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Characterisation of Antimicrobial Resistant *Escherichia coli* Isolated from a Dairy Farm

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Dissertation submitted to the University of Nottingham for the degree of Masters of Research

September 2015

Word count: 34,017

Abstract

Antibiotic resistance is the greatest challenge facing modern medical practice with the prospect of completely resistant untreatable pathogens a potential outcome. One driver of this resistance is the widespread use of clinical antibiotics on livestock farms. This project investigated the antimicrobial resistance profiles of *Escherichia coli* isolated from a dairy farm, which had recently altered their antibiotic treatment regime. Strains of *E. coli* were selectively isolated from cow slurry and their antimicrobial resistance profiles compared to previously isolated strains from the same farm. No strains could be isolated using the antibiotic cefotaxime, which was previously successfully used for isolation. Three multidrug resistant *E. coli* strains, which showed a wider range of resistances than other strains were then sequenced to compare resistance genes presence with resistance phenotypes. The results of this study indicated that ESBL and multiple antibiotic resistant *E. coli* were significantly more difficult to isolate in 2014/15 compared to 2013, although multiple-drug resistant strains were still isolated. Phenotypic mercury chloride resistant strains were also more difficult to isolate in 2014/15 compared to 2013, however mercury chloride selective media were never used to isolate the strains. The carriage of mercury resistance genes correspondingly significantly decreased between the different strain groups. The antibiotic resistance genes were successfully shown within the three sequenced genomes residing on both mobile-genetic elements and chromosomally. The results of this project are consistent with the hypothesis that the reduced use of cephalosporins on this dairy farm positively affected the antibiotic resistance of the inherent *E. coli* population isolated using antibiotic selection.

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List of Abbreviations

ETEC	Enterotoxigenic *E. coli*
EPEC	Enteropathogenic *E. coli*
EHEC	Enterohaemorrhagic *E. coli*
APEC	Avian pathogenic *E. coli*
WHO	World Health Organisation
ExPEC	Extraintestinal pathogenic *E. coli*
UPEC	Uropathogenic *E. coli*
MNEC	Meningitis-associated *E. coli*
UTI	Urinary Tract Infection
LPS	Lipopolysaccharide
SCC	Somatic Cell Count
PBP	Penicillin-binding proteins
ESBL	Extended Spectrum β-lactamase
MLST	Multi-locus sequencing typing
PFGE	Pulsed-field Gel Electrophoresis
ERIC	Enterobacterial repetitive intergenic consensus
PCR	Polymerase Chain Reaction
tet	Tetracycline Resistance Genes
aadA1	3"-(9)-O-adenylyltransferase Gene
TEM	Temoneria family of β-lactamases
SHV	Sulphydryl variable family of β-lactamases
OXA	Oxacillin family of β-lactamases
CTX-M	Cefotaxime resistant family of β-lactamase
NDM-1	New Delhi metallo-β-lactamase 1
AmpC	Penicillin Resistance family of β-lactamase
amp	Ampicillin resistance genes
mer	Mercury resistance genes
tnp	Transposition module genes
IR	Terminal inverted repeat sequences
ST#	Strain Isolated from the Slurry Tank
BF#	Strain Isolated from the Barn Floor
TBX	Tryptone Bile X-Glucuronide Agar
LB	Luria-Bertani
MH	Muller-Hinton
MRD	Maximum Recovery Diluent
$HgCl_2$	Mercury Chloride
AMP	Ampicillin

CTX	Cefotaxime
IMP	Imipenem
C	Chloramphenicol
TET	Tetracycline
BLAST	Basic Local alignment search tool
NCBI	National Centre for Biotechnology Information
CAZ	Ceftazidime
EFT	Ceftiofur
CFQ	Cefquionme
AMC	Amoxicillin and Clavulanic Acid
ATM	Aztreonam
S10	Streptomycin
NA	Nalidixic Acid
CIP	Ciprofloxacin
ENR	Enrofloxacin
SXT	Trimethoprim/Sulfamethoxazole
S300	Sulphonamides
F	Nitrofurantoin
OT	Oxytetracycline
FOX	Cefoxitin
CV	Clavulanic acid
CX	Cloxacillin
GC	Guanine-Cytosine Percentage
RAST	Rapid Annotation using Subsystem Technology
blastP	Amino acid search of the NCBI database
blastN	Nucleotide search search of the NCBI database
Contig	Assembled Reads
Nodes	Reads within the Contigs
FEP	Cefepime
ESAC	Extended-Spectrum AmpC β-lactamase
Tn#	Transposon
In#	Intergon
int1	Class 1 Intergrase
IS#	Insertion Sequence Element
MDR	Multidrug Resistant
MRD	Maximum Recovery Diluent
CFU	Colony-forming Unit

1.0 Introduction

1.1 Background

Escherichia coli is a member of the Enterobacteriaceae and was discovered in 1885 by Theodor Escherich (Berg, 2008). It is closely related to the genus *Shigella* and is a Gram-negative non-sporing facultative anaerobic bacillus (Sussman, 1997). *E. coli* cells do not contain a nucleus or membrane enclosed organelles, but do possess pili and flagella, meaning they can attach to surfaces and are motile both of which are essential for pathogenicity (Berg, 2008).

The optimum temperature for *E. coli* growth is 37°C and it is able to grow on a wide range of media with its colonies typically smooth, circular and non-pigmented. Biochemically *E. coli* is indole positive and does not produce cytochrome c oxidase and it is capable of fermentative and respiratory metabolism which allows for both anaerobic and aerobic growth for survival both outside of and inside mammalian guts (Sussman, 1997).

E. coli is a common commensal of mammals whose typical habitat is the lower intestines of humans and other warm-blooded animals. In humans *E. coli* colonisation is either vertically via the birth canal or from the environment soon after birth (Bettelheim *et al*., 1974, O'Farrell *et al*., 1976). Unlike the majority of other bacteria present in the gut *E. coli* can survive in the presence of oxygen outside of the body until it finds another host, and consequently has been found in environments such as soil and water contaminated with faecal matter (Berg, 2008). *E. coli* is commonly used as an indicator organism for environmental faecal contamination (Edberg *et al*., 2000).

The majority of *E. coli* isolates are commensals, but pathogenic variants do exist that can cause disease amongst its varied hosts which are often non-

mammalian (Bélanger *et al*., 2011). Pathogenic *E.coli* pathotypes cause different diseases, which are classified as either intestinal or extraintestinal. The enterotoxigenic (ETEC), enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) pathotypes are known to cause intestinal pathologies (Bélanger *et al*., 2011). These pathotypes typically cause diarrhoea, which according to the World Health Organisation (WHO) is still the second leading cause of death in children below the age of 5. EHEC differs from the other diarrheagenic *E. coli* as these strains possess the *Shigella* Shiga toxin (Manning, 2010). 10% of EHEC infections have been known to lead to haemolytic uraemic syndrome (HUS) which is a life-threatening illness leading to death in 3-5% of cases (WHO, 2011). The main animal reservoir of the EHEC serotype O157:H7 is cattle. It does not cause disease in cattle as they lack the shiga toxin receptor, but can spread to humans via food products such as ground beef and milk (Pruimboom-Brees *et al*., 2000, Zachary and McGavin, 2012). EPEC is also a problem for humans typically in developing countries as it can often cause community outbreaks and is a leading cause of infantile diarrhoea. Atypical EPEC, which is not restricted to developing countries, causes less severe symptoms but the disease can persist significantly longer. ETEC is also not restricted to developing countries and is the most predominant diarrhoeal causing *E. coli* pathotype worldwide. Its transmission to children is usually via contaminated water and food. Although diarrhoea causing *E. coli* in humans is typically self-limiting it can cause severe disease and even death and is therefore a significant clinical pathogen (Manning, 2010).

Livestock can also be affected by *E. coli* intestinal pathologies, which can cause a range of disease such as colibacillosis and mastitis. Colibacillosis caused by ETEC most often affects calves and piglets causing diarrhoea, which can lead to a fatal infection. Edema disease is caused by ETEC and only occurs in pigs. This

disease is characterised by the formation of an oedema and causes death to all infected by an endotoxic shock like syndrome. EHEC has been shown to cause disease in humans and is infrequently reported in cattle and pigs.

The extraintestinal pathogenic *E. coli* (ExPEC) group includes uropathogenic (UPEC) and meningitis-associated (MNEC) *E. coli* (Bélanger *et al*., 2011). *E. coli* is one of the leading causes of neonatal meningitis after group B streptococci and together they account for 70-80% of neonatal bacterial meningitis in developed countries. Neonatal meningitis is not only highly linked to neurological sequelae but also to neonatal mortality (Bonacorsi and Bingen, 2005). In 20% of cases *E. coli* Neonatal meningitis occurs via the urinary tract and *E. coli* is the most preventable cause of UTI infections (Russon and Johnson, 2003, Bonacorsi and Bingen, 2005). The urinary tract is the most frequent extraintestinal site of infection associated with ExPECs with *E. coli* infection being the cause for 90% of all ambulatory UTI patients and UPEC stains for 70-90% of all community-acquired UTIs (Russon and Johnson, 2003, Kucheria *et al*., 2005, Jacobsen *et al*., 2008). ExPECs can also be carried by healthy animals and if aerosolized in animal faeces it can be inhaled resulting in fulminating necrohemorrhagic pleuropneumonia (Zachary and McGavin, 2012).

A major infection in cattle is the development of mastitis. There is increasing interest in "environmental mastitis" caused by *S. uberis* and *E. coli* (Burvenich *et al*., 2003). With *E. coli*-caused mastitis, the pathotype of the strain alone does not typically affect the outcome (Burvenich *et al*., 2003) Other factors such as the presence of an endotoxin and the type of lipopolysaccharide (LPS) carried by the *E. coli* strain is more important than the pathotypes alone (Burvenich *et al*., 2003). Mastitis is detected through a raised somatic cell count (SCC) in the milk, which is an immunological reaction to the infection. Although

70% of all cases are self-limiting and are not fatal, the milk from the teat cannot enter the food chain and is disposed of via drains or is poured into the slurry tank causing a considerable economic cost to the dairy farmers (Roberts, 2012). The wide-range of disease that *E. coli* can cause in both humans and animals shows its pathogenic potential and the need to characterise the bacteria in both animal reservoirs and humans.

The acquisition of antibiotic resistance by this pathogen makes *E. coli* one of the most clinically significant human and animal pathogens. Over time, frequency of antibiotic resistance amongst *E. coli* isolates has gradually increased and it is still a major pathogen, which can evade antibiotics due to the acquisition of β-lactamase genes, other antibiotic resistance genes, and mutation in penicillin binding proteins (PBP's) (Rice, 2008, Mayers, 2009). During the 1990's there was a worldwide spread of *E. coli* producing CTX-M β-lactamase and extended spectrum β-lactamase (ESBLs), which hydrolyse a wider range of antibiotics (French, 2010). This has resulted in the appearance of multidrug resistance *E. coli* outbreaks in both communities and hospitals. The increasing resistance to newer antibiotics is also concerning, for example the increase in *E. coli* blood isolates resistant to 3rd generation cephalosporins increased from 2% in 2001 to 12% in 2007 (French, 2010). Increasing resistance amongst coliforms to carbapenems, used clinically as a last resort drug, is also of great concern (Hong *et al*., 2005, French, 2010).

Livestock, as previously stated, are known reservoirs of potentially pathogenic *E. coli* and their resistance genes. They allow for the transfer of antibiotic resistance genes, not only amongst pathogens but also amongst the population of endogenous bacteria. Horizontal transfer of these resistance genes from large populations of endogenous bacteria to potential pathogens can then

disseminate, via environmental contamination or contaminated food products to the human population (Bélanger *et al*., 2011). The reduction of the use of antibiotics in farm animals is necessary in order to reduce the risk of the appearance of antibiotic resistance genes and their consequent dissemination (Affairs, 2013). This has been explored through the use of vaccines to reduce *E. coli* associated with specific virulence factors (Cox *et al*., 2014). Vaccines limit the number of *E. coli* with known virulence factors and prevent them from causing disease in the livestock. However this does not prevent antibiotics from being used for other bacteria and thus allows for the continued dissemination of antibiotic resistance genes in commensal *E. coli,* potentially pathogenic *E. coli* not vaccinated against and other bacteria present.

Therefore there is a continued reliance on antibiotics on livestock farms, including dairy farms, it is necessary to study their effect on the population of *E. coli* through the characterisation of the antibiotic resistance genes present. A single cow generates ~60kg of faeces a day and the liquid faeces is stored in vast slurry tanks along with any remaining antibiotic given to the animal. This along with antibiotic containing mastitic milk are stored together creating great potential for horizontal gene transfer and generation of antibiotic resistance genes. Characterisation of the effect antibiotic use has upon an *E. coli* population on a farm could lead to further insights in to the relationship between spread of antibiotic resistance and antibiotic use as well as the potential knock on effects to humans.

1.2 Profiling *E. coli*

Different typing of *E. coli* such as Multi-locus sequencing typing (MLST) and Pulsed-field Gel Electrophoresis (PFGE) exist to either categorise different strains or compare different isolates. MLST is commonly used to determine clonal relationship of bacteria isolated from hosts (Adiri *et al*., 2003). The 7 different housekeeping genes used in this project to form an MLST type were, *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase), and *recA* (ATP/GTP binding motif) (Wirth *et al*., 2006). The specific sequence for each is allocated a number which combine to make a unique Sequence Type (ST), which is linked to a sequence cluster. This allows for the identification of different *E. coli* ST-types, which can be linked to different *E. coli* characteristics such as pathogenicity and host specificity.

ERIC PRC is another profiling method. It relies on Enterobacterial repetitive intergenic consensus (ERIC) sequences, which are 127bp palindromic sequences. These sequences occur numerous times across a wide range of species, including *E. coli,* and are hypothesised to have mRNA stability function or act as RNA control elements (De Gregorio *et al*., 2005, Wilson and Sharp, 2006). ERIC sequences can be elucidated in isolates using PCR and gel electrophoresis which results in a distinct pattern of bands unique to the strain of *E. coli*; isolates that share the same fingerprint will be from the same strain (Versalovic *et al*., 1991). This method allows for the characterisation of genetic diversity among a group of *E. coli* isolates.

1.3 Antimicrobial Resistance in *E. coli*

There have been many antimicrobial methods throughout history to try and cure bacterial disease; one such method was the use of metal compounds as antimicrobials. Once antibiotics were developed they were increasingly used instead of other previously used antimicrobials. However through the use of these antimicrobials there has been a selective pressure on resistance genes and due to this antimicrobial resistance genes such as metal and antibiotic resistance genes have been selected for (Hobman and Crossman, 2015). Antimicrobial resistance genes can be chromosomal genes or can be carried on mobile genetic elements, which may be spread to different bacterial species.

1.3.1 Tetracycline Resistance

Tetracycline and oxytetracyline have been used as a growth promoter in livestock feed for cattle and is still commonly used in the USA today and marketed under trade names such as Terramycin® (Dupont and Steele, 1987, Landers *et al*., 2012). Resistance to tetracycline is however very common (De Briyne *et al*., 2014). Over 35 different tetracycline resistance genes (*tet*) have been characterised including *tetA*, *B*, *C*, *D*, *E*, *Y*, and, *I* in Gram-negative bacteria (Chopra and Roberts, 2001, Mayers, 2009). The genes *tetA* and *B* both encode a cell efflux mechanism and are the most frequent cause of tetracycline resistance in bacterial isolates from humans and animals (Dominguez *et al*., 2002, Lanz *et al*., 2003, Karami *et al*., 2006). These genes are also known to be carried on transposons causing spread in Enterobacteriaceae such as the *tet* carrying Tn*10* transposon in *Shigella flexneri* (Chalmers *et al*., 2000). Tetracycline use on farms

is a current issue as tetracycline is still used as a human clinical antimicrobial therapy, in fact it was one of the most widely used antibiotics in the UK between 2010 and 2013 (Public Health England, 2014).

1.3.2 Aminoglycoside resistance

Aminoglycosides such as streptomycin, spectinomycin and neomycin have been used on cattle previously to treat bacterial infections and are still used in products for treatment of cattle mastitis such as in Tetra-Delta™, Ubro Yellow™, and Multiject™ IMM (Guardabassi *et al*., 2008). Resistance to aminoglycosides is common in *E. coli* isolated from cattle farms and can be due to a number of resistance genes (Shin *et al*., 2014, Yamamoto *et al*., 2014). One protein that confers resistance to some aminoglycosides and has also been isolated from cattle is 3'-(9)-O-adenylyltransferase encoded by *aadA1* and confers resistance to streptomycin and spectinomycin (Bywater *et al*., 2004). This gene, similarly to other aminoglycoside resistance genes, is encoded on various plasmids in gene cassettes which, in Gram-negative bacteria, are mostly self-transmissible (Davies, 1983).

1.3.3 β-lactam Resistance

The discovery of penicillin in 1929 (Fleming, 1929) (currently called penicillin G) revolutionised the medical industry, becoming the first antibiotic. After further research Penicillin G went into mass production in the 1940's and, although it was mostly Gram-positive bacteria were susceptible, was heavily relied upon during the latter stages of World War II. The drug was made

available to the public in 1945 in the US, after the end of the war, and 1946 in the UK (Chain, 1980).

Structurally, penicillin is a β-lactam ring-containing compound (Figure 1), founding the β-lactam antibiotic group. The β-lactam ring binds to the periplasmic enzyme D-ala-D-ala-transpeptidase which is required for peptide cross-linking, an essential step in the biosynthesis of peptidoglycan. Inhibition of this transpeptidase enzyme prevents cell wall production thus has a bactericidal effect on the cell (Yocum *et al*., 1980).

In 1948 the first known case of penicillin resistance appeared in staphylococci and was due to the hydrolysis of the drug into an inactive form by a β-lactamase enzyme called Penicillinase (Bellamy and Klimek, 1948). These β-lactamase genes were shown to originate from Gram-negative bacilli and transferred rapidly to other strains via plasmids (Abraham, 1991). These serine-protease enzymes work by catalytically inactivating the amide bond in the β-lactam ring whilst remaining intact and still active after the process (Mayers, 2009). The introduction of new semi-synthetic penicillins such as ampicillin, carbenicillin and oxacillin (Acred, 1962) and the first generations of Cephalosporins, such as cefadroxil and cephalexin (Hara, 2003) overcame initial β-lactamase derived resistance (Franklin and Snow, 1989); however new β-lactamases were soon discovered that were resistant to these 1st generation cephalosporins. The β-lactam-β-lactamase inhibitors were discovered in 1981 and inhibitors such as clavulanic acid, sulbactam and tazobactam which bind and inactivate the β-lactamase enzyme active site, so the antibiotic is not inactivated rapidly became established in clinical therapy (Drawz and Bonomo, 2010). This cycle of antibiotic production and evolved resistance is called the 'β-lactamase cycle' (Salverda *et al*., 2010).

A new class of drugs called the extended spectrum cephalosporins overcame problems of resistance; however, sooner than predicted, Extended Spectrum β-Lactamase (ESBL) enzymes emerged. ESBL enzymes have the ability to hydrolyse this new group of cephalosporins and have threatened the use of this entire class (Mayers, 2009). β-lactamase antibiotics are widely used clinically in humans and are heavily relied upon in agriculture, making resistance a critical problem.

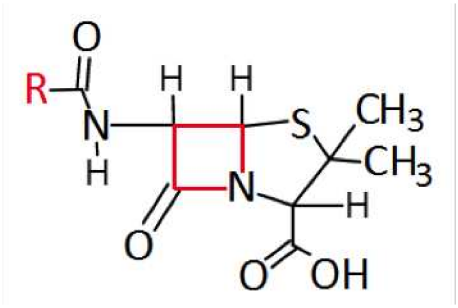


Figure 1-Chemical structure of Penicillin with the β-Lactam ring (red) and R representing the side change that is different for each type of Penicillin.

1.3.4 β-lactamases

As novel β-lactam agents were produced different β-lactamases showed different substrate specificities and hence resistance profiles, and were classified into functional groups: Cephalosporinases (Group 1), Penicillinases (Group 2a), β-lactamases (Group 2b), Carpbenicillinase (Group 2c), Cloxacillinase (Group D), Cephalosporinase inhibited by Clavulanic Acid (Group 2e), Carbapenemase (Group2f), Metalloenzymes (Group3), and Penicillinases not inhibited by

Clavulanic Acid (Group4) (Bush, 1989a, Bush, 1989b). There were different enzyme families within these groups such as TEM, SHV, OXA, and CTX.

1.3.5 TEM and SHV

TEM (Temoneria) and SHV (Sulphydryl variable) are two β-lactamase families generally classified into Group 2b, characterised by their ability to hydrolyse penicillins and narrow spectrum Cephalosporins and their inhibition by clavulanic acid (Bush, 1989a). These enzymes were swiftly horizontally transferred amongst the Enterobacteriaceae, via plasmids (Mayers, 2009), and after the introduction of Extended Spectrum Cephalosporins new ESBL, TEM and SHV enzymes appeared. These were derivatives of the original TEM-1 and SHV-1 broad-spectrum β-lactamases and contained slight alterations, usually resulting in amino acid substitutions. In the TEM-type these alterations conferred various different resistance profiles which now span to cover 2nd, 3rd and 4th generation cephalosporins, a wider range of monobactams and β-lactamase inhibitors (Salverda *et al*., 2010).

It was discovered that the level of expression of TEM type enzyme contributed to the level of resistance that was conferred (Martinez *et al*., 1987, Page *et al*., 1989, Wu *et al*., 1995). Hyper-production of the TEM β-lactamase, from *bla*$_{TEM}$, could be a result of the location of the gene on a high copy number plasmid or due to multiple copies of the gene, both causing higher expression due to higher gene dosage. Another mechanism of hyper-production is due to a more efficient promoter which causes the gene to simply be transcribed at a higher rate (Wu *et al*., 1995). The original non-ESBL TEM-1 was readily detected

with the promoter P3 (Briñas *et al*., 2002) which was found to be a weaker, less efficient promoter (Chen and Clowes, 1984) than other *bla*$_{TEM}$ promoters. The first more efficient promoters discovered were Pa and Pb, which were overlapping promoters whose high efficiency was caused by a single base pair mutation (Chen and Clowes, 1984, Chen and Clowes, 1987, Wu *et al*., 1995). Even more efficient promoters, P4 and P5, were discovered and when the same species of *E.coli* with the TEM gene *bla*$_{TEM\text{-}1b}$ utilised these different promoters, there was progressively higher activity from P3, to Pa/Pb, P4, and P5, respectively (Lartigue *et al*., 2002)

bla$_{SHV}$ encodes the SHV type β-lactamase and is most commonly found integrated into the chromosome of *Klebsiella pneumoniae* (Livermore, 1995). The first SHV- type, SHV-1, is a broad spectrum Group 1 Penicillinase, but ESBL phenotypes amongst SHV appeared rapidly (Gupta, 2007, Guenther *et al*., 2011). The first ESBL SHV-type β-lactamase was isolated in 1983 from a plasmid carried in *Klebsiella ozaenae*. The enzyme was thought to be a natural mutant of SHV-1 and was called SHV- 2. Other SHV-types have originated from these two (Knothe *et al*., 1983, Kliebe *et al*., 1985, Guenther *et al*., 2011). SHV-3, -4, and -5, also discovered in *Klebsiella*, differed from SHV-1 and -2 by point mutations yet conferred the ESBL phenotype (Jarlier *et al*., 1988, Gutmann *et al*., 1989, Arlet *et al*., 1994). One of the first cases where an *E. coli* isolate encoded an ESBL SHV β-lactamase was in North America where it was found to have a novel enzyme SHV-7. This was a clinically significant development as *E. coli* is a very common and highly susceptible human pathogen (Bradford *et al*., 1995). The *bla*$_{SHV}$ gene was found to be carried by a transposon on a number of different plasmids which enabled its dissemination throughout the Enterobacteriaceae, especially in *E. coli* (Nugent and Hedges, 1979). This was reflected by SHV-8 and -9 which were also

discovered in *E. coli* (Prinarakis *et al*., 1996, Rasheed *et al*., 1997). This evolution of extended-spectrum SHV presents massive problems for patients and their clinicians (Heritage *et al*., 1999).

1.3.6 CTX-M and NDM-1

The first emergence of the CTX-M family of β-lactamase enzymes was in 1989 in both Europe and South America (Bauernfeind *et al*., 1990, Bauernfeind *et al*., 1992). One of the enzymes, from an *E. coli* in Europe, was determined to be ESBL as it was resistant to cefotaxime which provided the name CTX-M-1, with the 'M' standing for Munich where the strain was isolated (Bauernfeind *et al*., 1990). The CTX isolates from South America were determined to share 84% homology with CTX-M-1 and were designated CTX-M-2 (Bauernfeind *et al*., 1996). During the 1990s more variants of CTX-M were discovered and it was determined that there were 5 different clusters of CTX-M variants. Each of these clusters has been linked back to the chromosomal β-lactamase genes in different *Kluyvera* species and corresponding literature points to this genus as the origin of CTX-M β-lactamases in Enterobacteriaceae. Mobilisation of the chromosomal *Kluyvera* β-lactamase genes is thought to have happened on multiple occasions causing its rapid spread (Cantón *et al*., 2012). The enzymes have suffered an extreme diversification since emergence onto the clinical scene and now over 60% of CTX-M variants confer resistance to both cefotaxime and ceftazidime (Novias *et al*., 2010). It has been suggested that the clinical use of ceftazidime has been a main factor in this extreme and swift diversification (Cantón *et al*., 2012).

