identifying the evolutionary history of the isolates [82].

Phylogenetic analysis

A frequent problem encountered by bacteriologists is to identify the evolutionary relationship between isolates. Very closely related isolates are sometimes called clones. The term clone does not have a clear definition, but can be loosely defined as isolates that with high probability originated from a recent common ancestor. However, the definition depends on the method used and previous knowledge about the circumstances in which the isolates were involved.

Phylogenetics is the field of study of evolutionary relationships among groups of organisms [82]. The most common way of representing phylogenetic relationships between organisms is a phylogenetic tree, exemplified in Figure 5.6. Every node in the tree represents the common ancestral state, and the leaves in the tree are the descendants. Trees like these may be calculated from many different types of data, and morphology has historically been frequently used to assess the relationship between the included organisms. However, today, molecular data is more commonly used to generate these trees. For example, an alignment of the core genome is regularly used to identify differences, i.e. single nucleotide polymorphisms (SNPs) between the included organisms. One method of creating these trees from the core genome alignment is the "character-state" method, where each position in the alignment (the "character") is independently analysed in regard to which nucleotide is present in that location (the "state") [83]. However, to properly make assumptions about the evolutionary relationship between isolates, a model of evolution has to be used in the analysis [84]. The choice of model indicate which assumptions are being made for the isolates included in the analysis, such as the rate of evolution, i.e. the mutation rate, for all included isolates. Many models exist, and finding the optimal model can be a difficult task. Therefore, software used for tree reconstruction sometimes has algorithms that find the optimal model to apply to your data to make this task easier. However, this selected model may not be optimal for a subset of the included isolates, since some lineages may evolve at a different rate [84].

Phylogenetic analysis is based on genealogy, and therefore assumes vertical inheritance. Horizontal gene transfers introduce genes that may have a different evolutionary history than the vertically inherited genome of the isolates. Recombination and horizontal gene transfer does not

seem to affect tree topology for *E. coli*, but it does have an effect on branch lengths [85], i.e. the representation of evolutionary time in the tree. Identifying and removing recombinant areas and horizontally transferred genes is therefore important, not only to prevent overestimated branch lengths, but also to ensure that only vertically inherited parts of the genome is included in the analysis.

Millions of different tree structures can describe the underlying data, and generating these trees is highly computationally demanding. Maximum likelihood (ML) methods are often implemented to handle these problems. Software that use ML methods apply a heuristic approach to identify a reasonably good tree by searching for the tree that maximizes the probability of observing the data, given the selected model of evolution [83]. Thus, multiple trees are generated, but only the one with the "best fit" to the data is selected.

6 Project Background and aims

Quinolone resistance among commensal *E. coli* from Norwegian food-producing animals is generally low. This is considered to be a consequence of good animal health and of the low usage of quinolones. After the introduction of a selective method in NORM-VET, QREC was detected in a high proportion of samples in broilers and pigs. Because of the low quinolone usage in these animal populations, the observed occurrence was somewhat unexpected and warranted further investigation into the characteristics and the origin of these bacteria. This project provides a unique opportunity to investigate other causes of quinolone resistance development than quinolone usage. Investigating the mechanisms conferring resistance may provide information on whether the majority is plasmid-mediated or chromosomal. This can, in turn, give indications on how the quinolone resistance is disseminated. Insights into the phylogenetic relationship between isolates may reveal information on possible dissemination within or between animal populations. It can also provide indications for persistence in these environments. This information is important to further understand how QREC may be disseminated or developed in animal populations, especially in countries with low antimicrobial usage. Furthermore, these data may be used to implement specific preventive strategies to reduce potential dissemination of QREC within production animal populations. These implementations can help maintain the favourable situation in Norway.

The aims of this study were to:

- Summarize and describe existing data on occurrence of quinolone resistance in animal populations
- Characterize genetic mechanisms that may explain the observed quinolone resistance
- Explore potential emergence and dissemination of quinolone resistance in *E. coli* in livestock populations

7 Materials and Methods

Here follows a summary of methods used - for details, see enclosed papers.

7.1 Laboratory methods

All *Escherichia coli* isolates included in this project were isolated through the NORM-VET programme, and the methods used for isolating *E. coli* and susceptibility testing are described in Section 5.1. An *E. coli* was categorized as quinolone resistant if the MIC value for ciprofloxacin or nalidixic acid was above 0.06 mg/L or 16 mg/L, respectively, based on ECOFF values defined by EUCAST (ECOFF values as of 01.08.2019, www.eucast.org).

Library preparation and sequencing service was provided by the Norwegian Sequencing Centre (NSC, www.sequencing.uio.no), a national technology platform hosted by the University of Oslo and supported by the "Functional Genomics" and "Infrastructure" programmes of the Research Council of Norway and the South-eastern Regional Health Authorities.

7.2 Bioinformatic methods

Bioinformatic analysis was mainly performed on the Abel Cluster, owned by the University of Oslo and Uninett/Sigma2, and operated by the Department for Research Computing at USIT, the University of Oslo IT-department (www.hpc.uio.no/).

Identification of resistance mechanisms

The program ARIBA (Antimicrobial Resistance gene Identification by Assembly) [86] was used to identify resistance genes with the Resfinder [87] database and mutations with the MEGARes database [88], as well as determining the sequence types of the isolates, with the *E. coli* scheme hosted by Enterobase [62]. The process by which ARIBA works is visualized in Figure 7.1.

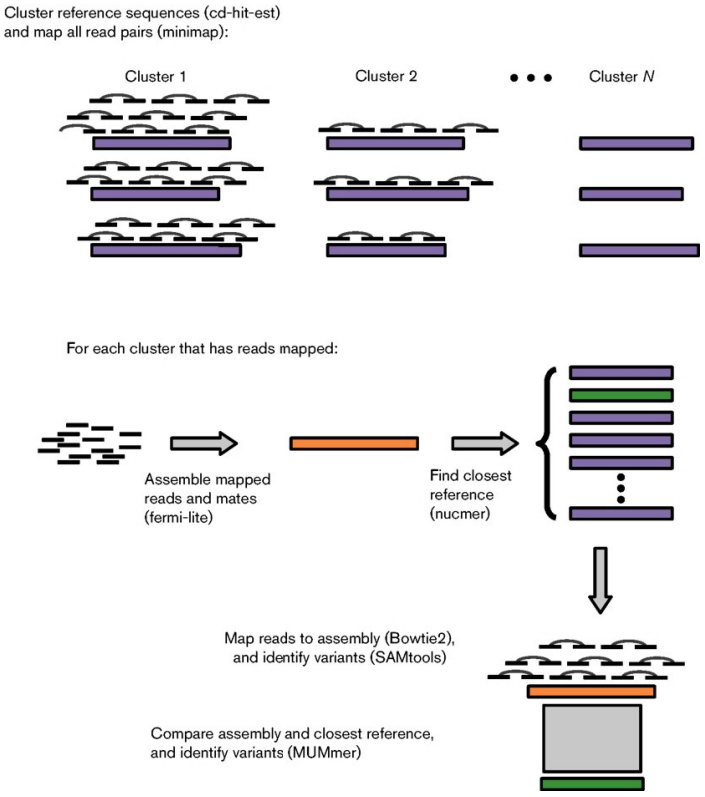

Figure 7.1: Overview of the ARIBA mapping and targeted assembly pipeline, reprinted from Hunt et al. 2017 [86] with permission from Microbiology Society. Program names are listed in brackets.

ARIBA is a read-based algorithm that works by clustering the reference sequences from the selected database (Figure 7.1). Then, reads and their pairs (mate in Figure 7.1) are mapped to the clusters and locally assembled. The closest reference to the assembled sequence is identified, and 11 different quality metrics are calculated, such as gene completeness and overall success of the local assembly [86]. The combination of all metrics is encoded into a flag, which is a

number given by ARIBA based on the underlying set of quality metrics for each predicted gene or mutation. Only one flag is given for each predicted mutation or gene, and each flag has a specific interpretation. In the current study, three of the 11 metrics were evaluated as TRUE, while four were evaluated as FALSE to ensure high quality of the predicted mutation or gene. The remaining four metrics could be either TRUE or FALSE. All allowed combinations of metrics are listed in Table 7.1. These criteria resulted in 16 different flags that were accepted. All genes or mutations that were predicted with a non-accepted flag were removed. An R script was used to select the genes or mutations that fulfilled these criteria (www.github.com/hkaspersen/VAMPIR).

Table 7.1: All metrics reported by ARIBA and their allowed values used in this study. All possible combinations of these metrics resulted in 16 different allowed flags. See github.com/sanger-pathogens/ariba/wiki/Task:-flag for a detailed description of each metric.

Metric	Value
Assembled	
95% of the reference sequence is identical to the assembly	TRUE
Assembled into one contig	
The gene is assembled into only one contig	TRUE
Region assembled twice	
< 3% of the reference has more than one match to the assembly	FALSE
Complete gene	
The gene is complete (from start to stop)	TRUE | FALSE
Unique contig	
Exactly one contig in the assembly matches the reference	TRUE
Scaffold graph bad	
Assembly graph ambiguity	FALSE
Assembly fail	
No output from assembler	FALSE
Variant suggests collapsed repeat	
Variant in position that matches to the reference	TRUE | FALSE
Hit both strands	
Two or more matches to the reference in opposite orientations	TRUE | FALSE
Has variant	
Variant present	TRUE | FALSE
Ref seq choose fail	
Error when selecting closest reference	FALSE

Assembly, annotation and pan-genome analysis

All software used for assembly, annotation and pan-genome analysis is presented in Figure 7.2.

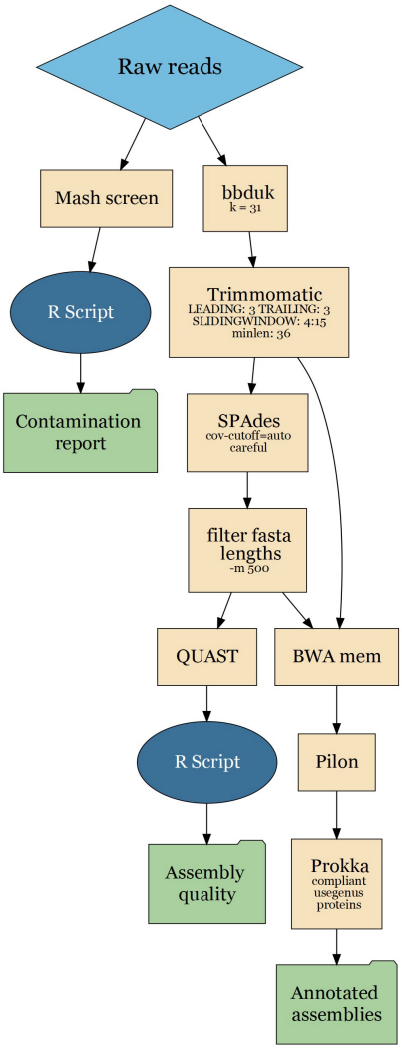

Figure 7.2: Flowchart of assembly and annotation pipeline. Light blue: input data. Yellow rectangle: name of the analysis tool used on the Abel cluster, with important program settings listed. Dark blue ellipses: analyses in R. Green folders: output data.

Phylogenetic analysis

Phylogenetic analysis methods are summarised in Figure 7.3. The left pathway was used to identify the overall relationship between all isolates in each paper. The right pathway was used to further analyse clades of interest with deeper resolution and recombination removal.

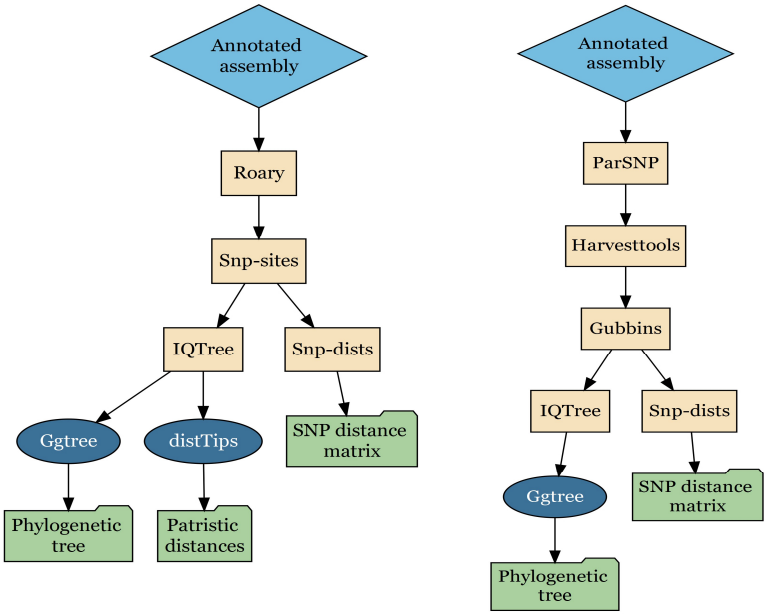

Figure 7.3: Flowchart of phylogenetic methods. Left: phylogenetic analysis for all isolates. Right: phylogenetic analysis for selected clades. Light blue: input data. Yellow rectangle: name of the analysis tool used on the Abel cluster. Dark blue ellipses: analyses in R. Green folders: output data.

7.3 Statistical methods

Statistical analysis was done in R version 3.6.1 (2019-07-05)[1]. Significant differences between groups were calculated by using χ^2-test. Confidence intervals were calculated by using the two-sided exact binomial test at 95% confidence level.

A non-parametric permutation test was used to assess significant aggregation of isolates in phylogenetic trees. Here, the median minimum SNP distance to the closest isolate within the

same animal species was calculated. Then, a permutation test was used to calculate the median minimum SNP distance for each iteration (n = 1000). Non-exact p-values were calculated based on the number of expected values that were lower than the observed value for all iterations.

Non-metric multidimensional scaling (NMDS) was used to cluster isolates based on presence/absence of quinolone resistance conferring substitutions and genes by using the vegan package [89]. NMDS is an ordination technique that has previously been used to relate the presence of acquired AMR genes to sample source or type [90, 91]. A stress plot (Shepard diagram) was calculated to determine how well the ordination represented the data [89].

8 Discussion

8.1 Methodological considerations

Selection bias

In the epidemiological study (paper I), the entire population of *E. coli* and QREC obtained through the NORM-VET programme from 2006 to 2016 was included. The sampling performed in NORM-VET from livestock animals at slaughterhouses is based on a proportional sampling according to the slaughter volume at each slaughterhouse. The sampling is done in a random week of the year, with only one sample per flock or herd. These samples can be considered as representative for the livestock populations in Norway. For the wild animals, the sampling is dependent on hunters or other people sending in carcasses to be included in the programme. Therefore, these samples may not be randomly distributed throughout Norway, even with efforts to request samples from the whole country. Regardless, the isolate collection from the wild animals is likely as representative for the actual *E. coli* population in the respective animal species as possible, and was regarded as such in the epidemiological study.

In paper II, a random selection of isolates was performed after grouping the isolates on phenotypic resistance patterns. This was done to ensure a high phenotypic diversity among the selected isolates. This selection may therefore not represent the actual QREC populations in each included animal species.

In paper III, the isolate selection included both QREC and quinolone susceptible (wild type) *E. coli*. Here, the goal was to further investigate dissemination, as well as to identify possible development of quinolone resistance in wild type isolates. Isolates were included if the production site was sampled at least three times, and at least one QREC isolate and one wild type isolate were detected in samples from the respective production site. The results may therefore be biased because of the uneven representation of each production site. In addition, not all production sites were represented, as the selection only covered 22 of 384 total registered production sites from 2006 to 2017.

Genomic analyses methods

The various software used in the genomic analysis were selected because they are internationally recognized programs that are regularly used for such analyses. The programs have a large user base. Thus, their behaviour and result characteristics are well known.

Contamination from non-*E. coli* isolates

MALDI-TOF was used to confirm the species as *E. coli*. The samples could nonetheless have been contaminated during DNA extraction. Colonies may grow on top of each other or have similar morphologies, making them difficult to distinguish. Implementing *in silico* screening for contaminants is therefore useful to prevent the inclusion of such contaminated data into further analysis. This was exemplified by the exclusion of four isolates in the current study. These samples were found to be contaminated with *Citrobacter* or *Enterobacter* using Mash. It is likely that contaminants were present in other included isolates as well. However, in those instances the potential contaminants were likely to be of low presence among the *E. coli* reads. Thus, the contaminant reads, if present, were likely assembled into small contigs. After the assembly, contigs smaller than 500 bp were filtered out of the data. This step probably removed most of these potential contaminant contigs.

Identification of genes and mutations

In this project, ARIBA [86] was used to identify resistance genes and mutations, and for sequence typing. For this work, ARIBA has multiple advantages, such as being able to detect both resistance genes and mutations. ARIBA is a read-based program. Using a read-based approach for the detection of AMR genes has been found to be superior to using assembled sequences [92]. The process of genome assembly is complicated, and if one gene is for example split over multiple contigs they may be missed [86, 92]. Another advantage of using ARIBA is the possibility of downloading newly updated databases of our choosing. Here, the ResFinder [87] database was used for identification of acquired genes. The MEGARes [88] database was used for the identification of mutations in chromosomal genes. Resfinder is a popular database for acquired resistance genes, which is manually curated and updated regularly. MEGARes was originally created for metagenomic characterization of AMR genes and mutations [88]. This database contains references from ResFinder, ARG-ANNOT, the Comprehensive Antibiotic Resistance Database (CARD), and the National Center for Biotechnology Information (NCBI) Lahey clinic β-lactamase archive. MEGARes is also manually curated, and is specifically annotated for

high-throughput data processing [88]. MEGARes was chosen as a database because it already encompasses other popular resistance databases. It is also easily downloaded through ARIBA. The results generated by ARIBA using the MEGARes database were easier to interpret than the data generated by using the CARD database alone. This is likely because the annotation is specifically designed for high throughput data processing.

Databases confer limitations on the resulting data generated by using them. As mentioned earlier, it is only possible to detect the genes that are present in the database, and potential novel genes are therefore missed. In this study, the flag accompanying each predicted gene or mutation were investigated. Genes or mutations that did not have sufficient quality, as presented in section 7.2, were removed. This could, for example, be because the gene was not adequately assembled, or that multiple contigs in the assembly matched the reference. Thus, some genes or mutations may be false negatives. In this project, only novel mutations were confirmed by identifying the same mutation using assembly methods. This was not done, however, for already known mutations, such as the mutation leading to the S83L amino acid substitution in GyrA. One way of checking for potential false negatives from the ARIBA results would be to confirm with assembly based methods. This could have been done with tools such as PointFinder [93].

Phylogeny

Phylogenetic methods were used to determine the evolutionary relationship between the isolates. Core genome SNP phylogeny is regarded as one of the methods with the highest resolution for inferring evolutionary relationships, compared to the clustering method core genome MLST (cgMLST). cgMLST is similar to MLST but utilize a scheme that includes over 2000 genes. Core genome SNP phylogeny and cgMLST have been regarded as complementary in an outbreak setting [94, 95]. This is likely not the case when the isolates are not assumed to be closely related, as reflected in the current project. Thus, a phylogenetic approach is appropriate to get the resolution necessary to be able to deduce the relationship between these isolates.

Genomes were assembled with SPAdes prior to phylogenetic analysis. When dividing each read into *k*-mers, the difficulty of putting them back together is increased by the *k*-mers that contain sequencing errors. These *k*-mers will create diverging paths in the de Bruijn graph, further complicating the assembly. A perfect eulerian path is not possible, as sequencing errors and repeats obscure the graph [76]. These errors may introduce or mask SNPs or indels in the assembly, but were likely removed during the assembly process. In this study, Pilon was used to correct potential errors in the assembly process by mapping the reads back to the assembly. Thus, sequencing errors were likely corrected.

Removing recombinant sites is an important step when analysing vertical inheritance, because these sites may have a different evolutionary history. Here, Gubbins was used in this regard. Gubbins have been found to be much faster than similar software [96], such as ClonalFrameML [97]. Recombinant sites are detected by scanning for loci that contain a higher density of base substitutions that the vertically inherited genome. This is indicative of horizontal transfer [96]. Here, assembly- or sequencing errors may influence the results, as Gubbins cannot distinguish between elevated densities generated by horizontal transfer or other causes [96]. Thus, some areas in the genome assemblies may have been erroneously categorized as recombinant and removed. However, the alignments that were used to create the phylogenetic trees were regarded as large enough that this probably did not change the phylogenetic structure in significant ways.

IQTree was selected as the ML algorithm to find the optimal phylogenetic tree. Comparative analyses have shown that the search strategy implemented in IQTree achieves higher likelihoods than similar algorithms [98]. IQTree also seem to find optimal trees faster. However, IQTree was not always the best algorithm when tested against RAxML and PhyML. Therefore, the authors recommended using all three programs [98]. In this study, only IQTree was used, which means that potential trees with higher likelihoods may have been missed.

An evolutionary model was selected by using ModelFinder plus [99] implemented in IQTree. ModelFinder finds the optimal evolutionary model for the data, based on, for example, Akaike's Information Critera (AIC) and Bayesian Information Criteria (BIC) [99]. Both AIC and BIC are values that describe how well the model fits the data [100]. Low values are preferred for both measures [100]. By using these criteria, ModelFinder plus identifies the model that best fit the data. However, using more than one model may be necessary when working with many isolates from potentially different lineages. Some lineages may evolve differently than others. The selected models in this study may therefore not be optimal in describing the evolutionary rate of all the isolates in one tree. This is also partly why phylogenetic analysis was performed separately on more closely related isolates, as exemplified by the ST117 isolates in paper II and ST355 isolates in paper III. Other more appropriate evolutionary models were selected for these trees.

Statistical considerations

A non-parametric iteration test was used to assess the aggregation of samples in phylogenetic trees based on SNP-distances. A non-parametric test was selected because the underlying distribution of SNP-distances did not follow a theoretical distribution, such as a normal distribution. Additionally, the data could not be regarded as independent observations.

Therefore, a test that does not rely on distribution assumptions or independent observations was selected.

Non-metric multidimensional scaling (NMDS) was used to cluster isolates based on presence / absence of resistance genes and mutations (paper II). NMDS is an ordination technique that differs from other ordination methods, such as principal component analysis (PCA), as NMDS does not assume a relationship between the samples [101]. Therefore, any distance measure is allowed in NMDS, which makes this method well suited for a wide variety of data. NMDS is an iterative method, and running the same analysis several times on the same data may yield a slightly different result each time. Therefore, the solution found in paper II may thus represent an adequate solution rather than the "best" one. A stress value is calculated for each iteration and represents how good the data is summarized by the ordination. In the present study, this stress value was low (< 0.05), which is regarded as a good representation [102, 103].

8.2 Main results and discussion

Occurrence of QREC

At the start of the project, the only information available on occurrence of QREC in Norwegian animals was in the NORM-VET reports. Here, occurrence data for each year was readily available. However, no overall occurrence comparison between animal species across the years had been performed. The difference in overall occurrence between animal species was therefore largely unknown. Compiling and analysing data from NORM-VET surveillance was therefore regarded as a good starting point, and could give pointers for where further investigation was needed. The results of this investigation showed an overall low occurrence in several animal species in Norway. An increasing trend of QREC occurrence was detected in broilers. The conclusion was that since little to no quinolones are used in these animal species, other factors could play a role in the occurrence of QREC (paper I).

In paper I, different levels of QREC occurrence were detected with the traditional method among commensal *E. coli* in different production animal species between 2006 and 2016. These animals are fed different feed, stay in different environments, have a different anatomy, and vastly different production site densities. All of these aspects and more may affect the QREC occurrence within these animal populations. No QREC were detected in horses or sheep in the current project. However, QREC were detected in 0.3% of commensal *E. coli* isolates from sheep in NORM-VET 2018 [7]. An overall QREC occurrence of 0.3% and 0.4% was identified among commensal *E. coli* from pigs and cattle, respectively. All of these occurrence levels are comparable to the levels reported by other Nordic countries, such as Sweden and Denmark, in the same time frame [104, 105], see Figure 8.1. The overall European level of ciprofloxacin resistant *E. coli* occurrence in pigs and cattle was in 2017 10.6% for both species [18]. This indicates that the Nordic countries have a much lower occurrence level than most other European countries. For broilers, the occurrence seems to be more varied among the Nordic countries. A slightly increasing trend of QREC occurrence has been observed in both Norway and Denmark. Contrastingly, the occurrence in Sweden has been decreasing since 2013 from 14% to 7% in 2018 [106, 107]. Regardless of these increasing and decreasing trends, the overall occurrence of QREC in the three countries from 2010 to 2018 is relatively similar, at 2.13% in Norway, 2.66% in Denmark, and 2.89% in Sweden based on data from the NORM-VET, SVARM and DANMAP reports [104, 105, 108]. These numbers correspond to a low occurrence in all three countries.

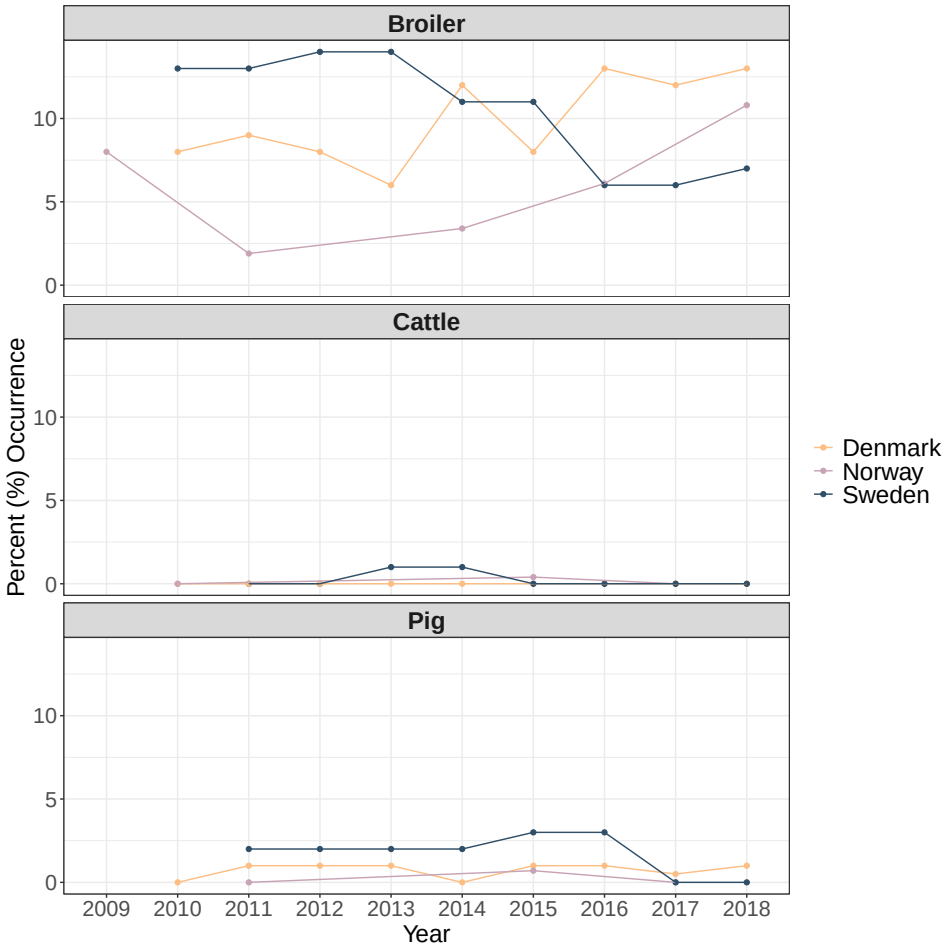

Figure 8.1: Overall QREC occurrence in broilers, pigs and cattle in Norway, Sweden and Denmark between 2009 and 2018. Data summarized from the NORM-VET, SVARM, and DANMAP reports, respectively [104, 105, 108].

