# Molecular epidemiology of extended-spectrum beta-lactamase (ESBL) carrying Enterobacteriaceae at ABM University Health Board.

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# Molecular epidemiology of extended-spectrum β-lactamase (ESBL) carrying *Enterobacteriaceae* at ABM University Health Board

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## Summary

Extended-spectrum beta-lactamases (ESBL) mediate resistance to 3rd generation cephalosporins and aztreonam in *Enterobacteriaceae* and pose major clinical problems. Screened *Enterobacteriaceae* were collected from PHW Microbiology ABM Swansea laboratory and were demonstrated phenotypically to be ESBL-producers by BSAC methods. Isolates were identified using the BD Phoenix™ Automated system and Bruker Daltonics MALDI Biotyper. 138 isolates were genetically defined as ESBL-producers (103 *Escherichia coli*, 32 *Klebsiella* spp., 2 *Enterobacter cloacae* and 1 *Citrobacter freundii*) and 4 isolates (2 *E. coli*, 1 *Enterobacter cloacae* and 1 *Morganella morganii*) were genetically confirmed as AmpC-producers.

PCR analysis revealed that the most prevalent ESBLs were CTX-M (n=133), predominantly Group 1 (n=128), of which 51% (66/128) contained the *IS*26-CTX-M-15 link region, which is characteristic for epidemic *E. coli* strain A. PFGE confirmed that these isolates had a clonal relatedness to epidemic *E. coli* strain A. Allele-specific PCR revealed that all *E. coli* positive for *IS*26-CTX-M-15 belonged to clone O25b-ST131 (found internationally), which has a high virulence potential and encompasses diverse PFGE patterns.

With the molecular epidemiology established; the sensitivity and performance of phenotypic screening and confirmatory assays were analysed so that optimal strategies to handle difficult-to-identify ESBL resistance traits could be determined. In this study, the sensitivity of ESBL screening increased to 100% when ceftazidime was used alongside cefpodoxime.

Isolates harbouring ESBL genes are often difficult to treat, as options are greatly limited. Susceptibility to various well-established antibiotics, along with temocillin and tigecycline, were investigated. Temocillin and tigecycline were effective against 98% and 89% of all isolates tested. The carbapenems were the most active antibiotics with 100% of isolates susceptible to imipenem and meropenem and 99% susceptible to ertapenem.

Biofilm production in *E. coli* was also investigated. The *pgaABCD* gene locus was detected in all ESBL and AmpC-producing *E. coli* isolates (n=105); however, only 38% of these produced a phenotypic biofilm.

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## Abbreviations

| | |
|---|---|
| **AST** | Antimicrobial susceptibility testing |
| **AUG** | Augmentin/co-amoxiclav |
| **bp** | Base pair |
| **BSAC** | British Society for Antimicrobial Chemotherapy |
| **CAZ** | Ceftazidime |
| **CFP** | Cefpirome |
| **CPD** | Cefpodoxime |
| **CPM** | Cefepime |
| **CTX** | Cefotaxime |
| **CXM** | Cefuroxime |
| **CV** | Clavulanic acid |
| **EUCAST** | European Committee on Antimicrobial Susceptibility Testing |
| **ESBL** | Extended-spectrum β-lactamase |
| **FOX** | Cefoxitin |
| **DNA** | Deoxyribonucleic acid |
| **dNTP** | Deoxynucleotide 5'-triphosphate |
| **$dH_2O$** | Deionised water |
| **EDTA** | Ethylenediaminotetraacetic acid |
| **g** | Grams |
| **HCl** | Hydrochloric acid |
| **IS** | Insertion sequence |

| | |
|---|---|
| **Kb** | Kilobase pairs |
| **L** | Litres |
| **LB** | Luria Bertani |
| **LMP** | Low melt point |
| **LPS** | Lipopolysaccharide layer |
| **MALDI-TOF/MS** | Matrix-assisted laser desorption ionization time-of-flight/mass spectrometry |
| **MIC** | Minimum inhibitory concentration |
| **ml** | Millilitres |
| **µg** | Micrograms |
| **µl** | Microlitres |
| **mM** | Millimolar |
| **mm** | Millimetres |
| **MLST** | Multilocus sequence typing |
| **NAMA** | *N*-acetylmuramic acid |
| **nm** | nanometres |
| **NaCl** | Sodium chloride |
| **NAG** | *N*-acetylglucosamine |
| **$NFH_2O$** | Nuclease Free Water |
| **PBPs** | Penicillin binding proteins |
| **PCR** | Polymerase chain reaction |
| **PFGE** | Pulsed-field gel electrophoresis |

| | |
|---|---|
| **PGA** | Poly-β-1, 6-N-acetyl-D-glucosamine |
| **RFLP** | Restriction fragment length polymorphism |
| **RPM** | Revolutions per minute |
| **TE** | Tris-EDTA buffer |
| **TAE** | Tris-Acetate-EDTA |
| **Tris** | Tris(hydroxymethyl)aminomethane |
| **UDP** | Uridine diphosphate |
| **w/v** | Weight by volume |

# Chapter 1: Introduction

The introduction chapter will give a fundamental overview of the aspects involved in this thesis. Each results chapter with then provide more indepth information along with discussion points.

## 1.1 *Enterobacteriaceae*

The *Enterobacteriaceae* are a large heterogeneous family of Gram-negative straight rods, with an approximate length of 1.5μm. They are non-spore forming bacteria that share many common characteristics. Generally, *Enterobacteriaceae* are catalase positive, oxidase negative organisms that can ferment many carbohydrates and reduce nitrate to nitrite (Mims, 1998, Brooks, 2007). They can be found with or without a capsule and be motile or non-motile (depending on the presence of flagella) (Brooks, 2007). The term *Enterobacteriaceae* was first established in 1937 by Otto Rahn to describe morphologically and biochemically similar organisms (Bacteriology, 1981, Janda, 2006). Common genera of *Enterobacteriaceae* include *Escherichia*, *Klebsiella*, *Enterobacter*, *Proteus*, *Salmonella*, *Shigella*, *Serratia*, *Citrobacter*, *Morganella*, *Yersinia*, *Hafnia* and *Providencia* (Mims, 1998). In 1984, there were 69 recognised species of *Enterobacteriaceae* (Farmer et al., 1985) whereas now there are in excess of 170. Primarily located (often as commensal flora) within the intestinal tract of human and animals, *Enterobacteriaceae* are one of the most abundant facultative anaerobes of this region making up approximately $10^9$ CFU/g of faeces (Mims, 1998).

*Enterobacteriaceae* can be serotyped based upon the presence of antigens. There are three main antigens outlined by Edwards and Ewing (1972) and these are known as the O (somatic), H (flagellar) and K (capsular). The O antigen is a heat-stable somatic antigen consisting of a phospholipid and polysaccharide complex. This complex is composed of approximately 60% polysaccharides, 20-30% lipids and 3.5-4.5% hexosamine. It is the nature of the terminal group along with the order found within the polysaccharide chain that determines the specificity of the O antigen. The H antigen or flagella are heat-labile proteins which are composed of flagellins. Specificity of the H antigen is determined by the amino acid content and order found within the flagellin (Edwards and Ewing, 1972). The flagella are

essential for bacterial motility (Cooke, 1974). The K antigen or capsule is made up of polysaccharides. It can be subdivided based upon physical and chemical properties (Edwards and Ewing, 1972). The capsule is outside of the cell wall, important serologically (Cooke, 1974) and is often associated with protecting the cell (Johnson, 1991).

### 1.1.1 *Escherichia coli*

The *Escherichia* genus was discovered by Theodor Escherich (1857-1911). He first described the bacterium *Bacterium coli commune*, which was later commonly known as *Escherichia coli* (Sussman, 1985). There are 7 species found within the genus of *Escherichia* (Table 1.1) (http://www.bacterio.cict.fr). However, *E. coli* is the species of medical importance. *E. coli* is a Gram-negative, mobile, non-fastidious, bile tolerant lactose fermenting organism, which is capable of growth at temperatures up to 44°C (Mims, 1998).

*E. coli* can be divided up into four phylogenetic groups known as A, B1, D and B2. It is thought that groups A and B1 are commensal-associated whereas groups D and B2 are associated with extraintestinal *E. coli* (Clermont et al., 2009, Picard et al., 1999). *E. coli* is usually located in the gut of humans and animals and is often found to colonise the lower end of the urethra and vagina. It is an opportunistic pathogen which is commonly associated with urinary-tract infections, wound infections following surgery, hospital-acquired pneumonia, infections of the gastrointestinal tract (diarrheal disease), neonatal meningitis and sepsis. Transmission of *E. coli* may be via the faecal-oral route or food-borne; infection may also be endogenously contracted (Mims, 1998).

Using serotyping, O (somatic), H (flagellar), K (capsular) antigens can be identified, as described above. Additionally, *E. coli* also have F (fimbrial) antigens (Mims, 1998). Fimbriae are associated with adhesion and conjugation but not motility (Cooke, 1974). The two main types of fimbriae found in *E. coli* are known as P fimbriae and type I fimbriae. These fimbriae are important in pathogenesis as they aid in the adherence of the bacteria to the host cell, thereby, permitting bacterial colonisation and stimulating inflammation. Additionally, colonising factors e.g. CFA I and II, K88 and K99 are adhesins associated with gastrointestinal tract infections

(Mims, 1998). In some *E. coli* strains, the capsule may also play a role in adhesion, as well as providing protection from phagocytosis (Johnson, 1991). The K1 capsular type is associated with neonatal meningitis (Mims, 1998). The polysaccharide capsule, associated adhesins and exopolysaccharide production e.g. colanic acid and poly-β-1, 6-N-acetyl-D-glucosamine (PGA) (Wang et al., 2004) can also play a role in the ability of *E. coli* to form a biofilm (Pratt and Kolter, 1998). Biofilms are a strategy of bacterial survival (Hall-Stoodley and Stoodley, 2009), as they play a role in protection (Costerton et al., 1999) and due to a complex structure, they can arguably prevent antimicrobial agents from penetrating (Jefferson et al., 2005) and can provide protection from phagocytes. Biofilms are discussed in Chapter 8.

Other virulence factors associated with *E. coli* include the production of haemolysin (capable of lysing some blood cells), siderophores e.g. aerobactin (promotes bacterial growth) and serum resistance (this allows the bacteria to escape killing by the innate immune system as it blocks the complement cascade) (Johnson, 1991). Additionally, some enteric *E. coli* are also associated with enterotoxins e.g. in ETEC (enterotoxigenic *E. coli*), a cholera-like heat-labile toxin (LT) is produced and in EHEC (enterohaemorrhagic *E. coli*), shiga-like cytotoxin or verotoxin associated with haemolytic uremic syndrome is produced (Mims, 1998, Nataro and Kaper, 1998).

Table 1.1 *Escherichia* species (http://www.bacterio.cict.fr)

| No. | ***Escherichia* species** |
|---|---|
| 1. | *Escherichia adecarboxylata* |
| 2. | *Escherichia albertii* |
| 3. | *Escherichia blattae* |
| 4. | *Escherichia coli* |
| 5. | *Escherichia fergusonii* |
| 6. | *Escherichia hermannii* |
| 7. | *Escherichia vulneris* |

### 1.1.2 *Klebsiella* spp.

Named after the German bacteriologist, Edwin Klebs (1834-1913) (http://www.bacterio.cict.fr), the genus *Klebsiella* are represented by Gram negative, non-motile, non-fastidious, oxidase negative, lactose fermenting encapsulated rod-shaped bacteria which have the capability of aerobic and anaerobic respiration (Mims, 1998). Originally, the taxonomy of *Klebsiella* divided isolates into three species according to the disease types caused. These were *K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis* (Podschun and Ullmann, 1998). However, there are now 12 species and 3 subspecies listed (Table 1.1.1) (http://www.bacterio.cict.fr).

There are currently three classification systems used to group the *Klebsiella* spp. known as the Cowan, Barcomb and Ørskov systems. Differences primarily lie with the classification of *K. pneumoniae*. The Ørskov system identifies *K. pneumoniae, K. ozaenae* and *K. rhinoscleromatis* to be subspecies of *K. pneumoniae* whereas the Cowan and Bascomb systems list them as individual species. In the UK and ex-Commonwealth countries, the Cowan system is used. However, in the USA and parts of Europe, the Ørskov system is followed (Podschun and Ullmann, 1998).

*Klebsiella* are opportunistic bacteria, which usually colonise the intestinal tract and often the nasalpharynx of humans. However, *Klebsiella* has also been found to inhabit the mucosal surfaces of some animals and is also ubiquitous in the environment e.g. in soil and water (Mims, 1998, Podschun and Ullmann, 1998).

*K. pneumoniae* is thought to the most medically important species of *Klebsiella* and infection is associated with hospitalised immunocompromised patients who often have underlying disorders such as diabetes mellitus and chronic obstructive pulmonary disease. *Klebsiella* can cause a number of medical conditions such as sepsis, pneumonia, urinary-tract infections and soft tissue infections (Podschun and Ullmann, 1998) and the transmission of infection is usually through contact spread or is endogenous (Mims, 1998).

Using serotyping, two main antigens can be identified for *Klebsiella* spp; the O (somatic) and K (capsular). Surrounding the bacterium is a well developed and

complex polysaccharide capsule, which gives it protection from phagocytosis and also its characteristic mucoid appearance. Also surrounding the bacterium are a number of pili (type 1 and type 3) adhesins, which allow the bacteria to adhere to host cells (Mims, 1998, Podschun and Ullmann, 1998). These virulence factors prepare the *Klebsiella* for causing infection.

Table 1.2 *Klebsiella* species and subspecies (http://www.bacterio.cict.fr)

| No. | ***Klebsiella* species and subspecies** |
|---|---|
| 1. | *Klebsiella granulomatis* |
| 2. | *Klebsiella mobilis* |
| 3. | *Klebsiella ornithinolytica* |
| 4. | *Klebsiella oxytoca* |
| 5. | *Klebsiella ozaenae* |
| 6. | *Klebsiella planticola* |
| 7. | *Klebsiella pneumonia* |
| | *Klebsiella pneumoniae* subsp. *ozaenae* |
| | *Klebsiella pneumoniae* subsp. *pneumonia* |
| | *Klebsiella pneumoniae* subsp. *rhinoscleromatis* |
| 8. | *Klebsiella rhinoscleromatis* |
| 9. | *Klebsiella singaporensis* |
| 10. | *Klebsiella terrigena* |
| 11. | *Klebsiella trevisanii* |
| 12. | *Klebsiella variicola* |

### 1.1.3 Other *Enterobacteriaceae*

As listed in 1.1, there are many genera associated with the *Enterobacteriaceae* family. In this study, *E. coli* and *Klebsiella* spp. were the main focus. However, *Enterobacter* spp., *Citrobacter* spp. and *Morganella morganii* were also investigated.

#### 1.1.3.1 *Enterobacter* spp.

The genus name of *Enterobacter* was proposed in 1960 by Hormaeche and Edwards (Hormaeche and Edwards, 1960). *Enterobacter* spp. are Gram-negative, lactose fermenting, bile tolerant, oxidase negative rod-shaped bacteria. *Enterobacter* spp. have non-fastidious growth and can grow aerobically and anaerobically. These bacteria usually colonise the gastrointestinal tract of humans and animals and can also be found in the environment. Infection with *Enterobacter* spp. is associated with the urinary and respiratory tracts (Mims, 1998). The *Enterobacter* genus can be divided into 24 species and 2 subspecies (Table 1.3) (http://www.bacterio.cict.fr).

Table 1.3 *Enterobacter* species and subspecies (http://www.bacterio.cict.fr)

| No. | The *Enterobacter* species and subspecies |
|---|---|
| 1. | *Enterobacter aerogenes* |
| 2. | *Enterobacter agglomerans* |
| 3. | *Enterobacter amnigenus* |
| 4. | *Enterobacter arachidis* |
| 5. | *Enterobacter asburiae* |
| 6. | *Enterobacter cancerogenus* |
| 7. | *Enterobacter cloacae* |
| | *Enterobacter cloacae* subsp. *cloacae* |
| | *Enterobacter cloacae* subsp. *dissolvens* |
| 8. | *Enterobacter cowanii* |
| 9. | *Enterobacter dissolvens* |
| 10. | *Enterobacter gergoviae* |
| 11. | *Enterobacter helveticus* |
| 12. | *Enterobacter hormaechei* |
| 13. | *Enterobacter intermedius* |
| 14. | *Enterobacter kobei* |
| 15. | *Enterobacter ludwigii* |
| 16. | *Enterobacter nimipressuralis* |
| 17. | *Enterobacter oryzae* |
| 18. | *Enterobacter pulveris* |
| 19. | *Enterobacter pyrinus* |
| 20. | *Enterobacter radicincitans* |
| 21. | *Enterobacter sakazakii* |
| 22. | *Enterobacter soli* |
| 23. | *Enterobacter taylorae* |
| 24. | *Enterobacter turicensis* |

### 1.1.3.2 *Citrobacter* spp.

The *Citrobacter* genus is thought to be closely related to the genera of *Escherichia* and *Salmonella*. Members of this genus are Gram negative, non-sporeforming, facultative anaerobes that are motile by the means of peritrichous flagella (Dworkin and Falkow, 2006, Werkman and Gillen, 1932). As the name suggests, *Citrobacter* spp. utilise citrate as their carbon source. The genus name *Citrobacter* and its species *C. freundii* was determined by Werkman and Gillen in 1932. These bacteria were found to produce trimethylene glycol from glycerol (Werkman and Gillen, 1932). This organism, along with *C. koseri* are opportunistic pathogens that have been isolated from urine, blood and wounds (Dworkin and Falkow, 2006). There are 11 species of *Citrobacter* (Table 1.4) (http://www.bacterio.cict.fr).

Table 1.4 *Citrobacter* species (http://www.bacterio.cict.fr)

| No. | The genus *Citrobacter* |
|---|---|
| 1. | *Citrobacter amalonaticus* |
| 2. | *Citrobacter braakii* |
| 3. | *Citrobacter farmeri* |
| 4. | *Citrobacter freundii* |
| 5. | *Citrobacter gillenii* |
| 6. | *Citrobacter koseri* |
| 7. | *Citrobacter murliniae* |
| 8. | *Citrobacter rodentium* |
| 9. | *Citrobacter sedlakii* |
| 10. | *Citrobacter werkmanii* |
| 11. | *Citrobacter youngae* |

### 1.1.3.3 *Morganella* spp.

The *Morganella* spp. are Gram negative rods, which are also motile by the means of a peritrichous flagella. They are also capable of the deamination of phenylalanine and tryptophan. *Morganella* spp. are related to other *Enterobacteriaceae* such as *Proteus* and *Providencia* (Dworkin and Falkow, 2006).

Typically, *Morganella* spp. are commensal organisms that inhabit the gut of humans and animals. However, they may also colonise the respiratory and urinary tract. They are opportunistic bacteria and for example, the colonisation of urinary catheters can lead to urinary tract infection. Infection with *Morganella* spp. is associated with immunocompromised and in particular, hospitalised patients (Dworkin and Falkow, 2006). There are 2 species of *Morganella* found within the genus and 2 subspecies (Table 1.5) (http://www.bacterio.cict.fr).

Table1.5 *Morganella* species and subspecies (http://www.bacterio.cict.fr)

| No. | ***Morganella* species and subspecies** |
|---|---|
| 1. | *Morganella morganii* |
| | *Morganella morganii* subsp. *morganii* |
| | *Morganella morganii* subsp. *sibonii* |
| 2. | *Morganella psychrotolerans* |

### 1.2 The Gram-negative cell wall

The Gram-negative cell wall is a structure capable of protecting the cell (Beveridge, 1999). It contains an outer membrane which has a bilayer of phospholipids and a number of proteins/lipoprotein structures e.g. porins along with the lipopolysaccharide layer (LPS). The LPS consists of components such as lipid A and the carbohydrate based O antigens (outlined in 1.2.1). The inner membrane (cytoplasmic membrane) is also made up of a bilayer of phospholipids and contains the penicillin binding proteins (PBPs). Between the outer and inner membrane a 2-3 nm layer of peptidoglycan which is loosely cross linked can be found within the gel-like matrix known as the periplasmic space (Mims, 1998, Walsh, 2003, Greenwood, 2000, Beveridge, 1999). Peptidoglycan is a polymer consisting of glycan strands cross linked through short peptides (Schleifer and Kandler, 1972).

#### 1.2.1 The lipopolysaccharide layer (LPS)

The LPS layer varies between bacteria and has many functions. It is essential for bacterial survival, can cause some human disease e.g. septic shock (Wang and Quinn, 2010) and is also responsible for preventing the entry of large hydrophilic molecules. However, small hydrophilic molecules have the ability to enter through aqueous channels and porins in the outer membrane (Mims, 1998).

The LPS layer can be divided into three main components: lipid A, O antigen and core polysaccharides (Wang and Quinn, 2010). Lipid A is a hydrophobic constituent of the LPS, which is often responsible for the toxic effects in bacteria (Raetz and Whitfield, 2002). The O antigen plays a protective role against the environment (Wang and Quinn, 2010). It consists of a polysaccharide structure and this structure defines the O antigen's serological specificity. The numbers of O antigens vary from species to species. For example in *E. coli*, there are 170 O serotypes (Nataro and Kaper, 1998, Raetz and Whitfield, 2002). Core polysaccharides can be divided into two types: the inner core and outer core. The outer core acts as an attachment site for the O antigen and often serves as a serological specificity marker in organisms where no O antigen is produced. The inner core is well conserved and is associated with the lipid A proximal region (Raetz and Whitfield, 2002).

### 1.2.2 Peptidoglycan synthesis

Peptidoglycan, also known as mucopeptide or murein, is an essential component of the cell wall. It protects the cell from osmotic lysis and determines the cell shape (Jacobs et al., 1997). Peptidoglycan is made up from alternating units of $\beta$-1, 4-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAMA) (Schleifer and Kandler, 1972, Walsh, 2003, Greenwood, 2000, Mims, 1998). The first step of peptidoglycan synthesis (Figure 1.1) takes place in the cytoplasm and involves the formation of uridine diphosphate (UDP)-NAMA from UDP-NAG by the addition of a lactic acid moiety derived from phosphoenolpyruvate. Next, UDP-NAMA receives three amino acids, *L*-alanine, *D*-glutamic acid and *meso*diaminopimetic acid (or *L*-lysine in Gram positive organisms). Two *D*-alanine (*D*-ala) residues are formed from *L*-alanine, via the enzymes alanine racemase and *D*-alanine synthetase. The *D*-ala-*D*-ala residue is subsequently added to the aforementioned three amino acids associated with the NAMA, thus forming the NAMA-pentapeptide. This is passed from the cytoplasmic membrane to a lipid carrier in the cell membrane whereby UDP-NAG transfers NAG to the NAMA-pentapeptide. This unit is then transported across the cell membrane where it is added to the existing peptidoglycan in the cell wall. Once transported, final cross-linking via transpeptidation by penicillin binding proteins (PBPs) takes place (Mims, 1998, Walsh, 2003, Greenwood, 2000). PBPs are found in most bacteria and in *E. coli* they are numbered 1A, 1B, 2, 3, 4, 5 and 6 (Selwyn et al., 1980) (Figure 1.2). PBPs are encoded chromosomally and mutations may affect their number and affinity to β-lactam antibiotics (Katzung, 1995).

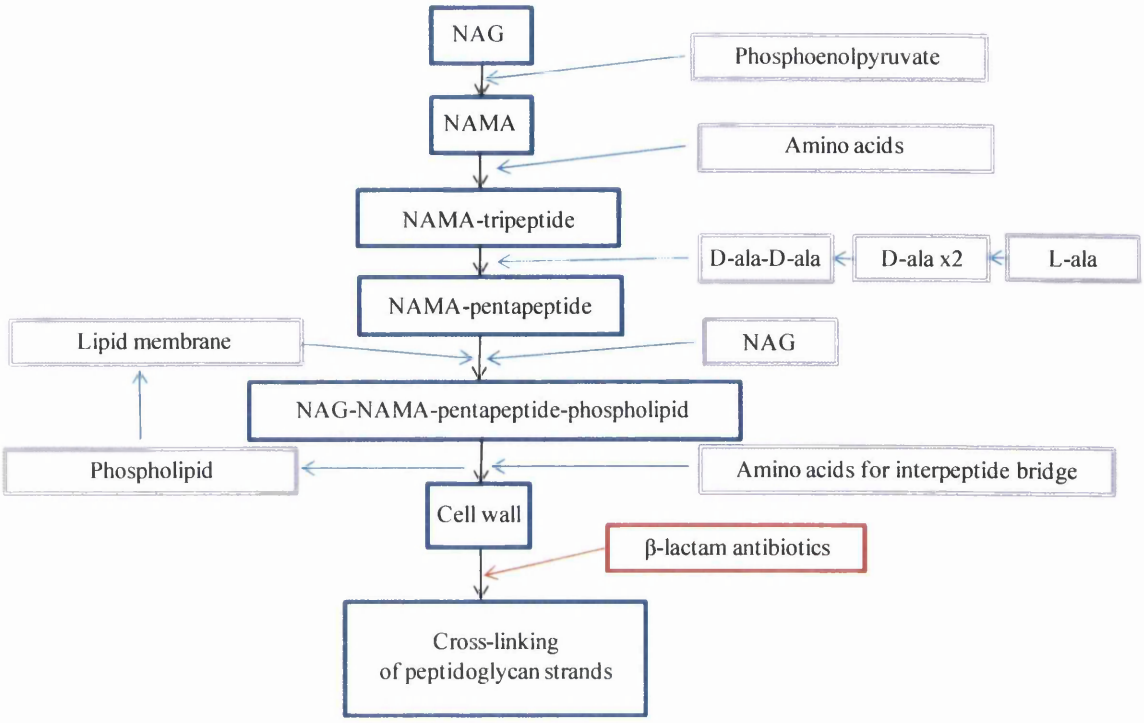



Figure 1.1 A simplified schematic diagram of bacterial cell wall synthesis and the site of action of β-lactam antibiotics. NAG – *N*-acetylglucosamine, NAMA – *N*-acetylmuramic acid and ala – alanine. Adapted from Greenwood (2000).

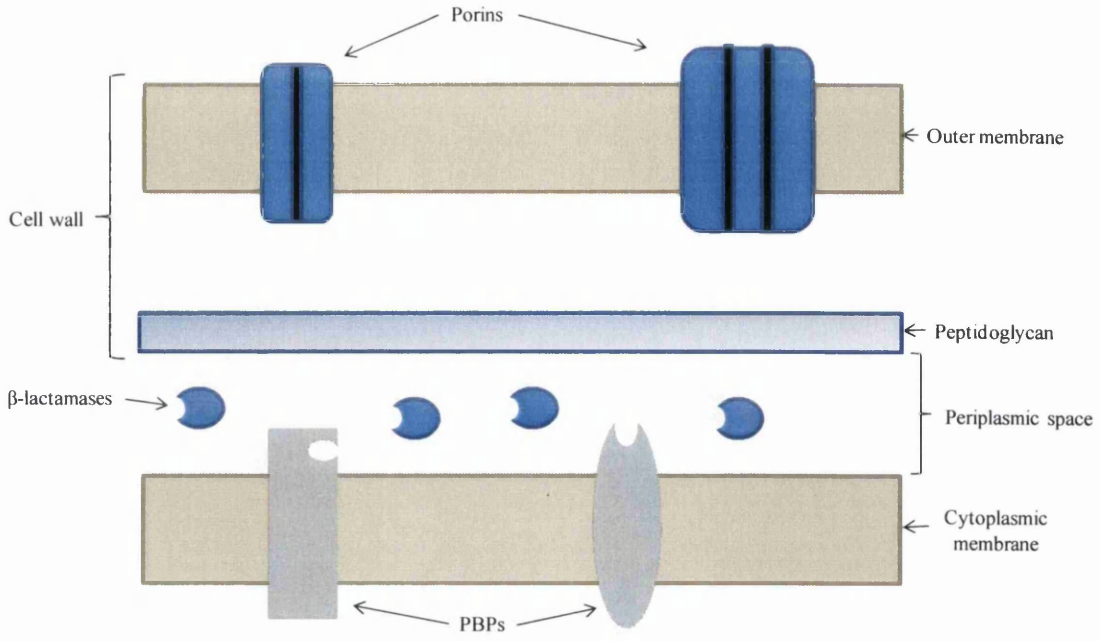



Figure 1.2 A simplified schematic diagram of the Gram-negative cell wall. The outer membrane is a lipid bilayer with hydrophilic proteins called porins. Beneath this membrane the thin peptidoglycan layer can be found sitting in the periplasmic space. The periplasmic space also contains β-lactamases. However, β-lactamases can also be found on the surface of the cytoplasmic membrane whereby penicillin binding proteins (PBPs) can also be found. Adapted from Katzung (1995).

### 1.3 β-lactam antibiotics

β-lactam antibiotics all contain a common β-lactam ring structure. Penicillins have a β-lactam ring that is fused to a 5 membered thiazolidine ring whereas the β-lactam ring in cephalosporins is fused to a 6 membered dihydrothiazine ring (Figure 1.3) (Greenwood, 2000, Samaha-Kfoury and Araj, 2003). The main focus of β-lactam antibiotics is to prevent the transpeptidation steps in the final stages of peptidoglycan synthesis (Mims, 1998, Katzung, 1995).

β-lactam antibiotics bind to bacterial cell receptors known as penicillin binding proteins (PBPs). It has been suggested that due to structural similarity between penicillins and cephalosporins to *D*-ala-*D*-ala, a component of peptidoglycan (see 1.2.2), the CO-N bond of the β-lactam ring in the antibiotic competes with the CO-N bond of *D*-ala-*D*-ala for the active site of the PBP transpeptidase enzyme (Katzung, 1995, Greenwood, 2000, Walsh, 2003).

Once the β-lactam antibiotic has bound to PBPs, the transpeptidase reaction is inhibited, as covalent penicilloyl complexes are formed between the PBPs and the β-lactam. As a result, the peptidoglycan lacks sufficient cross linkages (Selwyn et al., 1980, Katzung, 1995, Mims, 1998). Additionally, autolytic enzymes or hydrolases are activated in some microorganisms resulting in cell lysis in isotonic conditions or in the formation of spheroplasts in hypertonic conditions (Samaha-Kfoury and Araj, 2003, Mims, 1998).

#### 1.3.1 Penicillins

Obtained from the *Penicillium* mould, the original preparations of penicillins were found to contain a mixture of closely related compounds. These compounds were labelled as penicillin F, G, K and X (Selwyn et al., 1980). Developments in manufacturing along with the properties of the compound, allowed the exclusive extraction of penicillin G or benzylpenicillin from *Penicillium chrysogenum*. The use of penicillin was ground-breaking in the treatment of many bacterial infections such as scarlet fever, bacterial endocarditis, meningococcal meningitis, puerperal sepsis, staphylococcal sepsis, pneumococcal pneumonia, gonorrhoea, syphilis, anthrax and also in the treatment of some anaerobic infections (Katzung, 1995, Walsh, 2003, Greenwood, 2000).

The spectrum of activity for penicillins was broadened by the development of penicillin derivates i.e. the semi-synthetic modifications of side chain variants (Figure 1.3). This allowed the β-lactam antibiotic to have an increased potency and also aided in overcoming resistance (Walsh, 2003).

### 1.3.2 Cephalosporins

Cephalosporin antibiotics are produced by *Cephalosporium* fungi. It was observed that this fungus generated antibiotics that resembled the penicillins. From this fungus, cephalosporin C was obtained but never marketed. However, the common cephalosporin nucleus, 7-aminocephalosporanic acid, was derived from cephalosporin C and produced on a large scale (Katzung, 1995). From 7-aminocephalosporanic acid, an array of cephalosporins with different properties was developed. Cephalosporins are active against Gram-positive and Gram-negative bacteria and have an analogous mode of action to penicillins (Figure 1.3). However, the extra atom in the fused ring structure allowed for further semi-synthetic modifications (Katzung, 1995, Walsh, 2003, Greenwood, 2000).

#### 1.3.2.1 First generation cephalosporins

The first generation cephalosporins were available circa 1975 (Greenwood, 2000). These compounds had narrow spectrum activity and were effective against Gram-positive (not methicilin resistant *Staphylococcus aureus* - MRSA) and some Gram-negative organisms. First generation cephalosporins include cefadroxil, cefazolin, cephalexin, cephalothin, cephapirin and cephradine (Katzung, 1995).

#### 1.3.2.2 Second generation cephalosporins

The second generation cephalosporins were termed 'expanded spectrum' due to their activity (Walsh, 2003). These compounds were less active against Gram-positive organisms when compared to the activity of the first generation cephalosporins (Walsh, 2003, Katzung, 1995). Second generation cephalosporins were stable against β-lactamases and had a greater activity against Gram-negative organisms for example *E. coli* and other enterobacteria, *B. fragilis* and *H. influenza*. However, they have no activity against enterococci or *P. aeruginosa*. Second generation cephalosporins include cefaclor, cefamandole, cefonicid, ceforanide, cefoxitin,

cefmetazole, cefoetan, cefuroxime, cefprozil, loracarbef and cefpodoxime (Katzung, 1995).

### 1.3.2.3 Third generation cephalosporins

The third generation cephalosporins were termed 'broad spectrum' antibiotics (Walsh, 2003). These compounds had stability to many β-lactamases and improved activity to most Gram-negative bacteria. Some have pronounced activity against *P. aeruginosa*, for example ceftazidime. Third generation cephalosporins differed from their predecessors in that they were capable of reaching the central nervous system (Katzung, 1995). Third generation cephalosporins include cefoperazone, cefotaxime, ceftazidime, ceftriaxone, cefixime and moxalactam (Greenwood, 2000, Katzung, 1995).

### 1.3.2.4 Fourth generation cephalosporins

The properties of fourth generation cephalosporins are similar to those of the third generation. These compounds have a greater activity against Gram-negative organisms and *P. aeruginosa*. Fourth generation cephalosporins include cefepime and cefpirome (Walsh, 2003).

With each generation of cephalosporins, greater activity against Gram-negative bacteria can be demonstrated. This is due to better penetration through porins of the Gram-negative outer membrane (determined by acyl chains), an increased affinity for PBP targets and a decreased catalytic efficiency towards hydrolysis by β-lactamases (Walsh, 2003, Greenwood, 2000).

### 1.3.3 Cephamycins

Cephamycins were first isolated from *Streptomyces* spp. in 1972 (Stapley et al., 1972) and were found to share their antimicrobial properties with the cephalosporins owing to sharing the same cephem nucleus (Greenwood, 2000). Some examples of cephamycins include latamoxef flomoxef, loracarbef and cefoxitin (Greenwood, 2000).

### 1.3.4 Monobactams

Monobactams are based upon a monocyclic β-lactam ring structure with no associated fused ring system (Katzung, 1995). They are active against Gram-negative bacteria similarly to 3rd generation cephalosporins like cefotaxime and ceftriaxone but exhibit no activity against Gram-positive or anaerobic organisms (Katzung, 1995, Greenwood, 2000). Aztreonam is currently the only licensed monobactam. This compound contains the same acyl chain as that found in ceftazidime (Walsh, 2003).

### 1.3.5 Carbapenems

The carbapenems are also a group of β-lactam containing compounds, which are stable against most β-lactamases. This group of compounds exhibit the broadest spectrum of activity and so have increased activity against Gram-positive and Gram-negative organisms including the obligate anaerobes. Carbapenems include imipenem, meropenem, doripenem and ertapenem. It was found that imipenem was inactivated by dihydropeptidases in renal tubules and it is therefore administered with a dihydropeptidase inhibitor (Greenwood, 2000, Walsh, 2003, Katzung, 1995). The carbapenems are considered to be the antibiotics of choice for treating serious ESBL infection (Livermore and Hawkey, 2005, Peirano and Pitout, 2010, Dhillon and Clark, 2011). However, it has been noted that carbapenems should be used in optimal doses and restricted to appropriate patients in order to minimise resistance development (Edwards and Betts, 2000).

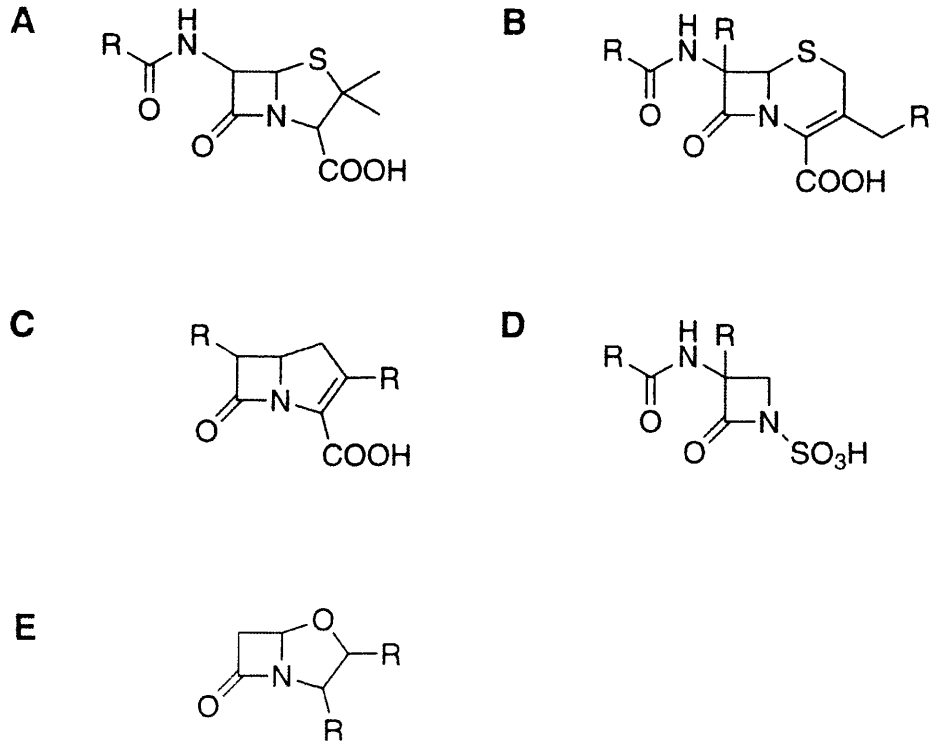



Figure 1.3 The structure of β-lactam antibiotics. A – penicillins, B – cephalosporins, C – carbapenems, D – monobactams and E – clavams. Figure reproduced from Walsh *et al.*, (2003) page 38.

### 1.4 β-lactamases

Often found in the periplasmic space or extracellularly (Bush, 1988) (Figure 1.2), β-lactamases are enzymes produced by certain bacteria that can prevent destruction by β-lactam antibiotics. The β-lactamase enzyme binds to the carbonyl moiety of the β-lactam ring, thus hydrolysing its amide bond. When the β-lactam ring is hydrolysed, penicillinoic acid, which is a stable product, is produced therefore inactivating the function of the β-lactam antibiotic (figure 1.4) (Greenwood, 2000, Katzung, 1995, Samaha-Kfoury and Araj, 2003, Sykes and Matthew, 1976).

The first β-lactamase was discovered in *E. coli* by Abraham and Chain in 1940, after the development of penicillin; penicillinases capable of hydrolysing penicillin were found in *S. aureus*. These *S. aureus* penicillinases were thought to be the 'scourge' of penicillin during the 1950s and so prompted research into alternative solutions such as the development of cephalosporins (Medeiros, 1997, Selwyn et al., 1980, Walsh, 2003). It is now estimated that over 850 β-lactamases have been identified (Drawz and Bonomo, 2010).

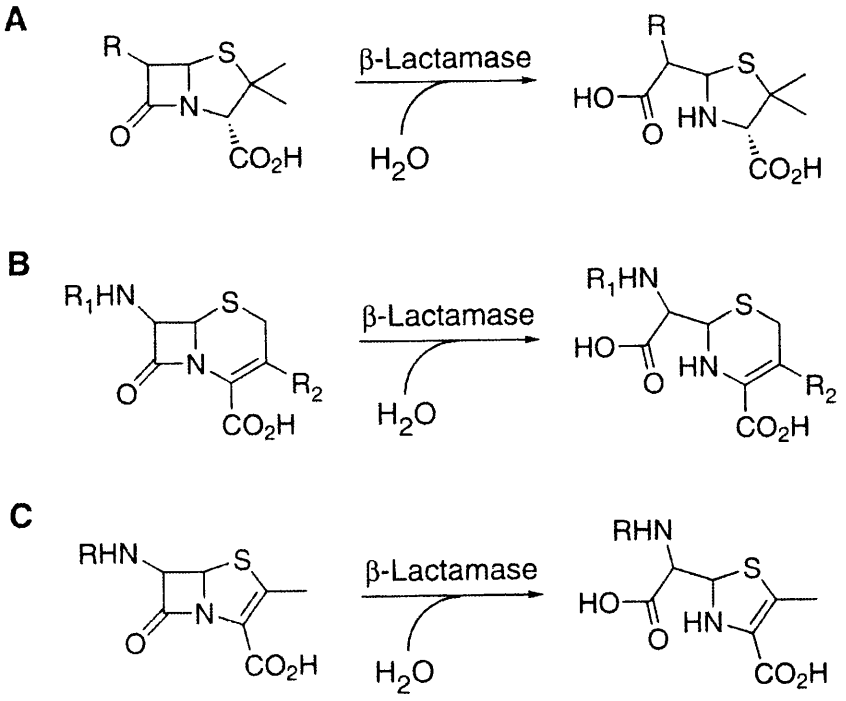



Figure 1.4 The inactivation of the β-lactam antibiotics by the β-lactamase enzyme. The figure illustrates the opening of the β-lactam ring in A – penicillins, B – cephalosporins and C – carbapenems. Figure reproduced from Walsh *et al.*, (2003) page 108.

### 1.4.1 β-lactamase inhibitors: overcoming resistance to β-lactam antibiotics

The β-lactamase inhibitors resemble β-lactam antibiotics and are structurally based on a clavam scaffold or a penicillinic acid sulfone. They are poor substrates for PBPs and therefore usually have no clinically useful antimicrobial activity (Drawz and Bonomo, 2010, Bush, 1988). However, they have β-lactamase inhibitor properties which protect β-lactam antibiotics from inactivation. They are capable of protecting the antibiotic by binding to the β-lactamases and so preventing the destruction of β-lactam antibiotics (Mims, 1998). Enzyme inhibitors can bind either reversibly or irreversibly. A reversible inhibitor can bind to an enzyme in such a way that the enzyme activity can be restored whereas the opposite is true for an irreversible inhibitor. Reversible inhibitors may be less effective than irreversible inhibitors as they are often hydrolysed as a substrate (Drawz and Bonomo, 2010, Bush, 1988).

Clavulanic acid, derived from *Streptomyces clavuligerus,* has a clavam structure. This was the first naturally occurring bicyclic β-lactam that did not possess a penicillin or cephalosporin nucleus (Reading and Cole, 1977). Other examples of β-lactamase inhibitors are sulbactam and tazobactam. These have penicillinic acid sulfone structures and were designed to have similar properties to clavulanic acid i.e. the inhibition of class A β-lactamases (Bush, 1988, Drawz and Bonomo, 2010).

## 1.5 The classification of β-lactamases

There are two major schemes of classification for β-lactamases, which are commonly currently utilised. These are known as the Ambler molecular classification scheme (Ambler, 1980) and the Bush-Jacoby functional classification system (Bush and Jacoby, 2010).

### 1.5.1 The Ambler molecular classification scheme

As the name suggests, the Ambler molecular classification scheme focuses on classifying β-lactamases at the molecular level. It uses protein homology, in particular amino acid similarity, as a basis for determining class (Ambler, 1980). There are four main classes within the Ambler classification scheme and these are labelled from A to D. β-lactamases belonging to classes A, C and D all have serine at their active site (Medeiros, 1997) whereas class B β-lactamases are known as metallo-β-lactamases and are often associated with zinc (Paterson and Bonomo,

2005). Class A, C and D β-lactamases share conserved amino acid homology to penicillin binding proteins and *D*-alanyl-*D*-alanine peptides which are the target of β-lactam antibiotics (Medeiros, 1997).

Class A is the largest group of β-lactamases. These have a high protein sequence similarity which is thought to be derived from a single ancestral gene. Although they have homologous protein structure, their enzymatic properties and substrate profiles differ greatly. Class A enzymes have a substrate preference of penicillin. Enzymes of this class can be cell bound, periplasmic or secreted and derived from plasmid or chromosomal genes and can belong to both Gram-positive and Gram-negative organisms (Ambler, 1980, Ambler et al., 1991).

The class B β-lactamases, or metallo-β-lactamases, are smaller than class A β-lactamases and require a metal co-factor e.g. zinc. These β-lactamases are inhibited by metal ion chelators and are not inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam (Bush, 1998, Wang et al., 1999).

The class C β-lactamases were discovered by Jaurin and Grundström (1981) in an AmpC-producing *E. coli* K12 strain. It was found to have a substrate preference for cephalosporins and had no homology with class A (Jaurin and Grundstrom, 1981).

The class D β-lactamases were discovered in 1988 after it was noted that the OXA group of enzymes were found to lack structural similarities to the class A, B and C β-lactamases. It was thought that they had a 'distinct evolutionary origin' and so the fourth class of β-lactamases were formed within the Ambler scheme (Huovinen et al., 1988).

### 1.5.2 The Bush-Jacoby functional classification system

The Bush-Jacoby functional classification system uses functional similarities, in particular substrate and inhibitor profiles, to classify β-lactamases (table 1.6) (Bush and Jacoby, 2010). Prior to the current Bush-Jacoby classification system and previous versions thereof, other functional schemes were devised to classify β-lactamases e.g. classification schemes by Sawai, Richmond and Sykes, Sykes and Matthew and Mitsuhashi and Inoue. However, unlike the Bush system(s), these

schemes did not combine functional properties i.e. substrate and inhibitor profiles with molecular structure (Bush et al., 1995).

The Bush-Jacoby system contains four main enzyme groups. Group 1 consists of cephalosporinases that are not fully inhibited by clavulanic acid, group 2 contain penicillinases, cephalosporinases and broad spectrum β-lactamases which are generally inhibited by β-lactamase inhibitors (active-site-directed), group 3 is made up of metallo-β-lactamases which are active against penicillins, cephalosporins and carbapenems and are poorly inhibited by β-lactamase inhibitors except EDTA and *p*-chloromercuribenzoate and finally, group 4 are penicilinases that are not inhibited by clavulanic acid (Bush, 1989a). However, differences between the 1989 and 1995 systems arose due to the variations in TEM and SHV β-lactamases. Therefore, three new subgroups were added to group 2 to further diversify the former 2b subgroup. These were named 2be, 2br and 2bf. Extended-spectrum β-lactamases (ESBLs) were added to the 2be group whereas enzymes with a reduced affinity to β-lactamase inhibitors were added to the 2br group. 2bf represents enzymes that hydrolyse carbapenems but are poorly inhibited by β-lactamase inhibitors (Bush, 1989a, Bush, 1989c, Bush, 1989b, Bush et al., 1995).

The functional classification system was reviewed once more in 2010 to incorporate more variations (the Bush-Jacoby system). Further subgroups were added within group 2. The 2ber subgroup represents ESBL TEM enzymes which are potentially resistant to clavulanic acid and the 2ce subgroup consists of the extended-spectrum carbenicillinase RTG-4 (CARB-10) with activity against cefepime and cefpirome. Other new additions to group 2 include 2de and 2df. The 2de subgroup consists of β-lactamases with extended spectrum ability to hydrolyse cloxacillin or oxacillin but not carbapenems whereas 2df contain β-lactamases that have the capability of hydrolysing carbapenems.

Additionally, subgroups were added to diversify group 1 and group 3. These new subgroups were named 1e, 3a and 3b respectively. Group 1e refers to variants of group 1 that have an increased activity towards ceftazidime. This type of variant is also known as an extended-spectrum AmpC or ESAC (Bush and Jacoby, 2010). Within group 3, 3a now refers to plasmid encoded metallo-β-lactamases e.g. VIM

and IMP (Queenan and Bush, 2007) whereas group 3b includes metallo-β-lactamases which preferentially hydrolyse carbapenems over penicillins or cephalosporins (Segatore et al., 1993). Another difference between the current Bush-Jacoby system and previous versions is the removal of group 4. It was stated that these enzymes could potentially belong to another group within the classification system if characterisation is undertaken to obtain more information (Bush and Jacoby, 2010).

Table 1.6 The classification of β-lactamases. Adapted from Bush *et al.*, 1995 and Bush and Jacoby 2010.

| **Bush-Jacoby System (2010)** | **Bush-Jacoby-Medieros System (1995)** | **Bush System (1989)** | **Molecular Classification** | **Characteristics** |
|---|---|---|---|---|
| 1 | 1 | 1 | C | Greater hydrolysis of cephalosporins than benzylpenicillin. Hydrolyses cephamycins. |
| 1e | NI | NI | C | Greater hydrolysis of ceftazidime and often other oxyimino-β-lactams. |
| 2a | 2a | 2a | A | Greater hydrolysis of benzylpenicillin than cephalosporins. |
| 2b | 2b | 2b | A | Similar hydrolysis of benzylpenicillin and cephalosporins |
| 2be | 2be | 2b' | A | Increased hydrolysis of cefotaxime, ceftazidime, ceftriaxone, cefepime and aztreonam. |
| 2br | 2br | NI | A | Resistant to clavulanic acid, tazobactam and sulbactam. |
| 2ber | NI | NI | A | Greater hydrolysis of oxyimino-β-lactams and resistance to β-lactamase inhibitors. |

NI – not included

Table 1.6...cont. The classification of β-lactamases. Adapted from Bush *et al.*, 1995 and Bush and Jacoby 2010.

| **Bush-Jacoby System (2010)** | **Bush-Jacoby-Medieros System (1995)** | **Bush System (1989)** | **Molecular Classification** | **Characteristics** |
|---|---|---|---|---|
| 2c | 2c | 2c | A | Greater hydrolysis of carbenicillin |
| 2ce | NI | NI | A | Greater hydrolysis of carbenicillin, cefepime and cefpirome. |
| 2d | 2d | 2d | D | Greater hydrolysis of cloxacillin or oxacillin. |
| 2de | NI | NI | D | Hydrolyses cloxacillin or oxacillin and oxyimino-β-lactams. |
| 2df | NI | NI | D | Hydrolyses cloxacillin or oxacillin and carbapenems. |
| 2e | 2e | 2e | A | Hydrolysis cephalosporins. Inhibited by clavulanic acid but not aztreonam. |
| 2f | 2f | NI | A | Greater hydrolysis of oxyimino-β-lactams, carbapenems and cephamycins |
| 3a | 3 | 3 | B | Hydrolysis of carbapenems but not monobactams. |
| 3b | 3 | 3 | B | Hydrolysis of carbapenems. |
| NI | 4 | 4 | Unknown | |

NI – not included

### 1.6 Extended-spectrum β-lactamases (ESBL)

ESBLs can be defined as β-lactamase enzymes capable of hydrolysing the penicillins, 1st, 2nd and 3rd generation cephalosporins and the monobactam aztreonam. They are unable to hydrolyze cephamycins and carbapenems. ESBLs are inhibited *in vitro* by the β-lactamase inhibitor clavulanic acid (Philippon et al., 1989, Paterson and Bonomo, 2005).

#### 1.6.1 The classification of extended-spectrum β-lactamases (ESBL) and AmpC

According to Ambler et al., (1991) ESBLs belong to molecular class A or D. TEM, SHV and CTX-M ESBLs are designated class A whereas, OXA ESBLs belong to class D (Huovinen et al., 1988, Bush and Jacoby, 2010, Paterson and Bonomo, 2005). AmpC β-lactamases belong to the Ambler class C (Jaurin and Grundstrom, 1981). However, classes A, C and D all have serine at their active site (Huovinen et al., 1988, Bush and Jacoby, 2010, Paterson and Bonomo, 2005, Jaurin and Grundstrom, 1981, Bradford, 2001).

The Bush-Jacoby system places ESBLs into the group 2be subgroup. This highlights that ESBLs are variants of group 2b enzymes such as TEM-1 and SHV-1 but have extended-spectrum activity (Bush and Jacoby, 2010, Bush et al., 1995). OXA enzymes belong to group 2d (Bush, 1989c). Using this classification system, AmpC β-lactamases are classified as being group 1 or group 1e (Bush and Jacoby, 2010, Bush, 1989a, Bush, 1989b, Bush et al., 1995).

#### 1.6.2 The diversity of ESBL enzymes

The types of ESBL enzymes are diverse. In the UK, there are three main types of ESBL enzyme namely TEM, SHV and CTX-M. However, other rarer ESBL enzymes also exist.

##### 1.6.2.1 TEM

The name TEM is derived from a Greek patient, Temoneira, from whom TEM-1 was first discovered in an *E. coli* isolate in 1965 (Paterson and Bonomo, 2005, Heritage et al., 1999). TEM-1 was found to have narrow-spectrum activity and was found to hydrolyse ampicillin at a greater rate than carbenicillin, oxacillin or cephalothin. Its

twin enzyme, TEM-2, shares the same properties as TEM-1 but varies in isoelectric point (Paterson and Bonomo, 2005, Jacoby and Medeiros, 1991).

The first ESBL-TEM enzyme was found in France in 1987. Originally named CTX-1, it was later renamed TEM-3 (Sirot et al., 1987, Paterson and Bonomo, 2005). TEM-3 differs from TEM-2 by two amino acid substitutions (http://www.lahey.org/studies). However, controversially, a report by Du Bios *et al.*, suggests that an ESBL-TEM enzyme was in fact possessed earlier by a *Klebsiella oxytoca* strain which was isolated in Liverpool in 1982 (Du Bois et al., 1995). An outbreak of TEM-1-producing *K. oxytoca* was treated with ceftazidime and subsequently, the *K. oxytoca* was found to be resistant. This indicates that ESBLs emerge as a response to selective pressure (Du Bois et al., 1995). There are currently 190 different TEM-type β-lactamase enzymes listed and their amino acid sequences vary in comparison to TEM-1 or TEM-2 (http://www.lahey.org/studies).

It has been noted that the point mutations leading to amino acid substitutions within the TEM-type β-lactamases are clustered within five areas of the enzyme and each adjacent to seven evolutionary conserved elements. These elements are located near to the active site and therefore increase the size available to accommodate oxyimino-components of cephalosporins thus allowing broad-spectrum resistance (Joris et al., 1991, Du Bois et al., 1995, Stürenburg and Mack, 2003).