CTX-M β-lactamase genes have not yet been known to confer resistance to carbapenems, the last resort β-lactam antibiotic (Cantón *et al*., 2012). Yet isolates that are resistant to carbapenems have been found to carry CTX-M and there are known links between the carriage of CTX-M and other mutations that confer carbapenem resistance (Leavitt *et al*., 2006, Cantón *et al*., 2012). An example of this was found by Gülmez *et al*. (2008) who determined that the loss of porins with the carriage of β-lactamase genes could confer carbapenem resistance. Due to wide-spread carriage of CTX-M the detection of *E. coli* with reduced porin expression and thus resistance to carbapenems is increasing (Girlich *et al*., 2009, Bennett *et al*., 2010, Cantón *et al*., 2012). This is extremely worrying as carbapenems are used clinically as a last resort antibiotic and increasing resistance is likely to lead to more untreatable diseases resulting in an increase in mortality for once treatable diseases.

Another β-lactamase enzyme, however, has been determined to confer specific carbapenem resistance. New Delhi metallo-β-lactamase 1 (NDM-1) was isolated from *K. pneumoniae* in a Swedish patient from India, and was determined to hydrolyse all β-lactam antibiotics and was only susceptible to monobactams and EDTA (Yong *et al*., 2009, Nordmann *et al*., 2011). This carbapenemase is now endemic in the Indian subcontinent and has been described in several other Gram-negative bacilli, however by 2012 there had only been two community-acquired cases outside of India and both were caused by *K. pneumoniae* (Cantón *et al*., 2012). The NDM-1 gene has previously been located on plasmids carrying multiple drug resistances and there has been an increasing worldwide spread of NDM-1 producing bacteria (Poirel *et al*., 2011, Nordmann *et al*., 2012, Cantón *et al*., 2012, Berrazeg *et al*., 2014). Another problem is that the main drug used to treat multi-drug resistant *K. pneumoniae*

such as NDM-1 carrying strains is colistin, and increasing resistance is again emerging against this last resort drug. Thus the spread of NDM-1 is becoming increasingly problematic as there are limited antibiotics available to treat these increasingly resistant isolates and the prospects of further drug discovery to treat these bacterial infections are slim (Poirel *et al*., 2011, Cantón *et al*., 2012).

1.3.7 OXA (Oxacillin)

The OXA family of β-lactamases were first discovered in the 1970s (Mayers, 2009). This family had a different resistance profile to the early TEM and SHV families; they were penicillinase enzymes that also conferred resistance to oxacillin therefore they were called OXA and assigned to a new group, Group 2d (Evans & Amyes, 2014). Although there were seven variants described by 1985 the OXA family enzymes used to be relatively rare and always plasmid mediated (Medeiros *et al*., 1985, Mayers, 2009). It is thought that the emergence of the OXA enzyme gene, *bla*$_{OXA}$ was associated with the widespread use of flucloxacillin and methicillin antibiotics against *Staphylococcus* (Evans and Amyes, 2014). The gene *bla*$_{OXA}$ is frequently found in *Pseudomonas aeruginosa* with some even mistaken for the novel β-lactamase group Pseudomonas Specific enzymes (PSE) such as OXA-10, which was previously called PSE-2 (Philippon *et al*., 1983, Hall *et al*., 1993, Bert *et al*., 2002). These OXA enzymes are plasmid encoded and transferrable so are now widespread amongst the Enterobacteriacae such as *E. coli* and *K. pneuomiae* (Naas and Nordmann, 1999, Mayers, 2009). The first case of an ESBL OXA was isolated in 1991, from a *Pseudomonas aeruginosa* patient in Turkey, with the gene being both multi-drug

resistant and transferrable (Hall *et al*., 1993). There were two amino acid substitutions that conferred the extra activity enhancing ceftazidime (3rd generation cephalosporin) binding to the active site for hydrolysis (Evans and Amyes, 2014). Since then other variants have been found and the OXA family of β-lactamases are increasingly being seen in Gram-negative bacteria (Walther-Rasmussen and Høiby, 2006, Rawat and Nair, 2010).

1.3.8 AmpC

AmpC is a β-lactamase enzyme found in most Enterobacteriaceae and non-fermenting Gram-negative bacilli. The *ampC* gene is located on the chromosome, and AmpC is produced at low levels. Expression of *ampC* is regulated by an array of genes such as *ampR, G, D* & *E* (Juan *et al*., 2005). Unlike other β-lactamases, AmpC is inducible and not constitutively expressed, but like other β-lactamases, AmpC enzymes hydrolyse the β-lactam ring and inactivate β-lactam based antibiotics (Mayers, 2009). AmpC inactivates cephalosporins, which classes it as a Group 1 Cephalosporinase. Mutations in genes responsible for regulating *ampC* expression enable hyper-production of AmpC; this combined with an antibiotic 'inducer', such as cefoxitin, broadens the resistance profile of AmpC to include penicillins, β-lactam β-lactamase inhibitor combinations, and aztreonam a monobactam (Juan *et al*., 2005, Mayers, 2009). The *ampC* gene has been found on plasmids and other mobile genetic elements, allowing for the spread of the genes to bacteria that either lack *ampC* or have a poorly expressed chromosomal *ampC* such as *E. coli* (Jacoby, 2009).

E. coli lacks the presence of regulatory *ampR* and other genes resulting in a non-inducible chromosomally encoded *ampC* gene. Hyper-production can still arise, either by gene amplification or mutations at the *ampC* attenuator or promoter regions (Olsson *et al*., 1983, Olsson *et al*., 1982, Fernández-Cuenca *et al*., 2005). An example of an *ampC* mutation increasing the rate of transcription was discovered by Fernández-Cuenca *et al*. (2005) who found a deletion mutation in the attenuator region of the *ampC* gene in a 1994 *E. coli* strain from a patient urine sample. It was postulated that the deletion undermined the stability of the hairpin loop in the attenuator region subsequently leading to *ampC* hyper-expression. Sequencing *ampC* in a known β-lactamase resistant strain of *E. coli* can lead to determining the potential expression of *ampC* along with its suspected resistance profile.

1.4 Mercury Resistance

Mercury resistance has been seen throughout history and is thought to be an ancient resistance (Barkay *et al*., 2010, Hobman and Crossman, 2015). Mercury resistance in *E. coli* however has been previously linked to antibiotic resistance and resistance plasmids (Skurnik *et al*., 2010). Antibiotic resistance genes have been found in remote environmental niches with little antibiotic exposure to necessitate their evolutionary presence. These environmental niches however are, or have been, exposed to mercury indicating that they are being carried on the same genetic element as mercury resistance (Grenet *et al*., 2004, Pallecchi *et al*., 2007). Mercury compounds are found in both the natural

environment and from man-made sources, and are extremely toxic to *E. coli* (Nies, 1999, Hobman and Crossman, 2015). Mercury has been used in agriculture (Huisingh, 1983) and medicines throughout recorded history (Hall, 1970, Nakahara *et al*., 1977, Hobman and Brown, 1997) and is still used in dental amalgam (Franke, 2007, Hobman and Crossman, 2015). Its widespread usage has resulted in the selection of bacteria resistant to its toxicity. One mechanism of resistance causes the reduction of toxic mercury ions to less toxic metallic mercury (Silver, 1996, Osborn *et al*., 1997). The mercury (*mer*) resistance operon is made up of several genes which include a gene encoding a regulator (*merR*), transport genes (*merT, P* and/or *merC, F*), and a gene encoding a mercury reductase (*merA*) (Barkay *et al*., 2003). The *mer* operon confers narrow spectrum resistance to inorganic mercury compounds such as $HgCl_2$. Sometimes *merB* is also present, conferring broad-spectrum mercury resistance, which includes resistance to organomercurials (Silver and Phung, 1996, Osborn *et al*., 1997). The *mer* operon is often carried by transposons or on plasmids, and is widely found in both Gram-negative and positive bacteria (Silver and Hobman, 2007). There are subgroups of transposons, which carry the *mer* operon in Gram-negative bacteria, the first carry *merC,* such as Tn*21* and the second lack *merC*, such as Tn*501* (Osborn *et al*., 1997).

Tn*21* is one of these genetic elements that carries the *mer* operon and is located on resistance plasmid R100, which was isolated in the 1950s (Nakaya *et al*., 1960, Liebert *et al*., 1999). R100 is known to carry resistance to tetracycline, chloramphenicol, sulphanomides, and aminoglycosides (Liebert *et al*., 1999). Another transposon known to carry both antibiotic resistance and the *mer* operon is Tn*1696,* which carries resistance to streptomycin, chloramphenicol and sulphonamides (Barbosa and Levy, 2000). There has been a correlation

between antibiotic resistance and positive *merA* gene isolates in *E. coli* isolated from humans, which also carry a complete *mer* operon and β-lactamase genes (Skurnik *et al*., 2010, Novais *et al*., 2010). It has been suggested that the continued carriage of the *mer* operon might be due to its association with antibiotic resistance genes and co-carriage or co-selection with these genes (Hobman and Crossman, 2015).

1.5 Tn*21*

Tn*21* (Figure 2) is a class II transposon, which is a subgroup of the Tn*3* transposon family (Mindlin and Petrova, 2013) and was originally isolated from the resistance plasmid R100 from *Shigella flexneri* (de la Cruz and Grinstead, 1982). This transposon is flanked by two terminal inverted repeats; IR_{tnp}, which is next to the transposition module and $IR_{mer,}$ which flanks the *mer* gene module (Liebert *et al*., 1999). The transposition module is made up of a transposase, *tnpA*, a resolvase, *tnpR,* and a putative *tnpM* gene (Halford *et al*., 1985, Hyde and Tu, 1985, Ward and Grinstead, 1987). After *tnpM* there are the *mer* genes *merEDACPTR.* Integrated between *tnpM* and *merE* is the integron In*2*. In*2* is a class 1 integron (Stokes and Hall, 1989) that includes the gene cassette, *aadA1*, and the insertion sequences IS*1326* and IS*1353* (Liebert *et al*., 1999). In*2* is made up of a transposition, *tni*, module a 5' conserved segment (CS) region and 3'CS region and the terminal IR's, IR_i flanks the 5'CS region and IR_t flanks the *tni* module (Liebert *et al*., 1999). The 5'-CS region contains an integrase, IntI1, which integrates different types of gene cassettes into the integron targeting the specific integration site *attI1* (Collis *et al*., 1998). In*2* has an *aadA1* gene cassette

integrated into it (Liebert *et al*., 1999). The *aadA1* gene, which confers resistance to aminoglycosides, carries an *attC* (59-be) site which is specific for IntI1 recombination and has a consensus simple site configuration (1L-2L and 1R-2R) (Liebert *et al*., 1999) .

The 3'CS region of In*2* contains *qacE*Δ*1*, encoding marginal resistance to quaternary ammonium compounds (Paulsen *et al*., 1993), *sulI1*, encoding resistance to sulphonamides (Swedberg, 1987) and *orf5*, of unknown function (Liebert *et al*., 1999). In the *tni* modules there are the transposition genes *tniAB*, but *tniB* is partially deleted and *tniA* alone cannot cause transposition of In*2* (Brown *et al*., 1996), so In2 is a defective transposable element. Inserted between the 3'CS region and the *tni* module is an insertion sequence IS*1326*, which carries two genes *istA* and *istB,* with a common promoter upstream of *istA* and flanking IR (Brown *et al*., 1996). However in Tn*21* there is another insertion sequence, IS*1353*, located between the -10 and -35 region of the IS*1326* promoter and encodes *orf*A and B (Liebert *et al*., 1999).

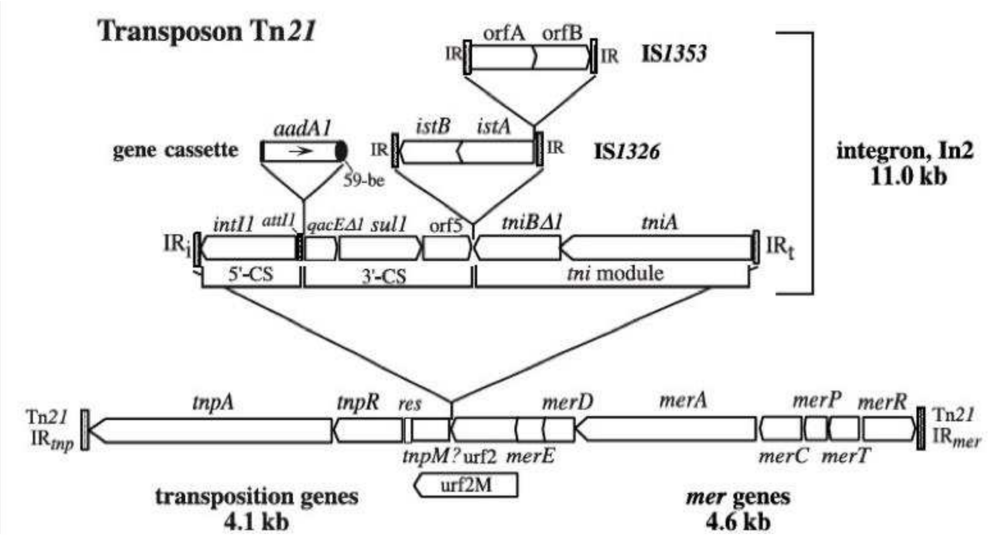


Figure 2-This figure is taken from Liebert *et al*. (1999) and shows the genes and elements within the transposon Tn*21*. It includes the transposition genes, the *mer* genes and the integrated In*2* integron.

1.6 The Project

All the bacterial strains characterised in this project were taken from a fully automated dairy farm at the University of Nottingham, which contained approximately 200 cattle. The cattle are milked by a milking robot that detects the somatic cell count (SCC) in the milk coming from each teat which is an indirect measurement of bacterial infection. If the SCC is greater than 222,000 cells/ml the cow is suspected of infection and the milk is not collected into the milk vat but added to the slurry tank. If the cattle with a raised cell count are diagnosed with mastitis then one of two antibiotic combination products are used. The first contains penicillin G, penethamate (a diethylaminoethyl ester of benzylpenicillin) and neomycin B (an aminoglycoside) and the second (Tetra-Delta™) contains penicillin G, streptomycin, neomycin and novobiocin. Previously, however, clinical mastitis was treated with products containing 1st and 4th generation cephalosporins (cephalexin and cefquinome, respectively) such as Cobactan™/Cobactan™ MC tubes (also known as Cephaguard LC), and Ubrolexin™. The treatment involved direct intramammary injection of antibiotic using a mastitis tube. The 4th generation cephalosporin intramammary mastitis treatment was stopped at the end of 2011 and the 1st generation cephalosporin products were stopped around the end of 2013. Systemic infection treatment is a Cobactan™ injection, which contains cefquinome. However due to a clinical decision made by the veterinary surgeons who oversee herd health that there was no justification to use cephalosporins, its use decreased during the end of 2012, with extremely occasional use after mid-2013. Another cefquinome product is also very occasionally used in dry cow therapy (Cephaguard dry), however the majority of the herd do not receive this treatment. Any contaminated milk, antibiotic contaminated milk, or faecal matter and urine, and

other farm wastes are deposited into the slurry tank. Any antibiotics that have been given to the cattle, along with any bacteria present, all accumulate in this slurry tank. The tank is emptied into the slurry lagoon every 10 weeks or spread directly onto fields as fertiliser. All isolates used in this project were taken from the barn floor or slurry tank and not from the lagoon.

E. coli isolated from this farm have previously been found to be multidrug resistant to a range of antibiotics from ampicillin to 3rd and 4th generation cephalosporins. The antibiotic resistance was a result of different resistance genes such as β-lactamases and ESBLs and was expected since antibiotics of this nature were used frequently on the farm (Delveen Ibrahim, Personal Communications). However since the decline in use of certain antibiotics, it is unknown whether the carriage of these resistance genes, along with the phenotypic resistance, has been affected. The aim of this project was to determine whether the *E. coli* isolates from this farm had both phenotypically and genotypically altered from previous populations. Determining the alteration throughout the decline in use of these antibiotics was also of interest.

2.0 Materials and Methods

2.1 Strains

This project used numerous isolates taken from the dairy farm at the University of Nottingham. Table 1 lists the isolates from the dairy farm and Table 2 lists the control strains for different experiments.

Strain	Year Isolated	From	Media Isolated on
A2.5	2012	Slurry	TBX
1’	2013	Slurry Tank	TBX + Tetracycline*
2’	2013	Slurry Tank	TBX + Tetracycline
3’	2013	Slurry Tank	TBX + Tetracycline
4’	2013	Slurry Tank	TBX + Tetracycline
5’	2013	Slurry Tank	TBX + Tetracycline
8’	2013	Slurry Tank	TBX + Tetracycline
20’	2013	Slurry Tank	TBX + Tetracycline
21’	2013	Slurry Tank	TBX + Tetracycline
22’	2013	Slurry Tank	TBX + Tetracycline
24’	2013	Slurry Tank	TBX + Tetracycline
28’	2013	Slurry Tank	TBX + Tetracycline
30’	2013	Slurry Tank	TBX + Tetracycline
35’	2013	Slurry Tank	TBX + Tetracycline
38’	2013	Slurry Tank	TBX + Tetracycline
39’	2013	Slurry Tank	TBX + Tetracycline
40'	2013	Slurry Tank	TBX + Tetracycline
45’	2013	Slurry Tank	TBX + Tetracycline
1’’	2013	Slurry Tank	TBX + Ampicillin**
2’’	2013	Slurry Tank	TBX + Ampicillin
4’’	2013	Slurry Tank	TBX + Ampicillin
5’’	2013	Slurry Tank	TBX + Ampicillin
6’’	2013	Slurry Tank	TBX + Ampicillin
7’’	2013	Slurry Tank	TBX + Ampicillin
10’’	2013	Slurry Tank	TBX + Ampicillin
13’’	2013	Slurry Tank	TBX + Ampicillin

Strain	Year Isolated	From	Media Isolated on
14''	2013	Slurry Tank	TBX + Ampicillin
15''	2013	Slurry Tank	TBX + Ampicillin
16''	2013	Slurry Tank	TBX + Ampicillin
21''	2013	Slurry Tank	TBX + Ampicillin
23''	2013	Slurry Tank	TBX + Ampicillin
25''	2013	Slurry Tank	TBX + Ampicillin
26''	2013	Slurry Tank	TBX + Ampicillin
27''	2013	Slurry Tank	TBX + Ampicillin
30''	2013	Slurry Tank	TBX + Ampicillin
31''	2013	Slurry Tank	TBX + Ampicillin
35''	2013	Slurry Tank	TBX + Ampicillin
39''	2013	Slurry Tank	TBX + Ampicillin
40''	2013	Slurry Tank	TBX + Ampicillin
42''	2013	Slurry Tank	TBX + Ampicillin
44''	2013	Slurry Tank	TBX + Ampicillin
46''	2013	Slurry Tank	TBX + Ampicillin
49''	2013	Slurry Tank	TBX + Ampicillin
BF1A	2014	Barn Floor	TBX + Ampicillin
BF2A	2014	Barn Floor	TBX + Ampicillin
BF3A	2014	Barn Floor	TBX + Ampicillin
BF4A	2014	Barn Floor	TBX + Ampicillin
BF5A	2015	Barn Floor	TBX + Ampicillin
BF6A	2015	Barn Floor	TBX + Ampicillin
BF7A	2015	Barn Floor	TBX + Ampicillin
BF9A	2015	Barn Floor	TBX + Ampicillin
BF5T	2015	Barn Floor	TBX + Tetracycline
BF6T	2015	Barn Floor	TBX + Tetracycline
BF7T	2015	Barn Floor	TBX + Tetracycline
BF8T	2015	Barn Floor	TBX + Tetracycline
BF9T	2015	Barn Floor	TBX + Tetracycline
ST1A	2014	Slurry Tank	TBX + Ampicillin
ST2A	2014	Slurry Tank	TBX + Ampicillin
ST3A	2014	Slurry Tank	TBX + Ampicillin
ST4A	2014	Slurry Tank	TBX + Ampicillin
ST5T	2015	Slurry Tank	TBX + Tetracycline
ST6T	2015	Slurry Tank	TBX + Tetracycline

Strain	Year Isolated	From	Media Isolated on
ST7T	2015	Slurry Tank	TBX + Tetracycline
ST8T	2015	Slurry Tank	TBX + Tetracycline
ST9T	2015	Slurry Tank	TBX + Tetracycline
ST5A	2015	Slurry Tank	TBX + Ampicillin
ST6A	2015	Slurry Tank	TBX + Ampicillin
ST7A	2015	Slurry Tank	TBX + Ampicillin
ST8A	2015	Slurry Tank	TBX + Ampicillin
ST9A	2015	Slurry Tank	TBX + Ampicillin
ST9I	2015	Slurry Tank	TBX + Imipenem***
MS	2015	Mastitis Milk	TBX + Ampicillin
SS	2015	Slurry Tank	TBX + Ampicillin

Table 1-The strains used in this project that were isolates from the Dairy Farm and the year, location and media they were isolates on. Strains MS and SS were isolates by Samiha Sokar (BSc Student) at the same time as this project.

*Tetracycline concentration was 10μg/ml

**Ampicillin concentration was 16μg/ml

***Imipenem concentration was 1μg/ml

2.1.1 Control strains

There were different strains used as positive and negative controls in this project for various experiments. They were used to ensure the experiment was working correctly and to limit the number of false positive and negative results. These strains are listed in Table 2.

Strain	Characteristics	Use	Reference or Patent
E. coli ATCC® 25922	Sensitive to all antibiotics used in antibiotic susceptibility testing method. This strain doesn't have any β-lactamases searched for in this paper other than Chromosomal *ampC*.	Used as a negative antibiotic control, ESBL control, *merAC* and *int1* control. Used as a positive chromosomal *ampC* control.	
E. coli NCTC 13353	An ESBL strain carrying CTX-M-15.	A positive control for CTX-M PCR.	Woodford *et al*. (2004)
E. coli NCTC 13352	This strain carries TEM-10.	Used as a positive control for TEM PCR.	Quinn *et al*. (1989)
Enterobacter cloacae ATCC 15337	Chromosomal *ampC* gene negative	Negative control for chromosomal AmpC PCR.	Hoffmann *et al*. (2005)
K. pneumoniae NCTC 13368	SHV-18 ESBL producing strain.	Positive control for the SHV PCR.	
E. coli J53	This strain is a derivative of K-12 from 1968. Used as a recipient strain for plasmids	Negative control for the plate and broth conjugation experiment.	Clowes and Hayes (1968)
E. coli J53 Azi^R	Spontaneous mutant of *E.coli* J53, which is resistant to sodium azide.	Used as the recipient strain for conjugation experiments, chromosomal marker sodium azide resistance.	Jacoby and Han (1996)

Strain	**Characteristics**	**Use**	**Reference or Patent**
E. coli J53 pMG101	*E. coli* J53 strain carrying silver resistance plasmid pMG101.	Used as the positive control for the plate and broth conjugation experiment.	Gupta *et al*. (1998)
E. coli 042	This strain contains the mercury resistance (*mer*) operon.	This strain was used as a positive control for the Mercury resistance gene profiling.	Nataro *et al*. (1985) & Chaudhuri *et al*. (2010)

Table 2-The control strains used in this project as well as their defining characteristics, a reference (if available) and the experiments they were used in as either a negative or positive control.

2.2 Oligonucleotide Primers

The primers used in this study (Table 3) were supplied by Eurofins (UK) and a 10pmol stock solution of each was made in sterile water, and stored at -20˚C.

Primer	Sequence	Reference
TEM-F	GCG GAA CCC CTA TTT G	Dierikx *et al*. (2012)
TEM-R	ACC AAT GCT TAA TCA GTG AG	
CTX-M-F	ATG TGC AGY ACC AGT AAR GTK ATG GC	
CTX-M-R	TGG GTR AAR TAR GTS ACC AGA AYS AGC GG	
SHV-F	TTA TCT CCC TGT TAG CCA CC	
SHV-R	GAT TTG CTG ATT TCG CTC GG	
CMY-2-F	ATG ATG AAA AAA TCG TTA TGC TGC	
CMY-2-R	GCT TTT CAA GAA TGC GCC AGG	
Chromosomal AmpC-F	AAT GGG TTT TCT ACG GTC TG	

Primer	Sequence	Reference
Chromosomal AmpC-R	GGG CAG CAA ATG TGG AGC AA	
TSO-T-F	CATTTCCGTGTCGCCCTTATTC	
TSO-T-R	CGTTCATCCATAGTTGCCTGAC	
ACC-F	CACCTCCAGCGACTTGTTAC	(Dallenne *et al.*, 2010, Sundsfjord *et al.*, 2004)
ACC-R	GTTAGCCAGCATCACGATCC	
MOX-F	GCAACAACGACAATCCATCCT	
MOX-R	GGGATAGGCGTAACTCTCCCAA	
DHA-F	TGATGGCACAGCAGGATATTC	
DHA-R	GCTTTGACTCTTTCGGTATTCG	
CIT-F	CGAAGAGGCAATGACCAGAC	
CIT-R	ACGGACAGGGTTAGGATAGY	
EBC-F	CGGTAAAGCCGATGTTGCG	
EBC-R	AGCCTAACCCCTGATACA	
FOX-F	CTACAGTGCGGGTGGTTT	
FOX-R	CTATTTGCGGCCAGGTGA	
Plasmid AmpC-F	GATCGTTCTGCCGCTGTG	
Plasmid AmpC-R	GGGCAGCAAATGTGGAGCAA	
ERIC1-F	ATGTAAGCTCCTGGGGATTCAC	Versalovic *et al*. (1991)
ERIC2-R	AAGTAAGTGACTGGGGTGAGCG	
merA-F	ACCATCGGGACCTGCGT	Liebert *et al*. (1997)
merA-R	ACCATCGTCAGGTAGGGGAACAA	
merC-F	CATCGGGCTGGGCTTCTTGAG	Liebert *et al*. (1997)
merC-R	CATCGTTCCTTATTCGTGTGG	
Int1-F	CCTCCCGCACGATGATC	Zhao *et al*. (2001)
Int1-R	TCCACGCATCGTCAGGC	

Table 3-Sequences of the primers used in this study

2.3 Media

All media was steam sterilised using an autoclave at 15psi reaching temperatures between 121ºC and 124ºC for ~15min.