Norway is, to the author's knowledge, the only country that has done selective screening for QREC among selected animal species. Comparison of occurrence data generated by this method to other countries is therefore difficult. However, the selective method supplies valuable data when used together with the traditional method. The two methods together provide a rough overview of the relative amount of QREC in the samples tested. For example, using the selective method, an occurrence of 2.4% was detected in samples from horses [35], 7.2% in samples from cattle

[33], and 9.6% in samples from sheep [7]. In contrast, the corresponding occurrence in broilers and pigs were 89.5% and 54.3%, respectively [32, 33]. Taken together with the occurrences generated with the traditional method presented above, there is an obvious difference in QREC occurrence between cattle, sheep and horses when compared to pigs and broilers. A hypothesis that the anatomy of ruminants might affect the occurrence of QREC in these animal species was investigated in the project. This hypothesis had previously been addressed in a Swedish study. There, they concluded that the prevalence of QREC was higher in calves less than 18 days of age compared to older calves [109]. Very young veal calves have not yet developed the intestinal microbiota. They are therefore regarded as monogastric up until the animal stops drinking milk from the mother and starts eating solid food. At this point, the animal is regarded as a ruminant. A small pilot study addressing this hypothesis was performed by collaborators. The temporal occurrence of QREC in calves and their mothers within two different farms were investigated (unpublished data). The calves were sampled frequently during the first three months of life, and the samples were subjected to selective screening of QREC. The results indicated that there were other factors than age alone that affected the occurrence of QREC. However, as this was only a small pilot study, additional studies are needed to further investigate this hypothesis.

In NORM-VET, pigs are sampled individually and the samples are not pooled together as they are for broilers. The occurrences detected with both methods indicate that QREC is present in a high proportion of samples from pigs, but at low levels. The occurrence of QREC in other Nordic countries is similarly very low [18]. A study is currently ongoing at the Institute where pig herds that have been medically remediated with quinolones against *Actinobacillus pleuropneumoniae* were sampled. These pig herds had been medically remediated only once between three to 25 years ago and were compared to control herds, where quinolones had, with a high certainty, not been used. A semi-quantitative method was used to identify the occurrence and relative presence of QREC in samples from both case and control herds. Preliminary results showed a significant difference between the case and control herds (unpublished data) and indicates persisting QREC in the production environment years after medical remediation with quinolones. However, it is unknown if these QREC isolates were already present before being exposed to the quinolones, or if commensal isolates developed resistance as a response to the exposure. The findings in this ongoing study may indicate that even with low quinolone usage, QREC may be selected for or develop in the gut of these animals and persist in the pig production environment.

In the current study, the occurrence of QREC was decidedly highest in broilers compared to the other production animals included. Samples from broilers are pooled before screening, which may have increased the probability of detecting QREC among the samples tested. Nonetheless, since quinolones are not used in the Norwegian broiler production, a question arose regarding their

origin. It was hypothesized that the observed occurrence in broilers was either due to introduction and dissemination of existing strains, or by spontaneous development of quinolone resistance in wild type *E. coli* in the gut of the animal due to unknown selective pressure. Similarly, because of the observed occurrence of QREC detected with the selective method in pigs, isolates from both species were whole genome sequenced to detect possible dissemination of similar isolates within each production chain. Additionally, QREC isolates from wild animals were included to identify isolates that may be introduced to these production chains from the outside. Moreover, which resistance mechanisms that were dominant among the QREC isolates from these animal populations was not known, thus a resistance mechanism characterization was performed.

Resistance mechanism characterization and comparative genomics

The results in paper II suggested that the majority of resistance mechanisms identified were chromosomally encoded. The major resistance mechanisms causing quinolone resistance among these isolates were mutations in *gyrA* and *parC*, which are well described mechanisms in the literature [110]. Novel mutations were identified in some of the other investigated chromosomal genes, but the effect of these mutations is still unknown. *In vitro* mutational studies are needed to further investigate if these mutations have an effect on MIC values toward quinolones. Transcriptomics may also give useful information on the expression levels of these proteins.

A relatively high occurrence of PMQR was detected among the pig isolates (26.6%) compared to the broiler isolates (4.6%). The low occurrence of PMQR in broilers is in concordance with other Nordic studies on QREC [111, 112], and may indicate that PMQR determinants are relatively rare in the Nordic broiler production chain. These concordant results emphasize the dominance of chromosomally encoded quinolone resistance mechanisms in the QREC isolates from the broiler production, and favour the hypothesis that QREC isolates are mainly vertically disseminated in the Nordic broiler production chain. Therefore, PMQR seem to be of minor importance in the development and dissemination of QREC in the Nordic broiler production chain. In contrast, PMQR genes, specifically *qnrB19* and *qnrS1*, were detected in a high proportion of the pig isolates. *qnrB19* have previously been identified on small, non-conjugative plasmids harbouring little to no other resistance genes conferring resistance toward other antimicrobials [113], while *qnrS1* has been associated with large, conjugative plasmids harbouring multiple resistance genes [114, 115]. Plasmid characterization has not been done in the current study, but if the *qnrB19* genes are indeed located on non-conjugative plasmids, then this may indicate that they are clonally disseminated in the pig production chain.

Phylogenetic analysis of QREC isolates from broilers and pigs revealed possible dissemination

within the respective production chains. Moreover, possible persistence in the broiler production chain was detected. Additionally, highly similar major sequence types among isolates from broilers were detected. These findings, together with the low occurrence of PMQR in broilers, suggested a clonal dissemination of QREC in the broiler production chain. The data thus suggested that dissemination was a major contributor to the observed occurrence of QREC in the broiler production chain. However, it was unknown if these strains were disseminated from higher levels of the production chain as suggested by a Swedish study [112], or if the resistance developed in wild type isolates somewhere in the Norwegian broiler production chain due to an unknown selective pressure. To investigate this further, both QREC and wild type *E. coli* were whole genome sequenced and compared (paper III).

The results in paper III further supported the hypothesis that QREC is disseminated through the Norwegian broiler production chain, and likely originate from imported breeding animals. This hypothesis has also been investigated in other Nordic countries both for QREC and cephalosporin-resistant *E. coli*, where the authors reach a similar conclusion as the present study [111, 112, 116–119]. Major QREC STs, the same as the ones identified in the current study, was reported in other Nordic countries[111], namely ST355, ST10, and ST349. These results, taken together with the similar levels of quinolone usage among these countries, provides strong evidence for implicating import of breeding animals as a major causative factor for the occurrence of QREC in the Norwegian broiler production. However, only a few samples from parent flocks were included in this project. To confirm that the major QREC lineages are introduced to the Norwegian broiler production through imported breeding animals, further sampling and characterization of QREC from parent flocks need to be performed. Also, studies comparing isolates from several Nordic and European countries would be of interest. To the authors' knowledge, no data on QREC occurrence or genetic characterization of QREC is available from the grandparent distributor in Scotland or Germany. This information would further provide much needed data on this hypothesized international dissemination.

The possibility of quinolone resistance development in wild type *E. coli* have, to the author's knowledge, not previously been investigated by comparing QREC and wild type isolates using a phylogenetic approach. The result of this analysis in paper III indicated that this rarely occurs in the Norwegian broiler production chain. However, one instance was observed where quinolone resistance was hypothesized to develop in a wild type *E. coli*, where the two isolates were isolated eight years apart, from broilers from different production sites. Because of the geographical distance, it was hypothesized that the wild type isolate may have either been disseminated to a lower level of the broiler production before developing resistance, or developed resistance at a higher level in the broiler production and were subsequently disseminated. However, it was

impossible to conclude on this from the current data. Because of the low occurrence of such resistance development among the isolates, it is likely that the hypothesized selective pressure is not very strong, as only a low level of QREC have been detected among the QREC positive samples. Taken together with the evidence for import and dissemination described above, the development of quinolone resistance in wild type *E. coli* seem to be of minor importance for the occurrence of QREC in the Norwegian broiler production.

Other than the work presented in paper II and in the upublished study, no further investigation of the presence of QREC in the Norwegian pig production was performed in this project. Since the Norwegian pig production is domestic, QREC is less likely to be introduced to the pig production chain in a similar manner as the broiler production, since little to no live pigs are imported each year. Feed has previously been found to introduce QREC strains to the broiler production chain on Iceland [120]. However, no QREC were identified in pig feed in Norway by using the selective method in 2016 [34], which makes it less likely as a source of QREC in the pig production. Further studies are needed to investigate the source of QREC in the Norwegian pig production chain.

9 Conclusions

The overall occurrence of QREC in Norwegian livestock animals is low. The resistance mechanisms identified in this project indicate that the majority of the mechanisms are being clonally disseminated. Horizontally transferrable quinolone resistance seems to be rare in the Norwegian broiler production environment. The results also show QREC dissemination throughout the Norwegian broiler production from higher in the production pyramid. Furthermore, the detection of the same major STs as in other studies on QREC from broilers in Scandinavia give strong indications that the QREC identified in the Norwegian broiler production originate from imported breeding animals. Also, little evidence for the development of quinolone resistance in wild type *E. coli* further support that dissemination is the major contributor to the occurrence of QREC in the Norwegian broiler production. These findings highlight the importance of biosecurity measures at a higher level in the pyramid, to prevent dissemination down through the Norwegian broiler production environment.

10 Future Perspectives

- In-depth characterization using comparative genomics of QREC isolates from broilers in Norway and other countries in Europe. This data is necessary to further investigate international dissemination of QREC in the broiler production chain. It would be interesting to identify and compare possible common sequence types, and also determine if ST355 and ST349 still are present. Also, sampling breeding and broiler flocks and investigating QREC occurrence from succeeding sampling of these would provide further data on dissemination and persistence of QREC in the production chain, and can possibly confirm if QREC is indeed disseminated from parent animals

- Further characterization of plasmids harbouring *qnr* genes. Identifying these plasmids *in silico* followed by conjugation experiments will provide necessary data when assessing their transferability. Characterizing the plasmids opens up the possibility to determine if these plasmids are similar to plasmids from similar production sites in other countries.

- Further investigate QREC in the Norwegian pig population. Since the origin and/or development of these isolates are largely unknown, further studies similar to the one described in paper III should be implemented on isolates from pigs. It would be interesting to determine if there is a higher occurrence of quinolone resistance development among commensal *E. coli* in pigs than in broilers that may explain the observed QREC occurrence.

- Quantification of QREC in livestock species. QREC was identified at low levels in a high proportion of samples, but the exact proportions of these bacteria are largely unknown. A combination of culture-based quantification and metagenomic quantification from the same samples may provide high quality data that can be used to deduce the amount of QREC in the gut of these animals.

11 Summary of scientific articles

1. Occurrence of quinolone resistant *E. coli* originating from different animal species in Norway

Håkon Kaspersen, Anne Margrete Urdahl, Roger Simm, Jannice Schau Slettemeås, Karin Lagesen, Madelaine Norström

The aim of the study was to describe and compare the occurrence of quinolone resistant *E. coli* in various animal species in relation to human population density. Data from 4568 *E. coli* isolates from the Norwegian monitoring programme for antimicrobial resistance in feed, food and animals from 2006 to 2016 was compiled and analysed. The isolates originated from broilers, layers, cattle, turkeys, dogs, wild birds, red foxes, reindeer, sheep, horses and pigs. Data on the geographical location of origin for the isolates was available for 4050 isolates, and was used to categorize the isolates depending on the human population density of the area. In total, 1.4 % of the isolates were regarded as quinolone resistant, where the highest occurrence was in broilers and wild birds. Human population density was not associated with the occurrence of quinolone resistant *E. coli*. In Norway, fluoroquinolones are not used prophylactically and in almost negligent amounts in various species. This, and the observed interspecies variation, suggests other factors than fluoroquinolone use may be important in the development of quinolone resistant *E. coli*.

2. Dissemination of quinolone resistant *Escherichia coli* in the Norwegian broiler and pig production chain, and possible persistence in the broiler production environment

Håkon Kaspersen, Camilla Sekse, Eve Zeyl Fiskebeck, Jannice Schau Slettemeås, Roger Simm, Madelaine Norström, Anne Margrete Urdahl, Karin Lagesen

In Norway, the use of quinolones in livestock populations is very low, and prophylactic use is prohibited. Despite this, quinolone resistant *E. coli* (QREC) are present at low levels in several animal species. The source of these QREC is unknown. The aim of this study was to characterize and compare QREC from different animal species to identify putative factors that may promote the occurrence of QREC. A total of 280 QREC isolates, from broilers, pigs, red foxes and wild birds, were whole genome sequenced and analysed. Well-known chromosomal and plasmid-mediated resistance mechanisms were identified. In addition, mutations in *marR*, *marA* and *rpoB* causing novel amino acid substitutions in their respective proteins were detected. Phylogenetic analyses were used to determine the relationships between the isolates. Quinolone resistance mechanism patterns appeared to follow sequence type groups. Similar QREC isolates with similar resistance

mechanism patterns were detected from the samples, and further phylogenetic analysis indicated close evolutionary relationships between specific isolates from different sources. This suggests dissemination of highly similar QREC isolates between animal species, and also persistence of QREC strains within the broiler production chain. This highlights the importance of both control measures at the top of the production chain, as well as biosecurity measures to avoid further dissemination and persistence of QREC in these environments.

3. Comparative genome analyses of wild type- and quinolone resistant *Escherichia coli* indicate dissemination of QREC in the broiler production pyramid and potential sporadic local resistance development

Håkon Kaspersen, Eve Zeyl Fiskebeck, Camilla Sekse, Jannice Schau Slettemeås, Anne Margrete Urdahl, Madelaine Norström, Karin Lagesen, Roger Simm

Quinolones are important antimicrobials for both humans and animals, and resistance towards these compounds is a serious threat to public health. In Norway, quinolone resistant *E. coli* (QREC) have been detected at low levels in a high proportion of broiler flocks, even without the use of quinolones in rearing of broilers. Due to the pyramidal structure of broiler breeding, QREC isolates may be disseminated from grandparent animals down through the pyramid. However, quinolone resistance can also develop in wild type *E. coli* through specific chromosomal mutations, and by horizontal acquisition of plasmid-mediated quinolone resistance genes. The goal of this study was to determine whether QREC is disseminated through the broiler breeding pyramid or developed locally at some stage in the broiler production chain. For this purpose, we whole genome sequenced wild type- and QREC isolates from broiler and parent flocks that had been isolated in the Norwegian monitoring program for antimicrobial resistance in feed, food and animals (NORM-VET) between 2006 and 2017, from 22 different production sites. The sequencing data was used for typing of the isolates, phylogenetic analysis and identification of relevant resistance mechanisms. Highly similar QREC isolates were identified within major sequence types from multiple production sites, suggesting dissemination of QREC isolates in the broiler production chain. The occurrence of potential resistance development among the wild type *E. coli* was low, indicating that this may be a rare phenomenon in the Norwegian broiler production. The results indicate that the majority of the observed incidence of QREC at the bottom of the broiler production pyramid originates from parent or grandparent animals. These results highlight the importance of surveillance at all levels of the broiler production pyramid and of implementation of proper biosecurity measures to control dissemination of QREC.

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Paper 1

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Occurrence of quinolone resistant *E. coli* originating from different animal species in Norway

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ABSTRACT

The aim of this study was to describe and compare the occurrence of quinolone resistant *Escherichia coli* (QREC) in various animal species in relation to human population density. Data from the Norwegian monitoring programme for antimicrobial resistance in feed, food and animals from 2006 to 2016 was compiled and analysed. In total, 4568 *E. coli* isolates were included in this study. The isolates originated from broilers, layers, cattle, turkeys, dogs, wild birds, red foxes, reindeer, sheep, horses and pigs. Data regarding the geographical location of sampling was obtained for 4050 of these isolates and used to categorize the isolates depending on the human population density of the area. In total, 1.4% of the isolates were categorized as quinolone resistant. Compared to most European countries, there was an overall low occurrence of QREC in various animal species in Norway, though with an interspecies variation with the highest occurrence in broilers and wild birds ($p < 0.05$). Human population density was not associated with the occurrence of QREC. Since fluoroquinolones are not used prophylactically and in almost negligent amounts in various species in Norway, the interspecies variation in the occurrence of QREC suggests that other factors than fluoroquinolone use may be important in the development of QREC.

1. Introduction

Quinolones and fluoroquinolones have been classified as critically important for human health by the World Health Organization (WHO, 2017). Resistance to these compounds has become widespread in Europe, and the occurrence of resistance has increased significantly from 2012 to 2015 (ECDC, 2016). Due to this rapid dissemination, the need for proper surveillance of antimicrobial resistance is paramount for both human and animal health alike (Queenan et al., 2016; Robinson et al., 2016).

The Norwegian monitoring programme for antimicrobial resistance in the veterinary sector (NORM-VET) was established in 2000 as part of a national strategy plan against antimicrobial resistance. The occurrence of quinolone resistance, as defined by epidemiological cut-off values (ECOFF; EUCAST, www.eucast.org), in indicator *E. coli* from healthy animal species has been monitored in NORM-VET since its beginning. Culturing and isolation methods have been used to identify *E. coli* from the samples collected. A random *E. coli* from each sample has been tested for the sensitivity to a range of substances, hereafter called the traditional method, of which the quinolones nalidixic acid and enrofloxacin (until 2005) or ciprofloxacin (from 2006) have been included. In 2014, an additional selective screening method for quinolone resistant *E. coli* (QREC) was introduced in the NORM-VET programme (NORM/NORM-VET, 2014, 2015, 2016). The selective method showed that QREC is present at low levels in a high proportion of the samples from some healthy animal species. The results from this screening indicated that there is a substantial difference in occurrence of QREC between animal species, with the most frequent findings being in broilers and pigs. Data from many years of sampling in different animals using the traditional method provides us with a unique opportunity to study the occurrence of QREC over time. Moreover, the data can be used to determine if the observed variation of QREC occurrence in various animal species could be detected as with the selective screening methodology. Furthermore, differences in occurrences over time can be compared to other factors such as human population density. This has previously been observed for QREC detected with the selective method in samples from red foxes (Mo et al., 2017).

The aim of this study was to describe and compare the occurrence of QREC in different animal species in relation to population density for a better understanding of the dissemination and occurrence of QREC in

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animals in Norway. Additionally, we wanted to see if the same species variation identified by selective screening for QREC was identifiable in indicator *E. coli*.

2. Materials and methods

2.1. Data sources and management

Data from the NORM-VET programme from 2006 to 2016 (NORM/NORM-VET, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016), including information about species, sampled material, date of sampling, and production site or sample site (municipality) was extracted from the internal recording system of the Norwegian Veterinary Institute.

Isolates for which municipality information could be obtained were further included in the analyses of possible relationships between human population density and occurrence of QREC. Some of the production animals had been sampled at slaughterhouses and for some samples the exact knowledge of the production site was missing. We chose to use the municipality of the slaughterhouses as the geographical localization since the production animals in Norway are mainly slaughtered in one of the slaughterhouses closely located to the production site. Isolates from two wild birds, two layers, two sheep, one pig, one turkey, and all the isolates from both horses and dogs were excluded due to either lack of information, anonymized samples (horses) or only the localization of the veterinary clinic (dogs) was known.

The categorization of population density was performed as previously described (Mo et al., 2017) into the following categories:

- Low population density (< five inhabitants per km^2)
- Medium population density (five–200 inhabitants per km^2)
- High population density (> 200 inhabitants per km^2)

Data regarding the population density in 2015 (number of inhabitants per km^2) were derived from Statistics Norway (www.ssb.no, accessed 19.09.2017).

Data management was performed in R version 3.4.2 (RCoreTeam, 2017) and in SAS SAS-PC system version 9.4 for Windows (SAS Institute inc., Cary, NC, USA).

2.2. Isolates and susceptibility testing

In total, 4568 isolates of indicator *E. coli* from various healthy animal species were included in this study (Table 1). Of these, information about municipality could be obtained for 4050 isolates. Sample material was either faecal, caecal or boot swabs. For broilers, all samples taken before 2014 were boot swabs, while after 2014 the samples were pooled caecal samples.

We did not have isolates from all species from all years due to the sample regimes in the NORM-VET surveillance programme, where only selected species are sampled each year. Due to this, only cattle, swine and broilers were analysed with regards to differences between years as they were sampled two, four and six times, respectively, over a ten year period. The isolation and identification of indicator *E. coli* in the NORM-VET programme follows standard guidelines used in general bacteriology. In short, putative *E. coli* colonies from faecal, caecal or boot swab samples are randomly selected on bromothymol-blue (in-house) or MacConkey agar (BD Biosciences, Le Pont de Claire, France), and species confirmation is done by indole, citrate and/or oxidase tests or by use of a matrix-assisted laser desorption/ionization time of flight apparatus (MALDI-TOF Microflex, Bruker Daltonik GmbH, Bremen, Germany). Minimum inhibitory concentration (MIC) values were determined by broth microdilution (VetMIC™, Dep. Of Antibiotics, National Veterinary Institute, Sweden, or SensiTitre®, TREK Diagnostics, LTD.). All isolates of *E. coli* isolated between 2006 and 2013 were tested with VetMIC™, while isolates from 2014 to 2016 were tested with SensiTitre®. MIC values of 0.016 mg/L from the VetMIC™ susceptibility testing system was grouped with the MIC value of 0.015 mg/L from the SensiTitre® system, assuming that this does not affect the results as these MIC values are below the ECOFF. Isolates were classified as resistant if the MIC-values were above the ECOFF values 0.06 mg/L for ciprofloxacin and/or 16 mg/L for nalidixic acid, as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). Detailed methodology can be found in the NORM-VET reports (NORM/NORM-VET, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016), and also in Supplementary material. Quinolone resistant isolates that were MIC-tested by VetMIC™ ($n = 31$) were re-tested with the SensiTitre® system to allow comparison with the ones previously tested on SensiTitre®. Further, we classified the occurrence of resistance and resistance levels in accordance with guidelines from the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) (EFSA and ECDC, 2016).

Table 1
Occurrence of quinolone resistant *E. coli* isolates per animal species collected within the Norwegian monitoring programme for antimicrobial resistance in feed, food and animals during 2006–2016. CI = confidence interval.

Species	Years isolated	Number of isolates	Number of QREC	QREC (%)	95 % CI
Wild Birds	2016	303	7	2.3	0.9–4.7
Cattle	2010, 2015	471	2	0.4	0.1–1.5
Dogs	2008, 2013	339	3	0.9	0.2–2.6
Broilers	2006, 2009, 2011, 2012[a], 2014, 2016	1059	38	3.6	2.6–4.9
Layers	2013	186	1	0.5	0.0–3.0
Horses	2009	171	0	0.0	0.0–2.1
Red Foxes	2010, 2016	489	7	1.4	0.6–2.9
Pigs	2007, 2008, 2011, 2015	918	3	0.3	0.1–1.0
Reindeer	2012	107	0	0.0	0.0–3.4
Sheep	2007	207	0	0.0	0.0–1.8
Turkey	2007, 2013, 2016	318	4	1.3	0.3–3.2
Total	2006–2016	4568	65	1.4	1.1–1.8

[a] Only breeders.

2.3. Statistical analysis

The total occurrence of QREC across all species, per species, and per year during the ten year period was calculated. To assess differences in QREC occurrences between species, different human population density areas, and each year, chi squared tests were used. First, each species was compared to each other. Species with statistically significant differences in QREC occurrence were pooled and tested against the rest of the species as a group. The occurrence of QREC in relation to human population density was assessed by chi squared tests. Confidence intervals were calculated by using exact binomial test. All statistical analyses was performed in R version 3.4.2 (RCoreTeam, 2017). R script can be accessed at github.com/hkaspersen/vetmic2018.

All isolates ($n = 4568$) were included in the MIC-distribution for ciprofloxacin and nalidixic acid. Thereafter, only the isolates classified as resistant to either ciprofloxacin, nalidixic acid or both were included in the MIC-distribution for the substances where monitoring data from the total study period was available: ampicillin, tetracycline, chloramphenicol, sulfamethoxazole, trimethoprim, gentamicin and cefotaxime. Pearson correlation was used to assess possible associations between MIC-values for nalidixic acid and ciprofloxacin.

Trends in broilers from 2011 to 2016 were analysed by calculating the annual percent increase in QREC occurrence.

Table 2
Minimum Inhibitory Concentration (MIC) distributions for nalidixic acid (NAL) and ciprofloxacin (CIP) for *E. coli* isolates (n = 4568) originating from all animal species included in the Norwegian monitoring programme for antimicrobial resistance in the years 2006–2016. The total number of isolates per year was 190 (2006), 458 (2007), 418 (2008), 333 (2009), 264 (2010), 400 (2011), 220 (2012), 474 (2013), 205 (2014), 532 (2015), and 1074 (2016). MIC values above the epidemiological cut-off values included before 2014 have been retested in the present study with SensiTitre®. The MIC value of 0.016 mg/L from the VetMIC™ system was grouped with the MIC value of 0.015 mg/L from the SensiTitre® system.