As outlined in 1.4.2, β-lactamase inhibitors such as clavulanic acid serve to protect its accompanying β-lactam antibiotic e.g. penicillins from hydrolysis by β-lactamase enzymes (Bush, 1988). However, resistance to these β-lactamase inhibitors, specifically clavulanic acid, was seen in *E. coli* isolates in the early 1990s. It was believed that this was due to an overproduction of the non-ESBL TEM-1 β-lactamase enzyme (Reguera et al., 1991). Bacteria exhibiting TEM-type enzymes and which are inhibitor resistant, often do not effectively hydrolyse 3$^{rd}$ generation cephalosporins and so are not classed as ESBL. Unfortunately, a number of ESBL-producing TEM were found to exhibit resistance to β-lactamase inhibitors. These have been referred to as 'complex mutants' (Fiett et al., 2000, Poirel et al., 2004, Sirot et al., 1997). Inhibitor resistant SHV have also been discovered (Prinarakis et

al., 1997, Manageiro et al., 2010). However, inhibitor resistant CTX-M remains to be seen (Ripoll et al., 2011).

### 1.6.2.2 SHV

The name SHV is derived from sulphydryl variable, which is a description of the biochemical properties of the SHV β-lactamase enzyme (Heritage et al., 1999, Paterson and Bonomo, 2005). In 1972, the first sulphydryl variable (SHV) β-lactamase was identified with the isolation of SHV-1 (Chaves et al., 2001). It was found to be the chromosomally encoded β-lactamase within the *Klebsiella* spp. SHV-1 was found to be a narrow-spectrum β-lactamase with the ability to hydrolyse penicillins (Heritage et al., 1999).

The first ESBL-SHV was isolated from isolates of *K. pneumoniae* (Heritage et al., 1999) and was designated SHV-2 (Kliebe et al., 1985). It was found that the SHV-2 enzyme had the same isoelectric point as SHV-1 (Kliebe et al., 1985). However, further analysis demonstrated a single amino acid substitution: a glycine to serine mutation at position 238 (Barthelemy et al., 1988). It was found that the cavity used by the β-lactam substrate of SHV-2 was of an increased size, thereby maximising the space used to accommodate the bulky chains of cephalosporins such as cefotaxime and ceftazidime (Joris et al., 1991). In doing so, there is an increased affinity during hydrolysis. However, as a disadvantage, the β-lactam is at an increased distance from the serine active site at position 70, thus decreasing enzyme activity (Heritage et al., 1999, Huletsky et al., 1993). To compensate for this, it is thought that mutations in the promoter region may increase the production of ESBLs (Nuesch-Inderbinen et al., 1995, Heritage et al., 1999). There are currently 141 SHV enzymes listed, each varying in the number and type of amino acid substitutions (http://www.lahey.org/studies).

SHV was initially only associated with *K. pneumoniae*. However, the first isolation of SHV enzymes from bacteria other than *K. pneumoniae* was discovered in *E. coli* isolates. By the mobilisation of transmissible plasmids, SHV is now also found in other *Enterobacteriaceae*, for example *C. diversus, K. oxytoca, M. morganii, P. aeruginosa* and *B. cepacia* (Heritage et al., 1999).

### 1.6.2.3 CTX-M

The name CTX-M is directly linked to the word cefotaxime signifying its hydrolysing ability. The 'M' of CTX-M is donated from the word Munich, where CTX-M was first isolated (Peirano and Pitout, 2010). Unrelated to TEM or SHV β-lactamases (Peirano and Pitout, 2010), CTX-M are presumed to be derived from the chromosome of the environmental bacteria *Kluyvera* spp. namely *K. cryocrescens*, *K. ascorbata* and *K. georgiana*. It is believed that these CTX-M enzymes emerged in other species via a genetic escape and mutation from this medically unimportant species (Poirel et al., 2002, Bonnet, 2004, Woodford et al., 2004) by genetic mobile elements such as IS*ESCp*1. CTX-M genes are carried on plasmids that are often associated with TEM-1, TEM-2, OXA-type and SHV-type β-lactamases (Bonnet, 2004). These plasmids are also often associated with resistance genes for other antibiotics e.g. aminoglycosides, chrloramphenicol and tetracycline (Peirano and Pitout, 2010).

There are currently 120 CTX-M enzymes listed (http://www.lahey.org/studies). These enzymes can be subdivided into 5 phylogenetic groups: 1, 2, 8, 9 and 25 (Bonnet, 2004). Within each group, members have approximately >94% amino acid homology and ≤90% amino acid homology between groups (Peirano and Pitout, 2010). For example, CTX-M group 1 includes CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15, CTX-M-23, CTX-M-32 and CTX-M-55 amongst others. CTX-M group 2 includes CTX-M-2, CTX-M-4, CTX-M-4L, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20 and Toho-1 amongst others. CTX-M group 9 includes CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-19, CTX-M-21, CTX-M-27 and Toho-2 amongst others. CTX-M group 25 includes CTX-M-25 and CTX-M-26 (Bonnet, 2004) and CTX-M group 8 includes CTX-M-8, CTX-M-40 and CTX-M-63 (Rossolini et al., 2008).

The CTX-M enzymes are less effective than TEM or SHV enzymes at hydrolysing penicillins. However, they show significant hydrolysis against cephalosporins such as cefepime and cefpirome (Bonnet, 2004).

### 1.6.2.4 Other ESBL enzymes

Aside from TEM, SHV and CTX-M, there are an abundance of rarer ESBLs in existence. Examples include TLA, BES-1, BEL-1, and SFO-1. However, although often uncommon, OXA, PER, VEB, GES are being identified more frequently (Naas et al., 2008).

Not all of the OXA class of β-lactamases have ESBL properties, as some are incapable of hydrolysing the oxyiminocephalosporins. Needless to say, a proportion of OXA enzymes do pertain to have ESBL activity. For example, OXA-45 was found in a *P. aeruginosa* strain capable of hydrolysing all antibiotics except polymyxin B (Toleman et al., 2003). OXA enzymes are predominantly associated with *P. aeruginosa* but have also been seen in *Enterobacteriaceae* (Naas et al., 2008).

Originally detected in *P. aeruginosa* (Nordmann et al., 1993), PER ESBLs are now found in *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Alcaligenes faecalis*, *S. enterica*, *Vibrio cholera* and *Acinetobacter* spp. (Paterson and Bonomo, 2005). The PER-1 β-lactamase is capable of hydrolysing penicillin and cephalosporins. It is inhibited by clavulanic acid (Nordmann et al., 1993).

VEB-1, or Vietnamese extended-spectrum β-lactamase, is thought to be homologous with the PER-1 enzyme (Naas et al., 2008, Bradford, 2001). It demonstrates activity against amoxicillin, ticarcillin, piperacillin, cefotaxime, ceftazidime and aztreonam and is inhibited by clavulanic acid (Poirel et al., 1999). GES, BES-1, BEL-1 and TLA-1 all hydrolyse broad-spectrum cephalosporins and are inhibited by β-lactamase inhibitors (Naas et al., 2008).

## 1.7 AmpC

*ampC* genes have been found chromosomally in a number of *Enterobacteriaceae* e.g. *Citrobacter freundii*, *Enterobacter* spp., *E. coli*, *Morganella morganii*, *Proteus rettigeri, Providencia stuartii, Serratia marcescens, Hafnia alvei* and *Yersinia enterocolitica.* They are also found in other species including *Acinetobacter* spp., *Aeromonas* spp., *Chromobacterium violaceum, Lysobacter lactemgenus, Ochrobactrum anthropi, Pseudomonas aeruginosa, Psychrobacter immobilis* and

*Rhodobacter sphaeroides* (Philippon et al., 2002). In these organisms, AmpC expression is regulated by AmpR (a member of the LysR transcriptional family). When peptidoglycan synthesis is interrupted by a β-lactam antibiotic, there is a build up of N-acetylglucosamine (NAG)-1,6-anhydro-N-acetylmuramic acid (NAMA) oligopeptides. The NAG moiety is removed thus forming 1,6-anhydro-N-acetylmuramic acid tri, tetra and pentapeptides. These oligopeptides compete with oligopeptides of UDP-NAMA for the binding site on AmpR. Displacement of UDP-NAMA leads to a conformational change in AmpR and therefore activates the transcription of *ampC*. However, to prevent the overexpression of AmpC, the AmpD enzyme removes stem peptides from 1,6-anhydro-N-acetylmuramic acid and N-acetylglucosamin-1,6-anhydro-N-acetylmuramic acid oligopeptides thereby decreasing their concentration (Jacoby, 2009, Jacobs et al., 1997). Mutation in this pathway can cause the overexpression of chromosomal AmpC (Jacoby, 2009, Philippon et al., 2002, Barlow and Hall, 2002).

AmpC have been found in naturally *ampC* negative isolates such as *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Salmonella* spp. and *P. mirabilis*. This is due to the transmission and acquisition of plasmid-mediated *ampC* genes. It is thought that *K. pneumoniae* for example, may have acquired its *ampC* genes from being close to an "ecological neighbourhood" of *ampC* carrying organisms e.g. other *Enterobacteriaceae* found in the intestine (Bauernfeind et al., 1999). However, it has been postulated that due to the position of the *ampC* genes within the host chromosome, *ampC* genes were not initially subject to the rapid dissemination which is usually demonstrated by the horizontal transmission of plasmid-borne genes (Barlow and Hall, 2002). Plasmid-mediated AmpC have now been discovered worldwide (Philippon et al., 2002).

Plasmid-mediated AmpC enzymes are named according to the resistance trait it exhibits e.g. CMY (cephamycin), FOX (cefoxitin), MOX (moxalactam) or LAT (latmoxef). Alternatively, they are so named according to the type of β-lactamase e.g. ACT (AmpC type), ACC (Ambler class C) or reflecting their site of discovery e.g. MIR-1 (Miriam Hospital in Providence) and DHA (Dhahram Hospital in Saudi Arabia) (Philippon et al., 2002).

The structures of AmpC enzymes are similar to the class A β-lactamases but possess a slightly more open active site to accommodate the bulkier side chains found within the cephalosporins (Jacoby, 2009). AmpC enzymes are capable of hydrolysing narrow and broad spectrum cephalosporins along with the cephamycins (Thomson, 2010). However, they are not affected by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Thomson, 2010, Philippon et al., 2002). Resistance to carbapenems has also been noted in association with AmpC enzyme production and it is thought that porin loss is partly responsible (Philippon et al., 2002, Stürenburg et al., 2002), thus impairing the entry of antibiotics into the cell (Edwards and Betts, 2000).

### 1.8 Carbapenemases

Carbapenemases can be described as enzymes capable of hydrolysing the carbapenem class of β-lactam antibiotics. However, they are often able to hydrolyse other β-lactam antibiotics too. Carbapenemases have disseminated into a number of species. Using the β-lactamase classification system outlined in 1.5 and Table 1.6, the carbapenemases are distributed amongst Ambler class A, B and D (Queenan and Bush, 2007).

#### 1.8.1 Carbapenemases found within Ambler class A

The carbapenemases that are found within this molecular class can be divided into chromosomal and plasmid mediated. The chromosomal carbapenemases are thought to be induced in response to cefoxitin and imipenem. Some examples of these are; SME (*S. marcescens*), IMI (imipenem hydrolysing β-lactamase) and NMC (not metallo-enzyme carbapenemase). The plasmid mediated carbapenemases are transferrable. The KPC (*K. pneumoniae* carbapenemase) has now also been found in *Enterobacter* spp. and *Salmonella* spp. (Queenan and Bush, 2007).

#### 1.8.2 Carbapenemases found within Ambler class B

As outlined in 1.5.1, the metallo-β-lactamases belong to Ambler class B and have zinc as opposed to serine in their active site (Bush, 1998, Wang et al., 1999). Some examples of Ambler class B carbapenemases include: IMP (active on imipenem), VIM (Verona integron-encoded metallo-β-lactamase), SPM (Sao Paolo metallo-β-lactamase), GIM (German metallo-β-lactamase), SIM (Seoul imipenemase)

(Queenan and Bush, 2007) and NDM-1 (New Delhi metallo-β-lactamase) (Kumarasamy et al., 2010, Yong et al., 2009).

### 1.8.3 Carbapenemases found within Ambler class D

Within the Ambler class D, the OXA β-lactamases are found. These are enzymes capable of hydrolysing oxacillin (Huovinen et al., 1988). Carbapenemases of the OXA class of β-lactamases are usually associated with *Acinetobacter baumannii* e.g. OXA-23 (Queenan and Bush, 2007, Perez et al., 2010).

## 1.9 The epidemiology of ESBLs in the UK

Initially, ESBLs in the UK were mostly mutants of TEM and SHV. Despite a number of outbreaks, ESBL-producing *Enterobacteriaceae* were not common. These ESBLs were predominantly found in nosocomial *Klebsiella* spp. (Potz et al., 2006). In 2001, the UK saw the first emergence of CTX-M-9 in a *K. oxytoca* strain (Alobwede et al., 2003). However, the first CTX-M related outbreak was seen in 2001 in a hospital in Birmingham with CTX-M-26 producing *K. pneumoniae* (Brenwald et al., 2003). CTX-M, particularly CTX-M-15, is now the most predominant UK ESBL type (Munday et al., 2004, Peirano and Pitout, 2010), followed by CTX-M-14 (Woodford et al., 2006). Cantón and Coque (2006) demonstrated that CTX-M enzymes were at endemic levels in Europe, Canada, South America, Asia, Africa and Australia (Canton and Coque, 2006). The epidemiology of ESBLs are discussed in Chapter 3.

The spread of *E. coli* harbouring CTX-M-15 is unexplained although suggestions have been put forward. It is thought that CTX-M-15 derives from India where it is the predominant CTX-M genotype. Therefore, it has been suggested that CTX-M-15 has spread through travelling and migration (Ensor et al., 2006, Warren et al., 2008a). A prospective Swedish study conducted in 2007 investigated the link between travel and ESBL colonisation. Volunteers were screened pre and post travel for ESBLs. Isolates harbouring ESBLs, particularly CTX-M types were found to colonise 24% of volunteers who were negative pre-travel. All travellers who had been to India were subsequently colonised with CTX-M-15 producing bacteria. Therefore these findings suggest that the ESBLs were acquired at the specific travel destination (Tangden et al., 2010).

Domestic animals have been suggested as a reservoir for ESBLs. For example, a study by O'Keefe et al. (2010), documented the presence of CTX-M (CTX-M-14 and CTX-M-15) and SHV-12 genes in dogs and cats suffering urinary tract infections (UTIs). Additionally, food animals can often act as a source of antibiotic-resistant organisms e.g. *Campylobacter jejuni* and *Salmonella enterica*, which can lead to gut colonisation. *S. enterica* is often a reservoir for CTX-M in food animals, such as poultry. Studies by Warren et al., (2008a) and Dhanji et al., (2010) found that chicken meat from Brazil and Argentina harboured CTX-M-2; although this enzyme is not common in the UK it is prevalent in Argentina, which could suggest that the genotype found in poultry reflects the genotype prevalence of the country of origin. However, Warren et al., (2008a) also analysed the prevalence of CTX-M in UK poultry and found that it harboured CTX-M-1 as opposed to CTX-M-15. It was suggested that this finding reflected the restrained use of antibiotics such as ceftiofur and enrofloxacin in UK poultry production (Warren et al., 2008a). The link between CTX-M and poultry has also investigated in Italy and Denmark. Bertolaia *et al* (2010b), identified CTX-M-1, CTX-M-12 and CTX-M-32 in Italian broiler flocks and *E. coli* harbouring CTX-M-1, CTX-M-2 and CTX-M-9 has been documented in Danish chickens (Bortolaia et al., 2010a). A study by Calbo et al., (2011) found that a single strain of SHV-1 and CTX-M-15-producing *K. pneumoniae* had caused infection within all wards of a hospital in Spain. However, after screening healthcare staff and the ward environment, no colonisation with this strain could be determined. Therefore it prompted an investigation into food borne transmission. It was found that the kitchen surfaces and food were contaminated, and that a number of food handlers were in fact, faecal carriers of the strain (Calbo et al., 2011). A study by Rodriguez-Bano et al., (2008) found that faecal carriers were often associated with patients (in particular those who were male or had a urinary catheter) with community-associated UTIs caused by ESBL-producing *E. coli*. In their study, UTIs caused by ESBL-producing *E. coli* derived from host flora in 50% of cases. It was suggested that person to person transmission or acquisition from a common source was likely in aiding the spread of ESBLs (Rodriguez-Bano et al., 2008). In addition, there are a number of factors associated with the acquisition of ESBLs. These can be divided into hospitalised and non-hospitalised and are outlined in Table 1.7 (Colodner et al., 2004).

In a hospital environment during an outbreak, infection control measures are put into place. Typically, these include isolation of the colonised patient, the use of gloves and aprons (barrier nursing), stringent hand washing and appropriate use of antibiotics. However, in a study by Calbo et al., (2011) in which an outbreak of *K. pneumoniae* harbouring SHV-1 and CTX-M affected all wards of a hospital, these infection control measures did not stop the outbreak. It was thought that by imposing these infection control measures that the horizontal transmission and wider dissemination of this strain could be prevented (Calbo et al., 2011).

The control of ESBL infection and the accurate detection of ESBLs are important as ESBLs are often associated with complications and a high mortality rate. Therefore, the correct treatment is imperative to ensure adequate patient care and to avoid a fatal outcome (Rodriguez-Bano et al., 2006, Stürenburg and Mack, 2003).

Table 1.7 Factors associated with ESBL colonisation in hospitalised and non-hospitalised patients. Adapted from Colodner et al., (2004).

| **Hospitalised patients** | **Non-hospitalised patients** |
|---|---|
| Length of hospital stay | Previous hospitalisation |
| Time in ICU | |
| Severity of illness | Underlying diseases e.g. diabetes, heart disease, genitourinary, neurological disease and malignancies |
| Intubation and mechanical ventilation | |
| Urinary or arterial catheterisation | |
| Previous exposure to antibiotics | Use of antibiotics in the last 3 months |
| UTI | Recurrent UTI |
| | Older age |
| | Male gender |

### 1.9.1 Epidemic *E. coli* Strains A-E

In the UK, five major epidemic *E. coli* strains have been identified based upon their different PFGE banding patterns. These strains are labelled A-E and all harbour CTX-M-15 (Lau et al., 2008a). However, epidemic *E. coli* Strain A has been found nationally and will therefore be focussed on in this study.

#### 1.9.1.1 Epidemic *E. coli* Strains A

In the UK, *E. coli* Strain A has been located in Shropshire, Ulster and parts of the South of England. However, the most prolific outbreak was seen in Shropshire during the latter part of 2002. During this time, 28 out of 105 patients died due to infection with *E. coli* with CTX-M-15. These fatalities brought about changes in antibiotic policies whereby a restriction was placed on the usage of quinolones and 3$^{rd}$ generation cephalosporins. The usage of carbapenems, particularly ertapenem, was increased in the treatment of patients suspected of or proven to have ESBL infection. GPs were also advised to prescribe trimethoprim as a first line treatment of urinary-tract infections. CTX-M-15-positive *E. coli* was also found in stool samples which could imply that CTX-M-15 is transferred through the food chain (Livermore and Hawkey, 2005).

Analysis of Strain A by Woodford et al., (2004) revealed that an insertion sequence, *IS*26, was found within the terminal inverted repeat of the IS*Ecp*1-like elements, which was positioned upstream of the CTX-M-15 allele therefore suggesting that *IS*26 separated CTX-M-15 from its promoter region. The *IS*26 element was found to be flanked by 8bp direct repeats of IS*Ecp*1 DNA, which is consistent with duplication after a transposition event (Woodford et al., 2004). This suggests that IS*Ecp*1 is involved in the dissemination of CTX-M-15 (Poirel et al., 2003).

### 1.9.2 International clone O25b-ST131

O25b-ST131 belongs to the B2, subgroup 1 phylogenetic group of *E. coli* and is associated with a specific O antigen, O25b. It also has the multilocus sequence type (MLST) 131 (Clermont et al., 2009). It is thought that the predominance of this specific sequence type ST131, particularly in urinary-tract infections, is due to the

ability to overcome host defence mechanisms by the acquisition of virulence factors (Lau et al., 2008b).

This clone encompasses the UK's epidemic *E. coli* strains A-E (Lau et al., 2008a) along with other ESBL types and includes isolates with diverse PFGE patterns (Clermont et al., 2009). The typing and characterisation of *E. coli* isolates are outlined in Chapter 5.

### 1.10 Laboratory detection of ESBLs

ESBL detection in the laboratory currently involves a two-step procedure whereby *Enterobacteriaceae* are firstly screened for ESBL production and then confirmed as ESBL producers. In the UK, guidelines for susceptibility testing are outlined by the British Society for Antimicrobial Chemotherapy whereby coliform isolates are screened using a cefpodoxime (CPD) disc at a concentration of 10μg (BSAC, 2009). If the isolate is found to be resistant to cefpodoxime ESBL status is confirmed routinely using a synergy test with clavulanic acid protected cefpodoxime discs. The presence of an ESBL can be confirmed where the zone of inhibition for the cephalosporin plus clavulanic acid is ≥ 5mm larger than the cephalosporin alone. The isolate is then usually identified to the species level e.g. using API 20E. However, this test is currently only recommended for *E. coli* and *Klebsiella* spp. (Black et al., 2005). The detection of ESBLs is detailed more extensively in Chapter 4.

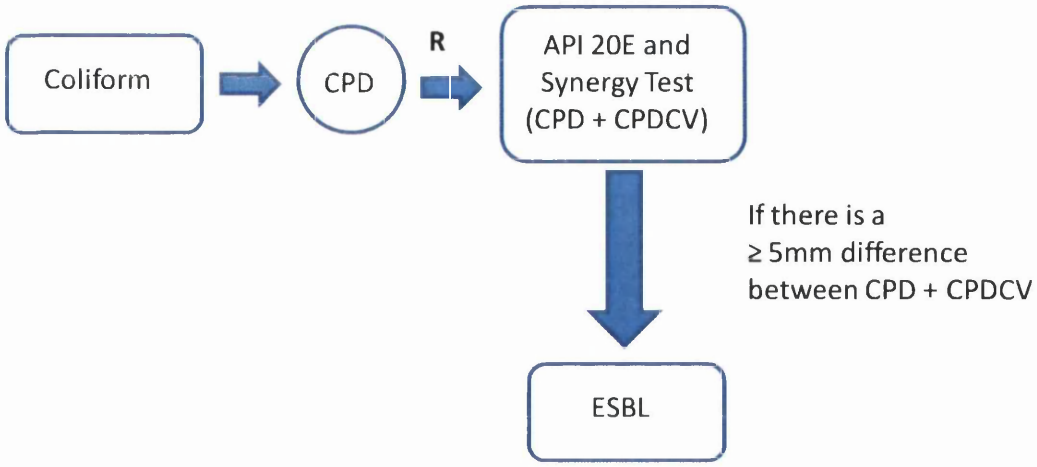



Figure 1.5 The detection of ESBLs within the clinical laboratory using a phenotypic screening and confirmation method with the cephalosporin cefpodoxime (CPD) and its augmented counterpart (CPDCV).

### 1.11 Project aims

The molecular epidemiology of ESBL-producing *Enterobacteriaceae* in Swansea will be investigated in order to give an insight into the current epidemiology of the area, which will enable a comparison to be made to the rest of the UK and other parts of the world. Accurate surveillance and ESBL detection allows for adequate and appropriate treatment regimens to be selected. Treatment options for infections with ESBL-producing organisms are limited, as ESBLs are often harboured alongside other resistance genes and are often also associated with clonal spread. The inappropriate treatment of infection with ESBL-producers can be associated with a higher mortality rate (Rodriguez-Bano et al., 2006). Therefore, briefly, the aims of this project included (the aims are outlined in more detail in each results chapter):

- Deduce the molecular epidemiology of ESBLs by the use of PCR amplification and DNA sequencing.
- Deduce the sensitivity of current phenotypic screening and confirmation methods.
- Evaluate the BD Phoenix Automated System and Mast AmpC and ESBL Detection set for the detection of ESBL and AmpC.

- Typing and characterisation to deduce any strain and or clonal relatedness within the *E. coli* and *Klebsiella* spp. populations by using PFGE and PCR based methodologies.

- Evaluate the use of temocillin and tigecycline along with other antibiotics.

- Evaluate the use of Matrix Assisted Laser Desorption Ionisation-Time of Flight mass spectrometry in the rapid identification of *Enterobacteriaceae*.

- To identify the *pgaABCD* locus in 3 different *E. coli* populations, to establish biofilm production phenotypically and to identify any correlation between biofilm formation and ESBL production.

- To evaluate the clinical epidemiology of ESBL-producing *Enterobacteriaceae*.

# Chapter 2: Materials and Methods

## 2.1 Bacterial Strains

Enterobacterial strains were obtained from the Public Health Wales (PHW) Microbiology Abertawe Bro-Morgannwg (ABM) University Healthboard, Swansea laboratory (Singleton Hospital), UK. Four-hundred and fifty-two isolates were phenotypically screened using cefpodoxime and of which, 164 isolates were retained for further characterisation, including:

- 117 *E. coli* isolates (102 from urine, 9 from blood, 3 from wound and 3 from faeces). 81/117 (69%) were hospital-associated and 36/117 (31%) were GP-associated.
- 38 *Klebsiella* spp. (33 from urine, 3 from blood and 2 from wound). 26/38 (68%) were hospital-associated and 12/38 (32%) were GP-associated.
- 9 other *Enterobacteriaceae* (5 from urine, 1 from blood and 3 from wound). 6/9 (67%) were hospital-associated and 3/9 (33%) were GP-associated.

*E. coli* strain A was a kind gift from the Health Protection Agency (HPA), Colindale, London, UK.

*E. coli* strains B-E were kind gifts from Dr. Mathew Upton, Manchester University, UK.

*E. coli* K12 was obtained from Dr. Alyson Bexfield, Swansea University, UK.

*E. coli* isolates sensitive to cefpodoxime (CPD) were kindly isolated by Michelle Reid from urine samples at the PHW Microbiology Laboratory, Singleton Hospital, ABM University NHS Trust, Swansea, UK.

NCTC 10418 was obtained from the PHW Microbiology ABM Swansea laboratory, Singleton Hospital, UK.

*E. coli* BL21 was purchased from Novagen, UK

Bovine *E. coli* isolates were obtained from Professor Martin Sheldon, Swansea University, UK (Sheldon et al., 2010).

Bacteria were stored in Microbank™ vials (Pro-Lab Diagnostics) and Glycerol (Sigma-Aldrich, UK). Glycerol stocks were made by using 300µl of liquid culture and 300µl of glycerol. All were stored at -80°C.

### 2.1.1 General Bacterial Growth Conditions

Enterobacterial isolates (that were screened to be resistant/intermediately resistant to cefpodoxime) were collected from the PHW Microbiology ABM Swansea laboratory, and were sub-cultured onto Cystine-Lactose-Electrolyte Deficient (C.L.E.D) agar or Luria Bertani (LB) agar and incubated at 37°C for 24 hours before use.

For liquid cultures, 2 colonies were suspended in 5ml LB/EC broth and incubated overnight at 37°C and aerated with 200 revolutions per minute (rpm).

## 2.2 Growth Media

### 2.2.1 Agar

Cystine-Lactose-Electrolyte Deficient (C.L.E.D) agar (Oxoid, UK)

Developed by Mackey and Sandys in 1966, C.L.E.D medium was found to support the growth of potential urinary pathogens. It is a non-inhibitory medium deficient in electrolytes therefore being capable of preventing *Proteus* spp. from swarming. The addition of cystine allowed the growth of cystine dependent coliforms. The addition of Bromothymol blue allows for a colour change depending on lactose-fermenting ability. Lacose fermenting bacteria produce yellow colonies whereas non-lactose fermenting bacterial colonies remain blue (Mackey and Sandys, 1966).

**Iso-sensitest agar** (Oxoid, Cambridge, UK)

Developed from Oxoid's sensitest media, iso-sensitest agar was designed specifically for antimicrobial testing and to overcome the limitations faced by Muller Hinton media.

**Luria-Bertani Agar**

LB was made using the recipe of Sambrook et al., (1989). 1% w/v bacto tryptone (Oxoid, Cambridge, UK), 0.5% yeast extract (Oxoid, Cambridge, UK), 1% Sodium

Chloride (Fisher Scientific) and 2% agar (Oxoid, Cambridge, UK) were dissolved in $dH_2O$ and autoclaved. The agar was cooled and aseptically poured into agar plates (10mm x 90mm) and allowed to set.

### 2.2.2 Broth

**Luria-Bertani Broth**

LB was made using the recipe of Sambrook et al., (1989). 1% w/v bacto tryptone (Oxoid, Cambridge, UK), 0.5% yeast extract (Oxoid, Cambridge, UK) and 1% sodium chloride (Fisher Scientific) were dissolved in $dH_2O$ and autoclaved before use.

**Tryptic Soy Broth (Oxoid, UK)**

30g TSB powder was dissolved in 1L $dH_2O$ and autoclaved before use.

**EC Broth** (Oxoid, UK)

EC broth was developed by Hajna and Perry (1943) to increase the selectivity of bacteria found within the coliform group and in particular, *E. coli*. It is a selective medium containing Bile salts No. 3 which enhance the growth of coliforms while inhibiting the growth of faecal streptococci.

37g of EC broth powder was dissolved in 1L of $dH_2O$ and autoclaved before use.

### 2.3 Chemicals and Reagents

| | |
|---|---|
| Tris HCl ($C_4H_{11}NO_3HCl$) | Fisher Scientific, UK |
| Tris Base ($C_4H_{11}NO_3$) | Fisher Scientific, UK |
| Sodium Chloride (NaCl) | Fisher Scientific, UK |
| Ethylenediaminetetraacetic acid (EDTA) | Fisher Scientific, UK |
| Magnesium Chloride ($MgCl_2$) | Fisher Scientific, UK |
| Dichlorodiphenyltrichloroethane (DDT, $C_{14}H_9Cl_5$) | Sigma-Aldrich, UK |
| Potassium Chloride (KCl) | Fisher Scientific, UK |

| | |
|---|---|
| Di-sodium hydrogen phosphate ($Na_2HPO_4$) | Fisher Scientific, UK |
| Potassium di-hydrogen phosphate ($KH_2PO_4$) | Fisher Scientific, UK |
| Alpha-4-cyano-hydrocycinnamic acid (HCCA, $C_{10}H_7NO_3$) | Sigma-Aldrich, UK |
| Tri-fluoro-acetic-acid (TFA, $CF_3CO_2H$) | Sigma-Aldrich, UK |
| Sodium N-lauryl sarcosine ($C_{15}H_{28}NNaO_3$) | Sigma-Aldrich, UK |
| Formic Acid ($CH_2O_2$) | Fisher Scientific, UK |
| Acetonitrile ($CH_3CN$) | Fisher Scientific, UK |
| Ethanol ($C_2H_5OH$) | Fisher Scientific, UK |
| D-Glucose ($C_6H_{12}O_6$) | Fisher Scientific, UK |
| Sucrose ($C_{12}H_{22}O_{11}$) | Fisher Scientific, UK |
| Sodium meta-periodate ($NaIO_4$) | Sigma-Aldrich, UK |
| Picric Acid ($C_6H_3N_3O_7$) | |
| Acetic Acid ($C_2H_4O_2$) | Fisher Scientific, UK |
| Formaldehyde ($CH_2O$) | Sigma-Aldrich, UK |
| Crystal Violet ($C_{25}H_{30}ClN_3$) | Sigma-Aldrich, UK |
| Ethidium Bromide (EtBr, $C_{21}H_{20}BrN_3$) | |
| SensiMix™ | Quantace, UK |
| 50x Sybr Green | Quantace, UK |
| 6X Tri Track Loading Dye Solution | Fermentas, UK |
| 100bp Molecular DNA marker | Promega, UK |
| 1Kb Molecular DNA marker | Promega, UK |
| 50 – 1000 Kb Pulse marker | Sigma-Aldrich, UK |

| | |
|---|---|
| Sybr Safe® | Invitrogen, UK |
| Primers | Invitrogen, UK |
| Deoxynucleotide Triphosphates (dNTPs) | Promega, UK |
| Agarose | Melford, UK |
| Low Melt Point (LMP) Agarose | Biorad, UK |
| Certified™ Molecular Biology Agarose | Biorad, UK |
| Glycerol | Sigma-Aldrich, UK |

**Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Matrix Preparation**

Matrix for MALDI-TOF was prepared by saturating 15mg of alpha-4-cyano-hydrocycinnamic acid (HCCA) in 500µl of 100% acetonitrile and 500µl of 5% tri-fluoro-acetic-acid (TFA) before ultra-sonicating for 10 minutes. The matrix solution was stored in the dark.

**Bouin's fixative**

Bouin's fixative was made by mixing 11.25ml of Picric Acid, 3.75ml of 40% Formaldehyde and 0.75ml of Acetic acid.

**Crystal Violet**

0.05g of Crystal Violet powder was dissolved in 100ml of $dH_2O$.

**2.3.3 Buffers**

**50X Tris-acetate-EDTA (TAE)**

2M Tris Base (pH 8.0) (Fisher Scientific), 1M glacial acetic acid (Fisher Scientific) and 0.05 M EDTA was made up to 1L with water.

**1X Tris-acetate-EDTA (TAE)**

20ml 50X TAE was made up to 1L with $dH_2O$.

**1M Tris HCl (pH 7.5)**

2.36g of Tris base and 12.70g Tris HCl was dissolved in 100ml of $dH_2O$ before being autoclaved before use.

**5M Sodium Chloride (NaCl)**

146.1g NaCl was dissolved in 500ml of $dH_2O$.

**1M Magnesium Chloride ($MgCl_2$)**

20.3g of $MgCl_2$ was dissolved in 100ml of $dH_2O$.

**0.5M Ethylenediaminetetraacetic acid (EDTA)**

93.06g $Na_4EDTA$ was dissolved in 400ml $dH_2O$. The pH was adjusted to 7.5 before adding an additional 100ml of $dH_2O$.

**SE Buffer**

15ml 5M NaCl and 50ml of 0.5M EDTA were made up to 1L with $dH_2O$.

**Tris-EDTA (TE) Buffer**

10ml 1M Tris HCl (pH 7.5) and 20ml 0.5M EDTA (pH 7.5) were made up to 1L with $dH_2O$.

**Proteinase K Buffer**

1L of 0.5M EDTA (pH 7.5) was added to10g Na-lauryl sarcosine before adjusting to pH to 9.

**0.5x Tris-Borate-EDTA (TBE)**

100ml of 10X TBE (Biorad) was made up to 2L with $dH_2O$.

**Phosphate Buffered Saline (PBS)**

3.2g NaCl, 0.08g KCl, 0.576g $Na_2HPO_4$ and 0.096g $KH_2PO_4$ were dissolved in 400ml $dH_2O$.

**Buffer D**

1x Buffer D was made using a recipe supplied by Promega. 6mM Tris HCl, 6mM $MgCl_2$, 150mM NaCl and 1mM DDT were mixed and the volume made up to 20ml with $dH_2O$. The pH was adjusted to 7.9.

**Buffer B**

Supplied by Promega, UK.

**PCR 10x Buffer**

Supplied by Invitrogen, UK.

**2.3.3.1 Buffers supplied by Qiagen, UK**

Genomic DNA extraction:

Buffer ATL

Buffer AL

Buffer AW1

Buffer AW2

PCR Purification:

Buffer PB

Buffer PE

**2.3.4 Antibiotics**

**2.3.4.1 Antibiotic discs**

Antibiotic discs are commercially available filter paper discs impregnated with the following antibiotics:

Cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP) (Mast, Merseyside, UK).

All antibiotic discs were supplied at a concentration of 30μg. However, cefpodoxime was used at a concentration of 10μg.

Discs containing amoxicillin and clavulanic acid known as co-amoxiclav or augmentin (AUG) (30 μg + 10 μg) and antibiotic discs (as described previously) with the addition of 10 μg clavulanic acid (CV) per disc were also utilised.

#### 2.3.4.2 E-test strips

E-test strips are commercially available plastic strips impregnanted with an antibiotic at various concentrations. Temocillin and tigecycline antibiotics were utilised (ABBiodisk, bioMérieux).

#### 2.3.4.3 Mast® AmpC and ESBL Detection set

A commercially available AmpC and ESBL detection set comprising of 4 discs. Each disc contained 10μg of cefpodoxime, 10μg of cefpodoxime plus ESBL inhibitor, 10μg of cefpodoxime plus AmpC inhibitor and 10μg of cefpodoxime plus ESBL inhibitor and AmpC inhibitor respectively (Mast, Merseyside, UK).

### 2.4 Identification of Bacteria

#### 2.4.1 BD Phoenix Automated Microbiology System

The BD Phoenix is a diagnostic system utilised in the rapid identification and antimicrobial susceptibility of organisms. The BD Phoenix panel (NMIC/ID) is split into two portions. One side determined bacterial identification (ID) and the other side deduced Antimicrobial Susceptibility Testing (AST) (BD, 2009).

**Bacterial Identification (ID)**

This portion of the BD Phoenix panel consisted of 51 wells, of which, 45 contained dried biochemical substrates. A series of fluoregenic, chromogenic and conventional biochemical tests were employed. The ability for the organism to utilise, degrade, hydrolyse or reduce each substrate was detected in order to determine its identification (BD, 2009).

**Antimicrobial Susceptibility Testing (AST)**

This portion of the BD Phoenix panel consisted of 85 wells, of which, 84 contained dried antimicrobial agents with a range of two-fold doubling concentrations. Antimicrobial susceptibility testing was based upon the principle of the microdilution method whereby the turbidity of bacteria was measured at each antimicrobial concentration. However, the BD Phoenix system utilised a redox AST indicator which allowed for the measurement of bacterial growth as well as bacterial tubidity (BD, 2009).

The following antimicrobial agents are contained within the BD Phoenix panel:

Amikacin, amoxicillin/clavulanate, ampicillin, aztreonam, cefotaxime, cefoxitin, ceftazidime, cefuroxime sodium, cephalexin, ciprofloxacin, colistin, ertapenem, gentamicin, imipenem, levofloxacin, meropenem, nitrofurantoin, piperacillin/tazobactam, tobramycin, trimeth/sulfamethoxazole (DIN) and trimethoprim.

A 0.5 McFarland suspension was made in Identification (ID) broth using a BD PhoenixSpec™ nephelometer. Of which, 25µl was aliquoted into BD Antibiotic Susceptibility Testing (AST) indicator broth along with one freefalling drop of AST indicator solution. The inoculated ID broth and BD Antibiotic Susceptibility Testing (AST) indicator was added to the appropriate sections of the BD Gram negative Phoenix panel (NMIC/ID) and placed into the BD Phoenix analyser. The BD Phoenix panel was incubated at 35°C and was systematically read by the instrument at 20 minute intervals. Results could be obtained up to 16 hours later. In order the detect ESBL, the Xpert system uses a screening technique and generates rules based upon antibiotic susceptibility (BD, 2009).

### 2.4.2 Bruker Daltonics MALDI Biotyper - Matrix-Assisted Laser Desorption/Ionization-Time of Flight/Mass Spectrometry (MALDI-TOF/MS)

Matrix Assisted Desorption/ionization-time of flight (MALDI-TOF) massspectrometry was performed with the Bruker Daltonics Microflex LT system (Bruker Daltonics, Germany).

The principles of MALDI-TOF are as follows. A laser beam is fired at the target plate thus causing the sample (which is overlayed with MALDI-TOF HCCA matrix) to ionise. The MALDI-TOF HCCA matrix protects the sample from complete degradation by absorbing most of the laser energy. Ionising the sample caused it to become vapourised. With the assistance of a strong electric field, the molecules enter a time of flight tube where they could be separated dependent on their mass to charge ratio (Stults, 1995). MALDI-TOF was first utilised for the identification of bacterial species in the 1990s (Holland et al., 1996).

In order to run MALDI-TOF, samples were prepared via two methods – a direct smear method or by ethanol-formic-acid extraction.

The target plate was placed in the Bruker microflex series MALDI-TOF whereby a spectrum could be achieved after 240 shots with a Nitrogen laser. The spectra were compared to the Bruker BioTyper database in order to determine the identification of the organism.

#### 2.4.2.1 Direct Smear Method

Using an inoculating loop, a bacterial colony was picked from an overnight plate culture and was thinly applied to the MALDI-TOF standard steel target plate. Depending on species, one colony of bacteria was sufficient to cover three sample spots of the steel target plate. The samples were overlayed with 2µl of MALDI-TOF matrix solution.

#### 2.4.2.2 Ethanol-formic-acid extraction

To prepare samples via ethanol-formic-acid extraction, approximately 3-5 colonies of bacteria was resuspended in 150µl of deionised water before adding 450µl of ethanol. The suspension was centrifuged at maximum speed for 2 minutes and the supernatant was discarded. The tubes were inverted and allowed to dry to ensure

complete removal of ethanol. The pellet was resuspended in 30µl of 70% formic acid. After a 10 minute incubation period, 30µl of 100% acetonitrile was added and the suspension vortexed before further centrifugation at maximum speed for 2 minutes. One microlitre of the supernatant was pipetted onto the MALDI-TOF target plate. The target plate was left to air dry at room temperature before overlaying with 2µl of MALDI-TOF matrix solution.

### 2.5 ESBL susceptibility tests

#### 2.5.1 Inoculation of Medium for Antibiotic Tests

For antibiotic testing, British Society for Antimicrobial Chemotherapy (BSAC) regulations were followed (BSAC, 2008). A colony of bacteria was added to 2ml of sterilised deionised water to create a suspension with a turbidity of 0.5 McFarland. This suspension was spread in a lawn fashion over iso-sensitest agar before the appropriate antibiotics were applied.

#### 2.5.2 Synergy Test

The synergy test is a phenotypic assay used to determine the presence of an ESBL. The principle is based upon the formation of synergy between a cephalosporin and a cephalosporin with clavulanic acid. ESBL production is determined when there is a difference of ≥5mm between the zone of inhibition of the cephalosporin and its augmented counterpart.

The cephalosporins cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefepime (CPM) and cefpirome (CFP) along with their augmented counterparts (CV) were divided into two batches (in their respected pairs) and dispensed on two inoculated agar plates along with cefuroxime (CXM) and cefoxitin (FOX). The discs were applied approximately 20-30mm apart and incubated for 24 hours at 37°C.

#### 2.5.3 Disc Approximation test

The Disc Approximation test is another phenotypic disc assay based upon the principle of demonstrating synergy. ESBL production can be confirmed when there is a distortion of the zone of inhibition between the cephalosporins and augmentin.

A disc of augmentin (AUG) was placed in the centre of an inoculated Iso-sensitest agar plate. CPD, CAZ, CTX were then applied 20-30mm apart in a triangular fashion around the augmentin disc. A second plate was set up using CFP and CPM. Plates were incubated for 24 hours at 37°C.

### 2.5.4 E-test

Iso-sensitest agar plates were inoculated as described in 2.5.1. Once the surface was dry, an E-test strip was applied using sterile forceps. Plates were incubated at 37°C for 18-24 hours before MIC readings were noted and interpreted.

### 2.5.5 Mast® AmpC and ESBL Detection set method

Iso-sensitest agar plates were inoculated as described in 2.5.1. Once the surface was dry, the four discs contained within the set were applied using sterile forceps. Plates were incubated at 37°C for 18-24 hours before being interpreted according to manufacturer's guidelines (Chapter 4).

## 2.6 Molecular Biology

### 2.6.1 Enzymes

*Nhe*I (10U/µl) (Promega, Southampton, UK)

A restriction enzyme used to cleave at the following site:

G▼CTAGC
C GATC▲G

*Xba*I (10U/µl) (Promega, Southampton, UK)

A restriction enzyme used to cleave at the following site:

T▼CTAGA
A GATC▲T

*Dde*I (10U/µl) (Promega, Southampton, UK)

A restriction enzyme used to cleave at the following site:

C▼TNAG

G ANT▲C

*Taq* Polymerase (5U/µl) (Invitrogen)

Utilised in PCR reactions, Taq polymerase is a heat stable enzyme isolated from *E. coli* cells expressing a *Thermus aquaticus* DNA polymerase gene.

Proteinase K (100mg/ml)

Isolated by Ebeling et al., (1974) from *Tritirachium album*, Proteinase K is a serine protease capable of aiding in the prevention of nucleic acid degradation.

All enzymes were stored at -20°C

### 2.6.2 DNA Extraction

#### 2.6.2.1 Crude DNA extraction

500µl of sterile deionised water was inoculated with two colonies from an overnight plate. The DNA was then extracted from bacterial cells by heating the suspension at 95°C for 10 minutes. The suspension was then centrifuged at 12, 000 rpm for 2 minutes.

#### 2.6.2.2 Genomic DNA extraction using Qiagen Kit

Genomic DNA was extracted from bacteria using the Qiagen DNeasy Blood & Tissue kit.

1ml of an overnight liquid culture was centrifuged at 7500 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 180µl Buffer ATL. 20µl of Proteinase K (supplied with Qiagen kit) was added and the suspension vortexed before incubating for 2 hours at 56°C. 200µl of Buffer AL (supplied with Qiagen kit) and 200µl of 100% ethanol were added. The suspension was thoroughly vortexed and pipetted into a DNeasy Mini spin column contained within a 2ml collection tube. The DNeasy mini spin column and its contents were centrifuged at 8000 rpm for 1 minute before discarding the flow through. The collection tube was replaced and 500µl of Buffer AW1 was added before centrifuging again at 8000 rpm for 1 minute – this step was repeated with the addition of Buffer AW2. To elute, the

DNeasy Mini spin column was placed in a clean 1.5ml microcentrofuge tube and 100µl of $dH_2O$ was directly added to the DNeasy membrane and centrifuged at 8000 rpm for 1 minute.

### 2.6.3 General PCR

PCR was carried out on all suspected ESBL-producers using a Corbett Rotorgene and BioRad Tetrad. The PCR master mix for one reaction consisted of 12.5µl 2x SensiMix™, 0.5µl each primer (10µM), 0.75µl 50x Sybr Green and 2µl crude DNA extract. Nuclease free water ($NFH_2O$) was added to make the total volume of master mix equal 25µl. To detect CTX-M and *ampC* genes respectively, multiplex PCR methods were used. Therefore, all the necessary primers were added together in one PCR reaction to determine which CTX-M group was present.

#### 2.6.3.1 Primers used

List of primers used in PCR reactions:

Table 2.1 Primers used in Multiplex reaction for identifying CTX-M groups (Woodford et al., 2006)

| CTX-M Group | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| 1 | 5′-AAAAATCACTGCGCCAGTTC-3′ | 415bp | 52°C |
| | 5′-AGCTTATTCATCGCCACGTT-3′ | | |
| 2 | 5′-CGACGCTACCCCTGCTATT-3′ | 552bp | |
| | 5′-CCAGCGTCAGATTTTTCAGG-3′ | | |
| 8 | 5′-TCGCGTTAAGCGGATGATGC-3′ | 666bp | |
| | 5′-AACCCACGATGTGGGTAGC-3′ | | |
| 9 | 5′-CAAAGAGAGTGCAACGGATG-3′ | 205bp | |
| | 5′-ATTGGAAAGCGTTCATCACC-3′ | | |
| 25/26 | 5′-GCACGATGACATTCGGG-3′ | 327bp | |
| | 5′-AACCCACGATGTGGGTAGC-3′ | | |

Table 2.2 Primers used to identify presence of SHV genes (Nuesch-Inderbinen et al., 1996)

| SHV | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| 1 | 5′-GCCCGGGTTATTCTTATTTGTCGC-3′ | 1017bp | 49 °C |
| 2 | 5′-TCTTTCCGATGCCGCCGCCAGTCA-3′ | | |

Table 2.3 Primers used to identify presence of TEM genes (Wiegand et al., 2007)

| TEM | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| T1 | 5′-ATTCTTGAAGACGAAAGGGCCTC-3′ | 1000bp | 55 °C |
| T3 | 5′-TTGGTCTGACAGTTACCAATGC-3′ | | |

Table 2.4 Primers used in a multiplex PCR to identify the presence of AmpC genes (Perez-Perez and Hanson, 2002)

| AmpC | | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| MOXMF | 5′-GCTGCTCAAGGAGCACAGGAT -3′ | 520bp | |
| MOXMR | 5′-CACATTGACATAGGTGTGGTGC-3′ | | |
| CITMF | 5′-TGGCCAGAACTGACAGGCAAA-3′ | 462bp | |
| CITMR | 5′-TTTCTCCTGAACGTGGCTGGC-3′ | | |
| DHAMF | 5′-AACTTTCACAGGTGTGCTGGGT-3′ | 405bp | 64°C |
| DHAMR | 5′-CCGTACGCATACTGGCTT-3′ | | |
| ACCMF | 5′-AACAGCCTCAGCAGCCGGTTA-3′ | 346bp | |
| ACCMR | 5′-TTCGCCGCAATCATCCCTAGC-3′ | | |
| EBCMF | 5′-TCGGTAAAGCCGATGTTGCGG-3′ | 306bp | |
| EBCMR | 5′-CTTCCACTGCGGCTGCCAGTT-3′ | | |
| FOXMF | 5′-AACATGGGGTATTCAGGGAGATG-3′ | 190bp | |
| FOXMR | 5′-CAAAGCGCGTAACCGGATTGG-3′ | | |

Table 2.5 Primers used for sequencing to type genes belonging to CTX-M group 1 (Stürenburg et al., 2004b)

| CTX-M Group 1 | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| F | 5′-TCTTCCAGAATAAGGAATCCC-3′ | 909bp | 58°C |
| R | 5′-CCGTTTCCGCTATTACAAAC-3′ | | |

Table 2.6 Primers used for sequencing to type genes belonging to CTX-M group 9 (Eckert et al., 2006)

| CTX-M Group 9 | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| M9 Upper | 5′-ATGGTGACAAAGAGAGTGCA-3′ | 1000bp | 52°C |
| M9 Lower | 5′-CCCTTCGGCGATGATTCTC-3′ | | |

Table 2.7 Primers used for sequencing to type genes belonging to CTX-M group 25/26. Designed using Primer 3 (http://frodo.wi.mit.edu/primer3/).

| CTX-M Group 25/26 | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| F | 5′-GCGATGTTAATGACGAGAGC-3′ | 806bp | |
| R | 5′-AACCGTCGGTGACAATTCTG-3′ | | |

### 2.6.2.2 General Reaction Conditions

Amplification conditions comprised of initial incubation step at 95°C for 15 minutes followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing for 20 seconds (temperatures shown in the appropriate Table) and a final extension at 72°C for 1 minute.

### 2.6.2.3 Restriction-Fragment Length Polymorphism (RFLP) Analysis of SHV positive PCR Products

RFLP analysis was carried out on all SHV positive isolates (PCR product approximately 1000bp) using the method developed by Nuesch-Inderbinen et al., (1996). A reaction mix consisting of 2µl DNA, 0.5µl of Buffer, 0.5µl of the restriction enzyme *Nhe*I (10U/µl) and 2µl of $dH_2O$ was incubated at 37°C for 2 hours. A second mix with additional $dH_2O$ in place of restriction enzyme was set up alongside as a control.

### 2.6.3 CTX-M-15 *IS*26 PCR

Table 2.8 Primers used to amplify the junction between *IS*26 and the CTX-M-15 allele (Woodford et al., 2004)

| CTX-M-15 *IS*26 | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| F | 5′-GCGGTAAATCGTGGAGTGAT-3′ | 400bp | 52 °C |
| R | 5′-AATCGGCAAGTTTTTGCTGT-3′ | | |

PCR cycling conditions consisted of 94°C for 5 minutes followed by 30 cycles of 94°C for 25 seconds, 52°C for 40 seconds 72°C for 50 seconds and a final extension at 72°C for 6 minutes.

### 2.6.4 O25b-ST131 PCR

O25b-ST131 PCR (Clermont et al., 2009) was carried out on all *E. coli* isolates to determine the presence of an intercontinental clone, O25b-ST131. In this allele-specific PCR, two primer sets are used (see table 2.9).

A PCR master mix for one reaction consisted of 8.25µl of $NFH_2O$, 2µl of 10x buffer (Invitrogen, UK), 0.5µl of dNTP mix (Promega, UK), 1.5mM $MgCl_2$ (Invitrogen, UK), 20 pmol of each 025pabBspe primer, 12 pmol of each trpA primer, 0.25µl of Taq polymerase (Invitrogen, UK) and 2µl of DNA bringing the total volume up to 20µl.

Table 2.9 Primers used to identify members of the intercontinental clone O25b-ST131 (Clermont et al., 2009)

| O25b-ST131 | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| trpA.F | 5′-GCTACGAATCTCTGTTTGCC-3′ | 427bp | 65°C |
| trpA.R | 5′-GCAACGCGGCCTGGCGGAAG-3′ | | |
| 025pabBspe.F | 5′-TCCAGCAGGTCTGGATCGT-3′ | 347bp | |
| 025pabBspe.R | 5′-GCGAAATTTTTCGCCGTAC-3′ | | |

The published amplification conditions comprised of 94°C for 4 minutes followed by 30 cycles of 94°C for 5 seconds, 65°C for 10 seconds and 72°C for 5 minutes. However, using these conditions on the Biorad Tetrad yielded no positive amplification products therefore, the method was optimised. Four experiments were set up in parallel. Each experiment had a different annealing time. The conditions were as follows:

94°C for 4 minutes, 30 cycles of 94°C for 20 seconds/30 seconds/40 seconds/1 minute, 65°C for 10 seconds and 72°C for 5 minutes. *E. coli* BL21 was used as a control isolate, as it is negative for *pab*B mutations. It was found that a 20 second annealing time was suitable for the specific nature of this PCR as a longer annealing time generated false-positive results.

#### 2.6.4.1. Digestion of positive *pabB* products

In order to identify that the products generated by *pabB* primers were not due to non-specific binding, enzymatic cleavage of the amplification product was carried out. A *pabB* gene sequence was analysed using NEBcutter V2.0 to determine a suitable enzyme along with digested fragment size. Cleavage with the restriction enzyme *Dde*I was employed. A reaction mix consisting of 2µl DNA, 0.5µl of Buffer, 0.5µl of the restriction enzyme *Dde*I (10U/µl) and 2µl of $dH_2O$ was incubated at 37°C for 2 hrs. A second mix with additional $dH_2O$ in place of restriction enzyme was set up alongside as a control.

