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CTX-M β-lactamases and associated integrons: their dissemination in Gram-negative bacteria.

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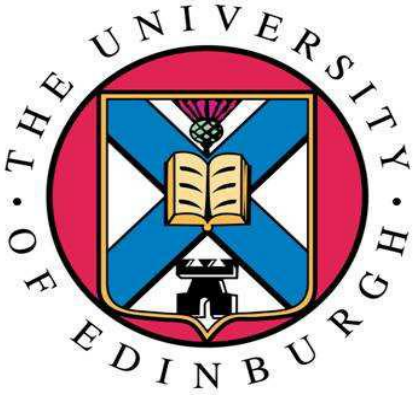

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Abstract

Gram-negative bacteria are able to cause many infections including blood stream infections (BSI).These bacteria may become resistant to antibiotics, often by acquiring genes in the presence of antibiotic selection pressure. Multi drug resistant Gram-negative bacteria have become an increasing problem worldwide. A study of antibiotic resistance in Gram-negative bacteria isolated from blood cultures from patients in the New Royal Infirmary of Edinburgh (NRIE) was performed. In addition, a study was performed on isolates from patients in an intensive care unit in Egypt. All isolates were investigated for susceptibility to an extensive range of antibiotics.

Gram-negative bacteria from Edinburgh found to be resistant to either cefotaxime or ceftazidime were investigated further. Among the cefotaxime/ceftazidime resistant isolates, Polymerase Chain Reaction (PCR) analysis revealed the presence of CTX-M-β-lactamases. Seven *E.coli* isolates were found to have CTX-M-15 β-lactamases while the CTX-M-14 β-lactamase was detected in six *Enterobacter cloacae.* The insertion sequence IS*Ecp1* was detected upstream of the $bla_{\text{CTX-M-15}}$ gene in some isolates while IS26 was found truncating the IS*Ecp1* in other isolates. Conjugation experiments found the $bla_{\text{CTX-M-15}}$ gene was transferable to *E. coli* J62-2. All the isolates had detectable plasmids, a plasmid ~260kb carried the $bla_{\text{CTX-M-15}}$ gene. Analysis of the CTX-M-containing isolates by PFGE shows that those carrying the CTX-M-14 β-lactamase were identical indicating cross infection within the hospital. The CTX-M-15 β-lactamase-containing isolates showed four isolates had ≥85% similarity but the others were diverse. Class 1 integrons were found in eight of the CTX-M β-lactamase-containing isolates with the associated gene cassette and *sul1* gene.

The isolates from Egypt were found to be resistant to carbapenem, which is the final mainstream antibiotic option in the treatment of multidrug resistant Gram-negative bacteria. Further analysis revealed all carried the CTX-M-14 β-lactamase and two additionally carried the VIM-4 metallo β-lactamase, which accounted for the resistance to the carbapenems. Furthermore, the insertion sequence IS*Ecp1* was found upstream of the $bla_{\text{CTX-M-14}}$ gene in two of the isolates. The $bla_{\text{VIM-4}}$ gene was found to be part of the gene cassette in the class 1 integron associate with complex IS*CR1*. Two of the Egyptian isolates had a detectable plasmid, ~300kb in size, which carried both $bla_{\text{CTX-M-14}}$ and $bla_{\text{VIM-4}}$ genes.

All the blood culture isolates were examined to ascertain the persistence of sulphonamide resistance despite the long-term prescribing reduction on this antibacterial. PCR was performed to detect *sul1*, *sul2* and *sul3* genes in all the isolates. Of the sulphonamide resistant isolates 25 carried the *sul1*, 27 carried the *sul2* and none carried the *sul3* genes. Eight isolates had both the *sul1* and *sul2* genes. Most of the isolates carried *sul1* had *Int1* as part of the same class 1 integron. Interestingly three isolates were PCR negative for *sul1* but positive for *sul2* and *int1*. *Int2* and *3* were found in 3 and 2 isolates respectively. The class 1 integron contained different insert gene cassettes; *dfrA* (*dfrA17*, *dfrA16*, *dfrA15*), *aadA* (*aadA5*, *aadA2*, *aadA1*) and $bla_{\text{OXA-1}}$ families in addition to the resident *sul* gene.

In conclusion this thesis shows the diversity of the genetic environment and carriers of the CTX-M β-lactamases within the same hospital. Sulphonamide resistance in Gram-negatives persists despite the prescribing reduction of this antibacterial in a Scottish hospital and the recommended constraint on the use of sulphonamide.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Dimude Juachi

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This dissertation is dedicated to Emeka Dimude-Love you

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Publications and presentations

Juachi u Dimude & S.G.B Amyes. Molecular diversity associated with the dissemination of CTX-M-15 β-lactamase gene in blood culture isolates of *Escherichia coli* from Edinburgh (2013).*Scandinavian Journal of Infectious Diseases* Vol 45 No 1 Pages 32 - 37.

Juachi Dimude & SGB Amyes. Molecular characterisation of CTX-M β–lactamase among blood stream isolate of *Enterobacteriaceae* September (2011). Presented at SGM York conference. Abstract YO07/04

Juachi Dimude & SGB Amyes. Characterisation of IncFIA, IncFIB, IncF11 and IncN plasmids carrying the CTX-M-15, TEM-1, OXA-1 β-lactamase genes and the acc (6')-IB-cr gene in Escherichia coli (2012). *Clinical Microbiology and Infection*. Vol 18 Issue Supllementry 3 page 734 Abstract no R2459. 22nd ECCMID Conference London

Juachi Dimude & SGB Amyes. Molecular characterization and diversity in *Enterobacter cloacae* from Edinburgh and Egypt carrying $bla_{\text{CTX-M-14}}$ and $bla_{\text{VIM-4}}$ β-lactamase genes (2013) International Journal of Antimicrobial Agent. Vol 41 Pages 574 - 577.

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Abbreviations

Ala	Alanine
AME	Aminoglycoside modifying enzyme
ATCC	American Type Culture Collection
bp	Base pairs
BSA	Bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
BSI	Bloodstream infection
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
Cfu	Colony forming unit
cm	Centimetres
Cip	Ciprofloxacin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ERT	Ertapenem
ESBL	Extended-spectrum β-lactamase
GES	Guiana extended spectrum
GIM	German Imipenemase
HGT	Horizontal gene transfer
ICU	Intensive care unit
IMI	Imipenem hydrolysing β-lactamase
IMP	Imipenem
ISCR	Insertion sequence common region
IST	Iso-sensitest

Kb	Kilo-base pairs
L	Litre
LB	Luria-Bertani
LPS	Lipopolysaccharide
M	Molar
MBL	Metallo β-lactamase
MDR	Multidrug resistant
MER	Meropenem
mg	Milligram
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
ml	Millilitre
mm	Millimetre
mM	Milli-molar
NCBI	National Center of Biotechnology Information
NCTC	National Collection of Type Cultures
NDM	New Delhi Metallo-β-lactamase
NMC	Non-metalloenzyme carbapenemase
OMP	Outer-membrane protein
PABN	Phenyl alanine arginyl b-naphtylamide
PBP	Penicillin-binding protein
PBRT	Plasmid based replicon typing
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Measure of the acidity or basicity of a solution

pmol	Pico-moles
rpm	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal-ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SIM	Seoul imipenemase
SME	Serratia marcescens enzyme
SPM	Sao Paulo metallo-β-lactamase
Spp	Species
ST	Sequence type
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TBE	Tris-borate-ethylenediaminetetraacetic acid
TE	Tris-EDTA
U	Units
V	Volts
VIM	Verona integron encoded metallo-β-lactamase
µg	Microgram
µl	Microlitre
µM	Micromolar

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Tables and Figures

1 Introduction

1.1 Antibiotics definition

Strictly speaking antibiotics are natural microbial products, however, the definition is usually now extended also to include synthetic chemotherapeutic agents. These compounds kill or inhibit the growth and survival of microorganisms without causing any serious toxicity to the host. The term Antibiotics was coined by Selman Waksman an Ukrainian- American Biochemist and Microbiologist in 1942 (Waksman 1947).

1.2 History and Development of Antibiotics

The term ‘Magic Bullet’ was first used by Paul Ehrlich to describe a compound which has a ‘magical’ effect on bacterial infections by targeting a specific pathogen. The first magic bullet-Salvarsan was discovered by Erhlich and Hata in 1907 and caused a great excitement because of its ability to cure syphilis and other spirochaetes infections (Ehrlich and Bertheim 1912). However, Salvarsan had severe toxicity problems associated with its use and was unfortunately only effective against a single group of pathogens, namely *Treponema* spp. causing syphilis. Continuing in the same line of research as Ehrlich in the development of synthetic compounds, Gerhard Domagk discovered an antimicrobial agent that cured infections when given to animals. He found that the dye Prontosil cured a disease caused by *Streptococcus pyogenea* in mice (Domagk 1935).

The discovery of natural antibiotics transformed medicine and began by accident in the morning of September 3rd 1928. Alexander Fleming was looking at some old plates and notices that colonies of *Staphylococcus aureus* had been lysed by the

product of a mould *Penicillium chrysogenum* in the adjacent area (Fleming 1929). Despite its early discovery, Penicillin was not available for commercial use until 1944 because of the difficulty in purification and production. In 1943, Selman Waksman and his group isolated another antimicrobial agent from the soil bacterium *Streptomyces griseus* (Schatz *et al*. 1944). The antibiotic, streptomycin, proved effective against several common infections, most notably those caused by Gram-negative bacteria, against which penicillin was ineffective, and especially the untouchable bacterium *Mycobacterium tuberculosis*, the microbe causing tuberculosis (Schatz and Waksman 1944). The use of streptomycin resulted in notable side effects (Kidney damage and deafness) that were absent with the use of penicillin. A second problem is bacteria developing resistance to the killing effect of streptomycin during therapy.

In the following years antibiotics became available to all ensuring that their use rose exponentially. In these early years several new classes of both man made and naturally occurring antibiotics were discovered including cephalosporins (1945), erythromycin (1952), methicillin (1960), ampicillin (1961), gentamicin (1963). Cephalosporins were introduced in 1964 with multiple second and third generation compounds becoming available by mid-1980s (Shlaes *et al*. 2004).

1.3 Antibiotics classes

All antibiotics are grouped into classes by their pharmacological properties. Antibiotics have different chemical compound and provide different action. Antibiotics can also be classified as either bacteriostatic or bactericidal as shown in Table 1.

Table 1 Major classes of antibiotics

Antibiotic Class	Sub-Class	Bacteriostatic/ Bactericidal	Examples
β-lactam	Penicillin		Amoxicillin
	Monobactam		Aztreonam
	Carbapenem	Bactericidal	Meropenem,Imipenem
	Carbacephems		Loracarbef
	Cephalosporine(1^{st}Gen)		Cefalexin,Cefadroxil
	(2^{nd}Gen)		Cefaclor,Cefuroxime
	(3^{rd}Gen)		Cefotaxime,Ceftazidime
	(4^{th}Gen)		Cefepime
	(5^{th} Gen)		Ceftobiprole
Polypepetides		Bactericidal	Polymixin-B, Colistin, Bacitracin
Lipopeptides		Bactericidal	Daptomicin
Glycopeptides		Bactericidal	Teichplanin,Vancomycin,Telavancin
Fosfomycin		Bactericidal	Fosfomycin
Aminoglycoside		Bactericidal	Gentamicin,Amikacin,Neomycin
Lincosamides		Bacteriostatic	Lincomysin,Clindamycin
Macrolides		Bacteriostatic	Erythromicin,Spectinomycin,
Quinolones		Bactericidal	Ciprofloxacin, Nalidixic acid
Sulphonamides		Bacteriostatic	Sulphonamidochrysoidine,Sulphametizole
Tetracyclines		Bacteriostatic	Tetracycline, Doxycycline
Oxazolidinoes		Bacteriostatic	Linezolides
Diaminopyrimidine		Bacteriostatic	Trimethoprim, Iclaprime
Nitroimidazole		Bactericidal	Metronidazole
Rifamycins		Bactericidal	Rifampicin,Rifabutin,

The table show the list of major antibiotic classes showing division between bacteristatic and bactericidal antibiotics with some example of each class.

Bacteriostatic agents prevent bacteria from reproducing, while not necessarily harming them otherwise. Bacteriostatic antibiotics limit the growth of bacteria usually by interfering with bacterial protein production. Bactericidal antibiotics kill the bacteria, often by interfering with DNA synthesis. However, there is not always a precise distinction between them and high concentrations of some bacteriostatic agents can also become bactericidal, whereas low concentrations of some bactericidal agents are bacteriostatic.

1.4 Antibiotics mechanism of action

Different antibiotics have different modes of action due to the nature of their structure and the level of affinity to the target site within the bacterial cells. The antimicrobial effect of these compounds can be shown through their mechanisms of action.

Inhibition of DNA synthesis: The most significant group of compounds acting against DNA synthesis are the fluoroquinolones. Fluoroquinolones act by inhibiting the DNA gyrases or topoisomerase IV required for supercoiling of DNA. Although some degree of overlap may exist, DNA gyrase tends to be the primary target for fluoroquinolones in Gram-negative organisms whereas topoisomerase IV is typically the primary target in Gram-positive bacteria (Hooper 1999). These two enzymes DNA gyrase and topoisomerases are trapped by fluoroquinolones on DNA as drug /enzyme/DNA complex which blocks progression of replication fork there by inhibiting DNA synthesis (Drlica 1999). DNA gyrase is composed of two *GyrA* and two *GyrB* monomeric subunits, which are encoded by the *gyrA* and *gyrB* genes,

respectively while the Topoisomerase IV is composed of four homologous monomeric subunits, two *ParC* subunits and two *ParE* subunits encoded by the *parC* and *parE* genes, respectively (Hooper 1999).

Metronidazole although not a classic inhibitor of DNA synthesis also has an effect on the DNA by producing metabolic cytotoxic by-products that disrupt DNA. Metronidazole inhibits anaerobic bacteria and protozoans. The nitro group of the drug undergoes reductive activation and is reduced by low redox potential electron transport proteins. The resulting active compound damages the cell through interaction with DNA (Neu and Gootz 1996).

Inhibition of RNA synthesis: Rifampicin is the main compound used clinically that acts against RNA synthesis. It's particular value is in the treatment of tuberculosis. It acts by inhibiting bacterial DNA-dependent RNA polymerase preventing RNA transcription and any subsequent translation. Rifampin binds noncovalently but strongly to a subunit of RNA polymerase and interferes specifically with the initiation process. DNA-dependent RNA polymerase is a complex enzyme with an α_2 β β‘ σ subunit structure. The binding site for rifampicin has been located at the β subunit encoded by the *rpoB* gene (Floss and Yu 2005). Because it prevents the formation of mRNA, it effectively disrupts protein synthesis and thus its action is often bacteriostatic. However, it has no effect once polymerization has begun (Neu and Gootz 1996).

Inhibition of protein synthesis: Enzymes and cellular structures are primarily made of proteins and, therefore, protein synthesis is an essential process necessary for the

multiplication and survival of all bacterial cells. Several antibiotics in these group act by binding to the 30S or 50S ribosomal RNA subunit preventing the binding of amino-acyl tRNA therefore inhibiting translation and normal cellular metabolism of the bacteria. Examples of antimicrobial agents that inhibit protein biosynthesis include aminoglycosides, macrolides, tetracyclines, chloramphenicol and lincomycins (Neu and Gootz 1996).

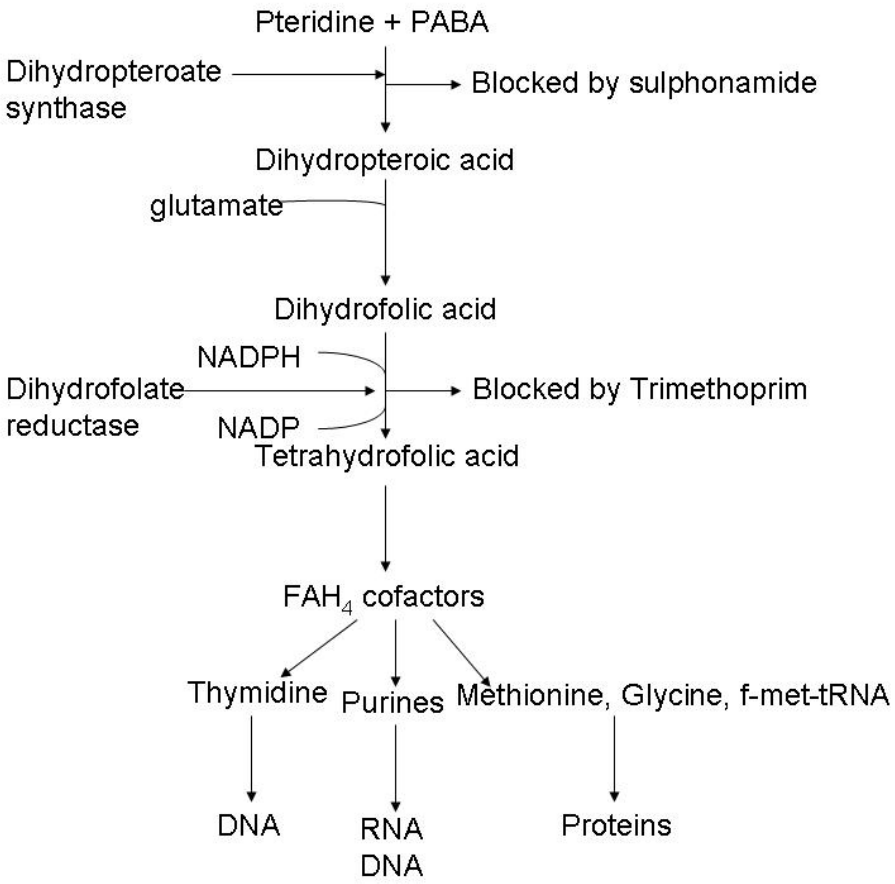
Inhibition of cell wall synthesis: The composition of bacterial cell is unique in nature and drugs that target cell walls can therefore selectively kill or inhibit bacterial. Examples are β-lactam antibiotics, which inhibit transpeptidase and peptidoglycansynthesis preventing the cross linking of the polysaccharide chains in the bacterial cell wall.

Inhibition of cell membrane function: A disruption or damage to cell membrane structure could result in leakage of important solutes essential for the cell's survival. Polymyxins act by interacting with lipopolysaccharide destroying the outer and inner membranes. The hydrophobic tail is important in causing the damage suggesting a detergent like mode of action.

Inhibition of metabolic pathway by acting on tetrahydrofolate synthesis: Sulphonamides and trimethoprim are the main compounds that act by inhibition of metabolic pathway. Sulphonamides are structural analogue of para amino benzoic acid (PABA) and compete with PABA. Sulphonamides competitively inhibit dihydropteroate synthetase resulting in the disruption of tetrahydrofolate synthetic pathway (Figure 1). It acts by blocking the conversion of pteridine and PABA to

dihydrofolic acid by the enzyme pteridine synthetase (Henry 1943). Trimethoprim inhibits dihydrofolate reductase, which is responsible for conversion of dihydrofolic to tetrahydrofolic acid leading to the synthesis of purines and ultimately DNA (Figure 1). Dihydrofolate reductase is essential for folic acid biosynthesis and thus trimethoprim inhibit the utilization of folic acid by bacteria (Darrell *et al*. 1968). Trimethoprim and sulphonamides inhibit with folate metabolism in Gram-negative bacterial cell and act as folate antagonists. Tetrahydrofolate is important for the proper synthesis of bacterial cell wall proteins, DNA, and RNA (Neu and Gootz 1996).

Figure 1 Sites of action of Sulphonamide and Trimethoprim in folic acid biosynthesis. (adapted from Scholar and Pratt 2000)

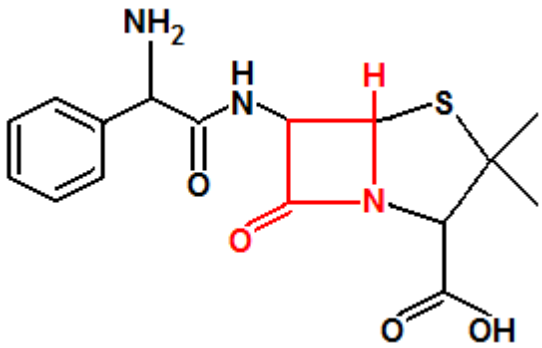


The figure show the site of action of sulphonamide and trimethoprim on the folic acid biosynthesis. Sulphonamide acts as a false substrate inhibitor of dihydropteroate synthase and trimethoprim acts by interfering with the action of bacteria dihydrofolate reductase

1.5 β-Lactam antibiotics

β-lactam antibiotics family are named and classified because of the presence of β-lactam ring (Figure 2) which is a heteroatomic ring structure comprising of three carbon atoms and one nitrogen atom.

Figure 2 Primary structure of Ampicillin with the β-lactam highlighted in red



The figure shows the β-lactam ring, highlighted in red, which is a four membered lactam with the nitrogen aton attached to the β-carbon relative to the carbonyl.

The β-lactams act in the final step of cell wall synthesis in which strands of peptidoglycan are cross linked via peptide side chains. The β-lactam antibiotics block the transpeptidation of the cell wall component peptidoglycan, through inhibition of transpeptidases called penicillin binding proteins (PBP). The PBPs forms a covalent penicilloyl-enzyme complex that blocks the transpeptidation which leads to irregularities in cell wall structure leading to cell lysis and eventually cell death (Wilke *et al*. 2005).

The β-lactam antibiotics family include penicillins, cephalosporins, cephamycins, monobactams, β-lactamase inhibitor and carbapenems (Champoux *et al*. 2004). β-lactams show activity against Gram-negative and Gram-positives organisms,

including anaerobes. They are the most widely used class of antibiotics (Mims *et al*. 2004). The cephalosporins are most often prescribed because of their broad spectrum of activity for more complicated and resistant infections. Due to increasing use of β-lactams antibiotics, there is development of resistance due to production and dissemination of β-lactamases (Livermore 1998). β-lactamases are the most common cause of Gram-negative bacterial resistance to β-lactam antibiotics (Livermore 1995). It seems that the β-lactamases have been co-evolving with β-lactam antibiotics ever since it came into use (Medeiros 1997). Among β-lactams, carbapenems are antibiotics with a broad spectrum of antibacterial activity, compared to other β-lactams such as penicillins and cephalosporins. A carbapenem is often used as a last resort drug to treat infections that are resistant to the cephalosporins. However carbapenems are attacked by few β-lactamases most notably the molecular class B enzyme (Livermore 1998). The attack to carbapenems has been reported to be caused by a combination of altered outer membrane protein, CTX-M-15 β-lactamases and upregulated efflux (Findlay *et al*. 2012).

1.6 Development of Resistance

Long before the discovery and use of antibiotics, bacteria where exhibiting resistance. This is largely because most antibiotics are produced by micro-organisms in the environment where bacteria encounter them naturally and develop a survival strategy. After the availability of antibiotics experts believed that infection would be a thing of the past, but Alexander Fleming was quoted in *New York Times* June 26, 1945 as saying “The greatest possibility of evil in self-medication is the use of too small doses

so that instead of clearing up infection the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed to other individuals and from them to others until they reach someone who gets a septicaemia or pneumonia which penicillin cannot save." In the early years of antibiotic development, bacteria resistance strains appear in hospitals where the antibiotics are used. Firstly, *Streptococcus pyogenes* resistant to sulphonamide appeared in military hospitals in 1930s (Delamater *et al*. 1946), *Staphylococcus aureus* resistant to penicillin appeared in the London hospitals after the introduction of the antibiotic in the 1940s (Barber and Rozwadowska-Dowzenko 1948). In a similar way soon after the discovery of streptomycin, *Mycobacterium tuberculosis* resistant to this antibiotic emerged in the community (Crofton and Mitchison 1948).From the review by Levy and Marshall, multi-drug resistance was first detected in Gram-negative bacteria-namely *Escherichia coli, Shigella* and *Salmonella spp*.-in the late 1950s to early 1960s (Levy and Marshall 2004). After the detection of resistant bacteria, the introduction of new antibiotics was used to prevent the damages caused by these bacteria. This pattern of introducing new antibiotics to counteract the resistance to previously-used drugs continued for almost all newly developed antibiotics. In recent years, however, the development of resistance appears to be outpacing the development of new antibiotics and is consequently resulting in the increasing emergence of multi-drug resistance (MDR) and Pan-drug resistance (PDR) strains (Levy and Marshall 2004).This is why treatment options are becoming limited and the pursuit of new antibiotics is of extreme importance.

1.7 Mechanisms of Resistance

Bacteria typically acquire resistance either through the ability of a gene in the bacterium to mutate or by the acquisition of resistance genes from other bacteria.

Several mechanisms have evolved in bacteria which confer resistance to antibiotics. These mechanisms include: drug inactivation or modification through the production of enzymes such as β-lactamases, alteration of the target site (PBP), alteration of a metabolic pathway, or reduced drug accumulation by efflux pump/decreased drug permeability (Figure 3) (Champoux *et al*. 2004). The most common mechanism is the enzymatic inactivation of the antibiotics.

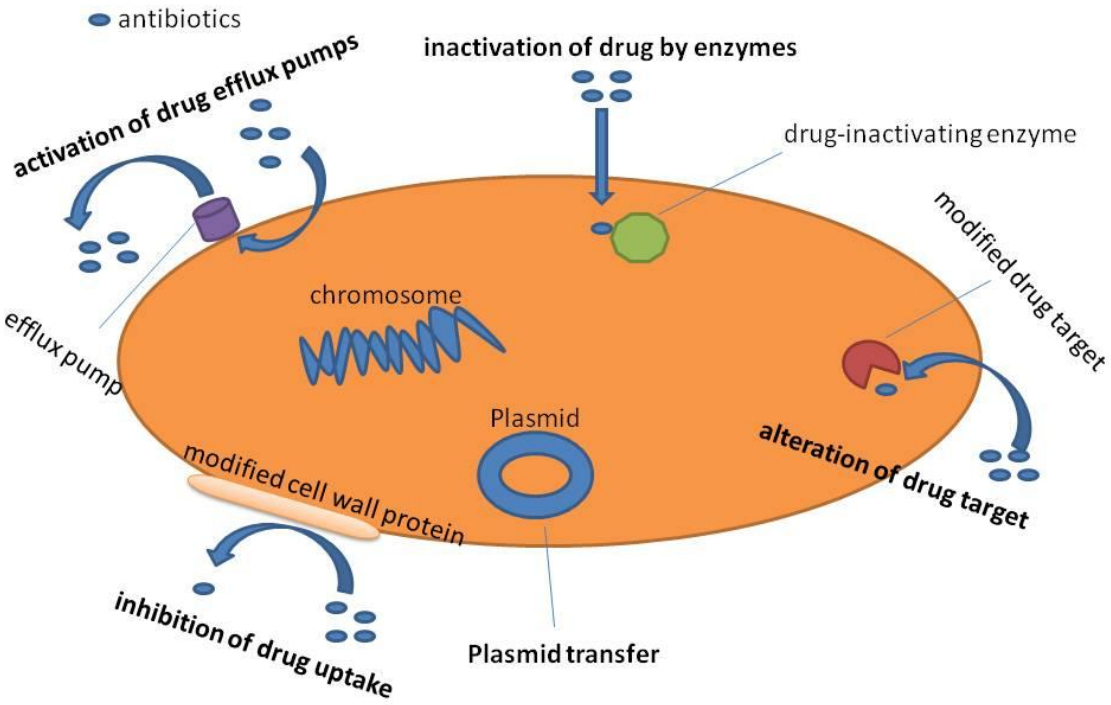


Figure 3 Mechanisms of resistance to Antibiotics: (modified from Coates *et al*. 2002)

This diagram shows the mechanism used by bacteria to confer resistance to antibiotics which includes inactivation of drug by bacteria enzymes,the activation of drug efflux pump and inhibition of drug uptake in cell

1.7.1 Resistance to β-lactams

Bacteria can avoid the bactericidal effect of β-lactam antibiotics by: active efflux pumps promoting the transport of the antibiotics out of the cell, alteration of the penicillin binding proteins (PBPs), lack/diminished expression of outer membrane proteins (OMPs) or finally production of β-lactamases that hydrolyses the β-lactam ring.

1.7.1.1 Penicillin-binding protein

PBP are the primary targets of β-lactam antibiotics. PBP catalyze an important step in bacterial cell wall synthesis [a transpeptidase reaction which removes a terminal alanine in a cross linking reaction with a nearby peptide]. There are several PBP mediated mechanisms of β-lactam resistance including acquisition of a new less-sensitive enzyme, a mutation of an endogenous PBP which lesses the reaction with the β-lactam,while maintaining some transpeptidase activity or up regulation of PBP expression (Wilke *et al*. 2005). Alteration of the PBP so that the β-lactam antibiotics cannot bind to them is a principal resistance mechanism observed in Gram-positive bacterial species (Zapun *et al*. 2008). This alteration in the PBP is responsible for the emergence of methicillin resistance in *Staphylococci aureus* (MRSA) and penicillin resistance in pneumococci. The *mec*A gene which codes for an additional PBP in MRSA is present in the chromosome. It is important to note that the majority of the MRSA produce β-lactamase (Mims *et al*. 2004). Although PBP alteration has be found in Gram-negatives (Zapun *et al*. 2008), their resistance to β-lactams is essentially due to the other mechanisms.

1.7.1.2 Outer membrane permeability

The outer membrane of Gram negative bacteria play a vital role in providing protection as well as exchanging material required for sustaining life. The membrane is made up of a combination of hydrophobic lipids, lipopolysacharides and proteins (porins) acting as a selective barrier (Delcour 2009). Porins facilitate the transport of β-lactam antibiotic molecules across the cell membrane therefore loss of porins confers reduced susceptibility to these antibiotics. Loss or functional changes in porins contribute to the overall resistance associated with β–lactamase production. There are reports of porin deficiency contributing to β-lactam antibiotics resistance in organisms such as, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Neisseria gonorrhoeae*, *E. coli* and *Klebsiella pneumoniae* (Crowley *et al*. 2002, Gayet *et al*. 2003). In *K. pneumoniae* exhibiting resistance to carbapenem, the loss OmpK36 has been associated with carbapenem resistance (Kaczmarek *et al*. 2006).

1.7.1.3 Efflux pump

Expression of efflux pump is another mechanism involved in resistance to β-lactam antibiotics (Figure 3). Efflux pumps are proteins involved in the transport of toxic substrates and antibiotics across the cell membrane into the external environment.As reviewed by Poole, an efflux pump was first described as a mechanism of resistance to tetracycline in *E. coli* (Poole 2005) and since then efflux pumps have been implicated as the cause of antibiotic resistance within numerous clinically relevant bacteria species such as *K. pneumoniae* and *Pseudomonas aeruginosa* (Webber and Piddock 2003). Bacterial efflux systems capable of accommodating antimicrobial generally fall into five classes, the major facilitator (MF) superfamily, the ATP-

binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family [a member of the much larger drug/metabolite transporter (DMT) superfamily] and the multidrug and toxic compound extrusion (MATE) family (Poole 2005). The presence of efflux is often gauged *in vitro* with the use of efflux pump inhibitors such as Carbonyl cyanide m-chlorophenylhydrazone (CCCP), Phenyl alanine arginyl b-naphtylamide (PABN) and reserpine. These efflux pump inhibitors are used alongside the relevant antibiotics to result in increased susceptibilities, when compared to the antibiotic alone, in the presence of active efflux.

Efflux systems are able to confer resistance to a wide range of antibiotics and are common among Gram-negative bacteria. Decreased susceptibility due to efflux has been shown in *P. aeruginosa* to many class of antibiotics (Li *et al*. 1995), *E. coli* (Poole 2000), and *Salmonella typhimurium*, (Nikaido 2001) Two of the most studies MDR efflux are AcrAB-TolC of *E. coli* and the several Mex-Opr pumps of *P. aeruginosa* (Poole 2000). Antibiotic efflux is mostly found to confer high level resistance in bacteria in association with other mechanisms such as β-lactamases. A good example is the cooperation between the penicillin efflux pumps and the β-lactamases in Gram-negative bacteria showing a phenotype of high-level resistance without being high-level producers of β-lactamase (Masuda *et al*. 1999, Nakae *et al*. 1999, Lakaye *et al*. 1999). It is important to note that high level resistance to antibiotics do not occur in the presence of efflux alone.

1.7.2 Production of β-lactamases

β-lactamases are the most important mechanism of resistance to β-lactam antibiotics. They are enzymes produced by many species of bacteria and were originally produced

to improve the chances of bacterium's survival in the soil so it could competed with organisms producing β-lactam antibiotics. These enzymes are capable of conferring resistance to β -lactam antibiotics via the hydrolysis of the amide bond of β -lactam ring. The β-lactamases are classified by their protein sequence into four molecular classes A, B, C and D as shown in table 2. These classes are split into two groups: those that contain a serine residue at their active site and those that require the metal ion, usually zinc, as a co-factor, known as metallo β-lactamases. Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate β-lactam hydrolysis (Bush and Jacoby 2010).These molecular classes can further be spilt into functional classes.