2.3.1 Luria-Bertani (LB) Broth

20 grams of LB broth powder (Fisher Scientific Cat No. BP9723-500) was dissolved into 1L Reverse Osmosis (RO) water. The 20g contained: 10g of Tryptone, 5g of Yeast Extract, 5g of Sodium Chloride.

2.3.2 Luria-Bertani (LB) Agar

To make LB Agar, 15g of Agar (Oxoid, Product No. CM1021) was added to 1L of LB Broth before autoclaving (ingredients shown in section 2.3.1). Solutions or antibiotics were added, if required, after autoclaving once the medium had cooled to below 50°C and 20ml was poured onto 90mm petri dishes.

2.3.3 Tryptone Bile X-Glucuronide (TBX) agar

A selective and chromogenic medium was used to isolate *E. coli*, which is a Tryptone bile agar (Anderson and Baird-Parker, 1975) with the addition of X-glucuronide (Oxoid, Product No. CM0595), a selective marker which when reduced by *E. coli* turns the colony green (Jefferson *et al*., 1987). Dissolved into 1L of RO water was 20g Peptone, 1.5g Bile Salts No. 3, 10g Agar, and 0.075g X-β-D-Glucuronide. After sterilisation when the medium had cooled to at least 50°C, antibiotics were added if required. 20ml of the medium was then poured into 90mm petri dishes.

2.3.4 Muller-Hinton (MH) Broth

21g of MH broth powder (Oxoid, Product No. CM0405) containing, 300g beef, dehydrated infusion form, 17.5g Casein hydrolysate, and 1.5g Starch, was added to 1L of RO water and autoclaved.

2.3.5 Muller-Hinton (MH) Agar

17g of agar was added to MH broth (Oxoid, Product No. CM0337), shown in section 2.3.4, and dissolved in 1L of RO water, and autoclaved. 25ml of the medium was poured onto 90mm petri dishes or 50ml onto 120mm square/round petri dishes.

2.3.6 Tryptone Water

1L of Tryptone Water (Oxoid, Product No. CM0087) was made from 10g of tryptone and 5g of sodium chloride added to 1L of RO water and autoclaved.

2.3.7 Maximum Recovery Diluent (MRD)

9.5g of MRD (Oxoid, Product No. CM0733), containing 1g of peptone and 8.5g of sodium chloride, was dissolved into 1L of RO water and autoclaved.

2.4 Solutions

2.4.1 Sodium Azide

0.625g of Sodium Azide (≥99.5%) (Sigma-Alrich, Product No. S-2002) was dissolved into 25ml of sterile water and filter sterilized using 32mm sterile syringe filters with 0.45µm pore size. This gave a stock solution of 25mg/ml; the stock was then diluted to give a final concentration on 250µg/ml.

2.4.2 TE Buffer

0.12g of TRIS and 0.029g EDTA (or 0.0372g disodium EDTA) was dissolved in 100ml of distilled water, and autoclaved.

2.4.3 Kovacs Reagent

The Kovacs Reagent (Pro-Lab Diagnostics, Product No. PL.375) contains 1-pentanol and hydrochloric acid (Diagnostics, 2012). It was covered in aluminium foil to keep it out of direct sunlight and stored at room temperature.

2.4.4 Mercury Chloride

A 0.1M solution of mercury chloride (ACS Reagent) was prepared by dissolving 0.271g of mercury chloride powder (Sigma-Aldrich, Product No. 230405) in 10ml of deionised water. The powder was added slowly with frequent vortexing to ensure it dissolved. This was then stored at room temperature wrapped in paper to avoid sunlight.

2.5 Antibiotics

Antibiotic stock solutions were stored in 1ml aliquots at -20°C and prepared at differing working concentrations:

Ampicillin (Fisher Scientific, Product No BP1760): 0.1g of ampicillin sodium salt was dissolved in 9.9ml sterile water to give a stock of 10mg/ml. The final concentration used was 16µg/ml.

Cefotaxime (Sigma Aldrich, Product No C7039): 40mg was dissolved into 10ml sterile water to give a solution of 4mg/ml. The final concentration used was 2µg/ml.

Imipenem (Sigma-Aldrich, Product No I0160): 5mg was dissolved into 2ml sterile water to create a 2.5mg/ml stock solution. 1µg/ml was the final concentration used.

Chloramphenicol (Sigma-Aldrich, Product No C0378): 25mg/ml was dissolved into 100% ethanol. The final concentration was 25µg/ml.

Tetracycline (Sigma-Aldrich, Product No T3383): A prepared stock solution of 8mg/ml was used and the final concentration used was 10µg/ml.

2.6 Software

The genomic assembly and analysis required the use of different software for various objectives. The software used are listed in Table 4.

Software	Version	Use	Citation	Download link
FastQC	v.0.11.3	A quality control tool that was used to run quality checks on the raw reads that were the result of sequencing to provide insight into how to improve the resulting assembly	Andrews (2010)	http://www.bioinformatics.babraham.ac.uk/projects/fastqc
CutAdapt	1.8.1	CutAdapt removes any adaptor sequences that have not been removed after sequencing	Martin (2011)	https://pypi.python.org/pypi/cutadapt/
PRINSEQ	0.20.4	PRINSEQ is trimming software; it was used to trim the raw reads to result in higher quality reads.	Schmieder and Edwards (2011)	http://sourceforge.net/projects/prinseq/files
K-merGenie	1.6982	Software used to predict the best k-mer length for a genome assembly. It estimates a k-mer value for both Forward and Reverse reads.	Chikhi and Medvedev (2013)	http://k-mergenie.bx.psu.edu
SPAdes	3.5.0	SPAdes is a genome assembler.	Nurk *et al*. (2013)	http://bioinf.spbau.ru/en/content/spades-download-0
BBMap (bbrename)	35.14	A short read aligner. However the only function used here was repair function in order to create suitable naming system to run in BWA.	Busnell (2014)	http://sourceforge.net/projects/bbmap/files/?source=navbar
BWA	0.7.12	Software was used to map the reads back to the assembled contigs to provide contig coverage data.	Li and Durbin (2009)	http://sourceforge.net/projects/bio-bwa/files

Software	Version	Use	Citation	Download link
SAMtools	1.2	Software used to convert the SAM file format created by BWA to a BAM file type which could be opened by QualiMap	Li *et al*. (2009)	http://sourceforge.net/projects/samtools/files/
QualiMap	V2.1.1	QualiMap was used to open and read the data provided by BWA, and create a report.	García-Alcalde *et al*. (2012)	http://qualimap.bioinfo.cipf.es/
Prokka	V1.11	Genome annotator software.	Seemann (2014)	http://www.vicbioinformatics.com/software.prokka.shtml
RAST	-	Online genome annotation tool.	Aziz *et al*. (2008), Overbeek *et al*. (2014), Brettin *et al*. (2015)	http://rast.nmpdr.org/
Mauve	2015-02-25	A genome alignment tool used to align assembled genomes to different reference genomes.	Darling *et al*. (2004)	http://darlinglab.org/mauve/download.html
Artemis	-	Artemis is a genome browser used to visualise the sequence and allows for sequence annotation.	Rutherford *et al*. (2000)	https://www.sanger.ac.uk/resources/software/artemis/#download

Software	Version	Use	Citation	Download link
BLAST	-	Basic Local Alignment Search Tool was used to align sequences against the NCBI database or specified sequences.	Altschul *et al*. (1990)	http://blast.ncbi.nlm.nih.gov/Blast.cgi

Table 4-Table showing the different software and version that was used to generate the assembled annotated genomes, along with the reference and the site they can be downloaded from.

3.0 Methods

3.1 Isolation of *E. coli* from Faecal Samples

The slurry/faecal matter was collected into sterile 15ml plastic tubes. 1g of the sample was mixed with 9ml of sterile LB broth; the mixture was then incubated at 37°C overnight. Each sample was grown under four separate LB broth conditions: no antibiotic, with 2µg/ml Cefotaxime (CTX), with 16µg/ml ampicillin (AMP) or with 1µg/ml imipenim (IMP).

After incubation, 100µL of each sample was plated onto a corresponding Tryptone Bile X-Glucuronide (TBX) agar plate containing the same antibiotic selection as in the LB broth above and incubated overnight (37°C). The blue/green presumptive *E. coli* colonies were selected for further purification. Purification was achieved by re-plating until single *E. coli* colonies could be selected.

3.2 Identification of *E. coli*

3.2.1 Indole Production Confirmation of *E. coli*

A single colony of presumptive *E. coli* subcultured from TBX was picked from a non-selective LB agar plate and was inoculated into 9ml of tryptone water and incubated at 37°C for 24-48 hours. After incubation 4-5 drops of Kovac's Reagent (Pro-Lab Diagnostics) was added, if a red to pink colour formed on the top layer of the mix then the strain was positive for indole production. No colour change or a yellow colour change was negative.

3.2.1 Oxidase Confirmation Test for *E. coli*

A part of a single colony, from an LB plate, was spread onto an Oxidase Identification strip (Oxoid, UK) using a sterile plastic loop. If the strip turned purple within 5 seconds the colony was oxidase positive. No colour change was a negative outcome.

3.3 Gram-stain

Presumptive *E. coli* were further tested using a single bacterial colony diluted, into a drop of sterile water, on a microscope slide via a sterile loop and heat fixed. The slide was covered in crystal violet (Prolab, UK) for 1 minute, Lugol's iodine solution (Prolab, UK) for 30 seconds and then ethanol for 1 minute. The last step the slide was placed in carbol fuchsin (Prolab, UK) for 30 seconds. After each step excess solution was washed off by immersing the slide into water. Once completed, the slide was left to dry and viewed using a light microscope via oil immersion under X100 objective lens.

3.4 Antibiotic resistance profiling by disc diffusion assay

An antibiotic resistance profile for each isolate was generated using the Kirby-Bauer Disc Method (Bauer *et al*., 1966), using 17 different antibiotics (Table 5) at different concentrations (Table 6). One colony of an isolate was incubated in 5ml of MH broth for 3h and then 200µL of this was added to 4ml of sterile water creating a similar turbidity and concentration to the McFarland standard reference 0.5. If the turbidity differed then either more water or more sample was added to correct it. 100µL of this mixture was then spread onto a 90mm petri dish or 200µL on to a 120mm petri dish of MH Agar. For one sample

4 different 90mm MHA agar petri dishes were required or 2x120mm petri dishes. A sterile swab was used to spread the sample 3 times, turning the plate by a quarter each time. The antibiotic discs were placed onto the plate within 15 minutes so the bacterial growth did not affect the antibiotic sensitivity results. Ethanol sterilised forceps were used to place the discs on the media, applying pressure to the disc to ensure it wouldn't move and the forceps were re-sterilised after each sample or if they accidentally touched the media. The plates were incubated in an inverted position at 37°C overnight. The plates were read after incubation by placing the plate on black paper and, using a ruler, the resistance profile was interpreted based on the zone inhibition size provided by CLSI (CLSI, 2014) shown in Table 6.

Antibiotic Class	Antibiotics	Symbol
Pencillin	Ampicillin	AMP
Penicillin Combination	Amoxicillin and Clavulanic acid	AMC
Cephalosporin 3rd	Cefotaxime	CTX
	Ceftazidime	CAZ
	Ceftiofur	EFT
Cephalosporin 4th	Cefquinome	CFQ
Monobactam	Aztreonam	ATM
Carbapenem	Imipenem	IMP
Aminoglycoside	Streptomycin	S10
Quinolone	Nalidixic Acid	NA
Fluoroquinolones 2nd generation	Ciprofloxacin	CIP
Fluoroquinolones Veterinary use only	Enrofloxacin	ENR
Antifolates combination	Trimethoprim/Sulfamethoxazole	SXT
Sulphonamide	Sulphonamides	S300
Chloramphenicol	Chloramphenicol	C
Nitrofuran Derivatives	Nitrofurantoin	F
Tetracycline	Oxytetracycline	OT

Table 5- Antibiotics used for the resistance profiling of the farm isolate *E. coli* strains, their corresponding class and identifying symbol

Antibiotics	Concentration (μg)	Inhibition Zone Diameter (mm)		
		Sensitive	Intermediate	Resistant
Ampicillin	10	≥17	14–16	≤13
Amoxicillin and Clavulanic acid	20 10	≥18	14–17	≤13
Cefotaxime	30	≥26	23–25	≤22
Ceftazidime	30	≥21	18–20	≤17
Aztreonam	30	≥21	18–20	≤17
Imipenem	10	≥23	20–22	≤19
Streptomycin	10	≥15	12–14	≤ 11
Ciprofloxacin	5	≥21	16–20	≤15
Nalidixic Acid	30	≥19	17–18	≤ 16
Trimethoprim/Sulfamethoxazole	1.25/ 23.75	≥16	11–15	≤10
Sulfonamides	300	≥17	13–16	≤12
Chloramphenicol	30	≥18	13–17	≤12
Nitrofurantoin	300	≥17	15–16	≤14
Oxytetracycline	30	≥19	15–18	≤14
Enrofloxacin	5	≥21	17–20	≤ 16
Ceftifur	30	≥21	18–20	≤17
Cefquinome	30	≥23	20-22	≤19

Table 6- Antibiotic concentration (μg) per disc, and the criteria for defining sensitive, resistant or having intermediate resistance for that antibiotic based on the size of the zone of growth inhibition around the disc. All zone criteria were taken from CLSI 2014 (CLSI, 2014).

3.5 *ampC* Confirmation

Any strain resistant to cefotaxime or ceftazidime was tested for resistance to cefoxitin. Any resistance to cefoxitin (Table 7) required a further AmpC Confirm Kit 98007 from ROSCO DIAGNOSTICA to confirm the existence of *ampC*. The kit utilised four separate antibiotic containing discs: a Cefotaxime containing disc (CTX30), a Ceftazidime containing disc (CAZ30), a Cloxacillin and Cefotaxime containing disc (CTXCX) and a Cloxacillin and Ceftazidime containing disc (CAZCX). All of the antibiotics were at a concentration of 30 μg. The kit was used as per the manufacturer's instructions. The zone size from the combination disc was compared to the zone size from the antibiotic only disc; any size difference of ≥5mm was considered a positive AmpC result. Zone sizes that were within ≤3mm were considered a demonstration of a lack of AmpC activity. All positive results were followed up with a chromosomal and plasmid AmpC PCR.

Antibiotic	**Symbol**	**Inhibition Zone Diameter (mm)**		
		Sensitive	**Intermediate**	**Resistant**
Cefoxitin	FOX	≥18	15-17	≤14

Table 7- Criteria for defining sensitive, resistant or having intermediate resistance for cefoxitin based on the size of the zone of growth inhibition around the disc. Information taken from CLSI 2014 (CLSI, 2014).

3.6 ESBL Confirmation

Any disc diffusion results that were Cefotaxime or Ceftazidime resistant were also tested for the presence of Extended Spectrum β-Lactamase (ESBL) genes. This was carried out using a ROSCO DIAGNOSTICA Total ESBL Confirm Kit 98014, and the disc diffusion method (Section 3.4).

This kit contained 6 antibiotic discs (Table 8) all containing 30 μg of the appropriate antibiotic or antibiotic/β-lactamase inhibitor combination. Any zone size differences of ≥5mm between the combination disc and the corresponding antibiotic only disc was considered a positive demonstration of ESBL activity. Only one positive result was enough for this and not all three combinations were required to be positive. All comparative zone sizes within ≤3mm were a negative demonstration of ESBL activity. Any positive results were followed up with an ESBL PCR.

Antibiotic discs	**Symbol**	**Concentration of Antibiotic (μg)**
Cefotaxime	CTX30	30
Cefotaxime and Clavulanate	CTXCV	30
Ceftazidime	CAZ30	30
Ceftazidime and Clavulanate	CAZCV	30
Cefepime	FEP30	30
Cefepime and Clavulanate	FEPCV	30

Table 8- The antibiotics in the ROSCO DIAGNOSTICA Total ESBL Confirm Kit, their symbol and the concentration of antibiotic.

3.7 Phenotypic Mercury resistance

LB agar and Mercury chloride ($HgCl_2$) plates were made at two differing $HgCl_2$ concentrations: 20μM and 50μM. The plates were made in the same way as the antibiotic plates, where sterile melted agar was cooled down to approximately 40°C and then the $HgCl_2$ was mixed into the liquid agar, and then 20ml was poured into each petri dish.

A negative control, *E. coli* ATCC 25922 and positive control *E. coli* 042 were used to ensure no false positives. False positive results were highly likely if too much bacterial inoculum was inoculated onto the media and thus bacterial inoculum was limited by using a single 5μl loop, as no CFU/ml limit was determined.

3.8 DNA extraction

100μL of TE buffer was mixed with a single colony of bacteria, from an overnight LB culture, in a 1.5ml microcentrifuge tube. The microcentrifuge tube was then heated to 99°C in a dry heating block for 30 minutes. Then the mixture was centrifuged for 15 min at 13000G. The supernatant, containing the DNA, was then transferred to another eppendorf and stored at -20°C. The NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific) was used to confirm DNA concentration and purity, any DNA sample with an 260/280nm absorbance ratio below 1.5 was assumed contaminated and DNA extraction was repeated.

3.9 Polymerase Chain Reaction

All polymerase chain reactions were carried out using a Thermocycler C300 and using molecular grade nuclease free water (Fisher Scientific). All primers used in the PCR reaction mix had the concentration of 10 pmol and are shown in Table 3 (Section 2.2). In all reactions the concentration of DNA used was between 50-100 ng/µL, if the sample was lower than 50ng/µL than 2uL of DNA was used to ensure the concentration was between 50-100ng/µL.

3.9.1 ERIC PCR

The PCR reaction mix was composed of 12.5µL DreamTaq Green (2x) PCR master mix (Life Technologies), 9 µl nuclease free water, 1µL of forward ERIC primer, 1µL of Reverse ERIC primer and either 1or 2µL DNA (depending on sample concentration). The reaction mix was then made up to 25µL using nuclease free water.

The PCR cycle consisted of an initial denaturing step of 3 minutes at 94°C and then 34 cycles of 94°C (30s), 52°C (60s), 65°C (4 mins) and then a final extension at 65°C for 8 minutes (Versalovic *et al*., 1991). The final extension temperature is lower than the recommended for this enzyme as the protocol was originally designed for another DNA polymerase (Versalovic *et al.*, 1991) and not altered for the DreamTaq polymerase.

3.9.2 Multiplex Plasmid *ampC* PCR

This PCR reaction mix consisted of 25µL of DreamTaq Green (2x) PCR master mix (Life Technologies), 1µL of Acc forward primer, 1µL Acc Reverse primer, 2.5µL Fox forward primer, 2.5µL of Fox reverse primer, 1µL Mox forward

primer, 1µL Mox reverse primer, 2.5µL DHA forward primer, 2.5µL DHA reverse primer, 1µL CTT forward primer, 1µL CTT reverse primer, 1µL EBC forward primer, 1µL EBC reverse primer, 2µL of template DNA. The reaction mix was then made up to 50µL using 5µL of nuclease free water.

The PCR cycle started with an initial denaturing step of 10 minutes at 94°C and then 30 cycles of 94°C (40s), 60°C (40s) and 72°C (60s). The PCR reaction ended with a final extension step of 72°C for 7 minutes (Dierikx *et al*., 2012).

3.9.3 Chromosomal *ampC* PCR

This PCR reaction mix consisted of 25µL DreamTaq Green (2x) PCR master mix (Life Technologies), 2.5µL of Chromosomal AmpC forward primer, 2.5µL of Chromosomal AmpC reverse primer and 2µL of template sample DNA. The reaction mixture was made up to 50µL with 18µL of nuclease free water.

The PCR cycle used an initial denaturing step of 94°C for 3 minutes and then 30 cycles of 94°C (90s), 57°C (30s), 72°C (30s). The reaction ended with a final extension step of 72°C for 7 minutes (Dierikx *et al*., 2012).

3.9.4 ESBL PCR

Three separate PCR reactions were used to amplify the TEM, SHV or CTX genes which produce the ESBLs. The reaction mix consisted of 12.5µL DreamTaq Green (2x) PCR master mix (Life Technologies), 1µL Forward Primer, 1µL Reverse Primer, 2µL template DNA. The reaction mix was made up to 20µL with 8.5µL of nuclease free water. Each PCR reaction used primers specific to the target gene. SHV PCR used SHV reverse and SHV forward primers, TEM PCR

used TEM reverse and TEM forward primers and CTX used CTX reverse and CTX forward primers.

The PCR cycle used an initial denaturing step of 94°C for 3 minutes and then 30 cycles of 94°C (90s), 57°C (30s), 72°C (30s) and a final extension step of 72°C for 7 minutes (Dierikx *et al*., 2012).

3.9.5 Multiplex *merA* and *merC* PCR

This PCR reaction mix consisted of 12.5µL ReddyMix PCR Master Mix (Thermo Scientific), 0.5µL MerC forward Primer, 0.5µL MerC Reverse Primer, 0.25 µL MerA Forward Primer, 0.25 µL MerA Reverse Primer, 9µL nuclease free water and 1µL of template sample DNA.

The PCR cycle utilised an initial denaturing step of 95°C for 5 minutes and then 30 cycles of 95°C (60s), 66°C (2 minutes), 72°C (3 minutes) and then a final extension step of 72°C for 10 minutes (Liebert *et al*., 1997).

3.9.6 *Int1* PCR

This PCR reaction mix consisted of 12.5µL of ReddyMix PCR master mix (ThermoScientific), 0.5µL Int1 Forward Primer, 0.5µL Int1 Reverse Primer, 1µL template sample DNA and 5.5µL nuclease free water.

The PCR cycle utilised an initial denaturing step of 95°C for 2 minutes and then 30 cycles of 95°C (25 s), 57.6°C (35 s), 72°C (17 s). The reaction finished with a final extension step of 72°C for 5 minutes (Zhao *et al*., 2001).

3.10 Plasmid Extraction

Plasmids were extracted from *E. coli* strains using Qiagen kit QIAprep® Spin Miniprep kit. A single colony was selected from an LB plate and incubated in 5ml of LB broth at 37°C for no more than 16h. Plasmid extraction was then performed using the Qiagen kit according to the manufacturer's instructions including the recommended low-copy plasmid extraction PB wash step.

After the extraction gel electrophoresis was performed to visualise the results. A 0.7% agarose gel was used under low voltage running for 2 hours, if a mini gel was used then the voltage was 40v and for a midi gel 60v. 7µL of a 1kb DNA extend ladder (BioLabs) was used to measure the band sizes and 1µL of 6X loading dye (BioLabs) was mixed with 5µL of the extraction product and loaded into the gel wells. A NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific) was then used to quantify the DNA yield.

3.11 Plate and Broth Conjugation

Self-transmissible plasmids were detected using conjugation experiments. Donor cell bacteria and recipient bacteria were separately grown in 5ml of LB broth over night at 37°C. 0.5ml of each were mixed in 4.5ml of LB broth, 100µL of this mix was plated directly onto an LB plate and incubated at 37°C overnight.

On the second day 1ml of MRD solution was plated onto the mixed LB plate to re-suspend the bacteria. 100µL of the re-suspension was plated onto an LB plate with 250µg/ml Sodium Azide mixed with either 25µg/ml Chloramphenicol, 10µg/ml Tetracycline, or 16µg/ml Ampicillin and incubated overnight at 37°C.

For the broth conjugation 0.5ml of each bacterial culture was mixed in 4.5mL LB broth and not plated but incubated over night at 37°C. Then the next day 100µL of this was plated onto LB with the same selection as the plate conjugation. Any growth is a positive result and was re-plated onto the same LB selective media and incubated for up to 2 days at 37°C to confirm this.

The recipient strain was J53 Sodium Azide resistant *E. coli*, the positive control was *E. coli* J53 pMG101 and the negative control was *E. coli* strain J53.

3.12 DNA extraction for sequencing

Total DNA was extracted from each strain using Qiagen® DNeasy Blood and Tissue Kit as it allowed for high-quality DNA to be extracted for sequencing. The protocol used was for Gram-Negative bacteria and the optional step of adding 4µL of RNase A was carried out, as we required RNA free DNA. The final flow through was saved and concentration, purity and quality of the DNA were checked using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). The DNA was stored in a 1.5ml Eppendorf tube at -20°C.

3.13 DNA Sequencing

DNA sequencing was carried out for two isolates for genomic characterisation and they were sequenced at Deep Seq (UoN) using the Illumina MiSeq 300PE machine. First the isolates underwent DNA Resequencing Library prep for the Illumina platform and then were sequenced generating approximately 50x coverage per isolate.

3.14 Genomic Assembly and Analysis

Genomic assembly and analysis was required to characterise the sequenced isolates. This was done using a variety of software, online services and databases. The overall steps each isolate underwent were the same and shown in Figure 3.