Upon digestion, positive fragments were cleaved to 216 bp and 131 bp.

### 2.6.4.2 *pabB* Primers

Primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) to amplify a larger region of the *pabB* gene in order to confirm the presence of mutations in selected positive fragments.

Table 2.10 Primers used to amplify the *pabB* gene

| *pabB* | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| F | 5′-ATGCCGATCATCCGTATAAGC-3′ | 597bp | 65°C |
| R | 5′-TGGCCTGATTAAGCTGAAGG-3′ | | |

### 2.6.5 *pgaABCD* PCR

Table 2.11 Primers used to identify the presence of the *pgaABCD* gene locus (Cerca et al., 2007).

The *pgaABCD* gene locus was found to be associated with the promotion of surface binding, intercellular adhesion through the production of poly-β-1, 6-N-acetyl-D-glucosamine. This is thought to be an important factor associated with *E. coli* biofilm formation (Wang et al., 2004).

| *pga*ABCD | Primers | Expected Amplicon size (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| *pgaA* F | 5′-GGCTTTGAAACTTCTTACTGC-3′ | 200bp | 50°C |
| *pgaA* R | 5′-CCTGTTTATCTTGCCCGGCC-3′ | | |
| *pgaC* F | 5′-ATGATTAATCGCATCGTATCG-3′ | 550bp | |
| *pgaC* R | 5′-CATCGGTTCCACAATATATGC-3′ | | |

PCR cycling conditions consisted of 94°C for 5 minutes followed by 32 cycles of 94°C for 30 seconds, 50°C for 30 seconds 72°C for 45 seconds and a final extension at 72°C for 5 minutes.

### 2.6.6 Agarose Gel Electrophoresis

PCR reaction products or DNA fragments were separated by agarose gel electrophoresis. 1-3% (w/v) agarose gels in TAE were made and submerged in TAE electrophoresis buffer. DNA samples were mixed with one fifth their volume of 6 × DNA loading buffer and $NFH_2O$, then loaded into wells in the gel formed by insertion of the appropriate comb prior to gel solidification. Electrophoresis was carried out at 90V for 30 minutes (1-2%) or at 110V for approximately 1 hour (3%). Amplification products were then stained and visualised using SybrSafe® and UV light. The size and concentration of DNA fragments were estimated by co-electrophoresing 7µl Promega 100bp DNA Ladder as size standards. A permanent record of agarose gels was obtained by photographing the SybrSafe® stained gels illuminated with incident UV light on a Bio-Doc It UV Transilluminator system.

### 2.6.7 DNA Purification – QIAquick PCR Purification Kit

PCR products were purified using the QIAquick PCR purification kit.

100µl of PB buffer was added to 20µl of PCR product. Once mixed, the sample was applied to a QIAquick spin column and centrifuged at 13,000 rpm for 30 seconds in order to bind the DNA. The flow-through was discarded and 750µl of Buffer PE was added before centrifuging at 13,000 rpm for 30 seconds. After the washing with Buffer PE, the flow-through was discarded once more and residual ethanol was removed by centrifuging the column at 13,000 rpm for 1 minute. The column was placed into a clean 1.5ml microcentrifuge tube and DNA was eluted using 30µl of $H_2O$ and centrifuging at 13,000 rpm for 1 minute.

### 2.6.8 DNA Sequencing

PCR products were purified as described in 2.6.7 before being quantified using a NanoDrop spectrophotometer. The purified DNA was diluted to a specific concentration in 15µl as stated by MWG Operon. The samples, along with 2pmol of each primer were sent to MWG operon for sequencing. The results were analysed

using Basic Local Alignment Search Tool (BLAST), ClustalX (Thompson et al., 1997) and Bioedit (Hall, 1999).

### 2.6.9 Pulsed Field Gel Electrophoresis (PFGE)

#### 2.6.9.1 Sample preparation

2-3 colonies of bacteria were suspended in 5ml of LB broth and incubated overnight at 37°C. 1.6ml of culture was aliquoted into microcentrifuge tubes and centrifuged for 2 minutes at 12,000 rpm. The pellet was resuspended in 1ml of SE buffer and the procedure was repeated twice. 125µl of washed bacterial suspension was added to 125µl of 1.6% Low Melt Point (LMP) agarose before loading approximately 100µl of the mixture into Biorad PFGE plug moulds and leaving at 4°C for 10-30 minutes to set.

The agarose plugs were removed from the moulds and placed into 20ml universal tubes. One mililitre of Proteinase K lysis solution was pipetted into each tube and allowed to incubate overnight at 56°C. The Proteinase K lysis solution was removed and the agarose plugs were washed four times with 10ml of TE Buffer for 1 hour with shaking. The TE buffer was discarded and the agarose plugs were equilibrated in 800µl of 1x Buffer D (Promega, UK) for 1 hour at room temperature. The restriction buffer solution was removed and replaced with 100µl of fresh Buffer D and 4µl of *Xba*I (10U/µl) (Promega, UK).

#### 2.6.9.2 Gel preparation

Two litres of 0.5x TBE was cooled at 4°C and 160ml was used to make a 1% (w/v) agarose gel using Certified™ Molecular Biology Agarose. The gel was allowed to set for approximately 30 minutes before placing each agarose mould into the wells. Molecular markers (Sigma Aldrich pulse-field DNA marker – 50-1000Kb) were placed at each end of the gel and a layer of LMP was pipetted onto each sample well.

#### 2.6.9.3 PFGE Settings

The remaining 0.5x TBE buffer was placed into the CHEF-DR® III Variable Angle System and allowed to circulate at 75L/minute. The buffer was equilibrated to 14°C before placing the gel inside the tank.

Settings:

Block 1: 5 seconds initial switch, 15 seconds final switch – run time 10 hours.

Block 2: 15 seconds initial switch, 60 seconds final switch – run time 13 hours.

6.0 V/cm, angle 120°.

The current was maintained between 115 and 135 V by adding or subtracting the appropriate volume of 0.5x TBE buffer.

Once the PFGE was complete, the gel was removed from the tank and stained in 1% Ethidium bromide for 20 minutes with shaking. The Ethidium bromide solution was removed and the gel was destained in 400ml of deionised water for 1-3 hours. After destaining the gel, the PFGE profile was visualised using UV light on a Bio-Doc It UV Transilluminator system.

**2.7 Biofilm Assay**

Overnight liquid cultures were diluted with fresh media in a ratio of 1:100 and 200µl aliquots were inoculated into the wells of 96-well tissue culture plate (Nunc, ThermoFisher Scientific, Denmark). Each sample was added in triplicate. After incubation at 37°C for 18-24 hours, the media was carefully removed and the wells were washed three times with PBS (200µl per well). 150µl of Bouin's Fixative was added to each well and left for 15 mins in order to fix adherent bacteria. The fixative was removed and the plate was washed once more in PBS (200µl per well). The plate was allowed to airdry at room termperature and later stained with crystal violet. The optical density of the biofilm was measured at 600 nm using a FLUOstar Optima plate reader (BMG, Germany).

# Chapter 3: Molecular characterisation and epidemiology of ESBL-producing *Enterobacteriaceae* isolated from the PHW Microbiology ABM Swansea laboratory

## 3.1 Introduction

β-lactam antibiotics are 'front line antibiotics' which account for approximately 50% of antibiotics used worldwide (Barlow and Hall, 2002). All β-lactam antibiotics are composed of a β-lactam ring fused to either a five or six-membered ring (Greenwood, 2000). β-lactam antibiotics function as bacterial cell wall synthesis inhibitors, which incorporate into the cell wall of the bacteria to bind to penicillin binding proteins (PBPs). PBPs are enzymes responsible for the final stages of peptidoglycan synthesis. Additionally, inhibition of PBPs causes the activation of cell wall hydrolases to stimulate the autolysis mechanism, thus resulting in cell lysis (Mims, 1998, Samaha-Kfoury and Araj, 2003). Certain bacteria possess an enzyme that can prevent the destruction caused by β-lactam antibiotics. The β-lactamase enzyme hydrolytically cleaves the β-lactam ring of the antibiotic thus inactivating its function (Samaha-Kfoury and Araj, 2003, Katzung, 1995, Greenwood, 2000).

The first plasmid-mediated β-lactamase in *E. coli*, TEM-1, was isolated in Greece in 1965 (Bradford, 2001). Due to its transferable capabilities e.g. plasmids or transposons, TEM-1 has been found in other *Enterobacteriaceae* and other bacterial species such as *Pseudomonas* spp. and *Neisseria gonorrhoeae* (Bradford, 2001). In 1972, the first sulfhydryl variable (SHV) β-lactamase was identified with the isolation of SHV-1 (Chaves et al., 2001). SHV-1 which is commonly chromosomally encoded in *K. pneumoniae* also spreads via plasmids and is now additionally common in *E. coli* (Bradford, 2001). According to Bush et al., these non-ESBL enzymes belong to the classification group 2b which states that they have a spectrum of activity whereby penicillins and some cephalosporins can be hydrolysed (Bush, 1989b).

The first report of a plasmid-mediated β-lactamase capable of hydrolysing broad-spectrum cephalosporins was published in 1983 (Paterson and Bonomo, 2005). Kliebe et al (1985), described plasmid-mediated resistance to broad-spectrum

cephalosporins in *Klebsiella ozaenae* isolates. Sequencing showed that this enzyme differed to SHV-1 as there was a glycine to serine mutation at position 238 which conferred extended spectrum properties. Owing to this difference, the enzyme was named as SHV-2 (Kliebe et al., 1985). Soon after, more variants of TEM-1 and SHV-1 with the ability to hydrolyse broad-spectrum cephalosporins were discovered and so the phrase 'extended-spectrum β-lactamase' was coined to describe this phenomenon (Philippon et al., 1989, Paterson and Bonomo, 2005). Since the discovery of TEM-1 and SHV-1, over 190 TEM and 141 SHV variants have been identified (www.lahey.org/studies).

Described as CTX-M owing to its ability to hydrolyse cefotaxime, a non-TEM, non-SHV ESBL found in *E. coli* was isolated simultaneously in Germany and Argentina in 1989 (Bonnet, 2004, Canton et al., 2008). Found to only have a 40% homology to TEM and SHV enzymes (Tzouvelekis et al., 2000), CTX-M enzymes emerged via a genetic escape and mutation from the medically unimportant *Kluyvera* spp. (Poirel et al., 2002, Bonnet, 2004, Woodford et al., 2004). Approximately 120 CTX-M variants have since evolved (www.lahey.org/studies) and it is now the most common ESBL type (Munday et al., 2004). CTX-M, specifically CTX-M-9 was first isolated in the UK in 2001 (Alobwede et al., 2003) and during the same year, the UK had its first ESBL outbreak caused by a CTX-M-26 producing *K. pneumoniae* in Birmingham (Brenwald et al., 2003). However in 2003, CTX-M outbreaks caused by CTX-M-15 (Mushtaq et al., 2003) were being reported. CTX-M-15 is now the most common CTX-M type in the UK (Munday et al., 2004). Aside from TEM, SHV and CTX-M, there are also minor or rarely identified ESBL types such as PER, VEB and OXA (Naas et al., 2008).

Another class of important β-lactamases detected in this study are the AmpC β-lactamases. Like ESBL enzymes these β-lactamases mediate resistance to cephalosporins, oxyiminocephalosporins and aztreonam (Barlow and Hall, 2002); however they are not inhibited by clavulanic acid (Bush, 1989a). The transfer of an *ampC* gene via a plasmid can assist in the spread of multiresistant *E. coli*, *K. pneumoniae* as well as other *Enterobacteriaceae* (Bauernfeind, 1999). AmpC production (either plasmid mediated or inducible chromosomal) can often be

difficult to distinguish from ESBL production (Thomson, 2001) especially if an organism produces both ESBL and AmpC enzymes. Identification of the β-lactamase type can be mistaken phenotypically (Perez-Perez and Hanson, 2002).

Variants of β-lactamases have been selected for by the selective pressure caused by the use and overuse of antibiotics in the treatment of patients (Bradford, 2001). Already in 1985, after the discovery of SHV-2, Kliebe noted that the use of broad-spectrum cephalosporins should be handled carefully and warned that resistance should be monitored (Kliebe et al., 1985).

The objective of this study was to deduce the molecular epidemiology of ESBLs by the use of PCR amplification and DNA sequencing.

## 3.2 Results

### 3.2.1 Screening for ESBL using Cefpodoxime Synergy

Four hundred and fifty-two enterobacterial isolates were collected from the PHW Microbiology ABM Swansea laboratory in Singleton Hospital. Isolates were screened for ESBL production using cefpodoxime (CPD) and cefpodoxime plus clavulanic acid (CPDCV).

Using BSAC guidelines (BSAC, 2007), isolates are said to be resistant to cefpodoxime when the zone of inhibition is ≤19mm. Susceptible isolates have a zone of inhibition of ≥20mm. Of the 452 isolates, 157 isolates were completely resistant to cefpodoxime, of which, 141 isolates were found to have a positive synergy result with cefpodoxime and cefpodoxime plus clavulanic acid. Seven isolates that were classified as susceptible with zones of inhibition of between ≥20mm and ≤25mm and with no cefpodoxime synergy were also collected. Therefore in total, 164 isolates were retained for molecular characterisation and further phenotypic analysis.

## 3.3 Prevalence of ESBL and AmpC-producing *Enterobacteriaceae*

### 3.3.1 TEM PCR and sequencing analysis

*bla*$_{TEM}$ specific PCR (Wiegand et al., 2007) revealed that 64 out of 164 potential ESBL-producing isolates (45%) were found to be TEM positive thus generating a 1000 bp amplicon (Fig. 3.1).

To deduce ESBL TEM-status, DNA sequencing (MWG Operon) was carried out on the PCR amplification products in order to identify single point-mutations that can lead to amino acid substitutions. Using ClustalX (Thompson et al., 1997) the DNA sequences obtained were aligned and exported into BioEdit (Hall, 1999) where they were translated into amino-acid sequences for further analysis. In this study, TEM-33 (L69M), TEM-116 (I84V, V184A) and TEM-52 (K104E, T185M, S238G) were found alongside TEM-1. A representation of these results can be seen in Figure 3.2.

Currently, the Lahey Clinic has the amino acid substitutions listed for approximately 190 different TEM enzymes (www.lahey.org/studies). Using these guidelines, 8

TEM positive amplicons were defined as ESBL (Table 3.1). In total 6 TEM-116 (75%), 1 TEM-33 (12.5%) and 1 TEM-52 (12.5%) were distributed amongst 5 *E. coli* isolates (3 TEM-116, 1 TEM-33 and 1 TEM-52), 2 *K. pneumoniae* (2 TEM-116) and 1 *Citrobacter freundii* (1 TEM-116). TEM-1 (non-ESBL) was found in 56 isolates (88%) and of which, 36 (64%) were found in *E. coli*, 18 (32%) were found in *Klebsiella* spp. and 2 (4%) were found in other *Enterobacteriaceae* namely *C. freundii* and *Enterobacter cloacae*.

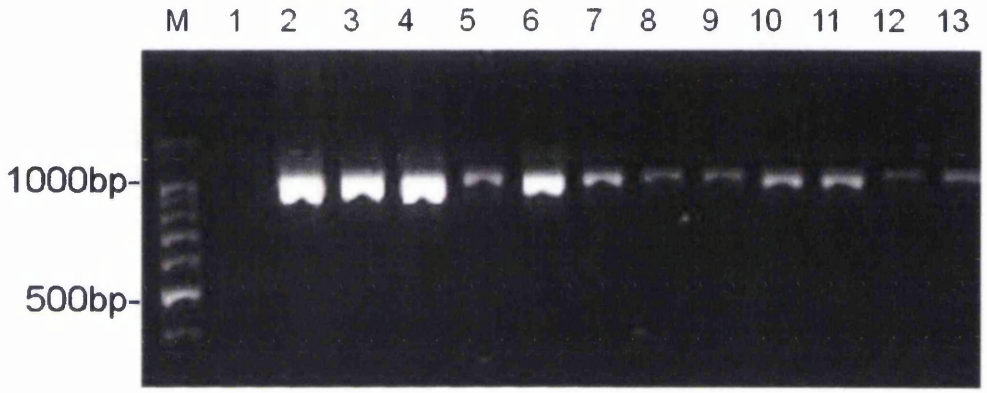
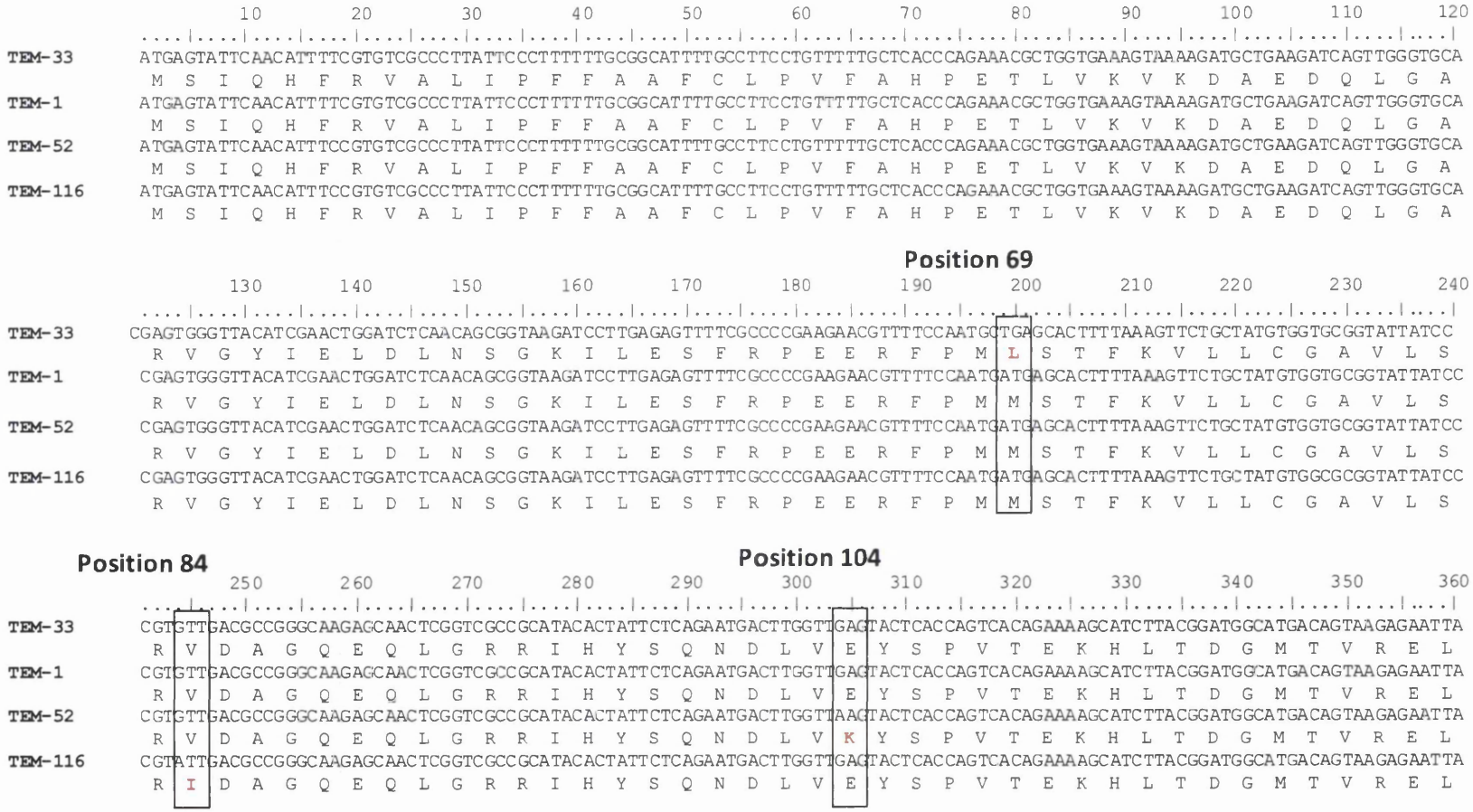



Figure 3.1. Agarose gel showing *bla*TEM specific PCR. Lanes 2-13 have a positive product of approximately 1000 bp. M -100 bp DNA marker. Lane 1: negative amplification control.

Table 3.1. Prevalence and distribution of $bla_{TEM}$ genes found by DNA sequencing within 64 TEM-producing isolates

| | TEM-116 | TEM-33 | TEM-52 | TEM-1 |
|---|---|---|---|---|
| *E. coli* (n=41) | 3 | 1 | 1 | 36 |
| *Klebsiella* spp. (n=20) | 2 | | | 18 |
| Other (n=3) | 1 | | | 2 |


10 20 30 40 50 60 70 80 90 100 110 120
TEM-33 ATGAGTATTCAACATTTTCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA
M S I Q H F R V A L I P F F A A F C L P V F A H P E T L V K V K D A E D Q L G A
TEM-1 ATGAGTATTCAACATTTTCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA
M S I Q H F R V A L I P F F A A F C L P V F A H P E T L V K V K D A E D Q L G A
TEM-52 ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA
M S I Q H F R V A L I P F F A A F C L P V F A H P E T L V K V K D A E D Q L G A
TEM-116 ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCA
M S I Q H F R V A L I P F F A A F C L P V F A H P E T L V K V K D A E D Q L G A
Position 69
130 140 150 160 170 180 190 200 210 220 230 240
TEM-33 CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGCTGAGCACTTTTAAAGTTCTGCTATGTGGTGCGGTATTATCC
R V G Y I E L D L N S G K I L E S F R P E E R F P M L S T F K V L L C G A V L S
TEM-1 CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGTGCGGTATTATCC
R V G Y I E L D L N S G K I L E S F R P E E R F P M M S T F K V L L C G A V L S
TEM-52 CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGTGCGGTATTATCC
R V G Y I E L D L N S G K I L E S F R P E E R F P M M S T F K V L L C G A V L S
TEM-116 CGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCC
R V G Y I E L D L N S G K I L E S F R P E E R F P M M S T F K V L L C G A V L S
Position 84
Position 104
250 260 270 280 290 300 310 320 330 340 350 360
TEM-33 CGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTA
R V D A G Q E Q L G R R I H Y S Q N D L V E Y S P V T E K H L T D G M T V R E L
TEM-1 CGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTA
R V D A G Q E Q L G R R I H Y S Q N D L V E Y S P V T E K H L T D G M T V R E L
TEM-52 CGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTAAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTA
R V D A G Q E Q L G R R I H Y S Q N D L V K Y S P V T E K H L T D G M T V R E L
TEM-116 CGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTA
R I D A G Q E Q L G R R I H Y S Q N D L V E Y S P V T E K H L T D G M T V R E L

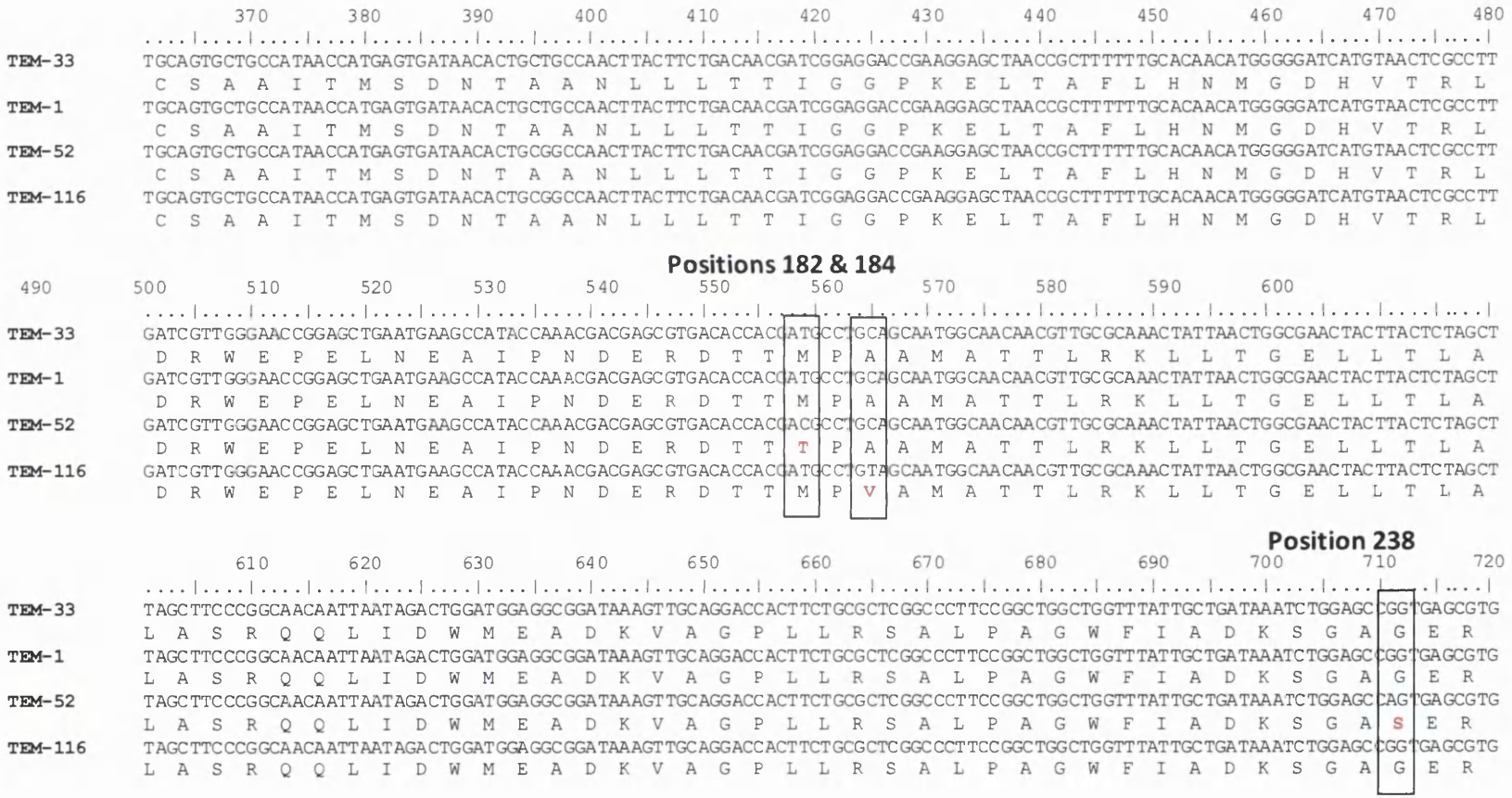



Figure 3.2 Sequence alignments of TEM ESBL genes found in this study versus non-ESBL TEM-1. Amino acid substitutions and positions are highlighted.

### 3.3.2 SHV RFLP and sequencing analysis

38 out of 164 potential ESBL producing isolates (23%) were identified as positive by PCR for the $bla_{SHV}$ gene by amplification of a 1017 bp amplicon. Positive amplification products were distributed amongst 5 *E. coli* (13%), 31 *Klebsiella* spp. (82%) and 2 *E. cloacae* (5%).

To determine ESBL status, RFLP analysis was carried out using *Nhe*I (Nuesch-Inderbinen et al., 1996).

In some SHV genes, this restriction site occurred through the formation of point-mutations and in turn led to a glycine to serine amino acid substitution at position 238. Of the 38 SHV-positive PCR products, 5 (13%) were cleaved with *Nhe*I to 770bp and 247bp and were deduced SHV-ESBL positive. The 5 ESBL-SHV genes were found within 2 *E. coli* (40%), 2 *K. pneumoniae* (40%) and 1 *E. cloacae* (20%) isolates.

At present the Lahey Clinic has listed approximately 141 different SHV enzymes (www.lahey.org/studies) and therefore the *Nhe*I methodology is not applicable for the identification of all SHV-ESBLs anymore. In order to identify the specific SHV genes found in this study, all 38 positive PCR amplification products were sent for DNA sequencing. The DNA sequences were analysed using ClustalX and BioEdit as described in section 3.3.1. A representation of these results can be viewed in figure 3.4, in which DNA sequences have been aligned to highlight amino acid substitutions between different SHV genes. In total, 33 non-ESBL SHV genes were found. The non-ESBL SHV genes consisted of SHV-1, SHV-11 (L35Q) (Nuesch-Inderbinen et al., 1997) and SHV-89 (L35Q, M129V) (Li et al., 2009). In total, 10 SHV-1 (26%), 17 SHV-89 (45%) and 6 SHV-11 (16%), were identified in 3 *E. coli* (1 SHV-1, 1 SHV-89, 1 SHV-11), 29 *Klebsiella* spp. (8 SHV-1, 16 SHV-89, 5 SHV-11) and 1 *E. cloacae* (1 SHV-1). The presence of SHV-2 (G238S) was confirmed by DNA sequencing in the 5 aforementioned *Nhe*I cleavable ESBL-SHV isolates (Table 3.2).

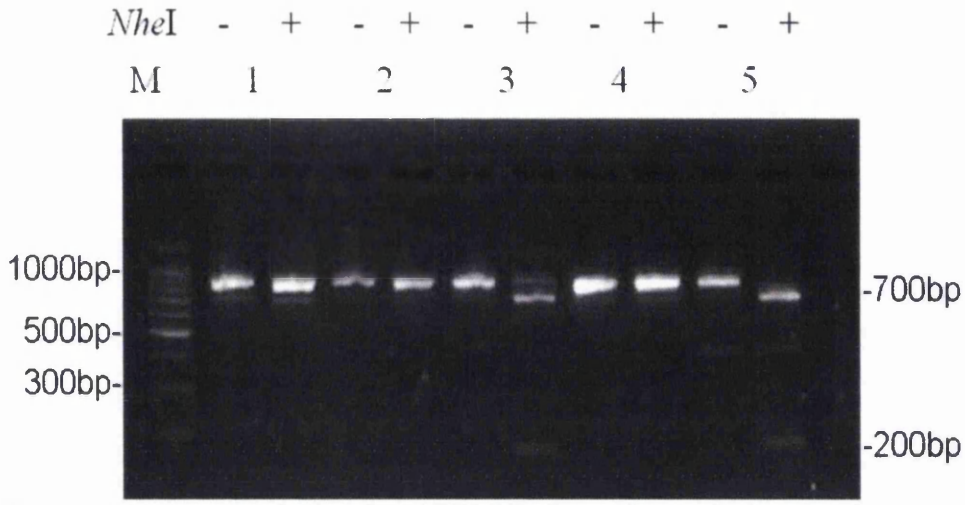
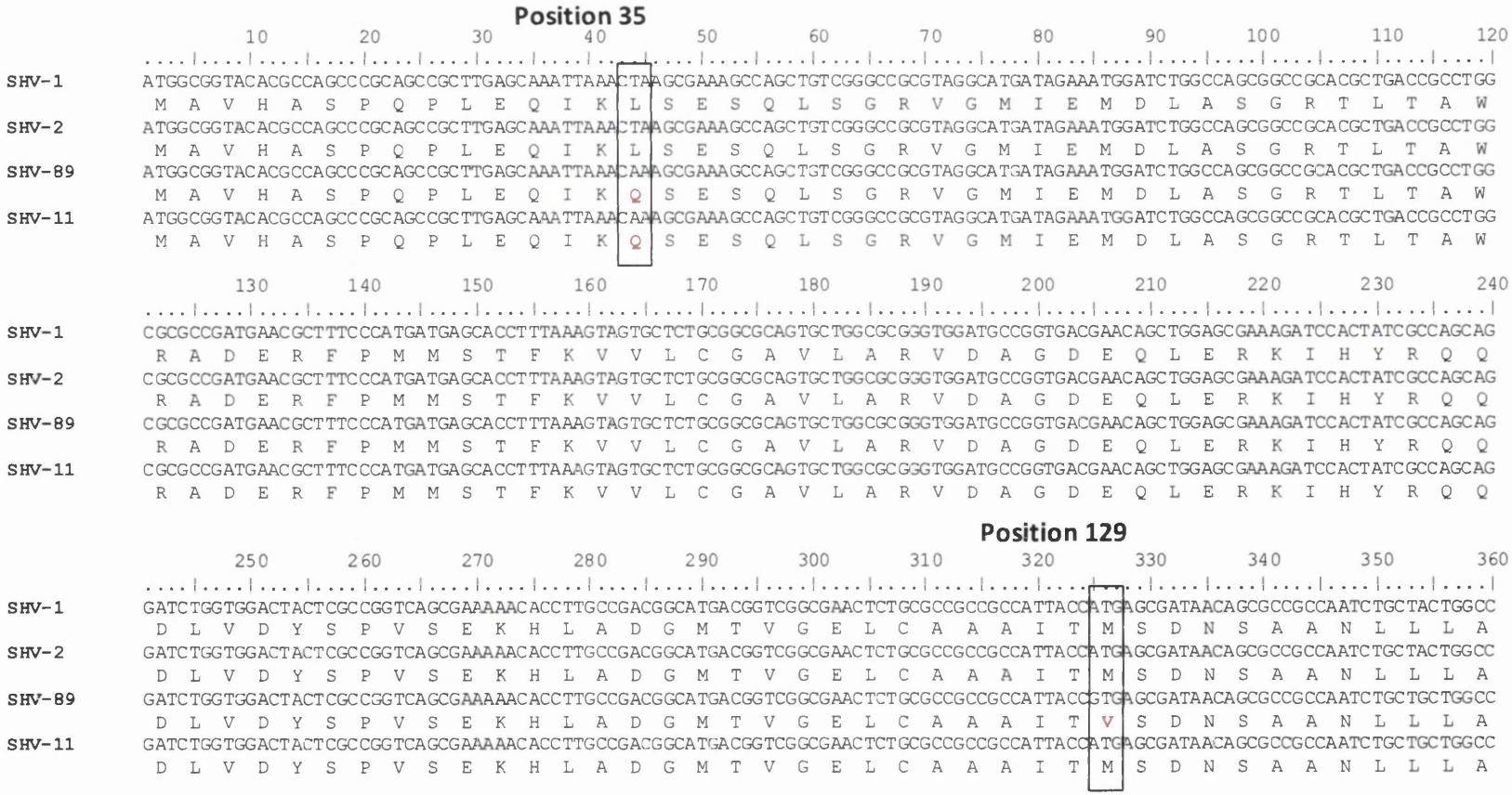



Figure 3.3 Agarose gel showing SHV-RFLP results. +/- denotes the presence or absence of Nhe1. Isolates 3 and 5 are SHV-ESBL positive as the 1000 bp fragment was cleaved to 770 bp and 247 bp. M – 100 bp DNA marker.

Table 3.2 Prevalence and distribution of $bla_{SHV}$ genes found by DNA sequencing within 38 SHV-producing isolates

| | **ESBL-SHV genes** | **Non-ESBL SHV genes** | | |
|---|---|---|---|---|
| | SHV-2 | SHV-1 | SHV-89 | SHV-11 |
| *E. coli* (n=5) | 2 | 1 | 1 | 1 |
| *Klebsiella* spp. (n=31) | 2 | 8 | 16 | 5 |
| Other (n=2) | 1 | 1 | | |


Position 35
Position 129
SHV-1
SHV-2
SHV-89
SHV-11

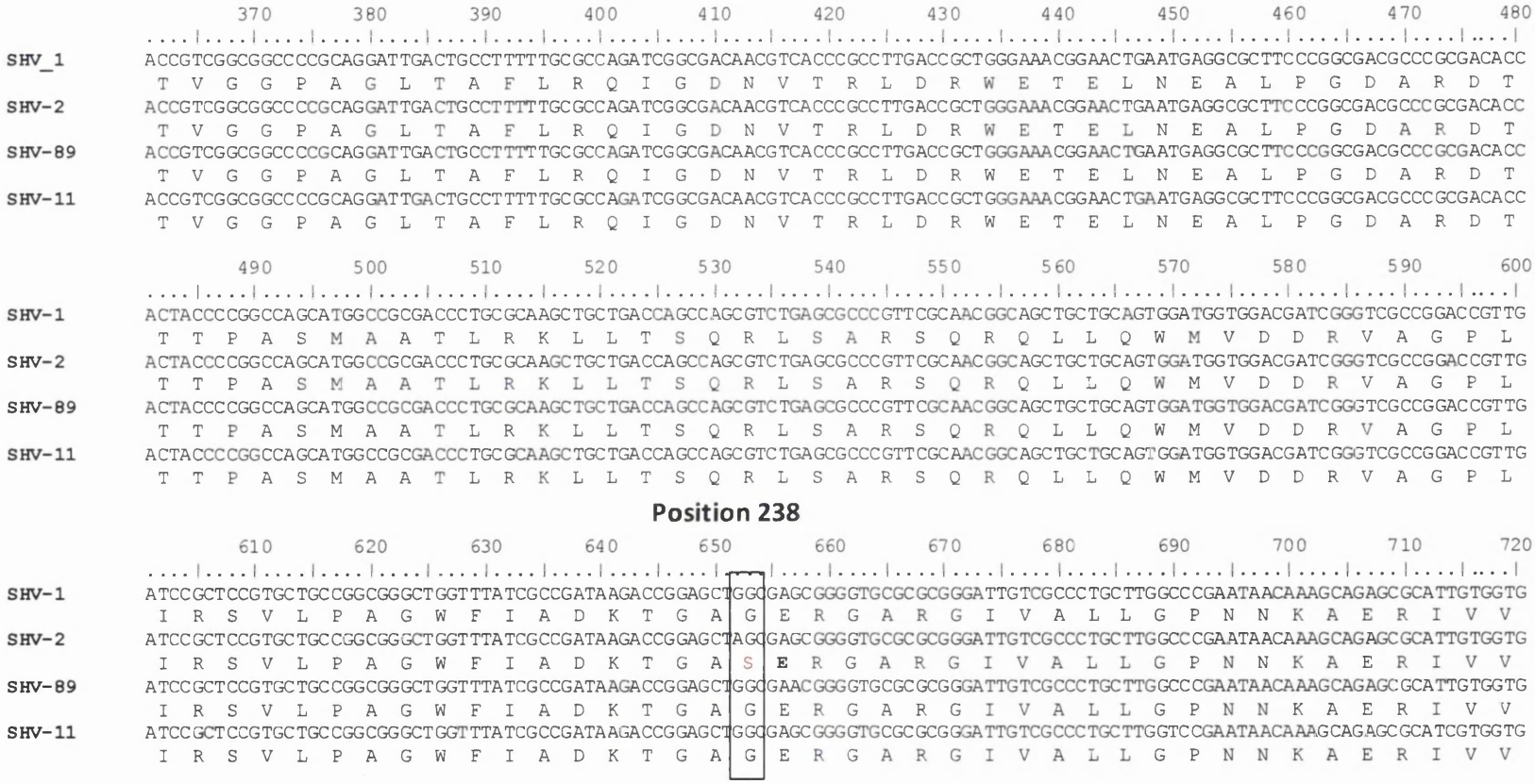



Figure 3.4 Sequence alignments of SHV genes found in this study: ESBL SHV-2 and non-ESBL SHV-11 and -89. Amino acid substitutions and positions are highlighted.

### 3.3.3 CTX-M PCR and Sequence Analysis

#### 3.3.3.1 CTX-M PCR Results

A multiplex PCR system was devised by Woodford *et al.* (2006) in order to allow the rapid identification of 5 CTX-M phylogenetic groups. Prior to this multiplex system, the characterisation of CTX-M alleles would often require two individual PCR runs firstly with universal primers and secondly with specific primers. Due to the increasing prevalence of CTX-M ESBLs, rapid identification facilitated CTX-M monitoring (Woodford et al., 2006).

Using this system, 138 out of 164 potential ESBL-producing isolates (81%) were initially found to be positive for a CTX-M gene (Figure 3.2) and were distributed within 102 *E. coli* (77%), 32 *Klebsiella* spp. (24%) and 5 other isolates (3%) specifically 4 *E. cloacae* and 1 *C. freundii*. 128 isolates were positive for a CTX-M group 1 gene (92%) and were found within 96 *E. coli* (75%), 30 *Klebsiella* spp. (23%) and 2 *E. cloacae* (2%) isolates. In addition, 5 *E. coli* isolates (4%) were positive for CTX-M group 9 genes, 4 isolates (2 *K. pneumoniae*, 1 *E. cloacae* and 1 *C. freundii*) (3%) were positive for CTX-M group 25/26 genes and 1 *E. cloacae* isolate (1%) was found to possess CTX-M group 2 genes. However, to ensure that the results obtained with this multiplex PCR system were accurate, PCR using the primers in a simplex fashion and later DNA sequencing was carried out (described in section 3.3.3.2). Upon doing this, the previously demonstrated CTX-M group 2 gene and 4 CTX-M group 25/26 genes were deemed false positive results (see in Figure 3.5) and so therefore making a total of 133 isolates positive for CTX-M genes.

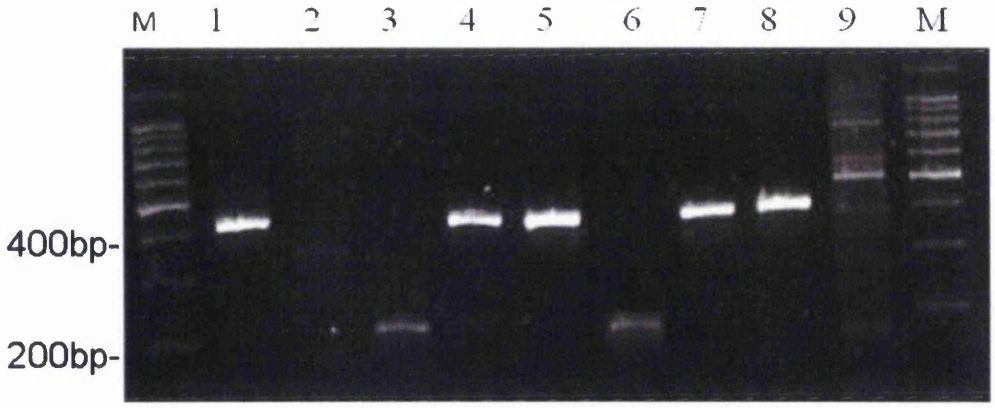



Figure 3.5 Agarose gel showing the prevalence of CTX-M genes obtained from CTX-M specific multiplex PCR. Lanes 1, 4, 5, 7, 8 - group 1 (415 bp); lane 2 - group 25/26 (327 bp); lane 3 – group 9 (205 bp); lane 9 - group 8 (552 bp);, M – 100 bp DNA marker.

Table 3.3 Prevalence of $bla_{CTX-M}$ genes found by multiplex PCR and confirmed using simplex PCR and sequencing within 164 potential ESBL-producing isolates.

| | **CTX-M Positive** | **CTX-M Group** | |
|---|---|---|---|
| | | **1** | **9** |
| *E. coli* (n=117) | 101 | 96 | 5 |
| *Klebsiella* spp. (n=38) | 30 | 30 | |
| Other (n=9) | 2 | 2 | |

### 3.3.3.2 CTX-M Sequence Analysis

To further characterise isolates with a positive CTX-M PCR product, DNA sequencing was carried out using primers that amplify a larger proportion of the gene of interest.

#### 3.3.3.2.1 CTX-M Group 1 Sequence Analysis

DNA amplification products positive for a CTX-M group 1 gene (a selection of 48 *E. coli* and 16 *Klebsiella* spp. isolates) were sequenced using primers as described by Stürenburg *et al.* (2004a). Table 3.4 outlines the prevalence of CTX-M genes found in this study. 38/43 (88%) *E. coli* isolates were positive for CTX-M-15, 2/43 (5%) were positive for CTX-M-1, 2/43 (5%) were positive for CTX-M-32 and 1/43 (2%) were positive for CTX-M-55. Sequencing revealed that 16 out of 16 (100%) of *Klebsiella* spp. with a positive CTX-M group 1 PCR product contained CTX-M-15. When compared to the DNA sequence of CTX-M-1, CTX-M-15 has an A to C mutation at position 138, T to C mutation at 239, T to C mutation at 313, G to T mutation at 315, G to A mutation at 349, G to T mutation at 427, T to G mutation at 582, T to C mutation at 609 and A to G mutation 725. CTX-M-55 shares all of these mutations except for the T to C mutation at position 239. CTX-M-32 only has one DNA mutation when compared to CTX-M-1 – an A to G mutation at position 325 (Figure 3.6).

Table 3.4 Prevalence of CTX-M group 1 genes found within a selection of *E. coli* and *Klebsiella* spp.

| | **CTX-M Group 1 Genes** | | | |
|---|---|---|---|---|
| | **CTX-M-15** | **CTX-M-1** | **CTX-M-32** | **CTX-M-55** |
| *E. coli* (n=43) | 38 | 2 | 2 | 1 |
| *Klebsiella* spp. (n=16) | 16 | 0 | 0 | 0 |

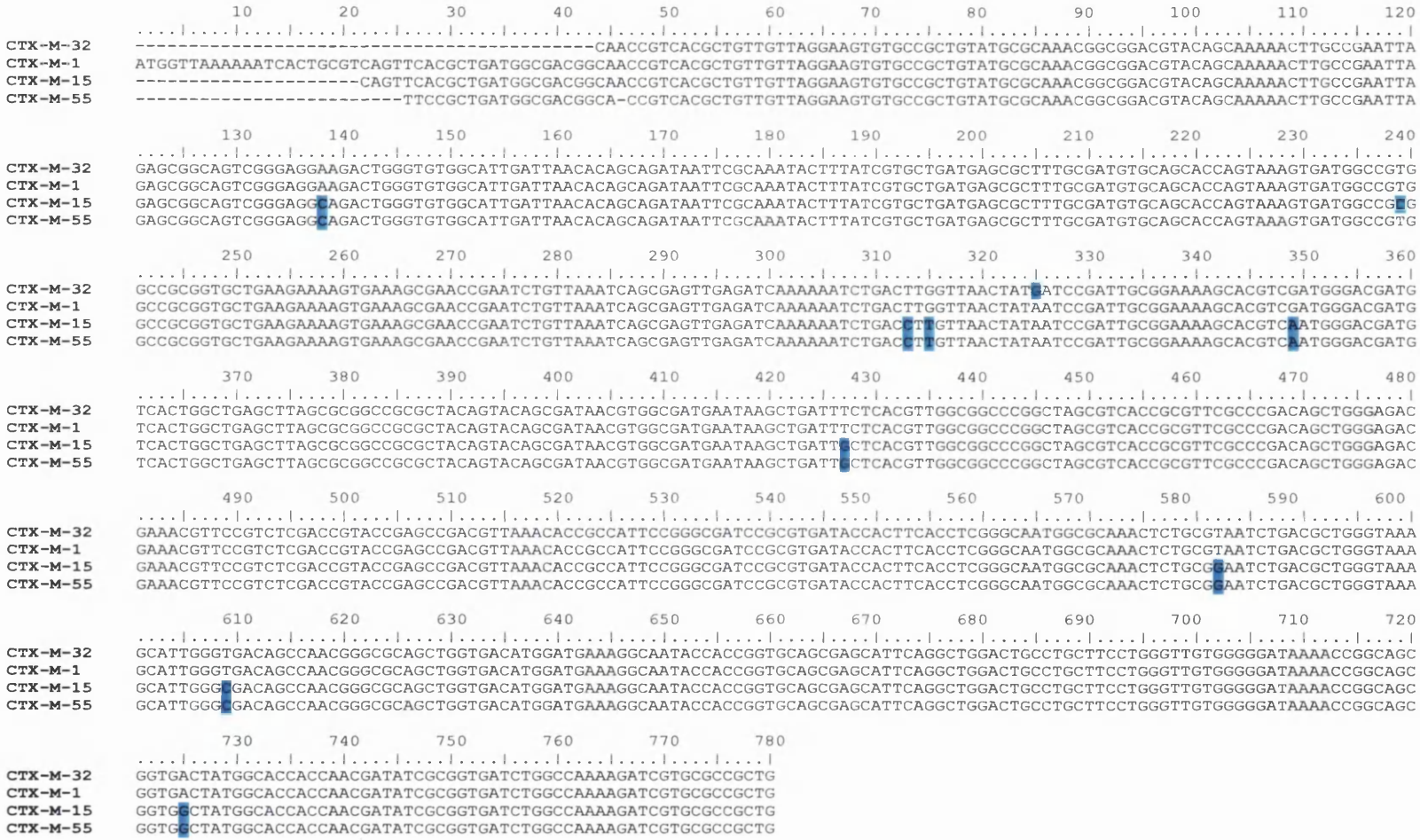



Figure 3.6 DNA sequences of CTX-M group 1 genes. CTX-M-1, CTX-M-15, CTX-M-32 and CTX-M-55 were found in this study. This figure illustrates the point mutations found within each type.

#### 3.3.3.2.2 CTX-M Group 9 Sequence Analysis

PCR amplification products positive for CTX-M group 9 were sent for DNA sequencing (MWG Operon) with primers of Eckert et al., (2006). The DNA sequences were analysed by initially performing a BLAST search to identify the most homologous sequences and then aligning using ClustalX and BioEdit. Out of 5 positive CTX-M group 9 *E. coli* isolates, 2 CTX-M-14 (40%), 2 CTX-M-14b (40%) and 1 CTX-M-27 (20%) were identified (Table 3.5). When compared to the DNA sequence of CTX-M-14, CTX-M-27 has a G to A mutation at position 372, A to G mutation at position 702 and A to G mutation at position 725; whereas, CTX-M-14b has a G to A mutation at position 570 (Figure 3.7).

Table 3.5 Prevalence of CTX-M group 9 genes found by DNA sequencing within *E. coli*.

| | **CTX-M Group 9 Genes** | | |
|---|---|---|---|
| | **CTX-M-14** | **CTX-M-14b** | **CTX-M-27** |
| E. coli (n=5) | 2 | 2 | 1 |

#### 3.3.3.2.3 CTX-M Group 25/26 Sequence Analysis

As amplification of the CTX-M group 25/26 genes could not be achieved by singleplex PCR, primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) in order to amplify a larger portion of the gene for analysis. However, no positive amplification was yielded using these primers. Therefore the positive amplification products generated through the multiplex PCR system were sent for DNA sequencing.

A BLAST search revealed that 2 of these PCR products from *Klebsiella* spp. isolates were sorbose-specific phosphotransferase enzyme IIB components also known as the phosphotransferase system (PTS) sorbose-specific EIIB component. The PTS system is a major carbohydrate active-transport system which phosphorylates and

translocates sugars (in this instance sorbose) across the cell membrane (Bramley and Kornberg, 1987).

The remaining 2 amplification products from isolates of *E. cloacae* and *C. freundii* had homology to an "uncultured bacterium pJM6 unknown genes".

The positive PCR products generated could have arisen from non-specific annealing within the multiplex PCR system. These isolates could not be assigned a CTX-M subgroup and were so deemed false positive.

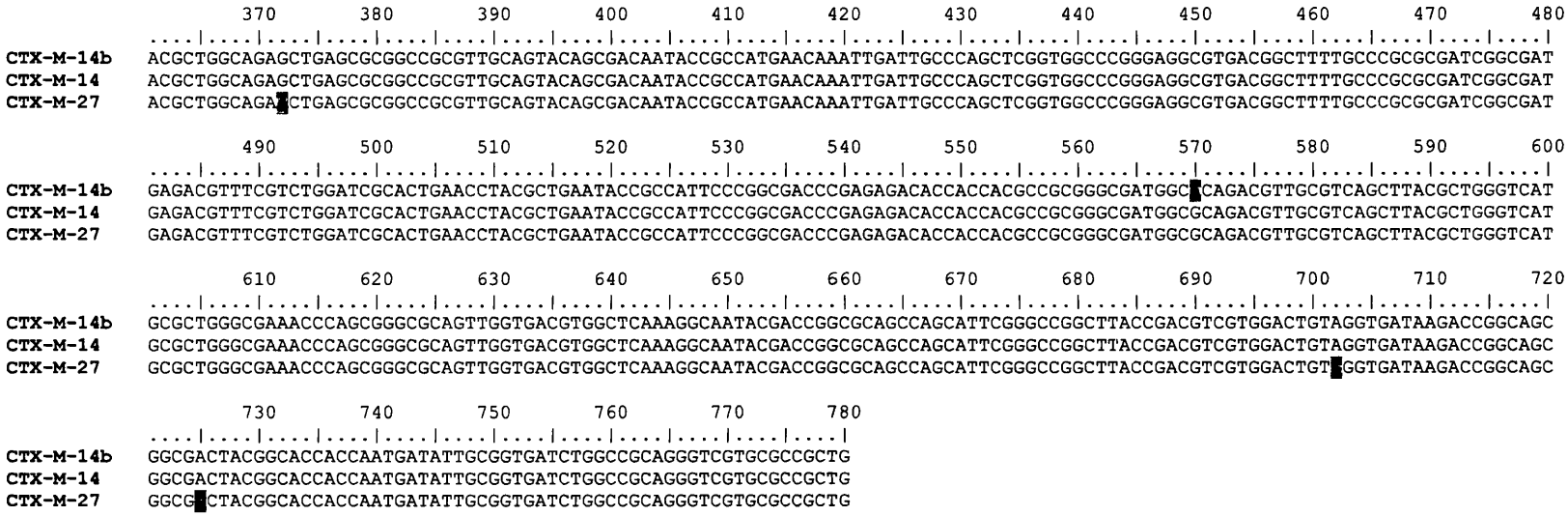



Figure 3.7 DNA sequences of CTX-M group 9 genes. CTX-M-14, CTX-M-14b, and CTX-M-27 were found in this study. This figure illustrates the point mutations found within each type.

### 3.3.4 Amp C prevalence

A multiplex PCR methodology devised by Pérez-Pérez and Hanson (2002) was used in order to detect family-specific plasmid-mediated *ampC* genes. The detection and differentiation of AmpC enzymes from ESBL enzymes is necessary for surveillance, epidemiology and infection control issues (Perez-Perez and Hanson, 2002).

Therefore in this study, this PCR system was used to determine genes associated with the *ampC* class of β-lactamases. In order to ensure that false-positive results were not obtained through the use of the multiplex PCR system, singleplex PCR was carried out on isolates which generated positive amplification products with the multiplex system. Of the 164 isolates collected, 5 isolates (3%) had *ampC* genes present. One *C. freundii* isolate was positive with CIT primers, 1 *M. morganii* was positive with MOX, 1 *E. cloacae* was positive with EBC and 2 *E. coli* isolates were positive for FOX primers. The CIT, MOX and EBC primers encode various specific target sequences (Table 3.6).