Table 2 β-lactamases classification schemes adapted from Bush and Jacoby (2010)

Molecular class	Bush-Jacoby group	Distinctive substrates	Representative enzymes
A	2a	Penicillin	PC1
	2b	Penicillin, early cephalosporins	TEM-1,TEM-2,SHV-1
	2be	Extended-spectrum cephalosporins, monobactams	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
	2br	Penicillin	TEM-30,SHV-10
	2ber	Extended-spectrum cephalosporins, monobactams	TEM-50
	2c	Carbenicillin	PSE-1 CARB-3
	2ce	Carbenicillin, cefepime	RTG-4
	2e	Extended-spectrum cephalosporins	CepA
	2f	Carbapenems	KPC-2, IMI-1, SME-1
B(B_1)	3a	Carbapenems	IMP-1,VIM-1, CcrA, IND-1
B(B_3)			L1,CAU-1,GOB-1,FEZ-1
B	3b	Carbapenems	CphA, Sfh-1
C	1	Cephalosporin	AmpC, p99, ACT-1, CMY-2, FOX-1, MIR-1
	1e	Cephalosporin	GC1, CMY-37
D	2d	Cloxacillin	OXA-1 OXA-10
	2de	Extended-spectrum cephalosporins	OXA-11OXA-15
	2df	Carbapenems	OXA-23 OXA-48
unknown	4	Penicillin not inhibited by Clavulanic acid	Penicillinase from *Pseudomonas cepacia*

The table show the comparison of the different classification schemes of β-lactamases with the distictive substrates and representative enzymes.

1.8 The clinically most important β-lactamases

1.8.1 Extended Spectrum β-Lactamases (ESBLs)

ESBLs are β-lactamases that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam, aztreonam. Thus ESBLs confer resistance to these antibiotics and related oxyimino-β-lactams. This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes. Types of ESBLs include TEM, SHV, OXA, and CTX-M. Other plasmid-mediated ESBLs such as PER, VEB, GES, SFO and IBC β-lactamases have been described but are uncommon (Nordmann *et al*. 1993, Poirel *et al*. 1999, Poirel *et al*. 2000a, Matsumoto and Inoue 1999). An increasing number of ESBLs, not of TEM or SHV lineage, have been described. The ESBLs are frequently plasmid encoded usually alongside other resistance determinants (Paterson *et al*. 2003).

Since the plasmid-mediated extended spectrum β-lactamases were first detected in a *Klebsiella pneumoniae* isolate in 1983 in Germany (Kliebe *et al*. 1985), they have been increasingly reported worldwide (Bradford 2001).The classical ESBLs are those derived from the broad spectrum enzyme TEM-1,TEM-2 and SHV-1 by the acquisition of specific point mutations which expand their spectrum of hydrolysis to oxyimino-cephalosporins and aztreonam (Bush *et al*. 1995). Nevertheless, the most wide spread plasmid-mediated ESBLs nowadays are variations of CTX-M enzymes (reviewed by (Canton *et al*. 2012) and will be further discussed . Most of the ESBLs are found in Gram-negative organisms including *Klebsiella* spp., *E. coli, P. aeruginosa, Enterobacter* spp. and *Salmonella* spp. (Bradford 2001).

1.8.1.1 TEM

The ESBLs family TEM named after a patient Temoniera was first reported in 1965 from an *E .coli* isolate (Datta and Kontomichalou 1965). The TEM-l β-lactamase, which is not an ESBL, is able to hydrolyse ampicillin and early cephalosporins such as cephalothin and cephaloridine. Because of its location in the plasmid, TEM-1 spread worldwide and can be found in many different members of the family *Enterobacteriaceae* (Bradford 2001). The TEM-2 β-lactamase is the first derivative of TEM-1 but is still not considered an ESBL having a substrate profile identical to TEM-1. TEM-2 had a single amino acid substitution at position 39 (Gln39→Lys) from the original TEM-1 β-lactamase. TEM-3 first isolated from *Klebsiella pneumonia* in 1987, is the first TEM-β-lactamase to display the ESBL phenotype (Sirot *et al*. 1987). The sequence of TEM-3 shows that it is related to TEM-2 with the difference in two amino acid changes at position 104 (Lys→Glu) and 238 (Ser→Gly) (Sougakoff *et al*. 1988). TEM-3 has an unusual resistance phenotype towards cefotaxime which is not shown in TEM-1 and -2. The mutation at position Gly238 with serine is critical for altering substrate specificity for cefotaxime hydrolysis (Cantu and Palzkill 1998). There are a number of amino acid residues especially important for producing the ESBL phenotype and extending its hydrolytic spectrum. They include amino acid substitutions at: glutamate to lysine at position 104, arginine to either serine or histidine at position 164, glycine to serine at position 238, and glutamate to lysine at position 240 (Bradford 2001). Currently, 190 TEM type β-lactamase enzymes have been described.

1.8.1.2 SHV

The acronym SHV refers to Sulfhydryl Variable designated because it was thought that SHV hydrolysed cephaloridine but not benzylpenicillin in the presence of p-chloromecuribenzoate inhibitory binding chemical (Tzouvelekis and Bonomo 1999). The SHV-1 β-lactamase was first described in 1972 (Pitton 1972) and confers resistance to ampicillin, amoxicillin, carbenicillin, ticarcillin. SHV-1 share 68% amino acid similarity with TEM-1 and has similar tertiary structure. The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at position 238. A number of variants related to SHV-5 also have a substitution of lysine for glutamate at position 240. It is interesting to note that both the Gly238Ser and Glu240Lys amino acid substitutions mirror those seen in TEM-type ESBLs (Bradford 2001). Both Gly238Ser and Glu240Lys amino acid substitutions is needed for strong hydrolysis of ceftazidime where as Gly238Ser is critical for cefotaxime hydrolysis (Huletsky *et al*. 1993). SHV type ESBL are found mostly in strains of *K. pneumoniae* but these enzymes has been found in other Gram-negative bacteria including *E. coli*, as plasmid mediated β-lactamases (Sabaté *et al*. 2002). Currently over 130 variants of SHV are known.

1.8.1.3 OXA

This β-lactamase differ from TEM and SHV in that they belong to molecular class D and functional group 2d (Bush *et al*. 1995). They have the ability to hydrolyse and confer resistance to cloxacillin and penicillin. bla_{OXA} genes comprise the chromosomal β-lactamases of *Acinetobacter* spp, known as OXA-51-like (Evans *et al*. 2007).. Some of these genes have migrated out of *Acinetobacter* spp into *P.*

aeruginosa and to a lesser extent into members of the Enterobacteriaceae including *E. coli* and *K. pneumoniae* (Naas and Nordmann 1999). These transfers have occurred on plasmids and outside Acinetobacter spp, bla_{OXA} genes are almost invariably plasmid-encoded.

The OXA ESBLs were originally isolated in *P. aeruginosa* isolates from a hospital in Ankara, Turkey (Hall *et al*. 1993). Currently OXA ESBLs are derived from the OXA-1,-10, and -2 β-lactamases. Among the enzymes related to the OXA-10 β-lactamase, there are one or two amino acid substitutions: an asparagine substitution for serine at position 73, or an aspartate substitution for glycine at position 157. In particular, the Gly157Asp substitution may be necessary for high-level resistance to ceftazidime (Bradford 2001). There are currently over 200 OXA gene derivatives, 16 of which are ESBLs derived from OXA-2 and OXA-10 (www.lahey.org/studies/webt.asp.).

1.8.1.4 CTX-M β-lactamases

The name 'CTX-M' is an abbreviation for 'cefotaximases' and refers to the more potent hydrolytic activity of these enzymes against cefotaxime than other oxyimino-β-lactam substrates. However, CTX-M enzymes that are able to hydrolyze ceftazidime efficiently have also been describe (Bonnet 2004). CTX-M enzymes are distinct class of ESBLs, which arose by gene escape from genus *Kluyvera,* (Canton 2008) which is closely related to *E. coli*. CTX-M-type β-lactamases constitute a group of class A plasmid-encoded enzymes, which are capable of hydrolyzing broad-spectrum cephalosporins and are inhibited by clavulanate and tazobactam. In that respect, CTX-M-type enzymes are typical ESBLs and have therefore been classified in the

functional group 2be of the Bush, Jacoby and Medeiros classification scheme (Table 2), which also contains the Extended Spectrum derivatives of TEM-1/2 and SHV-1 β-lactamases (Bush *et al*. 1995).

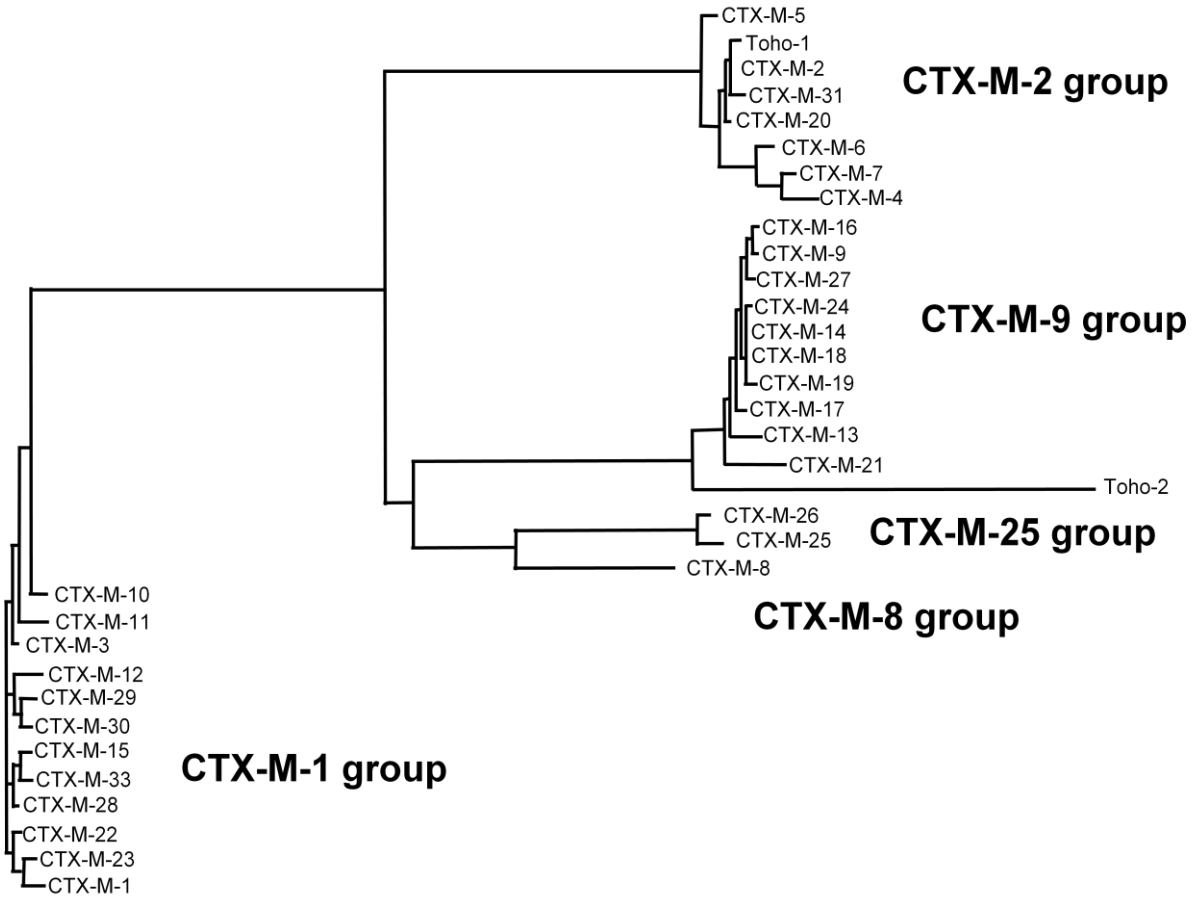


Figure 4: Dendrogram of the CTX-M gene clusters

This dendrogram shows the relative distances between the primary amino acid structure of the five CTX-M groups as well as the relative distances within each group.

Unlike the TEM and SHV enzymes, the CTX-M enzymes have not evolved from point mutations from the parent strain rather they originate from chromosomal *bla* gene of *Kluyvera spp* (Bonnet 2004). Subsequently, mutation has contributed to the diversification of the CTX-M family. CTX-M β-lactamases were cefotaximases but these subsequent mutations gave rise to expanded hydrolytic activity to ceftazidime. An example is the CTX-M-15 which was derived from CTX-M-3 by Asp240→Gly substitution which increases the catalytic activity to Ceftazidime (Poirel *et al*. 2002a) . Currently more CTX-Ms are able to hydrolyse both cefotaxime and ceftazidime.

The CTX-M groups are sub-classified by amino acid similarities into five different: CTX-M-1 group, CTX-M-2 group, CTX-M-9 group, CTX-M-8 group and CTX-M-25 group. The members of each group share 94% amino acid sequence similarities and 90% similarities between groups. CTX-M β-lactamase is natural in the chromosome of *Kluyvera cryocrescens*, *Kluyvera ascorbata*, and *Kluyvera georgiana* (Bonnet 2004). bla_{kluC} gene from *Kluyvera cryocrescens* is considered the ancestor for CTX-M-1 group (Decousser *et al*. 2001), bla_{kluA} from *Kluyvera ascorbata* for CTX-M-2 group (Humeniuk *et al*. 2002). There are three genes present in *Kluyvera georgiana* designated $bla_{KluG,}$ $bla_{KluY,}$ and $bla_{CTX\text{-}M\text{-}78.}$ bla_{KluG}, is considered the origin of CTX-M- 8,(Poirel *et al*. 2002b) bla_{KluY} for CTX-M-9 cluster (Olson *et al*. 2005) and $bla_{CTX\text{-}M\text{-}78}$ for CTX-M-25 cluster (Margarita Rodriguez *et al*. 2010). This suggests the mobilisation of this type of β-lactamase from the chromosome to the plasmids. Thus CTX-M β-lactamases originated from *Kluyvera* Spp chromosomal gene. Over 130 different variant of CTX-M have been described so far

(www.Lahey.org/studies/webt.asp - last accessed 3 December 2012).

1.8.1.4.1 CTX-M Emergence

Matsumoto *et al* reported in 1988 a non-TEM, non-SHV ESBL, designated FEC-1, in a cefotaxime-resistant *Escherichia coli* strain isolated from the faecal flora of a laboratory dog, which was used for pharmacokinetic studies of β-lactam antibiotics in Japan (Matsumoto *et al*. 1988). A few years later, CTX-M-1 in reference to its hydrolytic activity against cefotaxime was first detected from an *Escherichia coli* strain in 1989 in Germany (Bauernfeind *et al*. 1990). At the same time in 1990, an explosive dissemination of a cefotaxime-resistant *Salmonella* strain began in Argentina which was plasmid mediated named CTX-M-2 (Bauernfeind *et al*. 1992). The β-lactamases responsible for the resistance to cefotaxime observed in Argentina had alkaline pI values, conferred a high level of resistance to cefotaxime rather than ceftazidime and were susceptible to the inhibitors sulbactam, clavulanic acid and tazobactam. In 1992, the same type of ESBL, designated MEN-1, was reported in a clinical *E. coli* strain MEN, isolated in 1989 in France from a patient hospitalised at French anti-cancer centre, who was an Italian national (Bernard *et al*. 1992). A few years later, there was a report of a MEN-1 related enzyme, designated Toho-1, which was produced by a cefotaxime-resistant *E. coli* strain isolated in 1993 in Japan (Ishii *et al*. 1995). Analysis of the sequences of the two non-TEM, non-SHV ESBL-encoding genes revealed that CTX-M-1 was identical to MEN-1 and is a variant of Toho-1 (Bauernfeind *et al*. 1996).

Subsequently in Poland, a report emerged of a variant of CTX-M-1, designated CTX-M-3, in different members of the family *Enterobacteriaceae* isolated in 1996 (Gniadkowski *et al*. 1998). Since then, CTX-M enzyme have formed a rapidly growing family of ESBLs distributed both over wide geographic areas and among a

wide range of clinical bacteria, in particular, member of the family of *Enterobacteriaceae (*Canton *et al*. 2012*).*

There has been a dramatic increase in the number of organisms reported in the literature that produce CTX-M β-lactamases. This class of β-lactamases has been recognised worldwide as an important mechanism of resistance to oxyimino cephalosporin used against Gram-negative pathogens. In most cases, the bacteria producing these enzymes display higher levels of resistance to cefotaxime and cefriaxone than ceftazidime (Bonnet 2004). However some point mutations produced enzymes with a much stronger activity against ceftazidime. For instance, CTX-M-15 was derived from CTX-M-3 by an Asp-240→Gly substitution which increased the catalytic activity of Ceftazidime (Poirel *et al*. 2002a). The Asp-240→Gly substitution were also observed in CTX-M-16 and CTX-M-27 also presenting a high catalytic activity against Ceftazidime (Bonnet *et al*. 2001).Also responsible for increased ceftazidime resistance is the Pro-167→Ser mutation which differentiates CTX-M-9 from CTX-M-14 (Poirel *et al*. 2001).

The CTX-M-15 and CTX-M-14 are the most important CTX-M enzymes which can be found in human, animal as well as in the environment all over the globe (Canton *et al*. 2008, Hawkey and Jones 2009, Dolejska *et al*. 2011, Hiroi *et al*. 2012). CTX-M-15 was first described in 1999 from enteric isolates recovered from a hospital in New Delhi India (Karim *et al*. 2001). However a retrospective study in Poland showed the presence of CTX-M-15 in *E.coli* and *Serratia marcescens* before the first description of this enzyme in India with sequence and plasmid analysis revealing a potential

evolution from CTX-M-3 (Baraniak *et al*. 2002). Currently CTX-M-15 is found to have disseminated all over the world (Canton *et al*. 2012).

CTX-M-14 was first reported in 2001 from an isolate obtained in 1996 from a stool isolate of *Shigella sonnei*,and blood isolates of *Escherichia coli* and *Klebsiella pneumoniae* from different parts of Korea (Pai *et al*. 2001). This was followed by a rapid dissemination all over the world including China (Chanawong *et al*. 2002), Taiwan (Yu *et al*. 2002) and France (Dutour *et al*. 2002)

Highly transmissible plasmids often encode CTX-M enzymes and enable the gene to be transferred from one species to another and one genus to another. The bla_{CTX-M} genes are encoded in the plasmids belonging to the narrow host range incompatibility types (IncF1, IncFII, IncH12 and IncI) or the broad host range incompatibility types (IncN, IncP1, IncL/M and IncA/C) (Novais *et al*. 2007, Carattoli 2009, Coque *et al*. 2008b).

1.8.1.4.2 Prevalence of CTX-M among Enterobacteriaceae in UK

Antimicrobial resistance among *Enterobacteriaceae* has become a growing problem in the UK. In the UK isolation of CTX-M was rare before 2001. Several papers have reported the growing incidence of ESBLs among Enterobacteriaceae in the UK, not least those of the CTX-M-like genotype (Livermore and Hawkey 2005, Woodford *et al*. 2007).The most prevalent CTX-M members among all the reported cases in the UK are CTX-M-14 and CTX-M-15 although CTX-M-26 and CTX-M-9 have also been reported (Tarrant *et al*. 2007).

However, the first CTX-M identified in UK was from a clinical isolate of *Klebsiella oxytoca* recovered from a stool sample of a 6-year-old child in May 2000, producing CTX-M-9 (Alobwede *et al*. 2003). This was followed, in 2001 by an outbreak in a Birmingham hospital, involving 30 patients and caused by a *Klebsiella pneumoniae* strain with CTX-M-26 β-lactamases (Brenwald *et al*. 2003). Also in 2001, a survey by Mushtaq *et al* examined over 200 *Enterobacteriaceae* from 26 hospitals in the UK and Ireland and recorded four isolates with CTX-M-15 enzyme (Mushtaq *et al*. 2003). A study in 2003 at York, UK showed that out of 22 ESBL positive *Enterobacteriaceae* isolates, 17 strains were positive for CTX-M (Munday *et al*. 2004a). In late 2004, a prospective survey was undertaken covering 16 laboratories in London (8) and SouthEast England (8) seeking up to 100 *Enterobacteriaceae* per site. This yielded 1122 out of 1253 isolates with confirmed cephalosporin resistance out of which 502 (44.7%) carried a CTX-M enzyme (Potz *et al*. 2006). Furthermore, a report in 2007 also found that out of 33 *E. coli*, 23 possessed $bla_{\text{CTX-M-15}}$, which were genetically linked to *ISEcp*1(Tarrant *et al*. 2007).

The rapid emergence of the CTX-M as the predominant ESBL type in the UK is not an isolated occurence. A report on ESBL types in *Enterobacteriaceae* in Argentina found that CTX-M accounted for roughly 54% of all ESBLs found in that country (Quinteros *et al*. 2003). Similarly studies in Japan, China, Taiwan and Spain showed the same high incidence of CTX-M ESBLs (Yamasaki *et al*. 2003, Yu *et al*. 2002, Canton *et al*. 2002, Munday *et al*. 2004b). The CTX-M-1 group enzyme mainly CTX-M-15 accounted for most CTX-M β-lactamases found in the UK followed by CTX-M-9 group, mainly CTX-M-9 and to a lesser extent CTX-M-14.

(Mushtaq *et al*. 2003, Woodford *et al*. 2004, Dimude and Amyes 2013). The CTX-M-15 isolates from the UK were particularly associated with the *E.coli* clone A to F with clone A later identified as belonging to O25:H4-ST131 clone - that has been isolated all over the world (Clermont *et al*. 2008, Coque *et al*. 2008b, Lau *et al*. 2008b, Lau *et al*. 2008a). Over the years in the UK CTX-M enzymes has been detected in isolates from hospitalised patients, patients in the community and from animals - with CTX-M-15 being the most prevalent (Dhanji *et al*. 2011, Younes *et al*. 2011, Woodford *et al*. 2004, Horton *et al*. 2011).

Furthermore most of the CTX-M producers are found also to be resistant to the fluoroquinolones, with the resistance found to be associated with the new variant of aminoglycoside modifying enzyme *aac(*6*')-Ib-cr* (Machado *et al*. 2006, Cerquetti *et al*. 2010). There is an observation of a strong relationship between $bla_{\text{CTX-M-15}}$ and *aac (*6*')-Ib-cr* in the same strain. This led to the study of this relationship by Jones *et al* in UK strains which shows that *aac(*6*')-Ib-cr* occured in the UK preceding the acquisition of $bla_{\text{CTX-M-15}}$ (Jones *et al*. 2008).

1.8.1.4.3 Prevalence of CTX-M in Egypt

Before the first detection of CTX-M in Egypt, there has been reports of a high proportion of ESBLs in *E.coli* (El Kholy *et al*. 2003, Borg *et al*. 2008). The presence of CTX-M-15 was reported in 2006, which found 28 ESBL- producing E.coli isolates having a CTX-M type gene (Mohamed Al-Agamy *et al*. 2006). Among the 28 CTX-M isolates 18 were found to harbour $bla_{\text{CTX-M-15,}}$ 7 harboured $bla_{\text{CTX-M-27}}$ and 3 had $bla_{\text{CTX-M-14.}}$ At the time of this finding there was no information present on the spread of CTX-M in Egypt with the author expressing that it's not unlikely that these strains producing CTX-M could spread in a population where antibiotics are available

without prescription. This study was followed by the first report of CTX-M-14 in *K. pneumonia* and *Ent. cloacae* (Khalaf *et al*. 2009). A more thorough epidemiological study on both isolates from the hospital and community setting in 2010 found 74 isolates with $bla_{\text{CTX-M-15}}$ gene out of the 83 ESBLs producers showing a high proportion of this gene (Fam *et al*. 2011). At the same time a report of plasmid mediated CTX-M-14 in *Salmonella serovar Typhimurium* was reported from Egypt (AbdelGhani *et al*. 2010). The data from Egypt is not surprising because antimicrobial can be purchased without prescription leading to misuse likely to encourage the selection of resistant strains.

1.8.2 Carbapenemases

Carbapenemases are capable of hydrolysing not only the cephalosporins and oxyimino-cephalosporins but also the carbapenem antibiotics. The carbapenemases are the group of β-lactamases that are of great concern in Gram-negative bacteria because the carbapenem antibiotics are the last mainstream drugs of resort for the treatment of MDR infection caused by Gram-negative bacteria. The carbapenamases can be found within each of the four molecular classes of β-lactamases. The molecular class A carbapenemases include the plasmid mediated *Klebsiella pneumoniae* carbapenemase (KPC), the Guiana extended spectrum (GES), the chromosomally encoded *Serratia marcescens* enzyme (SME), the related NMC carbapenemases and the imipenem-hydrolysing (IMI) β-lactamases

The molecular class B Metallo β-lactamases (MBL) includes: active on imipenem (IMP) (Watanabe *et al*. 1991), Verona integron-encoded metallo-β-lactamase (VIM) (Lauretti *et al*. 1999), Sao Paulo metallo-β-lactamase (SPM) (Toleman *et al*. 2002),

Seoul imipenemase (SIM) (Lee *et al*. 2005), German imipenemase (GIM) (Castanheira *et al*. 2004) and New Delhi MBL (NDM) (Yong *et al*. 2009).

The molecular class C is the CMY-10 type first isolated from *Enterobacter aerogenes* in 2003 and one of only a few that belongs to β-lactamase molecular class C (S. H. Lee *et al*. 2003). However, the CTX-M-15 β-lactamases has also been identified as having carbpanemase activity (Findlay *et al*. 2012). Resistance to carbapenem by CTX-M producing Enterobacteriaceae has been reported with a combination of other resistance mechanisms such as selection of mutations leading to lack of porin expression, upregulated efflux or OMP alteration (Mena *et al*. 2006, Oteo *et al*. 2008).These resistance mechanism cannot confer resistance to carbapenem on its own but can result in resistance together CTX-M enzymes. The molecular class D carbpanemases are the Oxacillinase OXA enzyme type. They are mainly found in found in *Acinetobacter baumannii* (reviewed by (Evans *et al*. 2007) and *P.aeruginosa* (Kong *et al*. 2005).

1.8.2.1 VIM β-lactamases

VIM is a second growing family of MBL apart from IMP. VIM-1 metallo β-lactamases was first reported in Verona Italy from an isolate of *P.aeruginosa* in 1997 (Lauretti *et al*. 1999). The VIM-1 β-lactamase shares less than 30% amino acid similarities with the IMP group of transferable carbapenemases that are largely confined to Japan: however, it is more closely related to BCll from *B. cereus* sharing 39% amino acid identity(Lauretti *et al*. 1999). bla_{VIM} genes are found as part of the gene cassette inserted into an integron in addition to an *aacA*4 gene cassette encoding resistance to the aminoglycosides. Shortly after the identification of VIM-1 β-

lactamase, a closely related β-lactamase, VIM-2, was isolated in the south of France from a *P. aeruginosa* isolated from a blood culture taken from a neutropenic patient in 1996 (Poirel *et al*. 2000b). VIM-2 shares 90% amino acid similarity the VIM-1 β-lactamase and is also encoded in an integron. There is usually a sulphonamide resistance gene found in the 3´element of the integron. Subsequently, VIM-2 producing *P. aeruginosa* isolates were found to be associated with outbreaks in hospitals in Italy and Greece (Lagatolla *et al*. 2004, Pournaras *et al*. 2003). Identification of the VIM-3 β-lactamase in *P. aeruginosa* from Taiwan showed the amino acid sequence differed from that of VIM-2 by two amino acid changes (Yan *et al*. 2001).This was the first identification of VIM outside Europe indicating worldwide spread of this β-lactamase group.

1.8.3 Resistance to trimethoprim- sulphamethoxazole

Resistance to trimethoprim is mediated with the acquisition of *dhfr* genes (Amyes and Smith 1974) while resistance to sulphonamides is mediated with the acquisition of *sul* genes (Skold 1976). The prevalence of resistance to trimethoprim-sulphamethoxazole in Gram-negative bacteria is high (Huovinen 2001). The sulphonamide resistance gene *sul* is often found be fused to the *qac* gene in the 3´conserved segment of integrons (Stokes and Hall 1989). Similarly the *dhfr* gene coding for trimethoprim resistance is often found as gene cassette in the variable region of integron (Partridge *et al*. 2009). Integrons are very common in multi drug resistance Gram-negative bacteria (Byarugaba 2010). Use of sulphamethoxazole in combination with trimethoprim is shown to have a synergistic effect with the first description both *in vivo* and *in vitro* experiments published in the 1960s (Bohni 1969, Bushby and Hitching 1968).

1.8.4 Resistance to Aminoglycosides

Bacteria continually develop a variety of mechanisms to evade the lethal effect of antibiotics. Resistance to aminoglycosides is usually causes by Aminoglycoside Modifying Enzymes (AME) that render the aminoglycosides incapable of binding to its ribosomal target (Reviewed by Ramirez and Tolmasky 2010). The AME enzymes inactivate aminoglycosides by transferring a functional group to the aminoglycoside structure. This makes the aminoglycoside unable to interact with the ribosome effectively. All of the major classes of enzymes conferring resistance to amino-glycosides have been described, these include: acetyltransferases, nucleotidyltransferases, and phosphotransferases (Shaw *et al*. 1993). These amino acid modifying enzymes are often located on the plasmids or transposons with other resistance genes encoding resistance to other classes of antibacterials. Consequently the consumption of non-aminoglycoside antibiotics can influence the epidemiology of aminoglycoside resistance. The presence of these genes on mobile genetic elements facilitates their dissemination into various bacteria populations. All these enzymes conferring resistance to aminoglycosides have been described in Gram-negative bacteria (Davies and Wright 1997, Mingeot-Leclercq *et al*. 1999, Kettner *et al*. 1981, Kallova *et al*. 1997, Dornbusch *et al*. 1990, Vatopoulos *et al*. 1992).

1.9 Mechanisms of dissemination of resistance genes.

Antibiotic resistance in bacteria has been spreading and evolving in a complex process involving a variety of mechanisms. There are different ways in which dissemination of resistance gene can occur such as: clonal spread, plasmids or mobile genetic elements (reviewed by Courvalin, 2005). Mobile genetic elements (MGEs) are segments of DNA that encode enzymes and other proteins that mediate the movement

of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility) (Frost *et al*. 2005). There are two main categories of mobile genetic elements: elements that can move from one bacterial cell to another, namely plasmids and bacteriophages; and those that can move from one genetic location to another within the same cell- such as transposons ,integrons, insertion sequence common region (ISCR)-promoted gene mobilisation (Bennett 2008).

1.9.1 Plasmids

Plasmids are transferable genetic elements capable of self replication within a suitable host. Bacterial plasmids can act as a scaffold upon which gene arrays can be built, often encoding antibiotic resistance genes, by the incorporation of transposable elements and integron gene cassettes. A resistance plasmid is one which encodes resistance genes to one or more antibiotics (Bennett 2008). Many of the resistance plasmids encode the functions necessary to promote cell-to-cell DNA transfer which can be attributed to the global dissemination of plasmid mediated CTX-Ms among Gram-negative bacteria. CTX-Ms have been described on plasmids, most especially CTX-M-15 (Dimude and Amyes 2013, Naseer *et al*. 2009, Woodford *et al*. 2009). Plasmid replicon typing has identified different replicon types associated with specific $bla_{\text{CTX-M.}}$ It has been observed that $bla_{\text{CTX-M-15}}$ is associated with incompatibility group IncFII plasmids with in some cases also found on multireplicon plasmids harbouring additional FIA and FIB (Coque *et al*. 2008b, Novais *et al*. 2007, Hopkins *et al*. 2006). On the other hand a broad range of replicon plasmids such as IncN (Shen *et al*. 2008), IncL/M (Naas *et al*. 2011), IncK (Valverde *et al*. 2009), IncH12 (Garcia Fernandez *et al*. 2007) and IncI1 (Woodford *et al*. 2009) have all been involved in the dissemination of CTX-M genes

1.9.2 Transposons

Transposons, also known as 'jumping genes', are elements that can move from one location on the genome to another by transposition. A transposon contains a number of genes coding for antibiotic resistance, flanked on both sides by insertion sequences (IS) encoding a transposase enzyme. The transposase can be found in both Gram-positive and Gram-negative bacteria and are identified by the presence of inverted repeated sequences at both ends of the element. Transposons are an essential mechanism in bacteria responsible for the dissemination of antibiotics resistance genes. CTX-M genes have been found to be associated with transposons such as Tn2 (Roy Chowdhury *et al*. 2011), Tn2051 (Potron *et al*. 2012) Tn402 (Novais *et al*. 2006), and Tn21 (Valverde *et al*. 2006).Transposons carrying antibiotic resistance genes have the ability to enter either a conjugative plasmid or chromosome (Norman *et al*. 2009).