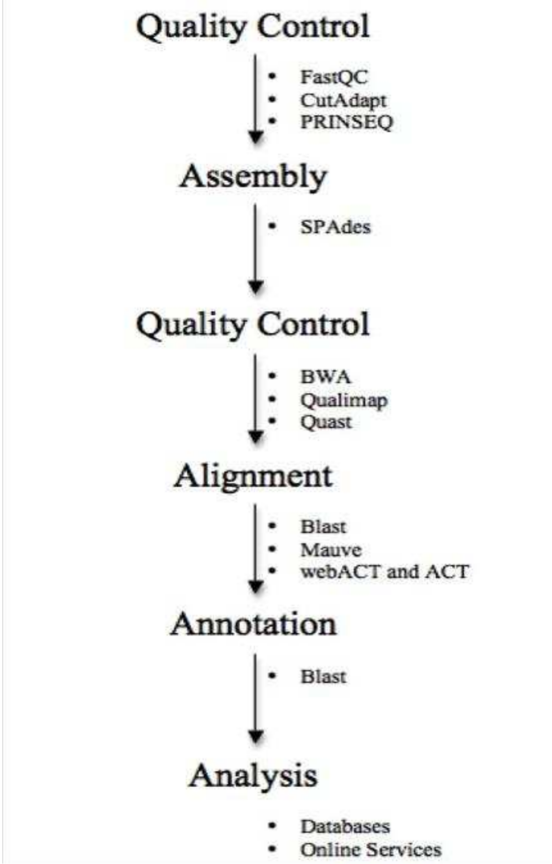


Figure 3-Bioinformatic pipline of genomic sequences analysis, and tools used.

3.14.1 Quality Control

The quality of each read was analysed by FastQC (Andrews, 2010) to determine their overall quality. Of interest were any fails or warning symbols shown through a cross and exclamation mark respectively. From here it was seen how the reads could be improved for example if the quality of the bases dropped off after a certain position, shown by 'Per base sequence quality', then it became evident that the read length would need trimming to increase the quality. This software was also used to generate overall statistics of the reads such as GC% and total sequence length. Any sequences that were over-represented in the genome, such as left over adaptor sequences, were identified by FastQC and fixed via trimming (3.14.2).

3.14.2 Trimming

Trimming is a technique to cut out DNA bases from reads that are of low quality so as to increase the overall quality of the read and thus assembly. The first trimming carried out was the trimming of over-represented adaptor sequence if present. If there were no over-represented sequences then this step was not carried out. This trimming step was carried out using a program called CutAdapt (Martin, 2011), using options shown in Table 9.

Subsequent trimming was to the 3' and 5' end of the reads in order to increase their quality and this was carried out using PRINSEQ (Schmieder and Edwards, 2011). PRINSEQ trimmed each end by quality score meaning that each read would be trimmed until the quality of the end bases reached a specified amount. It also allowed for the trimming of each end by a specified number of bases for example if the quality was low at the 3' end of the read than any specified number of bases from this end e.g. 5bp could be trimmed away to try and increase the quality.

CutAdapt		**PRINSEQ**	
Isolate	**Options**	**Isolate**	**Options**
24'	-a GATCGGAAGAGCACACGT CTGAACTCCAGTCACAGT CAACAATCTCGTAT	24'	-trim_right 100 -trim_qual_right 30 -trim_qual_left 30
		39'	-trim_right 100 -trim_left 5 -trim_qual_right 30 -trim_qual_left 30

Table 9-Options used for the software CutAdapt (Martin, 2011) and PRINSEQ (Schmieder and Edwards, 2011) for isolates 24' and 39'. Note that 39' did not require Cutadapt as no over-represented sequences were identified.

3.14.3 K-mer estimation

A k-mer value is required for some software to increase the quality of genome assembly. This is a fixed number of bases (K) in a short DNA sequence which occurs twice or more in a genome and the k-mer value is the number of times it occurs in the sequence; estimating a value allows for more accurate genomic assembly. The estimation was carried out using KmerGenie (Chikhi and Medvedev, 2013), the highest k-mer value considered by this software was increased to 200 by using the function `Make Clean` then `MAKEMAXK-MER=200`.

3.14.4 Assembly

Assembling the reads into contigs is necessary in order to analyse the genome and contigs. First the reads are assembled in to nodes and then the shortest paths between nodes are determined; both the nodes and the paths are grouped into contigs. The assembling software, SPAdes (Nurk *et al*., 2013)was used specifying the options `--careful` and setting `--cov-cutoff` to auto. The first option allows the software to try and reduce the number of mismatches and short indels. It also activates a pre-installed MismatchCorrector a tool from BWA software (Li and Durbin, 2009), however this software did not successfully run for both genomes and due to time limitations was not corrected. `--cov-cutoff` specified the software to automatically compute a coverage cutoff value using a conservative strategy. The k-mer value was also set specifying the estimated k-mer value output for both the forward and reverse reads from K-merGenie. The assembled output file was in fasta format and statistics for the output files are shown in appendix 3.0.

3.14.5 Contig Coverage

In order to estimate to the percentage of coverage for each contig, which provides information on possible plasmids present in the genome, the reads from the assembly output were mapped back to the assembled contig. The software BWA (Li and Durbin, 2009) was used for this. BWA first required an index to be created using the `bwa index` and the option `-a is` invoked an IS linear-time algorithm and the contig.fasta SPAdes (Nurk *et al*., 2013) output file and both read files were specified as inputs. After this the function `bwa mem` was used with the number of threads option, `-t`, specified to 4 and the output file specified to be in the SAM file format. This SAM file was then converted into a BAM file format using the software SAMTools (Li *et al*., 2009). This was done using the functions `view` option `-b` (bam file), `sort` specifying the format as `out.s`, and `index` it as `out.s.bam`. This file was then opened using Qualimap (García-Alcalde *et al*., 2012), File>New Analysis>BAM QC. From here the contig coverage can be seen in the 'Chromosome stats' section, which displayed all the reads have been successfully mapped. The contig coverage for isolate 24' and 39' are shown in appendix 5.

3.14.6 Genome alignment

Aligning a genome to another assembled genome results in aligning of contigs, this allows for higher quality analysis as it provides a more informed genome structure. The top 10 largest contigs were uploaded to BLAST (Altschul *et al*., 1990) to search for a reference strain using blastN. The top 5 closest full genome matches, in terms of the largest Query Coverage, Identity percentage and e-value for each were tallied and the 2 most frequent were used as a reference

genome for contig alignment in Mauve (Darling *et al*., 2004). These reference genomes were *E. coli* APEC strain O78 and APEC strain O78-9 (Mangiamele *et al*., 2013, Huja *et al*., 2015).

In Mauve (Darling *et al*., 2004) the option Tools>Move Contigs was used to align the assembled contigs.fasta file to the reference genome. The contigs.fasta file in the highest number alignment output folder is the ordered contigs file.

3.14.7 Annotation

Annotation of genes allows for easy analysis of the potential genes carried by the organism. The ordered contig.file from Mauve was uploaded to the Rapid Annotation using Subsystem Technology (RAST) server. The taxonomy of the uploaded file was specified using the Taxonomic ID number 562 *Escherichia coli*. After annotation two file formats were downloaded, these were the amino acid file format and the merged Genbank file format, which can be opened in Artemis.

3.14.8 Multi-locus Sequence Typing and Phylo-typing

The phylo-typing method used here was the Clermont method (Clermont *et al*., 2013) for *E. coli*. Primers were used (Table 10) to search the genomic sequence using the Navigator in Artemis (Rutherford *et al*., 2000) with the 'Find base pattern' option. The genes searched for were *chuA, yjaA, arpA*, DNA fragment TspE4.C2 and if required *trpA* and *arpA* group E (*arpA* GE) (Table 10). If the sequence between the primers plus the primers resulted in the correct

gene length (Table 10) then the isolate was determined positive for that gene. If there was no match for the primers or only one primer matched then the isolate was said to be negative for that gene. The pattern of genes possessed is linked to seven different phylogroups, A, B1, B2, C, D, E and F (Table 11) and determined for isolates 24', 39' and A2.5 (appendix 4).

The Multilocus Sequence Typing (MLST) was determined using the MLST service from the Centre for Genomic Epidemiology (Larsen *et al*., 2012) using the organism option *E. coli* #1. The read format selected was the 'Assembled Genome/Contigs' format option uploading the assembled Contigs fasta file. The service searches for the different housekeeping genes allocating a number to each and provides the Sequence Type (ST) and sequence cluster shown in appendix 4.0 and 4.1

Target	Primer ID	Primer Sequence 5'-3'	Product Size (bp)	Reference
chuA	chuA.1b	ATGGTACCGGACGAACCAAC	288	Clermont *et al*. (2013)
	chuA.2	TGCCGCCAGTACCAAAGACA		Clermont *et al*. (2000)
yjaA	yjaA.1b	CAAACGTGAAGTGTCAGGAG	211	Clermont *et al*. (2013)
	yjaA.2b	AATGCGTTCCTCAACCTGTG		
TspE4.C2	TspE4C2.1b	CACTATTCGTAAGGTCATCC	152	Clermont *et al*. (2013)
	TspE4C2.2b	AGTTTATCGCTGCGGGTCGC		
arpA	AceK.f	AACGCTATTCGCCAGCTTGC	400	Clermont *et al*. (2013)
	ArpA1.r	TCTCCCCATACCGTACGCTA		Clermont *et al*. (2004)
trpA	trpAgpC.1	AGTTTTATGCCCAGTGCGAG	219	Lescat *et al*. (2012)
	trpAgpC.2	TCTGCGCCGGTCACGCCC		
arpA GE	ArpAgpE.f	GATTCCATCTTGTCAAAATATGCC	301	Lescat *et al*. (2012)
	ArpAgpE.r	GAAAAGAAAAAGAATTCCCAAGAG		

Table 10-Genes searched for in the phylo-typing method and the primers associated with them along with the product length and the reference for the primers. The information was taken from Clermont *et al*. (2013).

Phylogroup	***arpA***	***chuA***	***yjaA***	**TspE4.C2**	**Other**
A	+	-	-	-	
A	+	-	+	-	
B1	+	-	-	+	
C	+	-	+	-	*trpA*+
E	+	+	-	-	*arpA* GE+
E	+	+	-	+	*arpA* GE+
E	+	+	+	-	*arpA* GE+
D	+	+	-	-	
D	+	+	-	+	
F	-	+	-	-	
B2	-	+	-	-	
B2	-	+	-	+	
B2	-	+	+	+	

Table 11- Pattern of positive and/or negative genes and DNA fragment that match up to the different phylogroups. A positive gene is depicted by a '+' symbol and a negative by '-' sign. If the patterns overlap they are tested for the genes in the 'Other' column. All information is taken from Clermont *et al*. (2013).

3.14.9 Gene Analysis

In order to confirm the identity of genes annotated by RAST the gene under went further identification using the NCBI database basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). The amino acids and/or the nucleotides were searched against the NCBI database using blastP or blastN, respectively and the search settings were not altered from default unless specified. Comparison of two or more genes/sequences was carried out using the 'Align two or more sequences' setting with the query gene/sequence always going in the top box on both blastP and blastN.

3.14.10 Databases and Services

Analysis of the entire genome can also be carried out using different databases and services. Those used are shown in Table 12, with a description of the service or database and the options used along with its online link and its reference.

Service	Description	Link	Reference
PlasmidFinder	Assembled Contigs were used to identify full or partial plasmids in the sequence using a 95% ID threshold and the option Enterobacteriaceae.	https://cge.cbs.dtu.dk//services/PlasmidFinder/	Carattoli *et al*. (2014)
ResFinder	Assembled contig files are searched for antibiotic resistance genes at a 95% threshold with at least 60% gene coverage.	https://cge.cbs.dtu.dk/services/ResFinder/	Zankari *et al*. (2012)

Table 12-Databases and services used to analyse the genomes in this project. A description of the service, a link and reference are also provided in the table.

4.0 Results

4.1 Isolation of *E. coli* Strains from Faecal Samples

The *E. coli* strains used in this project were all isolated on TBX media using Ampicillin or Tetracycline for selection. Cefotaxime and imipenem TBX plates were also used for selection. However, only one strain of *E. coli* grew on the imipenem plate and due to its subsequent phenotypic lack of any antibiotic resistance (Table 13) it was concluded that the phenotype was unstable. No strains were isolated on TBX containing cefotaxime. Seventeen strains were successfully isolated using ampicillin and 10 strains using tetracycline (Table 1). These strains were all confirmed to be *E. coli* as they were all Gram-negative rods, oxidase negative and indole positive.

4.2 Antibiotic Resistance Profiles of the 2014/15 Isolates

Overall the 2014/15 isolate group showed resistance to 8 of the 17 antibiotics tested, with over half the isolates being resistant to oxytetracycline, sulphonamides or ampicillin. Of the isolates that carry a resistance, on average they carried resistances to just over 4 antibiotics (Table 13), which most often included resistance to chloramphenicol, streptomycins, amoxicillin-clavulanic acid, or trimethoprim/sulfamethoxazole. Thus these isolates would qualify as mulitdrug resistant strains (MDR) according to the current accepted definition of MDR (Magiorakos *et al*., 2012). There was no resistance or intermediate resistance to the carbapenem antibiotic, imipenem, the quinolone antibiotic

nalidixic acid, the monobactam antibiotic, aztreonam, the 3rd generation cephalosporins, ceftifur, cefotaxime, ceftazidime, the 4th generation cephalosporin cefquinome and the fluoroquinolones enrofloxacin, and ciprofloxacin.

The resistance profiles of strains isolated from the farm in 2013 was kindly provided by Delveen Ibrahim (PhD Student) and Jack Marsland (BSc Student) and shown in Appendix 1. Any isolate that showed resistance to cefotaxime and ceftazidime from both the 2013 and 2014/15 isolates were tested for ESBL activity, using the ROSCO DIAGNOSTICA Total ESBL Confirm Kit, and resistance against cefoxitin to determine plasmid *ampC* activity (Table 14). If resistant to cefoxitin, plasmid *ampC* activity was confirmed using a ROSCO AmpC confirmation kit (Table 14).

There were three positive ESBL isolates in the 2013 isolate group and none in the 2014/15 Isolate group. There were also 7 plasmid *ampC* positive isolates in the 2013 group and none in the 2014/15 group. Thus these results show a higher frequency of both ESBL and plasmid *ampC* presence in the 2013 samples. However the difference was quite small, with 7.9% of the 2013 isolates ESBL positive and 16.7% of the isolates positive for plasmid *ampC* compared to 0 isolates in the 2014/15 group for both.

Isolate	Antibiotics																	No. Resistances
	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP	
BF1A	6	6	20	6	26	8	29	24	22	6	24	31	6	7	28	27	32	7
BF2A	24	6	21	27	30	6	29	23	26	6	36	33	6	26	29	32	32	4
BF3A	22	6	20	27	30	6	28	24	25	6	34	32	6	26	31	30	32	4
BF4A	23	6	20	24	30	17	28	24	25	6	33	31	6	26	30	31	31	3
BF1I	27	17	20	26	29	15	29	24	28	8	35	35	15	26	33	32	35	1
BF5A	10	16	25	6	32	15	27	24	25	6	32	31	10	6	30	30	32	5
BF6A	24	20	23	6	30	6	29	24	28	6	35	31	6	26	32	32	30	4
BF7A	10	16	22	6	30	16	29	23	27	6	36	31	6	6	30	32	32	5
BF9A	31	25	22	25	35	17	30	27	30	26	34	35	22	30	30	35	32	0
BF5T	30	22	22	24	32	16	30	24	28	27	35	32	18	25	28	34	35	0
BF6T	30	26	20	22	30	15	30	25	28	24	40	34	22	25	31	34	35	0
BF7T	27	21	20	24	30	15	29	24	28	23	34	30	20	24	32	33	35	0
BF8T	29	19	21	22	25	14	27	24	25	25	33	30	17	24	31	29	35	0
BF9T	29	21	21	24	30	15	29	25	25	25	33	34	20	25	31	32	36	0
ST1A	26	6	21	6	31	16	27	20	26	10	32	33	6	24	32	33	33	4
ST2A	21	6	20	6	32	16	31	22	25	6	35	33	6	25	33	34	33	4
ST3A	6	6	21	6	30	6	30	20	27	6	35	33	6	9	23	34	25	7
ST4A	6	6	20	6	28	11	28	20	27	6	34	34	6	8	23	32	23	7

Isolate	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP	No. Resistances
ST5T	24	23	18	28	32	17	31	23	30	6	37	37	22	28	36	35	43	1
ST6T	26	24	19	30	31	17	32	26	29	15	38	35	24	30	42	36	46	0
ST7T	27	23	17	26	33	20	32	26	30	17	40	32	20	29	40	37	42	0
ST8T	27	21	24	6	32	14	34	29	27	6	38	34	6	6	35	39	38	4
ST9T	27	23	20	24	27	15	27	22	25	25	32	29	19	29	29	28	30	0
ST5A	27	19	21	6	30	13	29	26	26	6	35	33	6	6	36	34	36	4
ST6A	26	18	20	6	28	13	27	24	25	6	34	32	6	7	32	31	40	4
ST7A	25	20	20	6	33	12	31	21	28	6	37	39	6	8	40	35	50	4
ST8A	21	21	24	6	32	14	32	29	27	6	35	34	6	6	35	35	38	4
ST9A	19	18	18	6	30	6	29	23	21	6	35	32	6	7	34	33	30	5
ST9I	30	25	22	26	33	17	33	25	30	21	37	35	21	29	40	33	42	0
MS	R	S	R	R	S	R	S	S	S	S	S	S	R	R	S	S	S	0
SS	R	R	R	R	S	R	S	S	S	S	S	S	-	R	S	S	S	0

Table 13- Antibiotic sensitivity results for the 2014/15 isolates resistances are highlighted by a red box, intermediate resistance by a yellow box and sensitivity by no colour. MS and SS *E.coli* strain (Section 2.1) results were carried out, and provided, by Samiha Sokar (BSc Student). The numbers represent the zone of clearing around the disc, the smaller the no. means the more resistant the isolates is to the antibiotic. The cell marked '-' reflects data not available.

	ESBL Disc Test Antibiotics									Amp C	AmpC Disc Test					
Isolate	FEP	FEP+C	Result	CTX	CTX+C	Result	CAZ	CAZ+C	Result	FOX	CTX30	CTXCX	Result	CAZ30	CAZCX	Result
4'	33	33	0	33	33	0	29	31	2	25						
21'	30	28	-2	22	23	1	19	22	3	13	22	30	8	18	25	7
24'	31	31	0	21	23	2	16	21	5	9	24	30	6	18	25	7
28'	30	31	1	21	23	2	16	20	4	10	25	31	6	19	29	10
38'	32	35	3	33	33	0	28	29	1	26						
39'	27	30	3	22	24	2	17	21	4	11	23	30	7	18	28	10
1''	33	32	-1	31	32	1	28	30	2	26						
4''	Data previously collected-ESBL negative									8	26	34	8	20	31	11
7''	Data previously collected-ESBL negative									15						
14''	Data previously collected-ESBL positive									27						
16''	Data previously collected-ESBL positive									26						
25''	30	30	0	21	25	4	16	20	4	14	23	25	2	18	24	6
26''	29	29	0	21	23	2	17	20	3	9	22	28	6	18	26	8
31''	33	31	-2	30	32	2	28	28	0	25						
49''	31	30	-1	32	31	-1	26	30	4	25						
BF1A	25	30	5	29	28	-1	27	28	1	25						
ST3A	32	33	1	34	31	-3	28	30	2	25						
ST4A	30	27	-3	30	35	5	28	29	1	24						

Table 14- ESBL and plasmid *ampC* activity for different isolates. Resistance was detected by measuring zones of clearing (mm) around antibiotic discs from the ROSCO DIAGNOSTICA Total ESBL Confirm Kit antibiotic discs and the AmpC confirm kit from ROSCO, respectively. These kits were used as per manufacturer's instructions, the clearing zone between the antibiotic and antibiotic plus combination of either clavulanate (C) (inhibits ESBLs) or cloxacillin (CX) (inhibits plasmid *ampC*) were compared. A result of ≥5mm is positive (red). The two yellow boxes show false positives as the initial zones of inhibition are too large to be considered. Only isolates resistant to cefoxitin (FOX) were tested for plasmid *ampC* activity. The boxes that indicated the 'data was previously collected' was collected and provided by Delveen Ibrahim (PhD student).

4.3 ESBL and *ampC* PCR of All Isolates

Separate PCR reactions to detect ESBL and plasmid or chromosomal *ampC* (Section 3.9) were carried out on all available isolates to confirm the genotype identified by the ESBL and plasmid *ampC* disc assays. Only isolates 21', 24', 28' and 39', showed carriage of the chromosomal *ampC* gene (Figure 4) and were negative for plasmid *ampC* (Results not shown). Only isolate 24' was tested by ESBL PCR as permission to use the other positive isolates, isolates 14" and 16" (Table 14, Section 4.2) was retracted. Isolate 24' was not positive for any ESBL genes that the PCR was designed to detect, and as the controls were positive it suggests that either the ESBL gene present in isolate 24' was different than those detected by the PCR, or the result from the confirmation ESBL disc test was a false positive. Another possibility is that the phenotypic ESBL results were due to a mutated chromosomal *ampC* as its phenotypic antibiotic resistance is similar to the ESBL and plasmid *ampC* phenotype (Jacoby, 2009).

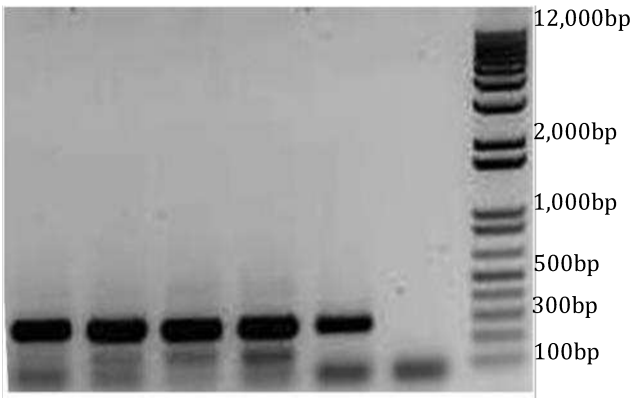


Figure 4-Agarose gel electrophoresis of the chromosomal *ampC* PCR results. From left to right are, the isolates 21', 24', 28', 39', positive control (*E. coli* ATCC® 25922), negative control (*E. cloacae* ATCC 15337) and a 1kb plus ladder (Thermo Fisher Scientific). All isolates are positive for this gene. The lower size band for isolates 24', 28', and 39' is non-specific binding.

4.4 Transferability of antibiotic resistance from the 2013 and 2014/15 isolates

The carriage of plasmids in 2013 and 2014/15 isolates was also analysed through selection of a subset of both sample groups to determine if any antibiotic resistances were plasmid borne. Nine isolates from 2013, and 8 isolates from 2014/15, were chosen as a result of their similar 'limited' resistance profiles which are also known as 'environmental resistances' (Appendix 1.1 & Table 11). These 'limited' resistances where to chloramphenicol, streptomycins, amoxicillin-clavulanic acid, sulphamonides, ampicillin and oxytetracycline, which were seen in strains from both 2013 and 2014/15 and therefore isolates with only these resistances were chosen for the conjugation experiments. These resistances were designated as 'limited' resistances because they are common among both groups of isolates and frequently isolated clinically (De Briyne *et al*., 2014, Shin *et al*., 2014, Yamamoto *et al*., 2014).

Plasmid extraction showed that MS, SS, 1', 3', 22', 30', 35', 38', ST2A, ST3A, ST4A, BF5A, BF7A all have plasmids (Figure 5, Results for 3', 35', ST4A not shown). After conducting the conjugation experiments the results showed that ST3, BF5A, BF7A, ST2A, 35' and 3' plasmids' were transmissible (Table 15), which was confirmed using plasmid extraction on the recombinant to demonstrate that the plasmid had transferred (Figure 6). As shown in Table 15, all plasmids were transferrable on both plate and broth medium except the plasmid in isolate 35', which only transferred using the plate method. It should be noted however, that there was heavier growth for all isolates using the plate conjugation method. There was evidence of a transfer of phenotypic characteristics from isolates MS, SS, 1', 22', 30', 38' and ST4, however no genotypic evidence of plasmid transfer. This suggests that too much of either

isolate was inoculated causing an overloading of cell mass on the media allowing the recipient isolate to grow with no plasmid, even in the presence of a lethal antibiotic dosage. Another possibility is that there were low-copy plasmids that were transferred however the necessary step to extract low-copy plasmids was carried out during plasmid extraction. It was also noted that the band pattern for isolate 1' differed between Figure 5 and 6 and thus was not used here further due to this inconsistency.

These isolates with transferred plasmids were tested to determine the antibiotic resistances that they may carry, which showed that on average there was a frequency of just over 6 resistances out of 10 being carried over (Table 16). This suggests that the antibiotic resistances shown by these isolates are plasmid-borne.

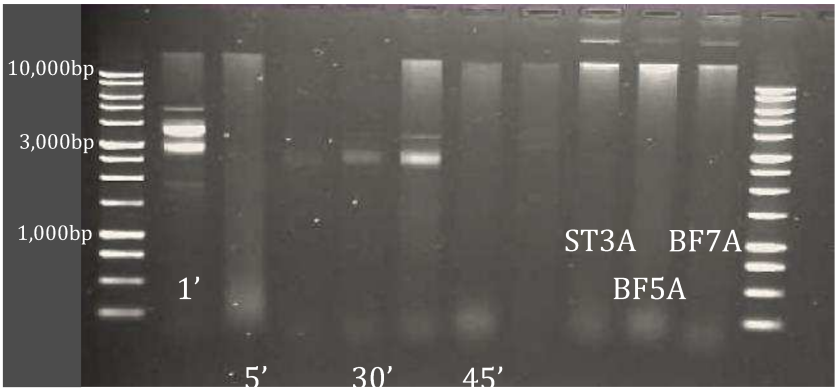


Figure 5-Plasmid preparations from some of the isolates. The two negatives are 5' and 45' whose lanes only show a genomic DNA smear.