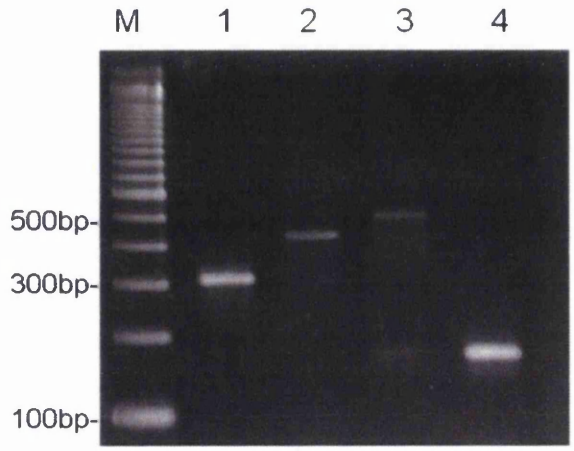



Figure 3.8 Agarose gel showing *ampC* specific multiplex PCR. Lane 1 - positive for EBC (302bp); lane 2 - positive for CIT (462bp); lane 3 positive for MOX (520bp); lane 4 positive for FOX (190bp). M -100bp DNA marker.

Table 3.6 Prevalence of *ampC* gene families and their respective target genes.

| | *ampC* gene family | **Target genes** |
|---|---|---|
| *E. coli* (n=2) | FOX | FOX-1 to FOX-5b |
| *E. cloacae* (n=1) | EBC | MIR-1T ACT-1 |
| *C. freundii* (n=1) | CIT | LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1 |
| *M. morganii* (n=1) | MOX | MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 |

### 3.3.5 General summary of ESBL and AmpC prevalence in *Enterobacteriaceae*

Using primers specific for CTX-M group 1 79% of *E. coli*, 76% of *Klebsiella* spp. and 11% of other *Enterobacteriaceae* selected for this study gave positive amplification. Of these CTX-M group 1 positive isolates, 92% (54/59) of the *E. coli* and *Klebsiella* spp. tested were found carry CTX-M-15.

Detection of CTX-M group 9 genes by PCR generated 5 positive amplification products, all within *E. coli*. Therefore 4% of all *E. coli* in this study were positive for CTX-M group 9. The most common CTX-M group 9 types found were CTX-M-14 and CTX-M-14b.

In this study, only 1 isolate of *E. coli* was found to possess a TEM ESBL gene (TEM-52) alone. In addition, the non-ESBL TEM-1 was found alone in 9 *E. coli* isolates, 3 *Klebsiella* spp. and 3 other *Enterobacteriaceae*. Two SHV-2 enzymes were found independently of other ESBL genes amongst 1 *E. coli* and 1 *Klebsiella* spp. isolates. The non-ESBL SHV-1 was found alone in 1 *E. coli* isolate whereas SHV-11 was found in 1 *Klebsiella* spp. The remaining TEM or SHV non-ESBL genes were all found in conjunction with a CTX-M gene. However, 8/12 genetically ESBL or AmpC negative *E. coli*, 3/6 genetically ESBL or AmpC negative *Klebsiella* spp., and 2/4 genetically ESBL or AmpC negative other *Enterobacteriaceae* possessed TEM-1. 1/6 genetically ESBL or AmpC negative *Klebsiella* spp. harboured SHV-11.

Four *ampC* genes were found alone in this study. These were distributed amongst 2 *E. coli* and 2 other *Enterobacteriaceae* isolates.

Eight isolates were found to possess multiple ESBL and *ampC* genes. These are described in section 3.3.6.

Within 22 isolates, of which 5 isolates had a positive synergy test with cefpodoxime, no genetic ESBL or AmpC mechanism could be ascertained. 10% *E. coli*, 16% *Klebsiella* spp. and 4% other *Enterobacteriaceae* isolates did not give positive amplification with any of the primers used in this study.

Table 3.7 Prevalence of ESBL, non-ESBL and *ampC* genes amongst *Enterobacteriaceae*.

| | **CTX-M Group 1 (alone)** | **CTX-M Group 9 (alone)** | **TEM (alone)** | **SHV (alone)** | **AmpC (alone)** | **Multiple ESBL/AmpC genes** | **No genetic ESBL/AmpC mechanism** |
|---|---|---|---|---|---|---|---|
| *E. coli* (n=117) | 92 | 5 | 1 TEM-52 | 1 SHV-2 | 2 (FOX) | 4 | 12 |
| *Klebsiella* spp. (n=38) | 29 | 0 | 0 | 1 SHV-2 | 0 | 2 | 6 |
| Other *Enterobacteriaceae* (n=9) | 1 | 0 | 0 | 0 | 2 (CIT, EBC) | 2 | 4 |

| | **Non-ESBL TEM-1 (alone)** | **SHV non-ESBL (alone)** |
|---|---|---|
| *E. coli* (n=117) | 9 | 1 SHV-1 |
| *Klebsiella* spp. (n=38) | 3 | 1 SHV-11 |
| Other *Enterobacteriaceae* (n=9) | 3 | 0 |

### 3.3.6 Multiple ESBL and AmpC-producing *Enterobacteriaceae*

After conducting TEM, SHV, CTX-M and AmpC PCR, 8 isolates were found to harbour more than one ESBL/*ampC* gene (Table 3.8). Four out of 105 (3%) ESBL and AmpC-producing *E. coli* were found to have a TEM gene, of which, 3 (75%) were TEM-116 and the remaining 1 (25%) was TEM-33. All were associated with a CTX-M group 1 gene. One of these four *E. coli* isolates harboured 3 β-lactamase genes: CTX-M group 1, TEM-116 and SHV-2. Two of 32 (6%) ESBL-producing *Klebsiella* spp. were found to contain two ESBL genes. Both (100%) were *K. pneumoniae* isolates carrying TEM-116. However, one isolate was found to harbour a CTX-M group 1 gene, whereas the other harboured SHV-2. Two of the 5 (40%) other ESBL/AmpC-producing *Enterobacteriaceae* specifically *C. freundii* and *E. cloacae*, were found to harbour multiple genes. The *C. freundii* was TEM-116 and MOX positive whereas, the *E. cloacae* harboured CTX-M group 1 and SHV-2 genes.

Table 3.8 Prevalence of multiple ESBL/*ampC* genes within *Enterobacteriaceae*

| | **ESBL Type** | | | |
|---|---|---|---|---|
| | **CTX-M** | **TEM** | **SHV** | **AmpC** |
| *E. coli* | Group 1 | TEM-33 | | |
| *E. coli* | Group 1 | TEM-116 | | |
| *E. coli* | Group 1 | TEM-116 | | |
| *E. coli* | Group 1 | TEM-116 | SHV-2 | |
| *K. pneumoniae* | Group 1 | TEM-116 | | |
| *K. pneumoniae* | | TEM-116 | SHV-2 | |
| *C. freundii* | | TEM-116 | | MOX |
| *E. cloacae* | Group 1 | | SHV-2 | |

### 3.4 Discussion

ESBLs were first reported in 1983 and until 2001 ESBLs in the UK were rare and mostly mutants of TEM and SHV which were predominantly found in *Klebsiella* spp. (Potz et al., 2006). However, the emergence of CTX-M since 2001 (Alobwede et al., 2003) has led to CTX-M becoming the most predominant ESBL type in the UK (Munday et al., 2004). This study provides an insight into the current epidemiology of ESBL-producing *Enterobacteriaceae* in Swansea, South West Wales. The molecular characterisation of isolates allows for the prevalence of ESBL and *ampC* genes within a particular area to be determined. The monitoring of ESBL enzyme types is fundamental in tracking changes within ESBL epidemiology i.e. emergence and dissemination of prevalent genes.

In this study, a multiplex PCR system was used to detect CTX-M genes belonging to 5 different phylogenetic groups namely CTX-M group 1, group 2, group 8, group 9 and group 25/26 (Woodford et al., 2006). Although this method provided a rapid identification of CTX-M genes, it was found to have reliability limitations. Fragments resembling group 2 and group 25/26 genes were amplified using the multiplex system but were later deduced to be false-positives. However, Woodford et al., (2006) utilised the primers on control isolates harbouring CTX-M groups 25/26 and 2 which had been previously sequenced. In their study, 633 isolates were analysed by this multiplex PCR system to find that no isolates harboured CTX-M group 25/26 genes and only 3 isolates were found to be CTX-M group 2.

The predominant ESBL genotype found was CTX-M. CTX-M-type enzymes were also prevalent in a study conducted in London and the South East of England by Potz et al., (2006). In Potz's study, CTX-M (phylogenetic groups not specified) was found in 50.7% of *E. coli*, 81.5% of *Klebsiella* spp. and 4% of *Enterobacter* spp. A study by Warren et al., (2008b) conducted in Shropshire between 2003 and 2006 demonstrated that 86% of ESBLs carried by *E. coli* were CTX-M, particularly CTX-M-15. This was also demonstrated by Tarrant et al., (2007) whereby CTX-M-15 was found in 70% of isolates from the South West of England. Results in this chapter demonstrate that CTX-M group 1 was found in 92% of isolates, comparable to figures recorded by Woodford et al., (2004) in whose study 95% (279/291) of isolates had a CTX-M group 1 gene. CTX-M group 1 was also prevalent during a

'Cornish outbreak' between 2004 and 2005. During this outbreak, 65% (45/69) *E. coli* isolates were found to possess CTX-M group 1 genes. This outbreak strain was found to be different by PFGE to any of the UK's 5 epidemic *E. coli* strains, A-E (Woodford et al., 2007b).

A report by Cantón and Coque (2006) demonstrated that CTX-M enzymes were at endemic levels in Europe, Canada, South America, Asia, Africa and Australia (Canton and Coque, 2006). In Europe, the rate of ESBL carriage is generally increasing with CTX-M and TEM ESBLs being the most prevalent enzymes (Coque et al., 2008a): CTX-M-15 enzymes are now the most commonly reported ESBL in Europe (Peirano and Pitout, 2010). Nordic countries maintain the lowest ESBL rates (Coque et al., 2008a). The rate of ESBLs in Europe is less than in South America and Asia (Dhillon and Clark, 2011).

Rates of ESBLs in South America are some of the highest in the world (Dhillon and Clark, 2011). There, prevalence of ESBL in *Klebsiella* spp. ranges from 45%-51% and in *E. coli* isolates from 8.5% to 18%. CTX-M is the predominant type of enzyme (Turner et al., 1999, Winokur et al., 2001) with CTX-M-2 and CTX-M-9 being most prevalent (Villegas et al., 2008).

In Africa, especially North Africa, CTX-M also appears to be the most prevalent ESBL type whereas it is suggested that TEM and SHV ESBLs are more frequent in South Africa (Bell et al., 2002, Dhillon and Clark, 2011). However, Egypt was found to be the African country with the highest rate of ESBLs with 72% of isolates exhibiting resistance to 3$^{rd}$ generation cephalosporins (Borg et al., 2006).

Rates of ESBLs are high in Asia and across the region variation in prevalence and ESBL types occur (Bell et al., 2002, Dhillon and Clark, 2011). Rates were found to be particularly high in China with 40% of *E. coli* and 20-60% of *Klebsiella* spp. demonstrating an ESBL profile (Bell et al., 2002). CTX-M-14 and CTX-M-9 were found to be the most common enzymes in China (Hawkey, 2008). A study carried out in India demonstrated ESBL rates to be at 48% (Tankhiwale et al., 2004) and a CTX-M genotype notably CTX-M-15 was the most prevalent (Ensor et al., 2006). CTX-M enzymes, particularly CTX-M-15, were also found to be most common in *E. coli* and *Klebsiella* spp. isolates in Australia (Zong et al., 2008).

In the USA, TEM and SHV ESBLs were once the most predominant ESBL type (Cornaglia et al., 2008, Livermore et al., 2007). However, it is thought that CTX-M-15 is, by now, the most commonly found ESBL genotype. This is comparable to the ESBL epidemiology of Canada whereby CTX-M-producing *Klebsiella* spp. have been reported (Melano et al., 2006). It was proposed that the spread of CTX-M from Canada to the USA was a potential threat (Dhillon and Clark, 2011).

In this study, CTX-M-15 was found to be the most common ESBL genotype in Swansea with 92% of sequenced *E. coli* and *Klebsiella* spp. possessing this gene. As mentioned above, CTX-M-15 enzymes are now the most commonly reported ESBL in Europe (Peirano and Pitout, 2010) and CTX-M-15 has also been reported in Canada (Pitout et al., 2007, Pitout et al., 2005a, Boyd et al., 2004), Asia (Ensor et al., 2006, Hawkey, 2008), Africa (Gangoue-Pieboji et al., 2005), South America (Villegas et al., 2008) and Australia (Zong et al., 2008).

The previously recorded changes in ESBL epidemiology can be clearly supported by this study. As previously mentioned, TEM and SHV were once the most frequently found ESBLs in the UK before the emergence of CTX-M. In this study, only one isolate of *E. coli* was found to possess a TEM gene independent of any other ESBL genes and only two isolates (1 *E. coli* and 1 *Klebsiella* spp.) were found to harbour SHV-2 as a sole ESBL gene.

In this chapter, 8 isolates were found to possess more than one ESBL/*ampC* gene suggesting the acquisition of plasmids with multiple-ESBL genes present. In a study by Woodford et al., (2006) 1 isolate (1/633) was found to possess a CTX-M group 1 gene alongside a CTX-M group 9 gene. However, in a study by Romero et al., (2007) multiple ESBLs were found more frequently. Out of 154 isolates, 7.8% were found to possess CTX-M and TEM genes, 4.6% possessed TEM and SHV genes, 4.6% possessed CTX-M, TEM and SHV genes and 1.3% possessed CTX-M and SHV genes (Romero et al., 2007). Additionally to note, AmpC production (either plasmid mediated or inducible chromosomal) can be difficult to distinguish from ESBL enzymes. If an organism is an ESBL and AmpC-producer, then identification of the β-lactamase type can be mistaken phenotypically thus potentially leading to the incorrect usage of antibiotics for treatment (Perez-Perez and Hanson, 2002).

In this study, 4 isolates were found to possess an *ampC* gene independent of an ESBL gene. The findings of positive PCR amplifications by EBC primers in *E. cloacae* and CIT primers in *C. freundii* are not unexpected as these isolates are the source for EBC and CIT *ampC* groups. However, the presence of MOX in *M. morganii* suggested a plasmid-acquisition of *ampC* genes as the expected AmpC group associated with *M. morganii* is DHA (Perez-Perez and Hanson, 2002). In this thesis, *ampC* amplification products were not sequenced and so could be seen as a limitation as false positive results could arise through PCR. In addition, to the 4 isolates harbouring *ampC* genes, 1 isolate had an *ampC* gene in conjunction with a TEM-116 gene. A study by Kao *et al.*, (2010) found that *ampC* genes were commonly found in association with a TEM gene. 26/52 *E. coli* isolates possessed an *ampC* and TEM combination (Kao et al., 2010).

Additionally, the presence of ESBL genes is linked to antibiotic resistance. For example, isolates producing TEM and SHV enzymes are usually resistant to ceftazidime but exhibit variable resistance to cefotaxime whereas isolates harbouring CTX-M are often resistant to cefotaxime and exhibit variable resistance to ceftazidime (Livermore and Brown, 2001). It has also been described that CTX-M-15-producing isolates are more resistant to ESBL inhibitor combinations e.g. piperacillin/tazobactam (Livermore and Hawkey, 2005) and that epidemic *E. coli* strain A is more susceptible to gentamicin (Woodford et al., 2004).

The numbers of ESBLs are continuously expanding and, as described, have been detected across the world. The concern with the vast spread of CTX-M genes lies with its impact on the clinical setting. With such an increase in CTX-M prevalence, treatment options become limited, and so even minor infections like UTI may require the use of carbapenems (Livermore and Hawkey, 2005, Peirano and Pitout, 2010, Dhillon and Clark, 2011). As a result of this increased selective pressure, the next threat, in the form of increasing rates of carbapenemases, in on the horizon (Livermore, 2009).

# Chapter 4: Evaluation of phenotypic screening and confirmatory assays for detection of ESBL-producing *Enterobacteriaceae*

## 4.1 Introduction

Extended-spectrum β-lactamase (ESBL) producers are problematic amongst multidrug resistant *Enterobacteriaceae* as they can hydrolyse extended-spectrum β-lactam antibiotics including 1st, 2nd, 3rd generation cephalosporins and the monobactam aztreonam. This leads to antibiotic resistance therefore causing problems as cephalsporins are key to many clinical antibiotic treatment regimens. There are many types of ESBL and their prevalence is increasing world-wide (Paterson and Bonomo, 2005, Bonnet, 2004, Livermore and Hawkey, 2005, Bradford, 2001).

In the clinical setting, the detection of ESBL is essential as complication and death can arise when ESBL-associated infections are treated with cephalosporins, to which they are resistant or even susceptible to *in vitro*. It is imperative to treat ESBL infections with the appropriate agents (Paterson et al., 2001, Crowley, 2001, Stürenburg and Mack, 2003).

The detection methods employed must be sensitive and accurate as ESBL-producers can often appear susceptible to extended-spectrum cephalosporins at a standard inoculum size of $10^5$ (Spencer et al., 1987, Jacoby and Medeiros, 1991). Also important to note is the need for a rapid and reliable turnaround time which is cost-effective.

### 4.1.1 Laboratory detection of ESBL producing organisms

ESBL detection in the PHW Microbiology ABM Swansea laboratory currently involves a two-step procedure whereby *Enterobacteriaceae* are firstly screened for ESBL production and then confirmed as ESBL producers in line with national recommendations e.g. those produced by BSAC. Coliform isolates are screened using a cefpodoxime (CPD) disc at a concentration of 10μg. If the isolate is found to be resistant to cefpodoxime then it is identified using API 20E in parallel to carrying out a synergy test. If the synergy test is found to be positive then the isolate can be

confirmed as ESBL. However, this test is currently recommended for *E. coli* and *Klebsiella* spp. only (Black et al., 2005).

#### 4.1.1.1 Screening for ESBL

The choice of antibiotics used for ESBL screening is important since different ESBL enzymes confer different levels of resistance amongst the cephalosporins *in vitro*. The BSAC recommends that all *Enterobacteriaceae* are screened for ESBL using an indicator cephalosporin as part of primary susceptibility testing (Andrews, 2004). Cefpodoxime tends to be used as the indicator of choice, as TEM and SHV are resistant to ceftazidime but exhibit variable resistance to cefotaxime whereas CTX-M are resistant to cefotaxime and exhibit variable resistance to ceftazidime. However in some situations, ceftazidime and cefotaxime can be used instead of cefpodoxime. If the tested organism is found to be resistant to the indicator cephalosporin then the isolate will undergo confirmation testing (Livermore and Brown, 2001).

BSAC produce disc susceptibility testing guidelines for determining whether *Enterobacteriaceae* are sensitive, intermediate or resistant to each antibiotic. Briefly, an iso-sensitest agar plate is swabbed with a 0.5 McFarland inoculum of the test organism. This step is important as the inoculum should give semi-confluent growth. The antibiotic discs are dispensed onto the inoculated agar and the plate is incubated at 37°C for 18 hours. After this time has elapsed, the diameters of the zones of inhibition are measured in millimetres and can therefore be interpreted. Table 4.1 outlines the susceptibility profile for each indicator cephalosporin tested in this study based upon its zone of inhibition (BSAC, 2007).

Table 4.1 Interpretation of susceptibility based upon the zone of inhibition created when tested with various cephalosporins.

| | | Zone of Inhibition (mm) | | |
|---|---|---|---|---|
| **Cephalosporin** | **Concentration** | **Resistant ≤** | **Intermediate** | **Sensitive ≥** |
| Cefpodoxime (CPD) | 10μg | 19 | | 20 |
| Ceftazidime (CAZ) | 30μg | 29 | | 30 |
| Cefotaxime (CTX) | 30μg | 29 | | 30 |
| Cefepime (CPM) | 30μg | 26 | 27-31 | 32 |
| Cefpirome (CFP) | 30μg | 24 | | 25 |
| Cefuroxime (CXM) | 30μg | 19 | | 20 |
| Cefoxitin (FOX) | 30μg | 19 | | 20 |

#### 4.1.1.2 Confirmation of ESBL

The production of an ESBL enzyme is confirmed phenotypically via three different methods.

##### 4.1.1.2.1 Synergy testing

This disc test involves the comparison of the zones of inhibition between an indicator cephalosporin and its augmented (i.e. with clavulanic acid) counterpart (Figure 4.1). The presence of an ESBL can be confirmed where the zone of inhibition for the cephalosporin plus clavulanic acid is ≥ 5mm larger than the cephalosporin alone.

In 2000, M'Zali et al. calculated a ratio of the diameter of the zone of inhibition with or without clavulanic acid using the formula:

$$\frac{\text{Cephalosporin/clavulanic acid}}{\text{Cephalosporin}} \geq 1.5$$

Therefore, a ratio of ≥ 1.5 can also confirm the presence of an ESBL (M'Zali et al., 2000).

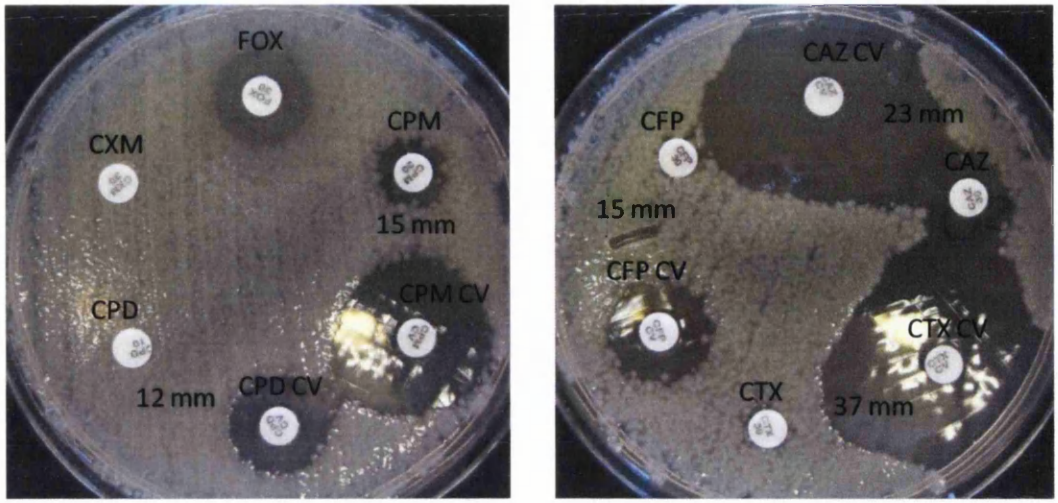



Figure 4.1 Principles of the synergy test. Synergy can be seen between each cephalosporin and clavulanic acid augmented counterpart, as there is a clear difference of ≥5mm between the diameters of the zones of inhibition. Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP) along with the respective augmented discs – clavulanic acid (CV).

#### 4.1.1.2.2 Disc Approximation Test

The Disc Approximation Test utilises co-amoxiclav (amoxicillin-clavulanic acid) as a source of clavulanic acid. The co-amoxiclav disc is placed in the centre of an inoculated agar plate surrounded by discs containing cephalosporins alone e.g. cefpodoxime, ceftazidime, cefotaxime at a distance of 25-30 mm apart (Fig 4.2) (Thomson and Sanders, 1992). ESBL presence is confirmed where there is a distortion to the zone of inhibition between the cephalosporin and co-amoxiclav discs.

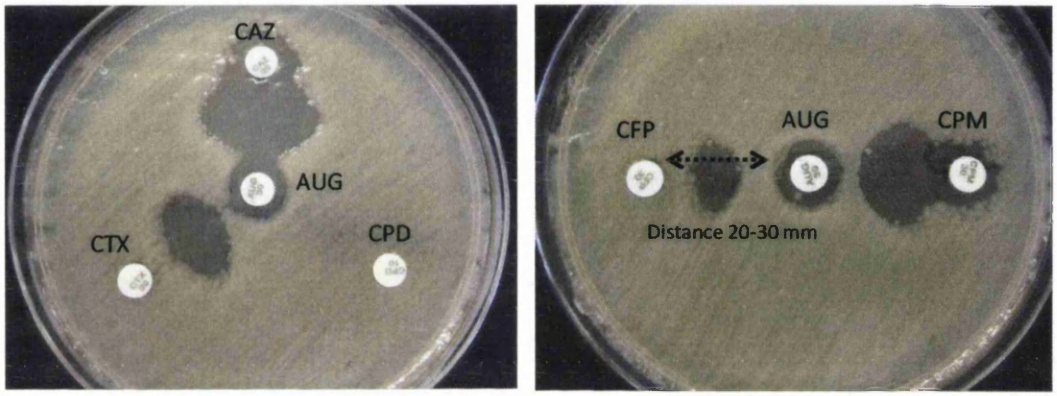



Figure 4.2 Representation of the Disc Approximation Test. Co-amoxiclav (AUG) has created a distortion and expansion in the zones of inhibition around the indicator cephalosporins. However, no distortion is seen between co-amoxiclav and cefpodoxime (CPD). Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefepime (CPM) and cefpirome (CFP) along with the co-amoxiclav (AUG).

#### 4.1.1.2.3 ESBL E-test

The ESBL E-test utilises a plastic strip impregnated at one end with a cephalosporin e.g. ceftazidime with a concentration range of 0.25–16mg/L. Its clavulanic acid counterpart e.g. ceftazidime with 4mg/L clavulanic acid with a concentration range of 0.064–4mg/L is found on the opposite end. The respective MIC can be read from the scale where the ellipse of the inhibition zone cuts the strip. ESBL production is confirmed if the ratio of the MIC of cephalosporin alone to the MIC of cephalosporin plus clavulanate is ≥8 (Cormican et al., 1996). The deformation of the zone of inhibition ellipse or the formation of a 'phantom zone' of inhibition is also characteristic of ESBL production (Stürenburg et al., 2004b).

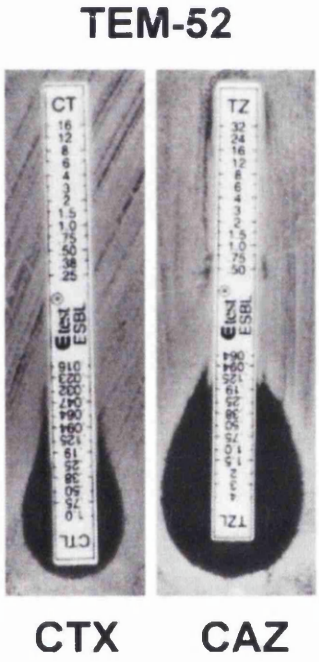



Figure 4.3 The ESBL E-test. The indicator cephalosporin is found at the upper half of the E-test strip in this case, CT is cefotaxime and TZ is ceftazidime. The lower half of the E-test strip contains the indicator cephalosporin plus clavulanic acid. Both are within an MIC gradient. In this example, a TEM-52 producing *E. coli* has a ceftazidime reading of >32µg/ml and a ceftazidime plus clavulanic acid reading of 0.125µg/ml. As the ratio between the two concentrations are ≥8, which is ≥ three MIC dilution steps, the isolate is confirmed to be ESBL producing. Reproduced from Stürenburg and Mack (2003).

#### 4.1.1.3 Detection of ESBL in strains harbouring inducible AmpC

In the clinical laboratory, the presence of an inducible AmpC may hinder the detection of ESBL production in strains harbouring both AmpC and ESBL enzymes. AmpC is poorly inhibited by clavulanic acid thus it allows the AmpC β-lactamase to attack the cephalosporin. This process can disguise synergy caused by the inhibition of the ESBL therefore preventing its detection. To overcome this problem, cefepime or cefpirome may be used. These antibiotics are stable to inducible AmpC and are therefore not hydrolysed as successfully (Tzouvelekis et al., 1999).

### 4.1.2 Phenotypic detection of AmpC

Currently in clinical laboratories, plasmid-mediated AmpC β-lactamases are not routinely screened for (Black et al., 2005). However, all *Enterobacter* spp., *C. freundii*, *Serratia* spp., *Providencia* spp., and *M. morganii* (not isolated from urine samples) are recommended to be reported as resistant to all 3$^{rd}$ generation cephalosporins, as they all possess chromosomal *ampC* genes which can be induced by certain β-lactam antibiotics (Livermore et al., 2001). In many clinical isolates of these species *ampC* expression becomes derepressed leading to resistance to all cephalosporins with exception of 4$^{th}$ generation cephalosporins and carbapenems.

There are methods available to phenotypically determine AmpC production. Four examples have been illustrated below. Of these, three use cefoxitin as an indicator as its susceptibility profile can be used to predict the inducibility of AmpC enzymes (Moritz and Carson, 1986). However, in this study the Mast® AmpC and ESBL Detection set was evaluated. This is a commercially available phenotypic kit which is also described here.

#### 4.1.2.1 Cefoxitin/cefotaxime antagonism

This method is a modified Disc Approximation Test (Sanders et al., 1982). A 30μg disc of cefoxitin is placed 25-30mm away from a 30μg disc of cefotaxime on an Iso-sensitest agar plate inoculated using BSAC guidelines. AmpC presence can be inferred when there is an antagonism between the two discs i.e. if the zone of inhibition radius between cefoxitin and cefotaxime is smaller by 4mm than the zone of inhibition produced by cefotaxime alone. Alternatively, antagonism can be

identified by a 'blunting' of the cefotaxime zone of inhibition adjacent to the cefoxitin disc (Livermore and Brown, 2001).

#### 4.1.2.2 Boronic acid disc test

In 2005, both Brenwald *et al.* and Coudron discussed the use of benzo(b)thiophene-2-boronic acid (Boronic acid) in combination with a cephalosporin to determine AmpC production. Boronic acid is an AmpC inhibitor (Beesley et al., 1982). Using BSAC guidelines, iso-sensitest agar is inoculated and discs containing a cephalosporin, cephalosporin plus clavulanic acid (clavulanic acid induces AmpC), either with or without Boronic acid, are placed on the surface and allowed to incubate overnight. A positive result is defined as a difference of $\geq$ 5mm in the zone of inhibition around the combined disc in comparison to the cephalosporin alone (Brenwald et al., 2005) The method utilised by Coudron differs in that cefotetan (CTT) is used along with phenylboronic acid (Coudron, 2005). Cefotetan is a strong inducer of *ampC* β-lactamases.

#### 4.1.2.3 Mast® AmpC and ESBL Detection Set

This method is capable of detecting the presence of an ESBL, AmpC or a combination of both. The detection set comprises of four discs; A, B, C and D. The discs contain 10μg of cefpodoxime, 10μg of cefpodoxime plus ESBL inhibitor, 10μg of cefpodoxime plus AmpC inhibitor and 10μg of cefpodoxime plus ESBL inhibitor and AmpC inhibitor, respectively. The diameters of the zones of inhibition (Z) are measured and a series of calculations (Table 4.2) performed to deduce the presence of ESBL, AmpC or both as recommended by the manufacturer.

Table 4.2 Zone calculations to determine presence of ESBL and/or AmpC

| Interpretation | Zone calculation | Result |
|---|---|---|
| ESBL positive | ZB - ZA and ZD – ZC | ≥ 5mm |
| | ZD - ZB and ZC - ZA | < 5mm |
| AmpC positive | ZB - ZA and ZD – ZC | < 5mm |
| | ZD - ZB and ZC - ZA | ≥ 5mm |
| ESBL and AmpC positive | ZD – ZC | ≥ 5mm |
| | ZB - ZA | < 5mm |
| AmpC and ESBL negative | | All zones differ by ≤ 2mm |

### 4.1.3 ESBL detection using semi automated systems

In order to decrease laboratory turnaround time and provide a cost-effective result, semi automated systems are routinely used to identify bacteria and to provide antimicrobial susceptibility testing (Wiegand et al., 2007). Some of these commercially available instruments also have algorithms capable of detecting ESBL e.g. BD Phoenix, Vitek (bioMerieux, France), MicroScan Walkway System (Siemens, Germany) and Sensititre ARIS (Automatic Reading and Incubation System). In this study the BD Phoenix Automated System was used and the Phoenix Xpertsystem evaluated with respect to ESBL identification. The methodology is detailed in Chapter 2. However, bacterial identifications provided by the BD Phoenix Automated System were compared with identifications using the Bruker Daltonics MALDI-Biotyper system. Results of this evaluation are outlined in detail in Chapter 7.

In facing the changing epidemiology of ESBL-types encountered in *Enterobacteriaceae* it is necessary to validate the performance of the diagnostic armamentarium with recent molecularly confirmed isolates. The aims of the experiments described in this chapter therefore included:

- Bacterial species identification by the BD Phoenix Automated System

- Detection of ESBL using the BD Phoenix Xpert System and rule 1505
- Sensitivity of ESBL screening using indicator cephalosporins (a comparison of BSAC and HPA QSOP51 guidelines)
- Sensitivity of confirmation using indicator cephalosporins (a comparison of the Synergy Test and Disc Approximation Test)
- Sensitivity of cefoxitin for the detection AmpC
- The evaluation of the Mast® AmpC and ESBL Detection set.

## 4.2 Results

### 4.2.1 Bacterial species identification

In order to identify the *Enterobacteriaceae* collected from the PHW Microbiology ABM Swansea laboratory, the BD Phoenix Automated system and Bruker Daltonics MALDI Biotyper were used (latter results reported in Chapter7).

#### 4.2.1.1 Identification of isolates by BD Phoenix automated system

The BD Phoenix Automated System was used to provide an identification of the *Enterobacteriacae* collected. The BD Phoenix system and method utilised are described in Chapter 2 section 2.4.1. One hundred and sixty-four isolates were subjected to identification by this semi-automated system and the identifications are shown in Table 4.3.

Table 4.3 Bacterial identification using BD Phoenix Automated System

| **Identification by BD Phoenix** | **Number of isolates (n=164)** |
|---|---|
| *E. coli* | 117 |
| *Klebsiella pneumoniae* | 30 |
| *Klebsiella pneumoniae* ssp. *ozaenae* | 4 |
| *Klebsiella oxytoca* | 4 |
| *Enterobacter cloacae* | 6 |
| *Citrobacter freundii* | 1 |
| *Citrobacter koseri* | 1 |
| *Morganella morganii* | 1 |

#### 4.2.1.2 Identification of ESBL by BD Xpert System (BD Phoenix)

Using antimicrobial susceptibility testing (AST) results, the BD Xpert system was able to deduce ESBL production. The BD Xpert System delegates rules depending on the resistance profile produced.

Table 4.4 BD Xpert system rules

| Rule | Definition |
|---|---|
| **1505** | *"Isolate is confirmed positive for Extended-Spectrum Beta-Lactamase (ESBL). Consultation with an infectious disease practitioner is recommended."* |
| **106** | *"Screening tests suggest a possible Extended-Spectrum Beta-Lactamase (ESBL) producer. Confirmatory testing is recommended before reporting results for penicillins, extended-spectrum cephalosporins or aztreonam"* |
| **1433** | *"Isolate exhibits unusual resistance to third generation cephalosporins. Additional confirmatory testing for possible ESBL or AmpC hyperproduction is recommended before reporting results for beta-lactams."* |

Table 4.5 outlines the BD Phoenix rules found within the 164 isolates tested. 88.4% (145/164) of all isolates were delegated rule no. 1505, 3.1% (5/164) generated rule no. 106, 1.2% (2/164) generated rule no. 1433 and 7.3% (12/164) had no ESBL related rule associated to their resistance profile. 84% (138/164) of isolates were molecularly confirmed as ESBL (see Chapter 3). Of these 138 isolates, 134 generated rule no. 1505 suggesting an overall sensitivity of 97% in detecting ESBL, for the BD Phoenix system. However, if the other species of *Enterobacteriaceae* are disregarded from this figure so that only ESBL *E. coli* and *Klebsiella* spp. are counted, then the sensitivity of the BD Phoenix automated system increases to 99%.

93% (109/117) of all *E. coli* generated rule no. 1505. 4% (5/117) generated rule no. 106 and 3% (4/117) generated no ESBL related rule. 88% (103/117) *E. coli* were molecularly confirmed to be ESBL-producing, of which, 102 (99%) were ascribed as rule no. 1505. One (1%) molecularly confirmed *E. coli* isolate (CTX-M-32) was delegated rule no. 106 by the BD Xpert System. The BD Xpert System generated

rule no. 106 for 2 AmpC producing *E. coli*, which were found independently of an ESBL gene.

95% (36/38) of all *Klebsiella* spp. generated rule no. 1505 by the BD Xpert System. 5% (2/38) of *Klebsiella* spp. did not have any ESBL related rule. However, one *Klebsiella* spp. possessed rule 2004 and another *Klebsiella* spp. generated rule 1503. These are β-lactamase related rules and are as follows: rule no. 2004 *"Enterobacteriaceae, resistant to any second generation cephalosporins, are resistant to first generation cephalosporins, because they are likely to have potent beta-lactamase."* and rule no. 1503 *"When K1 hyperproduction phenotype (a pattern of intermediate or resistant to aztreonam) is detected in Klebsiella oxytoca, an interpretation of susceptible for beta-lactams, except carbapenems, cephamycins, fourth generation cephalosporins, ceftazidime, or moxalactam should be changed to resistant."* 82% (31/38) *Klebsiella* spp. were molecularly confirmed as ESBL, all of which (100%) were delegated rule no. 1505.

Rule no. 1505 was generated in seven non-molecularly confirmed *E. coli* isolates and 4 *Klebsiella* spp.

Apart from *E. coli* and *Klebsiella* spp., rule no. 1505 was not generated in any other species. However, an alternative rule, 1433 was generated in 3/9 remaining *Enterobacteriaceae* isolates (all of which were *E. cloacae*). 67% (2/3) were molecularly confirmed as ESBL-producers. Although possessing rule no. 1433, the remaining isolate (33%) was not genetically confirmed as carrying ESBL or AmpC.

One isolate generated rule no. 106. This isolate was identified as *E. cloacae* and was genetically confirmed to be an AmpC-producer. The BD Xpert system did not generate a rule for the second genetically confirmed AmpC-producing isolate.

In conclusion it appears that the BD Phoenix Xpert system screened and confirmed ESBL presence in the molecularly confirmed *E. coli* and *Klebsiella* spp. with high sensitivity.

Table 4.5 Detection of ESBL-producing organisms based on specific rules generated by BD Xpert System (BD Phoenix)

| | BD Phoenix Rule | | | |
|---|---|---|---|---|
| | **1505** | **106** | **1433** | **No ESBL or AmpC rule** |
| Total (n=164) | 145 | 5 | 2 | 12 |
| All *E. coli* (n=117) | 109 | 4 | | 4 |
| Molecular ESBL *E. coli* (n=103) | 102 | 1 | | |
| *E. coli* IS26 Postive (n=66) | 66 | | | |
| *E. coli* IS26 Negative (n=37) | 36 | 1 | | |
| AmpC positive *E. coli* (n=2) | | 2 | | |
| All *Klebsiella spp.* (n=38) | 36 | | | 2 |
| Molecular ESBL *Klebsiella* spp. (n=32) | 32 | | | |
| All Other (n=9) | | 1 | 3 | 5 |
| ESBL Other (n=3) | | | 2 | 1 |
| AmpC Other (n=2) | | 1 | | 1 |

### 4.3 Sensitivity of ESBL screening using BSAC and HPA QSOP 51 guidelines

The choice of inhibitor cephalosporin is crucial in screening for presence of ESBL. The sensitivity of screening has been analysed using two sets of guidelines – BSAC and HPA QSOP51. The only difference between these guidelines is the adjustment of ceftazidime breakpoints. BSAC list a screening breakpoint of 17mm and the HPA QSOP51 guidelines propose increasing the breakpoint to 21mm. The screening results for all isolates along with all molecularly confirmed ESBL and AmpC isolates using BSAC guidelines and HPA QSOP 51 guidelines are outlined in tables 4.6 and 4.7.

Using cefpodoxime, 157/164 (96%) of all isolates were found to be resistant giving an overall sensitivity of 99% when taking all molecularly confirmed ESBL carriers as a baseline for true positives. The sensitivity of screening for ESBL-producing *E. coli* was 99% whereas it was 100% sensitive for *Klebsiella* spp. and other *Enterobacteriaceae*. Cefotaxime was the next most sensitive indicator cephalosporin, with an overall sensitivity of 91% for all molecularly confirmed ESBL isolates. Cefotaxime was found to have a better sensitivity in detecting ESBL-carrying *Klebsiella* spp. (100%) than *E. coli* (88%). Two different guidelines were utilised in the evaluation of ceftazidime; BSAC and HPA QSOP51. Using BSAC guidelines, a sensitivity of 36% was achieved for all molecularly confirmed ESBL isolates whereas this increased to 41% using HPA QSOP51 guidelines. Sensitivity for ESBL-producing *E. coli* isolates was 18% using BSAC guidelines and this percentage increased to 25% using HPA guidelines. An difference of 4% in sensitivity was demonstrated between the two guidelines for *Klebsiella* spp.; 84% sensitivity with BSAC and 88% sensitivity with HPA QSOP51 guidelines. The sensitivity of cefepime screening was also quite poor, with an overall sensitivity of 48% for molecularly confirmed ESBL isolates. However, it was found to be more sensitive at screening ESBL-producing *Klebsiella* spp. (84%) than *E. coli* isolates (37%). Using cefpirome, a screening sensitivity of 69% was achieved for all molecularly confirmed ESBL isolates. Again it was also found to be more sensitive at screening for ESBL-producing *Klebsiella* spp. (88%) than *E. coli* isolates (67%)

In summary, cefpodoxime and cefotaxime are the most suitable indicator cephalosporins as a sensitivity of 99% and 91% was attained.

Using a combination of ceftazidime and cefotaxime gave overall sensitivities for molecularly confirmed ESBL-producing isolates of 91% and 92% using BSAC and HPA QSOP51 guidelines, respectively. However by using a combination of cefpodoxime and ceftazidime along with the HPA QSOP51 guidelines, gave 100% sensitivity of ESBL screening. Using BSAC guidelines meant that a sensitivity of 99% was achieved. This pattern is followed throughout as more disc combinations are investigated. For example, using a 3, 4 or 5 disc combination would give either a 99% or 100% sensitivity depending on the guidelines followed.

Table 4.6 Sensitivity of BSAC and HPA NDM QSOP 51 ESBL screening using cephalosporin discs

| | CPD | | CTX | | CAZ | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | BSAC | | HPA | |
| | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity |
| All *E. coli* (n=117) | 110 (94%) | 99% | 98 (84%) | 89% | 22 (19%) | 18% | 29 (25%) | 25% |
| ESBL *E. coli* (n=103) | 102 (99%) | 99% | 91 (88%) | 89% | 19 (18%) | 18% | 26 (25%) | 25% |
| AmpC E. coli (n=2) | 2 (100%) | | 2 100% | | 2 (100%) | | 2 (100%) | 100% |
| All Klebsiella spp. (n=38) | 37 (97%) | 100% | 37 (97%) | 100% | 31 (82%) | 84% | 32 (84%) | 88% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 32 100% | 100% | 27 (84%) | 84% | 28 (88%) | 88% |
| All Others (n=9) | 7 (78%) | 100% | 4 (44%) | 100% | 4 (44%) | 100% | 5 (56%) | 100% |
| Molecularly confirmed ESBL Others (n=3) | 3 (100%) | 100% | 3 (100%) | 100% | 3 (100%) | 100% | 3 (100%) | 100% |
| Molecularly confirmed AmpC Others (n=2) | 2 (100%) | | 0 (0%) | | 0 (0%) | | 1 (50%) | |
| Total of all isolates (n=164) | 157 (96%) | 99% | 139 (85%) | 91% | 57(35%) | 36% | 66 (40%) | 41% |
| Total of molecularly confirmed ESBL (n=138) | 137 (99%) | 99% | 126 (91%) | 91% | 49 (36%) | 36% | 57 (41%) | 41% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.6 cont...Sensitivity of BSAC and HPA NDM QSOP 51 ESBL screening using cephalosporin discs

| | CPM | | CFP | |
|---|---|---|---|---|
| | **No. resistant (%)** | **Sensitivity** | **No. resistant (%)** | **Sensitivity** |
| All *E. coli* (n=117) | 40 (34%) | 37% | 66 (56%) | 63% |
| ESBL *E. coli* (n=103) | 38 (37%) | 37% | 64 (62%) | 62% |
| AmpC *E. coli* (n=2) | 1 (50%) | | 2 (100%) | |
| All Klebsiella spp. (n=38) | 31 (82%) | 84% | 32 (84%) | 88% |
| ESBL *Klebsiella* spp. (n=32) | 27 (84%) | 84% | 28 (88%) | 88% |
| All Others (n=9) | 0 (0%) | 0% | 6 (67%) | 100% |
| Molecularly confirmed ESBL Others (n=3) | 0 (0%) | 0% | 3 (100%) | 100% |
| Molecularly confirmed AmpC Others (n=2) | 0 (0%) | | 1 (50%) | |
| Total of all isolates (n=164) | 71 (43%) | 48% | 104 (63%) | 69% |
| Total of molecularly confirmed ESBL (n=138) | 65 (47%) | 48% | 95 (69%) | 69% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.7 Sensitivity of BSAC and HPA NDM QSOP 51 ESBL screening using combinations of cephalosporin discs

| | CPD+CAZ | | | | CPD+CTX | |
|---|---|---|---|---|---|---|
| | BSAC | | HPA | | | |
| | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity |
| All *E. coli* (n=117) | 112 (96%) | 99% | 113 (97%) | 100% | 111 (95%) | 99% |
| ESBL *E. coli* (n=103) | 102 (99%) | 99% | 103 (100%) | 100% | 102 (99%) | 99% |
| AmpC *E. coli* (n=2) | 2 (100%) | | 2 (100%) | | 2 (100%) | |
| All Klebsiella spp. (n=38) | 37 (97%) | 100% | 37 (97%) | 100% | 37 (97%) | 100% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 32 (100%) | 100% | 32 (100%) | 100% |
| All Others (n=9) | 8 (89%) | 100% | 8 (89%) | 100% | 8 (89%) | 100% |
| Molecularly confirmed ESBL Others (n=3) | 3 (100%) | 100% | 3 (100%) | 100% | 3 (100%) | 100% |
| Molecularly confirmed AmpC Others (n=2) | 2 (100%) | | 2 (100%) | | 2 (100%) | |
| Total of all isolates (n=164) | 157 (96%) | 99% | 158 (96%) | 100% | 156 (95%) | 99% |
| Total of molecularly confirmed ESBL (n=138) | 137 (99%) | 99% | 137 (99%) | 100% | 137 (99%) | 99% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.7 cont...Sensitivity of BSAC and HPA NDM QSOP 51 ESBL screening using combinations of cephalosporin discs

| | CAZ+CTX | | | |
|---|---|---|---|---|
| | BSAC | | HPA | |
| | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity |
| All *E. coli* (n=117) | 99 (85%) | 88% | 100 (85%) | 89% |
| ESBL *E. coli* (n=103) | 91 (88%) | 88% | 92 (89%) | 89% |
| AmpC *E. coli* (n=2) | 2 (100%) | | 2 (100%) | |
| All Klebsiella spp. (n=38) | 37 (97%) | 100% | 37 (97%) | 100% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 32 (100%) | 100% |
| All Others (n=9) | 4 (44%) | 100% | 5 (59%) | 100% |
| Molecularly confirmed ESBL Others (n=3) | 3 (100%) | 100% | 3 (100%) | 100% |
| Molecularly confirmed AmpC Others (n=2) | 0 (0%) | | 1 (50%) | |
| Total of all isolates (n=164) | 140 (85%) | 91% | 142 (87%) | 92% |
| Total of molecularly confirmed ESBL (n=138) | 126 (91%) | 91% | 135 (98%) | 92% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.7 cont...Sensitivity of BSAC and HPA NDM QSOP 51 ESBL screening using combinations of cephalosporin discs

| | CPD+CAZ+CTX | | | | CPD+CAZ+CTX+CPM | | | |
|---|---|---|---|---|---|---|---|---|
| | BSAC | | HPA | | BSAC | | HPA | |
| | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity |
| All *E. coli* (n=117) | 110 (94%) | 99% | 113 (97%) | 100% | 113 (97%) | 99% | 113 (97%) | 100% |
| ESBL *E. coli* (n=103) | 102 (99%) | 99% | 103 (100%) | 100% | 102 (99%) | 99% | 103 (100%) | 100% |
| AmpC *E. coli* (n=2) | 2 (100%) | | 2 (100%) | | 2 (100%) | | 2 (100%) | |
| All *Klebsiella* spp. (n=38) | 37 (97%) | 100% | 37 (97%) | 100% | 37 (97%) | 100% | 37 (97%) | 100% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 32 (100%) | 100% | 32 (100%) | 100% | 32 (100%) | 100% |
| All Others (n=9) | 8 (89%) | 100% | 8 (89%) | 100% | 8 (89%) | 100% | 8 (89%) | 100% |
| Molecularly confirmed ESBL Others (n=3) | 3 (100%) | 100% | 3 (100%) | 100% | 3 (100%) | 100% | 3 (100%) | 100% |
| Molecularly confirmed AmpC Others (n=2) | 2 (100%) | | 2 (100%) | | 2 (100%) | | 2 (100%) | |
| Total of all isolates (n=164) | 155 (95%) | 99% | 158 (96%) | 100% | 158 (96%) | 99% | 158 (96%) | 100% |
| Total of molecularly confirmed ESBL (n=138) | 137 (99%) | 99% | 137 (99%) | 100% | 137 (99%) | 99% | 137 (99%) | 100% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.7 cont...Sensitivity of BSAC and HPA NDM QSOP 51 ESBL screening using combinations of cephalosporin discs

| | CPD+CAZ+CTX+CFP+CPM | | | |
|---|---|---|---|---|
| | BSAC | | HPA | |
| | No. resistant (%) | Sensitivity | No. resistant (%) | Sensitivity |
| All *E. coli* (n=117) | 113 (97%) | 99% | 113 (97%) | 100% |
| ESBL *E. coli* (n=103) | 102 (99%) | 99% | 103 (100%) | 100% |
| AmpC *E. coli* (n=2) | 2 (100%) | | 2 (100%) | |
| All *Klebsiella* spp. (n=38) | 37 (97%) | 100% | 37 (97%) | 100% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 32 (100%) | 100% |
| All Others (n=9) | 8 (89%) | 100% | 8 (89%) | 100% |
| Molecularly confirmed ESBL Others (n=3) | 3 (100%) | 100% | 3 (100%) | 100% |
| Molecularly confirmed AmpC Others (n=2) | 2 (100%) | | 2 (100%) | |
| Total of all isolates (n=164) | 158 (96%) | 99% | 158 (96%) | 100% |
| Total of molecularly confirmed ESBL (n=138) | 137 (99%) | 99% | 137 (99%) | 100% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

### 4.3.3 Sensitivity of cefoxitin screening for AmpC detection

Coudron et al. used cefoxitin as an initial screening method for the detection of AmpC. A zone diameter of ≤18mm is used as a breakpoint for determining resistance (Coudron et al., 2000, Manchanda and Singh, 2003). However, BSAC utilise a breakpoint of ≤19mm (BSAC, 2009) and it is this measurement that is used in this study.

Table 4.8 Cefoxitin resistance amongst molecularly confirmed ESBL and AmpC

| | **Cefoxitin Susceptibility** | |
|---|---|---|
| | **Resistant** | **Susceptible** |
| ESBL *E. coli* (n=103) | 9% | 91% |
| AmpC *E. coli* (n=2) | 100% | 0% |
| ESBL *Klebsiella spp.* (n=32) | 25% | 75% |
| Others ESBL (n=2) | 100% | 0% |
| Others AmpC (n=2) | 100% | 0% |
| Others ESBL and AmpC (n=1) | 100% | 0% |
| Total (n=142) | 17% | 83% |

As outlined in Table 4.8, it is clear that amongst the ESBL-producing *Enterobacteriaceae*; cefoxitin resistance is low i.e. 9% for ESBL *E. coli* and 25% with *Klebsiella* spp. This has been noted before as an indicator for presence of ESBL in this species when there was a discrepancy between 3$^{rd}$ generation cephalosporin resistance paired with cefoxitin susceptibility in these species (Stürenburg and Mack, 2003). However, it is interesting to mention that all of the molecularly confirmed AmpC were in fact completely resistant to cefoxitin.

## 4.4 Sensitivity of ESBL confirmation using Synergy and Disc Approximation tests

### 4.4.1 Sensitivity of the Synergy Test in confirming ESBL in molecularly confirmed isolates

Figure 4.1 provides an example of how the synergy test is applied to confirming ESBL within *Enterobacteriaceae*. This figure displays synergy between all of the indicator cephalosporins and their respective clavulanic acid counterparts for this particular isolate.

However, by looking at the confirmatory assay, it is also important to note how effective each cephalosporin is, both individually and in combination, at successfully confirming the presence of an ESBL. Results are compiled in tables 4.9 and 4.10

Using cefpodoxime, 138/164 (84%) of all isolates gave a positive synergy test and of these 132/138 (96%) molecularly confirmed ESBL-producing isolates were positive by synergy thereby giving a sensitivity of 96%. Cefpodoxime was 97% sensitive at confirming ESBL in *E. coli* and 94% in *Klebsiella* spp. Using cefotaxime, an overall sensitivity for molecularly confirmed ESBL-producing isolates was 77% and similar sensitivities were observed for *E. coli* and *Klebsiella* spp.; 77% and 78%, respectively. The use of ceftazidime gave an overall sensitivity of 71% for confirming molecularly confirmed ESBL isolates. Ceftazidime was more sensitive at confirming ESBL in *Klebsiella* spp. (94%) than *E. coli* (64%). Cefepime and cefpirome had a poorer sensitivity for confirming ESBLs as overall for all molecularly confirmed ESBLs, sensitivities of 51% and 62% were found. However a difference could be ascertained between molecularly confirmed ESBL-producing *E. coli* and *Klebsiella* spp. (Table 4.9).