1.9.3 Insertion sequence

Insertion sequences are small genetic elements flanked by short terminal inverted-repeat sequence (IR) of 10-40bp and are able to insert at multiple sites in target DNA (Mahillon and Chandler 1998). Insertion sequences carry the information necessary for insertion functions but do not carry any accessory genes, such as antibiotic resistance genes. Insertion sequences play a role in the expression of $bla_{\text{CTX-M}}$ gene in Gram-negative bacteria. IS*Ecp*1 has been found upstream of $bla_{\text{CTX-M}}$ genes and has been shown to be involved in dissemination and over-expression of the gene leading to cephalosporin resistance (Poirel *et al*. 2003, Eckert *et al*. 2006, Saladin *et al*. 2002). The presence of insertion sequence IS*903* downstream of $bla_{\text{CTX-M}}$ genes has been

reported in several papers (Pai *et al*. 2001, Lartigue *et al*. 2004, Chanawong *et al*. 2002). Analysis of the promoter region in bacteria producing plasmid mediated β-lactamases revealed insertion elements IS*26*, IS*903*, IS*Ecp1* and these elements could play an important role in dissemination and selection of such ESBLs (Eckert *et al*. 2006). IS*Ecp1* mobilizes genes by transposing adjacent to them, and then misreading one of its cognate ends in a second transposition event, with the effect of moving the adjacent gene. This process has been carried out experimentally for CTX-M gene from chromosome of *Kluyvera spp*. (Lartigue *et al*. 2006).

In addition the same $bla_{\text{CTX-M}}$ has been found to be linked to the insertion sequence common region 1(*ISCR*1formerly *orf*513) upstream of the gene (Di Conza *et al*. 2002, Eckert *et al*. 2006). The ISCR1 is found at 3'CS on a class 1 integron suggesting participation in gene mobilisation by a rolling circle replication in addition to providing a putative promoter for high level expression.(Rodriguez-Martinez *et al*. 2006, Toleman *et al*. 2006)

1.9.4 Integrons and gene cassettes

Integrons are DNA elements with the ability to capture genes by site specific recombination and are not self transmissible. Integrons contain an integrase gene (*intl*) to mediate the excision and integration of DNA into the integron. Integrons often reside within transposons and so represent a mechanism by which new antibiotic resistance genes are added to the complement of a transposon and are subsequently disseminated (Figure 4)(reviewed by Cambray *et al*. 2010).

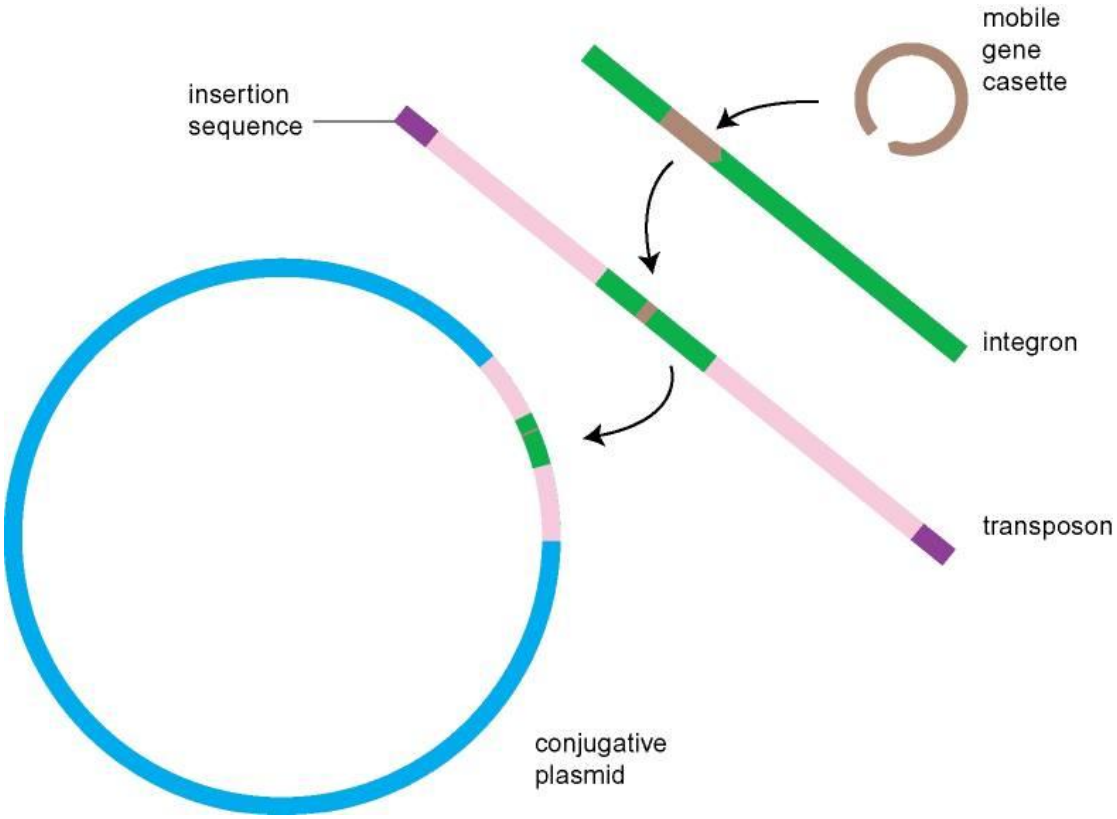


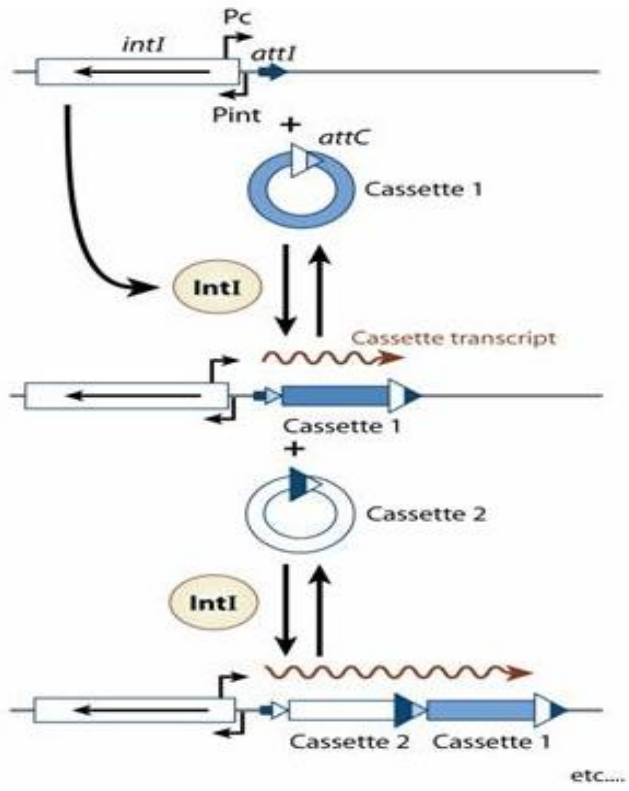
Figure 5 Integron may be inserted into transposons (taken from Norman *et al.* 2009)

The figure show how integron with resistance gene cassete may be inserted into the transposon and subsequently to a conjugative plasmid which can be disseminated between bacteria.

All integrons characterised to date have these essential components: the *intl* gene encoding an integrase belonging to the tyrosine-recombinase gene family. In the integron there is a recombination site *att* , which is recognised by the integrase (Figure 5) and is a receptor site for incoming gene cassettes, as well as an outward oriented promoter(Pc), which transcribes the inserted gene cassettes (Mazel 2006). Currently, five classes of mobile integrons are known to have a role in the dissemination of antibiotic resistance genes. These classes have been defined based on the sequence of

the encoded integrases, which show 40–58% identity between themselves (Mazel 2006). There are three separate classes of integron involved in antibiotic resistance, designated class 1, class 2 and class 3 (reviewed by (Cambray *et al*. 2010).

Figure 6 Functional platform of an integron (Cambray *et al*. 2010)



The figure show the general structure of an integron with the integrase gene *Intl* , the promoter region and the gene cassettes.

Class 1 integrons were first described by Stokes and Hall (Stokes and Hall 1989). This class of integron is associated with functional and non-functional transposons derived from transposon Tn*402* that can be embedded in larger transposons, such as Tn*21* (Mazel 2006). Class 1 integrons are found extensively in clinical isolates, and the majority of known antibiotic resistance gene cassettes belong to this class. Over 80

different gene cassettes, which form this class, have been described which confer resistance to β-lactams, all aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampicin, erythromycin, fosfomycin, lincomycin and antiseptics of the quaternary-ammonium-compound family (Rowe-Magnus and Mazel 2002, Fluit and Schmitz 2004). The class I integrons are also characterised by the 5′ and distal 3′CS conserved segment (CS). The 3′CS often consists of *qacEΔ1* gene, which represents a truncated version of the gene cassette *qacE*, which encodes resistance to quaternary ammonium compounds, the *sul1*sulphonamide resistance determinant and ORF6, which has no known biological function.

Class 2 integrons are found to be associated with Tn*7* derivatives and, in contrast, to class 1 integrons, only six different resistance cassettes have been found associated with them (Ramirez *et al*. 2005, Biskri and Mazel 2003). This can be attributed to the fact that the gene encoding the integrase in class 2 integrons contains a nonsense mutation in codon 179 (ochre 179), thereby yielding a truncated, non-functional protein (Hansson *et al*. 2002).

Class 3 integrons are thought to be located on a transposon inserted on an, as yet, uncharacterised plasmid (Mazel 2006). Class 3 integrons were first described in Japan (Arakawa *et al*. 1995) and have been involved in antibiotic resistance in clinical isolates from Portugal and Canada (Arakawa *et al*. 1995, Xu *et al*. 2007, Correia *et al*. 2003).

The other two classes of mobile integrons, class 4 and class 5 have been described in *Vibrio cholerae* and *Vibrio salmonicida* respectively (Mazel 2006). It is interesting to

note that all these classes of integron have been found in Gram-negative bacteria as a means of dissemination of antibiotics resistance genes.

1.10 Antibiotic resistance in Gram-negative organisms: combating resistance and surveillance

Gram-negative bacteria are among the most important cause of serious infection in humans and antibiotic resistance in this group of pathogens has become an increasingly health problem worldwide (Hawkey and Jones 2009). Antibiotic resistance is now considered a global health problem that increases morbidity, mortality and treat infectious disease (Livermore 2009, Hawkey and Jones 2009). Specifically antibiotic resistance leads to longer hospital stays, increased exposure of others to drug-resistant isolates, and therefore to higher hospital costs, when compared with infections associated with susceptible isolates. β-lactam antibiotics are arguably the most important antibiotic class used in the treatment of infection caused by Gram-negative bacteria. There have been reported cases of resistance against β-lactam antibiotics in Gram-negatives (Rahal 2009).

Initialising steps towards combating the rise of resistance must include but not limited to surveillance to provide information necessary for the management of these pathogens to minimise morbidity and mortality. Surveillance studies on antibiotic resistance allow us to gather information regarding: 1) existing trends in pathogen incidence and antibiotic resistance mechanisms; 2) the appearance of novel resistance types; and 3) the prediction of future trends in antibiotic resistance.

Furthermore infection control measures are vital to help prevent transmission and persistence of bacteria in general and MDR bacteria specifically within the hospital environment and larger community. Health care professionals should be made aware of important infection control measures such as reduced patient contact and appropriate hand-hygiene can be, and there is evidence for such measures successfully controlling outbreaks (Enoch *et al*. 2008). The combined use of alcohol-based hand gels and improved infection control education are expected to and have limited the spread of nosocomial infections.

Additionally, the large scale spread of resistance to antibiotics has been attributed, at least in part, to the inappropriate prescribing and administration of antibiotics (Goossens *et al*. 2005, Goossens 2009). A more rational and controlled use of antibiotics is required to reduce the selective pressure that they provide. In order to combat this health authorities have introduced antibiotic prescription guidelines alongside continuous monitoring of infections and resistance in order to detect the early development of any patterns of what so that appropriate interventions can be put in place to limit their spread. Continual non-sensational education is important to maintain and increase public awareness of the issues, and encourage both patients and clinicians against the misuse of antibiotics.

Above all increasing our understanding of bacterial resistance development,is essential to pinpoint new targets for antibiotic agents. It is also vital to enable the implementation of effective methods to slow the seemingly relentless rise of resistance, and to warn against the development of potential new resistance

mechanisms. All these when used in conjunction with one another will help developed a strategy to protect the public health both now and in the future.

1.11 Bacteria species investigated

1.11.1 *Escherichia coli*

Escherichia coli is named after the original discoverer Theodor Escherich. *E. coli* are facultative anaerobic, Gram-negative rods which are motile and may appear with or without a capsule. Most strains of *E.coli* ferment lactose with the production of acid and gas within 24-48 hours, while some do so only after extended incubation or are non- lactose fermenters. Although the normal habitat of *E. coli* is the gut of man and animals, it may colonise the lower end of the urethra and vagina. Infection with *E. coli* can be spread by contact, ingestion (Faecal-oral-route) and may be associated with food. Diseases caused by *E. coli* include urinary tract infections, diarrhoeal diseases, and neonatal meningitis.

1.11.2 *Klebsiella* spp

The organism *Klebsiella* was named in honour of Edwin Klebs a 19th century German-Swiss microbiologist. *Klebsiella* spp are Gram-negative rods belonging to the family *Enterobacteriaceae* family. They are facultative anaerobes but growth under strict anaerobic condition is poor. The capsule is often pronounced and can be produced in greater amount in carbohydrate rich media. *Klebsiella* spp are non-motile but most strains are fimbriated. The normal habitat of this species is the gut of man and animals and moist inanimate environments, especially soil and water. Infection

may be endogenous or acquired by contact spread. *Klebsiella* spp are considered to be an opportunistic pathogen causing infection in immuno-compromised patients and those with underlying health conditions such as alcoholics, those with lung disease, diabetics, etc. *Klebsiella* spp are responsible for 7-10% of hospital associated bloodstream infections in Europe, Latin America, and North America (Biedenbach *et al*. 2004).

1.11.3 *Enterobacter* spp

Enterobacter spp are Gram-negative rod-shaped bacteria that are facultatively anaerobic. They are motile and belong to the family Enterobacteriaceae. *Enterobacter* species are found in the natural environment in habitats such as water, sewage, vegetables, and soil. The most clinically important are the *Ent. Cloacae* and *Ent. aerogenes,* which can cause opportunistic infection in immuno-compromised patients. Cephalosporins are contra-indication because of the risk of treatment failure due to inducible β-lactamases.

1.11.4 *Serratia marcescens*

Serratia is a genus of Gram-negative, facultative anaerobic rod shaped bacteria of *Enterobacteriaceae* family. *Serratia marcescens* is the species, in this genus, most often encountered in clinical specimens (Greenwood *et al*. 1992). Most *Serratia* are motile by means of peritrichous flagella and are facultative anaerobe. They belong to *Enterobacteriaceae* family. *Serratia* are distributed ubiquitously in soil, freshwater and sea water. *Serratia marcescens* thrives in moist environments and human disease caused by this organism are often assocaited with septicaemia, wound and respiratory tract infections has been reported (Hejazi and Falkiner 1997). *Serratia* strains are

commonly resistant to cephalosporins and an aminoglycoside is usually the most reliable drug of choice.

1.11.5 *Morganella morganii*

Morganella morganii is a Gram-negative rod that is a facultative anaerobe. It is found in the faeces and the intestines of humans, dogs, and other mammals. It is known to be a causative organism of opportunistic infections in the respiratory tract, the urinary tract, and in wound infections. This organism is characteristically resistant to many β-lactam antibiotics, which may lead to delays in proper treatment (Mugnaioli *et al*. 2005).

1.11.6 *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia is an aerobic, non fermentative Gram-negative bacterium and human infection caused by this organism is difficult to treat. They can occur almost in any aquatic or humid environment including the drinking water supply (Hoefel *et al*. 2005, Cervia *et al*. 2008). *S. maltophilia* is recognized as an opportunistic pathogen causing severe infection in immunocompromised patients (Safdar and Rolston 2007, Tan *et al*. 2008). *S. maltophilia* colonization rates in individuals with cystic fibrosis have been increasing (Waters *et al*. 2007). *S. maltophilia* is naturally resistant to many broad spectrum antibiotics including carbapenems due to the production of inducible chromosomal metallo-β-lactamases (L1 and L2) and these enzymes make treatment difficult (Denton and Kerr 1998).

1.12 Aims of this study:

- To determine the susceptibility pattern of Gram-negative blood culture isolates.
- To determine the molecular diversity associated with the dissemination of ESBL genes, particularly *bla*$_{\text{CTX-M-15}}$, in blood culture isolates of *E. coli.*
- Comparison of the diversity and ESBLs of *Enterobacter cloacae* isolated in Edinburgh with those isolated in Cairo, (an area that has had a bigger problem with resistance). The seldom studied *Ent cloacae*,carrying the *bla*$_{\text{CTX-M-14}}$ gene was also investigated.
- Sulphonamide resistance genes are closely associated with *bla*$_{\text{CTX-M-15}}$ genes. The final aim was to study integrons and the persistence of these sulphonamide resistance genes in blood culture isolates in a population where the use of this antimicrobial had been restricted.

2 Materials and methods

2.1 Bacterial strains

A total of 100 Gram-negative isolates which had been assumed to be resistance to carbapenems were collected from New Royal Infirmary of Edinburgh. The period of sample collection were between July 2009 and December 2009. The isolates comprise 65 *E.coli,* 11 *Enterobacter cloacae*, 8 *K. pneumoniae*, 4 *K. oxytoca,* 3 *Serratia marcescens*, 2 *Morganella morganii,* and 1 *Enterobacter asburiae,* 5 *Stenotrophomonas maltiphilia* and 1 *Cupriavidus pauculus.* All isolates were from blood cultures. Also 3 *Enterobacter cloacae* isolates from Egypt were examined in addition. The first was isolated from a male in ICU in March 2010. The second was isolated from sputum of a female in ICU in March 2010 and the third from a female also in ICU in March 2011.

2.1.1 Control strains

The control strains used for this study were *E.coli* NCTC 10418, *Staphylococcus aureus* NCTC 6571, and *Pseudomonas aeruginosa* NCTC 10662.

2.2 Identification of isolates

Isolates were initially identified in the hospital using standard microbiology techniques. The isolates were confirmed by me using API (20E/20NE) methods according to manufacturer's instructions and PCR molecular identification of strains by sequencing of approximately 500bp fragment of the 16S rRNA gene using

previously described universal primers(Kommedal *et al*. 2008). Sequences were then compared to those in the GenBank database using BLASTN to find species with similar sequences.

2.3 Media broths, agars and normal saline

All the media used in this study were from Oxoid (Basingstoke, UK) and were reconstituted with distilled water in the laboratory according to manufacturer's instruction and autoclaved before use to ensure sterility. The normal saline used for the study was prepared by dissolving 0.85g NaCl (Sigma ultra USA) in 100ml of distilled water followed by autoclaving.

2.4 Antimicrobial susceptibility testing.

2.4.1 Disc Susceptibility Testing

Initial antimicrobial susceptibilities were determined by the disc diffusion method on Iso-sensitest Agar (IST Agar) and the susceptibility breakpoints were estimated as recommended by BSAC Methods for Antimicrobial Susceptibility Testing version 10 (Andrews *et al*. 2011). The antibiotic discs used were obtained from Oxoid (Basingstoke, UK). Briefly an overnight broth culture was diluted 1: 500 in sterile 0.8% saline; the inoculum was spread over the surface of an agar plate in two dimensions. An antibiotics disc was applied to the surface of the agar within 15 minutes of inoculation.

2.4.2 Minimum Inhibitory concentration (MIC)

The MICs of the antibiotics were determined using agar dilution method according to the guideline of British Society for Antimicrobial Chemotherapy (BSAC) Working Party and the MIC concentrations were between 0.008-256mg/L(Andrews *et al*. 2011). Briefly, Iso-sensitest (IST) agar was allowed to cool to 50°c before the addition to the antibiotic containing plate, then allowed to set and dried at room temperature. An overnight broth culture was diluted 1:500 in sterile 0.8% saline and approximately 10^4 of the organism inoculated using multipoint inoculators (Denley: surrey.UK) onto the antibiotic containing media. Inoculated plates were incubated overnight at 37°c.The MIC is the lowest antibiotic concentration to inhibit all visible growth.

Table 3 Antimicrobial agents used

Antimicrobial Agent	Supplier/ Manufacturer
Kanamycin	Sigma-Aldrich (Dorset,UK)
Aztreonam	Sigma-Aldrich (Dorset,UK)
Cefepime	Sigma-Aldrich (Dorset,UK
Cefotaxime	Sigma-Aldrich (Dorset,UK
Ceftazidime	Sigma-Aldrich (Dorset,UK)
Gentamicin	Sigma-Aldrich (Dorset,UK)
Ampicilin	Sigma-Aldrich (Dorset,UK)
Piperacilin	Sigma-Aldrich (Dorset,UK)
Streptomycin	Sigma-Aldrich (Dorset,UK)
Spectinomycin	Sigma-Aldrich (Dorset,UK
Co-trimoxazole	Sigma-Aldrich (Dorset,UK
Ciprofloxacin	Sigma-Aldrich (Dorset,UK)
Nalidixic acid	Sigma-Aldrich (Dorset,UK
Imipenem	Merck (Nottingham, UK)
Meropenem	Astra Zeneca (London, UK
Ertapenem	Merck (Nottingham, UK)
Cefoxitin	Sigma-Aldrich (Dorset,UK)
Rifampicin	Sigma-Aldrich (Dorset,UK)
Pottasium Clavulanate	Sigma-Aldrich (Dorset,UK)
Cefoperazone	Sigma-Aldrich (Dorset,UK
Cefuroxime	Sigma-Aldrich (Dorset,UK

A list of the antimicrobials used and their suppliers.

2.5 ESBLs detection by double disc synergy test

A plate is inoculated as the disc susceptibility above. Synergy between cefotaxime and amoxicillin/clavulanic acid were detected by placing the disc 30mm apart and incubated at 37°c overnight. ESBLs production is confirmed when a clear cut extension of the cefotaxime inhibition zone towards the disc with clavulanic acid which indicates the synergy.

2.6 Polymerase Chain Reaction (PCR) Amplification

2.6.1 Extraction of DNA

DNA was extracted by suspending 1 loopful of culture (from a plate incubated overnight) in 50µl sterile distilled water. The mixture was then boiled for 10 minutes and centrifuged at 13,000 rpm for 1min using eppendorf micro centrifuge 5415D. The supernatant was taken and used as template DNA.

Genomic DNA was extracted using Promega DNA isolation kit according to manufacturer's instructions.

2.6.2 PCR reagents

PCR reagents were provided from (Promega, Southampton, UK). The reagents used for PCR were: 0.02µm of each primer, 5x green or colourless buffer, Deoxynucleoside triphosphates (dNTP) / PCR nucleotide mix (10 mM each), Go Taq DNA Polymerase (5u/µl), and $MgCl_2$ solution (25 mM).

2.6.3 PCR reactions

The final volume of the PCR reaction mixture was 50 µl. The reaction mixture had 1µl dNTPs, 3µl MgCl2, 0.2 µl of Taq DNA polymerase, 5µl green /colourless buffer, 1µl template DNA, 1µl each primer. Sterile Distilled water (38.8µl) was used as diluents for the reaction.

2.6.4 PCR Primers

The primers used were manufactured by Eurofins MWG Operon and Invitrogen life technology. The primers used in this study were either from published papers or designed using biotools software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Table 4 lists all the primers used in this study.

Table 4 Primers used in this study

Primers	Forward Sequence(5'-3')	Reverse sequence(5'-3')	Reference
CTX-M	TTTGCGATGTGCAGTACCAGTAA	CGATATCGTTGGTGGTGCCATA	(Edelstein *et al*. 2003)
CTX-M-1	AAAAATCACTGCGCCAGTTC	AGCTTATTCATCGCCACGTT	(Woodford *et al*. 2006)
CTX-M-2-F	CGACGCTACCCCTGCTATT	CCAGCGTCAGATTTTTCAGG	(Woodford *et al*. 2006)
CTX-M-9-F	CAAAGAGAGTGCAACGGATG	ATTGGAAAGCGTTCATCACC	(Woodford *et al*. 2006)
CTX-M-8F	TCGCGTTAAGCGGATGATGC	AACCCACGATGTGGGTAGC	(Woodford *et al*. 2006)
CTX-M-25F	GCACGATGACATTCGGG	AACCCACGATGTGGGTAGC	(Woodford *et al*. 2006)
CTX-M1A	CTTCCAGAATAAGGAATC	CCGTTTCCGCTATTACAA	(Dutour *et al*. 2002)
*Isecp*1	GCAGGTCTTTTTCTGCTCC	TTTCCGCAGCACCGTTTGC	(Poirel *et al*. 2003)
Is26	GCGGTAAATCGTGGAGTGAT	ATTCGGCAAGTTTTTGCTGT	(Woodford *et al*. 2004)
CTX-M-14	AACACGGATTGACCGTATTG	TTACAGCCCTTCGGCGAT	(Bou *et al*. 2002)
IS903	CATATGAAATCATCTGCGC	CCGTAGCGGGTTGTGTTTTC	(Poirel *et al*. 2003)
aac(6')-Ib-cr	TTGCGATGCTCTATGAGTGGCTA	CTCGAATGCCTGGCGTGTTT	(Park *et al*. 2006)
orf-513	CTTTTGCCCTAGCTGCGG	CTCACGCCCTGGCAAGGTTT	(Lartigue *et al*. 2004)
orf-477	GCGCATAGTCATCGGCAG	CCTGGGACCTACGTG	(Literacka *et al*. 2009)
*Sul*1	CGGCGTGGGCTACCTGAACG	GCCGATCGCGTGAAGTTCCG	(Kerrn *et al*. 2002)
*Sul*2	GCGCTCAAGGCAGATGGCATT	GCGTTTGATACCGGCACCCGT	(Kerrn *et al*. 2002)
*intl*1	GCCACTGCGCCGTTACCACC	GGCCGAGCAGATCCTGCACG	(Kerrn *et al*. 2002)
hep58 & 59	TCATGGCTTGTTATGACTGT	GTAGGGCTTATTATGCACGC	(White *et al*. 2000)
*Sul*3	GAGCAAGATTTTTGGAATCG	CATCTGCAGCTAACCTAGGGCTTTGGA	(Perreten and Boerlin 2003)
16s rDNA	CGGCCCAGACTCCTACGGGAGGCAGCA	GCGTGGACTACCAGGGTATCTAATCC	(Kommedal *et al*. 2008)
VIM-1	TTATGGAGCAGCAACGATGT	CAAAAGTCCCGCTCCAACGA	(Yan *et al*. 2001)
*Intl*2 CS	TGGGCTGAGAGAGTGGT	TTTTTGTGCTGCCATATCCGTG	(Ramírez *et al*. 2005)
*aadA*2	TGTTGGTTACTGTGGCCGTA	GATCTCGCCTTTCACAAAGC	(Randall *et al*. 2004)
F11	CTGATCGTTTAAGGAATTTT	CACACCATCCTGCACTTA	(Villa *et al*. 2010)
F1B	TCTGTTTATTCTTTTACTGTCCAC	CTCCCGTCGCTTCAGGGCATT	(Villa *et al*. 2010)
F1C	GTGAACTGGCAGATGAGGAAGG	TTCTCCTCGTCGCCAAACTAGAT	(Villa *et al*. 2010)
O25pabBspe	TCCAGCAGGTGCTGGATCGT	GCGAAATTTTTCGCCGTACTGT	(Clermont *et al*. 2009)
TspE4C2	GAGTAATGTCGGGGCATTCA	CGCGCCAACAAAGTATTACG	(Clermont *et al*. 2000)
ChuA	GACGAACCAACGGTCAGGAT	TGCCGCCAGTACCAAAGACA	(Clermont *et al*. 2000)
YjaA	TGAAGTGTCAGGAGACGCTG	ATGGAGAATGCGTTCCTCAAC	(Clermont *et al*. 2000)
trpA	GCTACGAATCTCTGTTTGCC	GCAACGCGGCCTGGCGGAAG	(Clermont *et al*. 2009)
Oxa-1 like	GGATAAAACCCCCAAAGG AA	TGCACCAGTTTTCCCATACA	(Karisik *et al*. 2006)
TEM	ATAAAATTCTTGAAGACGAAA	GACAGTTACCAATGCTTAATC	(K. Lee *et al*. 2003)
IncF1A	CCATGCTGGTTCTAGAGAAGGTG	GTATATCCTTACTGGCTTCCGCAG	(Villa *et al*. 2010)
IncF1B	TCTGTTTATTCTTTTACTGTCCAC	CTCCCGTCGCTTCAGGGCATT	(Villa *et al*. 2010)
IncF1C	GTGAACTGGCAGATGAGGAAGG	TTCTCCTCGTCGCCAAACTAGAT	(Villa *et al*. 2010)
IncF11	CTGATCGTTTAAGGAATTTT	CACACCATCCTGCACTTA	(Villa *et al*. 2010)
IncN	GTCTAACGAGCTTACCGAAG	GTTTCAACTCTGCCAAGTTC	(Carattoli *et al*. 2005)
IMP	GGAATAGAGTGGCTTAAYTCTC	CCAAACYACTASGTTATCT	(Ellington *et al*. 2007)
SIM	TACAAGGGATTCGGCATCG	TAATGGCCTGTTCCCATGTG	(Ellington *et al*. 2007)
SPM	AAAATCTGGGTACGCAAACG	ACATTATCCGCTGGAACAGG	(Ellington *et al*. 2007)
GIM	TCGACACACCTTGGTCTG	AACTTCCAACTTTGCCATGC	(Ellington *et al*. 2007)
*intl*2	CACGGATATGCGACAAAAAGGT	GTAGCAAACGAGTGACGAAATG	(Machado *et al*. 2005)
intl 3	AGTGGGTGGCGAATGAGTG	TGTTCTTGTATCGGCAGGTG	(Machado *et al*. 2005)

A list of primers used in this study and their original sources.

2.7 Agarose gel electrophoresis of PCR product

2.7.1 10X TAE buffer

The constituents of 10X TAE buffer were: 3.72g of EDTA (Ethylene Diamine Tetra acetic Acid) Sodium salt dehydrate 99% (Aldrich produce of USA), 11.4 ml glacial acetic acid (Bio keystone Co LLC, California, USA). The final volume of the buffer was made up to one litre and the pH was adjusted to 8.0. Just before use, the buffer was diluted to1:10 to make 1X TAE buffer.

2.7.2 Visualisation of PCR product

After amplification reaction, the PCR product were analysed in 1.5% agarose gel (Fisher Bioreagent) using the following electrophoresis conditions: 100v, 30min on 1X TAE buffer at room temperature. The molecular weight markers used were 100bp and Ikb ladder. After running, the gel was stained with Gel red and then visualise under UV Tran illuminator Bio-Rad Gel Doc 2000 (Bio-Rad, Hemel Hemstead, UK)

2.8 Sequencing of PCR product

PCR products were purified using QIAquick purification Kit (Qiagen, West Sussex.UK) according to the manufacturer's instructions. Primers were diluted to 3.2 picamole.1µl of the diluted primer and 5µl of the purified PCR product were sent for sequencing .The sequencing was carried out in the Gene pool Genomics facility at the

University of Edinburgh (http://genepool.bio.ed.ac.uk/). The DNA sequence result were analysed using BLAST and compared with published sequence on the website http//www.blast.ncbi.nlm.nih.gov/genbank. Protein sequences were obtained using Expasy translate website (http//www.expasy.ch/tools/dna.html).

