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Characterization of Relationships Between Fluoroquinolone-Resistant *E. coli* from Humans, Dogs, and Dairy Cattle Living in South West England

Oliver Jon Mounsey

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of PhD in the Faculty of Biomedical Sciences

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Abstract

Escherichia coli are a common component of the mammalian gut microflora. *E. coli* are also frequent intestinal and extraintestinal pathogens, which cause diarrhoea, urinary tract infection, cystitis, pyelonephritis, sepsis, and meningitis, in humans, as well as mastitis, in dairy cattle, and severe respiratory and systemic disease in chickens, and which frequently carry multiple antibacterial resistance (ABR) genes. This project sought to investigate the transmission of fluoroquinolone resistant (FQ-R) *E. coli* between three important reservoirs – humans, dairy cattle, and companion animals (16-week-old dogs) within a 50 x 50 km study region in the South West of England. To do this, *E. coli* isolated from faecally-contaminated near-cattle environments, human urine samples submitted for diagnostic microbiology, and puppy faeces, were subjected to detailed molecular epidemiology investigation. Risk factors for the carriage of (FQ-R) *E. coli* in dogs and dairy cattle were also investigated.

Isolates from all three reservoirs were analysed by multiplex PCR and a subset were subjected to whole genome sequencing (WGS). This enabled a thorough analysis of ABR and virulence gene carriage, sequence typing (ST), and mutations in the quinolone resistance determining regions (QRDR) of *gyrA/B* and *parC/E*. Whole-genome sequencing also enabled single nucleotide polymorphism variant calling for SNP-distance and phylogenetic analysis.

Significant associations were found in the human urinary isolates between carriage of genes associated with ABR (*aac(6')Ib-cr*, bla_{OXA-1}, $bla_{CTX-M-15}$, *tet(A)*, and *aac(3)-IIa*) and virulence (*cnf1*, *iss*, and *nfaE*). Raw feeding was identified as a significant risk factor for carriage of FQ-R *E. coli* in dogs. Fluoroquinolone use on a farm was positively associated with the odds of finding FQ-R *E. coli* in faeces from cattle suggesting that reducing use may lead to a reduction in FQ-R in dairy farms. Dry cow therapy – antibacterial injected into the udder – was found to be negatively associated with FQ-R *E. coli* in dairy cattle, and we showed that this was likely to be because FQ-R *E. coli* are rarely resistant to the antibacterial agents found within dry cow therapy. Hence, dry cow therapy could be manipulated to reduce the prevalence of other types of resistance.

Most importantly, closely related FQ-R *E. coli*, differing by <30 SNPs, were found in all three reservoirs, which is suggestive of sharing between dogs, humans, and dairy cattle within the study region. This was only true for certain STs, particularly important are ST162 and ST744.

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Finally, thank you to my wonderful fiancée, Shona Thomson, for your love and support, and for accepting to do all of the housework in the runup to my submission deadline (I promise I will make it up to you).

Dedication

I dedicate this thesis to the memory of my grandma, Nancy Lovell, who always believed in me.

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: DATE: 21/05/2018

Publications

Mounsey O, Wareham K, Hammond A, Findlay J, Gould VC, Morley K, et al. Evidence that faecal carriage of resistant Escherichia coli by 16-week-old dogs in the United Kingdom is associated with raw feeding and that E. coli from these dogs are shared with humans and cause opportunistic infections. bioRxiv. 2021:2021.04.17.440283.

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Schubert H, Morley K, Puddy EF, Arbon R, Findlay J, **Mounsey O**, et al. Reduced Antibacterial Drug Resistance and blaCTX-M β-Lactamase Gene Carriage in Cattle-Associated Escherichia coli at Low Temperatures, at Sites Dominated by Older Animals, and on Pastureland: Implications for Surveillance. Applied and Environmental Microbiology. 2021;87(6):e01468-20.

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List of Abbreviations

3GC	3rd generation cephalosporin
ABU	Antibacterial usage
ABR	Antibacterial resistance
AIEC	Adherent invasive *Escherichia coli*
AMR	Antimicrobial resistance
APEC	Avian pathogenic *Escherichia coli*
BSI	Bloodstream infection
CIP	Ciprofloxacin
CLIMB	Cloud Infrastructure for Big Data Microbial Bioinformatics
CLSI	Clinical and Laboratory Standards Institute
CTX	Cefotaxime
DAEC	Diffuse Adherent *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended spectrum β-lactamase
ETEC	Enterotoxigenic *Escherichia coli*
EUCAST	European Society of Clinical Microbiology and Infectious Diseases
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
FQ	Fluoroquinolone
GTR	General time-reversible
NJ	Neighbour-joining
NMEC	New-born Meningitis *Escherichia coli*
PMQR	Plasmid-mediated quinolone resistance
QRDR	Quinolone resistance determining region
RAxML	Random ‘Axelerated’ Maximum-Likelihood
SEPEC	Sepsis-associated *Escherichia coli*
SNP	Single nucleotide polymorphism
STEC/EHEC	Shigellatoxigenic *Escherichia coli* / enterohaemorrhagic *Escherichia coli*
TBX	Tryptone bile X-glucuronide
UPEC	Uropathogenic *Escherichia coli*
WGS	Whole-genome sequence/sequencing

1. Introduction

1.1. The project in context

This thesis is an extension of work carried out as part of the One Health Selection and Transmission of Antimicrobial Resistance (OH-STAR) consortium project. This was a multidisciplinary group endeavour which sought to estimate the transmission of *Escherichia coli* resistant to a range of different antimicrobials between farmed animals (dairy cattle), companion animals (dogs), and humans within a defined geographical region centred on Bristol. Furthermore, to define the behavioural risk factors associated with finding resistant *E. coli* in these three compartments. *E. coli* isolates have been obtained from three distinct reservoirs: dog faecal samples from a national survey, including additional recruitment from dogs located close to Bristol; near-animal environment dairy cattle samples collected on farms in the South West; human urinary isolates sourced by Severn Pathology, a regional diagnostic laboratory located at Southmead hospital, in Bristol. The work reported in this thesis will focus primarily on resistance to one critically important class of antimicrobials, fluoroquinolones, with some additional analysis of 3rd generation cephalosporin (3GC) resistance, which came about as part of a collaboration.

OH-STAR, and so the work reported in this thesis, exclusively studied *E. coli*, a facultative anaerobe and common human commensal organism found in faecal microbiota, and a strong indicator of faecal contamination in the environment (1). *E. coli* are not only present in significant amounts in the faeces of the majority of mammals but are also the most common gut colonising bacterium to cause opportunistic infections in humans, including surgical site infections, urinary tract infections (UTI) and bloodstream infections (BSI), causing 40,000 instances of bacteraemia between 2014 and 2015 (2, 3). *E. coli* are also an important reservoir of antimicrobial resistance (AMR) genes and, relevant to this project, antibacterial drug resistance (ABR) genes, capable of transferring resistance horizontally between strains and into other disease-causing coliform bacteria, including *Salmonella* spp., *Klebsiella* spp. and *Shigella* spp. (4). Whilst *E. coli* are typically the most populous aerobic commensal Gram-negative organism in the mammalian gut, other bacteria, particularly *Bacteroides spp.*, are more abundant in the human gut microbiome than *E. coli* but do not have the same relevance to disease and are not as persistent or stable outside of the gut, due to their obligate anaerobicity (5). Other gut flora known to persist outside of the body and cause infection include other members of the Enterobacterales order, especially but not exclusively members of the Enterobacteriaceae family, such as *Klebsiella spp.*, *Salmonella spp.* and

Shigella spp. (6); *Campylobacter spp.*, especially *Campylobacter monocytogenes* (7); and Enterococcus sp., especially *Enterococcus faecium* and *Enterococcus faecalis* (8).

1.2. Escherichia coli

E. coli was discovered by Theodor Escherich and initially named *Bacterium coli commune* in a presentation in Munich to the Society for Morphology and Physiology, in 1885 (9), and not officially renamed until 1958 (10). At a glance, the organism does not appear to be particularly interesting. It is a typical member of its taxonomic family, Enterobacterales; most strains do not typically cause disease and it grows well on a variety of media, though not at extreme temperatures or pH. However, whilst none of its characteristics make it stand out as exceptional, the ease with which it can be cultured and the ubiquity with which it is found are what have made it such a popular organism to work with over the past 135 years (11) and led it to become one of humanity's most studied organisms (11, 12).

The Enterobacterales family initially diverged between 300-500 million years ago (13), leading to the contemporary formation of six distinct clades, and one pseudo-clade yet to be resolved (14). One of these clades, termed the *Escherichia* clade, contains the genera, *Citrobacter*, *Escherichia*, *Salmonella,* and *Shigella*. Outside of its complex relationship with *Shigella*, the next closest relative of the *Escherichia* genus is *Salmonella*, which diverged from *Escherichia* approximately 140 million years ago (15). This divergence and the subsequent diversification of these two important groups of organisms may have coincided with the emergence of birds (16, 17), and mammals (18). Perhaps then the proverbial early bird caught not just the worm but also gastroenteritis.

The *Escherichia* genus later gave rise to distinct species, which share a common ancestor thought to have lived approximately 60 million years ago and which gave rise to all extant *E. coli* 19-31 million years ago (19). The highly pathogenic *Shigella* spp. are, from a phylogenetic perspective, subspecies of *E. coli*, having emerged in three groups between 35,000-270,000 years ago and converging phenotypically (20). They remain, nonetheless, closely related to *E. coli* and indeed some strains of *E. coli* are more closely related to *Shigella* spp. than they are to other strains of *E. coli* (21).

In the time since *E. coli* emerged, it has spread across the globe and colonised the guts of most warm-blooded species, as well as many fish (22) and reptiles (23), and is also known to be able to persist for long periods of time in temperate soils (24). It is sometimes claimed that *E. coli* are found in all mammals (25). However, a study of Australian vertebrates found *E. coli* in the faeces of only 56% of mammals (and 23% of birds). Conversely, it was found in 90% samples from humans. The study identified taxonomic rank, diet, climate, and body

mass all to be predictive indicators of the carriage of *E. coli*, with an omnivorous diet, temperate climate, and larger body mass each having the strongest positive association with carriage and a carnivorous diet, hot climate (tropical or desert), and low body mass each having the strongest negative association (26).

The spread of *E. coli* has coincided with massive diversification (27, 28), leading to the situation we see today, in which the pan-genome of extraintestinal pathogenic *E. coli* (ExPEC) alone comprises over 16,000 genes (29). The pan-genome includes all non-orthologous genes found in a given species, it comprises both the core-genome (found in all wild strains of a species) and the accessory genome (found in some strains but not all) (28, 30). This has come about due to a high propensity for sharing accessory genes – an attribute of which *E. coli* has been found to be exceptional and which may be the secret to its ubiquitous nature (30).

It could be argued that the large degree of variety seen within the *E. coli* species is evidence that it should be considered more than one species or more definitively broken into subspecies (ignoring for a moment the issue of the *Shigella* groups within the *E. coli* clade). However, the variation seen within closely related clades makes this difficult to justify (31) and would inevitably lead to a situation in which the members of some species or subspecies are more similar to other species or subspecies than they are to organisms with which they share a common ancestry (30). And despite the wide diversity of genes across the *E. coli* pan-genome, a detailed comparison of two distantly related *E. coli* strains – the common lab strain, K12, and the highly pathogenic O157:H7 – reveals a remarkably conserved core genome of over 4 Mb, with 98.31% conserved identity (32). Ultimately, though, it must be acknowledged that, as things stand, the distinction between bacterial taxonomic groups is a human convention for human convenience. The phylogenetic depth of taxa ranges wildly across the bacterial domain (33) and some species are placed in the middle of other taxons, based on phenotypic difference, as is the case for *Shigella* spp.

1.3. Methods for grouping *E. coli* isolates

Some researchers have sought to order *E. coli* into different groups. There are multiple methods for differentiating strains of bacteria, which can broadly be divided into phenotypic and genotypic methods (34). Phenotypic differentiation involves separating different strains on the basis of one or more observable traits and, from a pragmatic perspective, is appealing, as it enables a simple discrimination between important characteristics, such as virulence factors, antigen presentation or ABR (35). However, methods for defining these phenotypes tend to be laborious and time consuming and often fail to distinguish between

different strains or even species, particularly when the result is polyphyletic grouping of distantly related strains which share traits due to horizontal gene transfer (36).

Genotypic analysis is more suitable for determining how closely related multiple organisms are and typically utilises one of three techniques: DNA hybridization, “fingerprint” analysis, and direct sequence analysis. DNA hybridisation is a technique in which a fragmented DNA sequence is hybridised to an array of fluorescently labelled oligonucleotide probes, whilst fingerprint analysis sees a fragmented DNA sequence separated by fragment size, usually by agarose gel or capillary electrophoresis. Direct sequence analysis offers the highest potential for accuracy but usually requires the sequencing and comparison of multiple genes or even whole genomes (34).

RAPD (random amplified polymorphic DNA) PCR is a method of DNA fingerprint analysis, in which segments of genomic DNA are amplified with non-specific primers in low annealing temperature PCR (37). The primers reproducibly anneal to random (in the sense that they are not known in advance) sites in the genome and produce a distinct and reproducible banding pattern when the resulting PCR products are subjected to agarose gel electrophoresis. Theoretically, the binding positions of primers to the genome varies between different bacteria so that different bacteria produce different banding patterns (38, 39). RAPD can be very useful as a fast and simple way of differentiating between bacteria and was used in this project to differentiate isolates cultured from a single sample. However, its sensitivity to initial conditions – e.g. concentration of dNTPs and template DNA, and exact polymerase used – make it a poor objective measure of strain relatedness, and identical banding patterns are not diagnostic of clonality in the absence of other tests (40).

There are many types of direct sequence analysis (34). Some of the most widely used are the multi locus sequence typing (MLST) systems, in which different combinations of alleles of 7-8 housekeeping genes are assigned a sequence type (ST) number (41). MLST can be performed through analysis of whole genome sequence (WGS) data, or using direct PCR sequencing of the genes being analysed following their amplification by PCR. There are three different MLST systems for *E. coli*, which all use a different combination of housekeeping genes, with the isocitrate dehydrogenase gene (*icd*) being the only one that all three systems share in common (42). Of these, the most widely and thus the most reliable for comparison with other studies – and the one used throughout the work reported in this thesis – is the Warwick system, sometimes referred to as the *E. coli* 1 system, which compares the alleles for of the following genes, the products of which are shown in brackets: *adk* (Adenylate kinase), *fumC* (Fumarate hydratase), *gyrB* (DNA gyrase subunit B), *icd* (Isocitrate dehydrogenase), *mdh* (Malate dehydrogenase), *purA* (Adenylosuccinate synthetase), and *recA* (RecA – a protein involved in DNA maintenance and repair) (43).

Another MLST system, the Pasteur Institute scheme – which utilizes the *dinB* (DNA polymerase IV), *icd* (Isocitrate dehydrogenase), *pabB* (aminodeoxychorismate synthase component 1), *polB* (DNA polymerase II), *putP* (Sodium/proline symporter), *trpA* (Tryptophan synthase alpha chain), *trpB* (Tryptophan synthase beta chain), and *uidA* (Beta-glucuronidase) genes – offers greater differentiation between strains but is less commonly used and recognised so was not utilized here (42). The Pasteur system The When higher resolution of within-ST subgroups is required, other options are available, such as *fimH* typing which distinguishes isolates based on *fimH* allele variability (44).

Much work has been done looking at the prevalence of particular *E. coli* STs in different reservoirs and several conclusions can be drawn (29, 45, 46). Isolates of a particular ST tend to occupy a particular niche within a distinct host species or group of related species (29, 47-51). For example, a group of closely related ST131 isolates have emerged over the past decade as the dominant multidrug resistant *E. coli* ST found in human-derived samples across multiple countries and continents (29, 50, 52-55). However, whilst particular STs are of much higher prevalence within particular reservoirs, there are also instances in which those STs have been found outside of their usual host, for example, ST131 has been found to be carried by fruit bats in the Republic of Congo (56) and by poultry in Bangladesh (57). This can also be seen for *E. coli* ST744, which has been found in animals in multiple countries, including, Austria, Germany, Australia and Bangladesh (48, 57, 58), but has also been identified as a human pathogen in Laos (59). Other important STs are discussed in the various results chapters in this thesis.

Whilst MLST systems are useful for grouping together closely related strains, and identifying instances of suspected clonal transfer, the considerable number of STs that have been identified (10,946 as of August 2021 for the Warwick [*E. coli* 1] system (60)) can make it difficult to infer the relatedness of different isolates (refer to phylogenetic tree in Figure 1). Additionally, considerable diversity has been observed within some STs, suggesting a need for more detailed examination (61). The original paper describing the Warwick sequence typing system identified 278 unique STs (43), and this has since been expanded considerably (62). This original paper identified and numbered 'clonal complexes' of related STs, defined as instances in which at least three STs share alleles for two or more of the ST-defining housekeeping genes (43).

As well as individual STs, *E. coli* can be split into a small number of general but distinct clades based on phylogenetic grouping, initially characterised by Selander *et al.* in 1987 (63), and further developed by Herzer *et al.* in 1990 (64). The first tree was divided into three large clades, labelled phylogenetic groups (or phylogroups) A, B and D; with the addition of a small unlabelled clade and the division of the B group into B1 and B2, by Herzer *et al.* (65).

This has since been built upon considerably, particularly by Clermont *et al.*, and there are now recognised seven primary phylogroups – A, B1, B2, C, D, E, F – and an additional cryptic clade (I), which is distantly related to the others but which nonetheless shares the phenotypical characteristics of *E. coli* (Figure 1) (66), with other subgroups also characterised (67). Somewhat confusingly, phylogroups A, B1, C and E are all more closely related to one another than any is to either phylogroup D or B2 (42). *Shigella spp.* are placed in different locations within the *E. coli* phylogenetic tree, depending on how the tree is constructed – usually between or adjacent to phylogroups A and B1 (21, 68, 69) – and the species *Escherichia fergusonii* is placed within clade I (66).

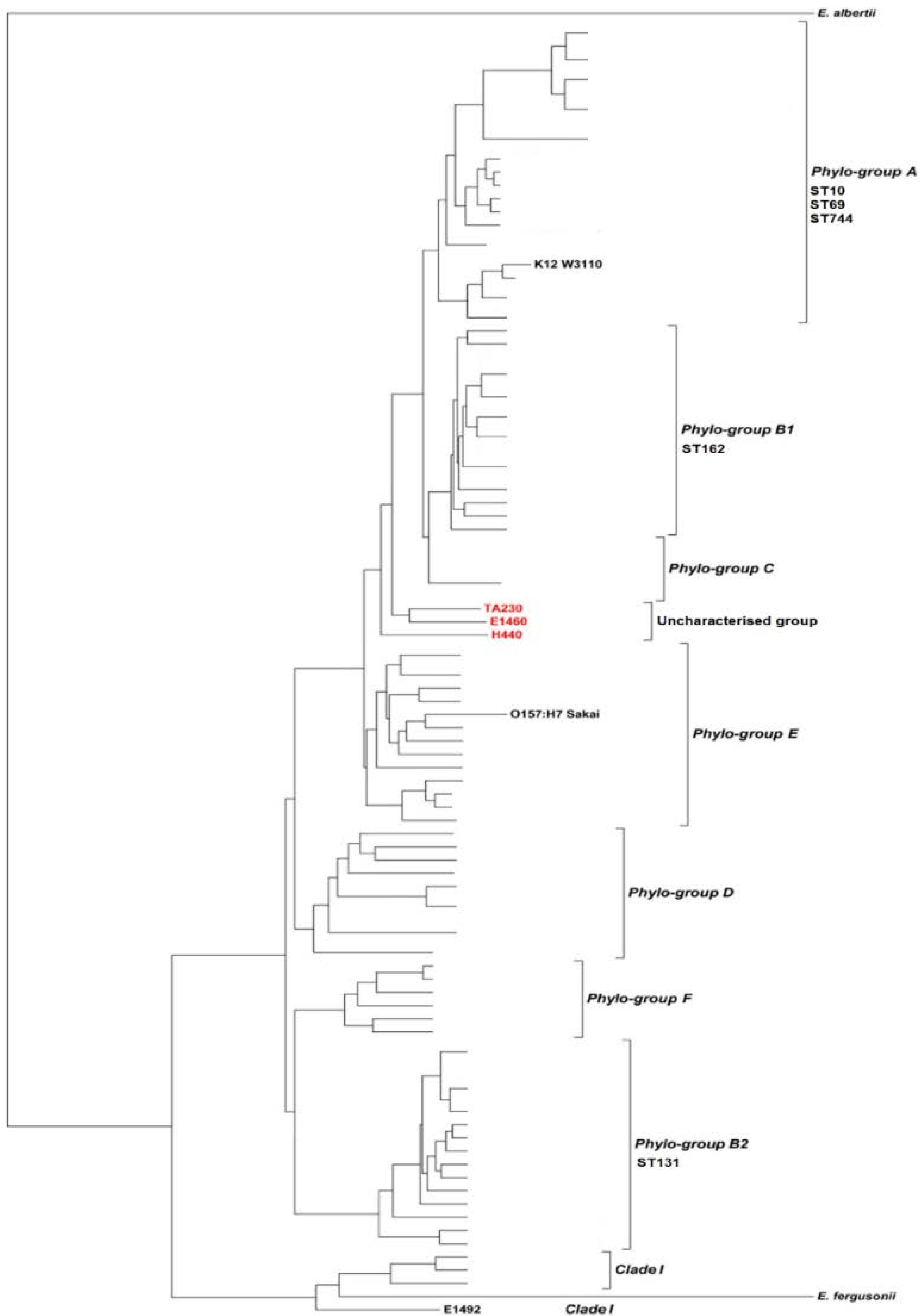


Figure 1. Phylogenetic tree adapted, with permission, from Clermont et al. (2013). The major phylogroups of *E. coli* and with some important strains and STs within those groups highlighted. Also shown, outside of the *E. coli* group, providing a for root the tree, is *Escherichia albertii*.

The placement of an isolate within a particular phylogroup can be determined through sequence analysis by the presence or absence of three genes – *arpA* (Ankyrin repeat protein A), *chuA* (Hemin TonB-dependent receptor) and *yjaA* (Uncharacterized protein YjaA) – and a DNA fragment designated TspE4.C2. These can be detected through a quadruplex PCR (65, 66) or searched for within WGS data. Alternatively, because the phylogroup associated with many STs is already known (29, 43, 50, 62), it is possible to determine from the literature in which phylogroup many STs belong, and to infer a degree of relatedness on this basis. Caution should be taken, however, as there have been documented instances of isolates with the same ST being placed in different phylogroups (29, 42).

1.4. Phylogenetic analysis

An exact degree of relatedness between bacteria can be determined by phylogenetic analysis, whereby the genetic sequences of multiple isolates are aligned and this is used to construct a dendrogram showing degree of relatedness between them, also known as a phylogenetic tree (Figure 1) (70). Genetic recombination can, however, make this troublesome and, although phylogenetic groupings mostly overlap with groupings based on recombinant DNA, there are exceptions. Wirth *et al*. therefore recommend removing recombinant regions to produce consistent phylogenetic trees (43), and that was the approach followed during this project.

From a phylogenetic tree, inferences can be drawn about whether acquired phenotypes have recently arisen and been selected for in bacteria across a range of environments (convergent evolution), or if the mutations were present in some pre-existing organism and have since been propagated and selected for. An example where this question is relevant is the emergence of bacteria carrying DNA topoisomerase mutations that are selected for by the use of fluoroquinolones, which forms a major research question discussed later in this thesis. Phylogenetic analysis also allows closer examination of the relationships between isolates from different STs – illustrating distinct clades and highlighting correlations between clade and, for example, ABR genotype – and provides the potential to calculate approximately how long ago isolates diverged from one another (71).

In order to construct a phylogenetic tree, sequences must first be aligned, and single nucleotide polymorphism (SNP) variants identified. Aligned sequences can then be compared in one of multiple ways in order to determine degree of relatedness. One of the simplest ways is through a basic neighbour joining (NJ) tree, in which the number of base pairs (bp) shared between each sequence is used to determine which sequences are more similar or less similar to one another and a tree is constructed accordingly (72). NJ trees are

a fast and simple way to explore the relationship between multiple isolate sequences. A popular program for constructing an NJ tree is RapidNJ (73). Unfortunately, however, NJ trees lack rigour; there can be multiple ways of constructing a single NJ tree, with no indication as to which most accurately represents the true evolutionary history of the isolates from which the sequences are derived, with most applying only a simple heuristic for estimating the relative frequencies of different base substitutions (70, 72-74).

The earliest model of base substitution assumed equal frequencies of each base substitution (e.g. A>T versus A>G) and therefore gave an equal probability to each type of substitution (75). This was later improved using a model which accounted for the fact that a change from a purine base to another purine, or a pyrimidine base to another pyrimidine, known as a transition, is considerably more likely than the change from a purine to a pyrimidine, or vice versa, known as a transversion; hence, some substitutions occur more frequently than others and this should be factored in when considering evolutionary relationships (74). Over the following decades, many more models of base substitution were proposed and today the generalised time-reversible (GTR) model is considered a standard tool for phylogenetic analysis (76).

GTR uses the frequencies of bases in the aligned sequences to estimate the probability of relationship between two or more sequences (76, 77). This may change in time, however, as the GTR model is not without its faults, and can sometimes yield incorrect results (78, 79). The GTR model assumes that the rate at which base substitution occurs remains constant across different sites in the genome (also known as rate homogeneity), though this is obviously not the case *in vivo*. DNA displays what is known as rate heterogeneity among sites – which is to say the mutation frequency varies across (and between) sequences (80). To overcome this, most models incorporate a discrete gamma (Γ) distribution model, which samples and averages a range of mutation probabilities across a Γ distribution and estimates from this a frequency rate for mutation (81). The alternative to this is to factor in the rate of mutation at each site. However, this raises concerns about overfitting of the data, and is computationally intensive, though some programs offer a compromise, as discussed below (77).

There are many programs available to construct phylogenetic trees; one example, and the one that is utilised here, is RAxML (short for Random ‘Axelerated’ Maximum Likelihood). RAxML constructs phylogenetic trees from aligned sequences using a maximum likelihood based GTR model, incorporating a Γ distribution model to estimate rate heterogeneity (though it also allows other models to be used, this is the default) (77, 82-84). Initially, random sequences are joined to form a starting tree based on parsimony – that is to say, they produce trees which factor in the number of mutations between each branch (84, 85).

The models discussed above are then used to rearrange the branches of the tree based on a likelihood value, or probability, that the new arrangement is representative of how the sequences have diverged. In addition, RAxML also offers, as an alternative to Γ distribution model, the CAT model of rate heterogeneity analysis. This sees mutation rates divided into distinct categories (which 'CAT' is short for) and the rate for each used as a basis for comparing sequences (77).

Finally, the program will then compare multiple trees, potentially hundreds or even thousands of times, and identify those with the maximum likelihood, in a process known as 'bootstrapping' (70, 82, 86). Once constructed, phylogenetic trees are stored as Newick files (.nwk), which can be visualised as dendrograms using other software, such Microreact, as used here (87).

1.5. *E. coli* in the environment

Previous analyses of the species *E. coli* paints a picture of a diverse and gregarious organism which regularly exchanges genetic information with its neighbours – mainly other *E. coli* but also members of other species, particularly within the Enterobacterales family (88). It has been suggested that the tendency of humans to share and trade culture, tools, and information is what has allowed us to thrive (89). And, likewise, the reason for the success and ubiquity of *E. coli* may be its propensity for sharing genetic information, though its ability to colonise the human gut must surely have helped.

E. coli are regularly expelled from their hosts into the environment where they must survive considerable variation in temperature and humidity, as well as exposure to ultraviolet light from the sun, and it has long been known that *E. coli* can survive outside of a host, sometimes for considerable amounts of time and, indeed, they have often been used as species indicative of faecal contamination of the environment. Despite this, until recently it was believed that *E. coli* were reliant on a biological host in order to grow and thrive. More recently, however, it has been demonstrated that some strains of *E. coli* are capable not just of persisting in the environment but of actively colonising it (90, 91). Importantly though, these *E. coli* strains have been found to be distinct from, and are not thought to commonly interact with, their gut-colonising relatives (92). Nonetheless, it is clear that even *E. coli* which are native to the human gut are typically able to persist in the environment for a considerable amount of time, and from there may acquire new hosts. Whilst outside they will likely encounter their environment-restricted relatives and exchange genetic information (19, 91).

1.6. *E. coli* as a commensal organism

The *E. coli* we are most familiar with is that which inhabits the gut. Unlike its cousin, *Salmonella*, which is an intracellular pathogen (93), most *E. coli* strains are either commensal or mutualistic (94). They inhabit the gut and feed off faecal matter as it passes through, sometimes also giving some benefit to their host by providing key nutrients, such as vitamin k, and occupying a niche which might otherwise be taken up by a more deleterious organism (3).

As previously discussed, *E. coli* are widely disseminated and highly diverse. Yet, with some exceptions, this diversity has not led to a branching into large pathogenic and non-pathogenic clades. Whilst some phylogroups are more associated with disease than others, pockets of pathogenicity can be found across the *E. coli* phylogenetic tree (95, 96). Likewise, as will be discussed below, phylogroups do not tend to target a particular host but can pass between and colonise diverse groups of organisms (26). Moreover, *E. coli* animal pathogens have been found to be closely related to isolates found to cause disease in humans (97, 98).

1.7. *E. coli* as a pathogen

It could be supposed that diarrhoea caused by enterovirulent *E. coli* will lead to contamination of the urogenital area and that this will then lead to urinary tract infection (UTI) with the bacterium that caused the gastroenteritis, however this is almost never the case (94). Almost all pathogenic *E. coli* can be divided into three groups: those which do not colonise, but on entry cause disease of the gut (enterovirulent or intestinal pathogenic *E. coli*); those which colonise the gut and are associated with disseminating infection (ExPEC), and those which colonise the gut and cause entirely opportunistic infection, for example, following bowel rupture, through a surgical wound, or via a central venous or urinary catheter (99-101).

Enterovirulent *E. coli* infections are probably what most lay people think of when they hear '*E. coli*', despite the diseases attributed to them being orders of magnitude less common than ExPEC and opportunistic *E. coli* infections in the developed world (99, 102). Infections with enterovirulent *E. coli* are typically transient and self-limiting, though some can be severe and even deadly without treatment. These are often considered outbreak organisms, which manifest as a sudden spike in cases restricted to a particular geographical area and/or source before being contained (103).

There are seven different varieties of enterovirulent *E. coli*, known as pathotypes, defined by the presence of particular virulence factors (Table 1). Infection with *Shigella* spp. displays

many of the same traits and symptoms as enterovirulent *E. coli*, particularly EIEC, and due to its close relation to *E. coli*, could arguably be included in this category, though typically it is not (99, 102, 104). A subset of DAEC strains is an exception to the division between enterovirulent and ExPEC outlined above. This *E. coli* type is associated both with diarrhoeal disease and UTI (102).

Table 1. Types of enterovirulent *E. coli* (102, 104-106)

	Characterisation	Typical presentation
Enteroaggregative (EAEC)	Adhere to Hep-2 cells and don't secrete labile (LT) or stable (ST) enterotoxins.	Watery mucoid, occasionally bloody, diarrhoea.
Enteroinvasive (EIEC)	Very similar and closely related to *Shigella* but typically non-toxin-producing. They can be distinguished from *Shigella* by acetate and mucate fermentation.	Transitive watery diarrhoea and occasionally dysentery.
Enteropathogenic (EPEC)	Attaching and effacing histopathology. Attachment to host Intimate receptor triggers structural rearrangement and effacing of the epithelium.	Watery diarrhoea
Enterotoxigenic (ETEC)	Defined by the production of two toxins, LT and/or ST.	Transitive watery diarrhoea
Shigatoxin-producing/enterohaemorrhagic (STEC/EHEC)	Produces a phage-encoded shiga toxin.	Severe abdominal pain and watery diarrhoea, followed by bloody diarrhoea. Illness is sometimes called haemorrhagic colitis
Diffusely-adhering (DAEC)	A distinct adherence pattern to human Hep-2 and HeLa cells.	Occasionally diarrhoea but often asymptomatic initially. Long-term carriage associated with inflammatory diseases of the gut.
Adherent-invasive (AIEC)	Adhesion to differentiated Caco-2 cells and/or undifferentiated I-407 cells, invasion of I-407 cells, utilize active and microtubule recruitment for uptake, do not have other known invasion determinants, and can survive and replicate within J774-A1 macrophages.	Associated with Crohn's Disease.

ExPEC are facultative pathogens which can infect multiple sites outside of the gut but, in humans, the most common are uropathogenic *E. coli* (UPEC), associated with UTI (96, 107). It is not possible to identify an ExPEC based on genotype alone, as many commensal *E. coli*, and those that cause opportunistic infections carry virulence genes associated with ExPEC (96, 108). Indeed, Tenaillon *et al.* argue that there is no distinction between the

commensal/opportunistic *E. coli* and ExPEC, that the 'virulence genes' associated with ExPEC infections, have actually evolved to facilitate adaptation to a local commensal environment, and that the more a bacterium colonises, the more likely it is to cause opportunistic infection (3). However, whilst it may be difficult to genotypically distinguish between commensal/opportunistic and ExPEC, it is clear that particular virulence genes are disproportionately associated with the progression of certain forms of ExPEC disease (95, 109).

Some ExPEC genes, such as *fimC* and *iroN*, which code for a fimbrial adhesin and siderophore production (109, 110), respectively, are widespread and somewhat evenly distributed among ExPEC associated with different types of disease (109). Whereas others, such as the *iss* gene, which codes for a surface protein that gives serum resistance, is strongly associated with new-born meningitis *E. coli* (NMEC) and avian pathogenic *E. coli* (APEC), and less so with UPEC (111). Another important ExPEC gene, *iha*, which codes for a siderophore receptor (112), is commonly found in UPEC and NMEC isolates, but rarely in APEC (95, 109).

Several toxin genes are also commonly associated with ExPEC and, again, these are more often associated with some types of ExPEC than others. The *astA* gene, for example, which codes for the enteroaggregative *E. coli* heat-stable enterotoxin (EAST-1) is commonly found in APEC, but rarely in UPEC. Conversely, the *cnf* and *sat* genes, which code for cytotoxic necrotizing factor (113) and secreted autotransporter toxin (114), respectively, are commonly found in UPEC and NMEC, but not APEC (95, 109, 114). The latter may also play a role in the development of complicated UTI (114). Others still have been associated with UPEC, APEC and NMEC (109, 115), such as *vat*, which codes for vacuolating autotransporter toxin, and may play a role in the development of sepsis (115).

In humans, BSI leading to sepsis is commonly caused by non-ExPEC colonisers/opportunists. However, around 40% of sepsis begins with colonisation of the urinary tract with UPEC in the community or healthcare setting and the establishment of UTI. From there, *E. coli* can disseminate to other locations causing pyelonephritis (also known as complicated UTI) and more severe disease. Severe, disseminating infections are particularly associated with certain ExPEC types. For example, sepsis (SEPEC), and meningitis, (NMEC) (96), though NMEC has also been observed to enter the bloodstream directly from the gut (116). As is often the case for infectious disease, both the virulence of the pathogen and the vulnerability of the patient are important factors in the likelihood of disease progression and the final prognosis of disease (96).

Isolates from *E. coli* phylogroups B2 and D have been found to be particularly associated with ExPEC but nonetheless many isolates from these phylogroups commensal/opportunists. Indeed, whether ExPEC should be considered facultative or opportunistic pathogens has been subject to some debate. It could be argued that ExPEC are commensal bacteria that have merely happened to find themselves in the wrong place and made a good situation of it. Indeed, the virulence factors mentioned above, which are associated with certain ExPEC types also aid in bacterial competition, survival, and fitness in the gut (96).

Like humans, cats and dogs occasionally contract urinary tract infections and again the principal causative agent is *E. coli* (117). Furthermore, sharing has been noted of UTI-associated *E. coli* between humans and co-habiting dogs and cats has been reported (118). ExPEC also cause disease in food-producing animals, perhaps the most important example being APEC, a major burden on the poultry industry, but *E. coli* also causes a large percentage of bovine mastitis, which has significant welfare and economic implications (119). As discussed above, considerable overlap has been seen in the carriage of genes associated with APEC and with those associated with human UPEC, NMEC and SEPEC (120). *E. coli* mastitis in cattle does not appear to be associated with a specific set of virulence genes and as such might be considered commensal/opportunistic (121).

1.8. Zoonotic transmission

It remains contentious as to how significant in healthcare terms transmission of *E. coli* between food-producing animals and the human population is and by what route this transmission occurs (109, 122, 123). One aim of the OH-STAR project was to compare the specific *E. coli* strains and ABR mechanisms found in cattle versus humans and companion animals (dogs) within a specific geographical area. With the intention of highlighting instances of commonality, which may indicate significant transmission between the three, and potentially factors that increase the risk of transmission occurring, and so potential interventions that might reduce it.

Transmission or sharing of bacteria – especially virulent and ABR bacteria – between animals and humans is a controversial, contested and much debated subject; often associated with debates about to the use of antimicrobials in agriculture, the wider impacts of meat production, and the impacts of farming on the environment (124, 125). Investigations looking for evidence of zoonotic transmission of ExPEC identified two principal sources, pigs and especially poultry through the contamination of meat, likely during slaughter (126).

Transmission and zoonosis of *E. coli* is complicated. Whilst some strains appear to be highly specific to certain animal species, others have shown high degrees of transmissibility (97).

Whilst this is usually framed in the context of animal-derived bacteria contaminating the human population, it is becoming increasingly recognised that bacteria from human populations can be passed to animal populations (reverse zoonotic transmission – also known as zooanthroponosis), that direction of transmission is not always obvious, and that it makes more sense to speak of sharing bacteria between populations (127). This has been seen in *E. coli* when, for example, the now widely discussed ExPEC ST131 strain first emerged in the human population and was also found in a small number of companion animals (128, 129). This new framing is at the heart of the push towards a so-called 'One Health' approach, bringing together environmental, human, and animal health (130).

Whilst the transmission of ExPEC between animals and humans has been identified, it should be remembered that a large amount of serious disease in humans is caused by commensal/opportunistic *E. coli* as discussed above. In this context, acquisition of any *E. coli* by a human from an animal can lead to future problems. Specifically, the issue is that ABR *E. coli* that have evolved on farms might colonise a human and then cause a resistant opportunistic infection in the future. Alternatively, the zoonotic ABR *E. coli* might pass on ABR genes to commensal ExPEC bacteria in the human gut (or even the wider environment) and this might also fuel ABR ExPEC infections in humans. Understanding this issue is a key objective of the OH-STAR project, and the work reported in this thesis.

1.9. Antibacterial drugs and ABR

ABR broadly comes in two forms. A gene coding for a protein which prevents the antimicrobial from reaching its intended target – by, for example, preventing entry into the cell, competing for space at the binding site, or by actively modifying the drug – or a change to the drug's target, or acquisition of an insensitive target, that reduces the drug's effectiveness. The principal mechanisms of resistance to β-lactam and fluoroquinolone antibacterials in *E. coli* provides good examples of each, respectively. β-Lactamase enzymes break apart the characteristic β-lactam ring, thus giving resistance to β-lactams (131), whereas mutations in the type II topoisomerases genes, *gyrA* and *parC*, reduce the binding coefficient of fluoroquinolones to their intended targets, DNA gyrase and Topoisomerase IV, thus giving fluoroquinolone resistance (FQ-R) (132). Transmission of ABR typically refers to the transmission of a resistant organism from one environment or animal host into another (133). However, as was observed above, resistance to many

antimicrobials is derived from genes, which can become mobilised on plasmids, and thus the ABR mechanism itself can be transmitted from one bacterium to another.

Whilst it is not impossible for mutational ABR to be passed from one *E. coli* to another, it has only rarely been reported, as will be discussed later. Conversely, genes which give ABR are often found mobilised on plasmids (45, 134, 135). Analysis of these genes often reveals a widespread distribution in diverse bacteria, their hosts and in many environments owing to ancient origins (136, 137), often arising from a small group of organisms or a single species prior to mobilisation and dissemination (138, 139).

At a glance, the role of selective pressure in the spread of plasmid-mediated ABR genes would seem simple and obvious. Use of a particular antibacterial eliminates competing organisms, including the normal gut flora, which are susceptible owing to a lack of ABR genes, and allows organisms carrying those genes to colonise, proliferate, and spread. If usage of a particular antimicrobial is reduced, then it is possible that the increased fitness cost of producing ABR proteins will lead to ABR bacteria being outcompeted by susceptible ones and rates of ABR falling. However, the situation is considerably more complex than it first appears. The presence of multiple ABR genes on a particular plasmid – including genes which code for mechanisms that resist heavy metals and general antiseptics, including general efflux pumps which mitigate the effects of multiple classes of antimicrobials – means that selective pressure can remain even when use of a particular antibacterial is reduced (140, 141). Furthermore, it is not uncommon for plasmids to possess genes which increase fitness in the absence of antibacterial (sometimes even ABR genes themselves) (142, 143). Despite this, studies aimed at investigating the effects of antibacterial discontinuation on ABR have found some positive outcome in a reduction of ABR to the discontinued drug (144). This underscores the importance of responsible management and stewardship of antibacterials.

1.10. β-Lactams and β-lactamases

Alexander Fleming's penicillin discovery in 1928 hailed the start of the antibiotic age. However, it would not be for another 13 years and the help of numerous collaborators – most notably Howard Florey and Ernst Chain – before the first purified penicillin drugs began clinical use, and it was not until 1946 that they became widely available (145). In the following years, the molecular structure of the penicillin, 6-aminopenicillanic acid, was discovered and the active β-lactam ring identified (Figure 2) (145, 146). The next four decades saw a golden age of discovery and invention for new antimicrobials, including four new subclasses of β-lactam and a multitude of different individual compounds. This came

alongside the elucidation of the β-lactam mechanism of action but also the discovery of a plethora of β-lactamases, enzymes which break apart the β-lactam ring, rendering the compound inactive (145).

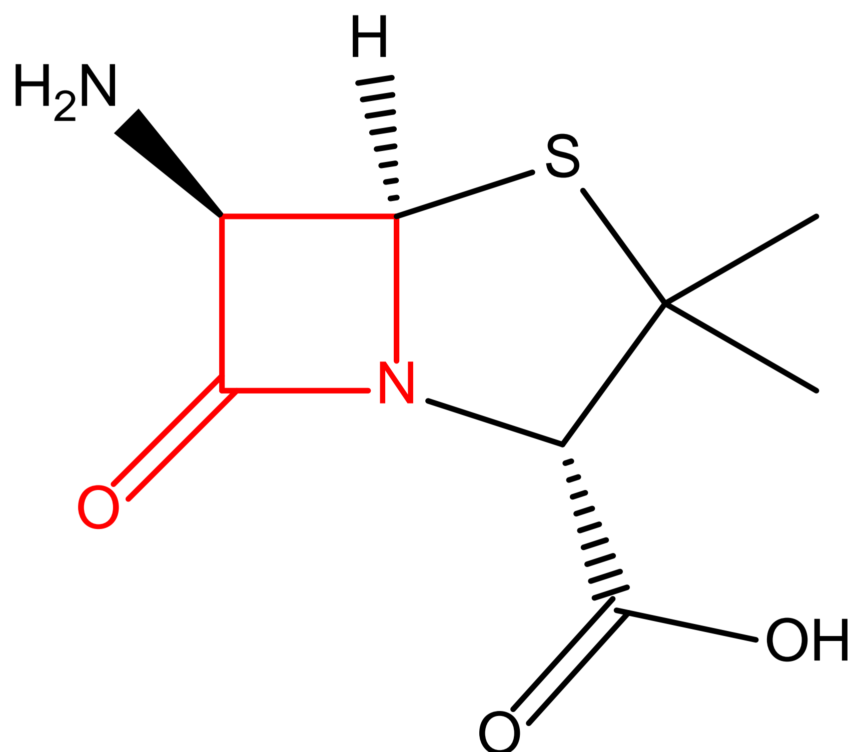


Figure 2. Skeletal formula of 6-aminopenicillanic acid.
The first penicillin structure to be elucidated, drawn in Chemdraw 20.0. The core β-lactam ring, common to all β-lactam antimicrobials, is shown in red. The amino group in the top left is often tagged with additional moieties to give different penicillin compounds and the thiazolidine ring on the right is common to all penicillins, but is modified, usually with different ring structures, to give other classes of β-lactam compound.

Today there are five widely used subclasses of β-lactam, characterised by a secondary ring structure joined to the β-lactam ring (or lack thereof): Penams (penicillins), penems, monobactams, cephalosporins and carbapenems, and many example compounds within each class (Figure 3) (145). All of these are built on the same β-lactam ring core, which mimics the shape of the D-alanyl-D-alanine peptide bond involved in the formation of crosslinks within the bacterial cell wall (147).

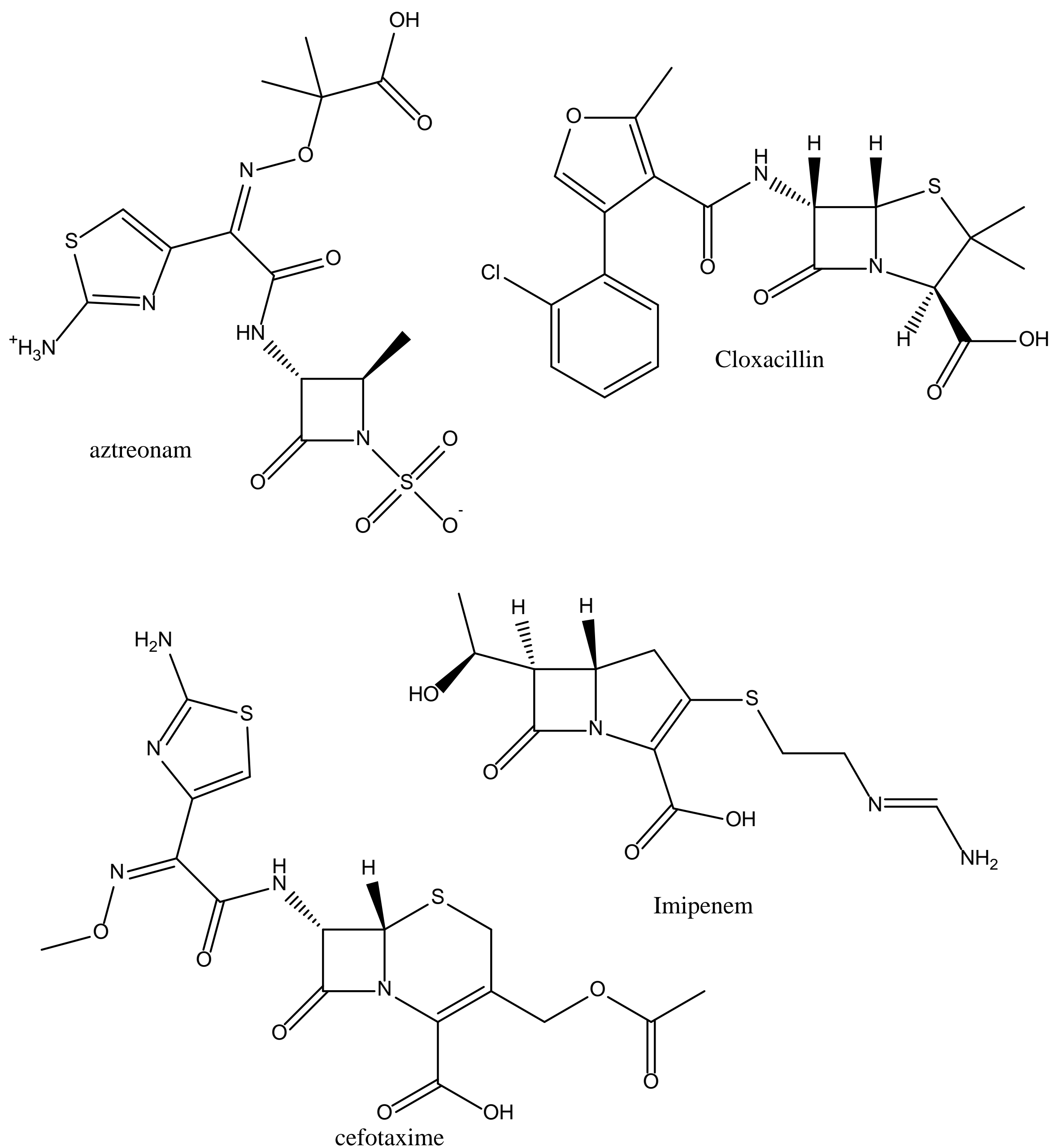


Figure 3. Structures of four important β-lactams.

Each compound represents a member of an important subclass of β-lactam: Aztreonam (monobactams), cloxacillin (penicillins), cefotaxime (cephalosporins), and imipenem (carbapenems). Note the different rings structures attached to the β-lactam ring.

Like multicellular organisms, a bacterium's first form of defence is a protective outer layer. This envelope protects the bacterium from harmful physical, chemical, and biological agents, bacteria have an inner lipid membrane and cell wall composed of peptide-sugar polymer called peptidoglycan, strands of which are crosslinked to increase strength (147). Gram-negative bacteria also have an additional protective (outer) membrane, discussed below,

which β-lactams must cross if they are to have any effect, forming an extra layer of defence (148).

1.11. Mechanism of action of β-lactams

Penicillin-binding proteins (PBPs) are a ubiquitous family of enzymes, found across the bacterial domain, which carry out a range of different functions. One of these functions, transpeptidation, is the crosslinking of penta-peptide sidechains attached to the sugar backbones of peptidoglycan strands, (which are composed of repeating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) subunits), where a penta-peptide chain ends in two D-alanine residues (D-alanyl-D-alanine) (Figure 4). The PBP contains a serine residue in its active site which temporarily forms a covalent bond to the inner D-alanine and releases the outer residue in the process. The remaining D-alanine is then joined to the DAP residue (in Gram negative bacteria such as *E. coli*) in the third position of an adjacent penta-peptide sidechain, replacing the active serine and releasing the transpeptidase PBP, thus crosslinking the two peptidoglycan strands (131).

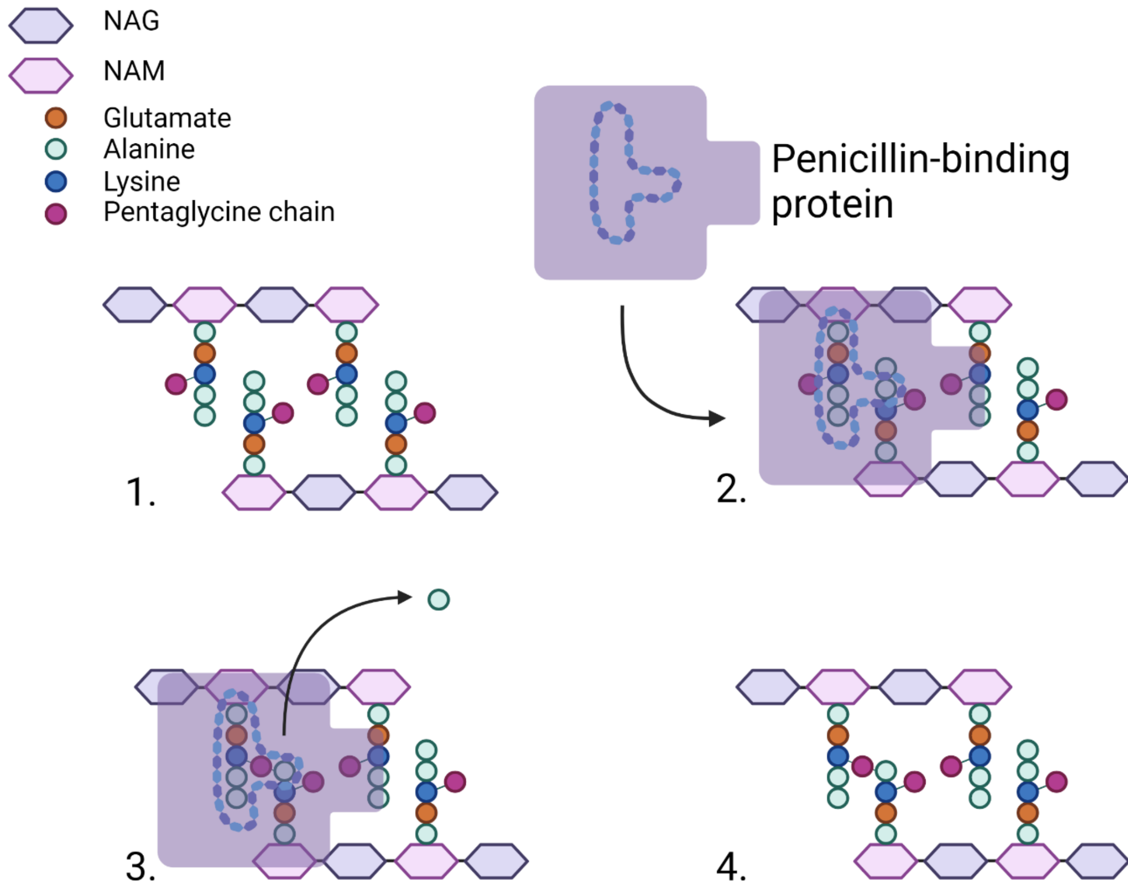


Figure 4. Crosslinking of peptidoglycan chains by penicillin-binding proteins
Peptidoglycan is composed of chains of alternating sugar residues, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). **1.** These chains are initially unlinked, providing little structural rigidity. **2.** Penicillin-binding protein (PBP) attaches to the amino acid chains which protrude from the alternating residues of NAM, targeting the D-alanyl-D-alanine moiety. **3.** PBP facilitates the crosslinking of adjacent amino acid chains by a 5-part chain of glycine residue (pentaglycine) and releases a D-alanine residue from one of the apposed chains. **4.** Crosslinked peptidoglycan. Multiple crosslinks between structures forms a lattice structure. Drawn using Biorender software.

β-Lactams have a similar shape to that of D-alanyl-D-alanine and thus fit into the active site of PBPs. In a β-lactam sensitive organism, the PBP then binds covalently to the β-lactam at its active serine residue, as it would with D-alanyl-D-alanine but is unable to cleave the β-lactam ring efficiently, so it remains in the binding pocket, blocking PBP activity, and thus crosslinking of peptidoglycan strands (131). The bacterial cell wall is under a constant balance of production and disassembly, so when synthesis is inhibited this balance is disrupted and the cell lyses (145). In *E. coli,* different β-lactam compounds have varying

binding affinities for different transpeptidase PBPs, usually inhibiting multiple enzymes making target site mutation usually ineffective as a mechanism of β-lactam resistance (147), whilst in *Staphylococcus aureus* the only target is PBP2, meaning that an alternative PBP (PBP 2a) confers resistance to a range of β-lactam compounds from diverse subclasses (149).

