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Phenotypic and genotypic study of multidrug resistant, extended spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolated from a dairy farm

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ABSTRACT

Approximately 400 tonnes of antibiotics (including synthetic antibiotics) are used every year in treating infections in farm animals, and as prophylactics against infection. Antimicrobial resistance is a crucial problem that is now of great concern in public health, with food and food producing animals as a potential route for spread of these resistances, especially resistance to cephalosporins, which is increasing. The main aim of this study was to determine the prevalence and range of multidrug resistance (MDR) and extended spectrum β-lactamase (ESBL) or ampicillin C (AmpC) β-lactamase producing *Escherichia coli* within a commercial dairy farm, to understand the diversity of resistance to β-lactam antibiotics, and to determine if co-carriage of other antimicrobial resistance (AMR) was associated with ESBL/AmpC producers. This would allow a better understanding of the contributions that farms and farm slurry may make to the presence of AMR in the environment, and the reservoir of resistance in agriculture. In this study, *E. coli* strains were isolated from a single dairy farm (East Midlands, England, United Kingdom) on two visits, a preliminary isolation using TBX agar in 2012 and more targeted isolation using antibiotic supplemented TBX media in 2014. Confirmed *E. coli* (126 out of 155 selected strains) were genotyped using ERIC-PCR and analysis of the ERIC profiles showed that, in comparison to the 2014 isolates, the 2012 isolates were a quite distinct genetic population. Antimicrobial sensitivity tests were performed using a disk diffusion test for all the strains against 17 antimicrobials representing seven different antimicrobial groups: β-lactams, aminoglycosides, tetracyclines, sulphonamides, chloramphenicols, nitrofuran derivatives and quinolones. Antimicrobial resistance profiling showed 92% of isolates showed resistance to at least 1 antimicrobial, of which

27.8% of the isolates were isolated without antibiotic selection, and 57.9% of the isolates were multidrug resistant to between 3 and 15 antimicrobials, of which 43.6% of the isolates were isolated using antibiotic supplemented media. Two strains showed resistance to imipenem which appeared to be an unstable phenotype and was subsequently lost. The finding was unexpected and of concern as imipenem is not used in veterinary medicine. *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{OXA}$ genes were detected by PCR among the cephalosporin resistant strains. No plasmid *ampC* genes were detected. Four strains were fully sequenced and the genetic/genomic environment surrounding β-lactamase genes and analysis of some other AMR genes showed these genes are associated with transposable elements, such as IS*Ecp1,* IS*CR2*, IS*26*-IS*26*, Tn*2*, Tn*10* or within a class I integron carried by a Tn-*21* like transposon. The association of AMR genes with these transposable elements might make the dissemination rate of these genes greater. Some of the insertion sequence-AMR gene combinations are thought to be novel, such as the unique insertion of IS*Ecp1*- *bla*$_{CTX_M14}$ unit into the *fdeC* chromosomal gene.

This is the first study of this type performed on this dairy farm; the data showed a diverse range of resistance genes present in the *E. coli* population in the farm, including resistance to historically used antimicrobials as well as cephalosporins in contemporary use, and a high level of multidrug resistance. The spread of such highly resistant strains to the environment and possibly to humans could present a real threat to human health especially if they are pathogenic.

Conferences and publications

SfAM Activated Sludge Meeting "Control of water-borne disease: A century of the activated sludge sewage treatment process". The Lancashire County Cricket Club, Manchester, UK on 1 and 2 April 2014, attendance.

24th International ICFMH conference, FOOD MICRO 2014, Nantes, France. **Presence of antibiotic resistance in *E. coli* from cattle slurry in a dairy unit in Nottingham**, poster presentation

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3rd International Symposium on the Environmental Dimension of Antibiotic Resistance which held in 17 May - 21 May 2015 – Wernigerode/Germany. **Multidrug resistant and ESBL-producing *E. coli* from cattle slurry in a dairy unit in Nottingham**, presented by Prof Christine Dodd.

Antibiotic resistance and Antibiotic alternatives conferece by Euroscicon, 3rd of November 2015, O2 Peninsula Square London, United Kingdom. **Multidrug resistant and ESBL-producing *E. coli* from cattle slurry in a dairy unit in Nottingham**, poster presentation.

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LIST OF ABBREVIATIONS

Abbreviation	Description
°C	Degrees centigrade
ΦPT	Measure of population differentiation for binary data
µg	Microgram
µM	Micrometre
3'	3 prime end of DNA
5'	5 prime end of DNA
16S	Subunit of 30S unit of the prokaryote ribosome; S is for Svedberg unit
A	Adenine
aa	Amino acid
aac	Aminoglycoside acetyltransferase gene
aac (6')-Ib-cr	A gene variant for aminoglycoside acetyltransferase that confers reduced susceptibility to quinolones
aad	Aminoglycoside adenylytransferase gene
ABI	Applied Biosystems™
ACC	Ambler class C [β lactamase]
ACDP-CL2	Advisory Committee on Dangerous Pathogens-Containment Level 2
AcrAB	Acridine resistance complex
acrR	DNA-binding transcriptional repressor
ACT	Artemis comparison tool
ADB	Agarose dissolving buffer
Afa/Dr adhesion	Afimbrial/drori blood group antigen adhesion
AMC	Amoxicillin-clavulanic acid
AMOVA	Analysis of molecular variance
AMP	Ampicillin
AmpC	Ampicillin C
ampD	N-acetylmuramoyl-L-alanine amidase gene
ampG	Transmembrane transport protein encoding gene
ampR	Gene for transcriptional regulator of *ampC*
AMR	Antimicrobial resistance
AMS	Automated milking system
ANOVA	Analysis of variance
aphAI	Aminoglycoside 3'-phosphotransferase gene
APHA	The Animal and Plant Health Agency
API	Analytical profile index
ars	Arsenic resistance gene cluster
ASTM	American Society of the International Association for Testing and Materials
ATM	Aztreonam
ATP	Adenosine triphosphate
attC	Specific cassette recombination site
attI	Site-specific recombination site
Azi^R	Azide resistance

Abbreviation	Description
B.C.	Before Christ
BacMet	Antibacterial biocide- and metal-resistance genes
BAM	Bacterial analytical manual
bla	Beta-lactamase gene
BLAST	Basic local alignment search tool
blastp	BLAST search using protein sequence
bp	Base pair
C	Cytosine
Ca^{2+}	Calcium ion
cat	Chloramphenicol acetyltransferase gene
CAZ	Ceftazidime
CAZ+C	Ceftazidime and clavulanate
CAZCX	Ceftazidime plus cloxacillin
CDC	Centre for Disease Control and Prevention
CFQ	Cefquinome
CFU	Colony forming unit
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
cml	Chloramphenicol efflux gene
CMY	Cephamycinase
COIPARS	Colombian Integrated Program for Antimicrobial Resistance Surveillance
CRM	Constant relative mutability
CTn	Conjugative transposon
CTX	Cefotaxime
CTX+C	Cefotaxime and clavulanate
CTXCX	Cefotaxime plus cloxacillin
CTX-M	Cefotaximase
czc	Cobalt-zinc-cadmium resistance genes
DAEC	Diffuse adhering *E. coli*
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
DEFRA	Department for Environment, Food & Rural Affairs
DF	Degrees of freedom
dfr	Dihydrofolate reductase gene
DHA	Dhahran Hospital in Saudi Arabia
DMEM	Dulbecco's Modified Eagle’s medium
DNA	Deoxyribonucleic acid
DR	Direct repeat
eae	Intimin adherence gene
EAggEC	Enteroaggregative *E. coli*

Abbreviation	Description
EBC	Plasmid beta-lactamase from *Enterobacter cloacae*
ECC	*E. coli* count
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EFT	Ceftiofur
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMBL-EBI	European molecular biology laboratory- European bioinformatics institute
emrD	Multidrug efflux system encoding gene
ENR	Enrofloxacin
EPEC	Enteropathogenic *E. coli*
ERIC	Enterobacterial repetitive intergenic consensus
ESBL	Extended spectrum β-lactamase
ESC	Extended spectrum cephalosporin
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
F	Nitrofurantoin
FDA	Food and Drug Administration
fdeC	Intimin-like adhesin gene
FEP+C	Cefepime and clavulanate
finO	Conjugal transfer fertility inhibition
fliC, *flkA*, *fllA*, *flmA*, and *flnA*	Flagellin genes
floR	Chloramphenicol/florfenicol efflux MFS transporter gene
FOX	Cefoxitin
g	Grams
G	Guanine
g	*g*-force
GC content	Guanine-cytosine content
glmM	Phosphoglucosamine mutase gene
gyrA/B	DNA gyrase subunit A/B genes
h	Hour
HCl	hydrochloric acid
Hfr	High frequency recombinant
HGT	Horizontal gene transfer
HUS	Haemolytic uraemic syndrome
ICE	Integrative conjugative elements
IMP	Imipenem
Inc	Incompatability group
*intI*1	Class 1 integron integrase gene

Abbreviation	Description
IR	Inverted repeat
IS	Insertion sequence
jemA	Sodium/glutamate symporter gene
jemB	Gene of unknown function on Tn*10*
jemC	Transcription regulator gene on Tn*10*
kbp	Kilo base pair
kg	Kilogram
K-O antigen	Capsule- oligosaccharides antigen
L	Litre
LB	Luria Bertani
LB AzideCTX agar	LB agar containing 200 µg ml^{-1} of sodium azide and 1 µg ml^{-1} of CTX
LGT	Lateral gene transfer
Log_{10}	Logarithm to the base 10
LT-I and LT-II	Heat labile toxin I and II
M	Molar
MAC	MacConkey agar
MAC+CTX	MacConkey agar supplemented with cefotaxime
marR	MarR family transcriptional regulator
marRAB	Multiple antibiotic resistance operon
MBC	Minimum bactericidal concentration
MCP	Methyl-accepting chemotaxis protein
MDR	Multidrug resistant
mdtG	Drug efflux system protein-encoding gene
mer	Mercury resistance genes
mg	Milligram
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
min	Minute
MIR	Miriam Hospital in Providence
ml	Millilitre
MLST	Multi locus sequence typing
mM	Millimolar
mm	Millimeter
MOX	Moxalactam
MRD	Maximum recovery diluent
MRI	Multi-resistance integrons
MUG	4-methylumbelliferyl-β-D-glucuronide
NA	Nalidixic acid
NAT	N-acetyltransferase
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
ng	Nano-gram

Abbreviation	Description
NGS	Next generation sequencing
NMEC	Neonatal meningitis *E. coli*
NRP	Norwich Research Park
ONPG	O-nitrophenol-β-D-galactopyranoside
ORF	Open reading frame
*orf*5	Gene of unknown function
orfI	Methyl-accepting chemotaxis protein encoding gene
*ori*IS	Origin of IS*CR* elements
oriT	Origin of conjugative transfer
OrthoMCL	A program that aids in the identification of orthologs using Markov Cluster Algorithm
OT	Oxytetracycline
OXA	Oxacillinase
P value	Probability value
PBP	Penicillin binding protein
PBRT	PCR based replicon typing
Pc/Pcw/P2m3	Gene cassette promoters
P3	Promoter for bla_{TEM}
PCR	Polymerase chain reaction
PCU	Population correction unit
pecM	Multidrug transporter gene
PET	Plasmid-encoded toxin
PFGE	Pulsed-field gel electrophoresis
pMLST	Plasmid multi locus sequence type
pmol	Picomole
Psi	Pounds per Square Inch
qacEΔ1	Quaternary ammonium compound export protein encoding gene
qnr	Quinolone resistance gene
rDNA	Ribosomal DNA
REP	Repetitive extragenic palindromic
repA/repC	Genes encoding for replication regulatory proteins
repB	Replication protein B gene
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RO	Reverse osmosis
ROAR	Reservoirs of Antibiotic Resistance Network
rpm	Revolutions per minute
Rrf2	Transcriptional regulatory protein
s	Second
S1	Single-strand-specific nuclease
S10	Streptomycin
S300	Sulphonamide

Abbreviation	Description
sav1886	A putative multidrug transporter gene
SHV	Sulphydryl variable
SI	Synteny index
ST	Sequence type
STa and STb	Heat stable toxin a and b
STEC	Shiga-toxin producing *E. coli*
strA and *strB*	Streptomycin phosphotransferase genes A and B
STX	Shiga toxin
sul	Sulphonamide-resistant dihydropteroate synthase gene
SXT	Trimethoprim/sulfamethoxazole
T	Thymine
T6ss	Type VI secretion system
TAE	Tris-acetate-EDTA
TBX	Tryptone bile x-glucuronide
TBX+CTX	TBX agar supplemented with cefotaxime
TCC	Total coliform count
tccA	Relaxase gene
TE	Tris-EDTA
TEM	Temoneria
*ter*IS	Terminus of IS*CR* elements
tet	Tetracycline resistance gene
Tn	Transposon
tnpA	Transposase gene
tnpM	Transposase modulator gene
tnpR	Resolvase gene
tra	Plasmid transfer gene
UK	United Kingdom
UPEC	Uropathogenic *E. coli*
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
US	United States
UTI	Urinary tract infection
UV	Ultra violet
V	Volts
v/v	Volume/volume
VARSS	Veterinary Antibiotic Resistance and Sales Surveillance
vatC	Virginiamycin A acetyltransferase gene
VMD	Veterinary Medicines Directorate
VTI and VTII	Verotoxin I and II
w/v	Weight/volume
WHO	World Health Organization
WWTP	Wastewater treatment plant
wzx, *wzy*, *wzm*, *wzt*	O-antigen genes
EAST-1	Enteroaggregative *E. coli* heat stable toxin 1
FEC-1	Faecal *E. coli* beta-lactamase-1

1 . Introduction

1.1 Overview

Antimicrobial resistance (AMR) is a worldwide agricultural and clinical problem, indicating the success and speed of bacterial adaptation to lethal selection in different environments. In spite of the fact that antibiotics have been in clinical use for a little over six decades, they have existed on Earth for about three billion years, and bacteria have developed protection mechanisms towards antibiotics and other toxic chemicals (Bennett, 2008). Enteric Gram-negative bacteria inhabit the gut of man and animals, can be found in the environment, and are considered as reservoirs of multidrug resistance. Those bacteria are not restricted to an isolated environment but are easily dispersed to other environments, for example: from humans and animals to water and soil and *vice versa*. That is why the spread of AMR is regarded as a major challenge to human and animal health that needs to be controlled (Wellington et al., 2013).

Escherichia coli (*E. coli*) is a Gram-negative bacterium found as part of the commensal microflora in the human and animal gastrointestinal tract. It is a member of the Enterobacteriaceae and whilst most *E. coli* strains are harmless, or beneficial, members of the gut microflora, some pathotypes can cause nosocomial and community-acquired infections. *E. coli* is the most common cause of bacterial infections (Torres et al., 2010) and its prevalence in causing infections is due to being the most non-fastidious bacterium in the gut. These infections include diarrhoeal disease for which at least six different categories have been recognized and characterized (Talaro and and Chess, 2012). *E. coli* can also cause extraintestinal

infections (extraintestinal pathogenic *E. coli*, ExPEC). Uropathogenic *E. coli* (UPEC) are one example of these which are the predominant causative organisms of urinary tract infections (UTI), and ExPEC can cause other serious infections such as neonatal meningitis, bacteraemia, and pneumonia (Murray et al., 2009, Torres et al., 2010, Talaro and and Chess, 2012).

Between the early 1950s to early 1960s*, E. coli*, *Shigella* and *Salmonella* were the first enteric bacteria that were identified as multiply antimicrobial resistant bacteria (Watanabe, 1963, Levy, 2001). These strains posed serious clinical problems and cost lives, mainly in developing countries, then in industrialized countries. For example, in developed countries bacillary dysentery does not present a serious problem compared with countries with less developed public health, but post-war in Japan *Shigella* dysentery was a major problem (Watanabe, 1963).

Some *E. coli* strains are now resistant to members of six antimicrobial families including the more recently introduced fluoroquinolones (Karlowsky et al., 2002, Zervos et al., 2003). *E. coli* can serve as a sentinel species for antimicrobial resistance in different animals because it is a common type of enteric bacteria and easily acquires resistance and this is important because the resistance modification of this bacterium can serve as an 'early warning system' for resistance in other pathogenic bacteria (Aarestrup et al., 1998, van den Bogaard et al., 2000).

Resistance genes can arise amongst bacteria by two routes: spontaneous mutation and horizontal gene transfer (HGT) (Eisen, 2000, Ochman et al.,

2000, Silbergeld et al., 2008). To acquire these resistance genes, bacteria need a range of mechanisms that depend on different gene transfer systems such as bacterial conjugative plasmids and transposable elements (mobilizable plasmid and transposons) and integrons. Bacterial plasmids can collect arrays of antimicrobial resistance genes, by transposition (transposable elements) and site-specific recombination mechanisms (integron gene cassettes) (Bennett, 2008), leading to transferable multidrug resistance.

Using antimicrobials in agriculture and animal husbandry is a crucial issue in the UK. From 2006-2011, about 400 tonnes of antibiotics were used annually in food-producing animals (Wellington et al., 2013). Moreover, it has been estimated that annually, about 70 million tonnes of animal manure waste are used as fertilizer on agricultural land in the UK (Hutchison et al., 2004). Continuous antimicrobial selection in the soil environment enables recruitment of new genes by foreign bacteria from indigenous soil bacteria (Gaze et al., 2008).

1.2 Antimicrobials

Development of AMR in bacteria can be due to important changes in genes (and consequently the proteins they encode), and/or in changes in regulation of gene expression. Of these the former has more serious implications for public health, because the regulatory changes to expression levels of genes that encode, for example, membrane-bound efflux pumps, are somewhat rare. Bacteria usually express relatively low-level resistance to antimicrobials, unless there are changes in expression

of genes. On the other hand, genetic changes that alter protein sequences which can persist and transfer amongst bacteria and confer high-level resistance are of more serious concern (Tenover, 2006).

1.2.1 Definition

The term antimicrobial can refer to antibacterial, antiviral and antifungal agents each one of which has a specific action against specific groups of microorganisms, and in many instances the term is used synonymously with antibiotic (Giguère et al., 2013). An antimicrobial can be defined as any substance of natural, semisynthetic, or synthetic origin that kills or inhibits the growth of a microorganism, whilst an antibiotic is a substance produced by a microorganism that at low concentrations inhibits or kills other microorganisms (Giguère et al., 2013, Phoenix et al., 2014). Somewhat confusingly fully synthetic antibiotics such as ciprofloxacins are referred to as antibiotics, even though they do not fit with these definitions. It is believed that antibiotics, which are particularly linked to soil microorganisms, for example *Streptomyces* species, provide a selective advantage for organisms in their competition for nutrients and space in the natural environment (Greenwood, 2008, de Lima Procópio et al., 2012). It is also believed that antibiotics might actually act as signal molecules in natural environments, facilitating intra- or inter-species interactions within microbial communities (Romero et al., 2011).

1.2.2 History

Herbal remedies, toxic metals and currently used antimicrobials all have been used through human history to treat infections. Herbal remedies have been used for thousands of years by different cultures. Plants such

as hollyhock were thought to be used by Neanderthals living 60,000 years ago in the Shanidar caves in northern Iraq and are still being used by different people all over the world in traditional medicines (Stockwell, 1988). About 400 medicinal plants were mentioned by Hippocrates in the late fifth century B.C. (Cowan, 1999). Chinese, Indian, Northern American, African and European peoples all have a history of using plants for medicinal purposes. Herbal medicinal products can be derived from plants by consuming a part of the plant such as a leaf as a tea or as an infusion, or can be extracted from the plants as essential oils (Edwards et al., 2015). Herbal remedies are used as antimicrobials or as chemotherapy (Lai and Roy, 2004). There are a wide range of researchers who have demonstrated that commonly used herbs and spices, garlic for example, can be used therapeutically as they have antimicrobial properties (Lai and Roy, 2004), and crude antibiotic preparations appear to have been used sporadically by ancient cultures (Aminov, 2010).

With regards to use of metals and metalloids, it is known that mercury (Hg), copper (Cu), silver (Ag), and arsenic (As) were the most toxic metals used in both medicine and agriculture (Hobman and Crossman, 2015). Organic mercury has been used in agriculture for plant treatment since the early 20th Century until the 1970s (Huisingh, 1974) while inorganic mercury compounds have been used in different ways in medicines including laxatives, diuretics, antidepressants, to treat skin disorders, syphilis, and as a topical antimicrobial since at least 1400 (Hobman and Brown, 1997). In the eighteenth-century copper sulphate solutions were used as an antifungal treatment for seed grains, and are

still used as antifungal sprays, and currently copper-containing surfaces are being used as antimicrobial solid surfaces to decrease microbial contamination and transmission of hospital-acquired infections, and copper sulphate is widely used in agriculture (Hobman and Crossman, 2015). It has been reported that more than 2000 years ago silver was used in drinking water vessels as an antibacterial (Silver et al., 2006), while recently, using silver nanoparticles as an antimicrobial in products is growing (Chaloupka et al., 2010) such as in household products (Hobman and Crossman, 2015).

The first modern chemotherapeutic antimicrobial agent was Salvarsan, an organic arsenic compound ('the arsenic that saves'), a remedy for syphilis that was synthesized by Paul Ehrlich's research group in 1910. This product was developed with different formulae until its final formula, Mapharsen, which was used as the drug of choice in syphilis treatment until the wide scale availability of penicillin (Bosch and Rosich, 2008). In the early 1930s, sulphonamides, (synthetic chemical inhibitors of folate synthesis) were discovered by Domagk and other researchers in Germany, and were the first clinically successful antibacterial agents. In 1928, Alexander Fleming discovered penicillin, a fungal metabolite. However, the antimicrobials revolution only occurred after the subsequent development of efficient penicillin production by Chain and Florey during World War II. This was later followed by the discovery of many other antibiotics such as streptomycin, tetracycline and chloramphenicol, all from soil bacteria. Streptomycin, for example, which has activity against Gram-negative bacteria, was isolated from *Streptomyces griseus* in the

late 1940s by Selman Waksman. Subsequently, the development of semisynthetic and synthetic antimicrobial agents, has resulted in increasingly important compounds for infectious disease treatment (Saga and Yamaguchi, 2009, Giguère et al., 2013).

Figure 1.1 provides a skeleton of the history of antimicrobial agents, and thereafter describes the development of resistance in many target microorganisms. Usually, the use of those antimicrobial agents in humans was followed by use for veterinary applications. However, there are some antimicrobials which have been developed specifically for animal health and production for example: tiamulin, tilmicosin, ceftiofur, and tylosin. But even though an antibiotic such as ceftiofur is used for veterinary purposes (Yancey Jr et al., 1987), this is a third generation cephalosporin and the same group of antibiotics is used in human treatment as well, for example cefotaxime. Thus, development of resistance to one antibiotic could give cross-resistance to another in a chemically similar antibiotic class, even where the antibiotic has had little or no use.

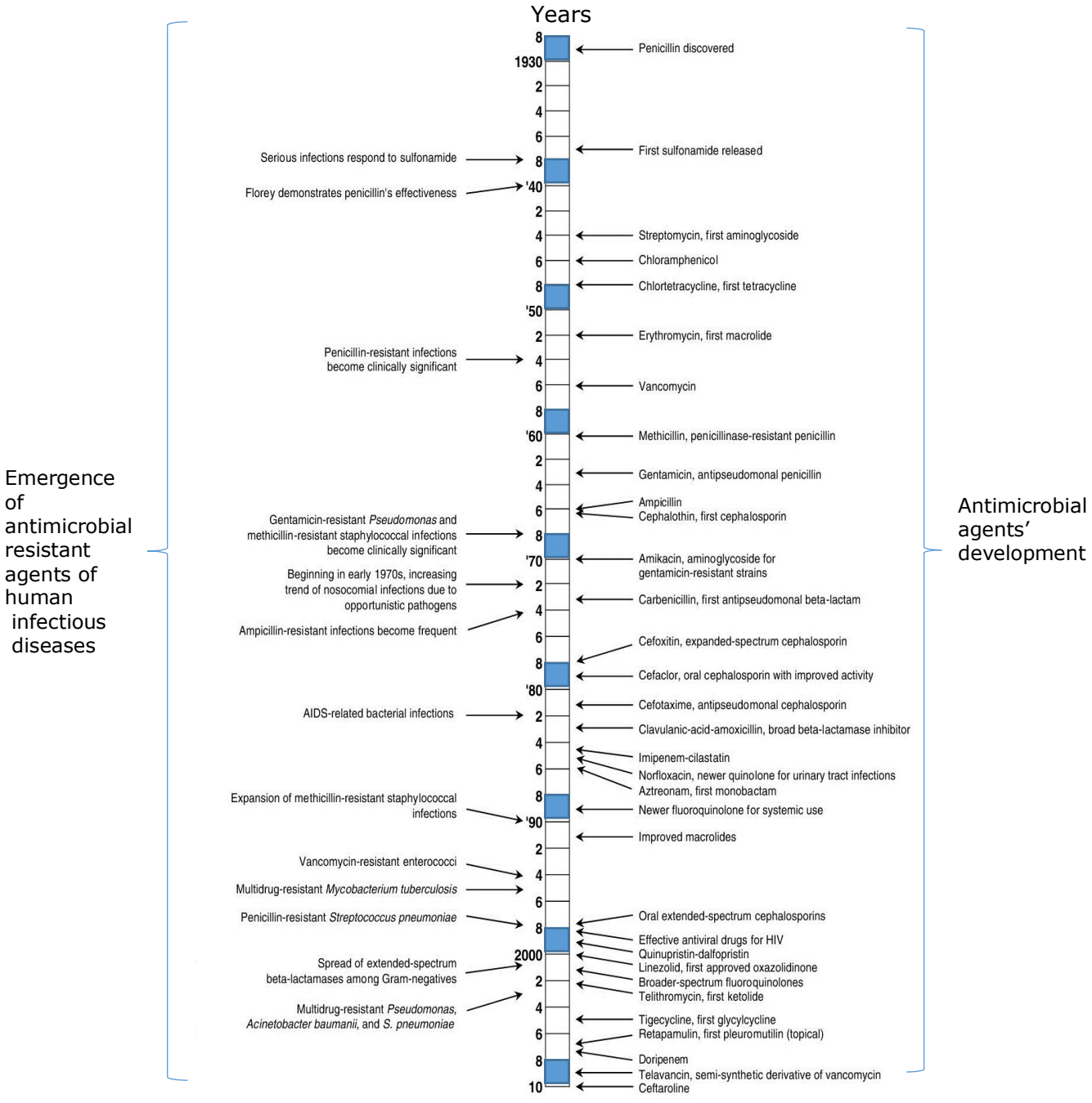


Figure 1-1: Time course of the history of antimicrobial agents in the twentieth century, and the development of resistance in many target microorganisms.

The development of the antimicrobial agent is shown in the right hand panel; the emergence of antimicrobial resistance to these agents in human infectious disease is shown in the left hand panel, and the year is indicated in the middle, adapted from Giguère et al., 2013.

1.2.3 Classification of antimicrobials

An antimicrobial can be classified in several ways. First, the target microorganism of the compound would determine its classification as an antibacterial, antifungal or antiviral. Second, depending on their activity spectrum, antimicrobials can be divided into broad-spectrum drugs which inhibit/kill both Gram-positive and Gram-negative bacteria, or narrow spectrum drugs which inhibit/kill only Gram-positive or Gram-negative bacteria or exceptionally ones which target very specific groups such as antimicrobials affecting *Mycobacterium tuberculosis*. Antibacterials can be classified depending on their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) into bacteriostatic or bactericidal (Gualerzi et al., 2013, Giguère et al., 2013). A bacteriostatic agent prevents the growth of bacteria while bactericidal agents kill the bacteria; but this is not an absolute definition, because some antibacterials can be -cidal at one concentration and -static at a lower one. The definition of bacteriostatic or bactericidal activity for an antibacterial agent varies between the results of a microbiological *in vitro* test compared to the clinical definition. For the microbiological definition, this can vary depending on many factors such as duration of the test, concentration of the agent tested, growth conditions and bacterial cell density. In the clinical definition the agent potentially can be bactericidal or bacteriostatic (Pankey and Sabath, 2004). This distinction is significant under certain clinical conditions but it is not absolute, for example, benzyl penicillin is bacteriostatic at low concentrations but bactericidal at normal therapeutic concentrations *(*Giguère et al*.*, 2013). Actually, the drug

concentration at the site of infection and the microorganism both contribute to the nature of the activity. Standardization of laboratory tests for antimicrobial activity is important because it reduces the error with the data obtained from antimicrobial sensitivity testing. That is why a consistent method should be used including the protocol and interpreting the results. Many protocols are available provided by many organizations such as Clinical and Laboratory Standards Institute (CLSI, 2013a), the European Committee on Antimicrobial Sensitivity Testing (Giske et al., 2013), and the British Society for Antimicrobial Chemotherapy (Andrews and Howe, 2011).

Antimicrobials can be classified on the basis of their mechanism of action into four main groups depending on their bacterial targets (Hooper, 2001): 1) Agents that interfere with cell wall synthesis such as β-lactams: penicillins, cephalosporins, carbapenems, monobactams and glycopeptides (Neu, 1992, Tenover, 2006); 2) Protein synthesis inhibitors which can be divided into two subgroups: agents that bind to the 50S ribosomal subunit such as macrolides, chloramphenicol, clindamycin, lincosamides, streptogramins, and linezolid; and agents that bind to the 30S ribosomal subunit such as aminoglycosides and tetracyclines (Tenover, 2006, Giguère et al., 2013); 3) Inhibitors of certain metabolic pathways such as sulphonamides and folic acid analogues (Anderson et al., 2012); 4) Nucleic acid synthesis inhibitors and gyrase inhibitors such as fluoroquinolones which inhibit DNA synthesis and rifampicin which inhibits RNA synthesis (Drlica and Zhao, 1997, Tenover, 2006). Figure 1.2 shows the target and pathways of different antimicrobial agents.

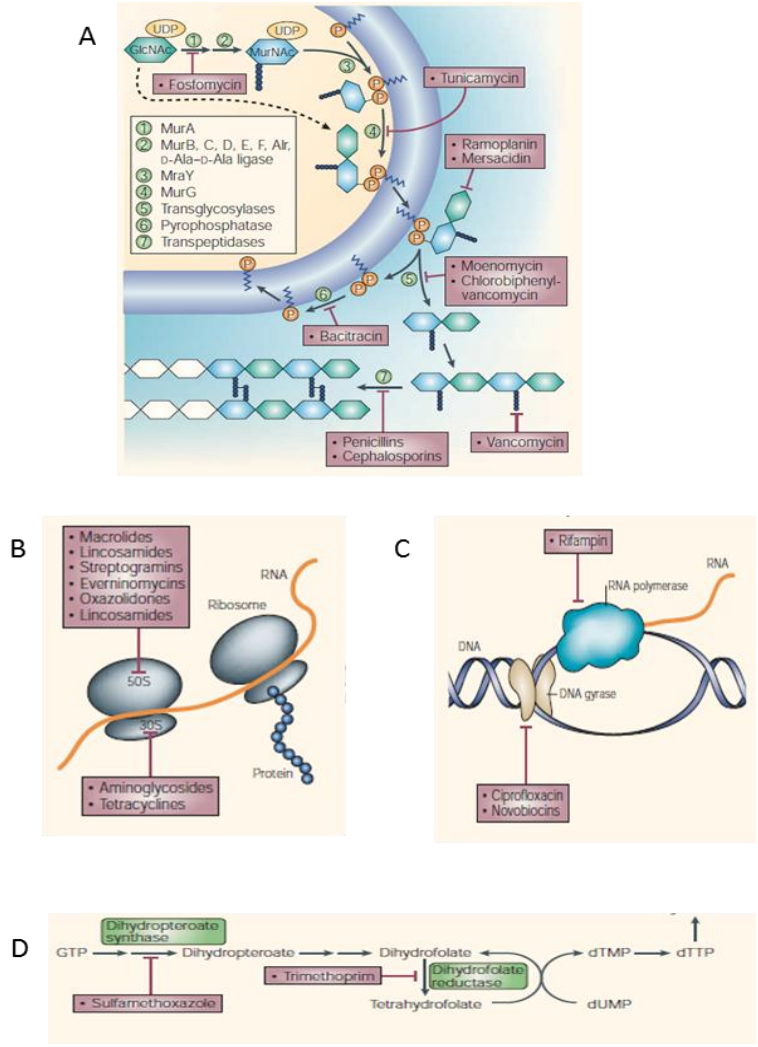


Figure 1-2: Principal mechanisms of action and cellular targets for antimicrobial agents.

A: Cell wall biosynthesis inhibitors: different antibacterial agents inhibit different steps in cell wall synthesis, starting from the inhibition of MurA (UDP-N-acetylglucosamine 1-carboxyvinyltransferase) and MurG (UDP-diphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase) inside the cytoplasm (steps 1-4), then in the periplasmic space the inhibition of transglycosylation and pyrophosphorylation (steps 5-6), and finally inhibition of transpeptidation. B: protein synthesis inhibition e.g.: by interfering with protein synthesis initiation (30S), or peptide chain elongation (50S). C: DNA synthesis inhibition by interfering with DNA gyrase which provides negative supercoiling for the DNA during DNA replication; RNA synthesis inhibition by interfering with DNA-dependent RNA polymerase. D: Folate metabolism inhibitors, interfering with 2 important enzymes in the folic acid synthase pathway which are: dihydropteroate synthase and dihydrofolate reductase, adapted from Walsh 2003.

1.3 β-lactam antibiotic mechanism of action and bacterial resistance

The name of the β-lactam group of antibiotics comes from the presence of a β-lactam ring in their structure; this ring is vital for antimicrobial activity (Figure 1.3). The β-lactam antibiotics include penicillins, cephalosporins, carbapenems and monobactams (Gualerzi et al., 2013). The mechanism of action of this group of antibiotics is based on their ability to interfere with bacterial cell wall synthesis. The β-lactam antibiotic inhibits the formation of a peptide bond during the biosynthesis of the peptidoglycan layer. The cross-linking of adjacent glycan strands is therefore prevented by the incorporation of the ß-lactam ring. The β-lactam ring binds to the transpeptidase (penicillin binding protein) and thereby stops formation of the peptide bond and destabilizes the cell wall (Sköld, 2011; Figure 1.4).

The most common resistance mechanism to ß-lactams is by the production of β-lactamase enzymes which hydrolyze the β-lactam ring which results in inactivation of the antibiotic (Bush, 1989, Walsh, 2003, Zapun et al., 2008). Other resistance mechanisms have also been reported such as reduced accumulation of the antibiotic. This reduced accumulation can occur as a result of alterations in cell wall permeability (for example, the loss of porins may enable a reduction in susceptibility, due to a reduced uptake of antimicrobials (Jaffe et al., 1982, Martinez-Martinez, 2008), or due to the action of efflux pumps. Different efflux pumps are thought to play a role in lowering the drug concentration within the bacterial cell (Fernandez and Hancock, 2012).

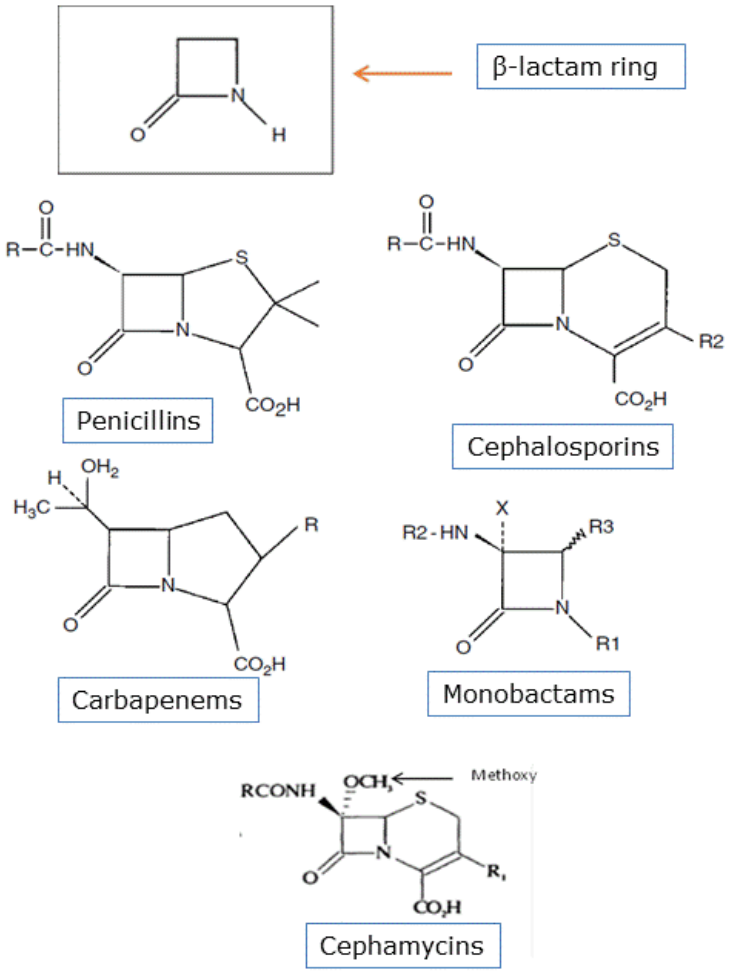


Figure 1-3: The core structure for β-lactam antibiotic groups.

This figure is adapted from Page (2012) and Giguère et al. (2013). The entire group of β-lactam antibiotics (penicillins, cephalosporins, carbapenems and monobactams) share the core structural feature of the β-lactam ring. They differ by the structure of the five or six membered rings they are fused to (and numbers of the side chains in each different group). Monobactams are β-lactams that are not fused to any other ring. Cephamycins are second generation cephalosporins that differ from the other cephalosporins as they contain a 7-α-methoxy group. This group of antibiotics can inhibit most ESBLs but not the AmpC enzyme.

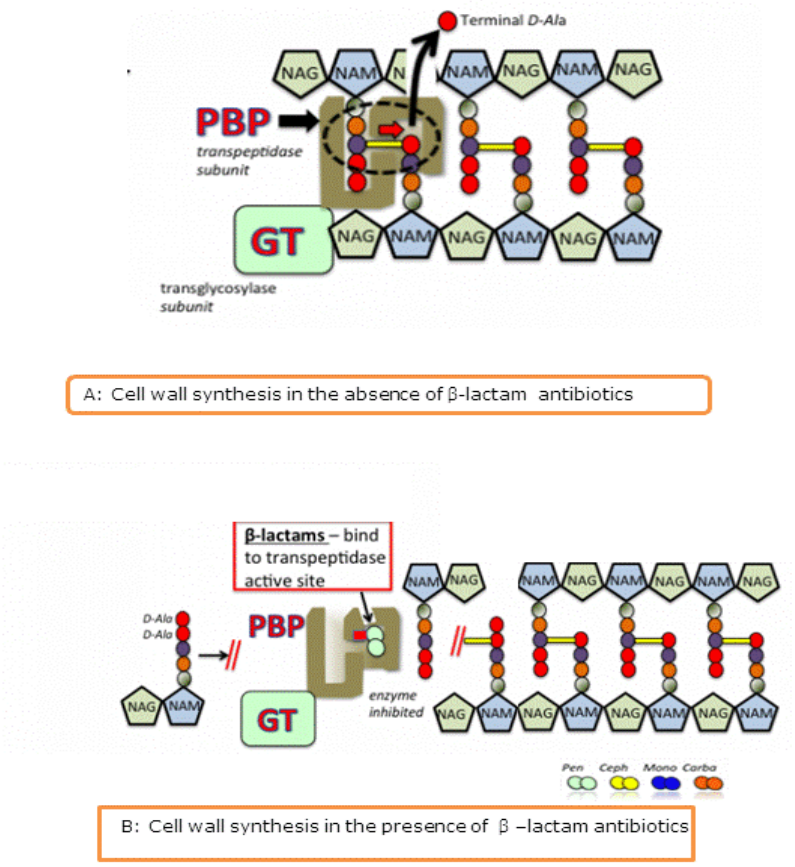


Figure 1-4: Mechanism of action of β-lactam antibiotics.

The figure shows (A): Cell wall formation in the absence of β-lactam antibiotics. (B): the inhibitory effects of penicillins (Pen), cephalosporins (Ceph), monobactams (Mono) and carbapenems (Carba) on cell wall synthesis. All β-lactam antibiotics interfere with cell wall synthesis by tightly binding to the active site of the transpeptidase (PBP - known alternately as Penicillin Binding Protein). GT denotes Glycosyltransferases, which exist as either separate subunits, or tightly associated with transpeptidases and which create covalent bonds between the adjacent NAG (N-acetylglucosamine) and NAM (N-acetylmuramic acid) sugar molecules. D-Ala denotes D-alanine, one of the 4-5 amino acids attached to NAG, adapted from (http://tmedweb.tulane.edu/pharmwiki/doku.php/betalactam).

1.3.1 β-lactamases

Penicillinase was the first described β-lactamase and acts by hydrolysing the β-lactam ring (Vu and Nikaido, 1985, Saga and Yamaguchi, 2009). The enzyme was first described in *E. coli* in 1940 (Abraham and Chain 1940) in the same year as the clinical introduction of penicillin; Thereafter many other β-lactamases have been identified which has led to a requirement to develop a classification system. Many classification systems were introduced, of which two are widely accepted: The Bush, Jacoby and Medeiros classification based on functional characteristics of the enzymes in relation to their molecular structure, and the older one by Ambler based on the structure of the β-lactamases (Hall and Barlow, 2005, Bush and Jacoby, 2010).

According to the Ambler classification β-lactamases can be divided into 4 classes. The first 2 classes were originally specified Ambler Class A which includes the active-site serine β-lactamases, and Class B which includes metallo-β-lactamases. Then another 2 classes were added: Class C which includes members of the AmpC group of β-lactamases; and Class D the OXA β-lactamases, Table 1.1 (Ambler, 1980, Jaurin and Grundström, 1981, Ouellette et al., 1987, Hall and Barlow, 2005). The other system is the Bush, Jacoby and Mederios classification based on enzyme functionality. This system was introduced in 1995 and updated in 2010. The current classification divides the β-lactamases into three major groups. Group 1 are cephalosporinases such as AmpC, which are poorly inhibited by clavulanic acid. Group 2 is the biggest group and has many subdivisions; it includes penicillinases, early cephalosporinases and

extended spectrum cephalosporinases and serine carbapenemases, of which most are inactivated by β-lactamase inhibitors such as clavulanic acid. Group 3 includes metallo β-lactamases, which hydrolyse carbapenems. These enzymes are not inhibited by clavulanic acid or tazobactam and have poor hydrolytic activity against monobactams (Bush et al., 1995, Bush and Jacoby, 2010; Table 1.1).

Table 1-1: Some selected β-Lactamases from Gram-Negative Bacteria

β- Lactamase	Molecular Class	Substrates	Inhibition by clavulanic acid	Examples
Broad-spectrum	A	Benzylpenicillin (penicillin G), aminopenicillins (amoxicillin and ampicillin), Carboxypenicillins (carbenicillin and ticarcillin), ureidopenicillin (piperacillin), narrow-spectrum cephalosporins (cefazolin, cephalothin, cefamandole, cefuroxime, and others)	+++	TEM-1, TEM-2, SHV-1
	D	Substrates of the broad-spectrum group plus cloxacillin, methicillin, and oxacillin	+	OXA family
Expanded-spectrum	A	Substrates of the broad-spectrum group plus oxyimino-cephalosporins (cefotaxime, cefpodoxime, ceftazidime, and ceftriaxone) and monobactam (aztreonam)	++++	TEM family and SHV family
	A	Same as for TEM family and SHV family	++++	Others (BES-1, GES/IBC family, PER-1, PER-2, SFO-1, TLA-1, VEB-1, and VEB-2)
	A	Substrates of the expanded-spectrum group plus, for some enzymes, cefepime	++++	CTX-M family
	D	Same as for CTX-M family	+	OXA family

Table 1-1 continued

β- Lactamase	Molecular Class	Substrates	Inhibition by clavulanic acid	Examples
AmpC	C	Substrates of expanded-spectrum group plus cephamycins (cefotetan, cefoxitin, and others)	0	ACC-1, ACT-1, CFE-1, CMY family, DHA-1, DHA-2, FOX family, LAT family, MIR-1, MOX-1, and MOX-2
Carbapenm-ase	B	Substrates of the expanded-spectrum group plus cephamycins and carbapenems (ertapenem, imipenem, and meropenem)	0	IMP family, VIM family, GIM-1, and SPM-1
	A	Same as for IMP family, VIM family, GIM-1, and SPM-1	+++	KPC-1, KPC-2, and KPC-3
	D	Same as for IMP family, VIM family, GIM-1, and SPM-1	+	OXA-23, OXA-24,OXA-25, OXA-26, OXA-27, OXA-40, and OXA-48

+: denotes relative sensitivity to inhibition; 0: denotes resistance to inhibition; A: Ambler Class A which includes the active-site serine β-lactamases; B: Class B which includes metallo-β-lactamases; C: Class C which includes members of the AmpC group of β-lactamases; D: Class D include the OXA β-lactamases; adapted from Jacoby and Munoz-Price, 2005.

1.3.2 ESBL/AmpC

A brief description of two important types of ß-Lactamases: Extended Spectrum ß-Lactamases (ESBL), referring to the expanded substrate spectrum of enzymes derived from narrower-spectrum β-lactamases (Jacoby and Munoz-Price, 2005), and AmpC ß-Lactamases, and the main differences between them will be given.

Both ESBLs and AmpC ß-Lactamases can confer resistance to third generation cephalosporins, penicillins and monobactams. However, these two groups of enzymes are very distinct. Phenotypically these enzymes can be differentiated by their different susceptibility to β-lactam site-specific inhibitors such as clavulanic acid, fourth generation cephalosporins such as cefepime, and cephamycins (second generation cephalosporins which have 7-α-methoxy group) such as cefoxitin (Figure 1.3). ESBL-type enzymes can be inhibited by β-lactamase inhibitors, can confer resistance to 4^{th} generation cephalosporins, but cannot hydrolyze cephamycins. AmpC-type enzymes can hydrolyze cephamycins, and are not inhibited by β-lactamase inhibitors, but cannot hydrolyze 4^{th} generation cephalosporins (Moritz and Carson, 1986, Livermore et al., 2001, Bush and Jacoby, 2010, CLSI, 2013a).

ESBL resistance genes are mostly located on mobile genetic elements such as plasmids, but can be situated on the chromosome as well. There are many gene families within the group of ESBLs e.g. *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$ and *bla*$_{VEB,}$ but also many others exist. Genes encoding broad-spectrum β-lactamases like TEM-1 (from Temoniera, the name of the patient) and SHV-1 (for sulphydryl variable) were discovered in the early

1960s (Medeiros, 1984, Bradford, 2001). Other ESBLs evolved from TEM-1, TEM-2, SHV-1, and OXA-10 due to mutations in the active site of the enzyme, which led to an increased spectrum of activity, especially against the oxyimino-cephalosporins (Kliebe et al., 1985, Paterson and Bonomo, 2005, Canton et al., 2012). Comparison of the DNA sequence of genes encoding SHV type ESBLs has shown that the new variant SHV-2 has a glycine to serine substitution at amino acid 238 compared to SHV-1 (Paterson et al., 2003). Similar mutations have occurred in TEM ESBLs where a change of two amino acids in TEM-3 compared to TEM-2 means that bacterial strains carrying TEM-3 are ESBLs and can hydrolyse 3rd generation cephalosporins. The sequence of TEM-3 differs in two positions compared to TEM-2: one mutation is at position 104 in which glutamic acid (TEM-2) has been replaced by lysine (TEM-3). The other point mutation means glycine (TEM-2) has been replaced by serine (TEM-3) at amino acid 238 (Sougakoff et al., 1988, Paterson and Bonomo, 2005). Similarly, OXA type ESBL enzymes have also been generated from mutations to the parent OXA-type enzyme such as OXA-10. OXA-28 which has ESBL activity differs from OXA-10 by 10 amino acid changes including a glycine to tryptophan substitution at position 164, which is likely to be important in its ability to hydrolyze ceftazidime (Poirel et al., 2001).

In the 1980s, new types of ESBLs were discovered. The first, FEC-1, was reported in Japan from *E. coli* isolated from a dog faecal sample (Matsumoto et al., 1988), and the other is represented by CTX-M, which is currently the most widely reported family of enzymes which confer extended spectrum cephalosporin (ESC) resistance in Enterobacteriaceae (Rossolini et al.,

2008). The first isolates of the CTX-M family of β-lactamase enzymes were found in both Europe and South America in 1989 (Bauernfeind et al., 1990, Bauernfeind et al., 1992, Canton et al., 2012). The name CTX-M is based on the finding that the enzyme conferred resistance to cefotaxime, with the 'M' standing for Munich where the strain was isolated (Canton et al., 2012). Subsequent sequencing of FEC-1 showed that this enzyme differed from the CTX-M-3 enzyme by a 2 amino acid substitution (Bonnet, 2004), CTX-M-3 was first described in Poland in different members of the family Enterobacteriaceae in 1996 (Gniadkowski et al., 1998). The CTX-M family can be classified into five main groups, based on similarity at the amino-acid sequence level: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 (Bonnet, 2004); a further group, group six (CTX-M-45) was added to the classification later. Each group includes minor allelic variants that differ from each other by a single or few amino acids (Rossolini et al., 2008). A number of environmental species of the genus *Kluyvera* with little or no pathogenic activity against humans is recognized as being the original source of genes encoding CTX-M-type β-lactamases, which in *Kluyvera* are chromosomally located genes (Rossolini et al., 2008). Up-to-now, more than 160 CTX-M variants, 220 TEM variants and 190 SHV variants have been described (http://www.lahey.org/Studies/).

AmpC is a β-lactamase enzyme found in most Enterobacteriaceae (Philippon et al., 2002). AmpC when first detected in 1940 was called penicillinase as it has the ability to destroy penicillin (Abraham and Chain, 1940). The *ampC* gene is a structural gene located on the chromosome, which is expressed at low levels and expression of which is regulated by an array of genes such as

ampR, G and *D* (Jacobs et al., 1997, Wiedemann *et al*., 1998). AmpC enzymes, like other β lactamases, hydrolyse the β-lactam ring, and inactivate β-lactam based antibiotics. However, unlike other β-lactamases, AmpC production is inducible. In *E. coli* the *ampC*-system lacks the presence of the regulatory *ampR* and other genes, resulting in a non-inducible chromosomally-encoded *ampC* gene (Honore et al., 1986, Philippon et al., 2002, Jacoby and Munoz-Price, 2005, Jacoby, 2009). However, hyper-production can still arise, either by mutations at the *ampC* attenuator or promoter regions or by gene amplification (Caroff et al., 1999, Nelson and Elisha, 1999, Philippon et al., 2002). Bacterial species lacking the hyperproduction of AmpC e.g. *E. coli*, or which do not have the chromosomal *ampC*-gene, such as *Salmonella*, can become AmpC producers by the acquisition of plasmid-mediated *ampC*-genes. Many different families of plasmid-mediated AmpC β-lactamases have been described such as CMY, ACC, ACT, MOX, DHA, MIR and FOX. The most common plasmid-mediated *ampC*-gene is the CMY-2 gene belonging to the CMY-family (Philippon et al., 2002, Jacoby, 2009).

Nowadays, more than 230 variants from the different plasmid AmpC families are known, 136 of which belong to the CMY family (http://www.lahey.org/studies/other.asp#table1).

1.4 Monitoring antimicrobial resistance

In the last six decades, the discovery and the clinical use of antibiotics (including the synthetic antibiotics), together with improvements in immunization, have radically reduced human suffering from infectious

diseases. Beyond treatment of infections, surgical operations, transplants and chemotherapy rely on other antimicrobials, including disinfectants and antiseptics, and not just antibiotics. The crisis posed by antimicrobial resistance is so alarming because antimicrobial agents are widely relied upon in clinical and veterinary medicine. There is increasing evidence to show that a consequence of this heavy usage of antimicrobials has been the emergence of resistance (Davies, 1994, Courvalin, 1996, Meek et al., 2015). A case in point is data from the Centre for Disease Control and Prevention (CDC) in the United States which demonstrate that the appearance of antimicrobial resistant bacteria is associated with increased use of the antimicrobials (Control and Prevention, 2013). Antimicrobial resistance has been widely recognized as a growing problem and many organizations and networks monitor antimicrobial resistance trends locally and internationally. Most of these networks are monitoring resistance in clinical isolates. However, the Reservoirs of Antibiotic Resistance Network (ROAR) is an exception, as it focuses on antimicrobial resistance in commensal bacteria in animals, and in natural environments, such as soil and water (Nwosu, 2001, Davis and Rutkow, 2011). It is now widely recognized that the large amounts of antimicrobials which are used worldwide to increase the animal production of milk and meat, through the use of antimicrobials as additives in livestock feeds, have led to an increase in antimicrobial-resistant pathogens that infect humans resulting from the food chain (Silbergeld et al., 2008).

As a consequence, since 1997 some organizations such as the World Health Organization (WHO), the International Organization for Epizootics

and the Food and Agriculture Organization, have suggested limitations on the use of antimicrobials, with this restriction applying especially to the antimicrobials that are used in agriculture for non-therapeutic purposes, as well as veterinary oversight on the use of antimicrobials in food livestock (WHO., 2001). Later, many other organizations have developed a surveillance system to monitor the antibiotic resistance trends, such as the Veterinary Medicines Directorate (VMD) providing detailed reports of UK veterinary antibiotic resistance and sales (https://www.gov.uk). The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP, 2012) is another example of such a monitoring programme and more recently the Colombian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS) was established (Donado-Godoy et al., 2015).

1.5 Resistance gene transfer in bacteria

As already discussed, resistance genes can arise among bacteria as a result of spontaneous mutation or as a result of transfer from other bacteria which can lead to rapid development towards a resistant phenotype by horizontal gene transfer (HGT) (Eisen, 2000, Ochman et al., 2000, Silbergeld et al., 2008). There are three ways in which HGT can occur: transduction, conjugation and transformation (Davison, 1999). Horizontal gene transfer or lateral transfer has more effect on bacterial evolution than the sequential modification by mutation in terms of the capacity of bacteria to exploit novel environments and to reply to a new selective pressure (Syvanen, 1994). The transfer of antibiotic resistance genes which occurs due to lateral transfer and the selective pressure that

increases by accumulation of the amount of an antibiotic in a specific environment is an example of this (Davies, 1994, Salyers and Shoemaker, 1996, Meek et al., 2015).

It has been found that the phenotypic traits used to differentiate between *E. coli* and *Salmonella enterica* are due to lateral gene transfer, and not point mutations (Ochman et al., 2000). The complete genome sequence of *E. coli* K-12 MG1655 has given an indication of the volume of horizontal gene transfer between *E. coli* and other bacteria. For example, it has been shown that 17.6% of the genes in MG1655 (755 of the 4288 ORFs) have been acquired by horizontal transfer since this lineage separated from *S. enterica* with at least 234 lateral transfer events (Lawrence and Ochman, 1998).

Nwosu (2001) estimated that less than 5% of antibiotic resistance is due to chromosomal-based determinants while the rest is due to mobile genetic elements including plasmids, transposons, integrons, gene cassettes, and bacteriophages (Nwosu, 2001). This has been noticed with antibiotic resistant *E. coli* strains that have integrons harbouring more than one resistance gene which have been isolated from consumer meat products (Sunde and Norstrom, 2006). Antibiotic resistance is the best example of the influence of horizontal transfer on bacterial adaptation (Rowe-Magnus et al., 2002). Bioinformatic methods have been developed to detect HGT between even very closely related bacteria, such as Phylo SI (*synteny index*) based on the conservation of gene order among species besides considering the events of gene gain/loss (Shifman et al., 2014).

Adato and co-workers (2015) have developed another method named Near HGT based on SI to identify HGT events between closely related strains. This approach is based on the concept of constant relative mutability (CRM), where genes conserve their relative evolution rate along lineages. They have shown that this method is very sensitive and can be applied even inter-species. They have applied this method to eight *E. coli* strains and identified the HGT events between them. The strains were *E. coli*: CFT073, EDL933, MG1655 (K-12 derivative), enteroaggregative *E. coli* 042 (denoted 042 in Figure 1.5), uropathogenic *E. coli* 536 (denoted 536), enterotoxigenic *E. coli* W (denoted W), enterohaemorrhagic *E. coli* O157:H7 str. TW14359 (denoted TW14359) and enteropathogenic *E. coli* O55:H7 str. CB9615 (denoted CB9615) (Adato et al., 2015). The number of HGT events between pairs of the tested strains is given and represented as a part of the pie chart, Figure 1.5.

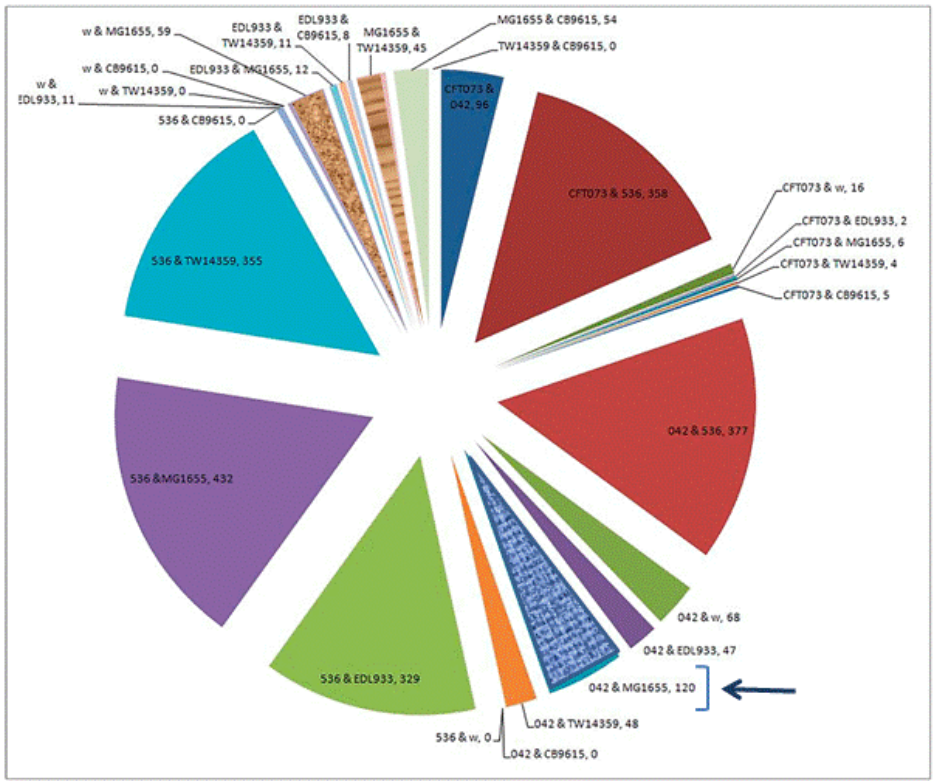


Figure 1-5: Near HGT method used to detect horizontal gene transfer (HGT) between 8 strains of *E. coli*.

This figure shows the application of Near HGT to 8 *E. coli* strains to detect HGT events per strain pair. Each part of the pie chart represents two strains (e.g. O42 & MG1655; indicated by an arrow within the figure) and the number of identified HGT events (e.g. 120); adapted from Adato et al. (2015).

Horizontal transfer occurs among *E. coli* species by conjugation by different mechanisms, such as transfer of self-transmissible conjugative plasmids (e.g. F-plasmid), mobilization (e.g. mobilization of the IncQ plasmid RSF1010 by conjugative IncP1 plasmids such as RP4) (Davison, 1999) and cointegration. Chromosomal gene transfer is a specialized form of cointegrate formation, for example via the formation of a Hfr (high frequency recombinant) by the F-plasmid of *E. coli* (Clewell et al., 1995).

Transduction is another mechanism of horizontal gene transfer between different types of bacteria, due to the integration of the bacterial gene into the genome of a bacteriophage particle and transfer to another bacterial species. In spite of the fact that bacteriophage are widespread in the environment, there are some reasons for the limitation of their transfer to some types of bacteria (Jiang and Paul, 1998). A case in point is mutation that would make the phage unable to attach to the bacteria and would result in bacterial resistance to that phage. Some bacteriophage have a very narrow host range and are able to infect a specific bacterial species or specific strains of that species only. With respect to *E. coli*, it has been shown that phage that encodes for the shiga toxin in *E. coli* O157:H7 are common in sewage (Muniesa and Jofre, 1998).

Moreover, horizontal gene transfer can arise due to the incorporation of naked DNA from the environment into the bacterial chromosome by homologous recombination. This is known as genetic transformation (Baur et al., 1996). DNA is quite common in nearly all environments regardless of its sensitivity to nucleases, and it can be excreted by living bacteria or

liberated through autolysis. There are two types of transformation, natural transformation and artificial transformation (Lorenz and Wackernagel, 1994). Many bacteria can develop natural transformation, however there are differences between them; some bacteria become competent in the natural course of their life cycle such as *Streptococcus pneumoniae* whereas others are always in a competent state such as *Neisseria gonorrhoea* (Lorenz and Wackernagel, 1994, Lunsford, 1998). Another species, e.g. *E. coli,* can exhibit both natural and artificial transformation. *E. coli* is not assumed to be naturally transformable. However, it has been reported that *E. coli* can express modest genetic competence in specific conditions that may arise in its environment (Etchuuya et al., 2011). Bauer et al. (1996) confirmed that *E. coli* can become naturally competent for transformation in spring water, river water, and mineral water which contains between 0 and 11 mM Ca^{2+}. In addition to that, transformation in *E. coli* can be induced by many physical or chemical processes to accept free DNA (Baur et al., 1996). Artificial transformation is an *in vitro* procedure used to introduce DNA molecules into bacterial cells (Lorenz and Wackernagel, 1994).

1.6 Mobile genetic elements involved in transferring resistance genes

Transposable genetic elements can be transferred between bacteria from different phyla, so resistance genes can be acquired from many sources. However, it is more common that mobile elements transfer between species that share similar niches, being drawn from related gene pools. Mobile genetic elements that connect Enterobacteriaceae in human beings,

animals, and terrestrial and aquatic environments are especially associated with transposons, integrons and gene cassettes (Hutchison et al., 2004). These elements can become linked with a conjugative transfer system, such as plasmids, which will further increase the mobility of the gene (Osborn and Boltner, 2002).

1.6.1 Bacterial Plasmids

Most bacteria contain self-replicating multiple copies of small extra chromosomal DNA called plasmid DNA. These are mostly circular, double stranded DNA (dsDNA) molecules that are separate from chromosomal DNA (Lederberg, 1952, Lodish et al., 2000, Frost et al., 2005). Plasmid DNA is very important for bacteria because it carries genes that may be useful periodically to enable bacteria to exploit particular environmental conditions. Plasmids accumulate antibiotic resistance genes as a consequence of recombination activities caused by transposons and integrons (Bennett, 2008). Plasmid-encoded resistance genes can protect bacteria against certain antibiotics, and plasmids may also carry genes that enable bacteria to live in the presence of toxic metals such as mercury, cadmium and silver, detergents and other antimicrobials. These plasmids are called resistance or 'R' plasmids. Plasmids may also carry virulence determinants that allow the bacteria to survive and invade human and animal systems, which are called virulence plasmids (Lodish et al., 2000, Smillie et al., 2010).

Plasmids are widespread in most bacterial species that have been investigated up to now; some can carry more than one plasmid, encoding a large pool of transportable genetic information. Plasmids can transfer many bacterial genes from one cell to another, with conjugative plasmids able to

support their own transfer and the transfer of other mobilizable plasmids from one bacterial cell to another (Frost et al., 2005, Bennett, 2008).

Many of these conjugative plasmids encode resistance to most types of antimicrobials, such as fluoroquinolones, cephalosporins and aminoglycosides. Other plasmids that encode antimicrobial resistance are mobilizable. Unlike the conjugative plasmids, mobilizable plasmids do not have the genes that encode the conjugation ability which enables cells to mate prior to DNA transfer; they just encode the functions required for transfer of their own DNA. Moreover, the minimum size of mobilizable resistance plasmids is smaller than conjugative plasmids, about 10 kbp compared to 30 kbp, for conjugative plasmids. This is due to the sizeable amount of DNA that is essential to encode the conjugation functions mainly between Gram-negative bacteria. Conjugation is a replicative method that gives both the donor and recipient cells a copy of the plasmid (Francia et al., 2004, Smillie et al., 2010). Such conjugation generally needs an external filamentous appendage called the sex pilus that connects the donor and recipient cells (Lawley et al., 2003, Frank et al., 2005). Some conjugative plasmids can exhibit a narrow host range in which the transfer is limited to and between small numbers of similar bacterial species; IncF type plasmids which carry CTX-M ESBLs are an example of this as they are large, narrow host range plasmids with low copy number (Brolund, 2014). On the other hand, broad host range plasmids can transfer between broadly diverse bacterial species (Sen et al., 2011, Heuer and Smalla, 2012, Brolund, 2014). In Gram-negative bacteria, some of these show no host limitation within the division such as IncA/C, IncL/M, IncN and IncI plasmids

which are broad host range and regarded as endemic resistance plasmids in Enterobacteriaceae. The resistance plasmid RP1 (also known as RP4) is an example of a broad host range plasmid which was recognized in a clinical strain of *Pseudomonas aeruginosa* for the first time and it has the ability to transfer effectively to most, if not all, Gram-negative bacteria (Bennett, 2008).

There are different methods to characterise plasmids. One of these is determining the incompatibility group, which is based on the plasmid replicon type, as plasmids belonging to the same incompatibility group are unable to be propagated stably in the same bacterial cell (Novick, 1987, Carattoli, 2009). Currently, a PCR-based replicon typing (PBRT) scheme for the determination of different replicon types for plasmids can be used for characterization of 18 different incompatibility groups (Carattoli et al., 2005).

Another PCR-based method for plasmid characterisation depends on the amplification of the relaxase gene as it is the common component in all transmissible (conjugative and mobilizable) plasmids (Alvarado et al., 2012). Plasmid multi locus sequence type (pMLST) is a sequence-based typing scheme which is available for characterization of five different incompatibility groups, IncF, IncI1, IncN, IncHI2, and IncHI1 (Phan et al., 2009, Garcia-Fernandez and Carattoli, 2010, Villa et al., 2010). Currently, this method can be used directly by using whole genome sequencing data in raw data form and uploading this to a specific on-line data base (https://cge.cbs.dtu.dk/services/pMLST/) (Carattoli et al., 2014). The determination

of the full-length DNA sequences is the most accurate method to characterize a plasmid, and currently, more than 800 plasmids from *Gammaproteobacteria* have been fully sequenced which has contributed to the identification of novel plasmid families (Carattoli, 2009).

With regard to the determination of the exact plasmid size, using electrophoresis methods, especially for big plasmids, can be difficult because the supercoiled nature of plasmid DNA causes it to have different mobility under agarose gel electrophoresis than the linear DNA typically used as a size marker, making sizing inaccurate if this is not addressed. In addition, large plasmids can be difficult to separate from linear chromosomal DNA. One approach which can be used to overcome these issues is demonstrated by the S1 PFGE method (Barton et al., 1995). This method is based on the conversion of supercoiled plasmids into full-length linear molecules by digesting with S1 nuclease and then separation of these fragments by pulsed field gel electrophoresis which produces better separation of large linear fragments. The sizes of the plasmids can then be estimated by comparison of fragments with a linear DNA ladder (Barton et al., 1995). Restriction digestion with some restriction enzymes is another way to determine plasmid size, as this also produces readily separable linear fragments, (Nauerby et al., 2000) and is especially useful if it is known that the strain has one plasmid, but this method is somewhat problematic in strains with multiple plasmids.

IncF, IncN, IncN2, IncI1, IncHI2, IncL/M, IncA/C, IncK, IncX4, and IncU are examples incompatibility groups of plasmids which encode resistance in the

Enterobacteriaceae and are associated with genes encoding CTX-M enzymes (Valverde et al., 2009, Chouchani et al., 2012, Carattoli, 2013, Yang et al., 2015). This can explain the wide dissemination of *bla*$_{CTX-M}$ genes between different genera of Enterobacteriaceae and from different hosts, as these genes can be carried by most plasmid replicon types (Carattoli, 2009). For example, the widespread distribution of *bla*$_{CTX-M-15}$ is important to the current epidemiology of ESBLs worldwide (Paterson and Bonomo, 2005, Livermore et al., 2007). Some authors have highlighted the dissemination of the highly virulent and internationally prevalent clone *E. coli* O25:H4-ST131 as responsible for the pandemic dissemination of this gene (Nicolas-Chanoine et al., 2008). However, the role of mobile genetic elements has also been recognised. The *bla*$_{CTX-M-15}$ gene has been found to be carried mainly by plasmids belonging to the IncF group, and *bla*$_{CTX-M-15}$ is often associated with the *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, and *aac(6)-Ib-cr* resistance genes (Woodford et al., 2009). The IncF plasmids are low-copy-number plasmids, often carrying more than one replicon (Carattoli, 2009). *E. coli* with different ST such as ST405, ST354, ST28, and ST695, were found to contain IncF plasmids carrying *bla*$_{CTX-M-15}$ (Coque et al., 2008). Besides, *bla*$_{CTX-M-15}$ has been found in other Enterobacteriaceae; in *Salmonella enterica* serovar Enteritidis in the United Kingdom, in a *Shigella sonnei* strain isolated from a Czech patient, and in a *Klebsiella pneumoniae* strain isolated in Spain (Coque et al., 2008, Hrabák et al., 2008, Diestra et al., 2009).

The *bla*$_{VEB-1}$ gene is another example of worldwide spread of a resistance gene. It was first reported in an *E. coli* isolate from a Vietnamese patient in 1996 (Naas et al., 2008). This gene has subsequently been identified in

many Gram-negative bacteria, including Enterobacteriaceae, *P*. *aeruginosa*, and *A*. *baumannii*. The *bla*$_{VEB-1}$ gene was found to be located on a single IncA/C group plasmid in strains of Enterobacteriaceae isolated from Canada, France, Thailand, and Turkey. Interestingly, *P. aeruginosa* carrying *bla*$_{VEB-1}$ from the same countries mentioned above was negative for the IncA/C plasmid type, which ruled out the idea that these plasmids were also the origin of *bla*$_{VEB-1}$ acquisition in *P. aeruginosa* (Poirel et al., 2007, Carattoli, 2009). Plasmids carrying several drug resistance genes are generally large and therefore have a low copy number (DeNap and Hergenrother, 2005). Table 1.2 shows some examples of plasmids carrying ESBL genes from *E. coli* with some of their characteristics such as the size, replicon type and the isolation sources; a lot of them are all from cattle sources.

Table 1-2: Examples of plasmids carrying ESBL genes from *E. coli* isolated from different hosts.

Plasmid or strain ID*	Size(bp) / Replicon (Inc) type	Resistance genes	Notes	Species/ Hosts	References
pCT	93,629/K	*bla*$_{CTX-M-14}$	This plasmid was found in the UK, isolated from *E. coli* from cattle, but found to be disseminated broadly between bacteria in animal and human ecosystems	*E. coli*/cattle	(Cottell et al., 2011)
pSAM7	35,341/ X4	*bla*$_{CTX-M-14b}$	Has a 99% similarity to pJIE143 from a human isolate in Australia	*E. coli* and *Enterobacter cloacae*/cattle	(Stokes et al., 2013)
pEK204	93,732/ I1	*bla*$_{CTX-M-3}$ and *bla*$_{TEM-1}$	It is thought that this plasmid arose by the transposition of Tn*3* and IS*Ecp1*-*bla*$_{CTX-M-3}$ elements into a pCOLIb-P9-like plasmid	O25:H4-ST131 *E. coli*/human	(Woodford et al., 2009)
Tc-19302	150,000/ FIIl FIA/ FIB	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$ *aac(6')-Ib-cr*	*bla*$_{CTX-M-15}$-carrying plasmids from cattle-derived non-ST131 *E. coli* isolates were highly similar to those found in ST131 *E. coli* isolates commonly reported in humans	*E. coli*/cattle	(Madec et al., 2012)
OT-ESBL-0163	40,000/ Inc (nt)**	*bla*$_{CTX-M-32}$	ESBL/AmpC-producing *E. coli* isolated from veal calves at slaughter	*E. coli*/cattle	(Hordijk et al., 2013b)

Table 1-2 continued

Plasmid or strain ID*	Size(bp)/ replicon (Inc) type	Resistance genes	Notes	Species/ Hosts	References
pIMT23463	143,193/ FIB	*bla*$_{CTX-M-14}$ *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *tetA, sul2*, *strA*, *strB*, *aadA*, *aac(3)-IId*, *aac(6 ')-Ib-cr*, *dfrA17*	Isolated from ESBL-producing D-ST648 *E. coli* strain from Black Vulture in Mongolia	*E. coli*/black vulture	(Ewers et al., 2014, Schaufler et al., 2016)
pIMT17433	122,823/ FIA/FII	*bla*$_{CTX-M-15}$ *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *tetA, tetR*, *aadA*, *aac(6 ')-Ib-cr*, *catB4*	Isolated from ESBL-producing B2-O25b-ST131 *E. coli* strain from dog UTI infection in Germany	*E. coli*/dog	(Schaufler et al., 2016, Ewers et al., 2010)
pHHA45	40,000/ N	*bla*$_{CTX-M-1}$	ST1 pHHA45 plasmid isolated from *E. coli* from pigs in Denmark: the study showed the distribution of specific IncN plasmids disseminating *bla*$_{CTX-M-1}$ gene among animals and humans in Europe	*E. coli*/pig	(Dolejska et al., 2013)

* All the names starting with (p) indicate the plasmid designation. For plasmids with undefined names, the strain name is given,
** Plasmid Inc was not defined; different variants for β-lactamases start with *bla; strA*, *strB*, *aadA* and *aac (3)-IId* are aminoglycoside resistance genes; *aac (6')-Ib-cr,* a gene variant for aminoglycoside acetyltransferase that confers reduced susceptibility to quinolones; *tetA* and *tetR* are tetracycline resistance genes; *catB4* confers resistance to chloramphenicol; *sul2* is a sulphonamide resistance gene and *dfrA17* confers high level resistance to trimethoprim. Information on copy number of plasmids was not available from the literature.

1.6.2 Insertion sequences (IS)

Insertion sequences (IS) are the simplest mobile genetic elements, often found in multiple copies both on chromosomal and plasmid DNA, and which have a major role in genomic rearrangements. The classic IS sequence structures are very similar to composite transposons with the exception that an IS does not carry resistance genes (Figure 1.6 A). They consist of a transposase gene flanked by inverted repeats (IR) in which the right side is designated as IRR and the left IR is IRL. Direct repeats (DR) at the IS termini range in size from 2-14 bp and are generated when an IS inserts into a target site (Mahillon and Chandler, 1998, Frost et al., 2005, Partridge, 2011). However, IS*CR* (Insertion Sequence Common Region) elements have a different structure, they do not create DR; they are bounded by an origin (*ori*IS) downstream and a terminus (*ter*IS) upstream, rather than IR, Figure 1.6 C (Partridge, 2011).

To date, twenty families of IS are known, these families were classified on the basis of several common features as an IS family is a group of IS with related transposases, strong conservation of the catalytic site, conservation of organisation and similar ends (Siguier et al., 2006). These small, genetically compact DNA sequences are usually less than 2.5 kbp in length. The classic ones do not have the ability to capture and transfer genes but they can only encode functions involved in their translocation, and transpose both within and between genomes (Mahillon et al., 1999). However, as a part of a composite transposon, two copies of the same IS

that happen to insert either side of a gene can capture it, which can then move as a unit.

IS carry 'passenger' genes mostly of unknown function but others carrying antibiotic resistance genes have been identified, which confuses the distinction between IS and transposons. A prefix(t) has been added to such IS sequences to make them distinct from the other members of the IS families and have been designated as tIS such as tIS*Sag10* from *Streptococcus agalactiae* which carries *lin* which is responsible for lincosamide resistance (Siguier et al., 2009).

There are some unusual types of IS, such as IS*Ecp1* (an IS weakly related to other IS elements and belonging to the IS*1380* family), which have the ability to control the expression of genes adjacent to them, acting as a promoter for a gene external to the IS element, e.g. $bla_{\text{CTX-M}}$ as IS*Ecp1* is commonly found upstream of this gene, Figure 1.6 B. Furthermore, IS*Ecp1*-like elements have the ability of transforming adjacent genes, even with a single copy. This is because sometimes IS transposases fail to recognise the right inverted repeat (IRR) and instead utilize an alternative (IRalt). If this lies on the other side of a resistance gene then the gene is mobilised with the IS (Mahillon and Chandler, 1998, Poirel et al., 2005, Partridge, 2011).

1.6.3 Transposons

Transposons are one of the transposable elements that can move from one genomic site to another. They have contributed to the evolution of most organisms by genomic rearrangements (Kazazian, 2004, Aziz et al., 2010).

They are thought to be an essential element in evolution due to their mobile nature, which not only widely distributes transposable elements within and between genomes, but also leads to mutations and rearrangements that can accelerate biological diversification (Aziz et al., 2010). Transposable elements also have another role which is unrelated to transposition; they confer a selectable phenotype on the host cell, such as resistance to an antibiotic. These resistance transposons integrate a resistance gene within the transposable element. They are diverse, with different structures and different mechanisms of transposition (Bennett, 2008). There are two groups of transposons: composite and non-composite (complex or unit transposons, respectively) (Partridge, 2011). The difference between the two types is in their modular structure; a non-composite transposon is more comparable to an insertion sequence (IS) element than to a composite transposon. A composite transposon is composed of several modules: a central distinctive DNA sequence not involved in transposition, which is surrounded by two copies of an IS element or an inverted repeat (IR) (Figure 1.6 D). One or both of these possesses the functions required for the transposition of the composite structure (Bennett, 2008). On the other hand, a non-composite (unit) transposon has no clear modular structure. They are larger than IS and carry antibiotic resistance and/or other genes in addition to genes encoding transposition functions (Partridge, 2011). Generally the whole gene sets are flanked by short IRs, and unlike the composite one, no component of a non-composite transposon has the ability to transpose independently (Bennett, 2004). Transposons can move both intra and

inter-molecularly, that is they can jump from a bacterial chromosome to a plasmid and *vice versa* or from one plasmid to another. DNA homology between the transposon element and the sites of insertion is not important, with some exceptions of specific transposons that favour a particular nucleotide sequence at an insertion site. Tn*7,* for example, inserts into a specific site (*att*Tn*7* site) in the bacterial chromosome (Craig, 1997). Examples of composite transposons that are found particularly in members of the Enterobacteriaceae and other Gram-negative bacteria, are: Tn*10* which encodes resistance to tetracycline (Hillen and Berens, 1994), and Tn*5* which encodes resistance to aminoglycosides such as kanamycin and neomycin (de Lorenzo and Timmis, 1994). Chance has an important role in producing such a compound element which confers a specific survival advantage, and then the establishment of copies of that element in a group of cells as a result of a selective pressure that is acting on the bacterial flora. For instance, in the case of Tn*10* and Tn*5*, this would be bacterial exposure to tetracycline or kanamycin and neomycin, respectively (Bennett, 2008). With regard to non-composite transposons that are present in plasmids of some Enterobacteriaceae, Tn*3*-like transposons and Tn*21*-like transposons are examples of such transposon types, Tn*3* encoding resistance to a group of β-lactam antibiotics, such as ampicillin (Figure 1.6 E). Tn*21* encodes resistance to mercuric ions, streptomycin and sulphonamides, and disinfectants (Figure 1.6 F) (Liebert et al., 1999, Essa et al., 2003, Partridge, 2011). Tn*2411* which is a Tn*21*-like transposon is a chromosomally-located transposon in *E. coli* O42. This transposon includes a class 1 integron, In*2,* that carries antimicrobial

resistance cassettes *aadA1* (streptomycin and spectinomycin), *sulI* (sulphonamide) and *emrE/QacE1* (ethidium bromide/antiseptic efflux pump). Besides this, it is surrounded by genes that encode resistance for tetracycline (*tetA*) on the right side and chloramphenicol (*cat*) on the left side (Chaudhuri et al., 2010).

Conjugative transposons (CTn) and integrative conjugative elements (ICE) differ from the other transposons, as they possess the characteristics of plasmids, transposons and phage; they have the ability to transfer DNA between different species and genera (Salyers et al., 1995, Wozniak and Waldor, 2010). These elements have an important role in the mobilisation of resistance genes (Wozniak and Waldor, 2010). ICE activity is associated with the stress response, which can be stimulated by exposure to antimicrobials and contaminants in the environment (Wellington et al., 2013).

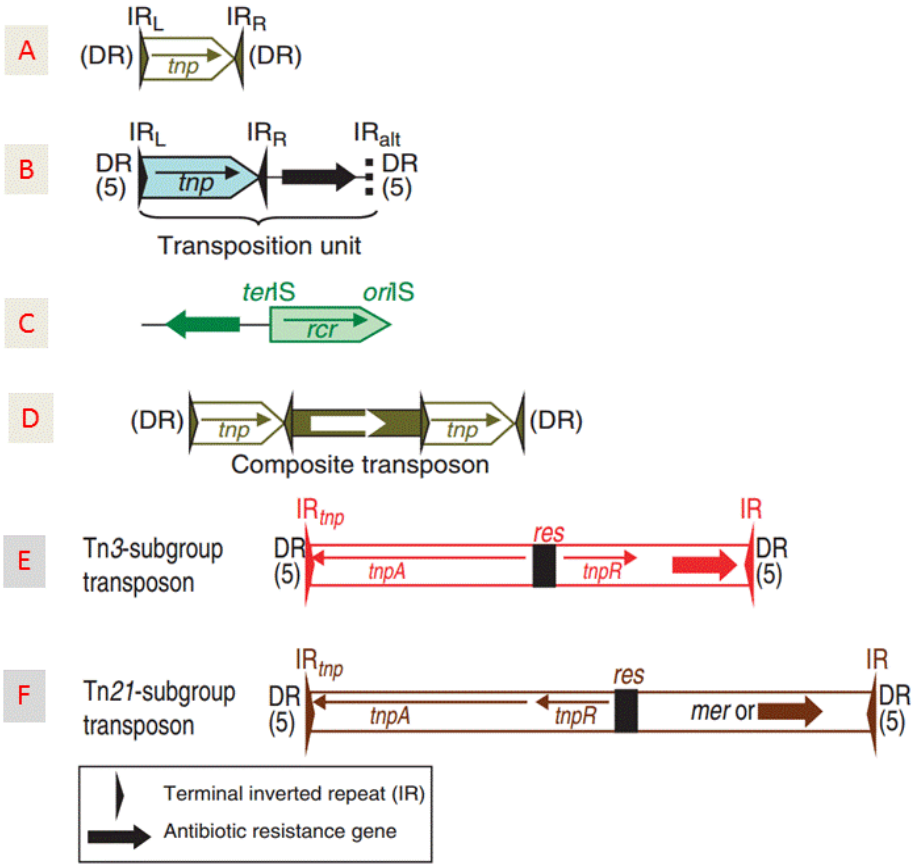


Figure 1-6: Characteristics of some Gram-negative mobile elements which are involved in the capture and mobilization of antibiotic resistance genes.

A: classic insertion sequence (IS); B: IS*Ecp1* structure; C: IS*CR* structure; D: composite transposon; E and F non-composite transposons. *tnpR*: resolvase; *tnpA*: transposase*;* IRL and IRR, left and right inverted repeats; IRalt, alternative IR; *rcr*, rolling circle replicase; *ori*IS, origin of IS*CR* elements; *ter*IS, terminus of IS*CR* elements; *res*, resolution site. Elements that create Direct repeats (DR) when inserting into their target are indicated and the DR length given, except for IS, where the DR length varies for different elements. (5): 5 base pairs (bp) length of the direct repeat. Tn*21*-subfamily transposons may carry resistance genes such as mercury (*mer*) or antibiotic resistance genes (represented by a thick arrow) as part of class 1 integrons inserted in or near the *res* site; adapted from Partridge (2011).

1.6.4 Integrons and gene cassettes

At the core of antibiotic resistance gene acquisition is the distinctive action of integrons. An integron is a genetic element that has three essential components: an *attI* site that a gene cassette can be integrated into by site-specific recombination, and the *intI* gene, an integrase gene of the tyrosine recombinase family that facilitates the site-specific recombination. *attI* is located adjacent to the *intI* gene. A promoter (Pc) is the third part of the integron, which leads to transcription of the cassette-borne genes, and lies within the *intI* gene, Figure 1.7. Furthermore, a gene cassette accommodates a short sequence called *attC* or a 59-base element that works as the specific cassette recombination site (Stokes and Hall, 1989, Recchia and Hall, 1995, Nunes-Duby et al., 1998, Carattoli, 2001, Bennett, 2008). A gene cassette is a separate genetic element that frequently contains one or more genes that encode antibiotic resistance or other genes, and it is present as a free circular non-replicating double stranded DNA molecule when transferring from one site to another, from one location in an integron to another in the same integron, or from one integron to another. However, a gene cassette can exist in a linear form when it is a part of another DNA element, such as a bacterial chromosome or mobile elements such as transposons and plasmids (Recchia and Hall, 1995, Hall and Collis, 1998, Holmes et al., 2003, Bennett, 2004). Indeed, it is not necessary that an integron should contain a gene cassette; for example, a cassette-free class 1 integron has been found in nature (Hall and Collis, 1998).