2.9 Pulsed-field Gel Electrophoresis (PFGE)

2.9.1 Agarose plug preparation

PFGE was applied to assess the clonality of the bacteria isolated. Strains were grown overnight in 5ml LB broth at 37^{o}C and harvested by centrifugation at 3000g for 20 mins using Sorval RT7 plus centrifuge. The supernatant was removed and the pellet suspended in 1ml cell suspension Buffer (100mM Tris-HCL, 100mM EDTA pH 8). Each cell suspension was adjusted to cell density between 1.3-1.4 absorbance at 610nm wavelength using a spectrophotometer. 500µl cell suspensions were incubated at 55^{o}C for 10mis in a water bath. Aliquots of 25µl of Proteinase K (20mg/l) were added and the suspension mixed gently by inversion. An equal volume of melted 1% Seakem Gold agarose: 1%SDS in TE buffer (10mM Tris, ImM EDTA, pH 8) was added to the suspension and mixed gently by inverting the tube 12 times. The mixture was immediately dispensed into wells of plug mold and allowed to solidify at room temperature for 5mins and at 4^{o}C for 10mins. The plugs were removed from plug mold and transferred to 2ml cell lysis solution-1 (50mM Tris, 50mM EDTA, 2.5mg/ml lysozyme, 1.5mg/mls Proteinase K pH 8) and placed in a water bath at 37^{o}C for 1hr. The plugs were transferred into 2ml cell suspension buffer-2 (0.5 EDTA, 1% Sarcosyl, and Proteinase K 400µg/ml) and places in a 55^{o}C water bath for 2hrs.The plugs were removed from the lysis solution and washed 3 timed in 4ml distilled water

at 55^{o}C for 15mins each and then 3 times in TE buffer(10mM Tris-HCL,0.1mM EDTA pH 7.6) at 55^{o}C for 15mins each .After the last wash, TE buffer was added and the plug stored at 4^{o}C until use.

2.9.2 Agar rose plug digestion

The plugs prepared above were equilibrated in 200µl of the restriction endonuclease buffer for 45mins at 37^{o}C. The restriction buffer was replaced with another 100µl buffer containing 30u of *Xba*l (PromegaUK) restriction enzyme and 1ul BSA. The tubes were incubated in a water bath at 37^{o}C for 18hrs.

2.9.3 Gel preparation and running conditions

A 1% pulsed-field certified agarose gel was prepared in 0.5x TBE buffer (0.089M Tris base, 0.089M Boric acid.2.5mM EDTA). Plugs were loaded into the wells of the PFGE agarose gel. Electrophoresis was performed in a CHEF-DRII system for 22hrs at 6.0volt/cm with a pulse time 5-45 seconds at 13^{o}C in 0.5x TBE buffer. A lambda ladder PFGE maker (New England Biolabs) was used as the size standard. Gels were stained in Gel Red for 30 mins and visualised under UV with Bio-Rad GelDoc system.

2.9.4 Interpretation of the PFGE

Cluster analysis was performed using Bio Numeric Software version 4 by the unweighted pair group method with arithmetic averaging (UPGMA). DNA relatedness was calculated by using the band-based Dice co-efficient with a tolerance

setting of 1.5% band tolerance and 1.5% optimization setting for the whole profile. Isolates which cluster together with a similarity of ≥ 85% were considered to belong to the same PFGE type and the results were obtained in a phylogenetic tree.

2.10 Plasmid DNA isolation

Several methods of plasmid extraction were used in this study including Qiagen midi and mini kit. Plasmid DNA was also extracted and isolated by a modification of the alkaline lysis method of described by Kado and Liu (Kado and Liu 1981). Briefly a loopful of bacteria grown on a plate was suspended in 60µl suspending buffer (50mM Tris-HCL,1mM EDTA, pH 8) ensuring suspension are uniform. 600µl of lysis buffer (50mM Tris base, 3%SDS, pH12.5) were added and mixed briefly. The mixture was incubated at 56^{o}C for 1hr. Three hundred µl of a mixture of phenol, chloroform, and isoamylalcohol in the proportion of 25:24:1 was added and mixed well. The mixture was centrifuged for 30 mins. The upper aqueous phase containing the plasmid was transferred to a fresh tube.

Plasmid DNA was also extracted using the Qiagen Midi prep kit according to manufacture's instructions. Plasmids were stored at -80^{o}C. This was used for the transformation assay.

2.10.1 Plasmid Profile by S1 Nuclease digestion

S1 is an endonuclease that is more active against DNA than RNA. This procedure converts the circular DNA to a linear form to determine the molecular sizes of the plasmids. The closed circular super coiled form moves very slowly, the naked plasmid

remains trapped in the wells. The protocol was modified from a previously used method by Barton *et al*, 1995 (Barton *et al*. 1995). The plugs slices were prepared as in the PFGE protocol and were equilibrated into 200µl 1x S1 nuclease buffer (Promega UK) at 37^{o}C for 45mins. The buffer was replaced with 1x S1 nuclease buffer containing 10 units of S1 nuclease and incubated for 45mins at 37^{o}C. The reaction was stopped with 0.5M EDTA (pH8). The gels were run and stained as described previously for PFGE. The plasmid sizes were determined by comparison with the PFG lambda ladder.

2.11 Conjugation assay by Broth Method

Conjugation assay were performed in broth with *E.coli J*62-2(Rif ^{R}) as the recipient. Overnight LB broth culture of the donor and recipient strains were mixed in the ratio of 1:10 in LB broth and incubated at 37^{o}C overnight. The cells were collected and resuspended in 5.6ml of sterile saline .100µl of the cells were dispensed onto plates containing rifampicin with the selective antibiotic and spread with the spreader. The plates were incubated at 37^{o}C overnight and growths of the transconjugants were observed. Controls of each strain were inoculated on the same rifampicin/antibiotic containing plate and incubated at 37^{o}C overnight.

2.12 Transformation

2.12.1 Preparation of competent cells

An overnight broth culture of *E.coli* DH5α was inoculated (1:100) into 50mls of fresh LB broth in a sterile 250ml flask at 37^{o}C on the shaker until approximately 0.5 absorbance at 600nm wavelength. The culture was placed on ice for 30mins. The cells were pelleted by centrifugation at 4^{o}C for 10mins. The supernatant was removed and

the pellets resuspended in 25mls of sterile ice cold 0.1M $CaCl_2$ and placed on ice for 30min. The cell suspensions were centrifuged at 4^{o}C for 10mins and the supernatant removed. The cell pellet was resuspended in 1ml of sterile ice cold 0.1M $CaCl_2$ and 200µl of 90% glycerol added. The cells were stored at 100µl aliquots at -80^{o}C.

2.12.2 Transformation via heat shock

The aliquot of the above competent *E.coli* DH5α cell were thawed on ice. 2µl of purified plasmid was added to the cell, mixed and chilled on ice for 30mins. The cells were placed in a water bath set at 42^{o}C for exactly 1 minute then placed on ice immediately for 2min. One ml of fresh LB broth was added to the cells which were then transferred into sterile 15ml centrifuge tube and incubated at 37^{o}C in a shaker for 1hr. Cells were then spread on LB agar containing the selective antibiotics and incubated overnight at 37^{o}C.

2.13 Assessment of transconjugants and transformants

Transconjugants and transformants were assessed by susceptibility, plasmid extraction and S1 nuclease digestion.

2.14 Gene Expression analysis

2.14.1 RNA extraction

Bacterial cultures were grown to an optical density of 0.5-0.8 at 600nm wavelength and total RNA extracted using Ribopure Bacteria RNA kit according to the manufacturer's instructions. Extracted RNA was treated with DNase 1 for 30 mins at 37^{o}C to remove any traces of genomic DNA.

2.14.2 Estimation of RNA concentration

The RNA concentrations were estimated using the Nanodrop 1000 spectrophotometer (Thermo scientific, Cramlington, UK). One µl of the extracted RNA sample were read by spectrophotometer and 260/280 absorbance reading were noted .The sample that fell between 1.8 and 2.1 were deemed suitable to the cDNA synthesis and reverse transcription.

2.14.3 Reverse transcription (RT) PCR

RT PCR was done with 1pg-1µg RNA as template, using Access quick RT PCR System (Promega) according to the manufacturer's instructions. The numbers of cycles for each PCR were 30 cycles, except for 16S rRNA gene, which was for 15 cycles.

2.14.4 Gel analysis

RT PCR products were analysed using the Bio-Rad Geldoc 2000 software, Quantity one. After electrophoresis on agarose gel and staining, Quantity one was used to semi quantitatively assess gene expression. The intensity of the PCR product bands were measured and normalised to the intensity of the 16S band before being used to compare expression levels between strains. The following setting and conditions were used: manual band selection, background subtracted, peak intensity readings measured, rolling disc value of 10 used.

2.15 Inverse PCR

Purification of DNA was performed on overnight culture using DNA purification kit (Promega). Restriction reaction were set up with EcoR1 (Promega) according to manufacturer's instruction and incubated for 4hrs at 37^{o}C. The enzyme was inactivated by heating at 65^{o}C for 20 minutes. A ligation reaction was set up using T4 DNA Ligase (Promega). T4 DNA ligase catalyses the joining of two strands of DNA between the 5'-phosphate and the 3'hydroxyl groups adjacent in either the cohesive-ended or the blunt-ended configuration. This gives rise to circularised DNA. The reaction was incubated at room temperature for 16 hours. A PCR reaction was set up using the primers designed for inverse PCR (Table 5) under the following conditions: 94^{o}C for 5min, followed by 30 cycles of 94^{o}C for 1min, 58^{o}C for 1 min, and 72^{o}C for 1 min then final extension of 72^{o}C for 5minutes. The PCR products were run on a 1.5% agarose gel.

Table 5 Primers used for inverse PCR

Primers	Forward Sequence(5'-3')	Reverse sequence(5'-3')	Reference
VIM OUT	GCGGTCTAGACTTGCTCCAGCACACAG	ACGGACTTGCGACAGCCATGACAG	This work
CTX-M-14 OUT	GCCGCTGGTTCTGGTGACCTATTTTACCCAG	CTTTTCTCCAGCGCCGCCAGCTTTTG	This work
ISCR1 OUT	GCATCTGAAACAGAAAACAGCCAAGGCGAATG	TGGGGATGGCGAGCCAGCAAAAAG	This work

2.16 PCR bases plasmid replicon sequence typing of IncF

Plasmid replicon sequence typing of IncF plasmid was performed according to the protocol described by Villa *et al* (Villa *et al*. 2010). The genes encoding replicons FII, FIA and FIB were sorted by PCR with the primers as listed in the primer table and the sequence was carried out in the Gene pool Genomics facility at the University of

Edinburgh (http://genepool.bio.ed.ac.uk/). The alleles were assigned by submitting the amplicon sequence to the multilocus sequence typing database (www.pubmlst.org/Plasmid). The allele number was used to get the FAB formular; F for FII replicon, A for FIA replicon and B for FIB replicon.

3 Antimicrobial susceptibility pattern of blood culture isolates due to Gram-negatives

3.1 Introduction

Blood culture is a essential tool in the diagnosis of severe bacterial infections and it is the only tool to detect blood stream infection. Usually the blood stream is sterile. Blood stream infection (BSI) is a major cause of morbidity and mortality worldwide and one of the most common healthcare- associated infections (Mylotte and Tayara 2000). Microbiological diagnosis and treatment of BSI is dependent on isolating the offending pathogen and determining it susceptibility pattern. The result of the susceptibility testing will assist in the choice of antimicrobial agent. Studies have shown that BSI due to Gram-negative organisms are associated with increased mortality when compared with Gram-positives (Diekema *et al*. 1999). BSI if caused by multidrug resistant bacteria results in a reduction in therapeutic options and will need immediate attention. Increasing multidrug resistance is a worldwide concern, therefore to tackle this resistance problem, appropriate surveillance by monitoring the resistance trend and implementing measures are needed. Appropriate treatment of BSI will be enhanced by the knowledge of the antibiotic susceptibility profile of the most common bacteria encountered locally in such infection. This will increase the probability of selecting an effective antimicrobial for empirical treatment. Appropriate antimicrobial therapy has been shown to reduce the mortality among patients with Gram-negative bacteria when initiated early (Kang *et al*. 2003, Leibovici *et al*. 1997, Vallés *et al*. 2003)

Therefore in this study I aim to evaluate the antimicrobial susceptibility pattern of the Gram-negative isolates obtained from blood culture with the aim of improving management by choice of therapy and surveillance.

3.2 Results

The Gram-negative isolates studied were all from blood cultures and consisted of 65 *E. coli*, 11 *Enterobacter cloacae*, 8 *K. pneumoniae*, 4 *K. oxytoca*, 3 *Serratia marcescens*, 2 *Morganella morganii*, as well as 1 *Enterobacter asburiae* 5 *Stenotrophomonas maltophilia* and 1 *Cupriavidus pauculus. E. coli* was the most frequently isolated followed by *Klebsiella* and *Enterobacter* spp. The MICs determined for all these isolates is in the appendix. There was a high level of resistance (78%) to ampicillin (breakpoint ≥8 mg/L) among the Gram-negative isolates tested. Overall resistance to cephalosporins was high with 22% resistance to cefepime (break point ≥ 2 mg/L) 23% to cefotaxime (breakpoint ≥ 2 mg/L) and 23% to ceftazidime (breakpoint ≥ 4 mg/L) meropenem (breakpoint ≥4 mg/L) and imipenem (breakpoint ≥4 mg/L) were the most active antibiotics against these Gram-negative bacteria. Intrinsic resistance within various bacteria species may produce some doubts; therefore the resistance pattern was determined within each species as seen in Table 6 which show the activities of the antimicrobial agent chosen against the Gram-negative isolates.

Among the *E.coli*, 44/65 were resistant to ampicillin and 42/65 to piperacillin (breakpoint ≥ 16 mg/L). This level of resistance was high and these penicillins were the least effective antimicrobials against these pathogens. The aminoglycosides (kanamycin, spectinomycin and streptomycin) exhibited a slightly higher level of resistance than the cephalosporins tested. The *E. coli* strains resistance to cefotaxime

or ceftazidime were investigated further in this thesis. The carbapenems: meropenem and imipenem, were active against *E. coli* with no resistance to these drugs from any strains of *E.coli* tested and only 2 (3.1%) *E.coli* were resistant to Ertapenem (break point ≥ 1mg/L).

Among the *Klebsiella* spp: 8/12 were resistant to ampicillin and 6/12 were resistant to piperacillin. It is important to note that it is generally accepted that *Klebsiella* spp are intrinsically resistant to ampicillin due to the production of β-lactamase (Fu *et al*. 2007). There was no resistance to the cephalosporins tested. Again there was no resistance to the carbapenems tested among the *Klebsiella* spp.

The *Enterobacter* spp were found to be resistant to the penicillins (ampicillin 75%, piperacillin 83.3%) and most of the antibiotics tested. There were no resistance to the carbapenem, imipenem and meropenem although 50% were found to be resistant to ertapenem. The *Enterobacter* spp resistance to cefotaxime and ceftazidime was investigated further in this thesis. *Enterobacter* spp are known to produce inducible cepharosporinase.

The *M. morganii* were susceptible to all the antibiotics tested. The *S. marcescens* were susceptible to all the antibiotics tested except ampicillin. The *S. maltophilia* were resistant to all the antibiotics tested, although it is known to produce an inducible chromosomal metallo β-lactamases. The *C. pauculus* tested was resistant to all the antibiotics tested

Table 6 Antimicrobial susceptibility pattern of the Gram-negative isolates. Figures show number and percentages resistant

Antibiotics	*E.coli* (n=65) (%)	*Klebsiella* spp (n=12) (%)	*Enterobacter* spp (n=12) (%)	*S. maltiphilia* (n=5)	*Serratia marcescens* (n=3)	*M .morganii* (n=2)	*Cupriavidus pauculus* (n=1)	%resistance
Ampicillin	44 (67.69)	12 (100)	12 (100)	5	3	1	1	78
Aztreonam	8 (12.31)	0	8 (66.7)	5	0	0	1	22
Cefepime	8(12.31)	0	8 (75)	5	0	0	1	22
Gentamicin	8 (12.31)	0	0	4	0	0	1	13
Cefotaxime	9 (13.9)	0	8 (66.7)	5	0	0	1	23
Ceftazidime	9 (13.9)	0	8 (66.7)	5	0	0	1	23
Ertapenem	2 (3.1)	0	6 (50)	5	0	0	1	14
Meropenem	0	0	0	5	0	0	1	6
Imipenem	0	0	0	5	0	0	1	6
Piperacillin	42 (64.62)	9 (75)	10 (83.3)	5	3	0	1	71
Kanamycin	21 (32.31)	0	7 (58.3)	5	0	0	1	34
Co-trimoxazole	32 (49.23)	1 (8.33)	10 (83.3)	5	0	0	0	48
Spectinomycin	17 (26.2)	1(8.33)	9 (75)	5	0	0	1	33
Streptomycin	21 (32.31)	1(8.33)	4(33.33)	5	0	0	1	32

The table show the number of isolates within each species/genus resistant to the antimicrobial agent tested with the percentage of resistant

Table 7 MIC_{50} and MIC_{90} values for *E. coli*

	mg/L										
	SXT	PIP	CAZ	CTX	ATM	GEN	FEP	AMP	MER	IMP	ERT
MIC_{50}	2	16	0.12	0.12	0.12	0.12	0.12	32	0.03	0.06	0.06
MIC_{90}	>128	128	16	64	>128	8	32	>128	0.25	0.12	0.12

Abbreviations: CTX = cefotaxime; CAZ = ceftazidime; FEP = cefepime; PIP = piperacillin; SXT = co-trimoxazole; GEN = gentamicin; ERT = ertapenem; IMI = imipenem; MER = meropenem; ATM=aztreonam; AMP=ampicillin

MIC_{50} and MIC_{90} defined as the antimicrobial concentration that inhibits growth of 50% and 90% respectively of all isolates tested for a particular species of organism. The MIC_{50} and MIC_{90} were calculated for the *E. coli* isolates. This was not calculate for the other species because the sample sizes were too small. Strains with high MICs will have a disproportionately high influence on the MIC_{50} and MIC_{90} values. MIC_{50} and MIC_{90} are shown in table 7.

According to the MIC_{90} the carbapenems (meropenem-0.25; imipenem-0.12 and ertapenem-0.12) were found to be the most active antimicrobial agent followed by the aminoglycoside gentamicin.

3.3 Discussion

The presence of bacteria in the blood of a patient has substantial clinical importance. The goal of this study is to characterise the antibiotic susceptibility profile of the isolated Gram-negative bacteria from blood cultures, which will help in appropriate therapy to increase the chance of survival for patients with such infections. The most frequently isolated Gram-negative species from this study is *E. coli* followed by *Enterobacter* spp and *Klebsiella* spp. *E.coli* is the leading cause of BSI involving Gram-negative bacteria (Weinstein *et al*. 1997) and multi-drug resistant *E. col* , particularly those with ESBLs, are endemic in many healthcare settings. Multidrug resistant organisms are bacteria that are resistant to three or more class of antibiotics. With this definition some of these isolates in this study show the characteristics of multi drug resistance. Analysis of the antibiogram infers that some isolates were ESBL producers, which are marked by resistance to cefotaxime, ceftazidime or aztreonam. They were investigated further in this thesis. It has been shown that ESBLs producers are often associated with multidrug resistance (Dimude and Amyes 2013). Most isolates in this study were sensitive to the carbapenems tested except the *S. maltophilia*, *Enterobacter spp*, two *E.coli* and *Cupriavidus pauculus.* The resistance in *S. maltophilia* could be attributed to the production of two chromosomal L1 and L2 β-lactamases produced by this species. The resistance the *E. coli* to ertapenem is attributed to efflux mechanism or change in outer membrane permeability. These two *E. coli* isolates were not resistant to imipenem and meropenem unlike those isolated in some parts of Europe (Rossolini and Mantengoli 2008). Resistance to ampicillin and piperacillin was recorded amongst *E. coli* isolates. Some multidrug resistance *Enterobacter* spp were also seen in this study. The high rates of ceftazidime resistance among *Enterobacter* spp suggests a high prevalence of

stably derepressed AmpC cephalosporinase-producing strains (Livermore and Woodford 2006), however this was outside the remit of this study.

The susceptibility pattern collected from this study shows that some antibiotics may have very limited usefulness in the treatment of BSIs caused by some of these Gram-negative bacteria; for instance the large numbers of *E. coli* isolates were found to be resistant to ampicillin and piperacillin. *E. coli* has been found to be frequently resistant to penicillins but rarely to this degree (Oteo *et al*. 2005). The carbapenems, imipenem and meropenem, stand out as the most potent agents for treatment of BSIs caused by these Gram-negative bacteria; however the use of these drugs will inevitably make bacteria resistant to this class of antibiotics.

4 Molecular diversity associated with the dissemination of CTX-M-15 β-lactamase gene in blood culture isolates of *Eschericia coli* from Edinburgh

4.1 Introduction

CTX-M extended-spectrum β-lactamases (ESBLs) are now widely distributed in multiresistant *Enterobacteriaceae* worldwide (Bonnet 2004, Nicolas-Chanoine *et al*. 2008, Woodford *et al*. 2004). In *E.coli*, the ST131 clone is mainly responsible for carrying plasmid-encoded CTX-M-15 β-lactamse gene (Coque *et al*. 2008b, Woodford *et al*. 2009). In England, the CTX-M β-lactamase was first identified in a *Klebsiella* isolate in 2000 (Alobwede *et al*. 2003), followed by an outbreak in a Birmingham Hospital (Brenwald *et al*. 2003). Since then, the CTX-M-15 β-lactamases rapidly became the most prominent CTX-M enzyme in England (Mushtaq *et al*. 2003).

Previous studies in our laboratory on *Klebsiella* spp isolated in Edinburgh showed five different clones carrying the $bla_{\text{CTX-M-15}}$ gene (Younes *et al*. 2011), indicating that plasmid transfer was likely to be an important agent of Horizontal Gene Transfer (HGT). The PCR-based replicon typing (PBRT) can identify the relationship between different plasmids and the epidemiology of HGT (Carattoli *et al*. 2005). By this technique it has been shown that plasmids of the IncF (FIA, FIB, FIC, FII) incompatibility family are the most widely disseminated and are commonly found in *E. coli* (Johnson *et al*. 2007, Marcadé *et al*. 2009). Furthermore individual *E coli* clones, such as ST131, have co-linkage of the $bla_{\text{CTX-M-15}}$ with other β-lactamase

genes such as *bla*$_{TEM-1}$, *bla*$_{OXA-1}$ and the fluoroquinolone resistance gene *aac(6')-Ib-cr* (Karisik *et al*. 2006, Coque *et al*. 2008b, Woodford *et al*. 2009).

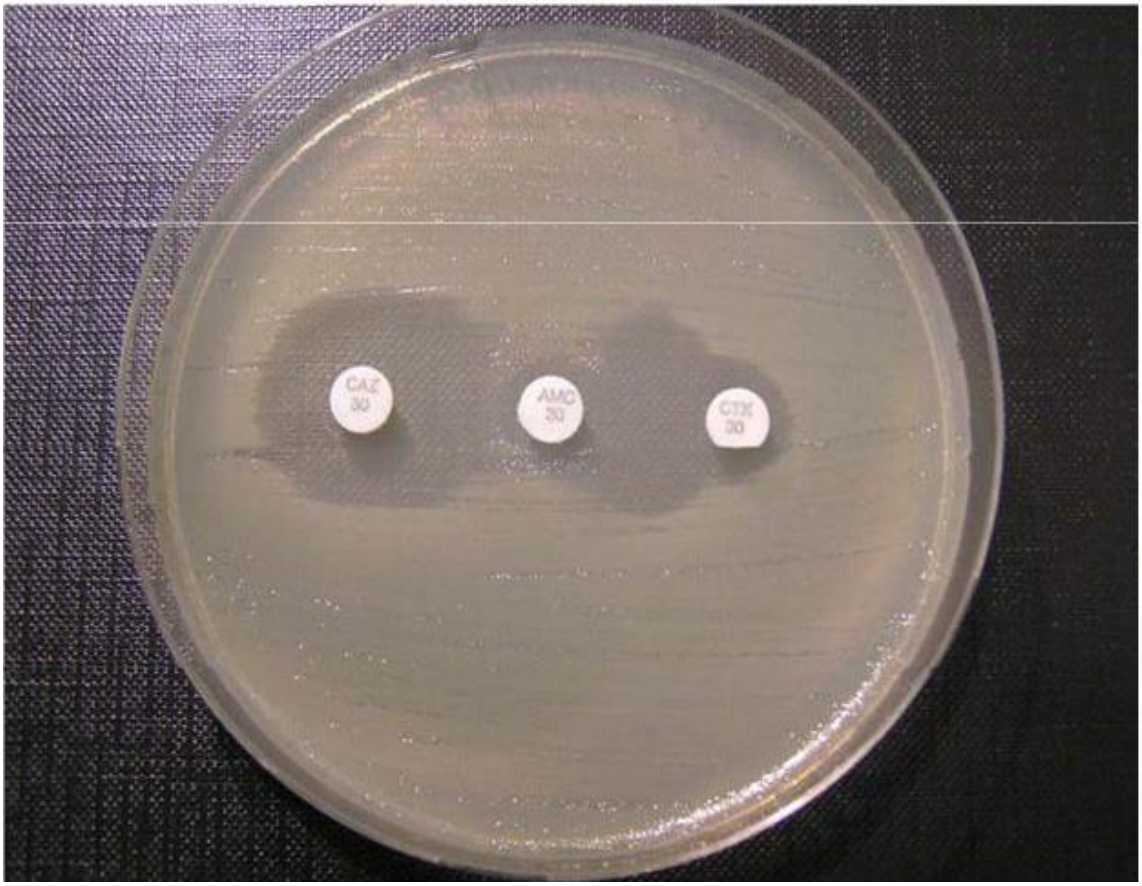
Therefore, to examine the fluidity of the *bla*$_{CTX-M-15}$ in a major Scottish teaching hospital, I have identified the plasmid incompatibility group, replicon sequence type and the total plasmid content of each of the strains carrying the *bla*$_{CTX-M-15.}$ gene. I assessed the clonal relationship between the isolates and characterised the genetic environment of the *bla*$_{CTX-M-15}$ gene to determine whether these genes derived from the same source and tracked their mobility.

4.2 Results

4.2.1 Susceptibility profile and characterisation of the resistance genes

Seven *E. coli* and six *E. cloacae* strains were positive with double disc and combination disc tests, characteristic for the carriage of ESBLs. This is detected by clavulanic acid synergy with either cefotaxime and/or ceftazidime as shown in figure 7. For the purpose of this chapter, only seven *E. coli* isolates will be discussed. All seven *E. coli* strains were found to be multidrug resistant. These isolates were resistant to all antibiotics tested with MIC of ≥128mg/L for ampicillin, piperacillin, co-trimoxazole, nalidixic acid and aztreonam. The MICs for the remaining antibiotics are in Table 8.

Figure 7: Double disc test showing clavulanic acid synergy with cefotaxime and ceftazidime.



The clavulanic acid containing disc in the middle is inhibiting the β-lactamase, thus causing a bowing of the zone,identified as synergy.

Table 8 MIC of antibiotics for the *E.coli* clinical isolates harbouring ESBLs

Isolate No	MIC (mg/l)							
	CTX	CAZ	FEP	FOX	CPZR	CXM	CIP	GEN
35	128	32	32	4	>128	>128	64	8
58	>128	64	64	8	>128	>128	>128	64
71	>128	64	64	8	>128	>128	>128	64
202	64	16	16	16	128	128	>128	8
270	>128	128	64	8	>128	>128	>128	64
274	>128	128	>128	8	>128	>128	>128	64
280	>128	128	128	8	>128	>128	>128	64

Abbreviations: CTX=cefotaxime; CAZ=ceftazidime; FEP=cefepime; FOX=cefoxitin; CPZR=cefoperazone; CXM=cefuroxime; CIP=ciprofloxacin; GEN=gentamicin

The table show the resistance pattern of the *E. coli* CTX-M-15 containing isolates

A multiplex PCR was done to detect the CTX-M groups of the isolates with ESBLs characteristics. $bla_{\text{CTX-M}}$ genes were detected in all seven of the *E. coli* isolates and six *E. cloacae* isolates by the multiplex PCR. Figure 8 shows an example gel with results of multiplex PCR showing the presence of CTX-M-1 groups and CTX-M-9 group.

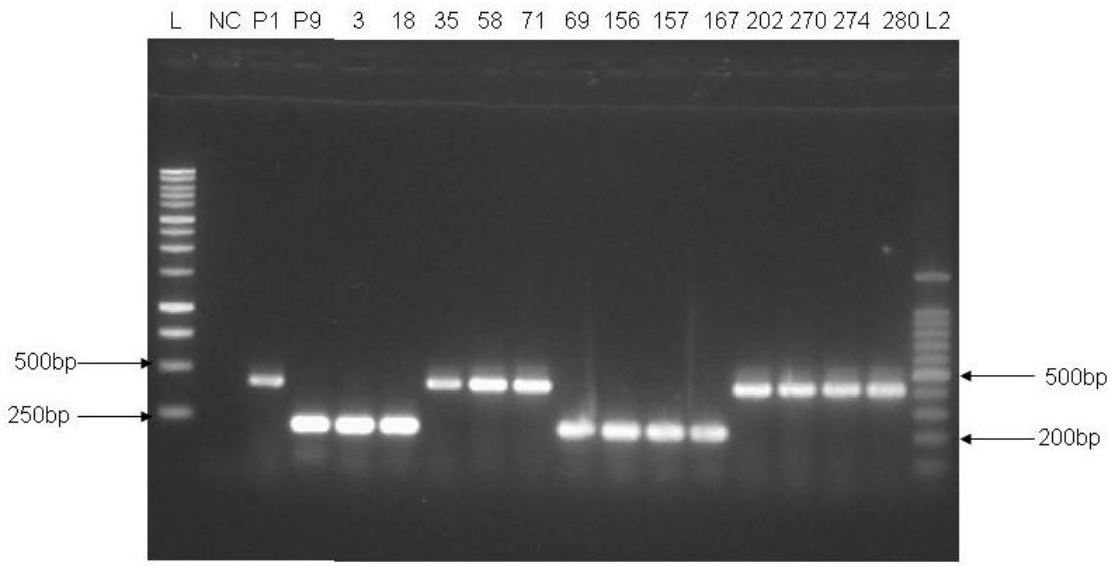


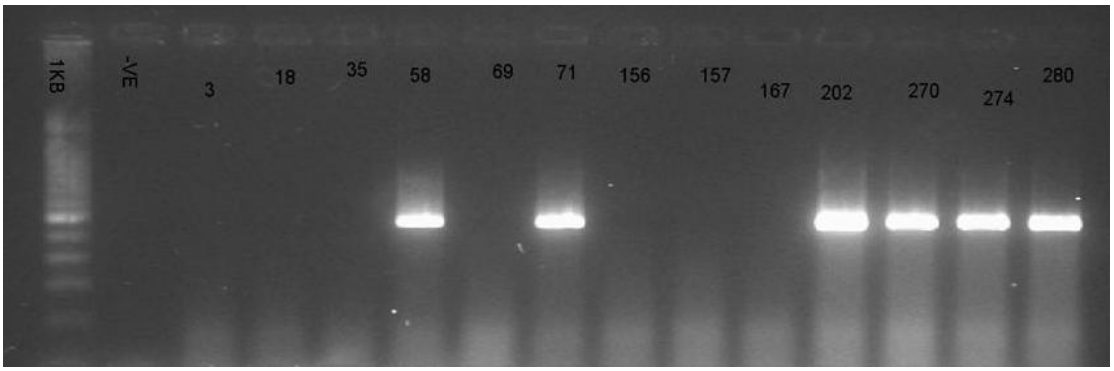
Figure 8 Agarose Gel Electrophoresis of a multiplex PCR shows CTX-M group 1 and 9 bands (L1-Molecular Weight marker 1kb, L2-Molecular weight Marker 100bp, NC-Negative control, P1-Positive group 1 control =415bp, P9-Positive group 9 control =205bp

The gel show a multiplex PCR for the detection of CTX-M group 1 and 9

For the purpose of this chapter only the six *E. coli* isolates showing positive for CTX-M-1 group will be discussed. The whole gene was amplified by PCR and when sequenced, all the isolates were confirmed of having $bla_{\text{CTX-M-15}}$ by comparison of the sequence in BLAST software of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

All the isolates, but one, were found to possess the gene for the OXA-1 β-lactamase and fluoroquinolone acetylating enzyme (*aac(6')-Ib*) when amplified by PCR. The PCR gel showing the 482 product base pair detection of *aac(6')-Ib* gene is as shown in figure 9.

Figure 9 PCR for *aac(6')-Ib-cr* gene



aac(6')-Ib-cr gene = 482bp

The band on the PCR gel was measured at 482bp and shows the detection of *aac(6')-Ib-cr* gene in the six *E.coli* isolates which confers resistance to fluoroquinolones

Upon sequencing, the *aac(6')-Ib* variant was found to be *aac(6')-Ib-cr.* The cr in *aac(6')-Ib-cr* means ciprofloxacin (fluoroquinolone) resistance showing that this variant can induce resistance against fluoroquinolones.