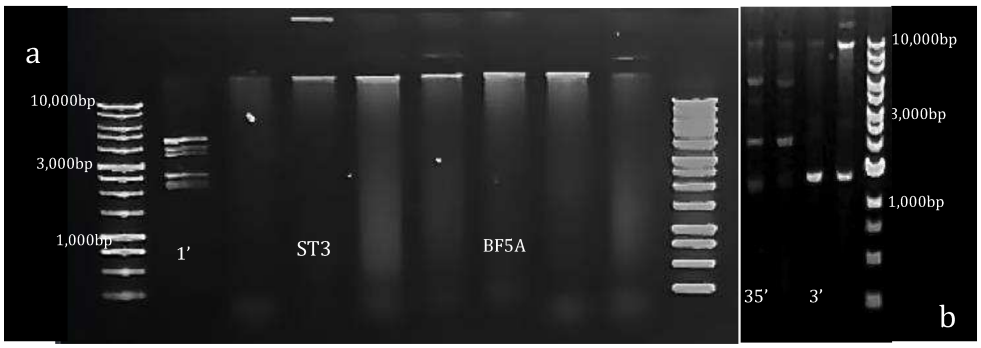


Figure 6-Agarose gel electrophoresis of originally extracted plasmid from isolates next to plasmid extracted from recipient J53 AziR conjugated with the same isolate using different media either plate(P) or broth(B) and selections (TET, AMP, or C). (a) Isolates 1', ST3, and BF5A in the 2nd, 4th and 7th column respectively. With the transconjugants for each isolate in 3rd (P + TET), 5th (P +TET) + 6th (P+AMP) and 8th (B+AMP) and 9th (B+C) columns respectively. (b) Original plasmid from isolates 35' and 3' in the 1st and 3rd columns with transconjugant in 2nd and 4th column, respectively. A 1kb ladder (Promega) was used in the (a) 1st, 10th and (b) 5th column.

Isolate	Solid Media Donor With Antibiotics			Liquid Media Donor With Antibiotics		
	C	AMP	TET	C	AMP	TET
ST2A	-	+	+	-	+	+
ST3A	-	+	+	-	+	-
BF5A	-	+	-	+	+	+
BF7A	+	-	-	+	-	+
3’	-	+	-	+	+	-
35’	+	-	+	-	-	-

Table 15-Resistances to selected antibiotics of isolates that were positive plasmid conjugations (confirmed by plasmid extraction) on the specific selective media.

Sample			Antibiotic resistance								Percentage (%) of donor isolates antibiotic resistances seen in the recipient
Isolate	**Media**	**Antibiotic**	**C**	**SXT**	**S300**	**AMC**	**AMP**	**OT**	**NA**	**S10**	
ST2A	Plate	TET	-	-	S	R	R	R	-	-	25
ST3A	Plate	TET	S	S	R	R	R	R	-	R	71
ST3A	Plate	AMP	S	S	R	R	R	S	-	R	57
BF5A	Broth	C	R	R	R	R	R	R	-	-	100
BF7A	Broth	AMP	S	S	S	R	R	R	-	-	50
3’	Plate	AMP	R	S	R	I	R	R	-	R	70
35’	Plate	TET	R	S	S	NG	R	R	NG	R	67
						Average total frequency					63.1

Table 16-Conjugates from specific selective media are shown with their antibiotic resistance profiles. The percentage of donor isolates’ antibiotic resistance that is also seen in the conjugate is shown, along with the total frequency of antibiotic resistances that have passed to the recipient from donor isolate. ‘-’ Depicts that this isolate wasn’t tested against the antibiotic as it was already sensitive to it. NG depicts no growth of the organism.

4.5 Mercury Chloride Phenotypic Resistance in 2013 and 2014/15 Isolates

Mercury chloride resistance was analysed via growth on LB media containing 50μM of $HgCl_2$ for both 2013 and 2014/15 isolates (Table 17). The possession of mercury resistance genes *merA* and *merC* as well as *intI1* was already known for the 2013 isolates (Appendix 2). Any isolates that had both of these genes and did not grow on 50μM of $HgCl_2$ was also tested on 20μM of $HgCl_2$ to determine if there was lower level mercury chloride resistance (Table 17).

The results show that one 2014/15 isolate, MS, is resistant to $HgCl_2$ at 50μM whereas 16 of the 2013 isolates are resistant at either 20μM or 50μM. Excluding those 2013 isolates resistant at 20μM and not at 50μM there is still a significant difference between the two groups, when using a chi square test at $P<0.05$ as the p value is 0.0003. However there were 4 isolates that grew on the 50μM $HgCl_2$ media which were only positive for *merC* (Appendix 2), and this resistance was probably due to overloading the plate with cell mass that absorbed the mercury and allowed the isolate to grow, or the isolates did have the gene but it had a different sequence than the one searched for using the *merAC* PCR.

2014/15 Isolates	50μM	2013 Isolates	20μM	50μM
ST1A	-	1’		+
ST2A	-	2’		-
ST3A	-	3’		-
ST4A	-	4’		+
ST5A	-	5’		+
ST5T	-	8’		+
ST6A	-	20’		+
ST6T	-	21’	+	-
ST7A	-	22’		-
ST7T	-	24’		-
ST8A	-	28’		-
ST8T	-	30’		-
ST9A	-	35’		-
ST9T	-	38’		-
ST9I	-	39’		-
BF1A	-	40’		+?
BF2A	-	45’		-
BF3A	-	2’’		+
BF4A	-	4’’		+
BF5A	-	5’’		-
BF5T	-	6’’		-
BF6A	-	7’’		+
BF6T	-	10’’		-
BF7A	-	13’’		+
BF7T	-	14’’		+
BF8T	-	16’’		-
BF9A	-	21’’		+?
BF9T	-	23’’		-
MS	+	25’’		-
SS	-	26’’		-
		27’’		-
		30’’		+?
		31’’		-
		35’’	+	-
		40’’		-
		42’’		+
		44’’		+?

2013 Isolates	20μM	50μM
46''		+
49''		-

Table 17-Mercury chloride resistance phenotype of isolates from 2013 and 2014/15 on 50μM $HgCl_2$ LB media. Positive resistance is shown by '+' and Mercury chloride sensitivity via '-'. Any 2013 isolate that was not resistant but carried the mercury resistance genes *merA* and *merC* (appendix 1.2) were tested on 20μM $HgCl_2$ LB media. Any '?' depicts isolates that did not possess *merA* and *merC* but were phenotypically resistant to 50μM $HgCl_2$.

4.6 Mercury Chloride Resistance Genes PCR for 2014/15 Isolates

PCR was used to determine presence of the mercury chloride resistance genes *merA* and *merC* and the class 1 integron integrase gene *intI1* for the 2014/15 isolates (Figure 7 &8, Table 18). This was done to confirm their phenotypic profiles shown in Table 17 and compare to the 2013 isolates whose PCR was previously carried out by Delveen Ibrahim (PhD Student) and Jack Marsland (BSc Student) shown in appendix 2.

The results show one of the 2014/15 isolates was *merA* positive, MS, whereas 13 % carried *merC* and 20% were *intI1* positive (Figure 7&8). The only isolate that was positive for *merA* and *merC,* MS, did show corresponding phenotypic resistance by growing on 50μM $HgCl_2$ LB media (Table 17). This isolate was isolated from a milk sample of a mastitic cow, not faecal matter however it is still a relevant isolate as mastitic milk is collected into the slurry tank along with faecal matter.

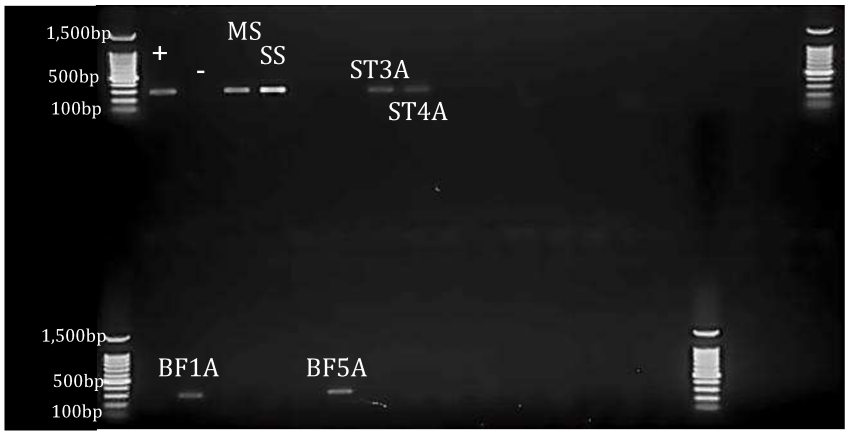


Figure 7- Agarose gel showing the results of the *intI1* PCR on the 2014/15 Isolates. Those that have a band are positive, MS, SS, ST3A ST4A, BF1A, BF5A. The ladder used was a 100bp from Promega. The positive control is indicated as a '+' and negative by '-'(Table 2).The band size is around the 300bp band, as the gene is 280bp.

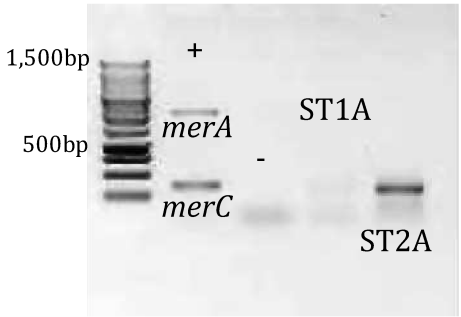


Figure 8- Agarose gel of the results from the *merA* and *merC* PCR for the 2014/15 Isolates. This portion shows the only positive results ST1A which shows a very faint band of the correct size for *merC*, just above the 300bp band size and ST2A who shows a much stronger band of the same size. The positive '+' and negative '-'control in the 2nd and 3rd columns respectively. The smears at the bottom of the gel are extra primer.

2014/15 Isolates	*merA*	*merC*	*int1*	2014/15 Isolates	*merA*	*merC*	*int1*
ST1A	-	+	-	BF1A	-	-	+
ST2A	-	+	-	BF2A	-	-	-
ST3A	-	-	+	BF3A	-	-	-
ST4A	-	-	+	BF4A	-	-	-
ST5A	-	-	-	BF5A	-	-	+
ST5T	-	-	-	BF5T	-	-	-
ST6A	-	-	-	BF6A	-	-	-
ST6T	-	-	-	BF6T	-	-	-
ST7A	-	-	-	BF7A	-	-	-
ST7T	-	-	-	BF7T	-	-	-
ST8A	-	-	-	BF8T	-	-	-
ST8T	-	-	-	BF9A	-	-	-
ST9A	-	-	-	BF9T	-	-	-
ST9T	-	-	-	MS	+	+	+
ST9I	-	-	-	SS	-	+	+

Table 18-Results from the *merA, merC* and *int1* PCR depicted as a + for positive and – for negative of the 2014/15 isolates.

4.7 ERIC Profiles of the 2014/15 isolates

ERIC PCR creates a distinctive amplicon profile for each isolate and similar isolates have the same profile, based upon amplification of genomic DNA between repetitive ERIC sequences. This can be used as an alternative method to MLST and PFGE and compares isolates within a population. The data were collected for the 2014/15 isolates to estimate the group's diversity and compare to their antibiotic resistance profiles.

The ERIC profile of all the 2014/15 isolates was determined through ERIC PCR (Figure 9). Due to time constraints cluster analysis of the ERIC PCR results was not carried out however it is evident that some of the isolates had unique ERIC profiles whereas other isolates had similar profiles. There was a clustering of isolates with similar profiles such as ST2A and BF5A, which had a similar ERIC profile and the isolates BF9T, BF9A and BF7A, which also had similar profiles. However isolates BF9A and BF9T could be the same strain as they were isolated from the same faecal sample just using a different antibiotic for selection. These profiles where different from ST1A, ST8A, ST7T, BF2A and BF3A, which all had similar profiles. The isolates ST4, MS, and SS also had comparable profiles that were distinct to ST6T and ST7As profile.

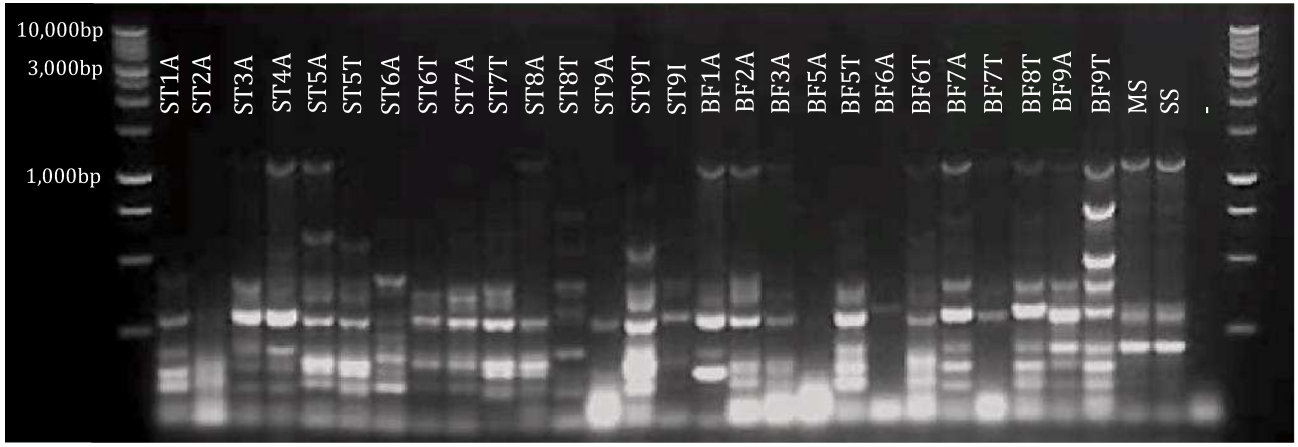


Figure 9-ERIC PCR profiles of all the 2014/15 isolates. The ladder used was a 1kb ladder from Promega (UK).

4.8 Sequencing Results of Isolates A2.5, 24' and 39'

The isolates chosen for sequencing were isolates 24' and 39', as these strains shared similar antibiotic resistance profiles that included 2nd and 3rd generation cephalosporin resistance. They also possessed the plasmid *ampC* phenotype yet were plasmid *ampC* PCR negative and isolate 24' showed phenotypic evidence of ESBL carriage, which could not be confirmed through PCR analysis. The sequence and assembly data are shown in appendix 3.

Another isolate A2.5, which was isolated from the slurry tank of the dairy farm in 2012 had previously been sequenced but limited analysis of its genome had been carried out. Due to its similarity in resistance of 3rd and 4th generation cephalosporins compared to isolates 24' and 39', A2.5 isolate was also included in the sequence analysis.

4.8.1 Mobile Antibiotic Resistance

Isolates 24' and 39' also underwent plasmid extraction and were shown to carry plasmids although the isolates did not have the same resistance profiles to those examined in Section 4.4. After plate and broth conjugation only isolate 24' successfully transferred the selected tetracycline resistance phenotype to the donor strain using the plate conjugation method. There was no available experimental data on plasmids within A2.5 and it was unavailable for testing, therefore there was no further analysis of its plasmid carrying potential. In order to locate these plasmids in the genomes of isolates 24' and 39' their reads were mapped back to the assembled contigs to provide a mean coverage. If above average then the specific node in the contig was suspected of carrying a plasmid and its sequence was used to search the NCBI database using blastN (Altschul *et*

al., 1990). PlasmidFinderDB (Carattoli *et al*., 2014) was also used to search for potential plasmids in the genome sequences of the sequenced isolates (Table 19). Overall it was determined that isolates 24' and 39' contain a plasmid that is similar to the plasmids pSH696_117 and _120 from *S. enterica* (Han *et al.*, 2012) (Table 19). However most of the antimicrobial resistance that was carried on this plasmid such as *tetACR*, *aph*(3')-I, and *sulI* are not present in this contig although the plasmid could span over more than a single contig. In isolate 39' node 78 seemed to match a plasmid, YU39_2.7 from *S. enterica* as does node 66 in isolate 24' (Calva *et al*., 2015) (Table 19). However this is a small plasmid of 2.6kb carrying 4 proteins and carries no antimicrobial resistance genes. Thus using these genomic methods the plasmid containing the tetracycline resistance in isolate 24' that transferred experimentally could not be confirmed and a more thorough examination was not undertaken due to time constraints. These two isolates did not provide evidence for the plasmid-borne antibiotic resistance shown experimentally. However they had a wider range of antibiotic resistances than those used in the conjugation experiments and this might be why there were no detected plasmids of similar characteristics to those seen in the conjugation experiment.

Contig Name	Organism Match is from	Plasmid Match Name	Match Accession no.	Query Coverage (%)	Identity (%)	E-value
24'						
Node_66	*S. enterica*	YU39_2.7	CP011435.1	100	99	0
	E. coli	pEC34A	HQ622575.1	42	95	0
		pHUSEC41-4	HE603113.1	31	94	0
		pColE1-H22	AY913943.1	31	94	0
		p9705	AB040037.1	56	94	0
Node_11	*S. enterica*	pSH163_120	JN983046.1	62	99	0
		pSH696_117	JN983047.1	64	99	0
	E. coli	pCA14	CP009231.1	42	95	0
39'						
Node_78	*S. enterica*	YU39_2.7	CP011435.1	100	99	0
	E. coli	pEC34A	HQ622575.1	42	92	0
		pColE1-H22	AY913943.1	31	92	0
		pHUSEC41-4	HE603113.1	31	92	0
	Citrobacter rodentium	pCRP3	AF311902.1	56	92	0

Table 19-Contigs in isolates 24' and 39' with high mean coverage or suspected of containing plasmids are shown above as well as potential plasmid matches when searching using the NCBI database using blastN. The matches are shown with the query coverage (%), identity (%) and e-value as well as an accession number for the matched plasmid.

4.8.2 Antibiotic Resistance Genes in Isolates A2.5, 24' and 39'

The antibiotic resistance profile of isolate A2.5 (Appendix 1), shows a similar resistance profile to that of the 2013 isolates 24' and 39' (Table 20 and Figure 4). Using a ResFinder (Zankari *et al*., 2012) the antibiotic resistance genes in A2.5 along with isolates 24' and 39' was determined (Table 20). All three isolates had very similar antibiotic resistance genes present, which would potentially result in similar antibiotic resistance profiles. One significant difference between the isolates is that A2.5 had two β-lactamase genes. It is also evident that there is a clustering occurring in all three isolates of certain resistance genes in the same nodes. *aadA1, florR, sul1,* and *dfrA1* all cluster in 24' and 39' in the nodes 39 and 24 respectively and the same cluster including *bla*OXA-1 minus *dfrA1* occurs in isolates A2.5 in node 65 (Table 20). There is also a clustering of *strAB* and *aph(3')-lc* in isolates 24' and 39' and *tet(L)* and *bla*TEM-1A in isolate A2.5 (Table 20). These suggest areas of interest for further analysis, which could be larger antibiotic resistance carrying elements.

Resistance gene	Identity (%)	Coverage (%)	39' Location	24' Location	A2.5 Location	Accession number
Predicted Aminoglycoside Resistance Genes						
strB	100	100	Node 52	Node 47	Node 119	M96392
aph(3')-lc	99.02	100	Node 52	Node 47	-	X62115
strA	100	100	Node 52	Node 47	Node 119	M96392
aadA1	100	100	Node 43	Node 39	Node 65 (ID 99.75%)	JQ414041
Predicted Phenicol Resistance genes						
floR	98.19	99.9	Node 43	Node 39	Node 65 (ID 98.27%)	AF118107
Predicted Sulphonamide Resistance Gene						
sul2	100	100	Node 116	Node 44	Node 161	GQ421466
sul2	100	100	-	Node 11	-	GQ421466
sul1	100	100	Node 43	Node 39	Node 65	CP002151

Resistance gene	Identity (%)	Coverage (%)	39' Location	24' Location	A2.5 Location	Accession number
Predicted Tetracycline Resistance Gene						
tetB	99.92	100	Node 58	Node 27	Node 87 (ID 100%)	AP000342
Tet(L)	100	100	-	-	Node 90	M29725
Predicted Trimethoprim Resistance Gene						
dfrA1	100	100	Node 43	Node 39	-	JQ690541
Predicted Beta-lactam Resistance Gene						
blaTEM-1A	100	100	-	-	Node 90	HM749966
blaOXA-1	99.88	100	-	-	Node 65	J02967

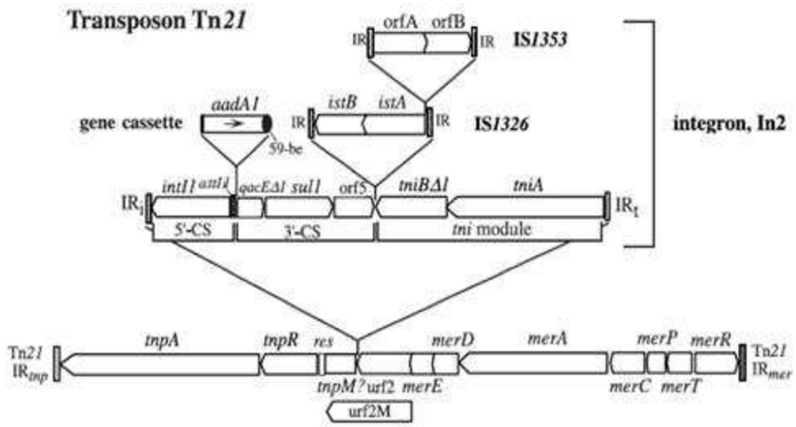
Table 20- Results from the ResFinder database (Zankari *et al*., 2012) for isolates 24' 39 and A2.5, including the antibiotic resistance gene present, its identity and query coverage the location in each isolate and an accession number. A node is a group of reads within a contig. Those with '-' do not carry the gene.

4.8.3 Tn*21*-like Element

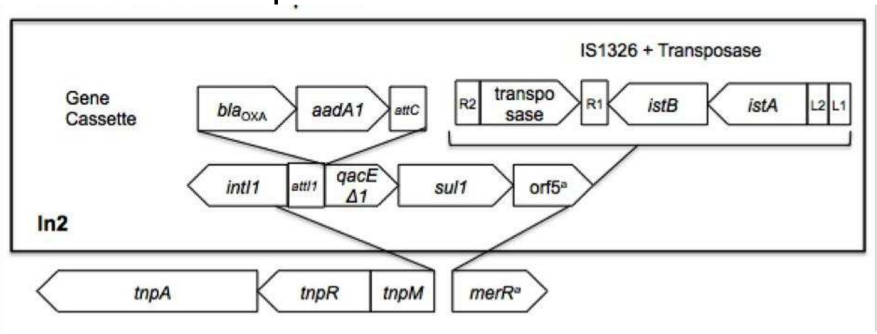
Although no antibiotic resistance plasmids could be located in the total DNA sequence of isolates 24' and 39', antibiotic resistance carried on mobile-genetic elements such as transposons was discovered. A Tn*21* resolvase in isolates 24' and 39' was annotated by RAST (Aziz *et al*., 2008) within Nodes 39 and 43 respectively. The Tn*21* reference sequence (AF071413) was used to search the region using BlastN (Altschul *et al*., 1990). This determined that the region in both isolates contained 54% of this reference sequence with 99% identity and e-value of 0. Specific Tn*21* transposon elements located in the region included the terminal imperfect inverted repeats of Tn*21* (IR_{tnp} and IR_{mer}), the inverted repeats (IR_i and IR_t) of the integron In*2* as well as the 5bp duplication as a result of the integron insertion. The inverted repeats (IR) of IS*1326* and IS*1353* were also searched for. The results determined that this was a Tn*21*-like transposon as all the necessary elements were present except the IR of IS*1353* and the IR_t of In*2* (Figure 10).

The genes of Tn*21* were also searched for in the area using BlastP (Altschul *et al*., 1990). The region between *orf*5 and *tniBΔ1* should include two insertion sequences, IS*1326* carrying *istB* and *istA* and IS*1353* carrying *orf*AB (Liebert *et al*., 1999). In both isolates the *istBA* genes are present and using ISFinder (Siguier *et al*., 2006) there was a 100% identity match to the full IS*1326* element (U38187). The flanking inverted repeats can also be seen, however R2 is further away than expected (Figure 10b) and there is another transposase annotated by RAST in between the R1 and R2 direct repeats. This suspected transposase contains a region of partial match for *orf*AB. The current profile of this region is shown in Figure 18b. The same region was also found in isolate

A2.5, which had 99% identity over 88% of the area to the Tn*21*-like transposon in isolates 24' and 39', with an e-value of 0 (Figure 10c), there is also an 85% query cover with 99% Identity match to the same area with a MDR region of a chromosome in *S. flexneri* YSH6000 (Rajakumar *et al*., 1997). This chromosomal region also carries the $bla_{\text{OXA-1}}$ gene cassette as seen in A2.5 however there is no match to the insertion sequence elements around this gene cassette that is seen in *S. flexneri* YSH6000 (Rajakumar *et al*., 1997). Although *S. flexneri* YSH6000 is also mercury chloride sensitive similar to isolate A2.5 there is still more of Tn*21* in this isolate than the MRD region seen in *S. flexneri* YSH6000. Both these matches also carry the genes *aadA1* and *sul1* which were in all three isolates and codes for resistance to aminoglycosides and sulphonamides respectively (Swedberg, 1987, Liebert *et al*., 1999). It also carries *qacE*Δ*1I* which encodes partial resistance to quaternary ammonium compounds (Paulsen *et al*., 1993). These isolates therefore show evidence of antibiotic resistance genes being carried on mobile elements such as integrons and continued presence of related transposons.