1.12. β-Lactamases

Bacteria have evolved a plethora of defence mechanisms of ABR, some of which are more specific than others. The principal mechanism of resistance to β-lactams are β-lactamases, enzymes which are able to hydrolyse the β-lactam ring and thus disable the compounds' PBP-inhibiting activity. β-Lactamases can be divided into four classes – A to D, based on their sequence homology – according to the Ambler classification (150). Three of these classes, A, C and D are serine β-lactamases, which use a serine residue in their active site like the transpeptidase PBPs, but are very distantly related, having evolved over a billion years ago (151). The fourth class, B, are metallo-β-lactamases, which use zinc ions in their active site to catalyse the hydrolysis of the β-lactam ring (137). Alternatively, β-lactamases are sometimes discussed in terms of their hydrolytic activity against a particular class of β-lactam – especially penicillins (penicillinases), carbapenems (carbapenemases) and cephalosporins (cephalosporinases) – or multiple classes (extended spectrum β-lactamases [ESBLs] hydrolyse penicillins, oxyimino cephalosporins, and monobactams) (152).

Fortunately, in their efforts to identify novel β-lactams, researchers discovered compounds which inhibited β-lactamases. The first of these, clavulanic acid, soon gave way to others, including sulbactam and tazobactam (153). These were found to be particularly effective against the class A β-lactamases, including ESBLs, but less effective against enzymes from classes B, C and D (138, 139, 152). Importantly, whilst some class A variants have been found to be inhibitor resistant, there tends to be a trade-off between inhibitor resistance and the spectrum of β-lactamase activity (152).

Class A β-lactamases are the most common form of plasmid-mediated β-lactamase with TEM β-lactamases being the most prevalent example of this class found in Gram negative bacteria (154). TEM-1 was first described over 50 years ago and is named after the first patient from which an isolate carrying TEM was found (Temoniera) (155). Since that time many variants have been characterised with a range of activities against different β-lactam compounds, including some which are ESBLs. However, the narrow spectrum TEM-1 remains by far the most prevalent, perhaps owing to its higher enzyme activity in hydrolysing the widely used penicillin derivatives than ESBL variants (152).

After TEM, the next most common mobile class A β-lactamases in Gram negative bacteria are SHV and CTX-M. Like TEM-1, SHV-1 has a narrow spectrum of hydrolytic activity. Despite there being fewer SHV variants, however, most identified so far are ESBLs, e.g. SHV-12 (152). SHV is named for the fact that it was initially thought to be a 'sulfhydryl variant' of TEM (155).

The final class A β-lactamase commonly found in *E. coli* is the ESBL, CTX-M, which gets its name from its considerably higher hydrolytic activity against cefotaxime that other cephalosporins, particularly ceftazidime. Unlike TEM- and SHV-derived ESBLs, one of the defining features of CTX-M when it was first discovered was the ability to hydrolyse the 3GC cefotaxime better than other cephalosporins (152). CTX-M is a broad family of enzymes, usually split into six groups, based on sequence homology, each named after the first CTX-M of that group to be identified: group 1, group 2, group 8, group 9, group 25, and group 45. Of particular significance in the UK are CTX-M-1 and CTX-M-15, both of which belong to group 1. Activity against different β-lactams vary between different CTX-Ms but generally they share in common a high affinity for cefotaxime and other smaller-sidechain oxyimino-cephalosporins, and lower activity against bulkier compounds like ceftazidime (156). Whilst the vast majority of CTX-M β-lactamases are ESBLs, CTX-M-54 is not, as it does not confer resistance to the monobactam aztreonam (156, 157).

Metallo-β-lactamases (MBL) can further be divided into three subclasses: B1, B2 and B3, with the first two being more closely related to one another than either is to B3. Like the serine β-lactamases, MBLs are thought to have evolved over a billion years ago (136). Most MBLs are found on the chromosomes of diverse families of bacteria (and one known archaeal species) but three groups, all belonging to class B1, have become mobilised on plasmids and are considered clinically significant: VIM, IMP and NDM, each capable of hydrolysing all classes of β-lactams, except aztreonam, including perhaps most importantly carbapenems, which most β-lactamases cannot hydrolyse (136, 158). Fortunately, prevalence of these mobile MBLs is very low in the UK, with a recent study conducted by Trepanier et al. finding carbapenemase-non-susceptible *E. coli* representing only 47 of 98,444 isolates investigated (0.005%) (158).

Class C β-lactamases, also known as AmpC-type enzymes due to their ability to hydrolyse ampicillin, are a ubiquitous family of enzymes found on the chromosomes of diverse groups of bacteria, which are also sometimes found mobilised on plasmids. They typically have high activity against penicillins and cephalosporins and are only weakly affected by the β-lactamase inhibitors, clavulanic acid, sulbactam, and tazobactam, but are inhibited by and the penicillins, cloxacillin and oxacillin (139).

AmpC production is often regulated by a complex inducer system but this is not the case for *E. coli*. Loss of the system's *ampR* gene means that *ampC* is constitutively expressed but not usually at a high enough level to give clinical resistance. However, mutations particularly in the upstream *ampC* promotor region can give rise to a hyperexpression phenotype which is resistant to cephalosporins and some penicillins (139).

In addition to chromosomal AmpC hyper-production, some *E. coli* can gain cephalosporin resistance through the acquisition of mobile *ampC* genes. Several have been identified but of particular importance to *E. coli* are the DHA (which is named for the hospital in which it was discovered – Dhahran hospital in Saudi Arabia) and CMY (which is named for its conferring resistance to cephamycins) types (139, 159, 160). Like their chromosomally-located counterparts, mobile AmpC β-lactamases typically confer resistance to a subset of cephalosporins and penicillins, but also often aztreonam, with susceptibility to fourth-generation cephalosporins and carbapenems remaining (139).

The final class of β-lactamases, class D, is OXA. Named for their high activity against oxacillin and cloxacillin. Like classes A and C, this broad group of enzymes uses an active serine residue for β-lactam hydrolysis but is nonetheless structurally distinct from these, though some OXA β-lactamases are quite distinct from each other, sometimes sharing as little as 20% sequence identity. (152). As might be expected from this, variants display a range of activity; some are ESBLs, some are carbapenemases, and most are inhibitor-resistant. Whilst typically less common in *E. coli* than other organisms, particularly *Klebsiella pneumoniae*, two variants of OXA show particular clinical importance for *E. coli*: OXA-48, a carbapenemase, and OXA-1, a strict penicillinase which is resistant to clavulanic acid. Both are often found on plasmids with other β-lactamases, including ESBLs (161, 162).

1.13. Quinolones and Fluoroquinolones (and Nalidixic Acid)

The first clinically available 'quinolone' antimicrobial, nalidixic acid, was not actually a quinolone but a naphthyridone (Figure 5). It was discovered 1962 as a biproduct of the synthesis of the antimalarial drug, chloroquine, and nonetheless gave rise to the quinolones and later the fluoroquinolones (163).

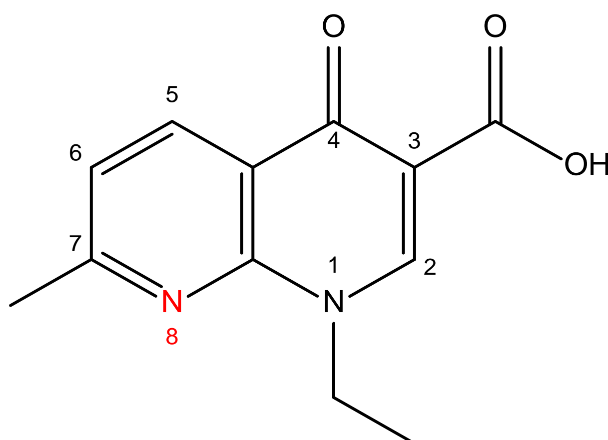


Figure 5. Structure of nalidixic, a 1,8-naphthyridone

Unlike quinolones and fluoroquinolones, nalidixic acid has two nitrogen atoms in its fused ring structure. Drawn in chemdraw 20.0.

Nalidixic acid was found to have a narrow spectrum of activity and was initially almost exclusively used in the treatment of uncomplicated UTI. However, it was later discovered that modifications to the compound – including the addition of a fluorine at the sixth carbon and a piperazinyl moiety at the seventh carbon and changing the 8-nitrogen to carbon – broadened the spectrum of activity and increased efficacy against Gram positive bacteria. These new, second generation, modified quinolones were named fluoroquinolones. The first of these, norfloxacin, began use clinically in the mid-1980's (163, 164).

Further modifications were found to improve activity and expand the range of infections treatable with fluoroquinolones, giving rise to a second class of second generation fluoroquinolones (164). These modifications included an additional carbon to form a cyclo-propyl ring structure at the 1-nitrogen position, producing ciprofloxacin (Figure 6). Other modifications included the fusing of the first carbon in the 1-nitrogen ethyl moiety to the 8-carbon in the core quinolone component with an oxygen atom, to form a more rigid three-membered methoxy ring structure, along with methylating the piperazinyl moiety, producing ofloxacin (165).

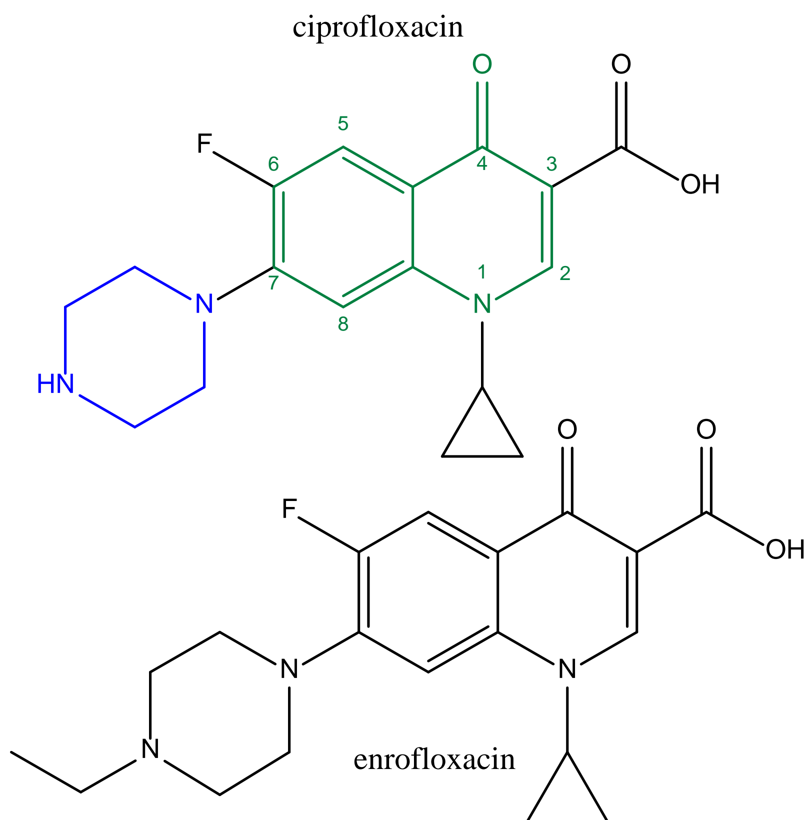


Figure 6. Structures of two commonly used fluoroquinolone antimicrobials.
The core 4-quinolone part of the structure is shown in green, for ciprofloxacin. Note that these structures have a carbon atom at position 8 (shown for ciprofloxacin), whereas nalidixic acid, shown in Figure 5, has a nitrogen at this position. The exposed piperazinyl moiety of ciprofloxacin, which is ethylated on enrofloxacin, is shown in blue. This enables acetylation by the aminoglycoside acetyltransferase, AAC(6')Ib-cr, discussed later. Aside from this difference, these two molecules are otherwise identical. Drawn in chemdraw 20.0.

Ofloxacin is a racemic mixture of two enantiomers, dextrofloxacin and levofloxacin (Figure 7) (166). The purified levofloxacin enantiomer was found to have enhanced activity against particular pathogens, especially *Streptococci*, and is an example of a third generation fluoroquinolone (164). These are characterised by an expanded utility in their treatment indication, especially for their use in treating respiratory tract infections (163, 164). Finally, fourth generation fluoroquinolones, such as trovafloxacin have further enhanced activity against anaerobic and Gram positive organisms (164).

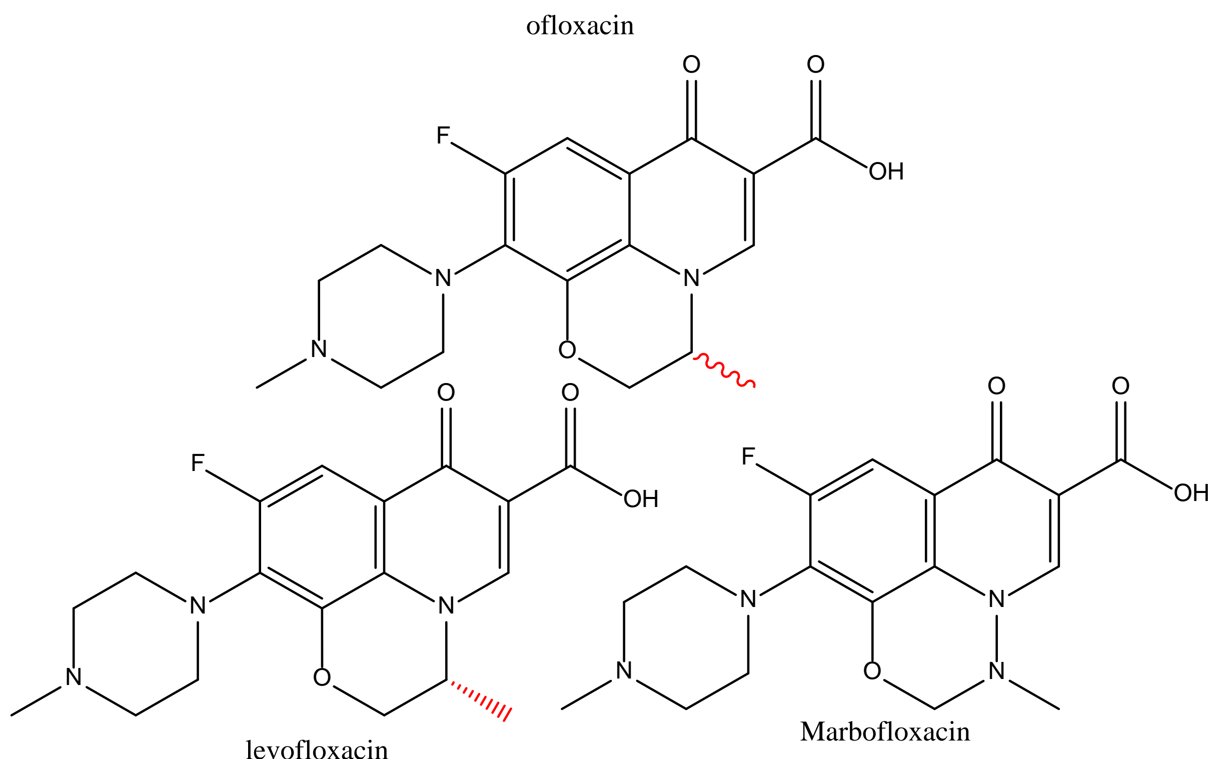


Figure 7. Structures of three more commonly used fluoroquinolones.
Ofloxacin and levofloxacin are identical apart from the wavy bond and dashed bond in each structure, respectively (shown in red on each). Ofloxacin is a racemic mixture, of which levofloxacin is one of its enantiomers. Marbofloxacin is very similar but is only licenced for use in animals, whereas levofloxacin and ofloxacin are only licenced for use in humans. Drawn in chemdraw 20.0.

Five fluoroquinolones are commonly administered to humans in the UK; ciprofloxacin, norfloxacin, ofloxacin, levofloxacin and moxifloxacin (167), though their use as a first-line treatment is currently being restricted (168). There are five fluoroquinolones currently licensed for veterinary use in the UK: marbofloxacin, enrofloxacin, pradofloxacin, danofloxacin and orbifloxacin, with most fluoroquinolone formulations containing either marbofloxacin or enrofloxacin. Pradofloxacin is licensed exclusively for cats and dogs, and danofloxacin exclusively for cattle and pigs. Only a single orbifloxacin formulation is under licence, a suspension for use as ear drops for dogs (169). Enrofloxacin, marbofloxacin and danofloxacin are all second generation fluoroquinolones, whilst orbifloxacin is third generation (170). Marbofloxacin closely resembles ofloxacin, except that the carbon to which the methyl group is attached in the methoxy ring structure is replaced with a nitrogen (Figure 7). This allows only a single orientation for the methyl group, meaning that unlike ofloxacin and its two enantiomers, marbofloxacin is not racemic. Enrofloxacin is a prodrug which

through deethylation is converted to ciprofloxacin. Prior to deethylation, the only difference between the two compounds is an ethyl group attached to the exposed nitrogen on the piperazinyl ring (171, 172).

1.14. Mechanism of action of quinolone antimicrobials

All of these fluoroquinolones share a common mechanism of action, causing bacterial lysis by disrupting the complex formed between DNA and the type II topoisomerase enzymes: topoisomerase IV and DNA gyrase (132). Type II topoisomerases bind to the DNA helix and cut it, allowing the DNA to loop around and pass through the cut, before ligating the two cut strands back together. They thus facilitate the relaxation of positive supercoils and the introduction of negative supercoils, which is important in overcoming the supercoiling generated, for example, by an expanding replication fork during DNA replication (this is a function of DNA gyrase). Fluoroquinolones intercalate with the DNA-topoisomerase complex and inhibit the ligation activity of the enzyme, leading to the release of DNA with double strand breaks and subsequent cell death (173). Whilst fluoroquinolones have been shown to disrupt the activity of both DNA gyrase and topoisomerase IV, the principal target in *E. coli* is gyrase (174).

1.15. Fluoroquinolone Toxicity

Over the past decade or more, it has become increasingly clear that in rare cases fluoroquinolones can cause severe and long-term adverse effects, from tendon and joint inflammation restricting mobility, to neuropathy affecting cognition. These adverse effects have been attributed to mitochondrial disturbance but the exact mechanism or the reason it only occurs in a small minority of patients remains unclear (175, 176). As a consequence of this, fluoroquinolone use in humans has been seen considerable reduction and restriction in both the UK, and the EU (167, 168). Despite this, however, it remains on the World Health Organisation's list of critically important antimicrobials (177). At the same time, it has also seen considerable restriction in its use in farming, particularly in the UK (125).

1.16. Fluoroquinolone Resistance (FQ-R)

FQ-R in *E. coli* comes principally from mutations in the quinolone resistance determining regions (QRDR) of the type II topoisomerase enzymes: encoded by the DNA-gyrase gene, *gyrA*, and the topoisomerase IV gene, *parC* and, to a lesser extent, a second gyrase subunit gene, *gyrB* and a second topoisomerase IV gene, *parE* (178). Two mutations in *gyrA* and

one in *parC* is sufficient for clinical resistance in *E. coli* (179). However, FQ-R can also be achieved through fewer mutations in the target enzymes with the addition of a plasmid mediated quinolone resistance (PMQR) gene or mutations in genes affecting bacterial envelope permeability and efflux (180-182). This mutational resistance cannot easily be transmitted between organisms. However, since single gyrase mutations are not sufficient to confer FQ-R, transfer of PMQRs into susceptible isolates with single gyrase mutations is a viable way of achieving resistance. There is reason to believe, though, that many FQ-R *E. coli* isolates are closely related and recently descended from a resistant common ancestor (183-185), suggesting that PMQR transmission is less important for the expansion of FQ-R in this species than clonal dissemination. Despite this, PMQR mechanisms are increasingly found on plasmids carrying multiple resistance genes and may one day become a more importance factor in FQ-R (186, 187).

There are lots of natural quinolone compounds but none discovered so far have the 3-carboxy group necessary to disrupt the complex between DNA and type IIA topoisomerases (188). However, given the fact that resistance-giving nucleotide polymorphisms within the QRDR are found in diverse and distantly related groups of *E. coli*, and many other species, it would seem plausible that some natural antimicrobial 3-carboxy quinolone either existed in the past or has yet to be discovered. Alternatively, it may be that multiple topoisomerase alleles have evolved to evade the action of antibacterial peptides, such as Microcin B17, which target DNA gyrase, and some of these alternative alleles happen to also give resistance to fluoroquinolones (189).

PMQR genes include the *qnr* genes, *qnrA*, *qnrB, qnrC qnrD* and *qnrS*, the products of which interfere with the gyrase-DNA complex*;* the efflux pump genes, *oqxAB* and *qepA*; and the fluoroquinolone-modifying variant aminoglycoside acetyltransferase gene, *aac(6')-Ib-cr*, the product of which only affects ciprofloxacin and norfloxacin (190). As discussed above, enrofloxacin has an active metabolite, ciprofloxacin, which would suggest that it too is affected by AAC(6')-Ib-cr *(172)*.

The *qnr* genes code for pentapeptide repeat proteins, with the pattern (A/C/S/T/V)(D/N)(L/F)(S/T/R)(G/R), which interfere with the binding of fluoroquinolones to the DNA-topoisomerase complex, thus reducing fluoroquinolone susceptibility (191). The *qnr* variants: A, B, C, D, and S, are diverse and likely emerged separately, in multiple organisms. Suspected origin species include *Shewanella algae* and members of the Vibrionaceae family (192-194). These may have evolved as a defence mechanism against bacteriocins, as related pentapeptide repeat proteins have been found to protect against the activity of bacterial toxins, such as microcin B17, a DNA replication inhibitor (191, 195).

Finally, there are two mobile efflux pumps which reduce susceptibility to fluoroquinolones and have been found, rarely, on plasmids harboured by clinical *E. coli* isolates (196, 197). OqxAB is an efflux pump of the resistance nodule division (RND) family (which also includes the native *E. coli* AcrAB efflux pump) and is believed to have originated in *K. pneumoniae* (196, 198). The gene for QepA, conversely, shows patterns of codon usage similar to those seen in the major facilitator superfamily (MFS) of efflux pumps found in diverse species of Actinomycetes (197).

1.17. Reduced envelope permeability as a mechanism of resistance in *E. coli*.

As alluded to above, the first form of defence for Gram negative bacteria is their double cell-membrane and peptidoglycan cell wall. To have any effect antibacterials must first bypass these barriers; for β-lactams only the outer membrane needs to be crossed but fluoroquinolones and most other antibacterials, entry into the cytoplasm is needed. Fortunately, bacteria require channels called porins to take up nutrients and antibacterial compounds can pass through these and into the cell (199).

However, the bacterial cell wall and double-membrane is not a fixed and static entity. A complex and intricate system of regulation controls a dynamic network of porins and efflux pumps – transmembrane proteins which act, not as passive channels, but which actively work to remove deleterious compounds from the cell – which change in response to a myriad of stressors and to nutrient availability (199).

In addition to the PMQR efflux pumps, discussed above, in *E. coli*, chromosomally-encoded porins and efflux pumps are also associated with ABR when differentially expressed. Expression of these genes is principally controlled by transcriptional regulators – for example, MarA, which is encoded on the *mar* (multiple antimicrobial resistance) operon, though there are many other factors which can affect the expression of individual or multiple porins and efflux pumps. MarA is activated by the presence of a wide variety of compounds, including many antibacterials, and is regulated itself at a transcriptional level by the repressor MarR. Disruption of the *marR* gene by mutation is thus associated with overproduction of MarA and a resistant phenotype due to increased transcription of the *acrAB-tolC* efflux pump operon, and increased expression of *micF*, an antisense RNA that blocks translation of the outer membrane protein, *ompF,* porin mRNA. The end result is reduced entry and increased efflux of a wide variety of compounds, including fluoroquinolones and some cephalosporins (200). Upregulation of the AcrAB-TolC efflux pump and downregulation of the porins, OmpC and OmpF, by other general and specific mechanisms (e.g. OmpR/EnvZ controlling porin production or AcrR mutation controlling

efflux pump production) is particularly associated with reduced susceptibility to fluoroquinolones and β-lactams, though changes in the expression of other efflux pumps and porins can also affect susceptibility (180, 201).

1.18. The Research in Context and Specific Aims

The work reported in this thesis was conducted as part of my role as a research technician within the OH-STAR consortium. The initial OH-STAR project aims were:

1: To survey for ABR *E. coli* from dairy farms in the South West of England. Samples were collected in 2017-18 by veterinary researchers, and processed microbiologically by me and another technician, Katy Morley.

2: To use the microbiological data collected in part by me, above, alongside farm management practice survey data and medicines usage data collected in parallel by the veterinary researchers, to identify "risk factors" for finding ABR *E. coli* on dairy farms. This analysis was performed by Dr Hannah Schubert.

3: To perform a survey of the carriage of ABR *E. coli* in the faecal flora of 16-week-old puppies, some of which being located in the South West of England, but most from a national cohort study "Generation Pup" run by the Dogs' Trust charity. Recruitment and commissioning of the survey was done by Masters' student Kezia Wareham, and microbiological processing of samples, provided in 2017-18 was shared between Kezia and myself.

4: To use the microbiological data collected in part by me, above, alongside puppy owner surveys commissioned by Kezia Wareham, to identify risk factors for finding ABR *E. coli* in the faecal flora of 16-week-old puppies. This analysis was performed by Dr Ashley Hammond.

5: To perform a comparison between the change in use of antibiotics in primary care between 2013 and 2016 across 146 GP practices in Bristol and surrounding regions (approx. 1.5 million population), and the levels of ABR *E. coli* within urinary samples submitted through primary care to the Severn Pathology diagnostic microbiology lab at Southmead hospital Bristol (>100,000 samples a year). This analysis was performed by Dr Ashley Hammond.

6: To characterise mechanisms of 3GC-R present in *E. coli* found in urine samples referred from 146 GP practices and processed at the Southmead hospital lab in 2017-18, and on the dairy farms as described above. This analysis included PCR and WGS and was performed by Dr Jackie Findlay.

7: To use comparative genomics to assess phylogenetic relationships between 3GC-R *E. coli* from dairy farms and from human urine identified in the above surveys, with an intention of identifying potential transmission between these compartments. This analysis was performed by me and will be described in this chapter alongside a narrative account of the key findings of the work reliant on microbiology data that I generated.

In the first results chapter, work relevant to aims 1, 2, and 7, above will be discussed. The overall objective here is to consider the selection and transmission of 3GC-R *E. coli* on dairy farms and the influence of this on human infections. Our focus on UTI as a source of human samples, was designed to include both ExPEC and commensal/opportunistic *E. coli* types, but the fact that the samples came from the community perhaps made ExPEC types more likely. Therefore, research questions were: Are 3GC-R ExPEC types found in dairy farms, and if not, what evidence is there that 3GC-R *E. coli* from dairy farms are contributing to UTI via the commensal/opportunistic route; also, is there evidence of plasmids found in 3GC-R *E. coli* from dairy farms penetrating into the ExPEC and commensal/opportunistic *E. coli* that have caused UTI in humans.

In addition, results chapters 2 and 3 will describe work I initiated and performed characterising FQ-R in *E. coli* found in samples collected from dairy farms and human urine addressing similar research questions to those set out for 3GC-R *E. coli*, above. Results chapter 4 will describe an analysis of 3GC-R and FQ-R *E. coli* from puppies, collected as part of OH-STAR aim 3, above, addressing the question of whether dogs impact on ABR in humans, and/or form an intermediate host for transmission of ABR between farm animals and humans.

The overall objective of this work is to apply a One Health research framework to the issue of ABR *E. coli* transmission within a defined geographical region, and to identify interventions that might reduce the acquisition of ABR *E. coli* by cattle and dogs, and the zoonotic threat that this poses to humans.

2. Methods and Materials

2.1. Farm recruitment, sampling and sample processing

A convenience sample of 53 dairy farms was recruited by the OH-STAR consortium and farms were visited monthly between January 2017 and December 2018 for sample collection as described in Schubert et al. (202). Briefly, samples were collected using sterile overshoes (over-boot socks) traversing farm areas by colleagues, Dr Hannah Schubert and Emma Puddy. Samples were refrigerated from collection to processing, were transferred into individual labelled sterile stomacher bags, and suspended in 10 mL/g of phosphate buffered saline (PBS Dulbecco A; Oxoid, Basingstoke, UK). Samples were then mixed for one min in a stomacher (Stomacher 400, Seward, Worthing, UK). Samples were mixed 50:50 with 100% sterile glycerol and aliquots stored at -80°C. Sample processing and storage was carried out by me and colleague, Katy Morley.

2.2. Dog recruitment

Dog owners were recruited to take part in the study in two ways, as described in Mounsey *et al.* (203). Briefly, (i) 236 were already recruited to the Dogs Trust "Generation Pup" project, a longitudinal study examining the health, welfare and behaviour of dogs across the UK (204), and (ii) 59 were locally recruited by colleague, Kezia Wareham, via word-of-mouth advertisement to clients bringing young dogs in for routine checks to veterinary practices in Somerset and Bristol, via puppy socialisation classes and via social media as well as local media advertisement.

2.3. Dog sample processing

All dog owners were supplied with a sample collection pack comprised of a specimen bottle, gloves, biohazard bag and a freepost envelope. Faecal samples were sent by post to the University of Bristol's Veterinary School alongside the consent form and, for locally recruited dogs, a questionnaire. To process each faecal sample, approximately 0.1-0.5 g of faeces was taken and weighed. Ten millilitres per gram of phosphate buffered saline (PBS) was added to the sample and the mixture vortexed. Next, 0.5 mL of the faecal/PBS homogenate was added to 0.5 mL of 50% v/v sterile glycerol and processed as below. This was carried out by me and colleague, Kezia Wareham.

2.4. Ethical Information

Ethical approval for the dog work was granted by the University of Bristol Health Sciences Student Research Ethics Committee (56783). Ethical approval for the dairy farm work was obtained from the University of Bristol's Faculty of Health Sciences Research Ethics Committee (41562).

2.5. Microbiology and PCR analysis

Twenty microlitres of faecal homogenate (diluted 1:10) were spread onto tryptone bile X-glucuronide agar (TBX; Scientific Laboratory Supplies); 20 µL of undiluted sample were spread onto TBX agar containing 16 mg/L tetracycline, 8 mg/L amoxicillin, 0.5 mg/L ciprofloxacin, 16 mg/L streptomycin or 16 mg/L cephalexin. Plates were incubated at 37°C, and the number of blue-green colonies (*E. coli*) counted. Data were collapsed into a binary "positive/negative" outcome for the homogenate derived from each faecal sample. Samples yielding no *E. coli* colonies on antibiotic-free agar were excluded from further analysis. Concentrations were chosen as those which define clinically relevant resistance in humans according to EUCAST (205). Up to five *E. coli* isolates from each cephalexin (16 mg/L) TBX agar plate were transferred onto the 3GC cefotaxime (2 mg/L) TBX agar by colleague, Dr Jackie Findlay, for further analysis of 3GC-R *E. coli*, as described in Schubert *et al.* for the cattle isolates (202) and Mounsey *et al.* for the canine isolates (203). Similarly, up to five *E. coli* from each ciprofloxacin (0.5 mg/L) TBX plate were transferred onto ciprofloxacin (0.5 mg/L) TBX agar by me to confirm resistance.

2.6. Multiplex PCR

PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, *oqxAB*, and *qepA*) were identified by multiplex PCR as previously described (206), primers and PCR product sizes are detailed (Table 2). The function of these genes is discussed in Introduction – 1.16.

Table 2. Fragment sizes and primer sequences for PMQR PCR.

Taken from Ciesielczuk et al. *(206)*.

Target	Primer	Sequence (5′→3′)	Fragment size (bp)	Reference
qnrA	F	CAGCAAGAGGATTTCTCACG	630	*Ciesielczuk et al. (2013) (206)*
	R	AATCCGGCAGCACTATTACTC		
qnrD	F	CGAGATCAATTTACGGGGAATA	581	Cavaco *et al.* (2009) (207)
	R	AACAAGCTGAAGCGCCTG		
qnrB	F	GGCTGTCAGTTCTATGATCG	488	*Ciesielczuk et al. (206)*
	R	GAGCAACGATGCCTGGTAG		
(degenerate)	R	SAKCAACGATGCCTGGTAG		
qnrS	F	GCAAGTTCATTGAACAGGGT	428	Cattoir *et al.* (2007) (208)
	R	TCTAAACCGTCGAGTTCGGCG		
oqx AB	F	CCGCACCGATAAATTAGTCC	313	*Ciesielczuk et al. (2013) (206)*
	R	GGCGAGGTTTTGATAGTGGA		
aac(6')-Ib-cr	F	TTGGAAGCGGGGACGGAM	260	Wareham *et al.* (2010) (209)
	R	ACACGGCTGGACCATA		
qepA	F	GCAGGTCCAGCAGCGGGTAG	218	Yamane *et al.* (2008) (210)
	R	CTTCCTGCCCGAGTATCGTG		
qnrC	F	GCAGAATTCAGGGGTGTGAT	118	*Ciesielczuk et al. (2013) (206)*
	R	AACTGCTCCAAAAGCTGCTC		

2.7. Antimicrobial disc susceptibility testing

Antimicrobial disc susceptibility testing was carried out according the Kirby-Bauer disc diffusion method (211).

2.8. Whole Genome Sequencing and analysis

Representative 3GC-R isolates were selected for WGS by colleague, Dr Jackie Findlay, based on resistance phenotype, β-lactamase gene carriage and farm of isolation, as described in Schubert *et al.* (202). WGS was performed by MicrobesNG (https://microbesng.uk/) as previously described (212, 213). Resistance genes were assigned using ResFinder 2.1 (214), STs were assigned using MLST 2.0 (41), virulence genes were detected using VirulenceFinder 1.2 (215), *fimH* typing was carried out using FimTyper (44), and QRDR point mutations were found using PointFinder (https://bitbucket.org/genomicepidemiology/pointfinder_db/src/master/). All were run using the Centre for Genomic Epidemiology pipeline service (http://www.genomicepidemiology.org/) platform (216).

2.9. Phylogenetics

Phylogenetic analysis was carried out using the Bioconda environment (217) on the Cloud Infrastructure for Microbial Bioinformatics (218). The reference sequences were ST131 isolate EC958 (accession: HG941718) , ST744 isolate EC590 (accession: NZ_CP016182), and ST162 isolate W2-5 (accession: NZ_CP032989). Sequence alignments were with Snippy and Snippy-Core (https://github.com/tseemann/snippy). SNP-distances between isolates were calculated using SNP-dists software (https://github.com/tseemann/snp-dists). Maximum likelihood phylogenetic trees were constructed using RAxML, utilising the GTRCAT model of rate heterogeneity and the software’s autoMR and rapid bootstrap (82, 84, 219). Trees were illustrated using Microreact (87).

2.10. Chi squared analysis

Variables were condensed into binary categories for 2 x 2 contingency tables, and from this expected values were inferred for each category. χ^2 analyses were then performed to evaluated statistically significant associates:

$$\frac{(O-E)^2}{E}$$

Where O = observed and E = expected. The results for each observed and expected value in the contingency table was calculated and summed to give a χ^2 value and the University of Illinois at Urbana-Champaign's online p value calculator for χ^2 distribution (http://courses.atlas.illinois.edu/spring2016/STAT/STAT200/pchisq.html) was used to calculate p (220).

2.11. Phi coefficient

Because a X^2 analysis can establish statistical significance but cannot measure the strength of associated between variables (220), the phi coefficient (φ) calculated for each χ^2 analysis, as follows (221):

$$\varphi = \sqrt{(\frac{\chi^2}{n})}$$

Where n = the number of observations (frequency of variables) in the 2 x 2 contingency tables.

2.12. Risk factor analyses and multilevel, multivariable logistic regression analyses

Risk factor analyses and multilevel, multivariable logistic regression analyses were carried out by colleague, Dr Hannah Schubert, as described in Schubert *et al.* (202), or Dr Ashley Hammond, as described in Mounsey *et al.* (222).

2.13. Bacterial strains

Four *K. pneumoniae* control strains, containing genes included in multiplex PCR were provided by colleague, Dr Wan Ahmad Kamil Wan Nur Ismah. All harbour *oqxAB*, which is found on the *K. pneumoniae* chromosome. Additional PMQR gene carriage is indicated in brackets:

R4 (*qnrS* and *aac(6')-Ib-cr*)

R5 (none)

R20 (*qnrB)*

R27 (*aac(6')-Ib-cr* only)

An additional control strain, *E. coli* Top10 transformed with a plasmid harbouring *qnrA* was provided by colleague, Dr Juan Carlos Jiménez-Castellanos.

3. Results Chapter 1 – No evidence for sharing of 3GC-R *E. coli* between human urinary tract and cattle in South West

3.1. Introduction

The data collected by the OH-STAR consortium has, at time of writing, led to the publication of five papers, of which three included work that I performed on 3GC-R *E. coli* from dairy farms and human urine, (202, 213, 223). Included in this chapter is a summary and extracts of the three published papers highlighting the contribution I made to each. Principally in the dairy cattle primary sample processing and in the phylogenetic and SNP-distance analyses comparing human and cattle isolates.

Many countries are implementing plans to reduce the use of antibacterial drugs in food-producing animals. For example, the most recent UK five-year National Action Plan includes a target to reduce antibacterial use (ABU) in the treatment of food-producing animals by 25% (224). In Europe, antibacterial sales for food-producing animals fell by 20% from 2011 to 2016 (225). In the UK dairy industry, overall ABU dropped from 24 mg/kg in 2015 to 17 mg/kg in 2018 (226, 227). In 2018, additional industry-led policies were enforced in the UK that aimed to almost eliminate the use of highest-priority critically important antimicrobials (HP-CIAs) such as 3GCs and fourth-generation cephalosporins (4GCs) as well as fluoroquinolones on dairy farms. One reason for reducing ABU in farming is to reduce the prevalence of ABR bacteria carried by farm animals. However, there is a need for better data on drivers of ABR in farming. More granularity of understanding is required concerning the risks of using individual ABs and other management practices. This is especially important in terms of drivers of HP-CIA resistance. A particular focus among HP-CIAs is on 3GC-R and FQ-R in *E. coli* (123).

3GC-R is increasingly prevalent in *E. coli* causing infections in humans (228) and is also found in farmed and domestic animals around the world (229). The production of CTX-M (an ESBL) is the most common mechanism of 3GC-R in *E. coli* in humans in the UK; for example, in the OH-STAR survey of urinary *E. coli* from humans in South West England, 82.2% of 3GC-R isolates carried $bla_{\text{CTX-M}}$ (212).

The aim of the work reported in this chapter was to characterise the prevalence of ABR *E. coli* in samples from dairy farms in the South West of England, and to consider whether 3GC-R isolates that have various mechanisms of 3GC-R are a zoonotic threat to humans living in the same geographical region.

3.1.1. Results 1: A Microbiological survey of resistant *E. coli* in dairy farms, which allowed risk factor analysis.

OH-STAR objective 1 was to describe the prevalence of 3GC-R *E. coli* carrying *bla*$_{CTX-M}$ – as well as *E. coli* resistant to amoxicillin, tetracycline, streptomycin, cephalexin, cefotaxime and the fluoroquinolone, ciprofloxacin – found in faecally contaminated environments of dairy cattle in a geographically restricted population of UK dairy farms in South West England. To achieve this, samples were collected from dairy farms monthly between January 2017 and December 2018. Samples were processed by myself and Katy Morley (Methods and Materials 2.1).

4581 boot-sock-pair samples from faecally contaminated sites were collected from 53 dairy farms and, following sample-preparation (Methods and Materials 2.1), samples were plated on TBX agar, either with or without one of five selective antibacterials (Methods and Materials 2.1). 4145 samples were positive for growth of *E. coli* on non-selective agar (Table 3). Prevalence of resistance to the six test antibacterials at farm and sample level are presented in Table 3.

Table 3. Farm and sample-level prevalence of resistance in faecal *E. coli*
4581 samples were taken from faecally-contaminated near-animal cattle environment

Antibacterial drug	Farm level resistance	Sample level resistance
Amoxicillin	53/53 (100%)	2754/4145 (66%)
Ciprofloxacin	49/53 (92%)	263/4145 (6%)
Streptomycin	53/53 (100%)	1475/4145 (36%)
Tetracycline	53/53 (100%)	2874/4145 (69%)
Cephalexin	53/53 (100%)	1263/4145 (30%)
Cefotaxime	47/53 (88.7%)	384/4145 (9.3%)

As part of this survey, 384 samples, collected across 47 farms, were found to be positive for the detectable growth of 3GC-R *E. coli* isolates, based on growth of cefotaxime. (Table 3). Colleague Dr Jackie Findlay used PCR to show that 648/1226 3GC-R *E. coli* isolates screened from these 384 samples contained a *bla*$_{CTX-M}$ gene, which represents 224/4145

samples and 42/53 farms. Interestingly, 566/1226 of these CTX-R *E. coli* isolates were PCR negative for mobile cephalosporinases and so were presumed to be chromosomal AmpC hyperproducers (202). If this presumption was correct (see section 3.1.2), AmpC hyperproduction was the mechanism of resistance in 46.2% of 3GC-R *E. coli* from dairy cattle in this region of the UK. This figure is comparable with the 42.9% presumed AmpC hyperproducers seen in 3GC-R *E. coli* from dairy cattle in a recent nationwide Dutch study (230) and contrasts with the 3.8% of AmpC hyperproducers seen in 3GC-R isolates in the OH-STAR survey of human urinary *E. coli* (212).

The data generated by the primary sample testing and $bla_{\text{CTX-M}}$ PCR combined with other data obtained on treatment, ABU, and environmental conditions enabled the conducting of a multilevel, multivariable logistic regression analysis, carried out by my colleague, Dr Hannah Schubert. This culminated in a paper which found that low average monthly temperature was associated with reduced odds of $bla_{\text{CTX-M}}$ *E. coli* positivity in samples and to reduced odds of finding *E. coli* resistance to each of the four tested non-cephalosporin antibacterials (amoxicillin, tetracycline, streptomycin, and ciprofloxacin). This was additional to the effect of temperature on total *E. coli* density. Furthermore, samples collected close to calves had increased odds of having *E. coli* resistance to each antibacterial or positive for $bla_{\text{CTX-M}}$. Samples collected on pastureland had reduced odds of having *E. coli* resistant to amoxicillin or tetracycline, and being positive for $bla_{\text{CTX-M}}$. These findings have profound implications for the design of routine surveillance and for surveys carried out for research. They provide important evidence that sampling single time-point and/or single locations on a farm are unlikely to be adequate to accurately determine the status of the farm with regard to the presence or number of resistant *E. coli* (202).

In addition, because ABR was found to be particularly prevalent in samples from around calves, it was possible to identify specific risk factors associated with finding critically important ABR in these calf samples. FQ-R will be discussed in results chapter 3. But one key finding in the context of 3GC-R *E. coli*, which will also be discussed in results chapter 3, was that antibiotic dry cow therapeutic choice (specifically the use of cefquinome or framycetin) was associated with increased odds of $bla_{\text{CTX-M}}$ positivity. Dry cow therapy is a treatment whereby udders are injected with an antibacterial, sometimes in combination with an internal teat sealant and is discussed more thoroughly in results chapter 3 – 5.1. Since CTX-M confers cefquinome (a 4GC) resistance, this is an example of direct selection. Framycetin (neomycin B) in an aminoglycoside, so it cannot directly select for CTX-M production, but framycetin resistance is commonly plasmid mediated, so it is possible that this represents an example of co-selection. And this will be discussed later in the light of WGS analysis in results chapter 3.

3.1.2. Results 2: Phylogenetic analysis shows no evidence for sharing of AmpC hyper-producing *E. coli* in humans and cattle collected at the same time in the same geographical region.

E. coli typically produce a class 1 cephalosporinase, encoded by the *ampC* gene, which is chromosomally located. Expression of *ampC* in WT cells is low and not enough to confer clinically relevant resistance to β-lactam antibiotics. Many mutations, insertions and gene duplication events have been shown to cause *ampC* hyperexpression and this leads to varying spectra of β-lactam resistance, dependent on the actual amount of AmpC produced (139). AmpC hyperproduction was first seen in *E. coli* from human clinical samples in 1979 (231) and for a period before the emergence of plasmid-mediated ESBLs, AmpC hyperproduction was the dominant mechanism of 3GC-R in *E. coli* from humans (139). This is no longer the case, however. For example, as discussed above, the recent OH-STAR survey of 3GC-R *E. coli* from urine collected from people living in South West England, only 24/626 isolates (3.8%) were presumed to be AmpC hyperproducers because of their lack of horizontally acquired β-lactamase genes; WGS confirmed that 13/13 sequenced isolates had *ampC* promoter mutations typical of AmpC hyperproducers (212).

AmpC is typical of class 1 β-lactamases in that it does not confer resistance to the 4GCs (139). However, *ampC* structural variants in *E. coli*, expanding AmpC activity to include cefepime, for example, have been identified from humans (232-235) and cattle (236). These are dominated by isolates from phylogroup A and particularly ST88 (233, 236). This is probably because expanded-spectrum activity evolves from existing AmpC hyperproducers, among which ST88 isolates are particularly common (237).

As discussed above, 42.9% of 3GC-R *E. coli* from our survey of dairy farms were negative for mobile cephalosporinases and so were presumed to be AmpC hyperproducers. This was far higher than the 3.8% of presumed AmpC hyperproducers seen in 3GC-R isolates in the OH-STAR survey of human urinary *E. coli*. A fellow PhD student, Maryam Alzayn, studied in detail 20 of these presumed AmpC hyperproducing urinary *E. coli* and 25 presumed AmpC hyperproducer *E. coli* from the OH-STAR cattle survey. One isolate was selected from each of 25 farms located in a 50 x 50 km sub-region of the OH-STAR study that also included the homes of the people providing urine samples for the OH-STAR urinary survey. Maryam was able to show that these isolates do hyper-produce AmpC due to *ampC* promoter mutations (223).

My role, in parallel, was to analyse WGS data for these 20 human and 25 cattle AmpC hyperproducers. This revealed the dominance of ST88 (n=11 – 24%) amongst farm isolates (Table 4), as has been previously reported from cattle (237).

Table 4. STs of AmpC-hyperproducing isolates

Information for 45 isolates, representing 25 dairy farms and 20 human urine samples, including ST and phylogroup, as defined in Introduction – 1.3.

Isolate	ST	Phylogroup
Farm-1	641	B1
Farm-2	88	C
Farm-3	88	C
Farm-4	388	B1
Farm-5	88	C
Farm-6	75	B1
Farm-7	641	B1
Farm-8	23	C
Farm-9	162	B1
Farm-10	88	C
Farm-11	2522	B1
Farm-12	88	C
Farm-13	278	B1
Farm-14	641	B1
Farm-15	88	C
Farm-16	278	B1
Farm-17	661	B1
Farm-18	88	C
Farm-19	88	C
Farm-20	278	B1
Farm-21	345	B1
Farm-22	641	B1
Farm-23	88	C
Farm-24	278	B1
Farm-25	88	C
UTI-1	141	B2
UTI-2	75	B1
UTI-3	200	B1
UTI-4	155	B1
UTI-5	73	B2
UTI-6	73	B2
UTI-7	200	B1
UTI-8	54	B1
UTI-9	73	B2
UTI-10	73	B2
UTI-11	405	D
UTI-12	131	B2
UTI-13	1499	C
UTI-14	200	B1
UTI-15	75	B1
UTI-16	73	B2
UTI-17	200	B1
UTI-18	428	B2
UTI-19	88	C
UTI-20	448	B1

The final aim of this work was to identify whether there was any evidence of sharing AmpC-hyperproducing *E. coli* between humans and cattle, since dominance of ST88 has previously been reported in humans in Northern Europe (237) and we found an over-representation of

ST88 on our farms (Table 4). A phylogenetic tree drawn based on core genome comparison showed that the cattle and human isolates were intermixed only to a small extent, with only one human ST88 isolate found (Table 4, Figure 8). Importantly, all 10 ST88 cattle isolates were 15 or fewer SNPs apart, suggesting very recent farm-to-farm transmission. Whereas the human ST88 isolate (UTI-19) was, at its closest distance, 1279 SNPs different from the cattle isolates. The two other examples where isolates from the same ST were found in farm and human samples painted the same picture (Figure 8): for ST75, the two human isolates (UTI-2 and UTI-15) were 60 SNPs apart, but the cattle isolate (Farm-6) was 1972 SNPs different at best. For ST23, the human and cattle isolates (UTI-13 and Farm-8, respectively) were 2754 SNPs different. Otherwise, there was no ST sharing and all cattle isolates fell into phylogroups B1 and C, with 8/20 human isolates falling into the highly pathogenic phylogroup B2, including a cluster of ST73 isolates (Table 4), of which three were only two SNPs apart (Figure 8). A core genome SNP distance of 30 or fewer is commonly seen in Enterobacteriales isolates that are confirmed to be part of an acute outbreak of foodborne illness (238).

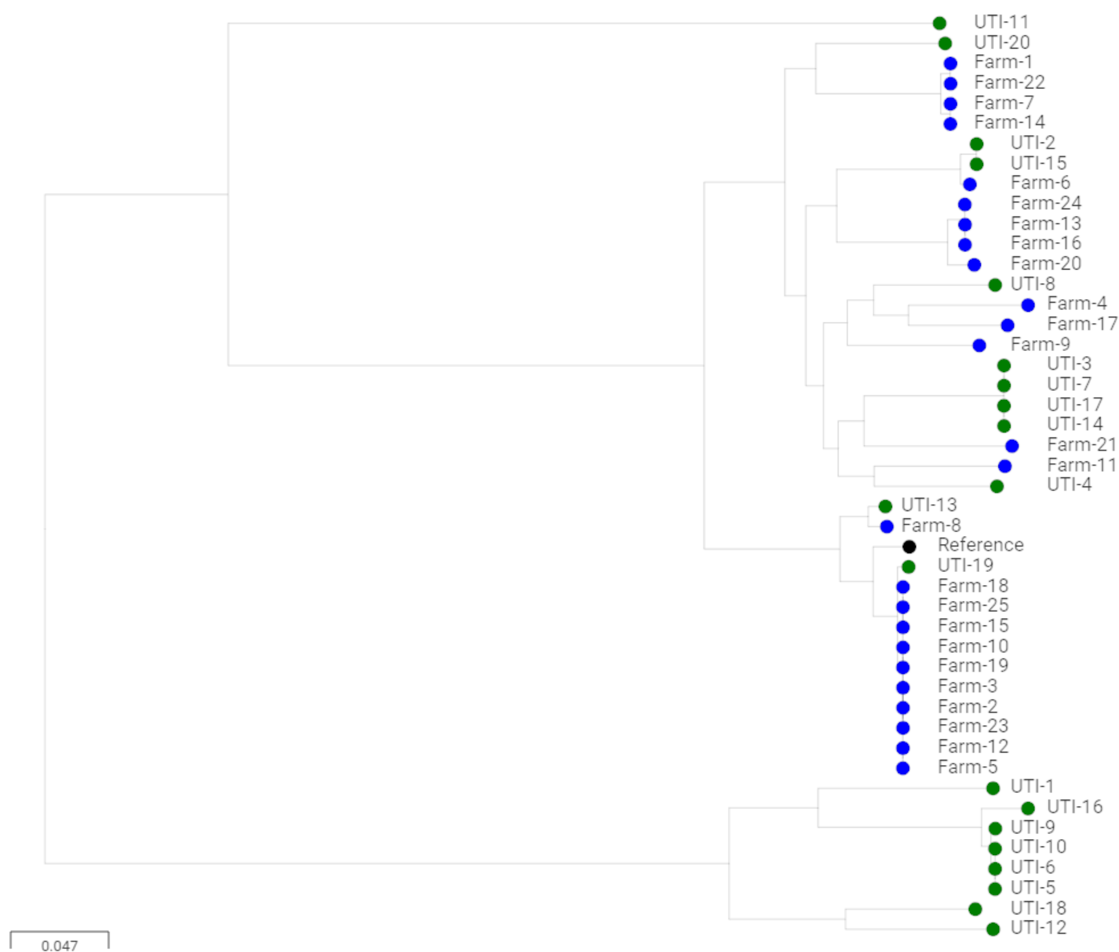


Figure 8. Phylogenetic tree of farm and human urinary AmpC-hyperproducing *E. coli*. The phylogenetic tree was illustrated using the Microreact program using a maximum likelihood tree generated from core genome alignments as described in Methods and Materials – 2.9. Isolates are coloured green (human urinary) and blue (farm). The ST88 finished reference genome (Accession: NZ_CP031546.1) used to generate the alignments is coloured black.

We concluded from these analyses that AmpC hyperproduction is a remarkably common mechanism of 3GC resistance in *E. coli* from dairy farms in the OH-STAR study region – a finding similar to that of a national survey in The Netherlands (230). However, comparison between AmpC-hyperproducing farm and human urinary *E. coli* in the same region provided no evidence of local sharing of AmpC hyperproducers between farms and the local human population. Accordingly, whilst reducing the on-farm prevalence of AmpC-hyperproducing *E. coli* should be an important aim, the primary reason for achieving this would be to reduce the

likelihood of difficult-to-treat infections in cattle rather than because of any direct zoonotic threat.

3.1.3. Results 3: No evidence for transmission of CTX-M and pAmpC β-lactamase producing *E. coli* from dairy farms to humans in the same geographical region

As discussed above, Dr Jackie Findlay's PCR analysis showed that 648/1226 (52.7%) of 3GC-R *E. coli* collected in the OH-STAR Dairy farm survey carried bla_{CTX-M} and 13/1226 (1.1%) carried a plasmid-derived AmpC (pAmpC) gene (202). A representative subset of these isolates, collected from dairy farms within the full OH-STAR study region (n=138) was taken forward for WGS analysis by Jackie. This analysis identified bla_{CTX-M} (131 isolates: encoding CTX-M-1, -14, -15, -32 and the novel variant, CTX-M-214), bla_{CMY-2} (6 isolates) and bla_{DHA-1} (one isolate).

Core genome maximum likelihood phylogenetic analysis was then carried out by myself using WGS data from 112 out of the 138 bla_{CTX-M} or pAmpC positive isolates sequenced by Jackie (i.e. those from dairy farms located in the 50 x 50 core OH-STAR study region, described above) and 212 human urinary *E. coli* isolates (from people living in this same region) carrying acquired 3GC-R genes, from the OH-STAR survey of 3GC-R urinary *E. coli* (212). This analysis (Figure 9) revealed only four STs including examples of both farm and human isolates. In no case was the SNP difference between any pair of human and farm isolate core genomes suggestive of recent transmission. ST10 was the closest (≥205 SNPs different between human and cattle isolates; Figure 9 insert); the others were ST540 (929 SNPs different), ST58 (≥1388 SNPs different) and ST69 (≥831SNPs different). In contrast, there was clear evidence of recent farm-to-farm transmission of isolates from multiple STs (e.g. ST10 and ST69 where, in both cases, there was a 3 SNP minimum distance between pairs of isolates representing two farms) (Figure 9). AmpC hyper-producing *E. coli* isolates from these same farms (discussed above) showed a similar pattern: no evidence of strain sharing between dairy farms and humans but strong evidence for recent farm-to-farm transmission (223). We conclude therefore that 3GC-R isolates from farms, do not readily circulate amongst the local human population and cause UTI.