TEM ESBL sort by PCR was found in five isolates as shown in the representative gel in figure 10. The TEM primers amplified a 1080 product base pair. The sequence analysis confirmed the gene to be TEM-1

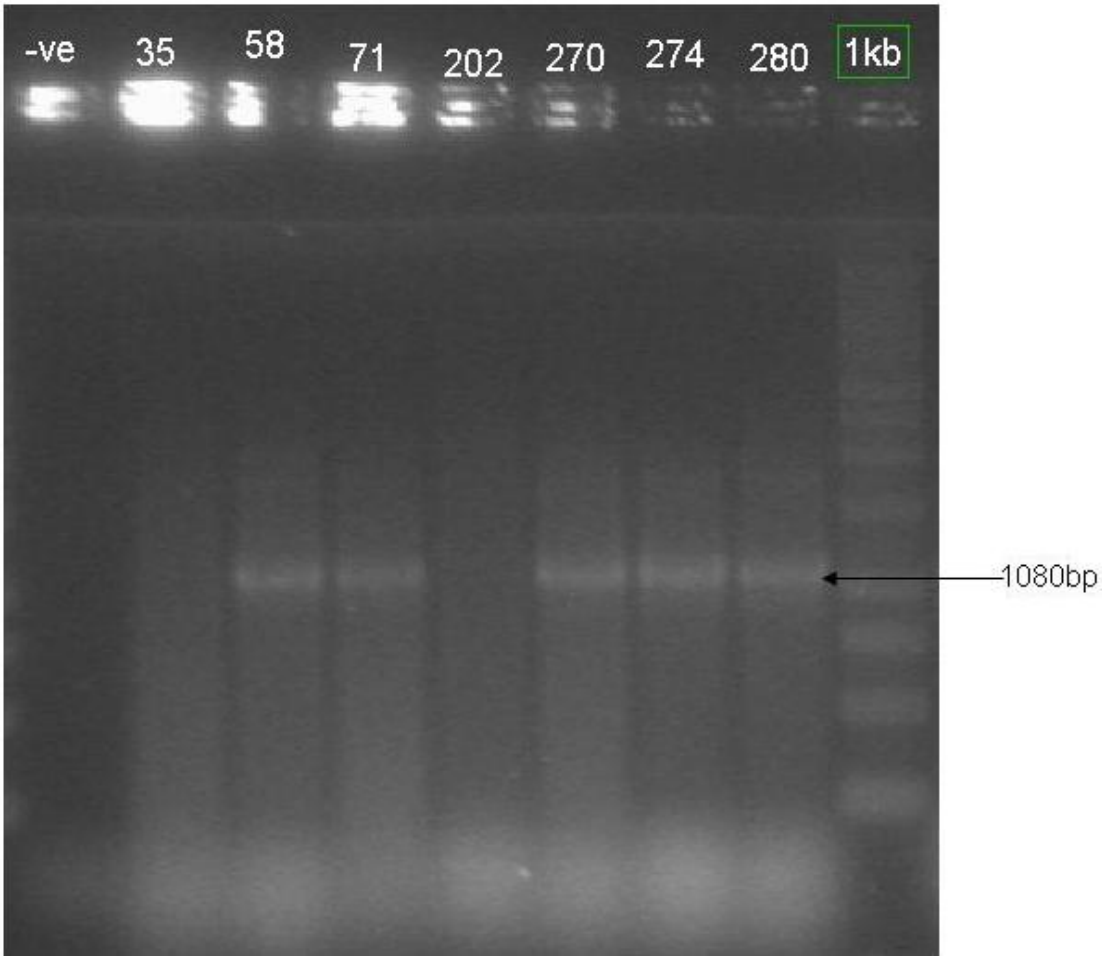


Figure 10 Representative TEM PCR gel **TEM = 1080bp**

The PCR show the detection of TEM-1 ESBL in five of the *E.coli* isolates.

The summary of the characteristics of each isolate is as summarised in table 9 showing different resistance genes identified.

Table 9 Resistance genes and their genetic location and environment

Isolate No	β-lactamase genes				*Int1*	*Sul1*	*Sul2*	Genetic environment of *bla* CTX-M-15		Incompatibility Group(Inc)	FAB Formula*	Conjugation
	CTX-M-15	TEM-1	OXA-1	aac(6')-ib-cr				Upstream	Downstream			
35	+	-	-	-	+	+	-	*IS26,IsEcp1*	*IS*26	FIA,N,FII	F2:A1:B-	-
58	+	+	+	+	-	+	+	*ISEcp1*	*ORF*477	FIB,FIA,N,FII	F33:A1:B26	+
71	+	+	+	+	-	+	+	*ISEcp1*	*ORF*477	FIB,FIA,N,FII	F33:A1:B26	+
202	+	-	+	+	+	+	-	*IS26,ISEcp1*	*ORF*477	FIA,N,FII	F2:A1:B-	+
270	+	+	+	+	-	+	+	*ISEcp1*	*ORF*477	FIB,FIA,N,FII	F33:A1:B26	+
274	+	+	+	+	-	+	+	*ISEcp1*	*ORF*477	FIB,FIA,N,FII	F33:A1:B26	+
280	+	+	+	+	-	+	+	*ISEcp1*	*ORF*477	FIB,FIA,N,FII	F33:A1:B26	+

* The replicon sequence types of the IncF plasmid were analysed for genes encoding replicons FIA, FIB, and FII following the protocol at http://pubmlst.org/plasmid/

The table show the genes identified in each *E. coli* isolate and the genetic environment of the CTX-M-15 gene. The result of the plasmid replicon typing for Incopatibility group show the detection of FIB,FIA,FII and N plasmids.

..

4.2.2 Conjugation

Conjugative transfers of the resistant determinant (cefotaxime resistance) of *bla*$_{\text{CTX-M-15}}$ into *E. coli* J62-2 strain were achieved in six out of the seven isolates; the final strain would not transfer *bla*$_{\text{CTX-M-15}}$ despite several attempts (Table 9).

4.2.3 Plasmid Profiling

Two different methods were used to analyse the plasmid content: plasmid extraction followed by S1 nuclease plasmid PFGE and replicon sequence typing. S1 nuclease PFGE and plasmid extraction revealed that all the isolates contain detectable plasmid DNA. Four of the isolates (58, 71, 274, 280) possessed seven plasmids, one isolate (270) had eight plasmids and two isolates (35, 202) had four plasmids. The gel picture of the plasmid profile is in the appendix. The plasmid sizes ranged from ~3kb to 300kb. After the conjugation experiment, two plasmids were transferred from five isolates and three plasmids transferred from one isolate (figure 11).

The transferred plasmids were cut out from the gel, extracted and cleaned. This was used as a DNA template to determine which plasmid carried the *bla* $_{\text{CTX-M-15}}$ gene. After performing PCR on all the extracted plasmids, the *bla*$_{\text{CTX-M-15}}$ was found to be located on ~260kb plasmids in all the transconjugants.

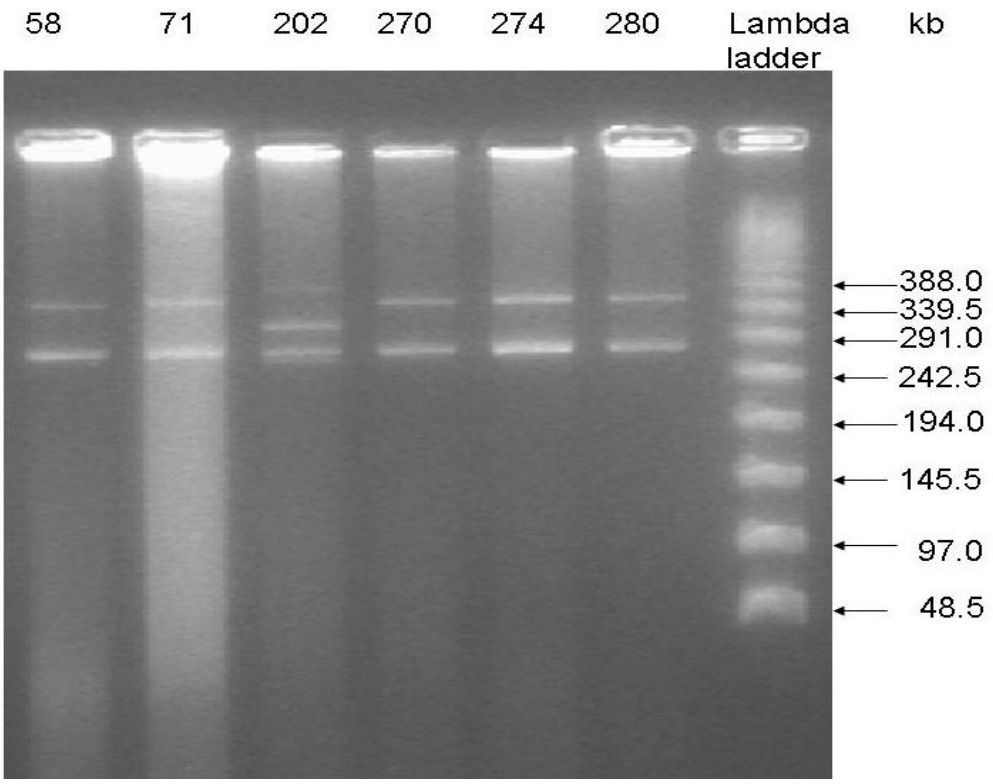


Figure 11 Plasmid profile of the transconjugants

The gel show the transfered plasmid with five of the *E. coli* having two plasmid and one isolate having three plasmid. One of the *E. coli* isolate could not transfer plasmid.

PCR was used for the amplification of the gene encoding replicons IncFIA (product 462 bp), IncFIB (product 683 bp), IncFII (product 262 bp) and IncN (product 559 bp). IncFIC was not detected in any of my isolates. The replicon typing of the isolates revealed IncFIA (n=7), IncFIB (n=5), IncFII (n=7) and IncN (n=7) as shown in Table 9. The representative PCR gel is as shown in figure 12. The PCR products were sequenced.

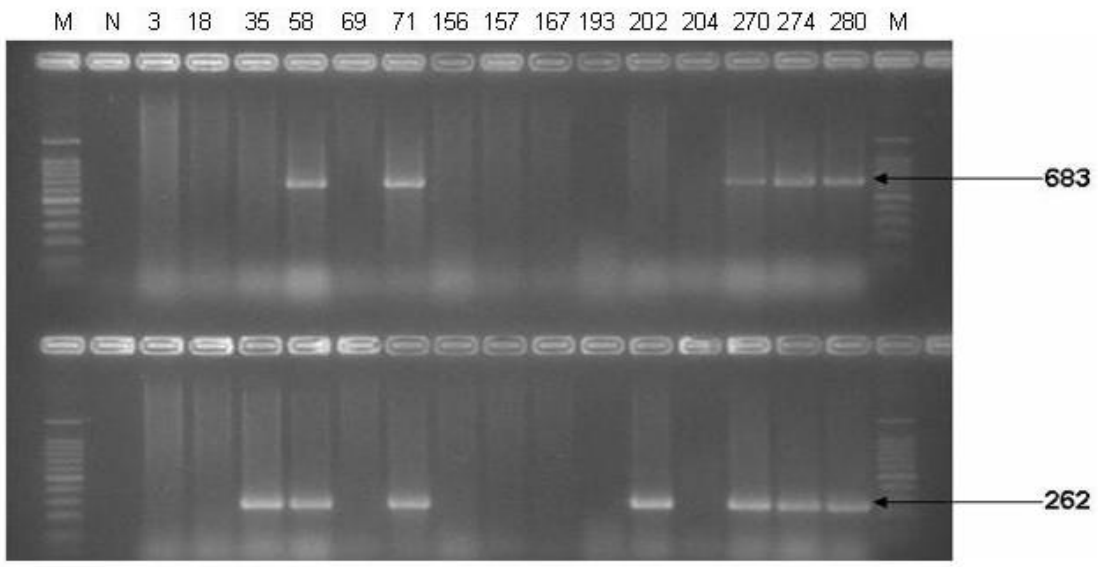


Figure 12 PCR of IncFIB up and Inc FII down showing the presence of the plasmid replicons.

M = 100bp ladder; N = Negative control; Inc FIB = 683bp; Inc FII = 262bp

The PCR show the detection of the Inc FII and Inc FIB in the *E. coli* isolates

Sequence analysis of the IncF amplicons was done using software on the website - http://pubmlst.org/plasmid. Within the IncFII replicon, F2 and F33 alleles were identified in two and five isolates respectively. The sequence of the F2 and F33 allele were compared on the multialin website (http://multalin.toulouse.inra.fr/multalin/) to show the nucleotide difference.

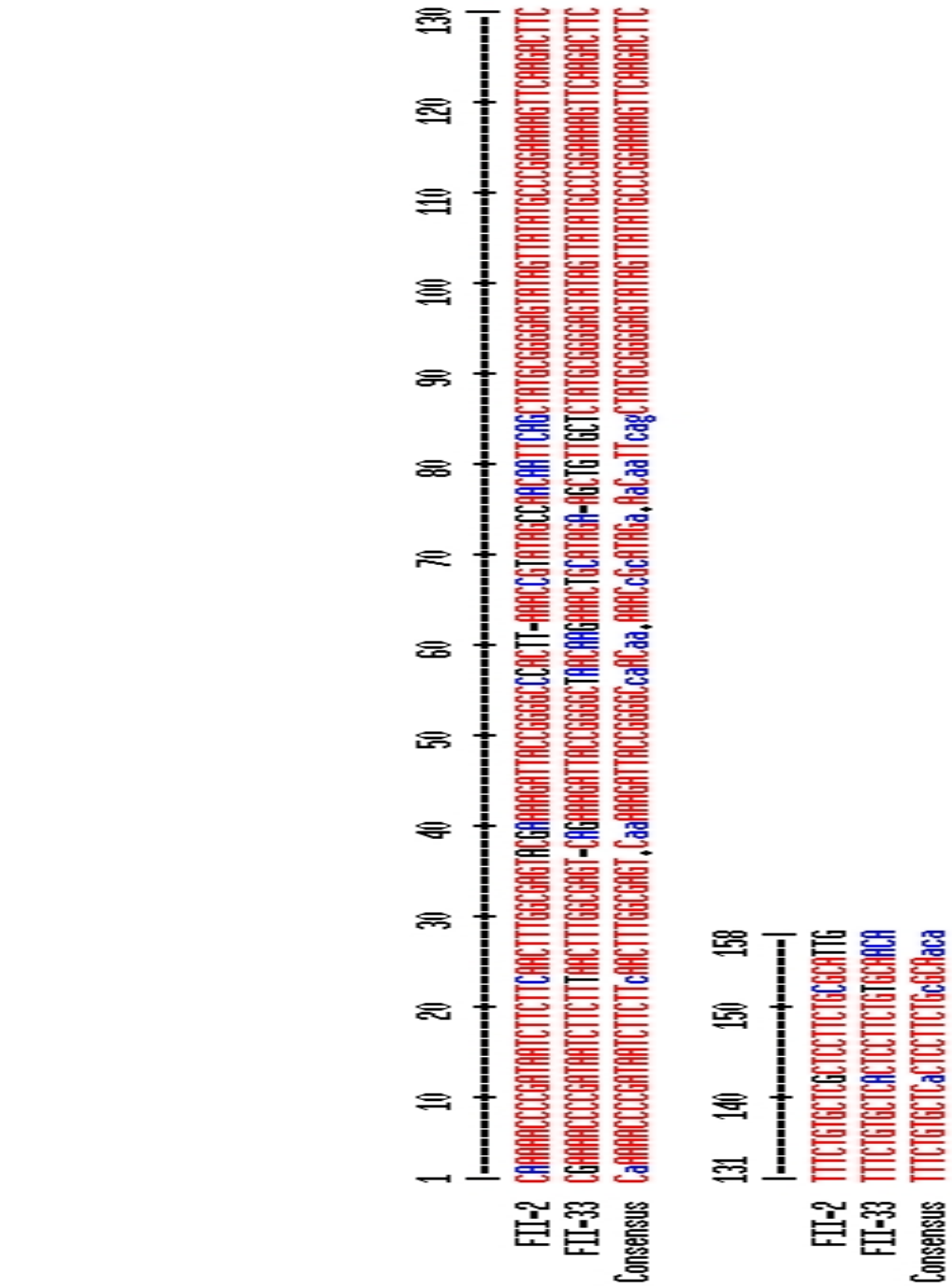


Figure 13 Sequence analyses of IncFII alleles

The sequence show the differences in nucleotide between FII-2 and FII-33.

The difference in nucleotide sequence between F2 and F33 can be seen in the alignment in figure 13.

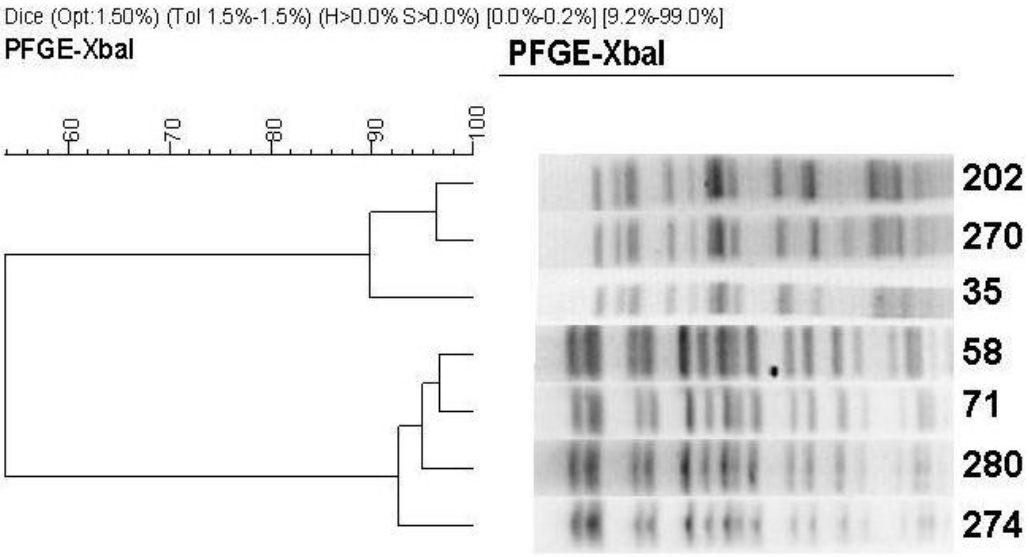
Within the FIA replicon, only the A1 allele was identified in all isolates. Also within FIB replicon, only B26 was identified in five isolates. All the sequences of the IncF replicon alleles found in this study can be found in the appendix.

IncF replicon sequence typing was now arranged using the FAB (FII FIA FIB) formulae discriminating IncF plasmid variants according to the allele type and number identified for each replicon. This shows two types; F2:A1: B- plasmid was detected in two of the isolates while F33:A1:B26 was detected in the remaining five isolates (Table 9).

4.2.4 PFGE, ST131 determination and phylogenetic grouping

PFGE showed ≥85% similarity was observed in four isolates (58, 71,274,280), but all the others were more diverse, though related, as shown in Figure 14. Isolates 58, 71, 280, and 270 were found to be in one cluster with ≥92% similarity. Isolates 202, 270, and 35 were found to be in a different cluster with ≥90 similarity among them.

Figure 14 Pulsed-field gel electrophoresis of isolates harbouring the *bla*$_{\text{CTX-M-15}}$ gene



The figure show the PFGE dendogram showing the genetic relatedness among the CTX-M-15 isolates.

The phylogenetic analysis was determined by a triplex PCR assay using a combination of three DNA markers (*chuA, yjaA* and *TspE4C2*). The PCR revealed

that all the isolates were positive for *chuA* (279 product bp), *yjaA* (211 product bp) and *TspE4C2* (152 product bp) as shown in figure 15.

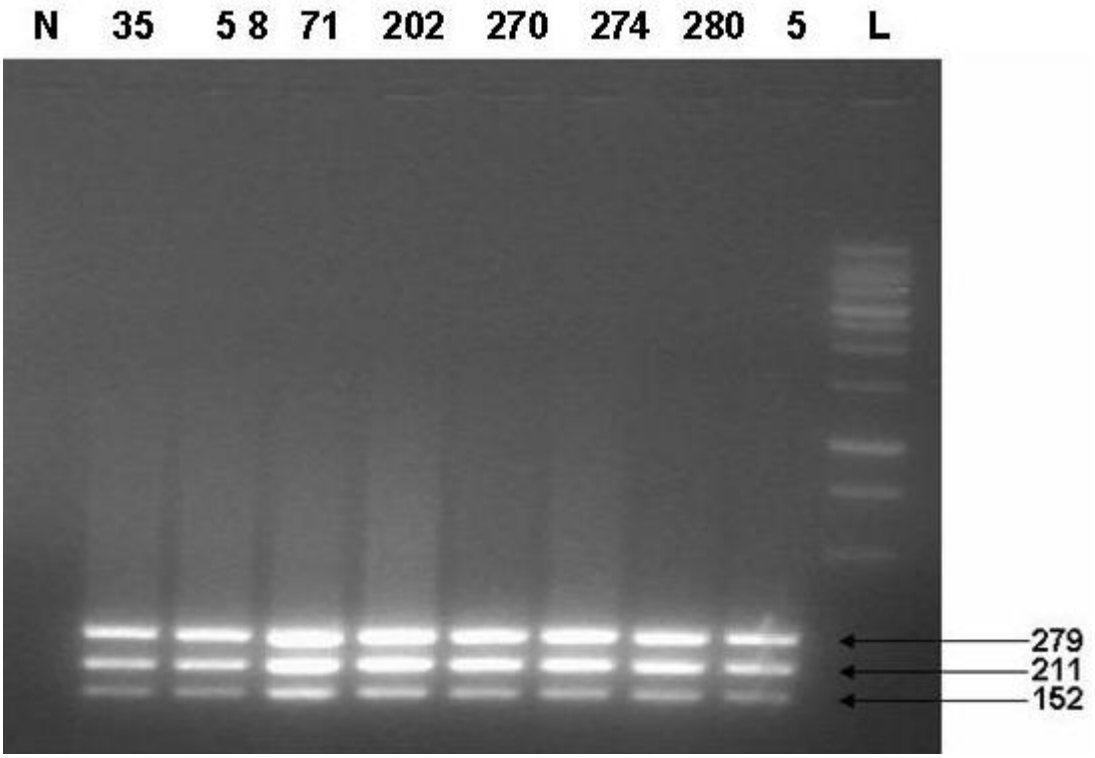
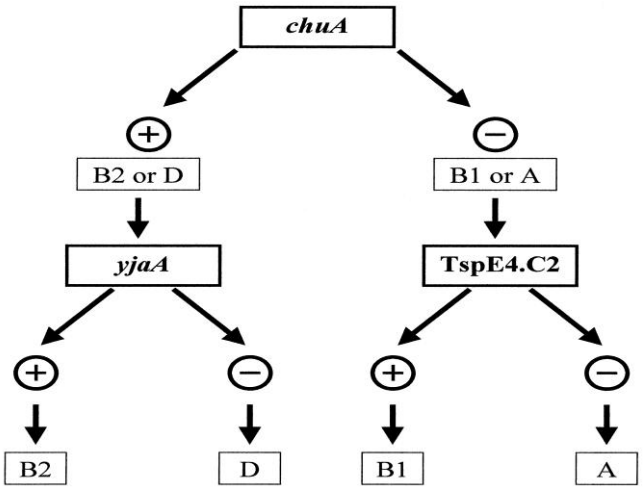


Figure 15 Triplex PCR showing Phylogenetic grouping N=negetive control L=1kb ladder

The PCR show the isolates were positive for the DNA markers and were used to detect the phylogenetic group.

The decision tree below was used to determine the phylogenetic group of the *E.coli* by using the result of the PCR amplification above.

Figure 16 Decision tree used to determine the phylogenetic group.



From the analysis using the tree in figure 16 above, all the isolates belong to the phylogenetic group B2.

PCR with 025pabBspe primers was used to amplify a 347bp fragment of the *pabB* gene. This fragment on the gene is specifically found in isolates belonging to the 025b-ST131 clone.

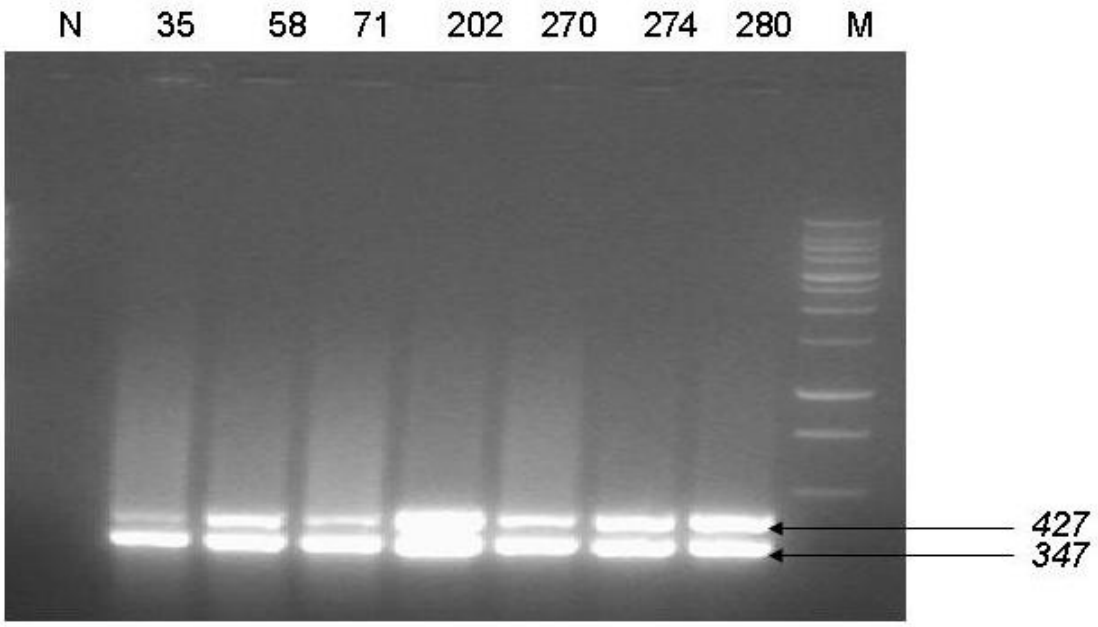


Figure 17 PCR detection of the 025b-ST131 clone

N = negative control; M = ladder; *pabB* =347; *trpA* =427

The PCR gel show the detection of the gene fragment specifically associated wit ST131 clone.

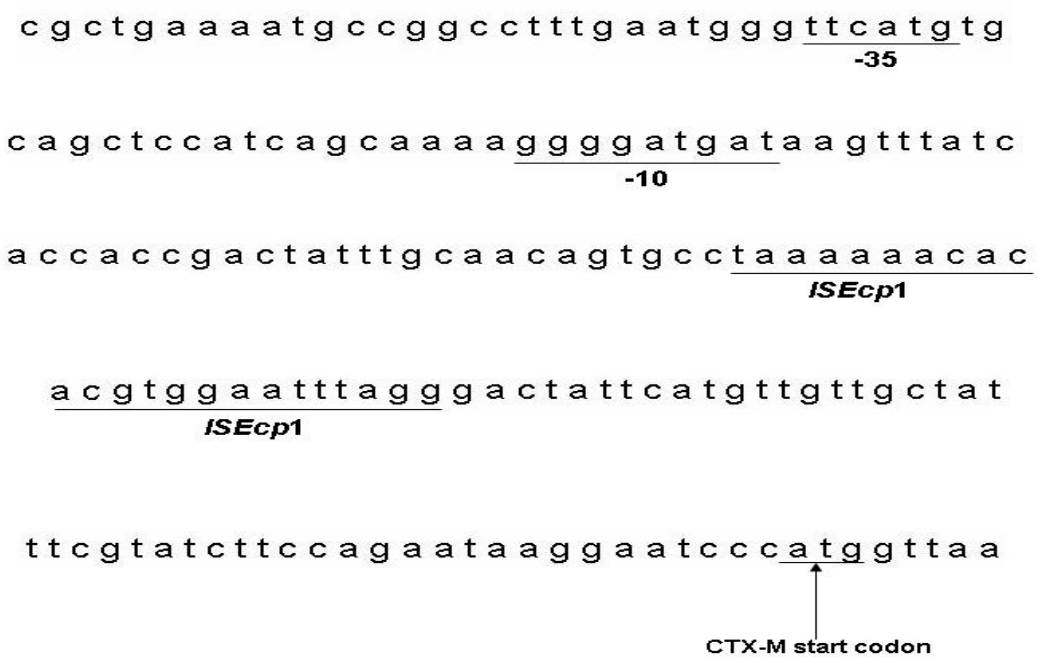
The control for this PCR targeted a 427bp fragment of the *trpA* gene to confirm that any amplification failure with the *pabB* was not due to poor DNA quality or failure of the PCR itself. This allele specific PCR discriminating the O25b-ST131 were positive for single nucleotide polymorphisms in the *pabB* gene for all seven isolates.

4.2.5 Genetic Environment of *bla*$_{\text{CTX-M-15}}$

PCR amplification and subsequent sequencing identified the Insertion sequence IS*Ecp1* upstream of *bla*$_{\text{CTX-M-15}}$ in five of the isolates while, in two isolates, IS*Ecp1* was truncated with IS*26.* The diagram in Figure 20 shows the arrangement of the

upstream region found in this study. Analysis of the sequence of the two isolates with *IS*26 truncation shows the putative -35 and -10 promoters located within the *IS*26 with the -35 (t t c a t g) and -10 (g g g g a t g a t) lying 140bp and 115bp respectively upstream of the *bla*$_{\text{CTX-M-15}}$ start codon. The locations of these putative promoters are shown in figure 18. Also 48bp intergenic region was found between the IS*Ecp1*and *bla*$_{\text{CTX-M-15.}}$

Figure 18 Sequence showing the promoter region



In the other five isolates, the typical promoters regions – 35 TTGAAA and -10 TACAAT were found within the 3' terminus of the insertion sequence IS*Ecp1*.

The PCR amplification of downstream region for *orf*477 showed a PCR product band in isolate 35 higher than the others as seen in figure 19. The sequences were subsequently analysed and revealed *orf*477 in all the isolate but one.