The mobility of integrons requires capture by transposons, insertion sequences, or conjugative plasmids (Hall and Collis, 1995, Holmes et al., 2003), while the gene cassette's mobility in and out of integrons occurs randomly, which is like most other genetic rearrangements in bacteria. Nevertheless, not all of these rearrangements survive; this depends on how much these gene combinations benefit the host, so if these rearrangements are of advantage to the host, it will be selected and established in the population by clone amplification (Bennett, 2004). More than 65 different antimicrobial resistance genes, comprising resistance to most classes of antimicrobials currently in use, have been reported to be organized as gene cassettes. This is not indicating that only gene cassettes confer resistance to antimicrobials, but actually reflects the extreme interest of microbiologists in resistance genes for the past 30 years (Hall and Collis, 1998, Bennett, 2004).

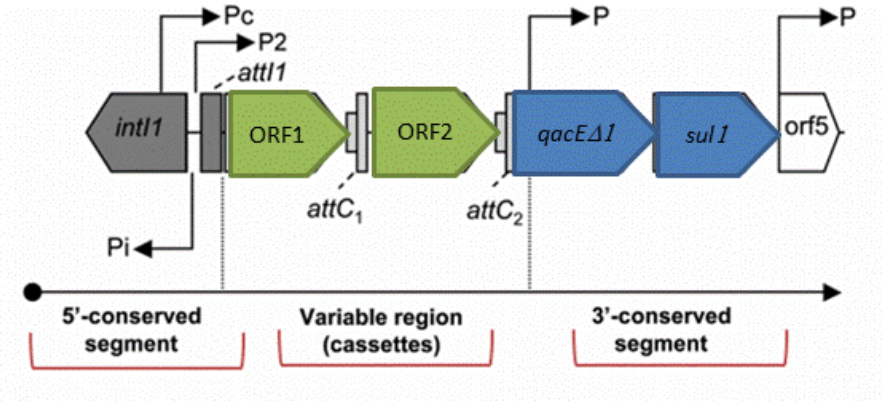


Figure 1-7: The composition of a class I integron.

P, promoters (Pc and P2 are the gene cassette promoters, Pi is the integrase promoter); ORF1 and ORF2: antimicrobial resistance gene cassette; *intI1*, integrase gene; *attI1* site-specific recombination site; *attC*1 and *attC*2, specific cassette recombination site; *qacEΔ1*, antiseptic/quaternary ammonium compound resistance gene; *sul1*, sulphonamide resistance gene; *orf*5, gene of unknown function; adapted from Léon et al. (2010).

Multi-resistance integrons (MRIs) are currently regarded as a real problem in the treatment of infectious diseases, as integrons holding up to eight resistance gene cassettes have been found in clinical isolates (Naas et al., 2001). The codon usage amongst resistance cassettes is notably different within the same MRI, showing that the antibiotic resistance elements are of dissimilar origin (Rowe-Magnus et al., 2002).

Up to the present time, at least four distinct integron classes have been found based on the sequence of the encoded integrases (Goldstein et al., 2001, Collis et al., 2002, Cambray et al., 2010). Most resistance integrons belong to the class 1 integron family; this class has also been recognized on transposable elements as in Tn*21,* a mercury resistance transposon (Grinsted et al., 1990). Tn*7* is an example of a transposon containing a Class 2 integron and it contains a dihydrofolate reductase gene cassette (Goldstein et al., 2001). Class 3 integrons have been characterized in *Serratia marcescens*, and contain a gene which encodes for broad-spectrum β-lactam antibiotic resistance (Arakawa et al., 1995). *Vibrio cholerae* contains Class 4 integrons that encompass the *intI4* gene, which has a role in the evolution of trimethoprim resistance in *Vibrio* species (Mazel et al., 1998, Heidelberg et al., 2000, Cambray et al., 2010). Classes 1 and 2 are often the most detected in many bacterial species, however, all classes are associated with determinants of antimicrobial resistance (Cambray et al., 2010).

1.7 *Escherichia coli*

The species *Escherichia coli* was named after Theodor Escherich who first described it in 1885. *E. coli* is a Gram-negative, non-spore forming facultative anaerobic bacilli organism. The optimum temperature for *E. coli* growth is 37 °C; it is a part of the Enterobacteriaceae family, which includes pathogenic species such as *Shigella*, *Salmonella*, *Klebsiella* and *Yersinia* (Murray et al., 2009, Torres et al., 2010). *E. coli* is closely related to *Shigella* sp. (Sussman, 1997). *E. coli* usually have peritricious flagella but they can be non-motile as well (Wright, 1955, Feng et al., 1996). It often has a capsule and is often fimbriated. The colonies on solid growth medium are usually circular, non-pigmented and can be smooth or rough; and in some cases the colonies can be mucoid. *E. coli* can utilise multiple carbohydrate sources, including lactose, glucose, D-mannitol and D-mannose and many other sugars, in the presence and absence of oxygen, with little variation in ability to ferment some sugars shown between different strains. *E. coli* is a versatile bacterium as it can adapt to the host environment and survive in the wider environment (Sussman, 1997).

1.7.1 *E. coli* Pathogenicity

In general, *E. coli* is one of the commensal bacteria that live harmlessly in the gastrointestinal tract. It has been considered as a commensal species, however, the acquisition of chromosomal or extra-chromosomal virulence loci, or random point mutations that increase adaptation for pathogenic environments can make this commensal become an extremely adapted pathogen (Torres et al., 2010). *E. coli* is an extraordinary and diverse

bacterium that has the ability to cause a range of infections such as diarrhoeal diseases, urinary tract infection, septicaemia, and meningitis (Croxen and Finlay, 2010, Talaro and and Chess, 2012).

E. coli has been implicated in a variety of infections which affect both humans and animals. So far, eight pathovars of *E. coli* have been extensively studied, and their virulence factors and pathogenicity mechanisms have been determined. Additionally, many other pathovars have been recognized. To start with, there are six types of *E. coli* pathovar that cause different categories of diarrhoeal diseases. These are entero-haemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAggEC) (Nataro and Kaper, 1998). Furthermore, there are two other pathovars that cause extraintestinal infections such as neonatal meningitis *E. coli* (NMEC) and uropathogenic *E. coli* (Croxen and Finlay, 2010) often collectively referred to as extra-intestinal pathogenic *E. coli*.

Gastroenteritis or diarrhoeal diseases that are caused by different *E. coli* pathovars differ in the pathogenesis mechanisms, symptoms and severity of the disease according to the causative type. One of these pathotypes is enteropathogenic *E. coli* (EPEC) which is a common cause of infant diarrhoea in developing countries. Similarly, it is a causative agent of diarrhoea in nurseries in developed countries. This is due to the incomplete immune system in infants (Murray et al., 2009). The infection occurs in two steps: initial adherence of *E. coli* to the mucosal cells of the small

intestine by bundle forming pili (plasmid encoded) and subsequent destruction of the microvillus and pedestal formation ('attaching and effacing' mechanism) coded by a chromosomal pathogenicity island; sometimes invasion of *E. coli* into the mucosal cells occurs. The symptoms of this infection range from mild to severe diarrhoea (Brooks et al., 2010).

Enterotoxigenic *E. coli* (ETEC) is also one of the causative agents of infant diarrhoea, but it is mainly responsible for causing travellers' diarrhoea. The consumption of contaminated water and food can cause the infection. This type of diarrhoea is similar to cholera diarrhoea, but it is milder (Nataro and Kaper, 1998, Strelkauskas, 2010). Significant symptoms can be shown by the infected host, such as watery diarrhoea, and abdominal cramp, nausea, and vomiting in some cases. ETEC strains have two specific toxins: heat labile toxin (LT-I and LT-II) and heat stable toxin (STa and STb). LT-I is similar to cholera toxin in terms of structure and function, STa is found in other Gram negative bacteria such as *Yersinia enterocolitica* but STb is associated primarily with ETEC strains isolated from pigs; human ETEC isolates expressing STb have been reported as well. Both toxin types stimulate fluid secretion in the intestinal epithelial cells, especially in infants. The strains which have both types of enterotoxins cause more severe diarrhoea (Murray et al., 2009, Brooks et al., 2010, Talaro and and Chess, 2012). The toxin genes are always plasmid-encoded and plasmids may also carry a fimbrial gene which allows attachment to the epithelial surface (Brooks et al., 2010). The nature of these fimbriae can give host specificity and produce greater levels of disease (Nataro and Kaper, 1998).

Enterohaemorrhagic *E. coli* (EHEC) is another pathovar that can cause a significant threat to human health: especially serotype O157:H7, which can cause severe diarrhoea in both adults and children that may develop into haemorrhagic colitis, and many cases have been recorded especially in developed countries. EHEC infection can lead to the potentially fatal syndrome: haemolytic uraemic syndrome (HUS). This *E. coli* pathovar can be transferred from cattle, which is the main reservoir for it, to humans through contaminated water and food (Croxen and Finlay, 2010). EHEC are also known as Shiga-toxin producing *E. coli* (STEC) because the key virulence factor for this type is a verotoxin related to the Shiga toxin (STX), which was originally identified in *Shigella.* There are two variants of the verotoxin: VTI is almost identical to Shiga toxin (one amino acid difference) and VTII is 60% similar to Shiga toxin; both are transferred horizontally via a bacteriophage which integrates into the STEC chromosome as a prophage (Nataro and Kaper, 1998, Murray et al., 2009). It is the verotoxin which is responsible for the pathology (particularly kidney failure) associated with HUS. EHEC also have the attaching and effacing ability of EPEC strains so have a combination of pathogenicity factors (Nataro and Kaper, 1998).

Enteroinvasive *E. coli* (EIEC) is a type of *E. coli* which has can invade and destroy the epithelial cells of the colon and causes watery diarrhoea which then progresses to dysentery and in some cases to colon ulceration. Fever, abdominal cramps and bloody diarrhoea are amongst the main symptoms. This type of *E. coli* is very closely related to *Shigella* because it has the

same invasion pathogenesis mechanism (Murray et al., 2009). In EIEC, virulence factors are carried on a plasmid, pInv, which encodes functions that promote invasion, cell survival and apoptosis of macrophages (Nataro and Kaper, 1998, Ogawa et al., 2008, Schroeder and Hilbi, 2008, Croxen and Finlay, 2010).

Enteroaggregative *E. coli* (EAggEC) is another pathovar that is responsible for causing traveller's diarrhoea in either developed or developing countries and is responsible for causing international epidemic and endemic outbreaks of diarrhoea. Although it is an emerging pathogen it has significant effects on human health (Croxen and Finlay, 2010). EAggEC colonize both the small and large intestine causing watery diarrhoea, sometimes accompanied by mucus and blood, due to inflammation in the colon and the secretion of enterotoxin and cytotoxins (Nataro et al., 1998, Nataro and Kaper, 1998). A feature of this pathovar is the auto-agglutination of bacterial cells in a stacked-brick configuration on the host cell surface, a feature which is thought to aid retention. The fimbrial genes which enable this are plasmid-derived (pAA plasmid) as are the Pet and EAST-1 toxin genes.

Besides the above pathovars there is another pathovar called diffusely adherent *E. coli* (DAEC) which can adhere to tissue culture cells such as HeLa cells. It is recognized by having Afa/Dr adhesion that reacts with the host cells, and it colonizes the small intestine, and has been implicated in diarrhoea in children between 1-5 years. It can also cause urinary tract infections in adults (Nataro and Kaper, 1998, Servin, 2005).

Finally, there are other pathogenic types of *E. coli* (ExPEC) that cause extraintestinal infections. The main virulence factors can be represented by the K-O antigen, haemolysin and iron sequestration systems and the presence of the adhesins for uroepithelial cells (type 1 and P fimbriae) (Bergsten et al., 2005). Examples of ExPEC include uropathogenic *E. coli* that result in the majority (~80%) of urinary tract infections (Alteri et al., 2009), and neonatal meningitis *E. coli* that transfer from the intestine to the blood stream and reach the central nervous system after passing the blood brain barrier. NMEC cause meningitis in infants that is very dangerous as the fatality rate is estimated at about 40% (Wooster et al., 2006, Croxen and Finlay, 2010). Approximately 75% of NMEC have the K1 capsule antigen, which has a role in invasion by preventing lysosomal fusion and thus allowing delivery of live bacteria across the blood–brain barrier (Brooks et al., 2010, Croxen and Finlay, 2010).

From what has been described above about the different patterns of *E. coli* pathogenicity, it can be observed that there are different mechanisms of infection depending on the virulence factor that it has which helps the bacteria to attack the host cell machinery.

1.7.2 Antimicrobial resistance in *E. coli*

The extensive use of antimicrobial agents in human medicine and agriculture has likely induced considerable changes in the human microbiome (Gill et al., 2006, Jia et al., 2008). *E. coli* as a commensal bacterium in the human microbiome is one of many that has recently emerged as a multidrug resistant bacterium. This bacterium acquires

antimicrobial resistance faster than other bacteria (Torres et al., 2010). It is due to this that it has been regarded as an indicator, or sentinel, species which helps us to understand what is happening with other pathogenic bacteria (von Baum and Marre, 2005).

Although commensal intestinal *E. coli* which carries antimicrobial-resistance genes is not a human health hazard in itself unless it enters others tissue through an accident or operation, it has been shown that the intestinal microflora can serve as a reservoir of antimicrobial resistant strains even if they are isolated from the faeces of a healthy person (Reinthaler et al., 2003), and these strains could give rise to multidrug resistant (MDR) bacterial infections with limited treatment options, increasing the risk of treatment failure.

Members of the Enterobacteriaceae have developed resistance to a variety of antimicrobials over time. Firstly, In the 1950s, MDR *Shigella* were reported by Japanese scientists (Davies, 2007). In the early 1970s plasmid-encoded β-lactamases significantly undermined the therapeutic advantage of semi-synthetic penicillins such as ampicillin, and their following combination with β-lactamase inhibitors such as the combination of amoxicillin and clavulanic acid (Galvin et al., 2010). This resulted in increased use of alternatives such as cephalosporins, fluoroquinolones and aminoglycosides. Plasmid-mediated amino-glycoside resistance emerged later which led to the increased use of third-generation cephalosporins and quinolones (Wellington et al., 2013).

Besides this, around ten years ago in Europe there was another worrying phenomenon in antimicrobial resistance which emerged. There was a huge increase in ESBLs in clinical cases from human infection (Livermore et al., 2007, Hawkey and Jones, 2009). In terms of *E. coli*, in Europe the rate of ESBL carriage for *E. coli* from human clinical isolates increased from 10% in 2004 to 17.6% in 2008 (Hawser et al., 2011). Furthermore, it has been reported by Liu and co-workers that an outbreak of *Klebsiella* and *E. coli* producing CTX-M-15 emerged from Southern China, despite this genotype being extremely rare prior to this outbreak (Liu et al., 2009).

1.7.3 *E. coli* as a reservoir for antimicrobial resistance genes in animals

Many bacteria develop resistance to antimicrobial agents due to exposure to different types of antimicrobials, especially in animals due to the intensive use of antimicrobials as animal feed additives and for treatment, especially in food production animals such as cattle, swine, poultry, and aquaculture species which are exposed to a variety of antimicrobial agents. Pets such as dogs, cats and birds have also received antimicrobial treatment but farm animals have more potential effects on human health due to the importance of food as a vehicle that expose humans to antimicrobial residues and AMR bacteria (Vidaver, 2002, Keen and Montforts, 2011). Besides, manure from livestock is another important source of antimicrobial resistant bacteria (Aarestrup et al., 1996), Figure 1.8.

E. coli has been used widely in antimicrobial resistance surveillance research as a Gram-negative indicator of antimicrobial selection pressure (McEwen and Fedorka-Cray, 2002). In terms of food-producing animals, some *E. coli* strains cause respiratory disease and mastitis in cattle, diarrhoea in pigs, and cellulitis and other infections in poultry (White et al., 2002). Dunlop and others showed that the use of several types of antimicrobials in pig feed at the finishing stages of production had a positive relationship with resistance to the same as well as unrelated antimicrobials in *E. coli* from finisher pigs (Dunlop et al., 1998). Furthermore, some infections in humans such as urinary tract infections and probably bloodstream infections can result from antimicrobial-resistant *E. coli*, which are present in food of animal origin (Manges et al., 2007, Jakobsen et al., 2010). Isolates of *E. coli* from urinary tract infections in the United States were very similar to those isolated from retail poultry (Johnson et al., 2007). Indeed, urinary tract infections caused by *E. coli* is considered a real problem in some countries especially in some parts of south-east Asia, China, the United States and other industrialized countries. This is due to the increase in antimicrobial resistance to a wide range of antimicrobial agents including the newer fluoroquinolones that are the suggested treatment for those infections (Karlowsky et al., 2002, Zervos et al., 2003). Additionally, research has shown that gentamicin resistant *E. coli* is more likely to infect poultry house workers than other people (Price et al., 2007). This is likely to be as a result of using the veterinary antibiotic apramycin (an antibiotic used in poultry production as a growth promoter but not in human medicine) which gives cross

resistance towards the important human antibiotic gentamicin (Marshall and Levy, 2011).

The recent report of transferable colistin resistance in commensal *E. coli* from farm animals and the presence of the same resistance genes in isolates from meat and *E. coli* and *K. pneumoniae* from patients has highlighted the threat that resistance genes originating in animals can subsequently be spread to humans (Liu et al., 2015). The same case has been reported in the UK as well; tests which were performed by the Public Health England showed 15 of 24,000 bacterial samples including *Salmonella* and *E. coli* (kept on record from cases between 2012 and 2015) were resistant to colistin. Colistin-resistant bacteria in samples from three pig farms have been reported by The Animal and Plant Health Agency (APHA) (http://www.bbc.co.uk/).

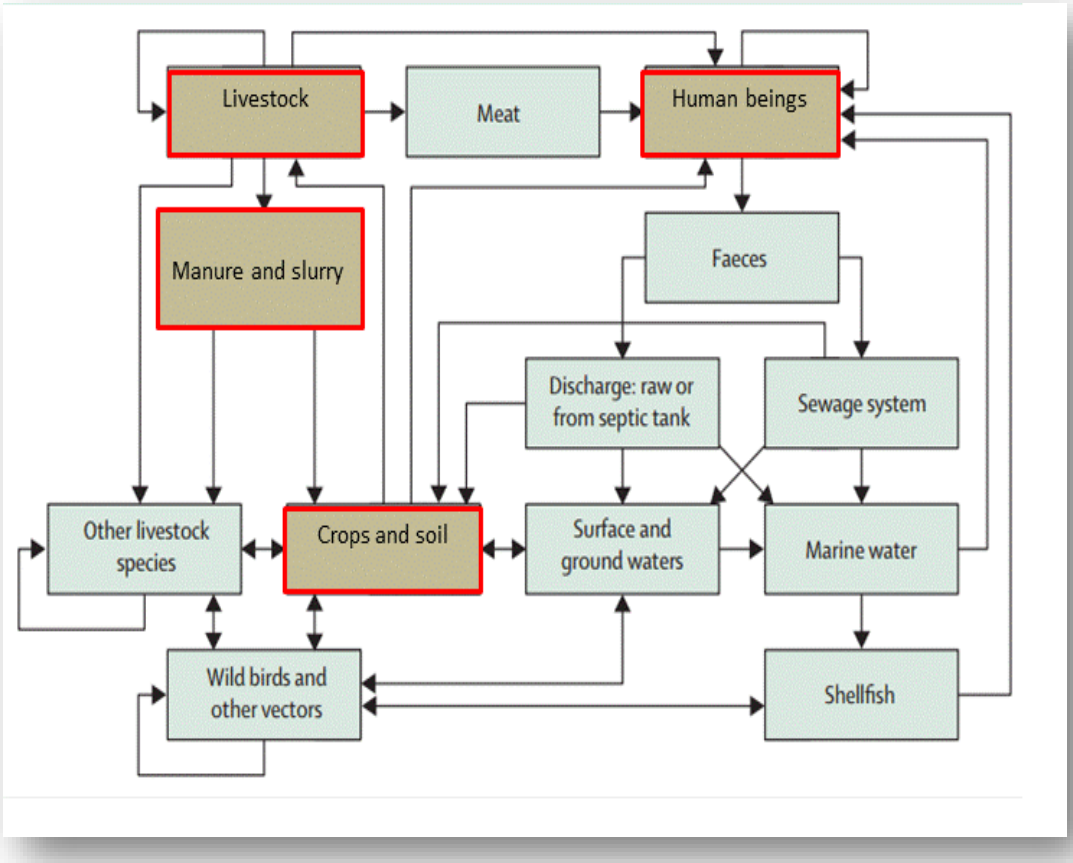


Figure 1-8: Possible routes for the transfer of antibiotic resistance bacteria or genes between livestock animals and humans.

The figure shows the associations and potential routes of transmission between potential sources of antibiotic-resistant bacteria. Manure and slurry from livestock animals represent an important reservoir for the antibiotic resistance bacteria/genes, which can transfer to livestock itself and to the human beings via many routes such as the crops and soils. Bacteria that can be transferred include pathogenic or normal intestinal flora, which are antibiotic resistant or susceptible; adapted from Wellington et al. (2013).

1.7.4 Antimicrobial resistance in *E. coli* in sewage and activated sludge

The presence and persistence of antimicrobial resistant bacteria in the environment such as sewage, surface water, soil and drinking water is a rising public health concern. It has been reported that sewage sludge contains pathogenic bacteria and antimicrobial resistance genes as well as antibiotic residues and their metabolites, so the extent of this dilemma and the possibility of resistance genes transferring to soil bacteria should be studied intensively (Wellington et al., 2013).

Activated sludge is considered as a hot spot for lateral gene transfer among bacteria owing to its nutrient fertility and high bacterial densities (Marcinek et al., 1998); wastewater treatment plants are a source of conjugative antimicrobial resistance plasmids and these plants can collect sewage from different sources such as farms and hospitals which can result in the rapid distribution of the resistance genes (Droge et al., 2000). However, it has been found recently that the antimicrobial resistance genes in wastewater treatment plants (WWTP) are rarely found in bacteria outside, which suggests that the treatment plants do not pass on resistance genes to pathogenic bacteria. The researchers found that the core resistome is unique to the WWTP environment, with less than 10% of the resistance genes found outside the WWTP environment, so according to these findings the existence of clinically important resistance gene exchange in the WWTP may happen but only at very low levels (Munck et al., 2015).

Many studies have shown the presence of an antimicrobial resistant bacterial strain in sewage sludge even after it has undergone intensive treatment. One of these studies showed that *E. coli* can persist in treated sewage up to a level of 10^3 CFU ml^{-1}, so even treated sewage can lead to an increase in resistant bacteria spread in the environment (Stelzer and Ziegert, 1988).

In addition, ESBL-producing *E. coli,* including CTX-M groups 1 and 9, survived the wastewater treatment process of a contemporary secondary treatment facility in Ireland (Galvin et al., 2010), and it has been found in a field experiment, where sewage sludge was applied to agricultural land, that fluoroquinolone residues remain in sludge-treated soils for a long time (Golet et al., 2003). This is alarming, as quinolone resistance genes (*qnr*) are located in the class 1 integron that also carries ESBL resistance genes, and the persistence of fluoroquinolones would appear to co-select for class 1 integrons and integron-borne ESBL genes (Nordmann and Poirel, 2005).

1.7.5 Distribution of ESBLs (mainly from *E. coli*) and antibiotic resistance in UK farms and other places worldwide:

The prevalence of ESBLs in UK farms, the predominant ESBL sequence types, and the plasmids they are carried on have been investigated in many studies. The emergence and spread of ESBLs (carried by *E. coli* and other Gram-negative bacteria) from different farm animals such as cattle, chickens, and pigs has increasingly been reported from UK farm animals and from other countries (Duan et al., 2006, Liebana et al., 2006, Madec et al., 2008, Dierikx et al., 2010, Snow et al., 2011, Madec et al., 2012,

Watson et al., 2012, Hordijk et al., 2013a, Liebana et al., 2013). ESBLs have also been reported from companion animals (Carattoli, 2008, Dierikx et al., 2012). Recently, cefotaximases have been found to be among the most prevalent ESBLs worldwide (Bonnet et al., 2004, Livermore et al., 2007, Hawkey and Jones, 2009).

E. coli isolated from cattle and sheep faecal samples from North Wales and the West Midlands of England were screened for ESBL-producing strains. Out of 113 farms tested (92 cattle, 24 sheep, and three of both), 8 farms were shown to be ESBL-positive; six via the submission of faecal samples from cattle and 2 from the submission of faecal samples from sheep. As no positive farm submitted faecal samples from cattle and sheep together, it is not clear if one of the mixed farms had both species infected. Sequence typing of all of the CTX-M isolates revealed both group 1 and group 9 CTX-M enzymes corresponding to CTX-M-14, CTX-M-14B (group 9) and CTX-M-15/28 (group 1) (Snow et al., 2011). Between 2009 and 2010, 65 dairy farms were investigated for *E. coli* producing CTX-M ESBLs. This study was carried out on multiple dairy farms in North West England and North Wales. CTX-M ESBL *E. coli* were shown to carry different CTX-M variants belonging to group 1 (CTX-M-1, CTX-M-32, CTX-M-15, CTX-M-55), group 2 (CTX-M-2) and group 9 (CTX-M-14, CTX-M-14B, CTX-M-27) (Snow et al., 2012). This shows that ESBL-producing *E. coli* are widespread in different parts of England and Wales, in cattle and sheep farms, with the group 1 and 9 CTX-M variants predominating. Different CTX-M sequence types have been reported in cattle from different countries. These types are represented by CTX-M-1, CTX-M-2, CTX-M-3,

CTX-M-13, CTX-M-14, CTX-M-15, CTX-M-29 and CTX-M-32, and many others (Shiraki et al., 2004, Duan et al., 2006, Madec et al., 2008, Karczmarczyk et al., 2011, Kirchner et al., 2011, Madec et al., 2012, Hartmann et al., 2012, Watson et al., 2012, Tamang et al., 2013b, Timofte et al., 2014). This indicates the presence of various types of CTX-M variants in cattle.

In a study conducted in the UK by Toszeghy et al. (2012), CTX-M *E. coli* isolates from cattle, chickens and turkeys were characterized using microarrays with respect to CTX-M sequence type, the presence of other antibiotic resistances, fitness and virulence genes, replicon types, and ability to transfer plasmids. *E. coli* producing CTX-M-14 and CTX-M-15 were predominant in cattle. The most common plasmid replicon was type I1-λ and was seen in 23%, 95% and 50% of the isolates tested from cattle, chickens and turkeys respectively, whilst types F, FIA, FIB and K were common to isolates from cattle and turkeys only (Toszeghy et al., 2012). The presence of ESBLs in UK cattle was separately reported with resistance a result of $bla_{\text{CTX-M-14}}$ carriage on IncK and IncFII plasmids (Liebana et al., 2006, Stokes et al., 2012). Conjugative $bla_{\text{CTX-M-14}}$ IncK pCT-like plasmids (85-110 kbp) have been isolated from unrelated *E. coli* isolates from cattle, turkeys and humans in the UK (Stokes et al., 2012) (See characteristic of pCT plasmid in Table 1.2). CTX-M-14- producing *E. coli* (n=70) from cattle (n=33), turkeys (n=9), sheep (n=2) and humans (n=26) were screened for pCT genetic markers and 25 isolates were positive. All pCT-like plasmids were conjugative, and most were IncK

(n=21) and had the same local genetic environment flanking the *bla*$_{\text{CTX-M-14}}$ gene (n=23) (Stokes et al., 2012). More examples of ESBL-carrying plasmids from *E. coli* from cattle and other sources are described in Table 1.2; this shows the widespread nature of ESBL plasmids which can serve as vectors for horizontal dissemination of the ESBL genes to different *E. coli* isolates from different hosts.

Many studies have investigated the possibility of ESBL spread from animals to humans. Farm animals are known as important carriers and a significant potential reservoir of ESBL *Salmonella* and *E. coli* in some countries (Carattoli, 2008). CTX-M-2 ESBL-producing *E. coli* isolates (n= 629) from animals and humans and animal-derived food products were isolated from the UK, the Netherlands, and Germany. These isolates were compared and analysed using a microarray to determine their similarities. In addition, 313 isolates were further analysed by MLST. In all three countries, *bla*$_{\text{CTX-M-1}}$ which originated from animals and some human infections were dominant (66%); 23% of all isolates in this study contained both *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{OXA-1}}$-like genes, predominantly isolated from humans and UK cattle. There were no *bla*$_{\text{SHV}}$ genes detected in isolates from UK and German cattle, chicken, turkey and pig hosts. In contrast, 6% and 17% of Dutch isolates from chicken and cattle hosts respectively, were positive for *bla*$_{\text{SHV}}$. The most predominant ST amongst the *E. coli* strains were ST131 (n=45), ST10 (n=25), ST88 (n=24), ST405 (n=11), ST69 (n=10) and ST58 (n=10). Only 1.2% (3/258) of characterized *E. coli* from animals were similar to isolates from human isolates as they share a high similarity in

terms of their resistance gene profiles and share the same MLST clonal complex profile (Wu et al., 2013). In a more recent study, the diversity of plasmids, STs and ESBL genes among ESBL-producing *E. coli* from humans, animals and food in the UK, the Netherlands, and Germany were investigated. Among 353 isolates the most predominant ST for human isolates only was ST131 and ST405 with multiple IncF *bla*$_{CTX-M-15}$ carrying plasmids, whilst the predominant ST for animal isolates only was ST88 with IncI1-Iγ *bla*$_{CTX-M-1}$ carrying plasmids. Various genes were detected including *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$, but *bla*$_{CTX-M-1}$ was the most frequently detected ESBL gene, followed by *bla*$_{CTX-M-15}$, which was the most common ESBL gene in the human isolates. The study shows a potential for plasmid/ESBL gene transmission between *E. coli* from humans, animals and food as some combinations were found in isolates from all of these sources, such as ST10, ST58 and ST117 with IncI1-Iγ plasmids (Day et al., 2016).

With regards to antimicrobial resistance in UK farms, there is an annual report produced by the VMD, which gives very detailed reports of UK veterinary antibiotic resistance and sales (https://www.gov.uk). For example, in 2013 of the *E. coli* isolates collected by scanning surveillance (obtained from diagnostic submissions, which therefore may be pathogenic or commensal strains), resistance to 3rd and 4th generation cephalosporins was greatest in cattle, with 13.3% of isolates resistant to cefotaxime. The majority of resistant isolates reported via scanning surveillance were obtained from neonatal or pre-weaned animals and resistance was much less frequently observed in isolates from adults. In the same year, 11.9%

of *E. coli* isolates were resistant to enrofloxacin (fluoroquinolones) in cattle, 8% in pigs, 4% in sheep and only 2% in poultry (UK-VARSS, 2013). In 2015, *E. coli* isolates from sheep had lower levels of resistance to most antibiotics compared to those from pigs and cattle. Between 2013 and 2015 *E. coli* isolates from cattle samples showed high levels of resistance to ampicillin (79.1-80.9%), while resistance to cefotaxime was 13.3-15.6% and to ceftazidime was 7.2-8.5%, Table 1.3. A higher prevalence of resistance to cefotaxime versus ceftazidime was observed, which might reflect the occurrence of cefotaximase ESBL enzymes rather than ceftazidimases (UK-VARSS, 2015), more details about antimicrobial resistance in UK farms and other place in the world are demonstrated in Section 4.3.

Table 1-3: Resistance in all *E. coli from cattle (all ages) in England & Wales**

No. resistant / No. tested (Percentage resistant)			
Antimicrobials	2013	2014	2015
amikacin	4/706 (0.6)	2/492 (0.4)	2/441 (0.5)
amoxicillin/ clavulanic acid	385/780 (49.4)	271/533 (50.8)	223/494 (45.1)
ampicillin	617/780 (79.1)	431/533 (80.9)	392/494 (79.4)
apramycin	50/761 (6.6)	22/517 (4.3)	22/480 (4.6)
cefotaxime	94/707 (13.3)	77/495 (15.6)	46/443 (10.4)
ceftazidime	52/707 (7.4)	42/495 (8.5)	32/443 (7.2)
cefpodoxime	-	-	-
chloramphenicol	386/706 (54.7)	271/492 (55.1)	228/441 (51.7)
enrofloxacin	93/780 (11.9)	60/533 (11.3)	58/494 (11.7)
florfenicol	262/724 (36.2)	190/507 (37.5)	147/455 (32.3)
neomycin	332/761 (43.6)	250/517 (48.4)	217/480 (45.2)
spectinomycin	380/761 (49.9)	231/517 (44.7)	218/480 (45.4)
streptomycin	439/706 (62.2)	316/492 (64.2)	315/441 (71.4)
tetracycline	595/780 (76.3)	424/533 (79.5)	369/494 (74.7)
trimethoprim/sulfamethoxazole	359/780 (46)	261/533 (49)	224/494 (45.3)

*: The majority of isolates reported in this section were recovered from faeces or intestinal contents, and includes both pathogenic and commensal strains; adapted from UK-VARSS (2015).

1.7.6 *E. coli* typing

Discriminating different bacterial isolates of the same species using molecular typing methods is essential in understanding the epidemiology of an organism and also in outbreak investigations to allow the source of infection to be identified, which can result in better implementation of effective infection control programmes (Sabat et al., 2013). Sub-species typing was performed in this thesis to check if there was clonality between different isolates from different samples. There are a wide range of methods that can be used for profiling of *E. coli* from environmental and clinical samples. There are phenotypic methods, such as antibiogram, biotyping, serotyping and phage-typing, however these methods are labour intensive to use and do not always give the right level of discriminatory power. For example, serotyping of *E. coli* could identify an isolate as being an O157:H7 serotype, but further characterisation would be needed to show two isolates were the same strain of O157:H7. The methods that examine the relatedness of isolates at a DNA level have made a revolution towards the ability to differentiate among bacterial types and subtypes (Sabat et al., 2013). These methods depend on the genomic diversity of the different strains of *E. coli*, either based on chromosomal differentiation or the whole genome including chromosomal and plasmid DNA. One of these techniques is ERIC (Enterobacterial repetitive intergenic consensus) PCR which allows the characterisation of genetic diversity among a group of *E. coli* isolates based on the amplification from repetitive sequences, which are 127 bp highly conserved palindromic sequences; these sequences occur several times across the

chromosome in a wide range of Gram-negative bacteria including *E. coli*, and are hypothesised to have an mRNA stability function, or act as RNA control elements (Wilson and Sharp, 2006). ERIC-PCR amplifies up the regions between ERIC sequences and results in a distinct pattern of bands unique to the strain of *E. coli*; isolates that share the same fingerprint will be the same strain (Versalovic et al., 1991). The great advantages of ERIC-PCR, is that it is cost effective, rapid and less laborious compared to other methods such as PFGE (Rai, 2016). The main limitation of this method, as with the other PCR-based typing approaches, is that it can lack reproducibility due to variability in reagents, thermal cycling conditions, and gel electrophoresis systems (Sabat et al., 2013, Rai, 2016). Variability can be avoided if the method is standardized, for example: standardization of the electrophoresis conditions and use of appropriate software to analyse the agarose gel, including a normalization step to avoid gel to gel variation (Wei, 2013, Feng et al., 2013). Pulsed-field Gel Electrophoresis (PFGE) is another method for DNA fingerprinting; in this method, a highly purified genomic DNA sample is digested with a rare-cutting restriction endonuclease resulting in infrequently occurring restriction sites in the genome being cut. The genomic fragments that result are mostly large, and can be separated on an agarose gel by 'pulsed-field' electrophoresis. The pattern of bands can be compared later using gel comparing software (Sabat et al., 2013). One of the advantages of PFGE is that it is based on genomic DNA analysis (more than 90% of the genome is included in the analysis) which enables discrimination between different genomes which have been subject to deletion or insertion of mobile genetic elements or

large recombination events, as this will produce different band patterns for each strain. Because of the sequence specific cutting sites of the restriction enzymes, some point mutations may also be detected through band pattern changes. These patterns are usually very reproducible for a given strain and have been the basis for the development of comparative databases. However, this method is time consuming and technically demanding (Sabat et al., 2013). The presence of large plasmids (bigger than 50 kbp) carried by a strain being tested can result in a single band difference from the particular PFGE profiles (Goering, 2010).

Multi-locus sequence typing (MLST) is based on determination of the nucleotide sequences of the internal fragments of selected housekeeping genes, seven loci that are exposed to very slow and minor changes resulting in polymorphism and diversity. The sequence for each gene is assigned a number and the seven numbers combines to make a unique Sequence Type (ST). This allows for the identification of different *E. coli* ST (Enright and Spratt, 1999, Adiri et al., 2003). This method is very effective if used to compare certain strains with other globally spread strains from the same species, e.g. *E. coli*. As it is based on seven housekeeping genes the method is fully standardised for numerous bacterial species (Enright and Spratt, 1999, Wirth et al., 2006). However, this method is expensive, labour-intensive, time-consuming and insufficiently discriminating between some pathogens for routine use in outbreak investigations (Sabat et al., 2013). For example, MDR EHEC *E. coli* O104:H4 ST678 which caused the outbreak in Germany in 2011 could not be discriminated from another

E. coli O104:H4 ST678 strain which caused one case of HUS in Germany in 2001, as both strains had similar ST. However, whole genome sequencing showed that the 2011 outbreak strain differed substantially from the 2001 isolate in chromosomal and plasmid content, which demonstrates that traditional MLST cannot accurately resolve relationships among closely genetically related isolates that differ in their pathogenic potential (Mellmann et al., 2011, Hao et al., 2012).

Many other methods are also available for profiling *E. coli* such as phylogenetic group (A, B1, B2 and D) detection by PCR depending on three specific markers: namely *chuA* (the gene responsible for heme transport in enterohaemorrhagic O157:H7 *E. coli*), *yjaA* (gene with unknown function), and TSPE4C2 (an uncharacterized DNA fragment later characterized as a putative lipase esterase gene) (Clermont et al., 2000, Gordon et al., 2008). This method was developed later to cover more phylogenetic groups (Clermont et al., 2013). This method is simple, inexpensive, and time-limited depending on the quadruplex PCR reaction. It can classify *E. coli* into 8 groups (Clermont et al., 2000, Clermont et al., 2013). However, this method still fails to correctly assign some strains to the appropriate phylo-group due to unassignable strains that are extremely rare, or due to highly variable gene content that is driven by the frequent gain and loss of genes in *E. coli* (Touchon et al., 2009, Clermont et al., 2013). It is also not sufficiently discriminatory to allow strain identity to be established.

Whole genome sequencing using next generation sequencing (NGS) seems to be the most promising method for the future due to its power to identify

genome-wide variation. This method produces millions of reads which are assembled based on overlapping regions (*de novo* assembly), or comparisons with previously sequenced 'reference' genomes (resequencing). This method is becoming very attractive and powerful in epidemiological studies (Mellmann et al., 2011, Grad et al., 2012, Sabat et al., 2013). The limitation for this method is that it is still not a rapid method and a cost-effective approach. To sum up, there is currently no single ideal typing method available. Therefore, there might be one or more different typing methods that need to be applied depending on the type of research and location of strains being compared (local, national or international) (Sabat et al., 2013).

1.8 Hypothesis and aims of this study

The hypothesis of this study is that farm practice and farm waste may contribute to the presence and dissemination of AMR in the environment, and contribute to the reservoir of resistance genes in agriculture. This is based on the premise that *E. coli* is a common enteric bacterium which can be used as a sentinel species for the presence of AMR genes in the different slurry and faeces samples from the dairy farm. Furthermore, use of cephalosporins such as ceftiofur and cefquinome, which are antibiotics inclusively used for animal treatment (e.g. in treating cattle mastitis), was expected to drive the resistance to these antibiotics besides causing resistance to other cephalosporins, such as cefotaxime, which is a third generation cephalosporin used in human treatment. This hypothesis also needs to be tested for the other antimicrobials which are used on the farm,

such as aminoglycosides and oxytetracycline. In the dairy farm studied, the mastitic (and antibiotic-contaminated) milk was discarded into the slurry tank, which may potentially select for *E. coli* strains carrying ESBL.

The aims of this study were:

- To determine the prevalence of *E. coli* from the selected slurry and faeces samples using bacteriological media with and without antibiotic (cefotaxime) to estimate the percentages of the putative CTX-resistant bacteria, and to estimate the number that can enter the environment from spreading liquid and solid slurry directly to the land.
- To determine the phenotypic resistance profiles of the collected isolates and determine the MDR patterns by testing the strains against 17 antimicrobials representing different antimicrobial groups.
- To determine the genotypic diversity of these isolates using ERIC-PCR method.
- To investigate the presence of ESBL/AmpC producing *E. coli* within the farm using appropriate bacteriological media for isolation and to use phenotypic and genotypic tests to establish the extent and basis of the isolates' resistance.
- To make a link between phenotypic and genotypic diversity to understand the diversity of resistance.
- To study the genetic environment of the AMR genes with specific concentration on β-lactam genes for four selected strains using

whole genome sequencing to be able to understand if the genes are carried on transposable elements and the possibility of their transfer to other species or genera by HGT. From this to determine the possibility of the spread of these genes to the environment.

2 . Materials and Methods

2.1 Media

Unless otherwise stated, all media were sterilized by autoclaving at 121 °C and 10 psi pressure for 15 min.

2.1.1 Luria-Bertani Lennox (LB) Broth

20 g of LB Lennox broth powder (Fisher Scientific, UK, Cat. no. 12881650) was dissolved in 1 L Reverse Osmosis (RO) water, transferred into appropriate containers and autoclaved. The 20 g of LB broth powder contained 10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride.

2.1.2 Luria-Bertani Lennox (LB) Agar

32 g of LB Lennox agar powder (Fisher Scientific, UK, Cat. no. 22700025) was suspended in 1 L of RO water. The medium had the same composition as LB broth, but with an added 12 g of agar. After autoclaving, antibiotics or sodium azide were added, if required, to the mixture once it had cooled to below 50 °C and the molten agar was then poured into 90 mm Petri dishes (Bibby Sterilin, UK).

2.1.3 Tryptone Bile X-Glucuronide (TBX) agar

TBX agar is a tryptone bile agar containing X-glucuronide (Merck, Germany. Cat. no. 1.16122) and is used as a selective medium to isolate and detect *E. coli.* X-glucuronide is a substrate for glucuronidase which is carried by *E. coli* but no other coliforms. *E. coli* cells can take up colourless intact X-glucuronide. Intracellular glucuronidase cleaves the glucuronide-chromophore bond, releasing the coloured chromophore, which accumulates in cells turning an *E. coli* colony blue/green (Hansen and Yourassowsky, 1984). 31.6 g of TBX powder was dissolved in 1 L of

RO water, dispensed into suitable containers and autoclaved. This medium is composed of 20 g peptone, 1.5 g bile salts No. 3, 10 g agar-agar, and 0.075 g X-β-D-glucuronide. Antibiotics were added if required, after the sterile medium had cooled to at least 50 °C, mixed, and then poured into 90 mm Petri dishes.

2.1.4 MacConkey agar NO.3

51.5 g of this medium (Oxoid, UK. Product code CM0115) was suspended in 1 L of RO water and sterilized by autoclaving. The medium is composed of 20 g peptone, 10 g lactose, 1.5 g bile salts No.3, 5 g sodium chloride, 0.03 g neutral red, 0.001 g crystal violet and 15 g agar-agar.

2.1.5 CHROMagar ESBL

This medium (CHROMagar, France. Product code ES372) is made up of two components: A) CHROMagar Orientation (peptone and yeast extract 17 g; chromogenic mix 1 g; agar 15 g per litre) and B) CHROMagar ESBL supplement- which is a proprietary selective supplement used at a rate of 0.57 g per litre of medium. The medium was prepared as directed by the manufacturer: CHROMagar Orientation medium (33 g) was suspended in 1 L of RO water, the mixture was brought to boiling (100 °C) while swirling or stirring continuously, and when dissolved was aliquoted into appropriate containers and autoclaved. The ESBL supplement powder (570 mg) was rehydrated in 10 ml of sterile purified water, and added to 1 L of the sterilised CHROMagar Orientation medium when it had cooled to ≤50 °C. Volumes (20 ml) of the supplemented medium were then poured into 90 mm petri dishes. *E. coli* ESBL should appear as dark pink to reddish colonies, *Klebsiella, Enterobacter,* and *Citrobacter* should

appear as metallic blue colonies, and *Proteus* as brown with a brown halo.

2.1.6 Mueller-Hinton Broth

21 g of Mueller-Hinton Broth powder (Oxoid, Product No. CM0405) was dissolved in 1 L of RO water, dispensed, and autoclaved. This medium was composed of a dehydrated infusion from beef: 2 g, 17.5 g casein hydrolysate, and 1.5 g starch per litre.

2.1.7 Mueller-Hinton Agar

38 g of Mueller-Hinton agar (Oxoid, Product No.CM0337) was dissolved in 1 L of RO water. The medium was sterilised by autoclaving. This medium has the same composition as Mueller-Hinton broth with added 17 g agar-agar per litre.

2.1.8 Tryptone Water

15 g of Tryptone water powder (Oxoid, Product No. CM0087) was dissolved in 1 L of RO water, distributed into appropriate containers and autoclaved. This medium was composed of 10 g of tryptone and 5 g of sodium chloride.

2.1.9 Maximum Recovery Diluent (MRD)

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

2.1.10 Agarose gel

Molecular Biology grade agarose (Melford Laboratories, Suffolk, UK) was used for all the PCR analysis except for ERIC-PCR analysis as GelPilot® LE Agarose (Qiagen) was used. The agarose suspension in 1 x TAE buffer (40 mM Tris-acetate adjusted with glacial acetic acid to pH 8.3, 1 mM disodium ethylene diamine tetra acetic acid) was melted in a microwave oven to give a 1-2% (w/v) solution (the exact concentration will be stated for each agarose gel application later in this chapter). After cooling, ethidium bromide was added to the molten agarose solution to a final concentration of 0.4 µg ml^{-1} prior to pouring into a gel casting tray with an appropriately sized comb. The gel was allowed to set at room temperature followed by removal of the comb.

2.2 Solutions

2.2.1 TE Buffer

To prepare 10 mM Tris-HCl, 1 mM EDTA buffer, 0.0372 g of disodium EDTA and 0.12 g of Tris base were dissolved in 80 ml of distilled water. The pH was adjusted to 7.9 using concentrated HCl and the volume made up to 100 ml and autoclaved.

2.2.2 Cefotaxime stock solution

10 mg of cefotaxime (Sigma Aldrich, Product No C7039-100MG) was dissolved in 5 ml of sterile water to give a solution of 2 mg ml^{-1}. The final concentration used was 2 µg ml^{-1}. Antibiotic stock solutions were stored in 1 ml aliquots at -20 °C.

2.2.3 Sodium Azide stock solution

2.5 g of sodium azide (Sigma Aldrich, Product No. S-8032) was dissolved in 100 ml of sterile water and filter sterilized using a 0.22 μM pore size sterile syringe filter. This gave a stock solution of 25 mg ml^{-1}.

2.3 Samples

2.3.1 Dairy farm unit

The farm in this study had a herd of approximately 200 dairy cows. The animals are milked using an automated milking system (AMS), and consequently the milking cows are housed for the majority of the year inside barns. Each cow produces approximately 60 kg of waste per day, which is daily automatically scraped into a sump. Slurry solids are mechanically separated and deposited into a trailer, and go to a heap or are directly used as a fertilizer. The remaining liquid slurry, containing approximately 5% (w/v) solids, is pumped into an on-site slurry tank and stored for field spreading. The slurry tank has a capacity of 3 million litres and is generally emptied after circa 90 days, either into a slurry lagoon by means of a pipeline, or is pumped into a tanker and taken directly to fields for spreading. The major use of antibiotics on the farm is to treat mastitis infections, which occur at a rate of ~100 incidences per year, i.e. approximately one every 3 days. These infections at the time this study was undertaken were treated with cephalosporin antibiotics (cefquinome-4^{th} generation cephalosporin-veterinary use only) through intramammary infusion directly into the infected quarter; the withheld milk was disposed of in the slurry tank, alongside waste footbath contents, washing water and other cleaning solutions used in the dairy unit. Many of the products

used as a part of the farm dairy care requirements might have an adverse effect on the environment due to some of the components they contain and the way used for disposal. There are many examples, such as copper footbaths, which contain a final concentration of 5% (w/v) copper-sulphate and 5% (w/v) zinc-sulphate which are known for their anti-bacterial properties, and are used to treat digital dermatitis. Besides these, many other disinfectants are being used in general cleaning on the farm such as Iodel FD II, which contain sulphuric acid.

Other antibiotics have been used on the farm for treatment of other bacterial infections common to dairy cattle. Those in the farm inventory include: ceftiofur (3rd generation cephalosporin veterinary use only), cephalexin (1st generation cephalosporin), oxytetracycline, penicillins, aminoglycosides and aminocoumarin antibiotics.

2.3.2 Sample collection

2.3.2.1 Sampling rationale

Slurry samples were collected from different areas in the University of Nottingham Dairy Unit in October 2012 and in March 2014. In the preliminary study in 2012, samples were taken from the slurry tank (n=2), separated solid slurry (n=1), and from a sample of cow faeces from the animal housing: solid (n=1) and liquid exudate from cattle faeces (n=1). In this visit the aim primarily was to establish that there was carriage of resistance in the *E. coli* isolated from the slurry in the tank and, samples from the other sites were selected for comparison. For the purpose of this visit, only TBX agar without any antibiotic supplement

was used, as TBX is a microbiological selective medium for isolation of *E. coli*. Use of a medium without antibiotic selection ensured *E. coli* isolation and allowed an indication to be gained of the percentage of isolates which were resistant to antibiotics and which antibiotic resistances were present. The isolates from this visit were then used to establish the testing procedures for different tests (e.g. disc assays to check the phenotypic resistance profile and for the preliminary test to validate the ERIC-PCR). Depending on the results obtained from this preliminary study in 2012, a plan for taking more samples was made.

The sampling strategy for the second visit was to check the diversity of the isolated *E. coli* in terms of their AMR profiles, strain and genotypic profiles between the different sources of the slurry samples starting from the cow barn and with the end point of the slurry represented by the slurry tank and solid slurry. As CTX-resistant *E. coli* had been found from the first set of samples, TBX (as in 2012 sampling) and TBX supplemented with CTX (2 μg ml^{-1}) was used to isolate ESBL/AmpC producers and to check the prevalence of the presumptive CTX-resistant *E. coli* in the second set of samples. To check the prevalence of presumptive CTX-resistant coliforms, MacConkey agar with and without CTX (2 μg ml^{-1}) was used. In addition, CHROMagar ESBL was used as a medium specifically designed to isolate ESBL-producing *E. coli*. In the 2014 study, samples from liquid slurry were again taken from the slurry tank (n=3), separated solid slurry (n=3), and cow faeces from the animal housing ('cow barn'; n=3) and were used to isolate *E. coli*. As the first study had shown resistant strains were found at a high rate, the number

of samples collected was kept small to make characterisation of the isolates feasible. However, the liquid and solid slurry samples represented mixed accumulated sources and so should give a representation of the diversity of strains present within the herd.

2.3.2.2 Sample processing

Samples were kept at 4 °C and isolation of bacteria started within 48 h of collection. For the 2012 samples, the samples were diluted 1/2 and processed in the stomacher machine (Stomacher 400 circulator, Seward, UK) for 1 min at 230 rpm before plating. Filtered stomacher bags (Seward, UK) were used to get a clear sample and to get rid of any straw. For the 2014 samples, the samples were diluted 1 in 10 by adding 10 g (or 10 ml for the liquid) of the sample in 90 ml of MRD. The samples were processed in the stomacher machine and filtered as described above. Three biological replicates were taken from each filtered sample, with 10 ml of the sample taken for each replicate and transferred to a sterile universal. Each sample was serially diluted with 1 ml of the sample transferred to 9 ml of MRD, and serial dilutions up to 10^{-8} were made for each sample.

2.3.3 *E. coli* and total coliform count

For the 2012 samples, isolates were selected on TBX without any antibiotic selection: 100 µl of the 1/2 a diluted sample was plated in triplicate but no counting was performed. For the 2014 samples, 100 µl of each dilution was spread in duplicate onto TBX agar, and TBX plus CTX (TBX+CTX) for *E. coli* counts. In addition, MacConkey agar and

MacConkey agar containing CTX were used for total coliform counting and CHROMagar ESBL for ESBL selection. All plates were incubated overnight at 37 °C. *E. coli* NCTC 13353 and *E. coli* ATCC 25922 were used as ESBL positive and negative control strains, respectively.

2.3.4 Statistical analysis

Plate counts were converted to CFU per ml by multiplying by 10 to take into account sample volume plated (0.1 ml) and 1/dilution factor. The logarithmic count results (log_{10} CFU ml^{-1}) were used for statistical analysis. 2 way ANOVA was used to test the significant difference for both *E. coli* counts (ECC) and total coliform counts (TCC) comparing the source of the sample. The significance (P value) under Tukey's multiple comparisons test and ANOVA test, the means, and the standard deviation of the sample sets were processed in Prism Graphpad6. The data used in this analysis were from the sampling in 2014 only as no counting was performed in 2012.

2.3.5 Selection and Confirmation of *E. coli* strains

Where possible from each sample, five colonies were picked from the TBX and five colonies from TBX+CTX plates, and 35 well-isolated pink colonies (out of 48) from the CHROMagar ESBL plates. These isolates were selected based on their colony characteristics which matched the characteristics for the reference strain *E. coli* NCTC 13353. Isolates were purified by streaking a single well-isolated colony for single colonies on LB agar (Fisher Scientific, USA) and incubating at 37 °C for 24 h. The colonies from CHROMagar ESBL plates were further streaked on TBX, and only those giving green/blue colonies characteristic of *E. coli* were

selected. All purified isolates were tested for indole production (Section 2.3.5.2.) and oxidase activity (Section 2.3.5.1); and indole positive, oxidase negative strains were considered as *E. coli*. API 20E tests (Section 2.3.5.3) were used to confirm identification for selected isolates.

2.3.5.1 Oxidase test

The oxidase test was used to confirm that the selected colonies were *E. coli* using an oxidase strip (Oxidase Detection sticks, Oxoid Cat. no. MB0266). Several colonies were transferred to the diagnostic strip using a sterile plastic loop, and the results were read within 5 s. No colour change was indicated as a negative result and formation of a dark blue colour was viewed as a positive result.

2.3.5.2 Indole Test

The indole test was carried out by the method of Collins et al. (2008). Five ml of tryptone water (Oxoid, UK catalogue number CM0087) was inoculated with a single bacterial colony, and incubated for 48 h at 37 °C. Then, 0.2 ml of Kovacs reagent (Pro-lab Diagnostic, UK; Cat no. PL.375) was added and the formation of a red ring on the surface of the culture was regarded as a positive result.

2.3.5.3 API 20E test

An API 20E identification kit (bioMerieux, France) was used to confirm the presumptive identification of the *E. coli* isolates, and to compare strain phenotypic properties. The test was performed as specified by the manufacturer's manual. Then, the results were analysed using APIWEB™

software online from the API website (https:// apiweb. biomerieux. com / servlet/Authenticate?action=prepareLogin).

2.3.6 Strain codes and numbers

The variation between the numbers of isolates for each site was due to different strategies for sampling in the two visits (Section 2.3.2.1), the final confirmation of the selected colonies as *E. coli*, and the possibility of selecting 5 representative colonies from each sample (Section 2.3.5 and Section 3.2.3). Strains isolated from different samples were given different codes. Designations of strains from 2012 all started with the letter (A), followed by a number from 1-5; 1 and 2 designate strains isolated from the slurry tank; 3 for strains isolated from solid cow faeces; 4 for strains from liquid exudate and 5 for strains from the separated solid slurry. Isolates from 2014 were designated with the letter (B). Isolates starting with B were further designated either with L (liquid slurry), S (solid slurry), or C (cattle faeces from cow barn) and were all isolated on TBX with no other added selective agents, e.g. BL1, BC1 and BS1. If the strain designation was followed by (C), it meant the strain was isolated using TBX supplemented with cefotaxime, e.g. BLC1, BSC1 and BCC1. Finally, if the strain designation was followed by (E) it meant the strain was isolated using CHROMagar ESBL, for example BSE1 and BCE1. All the isolates are listed in Table 2.1.

Table 2-1: Isolates from the dairy farm (2012/2014)

Sample ID	Source	Media	Year Isolated
A1.1	Liquid Slurry	TBX	2012
A1.3	Liquid Slurry	TBX	2012
A1.4	Liquid Slurry	TBX	2012
A1.5	Liquid Slurry	TBX	2012
A2.1	Liquid Slurry	TBX	2012
A2.2	Liquid Slurry	TBX	2012
A2.3	Liquid Slurry	TBX	2012
A2.4	Liquid Slurry	TBX	2012
A2.5	Liquid Slurry	TBX	2012 (fully sequenced)
A3.4	cow faeces solid	TBX	2012
A3.5	cow faeces solid	TBX	2012
A4.1	liquid exudate*	TBX	2012
A4.2	liquid exudate	TBX	2012
A4.3	liquid exudate	TBX	2012
A4.4	liquid exudate	TBX	2012
A4.5	liquid exudate	TBX	2012
A5.1	Solid slurry	TBX	2012
A5.3	Solid slurry	TBX	2012
A5.4	Solid slurry	TBX	2012
A5.5	Solid slurry	TBX	2012
BL1	Liquid slurry	TBX	2014
BL2	Liquid slurry	TBX	2014
BL3	Liquid slurry	TBX	2014
BL4	Liquid slurry	TBX	2014
BL5	Liquid slurry	TBX	2014
BL6	Liquid slurry	TBX	2014
BL7	Liquid slurry	TBX	2014
BL8	Liquid slurry	TBX	2014
BL9	Liquid slurry	TBX	2014
BL10	Liquid slurry	TBX	2014
BL11	Liquid slurry	TBX	2014
BL12	Liquid slurry	TBX	2014
BL13	Liquid slurry	TBX	2014
BL14	Liquid slurry	TBX	2014
BL15	Liquid slurry	TBX	2014
BS1	Solid Slurry	TBX	2014
BS2	Solid Slurry	TBX	2014
BS3	Solid Slurry	TBX	2014
BS4	Solid Slurry	TBX	2014
BS5	Solid Slurry	TBX	2014
BS5b	Solid Slurry	TBX	2014
BS6	Solid Slurry	TBX	2014
BS7	Solid Slurry	TBX	2014 (fully sequenced)
BS8	Solid Slurry	TBX	2014
BS9	Solid Slurry	TBX	2014
BS10	Solid Slurry	TBX	2014
BS11	Solid Slurry	TBX	2014
BS12	Solid Slurry	TBX	2014
BS13	Solid Slurry	TBX	2014

BS14	Solid Slurry	TBX	2014
BS15	Solid Slurry	TBX	2014
BC1	Cow Barn**	TBX	2014
BC2	Cow Barn	TBX	2014
BC3	Cow Barn	TBX	2014
BC4	Cow Barn	TBX	2014
BC5	Cow Barn	TBX	2014
BC6	Cow Barn	TBX	2014
BC7	Cow Barn	TBX	2014
BC8	Cow Barn	TBX	2014
BC9	Cow Barn	TBX	2014
BC10	Cow Barn	TBX	2014
BC11	Cow Barn	TBX	2014
BC12	Cow Barn	TBX	2014
BC13	Cow Barn	TBX	2014
BC14	Cow Barn	TBX	2014
BC15	Cow Barn	TBX	2014
BLC1	Liquid Slurry	TBX+CTX	2014
BLC2	Liquid Slurry	TBX+CTX	2014
BLC3	Liquid Slurry	TBX+CTX	2014
BLC4	Liquid Slurry	TBX+CTX	2014
BLC5	Liquid Slurry	TBX+CTX	2014
BLC6	Liquid Slurry	TBX+CTX	2014
BLC7	Liquid Slurry	TBX+CTX	2014
BLC8	Liquid Slurry	TBX+CTX	2014
BLC9	Liquid Slurry	TBX+CTX	2014
BSC1	Solid Slurry	TBX+CTX	2014
BSC2	Solid Slurry	TBX+CTX	2014
BSC3	Solid Slurry	TBX+CTX	2014
BSC4	Solid Slurry	TBX+CTX	2014
BSC5	Solid Slurry	TBX+CTX	2014
BSC6	Solid Slurry	TBX+CTX	2014
BSC7	Solid Slurry	TBX+CTX	2014
BSC8	Solid Slurry	TBX+CTX	2014
BSC9	Solid Slurry	TBX+CTX	2014
BSC10	Solid Slurry	TBX+CTX	2014
BSC13	Solid Slurry	TBX+CTX	2014
BSC14	Solid Slurry	TBX+CTX	2014
BSC15	Solid Slurry	TBX+CTX	2014
BSC16	Solid Slurry	TBX+CTX	2014
BSC17	Solid Slurry	TBX+CTX	2014
BSC18	Solid Slurry	TBX+CTX	2014
BSC19	Solid Slurry	TBX+CTX	2014
BSC20	Solid Slurry	TBX+CTX	2014
BSC21	Solid Slurry	TBX+CTX	2014
BSC22	Solid Slurry	TBX+CTX	2014
BCC1	Cow Barn	TBX+CTX	2014
BCC2	Cow Barn	TBX+CTX	2014 (fully sequenced)
BCC3	Cow Barn	TBX+CTX	2014
BCC4	Cow Barn	TBX+CTX	2014
BCC5	Cow Barn	TBX+CTX	2014
BCC6	Cow Barn	TBX+CTX	2014
BCC7	Cow Barn	TBX+CTX	2014

BCC8	Cow Barn	TBX+CTX	2014
BCC9	Cow Barn	TBX+CTX	2014
BCC10	Cow Barn	TBX+CTX	2014
BCC11	Cow Barn	TBX+CTX	2014
BCC12	Cow Barn	TBX+CTX	2014
BCC13	Cow Barn	TBX+CTX	2014
BCC14	Cow Barn	TBX+CTX	2014
BCC15	Cow Barn	TBX+CTX	2014
BCC16	Cow Barn	TBX+CTX	2014
BSE1	Solid Slurry	CHROMagar ESBL	2014
BSE2	Solid Slurry	CHROMagar ESBL	2014
BSE3	Solid Slurry	CHROMagar ESBL	2014
BSE3B	Solid Slurry	CHROMagar ESBL	2014
BSE4	Solid Slurry	CHROMagar ESBL	2014
BSE5	Solid Slurry	CHROMagar ESBL	2014
BSE6	Solid Slurry	CHROMagar ESBL	2014
BSE7	Solid Slurry	CHROMagar ESBL	2014
BSE8	Solid Slurry	CHROMagar ESBL	2014
BSE9	Solid Slurry	CHROMagar ESBL	2014
BCE1	Cow Barn	CHROMagar ESBL	2014
BCE2	Cow Barn	CHROMagar ESBL	2014
BCE3	Cow Barn	CHROMagar ESBL	2014 (fully sequenced)
BCE4	Cow Barn	CHROMagar ESBL	2014
BCE5	Cow Barn	CHROMagar ESBL	2014

*: liquid exudate refers to liquid seepage from cow faeces; **: cow barn refers to cow faeces collected from the animal housing

2.3.7 Storage of the strains

All confirmed isolates were immediately stored as Microbank bead (Pro-Lab Diagnotics, UK. Cat no. PL.170/M) stocks at -80 °C, and were grown from frozen stocks for each subsequent characterization.

2.4 Antimicrobial sensitivity tests

The disc diffusion antimicrobial sensitivity tests were carried out according to the National Committee for Clinical Laboratory Standards (NCCLS) and Clinical & Laboratory Standards Institute (CLSI) guidelines with some modifications (NCCLS, 2002, CLSI, 2012). The inoculum was prepared using a direct colony suspension method; 2-4 colonies were isolated from an 18-24 h old incubated LB plate, and were suspended in 5 ml of Mueller-Hinton broth (Section 2.1.6). The suspension was then incubated for 2-6 h at 37 °C. The suspension volume was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard (Oxoid, UK product code R20421) which results in a suspension containing approximately 1 to 2 × 10^8 CFU ml^{-1}. This was performed visually, using adequate light to compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines. After dilution, 100 µl of the culture was pipetted onto the surface of a 90 mm Petri dish which contained 25 ml Mueller-Hinton agar (Section 2.1.7), and the inoculum was distributed by spreading using a sterile swab. The plates were left to dry at room temperature (for no more than 15 min), and 4-5 antimicrobial discs were placed on to the plate surface using sterile forceps (after applying each disc the forceps were ethanol/flame sterilized to prevent contamination) or a disc dispenser

(Oxoid, UK). The plates were then incubated at 37 °C for 18-20 h, and the results recorded by measuring the inhibition zone diameter across the disc. The size of the inhibition zone was measured using a ruler with the plate illuminated with a reflected light against a black background; the zone of clearing was interpreted according to standard measurement tables (CLSI, 2013a, CLSI, 2013b). The test was carried out using 17 antimicrobials from different antimicrobial groups. The disc assay test was performed once for each isolate, unless a specific resistance phenotype needed to be confirmed, in this case the test was repeated. All antimicrobials used in the tests are listed in Table 2.2 and their interpretive criteria in Table 2.3; the control strain used was *E. coli* ATCC 29522 which was sensitive to all the antimicrobial used in the test, to confirm the antimicrobial activity of the discs.

Table 2-2: List of the antimicrobials used for AMR profiling

Antimicrobial class	Antimicrobial	Symbol*
Penicillin	ampicillin	AMP**
Penicillin Combination with β-lactamase inhibitor	amoxicillin-clavulanic acid	AMC**
Cephalosporin 3rd generation	cefotaxime	CTX**
	ceftazidime	CAZ**
	ceftiofur	EFT
Cephalosporin 4th generation	cefquinome	CFQ
Monobactam	aztreonam	ATM**
Carbapenem	imipenem	IMP**
Aminoglycoside	streptomycin	S10
Nitrofuran Derivatives	nitrofurantoin	F
Tetracycline	oxytetracycline	OT
Antifolates combination	trimethoprim/ sulfamethoxazole	SXT**
Quinolone	nalidixic acid	NA
Fluoroquinolone	ciprofloxacin	CIP**
Fluoroquinolone (Veterinary use only)	enrofloxacin	ENR
Sulphonamides	sulphonamide	S300
Chloramphenicol	chloramphenicol	CHL**

The main classes of antibiotics and their abbreviated symbols which were used throughout this study are shown. *: symbol used is the same symbol provided on the antimicrobial disc by the manufacturer (Oxoid, UK), except for chloramphenicol a standard abbreviation was used (CHL) instead of the symbol provided on the antimicrobial disc (C) to be differentiated from the abbreviation for cytosine (C). **These symbols for the antimicrobials are recommended by the Journal of Antimicrobial Agents and Chemotherapy to be used as standard abbreviations (http://aac.asm.org/site/misc/journal-ita_abb.xhtml).

Table 2-3: Antimicrobial content (µg) per disc for the 17 antimicrobial discs used in this study, and the interpretive criteria for the zone of growth inhibition around the disc.

Antimicrobials	Antimicrobial quantity (µg)	Zone Diameter Interpretive Criteria (nearest whole mm)*		
		Sensitive	Intermediate	Resistant
ampicillin[2]	10	≥17	14–16	≤13
amoxicillin-clavulanic acid[2]	20/10	≥18	14–17	≤13
cefotaxime[1]	30	≥26	23–25	≤22
ceftazidime[1]	30	≥21	18–20	≤17
aztreonam[1]	30	≥21	18–20	≤17
imipenem[2]	10	≥16	14-15	≤13
streptomycin[2]	10	≥15	12–14	≤ 11
ciprofloxacin[1]	5	≥21	16–20	≤15
nalidixic acid[1]	30	≥19	14–18	≤ 16
trimethoprim /sulfamethoxazole[2]	1.25/ 23.75	≥16	11–15	≤10
sulphonamides[1]	300	≥17	13–16	≤12
chloramphenicol[2]	30	≥18	13–17	≤12
nitrofurantoin[2]	300	≥17	15–16	≤14
oxytetracycline[2]	30	≥19	15–18	≤14
enrofloxacin[2]	5	≥23	19–22	≤ 18
ceftiofur[2]	30	≥21	18–20	≤17
cefquinome[2]	30	≥23	20-22	≤19
cefoxitin[1]	30	≥18	15-17	≤14

*: The value for the integrative criteria were taken from (CLSI, 2013a)[1] and (CLSI, 2013b)[2].

2.4.1 Quality control reference strains

A number of quality control reference strains were used within this study as controls for different antimicrobial sensitivity tests. These strains are shown in Table 2.4.

Table 2-4: Quality control reference strains used in this study, uses and sources.

Strain	Characteristics and Use	Source
E. coli ATCC 25922	Quality control for the disc assay test, negative control for all the PCR reactions except for chromosomal AmpC	COSMOS BIOMEDICAL LTD, UK (CLSI, 2013a)
E. coli NCTC 13353	CTX-M-15 ESBL producer. Control strain for group 1 bla_{CTX-M} PCR assays and CHROMagar ESBL positive control	Public Health England, UK (Woodford et al., 2004)
E. coli NCTC 13352	TEM-10 ESBL – Transconjugant of original TEM-10 producer isolated in Chicago in 1988 Positive control for TEM PCR assay	Public Health England, UK (Quinn et al., 1989)
E. coli NCTC 13476	IMP resistance Disc assay control	Public Health England, UK (Giske et al., 2013)
Klebsiella pneumoniae NCTC 13368	SHV-18 (ATCC 700603) Positive control for SHV PCR assay	Public Health England, UK (Brown, 2000)
Enterobacter cloacae NCTC 13406	AmpC β-lactamase de-repressed (i.e. constitutive hyper-producing) mutant of NCTC 13405. Control for AmpC detection tests.	Public Health England, UK (Micalizzi, 2013)
E. coli J53 Azide^R	Recipient for conjugation experiment	Kindly provided by Dr. George Jacoby (Jacoby et al., 2003)

2.4.2 Clustering of resistance profiles (heatmap)

Clustering of antimicrobial resistance profiles was carried out using the heatmap.2 function in the gplots library (version 2.17.0) in the R software package (version 3.2.0). The heatmap shows a graphical representation of data using colours to indicate the level of resistance/sensitivity of each strain. The size of the zone of clearing around the antimicrobial discs was represented by the colour spectrum in the diagram created, with red representing no zone of clearing (resistance), red through to orange (resistant- zone of clearing 10-22 mm depending on antimicrobial), orange to yellow (sensitive-zone of clearing 17-27 mm depending on antimicrobial) and yellow to white representing a zone of clearing of 30-37 mm). A zone of >37mm indicates highly sensitive –white. Euclidean distance was used to calculate dissimilarity between each pair of bacterial strains (rows) to produce a dissimilarity matrix for all of the bacterial strains, and also for each pair of antimicrobials (columns) to produce a dissimilarity matrix for all of the antimicrobials. The clustering was carried out in both directions using complete linkage: strains were clustered according to their pattern of resistance to antimicrobials and antimicrobials are clustered according to their pattern of resistant strains. Clustering was used for data visualization, so no formal statistics were run. The creation of the heatmap was performed by Dr Dov Stekel.

2.5 Phenotypic confirmation of ESBL/AmpC producing *E. coli*

2.5.1 Rosco Diagnostica Total ESBL Confirmatory Kit 98014

All bacterial isolates that gave an inhibition zone indicating resistance or intermediate resistance to cefotaxime, and/or ceftazidime using standard antibiotic discs, were further tested using a combination of discs containing cefotaxime (CTX), ceftazidime (CAZ), and cefepime (FEP) alone and in combination with clavulanate (Table 2.5). Detection of ESBLs can be obscured by chromosomal AmpC producing bacteria. The test was performed using the total ESBL confirmatory Kit (Rosco Diagnostica, France. Cat. no. 98014) in accordance with the guidelines of the manufacturer. The disc assay was performed as described in Section 2.4. The Total ESBL Confirmatory kit contains cefepime which is not inactivated by chromosomally encoded AmpC to the same extent as other cephalosporins. An increase in the diameter of the zone of clearing around the disc by ≥ 5 mm for either antimicrobial agent tested in combination with clavulanate vs the diameter of the zone of clearing around a disc containing the agent when tested alone, indicated ESBL presence (CLSI, 2013a, Giske et al., 2013). In addition, a cefoxitin (FOX) disc (30 µg, Oxoid) was added to the test, to detect chromosomal AmpC phenotypes. The bacterial reference strains used were *E. coli* ATCC 25922 (ESBL negative control) and *E. coli* NCTC 13353 (ESBL positive control CTX-M-15).

Table 2-5: ROSCO DIAGNOSTICA Total ESBL Confirm Kit (98014).

Antimicrobial disc*	Symbol	Quantity of antimicrobial per disc (µg)
cefotaxime	CTX30	30
cefotaxime and clavulanate	CTX+C**	30
ceftazidime	CAZ30	30
ceftazidime and clavulanate	CAZ+C	30
cefepime	FEP30	30
cefepime and clavulanate	FEP+C	30

*: The antimicrobials and their identification symbol (as provided by the manufacturer), and amount of antimicrobial per disc. **: the quantity of clavulanate was not provided by the manufacturer.

2.5.2 Rosco Diagnostica AmpC Confirm Kit 98007

The presence of de-repressed/plasmid mediated AmpC β-lactamases was detected using the AmpC Confirm Kit (Rosco Diagnostica, France. Cat. no. 98007), where a ≥ 5 mm increase in the inhibition zone diameter of cefotaxime 30 µg + cloxacillin (CTXCX) or ceftazidime 30 µg + cloxacillin (CAZCX), compared with the inhibition zone for the antibiotics alone, indicated the presence of de-repressed/plasmid-encoded AmpC. Control strains used were *E. coli* ATCC 29522 (ESBL negative control) and *Enterobacter cloacae* NCTC 13406 (AmpC β-lactamase de-repressed). The disc assay was performed as in Section 2.4.

2.6 Genotyping of isolates

2.6.1 DNA extraction

2.6.1.1 Crude DNA

Bacterial DNA for PCR was extracted by a simple boiling method (Wei, 2013). A single colony of *E. coli* was picked from an overnight culture on LB agar into 100 µl of 1 x TE buffer (10 mM Tris-Cl, 1 mM EDTA buffer, pH 7.9). The suspension was heated to 99 °C for 30 min to disrupt bacterial cells (Eppendorf Thermomixer comfort, Germany) and the sample then centrifuged (Sigma1-16 bench-top centrifuge, UK) at 13,000 x *g* for 15 min. The crude DNA in the supernatants was transferred into sterile microcentrifuge tubes and stored at -20 °C until required. The DNA concentration and purity of each sample was measured using The NanoDrop® ND-1000 Spectrophotometer (Thermofisher, UK). Briefly, the spectrometer was initialized by adding 2 µl of water to the measurement pedestal surface, then the surface was cleaned and 2 µl of the blank

buffer loaded (the blank buffer here is TE buffer). Then, after cleaning the surface again, 2 µl of the DNA sample was loaded onto the measurement surface. The ratio of absorbance at 260/280 nm was used to assess the quantity and purity of the DNA. According to the manufacturer's guidelines, a ratio of ~1.8 is generally accepted as "pure" for DNA. The concentration of the DNA was given as ng μl^{-1}.

2.6.1.2 GenElute Bacterial Genomic DNA Kit

E. coli genomic DNA was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) following the manufacturer's instructions. All the buffers have a proprietary composition unless stated. Briefly, 1.5 ml of an overnight culture was centrifuged at 12,000 x *g* for 2 min at room temperature using a Sigma1-16 bench-top centrifuge. The pellet was then suspended in 180 µl of Lysis Solution T and 20 µl of RNase A solution was added followed by incubation at room temperature for 2 min. Then, 20 µl of a 20 mg ml^{-1} proteinase K solution (Sigma-Aldrich) was added, and the mixture was incubated for 30 min at 55 °C. Afterwards, 200 µl of Lysis Solution C was added and the mixture was thoroughly vortexed for 15 s to achieve a homogenised mixture followed by a further incubation at 55 °C for 10 min. During this time, a column was prepared by adding 500 µl of Column Preparation Solution and centrifuging at 12,000 x *g* for 1 min. The eluate was then discarded. After incubation, 200 µl of absolute ethanol (99% v/v) (Fisher Scientific) was added to the lysate and this was homogenised thoroughly by vortexing for 5-10 s. This mixture was then loaded onto the prepared column and was centrifuged at 6,500 x *g* for 1 min. The eluate and collection tube

were both discarded and the column was placed into a new 2 ml collection tube. Wash Solution 1 (500 µl), was added to the column and this was again centrifuged for 1 min. Next, the collection tube was discarded and replaced and 500 µl of Wash Solution Concentrate was added to the column for a second wash. The column was centrifuged at 13000 x *g* for 3 min to dry the column. The collection tube was discarded and replaced. Then, 200 µl of Elution Solution (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) was added directly to the centre of the column. The tube was then centrifuged at 6,500 x *g* for 1 min in order for the DNA to elute. The genomic DNA was stored at 4 °C for short-term storage and more immediate use and at -20 °C for long-term storage.

2.6.1.3 QIAGEN DNeasy blood & tissue kit

Bacterial DNA of high enough quality for sequencing was prepared using a QIAGEN DNeasy blood & tissue kit (Qiagen, UK. Cat. no. 69504). The kit was used according to the manufacturer's protocol, for purification of total DNA from Gram-negative bacteria, such as *E. coli*. The components of the buffers are proprietary, unless stated. A maximum of 2 x 10^9 cells were harvested in a microcentrifuge tube by centrifuging a sample of overnight culture for 10 min at 5000 x *g*. The bacterial pellet was resuspended in 180 µl of Buffer ATL, and then 20 µl of proteinase K solution was added and mixed thoroughly by vortexing. The resuspended pellet was incubated at 56 °C for 1.5 h in the thermomixer until the cells were completely lysed. 20 µl of RNase A solution (20 mg ml^{-1}) were added and incubated for 2 min at room temperature. The sample was vortexed for 15 s, and 200 µl Buffer AL and 200 µl of ethanol (96–100%

v/v) were added to the sample and mixed thoroughly by vortexing. The mixture was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 6000 x *g* for 1min. The flow-through was discarded and the Mini spin column placed into a new 2 ml collection tube. Then 500 µl of Buffer AW2 was added to the spin column, and this was centrifuged for 3 min at 20,000 x *g* to dry the DNeasy membrane. The collection tube was emptied and the spin column was centrifuged at 20,000 x *g* for another 1 min to remove any residual ethanol from the membrane. The DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and 100 µl of Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) pipetted directly onto the DNeasy membrane. The column was left to incubate at room temperature for 1 min, and then centrifuged for 1 min at 6000 x *g* to elute DNA.

2.6.1.4 Plasmid DNA extraction

A QIAprep Spin Miniprep Kit (Qiagen, UK. Cat. No. 27104) was used to purify plasmid DNA from strains. The components of the buffers are proprietary, unless stated. The manufacturer's protocol for low copy plasmid purification was performed with some modifications, as the copy number of any plasmids present was unknown. The modifications used were that 10 ml of a bacterial overnight culture was centrifuged at 4700 x *g* using a HERAEUS MEGAFUGE 40R (Thermo Scientific, UK) for 15 min, the pellets were then suspended in 500 µl of Buffer P1 and transferred into a sterile 2 ml microcentrifuge tube. Then 500 µl of Buffer P2 was added and mixed thoroughly by inverting the tube 4–6 times until the solution became clear. Within 2-4 min of the addition of P2, 700 µl of

Buffer N3 was added, and mixed immediately by inverting the tube 4–6 times. The tube was then transferred into a microcentrifuge (Sigma1-16) and centrifuged for 10 min at 17,900 x *g*. The supernatant was applied to the QIAprep spin column by pipetting and the column was centrifuged for 1 min. The flow-through was discarded, and 500 µl of PB wash buffer was added and the column centrifuged for 1 min. The flow-through was discarded, and 750 µl of PE wash buffer was added and centrifuged for 1 min. The flow-through was once again discarded and the tube was then subjected to an extra 1 min centrifugation performed to remove any residual wash buffer. The QIAprep spin column was then placed into a sterile 1.5 ml microcentrifuge tube. To elute bound DNA, 35 µl of EB buffer (10 mM Tris-Cl, pH 8.5) was added. After 2 min incubation at room temperature, a final centrifugation step was performed for 2 min. The concentration and the quality of the DNA was checked using a Nandrop spectrophotometer (as in Section 2.6.1.1), and DNA was stored at -20 °C until analysed.

2.6.1.5 Plasmid DNA analysis

To analyse plasmid DNA extracted from bacterial strains, 1 µl of the loading dye (6x) (Promega, UK) was mixed with 5 µl of purified DNA and loaded onto a 0.7% (w/v) agarose gel (Melford Laboratories, UK), containing ethidium bromide (0.4 µg ml^{-1}) in 1 x TAE running buffer (40 mM Tris-acetate, 1mM EDTA). A Quick-Load® 1 kb Extend DNA Ladder size marker (New England Biolabs, UK) was used on each gel. All the gels were imaged using a Bio-Rad Gel-Doc XR system. The gel was run for 3 h at 70 V.

2.7 Polymerase Chain reaction

All polymerase chain reactions (PCR) were carried out in a programmable C1000™Thermal cycler (Bio-Rad, UK). Either DreamTaq Green PCR mastermix (2X) (ThermoFisher Scientific, UK; product code K1081) (containing DreamTaq™ DNA polymerase, optimized DreamTaq Green buffer, 0.4 mM of each of the dNTPs, 4 mM $MgCl_2$) or HotStarTaq plus Mastermix (2X) (Qiagen, UK) were used, which will be stated in each PCR reaction assay. All oligonucleotides were synthesized by Eurofins MWG Operon, Germany. The final oligonucleotide concentration for use in PCR reactions was 10 pmol μl^{-1}.

2.7.1 ERIC-PCR

Genotypes were investigated using the ERIC-PCR method (Versalovic et al., 1991). The PCR mixture (25 µl) contained 12.5 µl of DreamTaq Green PCR master mix, 9.5 µl of nuclease-free water, ~1 µl DNA (50-100 ng) and 1 µl (10 pmol μl^{-1}) of each primer ERIC1 (forward) 5'–ATGTAAGCTC CTGGGGATTCAC–3' and ERIC2 (reverse) 5'–AAGTAAGTGACTGGGGTG AGCG–3', using the following conditions: one cycle of 3 min at 94 °C; 35 cycles of: 30 s at 94 °C, 1 min at 52 °C, and 4 min at 65 °C. The final cycle was for 8 min at 65 °C. The PCR product (7 µl) was loaded onto a 2% (w/v) TAE (GelPilot® LE Agarose, Qiagen) agarose gel (Section 2.1.10), and electrophoresed at 120 V for 2 h. A 1 kb plus DNA ladder™ (Invitrogen, UK) was included on every gel to allow cross gel comparison. All the gels were imaged using a Gel-Doc XR system (Bio-Rad, UK). A preliminary test was performed on 6 isolates to determined method

reproducibility; their strain codes and their concentrations of DNA are shown in Table 2.6.

Table 2-6: The selected isolates for preliminary study for ERIC-PCR.

Sample ID	Isolate code on the ERIC dendrogram	DNA (ng μl^{-1})
A2.5*	A1	51.2
A2.5	A2	58.9
A2.5	A3	69.3
A2.5	A4	50
A2.5	A5	50
A2.5	A6	50
A4.3	B1	70
A4.3	B2	74.1
A5.3	C1	59.9
A5.3	C2	42.5
EDL933	D1	90.5
EDL933	D2	31
A1.5	E1	83.9
A1.5	E2	91.4
A2.3	F1	38.1
A2.3	F2	74.4
A2.3	F3	50

*: A4, A5 and A6 are replicates of A1, A2 and A3 respectively with DNA concentration adjusted to 50 ng μl^{-1}.

2.7.1.1 Comparison of ERIC-PCR profiles and cluster analysis

After electrophoresis, the gel was imaged using a Gel-Doc XR system (Bio-Rad, UK) and the image analysed using FPQuest gel analysis software (Bio-Rad, UK). A dendrogram was obtained from the comparison of ERIC-PCR profiles, using the Dice coefficient, and clustered by the unweighted pair group method with arithmetic averages (UPGMA) with 1.5% of optimization and 1.5% of tolerance to display the dendrogram. Molecular variance framework (AMOVA) (Excoffier et al., 1992) was used to analyse the confidence of the selected similarity threshold and the significance of clusters. The AMOVA calculation was carried out using GenAlEx v6.5b5 software (Peakall and Smouse, 2006). The significance was examined with the calculation of ΦPT, a measure of population differentiation that suppresses intra-individual variation. In the case of AMOVA, the null hypothesis (H0; ΦPT = 0) meant that there was no genetic difference among the populations and the alternative hypothesis (H1; ΦPT >0) means there are genetic differences amongst the populations.

2.7.2 PCR detection of ESBL genes

All isolates that gave an inhibition zone indicating resistance or intermediate resistance to cefotaxime, and/or ceftazidime using standard antibiotic discs were further tested for the presence of ESBL genes regardless of the results of the phenotypic confirmation kit. *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-1}$ and *bla*$_{OXA-2}$ genes were detected using PCR as described by Dierikx and co-workers (2012). Total DNA was extracted as described in Section 2.6.1.1. Total DNA (2 µl) was used in a 25 µl

reaction mixture which contained 12.5 µl of DreamTaq Green PCR mastermix (2X), 8.5 µl of nuclease-free water, and 1 µl (10 pmol μl^{-1}) of each primer. The primers and expected product sizes are shown in Table 2.7. The reaction was carried out under the following conditions: one cycle of denaturation: 5 min at 94 °C; followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C, with a final extension of 7 min at 72 °C. The PCR product (7 µl) was loaded onto a 1% (w/v) agarose gel (Melford Laboratories, UK), and electrophoresed at 85 V for 1 h. A 100 bp plus DNA size ladder (Invitrogen, UK) was used on each gel.