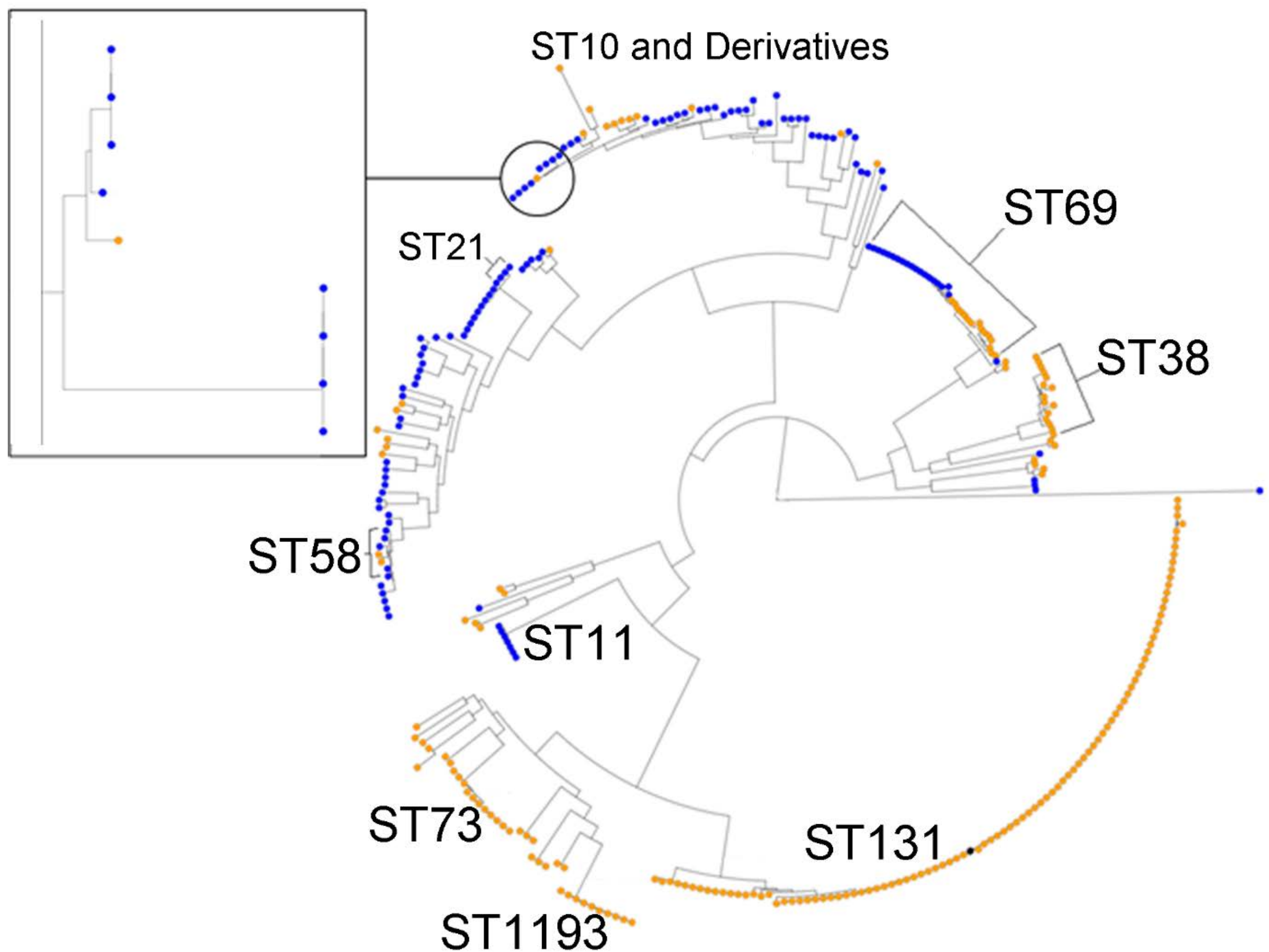


Figure 9. Phylogenetic analysis of *E. coli* from dairy farms and human UTI collected in parallel in a 50 x 50 km region.

Human isolates are noted in orange; cattle isolates are noted in blue. The reference ST131 isolate is noted in black. Certain key STs are highlighted, particularly STs with representatives from human and cattle isolates: ST21 (ST540), ST69, ST58 and ST10. The insert shows a more detailed analysis of ST10 isolates which represents the closest relationship between a human and a cattle isolate: 205 SNPs different across the core genome.

Overall, this analysis finds no evidence of recent, direct sharing of *E. coli* between farms and the local human population that have resulted in UTI. However, fellow PhD student Winnie Lee identified that three farm *E. coli* isolates carried a *bla*$_{CTX-M}$ or *bla*$_{CMY-2}$ plasmid almost identical to one of three plasmids found in urinary *E. coli* in the local human population, though these three plasmids are known to be widely disseminated in humans and animals across several continents, e.g., pCT (239). Furthermore, no human/farm plasmid pair shared 100% identity. So, whilst there is some general evidence of plasmids circulating between human and animal populations, as reported in a number of recent studies in Western countries (240-242), the level of overlap between UK dairy farm and human 3GC-R *E. coli* identified in this study was very small and not suggestive of any novel or recent zoonotic transmission events. In contrast, both recent and sustained farm-to-farm transmission of 3GC-R *E. coli*, and particularly an epidemic plasmid pMOO-32 across many different *E. coli* STs and dairy farms, was clearly seen by Jackie Findlay, when performing molecular epidemiology on *bla*$_{CTX-M}$ genes and their surrounding genetic contexts among the WGS data from the OH-STAR dairy farm survey (213). Identifying the vectors for this transmission will inform interventions that might facilitate a more rapid reduction in 3GC-R *E. coli* on dairy farms.

3.2. Conclusions

My involvement in the processing and plating samples collected in the OH-STAR dairy farm survey was key to the identification of multiple risk factors for the presence of ABR *E. coli* in faecally contaminated sites around dairy cattle in the UK. Whilst we found some evidence for specific antibacterial drug treatments affecting ABR *E. coli* carriage, by far the largest impact was of the average temperature (so seasonality) and the age of the animals being sampled. It then led to more detailed molecular epidemiological, and in my case core genome phylogenetic analysis of the differences between 3GC-R isolates found in humans and dairy cattle. Ultimately, whilst the same or similar resistance mechanisms and STs were identified in both reservoirs, an in-depth phylogenetic and SNP-distance based analysis, found no evidence of recent sharing of either AmpC hyperproducers (having resistance caused by chromosomal mutation) or isolates that are 3GC-R because they carry mobile resistance genes. Later work in the thesis will consider if the same applied for resistance to another critically important antibacterial class, the fluoroquinolones, and if domestic pets (dogs) might act as an intermediate host between farm animal and human resistant *E. coli* carriage.

4. Results Chapter 2. Characterisation of FQ-R urinary *E. coli* from people living in Bristol and the surrounding region.

4.1. Introduction

Fluoroquinolones are an important class of antibacterial drugs, included in the World Health Organisation's list of 'Highest Priority Critically Important Antimicrobials' (177). They are extensively used to treat infections in humans, companion animals, and farmed animals. These important medicines work by disrupting the activity of type II topoisomerases, causing the release of DNA that has double-strand breaks, leading to cell death (173).

In addition to safety concerns about fluoroquinolone use in humans (Introduction – 1.15) (243), bacteria such as *E. coli* are increasingly becoming FQ-R (244). Here, FQ-R is mainly caused by mutations altering the QRDRs of the primary target, DNA gyrase subunit A (GyrA), and the secondary target, DNA topoisomerase IV subunits (ParC) and, to a lesser extent, ParE (178, 179). However, additional mechanisms can also contribute to FQ-R, including regulatory mutations leading to AcrAB-TolC efflux pump over-production and expression of various PMQR genes. For example, the various *qnr* genes, *oqxAB* efflux pump genes, and *aac(6')Ib-cr*, which encodes a mutated aminoglycoside-modifying enzyme (178, 182).

Because most FQ-R in *E. coli* results from multiple mutations in target enzymes and because PMQRs cannot confer FQ-R alone, the spread of FQ-R *E. coli* typically involves vertical dissemination of resistant clones. Most commonly in humans, the widespread proliferation of an FQ-R subset of the ST131 complex (61), which is also frequently associated with 3GC-R (212). Additionally, the pandemic ST1193 group (245, 246), which is only occasionally 3GC-R (245, 247).

In secondary care, the fluoroquinolone ciprofloxacin is commonly used as an oral switch following intravenous therapy for serious infections caused by Gram negative bacteria (248). Accordingly, FQ-R is an important threat to human health, and particularly when found in urinary *E. coli* since a substantial proportion of sepsis has a urinary origin and *E. coli* are the leading cause of urosepsis (249). The use of fluoroquinolones in primary care in the UK has reduced in recent years, primarily due to changes in policies concerning prescribing for community urinary tract infections. For example, in the 1.5 million population centred on the city of Bristol, we recently demonstrated a 24% fall in dispensing of ciprofloxacin, by far the

most used fluoroquinolone in primary care, between 2013 and 2016. We noted a commensurate reduction in FQ-R in community-origin urinary *E. coli* in the same region (144).

The aim of the work reported in this chapter was to undertake a survey of FQ-R urinary *E. coli* from people in Bristol and surrounding regions. This was performed at the same time as the survey of 3GC-R urinary *E. coli* performed by Dr Jackie Findlay as part of the OH-STAR consortium project (see section 3.1.1 for details). As well as facilitating a molecular ecological assessment of FQ-R urinary *E. coli*, considering mechanism of resistance and population structure, the work was designed to allow comparisons with FQ-R isolates from cattle (results chapter 3) and dogs (results chapter 4). A considerable portion of the work presented in this chapter and the next is presented in a scientific paper currently in review, and preprinted on BioRxiv (222).

4.2. Results and discussion

4.2.1. Isolate selection and collection

As part of the OH-STAR project, 3GC-R urinary *E. coli* were collected first by Dr Jackie Findlay. Isolates were collected weekly, by visiting Severn Pathology diagnostic laboratory based at Southmead Hospital, Bristol, which serves 146 GP practices from the Bristol, Bath, South Gloucestershire and North Somerset region comprising a population of 1.5 million people. The procedure was as follows. Urine samples are automatically plated directly onto ChromAgar Orientation medium via a Becton Dickinson Kiestra automated laboratory system (to allow species identification), six antibacterial discs are placed directly, and the samples incubated and the susceptibility results read automatically by reference to zone of inhibition. Normally, the plates are then discarded, but during the time of the survey, laboratory staff kept plates flagged as cephalexin resistant and stored them at 4°C until collection and transportation to the University for further analysis. For each cephalexin resistant isolate, data were provided by the laboratory which allowed fluoroquinolone resistance/susceptibility to be defined, based on the original disc susceptibility testing via the Kiestra, in this case using a ciprofloxacin disc. Jackie Findlay picked all cephalexin resistant isolates onto the 3GC, cefotaxime, used at the EUCAST breakpoint concentration. All isolates that grew were designated 3GC-R. All 3GC-R isolates that were also designated as ciprofloxacin resistant were designated FQ-R and passed on to me for further analysis.

The aim was to analyse the molecular ecology of FQ-R/3GC-R dual resistant isolates, and this will be reported below. However, it was recognised that using FQ-R isolates which were also 3GC-R would constitute a biased selection and these could not be used as a sensible comparison to the dog and cattle isolates which did not undergo the same dual selection criterion (see results chapters 3 and 4). Furthermore, it was of interest to compare FQ-R isolates that were 3GC-R with those that were 3GC susceptible (3GC-S). Subsequent work was planned and conducted by myself. Plates were collected from Severn Pathology laboratory weekly, for six weeks. As described above, urinary *E. coli* that were flagged by the Kiestra as being FQ-R but 3GC-S. This latter status could be determined because a cefpodoxime (3GC) disc is also used in the Keistra workflow, so FQ-R isolates that flagged susceptible to cefpodoxime (3GC-S) could be requested specifically.

The aim in both surveys was to collect isolates sequentially. However, such was the voluntary nature of the help provided by busy diagnostic laboratory staff in setting aside plates for storage, it is entirely possible that some isolates were not retained. This was considered unlikely of itself to bias the survey, being essentially random.

All reportedly FQ-R urinary *E. coli* isolates provided on the initial selective agar having antimicrobial discs attached (e.g. as shown in Figure 10) were sub-cultured onto TBX, containing 0.5 mg/L ciprofloxacin, to confirm FQ-R status before further analysis. A total of 489 FQ-R urinary isolates were collected and, of these, approximately half (n=255) were also 3GC-R, whilst the remaining 234 were 3GC-S.

Figure 10. Example of urinary disc test plates obtained from Severn Pathology Laboratory

326 *E. coli* isolate-containing plates were collected and these were checked for resistance to ciprofloxacin confirmed to be *E. coli* by subculturing onto TBX agar containing 0.5 mg/L ciprofloxacin. The cefpodoxime disc (white arrow) zone of clearance was measured to confirm 3GC-S phenotype.

4.2.2. Multiplex PCR for PMQR genes

Collected isolates were analysed for the presence of PMQR genes using multiplex PCR (Methods and Materials 2.6). Positive controls were obtained and run alongside each sample (Figure 11).

Figure 11. Agarose gel showing positive controls for PMQR genes against Bioline HyperLadder™ 100bp.

Controls are positive for the following (from the bottom up): 1. *aac(6')-Ib-cr*, *oqxAB*, and *qnrS*; 2. *oqxAB* only; 3. *oqxAB* and *qnrB*; 4. *aac(6')-Ib-cr* and *oqxAB*; and 5. *qnrA* only. Ladder bands for the first 500 BP are also labelled, each other band represents an additional 100 BP.

489 isolates were analysed (e.g. a batch of these shown in Figure 12 for illustrative purposes) and PMQR genes were detected in 176 isolates, with a considerable difference noted between 3GC-S and 3GC-R isolates Table 5. No instances of *qnrA*, *qnrC*, *qnrD* or *qepA* were detected but a positive control was only available for one of these (*qnrA*).

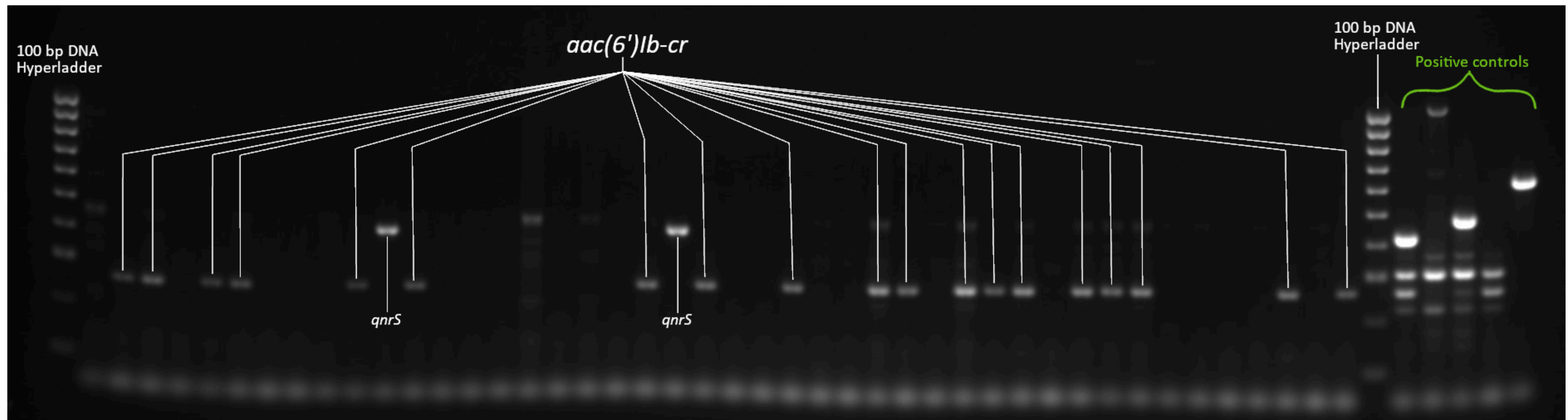


Figure 12. Agarose gel displaying PMQR multiplex PCR results for 44 urinary *E. coli* isolates.

Bioline HyperLadder™ 100bp and positive outcomes for *qnrS* and *aac(6')Ib-cr* are indicated in white, whilst positive controls, as shown in Figure 11. Suspected positive results for gene presence were run again to confirm outcome.

Table 5. PMQR genes detected in urinary isolates using multiplex PCR.
Differences were seen between 3GC-S and 3GC-R isolates in terms of frequency and percentage of carriage in 3GC-S or 3GC-R isolates. All isolates were confirmed non-susceptible to 0.5 mg/L CIP on TBX agar.

	3GC-S	**3GC-R**
aac(6')Ib-cr	26 (~11.1%)	135 (~52.9%)
qnrB	0	5 (~2.0%)
qnrS	5 (~2.1%)	9 (~3.5%)
oqxAB	0	2 (~0.78%)
PMQR total	31 (~13.2%)	151 (~59.2%)
No PMQR detected	203 (~86.8%)	107 (~42.0%)
Total	234	255

An association between carriage of any PMQR gene and 3GC-R/S status was observed (Table 5). To further investigate whether this association was statistically significant ($p<0.05$), a series of Pearson chi squared (χ^2) analyses were performed (Methods and Materials 2.10)(220) and a phi coefficient (φ) was calculated to determine the strength of the association (Methods and Materials 2.11). φ measures the strength of an association, determined by Pearson's χ^2 test, from 0 (no association) to 1 (perfect association). <0.25 is typically considered a weak associated, between 0.25 and 0.75 is considered moderate, and >0.75 is considered strong (220, 221).

The first of these investigated whether the presence or absence of a PMQR gene was significantly associated with 3GC-R/S status and determined what the strength of that association was. A moderate positive association was found between 3GC-R and carriage of any PMQR gene or, put another way, between 3GC-S and not carrying any PMQR gene (Table 6).

Table 6. χ^2 analysis exploring the association between carriage of a PMQR gene and 3GC-R/S.

Green highlighting indicating where a result is higher than that expected if the null hypothesis were true (there was no association) and blue highlighting indicating where a result is lower than expected. Degrees of freedom = (number of rows -1) x (number of columns -1) in the contingency table. The full analysis is shown here for illustrative purposes but results only will be shown from here on.

	PMQR	No PMQR	Total	Percentage
3GC-R	151	104	255	52.1
Expected	94.91	160.09		
3GC-S	31	203	234	47.9
Expected	94.91	160.09	489	
Total	182	307		
			χ^2:	110.35
	33.15	19.65	**φ:**	0.48
	36.13	21.42	**Degrees of freedom (DF):**	1
			P value:	<0.0001

The identification of a moderate statistically significant positive association between carriage of any PMQR gene and 3GC-R prompted a follow-up to explore whether the observed association was being driven by a more specific association with a particular PMQR gene. A similar, albeit slightly weaker, association was found between carriage of the PMQR gene, *aac(6')Ib-cr* and 3GC-R (χ^2=96.68, p<0.0001, φ=0.44). A weaker association was also identified between carriage of the much less common PMQR gene *qnrB* and 3GC-R. However, low prevalence of this gene led to an expected value of 2.6 in the χ^2 contingency table – an expected value of <5 renders the results invalid, due to the so called 'rule of five' (220). No other PMQR gene was present in sufficient frequency to draw significant conclusions about associations with other factors.

4.2.3. Mutations in the QRDR among FQ-R urinary *E. coli*

188 FQ-R urinary *E. coli* isolates were selected for WGS analysis. In total, 90 isolates were 3GC-R and 98 were 3GC-S. Sequences were analysed using the Center for Genomic Epidemiology (CGE) pipeline analysis, to detect presence of mobile resistance genes (ResFinder 2.1) and virulence genes (VirulenceFinder 1.2), and identify ST (MLST 1.6) (216). In addition, each isolate was analysed for QRDR mutations, known to increase fluoroquinolone MICs against *E. coli*, using the PointFinder program (Methods and Materials 2.8).

Only one isolate lacked a QRDR mutation, and the 187 other isolates all shared one specific QRDR mutation: *gyrA* pS83L. Two other QRDR mutations were also very common: *parC* pS80I (n=184) and *gyrA* pD87N (n=176). Overall analysis found 21 different patterns of QRDR mutation(s), referred to here as 'QRDR types' (Table 7).A large proportion of isolates (n=156) had one of three QRDR types, type 10, 12 or 15. All of these and most other QRDR types shared in common the same two gyrA mutations (D87N and S83L) and *parC* mutation (S80I) (n=176) and an additional six isolates shared mutations at these sites but with a different substitution at gyrA 87 – 5 D to H and one D to Y. Meaning only six FQ-R isolates lacked mutations at these 3 sites. This is not surprising given that these three mutations alone are sufficient for clinical resistance to ciprofloxacin and that is what was select for (179). However, 151 isolates had mutations in addition to these three sites, perhaps suggesting selective pressure from fluoroquinolone use is sufficient to necessitate a fitness advantage in the presence of fluoroquinolone that outweighs their cost when the compound is absent (250).

Table 7. QRDR mutation patterns

Assigned 'types' and frequency in 188 WGS urinary *E. coli* isolates

QRDR Mutations	Number of isolates.	Type	QRDR Mutations	Number of isolates.	Type
gyrA D87H, *gyrA* S83L, *parC* S80I	3	Type 1	*gyrA* D87N, *gyrA* S83L, *parC* S80I	28	Type 12
gyrA D87H, *gyrA* S83L, *parC* S80I, *parE* S458A	2	Type 2	*gyrA* D87N, *gyrA* S83L, *parC* S80I, *parE* E460D	1	Type 13
gyrA D87N, *gyrA* S83L, *parC* A56T, *parC* E84A, *parC* S80I	1	Type 3	*gyrA* pD87N, *gyrA* S83L, *parC* S80I, *parE* I464F	1	Type 14
gyrA D87N, *gyrA* S83L, *parC* A56T, *parC* S80I	4	Type 4	*gyrA* D87N, *gyrA* S83L, *parC* S80I, *parE* L416F	38	Type 15
gyrA D87N, *gyrA* S83L, *parC* A56T, *parC* S80I, *parE* I464F	1	Type 5	*gyrA* pD87N, *gyrA* S83L, *parC* S80I, *parE* S458A	5	Type 16
gyrA D87N, *gyrA* S83L, parC A56T, *parC* S80I, *parE* S458A	1	Type 6	*gyrA* pD87N, *gyrA* S83L, *parC* S80I, *parE* S458T	1	Type 17
gyrA D87N, *gyrA* S83L, *parC* E84G, *parC* S80I	1	Type 7	*gyrA* D87Y, *gyrA* S83L, *parC* S80I, *parE* L445H	1	Type 18
gyrA D87N, *gyrA* S83L, *parC* E84V, *parC* S57T, *parC* S80I	1	Type 8	*gyrA* S83L	2	Type 19
gyrA D87N, *gyrA* S83L, *parC* E84V, *parC* S80I	1	Type 9	*gyrA* S83L, *parC* S80I	2	Type 20
gyrA D87N, *gyrA* S83L, *parC* E84V, *parC* S80I, *parE* I529L	90	Type 10	*gyrA* S83L, *parE* I529L	1	Type 21
gyrA D87N, *gyrA* S83L, *parC* S57T, *parC* S80I	2	Type 11	No mutations	1	

4.2.4. Association between number of alternate QRDR SNPs and carriage of PMQR genes

We hypothesised that that the carriage of a PMQR gene would be negatively associated with the number of QRDR mutations present in an isolate. This is because as clinical FQ-R can be achieved with fewer QRDR mutations if a PMQR gene is also present (179). However, there was no significant association between PMQR gene carriage (of any type) and isolates having low numbers of QRDR mutations (χ^2=0.12, p=0.73). This did, however, highlight that isolates with large numbers of QRDR mutations, more than sufficient to confer FQ-R, commonly also carried PMQR genes, and so we set out to investigate the specifics of these associations.

An association was found between an isolate being of QRDR type10 and the carriage of the PMQR gene, *aac(6')Ib-cr* (χ^2=21.74, p<0.0001, φ=0.34). However, it was surmised that this might be due to an association between *aac(6')Ib-cr* carriage and the and a particular ST among which QRDR type 10 dominated. This was explored next.

4.2.5. ST analysis

Analysis of ST distribution revealed that, like QRDR type, FQ-R isolates were dominated by certain STs, particularly ST131 (n=94) and ST1193 (n=35), which together make up over two thirds of FQ-R isolates, and only four other STs had a frequency ≥ 5: ST162, ST38, ST69 and ST744 (Table 8).

Table 8. STs found in FQ-R urinary *E. coli* and the frequency with which they were presence.

ST	Freq.		ST	Freq.		ST	Freq.		ST	Freq.
ST10	2		ST209	1		ST405	2		ST69	12
ST101	1		ST224	1		ST443	1		ST744	5
ST1193	35		ST359	2		ST450	3		ST8312	1
ST12	1		ST361	1		ST4981	1		ST8313	1
ST131	94		ST369	1		ST4985	1		ST8467	1
ST155	1		ST38	6		ST648	2		ST93	3
ST162	6		ST393	1		ST6756	1		ST997	1

Considerable overlap was noticed between some of the QRDR types and STs, particularly between ST131 where 89/94 isolates had QRDR type 10 and between ST1193 where 35/35 isolates had QRDR type 15. χ^2 analyses, confirmed these associations (Table 9), but the low frequency of many STs and QRDR types meant that it was not possible to identify any other significant QRDR type/ST associations. Note that, as the φ value indicates, association was not perfect. There were 90 isolates with QRDR type 10, and 89 of these were ST131. Similarly, there were 38 isolates with QRDR type 15, and 35 of these were ST1193.The dominance of ST131 and ST1193 among urinary *E. coli* in this survey was not surprising given that STs 131 and 1193 are well known emergent pathogens which have rapidly disseminated in only the past few decades.

Table 9. Results of χ^2 analysis investigating the association between ST131 and the QRDR SNP pattern type10 and between ST1193 and QRDR type 15.

	χ^2	φ	P value
ST131 associated with QRDR type10	165.1	0.94	<0.0001
ST1193 associated with QRDR type 15	169.8	0.95	<0.0001

4.2.6. Comparing the individual isolate results for multiplex PCR and WGS ResFinder

The ResFinder-2.1 results agreed with the multiplex PCR results in 180/188 instances (95.7%). Of the eight cases where there was a mismatch between the two results, there were five instances where genes identified by ResFinder were not detected by multiplex PCR analysis – two instances of *aac(6')Ib-cr*, one instance of *qepA*, one instance of *qnrB* and one instance of a *qnrD*-like gene. Conversely, there were three instances where genes detected by multiplex PCR were not identified by ResFinder – all *aac(6')Ib-cr*. These are possibly instances where mutations or insertions, in *aac(6')Ib-cr* gene have led to ResFinder not detecting a match. Likewise, silent mutations in the primer binding sites may have led to a false negative from the multiplex PCR.

4.2.7. Exploring the carriage of other resistance genes among FQ-R isolates

ResFinder-2.1 analysis of WGS data was also used to explore the presence of mobile genes which confer resistance to other antimicrobials relevant to the OH-STAR project (Table 10): aminoglycosides (e.g. streptomycin), tetracycline, phenicols (e.g. chloramphenicol), and β-lactams (e.g. amoxicillin). 155 (82.4%) isolates carried at least one acquired β-lactamase gene (of which the 90 [47.9%] which were 3GC-R all carried at least one mobile 3GC-R gene), 143 (76.1%) carried at least one gene associated with aminoglycoside resistance, 123 (65.4%) carried a *tet* gene, conferring tetracycline resistance and 60 (31.9%) carried a gene associated with phenicol resistance. Many isolates had multiple resistance genes associated with resistance to each antimicrobial and 13 (6.9%) lacked any mobile resistance genes.

Table 10. Carriage of genes associated with resistance to non-fluoroquinolone antibacterials in FQ-R urinary *E. coli* isolates.

Aminoglycosides									
aac(3)-IIa	23	*aac(3)-IId*	25	*aadA1*	21	*aadA15*	1	*aadA17*	1
aadA2	12	*aadA24*	1	*aadA5*	95	*aadA8b*	1	*aph(3')-Ia*	13
strA	88	*strB*	87						
Tetracycline									
tet(A)	94	*tet(B)*	31	*tet(M)*	2	*tet(X)*	1		
Phenicols									
cmlA1	7	*catA1*	13	*catB3*	43	*floR*	4		
β-lactams									
bla$_{\text{CMY-2}}$	4	*bla*$_{\text{CMY-3}}$	1	*bla*$_{\text{CMY-42}}$	1	*bla*$_{\text{CMY-60}}$	1	*bla*$_{\text{CTX-M-1}}$	1
bla$_{\text{CTX-M-14}}$	2	*bla*$_{\text{CTX-M-15}}$	57	*bla*$_{CTX\text{-}M\text{-}27}$	22	*bla*$_{\text{CTX-M-3}}$	1	*bla*$_{\text{OXA-1}}$	41
bla$_{\text{SHV-12}}$	4	*bla*$_{\text{SHV-1}}$	1	*bla*$_{\text{TEM-1A}}$	2	*bla*$_{\text{TEM-1B}}$	82	*bla*$_{\text{TEM-1C}}$	2
bla$_{\text{TEM-33}}$	1								

Six aminoglycoside resistance genes were found in at least 10% of isolates (n>19): *aac(3)-IIa, aac(3)-IId, strA, strB, aadA1, aadA5*. The *aac(3)* enzymes code for aminoglycocide acetyltransferases, which modify and, thus give resistance to, gentamycin, tobramycin, dibekacin, netilmicin and sisomicin (251). The *strA* and *strB* genes, which were almost always found together in an isolate (n=86), code for streptomycin-modifying enzymes, and thus confer resistance to streptomycin (252). Finally, the *aadA* genes code for aminoglycoside adenylyltransferases, which typically adenylylate and confer resistance to streptomycin and spectinomycin (253). However, *aadA5* confers resistance to spectinomycin but not streptomycin (254). Notably, none of these enzymes affect amikacin which is, however, modified by *aac(6')Ib-cr* (255).

The *tet* genes, each give resistance to tetracycline, though through different mechanisms. The *tet(A)* and *tet(B)* genes code for efflux pumps, whilst, *tet(M)*, which is more typically found in Gram-positive bacteria, codes for a ribosomal protection protein (256). Finally, *tet(X)* codes for a tetracycline-inactivating enzyme, which has also been found to degrade tigecycline (257).

The *cat* genes, *catA1* and *catB3*, code for chloramphenicol acetyltransferases, whilst *cmlA1* codes for an efflux pump. All of which confer resistance to chloramphenicol. The *floR* gene also codes for an efflux pump which confers resistance to chloramphenicol, and also florfenicol (258). The β-lactamase genes are all discussed extensively in the Introduction

(section 1.10). Whilst not investigated in the OH-STAR project, interestingly one isolate, which was ST69, was found to carry the *mcr-1* gene, which confers resistance to the clinically important antimicrobial, colistin. Colistin is often reserved as a drug of last resort when all other treatment options have been exhausted, and *mcr-1* is the principal mechanism of mobile resistance to colistin (259).

4.2.8. Association between PMQR type and other mobile ABR genes

PCR analysis on the total collection of 489 FQ-R urinary isolates revealed a moderate positive association (φ=0.48 between 3GC-R status and carriage of *aac(6')Ib-cr* (discussed in 4.2.2). A χ^2 analysis using data obtained from WGS yielded a similar φ value of 0.47. It was hypothesised that carriage of *aac(6')Ib-cr* was not associated with 3GC-R status in general, but instead it was associated with the carriage of a particular 3GC-R gene – probably a $bla_{CTX\text{-}M}$ gene, since these are the principal cause of 3GC-R in the sequenced isolates (81/90 3GC-R isolates carried a $bla_{CTX\text{-}M}$ gene (none had more than one such gene). Only two $bla_{CTX\text{-}M}$ genes were present in sufficient frequency among the 90 sequenced FQ-R/3GC-R isolates to accurately test for an association with co-carriage of *aac(6')Ib-cr*: $bla_{CTX\text{-}M\text{-}15}$ (found in 57/90 sequenced isolates) and $bla_{CTX\text{-}M\text{-}27}$ (found in 22/90 sequenced isolates). χ^2 analyses were carried out looking for association between these carriage of blaCTX-M genes and all other resistance genes present in sufficient numbers to make an analysis possible, and carriage of *aac(6')Ib-cr* (Table 11).

Table 11. χ^2 analyses looking at association between carriage of *aac(6')Ib-cr* gene and other ABR genes. Results lacking statistical significance are highlighted in red.

aac(6')Ib-cr associated with carriage of:	χ^2	φ	Association	P value	n
β-lactamases:					
bla$_{CTX-M-15}$	81.95	0.66	Positive	<0.0001	188
bla$_{CTX-M-27}$	7.39	0.20	Negative	0.0066	188
bla$_{CTX-M-15}$ 3GC-only	29.48	0.57	Positive	<0.0001	90
bla$_{CTX-M-27}$ 3GC-only	22.27	0.50	Negative	<0.0001	90
bla$_{TEM-1b}$	7.37	0.20	Negative	0.0066	188
bla$_{OXA-1}$	155.16	0.91	Positive	<0.0001	188
Phenicol resistance:					
catB3	166.01	0.94	Positive	<0.0001	188
Tetracycline resistance:					
tet(A)	21.98	0.34	Positive	<0.0001	188
tet(B)	0.96	0.07	N/A	0.33	188
Aminoglycoside resistance:					
aac(3)-IIa	78.69	0.65	Positive	<0.0001	188
aac(3)-IId	0.77	0.06	N/A	0.38	188
StrA	0.15	0.03	N/A	0.70	188
StrB	0.44	0.05	N/A	0.51	188

A moderate positive association was found between carriage of *aac(6')Ib-cr* and *bla*$_{CTX-M-15}$ whilst, conversely, a negative association was found between carriage of *aac(6')Ib-cr* and *bla*$_{CTX-M-27}$. Strong positive associations were identified between carriage of *aac(6')Ib-cr* and carriage of genes which confer resistance to two other classes of antimicrobials, *catB3* and *bla*$_{OXA-1}$, whilst moderate positive associations were identified with an additional two genes, tet(A) and aac(3)-IIa. The *catB3* gene codes for a chloramphenicol acetyltransferase, which confers resistance to chloramphenicol (258) and *bla*$_{OXA-1}$ codes for the OXA-1 β-lactamase, an inhibitor-resistant enzyme which is discussed in Introduction (section 1.12). The *aac(3)-IIa* gene codes for an aminoglycoside acetyltransferase, which confers resistance to gentamycin, kanamycin, netilmicin and tobramycin (260) and the *tet(A)* codes for an efflux pump which confers resistance to tetracycline (261).

We further hypothesised that the association between carriage of *aac(6')Ib-cr* and carriage of bla_{OXA-1}, *catB3*, and $bla_{CTX-M-15}$ was due to physical linkage of $bla_{CTX-M-15}$ with the class 1 integron In*37*, carrying *aac(6')Ib-cr*, bla_{OXA-1} and *catB3* in the first three gene cassettes. Even given the difficulties of interpreting short-read WGS data, there was evidence in favour of this hypothesis. Of 11 isolates where the genomic locations of these genes could be definitively identified, in three, In*37* and $bla_{CTX-M-15}$ were both embedded (at separate locations) in contigs surrounded by known chromosomal genes. In eight, In*37* and $bla_{CTX-M-15}$ were immediately adjacent on the same contig, sometimes surrounded by known chromosomal genes, and sometimes not, suggesting a plasmid location. However, the small sample size is not sufficient to confirm that this is the reason for the association.

We next tested to see if there was an association between carriage of *aac(6')Ib-cr* and the isolate being from a particular ST. A positive association was found between ST131 and carriage of *aac(6')Ib-cr* (Table 12). However, this was not as strong as that observed between carriage of *aac(6')Ib-cr* and $bla_{CTX-M-15}$ (φ=0.66 – Table 11). Importantly, $bla_{CTX-M-15}$ is commonly found in ST131 (212), and we also confirmed a positive association between ST131 and carriage of $bla_{CTX-M-15}$ among our sequenced isolates (Table 12). So we conclude that the association between *aac(6')Ib-cr* carriage and $bla_{CTX-M-15}$ carriage is the primary association, and the fact that ST131 commonly carries $bla_{CTX-M-15}$ means that it also commonly carries *aac(6')Ib-cr* and, by extension, the other ABR genes associated with it (Table 11). Conversely, *aac(6')Ib-cr* was found to be negatively associated with ST1193 (χ^2=4.3, φ=0.15, p=0.038).

Table 12. Results of χ2 analyses looking at association between carriage of ABR genes, *aac(6')Ib-cr* and $bla_{CTX-M-15}$, and ST131.

ST131 associated with:	χ^2	φ	P value	n
aac(6')Ib-cr	15.95	0.29	<0.0001	188
$bla_{CTX-M-15}$	18.35	0.31	<0.0001	188
$bla_{CTX-M-15}$ in 3GC-R isolates	1.66	N/A	0.2	90

Taken together, we found that *aac(6')Ib-cr* is by far the most frequent PMQR gene among FQ-R urinary *E. coli*, despite the fact that in almost all isolates carrying this gene, FQ-R is being conferred by QRDR mutations. Of course, this PMQR gene is also an aminoglycoside resistance gene, which confers resistance to tobramycin, kanamycin, and amikacin (190). It may be that its presence is being selected by its ability to confer aminoglycoside resistance; whilst it was also found to be moderately (φ=0.63) associated with the the kanamycin and tobramycin resistance gene *aac(3)-IIa* gene (Table 11), this gene does not confer resistance to amikacin (262).

4.2.9. Virulence genes among FQ-R urinary isolates.

Given that isolates were urinary in origin, and that UPEC, and ExPEC in general, are associated with an array of virulence genes (95, 263, 264) it was expected that many of the isolates investigated would carry genes associated with extraintestinal virulence. Not only was this seen as an opportunity to explore association between the resistance genes discussed above and ExPEC virulence factors, but this also offered another avenue in which to explore the similarities and differences between the human-derived isolates and those obtained from cattle and canine samples, as will be set out in later chapters.

As discussed in the introduction (1.2), *E. coli* has an extensive accessory genome. This includes myriad virulence genes which are often associated with particular pathotypes (28). These are particularly important for disease progression and prognosis following colonisation of the urinary tract. WGS allowed for the carriage of virulence genes among the 188 sequence FQ-R urinary *E. coli* to be investigated, using VirulenceFinder 1.2 (215). In total, 36 separate virulence genes were identified, ranging in frequency among the 188 isolates from ~0.5% (one isolate carried the gene) to ~99.5% (187 isolates carried the *gad* gene) (Table 13). There were 17 genes, where each was found in at least 10 different isolates. The number of virulence genes found in a given isolate ranged from just one – always the *gad* gene – to 15. However, 8 of those 15 genes were involved in the production or regulation of the Aggregative Adhesion Fimbria (AAF), found in three ST38 isolates (265). Additionally, two other genes labelled ORF3 and ORF4 code for proteins thought to be involved in isoprenoid biosynthesis, due to their structural and sequence similarity with *trans*-isoprenyl phosphate synthases and isopentenyl isomerase enzymes, respectively. These have previously found to be strongly associated with the AAF system and are found in the same three ST38 isolates here (266). If these 10 AAF genes are treated as a single virulence factor then the highest number of virulence genes found in a single isolate is nine. Four ST69 isolates carried the same genes, one ST1193 and one ST12 had nine virulence genes.

The ST69 isolates and the ST1193 isolate shared seven genes in common: *eilA*, *gad*, *iha*, *iss*, *lpfA*, *sat*, *senB*. Below, I will discuss the ten most frequency identified virulence genes.

Table 13. Virulence gene frequencies in human urinary *E. coli* isolates

Gene	Protein	Freq.		Gene	Protein	Freq.
aap	Anti-aggregation protein	3		*iha*	Iha	147
aar	AggR-activated regulator	3		*ireA*	Iron-regulated outer membrane virulence protein	11
aatA	APEC autotransporter adhesin	3		*iroN*	Ferric enterobactin receptor	22
agg3B	Aggregative adherence fimbriae	3		*iss*	Increased serum survival	135
agg3C	Aggregative adherence fimbriae	3		*lpfA*	Fimbrial major protein	29
agg3D	Aggregative adherence fimbriae	3		*mchB*	Microcin H47	2
agg5A	Aggregative adherence fimbriae	3		*mchC*	MchC protein	2
aggR	Aggregative adherence regulator	3		*mchF*	Probable microcin-H47 secretion/processing ATP-binding protein MchF	18
air	Enteroaggregative immunoglobulin repeat	22		*mcmA*	Microcin M	3
astA	Enteroaggregative *E. coli* heat-stable enterotoxin (EAST-1)	14		*nfaE*	Chaperone protein NfaE	29
capU	Hexosyltransferase homologue	7		*ORF3*	*Trans*-isoprenyl phosphate synthase?	3
ccl	Cloacin DF13 protein	1		*ORF4*	Isopentenyl isomerase?	3
celb	Permease IIC component	10		*sat*	Secreted autotransporter toxin	140
cma	Colicin M	13		*senB*	Enterotoxin TieB protein	90
cnf1	Cytotoxic Necrotizing Factor 1	19		*sfaS*	S-fimbrial adhesin protein SfaS	1
eatA	ETEC autotransporter A	1		*sigA*	Serine protease autotransporter toxin SigA	1
eilA	HilA family transcriptional regulator	25		*tsh*	Temperature-sensitive hemagglutinin tsh autotransporter	6
gad	Glutamate decarboxylase	187		*vat*	Vacuolating autotransporter toxin	35

The glutamate decarboxylase gene (*gad*) was found in 187 isolates. *E. coli* typically carry two very similar glutamate decarboxylase genes, *gadA* and *gadB*, as a result of a gene duplication event early in the evolutionary history of the *Escherichia* genus (267). Whilst these have been suggested as pre-screening markers for the presence of pathogenic *E. coli*, this was to screen all *E. coli* before looking for virulent strains, specifically (268). Other genes, involved in the regulation of *gadA/B,* have also been named *gad*, such as *gadX*, which activates transcription of *gadA/B*, and thus increases acid resistance. This has been associated with enterovirulent *E. coli* (269). However, it does not appear to be the case that this is what the VirulenceFinder 1.2 program is identifying (if it were it presumably would not be so widespread). The paper associated with the program only lists the *gad* gene and describes it as glutamate decarboxylase, without any further justification for its apparent association with virulence (215). It will therefore be omitted from further consideration or discussion.

The *iha* gene codes for the iron-regulated gene homologue adhesin (Iha) (270). This adhesin and siderophore receptor is commonly found in UPEC isolates (95, 109), and has been found to aid in the colonisation of the urinary tract, in a murine model, through its activity as a catecholate siderophore receptor and adhesin (112, 270).

Like *iha*, the *sat* gene, which codes for the secreted autotransporter toxin (Sat) is commonly found in UPEC isolates (109, 114). Sat has been found to be cytotoxic to bladder and kidney cells, *in vitro*, so may play a role in the progression of disease from simple to complicated UTI. However, a murine model experiment was not able to demonstrate this, so whilst Sat may play a role in complicated UTI, other factors are likely necessary as well (114).

The *iss* gene codes for the increased serum survival protein (Iss), a surface protein which protects against phagocytosis (116). The *iss* gene has consistently been found in over 75% of APEC isolates (95, 109, 271), where it is often associated with a large virulence plasmid, ColV (116), but it is also commonly found in UPEC (95, 109), and is thought to play a significant role in disease progression in SEPEC and NMEC (116). Its significance in urinary isolates might appear a mystery but phagocytic leucocytes are excreted in urine during UTI (272), which explains why the *iss* gene, which codes for a membrane protein that confers resistance to phagocytosis, is so common in the urinary isolates (111).

The *senB* gene, codes for the TieB enterotoxin, and has been associated with EIEC (273), where it is purported to play a role in enterotoxicity (274). It has been associated with UTI but what exactly its role is remained unclear (273, 275)

The *vat* gene codes for the vacuolating autotransporter toxin, Vat – a cytotoxin, which appears to play a significant role in sepsis (115). It has also been found to greatly enhance ability to colonise the urogenital tract and is associated with NMEC (276).

The *nfaE* codes for an adhesin protein, NfaE, which is a member of the Dr family of adhesins (277). Dr adhesins are typically associated with DAEC, discussed in introduction (section 1.7). Some DAEC variants have been associated with diarrhoeal disease, whilst others have been found to cause UTI (102).

The long polar fimbriae (Lpf) gene, *lpfA*, is typically associated with STEC (278), and other diarrhoeagenic *E. coli* (279), and has been associated with gut colonisation (278). However, it has been previously found in UPEC as well, in 12.4% of isolates examined in one study (280).

The *eilA* gene codes for EAEC HilA homologue, EilA, which acts as a transcriptional activator of genes associated with adhesion in EAEC (281). It might be surmised, then, that the isolates carrying *eilA* are EAEC. However, only three isolates were found to carry the AAF genes, discussed above, which are characteristic of the EAEC phenotype (265) and whilst the *eilA* gene is found in these isolates, it is also found in 22 others. A review of the literature suggests it has not previously been found in ExPEC isolates. Whether or not it affects adhesion in non-EAEC isolates, or has some other function, is not clear.

The *iroN* gene codes for the catecholate siderophore receptor, IroN, which binds to and uptakes the salmochelin siderophore (282). It has previously been found to be strongly associated with ExPEC, especially APEC, though it is also very common in UPEC and NMEC isolates (95, 109).

Like *eilA*, the *air* gene, which codes for the enteroaggregative immunoglobulin repeat protein (Air), is usually associated with EAEC (281). These two genes were found to heavily overlap. All isolates which carried *air* also carried *eilA*.

4.2.10. Virulence genes and phylogroups

It has been known for some time that ExPEC typically belong to *E. coli* phylogroup B2 and, to a lesser extent, phylogroup D (96, 99) and, indeed, B2 was by far the most represented phylogroup (heavily dominated by STs 1193 and 131) among the 188 sequenced urinary FQ-R *E. coli*, distantly followed by phylogroup D (Table 14). Phylogroups A and B1 were found in the next highest frequency, whilst two group F isolates (both ST648), one group C (ST369) and one group E (ST997) were also present. Importantly, the isolates being sequenced were collected from urine samples. Many but not all of these will be from people

with active UTI. In other cases, bacteriuria was asymptomatic and picked up following routine screening. We do not have clinical data to determine the proportion of each type, but it was assumed that a majority would be from patients suffering symptomatic UTI.

It was hypothesised, therefore, that a higher virulence gene carriage rate is a factor in the successful colonisation and infection of the urinary tract and – given that all isolates are of urinary origin, if not actually causing disease – most would share a similar set of virulence genes, regardless of phylogroup. However, that was found not to be the case. The average number of virulence genes varied considerably across different phylogroups and was surprisingly low in phylogroup B2, which is typically associated with ExPEC (96). Lower in fact than the average across all phylogroups. The AAF-associated genes present in three phylogroup D (ST38) isolates are treated as one virulence gene here but if they are counted individually, the average number of virulence genes in group D rises to 8.2.

Table 14. Phylogroup frequency and average number of virulence genes carried.

Phylogroup	Number of isolates.	Avg. virulence genes		Phylogroup	Number of isolates.	Avg. virulence genes
A	18	3.4		F	2	5
B1	12	5.3		C	1	7
B2	133	5.1		E	1	8
D	21	7.1		**Total**	**188**	**5.2**

Interestingly, whilst the average number of virulence genes in any given phylogroup B2 isolate is relatively low, two of the three isolates found to have nine virulence genes (discussed above) are from phylogroup B2 (STs 1193 and 12) and one ST131 isolate has eight virulence genes. Indeed, there is surprising variation in the number of virulence genes carried by ST131 and ST1193 isolates, ranging from 2 to 8, and 3 to 9, respectively.

4.2.11. Association between virulence gene carriage and ST

Given the considerable variability seen in the carriage rate of virulence genes within STs 131 and 1193, it was hypothesised that virulence genes are frequently gained and lost and that there would not, therefore, be a great deal of association between ST and the carriage of particular genes. Again, χ^2 analyses were conducted to investigate this (Table 15 and Table 16).

Table 15. χ^2 analyses results. Virulence gene carriage versus ST131 or other ST.

Gene	Association	χ^2	φ	P value		Gene	Association	χ^2	φ	P value
cnf1	ST131	12.82	0.26	0.0003		***ireA***	Not ST131	11.93	0.25	0.0006
vat	Not ST131	43.93	0.48	<0.0001		***iss***	ST131	54.56	0.54	<0.0001
lpfA	Not ST131	35.03	0.43	<0.0001		***nfaE***	ST131	14.25	0.28	0.0002
iroN	Not ST131	13.57	0.27	0.0002		***mchF***	Not ST131	9.13	0.22	0.003
sat	ST131	50.56	0.52	<0.0001						

Table 16. χ^2 analyses results. Virulence gene carriage versus ST1193 or other ST

Gene	Association	χ^2	φ	P value		Gene	Association	χ^2	φ	P value
cnf1	Not ST1193	4.84	N/A	0.03		***ireA***	Not ST1193	0.70	N/A	0.4
vat	ST1193	162.53	0.93	<0.0001		***iss***	Not ST1193	101	0.73	<0.0001
lpfA	Not ST1193	5.21	N/A	0.02		***nfaE***	Not ST1193	7.84	0.20	0.005
iroN	Not ST1193	3.26	N/A	0.07		***mchF***	Not ST1193	4.55	N/A	0.03
sat	ST1193	11.63	0.25	0.0006						

Nine virulence genes were found to have significant association (positive or negative) with ST131 (Table 14). A moderate positive association was found with *cnf1*, *iss, nfaE*, and *sat*. It was hypothesised that these would be associated with the co-associating ABR genes, discussed in section 4.2.8. Carriage of two virulence genes, *vat* and *sat* were significantly associated with ST1193 (Table 15)but the association was very strong for *vat*.

Several genes were found to have a significant negative association with ST1193; these included *cnf1*, *iss* and *nfaE*, shown to be associated with ST131, above. But other genes, (*lpfA*, *iroN*, *ireA*, and *mchF*) were negatively associated with either ST1193 or ST131, suggesting there might be association instead with lower frequency STs, for which there were not enough isolates present in the analysis to draw significant conclusions.

4.2.12. Association between virulence factor gene carriage and 3GC-R/S phenotype

Given that *bla*$_{CTX-M-15}$ carriage is moderately associated with ST131 (Table 12, φ = 0.31), it was hypothesised that *bla*$_{CTX-M-15}$ carriage is driving the association between ST131 and the three virulence genes, *cnf1*, *iss*, and *nfaE*. To investigate this, χ^2 analyses were carried out for virulence genes found in >10 FQ-R isolates and where there appeared to be a difference between prevalence among 3GC-S and 3GC-R isolates (x1.5 frequency difference). These amounted to 10 virulence genes (*astA*, *cnf1*, *ireA*, *iroN*, *lpfA*, *mchF*, *nfaE*, *vat*, *iss*, and *celB*) (Table 17).

Table 17. χ^2 analyses results. Virulence gene carriage versus 3GC-R/S.

Gene	Association	χ^2	φ	P value		Gene	Association	χ^2	φ	P value
cnf1	3GC-R	11.18	0.24	0.0008		***ireA***	3GC-S	10.73	0.24	0.0001
vat	3GC-S	13.39	0.27	0.0003		***iss***	3GC-R	9.25	0.22	0.0023
nfaE	3GC-R	2.77	N/A	0.096		***astA***	N/A	1.63	N/A	0.20
iroN	N/A	1.32	N/A	0.25		***lpfA***	3GC-S	3.90	0.14	0.04
mchF	N/A	3.22	N/A	0.07		***celB***	N/A	0.26	N/A	0.06

vat was moderately associated with 3GC-S (Table 17), but this was a much weaker association than was observed between *vat* and ST1193 (Table 16), which is more often 3GC-S (n=30) than 3GC-R (n=6), suggesting that its association with ST1193 is driving the apparent association observed between *vat* and 3GC-S. A similar outcome was observed with the *iss* gene, which was moderately associated with ST131 (Table 15), and weakly associated with 3GC-R (when ST131 is commonly 3GC-R). The *cnf1* gene, on the other hand, showed approximately the same strength association with ST131 (Table 15) as was observed with 3GC-R, suggesting that in this case the virulence gene might be associated with a resistance gene that causes 3GC-R, so this was next investigated.

4.2.13. Association between virulence genes and ABR genes

In section 4.2.9 it was observed that *aac(6')Ib-cr* carriage is strongly associated with other ABR genes, *bla*$_{OXA-1}$ and *catB3* (likely as part of the class 1 integron In*37*) and moderately associated with *bla*$_{CTX-M-15}$, *tet(A)*, and *aac(3)-IIa*, likely due to their physical linkage with In*37* (Table 11). It was hypothesised that some virulence genes may share this association, and

so a final set of χ^2 analyses were carried out to explore this. These compared the presence of four virulence genes positively associated with ST131 or ST1193 (*cnf1*, *iss*, *nfaE*, and *vat* – Table 15 and Table 16), as well as three other virulence genes, present in more than a quarter of the isolates (n>47 – Table 13 *iha*, *senB* and *sat*), with carriage of *aac(6')Ib-cr* and other ABR genes which were either associated (positively or negatively) with *aac(6')Ib-cr* ($bla_{CTX-M-27}$, bla_{TEM-1b}, $bla_{CTX-M-15}$, bla_{OXA-1}, *catB3*, *tet(A)*, *aac(3)-IIa* – Table 11) or were found in more than a quarter of the isolates (*aadA5*, *strA*, *strB* – Table 10). Because many variables are compared, I have chosen to use a higher threshold for significance (p<0.01) and, for brevity, only statistically significant outcomes have been presented.

Table 18. Association between virulence genes and ABR genes found in FQ-R urinary *E. coli*

ABR Gene	Vir. Gene	χ^2	φ	P value	ABR Gene	Vir. Gene	χ^2	φ	P value
aac(6')Ib-cr	*vat*	7.51	0.20	0.006	$bla_{CTX-M-27}$	*iha*	6.95	0.19	0.008
$bla_{CTX-M-15}$	*cnf1*	23.66	0.35	<0.0001	$bla_{CTX-M-27}$	*senB*	14.79	0.28	0.0001
$bla_{CTX-M-15}$	*nfaE*	13.00	0.26	0.0003	$bla_{CTX-M-27}$	*sat*	8.54	0.21	0.003
$bla_{CTX-M-15}$	*vat*	9.63	0.23	0.002	bla_{OXA-1}	*nfaE*	7.70	0.20	0.006
aadA5	*iss*	10.06	0.23	0.002	*tet(A)*	*iss*	7.59	0.20	0.006
aadA5	*nfaE*	8.80	0.22	0.003	*tet(A)*	*vat*	12.67	0.26	0.0004
aadA5	*sat*	7.63	0.20	0.006	*strA*	*nfaE*	12.04	0.25	0.0005
aadA5	*vat*	13.18	0.26	0.0003	*strA*	*SenB*	12.07	0.25	0.0005
bla_{TEM-1B}	*cnf1*	6.66	0.19	0.01	*strB*	*nfaE*	11.63	0.25	0.0006
bla_{TEM-1B}	*iss*	6.64	0.19	0.01	*strB*	*senB*	11.05	0.24	0.0009
bla_{TEM-1B}	*vat*	8.54	0.21	0.003	*catB3*	*nfaE*	9.40	0.22	0.002
bla_{TEM-1B}	*sat*	7.40	0.20	0.007	*catB3*	*vat*	7.18	0.20	0.007

Five ABR genes were found to be significantly positively associated with *aac(6')Ib-cr* (table 7): $bla_{CTX-M-15}$, bla_{OXA-1}, *catB3*, *tet(A)*, and *aac(3)-IIa*. Most of these, and *aac(6')Ib-cr*, were also found to have a weak-to-moderate negative association with the *vat* gene which, in turn, is strongly positively associated with ST1193 (Table 16) and negatively associated with ST131 (Table 15 – zero ST131 isolates carry the *vat* gene). Accordingly, this particular ABR

gene/virulence gene association was expected. The relatively low-rate of *vat* carriage seen here (19%), compared to the 55-62% observed in other studies of UPEC isolates, suggests that, outside of ST1193 it is strongly negatively associated with FQ-R (95, 109).