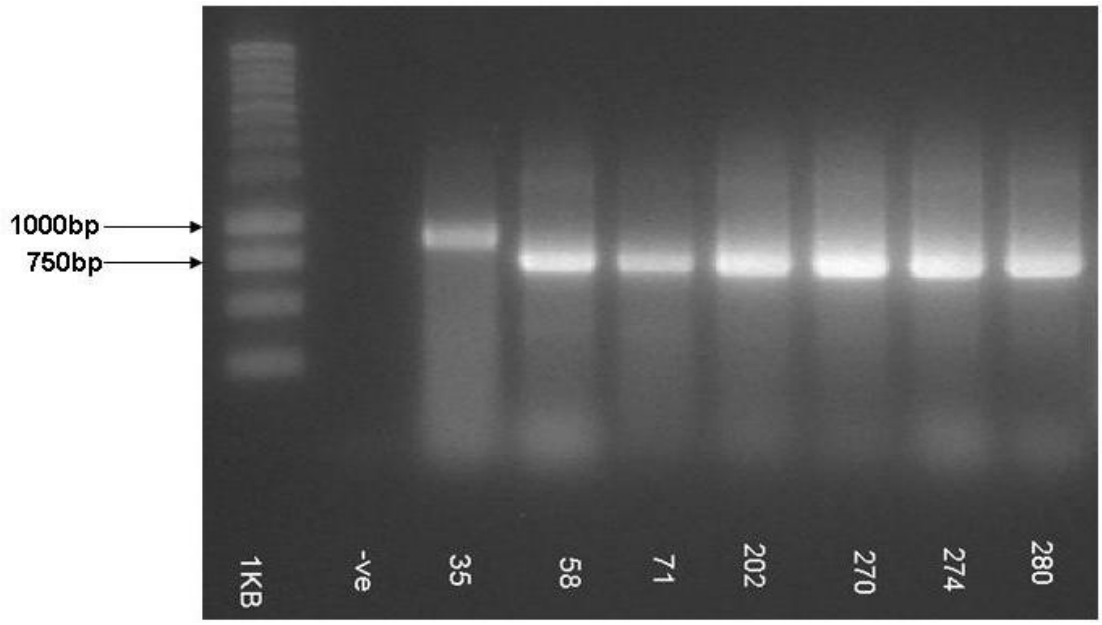


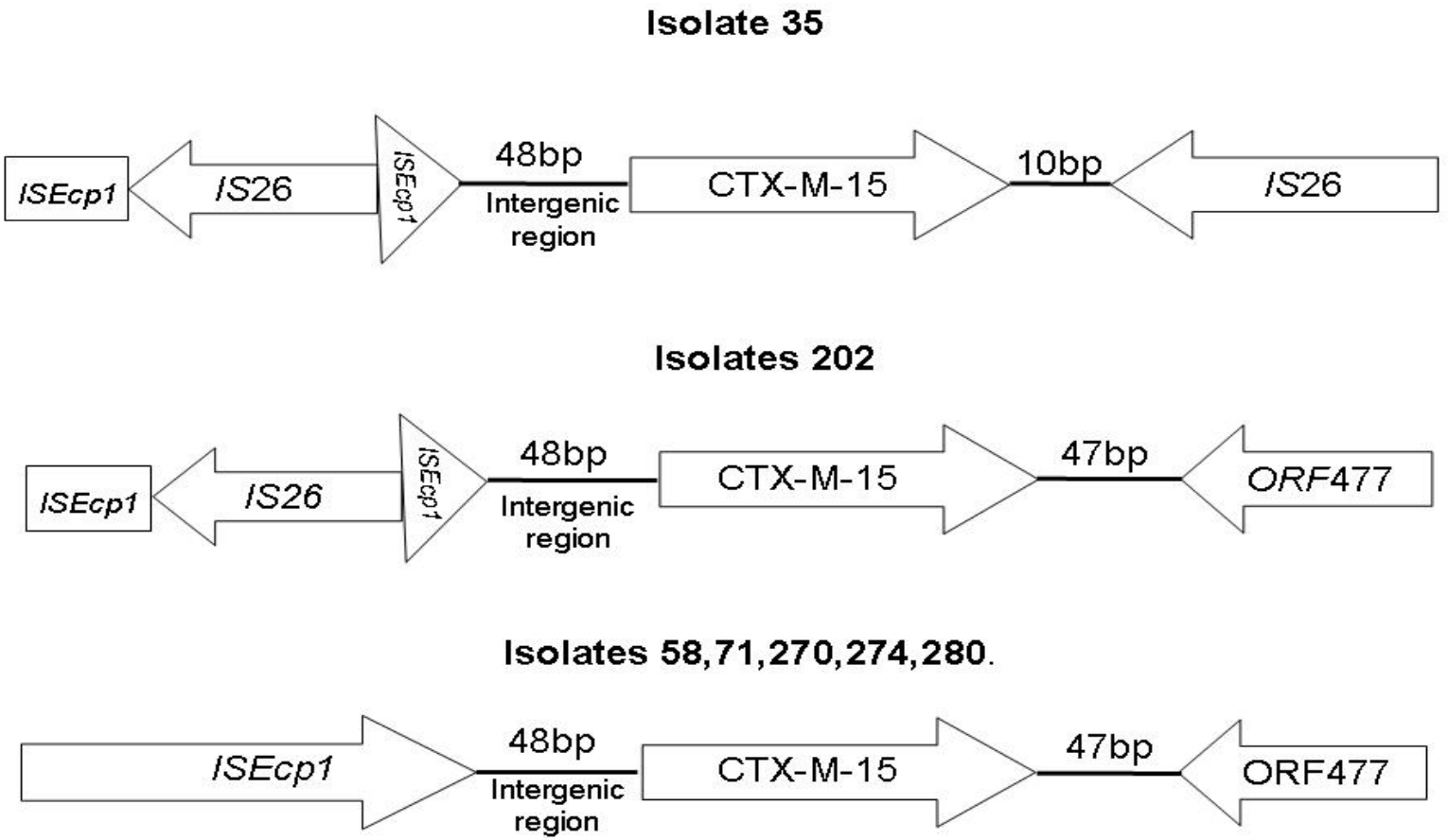
Figure 19 PCR amplification gel for downstream *bla*$_{\text{CTX-M-15}}$.

The PCR gel show the detection of the downstream region of the CTX-M-15 for the *orf*477. The PCR product was sequenced to analyse the gene.

The sequence showed a 47bp intergenic region between the *bla*$_{\text{CTX-M-15}}$ and *orf*477 in all the isolates but that same one. In isolate 35 the insertion sequence *IS*26 was found downstream of the *bla*$_{\text{CTX-M-15}}$ as opposed to the *orf*477 found in the other isolates. There is a 10bp intergenic region between the *bla*$_{\text{CTX-M-15}}$ and *IS*26

The general impression of the genetic environment upstream and downstream of *bla*$_{\text{CTX-M-15}}$ is as shown in Figure 20.

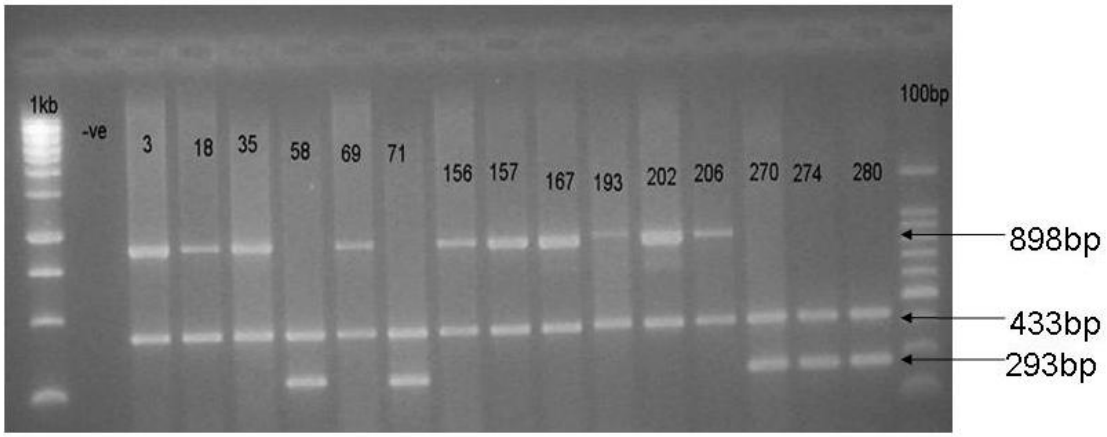
Figure 20 Alignment of ISEcp1 and IS26 upstream and *orf*477/*IS*26 down stream of *bla*$_{CTX-M-15}$



The figure show the genetic arrangement surrounding the CTX-M-15 gene with *ISecp*1/*IS*26 upstream of the gene and orf 477 downstream of the gene

PCR-based amplification examination of integron shows an 898bp amplicon in two (202, 35) out of the seven isolates for the *intI1* integrase gene (Table 9). The PCR gel representation of the PCR is as shown in figure 21. The *intI1* integrase gene was confirmed by sequencing and analysis on BLAST software.

Figure 21 PCR Gel Showing *intI1*, *sul1* and *sul2*



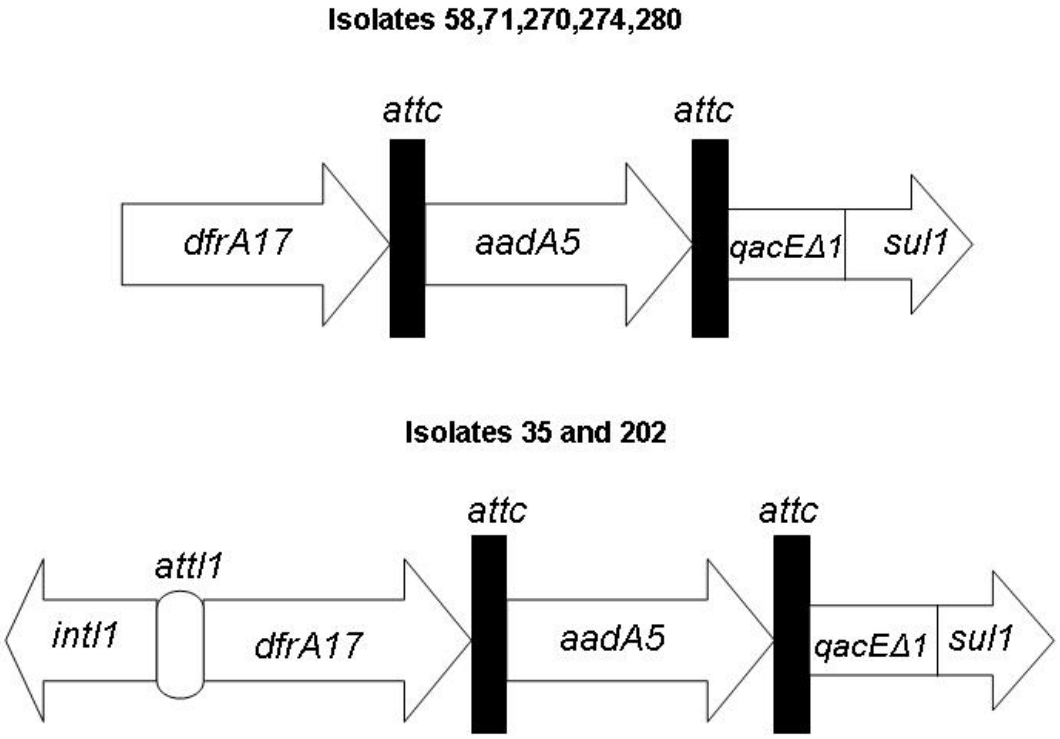
The PCR gel show a PCR for the detection of the *intI1(898bp), sul1*(433bp*)and sul2*(293bp) of the integron.

None of the isolates showed an amplification product with the *intI2* and *intI3* primers. Also *sul1* gene which is part of class 1 integron showed a 433bp product size as seen on the PCR gel in figure 21 and was found in all the isolates. The *sul2* gene was present showing a 293bp amplicon in five of the isolates. Furthermore, none of the isolates showed an amplification product with the *sul 3* primer. The *sul* genes were confirmed by sequencing and analysis on BLAST software.

The PCR for the detection of the variable region were done using the hep 58 and hep 59 primers and the amplicon sequenced. Upon examination of the sequence for the variable region containing the gene cassette, I found the *dfrA17* gene encoding a

trimethoprim-resistant dihydrofolate reductase and an *aadA5* gene encoding aminoglycoside-3-adenyltransferase in all isolates. This was also done using BLAST software of NCBI. The four isolated that do not have the *intI1* integrase gene was found to have this gene cassette which is of interest. The diagrammatic representation of the variable region showing the structural organisation of the gene cassette is shown in the figure 22.

Figure 22 The structural organisation of the gene cassette.



The class 1 integron show the *Intl* integrase with the associated gene cassette of *dfrA*17 and *aad*A5

4.2.6 Discussion

CTX-M β-lactamase penetrated and became prevalent in the United Kingdom displacing the SHV and TEM ESBL variants. This study provides data on CTX-M-15 carrying *E. coli* in blood culture isolates from Edinburgh. CTX-M-15 was first isolated in India (Karim *et al*. 2001) and emerged in the UK in 2001 (Mushtaq *et al*. 2003). All isolates belonged to phylogenetic group B2, often associated with extra-intestinal infection. CTX-M-15 producing *E. coli* from clinical isolates are often found to belong to phylogenetic group B2 (Machado *et al*. 2006, Pitout *et al*. 2005, Leflon-Guibout *et al*. 2004). Analysis of the resistance determinants shows the presence of $bla_{\text{CTX-M-15}}$ co-existed with bla_{TEM}, $bla_{\text{OXA-1}}$ and *aac(6')-Ib-cr* genes. The *aac(6')-Ib-cr* variant is known to induce resistance to fluoroquinolones and can be seen in this study,were isolates confer resistance to ciprofloxacin and nalidixic acid. Similar associations have been described in isolates from Europe and North America (Karisik *et al*. 2006, Boyd *et al*. 2004, Machado *et al*. 2006) but not, until now, in Scotland.

My analysis found that all four of these isolates were closely related if not identical and that all seven isolates were associated with the 025b-ST131 clone. The 025b-ST131 clone has been responsible for much of the dispersal of CTX-M-15 across the world (Nicolas-Chanoine *et al*. 2008); however, the strains described in this study were not all identical. The *aac(6')-Ib-cr* gene variant has been reported to be confined in *E. coli* ST131 and has therefore been linked to CTX-M-15 positive isolates (Coque *et al*. 2008a, Pomba *et al*. 2009). The ST131 *E. coli* clone has spread rapidly, probably as it may have a fitness advantage because of the group B2 genomic

backbone (Johnson *et al*. 2010) that is closely associated with extraintestinal infection in *E. coli* . The clone appears to combine resistance and virulence, which in *E. coli*, have often been somewhat mutually exclusive (Johnson *et al*. 2003).

This study shows the individual dissemination of the $bla_{\text{CTX-M-15}}$ within the microcosm of a major Scottish Teaching hospital. Four isolates had ≥85% similarity; the remaining three isolates 202, 270, and 35 were more closely related to each other than to the previous four. The PFGE results show the ST131 clone divides into multiple genotypes. Genomic diversification can occur within the ST131 lineage reflecting DNA rearrangement, mutation and integration of insertion sequence and other genetic elements (Lau *et al*. 2008a). The PFGE results show that the ST131 clone can be further differentiated into diverse genotypic groups. The use of sequence type (ST) to identify a clone appears to be questionable because of the variation in the PFGE. Therefore the use of PFGE is still needed and looks more dependable in the identification of a clone and helps in the study of clonal spread.

$bla_{\text{CTX-M-15}}$ genes in our isolates were found to be associated upstream with an IS*Ecp1* and two of our isolates has IS*Ecp1* truncated with a copy of IS*26* (Figure 20). IS*Ecp1* may provide high level expression promoter for the $bla_{\text{CTX-M-15}}$ (Poirel *et al*. 2005) and has been shown to mediate the transposition of $bla_{\text{CTX-M}}$ genes from the chromosome of *Kluyvera ascorbata* to plasmids (Lartigue *et al*. 2006). IS*Ecp1* identified upstream of $bla_{\text{CTX-M}}$ genes plays a vital role in the expression and mobilisation of this gene (Poirel *et al*. 2003, Eckert *et al*. 2006, Saladin *et al*. 2002). The truncating of the IS*Ecp1*insertion sequence may inhibit the mobilisation of this gene and confine its location to the specific genotype. IS*Ecp1* is a member of the

IS*1380* family and was first observed in the plasmid pST01 in *E. coli* (AJ242809). The *orf*477 was found downstream of the CTX-M-15 gene with one isolate having the IS*26* element downstream.

Integrons promote the dissemination of antibiotic resistance genes. Since *sul1* is located on class 1 integron, isolates with *sul1* should carry the associated integrase gene *intI1* but unusually this was not found in my study. An interesting observation was the presence of gene cassettes in isolates without *intI1*. The result correlates with the previous finding of a plasmid with a truncated *intI1* gene which could give similar results (Klockgether *et al*. 2004). Similarly, in our study the presence of *dfr* and *aadA* was the combination most frequently detected (Vinué *et al*. 2008).

In this investigation, an IncN plasmid was always present with IncFII when $bla_{\text{CTX-M-15}}$ was identified. Although IncN plasmids have been shown to carry $bla_{\text{CTX-M-15}}$ genes in *E. coli*, spread of this gene has been assumed to be related to IncFII plasmid (Coque *et al*. 2008b, Boyd *et al*. 2004, Hopkins *et al*. 2006, Novais *et al*. 2007). This was also true in our study. The IncFII plasmid carrying $bla_{\text{CTX-M-15}}$ has been shown also to carry *aac(6')-Ib-cr* gene and $bla_{\text{OXA-1}}$ in studies around the world (Hopkins *et al*. 2006, Lavollay *et al*. 2006). As in my study, $bla_{\text{CTX-M-15}}$ has been found previously on F2:A1:B- plasmid from England (Villa *et al*. 2010) but to my knowledge this is the first report of F33:A1:B26 plasmid carrying $bla_{\text{CTX-M-15}}$. Additionally, as far as I am aware, this is the first report of $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and PMQR *aac(6')-Ib-cr* genes co-existing in the same *E. coli* bacterial cell isolated from a patient in a Scottish hospital.

It is interesting to note that the upstream environment of *bla*$_{\text{CTX-M-15}}$ in five isolates was identical; four were closely related strains. The fifth isolate 270 had a genotype much more similar to isolates 35 and 202, both of which had a different upstream region. But these two isolates have a different downstream region, *IS*26 was found in isolate 35 where as *orf*477 was found in isolate 202 and *IS*26 could not be detected.

5 Molecular characterization and diversity in *Enterobacter cloacae* from Edinburgh and Egypt carrying *bla*$_{CTX-M-14}$ and *bla*$_{VIM-4}$ β-lactamase genes

5.1 Introduction

Enterobacter cloacae is an important nosocomial pathogen responsible for various infections. Management of these infections is often complicated by these organisms being multidrug resistant. (Fernández *et al*. 2011). Carbapenems are therefore important therapeutic agents for the treatment of multidrug resistant *Ent. cloacae*. However, the production of both ESBLs (Bell *et al*. 2003, Canton *et al*. 2002, Tzelepi *et al*. 2000) and metallo-β-lactamases (MBL)(Yan *et al*. 2002, Luzzaro *et al*. 2004, Sianou *et al*. 2012) by *Ent. cloacae* have been reported in different parts of the world. In particular, the production of MBLs by *Ent. cloacae* render them resistant to carbapenems, which are primary treatment options for multidrug resistant strains of this bacterium. CTX-M type β-lactamases are one of the most prevalent types of ESBLs globally (Pitout and Laupland 2008) while VIM β-lactamases are one of the most prevalent MBL types (Nordmann and Poirel 2002, Livermore and Woodford 2000). The genes encoding these ESBLs and carbapenemase are often located on plasmids, which play a role in the mobilisation of genetic material by there ability to capture and spread these resistance genes (Ktari *et al*. 2006, Miro *et al*. 2010). The dissemination of these resistance genes can also be attributed to clonal expansion of a bacterial host of *Enterobacteriaceae*.(Woodford *et al*. 2011)

The *bla*$_{CTX-M-14}$ gene was initially identified in clinical isolates from South Korea in 1995 (Pai *et al*. 2001). Since then the *bla*$_{CTX-M-14}$ gene has disseminated globally with the report of dissemination via plasmids (Valverde *et al*. 2009). There has been report

in the United Kingdom of *Enterobacteriaceae* such as *E. coli* with the $bla_{\text{CTX-M-14}}$ gene (Livermore *et al*. 2007) but there is less information on *Ent. cloacae* possessing $bla_{\text{CTX-M-14}}$ genes with only reports from China and Egypt (Khalaf *et al*. 2009, Liu *et al*. 2009).

This study compares the *Enterobacter cloacae* isolates from Edinburgh and Egypt to identify the degree of their relatedness or diversity and to characterise the genetic environment of the ESBL gene, the molecular mechanism of carbapenem resistance and their potential carriage on plasmids- to indicate their mode of spread.

5.2 Results

5.2.1 Susceptibility testing

The *Ent. cloacae* isolates from Egypt (n = 3) and six isolates that were positive for CTX-M-9 group from the multiplex PCR in section 4 figure 8 were examined further in this section. The MIC were done using the BSAC method and all isolates were found to be resistant to ampicillin, piperacillin, co-trimoxazole, nalidixic acid, and aztreonam (MIC ≥128 mg/L). The MICs of the remaining antibiotics tested are shown in the Table 10

Table 10 MIC of antibiotics for the *Enterobacter cloacae* clinical isolate harbouring ESBLs and Metallo β-lactamases

Isolate No	MIC (mg/L)								
	CTX	CAZ	FEP	FOX	CIP	GEN	ERT	IMI	MER
3	>128	8	4	>128	2	0.5	2	0.25	0.25
18	>128	8	4	>128	4	0.5	4	0.12	0.12
69	>128	4	4	>128	4	0.5	4	2	0.25
156	>128	4	4	>128	4	0.25	2	0.06	0.25
157	128	4	4	128	4	2	2	0.06	0.12
167	>128	16	4	>128	4	0.5	2	0.12	0.12
E1720	>128	64	8	>128	8	8	64	>128	64
E4293	>128	64	8	>128	8	8	32	>128	64
E4303	>128	32	8	>128	8	4	8	2	8

Abbreviations: CTX = cefotaxime; CAZ = ceftazidime; FEP = cefepime; FOX = cefoxitin; CIP = ciprofloxacin; GEN = gentamicin; ERT = ertapenem; IMI = imipenem; MER = meropenem

The table show the *E. cloacae* CTX-M-14 isolate showing resistance to the antibiotics with some isolates showing high resistance to carbapenems tested.

5.2.2 Efflux Pump activity

The MIC of Ertapenem was done with the addition of 10mg/L of the efflux pump inhibitor carbonyl cyanide*m*-chlorophenylhydrazone (CCCP).

Table 11 MIC with ertapenem (ERT) +10mg/L CCCP

Isolate No	ERT	ERT + CCCP
3	2	0.25
18	4	1
69	4	1
156	2	0.5
157	2	0.5
167	2	0.25
E1720	64	8
E4293	32	8
E4303	8	1

The table show the reduction in the MIC of ertapenem in the presence of the efflux pump inhibitor CCCP

There was a greater than or equal to two fold concentration decrease in MIC concentration for ertapenem in combination with 10mg/L of the efflux pump inhibitor CCCP. This indicates that an active efflux pump contributes to ertapenem resistance in these *Ent. cloacae* isolates

5.2.3 Amplification, sequencing of resistant determinant and genetic environment

The PCR amplification of the CTX-M-9 group isolates showed a 905bp amplicon size which was sequenced and analysed on the BLAST software of NCBI. A representative PCR gel is as shown in figure 23.

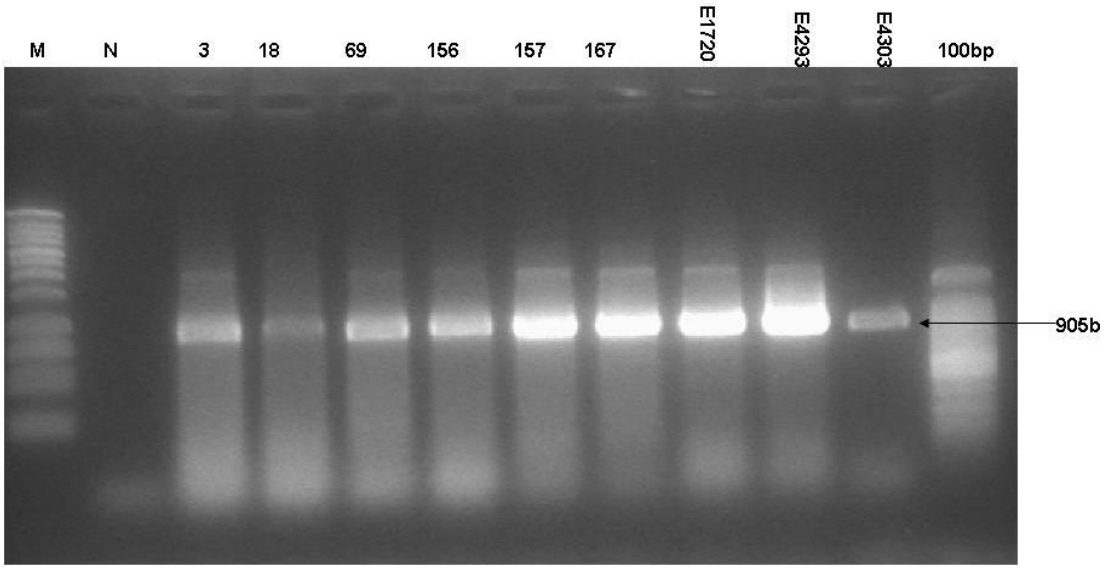


Figure 23 PCR gel for CTX-M-14

M = 1kb ladder; N = negative control; $bla_{\text{CTX-M-14}}$ = 905bp

The PCR gel show the detecton of the CTX-M-14 gene in the *Ent. cloacae* isolates and which were confirmed by sequencing.

The sequencing results confirmed the presence of $bla_{\text{CTX-M-14}}$ in all *Ent. cloacae* strains tested from Edinburgh and Egypt when analysed by BLAST software of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

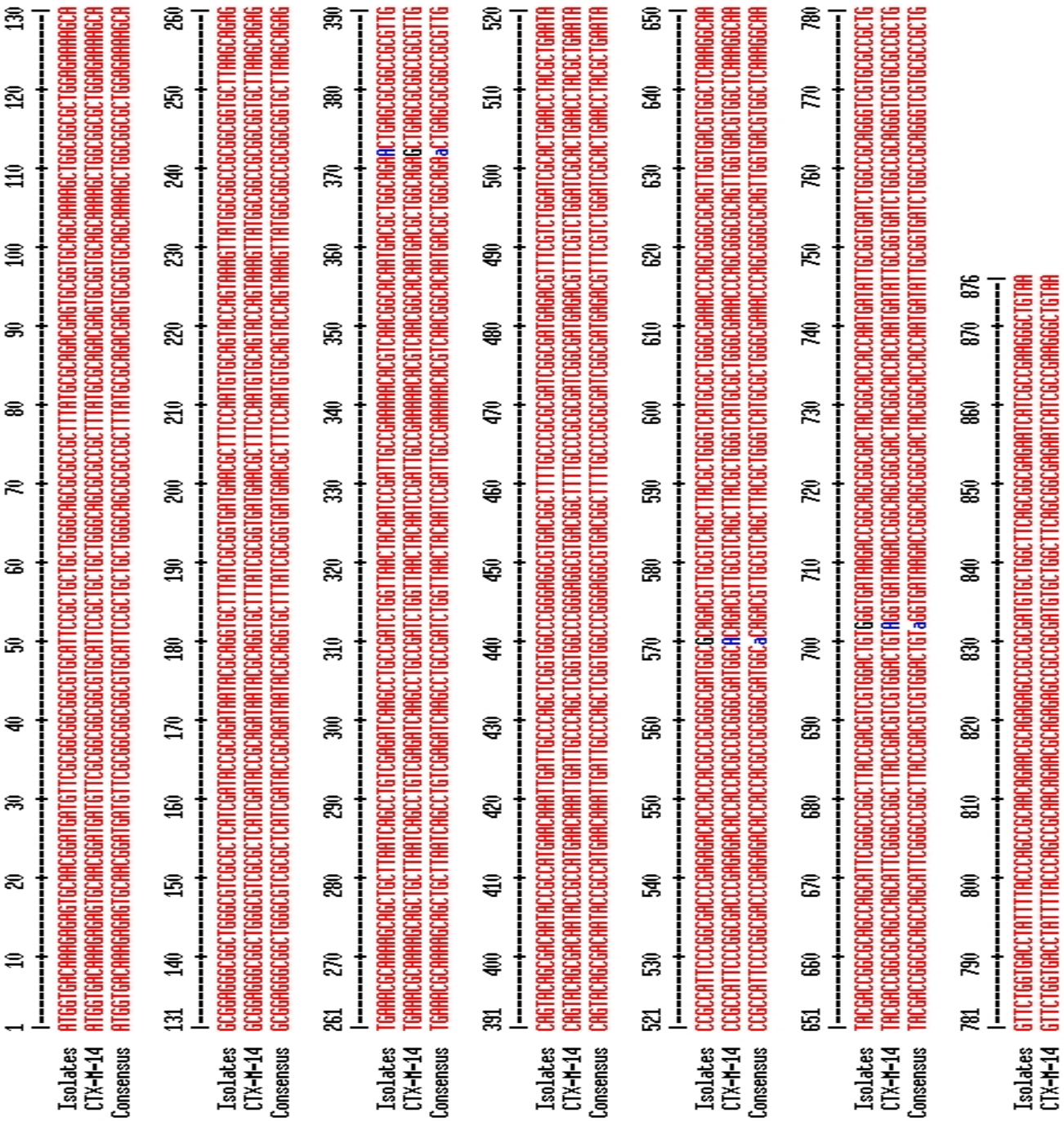


Figure 24 Nucleotide sequence of my Isolate aligned with CTX-M-14

A = Adenine T=Thymine G = Guanine C = Cytosine

The figure show the CTX-14 with changes at position 372 and 702.

The sequence was compared with a CTX-M-14 sequence from Genbank and there was a change from the original in the nucleotide sequence at positions 372 G→A, 570 A→G, and 702 A→G as shown in figure 24. However when the sequence was translated to amino acids, there was no change in the sequence. The amino acid sequence compared with the CTX-M-14 from gene bank is as shown in figure 25

Figure 25 Amino acid translated sequence of isolates aligned with CTX-M-14

The figure show the conversion to amino acid with no change in the sequence when compared with CTX-M-14.

The upstream regions of *bla*$_{CTX-M-14}$ were sorted by PCR. A PCR product amplicon greater than 1500bp was obtained in two isolates as shown in Figure 26. This was sequenced and analysed on BLAST software.

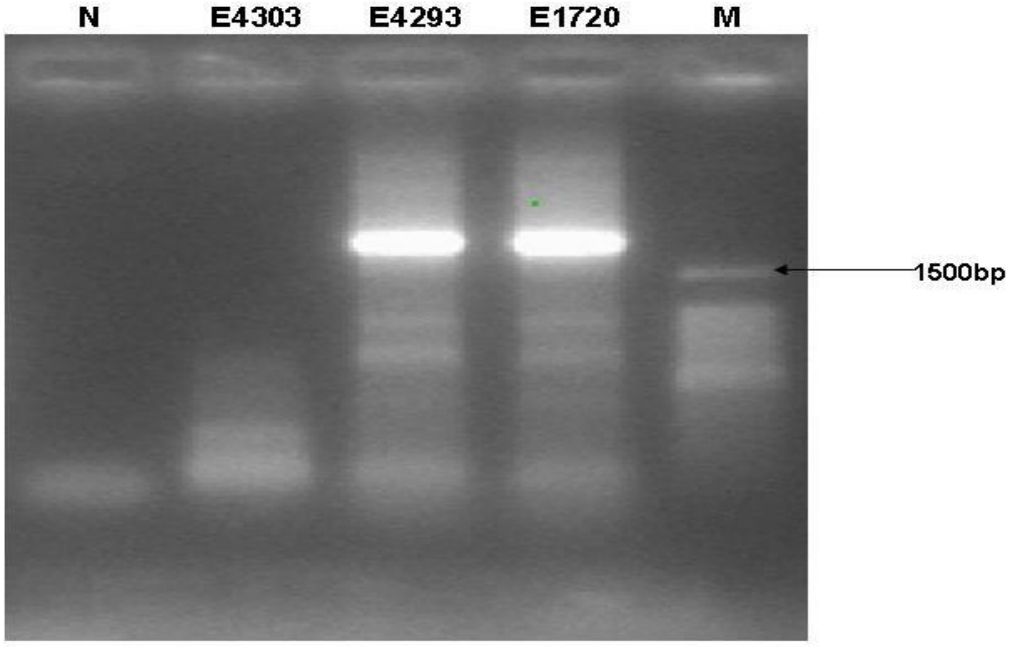


Figure 26 PCR gel for upstream region of *bla*$_{CTX-M-14}$

The PCR gel show the detection of ISecp1 upstream of the CTX-M-14 gene, which was confirmed by sequencing.

Analysis of the upstream region by PCR amplification shows, two of the isolates were found to possess the insertion sequence IS*Ecp1* upstream of the *bla*$_{CTX-M-14}$ while the remaining seven did not have this insertion element. A 43bp intergenic region was observed between the insertion sequence IS*Ecp1*and *bla*$_{CTX-M-14}$ gene.

A PCR was performed for the detection of carbapenemase by multiplex PCR which shows the presence of VIM. Primers to detect the whole VIM gene were used to perform a PCR. A 920bp amplicon product size was detected in two isolates and the representative PCR gel is shown in figure 27. The amplicon was sequenced followed

by analysis on the BLAST software of NCBI. Two of the isolates were found to possess the *bla*$_{\text{VIM-4}}$ metallo β-lactamase gene.