		Quinolone resistance		Distribution (%) of MIC values (mg/L)*														
Substance	Year	%	[95% CI]	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	> 128
NAL	2006	1.1	[0.13–3.75]								1.1	41.1	49.5	6.8	0.5	1.1			
	2007	0.4	[0.05-1.57]								2.2	64.8	32.1	0.4			0.4		
	2008	0.2	[0.01-1.33]								1.9	50.0	47.1	0.7					0.2
	2009	3.9	[2.09-6.58]								3.3	45.3	45.6	1.8		0.6	0.6	2.1	0.6
	2010	0.4	[0.01-2.09]								10.6	32.6	55.7	0.8			0.4		
	2011	1.0	[0.27-2.54]								4.2	61.5	32.8	0.2	0.2			0.2	0.8
	2012	0.5	[0.01-2.51]								6.4	56.8	35.0	1.4				0.5	
	2013	0.8	[0.23-2.15]								5.7	65.0	27.8	0.6		0.2	0.4		0.2
	2014	3.4	[1.38-6.91]										96.6			1.0	1.5	1.0	
	2015	0.8	[0.21-1.91]										98.1	1.1		0.4		0.2	0.2
	2016	2.2	[1.44-3.31]										96.8	0.9		0.1	0.1	0.4	1.7
CIP	2006	1.1	[0.13–3.75]		20.5	59.5	18.9	1.1											
	2007	0.4	[0.05-1.57]		10.3	39.5	49.8		0.4										
	2008	0.2	[0.01-1.33]	0.2	5.0	70.6	23.9								0.2				
	2009	3.9	[2.09-6.58]		6.3	65.2	24.6	0.9	1.8	1.2									
	2010	0.4	[0.01-2.09]		3.0	73.1	23.5	0.4											
	2011	1.0	[0.27-2.54]		0.5	62.3	36.2		0.2	0.8									
	2012	0.5	[0.01-2.51]		4.5	53.6	41.4		0.5										
	2013	0.8	[0.23-2.15]		3.4	52.5	43.2	0.4	0.4										
	2014	3.4	[1.38-6.91]		87.3	9.3		1.0	2.4										
	2015	0.6	[0.12-1.64]		98.3	1.1			0.4	0.2									
	2016	2.3	[1.51-3.42]		90.6	6.9	0.2		0.9	0.1	0.9			0.2	0.2				

*Bold vertical lines denote epidemiological cut-off values for resistance. CI = confidence interval. White fields denote range of dilutions tested for each antimicrobial agent. MIC values higher than the highest concentration tested are given as the lowest MIC value above the range. MIC values equal to or lower than the lowest concentration tested are given as the lowest concentration tested.

3. Results

In total, 65 isolates (1.4%, 95% CI: [1.1–1.8]) were classified as resistant to quinolones. QREC was not detected in reindeer, sheep or horses. The occurrence was highest in broilers and wild birds (Supplementary Fig. 1), and significantly higher than all other animal species in this study (chi square test, $p < 0.05$; Table 1). The total occurrence of QREC was significantly higher in 2009, 2014 and 2016 compared to the most other years (chi square test, $p < 0.05$; Table 2). This is mostly due to the high proportion of samples from broilers in those years.

The occurrence of QREC over time in broilers (Fig. 1) was significantly higher in 2009 and 2016 compared to 2006 and 2011 (chi square test, $p < 0.05$), with a mean QREC occurrence of 4.1%. The occurrence of QREC in broilers increased from 2011 to 2016, with an annual increase of 26.3%, excluding 2012 as only parent flocks were sampled that year. The occurrence of QREC in swine and cattle have been less than 1% during the whole study period (Supplementary Fig. 1).

The MIC-distributions for ciprofloxacin and nalidixic acid from all isolates (Table 2) shows a difference between the MIC-distributions as a result of the use of two different systems for susceptibility testing. There is a positive correlation between the MIC-values of ciprofloxacin and nalidixic acid (Pearson correlation coefficient = 0.475). The occurrence of resistance to ampicillin (21.5%), tetracycline (27.7%), trimethoprim (16.9%) and sulfamethoxazole (16.9%) was detected in the 65 quinolone resistant isolates (Table 3). Of these isolates, 21.5% were resistant to two different classes of antimicrobials, and 23.1% were resistant to three or more different classes. Two isolates had ciprofloxacin MIC-values above the ECOFF (MIC = 0.25 and 0.5 mg/L) and nalidixic acid MIC-values below the ECOFF (MIC = 4.0 and 8.0 mg/L; Fig. 2), and multi-drug resistance to ampicillin, tetracycline and trimethoprim was detected in one of these isolates that originated from turkeys. Additionally, two isolates had ciprofloxacin MIC-values below the ECOFF (MIC = 0.015 and 0.06 mg/L) and nalidixic acid MIC-values above the ECOFF (MIC = 32 mg/L).

A possible association between the occurrence of QREC and human population density was only detected for layers ($p = 0.011$; Table 4).

4. Discussion

This study is to our knowledge the first study comparing the occurrence of QREC originating from production animals, companion animals, and wildlife. Here, we aggregated and analysed data gained through the NORM-VET surveillance programme in Norway for a ten year period (NORM/NORM-VET, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016) to describe and compare the occurrence of QREC in different animal species, in relation to human population density and over time. Even though the sampling strategy, including sampled animal species, sample material and sampling location differed between the years, this study shows that the occurrence of QREC originating from healthy animals, including production-, wild- and

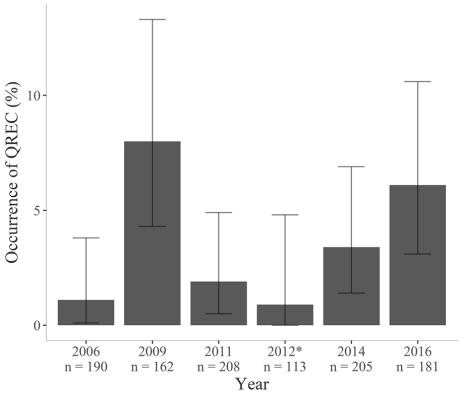

Fig. 1. Prevalence from 2006 to 2016 of quinolone resistant *E. coli* from broilers included in the Norwegian monitoring programme for antimicrobial resistance in feed, food and animals. Confidence intervals (95%) are denoted as black lines above and within the bars. *Breeders.

companion animals in Norway is low. In contrast, a similar study from Poland concerning only production animals reported a mean occurrence of 79.8% for broilers, 42.7% for layers, 61.0% for turkeys, 7.9% for pigs and 3.2% for cattle (Wasyl et al., 2013). EFSA reports a mean QREC occurrence of 64% in broilers sampled in 2014 from 28 different European countries (EFSA and ECDC, 2016), while Norway has an occurrence of 3.4%. Norway has a mean occurrence of QREC in broilers the last ten years at 6.3%, slightly lower than Sweden (SWEDRES/SVARM, 2015) and Denmark (DANMAP, 2015), with a mean occurrence of 11.0% and 9.5%, respectively. In 2015, Finland, Denmark and the Netherlands had similar occurrence of QREC in pigs as Norway, at 0.5%, 1.1%, 0.7% and 0.7%, respectively (EFSA and ECDC, 2017).

Our study indicates an interspecies variation in QREC occurrence, since there was a significantly higher occurrence of QREC in samples from broilers and wild birds than in other animals. This is consistent with the results of previous studies (Wasyl et al., 2013; Wasyl, 2014) and EFSA reports (EFSA and ECDC, 2010a, 2010b, 2016, 2017). The higher occurrence of QREC detected in broilers is also in accordance with the results obtained from the selective methodology used in NORM-VET since 2014. However, for further comparison of the interspecies variation detected by the traditional method, data from the same animal species and years need to be analysed simultaneously. In Sweden, a rapid increase in QREC occurrence from 5% to 15% from 2007 to 2010 was detected (SWEDRES/SVARM, 2015). Similarly, an increasing trend is observed in Norway as well. This increasing trend, and the higher prevalence in broilers compared to other species is currently unexplained. To our knowledge, there has been no major changes in management during this time period. There was, however, a change in sample material from boot swab to pooled caecal samples, which could have influenced the results. However, an increase was also observed from 2014 and 2016, which do not support sampling procedure as an explanation for the observed increase. It has been suggested that QREC may originate from imported breeding birds (Börjesson et al., 2015), and that vertical transmission of resistant bacterial clones to flocks downwards in the production pyramid can occur (EFSA and ECDC, 2016). This might be an explanation for the occurrence of QREC in Norwegian broilers and other poultry since Norway imports eggs for the production of parent flocks from Sweden, and Sweden import grandparent flocks from breeding companies in the UK. Even if the occurrence of QREC is initially low, dissemination and spread within a poultry flock may be rapid due to high density of animals. Additionally, broiler feed have been implicated in the dissemination of QREC in broiler farms on Iceland (Thorsteinsdottir et al., 2010) and also in Portugal (da Costa et al., 2007). In Norway, dry feed products for dogs, cattle and pigs have been screened for QREC without any QREC detection (NORM/NORM-VET, 2016). Broiler feed have not been examined and further investigations are needed to explore broiler feed as a source for QREC dissemination among broilers in Norway. However, multiple mechanisms may be responsible for the development of quinolone resistance. For example, bacterial stress factors have been shown to induce chromosomal mutations, which is typical for quinolone resistance (Qin et al., 2015). Further surveillance data of QREC occurrence in broilers is of importance to follow the situation in Norway. The higher occurrence of QREC in broilers compared to turkeys have in other European countries been suggested to depend on an overall higher exposure to fluoroquinolones through the use of this antimicrobial (EFSA and ECDC, 2016). To our knowledge, fluoroquinolones are not used in Norwegian broiler production (personal communication: Høy, T., The Norwegian Medicines Agency) and in almost negligible amounts in other animal species (NORM/NORM-VET, 2016). The difference in occurrence of QREC in the various animal species may indicate that the production environment may be an important factor. Broilers have the highest production density and also the highest QREC occurrence, while cattle have the lowest production density of the production animals and also one of the lowest QREC

Table 3
Minimum inhibitory concentration distributions for the quinolone resistant *E. coli* isolates ($n = 65$) for the antimicrobials AMP = ampicillin, TET = tetracycline, CHL = chloramphenicol, SMX = sulfamethoxazole, TMP = trimethoprim, GEN = gentamicin, and CTX = cefotaxime.

	Resistance (%)		Distribution (%) of MIC values (mg/L)*														
Substance	[95 % CI]		0.015	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	> 1024
TET	27.7	[17.3 – 40.2]						18.5	53.8				1.8	7.7	9.2		
CHL	1.5	[0.0 – 8.3]								43.1	53.8	1.5			1.5		
AMP	21.5	[12.3 – 33.5]					7.7	1.5	26.2	43.1				1.5	20		
CTX	1.5	[0.0 – 8.3]			41.5	56.9					1.5						
SMX	16.9	[8.8 – 28.3]								29.2	43.1	9.2	1.5				16.9
TMP	16.9	[8.8 – 28.3]			29.2	41.5	1.8	1.5						16.9			
GEN	1.5	[0.0 – 8.3]				26.2	36.9	32.3	3.1				1.5				
CIP	96.9	[89.3 – 99.6]	1.5	1.5	15.4	44.6	13.8	15.4			3.1	4.6					
NAL	96.9	[89.3 – 99.6]								1.5	1.5		15.4	16.9	24.6	40	

*Bold vertical lines denote epidemiological cut-off values for resistance. CI = confidence interval. White fields denote range of dilutions tested for each antimicrobial agent. MIC values higher than the highest concentration tested are given as the lowest MIC value above the range. MIC values equal to or lower than the lowest concentration tested are given as the lowest concentration tested.

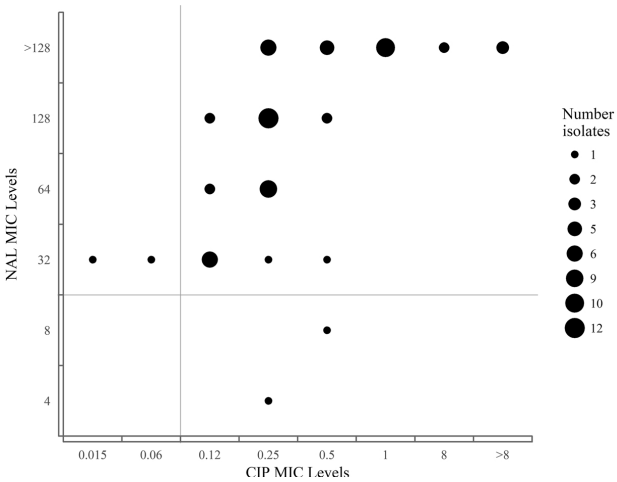

Fig. 2. The number of minimum inhibitory concentration (MIC) value combinations for ciprofloxacin (CIP) and nalidixic acid (NAL) for the quinolone resistant *E. coli* isolates in this study ($n = 65$). Vertical and horizontal lines indicate epidemiological cut off values for the respective antimicrobial compound. The MIC value of 0.016 mg/L from the VetMIC™ susceptibility testing system was grouped with the MIC value of 0.015 mg/L from the SensiTitre® system.

Table 4
Occurrence of quinolone resistant *E. coli* from different animal species sampled within three population densities. Resistance was defined by the epidemiological cut-off value for ciprofloxacin and nalidixic acid, as defined by EUCAST.

Animal species	Population density	Quinolone resistance (%)	95 % CI	*n*
Wild Birds	High	5.0	0.6–16.9	40
	Med	2.1	0.7–4.8	242
	Low	0.0	0.0–17.6	19
Cattle	High	0.0	0.0–3.0	121
	Med	0.4	0.0–2.1	268
	Low	1.2	0.0–6.6	82
Broilers	High	4.1	1.1–10.2	97
	Med	3.7	2.5–5.2	820
	Low	2.8	0.8–7.1	142
Layers	High	4.5	0.1–22.8	22
	Med	0.0	0–2.6	142
	Low	0.0	0–16.8	20
Red Foxes	High	2.9	0.4–10.2	68
	Med	1.5	0.5–3.6	323
	Low	0.0	0.0–3.7	98
Pigs	High	0.0	0.0–3.0	122
	Med	0.4	0.1–1.2	709
	Low	0.0	0.0–4.2	86
Reindeer	High	0.0	0.0	0
	Med	0.0	0.0	0
	Low	0.0	0.0–3.4	107
Sheep	High	0.0	0.0–33.6	9
	Med	0.0	0.0–3.0	123
	Low	0.0	0.0–4.9	73
Turkeys	High	0.0	0.0–12.3	28
	Med	1.6	0.5–4.2	243
	Low	0.0	0.0–7.7	46
All species	High	1.8	0.8–3.3	507
	Med	0.7	1.2–2.2	673
	Low	1.7	0.2–1.7	2870

occurrences. However, studies that link production density to the dissemination of antimicrobial resistance have to our knowledge not been performed but theoretical models show that the ability for an infectious agent to spread depends on the number of possible contacts (de Jong et al., 1995).

No selection pressure for quinolone resistance is expected in wild animals, as quinolones are not naturally found in nature. However, QREC may be disseminated to the environment from human and animal sources through wastewater, manure etc. In the present study, isolates from wild birds and dogs were found to have the highest MIC-values for ciprofloxacin (> 8 µg/ml, data not shown). Such a high MIC-value suggests multiple resistance mechanisms (Vila et al., 1994; Machuca et al., 2014). It has previously been suggested that wild birds may be reservoirs for quinolone resistance (Oh et al., 2016), and due to their freedom of movement may disseminate this resistance to other countries. Similar to wild birds, dogs may be exposed to sources of QREC in the environment in addition to being in close contact with humans. However, wild birds and dogs were only sampled in 2016, and 2008 and 2013, respectively, and care should therefore be taken when interpreting these results.

We hypothesized that there might be an influence of human activity on the occurrence of QREC. Earlier reports have suggested a link between human population density and the occurrence of antimicrobial resistance (Bruinsma, 2003; Mo et al., 2017). We therefore compared the occurrence of QREC in relation to human population density categorized in low, medium and high density areas. No association between the occurrence of QREC and human population density was detected, except for layers, where no QREC was identified in low and medium density categories and only one QREC isolate in the high density category. Even if this is a significant result, care should be taken when interpreting these results due to the small sample size. Human population density data from 2015 was used to identify the population categories for each municipality. Only the data from 2015 was used, as the demographic structure in Norway have been relatively stable the last ten years. The population categories for each municipality would therefore most likely stay the same if categorised for each year. In a recent report (Mo et al., 2017), where a selective screening of QREC from red foxes was performed, there was a higher occurrence in the high density category when compared to less populated areas. Production animals in Norway is mostly kept in close premises with little to no contact with human waste, sewage etc. that might contain resistant bacteria, therefore the influence from these sources is probably less for production animals than for other animals.

Evaluating the MIC-values for ciprofloxacin and nalidixic acid may give an indication regarding the underlying resistance mechanism in a given isolate where a decreased susceptibility to ciprofloxacin but increased susceptibility to nalidixic acid is indicative of plasmid mediated quinolone resistance (PMQR) and the presence of *qnr*-genes (Hooper and Jacoby, 2015). In this study, two *E. coli* isolates had increased susceptibility for nalidixic acid and decreased susceptibility for ciprofloxacin, indicating the presence of PMQR. These isolates were obtained from one wild bird and a turkey. Furthermore, the multi-drug resistance identified in the turkey isolate further supports this hypothesis. However, the presence of PMQR and the specific resistance genes needs to be confirmed by whole-genome sequencing.

5. Conclusion

The results indicate a low occurrence of QREC in various animal species in Norway. Fluoroquinolones are not used prophylactically in Norway, and almost negligent amounts have been used for treatment of animals. The source of this observed resistance is therefore unknown. However, there is a significant difference in the occurrence of QREC between the animal species, which might indicate that other factors than fluoroquinolone use may play a role in the occurrence of QREC. These factors are currently unknown, and further research is needed to investigate possible explanations.

Conflict of interest

None to declare.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.02.022.

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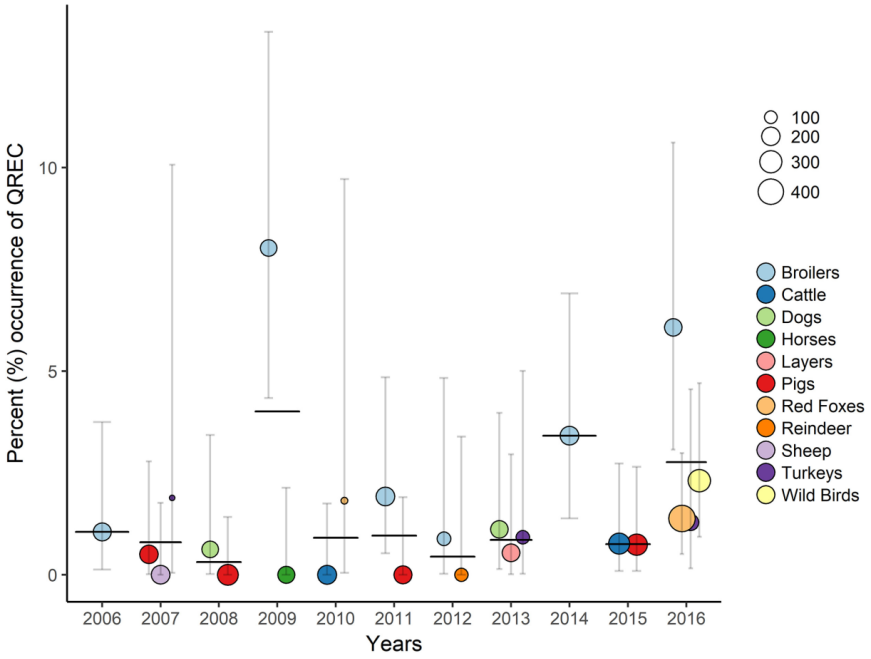
Supplementary Table 1: Changes in methods used in the Norwegian monitoring programme for antibiotic resistance for the isolation, identification and antimicrobial susceptibility testing (AST) of *E. coli* during the last ten years. BTB = Bromothymol blue. MALDI-TOF = Matrix-assisted laser desorption ionization time-of-flight.

	E. coli identification		
Year	Agar	Method	AST
2006	BTB[1]	Lactose-saccharose fermentation and indole	VetMIC™,[4]
2009	BTB	Lactose-saccharose fermentation, indole and oxidase	VetMIC™
2012	BTB	Lactose-saccharose fermentation, indole, oxidase, and citrate	VetMIC™
2014	MacConkey[2]	Lactose-saccharose fermentation, indole, oxidase, and citrate	SensiTitre®[5]
2015	MacConkey	MALDI-TOF[3]	SensiTitre®

[1]In-house, [2]BD Biosciences, Le Pont de Claire, France, [3]Bruker Daltonik, Bremen, Germany, [4]Dep. Of Antibiotics, National Veterinary Institute, [5]TREK Diagnostics, LTD



Supplementary Figure 1: 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 is represented as the amount of isolates for each respective animal species each year. Confidence intervals (95 %) are represented as black vertical lines.

Paper 2

Dissemination of quinolone resistant *Escherichia coli* in the Norwegian broiler and pig production chain, and possible persistence in the broiler production environment

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Keywords: QREC, AMR, quinolone, *E. coli*, livestock, wildlife

Abstract

In Norway, the use of quinolones in livestock populations is very low, and prophylactic use is prohibited. Despite this, quinolone resistant *E. coli* (QREC) are present at low levels in several animal species. The source of these QREC is unknown. The aim of this study was to characterize and compare QREC from different animal species to identify putative factors that may promote the occurrence of QREC. A total of 280 QREC isolates, from broilers, pigs, red foxes and wild birds, were whole genome sequenced and analysed. Well-known chromosomal and plasmid-mediated resistance mechanisms were identified. In addition, mutations in *marR, marA* and *rpoB* causing novel amino acid substitutions in their respective proteins were detected. Phylogenetic analyses were used to determine the relationships between the isolates. Quinolone resistance mechanism patterns appeared to follow sequence type groups. Similar QREC isolates with similar resistance mechanism patterns were detected from the samples, and further phylogenetic analysis indicated close evolutionary relationships between specific isolates from different sources. This suggests dissemination of highly similar QREC isolates between animal species, and also persistence of QREC strains within the broiler production chain. This highlights the importance of both control measures at the top of the production chain, as well as biosecurity measures to avoid further dissemination and persistence of QREC in these environments.

Significance of study

Since antimicrobial usage is low in Norwegian animal husbandry, Norway is an ideal country to study antimicrobial resistance in the absence of selective pressure from antimicrobial usage. In particular, the usage of quinolones is very low, which makes it possible to investigate the spread and development of quinolone resistance in natural environments. Comparing quinolone resistant *E. coli* (QREC) from livestock and wild animals in light of this low quinolone usage provides new insights into the development and dissemination of QREC in both natural- and production environments. With this information, preventive measures may be taken to prevent further dissemination within Norwegian livestock and between other animals, thus maintaining the favourable situation in Norway.

Introduction

Quinolones are broad-spectrum antimicrobial compounds that have been used to treat infections in both humans and animals all over the world, and are included in the highest priority group on the WHO's list of critically important drugs for human medicine. Unfortunately, extensive use of quinolones has resulted in emergence of quinolone resistant bacteria. As part of a combined effort to manage the increasing problem of antimicrobial resistance, national and international surveillance programmes have been established to monitor the occurrence and spread of resistant bacteria, including quinolone resistant *Escherichia coli* (QREC) in livestock animals (1, 2). The overall occurrence of quinolone resistance among commensal *E. coli* from broilers and fattening pigs in Europe in 2016 and 2017 was 64.0% and 10.6%, respectively, although the occurrence varies considerably between countries (1, 3). These values were based on the epidemiological cut off (ECOFF) values for ciprofloxacin defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). Similar resistance levels were reported for nalidixic acid. To our knowledge, no systematic surveillance has been done on wild animals at a European level.

The Norwegian monitoring programme for antimicrobial resistance in feed, food and animals (NORM-VET) has since 2000 monitored antimicrobial resistance in commensal *E. coli* from a range of animal species (4). In NORM-VET, antimicrobial susceptibility to a panel of substances, including quinolones, is determined by susceptibility testing randomly selected isolates using broth microdilution (4). In addition, a directly selective method for detecting QREC in samples from animals was introduced in 2014 (5). In Norway, the use of fluoroquinolones in livestock populations is very low (6), and prophylactic use is prohibited. This is reflected in a low occurrence of quinolone resistance among commensal *E. coli* as documented through NORM-VET reports. For example, the

overall occurrence of quinolone resistance among commensal *E. coli* from broilers, pigs, red foxes and wild birds during 2006-2017 was 1.8%, ranging from 0.3% in pigs, 1.24% in red foxes, 2.3% in wild birds, to 2.9% in broiler flocks (data retrieved from the NORM-VET database). QREC has nevertheless been detected with the selective method in a high proportion of samples from these animal species (5, 7, 8). The overall occurrence of QREC detected by selective screening performed in the years 2014 to 2017 among the previously mentioned animal species was 37.1%; ranging from 14.8% in red foxes, 20.4% in wild birds, 54.4% in pigs, to 79.2% in broilers (boot swab samples from broiler production breeder flocks were included in 2017). Although the number of positive samples from broilers seem higher than from pigs, it has to be taken into account that broiler samples are pooled samples of ten animals per flock, while pig samples are from individual animals representing the pig herd.

The broiler production system in Norway has a pyramidal structure with high levels of biosecurity. Grandparent eggs are imported from Scotland to Sweden before hatching. Eggs from these grandparent animals are then imported to Norway to become parent animals, whose day-old chickens are distributed to broiler farms across the country. In contrast, pig production in Norway is a purely domestic system with negligible import of live animals. Although the pig production also has a pyramidal structure, it has considerably more movement of animals between farms.

Quinolone resistance mechanisms in *E. coli* have been thoroughly characterized, and is for the most part mediated by chromosomal mutations in the quinolone resistance determining region (QRDR) of *gyrA, gyrB, parC* and/or *parE* (9). Mutations in several other chromosomally encoded regulatory genes (e.g. *marA, soxRS* and *robA*) or mutations in *rpoB* (RNA polymerase B) have also been implicated (10–13). Additionally, plasmid-mediated quinolone resistance (PMQR), such as the *qnr*-family of genes, *qepA, oqxAB,* and *aac(6')-Ib-cr,* have been described (14–17).

The aim of the present study was to compare QREC isolates originating from four different animal species (broilers, pigs, red foxes and wild birds), susceptibility tested within the framework of NORM-VET from 2006 to 2017. For these purposes, whole genome sequencing of the isolates and subsequent analyses were performed. The relationships between isolates were analysed by phylogenetic approaches with the intent to elucidate possible dissemination within and between animal species. In addition, genetic characterization of quinolone resistance and plasmid-mediated resistance toward other antimicrobials was performed.