A2.5 Tn-*21* like Transposon



c

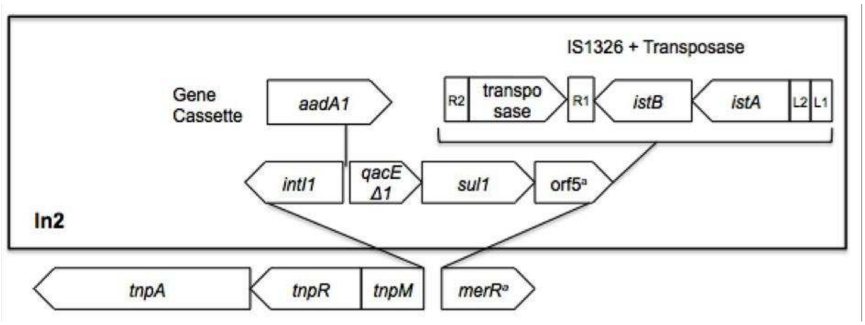


Figure 10-(a) Genetic organization of a Tn*21* transposon. (b) Diagram of the Tn*21*-like transposon in the isolates 24', 39', and (c) A2.5, with the embedded integron, In*2*, shown within the box. (b) The gene cassette does not have the *attI1* or *attC* site, and (b)(c) R1, 2, L1, and L2 represent the direct repeats of the IS*1356* element (Brown *et al*., 1996) inserted after *orf5*.

[a] represents an incomplete gene. This figure is modified from Liebert *et al*. (1999)

4.8.4 β-Lactamase genes

In the A2.5 sequence, two β-lactamase genes were annotated by RAST (Aziz *et al*., 2008) and ResFinder (Zankari *et al*., 2012). One of these β-lactamase genes, *bla*$_{\text{TEM-1A}}$, was confirmed by The Lactamase Engineering Database (Thai *et al*., 2009). The expression strength of this gene was predicted based on the promoter present. This isolate carried the promoter type P3 upstream of the gene; this is the weakest promoter for this gene, suggesting the gene is not hyper-expressed (Frédérique Lartigue *et al*., 2002). It is important to note that this gene is located near a Tn*10* transposon which carries tetracycline resistance genes (Chalmers *et al*., 2000). This could be a potential carrier of the β-lactamase gene. 100% of the Tn*10* sequence from *Shigella flexneri* (AF162223) matches the A2.5 sequence with 99% identity. This region does not seem to cover the *bla*$_{\text{TEM1A}}$ gene as it is a wild-type Tn*10* and only covers the tetracycline operon *tetRACD* and the Tn*10 Jem* genes (Chalmers *et al*., 2000). This transposon is usually flanked by the elements IS*10*L and IS*10*R, which are non-identical regions that encode a transposon however IS*10*L transposon differs to *IS10*R by 18 nucleotides rendering the transposon defective (Siguier *et al*., 2006). These are both missing from the end of Tn*10* in A2.5. There is an IS*10*R, with one coding region mutation and a highly mutated right and left end sequence, predicted to be 10,000bp away from the suspected Tn*10* region. However without the flanking regions present in the *bla*$_{\text{TEM1A}}$ gene area there is no evidence that the *bla*$_{\text{TEM1A}}$ gene was not carried by this Tn*10* transposon.

The second β-lactamase gene, *bla*$_{\text{OXA-1a}}$, was confirmed using the NCBI database using blastP (Altschul *et al*., 1990) which showed an identical match between the A2.5 OXA β-lactamase protein and a known OXA-1 protein (YP_006953880.1) from an *E. coli* strain. The gene was located on a Tn*21*

transposon region of *In2* in the *aadA1* gene cassette. The evidence that this gene is carried in the cassette is shown by the complete *attI*1 site before the β-lactamase gene (Partridge *et al*., 2000). The *attI1* site has the motif 'GTTRRRY' however the actual sequence in A2.5 is 'GTTGGGC' which has also been previously linked to *bla*$_{OXA-1}$ genes in these gene cassettes (Hansson *et al*., 1997, Hanau-Berçot *et al*., 2002). The 59-base element, *attC*, which is the IntI1 specific recombination site is also present downstream of the *aadA1* gene, suggesting a complete gene cassette (Partridge *et al*., 2000). The *aadA1* gene cassette promoter sequence was examined in order to determine the relative gene expression of *bla*$_{OXA-1}$. There are two promoters, one that lies within the integrase gene Pc, and another downstream of the gene P2. There are different variants of these promoters that confer various promoter strengths (Jové *et al*., 2010). A2.5 carries PcW, the weaker promoter and strong promoter P2m3 as there is a G→A mutation in the -10 signal of this promoter (Vinué *et al*., 2011). This region in the isolates 24' and 39' was also analysed and the *attI1* and the *attC* sites were found to be missing in the *aadA1* gene cassette, but the *aadA1* gene is still fully present. These sites are not located in any other sites in either of the genomes. The *aadA1* gene cassette promoter Pc and P2 are both present in 24' and 39', with the variants being determined as containing the same PcW and P2 promoters (Jové *et al*., 2010), however, the -35 and -10 signal sites of P2 only have a spacer of 14bp and thus it is not active (Lindstedt *et al*., 2003). There are no similarities of this area to the *bla*$_{OXA-1}$ gene however the loss of the *attI1* and *attC* site suggests that another gene could have been present and subsequently lost. If this gene was *bla*$_{OXA-1}$ it would demonstrate a genomic factor in antibiotic resistance reduction however this cannot be confirmed.

4.8.5 *ampC*

The presence of chromosomal *ampC* in the sequenced isolates 24' and 39' was shown though phenotypic and molecular characterisation experiments (Table 14). The resulting gene was also annotated by RAST (Aziz *et al*., 2008) in both genomes as a β-lactamase. Since the nucleotides in the coding region of both isolates were identical they were characterised together. The amino acid sequence from the suspected *ampC* genes of both isolates was compared with other proteins in the NCBI database via blastP (Altschul *et al*., 1990). This resulted in a group of matches with 99% query cover and 99% identity to the *ampC* β-lactamase genes from a group of *E. coli* clinical isolates described by Mammeri *et al*. (2006). They matched *ampC* genes from isolates EC9 (DQ092428), EC14 (DQ092433), EC17 (DQ091197) and EC16 (DQ092434), with an e-value of 0 (Mammeri *et al*., 2006). The *ampC* gene from isolates 24' and 39' were also compared to the reference strains *E. coli* KL, which produced extended-spectrum AmpC β-lactamase (ESAC) and *E. coli* K-12 MG1655 which has a wild-type *ampC* gene (Mammeri *et al*., 2004, Mammeri *et al*., 2006). Both the promoter/attenuator and protein-coding region of the gene from isolates 24' and 39' were found to have 28 nucleotide mismatches in the attenuator region when compared with *ampC* from *E. coli* K-12 MG1655. Seventeen of these mismatches were synonymous and 11 were non-synonymous, potentially altering the overall AmpC protein structure in 24' and 39'. *E. coli* KL *ampC* had a more similar coding-region with only 9 nucleotide mismatches, which resulted in 3 non-synonymous amino acid changes. However the *ampC* genes from the isolates EC9, EC14, EC17, and EC16, had only 1 nucleotide mismatch from 24' and 39' resulting in a different non-synonymous amino acid change in each; this

signifies a much closer match. From the amino acid sequence it can be seen that both 24' and 39' have a Proline at position 296 (Figure 11), which matches EC17. This specific amino acid has been linked to greater β-lactamase activity and greater resistance levels (Mammeri *et al*., 2006).

This gene was also annotated by RAST (Aziz *et al*., 2008) in A2.5. The A2.5 *ampC* protein-coding region (Figure 11) had 99% similarity over 99% of the query to isolates from the Mammeri *et al*. (2006) sample group. These isolates were *E. coli* EC10 (DQ092429), EC14 (DQ092433) and EC12 (DQ092431) however A2.5 matched closest with *E. coli* KL (Mammeri *et al*., 2006). There were 9 nucleotide differences in *ampC* between A2.5 and *E. coli* KL, which resulted in 5 different amino acids. When matched to *E. coli* EC12 there were 10 nucleotide mismatches, however only 4 non-synonymous amino acid changes. In comparison to EC14 and EC10 both had 14 nucleotide mismatches and 5 non-synonymous amino acids in each. One important similarity between the majority of these isolates and A2.5 was a Proline at position 296, previously stated as linked to AmpC extended hydrolysis activity (Mammeri *et al*., 2006).

Other areas known to affect production levels of AmpC are the promoter and attenuator regions (Olsson *et al*., 1982, Olsson *et al*., 1983, Fernández-Cuenca *et al*., 2005). Both areas from 24' and 39' were compared to the strain *E. coli* K12 MG1655, which has the wild-type promoter and attenuator. Both isolates had identical sequences for the promoter/attenuator region, which contained multiple mutations from wild-type promoter region (Figure 12). There are 6 amino acid variations at positions -88, -82, -42, -18, -1, and 58, which have been previously described by Olsson *et al*. (1983). The same mutations, with the exception of -42, were seen in the promoter/attenuator region of *ampC* in isolate A2.5. The -35 and -10 promoter region in all isolates remains intact, as does the

attenuator region shown in Figure 12. The mutations at -42 (C to T) and at -18 (G to A) are 7bp and 5bp upstream of the -35 and -10 promoter region respectively (Figure 12)(Olsson *et al*., 1982). The mutations downstream of these promoter regions are also near other important regions; the Thymine mutation (from C) at -1 occurs 1bp before the transcriptional start for the *E. coli* K12 promoter (Jaurin and Grundström, 1981). Although the hairpin attenuator is intact there is a downstream mutation at +58, 2bp upstream of the initiation codon for translation (Olsson *et al*., 1982). There are 3 deleted nucleotides, when compared to *E. coli* K12, two deleted adenine upstream of base +70 and one deleted thymine 8bp downstream of base +70. When compared to *E. coli* K12 an insertion of an adenine can be seen at base +110, however all of the deletions and the insertion occur after the hairpin attenuator, suggesting it might not have any effect on expression levels. Although these mutations occur after the start codon for translation (+60) there doesn't seem to be any effect shown at the amino acid level (Figure 11).

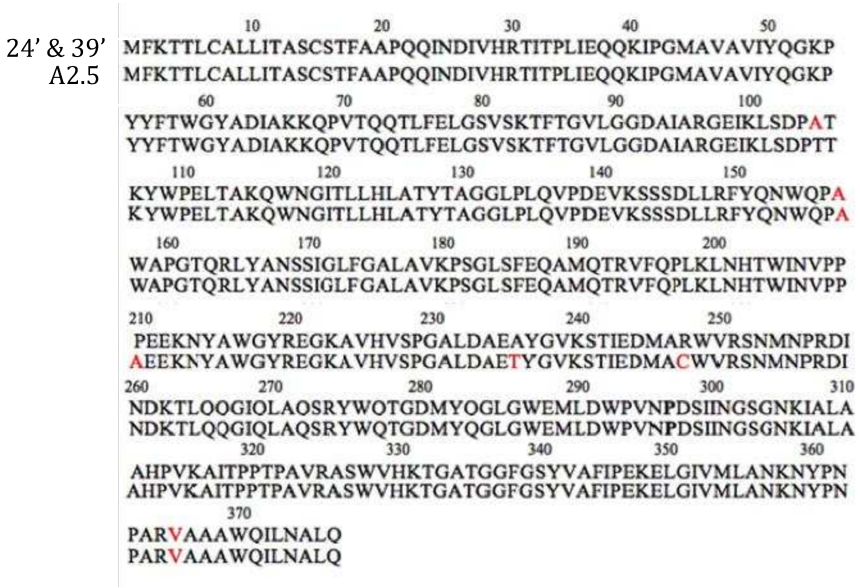


Figure 11- Amino acid sequence of the protein-coding region of the *ampC* gene in isolates 24' and 39' in the top row and A2.5 below. The letters in red show any different amino acids when compared with *E. coli* KL and the letter in bold at position 296 shows the amino acid that affects the hydroylsis range of the protein.



Figure 12- Nucleotides of the promoter/attenuator region of *ampC* from isolates 24', 39' and A2.5, which were compared to *E. coli* K12. Any point mutations are shown in red with their position away from the transcription start point (no.1) shown by the number above. The underlined bases convey the -35 and -10 hexamer promoter sequence. The underlined italic bases depict the hairpin attenuator region. The '-' is a deleted base pair and the base at position 110 is an insertion. The 'ATG' string of bases at position 60 is the initiation codon for translation.

5.0 Discussion

5.1 Antibiotic Resistance Decreases from 2013 to 2014/15

Investigation of the antibiotic resistance profiles of the 2014/15 isolates showed antibiotic resistance to oxytetracycline, sulphonamides, ampicillin, chloramphenicol, streptomycins, amoxicillin-clavulanic acid, and trimethoprim/sulfamethoxazole. High resistance rates to these antibiotics has previously been demonstrated in cattle (Guerra *et al*., 2003, Tadesse *et al*., 2012). The isolates from the 2013 group also showed these resistances but at a higher frequency (Appendix 1). This suggests that resistance to these antibiotics represents a common resistance profile of the *E. coli* population on the farm that already carry ampicillin and tetracycline resistance, as these were the selective markers used for isolation. The majority of these resistances are to antibiotics that are present on the farm, apart from chlorampenicol the use of which was restricted in 1971 and has since been completely phased out (Young *et al*., 1999). They also have a wider resistance profile compared to the 2014/15 isolates (Figure 13). This could suggest that these 'limited' resistances were co-carried or co-selected with other resistance genes and when lost from 2013 isolates to 2014/15 isolates the 'limited' resistance genes were also lost. If there was no link then the frequency of 'limited' resistances would be conserved from 2013 to 2014/15 isolates as they would be selected for together.

The 2013 group showed resistance to 16 of the 17 antibiotics, with only intermediate resistance seen for Imipenem (Appendix 1). One isolate showed

resistance to 10 different antibiotics and intermediate resistance to 2 other antibiotics. Comparing the number of resistant 2014/15 isolates with the 2013 isolates using a chi-squared test shows that, at a significance level of $P<0.05$, there is a significant difference between the frequency of resistances among the two groups. Since they were collected from the same environment (barn floor or slurry tank) and using the same isolation technique, it suggests that the resistance profile of this population of *E. coli* has altered over the year. The significant antibiotic related change on the farm was the reduction in the use of 1st and 4th generation cephalosporins on the cattle after 2011. It is probable that the reduction in frequency of antibiotic resistance among these *E. coli* isolates is a result of this antibiotic regimen change, an effect that has previously been shown (Dutil *et al*., 2010). The reduction of the use of these antibiotics was staggered from 2011 to mid-2013 and since then almost never used, this could explain why the resistance profiles differed between the 2013 isolates and the 2014/15 isolates. The reduction in 4th generation cephalosporin resistance within a year of the antibiotics withdrawal has been seen previously, the difference in this project results being that the genes encoding resistance were also no longer present (Dutil *et al*., 2010).

However it cannot be concluded that the reduction of cephalosporin use was the causal factor in the decrease of antibiotic resistance seen from 2013 to 2014/15. There is only evidence of a reduction in antibiotic resistance and to link it to the reduction of cephalosporin use that occurred on the farm in 2011, isolates would have needed to be sampled between 2010-2012 and their antibiotic resistance profile characterised. Another possibility is to reinstate the common practice of using 3rd and 4th cephalosporins on this farm and determine if there is a corresponding increase in antibiotic resistance.

A reduction was also seen in the number of ESBL and plasmid *ampC* positive isolates between the 2013 and 2014/15 isolates. The 2013 group had 3 ESBL positive isolates and 7 isolates positive for plasmid *ampC* whereas there was a complete absence of ESBL and plasmid *ampC* in the 2014/15 samples. However the molecular results do not show the same evidence; there were no ESBL genes found via PCR in isolate 24' which showed phenotypic carriage of ESBL. This could be due to the limited range of the primers choosen for the PCR, however genomic sequencing also displayed no evidence of an ESBL which is colllaborated by the fact that there were no unmapped reads. The majority of strains that phenotypically showed plasmid *ampC* type resistance were negative for a plasmid *ampC* gene when searched for by using PCR. This could again be due to the PCR primers not including all the possible plasmid *ampC* genes however since they were all chromosomal *ampC* positive it is also possible that a mutated chromosomal *ampC* could be responsible for the plasmid *ampC* phenotype. Thus a reduction in ESBL and plasmid *ampC* genes between the strains isolated from 2013 to 2014/15 could not be verified as there is no genetic evidence that these isolates actually carried these genes other than phenotypic evidence which could not be confirmed genotypically. It does however suggest that characterisation of the chromosomal *ampC* region in the plasmid *ampC* phenotypic positive 2013 and 2014/15 isolates is necessary in order to determine if this gene is linked to the phenotypes displayed. It would also be relevant to characterise other isolates from both groups to see if there is any change over the years such as degradation that could be linked to the decrease in antibiotic resistance previously shown. The region in three sequenced isolates was characterised further on in the project (see *ampC* section) however without more sequences the link to antibiotic reduction cannot be throughly established.

The 'low-level' antibiotic resistance was demonstrated to be plasmid borne through the positive transfer of antibiotic resistance in plasmid conjugation experiments (Table 16). The plasmid profile of the isolates showed that both groups carried plasmids at a similar frequency however the 2014/15 groups' plasmids transferred more frequently. It also showed on average 6 out of 10 resistances carried by the bacterial strain would be transferred, suggesting the majority of resistance from both isolate groups are plasmid borne (Table 16). However, this needs to be confirmed with a larger sample size to allow for a significant result. These resistances were to antibiotics that have been heavily used in agriculture, and to antibiotics bacteria have been resistant to for a long time, such as tetracycline and chloramphenicol. However isolates with a wider range of resistance were not tested in order to determine if other resistances were plasmid-borne, if this were carried out it could provide further insight into whether these other resistances were plasmid-borne or not.

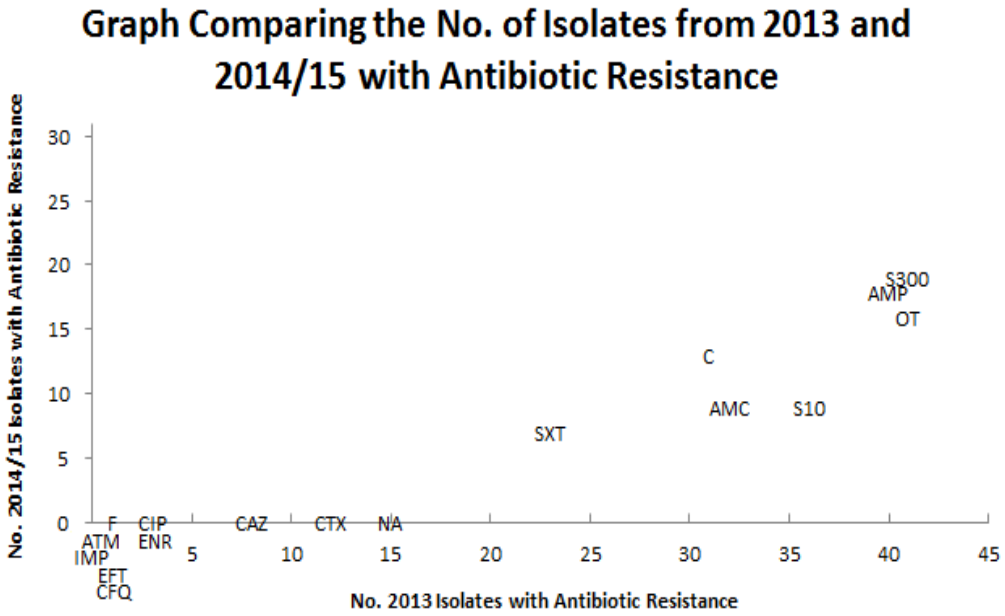


Figure 13-Difference in antibiotic resistance between the 2013 isolate group and the 2014/15 Isolate group. Each point represents an antibiotic the location is determined by the number of 2013 (x axis) and 2014/15 (y axis) isolates resistant to it.

5.2 Mercury Chloride resistance and gene carriage decreases from 2013 to 2014/15 isolates

The phenotypic mercury chloride resistance of the 2013 and 2014/15 isolates did show a significant difference, which was a significant decrease in mercury chloride resistance from 2013 to 2014/15. The same decrease was seen in the carriage of the mercury resistance genes *merA* and *merC* as well as the carriage of the gene *int1I* (Figure 14). The *merAC* and *int1I* PCR results for the 2013 group (Appendix 2 & Figure 14) showed that over half the isolates carried *merA* or *merC* and 86% of the isolates carried *int1*. In contrast 13% of the 2014/15 isolates carried *merC*, 20% carried *int1* and 1 carried *merA*. A chi-square test determined that the difference in number of isolates carrying *merA* between the 2013 and 2014/15 isolates was significant at $p<0.05$ with a P value of 0.002. The difference in carriage of *merC* and *int1I* between the two groups was also determined to be significant with P-values of 3.4E-05 and 0.0108, respectively.

Any isolates with the mercury resistance phenotype, at either 20μM or 50μM of $HgCl_2$, and also carried *merC*, or carried both *merA/C* showed common antibiotic resistance profiles. Of the 21 mercury resistant isolates, resistance was seen against amoxicillin and clavulanic acid (14 isolates), oxytetracycline (21), streptomycin (20), sulphonamides (20), ampicillin (19) and chloramphenicol (19). Isolates resistant to $HgCl_2$ have been known to share a similar resistance

profile to these isolates conferring resistance to ampicillin, tetracycline and chloramphenicol due to the presence of Tn*21* carrying the R100 plasmid (Summers *et al*., 1993, Liebert *et al*., 1999), or Tn*21*-like elements and other similar transposons such as Tn*2603* (Tanaka *et al*., 1982). The majority of these 21 isolates do not carry the class 1 integron integrase *int1* gene. This suggests that the resistance profile might not be due to an R100-like plasmid or Tn*21* but perhaps due to chromosomally acquired resistance or a different genetic element without a class 1 integron such as Tn*501* (Liebert *et al*., 1999). The 6 isolates that are positive for *int1, merA* and *merC* (1', 8', 22', 13'', 35'', MS) and their corresponding resistance profiles point towards the presence of a R100-like plasmid or sole carriage of a Tn*21*-like element (Liebert *et al*., 1999). Isolate 1' is positive for all three genes, is resistant to $HgCl_2$ and sulphonamide but not resistant to streptomycin, this suggests that it could still carry a R100-like plasmid or Tn*21*-like transposon and simply have deleted the *aadA1* gene. Isolate MS is not resistant to sulphonamide however it could still carry Tn*21* or R100 but have lost *sul1* from 3'-CS region of In*2*. The presence of other genes characteristically on R100-like plasmid and Tn*21*-like elements, including *aadA1* and *sul1*, will need to be amplified by PCR in order to determine conclusively the presence of this plasmid or transposon. This can then be used to further characterise the resistance mechanisms of these strains.

The link between the absence of mercury genes and integrase with the loss of antibiotic resistance is intriguing and it could be postulated that the two are linked. The presence of *merC* and *int1* remains in some of the 2014/15 isolates, which coincides with some phenotypic antibiotic resistance, which could again suggest a link between the carriage of these genes and antibiotic resistance. This had been suggested before by Hobman and Crossman (2015)

however there is no real evidence for this other than the co-decrease of the two between these groups.

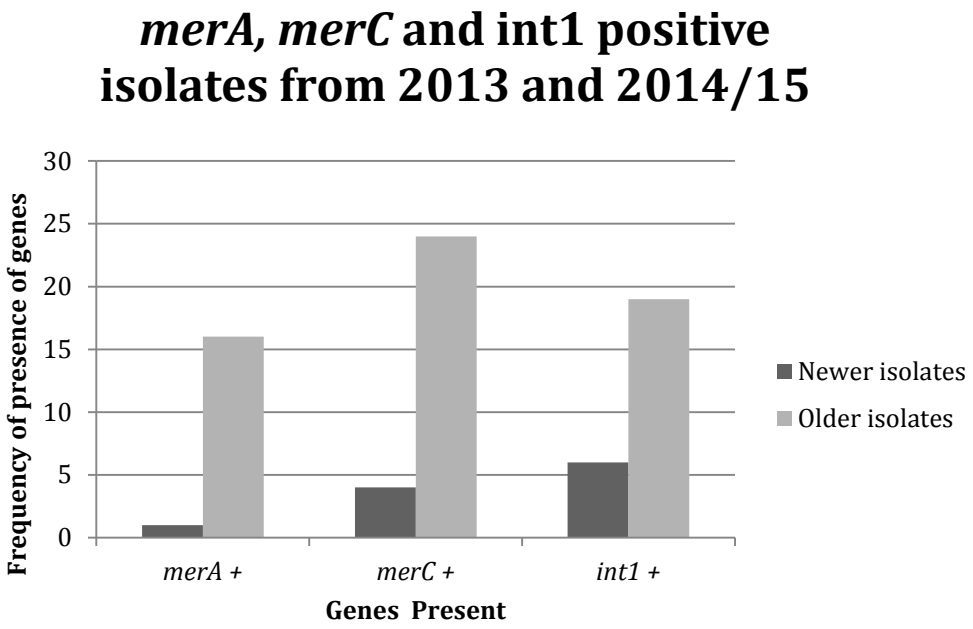


Figure 14-Difference in frequency of positive *merA, merC*, and *int1* genes between the 2014/15 and 2013 isolates. Delveen Ibrahim (PhD Student) and Jack Marsland (BSc student) previously collected the data for the 2013 Isolates and kindly provided them.

5.3 ERIC and Resistance Profiles Do not Correspond

The ERIC profiles of all the 2014/15 isolates were determined and those with similar profiles were analysed to determine whether they shared other similar characteristics, particularly antibiotic resistances. However due to time constraints only visual analysis of the ERIC could be carried out instead of a more accurate cluster analysis. ST2A and BF5A had a similar ERIC profile and also carry resistances to some of the same antibiotics (Table 11). BF9T, BF9A and BF7A again shared similar ERIC profiles. However, BF7A has a very different antibiotic resistance profile (Table 11) to the other two isolates, which were most likely the same strain and sensitive to all of the tested antibiotics. ST1A, ST8A, ST7T, BF2A and BF3A also had comparable ERIC profiles and they all carry resistances to the same three antibiotics apart from ST7T, which was fully sensitive. ST4, MS, and SS again shared similar profiles, which is also evident in their antibiotic resistance profiles (Table 11). ST6T and ST7A had the same ERIC profile yet different antibiotic resistance profiles. Since the ERIC profile is used to differentiate genetic diversity to distinguish lineage, the differences in resistance profiles of these isolates do not correspond to their ERIC profile suggesting that horizontal gene transfer of the antibiotic resistance genes has occurred. This could be also inferred using computation or phylogenetic analysis

of the antibiotic resistance gene to determine if they show signs of horizontal gene transfer such as different GC content. If the data for the 2013 isolates was available this could provide more evidence of a lack for similarity between ERIC and antibiotic resistance profiles and the genomes of isolates 24' and 39' could be analysed for the signs of horizontal gene transfer. The ERIC profiles did however show a diverse range of *E. coli* isolates on the farm and in this project.