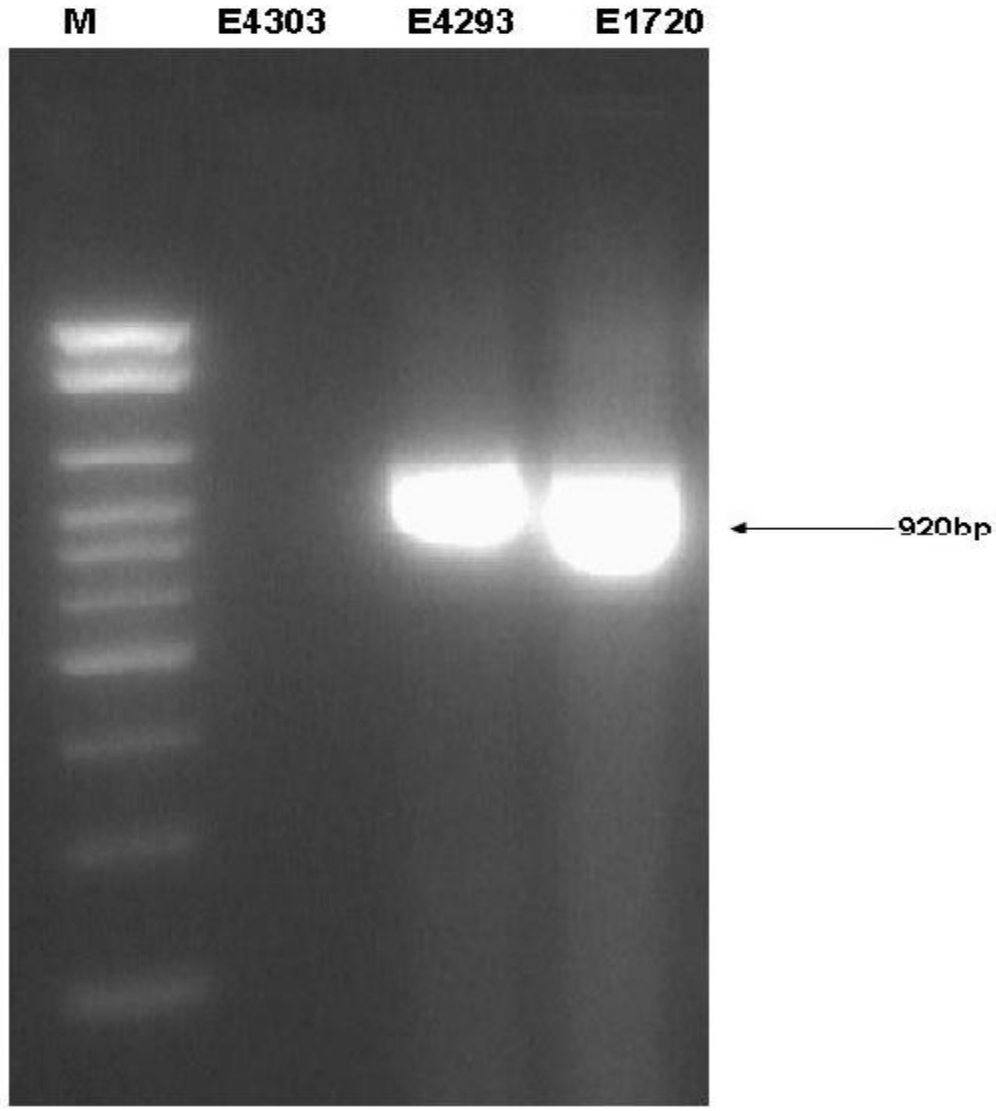


Figure 27 Representative PCR gel for detection of VIM-4

M = low range plus DNA ladder

The PCR gel electrophoresis show the detection of VIM-4 in the isolates from Egypt and which was sequenced to confirm the gene identity.

A PCR was also used for the detection of different types of integron genes. All the isolates were found to possess the *int11* integrase gene encoded in class 1 integron.

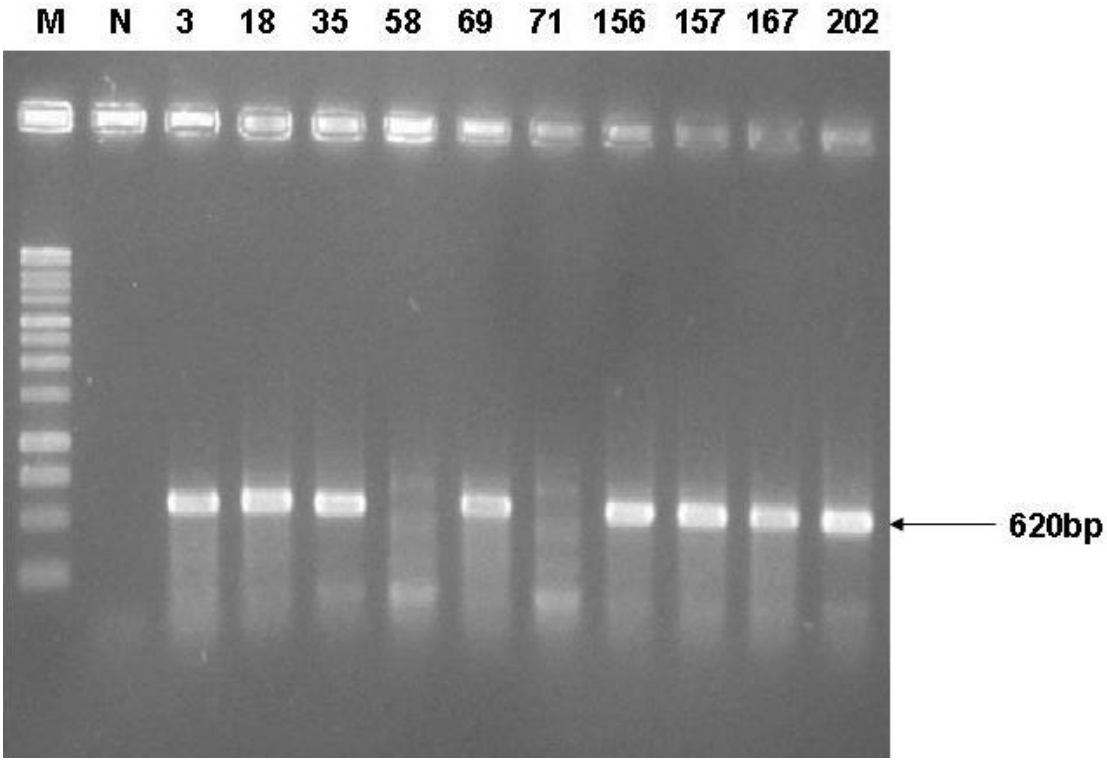


Figure 28 Representative PCR gel for class 1 integron

The PCR gel show the detection of Intl 1 integrase among the *E. cloacae* isolates and which was sequenced to confirm the gene.

The representative PCR gel to show the *int11* integrase is shown in figure 28. *IntI2* and *IntI3*, encoded in class 2 and 3 integrons respectively, were not found in any of the isolates. PCR with primers Hep 58 and 59 were used to detect the variable region, and the amplicon was sequenced and analysed using the BLAST software of the NCBI. Upon analysis of the gene cassette of the class 1 integron, the first cassette for six of the isolates contained *dfr*A17, which confers resistance to trimethoprim. The second cassette was *aad*A2, which confers resistance to the aminoglycosides.

In the remaining three isolates, one had no gene cassette while the remaining two isolates had $bla_{\text{VIM-4}}$ gene as part of a gene cassette in class 1 integron. The PCR gel

showing the combination primers used to detect and identify the arrangement of the cassette is as shown in figure 29

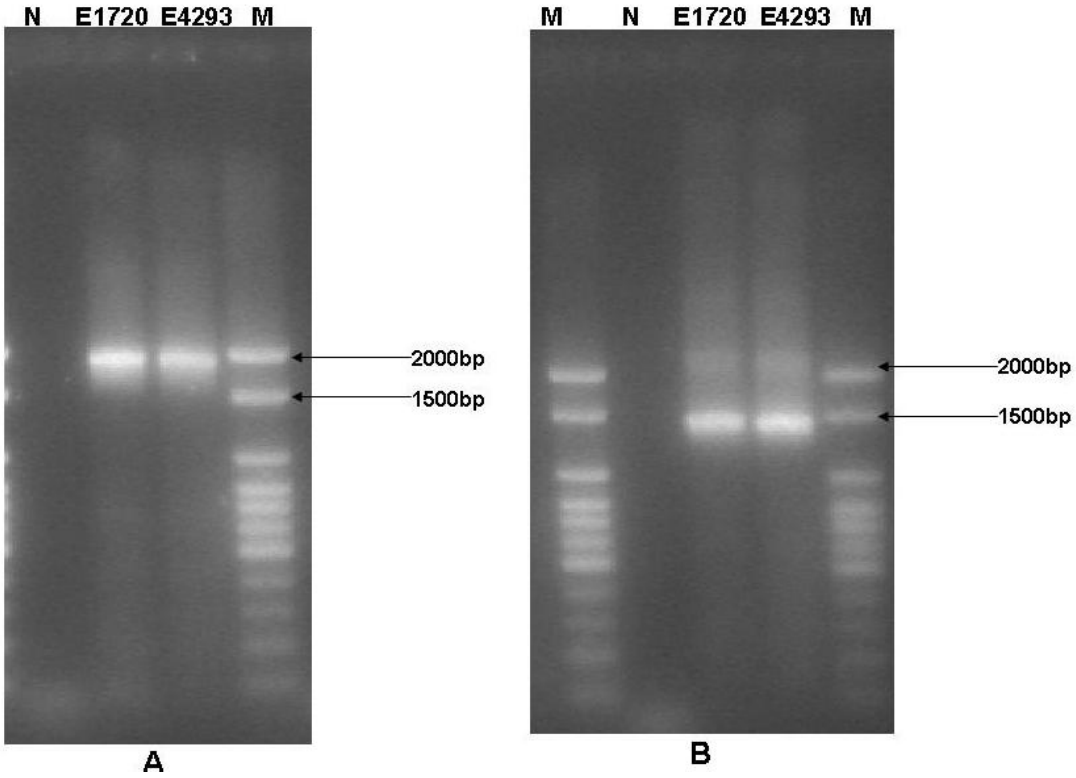
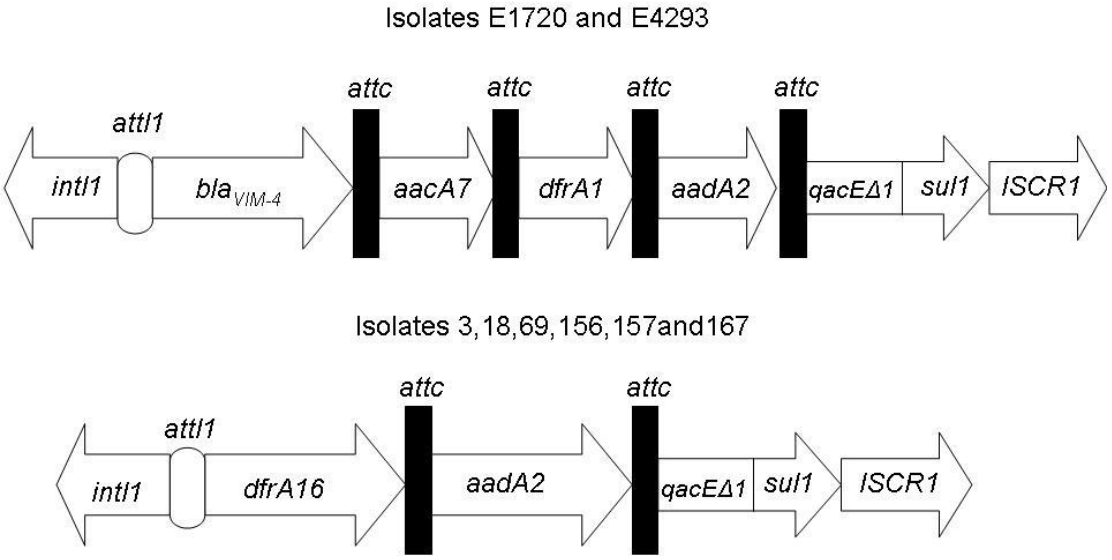


Figure 29 PCR gel for the detection of gene cassette.

The PCR gel shows the detection of the gene cassette and to know the arrangement of these genes in the cassette.

Figure 29A shows the PCR with *aadA*2 forward primer and *sul*1 reverse primer with PCR amplicon approximately 2000bp. Figure 29B shows a PCR with the VIM forward primer and *aadA*2 reverse primer with amplicon size approximately 1400bp. The cassette structure shows $bla_{\text{VIM-4}}$ was located downstream of the *attl1* recombination site, followed by the *aacA7* cassette, *dhfrA1* cassette, *aadA2* cassette and the 3-CS. The organisational structure of the gene cassette is as represented in Figure 30.

Figure 30 Structural organisations of gene cassettes in *Ent. cloacae*



The diagram show how the resistance genes are arranged within the integron structure with the isolated exhibiting different arrangement.

A PCR was also used to sort the 3'-CS end of the class 1 integron structure. A *sul*1 forward primer and *orf*513 reverse primer was used to get a PCR amplicon of approximately 1500bp shown in figure 31. This amplicon was sequenced and analysed using the BLAST software of NCBI.

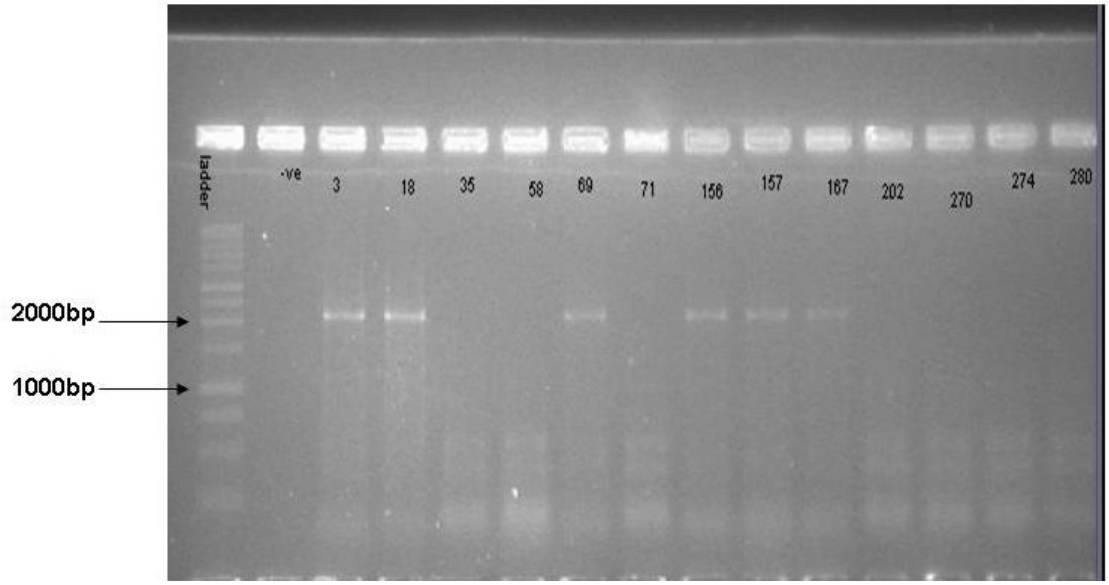


Figure 31 PCR gel for amplification of 3'CS

Ladder = 1kb

The PCR gel electrophoresis show the 3'-CS of the class 1 integron in the *E. cloacae* isolates

Interestingly the 3'-CS of the class 1 integron in the six isolates contained the *qacE*Δ1, *sul*1 genes and the complex *IS*CRI insertion element (previously called *orf*513).

5.2.4 Plasmid Profiling and Analysis:

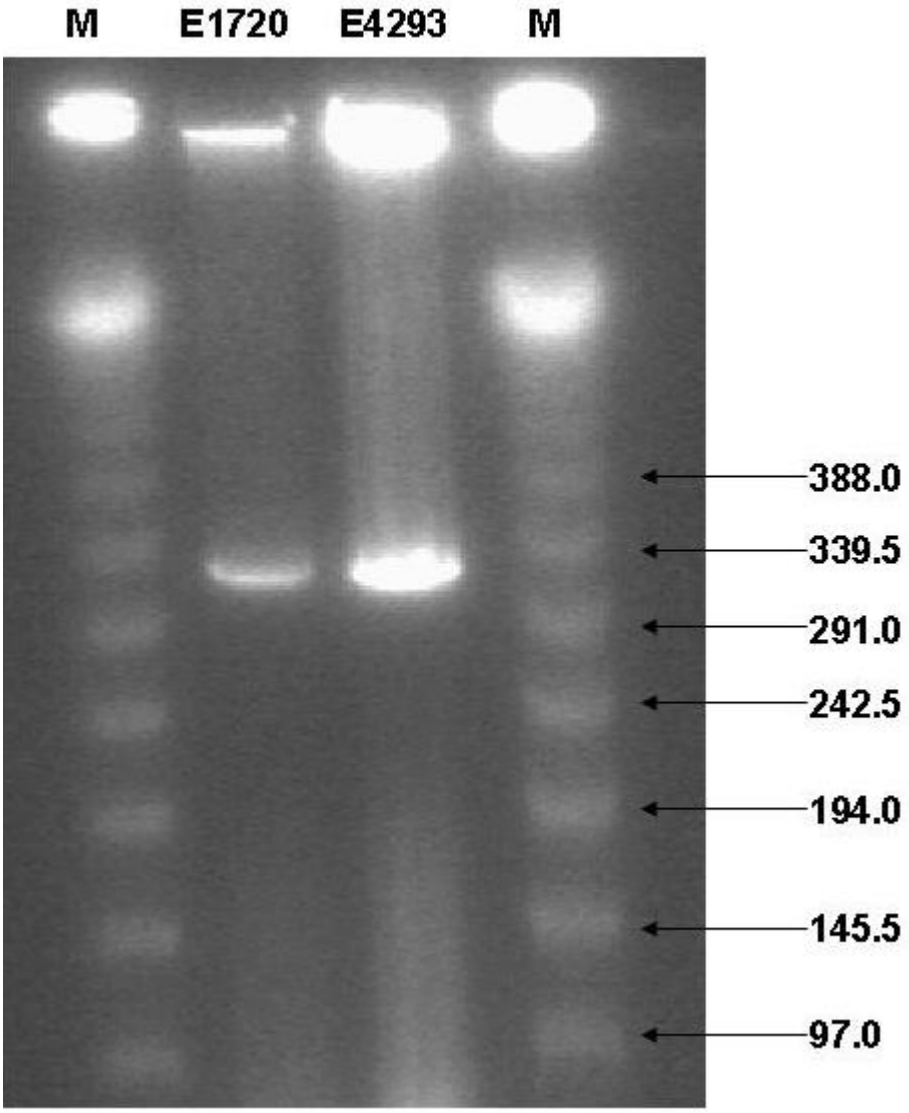
Plasmid analysis revealed that all isolates contained detectable plasmids (Figure in the appendix C). Six of the isolates possess four plasmids, two isolates possessed one plasmid and one isolate did not contain any detectable plasmids, despite several attempts to detect them. The plasmid sizes ranged from ~10kb to 300kb. Analysis of the extracted plasmids for the location of the genes revealed $bla_{\text{CTX-M-14}}$ and $bla_{\text{VIM-4}}$ integron structure on the ~300kb plasmid in two of the isolates from Egypt. The other

remaining isolates from Edinburgh did not show the *bla*$_{\text{CTX-M-14}}$ or the integron structure in any of the plasmids.

5.2.5 Conjugation and Transformation Experiment:

The plasmids in the six Edinburgh isolates could not be transferred or transformed despite several attempts. Conjugative transfers of *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{VIM-4}}$ into the *E. coli* J62-2 strain were achieved in two of the Egyptian isolates, which showed the transfer of a 300kb plasmid. Transformations of the same two plasmids were also achieved. The plasmid DNA was extracted from the gel and used as a DNA template for PCR. Analysis of the transconjugant and transformant extracted plasmid DNA showed that the *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{VIM-4}}$ genes in the two isolates were located on the same 300kb plasmid (Figure 32). The *bla*$_{\text{CTX-M-14}}$ in the other six isolates could not be detected in the extracted plasmids by PCR.

Figure 32 Plasmid profile of the E1720 and E4293 transconjugants

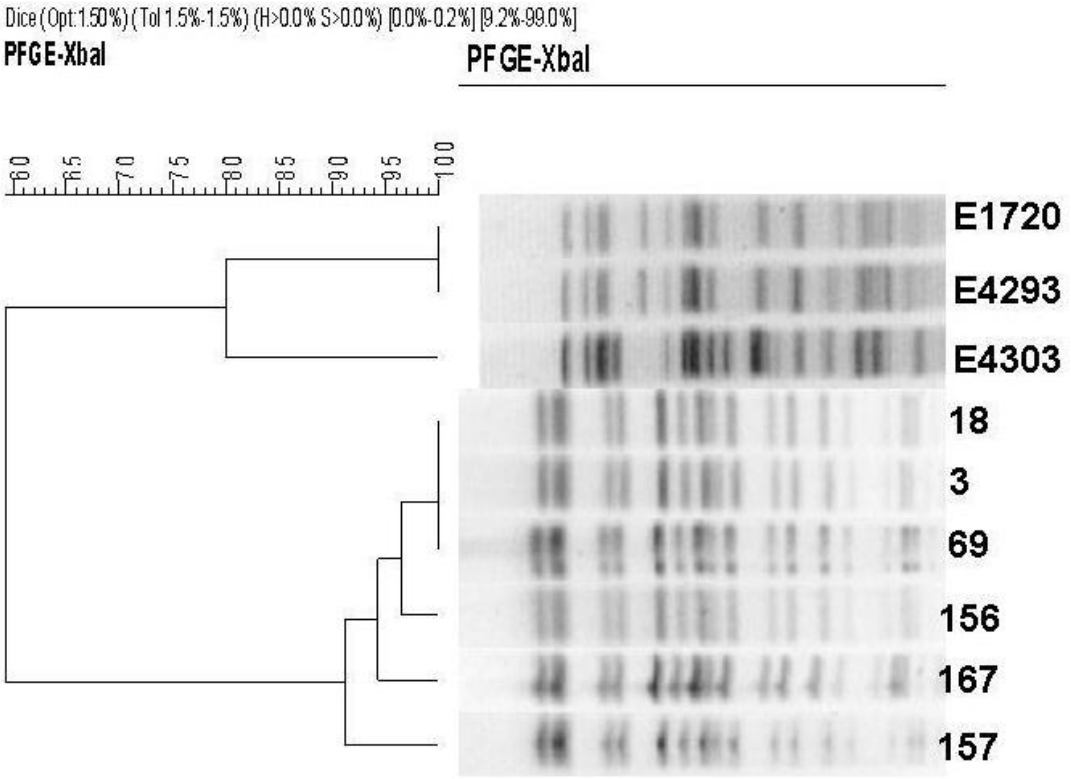


The gel show the plasmid profile of the isolates from Egypt with VIM-4 having similar plasmid profile where M is the Marker

5.2.6 Genetic relationship between isolates by PFGE analysis:

PFGE banding patterns were obtained for all the isolates, ≥85% similarities were observed for the six Edinburgh isolates. Also two of the Egyptian isolates were found to have ≥85% similarity while the remaining isolate did not have similarity with the other isolates (Figure 33).

Figure 33 Pulsed-field gel electrophoresis of all isolates



PFGE show the genetic finger printing of the *Ent. cloacea* isolates with the isolate from Egypt and Edinburgh. With the Edinburgh isolates showing ≥90% similarity.

5.3 Analysis of gene expression

Analysis of the CTX-M-14 and VIM-4 β-lactamase genes showed substantial expression of $bla_{\text{CTX-M-14}}$ and $bla_{\text{VIM-4}}$ in some isolates. The PCR products were quantified using the Bio-Rad Quantity One Software 4.6.1 (Bio-Rad). Normalization of DNA was done with the 16S-rRNA primers with the appropriate amounts of serially diluted RNA used for c-DNA synthesis. The results of the expression are shown in Table 12.

Table 12 Quantification of gene expression (Arbitrary units)

Isolate	Expression of 16s rRNA gene	Expression of $bla_{\text{CTX-M-14}}$	Expression of $bla_{\text{VIM-4}}$
3	252	189	-
18	249	108	-
69	255	152	-
156	255	255	-
157	251	255	-
167	255	255	-
E4303	255	212	200
E4293	255	255	212
E1720	255	255	-

- indicates no expression detected.

The table shows that the CTX-M-14 are expressed and the VIM-4 is expressed in two of the Egyptian isolate.

5.4 Discussion

I screened the *Ent. cloacae* isolates collected from Edinburgh and Egypt for their antibiotic resistance phenotype. The Edinburgh and Egyptian isolates differed in their susceptibility to gentamicin and the carbapenems tested. The Edinburgh isolates were susceptible to gentamicin, while the Egyptian isolates were resistant to this antibiotic. There also were increased MIC values of imipenem and meropenem in the Egyptian isolates compared to the MIC values in the Edinburgh isolates (Table 10). This can probably be attributed to the relative degree of regulation and restriction in the use of these antimicrobials in each area.Lack of infection control can also be the reason.

All isolates had MICs ≥128 mg/L to cefotaxime, which can be explained by the presence of the *bla*$_{\text{CTX-M-14}}$ found in all isolates irrespective of the source. This β-lactamase (*bla*$_{\text{CTX-M-14}}$) in *Ent. cloacae* has not been described previously in the Scotland or the UK but has been described only in Egypt (Khalaf *et al*. 2009) and in China (Liu *et al*. 2009). Analysis of the upstream region of *bla*$_{\text{CTX-M-14}}$ revealed, in isolates E1720 and E4293 from Egypt, the presence of the *ISEcp1* insertion sequence 42bp upstream of *bla*$_{\text{CTX-M-14.}}$ This insertion element contained a -10 and -35 putative promoter region able to control the expression of *bla*$_{\text{CTX-M-14}}$ (Poirel *et al*. 2003). The ISEcp1 insertion element found upstream of the *bla*$_{\text{CTX-M-14}}$ gene in the Egyptian isolates suggests it may be involved in the mobilisation of the gene. It is important to note the time of isolation of these two isolates was ten months apart. None of the isolates from Edinburgh possessed this insertion sequence upstream of *bla*$_{\text{CTX-M-14}}$ again emphasizing the differences in *Ent. cloacae* from the two regions.

Carbapenem resistance in Gram-negative bacteria is increasing globally. The high MIC of carbapenems tested in isolates E1720 and E4293, from Egypt, could be explained by the presence of the metallo-β-lactamase (MBL) gene, *bla*$_{\text{VIM-4.}}$ This is the first time that *bla*$_{\text{VIM-4}}$ and *bla*$_{\text{CTX-M-14}}$ genes have been described together in *Ent. cloacae* although VIM-1/ CTX-M-15 and VIM-1/ CTX-M-2 β-lactamases have been described in the same strains of *Klebsiella pneumoniae* isolated in Turkey and Spain respectively (Yildirim *et al*. 2007, Cendejas *et al*. 2010). The VIM-4 MBLs are normally found as part of a gene cassette in the integron structure (Nordmann and Poirel 2002) and we report a novel arrangement in the integron structure where the *aadA2* replaced the usual *aadA1* and the association with complex *ISCR1* insertion

element (Figure 30). The difference between the two types of integron structure identified in Figure 30 is that the first has insertion of the *bla*$_{\text{VIM-4}}$ and *aadA*7 cassette upstream of the *dfrA* genes. Another difference is the presence of the *dfrA1* gene in the first cassette and the related, but not identical, *dfrA16* gene in the second cassette. It is important to note that the first integron type is located in the plasmid while the second type is not on a plasmid. The Edinburgh isolates do not have the *bla*$_{\text{VIM-4}}$ in the integron structure, in contrast to the Egyptian isolates, confirming the low carbapenem MICs found in isolates from Edinburgh.

Interestingly the 300kb plasmids (Figure 32) were found to have both the *bla*$_{\text{VIM-4}}$ and *bla*$_{\text{CTX-M-14}}$ in the Egyptian isolates. This is the first report of the co-existence of these two genes in an *Ent cloacae* clinical isolate and on the same plasmid in any strain. This is a conjugative plasmid and can transfer to other bacteria facilitating the spread of these resistance genes. The plasmids from the Edinburgh isolates were neither transformable nor transferable in my hands and the analysis of the plasmid showed *bla*$_{\text{CTX-M-14}}$ and its integron structure indicated that it is not located in any of its plasmid. Therefore presumably it is in the chromosome.

Identical PFGE patterns were found for the *bla*$_{\text{VIM-4}}$/*bla*$_{\text{CTX-M-14}}$ producing *Ent cloacae.* The Edinburgh isolates were found to be related by PFGE with greater than 85% similarity. Contact transmission is a possible mechanism of acquisition of these stains in these patients with *bla*$_{\text{CTX-M-14}}$ producing *Ent. cloacae* in Edinburgh. However three isolates from Egypt show 100% relatedness in two isolates with 80% relatedness in the third. The three isolates from Egypt were isolated from the same ICU with the two identical isolates collected 10 months apart. There was no

significant genotypic similarity between the Edinburgh and Egyptian isolates as seen in Figure 33.

6 Integrons and the persistence of sulphonamide resistance genes in blood culture isolates, despite the apparent reduction in the use of this antimicrobial.

6.1 Introduction

Antibiotic resistance in bacteria depends on the resistance genes that are encoded in the bacteria. The quantity of antibiotics used and how they are used contributes to the development of resistance. There is an expectation that reduction in the antibiotic use and the consequent decrease in selective pressure will promote loss of the resistance determinant (Murray 1994). There have been reported successes in decreasing antibiotic resistance following implementation of antibiotics reduction policies in the UK (Barber *et al*. 1960, Ridley *et al*. 1970); for instance a withdrawal in the use of prophylactic and therapeutic antibiotics was followed by a reduction in drug resistant *Klebsiella aerogenes* in Glasgow (Price and Sleigh 1970). Furthermore, there are also reported successes of these policies world wide: USA (White *et al*. 1997), Japan (Fujita *et al*. 1994), Finland (Seppala *et al*. 1997), Germany (Klare *et al*. 1999), and the Netherlands (van den Bogaard *et al*. 2000).

Sulphonamides target the enzyme dihydropteroate synthase in the folic acid pathway in bacteria. There has been a decline in the use of sulphonamides in the UK. Sulphonamides were used alone until 1969 when sulphamethoxazole in combination with trimethoprim, as co-trimoxazole, was introduced and became widely used in clinical practice. Its use was restricted in 1995 because of the hypersensitivity reactions attributed to sulphamethoxazole (Enne *et al*. 2001). Consequently this study is designed to elusidate whether sulphonamide resistance genes still persist, in the absence of the use of this drug, in Gram-negative isolates from blood culture in

Edinburgh. In particular, the strength of association between *sul* genes and resistance to other antimicrobials were determined and the association of the resistance genes with integrons that play a key role in multiple antibiotics resistances.

6.2 Results

6.2.1 Prevalence of antimicrobial resistance

The MIC of all these isolates is as described previously and in the appendix. Under half of the Gram-negative isolates were sulphonamide resistant at break point ≥4mg/L. The resistance to other antimicrobials among isolates with sulphonamide resistance genes were analysed for strength of association and was demonstrated by an odds ratio shown in table 13. This statistical analysis was performed using Medcalc for windows, version 12.4.0 (Medcalc software,Ostend,Belgium)

Table 13 Relationship between sulphonamide resistance *sul* gene and resistance to antibiotics

Antimicrobials	*sul* gene present (n=45)	*sul* gene absent (n=55)	odd ratio(95% confidence interval)
Kanamycin	31 (68.9%)	3 (5.5%)	38.38(10.21 to 144.24)
Spectinomycin	30 (66.7%)	3(5.5%)	34.67(9.27 to 129.58)
Piperacillin	44 (97.8%)	27(49.1%)	45.63(5.87 to 355)
Streptomycin	29(64.4%)	3(5.5%)	30.21(8.11 to 112.55)
Ceftazidime	18(40%)	5(9.1%)	6.46(2.17 to 19.20)
Cefotaxime	19(42.2%)	4(7.3%)	9.32(2.87 to 30.24)
Aztreonam	18(40%)	4(7.3%)	8.20(2.53 to 26.61)
Gentamicin	11(24.4%)	2(3.6%)	8.74(1.82 to 41.84)
Cefepime	18(40%)	4(7.3%)	8.68(2.68 to 28.12)
Ampicillin	41(91.1%)	37(67.3%)	4.99(1.55 to 16.08)

The table show there is a relationship between the presence of sul gene and the resistance to an antibiotic

Specifically, resistances to all these antibiotics in table 13 were significantly ($P \leq 0.01$) associated with sulphonamide resistance (*sul*) genes

6.2.2 Association of Sulphonamide resistance with other resistance determinants

Sulphonamide resistance genes were detected in 45 out of the 100 Gram-negative isolates tested. PCR for detection of *sul1* show amplicon product size 822bp while *sul*2 showed 293bp amplicon. The representative PCR gel is as shown in figure 34 and 33 for *sul1*and *sul*2 respectively.

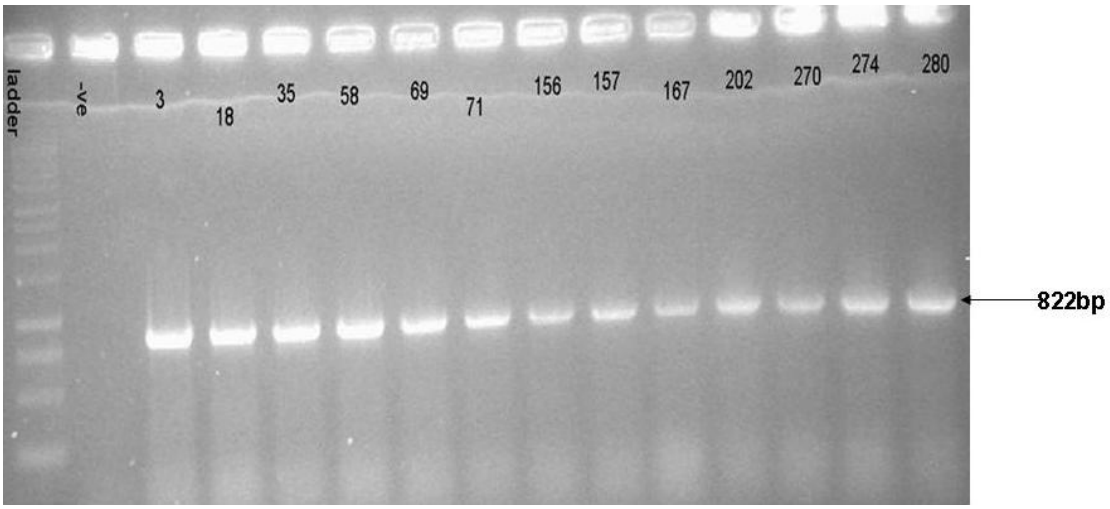


Figure 34 A representative PCR gel identifying the *sul*1 gene

The PCR gel show the detection of *sul*1 gene in the isolates with 822bp PCR product sequenced to confirm the gene.