Table 2-7: List of gene primers used in this study to detect ESBL genes.

Oligonucleotide name	Sequence (5`-3`)	Product size (bp)	Reference
TEM-F-	GCGGAACCCCTATTTG	964	(Dierikx et al., 2012)
TEM-R-	ACCAATGCTTAATCAGTGAG		
CTX-M-F-	ATGTGCAGYACCAGTAARGTKATGGC [a]	592	
CTX-M-R-	TGGGTRAARTARGTSACCAGAAYSAGCGG [a]		
SHV-F-	TTATCTCCCTGTTAGCCACC	796	
SHV-R-	GATTTGCTGATTTCGCTCGG		
OXA-1-F-	ATGAAAAACACAATACATATCAACTTCGC	820	
OXA-1-R-	GTGTGTTTAGAATGGTGATCGCATT		
OXA-2-F-	ACGATAGTTGTGGCAGACGAAC	601	
OXA-2-R-	ATYCTGTTTGGCGTATCRATATTC		

[a](R is a purine; Y is a pyrimidine; S is G or C)

2.7.3 Plasmid *ampC* PCR

The same isolates tested for ESBL genes (Section 2.7.2), were also tested for the presence of plasmid *ampC* and chromosomal *ampC* despite the results for the phenotypic confirmation kit. Primarily tests were carried out using a set of primers to detect the CMY type of plasmid AmpC (Dierikx et al., 2010); the PCR mixture (20 µl) contained 10 µl of HotStarTaq plus Mastermix (2X), 1 µl of each forward and reverse primer, 2 µl of coral load dye, 5 µl Dnase-free water and 1 µl of DNA. The PCR cycle consists of an initial denaturing step of 5 min at 95 °C and then 30 cycles of 94 °C (30 s), 58 °C (30 s) and 72 °C (60 s). The PCR reaction ended with a final extension step of 72 °C for 10 min.

Multiplex PCR was carried out as described in Dallenne et al. (2010) to detect plasmid AmpC; the reaction targeted the six families of AmpC including ACC, FOX, MOX, DHA, EBC and CIT. The PCR reactions were carried out in 50 µl of PCR mixture including 25 µl of DreamTaq Green PCR master mix (2X), 1 µl of each reverse and forward primer, for all except for FOX and DHA where 2.5 µl were used for each of the forward and reverse primers, 5 µl of nuclease-free water and 2 µl of DNA. The PCR cycle started with an initial denaturing step of 10 min at 94 °C and then 30 cycles of 94 °C (40 s), 60 °C (40 s) and 72 °C (60 s). The PCR reaction ended with a final extension step of 72 °C for 7 min. All the primers are listed in Table 2.8.

Table 2-8: List of gene primers used in this study to detect plasmid-mediated *ampC* and chromosomal *ampC*

Oligonucleotide name	Sequence (5`-3`)	Product size (bp)	References
MultiACC-F-	CACCTCCAGCGACTTGTTAC	346	(Dallenne et al., 2010)
MultiACC-R-	GTTAGCCAGCATCACGATCC		
MultiMOX-F-	GCAACAACGACAATCCATCCT	895	
MultiMOX-R-	GGGATAGGCGTAACTCTCCCAA		
MultiDHA-F-	TGATGGCACAGCAGGATATTC	997	
MultiDHA-R-	GCTTTGACTCTTTCGGTATTCG		
MultiCIT-F-	CGAAGAGGCAATGACCAGAC	538	
MultiCIT-R-	ACGGACAGGGTTAGGATAGY*		
MultiEBC-F-	CGGTAAAGCCGATGTTGCG	683	
MultiEBC-R-	AGCCTAACCCCTGATACA		
MultiFOX-F-	CTACAGTGCGGGTGGTTT	162	
MultiFOX-R-	CTATTTGCGGCCAGGTGA		
AmpC-F-	GATCGTTCTGCCGCTGTG	271	(Peter-Getzlaff et al., 2011)
AmpC-R-	GGGCAGCAAATGTGGAGCAA		
CMY-2-F-	ATGATGAAAAAATCGTTATGC TGC	1138	(Dierikx et al., 2012)
CMY-2-R	GCTTTTCAAGAATGCGCCAGG		

* Y is a pyrimidine

2.7.4 Chromosomal *ampC* PCR

PCR reaction mixes used to detect chromosomal *ampC* genes consisted of 25 µl DreamTaq Green (2x) PCR master mix, 2.5 µl of Chromosomal *ampC* forward primer, 2.5 µl of Chromosomal *ampC* reverse primer (Table 2.8) and 2 µl of template sample DNA. The reaction mixture was made up to 50 µl with 18 µl of nuclease free water. The PCR cycle used an initial denaturing step of 94 °C for 3 min followed by 30 cycles of 94 °C (90 s), 57 °C (30 s), and 72 °C (30 s). The reaction ended with a final extension step of 72 °C for 7 min. The PCR products were run on a TAE agarose gel and electrophoresed as described in Section 2.7.2.

2.7.5 16S rDNA V3 region PCR

PCR amplification of 16S rDNA was performed using a published protocol for amplification of the V3 variable region, using the gene primer set: V3F (5'-CCTACGGGAGGCAGCAG-3') and V3R (5'-ATTACCGCGGCTGCTGG-3') (Ercolini et al., 2003). The PCR mixture (25 µl) contained 12.5 µl of DreamTaq Green PCR master mix (2X), 9.5 µl of nuclease-free water, 1 µl DNA and 1 µl (10 pmol μl^{-1}) of each primer. The reaction was started by a denaturation step at 94 °C for 5 min. A touchdown PCR was then performed with the initial annealing temperature of 66 °C, which was decreased 1 °C every cycle for 10 cycles; finally, the remaining 20 cycles were performed with an annealing temperature of 56 °C. The extension for each cycle was carried out at 72 °C for 3 min, while the final extension was at 72 °C for 10 min. Aliquots (7 µl) of PCR products were checked on 1.5% (w/v) TAE agarose gels and purified as described in Section 2.7.6.

2.7.6 Purification of PCR products

PCR products were initially purified by separation of the DNA amplimers using a 1.5% (w/v) TAE Agarose gel run for 1 h at 85 V. The DNA band on the gel of the correct size was removed using a sterile scalpel blade, and transferred to a 1.5 ml microcentrifuge tube. DNA purification was performed using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA. Cat no. D4008), following the manufacturer's instructions. Briefly, 3 volumes of Agarose Dissolving Buffer (ADB) were added to each volume of agarose excised from the gel. The mixture was incubated at 55 °C for 5-15 min until the gel slice was completely dissolved. The melted agarose solution was then transferred to a Zymo-Spin™ Column in a collection tube, transferred to a microcentrifuge (Sigma), and centrifuged at 12000 x *g* for 1 min. The flow-through was discarded and 200 µl of the DNA wash buffer was added and centrifuged for 30 s. The washing step was repeated. The column was then placed into a 1.5 ml tube and 10 µl of DNA Elution Buffer was added directly to the column matrix and centrifuged for 1 min to elute DNA. Kit components are confidential to the manufacturer. Purity and quality of the DNA were checked using a NanoDrop® ND-1000 Spectrophotometer. The DNA was then stored at -20 °C until it was sent for sequencing.

2.7.7 Sequencing of PCR products

The purified PCR products were sequenced by Eurofins, using Sanger cycle sequencing technology (dideoxy chain termination/cycle sequencing) on Applied Biosystems™ (ABI) 3730XL sequencing machines using ABI BigDye® Terminator 3.1 chemistry.

2.8 Conjugation experiments

Broth mating, and plate mating conjugation experiments were performed for all the isolates with *bla*$_{CTX-M}$ resistance gene according to the method of (Walker and Pemberton, 1987) with some modification. *E. coli* K-12 J53Azi^{R} (provided kindly by Dr George Jacoby) was used as a plasmid recipient strain for broth and plate mating. The recipient strain was grown on LB agar containing 200 µg ml^{-1} of sodium azide (Jacoby et al., 2003, Wang et al., 2004), and the donor strains were grown up on LB agar containing 1 µg ml^{-1} of CTX. Separately, the donor and recipient were streaked on LB agar containing 200 µg ml^{-1} of sodium azide and 1 µg ml^{-1} of CTX (LB AzideCTX agar) as a control to confirm sensitivity of the donor and recipient strains to the selective agents. A loopful of each donor and recipient was transferred separately to 5 ml of LB broth; the cultures were incubated for 3 h with orbital shaking (100 rpm). Serial dilutions of the donor and recipient cultures separately were made in MRD. Plate counts were performed in triplicate to determine the number of bacteria.

2.8.1 Broth mating

Donor and recipient cultures were separately made in LB broth which was incubated for 3 h with shaking (Section 2.8). A 0.5 ml sample of the J53Azi^{R} recipient (8x10^{9} CFU ml^{-1}) and 0.5 ml of donor cells (ranged from 1.3x10^{8}-2.5x10^{9} CFU ml^{-1} depending on the tested strain) were taken from the cultures and mixed together in 4 ml of LB broth, and then incubated statically overnight at 37 °C. Serial dilutions of the donor and recipient cultures were made in MRD, and then 100 µl of the mixture was

plated in duplicate onto LB agar supplemented with 200 µg ml^{-1} of sodium azide and 1 µg ml^{-1} CTX. All the plates were incubated overnight at 37 °C.

2.8.2 Plate mating

Donor and recipient were cultured in LB broth as described in Section 2.8. A 0.5 ml sample of the J53Azi^R recipient and 0.5 ml of donor cells were mixed together in 4 ml of LB broth. 100 µl of the mixture was plated onto an LB agar plate containing no selective agents, which was incubated overnight at 37 °C. After incubation 1 ml of MRD was added to the plate; using a sterile L-shaped spreader, cells were mixed, and resuspended in the MRD. Resuspended bacterial cells were transferred to a sterile 1.5 ml microcentrifuge tube, and serial dilutions made of the resuspended mating mixture. 100 µl of the resuspended cells were plated on LB AzideCTX agar plates (in triplicate). Plates were incubated overnight at 37 °C. Any colonies growing on this medium were considered presumptive transconjugants.

2.8.3 Transconjugant selection

For both mating methods, any colonies growing on LB AzideCTX agar were considered presumptive transconjugants and representative colonies (one per isolate) were subcultured onto LB AzideCTX and used for subsequent confirmation tests (Section 2.8.4). The selected transconjugant was stored as described in Section 2.3.7.

2.8.4 Transconjugants confirmation

Presumptive transconjugant colonies were randomly selected from the LB AzideCTX agar (one representative colony per isolate) and the

selected transconjugant subcultured on LB AzideCTX agar (Section 2.8.3). The presence of the CTX-M gene was confirmed by PCR (Section 2.7.2). Further tests were performed by growing the selected transconjugants in DMEM (Dulbecco's Modified Eagle Medium) containing no methionine and no proline (Thermos Fisher Scientific, UK. Cat no. 21013-024). The recipient J53 is an auxotroph for these amino acids, so the recipient J53 (negative control), selected transconjugants and *E. coli* MG1655 (positive control) were grown in this medium. The donors (parents) were grown on this medium as well as a control. One colony of each isolate was transferred to 5 ml of DMEM and incubated at 37 °C overnight. If the bacteria grow, the colour of the medium turns from pink to yellow with turbidity, if not the colour does not change and no turbidity is seen.

2.9 Whole Genome DNA Sequencing

Whole genome DNA sequencing was carried out for 4 isolates for complete genomic characterisation. DNA was extracted as described in Section 2.6.1.3. The DNA concentration and purity of each isolate was measured using the NanoDrop® ND-1000 Spectrophotometer (Section 2.6.1.1). The extracted DNA then was sent for sequencing. One of the strains (A2.5) was sequenced in BGI Tech Solutions (Hong Kong) Co., Limited using an Illumina MiSeq machine, while the other strains (BCE3, BCC2 and BS7) were sequenced at DeepSeq (University of Nottingham) using an Illumina MiSeq 300PE machine. First the isolates underwent DNA Resequencing Library prep for the Illumina platform and were then sequenced, generating approximately 50x coverage per isolate.

2.9.1 Genomic Assembly and Analysis

2.9.1.1 Genome assembly

Reads were assembled initially with Spades (Bankevich et al., 2012) and low-level contaminating contigs were discarded. Subsequently reads were assembled with the Celera assembler (Berlin et al., 2015).

2.9.1.2 Genome annotation

The genome assemblies were annotated with Prokka (Seemann, 2014) with subsequent inclusions from ShoreLark (SequenceAnalysis.co.uk). Predicted proteins were searched with OrthoMCL (Li et al., 2003) against the protein versions of the BacMet and CARD databases. For three strains (BCC2, BCE3 and BS7) results were visualised across the draft genomes using a customised R script with ggplot2 to form a heatmap. Genome assembly and annotation and the methods related to them was provided and performed by Dr Lisa Crossman.

2.9.2 Gene analysis: Artemis and NCBI tools

The Artemis: Genome Browser tool (Rutherford et al., 2000) available from Wellcome Trust Sanger Institute (http://www.sanger.ac.uklresources/software/artemisD) was used in order to visualise the sequence and for confirmation of gene sequence annotation. In order to confirm the identity of annotated genes (Section 2.9.1.2), the gene underwent further identification using the NCBI database basic local alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the amino acid sequence.

2.9.3 The comprehensive antibiotic resistance database (CARD)

For the sequenced PCR products of CTX and TEM genes, the comprehensive antibiotic resistance database (CARD) (McArthur et al., 2013) was used along with NCBI database to identify the variant type using both amino acid and nucleotide sequence (http://arpcard.mcmaster.ca/ ?q=CARD/blast/0).

2.9.4 Resfinder database

The assembled DNA FASTA files were loaded into the Resfinder database to confirm the identification of antibiotic resistance genes detected by other databases (Zankari et al., 2012). The database is available from https://cge.cbs.dtu.dk/ services/ResFinder/.

2.9.5 Multi locus sequence type (MLST)

The MLST service from the Centre for Genomic Epidemiology (Larsen et al., 2012) was used to define the Multilocus Sequence Typing (MLST) for the sequenced strains. MLST identities were determined using *E. coli* #1 option (Wirth et al., 2006). The service searches for the different housekeeping genes allocating a number to each and provides the Sequence Type (ST) and sequence cluster. The database is available from https://cge.cbs.dtu.dk/services/MLST/.

2.9.6 Artemis Comparison Tool

Genome sequences were compared using the Artemis Comparison Tool (ACT) (http://www.sanger.ac.uk/science/tools/artemis-comparison-tool-act) from Wellcome Trust Sanger Institute. WebAct (http://www.webact.orglWebACT/home) was used to generate the pairwise comparison files

(Abbott et al., 2008). Comparison files were analysed and visualized using ACT (Carver et al., 2005).

2.9.7 Other software and websites

For aligning 2 specific sequences and determining mismatches and mutations, DNADynamo Sequence Analysis Software (http://www.bluetractorsoftware.co.uk/DynamoDemo.htm) was used; the NCBI alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was also used for the same purpose. The EMBL-EBI sequence translation tool was used to translate the nucleotide sequence for the sequenced PCR product into amino acid sequences (http://www.ebi.ac.uk/Tools/st/). Furthermore, PlasmidFinder was used to detect predicted plasmids within the total genome sequences. This software is used to identify plasmids in total or partially sequenced isolates of bacteria (https://cge.cbs.dtu.dk/services/PlasmidFinder/) (Carattoli et al., 2014). ISfinder was used to identify the insertion sequence types and their IR sequences (https://www-is.biotoul.fr)(Siguier et al., 2006).

3 . *E. coli* isolation, enumeration and characterization from cattle farm slurry and faeces

3.1 Introduction

The amount of animal manure used as fertiliser in UK agriculture has increased. In 2004 an estimated 70 million tonnes of animal manure, including solid manures and slurries, were spread onto agricultural land per year (Hutchison et al., 2004), while in 2013 it has been reported that an estimated 96 million tonnes of farm manure waste was spread onto land in the UK. Despite the benefits of using this manure as a fertilizer, it may contain pathogens such as *Listeria monocytogenes*, *Campylobacter jejuni* or *E. coli* pathotypes, which can cause food borne diseases (https://www.gov.uk/guidance/managing-nutrients-and- fertilisers), and may be responsible for the spread of antibiotic resistance genes and AMR bacteria.

The dissemination of multidrug resistant (MDR) *E. coli* in farms and food production units may occur via faecal cross-contamination within and between groups of animals such as cattle, pigs and chickens. Faecal carriage of MDR and ESBL-producing *E. coli* from food producing animals has been reported in many studies (Escudero et al., 2010, Horton et al., 2011, Geser et al., 2012). Even in healthy animals, commensal *E. coli* carrying ESBL genes have been found (Zheng et al., 2012).

With regards to the dissemination of ESBL-producing *E. coli* in dairy farms, some studies have tested the prevalence of certain ESBL *E. coli* types within dairy farms (Liebana et al., 2006, Watson et al., 2012, Schmid et al., 2013). Watson et al. (2012) commenced a study to observe the epidemiology of an ESBL *E. coli* (CTX-M-15) on a commercial

dairy farm located in the United Kingdom. The tested farm herd comprised milking and non-milking cows, calving cows, and calves. In the studied farm there were four different sites (A–D) which specialised in different husbandry activities in the cycle for milk production. One of the findings in this previous study was that the milking cow groups (located in site A, with other cattle groups with higher prevalence of CTX-M *E. coli* including calving cows, neonatal and sick calves) had the highest proportion of CTX-M *E. coli* positive samples, and were approximately 14 times more likely to test positive for CTX-M than the non-milking cow groups (located in site C with a low level of prevalence) (Watson et al., 2012). Using 3rd and 4th generation cephalosporins with other antimicrobials was highest on site A for the treatment and prophylaxis of mastitis, and other infections; this with the continual circulation of cattle between sites was thought to have a role in the epidemiology of CTX-M15 *E. coli*, and the increased prevalence of the CTX-M-15 *E. coli* in the milking cow group (Watson et al., 2012).

On the farm used in the current study there were around three cases of mastitis a week caused by *E. coli* and, at the time of sampling, these were treated with cefquinome which is a 4th generation cephalosporin. In addition, ceftiofur (3rd generation cephalosporin) and many other antimicrobials were used for treating cows with other infections (Section 2.3.1).

Cattle farms differ depending on the type of production methods used, and can contain dairy cattle and veal calves, which are housed under

different circumstances; the type of housing and farm management also varies. Usually, dairy farms are predominantly closed production systems (Hordijk et al., 2013c). Depending on the farm space and the farmer's choice, dairy farming in the UK can vary from small scale, extensive units where animals are exclusively grazed, to more intensive units, where cows may be housed for all or part of their lactation (http://www.dairyuk.org). In the dairy farm (Figure 3.1) used in this study the milking cows (approximately 200 dairy cows) are housed for the majority of the year inside barns, and animal waste (approximately 60 kg per day) is automatically scraped into a slurry handling system (Section 2.3.1).

As previously discussed in Sections 1.7.3 and 1.7.4, animal manure, sewage sludge and activated sludge may contain high levels of MDR bacteria. Mobile genetic elements are important reservoirs of resistance genes in the environment and can be co-selected by pollutants such as antibiotics and metals found in animal and human waste (Wellington et al., 2013). One piece of evidence that suggests increased antimicrobial use in farms is associated with increased resistance gene carriage in bacteria is the high frequency of bacteria carrying AMR genes in pigs compared to cattle or sheep, which is believed to be associated with the different amounts of antimicrobials used in the husbandry of these animal species (Enne et al., 2008).

The aims of the study for this chapter were:

- To isolate *E. coli* from slurry and faeces samples around the dairy farm which were to be used as an indicator of the levels and types of resistances on farm and to survey the level of the antimicrobial resistance in the slurry waste. Initial sampling was performed in 2012 using TBX agar only while in 2014, TBX+CTX and CHROMagar ESBL media were also used.
- To evaluate the prevalence of CTX-resistant strains amongst the *E. coli* and the total coliform populations in the slurry samples. The approach was to use antibiotic-selective and non-selective conditions to be able to determine the prevalence of the presumptive CTX-resistant *E. coli* and coliforms in these samples by comparing the number of *E. coli* isolates from TBX+CTX with the total from TBX and comparing the number from MAC+CTX with that of MAC only for the coliforms.
- To study the genotypic diversity amongst those isolates using ERIC-PCR methods, as it is one of the common methods used widely for the genotyping of *E. coli* and many other enteric bacteria. The genotyping was performed for two reasons: firstly, to see if there is clonality to the resistant population. Secondly to see if strains from the same genotype show differences in their AMR profile which might be suggestive of the potential for plasmid carriage; this will be clarified further in Chapter 4. ERIC-PCR was used as the method of typing because it is a relatively simple and

high throughput method which gives sufficient data to give a first evaluation of this.

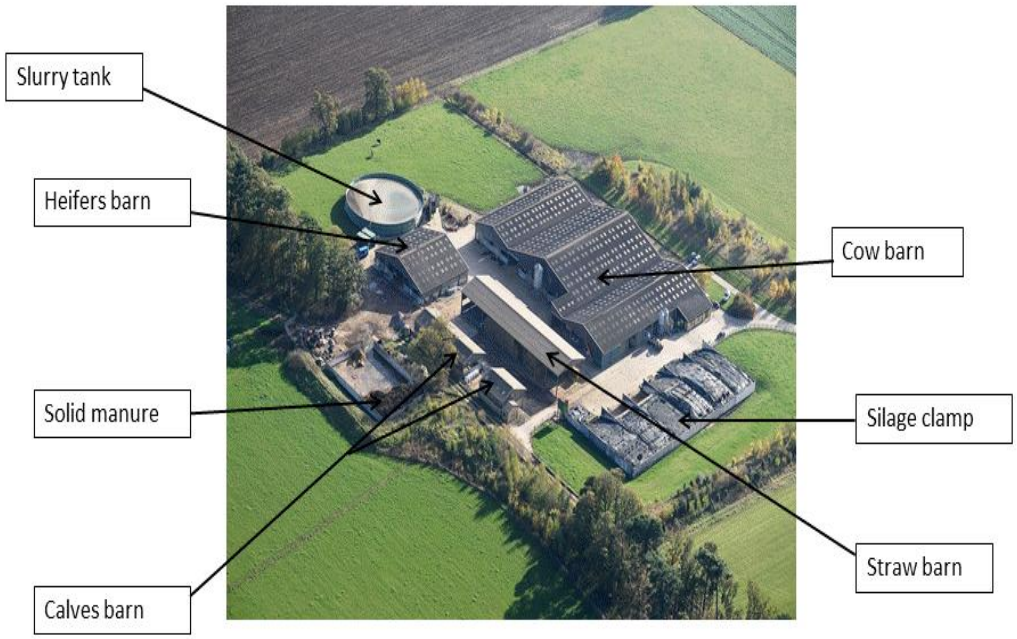


Figure 3-1: The dairy farm, where *E. coli* strains were isolated in this study.

The figure shows the 3 main locations used for sample collection: the main cow barn, the slurry tank and the separated solid slurry. The picture was taken by Mr. Mike Beard, (University of Nottingham, Sutton Bonington Campus photographer).

3.2 Results

3.2.1 *E. coli* isolation from 2012 samples

This initial study was carried out to establish the sampling and testing approaches to isolate *E. coli* from the dairy farm and determine the nature of antimicrobial resistance carriage. Five samples were collected from the dairy farm (Section 2.3.2.1) and plated on TBX without antimicrobial selection to ensure *E. coli* isolation and allow an indication of the percentage of isolates which were resistant to antimicrobials, and which antimicrobial resistances were present. Twenty-five (five per sample) presumptive *E. coli* isolates were isolated using TBX agar (Section 2.3.2.1). Twenty isolates were confirmed as *E. coli* by being oxidase negative and indole positive (Sections 2.3.5.1 and 2.3.5.2). API 20E identification kits were further used to confirm the identity of all the strains isolated. Good or a very good identification (85.5-99.8%) was obtained for all the 20 strains as *E. coli*, Appendix I. Therefore, confirmation of the *E. coli* strains isolated on TBX agar and tested using only the oxidase and indole tests was regarded as sufficient confirmation for colonies as *E. coli*. This approach was therefore used for the identification of the strains isolated in 2014 without routinely checking identification by API 20E (Section 3.2.3). Testing the antimicrobial resistance profile for these 20 isolates showed five isolates with CTX resistance; this led to the use of TBX plus CTX and CHROMagar ESBL media for specifically isolating ESBL/AmpC *E. coli* in the 2014 study. The characteristics of these isolates will be covered in later sections (Section 3.2.3- 3.2.6).

3.2.2 *E. coli* and total coliform counts for the samples from 2014

The second sampling study was carried out in 2014 with the primary aim of isolating and characterising resistance in further *E. coli* isolates. Because CTX-resistant *E. coli* had been found in the first study, this second study used TBX alone and TBX with cefotaxime and MacConkey agar with and without cefotaxime to allow benchmarking of the levels of coliforms and *E. coli* present, and to determine the frequencies and nature of the antimicrobial resistances these carried. As resistance carriage had been found to be frequent amongst the *E. coli* isolated in 2012, a limited number of representative faecal and slurry samples were collected as given in Section 2.3.2 and processed as in Section 2.3.2.2. In addition, CHROMagar ESBL was used for isolation as this is a commercially produced medium for isolation of ESBL-producing species.

The total mean counts (CFU ml^{-1}) of coliforms and *E. coli* on both MacConkey (MAC) and TBX with and without antibiotic selection (using CTX) are shown in Appendix II. Specific counts were made on the basis of counting those colonies showing colony morphologies characteristic for the target group on the medium and hence represent presumptive counts. Samples from solid slurry gave the highest value for total coliform counts (TCC) of 6.06±0.09 log_{10} CFU ml^{-1} for sample 3 on MAC only, and the highest *E. coli* count (ECC) on TBX which was 5.86±0.11 log_{10} CFU ml^{-1}. The highest value for the presumptive CTX-resistant coliforms on MAC+CTX was from solid slurry samples as well, with the highest count value of 3.60±0.03 log_{10} CFU ml^{-1} from sample 2, while the highest count

for the presumptive CTX-resistant *E. coli* on TBX+CTX was from the cow barn at 3.43 ±0.07 $\log_{10}$ CFU ml^{-1} from sample 1 (Appendix II).

A comparison of TCC ($\log_{10}$ CFU ml^{-1}) on MAC and on MAC+CTX between the different sources of samples was performed using the average of the total count value for the three replicate samples from each site (slurry tank, solid slurry and cow barn). Tukey's multiple comparisons test and the ANOVA test were used, see Section 2.3.4.

The TCC $\log_{10}$ CFU ml^{-1} on MAC were significantly different between the samples from the slurry tank and the other two sites ($P< 0.0001$, DF=91) with the counts for the coliforms from the slurry tank samples being less than those in the other samples; no significant differences were observed between solid slurry and the cow barn samples. TCC on MAC+CTX were also significantly different (lower) between the samples from the slurry tank and the other two sites ($P< 0.0001$, DF=91) with no significant difference between the count values from the cow barn samples and solid slurry detected (Figure 3.2). It should be noted here that the number of colonies for the TCC on MAC+CTX from the slurry tank, solid slurry (S1 and S3) and from S3 from the cow barn were less than the quantification limit for the plate counting method used (< 3.39 $\log_{10}$ CFU ml^{-1}; based on 25 colonies per plate and 2500 cells per ml). A summary of ANOVA analyses and multiple comparisons are illustrated in Appendix III.

The comparison of ECC $\log_{10}$ CFU ml^{-1} on TBX and TBX+CTX between the different sources of samples was performed using the average of the total

count value for the three replicate samples from each site (slurry tank, solid slurry and cow barn) from the samples collected in 2014. As with the TCC, Tukey's multiple comparisons test and the ANOVA test were used, see Section 2.3.4.

Significant differences ($P< 0.0001$, DF=86) were found when the average total count (log_{10} CFU ml^{-1}) for the ECC on TBX from the slurry tank were compared with the average total TBX count from the cow barn and the solid slurry, with the slurry tank samples again giving lower counts than those from the other two sources; again, no significant differences were found with regards to the ECC between the cow barn and solid slurry samples. The ECC from the slurry tank on TBX+CTX plates was also significantly lower ($P< 0.0001$, DF=86) from the respective ECC from the cow barn and solid slurry, and the ECC from the solid slurry was significantly lower ($P< 0.0001$, DF=86) to that from the cow barn, Figure 3.3. This reduction in *E. coli* and coliform counts for the samples from the slurry tank compared to the samples from other sources might be due to the processing of the cow faeces before entering the slurry tank and many other possible reasons (discussed in Section 3.3).

It should be mentioned here that the number of colonies for the ECC on TBX+CTX from the slurry tank, solid slurry and sample 2 and 3 from the cow barn were again less than the quantification limit for this study (< 3.39 log_{10} CFU ml^{-1}); a summary of ANOVA and multiple comparisons are illustrated in Appendix IV.

Based on a comparison of the counts obtained on selective agars with and without the presence of antibiotics, the percentage of the presumptive coliforms resistant to cefotaxime ranged from between 0.01-0.76% depending on sample, with the highest proportion of resistant strains seen in sample 2 from the liquid slurry, while the percentage of the presumptive *E. coli* resistant to cefotaxime ranged from 0.002-0.54 % with the highest proportion of resistant strains seen in sample 2 from the cow barn (Appendix II).

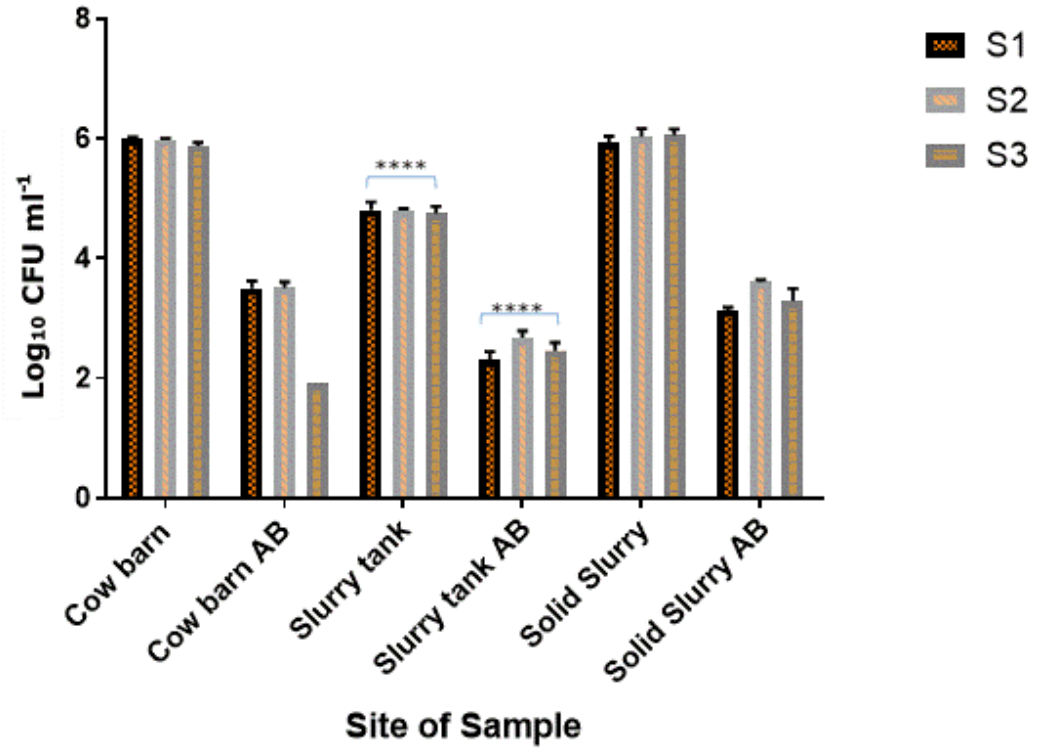


Figure 3-2: Comparison of total coliform counts (TCC) isolated on MAC and with cefotaxime on MAC+CTX for the samples from 2014.

The data are shown as Log_{10} CFU ml^{-1}. S: sample; S1, S2 and S3 are three different samples from each site (slurry from the cow barn, liquid slurry from the slurry tank, and solid slurry). Sites with the suffix AB give the TCC on MAC+CTX (with 2 µg ml^{-1} of CTX); all the samples were collected in 2014. The difference between TCC from the liquid slurry samples on MAC and on MAC+CTX compared with the other 2 sites were significantly different ($P< 0.0001$). For each sample, three biological replicates were plated in duplicate. The error bars indicate the standard deviation; two way ANOVA & Tukey's multiple comparison test were performed for the statistical analysis.

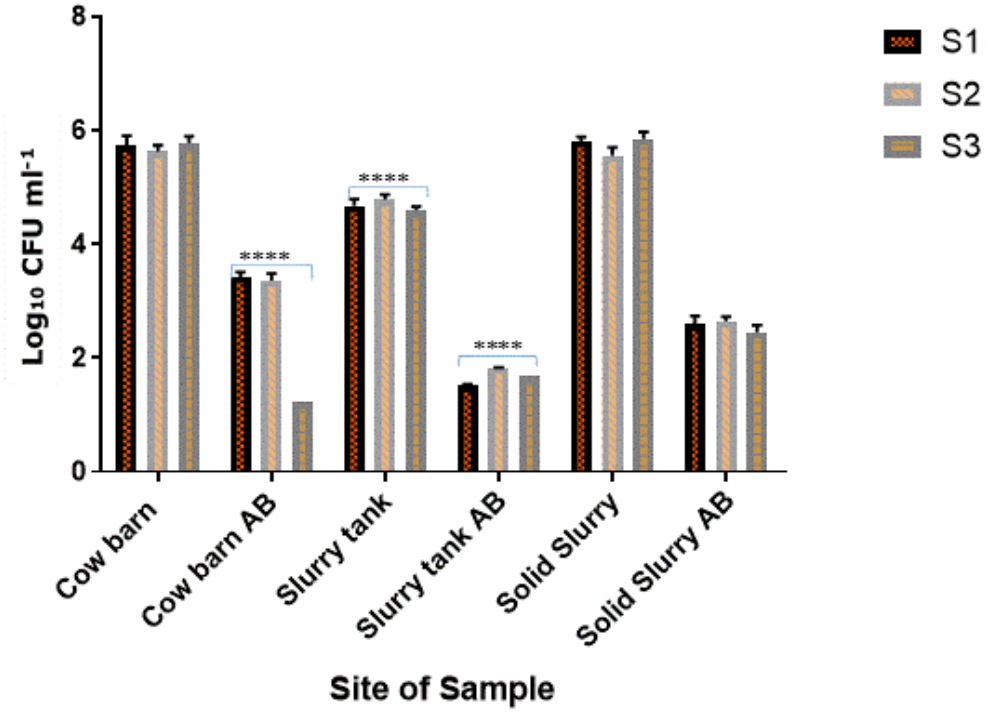


Figure 3-3: Comparison of *E. coli* counts (ECC) on TBX and with cefotaxime on TBX+CTX agar for the samples from 2014.

The data are shown as Log_{10} CFU ml^{-1}. S: sample; S1, S2 and S3 three different samples from each site (slurry from the cow barn, liquid slurry from slurry tank and solid slurry). Sites with a suffix AB have the ECC counts made using TBX+CTX (containing 2 µg ml^{-1} of CTX); all the samples were collected in 2014. The differences between ECC from the liquid slurry samples on TBX and on TBX+CTX compared with the other 2 sites were significantly different ($P< 0.0001$); the differences between mean value for ECC from the cow barn samples on TBX+CTX compared with that for solid slurry were significantly different ($P< 0.0001$). For each sample, three biological replicates were plated in duplicate. The error bars indicating the standard deviation are shown; two way ANOVA & Tukey's multiple comparison test were performed for the statistical analysis.

3.2.3 *E. coli* confirmation

Five presumptive *E. coli* colonies were selected from each sample where possible. This was possible using TBX for all the samples, but varied when using TBX+CTX and CHROMagar ESBL, for example not enough colonies grew from the liquid slurry sample using TBX+CTX and CHROMagar ESBL, and so fewer colonies were selected. With regards to the number of colonies selected from CHROMagar ESBL medium, a total of 35 pink colonies were selected from all samples.

The selected green/blue colonies from TBX and TBX+CTX were purified; pure cultures were obtained by selecting single colonies and streaking for single colonies on LB agar, and these were tested for oxidase activity (Section 2.3.5.1) and the ability to convert tryptophan into indole (Section 2.3.5.2) as previously established (Section 3.2.1).

In total 155 colonies were selected from different media: in 2012, 25 colonies from TBX; in 2014, 35 colonies from CHROMagar ESBL, 47 colonies from TBX and 48 colonies from TBX+CTX. 126 isolates were confirmed as *E. coli*. Nine isolates that had grown on TBX or TBX+CTX were either negative for the indole test or positive for oxidase. Twenty of the colonies isolated on the CHROMagar ESBL gave typical colony characteristics for *E. coli*, but the confirmation tests did not confirm this identification, and consequently they were excluded from further analysis. Strains were numbered indicative of source and medium of isolation: A designates strains from 2012 samples with isolation on TBX (no other

media were used in this preliminary study), from which 20 isolates were confirmed as *E. coli* (Section 3.2.1). B designates 2014 isolates of which 106 isolates were confirmed as *E. coli*. The full system for strain numbering is given in Section 2.3.6.

3.2.4 16S rDNA and API 20E for isolates not confirmed as *E. coli*

Putative *E. coli* isolates which did not give the characteristic oxidase and indole tests for *E. coli* were further characterised to determine which organisms give false-positive colonies. One isolate from 2012 that grew on TBX agar and was green-blue, but was positive in the oxidase test and therefore excluded from the *E. coli* grouping, was identified by API 20E as *Aeromonas hydrophila/caviae.* Many colonies on CHROMagar ESBL showed typical pink colonies but when subsequently grown on TBX did not produce blue/green colonies. 16S rDNA sequencing (Section2.7.5) of 3 isolates identified two as *Escherichia fergusonii* and one as *E. coli* but phenotypically this strain was lacking glucuronidase activity. As 3-4% of *E. coli* are glucuronidase-negative, notably *E. coli* O157 strains, it was not possible to proceed with any work on such isolates due to the laboratory being ACDP-CL2.

3.2.5 ERIC-PCR preliminary study

Many methods are available to carry out genotyping of *E. coli* from environmental samples. Both enteric bacteria and *Vibrio* genomes contain enterobacterial repetitive intergenic consensus (ERIC) sequences which are 127 bp imperfect palindromes that occur in multiple copies in the genome (Wilson and Sharp, 2006). There are about 30-50 copies of ERIC

sequences in *E. coli* (Sharples and Lloyd, 1990). The primers in the ERIC-PCR method bind to the ERIC sequences and then amplify the DNA between the ERIC sequences (Versalovic et al., 1991). However, due to some observations in the literature indicating a poor reproducibility of PCR-based typing methods (Sabat et al., 2013), a preliminary study to check the reproducibility of this method was performed before testing a large number of isolates. The experimental approach to determine reproducibility of the method was: to select some strains and to do DNA extraction for each isolate more than once; to subject the extracted DNA to ERIC-PCR, and to see if the same band pattern resulted from different DNA extractions of the same strain. So accordingly, six isolates were chosen to perform the test; five farm isolates (A1.5, A2.5, A2.3, A4.3 and A5.3), and *E. coli* O157:H7 EDL933 (EHEC) *stx*- was further selected as a bovine strain not related to the farm isolates. This strain had been isolated from Michigan ground beef linked to an outbreak in 1982 involving contaminated hamburgers (Perna et al., 2001), and originally had the *stx* genes, but these had been deleted. DNA was extracted from each isolate 2 or 3 times independently using a DNA extraction kit (Genelute kit, Sigma) and using the DNA preparation boiling method (Section2.6.1.1). DNA concentrations were measured using a Nanodrop ND100 to check the purity of DNA, and to determine if the presence of different DNA concentrations could affect the band pattern appearance for the selected strains (Table 2.6). For the same reason, the replicates of the extracted DNA for one of the selected isolates, A2.5 (A1, A2 and A3), were adjusted to 50 ng μl^{-1} (A4, A5 and A6, respectively; see Section

2.7.1). The extracted DNA was then subjected to ERIC-PCR. To calibrate the band sizes, a 1 kb plus DNA ladder™ (Invitrogen, UK) was used for the analysis of all the gels. This decision has made after comparing with two other DNA ladders which were 100 bp ladder™ (Invitrogen, UK) and Promega™ 1kb DNA ladder (Promega, UK) (Figure 3.4). The range of the 1 kb plus DNA ladder™ was suitable to cover the band range of the selected isolates. Three lanes of DNA ladder were load in each gel, to increase the optimization step during the gel analysis. Replicates (different PCR products from different DNA extractions for the same isolates) were run on the same gel and on different gels and these were compared together. All the isolates were run on three replicate gels (2% (w/v) agarose gel) at 120 V, (Section 2.7.1), gels 1 and 2 were run exactly for 2 hours and gel 3 was run for 2 h and 10 min (Figure 3.4). This latter alteration was performed to check if there were any variations in the band matching patterns with running time. Replication of the same isolates across different gels allowed the best setting for the FPQuest software for the gel analysis to be determined. Some variations in the density of some faint bands were noticed (Figure 3.4), but using the FPQuest software for the gel analysis gave all the replicates showing 100% similarity in their band pattern profiles on the same gel (Appendix V). A dendrogram was produced by FPQuest v4.5 with the UPGMA algorithm based on a Dice similarity coefficient with a 1% band position tolerance, the setting of the program was to exclude uncertain bands from the analysis. For comparing replicates between two gels, all the replicates of isolates showed a similarity between 96-100% when using

1% band position tolerance and 1% of optimization (Appendix VI). For the isolates run on 3 different gels, the similarity value was 92.8-100% for all the replicates using 1.5% for the optimization and band tolerance (Figure 3.5). Based on these analyses and this level of similarity from different gels, the method was considered robust. Using the FPQuest software to do the gel analysis, and using the 1.5% of optimization and band tolerance value, enabled the reduction of gel-to-gel variation and gave matches between the different replicates run on different gels. After analysis of the preliminary results, a standard method was set to follow during this study. The concentration of the DNA did not show an effect on the band intensity, but for consistency a concentration of 50-100 ng μl^{-1} was chosen as a standard concentration for the ERIC-PCR during the study. This was due to the fact that the DNA concentrations for approximately 80% of the replicate samples used in the preliminary study were within this range, Table 2.6. The PCR and gel electrophoresis conditions were all standardised and used throughout the study together with the dendrogram analysis as given in Section 2.7.1 and 2.7.1.1.

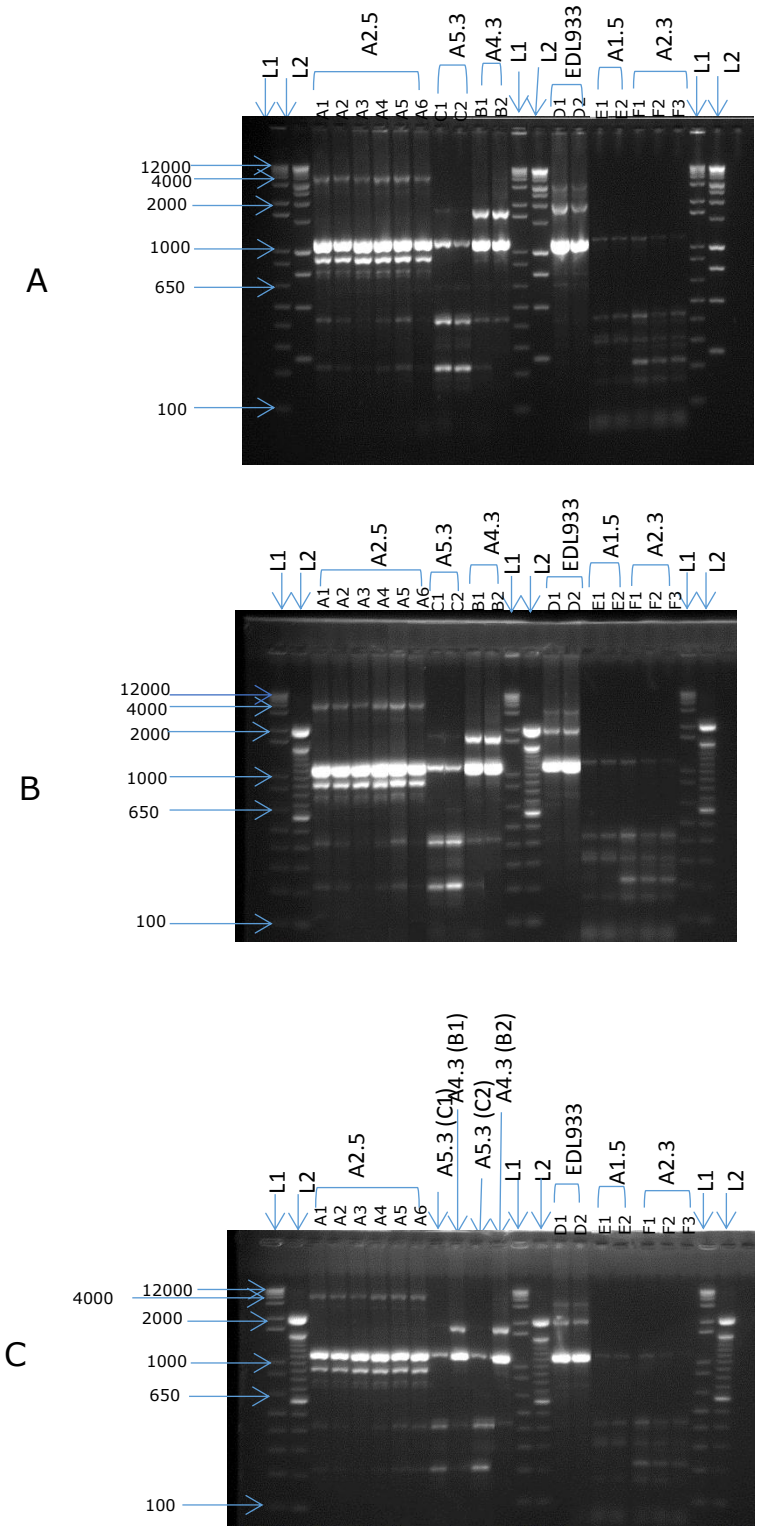


Figure 3-4: Agarose gel figures for the isolates which were used in preliminary ERIC-PCR analysis.

A, B and C represent gels 1, 2 and 3, respectively (three different gels for the same replicates used in the ERIC-PCR preliminary study) Gels were 2% (w/v) TAE agarose run at 120 V; gels 1 and 2 were run for 2 h; gel 3 run for 2 h and 10 min. L1 =1 kb plus DNA ladder™ (Invitrogen, UK), L2, gel1 : Promega™ 1kb DNA ladder; L2, gels 2 and 3: 100 bp ladder™ (Invitrogen, UK); all the replicates were subjected to the same PCR conditions, except for the DNA concentrations. The different DNA concentrations for each isolate have a different code, for example D1 and D2 for EDL933 means two different DNA extractions from the same isolate, the DNA concentration for A2.5 replicates are (in ng μl^{-1}), A1: 51.2; A2: 58.9; A3: 69.3; A4: 50; A5: 50; A6: 50. For A4.3 are, B1: 70 and B2: 74.1; for A5.3: C1: 59.9 and C2: 42.5; for *E. coli* EDL933 are D1: 90.5 and D2: 31; for A1.5 are E1: 83.9 and E2: 91.4 and for A2.4 are F1: 38.1, F2: 74.4 and F3: 50.

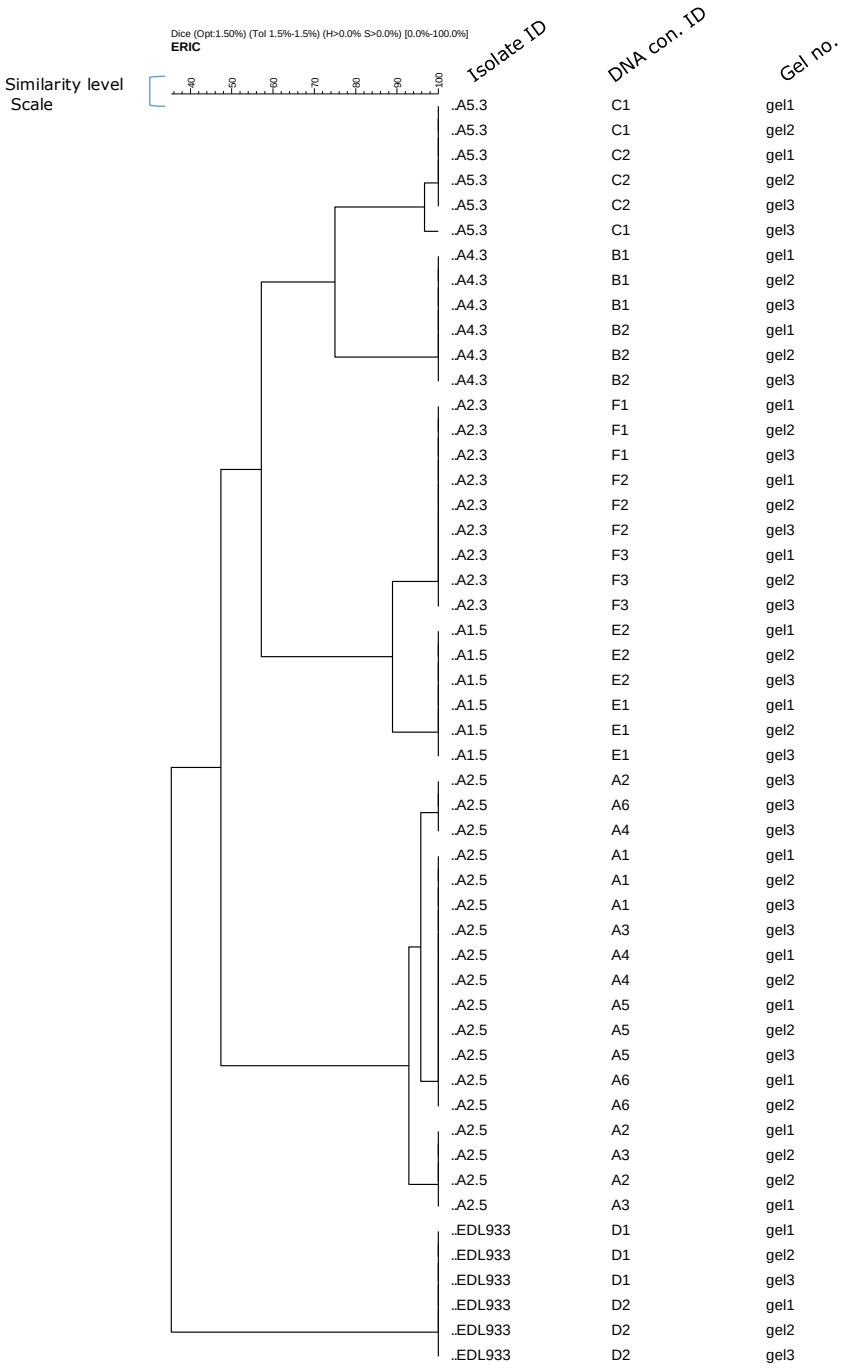


Figure 3-5: ERIC-PCR genotyping, preliminary study.

Replicate samples of the ERIC-PCR amplicons of different *E. coli* strains (n=6), were resolved on three agarose gels [gels 1 and 2, the isolates were run in a 2% (w/v) TAE agarose gel at 120 V for 2 h; gel 3 run at the same conditions but for 2 h and 10 min; 2-6 replicates for each isolate on each gel]. This dendrogram was produced using FPQuest v4.5 with the UPGMA algorithm based on a Dice similarity coefficient with a 1.5% band position tolerance. For each isolate, PCR

amplification was performed on independently extracted replicate DNA samples. The different DNA concentrations for each isolate have a different code, for example D1 and D2 for EDL933 means two different DNA extractions from the same isolate, the DNA concentration for A2.5 replicates are (in ng μl^{-1}), A1: 51.2; A2: 58.9; A3: 69.3; A4: 50; A5: 50; A6: 50. For A4.3 are, B1: 70 and B2: 74.1; for A5.3: C1: 59.9 and C2: 42.5; for *E. coli* EDL933 is D1: 90.5 and D2: 31; for A1.5 are E1: 83.9 and E2: 91.4 and for A2.4 are F1: 38.1, F2: 74.4 and F3: 50. The same PCR reaction amplimers were run on three different gels, the gel number is indicated to allow comparisons.

3.2.6 Genotyping of *E. coli* strains using ERIC-PCR

The similarity of the ERIC-PCR profiles of the 126 *E. coli* isolates from different sampling sites was analysed, and cluster analysis divided strains into 7 main groups at a 54% similarity level (Figure 3.6), group I (n =9), group II (n =41), group III (n=33), group IV (n =2), group V (n=12),group VI (n=7) and group VII (n=19), with 3 outlier strains BS7, BC3 and BSE3 which did not cluster with any other groups at a 54% similarity threshold. This threshold for group delineation was based on the cluster significance analysis ($\Phi PT=0.53$; $P<0.001$) (Appendix VII) as AMOVA statistical analysis using a higher % similarity threshold showed no significant difference amongst all the branches. At 54% similarity, all the groups were significantly different, except for group IV which overlapped with other groups; as this group is composed of 2 strains only it can be identified as an outlier more than a group. Group I included isolates mainly from the cow accommodation, which were isolated using CHROMagar ESBL or TBX+CTX, whilst group II was composed of mixed isolates from the solid slurry and the cow accommodation. In group III, the isolates were from mixed sources with no source distinction, but all

the isolates were from the TBX+CTX plates. Within group IV the 2 isolates were from solid slurry and the slurry tank. Within group V isolates from the slurry tank predominated. Most of the isolates in group VI were from the solid slurry and were isolated using TBX+CTX. With regard to group VII, the isolates represented a specific period of time (2012); those isolates were isolated from different sites on the farm (Section 2.3.2.1 and 2.3.6). In comparison to the 2014 isolates, the ERIC profiles of the 2012 isolates (A-strains) partitioned them into a separate group on the dendrogram, showing them to be a quite distinct genetic population; all these strains were isolated on TBX agar without antibiotic selection.

There were 3 outliers at this level of similarity (54%) which are very distinct from the other groups: BS7 which was isolated from the solid slurry, BC3 which was isolated from the cow barn and BSE3B which was isolated from solid slurry using CHROMagar ESBL medium. There was no significant association of clusters with source of isolation, with most groups containing isolates from all three sources: cow barns, slurry tank and solid slurry (discussed further in Section 3.3). Some solid slurry isolates did associate closely in a separate cluster, in group VI, suggesting this distinct genotype may be partitioning with the solids. The same was the case from the cow barn isolates, group I. Figure 3.7 shows some ERIC profile patterns for some strains from the different dendrogram groups.

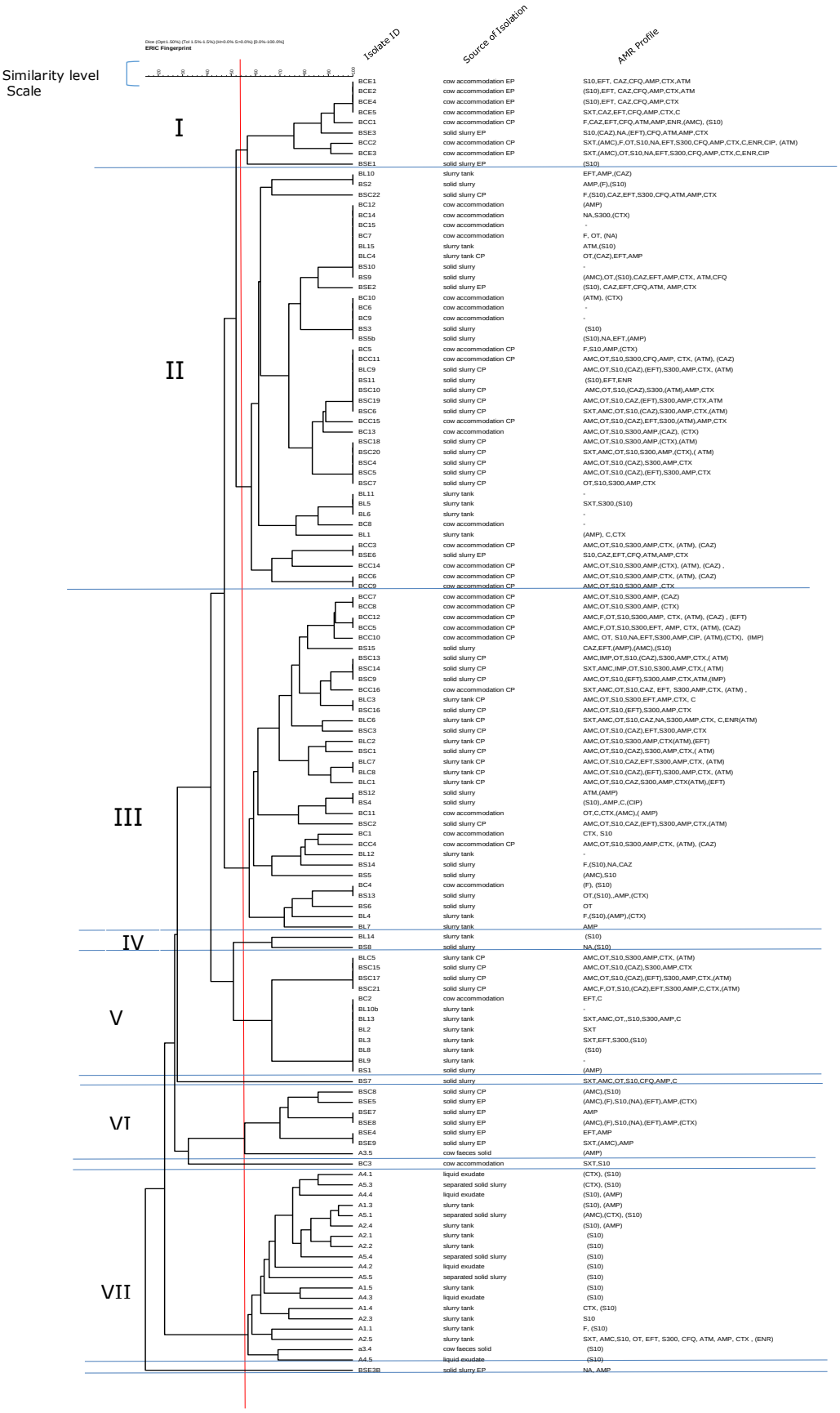


Figure 3-6: ERIC-PCR genotyping of 126 *E. coli* isolates from cattle slurry samples (clusters shown at 54% similarity level).

The dendrogram was produced using the UPGMA algorithm based on a Dice similarity coefficient. The first field gives the isolate ID, the second field gives the source of the isolate and the medium of isolation. Where the source shown is only slurry tank, solid slurry or cow accommodation it means the isolate was isolated from one of these sample using TBX. If the source shows slurry tank CP, solid slurry CP or cow accommodation CP, it means that the isolates were selected and purified from TBX+CTX medium. If the source shows slurry tank EP, solid slurry EP or cow accommodation EP, it means that the isolates were selected and purified from CHROMagar ESBL medium. For comparison, the last field shows the resistance profile for each strain: ampicillin (AMP), amoxicillin–clavulanic acid (AMC), cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM), cefquinome (CFQ), ceftiofur (EFT), streptomycin (S10), ciprofloxacin (CIP), enrofloxacin (ENR), nalidixic acid (NA), trimethoprim/sulfamethoxazole (SXT), sulphonamide (S300), chloramphenicol (CHL), oxytetracycline (OT), nitrofurantoin (F). If the antimicrobial is placed between brackets in the figure it means it shows intermediate resistance to that antimicrobial. The resistance data are presented in Chapter 4. The Roman numbers refer to the clusters numbers. Clusters I–VII were formed at 54% similarity level as designated using AMOVA analysis. The horizontal blue line divides each main group I–VII that clustered at 54% similarity threshold, which is indicated by the vertical red line.

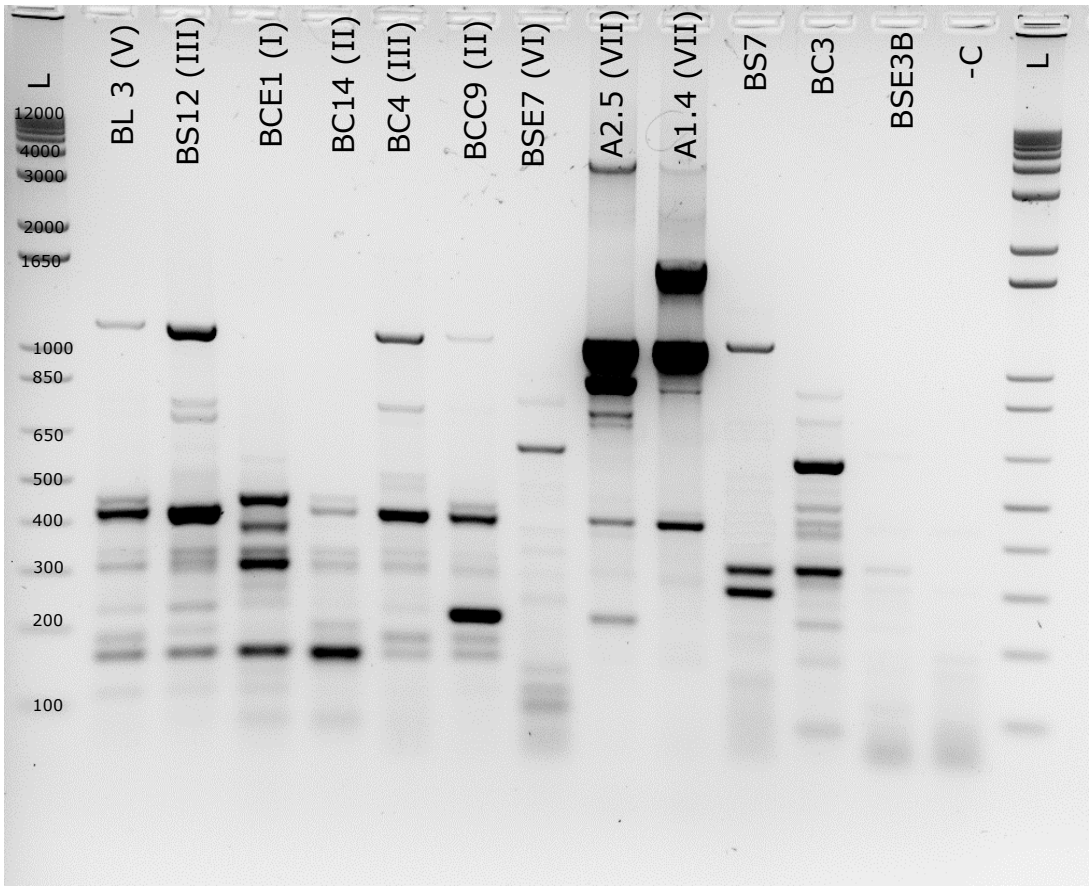


Figure 3-7: ERIC profiles for some of the selected *E. coli* strains representing some of the ERIC-PCR dendrogram clusters.

ERIC-PCR amplicons of the isolates were run in a 2% (w/v) TAE agarose gel at 100 V for 2 h. L =1 kb plus DNA ladder™ (Invitrogen, UK); –C, is a negative control. The Roman numbers refer to the ERIC-PCR dendrogram clusters which shown in Figure 3.6, those clusters formed at 54% similarity level. BL3 is from cluster V; BS12 and BC4 are from cluster III; BCE1 is from cluster I; BC14 and BCC9 are from cluster II; BSE7 is from cluster VI, A2.5 and A1.4 are from cluster VII; BS7, BC3 and BSE3B are outliers.

3.3 Discussion

Many methods are available to quantify bacteria in different sample types, such as food, water and slurry samples. Different selective and diagnostic media are available to perform counting of specific desired bacteria, so for the purpose of this study, plate counting has been performed using a selective diagnostic medium for *E. coli* (TBX) and selective medium for coliforms (MacConkey agar); the same media have been used with an antibiotic supplement to count selectively presumptive CTX-resistant *E. coli* and presumptive CTX-resistant coliforms (Section 2.3.3). In addition, CHROMagar ESBL was used to isolate ESBL-producing *E. coli*; this medium allows the detection of ESBL-producing Gram-negative bacteria while inhibiting the growth of other bacteria, including most of those carrying *ampC* type resistances. The chromogenic supplement allows species identification as different species appear with different colours; for example *E. coli* appears as dark pink colonies (http://www.chromagar.com).

The *E. coli* isolates were isolated using TBX agar, which is a chromogenic agar for the detection and enumeration of *E. coli* which is used widely in testing of animal food, human foodstuffs and water microbiology (ISO.16649-2, 2001). TBX in combination with CTX has been used as well for isolation of ESBL-producing *E. coli* from domestic pet faecal samples (Leite-Martins et al., 2014) and from surface and wastewaters (Franz et al., 2015). Bile salts are the selective agent against Gram-positive bacteria, particularly bacilli and faecal streptococci. TBX medium is

enhanced by the addition of a chromogenic agent, X-glucuronide to detect glucuronidase activity (Feng and Hartman, 1982). Most *E. coli* strains can be differentiated from other coliforms by the presence of the glucuronidase enzyme which is highly specific to *E. coli* (Hansen and Yourassowsky, 1984). This medium should be specific and the blue-green colonies which grow on it should be *E. coli* colonies, however out of 25 isolates obtained in 2012, 20 isolates were confirmed as *E. coli* after using the indole and oxidase confirmation tests; one of the five colonies which was not confirmed as *E. coli* appeared to be *Aeromonas* using API 20E with 67% identity (Appendix I). Further tests were performed on these 20 isolates using API 20E which gave confirmation that these isolates are *E. coli;* this suggests that the indole and oxidase tests are effective complementary tests to confirm that the blue-green colonies which were isolated from TBX plates were *E. coli.* This is in agreement with the findings of Wei (2013) who did a large study on isolating and detecting *E. coli* from pig samples using TBX agar and demonstrated that *Aeromonas* may be isolated as blue-green colonies which can be differentiated from *E. coli* by being oxidase positive (Wei, 2013).

Defined substrate technology depends on an enzymatic reaction for a specific enzyme produced by a specific species, and depending on this, the species can be detected. The commercial Colilert® system which is a type of defined substrate technology is used to identify total coliforms and especially *E. coli*. This system is based on the activity of two enzymes, β-galactosidase and β-glucuronidase. The first is present in all coliforms and

the second is specific for *E. coli*. The function of these enzymes is to convert separate chromogenic and fluorogenic substrates, o-nitrophenol-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG), into either a detectable colour or fluorescence. However, it has been found that other bacteria can give false positive results in this test e.g. *Aeromonas* (Edberg et al., 1988, Edberg et al., 1990, Katamay, 1990, Landre et al., 1998) again showing that some genera can be difficult to differentiate from *Escherichia* using these approaches and confirmatory testing is necessary.

CHROMagar ESBL was used to isolate ESBL-producing *E. coli* in this study. However, of the 35 presumptive isolates obtained, only 15 were subsequently shown to be *E. coli.* According to the information provided by the manufacturer, CHROMagar ESBL has 99.2% sensitivity and 89% specificity. These figures were obtained from a study (Lagacé-Wiens et al., 2010a) evaluating the sensitivity and specificity of this medium in detecting ESBL/AmpC-producer *E. coli* and *K. peumoniae* which had been initially screened using ceftazidime and/or ceftriazone (1 μg ml^{-1}). The study showed that for all the ESBL-producing *E. coli* the medium was 99.2% sensitive in the identification of those strains based on characteristic colour development of the colonies. This finding was obtained as only one ESBL isolate out of 122 ESBL-producing (114 ESBL-producing *E. coli* and 8 co-expressing ESBL and AmpC) isolates did not grow on this medium; the inhibited isolate was carrying TEM-12. With regard to the specificity comparing ESBL-producers with AmpC-producers,

the medium was 89% specific for ESBL-producers, as AmpC mutants could still grow on this medium. This finding was obtained as 10 AmpC producer isolates out of 91 AmpC producer *E. coli* isolates were able to grow on this medium. This lower specificity can also be due to mutations in non-ESBL encoding genes such as bla_{TEM-1} which can be overexpressed and give a false postive result (Lagacé-Wiens et al., 2010a). In another study CHROMagar ESBL medium was compared with four other media specified for the isolation of ESBL (ChromID, Brilliance, BD Drigalski, and HEGP media). In this study an automated system, a Wasp instrument, was used to screen 2,337 rectal screening samples, and it appeared that no medium was perfectly selective for ESBL-producing strains. The specificity and sensitivity varied for each medium, but CHROMagar ESBL and ChromID were more specific than the other media giving 79.3% and 79.9% specificity, respectively. The majority of non-ESBL-producing strains isolated on those media were Enterobacteriaceae overproducing AmpC β-lactamase and nonfermenting Gram-negative bacilli, mostly *Pseudomonas aeruginosa* (Grohs et al., 2013). According to the manfacturer's instructions for the CHROMagar ESBL medium, final identification may require further testing such as biochemical or immunological tests such as latex agglutination confirmation tests. It has also been mentioned that most AmpC-producing bacteria are inhibited but some may show some growth (www.chromagar.com).

E. fergusonii was isolated from CHROMagar ESBL medium as a putative *E. coli* (see Section 3.2.4). *E. fergusonii* appeared identical on the medium

to *E. coli* but the 16S rDNA sequencing showed it was not. *E. fergusonii* differs from *E. coli* by their ability to ferment cellobiose (Barrow and Feltham, 2004). However, *E. fergusonii* shares phenotypic traits with *E. coli* O157:H7 in having an inability to ferment sorbitol and their failure to produce β-glucuronidase (Farmer et al., 1985, Rice et al., 1992, Gaastra et al., 2014). Hence, they should appear as white colonies on TBX agar, and this was the case with the *E. fergusonii* isolated in this study. This is not the first report of such a finding. It has been previously reported that *E. fergusonii* was isolated from a patient with cystitis on CHROMagar Orientation medium. The *E. fergusonii* isolate produced *E. coli*-like pink colonies, which were later confirmed as ESBL-producing *E. fergusonii* (Lagacé-Wiens et al., 2010b). Hence the CHROMagar ESBL isolation medium used in the current study did not reliably differentiate the two *Escherichia* species, so the confirmation of the identity of ESBL-producing colonies on CHROMagar ESBL medium is very important for accurate characterization. A case of *E. fergusonii* associated with an acute pneumonia in cattle has been described before (Rimoldi and Moeller, 2013) which demonstrates that the presence of this isolate could be explained in the cattle manure.

The spread plating method has been used to enumerate the viable counts for *E. coli* and coliforms from the slurry samples. Statistical analyses were performed to compare the mean values for the *E. coli* counts from the 9 samples taken in 2014, and the same analyses were performed with the coliform counts. This was performed although the number of samples was

small, and might not represent enough data for robust statistical confirmation. However, the results can give an idea of whether there were differences in the prevalence percentages in the selected samples. With regard to the lower counts of the *E. coli* and the coliforms from liquid slurry compared to the two other sources, this might be due to several factors: more than 90% of solids are separated from the slurry prior to it being pumped into the slurry tank; a dilution effect that happens to the bacterial population in the slurry tank occurs due to the mixing of slurry with the waste milk, footbath, wash water, and rain water (as it is an open slurry tank). The difference might also be explained as the slurry in the slurry tank was being treated with "slurrybugs" (aerobic bacteria that had originated in soil and were introduced to reduce the crust and the smell in the slurry tank) while the solids were not. Liquid slurry may have less effect in dissemination of the bacteria of concern in the field after spreading because of these lower levels, while the solid separated slurry may have more effect when it goes directly to the field. There are many possible ways to treat slurry and minimise the spread of bacteria to the land before dissemination, such as heating over 55 °C or freezing, drying in sunlight and UV, acidic and alkaline conditions, increasing the time for storing the slurry; as some bacteria such as *E. coli* O157 can survive for months (https:// www.gov. uk/guidance/managing-nutrients-and-fertilisers). These conditions can affect the survival of microorganisms in manure, although there are significant operating costs involved in sludge treatments. In a study on the survival of *E. coli* O157:H7 and *S.* Typhimurium in manure it was concluded that the manure should be

stored for 105 days at 4 °C or 45 days at 37 °C in order to eliminate these pathogens (Himathongkham et al., 1999).

It was obvious there was a difference between the ECC isolated from the selective growth media with and without antibiotic and the same was true with TCC; this is due to the selective effect of the antibiotic (cefotaxime) in the media. From the data obtained in this study, it can be seen that the percentages of the presumptive CTX-resistant isolates were very low. In some samples, such as the liquid slurry, the viable count values on TBX+CTX and MAC+CTX were outside of the accepted countable range. The general ranges in common acceptance for countable numbers of colonies on a plate are 25-250 and 30-300 (Sutton, 2011) and for the current study the countable range chosen was between 25-250. The countable range value varies depending on the methods described by different organizations as each one specifies different values for the statistically accepted quantity. For example, the ASTM (American Society of the International Association for Testing and Materials) define the countable ranges on a plate using the spreading method as between 20-200, while for pour plates it is 30-300 for counting viable cells from water. However, the number of colonies should be recorded and reported as CFU ml^{-1} even if the sample has less than the countable range (lower than 20 for spreading method or lower than 30 for pour plate) of colonies on a plate (ASTM, 1998). Another example is the FDA (Food and Drug Administration) who recommend in their Bacterial Analytical Manual (BAM) 25-250 CFU per plate as a countable range using pour plate

methods for food samples (FDA, 2001). As the samples in our study are environmental, not food or water, reporting the value of the viable count for different sources is necessary for the purpose of this study even if it is less than the quantification limit, especially as this is related to the isolates selected on TBX+CTX and MAC+CTX. This enables us to find the estimated level of resistance carriage in selected groups from those samples. These results were obtained without using an enrichment step as the samples were directly diluted and spread on the different media. However, all the samples showed positive growth for presumptive CTX-resistant *E. coli*.

In a study performed by Horton et al. (2011), faecal samples from cattle were used to compare the prevalence of CTX-M positive *E. coli* (using CHROMagar CTX which is mainly selective for CTX-M-positive *E. coli*) with those in chicken and pig faecal samples. There was wide variation in the number of CTX-M-positive *E. coli* organisms detected; the median (range) CFU g^{-1} were 100 (100 to 1 x 10^6), 5,350 (100 to 3.1 x 10^6), and 2,800 (100 to 4.7 x 10^5) for cattle, chickens, and pigs, respectively. The percentage of presumptive CTX-M-positive *E. coli* isolates varied widely between the different sources; median (range) values were 0.013% (0.001 to 1%) for cattle, 0.0197% (0.00001 to 28.18%) for chickens, and 0.121% (0.0002 to 5.88%) for pigs (Horton et al., 2011). This was more than the range of the percentage of presumptive CTX-resistant *E. coli* that have been found by the present study compared to the range of resistant *E. coli* from cattle faecal samples. This might be due to the differences in

sample types as the samples for our study are cattle slurry, not pure cattle faeces. Besides, the media used for counting in both studies are different and selects for different populations of *E. coli*.

ERIC-PCR is used widely to genotype ESBL-producing *E. coli* and other enteric bacteria (Lim et al., 2009, Hussain et al., 2014, Durmaz et al., 2015). With regard to the genotyping of the *E. coli* isolates using ERIC-PCR, there was no absolute distinction between isolate sources (faeces from cow barn, liquid slurry and solid slurry), as different isolates from different sources were seen in most of the groups, except with group I and VI. This is not surprising as the isolates in slurry come from cattle faeces and therefore would be expected to show a direct relationship between the two sources. However, there was a significant difference ($P \leq 0.001$) between the isolates from different periods of time, i.e. between group VII (2012) and the others (2014). The point of doing the genotyping here is to distinguish between different *E. coli* strain genotypes rather than distinguishing the strain epidemiology, as all samples are from the same farm. It would therefore identify if there are genomically identical (clonal) isolates. Liquid slurry was collected from a 3 million litre tank in the farm in which the slurry has accumulated before being used as a fertilizer. The five sources from which the samples were taken are not from different animals (all are from cattle manure) or from different farms; the samples were all from one main source, cows' slurry and faeces on one farm. So this may be the reason for the clustering between the isolates from liquid slurry and other sources as this tank is

acting as a reservoir for all slurry sources. However, 7 groups were distinctly diverse. Despite disagreement about using ERIC-PCR for genotyping many types of bacteria besides enteric bacteria, it has been found that ERIC-PCR can work effectively with genotyping of *E. coli* and determining the host sources (McLellan et al., 2003). ERIC-PCR has been shown to be a reliable method to differentiate between different genotypes of *E. coli*, and preliminary experiments carried out in the current study showed it to be sufficiently reproducible to determine identical genotypes. Checking the reproducibility of ERIC-PCR has been performed before but with a slightly different method (Wei, 2013). In Wei's experiments 19 replicates of one *E. coli* strain were run with one *Salmonella* strain as a control on 2 different gels. The dendrogram was produced by FPQuest v5.1 with the UPGMA algorithm based on a Dice similarity coefficient with a 1.5% band position tolerance and 1.5% optimization. Less than 2% similarity was observed for the same strain compared with *Salmonella*, and more than ninety percent (90.5% - 100%) similarity was seen for the replicate samples on the same gel or on different gels (Wei, 2013). In our preliminary study, some variation in the intensities of the bands was observed. However, standardization of the method by using identical conditions for the experiment and using FPQuest software to analyse the bands and normalize the band position between different gels gave a reliable method for genotyping with 92.8-100% matching between the replicates run on different gels obtained. This could be further improved if a reference strain was chosen and an ERIC-PCR of this isolate performed alongside the test isolates to check the

band pattern reproducibility as a control throughout the study. Genotyping can give an indication of identical isolates, which might be clonal, and can help in explaining some variation in such strains that showed 100% identity at the genomic level, but had different antimicrobial resistance profiles, which will be discussed in the next chapter. This method is effective, time-saving and is reliable (Duan et al., 2009). According to a study conducted by McLellan and co-workers (2003), repetitive extragenic palindromic (REP), ERIC-PCR and pulsed-field gel electrophoresis (PFGE) produced comparable, but not identical, results in determining *E. coli* isolate host sources which were obtained from different sources of faecal pollution such as sewage and animals (gulls, humans, and dairy cattle). According to the previous study, PFGE offered a greater differentiation of closely related strains than both PCR techniques (McLellan et al., 2003). However, PFGE is a more labour intensive method and would be time-consuming to apply to a large number of strains. In another study on genotyping *E. coli* from pig samples, it has been found that with the normalisation facilities of the analysis software, ERIC-PCR gave stable and reproducible results. The ERIC pattern data showed a high diversity of *E. coli* types amongst the various slaughter stages (Wei, 2013). In a study aimed to indicate sources of *E. coli* aerosol pollution caused by pigs in swine houses, it was found that ERIC-PCR and REP-PCR were very effective techniques in discriminating between strains isolated from different locations in swine houses (Leung et al., 2004). From the results obtained in the current study it appears that ERIC-PCR is suitably robust to obtain the

information on diversity of *E. coli* isolates needed and is sufficiently simple and rapid a method to allow it to be applied to a large number of strains. In the next chapter the resistance profile for *E. coli* strains and their percentages will be discussed.

4. Multidrug resistant ESBL-producing *E. coli* from a dairy farm

4.1 Introduction

The use of antimicrobials in agriculture and animal husbandry is increasingly being considered a global health issue, both from the animal health and welfare aspect, and because of the development of antimicrobial resistance in animal pathogens (Davies et al., 2013, O'Neill, 2015). Sales of different groups of antimicrobials (e.g. aminoglycosides, tetracyclines and β-lactams) for animal treatment in the UK vary, but what is very clear is that sales of three groups of antimicrobials have predominated since 2006: tetracyclines, β-lactams and trimethoprim/ sulphonamide (DEFRA, 2012, Eckford et al., 2013).

Animal manure is a major source of antimicrobial resistant bacteria entering the environment, especially the soil (Aarestrup et al., 1996, Binh et al., 2007, Ghosh and LaPara, 2007), and it has been estimated that annually about 70 million tonnes of animal manure are used as fertilizer on agricultural land in the UK (Hutchison et al., 2004). Much of this will contain low levels of antimicrobials or antimicrobial metabolites/conjugates, and antimicrobial resistant bacteria.

E. coli is a common enteric commensal, can be a pathogen, and easily acquires resistance and therefore can act as a reservoir which can transfer resistance to other species. Therefore, this species serves as a sentinel organism for antimicrobial resistance development in different types of animals (Aarestrup et al., 1998, van den Bogaard et al., 2000, Allen et al., 2010, Ashbolt et al., 2013).

Cephalosporin antibiotics are commonly used in dairy veterinary medicine because they are effective in treating environmental mastitis caused largely by *E. coli* strains (Shpigel et al., 1997).

According to the UK Veterinary Antibiotic Resistance and Sales Surveillance report (UK-VARSS, 2014b), the antimicrobials which are recognised as critically important for human use are 3rd and 4th generation cephalosporins and fluoroquinolones. From the data provided in this report, an increase in the sales of this group can be noticed (Figure 4.1). Using mg PCU^{-1} (population correction unit), the sale in 2014 was 0.20 mg PCU^{-1} compared to 2013 (0.18 mg PCU^{-1}). This was more obvious with the fluoroquinolone group (the other critically important group of antimicrobials for humans) as the sale in mg PCU^{-1} increased clearly from 2010 (0.27 mg PCU^{-1}) to 2014 (0.34 mg PCU^{-1}).

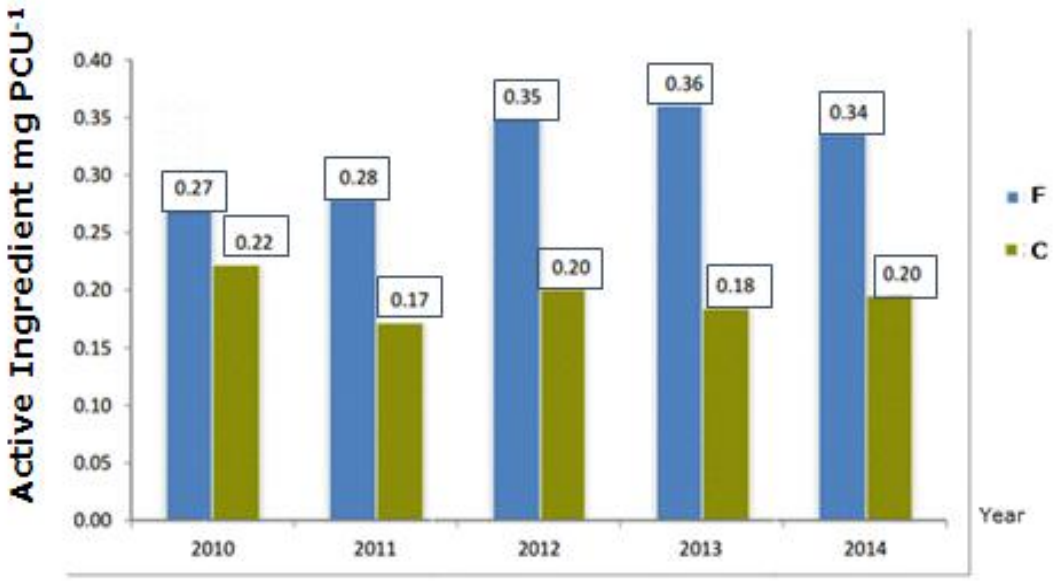


Figure 4-1: Fluoroquinolone and 3rd and 4th generation cephalosporin sales in the UK from 2010 -2014 in active ingredient mg PCU^{-1}.

This figure shows the sales of critically important groups of antimicrobials for humans (fluoroquinolones and 3rd/ 4th generation cephalosporins) used in veterinary medicine. F: fluoroquinolones; C: 3rd /4th generation of cephalosporins, adapted from UK-VARSS, 2014b.

In 2013-14, there was increased resistance to 3rd and 4th generation cephalosporins amongst *E. coli* isolated from cattle faeces or intestinal contents, including strains which are both animal pathogens and commensals (UK-VARSS, 2014a); Figure 4.2. This might be due to the increase in using this group of antibiotics.

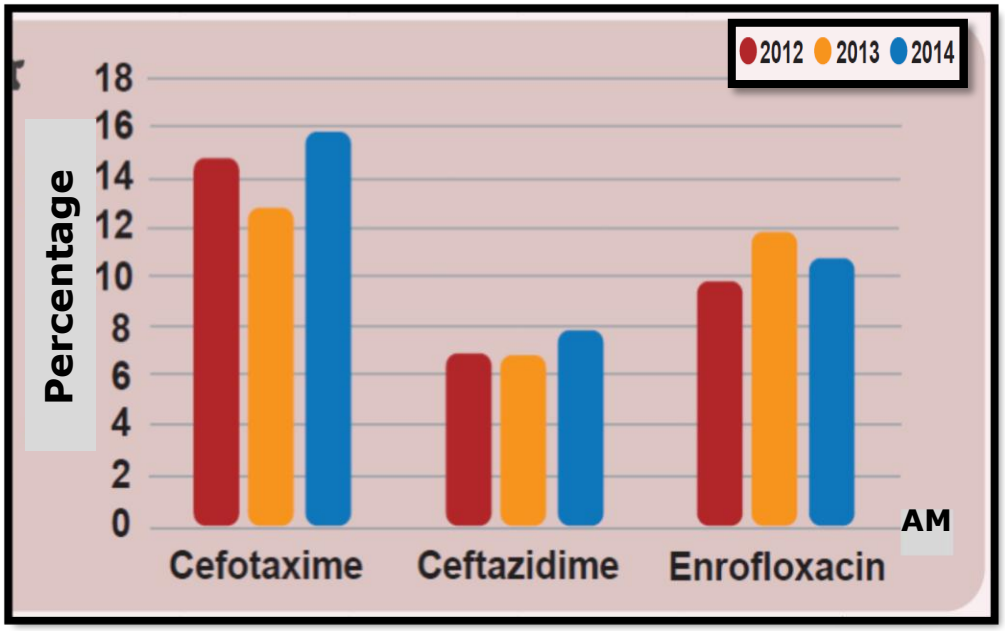


Figure 4-2: Percentage resistance in *E. coli* from cattle to highest-priority critically important antimicrobials for human health from 2012-2014.