5.4 Sequencing

5.4.1 General Resistances

The resistance genes that were carried by isolates 24' and 39' were very similar. The resistance genes present conferred antibiotic resistance to aminoglycosides, phenicols and tetracyclines. The genes *strB* and *strA* present in isolates 24' and 39' were also found in A2.5. It is very common in *E. coli* and confers strong resistance to aminoglycosides (Chiou and Jones, 1995, Enne *et al*., 2008). In isolates 39' and 24' there are two copies of *strA*, one of which is next to an aminoglycoside resistance gene *aph(3')-lc*, and a transposase resolvase (*tnpR*). The transposase matches Tn*5393*e and Tn*5393*c of *S. enterica* and its presence suggests the transposon is also present. However the rest of Tn*5393*e/c is not present except *strA* and the Tn*5393* terminal inverted repeats which do not encompass *tnpR* (L'Abée-Lund and Sørum, 2000, Cain and Hall, 2011). This suggests that a transposon similar to the Tn*5393*e/c was present and has undergone significant alterations including duplication of *strA* (Chiou and Jones, 1995, Enne *et al*., 2008). The second copy of *strA* is after the 3' end Tn*5393*-like terminal repeat and next to *strB*. A2.5 differs as it only has one copy of *strAB* and only the 3' terminal repeat region of the transposon Tn*5393*, again

suggesting that this transposon could have been present at one time. In order to determine a causal relationship between the aminoglycoside resistance and the expression of these genes, this expression analysis would be via transcriptional or protein experiments. This analysis would also need to be carried out for the aminoglycoside resistance gene *aadA1*which is also carried by all three isolates in the Tn*21*-like region of their genomes. These data also show that there is not a massive difference in the other resistance genes carried by these three strains yet there is a significant difference in the antibiotic resistance profile generated by A2.5 compared to isolates 24' and 39'. It suggests that the difference is mainly due to 2 reasons: the OXA β-lactamase gene (discussed in section 5.4.3) and the stronger promoter related to the chromosomal *ampC* (discussed in section 5.4.4). It is worrying that a significant increase in antibiotic resistance profile as seen from isolates 24' and 39' to isolate A2.5 is potentially generated by two changes.

5.4.2 Tn*21*-like element

A Tn*21*-like element which should carry the mercury resistance operon was located in isolates 24', 39' and A2.5. The elements were similar to each other in all three strains and to Tn*21* however they lack IS*1353* and *merTPCADE*. The IS*1326* element is intact including both terminal inverted repeats (IR) suggesting that IS*1353* was not lost but had never integrated. This can be assumed as if it had integrated and been lost it is likely that some of the 3' end of the IS*1326* would be affected. It is therefore more similar to the Tn*21* ancestor transposon Tn*2411* which IS*1353* never integrated into (Kratz *et al*., 1983, Brown *et al*., 1996). On the other hand the carriage of the β-lactamase gene *bla*$_{\text{OXA-1a}}$ gene in

the *aadA1* gene cassette of A2.5 has been found in other Tn*21*-like transposons such as Tn*2603*; yet this transposon does carry IS*1353* and is more similar to Tn*2411* in this sense (Tanaka *et al*., 1985, Ouellette *et al*., 1987, Liebert *et al*., 1999). The *bla*$_{OXA-1a}$ gene cassette next to the *aadA1* gene has also been discovered in the chromosome of *Shigella flexneri* YSH6000, which also has similar mercury chloride sensitivity and antibiotic resistance (Rajakumar *et al*., 1997). However there was no match to the Insertion Sequence elements surrounding the *bla*$_{OXA-1a}$ gene cassette from *S. flexneri* YSH6000 in isolate A2.5 and overall there was a greater match to Tn*21*-like elements (Rajakumar *et al*., 1997). It cannot be ruled out however that this chromosomal region in *S. flexneri* YSH6000 could be related to, or an ancestor of the element in A2.5. Until this is determined, the element in all three genomes is most likely Tn*2411*-like as they are not identical to Tn*2411.* They have a deletion just after the IR of IS*1326*, resulting in a loss of the *tni* module in In*2*, the IR$_t$ of In*2* and the majority of the *mer* genes, making the transposon Tn*2411*-like. The loss of these genes is reflected in the inability of the strains to grow at 50μM $HgCl_2$. Mercury is no longer used, as an antimicrobial, on farms and thus the presence of mercury resistance is not beneficial other than its link to antimicrobial resistance. These isolates do provide some evidence that the mercury chloride resistance has decreased with cephalosporin reduction, however isolate A2.5 shows significant antibiotic resistance without the *mer* operon showing perhaps an evolution of the mercury carrying transposon resulting in the carriage of only the beneficial resistance genes and not the mercury operon. The absence of the IS*1353* element and the loss of the same genes with the retention of a partial *merR* gene are common between the isolates, suggesting that the further loss of the *bla*$_{OXA-1}$ gene and cassette sites in 24' and 39' was the progression of this Tn*2411*-like

transposon seen in A2.5. To provide more information on the events, mercury sensitive isolates from 2011, 2012 and before cephalosporin reduction occurred need to be searched for a Tn*2411*-like transposon. This would determine if this novel Tn*2411*-like structure occurred due to the reduction of cephalosporin use or due to the unnecessary carriage of the *mer* operon on an otherwise beneficial resistance transposon.

5.4.3 β-lactamases

The β-lactamase genes found carried by isolate A2.5 were also located on or next to a transposon. *bla*$_{\text{OXA-1a}}$ was found to be within the *aadA1* gene cassette carried by a Tn*21*-like region. It was also found with a strong promoter P2m3, suggesting it is strongly up-regulated (Vinué *et al*., 2011). Although it did not have any mutations to increase its range of antibiotic hydrolysis the strong promoter does suggest significant hydrolysis within its normal antibiotic range of penicillins and oxacillin antibiotics. The second β-lactamase gene located in A2.5 is *bla*$_{\text{TEM-1a}}$ and located next to the tetracycline resistance carrying transposon Tn*10*. This Tn*10* transposon lacks the presence of the characteristic terminal inverted repeats, IS*10*L and IS*10*R, in the correct region; it cannot be confirmed that this transposon did not carry *bla*$_{\text{TEM-1a}}$. These transposon-borne antibiotic resistances are found in isolate A2.5 but not 24' and 39'. Thus they are a potential region of antibiotic resistance loss from A2.5 to isolates 24' and 39'

which were isolated a year after. However there is no way to determine if isolates 24' and 39' ever carried such genes and thus no way to confirm that there was a loss of antibiotic resistance between the years. Also although the *bla*$_{OXA-1a}$ was found with a stronger promoter and potentially had a wider range of antibiotic resistance it is important to note that neither of these genes are ESBL and *bla*$_{TEM-1a}$ shows no signs of up-regulation. Thus the loss of *bla*$_{TEM-1a}$ at least might not have a major impact on an isolate's antibiotic resistance profile unless it was linked to other genes that could be lost with it. The loss of *bla*$_{OXA-1a}$ might have a more significant effect as with a stronger promoter it probably contributes considerably to resistance, however it is important to note that without these two genes the isolates 24' and 39' can still be classified as MDR (Magiorakos *et al*., 2012).

5.4.4 *ampC*

The *ampC* gene in isolates 24', 39' and A2.5 show mutations in the promoter/attenuator region at positions -88, -81, -42, -18, -1 and +58 except for A2.5 which lacks the -42 mutation. The latter 4 have been shown to cause an increase in *ampC* gene expression by up to 40-fold through their effects in this region (Bergström and Normark, 1979). The roles of the mutations seen at -1 and +58 have been speculated about. It was thought that together these mutations could lead to an RNA polymerase pause or hesitation causing attenuator read-through causing constitutive AmpC expression (Olsson *et al*., 1982, Olsson *et al*., 1983, Caroff *et al*., 1999, Nelson and Gay Elisha, 1999, Peter-Getzlaff *et al*., 2011). It was previously thought that together the -42 and -18

mutations create a new stronger *ampC* promoter that overlaps the wild-type. However, it is now known that this novel promoter can be generated with only the mutation at -42 (Olsson *et al*., 1982, Olsson *et al*., 1983). The -42 and the -18 mutations generate novel hexamer sequences that are homologous to the consensus sequences of -35 and -10, respectively (Olsson *et al*., 1982, Hawley and McClure, 1983). It was thought that a partial cause of the relative weakness of the wild-type promoter was due to the unusual short distance between the -35 and -10 (Jaurin *et al*., 1981) and thus a stronger promoter is created by the novel hexamer sequences as they are a standard distance from each other (Nelson and Gay Elisha, 1999). Although the isolates 24' and 39' possess this stronger promoter and have a potential to express *ampC* at a higher level, its use cannot be concluded unless *in vitro* transcriptional analysis is used to confirm it. If attenuated leader transcript that is generated is longer than 41 nucleotides it can be concluded that the promoter is being used as transcription would start 4-5bp upstream of the wild-type promoter (Olsson *et al*., 1983). It is unlikely that without the -42 mutation there would be any effect on *ampC* expression, as it has previously been shown that a *Shigella* isolate with this identical promoter region had low *ampC* promoter expression (Olsson *et al*., 1983). The absence of the -42 mutation in isolate A2.5 should have low level expression of *ampC*, although the -18 mutation provides it with the potential to easily increase its ampicillin resistance to high levels with a single point mutation. This promoter may have been obtained via horizontal transfer from *Shigella* spp. as isolates were also found with the same novel promoter (Olsson *et al*., 1983). The pronounced difference from the *E. coli* K-12 wild-type promoter, and the fact that the *Shigella* promoter is often found with an Adenine at -18, the same as isolate A2.5, is evidence for this argument (Olsson *et al*., 1983). The origin of the novel

promoter is still questionable as *E. coli* easily could also be the origin. The -18 mutation alone was thought to distinguish between the *Shigella* promoter and the *E. coli* wild-type promoter (Olsson *et al*., 1983), however, A2.5 has this mutation without the -42 mutation suggesting either that this promoter is of *Shigella* origin or that the origin of these promoters are not so easily distinguished.

The analysis of the AmpC protein from 24', 39' and A2.5 also differs from that of *E. coli* K-12. This region shared more similarity with *E. coli* strain KL, which was isolated from a hospital patient in France in 2001 (Mammeri *et al*., 2004). This isolate harboured resistance to extended spectrum cephalosporins conferred by AmpC β-lactamase mutations (Mammeri *et al*., 2004). There are 3 amino acid differences between the region in isolates 24' and 39' and A2.5 when compared to *E. coli* KL, which could result in a very different AmpC phenotype. When these isolates were compared to other isolates, some of which produced extended-spectrum AmpC β-lactamase (ESAC) there was more similarity. Isolates 24' and 39' had 99% identity to *ampC* from isolates *E. coli* EC16, EC17, EC14 and EC9 where as A2.5 had the same similarity to EC10, EC12 and EC14 (Mammeri *et al*., 2006). These isolates, including *E. coli* KL, were all from phylogroups A or B1, the same as isolates 24', 39' and A2.5 (Section 3.14.8 and Appendix 4). Each isolate only differed by 1 amino acid, at differing positions, when compared with 24' and 39'; whereas A2.5 differed by at least 4 amino acids to EC12. It had been previously shown by Mammeri *et al*. (2006) that the ESAC activity for isolates EC16, 17, and 14 was due to different amino acid changes in *ampC*. In EC16 there was a Cysteine at 287, for EC17 a Proline at 296 and for EC14 a Leucine a 298 (Mammeri *et al*., 2006). Isolates 24', 39' and A2.5 all shared the mutation Proline at 296, which suggests it could have ESAC activity. However

analysis showed that this particular alteration did not have as great an affect as other mutations, resulting in minimal inhibitory concentrations 30-60- fold higher than wild-type *E. coli*, although not conferring resistance to cefepime, a 4th generation cephalosporin (Mammeri *et al*., 2006). This corresponds with the antibiotic resistance profile of 24' and 39', which had resistance to 2nd and one 3rd generation cephalosporin but was susceptible to other 3rd and 4th generation cephalosporins. This also corresponds with the 3rd generation cephalosporin and aztreonam resistance seen in A2.5 but not its resistance to cefquinome, a 4th generation cephalosporin (Mammeri *et al*., 2006). The transposon borne, potentially up regulated, *bla*$_{\text{OXA-1A}}$ and the *bla*$_{\text{TEM-1A}}$ genes also do not explain the resistance to cefquinome shown by A2.5. One explanation is the combination of production of all three β-lactamase genes produces this resistance, however this has not been described before in the literature. There could also have been an undiscovered factor that confers or contributes to this resistance, which is again unlikely as none of the annotation programmes or databases located one. A more plausible explanation is the loss of an element that conferred this resistance after the antibiotic profiling and before genomic sequencing. The results however do demonstrate the chromosomally based antibiotic resistance in all three isolates. They also convey a difference in *ampC* expression levels as only A2.5 has the potential to strongly express *ampC*, thus this could be the cause of the difference in antibiotic profiles between the isolates (appendix 1.1). In order to be able to clarify that a change in *ampC* gene could be a cause of antibiotic resistance decrease, the *ampC* gene from other isolates from 2013-2014/15 would need to be analysed for any differences or degradation in the area. Using these data however, it can be argued this gene in all three isolates is most likely the cause

of their 2nd, 3rd, and 4th generation cephalosporin resistance which could be tested by targeted mutation and complementation of the suspected genes.

6.0 Conclusion

Overall there were a few differences demonstrated between the strains isolated from this dairy farm in 2013 and 2014/15. These strains were isolated using the same techniques and selective markers from the same areas of the dairy farm located at the University of Nottingham. This farm decreased its use of cephalosporin antibiotics from 2011 until a virtual cessation of use in 2013. As there is a decline in breadth of antibiotic resistances and the frequency of resistance between the 2013 and 2014/15 isolates we could hypothesise that it is linked to the cessation of cephalosporin use, a phenomenon that has been seen previously (Dutil *et al*., 2010). However without characterisation of more isolates from 2010-2013 and the use of controls where cephalosporin use is being continued, this link cannot be validated.

There is no real evidence of a major decrease in ESBL and plasmid *ampC* genes from strains isolated in 2013 to those isolates in 2014/15. Although these strains were isolated after the majority of cephalosporin use was stopped and previous studies have found a decline in antibiotic resistance within a year, thus it might be prudent to characterise strains from before the cessation and one year after (Dutil *et al.,* 2010). If no strains from this time were stored, a reintroduction of the previous practice of cephalosporin use might be necessary or another dairy farm that still relies on cephalosporins could be the target of the investigation

There was also a decline in mercury chloride resistance between the 2013 isolates to 2014/15 isolates. Although mercury chloride is not used on this dairy farm resistance was common in the 2013 isolates, however it was rare in the 2014/15 isolates. Resistance has previously been shown to be common, even without $HgCl_2$ use, due to the linkage with antibiotic resistance (Skurnik *et al*., 2010, Novais *et al*., 2010, Hobman and Crossman, 2015). The difference between the isolate groups conveys a correlated decrease in mercury chloride resistance with antibiotic resistance. Although there was no difference in the mercury chloride resistance in the genomes of isolates 24', 39' and A2.5, which were all mercury chloride sensitive, these are only a small subset of the population of *E. coli* on the farm at these times. However the experimental characterisation of mercury chloride resistance in the 2013 and 2014/15 isolates might be a more accurate source of data than from the three sequenced genomes. They provide a greater insight into the population of ampicillin and tetracycline resistant *E. coli* on the farm as these were the selective markers used to isolate the strains.

The genomic information obtained from isolates A2.5, 24' and 39' suggests a genotype of isolates that are no longer resistant to mercury chloride yet still carry some antibiotic resistances. One possible transposon present, the Tn*2411*-like transposon, could be circulating in the isolates that are mercury chloride sensitive yet contain a class one integrase and retain some antibiotic resistances. It would be interesting to see if this transposon is still present in some 2014/15 isolates and determining if the *mer* operon was in fact deleted or simply moved onto another genetic element. Characterisation of isolates pre cephalosporin use as well as those from 2011 and 2012 would provide more insight into the apparent decline of mercury chloride resistance within this particular population of *E. coli.* It has previously been suggested that there might

be a link between antibiotic resistance and mercury chloride resistance; however this could not be shown in the genome of isolate A2.5 (Hobman and Crossman, 2015). It carried a potentially strongly expressed β-lactamase gene, *bla*$_{\text{OXA-1a}}$, within an element that is known to carry the *mer* operon. However this operon was no longer present in A2.5. The apparent co-decline of both antibiotic resistance and mercury chloride resistance in the isolates from 2013 and 2014/15 points towards a potential link between the characteristics, but characterisation of isolate from before the reduction of cephalosporin use and from 2011 and 2012 would provide more insight if the decline of both in this selected population occurred at the same time point and after the reduction of cephalosporin use.

The majority of the antibiotic resistance carried by isolates A2.5, 24' and 39' could be explained based on the chromosomal *ampC* gene, but, as the plasmid conjugation data showed, there were resistances that were also carried on mobile genetic elements. Although resistance-carrying plasmids could not be isolated from A2.5, 24' or 39', there were many resistances carried on transposons such as tetracycline resistance and potentially *bla*$_{\text{TEM-1A}}$ carried on a Tn*10*-like transposon, as well as aminoglycoside, sulphonamide, penicillin and oxacillin resistance carried by the Tn*2411*-like transposon. All the phenotypic resistances could be explained for each isolate based on chromosomal and mobile-borne resistance genes with the exception of the 4$^{\text{th}}$ generation cephalosporin resistance in A2.5, which hypothetically could be a result of a combination of strongly expressed *bla*$_{\text{OXA-1a}}$ and chromosomal *ampC.*

The ERIC profiles generated for the 2014/15 isolates did not correspond to the antibiotic resistance profile of the isolates. This suggested that horizontal gene transfer is a potential method of antibiotic resistance genes transfer in

isolates that have the same ERIC profile yet different resistances. However this could not be concluded and more isolates were needed to generate additional ERIC profiles or bioinformatic and phylogenetic analysis of the resistance genes. It does however suggest a potential method of resistance gene transfer within the *E. coli* population on the farm.

The final results of this project showed reduction in antibiotic resistance that correlated with reduction in mercury chloride resistance in the isolates selected from this farm using ampicillin and tetracycline. This decline occurs at a significant time in the farm's history where they implemented a reduction in use of 1st, 3rd, and 4th generation cephalosporins. Although there is a correlation between the decline in cephalosporin use and a decline in antibiotic resistance and mercury chloride there is not enough evidence to conclude causation. Further characterisation of isolates from before and just after the decline in cephalosporin use is required as well as a potential control farm that demonstrate it is not a normal fluctuation within a population of *E. coli*.

This project also determined a potential method of antibiotic resistance gene transfer via horizontal gene transfer. The resistances seen in the isolates in this project were demonstrated to be both chromosomally-based and on mobile genetic elements such as transposons and on plasmids. These results are significant for the farming community; they are consistent with the hypothesis that a reduction of cephalosporin use can positively alter the range and frequency of antibiotic resistance of select *E. coli* populations on a dairy farm. Any positive alteration in antibiotic resistance can have further beneficial implications as limiting the frequency of MDR strains could potentially enhance the lifespan of antibiotics. In an era where antibiotic discovery is low, extending the lifespan of current antibiotics could have a significant clinical impact.

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8.0 Appendix

8.1 Appendix 1

This shows the antibiotic resistance profiles of those strains isolated in 2013. The results were kindly provided by Delveen Ibrahim (PhD Student) and Jack Marsland (BSc Student).

	Antibiotics																
Isolate	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP
1''	6	13	17	6	23	6	21	8	19	6	24	21	6	21	20	22	26
2''	23	13	21	6	27	6	28	24	30	6	35	35	6	25	30	32	34
4''	22	6	21	6	27	11	17	20	19	6	32	19	6	6	30	22	33
5''	6	9	22	6	30	13	27	9	25	6	30	28	6	25	22	30	26
6''	6	15	19	6	28	6	24	8	23	6	34	30	6	25	24	30	32
7''	21	6	20	6	22	11	19	24	23	6	34	23	6	6	30	22	35
10''	30	9	28	6	31	9	25	28	29	30	31	30	6	6	32	30	34
13''	6	9	18	6	30	6	27	6	25	6	30	29	6	6	9	30	12
14''	23	6	21	6	26	6	17	22	18	6	31	20	6	6	30	20	30
15''	6	15	20	6	30	6	27	9	23	6	32	31	6	27	24	32	30
16''	6	15	21	22	25	13	15	21	8	6	15	15	6	21	22	10	28
21''	26	9	22	6	30	6	30	26	28	6	32	30	6	6	31	29	34
23''	6	9	27	6	29	6	28	8	26	6	32	30	6	26	24	30	32

Antibiotics																	
Isolate	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP
25''	6	6	22	6	30	8	17	22	20	6	32	25	6	7	30	22	30
26''	6	6	24	6	30	6	24	8	22	6	34	22	6	12	28	24	32
27''	6	9	17	6	30	6	27	9	27	6	34	30	6	20	24	30	31
30''	25	15	22	6	33	6	30	26	27	6	34	34	6	6	34	30	38
31''	6	15	19	6	26	6	25	8	20	6	30	29	6	24	22	27	28
35''	6	12	19	6	28	6	25	6	22	6	30	23	6	6	8	30	12
39''	22	8	21	6	32	6	30	25	28	6	34	33	6	6	35	30	34
40''	28	9	23	6	30	16	30	26	29	6	31	30	6	6	31	30	34
42''	22	15	21	6	28	6	26	22	25	6	30	30	6	6	32	31	34
44''	20	14	18	6	29	6	27	24	24	6	30	29	6	6	30	29	34
46''	22	9	20	6	34	6	32	29	30	6	34	32	6	6	30	30	34
49''	6	9	18	6	28	6	27	9	24	6	32	28	6	25	29	28	30
1'	6	19	22	6	30	15	31	26	26	6	35	34	22	6	35	32	34
2'	6	6	22	6	27	9	18	22	20	6	31	19	6	8	29	22	31
3'	25	6	21	6	31	6	30	21	26	6	32	31	6	6	34	33	36
4'	6	6	21	6	29	6	19	20	23	6	34	21	6	6	31	22	34
5'	21	6	23	6	28	6	31	25	26	6	33	32	6	25	30	32	36
8'	6	19	22	6	30	14	32	21	22	6	35	33	22	8	32	34	31
20'	24	6	22	6	31	6	26	24	23	6	34	30	6	6	33	32	40
21'	6	6	4	6	28	11	17	21	20	6	30	20	6	8	30	22	32

Antibiotics																	
Isolate	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP
22'	25	7	21	6	27	8	29	24	26	6	32	32	6	6	33	31	35
24'	6	6	22	6	27	12	17	20	20	6	30	19	6	8	27	20	30
28'	6	6	22	6	26	10	12	21	19	6	30	19	6	8	30	22	36
30'	28	8	20	6	29	8	28	24	25	6	34	31	6	6	31	33	36
35'	6	7	21	6	30	6	27	8	23	6	33	32	6	6	24	31	26
38'	22+	6	20	6	26	8	27	23+	25	6	32	30	6	6	26	30	35
39'	6	6	20	6	29	6	17	6	18	6	33	20	6	8	24	22	35
40'	25	21	21	6	31	6	29	6	26	6	34	32	6	6	6	32	12
45'	6	7	21	6	26	6	25	8	25	6	32	32	6	6	23	30	31
A2.5	6	12	22	6	30	6	27	25	8	6	17	14	6	23	20	12	30

8.2 Appendix 2

This table conveys the *merAC* and *int1I* PCR results for the 2013 isolates. These results were kindly provided by Delveen Ibrahim (PhD Student) and Jack Marsland (BSc Student).

2013 Isolates	*merA*	*merC*	*int1*	2013 Isolates	*merA*	*merC*	*int1*
1’	+	+	+	16’’	-	-	+
2’	-	-	+	21’’	-	+	-
3’	+	+	-	23’’	-	-	+
4’	+	+	-	25’’	-	-	-
5’	+	+	-	26’’	-	-	-
8’	+	+	+	27’’	-	-	+
20’	+	+	-	30’’	-	+	-
21’	+	+	-	31’’	-	-	+
22’	+	+	+	35’’	+	+	+
24’	-	-	+	40’’	-	-	-
28’	-	-	+	42’’	+	+	-
30’	-	+	+	44’’	-	+	-
35’	-	+	-	46’’	+	+	-
38’	-	+	+	49’’	-	-	+
39’	-	-	+				
40’	-	+	-				
45’	-	-	+				
2’’	+	+	-				
4’’	+	+	-				
5’’	-	-	+				
6’’	-	-	+				
7’’	+	+	-				
10’’	-	+	-				
13’’	+	+	+				
14’’	+	+	-				

8.3 Appendix 3

8.3.1. Appendix 3.1- Genomic Assembly Results of Isolates 24' and 39'

The assembly statistics are shown below; 24' had a mean coverage of 129, with a standard deviation of 55.41. Isolate 39' had a mean coverage of 130 with a standard deviation of 50.92. The mean mapping quality of 24' was 54.17 and 39' was 53.9 meaning that there was a $10^{-5.417}$ and $10^{-5.39}$ respective probability that the read is aligned incorrectly, which is a very low chance. This information suggests that the assembly of 39' was of slightly higher quality than 24'.