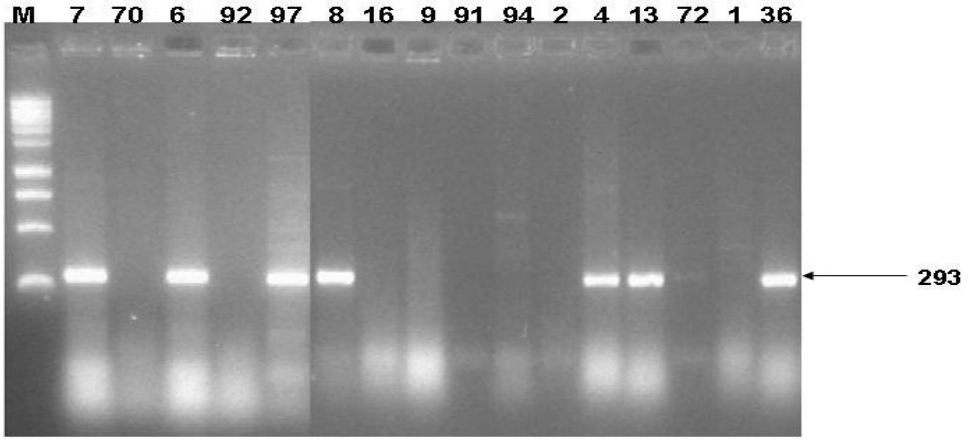


Figure 35 A representative PCR gel showing the *sul2* gene

M = 1kb ladder

The PCR gel show the detection of *sul*2 gene in the isolates with a 293bp PCR product sequenced to confirm the gene

The PCR amplicon were sequenced and the sequence analysed using the BLAST software of NCBI. *sul1* genes were detected in 26 strains, 27 strains had *sul2* gene and none of the isolates was found to have *sul3* gene. Eight of the isolates had both the *sul*1 and *sul*2 genes.

With a PCR followed by sequencing of the PCR product for integrase genes encoded in an integron were detected in 26 of the 100 isolates. *Intl*1 integrase genes were detected in 25 strains, *intl*2 gene in 3 and 2 of the isolates had *intl3* integrase gene. Two of the isolates had both *intl2* and *intl1* while one isolate had *intl1* and *IntI*3.

The variable zones were sorted by PCR and sequenced followed by analysis of the sequence on the BLAST software. Of the 25 *intl1* positive isolates, 20 harboured gene cassettes in the variable region (Table 14). Five different gene cassette arrangements were identified in the 20 isolates as follows, with the number of isolates shown in parentheses: *dfrA17* + *aadA5* (10), *dfrA16* + *aadA2* (6), *dfrA15* + *aadA1* (1), *aadA1*

(2) and *bla* $_{OXA-1}$ + *aadA1* (1). The structural organisation of the arrangement in the variable zone is as shown in figure 36.

Table 14 Types of integron detected and the gene cassette in isolates from blood culture

Class of integrase	Cassette array	Number of isolates
*intI*1	*dfrA*17-*aadA*5	10
*intI*1	*dfrA*16-*aadA*2	6
*intI*1	*dfrA*15-*aadA*1	1
*intI*1	*aadA*1	2
*intI*1	oxa-1-*aadA*1	1
*intI*1	-	5
*intI*2	*dfrA*1-*sat1*-*aadA*1	2
*intI*2	-	1
*intI*3	-	2

The table show the class of integron detected with the associated gene cassette and the number of isolates with the cassette arrangement.

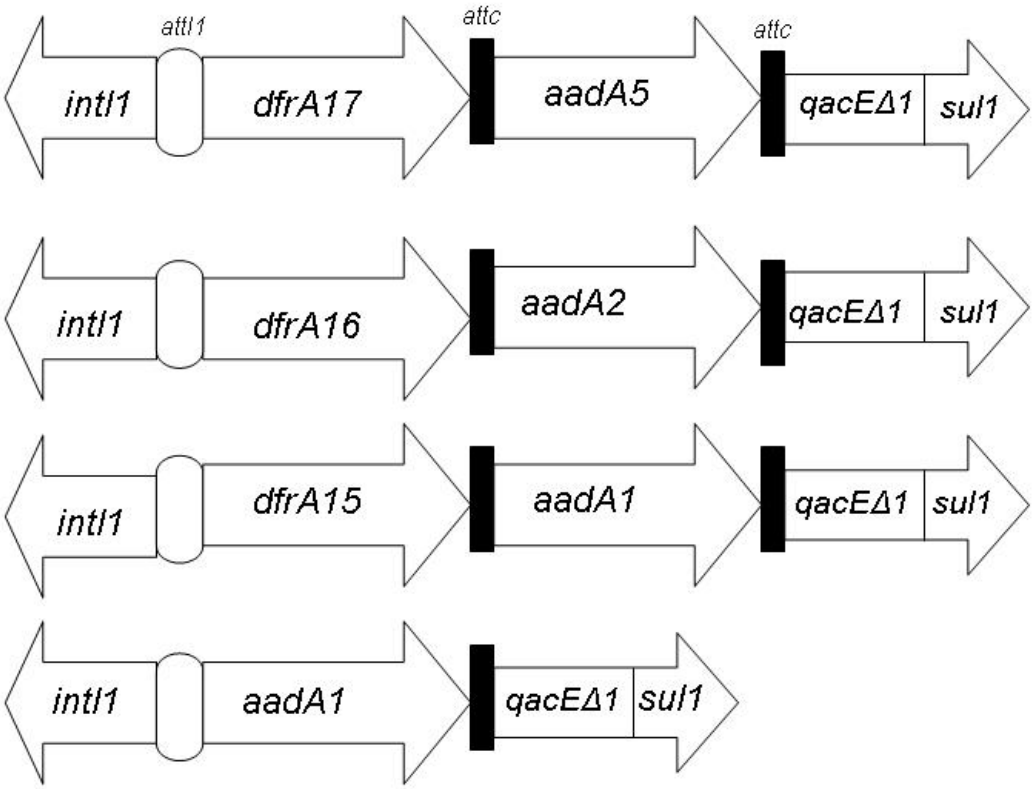


Figure 36 Structural organisation of the variable zone in class 1 integron showing gene cassettes.

The figure show the diagramatic arrangement of the class 1 integron with the gene cassette arrangement.

Two of the three *intl2* positive isolates presented the *dfrA1+ sat1 + aadA1* gene cassette arrangement (Table 14) and *tns*E while no gene cassette was detected in the remaining isolate. The diagrammatic representation of the variable region of the class 2 integron is as shown in the figure 37.

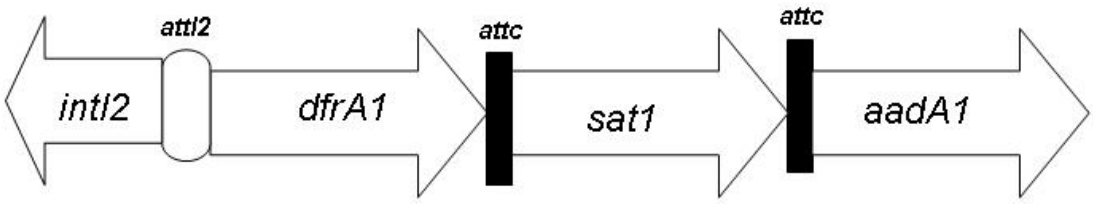


Figure 37 Structural organisation of the class 2 integron variable region

The figure show the class 2 integron with the gene cassette showing the resistance gene.

6.3 Discussion

There is still a high prevalence of sulphonamide resistance genes in our hospital isolates despite the reduction in the use of the antibacterial. Although there have been reported cases of successful decreases in antibiotic resistance after reduction or removal of the antibiotic (Klare *et al*. 1999, van den Bogaard *et al*. 2000, Seppala *et al*. 1997), the reason for this is still not totally clear because the studies did not consider other factors that may contribute to the decrease, such as the infection control measures. In this study there is a significant relationship between isolates with sulphonamide resistance genes and resistance with other antibiotics tested ($P \leq 0.01$); in other words isolates with sulphonamide resistance genes are usually resistant to other antibiotics (Table13).

Certain factors can influence the persistence of resistance in the event of antibiotics withdrawal, one explanation is the contamination from food which has been reported previously (Corpet 1988). Another explanation is the co-selection process were multiple resistance gene are found in the same plasmid, transposon or any mobile

element and therefore the use of any antibiotics will select the resistance of all the others. This is the case in this study where the *sul1* resistance gene is an integral part of a class one integron. Integrons are mobile genetic elements capable of collecting several resistance genes in the form of gene cassettes and dissemination of these genes among strains. Integrons play a key role in multiple antibiotic resistances posing potential health risk to the general public. Class 1 integrons were the most frequent type of integron detected in our study. Most of the genes found within the gene cassette of class I integron in our study correspond to $bla_{\text{OXA-1}}$, *dfrA* and *aadA* genes conferring resistance to β-lactam, trimethoprim and aminoglycosides respectively. The *dfrA17 + aadA5* combination was the most frequent gene cassette arrangement found in our study. The 3'CS of the class one integron just before the *sul1* gene contained the *qacEΔ1* that confers resistance to the quaternary ammonium compounds that are used as antiseptics and disinfectants. In this study most of the isolates with *sul1* had *intI1* but it is interesting to note that three isolates were negative for *sul1* but positive for *sul2* and *intI1* and a gene cassette was not detected in these isolates.

The class two integron gene cassettes had the *dfrA1+ sat1 + aadA1* combination, which is a common cassette often found in the variable zone (Kadlec and Schwarz 2008, Vinué *et al*. 2008). The *sat* gene, which encodes a streptothricin acetyltransferases, confers resistance to streptothricin. The transposition protein *tnsE* gene was also found in the class 2 integron structure. This arrangement of the class 2 integron is similar to integrons on the widely disseminated bacterial transposon Tn7.

The *sul2* gene is often associated with the resistance gene *strAB*, which is a streptomycin phosphotransferase enzyme conferring resistance to streptomycin (Enne *et al*. 2004). Although the *sul2* genes association with *strAB* is not regarded as mobile genetic element, it has been known to be associated with insertion elements such as *ISCR2* and *IS26* (Toleman *et al*. 2006, Parkhill *et al*. 2001). The data in this study shows the *sul* genes were often associated with a mobile genetic element and when an antimicrobial resistance is associated with a mobile genetic element, it may be difficult to eliminate (Enne *et al*. 2004).

7 Summary and Conclusion

- There was an unusually high resistance to penicillins in *Escherichia coli*.. There were nine isolates of *E. coli* resistant to cefotaxime. Additionally, all 12 *Enterobacter cloacae* isolates were resistant to penicillins and eight were resistant to cefotaxime.
- Most of the cefotaxime resistance was cause by the carriage and expression of the $bla_{\text{CTX-M-15}}$ gene.
- The $bla_{\text{CTX-M-15}}$ gene was predominantly carried on IncF plasmids and its spread was largely associated with the transfer of these plasmids from one isolate to another.
- Most *E. coli* isolates containing the $bla_{\text{CTX-M-15}}$ gene were from related but not identical variants of the ST131 clone.
- There was an absence of the *intI*1 integrase but this did not appear to exclude the presence of the gene cassette which was found in most of the $bla_{\text{CTX-M-15}}$ gene-containing isolates.
- This is the first report of $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and PMQR *aac(6')-Ib-cr* genes co-existing in the same *E. coli* bacterial cell in the United Kingdom.
- This is the first report of F33:A1:B26 plasmid carrying $bla_{\text{CTX-M-15.}}$
- This is also the first report of $bla_{\text{CTX-M-14}}$ in *Ent. cloacae* in the UK.
- This is the first report of $bla_{\text{CTX-M-14}}$ and $bla_{\text{VIM-4}}$ gene in complex with an IS*CR1* element to be identified in a *Ent. cloacae* clinical isolate anywhere in the World. The results suggest that there is microevolution of these strains in Egypt.
- This study further provides evidence that the huge reduction in the use of sulfonamides has not eliminated the presence of the *sul* gene. Rather the

association of this gene within an integron, which contains other genes that confer resistance to antibiotics currently used, contributes to the maintenance of *sul* gene through co-selection.

8 References

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Appendix A

Sequence of IncF allele.

FII-2

CAAAAACCCCGATAATCTTCTTCAACTTTGGCGAGTACGAAAAGATTACCGGGGCCC
ACTTAAACCGTATAGCCAACAATTCAGCTATGCGGGGAGTATAGTTATATGCCCGGA
AAAGTTCAAGACTTCTTTCTGTGCTCGCTCCTTCTGCGCATTG

FII-33

CGAAAACCCCGATAATCTTCTTTAACTTTGGCGAGTCAGAAAGATTACCGGGGCTAA
CAAGAAACTGCATAGAAGCTGTTGCTCTATGCGGGGAGTATAGTTATATGCCCGGAA
AAGTTCAAGACTTCTTTCTGTGCTCACTCCTTCTGTGCAACA

FIA-1

TTACCCTCAAATGACAGTCCTGTCTGTGACAAATTGCCCTTAACCCTGTGACAGATT
GCCCTCAGAAGAAGCTGTTTTTTCACAAAGTTATCCCTGCTTATTGACTCTTTTTTA
TTTAGTGTGACAATCTAAAAACTTGTCACACTTCACATGGATCTGTCATGGCGGAAA
CAGCGGTTATCAATCACAAGAAACGTAAAAATAGCCCGCGAATCGTCCAGTCAAACG
ACCTCACTGAGGCGGCATATAGTCTCTCCCGGGATCAAAAACGTATGCTGTATCTGT
TCGTTGACCAGATCAGAAAATCTGATGGCACCCTACAGGAACATGACGGTATCTGCG
AGATCCATGTTGCTAAATATGCTGAAATATTCGGATTGACCT

F1B-26

ATTCAGACATCAAAAAACTGTTCGGCGAGGTGGATAAGTCGTCCGGTGAGCTGGTGA
CACTGACACCAAACAATAACAACACCGTACAACCTGTGGCGCTGATGCGTCTGGGCG
TTTTTGTACCGACCCTTAAATCACTGAAGAACAGTAAAAAAAATACACTGTCACGTA
CTGATGCCACGGAAGAGCTGACACGTCTTTCCCTGGCCCGTGCTGAGGGATTCGATA
AGGTTGAGATCACCGGCCCCCGGCTGGATATGGATAACGATTTCAAGACCTGGGTGG
GGATCATTCATTCCTTTGCCCGCCATAACGTGATTGGTGACAAAGTTGAACTGCCTT
TTGTCGAGTTTGCAAAACTGTGTGGTATACC

Appendix B

Antimicrobial susceptibility of all the isolates in this study (MIC mg/L)

		Break point (Mg/L)											zone diameter (mm)		
		4	16	4	2	2	4	2	8	4	4	1	12	13	16
Isolates		SXT	PIP	CAZ	CTX	ATM	GEN	FEP	AMP	MER	IMP	ERT	STREP	SPEC	KAN
E.clo	3	>128	128	8	>128	4	0.5	4	>128	0.25	0.015	2	11	0	15
E.clo	18	>128	128	8	>128	64	0.5	4	>128	0.12	0.25	4	14	0	14
E.clo	69	>128	64	4	>128	4	0.5	4	>128	0.25	0.03	4	13	0	13
E.clo	156	>128	128	4	>128	>128	0.25	8	>128	0.25	0.06	2	14	0	13
E.clo	157	>128	64	4	>128	>128	2	4	>128	0.12	0.12	2	13	0	15
E.clo	167	>128	64	16	>128	>128	0.5	4	>128	0.12	0.06	2	14	0	15
E.coli	35	>128	128	32	128	>128	8	32	>128	0.03	0.03	0.015	13	15	16
E.coli	58	>128	128	64	>128	>128	64	64	>128	0.03	0.06	2	8	13	12
E.coli	71	>128	128	64	>128	>128	64	64	>128	0.03	0.03	1	0	0	11
E.coli	202	>128	64	16	64	>128	8	16	>128	<0.008	0.015	0.06	16	12	17
E.coli	270	>128	128	128	>128	>128	64	64	>128	0.03	0.06	0.015	8	13	12
E.coli	274	>128	>128	128	>128	>128	64	>128	>128	0.015	<0.008	0.12	8	12	12
E.coli	280	>128	>128	128	>128	>128	64	128	>128	0.015	0.12	0.03	8	13	12
E.coli	7	64	4	0.12	0.25	0.25	0.5	0.25	4	<0.008	0.03	0.12	10	16	14
E.coli	6	16	8	0.5	0.5	0.5	0.03	8	16	0.03	0.06	0.015	10	18	16
Cup.Var	204	2	128	32	4	16	16	4	>128	4	8	64	8	12	10
E.coli	116	8	128	0.25	0.12	0.12	0.015	0.25	>128	0.06	0.03	0.12	17	16	15
E.coli	265	4	128	0.03	0.06	0.12	0.06	0.015	>128	<0.008	0.015	0.03	10	18	17
E.coli	189	128	128	0.060	0.015	0.12	0.12	0.12	>128	0.015	0.06	0.12	10	11	18
E.coli	70	4	0.12	0.015	0.03	0.12	0.25	0.12	0.25	0.25	<0.008	0.015	16	8	18
E.coli	97	8	64	0.5	0.12	0.25	0.12	0.25	128	0.03	0.06	0.12	17	16	15
E.coli	252	128	64	4	0.25	0.25	0.03	0.015	>128	0.06	0.015	0.03	11	0	16
E.coli	155	64	128	0.12	0.015	0.25	0.25	0.12	>128	0.25	0.03	0.12	14	13	18
E.coli	128	4	128	0.03	0.25	0.12	0.12	0.12	>128	0.015	<0.008	0.06	9	19	0
E.asb	192	8	64	0.060	0.12	0.12	0.25	0.015	128	0.25	0.06	0.015	10	21	19
Ste.mal	8	>128	128	16	32	128	8	128	>128	4	128	8	0	13	10
K.pne	122	128	64	0.25	0.5	0.25	0.12	0.12	>128	0.25	0.03	0.06	16	11	17
E.coli	219	>128	0.25	0.5	0.25	0.25	0.03	0.015	1	0.03	0.06	0.03	16	13	15

		Break point (Mg/L)											zone diameter (mm)		
Isolates		4	16	4	2	2	4	2	8	4	4	1	12	13	16
		SXT	PIP	CAZ	CTX	ATM	GEN	FEP	AMP	MER	IMP	ERT	STREP	SPEC	KAN
E.clo	4	32	16	0.12	0.015	0.25	0.015	0.25	8	0.12	0.015	0.12	10	13	15
E.coli	92	64	64	0.03	0.12	0.12	0.06	0.015	>128	0.015	0.06	0.015	17	15	18
E.coli	13	128	64	0.060	0.03	0.25	0.12	0.12	128	0.25	0.12	0.12	0	10	0
E.coli	266	4	32	0.12	0.25	0.12	0.03	0.015	128	0.03	0.06	0.015	0	16	10
E.coli	267	4	64	0.03	0.12	0.03	0.015	0.25	128	0.12	0.015	0.12	9	13	18
E.coli	10	64	16	0.25	0.5	0.06	0.06	0.015	2	0.015	0.12	0.03	8	16	20
E.coli	36	4	64	0.5	0.015	0.015	8	0.12	128	0.06	0.03	0.12	10	15	15
E.coli	121	4	64	0.060	0.25	0.25	0.12	0.015	>128	0.25	0.12	0.06	18	17	15
E.coli	98	4	64	0.12	0.12	0.12	0.5	0.015	>128	<0.008	<0.008	0.015	9	15	15
E.coli	209	128	64	0.03	0.03	0.25	0.25	0.25	>128	0.25	0.06	0.12	0	9	15
E.coli	268	4	128	0.25	0.25	0.12	0.03	0.015	>128	0.03	0.015	0.03	9	13	20
E.coli	170	128	32	0.015	0.015	0.25	0.12	0.12	64	0.25	0.12	0.12	8	17	20
E.coli	5	32	16	4	4	0.12	0.015	0.015	4	<0.008	<0.008	0.06	9	13	15
E.coli	105	64	64	0.060	0.5	0.015	0.06	0.03	128	0.06	0.06	0.015	9	12	0
E.coli	117	4	64	0.03	0.12	8	0.12	0.25	>128	0.25	0.12	0.12	15	18	19
E.clo	193	128	128	32	32	>128	0.25	8	>128	0.015	0.015	0.03	7	0	18
E.clo	206	128	128	8	64	>128	0.03	4	>128	0.25	0.06	0.12	8	0	18
E.clo	176	0.060	8	0.25	0.5	0.12	0.25	0.015	16	0.03	0.03	0.12	17	21	17
E.clo	207	0.12	4	0.12	0.12	0.06	0.12	0.03	8	0.12	0.015	0.015	16	18	20
K.pne	72	0.5	16	0.5	0.25	0.12	0.03	0.12	32	0.015	0.12	0.06	19	19	17
K.pne	94	0.12	16	0.060	0.015	0.03	0.015	0.015	64	0.015	<0.008	0.12	20	21	19
K.pne	200	0.25	16	0.03	0.5	0.25	0.06	0.06	16	0.03	0.12	0.03	18	18	18
K.pne	201	0.12	16	0.015	0.03	0.06	0.03	0.015	32	0.015	0.06	0.12	19	19	20
K.pne	111	0.5	4	0.5	0.25	0.015	0.12	0.12	8	0.03	0.015	0.015	16	18	17
K.pne	261	0.060	32	0.12	0.06	0.06	0.015	0.25	64	0.015	0.12	0.06	17	20	20
K.pne	259	0.12	8	0.25	0.12	0.25	0.06	0.06	16	0.015	0.03	0.015	15	18	17
K.oxy	248	0.25	16	0.5	0.5	0.03	0.5	0.015	32	0.25	0.12	0.03	19	19	19
K.oxy	246	0.03	16	0.25	0.06	0.12	0.25	0.12	8	0.03	0.12	0.015	20	18	17

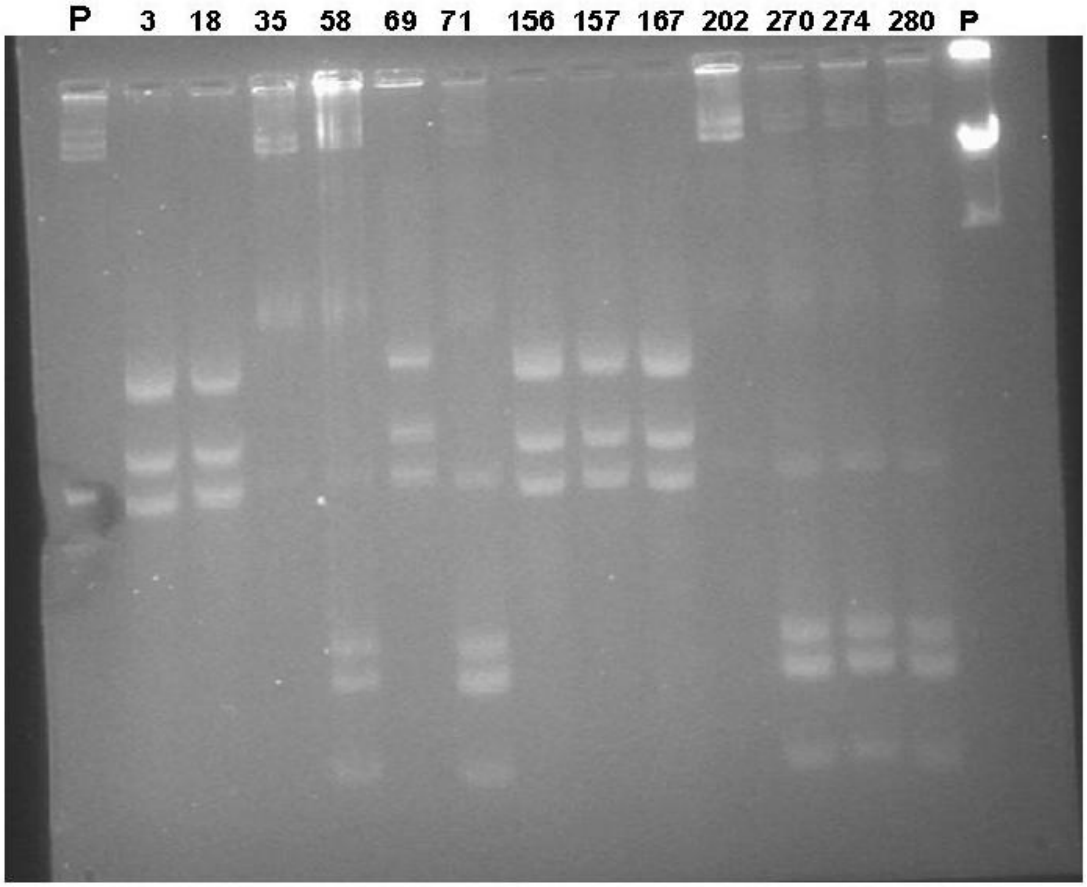
		Break point (Mg/L)											zone diameter (mm)		
		4	16	4	2	2	4	2	8	4	4	1	12	13	16
Isolates		SXT	PIP	CAZ	CTX	ATM	GEN	FEP	AMP	MER	IMP	ERT	STREP	SPEC	KAN
K.oxy	154	0.5	16	0.5	0.015	0.06	0.03	0.03	8	0.25	0.12	0.12	16	17	20
K.oxy	28	0.060	4	0.12	0.25	0.015	0.12	0.06	8	<0.008	0.5	0.015	17	19	17
E.coli	218	0.25	16	0.060	0.03	0.12	0.25	0.015	16	0.06	0.12	0.015	19	18	18
E.coli	220	0.12	32	0.03	0.06	0.06	0.015	0.12	64	0.12	0.06	0.03	18	20	17
E.coli	221	0.5	4	0.015	0.5	0.015	0.06	0.25	8	0.015	0.015	0.12	17	17	17
E.coli	226	0.25	16	0.060	0.12	0.03	0.03	0.12	32	0.25	0.06	0.03	16	19	20
E.coli	234	0.5	8	0.5	0.03	0.12	0.25	0.25	16	0.12	0.12	0.06	15	18	17
E.coli	91	1	1	0.12	0.25	0.25	0.12	0.06	2	<0.008	<0.008	0.015	18	21	19
E.coli	190	0.12	0.06	0.25	0.015	0.12	0.015	0.25	0.5	0.25	0.12	0.06	20	17	17
E.coli	177	0.060	0.25	0.060	0.12	0.03	0.06	0.015	0.5	0.03	0.015	0.03	19	19	18
E.coli	140	0.25	0.25	0.03	0.06	0.06	0.03	0.12	0.5	0.06	0.06	0.12	17	17	17
E.coli	9	1	0.12	0.5	0.03	0.015	0.25	0.25	0.5	0.25	0.03	0.06	16	18	20
E.coli	245	0.5	16	0.015	0.25	0.06	0.12	0.03	16	0.015	0.12	0.015	15	20	17
E.coli	101	2	32	0.25	0.06	0.12	0.25	0.06	64	0.25	0.12	0.06	18	17	20
E.coli	171	0.060	0.12	0.12	0.015	0.25	0.03	0.015	0.5	0.03	0.03	0.03	20	19	18
E.coli	208	0.25	8	0.5	4	0.06	0.25	0.12	16	0.25	0.12	0.12	16	18	17
E.coli	37	2	0.12	0.060	0.06	0.015	0.12	0.03	1	<0.008	<0.008	0.06	17	21	19
E.coli	63	0.5	16	0.03	0.5	0.06	0.25	0.25	16	0.12	0.12	0.015	20	17	17
E.coli	65	0.060	16	0.015	0.12	0.12	0.25	0.06	32	0.06	0.06	0.06	19	19	20
E.coli	15	0.12	0.5	0.03	0.25	0.03	0.03	0.25	1	0.25	0.015	0.03	17	18	19
E.coli	59	0.25	0.5	0.5	0.03	0.25	0.5	0.12	1	0.015	0.12	0.015	16	20	17
E.coli	60	2	16	0.060	0.5	0.06	0.25	0.03	32	0.25	0.03	0.03	15	17	18
E.coli	73	0.5	1	0.5	0.015	0.015	0.015	0.06	0.5	0.03	0.12	0.015	18	18	17
E.coli	52	2	16	0.12	0.06	0.06	0.06	0.25	32	0.12	<0.008	0.06	19	21	19
E.coli	17	0.060	0.25	0.25	0.25	0.12	0.03	0.015	0.5	0.25	0.06	0.12	16	17	17
E.coli	19	0.25	16	0.060	0.12	0.25	0.25	0.06	16	<0.008	0.12	0.06	17	20	17
E.coli	29	0.12	1	0.015	0.06	0.06	0.12	0.03	0.25	0.25	0.015	0.03	15	19	20
E.coli	257	0.5	1	0.03	0.5	0.25	0.25	0.015	0.5	0.06	0.12	0.015	20	21	17

Isolates		Break point (Mg/L)											zone diameter (mm)		
		4	16	4	2	2	4	2	8	4	4	1	12	13	16
		SXT	PIP	CAZ	CTX	ATM	GEN	FEP	AMP	MER	IMP	ERT	STREP	SPEC	KAN
E.coli	258	0.060	16	0.5	0.03	0.12	0.5	0.03	16	0.12	0.03	0.06	15	21	17
E.coli	260	0.25	16	0.25	0.06	0.06	0.03	0.12	32	0.015	0.12	0.12	16	17	18
E.coli	276	2	1	0.5	0.25	0.03	0.25	0.015	0.5	0.25	0.06	0.03	20	18	17
E.coli	166	0.5	2	0.12	0.06	0.12	0.015	0.06	1	0.03	<0.008	0.06	19	21	18
E.coli	182	0.060	16	0.5	0.12	0.06	0.06	0.015	16	<0.008	0.12	0.015	17	17	17
E.coli	47	0.25	1	0.060	0.03	0.015	0.03	0.12	0.25	<0.008	0.03	0.12	16	21	19
E.coli	281	0.12	32	0.03	0.015	0.06	0.5	0.03	64	0.25	0.06	0.06	15	17	17
Ser.mar	263	0.5	32	0.015	0.06	0.25	0.12	0.06	32	0.06	<0.008	0.12	18	20	18
Ser.mar	272	0.5	16	0.5	0.03	0.06	0.03	0.03	8	0.03	0.03	0.06	20	19	17
Ser.mar	264	1	32	0.25	0.5	0.12	0.015	0.12	64	<0.008	0.015	0.12	16	20	19
M.morg	279	0.5	2	0.12	0.25	0.03	0.06	0.06	4	0.06	<0.008	0.015	17	18	17
M.morg	247	1	16	0.25	0.12	0.06	0.03	0.12	32	0.015	0.06	0.03	19	20	20
Ste.mal	253	32	32	8	16	16	2	8	64	64	32	8	2	10	8
Ste.mal	1	32	16	16	32	8	8	16	32	4	128	16	4	7	9
Ste.mal	2	>128	32	8	16	4	16	32	32	4	64	4	5	9	10
Ste.mal	16	64	64	16	64	4	16	32	64	8	64	32	6	5	12
E.clo	E1720	>128	>128	64	>128	>128	8	8	>128	64	>128	64	-	-	-
E.clo	E4293	>128	>128	64	>128	>128	8	8	>128	64	>128	32	-	-	-
E.clo	E4303	>128	>128	32	>128	>128	8	8	>128	8	2	8	-	-	-

Abbreviations: CTX = cefotaxime; CAZ = ceftazidime; FEP = cefepime; PIP = piperacillin; SXT = co-trimoxazole; GEN = gentamicin; ERT = ertapenem; IMI = imipenem; MER = meropenem; ATM=aztreonam; AMP=ampicillin; STREP = streptomycin; SPEC = spectinomycin; KAN = kanamycin

Appendix C

A plasmid gel picture showing detectable plasmids.



Appendix D.

Published Papers.