Cefotaxime and ceftazidime are 3rd generation cephalosporins used in human medicine; enrofloxacin is a fluoroquinolone antimicrobial used in veterinary medicine. AM: antimicrobial, adapted from UK-VARSS, 2014a.

The emergence and spread of ESBL-producing *E. coli* associated with cattle and other farm animals has been of particular concern (Bush and Jacoby, 2010, Pfeifer et al., 2010). ESBL and AmpC β-lactamases are very distinct groups of enzymes. ESBL and AmpC β-lactamases can confer resistance to third generation cephalosporins, penicillins and monobactams. Strains that produce them can mainly be differentiated by different susceptibility patterns of the enzymes to β-lactam site-specific inhibitors such as clavulanic acid, which ESBL-producing *E. coli* are inhibited by, but AmpC- producing *E. coli* are not. AmpC enzymes confer resistance to cephamycins such as cefoxitin while ESBLs confer

resistance to fourth generation cephalosporins such as cefepime (Moritz and Carson, 1986, Livermore et al., 2001, Bush and Jacoby, 2010, CLSI, 2013a).

In the dairy farm studied, cephalosporins by intramammary infusion were used for treatment of environmental mastitis, and antimicrobial-containing mastitic milk was discarded into the slurry tank, which may potentially select for *E. coli* strains carrying ESBL genes.

In the work presented in this chapter, the aim was to determine the prevalence and range of multidrug resistance and particularly ESBL carriage in *E. coli* within the farm, the diversity of resistance to β-lactam antibiotics, and to determine if carriage of ESBL was associated with other antimicrobial resistances.

4.2 Results

4.2.1 Antimicrobial Resistance

The susceptibility of one hundred and twenty six confirmed *E. coli* isolates (from 2012/14 studies) from TBX, TBX + CTX and CHROMagar ESBL (Section3.2.3) to 17 antimicrobials (representing 7 antimicrobial groups) was tested using standard disc diffusion antimicrobial sensitivity tests (Section 2.4 and Table 2.2). The criteria for multidrug resistance (MRD) classification are not consistent between different organizations as each one gives a different definition for MDR (Schwarz et al., 2010, EFSA, 2015). So for this purpose, the definition by Magiorakos and co-authors (2012) has been used in this study, in which any isolates that are resistant to 3 antimicrobials from different classes, will be regarded as MDR (Magiorakos et al., 2012). Table 4.1 shows the number of isolates with each susceptibility type against a different number of antimicrobials tested. Of the 126 isolates tested, only 7.9% of the isolates were sensitive to all antimicrobials; 92% showed resistance to at least 1 antimicrobial, of which 27.8% of the isolates were isolated on TBX only and showed resistance to at least one antimicrobial; 57.9% of the isolates were MDR and showed resistance to 3 or more antimicrobials from different antimicrobial groups. The resistance profiles of MDR strains ranged from 3-15 antimicrobials and the percentage of strains within each group is shown in Figure 4.3. The highest percentages were for the 17 *E. coli* isolates that were resistant to 9 antimicrobials (13.49%) followed by 15 isolates that had resistance to 7 antimicrobials (11.9%). The isolate with the highest number of resistances (BCC2) was

resistant to all antimicrobials tested except for imipenem and ceftazidime. With regards to the percentages of resistance according to the source of the samples, the highest percentages for MDR isolates were from solid slurry 26.1% (33 isolates), then cow accommodation slurry 19.8% (25 isolates) and from liquid slurry 11.9% (15 isolates). With regards to the percentages of resistant isolates according to the media of isolation, 43.6% of MDR isolates (55 isolates) were strains that were isolated using extended spectrum cephalosporin selective media: these were obtained from TBX + CTX (44 isolates which represent 97.7% of all the TBX+CTX isolates) and CHROMagar ESBL (11 isolates which represent 73.3% of all the isolates from CHROMagar ESBL medium), whilst the percentage of MDR *E. coli* isolated on TBX without antibiotic selection was 14.2% (18 isolates which represent approximately 27% of all the TBX isolates). For MDR strains that had been isolated using TBX only, 17 MDR isolates were isolated from the 2014 sampling while only one MDR isolate was isolated in 2012.

Table 4-1: The number and percentages of 126 *E. coli* isolates showing resistance to different numbers of antimicrobials.

Number of Resistances	No. of isolates	Percentage
sensitive to all	10	7.93
resistance to 1	21	16.7
resistance to 2	18	14.3
resistance to 3	10	7.93
resistance to 4	8	6.34
resistance to 5	3	2.38
resistance to 6	4	3.17
resistance to 7	15	11.9
resistance to 8	11	8.73
resistance to 9	17	13.49
resistance to 10	3	2.38
resistance to 11	3	2.38
resistance to 12	1	0.79
resistance to 13	1	0.79
resistance to 14	0	0
resistance to 15	1	0.79
resistance to 16	0	0
resistance to 17	0	0
Total	126	100

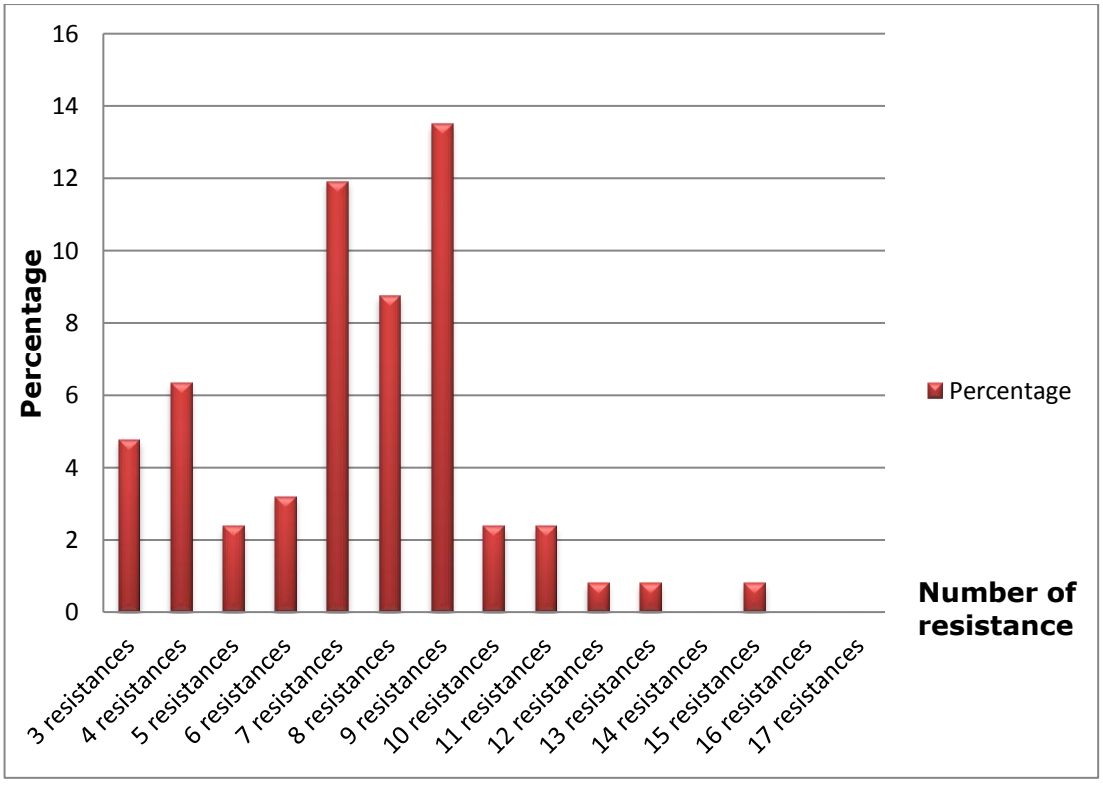


Figure 4-3: Percentage of MDR *E. coli* resistant to different numbers of antimicrobials.

The highest percentage of MDR type was for strains showing resistance to 9 antimicrobials while no strains showed resistance to 14, 16 and 17 antimicrobials.

With regard to the levels of resistance to each antimicrobial, amongst the strains tested, the highest percentage resistance was to ampicillin (56.3%), followed by oxytetracycline, streptomycin, sulphonamide and amoxicillin-clavulinc acid (41.2%, 39.6%, 38.8%, 33.3%, respectively). Dihydrofolate reductase inhibitors, such as trimethoprim, were tested in combination with sulphamethoxazole and 12.6% of the *E. coli* isolates were resistant to this combination, while resistance to chloramphenicol and nitrofurantoin was 8.7% for both. It is worth noting that ampicillin was one of the most widely used veterinary antimicrobials in the UK from 2006 to 2011, as well as tetracycline and trimethoprim/sulphonamides (DEFRA, 2012). Furthermore, the isolates showed a range of resistance to different antimicrobials from the cephalosporin family: 38%, 14.2% and 23% were resistant to cefotaxime, ceftazidime and ceftiofur, respectively, which are all third generation cephalosporins, while 11.1% of the isolates were resistant to cefquinome, which is a fourth generation cephalosporin. This is not surprising as approximately 47% of the isolates were directly selected on media containing β-lactam antimicrobial supplements (60 isolates were from selective media containing antimicrobials: 15 from CHROMagar ESBL and 45 from TBX+CTX). Quinolone antimicrobials were still largely effective against these strains, with only 3.1% of the *E. coli* isolates resistant to both ciprofloxacin (CIP) and enrofloxacin (ENR). With regards to imipenem (IMP) 1.6% (2) of the *E. coli* isolates were phenotypically resistant, with 3.1% (4) showing intermediate resistance, but subsequent characterization of the imipenem resistant strains showed this resistance was not stable. The highest

proportion of isolates with intermediate levels of resistance was to streptomycin (S10) (36.5%) and aztreonam (ATM) (23.8%). There was no intermediate resistance against any of the following: cefquinome, trimethoprim/sulfamethoxazole, sulphonamides, chloramphenicol and oxytetracycline (Table 4.2 and Figure 4.4). All the data from the disc assays is shown in Appendix VIII.

Table 4-2: The number of 126 *E. coli* isolates that were susceptible, resistant or with intermediate resistance to each antimicrobial type

	Number of isolates		
Antimicrobials	S*	I*	R*
ampicillin	43	12	71
amoxicillin-clavulanic acid	71	13	42
cefotaxime	60	18	48
ceftazidime	84	24	18
ceftiofur	84	13	29
cefquinome	112	0	14
aztreonam	82	30	14
imipenem	120	4	2
streptomycin	30	46	50
enrofloxacin	121	1	4
ciprofloxacin	121	1	4
nalidixic acid	113	4	9
trimethoprim/ sulfamethoxazole	110	0	16
sulphonamides	77	0	49
chloramphenicol	115	0	11
oxytetracycline	74	0	52
nitrofurantoin	113	2	11

*S: Susceptible, I: intermediate, R: resistant using CLSI (2013a and 2013b) definitions.

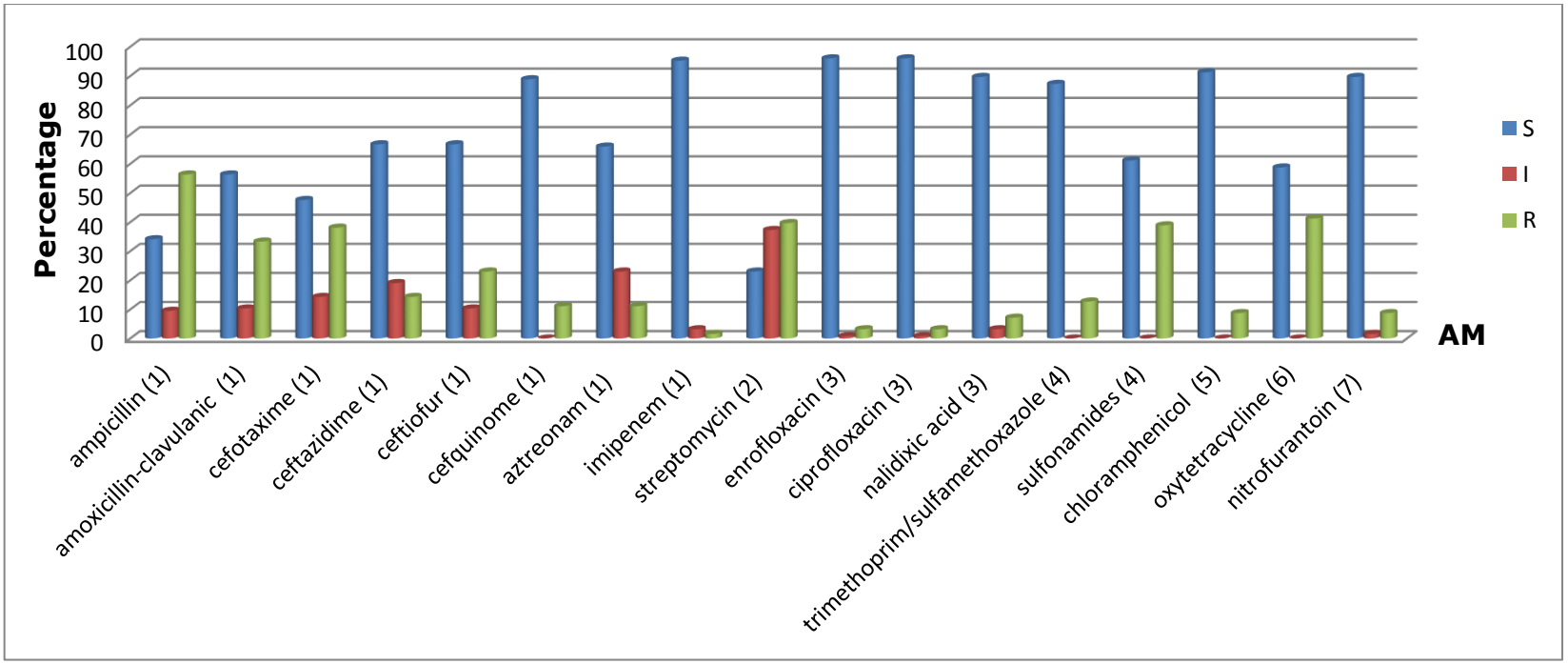


Figure 4-4: Percentage sensitivity to 17 antimicrobials for 126 *E. coli* farm isolates.

A blue bar indicates sensitive (S), a red bar indicates intermediate sensitivity (I), and a green bar indicates resistance (R) to the antimicrobial using CLSI (2013a and 2013b) definitions. The Y axis represents the percentage of isolates based on testing 126 *E. coli* isolates. AM: antimicrobials. (1) β-lactam, (2) aminoglycoside, (3) quinolone, (4) sulphonamide/complex, (5) phenicol, (6) tetracycline and (7) nitrofuran derivative**.**

4.2.2 Clustering of the antimicrobial resistance profile

When the antimicrobial resistance profile of each *E. coli* strain was clustered using the heatmap (Figure 4.5) function of the R statistical package (Section 2.4.2), the analysis showed that, with some exceptions, the isolates mainly clustered together based on the selective medium which was used to isolate them. The strains isolated on TBX medium and then tested for antimicrobial resistance were more diverse in their resistance patterns than those isolated on TBX+CTX. The latter clustered in two groups with the majority of isolates showing clustered resistance to ampicillin (AMP), streptomycin (S10), oxytetracycline (OT), amoxicillin-clavulanic acid (AMC) and sulphonamide (S300). At the bottom of the heatmap there was a separate cluster for 4 isolates including the positive control isolates, *E. coli* NCTC 13353 with BCE3, BCC2 and BCC10 which all showed resistance to ciprofloxacin besides the other antimicrobials. The majority of those isolated on CHROMagar ESBL formed a closely related resistance cluster, with ESBL resistance to cefquinome (CFQ), ceftiofur (EFT), ceftazidime (CAZ), cefotaxime (CTX) and aztreonam (ATM) (Figure 4.5). Several strains that were isolated on TBX containing cefotaxime (BCC1, BSC22) or TBX alone (BS9) clustered with the CHROMagar isolates, and showed this same cluster of resistances, while CHROMagar ESBL isolates which clustered in the TBX cluster (BSE1, 3B, 4, 5, 7, 8, 9) lacked them. All the isolates that were an exception in clustering (i.e. did not cluster with the other strains isolated on the same media type) are shown in blue to be differentiated from the others.

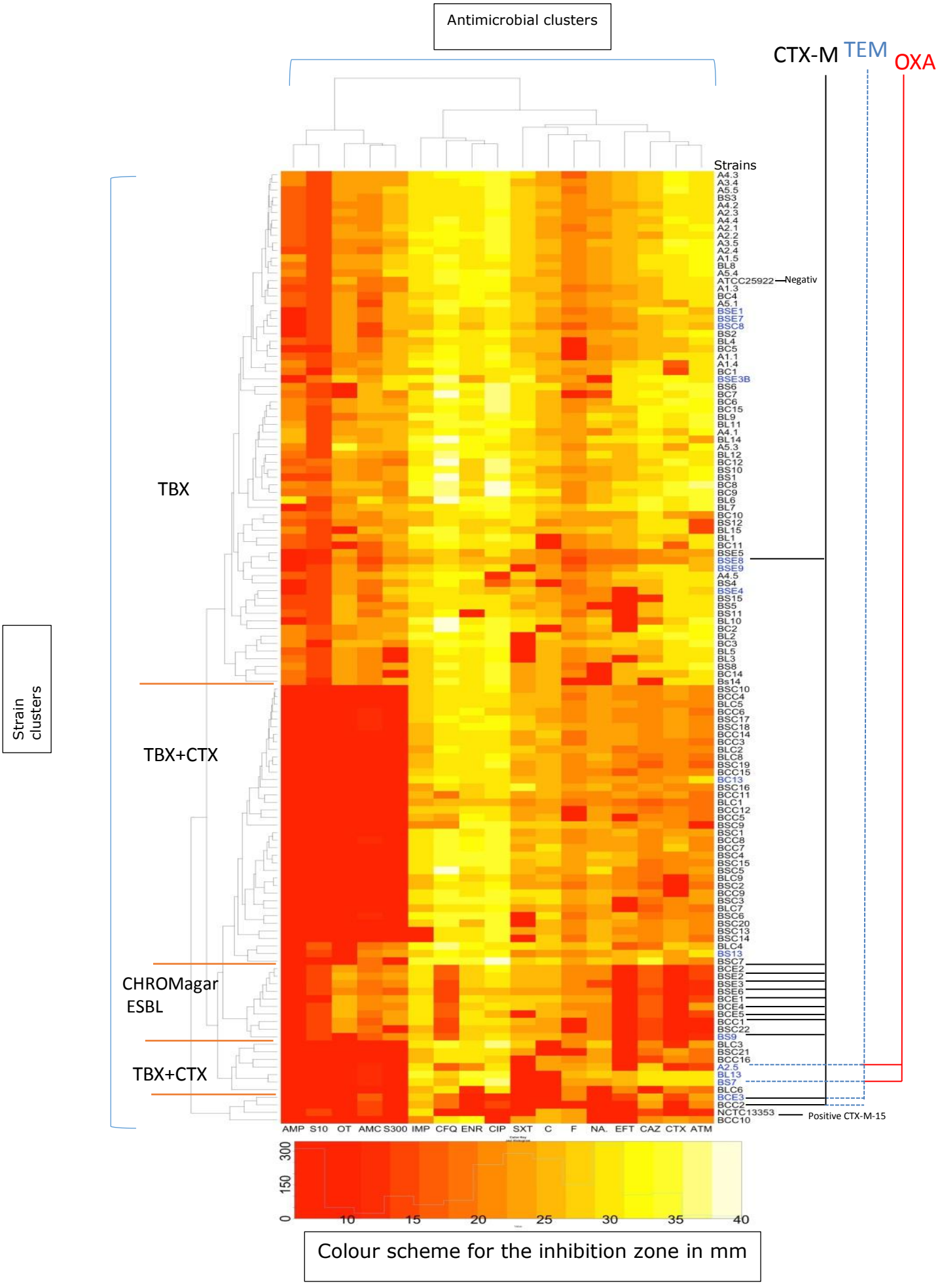

Antimicrobial clusters
CTX-M
TEM
OXA
Strains
Strain clusters
TBX
TBX+CTX
CHROMagar ESBL
TBX+CTX
Negativ
Positive CTX-M-15
AMP S10 OT AMC S300 IMP CFQ ENR CIP SXT C F NA. EFT CAZ CTX ATM
0 150 300
10 15 20 25 30 35 40
Colour scheme for the inhibition zone in mm

Figure 4-5: Heatmap representation of zone of growth inhibition surrounding antimicrobial assay discs to 17 antimicrobials for 126 *E. coli* isolates.

The figure shows a heatmap or graphical representation of antimicrobial sensitivity test results using colours to indicate the level of resistance. The size of the zone of clearing around the antimicrobial discs (in mm) is represented by the colour spectrum in the diagram, with red representing no zone of clearing (resistant), red through to orange (resistant- zone of clearing 10-22mm depending on antimicrobial), orange to yellow (sensitive-zone of clearing 17-27mm depending on the antimicrobial) and yellow to white (sensitive-zone of clearing of 30-37mm). A zone of >37mm is white and indicates that the strain was highly sensitive to the antimicrobial. Strains are clustered according to their pattern of resistance to antimicrobials (left-hand dendrogram) and antimicrobials are clustered according to their pattern of resistant strains (top dendrogram). Dissimilarity was calculated using Euclidean distance and cluster analysis using complete linkage. TBX, TBX + CTX (cefotaxime) and CHROMagar ESBL represent the media used to isolate the strains; the horizontal orange line divides each main group. Strain numbers in blue typeface indicate strains which were not isolated on the same medium as other strains in the cluster. *E. coli* ATCC 25922 and *E. coli* NCTC 13353 are negative and positive control reference strains for producing ESBLs, respectively. Presence of $bla_{\text{CTX-M}}$ or bla_{TEM} or bla_{OXA} is indicated by solid black lines (CTX-M), dotted blue lines (TEM), or solid red lines (OXA) respectively. Identification codes for antimicrobials: ampicillin (AMP), amoxicillin–clavulanic acid (AMC), cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM), cefquinome (CFQ), ceftiofur (EFT), streptomycin (S10), ciprofloxacin (CIP), enrofloxacin (ENR), nalidixic acid (NA), trimethoprim/sulfamethoxazole (SXT), sulphonamide (S300), chloramphenicol (CHL), oxytetracycline (OT), nitrofurantoin (F).

4.2.3 Phenotypic confirmation of ESBL- and /or AmpC-producing *E. coli*

ESBL/AmpC phenotypes were tested in the 52 strains that were resistant to cefotaxime/ceftazidime in the initial assays. Figure 4.6 shows the results using the ESBL/AmpC phenotypic confirmation kits (Section 2.5) for ESBL- and AmpC-producers. The confirmatory test kits showed that 25/52 (48.0%) of the strains were phenotypically ESBL producers (Table 4.3). Phenotypic AmpC-type resistance was identified in 35 isolates, initially by the antibacterial activity of cefepime against these strains, as AmpC β-lactamases do not inactivate cefepime. AmpC β-lactamases degrade penicillins, expanded-spectrum cephalosporins (with the exception of cefepime and cefpirome), cephamycins, monobactams, and β-lactam inhibitors. This was further confirmed using the AmpC confirmatory kit in addition to a cefoxitin (FOX) disc assay. Cefoxitin is a cephamycin and is susceptible to AmpC β-lactamases, but using only cefoxitin as a confirmatory test is not sufficient to confirm AmpC activity. This is because cefoxitin resistance can sometimes be due to decreased cellular permeability (Brenwald et al., 2005). Full phenotypic AmpC confirmation was if the strain was resistant to cefoxitin, and the inhibition zone diameter for CTX + cloxacillin, and CAZ + cloxacillin was ≥ 5-mm larger than the inhibition zone diameter from the discs containing CTX and CAZ alone, as AmpC activity against cephalosporins is inhibited by cloxacillin. Eight isolates (15.3%) had both ESBL and AmpC activity; those strains were BCC12, BCC7, BLC5, BSC3, BSC4, BSC9, BSC14 and BSC19 (Table 4.3 and Figure 4.7). Besides, the 52 cefotaxime/ ceftazidime resistant strains, the ESBL confirmatory kit was used to test

a further 14 isolates which initially showed resistance or intermediate resistance to cefotaxime or ceftazidime, but when the kit was used those isolates showed no resistance against cefotaxime or ceftazidime, so no further phenotypic confirmation was performed on those isolates (Appendix IX).

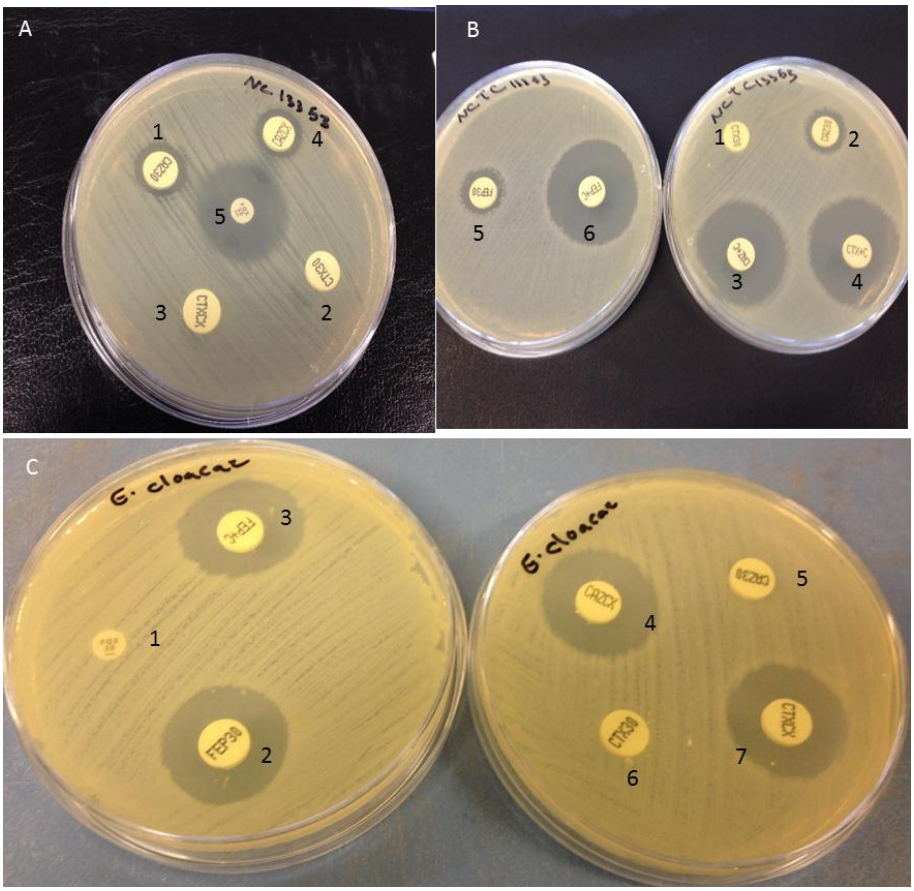


Figure 4-6: The ESBL/AmpC phenotypic confirmation test.

A) AmpC confirmation test for *E. coli* NCTC 13353, this strain is AmpC negative. 1: ceftazidime (CAZ30); 2: cefotaxime (CTX30); 3: cefotaxime plus cloxacillin (CTXCX); 4: ceftazidime plus cloxacillin (CAZCX); 5: cefoxitin (FOX). **B)** ESBL confirmation test for *E. coli* NCTC 13353, this is a positive control reference strain. 1: ceftazidime (CAZ30); 2: cefotaxime (CTX30); 3: ceftazidime plus clavulanate (CAZ+C); 4: cefotaxime plus clavulanate (CTX+C); 5: cefepime (FEP30); and 6: cefepime plus clavulanate (FEP+C). This shows the increased inhibition zone sizes seen in the presence of the clavulanate inhibitor. **C)** AmpC confirmation test for *Enterobacter cloacae* which is a positive control for this test. The plate on the left contains the 1: cefoxitin disc (FOX) which the AmpC producer is resistant to, 2: cefepime (FEP30) which the AmpC producer is sensitive to, and 3: cefepime plus clavulanate (FEP+C). The plate on the right is the AmpC confirmation kit; 4: ceftazidime plus cloxacillin (CAZCX); 5: ceftazidime (CAZ30); 6: cefotaxime (CTX30), and 7: cefotaxime plus cloxacillin (CTXCX); this demonstrates the sensitivity gained in the presence of the inhibitor cloxacillin.

Table 4-3: Phenotypic confirmation test of ESBL- and /or AmpC-producing *E. coli**

ESBL confirmation test (Inhibition Zone in mm)											AmpC confirmation test (Inhibition Zone in mm)						
Isolate ID	CTX	CTX+C	D	CAZ	CAZ+C	D	FEP	FEP +C	D		CTX	CTXCX	D	CAZ	CAZCX	D	FOX
BC13	20	23	3	20	25	5	30	30	0		20	26	6	20	25	5	15
BCC1	0	30	30	16	26	10	16	30	14		0	3	3	16	17	1	25
BCC11	21	21	0	19	20	1	26	30	4		22	26	4	19	26	7	18
BCC12	22	24	2	19	24	5	25	28	3		22	30	8	20	30	10	20
BCC14	24	25	1	21	24	3	30	31	1		25	31	6	22	29	7	18
BCC15	20	20	0	18	20	2	23	25	2		20	28	8	15	29	14	20
BCC16	20	22	2	18	22	4	25	25	0		20	30	10	18	30	12	20
BCC2	15	28	13	25	26	1	20	30	10		15	18	3	25	28	3	28
BCC3	24	26	2	21	24	3	30	31	1		24	29	5	20	25	5	16
BCC4	23	25	2	20	22	2	29	29	0		23	30	7	20	29	9	16
BCC5	23	24	1	21	23	2	29	29	0		22	27	5	20	26	6	16
BCC6	22	25	3	20	25	5	30	31	1		22	24	2	20	20	0	22
BCC7	23	24	1	20	25	5	30	30	0		23	30	7	20	30	10	18
BCC8	23	24	1	21	22	1	28	28	0		25	30	5	23	29	6	16
BCC9	22	24	2	19	24	5	27	28	1		23	25	2	21	25	4	18
BCE1C	0	30	30	15	30	15	18	30	12		0	3	3	15	17	2	25
BCE2	0	25	25	15	25	10	17	30	13		0	3	3	16	17	1	25
BCE3	14	25	11	23	25	2	16	28	12		14	18	4	23	27	4	25
BCE4	10	29	19	15	25	10	17	30	13		10	12	2	15	16	1	26
BCE5	11	29	18	16	25	9	15	29	14		11	15	4	16	19	3	35
BLC1	21	24	3	20	21	1	26	27	1		21	31	10	20	30	10	20
BLC2	21	23	2	18	22	4	27	29	2		21	27	6	18	25	7	17
BLC3	20	24	4	18	21	3	25	25	0		21	26	5	19	25	6	15
BLC4	28	29	1	21	28	7	25	25	0		28	28	0	21	25	4	20
BLC5	19	25	6	20	25	5	30	30	0		20	28	8	20	26	6	17
BLC6	20	20	0	18	20	2	25	25	0		20	24	4	19	27	8	20
BLC8	20	24	4	19	21	2	25	25	0		20	24	4	21	27	6	19
BLC9	21	21	0	18	21	3	25	27	2		21	30	9	20	30	10	20
BS9	10	30	20	17	28	11	19	32	13		10	14	4	17	18	1	22
BSC1	23	27	4	20	23	3	30	34	4		23	30	7	20	30	10	15
BSC10	24	26	2	21	25	4	31	31	0		26	33	7	23	31	8	16
BSC13	22	24	2	20	22	2	28	28	0		22	26	4	20	26	6	17
BSC14	23	26	3	20	25	5	28	28	0		23	31	8	20	30	10	16

Table 4.3 (continued)

ESBL confirmation test (Inhibition zone in mm)											AmpC confirmation test (Inhibition zone in mm)						
Isolate ID	CTX	CTX+C	D	CAZ	CAZ+C	D	FEP	FEP +C	D		CTX	CTXCX	D	CAZ	CAZCX	D	FOX
BSC15	22	24	2	20	20	0	28	30	2		20	25	5	18	24	6	20
BSC16	23	25	2	20	22	2	30	30	0		24	32	8	22	30	8	16
BSC17	23	24	1	20	24	4	28	28	0		23	30	7	21	28	7	14
BSC18	22	24	2	20	23	3	30	30	0		22	30	8	20	29	9	17
BSC19	20	25	5	20	24	4	26	28	2		20	30	10	20	30	10	18
BSC2	22	24	2	20	23	3	28	30	2		21	30	9	20	27	7	15
BSC20	24	27	3	20	25	5	30	30	0		25	30	5	20	29	9	14
BSC21	19	21	2	17	21	4	25	25	0		19	30	11	19	29	10	15
BSC22	10	30	20	15	21	6	22	30	8		10	12	2	15	17	2	25
BSC3	22	24	2	20	25	5	30	30	0		23	29	6	20	28	8	15
BSC4	22	25	3	20	25	5	28	31	3		24	32	8	20	31	11	15
BSC5	23	24	1	20	24	4	30	31	1		23	31	8	20	30	10	16
BSC6	22	25	3	20	24	4	30	30	0		22	27	5	20	24	4	18
BSC7	23	25	2	22	26	4	29	30	1		23	30	7	22	29	7	15
BSC9	21	25	4	20	24	4	24	28	4		21	30	9	21	29	8	16
BSE2	14	30	16	17	28	11	20	30	10		14	15	1	17	19	2	25
BSE3	10	27	17	14	25	11	19	27	8		10	14	4	14	18	4	27
BSE6	17	30	13	17	27	10	16	27	11		15	15	0	17	18	1	25
BSE8	20	30	10	26	28	2	30	32	2		20	24	4	26	30	4	24
E. coli NCTC13353	0	28	28	13	30	17	14	30	16		0	0	0	13	13	0	25
Enterobacter cloacae	9	11	2	9	11	2	25	25	0		9	24	15	9	24	15	6

*: Inhibition zone sizes (mm) for ESBL and/or AmpC-producing *E. coli* isolates using the ESBL (left columns: CTX to FEP +C) and AmpC confirm (right columns CTX to FOX) test kits. D: is the difference between the 2 previous values (or the difference between antibiotic alone and antibiotic with inhibitor); 5 mm or greater difference for D indicates positive results for the test (positive for ESBL or AmpC activity) and it has been highlighted by a red colour. Strain numbers in red typeface indicate both ESBL and AmpC activity. Identification codes for antimicrobial discs: CTX: cefotaxime; CTX+C: cefotaxime plus clavulanate; CAZ: ceftazidime; CAZ+C: ceftazidime plus clavulanate; CTXCX: cefotaxime plus cloxacillin; CAZCX: ceftazidime plus cloxacillin; FOX: cefoxitin, FEP: cefepime; and FEP+C: cefepime plus clavulanate.

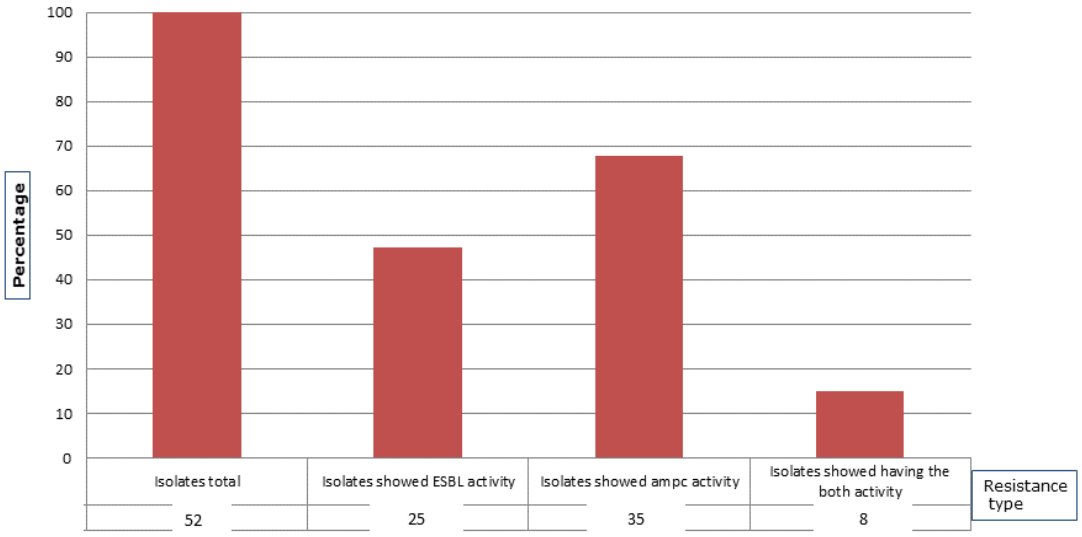


Figure 4-7: Percentage of ESBL and AmpC phenotypes amongst *E. coli* isolates resistant to cefotaxime and/or ceftazidime.

The total number of the isolates is 52; 25 isolates showed ESBL activity, 35 isolates showed AmpC activity and 8 isolates showed both activities.

4.2.4 PCR typing of ESBLs and β-lactamase genes

All of the strains which showed resistance or intermediate resistance to one of the cephalosporins were also tested by PCR for the presence of *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{OXA-2}$ and *bla*$_{SHV}$ β-lactamases (Section 2.7.2). No *bla*$_{SHV}$ β-lactamases were detected, but *bla*$_{CTX-M}$ (12 positive), and *bla*$_{TEM}$ (4 positive) were both detected in the ESBL-producing isolates, including examples of both genes in the same isolate (BCE3 and BCC2) (Figure 4.8). *bla*$_{OXA-1}$ was present in 2 isolates, but not in the ESBL-producing isolates, it was present in the BS7 and A2.5 strains (both of which are also positive for *bla*$_{TEM}$). BS7 is not a cephalosporin resistance strain but it was tested by PCR as sequencing analysis using whole genome sequencing (illustrated in Chapter 5; Section 5.6.2.3 and 5.6.3) showed the presence of *bla*$_{OXA}$ and *bla*$_{TEM}$ genes. PCR tests for the presence of chromosomal AmpC were positive for all of the isolates, and plasmid *ampC* genes from the six major families: ACC, FOX, MOX, DHA, EBC and CIT, were negative for all 35 of the phenotypically AmpC strains, suggesting that the AmpC phenotype in the farm *E. coli* isolates may be chromosomal hyper-production. Sequencing the CTX-M and TEM PCR products has defined most of the CTX-M types and all TEM variants (Table 4.4). The majority of the CTX-M gene sub-types were CTX-M-32 (6 of the 12 CTX-M positive strains). CTX-M-14 was found in two strains (BCE3 and BCC2) which also carried TEM-1b. A2.5 carried both OXA-1 and TEM-1, and BS7 carried OXA-1 and TEM-30. Not all of the CTX-M variants could be defined using the primers in this study, as the DNA sequence data

obtained from the CTX-M PCR products did not give an unambiguous identity when searched in different databases.

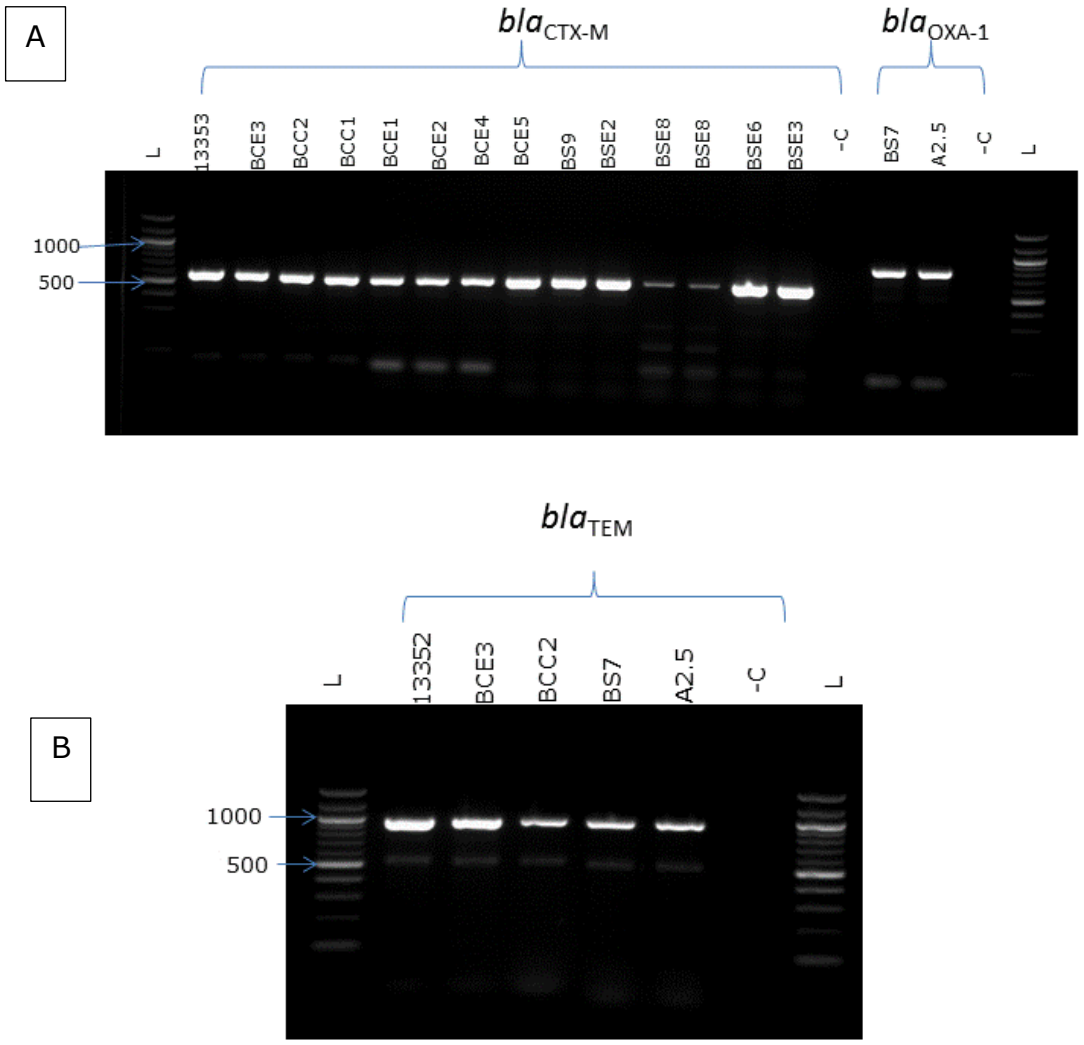


Figure 4-8: PCR amplification of (A) *bla*$_{CTX-M}$, *bla*$_{OXA-1}$ and (B) *bla*$_{TEM}$ genes from ESBL and non- ESBL-producing *E. coli* isolates from the dairy farm.

(A) L: is 100 bp DNA ladder size marker (NEB, UK); 13353: *E. coli* NCTC 13353 positive control for *bla*$_{CTX-M}$; positive strains for *bla*$_{CTX-M}$ start with BCE3 to BSE3, -C: negative control for *bla*$_{CTX-M}$. *bla*$_{OXA-1}$ positive strains are BS7 and A2.5 followed by –C negative control for *bla*$_{OXA-1}$. The product size for CTX-M is 592 bp, for OXA-1 it is 820 bp.

(B) L: is 100 bp DNA ladder size marker (NEB, UK); 13352: *E. coli* NCTC 13352 (positive control for *bla*$_{TEM}$); positive strains for *bla*$_{TEM}$ start with BCE3 to A2.5; -C: negative control for *bla*$_{TEM}$. The product size for TEM is 964 bp. *E. coli* ATCC 25922 is a negative control for all PCR reactions. The PCR products were electrophoresed in 1% (w/v) TAE agarose gel, 80 V for 1 h.

Table 4-4: β-lactamase and ESBL-producing *E. coli* strains, the β-lactamase genes they carry, the variant types, the phenotypic confirmation results and the resistance profile.

Isolate ID	β - lactamases	Gene sequence type	Accession No.*	ESBL Screening Test	Ampc Screening test	Antimicrobial resistance profile
BCE1	CTX	ND	ND	+	-	(S10), EFT, CAZ, CFQ, AMP, CTX, ATM
BCE2	CTX	CTX-M-32	KP634899.1	+	-	(S10), EFT, CAZ, CFQ, AMP, CTX, ATM
BCE3	TEM, CTX	CTX-M-14, TEM-1	WP_001617865.1, WP_000027057.1	+	-	SXT, (AMC), OT, S10, EFT, NA, CFQ, AMP, CTX, S300, CHL, ENR, CIP
BCE4	CTX	CTX-M-32	EU921825.1	+	-	(S10), EFT, CAZ, CFQ, AMP, CTX
BCE5	CTX	ND	ND	+	-	SXT, EFT, CAZ, CFQ, AMP, CTX, ATM
BSE2	CTX	CTX-M-32	EU921825.1	+	-	(S10), CAZ, EFT, CFQ, ATM, AMP, CTX
BSE3	CTX	CTX-M-32	EU921825.1	+	-	S10, (CAZ), NA, EFT, CFQ, ATM, AMP, CTX
BSE6	CTX	ND	ND	+	-	S10, CAZ, EFT, CFQ, ATM, AMP, CTX
BSE8	CTX	ND	ND	+	-	(AMC), (F), S10, (NA), (EFT), AMP, (CTX)
BS9	CTX	CTX-M-32	EU921825.1	+	-	(AMC), OT, (S10), CAZ, EFT, AMP, CTX, ATM, CFQ
A2.5	TEM, OXA	TEM1, OXA1	WP_000027057.1, WP_000881655.1	-	-	SXT, AMC, S10, OT, EFT, S300, ATM, AMP, CTX, (ENR)
BCC1	CTX	CTX-M-32	EU921825.1	+	-	F, CAZ, EFT, CFQ, ATM, AMP, ENR, (AMC), (S10)
BCC2	CTX, TEM	CTX-M-14, TEM-1	WP_001617865.1, WP_000027057.1	+	-	SXT, F, (AMC), OT, S10, EFT, NA, CFQ, AMP, CTX, ATM, S300, CHL, ENR, CIP
BS7	TEM, OXA	TEM30, OXA1	WP_021561477.1, KF914287.1	NP	NP	SXT, AMC, OT, S10, S300, AMP, CHL

*: Accession No. : accession number for the closest match in GenBank ; ND: not defined because it could not be distinguished using the primers in the current study; NP: the test not performed because the strain doesn't shows resistance to cefotaxime or ceftazidime; ampicillin (AMP), amoxicillin–clavulanic acid (AMC), cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM), cefquinome (CFQ), ceftiofur (EFT), streptomycin (S10), ciprofloxacin (CIP), enrofloxacin (ENR), nalidixic acid (NA), trimethoprim/sulfamethoxazole (SXT), sulphonamide (S300), chloramphenicol (CHL), oxytetracycline (OT), nitrofurantoin (F). If the antimicrobial is placed between brackets in the table it means the isolate shows intermediate resistance to that antimicrobial. -: negative for the test; +: positive for the test.

4.2.5 Conjugation experiments and plasmid profiles

All the isolates that carried *bla*$_{CTX-M}$ genes (BCC1, BCC2, BCE1, BCE2, BCE3, BCE4, BCE5, BSE2, BSE3, BSE6, BSE8 and BS9) were tested for their ability to transfer cephalosporin resistance by conjugation. Both broth and plate mating methods were used, and *E. coli* K-12 J53 resistant to sodium azide (J53 Azi^R) was used as the recipient strain (Section 2.8). No cephalosporin resistant putative transconjugants were obtained from two of the isolates (BSE3 and BSE6). The transfer rate was calculated from the solid mating experiment (data are not shown; further discussed in Section 4.3). One selected transconjugant colony was chosen from each successful mating, and PCR was used to confirm the presence of the CTX genes (Section 2.8.3). All the presumptive transconjugants were positive for CTX-M genes when tested by PCR. As a control, donor isolates were streaked on plates containing sodium azide and CTX; this step was to check the rate of mutation for the donor strains to sodium azide resistance as the *E. coli* K-12 J53Azi^R recipient strain was originally isolated as a spontaneous mutant (Jacoby and Han, 1996, Yi et al., 2012). At a concentration of 200 µg ml^{-1} of sodium azide, no growth of the donors was found to occur on the plates, so the presumptive transconjugants were expected not to be azide resistant mutants of the donor parents.

Plasmid profiling was performed (Section 2.6.1.5) for the parental strains above. However, for some strains (BCE5 and BCE2) the plasmid extraction using the Qiagen miniprep kit failed (see Section 4.3). Plasmid

profiling of the original strains using uncut plasmid DNA from the Qiagen miniprep kit showed that the donor parent strains BCE1, BCC1, BCE4, BS9 and BSE2 had apparently, the same plasmid profile with a single plasmid running at around 1 kbp when compared to a linear DNA ladder (Figure 4.9). BCC2 and BCE3 had a different profile from each other and from the previously mentioned isolates. They both carried one plasmid which ran at around 4 kbp but BCC2 also carried a second plasmid of around 10 kbp. It should be mentioned here that these sizes for the bands seen in these strains is nominal as these plasmids were extracted using a miniprep kit and there is no evidence if the plasmid is linear or not. The plasmid profiles for the presumptive transconjugants were tested as well (Figure 4.10). In each case this was the same as their respective donor parent, except for BS9T and BSE2T which showed no plasmids. There did appear to be discrete DNA bands which might be for a large plasmid running above what may be the chromosomal DNA band on the gel, this was the case with BCC1T, BCE1T and BCE4T. The DNA preparations from the BCE5 and BCE2 parent strains using the Qiagen miniprep kit method apparently contained no plasmid DNA, and the same was the case for their respective presumptive transconjugants, BCE5T and BCE2T (Figures 4.9 and 4.10).

To check if the selected transconjugants were real, and not a donor mutated for azide resistance, further confirmation testing for successful conjugation was performed by growing the presumptive transconjugants on minimal medium without methionine and without proline, as the recipient strain *E. coli* K-12 J53Azi^{R} is auxotrophic for these amino acids.

The results of testing for auxotrophy were that all the selected transconjugant colonies grew on that medium except for BCE1T and BCE2T. As expected, the J53Azi^R recipient strain did not grow on minimal media lacking methionine and proline, which shows that the colonies which grew were not true transconjugants as they were not auxotrophic for methionine and proline. The two which did not grow, BCE1T and BCE2T, were real transconjugants as the original donor parents for these isolates had the ability to grow on this medium while the transconjugants did not. Linking this to the plasmid profile, the selected presumptive transconjugants showed they have the same plasmid profile as the parent strain and this would be expected for most putative transconjugants given that the auxotrophic growth tests had shown they lacked the recipient strain's characteristics and so were probably donor strains.

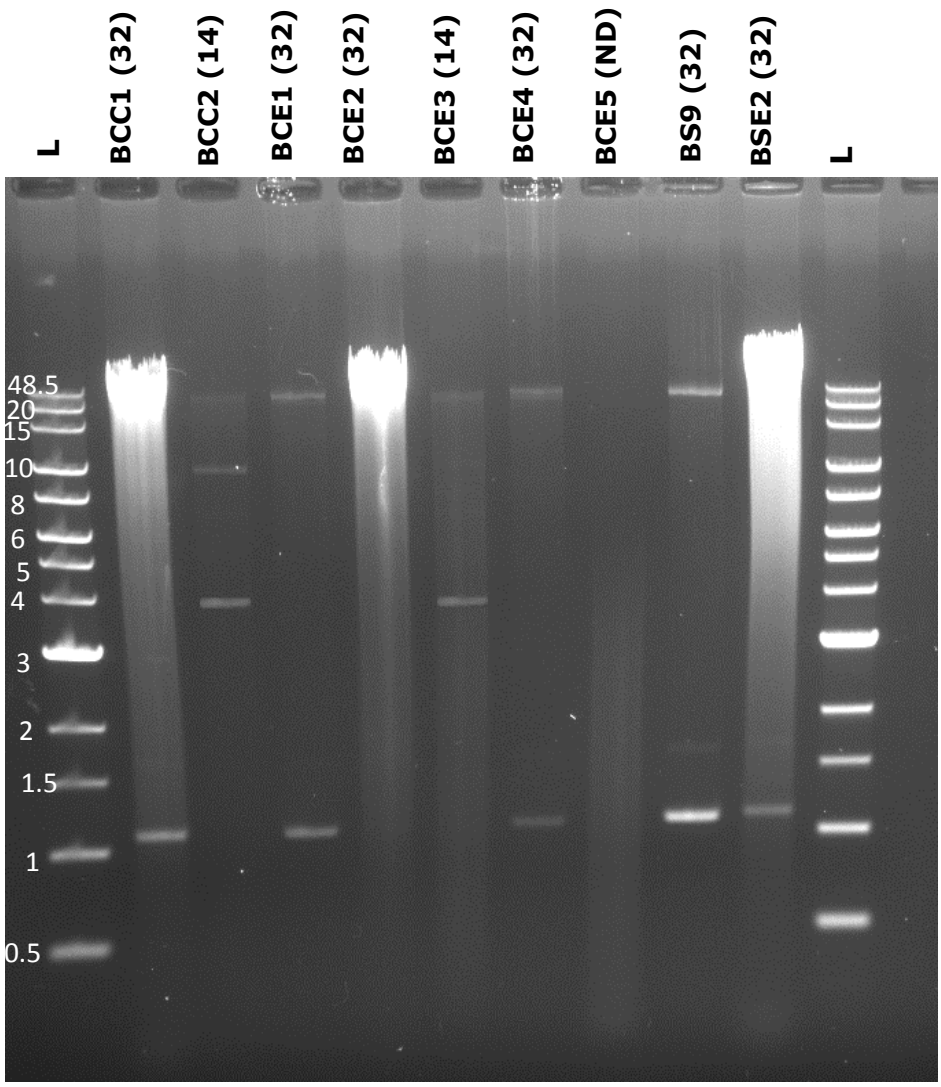


Figure 4-9: Plasmid profiles for some of the ESBL-producing *E. coli* strains.

L: Quick-Load® 1 kb Extend DNA Ladder (NEB, UK). 0.7% (w/v) TAE agarose gel, the gel was run for 3 h at 70 V. The sequence type for the CTX-M is indicated between brackets; BCC1, BCE1, BCE2, BCE4, BS9 and BSE2 have CTX-M-32 whilst BCC2 and BCE3 have CTX-M-14; ND: not defined CTX-M type. No plasmid DNA was obtained using the Qiagen miniprep kit for BCE2 and BCE5.

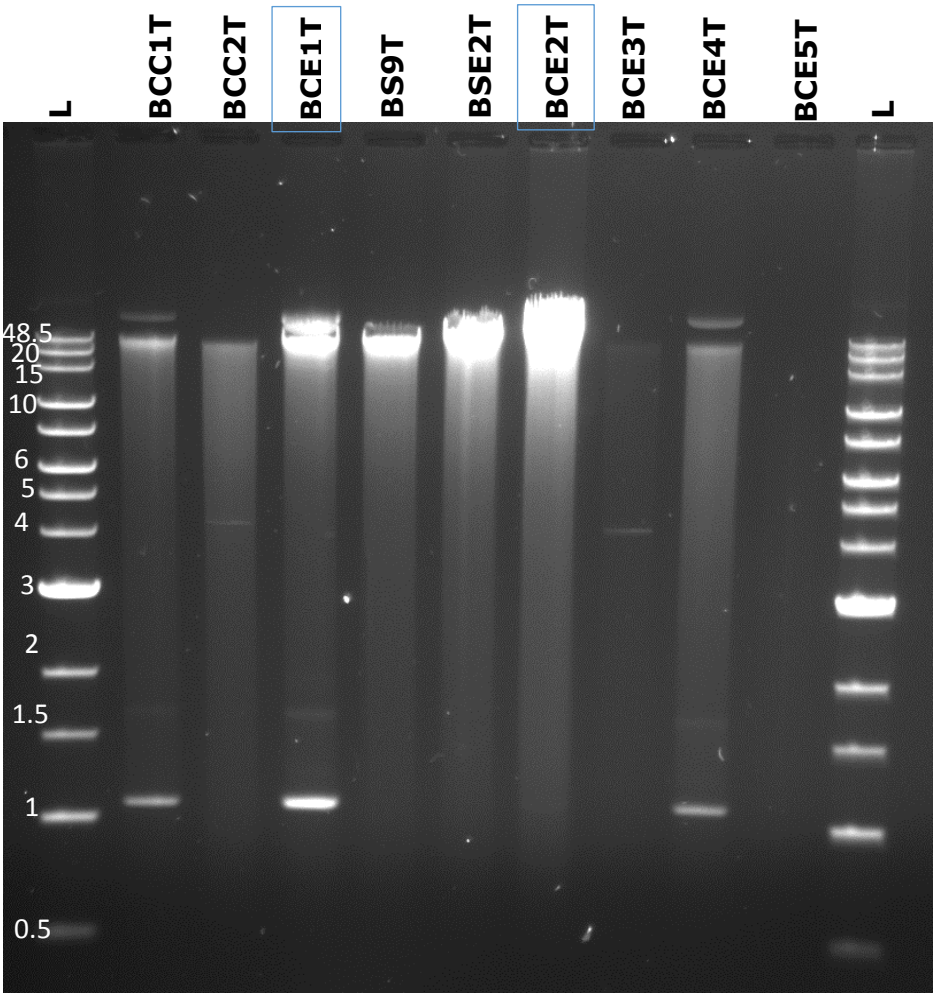


Figure 4-10: Plasmid profiles of the presumptive transconjugant isolates from the ESBL-producing *E. coli* conjugations.

L: Quick-Load® 1 kb Extend DNA Ladder (NEB, UK); 0.7% (w/v) TAE agarose gel, the gel was run for 3 h at 70 V. No plasmid DNA was obtained for BS9T, BSE2T, BCE2T and BCE5T using the Qiagen miniprep kit. BCE1T and BCE2T are the only confirmed transconjugants using growth on DMEM with no methionine and no proline to confirm the J53Azi^{R} recipient phenotype.

4.3 Discussion

Escherichia coli are common enteric strains which can be commensals or can cause human and animal disease. *E. coli* is one of the groups of seven bacterial species that the WHO has highlighted as of key AMR concern and serves as a sentinel organism for antimicrobial resistance development (WHO, 2014). Of particular concern has been the emergence and spread of ESBL-producing *E. coli* associated with cattle and other farm animals (Liebana et al., 2013). This has become of more concern recently when transferable colistin resistance has been reported in commensal *E. coli* from farm animals. The presence of the same resistance genes in isolates from meat and from patients has highlighted the threat that resistance genes originating in animal isolates can afterwards be spread to humans isolates (Liu et al., 2015).

On the farm that the samples were collected from (as will be the case in others worldwide), different types of antimicrobials are being given to the cattle, such as cefquinome, ceftiofur, oxytetracycline, penicillin, streptomycin sulphate and neomycin sulphate. The use of these antimicrobials is supposed to make a contribution to antimicrobial resistance gene selection and horizontal gene transfer among different species of bacteria. To our knowledge, similar studies on MDR *E. coli* from cattle slurry within one dairy farm have not been performed, or have been performed with different methodology or materials, such as the antimicrobials that were used to challenge the strains. Comparison between the results of this study with other studies would not essentially give the same patterns of resistance, as each farm has a different

management regime regarding housing the cattle, the type of cattle, their nutrition and veterinary treatments, disposal of antimicrobial-contaminated mastitic milk, use of disinfectants and other antimicrobials, and slurry handling. The source of the isolates in this study is a well-managed farm with records of the antimicrobial types that are being used for disease treatment (Section 2.3.1). The results of this study using *E. coli* has given us an indication of the reservoir of resistance that is occurring in the farm bacterial population.

This study showed that MDR *E. coli* were present in all samples. However, the faecal samples from the barns and the solid slurry carried a greater number of resistant isolates compared to samples from the liquid slurry. This might be due to the dilution that happens to the bacterial levels contained in the slurry tank, as wastewater from cleaning the barns and the milking equipment is disposed of into the slurry tank, and the slurry tank is open-topped and receives rain water.

Using different isolation media enabled the isolation of different subpopulations of resistant *E. coli* from the same samples. Using an extended spectrum cephalosporin antibiotic (i.e. CTX) in the selective medium, has isolated strains with the same multiple antimicrobial resistances more frequently (Figure 4.5).

The total percentage of MDR strains resistant to 3 or more antimicrobials was 57.9% (73 isolates) (including extended spectrum β-lactam resistance and resistance to other antimicrobials) of which 43.6% (55 isolates) were strains that were isolated using extended spectrum

cephalosporin selective media, whilst the percentage of MDR *E. coli* isolated on TBX without antibiotic selection was 14.2% (18 isolates). This difference might be due to the effectiveness of the direct selection method which selects for CTX-resistant *E. coli* of which approximately 97% were subsequently found to be MDR. This is further demonstrated by the heatmap results (Figure 4.5) where all strains isolated on TBX+CTX showed a consistent resistance to five antimicrobials. This might indicate the co-carriage of different resistance genes on one transmissible element (for example, a plasmid) and by selecting for one resistant phenotype the other resistant phenotypes can be selected.

Relative to the number of resistant isolates found using TBX+CTX, only a low level of isolation was made of ESBL-producing isolates using CHROMagar ESBL medium. There was no enrichment step used for isolating ESBL-producing *E. coli* in this study as the samples were directly plated on to CHROMagar ESBL selective medium. Enrichments are usually carried out using broth media supplemented with one of the cephalosporins, and selective enrichment methods for isolating ESBL-producing *E. coli* have been shown to increase the number of resistant isolates compared to the use of non-selective enrichment methods. The study by Schmid et al. (2013) showed that ESBL-producing *E. coli* can be isolated from cattle farms more than twice as often using enrichment procedures compared to direct plating onto selective solid media (Schmid et al., 2013). In another study, the use of an enrichment broth improved the detection of ESBL-producing Enterobacteriaceae in clinical stool samples (Jazmati et al., 2015). In a further study manure samples from

the input and output of a biogas unit were plated on CHROMagar ESBL with and without a selective enrichment step using 0.5 mg L^{-1} ceftazidime and 0.5 mg L^{-1} cefotaxime in LB broth. Selective enrichment increased the possibility of isolating ESBL-producing bacteria from the output samples (Schauss et al., 2015). According to the manufacturer's instructions no enrichment step is required when using the CHROMagar ESBL medium, but it seems that selective enrichment could be more appropriate as it appears to increase the number of ESBL enterobacteria which can be isolated, especially if they are in low numbers. Selective enrichment could increase isolation of ESBL-producing enterobacteria, especially if used with the samples from the slurry tank, as although MDR and CTX-resistant strains were isolated from the slurry tank samples, no ESBL-producing strains from that site were isolated using CHROMagar ESBL medium. This might be due to low numbers of such strains.

The presence of ESBL-mediated resistance in the isolates was not surprising given the use of 3rd and 4th generation cephalosporins for mastitis treatment in the herd. The same was true for the level of resistance carriage to older antimicrobials, with resistance to oxytetracycline and streptomycin seen in over a third of strains, as these antimicrobials are being used on the farm, and are widely used in UK agriculture. Co-acquisition of these resistance genes with ampicillin resistance was strongly indicated in TBX+CTX isolates (Figure 4.5) and may indicate the presence of a multi-resistance genetic element in these isolates. This is not surprising, as previously discussed in Section 4.1, tetracyclines, β-lactams and trimethoprim/sulphonamide are the most

heavily used antimicrobial groups in UK agriculture (DEFRA, 2012). There have been some fluctuations in sales of tetracyclines, trimethoprim/sulphonamides and ß-lactams, with sales of trimethoprim/sulphonamides and ß-lactams declining in 2013 compared to 2012, but increasing again in 2014. Tetracycline sales have the greatest variation observed between 2010 and 2011 and declined notably in 2013 and further in 2014 (UK-VARSS, 2014b) (Table 4.5).

Table 4-5: Sales (tonnes active ingredient) of total antimicrobial products (Food and Non-Food Animals) by chemical grouping, and total sales from 2010 –2014 in the UK.

	2010	2011	2012	2013	2014
ß-lactams	93	86	90	89	91
1st/2nd Generation Cephalosporins	6	5	6	5	6
3rd/4th Generation Cephalosporins (t)	1	1	1	1	1
(kg)*	1455	1172	1332	1189	1335
Penicillins**	17	20	8	20	12
Other Penicillins***	69	60	75	63	72
Tetracyclines	200	110	190	184	173
Trimethoprims/ Sulphonamides	75	72	80	61	70
Trimethoprims	12	12	13	10	11
Sulphonamides	63	60	66	51	59
Aminoglycosides	22	19	20	21	22
Streptomycins	8	7	8	8	7
Neomycin and framycetin	<1	<1	<1	<1	1
others****	14	12	12	13	14
Macrolides	35	37	41	43	48
Fluoroquinolones (t)	2	2	2	3	3
(kg)*	2,232	2,085	2,434	2,610	2,596
Other	20	20	21	19	22
Total	447	346	445	420	429

* The kg active substance is shown in addition to the rounded tonnage (t) because these are of the highest priority critically important antimicrobial classes. ** includes benzylpenicillin, benzathine penicillin, phenoxymethyl penicillin and procaine penicillin. ***includes amoxicillin (including in combination with clavulanic acid), ampicillin, cloxacillin and nafcillin. ****includes spiramycin, gentamycin, spectinomycin and kanamycin; adapted from UK VARSS 2014b.

Schmid et al. (2013) found ESBL-producing *E. coli* from some farms which did not use β-lactam antibiotics and they suggested that the presence of such isolates is due to use of other classes of antibiotics that can select for ESBLs as well. In a previous study of *E. coli* strains isolated from cattle, most strains were resistant to ampicillin (64%), tetracycline (74%), streptomycin (60%), and sulphonamide (76%), with a low occurrence (1%) of enrofloxacin resistance (EMEA, 1999). In a later study by Nováková et al. (2009) all the *E. coli* isolates from dairy calves and lambs showed multi-resistance to tetracycline, streptomycin and compound sulphonamides with less resistance to enrofloxacin *(*Nováková et al., 2009). In another study, 100 MDR *E. coli* strains were recovered from cattle and the farm environment in Ireland, and the most prevalent antimicrobial resistance identified was to streptomycin (100%), tetracycline (99%), sulphonamides (98%), and ampicillin (82%) but less resistance was found to ceftiofur (0%), chloramphenicol (9%), ciprofloxacin (0%), nalidixic acid (2%) (Karczmarczyk et al., 2011). In another study, 23 herds from central and south-central Pennsylvania were surveyed for antimicrobial-resistant Gram-negative enteric bacteria from the faeces of healthy lactating cattle. Gram-negative enteric bacteria were isolated from cattle (cow and faeces) and tested against different antimicrobial agents. *E. coli* strains were resistant to ampicillin (48%), ceftiofur (11%), chloramphenicol (20%), spectinomycin (18%), and tetracycline (93%) (Sawant et al., 2007). In a study conducted in New York State, 135 *E. coli* strains isolated from dairy cows with mastitis from two Holstein dairy herds, showed resistance profiles of the

following: 98.4% were resistant to ampicillin, 40.3% were resistant to streptomycin, 24.8% to tetracycline, 97.7% to aztreonam, 38% to nitrofurantoin and no resistance were observed to ciprofloxacin (Srinivasan et al., 2007).

Some of the resistance patterns observed in the current study mirror these findings, and resemble a historical record of antimicrobial use and development of resistance to them (Toleman and Walsh, 2011), and may represent a 'genetic archaeology' of the use of veterinary antimicrobials.

According to Jacoby and Sutton (1991), resistance determinants against aminoglycosides, tetracycline, sulphonamides and cephalosporins, are often situated on the same plasmid (Jacoby and Sutton, 1991). Many of the *E. coli* strains in this study carry this resistance profile (Figure 4.5, TBX+CTX clusters). Metagenomics, PCR and exogenous plasmid isolation studies of cow manure have also detected diverse resistance genes (Jacoby and Sutton, 1991, van Overbeek et al., 2002, Wichmann et al., 2014). Plasmids and transposons that carry multi-antibiotic resistance genes can also carry genes mediating resistance to toxic metals, virulence, and metabolic functions, for example Tn*21* encodes resistance to mercuric ions, streptomycin and sulphonamides (Liebert et al., 1997, Partridge, 2011). Tn*1691* is another example which confers resistance to mercury as well as to some antimicrobials: streptomycin, sulphonamides and chloramphenicol (Barbosa and Levy, 2000). This could indicate that there are factors other than veterinary medicines leading to retention of antimicrobial resistances within the dairy herd and the farm environment. Finding two strains that were resistant to imipenem was unexpected and

of concern. When confirmatory testing was performed, these isolates showed initial retention of imipenem resistance, but on further retesting the strains reverted to imipenem sensitivity; this phenomenon has been reported elsewhere (Villa et al., 2013) and can be associated with loss of plasmid carriage of genes. Resistance to carbapenems might also occur due to overexpression of AmpC in combination with porin loss (Hansen et al., 2012). As isolates BSC13 and BSC14 were both phenotypically positive for the AmpC confirmation test, this resistance to imipenem could be due to the second hypothesis, however this cannot be confirmed unless further tested as many other strains showed overexpression of AmpC but no investigation was made with regards to differences in the presence of porins.

The most frequently encountered ESBL genes in Enterobacteriaceae belong to the TEM, SHV and CTX-M families (Paterson and Bonomo, 2005), and the number of novel gene variants is still increasing as different genes are being described (http://www.lahey.org/studies/). This study showed the presence of β-lactamase and ESBL-producing *E. coli* in one dairy farm as $bla_{\text{CTX-M}}$ (12 strains), bla_{TEM} (4 strains), and bla_{OXA} (2 strains) were detected but not bla_{SHV}. Nine of the 12 $bla_{\text{CTX-M}}$ positive *E. coli* strains were isolated on CHROMagar ESBL, in which two of the isolates (BCE3 and BCC2) were also shown to be bla_{TEM} positive. With regards to the $bla_{\text{CTX-M}}$ variants, 6 strains were identified as CTX-M-32 type (five of them isolated from CHROMagar ESBL), 2 were CTX-M-14 type (one of them isolated from CHROMagar ESBL), and the other 4 were not differentiated using the primers in this study (all of which were

isolated using CHROMagar ESBL). The different types in each CTX group have evolved from point mutation events usually resulting in a single amino acid substitution. This mutation alters the ability of the bacteria to hydrolyse different substrates (Cartelle et al., 2004, Stürenburg et al., 2004, Bae et al., 2006). For example the CTX-M-1 group includes CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15, and CTX-M-32 and many others. The difference between CTX-M-1 and CTX-M-32 is an Asp240-Gly substitution due to a mutation which has led to increased hydrolytic activity of CTX-M-32 towards ceftazidime which was very low in CTX-M-1 (Cartelle et al., 2004). CTX enzyme types have more hydrolytic activity against cefotaxime than against ceftazidime, for this reason the responsible resistance genes are designated as CTX gene types (Cartelle et al., 2004). CTX-M-14 belongs to group 9 which also has many other variants such as CTX-M-9, CTX-M-16, CTX-M-19, CTX-M-21, and CTX-M-27 (Bonnet, 2004, Novais et al., 2008). The presence of different CTX-M variants has been reported in various places in the world. The prevalence of a specific CTX-M variant differs between different sources. CTX-M-14 and CTX-M-15 are among the most important CTX-M types due to them being found in *E. coli* strains which are found in humans and animals, as well as being dispersed in the environment worldwide (Hawkey and Jones, 2009, Canton et al., 2012). In cattle CTX-M-1, CTX-M-2, CTX-M-3, CTX-M-13, CTX-M-14, CTX-M-15, CTX-M-29 and CTX-M-32 have been reported in different places in the world (Shiraki et al., 2004, Duan et al., 2006, Madec et al., 2008, Kirchner et al., 2011, Hartmann et al., 2012, Watson et al., 2012, Cottell et al., 2013, Tamang et al., 2013a). The

variant types for *bla*$_{CTX-M}$ detected in this study (CTX-M-32 and CTX-M-14) are also reported from cattle sources elsewhere.

In a study published in 2012, CTX-M-14 *E. coli* were isolated from humans, cattle and turkeys (Stokes et al., 2012). In a study in Korea, a total of 84 ESBL-producing *E. coli* were isolated from cattle, the farm environment, and farm workers. All of the ESBL-producing isolates carried *bla*$_{CTX-M}$ genes that belonged to the CTX-M-1 group (n = 35) in which CTX-M-32 (n = 26) were predominant or the CTX-M-9 (n = 49) family in which all isolates carried CTX-M-14 (Tamang et al., 2013a). Both these studies (by Stokes et al. and Tamang et al.) are in line with the findings of the current study regarding the CTX variants found in cattle at the dairy unit. Both of these studies also showed that the same CTX-M variant types were present in humans associated with the farms, which suggests the dissemination of these genes in humans and different animal hosts. In the US *bla*$_{CTX-M-32}$ was detected in *E. coli* strains collected from a cohort of steers, of which 14 were treated with ceftiofur, four received chlortetracycline, and 11 received both treatments. This CTX type is not common in the US compared to Europe (Cottell et al., 2013). In the UK, a study was conducted on many dairy farms in North West England and North Wales. CTX-M ESBL *E. coli* isolated from different faecal sources on these farms carried different CTX-M types. Group 1 (CTX-M-15, CTX-M-55, CTX-M-1, CTX-M-32), group 2 (CTX-M-2) and group 9 (CTX-M-14, CTX-M-14B, CTX-M-27) CTX-M *E. coli* were recognized on the farms with some of the farms having both groups 1 and 9 (Snow et al., 2012). This mirrors the finding for the current study

as both group 1 and 9 CTX-M genes were isolated from the dairy unit in the current study.

With regards to *bla*$_{TEM}$ (1 and 30) and *bla*$_{OXA}$ (OXA-1) variants found in this study, they are broad spectrum β-lactamases not ESBLs. In a study performed on healthy pigs and cattle in Korea, 1212 faecal samples were tested for the prevalence of ESBL-producing *E. coli* and the *bla*$_{TEM-1}$ gene was associated with CTX-M β-lactamase in 55 *E. coli* isolates out of 121 isolates (Tamang et al., 2013b). In a previous study in Ireland, 79% of 100 multidrug resistant *E. coli* recovered from cattle and the farm environment carried *bla*$_{TEM}$ (Karczmarczyk et al., 2011). The findings from these other studies are broadly similar to the findings of the current study, and might indicate the commonness of *bla*$_{TEM}$ gene prevalence in cattle and their environments. However, the current study shows less prevalence of *bla*$_{TEM}$ compared to the other studies.

In a more recent study in Germany performed by Dahms et al., (2015), samples were collected from cattle, pig and poultry farms from the livestock and the workers, in order to isolate ESBL-producing *E. coli*. With regard to the cattle farms, pooled faecal samples were collected from each farm and the results showed that 6 out of 11 farms had *E. coli* populations which carried different ESBL genes. Eight isolates carried CTX-M, 3 harboured TEM, 2 harboured OXA and 1 harboured SHV. One of the isolates carried CTX-M, OXA and TEM, and one isolate carried CTX-M and TEM. This is similar to the findings of this study, as two of the isolates (BCC2 and BCE3) carry both *bla*$_{CTX-M}$ and *bla*$_{TEM}$. What was worrying in the Dahms et al. (2015) study was an indication that a

zoonotic transfer had occurred. One human isolate shared an identical MLST sequence type (ST3891) and CTX-M allele (CTX-M-1/-61) to the isolate found in the cattle faecal sample from the same farm. This is of concern because the isolates from the current study might have the ability to transfer to other hosts (i.e. humans) the same as the isolates in the Dahms et al. (2015) study.

Complex ESBL gene carriage patterns have been found elsewhere. In a study performed on a farm suffering from calf scour it was found that amongst *E. coli* isolates that were resistant to cefotaxime, isolates were positive for CTX-M (CTX-M-14/17) and TEM (TEM-35) and these genes have been found to be located on a transferable plasmid of the IncK type. But 5/48 calf and 28/60 cow isolates were $bla_{\text{CTX-M}}$ and bla_{TEM} negative, and sequence analysis confirmed that these had mutations in the promoter region of the chromosomal *ampC* gene (Liebana et al., 2006). This reflects the findings in the current study as 35 CTX and/or CAZ resistant isolates were $bla_{\text{CTX-M}}$ and bla_{TEM} negative and phenotypically had *ampC* activity which might be due to mutations in the *ampC* promoter region.

In a study carried out on *E. coli* from farms in the UK, it was found that *E. coli* transconjugants harbouring $bla_{\text{CTX-M}}$ genes from cattle, chicken and turkey isolates were carrying 10, 8 and 7 other antimicrobial resistance genes respectively, and these mainly encoded resistances to older antimicrobials. This study suggested that chicken and cattle CTX-M *E. coli* represent different populations (Toszeghy et al., 2012), and in a separate study, where there was the use of 3rd or 4th generation cephalosporin

(ceftiofur and cefquinome) in livestock for 12 months prior to the analysis, *E. coli* isolates were nearly four times more likely to be ESBL-positive (Snow et al., 2012). The different conclusions from different studies may reflect the fact that each farm has different antimicrobial usage policies, which can affect the prevalence of antimicrobial resistance.

In the current study thirty-five isolates were confirmed as having resistance phenotypes consistent with hyperproduction of AmpC. The phenotypic differentiation between plasmid-borne and chromosomal AmpC resistance in *E. coli* is difficult. Although the AmpC β-lactamase enzyme is normally encoded by a non-inducible chromosomal gene (Honore et al., 1986, Philippon et al., 2002, Jacoby and Munoz-Price, 2005, Jacoby, 2009), hyper-production can arise, either by mutations at the *ampC* attenuator or promoter regions, or by gene amplification (Caroff et al., 1999, Nelson and Elisha, 1999, Philippon et al., 2002). In bacteria that lack chromosomal *ampC*, phenotypic differentiation is easier, as the difference in β-lactamase activity between ESBL and plasmid-carried AmpC strains can be seen in the difference in sensitivity towards cefoxitin (to which an AmpC producer is resistant) and β-lactamase inhibitor combinations: clavulanic acid and EDTA (to which an ESBL producer is sensitive) (Black et al., 2005, Giske et al., 2013). However, because no plasmid AmpC was detected in isolates from this farm, resistance is likely to be due to mutations at the *E. coli* chromosomal *ampC* attenuator or promoter regions. In a study published in 2012 by Hansen et al., in which different phenotypic confirmation kits

from ROSCO (Neo-Sensitabs™) were used to differentiate between ESBL and AmpC, it was found that out of 66 ESBL-producer enteric isolates (*E. coli* and *K. pneumoniae*), 62 showed positive ESBL activity using cefotaxime and cefotaxime plus clavulanate combinations (CTX-CTX+C), 58 isolates showed positive ESBL activity using cefepime and cefepime plus clavulanate (FEP-FEP+C), and only 38 isolates showed positive activity using a ceftazidime and a ceftazidime plus clavulanate combination (CAZ-CAZ+C); and none of the AmpC strains showed activity against cefepime and cefepime plus clavulanate (Hansen et al., 2012). In the previous study, the researchers suggested that using CTX and CAZ alone and with clavulanic acid in combination is sufficient for the detection of ESBL activity, but the use of cefepime and a cefepime plus clavulanic acid combination disc in combination with either CTX-CTX+C or CAZ-CAZ+C can be a suboptimal test for the detection of ESBL activity. However, class D (OXA-type) ESBLs are poorly inhibited by clavulanic acid and therefore cannot be detected by the methods described above (Naas et al., 2008, Drieux et al., 2008).

E. coli, isolated from faecal samples from 100 veal calf herds, with non-wild type susceptibility to cefotaxime were detected and screened for ESBL/AmpC genes (Hordijk et al., 2013). The results showed that 66% of the herds were positive for *E. coli* with non-wild type susceptibility for cefotaxime. One isolate was selected from each tested herd and from a total of 100 isolates, 83% of the *E. coli* producing ESBL/AmpC carried $bla_{\text{CTX-M}}$ genes, of which $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ were the most predominant, but $bla_{\text{CTX-M-32}}$ was also detected. With regards to AmpC

type, one isolate was found with *bla*$_{CMY-2}$ and 6 isolates had mutations in the promoter region of the chromosomal *ampC*. The dominant plasmids were IncI1 and IncF-type plasmids. In one isolate which failed to give positive results either for plasmid-mediated ESBL/AmpC genes or for mutations in the promoter region of the chromosomal *ampC*, the β-lactamases *bla*$_{TEM-1c}$ and *bla*$_{OXA-1}$ were detected (Hordijk et al., 2013b). Some of the findings in the current study are in line with the findings from the study described above: *bla*$_{CTX-M14}$ and *bla*$_{CTX-M-32}$ were isolated in both studies from cattle faecal samples but these were from dairy cattle (current study) rather than veal calf cattle (previous study). One of the isolates in the current study (A2.5) which shows phenotypic resistance to cefotaxime also failed to give positive results for ESBL genes and plasmid-mediated *ampC*, but it was carrying *bla*$_{TEM-1}$ and *bla*$_{OXA-1}$ which is similar to the characteristics of one of the strains from the veal calf study except for the TEM variant type. However, the carriage of these two genes in A2.5 does not seem to be responsible for the phenotypic resistance for cefotaxime (this will be discussed further in the next chapter; Section 5.9).

With regards to the conjugation experiment results, the plasmid profiling methods performed for the parents and presumptive transconjugant strains were based on the use of a miniprep kit (QIAprep Spin Miniprep Kit), which has been used widely for isolation of plasmids carrying different ESBL genes (Preston et al., 2003, Bortolaia et al., 2010, Mnif et al., 2013) but is not specifically designed for isolation of large plasmids. In some cases there may have been a failure in plasmid isolation, for

example for BCE2, BCE2T, BCE5, BCE5T, BSE2T and BS9T. This could be because some strains originally did not carry plasmids (but this is not the case for BCE2 which produced real transconjugants), or a better method may be needed to isolate ESBL-carrying plasmids. From the literature (Section 1.6.1 and Table 1.2), ESBL-carrying plasmids are usually large (between 35-150 kbp for the examples provided) and other methods, such as using the large construct kit from Qiagen, might give better results than using the plasmid miniprep method. According to the information provided by the manufacturer, if the plasmid has a very low copy number or the plasmid is bigger than 50 kbp, other kits and protocols are necessary to isolate it (www.qiagen.com). The common method to determine the exact size for plasmids, especially for big plasmids, is using S1 PFGE methods (Barton et al., 1995); restriction digests can be used as well (Nauerby et al., 2000) but this method is problematic for strains with multiple plasmids. Comparing the plasmid profile for the parents and the transconjugants for BCE1 and BCE2 strains which were real transconjugants, it can be concluded that the small plasmids transferred for BCE1T (running around 1 kbp and 1.5 kbp) are not the plasmids carrying the *bla*$_{\text{CTX-M}}$ as many other strains have the same plasmids but were not confirmed as real transconjugants. This suggests that the actual plasmid carrying *bla*$_{\text{CTX-M}}$ is large and failed to be isolated using the miniprep method, as is probably the case for BCE2 and BCE2T. However, there appear to be discrete DNA bands running above what may be the chromosomal DNA band on the gel which is where large

plasmid bands would run. A further problem is that the plasmid might be linearized and run with the chromosomal DNA.

The *E. coli* K-12 J53Azi^R recipient strain (carrying a chromosomal mutation conferring resistance to sodium azide) was selected as it has been widely used in MDR plasmid conjugation experiments (Jacoby et al., 2003, Wang et al., 2004, Liu et al., 2007, Wang et al., 2008, Warnes et al., 2012, Bonnin et al., 2012). In all the published papers found where J53Azi^R was used as a recipient, the transconjugants were confirmed by performing PCR to check for the genes that were expected to transfer, for example *qnr* or *bla*$_{CTX-M}$. But none of these mentions how they confirmed that the transconjugants were not actually the original strain that had mutated to azide resistance. Sodium azide resistance is conferred by a single amino-acid mutation in the SecA protein. To check for the spontaneous mutation rate to sodium azide resistance, donors were plated onto LB agar containing sodium azide and CTX. This was a control to check if there is any mutation, as *E. coli* mutation rates to azide resistance can vary depending on the strain type (George Jacoby, personal communication). This confirmed that selection of azide resistant mutants did not occur at a rate likely to influence the conjugation experiments. But a question arose whether this was enough for confirmation, so a further test was performed and the selected transconjugant colonies were grown on minimal media without methionine and proline (George Jacoby, personal communication). Only two isolates failed to grow on the minimal medium while the other seven grew, which would indicate that the putative transconjugants which were

selected were donor strain mutants not transconjugants. This was unexpected given the low reversion rate when donors were plated onto plates containing 200 ug ml^{-1} sodium azide. Furthermore, two of the strains which produced presumptive transconjugants (BCE3 and BCC2), which were then not confirmed, were subsequently shown to have the *bla*$_{CTX-M14}$ gene located on the chromosome from whole genome sequence analysis (described later in Section 5.6.1). This suggested that all of the apparent transconjugants which had grown on LB AzideCTX were actually donor strains which had spontaneously mutated to become azide resistant. Due to this, the apparent transfer rate data were thought not to reflect the real transfer rate and as a consequence are not shown.