When using combinations of cephalosporins, it might be possible to confirm a greater number of ESBLs. For *E. coli*, the combination of cefpodoxime and cefotaxime meant that all ESBLs would be confirmed. As expected, neither of the AmpC producing *E. coli* demonstrated synergy using cephalosporin combinations. Combining cefpodoxime and ceftazidime confirmed 100% of *Klebsiella* spp. For all other *Enterobacteriaceae*, combinations of cephalosporins maintained a sensitivity of 67%.

In summary, the most suitable two disc approach for confirming the presence of ESBL was found either with cefpodoxime and cefotaxime or cefpodoxime and cefotaxime as synergy was exhibited in 98% of all the molecularly confirmed ESBL-producing *Enterobacteriaceae*. However, the addition of ceftazidime achieved 99% sensitivity. The combination of cefotaxime and ceftazidime as advocated by the CLSI was inferior in sensitivity for ESBL confirmation in the strain collection investigated in this thesis.

Table 4.9 Sensitivity of ESBL confirmation using individual cephalosporin discs in the synergy method

| | Synergy with CV | | | | | |
|---|---|---|---|---|---|---|
| | CPD | | CTX | | CAZ | |
| | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity |
| All *E. coli* (n=117) | 102 (87%) | 97% | 80 (68%) | 77% | 67 (57%) | 64% |
| *ESBL E. coli* (n=103) | 101 (98%) | 97% | 79 (77%) | 77% | 66 (64%) | 64% |
| AmpC *E. coli* (n=2) | 0 (0%) | | 0 (0%) | | 0% | |
| All *Klebsiella* spp. (n=38) | 34 (89%) | 94% | 29 (76%) | 78% | 35 (92%) | 94% |
| ESBL *Klebsiella* spp. (n=32) | 30 (94%) | 94% | 25 (78%) | 78% | 30 (94%) | 94% |
| All Others (n=9) | 2 (22%) | 67% | 2 (22%) | 67% | 2 (22%) | 67% |
| Molecularly confirmed ESBL Others (n=3) | 2 (67%) | 67% | 2 (67%) | 67% | 2 (67%) | 67% |
| Molecularly confirmed AmpC Others (n=2) | 0 (0%) | | 0 (0%) | | 0 (0%) | |
| Total of all isolates (n=164) | 138 (84%) | 96% | 111 (67%) | 77% | 104 (63%) | 71% |
| Total of molecularly confirmed ESBL (n=138) | 132 (96%) | 96% | 106 (77%) | 77% | 98 (72%) | 71% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.9 cont...Sensitivity of ESBL confirmation using individual cephalosporin discs in the synergy method

| | Synergy with CV | | | |
|---|---|---|---|---|
| | CPM | | CFP | |
| | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity |
| All *E. coli* (n=117) | 39 (33%) | 38% | 51 (44%) | 54% |
| *ESBL E. coli* (n=103) | 39 (38%) | 38% | 50 (49%) | 54% |
| AmpC *E. coli* (n=2) | 0% | | 0% | |
| All *Klebsiella* spp. (n=38) | 31 (82%) | 91% | 30 (79%) | 84% |
| ESBL *Klebsiella* spp. (n=32) | 29 (91%) | 91% | 27 (84%) | 84% |
| All Others (n=9) | 3 (33%) | 67% | 2 (22%) | 67% |
| Molecularly confirmed ESBL Others (n=3) | 2 (67%) | 67% | 2 (67%) | 67% |
| Molecularly confirmed AmpC Others (n=2) | 0 (0%) | | 0 (0%) | |
| Total of all isolates (n=164) | 72 (44%) | 51% | 83 (51%) | 62% |
| Total of molecularly confirmed ESBL (n=138) | 70 (50%) | 51% | 79 (57%) | 62% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.10 Sensitivity of ESBL confirmation using combinations of cephalosporin discs in the synergy method

| | Synergy with CV | | | | | |
|---|---|---|---|---|---|---|
| | CPD+CAZ | | CPD+CTX | | CAZ+CTX | |
| | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity |
| All *E. coli* (n=117) | 104 (89%) | 98% | 105 (90%) | 100% | 94 (82%) | 88% |
| *E. coli* (n=103) | 101 (98%) | 98% | 103 (100%) | 100% | 91 (88%) | 88% |
| AmpC *E. coli* (n=2) | 0 (0%) | | 0 (0%) | | 0 (0%) | |
| All *Klebsiella* spp. (n=38) | 37 (97%) | 100% | 35 (93%) | 94% | 36 (95%) | 97% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 30 (94%) | 94% | 31 (97%) | 97% |
| All Others (n=9) | 2 (22%) | 67% | 2 (22%) | 67% | 2 (22%) | 67% |
| Molecularly confirmed ESBL Others (n=3) | 2 (67%) | 67% | 2 (67%) | 67% | 2 (67%) | 67% |
| Molecularly confirmed AmpC Others (n=2) | 0 (0%) | | 0 (0%) | | 0 (0%) | |
| Total of all isolates (n=164) | 143 (87%) | 98% | 142 (87%) | 98% | 132 (80%) | 90% |
| Total of molecularly confirmed ESBL (n=138) | 135 (98%) | 98% | 135 (98%) | 98% | 124 (90%) | 90% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.10 cont...Sensitivity of ESBL confirmation using combinations of cephalosporin discs in the synergy method

| | Synergy with CV | | | | | |
|---|---|---|---|---|---|---|
| | **CPD+CAZ+CTX** | | **CPD+CAZ+CTX+CPM** | | **CPD+CAZ+CTX+CFP+CPM** | |
| | **No. Positive (%)** | **Sensitivity** | **No. Positive (%)** | **Sensitivity** | **No. Positive (%)** | **Sensitivity** |
| All *E. coli* (n=117) | 106 (91%) | 100% | 106 (91%) | 100% | 106 (91%) | 100% |
| *E. coli* (n=103) | 103 (100%) | 100% | 103 (100%) | 100% | 103 (100%) | 100% |
| AmpC *E. coli* (n=2) | 0 (0%) | | 0 (0%) | | 0 (0%) | |
| All *Klebsiella* spp. (n=38) | 37 (97%) | 100% | 37 (97%) | 100% | 37 (97%) | 100% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 32 (100%) | 100% | 32 (100%) | 100% |
| All Others (n=9) | 2 (22%) | 67% | 2 (22%) | 67% | 2 (22%) | 67% |
| Molecularly confirmed ESBL Others (n=3) | 2 (67%) | 67% | 2 (67%) | 67% | 2 (67%) | 67% |
| Molecularly confirmed AmpC Others (n=2) | 0 (0%) | | 0 (0%) | | 0 (0%) | |
| Total of all isolates (n=164) | 145 (88%) | 99% | 145 (88%) | 99% | 145 (88%) | 99% |
| Total of molecularly confirmed ESBL (n=138) | 137 (99%) | 99% | 137 (99%) | 99% | 137 (99%) | 99% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

### 4.4.2 Sensitivity of the Disc Approximation Test in confirming ESBL in molecularly characterised isolates

Figure 4.2 demonstrates a typical positive Disc Approximation Test. Distortion and expansion of the zones of inhibition is observed between the indicator cephalosporins and the central co-amoxiclav disc. Even though there is no zone of inhibition around cefpodoxime in this example, it is possible to state that synergy has still occurred due to the distortion and zone created in between the cefpodoxime and co-amoxiclav discs.

As with the Synergy Test, it is important to look at which indicator cephalosporins, both individually and in combinations, are the most sensitive at confirming ESBL status for the Disc Approximation Test.

The results are outlined in Table 4.11 and 4.12. Using cefpodoxime, 98/134 of molecularly confirmed ESBL-producing isolates exhibit distortion around the zone of inhibition, thereby giving a sensitivity of 73%. For *E. coli* and *Klebsiella* spp., sensitivities of 75% and 69% were found, respectively. Two molecularly confirmed ESBL-producing *E. cloacae* isolates were positive for distortion giving a sensitivity of 67%. In addition one AmpC-producing *E. cloacae* isolate was positive. Using cefotaxime, cefepime and cefpirome, overall sensitivities of 96% were achieved and all gave 100% sensitivity at confirming ESBL in *Klebsiella* spp. However, for ceftazidime, an overall sensitivity of 95% was found for molecularly confirmed ESBL-producing isolates as it confirmed ESBL in 94% of *Klebsiella* spp. Therefore, for the Disc Approximation Test, cefpodoxime is not the best indicator cephalosporin of choice (73%).

When using combinations of cephalosporins, it is possible to increase the sensitivity for confirming ESBL. For *E. coli*, the combination of cefpodoxime, ceftazidime, cefotaxime and cefepime increased sensitivity to 98%. However, for *Klebsiella* spp., the sensitivity can be increased to 100% with a combination of cefpodoxime and cefotaxime or ceftazidime. The sensitivity for the other *Enterobacteriaceae* remained at 67% regardless of combinations.

In summary, the best two-disc approach for confirming sensitivity when using the Disc Approximation Test is achieved through the use of either cefpodoxime and

ceftazidime or ceftazidime and cefotaxime, with sensitivities of 96%. By using four or five discs, the sensitivity increased to 98%.

Table 4.11 Sensitivity of ESBL confirmation using individual cephalosporin discs in the Disc Approximation method

| | Disc Approximation Test – Synergy with CV | | | | | |
|---|---|---|---|---|---|---|
| | CPD | | CAZ | | CTX | |
| | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity |
| All *E. coli* (n=113) | 76 (67%) | 75% | 99 (88%) | 96% | 100 (88%) | 96% |
| *ESBL E. coli* (n=99) | 74 (75%) | 75% | 95 (96%) | 96% | 95 (96%) | 96% |
| AmpC *E. coli* (n=2) | 0 (0%) | | 0 (0%) | | 0 (0%) | |
| All *Klebsiella* spp. (n=38) | 27 (71%) | 69% | 36 (95%) | 94% | 38 (100%) | 100% |
| ESBL *Klebsiella* spp. (n=32) | 22 (69%) | 69% | 30 (94%) | 94% | 32 (100%) | 100% |
| All Others (n=9) | 3 (33%) | 67% | 5 (56%) | 67% | 5 (56%) | 67% |
| Molecularly confirmed ESBL Others (n=3) | 2 (67%) | 67% | 2 (67%) | 67% | 2 (67%) | 67% |
| Molecularly confirmed AmpC Others (n=2) | 1 (50%) | | 1 (50%) | | 1 (50%) | |
| Total of all isolates (n=160) | 106 (66%) | 73% | 140 (88%) | 95% | 143 (89%) | 96% |
| Total of molecularly confirmed ESBL (n=134) | 98 (73%) | 73% | 127 (95%) | 95% | 129 (96%) | 96% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.11 cont...Sensitivity of ESBL confirmation using individual cephalosporin discs in the Disc Approximation method

| | Disc Approximation Test – Synergy with CV | | | |
|---|---|---|---|---|
| | CFP | | CPM | |
| | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity |
| All *E. coli* (n=113) | 96 (85%) | 95% | 99 (88%) | 96% |
| *ESBL E. coli* (n=99) | 94 (95%) | 95% | 95 (96%) | 96% |
| AmpC *E. coli* (n=2) | 0 (0%) | | 0 (0%) | |
| All *Klebsiella* spp. (n=38) | 38 (100%) | 100% | 38 (100%) | 100% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 32 (100%) | 100% |
| All Others (n=9) | 5 (56%) | 67% | 5 (56%) | 67% |
| Molecularly confirmed ESBL Others (n=3) | 2 (67%) | 67% | 2 (67%) | 67% |
| Molecularly confirmed AmpC Others (n=2) | 1 (50%) | | 1 (50%) | |
| Total of all isolates (n=160) | 139 (87%) | 96% | 142 (89%) | 96% |
| Total of molecularly confirmed ESBL (n=134) | 128 (96%) | 96% | 129 (96%) | 96% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.12 Sensitivity of ESBL confirmation using combinations of cephalosporin discs in the Disc Approximation method

| | Disc Approximation Test – Synergy with CV | | | | | |
|---|---|---|---|---|---|---|
| | CPD+CAZ | | CPD+CTX | | CAZ+CTX | |
| | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity |
| All *E. coli* isolates (n=113) | 100 (88%) | 96% | 100 (88%) | 96% | 100 (88%) | 96% |
| *E. coli* (n=99) | 95 (96%) | 96% | 95 (96%) | 96% | 95 (96%) | 96% |
| All *Klebsiella* spp. (n=38) | 35 (92%) | 94% | 38 (100%) | 100% | 38 (100%) | 100% |
| *Klebsiella* spp. (n=32) | 30 (94%) | 94% | 32 (100%) | 100% | 32 (100%) | 100% |
| All Others (n=9) | 5 (56%) | 67% | 5 (56%) | 67% | 5 (56%) | 67% |
| Molecularly confirmed ESBL Others (n=3) | 2 (67%) | 67% | 2 (67%) | 67% | 2 (67%) | 67% |
| Molecularly confirmed AmpC Others (n=2) | 1 (50%) | | 1 (50%) | | 1 (50%) | |
| Total of all isolates (n=160) | 140 (85%) | 95% | 143 (87%) | 96% | 143 (87%) | 96% |
| Total of molecularly confirmed ESBL (n=134) | 127 (95%) | 95% | 129 (96%) | 96% | 129 (96%) | 96% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

Table 4.12 cont...Sensitivity of ESBL confirmation using combinations of cephalosporin discs in the Disc Approximation method

| | Disc Approximation Test – Synergy with CV | | | | | |
|---|---|---|---|---|---|---|
| | CPD+CAZ+CTX | | CPD+CAZ+CTX+CPM | | CPD+CAZ+CTX+CFP+CPM | |
| | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity | No. Positive (%) | Sensitivity |
| All *E. coli* (n=113) | 100 (88%) | 96% | 102 (90%) | 98% | 102 (90%) | 98% |
| *ESBL E. coli* (n=99) | 95 (96%) | 96% | 97 (98%) | 98% | 97 (98%) | 98% |
| All *Klebsiella* spp. (n=38) | 38 (100%) | 100% | 38 (100%) | 100% | 38 (100%) | 100% |
| ESBL *Klebsiella* spp. (n=32) | 32 (100%) | 100% | 32 (100%) | 100% | 32 (100%) | 100% |
| All Others (n=9) | 5 (56%) | 67% | 5 (56%) | 67% | 5 (56%) | 67% |
| Molecularly confirmed ESBL Others (n=3) | 2 (67%) | 67% | 2 (67%) | 67% | 2 (67%) | 67% |
| Molecularly confirmed AmpC Others (n=2) | 1 (50%) | | 1 (50%) | | 1 (50%) | |
| Total of all isolates (n=160) | 143 (87%) | 96% | 143 (87%) | 98% | 143 (87%) | 98% |
| Total of molecularly confirmed ESBL (n=134) | 129 (96%) | 96% | 129 (96%) | 98% | 129 (96%) | 98% |

*sensitivity was calculated whereby true positives equal molecularly confirmed ESBL isolates with a positive phenotypic test. False negatives are molecularly confirmed ESBL isolates with a negative phenotypic test.

Antibiotics used: cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), cefepime (CPM) and cefpirome (CFP).

### 4.5 Mast AmpC and ESBL Detection Set

In order to evaluate the Mast® AmpC and ESBL Detection Set, an additional number of organisms were obtained from the PHW Microbiology ABM Swansea laboratory as the number of AmpC-producing *Enterobacteriaceae* collected in this thesis were few. In total 10 isolates were collected including 2 *E. coli*, 3 *Klebsiella* spp., 2 *Serratia marcescens*, 2 *C. freundii* and *1 E. cloacae*. PCR for TEM, SHV and CTX-M were carried out on all 10 isolates. However, no positive amplification for ESBL genes was found. Using the multiplex PCR for *ampC* genes described in Chapter 3, 6 of these isolates were found to be positive; 3 *Klebsiella* spp. gave positive amplification for the FOX primers whereas 1 *E. cloacae* and 2 *C. freundii* were positive with the CIT primers. They were therefore included as AmpC-producers in this section of study. As the remaining 2 *E. coli* and 2 *S. marcescens* were not molecularly characterised, they were not counted in this evaluation or in table 4.13. However, by using the Mast® AmpC and ESBL Detection Set, these isolates were phenotypically AmpC positive.

The Mast® AmpC and ESBL Detection Set was used on a subset of 64 *Enterobacteriaceae*. Of these 53 were ESBL, 10 were AmpC-producers and 1 contained both an ESBL and AmpC. The set detected ESBL (sensitivity 98%) and AmpC (sensitivity 100%) in *E. coli*, *Klebsiella* spp. and other *Enterobacteriaceae,* with the exception to detect the presence of ESBL in one *C. freundii* isolate harbouring TEM-166 and an *ampC* gene. In fact no phenotypic test used within this study was able to confirm the presence of ESBL in this particular isolate.

Table 4.13 Evaluation of Mast® AmpC and ESBL Detection set

| | **Mast® Disc Result** | | |
|---|---|---|---|
| | **ESBL** | **AmpC** | **ESBL+AmpC** |
| ESBL *E. coli* (n=32) | 100% (32) | | |
| AmpC *E. coli* (n=2) | | 100% (2) | |
| ESBL *Klebsiella spp.* (n=19) | 100% (19) | | |
| AmpC *Klebsiella spp.* (n=3) | | 100% (3) | |
| ESBL Other (n=2) | 100% (2) | | |
| AmpC Other (n=5) | | 100% (5) | |
| ESBL+AmpC Other (n=1) | 0% (0) | 100% (1) | 0% (0) |
| All (n=64) | 98% (53) | 100% (11) | 0% (0) |

### 4.6 Discussion

The quick and effective detection of ESBL producing *Enterobacteriaceae* is critical for patient treatment and for initiating correct infection control procedures to prevent the spread of infection (Stürenburg and Mack, 2003). With changes in molecular epidemiology and the emergence of new enzyme types, it is vital that continuous monitoring of current methods is undertaken.

The phenotypic screening and confirmation methods used in the detection of ESBL raise the question of whether the indicator cephalosporins currently used are still effective or not. By carrying out testing on a collection of molecularly confirmed ESBL, the sensitivity can be deduced in order to decide on which cephalosporin or indeed combinations of cephalosporins are the most suitable.

BSAC recommends that cefpodoxime is used as an indicator of choice (BSAC, 2007, BSAC, 2009) as variable resistance to cefotaxime and ceftazidime is seen (Livermore and Brown, 2001). This is particularly advisable since in this study it was found that the majority of ESBL enzymes are CTX-M and hence have a greater activity against cefotaxime. Table 4.7 shows that the additional use of ceftazidime (with HPA QSOP 51 guidelines) alongside cefpodoxime would detect 100% of ESBL-producing *Enterobacteriaceae.* With regards to ESBL confirmation, cefpodoxime is also the indicator cephalosporin of choice stipulated by BSAC, but for the Synergy Test the addition of cefotaxime would increase sensitivity from 96% to 98%. The further addition of ceftazidime would increase sensitivity to 99%. When using the Disc Approximation test, cefpodoxime is not the most suitable indicator cephalosporin at confirming ESBL as a sensitivity of only 73% was achieved. On average, for the remaining cephalosporins both individually and in combination a sensitivity of 95-96% is found. However, four discs are required to achieve 98% sensitivity. The Disc Approximation Test is simple and easy to carry out; however, the interpretation of results is subjective. This has been commented on previously (Vercauteren et al., 1997, Brown et al., 2000).

The laboratory detection of ESBL can often be problematic due to the presence of AmpC enzymes. In this study, one *C. freundii* isolate was found to possess TEM-116 and an *ampC* gene. Unfortunately, the isolate was negative to all phenotypic ESBL tests and would have therefore been overlooked as an ESBL carrier in the clinical setting. None of the AmpC-producing *Enterobacteriaceae* were phenotypically misidentified as ESBL. The Mast® AmpC and ESBL Detection Set was evaluated as a commercial kit which allowed for the detection of both AmpC and ESBL (both individually and in combination). In this study, the kit was effective at confirming presence of ESBL or AmpC with an overall sensitivity of 98%. However, the aforementioned *C. freundii* proved to be a challenge as the ESBL was not detected with this method also. This is because cefpodoxime is not a suitable indicator cephalosporin in this instance. A study by Ingram et al., (2011) found a sensitivity of 96% (71/74) and specificity of 98% (169/172) when using this detection set whereas it achieved 83.3% sensitivity in a study by Coyle et al., (2011).

The capability of the BD Phoenix Automated System to detect ESBL in the clinical laboratory is well established (Leverstein-van Hall et al., 2002, Stürenburg et al., 2003, Sanguinetti et al., 2003, Wiegand et al., 2007). Results in this chapter show that the BD Phoenix Automated System had 99% sensitivity at detecting ESBL, which is directly comparable to the BD Phoenix results found by Wiegand et al. in 2007. Leverstein-van Hall *et al.* (2002) had 89% sensitivity for identifying ESBLs whereas Sanguinetti et al. (2003) and Stürenburg et al. (2003), achieved 100% sensitivity for identifying ESBLs.

Although the population of AmpC-producing *Enterobacteriaceae* is small (n=4), the BD Phoenix generated rule no. 106 for 3 of these isolates. Thus it is difficult to state whether there are any problems associated with AmpC detection using the BD Phoenix. However, a collection of 22 isolates which were not molecularly characterised as ESBL or AmpC provided some interesting results whereby rule nos. 1505 (found in 7 non-molecularly confirmed *E. coli*, 4 *Klebsiella* spp.), 106 (1 *E. coli* isolate) and 1433 (1 *E. cloacae*) were obtained. Of these 13 isolates, only 4 of the *E. coli* and 4 *Klebsiella* spp. had positive phenotypic ESBL tests (Synergy Test and Disc Approximation Test) thereby possibly suggesting that isolates with a BD

Phoenix rule and positive confirmatory phenotypic tests may contain ESBL genes that are atypical and rarer. For the remaining 4 *E. coli* and 1 *E. cloacae*, which did not have positive phenotypic confirmatory ESBL tests and were not molecularly confirmed as ESBL-carriers, the BD Phoenix could have flagged false-positive ESBL results. Specificity of ESBL detection was also a problem noted in the study reported by Wiegand *et al.*, (2007). However, the strain collection in this thesis did not contain enough cephalosporin resistant, ESBL-negative isolates to properly evaluate the specificity of the BD Phoenix for ESBL detection.

In summary, analysis of the methods employed in this study give an indication of suitable ESBL detection strategies. It is clear that for ESBL screening, the addition of ceftazidime using HPA QSOP 51 guidelines could improve sensitivity to 100% as found in this chapter. For the confirmation of ESBL, although the Disc Approximation Test is simple to use, the subjectivity of interpretation could potentially lead to error. Therefore, the use of the Synergy Test with appropriate cephalosporins (to maximise sensitivity) is usually recommended. However, using cefpodoxime alone for confirmation has a high sensitivity of 96%, which could be improved to 100% by additionally using cefotaxime in combination. Adding further additional cephalosporins in combination would probably be considered unacceptable by most clinical microbiology laboratories.

The commercial AmpC and ESBL Detection Set was shown to be 98% effective overall and proved to be an adequate detection system for AmpC with a sensitivity of 100%. Only few independent evaluations have been performed of this assay, which indicated similar promising results (Ingram et al., 2011). However, an increased number of ESBL and AmpC producing *Enterobacteriaceae* would need to be evaluated and ideally such a collection should also contain challenge organisms without any of the two resistance mechanisms despite cephalosporin resistance to assess the specificity of the test.

# Chapter 5: Typing and characterisation of ESBL isolates

## 5.1 Introduction

Antibiotic resistance is an ongoing problem as limitations are faced with regards to treatment options (Paterson et al., 2000). ESBLs are often associated with multidrug resistances (Livermore and Hawkey, 2005) and thus subsequently pose greater treatment limitations. This problem is emphasised more so when clonal dissemination occurs. Courvalin et al., (2001) describes clonal dissemination as being "*due to chromosome replication, plasmid conjugation to replicative transfer and gene migration with replicative transposition*" (Courvalin and Trieu-Cuot, 2001).

Clonal dissemination can be associated with the spread of virulence factors as well as multidrug resistances (Coque et al., 2008b, Clermont et al., 2009). The ability to accurately identify strains and clones capable of causing infection is paramount to epidemiological surveillance and public health decisions. There are many methods for identifying genetic relatedness. However, discrepancies can occur when the results are not reproducible between laboratories. Therefore, molecular typing with good discrimination has been developed to overcome this problem (Maiden et al., 1998) and has been subsequently used in the context of infections caused by MRSA, *E. coli* O157: H7 and vancomycin-resistant enterococci (Enright and Spratt, 1999).

### 5.1.1 Pulsed-field gel electrophoresis and PCR based methods

The determination of clonal relatedness between isolates is a key component of disease outbreak investigation and epidemiological studies. Molecular techniques such as pulsed-field gel electrophoresis (PFGE) along with PCR based methods are now commonly executed.

#### 5.1.1.1 Determination of clonal relatedness by pulsed-field gel electrophoresis

Developed by Schwartz and Cantor (1984), PFGE was used to separate high molecular weight DNA. The principle of PFGE is the digestion of chromosomal DNA by a restriction enzyme. The DNA is then resolved by electrophoresis with alternately pulsed electrical fields. In doing so, the banding patterns generated by

PFGE give a highly specific 'fingerprint' for the organism, thus allowing the discrimination of strains for molecular genotyping. The PFGE banding pattern can be interpreted to deduce strain relatedness using specific guidelines i.e. those published by Tenover (1995). Tenover's guidelines set criteria (Table 5.1) that allow the recognition and analysis of strain relatedness in isolates which may have undergone genetic mutations leading to subsequent banding differences e.g. the loss of larger fragments into two smaller fragments.

PFGE is frequently used for the monitoring of *E. coli* O157: H7 (Tartof et al., 2005) and methicillin resistant *Staphylococcus aureus* (MRSA). However, despite its efficient discriminatory ability, PFGE is a time consuming, labour intensive procedure (Gautom, 1997).

Table 5.1 Tenover's criteria for interpreting PFGE (Tenover et al., 1995).

| **Category** | **No. of genetic differences** | **No. of fragment differences** | **Epidemiological interpretation** |
|---|---|---|---|
| Indistinguishable | 0 | 0 | Part of the outbreak |
| Closely related | 1 | 2-3 | Probably part of the outbreak |
| Possibly related | 2 | 4-6 | Possibly part of the outbreak |
| Different | ≥3 | ≥7 | Not part of the outbreak |

### 5.1.1.2 PCR for the detection of clonal relatedness to CTX-M-15 *IS*26 (UK epidemic strain A)

A CTX-M-15 insertion sequence 26 (*IS*26) PCR developed by Woodford et al., (2004) was used to identify a CTX-M-15 *IS*26 link-region sequence which is specific to epidemic *E. coli* strain A (Figure 5.1). Using specific primers for this region, a 400bp amplicon was generated upon positive amplification. The development of this

PCR allows for the rapid identification of *E. coli* strain A. This is especially important for screening during an infection outbreak (Woodford et al., 2004).

**AY462238**

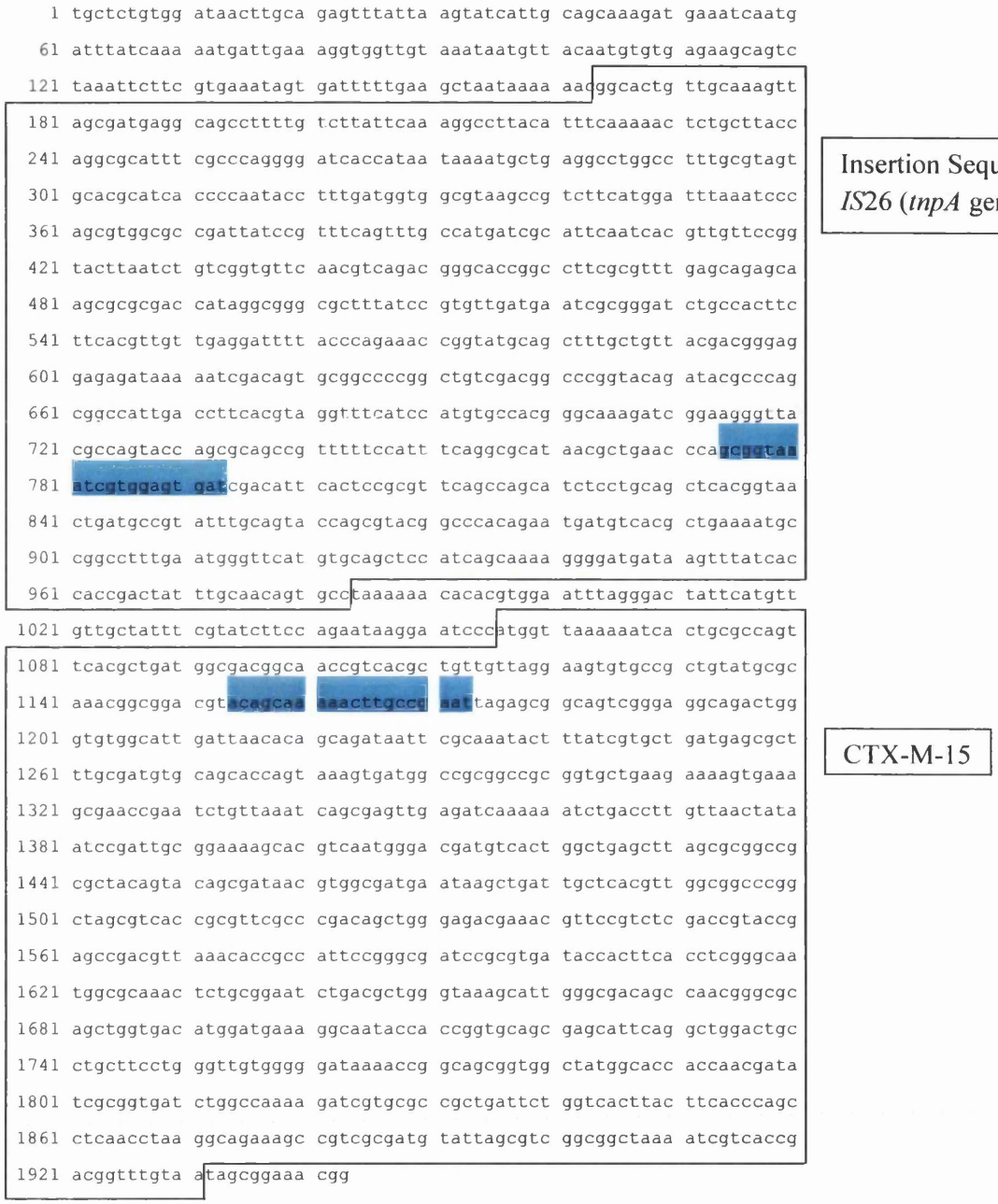



Figure 5.1 Positioning of CTX-M-15 IS26 primers. Primers (highlighted in blue) span the *IS*26 and CTX-M-15 link region which is characteristic of UK epidemic *E. coli* strain A.

### 5.1.2 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was developed by Maiden et al. in 1998 in order to identify virulent lineages of *N. meningitidis* and to overcome reproducibility issues faced with other typing methods (Maiden et al., 1998). The principles of MLST involve the determination of an allelic profile by the sequence analysis of seven or more housekeeping genes. The allelic profile defines the sequence type (ST). The sequence data obtained can be used to compare genetic relatedness. Clonal relationships can be visualised by various dendrograms or eBURST plots (Enright and Spratt, 1999, Maiden et al., 1998). This information gives an understanding into the population biology of important pathogens e.g. *E. coli* and *S. aureus* (Lau et al., 2008b). There are two different MLST typing schemes for *E. coli*. The scheme used to identify the sequence type ST131 frequently mentioned in this chapter was developed by Mark Achtmann and colleagues using 7 housekeeping genes including *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate synthetase), and *recA* (ATP/GTP binding motif) (Wirth et al., 2006, Lau et al., 2008b). The other typing scheme is proposed by the Institute Pasteur and uses 8 housekeeping genes including *dinB* (DNA polymerase), *icdA* (isocitrate dehydrogenase), *pabB* (p-aminobenzoate synthase), *polB* (polymerase PolII), *putP* (proline permease), *trpA* (tryptophan synthase subunit A), *trpB* (tryptophan synthase subunit B), *uidA* (beta-glucuronidase) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/primers_Ecoli.html).

This second scheme is of importance for this chapter as an allelic variant in the *pabB* gene, which is part of this MLST scheme, is used for rapid detection of the O25b-ST131 clone by PCR (see below; Clermont et al., 2009).

#### 5.1.2.1 Allele-specific PCR for the detection of clonal relatedness to O25b-ST131

Allele-specific PCR takes advantage of the many single nucleotide polymorphisms (SNPs) found within genomes. With such a high frequency, SNPs can be advantageously used as markers for genetic studies (Brookes, 1999).

In this study, allele-specific PCR was used within the *pab*B gene to rapidly identify members of the *E. coli* O25b-ST131 clone. Two primer sets were used. *trpA* primers

amplified a 427 bp section of the *trpA* gene and were used as a control to ensure that the DNA was of sufficient quality but also, to ensure the PCR was functioning correctly. The *pabB* primers amplified a 347bp fragment of the *pabB* gene and contained single nucleotide mutations at the 3' end. These mutations are specific for *E. coli* belonging to B2 subgroup I isolates containing O type 25b and so positive amplification should only occur if these mutations are present (Figure 5.4) (Clermont et al., 2009).

The aim of this chapter was to type and further characterise and to deduce any strain and/or clonal relatedness within the *E. coli* and *Klebsiella* spp. populations by using PFGE and PCR-based methodologies.

## 5.2 Results

### 5.2.1 Screening isolates for CTX-M-15 *IS*26

Out of 128 *Enterobacteriaceae* isolates positive for CTX-M group 1, 66 isolates (51%) generated a positive amplification product (400 bp) with primers specific for CTX-M-15 *IS*26 (Figure 5.2). All of these isolates were identified as *E. coli*. Therefore, out of 96 *E. coli* isolates positive for CTX-M group 1, 69% gave a positive PCR result. Positive amplification was not found in any other *Enterobacteriaceae* species. Additionally, as a control, this PCR was carried out on epidemic *E. coli* strains A-E. Only strain A gave a positive amplification (Figure 5.3).

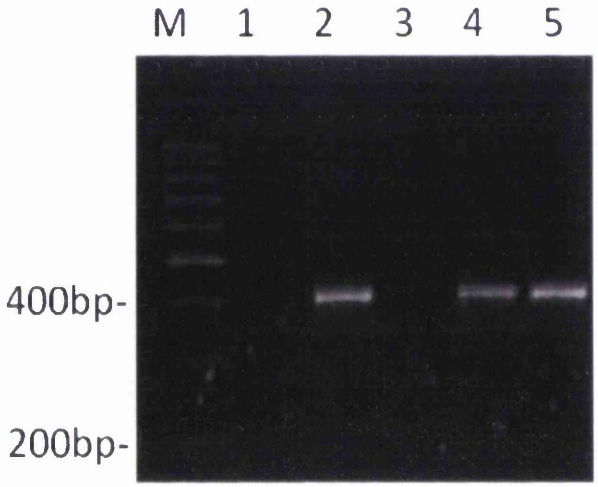



Figure 5.2 Gel electrophoresis of the CTX-M-15 IS26 link-region. Positive amplification products are 400 bp in size. M, 100 bp molecular ladder; Lanes 1 and 3 CTX-M-15 *IS*26 negative; Lanes 2, 4 and 5 CTX-M-15 *IS*26 positive.

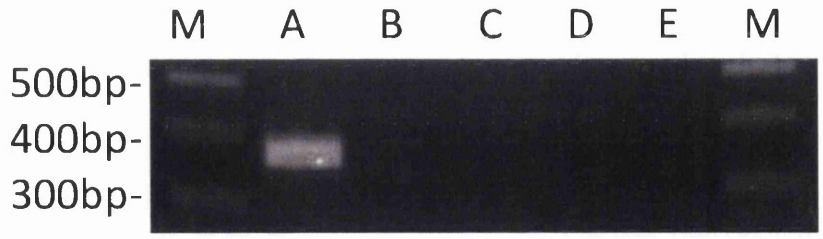



Figure 5.3 Prevalence of the CTX-M-15 IS26 link region within epidemic *E. coli* strains A-E. M, 100 bp molecular ladder; Lanes A-E represent epidemic *E. coli* strains A-E.

### 5.2.2 Detection of *E. coli* O25b-ST131 using allele-specific PCR

To identify members of the intercontinental clone *E. coli* O25b-ST131, a PCR developed by Clermont et al. (2009) and capable of identifying single-nucleotide polymorphisms (SNP) within the *pab*B gene was used.

100 (3 isolates could not be revived at this stage) ESBL-producing *E. coli* isolates were screened to identify members of O25b-ST131. A selection of 40 cefpodoxime-sensitive *E. coli* isolates obtained from urine samples were additionally screened as a control.

Eighty out of 100 *E. coli* isolates (80%) yielded positive amplification by PCR with primers specific for the *pab*B mutation, generating a 347bp amplicon (Figure 5.4). Of these, 100% of *E. coli* isolates (n=63) positive for CTX-M-15 *IS*26 and 46% of *E. coli* (n=37) isolates negative for CTX-M-15 *IS*26 generated positive amplification products with this PCR. Within the CTX-M-15 *IS*26 negative *E. coli*, one isolate harbouring CTX-M-27 was positive for the *pab*B mutation. Eight out of 40 non-ESBL control *E. coli* isolates (20%) gave a positive *pab*B amplification product.

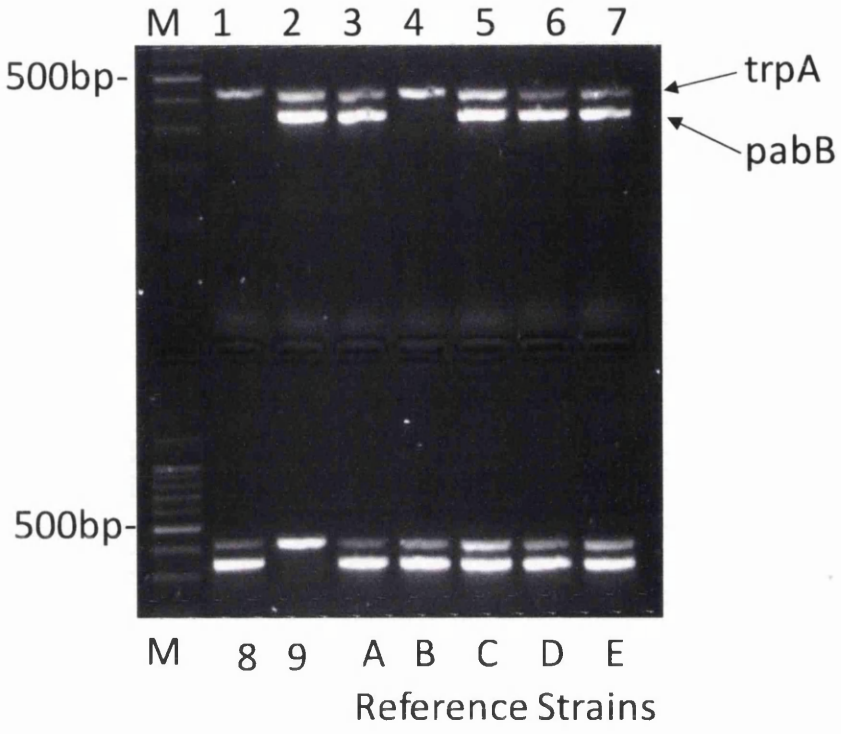



Figure 5.4 Prevalence of amplification products positive for pabB single-nucleotide polymorphisms (SNPs). In this instance, amplification of the *pab*B gene is a specific determinant of O25b-ST131. M, 100bp molecular ladder; Lanes 1, 4, 8, 9 CTX-M-15 *IS*26 negative isolates; Lanes 2, 3, 5, 6, 7, CTX-M-15 *IS*26 positive isolates. Reference strains epidemic *E. coli* A-E.

### 5.2.2.1 DNA sequencing of *pab*B positive amplicons

A sub-section of each group (10 *E. coli* isolates positive for CTX-M-15 *IS*26 and 10 *E. coli* isolates negative for CTX-M-15 *IS*26) were sent for DNA sequencing to confirm the sensitivity of this PCR and to validate the accuracy of the assay. All 20 isolates sequenced revealed the SNPs within the *pab*B gene therefore confirming that these isolates belong to the O25b-ST131 intercontinental clone (Figure 5.5).

DNA sequencing was also carried out on the 8 control *E. coli* isolates, which had generated a positive PCR product of 347bp. SNPs could not be detected in 4 (50%) isolates. Therefore, they were considered to be PCR false-positives. However, SNPs were detected in the other 4 (50%) isolates, suggesting that these isolates were related to the intercontinental clone (Table 5.2).

Table 5.2 Prevalence of isolates positive for a single-nucleotide polymorphism (SNP) within the *pab*B gene.

| | *pab*B positive |
|---|---|
| All ESBL positive *E. coli* (n=100) | 80 (80%) |
| *E. coli IS*26 positive (n=63) | 63 (100%) |
| *E. coli IS*26 negative (n=37) | 17 (46%) |
| Control ESBL negative *E. coli* (n=40) | 4 (10%) |


Figure 5.5 Demonstration of allele-specific PCR. Single-nucleotide polymorphisms (SNPs) on the 3' end of the primer are specific for mutations within the *pabB* gene of members of the intercontinental O25b-ST131 clone. These SNPs have been highlighted in the *pab*B positive sequence.

## 5.3 Detection of clonal relationships using Pulsed Field Gel Electrophoresis (PFGE)

### 5.3.1 Clonal relatedness by PFGE within *E. coli*

Pulsed field gel electrophoresis (PFGE) was carried out on 139 *Enterobacteriaceae* isolates in order to determine clonal patterns. The results were analysed in concordance with guidelines outlined by Tenover et al. (1995). Within the *E. coli* population (n=102) a distinct clonal pattern (Figure 5.6) could be distinguished within the CTX-M group 1 isolates (n=96). Using these guidelines, isolates that had been depicted as being CTX-M-15 *IS*26 positive by PCR (see section 5.2) could be termed 'closely related' and 'possibly related' as 54 isolates had 2-3 band differences and 12 isolates had 4-6 band differences when compared to epidemic *E. coli* strain A (Table 5.3). By Tenover's epidemiological interpretation, isolates with a 2-3 band difference are defined as 'probably part of the outbreak' and isolates with a 4-6 band difference are defined as 'possibly part of the outbreak'. This suggests that the CTX-M-15 *IS*26 positive *E. coli* isolates collected at Swansea had a closely related genetic background to that of *E. coli* strain A.

A second 'closely related' clone could be identified within 5 (5%) *E. coli* isolates (Figure 5.7). This was unrelated to any of the major UK epidemic strains A-E. The remaining 31 (31%) *E. coli* isolates were unrelated and had unique PFGE patterns.

Table 5.3 Comparison of *E. coli* obtained at Swansea to epidemic *E. coli* strain A by PFGE

| | **No. of fragment differences compared to epidemic *E. coli* Strain A** | | | |
|---|---|---|---|---|
| | **0** | **2-3** | **4-6** | **≥7** |
| *E. coli* (n=66) | 0 | 54 | 12 | 0 |

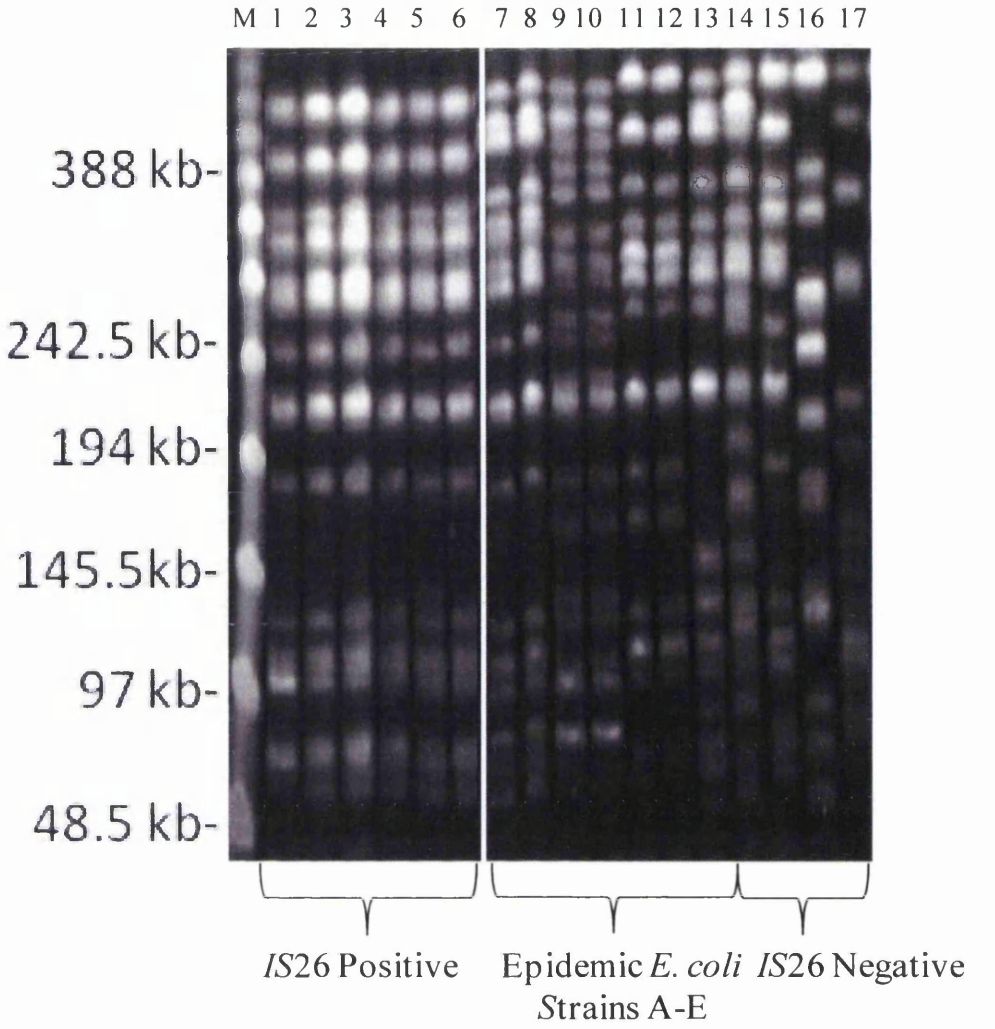



Figure 5.6 PFGE gel illustrating clonal relatedness between *E. coli* isolates from this study and epidemic *E. coli* strains A-E using *Xba*I. M, molecular marker; lanes 1-6 *E. coli IS*26 positive; lanes 7 and 8 *E. coli* strain A; lanes 9 and 10 *E. coli* strain B; lanes 11 and 12 *E. coli* strain C; lane 13 *E. coli* strain D; lane 14 *E. coli* strain E; lanes 15-17 *E. coli IS*26 negative (unique banding patterns).

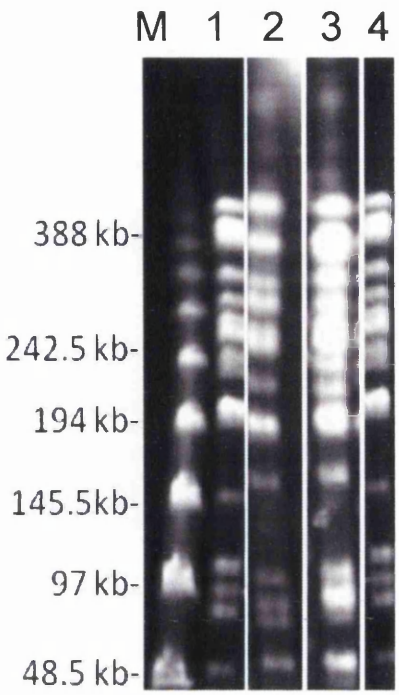



Figure 5.7 PFGE gel illustrating clonal relatedness between *E. coli* isolates using *Xba*I. A 'closely related' clone was identified in 5 *E. coli* isolates (four of which are shown here). This clone is unrelated to any of the UK epidemic *E. coli* strains A-E. M, molecular marker; lanes 1-4 *E. coli* isolates demonstrating a 'closely related' pattern.

### 5.3.2 Clonal relatedness within *Klebsiella* spp. by PFGE

Within the *Klebsiella* spp. (n=32), two distinct clones (Figure 5.8) could be identified using Tenover's guidelines (Tenover et al., 1995). Both clones were associated with *K. pneumoniae*, of which, 18 (58%) belonged to 'clone I' and 2 (6%) belonged to 'clone II' (Table 5.4). Additionally, 12 (38%) *Klebsiella* spp. isolates had unique PFGE banding patterns.

Table 5.4 Identification of clonally related *Klebsiella* spp. by PFGE.

| | **Clone I** | **Clone II** | **Unrelated** |
|---|---|---|---|
| *K. pneumoniae* (n=31) | 18 | 2 | 11 |
| *K. oxytoca* (n=1) | | | 1 |

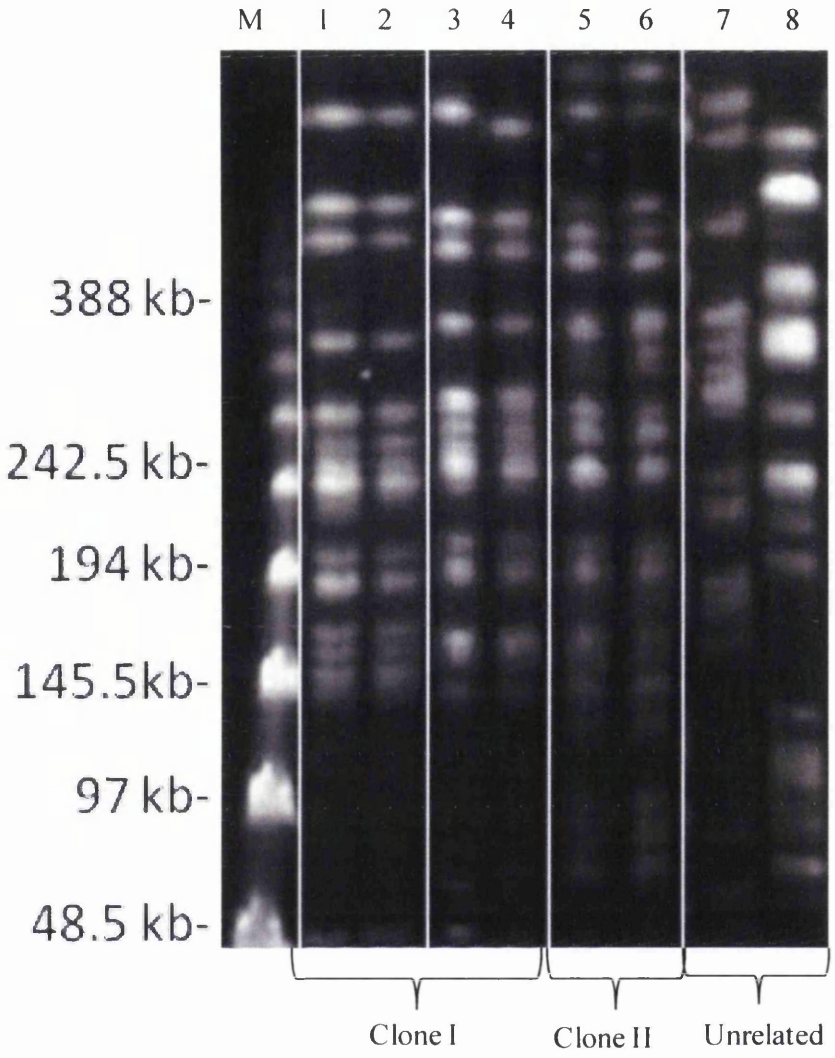



Figure 5.8 Clonal relatedness between isolates of *Klebsiella* spp. using *Xba*I. M, molecular marker; lanes 1-4 clone I; lanes 5 and 6 clone II; lanes 7 and 8 examples of unrelated banding patterns.

## 5.4 Discussion

The further characterisation and typing of isolates allows for a better understanding of the genetic variability and pathogenicity of bacterial isolates. It also allows tracing and the tracking of outbreaks and changes in molecular epidemiology.

Primers developed by Woodford et al., (2004) were used to amplify the CTX-M-15 *IS*26 link region, which is found in epidemic *E. coli* strain A. Strain A was first reported in the UK in 2003 after an epidemic *E. coli* outbreak in Shropshire (Woodford et al., 2004). However, strain A has also been found in Ulster and in areas of Southern England (Livermore and Hawkey, 2005). Upon screening all CTX-M group 1 *Enterobacteriaceae* isolates, 51% were found to possess this region, which were exclusively *E. coli*. This percentage changes to 68% (66/96) when comparing CTX-M-15 *IS*26-producing *E. coli* to all *E. coli* possessing a CTX-M group 1 gene. However, after aligning several CTX-M group 1 gene sequences in ClustalX, the reverse primer region was not conserved just for CTX-M-15 implying that several CTX-M group 1 genes paired with the *IS*26 element would be amplified. This is possible as the *IS*26 sequence is found on a mobile element. However, all CTX-M-15 *IS*26 positive *E. coli* isolates in this thesis followed up by CTX-M gene sequencing were revealed to carry CTX-M-15. In contrast a study conducted by Shahid (2010) found that CTX-M-15-producing *Citrobacter* spp. isolates generated the same 400bp amplicon when the CTX-M-15 *IS*26 PCR was used. Woodford et al., (2004) stated that this PCR is not an indicator of clonal relatedness but an additional aid in characterising *E. coli* strain A.

Within this study, it can be deduced that a large proportion of CTX-M group 1 positive *E. coli* isolates (69%) are 'closely related' and 'possibly related' to one of the UK's epidemic *E. coli* strains, notably strain A as deduced by PFGE, which is consistent with the presence of CTX-M-15 *IS*26 in these isolates. However looking at the larger picture, by further analysing these isolates it can be said that 100% of the positive CTX-M-15 *IS*26 isolates are in fact related to an intercontinental clone, O25b-ST131. This clone, which encompasses the UK's epidemic *E. coli* strains A-E (Lau et al., 2008a) amongst other ESBL types, takes into consideration isolates with diverse PFGE patterns (Clermont et al., 2009). In order to question the validity of the PCR assay used to identify members of the O25b-ST131 clones, a selection of 40

non-ESBL *E. coli* isolates obtained from urine samples were screened. SNPs or isolates with a positive *pab*B amplification product were found in a total 4 out of 40 (10%) isolates. Leflon-Guibout (2008), discussed that this clone had been found in 7% of non-ESBL producing *E. coli* isolates obtained from faecal samples in Paris. A further study conducted by Clermont et al., (2009) analysed the frequency of O25b-ST131 in non-ESBL producing *E. coli* isolates obtained from UTI, of which 3% were found to be positive. Therefore, Clermont et al., (2009) has predicted that 1.5% of UTI are caused by *E. coli* associated with the O25b-ST131 clone.