Conversely, at least one of the six co-associating ABR genes (Table 11), was found to be positively associated with *cnf1*, *iss*, *nfaE*, or *sa*t (Table 18). Associations between ABR genes and *iss* and *vat* are clearly driven by their primary association with ST131 which was considerably more strongly associated with *iss* (φ=0.54 – Table 15) and *sat* (φ=0.52 – Table 15) than with any of the ABR genes, and which was earlier found to be moderately positively associated with *aac(6')Ib-cr* (φ=0.29 – Table 12) and $bla_{\text{CTX-M-15}}$ (φ=0.31 Table 12). This was probably also the case for the *nfaE* gene, which had a slightly stronger association with ST131 (φ=0.28 – Table 15) than was observed between *nfaE* any of the ABR genes. This was not the case, however, with *cnf1*, which had a stronger association with $bla_{CTX\text{-}M\text{-}15}$ (φ=0.35), than was observed with either ST131 (φ=0.26 – Table 15) or 3GC-R (φ=0.24 – Table 17). A moderate association (φ=0.40) between *aac(6')Ib-cr* and *cnf1* was hinted at but the relatively low combined frequencies of these two genes led to an expected value of less than five (4.45) in the contingency table for the χ^2 analysis, invalidating the conclusion (220). A similar outcome was observed between $bla_{\text{OXA-1}}$ and *cnf1* (φ=0.29) and between *aac(3)-IIa* and *cnf1* (φ=0.57). This suggests that perhaps *cnf1* is genetically linked to these resistance genes among ST131 isolates.

Lastly, some significant but weak positive associations were also identified between other ABR genes and virulence genes. The $bla_{\text{TEM-1B}}$ gene was found to be positively associated (φ=0.21) with *vat* and *strA/B* was found to be positively (φ=0.24) associated with *senB*. Whilst *iss* and *iha* have previously been found to be positively associated with multiple drug resistance (108), a review of the relevant literature suggests that the associations identified here, between specific virulence and ABR genes, have not been detected previously, and may have important implications for treatment.

4.2.14. Phylogenetics comparing 3GC-R and 3GC-S FQ-R isolates

Sequenced FQ-R urinary *E. coli* isolates were aligned and SNP variants called using Snippy (Methods 2.9). This was used to construct a maximum likelihood phylogenetic tree using RAxML (Methods 2.9), comparing 3GC-S isolates with 3GC-R (Figure 13). As well as illustrating the dominance of ST131 (mainly 3GC-R, green markers) and ST1193 (mainly 3GC-S, orange markers) this tree also shows that clusters of isolates fell into ST744, ST162, ST69 and ST38, of which only the last had a substantial proportion of 3GC-R isolates. Interestingly, three ST131 isolates were found to form a distinct clade, separate from the

main ST131 clade (Figure 1). *FimH* typing revealed that two of these isolates contain the *fimH*41 allele, which is associated with ST131 clade A, whilst the third isolate contains the poorly characterised *fimH*42 (212). Each of these had a unique set of mutations in the QRDR, types 13 and 18 in the two *fimH*41 isolates, and type 21 in the *fimH*42 isolate.

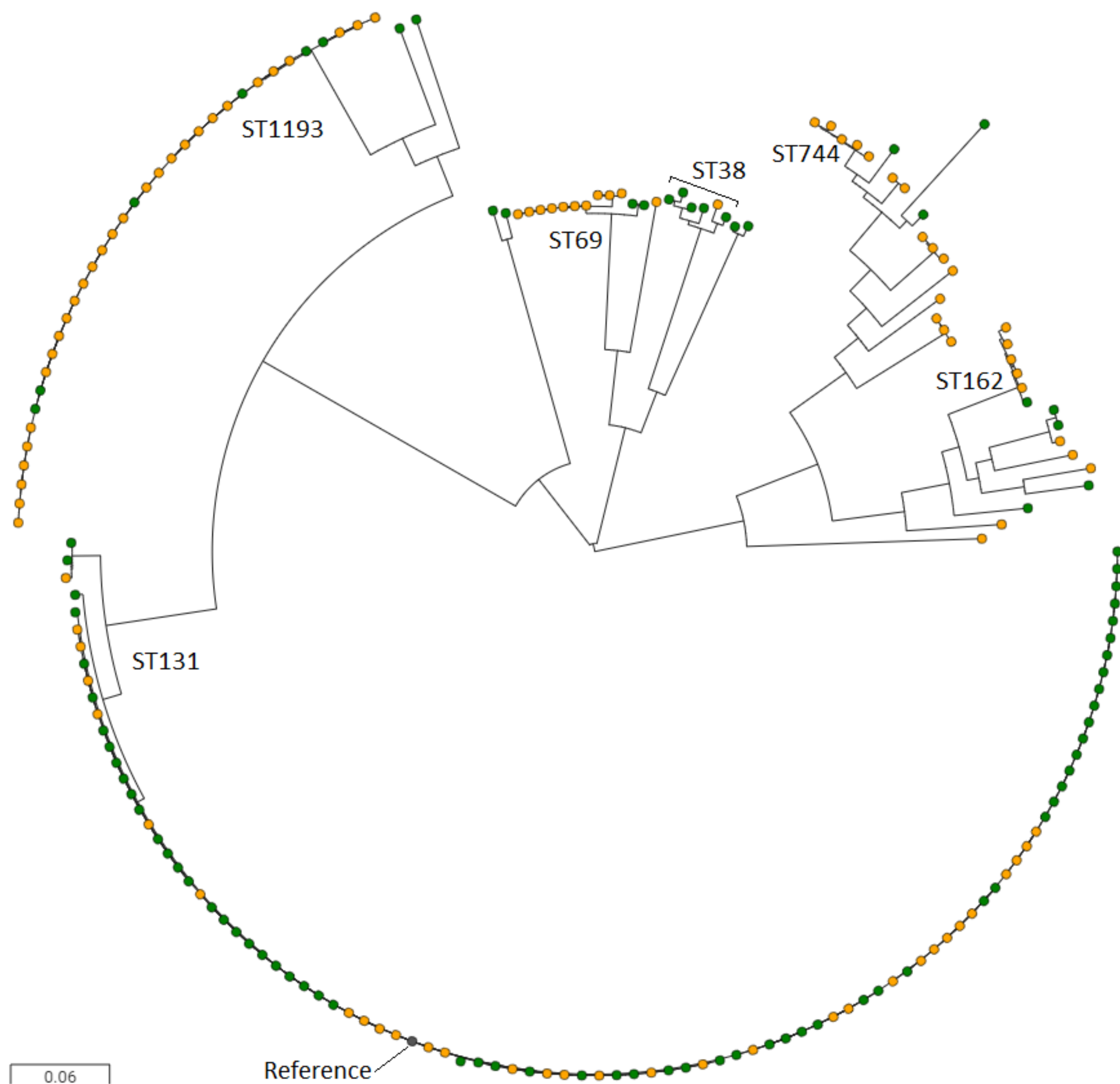


Figure 13. Maximum likelihood phylogenetic tree showing FQ-R urinary isolates. Green nodes were also 3GC-R, whilst yellow nodes were 3GC-S. Sequences were aligned against an ST131 reference (grey node). STs have been indicated where more than five isolates from that ST were present.

4.3. Discussion

3GC-R ST131 urinary *E. coli* isolates commonly carry *bla*$_{CTX-M-15}$ in this study region (212). Therefore, after finding a positive association between carriage of *bla*$_{CTX-M-15}$ and carriage of *aac(6')Ib-cr* among FQ-R urinary isolates studied here, it was not surprising to find a positive association between *aac(6')Ib-cr* carriage and ST131 and between *aac(6')Ib-cr* carriage and QRDR Type 10 (Table 9), which is the QRDR type dominated by ST131. However, of 32 FQ-R/3GC-S ST131 *E. coli*, only four carried *aac(6')Ib-cr*, confirming *aac(6')Ib-cr* was linked with *bla*$_{CTX-M-15}$ and not with ST131. We also showed that *bla*$_{CTX-M-15}$ and *aac(6')Ib-cr* were further linked to an additional four ABR genes, *bla*$_{OXA-1}$, *catB3*, *tet(A)*, and *aac(3)-IIa* (Table 11), which is likely to reflect common multi-drug resistance arrangements including In*37* and other genes.

In contrast, ST1193 isolates in this study were found mainly to be 3GC-S (n=28, versus n=6 for 3GC-R;), and there was a significant negative association between *aac(6')Ib-cr* carriage and the isolate being ST1193. There was also a negative association between carriage of *bla*$_{CTX-M-27}$ and carriage of *aac(6')Ib-cr* (Table 11). These associations reinforce the conclusion that In*37* (including *aac(6')Ib-cr*) and *bla*$_{CTX-M-15}$ are closely genetically linked in urinary *E. coli* in our study region.

Virulence factor genes were widespread and numerous and we did find some associations between certain genes and certain types, including an association with vat and ST1193, and between cfn1 and the *aac(6')Ib-cr*) and *bla*$_{CTX-M-15}$ ABR gene cluster. The relevance of these findings to disease and patient outcome are uncertain, but it does suggest at least that virulence might be co-selected with ABR and this would add to the reasons for reducing ABR in commensal *E. coli* with the potential to cause disease.

Future chapters will consider these findings in the context of animal-associated FQ-R *E. coli* and consider the potential for zoonotic transmission to humans.

5. Results Chapter 3. Fluoroquinolone resistance in dairy cattle *E. coli*; a potential source of zoonotic infection?

5.1. Introduction

As well as being widely used in human medicine (e.g. ciprofloxacin and levofloxacin, see results chapter 2), fluoroquinolones have been extensively used for the treatment of companion (283) and farmed animals (284) for decades. The principal compounds used in veterinary medicine (but never in human medicine) are marbofloxacin and enrofloxacin, but three others – pradofloxacin, danofloxacin and orbifloxacin – are also used (see Introduction – 1.13) (169).

Fluoroquinolone usage (mg/kg) has decreased in farmed animals in the UK over recent years, down by 81% from 2017 to 2019 (285), and voluntary use restrictions were introduced in June 2018 that mean fluoroquinolones are only used as a last resort, backed up by susceptibility testing (125) and, as of 2019, total fluoroquinolone sales for food-producing animals were down by 61% since 2015. However, whilst total fluoroquinolone use has decreased, orally administered fluoroquinolone use has actually increased – 61% of fluoroquinolone sales were for oral use in 2018 (285) – and 7.2% of cattle *E. coli* were found to be resistant to enrofloxacin in 2019, up from 6.6% in the previous year (286). There is still the possibility, therefore, that FQ-R *E. coli* that have been selected on farms might colonise humans and ultimately cause disease. These might be ExPEC or commensal/opportunistic *E. coli* types. There is also strong evidence that ABR *E. coli* are shared between companion animals and humans (287), which will be considered in results chapter 4. Hence, considering FQ-R in humans through the lens of the One Health research framework may help obtain a wider picture of selection and transmission.

Our primary aim in the work reported in this chapter was to test the hypothesis that FQ-R *E. coli* obtained as part of the OH-STAR survey (see results chapter 1) in 2017-18 from dairy farms located within a 50 x 50 km region of South West England were closely related to FQ-R human urinary *E. coli* collected in parallel from the same region (as described in results chapter 2), suggestive of transmission. Whilst our similarly motivated studies (set out in results chapter 1) of 3GC-R *E. coli* showed no evidence of recent sharing of isolates between dairy farms and humans (213, 223), we considered that the clonal nature of FQ-R *E. coli* might give a different outcome.

As well as having zoonotic potential, *E. coli* are a leading cause of mastitis – inflammation of the mammary glands (udder tissue), caused by infection – in dairy cattle. Mastitis is an important factor in dairy cattle morbidity, conveying a considerable economic burden on the

dairy industry through its effect on milk production and negatively impacting animal welfare (288). A leading source of mastitis-causing *E. coli* is bacteria deposited into the near-cattle environment in faeces. Dirtying of the udders in a faecally-contaminated environment has been strongly associated with an increased risk of clinical *E. coli* mastitis (289). Clinical *E. coli* mastitis is therefore typically considered an environment hazard, an opportunistic infection rather than an infectious disease spread from one animal to another (289, 290).

As with many ExPEC types (Introduction – 1.7), definition of the requirements for a mastitis-associated pathotype does not appear to stand up to scrutiny, but despite the seemingly opportunistic nature of mastitis, the existence of a mammary pathogenic *E. coli* (MPEC) has been proposed (290). No specific set of virulence genes appears to be required for the establishment of infection or is associated with mastitis (121, 290) but specific virulence genes have been associated with mastitis-causing *E. coli*, including the siderophore receptor, *fecA* (291), and long polar fimbriae gene, *lpfA*, which was also found to enable epithelial invasion (292) and has previously been associated with enterovirulent *E. coli* (279), but has also been found in UPEC isolates (280) so it is not clear how valuable the MPEC designation is. However, it does show that cattle pathogens can carry virulence factors associated with other ExPEC types, and this potentially adds to the zoonotic threat.

The cattle lactation cycle includes a critical 'dry period', during which cows are not milked. This is important to maintain high milk yields and allows time for the recovery from subclinical intramammary infection (IMI), that is an infection with little or no signs or symptoms, which can be a precursor of mastitis. However, cows are also at increased risk of the development of new infections during this time. To facilitate both the elimination of existing IMI and prevent new infection, a programme of 'dry cow therapy' is applied on many dairy farms, whereby udders are injected with an antibacterial, sometimes in combination with an internal teat sealant. To reduce antibacterial use, increasingly the approach is to select cows at greatest risk for dry cow therapy, so called "selective dry cow therapy", and different farms will therefore treat different percentages of their herd. Three types of antimicrobials are commonly used for this purpose: the aminoglycoside, framycetin (neomycin B); and the β-lactams, cloxacillin (a penicillin) and various cephalosporins (293, 294). As well as considering zoonotic potential, another aim of the work reported in this chapter was to consider in molecular ecology terms by analysis of WGS data, why certain antibacterial usage risk factors, including dry cow therapeutic use, have been associated during OH-STAR with an increased odds of finding FQ-R *E. coli* on farms.

5.2. Results and discussion

5.2.1. Antibacterial disc susceptibility testing

FQ-R *E. coli* isolates were selected from farm samples, as described in chapter 1 – 3.1.1. Of 4145 faecal samples collected from vicinities near animals, 263 were positive for FQ-R *E. coli*, representing 49 of 53 farms surveyed. A subset of the FQ-R isolates (n=113) were subjected to disc susceptibility testing (Methods and Materials 2.7), to confirm FQ-R phenotype and investigate which other antibacterials these FQ-R isolates were resistant to. The percentage of isolates which were also resistant to other antibacterials was calculated, using Clinical and Laboratory Standards Institute (CLSI) (human clinical) breakpoints to define resistance (295) (Table 19).

Based on data from Severn Pathology – the source of the human urinary isolates presented in results chapter 2 – of 144,855 urinary *E. coli* cultured in 2013-2016, 14,886 (10.3%) were FQ-R. Of these FQ-R isolates, 5,008 (33.6%) were also 3GC-R. Strikingly, of the FQ-R dairy farm isolates collected in the OH-STAR survey, only 3.54% were 3GC-R, based on cefotaxime disc susceptibility testing. (Table 19).

Table 19. Total number of isolates resistant, intermediate, or susceptible to each non-FQ antibacterial

Phenotypes were determined by antibacterial disc suscepbility testing, using CLSI breakpoints *(295)*.

	Tetracycline	Streptomycin	Chloramphenicol	Cefotaxime	Cefuroxime
Susceptible	8	16	36	95	97
Intermediate	2	17	2	3	1
Resistant	92	69	64	4	4
Percentage resistant:	90.2%	67.6%	62.7%	3.9%	3.9%

During the time of the study, the UK Veterinary Medicines Directorate (VMD), an Executive agency of the Department for Environment, Food and Rural Affairs (Defra) surveyed resistance to a range of antibacterials among *E. coli* from dairy cattle (285, 286). The breakpoints they used to assign resistance were different to those used by CLSI, above, but if their breakpoints are applied to the disc testing data gathered here for FQ-R *E. coli*, the

two sets of results can be more accurately compared. The most notable difference in the FQ-R isolates analysed here was that the VMD breakpoints put cefotaxime resistance at 10.8% (Table 20), whereas, the CLSI breakpoints put it at 3.9% (Table 19). For other antibacterials, resistance rates were almost identical when applying either breakpoint.

Table 20. Comparison of the rates of resistance found in dairy cattle *E. coli* across England and Wales

Comparing results gathered by VMD *(285, 286)*, with FQ-R *E. coli* found in the South West as part of OH-STAR and analysed as above. The total number of isolates tested (n) is also shown.

Percentage resistant	Tetracycline	Streptomycin	Chloramphenicol	Cefotaxime
FQ-R *E. coli* (OH-STAR)	91.2% (n=113)	70.6% (n=113)	65.7% (n=113)	10.8% (n=113)
VMD 2017	69.8% (n=285)	57.3% (n=206)	46.1% (n=206)	14.5% (n=207)
VMD 2018	65.1% (n=304)	53.8% (n=208)	43.8% (n=208)	12.0% (n=209)
VMD 2019	64.7% (n=278)	55.0% (n=140)	38.6% (n=140)	11.7% (n=145)
VMD Neonatal 2019	70.0% (n=150)	54.7% (n=117)	40.2% (n=117)	7.6% (n=118)
VMD Pre-weaning 2019	69.5% (n=59)	53.8% (n=13)	38.5% (n=13)	31.3% (n=16)
VMD Adult	43.3% (n=30)	n=0	n=0	100% (n=1)

Applying VMD breakpoints, the rate of tetracycline resistance amongst the FQ-R isolates found in the South West, as analysed above far exceeded rates observed by VMD in *E. coli* (i.e. not pre-selected to be FQ-R) from dairy cattle from across England and Wales in each year between 2017-2019. This over-abundance of resistance among FQ-R isolates from the South West versus the general VMD survey was also seen, though to a lesser degree, for streptomycin and chloramphenicol. However, the rate of cefotaxime resistance was very similar among FQ-R *E. coli* from our survey to that measured by VMD in every instance except specifically neonatal *E. coli* investigated in 2019. This would suggest that FQ-R *E. coli* are more likely to be tetracycline, streptomycin or chloramphenicol resistant than fluoroquinolone susceptible isolates, but equally likely to be 3GC-R, but this could be reflective of the situation on the 53 OH-STAR study farms.

5.2.2. Multiplex PCR and WGS for PMQR genes in FQ-R cattle *E. coli* isolates

Of 42 farms located within a 50 x 50 km region, which included the homes of the people providing the urinary samples discussed in results chapter 2, 245 FQ-R cattle *E. coli* isolates

were taken forward for further analyses, representing one unique isolate from each positive sample. Multiplex PCRs showed that *aac(6')Ib-cr* (found to be highly prevalent in the human urinary *E. coli* isolates) was entirely absent from the cattle isolates and only nine isolates (3.67%) carried a PMQR gene (5 *qnrS*, 2 *qnrA* and 2 *qnrD*), compared with 35.99% of the urinary isolates (chapter 2 – 4.2.2, Table 5).

WGS was performed, as for the urinary isolates (results chapter 2), for 42 FQ-R, cattle-associated *E. coli* which were selected so that at least one isolate represented each farm that was positive for FQ-R *E. coli* within the 50 x 50 km region that includes the homes of all the people that submitted urine samples for analysis (see results chapter 2). WGS confirmed the low number of PMQR genes in cattle isolates; only a single *qnrS* gene was identified among these FQ-R 42 isolates.

5.2.3. ST diversity and phylogenetic comparisons between human and cattle FQ-R *E. coli.*

ST distribution in the 42 FQ-R cattle isolates was analysed based on WGS data (Table 21), as it was on in the human urinary isolates in results chapter 2 – 4.2.5, Table 8. It was suspected that the cattle isolates would contain greater ST diversity than seen in the human isolates, because the urinary isolates were harvested from a niche environment – most *E. coli* do not typically colonise the human urinary tract, whereas *E. coli* are generally well known as colonisers of the mammalian gut.

Table 21. ST frequency in FQ-R cattle isolates.

ST	Freq.	ST	Freq.	ST	Freq.	ST	Freq.
ST744	21	ST155	2	ST2599	1	ST88	1
ST162	8	ST117	1	ST4988	1		
ST10	5	ST224	1	ST7593	1		

Comparing the number of STs identified divided by the number of isolates sequenced (a measure of ST diversity in a sample) in the 188 human urinary isolates revealed that the number of STs was 14.4% of the number of isolates examined. Amongst the 42 cattle isolates, conversely, ten STs were identified (23.8%). The large difference in sample size means that this difference is not significant, however, and it remains uncertain whether there is any greater ST diversity among cattle isolates. It may be that the commensal/opportunistic nature of many UTIs coupled with the niche situation of the cattle gut both contribute to this finding. Importantly, Five of the STs found in the cattle isolates (50%) were also represented in the human isolates: ST744, ST162, ST10, ST155 and ST224.

Importantly, phylogroups B2 and D, which are typically most associated with uropathogenic virulence (29) were completely absent from the FQ-R cattle isolates. However, ST744 and ST162 were common among cattle isolates, and also among FQ-R/3GC-S human isolates (results chapter 2 – 4.2.5 Table 8). Accordingly, because of this ST overlap, a phylogenetic tree was generated to consider relationships between the cattle FQ-R and human FQ-R/3GC-S *E. coli* collected in parallel within our 50 x 50 km study area (Figure 14). This tree suggested overlapping ST744 and ST162 isolates found on different farms, but, importantly, also an overlap between cattle isolates and human urinary isolates. Detailed trees generated using an ST744 or ST162 reference genome (Figure 15) confirmed this. For ST744, the closest relationship between two human urinary isolates was 931 SNPs, but the closest relationship between a human and a cattle isolate was 71 SNPs. For ST162, the closest two human isolates differed by 175 SNPs, whereas the closest relationship between a human and a cattle isolate was 63 SNPs. As mentioned in chapter 1, a core genome SNP distance of 30 or fewer is commonly seen in Enterobacteriales isolates that are confirmed to be part of an acute outbreak of foodborne illness (300).

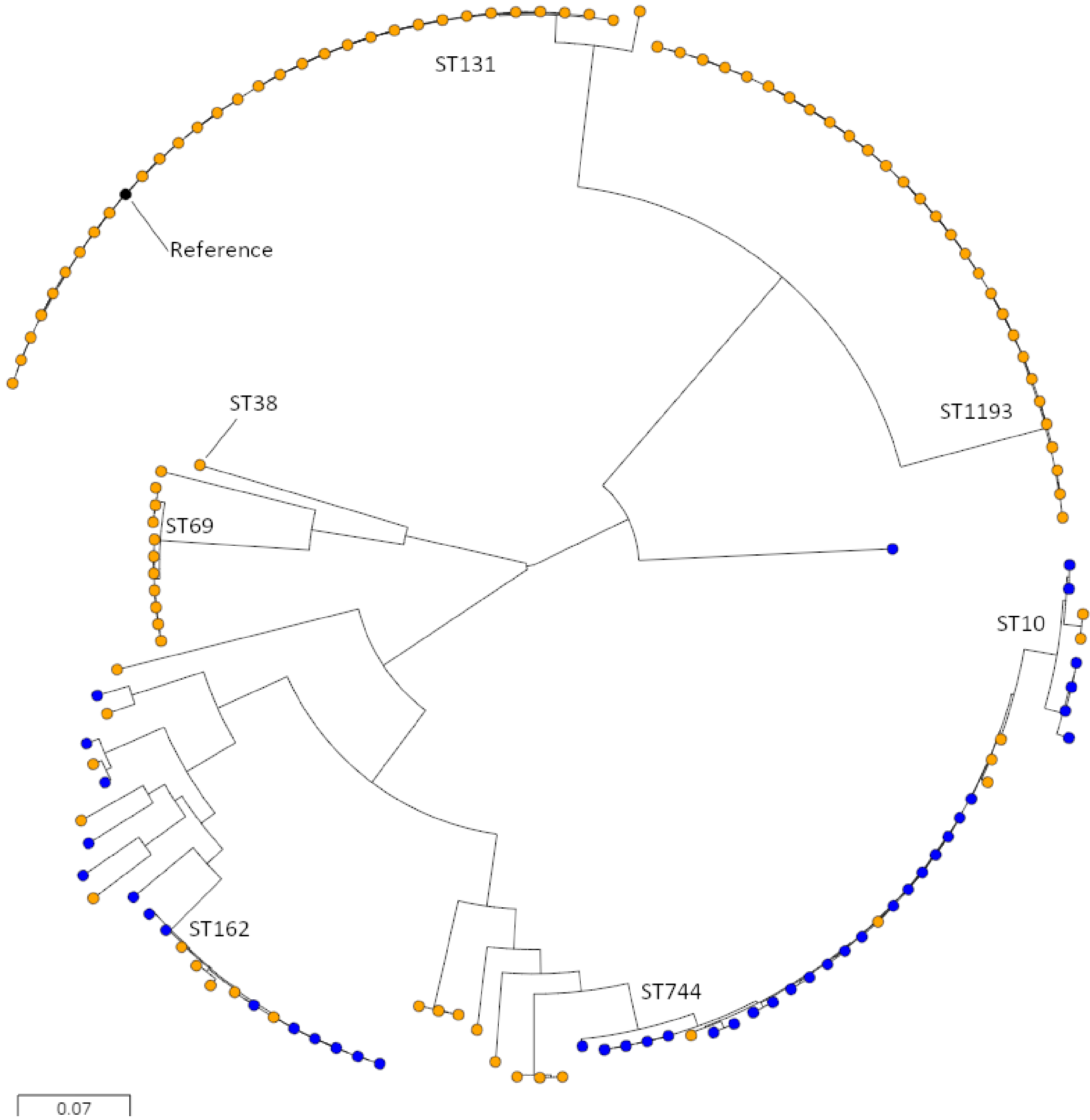


Figure 14. Maximum likelihood phylogenetic tree showing urinary and cattle isolates. FQ-R, 3GC-S urinary isolates (orange nodes) and cattle isolates (blue nodes), and prevalent STs are shown. Sequences were aligned against an ST131 reference (black node).

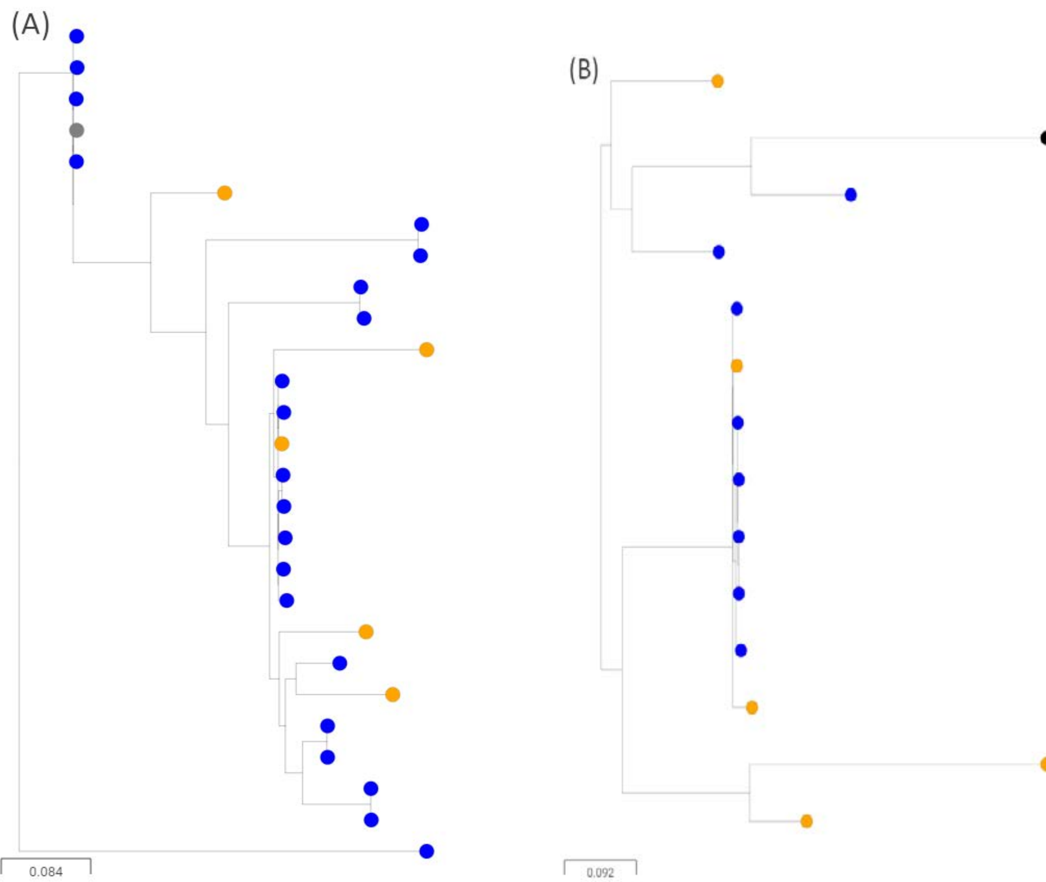


Figure 15. Maximum likelihood phylogenetic trees for (A) ST744 and (B) ST162 only. ST744 and ST162 isolates shown in Figure 14 were used to construct separate phylogenetic trees, after aligning against references of the same ST (grey node, ST744 – black node ST162). 3GC-S urinary isolates are represented by orange nodes and cattle isolates are represented by blue nodes.

5.2.4. Mutations in the QRDR found among cattle FQ-R *E. coli*

All of the QRDR mutation types found in the cattle isolates were also observed among the human urinary isolates (see chapter 2 – 4.2.3, Table 7) but there were just four QRDR types observed in total in the cattle isolates. The most common pattern, QRDR type 4, was almost exclusive to the ST744 isolates ($\chi^2 = 37.19$, $\varphi = 0.94$, $p<0.0001$), and was the same pattern observed in most of the human-derived ST744 isolates (the two human isolate exceptions to this each had this pattern plus a different mutation [in *parC*] in each isolate [types 3 and 5] – chapter 2 – 4.2.3, Table 7). Only one non-ST744 isolate also had this QRDR pattern, an ST162 isolate. All of the other ST162 isolates and all but two other isolates had the type 12 pattern, which was also the most common pattern outside of the ST131 and ST1193 urinary isolates, including the ST162 human urinary isolates. These two QRDR patterns differ by only a single mutation, *parC* A56T. One of the two remaining cattle isolates had a single *gyrA* mutation, pS83L, supplemented with *qnrS*. The final isolate was the only cattle-derived isolate with a *parE* mutation, S458A (QRDR type 16). As with type 4 (ST744), type 16 differs from the type 12 (ST162) pattern by only a single mutation.

5.2.5. Exploring the carriage of other ABR genes in FQ-R cattle *E. coli*

As with the human urinary isolates (chapter 2 – 4.2.7), the carriage of acquired ABR genes was explored in the FQ-R cattle isolates (Table 22). The carriage rate for any β-lactamase gene was found to be very similar in dairy cattle isolates and human urinary isolates (88.1% versus 82.4%, respectively). However, carriage of $bla_{\text{CTX-M}}$ (4.8%) and bla_{CMY} (7.1%) was considerably lower; this was expected though, given that half of the urinary isolates were selected for 3GC-R/FQ-R double resistance. The carriage rate for any gene associated with aminoglycoside resistance among cattle FQ-R *E. coli* was very high (92.9%) – higher than that observed in the human urinary isolates (76.1% – chapter 2, 4.2.7, Table 10) and much higher than the observed rate of streptomycin resistance seen among the cattle FQ-R *E.coli* according to disc testing (67.6% – Table 19). Indeed, even considering that two of the genes found in more than a quarter of the cattle isolates, *aph(3')-Ia* and *aadA5*, do not modify streptomycin, 88.1% isolates carried either *strA/B* or an *aadA* gene other that *aadA5*, which do (251, 254). The *aph(3')-I* genes do, however, confer resistance to framycetin which, as discussed in the chapter introduction, is used in dry cow therapy (296). The carriage rate for any *tet* gene (90.5%) was considerably higher than that observed in the human isolates (65.4% – 4.2.7, Table 10) and was close to the rate of phenotypic tetracycline resistance observed from disc testing (Table 19). Finally, carriage of any gene associated with phenicol resistance – all of which confer resistance to chloramphenicol – was 69.0%; not much higher than the rate of resistance to chloramphenicol resistance observed in the disc testing results

of a wider selection of FQ-R cattle *E. coli* than those subjected to WGS (62.7% – Table 19). This was much higher than the rate of phenicol resistance gene carriage observed in the human urinary isolates (31.9% – 4.2.7, Table 10). However, this was not surprising given that, with the exception of ear- and eye-drops, phenicols have not been used in human medicine for decades.

Table 22. Acquired ABR genes found in FQ-R cattle *E. coli* isolates

As detected by ResFinder-2.1

Aminoglycosides									
aac(3)-IId	1	*aadA1*	12	*aadA2*	3	*aadA24*	1	*aadA5*	21
aph(3')-Ia	13	*strA*	31	*strB*	31	*aph(3')-Ic*	2		
Tetracycline									
tet(A)	9	*tet(B)*	33	*tet(M)*	1				
Phenicols									
cmlA1	2	*catA1*	20	*floR*	12				
β-lactams									
bla$_{CMY-2}$	1	*bla*$_{CTX-M-15}$	1	*bla*$_{CTX-M-3}$	1	*bla*$_{OXA-1}$	3	*bla*$_{TEM-1A}$	5
bla$_{TEM-1B}$	25	*bla*$_{TEM-1C}$	1	*bla*$_{TEM84}$	1	*bla*$_{TEM-207}$	1		

Many of the *bla*$_{CTX-M}$ genes identified in 3GC-R cattle isolates in results chapter 1 – 3.1.1 (including *bla*$_{CTX-M-1}$, $_{-14,}$ the novel variant, *bla*$_{CTX-M-214}$, and *bla*$_{CTX-M-32}$, which was found on half of the farms included in the study) were notably absent from the FQ-R isolates collected from the same samples, explaining why rates of 3GC-R among FQ-R cattle isolates are low (Table 19). Two poorly characterised TEM genes, *bla*$_{TEM-84,}$ and *bla*$_{TEM-207}$, were found in the FQ-R cattle isolates. Isolates carrying each were found to be susceptible to cefotaxime and cefuroxime by disc susceptibility testing prior to sequencing so it is assumed these are basic penicillinases.

5.2.6. Exploring virulence gene carriage among FQ-R cattle *E. coli*

The carriage of virulence genes was next examined in the cattle isolates (Table 23). The most obvious difference from what was observed in the human urinary isolates was the total number of different virulence genes detected across the isolates. Among the cattle isolates was 14, compared with 36 in the urinary isolates. This difference becomes less pronounced, however, if genes reported to contribute to a single virulence factor – as in the AAF system discussed in chapter 2, section 4.2.9 and the F17 fimbrial genes – are treated as a single entity (13, versus 29), and fewer cattle isolates were examined than urinary isolates (42 versus 188), and it is true that among the urinary isolates many virulence genes were only found in a small number of isolates. It may be therefore, that finding more virulence genes could be a simple matter of probability, and if we had sequenced more cattle isolates, we would have found more genes.

When the number of virulence genes carried per isolate was compared, the human-derived isolates again come out on top. 1000 virulence genes were detected across all human isolates (5.3 per isolate, on average [standard deviation: 1.5]), compared to only 138 in the cattle isolates (3.1 per isolate, on average [standard deviation: 2.3]). However, considerable variance in the number of virulence genes carried in an isolates was observed in isolates found in both reservoirs (between one and nine in each).

Table 23. Virulence genes found in cattle *E. coli* isolates

As detected by VirulenceFinder 1.2

Gene	Protein	Freq.		Gene	Protein	Freq.
astA	Enteroaggregative *E. coli* heat-stable enterotoxin (EAST-1)	4		*iroN*	Ferric enterobactin receptor	13
celb	Permease IIC component	1		*iss*	Increased serum survival	19
cma	Colicin M	3		*lpfA*	Fimbrial major protein	15
f17A	F17 fimbrial protein	1		*mchF*	Probable microcin-H47 secretion/processing ATP-binding protein MchF	12
f17G	F17G adhesin subunit	1		*mcmA*	Microcin M	8
gad	Glutamate decarboxylase	41		*pic*	Serine protease pic autotransporter	1
ireA	Iron-regulated outer membrane virulence protein	10		*vat*	Vacuolating autotransporter toxin	1

Of course virulence genes are not all of equal importance and probably the most significant finding from the cattle virulence gene analysis was the complete absence of some of the most important UPEC-associated virulence genes, which were found to be widespread in the urinary isolates. These included the *iha* gene – which was present in 78.2% of urinary isolate (chapter 2 – 4.2.9, Table 13), the product of which has been shown to aid in the colonisation of the urinary tract (112). Also the *sat* gene, which was present in 74.5% of urinary isolates, the product of which has been found to be cytotoxic to bladder and kidney epithelial cells (114). However, three genes reported to be important virulence factors for ExPEC, the two iron acquisition genes *iroN, ireA* (297), and the increased serum survival gene, *iss* (111) were detected in the cattle isolates. It's important to note, though, that the latter two have been more strongly associated with APEC than UPEC (111, 264, 297), and neither *iroN* nor *ireA* were very common in the human urinary isolates analysed here (11.7% and 5.9% positive isolates, respectively – chapter 2 – 4.2.9, Table 13).

ST744 was one of two STs where potential sharing of isolates has been demonstrated, above. ST744 isolates from both cattle and urinary origin were found to share a similar array of virulence genes. That is, almost none. At most, an ST744 isolate was found to have only 3 virulence genes, which were different in the human and cattle isolates. In the cattle, two were genes for F17 fimbriae and one is the *gad* gene which, as is discussed in chapter 2 – 4.2.9, has a questionable association with virulence. In the humans the three genes found in a single ST744 isolate were *gad*, *astA* and *mchF*. Two of the ST744 human urinary isolates and one of the cattle isolates carry the *iss* gene, but as is discussed above and in chapter 2 – 4.2.9, *iss* is most commonly associated with APEC. One urinary isolate was <50 SNPs distant from two cattle isolates. One of the cattle isolates carried an *astA* gene and aside from that the only virulence-associated gene any of the three isolates carried was *gad*.

ST162 was also found to be shared between humans and cattle, above. Cattle ST162 isolates were found to carry mostly the same virulence genes as the human urinary ST162 isolates. Four genes were found in all ST162 isolates, regardless of source – *gad*, *iroN*, *iss*, and *lpfA*. Additionally, the *mchF* gene was found in all but one (human) ST162 isolate. The *mcmA* gene was common in the ST162 cattle isolates (present in 6/8 isolates) but present in only one of the ST162 urinary isolates. One of the ST162 urinary isolates also carried the *astA*, *celb* and *cma* genes; these were completely absent from the ST162 cattle isolates but were found in cattle isolates from other STs (Table 23). Two ST162 human urinary isolates were <50 SNPs distant from five of the cattle isolates. These all shared five virulence genes in common – *gad, iroN, iss, lpfA, mchF* – and three of the cattle isolates also carried the *ireA* and *mcmA* genes, additionally.

Two genes associated with enterovirulence, *pic* and *astA*, were identified among the 42 FQ-R cattle isolates. The latter of these codes for the Enteroaggregative *E. coli* heat-stable enterotoxin (EAST-1) and was also detected in 14 urinary isolates. Bacteriocin genes were found to be much more common in the cattle isolates, generally, than the human urinary isolates, mainly due to a high carriage rate in ST162 cattle isolates. At least one bacteriocin gene was found in 19/42 of the cattle isolates (45.2%) versus 24/188 in the human urinary isolates (12.8%).

Three different bacteriocin genes were detected among the 42 cattle isolates: the microcin genes, *mchF* and *mcmA*, and the colicin gene, *cma* (Table 23). Bacteriocins are peptide toxins which bacteria deploy to inhibit competitor bacteria (298). The *cma* gene codes for Colicin M (Cma), which causes *E. coli* lysis through inhibition of murein biosynthesis (299) The *mcmA* gene codes for siderophore microcin precursor, McmA, which is converted to the active form, MccM, with the addition of a siderophore moiety. MccM enters the cell through TonB-ExbBD regulated siderophore receptors, FepA, Fiu, Cir (300). The exact mechanism of its bactericidal activity is not known but impairment of cellular proton channels has been proposed (301). The *mchF* gene – along with *mchE*, which cannot be detected by VirulenceFinder 1.2 (215) – codes for an ABC transporter protein, involved in the secretion of another siderophore microcin, Microcin H47 (302). Curiously, the genes encoding the toxin component of the Microcin H47 system, *mchA-D* were not detected in *mchF* positive isolates. However, *mchEF* is highly homologous (92.1% sequence identity) with the *cvaAB* genes (303), which code for the CvaA and CvaB parts of the Colicin V (ColV) exporter (the remaining part being TolC) (304). Despite VirulenceFinder identifying *mchF*, GBK files for the isolates (assembled using Prokka and supplied by MicrobesNG – Methods and Materials 2.8), did not contain an open reading frame labelled as *mchF* but a gene labelled *apxIB_2* was found. A Blast search of the amino acid sequence found an entry with 100% sequence identity for 'microcin H47 export transporter peptidase/ATP-binding subunit MchF [*Escherichia coli*]' (accession: WP_000184924), supporting the VirulenceFinder identification. However, it also matched a database sequence named as CvaB with 99.86% sequence identity (accession: CAA0168846). Accordingly, there was some uncertainty whether this locus is the Microcin H47 or ColicinV exporter. However, in GBK files the *apxIB_2* (possibly *mchF*, possibly *cvaB*) gene in isolates from both cattle and humans, representing multiple STs is located between *cvaA*, which as mentioned above, codes for part of the ColV exporter system, and *cvaC*, which codes for the colicin toxin, Colicin-V (304), and which VirulenceFinder 1.2 does not identify (215). Accordingly, we conclude that these isolates are Cva positive not Mch positive, and therefore produce Colicin V and not

Microcin H47, which would also explain a lack of *mchA* (Microsin H47 structural genes) in these isolates.

The Colicin-V toxin kills by disrupting inner membrane potential (305). Colicin-V enters through outer membrane receptors which are regulated by the TonB-ExbBD system (304, 305). TonB-dependent receptors are typically involved in siderophore uptake (306), and whilst ColV has not been shown to be a siderophore bacteriocin (as far as can be determined by a review of the relevant literature), it seems plausible – especially considering its close relationship to the *mch* siderophore system, discussed above.

5.2.7. Fluoroquinolone use as a driver of FQ-R *E. coli* on dairy farms and the suppressive effect of dry cow therapy

Finally, this work enabled the conduction of an in-depth risk factor analysis, carried out by my colleague, Dr Hannah Schubert. Factors were considered that might influence the prevalence of FQ-R *E. coli* on farms, with the aim of identifying potential interventions that might reduce it, and so reduce any ongoing zoonotic threat. As explained in results chapter 1, the OH-STAR project's survey of resistance in dairy farms showed that the odds of a sample being positive for FQ-R *E. coli* was significantly greater if the sample came from the environment of heifer calves (202). This is important because dairy heifer calves are normally reared on the farm as replacement milking cows, so factors associated with the increased carriage of FQ-R *E. coli* in calves could have a long-term effect on the whole farm. Accordingly, Hannah Schubert performed a new risk factor analysis to identify management and ABU factors associated with the odds of finding FQ-R *E. coli*-positive samples in the environment of heifer calves.

Of 631 samples collected from the environments of heifer calves in the OH-STAR survey (results chapter 1), 103 (16.3%) were positive for FQ-R *E. coli*. Two variables were identified that were associated with sample-level positivity for FQ-R *E. coli*. One variable – the total usage of fluoroquinolones in the year the samples were collected - was positively associated with finding FQ-R *E. coli* in a sample (odds ratio 2.39, 95% credible interval [1.01, 6.02]). Another variable – the percentage of cows within the herd dried off using an antibacterial dry cow therapy (see introduction to this chapter) – was negatively associated with FQ-R *E. coli* positivity in samples from the heifer's environment (odds ratio 0.24, 95% credible interval [0.11, 0.50]).

5.3. Discussion

The main conclusion from examination of the dairy cattle *E. coli* isolates is that dual FQ-R and 3GC-R is uncommon and PMQR gene carriage was low in FQ-R isolates. However, there is some evidence to suggest recent sharing of FQ-R *E. coli* between humans and cattle.

We have shown in previous similarly powered work performed in parallel on the same study farms that there was no evidence of recent sharing of 3GC-R *E. coli* between farms and humans in the OH-STAR study region (as discussed in chapter 1) (213, 223). This agrees with the findings of similarly motivated studies from other groups (240, 307, 308). However, when considering FQ-R *E. coli* populations, we now report evidence for mixing of farm and human *E. coli*, and that the shared *E. coli* clones can cause bacteriuria. A core genome SNP distance of 30 or fewer is commonly seen in phylogenetic analyses of Enterobacterales isolates that are confirmed to be part of an acute outbreak of foodborne illness (238) and hospital studies frequently set a cut-off of <100 SNPs to define an outbreak (309). Finding FQ-R human/cattle isolates pairs differing by 71 (ST744) or 63 (ST162) SNPs is therefore suggestive of a situation where human and cattle isolates in this region do intermingle. However, this observation should be considered in the context of our finding that the closest isolates from two different farms were only three and seven SNPs apart for ST162 and ST744, respectively (Figure 15).

Generally, the shared ST744 and ST162 isolates did not carry virulence factors significantly associated with a UPEC phenotype, as opposed to APEC (95, 310). Accordingly, it is likely that bacteriuria results from commensal carriage of these cattle-associated isolates leading to opportunistic urinary colonisation. We conclude therefore, that whilst farm-related strains made up a small component of the total FQ-R urinary *E. coli* in our survey, and the isolates do not carry virulence factor genes associated with severe disease, there is evidence for zoonotic transmission to humans (Figure 15), which contrasts with studies considering 3GC-R *E. coli* (213, 223, 240, 307, 308). More work is needed to establish the exact routes of transmission, but even the minor zoonotic potential of cattle-associated FQ-R *E. coli* identified here should act as a stimulus to reduce the prevalence of such bacteria on farms. This work also suggests potential ways to achieve that objective.

As part of OH-STAR, Hammond *et al*. reported within our study region that reducing fluoroquinolone use in primary care was associated with a reduction in the proportion of *E. coli*-positive community urine samples where the isolate was FQ-R (144). Accordingly, it was interesting to find that overall fluoroquinolone use at farm level was positively associated with the odds of finding FQ-R *E. coli*-positive faecal samples in the environments around

dairy heifer calves. The implication is that reducing fluoroquinolone use on farms may well reduce the prevalence of FQ-R *E. coli* in heifer calves, which, as they join the milking herd, may lead to a general reduction of FQ-R *E. coli* on the farm.

Notably, in mid-2018, as our surveillance of resistant *E. coli* on study farms was ending, the use of fluoroquinolones was effectively stopped, except in the very rare instance where susceptibility testing confirmed that no other antibacterial treatment option was available (311). This Red Tractor farm assurance scheme regulation is applicable to the vast majority of UK dairy farms. Red Tractor are a not-for-profit company which runs a scheme called Red Tractor Assurance, to which UK farms can apply to if they meet and adhere to a set of regulator standards (https://redtractor.org.uk/about-red-tractor/).

A final key finding was that dry cow therapy use may expedite the reduction of FQ-R *E. coli* in heifer calves, which was unexpected. We hypothesised that the reason for the association between increased usage of dry cow therapy on a farm and reduced prevalence of FQ-R *E. coli* in the environments of heifer calves was that relatively few FQ-R *E. coli* from these farms were cross-resistant to the antibacterial ingredients of dry cow therapies. These antibacterials can be released in the colostrum and first milk from treated cows. Since this colostrum is usually fed to calves at birth (294, 312, 313), it is plausible that calves receiving colostrum from treated cows are protected from colonisation by FQ-R *E. coli* due to the dose of antibacterial inadvertently received shortly after birth.

Dr Hannah Schubert calculated that 83% (in terms of weight of active ingredient) of the dry cow therapy anti-Gram-negative antibacterials used on study farms during the period of our project (2017-2018) were cephalosporins or cloxacillin (3.44 kg). Framycetin (neomycin B) made up the remainder (0.69 kg). Notably, of the 42 FQ-R cattle *E. coli* isolates subjected to WGS, only two (4.8%) were resistant to both cephalosporins and cloxacillin, as inferred from WGS.

Whilst genetically inferred framycetin resistance (presence of an *aph* gene) was more common among sequenced FQ-R cattle *E. coli* isolates (15/42, 36%), it was far less common among FQ-R isolates than among CTX-M β-lactamase-positive 3GC-R *E. coli* isolates from these same farms collected in parallel (109/135, 81% of isolates) and analysed by Dr Jackie Findlay (213). Accordingly, there is evidence for suppression of FQ-R *E. coli* by dry cow therapy use, irrespective of active agent, because the FQ-R bacteria found on these farms are rarely resistant to the active agents used.

Much attention has been paid to reducing dry cow therapy use on dairy farms, as a way of reducing total ABU (294, 312, 313). We would not suggest a shift away from this approach, because inappropriate use might increase the selection of zoonotic threat of cattle *E. coli*

that are resistant to other antibacterials. Indeed, we have already shown, as reported in results chapter 1, a positive association between cefquinome dry cow therapy use on dairy farms and *E. coli* carrying CTX-M type β-lactamases (202), the most common cause of 3GC-R in *E. coli* isolated from community UTIs in the OH-STAR study region (212). Whilst use of cefquinome in dry cow therapy is also effectively stopped under the 2018 Red Tractor regulations (311), it is certainly possible that switching to the use of first-generation cephalosporins or cloxacillin dry cow therapy could maintain selection for CTX-M producers, which are resistant to these agents. Importantly, however, 50% of 3GC-R on our study farms was caused by chromosomally encoded AmpC hyper-production (results chapter 1 – 3.2) (213, 223), an enzyme inhibited by cloxacillin (314). We suggest, therefore, that a better alternative to cefquinome dry cow therapy might be cloxacillin, if all else is equal.

In contrast, a switch from cefquinome to framycetin-containing dry cow therapy might be less favoured because, as shown in results chapter 1, framycetin dry cow therapy use co-selects CTX-M positive strains (202). Furthermore, whilst analysis of WGS data from OH-STAR cattle isolates identified that framycetin resistance is less common in FQ-R *E. coli* than in $bla_{\text{CTX-M}}$ positive *E. coli* on farms (36% versus 81%), we found framycetin resistance to be more common than cloxacillin resistance in FQ-R *E. coli* (36% versus 4.8%), supporting the use of cloxacillin over framycetin or a first-generation cephalosporin as the best choice to drive down FQ-R *E. coli* whilst less strongly selecting for 3GC-R *E. coli* (i.e. actively selecting against the proportion caused by AmpC enzymes).

Overall, our One Health approach to investigating selection and transmission of critically important ABR *E. coli* within our study region highlights the rare but not insignificant zoonotic potential for cattle-associated FQ-R *E. coli* as a cause of bacteriuria in humans. Our results also highlight that reducing fluoroquinolone use on farms, whilst carefully selecting the most appropriate dry cow therapy active ingredient to match the ecology of resistance found on a particular farm, should most effectively reduce that zoonotic potential. Our work certainly demonstrates the foresight of the recently introduced Red Tractor regulations designed to effectively eliminate use of highest-priority critically important antibacterials on dairy farms in the UK (311).

6. Results chapter 4. Antimicrobial resistance in canine companion animals, with a focus on resistance to fluoroquinolones

6.1. Introduction

Antimicrobial resistance and particularly antibacterial resistance (ABR) has many negative impacts on the health and welfare of humans and animals including increased morbidity and mortality and an increase in treatment costs (315). ABR is linked across human populations, animal populations and the environment, and it is possible for ABR bacteria - or ABR genes that they carry - to be passed between these realms (316). Previous research has indicated that farmed animals act as reservoirs of ABR bacteria that can be transmitted to humans either through the food chain, through direct contact between humans and animals or via the environment (284, 317).

In many countries, particularly in urban areas, interaction between humans and farm animals – directly or via the environment – is limited. This may explain why studies using whole genome sequencing (WGS) have found little evidence that sharing of ABR bacteria between farmed animals and humans is a significant problem (213, 223, 240, 307). However, close interaction between humans and domestic animals is common in such areas. Accordingly, it may be that for many people around the world, a pet dog is a more likely source of ABR bacteria than are farmed animals. Indeed, ABR bacteria found in domestic pets and their owners are often indistinguishable (287, 318-320). A key ABR pathogen of relevance is *E. coli*, which is carried in the intestines of humans, farmed and companion animals, and causes a significant disease burden in all three, and especially in humans (321).

There are several ways that dogs may become colonised by ABR *E. coli* and so bring them into the home. Ingestion is an essential part of colonisation; therefore, ingestion of faeces or faecally contaminated food or water by dogs may be a key source of ABR bacteria derived from humans and farmed animals. For example, farm animal manure is often spread on pastureland where dogs might be exercised. Wastewater from farm run-off or from human sewage outlets may introduce *E. coli* to fresh and sea water where dogs might bathe (322, 323). Meat can be contaminated with animal faeces during slaughter, and if eaten in its raw form by a dog may lead to *E. coli* colonisation (324). Research has also suggested that dogs become colonised by ABR bacteria when visiting veterinary hospitals, which act as reservoirs for multi-drug resistant (MDR) organisms, and particularly if the dog receives antibacterial therapy (325-327). Recent research examining 374 veterinary practices in the UK estimated that during the two years investigated, around 25% of approximately one

million pet dogs registered received at least one antibacterial course. Of dog antibacterial usage in this study, 60% was classified as use of a 'critically important' medicine as defined by WHO criteria (283).

Overall, ABR bacteria have been detected in both healthy and sick adult dogs and associations have been found between increased carriage of ABR bacteria and exposure to antibacterials (327). Associations have also been found between increased carriage of ABR bacteria following veterinary healthcare in general as well as with coprophagia and with the feeding of raw poultry (328-332). Of direct relevance to the present study, two UK studies have identified associations between ABR in faecal *E. coli* of adult dogs and those dogs being fed raw meat (331, 332).