From these studies the J53Azi^R recipient strain is not ideal, as the azide resistance mutation has appeared readily in the donor strains used, making it hard to differentiate between a donor mutant and real transconjugants. Here significant work was required to confirm putative transconjugants have a proline/methionine auxotrophy typical of the recipient. Additional phenotypic markers would improve J53Azi^R as a recipient.

Linking the results from the heatmap (Figure 4.5) and ERIC dendrogram (Figure 3.6) we can see that most of the strains that carried CTX-M genes are in a related population represented by the first cluster in the ERIC dendrogram and the CHROMagar ESBL cluster in the heatmap, however there are some strains which did not cluster with this related population group in the ERIC dendrogram such as BS9, BSE2, BSE6 in cluster II and BSE8 in cluster VI. This might indicate the carriage of some

of these genes on transposable elements, especially when we compare a strain such as BSE8 with BSE7; both have a 100% similarity with their ERIC profile, but BSE8 is an ESBL strain while BSE7 is not. The same thing can be seen with BS9 which shares 100% similarity in its ERIC profile with seven other strains of which none is an ESBL; this is also the case with BSE6. Determining the diversity of resistance to the tested antimicrobials and the prevalence and range of MDR and particularly ESBL carriage in *E. coli* within the farm has been demonstrated in this chapter. The presence of ESBL genes clearly increases the range of β-lactam antibiotics which the *E. coli* strains are resistant to. However, the reason for some strains having multiple resistances to β-lactam antibiotics is still not clear and further investigation in the future will be needed especially with plasmid characteristics. The next chapter will provide more detail about the molecular variation between different strains using the whole genome analysis, and may give more explanation to the reasons for the variation in the phenotypic resistance profile.

5 .Whole genome sequencing and comparative analysis of MDR *E. coli* strains A2.5, BCE3, BCC2 and BS7

5.1 Introduction

The phenotypic diversity in AMR of *Escherichia coli* strains isolated in this study indicates the variety of AMR genes carried by the different strains. The identification of these AMR genes is important for understanding the underlying mechanisms for dissemination of these genes between the cattle *E. coli* community on the dairy farm. Developments in next generation sequencing (NGS) technology and decreasing costs of sequencing have made NGS a very attractive and powerful technique for understanding evolution, and in epidemiological studies. In addition, the development of many searchable databases and websites means that information can be obtained from the whole genome sequence easily: for example AMR genes (Zankari et al., 2013), virulence factors (Joensen et al., 2014), serotypes of *E. coli* (Joensen et al., 2015), and MLST grouping (Larsen et al., 2012) can be determined from sequence data.

Four *E. coli* strains were selected for whole genome sequencing: A2.5, BCC2, BCE3 and BS7. A2.5 is a strain from the 2012 collection isolated from the liquid slurry taken from the slurry tank. A2.5 is resistant to 3rd and 4th generation cephalosporins and a range of the other antimicrobials, (Table 4.4). This strain was sent for sequencing before collecting samples during 2014. BCE3, BCC2 and BS7 were isolated in 2014. BCE3 was isolated from slurry from the cow barn, this isolate was selected on CHROMagar ESBL medium, and showed resistance to 13 antimicrobials (Table 4.4). From the same site BCC2 was isolated but using TBX+CTX medium. This strain showed resistance to 15 out of the

17 antimicrobials used in this study (Table 4.4). These two isolates were from the same group in the ERIC dendrogram (Figure 3.6). Strain BS7 was isolated from solid slurry and showed resistance to a range of antimicrobials (but not cephalosporins), and had a unique ERIC profile that put it as an outlier, which did not cluster with the other strains at the 54% similarity threshold (Figure 3.6 and Table 4.4).

Whole genome sequences of *E. coli* isolates from cattle have been reported elsewhere. Some cattle *E. coli* isolates have been reported via *Genome Announcements* which does not provide enough information regarding the AMR genes found in those strains, as it only provides a short description for the source of these strains and some information about the sequencing methodology. Draft genome sequences of *E. coli* W26 (Kim et al., 2012) and *E. coli* AA86 (Yi et al., 2011), both isolated from healthy cattle faeces from South Korea, have been announced. An *E. coli* O157:H43 strain, designated T22, has also been sequenced and announced via *Genome Announcements* (Sváb et al., 2013); this strain has been isolated from healthy cattle in Hungary, and belongs to phylogenetic group B1, and sequence type 155 (ST155). Other studies have performed whole genome sequencing for a specific related group of *E. coli* strains to study the relatedness of that strain type. In a recent study by Norman et al., (2015), whole genome sequencing was performed on 182 epidemiologically unrelated O26 strains, including 109 human-derived strains and 73 non-human-derived strains of which 59 were from bovines. The outcome of the previous study was that the same strains can be present in humans and cattle, sharing the same

virulence genes and the same polymorphism-derived genotypes which suggested transmission between cattle and humans were possible (Norman et al., 2015). This was also observed in an earlier study when seven O26:H11 strains were compared using whole genome sequencing, and a cluster containing one human strain, four cattle strains and one swine strain was found (Ju et al., 2012).

The aim of the research in this chapter was to identify the different AMR genes found in the four representative strains, and to relate gene carriage to resistance phenotypes in order to understand the mechanism behind that resistance phenotype. The aims were also to study the genetic environment of these genes, how these resistance genes are clustered, and to identify if they were carried on transposable elements. Genome sequences were examined to see if there was any evidence for plasmid carriage, which would make the dissemination of these genes wider. Additional analyses were performed to determine if the sequence type and serotype of these strains have been reported from other studies, and if they were related to human strains. The sequence data were also used to confirm the previous results obtained using phenotypic and genotypic tests, the disc assay results, the ESBL confirmation kit, PCR results, and ERIC profile data.

5.2 Results

5.2.1 Genome analysis

A2.5 whole genome sequencing was performed by BGI Tech Solutions (Hong Kong), Co. Limited using an Illumina MiSeq machine in 2013. DNA from BCE3, BCC2 and BS7 was sequenced at DeepSeq (University of Nottingham) using an Illumina MiSeq 300PE machine in 2015. Data handling, assembly and annotation was performed by Dr Lisa Crossman from SequenceAnalysis.co.uk, NRP Innovation Centre, Norwich; (details about the genome sizes are provided in Appendix X, and a comparison circular figure is provided in Appendix XI). The annotated sequences for BCC2, BCE3, BS7 and A2.5 have been submitted to the Genbank and EMBL sequence databases. The final accession numbers have not been obtained yet, but the preliminary accession numbers for the assembled contigs are shown in Appendix X. This will put the sequenced strains in public domain databases, and will enable other researchers to use them for comparative studies.

5.3 MLST for the sequenced strains

The Multi Locus Sequence Type (MLST) for A2.5, BCC2, BCE3 and BS7 was determined using the Centre for Genomic Epidemiology MLST service (https://cge.cbs.dtu.dk//services/MLST/) (Larsen et al., 2012). MLST showed that the sequence type (ST) for the *E. coli* strains differed, but was the same ST for BCC2 and BCE3. A2.5 was assigned to ST56, BCE3 and BCC2 were both assigned to ST162, and BS7 was assigned to ST69, Appendix XII. Performing this analysis confirmed the relatedness of the strains as suggested by ERIC genotyping, as A2.5 and BS7 have very distinct genotype profiles compared with the other two sequenced strains, while BCE3 and BCC2 shared 91% similarity using ERIC genotyping and were from the same ERIC cluster (Figure 3.6). This difference in MLST not only reinforces ERIC-PCR typing results but also reflects the fact that these are complex populations on the farm which have a lot of variation in their genomes. A2.5 was isolated 1.5 years before the others, so it is likely to be unrelated. BS7 is an ERIC outlier with a distinct profile and from a different site to BCC2 and BCE3 which were both isolated from the same site. Identifying the ST of these strains makes comparisons with other studies possible (see Section 5.9).

5.4 Predicted Serotypes for the different strains

Serotypefinder 1.1 (https://cge.cbs.dtu.dk/services/SerotypeFinder/) was used to detect the predicted serotype for the different strains. Serotype detection depends on the presence of DNA sequences for the O-antigen genes: *wzx*, *wzy*, *wzm*, and *wzt*, and the flagellin genes: *fliC*, *flkA*, *fllA*, *flmA*, and *flnA*, from the whole genome sequence (Joensen et al., 2015). The predicted serotype for A2.5 was O3:H21, for BCC2 and BCE3 was O9:H9, and for BS7 was O17/O77:H18 (Appendix XIII). As discussed in Section 5.3, this further shows consistency in the results of sub-species typing methods between serotyping, and ERIC & MLST profiling, and enables a comparison with other strains reported by other studies (see Section 5.9).

5.5 AMR gene analysis for A2.5, BCC2, BCE3 and BS7

Using the whole genome sequence data for the sequenced strains, preliminary detection of the predicted AMR genes was performed using the CARD databases for antimicrobial resistance genes (Section 2.9.1.2). A heatmap of the differences in the AMR genes was produced from that information by Dr Lisa Crossman. The heatmap represents the difference between the selected strains (BCC2, BCE3 and BS7) (Figure 5.1 part A) and *E. coli* K-12. This heatmap was constructed based on the number of orthologues found in each genome; so these are protein orthologues found in both CARD and within the predicted genes from each genome. The number of hits represents the number of orthologues which are represented in different colours in the heatmap: white denotes absence of orthologues, a gradient blue colour represents 1-3 hits for presence of the orthologous protein. Due to the difference in number of hits (the number of orthologues) for each protein, the presence of these predicted genes in each genome was further confirmed using the Artemis genomic browser tool for gene sequence detection and NCBI blast search tool (using blastp) for gene type confirmation (Figure 5.1 part B). The gene length, direction, location, and gene description for each strain are listed in Appendix XV. Performing confirmation analyses gave an absolute confirmation of the presence or the absence of each gene. The heatmap was produced for only 3 of the sequenced strains, BS7, BCE3 and BCC2 as these strains were isolated at the same time (2014) and the sequencing and data handling performed at the same time (including the creation of the heatmap to compare them), while A2.5 was sequenced

before that and the analysis of some of the AMR genes had already been performed (Chapman, 2016). A modification of the heatmap was performed to show the differences between the selected strains (Figure 5.1A), and for comparison with the confirmation analysis for the presence/absence of the genes (Figure 5.1B), and the original heatmap including *E. coli* K12 shown in Appendix XIV. Even though A2.5 was not included in the heatmap, all of the predicted genes found for the 2014 strains were checked for A2.5 to determine if similar genes had been present in earlier populations (Appendix XV). The predicted AMR genes in all the strains were further checked with ResFinder databases, (Section 2.9.4). These provided a further confirmation of the heatmap.

A



B

Genes	BCC2	BCE3	BS7
yjiO	-	-	P
yjaA	-	-	-
ybtQ	P	P	-
ybtP	P	P	-
vatC	-	-	-
vanRF	-	-	-
ugd	-	-	P
triC	-	-	-
triA	-	-	-
tmrB	-	-	-
tetR	P	P	P
tetB/tetA	P	P	P
tet32	-	-	-
tet	-	-	-
terB	-	-	-
bla_{TEM}	P	P	P
tap	-	-	-
sulII	P	P	P
sulI	-	-	P
strB	P	P	-
strA	P	P	-
sitD	P	P	-
sitC	P	P	P
sitB	P	P	-
sitA	-	-	P
sav1886	-	-	-
sat1	-	-	-
qacEdelt1	-	-	P
pitA	P	P	P
perO	-	-	-
pduF	-	-	P
opnM			
nmpC	P	P	P
Omp36	-	-	-
mphA	-	-	-
mmR	-	-	-
merT	P	P	P
merR	P	P	P
merP	P	P	P
merG	-	-	-
merE	P	P	P
merD	P	P	P
merC	P	P	P
merA	P	P	P
marR	P	P	P
macB	P	P	P
macA	P	P	P
hpcR	P	P	-
gadA	P	P	P
ftr1	-	-	-
fmE	-	-	-
fpvA	-	-	-
floR	-	-	P
ygaA	P	P	P
evgS	P	P	P
envR	-	-	P
emrR	-	-	-
emrE	-	P	-
eefA	-	-	-
dfrA20	-	-	-
dfrAp17	P	P	-
dfraA1	-	-	-
bla_{CTX-M}	P	P	-
cphA1	-	-	-
catI	P	P	-
bla_{OXA-1}	-	-	P
bepC	-	-	-
arsP	-	-	-
arsD	-	-	P
arsA	-	-	P
aphAI	P	P	-
adeE	-	-	-
acn	P	P	P
aadA5	P	P	-
aadA1	-	-	P

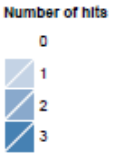


Figure 5-1: Heatmap for the differences in the predicted AMR genes and metal resistance genes between BCC2, BCE3 and BS7 strains.

The figure shows a modified heatmap (A) for AMR gene differences between 3 of the farm isolate *E. coli* strains (BCC2, BCE3 and BS7). The number of hits represents the number of orthologues found in each genome, so these are protein orthologues found in both CARD and within the predicted genes from each genome, white denotes absence of orthologues, a gradient blue colour represents (1-3) hits for presence of the orthologous protein. This heatmap was produced by Dr Lisa Crossman. (B) This part shows the results of the confirmation of the presence of these genes after searching the protein sequence for each of these predicted genes in the sequenced strains using the Artemis genomic browser tool. If the genes were present a further confirmation step was performed using the NCBI blast search tool (blastp) to confirm the gene ID. The presence of the confirmed genes is indicated by (P) and a red shaded colour, P: presence of the gene; -: absence of the gene. The locations, lengths and descriptions for the confirmed genes are listed in Appendix XV.

It can be seen from Figure 5.1A and B, that there are some genes which were predicted in the heatmap but which were not present in the tested strains when the confirmation analysis was performed. For example – *vatC* (virginiamycin A acetyltransferase). The percentages of the confirmed genes varies between the different strains, 49 genes were predicted to be present in BCC2 but only 67.3% of these genes were confirmed, whilst 50 genes were predicted to be present in BCE3 but only 68% were confirmed, and 46 genes were predicted to be present in BS7 but only 71.7% were confirmed to be present.

It can be seen from Figure 5.1A and B that each strain has multiple resistance genes that confer resistance to different antimicrobials including the β-lactam group, but also aminoglycoside, sulphonamide, phenicol and tetracycline resistances. In addition, many other genes are present which have a role in metal resistance such as *mer* (mercury) operon genes and *ars* (arsenic) genes. Many efflux pump genes, such as *mdtG*, also confer resistance to different antimicrobials, and are found within the genome of the sequenced strains.

The BCC2 and BCE3 strains have similar but not identical AMR genes, which reinforces the results of genotyping using ERIC-PCR as these 2 strains were shown to be 91% similar (Figure 3.6). BCE3 has *emrE* (multidrug transporter), while BCC2 does not (Figure 5.1). From Appendix XV it can be seen that the location and the orientation on the chromosome of the genes common between these two strains varies,

however these might not be the real locations and directions, see Section 5.9.

With regards to the AMR-related genes carried by BS7, there are some differences compared to the other 2 strains (BCC2 and BCE3). This was expected especially regarding the β-lactamase genes, as BCC2 and BCE3 are ESBL-producing strains while BS7 is not. Other AMR related genes were carried by BS7, but not carried by the other 2 strains, for example *sulI* (sulphonamide-resistant dihydropteroate synthase) and *qacEΔ1* (ethidium bromide and quaternary ammonium compound export protein), and vice versa some of the genes are carried by BCC2 and BCE3 but not by BS7 for example: *strA* and *strB* (streptomycin phospho-transferase), *dfrA17* (trimethoprim-resistant dihydrofolate reductase) and *catAI* (type A chloramphenicol O-acetyltransferase).

Regarding the AMR gene profile for A2.5; this strain is closer to BS7 than to BCC2 and BCE3 (Appendix XV). No ESBL genes were detected, but it carries the same bla_{OXA-1} as BS7. It also carries *floR* (chloramphenicol/florfenicol efflux MFS transporter) and *aadA1* (streptomycin adenyltransferase)*, qacEΔ1* and *sulI* (the same as BS7), but it carries *strA* and *strB* the same as BCC2 and BCE3. However, it has a unique profile regarding the *mer* genes, as it only has *merR* and lacks all the other *mer* genes present in the other strains.

This difference in the AMR gene profiles for BCC2 and BCE3 with that of BS7 and A2.5 reflects the difference at the genomic level found between

those strains using three typing methods (Figure 3.6, Sections 5.3 and 5.4).

In conclusion, confirming the presence of the predicted genes is very important. Firstly, it confirms the presence or the absence of these genes. For example, some genes such as *arsP* (the membrane transporter mediating resistance to organic arsenic), *vatC* and *sav1886* (a putative multidrug transporter), have been predicted to be present in the genome, but the confirmation step did not show that. Secondly, confirmation gives the exact variant types for the genes. For example; TEM-90 was predicted to be present in BCC2, BCE3 and BS7 giving the same possibility (1 hit for all), but the confirmation step showed the actual variant for this gene was $bla_{\text{TEM-1b}}$ for both BCC2 and BCE3 and $bla_{\text{TEM-30}}$ for BS7. The same case was seen with CTX-M-35, which was predicted to be present in BCC2 and BCE3, but the confirmation step showed it was $bla_{\text{CTX-M-14a}}$. However, for some other genes, such as *aadA1* and $bla_{\text{OXA-1}}$ in BS7, *aadA5* in BCC2 and BCE3, the variant of the genes was confirmed to be the same as that which had been predicted by the heatmap.

Analysis of the β-lactamase genes, including their genetic environment, will be described in the next section followed by the analysis for other genes responsible for the resistance against other groups of antimicrobials.

5.6 β-lactam resistance gene analysis

5.6.1 CTX-M region analysis

The *bla*$_{\text{CTX-M}}$ gene was present in only two strains: BCC2 and BCE3. The regions surrounding this gene were analysed to determine if the gene was carried by a transposable element. The variant type for both *bla*$_{\text{CTX-M}}$ genes was *bla*$_{\text{CTX-M-14}}$, which was confirmed by PCR typing for the gene (Section 4.2.4). However, the availability of the whole DNA sequence of the genes enabled further characterization of the variant type, as the sequence aligned with both *bla*$_{\text{CTX-M-14a}}$ (Accession no. AF252622.2) and *bla*$_{\text{CTX-M-14b}}$ (Accession no. DQ359215.1); these two variants differ by three nucleotides. For BCC2 and BCE3, 100% identity was achieved when the *bla*$_{\text{CTX-M-14}}$ genes from BCC2 and BCE3 was aligned with *bla*$_{\text{CTX-M-14a}}$. This is the predominant variant of *bla*$_{\text{CTX-M14}}$ (Valverde et al., 2009, Liao et al., 2015). However, *bla*$_{\text{CTX-M-14b}}$ carried on pSAM7, an IncX4 plasmid from *E. coli* from cattle, has been reported as well in the UK (Stokes et al., 2013).

In both strains the *bla*$_{\text{CTX-M-14a}}$ gene was located downstream of the transposable element IS*Ecp1* (Figure 5.2). A truncated IS*903B* (IS*903B'*) was located downstream of *bla*$_{\text{CTX-M-14a}}$. It seems that IS*Ecp1* carrying *bla*$_{\text{CTX-M-14a}}$ had inserted into the *fdeC* gene (intimin-like adhesin) and divided it into two parts, as one part of *fdeC* is located upstream of IS*Ecp1*, and the second part is located downstream of *bla*$_{\text{CTX-M-14a}}$. There are 42 base pairs between the IRR (inverted repeat right) for *ISEcp1* and the start codon of *bla*$_{\text{CTX-M-14a.}}$ This spacer has been found to be essential to confer high level resistance to cefotaxime (Ma et al., 2011). A putative

5 bp GGTTA DR (direct repeat) was found directly upstream of IRL (inverted repeat left) for IS*Ecp1* and at the junction between IS*903B*' and the second part of *fdeC*, suggesting that the putative IRR2 (inverted repeat right 2) might be the sequence upstream of the 5 bp DR (GGTTA) within the IS*903*` sequence (Figure 5.2). The carriage of *bla*$_{\text{CTX-M-14a}}$ by IS*Ecp1* with the presence of a complete IR and a putative IRR2 (alternative IR), may represent an active transposition unit. A similar arrangement of *bla*$_{\text{CTX-M-14}}$ with IS*Ecp1* and IS*903*`has been reported elsewhere in plasmid pKF3-70 from *K. pneumoniae*, which was thought to have originated in an R100-like plasmid from *E. coli* (Yi et al., 2010), but this transposable element was not inserted into the *fdeC* gene. The %GC content for IS*Ecp1* (33.9%) differs greatly to that of adjacent regions, and is much lower (60.5% for *bla*$_{\text{CTX-M14a}}$ and 52.38% for *fdeC*1) (Figure 5.3) which implies a different evolutionary origin, and this is generally recognised as suggesting it might have originated in a different host (Yi et al., 2010). The presence of *bla*$_{\text{CTX-M-14a}}$ located downstream of IS*Ecp1* has been reported widely. This mobile genetic element is carried on a pCT-like plasmid belonging to the IncK incompatibility group and has been isolated from cattle and humans in the United Kingdom (Table 1.2) (Cottell et al., 2011, Stokes et al., 2012).

Insertion of (IS*Ecp1*-*bla*$_{\text{CTX-M-14a}}$-IS*903B*') in the same place in the chromosome of both the BCC2 and BCE3 strains (which came from the same sample and are the same by 2 typing methods (MLST and serotyping) and 91% similar by ERIC-PCR typing) might suggest a common ancestry for both strains.

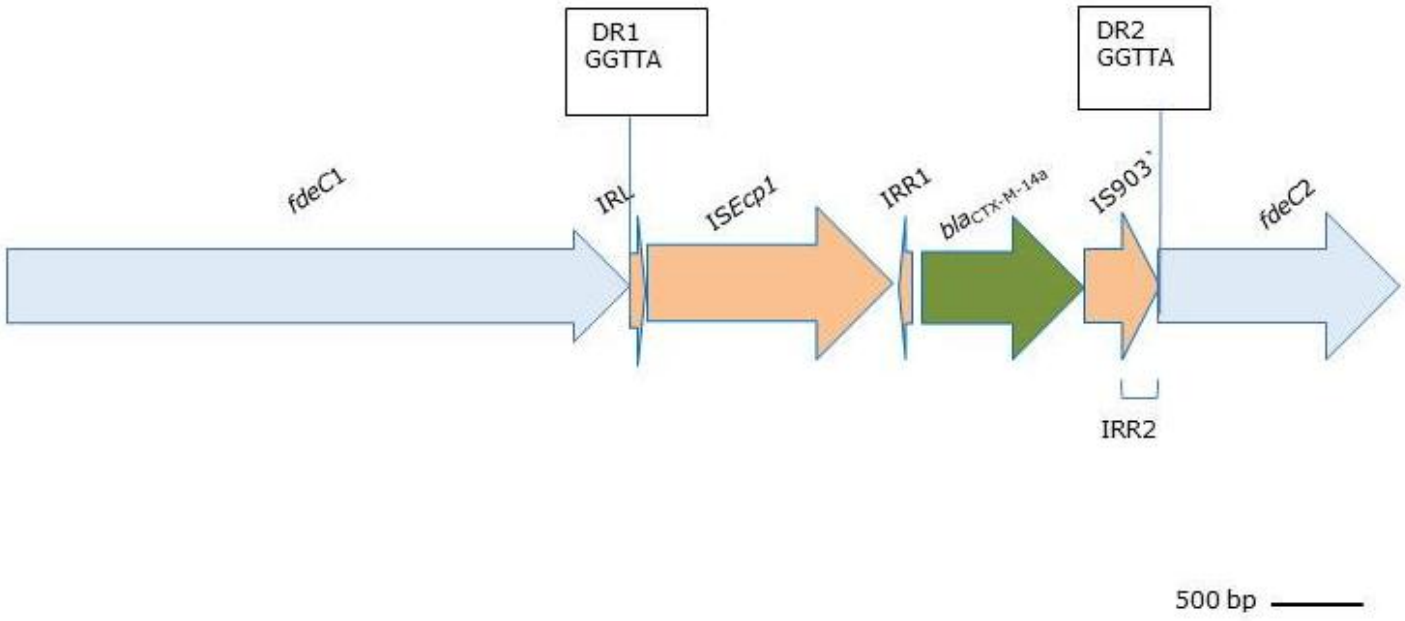


Figure 5-2: The *bla*$_{CTX-M-14a}$ region in BCC2 and BCE3 genomes.

IS*Ecp1;* insertion sequence elements*;* *bla*$_{CTX-M-14a}$: β-lactamase ; IS*903`*: insertion sequence element*;* IRL and IRR : inverted repeat left and right respectively; IRR2 is the putative right alternative IR for IS*Ecp1*; putative direct repeats (DR) are boxed above the Inverted Repeats; *fdeC* 1 and 2 : first and second part of *fdeC* (intimin like adhesion); green arrow indicates AMR genes, orange arrows indicate transposable elements; boxed arrows indicate the orientation of the gene; blue genes represent genes with other functions; all the elements in the figure are drawn to scale (500 bp) except for IRL and IRR due to their small length (14 bp).

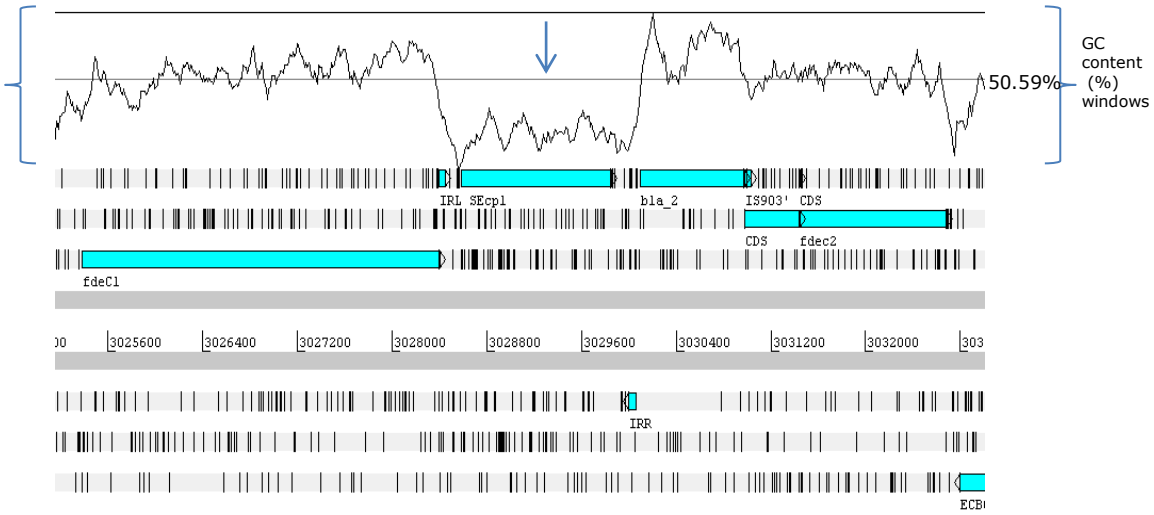


Figure 5-3: %GC content for IS*Ecp1* compared to the nearby region in BCC2.

There is a significant difference in the %GC content of IS*Ecp1* (33.9%) compared to the surrounding region (60.5% for bla-2 and 52.38% for *fdeC*1) which might indicate that it originated in a different host. Key: *fdeC*1 and *fdeC*2: first and second part of *fdeC* (intimin-like adhesion gene); bla-2: $bla_{CTX\text{-}M\text{-}14a}$ β-lactamase; IS*903*`: truncated insertion sequence element IS903; IS*Ecp1*: insertion sequence elements IS*Ecp1*; IRL and IRR: IS*Ecp1* inverted repeat left and right respectively. The figure was produced using the Artemis genomic browser, the upper panel (GC content windows surrounded by 2 brackets) is showing the GC content % graph for the viewed genes, the middle line which is indicated by arrow, is indicative for 50.59% of GC content.

5.6.2 *bla*$_{TEM}$ region analysis

All four MDR sequenced strains carried *bla*$_{TEM}$ genes. The *bla*$_{TEM}$ variant types from both the predicted protein sequence, and the nucleotide sequence were examined for all 4 strains. BCC2, BCE3 and A2.5 were known to carry this gene as it was detected by PCR (described in Section 4.2.4), so this analysis confirm the results that had been obtained previously from PCR typing of *bla*$_{TEM}$ variants. BS7 is not an ESBL strain but the sequence analysis showed that it carries *bla*$_{TEM}$ gene. The *bla*$_{TEM}$ type for the A2.5 strain was *bla*$_{TEM-1a}$, while BCC2 and BCE3 carry *bla*$_{TEM-1b}$; and BS7 carries *bla*$_{TEM-30}$. The difference between *bla*$_{TEM-1a}$ and *bla*$_{TEM-1b}$ is a substitution of 3 nucleotides at nucleotide positions: 226 -with C replaced by T (C=T), 436 -(C=T) and 604 -(G=T) (Goussard and Courvalin, 1999). The promoter type for all of the *bla*$_{TEM}$ genes was found to be P3, which is a weak promoter for *bla*$_{TEM}$ (Lartigue et al., 2002), and which has been previously found in many *bla*$_{TEM}$ variants (Brinas et al., 2002). The variant type and the promoter types for these genes might indicate that the expression of the *bla*$_{TEM}$ does not confer high level resistance to broad spectrum β-lactam antibiotics (Lartigue et al., 2002).

5.6.2.1 The *bla*$_{TEM}$ region in BCE3 and BCC2

The *bla*$_{TEM}$ region was analysed in both BCE3 and BCC2 to investigate if the *bla*$_{TEM}$ genes were carried by transposable elements. Tn*2* and Tn*3* are both composed of the *bla* gene, *tnpR* (resolvase) and *tnpA* (transposase), flanked by two 38 bp IRs. Tn*2* and Tn*3* are approximately 99% identical to each other over most of their 4.95 kbp length, but only share 83.1%

similarity in the resolution site (Partridge and Hall, 2005). Tn*2* carries *bla*$_{\text{TEM-1b}}$ and Tn*3* carries *bla*$_{\text{TEM-1a}}$ (Goussard and Courvalin, 1999). The DNA sequence of the transposon carrying *bla*$_{\text{TEM-1b}}$ in BCC2 and BCE3 was aligned with Tn*3* (GenBank accession no. V00613.1) (97% identity), and aligned with Tn*2* (GenBank accession no. AY123253.3) (100% identity) using BLAST alignment tool, and therefore it was concluded this was a Tn*2* transposon based on higher sequence homology. The Tn*2* transposon is located on one conting adjacent to the Tn*10* tetracycline resistance transposon in both BCC2 and BCE3 (Figure 5.4). Tn*2* found in BCC2 and BCE3 has also been found to be carried in many plasmids in different bacteria, such as pCAR10 in *Shigella dysenteriae* (GenBank accession no.: KT754161.1), pCAV1668-85 in *Enterobacter cloacae* (GenBank accession no.: CP011583.1) and the *bla*$_{\text{NDM-1}}$ plasmid in *Klebsiella pneumoniae* (GenBank accession no.: CP009116.1). The carriage of the *bla*$_{\text{TEM-1b}}$ on Tn*2* and adjacent to a Tn*10*, represents an MDR cluster in both strains; Tn*2* might represent an active unit transposon which is able to transfer *bla*$_{\text{TEM-1b}}$ intra- and inter- genomically.

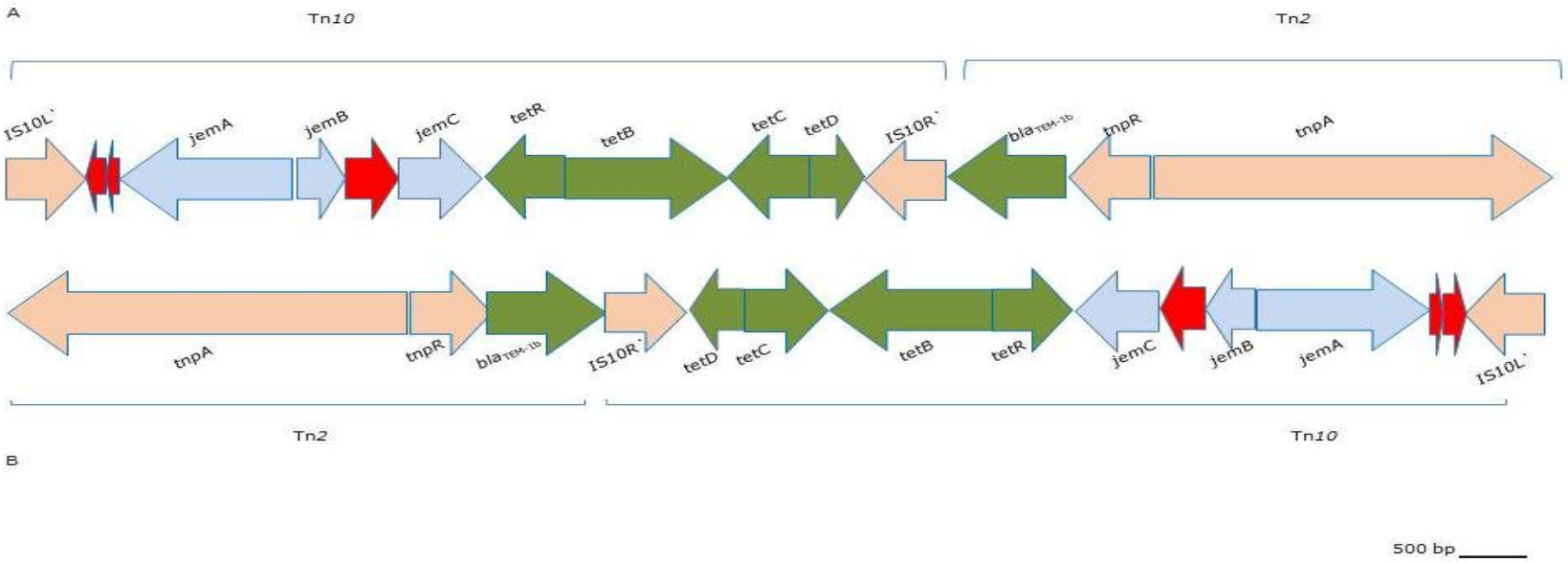


Figure 5-4: *bla*$_{\text{TEM-1b}}$ region in A) BCC2 and B) BCE3.

The figure shows *bla*$_{\text{TEM-1b}}$ carried by Tn*2*, and located adjacent to Tn*10* in BCC2 (A) and BCE3 (B). This represents a MDR cluster of 2 transposons carrying different MDR genes. Boxed arrows indicate the orientation of the gene; green arrows indicate AMR genes, orange arrows indicate transposition related elements, red arrows indicate hypothetical proteins within the Tn*10* transposon; *bla*$_{\text{TEM-b}}$: β-lactamase encoding gene; *tnpR* : resolvase; *tnpA*: transposase, IS*10*L` and IS*10*R`: truncated left and right insertion sequence for Tn*10*, respectively;.*tetB*: tetracycline resistance efflux pump; *tetR*: TetR family transcriptional regulator; *tetD* and *tetC*: transcription regulator genes in transposon Tn*10* which confer resistance to tetracycline ; *jemA* : sodium/glutamate symporter ; *jemB*: unknown function; *jemC*: ArsR (arsenic/antimony-responsive repressor) family transcriptional regulator; drawn to scale (500 bp).

5.6.2.2 *bla*$_{TEM}$ region in A2.5

The *bla*$_{TEM}$ region in A2.5 was examined to investigate if it was also carried by transposable elements. From sequence analysis the variant is *bla*$_{TEM-1a}$ which is located between *repB* and Tn*3* IRL, but no other components of the Tn*3* transposon are present. The *repB* gene has 100% identity with the RepB replication protein in *Streptococcus agalactiae* and *Bacillus subtilis* (Figures 5.5 and 5.8). The RepB sequence in A2.5 has a motif3 conserved sequence such as is found in pMV158 (NVENMYLYLTHESKD) (Del Solar et al., 1998). This broad host range plasmid replicon is the prototype for a family of replicons isolated from Gram-positive and Gram-negative bacteria (Solar et al., 1993). Plasmid pMV158, and its derivative pLS1, replicate and expresse tetracycline resistance in *Streptococcus pneumoniae*, *Bacillus subtilis* and *E. coli* (Lacks et al., 1986). In A2.5, the tetracycline resistance gene (*tetL*) was located on the same conting with *repB* and *bla*$_{TEM-1a}$, and it has a 99% identity with the tetracycline resistance MFS efflux pump in *Enterococcus*, *Staphylococcus* and *Bacillus*, which are the same features described before for pLS1 (Lacks et al., 1986). There is no evidence that the *bla*$_{TEM}$ gene in A2.5 was carried by a plasmid similar to pMV158 and to my knowledge such a case has not been reported before, but at the same time the opposite conclusion cannot be confirmed especially with the presence of *repB* and *tetL* adjacent to *bla*$_{TEM-1a}$. The failure to amplify the A2.5 *bla*$_{TEM}$ gene by PCR in experiments to confirm the genome

sequencing might indicate the presence of this gene on an unstable plasmid.

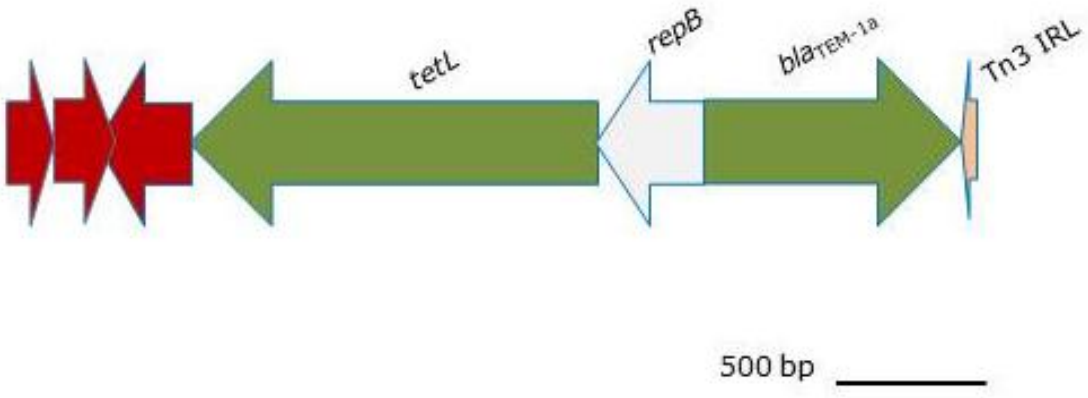


Figure 5-5: The *bla*$_{TEM}$ gene region in A2.5.

Boxed arrows indicate the orientation of the gene; green arrows indicate AMR genes, orange arrow indicates transposition related element; red arrows indicate a hypothetical protein; the *bla*$_{TEM-1a}$ (β-lactamase) is flanked by a Tn*3* IRL (inverted repeat left) and the *repB* (replication protein B); *tetL* is the tetracycline efflux pump; all the elements in the figure were drawn to scale (500 bp) except for IRL due to the small length (38 bp).

5.6.2.3 *bla*$_{TEM}$ region in BS7

As for the other *bla*$_{TEM}$ genes, the region surrounding *bla*$_{TEM}$ in BS7 was examined to see if the gene is carried by a transposable element. BS7 carries a different *bla*$_{TEM}$ variant (*bla*$_{TEM-30}$) compared to the other strains. This variant differs from *bla*$_{TEM-1}$ by a substitution at amino acid 241, where arginine is replaced by serine. This confers resistance to a β-lactam inhibitor (clavulanic acid) (Belaaouaj et al., 1994). *bla*$_{TEM-30}$ in BS7 is carried on a Tn*3*-like transposon. The transposon has 99% similarity with Tn*3* (GenBank accession no. V00613.1) and 97% similarity with Tn*2* (Genbank accession no. AY123253.3) and is classed as a Tn*3* transposon based on this greater level of similarity.

In BS7, transposon Tn*3* seems to have inserted into a mercury resistance, *mer,* operon as the Tn*3* transposon has interrupted *merP*. There are 2 regions of *merP* in BS7, each region carrying a part of the gene and these are found upstream and downstream of Tn*3*, which carries *bla*$_{TEM-30}$ (Figures 5.6, 5.7 and 5.8). This unique arrangement might indicate that gene acquisition has occurred in this strain. The carriage of *bla*$_{TEM-30}$ by this transposon might increase the possibility of transfer of this gene both inter- and intra- genomically, thus increasing the possibility of the transfer via HGT.

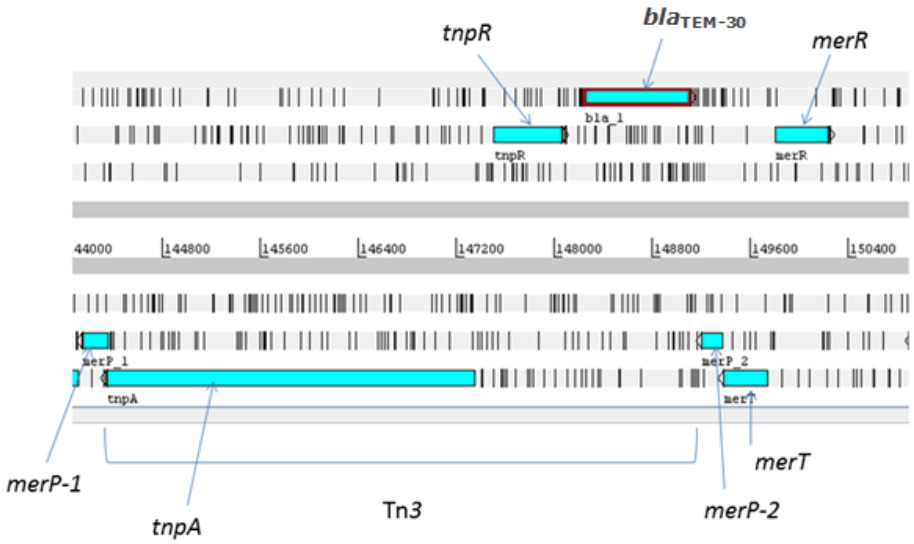


Figure 5-6: The *bla*$_{TEM-30}$ region in BS7.

The *bla*$_{TEM-30}$ gene is carried by a Tn*3*-like transposon, which also contains *tnpR* (resolvase) and *tnpA* (transposase), The Tn*3* region is indicated by the bracket; Tn*3* interrupted *merP* (mercuric transport protein periplasmic component) into 2 parts, *merp-1* and *merp-2; merR:* mercuric resistance operon regulatory protein; *merT*: the mercuric transport protein.

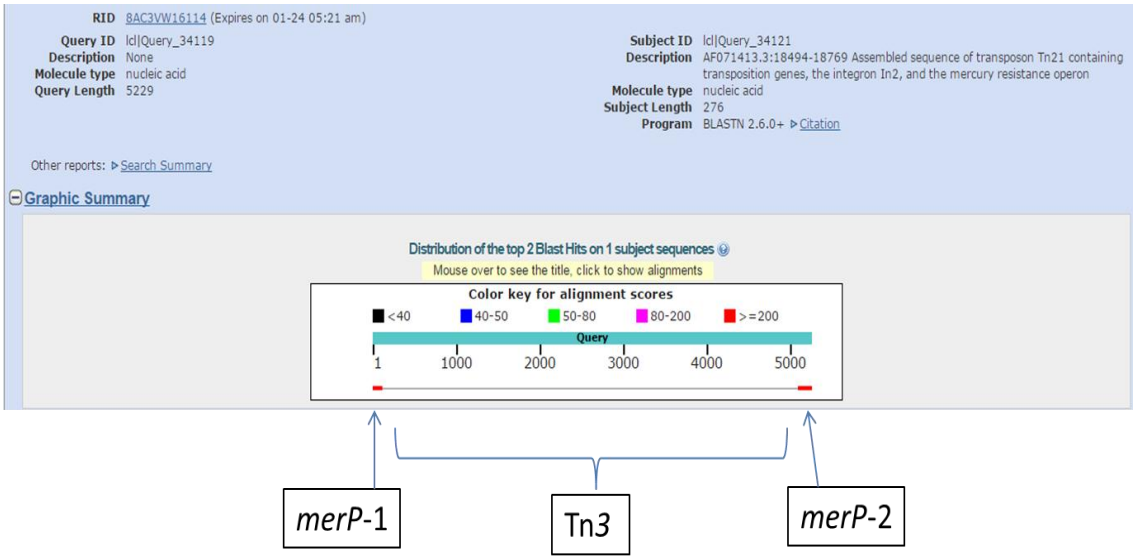


Figure 5-7: Alignment of the *merP-1* and *merP-2* region including the Tn*3* region with a complete *merP* nucleotide sequence from *E. coli* (Accession no: AF071413.3) using BLASTN alignment tool.

The query length shown is 5229 nucleotides, which is the length of the region starting from *merP-2* (168 nucleotides of which 115 nucleotides are for *merP*) to *merP-1* (207 nucleotides of which 169 nucleotides are for *merP*) in BS7; the subject length is 276 nucleotides which is the complete *merP* nucleotide sequence from *E. coli* (accession no: AF071413.3); the 2 parts of *merP* are the red line regions from the alignment, which are separated into 2 distinct areas due to the insertion of Tn*3* (transposon)*; merP* is the periplasmic mercuric transport protein.

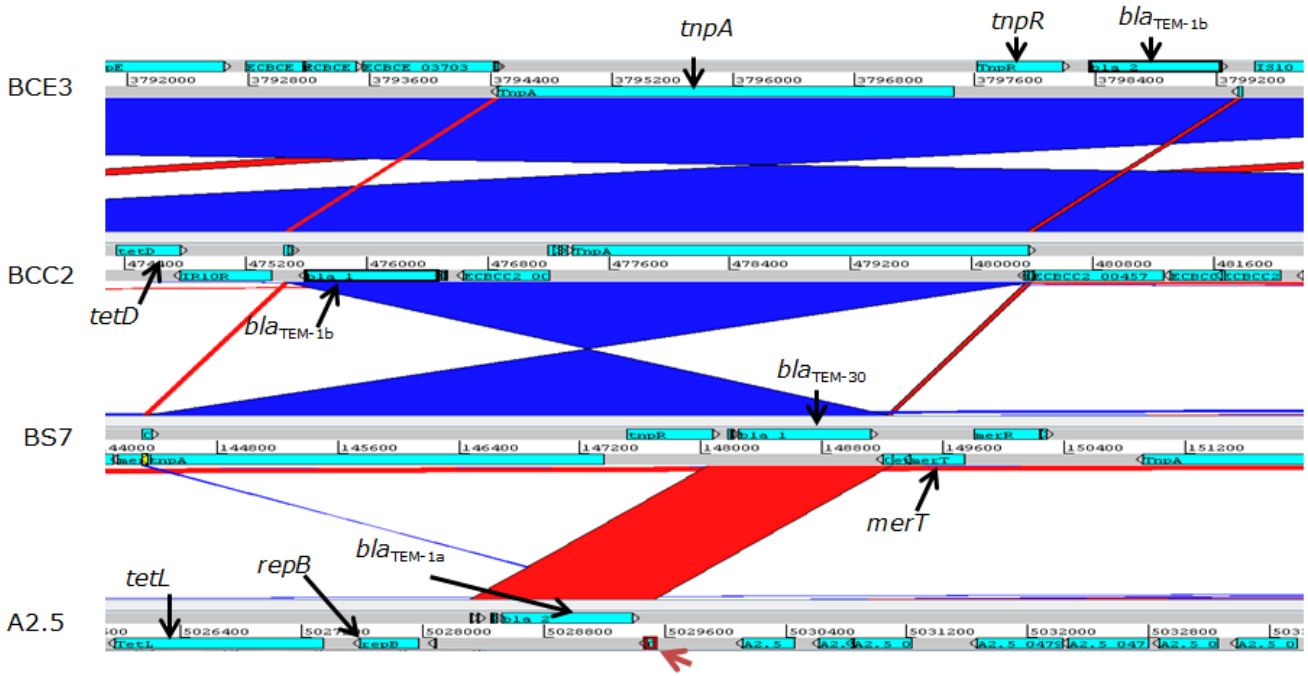


Figure 5-8: Sequence alignment comparison between BCE3, BCC2, BS7 and A2.5 *bla*$_{TEM}$ regions using the Artemis Comparison Tool (ACT).

The figure shows the environment of each *bla*$_{TEM}$ gene in BCE3, BCC2, BS7 and A2.5, and compares them using the sequence of each strain. The blue regions indicate DNA homology, but the gene order is in the reverse orientation compared to the gene region it is being aligned to; the red area indicates homology with the gene in the same direction to the gene it is being aligned to, whilst the white area indicates no DNA homology. The order of strains from the top is BCE3, BCC2, BS7 and A2.5; *bla*$_{TEM}$ variant types are labelled and shown on the figure. The *bla*$_{TEM-1b}$ genes in BCE3 and BCC2 are both carried by Tn*2* and share homology, but the gene order is in the reverse orientation. The *bla*$_{TEM-30}$ gene in BS7 is carried by Tn*3* which shares homology with Tn*2* from BCC2, but the gene order is in the reverse orientation. The *bla*$_{TEM-1a}$ gene in A2.5 shares the same orientation with *bla*$_{TEM-30}$ but is found in a completely different environment. *bla*$_{TEM-}$

$_{1a}$ gene in A2.5 is flanked by Tn*3* IRL (inverted repeat left) indicated by red arrow on the right side it, the other Tn*3* elements are missing or might not be covered by sequencing. Key: Tn*3* and Tn*2*- transposon, composed of *tnpR* (resolvase) and *tnpA* (transposase) and bla_{TEM} (β-lactamase) flanked by two IR; *merR:* mercuric resistance operon regulatory protein; *merT*: mercuric transport protein; *tetD*: transcription regulator genes in transposon Tn*10* which confer resistance to tetracycline; *tetL*: tetracycline efflux pump; *repB*: replication protein.

5.6.3 Analysis of the *bla*$_{OXA-1}$ region in strains A2.5 and BS7

The *bla*$_{OXA-1}$ gene sequence was present in only two strains which are A2.5 and BS7. The region surrounding this gene was analysed to check for carriage by a transposable element. In A2.5 the *bla*$_{OXA-1}$ gene is located in an In*2*-like integron with an *aadA1* gene in the same gene cassette (Figures 5.9 and 5.10). The In*2*-like integron is located within a Tn*21*–like mercury resistance transposon (Section 1.6.3 and Appendix XVI). The evidence that the *bla*$_{OXA-1}$ gene is carried in the same gene cassette as *aadA1* is shown by the complete *attI*1 site adjacent to *bla*$_{OXA-1}$ (Partridge et al., 2000), this is the case with *bla*$_{OXA-1}$ in A2.5. The same genetic organization surrounding *bla*$_{OXA-1}$ is found in BS7. The *bla*$_{OXA-1}$ gene is located within the same gene cassette with *aadA1*; the *attI*1 site is located upstream of *bla*$_{OXA-1}$ and the *attC* site is located downstream of *aadA1*. This gene cassette is also located on an In*2*-like integron on a Tn*21*–like transposon region, as in A2.5 (Figures 5.9 and 5.10). There are two promoters for this gene cassette, one that lies within the integrase gene (Pc), and another downstream of the integrase gene (P2). The promoter strength varies depending on the promoter variant (Jové et al., 2010). Both *bla*$_{OXA-1}$ genes have the weak (Pc) promoter which is (PcW). With regard to the second promoter (P2), the *bla*$_{OXA-1}$ gene in BS7 has the normal P2 promoter, while A2.5 has the strong promoter (P2m3) which is mutated from P2, as there is a G→A mutation in the -10 element of this promoter, and this doubles the gene expression (Vinué et al., 2011). The class I integron carrying this gene cassette arrangement has been described before in *E. coli* from cattle at a low level of occurrence (2%) in 100 isolates (Karczmarczyk et al., 2011).

The same arrangement has been identified in *Salmonella* from both food and human sources (Antunes et al., 2004). The carriage of bla_{OXA-1} and *aadA1* with other AMR genes (Figure 5.9) by a Tn*21*-like transposon, could facilitate the transfer of these genes inter- and intra-genomically, thus increasing the possibility of its transfer via HGT. A2.5 was isolated in October 2012 and BS7 was isolated in March 2014. The presence of a class I integron with the same gene cassettes and the carriage of this integron by a Tn*21*-like transposon, might represent the potential on-going circulation of this gene cluster in the *E. coli* community in the farm. However a more structured survey would be needed to investigate this possibility.

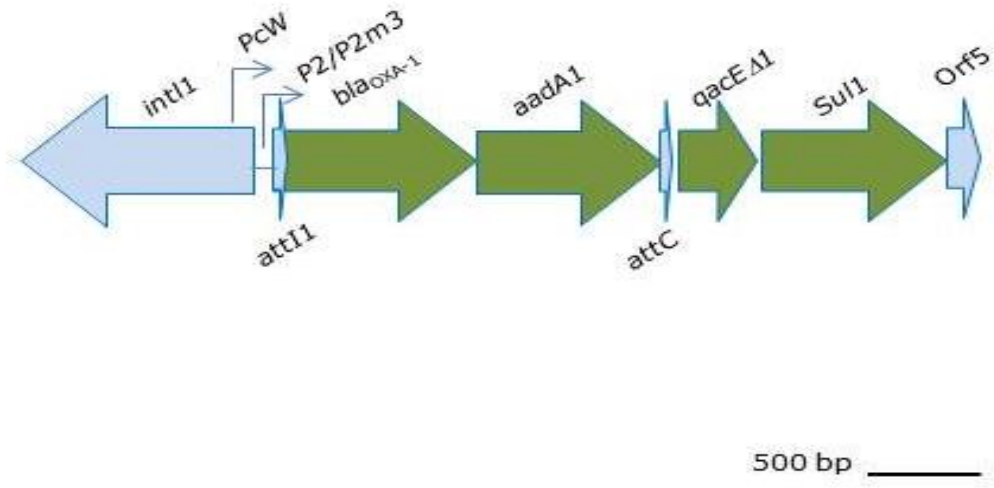


Figure 5-9: The *bla*$_{OXA-1}$ region in A2.5 and BS7

bla$_{OXA-1}$ located in a class I integron in both BS7 and A2.5. Boxed arrows indicate the orientation of the gene; green arrows indicate AMR genes, *intI1*: integrase gene; PcW and P2/P2m3: are gene cassette promoters (BS7 has P2 while A2.5 has P2m3); *attI*1: site-specific recombination site; *attC:* specific cassette recombination site; *Orf*5: gene of unknown function; *bla*$_{OXA-1}$: β-lactamase OXA-1; *aadA1*: streptomycin adenylyltransferase; *sul1*: sulphonamide-resistant dihydropteroate synthase; *qacEΔ1:* QacE family quaternary ammonium compound efflux*;* drawn to scale (500 bp) except for *attI1* due to the small length (38 bp).

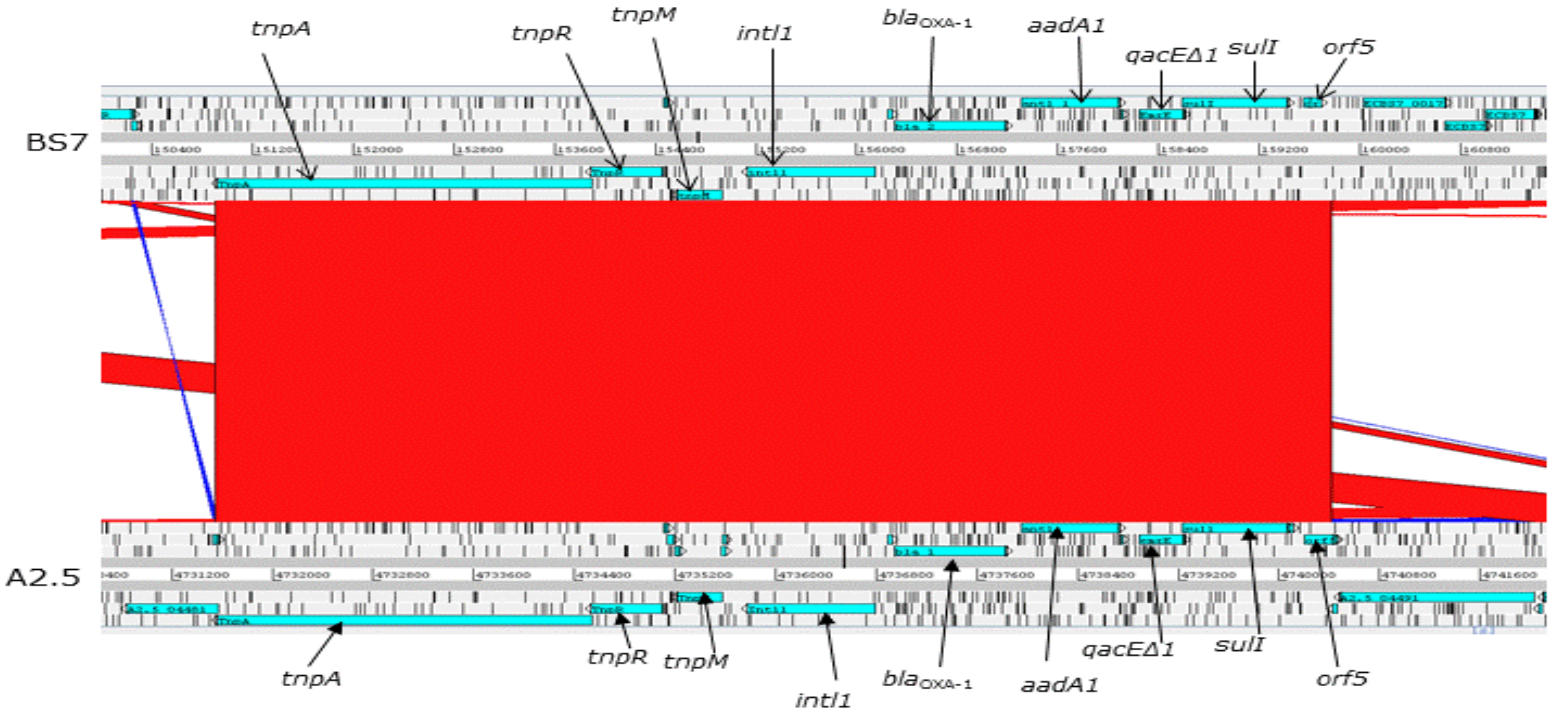


Figure 5-10: Sequence alignment comparison between BS7 and A2.5 *bla*$_{OXA-1}$ region using Artemis Comparison Tool.

Figure showing the region of homology between the integrons from BS7 (on the top) and A2.5 (on the bottom). The region of homology starts from *tnpA* to *orf5* and surrounds *bla*$_{OXA-1}$. The presence of this cluster in 2 strains isolated approximately 1.5 years apart suggests the potential on-going circulation of this gene cluster. The red area indicates homology with the genes being in the same orientation; the blue regions indicate DNA homology but the gene order is in the reverse orientation; the white area indicates no DNA homology; *bla*$_{OXA-1}$: β-lactamase OXA-1; *aadA1*: streptomycin adenylyltransferase; *sul1*: sulphonamide-resistant dihydropteroate synthase; *qacEΔ1:* QacE. family quaternary ammonium compound efflux; *intI1*: integrase gene; *orf*5: gene of unknown function: *tnpM*: transposase modulator; *tnpR*: resolvase; *tnpA*: transposase.

5.7 Analysis of other AMR genes

As there are many AMR genes within the genome of each strain, an illustration will be given of the arrangement of these resistance genes for selected examples providing resistance to different groups of antimicrobials in the four strains. The gene type, gene description, and the location have been listed for all the genes in Appendix XV. The purpose of this analysis is to investigate the possible carriage of these genes on a transposable element, so as to evaluate the possibility of their transfer to other bacteria from different genera or species within the farm bacterial community. This analysis includes AMR genes for aminoglycoside, sulphonamide and trimethoprim, phenicol and tetracycline classes of antimicrobials.

5.7.1 Aminoglycoside resistance genes

Resistance to streptomycin has been identified widely in the *E. coli* isolates in this study (39.6% were resistant to streptomycin). All the sequenced strains phenotypically showed resistance to streptomycin. To check whether aminoglycoside resistance genes are carried on transposable elements, the genomic environment for some of these genes was analysed. BCE3 and BCC2 have 4 genes which confer resistance to aminoglycosides; they both carry *strA*, *strB*, *aphAI* and *aadA5*. BS7 carries 2 copies of *aadA1*, while A2.5 carries *strA*, *strB* and *aadA1*.

One of the important resistance gene clusters that is found in Enterobacteriaceae is the IS26-*repA*-*repC*-*sul2*-*strA*-*strB*-IS26 cluster (Carattoli et al., 2014). This resistance gene cluster was found in BCC2 and BCE3 but in different orientations with regards to the location of the genes

on the forward and reverse DNA strand (Figure 5.11A and B) however, this location and orientation of these genes in the genome sequence might not be the same as physically found in the chromosome, see section 5.9. The *repA-repC-sul-strA-strB*- cluster has been found on the IncQ plasmid, RSF1010, a broad host non-conjugative plasmid from *Erwinia* (Palmer et al., 1997) and on the same plasmid in *E. coli* (EMBL accession no. M28829). This cluster has been shown to be mobilized by IS*26* (including a part of the IncQ1 replicon of the RSF1010 plasmid) as part of a composite transposon (Miriagou et al., 2006, Carattoli et al., 2014). This cluster has also been found on both the chromosome and in a plasmid in both *E. coli* and *Salmonella* (Chowdhury et al., 2015, Reid et al., 2015). This indicates the prevalence of this cluster within *E. coli* and other Enterobacteriaceae.

IS*26*-*aphA1*-IS*26* is a composite transposon carrying an AMR gene, and was found in BCC2 and BCE3, (Figure 5.11E). The *aphA1* gene conveys kanamycin resistance and has previously been found located within transposons Tn*2680*, Tn*4352* and Tn*4352*-derivatives, inserted between two copies in directly repeated orientation of IS*26* or IS*26*-like elements, and is a prevalent kanamycin resistance gene in Gram-negative species (Mollet et al., 1983, Mollet et al., 1985, Harmer and Hall, 2015). The DNA sequence of this transposon aligned with the sequence of Tn*4352* (GenBank: HM999791.1) and was a 100% identical to this transposon. The complete sequence of this transposon in both BCC2 and BCE3 strains might indicate an active composite transponson which might facilitate *aphA1* transfer between *E. coli* and other enterobacteria. However a more structured survey would be needed to investigate this possibility, because

BCC2 and BCE3 are just two strains within an *E. coli*/enterobacterial community.

The *aadA5* gene was located with *dfrA*17 in a class 1 integron in aTn*21*-like transposon in BCC2 and BCE3 (Figure 5.11 C & D, Figure 5.15 and Appendix XVI). The *dfrA17* gene confers trimethoprim resistance (Roberts et al., 2012) and *aadA5* confers spectinomycin resistance; such a cassette has been described recently in a class 1 integron in ESBL-producing *E. coli* isolated from diverse dairy farms in China (Ali et al., 2016). A class 1 integron carrying a gene cassette containing *aadA*5 and *dfrA*17 was found to be present in 38% of 139 trimethoprim/sulfamethoxazole resistant uropathogenic *E. coli* strains from women from a California community college (Solberg et al., 2006). This combination has been found in a clonal group of *E. coli* strains. The *dfrA17-aadA5* integron arrangement in the previous study was shown to have 100% sequence identity with that of *dfrA17-aadA5* from cow isolates (isolated in 1999) (Solberg et al., 2006). Class 1 integrons carrying the same gene cassette have also been reported in *E. coli* isolated from UTI infections from humans and dogs (Cocchi et al., 2007). These findings indicate the spread of this gene cassette carried by class I integrons among different hosts. As shown in Figure 5.11C and D, in both BCE3 and BCC2, this gene cassette was located between IS*26* and *intI1*. The 3`-conserved segment of a class I integron (including *qacE*Δ*1*, *sul1* and *orf5*; Figure 1.7) was lost and this is thought to be due to the insertion of IS*26*. This case has been reported widely before, with many different gene cassettes found within a class I integron (Dawes et al., 2010). In BCC2 there is insertion of 2 copies of IS*26* in the 3`-conserved

segment instead of one, which is different from the arrangement of a class I integron described previously in the literature. To my knowledge, this is the first time such an arrangement has been described. However, this should be confirmed by other methods, see Section 5.9, as each of the IS*26* elements was located on separate contigs, but both within the Tn*21*-like transposon. Integrons themselves are not mobile; however they have an important role in the emergence of multi-resistant bacteria. They are often carried by mobilizing elements such as conjugative plasmids, transposons or phage, which ensure their horizontal transfer (Toussaint and Merlin, 2002). The capture of AMR genes by IS-mediated recombination or by class I integrons can explain the successful dissemination of these resistance genes in the environment. The integron found in BCE3 and BCC2 is carried by a Tn*21*-like transposon and carriage on this transposon could increase their inter- and intra-genomic mobility.

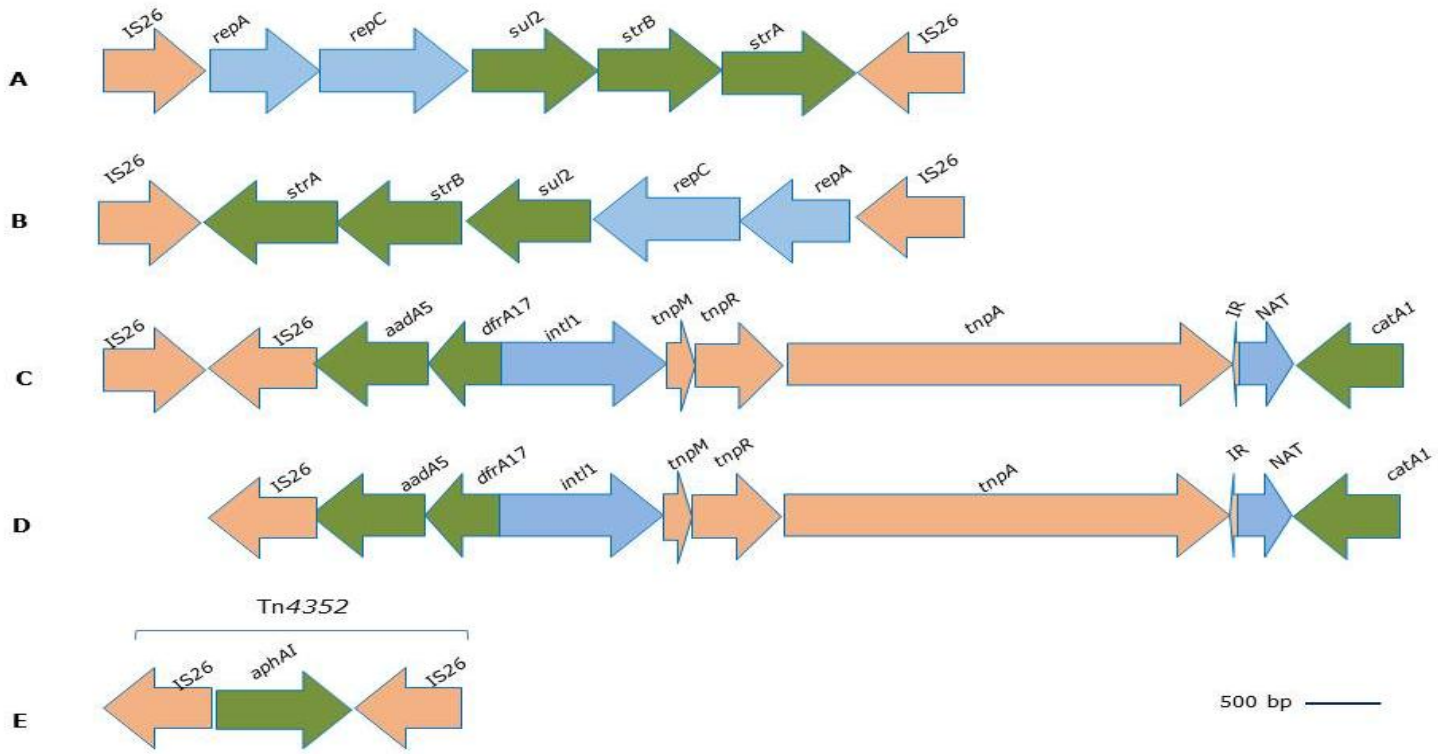


Figure 5-11: Some AMR gene arrangements in BCC2 and BCE3.

This figure shows different AMR genes associated with mobile element IS*26*. **A**: *strA*, *strB*, *sul*2 cluster in BCE3; **B**: *str*A, *str*B, *sul*2 cluster arrangement in BCC2; **C**: *aadA*5, *dfrA*17 and *catA1* arrangement in BCC2; **D**: *aadA*5, *dfrA*17 and *catA1* arrangement in BCE3; **E**: Tn*4352* (transposon) structure in BCE3 and BCC2. Key: boxed arrows indicate the orientation of the gene; green arrows indicate AMR genes, orange arrows indicate transposition relative elements; blue arrows indicate other genes; IS*26*: insertion sequence; *repA* and *repC*: replication regulatory proteins; *sul2*: sulphonamide-resistant dihydropteroate synthase; *strA* and *strB* : streptomycin phosphotransferase; *aadA5:* aminoglycoside adenyltransferase; *dfrA17:* dihydrofolate reductase; *tnpM*: transposase modulator; *tnpR*: resolvase; *tnpA*: transposase; *intI*1: integrase gene; NAT: N-acetyltransferase; *catA1:* chloramphenicol O-acetyltransferase; *aphAI:* aminoglycoside 3'-phosphotransferase; drawn to scale (500 bp) except for IR (Tn*21* inverted repeat) due to the small lengths (38 bp).

5.7.2 Sulphonamide and trimethoprim resistance genes

All of the sequenced strains showed phenotypic resistance to sulphonamides, and carried recognised genes (*sul*1 and/or *sul*2) for antimicrobial-resistant forms of the enzyme dihydropteroate synthase, which catalyses the conversion of *p*-aminobenzoic acid to dihydrofolic acid in the folic acid pathway (Sköld, 2001); *sul*2 was found in all four strains and *sul*1 in BS7 and A2.5 (Appendix XV). Analysis of the genetic context of these genes was performed to determine if they are carried by transposable elements. There are two copies of the *sul*1 genes in BS7 and one copy in A2.5; one of the copies in BS7 (158590-159429 bp, Appendix XV) and the one in A2.5 are both located in In*2* within the Tn*21*-like transposon (Figure 5.9).

In BCE3 and BCC2, the *sul*2 gene was clustered with *strA* and *strB* within a potential transposable complex as described in Section 5.7.1, (Figure 5.11 A and B). In the same strains (BCC2 and BCE3) the *dfrA17* gene which confers resistance to trimethoprim was located in a class I integron as described in Section 5.7.1.

In BS7 the *sul*2 gene was located downstream of a truncated IS*CR*2 (*orfA* or IS*Vsa*3) which belongs to the IS*91* family (Section 5.9) (Siguier et al., 2006). The *dfr* gene (dihydrofolate reductase), a transcriptional regulatory protein (Rrf2), and a phosphoglucosamine mutase (*glmM*), were located between *sul*2 and IS*CR*2 (Figure 5.12 A). The NCBI blastp search tool shows that the *dfr* gene shares 100% homology with a dihydrofolate reductase from *E. coli* MS 84-1 (accession no. EFJ89354.1)

and it shares 49% homology with *dfrA20* (a variant which confers resistance to trimethoprim) from *Pasteurella multocida* (Accession no. WP_ 03249 118 1.1; Appendix XVII). The same *dfr* gene was found in A2.5 (which shows phenotypic resistance to trimethoprim/ sulfamethoxazole) upstream of a transcriptional regulatory protein (Rrf2) on a small contig. IS*CR2* is often associated with the *sul*2 gene, and is also found carrying *dfr* genes such as *dfrA*9 and *dfrA*20 (both known gene variants which produce an altered enzyme which confers resistance to trimethoprim) and chloramphenicol/florfenicol resistance gene on many plasmids (Toleman et al., 2006). This MGE has an important role in the capture and mobilization of antibiotic resistance genes found in Gram-negative bacteria (Partridge, 2011).

In A2.5, *sul*2 is located on a small conting adjacent to the *czcA* and *czcB* (cobalt-zinc-cadmium) resistance genes, but there is no evidence of the presence of a transposable element close to these genes. The membrane-bound CzcCBA protein complex mediates heavy metal resistance (Rensing et al., 1997), but only *czcA* and *czcB* were found in strain A2.5; *czcC* was not present, (Figure 5.12 B). This two-gene system (*czcAB*) with *sul*2 has been found to be carried on the EC869 phage from *E. coli* EC869, a bovine-associated *E. coli* O157:H7 isolate (Eppinger et al., 2011). So these genes potentially could be carried by a similar prophage. However, the contigs that carried these genes are very small and such a suggestion would need to be confirmed through completing the gaps in sequencing using PCR.

It is clear from what has been described above that all the examples of the genes associated with sulphonamide resistance are carried by different transposable elements depending on the strains, except for *sul*2 in A2.5 where it was not possible to confirm if it was carried by a transposable element or not. This carriage associated with transposable elements could explain the prevalence of the resistance to sulphonamides carried by 38.8% of the isolates in this study.

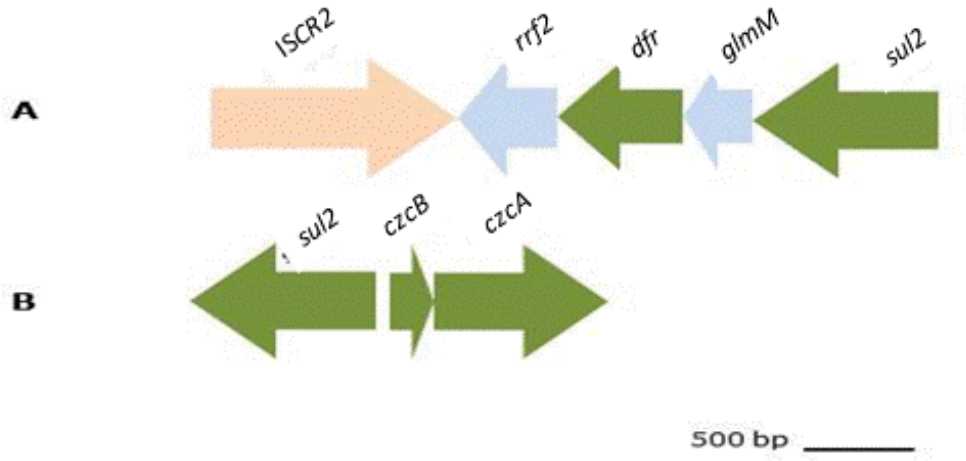


Figure 5-12: Arrangement of *sul*2 in BS7 (A) and in A2.5 (B).

The figure shows *sul2* and *dfr* genes associated with mobile element IS*CR2* in (A) and a unique arrangement of *czcA* and *czcB* which is only present on A2.5 in (B). Boxed arrows indicate the orientation of the gene; green arrows indicate AMR genes, orange arrows indicate transposition related elements; blue arrows indicate other genes; grey arrows indicate hypothetical proteins**.** IS*CR2*: insertion sequence from the IS*CR* family; *rrf2*: is a transcriptional regulator gene; *dfr:* dihydrofolate reductase; *glmM*: phosphoglucosamine mutase; *sul2*: sulphonamide-resistant dihydropteroate synthase; *czcA* and *czcB*: cobalt-zinc-cadmium resistance genes. ; Figure drawn to scale (500 bp).

5.7.3 Phenicol resistance

Two types of chloramphenicol resistance genes were detected in the sequenced strains: chloramphenicol acetyltransferase, *catA1,* and *floR* which confer resistance to florfenicol (exclusively in veterinary use) and chloramphenicol. To check if these genes were carried on transposable elements, the region surrounding the genes was analysed in the sequenced *E. coli* isolates.

A2.5 and BS7 were found to have a *floR* gene, and the *floR* region in BS7 was chosen for the analysis. In BS7 the gene was located upstream of a putative transposase and a LysR-like transcriptional regulator, and downstream of a type VI secretion protein (T6ss). The transposase type was found to be IS*CR*2 (IS*Vsa*3) from the IS*90* family (Figure 5.13). In *E. coli*, both plasmid and chromosomal locations of *floR* gene are known (Doublet et al., 2002, Schwarz et al., 2004, Doublet et al., 2005).

In Doublet et al., (2002) study, twenty-two bovine *E. coli* from France and Germany were examined for their chromosomal region containing *floR* and compared to that in a plasmid. The DNA sequences of the *floR*-flanking regions were found to be variable, and the variable RFLPs of *floR* gene-carrying restriction fragments suggested variable chromosomal integration sites (Doublet et al., 2002). *floR* carried on conjugative plasmids has been reported before in *E. coli* and *Salmonella* from cattle. This gene has been found to be flanked by two IS*CR*2, of which one was truncated (Cloeckaert et al., 2000).

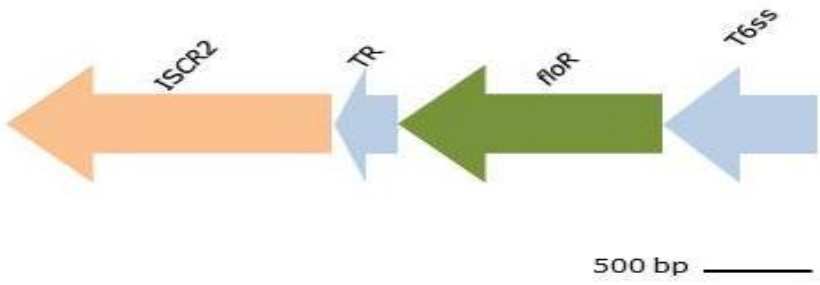


Figure 5-13: The organization of the *floR* gene cluster in BS7.

The figure shows *floR* gene associated with IS*CR2*. Boxed arrows indicate the orientation of the gene; a green arrow indicates an AMR gene, blue arrows indicate other genes; an orange arrow indicates transposition related elements; IS*CR2*: insertion sequence from IS*CR* family; *floR*: chloramphenicol/florfenicol efflux; TR: LysR-like transcriptional regulator; T6ss encodes for type 6 secretion system proteins, figure drawn to scale (500 bp).

IS*CR2* was also found to be associated with *floR* found on plasmids in *E. coli* isolated from pigs (Blickwede and Schwarz, 2004). This might indicate the ability of this kind of insertion element to transfer *floR* between the chromosome and a plasmid, and, as illustrated in Section 5.7.2, this MGE may contribute to the mobilization of different AMR genes. This could explain its dissemination in several bacterial species from different animal hosts.

Both BCC2 and BCE3 carry *catA1*, which belongs to the chloramphenicol acetyltransferase family, which can inactivate chloramphenicol. In BCC2 and BCE3, this gene is located adjacent to the IR for a Tn*21*-like

transposon (from the *tnp* side) separated by an *N*-acetyltransferase gene (Figures 5.11C and D). In NR1 (R100) plasmid, *catA1* has been found to be adjacent to a Tn*21* transposon, which was the original 1950s Tn*21* isolate from Japan (Figure 5.14). Using PlasmidFinder (which is a website for in silico plasmid detection and characterization of whole-genome sequence data from Enterobacteriaceae) (Carattoli et al., 2014) to analyse the BCC2 genome sequence indicates the presence of an IncFII plasmid within this strain, due to 95% sequence similarity to the IncFII RepA protein which is a replication initiation protein (Appendix XVIII). *repA* gene was located with other genes such as *repB, finO* (conjugal transfer fertility inhibition gene) and *tra* (the transfer genes) elements upstream of an IS*1b* transposase, which is located directly upstream of the terminal IR for a Tn*21*-like transposon. The sequence alignment between the *catA1* region and the surrounding environment (including a Tn*21*-like transposon region) with that of plasmid R100 (94.2 kbp in length) from *Shigella flexneri* (accession no. AP000342.1) was compared using the Artemis Comparison Tool (ACT) (Figure 5.15), with large areas of homology observed between both regions. Due to this, the presence of *catA1* with the other resistance genes carried by a Tn*21*-like transposon is very likely to be carried by an NR1-like plasmid. If this is so, the transfer of such a large AMR cluster between different *E. coli* strains and other species is very likely to occur, which might increases the possibility of the transmission of these genes to the environment.

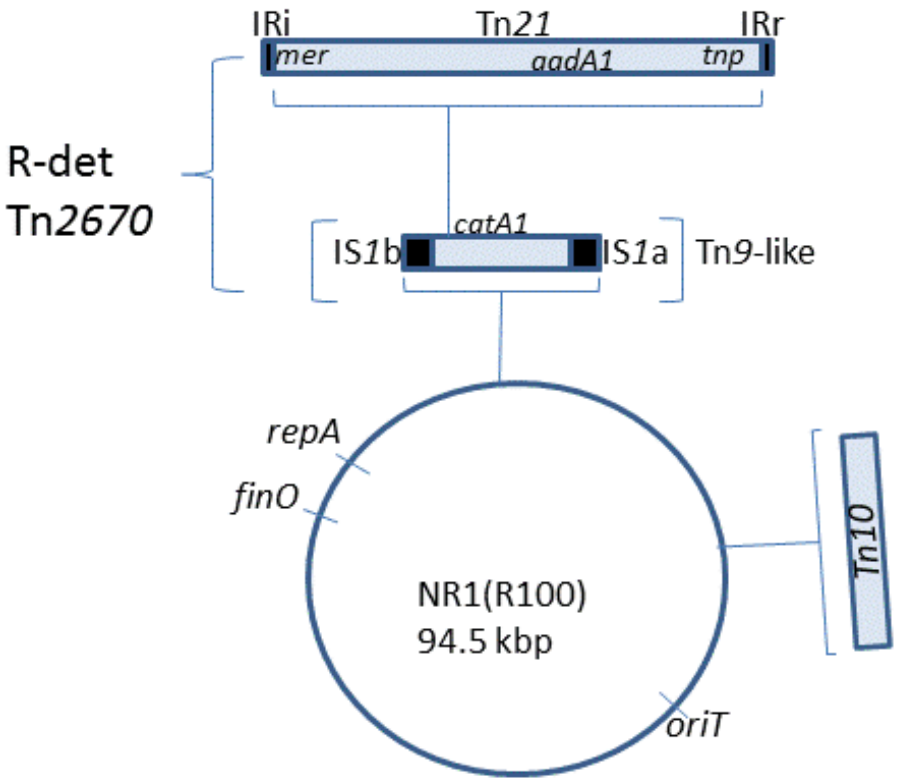


Figure 5-14: Structure of the R plasmid NR1 (R100); a self-transmissible, multiple-antibiotic resistance plasmid (adapted from Liebert et al., 1999).

The multiple-antibiotic resistance R plasmid NR1 (R100), is an IncFII, 94.5 kbp self-transmissible plasmid. The inserts indicate mobile genetic elements including Tn*10*, and Tn*9* and Tn*21* within Tn*2670;* R-det: resistance determinant, this composite of Tn*9*-like transposon, carrying the *catA1* gene, and transposon Tn*21*; IS*1*a and IS*1*b: insertion sequences from the IS*1* family flank the R-det and it is itself transposable as Tn*2670* ; *catA1*: chloramphenicol O-acetyltransferase; IRi and IRr: inverted repeat bound Tn*21*; *aadA1:* streptomycin adenylyltransferase; *tnp*: the transposition region for Tn*21*; *mer*: the mercury resistance operon in Tn*21*; Tn*10*: transposon carrying tetracycline resistance genes; *oriT:* origin of conjugative transfer; *finO*: conjugal transfer fertility inhibition gene; *repA*: encode for replication initiation protein.