In the Northwest of England, ST131 was found within 13.3% of *E. coli* and of which, 35% harboured CTX-M-15. A study conducted in the Calgary region of Canada demonstrated that the prevalence of ST131 associated *E. coli* isolated from blood had increased from 5% in samples collected between 2000-2003 to 41% in samples collected from 2004-2007 (Pitout et al., 2009b). ST131 positive isolates have also been found to be prevalent in the USA and Norway with 35% and 20% of isolates being positive for this sequence type, respectively (Naseer et al., 2009, Johnson et al., 2010b).

ST131 positive isolates have been found to have an increased resistance to antibiotics. A study by Johnson et al. (2010b) revealed that isolates were more resistant to levofloxacin, cefazolin, gentamicin and ampicillin. Johnson et al. (2010a) noted in their study that ST131 was associated with >50% of multidrug resistant isolates (Johnson et al., 2010a). In addition, it has been suggested that the ST131 group has a highly virulent potential. Ender et al. (2009) reported a clinical case whereby an adult woman contracted emphysematous pyelonephritis, renal absess, bacteraemia and septic shock two weeks after visiting her hospitalised father who was suffering with pyelonephritis. *E. coli* harbouring ST131 and CTX-M-15 was isolated from both individuals. It was revealed that even though she had limited contact with her father clinical evidence suggested that transmission had occurred either through host to host contact or from a common external source (Ender et al., 2009).

Carbapenemases have also been found to be associated with the ST131 clone. Morris et al. (2011) reported of a KPC-2 carbapenemase in an *E. coli* isolate belonging to the ST131 clone. It was isolated along with a *K. pneumoniae* isolate from the urine of an 84 year old woman who was a resident in a care home in Ireland. The woman had no recent foreign travel and had not been admitted to hospital within 6 months prior to the isolation of the organisms. Both isolates were found to be carbapenemase producers and harboured TEM-1 and CTX-M-15. The *K. pneumoniae* isolate was additionally SHV-12 positive (Morris et al., 2011). Peirano and collegues (2011) found that an *E. coli* isolate isolated from the urine of a 40 year old paraplegic man harboured the NDM-1 carbapenemase and was positive for the ST131 clone. It was revealed that the man had travelled from the USA to New Dehli, India, for a surgical procedure. He presented with symptoms 3-4 months later. The combination of carbapenemases and the ST131 clone poses a great concern for the future treatment of community associated infections (Peirano et al., 2011). Pitout et al. (2009b) noted that the ST131 sequence type was an important cause in community onset bacteraemia.

The O25b-ST131 clone has also spread from human to animal as Pomba et al., (2009) demonstrated its presence in a dog (Pomba et al., 2009). It has also been detected in municipal wastewater treatment plant effluents. Dolejska et al. (2011) collected 26 *E. coli* isolates. Of these, 19 were O25b-ST131 and 17 were found to be CTX-M-15 *IS*26 positive. It has been suggested that the conditions e.g. pH, temperature, nutrient concentrations and high bacterial biomass found in the plant effluents were ideal for gene transfer and spread of resistance. Additionally, the effluents also contained antibiotics which would select for resistant strains and support the acquisition of resistance genes (Dolejska et al., 2011).

However, it is notable to mention that false-positive PCR products were amplified in 50% (4/8) of the control *E. coli* isolates as sequencing revealed that these isolates did not contain the *pabB* SNP thereby suggesting a limitation in the reliability of this PCR method. In order to improve this experiement, all *E. coli* isolates with positive amplification products would need to be sequenced.

Aside from allele-specific PCR, alternative rapid identification methodologies for the detection of the O25b-ST131 clone are available. Lau et al., (2009) and Pitout et al., (2009a) have both published data using the DiversiLab system. This is a semi-automated method which utilises repetitive sequence based PCR (rep-PCR) to generate a fingerprint of the isolate in question. Both Lau et al., (2009) and Pitout *et al.,* (2009a) successfully and accurately identified *E. coli* O25b-ST131 in concordance with MLST, but Lau et al., (2009) found it to be less discriminatory than PFGE. Additionally, both agree that this method could be adopted as a front-line tool for the detection of clones as results are generated more quickly than by the use of PFGE and MLST. However, the cost implications of the DiversiLab system could potentially be an issue (Pitout et al., 2009a).

It was revealed that 18 *K. pneumoniae* isolates were clonally related by PFGE (Figure 5.8). There have been a number of studies reporting the incidence of *K. pneumoniae* clones harbouring genes for CTX-M-15 (Kamatchi et al., 2009, Damjanova et al., 2008, Dashti et al., 2010). Damjanova et al., (2008) described that the *K. pneumoniae* in their study were ciprofloxacin resistant, which posed a problem in Hungary and so an infection control strategy was adopted in hospitals.

In summary, *E. coli* and *Klebsiella* spp. isolates have been further characterised in order to identify clonal relatedness. The identification of clones, mechanisms and genes of resistance are important factors to consider for potential new antimicrobial agents and infection control procedures.

# Chapter 6: ESBLs and Antibiotics

## 6.1 Introduction

ESBLs are a widespread problem. Since the emergence of CTX-M in the UK in 2000 (Alobwede et al., 2003), CTX-M genes have rapidly disseminated. ESBLs are now found in both hospital and community environments (Bonnet, 2004, Woodford et al., 2004).

With ESBLs, resistance is conferred to oxyimino-cephalosporins. These are 'front line' antibiotics prescribed for urinary-tract infections, respiratory-tract infections and intra-abdominal infections (Potz et al., 2006, Livermore, 1995). However, in addition to the aforementioned antibiotics, ESBL plasmids often harbour genes for resistance to other antibiotics including aminoglycosides (e.g. gentamicin and amikacin), quinolones and trimethoprim-sulfamethoxazole (TMP-SMX) thus causing strains to be multidrug resistant (Paterson et al., 2000). The clonal spread of resistance genes also poses a great problem. Data obtained by Cagnacci et al., (2008) revealed a correlation between ciprofloxacin resistance and *E. coli* clones O15: K52 H1 and O25: H4 ST131 (predominantly ESBL-producers) in urinary-tract infections. Therefore, the treatment of infections caused by ESBL-producers is challenging and the choice of antimicrobial agent is important as inappropriate treatment is linked to an increased mortality (Rodriguez-Bano et al., 2006). Also as the patterns of resistance change, the demand for new antimicrobial agents increases.

With this in mind, the following questions will be posed and answered in this chapter. How resistant are *Enterobacteriaceae* with ESBLs to aztreonam, ceftazidime and piperacillin/tazobactam? How active are ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin and amikacin against these organisms? Are carbapenems the only option against ESBL carriers? Are 'newer' antibiotics such as temocillin and tigecycline active against ESBLs and could they be suitable alternatives?

### 6.1.1 Ceftazidime

Ceftazidime belongs to the 3rd generation cephalosporin class of antibiotics (Figure 6.1). Information regarding 3rd generation cephalosporins can be found in Section 1.3.2.3.

### 6.1.2 Aztreonam

Aztreonam is currently the only licensed monobactam antibiotic (Figure 6.1) and can be hydrolysed by ESBLs (Philippon et al., 1989, Paterson and Bonomo, 2005). Information regarding monobactams can be found in Section 1.3.4.

### 6.1.3 Piperacillin/tazobactam

Piperacillin/tazobactam is a combination antibiotic consisting of the penicillin-type antibiotic, piperacillin, along with the β-lactamase inhibitor, tazobactam. Information regarding β-lactamase inhibitors can be found in Section 1.4.1.

### 6.1.4 Gentamicin and amikacin

Gentamicin and amikacin are both belong to the aminoglycoside family of antibiotics (Figure 1) and are active against Gram-positive and Gram negative organisms. Gentamicin was originally isolated from *Micromonospora purpurea*; whereas, amikacin is a semisynthetic derivative of kanamycin. The mode of action of aminoglycoside antibiotics involves the irreversible inhibition of protein synthesis. The antibiotic penetrates the cell wall by active transport and binds to receptors on the 30S subunit of the bacterial ribosome thereby inhibiting ribosomal protein synthesis. By doing so, the 'initiation complex' of peptide formation is interfered thus causing the misreading of the mRNA template code. This allows the incorrect amino acid to be incorporated into the peptide. Aminoglycosides cause a breakup of polysomes into non-functional monosomes (Katzung, 1995).

### 6.1.5 Ciprofloxacin

Ciprofloxacin is a member of the quinolone family of antibiotics. It is a synthetic fluorinated analogue of nalidixic acid (Figure 6.1) and is active against Gram-positive and Gram-negative bacteria. Its mode of action is to block DNA synthesis by inhibiting DNA gyrase. This inhibition prevents the relaxation of supercoiled DNA, which is required for normal transcription and duplication (Katzung, 1995).

### 6.1.6 Trimethoprim/sulfamethoxazole

Trimethoprim/sulfamethoxazole, or co-trimoxazole, is a combination antibiotic containing trimethoprim and sulfamethoxazole (Figure 6.1). Both are used in combination as they sequentially block the same pathway. They have a synergistic affect, which improves drug activity. Trimethoprim inhibits specific enzymes in bacteria known as dihydrofolic acid reductases. These enzymes convert dihydrofolic acid into tetrafolic acid, a component of purine synthesis and eventually involved in the formation of DNA. Sulfamethoxazole is a sulphonamide antibiotic, which is a structural analogue of *p*-aminobenzoic acid (required to form folic acid for the production of purines and nucleic acid). The mode of action involves the competition of the sulphonamide with the enzyme dihydropteroate synthesis and results in the formation of non-functional analogues of folic acid, which prevents the bacteria from further growth (Katzung, 1995). Trimethoprim/sulfamethoxazole is active against Gram-positive and Gram-negative bacteria and is often used in the prophylaxis of post-renal transplant patients (Johnson et al., 2010b).

### 6.1.7 Ertapenem

Ertapenem is a carbapenem antibiotic (Figure 6.1) marketed for the use in severe community-acquired infections (Livermore et al., 2005). Information regarding the carbapenems can be found in Section 1.3.5.

### 6.1.8 Temocillin

Temocillin was first introduced into the UK by Beecham Pharmaceuticals in the 1980s but was later withdrawn due to poor commercial success (Livermore and Tulkens, 2009). It is a 6-α-methoxy derivative of ticarcillin (Slocombe et al., 1981) which has the ability to prevent the activation of serine (by impeding the entry of water) and therefore blocking the chemical events which lead to the hydrolysis of the β-lactam ring (Figure 6.1) (Livermore and Tulkens, 2009). Temocillin is active against Gram-negative bacteria but has no activity against *P. aeruginosa*, *Bacteroides fragilis*, (Spencer, 1990) *Campylobacter* spp. or *Acinetobacter* spp. (Rodriguez-Villalobos et al., 2006). It also has low activity against *Serratia* spp. It has been shown to have activity towards ESBL and AmpC enzyme producers (Livermore et al., 2006).

### 6.1.9 Tigecycline

Tigecycline was developed by Francis Tally (Projan, 2009) and is now marketed by Wyeth Pharmaceuticals. It belongs to a class of antibiotics known as the glycylcyclines, which were derived from tetracycline in order to overcome resistance (Pankey, 2005). It shares its 4 ring carbocyclic skeleton with the tetracyclines but has a substitution of N-alkyl-glycylamido on the D ring at the $9^{th}$ position (Figure 6.1) which is said to achieve a broader spectrum of activity (Stein and Craig, 2006). Tigecycline has been shown to have activity *in vitro* against Gram-positive and Gram-negative bacteria (aerobic and anaerobic) including ESBL and AmpC-producing *Enterobacteriaceae* (Hope et al., 2006). However, tigecycline has been found to be less effective against *P. aeruginosa* and *Proteus mirabilis* (Gales and Jones, 2000, Livermore, 2005).

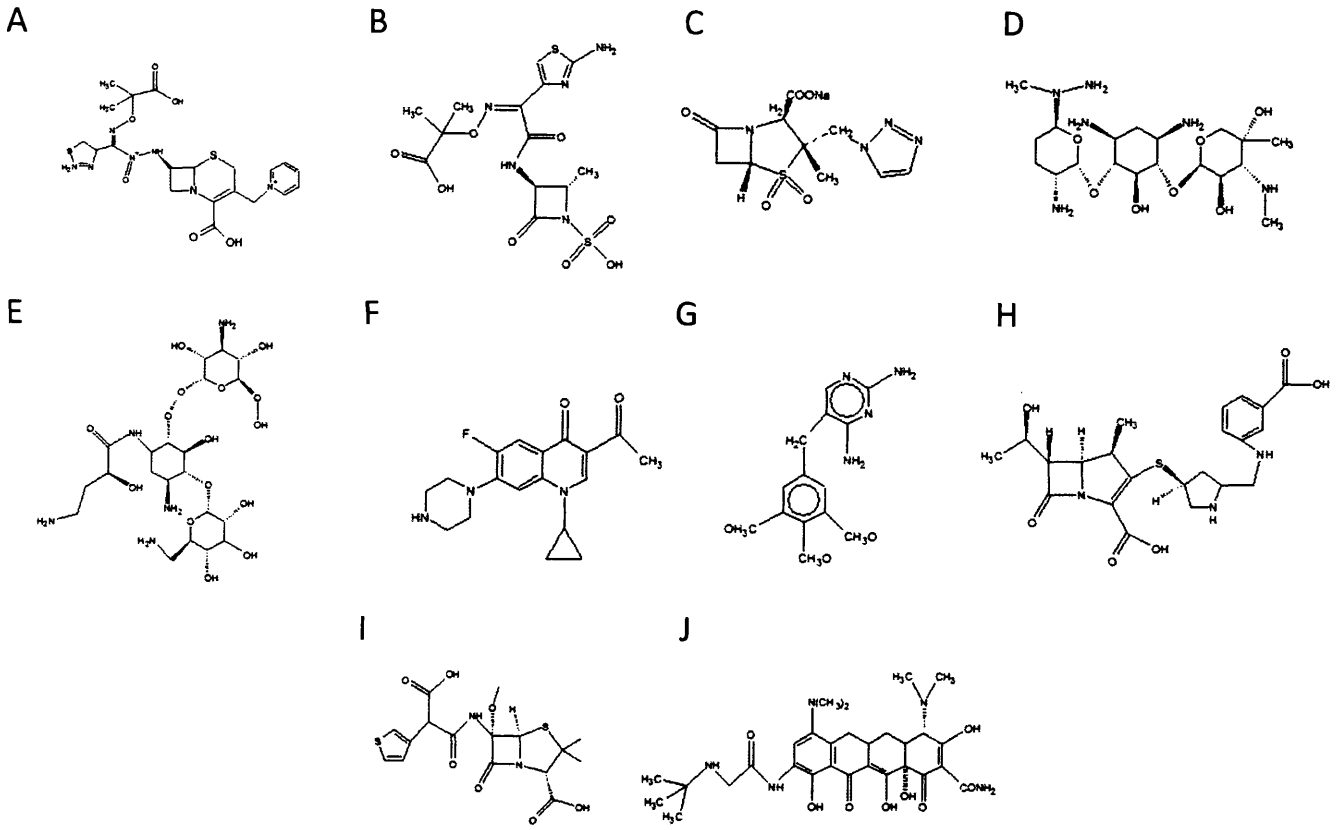



Figure 6.1. Antibiotic structures of A. ceftazidime, B. aztreonam, C. piperacillin/tazobactam, D. Gentamicin, E. Amikacin, F. ciprofloxacin G. trimethoprim/sulfamethoxazole, H. ertapenem, I temocillin J. tigecycline. Images produced using ChemSketch (ACD/Labs).

## 6.2 Results

### 6.2.1 Antibiotic Susceptibility

ESBL-producing *Enterobacteriaceae* were tested against antibiotics of the BD Phoenix Gram-negative panel along with temocillin and tigecycline E-test strips. The Minimum Inhibitory Concentrations (MIC) were determined and using breakpoints supplied by societies/institutions such as BSAC and the Clinical and Laboratory Standards Institute (CLSI), susceptibility profiles were generated.

#### 6.2.1.1 Antibiotic susceptibility profile generated by BD Phoenix

##### 6.2.1.1.1 MIC Distributions of Aztreonam, Ceftazidime and Piperacillin/Tazobactam

The activity of antibiotics against ESBL-producing strains was determined by the comparison of MIC values to breakpoints published by BSAC (BSAC, 2009) as outlined in table 6.1.

Table 6.1 BSAC MIC breakpoints of aztreonam, ceftazidime and piperacillin/tazobactam

| | BSAC MIC Breakpoints (µg/ml) | | |
|---|---|---|---|
| | **R** | **I** | **S** |
| Aztreonam | ≥8 | 2-8 | ≤1 |
| Ceftazidime | ≥8 | 2-8 | ≤1 |
| Piperacillin/Tazobactam | ≥16 | 16 | ≤8 |

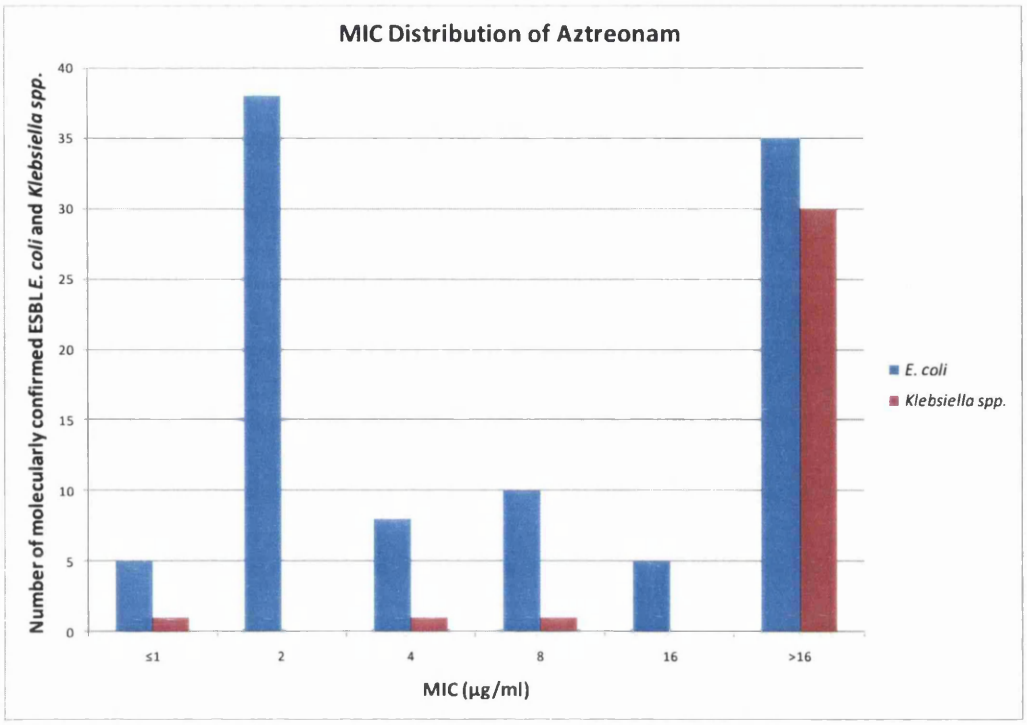



Figure 6.2 MIC Distribution of aztreonam for ESBL-producing *E. coli* (n=101) and *Klebsiella* spp. (n=32).

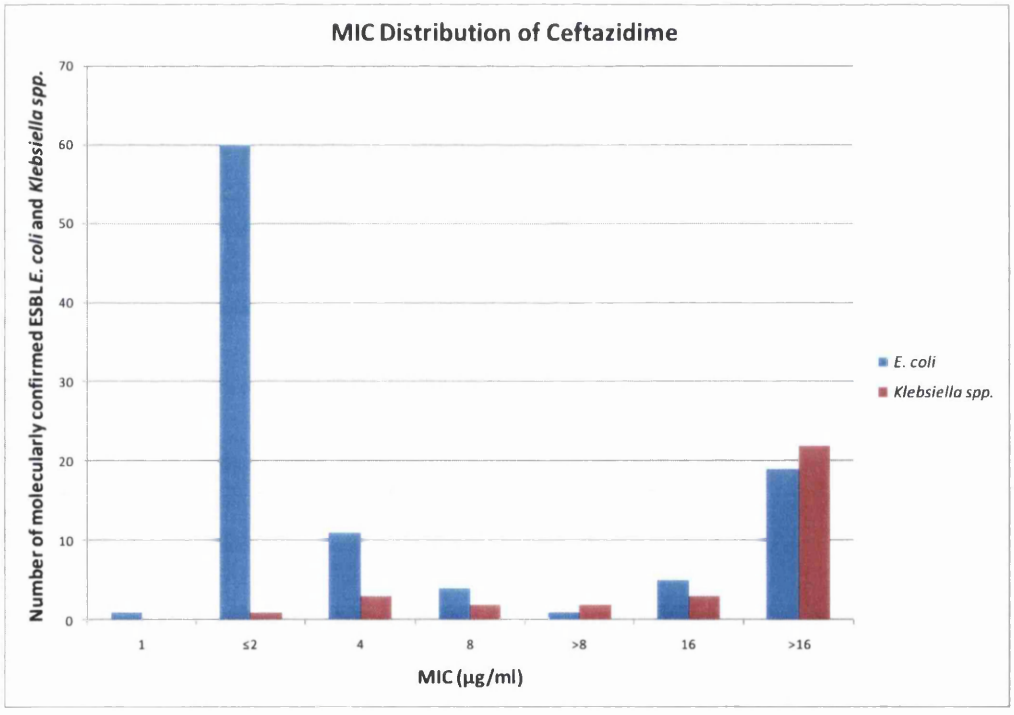



Figure 6.3 MIC distribution of ceftazidime for ESBL-producing *E. coli* (n=101) and *Klebsiella* spp. (n=32).

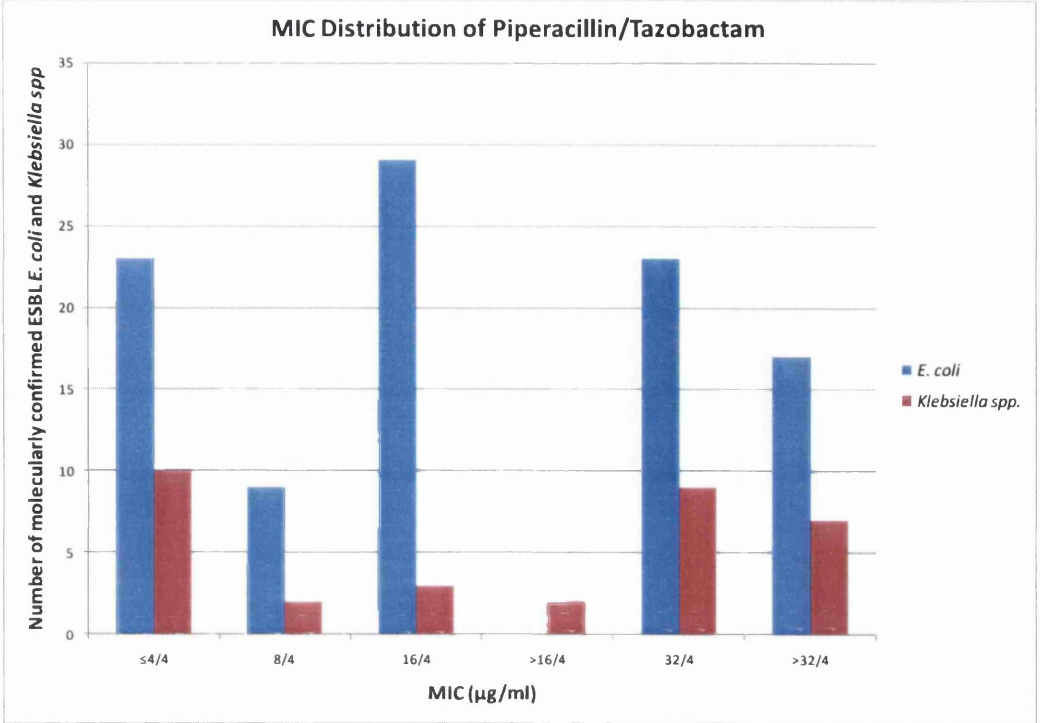



Figure 6.4 MIC distribution of piperacillin/tazobactam for ESBL-producing *E. coli* (n=101) and *Klebsiella* spp. (n=32).

#### 6.2.1.1.2 Resistance Profile of aztreonam, ceftazidime and piperacillin/tazobactam against ESBL-producing *Enterobacteriaceae* generated by BD Phoenix

Table 6.2 outlines the resistance profiles of ESBL-producing *Enterobacteriaceae* against aztreonam, ceftazidime and piperacillin/tazobactam. The BD Phoenix interprets MIC breakpoints according to BSAC guidelines. An initial MIC measurement by the BD Xpert system indicated that not all of the ESBL-producing *Enterobacteriaceae* were resistant to the aforementioned antibiotics. As outlined in Figures 6.2-6.4, the MIC distrubitions for each antibiotic was not only confined to the resistant ranges. For aztreonam (Figure 6.2), the MIC range for *E. coli* and *Klebsiella* spp. was found between ≤1 and ≥16μg/ml with a modal (most frequently occurring) MIC at >16μg/ml. For ceftazidime (Figure 6.3), the MIC range for *E. coli* was between 1μg/ml and >16μg/ml with a modal MIC of ≤2μg/ml and for *Klebsiella* spp. the MIC range was between ≤2μg/ml and >16μg/ml with a modal MIC of >16μg/ml. For piperacillin/tazobactam (Figure 6.4), the MIC ranges for *E. coli* and *Klebsiella* spp. were between ≤4 and >32μg/ml. A modal MIC of 16μg/ml was demonstrated for *E. coli*, whereas, a modal MIC of ≤4μg/ml was demonstrated for *Klebsiella* spp.

The Xpert system of the BD Phoenix interprets these cephalosporin MIC readings according to the respective resistance mechanisms anticipated, in this case ESBL, and the vast majority of susceptible isolates were given a final MIC interpretation of resistant.

Overall, the percentages of ESBL-producers resistant to aztreonam and ceftazidime were, according to MIC 54% and 39% respectively, but reported as 99% and 98% respectively. The interpretations for piperacillin/tazobactam did not change as tazobactam is thought to effectively protect the piperacillin from ESBL activity.

The greatest disparity between resistance as measured by MIC and reported results was seen for *E. coli*. MICs for aztreonam and ceftazidime demonstrated resistance in 40% and 25% of the strains respectively whereas the organisms were reported as resistant in 99% and 98% strains respectively. Within the *Klebsiella* spp., 84% had a resistant MIC to ceftazidime, which was then altered to resistant in 97% for the final

interpretation. For *Klebsiella* spp., 94% had a resistant MIC to aztreonam, however, all were reported as resistant (100%).

Those isolates which were not resistant by MIC measurement were split between 'susceptible' and 'intermediately resistant'. Using the initial BD Phoenix MIC measurement, 5% (6/101) of *E. coli* isolates were found to be susceptible to aztreonam by MIC whereas 55% (56/101) were found to be intermediately resistant. 6% (2/32) of *Klebsiella* spp. were initially found to be intermediately resistant to aztreonam according to MIC measurement before being reported as resistant. 60% (61/101) of *E. coli* isolates were found to be susceptible to ceftazidime. The reported value changed to 1% (2/101) susceptible after the Xpert system's interpretation. 15% (15/101) of *E. coli* isolates were intermediately resistant. For *Klebsiella* spp., 16% (5/32) were intermediately resistant to ceftazidime by MIC. 60% (61/101) of *E. coli* and 41% (13/32) of *Klebsiella* spp. were susceptible to piperacillin/tazobactam, which is important as the antibiotic may eventually be used in non-life-threatening infection in these cases.

Table 6.2 Resistance profiles of aztreonam, ceftazidime and piperacillin/tazobactam for ESBL-producing *Enterobacteriaceae*. MIC values were generated by the BD Phoenix Xpert System by MIC measurement. The final interpretation overrides the original interpretation in association with the designated ESBL rule.

| | % Resistant | | | | | |
|---|---|---|---|---|---|---|
| | **Aztreonam** | | **Ceftazidime** | | **Piperacillin/Tazobactam** | |
| | Measurement | Final Interpretation | Measurement | Final Interpretation | Measurement | Final Interpretation |
| *E. coli* (n=101) | 40% | 99% | 25% | 98% | 40% | 40% |
| *E. coli* IS26 Positive (n=64) | 20% | 100% | 6% | 100% | 48% | 48% |
| *E. coli* IS26 Negative (n=37) | 73% | 97% | 57% | 95% | 24% | 24% |
| *Klebsiella* spp. (n= 32) | 94% | 100% | 84% | 97% | 59% | 59% |
| Total (n=133) | 54% | 99% | 39% | 98% | 44% | 44% |

### 6.2.1.3 Susceptibility profile of ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin, amikacin and ertapenem against ESBL-producing *Enterobacteriaceae*

The MIC values in this section were interpreted according to BSAC guidelines. These are outlined in table 6.3.

Table 6.3 BSAC MIC breakpoints for ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin, amikacin and ertapenem.

| | BSAC MIC Breakpoints (μg/ml) | | |
|---|---|---|---|
| | R | I | S |
| Ciprofloxacin | ≥1 | 1 | ≤0.5 |
| Trimethoprim-sulfamethoxazole | ≥4 | 4 | ≤2 |
| Gentamicin | ≥4 | 4 | ≤2 |
| Amikacin | ≥16 | 16 | ≤8 |
| Ertapenem | ≥1 | 1 | ≤0.5 |

The results outlined in table 6.4 give an overview of the susceptibility profile of ESBL-producing *Enterobacteriaceae* against the following antibiotics: ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin, amikacin and ertapenem. Ninety percent of all isolates were resistant to ciprofloxacin (90% of *E. coli* and 91% of *Klebsiella* spp.). The remaining *E. coli* (10%) were susceptible whereas 3% of the remaining *Klebsiella* spp. were resistant and 6% were intermediately resistant.

Eighty-nine percent of all ESBL-producing *Enterobacteriaceae* were found to be resistant to trimethoprim-sulfamethoxazole with 94% of *E. coli* and 73% of *Klebsiella* spp. having MIC values of >4μg/ml. 6% of *Klebsiella* spp. isolates were found to be intermediately resistant.

Gentamicin and amikacin are both aminoglycoside antibiotics. 67% of the isolates were susceptible to gentamicin and 85% to amikacin. For gentamicin, 82% of *E. coli* were susceptible, 16% were resistant and 2% were intermediately resistant. The

*Klebsiella* spp. show a different profile as 21% were susceptible and 79% were resistant. For amikacin, 81% of *E. coli* were susceptible, 15% were resistant and 4% were intermediately resistant. With the *Klebsiella* spp., 97% of isolates were susceptible and 3% were intermediately resistant.

The carbapenems tested were meropenem, imipenem and ertapenem. 100% susceptibility was found for all isolates for meropenem and imipenem. However, the susceptibility for ertapenem was 99% as 1% (n=1) of *E. coli* was found to be resistant.

Table 6.4 The Susceptibility profiles of ciprofloxacin, piperacillin/tazobactam, trimethoprim-sulfamethoxazole, gentamicin, amikacin and ertapenem in ESBL-producing *Enterobacteriaceae*. MIC values were measured by the BD Phoenix Xpert system. Imipenem and meropenem were also tested and 100% of all isolates were susceptible.

| | **Ciprofloxacin** | | | **Trimethoprim-sulfamethoxazole** | | | **Gentamicin** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **S** | **I** | **R** | **S** | **I** | **R** | **S** | **I** | **R** |
| *E. coli* (n=101) | 10% | 0% | 90% | 6% | 0% | 94% | 82% | 2% | 16% |
| *E. coli IS*26 Positive (n=64) | 0% | 0% | 100% | 0% | 0% | 100% | 98% | 2% | 0% |
| *E. coli IS*26 Negative (n=37) | 24% | 0% | 76% | 86% | 0% | 14% | 54% | 2% | 44% |
| *Klebsiella* spp. (n= 32) | 3% | 6% | 91% | 21% | 6% | 73% | 21% | 0% | 79% |
| Total (n=133) | 8% | 1% | 90% | 10% | 1% | 89% | 67% | 2% | 31% |

Table 6.4 cont...The Susceptibility profiles of amikacin and ertapenem in ESBL-producing *Enterobacteriaceae*. MIC values were measured by the BD Phoenix Xpert system. Imipenem and meropenem were also tested and 100% of all isolates were susceptible.

| | **Amikacin** | | | **Ertapenem** | |
|---|---|---|---|---|---|
| | **S** | **I** | **R** | **S** | **R** |
| *E. coli* (n=101) | 81% | 4% | 15% | 99% | 1% |
| *E. coli IS*26 Positive (n=64) | 75% | 19% | 6% | 98% | 2% |
| *E. coli IS*26 Negative (n=37) | 92% | 0% | 8% | 100% | 0% |
| *Klebsiella* spp. (n= 32) | 97% | 0% | 3% | 100% | 0% |
| Total (n=133) | 85% | 4% | 11% | 99% | 1% |

### 6.3 Antibiotic Susceptibility Profile generated by E-test

E-test strips containing temocillin and tigecycline were used to test a total of 134 molecularly-confirmed ESBL-producers, namely 99 *E. coli* isolates, 32 *Klebsiella* spp. (30 *Klebsiella* spp. were tested against tigecycline) and 3 other *Enterobacteriaceae* isolates (2 *E. cloacae* and 1 *C. freundii*).

A selection of control isolates - *E. coli* strains A-E, *E. coli* K12 and *E. coli* NCTC 10418 - was also utilised. The method is described in Chapter 2 section 2.5.4.

MICs were interpreted using various breakpoint guidelines. For temocillin, BSAC UTI , BSAC systemic (BSAC, 2009) and Fuchs (Fuchs et al., 1985) breakpoints were used. BSAC UTI breakpoints have a specific stipulation associated with their usage. BSAC states: *"UTI recommendations are for organisms associated with uncomplicated urinary infections only. For complicated UTI systemic recommendations should be used."* For this study, both breakpoint sets were analysed for comparison.

To interpret tigecycline MIC values, BSAC and CLSI breakpoints were followed (BSAC, 2009), (ABbioMérieux, 2009). Tables 6.5 and 6.5.1 outline the breakpoints used.

Table 6.5 BSAC UTI and BSAC Systemic guidelines for the interpretation of temocillin MIC values

| **Temocillin MIC (μg/ml)** | **Breakpoints** | |
|---|---|---|
| | **R** | **S** |
| BSAC UTI | >32 | ≤32 |
| BSAC systemic | >8 | ≤8 |
| Fuchs | ≥32 | ≤16 |

Table 6.5.1 BSAC and CLSI (as stated by bioMérieux) breakpoints for the interpretation of tigecycline MIC values. EUCAST (European Committee Antimicrobial Susceptibility Testing) MIC values are the same as those outlined for BSAC.

| | Tigecycline Breakpoints (μg/ml) | | |
|---|---|---|---|
| | **R** | **I** | **S** |
| BSAC | >2 | 2 | ≤1 |
| CLSI | ≥8 | 4 | ≤2 |

### 6.3.1 Temocillin

#### 6.3.1.1 MIC Distributions

The temocillin E-test strips utilised had a concentration range of 0.125μg/ml - 256μg/ml (Figure 6.6). For all ESBL-producing *E. coli* isolates tested (n=99), the MIC values were within a concentration range of 2-32μg/ml (Table 6.6). 36% of *E. coli* isolates (n=36) had an MIC ≥12μg/ml of which 8% (n=3) had MIC values at the top end of the *E. coli* range (32μg/ml). 24% of *E. coli* isolates (n=24) had an MIC value of ≤6 μg/ml of which 12.5% (n=3) had an MIC of 2μg/ml. 38% of *E. coli* isolates (n=38) were found to have a modal (most frequently occurring) MIC of 8μg/ml (Figure 6.5). The $MIC_{50}$ and $MIC_{90}$ values represent the MIC at which 50% and 90% of organisms are inhibited, respectively. The values were calculated by cumulating the number of isolates inhibited and finding the MIC value at the respective percentage (Hamilton-Miller, 1991). The $MIC_{50}$ value for *E. coli* was 8μg/ml whereas the $MIC_{90}$ value was 16μg/ml. However, differences do occur within *E. coli* when isolates are analysed according to the presence of absence of an insertion element 26 (*IS*26). The modal MIC for *E. coli* containing *IS*26 was 8μg/ml (52%) whereas for *E. coli IS*26 negative isolates, the modal MIC was 12μg/ml (25%). The $MIC_{50}$ and $MIC_{90}$ values also differ as *E. coli IS*26 negative isolates have an $MIC_{50}$ of 12μg/ml and $MIC_{90}$ value of 24μg/ml. The $MIC_{50}$ value for *E. coli IS*26 positive isolates was 8μg/ml whereas the $MIC_{90}$ value was 12μg/ml.

Additionally, temocillin was tested against epidemic *E. coli* strains A-E, *E. coli* K12 and NCTC 10418. Within the 5 epidemic *E. coli* strains, the MIC distribution for temocillin was bimodal with values at 8μg/ml (strain A and C) and 12μg/ml (strain

D and E). Strain B had an MIC value of 24μg/ml. These MIC values were different from those measured for *E. coli* K12 and *E. coli* NCTC 10418, which had lower MIC values of 6μg/ml and 2μg/ml, respectively.

The MIC concentration range for *Klebsiella* spp. (n=30) was found to be 3-256μg/ml (Figure 6.5). 43% of *Klebsiella* spp. (n=13) had MIC values of ≥12μg/ml and of which, 1 *K. pneumoniae* isolate had an MIC value of 256μg/ml – the highest concentration found on the temocillin E-test strip. 30% of isolates (n=9) had an MIC of ≤6μg/ml. The modal MIC for temocillin within the *Klebsiella* spp. was found to be 8μg/ml (27%). The $MIC_{50}$ value for *Klebsiella* spp. was 8μg/ml and the $MIC_{90}$ value was 24μg/ml.

Two *E. cloacae* and 1 *C. freundii* isolate were tested against temocillin. The MIC range was found to be 2-12μg/ml. The MIC values of the two *E. cloacae* were 3 and 12μg/ml whereas *C. freundii* had the lowest MIC value of 2μg/ml.

Table 6.6 MIC distribution of temocillin against ESBL-producing *Enterobacteriaceae.*

| | **Temocillin** | | |
|---|---|---|---|
| | Range (μg/ml) | $MIC_{50}$ (μg/ml) | $MIC_{90}$ (μg/ml) |
| *E. coli* (n=99) | 2-32 | 8 | 16 |
| *E. coli IS*26 positive (n=63) | 2-32 | 8 | 12 |
| *E. coli IS*26 negative (n=36) | 2-32 | 12 | 24 |
| *Klebsiella spp.* (n=30) | 3-256 | 8 | 24 |

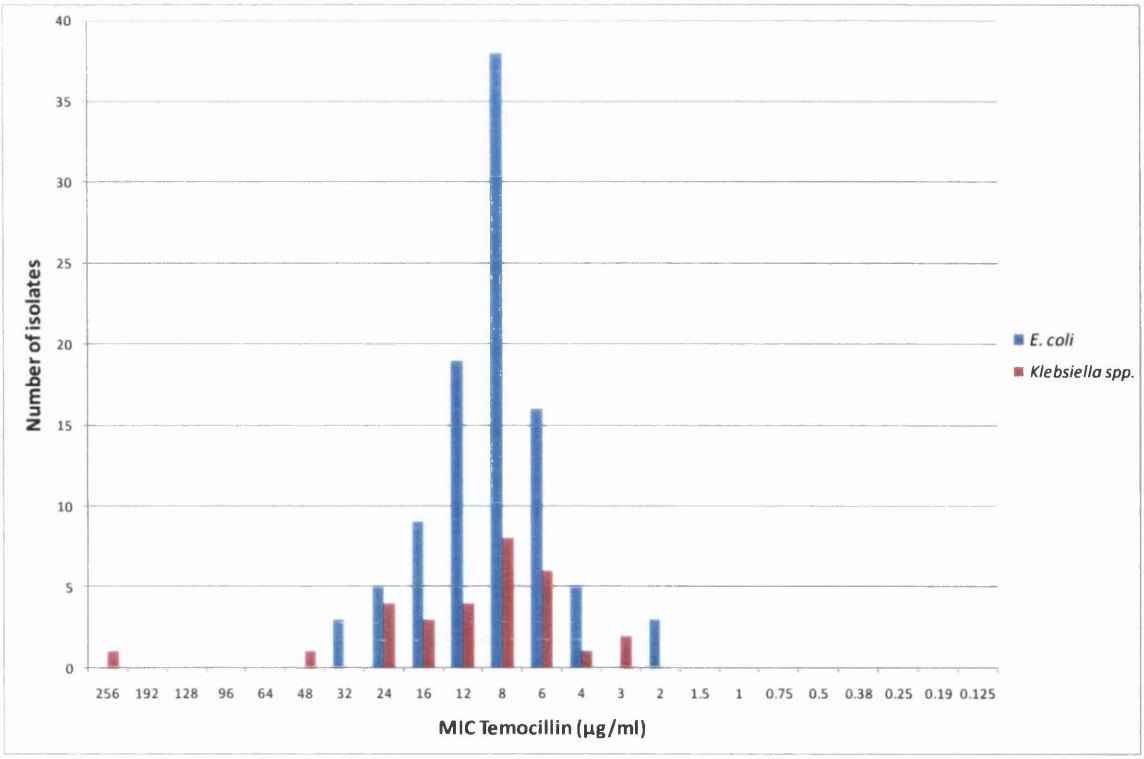



Figure 6.5 MIC distribution of *E. coli* and *Klebsiella* spp. for temocillin.

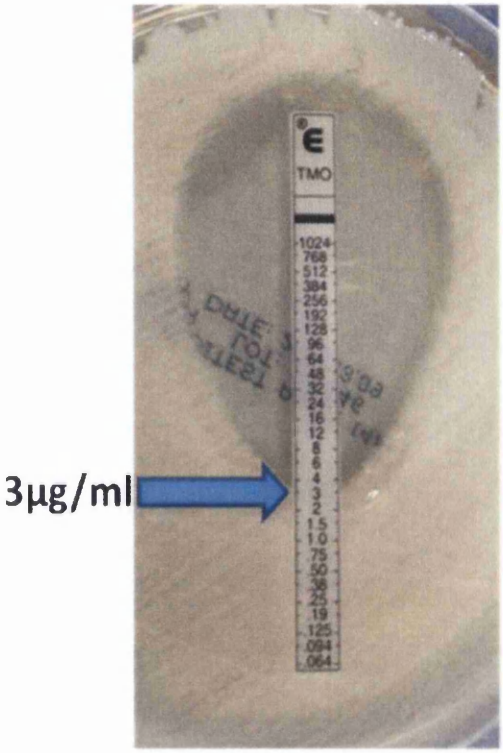



Figure 6.6 Temocillin E-test. E-test to determine the MIC of ESBL-producing isolates against temocillin.

### 6.3.1.2 Susceptibility profile of temocillin

100% (n=99) of *E. coli* and other *Enterobacteriaceae* (n=3) isolates tested against temocillin and interpreted using BSAC UTI breakpoints were found to be susceptible. 93% (n=28) of *Klebsiella* spp. were found to be susceptible whereas 7% (n=2) were found to be resistant. Therefore, in total temocillin was effective against 98% (n=130) of ESBL-producing *Enterobacteriaceae* using BSAC UTI breakpoints.

However, the susceptibility profile changes when interpreted using BSAC systemic breakpoints. The number of resistant isolates increased, with 37% of *E. coli*, 47% of *Klebsiella* spp. and 33% of other isolates being called resistant. Using these breakpoints, temocillin was only effective against 61% (n=81) of ESBL-producing *Enterobacteriaceae* with an approximately 25-fold increase in the number of resistant isolates (n=51).

Using Fuchs' breakpoints to interpret MIC, the susceptibility profile generated for temocillin is altered once more. Fuchs' guidelines share a similarity to BSAC UTI guidelines as they have the same breakpoint for determining resistance (>32 for BSAC UTI and ≥32 μg/ml for Fuchs). With Fuchs' breakpoints, the threshold for susceptible isolates is set between the susceptible breakpoints of BSAC UTI and BSAC systemic guidelines, at ≤16 μg/ml. Therefore, fewer susceptible isolates are found with Fuchs' guidelines than with BSAC UTI. Ninety-one percent of *E. coli* isolates and 80% of *Klebsiella* spp. were found to be susceptible to temocillin when using Fuchs' guidelines. Overall, Fuchs' guidelines identified 88% of all isolates as susceptible (Table 6.7).

Using BSAC UTI breakpoints, all seven of the control isolates used in this section of study were found to be susceptible to temocillin (Table 6.8). However, using the BSAC systemic breakpoints 43% (n=3) of isolates were found to be resistant.

Table 6.7 Susceptibility profile to temocillin amongst ESBL-producing *Enterobacteriaceae.*

| | **Temocillin BSAC UTI** | | **Temocillin BSAC systemic** | | **Temocillin Fuchs'** | | |
|---|---|---|---|---|---|---|---|
| | S | R | S | R | S | I | R |
| *E. coli* (n=99) | 100% | 0% | 63% | 37% | 91% | 5% | 4% |
| *E. coli IS*26 positive (n=63) | 100% | 0% | 75% | 25% | 96% | 2% | 2% |
| *E. coli IS*26 negative (n=36) | 100% | 0% | 41% | 59% | 83% | 11% | 6% |
| *Klebsiella* spp. (n=32) | 93% | 7% | 53% | 47% | 80% | 13% | 7% |
| Other (n=3) | 100% | 0% | 67% | 33% | 100% | 0% | 0% |
| Total (n=134) | 98% | 2% | 61% | 39% | 88% | 7% | 5% |

The MIC values were interpreted into susceptible (S) or resistant (R) using BSAC UTI, BSAC systemic and Fuchs' breakpoints.

Table 6.8 Resistance profile of temocillin for epidemic *E. coli* strains A-E, *E. coli* K12 and NCTC 10418 using BSAC UTI and BSAC systemic breakpoints.

| **Controls** | **Temocillin BSAC UTI** | **Temocillin BSAC systemic** |
|---|---|---|
| *E. coli* Strain A | Susceptible | Susceptible |
| *E. coli* Strain B | Susceptible | Resistant |
| *E. coli* Strain C | Susceptible | Susceptible |
| *E. coli* Strain D | Susceptible | Resistant |
| *E. coli* Strain E | Susceptible | Resistant |
| *E. coli* K12 | Susceptible | Susceptible |
| *E. coli* NCTC 10418 | Susceptible | Susceptible |

### 6.3.2 Tigecycline

#### 6.3.2.1 MIC Distributions

Tigecycline E-test strips have an MIC range of 0.64-256μg/ml (Figure 6.8). The MIC range for *E. coli* (n=99) was between 0.125 and 2μg/ml with 53% (n=53) of isolates being predominantly found at the lower end of the concentration scale (≤0.5μg/ml). 27% (n=27) of isolates had an MIC of ≥1μg/ml (Table 6.9). The modal MIC was 0.75μg/ml with 19% (n=19) of *E. coli* with this value (Figure 6.7). The $MIC_{50}$ and $MIC_{90}$ for *E. coli* were 0.5μg/ml and 1μg/ml respectively. However as with temocillin, differences occur when *E. coli* is categorised according to the presence or absence of *IS*26. *E. coli IS*26 positive isolates had an increased lower range from 0.125μg/ml to 0.19μg/ml whereas the $MIC_{50}$ and $MIC_{90}$ values for *E. coli IS*26 negative isolates were 0.38μg/ml and 1.5μg/ml respectively (Table 6.9).

Tigecycline was tested against epidemic *E. coli* strains A-E, *E. coli* K12 and NCTC 10418. The range of activity was 0.64μg/ml - 1μg/ml and the modal MIC was at 0.5μg/ml (43%). Once more, the MIC of *E. coli* K12 and *E. coli* NCTC 10418 were found at the lower end of the scale with values of 0.19μg/ml and 0.64μg/ml respectively.

The MIC range for *Klebsiella* spp. tested against tigecycline was found to be at a higher portion of the concentration gradient in comparison to *E. coli* with values between 0.5μg/ml - 8μg/ml with 40% (n=13) of isolates with an MIC of ≥2μg/ml and 38% (n=12) with an MIC of ≤1μg/ml. The modal MIC was 1.5μg/ml (22%). $MIC_{50}$ and $MIC_{90}$ values for *Klebsiella* spp. were 1.5μg/ml and 4μg/ml respectively. MIC values of 1.5μg/ml, 1μg/ml and 0.25μg/ml were found for the 2 *E.cloacae* and 1 *C. freundii*.

Table 6.9 MIC distribution of tigecycline against ESBL-producing *Enterobacteriaceae*.

| | Tigecycline | | |
|---|---|---|---|
| | Range (μg/ml) | $MIC_{50}$ (μg/ml) | $MIC_{90}$ (μg/ml) |
| *E. coli* (n=99) | 0.125-2 | 0.5 | 1 |
| *E. coli IS*26 positive (n=63) | 0.19-2 | 0.5 | 1 |
| *E. coli IS*26 negative (n=36) | 0.125-2 | 0.38 | 1.5 |
| *Klebsiella* spp. (n=32) | 0.5-8 | 1.5 | 4 |

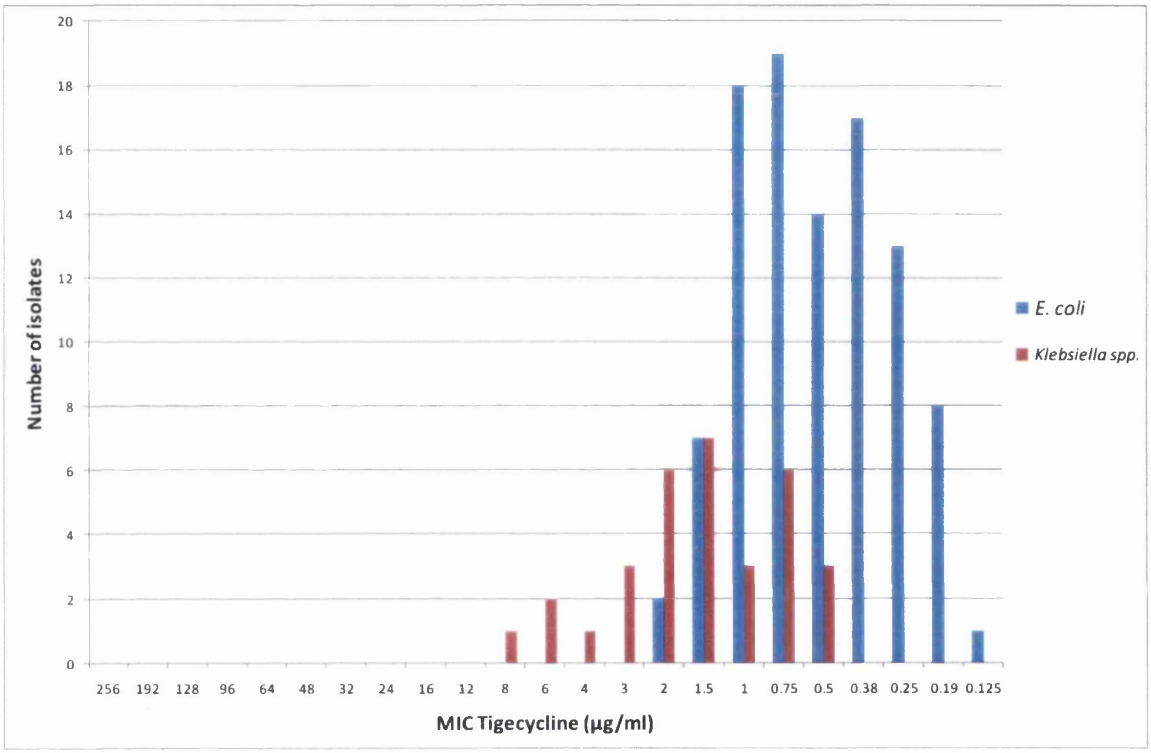



Figure 6.7 MIC distribution of *E. coli* and *Klebsiella* spp. for tigecycline.

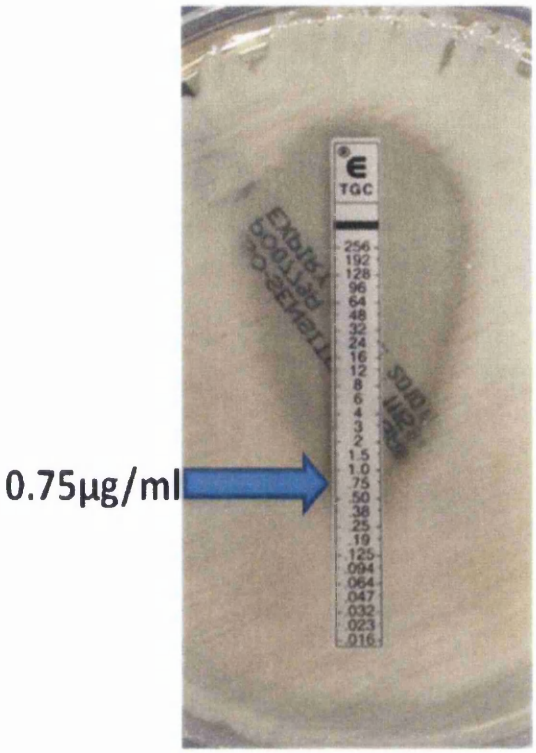



Figure 6.8 Tigecycline E-test. E-test to determine the MIC of tigecycline against ESBL-producing isolates.