Up to now, there has not been any published work reporting very early life risk factors for carriage of ABR *E. coli* in domestic pet dogs. In the UK, current recommendations are for juvenile dogs to be weaned onto solid food and receive a core vaccinations (for canine distemper virus (CDV), canine adenovirus (CAV), and canine parvovirus type 2 (CPV-2) and its variants) at six to eight weeks of age and then receive booster vaccinations every two to four weeks until 16 weeks of age (333). Dogs should stay with their mother until eight weeks of age, and owners are usually advised not to walk their dog outside in public places until after the dog has had its second vaccination (approximately 12 weeks of age).

In this chapter, risk factors are investigated to explore associations between various lifestyle factors and the detection of ABR *E. coli* in faecal samples taken from dogs at 16 weeks of age. Other studies looking at the presence of pathogenic and resistant bacteria in dog faeces have found a high prevalence of resistant *Enterococcus* spp. (e.g. 62% for *E. faecium*) in Italy (334) and *E. coli* (one study detected AMR *E. coli* in 260 (45%) of 580 samples tested) (332). Practices and behaviours that might increase ingestion of faecal bacteria from the environment or food were particularly considered.

Furthermore, WGS was used to characterise ABR isolates. The focus was specifically on resistance to critically important antibacterials: 3GCs (e.g., cefotaxime) and fluoroquinolones, (e.g., ciprofloxacin). 3GC-R and FQ-R *E. coli* carried by a sub-set of puppies were then compared with those cultured from human urinary tract (discussed in chapter 2) and bloodstream infections collected in parallel within the same 50 × 50 km region, to investigate whether there is evidence of transmission. Much of the work discussed in this chapter is presented in a journal paper, currently in preprint (203), on which I am co-lead author.

6.2. Results and Discussion

6.2.1. Risk factors for carriage of ABR *E. coli* in dogs at 16 weeks of age

In total, 295 dogs were recruited by Kezia Wareham as part of the OH-STAR consortium project, and with assistance from the Dog's Trust charity. Data for 223 dogs were included in the analysis. Submissions were excluded if the questionnaire was not fully completed (n=14) or because the faecal sample did not grow enough *E. coli* to be sure of ABR status as defined in Methods and Materials 2.2 (n=58). I played a leading role in faecal sample processing and determination of ABR status, with these two tasks being divided roughly evenly between myself and Kezia Wareham.

For each of the 223 included faecal samples, ABR *E. coli* carriage status was categorised as positive or negative for resistance to five test antibacterials: amoxicillin, cefalexin, ciprofloxacin, streptomycin, or tetracycline, as set out in Methods and Materials 2.5. In a preliminary χ^2 analysis performed by Kezia Wareham, the only significant risk factor identified for 16-week-old dogs providing faecal samples carrying *E. coli* resistant to at least one antibacterial was having been fed raw food ($p < 0.001$;Table 24). Subsequent univariable and multivariable logistic regression analyses performed by Dr Ashley Hammond showed a strong association between raw feeding and carriage of *E. coli* resistant to any one of the five antibacterials tested as well as individually with resistance to each of the antibacterials tested except cefalexin (Table 25).

Table 24. Baseline data for all 16-week-old dogs (n=223)

Associations with risk factors for carriage of *E. coli* resistant to at least one test antibacterial. p-values were calculated by Kezia Wareham using the Pearson χ^2 test (Stata/IC 15.1, StataCorp LLC, College Station, TX, USA). The bold figures show a p-value < 0.05.

Risk factor from questionnaire	Response to question	Response to question total (n=223)	Also resistant to at least one antibiotic (n=106)	*p*-value
Fed raw food	Yes	43	32/43	**<0.001**
	No	180	76/180	
Walked in town	Yes	181	84/181	0.21
	No	42	24/42	
Walked on farmland	Yes	142	69/142	0.95
	No	81	39/81	
Walked on beaches	Yes	103	52/103	0.57
	No	120	56/120	
Walked in the countryside	Yes	191	95/191	0.34
	No	32	13/32	
Walking near cattle	Yes	84	37/70	0.31
	No	139	71/139	
Swum/ paddled/ played in salt water	Yes	62	32/62	0.56
	No	161	76/161	
Swum/ paddled/ played in lake water	Yes	29	17/29	0.24
	No	194	91/194	
Swum/ paddled/ played in river water	Yes	66	33/66	0.76
	No	157	75/157	
Swum/ paddled/ played in pond water	Yes	65	38/65	0.06
	No	158	70/158	

Table 25. Univariable and multivariable logistic regression analyses

Conducted by Dr Ashley Hammond. Questionnaire and antibacterial-resistant *E. coli* data for 16-week-old dogs (n=223) were used. Presentation: Odds ratio (95% confidence interval) p-value. Only risk factors significantly associated with resistance (p-value < 0.05) are included.

Risk Factor	Univariable (n=223)	Multivariable for all samples (n=223)
Resistance to at least one antibacterial (n=108)		
Fed raw food	3.98 (1.89 to 8.40) <0.001	3.98 (1.89 to 8.40) <0.001
Resistance to ciprofloxacin (n=26)		
Fed raw food	12.42 (5.01 to 30.78) <0.001	12.42 (5.01 to 30.78) <0.001
Resistance to tetracycline (n=81)		
Fed raw food	4.47 (2.21 to 9.05) <0.001	4.47 (2.21 to 9.05) <0.001
Resistance to amoxicillin (n=93)		
Fed raw food	3.30 (1.64 to 6.63) 0.001	3.18 (1.57 to 6.42) 0.001
Swum/ paddled/ played in pond water	2.01 (1.12 to 3.61) 0.02	1.91 (1.05 to 3.48) 0.04
Resistance to cephalexin (n=34) No significant risk factors identified		
Resistance to streptomycin (n=51)		
Fed raw food	8.23 (3.95 to 17.15) <0.001	8.23 (3.95 to 17.15) <0.001

The most substantial risk associated with raw feeding in 16-week-old dogs was that of carriage of FQ-R *E. coli* (Table 25). This association has previously been reported in adult dogs in the UK; a study based on 445 dogs found that feeding raw poultry significantly increased the risk of carrying FQ-R *E. coli* in faeces (330). Raw poultry, especially chicken, often becomes contaminated with faeces during the slaughtering process (335). Findings from the present study extend these earlier studies to show that the impact of raw feeding on ABR *E. coli* carriage can be seen as early as 10 weeks after the first introduction of solid food. Faecal samples taken from broilers at a slaughterhouse commonly contain FQ-R *E. coli* (336) and raw chicken imported into (337) and produced in the UK (338) have been identified as contaminated with FQ-R *E. coli*. Feeding raw chicken could therefore be a source of FQ-R *E. coli* in our study, as has been seen with adult dogs (330), but this remains

to be confirmed. The risk of dogs acquiring ABR bacteria from meat would be mitigated simply by cooking that meat to reduce any contamination with faecal bacteria that occurs at slaughter and during processing.

6.2.2. Molecular epidemiology of 3GC-R *E. coli* from puppies

All of the work below was carried out by me working alone. Of faecal samples from 34 dogs that contained cefalexin-resistant *E. coli*, 27 gave 3GC-R isolates. In total, 29 unique isolates from these 27 dogs were analysed by WGS. Of these, seven isolates were also FQ-R (Table 26). WGS revealed a wide range of *E. coli* STs and 3GC-R mechanisms: ST88 (one isolate with CTX-M-1; three isolates with mutations in the *ampC* promoter known to be associated with hyper-expression) was dominant, followed by ST744 (three CIP-R isolates with CTX-M-1), ST963 (three isolates with CMY-2) and ST38 (two isolates with CTX-M-15). Seventeen additional isolates, each representing a unique ST, were found to be carrying CTX-M-1 (three isolates), CTX-M-15 (three isolates), CTX-M-65 (one isolate), CTX-M-14 (one isolate), CMY-2 (three isolates) DHA-1 (one isolate) and *ampC* promoter mutation (five isolates).

Table 26. Characterisation of 3GC-R *E. coli* from puppies using WGS.

Stars denote locally recruited dogs. Bold underlining denotes dogs fed raw food. Also shown are the QRDR types, as laid out in results chapter 2 – 4.2.3, Table 7. FQ-R mechanisms found in isolates which were not resistant given in brackets.

Dog ID	ST	CIP-R mechanism(s)	QRDR Type	3GC-R mechanism
DOG 1	ST372			CMY-2
DOG 2	ST10			CTX-M-1
DOG 3**	ST2179	*gyrA* S83L; *parC* S80I	Type 20	CTX-M-65
DOG 4	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4	CTX-M-1
DOG 5	ST38	(*gyrA* S83L; *aac(6')-Ib-cr*)	Type 19	CTX-M-15
DOG 6	ST58			*ampC* -42C>T
DOG 7	ST88			CTX-M-1
<u>DOG 8</u>	ST88			*ampC* -42C>T
DOG 9	ST38	(*qnrS1*)		CTX-M-15
DOG 10	ST963			CMY-2
DOG 11	ST1196	*gyrA* S83L; *gyrA* D87N; *parC* S80I; *qnrB4*	Type 12	DHA-1
DOG 12	ST215	(*qnrS1*)		CTX-M-15
DOG 13	ST973			CMY-2
DOG 15	ST6096			CMY-2
DOG 16	ST3889	(*qnrS1*)		CTX-M-15
<u>DOG 18</u>	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4	CTX-M-1
DOG 21**	ST69	(*qnrS1*)		CTX-M-14
DOG 21**	ST963			CMY-2
DOG 22**	ST963			CMY-2
DOG 23	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4	CTX-M-1
DOG 25	ST155			*ampC* -42C>T
<u>DOG 27</u>	ST88	(*gyrA* S83L)	Type 19	*ampC* -42C>T
<u>DOG 27</u>	ST1431	*gyrA* S83L; *gyrA* D87N; *parC* S80I; *qnrS1*	Type 12	*ampC* -42C>T
<u>DOG 28</u>	ST602			*ampC* -42C>T
DOG 29	ST4988	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12	CTX-M-15
<u>DOG 31</u>**	ST88			*ampC* -42C>T
DOG 42	ST1056			CTX-M-1
DOG 43	ST75			*ampC* -42C>T
DOG 44	ST961			CTX-M-1

Overall, therefore, AmpC-type β-lactamase-mediated resistance was found in 15/29 isolates and CTX-M was found in 14/29. This approximately 50:50 split was also seen in Jackie Findlay's analysis of 3GC-R *E. coli* from 53 dairy farms in South West England (as mentioned in results chapter 1), where amoxicillin/clavulanate use was associated with finding AmpC-mediated 3GC-R *E. coli* in farm samples (223). A study examining prescribing at small animal veterinary practices in the UK found that amoxicillin/clavulanate was the most common antibacterial prescribed, accounting for 36% of prescriptions (339), and it has been demonstrated that routine amoxicillin/clavulanate treatment selects for increased 3GC-R *E. coli* in the faeces of dogs (327). It could therefore be hypothesised that the reason why clavulanic acid-insensitive AmpC-type β-lactamases are so common in 3GC-R *E. coli* carried by dogs is because of high levels of amoxicillin/clavulanate usage in the canine population generally. However, whilst this study did not record veterinary treatments, it seems unlikely that antibacterial therapy was widespread in these puppies, given their age and exclusion of puppies that had been hospitalised. This finding of AmpC dominance is therefore suggestive of transmission into the juvenile dogs in the study. There was no positive association between raw feeding and the presence of 3GC-R isolates in general; only 6/29 3GC-R isolates were from raw-fed dogs (Table 25). However, among these, 5/8 of the AmpC hyper-producing isolates were from raw-fed dogs. Whilst these numbers are too small for clear conclusions to be drawn, it is plausible that raw feeding may selectively seed *ampC* hyper-producer *E. coli* carriage.

6.2.3. Molecular epidemiology of FQ-R *E. coli* from puppies

Carriage of FQ-R *E. coli* was strongly associated with raw feeding in puppies (Table 25). From 26 puppies that produced samples carrying FQ-R *E. coli*, 30 isolates were subjected to WGS (Table 27) in addition to the seven dual FQ-R/3GC-R isolates discussed above (Table 26). PMQR mechanisms were found in only 3/37 CIP-R isolates, and in only one ST58 isolate carrying *qnrS1* and a single *gyrA* mutation (Table 26) was there any suggestion that a PMQR gene was necessary for conferring FQ-R. The other two FQ-R isolates carrying PMQR were also 3GC-R (Table 26). These two were an ST1196 isolate carrying *qnrS1* and an ST1431 isolate carrying *qnrB4*, but in both there were also two mutations in *gyrA* and one in *parC*, sufficient to confer FQ-R in the absence of a PMQR gene (340). Indeed, many of the FQ-R isolates collected in this study carried identical mutations and no PMQR genes (Table 27). Interestingly, five of the 3GC-R isolates that were not FQ-R also carried PMQRs: four had a *qnrS1* gene and one ST38 isolate had an *aac(6)-Ib-cr* gene (Table 26). This supports previous conclusions, that carriage of these genes is not sufficient to confer FQ-R

in the absence of other mechanisms (340) and that *aac(6)-Ib-cr* carriage is associated with carriage of *bla*$_{CTX-M-15}$ (chapter 2 – 4.2.8, Table 11).

Of the CIP-R isolates sequenced, ST744 (12/37 isolates) dominated, followed by ST162 with 6/37 isolates (as was the case for the cattle isolates [chapter 3 – 5.2.3, **Table 21**]), 4/37 of the isolates were identified as ST1011, 3/37 were identified as ST224, 2/37 identified as ST1196 and individual examples of 10 other STs were also found (Table 26 and Table 27). Five of the STs identified (STs 744, 162, 10, 224 and 155) were also represented in both the FQ-R human isolates (chapter 2 – 4.2.5, Table 8) and the FQ-R cattle isolates (chapter 3 – 5.2.3, Table 21). Additionally, a single ST4988 isolate was also found in the cattle and this isolate also carried the *bla*$_{CTX-M-15}$ gene, like the canine ST4988. Both isolates also carried the streptomycin resistance genes, *strA/B,* and the tetracycline resistance gene, *tet(A).* Notably, a single ST1193 isolate was found in the canine isolates, which is an important clone currently emerging in human infections and of the most pathogenic phylogroup, B2 (246). Thus it was interesting to test relationships between 3GC-R and CIP-R isolates from locally recruited dogs with human urinary 3GC-R and CIP-R isolates from people living in the same geographical area as the locally recruited dogs (212, 222) whose infections occurred within the same six-month period as collection of the canine faecal samples yielding these isolates.

Table 27. Characterisation of FQ-R, 3GC-S *E. coli* from puppies using WGS.

Stars denote locally recruited dogs. Bold underlining denotes dogs fed raw food. Also shown are the QRDR types, as laid out in chapter 2 – 4.2.5, Table 8.

Dog ID	ST	FQ-R mechanism(s)	QRDR Type
DOG 4	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
<u>DOG 9</u>	ST162	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 10	ST224	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 14	ST162	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
<u>DOG 16</u>	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
<u>DOG 17</u>	ST453	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
<u>DOG 17</u>	ST58	*gyrA* S83L; *qnrS1*	Type 19
<u>DOG 17</u>	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
<u>DOG 18</u>	ST224	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
<u>DOG 19</u>	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
<u>DOG 20</u>	ST162	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 21**	ST10	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
<u>DOG 24</u>	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
<u>DOG 26</u>	ST1196	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 16
<u>DOG 26</u>	ST1011	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
<u>DOG 30</u>	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
<u>DOG 31</u>**	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
DOG 32**	ST162	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 33	ST542	*gyrA* S83L; *parC* S80I	Type 20
<u>DOG 34</u>	ST1011	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
<u>DOG 34</u>	ST6817	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 35	ST1011	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 35	ST224	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
<u>DOG 36</u>	ST155	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 37	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
DOG 37	ST162	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 38**	ST744	*gyrA* S83L; *gyrA* D87N; *parC* A56T; *parC* S80I	Type 4
DOG 39**	ST162	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12
DOG 40	ST1193	gyrA S83L; gyrA D87N; parC S80I; *parE* L416F	Type 15
DOG 41**	ST1011	*gyrA* S83L; *gyrA* D87N; *parC* S80I	Type 12

6.2.4. Mutations in the QRDR

As was observed in the FQ-R cattle isolates (chapter 3 – 5.2.4), summed together, QRDR types 4 and 12 were, by far, the highest represented among FQ-R canine isolates (Table 26 and Table 27), with 33/37 isolates (89.2%) having one of these QRDR types. However, QRDR type 4 was less common in the dogs (12/37, 32%) than in the cattle (22/42, 42%). This is because, as in the cattle (chapter 3 – 5.2.4 – $\chi^2 = 37.19$, $\varphi = 0.94$, $p<0.0001$), QRDR type 4 is strongly associated with ST744 in the dogs ($\varphi=1$, perfect association), and ST774 were less common in the dogs (12/37, 32%) than in the cattle (21/42 (50%). Of the four puppy isolates with a QRDR type that was not type 4 or 12, one had a single *gyrA* mutation (S83L), supplemented by *qnrS*, two had the same *gyrA* mutation, plus a *parC* mutation (S80I), and the final isolate had the same variations as seen in type 12, but with an additional *parE* mutation (L416F).

6.2.5. Evidence of faecal carriage of 3GC-R and FQ-R *E. coli* in puppies being clonally related to these causing urinary and bloodstream infections in humans in the same geographical area

A phylogenetic analysis of all the 3GC-R and FQ-R isolates from puppies subjected to WGS in this study was constructed (Figure 16). There were three clusters of isolates with chromosomal mutations conferring resistance: FQ-R isolates of ST162 and ST744 with multiple gyrase and topoisomerase mutations and a smaller ST88 cluster with chromosomal *ampC* promoter mutations conferring 3GC-R and amoxicillin/clavulanate resistance. In contrast, mobile resistance mechanisms were spread widely across the phylogenetic tree.

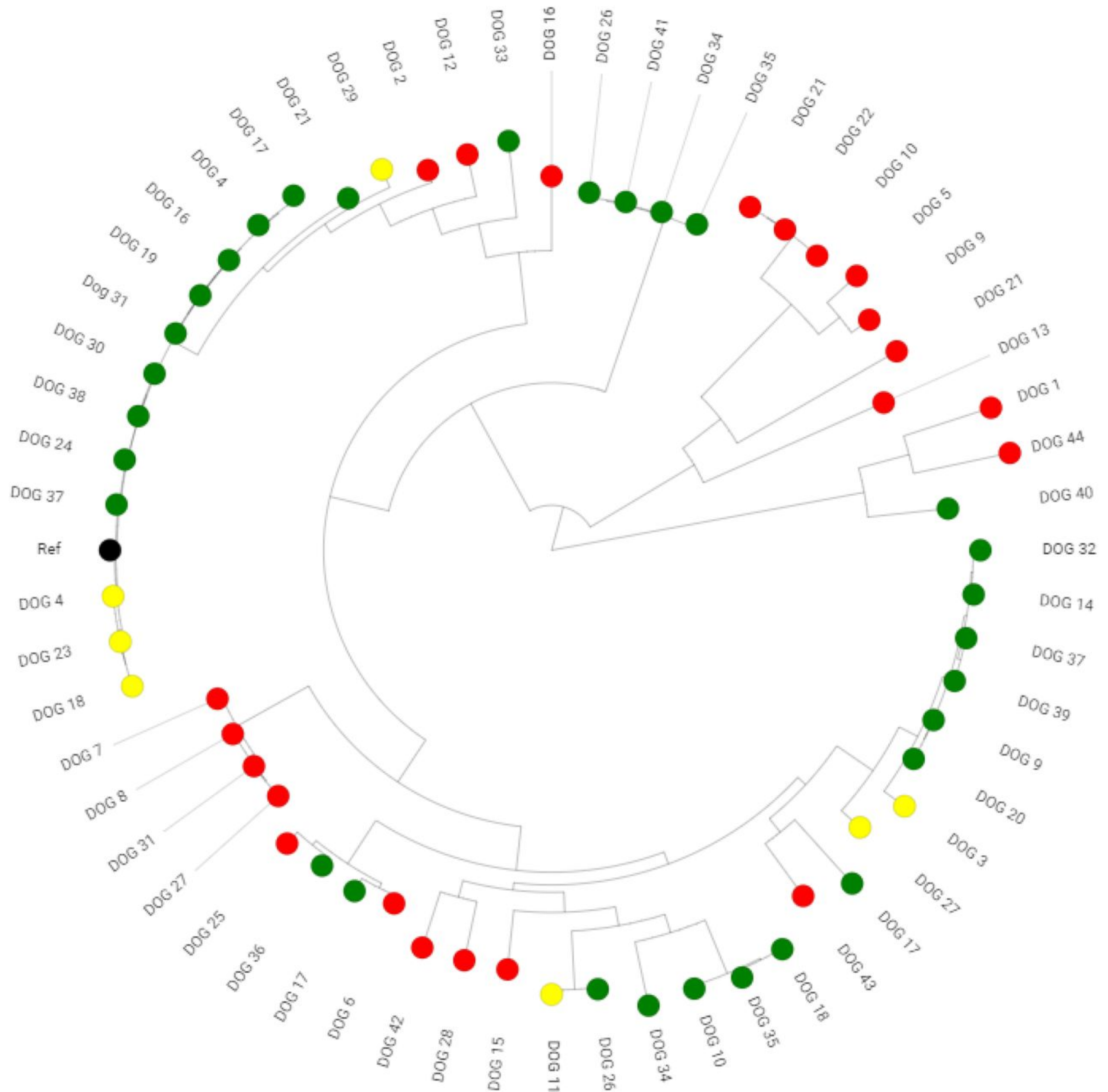


Figure 16. Core genome maximum likelihood phylogenetic analysis of antibacterial-resistant *E. coli* from puppies.

3GC-R isolates are labelled red, FQ-R isolates are labelled green and 3GC-R/FQ-R dual-resistant isolates are labelled yellow. The randomly assigned Dog ID relevant to each isolate is also labelled.

There were four 3GC-R isolates from locally recruited dogs; two of these (from two different dogs: Dog 21 and Dog 22) were ST963; the others were ST88 and ST2179 (Table 26). None of the 225 3GC-R urinary *E. coli* (212) in the comparison was ST2179 and a SNP distance analysis showed that the canine ST88 isolate was >1000 SNPs distant in the core genome from its closest ST88 human urinary isolate. To reiterate something mentioned in previous chapters, a core genome SNP distance of 30 or fewer is commonly seen in Enterobacteriales isolates that are confirmed to be part of an acute outbreak of foodborne illness (238). Hence, for these ST88 isolates, there was no evidence for sharing or transmission of isolates between dogs and humans, or *vice versa*. In contrast, the two canine ST963 isolates were 37 SNPs different from each other, suggesting recent sharing of the isolates. Significantly, however, the isolate from Dog 21 was <50 SNPs different from each of two human urinary ST963 isolates, and the isolate from Dog 22 was <65 SNPs from these same two human urinary isolates. Even more troubling, the isolate from Dog 21 was only 34 SNPs different from a 3GC-R ST963 bloodstream isolate, one of 82 3GC-R bloodstream isolates collected in parallel from clinical cases in the same geographical region at the same time and sequenced by Jackie Findlay, as part of a different study. The isolate from Dog 22 was 51 SNPs different from this bloodstream isolate. The urinary and bloodstream isolates were between 31 and 38 SNPs different from each other, so this is clear evidence that human and canine 3GC-R ST963 isolates in this region can be highly clonally related, suggestive of transmission. Each of these isolates (two canine, two urinary and one bloodstream) had a mobile bla_{CMY-2} gene embedded into the chromosome at the same position – proximal to *nhaRA*, *dnaJ* - which is further evidence of descent from a recent common ancestor. Most interestingly, another canine ST963 isolate was identified in this study, but not in a locally recruited dog (Dog 10, Figure 16). In this case, the isolate was 33, 35 and 21 SNPs different from the two urinary isolates and the bloodstream isolate, respectively, an even closer match than that seen with isolates from the two locally recruited dogs, suggesting an even closer clonal relationship. A similar result was observed with the ST1193 isolate found in Dog 40; this was observed to be just 21 SNPs from a human urinary isolate, which was just four SNPs more than the shortest SNP difference observed between two human ST1193 isolates, 17. Whilst Dogs 10 and 40 were not locally recruited, it is possible that they could still be based locally as address details for the nationally recruited dogs were not available for analysis.

Of the seven FQ-R isolates from locally recruited puppies (Table 26 and Table 27), five were of STs found amongst the 188 FQ-R urinary *E. coli* from people living in the same geographical area, described in results chapter 2. One of these was ST10 and two each were ST744 and ST162 (Table 27). One of the ST744 isolates was 47 SNPs different from a

human urinary isolate, which is suggestive of sharing, as defined above. Among the other four canine isolates, the lowest SNP difference from a human isolate was 324, which does not suggest sharing in these cases. Interestingly, the puppy carrying the seemingly shared ST744 isolate, Dog 31, was the only FQ-R *E. coli*-positive locally recruited dog reported to be fed raw meat (**Table 25**).

6.2.6. Antibacterial disc susceptibility testing

As with the FQ-R cattle isolates, FQ-R dog isolates were subjected to antimicrobial disc susceptibility testing. These were again used to determine the resistance rates, in FQ-R isolates, to other antibacterials important in the OH-STAR project (**Table 28**), and compared with the rates of resistance found in the FQ-R cattle, taken from results chapter 3 – 5.2.1, **Table 19**. Resistance to streptomycin and chloramphenicol was only marginally higher amongst the FQ-R canine isolates than those isolated from cattle. Resistance to cefotaxime and cefuroxime, meanwhile, was much greater, as would expected from the WGS results, which indicate a higher carriage rate of ESBLs amongst the dogs than the cattle. This may owe to the fact that cephalosporin use in dairy cattle is increasingly limited to instances where resistance to other antibacterials has been demonstrated (125). Tetracycline resistance, conversely, was more common among cattle isolates, likely due to its common use as a treatment for bovine respiratory disease, whereas its use in dogs has become less common in recent years (341).

Table 28. Susceptibility and resistance of FQ-R canine *E. coli* isolates to non-fluoroquinolone antibacterials

As determined by antimicrobial disc susceptibility testing, using CLSI breakpoints (295). Also shown are the resistance rates in FQ-R cattle, as described in chapter 3.

	Tetracycline	Streptomycin	Chloramphenicol	Cefotaxime	Cefuroxime
Susceptible	6	5	8	21	21
Intermediate	12	12	12	12	13
Resistant	19	20	17	4	3
Percentage resistant (dog isolates):	76.0%	80.0%	68.0%	16.0%	12.0%
Percentage resistant in cattle isolates:	90.2%	67.6%	62.7%	3.9%	3.9%

6.2.7. Exploring the carriage of non-PMQR resistance genes in FQ-R canine isolates

ResFinder-2.1 analysis of WGS data was also used to explore the presence of mobile genes which confer resistance to other antimicrobials relevant to the OH-STAR project (Table 29): aminoglycosides (e.g. streptomycin), tetracycline, phenicols (e.g. chloramphenicol), β-lactams (e.g. amoxicillin), and 3GC (e.g. cefotaxime). This was used to assemble a table comparing AMR gene carriage rates in FQ-R canine isolates, with those from humans and cattle, discussed in chapter 2 (4.2.7) and 3 (5.2.5), respectively (Table 30). Note that the 3GC-R genes are included in the general β-lactamase gene carriage rates but that the 3GC-R gene carriage in the human isolates cannot reasonably be compared with those of the cattle and canines, as the former were selected specifically for their resistance or sensitivity to 3GC.

Table 29. Carriage of genes associated with resistance to non-fluoroquinolone antimicrobials in FQ-R urinary *E. coli* isolates

Aminoglycosides									
aac(3)-IId	3	*aadA1*	7	*aadA2*	6	*aadA24*	1	*aadA5*	10
aph(3')-Ia	8	*aph(3')-Ic*	1	*strA/B*	24				
Tetracycline									
tet(A)	13	*tet(B)*	16						
Phenicols									
catA1	13	*cat2A*	1	*catB3*	1	*cmlA1*	3	*floR*	5
β-lactams									
bla$_{CTX\text{-}M\text{-}1}$	2	*bla*$_{CTX\text{-}M\text{-}15}$	1	*bla*$_{CTX\text{-}M\text{-}65}$	1	*bla*$_{TEM\text{-}1B}$	23	*bla*$_{TEM\text{-}1C}$	2
bla$_{OXA\text{-}1}$	1	*bla*$_{DHA\text{-}1}$	1	*bla*$_{CARB\text{-}2}$	1				

Table 30. Carriage rate on non-PMQR genes in FQ-R *E. coli* isolates from humans, cattle, and dogs

	Mobile β-lactam resistance gene carriage	Mobile 3GC resistance gene carriage	Mobile Aminoglycoside resistance gene carriage	Mobile Tetracycline resistance gene carriage	Mobile Phenicol resistance gene carriage	No mobile AMR genes detected
Human isolates	82.4%	47.9%	76.1%	65.4%	31.9%	6.9%
Canine isolates	78.4%	13.5%	78.4%	73.0%	54.1%	8.1%
Cattle isolates	88.1%	7.1%	92.9%	90.5%	69.0%	2.4%

In the carriage rate of the three non-β-lactam antimicrobial, the canine isolates were consistently between the human and cattle isolates. However, the results for β-lactamase carriage were different. Curiously, the cattle had the highest rates of mobile β-lactamase carriage, despite having the lowest rate of the mobile 3GC-R gene carriage – approximately

half what was observed in the canine isolates, and higher even than what was observed in the human urinary isolates. However, approximately half of the human-derived isolates were selected specifically for 3GC resistance or sensitivity and many of the human 3GC-R isolates had resistance owing to carriage of a single bla_{CTX-M} gene. Selection for 3GC-S, then, likely selected for no β-lactamase in many instances.

6.2.8. Virulence gene carriage

An analysis of virulence genes carried by the canine FQ-R isolates (**Table 31**) was performed. The total number of unique virulence genes was particularly high among canine isolates: 31 unique virulence genes were identified across just 37 isolates. This contrasts with the cattle isolates, in which only 14 unique virulence genes were identified across 42 isolates (chapter 3 – 5.2.6, **Table 23**). Meanwhile, the human-derived isolates carried 36 unique virulence genes (chapter 2 – 4.2.9, **Table 13**), but this was across 188 isolates. It should be noted that both the human and the canine isolates included multiple genes associated with aggregative adherence phenotype, characteristic of EAEC, which are usually carried together, and which represents a substantial number (8 in the human isolates and 7 in the canine isolates) of the genes identified. With these counted as a single virulence factor, the numbers fall to 29 in the human isolates, 25 in the canine isolates, and 13 in the cattle isolates.

We found an average of 3.9 virulence genes per isolate (standard deviation: 2.0) in the FQ-R canine isolates. This compares with 3.1 (standard deviation: 2.3) in the cattle isolates (chapter 3 – 5.2.6) and 5.2 (standard deviation: 1.5) in the human isolates (chapter 2 – 4.2.10, **Table 14**). So, despite the high degree of virulence factor diversity among the canine isolates, it would appear that in terms of virulence factors per isolate, the canine isolates lie between the cattle and the humans, but are more similar to the cattle isolates. This makes sense, given that the canine and cattle isolates were both faecal in origin, whereas the human-derived isolates were obtained from urine, of which most would be UTI.

That some FQ-R *E. coli* found in dog faeces carry virulence genes associated with diarrhoeal disease was not particularly surprising (342). What was less expected was the relatively high carriage rates of genes associated with ExPEC. The carriage rate for the increased serum survival gene, *iss*, and ferric enterobactin receptor, *iroN*, were both higher in the canine isolates than in the cattle (51.4% and 37.8% versus 45.2%, and 31.0%, respectively [chapter 3 – 5.2.6, **Table 23**]), and the latter also higher than in the human isolates (11.7% [chapter 2 – 4.2.9, **Table 13**]). Perhaps the most important finding, however, was carriage of the *iha* gene (present in 8.1% of canine isolates) which was absent in the

cattle isolates but found in 78.2% of human urinary isolates, and has been shown to be a significant factor in the establishment of urinary tract colonisation in a murine model (112).

Table 31. Canine FQ-R *E. coli* virulence gene analysis

Gene	Protein	Freq.		Gene	Protein	Freq.
aap	Anti-aggregation protein	1		*gad*	Glutamate decarboxylase	33
aar	AggR-activated regulator	1		*iha*	Iha	3
aatA	APEC autotransporter adhesin	1		*iroN*	Ferric enterobactin receptor	14
agg3C	Aggregative adherence fimbriae	1		*iss*	Increased serum survival	19
agg3D	Aggregative adherence fimbriae	1		*lpfA*	Fimbrial major protein	17
aggR	Aggregative adherence regulator	1		*mchB*	Microcin H47	2
air	Enteroaggregative immunoglobulin repeat	4		*mchC*	MchC protein	2
astA	Enteroaggregative *E. coli* heat-stable enterotoxin (EAST-1)	6		*mchF*	Probable microcin-H47 secretion/processing ATP-binding protein MchF	11
capU	Hexosyltransferase homologue	2		*mcmA*	Microcin M	4
cba	Colicin B	1		*ORF3*	*Trans*-isoprenyl phosphate synthase?	1
ccl	Cloacin DF13 protein	1		*ORF4*	Isopentenyl isomerase?	1
cdtB	Cytolethal distending toxin	1		*sat*	Secreted autotransporter toxin	1
cma	Colicin M	10		*senB*	Enterotoxin TieB protein	1
eilA	HilA family transcriptional regulator	4		*tsh*	Temperature-sensitive hemagglutinin tsh autotransporter	1
f17A	F17 fimbrial protein	3		*vat*	Vacuolating autotransporter toxin	1
f17G	F17G adhesin subunit	3				

Multiple other virulence-associated factors were found in both the canine- and human-derived isolates, but were absent from the cattle, including the EAEC-associated genes (*aap, aar, aatA, agg3C/D, aggR, air, capU, eilA, ORF3/4*), the toxin genes, *tsh* and *sat*, and the bacteriocin genes *ccl* and *mchB/C* (chapter 2 – 4.2.10, table 9). It is important to note, however, that many of these genes were found in only a very small number of both the canine-derive and human-derived isolates. It is plausible that, with a higher sample size, they would also have been detected in the cattle isolates. The *lpfA* gene, which was found to be most prevalent in the canine isolates (45.9%), followed by the cattle (35.7%) and the human isolates (15.4%), has been associated with enterovirulent *E. coli* (280), and also bovine mastitis (292).

In results chapter 2 (4.2.9, **Table 13**), the *lpfA*, *iroN* and *mchF* genes were all found to be significantly negatively associated with *E. coli* ST131. As these, and also *mcmA*, were often found in ST162, a series of χ^2 analyses were performed to investigate whether there was a significant association. In each case an association was hinted at (Table 32) but even including all ST162 isolates (n=20), there was not enough power to draw significant conclusions (in each case, one of the expected values in the contingency table was less than five, which as mentioned in chapter 2 is considered an invalidation of the χ^2 test (220)).

Table 32. Association between ST162 isolates from human, cattle and dogs, and carriage of virulence genes

Association between ST162 and …	χ^2	φ	p value
mchF	24.71	0.77	<0.0001
mcmA	20.06	0.69	<0.0001
lpfA	17.79	0.65	<0.0001
iroN	22.05	0.72	<0.0001

6.2.9. Phylogenetic analysis of canine, cattle, and human isolates.

Finally, a maximum likelihood phylogenetic tree was constructed, including the human, cattle and canine FQ-R *E. coli* isolates discussed in results chapters 2, 3 and above (Figure 17), to explore the relationships between isolates from the three reservoirs investigated in this and previous chapters. In this case, all canine isolates were included not only those from the locally recruited cohort. STs 744, 162, 10, 224 and 155 were found in all three reservoirs. Additionally, an ST4988 isolate was found in both the cattle and the canines (differed by just 3 SNPs), and an ST1193 isolate was found in both the canines and the humans (discussed above – 6.2.5).

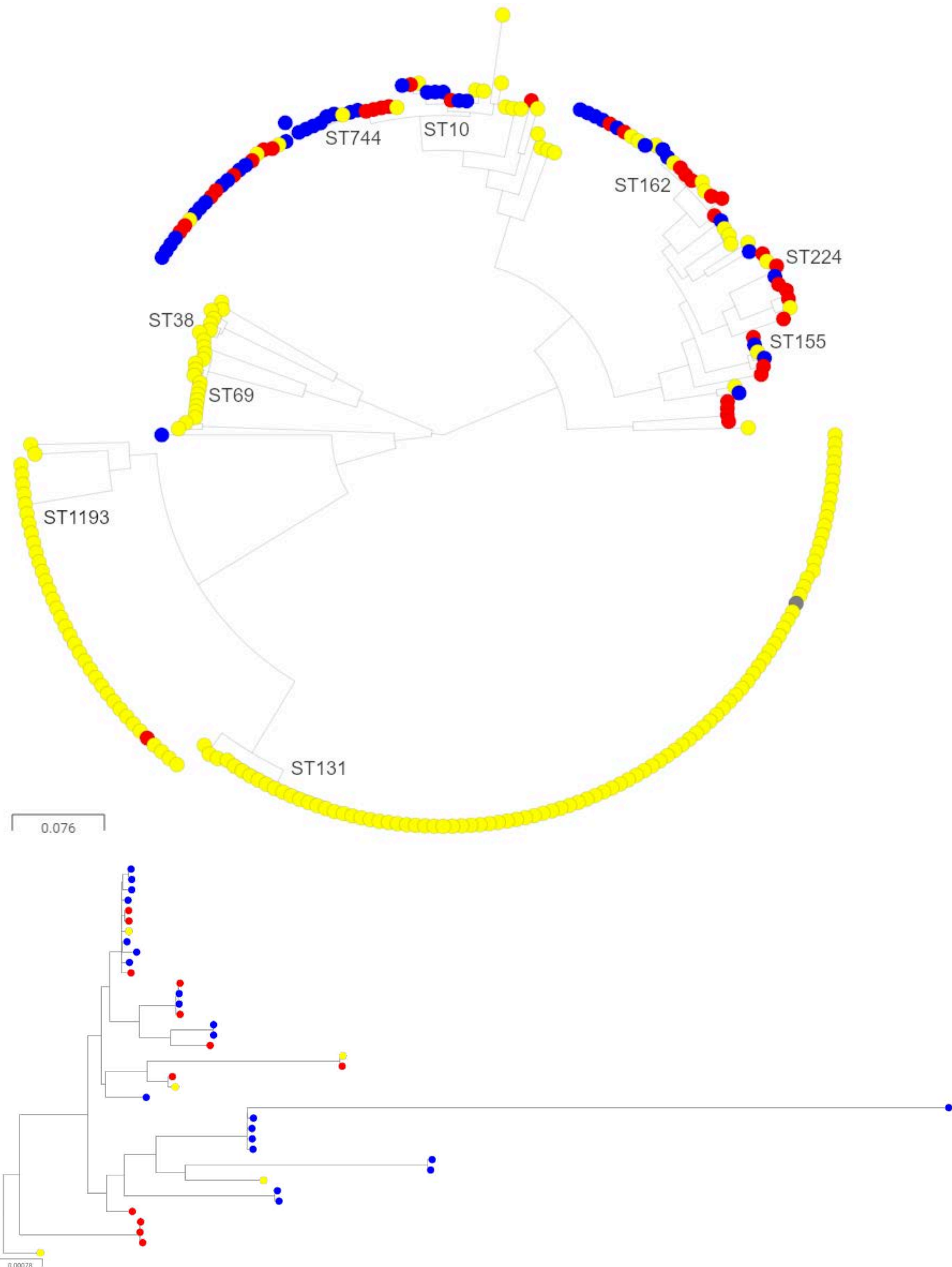


Figure 17. Core genome maximum likelihood phylogenetic analysis of all FQ-R *E. coli* included in this study, with ST744 subtree below

Human urinary isolates are represented by yellow nodes, canine faecal isolates by red nodes, and near-cattle environment isolates by blue nodes. Isolates were aligned against an ST131 reference (grey node). Regions of important STs represented in >5 isolates or found in all five reservoirs, are also indicated.

The core sequence alignment used for the phylogenetic tree was also used to examine the SNP distances between each of these STs in all three reservoirs (***Table 33***). No sharing (defined as above) was seen for ST155. Sharing of ST10 or ST244 was seen between cattle and dogs; sharing for ST244 was also seen between dogs and humans, but not between cattle and humans. Sharing of ST744 and ST162 was seen across all three reservoirs. Remarkably, the closest pair of humans isolates for ST744 different by 323 SNPs, but the closest human to dog or human to cattle match was 18 or 40 SNPs, respectively.

ST162 isolates were found to differ by <30 SNPs between all three reservoirs, strongly suggesting recent sharing, and importantly was commonly found to carry the *iss* gene, which was found in 71.8% of human urinary isolates (chapter 2 – 4.2.10, table 9). For ST744 and ST162, where there is evidence of sharing across all three reservoirs, confirming that these are the most important FQ-R *E. coli* STs from a One Health perspective.

Table 33. SNP distances for STs found in all three reservoirs

The shortest SNP distance is shown for each ST between and within isolates from each reservoir. N/A (grey) means there was only one isolate from that reservoir. Cases with less than 10 SNPs between isolates from those reservoirs are shown in pink, whilst cases of between 11-100 SNPs are shown in yellow and anything greater than 100 is shown in blue.

ST10	**Human**	**Canine**	**Cattle**
Human	73	2209	2204
Canine	2209	N/A	45
Cattle	2204	45	9
ST155	**Human**	**Canine**	**Cattle**
Human	N/A	1618	1558
Canine	1618	N/A	1231
Cattle	1558	1231	2465
ST162	**Human**	**Canine**	**Cattle**
Human	28	21	22
Canine	21	30	9
Cattle	22	9	2
ST224	**Human**	**Canine**	**Cattle**
Human	N/A	53	712
Canine	53	716	55
Cattle	712	55	N/A
ST744	**Human**	**Canine**	**Cattle**
Human	323	18	40
Canine	18	2	9
Cattle	40	9	2

6.3. Discussion and Conclusions

Raw meat feeding was identified as a risk factor for the excretion of ABR *E. coli* in the faeces of 16-week-old puppies, with particularly strong impact on excretion of isolates resistant to the critically important fluoroquinolones. If owners insist on feeding raw meat to their dog, it is essential that they fully understand this practice puts their dog at risk of becoming colonised with bacteria resistant to critically important antibacterials.

E. coli is the most clinically important opportunistic human bacterial pathogen (321). ABR *E. coli* infections are more difficult to treat, and result in more morbidity and higher mortality rates (321); there is also strong evidence that domestic pet dogs transmit ABR bacteria to humans (287, 310, 318-320, 343) and this study provides clear evidence of the faecal carriage within puppies of 3GC-R and FQ-R *E. coli* clonally related to those that have also caused urinary and bloodstream infections in humans living in the same geographical region collected within months of each other.

Therefore, if owners feed raw food to their dog, practices that mitigate the risk of onward transmission of ABR *E. coli* - which are more likely to be carried by these dogs – to humans should be encouraged. These include strict hygiene practices when anyone (particularly those vulnerable to bacterial infection) interacts with a raw-fed dog along with scrupulous disposal of the dog's faeces so that it cannot pose a risk to the general human population by contaminating the wider environment with ABR *E. coli*.

The hypothesis, that dogs act as transmission vectors for the spread of FQ-R *E. coli* between cattle and humans has been explored through three different routes – similarity in ABR gene carriage and resistance, virulence gene carriage, and direct relatedness. FQ-R canine isolates were found to be intermediate – in terms of prevalence of resistance and ABR gene carriage – between the cattle and human isolates. Canine isolates were also found to carry many of the same virulence genes that were identified in the majority human urinary isolates, and which have been found to be associated with UPEC. Five STs (744, 162, 10, 224 and 155) were represented in all three reservoirs, but one additional ST (4988) was represented in both a canine isolate and a cattle isolate, and another (ST1193) was represented in both a canine isolate, and is known as a widespread human pathogen (245). Finally, canine isolates were found to be closely related (<30 SNPs) both to human-derived isolates and cattle-derived isolates. More so than either is to the other. These results do support the hypothesis that dogs facilitate the transmission of *E. coli* between cattle and humans, and moreover that these *E. coli* are clinically relevant, both in terms of ABR and virulence. More evidence could be provided if the geographical location of each dog could be identified, so for example, those living in rural or urban communities. And it would be further

strengthened by analysing *E. coli* from their owners, and from people who do not own dogs that live in the same rural or urban environment as the dog owners. If dogs do act as a vector, bringing resistant bacteria from cattle into the home, and passing them onto humans, we would expect to see humans more likely colonised by *E. coli* closely related to cattle if they own a dog, and if they live in a rural area. Such a study could form a follow on to the one reported in this chapter, since we have recruited dog owners who could perhaps be persuaded to maintain their involvement in this research.

7. General discussion

The initial aims of the OH-STAR project are laid out in Introduction – 1.18. Whilst the project aimed to measure the prevalence of *E. coli* resistant to a variety of antibacterials in faeces from dogs and collected on dairy farms, in terms of detailed molecular ecology and comparisons between *E. coli* from humans and animals, the project exclusively focused on 3GC-R. Whilst my role as a technician allowed involvement in all aspects of this work, in order to develop a PhD project, I sought to develop something that would add value to the OH-STAR project, and so I chose to develop a molecular ecology project considering FQ-R *E. coli* from humans – which required a separate survey of urinary *E. coli* – and from dogs and cattle, as derived from OH-STAR samples.

The main aim of the work reported in this thesis was to examine the hypothesis that there is sharing of 3GC-R (a specific task of mine in OH-STAR) and FQ-R *E. coli* between companion animals (dogs), dairy cattle and humans within a relatively small (50 x 50 km) geographical region of Bristol and surrounding areas. Furthermore, it sought to investigate the risk factors associated with carriage of FQ-R *E. coli* in dairy cattle and dogs, and explore the carriage of other ABR mechanisms and virulence factors in FQ-R *E. coli* isolated from dogs, humans, and cattle in this region.

In results chapter 1, work carried out in collaboration with my colleagues, Hannah Schubert, Maryam Alzayn and Jackie Findlay, is presented. This was core OH-STAR work and has led to the publication of three papers in which I am named as an author (202, 213, 223). No evidence was found for the sharing of 3GC-R *E. coli* – either bacteria carrying mobile 3GC-R genes or those which carried mutations leading to AmpC hyperproduction – between dairy cattle and humans. These conclusions align with those of other similarly motivated studies from the UK, which also considered sharing of 3GC-R *E. coli* between various animal species (including those present on food for human consumption) and humans (240, 287, 307).

Indeed, it seems that for 3GC-R *E. coli*, the most likely source of colonisation for a person is contact with another person. Subsequent chapters describe a more detailed examination of FQ-R *E. coli* in human urinary isolates (chapter 2), near-animal environment dairy cattle isolates (chapter 3), and companion animal (dog) faecal isolates (chapter 4), and reach a conclusion about sharing of FQ-R *E. coli* between these three important reservoirs.

In results chapter 2, I report a survey of human urinary FQ-R *E. coli* designed and initiated by myself, which complemented the 3GC-R/FQ-R dual resistant isolates collected in OH-STAR and allowed interesting comparisons. Of 489 FQ-R isolates analysed, 36.0% were positive for at least one PMQR gene. A strong positive association was found between

PMQR carriage in an isolate and that isolate being 3GC-R. A detailed examination of 189 isolates which were subject to WGS followed, in which QRDR mutations, ST, ABR gene carriage (including PMQR), virulence gene carriage, and phylogenetic relationships between these human isolates were explored and associations between these different phenotypes and genotypes were investigated. Statistically significant associations were identified between ST131, ABR gene carriage (*aac(6')Ib-cr*, bla_{OXA-1}, $bla_{CTX-M-15}$, *tet(A)*, and *aac(3)-IIa*), and virulence gene carriage (*cnf1*, *iss*, and *nfaE*) – and between ST1193 and the virulence gene, *vat* – using χ^2 analyses and φ coefficient to establish statistical significance (p value) and strength of association, respectively. The accumulation of resistance to so many important antibacterials, and in combination with important virulence factors, in a notoriously pathogenic strain is deeply concerning and may soon lead to few available options for treating infection with ExPEC diseases, from UTI to sepsis.

Analysis of FQ-R *E. coli* in dairy cattle is presented in results chapter 3. Carriage of PMQR genes and mutations in the QRDR was investigated and compared with the urinary isolates discussed in chapter 2. Resistance rates to non-FQ antimicrobials was explored, using antimicrobial disc susceptibility testing, and compared with carriage rates of ABR genes identified through WGS. ABR and virulence gene carriage was also investigated and compared with the genes identified in the urinary isolates in chapter 2. Overall, virulence gene carriage was much lower than in the human urinary isolates, and rates of 3GC-R were typically lower than has been observed in general (285, 286). No isolates from phylogroups B2 or D – the most significant phylogroups for ExPEC (96) – were found in the cattle. Finally, relatedness (in terms of SNP distance and phylogenetic distance) between isolates found in the dairy cattle and human urine was analysed, and evidence of recent sharing was identified. This work also enabled an in-depth risk factor analysis for carriage of FQ-R *E. coli* – carried out by my colleague, Dr Hannah Schubert – which showed that the use of fluoroquinolones drives emergence of FQ-R *E. coli* on dairy farms, and that dry cow therapy was negatively associated with FQ-R *E. coli* carriage. There are significant implications here for the approach that might be taken to reduce the abundance of FQ-R *E. coli* on dairy farms, and given the small but not zero zoonotic threat in the context of human bacteriuria, antimicrobial usage changes should be encouraged to do this.

Finally, in chapter 4 *E. coli* isolates from canine faecal samples are investigated and it is found that puppies carry FQ-R and 3GC-R *E. coli* that are closely phylogenetically related to those which have caused urinary and bloodstream infections in people living the same geographical area as the dogs. This work also enabled an in-depth risk factor analysis for carriage of FQ-R *E. coli* – carried out by my colleague, Dr Ashley Hammond – which showed that feeding puppies raw meat is positively associated with FQ-R *E. coli* carriage.

Differences and similarities are noted between FQ-R dog isolates and those examined in previous chapters (2 and 3), in terms of ST prevalence, QRDR mutations, ABR, and both ABR- and virulence-gene carriage. Evidence was found of FQ-R *E. coli* sharing between the three reservoirs and the results found that the FQ-R dog isolates were intermediate between the human and the cattle isolates in terms of STs, frequency of 3GC-R phenotype, ABR gene carriage, virulence gene abundance and, perhaps most importantly, SNP distance for those STs found in all three reservoirs. It is concluded, therefore, that dogs likely act as vectors for the sharing of clinically relevant *E. coli* between humans and dairy cattle. It has recently been shown that companion animals in Australia often carry the same clinically relevant FQ-R *E. coli* isolates as are found in humans (320). The work presented here comes to the same conclusion but, additionally, advances current knowledge about FQ-R transmission. It is possible – and the data obtained here does not exclude this possibility – that dogs may act as an intermediate host, bringing environmental bacteria, e.g. those derived from cattle faeces, into the home, resulting in colonisation by humans. However, to test this hypothesis properly would require a very targeted and properly controlled survey project, looking at humans, their pets, and the environments that their pets interact with during exercise.

At points in the project, it was necessary for me to make key decisions about what tools and processes to utilise in order to explore the question of FQ-R *E. coli* sharing between the three reservoirs. For example, whilst the VirulenceFinder 2.1 analysis was included in the default pipeline analysis run by the Centre for Genomic Evolution's (CGE) web service for analysis of WGS data, I made the decision to investigate virulence factor gene carriage further, as a way to analyse the differences and similarities between genes (both in number and type) found in isolates from each reservoir and to explore association with AMR gene carriage. I also made the decision to take on the phylogenetic and SNP distance analysis in order to quantify the relatedness between isolates, both for the OH-STAR project and for this PhD. Because I had no prior experience of this, I initiated contact with Professor Ed Feil of the Milner Centre for Evolution, at Bath University for that purpose. Professor Feil introduced me to Nicola Coyle, who set me up with an account on an Ubuntu-based server run by the Cloud Infrastructure for Big Data Microbial Bioinformatics (CLIMB) project (218). The server uses a BASH interface, through which directories can be navigated and programs run, which I had no prior experience with. I have since become proficient with BASH and in the use of some of the software that can be used for SNP variant calling (Snippy) and phylogenetic analysis (RAxML), for the benefit of multiple publications. Furthermore, I have been able to share this knowledge and teach colleagues how to interact with and use this software.

A maximum likelihood approach, utilising RAxML, was used for the phylogenetic analyses throughout this work. This was recommended by the Feil group in Bath and is widely regarded as a robust and rigorous method of estimating phylogeny (70). SNP distance has frequently been used here to quantify relatedness between isolates and a reasonable question therefore might be, why bother with the phylogenetic analysis if you have the SNP distance? The answer is that phylogenetic analysis, when inferred by maximum likelihood, is much more accurate, especially when comparing isolates which are distantly related (70). SNP distance is a simple measure of the number of SNPs that are different in the core genome between two isolates and thus does not account for rate heterogeneity – the fact that some nucleotide substitutions are more likely to happen than others – so while useful for comparing closely related isolates, would not usually be appropriate for comparing, for example, isolates of different ST (344). In the work reported in this thesis, we needed to perform both types of analysis.

The work shared here is not without its limitations. Whilst I am confident that the conclusions reached are supported by the data presented, there are some areas in which things could have been improved or done better. For example, most of the dog isolates were sent from unknown locations across the UK. Only seven FQ-R isolates were known to have come from the OH-STAR study region, so it would not be reasonable to make strong inferences about a lack of similarity, if that was what was found between dog isolates and those from other sources. However, the fact that evidence of sharing between dogs and humans and between dogs and cattle was found despite the small sample size of dog isolates, suggests that a wider survey would likely find more sharing between reservoirs, though this is not guaranteed, and we might have simply been lucky to find it. Hence we can say sharing involving dogs happens but not how much it happens.

As with the geographical location problem found with the canine isolates, it would be fair to argue that the human isolates are not entirely equivalent (given the strong population bias towards the city, where there are no dairy farms, for example) and therefore little could be reasonably concluded from an observed lack of sharing of isolates between cattle and humans. This criticism could be applied to the results in chapter 1, which indicate a lack of sharing 3GC-R *E. coli* between cattle and humans, but the strong evidence of sharing observed between the FQ-R *E. coli* between dairy cattle and humans, showing that the study had the correct design to identify sharing when it happens and adds legitimacy to the results presented in chapter 1.