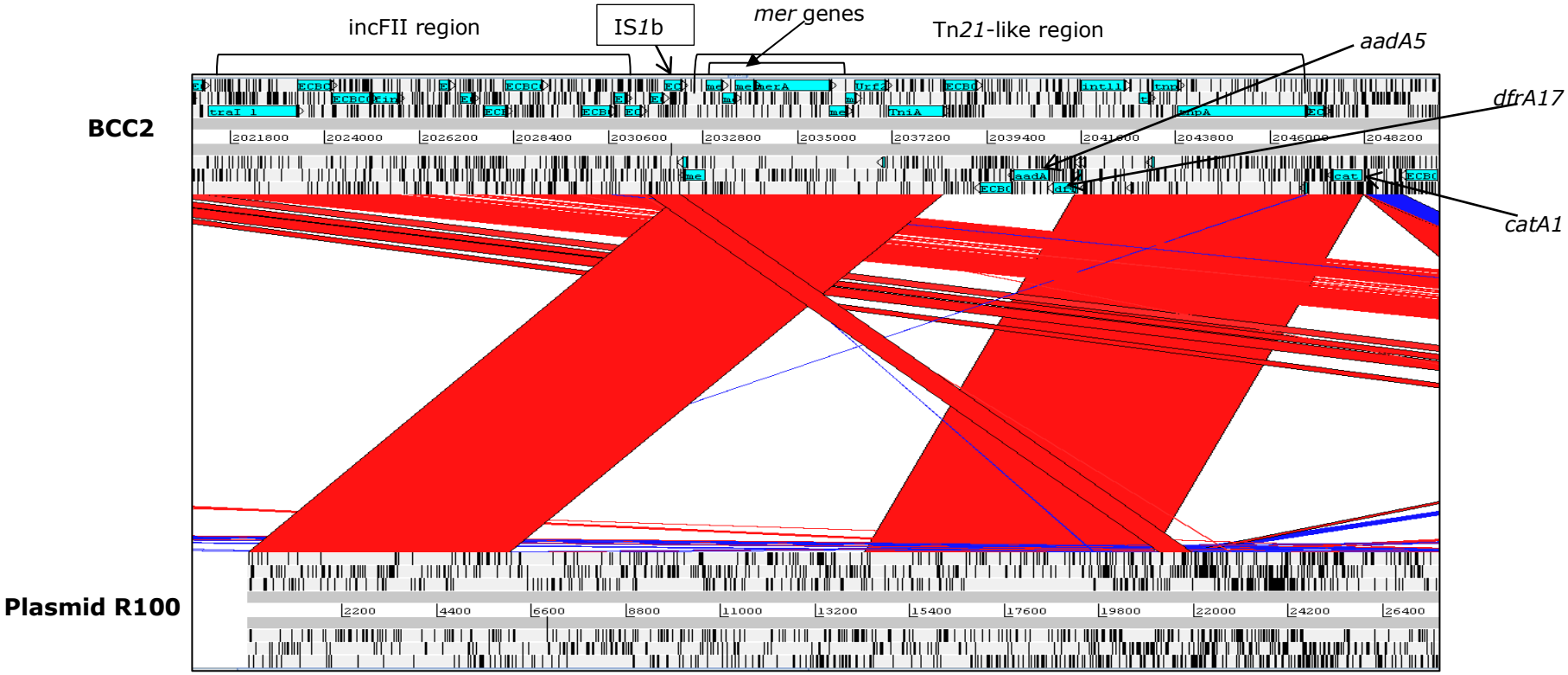


Figure 5-15: Comparison of *catA1* and the adjacent region, between BCC2 and plasmid R100 from *Shigella flexneri*. (Accession no. AP000342.1) using the Artemis Comparison Tool (ACT).

The diagram shows a large area of homology for *catA1*, the Tn*21*-like transposon and the IncFII region in BCC2 and that in plasmid R100. This might indicate the possibility of the carriage of *catA1* and other AMR genes (*mer* genes, *dfrA17* and *aadA5*) located in a Tn*21*-like transposon by an R100-like plasmid in BCC2. The specific regions of interest have been indicated, as the alignment covers approximately 26 kbp, so the annotation of all the genes is hard to see. BCC2 DNA sequence is at the top, and plasmid R100 is on the bottom. The red areas indicates homology with the genes being in the same direction, the blue regions indicate DNA homology but the gene order is in the reverse orientation; the white area indicates no DNA homology; *mer*: mercury resistance; *aadA5*: aminoglycoside adenyltransferase; *dfrA17*: trimethoprim-resistant dihydrofolate reductase; *catA1*: chloramphenicol O-acetyltransferase; IS*1b*: insertion sequence from IS*1* family.

5.7.4 Tetracycline resistance genes

Resistance to tetracycline is widely disseminated in *E. coli,* where it is generally mediated by tetracycline efflux pumps (Møller et al., 2016) and, like the other facultatively anaerobic Gram-negative bacteria, efflux genes are carried on classic transposons and integrons, which are located on a diverse group of plasmids, as well as in the chromosome (Chopra and Roberts, 2001). As the tetracycline resistance genes were found in all of the sequenced strains, the regions surrounding these genes were examined to determine whether they are carried by transposable elements. Tetracycline resistance genes in A2.5, BCC2 and BCE3 were found to be carried on a Tn*10* transposon. The organization of a standard Tn*10* transposon is IS*10*L (insertion sequence), *jemA* (sodium/glutamate symporter), *jemB* (unknown function); *jemC* (ArsR, arsenic/antimony-responsive repressor, family transcriptional regulator), *tet*-RACD (genes contributing to tetracycline resistance) and IS*10*R (Chalmers et al., 2000), Figure 5.16A.

All of the three strains were found to carry *tetB* (tetracycline efflux) instead of *tetA*; the latter confers resistance to tetracycline only, while *tetB* encodes a pump that is able to extrude both tetracycline and minocycline (Tuckman et al., 2007, Coyne et al., 2011). In BCC2 and BCE3 Tn*10* was defective; all the elements for a Tn*10* transposon are present but with truncated IS*10*L and IS*10*R regions (Figure 5.4), while in A2.5, which also carries a defective Tn*10*, Tn*10* lacks the complete sequence of both IS*10*L and IS*10*R except for the IRR for both. These IRs are flanking the other Tn*10* components in A2.5 (Figure 5.16B). The

incomplete sequence of the IS*10* transposase might indicate that this transposon cannot transpose and thus not spread the *tet* genes to other bacteria, unless the whole Tn*10* is carried on a plasmid and transfers as a part of that plasmid. It has been found that Tn*10* which carries the tetracycline resistance genes was associated with streptomycin and sulfadiazine resistance and was widely distributed in *E. coli* from 18 different cattle farms in Washington (Khachatryan et al., 2008).

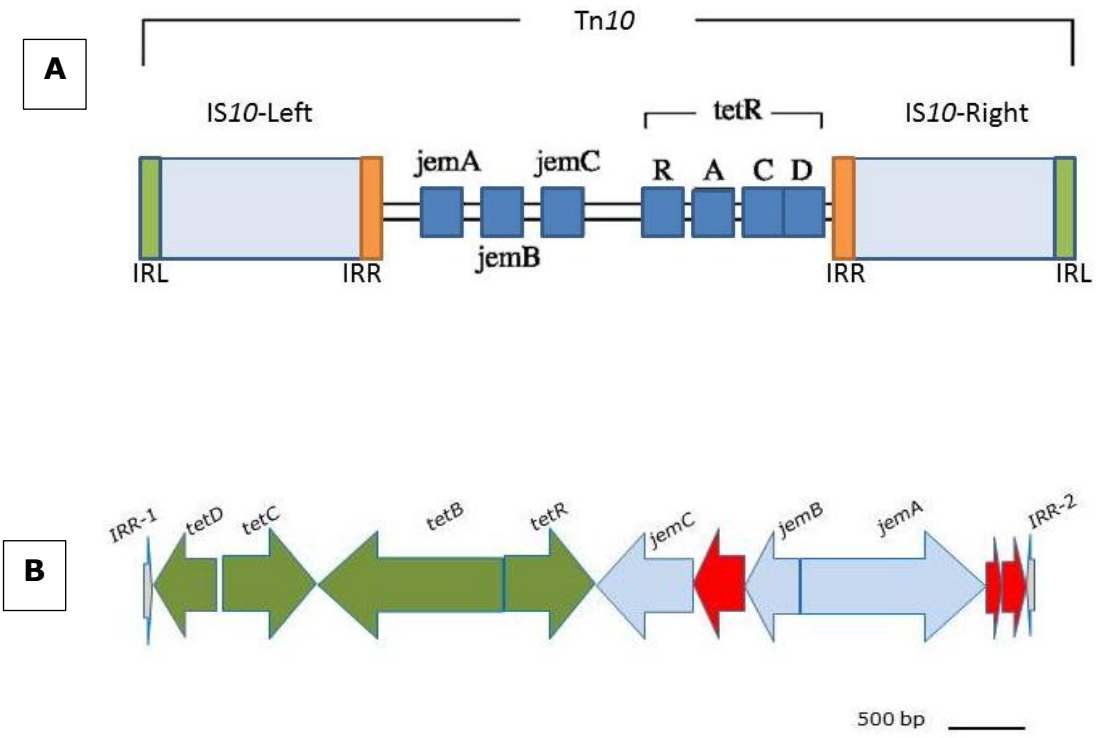


Figure 5-16: Tn*10* map (A)* and Tn*10* arrangement in A2.5 (B).

A: standard Tn*10* structure adapted from Wardle et al. (2005), IS*10*: insertion sequences, IRR: right inverted repeat, IRL: left inverted repeat, *tetR*: genes contributing to tetracycline resistance which include, R (*tetR*), A (*tetA*), C (*tetC*) and D (*tetD*). *tetA* and *tetB*: tetracycline resistance efflux pump; *tetR*: TetR family transcriptional regulator; and *tetC*: transcription regulator gene in transposon Tn*10* which confers resistance to tetracycline; *jemA*: sodium/glutamate symporter; *jemB*: unknown function; *jemC*: ArsR (arsenic/antimony-responsive repressor) family transcriptional regulator. **B**: The boxed arrows indicate the orientation of the gene; green arrows indicate genes which contribute to tetracycline resistance, orange arrows indicate transposition related elements, red arrows indicate a hypothetical protein; blue arrows indicate genes with different functions; IRR-1: right inverted repeat for IS*10*R; IRR-2: right inverted repeat for IS*10*L; the designation for the other genes is as described for A; all the elements in the figure were drawn to scale (500 bp) except for IRR-1 and IRR-2 due to the small length (22 bp).

In BS7, 2 copies of *tetR* and *tetA* were present in two different locations, so an example was chosen for the analysis. One copy of *tetR* and *tetA* was found to be carried by a Tn*1721*-like transposon. Standard Tn*1721* is composed of three 38bp IR, a *tet* region conferring resistance to tetracycline (*tetR* and *tetA*), a truncated *tnpA'*, a relaxase gene *tccA*, and Tn*1722* which contains *tnpA* , *tnpR* and the *res* (resolution) site in addition to *orf*I which encodes methyl-accepting chemotaxis protein (MCP) (Allmeier et al., 1992), Figure 5.17 A.

In BS7, a Tn*1721*-like transposon composed of *tnpA'*, the *tet* region*; pecM* (a multidrug transporter) was found but with only one of the 38 bp IRR for Tn*1721* which is located downstream of *tnpA`*, Figure 5.17 B. BS7 lacks Tn*1722;* this finding is based on the use of the sequence of Tn*1722* from *E. coli* ST131 plasmid pKC396 (Accession no: HM138653) (Zong et al., 2011) for the sequence searches. The Tn*1721*-like transposon sequence from BS7 has high homology to that characterized in *K. pneumoniae* plasmid pK1HV (NC_020087.1 gene ID: 15152875); this was compared using ACT tool (Figure 5.18). In a published study, *E. coli* isolated from cattle from an Irish cattle farm were found to be carrying Tn*1721* containing *tet*A in 10 out of 100 isolates , (Karczmarczyk et al., 2011). The presence of Tn*1721* in different species might indicate the active role for this transposon in tetracycline dissemination.

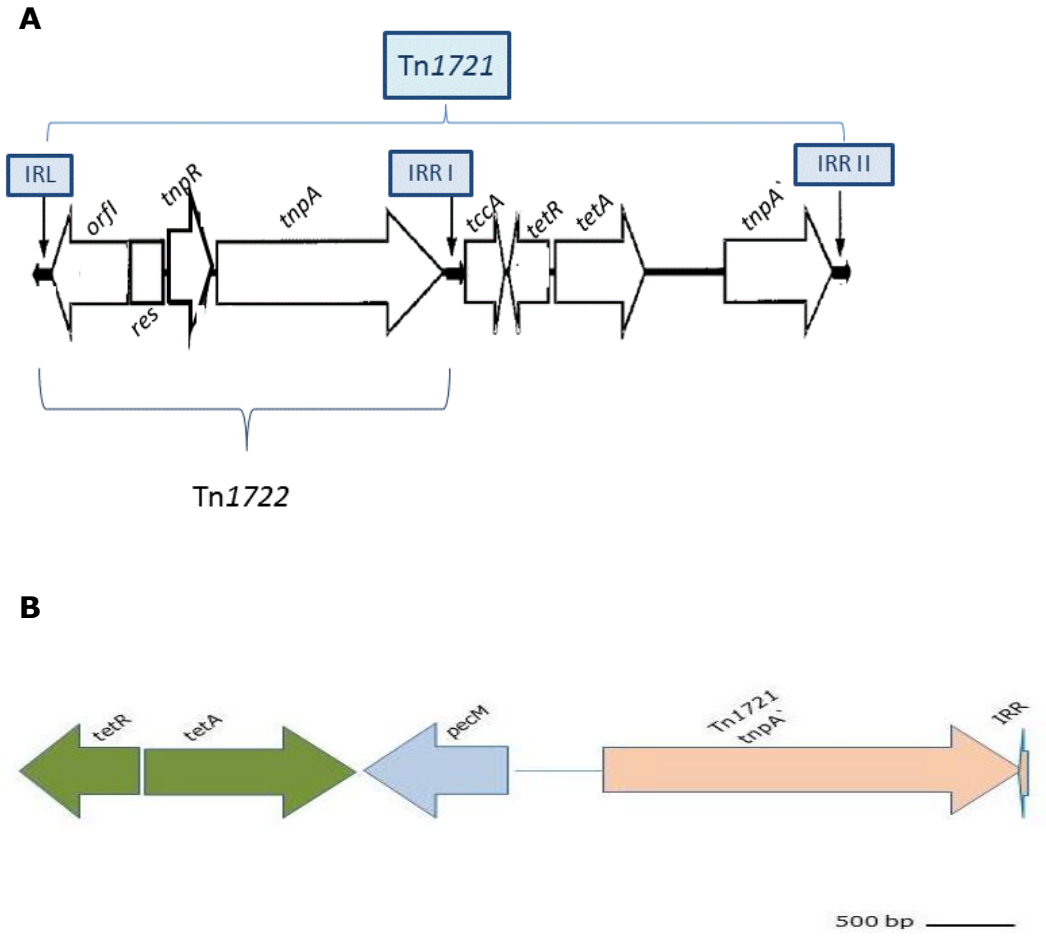


Figure 5-17: The genetic structure of transposon Tn*1721* (A) and Tn*1721*-like transposon in BS7 (B).

A) The genetic structure of transposon Tn*1721* (length, 11,139 bp). The designation, position, and direction of transcription of genes are shown by the arrowed boxes; IRL: inverted repeats left; IRR-I: the first right inverted repeat; IRR-II: the second right inverted repeat; *tccA*: relaxase gene; *tetR* and *tetA:* a *tet* region conferring resistance to tetracycline, *tnpA'*: a truncated transposase; *tnpA*: encoding a transposase; *tnpR*: encoding a resolvase; *res* site: resolution site; *orf*I: encodes methyl-accepting chemotaxis protein (MCP); adapted from Rhodes et al. (2000). **B)** the structure of the Tn*1721*-like transposon in BS7; *pecM*; a multidrug transporter; IRR: inverted repeat right; boxed arrows indicate the orientation of the gene; green arrows indicate AMR genes, orange arrows indicate transposition relative elements, rest of the gene designations are as described for part A, figure drawn to scale (500 bp) except for IRR due to the small length (38 bp).

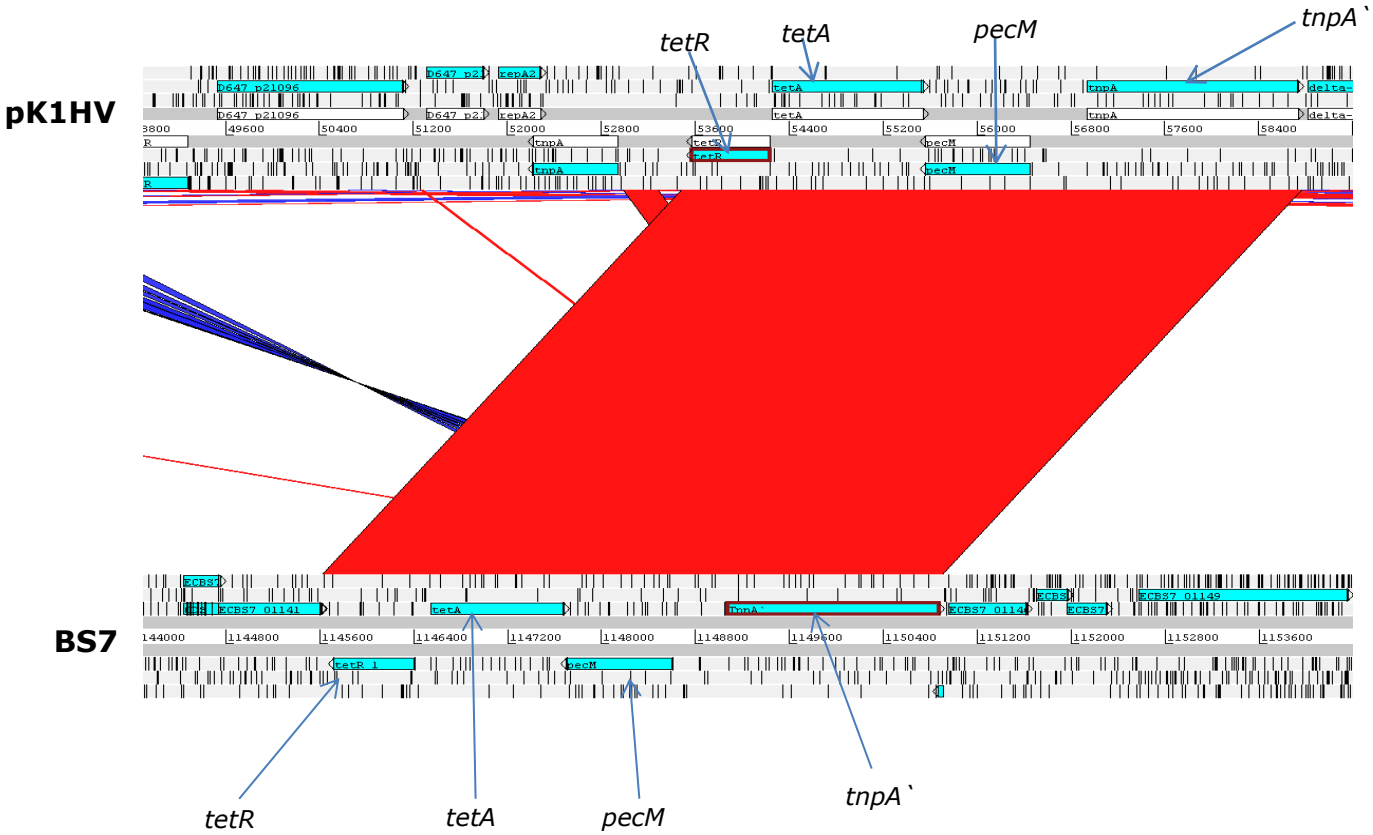


Figure 5-18: Sequence alignment of the Tn*1721*-like transposon from BS7 with the Tn*1721*-like transposon from *K. pneumoniae* plasmid pK1HV.

The figure shows the homology between the structure and sequence of both Tn*1721*-like transposons. Order of sequences from the top is *K. pneumoniae* plasmid pK1HV (accession no.:NC_020087.1) and BS7 on the bottom; the red area indicates homology with the gene in the same orientation; the blue regions indicate DNA homology but the gene order is in the reverse orientation; the white area indicates no DNA homology; the big area of homology represents the Tn*1721*-like region. *tetR* and *tetA:* a *tet* region conferring resistance to tetracycline, *tnpA'*: a truncated transposase; *pecM*; a multidrug transporter. The figure was produced using the Artemis Comparison Tool.

5.8 Detection of mutation in some genes related to AMR

In some strains showing phenotypic resistance to certain antimicrobials no known AMR genes were detected. It is widely known that mutation in some genes and effluxers can result in AMR, especially by the overexpression of these genes (Yaron et al., 2003, Mulvey et al., 2005, Tavio et al., 2014). As a wide investigation has been performed to analyse the presence of acquired AMR genes in the different strains, some examples will be given to show the contribution that mutation in some genes is making to AMR.

5.8.1 Mutations in the *ampC* promoter/attenuator region

The *ampC* gene is a structural gene located on the chromosome, which is expressed at low levels; this gene was present in the genome sequence of all the sequenced strains. Strain A2.5 showed resistance to cephalosporins in the phenotypic disc assay in the initial tests. However, the phenotypic confirmation tests using the ESBL and AmpC confirmation kits (Section 2.5) showed no ESBL or AmpC β-lactamase phenotype and no ESBL genes were detected by PCR (Section 2.4.2). It is widely known that mutation in the *ampC* promoter/attenuator region (Figure 5.19) can be responsible for cephalosporin resistance, so the alternative hypothesis was that the resistance phenotype of A2.5 was due to a mutation resulting in overproduction of AmpC. Analysis of the chromosomal AmpC promoter/attenuator and protein coding region for the chromosomal *ampC* gene in A2.5 was performed previously by Sarah Chapman (Chapman, 2016) and confirmed by me. Three mutations were observed at -18 (G=A), -1(C=T) and +58(C=T). This combination of mutations has been described

before (Mulvey et al., 2005, Tracz et al., 2007), but does not seem to make a big difference in increasing *ampC* expression over the wild type as the contribution to *ampC* overexpression by mutations at -1 and +58 is negligible; mutation at the -18 position was found to increase the *ampC* expression 1.5–3.7-fold only, while a mutation at position -42 with a mutation at the -18 position can increase the expression of *ampC* up to 60 fold (Tracz et al., 2007).

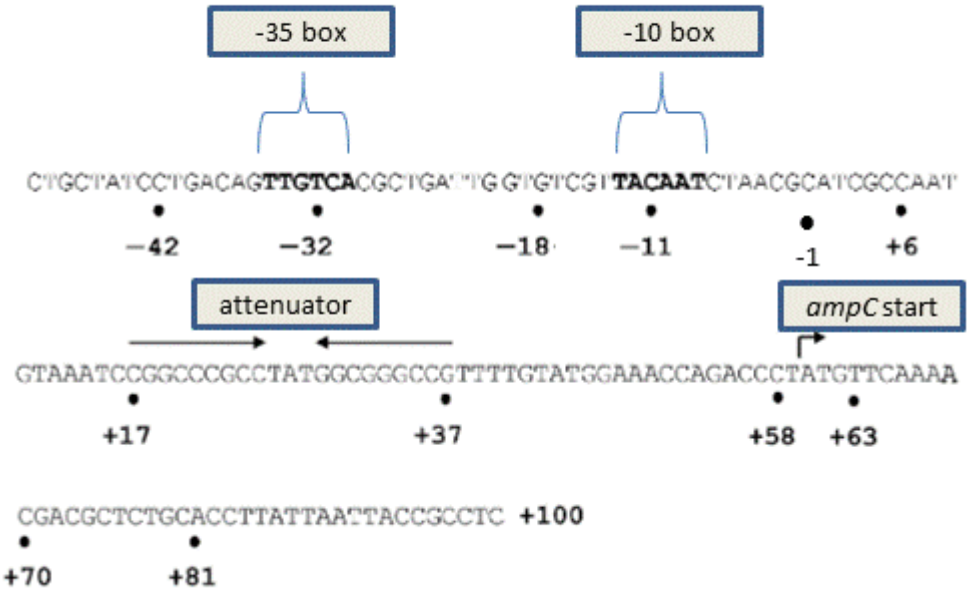


Figure 5-19: The wild type sequence of the *E. coli* ATCC 25922 *ampC* promoter region.

The figure is adapted from Tracz et al., (2007). Numbering is according to Jaurin and Grundström (1981); the numbers highlighted by bullet points are for point mutation positions detected within a number of strains in different studies, including the reference for this figure. These mutations are within the promoter and coding region for *ampC*. The bases in bold convey the -35 and -10 promoter sequences. The bases for the attenuator region are shown by the two arrows; the elbow arrow indicates the ATG start codon for *ampC*.

The *ampC* promoter/attenuator region was also analysed for BCC2 and BCE3 and checked for mutations, as these strains are resistant to cephalosporins. The same mutations detected in the A2.5 *ampC* promoter/attenuator region were also detected in BCC2 and BCE3 (see the example in Appendix XIX). However, due to the fact that these strains (BCC2 and BCE3) already carry ESBL genes, it is not known whether these mutations have any effect on cephalosporins resistance and therefore these findings cannot be used to support the conclusions for A2.5. The analysis of *ampC* region confirms the findings of the AmpC phenotypic confirmation test for A2.5, BCC2 and BCE3, as all were phenotypically negative for AmpC activity (Table 4.4). BS7 did not show resistance to any of the cephalosporins, and genetic analysis showed a 100% similarity with the wild type promoter/attenuator region of *ampC* with *E. coli* K-12; the same sequence (*E. coli* K-12) was used for the comparison with the promoter/attenuator region of the other sequenced strains. So, the conclusion from this analysis is that A2.5, BCE3 and BCC2 share the same point mutations in the promoter region for *ampC,* which do not seem to have a role in their resistance to cephalosporins, so the phenotypic cephalosporin resistance in A2.5 does not seem to be due to a mutation in this region (discussed further in Section 5.9).

5.8.2 Quinolone resistance due to mutation in gyrase A

BCC2 and BCE3 both showed resistance to nalidixic acid, enrofloxacin and ciprofloxacin, but no known quinolone resistance genes, such as *qnrA* and *qnrB,* were detected. An alternative explanation for quinolone resistance is a mutation in one of the gyrase genes that could be responsible for this

resistance phenotype. This was the motivation to check the *gyrA* sequence to see if this was the reason for this resistance phenotype. A double mutation in *gyrA* was detected in both strains (originally by Dr Lisa Crossman and confirmed by me), and was thought to be responsible for this resistance. The mutations produced a change in serine to leucine at the 83 aa position and aspartic acid to asparagine at the 87 aa position (Figure 5.20). This combination of mutations has been found to be responsible for high levels of resistance to fluoroquinolone in *E. coli* as a single mutation in codon 83 of *gyrA* was found to be associated with low levels of resistance to fluoroquinolones (Heisig et al., 1993, Weigel et al., 1998). However, mutation in only 83 aa position has been found to be responsible for high levels of resistance to nalidixic acid (Yoshida et al., 1990). So the two mutations in *gyrA* are likely to be the reason for the resistance phenotypes for both strains.

A2.5 shows intermediate resistance to enrofloxacin but, similarly to BCC2 and BCE3, no AMR genes related to quinolone resistance were detected. *gyrA* was checked for mutations, but the *gyrA* sequence was 100% identical with *gyrA* from *E. coli* K-12. This means that another mechanism was responsible for this resistance phenotype which might be a hyper-expression of one of the multidrug efflux pumps (see Section 5.9).

```
Query  1    MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWN  60
            MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWN
Sbjct  1    MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWN  60

Query  61   KAYKKSARVVGDVIGKYHPHGDLAVYNTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAAM  120
            KAYKKSARVVGDVIGKYHPHGD AVY+TIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAAM
Sbjct  61   KAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAAM  120

Query  121  RYTEIRLAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMAT  180
            RYTEIRLAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMAT
Sbjct  121  RYTEIRLAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMAT  180

Query  181  NIPPHNLTEVINGCLAYIDDEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKV  240
            NIPPHNLTEVINGCLAYIDDEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKV
Sbjct  181  NIPPHNLTEVINGCLAYIDDEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKV  240

Query  241  YIRARAEVEVDAKTGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDESDKDG  300
            YIRARAEVEVDAKTGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDESDKDG
Sbjct  241  YIRARAEVEVDAKTGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDESDKDG  300

Query  301  MRIVIEVKRDAVGEVVLNNLYSQTQLQVSFGINMVALHHGQPKIMNLKDIIAAFVRHRRE  360
            MRIVIEVKRDAVGEVVLNNLYSQTQLQVSFGINMVALHHGQPKIMNLKDIIAAFVRHRRE
Sbjct  301  MRIVIEVKRDAVGEVVLNNLYSQTQLQVSFGINMVALHHGQPKIMNLKDIIAAFVRHRRE  360

Query  361  VVTRRTIFELRKARDRAHILEALAVALANIDPIIELIRHAPTPAEAKTALVANPWQLGNV  420
            VVTRRTIFELRKARDRAHILEALAVALANIDPIIELIRHAPTPAEAKTALVANPWQLGNV
Sbjct  361  VVTRRTIFELRKARDRAHILEALAVALANIDPIIELIRHAPTPAEAKTALVANPWQLGNV  420

Query  421  AAMLERAGDDAARPEWLEPEFGVRDGLYYLTEQQAQAILDLRLQKLTGLEHEKLLDEYKE  480
            AAMLERAGDDAARPEWLEPEFGVRDGLYYLTEQQAQAILDLRLQKLTGLEHEKLLDEYKE
Sbjct  421  AAMLERAGDDAARPEWLEPEFGVRDGLYYLTEQQAQAILDLRLQKLTGLEHEKLLDEYKE  480

Query  481  LLDQIAELLRILGSADRLMEVIREELELVREQFGDKRRTEITANSADINLEDLITQEDVV  540
            LLDQIAELLRILGSADRLMEVIREELELVREQFGDKRRTEITANSADINLEDLITQEDVV
Sbjct  481  LLDQIAELLRILGSADRLMEVIREELELVREQFGDKRRTEITANSADINLEDLITQEDVV  540

Query  541  VTLSHQGYVKYQPLSEYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGRVY  600
            VTLSHQGYVKYQPLSEYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGRVY
Sbjct  541  VTLSHQGYVKYQPLSEYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGRVY  600

Query  601  SMKVYQLPEATRGARGRPIVNLLPLEQDERITAILPVTEFEEGVKVFMATANGTVKKTVL  660
            SMKVYQLPEATRGARGRPIVNLLPLEQDERITAILPVTEFEEGVKVFMATANGTVKKTVL
Sbjct  601  SMKVYQLPEATRGARGRPIVNLLPLEQDERITAILPVTEFEEGVKVFMATANGTVKKTVL  660

Query  661  TEFNRLRTAGKVAIKLVDGDELIGVDLTSGEDEVMLFSAEGKVVRFKESSVRAMGCNTTG  720
            TEFNRLRTAGKVAIKLVDGDELIGVDLTSGEDEVMLFSAEGKVVRFKESSVRAMGCNTTG
Sbjct  661  TEFNRLRTAGKVAIKLVDGDELIGVDLTSGEDEVMLFSAEGKVVRFKESSVRAMGCNTTG  720

Query  721  VRGIRLGEGDKVVSLIVPRGDGAILTATQNGYGKRTAVAEYPTKSRATKGVISIKVTERN  780
            VRGIRLGEGDKVVSLIVPRGDGAILTATQNGYGKRTAVAEYPTKSRATKGVISIKVTERN
Sbjct  721  VRGIRLGEGDKVVSLIVPRGDGAILTATQNGYGKRTAVAEYPTKSRATKGVISIKVTERN  780

Query  781  GLVVGAVQVDDCDQIMMITDAGTLVRTRVSEISIVGRNTQGVILIRTAEDENVVGLQRVA  840
            GLVVGAVQVDDCDQIMMITDAGTLVRTRVSEISIVGRNTQGVILIRTAEDENVVGLQRVA
Sbjct  781  GLVVGAVQVDDCDQIMMITDAGTLVRTRVSEISIVGRNTQGVILIRTAEDENVVGLQRVA  840

Query  841  EPVDEEDLDTIDGSAAEGDDEIAPEVDVDDEPEEE  875
            EPVDEEDLDTIDGSAAEGDDEIAPEVDVDDEPEEE
Sbjct  841  EPVDEEDLDTIDGSAAEGDDEIAPEVDVDDEPEEE  875
```

Figure 5-20: Protein sequence alignment for *gyrA* between BCC2 and *E. coli* K-12.

The query strand (on top) is for BCC2 and the subject strand (bottom strand) is for *E. coli* K-12, the middle line shows the substitution scoring; +: indicates conservative substitution. The mutation in BCC2 is serine (S) to leucine (L) at the 83 aa position and aspartic acid (D) to asparagine (N) at the 87 aa position, the position of these substitutions is determined by a red box. The alignment was performed using blastp from NCBI.

5.9 Discussion

The existence of different databases relating the phenotype and genotype of *E. coli* makes the typing of different strains very straightforward, such as determining the ST, phylotype and serotype. Using MLST and Serotypefinder 1.1 databases for the sequenced strains in this study gave a confirmation of the genomic diversity and relatedness for these strains which had previously been determined by ERIC-PCR. BCC2 and BCE3 were from the same cluster in the ERIC profile dendrogram and they were shown to have the same ST and serotype. BS7 was an outlier and A2.5 was from a different cluster and these strains were shown to be different ST and serotypes from each other and from BCC2 and BCE3.

E. coli O17/O77: H18 (ST69) has been reported to cause 11% of all *E. coli* UTIs and 49% of all trimethoprim/sulfamethoxazole-resistant *E. coli* UTIs in one Californian community over a 4-month period and has also been isolated from retail pork meat (Manges et al., 2001, Vincent et al., 2010). The profile of this strain matches the ST and serotype profile of BS7 (Section 5.4), this strain is resistant to trimethoprim/sulfamethoxazole, the same as BS7 (Table 4.4). This might indicate the wide spread distribution of such strains and the possibility of the transmission of such a strain between humans and animals.

ST162 *E. coli,* which was the MLST group of BCC2 and BCE3, has been reported before from livestock (pigs, chickens and cows) in a study commenced in Germany comparing the ST of *E. coli* from livestock and farm workers (Dahms et al., 2015). However, the same ST has been reported in

Greece from *E. coli* which was associated with community-acquired infections (Mavroidi et al., 2012). In the latter study, an *E. coli* strain with ST162 shared some features with BCC2 and BCE3. It was resistant to ampicillin, trimethoprim/sulfamethoxazole, and ciprofloxacin, has $bla_{TEM-1,}$ had a mutation in *gyrA* in the same amino acid positions as in BCC2 and BCE3 (Section 5.8.2), but unlike BCC2 and BCE3, it had *aac (6')-Ib-cr* and did not carry any ESBL genes (Mavroidi et al., 2012). There was no mention about the serotype group of this ST in either of the studies above, so it is not known if any of those strains has a similar serotype to BCC2 and BCE3. However, the presence of this ST in both humans and animals might show the possible distribution within different hosts.

The same ST of A2.5, ST56, has been reported from a group of food-associated Shiga toxin-producing *E. coli* (STEC) and haemolytic-uremic syndrome (HUS)-associated enterohaemorrhagic *E. coli* (HUSEC) strains which lacked *eae*. Amongst these strains, known pathogen serotypes O91:H21 and O113:H21 were present (Hauser et al., 2013). However, A2.5 has the serotype O3:H21, which was not reported within the serotypes group for ST56 strains in the study above. The presence of this ST from different sources might represent another example of the possibility of this bacteria transferring amongst different hosts.

From what has been shown above about the distribution of *E. coli* strains with the same ST and sometimes the same serotypes among different hosts, it could be concluded that there are no barriers to the spread of bacteria through the environment or through close contact to the livestock,

as the risk of *E. coli* transfer from livestock to people has been indicated before.

MLST typing and serotyping of the sequenced strains gave another advantage over using ERIC-PCR typing, as it enables comparison of the sequenced strains with *E. coli* strains reported in other studies (as illustrated for each strain above). However, ERIC-PCR did discriminate between BCC2 and BCE3, as it showed they are similar (91% similarity depending on ERIC-PCR typing) but not identical, while the other typing methods (MLST and serotype) showed they have the same ST and serotype. As has been described before (Section 1.7.6) MLST cannot always accurately resolve relationships among genetically related isolates, for example the ability to differentiate between isolates that vary in their pathogenic potential (Mellmann et al., 2011, Hao et al., 2012). This could lead to the conclusion that it would be better to use more than one typing method to discriminate between bacteria, especially for epidemiological studies.

Further to the advantage of using database search tools for bacterial typing, other advantages can be shown by the AMR gene heatmap. This heatmap provides a great advantage for searching for AMR genes. Detecting the presence of these genes using databases like CARD, saves time in analysis. However, as the heatmap represents just the difference between the selected strains (BCC2, BCE3 and BS7) and *E. coli* K-12, some other genes could be present but not listed in the heatmap. This can happen if the same genes are present in all 4 strains, for example *emrD* which encodes for a multidrug efflux system protein. In addition, the heatmap only gives a

prediction of the AMR genes present which makes the confirmation step necessary to perform (Section 5.5). The confirmation step can confirm the presence/absence of the genes predicted by the heatmap and confirm the variant type for some genes, such as *bla*$_{TEM}$ and *bla*$_{CTX-M}$.

Further to the confirmation analysis for the presence/absence of the AMR genes, another advantage of this genomic analysis method is that it notes information such as the location and the direction of the genes on the forward and reverse DNA strands (Appendix XV), this provides a record about this information and facilitates the process of finding these genes in case they are used in a comparison study in the future. However, this information might not represent the real locations and orientation of the genes on the chromosome. As the sequences are not complete, there are some gaps between contigs and the arrangement and order of the genes is determined by the programme based on contig size in the assembly rather than their real physical location on the chromosome. In BCC2 and BCE3, which share similar AMR gene profiles, the direction of the same AMR genes on the reverse and forward DNA strands in the genome assembly of the strains varies; some of the genes have similar orientations such as *bla*$_{CTX-M14}$, while many others are oriented in the opposite direction such as *bla*$_{TEM-1}$. This might not be the real situation on the chromosome; this cannot be known from the current assembly which is not fully completed. One method to check this is by aligning them using a fully sequenced genome as a scaffold but as mobile genetic elements are known to change position and orientation during bacterial growth in the case of genes being on short contigs this approach might not be of value. Strand-specific RNA sequencing

is another method which could be used to know if a gene is transcribed on one strand or the other (Dr Lisa Crossman, personal communication).

Using whole genome sequence data for the selected strains gave an explanation for the different AMR phenotypes seen between the strains and confirms the phenotypic and PCR detection results for specific genes i.e. ESBL. BCE3 and BCC2 which have an almost identical AMR phenotype (Table 4.4) seem to have the similar AMR gene profiles. Both strains possess *bla*$_{\text{CTX-M-14a}}$ which confers resistance to different β-lactams, including ampicillin and resistance to different 3rd and 4th generation cephalosporins, especially cefotaxime, but gives poor resistance to ceftazidime and aztreonam. The same phenotype has been seen before with bacteria carrying this gene (Ma et al., 2002, Yi et al., 2010). The carriage of this gene by a complete IS*Ecp1* with the complete sequence for IS*Ecp1* IR and the presence of a putative IRR2 (alternative IR), may represent a putative active transposition unit. The position of this gene interrupting *fdeC* seems to have occurred due to transposition from another place which might be a plasmid or chromosome (Figure 5.2 and Figure 5.3). The data from genome sequencing regarding the location of *bla*$_{\text{CTX-M-14a}}$ in BCC2 and BCE3 are in line with the results from the plasmid conjugation experiment (Section 4.2.5). Putative trans-conjugants from BCC2 and BCE3, were found to be azide resistant mutants of the donor strain, not true transconjugants, and the genome sequence data confirms that the position of these genes is not on a plasmid but these are inserted in the chromosome. The presence of *bla*$_{\text{CTX-M_14a}}$ on this possible transposition unit without the presence of any other AMR genes might indicate that the constant use of β-lactams in the herd is

the selective pressure. The co-selection associated with selective pressure from other antibiotics is unlikely to be the reason. This hypothesis was supported by the lack of detection of this and other β-lactamase genes after 2014 from *E. coli* isolates from same farm (Chapman, 2016) when the use of cephalosporins had been reduced on the farm.

Strains BCC2 and BCE3 were phenotypically resistant to streptomycin, sulphonamide, tetracycline, trimethoprim/sulfamethoxazole and chloramphenicol and the phenotype was confirmed by detecting different known AMR genes conferring resistance to these antimicrobials as has been discussed previously (Sections 5.7.1-5.7.4). Resistance to nalidixic acid, enrofloxacin and ciprofloxacin was likely due to a mutation in *gyrA*, rather than the presence of one of the quinolone resistance genes. This is concluded due to the absence of any AMR genes related to quinolone resistance and the fact that there are mutations in *gyrA* known to convey resistance to quinolones. This represents another similarity between those two strains which have a lot of features in common, including the AMR genes and their environments, the carriage by the same insertion elements and transposons, all suggesting these strains share a similar origin and might have the same ancestor.

Genome analysis of A2.5 did not identify the reason for the observed phenotypic resistance to 3rd and 4th generation cephalosporins in this isolate. The carriage of *bla*$_{OXA-1}$ and *bla*$_{TEM-1a}$ by A2.5 strain does not confer resistance to 3rd and 4th generation cephalosporins, because these genes confer resistance to broad spectrum β-lactams and not extended spectrum β-lactams. A2.5 was found to carry *bla*$_{OXA-1}$ with the strong promoter P2m3

in addition to PcW (Section 5.6.3). The P2m3 promoter in A2.5 is known to double *bla*$_{OXA-1}$ gene expression. The hyperproduction of this gene can increase resistance to substrate antibiotics, as *bla*$_{OXA-1}$ confers resistance to different broad spectrum penicillins, cloxacillin, methicillin, and oxacillin but not extended spectrum cephalosporins (Jacoby and Munoz-Price, 2005). A2.5 has *bla*$_{TEM-1a}$ with the weak promoter P3 (Section 5.6.2), as mentioned above this gene is a broad spectrum β-lactamases not an ESBL, so it is not likely to be the reason for this phenotype. The mutation in the *ampC* promoter/attenuator region found in A2.5 does not produce strong overexpression of *ampC* (Section 5.8.1) and so is unlikely to be the reason for the cephalosporin resistance phenotype for this strain and especially resistance to 4th generation cephalosporins. So the alternative explanation that can be given for the phenotype of this strain is that it might be due to overexpression of a multidrug efflux pump which can confer β-lactam resistance such as AcrAB (acridine resistance complex)–TolC multidrug efflux pump. It has been shown before that resistance to ceftazidime in *E. coli* is associated with mutations in *acrR* (DNA-binding transcriptional repressor), *marR* (MarR family transcriptional regulator) and PBP3 (penicillin-binding protein 3) (Tavio et al., 2014). Other mutations in the *marRAB* (multiple antibiotic resistance operon) system seem to confer some degree of resistance to penicillins, cephalosporins, fluoroquinolones, tetracycline, and chloramphenicol (Alekshun and Levy, 1997, Yaron et al., 2003). A2.5 shows phenotypic resistance to enrofloxacin but no resistance genes against this antimicrobial have been found in the genome of this strain, and no mutation in *gyrA* detected. This indicate that the reason for this resistance phenotype might be due to mutation in one of the efflux

systems or, as mentioned above, it might be due to mutation in *marRAB*. The efflux systems have a broad substrate range which is of concern, as usually over-expression of a pump will result in resistance to antibiotics of more than one class as well as some detergents, dyes and disinfectant (Webber and Piddock, 2003). The other resistance phenotypes in A2.5 to streptomycin, oxytetracycline and sulphonamide can be explained by the presence of the specific genes that confer resistance to these antimicrobials.

BS7 did not show resistance to cephalosporins, but shows resistance to ampicillin and amoxicillin–clavulanic acid which can be due to the carriage of $bla_{\text{oxa-1}}$ and $bla_{\text{TEM-30,}}$ (Section 5.6.2.3 and 5.6.3). Many genes present in this strain's genome would explain the phenotypic resistance patterns for this strain against chloramphenicol, streptomycin, oxytetracycline and sulphonamide. Some of these resistance genes are present in more than one copy in the genome of this strain, for example 2 copies of *sul1* and *tetA* were found in the genome of BS7 (Appendix XV).

Both BS7 and A2.5 show phenotypic resistance to trimethoprim, but they only carry a *dfr* gene. It is not known if this *dfr* gene (in BS7 and A2.5) confers resistance to trimethoprim or not as it does not match any of the known trimethoprim resistance *dfr* variants (Section 5.7.2). In order to determine the relationship between the trimethoprim resistance and the expression of this gene, transcriptional or protein experiments could be used.

One of the important findings (in the current study) about the AMR genes in such a community of *E. coli* is the fact that there is evidence that most of

these are associated with transposable elements such as insertion elements, transposons and putative plasmids, which might enable the transfer of these genes to other *E. coli* strains or even potentially to other species, which is common via HGT. Most of the examples chosen for analysis in this study seem to be active in transposition as they possess a complete transposition sequence with complete IR, such as IS*Ecp1* carrying $bla_{\text{CTX-M-14a}}$, Tn*2* and Tn*3* carrying bla_{TEM} genes. Tn*21*-like transposons carrying class I integrons were present in all the sequenced strains, but there was variation from one strain to another in terms of carrying different gene cassettes. BS7 and A2.5 class I integron carries $bla_{\text{OXA-1}}$ and the *aadA1* gene cassette (Figure 5.9), while BCE3 and BCC2 carry *dfra*17 and *aadA5* gene cassettes (Figure 5.11C and D). In BS7 , the insertion of Tn*3* into the Tn*21*-like transposon might indicate that the insertion is co-evolved, so the whole AMR cluster (including $bla_{\text{TEM-30}}$ within Tn*3*, and $bla_{\text{OXA-1,}}$ *aadA1*, *sul1* and *qacEΔ1* within the In*2*-like integron, which are all part of a Tn*21*-like transposon) might possibly transfer both inter- and intra- genomically. The *strA* and *strB* genes and *aphA1* all seem to be capable of transposition due to their association with two complete IS*26* elements.

Some MGEs are not complete in their elements, for example absence of IR, having a truncated transposase or complete loss of transposase which make these transposable elements unable to transpose; $bla_{\text{TEM-1a}}$ in A2.5 is such an example as it seems to be carried putatively on a plasmid and is flanked by the left IR for Tn*3* with the other elements of Tn*3* missing, which would mean it could no longer transpose. Completing the sequence analysis of this strain by filling the gaps between the contigs that were not covered

previously by sequencing would allow a better understanding of how this gene was carried. Completing the sequence can be performed by PCR analysis including primer design, PCR to fill gaps between the different contigs using the designed primers and then sequencing the PCR product. Smaller gaps might be closed by standard PCR, if information concerning the contig ends is available (Frohme et al., 2001) while for large gaps long-range PCR can be used (Jia et al., 2014). In the same strain (A2.5) there is a truncated Tn*10* transposon which carries the tetracycline resistance genes; this transposon lacks the IS*10*L and IS*10*R except for the IRR for both of them, which again suggests it is not an active transposon, as it lacks both transposase elements. BCC2 and BCE3 have Tn*10* as well, but with truncated IS*10*L and IS*10*R, which might prevent transposition of this transposon. In a standard Tn*10*, IS*10*R is a structurally and functionally intact transposase and it encodes a single transposase protein which mediates transposition, while IS*10*L is a functionally defective transposase (Kleckner et al., 1996).

IS*CR*2 which belongs to the IS*91* family was found associated with 2 AMR genes in BS7; *sul2* and *floR*. IS*91* is an insertion sequence that transposes by a rolling-circle replication mechanism, which is thought to play an important role in the dissemination and evolution of virulence, antibiotic resistance and pathogenicity types of genes (Garcillán-Barcia and de la Cruz, 2002).

Streptomycin resistance is expected to be high within the isolates as a result of the widespread use of this antibiotic in treating different infections in cattle. The presence of a class I integron with associated AMR genes in

each of the sequenced strains (within different gene cassettes but all having a streptomycin resistance gene), and the carriage of these integrons by Tn*21*-like transposons (Appendix XVI) indicates the potential for co-selection. For example, genes encoding resistance to broad spectrum β-lactams, streptomycin and sulphonamide were present in a class I integron in BS7 and A2.5. In this case, using one of these antimicrobials for example, streptomycin in treating the cattle, can select for the emergence of streptomycin resistance genes which if present in the class I integron will co-select for the other resistance genes (β-lactamase and *sul* genes), because all the resistance genes are carried on the same transposable elements. This is really of concern especially with ESBL genes being located in a class I integron, even if such a combination was not found in this study. ESBL encoding genes located within class I integrons have been described elsewhere in *E. coli* and Enterobacteriaceae including $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-14}}$ (Di Conza et al., 2002, Arduino et al., 2002, Bae et al., 2007, Casella et al., 2015). As just four strains were sequenced in the current study, it is not known if such combinations are present within the other strains which had different $bla_{\text{CTX-M}}$ variants. In this study, a β-lactamase gene ($bla_{\text{Oxa-1}}$) was located in a class I integron, however it was not an ESBL gene.

The diversity between the different strains is very clear with regards to the β-lactam gene carriage and other genes. The presence of such diversity of AMR genes in this *E. coli* community is of concern, but it represents only a pointer as to what could be found on other farms; indeed the farm from which the samples were collected in this study is a well-managed farm and on other farms the situation might prove worse. The transfer of these

strains via the food chain or environment to humans might be possible as the sequenced strains A2.5 (ST56), BCC2 and BCE3 (ST162) and BS7 (ST69) are sequence types which can be found in humans and animals (Vincent et al., 2010, Mavroidi et al., 2012, Hauser et al., 2013). Controlling the spread of AMR from the environment might be the key to controlling the spread of these genes to humans.

6 . General discussion

6.1 General discussion

The aim of this research was: 1) To determine the prevalence of *E. coli* from selected slurry samples from the dairy farm and to estimate the percentages of the putative CTX-resistant *E. coli*, and to estimate the number of resistant bacteria that can enter the environment from spreading slurry on to the land; 2) To determine the phenotypic resistance profiles of the collected *E. coli* isolates and determine the MDR patterns against different antimicrobials (17 antimicrobials) representing different antimicrobial groups; 3) To investigate the presence of ESBL/AmpC producing *E. coli* within the farm; 4) To determine the genotypic diversity of the collected *E. coli* strains, with the aim of: 5) Making a link between the phenotypic resistance profile and genotypic diversity in order to understand the diversity of the resistance. Furthermore, this study aimed to: 6) Determine the genetic environment of the AMR genes for four selected *E. coli* strains and especially the β-lactamase genes, and to determine the possibility of spread of such genes to the environment. On the basis of the results, possible recommendations can be made concerning the control of MDR bacteria and their spread to the environment.

It is important to understand the reasons that lead bacteria to develop antimicrobial resistance to estimate the crisis that has been produced by using antimicrobials widely in medicine and animal husbandry. One of the most important reasons is that bacteria over thousands of years have tried to protect themselves from different circumstances including naturally occurring antimicrobial agents (Wright, 2007, Djordjevic et al., 2013). Natural resistance selection may occurs due to bacterial exposure to

sublethal concentrations of antimicrobial agents. This results in resistant strains that can reproduce and spread easily through the bacterial community and express their resistance rapidly (Silbergeld et al., 2008). Once the AMR genes exist they can be exchanged from commensal to pathogenic bacteria and vice versa (Rowe-Magnus et al., 2002, Summers, 2006). It should be mentioned here that there are many sources that lead to dissemination of the different antimicrobials in the environment (manufacture, disposal, and excretion of metabolites and conjugates from treated humans and animals) and thus co-selection for the resistance genes. Obtaining accurate data about the pharmaceutical industries, in term of the quantities of antimicrobials they produce, is not easy. However, it can be estimated that many millions of metric tons of antimicrobials compounds have been released into the biosphere over the last half-century (Davies and Davies, 2010). Antimicrobials that enter the environment during the drug manufacturing process are believed to release the vast majority of unmetabolized antimicrobials in the biosphere (Davies and Davies, 2010). In India and China, this is considered as a real problem. According to data produced by a monitoring study in India, many antimicrobials (ciprofloxacin, enrofloxacin, ofloxacin and trimethoprim) were present in river water downstream of a wastewater treatment plant which received wastewater from more than 85 bulk drug manufacturers in different concentrations; for example, very high concentrations of ciprofloxacin (14 mg L^{-1}) were found in the effluent of the treatment plant (Fick et al., 2009). In addition, resistance may be selected for even after antimicrobials are no longer used; for instance, tetracyclines and fluoroquinolones persist in the environment for months to years (Boxall et al., 2006, Monteiro and Boxall, 2010). It has

been shown that even if antimicrobials are removed from animal feeds and the level of resistance in bacteria decreased significantly, resistant bacteria would still be detected in animal houses, waste, and food products in Europe (Sorum et al., 2006).

There are many potential mechanisms that contribute to the spread of antimicrobial-resistant bacteria from animals to humans or vice-versa. These can be explained mainly by transfer via direct contact, the spread of bacteria and antimicrobial resistance genes to the environment via manure and thus to humans, and the horizontal transmission of resistance genes between bacterial species and genera from animals to the environment and to humans (Figure 1.8). Thus the environment is one of the most important reservoirs of resistant bacteria: it reintroduces resistant bacteria into the food-animal and human reservoirs (Aarestrup et al., 1998, Barza, 2002, Jensen et al., 2004, Hammerum and Heuer, 2009, Wegener, 2012).

In this study, *E. coli* was used as an indicator for the presence of AMR genes in the different slurry and faeces samples from the dairy farm, being a common enteric commensal strain of which some pathotypes can cause human and animal disease and is one of the groups of seven bacterial species that the WHO has underlined as of key AMR concern: *E. coli* can serve as a sentinel organism for antimicrobial resistance development (WHO, 2014). It can be used as an indicator for the transmission of resistance between animals, the environment and humans: and, of great concern, the transmission of ESBL resistance genes. The results obtained in this study have been discussed in each chapter: Chapter 3 shows the prevalence of *E. coli* and coliforms in different slurry and faecal samples, the

percentages of putative CTX-resistant *E. coli* isolated using a selective agent (CTX) in combination with TBX medium; and the genotyping of the selected strains using ERIC-PCR. Chapter 4 discusses the phenotypic AMR profile of each isolate tested using 17 antimicrobial discs, the percentages of resistant isolates to each tested antimicrobial, phenotypic confirmation of ESBL/AmpC producer *E. coli* strains, detection of some β-lactamses genes including ESBL and *ampC* genes from CTX-resistant *E. coli* using PCR, and determination of gene variant types and testing the possibility of $bla_{\text{CTX-M}}$ gene transfer using a conjugation method. Chapter 5 examines the diversity in AMR genes and the genetic environments for these genes for 4 sequenced MDR *E. coli* strains isolated and characterized in Chapters 3 and 4. However an overview of some of these results will be highlighted further in this chapter.

Estimation of the numbers of *E. coli* present in the collected samples was performed using the spread plate count method. The difference between *E. coli* counts (ECC) isolated from the selective growth media with and without antibiotic was very obvious, and that is due to the selective effect of the antibiotic (cefotaxime) in the media, as was also the case for total coliform counts (TCC). The viable counts for *E. coli* and coliforms from the slurry samples were significantly lower for the ECC and TCC from liquid slurry (4.6-4.79 and 4.76-4.82 $\log_{10}$ CFU ml^{-1}, respectively) compared to the ECC and TCC from the cow barn slurry (5.64-5.78 and 5.88-5.99 $\log_{10}$ CFU ml^{-1}, respectively) and ECC and TCC from the solid slurry samples (5.75-5.86 and 5.94-6.06 $\log_{10}$ CFU ml^{-1}, respectively). CTX-resistant *E. coli* were present in all samples that have been collected in different places from the dairy farm,

but the numbers were less in the liquid slurry sample (P< 0.0001, DF=86) compared to the faecal samples from the barns and the solid slurry. This is expected to be due to a dilution effect that happens to the bacterial population in the slurry tank. This led to a conclusion that liquid slurry might have less effect in dissemination of these bacteria in the field after spreading, while the separated solid slurry may have more effect when it is applied directly to the field as it carries larger numbers of resistant bacteria.

TBX+CTX and CHROMagar ESBL were effective at isolating ESBL/AmpC producing *E. coli*. 43.6% of the MDR *E. coli* isolates showed phenotypic resistance to cefotaxime and/or other cephalosporins used in this study; these had been isolated using selective media, while only 14.2% of the isolates showed MDR without using antibiotic selection. The level of resistance to each of the 17 antimicrobials used in this study varied widely (Section 4.2.1), but the highest percentage of resistance was to AMP (56.3%), OT (41.1%), S10 (39.6%), CTX (38%), S300 (38.8%), AMC (33.3%), EFT (23%), and CAZ (14.2%). The presence of this level of resistance to β-lactams, oxytetracycline and streptomycin in those isolates was not surprising given the use of cephalosporins for mastitis treatment in the herd, besides the use of other antimicrobials (oxytetracycline, streptomycin and neomycin). What was surprising is the high level of sulphonamide resistance as it was not used with the other antimicrobials. Isolates sharing a resistance phenotype to β-lactam group antibiotics represented by resistance to AMP and AMC and aminoglycoside resistance represented by resistance to S10, tetracycline resistance represented by resistance to OT, and sulphonamide resistance represented by resistance to

S300 shows a close relationship with the TBX+CTX cluster, as can be seen from Figure 4.5. This may have been due to the presence of a multi-resistance genetic element present in these isolates or it may have been due to the presence of different genes carried on different elements. Sequence analysis for some selected strains showed the latter, however these selected strains were not located within this cluster so the resistance genes might have a different arrangement.

Different CTX-M variants were found in this study. CTX-M-32 was the dominant type in the strains that were studied; CTX-M-14 was present in two strains while some of the other CTX-M variant types could not be identified using the PCR primers used in this study: many possibilities were given when sequences were searched using the NCBI blast search and CARD databases, and no specific variant could be identified. These strains with many other CTX-M variants have been reported elsewhere in cattle (Shiraki et al., 2004, Duan et al., 2006, Madec et al., 2008, Kirchner et al., 2011, Hartmann et al., 2012, Watson et al., 2012, Cottell et al., 2013, Tamang et al., 2013a). The occurrence of *bla*$_{TEM}$ (1a and 30) was found in combination with *bla*$_{OXA}$ (OXA-1) in two of the farm isolates (A2.5 and BS7) in the current study; these genes have been widely reported in *E. coli* from cattle faeces, either as separate genes or in combination with each other, or with other β-lactam resistance genes (Karczmarczyk et al., 2011, Tamang et al., 2013a, Timofte et al., 2014, Dahms et al., 2015).

Genotyping using ERIC-PCR (Figure 3.6), showed that most of the strains that carried CTX-M genes were in a closely related cluster (represented by cluster I in the dendrogram), however there were some strains that did not

cluster with this closely related cluster in the ERIC dendrogram such as strains BS9, BSE2, and BSE6 in cluster II and strain BSE8 in cluster VI. This might be due to the carriage of some of these genes on mobile elements which make such genes spread into diverse *E. coli* strains. What supports this hypothesis further are the different resistance profiles for some strains which shared a 100% similarity in their ERIC profiles. Strains BSE8 and BSE7 share an identical ERIC profile, but BSE8 is an ESBL strain, while BSE7 is not. The same thing occurs with strain BS9 (which carries $bla_{\text{CTX-M-32}}$), which shares an identical ERIC profile with seven other strains, none of which is an ESBL strain carrying $bla_{\text{CTX-M-32,}}$ except for BLC4 which is phenotypically ESBL due to resistance to CAZ but had no detectable ESBL genes. The same is the case with BSE6 which shared a 100% similarity with BCC3, a phenotypically AmpC strain but which does not carry any ESBL genes. Comparing this genetic clustering arrangement to the clustering of these stains based on their resistance profiles in the heatmap, another organization can be seen as almost all the ESBL-producing strains cluster in one group which is the "CHROMagar ESBL" group, as this depends on the phenotypic profile for these strains, but exceptions such as BCC2 and BCE3 can also be seen. The different clustering for these 2 strains (BCC2 and BCE3) is due to the difference in their phenotypic profiles as they do not show phenotypic resistance to CAZ but the other ESBL-producing *E. coli* strains do. This might be due to the different CTX-M variant that these two strains have (CTX-M-14) which has more hydrolytic activity against cefotaxime than ceftazidime (Yin et al., 2009). BCC2 and BCE3 strains also show resistance to ciprofloxacin and enrofloxacin while the other strains do not. The other 6 strains carry CTX-M-32, exhibiting hydrolytic activity

towards cefotaxime and ceftazidime, this hydrolytic activity against ceftazidime was very low in its progenitor CTX-M-1 (Cartelle et al., 2004).

Chapter 5 provides a significant contribution towards a better understanding of the diversity of multiple AMR genes in the *E. coli* population in this study. Sequencing the whole genome of ESBL and non-ESBL strains enabled understanding of the genetic/genomic environments for different genes, and in particular ESBL genes, leading to a greater understanding of the spread of resistance. The finding that most of the AMR genes were found on transposable elements is really of concern and in particular the presence of $bla_{\text{CTX-M-14a}}$ with an IS*Ecp1* related transposable element. Despite the fact that this gene was inserted into *fdeC* in the chromosome, the complete sequence of it with the presence of the IRs might represent active transposition units as discussed in Chapter 5; transfer to another position in the chromosome or to a plasmid is possible. The presence in individual *E. coli* strains of this element and other elements such as IS*CR2*, a Tn*21*-like transposon, Tn*2*, a putative plasmid, and composite transposons such as the one formed by 2 copies of IS*26,* all carrying different AMR genes, can increase the possibility of the spread of these genes to the environment via the dissemination of these genes to other bacteria which might be from the same species or to different genera. This can mainly happen if these transposable elements are carried by a plasmid as it will facilitate the transfer of these genes via HGT. *E. coli* carrying the same plasmid with ESBL genes, which were found to be disseminated in both animals and humans, has been described before (Stokes et al., 2012, Madec et al., 2012). Once the AMR genes spread into the environment they can be

disseminated into different hosts, because bacteria with the same ST are present in humans and animals, and this can be seen from the results of the present study for, as an example, BS7 (ST69) and A2.5 (ST56) both of which sequence types have been described previously in humans and animals (Vincent et al., 2010, Hauser et al., 2013, Dahms et al., 2015). The sequenced strains represent 3 different clusters of bacteria with regards to their ERIC profiles; BCC2 and BCE3 share approximately the same AMR phenotype and genotype and both are ESBL-producing strains. BS7 was an outlier and is non-ESBL, and A2.5 was from a distinct cluster and represents an isolate from a different time period compared to the other strains and the non–ESBL producing strain. However from the selected gene combination clusters we can see that similar gene clusters can be present in those different strains, such as $bla_{\text{OXA-1}}$ and *aadA1* within a class I integron carried by the Tn*21*-like transposon in BS7 and A2.5. Tetracycline resistance genes were carried on Tn*10* in BCC2, BCE3 and A2.5. The presence of these different clusters of AMR genes in these non-clonal strains (except for BCC2 and BCE3) might suggests a major role of horizontal gene transfer. Diversity of the gene cassettes detected in class I integrons which were mainly associated with Tn*21*-like transposons in all the strains, shows that these systems are structurally different and represent complex genetic backgrounds. The capture of AMR genes by class I integrons or through IS-mediated recombination can explain the successful dissemination of these resistance genes in the environment.

The contribution of the animal reservoir of AMR genes to the burden of antimicrobial resistance should be taken into consideration especially

regarding the antimicrobial agents that are used for treatment of infections in humans (Hammerum and Heuer, 2009). The reduction of antibiotic use can reduce the resistance toward these antibiotics; this has mainly been seen with cephalosporins in the farm, the source of strains in this study. The use of cephalosporins was reduced in 2012/2013, and in 2015 no ESBL-producing *E. coli* were isolated from slurry samples taken from the same farm (Chapman, 2016). The reduction of the use of cephalosporins and its effect to reduce the presence of ESBL-producing bacteria has been observed in many other studies (Bamberger and Dahl, 1992, Agerso and Aarestrup, 2013, Chantziaras et al., 2014, NethMap, 2014).

In 1997 organizations such as the World Health Organization (WHO), the International Organization for Epizootics and the Food and Agriculture Organization, suggested limitations on the use of antimicrobials, especially for antimicrobials that are used in agriculture for non-therapeutic purposes, and for greater veterinary oversight on the use of antimicrobials in food animals (WHO., 2001). In 2012, EFSA (European Food Safety Authority) stated that reports on the occurrence of antimicrobial resistance in the indicator organism, *E. coli*, should become mandatory from 2013 onwards, and should be included in each surveillance programme of an EU member state (Chantziaras et al., 2014). In the UK, Veterinary Medicines Directorate (VMD) have developed a surveillance system to monitor antimicrobial resistance trends and providing detailed reports of UK veterinary antimicrobial resistance and sales (https://www.gov.uk).

The consequence of using antimicrobials in animal husbandry has been studied from the economic point of view as well; Van Boeckel and co-

workers used Bayesian statistical models to model antimicrobial use in food animals for 2010 to 2030. This study includes high-income countries based on current estimations of antimicrobial consumption, combining maps of livestock densities and economic estimates of demand for meat products. They found the models suggested consumption of antimicrobials will increase by 67% by 2030 (Van Boeckel et al., 2015).

In conclusion, this study showed the presence of MDR and ESBL-producing *E. coli* in the farm environment, the source of the collected samples. The prevalence of these strains varied depending on source; more *E. coli* were isolated from solid slurry than liquid slurry samples, which indicates the potentially higher risk of spreading of this directly to the land; however the risk from spreading liquid slurry should not be ignored. The genotypic diversity of these strains and their relation to their AMR phenotype indicates the presence of clonal and non-clonal relationships between those different strains. This might indicates the presence of these genes on transposable elements which could enable the spread of these genes to the environment via HGT. The presence of different variants of ESBL genes, represented by CTX-M sequence types, indicates the presence of more than one genetic environment for these genes, which is very likely to be on a plasmid due to the successful plasmid transfer for two selected strains (BCE1 and BCE2) using plate and broth mating, confirmed using different confirmation tests. The comparative genomic analysis for the four selected genome-sequenced strains shows that most of AMR genes were found to be carried on transposable elements and putatively on a plasmid. Using whole genome sequencing allows us to understand the different AMR phenotypes of the

different strains. The presence of 4 strains with an ST which has been reported previously in both humans and animals might indicate the success of these strains in surviving in different environments.

6.2 Future work

Strains isolated in this study represent a potential reservoir of AMR resistance genes, and further work will be of great value in understanding the nature of multi-resistance for these strains. One of the important aspects of this study that could be tested more is detection of plasmid-borne CTX-M genes, and what other AMR genes are carried on the same plasmid. Due to time limitations only a preliminary test was performed to check the presence of *bla*$_{CTX-M}$ on plasmids using conjugation experiments. The problem with discriminating between real transconjugants and azide resistant spontaneous mutants of the donor strains was not expected, and not reported by other studies using the protocols followed (Jacoby et al., 2003, Wang et al., 2004, Liu et al., 2007, Wang et al., 2008, Bonnin et al., 2012, Warnes et al., 2012). A better recipient should be chosen or developed, which can be differentiated phenotypically from the donor strain, such as *Salmonella* sp., or *E. coli* strains with other non-antibiotic resistance phenotypic markers. In addition, using a better method to isolate plasmid DNA and determining the plasmid size and incompatibility group would be very helpful especially for studies on spread of plasmids. Understanding spread to different places in the farm including the workers and the animals, and into the soil that the slurry has been spread on would allow us to follow to what extent these bacteria can spread into different hosts and survive. At the same time the presence of specific genes in such an environment carried by different bacterial genera could be studied.

Using whole genome sequence for just four selected strains gave an indication about the prevalence of transposable elements in these strains,

however more analysis is need. The bioinformatics analysis concentrated exclusively on the genomic environment of the AMR genes, however further analysis on the different efflux systems and checking for mutations in the genes for each efflux system would be very helpful to understand some phenotypic resistance profiles which do not seem to be due to the presence of a specific known resistance gene.

Initial work was started to determine the complete differences between the different Tn*21*-like transposons in the four sequenced strains. Once completed this will be used for publication with other data related to the identification of class I integrons, *merA* and *merC* as a marker for the presence of Tn*21*-like transposons which was not presented in this thesis. Similarly the data for 35 *E. coli* strains, isolated from different human infection sites, for which all the tests which were performed for the farm isolates were performed, have not been included in this thesis. Two of these strains (H5B and H22U) were fully sequenced, but, due to the time limitations, analysis of the results will be performed later and also used in a publication.

Detecting the role of phage to transfer AMR genes in these strains will be of great importance which can aid our understanding of the role of such elements to transfer AMR in different hosts.

Although these are commensal strains, and when selected on TBX the system used excludes the possibility of isolating *E. coli* O157:H7, detecting the pathogenicity of the strains via searching some virulence elements

would be very helpful to link between the AMR type and the pathogenicity possibility for these strains even if they are not Shiga toxins producers.

References

AARESTRUP, F. M., AHRENS, P., MADSEN, M., PALLESEN, L. V., POULSEN, R. L. & WESTH, H. 1996. Glycopeptide susceptibility among Danish *Enterococcus faecium* and *Enterococcus faecalis* isolates of animal and human origin and PCR identification of genes within the *vanA* cluster. *Journal of Antimicrobial Chemotherapy,* 40**,** 1938-40.

AARESTRUP, F. M., BAGER, F., JENSEN, N., MADSEN, M., MEYLING, A. & WEGENER, H. C. 1998. Resistance to antimicrobial agents used for animal therapy in pathogenic-, zoonotic-and indicator bacteria isolated from different food animals in Denmark: a baseline study for the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP). *Apmis,* 106**,** 745-770.

ABBOTT, J. C., AANENSEN, D. M. & BENTLEY, S. D. 2008. WebACT: an online genome comparison suite. *Comparative Genomics***,** 57-74.

ABRAHAM, E. P. & CHAIN, E. 1940. An enzyme from bacteria able to destroy penicillin. *Nature,* 146**,** 837.

ADATO, O., NINYO, N., GOPHNA, U. & SNIR, S. 2015. Detecting Horizontal Gene Transfer between Closely Related Taxa. *PLoS Computational Biology,* 11**,** e1004408.

ADIRI, R. S., GOPHNA, U. & RON, E. Z. 2003. Multilocus sequence typing (MLST) of *Escherichia coli* O78 strains. *FEMS Microbiology Letters,* 222**,** 199-203.

AGERSO, Y. & AARESTRUP, F. M. 2013. Voluntary ban on cephalosporin use in Danish pig production has effectively reduced extended-spectrum cephalosporinase-producing *Escherichia coli* in slaughter pigs. *Journal of Antimicrobial Chemotherapy,* 68**,** 569-72.

ALEKSHUN, M. N. & LEVY, S. B. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. *Antimicrobial Agents and Chemotherapy,* 41**,** 2067-2075.

ALI, T., UR RAHMAN, S., ZHANG, L., SHAHID, M., ZHANG, S., LIU, G., GAO, J. & HAN, B. 2016. ESBL-Producing *Escherichia coli* from Cows Suffering Mastitis in China Contain Clinical Class 1 Integrons with CTX-M Linked to IS*CR1*. *Frontiers in Microbiology,* 7.

ALLEN, H. K., DONATO, J., WANG, H. H., CLOUD-HANSEN, K. A., DAVIES, J. & HANDELSMAN, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology,* 8**,** 251-259.

ALLMEIER, H., CRESNAR, B., GRECK, M. & SCHMITT, R. 1992. Complete nucleotide sequence of Tn*1721*: gene organization and a novel gene product with features of a chemotaxis protein. *Gene,* 111**,** 11-20.

ALTERI, C. J., SMITH, S. N. & MOBLEY, H. L. 2009. Fitness of *Escherichia coli* during urinary tract infection requires gluconeogenesis and the TCA cycle. *PLoS Pathog,* 5**,** e1000448.

References

ALVARADO, A., GARCILLáN-BARCIA, M. P. & DE LA CRUZ, F. 2012. A Degenerate Primer MOB Typing (DPMT) Method to Classify Gamma-Proteobacterial Plasmids in Clinical and Environmental Settings. *PLoS ONE,* 7**,** e40438.

AMBLER, R. P. 1980. The structure of beta-lactamases. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 289**,** 321-31.

AMINOV, R. I. 2010. A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Frontiers in Microbiology,* 1**,** 134.

ANDERSON, R., GROUNDWATER, P. & TODD, A. 2012. *Antibacterial Agents : Chemistry, Mode of Action, Mechanisms of Resistance and Clinical Applications,* Hoboken, NJ, USA, John Wiley & Sons.

ANDREWS, J. & HOWE, R. 2011. BSAC standardized disc susceptibility testing method (version 10). *Journal of Antimicrobial Chemotherapy,* 66**,** 2726-2757.

ANTUNES, P., MACHADO, J., SOUSA, J. C. & PEIXE, L. 2004. Dissemination amongst humans and food products of animal origin of a *Salmonella typhimurium* clone expressing an integron-borne OXA-30 β-lactamase. *Journal of Antimicrobial Chemotherapy,* 54**,** 429-434.

ARAKAWA, Y., MURAKAMI, M., SUZUKI, K., ITO, H., WACHAROTAYANKUN, R., OHSUKA, S., KATO, N. & OHTA, M. 1995. A novel integron-like element carrying the metallo-beta-lactamase gene bla_{IMP}. *Antimicrobial Agents and Chemotherapy,* 39**,** 1612-5.

ARDUINO, S. M., ROY, P. H., JACOBY, G. A., ORMAN, B. E., PINEIRO, S. A. & CENTRON, D. 2002. $bla_{CTX-M-2}$ is located in an unusual class 1 integron (In*35*) which includes *orf513*. *Antimicrobial Agents and Chemotherapy,* 46**,** 2303-6.

ASHBOLT, N. J., AMEZQUITA, A., BACKHAUS, T., BORRIELLO, P., BRANDT, K. K., COLLIGNON, P., COORS, A., FINLEY, R., GAZE, W. H. & HEBERER, T. 2013. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environmental Health Perspectives,* 121**,** 993-1001.

ASTM 1998. Standard Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods, D5465-93.

AZIZ, R. K., BREITBART, M. & EDWARDS, R. A. 2010. Transposases are the most abundant, most ubiquitous genes in nature. *Nucleic Acids Research,* 38**,** 4207-17.

BAE, I. K., LEE, B. H., HWANG, H. Y., JEONG, S. H., HONG, S. G., CHANG, C. L., KWAK, H.-S., KIM, H. J. & YOUN, H. 2006. A novel ceftazidime-hydrolysing extended-spectrum β-lactamase, CTX-M-54, with a single amino acid substitution at position 167 in the omega loop. *Journal of Antimicrobial Chemotherapy,* 58**,** 315-319.

BAE, I. K., LEE, Y. N., LEE, W. G., LEE, S. H. & JEONG, S. H. 2007. Novel complex class 1 integron bearing an IS*CR1* element in an *Escherichia coli* isolate carrying the $bla_{CTX-M-14}$ gene. *Journal of Antimicrobial Chemotherapy,* 51**,** 3017-9.

References

BAMBERGER, D. M. & DAHL, S. L. 1992. Impact of voluntary vs enforced compliance of third-generation cephalosporin use in a teaching hospital. *Archives of Internal Medicine,* 152**,** 554-557.

BANKEVICH, A., NURK, S., ANTIPOV, D., GUREVICH, A. A., DVORKIN, M., KULIKOV, A. S., LESIN, V. M., NIKOLENKO, S. I., PHAM, S. & PRJIBELSKI, A. D. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology,* 19**,** 455-477.

BARBOSA, T. M. & LEVY, S. B. 2000. The impact of antibiotic use on resistance development and persistence. *Drug Resistance Updates,* 3**,** 303-311.

BARROW, G. & FELTHAM, R. K. A. 2004. *Cowan and Steel's manual for the identification of medical bacteria*, Cambridge university press.

BARTON, B. M., HARDING, G. P. & ZUCCARELLI, A. J. 1995. A general method for detecting and sizing large plasmids. *Analytical Biochemistry,* 226**,** 235-40.

BARZA, M. 2002. Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals. *Clinical Infectious Diseases,* 34**,** S123-S125.

BAUERNFEIND, A., CASELLAS, J. M., GOLDBERG, M., HOLLEY, M., JUNGWIRTH, R., MANGOLD, P., ROHNISCH, T., SCHWEIGHART, S. & WILHELM, R. 1992. A new plasmidic cefotaximase from patients infected with *Salmonella typhimurium*. *Infection,* 20**,** 158-63.

BAUERNFEIND, A., GRIMM, H. & SCHWEIGHART, S. 1990. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. *Infection,* 18**,** 294-8.

BAUR, B., HANSELMANN, K., SCHLIMME, W. & JENNI, B. 1996. Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. *Applied and Environmental Microbiology,* 62**,** 3673-8.

BELAAOUAJ, A., LAPOUMEROULIE, C., CANICA, M. M., VEDEL, G., NEVOT, P., KRISHNAMOORTHY, R. & PAUL, G. 1994. Nucleotide sequences of the genes coding for the TEM-like β-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). *FEMS Microbiology Letters,* 120**,** 75-80.

BENNETT, P. M. 2004. Transposable elements. In: Schaechter M (ed) The Desk Encyclopedia of Microbiology. Elsevier Academic Press: San Diego, CA, pp 1025–1041.

BENNETT, P. M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology,* 153 Suppl 1**,** S347-57.

BERGSTEN, G., WULLT, B. & SVANBORG, C. 2005. *Escherichia coli*, fimbriae, bacterial persistence and host response induction in the human urinary tract. *International Journal of Medical Microbiology,* 295**,** 487-502.

BERLIN, K., KOREN, S., CHIN, C.-S., DRAKE, J. P., LANDOLIN, J. M. & PHILLIPPY, A. M. 2015. Assembling large genomes with single-molecule sequencing and locality-sensitive hashing. *Nature Biotechnology,* 33**,** 623-630.

References

BINH, C. T., HEUER, H., GOMES, N. C., KOTZERKE, A., FULLE, M., WILKE, B. M., SCHLOTER, M. & SMALLA, K. 2007. Short-term effects of amoxicillin on bacterial communities in manured soil. *FEMS Microbiology Ecology,* 62**,** 290-302.

BLACK, J. A., MOLAND, E. S. & THOMSON, K. S. 2005. AmpC Disk Test for Detection of Plasmid-Mediated AmpC β-Lactamases in Enterobacteriaceae Lacking Chromosomal AmpC β-Lactamases. *Journal of Clinical Microbiology,* 43**,** 3110-3113.

BLICKWEDE, M. & SCHWARZ, S. 2004. Molecular analysis of florfenicol-resistant *Escherichia coli* isolates from pigs. *Journal of Antimicrobial Chemotherapy,* 53**,** 58-64.

BONNET, R. 2004. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial Agents and Chemotherapy,* 48**,** 1-14.

BONNIN, R. A., POIREL, L., CARATTOLI, A. & NORDMANN, P. 2012. Characterization of an IncFII Plasmid Encoding NDM-1 from *Escherichia coli* ST131. *PLoS ONE,* 7**,** e34752.

BORTOLAIA, V., GUARDABASSI, L., TREVISANI, M., BISGAARD, M., VENTURI, L. & BOJESEN, A. M. 2010. High diversity of extended-spectrum β-lactamases in *Escherichia coli* isolates from Italian broiler flocks. *Antimicrobial Agents and Chemotherapy,* 54**,** 1623-1626.

BOSCH, F. & ROSICH, L. 2008. The contributions of Paul Ehrlich to pharmacology: a tribute on the occasion of the centenary of his Nobel Prize. *Pharmacology,* 82**,** 171-9.

BOXALL, A. B., JOHNSON, P., SMITH, E. J., SINCLAIR, C. J., STUTT, E. & LEVY, L. S. 2006. Uptake of veterinary medicines from soils into plants. *Journal of Agricultural and Food Chemistry,* 54**,** 2288-97.

BRADFORD, P. A. 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews,* 14**,** 933-51.

BRENWALD, N. P., JEVONS, G., ANDREWS, J., ANG, L. & FRAISE, A. P. 2005. Disc methods for detecting AmpC β-lactamase-producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy,* 56**,** 600-601.

BRINAS, L., ZARAZAGA, M., SáENZ, Y., RUIZ-LARREA, F. & TORRES, C. 2002. β-Lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. *Antimicrobial Agents and Chemotherapy,* 46**,** 3156-3163.

BROLUND, A. 2014. Overview of ESBL-producing Enterobacteriaceae from a Nordic perspective. *Infection Ecology and Epidemiology,* 4.

BROOKS, G. F., CARROLL, K. C., BUTEL, J. S., MORSE, S.T. & AND MIETZNE, T. A. 2010. *Jawetz , Melnick & Adelberg's Medical Microbiology.* 25th ed.USA: McGraw-Hill

References

BROWN, D. 2000. Quality assurance of antimicrobial susceptibility testing. *Quality assurance: principles and practice in the microbiology laboratory. Public Health Laboratory Service,* London, England**,** 151-168.

BUSH, K. 1989. Characterization of beta-lactamases. *Antimicrobial Agents and Chemotherapy,* 33**,** 259.

BUSH, K. & JACOBY, G. A. 2010. Updated functional classification of beta-lactamases. *Antimicrobial Agents and Chemotherapy,* 54**,** 969-76.

BUSH, K., JACOBY, G. A. & MEDEIROS, A. A. 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy,* 39**,** 1211-33.

CAMBRAY, G., GUEROUT, A. M. & MAZEL, D. 2010. Integrons. *Annual Review of Genetics,* 44**,** 141-66.

CANTON, R., GONZALEZ-ALBA, J. M. & GALAN, J. C. 2012. CTX-M Enzymes: Origin and Diffusion. *Frontiers in Microbiology,* 3**,** 110.

CARATTOLI, A. 2001. Importance of integrons in the diffusion of resistance. *Veterinary Research,* 32**,** 243-59.

CARATTOLI, A. 2008. Animal reservoirs for extended spectrum β-lactamase producers. *Clinical Microbiology and Infection,* 14**,** 117-123.

CARATTOLI, A. 2009. Resistance plasmid families in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy,* 53**,** 2227-2238.

CARATTOLI, A. 2013. Plasmids and the spread of resistance. *International Journal of Medical Microbiology,* 303**,** 298-304.

CARATTOLI, A., BERTINI, A., VILLA, L., FALBO, V., HOPKINS, K. L. & THRELFALL, E. J. 2005. Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods,* 63**,** 219-228.

CARATTOLI, A., ZANKARI, E., GARCíA-FERNáNDEZ, A., LARSEN, M. V., LUND, O., VILLA, L., AARESTRUP, F. M. & HASMAN, H. 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrobial Agents and Chemotherapy,* 58**,** 3895-3903.

CAROFF, N., ESPAZE, E., BERARD, I., RICHET, H. & REYNAUD, A. 1999. Mutations in the *ampC* promoter of *Escherichia coli* isolates resistant to oxyiminocephalosporins without extended spectrum beta-lactamase production. *FEMS Microbiology Letters,* 173**,** 459-65.

CARTELLE, M., DEL MAR TOMAS, M., MOLINA, F., MOURE, R., VILLANUEVA, R. & BOU, G. 2004. High-level resistance to ceftazidime conferred by a novel enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240-Gly substitution. *Antimicrobial Agents and Chemotherapy,* 48**,** 2308-2313.

CARVER, T. J., RUTHERFORD, K. M., BERRIMAN, M., RAJANDREAM, M.-A., BARRELL, B. G. & PARKHILL, J. 2005. ACT: the Artemis comparison tool. *Bioinformatics,* 21**,** 3422-3423.

References

CASELLA, T., RODRIGUEZ, M. M., TAKAHASHI, J. T., GHIGLIONE, B., DROPA, M., ASSUNCAO, E., NOGUEIRA, M. L., LINCOPAN, N., GUTKIND, G. & NOGUEIRA, M. C. 2015. Detection of *bla*$_{CTX-M}$-type genes in complex class 1 integrons carried by Enterobacteriaceae isolated from retail chicken meat in Brazil. *International Journal of Food Microbiology,* 197**,** 88-91.

CHALMERS, R., SEWITZ, S., LIPKOW, K. & CRELLIN, P. 2000. Complete nucleotide sequence of Tn*10*. *Journal of Bacteriology,* 182**,** 2970-2972.

CHALOUPKA, K., MALAM, Y. & SEIFALIAN, A. M. 2010. Nanosilver as a new generation of nanoproduct in biomedical applications. *Trends in Biotechnology,* 28**,** 580-8.

CHANTZIARAS, I., BOYEN, F., CALLENS, B. & DEWULF, J. 2014. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *Journal of Antimicrobial Chemotherapy,* 69**,** 827-834.

CHAPMAN, S. M. O. 2016. Characterisation of antimicrobial resistant *Escherichia coli* isolated from a dairy farm. MRes thesis, University of Nottingham.

CHAUDHURI, R. R., SEBAIHIA, M., HOBMAN, J. L., WEBBER, M. A., LEYTON, D. L., GOLDBERG, M. D., CUNNINGHAM, A. F., SCOTT-TUCKER, A., FERGUSON, P. R., THOMAS, C. M., FRANKEL, G., TANG, C. M., DUDLEY, E. G., ROBERTS, I. S., RASKO, D. A., PALLEN, M. J., PARKHILL, J., NATARO, J. P., THOMSON, N. R. & HENDERSON, I. R. 2010. Complete genome sequence and comparative metabolic profiling of the prototypical enteroaggregative *Escherichia coli* strain 042. *PLoS One,* 5**,** e8801.

CHOPRA, I. & ROBERTS, M. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology reviews,* 65**,** 232-260.

CHOUCHANI, C., EL SALABI, A., MARRAKCHI, R., FERCHICHI, L. & WALSH, T. R. 2012. Characterization of IncA/C conjugative plasmid harboring bla TEM-52 and *bla*$_{CTX-M-15}$ extended-spectrum beta-lactamases in clinical isolates of *Escherichia coli* in Tunisia. *European Journal of Clinical Microbiology and Infectious Diseases,* 31**,** 1081-7.

CHOWDHURY, P. R., CHARLES, I. G. & DJORDJEVIC, S. P. 2015. A Role for Tn*6029* in the Evolution of the Complex Antibiotic Resistance Gene Loci in Genomic Island 3 in Enteroaggregative Hemorrhagic *Escherichia coli* O104:H4. *PLoS ONE,* 10**,** e0115781.

CLERMONT, O., BONACORSI, S. & BINGEN, E. 2000. Rapid and Simple Determination of the *Escherichia coli* Phylogenetic Group. *Applied and Environmental Microbiology,* 66**,** 4555-4558.