### 6.3.2.2 Susceptibility profile of tigecycline

Using BSAC guidelines, 98% (n=97) of *E. coli* isolates were susceptible to tigecycline, 2% (n=2) were intermediate (Table 6.10). This varies slightly when divided between *IS*26 positive and negative strains as *E. coli IS*26 negative isolates are 97% (n=35) susceptible and 3% (n=1) intermediate. Using CLSI breakpoints, 100% (n=99) of *E. coli* isolates were found to be susceptible.

56% (n=18) of *Klebsiella* spp. were susceptible using BSAC breakpoints, 19% (n=6) were intermediate and 25% (n=8) were resistant. In comparison, using CLSI breakpoints generated more susceptible MIC values than BSAC breakpoints with 88% (n=28) of *Klebsiella* spp. being susceptible, 3% (n=1) intermediate and 9% (n=3) resistant. 100% of other *Enterobacteriaceae* species were found to be susceptible using both BSAC and CLSI breakpoints.

In total, tigecycline was effective against 89% of all *Enterobacteriaceae* using BSAC breakpoints and 97% effective using CLSI breakpoints. However, despite the higher number of susceptible MIC values, the CLSI breakpoints reveal more resistant values (5%) than the BSAC breakpoints (2%). BSAC breakpoints have a greater number of intermediate MIC values (6%) than CLSI (1%).

Using both BSAC and CLSI breakpoint to interpret results, all seven control isolates were found to be susceptible to tigecycline.

Table 6.10 Susceptibility profile of tigecycline against ESBL-producing *Enterobacteriaceae*. The MIC values were interpreted as susceptible (S), intermediate (I) or resistant (R) using BSAC and CLSI (as provided by bioMérieux) breakpoints.

| | Tigecycline BSAC | | | Tigecycline CLSI | | |
|---|---|---|---|---|---|---|
| | S | I | R | S | I | R |
| *E. coli* (n=99) | 98% | 2% | 0% | 100% | 0% | 0% |
| *E. coli IS*26 positive (n=63) | 98% | 2% | 0% | 100% | 0% | 0% |
| *E. coli IS*26 negative (n=36) | 97% | 3% | 0% | 100% | 0% | 0% |
| *Klebsiella* spp. (n=32) | 56% | 19% | 25% | 88% | 3% | 9% |
| Other (n=3) | 100% | 0% | 0% | 100% | 0% | 0% |
| Total (n=134) | 89% | 6% | 2% | 97% | 1% | 5% |

## 6.4 Discussion

This chapter has evaluated the susceptibility of ESBL-producing *Enterobacteriaceae* to various antibiotics. Clinically, ESBL-producers are considered to be resistant to aztreonam and ceftazidime (Paterson and Bonomo, 2005). However, from this study it is clear to see that, according to measurement of MIC, this is not the case for all ESBL-producing *Enterobacteriaceae*. Within the *E. coli* population, only 40% of isolates have resistant MIC values to aztreonam and only 25% of isolates were resistant to ceftazidime. This highlights the importance of detection of the ESBL resistance mechanisms, as these antibiotics would otherwise eventually be recommended for therapy.

Resistance to front line antibiotics poses a great limitation from the perspective of treatment options (Paterson et al., 2000). Ciprofloxacin is commonly used to treat urinary-tract infections. In this study, 90% of all *Enterobacteriaceae* were resistant to ciprofloxacin. Breaking this down into species level, 90% of *E. coli* and 91% of *Klebsiella* spp. were resistant. A study conducted by Hwang et al., (2009) observed 52% and 50% resistance with ESBL-producing *E. coli* and *Klebsiella* spp., respectively. Cagancci et al., (2008) carried out a study on non ESBL-producing *E. coli* associated with urinary-tract infections, showing that only 10% were resistant to ciprofloxacin. Paterson et al., (2000) described a correlation between ciprofloxacin resistance and ESBL production, stating that 60% of isolates in the study were in fact ESBL-producing.

Piperacillin/tazobactam is a combination antibiotic containing the broad-spectrum penicillin piperacillin and the β-lactamase inhibitor tazobactam. In theory, owing to the presence of a β-lactamase inhibitor, the large majority of isolates should be susceptible. However, it was only effective against 56% of isolates: 60% of *E. coli* and 42% *Klebsiella* spp. were susceptible. This result appears to be at odds once more with the findings of by Hwang et al., (2009) who observed that 90% of ESBL-producing *E. coli* and 73% of ESBL-producing *Klebsiella* spp. were susceptible. However, CLSI guidelines were followed in their study.

As stated in the introduction to this chapter (section 6.1), plasmids encoding ESBL genes often harbour genes for other resistance mechanisms e.g. aminoglycosides and

trimethoprim-sulfamethoxazole. In this study, the effectiveness of gentamicin, amikacin and trimethoprim-sulfamethoxazole was analysed. 31% of all isolates were resistant to gentamicin whereas 11% were resistant to amikacin. 89% of all *Enterobacteriaceae* isolates were found to be resistant to trimethoprim-sulfamethoxazole of which 94% were *E. coli*. A study by Cagnacci et al., (2008) on a population of non-ESBL-producing *E. coli* stated that only 30.2% were resistant.

The carbapenems are active against Gram-negative organisms and are reserved primarily for severe infections with organisms producing ESBL and high level AmpC (Woodford et al., 2007a). In this study, 100% of isolates were fully susceptible to meropenem and imipenem. When evaluating the use of ertapenem only one *E. coli* (n=101) isolate was found to be resistant with an MIC value of 4μg/ml. The *E. coli* isolate in question carried CTX-M-15. Comparing the results to those of Hwang et al., (2009), all isolates in their study were susceptible.

Temocillin has been shown to have an increased stability to ESBL- and AmpC-producing *Enterobacteriaceae*. In this study, using BSAC UTI breakpoints, temocillin was effective against 98% of all isolates tested. This is comparable with the sensitivity rate (99%) obtained by Livermore et al., (2006) in the South East of England.

Rodriguez-Villalobos et al., (2006) Rodriguez-Villalobos et al., (2009) and Glupczynski et al., (2007) used Fuchs' guidelines (Fuchs et al., 1985) to interpret temocillin MIC breakpoints. Rodriguez-Villalobos et al., (2006) demonstrated that 92% of *E. coli* were susceptible to temocillin and Rodriguez-Villalobos et al., (2009) found that 95.4% of *E. coli* were susceptible to temocillin. Glupczynski et al., (2007) stated that 97.5% of *E. coli* were susceptible. In this thesis, 97% of *E. coli* strains were susceptible using Fuchs' breakpoints. Glupczynski et al., (2007) also stated that 88% of ESBL-producing *Klebsiella* spp. were susceptible to temocillin whereas in this study, 93% were found to be susceptible. The percentage of susceptible isolates decreases when BSAC systemic breakpoints are used. Using these guidelines, temocillin was effective against 61% of all isolates including 63% *E. coli*, 53% *Klebsiella* spp. and 67% of other *Enterobacteriaceae*. This is interesting to note as community-acquired bacteraemia can be associated with localised

infections such as those found in the urinary tract (Hwang et al., 2009). However, temocillin has high activity in urinary-tract infections against most ESBL-producing organisms.

Tigecycline has generally exhibited good *in vitro* activity against *Enterobacteriaceae* in previous studies (Hope et al., 2006, Morosini et al., 2006, Lu et al., 2008, Jones et al., 2009). A study carried out by Hope et al., (2006) in the South East of England demonstrated 100% sensitivity when testing tigecycline against ESBL-producing *E. coli* and 52% sensitivity against ESBL-producing *Klebsiella* spp. The results shown in this study are comparable as 98% of ESBL-producing *E. coli* and 56% of *Klebsiella* spp. were susceptible when using BSAC guidelines. However, these results contrast with those obtained by Naesens et al., (2009) who found that only 65% of ESBL-producing *E. coli* and 0% of ESBL-producing *Klebsiella* spp. were susceptible to tigecycline. In this chapter, using CLSI guidelines increased the percentage of susceptible *E. coli* isolates to 100% and *Klebsiella* spp. to 88% giving an overall susceptibility of 97%. This result exactly supports the findings of Jones et al., (2009) and is also comparable to the results (99% susceptible) of Lu et al., (2008).

In summary, resistance is commonly found against the most established antibiotics. In this study amikacin (11% of isolates resistant) and the carbapenems (ertapenem 1% of isolates resistant) have proved to be the most active of the established antibiotics against ESBL-producing *Enterobacteriaceae*. Also the 'new' alternatives temocillin and tigecycline have demonstrated good *in vitro* activity. However with temocillin, considerations need to be made when choosing the most suitable MIC breakpoint guidelines and type of infection involved, as the susceptibility profile varies between each considerably.

# Chapter 7: Identification of *Enterobacteriaceae* isolates by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

## 7.1 Introduction

Within the clinical laboratory, bacteria are identified using conventional phenotypic methods such as growth on various media, colony morphology, Gram stain and other biochemical tests. However, although accurate, these typical tests are often time consuming and costly (Bizzini et al., 2003). As bacterial identification can take between 24 and 36 hours (Cherkaoui et al., 2010), the correct treatment of patients by empirical antibiotic therapy is often hampered (Seng et al., 2009). In order to improve patient care, the need for a rapid and accurate identification of bacteria is essential (Carbonnelle et al., 2011, Seng et al., 2009). Molecular and genotypic methods e.g. PCR and nucleotide analysis have been developed but they are often technically demanding and expensive (Conway et al., 2001). The use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) has been demonstrated by a number of studies to have the capability of rapidly identifying bacteria to species level e.g. enterobacteria (Lynn et al., 1999, Conway et al., 2001), staphylococci (Carbonnelle et al., 2007), listeria (Barbuddhe et al., 2008) and clostridia (Grosse-Herrenthey et al., 2008).

MALDI-TOF/MS utilises a 'proteomic' approach to bacterial identification by creating a protein 'fingerprint' which can be compared to a database (Carbonnelle et al., 2011, Bizzini et al., 2003). Sample preparation for MALDI-TOF is relatively simplistic. Intact cells can be directly applied (smear method) to a target plate or alternatively, an extraction procedure can be adopted to disrupt the cell (particularly the cell wall) thus causing the release of protein markers. The samples are overlaid with a matrix, before being subjected to laser radiation. Protein and peptide ions, mainly from ribosomal proteins, enter a time of flight tube where they can be separated dependent on their mass to charge ratio (*m/z*) (Stults, 1995). The mass to charge ratio values form mass spectral peaks which indicate the respective components detected in the sample (Cherkaoui et al., 2010). In this study the Bruker Daltonics Microflex LT Benchtop MALDI Mass Spectrometer (Bruker Daltonics,

Germany) was utilised and spectra were analysed using MALDI Biotyper 2.0 software.

MALDI-TOF/MS was first utilised in the 1990s for the identification of bacteria (Holland et al., 1996, Krishnamurthy and Ross, 1996). However, its usage in the clinical setting has only been investigated in recent years. MALDI-TOF/MS methods capable of directly detecting bacteria from the source sample e.g. blood (La Scola and Raoult, 2009, Stevenson et al., 2010) and urine (Ferreira et al., 2010) are being established to reduce time and maximise efficiency.

Additionally, as well as bacterial identification, MALDI-TOF/MS has been utilised in studies for the detection of methicillin-resistant *Staphylococcus aureus*, MRSA versus methicillin sensitive *S. aureus*, MSSA (Edwards-Jones et al., 2000). MALDI-TOF/MS has also been used to subtype clonal lineages of MRSA (Wolters et al., 2010) and *Staphylococcus epidermidis* (Harris et al., 2010). It has also been shown to have the capability to differentiate *E. coli* O157: H7 from wild type *E. coli* strains (Parisi et al., 2008).

In this chapter, the aim was to rapidly identify 160 *Enterobacteriaceae* isolates and to compare the findings with the BD Phoenix Automated system which is already established as an automated identification tool within the clinical laboratory.

## 7.2 Results

### 7.2.1 Identification of bacterial isolates by the Bruker Daltonics MALDI-Biotyper

Mass spectra of *Enterobacteriaceae* were analysed by the MALDI Biotyper 2.0 software (Bruker Daltonik, Bremen,Germany). Figure 7.1 and 7.2 demonstrate spectra of *E. coli* and *K. pneumoniae*, respectively. The spectra demonstrate mass ion intensity over a mass to charge ratio (m/z) range of 2000 to 20000. Differences between the spectra are clearly distinguished, which support this instrument's capability in the identification of bacteria. For each sample tested, the mass ion peaks were compared to the spectra contained within MALDI Biotyper 2.0 database. The Bruker MALDI Biotyper generated the 'Best Match' identification along with a 'Score Value'. It also gave a 'Second Best Match' option and 'Score Value'. The score value is a number generated to give an indication of how successful and effective the identification process had been. The score value scale ranged from 0 (no reliable identification) to 3 (highly probably species identification). Overall, there are four score value groups as outlined in Table 7.2.

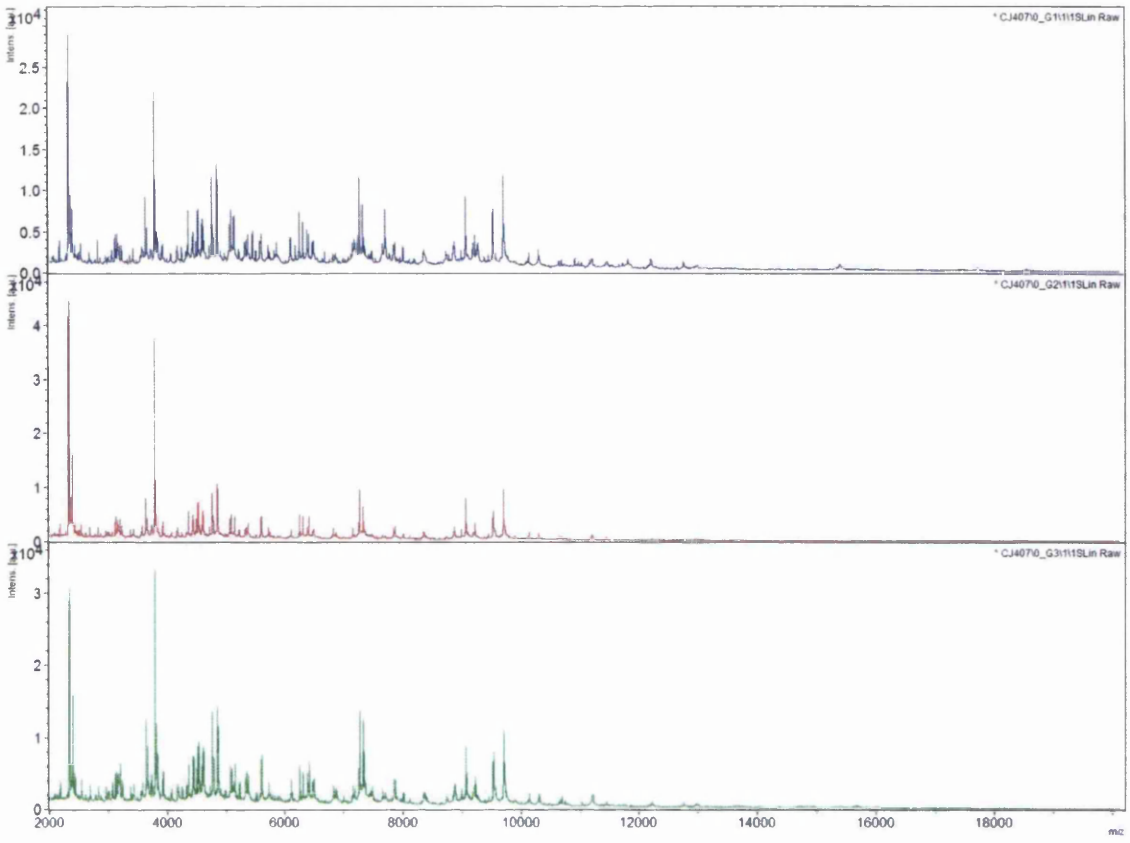



Figure 7.1 Mass spectra of three replicates of an *E. coli* isolate.

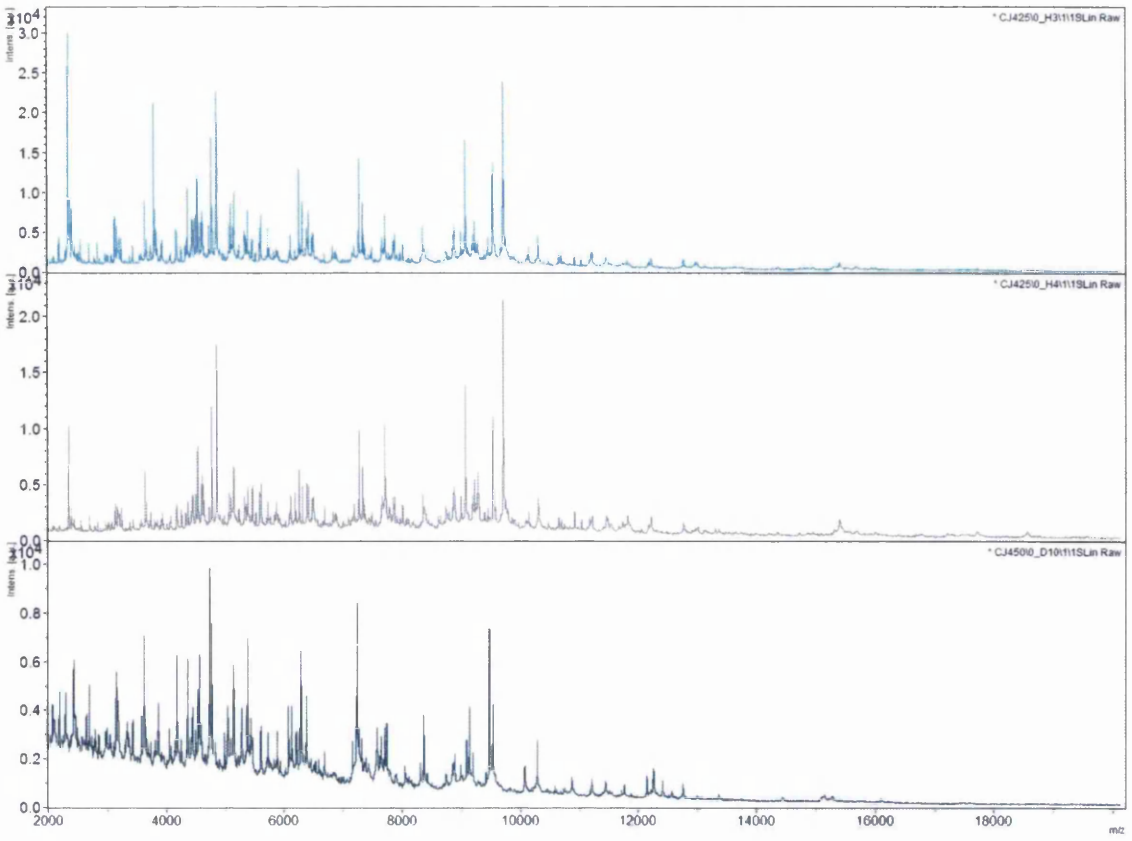



Figure 7.2 Mass spectra of three replicates of a *K. pneumoniae* isolate.

#### 7.2.1.1 The use of the smear method versus extraction method in the preparation of samples

Two approaches were utilised in the preparation of samples for MALDI-TOF/MS. The direct smear method and the extraction method were both used on 4 isolates of *E. coli* and *Klebsiella* spp. and 6 replicates of each were carried out. All had a score value of >2.0 and 42/48 (88%) had a score value of ≥2.3.

Using the smear method, 19/24 (79%) of *E. coli* isolates were found to have a score value of >2.3. 5/24 (21%) of isolates had a score value of 2.2. Within the *Klebsiella* spp., 23/24 (96%) isolates had a score value of >2.3.

Using the extraction method, 23/24 (96%) of *E. coli* isolates had a score value of >2.3 and 1/24 (4%) of isolates had a score value of 2.2. Within the *Klebsiella* spp., 24/24 (100%) isolates had a score value of >2.3.

The extraction method was utilised as the score values were generally higher. It was also suggested by the manufacturer that the extraction method generated more detailed spectra.

### 7.2.2 The identification of bacterial isolates using the extraction preparation method for MALDI-TOF/MS

One hundred and sixty isolates were identified using Bruker Daltonics MALDI-Biotyper. The methodology employed is described in Chapter 2 (2.4.2). Unfortunately, four of the original 164 isolates could not be revived for testing at this point.

As outlined in table 7.1, the MALDI Biotyper identified 114 *E. coli* (71.25%), 34 *Klebsiella pneumoniae* (21.25%), 4 *Klebsiella oxytoca* (2.5%), 4 *Enterobacter cloacae* (2.5%), 1 *Enterobacter aerogenes* (0.625%), 1 *Citrobacter freundii* (0.625%), *Citrobacter koseri* (0.625%) and 1 *Morganella morganii* (0.625%).

97% (111/114) of all *E. coli* isolates were identified with a score value of greater than 2.3. 3% (3/114) had a score of less than 2.3. However, those isolates with a score value of less 2.3 all had a score value of >2.2, which still suggests that the correct identification had been achieved as a secure genus and probable species identification.

All *Klebsiella* spp. were identified with a score value of greater than 2.3. 89% (8/9) of all other *Enterobacteriaceae* were identified with a score value of greater than 2.3 and only one isolate (*E. cloacae*) had a score value of less than 2.3 with a value of 2.1.

Using the MALDI Biotyper 2.0 software, a dendrogram of all samples identified was generated. This allowed for *Enterobacteriaceae* to be clustered according to species. The MALDI Biotyper 2.0 software correctly grouped isolates of the same species together (Figure 7.3).

Table 7.1 Bacterial identification using Bruker MALDI Biotyper

| | Score Value | |
|---|---|---|
| **Identification with MALDI Biotyper** | **<2.3** | **>2.3** |
| *E. coli* (n=114) | 3 | 111 |
| *Klebsiella pneumoniae* (n=34) | | 34 |
| *Klebsiella oxytoca* (n=4) | | 4 |
| *Enterobacter cloacae* (n=4) | 1 | 3 |
| *Enterobacter aerogenes* (n=1) | | 1 |
| *Citrobacter freundii* (n=1) | | 1 |
| *Citrobacter koseri* (n=1) | | 1 |
| *Morganella morganii* (n=1) | | 1 |

Table 7.2 Score Value groupings and interpretations using Bruker MALDI Biotyper software for the identification of organisms

| Range | **Description** |
|---|---|
| 2.300 - 3.000 | Highly probable species identification |
| 2.000 - 2.299 | Secure genus identification, probable species identification |
| 1.700 - 1.999 | Probable genus identification |
| 0.000 - 1.699 | No reliable identification |

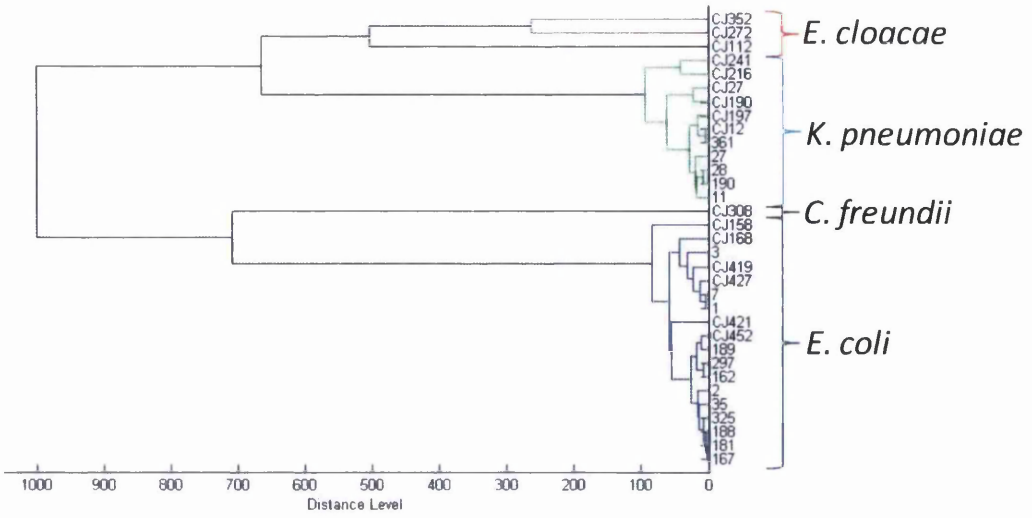



Figure 7.3 Species dendrogram produced by Bruker MALDI Biotyper. Selections of isolates of different species were analysed with the MALDI BioTyper software in order to create a dendrogram. Isolates of the same species were grouped together.

### 7.3 Concordance of MALDI-TOF identification with BD Phoenix

The bacterial identifications provided by the Bruker MALDI Biotyper were compared to the bacterial identifications achieved via the use of the BD Phoenix (data outlined in Chapter 4). Between the two methods, there is a 97% agreement between bacterial identification.

Discrepancies occurred between the two instruments as the BD Phoenix identification of *K. pneumoniae* ssp. *ozaenae* (n=4) differed to the Bruker MALDI Biotyper. The Bruker MALDI Biotyper identified these isolates as just being *K. pneumoniae* with no further sub-speciation. Another discrepancy occurred with an *Enterobacter* spp. The BD Phoenix defined this isolate as *E. cloacae* whereas the Bruker MALDI Biotyper identified it as *E. aerogenes*.

## 7.4 Discussion

Preliminary experiments were set up using a selection of 8 isolates, 4 *E. coli* and 4 *Klebsiella* spp. (6 replicate tests of each), to determine which preparation method (smear versus extract) was most suitable using the Bruker Daltonics Microflex LT Benchtop MALDI Mass Spectrometer. Using the smear method 19/24 (79%) of *E. coli* and 23/24 (96%) of *Klebsiella* spp. had a score value of ≥2.3. However, upon using the extract method, 23/24 (96%) *E. coli* isolates and 24/24 (100%) of *Klebsiella* spp. had a score value of ≥2.3. The smear method was very simple and easy and had a very quick preparation time. Minimal consumables were used meaning it had very low consumable costs. The extraction method took slightly longer to prepare the samples as there are 2 centrifugation steps and 2 incubation steps (waiting for pellet to dry and for cell lysis after the addition of formic acid). Additionally, more reagents were used in this method suggesting it may be slightly more expensive not only due to the longer hands on time but also for the used consumables. However, it is thought that more detailed spectra are achieved through the extract method which could in turn lead to more accurate and reliable identifications (Bohme et al., 2010).

In this study, 160 *Enterobacteriaceae* isolates were identified by MALDI-TOF/MS. These isolates had previously been identified using the BD Phoenix Automated System. Overall, the MALDI Biotyper generated a score value reading of 2.3 for 98% of all isolates tested. The remaining 2% of isolates had score values of ≥2.0. A score value of 2.0 is recommended by the manufacturers as cut-off for probable species identification and this suggests that the MALDI Biotyper is a reliable tool for genus and species level identification within *Enterobacteriaceae* tested in this study.

A concordance of 97% was found between the use of the Bruker MALDI Biotyper and the BD Phoenix Automated System as 5 discrepant results were obtained. Discrepancies in identification have been found in other studies. In a study by Seng et al., (2009) a large number of samples were identified (n=1660) and 95.4% were found to be correctly identified. A number of isolates could not be correctly identified due to limitations in the database. This was also found by Besséde et al., (2010) who achieved 98% identification and suggested that the database should be

expanded to include a greater range of organisms. A study by van Veen et al., (2010) demonstrated correct identification for 97.8% of all bacteria and yeast tested (n=980) and their results show that 96.6% of *Enterobacteriaceae* were correctly identified to species level using MALDI Biotyper whereas 100% were correctly identified to genus level. 81.8% of non-fermentative Gram-negative bacteria were identified to genus level and of which, 74.5% were also identified to species level.

An explanation for the discrepancies found within the identification of *Klebsiella* spp. could be attributed to the *Klebsiella* spp. classification systems. As outlined in Chapter 1 (section 1.1.2), there are 3 main classification systems for *Klebsiella* spp. The Ørskov system (used in the USA and parts of Europe) identifies *K. ozaenae* to be a subspecies of *K. pneumoniae* whereas the Cowan (used in the UK) and Bascomb systems list them as individual species (Podschun and Ullmann, 1998). However despite database issues, the MALDI Biotyper proved to be a much more rapid identification tool when compared to the BD Phoenix Automated System as bacteria on a 96 well plate could be identified in approximately 90 minutes-2 hours.

Aside from bacterial identification, MALDI-TOF/MS has been shown to be a rapid alternative to current typing methods e.g. PFGE, spa typing, MLST as well as other biochemical typing methods (Barbuddhe et al., 2008, Dieckmann et al., 2008, Harris et al., 2010, Wolters et al., 2010). In 2000, Edwards-Jones et al., demonstrated that some MRSA strains could be discriminated from MSSA strains (Edwards-Jones et al., 2000) and this has been developed further so that five major MRSA clonal lineages can now be discriminated using MALDI-TOF/MS (Wolters et al., 2010). Dubois et al., (2009) and Harris et al., (2010) also demonstrated this principle for *S. epidermidis*. Dubois et al., (2009) showed that clinical *S. epidermidis* could be discriminated from environmental *S. epidermidis* whereas Harris et al., (2010) found that isogenic mutants of *S. epidermidis* could be paired to their respective parent strain using the Bruker Daltonics MALDI Biotyper 2.0 software.

The use of MALDI-TOF/MS as a typing tool has been applied to other bacterial species besides staphylococci. For instance, Dieckmann *et al.*, (2008) found that *Salmonella* spp. could be rapidly typed using MALDI-TOF/MS. Usually, isolates of

*Salmonella* spp. have to undertake long incubation periods through biochemical typing tests and so the result is often delayed as well as being limited by subjectivity and specificity. However, the MALDI-TOF/MS approach was found to resolve these issues and provide results comparable to DNA-sequence methods (Dieckmann et al., 2008). Another example is outlined by the subtyping of *Listeria* spp. Clonal lineages were discriminated due to spectral peak differences therefore allowing various serotypes to be identified (Barbuddhe et al., 2008). In addition, MALDI-TOF/MS has been able to discriminate strains of *E. coli* O157: H7 due to differences in mass spectra peaks (Parisi et al., 2008).

Clonal dissemination of an *E. coli* clone, O25b-ST131 has been reported internationally (Clermont et al., 2009, Lau et al., 2009, Lau et al., 2008a, Pitout et al., 2009a, Rogers et al., 2011, Johnson et al., 2010a, Peirano and Pitout, 2010). The use of MALDI-TOF/MS in the rapid discrimination of this clone could be potentially adopted. A current literature search yielded one result whereby MALDI-TOF/MS had been used to identify clonal relatedness. Using MALDI Biotyper software, 2 ST131 positive *E. coli* isolates originating from poultry (n=1) and calf (n=1) were found to be related and it was found that ST131 positive *E. coli* isolates obtained from humans were unrelated (Kmet et al., 2011). The development of such a method would allow epidemiologically relevant and related strains to be identified rapidly (within 2 hours) and in doing so improvements could be made with regards to clinical decisions and outbreak management to prevent further clonal dissemination (Harris et al., 2010, Wolters et al., 2010).

The use of MALDI-TOF/MS could one day replace current time consuming phenotypic testing (Carbonnelle et al., 2011) as it has been demonstrated to be a robust and successful identification tool within this study and others. The potential for MALDI-TOF/MS to be implemented as a typing tool appears promising. It has been suggested that further studies using more clonally related strains, along with an assessment of laboratory reproducibility should be carried out (Wolters et al., 2010). However current literature suggests that it has good discriminatory power akin to current typing methods, which could potentially be developed for the identification of clonal lineages in other species.

# Chapter 8: Biofilm formation in *Escherichia coli* isolates

## 8.1 Introduction

Costerton *et al.*, (1999) defines a bacteria biofilm as "*a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface*".

Biofilms are formed over five different stages (Van Houdt and Michiels, 2005) (Figure 8.1).

1. Attachment of planktonic bacteria (reversible)
2. Irreversible attachment by the production of extracellular polymers or adhesins.
3. Development of biofilm structure by formation of microcolonies.
4. Development of mature biofilm with water channels and pores from microcolonies. Extracellular polymeric matrix is produced for adhesion and to trap nutrients for continuing growth.
5. Dispersion of bacteria from the biofilm into the environment (return to planktonic form).

There are many surface determinants associated with *E. coli* biofilm formation and some examples are outlined below.

The flagella play a role in the motility of bacteria (Cooke, 1974). There are four main rationales linking flagella to biofilm formation. Firstly, it is thought that flagella play a role in the attachment of the cell to a surface (Pratt and Kolter, 1998). Flagella are believed to facilitate the movement of the bacteria to the surface and to also play a role in the motility of bacteria along the surface in order to allow for growth and spread during the developing stages (Pratt and Kolter, 1998). Lastly, flagella are associated with chemotaxis whereby bacteria move towards nutrients that are situated at the biofilm surface (Pratt and Kolter, 1998). However, experiments associated with flagella mutants have been carried out by Pratt and Kolter, (1998). It was found that bacteria lacking flagella or with flagella with insufficient motility

were defective in forming a biofilm as the initial stages of biofilm formation were hindered. Interestingly, it was also found that non-chemotactic bacteria still formed a biofilm (Pratt and Kolter, 1998).

Type I pili, also known as fimbriae, are associated with host tissue adhesion in pathogenic bacteria strains (Finlay and Falkow, 1997). In *E. coli*, the *fim* gene operon is associated with encoding type I pili. Within the *fim* operon, *fimA* encodes for the major fimbrin subunit, *fimH* encodes the mannose specific adhesin and *fimC* and *fimD* are associated with assembly (Fernandez and Berenguer, 2000). Mutations in these *fim* genes are associated with isolates whereby pili do not attach to surfaces (Pratt and Kolter, 1998). More specifically, it has been shown that mutations in *fimH* alter the structure of pili, which therefore leads to the interference of normal attachment (Pratt and Kolter, 1998). Type 3 fimbriae have also been shown to be involved with biofilm formation in uropathogenic *E. coli* (Ong et al., 2008).

Antigen 43 is an outer membrane protein found in *E. coli* (Owen et al., 1987), which is important for cell to cell and cell to surface attachment. It is encoded by the *agn43* gene and mutations of this gene can affect the autoaggregation of *E. coli* in liquid media. Cells deficient in antigen 43 were found to be defective at forming mature biofilms. However, these mutants are capable of forming closely packed cell clusters (Danese et al., 2000a).

Curli are important for surface attachment and colonisation (Vidal et al., 1998). The *csgA* gene is associated with encoding curli subunits. It has been demonstrated that no biofilm formation occurs in bacteria whereby a *csgA* null mutation has occurred. Curli formation is regulated by the OmpR protein. Biofilm formation is hindered in bacteria with an *ompR* null mutation, as adhesion is suppressed (Vidal et al., 1998).

Exopolysaccharide production e.g. colanic acid or M antigen was found to play a role in giving the biofilm its protective outer capsule. Colanic acid was not found to play a role in initial attachment but it is associated with the formation of the three-dimensional structure (Danese et al., 2000b, Prigent-Combaret et al., 2000). Another form of exopolysaccharide is poly-β-1, 6-N-acetyl-D-glucosamine (PGA), which is involved with the attachment of cells to abiotic surfaces, intercellular adhesion and biofilm formation (Wang et al., 2004).

Biofilms are a part of bacterial survival (Hall-Stoodley and Stoodley, 2009) and are often difficult to eradicate (Lewis, 2001). In medicine biofilms can complicate infections which are chronic or difficult to treat for example prostatitis (Kanamaru et al., 2006) and urinary catheter cystitis caused by *E. coli* (Costerton et al., 1999, Jackson et al., 2002). Biofilms have a protective phenotype (Costerton et al., 1999). Antimicrobial agents often fail to penetrate the complex biofilm structure (Jefferson et al., 2005) and also, due to nutrient limitations some bacteria exist in a slow-growing state (Brown et al., 1988).

As well as aiding to medical problems, biofilms are also present in nature and are able to cause environmental problems (Costerton et al., 1999).

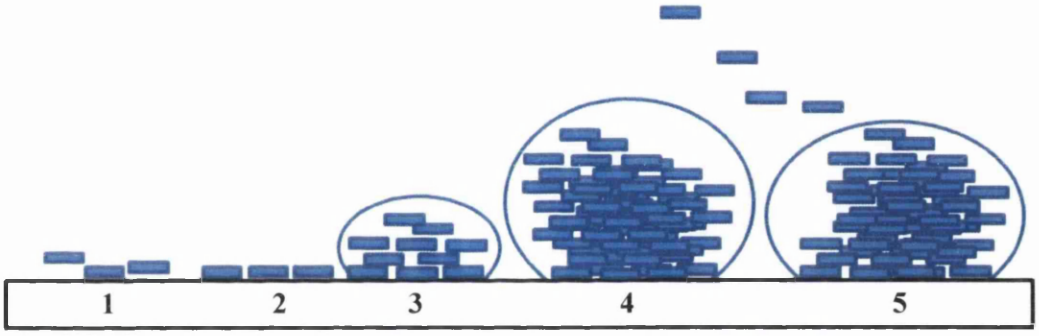



Figure 8.1 The development of a bacterial biofilm encompassing the 5 stages of formation. Adapted from (Van Houdt and Michiels, 2005).

### 8.1.1 Significance of the *pgaABCD* gene locus

Wang et al., (2004) identified a gene locus in *E. coli* known as *pgaABCD*. This locus, which can be horizontally transferred, was found to be associated with the promotion of surface binding, intercellular adhesion through the production of poly-β-1, 6-N-acetyl-D-glucosamine (PGA) (known as polysaccharide intercellular adhesin (PIA) in *Staphylococcus epidermidis* (Mack et al., 1996)) and biofilm formation.

The transcription of *pgaABCD* is activated by NhaR (DNA binding protein) belonging to the LysR family of transcriptional regulators (Goller et al., 2006). *pgaABCD* was found to have homology to the *hmsHFRS* locus found in *Y. pestis*

(Hare and McDonough, 1999) and the *icaADBC* gene locus found in *S. epidermidis* (Heilmann et al., 2006). Each gene within the locus has a specific function (Wang et al., 2004).

PgaC is a glycosyltransferase and is homologous to IcaA found in *S. epidermidis*.

PgaB is a lipoprotein with homology to IcaB from *S. epidermidis*.

PgaA is an outer membrane protein associated with the translocation and docking of PGA to the cell surface. It has no homologue in staphylococci.

PgaD is an inner membrane protein which is a potential functional (not sequence) homologue of IcaD of *S. epidermidis*.

Each gene is involved with the production and function of PGA. *pgaC* and *pgaD* are involved in PGA synthesis whereas *pgaA* and *pgaB* have a function in the export of PGA (Itoh et al., 2008).

### 8.1.2 Biofilms and ESBLs

Bacterial conjugation is a mechanism by which genetic information can spread. Gene transfer occurs frequently in biofilms as their complex network of communities is ideally suited to the role (Hausner and Wuertz, 1999). However owing to the connection of conjugation and biofilm, medically relevant plasmid-bearing strains could potentially form biofilms thus increasing the chance of virulence spread and biofilm related infection. Conjugation also plays a role in the horizontal transfer of antibiotic resistance and virulence factors. It has been suggested that a consequence of using antimicrobial agents in medicine and the environment may have been a selection of plasmid bearing-strains capable of biofilm formation (Ghigo, 2001).

With this information in mind, it was interesting to investigate whether ESBL-producing *E. coli* isolates in this study had a greater likelihood of forming biofilms.

The aim of this chapter was to identify the prevalence of *pgaABCD* locus in 3 different *E. coli* populations, to establish biofilm production phenotypically and to identify any correlation between biofilm formation and ESBL production.

## 8.2 Results

### 8.2.1 Screening ESBL and AmpC-producing *E. coli* isolates for *pgaABCD*

Using primers specific for amplifying *pgaA* and *pgaC* of the *pgaABCD* gene locus (Cerca et al., 2007), 105 (100%) ESBL and AmpC-producing *E. coli* (largely belonging to phylogenetic subgroup B2), were found to give positive amplification products (Table 8.1, Figure 8.2). As a control, *E. coli* strain A and *E. coli* K12 were used and both possessed the target genes. Additionally, several isolates of *Klebsiella* spp. were screened. No amplification products were generated. Therefore, in positive strains, both targets were consistently found.

#### 8.2.1.1 Screening control *E. coli* isolates for *pgaABCD*

Forty cefpodoxime sensitive *E. coli* isolates obtained from urine specimens were screened for the *pgaABCD* locus to serve as a control population. 33/40 (83%) were found to be positive (Table 8.1).

#### 8.2.1.2 Screening bovine *E. coli* isolates for *pgaABCD*

As *E. coli* isolates in this thesis were found to be largely related to one clonal type, characterised bovine *E. coli* isolates were used to act as a non-human control population. Therefore, 124 *E. coli* isolates obtained from bovine endometrium were also used in this experiment. Of these 124 bovine *E. coli* isolates, 114 had previously been characterised by Triplex PCR into phylogenetic groups; 37/114 (32%) were group A, 51/114 (45%) were group B1, 3/114 (3%) were group B2 and 23/114 (20%) were group D. MLST revealed that many of these isolates belonged to 4 distinct clonal clusters (Sheldon et al., 2010).

Using the *pgaABCD* PCR, 114/124 (92%) were found to be positive for *pgaA* and *pgaC* whereas 10/124 (8%) were negative (Table 8.1). These isolates had additionally been screened for ESBL genes by multiplex PCR as described in chapter 3; however no positive amplification was generated.

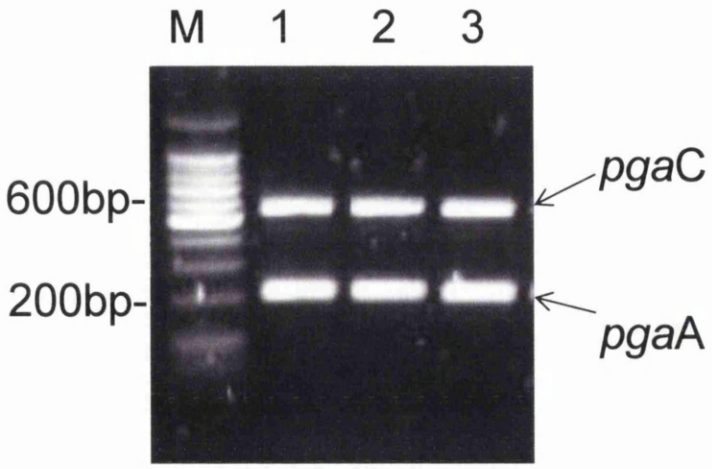



Figure 8.2 Amplification of *pgaA* and *pgaC*. M, 100bp molecular ladder; Lanes 1-3 *E. coli* isolates positive for the gene locus.

Table 8.1 Prevalence of *pgaABCD* locus amongst *E. coli* isolates from various sources

| | **Human *E. coli* ESBL and AmpC (n=105)** | | **Human *E. coli* control (n=40)** | | **Bovine *E. coli* (n=124)** | |
|---|---|---|---|---|---|---|
| | + | - | + | - | + | - |
| *pgaABCD* | 105 (100%) | 0 (0%) | 33 (83%) | 7 (17%) | 114 (92 %) | 10 (8%) |

### 8.3 Detection of biofilms in *E. coli* isolates using a biofilm assay

Although the prevalence of the *pgaABCD* locus was established, isolates were phenotypically screened for the capability to produce a biofilm using a semi-quantitative microtitre plate biofilm assay (Chapter 2) for *Staphylococcus* spp. (Mack et al., 1996, Christensen et al., 1985). *S. epidermidis* 1457 grown in TSB media was used as a positive control.

#### 8.3.1 Method optimisation

The standard protocol did not yield a significant level of biofilm formation of *E. coli* when compared to the absorbance values generated by *S. epidermidis* so a number of variables including medium, time of incubation, etc. were varied in order to optimise the experiment for *E. coli* isolates. All optimisation experiments were carried out on *E. coli* K12; *E. coli* isolates 35 and 231 (CTX-M-15 *IS*26 positive and negative, respectively) and *S. epidermidis* 1457. As a negative control and to establish background absorbance values for biofilm positive isolates, wells were also incubated with medium without the addition of bacteria. This generally yielded background absorbance values of approximately 0.048-0.056.

##### 8.3.1.1 Time

Using LB media for *E. coli*, the time of incubation of the 96-well plate at 37°C was varied (24 hours, 48 hours and 72 hours) to determine any effect on biofilm formation.

It is clear to see that an incubation time of 24 hours yields an increase in biofilm formation when compared to 48 hours and 72 hours (Figure 8.3) as absorbance value for *E. coli* K12 was approximately 0.13 ± 0.008. The ability to form biofilm appears to decrease using longer times. However, using the student t-test, no statistically significant difference was observed between 48 and 72 hours ($p=0.6622$)

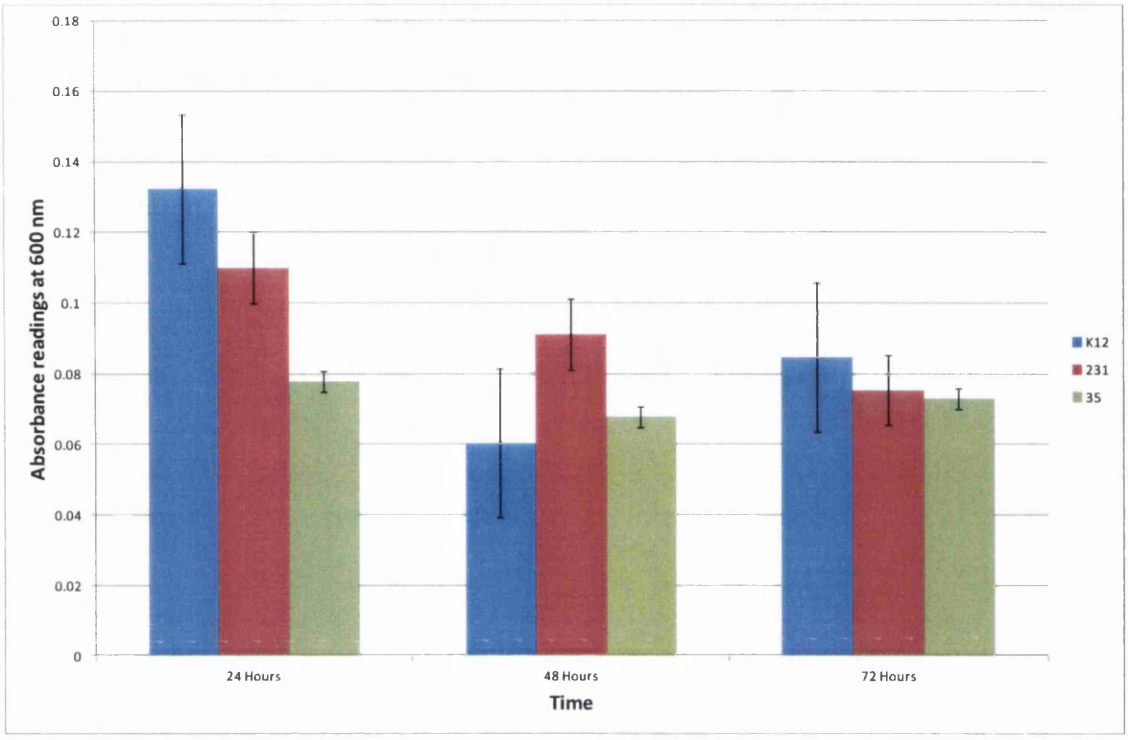



Figure 8.3 Effect of time (24, 48 and 72 hours respectively) on biofilm formation using LB medium and *E. coli* K12; *E. coli* isolates 35 and 231 (CTX-M-15 *IS*26 positive and negative respectively).

### 8.3.1.2 Temperature

Using LB media for *E. coli*, the incubation temperature was lowered to 19°C (using a 19°C incubator to maintain a constant temperature) and 30°C for 24 hours was altered to determine any effect on biofilm production. From the data for *S. epidermidis*, it can be determined that by using a higher temperature of 30°C, absorbance values of approximately 0.55 ±0.018 were generated whereas with a temperature of 19°C, an absorbance value of less than 0.1 ±0.0087 was achieved. Absorbance values were much lower for the *E. coli* K12 and the clinical *E. coli* isolates (less than 0.1) using both temperatures (Figure 8.4).

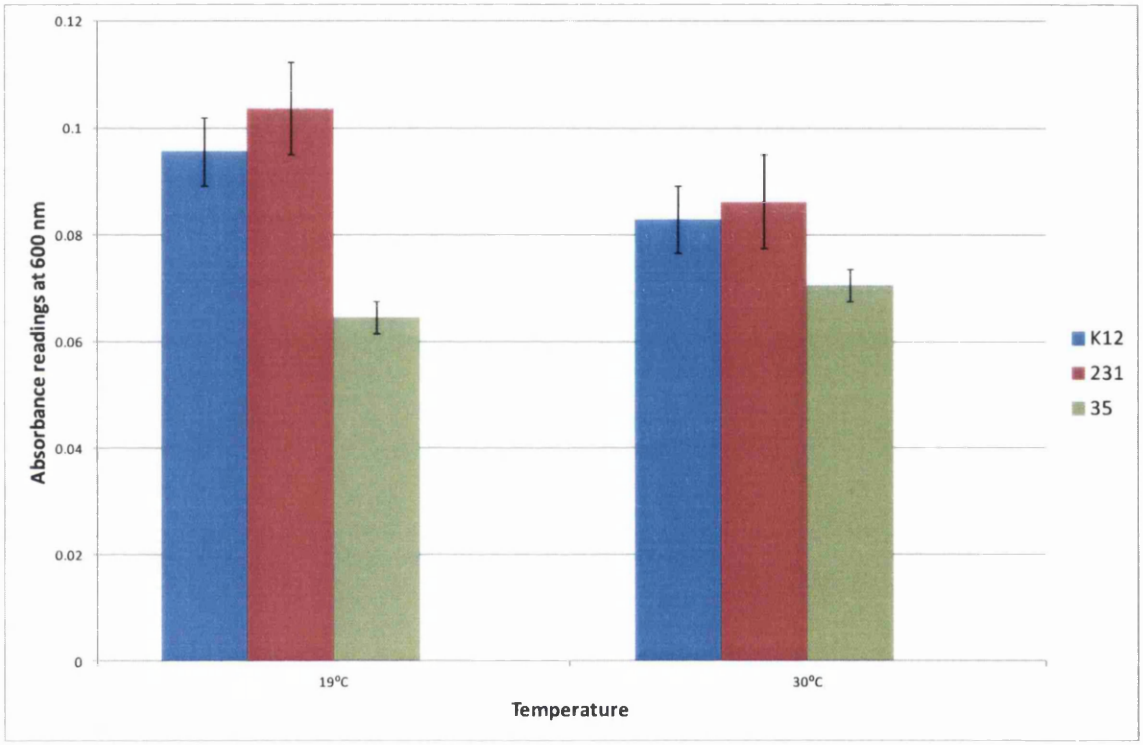



Figure 8.4 Effect of temperature (19°C and 30°C) on biofilm formation using *E. coli* K12; *E. coli* isolates 35 and 231 (CTX-M-15 *IS*26 positive and negative respectively) and *S epidermidis* 1457.

### 8.3.1.3 Media

As all the previous experiments were carried out in LB broth, another variable to consider was to supplement the media and also to test how well biofilms form in different media. Supplementing media with glucose, ethanol and NaCl has been shown to increase PGA production (Cerca and Jefferson, 2008) therefore a series of experiments were set up using LB supplemented with 1% glucose, 2% glucose, 1% sucrose, 2% sucrose, 1% glucose and sucrose, 4% NaCl and 4% ethanol. Also, TSB, BHI (brain heart infusion) media and EC broth were used as alternative media to aid in the determination of which media best supported biofilm production.

From the experiment carried out with LB, it was clearly seen that 2% sucrose had the greatest positive effect as the absorbance value for *E. coli* K12 is approximately 0.2 ±0.39. The use of 4% ethanol and 4% NaCl appeared to have a negative effect on biofilm formation as absorbance values are below 0.1 for *E. coli* K12 (±0.009 and ±0.008, respectively). However, the most significant increase in biofilm formation could be seen with the use of EC broth. Absorbance values of approximately 0.275 ±0.08 were obtained with *E. coli* K12 whereas for the clinical isolates, values exceeded 0.15 (±0.01, ±0.05 and ±0.007, respectively).

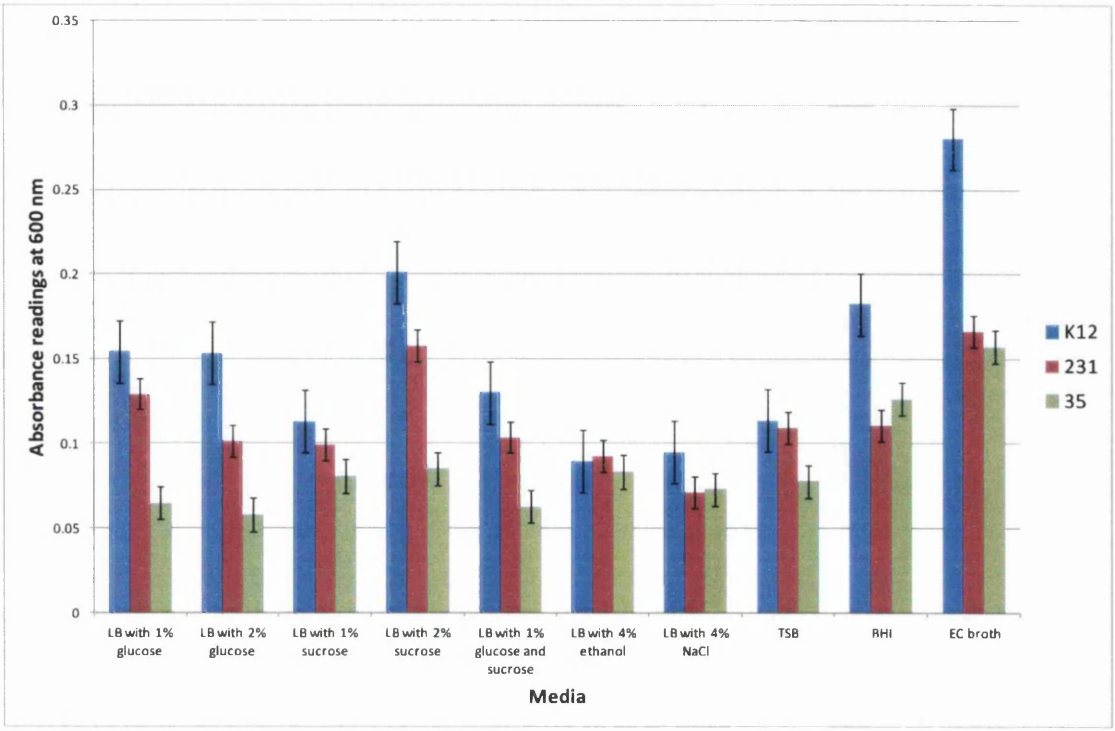



Figure 8.5 Effects of different media and media supplements including varying concentrations of glucose, sucrose, ethanol and NaCl on biofilm formation using *E. coli* K12; *E. coli* isolates 35 and 231 (CTX-M-15 *IS*26 positive and negative respectively).