The study compared *E. coli* derived from near-animal environmental cattle samples – which were often contaminated with faeces – and *E. coli* derived directly from dog faeces, with *E. coli* isolated from human urine. The dog and cattle isolates, then, are somewhat equivalent,

being essentially commensal organisms, of which there may be a variety in each sample. However, the human isolates are different, being likely clonal pathogens inhabiting a specific (and not entirely welcoming) niche. Ethical barriers mean it is much harder to obtain faeces from humans than from dogs or cattle, and we lacked the permissions to process human faeces in the laboratory, so this was not pursued. Indeed, using urinary isolates allowed us to highlight the fact that shared isolates are clinically relevant. Whilst bacteriuria is not necessarily indicative of UTI – one study found that 18.9% of nursing home residents had asymptomatic bacteriuria (345) – the presence of bacteriuria is nonetheless a cause for concern. People with asymptomatic bacteriuria have been found to have significantly elevated levels of complement component C3, relative to creatinine, indicating an increased immune response to bacteriuria (345). It would also have been advantageous to have *E. coli* from healthy humans particularly to allow consideration of virulence gene carriage in the context of commensal *E. coli* in humans, because this was perhaps the analysis most directly affected by the fact that two sets of animal commensals were being compared with a set of human isolates dominated by pathogens . A study on the carriage of virulence genes in *E. coli* from healthy adults and children living in Poland found many of the virulence genes we detected in the urinary isolates (346). Some of the urinary isolates we collected carried very few virulence genes associated with UPEC. This may highlight the fact that these were urinary isolates, collected as part of routine diagnosis. Accordingly the abundance of bacteria in the sample was sufficient to warrant processing in the lab, but the person providing the sample did not necessarily have UTI. Bacteriuria is now known to be quite common, especially amongst the elderly (345). However it may also highlight that virulence is not absolutely required for the induction of UTI, and that infection is simply opportunistic in nature, arising, by chance, from the commensal flora.

It might seem that a reasonable explanation for the similarities observed between the dog and cattle isolates, is that they are both faecal in origin; whereas the human isolates were selected for their presence in urine, and many were likely causing UTI. Indeed, the large differences observed in virulence gene carriage would support this hypothesis. It could also be suggested that the human and the canine isolates are similar due to an at least somewhat similar diet and intestinal physiology. However, some of the isolates – especially those which were STs 162 and 744, but also STs 10 and 224 – were not just similar but extremely closely related, sometimes differing by less than 20 SNPs across the three reservoirs. A core genome SNP distance of 30 or fewer is commonly seen in Enterobacterales that are confirmed part of an acute outbreak of foodborne illness (238). For further context, however, consider ST1193. ST1193 is well recognised as a clonal FQ-R ExPEC group which came about via multiple large simultaneous homologous recombination

events (246) and emerged globally in humans approximately 25 years ago (245), but which spread significantly into hospitals around the world only in the last ten years (247). ST1193 was a commonly represented ST found in the human isolates and is discussed in chapter 2. A SNP distance of 40-80 was commonly observed between ST1193 isolates and some ST1193 isolates differed by as much as 250-300 SNPs. Accordingly, some of the three-reservoir "triplets" of FQ-R *E. coli* found in this study are more closely related than almost all examples of a known epidemic human clone (all collected within a few months) in the OH-STAR study region.

Analysis of virulence gene carriage using VirulenceFinder 1.2 highlighted some interesting differences and similarities between the isolates from each reservoir. However, as a tool the program was not without its shortcomings. As discussed in results chapter 2, its identification as *gad* as a virulence gene is questionable, not least because it seems to be almost ubiquitous. Additionally, there are some important virulence-associated genes which it did not identify, such as the capsule-encoding gene *neuC*, which is much more common in NMEC strains than UPEC or APEC, or the iron acquisition gene, *fyuA*, which is commonly associated with ExPEC strains in general (95, 109). A full list of the genes which VirulenceFinder 1.2 identifies can be found in Joensen *et al.* (215), whilst more expansive lists of genes associated with ExPEC – focusing on UPEC, NMEC and APEC, in particular – can be found in Ewers *et al.* (109), and Johnson *et al.* (95). There is now an updated version of VirulenceFinder (2.0) (264), which has a more expansive list of genes but, unfortunately, this is not yet integrated into the pipeline analysis service run by Center for Genomic Epidemiology (CGE) (216).

The question remains as to which virulence genes, if any, are essential for the establishment of UTI, and for progression into complicated UTI, sepsis, and new-born meningitis. Enhanced adhesion, protectins, toxin-production, and iron acquisition are all factors commonly associated with UPEC. With the help of tools like the updated VirulenceFinder 2.0, it might soon be possible to know whether or not a particular isolate has a strong capability of causing one or more of these important diseases. Some of the human urinary isolates carried virulence genes which are typically associated with enterovirulence, such as fimbrial protein gene, *lpfA*, and the EAST-1 toxin gene, *astA* (chapter 2, table 12). This has been described previously (279, 280), but notably *astA*-carrying *E. coli* have been isolated from patients who were not admitted to hospital due to diarrhoea (347).

One minor shortcoming for the project was that I did not put more effort into obtaining positive controls for *qnrC* and *qnrD* genes and *qepA*. Both *qepA* and a *qnrD*-like gene were detected in the WGS results by ResFinder 2.1 but missed by the multiplex PCR. A positive control for these genes would have ensured that the multiplex PCR analysis was functioning

correctly. However, given analysis of hundreds of sequenced isolates identified one or two of each of these genes, the impact of this on a PCR survey of 489 isolates is very small.

FQ-R comes principally from mutations in the QRDR region, sometimes augmented by carriage of a PMQR (173). These mechanisms were sufficient to account for resistance in the vast majority of isolates investigated. However, 8 out of 267 isolates, across all three reservoirs, did not have the necessary QRDR mutations and/or PMQR genes to explain FQ-R. It is suspected that resistance in these isolates was due to altered permeability or efflux, possibly due to disruption of regulatory genes, as discussed in Introduction – 1.17. Unfortunately, PointFinder does not identify mutations in regulatory genes, such as *marR*, which affect permeability and efflux and can confer reduced susceptibility to multiple antimicrobials. Nonetheless I would have liked to explore this further but it was given low priority, as it was only known to be relevant for 3% of the isolates studied. ResFinder has recently been updated to version 4.1, and now incorporates PointFinder into its search function but unfortunately this still does not identify disruptions in regulatory genes associated with reduced permeability/efflux and is also not yet integrated into CGE's pipeline analysis. As this project focused principally on FQ-R *E. coli* and did not investigate isolates which were grown without antibacterial selection, it was not possible to assess rates of PMQR gene carriage in FQ-R relative FQ-S *E. coli* (except for those which were 3GC-R, collected in OH-STAR, which would be yet another biased sample). It was also not possible to investigate the prevalence of QRDR mutations insufficient to give resistance among FQ-S isolates. It would have been interesting to see how prevalent single *gyrA* or *parC* mutants were in FQ-S isolates, as these could potentially be only a single step away from an FQ-R phenotype (348).

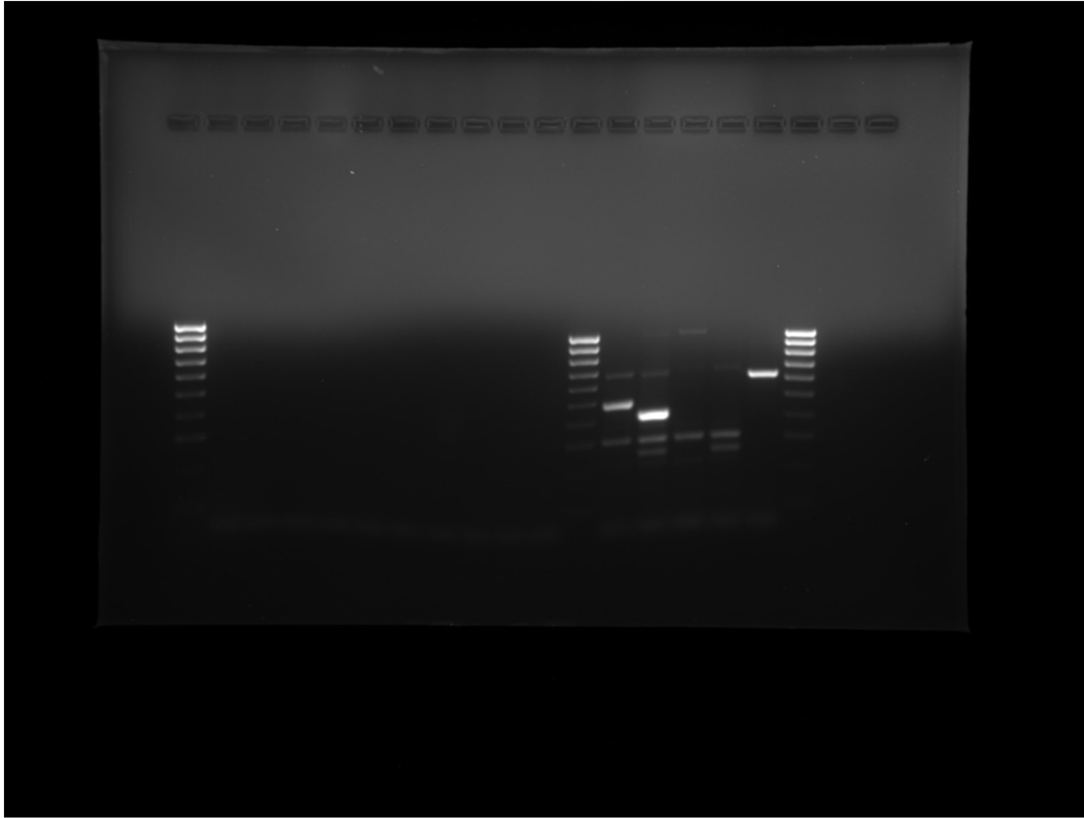
The significance of the AAC(6')Ib-cr enzyme for FQ-R *E. coli* remains an open question. The gene which codes for it was found in 43 FQ-R isolates across all three reservoirs (16.1%), though was notably absent from the dairy cattle isolates. In all but one instance those isolates had QRDR mutations sufficient to give clinical FQ-R. One explanation is that AAC(6')Ib-cr, despite having the two amino acid changes differentiating it from AAC(6')Ib and necessary to modify ciprofloxacin and norfloxacin (349), is still principally selected for by amikacin usage. Indeed, this would explain its absence from cattle isolates (amikacin is not authorised for use in animals in the UK) (169). Indeed, *aac(6')-Ib-cr* has been found in US dairy calves (350), and amikacin is sometimes used in US cattle (351). Interestingly, carriage of *aac(6')-Ib-cr* was found to be significantly associated with enrofloxacin usage in the US dairy calf study (350). AAC(6')Ib-cr is widely understood to affect only two commonly used fluoroquinolone antimicrobials, ciprofloxacin and norfloxacin (181, 349). However, as discussed in the Introduction – 1.14, it has been shown that enrofloxacin – one of the few

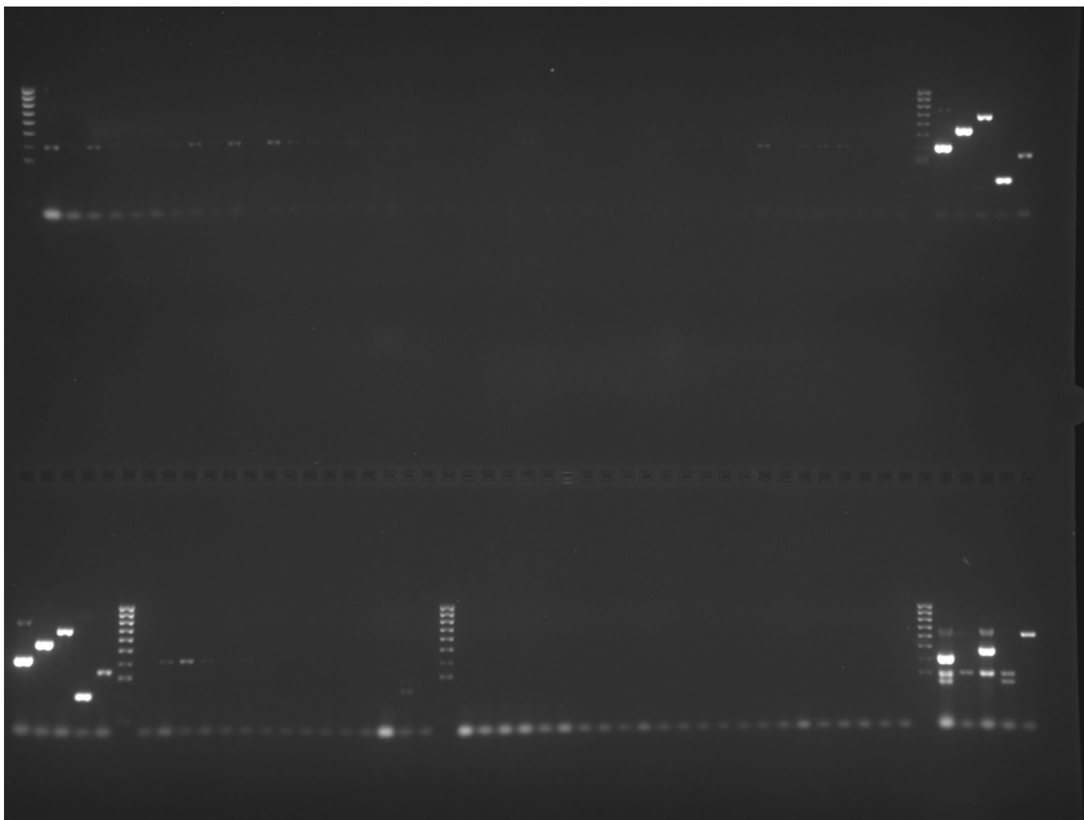
fluoroquinolone drugs indicated for use in veterinary medicine – is actually a prodrug and is converted into an active metabolite, ciprofloxacin (172). It should be expected, then, that enrofloxacin MIC is affected by AAC(6')Ib-cr. I hope to test this hypothesis soon.

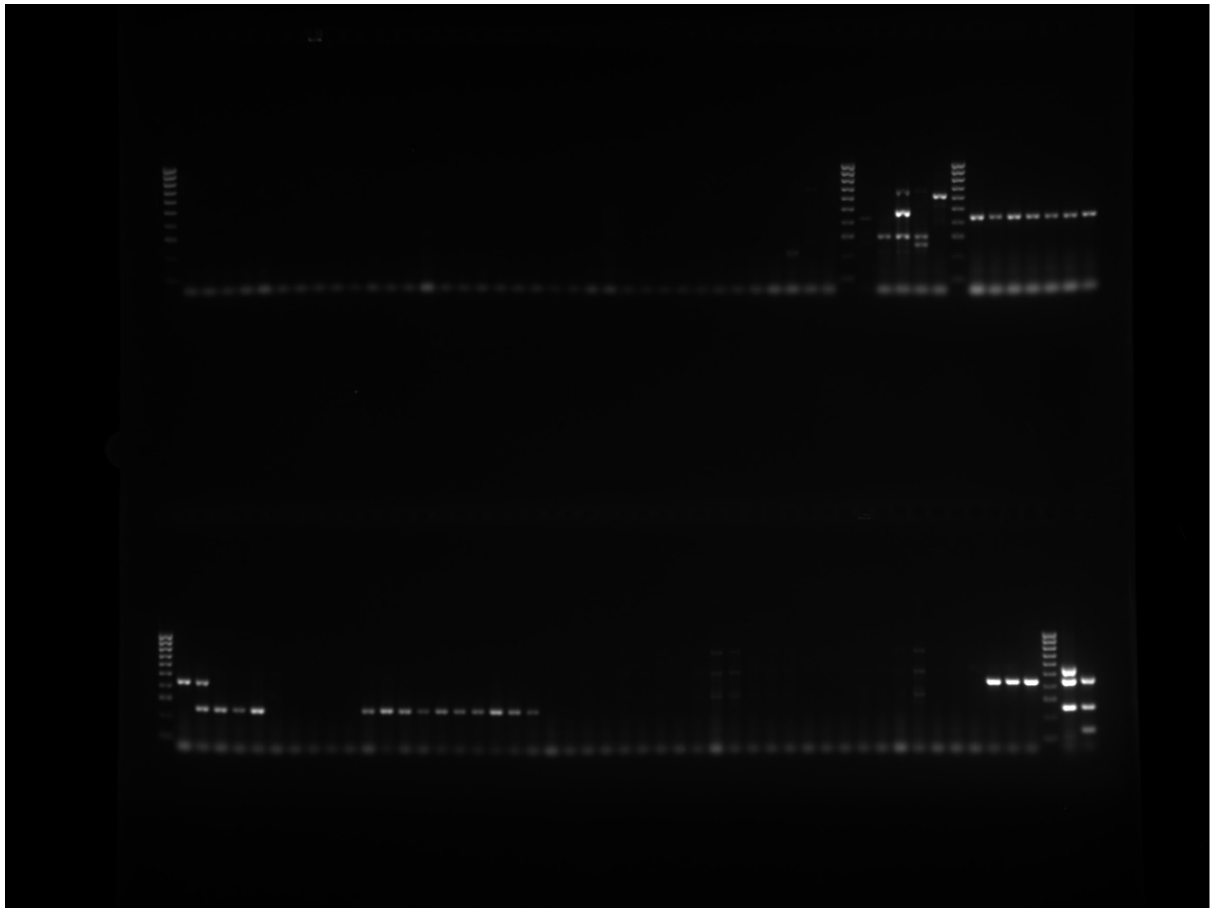
The current situation sees high-income countries, where food-producing animals rarely mix with the human population, often maintaining rigorous ABR surveillance programmes and antibacterial usage management. Whereas lower- and middle-income countries (LMICs) – where people are more likely to live in close proximity to food-producing animals and thus where novel pathogens and AMR-mechanisms are more likely to emerge – understandably consider antimicrobial surveillance and stewardship a lower priority, and lack the funding necessary to maintain such schemes anyway. This situation is improving, however, as multinational organisations initiate greater global surveillance and stewardship and as global partnership projects between high-income countries and LMICs are given higher priority. The Avison group are pioneering this, with two projects currently on-going – the One Health Drivers of ABR in rural Thailand (OH-DART) project, and the Future-proofing Antimicrobial resistance Risk Management Surveillance and Stewardship in Argentinian Farming Environment (FARMS-SAFE) project. People are increasingly understanding that infectious disease and ABR are global problems which begin in a local environment but can quickly spread. The SARS-CoV-2 pandemic has highlighted this and will hopefully encourage greater consideration and respect for the problem of infectious disease and AMR in the future.

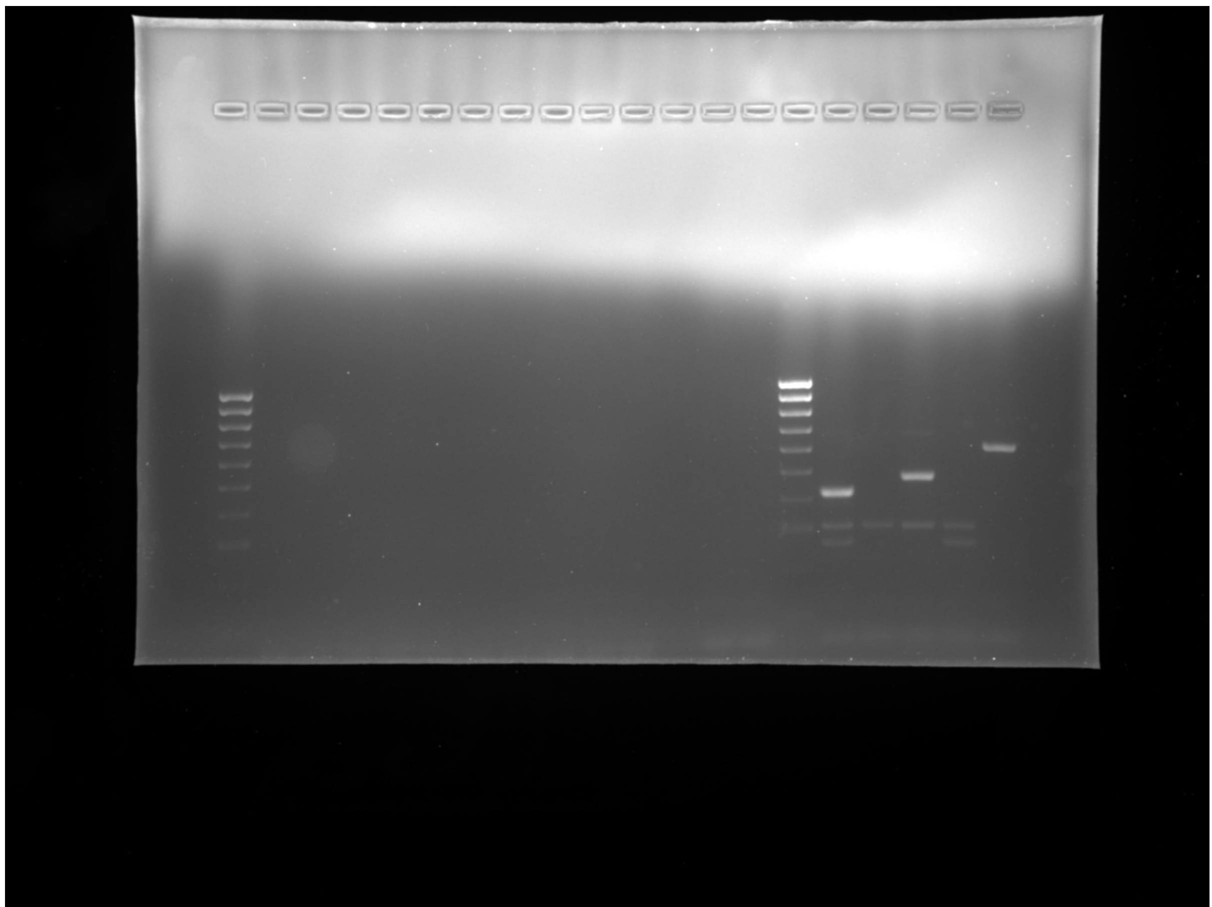
Appendix

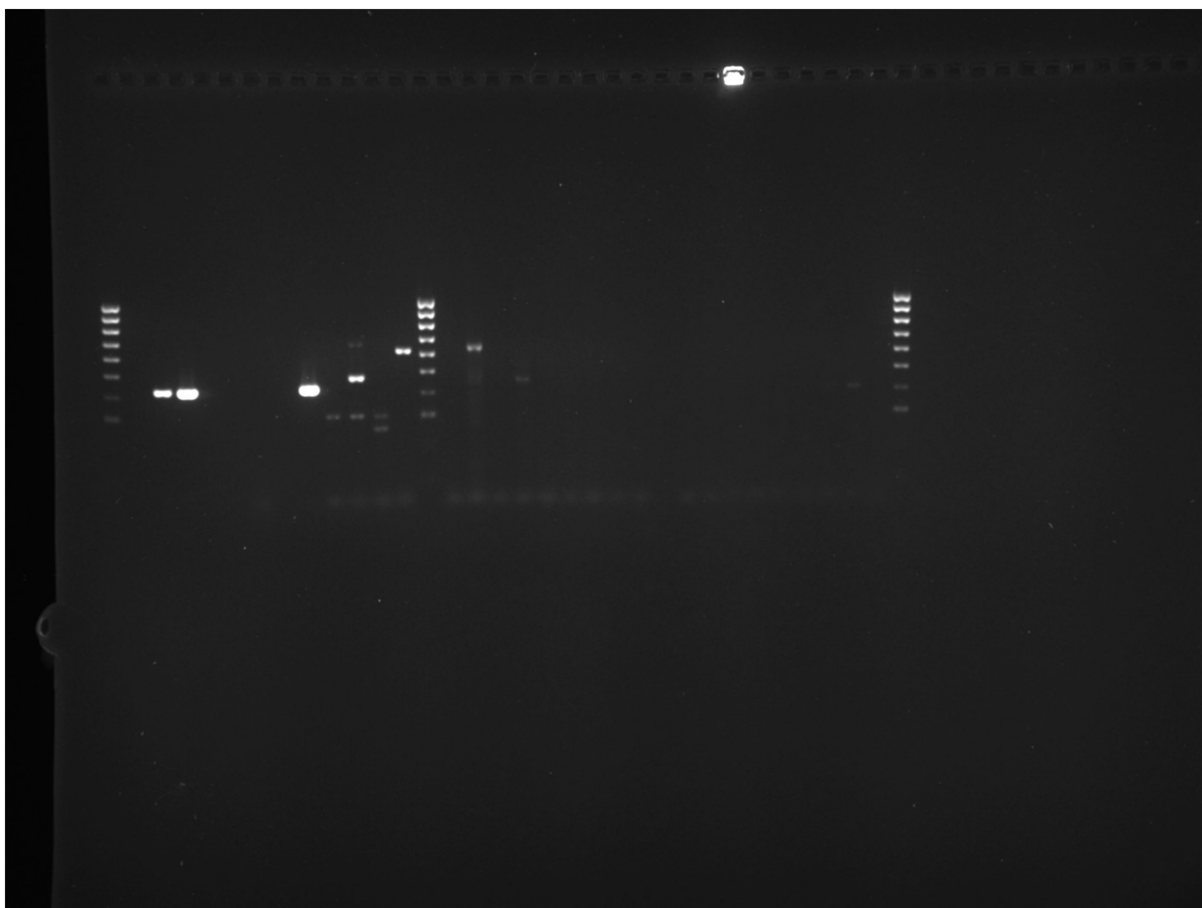
Agarose gel electrophoresis images from PMQR multiplex PCRs:

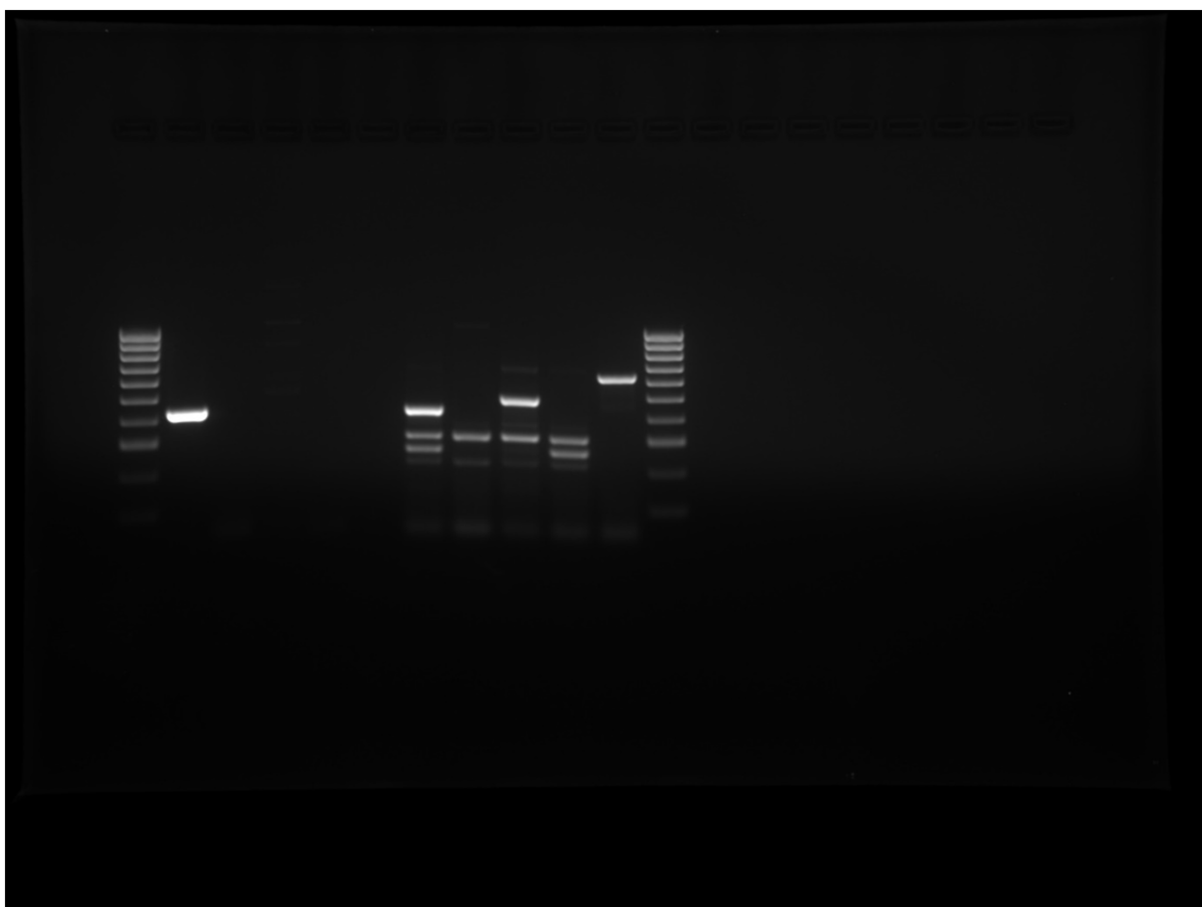


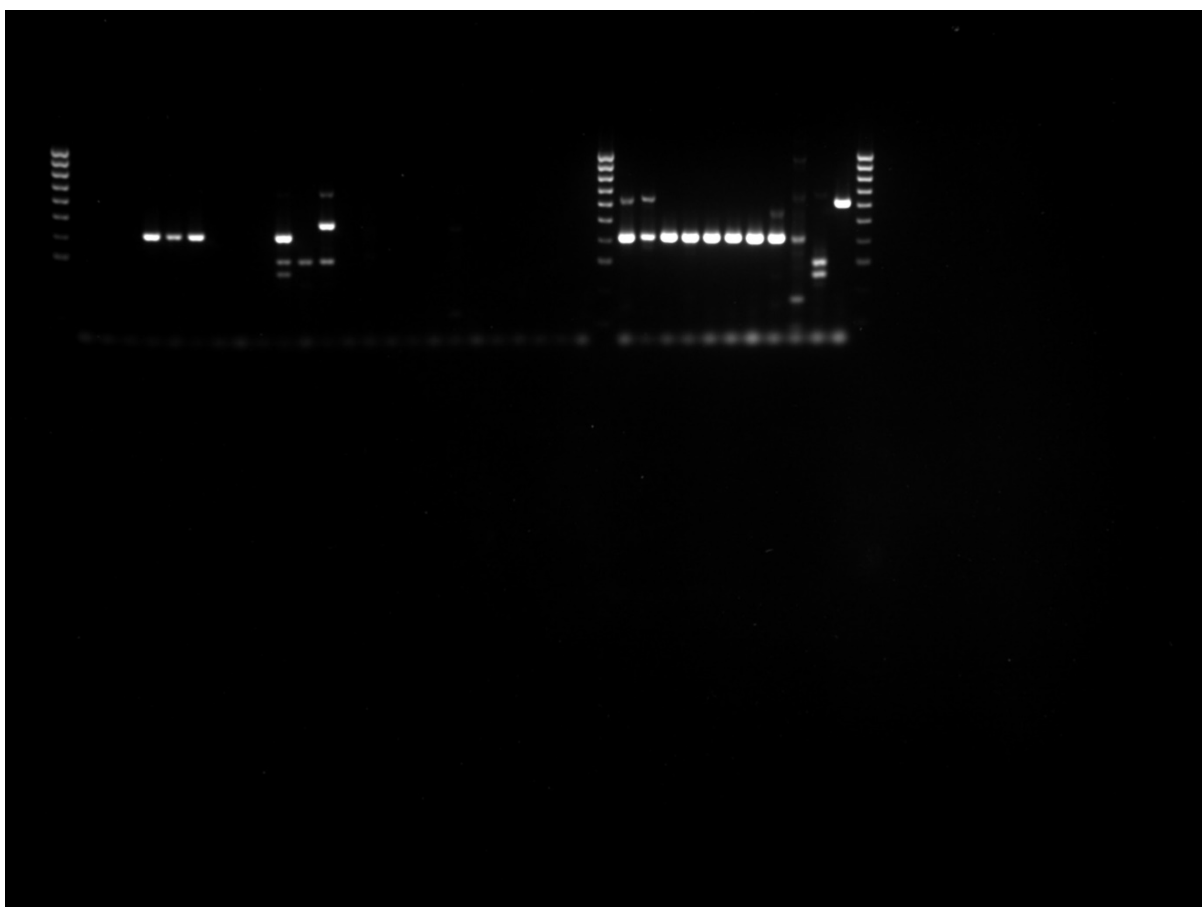


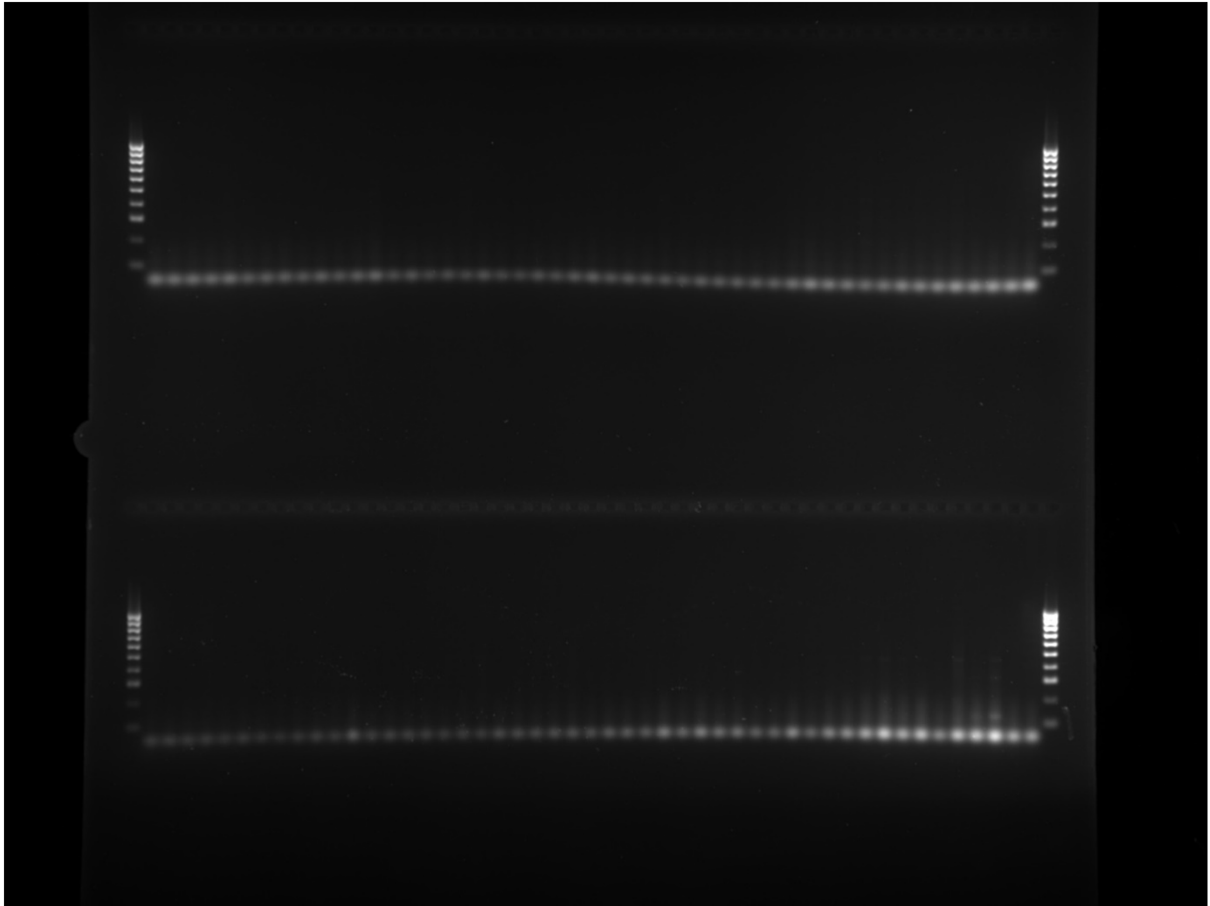


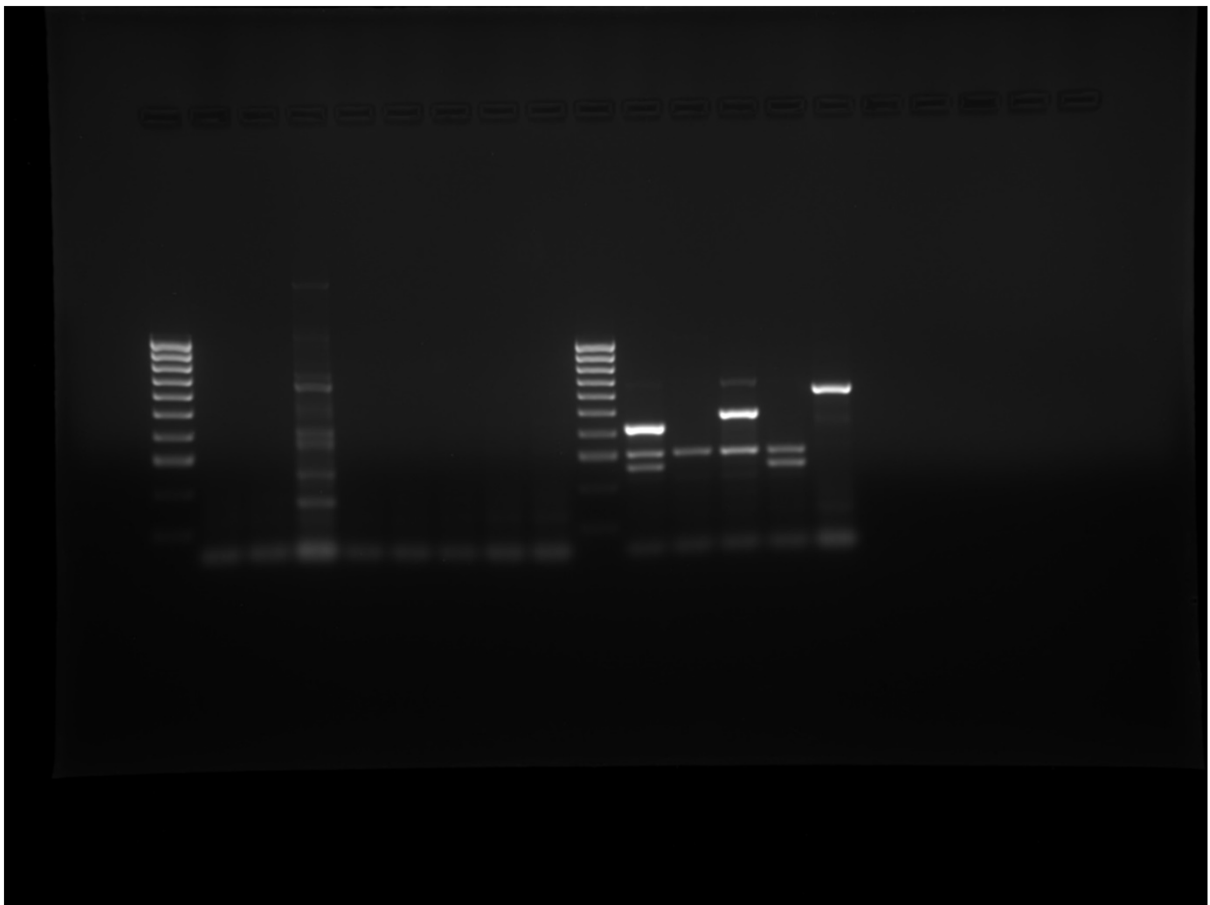


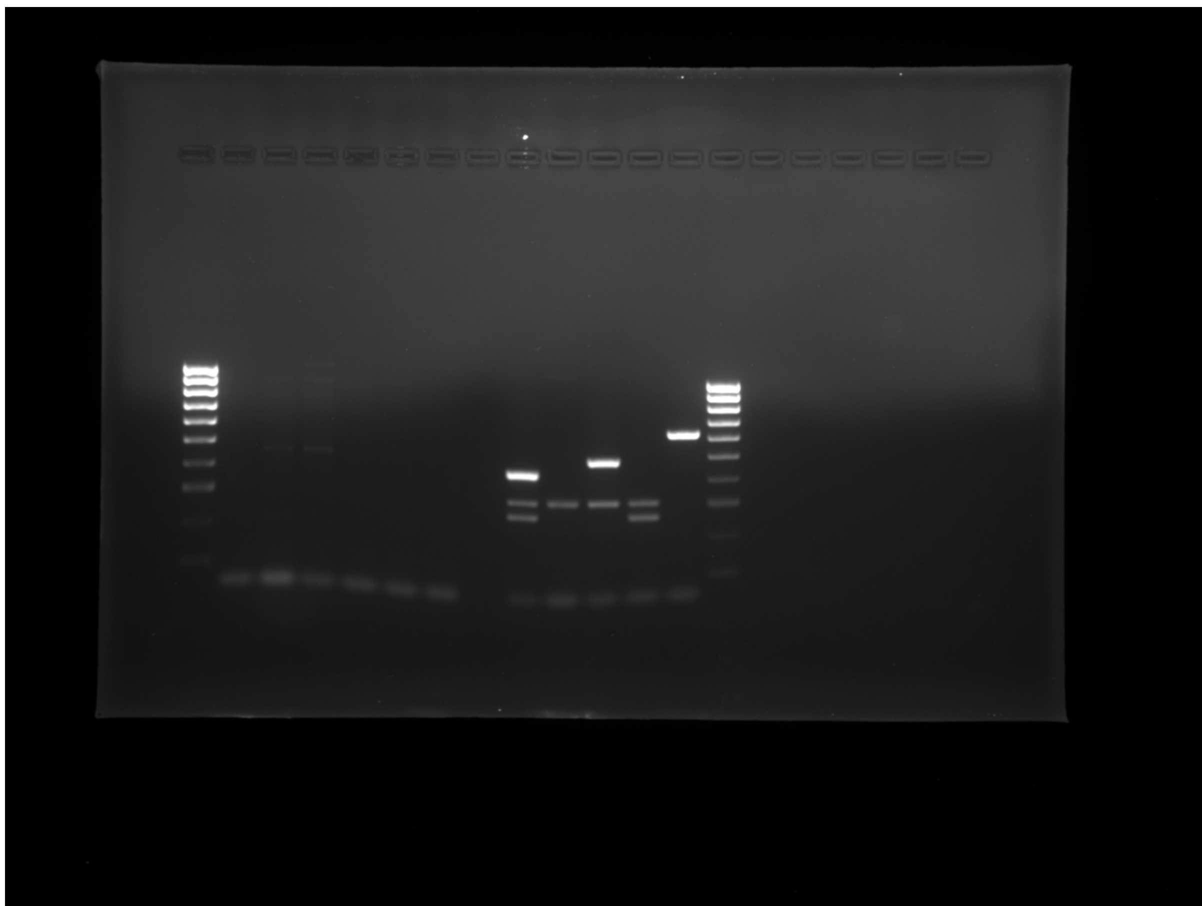


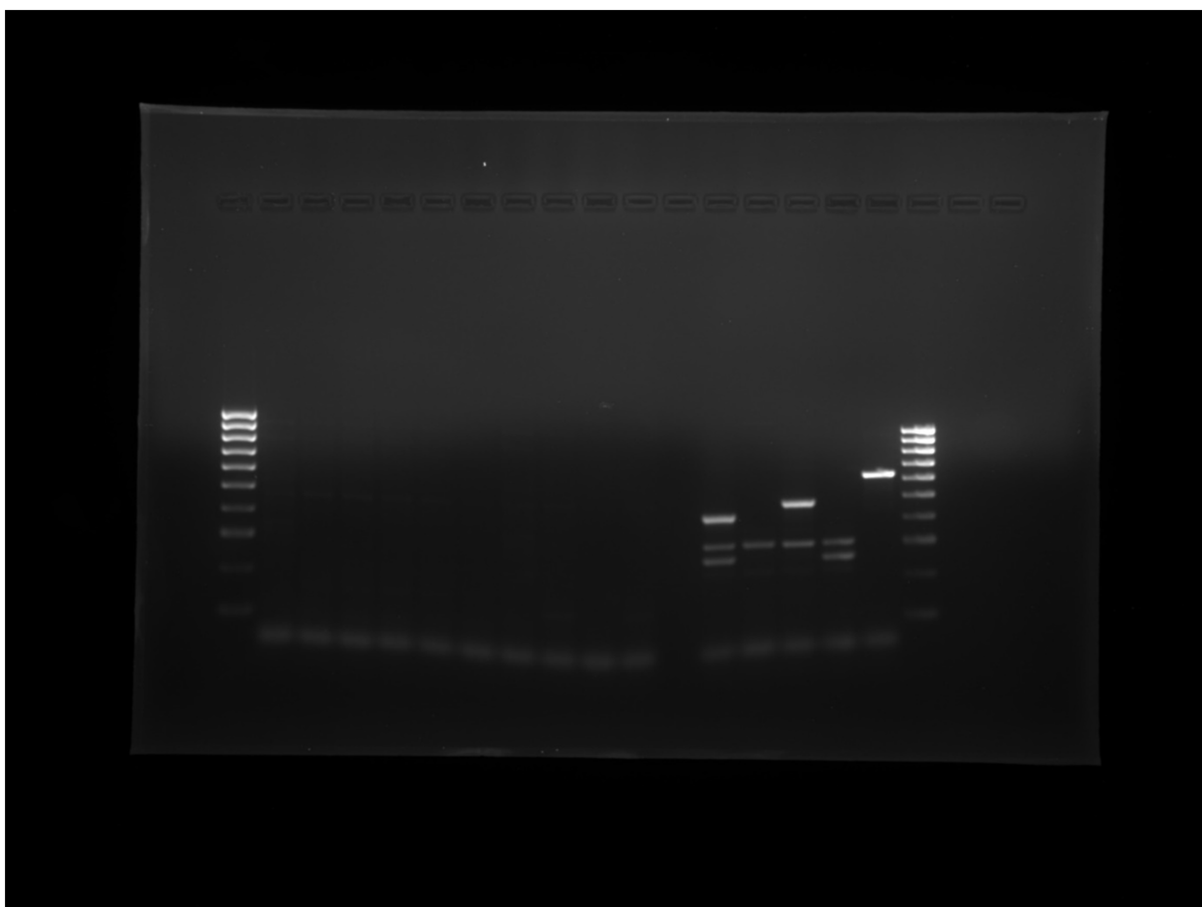


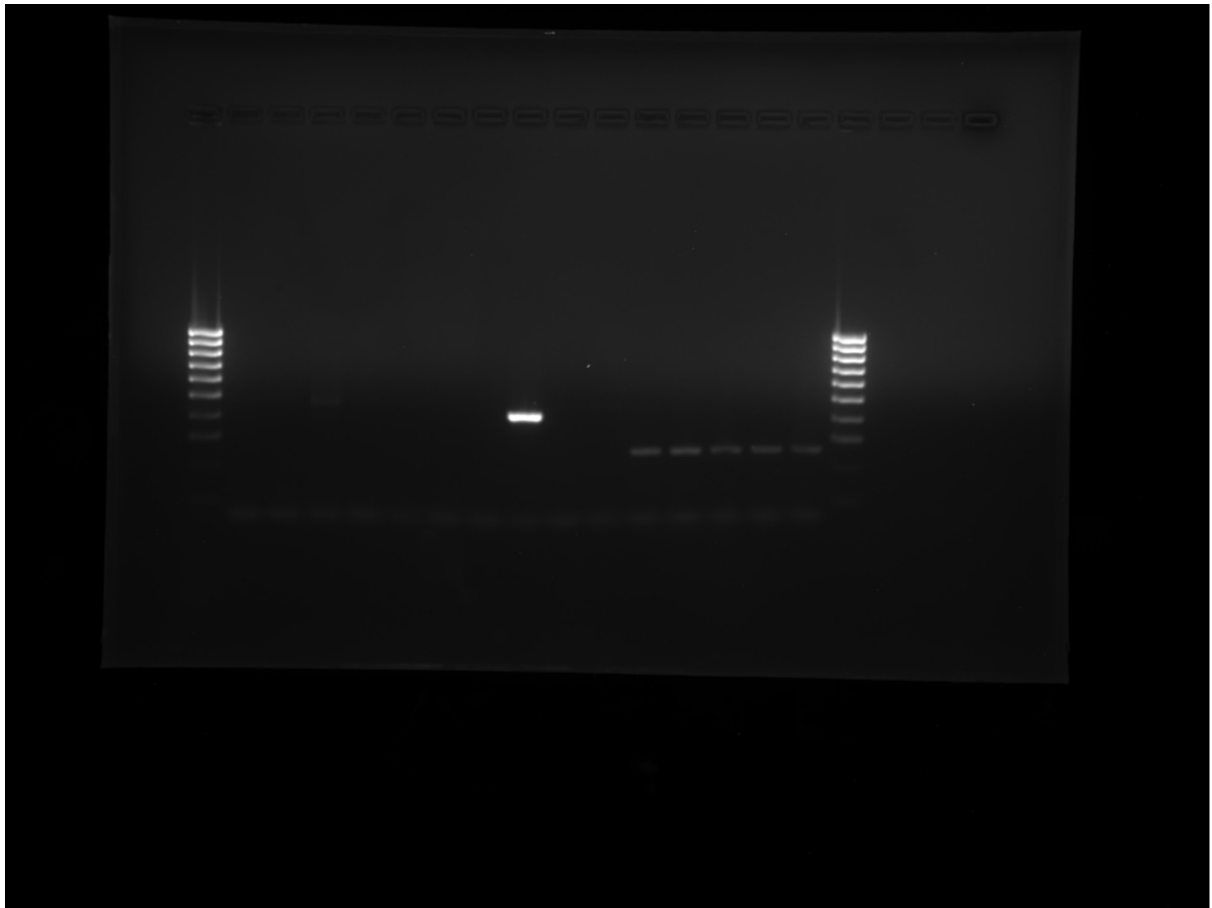


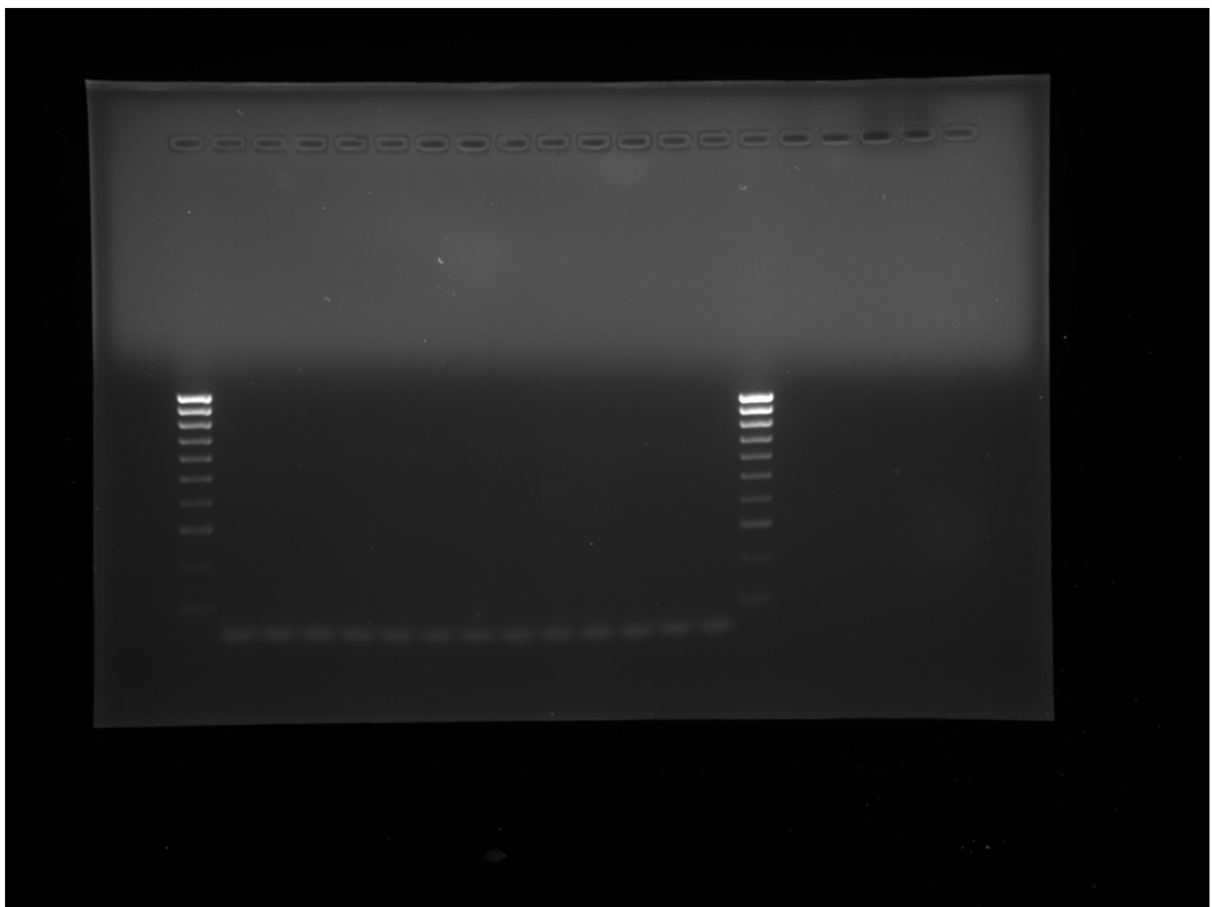


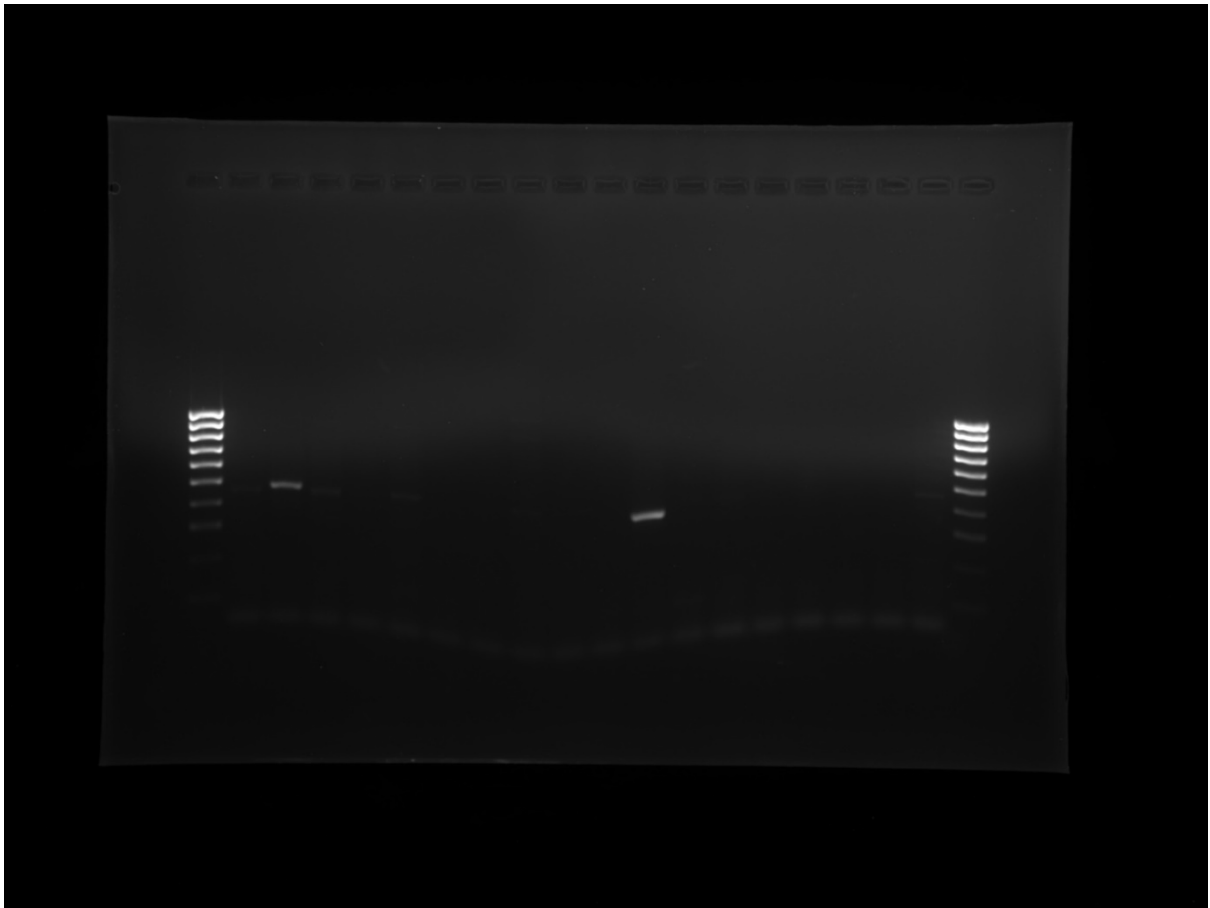


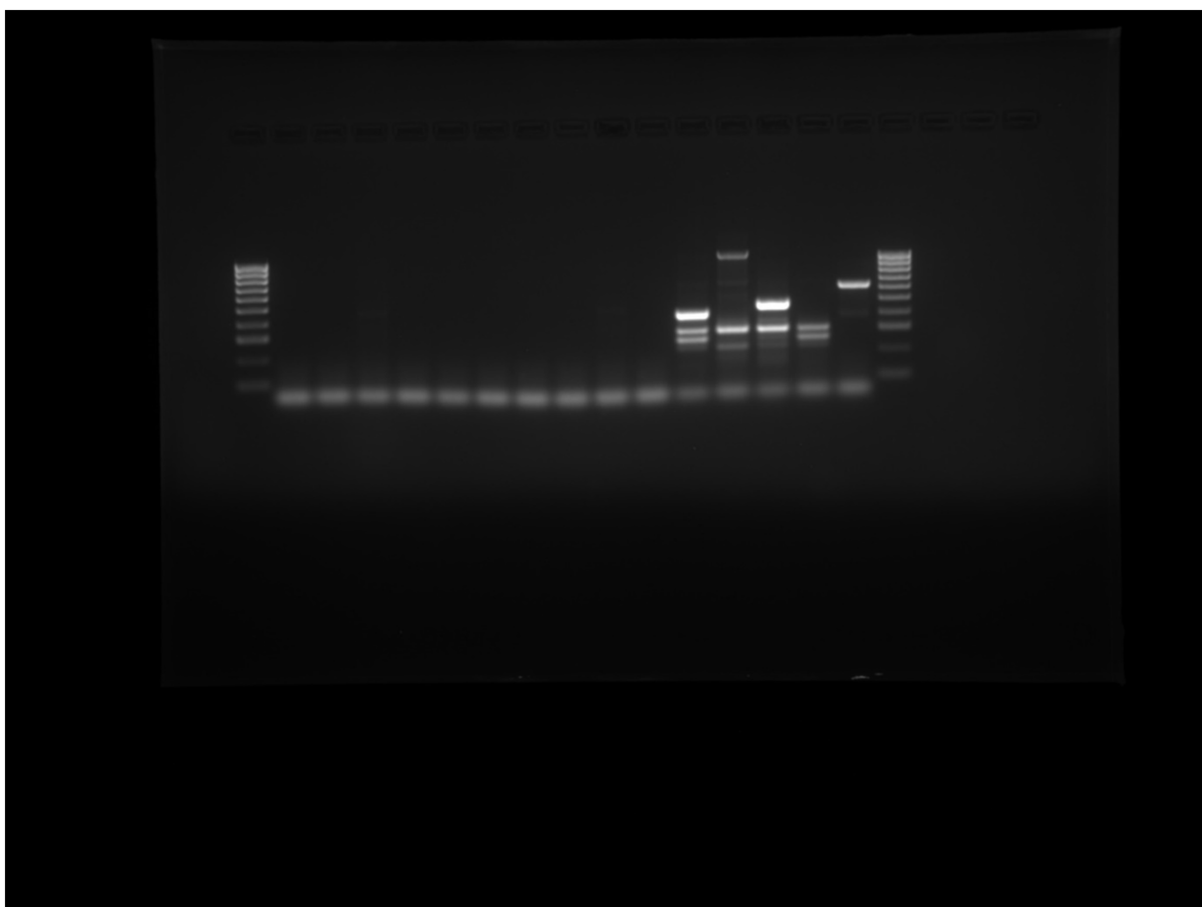


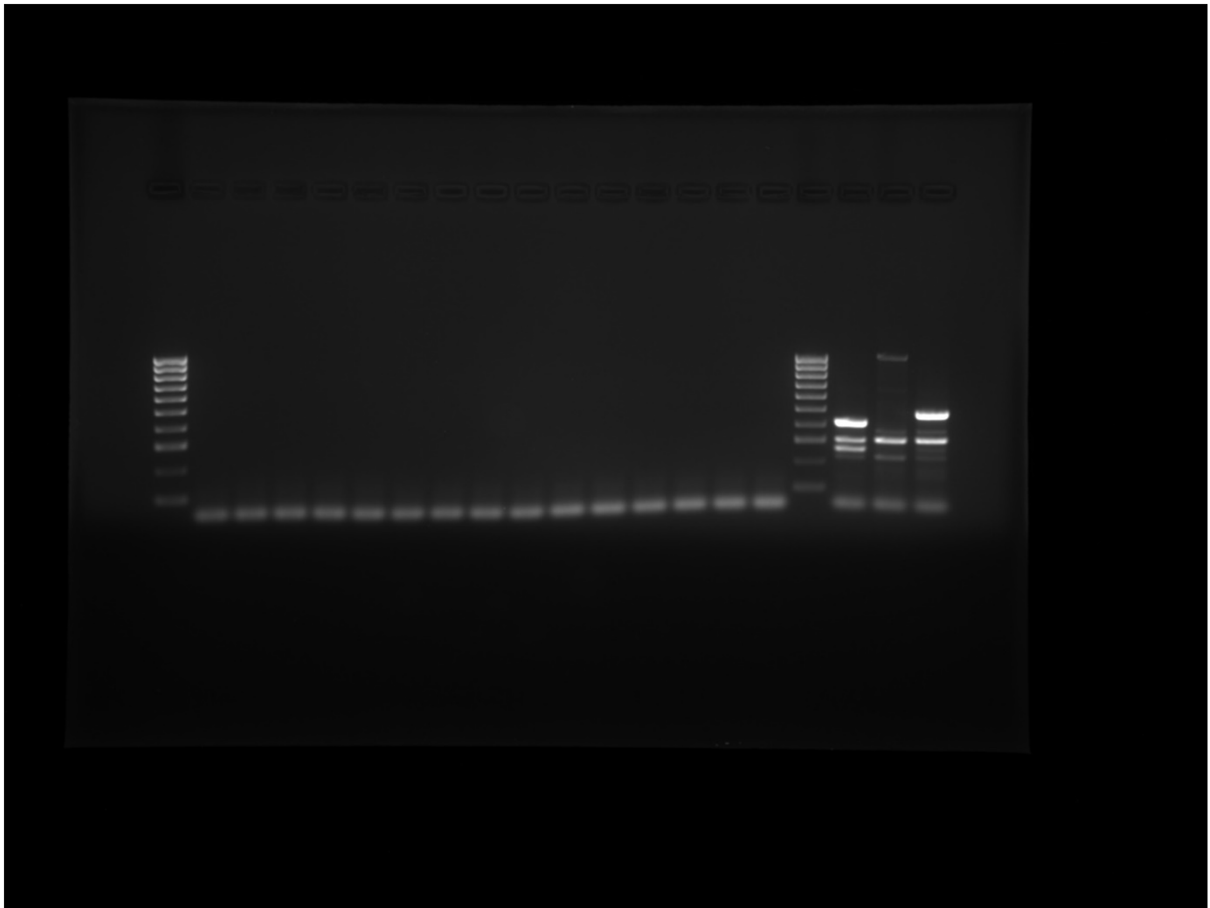


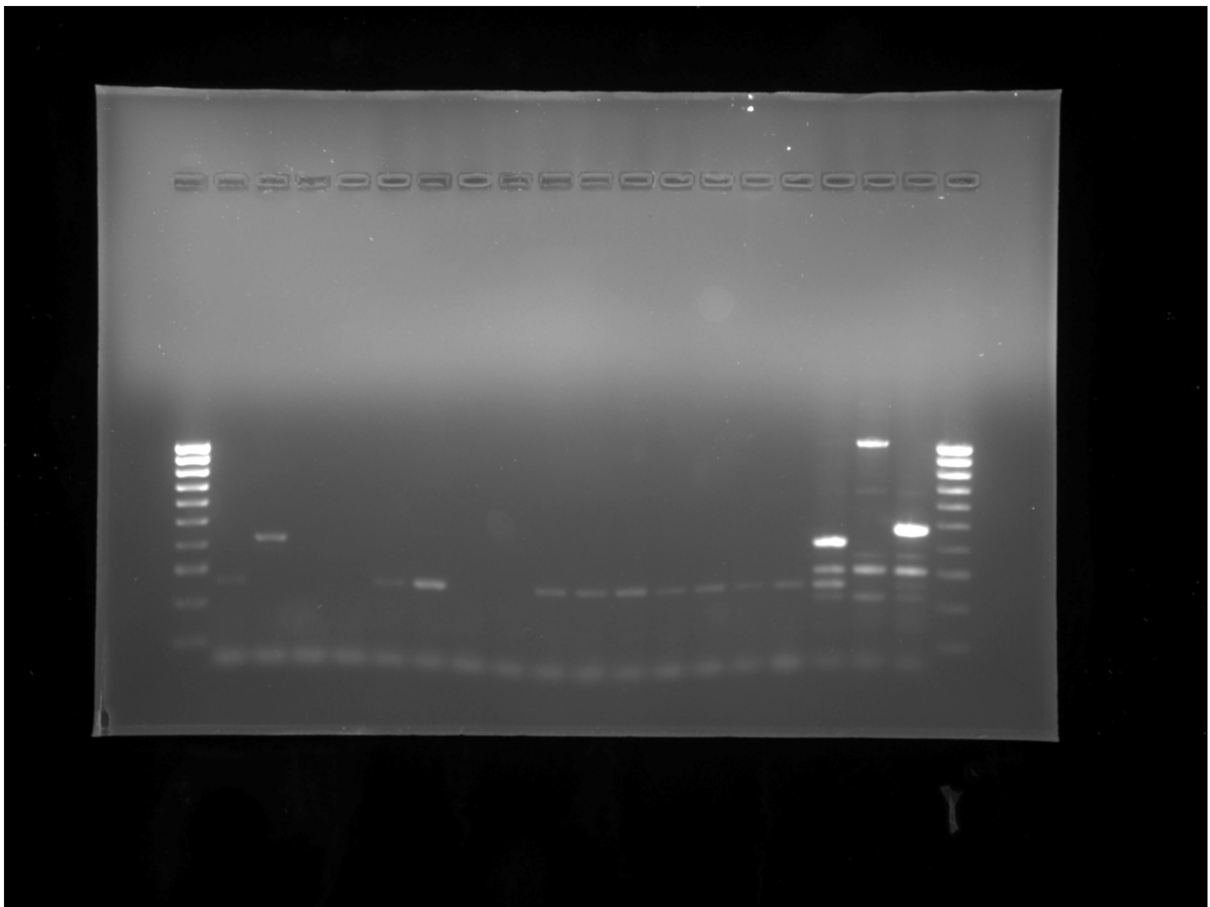


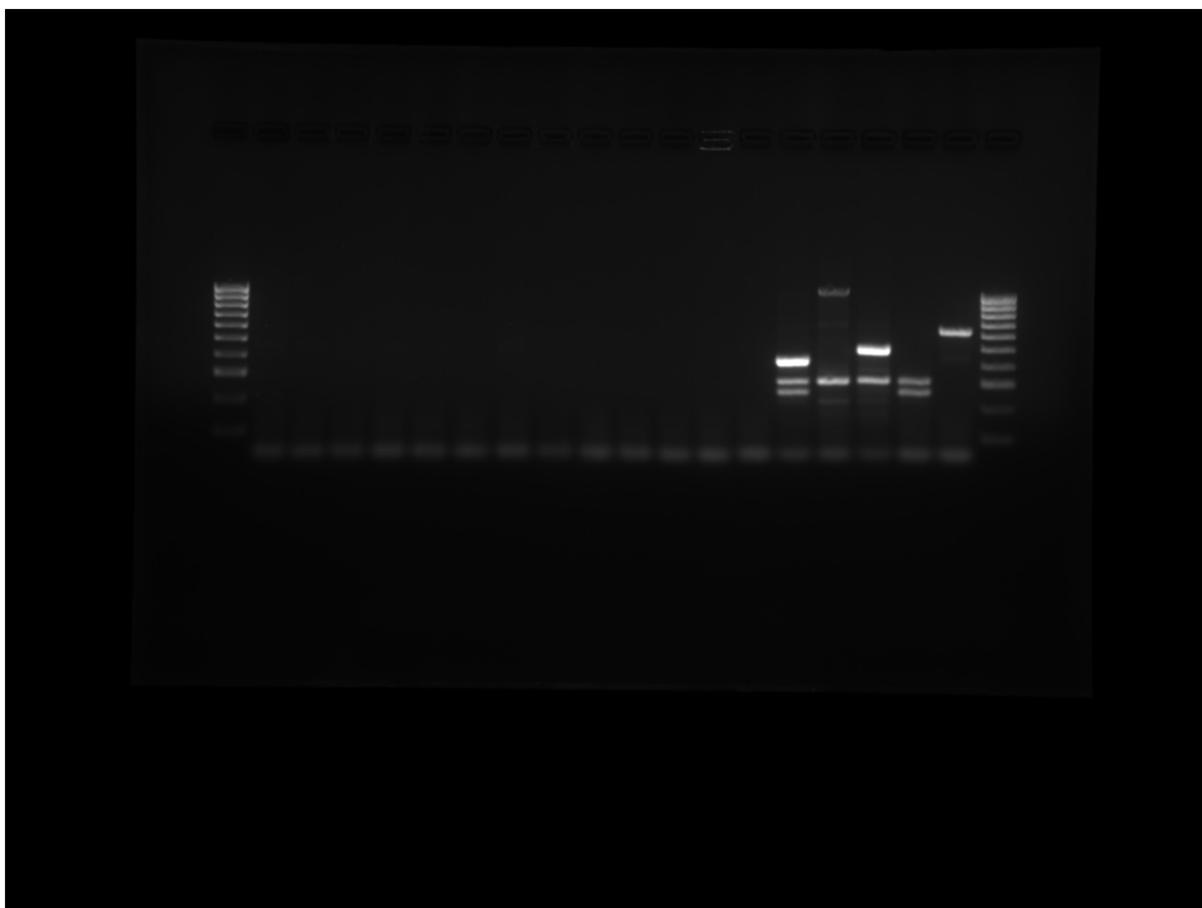


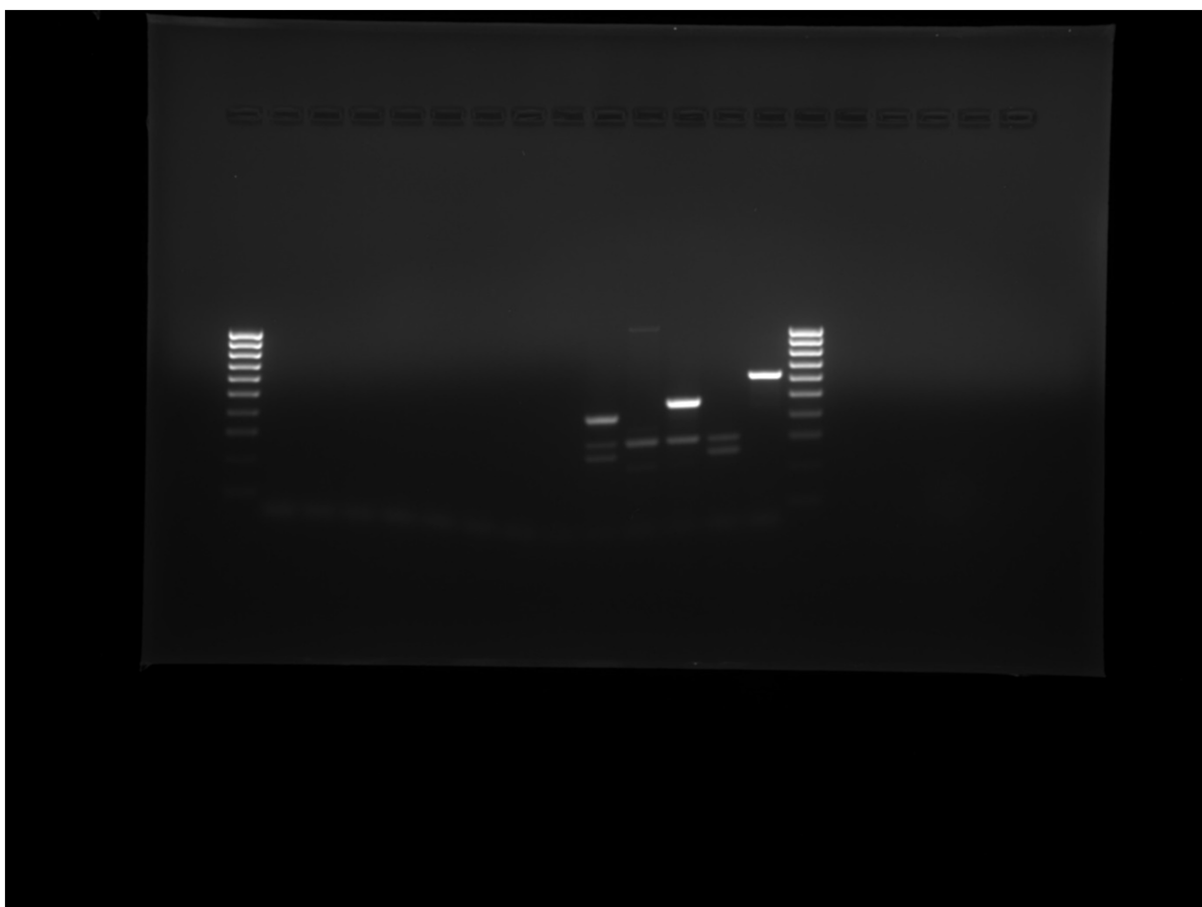


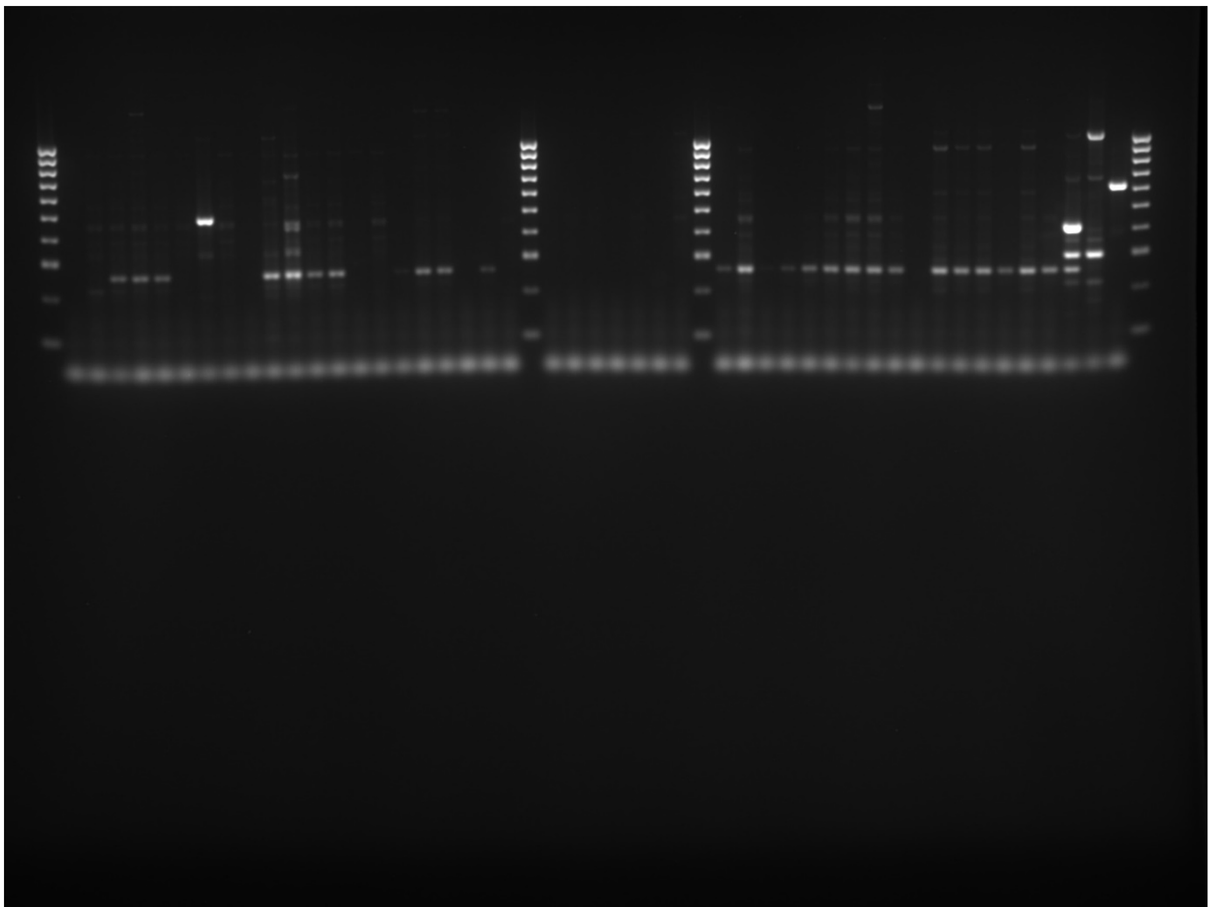


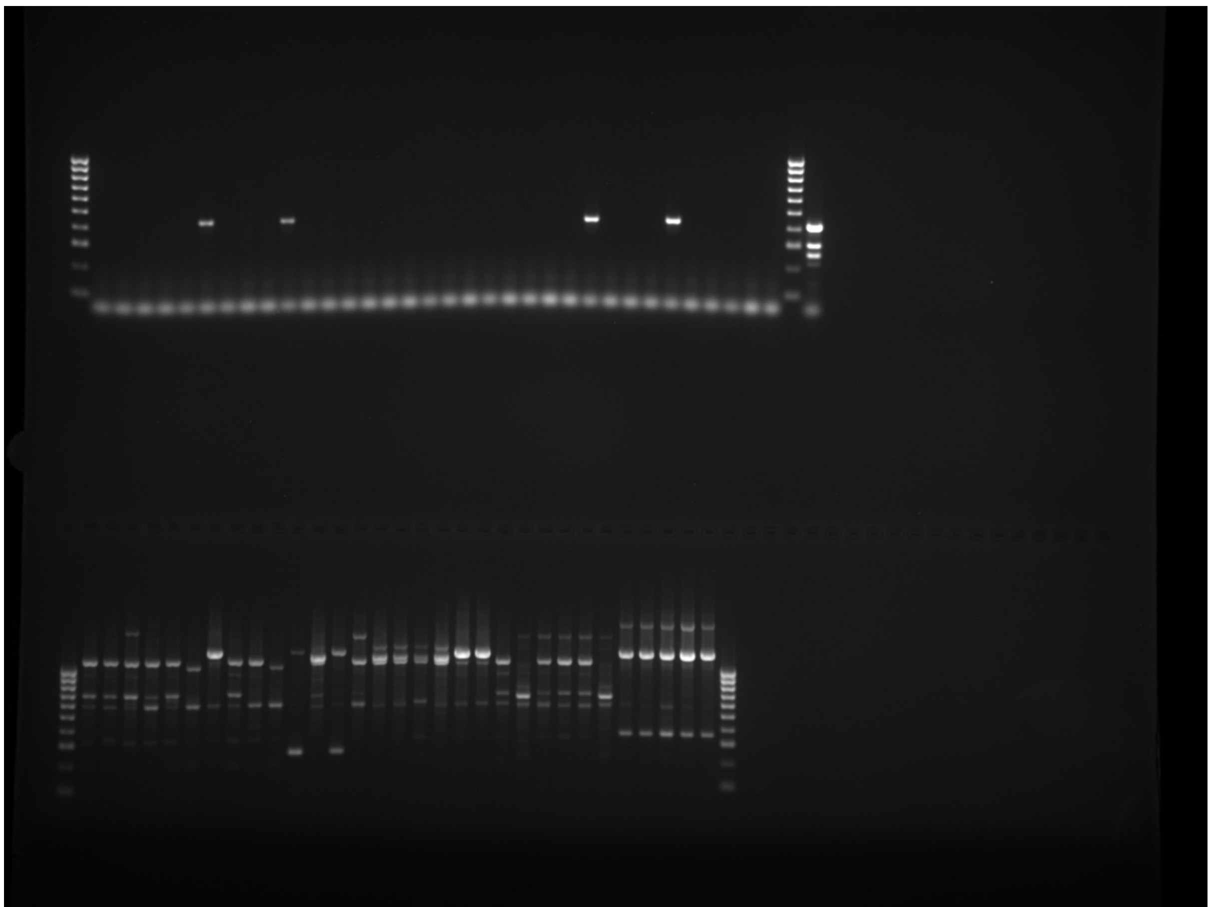


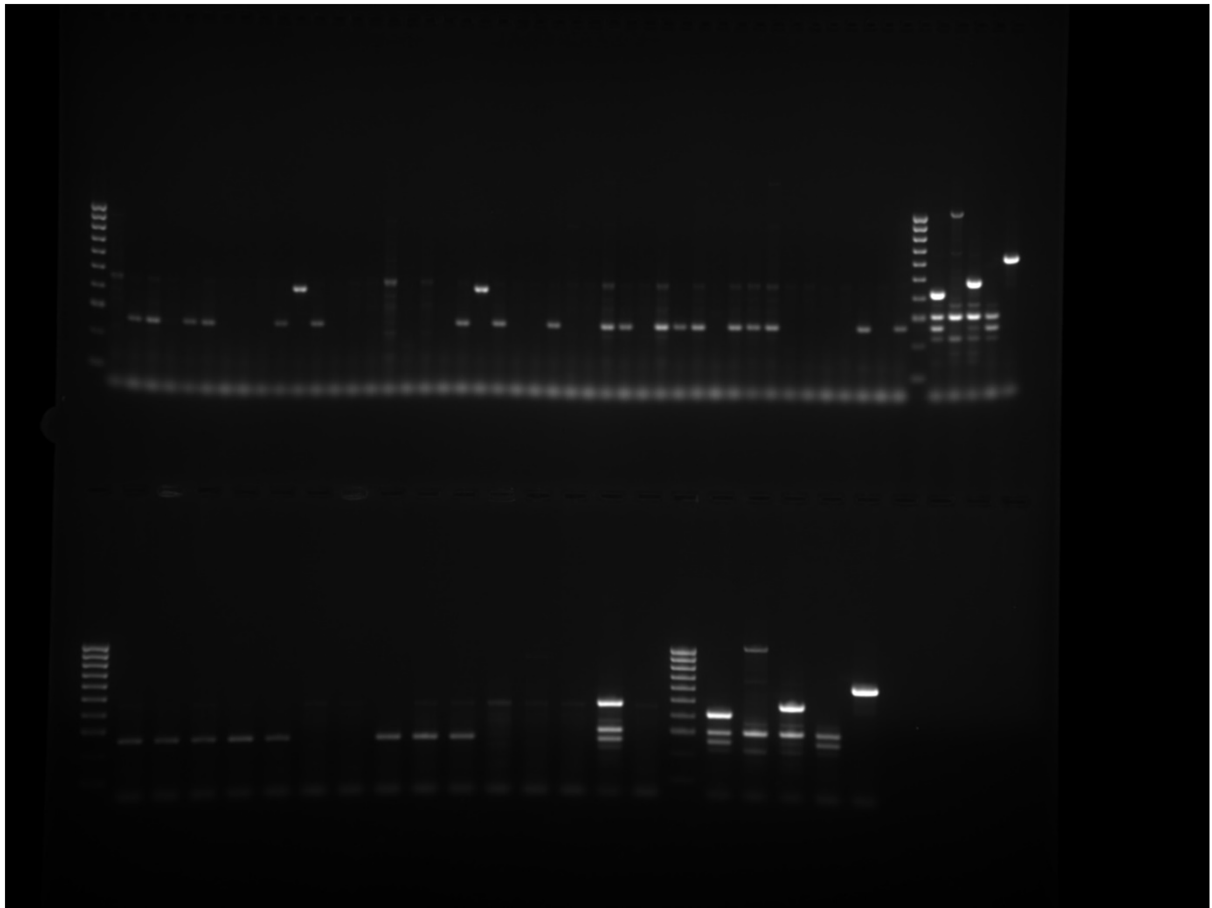


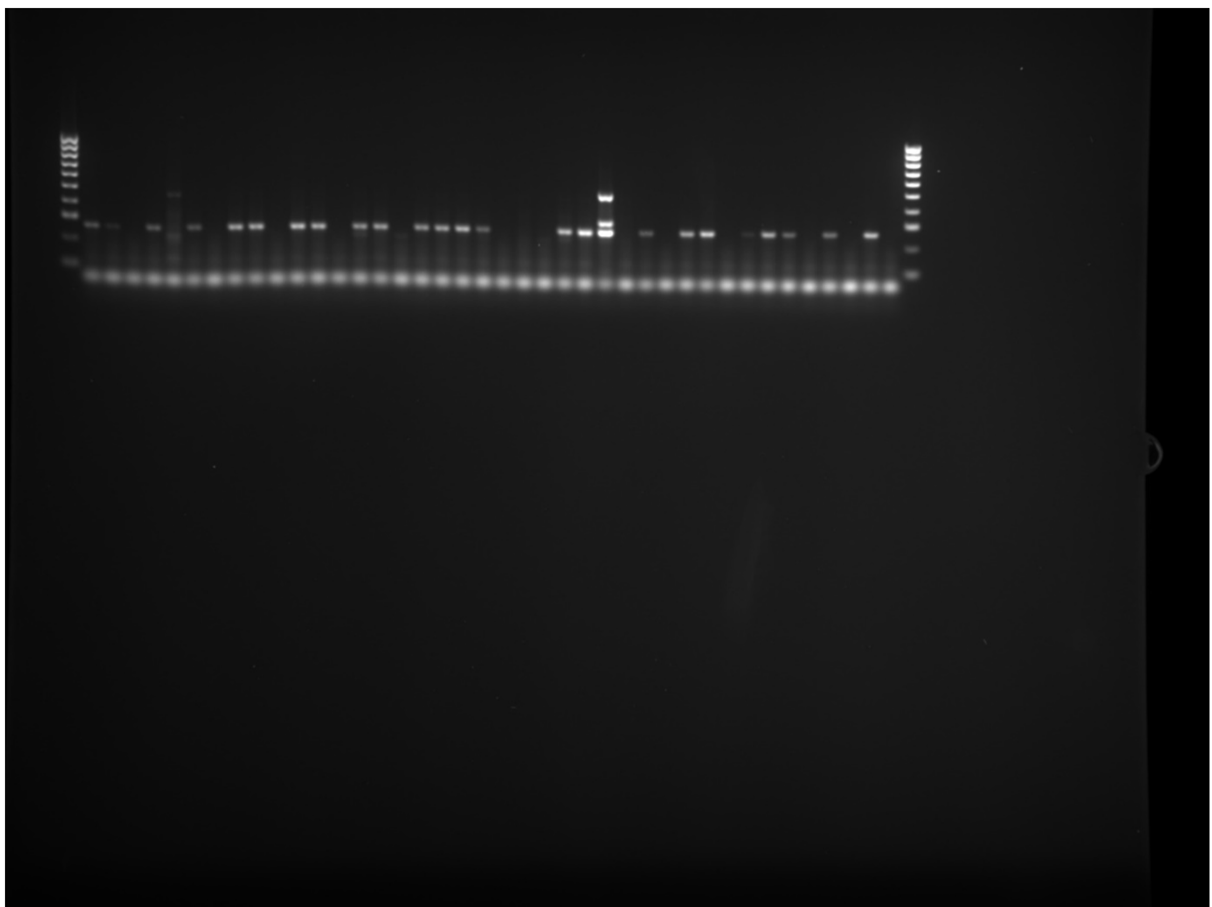


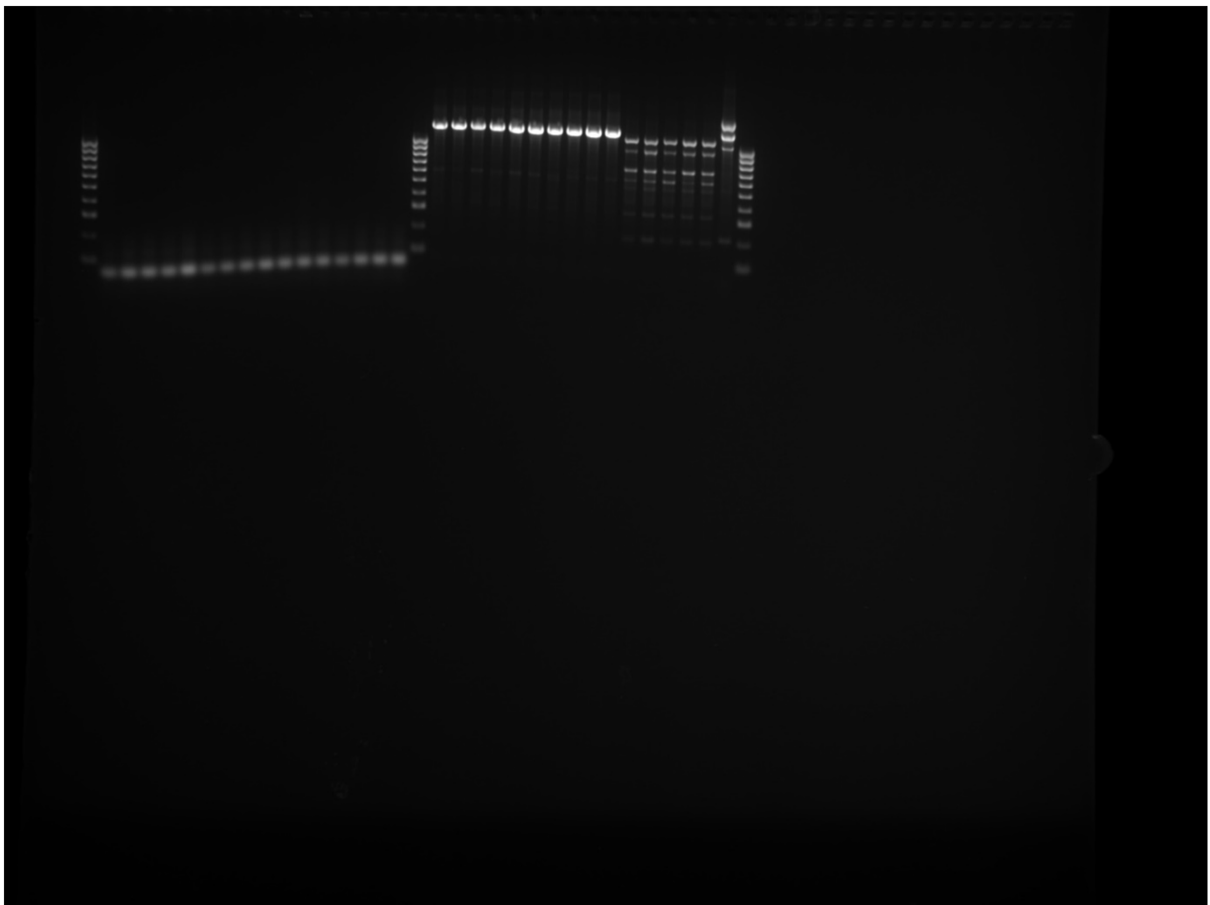


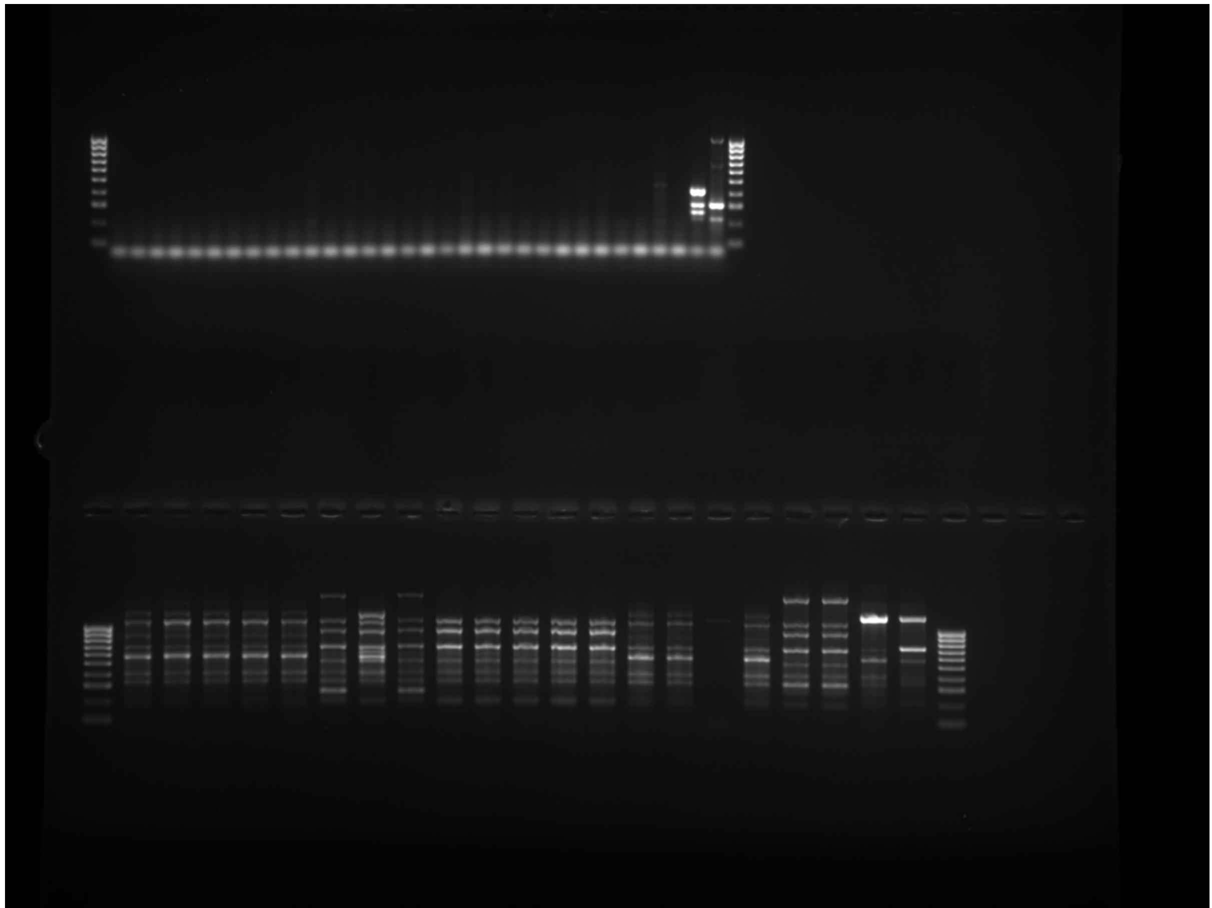


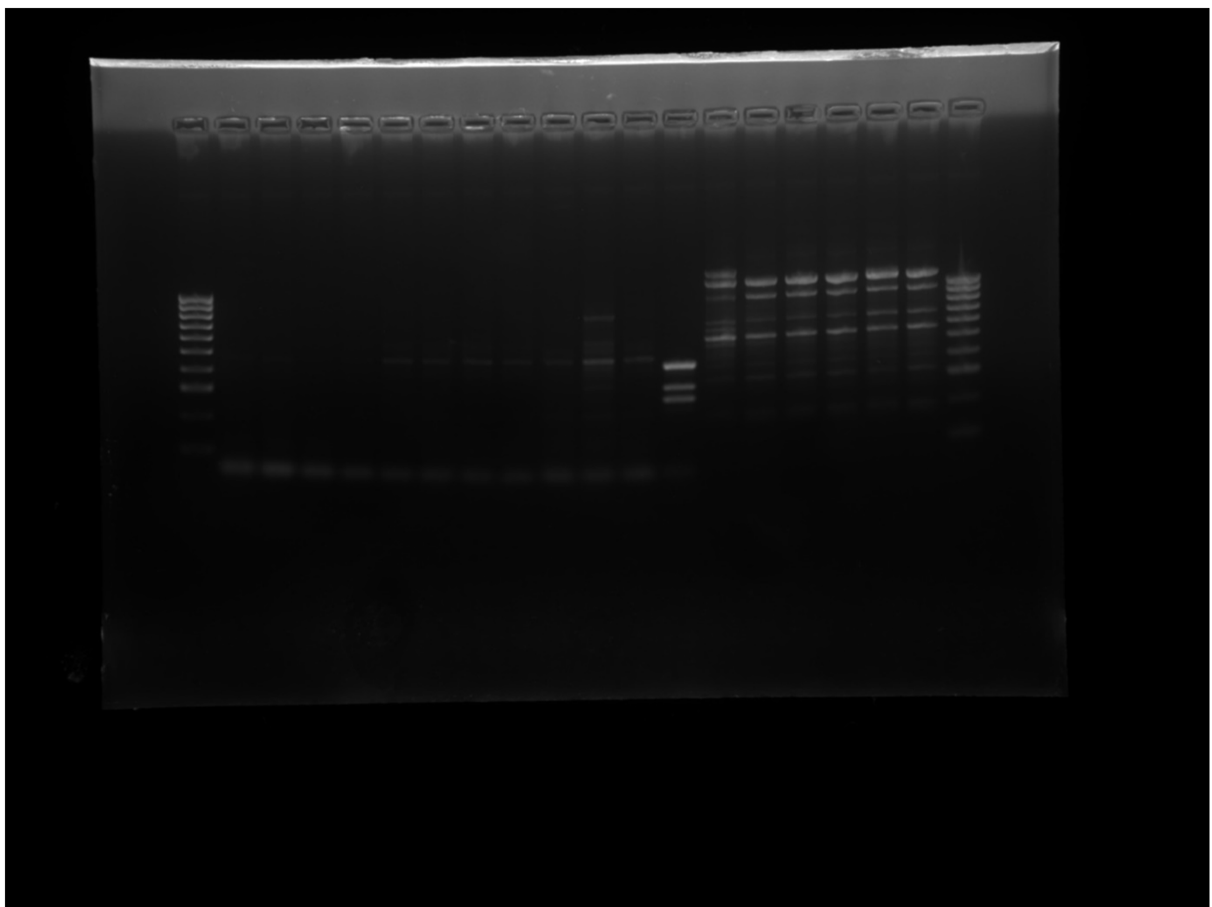