CLERMONT, O., CHRISTENSON, J. K., DENAMUR, E. & GORDON, D. M. 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environmental Microbiology Reports,* 5**,** 58-65.

CLEWELL, D. B., FLANNAGAN, S. E. & JAWORSKI, D. D. 1995. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. *Trends in Microbiology,* 3**,** 229-36.

References

CLOECKAERT, A., BAUCHERON, S., FLAUJAC, G., SCHWARZ, S., KEHRENBERG, C., MARTEL, J.-L. & CHASLUS-DANCLA, E. 2000. Plasmid-mediated florfenicol resistance encoded by the *floR* gene in *Escherichia coli* isolated from cattle. *Antimicrobial Agents and Chemotherapy,* 44**,** 2858-2860.

CLSI 2012. Methods for Dilution Antimicrobial Susceptibility Tests f or Bacteria That Grow Aerobically Approved St andard—Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI 2013a. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-third Informational Supplement. CLSI document M100-S23.

CLSI 2013b. (M31-A4)Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; draft, 2013, and M31-S2 (2nd Informational Supplement), provided by NEO-SENSITABS™ Document: 9.1.0 Veterinary practice according to CLSI breakpoints.

COLLIS, C. M., KIM, M.-J., PARTRIDGE, S. R., STOKES, H. W. & HALL, R. M. 2002. Characterization of the Class 3 Integron and the Site-Specific Recombination System It Determines. *Journal of Bacteriology,* 184**,** 3017-3026.

CONTROL, C. F. D. & PREVENTION 2013. Antibiotic resistance threats in the United States, 2013, *Centres for Disease Control and Prevention, US Department of Health and Human Services*.

COQUE, T. M., NOVAIS, Â., CARATTOLI, A., POIREL, L., PITOUT, J., PEIXE, L., BAQUERO, F., CANTóN, R. & NORDMANN, P. 2008. Dissemination of Clonally Related *Escherichia coli* Strains Expressing Extended-Spectrum β-Lactamase CTX-M-15. *Emerging Infectious Diseases,* 14**,** 195-200.

COTTELL, J. L., KANWAR, N., CASTILLO-COURTADE, L., CHALMERS, G., SCOTT, H. M., NORBY, B., LONERAGAN, G. H. & BOERLIN, P. 2013. $bla_{\text{CTX-M-32}}$ on an IncN plasmid in *Escherichia coli* from beef cattle in the United States. *Antimicrobial Agents and Chemotherapy,* 57**,** 1096-1097.

COTTELL, J. L., WEBBER, M. A., COLDHAM, N. G., TAYLOR, D. L., CERDENO-TARRAGA, A. M., HAUSER, H., THOMSON, N. R., WOODWARD, M. J. & PIDDOCK, L. J. 2011. Complete sequence and molecular epidemiology of IncK epidemic plasmid encoding $bla_{\text{CTX-M-14}}$. *Emerging Infectious Diseases,* 17**,** 645-52.

COURVALIN, P. 1996. The Garrod Lecture. Evasion of antibiotic action by bacteria. *Journal of Antimicrobial Chemotherapy,* 37**,** 855-69.

COWAN, M. M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews,* 12**,** 564-82.

COYNE, S., COURVALIN, P. & PERICHON, B. 2011. Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrobial Agents and Chemotherapy,* 55**,** 947-953.

CRAIG, N. L. 1997. Target site selection in transposition. *Annual Review of Biochemistry,* 66**,** 437-74.

CROXEN, M. A. & FINLAY, B. B. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. *Nature Reviews Microbiology,* 8**,** 26-38.

References

DAHMS, C., HUBNER, N. O., KOSSOW, A., MELLMANN, A., DITTMANN, K. & KRAMER, A. 2015. Occurrence of ESBL-Producing *Escherichia coli* in Livestock and Farm Workers in Mecklenburg-Western Pomerania, Germany. *PLoS One,* 10**,** e0143326.

DALLENNE, C., DA COSTA, A., DECRE, D., FAVIER, C. & ARLET, G. 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy,* 65**,** 490-5.

DAVIES, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. *Science,* 264**,** 375-82.

DAVIES, J. 2007. Microbes have the last word. *EMBO reports,* 8**,** 616-621.

DAVIES, J. & DAVIES, D. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews,* 74**,** 417-33.

DAVIES, S.C., FOWLER, T., WATSON, J., LIVERMORE, D.M. AND WALKER, D., 2013. Annual Report of the Chief Medical Officer: infection and the rise of antimicrobial resistance. *The Lancet*, 1606-1609.

DAVIS, M. F. & RUTKOW, L. 2011. Regulatory Strategies to Combat Antimicrobial Resistance of Animal Origin: Recommendations for a Science-Based US Approach. *Tulane Environmental Law Journal,* 25**,** 327.

DAVISON, J. 1999. Genetic exchange between bacteria in the environment. *Plasmid,* 42**,** 73-91.

DAWES, F. E., KUZEVSKI, A., BETTELHEIM, K. A., HORNITZKY, M. A., DJORDJEVIC, S. P. & WALKER, M. J. 2010. Distribution of Class 1 Integrons with IS*26*-Mediated Deletions in Their 3′-Conserved Segments in *Escherichia coli* of Human and Animal Origin. *PLoS ONE,* 5**,** e12754.

DAY, M. J., RODRIGUEZ, I., VAN ESSEN-ZANDBERGEN, A., DIERIKX, C., KADLEC, K., SCHINK, A. K., WU, G., CHATTAWAY, M. A., DONASCIMENTO, V., WAIN, J., HELMUTH, R., GUERRA, B., SCHWARZ, S., THRELFALL, J., WOODWARD, M. J., COLDHAM, N., MEVIUS, D. & WOODFORD, N. 2016. Diversity of STs, plasmids and ESBL genes among *Escherichia coli* from humans, animals and food in Germany, the Netherlands and the UK. *Journal of Antimicrobial Chemotherapy,* 71**,** 1178-82.

DE LIMA PROCóPIO, R. E., DA SILVA, I. R., MARTINS, M. K., DE AZEVEDO, J. L. & DE ARAúJO, J. M. 2012. Antibiotics produced by *Streptomyces*. *The Brazilian Journal of Infectious Diseases,* 16**,** 466-471.

DE LORENZO, V. & TIMMIS, K. N. 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. *Methods in Enzymology.* Academic Press.

DEFRA 2012. Sales of antimicrobial products authorised for use as veterinary medicines in the UK in 2011. URL http://www.vmd.defra.gov.uk/pdf/ salesanti11.pdf.

DEL SOLAR, G., GIRALDO, R., RUIZ-ECHEVARRíA, M. J., ESPINOSA, M. & DíAZ-OREJAS, R. 1998. Replication and control of circular bacterial plasmids. *Microbiology and Molecular Biology Reviews,* 62**,** 434-464.

References

DENAP, J. C. & HERGENROTHER, P. J. 2005. Bacterial death comes full circle: targeting plasmid replication in drug-resistant bacteria. *Organic and Biomolecular Chemistry,* 3**,** 959-966.

DI CONZA, J., AYALA, J. A., POWER, P., MOLLERACH, M. & GUTKIND, G. 2002. Novel Class 1 Integron (InS*21*) Carrying *bla*$_{CTX-M-2}$ in *Salmonella enterica* Serovar Infantis. *Antimicrobial Agents and Chemotherapy,* 46**,** 2257-2261.

DIERIKX, C., VAN ESSEN-ZANDBERGEN, A., VELDMAN, K., SMITH, H. & MEVIUS, D. 2010. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Veterinary Microbiology,* 145**,** 273-278.

DIERIKX, C. M., VAN DUIJKEREN, E., SCHOORMANS, A. H., VAN ESSEN-ZANDBERGEN, A., VELDMAN, K., KANT, A., HUIJSDENS, X. W., VAN DER ZWALUW, K., WAGENAAR, J. A. & MEVIUS, D. J. 2012. Occurrence and characteristics of extended-spectrum-beta-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. *Journal of Antimicrobial Chemotherapy,* 67**,** 1368-74.

DIESTRA, K., JUAN, C., CURIAO, T. N., MOYá, B., MIRó, E., OTEO, J., COQUE, T. M., PEREZ-VáZQUEZ, M., CAMPOS, J. & CANTóN, R. 2009. Characterization of plasmids encoding *bla*$_{ESBL}$ and surrounding genes in Spanish clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy,* 63**,** 60-66.

DJORDJEVIC, S. P., STOKES, H. W. & CHOWDHURY, P. R. 2013. Mobile elements, zoonotic pathogens and commensal bacteria: conduits for the delivery of resistance genes into humans, production animals and soil microbiota. *Frontiers in Microbiology,* 4**,** 1-12.

DOLEJSKA, M., VILLA, L., HASMAN, H., HANSEN, L. & CARATTOLI, A. 2013. Characterization of IncN plasmids carrying *bla*$_{CTX-M-1}$ and qnr genes in *Escherichia coli* and *Salmonella* from animals, the environment and humans. *Journal of Antimicrobial Chemotherapy,* 68**,** 333-339.

DONADO-GODOY, P., CASTELLANOS, R., LEóN, M., AREVALO, A., CLAVIJO, V., BERNAL, J., LEóN, D., TAFUR, M. A., BYRNE, B. A., SMITH, W. A. & PEREZ-GUTIERREZ, E. 2015. The Establishment of the Colombian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS): A Pilot Project on Poultry Farms, Slaughterhouses and Retail Market. *Zoonoses and Public Health,* 62**,** 58-69.

DOUBLET, B., SCHWARZ, S., KEHRENBERG, C. & CLOECKAERT, A. 2005. Florfenicol resistance gene *floR* is part of a novel transposon. *Antimicrobial Agents and Chemotherapy,* 49**,** 2106-2108.

DOUBLET, B., SCHWARZ, S., NUßBECK, E., BAUCHERON, S., MARTEL, J.-L., CHASLUS-DANCLA, E. & CLOECKAERT, A. 2002. Molecular analysis of chromosomally florfenicol-resistant *Escherichia coli* isolates from France and Germany. *Journal of Antimicrobial Chemotherapy,* 49**,** 49-54.

DRIEUX, L., BROSSIER, F., SOUGAKOFF, W. & JARLIER, V. 2008. Phenotypic detection of extended-spectrum beta-lactamase production in Enterobacteriaceae: review and bench guide. *Clinical Microbiology and Infection,* 14 Suppl 1**,** 90-103.

References

DRLICA, K. & ZHAO, X. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiology and Molecular Biology Reviews,* 61**,** 377-392.

DROGE, M., PUHLER, A. & SELBITSCHKA, W. 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Molecular and General Genetics MGG,* 263**,** 471-82.

DUAN, H., CHAI, T., LIU, J., ZHANG, X., QI, C., GAO, J., WANG, Y., CAI, Y., MIAO, Z., YAO, M. & SCHLENKER, G. 2009. Source identification of airborne *Escherichia coli* of swine house surroundings using ERIC-PCR and REP-PCR. *Environmental Research,* 109**,** 511-517.

DUAN, R. S., SIT, T. H., WONG, S. S., WONG, R. C., CHOW, K. H., MAK, G. C., YAM, W. C., NG, L. T., YUEN, K. Y. & HO, P. L. 2006. *Escherichia coli* producing CTX-M beta-lactamases in food animals in Hong Kong. *Microbial Drug Resistance,* 12**,** 145-8.

DUNLOP, R. H., MCEWEN, S. A., MEEK, A. H., CLARKE, R. C., BLACK, W. D. & FRIENDSHIP, R. M. 1998. Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. *Preventive Veterinary Medicine,* 34**,** 283-305.

DURMAZ, S., BUYUKUNAL BAL, B. E., GUNAYDıN, M., YULA, E. & PERCIN, D. 2015. Detection of β-lactamase genes, ERIC-PCR typing and phylogenetic groups of ESBL producing quinolone resistant clinical *Escherichia coli* isolates. *Biomedical Research*, *26*(1).

ECKFORD, S., GRACE, K., HARRIS, C., REEVES, H. & BORRIELLO, S. 2013. UK Veterinary Antibiotic Resistance and Sales Surveillance: UK-VARSS 2013.

EDBERG, S. C., ALLEN, M. J. & SMITH, D. B. 1988. National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: comparison with the standard multiple tube fermentation method. *Applied and Environmental Microbiology,* 54**,** 1595-1601.

EDBERG, S. C., ALLEN, M. J., SMITH, D. B. & KRIZ, N. J. 1990. Enumeration of total coliforms and *Escherichia coli* from source water by the defined substrate technology. *Applied and Environmental Microbiology,* 56**,** 366-369.

EDWARDS, S.E., DA COSTA ROCHA, I., WILLIAMSON, E.M. AND HEINRICH, M., 2015. *Phytopharmacy: An Evidence-Based Guide to Herbal Medicinal Products*. John Wiley & Sons.

EFSA 2015. EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. *EFSA Journal*, *13* (2):p. 4036.

EISEN, J. A. 2000. Horizontal gene transfer among microbial genomes: new insights from complete genome analysis. *Current Opinion in Genetics and Development,* 10**,** 606-11.

References

EMEA 1999. Antibiotic Resistance in the European Union Associated with the Therapeutic Use of Veterinary Medicines. European Agency for the evaluation of medicinal products. *Veterinary Medicines Evaluation Unit*, London.

ENNE, V. I., CASSAR, C., SPRIGINGS, K., WOODWARD, M. J. & BENNETT, P. M. 2008. A high prevalence of antimicrobial resistant *Escherichia coli* isolated from pigs and a low prevalence of antimicrobial resistant *E. coli* from cattle and sheep in Great Britain at slaughter. *FEMS Microbiology Letters,* 278**,** 193-9.

ENRIGHT, M. C. & SPRATT, B. G. 1999. Multilocus sequence typing. *Trends in Microbiology,* 7**,** 482-487.

EPPINGER, M., MAMMEL, M. K., LECLERC, J. E., RAVEL, J. & CEBULA, T. A. 2011. Genome Signatures of *Escherichia coli* O157:H7 Isolates from the Bovine Host Reservoir. *Applied and Environmental Microbiology,* 77**,** 2916-2925.

ERCOLINI, D., HILL, P. J. & DODD, C. E. 2003. Bacterial community structure and location in Stilton cheese. *Applied and Environmental Microbiology,* 69**,** 3540-3548.

ESCUDERO, E., VINUE, L., TESHAGER, T., TORRES, C. & MORENO, M. A. 2010. Resistance mechanisms and farm-level distribution of fecal *Escherichia coli* isolates resistant to extended-spectrum cephalosporins in pigs in Spain. *Research in Veterinary Science,* 88**,** 83-7.

ESSA, A. M., JULIAN, D. J., KIDD, S. P., BROWN, N. L. & HOBMAN, J. L. 2003. Mercury resistance determinants related to Tn*21*, Tn*1696*, and Tn*5053* in enterobacteria from the preantibiotic era. *Antimicrobial Agents and Chemotherapy,* 47**,** 1115-1119.

ETCHUUYA, R., ITO, M., KITANO, S., SHIGI, F., SOBUE, R. & MAEDA, S. 2011. Cell-to-cell transformation in *Escherichia coli*: a novel type of natural transformation involving cell-derived DNA and a putative promoting pheromone. *PLoS One,* 6**,** e16355.

EWERS, C., BETHE, A., STAMM, I., GROBBEL, M., KOPP, P. A., GUERRA, B., STUBBE, M., DOI, Y., ZONG, Z. & KOLA, A. 2014. CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence? *Journal of Antimicrobial Chemotherapy***,** 69**,**1224-1230.

EWERS, C., GROBBEL, M., STAMM, I., KOPP, P. A., DIEHL, I., SEMMLER, T., FRUTH, A., BEUTLICH, J., GUERRA, B. & WIELER, L. H. 2010. Emergence of human pandemic O25: H4-ST131 CTX-M-15 extended-spectrum-β-lactamase-producing *Escherichia coli* among companion animals. *Journal of Antimicrobial Chemotherapy,* 65**,** 651-660.

EXCOFFIER, L., SMOUSE, P. E. & QUATTRO, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics,* 131**,** 479-91.

FARMER, J. J., 3RD, FANNING, G. R., DAVIS, B. R., O'HARA, C. M., RIDDLE, C., HICKMAN-BRENNER, F. W., ASBURY, M. A., LOWERY, V. A., 3RD & BRENNER, D. J. 1985. *Escherichia fergusonii* and *Enterobacter taylorae*, two

new species of Enterobacteriaceae isolated from clinical specimens. *Journal of Clinical Microbiology,* 21**,** 77-81.

FDA 2001. FDA, Bacterial Analytical Manual, Maturin, LJ and JT Peeler. Chapter 3: Aerobic Plate Count. *US Department of Health and Human Services*.

FENG, J.-L., LIN, J.-Y., JIANG, X.-B., ZHANG, O., ZHU, J.-F., HU, J., SHI, L. & CHEN, Q. 2013. Development of an ERIC sequence typing scheme for Laribacter hongkongensis, an emerging pathogen associated with community-acquired gastroenteritis and travellers' diarrhoea. *Journal of Medical Microbiology,* 62**,** 701-707.

FENG, P., FIELDS, P. I., SWAMINATHAN, B. & WHITTAM, T. S. 1996. Characterization of nonmotile variants of *Escherichia coli* O157 and other serotypes by using an antiflagellin monoclonal antibody. *Journal of Clinical Microbiology,* 34**,** 2856-2859.

FENG, P. C. & HARTMAN, P. A. 1982. Fluorogenic assays for immediate confirmation of *Escherichia coli*. *Applied and Environmental Microbiology,* 43**,** 1320-9.

FERNANDEZ, L. & HANCOCK, R. E. 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clinical Microbiology Reviews,* 25**,** 661-81.

FICK, J., SODERSTROM, H., LINDBERG, R. H., PHAN, C., TYSKLIND, M. & LARSSON, D. G. 2009. Contamination of surface, ground, and drinking water from pharmaceutical production. *Environmental Toxicology and Chemistry,* 28**,** 2522-7.

FRANCIA, M. V., VARSAKI, A., GARCILLAN-BARCIA, M. P., LATORRE, A., DRAINAS, C. & DE LA CRUZ, F. 2004. A classification scheme for mobilization regions of bacterial plasmids. *FEMS Microbiology Reviews,* 28**,** 79-100.

FRANK, A. C., ALSMARK, C. M., THOLLESSON, M. & ANDERSSON, S. G. 2005. Functional divergence and horizontal transfer of type IV secretion systems. *Molecular Biology and Evolution,* 22**,** 1325-36.

FRANZ, E., VEENMAN, C., VAN HOEK, A. H., DE RODA HUSMAN, A. & BLAAK, H. 2015. Pathogenic *Escherichia coli* producing Extended-Spectrum beta-Lactamases isolated from surface water and wastewater. *Scientific Reports,* 5**,** 14372.

FROHME, M., CAMARGO, A. A., CZINK, C., MATSUKUMA, A. Y., SIMPSON, A. J., HOHEISEL, J. D. & VERJOVSKI-ALMEIDA, S. 2001. Directed gap closure in large-scale sequencing projects. *Genome Research,* 11**,** 901-903.

FROST, L. S., LEPLAE, R., SUMMERS, A. O. & TOUSSAINT, A. 2005. Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology,* 3**,** 722-732.

GAASTRA, W., KUSTERS, J. G., VAN DUIJKEREN, E. & LIPMAN, L. J. 2014. *Escherichia fergusonii*. *Veterinary Microbiology,* 172**,** 7-12.

GALVIN, S., BOYLE, F., HICKEY, P., VELLINGA, A., MORRIS, D. & CORMICAN, M. 2010. Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary

treatment facility sources. *Applied and Environmental Microbiology,* 76**,** 4772-9.

GARCIA-FERNANDEZ, A. & CARATTOLI, A. 2010. Plasmid double locus sequence typing for IncHI2 plasmids, a subtyping scheme for the characterization of IncHI2 plasmids carrying extended-spectrum beta-lactamase and quinolone resistance genes. *Journal of Antimicrobial Chemotherapy,* 65**,** 1155-61.

GARCILLáN-BARCIA, M. P. & DE LA CRUZ, F. 2002. Distribution of IS*91* family insertion sequences in bacterial genomes: evolutionary implications. *FEMS Microbiology Ecology,* 42**,** 303-313.

GAZE, W., O'NEILL, C., WELLINGTON, E. & HAWKEY, P. 2008. Antibiotic resistance in the environment, with particular reference to MRSA. *Advances in Applied Microbiology,* 63**,** 249-80.

GESER, N., STEPHAN, R. & HACHLER, H. 2012. Occurrence and characteristics of extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae in food producing animals, minced meat and raw milk. *BMC Veterinary Research,* 8**,** 21.

GHOSH, S. & LAPARA, T. M. 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *The ISME Journal,* 1**,** 191-203.

GIGUERE, S., PRESCOTT, J. F. & DOWLING, P. M. 2013. *Antimicrobial Therapy in Veterinary Medicine*. 5 ed. Somerset: Wiley.

GILL, S. R., POP, M., DEBOY, R. T., ECKBURG, P. B., TURNBAUGH, P. J., SAMUEL, B. S., GORDON, J. I., RELMAN, D. A., FRASER-LIGGETT, C. M. & NELSON, K. E. 2006. Metagenomic analysis of the human distal gut microbiome. *Science.* United States.

GISKE, C. G., MARTINEZ-MARTINEZ, L., CANTóN, R., STEFANI, S., SKOV, R., GLUPCZYNSKI, Y., NORDMANN, P., WOOTTON, M., MIRIAGOU, V. & SIMONSEN, G. 2013. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. *EUCAST*.

GNIADKOWSKI, M., SCHNEIDER, I., PAL, A., JUNGWIRTH, R., MIKIEWICZ, B. & BAUERNFEIND, A. 1998. Cefotaxime-resistant enterobacteriaceae isolates from a hospital in warsaw, poland: identification of a New CTX-M-3 cefotaxime-hydrolyzing β-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. *Antimicrobial Agents and Chemotherapy,* 42**,** 827-832.

GOERING, R. V. 2010. Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. *Infection, Genetics and Evolution,* 10**,** 866-875.

GOLDSTEIN, C., LEE, M. D., SANCHEZ, S., HUDSON, C., PHILLIPS, B., REGISTER, B., GRADY, M., LIEBERT, C., SUMMERS, A. O., WHITE, D. G. & MAURER, J. J. 2001. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrobial Agents and Chemotherapy,* 45**,** 723-6.

References

GOLET, E. M., XIFRA, I., SIEGRIST, H., ALDER, A. C. & GIGER, W. 2003. Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. *Environmental Science and Technology,* 37**,** 3243-9.

GORDON, D. M., CLERMONT, O., TOLLEY, H. & DENAMUR, E. 2008. Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. *Environmental Microbiology,* 10**,** 2484-96.

GOUSSARD, S. & COURVALIN, P. 1999. Updated sequence information for TEM β-lactamase genes. *Antimicrobial Agents and Chemotherapy,* 43**,** 367-370.

GRAD, Y. H., LIPSITCH, M., FELDGARDEN, M., ARACHCHI, H. M., CERQUEIRA, G. C., FITZGERALD, M., GODFREY, P., HAAS, B. J., MURPHY, C. I. & RUSS, C. 2012. Genomic epidemiology of the *Escherichia coli* O104: H4 outbreaks in Europe, 2011. *Proceedings of the National Academy of Sciences,* 109**,** 3065-3070.

GREENWOOD, D. 2008. *Antimicrobial drugs: chronicle of a twentieth century medical triumph*, Oxford University Press.

GRINSTED, J., DE LA CRUZ, F. & SCHMITT, R. 1990. The Tn*21* subgroup of bacterial transposable elements. *Plasmid,* 24**,** 163-89.

GROHS, P., TILLECOVIDIN, B., CAUMONT-PRIM, A., CARBONNELLE, E., DAY, N., PODGLAJEN, I. & GUTMANN, L. 2013. Comparison of Five Media for Detection of Extended-Spectrum Beta-Lactamase by Use of the Wasp Instrument for Automated Specimen Processing. *Journal of Clinical Microbiology,* 51**,** 2713-2716.

GUALERZI, C. O., BRANDI, L., FABBRETTI, A. & PON, C. L. 2013. *Antibiotics: targets, mechanisms and resistance*, John Wiley & Sons.

HALL, B. G. & BARLOW, M. 2005. Revised Ambler classification of beta-lactamases. *Journal of Antimicrobial Chemotherapy.* England.

HALL, R. M. & COLLIS, C. M. 1995. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Molecular Microbiology,* 15**,** 593-600.

HALL, R. M. & COLLIS, C. M. 1998. Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resistance Updates,* 1**,** 109-119.

HAMMERUM, A. M. & HEUER, O. E. 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clinical Infectious Diseases,* 48**,** 916-21.

HANSEN, F., HAMMERUM, A. M., SKOV, R. L., GISKE, C. G., SUNDSFJORD, A. & SAMUELSEN, O. 2012. Evaluation of ROSCO Neo-Sensitabs for phenotypic detection and subgrouping of ESBL-, AmpC- and carbapenemase-producing Enterobacteriaceae. *APMIS,* 120**,** 724-32.

HANSEN, W. & YOURASSOWSKY, E. 1984. Detection of beta-glucuronidase in lactose-fermenting members of the family Enterobacteriaceae and its presence in bacterial urine cultures. *Journal of Clinical Microbiology,* 20**,** 1177-9.

References

HAO, W., ALLEN, V. G., JAMIESON, F. B., LOW, D. E. & ALEXANDER, D. C. 2012. Phylogenetic incongruence in *E. coli* O104: understanding the evolutionary relationships of emerging pathogens in the face of homologous recombination. *PloS One,* 7**,** e33971.

HARMER, C. J. & HALL, R. M. 2015. IS*26*-Mediated Precise Excision of the IS*26*-*aphA1a* Translocatable Unit. *MBio,* 6**,** e01866-15.

HARTMANN, A., LOCATELLI, A., AMOUREUX, L., DEPRET, G., JOLIVET, C., GUENEAU, E. & NEUWIRTH, C. 2012. Occurrence of CTX-M Producing *Escherichia coli* in Soils, Cattle, and Farm Environment in France (Burgundy Region). *Frontiers in Microbiology,* 3**,** 83.

HAUSER, E., MELLMANN, A., SEMMLER, T., STOEBER, H., WIELER, L. H., KARCH, H., KUEBLER, N., FRUTH, A., HARMSEN, D. & WENIGER, T. 2013. Phylogenetic and molecular analysis of food-borne shiga toxin-producing *Escherichia coli*. *Applied and Environmental Microbiology,* 79**,** 2731-2740.

HAWKEY, P. M. & JONES, A. M. 2009. The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy,* 64 Suppl 1**,** i3-10.

HAWSER, S., HOBAN, D., BOUCHILLON, S., BADAL, R., CARMELI, Y. & HAWKEY, P. 2011. Antimicrobial susceptibility of intra-abdominal gram-negative bacilli from Europe: SMART Europe 2008. *European Journal of Clinical Microbiology and Infectious Diseases,* 30**,** 173-9.

HEIDELBERG, J. F., EISEN, J. A., NELSON, W. C., CLAYTON, R. A., GWINN, M. L., DODSON, R. J., HAFT, D. H., HICKEY, E. K., PETERSON, J. D., UMAYAM, L., GILL, S. R., NELSON, K. E., READ, T. D., TETTELIN, H., RICHARDSON, D., ERMOLAEVA, M. D., VAMATHEVAN, J., BASS, S., QIN, H., DRAGOI, I., SELLERS, P., MCDONALD, L., UTTERBACK, T., FLEISHMANN, R. D., NIERMAN, W. C., WHITE, O., SALZBERG, S. L., SMITH, H. O., COLWELL, R. R., MEKALANOS, J. J., VENTER, J. C. & FRASER, C. M. 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature,* 406**,** 477-83.

HEISIG, P., SCHEDLETZKY, H. & FALKENSTEIN-PAUL, H. 1993. Mutations in the *gyrA* gene of a highly fluoroquinolone-resistant clinical isolate of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy,* 37**,** 696-701.

HEUER, H. & SMALLA, K. 2012. Plasmids foster diversification and adaptation of bacterial populations in soil. *FEMS Microbiology Reviews,* 36**,** 1083-104.

HILLEN, W. & BERENS, C. 1994. Mechanisms underlying expression of Tn*10* encoded tetracycline resistance. *Annual Reviews in Microbiology,* 48**,** 345-369.

HIMATHONGKHAM, S., BAHARI, S., RIEMANN, H. & CLIVER, D. 1999. Survival of *Escherichia coli* O157: H7 and *Salmonella typhimurium* in cow manure and cow manure slurry. *FEMS Microbiology Letters,* 178**,** 251-257.

HOBMAN, J. L. & BROWN, N. L. 1997. bacterial mercury-resistance genes. *Metal Ions in Biological Systems,* 34**,** 527-68.

HOBMAN, J. L. & CROSSMAN, L. C. 2015. Bacterial antimicrobial metal ion resistance. *Journal of Medical Microbiology,* 64**,** 471-497.

References

HOLMES, A. J., GILLINGS, M. R., NIELD, B. S., MABBUTT, B. C., NEVALAINEN, K. M. & STOKES, H. W. 2003. The gene cassette metagenome is a basic resource for bacterial genome evolution. *Environmental Microbiology,* 5**,** 383-94.

HONORE, N., NICOLAS, M. H. & COLE, S. T. 1986. Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*. *The EMBO Journal,* 5**,** 3709-14.

HOOPER, D. C. 2001. Mechanisms of action of antimicrobials: focus on fluoroquinolones. *Clinical Infectious Diseases,* 32**,** S9-S15.

HORDIJK, J., MEVIUS, D. J., KANT, A., BOS, M. E., GRAVELAND, H., BOSMAN, A. B., HARTSKEERL, C. M., HEEDERIK, D. J. & WAGENAAR, J. A. 2013a. Within-farm dynamics of ESBL/AmpC-producing *Escherichia coli* in veal calves: a longitudinal approach. *Journal of Antimicrobial Chemotherapy,* 68**,** 2468-76.

HORDIJK, J., WAGENAAR, J. A., KANT, A., VAN ESSEN-ZANDBERGEN, A., DIERIKX, C., VELDMAN, K., WIT, B. & MEVIUS, D. 2013b. Cross-sectional study on prevalence and molecular characteristics of plasmid mediated ESBL/AmpC-producing *Escherichia coli* isolated from veal calves at slaughter. *PLoS One,* 8**,** e65681.

HORDIJK, J., WAGENAAR, J. A., VAN DE GIESSEN, A., DIERIKX, C., VAN ESSEN-ZANDBERGEN, A., VELDMAN, K., KANT, A. & MEVIUS, D. 2013c. Increasing prevalence and diversity of ESBL/AmpC-type beta-lactamase genes in *Escherichia coli* isolated from veal calves from 1997 to 2010. *Journal of Antimicrobial Chemotherapy,* 68**,** 1970-3.

HORTON, R. A., RANDALL, L. P., SNARY, E. L., COCKREM, H., LOTZ, S., WEARING, H., DUNCAN, D., RABIE, A., MCLAREN, I., WATSON, E., LA RAGIONE, R. M. & COLDHAM, N. G. 2011. Fecal carriage and shedding density of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* in cattle, chickens, and pigs: implications for environmental contamination and food production. *Applied and Environmental Microbiology,* 77**,** 3715-9.

HRABáK, J., EMPEL, J., GNIADKOWSKI, M., HALBHUBER, Z., REBL, K. & URBáŠKOVá, P. 2008. CTX-M-15-producing *Shigella sonnei* strain from a Czech patient who traveled in Asia. *Journal of Clinical Microbiology,* 46**,** 2147-2148.

HUISINGH, D. 1974. Heavy metals: implications for agriculture. *Annual Review of Phytopathology,* 12**,** 375-388.

HUSSAIN, A., RANJAN, A., NANDANWAR, N., BABBAR, A., JADHAV, S. & AHMED, N. 2014. Genotypic and phenotypic profiles of *Escherichia coli* isolates belonging to clinical sequence type 131 (ST131), clinical non-ST131, and fecal non-ST131 lineages from India. *Antimicrobial Agents and Chemotherapy,* 58**,** 7240-7249.

HUTCHISON, M. L., WALTERS, L. D., MOORE, A., CROOKES, K. M. & AVERY, S. M. 2004. Effect of length of time before incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural soil. *Applied and Environmental Microbiology,* 70**,** 5111-8.

References

ISO.16649-2 2001. Part 2: Colony-count technique at 44 °C using 5-bromo-4-chloro-3-indolyl-D-glucuronide. The International Organization for Standardization.

JACOBS, C., FRERE, J. M. & NORMARK, S. 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. *Cell,* 88**,** 823-32.

JACOBY, G. A. 2009. AmpC beta-lactamases. *Clinical Microbiology Reviews,* 22**,** 161-82.

JACOBY, G. A., CHOW, N. & WAITES, K. B. 2003. Prevalence of Plasmid-Mediated Quinolone Resistance. *Antimicrobial Agents and Chemotherapy,* 47**,** 559-562.

JACOBY, G. A. & HAN, P. 1996. Detection of extended-spectrum beta-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli. Journal of Clinical Microbiology,* 34**,** 908-11.

JACOBY, G. A. & MUNOZ-PRICE, L. S. 2005. The New β-Lactamases. *New England Journal of Medicine,* 352**,** 380-391.

JACOBY, G. A. & SUTTON, L. 1991. Properties of plasmids responsible for production of extended-spectrum beta-lactamases. *Antimicrobial Agents and Chemotherapy,* 35**,** 164-169.

JAFFE, A., CHABBERT, Y. A. & SEMONIN, O. 1982. Role of porin proteins OmpF and OmpC in the permeation of beta-lactams. *Antimicrobial Agents and Chemotherapy,* 22**,** 942-8.

JAKOBSEN, L., KURBASIC, A., SKJOT-RASMUSSEN, L., EJRNAES, K., PORSBO, L. J., PEDERSEN, K., JENSEN, L. B., EMBORG, H. D., AGERSO, Y., OLSEN, K. E., AARESTRUP, F. M., FRIMODT-MOLLER, N. & HAMMERUM, A. M. 2010. *Escherichia coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs share phylogroups and antimicrobial resistance with community-dwelling humans and patients with urinary tract infection. *Foodborne Pathogens and Disease,* 7**,** 537-47.

JAURIN, B. & GRUNDSTRöM, T. 1981. ampC cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of beta-lactamases of the penicillinase type. *Proceedings of the National Academy of Sciences,* 78**,** 4897-4901.

JAZMATI, N., HEIN, R. & HAMPRECHT, A. 2015. Use of an enrichment broth improves the detection of ESBL producing Enterobacteriaceae in clinical stool samples. *Journal of Clinical Microbiology*.

JENSEN, V. F., NEIMANN, J., HAMMERUM, A. M., MøLBAK, K. & WEGENER, H. C. 2004. Does the use of antibiotics in food animals pose a risk to human health? An unbiased review? *Journal of Antimicrobial Chemotherapy,* 54**,** 274-275.

JIA, H., GUO, Y., ZHAO, W. & WANG, K. 2014. Long-range PCR in next-generation sequencing: comparison of six enzymes and evaluation on the MiSeq sequencer. *Scientific reports,* 4**,** 5737.

References

JIA, W., LI, H., ZHAO, L. & NICHOLSON, J. K. 2008. Gut microbiota: a potential new territory for drug targeting. *Nature Reviews Drug Discovery.* England.

JIANG, S. C. & PAUL, J. H. 1998. Gene transfer by transduction in the marine environment. *Applied and Environmental Microbiology,* 64**,** 2780-7.

JOENSEN, K. G., SCHEUTZ, F., LUND, O., HASMAN, H., KAAS, R. S., NIELSEN, E. M. & AARESTRUP, F. M. 2014. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli. Journal of Clinical Microbiology,* 52**,** 1501-1510.

JOENSEN, K. G., TETZSCHNER, A. M., IGUCHI, A., AARESTRUP, F. M. & SCHEUTZ, F. 2015. Rapid and easy in silico serotyping of *Escherichia coli* using whole genome sequencing (WGS) data. *Journal of Clinical Microbiology***,** JCM. 00008-15.

JOHNSON, J. R., SANNES, M. R., CROY, C., JOHNSTON, B., CLABOTS, C., KUSKOWSKI, M. A., BENDER, J., SMITH, K. E., WINOKUR, P. L. & BELONGIA, E. A. 2007. Antimicrobial drug-resistant *Escherichia coli* from humans and poultry products, Minnesota and Wisconsin, 2002-2004. *Emerging Infectious Diseases,* 13**,** 838-46.

JU, W., CAO, G., RUMP, L., STRAIN, E., LUO, Y., TIMME, R., ALLARD, M., ZHAO, S., BROWN, E. & MENG, J. 2012. Phylogenetic analysis of non-O157 Shiga toxin-producing *Escherichia coli* strains by whole-genome sequencing. *Journal of Clinical Microbiology,* 50**,** 4123-7.

KARCZMARCZYK, M., WALSH, C., SLOWEY, R., LEONARD, N. & FANNING, S. 2011. Molecular Characterization of Multidrug-Resistant *Escherichia coli* Isolates from Irish Cattle Farms. *Applied and Environmental Microbiology,* 77**,** 7121-7127.

KARLOWSKY, J. A., KELLY, L. J., THORNSBERRY, C., JONES, M. E. & SAHM, D. F. 2002. Trends in antimicrobial resistance among urinary tract infection isolates of *Escherichia coli* from female outpatients in the United States. *Antimicrobial Agents and Chemotherapy,* 46**,** 2540-5.

KATAMAY, M. 1990. Assessing defined-substrate technology for meeting monitoring requirements of the total coliform rule. *Journal of the American Water Works Association,* 82**,** 83-87.

KAZAZIAN, H. H., JR. 2004. Mobile elements: drivers of genome evolution. *Science,* 303**,** 1626-32.

KEEN, P. L. & MONTFORTS, M. H. M. M. 2011. *Antimicrobial Resistance in the Environment,* Hoboken, NJ, USA, Wiley-Blackwell.

KHACHATRYAN, A. R., BESSER, T. E. & CALL, D. R. 2008. The streptomycin-sulfadiazine-tetracycline antimicrobial resistance element of calf-adapted *Escherichia coli* is widely distributed among isolates from Washington state cattle. *Applied and Environmental Microbiology,* 74**,** 391-395.

KIM, M., YI, H., CHO, Y.-J., JANG, J., HUR, H.-G. & CHUN, J. 2012. Draft Genome Sequence of *Escherichia coli* W26, an Enteric Strain Isolated from Cow Feces. *Journal of Bacteriology,* 194**,** 5149-5150.

References

KIRCHNER, M., WEARING, H., HOPKINS, K. L. & TEALE, C. 2011. Characterization of plasmids encoding cefotaximases group 1 enzymes in *Escherichia coli* recovered from cattle in England and Wales. *Microbial Drug Resistance,* 17**,** 463-70.

KLECKNER, N., CHALMERS, R., KWON, D., SAKAI, J. & BOLLAND, S. 1996. Tn*10* and IS*10* transposition and chromosome rearrangements: mechanism and regulation in vivo and in vitro. *Transposable elements.* Springer.

KLIEBE, C., NIES, B. A., MEYER, J. F., TOLXDORFF-NEUTZLING, R. M. & WIEDEMANN, B. 1985. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. *Antimicrobial Agents and Chemotherapy,* 28**,** 302-7.

LACKS, S. A., LOPEZ, P., GREENBERG, B. & ESPINOSA, M. 1986. Identification and analysis of genes for tetracycline resistance and replication functions in the broad-host-range plasmid pLS1. *Journal of Molecular Biology,* 192**,** 753-765.

LAGACE-WIENS, P., TAILOR, F., BAURDRY-SIMNER, P., ZHAREL, G. G. & HOBAN, D. J. 2010a. Evaluation of a chromogenic medium for extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae. University of Manitoba, Canada. ECCMID Poster 2010.

LAGACE-WIENS, P. R., BAUDRY, P. J., PANG, P. & HAMMOND, G. 2010b. First Description of an Extended-Spectrum-β-Lactamase-Producing Multidrug-Resistant *Escherichia fergusonii* Strain in a Patient with Cystitis. *Journal of Clinical Microbiology,* 48**,** 2301-2302.

LAI, P. K. & ROY, J. 2004. Antimicrobial and chemopreventive properties of herbs and spices. *Current Medicinal Chemistry,* 11**,** 1451-60.

LANDRE, J., GAVRIEL, A. & LAMB, A. 1998. False-positive coliform reaction mediated by *Aeromonas* in the Colilert defined substrate technology system. *Letters in Applied Microbiology,* 26**,** 352-354.

LARSEN, M. V., COSENTINO, S., RASMUSSEN, S., FRIIS, C., HASMAN, H., MARVIG, R. L., JELSBAK, L., SICHERITZ-PONTEN, T., USSERY, D. W., AARESTRUP, F. M. & LUND, O. 2012. Multilocus sequence typing of total-genome-sequenced bacteria. *Journal of Clinical Microbiology,* 50**,** 1355-61.

LARTIGUE, M. F., LEFLON-GUIBOUT, V., POIREL, L., NORDMANN, P. & NICOLAS-CHANOINE, M.-H. 2002. Promoters P3, Pa/Pb, P4, and P5 upstream from bla_{TEM} genes and their relationship to β-lactam resistance. *Antimicrobial Agents and Chemotherapy,* 46**,** 4035-4037.

LAWLEY, T. D., KLIMKE, W. A., GUBBINS, M. J. & FROST, L. S. 2003. F factor conjugation is a true type IV secretion system. *FEMS Microbiology Letters,* 224**,** 1-15.

LAWRENCE, J. G. & OCHMAN, H. 1998. Molecular archaeology of the *Escherichia coli* genome. *Proceedings of the National Academy of Sciences,* 95**,** 9413-7.

LEDERBERG, J. 1952. Cell genetics and hereditary symbiosis. *Physiological Reviews,* 32**,** 403-30.

References

LEITE-MARTINS, L. R., MAHú, M. I. M., COSTA, A. L., MENDES, Â., LOPES, E., MENDONCA, D. M. V., NIZA-RIBEIRO, J. J. R., DE MATOS, A. J. F. & DA COSTA, P. M. 2014. Prevalence of antimicrobial resistance in enteric *Escherichia coli* from domestic pets and assessment of associated risk markers using a generalized linear mixed model. *Preventive Veterinary Medicine,* 117**,** 28-39.

LEON, G., QUIROGA, C., CENTRóN, D. & ROY, P. H. 2010. Diversity and strength of internal outward-oriented promoters in group IIC-attC introns. *Nucleic Acids Research,* 38**,** 8196-8207.

LEUNG, K. T., MACKERETH, R., TIEN, Y.-C. & TOPP, E. 2004. A comparison of AFLP and ERIC-PCR analyses for discriminating *Escherichia coli* from cattle, pig and human sources. *FEMS Microbiology Ecology,* 47**,** 111-119.

LEVY, S. B. 2001. Antibiotic resistance: consequences of inaction. *Clinical Infectious Diseases,* 33 Suppl 3**,** S124-9.

LI, L., STOECKERT, C. J. & ROOS, D. S. 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Research,* 13**,** 2178-2189.

LIAO, X.-P., XIA, J., YANG, L., LI, L., SUN, J., LIU, Y.-H. & JIANG, H.-X. 2015. Characterization of CTX-M-14-producing *Escherichia coli* from food-producing animals. *Frontiers in Microbiology,* 6.

LIEBANA, E., BATCHELOR, M., HOPKINS, K. L., CLIFTON-HADLEY, F. A., TEALE, C. J., FOSTER, A., BARKER, L., THRELFALL, E. J. & DAVIES, R. H. 2006. Longitudinal Farm Study of Extended-Spectrum β-Lactamase-Mediated Resistance. *Journal of Clinical Microbiology,* 44**,** 1630-1634.

LIEBANA, E., CARATTOLI, A., COQUE, T. M., HASMAN, H., MAGIORAKOS, A. P., MEVIUS, D., PEIXE, L., POIREL, L., SCHUEPBACH-REGULA, G., TORNEKE, K., TORREN-EDO, J., TORRES, C. & THRELFALL, J. 2013. Public health risks of enterobacterial isolates producing extended-spectrum beta-lactamases or AmpC beta-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. *Clinical Infectious Diseases,* 56**,** 1030-7.

LIEBERT, C. A., HALL, R. M. & SUMMERS, A. O. 1999. Transposon Tn*21*, flagship of the floating genome. *Microbiology and Molecular Biology Reviews,* 63**,** 507-22.

LIEBERT, C. A., WIREMAN, J., SMITH, T. & SUMMERS, A. O. 1997. Phylogeny of mercury resistance (*mer*) operons of gram-negative bacteria isolated from the fecal flora of primates. *Applied and Environmental Microbiology,* 63**,** 1066-76.

LIM, K.-T., YASIN, R., YEO, C.-C., PUTHUCHEARY, S. & THONG, K.-L. 2009. Characterization of multidrug resistant ESBL-producing *Escherichia coli* isolates from hospitals in Malaysia. *BioMed Research International,* 2009.

LIU, J. H., WEI, S. Y., MA, J. Y., ZENG, Z. L., LU, D. H., YANG, G. X. & CHEN, Z. L. 2007. Detection and characterisation of CTX-M and CMY-2 beta-lactamases among *Escherichia coli* isolates from farm animals in Guangdong Province of China. *International journal of antimicrobial agents,* 29**,** 576-81.

References

LIU, W., CHEN, L., LI, H., DUAN, H., ZHANG, Y., LIANG, X., LI, X., ZOU, M., XU, L. & HAWKEY, P. M. 2009. Novel CTX-M beta-lactamase genotype distribution and spread into multiple species of Enterobacteriaceae in Changsha, Southern China. *Journal of Antimicrobial Chemotherapy.* England.

LIU, Y.-Y., WANG, Y., WALSH, T. R., YI, L.-X., ZHANG, R., SPENCER, J., DOI, Y., TIAN, G., DONG, B., HUANG, X., YU, L.-F., GU, D., REN, H., CHEN, X., LV, L., HE, D., ZHOU, H., LIANG, Z., LIU, J.-H. & SHEN, J. 2015. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases,* DOI: 10.1016/S1473-3099(15)00424-7.

LIVERMORE, D. M., CANTON, R., GNIADKOWSKI, M., NORDMANN, P., ROSSOLINI, G. M., ARLET, G., AYALA, J., COQUE, T. M., KERN-ZDANOWICZ, I. & LUZZARO, F. 2007. CTX-M: changing the face of ESBLs in Europe. *Journal of Antimicrobial Chemotherapy,* 59**,** 165-174.

LIVERMORE, D. M., WINSTANLEY, T. G. & SHANNON, K. P. 2001. Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. *Journal of Antimicrobial Chemotherapy,* 48 Suppl 1**,** 87-102.

LODISH, H., BERK, A., ZIPURSKY, S., MATSUDAIRA, P., BALTIMORE, D. & J., A. D. 2000. *Molecular Cell Biology*. 4th edition. New York: W. H.

LORENZ, M. G. & WACKERNAGEL, W. 1994. Bacterial gene transfer by natural genetic transformation in the environment. *Microbiological reviews,* 58**,** 563-602.

LUNSFORD, R. D. 1998. Streptococcal transformation: essential features and applications of a natural gene exchange system. *Plasmid,* 39**,** 10-20.

MA, L., ISHII, Y., CHANG, F.-Y., YAMAGUCHI, K., HO, M. & SIU, L. 2002. CTX-M-14, a plasmid-mediated CTX-M type extended-spectrum β-lactamase isolated from *Escherichia coli. Antimicrobial Agents and Chemotherapy,* 46**,** 1985-1988.

MA, L., SIU, L. K. & LU, P. L. 2011. Effect of spacer sequences between bla_{CTX-M} and IS*Ecp1* on bla_{CTX-M} expression. *Journal of Medical Microbiology,* 60**,** 1787-92.

MADEC, J.-Y., POIREL, L., SARAS, E., GOURGUECHON, A., GIRLICH, D., NORDMANN, P. & HAENNI, M. 2012. Non-ST131 *Escherichia coli* from cattle harbouring human-like $bla_{CTX-M-15}$-carrying plasmids. *Journal of Antimicrobial Chemotherapy,* 67**,** 578-581.

MADEC, J. Y., LAZIZZERA, C., CHATRE, P., MEUNIER, D., MARTIN, S., LEPAGE, G., MENARD, M. F., LEBRETON, P. & RAMBAUD, T. 2008. Prevalence of fecal carriage of acquired expanded-spectrum cephalosporin resistance in Enterobacteriaceae strains from cattle in France. *Journal of Clinical Microbiology,* 46**,** 1566-7.

MAGIORAKOS, A. P., SRINIVASAN, A., CAREY, R., CARMELI, Y., FALAGAS, M., GISKE, C., HARBARTH, S., HINDLER, J., KAHLMETER, G. & OLSSON-LILJEQUIST, B. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection,* 18**,** 268-281.

References

MAHILLON, J. & CHANDLER, M. 1998. Insertion sequences. *Microbiology and Molecular Biology Reviews,* 62**,** 725-774.

MAHILLON, J., LEONARD, C. & CHANDLER, M. 1999. IS elements as constituents of bacterial genomes. *Research in Microbiology,* 150**,** 675-687.

MANGES, A. R., JOHNSON, J. R., FOXMAN, B., O'BRYAN, T. T., FULLERTON, K. E. & RILEY, L. W. 2001. Widespread distribution of urinary tract infections caused by a multidrug-resistant *Escherichia coli* clonal group. *New England Journal of Medicine,* 345**,** 1007-1013.

MANGES, A. R., SMITH, S. P., LAU, B. J., NUVAL, C. J., EISENBERG, J. N., DIETRICH, P. S. & RILEY, L. W. 2007. Retail meat consumption and the acquisition of antimicrobial resistant *Escherichia coli* causing urinary tract infections: a case-control study. *Foodborne Pathogens and Disease,* 4**,** 419-31.

MARCINEK, H., WIRTH, R., MUSCHOLL-SILBERHORN, A. & GAUER, M. 1998. *Enterococcus faecalis* gene transfer under natural conditions in municipal sewage water treatment plants. *Applied and Environmental Microbiology,* 64**,** 626-32.

MARTINEZ-MARTINEZ, L. 2008. Extended-spectrum beta-lactamases and the permeability barrier. *Clinical Microbiology and Infection,* 14 Suppl 1**,** 82-9.

MATSUMOTO, Y., IKEDA, F., KAMIMURA, T., YOKOTA, Y. & MINE, Y. 1988. Novel plasmid-mediated beta-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. *Antimicrobial Agents and Chemotherapy,* 32**,** 1243-6.

MAVROIDI, A., MIRIAGOU, V., LIAKOPOULOS, A., TZELEPI, E., STEFOS, A., DALEKOS, G. N. & PETINAKI, E. 2012. Ciprofloxacin-resistant *Escherichia coli* in Central Greece: mechanisms of resistance and molecular identification. *BMC Infectious Diseases,* 12**,** 371.

MAZEL, D., DYCHINCO, B., WEBB, V. A. & DAVIES, J. 1998. A distinctive class of integron in the *Vibrio cholerae* genome. *Science,* 280**,** 605-8.

MCARTHUR, A. G., WAGLECHNER, N., NIZAM, F., YAN, A., AZAD, M. A., BAYLAY, A. J., BHULLAR, K., CANOVA, M. J., DE PASCALE, G., EJIM, L., KALAN, L., KING, A. M., KOTEVA, K., MORAR, M., MULVEY, M. R., O'BRIEN, J. S., PAWLOWSKI, A. C., PIDDOCK, L. J., SPANOGIANNOPOULOS, P., SUTHERLAND, A. D., TANG, I., TAYLOR, P. L., THAKER, M., WANG, W., YAN, M., YU, T. & WRIGHT, G. D. 2013. The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy,* 57**,** 3348-57.

MCEWEN, S. A. & FEDORKA-CRAY, P. J. 2002. Antimicrobial use and resistance in animals. *Clinical Infectious Diseases.* United States.

MCLELLAN, S. L., DANIELS, A. D. & SALMORE, A. K. 2003. Genetic Characterization of *Escherichia coli* Populations from Host Sources of Fecal Pollution by Using DNA Fingerprinting. *Applied and Environmental Microbiology,* 69**,** 2587-2594.

MEDEIROS, A. A. 1984. Beta-lactamases. *British Medical Bulletin,* 40**,** 18-27.

References

MEEK, R. W., VYAS, H. & PIDDOCK, L. J. 2015. Nonmedical Uses of Antibiotics: Time to Restrict Their Use? *PLoS Biology,* 13**,** e1002266.

MELLMANN, A., HARMSEN, D., CUMMINGS, C. A., ZENTZ, E. B., LEOPOLD, S. R., RICO, A., PRIOR, K., SZCZEPANOWSKI, R., JI, Y. & ZHANG, W. 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104: H4 outbreak by rapid next generation sequencing technology. *PloS one,* 6**,** e22751.

MICALIZZI, G. 2013. Beta-lactamases in *Salmonella enterica* isolated in Australia. *Communicable Diseases Intelligence Quarterly Report*, *37*(1)

MIRIAGOU, V., CARATTOLI, A. & FANNING, S. 2006. Antimicrobial resistance islands: resistance gene clusters in *Salmonella* chromosome and plasmids. *Microbes and Infection,* 8**,** 1923-1930.

MNIF, B., HARHOUR, H., JDIDI, J., MAHJOUBI, F., GENEL, N., ARLET, G. & HAMMAMI, A. 2013. Molecular epidemiology of extended-spectrum beta-lactamase-producing *Escherichia coli* in Tunisia and characterization of their virulence factors and plasmid addiction systems. *BMC Microbiology,* 13**,** 147.

MøLLER, T. S. B., OVERGAARD, M., NIELSEN, S. S., BORTOLAIA, V., SOMMER, M. O. A., GUARDABASSI, L. & OLSEN, J. E. 2016. Relation between *tetR* and *tetA* expression in tetracycline resistant *Escherichia coli. BMC Microbiology,* 16**,** 39.

MOLLET, B., CLERGET, M., MEYER, J. & IIDA, S. 1985. Organization of the Tn*6*-related kanamycin resistance transposon Tn*2680* carrying two copies of IS*26* and an IS*903* variant, IS*903*. B. *Journal of Bacteriology,* 163**,** 55-60.

MOLLET, B., IIDA, S., SHEPHERD, J. & ARBER, W. 1983. Nucleotide sequence of IS*26*, a new prokaryotic mobile genetic etement. *Nucleic Acids Research,* 11**,** 6319-6330.

MONTEIRO, S. C. & BOXALL, A. B. 2010. Occurrence and fate of human pharmaceuticals in the environment. *Reviews of Environmental Contamination and Toxicology,* 202**,** 53-154.

MORITZ, V. A. & CARSON, P. B. 1986. Cefoxitin sensitivity as a marker for inducible beta-lactamases. *Journal of Medical Microbiology,* 21**,** 203-7.

MULVEY, M. R., BRYCE, E., BOYD, D. A., OFNER-AGOSTINI, M., LAND, A. M., SIMOR, A. E., PATON, S., COMMITTEE, C. H. E. & PROGRAM, C. N. I. S. 2005. Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. *Antimicrobial Agents and Chemotherapy,* 49**,** 358-365.

MUNCK, C., ALBERTSEN, M., TELKE, A., ELLABAAN, M., NIELSEN, P. H. & SOMMER, M. O. 2015. Limited dissemination of the wastewater treatment plant core resistome. *Nature communications,* 6.

MUNIESA, M. & JOFRE, J. 1998. Abundance in sewage of bacteriophages that infect *Escherichia coli* O157:H7 and that carry the Shiga toxin 2 gene. *Applied and Environmental Microbiology,* 64**,** 2443-8.

MURRAY, P. R., ROSENTHAL, K. S. & AND PFALLER, M. 2009. *Medical Microbiology.* 6th edn. Philadelphia: Mosby Elsevier.

References

NAAS, T., MIKAMI, Y., IMAI, T., POIREL, L. & NORDMANN, P. 2001. Characterization of In*53*, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. *Journal of Bacteriology,* 183**,** 235-49.

NAAS, T., POIREL, L. & NORDMANN, P. 2008. Minor extended-spectrum beta-lactamases. *Clinical Microbiology and Infection,* 14 Suppl 1**,** 42-52.

NATARO, J. P. & KAPER, J. B. 1998. Diarrheagenic *Escherichia coli. Clinical Microbiology Reviews,* 11**,** 142-201.

NATARO, J. P., STEINER, T. & GUERRANT, R. L. 1998. Enteroaggregative *Escherichia coli. Emerging infectious diseases,* 4**,** 251.

NAUERBY, B., PEDERSEN, K., DIETZ, H. & MADSEN, M. 2000. Comparison of Danish Isolates of *Salmonella enterica* Serovar Enteritidis PT9a and PT11 from Hedgehogs (*Erinaceus europaeus*) and Humans by Plasmid Profiling and Pulsed-Field Gel Electrophoresis. *Journal of clinical microbiology,* 38**,** 3631-3635.

NCCLS 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 2nd ed. NCCLS document M31-A2. National Committee for Clinical Laboratory Standards. Wayne, USA.

NELSON, E. C. & ELISHA, B. G. 1999. Molecular basis of AmpC hyperproduction in clinical isolates of *Escherichia coli. Antimicrobial Agents and Chemotherapy,* 43**,** 957-9.

NethMap, S.W.A.B. 2014. Nethmap 2014, consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands. *SWAB NETHMAP.*

NEU, H. C. 1992. The crisis in antibiotic resistance. *Science,* 257**,** 1064-1073.

NICOLAS-CHANOINE, M.-H., BLANCO, J., LEFLON-GUIBOUT, V., DEMARTY, R., ALONSO, M. P., CANICA, M. M., PARK, Y.-J., LAVIGNE, J.-P., PITOUT, J. & JOHNSON, J. R. 2008. Intercontinental emergence of *Escherichia coli* clone O25: H4-ST131 producing CTX-M-15. *Journal of Antimicrobial Chemotherapy,* 61**,** 273-281.

NORDMANN, P. & POIREL, L. 2005. Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy,* 56**,** 463-9.

NORMAN, K. N., CLAWSON, M. L., STROCKBINE, N. A., MANDRELL, R. E., JOHNSON, R., ZIEBELL, K., ZHAO, S., FRATAMICO, P. M., STONES, R., ALLARD, M. W. & BONO, J. L. 2015. Comparison of whole genome sequences from human and non-human *Escherichia coli* O26 strains. *Frontiers in Cellular and Infection Microbiology,* 5**,** 21.

NOVAIS, A., CANTON, R., COQUE, T. M., MOYA, A., BAQUERO, F. & GALAN, J. C. 2008. Mutational events in cefotaximase extended-spectrum beta-lactamases of the CTX-M-1 cluster involved in ceftazidime resistance. *Antimicrobial Agents and Chemotherapy,* 52**,** 2377-82.

References

NOVáKOVá, I., KAČáNIOVá, M., HAŠČíK, P., PAVLIČOVá, S. & HLEBA, L. 2009. The resistance to antibiotics in strains of *E. coli* and *Enterococcus* sp. isolated from rectal swabs of lambs and calves. *Scientific Papers Animal Science and Biotechnologies,* 42**,** 322-326.

NOVICK, R. P. 1987. Plasmid incompatibility. *Microbiological Reviews,* 51**,** 381-395.

NUNES-DUBY, S. E., KWON, H. J., TIRUMALAI, R. S., ELLENBERGER, T. & LANDY, A. 1998. Similarities and differences among 105 members of the Int family of site-specific recombinases. *Nucleic Acids Research,* 26**,** 391-406.

NWOSU, V. C. 2001. Antibiotic resistance with particular reference to soil microorganisms. *Research in Microbiology,* 152**,** 421-430.

O'NEILL, J. 2015. Antimicrobials in agriculture and the environment: reducing unnecessary use and waste. *The Review on Antimicrobial Resistance.*

OCHMAN, H., LAWRENCE, J. G. & GROISMAN, E. A. 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature,* 405**,** 299-304.

OGAWA, M., HANDA, Y., ASHIDA, H., SUZUKI, M. & SASAKAWA, C. 2008. The versatility of *Shigella* effectors. *Nature Reviews Microbiology,* 6**,** 11-16.

OSBORN, A. M. & BOLTNER, D. 2002. When phage, plasmids, and transposons collide: genomic islands, and conjugative- and mobilizable-transposons as a mosaic continuum. *Plasmid,* 48**,** 202-12.

OUELLETTE, M., BISSONNETTE, L. & ROY, P. H. 1987. Precise insertion of antibiotic resistance determinants into Tn*21*-like transposons: nucleotide sequence of the OXA-1 beta-lactamase gene. *Proceedings of the National Academy of Sciences,* 84**,** 7378-82.

PAGE, M. I. 2012. *The chemistry of β-lactams*, Springer Science & Business Media.

PALMER, E., TEVIOTDALE, B. & JONES, A. 1997. A relative of the broad-host-range plasmid RSF1010 detected in *Erwinia amylovora*. *Applied and Environmental Microbiology,* 63**,** 4604-4607.

PANKEY, G. & SABATH, L. 2004. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clinical Infectious Diseases,* 38**,** 864-870.

PARTRIDGE, S. R. 2011. Analysis of antibiotic resistance regions in Gram-negative bacteria. *FEMS Microbiology Reviews,* 35**,** 820-855.

PARTRIDGE, S. R. & HALL, R. M. 2005. Evolution of transposons containing bla_{TEM} genes. *Antimicrobial Agents and Chemotherapy,* 49**,** 1267-1268.

PARTRIDGE, S. R., RECCHIA, G. D., SCARAMUZZI, C., COLLIS, C. M., STOKES, H. W. & HALL, R. M. 2000. Definition of the *attI1* site of class 1 integrons. *Microbiology,* 146 (11)**,** 2855-64.

PATERSON, D. L. & BONOMO, R. A. 2005. Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews,* 18**,** 657-86.

PATERSON, D. L., HUJER, K. M., HUJER, A. M., YEISER, B., BONOMO, M. D., RICE, L. B. & BONOMO, R. A. 2003. Extended-spectrum beta-lactamases in

Klebsiella pneumoniae bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type beta-lactamases. *Antimicrobial Agents and Chemotherapy,* 47**,** 3554-60.

PEAKALL, R. O. D. & SMOUSE, P. E. 2006. genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes,* 6**,** 288-295.

PERNA, N. T., PLUNKETT, G., 3RD, BURLAND, V., MAU, B., GLASNER, J. D., ROSE, D. J., MAYHEW, G. F., EVANS, P. S., GREGOR, J., KIRKPATRICK, H. A., POSFAI, G., HACKETT, J., KLINK, S., BOUTIN, A., SHAO, Y., MILLER, L., GROTBECK, E. J., DAVIS, N. W., LIM, A., DIMALANTA, E. T., POTAMOUSIS, K. D., APODACA, J., ANANTHARAMAN, T. S., LIN, J., YEN, G., SCHWARTZ, D. C., WELCH, R. A. & BLATTNER, F. R. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature,* 409**,** 529-33.

PETER-GETZLAFF, S., POLSFUSS, S., POLEDICA, M., HOMBACH, M., GIGER, J., BöTTGER, E., ZBINDEN, R. & BLOEMBERG, G. 2011. Detection of AmpC beta-lactamase in *Escherichia coli:* comparison of three phenotypic confirmation assays and genetic analysis. *Journal of Clinical Microbiology***,** *JCM*. 00091-11.

PFEIFER, Y., CULLIK, A. & WITTE, W. 2010. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. *International Journal of Medical Microbiology,* 300**,** 371-9.

PHAN, M. D., KIDGELL, C., NAIR, S., HOLT, K. E., TURNER, A. K., HINDS, J., BUTCHER, P., COOKE, F. J., THOMSON, N. R., TITBALL, R., BHUTTA, Z. A., HASAN, R., DOUGAN, G. & WAIN, J. 2009. Variation in *Salmonella enterica* serovar typhi IncHI1 plasmids during the global spread of resistant typhoid fever. *Antimicrobial Agents and Chemotherapy,* 53**,** 716-27.

PHILIPPON, A., ARLET, G. & JACOBY, G. A. 2002. Plasmid-determined AmpC-type beta-lactamases. *Antimicrobial Agents and Chemotherapy,* 46**,** 1-11.

PHOENIX, D. A., HARRIS, F. & DENNISON, S. R. 2014. *Novel Antimicrobial Agents and Strategies*, John Wiley & Sons.

POIREL, L., GIRLICH, D., NAAS, T. & NORDMANN, P. 2001. OXA-28, an extended-spectrum variant of OXA-10 β-lactamase from *Pseudomonas aeruginosa* and its plasmid-and integron-located gene. *Antimicrobial Agents and Chemotherapy,* 45**,** 447-453.

POIREL, L., LARTIGUE, M.-F., DECOUSSER, J.-W. & NORDMANN, P. 2005. IS*Ecp1B*-Mediated Transposition of bla_{CTX-M} in *Escherichia coli. Antimicrobial Agents and Chemotherapy,* 49**,** 447-450.

POIREL, L., VILLA, L., BERTINI, A., PITOUT, J. D., NORDMANN, P. & CARATTOLI, A. 2007. Expanded-spectrum β-lactamase and plasmid-mediated quinolone resistance. *Emerging Infectious Diseases,* 13**,** 803.

PRESTON, K. E., GRAFFUNDER, E. M., EVANS, A. M. & VENEZIA, R. A. 2003. Survey of plasmid-associated genetic markers in Enterobacteriaceae with reduced susceptibilities to cephalosporins. *Antimicrobial Agents and Chemotherapy,* 47**,** 2179-2185.

References

PRICE, L. B., GRAHAM, J. P., LACKEY, L. G., ROESS, A., VAILES, R. & SILBERGELD, E. 2007. Elevated risk of carrying gentamicin-resistant *Escherichia coli* among U.S. poultry workers. *Environmental Health Perspectives,* 115**,** 1738-42.

QUINN, J. P., MIYASHIRO, D., SAHM, D., FLAMM, R. & BUSH, K. 1989. Novel plasmid-mediated beta-lactamase (TEM-10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy,* 33**,** 1451-6.

RAI, V. R. 2016. *Advances in food biotechnology*, 1st ed. Wiley-Blackwell.

RECCHIA, G. D. & HALL, R. M. 1995. Gene cassettes: a new class of mobile element. *Microbiology,* 141 (12)**,** 3015-27.

REID, C. J., CHOWDHURY, P. R. & DJORDJEVIC, S. P. 2015. Tn*6026* and Tn*6029* are found in complex resistance regions mobilised by diverse plasmids and chromosomal islands in multiple antibiotic resistant Enterobacteriaceae. *Plasmid,* 80**,** 127-137.

REINTHALER, F. F., POSCH, J., FEIERL, G., WUST, G., HAAS, D., RUCKENBAUER, G., MASCHER, F. & MARTH, E. 2003. Antibiotic resistance of *E. coli* in sewage and sludge. *Water Research,* 37**,** 1685-1690.

RENSING, C., PRIBYL, T. & NIES, D. H. 1997. New functions for the three subunits of the CzcCBA cation-proton antiporter. *Journal of Bacteriology,* 179**,** 6871-6879.

RHODES, G., HUYS, G., SWINGS, J., MCGANN, P., HINEY, M., SMITH, P. & PICKUP, R. W. 2000. Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn*1721* in dissemination of the tetracycline resistance determinant Tet A. *Applied and Environmental Microbiology,* 66**,** 3883-3890.

RICE, E. W., SOWERS, E. G., JOHNSON, C. H., DUNNIGAN, M. E., STROCKBINE, N. A. & EDBERG, S. C. 1992. Serological cross-reactions between *Escherichia coli* O157 and other species of the genus *Escherichia*. *Journal of Clinical Microbiology,* 30**,** 1315-1316.

RIMOLDI, G. M. & MOELLER, R. B., JR. 2013. *Escherichia fergusonii* Associated with Pneumonia in a Beef Cow. *Journal of Veterinary Medicine,* 2013.

ROBERTS, M. C., SCHWARZ, S. & AARTS, H. J. 2012. Erratum: Acquired antibiotic resistance genes: an overview. *Frontiers in Microbiology,* 3**,** 384.

ROMERO, D., TRAXLER, M. F., LóPEZ, D. & KOLTER, R. 2011. Antibiotics as Signal Molecules. *Chemical Reviews,* 111**,** 5492-5505.

ROSSOLINI, G. M., D'ANDREA, M. M. & MUGNAIOLI, C. 2008. The spread of CTX-M-type extended-spectrum beta-lactamases. *Clinical Microbiology and Infection,* 14 Suppl 1**,** 33-41.

ROWE-MAGNUS, D. A., GUEROUT, A. M. & MAZEL, D. 2002. Bacterial resistance evolution by recruitment of super-integron gene cassettes. *Molecular Microbiology.* England.

References

RUTHERFORD, K., PARKHILL, J., CROOK, J., HORSNELL, T., RICE, P., RAJANDREAM, M.-A. & BARRELL, B. 2000. Artemis: sequence visualization and annotation. *Bioinformatics,* 16**,** 944-945.

SABAT, A., BUDIMIR, A., NASHEV, D., Sá-LEãO, R., VAN DIJL, J., LAURENT, F., GRUNDMANN, H., FRIEDRICH, A. & MARKERS, E. S. G. O. E. 2013. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Euro Surveill,* 18**,** 20380.

SAGA, T. & YAMAGUCHI, K. 2009. History of antimicrobial agents and resistant bacteria. *JMAJ,* 52**,** 103-08.

SALYERS, A. A. & SHOEMAKER, N. B. 1996. Resistance gene transfer in anaerobes: new insights, new problems. *Clinical Infectious Diseases,* 23 Suppl 1**,** S36-43.

SALYERS, A. A., SHOEMAKER, N. B., STEVENS, A. M. & LI, L.-Y. 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiological Reviews,* 59**,** 579-590.

SAWANT, A. A., HEGDE, N. V., STRALEY, B. A., DONALDSON, S. C., LOVE, B. C., KNABEL, S. J. & JAYARAO, B. M. 2007. Antimicrobial-resistant enteric bacteria from dairy cattle. *Applied and Environmental Microbiology,* 73**,** 156-163.

SCHAUFLER, K., SEMMLER, T., PICKARD, D. J., DE TORO, M., DE LA CRUZ, F., WIELER, L. H., EWERS, C. & GUENTHER, S. 2016. Carriage of Extended-Spectrum Beta-Lactamase-Plasmids Does Not Reduce Fitness but Enhances Virulence in Some Strains of Pandemic *E. coli* Lineages. *Frontiers in Microbiology,* 7**,** 336.

SCHAUSS, T., GLAESER, S. P., GUTSCHOW, A., DOTT, W. & KäMPFER, P. 2015. Improved Detection of Extended Spectrum Beta-Lactamase (ESBL)-Producing *Escherichia coli* in Input and Output Samples of German Biogas Plants by a Selective Pre-Enrichment Procedure. *PLoS ONE,* 10**,** e0119791.

SCHMID, A., HöRMANSDORFER, S., MESSELHäUSSER, U., KäSBOHRER, A., SAUTER-LOUIS, C. & MANSFELD, R. 2013. Prevalence of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* on Bavarian Dairy and Beef Cattle Farms. *Applied and Environmental Microbiology,* 79**,** 3027-3032.

SCHROEDER, G. N. & HILBI, H. 2008. Molecular pathogenesis of *Shigella* spp.: controlling host cell signaling, invasion, and death by type III secretion. *Clinical Microbiology Reviews,* 21**,** 134-56.

SCHWARZ, S., KEHRENBERG, C., DOUBLET, B. & CLOECKAERT, A. 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiology Reviews,* 28**,** 519-542.

SCHWARZ, S., SILLEY, P., SIMJEE, S., WOODFORD, N., VAN DUIJKEREN, E., JOHNSON, A. P. & GAASTRA, W. 2010. Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *Journal of Antimicrobial Chemotherapy***,** dkq037.

SEEMANN, T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics,* 30**,** 2068-9.

References

SEN, D., VAN DER AUWERA, G. A., ROGERS, L. M., THOMAS, C. M., BROWN, C. J. & TOP, E. M. 2011. Broad-host-range plasmids from agricultural soils have IncP-1 backbones with diverse accessory genes. *Applied and Environmental Microbiology,* 77**,** 7975-83.

SERVIN, A. L. 2005. Pathogenesis of Afa/Dr diffusely adhering *Escherichia coli. Clinical Microbiology Reviews,* 18**,** 264-92.

SHIFMAN, A., NINYO, N., GOPHNA, U. & SNIR, S. 2014. Phylo SI: a new genome-wide approach for prokaryotic phylogeny. *Nucleic Acids Researches,* 42**,** 2391-404.

SHIRAKI, Y., SHIBATA, N., DOI, Y. & ARAKAWA, Y. 2004. *Escherichia coli* producing CTX-M-2 beta-lactamase in cattle, Japan. *Emerging Infectious Diseases,* 10**,** 69-75.

SHPIGEL, N., LEVIN, D., WINKLER, M., SARAN, A., ZIV, G. & BöTTNER, A. 1997. Efficacy of cefquinome for treatment of cows with mastitis experimentally induced using *Escherichia coli. Journal of Dairy Science,* 80**,** 318-323.

SIGUIER, P., FILEE, J. & CHANDLER, M. 2006. Insertion sequences in prokaryotic genomes. *Current Opinion in Microbiology,* 9**,** 526-531.

SIGUIER, P., GAGNEVIN, L. & CHANDLER, M. 2009. The new IS*1595* family, its relation to IS1 and the frontier between insertion sequences and transposons. *Research in Microbiology,* 160**,** 232-41.

SILBERGELD, E. K., GRAHAM, J. & PRICE, L. B. 2008. Industrial food animal production, antimicrobial resistance, and human health. *Annual Reviews Public Health,* 29**,** 151-69.

SILVER, S., PHUNG, L. T. & SILVER, G. 2006. Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. *Journal of Industrial Microbiology and Biotechnology,* 33**,** 627-634.

SKÖLD, O., 2011. *Antibiotics and antibiotic resistance*. John Wiley & Sons.

SKÖLD, O. 2001. Resistance to trimethoprim and sulfonamides. *Veterinary research,* 32**,** 261-273.

SMILLIE, C., GARCILLAN-BARCIA, M. P., FRANCIA, M. V., ROCHA, E. P. & DE LA CRUZ, F. 2010. Mobility of plasmids. *Microbiology and Molecular Biology Reviews,* 74**,** 434-52.

SNOW, L. C., WARNER, R. G., CHENEY, T., WEARING, H., STOKES, M., HARRIS, K., TEALE, C. J. & COLDHAM, N. G. 2012. Risk factors associated with extended spectrum beta-lactamase *Escherichia coli* (CTX-M) on dairy farms in North West England and North Wales. *Preventive Veterinary Medicine,* 106**,** 225-34.

SNOW, L. C., WEARING, H., STEPHENSON, B., TEALE, C. J. & COLDHAM, N. G. 2011. Investigation of the presence of ESBL-producing *Escherichia coli* in the North Wales and West Midlands areas of the UK in 2007 to 2008 using scanning surveillance. *The Veterinary Record,* 169**,** 656.

References

SOLAR, G., MOSCOSO, M. & ESPINOSA, M. 1993. Rolling circle-replicating plasmids from Gram-positive and Gram-negative bacteria: a wall falls. *Molecular Microbiology,* 8**,** 789-796.

SOLBERG, O. D., AJIBOYE, R. M. & RILEY, L. W. 2006. Origin of class 1 and 2 integrons and gene cassettes in a population-based sample of uropathogenic *Escherichia coli. Journal of Clinical Microbiology,* 44**,** 1347-1351.

SORUM, M., JOHNSEN, P. J., AASNES, B., ROSVOLL, T., KRUSE, H., SUNDSFJORD, A. & SIMONSEN, G. S. 2006. Prevalence, persistence, and molecular characterization of glycopeptide-resistant enterococci in Norwegian poultry and poultry farmers 3 to 8 years after the ban on avoparcin. *Applied and Environmental Microbiology.* United States.

SOUGAKOFF, W., GOUSSARD, S., GERBAUD, G. & COURVALIN, P. 1988. Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes. *Review of Infectious Diseases,* 10**,** 879-884.

SRINIVASAN, V., GILLESPIE, B. E., LEWIS, M. J., NGUYEN, L. T., HEADRICK, S. I., SCHUKKEN, Y. H. & OLIVER, S. P. 2007. Phenotypic and genotypic antimicrobial resistance patterns of *Escherichia coli* isolated from dairy cows with mastitis. *Veterinary Microbiology,* 124**,** 319-328.

STELZER, W. & ZIEGERT, E. 1988. The occurrence of antibiotic resistant coliforms in the waste water of a water treatment plant. *Zentralblatt fur Mikrobiologie,* 143**,** 415-23.

STOCKWELL, C. 1988. Nature's pharmacy. London, United Kingdom: Century Hutchinson Ltd. Cited in COWAN, M. M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12, 564-82.

STOKES, H. T. & HALL, R. M. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Molecular Microbiology,* 3**,** 1669-1683.

STOKES, M. O., ABUOUN, M., UMUR, S., WU, G., PARTRIDGE, S. R., MEVIUS, D. J., COLDHAM, N. G. & FIELDER, M. D. 2013. Complete sequence of pSAM7, an IncX4 plasmid carrying a novel *bla*$_{\text{CTX-M-14b}}$ transposition unit isolated from *Escherichia coli* and Enterobacter cloacae from cattle. *Antimicrobial Agents and Chemotherapy,* 57**,** 4590-4.

STOKES, M. O., COTTELL, J. L., PIDDOCK, L. J., WU, G., WOOTTON, M., MEVIUS, D. J., RANDALL, L. P., TEALE, C. J., FIELDER, M. D. & COLDHAM, N. G. 2012. Detection and characterization of pCT-like plasmid vectors for *bla*$_{\text{CTX-M-14}}$ in *Escherichia coli* isolates from humans, turkeys and cattle in England and Wales. *Journal of Antimicrobial Chemotherapy,* 67**,** 1639-44.

STRELKAUSKAS, A. P. 2010. *Microbiology: a clinical approach.* New York: Garland Science.

STURENBURG, E., KUHN, A., MACK, D. & LAUFS, R. 2004. A novel extended-spectrum β-lactamase CTX-M-23 with a P167T substitution in the active-site omega loop associated with ceftazidime resistance. *Journal of Antimicrobial Chemotherapy,* 54**,** 406-409.

References

SUMMERS, A. O. 2006. Genetic linkage and horizontal gene transfer, the roots of the antibiotic multi-resistance problem. *Animal Biotechnology.* United States.

SUNDE, M. & NORSTROM, M. 2006. The prevalence of, associations between and conjugal transfer of antibiotic resistance genes in *Escherichia coli* isolated from Norwegian meat and meat products. *Journal of Antimicrobial Chemotherapy,* 58**,** 741-7.

SUSSMAN, M. 1997. *Escherichia coli: mechanisms of virulence*, Cambridge University Press.

SUTTON, S. 2011. Accuracy of plate counts. *Journal of Validation Technology,* 17**,** 42.

SVáB, D., HORVáTH, B., SZŰCS, A., MARóTI, G. & TóTH, I. 2013. Draft Genome Sequence of an *Escherichia coli* O157:H43 Strain Isolated from Cattle. *Genome Announcements,* 1**,** e00263-13.

SYVANEN, M. 1994. Horizontal gene transfer: evidence and possible consequences. *Annual Review of Genetics,* 28**,** 237-61.

TALARO, K. P. & AND CHESS, B. 2012. *Foundation in Microbiology*. 8th ed. New York: McGraw-Hill.

TAMANG, M. D., NAM, H. M., GURUNG, M., JANG, G. C., KIM, S. R., JUNG, S. C., PARK, Y. H. & LIM, S. K. 2013a. Molecular characterization of CTX-M beta-lactamase and associated addiction systems in *Escherichia coli* circulating among cattle, farm workers, and the farm environment. *Applied and Environmental Microbiology,* 79**,** 3898-905.

TAMANG, M. D., NAM, H. M., KIM, S. R., CHAE, M. H., JANG, G. C., JUNG, S. C. & LIM, S. K. 2013b. Prevalence and molecular characterization of CTX-M beta-lactamase-producing *Escherichia coli* isolated from healthy swine and cattle. *Foodborne Pathogens and Disease,* 10**,** 13-20.

TAVIO, M. M., AQUILI, V. D., VILA, J. & POVEDA, J. B. 2014. Resistance to ceftazidime in *Escherichia coli* associated with AcrR, MarR and PBP3 mutations and overexpression of *sdiA*. *Journal of Medical Microbiology,* 63**,** 56-65.

TENOVER, F. C. 2006. Mechanisms of antimicrobial resistance in bacteria. *The American Journal of Medicine.* United States.

TIMOFTE, D., MACIUCA, I. E., EVANS, N. J., WILLIAMS, H., WATTRET, A., FICK, J. C. & WILLIAMS, N. J. 2014. Detection and Molecular Characterization of *Escherichia coli* CTX-M-15 and *Klebsiella pneumoniae* SHV-12 β-Lactamases from Bovine Mastitis Isolates in the United Kingdom. *Antimicrobial Agents and Chemotherapy,* 58**,** 789-794.

TOLEMAN, M. A., BENNETT, P. M. & WALSH, T. R. 2006. IS*CR* elements: novel gene-capturing systems of the 21st century? *Microbiology and Molecular Biology Reviews,* 70**,** 296-316.

TOLEMAN, M. A. & WALSH, T. R. 2011. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiology Reviews,* 35**,** 912-935.

References

TORRES, A. G., ARENAS-HERNáNDEZ, M. M. & MARTíNEZ-LAGUNA, Y. 2010. *in Pathogenic Escherichia coli in Latin America, edited by Alfredo G Torres,* Sharjah, UAE, Bentham Science Publishers.

TOSZEGHY, M., PHILLIPS, N., REEVES, H., WU, G., TEALE, C., COLDHAM, N. & RANDALL, L. 2012. Molecular and phenotypic characterisation of extended spectrum beta-lactamase CTX-M *Escherichia coli* from farm animals in Great Britain. *Research in veterinary science,* 93**,** 1142-50.

TOUCHON, M., HOEDE, C., TENAILLON, O., BARBE, V., BAERISWYL, S., BIDET, P., BINGEN, E., BONACORSI, S., BOUCHIER, C. & BOUVET, O. 2009. Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. *PLoS Genetics,* 5**,** e1000344.

TOUSSAINT, A. & MERLIN, C. 2002. Mobile elements as a combination of functional modules. *Plasmid,* 47**,** 26-35.

TRACZ, D. M., BOYD, D. A., HIZON, R., BRYCE, E., MCGEER, A., OFNER-AGOSTINI, M., SIMOR, A. E., PATON, S., MULVEY, M. R. & PROGRAM, C. N. I. S. 2007. ampC gene expression in promoter mutants of cefoxitin-resistant *Escherichia coli* clinical isolates. *FEMS Microbiology Letters,* 270**,** 265-271.

TUCKMAN, M., PETERSEN, P. J., HOWE, A. Y., ORLOWSKI, M., MULLEN, S., CHAN, K., BRADFORD, P. A. & JONES, C. H. 2007. Occurrence of tetracycline resistance genes among *Escherichia coli* isolates from the phase 3 clinical trials for tigecycline. *Antimicrobial Agents and Chemotherapy,* 51**,** 3205-3211.

UK-VARSS 2013. UK Veterinary Antibiotic Resistance and Sales Surveillance Report. URL https://www.gov.uk/government/publications/veterinary-antimicrobial-resistance-and-sales-surveillance-2013.

UK-VARSS 2014a. High level summary of the Veterinary Antibiotic Resistance and Sales Surveillance Report (UK-VARSS). URL https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/478014/VARSS_Summary.pdf.

UK-VARSS 2014b. UK Veterinary Antibiotic Resistance and Sales Surveillance Report. URL https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/477788/OptimisedversionVARSS_Report_2014__Sales___Resistance_.pdf.

UK-VARSS 2015. UK Veterinary Antibiotic Resistance and Sales Surveillance Report. URL https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/582341/1051728-v53-UK-VARSS_2015.pdf

VALVERDE, A., CANTON, R., GARCILLAN-BARCIA, M. P., NOVAIS, A., GALAN, J. C., ALVARADO, A., DE LA CRUZ, F., BAQUERO, F. & COQUE, T. M. 2009. Spread of $bla_{\text{CTX-M-14}}$ is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. *Antimicrobial Agents and Chemotherapy,* 53**,** 5204-12.

VAN BOECKEL, T. P., BROWER, C., GILBERT, M., GRENFELL, B. T., LEVIN, S. A., ROBINSON, T. P., TEILLANT, A. & LAXMINARAYAN, R. 2015. Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences,* 112**,** 5649-54.

References

VAN DEN BOGAARD, A. E. J. M., LONDON, N. & STOBBERINGH, E. E. 2000. Antimicrobial resistance in pig faecal samples from The Netherlands (five abattoirs) and Sweden. *Journal of Antimicrobial Chemotherapy,* 45**,** 663-671.

VAN OVERBEEK, L. S., WELLINGTON, E. M., EGAN, S., SMALLA, K., HEUER, H., COLLARD, J.-M., GUILLAUME, G., KARAGOUNI, A. D., NIKOLAKOPOULOU, T. L. & VAN ELSAS, J. D. 2002. Prevalence of streptomycin-resistance genes in bacterial populations in European habitats. *FEMS Microbiology Ecology,* 42**,** 277-288.

VERSALOVIC, J., KOEUTH, T. & LUPSKI, J. R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research,* 19**,** 6823-31.