Materials and Methods

Isolate selection

Isolates included in this study were collected in the NORM-VET programme from 2006 to 2017 (5–8, 18–24). Isolate metadata can be downloaded as described in Supplementary Section 3.1. In NORM-VET the procedure for isolation were either traditionally by plating faecal, caecal or boot swab samples on MacConkey agar (BD Biosciences, Le Pont de Claire, France), or selectively by plating on MacConkey agar with 0.06 mg/l ciprofloxacin (0.12 mg/l in 2014). For both methods, a random *E. coli* colony was selected from the plate and confirmed as *E. coli* either by citrate, indole and/or oxidase tests or by matrix-assisted laser desorption ionization time of flight (MALDI-TOF, Microflex, Bruker Daltonik GmbH). The selected isolate was then susceptibility tested by a broth microdilution assay (EUVSEC, SensiTitre®, TREK Diagnostics, LTD.), which include the quinolones ciprofloxacin and nalidixic acid. Isolates were classified as resistant if they grew on or above the ECOFF values for ciprofloxacin (R > 0.06 mg/l) and/or nalidixic acid (R > 16 mg/l) as defined by the EUCAST (ECOFF values as of 01.08.2019). In addition, all isolates were susceptibility tested for the following substances: tetracycline, ampicillin, sulfamethoxazole, trimethoprim, chloramphenicol, cefotaxime, ceftazidime, gentamicin, azithromycin, meropenem, colistin, and tigecycline. Azithromycin was excluded from further data analyses, as no ECOFF has not yet been defined for this compound. In the present study QREC isolates from two livestock species and two wild animal species, specifically broilers, pigs, wild birds, and red foxes were included. Broiler and pig isolates were chosen due to their relatively high number of samples positive for QREC by the selective screening compared to other Norwegian livestock species (25), as well as the number of available isolates. Isolates were grouped according to minimum inhibitory concentration (MIC) values for ciprofloxacin and nalidixic acid, and to the total number of antimicrobial substances they were resistant to based on the EUVSEC panel, resulting in 86 groups (Table S1). A random selection within each group was done, representing each animal species where available. This grouping ensured phenotypic diversity among the isolates. Year of isolation and geographical location data for each isolate was collected where available. The resulting data set was composed of 285 isolates, where 88 isolates were from broilers, 75 from pigs, 70 from wild birds, and 52 from red foxes. The overall occurrence of antimicrobial resistance among the isolates and per animal species included in this study is available in Table S2.

DNA extraction

Isolates stored at -80 °C were plated onto MacConkey agar with 0.06 mg/L ciprofloxacin to confirm resistance. DNA was extracted from colonies on the plate with the QIAmp DNA mini kit (Qiagen), according to the manufacturer's instructions. DNA concentration was determined by the broad-range DNA Qubit assay (Qiagen), and DNA quality was assessed by the NanoDrop™ One spectrophotometer (Thermo Scientific). A Fragment Analyser™ Automated CE System instrument (FSV2-DE2-100, Advanced Analytical) and gel electrophoresis were used to determine DNA integrity.

Library preparation and sequencing

Quality controlled DNA (n = 212) was used for Nextera Flex (Illumina) library preparation and sequenced on two lanes in HiSeq 3000 (Illumina), spiked with PhiX for sequencing quality control, resulting in paired-end reads of 150 bp. The sequencing service was provided by the Norwegian Sequencing Centre (sequencing.uio.no). The remaining isolates were previously sequenced at the same facility with Nextera XT library preparation on HiSeq 2000 (n = 29) or HiSeq 2500 rapid run (n = 44), resulting in paired-end read lengths of 125 and 250 bp, respectively. For this last group, each sample was sequenced on two lanes, resulting in four fastq files per sample. Raw reads have been uploaded to ENA with the bioproject numbers PRJEB33043, PRJEB33046, and PRJEB33048.

Quality control and contaminant screening

Sequences were quality controlled using fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) version 0.11.7. Potential contaminants were screened for using Mash (26) version 1.1. A minimum identity value was set at 0.95. Bacterial species other than *E. coli* above this threshold were deemed a significant contaminant. This excluded four isolates from all further analyses due to contamination with *Citrobacter* or *Enterobacter* reads. See Supplementary Sections 3.2 and 3.3 for results.

Antimicrobial resistance gene identification and multi locus sequence typing

In total, 19 different plasmid-mediated and chromosomal genes associated with quinolone resistance were investigated (chromosomal genes: *gyrA, gyrB, parC, parE, marR, marA, soxR, robA* and *rpoB*. Plasmid-mediated genes: *qnrA, qnrB, qnrC, qnrD, qnrS, qnrE, qnrVC, oqxAB, qepA,* and *aac(6')-Ib-cr*). The genes were selected based on their description in the literature as well as their presence in the antimicrobial resistance gene databases described below. Possible co-selection of antimicrobial resistance was investigated by including all additional plasmid-mediated genes related to other antimicrobial resistance types in the database used.

The genes *gyrA, gyrB, parC and parE* were screened for mutations in the QRDR (27). Specifically, the QRDR of GyrA is located between amino acid 67 and 106 (28). Based on alignments of QRDR from another study (27) to *E. coli* K12 versions of the genes, this region was in the other proteins defined to be between amino acid 333 and 481 for GyrB, between amino acid 51 and 170 for ParC, and between amino acid 366 and 523 for ParE. See Supplementary Section 3.4 for reference sequences. The remaining chromosomal genes were investigated for mutations in the whole gene. Only mutations that lead to amino acid substitutions, hereafter called substitutions, were of interest. Only presence/absence was considered for plasmid-mediated genes. Phenotypic resistance patterns were compared to the genotype identified for each animal species.

Antimicrobial resistance gene detection and sequence type (ST) determination was done by analysing raw reads with *Antimicrobial resistance identification by assembly* (ARIBA) (29) version 2.12.1. Presence of plasmid-mediated genes was determined by comparing to the Resfinder (30) database (downloaded 4th of September 2018), while mutations in chromosomal genes were determined by comparing to the MegaRes (31) database (downloaded 4th of September 2018), see Supplementary Section 3.5 for reference sequences. An R script was used to extract the previously mentioned genes from the ARIBA results (https://tinyurl.com/y3f35mj2). Flags reported by ARIBA were used to quality check the reported variant or gene (Supplementary Section 3.6). Each novel substitution reported by ARIBA was verified by comparing to their subsequent assemblies.

STs were determined using the MLST scheme hosted by EnteroBase (32). Isolates with STs that were not possible to identify were uploaded to EnteroBase for manual identification (https://enterobase.warwick.ac.uk/).

Assembly, annotation and core gene analysis

Residual PhiX was removed with BBduk version 38.20 (https://jgi.doe.gov/data-and-tools/bbtools/) by mapping kmers to the PhiX genome (accession number NC_001422.1), using a k-mer size of 31. Trimmomatic version 0.38 (33) was subsequently used to trim adapter sequences and low-quality nucleotides using a minimum length setting of 36 bp and a sliding window of 4:15, with the Trimmomatic NexteraPE-PE adapter file. SPAdes (34) version 3.12.0 was used to assemble genomes with the settings “careful” and “coverage cutoff auto”. Both the paired and singleton reads from Trimmomatic were used. Assembly error correction was performed with Pilon (35) version 1.22 by mapping the trimmed reads back to the assembly with BWA mem version 0.7.17 (http://bio-bwa.sourceforge.net/). Prokka (36) version 1.13 was utilized for gene annotation, with the genus setting at “*Escherichia*”, species setting at “*coli*”, and kingdom setting as “Bacteria”. Five complete *E. coli* reference genomes were downloaded from the National Center for Biotechnology Information

(NCBI) and used as annotation reference (Table S3). Pan-genome analysis was performed with Roary (37) version 3.12.0 using the MAFFT aligner. QUAST (38) version 4.6.3 was used to evaluate the assemblies, see Supplementary Section 3.7 for results. One isolate was excluded due to low assembly quality, in addition to the four previously mentioned which were removed due to contamination. The final data set was thus composed of 280 isolates, 87 of which were from broilers, 75 from pigs, 52 from red foxes, and 66 from wild birds.

Phylogenetic analysis

Snp-sites (39) version 2.4.1 was used to concatenate single nucleotide polymorphism (SNP) sites in the core gene alignment from Roary. The resulting SNP sites alignment was used to reconstruct a maximum likelihood (ML) tree with IQTree (40) version 1.6.8. Branch supports were obtained using the Ultrafast Bootstrap approximation (UFBoot) (41) with 1000 bootstrap replicates. ModelFinder (42) and ascertainment bias correction (ASC) (43) was used to determine the best fitted evolutionary model. ASC was used to avoid branch length overestimation due to the absence of invariant sites in our dataset. Annotation and tree visualization was done with ggtree (44). Snp-dists (https://github.com/tseemann/snp-dists) version 0.6.3 was used to identify the number of SNP differences between all isolates.

The phylogenetic tree was inspected to identify major clades with isolates showing low genetic divergence. To quantify the amount of genetic change, patristic distances were calculated from the total tree in R with the «distTips» function from the adephylo package (45). The patristic distance cutoff was set to 0.003 because it resulted in clades that predominantly contained isolates from a single ST (Figure S1). Clades deemed of interest were selected based on the presence of isolates from different animal species, or same animal species but from different geographic locations, resulting in six clades.

New phylogenetic trees were created for each of the six clades by first aligning the pilon-corrected assemblies using ParSNP (46) version 1.2 to identify the core genome SNPs for the isolates in each clade. Harvesttools (46) version 1.2 was used for format conversion, followed by Gubbins (47) version 2.3.2 to screen for and remove possible recombinant sequence from the core SNP multifasta alignment using the GTRGAMMA model with RaxML as the treebuilder. IQTree was subsequently used to generate a ML tree from the filtered polymorphic sites alignment using UFBoot and ModelFinder with ASC. SNP distances were calculated from the filtered polymorphic sites alignment from Gubbins with snp-dists. Additionally, the fraction of shared genome for isolate pairs differing with <20 SNPs was calculated with ParSNP. Isolates sharing >90% were regarded as clones and were further investigated to uncover possible dissemination.

Statistical analyses

Statistical analyses, figures and tables was generated with R version 3.6.1 (48).

Significance of differences between the observed and expected occurrence of resistance mechanisms between the four animal species were determined by χ^2 tests. Correlations between the presences of specific genes were calculated using a Pearson correlation test, with a significance level of 0.05.

Basic summary statistics were calculated on the SNP distances for isolates within each animal species, and for isolates within the selected clades. To determine whether isolates from one animal species clustered more closely than isolates within other animal species, the median of the minimum pairwise SNP distance for isolates belonging to the same animal species was calculated. To evaluate if isolates belonging to each host species were more aggregated in the tree, i.e. had shorter distance to another isolate from the same species than randomly expected, we performed a randomization test with 1000 permutations. The median minimum pairwise SNP distance for isolates belonging to the same animal species was calculated for each iteration. *P*-values were calculated on the basis of how many expected values from x iterations were below the observed values.

Non-metric multidimensional scaling (NMDS) was used to identify the distribution of quinolone resistance mechanisms within each major ST cluster based on presence (1) and absence (0) of quinolone resistance conferring substitutions and genes. Only isolates from the dominant STs were included (n > 9). Distances were calculated from the presence/absence data with the "dist" function using the method "binary". The NMDS analysis was performed with the "metaMDS" function from the "vegan" package (49), with 200 random starts. A stressplot was calculated to determine how well the ordination represented the data (Figure S2).

Results

Quinolone resistance gene identification

Chromosomal genes

Mutations resulting in amino acid substitutions were detected in seven of the nine chromosomal genes investigated. In total, 229 of the 280 isolates had substitutions in the QRDR of GyrA*,* 43 isolates in ParC*,* and 29 isolates in ParE (Table 1). No mutations giving rise to substitutions in the QRDR of GyrB were detected. Six different substitutions were identified in GyrA and ParC, while seven were identified in ParE (Table S4). Isolates from broilers had the highest occurrence of substitutions in GyrA and ParE, while isolates from wild birds had the highest occurrence of

substitutions in ParC (Table 1). The most frequent substitutions in the respective proteins were S83L in GyrA, S80I in ParC, and D475E in ParE (Table S4). The S83L substitution in GyrA and the D475E substitution in ParE were most often identified in isolates from broilers (Table S5), while the S80I substitution in ParC was most often identified in isolates from wild birds. A total of 231 isolates had substitutions in the QRDR of at least one of GyrA, ParC or ParE. The most abundant combination of substitutions in the QRDR of GyrA, ParC and ParE was S83L in GyrA alone, found in 141 isolates. The substitutions S83L and D87N in GyrA combined with the S80I substitution in ParC occurred in a total of 33 isolates, of which 16 only had the S80I substitution, eight had the S80I combined with A56T, and one had S80I combined with E84V. The remaining eight isolates had the S80I substitution in ParC combined with substitutions in ParE. Regarding all three genes combined, eight isolates had substitutions in GyrA, ParC and ParE. Considering the other chromosomal genes, 212 isolates had substitutions in MarR, 71 in SoxR, 48 in RpoB, and 34 in MarA. No substitutions were identified in RobA (Table 1). The most common substitutions in each gene were S127N in MarA, G103S combined with Y137H in MarR, E320D in RpoB, and T38S combined with G74R in SoxR (Table S6). Substitutions in RpoB occurred significantly more often in isolates from broilers compared to pigs χ^2 (1,N = 163) = 10.95, $p = 0.001$; and wild birds; χ^2 (1,N = 153) = 5.73, $p = 0.017$. Substitutions in MarA always accompanied substitutions in MarR.

PMQR genes

Plasmid-mediated quinolone resistance was identified in 59 of the 280 isolates, and only one PMQR gene type was found for each isolate. See Table 1 for presence of PMQR positive isolates in different animal species and the specific PMQR genes present. The occurrence of PMQR was significantly lower in isolates from broilers compared to isolates from pigs χ^2 (1,N = 163) = 15.78, $p < 0.05$, red foxes χ^2 (1,N = 140) = 9.42, $p = 0.002$, and wild birds χ^2 (1,N = 153) = 26.21, $p < 0.05$. The most common identified PMQR genes were *qnrS1* and *qnrB19*, identified in isolates from all animal species (Table 1). Isolates from pigs had a significantly higher occurrence of *qnrB19* than isolates from broilers; χ^2 (1,N = 163) = 10.87, $p = 0.001$ and red foxes; χ^2 (1,N = 127) = 3.91, $p = 0.048$. The occurrence of *qnrS1* was significantly higher in wild birds compared to isolates from broilers; χ^2 (1,N = 153) = 12.44, $p < 0.05$ and pigs; χ^2 (1,N = 140) = 5.21, $p = 0.022$. A strong negative correlation between the presence of *qnr* and substitutions in GyrA was observed (-0.92, $p < 0.05$); 49 of the 58 isolates carrying *qnr* did not have substitutions in the QRDR of either GyrA, ParC or ParE (Table S7).

Co-resistance

In total, the presence of 42 different genes encoding resistance towards gentamicin, cefotaxime, chloramphenicol, tetracycline, trimethoprim and sulfamethoxazole was identified (Table S8), in addition to the PMQR genes described above. Six genes did not have a corresponding antimicrobial compound in the panel of substances for which all the isolates had previously been tested, and were therefore not considered when comparing genotype to resistance phenotype. Except for a few cases, the genotype corresponded to the phenotype (Figure 1).

In the 59 PMQR positive isolates, *qnr* was observed as the only plasmid-mediated gene in 14 of the isolates (Table S9). Of these 14 isolates, 12 harboured *qnrB19* and two harboured *qnrS2.* Among the 29 *qnrS1* positive isolates, 22 harboured *tetA* and 21 harboured $bla_{\text{TEM-1B}}$, while among the 21 *qnrB19* positive isolates, only four isolates carried *tetA,* and six carried both *aph3-Ib* and *aph6-Id*.
A significant positive correlation between the presence of *qnrS1* and *tetA* (0.36, n = 22), *dfrA14* (0.31, n = 8), $bla_{\text{CTX-M-55}}$ (0.31, n = 3), $bla_{\text{TEM-1B}}$ (0.26, n = 21), *floR* (0.22, n = 3), and *aac(3')-IId* (0.12, n = 3) was observed ($p < 0.05$). For *qnrB19*, a significant positive correlation was identified with $bla_{\text{TEM-1A}}$ (0.14, $p < 0.05$), but the two genes were only observed together in one isolate. For the 221 PMQR negative isolates, 72 isolates had no identified plasmid-mediated resistance genes. Except for ParC, a negative correlation was observed between the presence of plasmid-mediated resistance genes and mutations in chromosomal genes (Figure S3).

Isolate diversity

In total, 83 unique STs were identified, with each animal species containing between 26 and 33 different STs. The most abundant STs were ST10 (*n* = 38), ST162 (*n* = 24), ST58 (*n* = 20), ST355 (*n* = 15), ST117 and ST155 (*n* = 13). ST10 and ST155 isolates were identified in all animal species. ST162 isolates were identified in all but pigs, and ST58 isolates were identified in all but broilers. ST355 isolates were identified in broilers and red foxes, while ST117 isolates were identified in broilers and pigs (Figure 2). A total of 59 STs were only present in one animal species.

Based on the core gene SNP alignment, isolates from broilers had the lowest median minimum pairwise distance compared to the other animal species, indicating smaller differences between isolates from broilers than the other species (Table S10). The randomization test revealed that isolates from broilers aggregated more closely than isolates within other animal species ($p < 0.01$, Figure S4).

Six clades were selected for deeper phylogenetic analysis, as they contained isolates with low genetic divergence and were either from different animal species or the same animal species but

different geographic locations: Clade A (ST162 subclade A), Clade B (ST162 subclade B), Clade C (ST744), Clade D (ST10), Clade E (ST355) and Clade F (ST117) (clade selection shown in Figure S1). The trees for clades A, C, D and E had low bootstrap supports, and were not considered further since the topology within each clade was judged to be uncertain (Figure S5 – S8, respectively). Clade B (Figure 3) consisted of isolates from broilers, red foxes and wild birds, sampled in 2014 and 2016. This clade contained two pairs of isolates that were especially similar. The first pair consisted of one isolate from a broiler and one from a red fox, these had a SNP difference of 13. The host species originated from geographically distant locations, and were also sampled in different years. The two isolates shared >90% of their genome (Table 2). The second pair of isolates were from broilers in different locations in 2014. They had a SNP distance of 14 and shared >90% of their genome. Clade F (Figure 4) consisted of isolates from broilers and pigs, sampled in the years 2006, 2007, 2012, 2014 and 2015. All annotated isolate pairs in Figure 4 were from pigs sampled in 2015, and had a SNP distance of eight, three, and 11 to the other isolate in the same same pair. Two of these pairs shared >90% of their genome. These two isolate pairs were from the same county but not the same municipality, while in the third pair the isolates were from different counties. All pairs of isolates investigated had identical phenotypic and genotypic resistance patterns.

NMDS clustering of isolates based on presence/absence of quinolone resistance mechanisms in isolates from major ST groups showed that ST355, ST155, ST117, and ST162 were relatively homogenous in their distribution of quinolone resistance mechanisms, while ST10 and ST58 were not (Figure 5).

Discussion

The present study is to our knowledge the first study using whole genome sequencing to characterize and compare such a large number of QREC isolates from different animal species obtained through a monitoring programme on antimicrobial resistance in animals. Although there was a high diversity of STs among the isolates and animal species, we show that phylogenetically similar QREC isolates were shared both between animal species and between locations. Moreover, the genetic quinolone resistance determinants found in this study predominantly clustered within STs. Taking this clustering pattern into consideration, the phylogenetic structure indicate dissemination in the broiler and in the pig production chains, and potential persistence in the broiler production chain.

We detected some novel substitutions, one in MarR and two in MarA and RpoB, which to our knowledge have not been previously described. As it is outside the helix-turn-helix DNA binding motifs, the observed D118N substitution in MarR probably does not affect DNA binding directly (50). However, follow-up studies are needed to examine if these novel substitutions affect quinolone susceptibility. In addition, the observed co-occurrence of substitutions in MarA with substitutions in MarR and the significantly higher occurrence of substitutions in RpoB in broilers should be further investigated.

PMQR determinants were identified in 21.1% of the 280 selected isolates, with the highest occurrence of PMQR genes among the wild bird isolates (36.7%), and with *qnrS1* being the most common determinant. The high occurrence of *qnrS* in wild birds is in concordance with previously published data (51, 52). A positive correlation was observed between *qnrS1* and genes related to tetracycline, gentamicin, trimethoprim, chloramphenicol, ampicillin and cefotaxime resistance. Resistance to these antimicrobials has previously been associated with qnrS1 (53). *qnrS1* have previously been identified on large conjugative plasmids harbouring $bla_{\text{TEM-1B}}$ and *tetA* (54, 55), which supports the significant positive correlations between *qnrS1,* $bla_{\text{TEM-1B}}$ and *tetA*. On the other hand, *qnrB19* have been encoded on small, non-conjugative plasmids without any other resistance genes (56). In our data, only $bla_{\text{TEM-1A}}$ had a significant positive correlation with *qnrB19*, but were only observed together in a single isolate. Furthermore, most *qnrB19* positive isolates harboured no other plasmid-mediated genes. These findings may suggest that we have two main types of plasmids in our isolates, one conjugative plasmid with *qnrS1* and other resistance genes, and another non-conjugative plasmid with mostly only *qnrB19.* The presence of these plasmid types mainly appeared to cluster within sequence types. However, further studies characterizing the plasmids from these isolates are needed to confirm these findings, but are not performed here, as this was outside the scope of this study. The occurrence of PMQR in wild birds was noticeably higher than what has been reported in other studies (53, 57, 58). However, comparing to other studies is difficult due to differences in sampling and study design. For instance, the wild bird isolates selected in this study were not representative for the wild bird population in Norway as the sampling was perfomed in four regions only. These isolates can therefore not be regarded as epidemiologically unrelated. PMQR was only detected in four isolates from broilers. This low occurrence may be due to the high biosecurity in the broiler production, with little to no contact with the outside environment. The predominance of chromosomally encoded resistance indicates that PMQR play a minor role in the occurrence of QREC in the broiler production chain. In contrast, PMQR determinants were detected in 20 isolates from pigs, the most common one being *qnrB19*, indicating a higher occurrence of

PMQR among QREC in the Norwegian pig production environment. Further studies are needed to elucidate the origins of these plasmids.

An overall correspondence between genotype and phenotype was observed in our data, except for two isolates with decreased susceptibility toward cefotaxime. Further investigation using PointFinder (59) identified a mutation in the *ampC* promotor region in one of these isolates (data not shown), but the decreased susceptibility remains unexplained in the other isolate. Isolates harbouring *qnr* in addition to substitutions in GyrA were identified in four broiler isolates. Three of these had the same sequence type and contained *qnrS1*, indicating that the containing plasmids are being clonally disseminated. In contrast, only one *qnr* positive isolate each from pigs, red foxes and wild birds had substitutions in GyrA. Six out of seven of these isolates showed elevated MIC values above the clinical breakpoint for ciprofloxacin (1 – 16 mg/L) and nalidixic acid (64 – 256 mg/L), corresponding to an additive effect of multiple quinolone resistance mechanisms. High MIC values from such an additive effect is a common finding in regards to quinolone resistance in *E. coli* (60, 61). Such elevated MIC values were not observed for the rest of the *qnr* positive isolates, highlighting the need for chromosomal mutations to gain a high MIC value.

A strong negative correlation between the presence of *qnr* genes and substitutions in GyrA was observed, indicating that the two mechanisms rarely coincide. This may be explained by the hypothesized protective effect of *qnr* genes on the quinolone targets, which allows for other resistance mechanisms to be developed instead of mutations in the QRDR of these genes (62). The majority of isolates that carried *qnr* genes without substitutions in GyrA, ParC or ParE had substitutions in MarR, which may be a consequence of this protective effect. Negative correlations were also observed for most of the investigated chromosomal genes and the plasmid-mediated resistance genes, indicating that co-selection of these are not common in QREC from animal sources in Norway. However, further studies regarding plasmid characterization and co-resistance are needed to confirm these findings.

We identified a high diversity of STs, which has also been reported by others (53, 63, 64). Among these were STs previously associated with quinolone resistance, such as ST10, ST162, ST355 and ST349 (53, 65). Moreover, the results show that the distribution of resistance mechanisms was relatively homogenous within most STs, supporting a clonal distribution of these mechanisms. Isolates from broilers were overall more similar to eachother than the isolates from the other animal species, as shown in the core gene SNP tree and supported by the permutation test. This may be due to the centralized distribution of broilers, permitting dissemination of QREC isolates to the entire production chain. Although there is a centralized distribution of animals in the pig production as

well, such an overall similarity was not observed among the QREC isolates from pigs. However, we did identify two phylogenetically related pig isolates from geographically distant locations indicating that dissemination of QREC isolates in the pig production chain may occur. Persistence of antimicrobial resistant bacteria in broiler production environments, despite short production cycles, cleaning and disinfection between each flock is known from other studies (66, 67). Vertical dissemination of QREC and cephalosporin resistant *E. coli* to all levels of the broiler production pyramid have previously been described, both for QREC and cephalosporin-resistant *E. coli* (68–71) both in Norway and in neighboring countries. Our results, which show close phylogenetic relationships between QREC isolates from broilers, strengthen the hypothesis that dissemination within the broiler industry originate from imported breeding animals, as suggested by Börjesson et al. (68).

Isolates from red foxes had the highest SNP distances to other isolates within the same animal species. In a previous study, Mo *et al.* showed that the occurrence of QREC in red foxes was low in areas with low human population density and higher in areas with medium or high human population density (72). Mo et al. suggested that the red foxes in urban areas have been exposed to different kind of indirect human exposures. This could contribute to the high diversity observed among the red fox isolates.

Interestingly, we identified phylogenetically related ST162 isolates with the same resistance mechanism patterns shared between a broiler and a red fox from geographically distant locations. One plausible explanation to this is a combination of distribution of similar isolates through the broiler production chain and that the red fox for instance came in contact with the isolate through broiler fecal matter used to fertilize crop fields. The two isolates in question were from different years, which may indicate persistence of QREC in the broiler production environment. Although dissemination from red foxes to broilers cannot be ruled out, the opposite direction is more likely due to the biosecurity measures in broiler production facilities.

To summarize, this study revealed high diversity in the QREC population in the four studied animal species. Nevertheless, QREC isolates that were phylogenetically related were found, both within and between host species. The phylogenetic structure also revealed that the quinolone resistance mechanisms are mostly clonal. While the origins of quinolone resistance in these populations remains unclear, these results indicate that QREC isolates in a livestock production chain may be disseminated down through the production pyramid. This highlights the importance of biosecurity focused control measures at the top of the production chain to prevent dissemination and persistence of QREC and PMQR in these environments.