	24'	39'
Total Contigs (#)	108	141
Total Length (bp)	5,231,243	5,136,899
Mapped reads (%)	100	100
Largest contig (bp)	65,6736	47,9960
N50	167788	184612
GC Content (%)	50.63	50.62

8.3.1 Appendix 3.2-Genomic Assembly Results of Isolate A2.5

The genome of A2.5 was isolated and sent to SequenceAnalysis (www.sequenceanalysis.co.uk) for genome sequencing, assembly and primary genomic characterisation. The Table below shows the statistics of the assembly, which was provided by the company as well as preliminary genomic analysis.

A2.5	Scaffold	Contigs
Total Number (#)	210	235
Total Length (bp)	5,233,959	5,233,441
Max Length (bp)	351,328	351,328
Min Length (bp)	502	287
GC Content (%)	50.51	50.51

8.4 Appendix 4

8.4.1 Appendix 4.1-MLST and Phylotype of Isolates 24' and 39'

The MLST of 24' and 39' was determined using Centre for Genomic Epidemiology MLST service (Larsen *et al*., 2012). The results show that both isolates have the same Sequence type, ST-88. The phylo-type was determined using the Clermont *E. coli* phylotyping method (Clermont *et al*., 2013), which determined both 24' and 39' to be phylotype A.

	24'			39'		
Locus	**Identity (%)**	**Gaps**	**Allele**	**Identity (%)**	**Gaps**	**Allele**
adk	100	0	*adk-6*	100	0	*adk-6*
fumc	100	0	*fumc-4*	100	0	*fumc-4*
gyrb	100	0	*gyrb-12*	100	0	*gyrb-12*
icd	100	0	*icd-1*	100	0	*icd-1*
mdh	100	0	*mdh-20*	100	0	*mdh-20*
pura	100	0	*pura-12*	100	0	*pura-12*
reca	100	0	*reca-7*	100	0	*reca-7*
			ST-88			**ST-88**

8.4.2 Appendix 4.2-MLST and Phylotype of Isolate A2.5

The MLST results for this genome determined that it was ST-56, part of Complex ST155. As it lacked genes *chuA, yjaA,* and was positive for *arpA*, and DNA fragment TspE4.C2 at the correct fragment length this isolate was assigned the phylogenetic group, B1 (Clermont *et al*., 2013).

Locus	Identity (%)	Gaps	Allele
adk	100	0	*adk-6*
fumc	100	0	*fumc-4*
gyrb	100	0	*gyrb-4*
icd	100	0	*icd-18*
mdh	100	0	*mdh-24*
pura	100	0	*pura-5*
reca	100	0	*reca-14*
		ST	56

8.5 Appendix 5

8.5.1 Appendix 5.1-

This table displays the statistic for each assembled nodes in isolate 24'.

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_169	120	110	108	0.92	0.28
NODE_168	120	0	78	0	0
NODE_165	122	305	82	2.5	0.99
NODE_163	128	0	172.7	0	0
NODE_160_	137	0	34.2	0	0
NODE_159	142	0	90.1	0	0
NODE_158	143	558	191.9	3.9	0.42
NODE_157	144	255	116.5	1.77	0.5
NODE_156	146	0	61.3	0	0
NODE_154	149	290	849	1.95	0.23
NODE_152	155	1829	51.9	11.8	2.01
NODE_148	179	627	312.8	3.5	0.78
NODE_147	182	0	23.4	0	0
NODE_143	194	7007	81.1	36.12	6.29
NODE_139	204	7253	29.3	35.55	4.71
NODE_137	215	20537	101.4	95.52	16.17

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_136	217	29830	87.3	137.47	28.91
NODE_135	237	10175	32.7	42.93	5.79
NODE_134	239	9715	47.9	40.65	10.22
NODE_133	244	7466	46.9	30.6	6.9
NODE_131	252	44999	150.7	178.57	49.37
NODE_144	192	6823	37.6	35.54	4.39
NODE_141	198	24180	215.7	122.12	17.89
NODE_130	258	21214	106.4	82.22	22.96
NODE_129	267	39633	113.9	148.44	42.25
NODE_127	322	23426	47	72.75	27.98
NODE_164	122	0	85.7	0	0
NODE_128	287	52423	180	182.66	62.7
NODE_126	334	57335	156.8	171.66	71.88
NODE_124	347	48971	106.6	141.13	55.18
NODE_123	348	45578	80.4	130.97	47.5
NODE_155	149	149	120.8	1	0
NODE_122	368	75509	113.8	205.19	77.54
NODE_167	120	0	84	0	0
NODE_121	393	35977	39	91.54	38.88
NODE_161	133	120	211.8	0.9	0.3
NODE_149	170	1380	78.3	8.12	1.34
NODE_142	197	4281	168.7	21.73	4.6
NODE_118	435	55195	85.6	126.89	32.63
NODE_117	451	67556	82.1	149.79	61.39
NODE_116	459	82575	91.7	179.9	66.77
NODE_115	462	64521	56.2	139.66	60.12
NODE_114	471	41876	47	88.91	34.6
NODE_113	479	48362	48.1	100.96	38.03
NODE_111	485	43705	35.6	90.11	34.58
NODE_119	425	35729	40.4	84.07	31.1
NODE_110	497	58601	54	117.91	43.49
NODE_51	7199	869331	43	120.76	20.4
NODE_138	215	21147	75.3	98.36	16.26
NODE_46	11070	1484890	51	134.14	16.91
NODE_45	11200	1553108	52.8	138.67	41.85

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_30	43647	5451414	46.3	124.9	14.47
NODE_120	415	24786	33.3	59.73	31.2
NODE_44	12094	1457662	54.7	120.53	40.4
NODE_171	120	34557	4027	287.98	45.3
NODE_43	12471	1385126	41.7	111.07	15.24
NODE_38	20785	2653296	47.1	127.65	19.97
NODE_79	1545	200691	42.6	129.9	33.44
NODE_109	497	106225	100.3	213.73	78.24
NODE_35	37119	4718508	47.3	127.12	18.69
NODE_13	124223	16275885	59.4	131.02	19.02
NODE_33	42143	5681118	50.8	134.81	19.21
NODE_39	17380	2290007	47.3	131.76	16.76
NODE_108	518	58594	43.1	113.12	37.98
NODE_146	185	8054	105.4	43.54	4.67
NODE_12	126545	16119175	48.9	127.38	16.35
NODE_50	7218	843117	45.9	116.81	18.59
NODE_150	164	3866	102.3	23.57	1.26
NODE_112	479	51142	58.4	106.77	40.45
NODE_20	72916	9479861	47.5	130.01	16.79
NODE_41	16240	2071287	46.8	127.54	16.24
NODE_17	87851	11096056	46.4	126.31	15.33
NODE_74	1933	253956	52.9	131.38	27.7
NODE_40	17179	2187057	47.3	127.31	21.37
NODE_28	44487	5886457	67.7	132.32	23.42
NODE_166	120	0	80	0	0
NODE_162	132	0	91.3	0	0
NODE_145	189	9891	77.7	52.33	7.71
NODE_125	335	58284	101.9	173.98	64.23
NODE_47	10954	1367312	70.6	124.82	54.85
NODE_27	46454	5995599	71.9	129.07	49.77
NODE_31	42997	5493979	46.8	127.78	16.24
NODE_49	7599	1008907	50.6	132.77	23.09
NODE_23	54935	6959481	88.1	126.69	32.64
NODE_19	76159	9789488	47.9	128.54	17.22
NODE_1	656736	83912648	49.1	127.77	17.91

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_103	546	47442	46.3	86.89	35.82
NODE_29	43715	5626697	47.2	128.71	16.81
NODE_36	32946	4151702	46.3	126.02	17.19
NODE_170	120	0	102	0	0
NODE_3	461233	58454238	46.8	126.73	16.17
NODE_52	7169	937200	55.2	130.73	31.81
NODE_4	375204	48632829	48.4	129.62	19.19
NODE_48	9005	772159	57.2	85.75	27.25
NODE_57	5226	599312	41.7	114.68	16.65
NODE_6	188464	24049947	46.7	127.61	16.71
NODE_98	611	171760	65.6	281.11	128.11
NODE_102	560	44955	39	80.28	27.88
NODE_9	158373	20112383	46.7	126.99	15.29
NODE_10	142908	18553036	47.3	129.83	15.6
NODE_100	569	102482	93.3	180.11	68.99
NODE_5	296927	38155849	47.1	128.5	16.28
NODE_93	769	153054	445.4	199.03	74.18
NODE_132	251	6905	30.1	27.51	7.41
NODE_15	97303	12568269	47.6	129.17	18.54
NODE_55	6140	839796	53.4	136.77	21.93
NODE_34	38235	4975112	48.1	130.12	16.15
NODE_11	141653	16457280	53.1	116.18	36.15
NODE_76	1708	194188	324.3	113.69	45.35
NODE_24	53229	6902202	48.6	129.67	18.31
NODE_87	1172	130452	47.6	111.31	39.38
NODE_22	59784	5726294	46.1	95.78	52.06
NODE_42	15329	1850791	44.3	120.74	16.39
NODE_16	92118	11850433	61.4	128.64	25.7
NODE_7	167788	21630267	47.6	128.91	15.37
NODE_106	518	70441	72.5	135.99	52.37
NODE_61	4377	532557	44.9	121.67	24.95
NODE_21	70752	9090379	47.9	128.48	17.45
NODE_107	518	54220	45.8	104.67	35.83
NODE_54	7025	838483	47.1	119.36	48.93
NODE_99	610	75208	49.5	123.29	58.05

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_56	5530	734271	51.4	132.78	19.7
NODE_97	637	64121	45.6	100.66	35.46
NODE_53	7142	850611	43.5	119.1	20.41
NODE_58	4939	619172	49.2	125.36	24.1
NODE_59	4500	253678	53.5	56.37	15.7
NODE_60	4444	477139	40.5	107.37	17.88
NODE_62	4249	591359	57.5	139.18	38.84
NODE_37	26252	3275097	45.9	124.76	16.24
NODE_2	520193	66418842	48.8	127.68	20.19
NODE_86	1195	151122	51.7	126.46	28.12
NODE_63	3496	415364	45.3	118.81	25.64
NODE_140	199	33419	129.9	167.93	22.19
NODE_68	2283	285318	48.4	124.98	26.2
NODE_64	3301	421979	49.8	127.83	19.83
NODE_65	3003	523078	71.5	174.19	88.3
NODE_66	2795	6328098	883.2	2,264.08	427.74
NODE_151	159	2184	100.3	13.74	0.58
NODE_69_	2210	334608	61.7	151.41	34.76
NODE_70	2191	335268	52.1	153.02	43.48
NODE_71	2174	283862	45.8	130.57	34.94
NODE_72	2141	819245	142	382.65	79.95
NODE_105	536	59032	44.1	110.13	35.04
NODE_73	2091	233615	44.6	111.72	24.22
NODE_32	42457	5660122	49.5	133.31	16.36
NODE_75	1858	232741	50.5	125.26	26.05
NODE_77	1626	233445	52.9	143.57	35.73
NODE_78	1582	216167	49.2	136.64	36.37
NODE_14	105355	13694878	47.8	129.99	15.43
NODE_18	86596	10791319	45.4	124.62	15.22
NODE_95	695	146820	94.9	211.25	63.22
NODE_153	153	602	90.1	3.93	0.27
NODE_96	657	60758	41.1	92.48	31.98
NODE_25	48913	6388004	48.2	130.6	17.28
NODE_80	1526	202334	55.8	132.59	30.33
NODE_92	788	80843	41.6	102.59	27.5

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_81	1360	161159	47.7	118.5	29.48
NODE_82	1345	325271	90.2	241.84	57.96
NODE_83	1343	150175	44.8	111.82	26.59
NODE_84	1343	147038	43.7	109.48	26.23
NODE_67	2354	585803	87.5	248.85	46.6
NODE_85	1264	312305	102.4	247.08	64.52
NODE_88	1169	116933	39.4	100.03	25
NODE_8	162072	20991193	47.6	129.52	16.34
NODE_101	560	48750	41.1	87.05	28.45
NODE_26	46788	5896678	46.1	126.03	16.22
NODE_89	1078	282273	111.3	261.85	60.15
NODE_90	1073	132293	41.6	123.29	38.88
NODE_91	983	104472	40	106.28	24.15
NODE_94	766	84091	39.3	109.78	39.72
NODE_104	538	109901	99.2	204.28	70.25

8.5.2 Appendix 5.2

This table displays the statistic for each assembled nodes in isolates 39'.

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_235	128	0	201	0	0
NODE_233	128	0	94	0	0
NODE_232	128	0	84	0	0
NODE_231	128	0	78	0	0
NODE_230	128	0	97	0	0
NODE_227	128	0	114	0	0
NODE_104	1204	126020	38.6	104.67	26.39
NODE_172	245	22271	69.5	90.9	22.51
NODE_98	1561	208571	39.3	133.61	31.73
NODE_122	705	140712	81.7	199.59	74.31
NODE_97	1643	173209	38	105.42	20.85
NODE_96	1643	195338	43.1	118.89	29.26
NODE_102	1329	1057154	303.2	795.45	190.98

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_229	128	0	79	0	0
NODE_14	105371	13843187	44	131.38	15.18
NODE_208	143	203	176.6	1.42	0.52
NODE_236	128	44846	4939	350.36	68.68
NODE_92	1873	213529	40.4	114	26.22
NODE_125	670	137627	85	205.41	73.74
NODE_90	1949	258705	48.5	132.74	29.26
NODE_75	3659	456437	42	124.74	22.87
NODE_89	2034	288131	48.2	141.66	31.44
NODE_62	5594	726346	42	129.84	28.35
NODE_137	534	46758	30.9	87.56	30.4
NODE_93	1787	238118	43.6	133.25	33.85
NODE_91	1874	227621	45.4	121.46	29.07
NODE_82	2299	294648	45.5	128.16	26.74
NODE_78	2803	4789640	604	1,708.75	306.18
NODE_77	3317	404698	43.5	122.01	19.78
NODE_74	3819	489134	45.7	128.08	22.7
NODE_54	7204	956882	46.1	132.83	27.1
NODE_111	1031	205565	77	199.38	50.3
NODE_106	1185	129248	39.6	109.07	26.27
NODE_73	4064	514281	45.7	126.55	24.89
NODE_66	4955	622667	44.6	125.66	20.46
NODE_67	4947	682750	50.8	138.01	19.96
NODE_13	142924	18510598	43	129.51	16.19
NODE_153	320	34182	87.3	106.82	42.75
NODE_64	5289	631725	41.2	119.44	22.5
NODE_200	156	2221	96.8	14.24	0.81
NODE_72	4100	583962	50.7	142.43	28.68
NODE_223	128	0	127	0	0
NODE_95	1655	1299043	282.3	784.92	133.52
NODE_71	4367	536519	42.6	122.86	19.9
NODE_43	17396	2273335	42.7	130.68	15.92
NODE_57	6961	917427	46	131.8	19.3
NODE_29	38196	4881119	42.3	127.79	17.36
NODE_56	7150	926174	40.5	129.53	29.6

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_55	7158	823905	38	115.1	16.3
NODE_193	177	20678	254.5	116.82	8.92
NODE_21	72932	9209372	41.8	126.27	16.84
NODE_60	6571	843392	43.9	128.35	21.62
NODE_20	75383	9539140	42.6	126.54	14.93
NODE_161	268	45950	124.7	171.46	53.31
NODE_25	43663	5520721	42.4	126.44	16.51
NODE_159	275	27541	133.7	100.15	31.44
NODE_216	133	139	164.2	1.05	1.35
NODE_36	28255	3610319	43.7	127.78	18.43
NODE_28	40191	5113902	42.3	127.24	16.33
NODE_17	86883	10982605	42.3	126.41	15.33
NODE_88	2107	247784	42.5	117.6	24.7
NODE_105	1193	144832	44.8	121.4	27.71
NODE_218	129	0	183	0	0
NODE_103	1304	160822	46.8	123.33	32.6
NODE_44	16256	2036163	42.1	125.26	14.56
NODE_39	20424	2692660	44.7	131.84	19.09
NODE_16	87943	11091022	42.1	126.12	15.03
NODE_167	254	35608	105.5	140.19	35.62
NODE_196	164	1414	76	8.62	1.03
NODE_9	169784	21613565	42.4	127.3	16.4
NODE_113	940	107757	43.6	114.64	36.36
NODE_84	2207	327948	49.8	148.59	30.71
NODE_83	2226	328909	55.5	147.76	35.77
NODE_160	275	38651	97.2	140.55	45.71
NODE_109	1097	220953	72.7	201.42	54.77
NODE_24	43980	5463503	41.1	124.23	14.36
NODE_133	562	55748	46.6	99.2	33.87
NODE_1	479960	62369985	43.3	129.95	43.68
NODE_33	32036	4091938	43	127.73	17.78
NODE_11	162088	20439001	42	126.1	16.21
NODE_194	164	1781	81.1	10.86	0.49
NODE_5	297642	38492905	43	129.33	16.11
NODE_149	355	48063	93.5	135.39	51.62

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_211	133	0	67.8	0	0
NODE_85	2206	485866	75.8	220.25	40.97
NODE_181	213	31101	183/8	146.01	25.45
NODE_10	165707	21226306	42.9	128.1	16.92
NODE_58	6666	915775	48.4	137.38	21.62
NODE_32	32929	4103441	41.7	124.61	16.38
NODE_87	2149	808815	128.5	376.37	83.51
NODE_7	186370	23665130	42.3	126.98	16.13
NODE_6	265552	33949661	42.7	127.85	17.55
NODE_173	243	22918	72.2	94.31	22.12
NODE_3	396671	50358461	42.5	126.95	15.71
NODE_27	41252	5150992	42	124.87	15.86
NODE_19	86508	10855525	41.9	125.49	14.64
NODE_2	402199	52310875	43.3	130.06	26.23
NODE_213	133	183	27.2	1.38	1.43
NODE_23	64162	8321186	43	129.69	15.68
NODE_61	6156	829458	48.1	134.74	20
NODE_40	19471	2499607	42.2	128.38	17.16
NODE_119	765	60380	38	78.93	35.54
NODE_59	6640	845920	44.1	127.4	21.76
NODE_79	2653	2083239	270.8	785.24	133.16
NODE_212	133	0	148.7	0	0
NODE_51	8522	1015295	40.4	119.14	19.73
NODE_145	451	72527	79.5	160.81	68.51
NODE_48	11086	1503115	46.2	135.59	19.26
NODE_152	335	52085	83.5	155.48	59.97
NODE_101	1345	335650	87.3	249.55	61.17
NODE_26	42159	5663235	46.1	134.33	18.82
NODE_31	32962	4036910	40.7	122.47	14.88
NODE_50	9239	1181033	42.3	127.83	17.51
NODE_99	1443	325449	88.8	225.54	58.04
NODE_4	358691	46329487	43.3	129.16	17.12
NODE_142	487	45221	43.3	92.86	31.08
NODE_34	30966	3949841	42.9	127.55	16.79
NODE_76	3512	423787	41.5	120.67	19.44

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_65	5242	615447	39.2	117.41	20.72
NODE_8	184612	23130016	41.2	125.29	15.44
NODE_46	14452	1854161	43.5	128.3	18.16
NODE_37	24812	3222027	43.7	129.86	16.67
NODE_176	237	11580	40.1	48.86	6.86
NODE_143	467	85712	92.4	183.54	67.81
NODE_219	129	0	136.5	0	0
NODE_100	1421	188024	50.5	132.32	35.94
NODE_80	2451	278308	40.4	113.55	21.92
NODE_131	571	60370	47.9	105.73	34.28
NODE_68	4575	531237	39.7	116.12	18.83
NODE_207	143	188	215.1	1.31	0.46
NODE_38	20801	2606956	41.9	125.33	16.29
NODE_187	194	18248	108.5	94.06	8.35
NODE_42	17894	2273871	43	127.07	20.7
NODE_52	7439	897208	39.3	120.61	19.94
NODE_183	204	17048	101.8	83.57	7.22
NODE_45	15653	1964018	41.3	125.47	16.2
NODE_135	552	54702	34.4	99.1	24.95
NODE_180	215	19197	61.5	89.29	15.81
NODE_53	7234	989443	47.5	136.78	20.28
NODE_49	10887	1375000	44.1	126.3	16.71
NODE_110	1078	265046	97.2	245.87	63.78
NODE_63	5546	725222	45.9	130.76	19.54
NODE_112	999	113799	38.2	113.91	28.36
NODE_115	866	314832	135.7	363.55	106.94
NODE_114	940	95130	40.7	101.2	32.99
NODE_116	863	211261	90.5	244.8	59.95
NODE_117	791	91892	50.6	116.17	33.28
NODE_69	4393	541004	41.5	123.15	24.65
NODE_203	153	0	38.9	0	0
NODE_163	260	17395	81.5	66.9	21.66
NODE_35	29725	4853005	55.5	163.26	119.52
NODE_118	782	95373	40.1	121.96	41.25
NODE_198	159	935	90.7	5.88	0.45

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_120	719	71260	39.9	99.11	27.71
NODE_123	703	123766	69.6	176.05	55.33
NODE_124	673	65122	40.7	96.76	31.53
NODE_127	625	54939	32.1	87.9	33.07
NODE_156	290	30825	57.8	106.29	34.06
NODE_199	157	314	5.3	2	0
NODE_129	576	51697	39.7	89.75	30
NODE_130	576	57701	41.6	100.18	31.7
NODE_70	4370	543081	43.2	124.27	22.61
NODE_132	571	52080	39.4	91.21	26.57
NODE_141	513	56017	44.4	109.19	41.66
NODE_204	152	0	49.4	0	0
NODE_138	534	50936	37.1	95.39	36.87
NODE_228	128	0	85	0	0
NODE_94	1686	174816	38.2	103.69	26.3
NODE_139	526	80206	71.4	152.48	64.16
NODE_12	158161	20231202	41.9	127.92	16.23
NODE_140	513	110806	93.7	216	83.54
NODE_144	466	174229	192.7	373.88	145.41
NODE_148	391	63641	85.6	162.76	71.6
NODE_186	196	24494	158.1	124.97	15.26
NODE_190	188	1820	41.6	9.68	1.13
NODE_146	432	43852	50.7	101.51	42.15
NODE_169	253	11894	33	47.01	6.37
NODE_121	718	72888	40.4	101.52	27.58
NODE_147	419	65640	80.4	156.66	69.71
NODE_182	212	15663	76.5	73.88	11.22
NODE_150	341	99563	157.5	291.97	111.63
NODE_151	341	59649	106.5	174.92	67.06
NODE_158	287	78132	185.2	272.24	89.93
NODE_47	12487	1620263	44	129.76	18.05
NODE_154	302	41562	84.4	137.62	50.55
NODE_155	295	35781	87.9	121.29	41.11
NODE_157	287	28612	100.2	99.69	35.93
NODE_162	267	7130	17.8	26.7	9.17

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_164	258	122399	353	474.41	131.56
NODE_185	198	2040	45.7	10.3	1.73
NODE_188	193	5839	82	30.25	4.54
NODE_166	255	60611	138.8	237.69	65.33
NODE_81	2354	550915	74.7	234.03	48.91
NODE_197	163	481	100	2.95	0.31
NODE_41	19190	2383017	40.5	124.18	18.02
NODE_168	254	68396	189.6	269.28	72.43
NODE_224	128	0	84	0	0
NODE_18	86612	10748218	41.2	124.1	14.7
NODE_170	246	28627	94.5	116.37	26.62
NODE_171	245	4083	12.1	16.67	4.38
NODE_86	2190	246077	40	112.36	22.36
NODE_108	1100	132568	45.7	120.52	29.69
NODE_174	242	21182	82.3	87.53	19.17
NODE_177	233	28353	104.3	121.69	24.69
NODE_178	233	32026	75.1	137.45	31.68
NODE_22	70953	9057379	43.1	127.65	15.65
NODE_179	220	8389	20.4	38.13	8.36
NODE_234	128	0	80	0	0
NODE_184	199	33544	121.8	168.56	20.41
NODE_107	1165	136654	37.8	117.3	41.49
NODE_189	188	13750	120.9	73.14	5.76
NODE_30	37135	4768761	43.1	128.42	15.6
NODE_191	184	7366	105.4	40.03	5.8
NODE_126	637	57427	41.3	90.15	41.17
NODE_136	538	102424	85.7	190.38	69.03
NODE_192	180	29225	330.9	162.36	11.87
NODE_195	164	1972	72.7	12.02	1.02
NODE_15	97501	12802223	43.9	131.3	16.5
NODE_222	128	70	118	0.55	0.5
NODE_202	155	300	40.1	1.94	0.25
NODE_175	241	54965	197.2	228.07	53.01
NODE_201	155	297	83.4	1.92	0.28
NODE_205	151	0	85.3	0	0

Name	Length	Mapped bases	Coverage	Mean coverage	Standard deviation
NODE_206	146	0	75.5	0	0
NODE_134	558	166756	140.5	298.85	99.01
NODE_217	130	0	124.7	0	0
NODE_215	133	66	40.8	0.5	0.86
NODE_128	578	111034	82.9	192.1	68.04
NODE_209	142	45	105.1	0.32	0.47
NODE_210	138	0	118	0	0
NODE_226	128	0	185	0	0
NODE_165	257	25600	85.6	99.61	27.59
NODE_214	133	114	117	0.86	0.35
NODE_220	129	0	62.5	0	0
NODE_221	129	0	110.5	0	0
NODE_225	128	0	89	0	0