VIDAVER, A. K. 2002. Uses of antimicrobials in plant agriculture. *Clinical Infectious Diseases,* 34 Suppl 3**,** S107-10.

VILLA, L., CAPONE, A., FORTINI, D., DOLEJSKA, M., RODRIGUEZ, I., TAGLIETTI, F., DE PAOLIS, P., PETROSILLO, N. & CARATTOLI, A. 2013. Reversion to susceptibility of a carbapenem-resistant clinical isolate of *Klebsiella pneumoniae* producing KPC-3. *Journal of Antimicrobial Chemotherapy,* 68**,** 2482-6.

VILLA, L., GARCIA-FERNANDEZ, A., FORTINI, D. & CARATTOLI, A. 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. *Journal of Antimicrobial Chemotherapy,* 65**,** 2518-29.

VINCENT, C., BOERLIN, P., DAIGNAULT, D., DOZOIS, C. M., DUTIL, L., GALANAKIS, C., REID-SMITH, R. J., TELLIER, P. P., TELLIS, P. A. & ZIEBELL, K. 2010. Food reservoir for *Escherichia coli* causing urinary tract infections. *Emerging Infectious Diseases,* 16**,** 88-95.

VINUE, L., JOVE, T., TORRES, C. & PLOY, M.-C. 2011. Diversity of class 1 integron gene cassette Pc promoter variants in clinical *Escherichia coli* strains and description of a new P2 promoter variant. *International Journal of Antimicrobial Agents,* 38**,** 526-529.

VON BAUM, H. & MARRE, R. 2005. Antimicrobial resistance of *Escherichia coli* and therapeutic implications. *International Journal of Medical Microbiology,* 295**,** 503-11.

VU, H. & NIKAIDO, H. 1985. Role of beta-lactam hydrolysis in the mechanism of resistance of a beta-lactamase-constitutive *Enterobacter cloacae* strain to expanded-spectrum beta-lactams. *Antimicrobial Agents and Chemotherapy,* 27**,** 393-8.

WALKER, M. & PEMBERTON, J. 1987. Construction of a transposon containing a gene for polygalacturonate trans-eliminase from *Klebsiella oxytoca*. *Archives of Microbiology,* 146**,** 390-395.

WALSH, C. 2003. Where will new antibiotics come from? *Nature Reviews Microbiology,* 1**,** 65-70.

WANG, A., YANG, Y., LU, Q., WANG, Y., CHEN, Y., DENG, L., DING, H., DENG, Q., ZHANG, H., WANG, C., LIU, L., XU, X., WANG, L. & SHEN, X. 2008. Presence of *qnr* gene in *Escherichia coli* and *Klebsiella pneumoniae* resistant to

ciprofloxacin isolated from pediatric patients in China. *BMC Infectious Diseases,* 8**,** 68.

WANG, M., SAHM, D. F., JACOBY, G. A. & HOOPER, D. C. 2004. Emerging plasmid-mediated quinolone resistance associated with the qnr gene in *Klebsiella pneumoniae* clinical isolates in the United States. *Antimicrobial Agents and Chemotherapy,* 48**,** 1295-9.

WARDLE, S. J., O'CARROLL, M., DERBYSHIRE, K. M. & HANIFORD, D. B. 2005. The global regulator H-NS acts directly on the transpososome to promote Tn*10* transposition. *Genes and Development,* 19**,** 2224-2235.

WARNES, S. L., HIGHMORE, C. J. & KEEVIL, C. W. 2012. Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: implications for public health. *MBio,* 3**,** e00489-12.

WATANABE, T. 1963. Infective heredity of multiple drug resistance in bacteria. *Bacteriological Reviews,* 27**,** 87-115.

WATSON, E., JECKEL, S., SNOW, L., STUBBS, R., TEALE, C., WEARING, H., HORTON, R., TOSZEGHY, M., TEARNE, O., ELLIS-IVERSEN, J. & COLDHAM, N. 2012. Epidemiology of extended spectrum beta-lactamase *E. coli* (CTX-M-15) on a commercial dairy farm. *Veterinary Microbiology,* 154**,** 339-46.

WEBBER, M. & PIDDOCK, L. 2003. The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy,* 51**,** 9-11.

WEGENER, H. C. Antibiotic resistance—Linking human and animal health. Improving Food Safety Through a One Health Approach: Workshop Summary, 2012. *National Academies Press*, 331.

WEI, S. H. 2013. *Escherichia coli contamination of pork carcasses in UK slaughterhouses.* University of Nottingham.

WEIGEL, L. M., STEWARD, C. D. & TENOVER, F. C. 1998. *GyrA* mutations associated with fluoroquinolone resistance in eight species of Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy,* 42**,** 2661-2667.

WELLINGTON, E. M. H., BOXALL, A. B. A., CROSS, P., FEIL, E. J., GAZE, W. H., HAWKEY, P. M., JOHNSON-ROLLINGS, A. S., JONES, D. L., LEE, N. M., OTTEN, W., THOMAS, C. M. & WILLIAMS, A. P. 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases,* 13**,** 155-165.

WHITE, D. G., ZHAO, S., SIMJEE, S., WAGNER, D. D. & MCDERMOTT, P. F. 2002. Antimicrobial resistance of foodborne pathogens. *Microbes and Infection,* 4**,** 405-412.

WHO 2014. *Antimicrobial resistance: global report on surveillance*, World Health Organization.

WHO. 2001. WHO global strategy for containment of antimicrobial resistance. URL http://www.who.int/csr/resources/publications/drugresist/WHO

WICHMANN, F., UDIKOVIC-KOLIC, N., ANDREW, S. & HANDELSMAN, J. 2014. Diverse antibiotic resistance genes in dairy cow manure. *MBio,* 5**,** e01017-13.

References

WIEDEMANN, B., DIETZ, H. & PFEIFLE, D. 1998. Induction of beta-lactamase in *Enterobacter cloacae*. *Clinical Infectious Diseases,* 27 Suppl 1**,** S42-7.

WILSON, L. A. & SHARP, P. M. 2006. Enterobacterial repetitive intergenic consensus (ERIC) sequences in *Escherichia coli:* Evolution and implications for ERIC-PCR. *Molecular Biology and Evolution,* 23**,** 1156-68.

WIRTH, T., FALUSH, D., LAN, R., COLLES, F., MENSA, P., WIELER, L. H., KARCH, H., REEVES, P. R., MAIDEN, M. C., OCHMAN, H. & ACHTMAN, M. 2006. Sex and virulence in *Escherichia coli:* an evolutionary perspective. *Molecular Microbiology,* 60**,** 1136-51.

WOODFORD, N., CARATTOLI, A., KARISIK, E., UNDERWOOD, A., ELLINGTON, M. J. & LIVERMORE, D. M. 2009. Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major *Escherichia coli* lineages from the United Kingdom, all belonging to the international O25: H4-ST131 clone. *Antimicrobial Agents and Chemotherapy,* 53**,** 4472-4482.

WOODFORD, N., WARD, M. E., KAUFMANN, M. E., TURTON, J., FAGAN, E. J., JAMES, D., JOHNSON, A. P., PIKE, R., WARNER, M., CHEASTY, T., PEARSON, A., HARRY, S., LEACH, J. B., LOUGHREY, A., LOWES, J. A., WARREN, R. E. & LIVERMORE, D. M. 2004. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases in the UK. *Journal of Antimicrobial Chemotherapy,* 54**,** 735-43.

WOOSTER, D. G., MARUVADA, R., BLOM, A. M. & PRASADARAO, N. V. 2006. Logarithmic phase *Escherichia coli* K1 efficiently avoids serum killing by promoting C4bp-mediated C3b and C4b degradation. *Immunology,* 117**,** 482-93.

WOZNIAK, R. A. & WALDOR, M. K. 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nature Reviews Microbiology,* 8**,** 552-563.

WRIGHT, G. D. 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Reviews Microbiology.* 5, 175-186

WRIGHT, J. 1955. Non-motile *Escherichia coli* O55, B5 Strains. *Journal of General Microbiology,* 12**,** 157-161.

WU, G., DAY, M. J., MAFURA, M. T., NUNEZ-GARCIA, J., FENNER, J. J., SHARMA, M., VAN ESSEN-ZANDBERGEN, A., RODRIGUEZ, I., DIERIKX, C., KADLEC, K., SCHINK, A. K., CHATTAWAY, M., WAIN, J., HELMUTH, R., GUERRA, B., SCHWARZ, S., THRELFALL, J., WOODWARD, M. J., WOODFORD, N., COLDHAM, N. & MEVIUS, D. 2013. Comparative analysis of ESBL-positive *Escherichia coli* isolates from animals and humans from the UK, The Netherlands and Germany. *PLoS One,* 8**,** e75392.

YANCEY JR, R., KINNEY, M., ROBERTS, B., GOODENOUGH, K., HAMEL, J. & FORD, C. 1987. Ceftiofur sodium, a broad-spectrum cephalosporin: evaluation in vitro and in vivo in mice. *American Journal of Veterinary Research,* 48**,** 1050-1053.

YANG, Q.-E., SUN, J., LI, L., DENG, H., LIU, B.-T., FANG, L.-X., LIAO, X.-P. & LIU, Y.-H. 2015. IncF plasmid diversity in multi-drug resistant *Escherichia coli* strains from animals in China. *Frontiers in Microbiology,* 6**,** 964.

References

YARON, S., WHITE, D. G. & MATTHEWS, K. R. 2003. Characterization of an *Escherichia coli* O157: H7 *marR* mutant. *International Journal of Food Microbiology,* 85**,** 281-291.

YI, H., CHO, Y.-J., HUR, H.-G. & CHUN, J. 2011. Genome sequence of *Escherichia coli* AA86, isolated from cow feces. *Journal of Bacteriology,* 193**,** 3681-3681.

YI, H., CHO, Y.-J., YONG, D. & CHUN, J. 2012. Genome Sequence of *Escherichia coli* J53, a Reference Strain for Genetic Studies. *Journal of Bacteriology,* 194**,** 3742-3743.

YI, H., XI, Y., LIU, J., WANG, J., WU, J., XU, T., CHEN, W., CHEN, B., LIN, M. & WANG, H. 2010. Sequence analysis of pKF3-70 in *Klebsiella pneumoniae*: probable origin from R100-like plasmid of *Escherichia coli. PloS One,* 5**,** e8601.

YIN, J., CHENG, J., SUN, Z., YE, Y., GAO, Y.-F., LI, J.-B. & ZHANG, X.-J. 2009. Characterization of two plasmid-encoded cefotaximases found in clinical *Escherichia coli* isolates: CTX-M-65 and a novel enzyme, CTX-M-87. *Journal of Medical Microbiology,* 58**,** 811-815.

YOSHIDA, H., BOGAKI, M., NAKAMURA, M. & NAKAMURA, S. 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli. Antimicrobial Agents and Chemotherapy,* 34**,** 1271-1272.

ZANKARI, E., HASMAN, H., COSENTINO, S., VESTERGAARD, M., RASMUSSEN, S., LUND, O., AARESTRUP, F. M. & LARSEN, M. V. 2012. Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy,* 67**,** 2640-4.

ZANKARI, E., HASMAN, H., KAAS, R. S., SEYFARTH, A. M., AGERSO, Y., LUND, O., LARSEN, M. V. & AARESTRUP, F. M. 2013. Genotyping using whole-genome sequencing is a realistic alternative to surveillance based on phenotypic antimicrobial susceptibility testing. *Journal of Antimicrobial Chemotherapy,* 68**,** 771-7.

ZAPUN, A., CONTRERAS-MARTEL, C. & VERNET, T. 2008. Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiology Reviews,* 32**,** 361-85.

ZERVOS, M. J., HERSHBERGER, E., NICOLAU, D. P., RITCHIE, D. J., BLACKNER, L. K., COYLE, E. A., DONNELLY, A. J., ECKEL, S. F., ENG, R. H., HILTZ, A., KUYUMJIAN, A. G., KREBS, W., MCDANIEL, A., HOGAN, P. & LUBOWSKI, T. J. 2003. Relationship between fluoroquinolone use and changes in susceptibility to fluoroquinolones of selected pathogens in 10 United States teaching hospitals, 1991-2000. *Clinical Infectious Diseases. 37***,** 1643-1648.

ZHENG, H., ZENG, Z., CHEN, S., LIU, Y., YAO, Q., DENG, Y., CHEN, X., LV, L., ZHUO, C., CHEN, Z. & LIU, J. H. 2012. Prevalence and characterisation of CTX-M beta-lactamases amongst *Escherichia coli* isolates from healthy food animals in China. *International Journal of Antimicrobial Agents,* 39**,** 305-10.

ZONG, Z., YU, R., WANG, X. & LUE, X. 2011. *bla*$_{\text{CTX-M-65}}$ is carried by a Tn*1722*-like element on an IncN conjugative plasmid of ST131 *Escherichia coli. Journal of Medical Microbiology,* 60**,** 435-441.

Appendices

Appendix I: API 20E results for 21 isolates of presumptive *E. coli* isolated in 2012 from the farm.

REFERENCE	API 20 E V4.1	Profile	Identification comment	Taxon	% ID
A1.1		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.5
		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Kluyvera spp*	0.4
A1.3		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.5
		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Kluyvera spp*	0.4
A1.4		+-+-----+--++-++-+-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.8
		+-+-----+--++-++-+-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 2	0.1
A1.5		+++-----+--++-++---+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	85.4
		+++-----+--++-++---+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 2	14.3
		+++-----+--++-++---+-	VERY GOOD IDENTIFICATION	*Salmonella choleraesuis*	0.1
A2.1		++++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.8
		++++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Salmonella choleraesuis*	0.1
A2.2		++++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.8
		++++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Salmonella choleraesuis*	0.1
A2.3		++++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.8
		++++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Salmonella choleraesuis*	0.1
A2.4		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.5
		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Kluyvera spp*	0.4
A2.5		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.5
		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Kluyvera spp*	0.4
A3.4		+-+-----+--++-++-+++-	GOOD IDENTIFICATION	*Escherichia coli* 1	94.7
		+-+-----+--++-++-+++-	GOOD IDENTIFICATION	*Kluyvera spp*	2.1
A3.5		+-+-----+--++-+--+-+-	GOOD IDENTIFICATION	*Escherichia coli* 1	98.1
		+-+-----+--++-+--+-+-	GOOD IDENTIFICATION	*Escherichia coli* 2	1.8
A4.1		+-+-----+--++-++-+-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.8
		+-+-----+--++-++-+-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 2	0.1
A4.2		+-+-----+--++-++-+++-	GOOD IDENTIFICATION	*Escherichia coli* 1	94.7
		+-+-----+--++-++-+++-	GOOD IDENTIFICATION	*Kluyvera spp*	2.1
A4.3		+-+-----+--++-++---+-	EXCELLENT IDENTIFICATION T	*Escherichia coli* 1	85.5
		+-+-----+--++-++---+-	EXCELLENT IDENTIFICATION T	*Escherichia coli* 2	14.4
		+-+-----+--++-++---+-	EXCELLENT IDENTIFICATION T	*Citrobacter youngae*	0.1
A4.4		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.5
		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Kluyvera spp*	0.4
A4.5		+-+-----+--++-++-+++-	GOOD IDENTIFICATION	*Escherichia coli* 1	94.7
		+-+-----+--++-++-+++-	GOOD IDENTIFICATION	*Kluyvera spp*	2.1
A5.1		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.5
		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Kluyvera spp*	0.4
A5.3		+-+-----+--++-++-+-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.8
		+-+-----+--++-++-+-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 2	0.1
A5.4		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Escherichia coli* 1	99.5
		+-++----+--++-++++-+-	VERY GOOD IDENTIFICATION	*Kluyvera spp*	0.4
5.5		+-+-----+--++-++-+++-	GOOD IDENTIFICATION	*Escherichia coli* 1	94.7
		+-+-----+--++-++-+++-	GOOD IDENTIFICATION	*Kluyvera spp*	2.1
A3.1		++------+-+++---+-+++	LOW DISCRIMINATION	*Aeromonas hydrophila /*	67
		++------+-+++---+-+++	LOW DISCRIMINATION	*Vibrio fluvialis*	32.8
		++------+-+++---+-+++	LOW DISCRIMINATION	*Aeromonas hydrophila/*	0.1

Appendix II: TCC and ECC (log_{10} CFU ml^{-1}) counts with and without cefotaxime selection from 2014 samples

		MacConkey (TCC)*		
Source	Sample no.	log_{10} CFU ml^{-1} _no CTX	log_{10} CFU ml^{-1} _with CTX	Resistance percentage
Cow barn	S1	5.99	3.51	0.33
Cow barn	S2	5.98	3.52	0.34
Cow barn	S3	5.88	1.92	0.01
Liquid slurry	S1	4.82	2.30	0.30
Liquid slurry	S2	4.79	2.68	0.76
Liquid slurry	S3	4.76	2.47	0.52
Solid slurry	S1	5.94	3.13	0.15
Solid slurry	S2	6.04	3.60	0.36
Solid slurry	S3	6.06	3.30	0.17
		TBX (ECC)*		
Source	Sample no.	log_{10} CFU ml^{-1} _no CTX	log_{10} CFU ml^{-1} _with CTX	Resistance percentage
Cow barn	S1	5.76	3.43	0.46
Cow barn	S2	5.64	3.37	0.54
Cow barn	S3	5.78	1.22	0.002
Liquid slurry	S1	4.68	1.52	0.06
Liquid slurry	S2	4.79	1.82	0.10
Liquid slurry	S3	4.60	1.69	0.12
Solid slurry	S1	5.81	2.61	0.06
Solid slurry	S2	5.57	2.65	0.11
Solid slurry	S3	5.86	2.47	0.04

*TCC: the total coliform count (log_{10} CFU ml^{-1}) averaged over three samples isolated on MacConkey agar with and without CTX selection (using 2 µg ml^{-1} of CTX); ECC: the total *E. coli* count (log_{10} CFU ml^{-1}) averaged over three samples on TBX with and without CTX (using 2 µg ml^{-1} of CTX). These data are for the three samples (S1, S2 and S3) from each site as sampled in 2014.

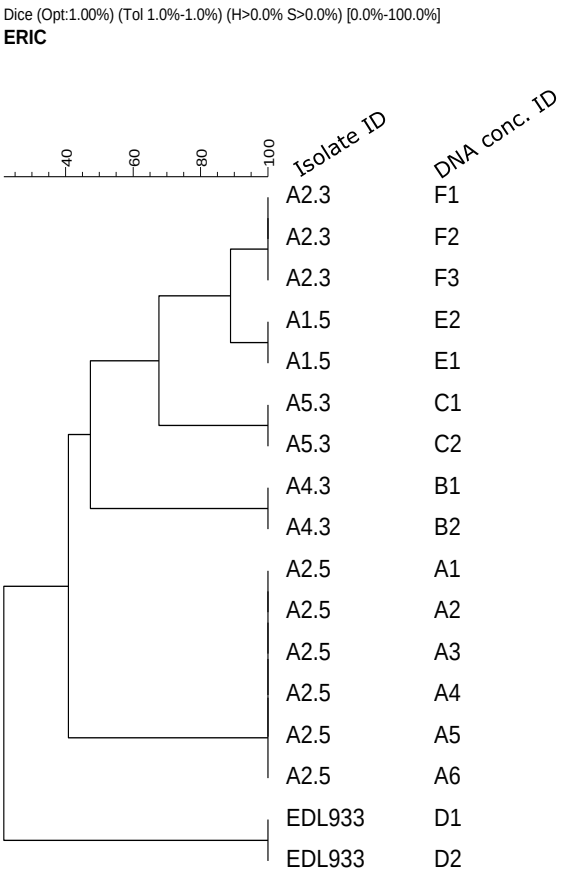
Appendix III: Comparison of TCC on MAC and MAC+CTX using the overall average for each sample in each site (using 2 way ANOVA multiple comparison)

Number of families	1				
Number of comparisons per family	15				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Cow barn:MAC vs. Cow barn:MAC+CTX	2.66	2.403 to 2.916	Yes	****	<0.0001
Cow barn:MAC vs. Slurry tank:MAC	1.168	0.9289 to 1.408	Yes	****	<0.0001
Cow barn:MAC vs. Slurry tank:MAC+CTX	3.355	3.099 to 3.611	Yes	****	<0.0001
Cow barn:MAC vs. Solid Slurry:MAC	-0.05287	-0.2925 to 0.1867	No	ns	0.9874
Cow barn:MAC vs. Solid Slurry:MAC+CTX	2.553	2.306 to 2.8	Yes	****	<0.0001
Cow barn:MAC+CTX vs. Slurry tank:MAC	-1.491	-1.747 to -1.235	Yes	****	<0.0001
Cow barn:MAC+CTX vs. Slurry tank:MAC+CTX	0.6955	0.4238 to 0.9671	Yes	****	<0.0001
Cow barn:MAC+CTX vs. Solid Slurry:MAC	-2.712	-2.969 to -2.456	Yes	****	<0.0001
Cow barn:MAC+CTX vs. Solid Slurry:MAC+CTX	-0.1068	-0.3698 to 0.1563	No	ns	0.8445
Slurry tank:MAC vs. Slurry tank:MAC+CTX	2.187	1.93 to 2.443	Yes	****	<0.0001
Slurry tank:MAC vs. Solid Slurry:MAC	-1.221	-1.461 to -0.9817	Yes	****	<0.0001
Slurry tank:MAC vs. Solid Slurry:MAC+CTX	1.384	1.137 to 1.631	Yes	****	<0.0001
Slurry tank:MAC+CTX vs. Solid Slurry:MAC	-3.408	-3.664 to -3.152	Yes	****	<0.0001
Slurry tank:MAC+CTX vs. Solid Slurry:MAC+CTX	-0.8022	-1.065 to -0.5392	Yes	****	<0.0001
Solid Slurry:MAC vs. Solid Slurry:MAC+CTX	2.606	2.359 to 2.853	Yes	****	<0.0001

Appendix IV: Comparison of ECC on TBX and TBX+CTX using the overall average for each sample in each site (using 2 way ANOVA multiple comparison).

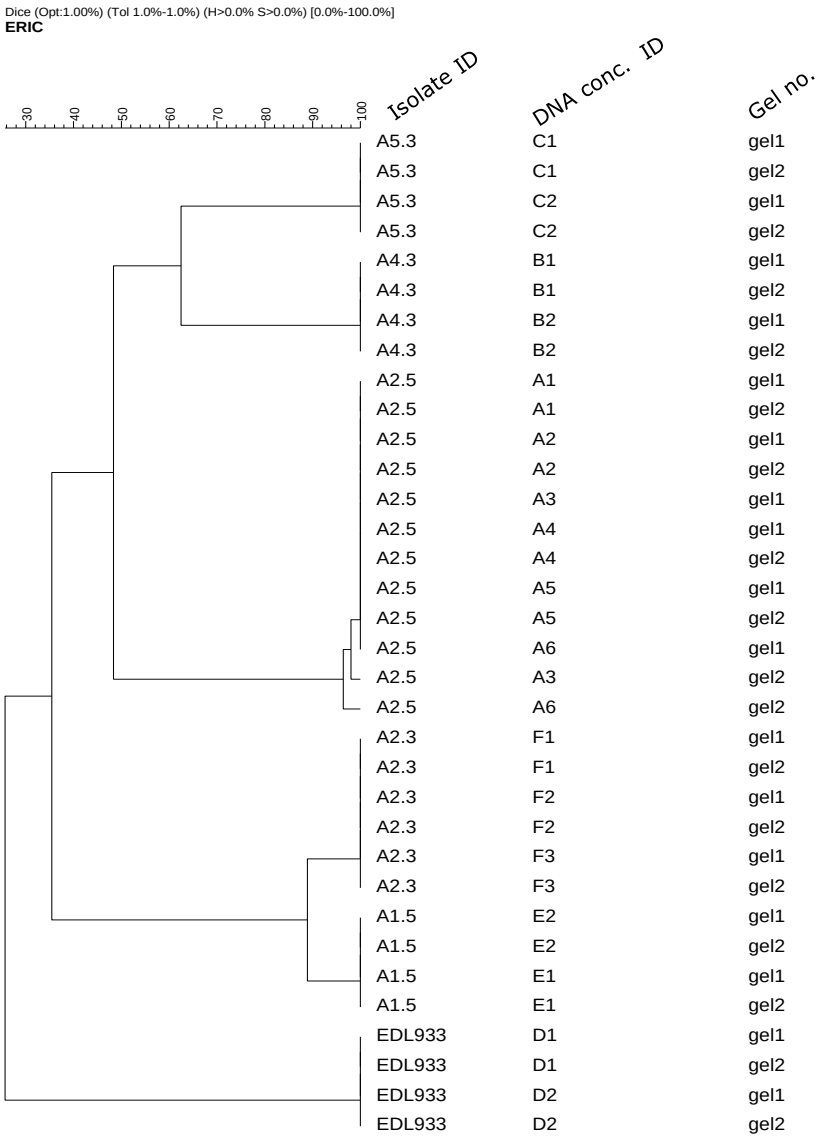
Number of families	**1**				
Number of comparisons per family	15				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Cow barn :TBX vs. Cow barn :TBX+CTX	2.429	2.222 to 2.636	Yes	****	<0.0001
Cow barn :TBX vs. Slurry tank:TBX	1.026	0.8366 to 1.215	Yes	****	<0.0001
Cow barn :TBX vs. Slurry tank:TBX+CTX	3.714	3.482 to 3.946	Yes	****	<0.0001
Cow barn :TBX vs. Solid Slurry:TBX	-0.02746	-0.2168 to 0.1619	No	ns	0.9982
Cow barn :TBX vs. Solid Slurry:TBX+CTX	3.092	2.897 to 3.288	Yes	****	<0.0001
Cow barn :TBX+CTX vs. Slurry tank:TBX	-1.403	-1.61 to -1.196	Yes	****	<0.0001
Cow barn :TBX+CTX vs. Slurry tank:TBX+CTX	1.285	1.039 to 1.532	Yes	****	<0.0001
Cow barn :TBX+CTX vs. Solid Slurry:TBX	-2.457	-2.663 to -2.25	Yes	****	<0.0001
Cow barn :TBX+CTX vs. Solid Slurry:TBX+CTX	0.6632	0.4511 to 0.8754	Yes	****	<0.0001
Slurry tank:TBX vs. Slurry tank:TBX+CTX	2.688	2.456 to 2.92	Yes	****	<0.0001
Slurry tank:TBX vs. Solid Slurry:TBX	-1.053	-1.243 to -0.8641	Yes	****	<0.0001
Slurry tank:TBX vs. Solid Slurry:TBX+CTX	2.066	1.871 to 2.261	Yes	****	<0.0001
Slurry tank:TBX+CTX vs. Solid Slurry:TBX	-3.742	-3.974 to -3.51	Yes	****	<0.0001
Slurry tank:TBX+CTX vs. Solid Slurry:TBX+CTX	-0.622	-0.8587 to -0.3853	Yes	****	<0.0001
Solid Slurry:TBX vs. Solid Slurry:TBX+CTX	3.12	2.925 to 3.315	Yes	****	<0.0001

Appendix V: ERIC-PCR genotyping, preliminary study; replicates run in one gel.



The ERIC-PCR amplicons of different *E. coli* strains (n=6) performed on replicate DNA samples were run in 2% (w/v) TAE agarose gel at 120 V for 2 h; 2-6 replicates were performed for each isolate. This dendrogram was produced using FPQuest v4.5 with the UPGMA algorithm based on a Dice similarity coefficient with a 1.0% band position tolerance. The different DNA concentrations for each isolate have a different code, the DNA concentration (ng μl^{-1}) for replicates are, for A2.5, A1: 51.2; A2: 58.9; A3: 69.3; A4: 50; A5: 50; A6: 50. For A4.3 are, B1: 70 and B2: 74.1; for A5.3: C1: 59.9 and C2: 42.5; for *E.coli* EDL933 is D1: 90.5 and D2: 31; for A1.5 are E1: 83.9 and E2: 91.4 and for A2.4 are F1: 38.1, F2: 74.4 and F3: 50.

Appendix VI: ERIC-PCR genotyping, preliminary study; replicates run in two gels.



The ERIC-PCR amplicons of different *E. coli* strains (n=6) performed on replicate DNA samples were resolved by two agarose gels (gel 1 and 2), the isolates were run in a 2% (w/v) TAE agarose gel at 120 V for 2 h; the dendrogram was produced using FPQuest v4.5 with the UPGMA algorithm based on a Dice similarity coefficient with a 1.0% band position tolerance. The different DNA concentrations for each isolate have a different code, the DNA concentration (ng μl^{-1}) for A2.5 replicates are A1: 51.2; A2: 58.9; A3: 69.3; A4: 50; A5: 50; A6: 50. For A4.3 are, B1: 70 and B2: 74.1; for A5.3: C1: 59.9 and C2: 42.5; for *E.coli* EDL933 is D1: 90.5 and D2: 31; for A1.5 are E1: 83.9 and E2: 91.4 and for A2.4 are F1: 38.1, F2: 74.4 and F3: 50. The same PCR reaction amplimers were run on 2 different gels, the gel number is indicated to allow comparisons.

Appendix VII: AMOVA statistical analysis for the 7 clusters of ERIC dendrogram at 54% similarity threshold; this includes 123 strains, outlier not included.

Data Sheet	**54% similarity**					
Data Title						
No. Samples	123					
No. Pops	7					
No. Regions	1					
No. Permutations	999	No. PW Pop Permutations	999			
Pairwise Matrix as Table						

Pop1	Pop2	PhiPT	#Pop1	#Pop2	P(rand >= data)	No. PW Pm
C1	C2	0.444	9	41	0.001	999
C1	C3	0.504	9	33	0.001	999
C2	C3	0.377	41	33	0.001	999
C1	C4	0.667	9	2	0.020	999
C2	C4	0.497	41	2	0.003	999
C3	C4	0.433	33	2	0.002	999
C1	C5	0.754	9	12	0.001	999
C2	C5	0.502	41	12	0.001	999
C3	C5	0.560	33	12	0.001	999
C4	C5	0.617	2	12	0.011	999
C1	C6	0.572	9	7	0.001	999
C2	C6	0.582	41	7	0.001	999
C3	C6	0.514	33	7	0.001	999
C4	C6	0.550	2	7	0.026	999
C5	C6	0.725	12	7	0.001	999
C1	C7	0.635	9	19	0.001	999
C2	C7	0.617	41	19	0.001	999
C3	C7	0.469	33	19	0.001	999
C4	C7	0.557	2	19	0.006	999
C5	C7	0.680	12	19	0.001	999
C6	C7	0.582	7	19	0.001	999

Appendix VIII: Antimicrobial sensitivity results for 2012 /2014 strains for 17 antimicrobials. Numbers in black with no highlight show sensitivity, numbers highlighted with yellow show intermediate resistance and numbers highlighted with red show resistance.

	Antibiotics																
Isolate ID	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP
A1.1	27	20	0	20	30	13	25	20	24	25	32	30	17	24	30	30	34
A1.3	25	18	19	20	30	13	25	20	24	20	30	30	15	25	31	30	33
A1.4	27	22	22	21	31	14	30	24	28	25	34	30	20	25	30	16	32
A1.5	28	23	20	22	30	14	30	24	25	27	34	33	22	24	29	30	32
A2.1	28	20	18	22	30	14	28	23	26	27	32	31	17	24	30	30	35
A2.2	27	22	20	22	31	14	24	21	25	26	32	30	17	24	32	30	34
A2.3	27	21	20	24	30	13	28	20	25	25	30	30	17	25	30	29	32
A2.4	27	18	20	21	31	14	27	24	25	25	30	31	15	26	30	32	34
A2.5	0	12	22	0	30	0	27	25	8	0	17	14	0	23	20	12	30
A3.4	26	22	20	22	30	13	27	23	25	23	30	30	20	24	31	32	34
A3.5	27	20	20	21	29	15	28	25	25	24	31	32	16	25	30	34	35
A4.1	28	24	22	22	30	14	30	21	28	26	31	32	25	24	30	25	34
A4.2	28	21	21	21	29	14	26	23	24	25	30	30	17	24	30	30	34
A4.3	29	23	17	24	30	13	27	24	25	24	31	30	20	24	31	32	34
A4.4	28	21	20	21	30	14	26	24	25	25	35	30	16	24	30	31	35
A4.5	27	23	20	22	30	13	27	22	27	28	32	30	17	24	32	30	11
A5.1	28	14	20	24	30	13	26	22	25	25	35	29	17	25	31	24	34
A5.3	28	25	21	34	32	14	30	26	25	25	34	33	20	25	32	24	30
A5.4	27	20	20	20	30	14	27	24	25	30	35	33	20	24	32	30	35
A5.5	27	22	19	23	30	13	27	22	25	27	30	30	17	25	30	35	35
E.coli ATCC 25922	26	19	20	25	30	15	25	24	26	20	34	30	17	26	31	30	32
BC1	30	20	20	24	26	0	30	23	30	26	32	30	19	25	29	14	30
BC10	30	21	22	24	26	16	30	24	23	26	30	20	18	26	30	23	32
BC11	30	17	20	0	30	15	30	24	26	24	32	29	16	0	29	17	30
BC12	27	20	23	24	30	19	28	25	25	18	40	30	16	27	28	34	36
BC13	25	0	21	0	29	8	20	22	22	0	31	29	0	26	29	24	29
BC14	22	21	20	22	28	15	25	10	25	0	30	29	18	25	30	25	35

Isolate ID	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP
BC15	29	25	20	22	28	15	31	25	26	25	31	32	21	25	30	32	36
BC2	30	22	24	21	30	16	32	26	0	22	40	33	20	0	31	30	34
BC3	0	25	25	26	30	0	25	27	28	21	34	34	20	26	32	29	35
BC4	27	17	20	23	28	14	25	24	22	25	30	29	17	23	31	29	30
BC5	25	18	0	23	27	11	25	21	24	25	33	28	12	26	29	25	33
BC6	30	26	20	22	30	16	30	24	30	23	34	33	21	26	31	34	37
BC7	30	25	8	0	30	15	31	14	29	20	40	35	18	25	30	30	37
BC8	30	25	21	25	35	18	30	25	30	21	37	37	21	25	30	35	40
BC9	31	24	20	24	34	19	30	25	27	20	36	35	18	30	28	34	40
BCC10	24	9	22	0	20	8	21	10	0	0	30	20	0	25	31	24	0
BCC11	24	10	20	0	26	8	20	20	22	0	18	19	0	25	28	22	30
BCC12	24	10	0	0	27	8	19	24	20	0	28	19	0	24	30	21	30
BCC14	24	9	19	0	26	8	20	24	22	0	29	20	0	27	30	23	30
BCC15	23	0	20	0	28	8	20	20	17	0	30	20	0	25	30	21	30
BCC16	8	9	20	0	25	9	15	23	0	0	29	19	0	24	30	21	27
BCC1	25	16	0	22	30	13	15	21	8	22	15	10	0	24	29	10	30
BCC2	0	14	0	0	25	0	24	0	0	0	18	20	0	8	6	0	14
BCC3	22	8	19	0	25	8	20	24	22	0	30	19	0	25	30	21	30
BCC4	25	10	19	0	28	8	20	24	21	0	30	20	0	26	29	22	30
BCC5	22	0	0	0	25	7	20	24	0	0	23	18	0	25	31	21	30
BCC6	26	11	20	0	28	7	20	24	21	0	30	19	0	24	32	22	28
BCC7	24	7	23	0	29	7	20	24	22	0	34	21	0	30	31	27	35
BCC8	26	11	20	0	30	8	21	24	24	0	34	21	0	25	31	25	34
BCC9	30	9	22	0	29	9	21	25	22	0	34	23	0	29	30	10	35
BCE1	28	20	24	26	30	8	17	22	0	22	14	13	0	27	30	8	27
BL1	30	21	20	23	30	15	27	24	26	25	34	30	16	0	29	25	35
BL10	30	24	24	25	35	15	19	28	0	24	40	34	0	27	30	27	34
BL10b	28	24	20	21	30	18	29	24	26	24	35	33	21	25	32	34	34
BL11	33	25	23	22	27	15	32	28	25	26	35	34	22	25	35	29	33

Isolate ID	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP
BL12	30	22	24	26	30	15	30	30	26	20	36	30	19	27	32	30	34
BL13	0	12	25	0	35	0	30	28	26	0	27	30	0	0	35	30	34
BL14	28	24	23	26	35	14	31	30	30	26	39	24	25	26	33	30	33
BL15	26	25	25	0	35	13	30	27	26	24	34	15	20	25	27	30	30
BL2	0	24	21	21	35	16	30	24	27	25	32	33	21	27	30	34	34
BL3	0	20	21	24	28	14	22	22	0	8	30	30	19	26	30	29	31
BL4	26	19	0	24	26	14	26	22	24	25	30	30	16	25	30	25	30
BL5	8	20	21	24	28	13	25	24	26	10	32	30	17	25	30	30	32
BL6	27	30	21	20	25	15	35	25	30	24	40	34	30	26	34	30	35
BL7	30	26	25	24	30	15	34	30	30	26	30	35	0	28	33	30	34
BL8	28	22	20	24	30	14	29	24	26	27	33	32	18	26	30	29	30
BL9	30	24	22	19	34	15	32	30	28	24	35	34	20	26	30	30	34
BLC2	27	8	24	0	24	8	21	24	19	0	31	20	0	24	30	20	30
BLC3	27	10	21	0	30	0	21	27	0	0	34	23	0	0	30	12	36
BLC4	29	18	25	0	31	17	18	27	17	20	36	25	0	28	31	27	30
BLC5	25	10	21	0	28	8	21	24	22	0	30	20	0	25	30	21	30
BLC6	0	0	20	0	25	10	8	0	21	0	29	19	0	0	12	20	30
BLC7	24	0	20	0	25	8	17	24	0	0	30	19	0	25	30	21	32
BLC8	25	0	21	0	26	9	18	25	20	0	30	18	0	25	30	21	33
BLC9	24	0	20	0	25	9	18	24	20	0	31	20	0	25	28	9	30
BS1	27	20	22	22	32	15	28	24	25	24	39	33	14	26	28	30	35
BS10	30	21	20	24	32	16	30	24	25	24	34	33	19	23	28	30	37
BS11	27	18	19	25	28	14	25	23	0	25	32	30	17	25	0	30	29
BS12	30	21	22	21	25	15	30	24	25	23	30	13	16	20	28	30	28
BS13	20	21	20	0	28	13	27	22	25	23	33	30	0	24	29	24	33
BS14	23	20	0	24	28	12	8	0	25	25	34	30	18	25	29	30	35
BS15	26	17	21	25	29	14	11	21	0	25	30	29	15	22	29	30	30
BS2	28	14	20	23	30	14	26	23	25	24	33	32	0	25	30	30	33

Isolate ID	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP
BS3	27	20	20	24	29	14	28	24	25	25	30	30	17	25	30	30	34
BS4	23	18	19	24	27	14	25	24	25	24	32	29	0	0	28	30	17
BS5	30	18	20	25	27	14	27	22	26	27	33	30	20	26	31	30	34
BS5b	24	20	21	25	28	13	25	0	0	20	30	29	15	25	30	30	30
BS6	30	25	21	0	26	15	30	24	30	30	34	35	20	30	31	34	36
BS7	0	12	22	0	29	8	29	24	25	0	26	30	0	0	33	30	36
BS8	23	21	19	24	28	14	30	0	25	24	34	35	20	25	30	30	35
BS9	25	16	20	0	27	13	15	20	0	20	16	11	0	25	28	8	30
BSC10	24	9	20	0	28	8	20	24	21	0	30	20	0	25	30	22	31
BSC13	23	8	20	0	0	0	20	25	21	0	30	20	0	25	30	22	34
BSC14	0	8	18	0	0	9	21	25	22	0	30	19	0	28	30	23	34
BSC15	25	9	22	0	30	8	19	25	25	0	35	21	0	28	35	21	35
BSC16	35	10	22	0	22	9	22	25	20	0	30	20	0	25	27	21	30
BSC17	27	12	20	0	27	10	20	23	20	0	31	20	0	23	30	22	31
BSC18	27	11	22	0	26	0	21	24	22	0	30	20	0	25	31	24	32
BSC19	23	0	20	0	25	9	17	22	20	0	30	17	0	25	30	21	34
BSC1	24	10	20	0	30	10	20	23	25	0	35	22	0	26	30	24	35
BSC20	0	10	21	0	27	8	21	25	24	0	34	20	0	25	26	24	35
BSC21	22	0	0	0	25	9	18	23	0	0	30	18	0	0	30	22	30
BSC22	24	19	9	23	26	14	13	20	10	0	15	10	0	23	27	8	28
BSC2	24	8	19	0	25	8	17	24	20	0	30	19	0	24	30	9	31
BSC3	28	10	24	0	30	0	19	26	0	0	35	23	0	30	35	20	34
BSC4	27	10	24	0	30	8	20	28	25	0	35	23	0	28	34	21	34
BSC5	26	8	24	0	30	0	19	30	20	0	40	24	0	24	30	20	35
BSC6	0	12	18	0	32	0	18	23	25	0	34	20	0	30	35	21	35
BSC7	27	18	24	0	34	8	35	27	25	0	34	21	0	30	35	22	40
BSC8	25	14	17	23	25	14	25	21	21	25	30	25	0	21	26	27	30
BSC9	27	9	25	0	20	8	22	25	20	0	20	0	0	28	34	21	34
BSE1	27	18	19	23	28	14	30	20	22	26	34	26	0	22	28	30	30

Isolate ID	SXT	AMC	F	OT	IMP	S10	CAZ	NA	EFT	S300	CFQ	ATM	AMP	C	ENR	CTX	CIP
BSE2	26	20	21	25	30	14	16	21	8	22	16	13	0	25	27	8	30
BSE3	34	23	23	28	33	19	33	0	30	30	37	30	0	24	24	31	30
BSE9	0	17	17	22	27	15	30	21	24	25	30	25	0	23	28	30	30
BSE4	27	19	18	24	30	15	26	21	0	28	30	28	0	22	27	30	26
BCE2	26	19	21	24	30	14	16	21	8	20	16	13	0	26	28	9	31
BCE3	0	17	21	0	28	0	26	9	13	0	19	22	0	0	7	15	11
BSE3	26	19	20	24	30	14	16	15	0	20	14	12	0	25	28	10	30
BCE4	27	20	21	25	29	14	16	20	10	21	17	24	0	25	27	10	30
BCE5	8	19	22	25	30	15	16	21	7	24	17	14	0	26	30	10	25
E.coli NCTC13353	0	14	24	0	30	14	13	0	0	0	10	11	0	25	0	0	0
BSE8	24	15	15	20	24	12	21	18	19	20	27	25	0	20	26	21	25
BSE7	28	18	20	23	26	15	26	20	22	26	30	27	0	22	28	28	30
BSE6	24	18	20	21	27	13	13	20	8	17	15	11	0	22	25	9	26
BSE5	24	17	16	22	26	11	21	18	19	22	28	22	0	20	24	24	30
BLC1	21	9	19	0	24	9	16	21	19	0	26	18	0	22	26	19	25

key: ampicillin (AMP), amoxicillin–clavulanic acid (AMC), cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM) , cefquinome (CFQ), ceftiofur (EFT), streptomycin (S10), ciprofloxacin (CIP), enrofloxacin (ENR), nalidixic acid (NA), trimethoprim/sulfamethoxazole (SXT), sulphonamide (S300), chloramphenicol (CHL), oxytetracycline (OT), nitrofurantoin (F).

Appendix IX: Phenotypic confirmation test of ESBL- and /or AmpC-producing *E. coli* in isolates which showed initial resistance to cefotaxime or ceftazidime.

Inhibition zone sizes for ESBL *E. coli* isolates using the ESBL (columns: CTX to FEP +C) test kits. D is the difference between the 2 previous values (or the difference between antibiotic alone and antibiotic with supplement); 5 mm or greater difference for D indicates positive results for the test.

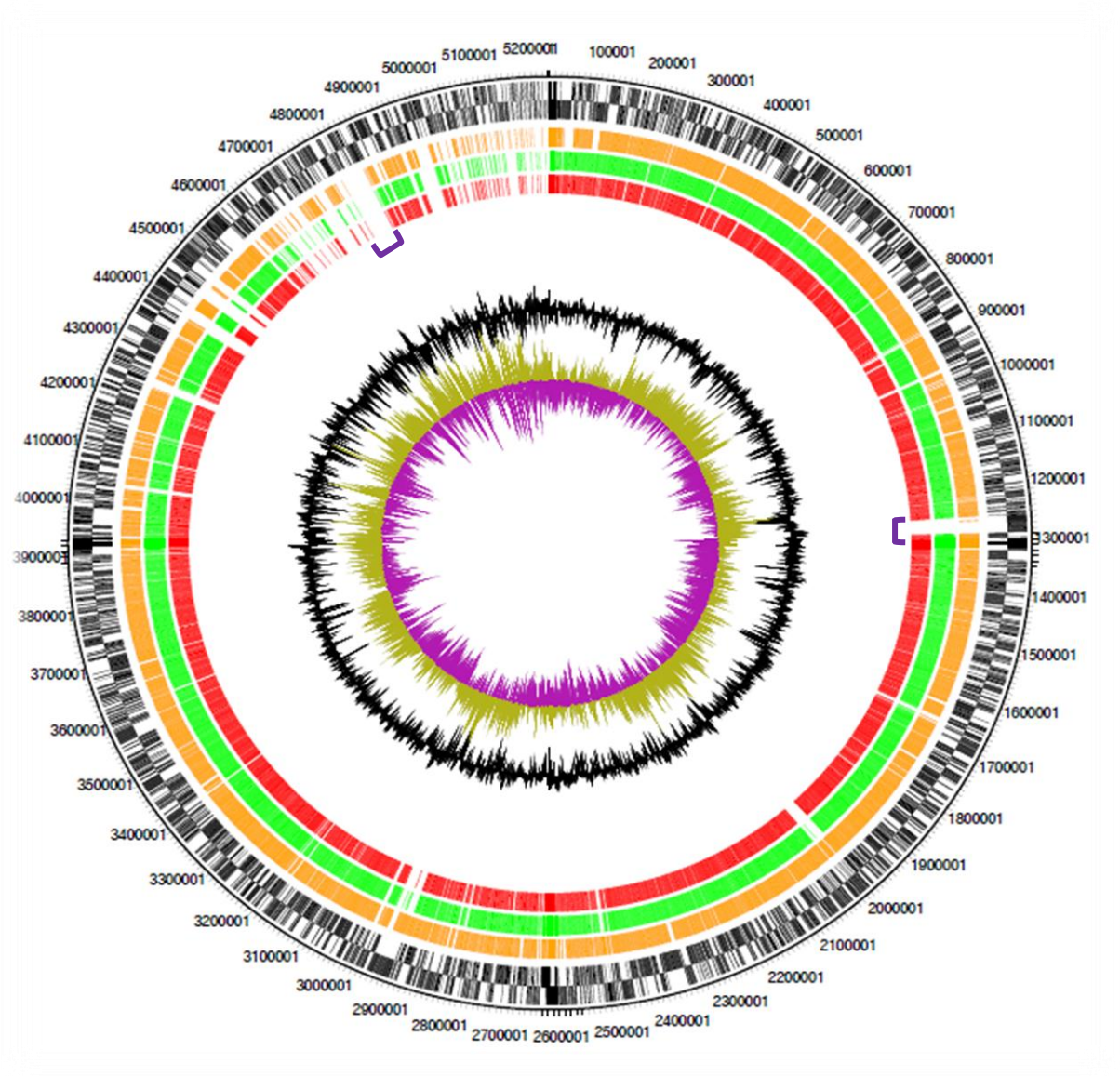
Isolate ID	Initial cephalosporin resistance (antibiotics/inhibition zone in mm)	CTX	CTX+C	D1	CAZ	CAZ+C	D2	FEP	FEP +C	D3
A1.4	CTX/16	30	32	2	30	30	0	30	30	0
A2.5	CTX/ 12	30	32	2	30	30	0	30	30	0
A4.1	CTX/ 25	31	33	2	30	31	1	29	30	1
A5.3	CTX/ 24	31	32	1	31	32	1	30	30	0
BC1	CTX/14	30	30	0	26	28	2	30	30	0
BC10	CTX/ 23	30	31	1	30	33	3	30	31	1
BC11	CTX/ 17	29	29	0	25	28	3	30	30	0
BC14	CTX/ 25	29	30	1	30	30	0	33	34	1
BC5	CTX/ 25	30	31	1	30	30	0	30	31	1
BL1	CTX/ 25	30	31	1	30	31	1	29	30	1
BL4	CTX/ 25	30	31	1	30	30	0	32	33	1
BS13	CTX/ 24	31	32	1	31	33	2	30	32	2
BS14	CAZ/ 8	30	32	2	30	30	0	28	30	2
BSE5	CTX/24	30	31	1	28	30	2	32	33	1

Appendix X: Some assembly statics and information for the sequenced strains (BS7, BCC2, BCE3 and A2.5), provided by Dr. Lisa Crossman

	BS7	BCC2	BCE3	A2.5
Total contigs	206	148	133	235
average length	26220	34762	38675	NP
median	1007	2230.5	3456	NP
>=100	199	146	131	NP
shortest contig	92	92	92	287
largest contig	731431	582240	582262	351328
total length	5.401 Mb	5.144 Mb	5.144 Mb	5.233 Mb
assembly accessions no.	FLMS01000001 - FLMS01000076	FLMT01000001 - FLMT01000075	FLMV01000001 - FLMV01000065	FLMX01000001 - FLMX01000235

NP: not provided

Appendix XI: A circular figure comparing the genome content for A2.5 with BS7, BCC2 and BCE3 using A2.5 as the reference strain.



Circles from the outermost to innermost circles indicate genes in forward and reverse orientation, in separate tracks, of the reference genome (in black). The next circular tracks are coloured by genome (BS7, BCC2 and BCE3 are coloured orange, green and red respectively) are the orthologous genes that are shared with the reference genome. The inner circles in the centre are GC content and GC skew in magenta and yellow respectively. GC skew represents the difference in GC content between the leading strand and the lagging strand of the genomic DNA. Some of the sizeable genomic islands on the A2.5 plot do not have a counterpart in the other genomes e.g. at just before 1300001 is a phage island (Mu phage); another example is the region just before 4800001 (HK97 family phage) both region are labelled by the purple bracket, this figure was created by Dr Lisa Crossman.

Appendix XII: MLST results for A2.5, BCE3, BCC2 and BS7

Locus	% Identity	Allele			
		A2.5	BCE3	BCC2	*BS7*
adk	100	*adk-6*	*adk-9*	*adk-9*	*adk-21*
fumc	100	*fumc-4*	*fumc-65*	*fumc-65*	*fumc-35*
gyrb	100	*gyrb-4*	*gyrb-5*	*gyrb-5*	*gyrb-27*
icd	100	*icd-18*	*icd-1*	*icd-1*	*icd-6*
mdh	100	*mdh-24*	*mdh-9*	*mdh-9*	*mdh-5*
pura	100	*pura-5*	*pura-13*	*pura-13*	*pura-5*
reca	100	*reca-14*	*reca-6*	*reca-6*	*reca-4*
ST		ST56	ST162	ST162	ST69

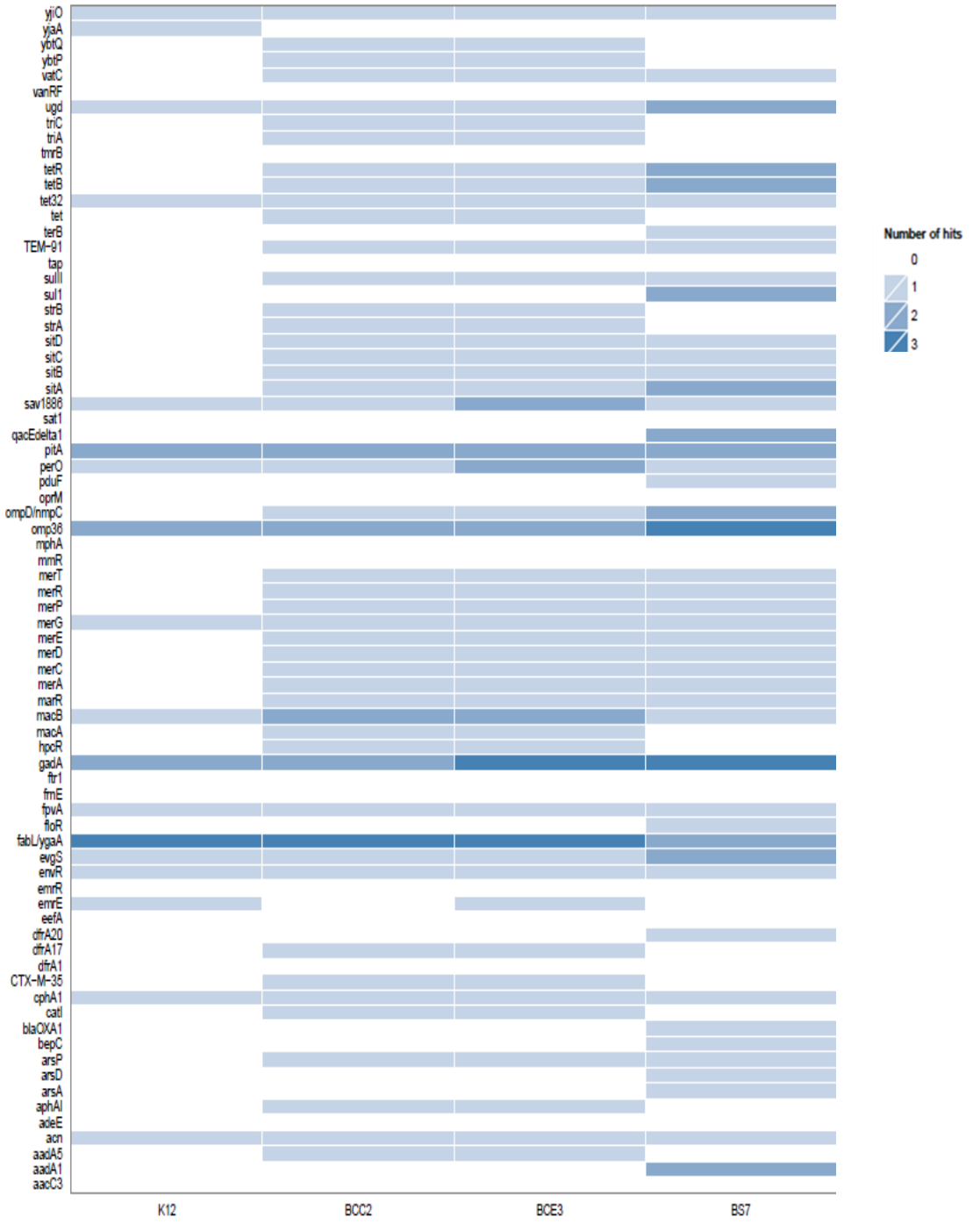
Appendix XIII: Predicted serotypes for A2.5, BCC2, BCE3 and BS7 using serotype finder databases provided by the Centre for Genomic Epidemiology.

	H type						
Strain ID	**Serotype gene**	**%Identity**	**Query/HSP length***	**Contig**	**Position in contig**	**Predicted serotype**	**Accession number**
A2.5	*fliC*	98.64	1476 / 1476	A2.5__04022014.gff	2464804..2466279	H21	AIHL01000060
BCE3	*fliC*	100.00	2013 / 2013	ECBCE_contig01	1152399..1154411	H9	AY249994
BCC2	*fliC*	100.00	2013 / 2013	ECBCC2_contig01	633995..636007	H9	AY249994
BS7	*fliC*	100.00	1665 / 1665	ECBS7_contig001	1964433..1966097	H18	AY250001

	O type						
Strain ID	**Serotype gene**	**%Identity**	**Query/HSP length**	**Contig**	**Position in contig**	**Predicted serotype**	**Accession number**
A2.5	*wzx*	99.87	1494 / 1494	A2.5__04022014.gff	2932950..2934443	O3	EU694097
	wzy	100.00	1086 / 1086	A2.5__04022014.gff	2935591..2936676	O3	EU694097
BCE3	*wzt*	99.92	1296 / 1296	ECBCE_contig01	3736577..3737872	O9	AB010293
	wzm	99.87	786 / 786	ECBCE_contig01	3737875..3738660	O9	atsushi
BCC2	*wzm*	99.87	786 / 786	ECBCC2_contig01	4734389..4735174	O9	atsushi
	wzt	99.92	1296 / 1296	ECBCC2_contig01	4735177..4736472	O9	AB010293
BS7	*wzx*	100.00	1236 / 1236	ECBS7_contig001	2067076..2068311	O17/O77	Og17O77
	wzy	100.00	1332 / 1332	ECBS7_contig001	2068353..2069684	O17/O44	DQ000314

* HSP: high-scoring segment pair

Appendix XIV: heatmap for the differences in the predicted AMR genes and metal resistance genes between BS7, BCC2 and BCE3 comparing with reference strain *E. coli* K12.



The number of hits represents the number of orthologues found in each genome, so these are protein orthologues found in both CARD and within the predicted genes from each genome, white denotes absence of orthologues, a gradient blue colour represents (1-3) hits for presence of the orthologous protein. This heatmap was produced by Dr Lisa Crossman.

Appendix XV: Genes related to AMR carried by BCC2, BCE3, BS7 and A2.5.

The genes are divided into groups according to their antibiotic resistance classes. The final group are genes which might be related to AMR or metal resistance. Genes location and direction determined using Artemis genome browser tool.

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
β-lactam antibiotic resistance genes							
bla$_{TEM-1}$	475597-476457 (R)	3798357-3799217 (F)	-	5028949-5029809 (F)	861	β-lactamase TEM-1	WP_000027057.1
bla$_{TEM-30}$	-	-	148249-149109 (F)	-	861	β-lactamase TEM-30	WP_021561477.1
bla$_{CTX-M-14}$	3030094-3030969 (F)	1490642-1491517 (F)	-	-	876	β-lactamase CTX-M-14	WP_001617865.1
bla$_{OXA-1}$	-	-	156306-157181 (F)	4736949-4737824 (F)	876	β-lactamase OXA-1 precursor	WP_000881655.1
Aminoglycoside resistance genes							
strB	927422-928258 (R)	64752-65588 (F)	-	5119113-5119949 (F)	837	Streptomycin phosphotransferase	WP_024211122.1
strA	928258-929061 (R)	63949-64752 (F)	-	5118310-5119113 (F)	804	Aminoglycoside phosphotransferase	WP_001696862.1

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
aphAI	2050010-2050825 (F)	67329-68144 (F)	-	-	816	Aminoglycoside 3'-phosphotransferase	WP_012846873.1
aadA1	-	-	157306-158085 (F) 3146312-3147091 (R)	4738242-4739021 (F)	780	Streptomycin adenylyltransferase	ACI43574.1
aadA5	2040038-2040826 (R)	4060158 - 4060946 (R)	-	-	789	Streptomycin and spectinomycin resistance aminoglycoside adenyltransferase	AF137361.1.gene1
Sulphonamide resistance genes							
sul1	-	-	158590-159429 (F) 3144968-3145807 (R)	4739526-4740365 (F)	840	Sulphonamide-resistant dihydropteroate synthase	WP_000451390.1
sul2	929122 929937 (R)	63073-63888 (R)	15440-16255 (R)	5180873-5181688 (R)	816	Sulphonamide-resistant dihydropteroate synthase	WP_053896540.1

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
Phenicol resistance							
catAI	2047454-2048113 (R)	4067574-4068232 (R)	-	-	660	Type A chloramphenicol O-acetyltransferase	WP_050436713.1
floR	-	-	3678821-3680035 (R)	4729408-4730622 (R)	1215	Chloramphenicol/florfenicol efflux MFS transporter FloR	WP_000214123.1
Tetracycline resistance genes							
tetB	472346-473551 (F)	3801263-3802468 (R)	-	5011826-5013031 (R)	1206	TetB tetracycline resistance MFS efflux pump	WP_012600019.1
tetA	-	-	1146543-1147670 (F) 2506693-2507820 (F)	-	1129	TetA family tetracycline resistance MFS efflux pump	WP_013279371.1
tetL	-	-	-	5026392-5027768 (R)	1377	Tetracycline efflux MFS transporter	WP_001574277.1

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
tetR	471641-472264 (R)	3802550-3803173 (F)	2505866-2506543 (R)** 1145716-1146393 (R)**	-	678** 624	TetR family transcriptional regulator (class A protein)	WP_004193135.1
Trimethoprim resistance genes							
dfrA17	2040957-2041430 (R)	4061077-4061550 (R)	-	-	474	Trimethoprim-resistant dihydrofolate reductase DfrA	WP_000752532.1
dfr (unknown)	-	-	14476-14988 (R)	5129781-5130293(F)	513	Dihydrofolate reductase	WP_000949574.1
Mercury resistance genes							
merR	2032382-2032816 (R)	6384-6818 (F)	149807-150241 (F)	4744789-4745037 (F)**	435 249**	Mercuric responsive transcriptional regulator/ putative mercuric resistance operon regulatory protein	KKA59047.1 EFJ85572.1
merT	2032888-2033238 (F)	5962-6312 (R)	149385-149735 (R)	-	351	Mercuric transport protein	EFO55295.1

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
merP	2033252-2033527 (F)	5673-5948 (R)	144143-144349 (R)** 149204-149371 (R)**	-	276 207** 168**	Mercuric transporter periplasmic component	WP_021538482.1
merA	2034037-2035731 (F)	3469 5163 (R)	141939-143633 (R)	-	1695	Mercury(II) reductase	WP_042066333.1
merD	2035749-2036111 (F)	3089-3451 (R)	141559-141921(R)	-	363	Transcriptional regulator	WP_012644013.1
merE	2036108-2036344 (F)	2856-3092 (R)	141326-141562 (R)	-	237	Mercury ion transmembrane transporter	EFO55297.1
merC	2033563-2033985(F)	5215-5637 (R)	143685-144107 (R)	-	423	Mercury transport protein -	WP_050858778.1
others							
mdtG	-	-	-	333036-334262 (F)	1227	Drug efflux system protein	EFF12375.1

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
sitA	-	-	421705-422619 (R)	-	915	Iron ABC transporter substrate-binding protein [Enterobacteriaceae]	WP_000555630.1
sitC	402093-402950 (F)	3820981-3821838 (R)	4457983-4458840 (F)	-	858	Iron/manganese ABC transporter, permease protein	ESD82615.1
qacEΔ1	-	-	158249-158596 (F) 3145801-3146148 (R)	4739185-4739532 (F)	348	Ethidium bromide and quaternary ammonium compound export protein	EGB77378.1
pitA	1697074-1698573 (R)	750971-752470 (F)	1255465-1256964 (R)	-	1500	Phosphate transporter PitA	WP_058055539.1
pduF	-	-	2030535-2031344 (R)	-	810	Aquaporin	WP_012602821.1
nmpC	2181359-2182456 (F)	3868582-3869679 (F)	163957-165039 (R)**	4678033-4679130 (F)	1098 1083**	Outer membrane porin protein	ESS89391.1 ABE06783.1 (bs7)

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
ompC	2428625-2429728 (R)	4928756 - 4929859 (F)	2275264-2276367 (R)	736488-737612 (F)**	1104 1125**	Outer membrane protein C	ACV88153.1 WP_001766154.1
emrE	-	3877951 - 3878175 (R)	-	-	225	Multidrug transporter *emrE*	EFZ69307.1
ybtQ	3433387-3435189 (F)	4239308 - 4241110 (R)	-	-	1803	Lipid ABC transporter permease/ATPase	WP_060684434.1
ybtP	3431688-3433400 (F)	4241276 - 4242898 (R)	-	-	1713	Lipid ABC transporter permease/ATPase	EQV41139.1
ugd	-	-	2064101-2065267 (R)	-	1167	UDP-glucose 6-dehydrogenase	WP_000704862.1
sitD	-	-	25733-26425 (F)	-	693	Iron ABC transporter permease	WP_013362825.1

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
sitD	402947-403804 (F)	3820127-3820984 (R)	-	-	858	Iron ABC transporter permease	WP_001514241.1
sitB	401269-402096 (F)	3821835-3822662 (R)	-	-	828	Manganese/iron transport system ATP-binding protein	WP_021517436.1
envR	-	-	4475058-4475720 (F)	-	663	*acrEF*/*envCD* operon repressor	WP_001129506.1
arsA	-	-	1243720-1245471 (R)	-	1752	Arsenical pump-driving ATPase	WP_012602880.1
arsD	-	-	1245489-1245851 (R)	-	363	Arsenical resistance operon trans-acting repressor	EGI39290.1
arsR	-	-	1245899-1246252 (R)	1463214-1463567 (F)	354	Arsenical resistance operon transcriptional repressor	WP_057108393.1
bepC	-	-	2609764-2611119 (R)	-	1356	Type I secretion outer membrane protein	WP_001601792.1

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
evgS	2578562-2582155 (F)	4776334-4779927 (R)	403742-405484 (R) 405559-407334 (R)**	-	3594 (1743 &1776)**	Sensor histidine kinase/response regulator	ABV18742.1
gadA	2982750-2984150 (F)	3559411-3560811 (R)	1215734-1217134 (F)	-	1401	Glutamate decarboxylase alpha	AAN82764.1
	-	780797-782197 (R)	4271760-4273160 (R)	-	1401	Glutamate decarboxylase beta	CDN82039.1
ompF	164039-165127(R)	2509292-2510380 (F)	3705433-3706521(F)	2687634-2688722 (F)	1089	Outer membrane protein F	WP_032221014.1
marR	1453866-1454300 (R)	833034-833468 (F)	4331781-4332215 (F)	3941291-3941725 (R)	435	Multiple antibiotic resistance protein	WP_000843419.1
marA	1453463-1453846 (R)	833488-833871 (F)	4332235-4332618 (F)	3940888-3941271 (R)	384	Multiple antibiotic resistance protein	EFI19621.1
macA	97168-98283 (F)	2576134-2577249 (R)	3803325-3804440 (R)	1486830-1487945 (R)	1116	Macrolide transporter membrane fusion protein (MFP) component	WP_029487409.1

Resistance gene	BCC2	BCE3	BS7	A2.5	Length (bp)	Gene description	Accession number
	Genomic Location (direction)						
envR	-	-	4475058-4475720 (F)	-	663	*acrEF*/*envCD* operon repressor	WP_001129506.1
acn	1078601-1081276 (F)	2125990-2128665 (F)	553932-556607 (F)	130452-133127 (R)	2676	Aconitate hydratase	WP_000099530.1
ygaA	5111939-5113453 (F)	3398899-3400017 (F)**	3259055-3260569 (R)	1729267--1730781 (R)	1515 1119**	anaerobic nitric oxide reductase DNA-binding transcriptional activator	WP_000010723.1
macB	98280-100226 (F)	2574191-2576137 (R)	3801382-3803328 (R)	1484887-1486833 (R)	1947	Macrolide ABC transporter permease /ATPase	WP_000188174.1
hpcR	4379777-4380223 (F)	3009910-3010356 (R)	-	2585612-2586058 (R)	447	Homoprotocatechuate degradative operon repressor	WP_024234247.1

*F: forward DNA strand; R: reverse DNA strand; **: indicate the gene with different length (shorter, longer or interrupted into two parts) comparing to the other genes from the other strains which might cause gene dysfunction.

Appendix XVI: Tn*21* elements within BS7, BCC2 and A2.5 genome

Elements*	Genomic location-direction		
	BS7	**BCC2**	**A2.5**
38bp terminal imperfect IR of transposon Tn*21* (front)	3680943-3680980 -F	2046805-2046842 -R	4731530-4731567 -F
tnpR	3683945-3684505 -R	2043280-2043840 -F	4734532-4735092-R
tnpA	3680976-3683942-R	2043843-2046809 -F	4731563-4734529 -R
Resolvase binding site III	154462-154491 -F	2043238-2043267 -R	4735105-4735134 -F
Resolvase binding siteII	154496-154538 -F	2043191-2043233 -R	4735139-4735181 -F
Resolvase binding siteI	154554-154590 -R	2043139-2043175 -R	4735197-4735233 -F
tnpM	3684634-3684981-R	2042954-2043154 -F	4735221-4735568 -R
Terminal imperfect IRi of In2	154926-154950	2042779-2042803	4735569-4735593 -F
attI1	156248-156282 -F	2041448-2041484 -R	4736891-4736925 -F
IntI1	155128-156141 -R	2041588-2042601 -F	4735771-4736784 -R
aadA1	157306-158085 -F	2040038-2040826 –R**	4737949-4738728 -F
Central imperfect repeat within 59-base element	158099-158115 -F	-	4738742-4738758 -F
SulI	158590-159429 -F	-	4739233-4740072 -F
qacEΔ1	158249-158596 -F	-	4738892-4739239 -F
Orf5	159547-159686 -F	-	4740200-4740433 -F
terminal of IR of IS*1326*	3144585-3144612 –R	-	4740428-4740453 -R
istB	135241-136026 -R	-	4742106-4742891 -R
istA	136013-137527 -R	-	4742878-4744392 -R
terminal IR of IS*1353*	-	-	-
orfAB	-	-	-
Terminal IR of	-	-	-

IS*1353*			
terminalIR of IS*1326*	137594-137643- R	-	4744483-4744508 -R
tniBΔ1	137638-138498 -R	-	-
tniA	138501-139664 -R	-	-
Urf2	140622-141329 -R	-	-
Terminal imperfect IRt of In*2*	140664-140688	2036982-2037006	-
merE	141326-141562 -R	2036108-2036344 -F	-
merD	141559-141921-R	2035749-2036111 -F	-
merA	141939-143633 -R	2034037-2035731 -F	-
merC	143685-144107 -R	2033563-2033985-F	-
merP	144143-144349 -R 149204-149371 -R	2033252-2033527 -F	-
merT	149385-149735 -R	2032888-2033238 -F	-
merR	149807-150241 -F	2032382-2032816 -R	4744789-4745037 -F
38bp terminal imperfect IR of Tn*21*	150237-150274-F	2032349-2032386-R	4744740-4744777 -F

*The sequence of the transposition genes, In*2* integron and *mer* genes for Tn*21* were checked using Tn*21* elements provided by NCBI *GenBank*: AF071413.3 ; ** It has *aadA5* not *aadA1*; _: element not present

Appendix XVII: Protein sequence alignment of the *dfr* gene from BS7 and *dfrA20* from *Pasteurella multocida*

```
Query  1    MKVSLIVAMDLEKGIGKNNDLMWHLPADMLFFKETTLNHIVVMGRKNFESIPERFRPLPN  60
            +K SLIVA+   + +G +NDL+WHLP DM FFKETT  H VVMGRK++ESIP+++RPLPN
Sbjct  3    IKYSLIVAIGKHREMGADNDLLWHLPRDMQFFKETTTGHAVVMGRKSWESIPQKYRPLPN  62

Query  61   RENAILTRNTAFEAPNCTVFHSMEGCLKHYENEDKRTVFIIGGGQIYEEALEKNRVDEMF  120
            R N +LTR+  + A   TV + ++   +H E ++  T FIIGG QIY+ ALE   ++EM+
Sbjct  63   RLNFVLTRDKNYSAEGATVIYDLKEVAQHLEGKN-LTCFIIGGAQIYQLALETGLLNEMY  121

Query  121  ITFVDHTF-GADTFFPSIDFSLWNEEVLRVHEADSKNAYNFTVKKFTK  167
            +T V +TF  ADTFFP +++  W EE +   + D K+ Y+F +KKFT+
Sbjct  122  VTQVHNTFEEADTFFPFVNWGEWEEEDILEQDKDEKHLYSFNIKKFTR  169
```

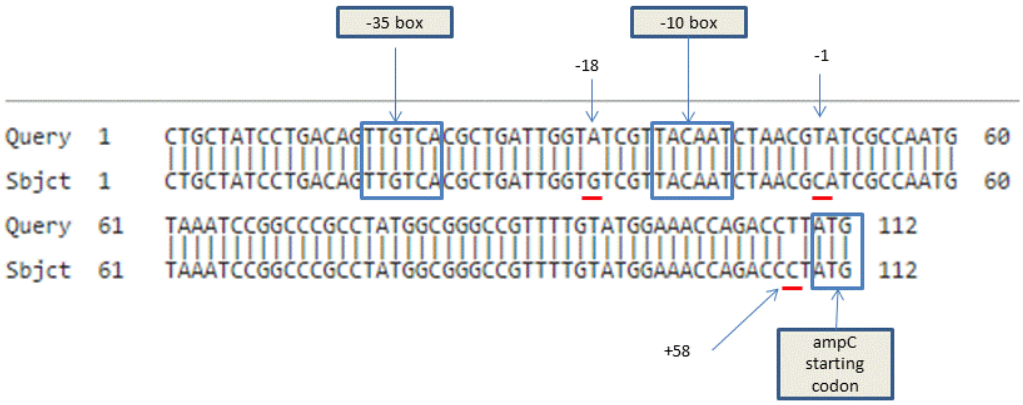
The protein query sequence is for *dfr* gene from BS7. The query length is 167aa, the subject length for *dfrA20* from *Pasteurella multocida* (Accession no. WP_032491181.1) is 169aa. The query has 49% identity with *dfrA20*, the middle line showing the substitution scoring; +: indicates conservative substitution

Appendix XVIII: predicted plasmid within BCC2 genome using PlasmidFinder–1.3 server.

Plasmid	%Identity	Query/HSP length*	Contig	Position in contig	Accession number
IncFIC(FII)	95.59	499 / 499	ECBCC2_contig01	2027227..2027723	AP001918
IncI1	99.30	142 / 142	ECBCC2_contig01	3739864..3740005	AP005147
IncFIB(AP001918)	98.39	682 / 682	ECBCC2_contig01	375260..375941	AP001918
IncI2	97.83	322 / 316	ECBCC2_contig01	4290181..4290502	AP002527
IncQ1	100.00	450 / 450	ECBCC2_contig01	931276..931725	HE654726

* HSP: high-scoring segment pair

Appendix XIX: Nucleotides sequence alignment of the promoter region of *ampC* from isolates BCE3 comparing to *E. coli* K12.



The nucleotide query sequence is for *ampC* promoter region for BCE3, the subject sequence is the nucleotide sequence for the promoter region for *E. coli* K12 (GenBank: U00096.3). Any point mutations are underlined with red line with their position and numbers (Numbering is according to Jaurin et al. 1981). The bases within boxes convey the -35 and -10 promoter sequence. The 'ATG' string of bases is the start codon for *ampC*.

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Research Article

RESEARCH ARTICLE

Multidrug resistant, extended spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolated from a dairy farm

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One sentence summary: Multidrug resistant E. coli were isolated from a dairy farm at high frequency.

Editor: Pascal Simonet

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ABSTRACT

Escherichia coli strains were isolated from a single dairy farm as a sentinel organism for the persistence of antibiotic resistance genes in the farm environment. Selective microbiological media were used to obtain 126 E. coli isolates from slurry and faeces samples from different farm areas. Antibiotic resistance profiling for 17 antibiotics (seven antibiotic classes) showed 57.9% of the isolates were resistant to between 3 and 15 antibiotics. The highest frequency of resistance was to ampicillin (56.3%), and the lowest to imipenem (1.6%), which appeared to be an unstable phenotype and was subsequently lost. Extended spectrum β-lactamase (ESBL) resistance was detected in 53 isolates and bla_{CTX-M}, bla_{TEM} and bla_{OXA} genes were detected by PCR in 12, 4 and 2 strains, respectively. Phenotypically most isolates showing resistance to cephalosporins were AmpC rather than ESBL, a number of isolates having both activities. Phenotypic resistance patterns suggested co-acquisition of some resistance genes within subsets of the isolates. Genotyping using ERIC-PCR demonstrated these were not clonal, and therefore co-resistance may be associated with mobile genetic elements. These data show a snapshot of diverse resistance genes present in the E. coli population reservoir, including resistance to historically used antibiotics as well as cephalosporins in contemporary use.

Keywords: cattle slurry; *Escherichia coli*; extended spectrum beta-lactamase resistance; multidrug resistance

Presence of Antibiotic Resistance In *E. coli* From Dairy Unit Cattle Slurry

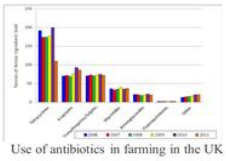
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BACKGROUND

- A significant amount of antibiotics are used in treating farm animals and as prophylactics to protect against different types of infection.
- Enteric Gram-negative bacteria inhabit the gut of man and animals and can be found in the environment. They are considered as reservoirs of antimicrobial resistance genes (AMR).
- The slurry from food producing animals is a potential route for spread of AMR into the environment.



Use of antibiotics in farming in the UK (DEFRA,2012)

AIMS

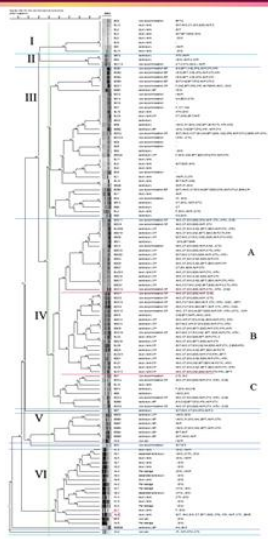
To investigate antibiotic resistance genes in the environment, using *E. coli* as an indicator organism, with a specific focus on the persistence of antibiotic resistance genes in cattle manure/slurry on a UK farm.

MATERIALS & METHODS

- **Samples**: • Liquid slurry • Solid slurry • Cow accommodation slurry
- **Media**: • TBX, TBX+ Cefotaxime • MacConkey • CHROMagar ESBL
- **Phenotyping**: • Oxidase test • Indole test • API 20E
- **Genotyping**: • ERIC PCR • *merA*, *merC* & *intI*1
- **Antibiotics Sensitivity Test**: • Test resistance to 17 antibiotics representing different antibiotic groups

- 3 samples of slurry have been taken from each site.
- 126 isolates were confirmed as *E. coli*, selected from TBX and TBX+Cefotaxime and CHROMagar ESBL .
- Antibiotic sensitivity tests were carried out according to CLSI, 2013.

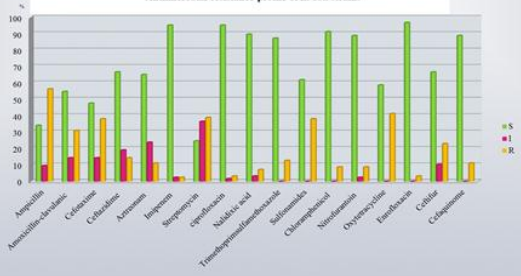
RESULTS



ERIC-PCR genotyping of *E. coli* isolates from cow slurry samples (similarity≥ 50%). The dendrogram was produced using the UPGMA algorithm based on a Dice similarity coefficient with a 1.5% band position tolerance

- The ERIC-PCR profiles were divided into 6 groups, at a 50% similarity threshold (cluster significance ΦPT=0.48; P<0.001)
- All the groups are significantly different at this level of similarity except groups I and II , which overlap with each other.
- Group VI contains the majority of isolates from 2012 (A strains) with those isolated in 2014 in other groups.
- Group IV dived to 3 significant different subgroup (A, B, and C) at 61% similarity threshold
- Other groups were from mixed sources with no source distinction

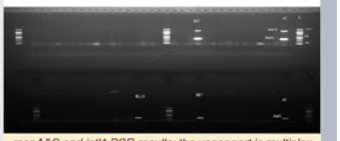
Antimicrobial resistance profile of *E. coli* strains



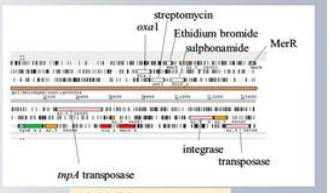
- 61% of the isolates showed multiple resistance to the tested antibiotics ranging from resistance to 2-13 antibiotics.
- 27.1% showed MAR resistance to 5-13 antibiotics .
- 74.5% of the isolates showed intermediate resistance to 1-4 antibiotics
- Highest resistance was showed against Ampicillin (56.3%)
- Lowest resistance showed against Imipenem (2.3%)

Mer* genes and *Intl1

- **Tn21-family mercury resistance transposons carry integrons which are involved in acquisition and carriage of antibiotic resistance genes**
- **Screening for Tn*21*-like transposable elements amongst the MDR *E. coli* strains by amplifying *mer* genes and *intI*1 using PCR, has shown that among 114 isolates, there were 2 isolates that carry *intI*1 alone, and one isolate carrying the *merA* and *merC* and *intI1*.**
- **Isolate A2.5 has been fully sequenced and shows the presence of a Tn*21* family transposon but just Mer R.**



mer A&C and intI1 PCR results: the upper part is multiplex PCR to detect mer A&C; the lower part is for detecting Intl1;+C is E.coli O42 control.



A 2.5 MDR transposon

CONCLUSIONS

- *E. coli* with different genotypes are present in different slurry sources.
- Antibiotic sensitivity tests showed that there were differences in sensitivities between *E. coli* isolates to a variety of antibiotics.
- Different antibiotic resistance profiles for identical ERIC PCR genotype isolates suggest plasmid involvement in resistance.
- An isolate carrying *mer* genes and *intI1* has been found despite the fact that mercury has not being used in animals for a long time.

REFERENCES

1. DEFRA 2012. Sales of antimicrobial products authorised for use as veterinary medicines in the UK in 2011.
2. WELLINGTON, E. M. H., BOXALL, A. B. A., CROSS, P., FEIL, E. J., GAZE, W. H., HAWKEY, P. M., JOHNSON-ROLLINGS, A. S., JONES, D. L., LEE, N. M., OTTEN, W., THOMAS, C. M. & WILLIAMS, A. P. 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. The Lancet Infectious Diseases, 13, 155-165.

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Multi Drug Resistant ESBL *E. coli* from Cattle Slurry in a Dairy Unit in Nottingham

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Background

- In dairy cattle antibiotics are used in treating infections particularly mastitis
- Extend spectrum cephalosporin are commonly used in animal treatment
- Milk from mastitic udders carrying contaminant bacteria and antibiotic residues is discarded into the slurry system
- Slurry is periodically spread onto land providing a route for dissemination into the environment



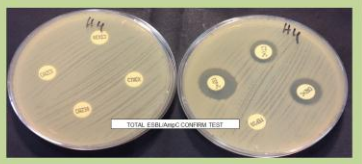
Aims

- To investigate presence of multi antimicrobial resistance in *E.coli* from cattle slurry, focusing on extended spectrum β-lactamase (ESBL)types.
- Determine the genetic determinants involved in resistance.



Methods

- Liquid slurry, solid slurry and slurry from the cow accommodation were collected from a dairy unit of ~200 cows living in barns in 2012 and 2014
- *E. coli* was isolated using TBX, TBX+ Cefotaxime (2 mg l^{-1}), MacConkey and CHROMagar ESBL.
- Phenotypic confirmation of *E. coli* was performed using indole and oxidase tests and API 20E.
- Disc sensitivity tests were used to test the resistance to seventeen antibiotics
- Total ESBL+AmpC Confirm kit was used to confirm the mechanism by which resistance to specific antimicrobial agents was gained.
- Genotyping of isolates was by ERIC PCR.
- Specific PCRs were used to detect some of the ESBL/*ampC* genes.

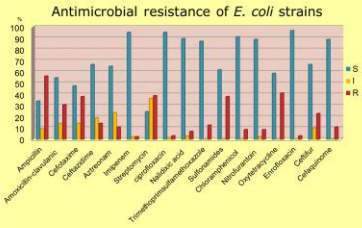


Results

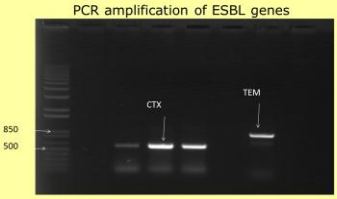
- Of 160 confirmed *E. coli* isolates, >55% showed multiple resistance to the tested antibiotics ranging from resistance to 2-13 antibiotics
- Highest resistance was shown against Ampicillin (56.3%), lowest resistance against Imipenem (2.3%).
  - Isolate A2.5 (slurry tank 2012) SXT, AMC, S10, OT, EFT, S300, CFQ, AMP, CTX, (ENR), ATM
  - Isolate BCE3 (cow accommodation 2014) SXT, (AMC), S10, OT, EFT, S300, CFQ, AMP, CTX, ENR, CIP, NA, C
- 28 isolates were ESBL positive, 58 were AmpC positive and 8 isolates were positive for both using Total ESBL/AmpC Confirm kit.
- Using PCR, 15 isolates were CTX positive, 5 were TEM positive and none carried SHV.
- Correlation of ERIC PCR genotyping and resistance shows some antibiotics appear to be co-acquired which may indicate a linked genetic acquisition.

Results

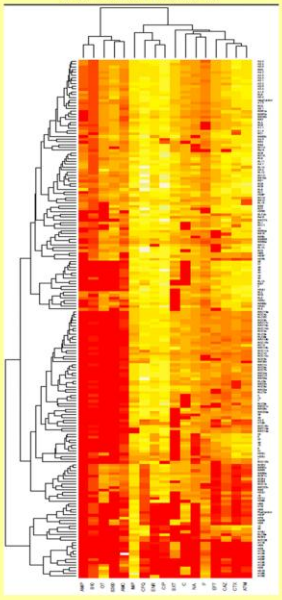
Antimicrobial resistance of *E. coli* strains



PCR amplification of ESBL genes



Heatmap of antimicrobial resistance



Significance of Work

- Initial sequencing has identified four different beta-lactamase genes, two of which are carried on different transposable elements.
- Even where antibiotics used for veterinary applications are not used in humans, resistance against antibiotics that are used in humans is widespread and retained in the population without known selection.

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