AUTHOR STATEMENTS

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Conflicts of interest

None to declare

ABBREVIATIONS

ECOFF – Epidemiological cut-off

E. coli – *Escherichia coli*

EUCAST – European committee of antimicrobial susceptibility testing

MLST – Multi locus sequence typing

ML – Maximum likelihood

PMQR – Plasmid-mediated quinolone resistance

QRDR – Quinolone resistance determining region

QREC – Quinolone resistant *Escherichia coli*

ST – Sequence type

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FIGURES AND TABLES

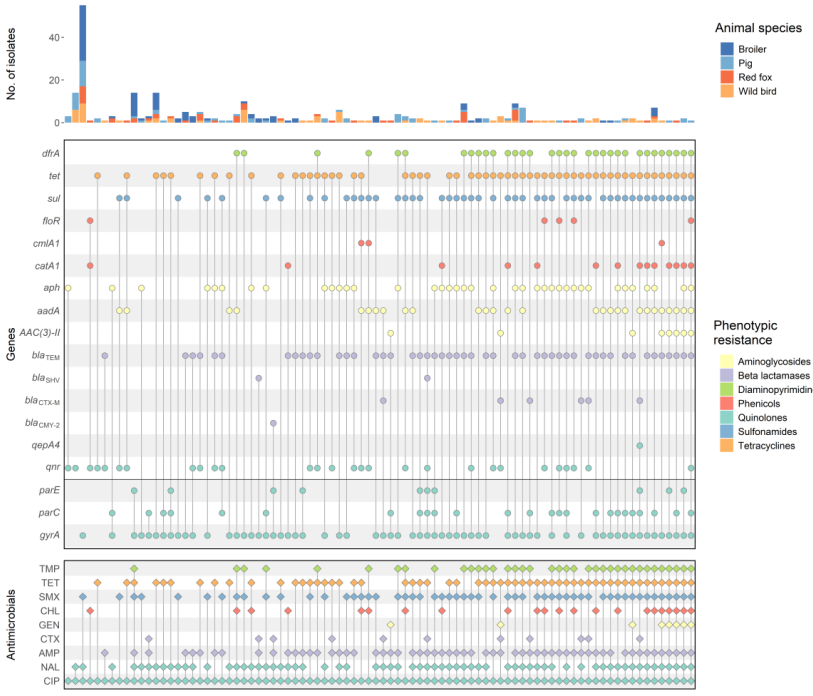
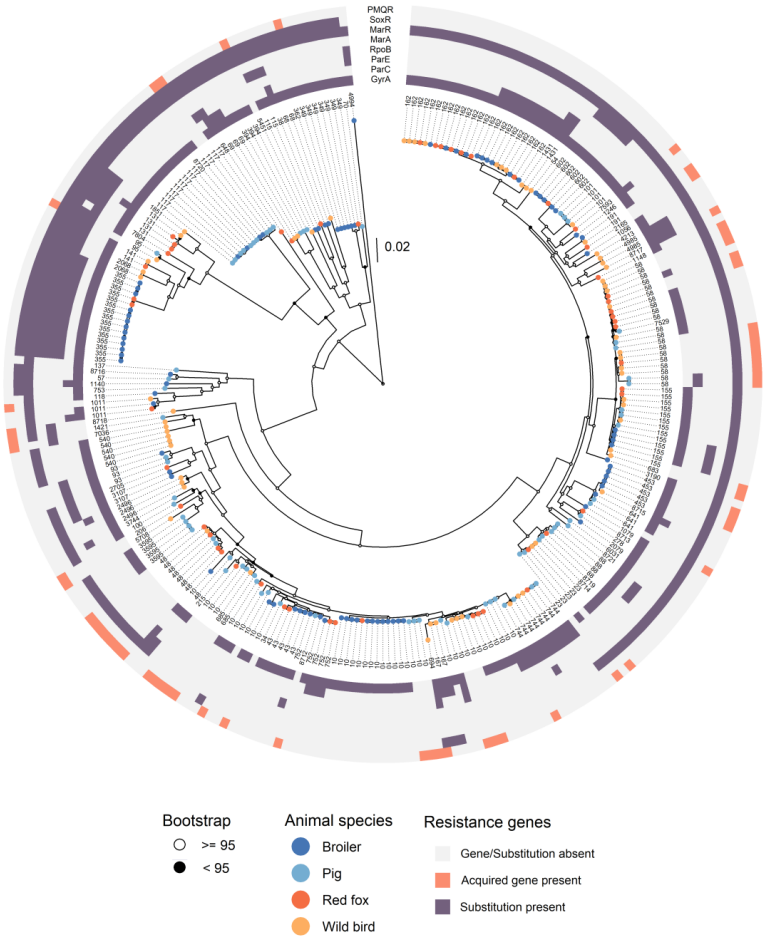
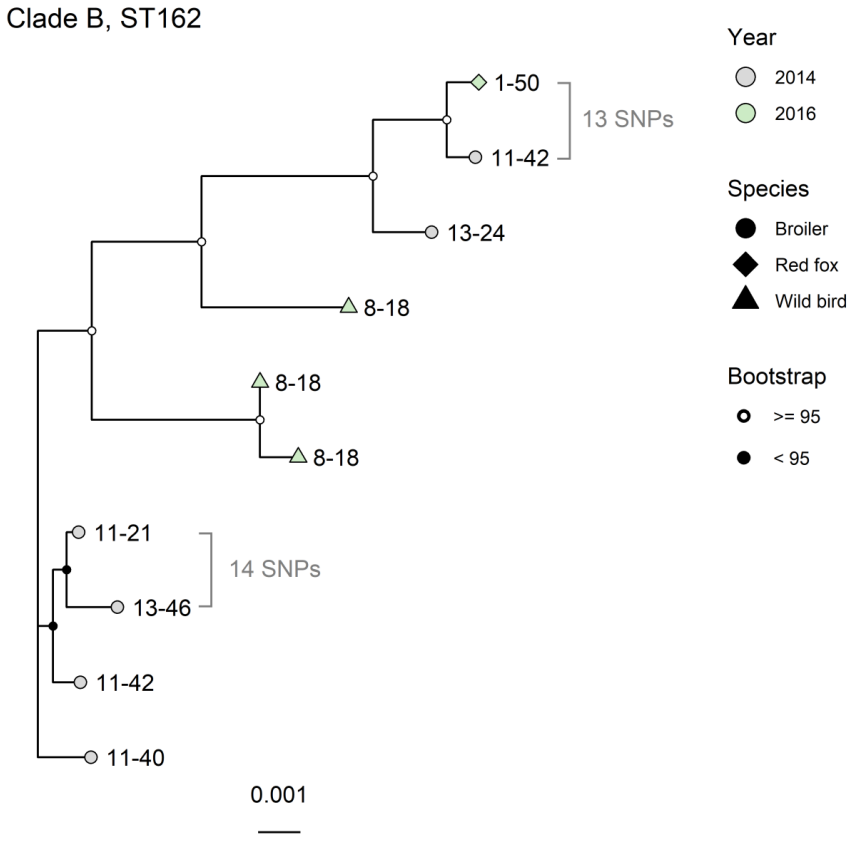


Figure 1: Phenotypic and genotypic resistance patterns for all plasmid-mediated resistance genes and *gyrA*, *parC* and *parE*. The top plot represents the number of isolates per group. The middle plot represents presence/absence of plasmid-mediated genes and chromosomal mutations (below the horizontal line). The bottom plot represents the phenotype of the respective gene/mutation combination. Meropenem and colistin were excluded as resistance was not observed among any isolates, and ceftazidime was excluded as cephalosporin resistance was already represented by cefotaxime. Tigecycline was excluded due to almost no resistance observed among the isolates. Colours represent animal species and resistance phenotypes. TMP = trimethoprim, TET = tetracycline, SMX = sulfamethoxazole, CHL = chloramphenicol, GEN = gentamicin, CTX = cefotaxime, AMP = ampicillin, NAL = nalidixic acid, CIP = ciprofloxacin. The genes in the middle plot are grouped based on gene family: *dfrA* = *dfrA1, dfrA5, dfrA8, dfrA12, dfrA14*, and *dfrA17*. *tet* = *tetA, tetB*, and *tetD*. *sul* = *sul1 - 3*. *aph* = *aph3Ia, aph3Ib,* and *aph6Id*. *aadA* = *aadA1, aadA2, aadA5, aadA12, aadA13*, and *aadA22*. *AAC(3)-II* = *AAC(3)-IIa* and *AAC(3)-IId*. bla_{TEM} = bla_{TEM-1A} – bla_{TEM-1C}. bla_{SHV} = bla_{SHV-2} and bla_{SHV-12}. bla_{CTX-M} = $bla_{CTX-M-1}$, $bla_{CTX-M-15}$, $bla_{CTX-M-32}$, and $bla_{CTX-M-55}$. *qnr* = *qnrA1, qnrB19, qnrS1, qnrS2*, and *qnrS4.*



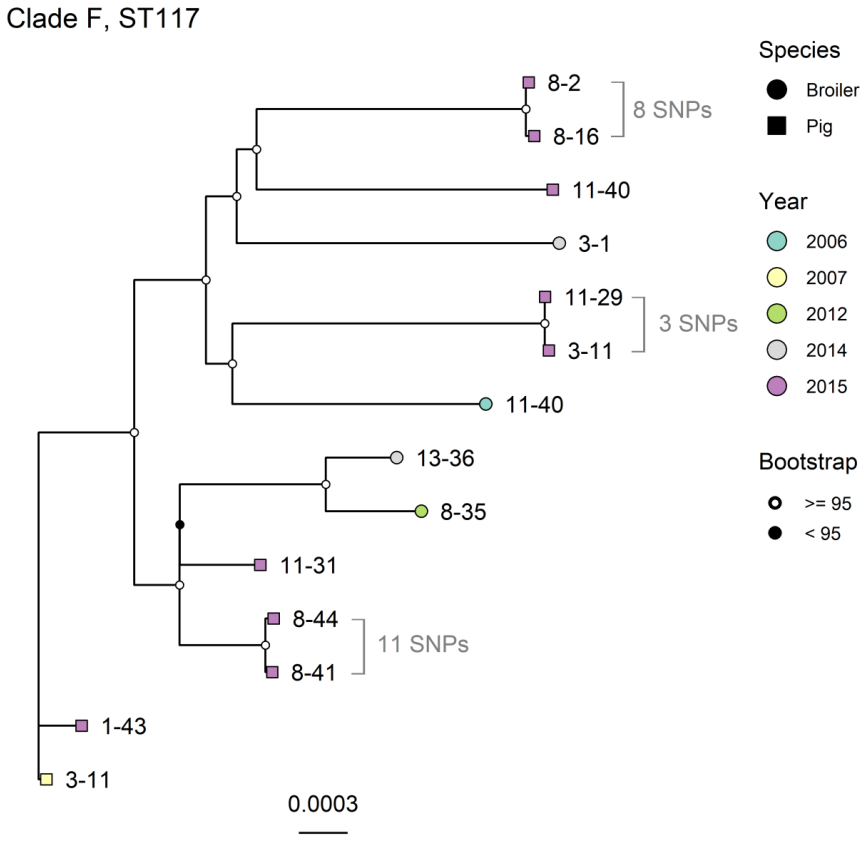
699

700 **Figure 2:** Maximum likelihood core gene SNP tree of all isolates. Branch supports (Ultra Fast
701 bootstrap approximation) are denoted as black or white nodes. The colored tips on the tree denote
702 animal species of origin, and the tip labels the sequence type from the MLST typing scheme hosted
703 by EnteroBase. The coloring on the outer rings denote presence/absence of mutations leading to
704 amino acid substitutions in chromosomal genes (purple) and presence/absence of plasmid-mediated
705 genes (orange). The tree was generated with IQTree from SNPs in core genes from Roary aligned
706 with MAFFT. Evolutionary model: GTR+F+ASC+R9. The tree is midpoint rooted for better
707 visualization.



708

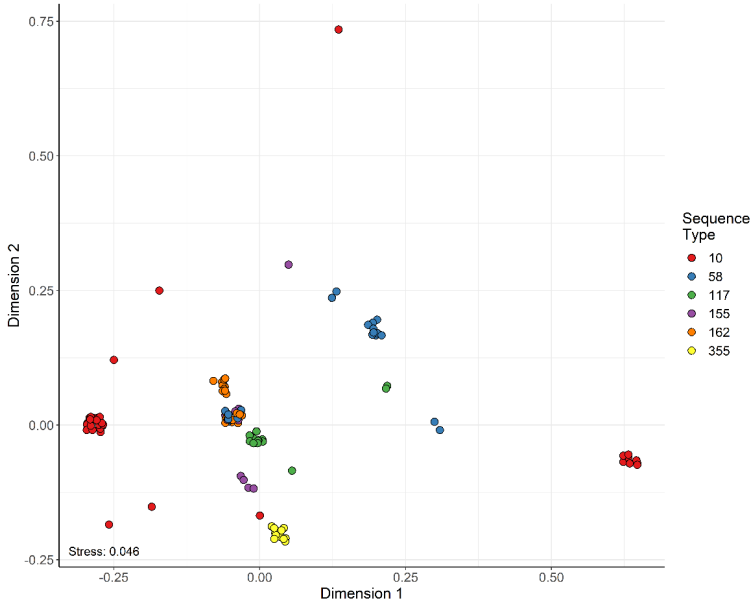
709 **Figure 3:** Maximum likelihood core genome tree of Clade B, containing ten ST162 isolates. Tip labels
710 denote the location of the isolate by county-municipality. Core genome SNPs were called with
711 ParSNP, recombinant sites removed with Gubbins, and the tree was generated with IQTree.
712 Evolutionary model: TIMe+ASC+R2. Shared genome among all isolates: 86%. The highly similar
713 isolates from wild birds in this tree (location 8-18, 2016) were disregarded as they were from the
714 same sample; one isolated by the traditional method and the other by the selective method.



715

716 **Figure 4:** Maximum likelihood core genome SNP tree of Clade F, containing both ST117 (n = 13) and
717 ST8720 (n = 1, from 2012) isolates. Tip labels denote the location of the isolate by county-
718 municipality. Core genome SNPs were called with ParSNP, recombinant sites removed with Gubbins,
719 and the tree was generated with IQTree. Evolutionary model: K3P+ASC+G4. Shared genome among
720 all isolates: 83.6%.

721



722

723 **Figure 5:** Non-metric multidimensional scaling (NMDS) analysis of presence/absence of quinolone
724 resistance mechanisms, both plasmid-mediated and chromosomal. The colors denote sequence
725 types. The points are jittered for easier interpretation.

726

Table 1: Number of isolates with mutations leading to amino acid substitutions in included chromosomal genes and presence/absence of plasmid-mediated genes per animal species. The percentage is relative to the total number of isolates (280).

Type	Gene	Number of isolates					Percent
		Broiler *n = 87*	Pig *n = 75*	Red fox *n = 52*	Wild bird *n = 66*	Sum *n = 280*	
Chromosomal	*gyrA*	87	56	42	44	229	81.8
	gyrB	0	0	0	0	0	0
	marA	19	2	7	6	34	12.1
	marR	66	52	40	54	212	75.7
	parC	8	9	10	16	43	15.4
	parE	14	5	3	7	29	10.4
	robA	0	0	0	0	0	0
	rpoB	25	6	9	8	48	17.1
	soxR	29	18	11	13	71	25.4
Plasmid-mediated	*qepA4*	0	0	0	1	1	0.4
	qnrA1	0	0	1	0	1	0.4
	qnrB19	1	11	2	7	21	7.5
	qnrS1	3	6	6	14	29	10.4
	qnrS2	0	3	1	2	6	2.1
	qnrS4	0	0	1	0	1	0.4

Table 2: Overview of isolates of interest from ST162 (Clade B) and ST117 (Clade F). The location ID represent county – municipality. The pairs correspond to the annotated clades in Figure 3 and 4.

ST	Isolate	No. of SNPs	Fraction similar genome	Source	Year	Location
162	1	13	90.8%	Red fox	2016	1-50
	2			Broiler	2014	11-42
	1	14	90.9%	Broiler	2014	11-21
	2			Broiler	2014	13-46
117	1	3	95.4%	Pig	2015	11-29
	2			Pig	2015	3-11
	1	8	74.1 %	Pig	2015	8-2
	2			Pig	2015	8-16
	1	11	91.0%	Pig	2015	8-44
	2			Pig	2015	8-41

Supplementary material

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27.11.2019

Contents

Section 1: Supplementary tables

Table S1: Group listing

The table presents the different phenotypic groups used in the isolate selection, and the number of isolates from each animal species in each group. The number of resistances within each group is listed in the column "No. of resistances".

Group	No. of resistances	CIP	NAL	Isolates per species	Total isolates
1	1	0.06	32	Pig (3), Wild bird (1), Red fox (2)	6
2	1	0.06	64	Red fox (1)	1
3	1	0.25	4	Pig (1), Broiler (1)	2
4	1	0.25	8	Pig (3), Wild bird (1)	4
5	1	0.25	16	Pig (4)	4
6	1	0.5	4	Pig (1)	1
7	1	0.5	8	Wild bird (2)	2
8	1	0.5	16	Pig (2), Wild bird (3)	5
9	2	0.06	32	Pig (1)	1
10	2	0.12	32	Broiler (4), Wild bird (1)	5
11	2	0.12	64	Broiler (1), Wild bird (1), Red fox (1)	3
12	2	0.12	128	Broiler (5), Wild bird (1)	6
13	2	0.25	8	Pig (1), Wild bird (1), Red fox (2)	4
14	2	0.25	32	Broiler (1)	1
15	2	0.25	64	Pig (2), Broiler (5)	7
16	2	0.25	128	Broiler (11), Pig (2), Wild bird (1), Red fox (3)	17
17	2	0.25	256	Broiler (1), Pig (4), Wild bird (4), Red fox (3)	12
18	2	0.5	8	Pig (1)	1
19	2	0.5	16	Wild bird (1)	1
20	2	0.5	32	Pig (1)	1
21	2	0.5	128	Broiler (2)	2
22	2	0.5	256	Broiler (3)	3
23	2	1	256	Broiler (1), Wild bird (1), Red fox (2)	4
24	2	4	256	Wild bird (1)	1
25	2	8	256	Red fox (1)	1
26	3	0.12	32	Broiler (2)	2
27	3	0.12	64	Red fox (3), Broiler (2)	5
28	3	0.12	128	Broiler (3), Pig (1)	4
29	3	0.25	4	Wild bird (1)	1
30	3	0.25	8	Pig (1), Red fox (1)	2
31	3	0.25	32	Broiler (1)	1
32	3	0.25	64	Broiler (4), Pig (1)	5
33	3	0.25	128	Broiler (10), Pig (4), Wild bird (4), Red fox (1)	19
34	3	0.25	256	Broiler (3), Pig (1), Wild bird (4), Red fox (5)	13
35	3	0.5	8	Pig (1)	1
36	3	0.5	16	Pig (1), Red fox (3)	4
37	3	0.5	256	Pig (1)	1
38	3	1	64	Broiler (1)	1
39	3	2	128	Broiler (1)	1
40	3	16	256	Pig (1), Wild bird (2), Red fox (1)	4

(continued)

Group	No. of resistances	CIP	NAL	Isolates per species	Total isolates
41	4	0.03	64	Pig (1)	1
42	4	0.12	64	Pig (1)	1
43	4	0.12	128	Broiler (2), Wild bird (2), Red fox (1)	5
44	4	0.12	256	Wild bird (1)	1
45	4	0.25	4	Wild bird (1), Red fox (3)	4
46	4	0.25	8	Wild bird (4)	4
47	4	0.25	16	Pig (1), Red fox (1)	2
48	4	0.25	128	Broiler (3), Pig (2)	5
49	4	0.25	256	Broiler (1), Pig (2), Wild bird (3), Red fox (1)	7
50	4	0.5	16	Wild bird (2)	2
51	4	0.5	128	Pig (1)	1
52	4	0.5	256	Broiler (3), Pig (3), Wild bird (3)	9
53	4	1	256	Red fox (1)	1
54	4	4	256	Broiler (1)	1
55	4	8	256	Broiler (1)	1
56	5	0.12	128	Pig (2), Red fox (1)	3
57	5	0.12	256	Broiler (1)	1
58	5	0.25	4	Red fox (1)	1
59	5	0.25	64	Broiler (1), Red fox (2)	3
60	5	0.25	128	Broiler (2), Pig (4), Wild bird (1)	7
61	5	0.25	256	Pig (1), Red fox (1)	2
62	5	0.5	16	Pig (1), Wild bird (4)	5
63	5	0.5	64	Broiler (1)	1
64	5	0.5	128	Broiler (1), Red fox (1)	2
65	5	0.5	256	Broiler (1)	1
66	5	1	64	Broiler (1)	1
67	5	4	256	Pig (1)	1
68	5	16	256	Pig (1), Wild bird (3), Red fox (1)	5
69	6	0.12	32	Pig (1), Wild bird (1)	2
70	6	0.12	64	Pig (2)	2
71	6	0.25	4	Pig (1)	1
72	6	0.25	64	Pig (1)	1
73	6	0.25	128	Broiler (1), Pig (1), Wild bird (2), Red fox (1)	5
74	6	0.25	256	Pig (4), Red fox (3)	7
75	6	0.5	16	Wild bird (1), Red fox (1)	2
76	6	1	128	Broiler (1)	1
77	6	1	256	Wild bird (1), Red fox (1)	2
78	6	8	256	Broiler (1), Pig (1), Wild bird (5)	7
79	6	16	256	Pig (1), Red fox (1)	2
80	7	0.25	128	Red fox (1)	1
81	7	8	256	Broiler (4), Wild bird (1), Red fox (1)	6
82	7	16	256	Wild bird (3)	3
83	8	0.25	64	Wild bird (1)	1
84	8	0.5	128	Pig (3)	3
85	8	8	256	Red fox (1)	1
86	8	16	256	Pig (1)	1

Table S2: Occurrence of antimicrobial resistance among the selected quinolone resistant isolates.

The table presents the percent (%) occurrence of antimicrobial resistance among the selected isolates (n = 280) identified through minimum inhibitory concentration values in the Norwegian monitoring programme from 2006 to 2017. The epidemiological cut off values used were defined by EUCAST. Azithromycin was excluded as no epidemiological cutoff value is currently available.

Substance	Broiler[a]	Pig[b]	Red fox[c]	Wild bird[d]	Total[e]
Ciprofloxacin	100.0	93.3	94.2	98.5	96.8
Nalidixic acid	100.0	74.7	76.9	68.2	81.4
Tetracycline	26.4	49.3	46.2	60.6	44.3
Ampicillin	36.8	40.0	51.9	51.5	43.9
Sulfamethoxazole	28.7	50.7	36.5	36.4	37.9
Trimethoprim	17.2	36.0	44.2	36.4	31.8
Chloramphenicol	5.8	10.7	11.5	16.7	10.7
Cefotaxime	4.6	2.7	5.8	9.1	5.4
Ceftazidime	3.4	2.7	5.8	5.8	5.4
Gentamicin	0.0	5.3	3.8	9.1	4.3
Meropenem	0.0	0.0	0.0	0.0	0.0
Colistin	0.0	0.0	0.0	0.0	0.0
Tigecycline	0.0	1.3	0.0	0.0	0.4

[a] 87 isolates
[b] 75 isolates
[c] 52 isolates
[d] 66 isolates
[e] 280 isolates

Table S3: Prokka reference genomes

The genomes listed below were used as references for the Prokka annotation, and downloaded with ncbi-genome-download (https://github.com/kblin/ncbi-genome-download) with the following commands:

–refseq-category reference

–assembly-level complete

–genus "Escherichia coli" bacteria

Accession number	Information
GCF_000005845.2_ASM584v2	*E. coli* K12
GCF_000008865.2_ASM886v2	*E. coli* O157:H7 Sakai
GCF_000026345.1_ASM2634v1	*E. coli* IAI39
GCF_000183345.1_ASM18334v1	*E. coli* O83:H1 NRG 857C
GCF_000299455.1_ASM29945v1	*E. coli* O104:H4 2011C-3493

Table S4: Identified amino acid substitutions in the QRDR of GyrA, ParC and ParE.

The table presents the different amino acid substitutions identified in GyrA, ParC and ParE and percent occurrence in total for each amino acid substitution.

AA substitution	n	Total	Percent
GyrA			
S83L	170	280	60.7
None	51	280	18.2
S83L, D87N	33	280	11.8
D87Y	11	280	3.9
S83A	9	280	3.2
D87N	3	280	1.1
D87G	2	280	0.7
D87H	1	280	0.4
GyrB			
None	280	280	100.0
ParC			
None	237	280	84.6
S80I	28	280	10.0
A56T, S80I	8	280	2.9
S57T	2	280	0.7
S58I	2	280	0.7
S80R	2	280	0.7
S80I, E84V	1	280	0.4
ParE			
None	251	280	89.6
D475E	15	280	5.4
S458A	10	280	3.6
D463N	1	280	0.4
H516Y	1	280	0.4
L416F	1	280	0.4
L488M, A512T	1	280	0.4

Table S5: QRDR substitutions in GyrA, ParC and ParE per animal species.

The table presents each identified amino acid substitution in GyrA, ParC and ParE and their occurrence in isolates from each animal species. The percentage is based on the number of isolates per species with the mutation in question (n) in relation to the total number of isolates per animal species (Total).

Protein	AA substitution	n	Total	Percent
Broiler				
GyrA	S83L	75	87	86.2
GyrA	S83L, D87N	7	87	8.0
GyrA	D87N	2	87	2.3
GyrA	D87Y	2	87	2.3
GyrA	D87H	1	87	1.1
GyrB	None	87	87	100.0
ParC	None	79	87	90.8
ParC	S80I	8	87	9.2
ParE	None	73	87	83.9
ParE	D475E	13	87	14.9
ParE	L488M, A512T	1	87	1.1
Pig				
GyrA	S83L	45	75	60.0
GyrA	None	19	75	25.3
GyrA	S83L, D87N	5	75	6.7
GyrA	S83A	4	75	5.3
GyrA	D87Y	2	75	2.7
GyrB	None	75	75	100.0
ParC	None	66	75	88.0
ParC	A56T, S80I	4	75	5.3
ParC	S57T	2	75	2.7
ParC	S80R	2	75	2.7
ParC	S80I	1	75	1.3
ParE	None	70	75	93.3
ParE	S458A	3	75	4.0
ParE	D463N	1	75	1.3
ParE	L416F	1	75	1.3
Red fox				
GyrA	S83L	31	52	59.6
GyrA	None	10	52	19.2
GyrA	S83L, D87N	6	52	11.5
GyrA	D87G	2	52	3.8
GyrA	S83A	2	52	3.8
GyrA	D87Y	1	52	1.9
GyrB	None	52	52	100.0
ParC	None	42	52	80.8
ParC	S80I	8	52	15.4
ParC	A56T, S80I	1	52	1.9
ParC	S80I, E84V	1	52	1.9
ParE	None	49	52	94.2
ParE	D475E	2	52	3.8
ParE	S458A	1	52	1.9
Wild bird				

(continued)

Protein	AA substitution	n	Total	Percent
GyrA	None	22	66	33.3
GyrA	S83L	19	66	28.8
GyrA	S83L, D87N	15	66	22.7
GyrA	D87Y	6	66	9.1
GyrA	S83A	3	66	4.5
GyrA	D87N	1	66	1.5
GyrB	None	66	66	100.0
ParC	None	50	66	75.8
ParC	S80I	11	66	16.7
ParC	A56T, S80I	3	66	4.5
ParC	S58I	2	66	3.0
ParE	None	59	66	89.4
ParE	S458A	6	66	9.1
ParE	H516Y	1	66	1.5

Table S6: AA substitutions in MarA, MarR, RobA, RpoB and SoxR.

The table presents the identified amino acid substitutions in MarA, MarR, RobA, RpoB and SoxR. The column "n" denote the amount of isolates with the respective AA substitution. The percentage is relative to the total amount of isolates.