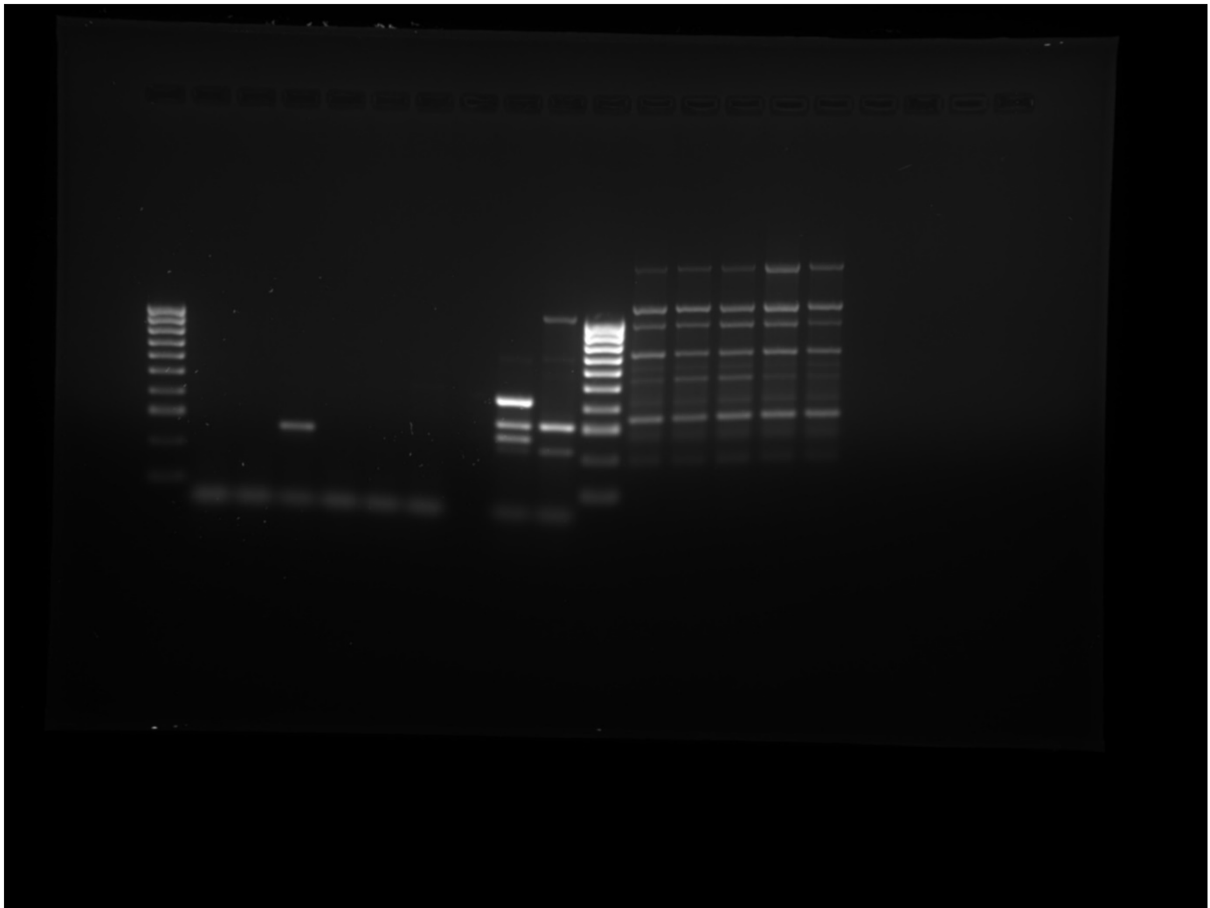


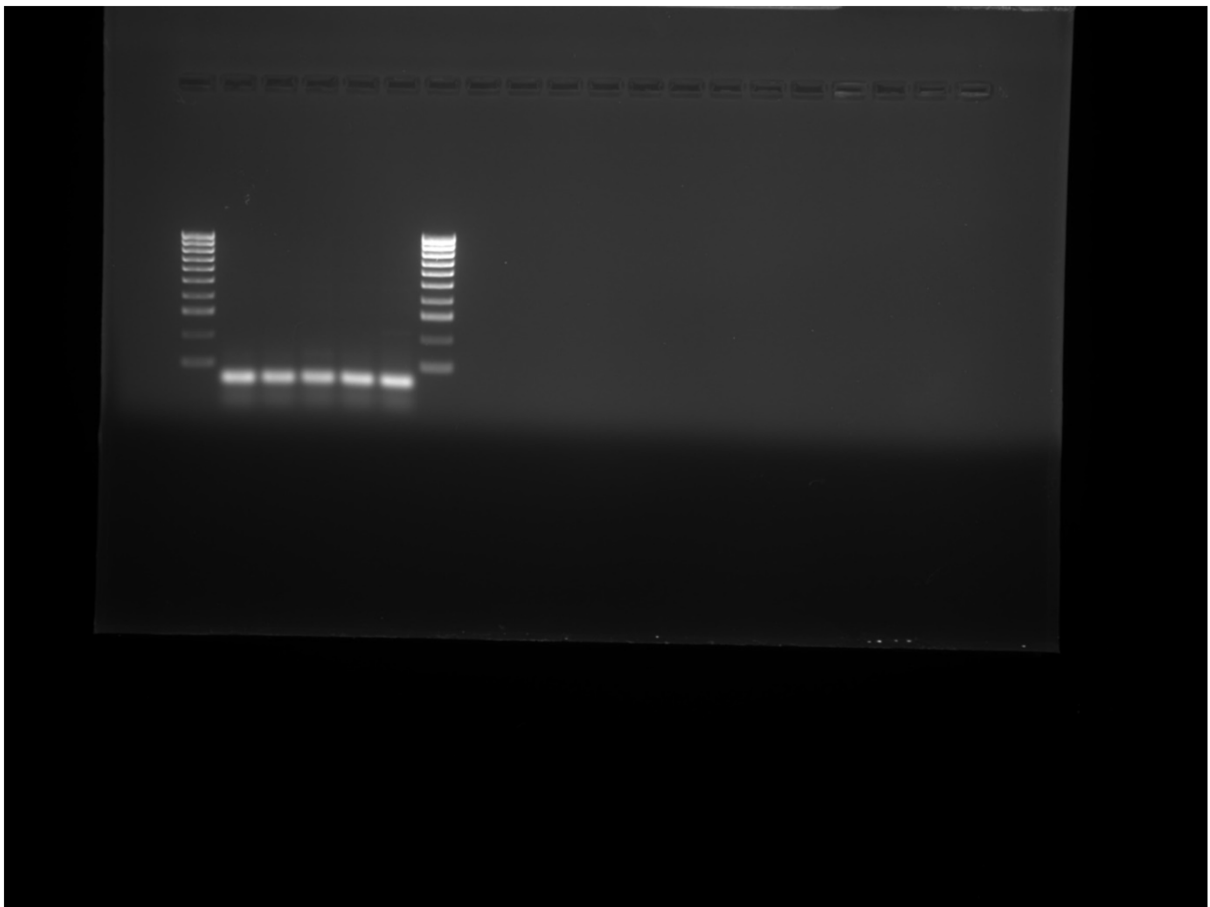


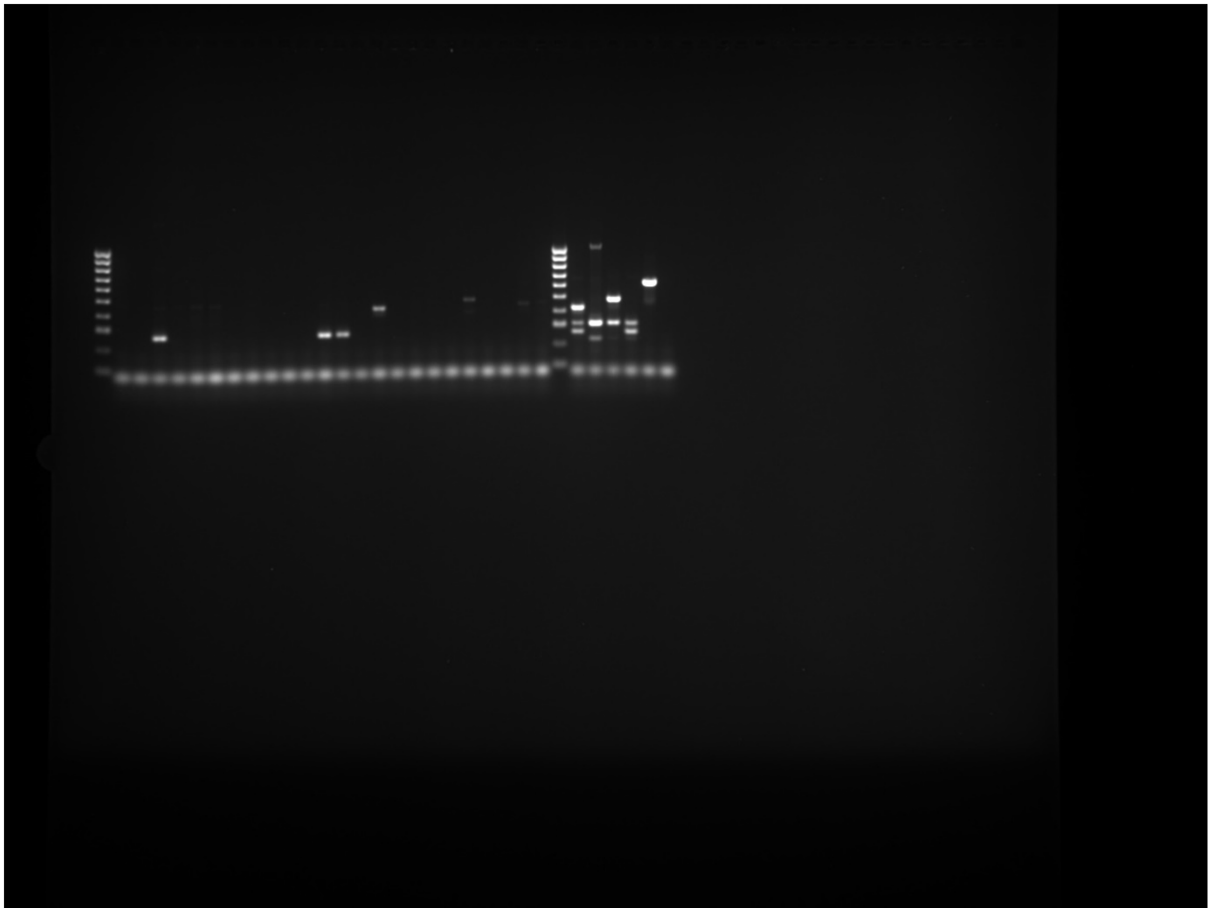


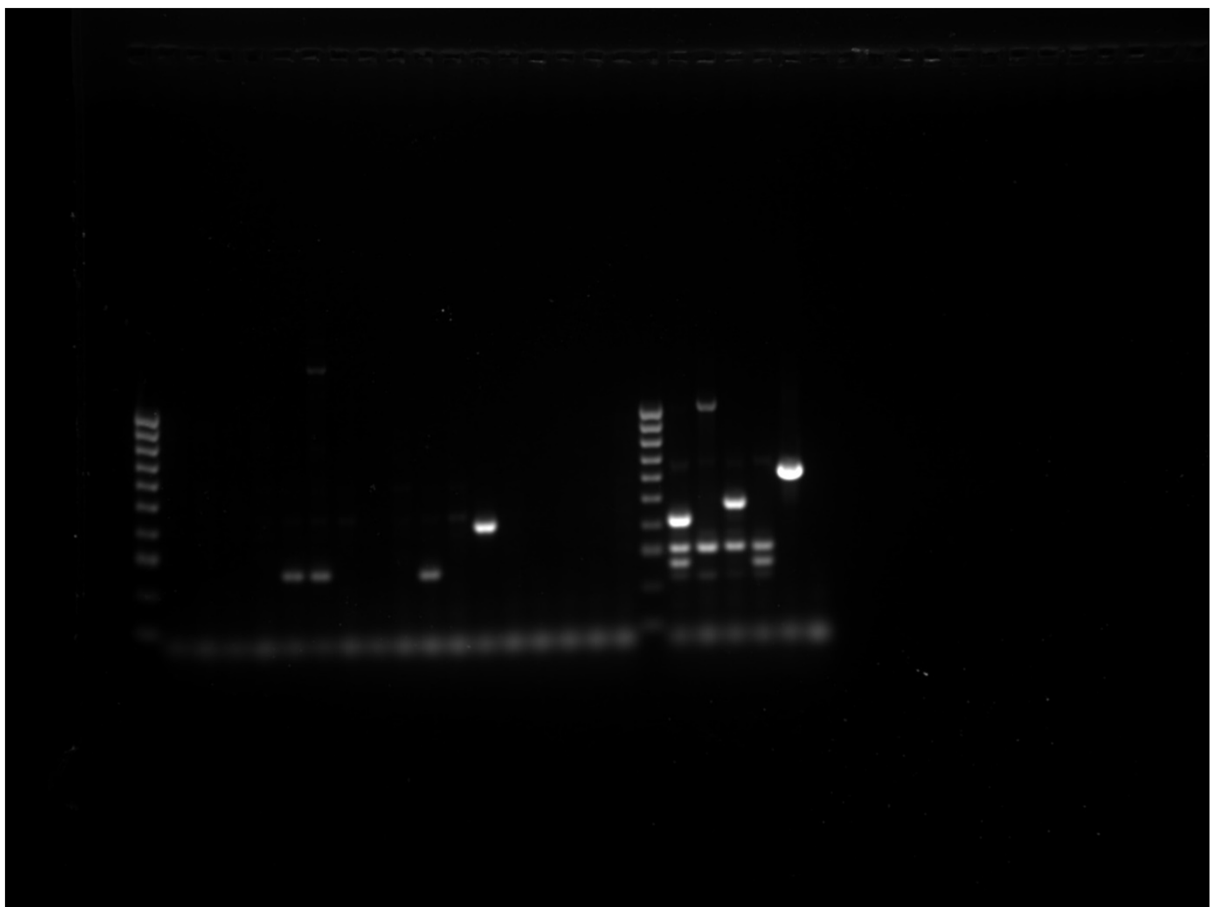


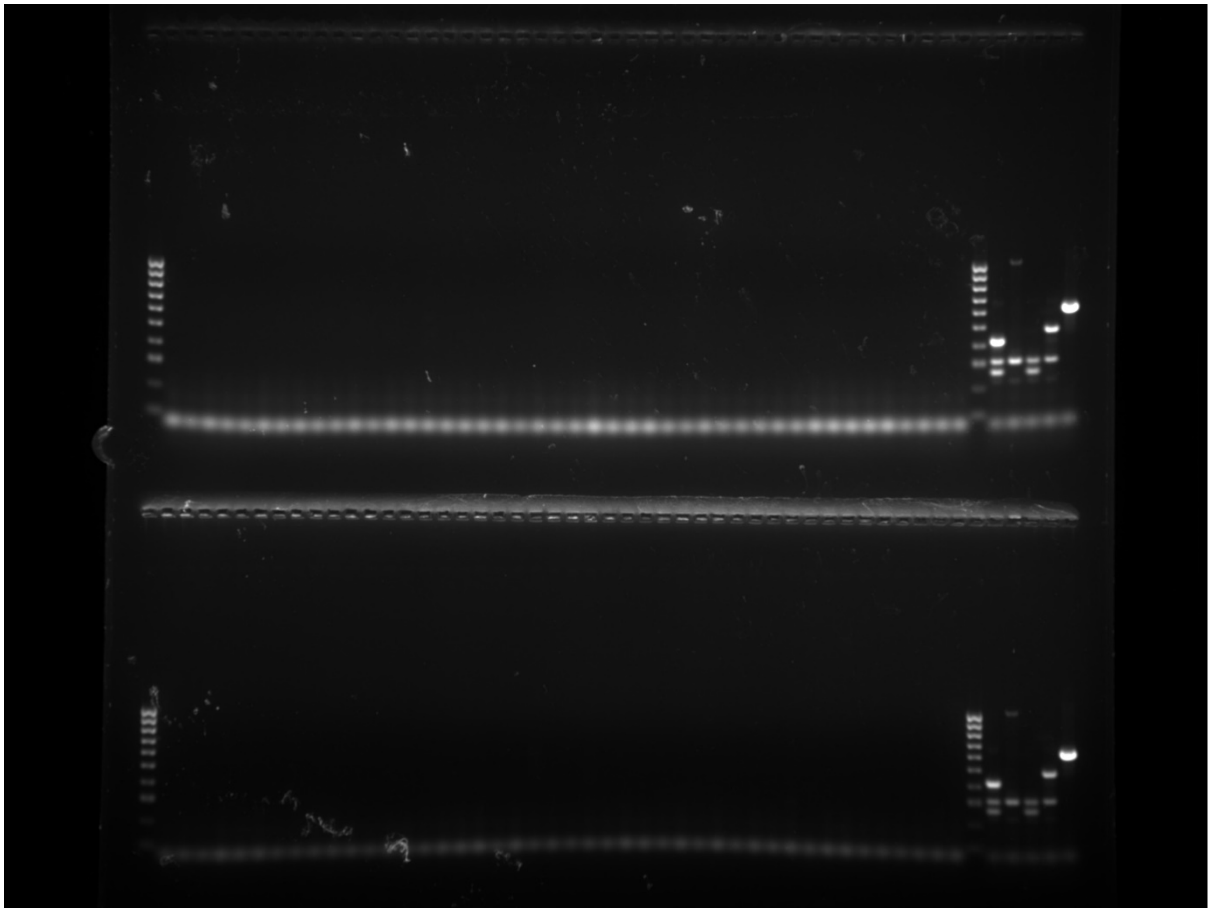


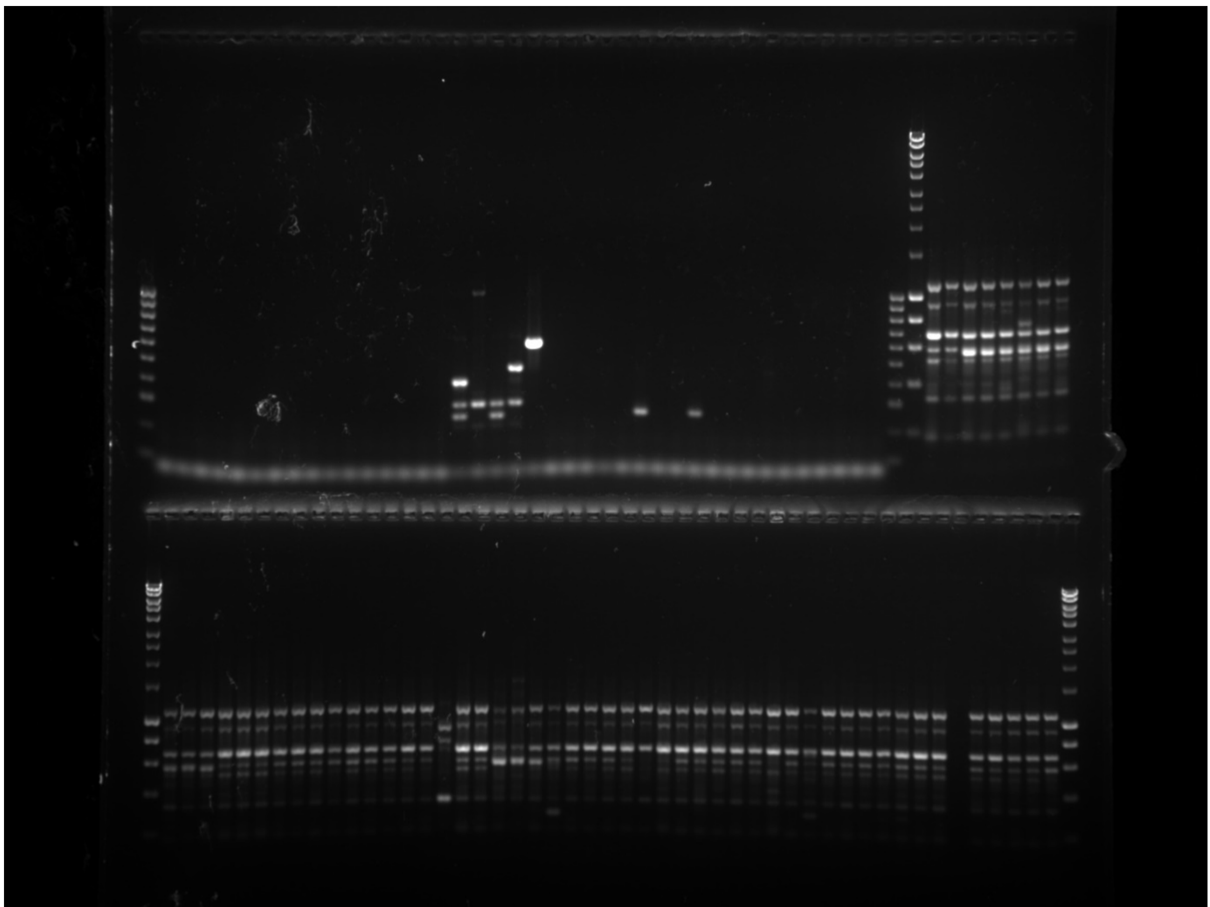


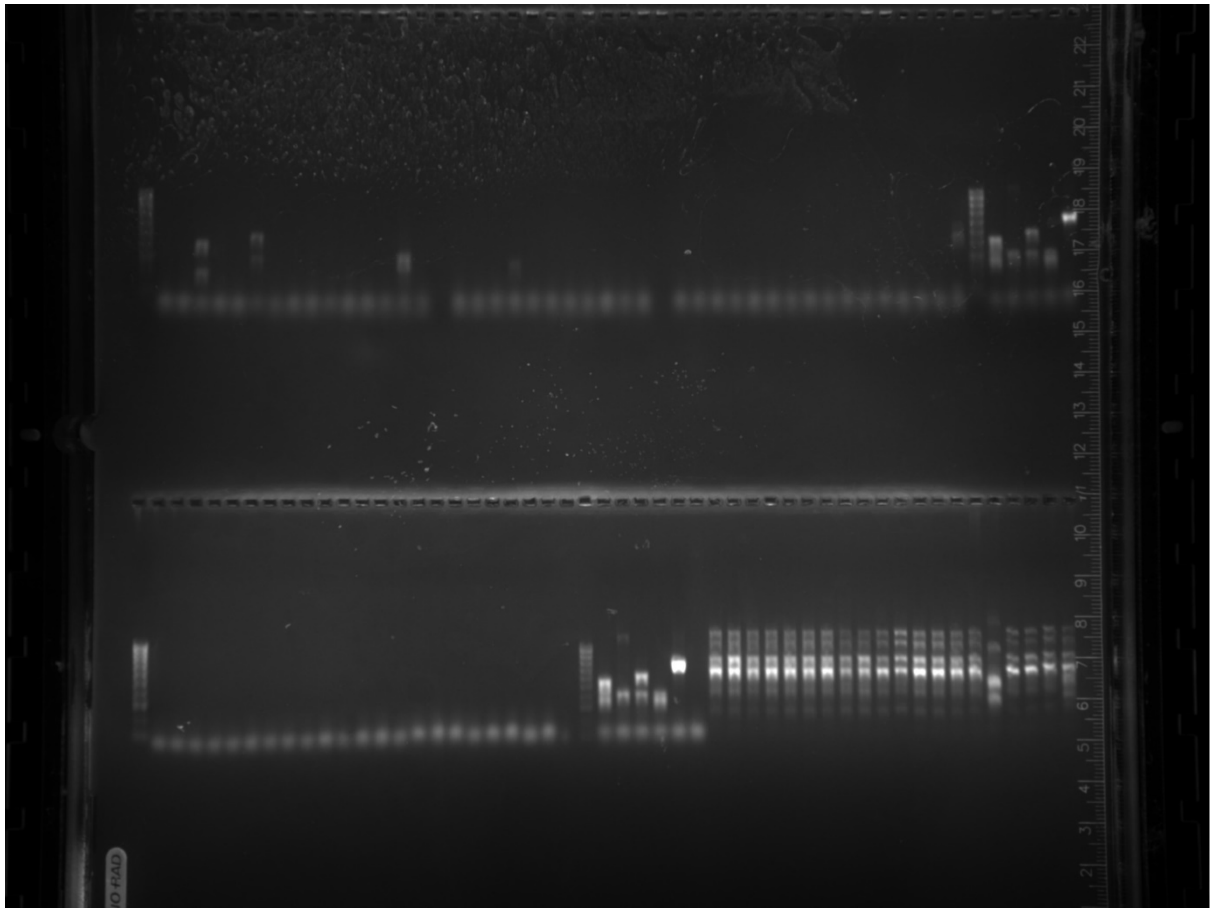


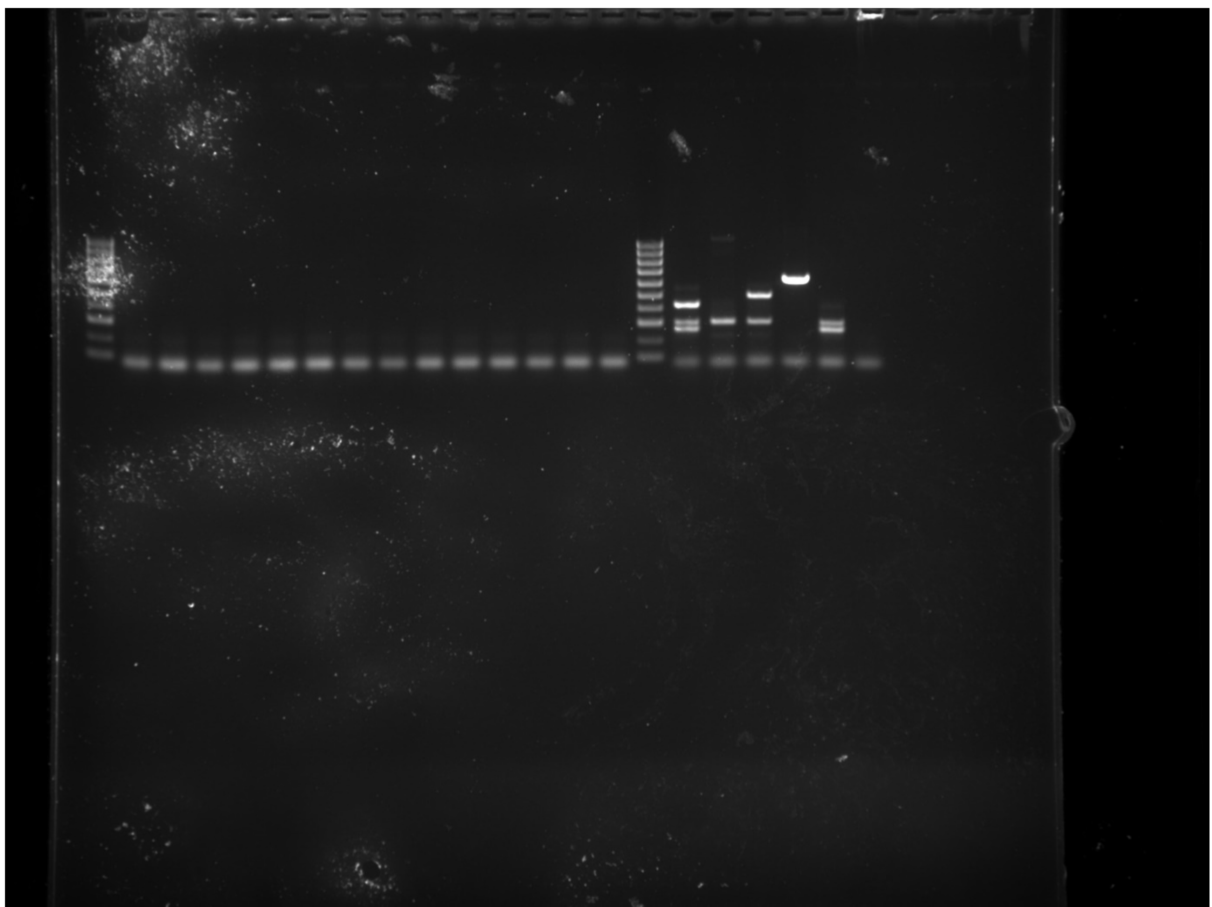


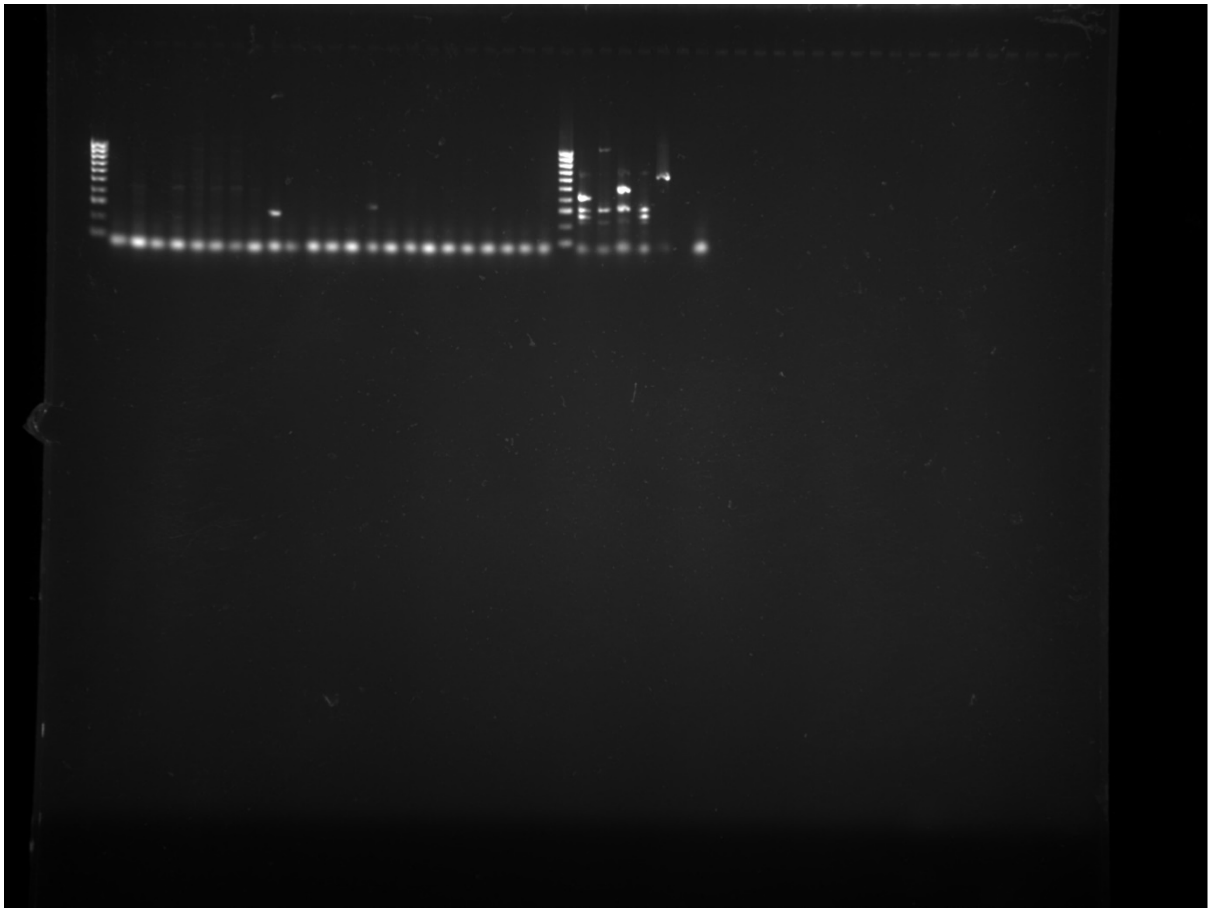


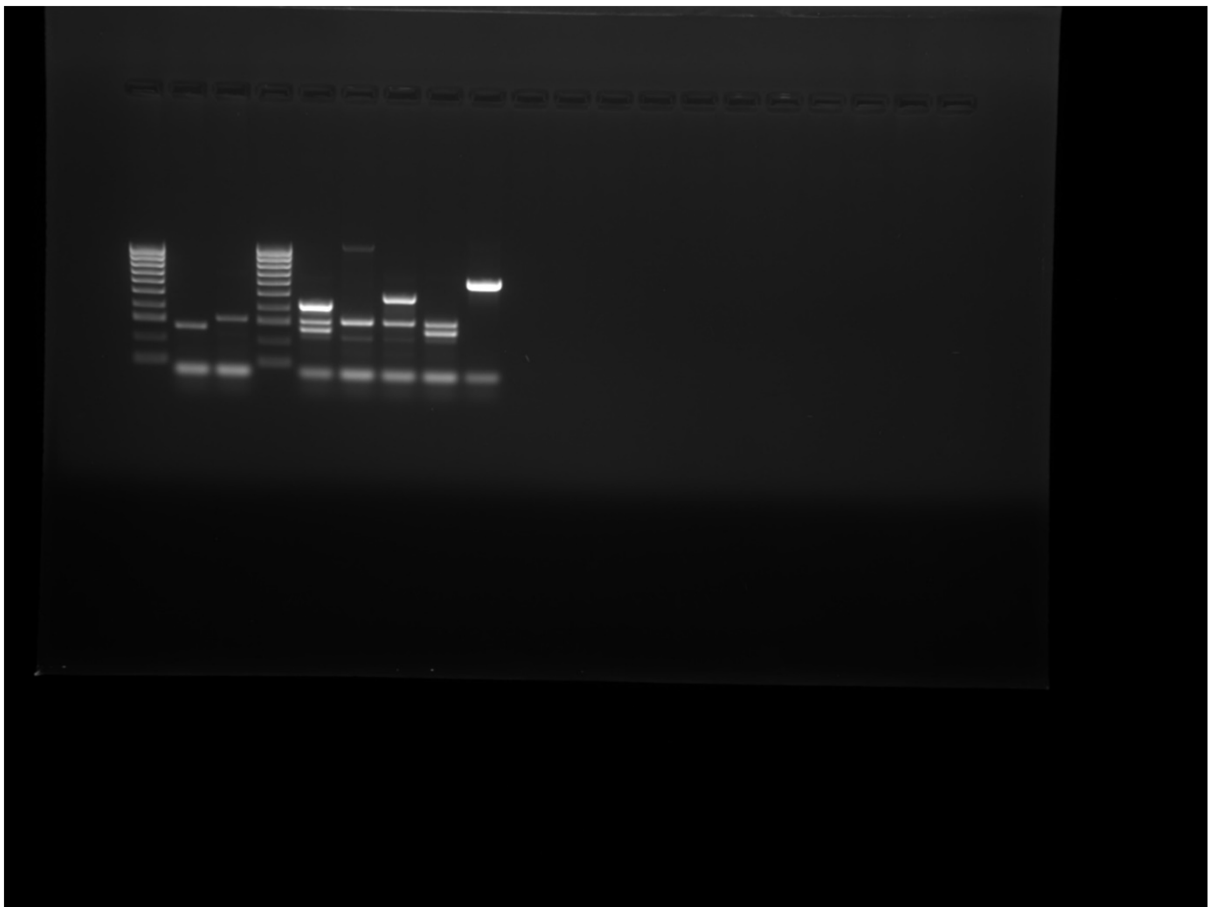


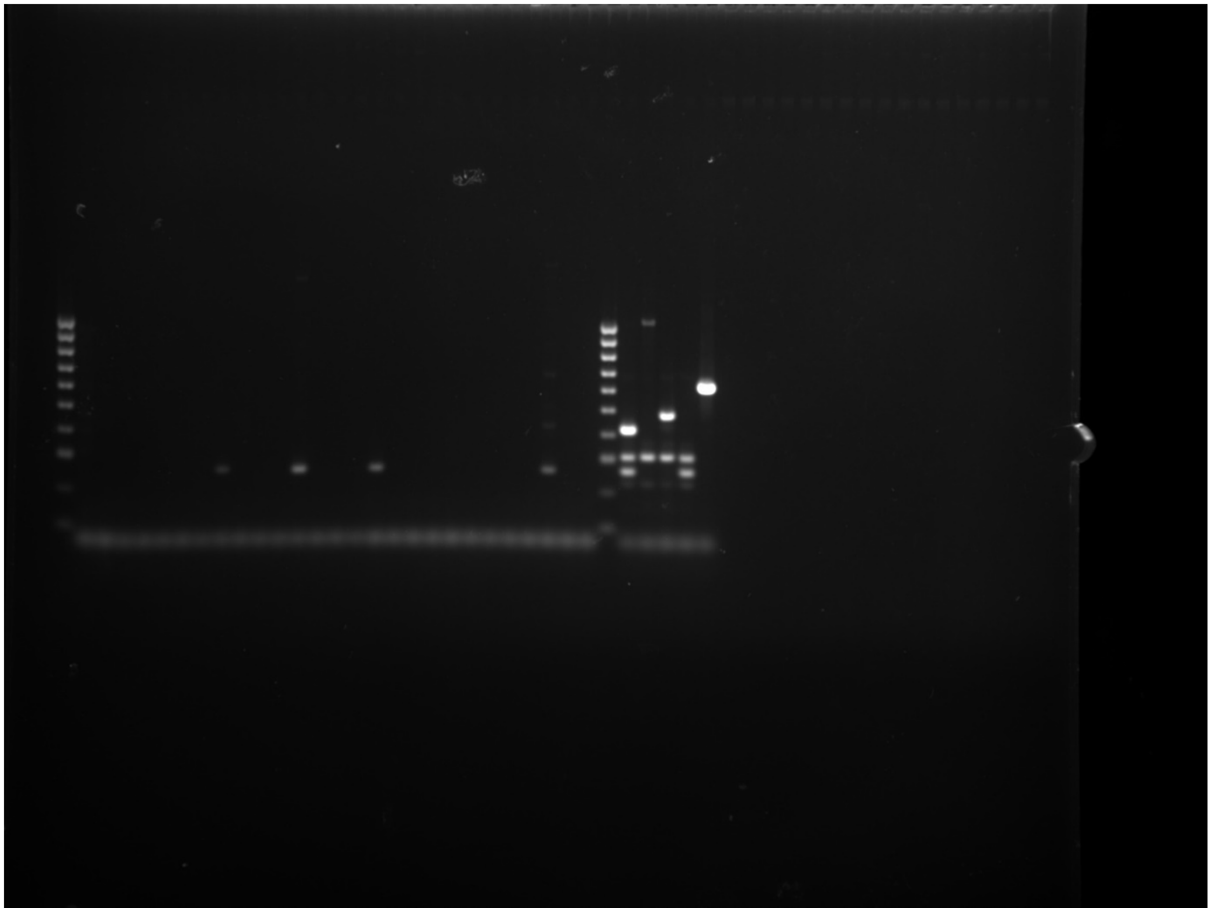


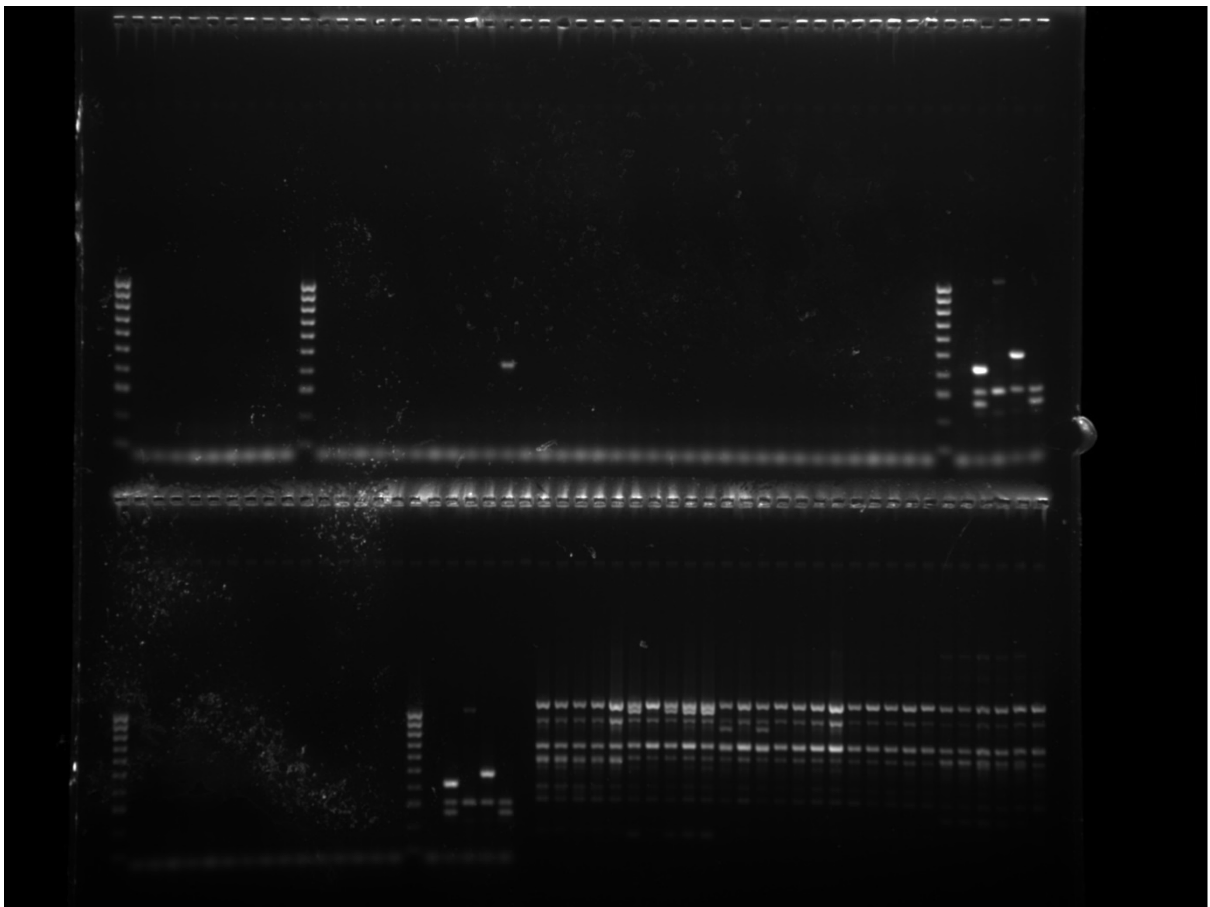


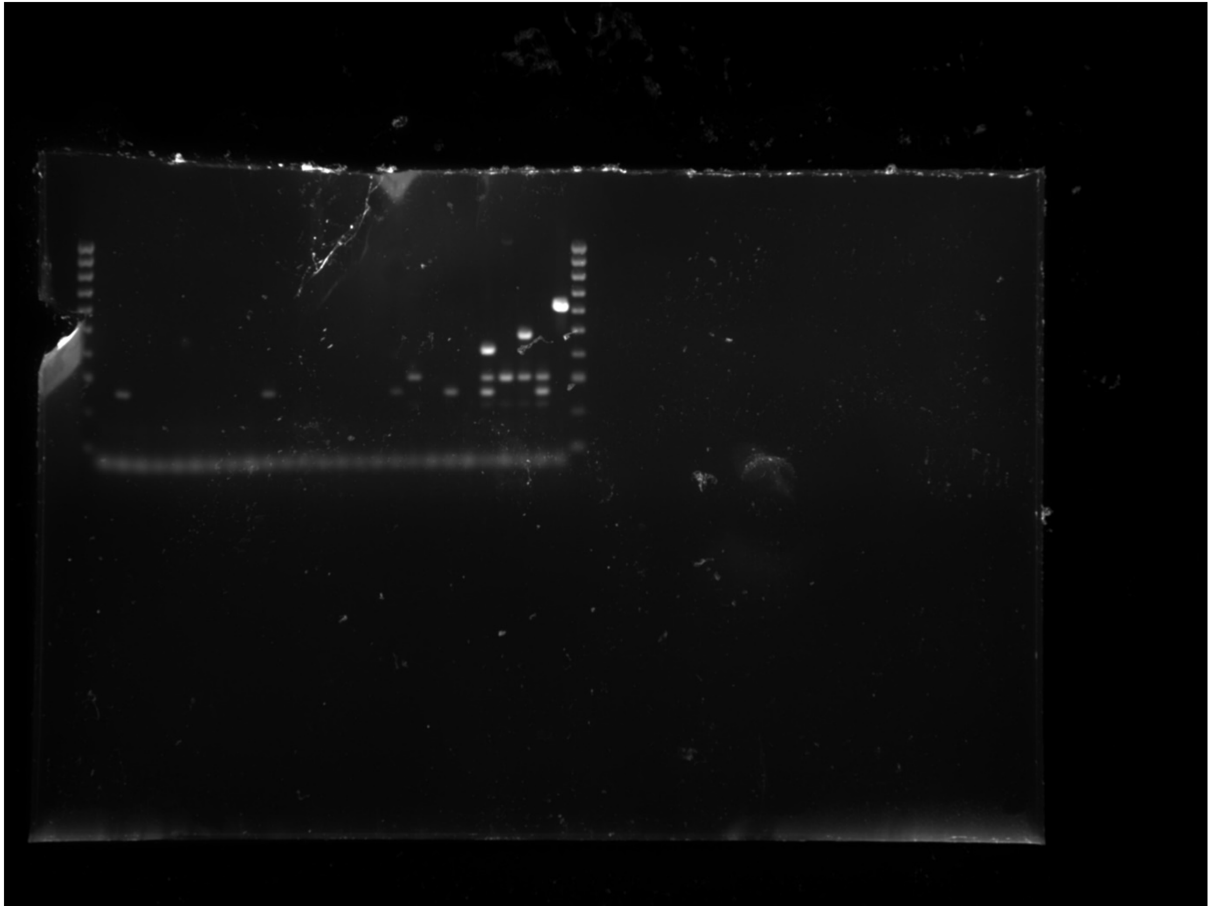


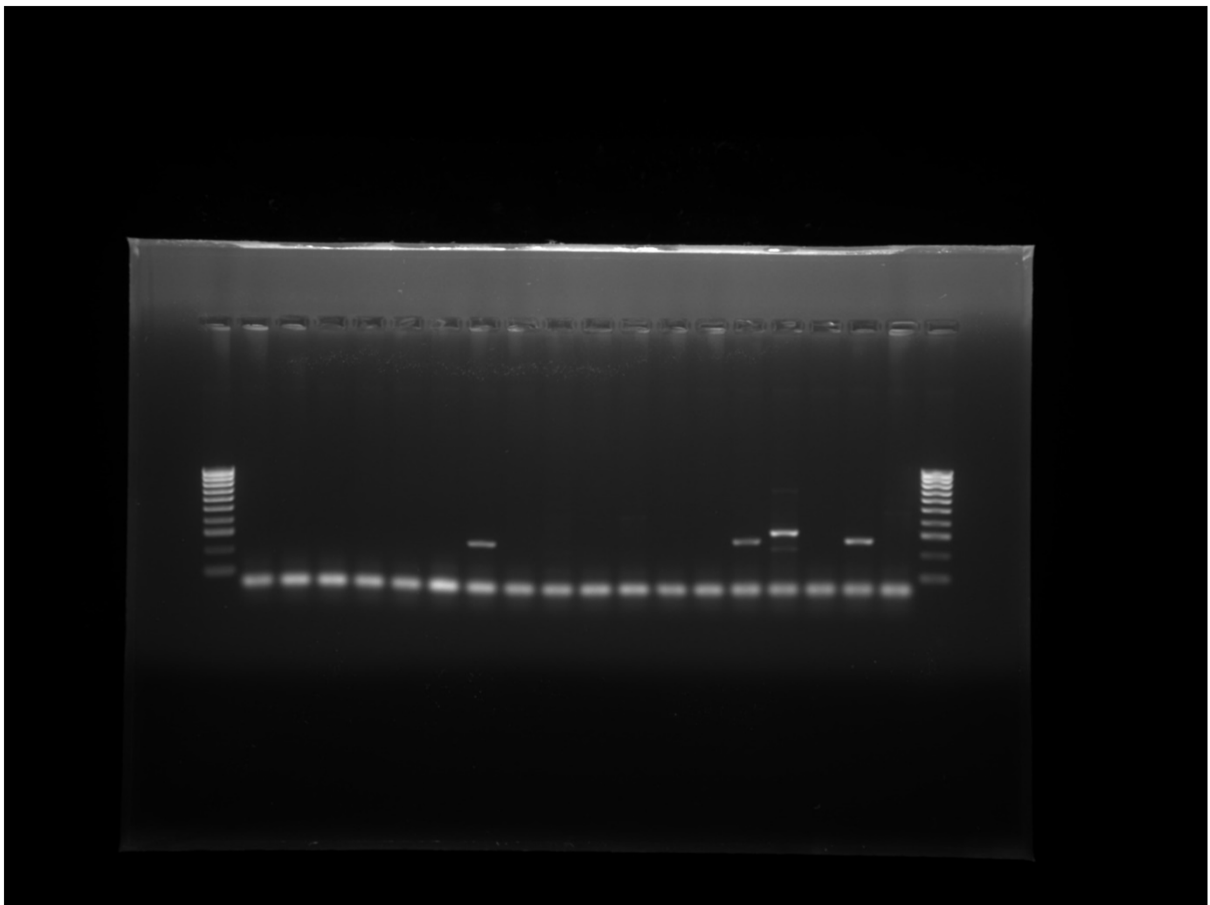


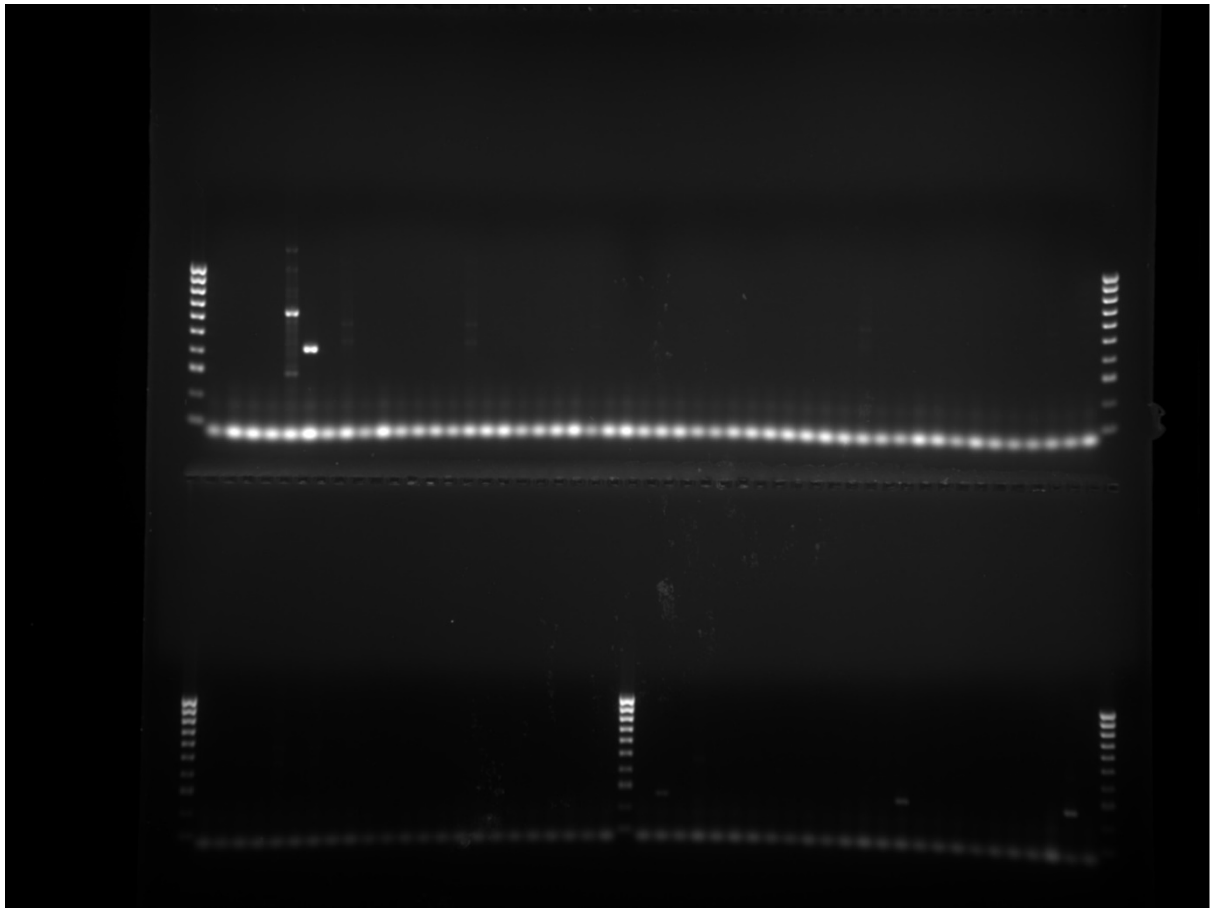


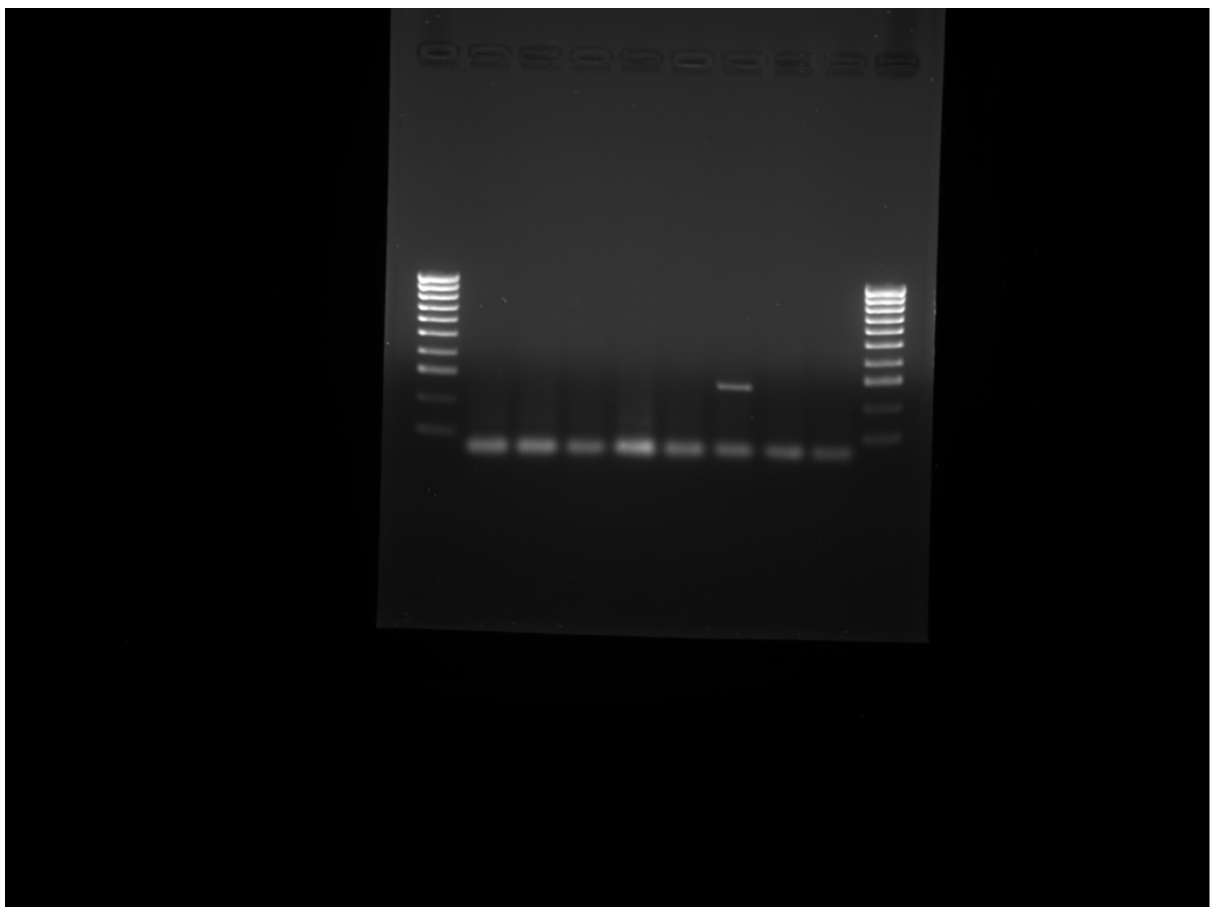


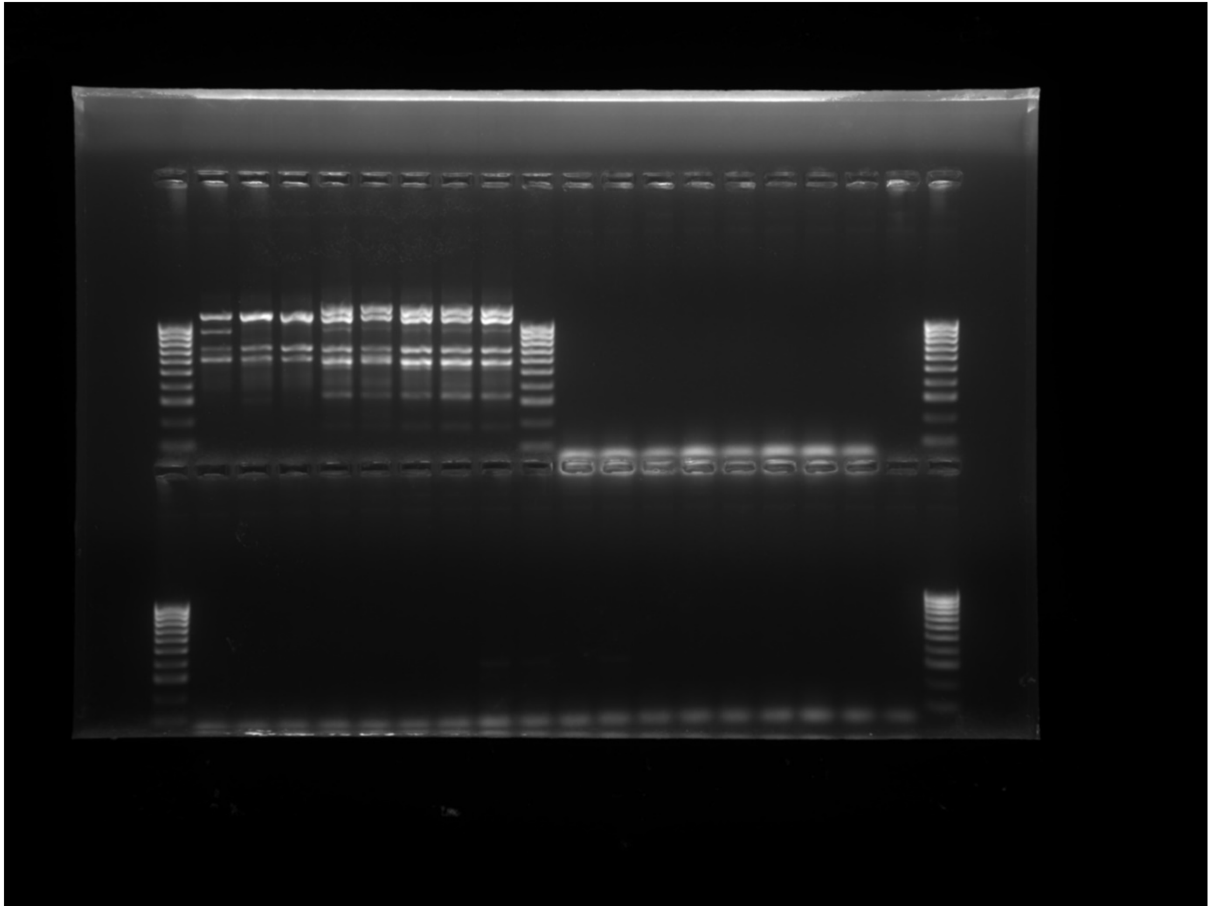


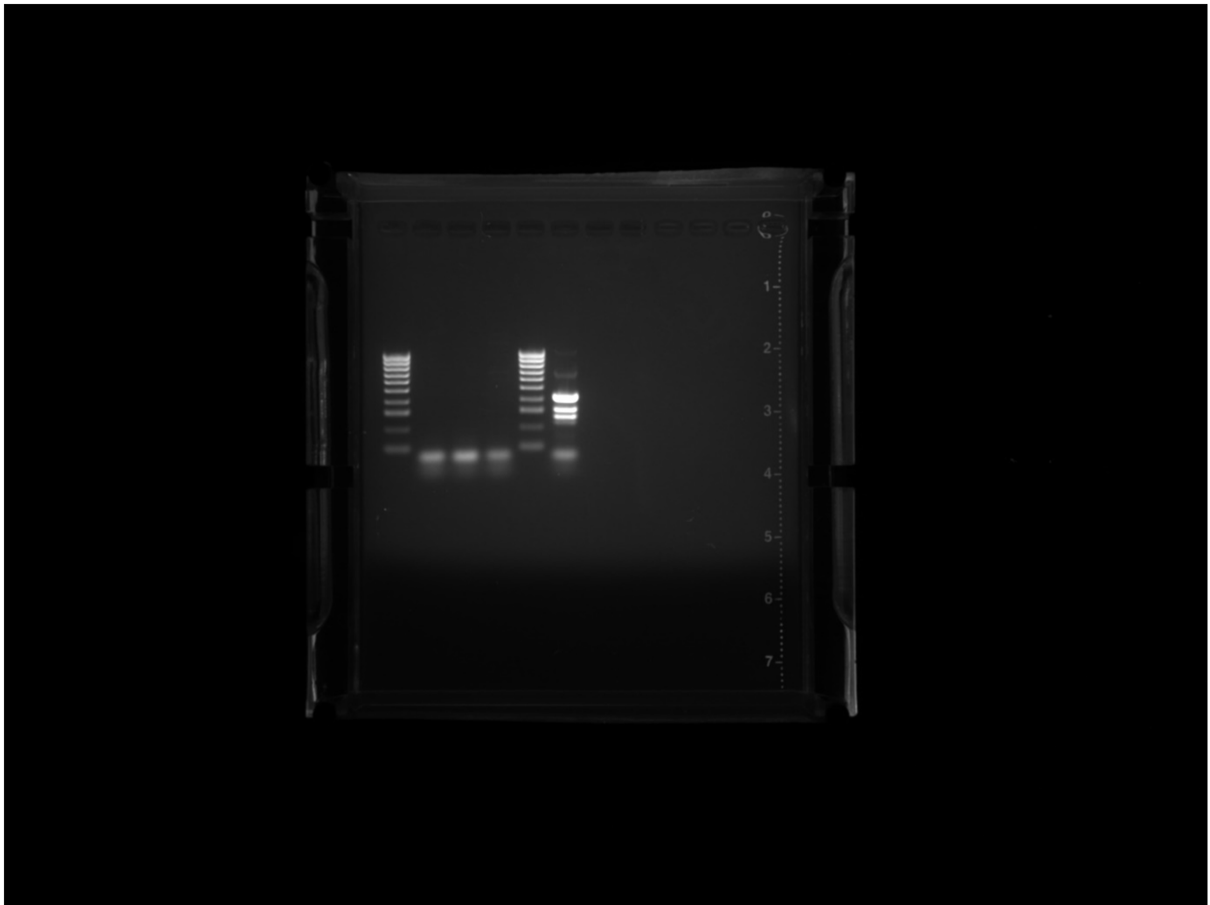








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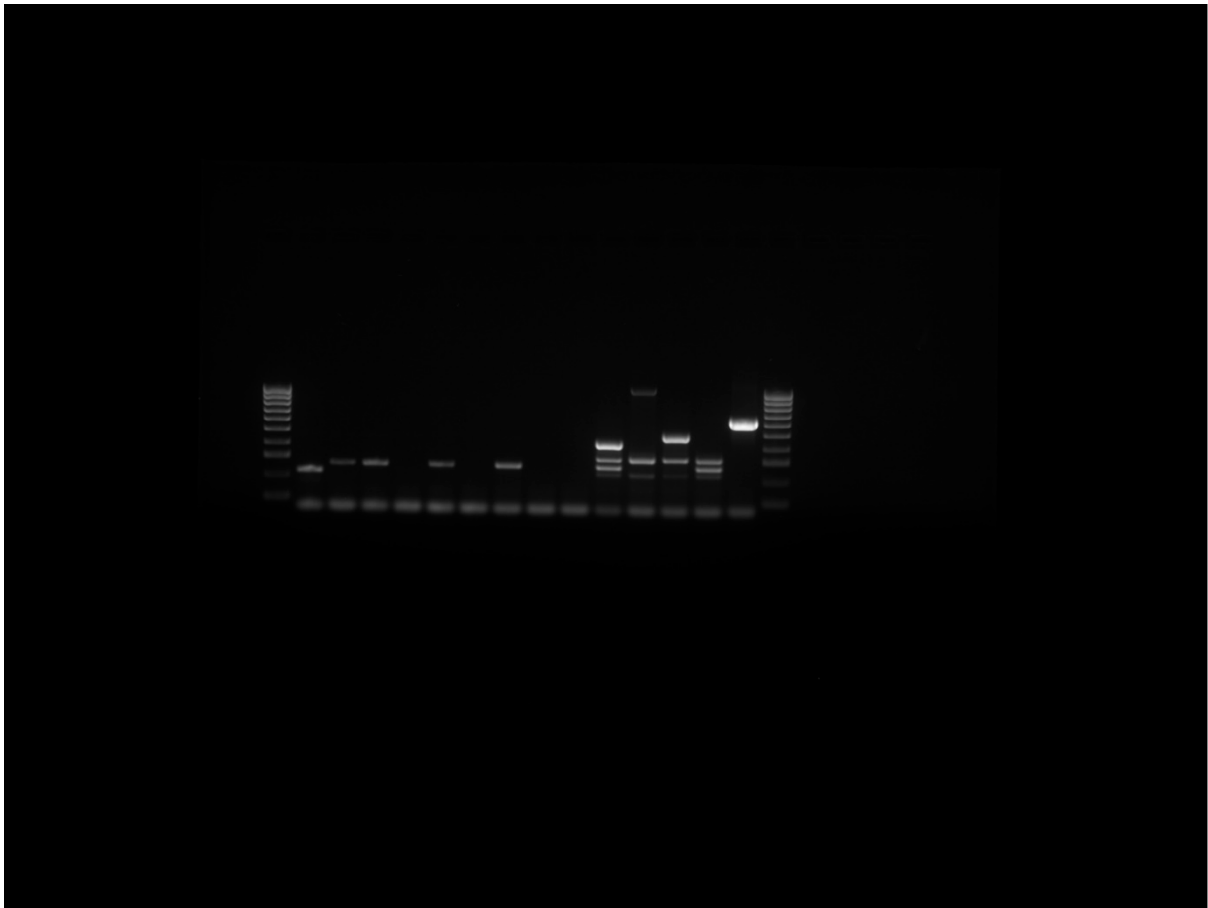

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