AA substitution	n	Total	Percent
MarA			
None	246	280	87.9
S127N	26	280	9.3
L78F	6	280	2.1
E33V	1	280	0.4
P76S	1	280	0.4
MarR			
G103S, Y137H	192	280	68.6
None	68	280	24.3
K62R, G103S, Y137H	6	280	2.1
S3N, G103S, Y137H	6	280	2.1
G103S, D118N, Y137H	3	280	1.1
A53E, G103S, Y137H	1	280	0.4
A53S, G103S, Y137H	1	280	0.4
E131K	1	280	0.4
G103S, D118N, H120K, Y137H	1	280	0.4
T102P, G103S, Y137H	1	280	0.4
RobA			
None	280	280	100.0
RpoB			
None	232	280	82.9
E320D	33	280	11.8
V261A	4	280	1.4
E320D, D485E	2	280	0.7
G1318S	2	280	0.7
D320E	1	280	0.4
E320D, D393E	1	280	0.4
E320D, F464C	1	280	0.4
E412D, T595I	1	280	0.4
H165Y	1	280	0.4
P847S	1	280	0.4
V980L	1	280	0.4
SoxR			
None	209	280	74.6
T38S, G74R	33	280	11.8
G74R	26	280	9.3
I40V	4	280	1.4
A111T	3	280	1.1
T38S, N45D, G74R	3	280	1.1
A24T, G74R	1	280	0.4
E115K	1	280	0.4

Table S7: Mechanisms in isolates without substitutions in QRDR

This table presents the mechanisms identified in isolates with no amino acid substitutions in either of GyrA, GyrB, ParC and/or ParE.

marA	marR	robA	rpoB	soxR	PMQR	n
0	0	0	0	0	qnrB19	12
0	0	0	0	0	qnrS2	4
0	0	0	1	0	qnrS1	1
0	1	0	0	0	qnrA1	1
0	1	0	0	0	qnrB19	5
0	1	0	0	0	qnrS1	17
0	1	0	0	0	qnrS2	2
0	1	0	0	0	qnrS4	1
0	1	0	0	1	qnrB19	1
0	1	0	0	1	qnrS1	2
0	1	0	1	0	qnrB19	1
0	1	0	1	0	qnrS1	1
1	1	0	1	1	qnrB19	1

Table S8: Other plasmid mediated genes

The table presents the plasmid mediated genes related to other resistance phenotypes among the isolates.

Gene	Absent	Present	Total	Percent	Included
bla$_{\text{TEM-1B}}$	182	98	280	35.00	Yes
aph6Id	192	88	280	31.43	Yes
aph3Ib	194	86	280	30.71	Yes
tetA	202	78	280	27.86	Yes
sul2	207	73	280	26.07	Yes
tetB	231	49	280	17.50	Yes
sul1	253	27	280	9.64	Yes
aadA1	254	26	280	9.29	Yes
dfrA5	255	25	280	8.93	Yes
catA1	257	23	280	8.21	Yes
dfrA17	259	21	280	7.50	Yes
aadA5	260	20	280	7.14	Yes
dfrA1	261	19	280	6.79	Yes
dfrA14	263	17	280	6.07	Yes
aph3Ia	266	14	280	5.00	Yes
mphA	268	12	280	4.29	No
aac(3')-IId	270	10	280	3.57	Yes
aadA13	271	9	280	3.21	Yes
sul3	271	9	280	3.21	Yes
bla$_{\text{TEM-1C}}$	273	7	280	2.50	Yes
aadA2	275	5	280	1.79	Yes
dfrA12	275	5	280	1.79	Yes
floR	275	5	280	1.79	Yes
bla$_{\text{CMY-2}}$	277	3	280	1.07	Yes
bla$_{\text{CTX-M-15}}$	277	3	280	1.07	Yes
bla$_{\text{CTX-M-55}}$	277	3	280	1.07	Yes
cmlA1	277	3	280	1.07	Yes
mphB	277	3	280	1.07	No
aac(3')-IIa	278	2	280	0.71	Yes
aadA12	278	2	280	0.71	Yes
aadA22	278	2	280	0.71	Yes
bla$_{\text{CTX-M-1}}$	278	2	280	0.71	Yes
bla$_{\text{SHV-2}}$	278	2	280	0.71	Yes
bla$_{\text{TEM-1A}}$	278	2	280	0.71	Yes
dfrA8	278	2	280	0.71	Yes
mphE	278	2	280	0.71	No
msrE	278	2	280	0.71	No
bla$_{\text{CTX-M-32}}$	279	1	280	0.36	Yes
bla$_{\text{SHV-12}}$	279	1	280	0.36	Yes
ermB	279	1	280	0.36	No
lnuF	279	1	280	0.36	No
tetD	279	1	280	0.36	Yes

Table S9: Co-resistance

The table presents the number of isolates with each combination of plasmid mediated resistance genes, excluding combinations represented by only a single isolate.

Combination of genes	Number of isolates
PMQR Negative	
tet	18
sul	2
dfrA	10
bla_{TEM}	5
bla_{SHV}	2
bla_{CMY}	3
aph	5
sul, *tet*	2
bla_{TEM}, *tet*	3
aph, *tet*	4
aph, *sul*	4
aadA, *dfrA*	4
bla_{TEM}, *sul*, *tet*	2
aph, *sul*, *tet*	2
aph, *dfrA*, *sul*	4
aph, bla_{TEM}, *tet*	6
aph, bla_{TEM}, *sul*	2
aadA, bla_{TEM}, *sul*	3
aph, bla_{TEM}, *sul*, *tet*	2
aph, bla_{TEM}, *dfrA*, *sul*	9
aph, $bla_{\text{CTX-M}}$, *sul*, *tet*	2
aadA, *dfrA*, *sul*, *tet*	2
aadA, bla_{TEM}, *sul*, *tet*	2
aadA, bla_{TEM}, *dfrA*, *tet*	2
aph, bla_{TEM}, *dfrA*, *sul*, *tet*	10
aadA, bla_{TEM}, *dfrA*, *sul*, *tet*	2
aac(3')-II, *aph*, bla_{TEM}, *sul*, *tet*	2
catA, *aadA*, *dfrA*, *sul*, *tet*	2
catA, *aadA*, bla_{TEM}, *dfrA*, *tet*	2
aadA, *aph*, bla_{TEM}, *dfrA*, *sul*, *tet*	9
catA, *aadA*, *aph*, bla_{TEM}, *dfrA*, *sul*, *tet*	8
catA, *aac(3')-II*, *aadA*, bla_{TEM}, *dfrA*, *sul*, *tet*	2
catA, *aac(3')-II*, *aadA*, *aph*, bla_{TEM}, *dfrA*, *sul*, *tet*	2
PMQR Positive	
qnr	14
qnr, *tet*	2
bla_{TEM}, *qnr*	4
aph, *qnr*	3
bla_{TEM}, *qnr*, *tet*	5
bla_{TEM}, *dfrA*, *qnr*, *tet*	4
aph, bla_{TEM}, *qnr*, *tet*	2
aac(3')-II, $bla_{\text{CTX-M}}$, *qnr*, *tet*	3
aadA, *dfrA*, *qnr*, *sul*, *tet*	3
aph, bla_{TEM}, *dfrA*, *qnr*, *sul*, *tet*	2

Table S10: SNP distance statistics on isolates from the same species.

The table presents the summary statistics on the minimum SNP distance to the closest isolate from same animal species.

Statistic	Broiler	Pig	Red fox	Wild bird
Min	0	0	0	0
1st.Q	2	2	9	1
Median	12	212	1388	53
Mean	3193	4310	7210	5323
3rd.Q	52	3471	12945	7922
Max	75534	47826	46955	33629

Section 2: Supplementary figures

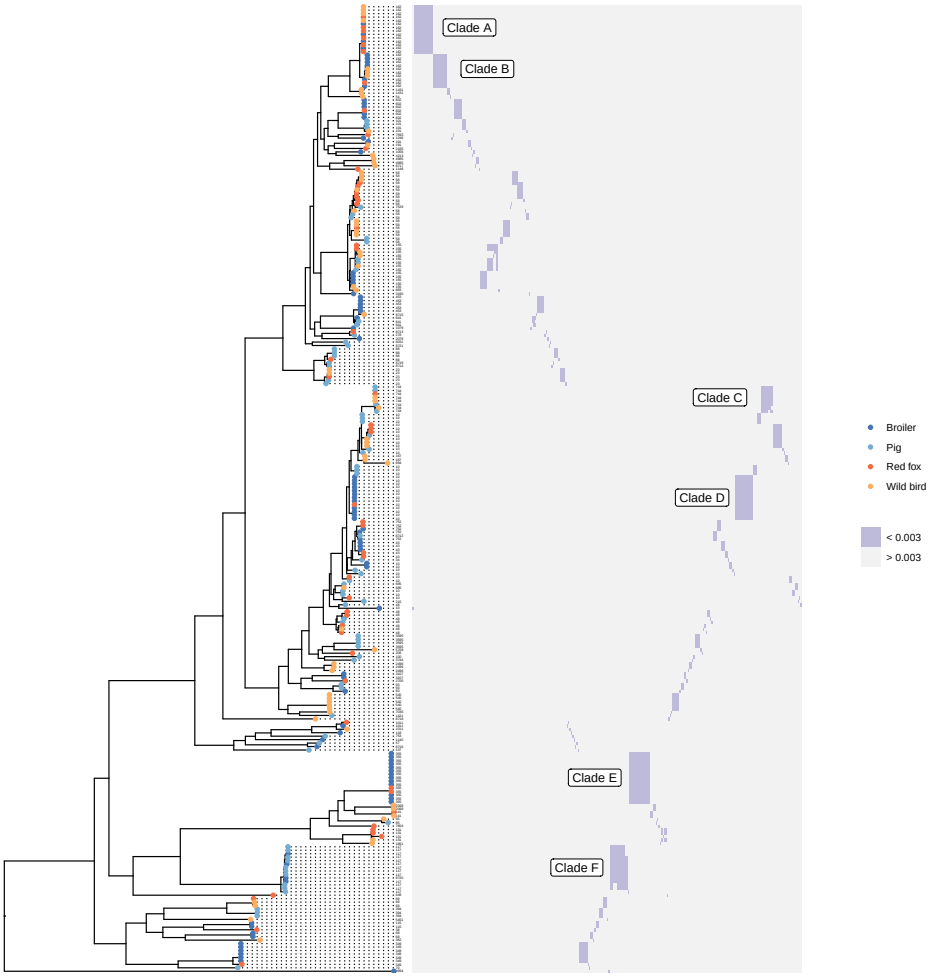

Figure S1: Clade selection based on patristic distances

The figure presents the overall phylogenetic tree (left) and the calculated patristic distances (right) as less than (<) or higher than (>) the specified cutoff value (0.003). The denoted clades were further investigated with phylogenetic analyses. Patristic distances were calculated from the core gene SNP tree.

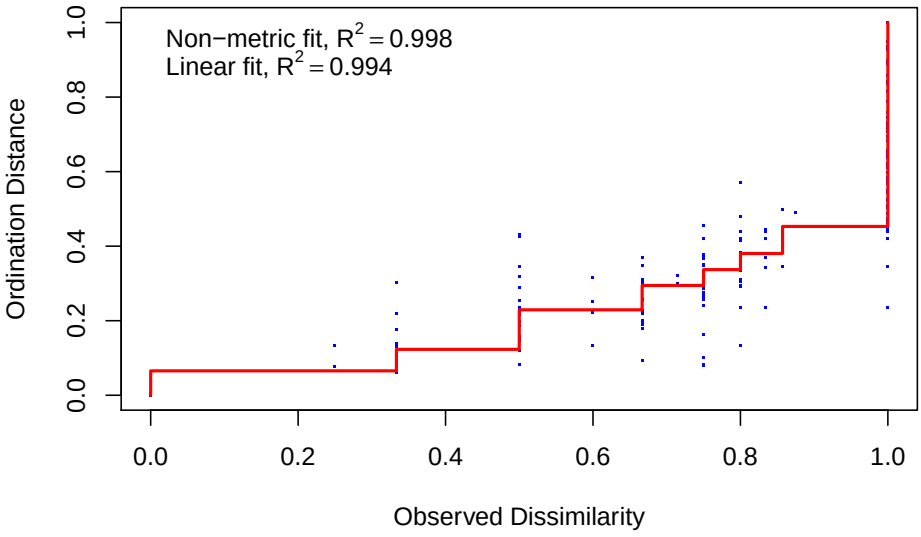

Figure S2: Stressplot

Non-metric multidimensional scaling (NMDS) was used to see if the distribution of quinolone resistance mechanisms from some sequence types were more homogenous than in other sequence types. The stressplot visualizes the goodness of fit.

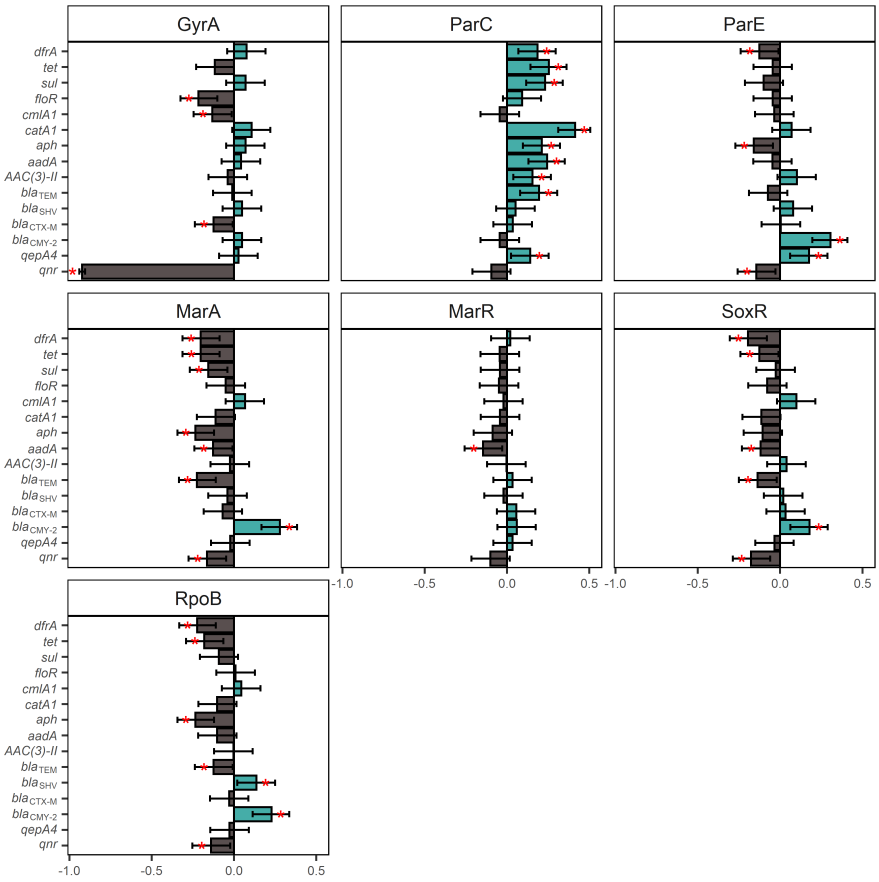

Figure S3: Correlations between chromosomal genes and plasmid mediated genes

The figure presents the Pearson correlation values for amino acid substitutions in GyrA, ParC, ParE, MarA, MarR, RpoB, and SoxR against all plasmid mediated genes identified. Grey = negative correlation, green = positive correlation. Black lines denote 95% confidence intervals. Red stars denote significant correlations. The plasmid mediated genes are grouped based on gene family: *dfrA* = *dfrA1*, *dfrA5*, *dfrA8*, *dfrA12*, *dfrA14*, and *dfrA17*. *tet* = *tetA*, *tetB*, and *tetD*. *sul* = *sul1* - *3*. *aph* = *aph3Ia*, *aph3Ib*, and *aph6Id*. *aadA* = *aadA1*, *aadA2*, *aadA5*, *aadA12*, *aadA13*, and *aadA22*. *AAC(3')-II* = *AAC(3')-IIa* and *AAC(3')-IId*. bla_{TEM} = $bla_{\text{TEM-1-A}}$ – $bla_{\text{TEM-1-C}}$. bla_{SHV} = $bla_{\text{SHV-2}}$ and $bla_{\text{SHV-12}}$. $bla_{\text{CTX-M}}$ = $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-32}}$, and $bla_{\text{CTX-M-55}}$. *qnr* = *qnrA1*, *qnrB19*, *qnrS1*, *qnrS2*, and *qnrS4*.

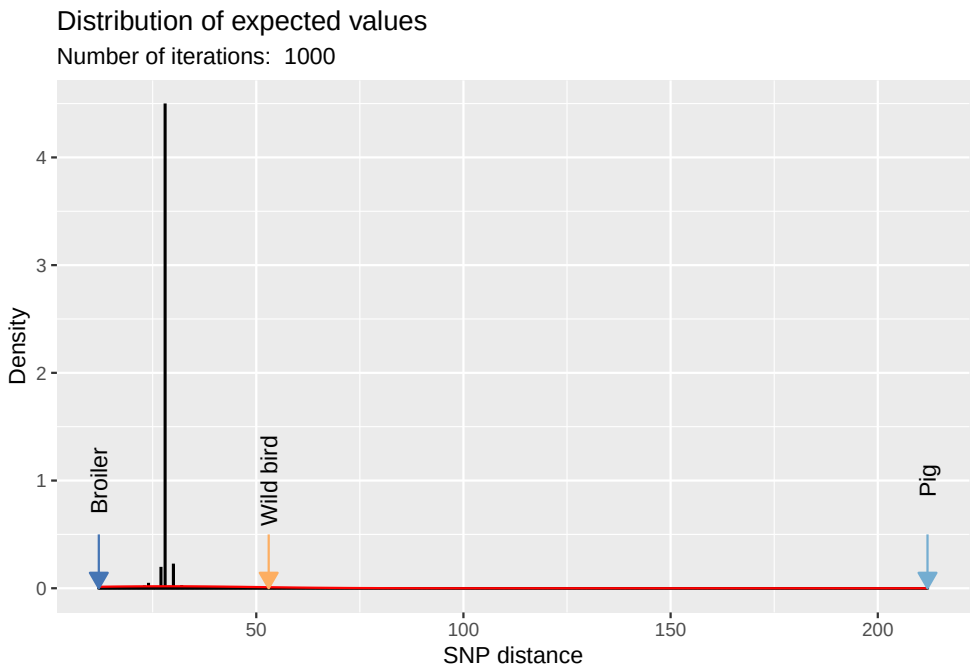

Figure S4: Iteration analysis results

The figure presents the observed median minimum SNP distance values for each animal species (coloured arrows) in relation to the expected values from the iteration analysis. Red fox results is excluded from the plot as the observed distance values were too high to visualize. The figure shows that isolates from broilers are more closely aggregated in the phylogenetic tree than what is randomly expected.

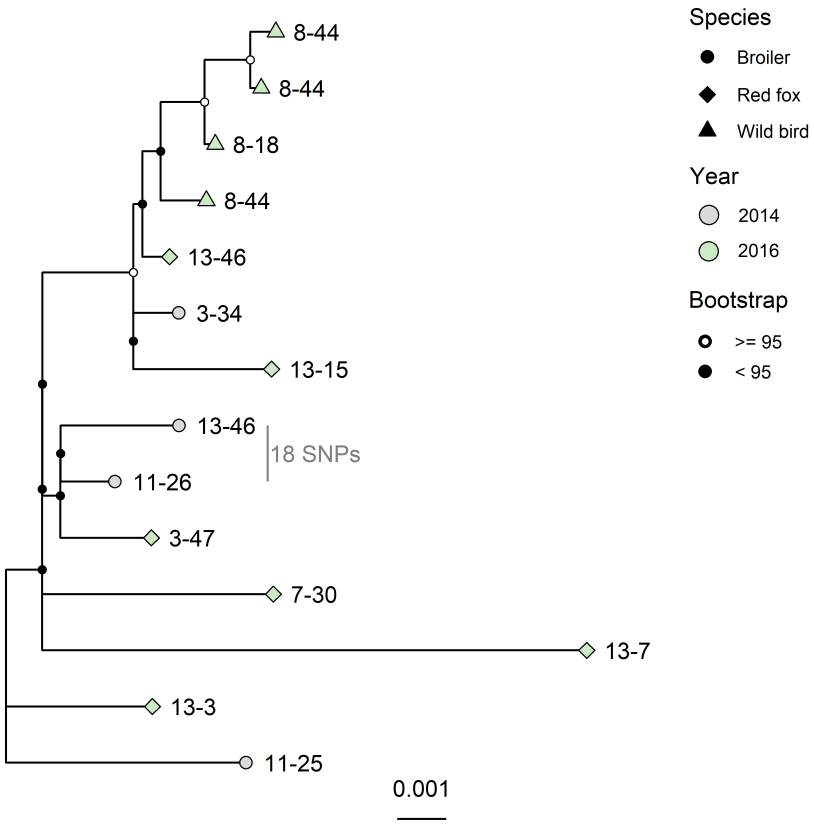

Figure S5: Maximum likelihood tree, Clade A

The values on each node is the UF Bootstrap values calculated by IQTree. The tip point shape represent the animal species of origin, and the color the year of isolation. The tip labels represent the location of the isolate; the first number represent the county, and the second number the municipality. Evolutionary model: K2P+ASC+R2. Shared genome: 88.4%.

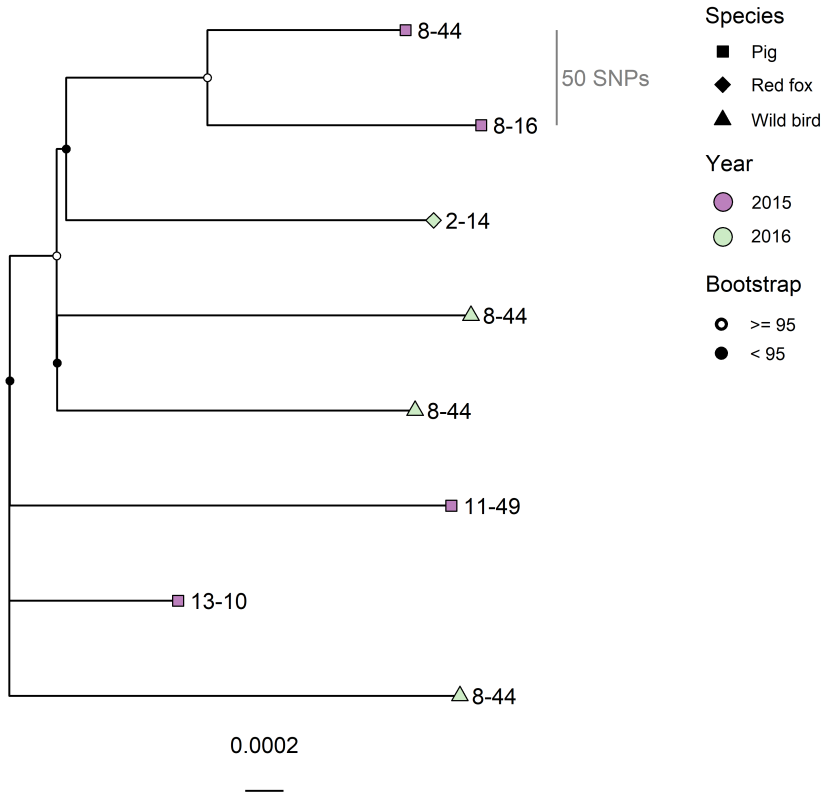

Figure S6: Maximum likelihood tree, Clade C

The values on each node is the UF Bootstrap values calculated by IQTree. The tip point shape represent the animal species of origin, and the color the year of isolation. The tip labels represent the location of the isolate; the first number represent the county, and the second number the municipality. Evolutionary model: K3P+ASC. Shared genome: 88.4%.

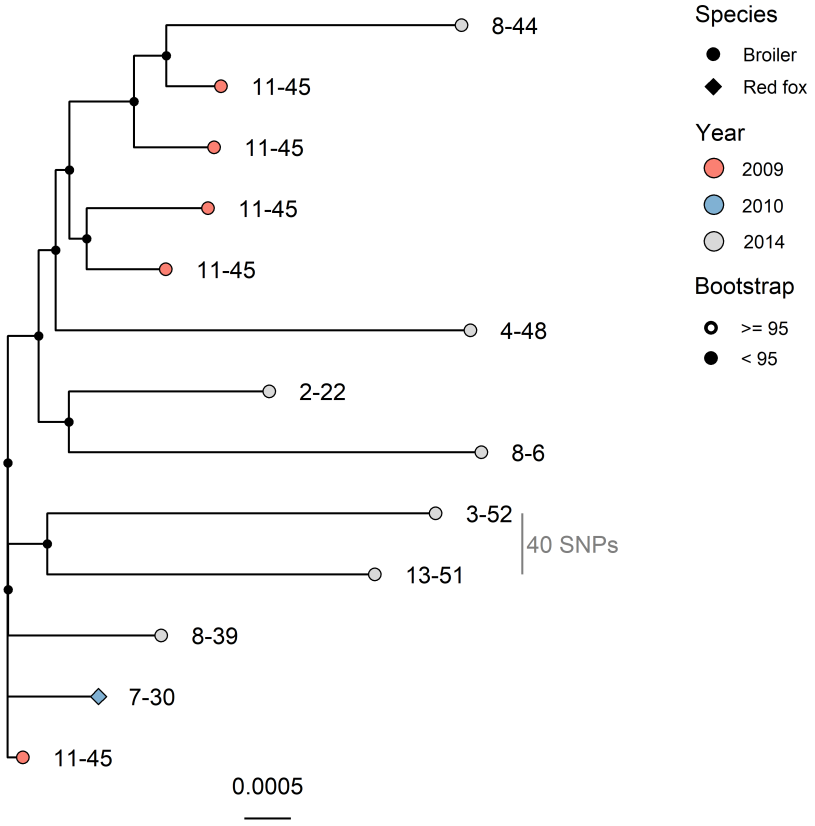

Figure S7: Maximum likelihood tree, Clade D

The values on each node is the UF Bootstrap values calculated by IQTree. The tip point shape represent the animal species of origin, and the color the year of isolation. The tip labels represent the location of the isolate; the first number represent the county, and the second number the municipality. Evolutionary model: K2P+ASC+R2. Shared genome: 87.2%.

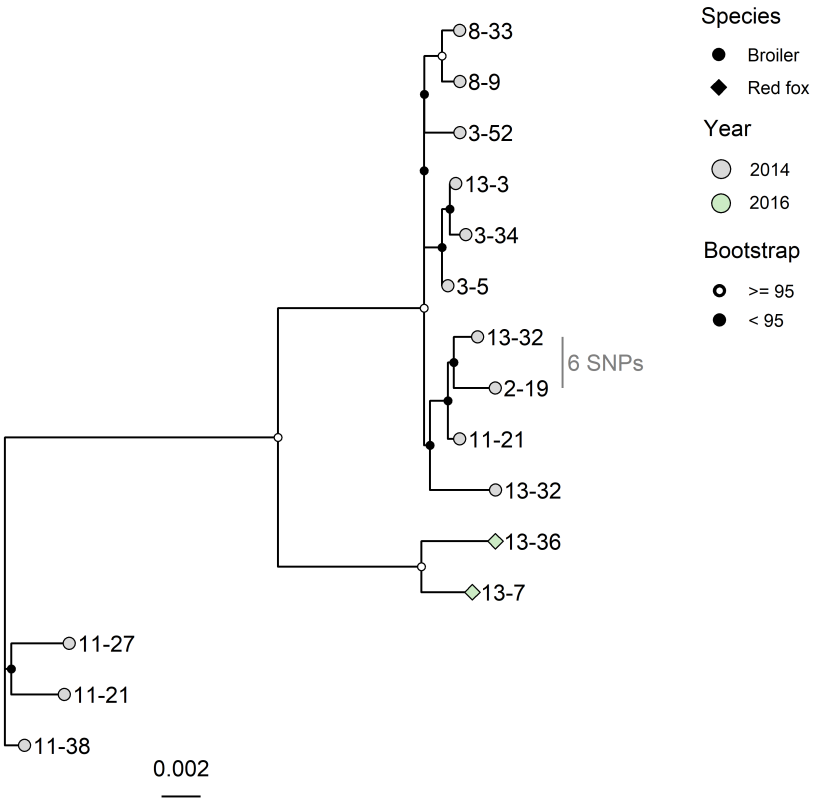

Figure S8: Maximum likelihood tree, Clade E

The values on each node is the UF Bootstrap values calculated by IQTree. The tip point shape represent the animal species of origin, and the color the year of isolation. The tip labels represent the location of the isolate; the first number represent the county, and the second number the municipality. Evolutionary model: K3P+ASC. Shared genome: 90.2%.

Section 3: Metadata and reference sequences

3.1: Isolate information and metadata

Metadata for all 280 included isolates can be downloaded here:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/total_data_new.xlsx

3.2: FastQC analysis results

Raw read quality control results can be accessed here:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/notebooks/fastqc_analysis.html

3.3: Mash screen results

Contaminant screening results can be accessed here:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/notebooks/mash_analysis.html

3.4: QRDR determination of GyrB, ParC and ParE

The K12 reference sequence on the whole protein is listed as the first entry in each file. The rest of the fasta entries are taken from Jiménez Gómez et al. 2004 [1].

GyrB:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/reference_genes/gyrB_QRDR_ref.fasta

ParC:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/reference_genes/parC_QRDR_ref.fasta

ParE:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/reference_genes/parE_QRDR_ref.fasta

3.5: ARIBA flag selection

Data on ARIBA flag selection can be downloaded here:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/ariba_flag_selection.xlsx

3.6: MEGARes and ResFinder reference sequences

MEGARes:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/reference_genes/total_megares_references.fa

ResFinder:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/reference_genes/total_resfinder_references.fa

3.7: QUAST results

Assembly metrics can be accessed here:

https://norwegianveterinaryinstitute.github.io/qm_wgs_4species/notebooks/assembly_metrics.html

References

[1] P.A. JG, J.E. G de los R, A. RM, P. de PR, R. GA. 2004. Molecular basis of quinolone resistance in Escherichia coli from wild birds. Canadian Journal of Veterinary Research 68:229–231

Paper 3

Comparative genome analyses of wild type- and quinolone resistant *Escherichia coli* indicate dissemination of QREC in the Norwegian broiler breeding pyramid

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Keywords: QREC, wild type, broiler, quinolone, resistance, dissemination.

Number of words:

Number of figures: 3

Abstract

Quinolones are important antimicrobials for both humans and animals, and resistance towards these compounds is a serious threat to public health. In Norway, quinolone resistant *E. coli* (QREC) have been detected at low levels in a high proportion of broiler flocks, even without the use of quinolones in rearing of broilers. Due to the pyramidal structure of broiler breeding, QREC isolates may be disseminated from grandparent animals down through the pyramid. However, quinolone resistance can also develop in wild type *E. coli* through specific chromosomal mutations, and by horizontal acquisition of plasmid-mediated quinolone resistance genes. The goal of this study was to determine whether QREC is disseminated through the broiler breeding pyramid or developed locally at some stage in the broiler production chain. For this purpose, we whole genome sequenced wild type- and QREC isolates from broiler and parent flocks that had been isolated in the Norwegian monitoring program for antimicrobial resistance in feed, food and animals (NORM-VET) between 2006 and 2017, from 22 different production sites. The sequencing data was used for typing of the isolates, phylogenetic analysis and identification of relevant resistance mechanisms. Highly similar QREC isolates were identified within major sequence types from multiple production sites, suggesting dissemination of QREC isolates in the broiler production chain. The occurrence of potential resistance development among the WT *E. coli* was low, indicating that this may be a rare

phenomenon in the Norwegian broiler production. The results indicate that the majority of the observed QREC at the bottom of the broiler production pyramid originates from parent or grandparent animals. These results highlight the importance of surveillance at all levels of the broiler production pyramid and of implementation of proper biosecurity measures to control dissemination of QREC.

1 Introduction

Quinolones and fluoroquinolones, hereafter collectively referred to as quinolones, are vital antimicrobials included in the World Health Organization list of essential medicines (WHO, 2019), and are regarded as last-line antimicrobials in both human and veterinary medicine. Use of quinolones confers a selection pressure that results in enrichment of a resistant subpopulation of bacteria. In line with this, use of quinolones has been linked to increased occurrence of resistant bacteria in both human and veterinary sectors (Teuber, 2001; Terahara and Nishiura, 2019). Quinolone resistance most often develop in bacteria as a result of spontaneous chromosomal mutations in the quinolone resistance determining region (QRDR) of the genes encoding DNA gyrase or topoisomerase IV (Gosling et al., 2012; Hooper and Jacoby, 2015). Resistance can also develop from mutations of regulatory elements resulting in reduced influx or increased efflux of quinolones (Tavío et al., 1999; Kern et al., 2000) or through acquisition of plasmid mediated quinolone resistance (PMQR) determinants, including *qnr*, *oqxAB*, *qepA* or *aac(6')-Ib-cr* (Gosling et al., 2012; Machuca et al., 2014, 2016; Yamasaki et al., 2015). Additionally, PMQR determinants have been shown to coexist with resistance genes causing resistance towards other antimicrobials, which enables co-selection (Huang et al., 2012; Slettemeås et al., 2019). Quinolone resistance frequently develop in a stepwise fashion, where a single mutation in *gyrA* is often the initial step (Huseby et al., 2017). Additional mutations in either the same gene or other potential quinolone resistance genes, e.g. *parC* or *marR*, can confer increased resistance towards quinolones, but can also be associated with a fitness cost (Marcusson et al., 2009). However, some mutation combinations have been shown to increase both relative fitness and resistance levels, suggesting that resistant mutants may have an advantage whether quinolones are present or not (Marcusson et al., 2009; Huseby et al., 2017).

Quinolone resistance in *E. coli* have been monitored through the Norwegian monitoring program for antimicrobial resistance in feed, food and animals (NORM-VET) since the start in 2000. A selective method for detecting quinolone resistant *E. coli* (QREC) was implemented in 2014 (NORM/NORM-

VET, 2014). Using this selective method together with traditional screening for quinolone resistance among commensal *E. coli*, QREC was detected at low levels in a high proportion of samples from broiler flocks. Since quinolones are not used in Norwegian broiler production, this raised the question why QREC is a common finding in the Norwegian broiler population.

The Norwegian broiler production has a pyramidal structure, with the purebred pedigree at the top, breeding animals (parent and grandparent animals) in the middle, and meat-producing broilers at the bottom, as illustrated in Mo et al. 2014 (Mo et al., 2014). Day-old grandparent animals are imported from Scotland or Germany to Sweden. Eggs from grandparent animals are imported to Norway and hatched to become parent animals, which lay eggs that become broilers. There is no contact between broiler flocks at the bottom of the pyramid. QREC can, as indicated by our previous study (Kaspersen et al., 2019), be introduced to the production pyramid by breeding animals and then be disseminated clonally down the production pyramid. Another possibility is that QREC develop from wild type (WT) *E. coli* at different locations within the production pyramid. Here, WT *E. coli* may either be disseminated from higher in the breeding pyramid to several production sites and subsequently develop resistance, or may develop resistance at a higher level in the pyramid and subsequently disseminate down the pyramid.

In this study, we used comparative genomics to determine whether QREC is disseminated in the broiler breeding pyramid or develops from WT *E. coli*. The aim was to understand if there is an unknown selective pressure in the broiler houses that can, at least partially, explain the observed occurrence of QREC in broilers.

2 Materials and methods

2.1 Study design and isolate selection

E. coli from chicken has been susceptibility tested in the NORM-VET program since it started in 2000. Isolation of *E. coli* has in general been done from fecal, boot swab or cecal samples from broiler chickens on a biannual basis. However, occasionally samples from layer hens and parent flocks have been included in the program. Each flock is only sampled once per year, and only one random *E. coli* isolate has been obtained from each sample.

The isolates used in the present study are a subset of the *E. coli* isolates that have been isolated in the NORM-VET program and have been stored in the biobank of the Norwegian Veterinary Institute. Isolates were included in the present study based on the following criteria: I) the production site had

been sampled at least three times between 2006 and 2017 and II) at least one QREC and one WT *E. coli* had been isolated from chickens originating from each production site in this time period. This selection resulted in a total of 106 isolates from 22 production sites, comprising 41 QREC and 65 WT *E. coli*, sampled in the years 2006, 2014, 2016 and 2017. Broiler flocks were sampled in 2006, 2014, and 2016 (n = 100), whereas in 2017 only parent flocks were sampled (n = 6). In total, each production site was represented by four to eight isolates (Table 1). However, it is not known if the isolates were sampled from the same broiler house each time.

All isolates had been susceptibility tested by the broth microdilution assay as part of the NORM-VET program, either using panels from VETMIC™ (Dep. Of Antibiotics, National Veterinary Institute, Sweden) in the years 2006-2013 or Sensititre® (TREK Diagnostics, LTD.) from 2014. The panels contain different antimicrobial agents, and only the compounds represented in both panels were considered. In addition to ciprofloxacin and nalidixic acid, the panels included ampicillin, tetracycline, gentamicin, chloramphenicol, trimethoprim, cefotaxime, and sulfamethoxazole. In this study, isolates with a minimum inhibitory concentration (MIC) value >0.06 mg/L for ciprofloxacin and/or >16 mg/L for nalidixic acid were defined as QREC, according to epidemiological cut-off (ECOFF) values defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[1]. Isolates with MIC below these values are referred to as WT.

2.2 DNA extraction and sequencing

QREC isolates were plated onto MacConkey agar with ciprofloxacin (0.06 mg/L) to confirm resistance, while WT isolates were plated onto MacConkey agar. Following incubation at 41.5 °C for 21 hours, bacteria were harvested directly from the agar plates and DNA was extracted with the QIAmp DNA mini kit (QIAGEN), according to the manufacturer's instructions. The DNA concentration and purity was determined using a Qubit (QIAGEN) and NanoDrop ONE spectrophotometer (Thermo Scientific), respectively. Gel electrophoresis was used to determine the DNA integrity.

A total of 95 isolates were sequenced in this study, using Nextera DNA Flex library preparation (Illumina) followed by sequencing on HiSeq X (Illumina) spiked with PhiX. The remaining 11 isolates were previously sequenced using Nextera XT and HiSeq 2000 (n = 4) or HiSeq 2000R (n =

[1] www.eucast.org, ECOFFs as of 01.08.2019

3), or Nextera DNA Flex and HiSeq 3000 (n = 1) or HiSeq X (n = 3). Library preparation and sequencing was done at the Norwegian Sequencing Centre[2].

2.3 Quality control of raw reads

All fastq files were quality controlled by fastQC[3] version 0.11.7. Mash (Ondov et al., 2016) version 1.1 was used to identify contaminants in the fastq files, by using a database of all complete bacterial genomes downloaded from RefSeq. Significant contaminants were defined as hits to other bacteria than *E. coli* with an identity value above 0.95. Residual PhiX (accession number NC_001422.1) was removed with bbduk[4] version 38.20 with a k-mer size of 31, followed by Trimmomatic (Bolger et al., 2014) version 0.38 to trim low-quality nucleotides using the NexteraPE-PE adapter file, a minimum length setting of 36 and a sliding window of 4:15.

2.4 MLST and resistance mechanism identification

Antimicrobial resistance gene identification by assembly (ARIBA) was used for multi-locus sequence typing (MLST), with the scheme hosted by EnteroBase (Wirth et al., 2006). Genomes with novel or uncertain sequence types (STs) were uploaded to EnteroBase for ST assignment.

Mutations in chromosomal genes related to quinolone resistance and plasmid mediated resistance genes were identified with ARIBA using the MEGARes (Lakin et al., 2017) and ResFinder (Zankari et al., 2012) databases, respectively. For the chromosomal genes, only mutations in the QRDR of *gyrA*, *gyrB*, *parC* and *parE* that led to amino acid substitutions in each encoded protein were included. For the plasmid mediated genes, all genes in the ResFinder database were included in the analysis. An R script[5] was used to filter the results based on flags reported by ARIBA to ensure high quality of the predicted variant or gene.

2.5 Assembly, annotation and pan genome analysis

[2] www.sequencing.uio.no

[3] https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed 29.09.2019

[4] https://jgi.doe.gov/data-and-tools/bbtools/ accessed 29.09.2019

[5] https://github.com/hkaspersen/VAMPIR, commitid 54d687a (12th of May)

SPAdes (Bankevich et al., 2012) version 3.12.0 was used to assemble the trimmed reads with "coverage cutoff" set to auto in addition to the "careful" setting. To maximise coverage, both the paired and singleton reads from Trimmomatic were used. Assemblies were error corrected with Pilon (Walker et al., 2014) version 1.22 by mapping the trimmed reads back to the assembly with BWA mem[6] version 0.7.17. Quast (Gurevich et al., 2013) version 4.6.3 was used for assembly evaluation. Prokka (Seemann, 2014) version 1.13 was used for gene annotation, with five complete *E. coli* genomes used as an annotation reference (Supplementary Table 1). Roary (Page et al., 2015) version 3.12.0 was used for pan-genome analysis.

2.6 Phylogenetic analysis

To investigate the overall phylogenetic relationship between the isolates, a core gene single nucleotide polymorphism (SNP) tree was calculated. First, SNP sites in the core gene alignment from Roary were concatenated with snp-sites (Page et al., 2016) version 2.4.1. The resulting concatenated SNPs were used in IQ-Tree (Nguyen et al., 2015) version 1.6.8 to create a maximum likelihood (ML) tree. The optimal evolutionary model was selected by using ModelFinder plus (Kalyaanamoorthy et al., 2017) in addition to the ascertainment bias correction (Lewis, 2001). Branch supports were generated with UltraFast bootstrap approximation (Hoang et al., 2018).

Major clades (n > 4) that were represented by either quinolone resistant isolates only, WT isolates only, or both, were further analyzed separately. First, ParSNP (Treangen et al., 2014) version 1.2 was used to align the pilon-corrected assemblies and identify core genome SNPs. The resulting alignment was format converted by using Harvesttools (Treangen et al., 2014) version 1.2. Then, Gubbins (Croucher et al., 2015) version 2.3.2 was used to remove recombinant sites in the multifasta alignment by using RAxML as treebuilder with the GTRGAMMA model. IQTree was subsequently used to calculate a ML tree from the resulting alignment, using the same settings as described above. All phylogenetic trees were visualized in R using ggtree (Yu et al., 2017). STs that contained both WT and QREC isolates were analyzed in regards to genome similarity using ParSNP.

2.7 Data management

[6] http://bio-bwa.sourceforge.net/, accessed 29.09.2019

Figures and tables were generated in and data management was done using R version 3.6.2 (RCoreTeam, 2018).

3 Results

3.1 Resistance patterns and mechanisms

Depending on the year of sampling, the isolates had previously been tested against one of two different panels of antimicrobials in the NORM-VET program. The resistance pattern of the isolates included in this study was summarized for each of the nine antimicrobials that were included in both panels (Supplementary Table 2). Overall, a low occurrence of resistance was observed for all tested antimicrobials except against ciprofloxacin and nalidixic acid. All QREC isolates were resistant to ciprofloxacin and nalidixic acid, 12% were resistant to ampicillin and sulfamethoxazole, 10% to trimethoprim, 7% to tetracycline and 2% to chloramphenicol. Resistance to gentamicin or cefotaxime was not observed. For the WT isolates, resistance towards ampicillin and sulfamethoxazole was observed in 9% of the isolates, 6% were tetracycline resistant, 3% were trimethoprim resistant and 2% were cefotaxime resistant. All WT isolates were susceptible to chloramphenicol and gentamicin.

Amino acid substitutions in the QRDR of GyrA were only observed in QREC isolates (Table 2), all of which had the S83L substitution. Two QREC isolates had an additional D87N substitution in GyrA. No substitutions in the QRDR of GyrB was observed among the QREC isolates. Some QREC had additional amino acid substitutions in ParC or ParE (Table 2). Four WT isolates had substitutions in the QRDR of either GyrB, ParC or ParE.

PMQR genes were not detected in any of the isolates, but plasmid mediated resistance genes conferring resistance to other antimicrobials were detected (Supplementary Table 3). The most abundant plasmid mediated resistance genes among the QREC and WT isolates were *aph3Ib* (9.8% and 3.1%), *aph6Id* (9.8% and 3.1%), $bla_{\text{TEM-1b}}$ (9.8% and 4.6%), *sul2* (7.3% and 4.6%), *dfrA5* (4.9% and 1.5%), *tetA* (4.9% and 4.6%) and *aadA1* (2.4% and 3.1%). Overall, the genotype corresponded to the observed phenotype, except for the *aph* and *aadA* genes, since gentamicin resistance was not observed in the isolates.

3.2 Sequence type diversity and phylogenetic analyses

In total, 37 different STs were detected among the 106 isolates. There were 31 different STs among the 65 WT isolates, and 13 different STs among the 41 QREC isolates (Table 2). Seven different STs contained both quinolone resistant and WT isolates, namely ST752, ST10, ST602, ST191, ST355,

ST117 and ST115 (Figure 1). ST10 and ST5825 represented the major STs for WT isolates, while ST349 and ST355 represented the major STs for QREC isolates (Figure 1).

The number of isolates and unique STs varied from year to year (Table 3). In 2006 each identified ST only consisted of a single isolate. Four major STs (ST10, ST349, ST355 and ST5825) were identified in 2014 and constituted 57% of the isolates for that year, whereas in 2016, ST10 alone accounted for 41% of the isolates. Both the number of isolates and unique STs were reduced in 2017 when parent flocks were sampled. No ST was overrepresented among these isolates. Only one production site had QREC and WT isolates belonging to the same ST (ST10, Table 1).

The four major clades (n > 4), illustrated as A – D in Figure 1, were further investigated with higher resolution phylogenetic methods. Clade A (Figure 2) consisted of ST10 (*n* = 22), ST752 (*n* = 3), and ST9424 (*n* = 1) from 15 different production sites. Most of these isolates were isolated in 2014 and 2016, and one in 2006. Most of the ST10 isolates clustered together in the topmost clade, all of which were WT isolates. As demonstrated by subclades 1- 3, phylogenetically related WT isolates were detected from different production sites and years. In addition, two QREC ST10 isolates from the same year but different production sites (Subclade 4 in Figure 2) were seen. Clade C was represented by 15 ST355 isolates from 2014 (n = 12), 2017 (n = 2) and 2006 (n = 1), from 12 different production sites (Figure 3). A majority of the isolates from 2014 (Figure 3, grey box) were separately analyzed in regards to shared genome fraction, and shared 92.5% of their genomes. These isolates had a median SNP distance of 13. The tree topology in clade B (Supplementary Figure 2) and D (Supplementary Figure 3) were judged to be uncertain due to low bootstrap values. Therefore, specific isolates within the trees were not compared, only the tree as a whole. Clade B was represented by ten ST5825 WT isolates from nine different production sites from 2014 (n = 7) and 2016 (n = 3). These shared 91.7% of their genomes and had a median SNP distance of 18. Finally, clade D was represented by eight QREC isolates of ST349, all isolated in 2014 from four different production sites. Here, a median SNP distance of nine was calculated, and the isolates shared 92.4% of their genomes.

In the seven STs containing both WT and QREC isolates, the two most similar WT and QREC isolates were compared with regards to resistance mechanisms, shared genome, and SNP distances based on the core gene alignment (Table 4). The lowest core gene SNP difference (40 SNPs) was observed between the ST191 isolates, which shared 84.2% of their genomes. Similarly, in ST355 the

WT isolate and the closest QREC isolate had a core gene SNP difference of 66, and shared 84.2% of their genomes.

4 Discussion

This is the first study using phylogenetic methods to compare both QREC and WT isolates from the Norwegian broiler production chain isolated under the auspices of the NORM-VET program. Here, we identified phylogenetically related QREC isolated from geographically distant production sites, indicating vertical dissemination of QREC in the broiler breeding pyramid. Our data also suggest potential rare sporadic development of quinolone resistance in WT isolates at different locations in the broiler production chain. Taken together, our data and the previously reported low-level occurrence of QREC in a high proportion of samples suggest that any unknown selective pressure, if present, is a minor contributor to the total occurrence of QREC observed in the broiler production chain.

In regards to SNP distances, isolates of the same ST from the same production site seemed to be more often phylogenetically related than isolates of the same ST from different production sites. ST355 and ST349 formed major clades of QREC in the phylogenetic tree in the present study. The relatively high similarity of isolates sampled from different production sites within these two STs (Figure 1, Table 1) suggests that they have a common origin. Occurrence of highly similar QREC ST355 isolates has recently been reported from Iceland and Norway in a study comparing ESBL and QREC isolates from the broiler production chains of Iceland, Norway and Sweden sampled in 2011 – 2014 (Myrenås et al., 2018). Furthermore, there were also highly similar QREC isolates of ST349 from Sweden and Norway (Myrenås et al., 2018). Since Norway and Iceland both import eggs from Sweden that subsequently become parent animals in the respective countries (Myrenås et al., 2018), this strongly suggests that QREC of ST349 and ST355 have been disseminated from a higher level in the broiler breeding pyramid in this time period. It is noteworthy that QREC of ST349 and ST355 were not detected in samples from the broiler houses in 2016. However, the sample set consisted of only three QREC from this year, and we cannot conclude if this is a trend or sampling bias. Interestingly, while internally related, the ST355 isolates from 2014 marked in grey in Figure 3 were all phylogenetically distinct from the ST355 isolates from parent animals in 2017.

Findings of ST349 and ST355 QREC isolates in the broiler production environment in several Nordic countries indicate that they are highly successful clones. The quinolone usage among terrestrial livestock in these countries is low (EMA, 2019). This indicates that the presence of the substitutions

detected among these isolates may provide a fitness benefit, even in the absence of quinolones. However, this fitness benefit may also be attributed to the QREC lineage itself rather than the specific mutation. All QREC isolates from both STs were found to have the S83L substitution in GyrA, while the ST355 isolates in addition have the D475E substitution in ParE. Isolates with only the S83L substitution have previously been linked with increased fitness (Machuca et al., 2015; Huseby et al., 2017; Wang et al., 2017), which may explain the apparent success of these lineages. The substitutions identified in ParE among the ST355 isolates does not seem to affect the MIC value towards ciprofloxacin and nalidixic acid, as the ST355 and ST349 QREC isolates had the same MIC values.

Occurrence of highly similar isolates of WT ST10 in 2006, 2014 and 2016 suggests that this ST was introduced into the broiler houses from a common source and have persisted in broiler flocks. This is supported by the close clustering of isolates from the same location sampled in different years. QREC isolates of ST10 that were sampled in 2012 – 2014 in Sweden and 2014 in Norway were predicted to have a potential common origin (Myrenås et al., 2018). Interestingly, two QREC isolates of ST10 were identified in our samples from 2014. These isolates clustered separately from most of the ST10 WT isolates (Figure 2). This supports the notion that the WT ST10 had been introduced separately a long time ago and have persisted in the broiler houses. It is similarly also possible that ST5825 with highly similar isolates sampled in 2014 and 2016 have persisted in the broiler houses, and may also suggest that these STs were (re)-introduced from parent animals.

Wild type *E. coli* and QREC isolates were compared phylogenetically to identify possible development of quinolone resistance among WT *E. coli.* Overall, we regarded the genetic distance between the QREC and WT *E. coli* belonging to the same STs as too high to assume a recent common ancestor, based on previous thresholds (Jagadeesan et al., 2019). However, one QREC/WT isolate pair of ST191 had a relatively low genetic distance (40 SNPs) based on the core gene alignment generated with Roary, were isolated eight years apart, and were from different production sites. Under relatively stable conditions with no apparent selective pressure, *E. coli* have been predicted to develop approximately 80 SNPs over a period of 20 years, given a low rate of horizontal transfer and recombination (Tenaillon et al., 2016). Thus, a difference of 40 SNPs between the ST191 QREC and WT isolates may be expected over eight years, and indicates phylogenetic relatedness. However, the two isolates only shared 84.2% of their genomes. Horizontal gene transfer and recombination over time may account for this difference. It should be mentioned that the SNP

distances mentioned above is based on the alignment of the 2931 core genes. Deeper phylogenetic analysis covering a larger portion of the genomes of the ST191 isolate pair is needed to conclude if these isolates indeed are phylogenetically related. This is evident in the investigated ST355 isolate pair, where 66 SNPs were detected using the core gene alignment described above, while 100 SNPs were detected in the core genome alignment used to create the tree in Figure 3. Although the environment of the broiler houses provide relatively consistent environmental conditions, there is a high turnover of animals and regular disinfection and washing between insets. One possible explanation for the development of quinolone resistance in WT isolates observed in our data may be exposure to such cleaning and disinfection agents. Exposure to disinfectants and detergents have previously been shown to induce stress responses in *E. coli*, which, among other things, may result in mutations in the QRDR of *gyrA* (Buffet-Bataillon et al., 2016). Consequently, stress-driven evolution of a persistent ST191 population may have resulted in development of quinolone resistance in WT isolates. However, conclusions on where this potential resistance development happened is impossible to draw based on our current data. It is therefore unknown if the WT isolate was disseminated before developing resistance, or developed resistance at a higher level in the broiler production pyramid and was subsequently disseminated as QREC down the pyramid. Taken together, the occurrence of resistance development among WT *E. coli* was low in our data. This indicates that such development of resistance is a rare phenomenon in the broiler production environment. As such, our results indicate that *E. coli* are (re)introduced into the broiler houses by dissemination through the breeding pyramid and that some STs can persist in this environment. Given our contention that QREC are mainly disseminated vertically in the broiler breeding pyramid is true, these findings can be confirmed by further investigating QREC from parent flocks.

Wild type isolates with substitutions in GyrB, ParC or ParE were identified. These substitutions have previously been described (Komp Lindgren et al., 2003; Saenz, 2003), and the S463A substitution in GyrB has been identified in *Klebsiella oxytoca* (Lascols et al., 2007). The presence of these substitutions in WT *E. coli* suggest that they alone are not enough to gain a quinolone resistant phenotype. No PMQR determinants were identified in any of the included isolates. This finding is in concordance with previous studies, where a very low occurrence of PMQR were reported (Börjesson et al., 2015; Myrenås et al., 2018), and suggests that PMQR may be a rare finding in the breeding animals that are imported from Scotland or Germany to Sweden. However, some plasmid mediated genes conferring resistance towards cefotaxime, ampicillin, trimethoprim, tetracycline, sulfamethoxazole and chloramphenicol were identified in the present study with low occurrence,

mostly in QREC isolates. The presence of these genes in QREC isolates may indicate the possibility of co-selection with the use of other antimicrobial compounds. However, antimicrobial usage in the Norwegian broiler production is very low (NORM/NORM-VET, 2018), and it is unlikely that this is the explanation for the occurrence of QREC in Norwegian broilers. The levels of quinolone usage in the grandparent production in Scotland or Germany is however currently unknown. Thus, conclusions based on potential selection of quinolone resistance at the highest levels of the broiler breeding pyramid cannot be drawn.

This study identified major QREC lineages of phylogenetically related isolates across multiple broiler production sites, suggesting vertical dissemination of quinolone resistance in the broiler breeding pyramid. The seemingly low occurrence of quinolone resistance development among WT *E. coli* together with the fact that QREC are found at low levels in a high proportion of samples, suggest that there is no major unknown pressure selecting for quinolone resistant bacteria. Instead, our data indicates that the major contributor to QREC occurrence in the broiler production chain is dissemination of strains originating from parent or grandparent animals. Measures to control occurrence of QREC in broilers should therefore be focused on the higher levels of the broiler breeding pyramid.

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7 Author contributions

AU, MN, KL, RS, CS, JS and HK conceptualized and designed the study. HK, EF, KL and RS analyzed the data. EF and KL advised and assisted the phylogenetic analysis. HK, KL, and RS wrote the main body of the article. All authors contributed to manuscript revisions, interpretation of results, and manuscript approval.

8 Conflict of interest

None to declare

9 Data availability statement

The datasets analyzed for this study can be found in the European Nucleotide Archive (To be uploaded).

10 Ethics statement

The study has been conducted in accordance with the Frontiers guidelines on study ethics, but did not encompass animal or human trials. Ethical approval from a committee was not relevant as the study only involved bacterial strains.

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514 **Table 1:** Number of sequence types per phenotype and location. Location: Broiler production site.
515 Numbers in parentheses denote number of isolates for STs with more than one isolate.

	Sequence types		
Location	Quinolone resistant	Wild type	Total
A	162	10, 442, 5825	4
B	131, 349	10, 1286	4
C	162	355, 2040, 9424	4
D	355, 641, 4994	10, 5375	5
E	155	10, 1286, 5825	4
F	117	328, 5825, 9427	4
G	162	10 (3)	4
H	355	10 (2), 5825	4
I	355	48, 189, 1730, 5825	5
J	349 (2)	648, 1266	4
K	349, 355, 752 (2)	10, 4537, 5825 (2)	8
L	131*, 355 (2)*, 355	117*, 189, 9425*, 9426*	8
M	349	10 (3), 1266	5
N	131, 349	1286, 5825	4
O	10, 349 (2)	756, 1266, 2178, 5375	7
P	355 (2)	115, 3107	4
Q	131, 355	1594, 5825	4
R	10, 355	10 (2), 5825	5
S	355	10 (2), 191	4
T	355, 602	10, 1056	4
U	191, 355	10, 752, 1251, 6726	6
V	115, 162	10, 69, 602	5

516 * Parent flocks

517 **Table 2**: Number of isolates with the respective amino acid substitution in GyrA, GyrB, ParC and
518 ParE per phenotype.

Protein	AA Substitution	Quinolone resistant	Wild type
GyrA	D87N	2	0
	S83L	41	0
GyrB	S463A	0	2
ParC	S57T	0	1
	S80I	4	0
ParE	A512T	1	0
	D475E	14	1
	L488M	1	0

519

520 **Table 3:** Number of sequence types per year of isolation and phenotype. The columns "*n*" denote the
521 number of unique STs for each phenotype per year. The "Sum" column denote the total number of
522 unique STs per year. "*n* isolates" denote the number of isolates per year. Numbers in parentheses
523 denote number of isolates for STs with more than one isolate. Major STs are denoted in bold.

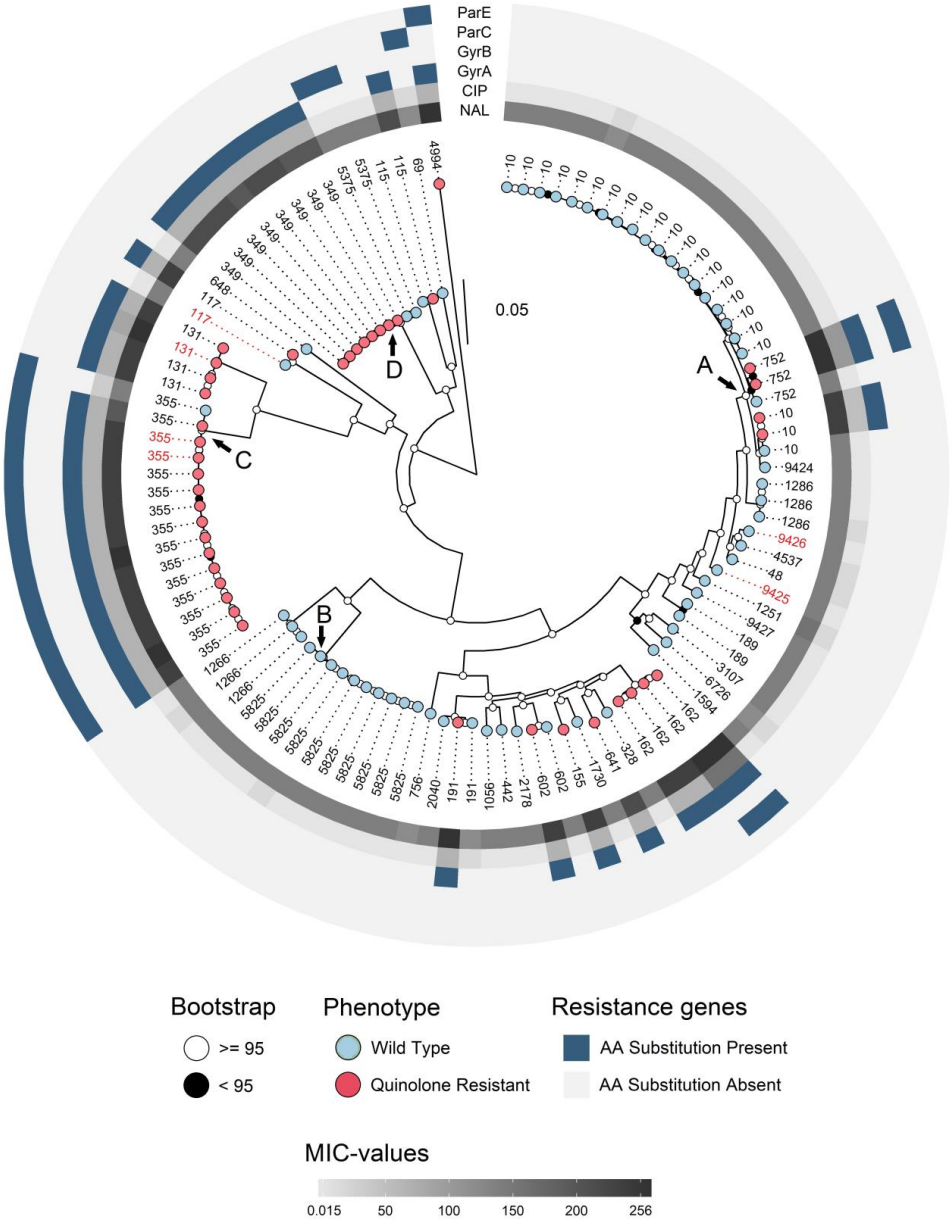
	Sequence types					
Year	Quinolone resistant	*n*	Wild type	*n*	Sum	*n* isolates
2006		0	10, 48, 69, 191, 355, 756, 1251, 1286, 4537, 6726	10	10	10
2014	**355 (12), 349 (8),** 162 (4), 131 (3), **10 (2),** 115, 117, 155, 191, 602, 4994	11	**10 (10), 5825 (7)**, 5375 (2), 115, 189, 442, 602, 752, 1056, 1266, 1286, 1594, 2040, 2178, 3107, 9424, 9427	17	25	68
2016	752 (2), 641	2	**10 (9), 5825 (3),** 1266 (2), 189, 328, 648, 1286, 1730,	8	10	22
2017*	**355 (2),** 131	2	117, 9425, 9426	3	5	6
Total		15		38	50	106

524 * Parent flocks

Table 4: Overview of quinolone resistant *E. coli* and wild type *E. coli* isolate pairs of the same sequence type. Two isolates are included for each ST, where the topmost isolate in each row is quinolone resistant and the bottom one the wild type isolate. Amino acid substitutions are listed in columns "GyrA", "ParC" and "ParE". The shared genome is calculated based on analysis with ParSNP on the two genomes, with results listed as "Clade shared genome (%)". If the clade was comprised of only the wild type and quinolone resistant isolate, this value is based on the shared genome between those two isolates only. The "SNP distance" column represents the SNP distances between the two respective isolates derived from the core gene alignment. CIP MIC = ciprofloxacin minimum inhibitory concentration. Location represents production site.

ST	GyrA	ParC	ParE	Year	Clade shared genome (%)	SNP distance	CIP MIC	Location
752	S83L	S80I		2016	83.4 [a]	2634 [d]	1	K
				2014			0.015	U
10	S83L			2014	80.1 [b]	635 [e]	0.12	R
				2014			0.015	S
602	S83L			2014	86.4	1795	0.25	T
				2014			0.015	V
191	S83L			2014	84.2	40	0.25	U
				2006			0.03	S
355	S83L		D475E	2014	85.4 [c]	66 [f]	0.25	Q
			D475E	2006			0.06	C
117	S83L			2014	88.2	2950	0.25	F
				2017*			0.015	L
115	S83L			2014	85.2	397	0.25	V
				2014			0.015	P

[a] For closest resistant and sensitive isolate: 84.0%
[b] For closest resistant and sensitive isolate: 82.0%
[c] For closest resistant and sensitive isolate: 84.2%
[d] Core genome SNP distance: 104
[e] Core genome SNP distance: 254
[f] Core genome SNP distance: 100
* Parent flock

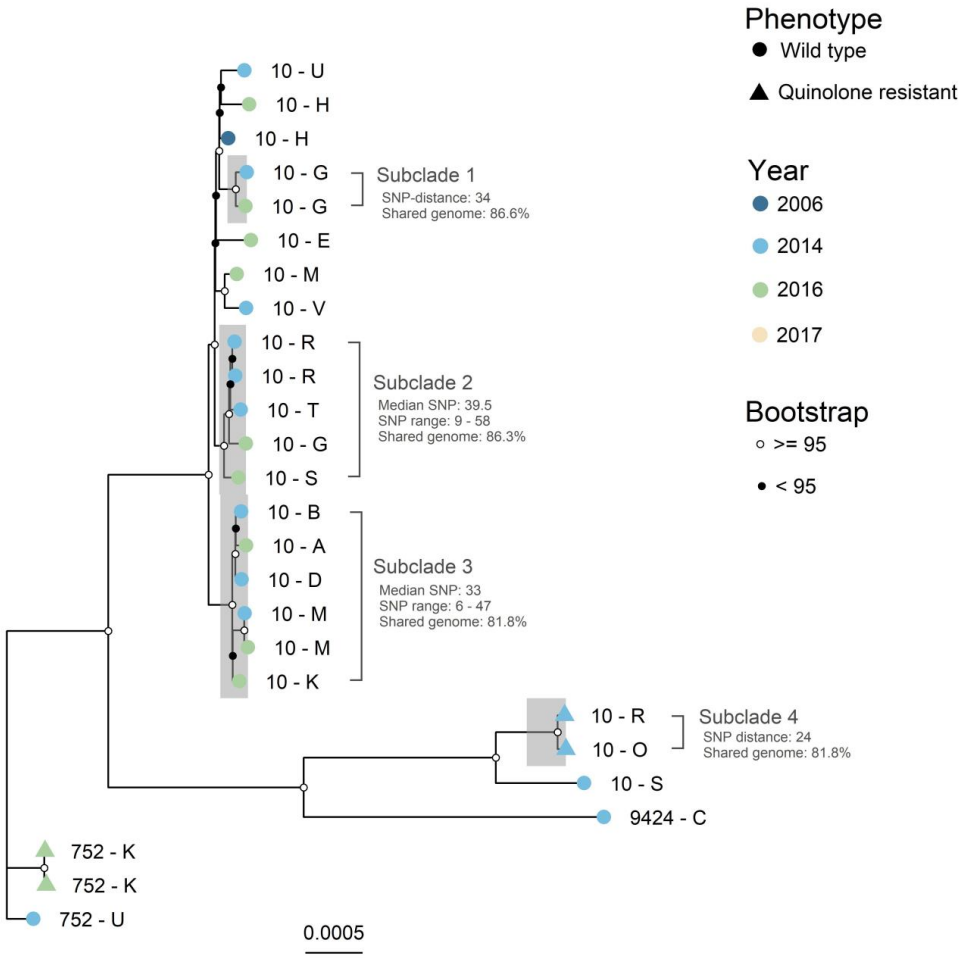


542

543

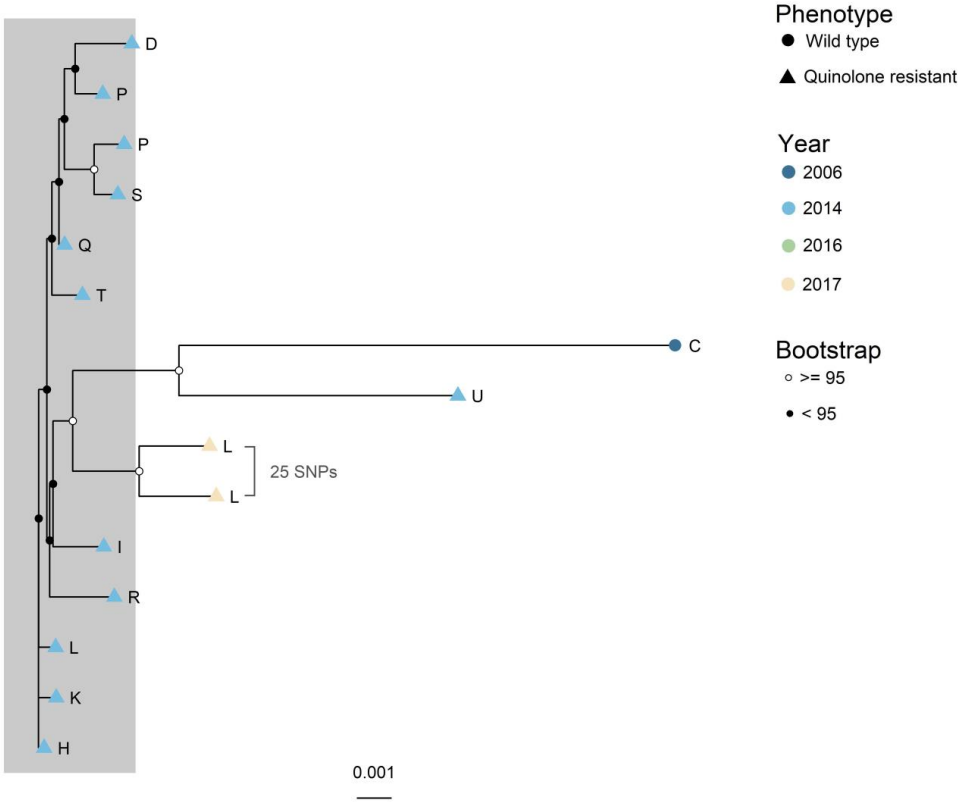
Figure 1: Maximum likelihood SNP tree calculated with IQTree, based on the 2931 core genes identified with Roary. The tree is midpoint rooted for better visualization. Bootstrap values are represented as black (< 95) and white (>= 95) circles on the nodes. Phenotype is represented as blue (wild type) and red (quinolone resistant) on the tip points. Sequence types are denoted as tip labels, red labels represent parent animals. MIC-values for ciprofloxacin (CIP) and nalidixic acid (NAL) are represented as increasing grey color in the innermost circles. Amino acid substitutions in the four genes related to quinolone resistance is denoted as blue in the surrounding circles. Arrows denote clades further investigated. Evolutionary model: GTR+F+ASC+R5.

553



554

555 **Figure 2**: Maximum likelihood core gene SNP tree generated with IQTree for Clade A. Core genome
556 SNPs were identified with ParSNP, and recombinant sites were removed with Gubbins. Phenotype is
557 represented by the tip point shapes, and year of isolation represented by the tip point color. Bootstrap
558 values are represented as black and white circles on the internal nodes. Tip labels represent the ST
559 (number) and production site (letter) of each isolate. Subclades of interest are highlighted in grey.
560 Evolutionary model: TVMe+ASC+R2. Total shared genome: 78.3%.



561

562 **Figure 3**: Maximum likelihood core gene SNP tree generated with IQTree for clade C (ST355). Core
563 genome SNPs were identified with ParSNP, and recombinant sites were removed with Gubbins.
564 Phenotype is represented by the tip point shapes, and year of isolation represented by the tip point
565 color. Isolates from 2017 were from parent flocks. Bootstrap values are represented as black and
566 white circles on the internal nodes. Especially similar isolates are marked with a grey box. These had
567 a median SNP distance of 13 and shared 92.5% of their genomes. Evolutionary model:
568 K3P+ASC+R2. Total shared genome: 85.4%.

569

Supplementary Material

1 Supplementary Data

Supplementary Data Sheet 1: Total data on all included isolates (See Excel sheet). Information includes year of isolation, production site of origin, phenotype, minimum inhibitory concentrations values, sequence types, amino acid substitutions in GyrA, GyrB, ParC and ParE, and all detected plasmid mediated resistance genes.

2 Supplementary Tables and Figures

2.1 Supplementary Tables

Supplementary Table 1: Complete *E. coli* genomes used with Prokka. The table presents the complete reference genomes used when annotating the draft genomes. These were selected as references since they are regarded as highly curated *E. coli* genomes, and were complete assemblies.

Accession number	Information
GCF_000005845.2_ASM584v2	*E. coli* K12
GCF_000008865.2_ASM886v2	*E. coli* O157:H7 Sakai
GCF_000026345.1_ASM2634v1	*E. coli* IAI39
GCF_000183345.1_ASM18334v1	*E. coli* O83:H1 NRG 857C
GCF_000299455.1_ASM29945v1	*E. coli* O104:H4 2011C-3493

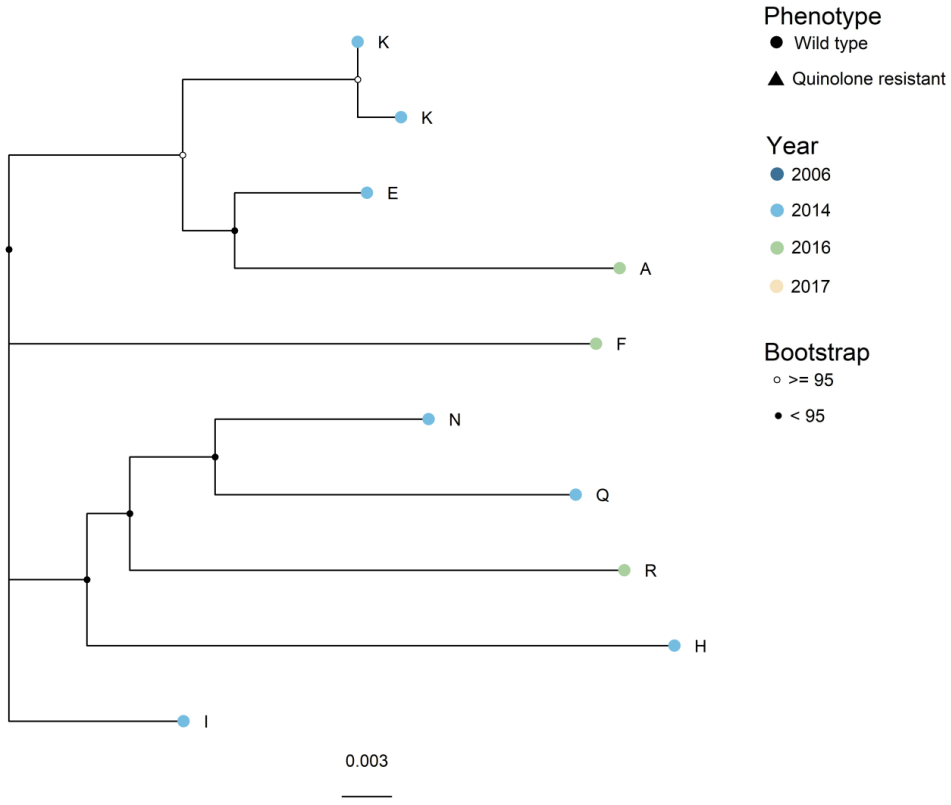
Supplementary Table 2: Resistance patterns among included isolates. The table presents the percent resistance among the isolates for the antimicrobials CIP = ciprofloxacin, NAL = nalidixic acid, AMP = ampicillin, SMX = sulfamethoxazole, TET = tetracycline, TMP = trimethoprim, CHL = chloramphenicol, CTX = cefotaxime, and GEN = gentamicin.

Antimicrobial	QREC (*n* = 41)	WT (*n* = 65)	Total (*n* = 106)
CIP	100	0	39
NAL	100	0	39
AMP	12	9	10
SMX	12	9	10
TET	7	6	7
TMP	10	3	6
CHL	2	0	1
CTX	0	2	1
GEN	0	0	0

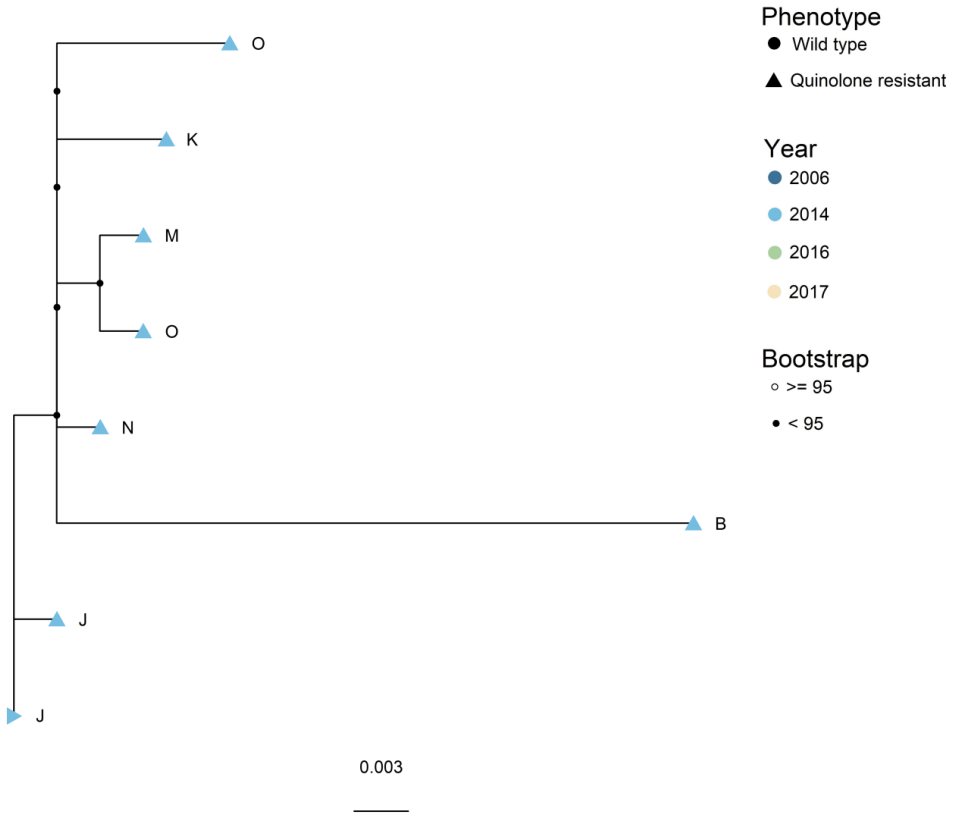
Supplementary Table 3: Occurrence of plasmid mediated resistance genes. The table presents the percent occurrence of all plasmid mediated resistance genes identified among the quinolone resistant (QREC) and wild type isolates.

Gene	QREC (*n* = 41)	Wild type (*n* = 65)
aph3Ib	9.8	3.1
aph6Id	9.8	3.1
$bla_{\text{TEM-1B}}$	9.8	4.6
sul2	7.3	4.6
dfrA5	4.9	1.5
tetA	4.9	4.6
aadA1	2.4	3.1
aadA5	2.4	0.0
bla_{CMY2}	2.4	1.5
catA1	2.4	0.0
dfrA1	2.4	1.5
dfrA17	2.4	0.0
sul3	2.4	0.0
tetB	2.4	1.5
$bla_{\text{TEM-1A}}$	0.0	1.5
$bla_{\text{TEM-220}}$	0.0	1.5
dfrA14	0.0	1.5
fosA7	0.0	1.5
sul1	0.0	1.5

2.2 Supplementary Figures



Supplementary Figure 1: Maximum likelihood core genome SNP tree generated with IQTree for clade B (ST5825). Core genome SNPs were identified with ParSNP, and recombinant sites were removed with Gubbins. Phenotype is represented by the tip point shapes, and year of isolation represented by the tip point colour. Bootstrap values are represented as black and white circles on the nodes. Median SNP distance for whole tree: 18 SNPs, with a range of 1 – 28 SNPs. Evolutionary model: K2P+ASC. Shared genome: 91.7%.



Supplementary Figure 2: Maximum likelihood core genome SNP tree generated with IQTree for clade D (ST349). Core genome SNPs were identified with ParSNP, and recombinant sites were removed with Gubbins. Phenotype is represented by the tip point shapes, and year of isolation represented by the tip point colour. Bootstrap values are represented as black and white circles on the nodes. Median SNP distance for whole tree: 9 SNPs, with a range of 2 – 37 SNPs. Evolutionary model: TIM3e+ASC. Shared genome: 92.4%.

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