#### 8.3.1.3.1 EC broth

As EC broth stimulated significantly higher absorbance readings for the clinical *E. coli* isolates which had not been achieved with any other media, further work was carried out to elucidate optimal conditions. EC broth was supplemented with 1% glucose, 2% glucose, and 1% glucose and 1% sucrose to elucidate any affect on biofilm formation. It is clear that supplementing the media had a positive effect on biofilm formation (Figure 8.6). In the case of *E. coli* K12, a significant difference was found with the addition of 1% glucose, 2% glucose, and 1% glucose and 1% sucrose as it was clear to see the absorbance values increase from approximately 0.3 ±0.08 with EC broth alone to 1.625 ±0.5 with EC plus 1% glucose and 1% sucrose. However, when statistical analysis was applied using student t test, no significant difference was found on biofilm formation when using 2% glucose or 1% glucose and 1% sucrose for the clinical *E. coli* isolates.

#### 8.3.1.3.2 EC broth without bile salts

The ingredients of EC broth list bile salts no. 3 as a component. However, the literature suggests that bile salts are associated with biofilm formation in *Bacteroides fragilis* (Pumbwe et al., 2007). Therefore a second batch of EC broth was made without bile salts to see if there was any effect on biofilm formation with *E. coli* isolates from this collection. Clearly absorbance values were lower when bile salts were removed. For instance, values for *E. coli* K12 decreased from approximately 0.275 ±0.08 to below 0.1 ±0.003 when comparing EC broth with bile salts and EC broth without bile salts (Figure 8.6).

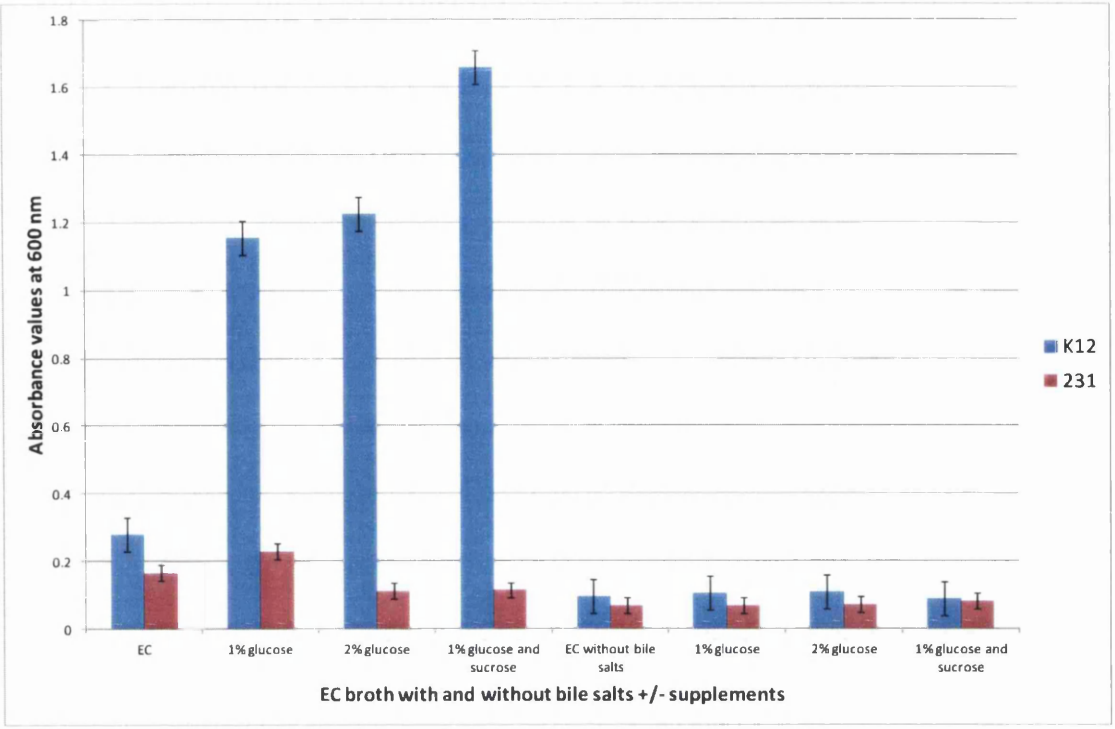



Figure 8.6 Effect of EC broths with and without bile salts on biofilm formation. EC broths with and without bile salts were also supplemented with glucose and sucrose. *E. coli* K12 and *E. coli* isolate 231 (CTX-M-15 *IS*26 negative) was used.

### 8.3.2 Prevalence of phenotypic biofilm production in human and bovine isolates

Using the optimised phenotypic biofilm assay consisting of EC broth (with and without 1% glucose) along with an incubation temperature of 24 hours at 37°C, 105 ESBL and AmpC-producing *E. coli* were screened. Of these, 38% (40/105) were found to produce biofilm with an absorbance reading of >0.1. 62% (65/105) were found have an absorbance reading of <0.1 and were deemed biofilm negative (Table 8.2). Notably, epidemic *E. coli* strain A is a successful biofilm producer with an absorbance value of 1.1.

From the non-ESBL producing *E. coli* control collection (n=40), 44% (15/40) were found to be positive for a phenotypic biofilm with an absorbance reading of >0.1 whereas 56% (19/40) were negative.

Analysis of the bovine *E. coli* isolates show that 53% (66/124) were phenotypic biofilm producers whereas 47% (58/124) were not.

Figure 8.7 outlines a box plot of all biofilm positive isolates (absorbance values >0.1) and biofilm negative isolates (absorbance vales <0.1) within the three *E. coli* populations. It is interesting to note that the ESBL and AmpC-producing *E. coli* population has a greater range of absorbance values (approximately 0.1-0.75) than the non-ESBL *E. coli* human and bovine populations which demonstrated ranges of approximately 0.1-0.65 and 0.1-0.325, respectively. However, the box size of the ESBL and AmpC-producing *E. coli* population demonstrated that the majority of biofilm positive isolates had absorbance values within that range whereas the box sizes were larger for the other two populations.

Table 8.2 Phenotypic biofilm production within *E. coli* isolates.

| | **Human *E. coli* ESBL and AmpC (n=105)** | | **Human *E. coli* control (n=40)** | | **Bovine *E. coli* (n=124)** | |
|---|---|---|---|---|---|---|
| | + | - | + | - | + | - |
| Biofilm (Abs >0.1) | 40 (38%) | 65 (62%) | 15 (44%) | 19 (56%) | 66 (53%) | 58 (47%) |

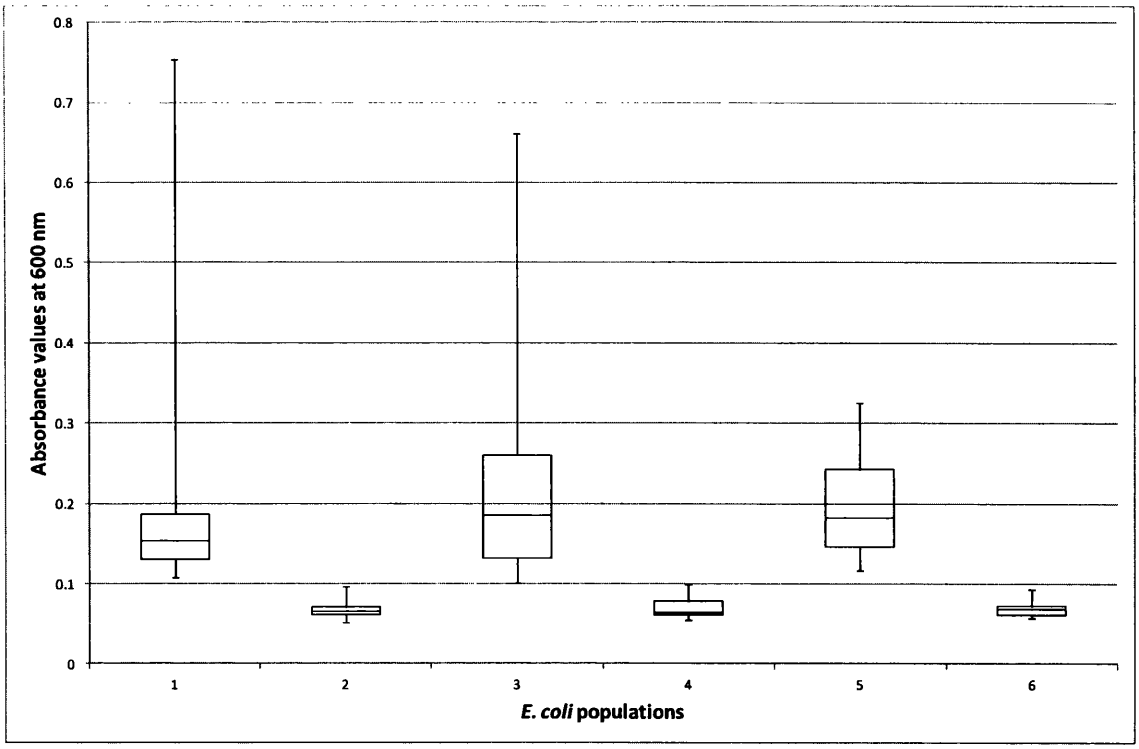



Figure 8.7 A box plot representation of biofilm positive isolates (absorbance values >0.1) and biofilm negative isolates (absorbance values <0.1) for 3 different *E. coli* populations. 1 represents the ESBL and AmpC biofilm positive *E. coli* isolates, 2 represents the ESBL and AmpC biofilm negative *E. coli* isolates, 3 represents the biofilm positive bovine *E. coli* isolates, 4 represents the biofilm negative *E. coli* bovine isolates, 5 represents the non-ESBL control biofilm positive *E. coli* isolates and 6 represents the non-ESBL control biofilm negative *E. coli* isolates.

## 8.4 Discussion

Biofilms are complex microbial communities that form to enhance survival under hostile conditions (Wang et al., 2004). Infections associated with biofilms are often complicated as they are not easily eradicated (Lewis, 2001). In this study, *E. coli* isolates were screened for the *pgaABCD* gene locus and assessed for phenotypic biofilm formation.

In ESBL and AmpC producing isolates (n=105), all *E. coli* isolates were found to possess the *pgaABCD* gene locus. However, in the non-ESBL producing control *E. coli* isolates and the uterine bovine *E. coli* isolates not all isolates gave positive amplification products (7/40 and 10/124, respectively) and so these negative isolates were reconfirmed as being *E. coli* using API 20E strips. In a study conducted by Cerca et al., (2007), 30 clinical *E. coli* isolates obtained from urinary tract and neonatal blood stream infections were screened for the *pgaABCD* locus. Of which 26 out of 30 possessed *pgaABCD*. The remaining 4 were negative and were found not to produce PGA (Cerca et al., 2007). In this thesis, isolates that did not amplify *pgaA* and *pgaC* did not form a biofilm under the optimal conditions of EC broth supplemented with and without 1% glucose.

To elucidate whether the *E. coli* isolates produced a biofilm, a phenotypic biofilm assay was carried out. However, in order to increase the absorbance values obtained so that the identification of a positive biofilm could be achieved, a number of variables were altered to identify optimal in vitro conditions. An initial experiment involved increasing the time of incubation at 37°C from 24 hours to 48 hours and 72 hours. In doing so, it can be clearly seen that biofilm formation decreases with time. A suggestion for this is that the biofilm is in its maturation stage whereby bacterial cells are dispersing and planktonic status is returned. The next variable to be altered was incubation temperature. Lower temperatures of 19°C, 30°C were used and were found to not support biofilm formation and thus generate absorbance values as high as those found when using 37°C. This agrees with the fact that 37°C is the optimum temperature for bacterial growth and biofilm formation. So once establishing the optimum time and temperature for biofilm formation, different media types were sought and tested for their capacity to support biofilm formation in *E. coli*. All the previous work had been carried out using LB broth (Sambrook et al., 1989) and so

the LB media was supplemented with glucose, sucrose, ethanol and NaCl. Cerca et al., (2008) had established that PGA production significantly increased in the presence of glucose whereas there was a slight increase with the addition of ethanol and NaCl. Statistical analysis was used for *E. coli* K12 (as this generally produced higher absorbance readings when compared to clinical isolates tested) to determine significant differences using different media supplemens. Using the student t-test no significant difference was found in this study when using LB supplemented with 1% glucose ($p=0.2247$) or 2% glucose ($p=0.2253$). A negative impact was found with the addition of ethanol and NaCl. The addition of 4% ethanol or 4% NaCl had a statistically significantly ($p=0.0134$ and $p=0.0188$, respectively) affect, as biofilm formation decreased.

Alongside LB; TSB, BHI and EC broth were also utilised. EC broth was found to generate absorbance values which were consistently higher and a significant difference was found when compared to LB ($p=0.0418$). It was therefore chosen as the optimal media to support biofilm formation. In parallel, the addition of 1% glucose, 2% glucose and 1% glucose and sucrose supplemented the EC broth based upon the findings that glucose increased PGA production (Cerca and Jefferson, 2008). It was found that the use of 1% glucose had a significant difference ($p=0.0041$) on biofilm formation when supplementing EC broth.

The contents of EC broth were investigated and the addition of bile salts no. 3 was observed. Bile salts, particularly bile salt stress had been associated with the enhanced adhesion in enteropathogenic *E. coli* isolates (de Jesus et al., 2005, Torres et al., 2007). Bile salts have also been implicated in biofilm formation in *Bacteroides fragilis* (Pumbwe et al., 2007). Therefore in order to deduce whether bile salts had any effect on *E. coli* isolates in this study, EC broth was made without bile salts. It was observed that the removal of bile salts greatly reduced biofilm formation. The average absorbance value for EC broth was 0.145, when bile salts were removed this value decreased to 0.07. Thus the removal of bile salts generated a statistically significant difference ($p=0.0205$).

In this study, biofilm formation was found in 38% ($n=40$) of ESBL and AmpC-producing *E. coli*, 44% ($n=15$) of non-ESBL control *E. coli* isolates and 53% ($n=66$)

of uterine bovine *E. coli* isolates. Potential rationales for this could be due to the fact that *E. coli* biofilms are more difficult to achieve *in vitro* or that the conditions in this study were not ideal for doing so in clinical isolates. Another suggestion to consider as to why not all *pgaABCD* positive isolates express a phenotypic biofilm could be related to other factors associated with *E. coli* biofilm formation e.g. surface determinants such as flagella, fimbriae (Pratt and Kolter, 1998), autotransporter proteins e.g. antigen 43 (Danese et al., 2000a), curli and pili (Pratt and Kolter, 1998). In addition, monoculture biofilms are rarely found in the natural environment; therefore, biofilm production and development may depend on interactions from other organisms (Blaschek et al., 2007).

With regards to investigating the link between ESBL and biofilm formation, it can be noted that all ESBL *E. coli* isolates possessed the *pgaABCD* gene locus but not all produced a phenotypic biofilm. Clermont *et al.*, (2008) reports of a CTX-M-15 clone prevalent in Europe and Africa having biofilm formation ability but stipulates that the CTX-M plasmid does not support biofilm formation. However, biofilm formation could aid in the persistence of clones (Clermont et al., 2008, Ghigo, 2001). Yang *et al.*, (2008) published data indicating that biofilm forming *K. pneumoniae* strains in their collection had an increased likelihood of also possessing ESBL enzymes.

The bovine *E. coli* isolates used in this chapter were additionally screened for ESBL genes. No positive amplification products were generated. However, ESBL-producing organisms have been associated with cattle. A study by Madec *et al.*, (2008) detected CTX-M-1, CTX-M-16, CTX-M-14 and TEM-126 in the faeces of sick cattle. ESBL genes have been found in cattle in the UK. Liebana et al., (2006) reported CTX-M genes associated with cattle at a dairy farm.

In summary, the *pgaABCD* gene locus was found in all ESBL and AmpC-producing *E. coli* but not in all other *E. coli*. In total, 10 isolates were found to be negative. *pgaABCD* negative isolates did not form a phenotypic biofilm. However, biofilm formation was demonstrated in approximately 45% of all *E. coli* isolates using optimum conditions i.e. a 24 hour incubation time at 37°C using EC broth (with and without 1% glucose).

# Chapter 9: Clinical epidemiology of ESBL-producing *Enterobacteriaceae* isolated from the PHW Microbiology ABM Swansea laboratory

## 9.1 Introduction

Located in the gut of humans and animals, *E. coli* is an opportunistic pathogen which is commonly associated with urinary-tract infections, wound infections following surgery, hospital-acquired pneumonia, infections of the gastrointestinal tract (diarrheal disease), neonatal meningitis and septicaemia (Mims, 1998, Nataro and Kaper, 1998). As the name suggests, UTIs are bacterial infections that affect any part of the urinary tract. Urine is the most commonly received specimen in the clinical laboratory with a large number of routine antimicrobial sensitivity testing and results being prepared and issued each day (Barrett et al., 1999). Therefore UTIs are one of the most common infections encountered in clinical practice. It is thought that *E. coli* is the causative bacteria in 70-95% of community-acquired cases and 50% of nosocomial cases (Chomarat, 2000, Kucheria et al., 2005).

UTIs can be divided up into uncomplicated and complicated infections. A complicated UTI is an infection associated with a condition that increases the risk of treatment failure. Factors associated with complicated UTI include: abnormalities of the urinary-tract e.g. renal stone, foreign bodies e.g. catheters, immunosuppression and the presence of multidrug resistant bacteria. An example of complicated UTI is pyelonephritis. Uncomplicated UTI are infections which can be diagnosed and treated more readily e.g. acute cystitis (Hooton, 2000). In the UK, complicated UTIs are often treated with parenteral cephalosporins and aminoglycosides whereas uncomplicated UTIs are treated frequently with trimethoprim or nitrofurantoin (Bean et al., 2008).

UTIs are associated with both the community and hospital setting and in recent years antibiotic resistance has become problematic (Chomarat, 2000). There has been an increase in the occurrence of cephalosporin-resistant *E. coli* which cause UTI and invasive infection. These are also often resistant to fluroquinolones and trimethoprim and therefore the treatment of this type of UTI is limited (Paterson and Bonomo, 2005).

UTIs due to *E. coli* can progress to bacteraemia, which can often be linked to a higher mortality (Lau et al., 2009, Melzer and Petersen, 2007). Data from the HPA demonstrates an increasing number of reports of bacteraemias caused by *Klebsiella* spp., as in 2005; there were 5150 reports of *Klebsiella* spp. bacteraemia whereas in 2009 there were 6160 reports (http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1287142531496).

Cephalosporins and fluoroquinolones are typically recommended in the front-line treatment of both community and hospital-associated bacteraemia (Rodriguez-Bano et al., 2006).

The aim of this chapter is to deduce the clinical epidemiology of ESBL-producing *Enterobacteriaceae*, primarily in isolates obtained from urine samples.

## 9.2 Results

### 9.2.1 Clinical epidemiology

In this section, the clinical epidemiology of isolates was analysed. This included the source of sample, distribution of hospital and community-associated isolates, gender distribution and average age. All isolates were analysed, including those that did not have ESBL or AmpC genotypes.

#### 9.2.1.1 All isolates

71.2% (117/164) of all isolates obtained were *E. coli*. Of which, 69% (81/117) were isolated from patients who had been admitted into hospital. The remaining 31% (36/117) of *E. coli* were isolated from samples that were submitted by a GP (community-associated). Of the 117 *E. coli* isolates, 90% (105/117) were found to possess ESBL/AmpC genes. 69% (72/105) were hospital associated and the remaining 31% (33/105) were community associated.

Within the hospital-associated *E. coli* population, 33% of isolates were isolated from males with an average age of 72.8 years whereas 67% were isolated from females with an average age of 65.6 years. For the ESBL and AmpC *E. coli* isolates, 35% were from males with an average age of 73.1 and 65% were females with an average age of 62.3. Amongst the community-associated *E. coli* isolates, 19% of isolates were obtained from males with an average age of 73.5 years whereas 81% were isolated from females with an average age of 53.9 years. For the ESBL and AmpC *E. coli* isolates, 21% were from males with an average age of 73.3 and 79% were from females with an average age of 53.9 (Table 9.1).

In total, 87% (102/117) *E. coli* were isolated from urine samples and of which, 24% were isolated from males with an average age of 73.5 years. 76% were isolated from females with an average age of 62.8 years. 88% (72/81) of these urinary isolates were obtained from hospital-associated *E. coli* isolates and 42% (34/81) were urine *E. coli* isolated from the community. For the ESBL and AmpC *E. coli* isolates, 88% (90/102) were obtained from urine samples and of which, 26% were from males with an average age of 73.9 years and 74% were from females with an average age of 60.4 years.

Blood culture isolates accounted for 13% (9/117) of all *E. coli* isolates and of these 67% were obtained from males with an average age of 76.2 years and 33% were obtained from females with an average age of 32.7 years. 5% (6/117) of all *E. coli* were obtained from other sources including wounds (3/6, 50%) and faeces (3/6, 50%). 60% (3/5) of these samples were obtained from males with an average age of 61.3 years. 40% (2/5) were from females with an average age of 35.5 years. Of these 'other' samples, 6% (3/4 wound and 1/4 faeces samples) were hospital-associated and 3% (2 faeces samples) were community-associated (Tables 9.2 and 9.3). All of these non-urine related isolates were found to be ESBL/AmpC-producing. For one isolate, no information could be obtained.

After *E. coli*, the next largest strain population in this study were *Klebsiella* spp. accounting for 23% (38/164) of all isolates. 68% (26/38) were hospital-associated. Of which, 38% (10/26) were obtained from males with an average age of 62.2 years whereas 62% (16/26) were from females with an average age of 69.5 years. 32% (12/38) of *Klebsiella* spp. were community-associated and 58% (7/12) of these were from males with an average age of 70.3 years whereas 42% (5/12) were obtained from females with an average age of 84.6 years (Table 9.1). For the ESBL and AmpC *Klebsiella* spp., 69% (22/32) were hospital-associated, of which, 40% (9/22) were obtained from males with an average age of 60.4 years whereas 60% (13/22) were from females with an average age of 76.5 years. 31% (10/32) of *Klebsiella* spp. were community-associated and 60% (6/10) were from males with an average age of 70.5 years and 40% (4/10) were obtained from females with an average age of 88 years (Table 9.1).

As with the *E. coli* population, the majority (33/38 – 87%) of *Klebsiella* ssp. isolates were obtained from urine samples. All 12 (100%) community-associated *Klebsiella* spp. were isolated from urine. 44% (15/33) of *Klebsiella* spp. obtained from urine samples were isolated from males with an average age of 65.7 years and 56% (18/33) were isolated from females with an average age of 73.3 years. For the ESBL-producing *Klebsiella* spp., 88% (28/32) were obtained from urine samples, of which, 85% (18/22) were hospital-associated whereas 100% (10/10) were community associated. 50% (14/28) of *Klebsiella* spp. urine isolates were isolated from males with an average age of 65.5 years whereas the other half (14/28) were

isolated from females with an average age of 80.5 years. Three *Klebsiella* spp. (8%) were isolated from blood cultures taken in hospital. Of which, 67% (2/3) were isolated from males with an average age of 64 years and these were both ESBL-producing. The remaining *Klebsiella* spp. (1/3 – 33%) was isolated from the blood of an 82 year old female. Aside from urine and blood cultures, two *Klebsiella* spp. (5%) were isolated from wound related sources. Both were hospital-associated and isolated from females with an average age of 48.5 years (Tables 9.2 and 9.3).

Nine other *Enterobacteriaceae* i.e. not *E. coli* and *Klebsiella* spp. were isolated. These included 6 *E. cloacae*, 1 *C. freundii*, 1 *C. koseri* and 1 *M. morganii*. 67% (6/9) were hospital-associated and 33% (3/9) were community-associated. Of the hospital-associated isolates, 67% (4/6) were isolated from males with an average age of 74 years and 33% were isolated from females with an average age of 78 years. Of the 3 community-associated isolates, one was isolated from a male aged 56 years and 67% (2/3) were isolated from females with an average age of 28.5 years (Table 9.1). As demonstrated by the *E. coli* and *Klebsiella* spp. populations, the most frequent source for these other isolates were urine samples (56% - 5/9). 60% (3/5) of the urine samples were obtained from males with an average age of 73 and 40% (2/5) were from females with an average age of 67.5 years. One *E. cloacae* isolate (11%) was from a blood culture. This was isolated from a 77 year old male. Three *E. cloacae* (33%) were isolated from wound samples. Of which, 33% (1/3) was isolated from a 56 year old male and 67% (2/3) were isolated from females with an average age of 39 years (Tables 9.1.1 and 9.1.2). 56% (5/9) were found to be ESBL-producing *Enterobacteriaceae*. For these ESBL-producing isolates, 67% (4/6) were found to hospital-associated and of which, 75% were from males with an average age of 77 years and 25% were from females with an average age of 78 years. The remaining 1 isolate was community-associated and isolated from a female with an age of 57 years. In total, 4 isolates were obtained from urine samples and 50% were obtained from males with an average age of 73 years and 50% were from females with an average age of 67.5 years. 1 isolate was obtained from other sources and was isolated from a female with an average age of 78 years.

Table 9.1 Gender and age distribution amongst hospital and community derived isolates

| | | | Age Range | | Average Age (Years) | | |
|---|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** | **Total Average** |
| All Hospital *E. coli* (n=80) | 26 (33%) | 54 (67%) | 34-90 | 3-95 | 72.8 | 65.6 | 68 |
| Hospital ESBL *E. coli* (n=72) | 25 (35%) | 47 (65%) | 34-90 | 3-90 | 73.1 | 62.3 | 66.1 |
| All Community *E. coli* (n=36) | 7 (19%) | 29 (81%) | 71-89 | 3-87 | 73.5 | 53.9 | 57.2 |
| Community ESBL *E. coli* (n=33) | 7 (21%) | 26 (79%) | 71-89 | 3-87 | 73.3 | 53.3 | 57.5 |
| All Hospital *Klebsiella spp.* (n=26) | 10 (38%) | 16 (62%) | 32-78 | 0-99 | 62.2 | 69.5 | 66.7 |
| Hospital ESBL *Klebsiella spp.* (n=22) | 9 (40%) | 13 (60%) | 32-78 | 60-99 | 60.4 | 76.5 | 70.7 |
| All Community *Klebsiella spp.*(n=12) | 7 (58%) | 5 (42%) | 68-76 | 71-99 | 70.3 | 84.6 | 76.3 |
| Community ESBL *Klebsiella spp.* (n=10) | 6 (60%) | 4 (40%) | 68-76 | 79-99 | 70.5 | 88 | 77.5 |
| All Hospital Other *Enterobacteriaceae* (n=6) | 4 (67%) | 2 (33%) | 65-89 | 78 | 74 | 78 | 75.3 |
| Hospital ESBL Other *Enterobacteriaceae* (n=4) | 3 (75%) | 1 (25%) | 65-89 | 78 | 77 | 78 | 77.4 |
| All Community Other *Enterobacteriaceae* (n=3) | 1 (33%) | 2 (67%) | 56 | 0-57 | 56 | 28.5 | 37.7 |
| Community ESBL Other *Enterobacteriaceae* (n=1) | 0 (0%) | 1 (100%) | 0 | 57 | 0 | 57 | 57 |

Table 9.2 Distribution of hospital and community based isolates amongst varying infection sources.

| | Isolate Source | | |
|---|---|---|---|
| | **Urine** | **Blood** | **Other** |
| All Hospital *E. coli* (n=81) | 68 (84%) | 9 (11%) | 4 (5%) |
| Hospital ESBL and AmpC *E. coli* (n=72) | 59 (81%) | 9 (13%) | 4 (6%) |
| All Community *E. coli* (n=36) | 34 (94%) | 0 (0%) | 2 (6%) |
| Community ESBL and AmpC *E. coli* (n=33) | 31 (94%) | 0 (0%) | 2 (6%) |
| All Hospital *Klebsiella spp.* (n=26) | 21 (81%) | 3 (12%) | 2 (7%) |
| Hospital ESBL *Klebsiella spp.* (n=22) | 18 (82%) | 2 (9%) | 2 (9%) |
| All Community *Klebsiella spp.* (n=12) | 12 (100%) | 0 (0%) | 0 (0%) |
| Community ESBL *Klebsiella spp.* (n=10) | 10 (100%) | 0 (0%) | 0 (0%) |
| All Hospital Other *Enterobacteriaceae* (n=6) | 4 (66%) | 1 (17%) | 1 (17%) |
| Hospital ESBL Other *Enterobacteriaceae* (n=4) | 3 (75%) | 0 (0%) | 1 (25%) |
| All Community Other *Enterobacteriaceae* (n=3) | 1 (33%) | 0 (0%) | 2 (67%) |
| Community ESBL Other *Enterobacteriaceae* (n=1) | 1 100%) | 0 (0%) | 0 (0%) |

Table 9.3 Gender and age distribution amongst isolates from varying infection sources.

| | Gender | | Age Range (Years) | | Average Age (Years) | | |
|---|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** | **Total Average** |
| All *E. coli* Urine (n=102) | 24 (24%) | 78 (76%) | 34-90 | 3-95 | 73.5 | 62.8 | 65.4 |
| ESBL and AmpC *E. coli* Urine (n=90) | 23 (26%) | 67 (74%) | 34-90 | 3-90 | 73.9 | 60.4 | 63.8 |
| All *E. coli* Blood (n=9) | 6 (67%) | 3 (33%) | 54-88 | 24-39 | 76.2 | 32.7 | 61.6 |
| ESBL *E. coli* Blood (n=9) | 6 (67%) | 3 (33%) | 54-88 | 24-39 | 76.2 | 32.7 | 61.6 |
| All *E. coli* Other Source (n=5) | 3 (60%) | 2 (40%) | 41-72 | 46-65 | 61.3 | 55.5 | 59 |
| ESBL *E. coli* Other Source (n=5) | 3 (60%) | 2 (40%) | 41-72 | 46-65 | 61.3 | 55.5 | 59 |
| All Klebsiella spp. Urine (n=33) | 14 (42%) | 18 (56%) | 32-78 | 0-99 | 65.7 | 75.3 | 71 |
| ESBL *Klebsiella spp.* Urine (n=28) | 14 (50%) | 14 (50%) | 32-78 | 60-99 | 65.5 | 80.5 | 73 |
| All Klebsiella spp. Blood (n=3) | 2 (67%) | 1 (33%) | 50-78 | 82 | 64 | 82 | 70 |
| ESBL *Klebsiella spp.* Blood (n=2) | 2 (100%) | 0 (0%) | 50-78 | | 64 | | 64 |
| All *Klebsiella spp.* Other Source (n=2) | 0 (0%) | 2(100%) | | 46-51 | | 48.5 | 48.5 |
| ESBL *Klebsiella spp.* Other Source (n=2) | 0 (0%) | 2 (100%) | | 46-51 | | 48.5 | 48.5 |
| All Other *Enterobacteriaceae* Urine (n=5) | 3 (60%) | 2 (40%) | 65-89 | 57-78 | 73 | 67.5 | 70.8 |
| ESBL Other *Enterobacteriaceae* Urine (n=4) | 2 (50%) | 2 (50%) | 65-89 | 57-78 | 77 | 67.5 | 72.3 |
| All Other *Enterobacteriaceae* Blood (n=1) | 1 (100%) | 0 (0%) | 77 | | 77 | | 77 |
| ESBL *Enterobacteriaceae* Other Blood (n=0) | 0 (0%) | 0 (0%) | | | | | |
| All Other *Enterobacteriaceae* Other Source (n=3) | 1 (33%) | 2 (67%) | 56 | 0-78 | 56 | 39 | 44.6 |
| ESBL Other *Enterobacteriaceae* Other Source (n=1) | 0 (0%) | 1 (100%) | | 78 | | 78 | 78 |

### 9.2.2 Epidemiology of urine isolates

As urine isolates predominated the strain collection this section focuses on isolates obtained from urine specimens to avoid selection bias. Table 9.3 demonstrates that 122 ESBL/AmpC-producing *Enterobacteriaceae* were isolated from urine samples. Of which, 80 (66%) isolates were found to be hospital-associated whereas 42 (34%) were community-associated. Of these, 28/37 (76%) were hospitalised males and 52/85 (61%) hospitalised females. 9/37 (24%) of isolates were from males in the community and 33/85 (39%) from females in the community. It is interesting to note in table 9.4, that *E. coli* are predominately isolated from women whereas amongst the *Klebsiella* spp., the numbers are more evenly distributed.

#### 9.2.2.1 Hospital-associated isolates

Of the 80 hospital-associated *Enterobacteriaceae* isolates, 59 (74%) isolates were *E. coli*, 18 (23%) were *Klebsiella spp.* and 3 (3%) were other *Enterobacteriaceae*. Fifty-six (94%) *E. coli* isolates contained a CTX-M group 1 gene, 2 (3%) isolates had a CTX-M group 9 gene and 2 (3%) isolates harboured a TEM gene (TEM-116 and TEM 52). It must be noted that the isolate harbouring TEM-116 also carries a CTX-M group 1 gene. It was demonstrated that 18/59 (31%) hospital-associated *E. coli* isolates were obtained from males and 41/59 (69%) from females. Within the male population, 17/18 (94%) *E. coli* isolates contained a CTX-M group 1 gene and 1 (6%) isolates had a CTX-M group 9 gene. Within the female population, 39/41 (95%) *E. coli* isolates contained a CTX-M group 1 gene, 1 (2%) isolates had a CTX-M group 9 gene and 2 (2%) isolates harboured a TEM gene.

Sixteen (89%) *Klebsiella* spp. isolates contained a CTX-M group 1 gene and 1 (5%) isolates harboured an SHV gene. It was demonstrated that 8/18 (44%) hospital-associated *Klebsiella* spp. isolates were obtained from males and 10/18 (56%) from females. Within the male population, 8/8 (100%) *Klebsiella* spp. isolates contained a CTX-M group 1 gene. Within the female population, 9/10 (90%) *Klebsiella* spp. isolates contained a CTX-M group 1 gene, and 1 (10%) isolates harboured a SHV gene.

1/3 other *Enterobacteriaceae* isolates (*E. cloacae*) contained a CTX-M group 1 gene and an SHV-2 gene, 1/3 (C. *freundii*) had a TEM-116 gene. 2/3 harboured *ampC*

genes. It was demonstrated that 2/3 hospital-associated *Klebsiella* spp. isolates were obtained from males and 1/3 from females. Within the male population (n=2), 1 other *Enterobacteriaceae* isolate (*E. cloacae*) contained a CTX-M group 1 gene, 1 *E. cloacae* isolate harboured an SHV-2 gene and had an *ampC* gene. Within the female population, one isolate harboured a TEM-116 and *ampC* gene (C. *freundii*).

#### 9.2.2.2 Community-associated isolates

Within the 42 community-associated *Enterobacteriaceae* isolates, 31 (74%) *E. coli*, 10 (24%) *Klebsiella spp.*, and 1 (2%) other *Enterobacteriaceae* were found. Twenty-seven (87%) *E. coli* isolates contained a CTX-M group 1 gene, 3 (10%) isolates had a CTX-M group 9 gene and 3 (10%) isolates harboured a TEM gene (2 TEM-116 and 1 TEM-52). One *E. coli* isolate was found to harbour an *ampC* gene. It was demonstrated that 5/31 (16%) community-associated *E. coli* isolates were obtained from males and 26/31 (84%) from females. Within the male population, 4/5 (80%) *E. coli* isolates contained a CTX-M group 1 gene and 1 (20%) isolates had a CTX-M group 9 gene. Within the female population, 23/26 (88%) *E. coli* isolates contained a CTX-M group 1 gene, 2 (8%) isolates had a CTX-M group 9 gene, 3 (11%) isolates harboured a TEM gene and 1 (4%) isolate had an *ampC* gene.

8/10 (80%) *Klebsiella* spp. isolates associated with the community contained a CTX-M group 1 gene, 2 (20%) isolates had a TEM gene (TEM-116) and 1 (10%) isolate harboured an SHV-2 gene. A higher proportion 6/10 (60%) community-associated *Klebsiella* spp. isolates were obtained from males and 4/10 (40%) from females. Within the male population, all 6 *Klebsiella* spp. isolates contained a CTX-M group 1 gene and 1 contained a CTX-M group 9 gene. Within the female population, 3/4 (75%) *Klebsiella* spp. isolates contained a CTX-M group 1 gene and 1 isolate harboured a TEM (TEM-116) gene and a SHV-2 gene.

One community associated *E. cloacae* isolate was found. This was isolated from a female patient and exhibited a CTX-M group 1 gene.

Table 9.4 Distribution of ESBL and *ampC* genes amongst hospital and community associated urine isolates.

| | CTX-M Grp 1 | CTX-M Grp 9 | TEM-116 | TEM-33 | TEM-52 | SHV-2 | AmpC |
|---|---|---|---|---|---|---|---|
| **All *E. coli*** (n=90) | 83 | 5 | 3 | 2 | 2 | 0 | 1 |
| Hospital-associated *E. coli* (n=59) | 56 | 2 | 1 | 0 | 1 | 0 | 0 |
| Male (n=18) | 17 | 1 | 0 | 0 | 0 | 0 | 0 |
| Female (n=41) | 39 | 1 | 1 | 0 | 1 | 0 | 0 |
| Community-associated *E. coli* (n=31) | 27 | 3 | 2 | 0 | 1 | 0 | 1 |
| Male (n=5) | 4 | 1 | 0 | 0 | 0 | 0 | 0 |
| Female (n=26) | 23 | 2 | 2 | 0 | 1 | 0 | 1 |
| **All *Klebsiella* spp.** (n=28) | 26 | 0 | 2 | 0 | 0 | 2 | 0 |
| Hospital-associated *Klebsiella* spp. (n=18) | 16 | 0 | 0 | 0 | 0 | 1 | 0 |
| Male (n=8) | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| Female (n=10) | 9 | 0 | 0 | 0 | 0 | 1 | 0 |
| Community-associated *Klebsiella* spp.(n=10) | 9 | 0 | 2 | 0 | 0 | 1 | 0 |
| Male (n=6) | 6 | 0 | 1 | 0 | 0 | 0 | 0 |
| Female (n=4) | 3 | 0 | 1 | 0 | 0 | 1 | 0 |
| **All Other *Enterobacteriaceae*** (n=4) | 2 | 0 | 1 | 0 | 0 | 1 | 2 |
| Hospital-associated Other *Enterobacteriaceae* (n=3) | 1 | 0 | 1 | 0 | 0 | 1 | 2 |
| Male (n=2) | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| Female (n=1) | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Community-associated Other *Enterobacteriaceae* (n=1) | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Male (n=0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Female (n=1) | 1 | 0 | 0 | 0 | 0 | 0 | 0 |

N.B. Eight isolates contain multiple genes but have been counted individually in this table.

### 9.2.3 The clinical epidemiology of *E. coli* isolates in relation to CTX-M-15 IS26

Table 9.5 outlines the distribution of CTX-M-15 *IS*26 (see Chapter 5) amongst hospital and community-associated *E. coli* isolates. By PCR, 64% (66/103) *E. coli* isolates were found to obtain CTX-M-15 IS26. Of these positive *E. coli* isolates, 66% were found to be hospital associated and 34% were associated with a GP practice. 31% were obtained from males and 69% from females. 36% of *E. coli* isolates did not yield a positive amplification for CTX-M-15 IS26 and were so named IS26 negative. Of these IS26 negative isolates, 72% were hospital associated and 28% were community associated. 34% were obtained from males and 72% were from females.

To coincide with section 9.2.2, isolates obtained from urine specimens were analysed independently to avoid selection bias. In total, 88 ESBL-producing *E. coli* isolates were obtained from urine specimens and of these, (59/88) 67% were positive for CTX-M-15 IS26 and 33% were negative for CTX-M-15 IS26. Within the CTX-M-15 IS26 positive isolates obtained from urine specimens, 62% were hospital associated and 38% were community associated. 26% were obtained from males and 74% were from females. Of the 33% of *E. coli* isolates negative for CTX-M-15 IS26, 72% were hospital-associated and 28% were community-associated. Of which, 28% were obtained from males and 72% from females.

Table 9.5 Distribution of CTX-M-15 *IS*26 amongst hospital and community-associated ESBL *E. coli* isolates

| | Source | | Gender | | Age Range (Years) | |
|---|---|---|---|---|---|---|
| | **Hospital** | **Community** | **Male** | **Female** | **Male** | **Female** |
| All *E. coli* CTX-M-15 *IS*26 positive (n=66) | 66% | 34% | 31% | 69% | 34-90 | 3-90 |
| *E. coli* CTX-M-15 *IS*26 positive urine (n=59) | 62% | 38% | 26% | 74% | 34-90 | 3-90 |
| All *E. coli* CTX-M-15 *IS*26 negative (n=37) | 72% | 28% | 34% | 66% | 41-89 | 4-90 |
| *E. coli* CTX-M-15 *IS*26 negative urine (n=29) | 72% | 28% | 28% | 72% | 54-89 | 4-90 |

### 9.2.4 The clinical epidemiology of *E. coli* isolates in relation to O25b-ST131

Table 9.6 outlines the distribution of the O25b-ST131 clone (see Chapter 5) within *E. coli* isolates. In total, 80 *E. coli* isolates were found to possess a SNP in the *pab*B gene which is indicative of the O25b-ST131 clone (Clermont et al., 2009). Of these isolates, 70% were hospital-associated and 30% were community-associated. 88% (70/80) of the *pab*B positive *E. coli* isolates were obtained from urine specimens. Within these *E. coli* isolates isolated from urine specimens, 67% were hospital-associated and 33% were found to be community-associated, of which, 23% were obtained from males and 77% from females.

To further evaluate the epidemiology of the O25b-ST131 clone, the distribution of O25b-ST131 with and without CTX-M-15 IS26 was analysed. Unfortunately, only 63/66 of the *E. coli* CTX-M-15 IS26 positive could be revived for testing with the O25b-ST131 allele specific PCR. Results for *E. coli* CTX-M-15 IS26 with the SNP in the *pab*B gene can be seen in table 9.6.

46% of isolates positive for the O25b-ST131 clone were CTX-M-15 IS26 positive and 82% were hospital-associated, whereas 18% were community-associated. 29% were obtained from males and 71% from females. 38% (14/37) of these isolates were obtained from urine specimens. Within these *pab*B positive CTX-M-15 IS26 negative *E. coli*, 86% were hospital-associated and 14% community-associated. 21% were obtained from males and 79% from females. This differs to distribution seen by *E. coli* CTX-M-15 IS26 negative O25b-ST131 negative, as 60% were hospital associated and 40% were community associated. 35% were obtained from males and 65% were from females.

Table 9.6 Distribution of O25b-ST131 amongst hospital and community-associated *E. coli* isolates.

| | Hospital | Community | Male | Female |
|---|---|---|---|---|
| All *pab*B positive *E. coli* (n=80) | 70% | 30% | 31% | 69% |
| All *pab*B positive *E. coli* from urine (n=70) | 67% | 33% | 26% | 77% |
| *pabB* positive CTX-M-15 IS26 positive *E. coli* (n=63) | 67% | 33% | 32% | 68% |
| *pab*B positive CTX-M-15 IS26 positive *E. coli* from urine (n=56) | 63% | 38% | 27% | 73% |
| *pab*B positive CTX-M-15 IS26 negative *E. coli* (n=17) | 82% | 18% | 29% | 71% |
| *pab*B positive CTX-M-15 IS26 negative *E. coli* from urine (n=14) | 86% | 14% | 21% | 79% |

**pabB* positive isolates are those whereby a SNP has been detected in the *pabB* gene. This SNP is characteristic of the intercontinental clone, O25b-ST131 (see chapter 5).

## 9.3 Discussion

The aim of this chapter was to identify the clinical epidemiology associated with ESBL-producing *Enterobacteriaceae*. It is clearly demonstrated in this study, *E. coli* followed by *Klebsiella* spp. were the most common types of isolates containing ESBL and AmpC genes. These isolates were most commonly isolated from urine samples from females with an average age of 60 years. This is not an unexpected result as UTIs are most commonly associated with women. There are several factors that predispose women to UTIs including: urethra length and shorter distance between urethra and the anus, which is the source of microorganisms capable of causing UTIs (Hooton, 2000). It is also thought that the reduced levels of the hormone oestrogen in postmenopausal women contributes to the occurrence of UTIs (Hooton, 2000).

As the number of isolates collected from urine samples exceeded others, the urine population was analysed independently. 66% of all ESBL and AmpC-producing *Enterobacteriaceae* in the urine collection were hospital-associated and 34% were community-associated. CTX-M group 1 was found to be the most prevalent ESBL type found, as 92% gave positive amplification by PCR and of which, 66% were hospital-associated and 33% were community-associated. In total, CTX-M group 1 was found in 93% (74/80) of hospital-associated and 90% (37/42) of community-associated *Enterobacteriaceae*. ESBLs, particularly CTX-M, have been increasingly prevalent in the community. A study conducted by Woodford *et al.*, (2004), demonstrated that 25% of CTX-M-producing isolates were obtained from the community. This result is similar to the 21% of community associated ESBL infection found by Kang et al., (2004). Rodriguez-Bano et al., (2006) demonstrated that 19% of bacteraemia due to ESBL-producing *E. coli* was community-associated. However, Melzer and Peterson, (2007) and Bean et al., (2008) observed lower values for ESBLs associated with the community. Melzer and Peterson, (2007) found that 6.6% of isolates in their ESBL bacteraemia study were community-acquired and Bean et al., (2008) demonstrated that 5.7% of their isolates which were ESBL-producing *E. coli* from UTIs were obtained from the community.

The prevalence of CTX-M-15 *IS*26 *E. coli* from the community in this isolate collection was evaluated. It was found that 66% of isolates were hospital-associated

whereas 34% were community-associated and in isolates obtained from urine specimens, these values change to 62% hospital-associated and 38% community associated.

The dissemination of ESBLs, particularly CTX-M, into the community could be associated with the presence of mobile genetic elements. CTX-M genes are often found alongside IS*Ecp1* or type 1 integrons (Pitout et al., 2005b, Poirel et al., 2003). It has also been implied that due to the presence of isolates carrying CTX-M in animals, that food-producing animals and domestic pets could act as a reservoir (Pitout et al., 2005b).

Additionally, the prevalence of the international clone, O25b-ST131 was also analysed. This clone was present in 70% of *E. coli* which were hospital-associated and 30% community-associated. Again, these numbers change to 67% and 33%, respectively in *E. coli* isolates isolated from urine specimens. A study by Lau et al., (2008b) found that 84% of hospital infection and 64% and community infection was associated with ST131. The dissemination of such clones could also play a role in facilitating the spread of ESBLs in the community.

With the spread of CTX-M, particularly CTX-M-15 into the community setting, treatment options are limited (Woodford et al., 2004). Isolates harbouring CTX-M genes often harbour resistance genes for other types of antibiotics e.g. trimethoprim-sulfamethoxazole, aminoglycosides and fluoroquinolones (Rodriguez-Bano et al., 2006). Therefore, nitrofurantoin or fosfomycin (not currently marketed in the UK) have been recommended as treatment options. However, it has been suggested that these are not ideal (Woodford et al., 2004).

UTIs caused by *E. coli* often progress to bacteraemia (Lau et al., 2009, Melzer and Petersen, 2007). A recent report by the Health Protection Agency (HPA) demonstrated that the rate of bacteraemia caused by *E. coli* has increased from 18,593 in 2005 to 25,532 in 2009. The report also stated that a reduction in the prevalence of CTX-M in *E. coli* was noted by BSAC (HPA, 2010). However, in the instance that the bacteraemia is due to ESBL-producing *E. coli*, problems with treatment options could be faced. A study by Rodriguez-Bano et al., (2006) found that empirical therapy with cephalosporins or fluoroquinolones was associated with a

higher mortality rate and found that 4/10 patients who received therapy with a cephalosporin died. Therefore it was suggested that carbapenems are the most appropriate option for treating bacteraemia caused by ESBL-producing *E. coli* (Rodriguez-Bano et al., 2006).

# Chapter 10: Discussion

## 10.1 General discussion

ESBLs can be defined as β-lactamase enzymes capable of hydrolysing the penicillins, 1st, 2nd and 3rd generation cephalosporins and the monobactam aztreonam. They are unable to hydrolyze cephamycins and carbapenems. ESBLs are inhibited *in vitro* by the β-lactamase inhibitor clavulanic acid (Philippon et al., 1989, Paterson and Bonomo, 2005). ESBLs were first reported in 1983 (Paterson and Bonomo, 2005) and until 2001 with the emergence of CTX-M, ESBLs in the UK were mostly mutants of TEM and SHV which were predominantly found in *Klebsiella* spp. (Potz et al., 2006). Resistance to front line antibiotics poses a great limitation from the perspective of treatment options (Paterson et al., 2000). The choice of antimicrobial agent is important as inappropriate treatment is linked to an increased mortality (Rodriguez-Bano et al., 2006). Therefore it is imperative to correctly and accurately identify ESBL-producing organisms to ensure that the appropriate antimicrobial therapy is delivered to the patient.

The rationale of this study was to establish the molecular epidemiology of ESBL-producing *Enterobacteriaceae* within the Abertawe Bro Morgannwg (ABM) University Health Board. *Enterobacteriaceae* were collected from the Public Health Wales (PHW) Microbiology ABM Swansea laboratory based upon resistance to cefpodoxime. With a selection of molecularly confirmed ESBL-producing *Enterobacteriaceae*, it was possible to analyse the sensitivity of current phenotypic laboratory ESBL detection procedures thus allowing suggestions to be made where relevant. Isolates were then further characterised and typed in order to identify clonal relationships. In doing so, a greater understanding of ESBLs types within the ABM University Health Board could be achieved. The activity of well established and 'newer' antibiotics towards ESBLs, and subsequently, ESBLs which were associated with clones that often harbour multidrug resistances could be analysed. The evaluation of this information could potentially contribute to decisions made regarding treatment options. Alongside the identification of clones, a hypothesis regarding a link between the spread of ESBL genes and *E. coli* biofilm formation was also investigated.

The three main UK ESBL types; TEM, SHV and CTX-M were detected by PCR and identified using DNA sequencing. The predominant ESBL genotype identified was CTX-M group 1, which was found in 92% of isolates. Sequence analysis revealed that CTX-M-15 was found to be the most common ESBL genotype with 92% of all *E. coli* and *Klebsiella* spp. analysed possessing this gene. CTX-M-15 enzymes are now the most commonly reported ESBL in Europe (Peirano and Pitout, 2010) and have also been reported in Canada (Pitout et al., 2007, Pitout et al., 2005a, Boyd et al., 2004), Asia (Ensor et al., 2006, Hawkey, 2008), Africa (Gangoue-Pieboji et al., 2005), South America (Villegas et al., 2008) and Australia (Zong et al., 2008).

ESBLs, particularly CTX-M, have been increasingly prevalent in the community owing to urinary-tract infections which may progress to bacteraemia. The HPA recently released a report stating that the number of reported bacteraemias in England, Wales and Northern Ireland caused by *E. coli* has increased from 18,593 in 2005 to 25,532 in 2009 (HPA, 2010). A study conducted by Woodford *et al.*, (2004), demonstrated that 25% of CTX-M-producing isolates were obtained from the community. In this study, CTX-M group 1 was found in 93% (74/80) of hospital-associated and 90% (37/42) of community-associated *Enterobacteriaceae*. In contrast, while the rate of *E. coli* bacteraemia has increased, efforts to decrease hospital-acquired infections caused by methicilin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* have resulted in a significant decrease in those infections in this time period. In 2003, approximately 6000 cases of MRSA bacteraemia were reported and in 2010, less than 2000 cases were reported (http://www.hpa.org.uk/hpr/infections/bacteraemia.htm#saur). For *C. difficile* infections, the baseline quarterly average number of reported cases has decreased from approximately 13,000 in 2008 to 4827 cases in 2011 (http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1284473407318). This implies that measures successful in reducing MRSA bacteraemia in the UK do not affect *E. coli* bacteraemia, which may be related to the increasing number of ESBL carrying *E. coli* in the community, which resist empiric front-line treatment and therefore cause bacteraemia as a complication of community- and hospital-acquired urinary tract infection with increasing frequency. Consequently the Wales Healthcare Associated Infection Programme (WHAIP) has introduced new mandatory

surveillance of *E. coli* community and hospital acquired bacteraemias, with the first data to be published in October 2011, in addition to its existing requirements on *C. difficile* infection and *S. aureus* bacteraemias.

The further characterisation and typing of isolates allows for a better understanding of the genetic variability and pathogenicity of bacterial isolates and allows changes in epidemiology to be monitored. Strain A was first reported in the UK in 2003 after an epidemic *E. coli* outbreak in Shropshire and was found to have a characteristic CTX-M-15 *IS*26 link region (Woodford et al., 2004). As shown in Chapter 3, upon screening all CTX-M group 1 *Enterobacteriaceae* isolates by PCR for this link region, 51% generated positive amplification products and all of which were found to be *E. coli*. It was found by PFGE that the isolates containing the CTX-M-15 *IS*26 link region were 'closely related' and 'possibly related' to epidemic *E. coli* strain A. The prevalence of CTX-M-15 *IS*26 *E. coli* in the community was evaluated and it was found that 66% of isolates were hospital-associated whereas 34% were community-associated. However, Lau *et al.,* (2008a) found that the UK's epidemic *E. coli* strains A-E belonged to an international clone, O25b-ST131. As outlined in Chapter 5, all isolates positive for the CTX-M-15 *IS*26 link region along with a number of isolates that did not possess this region, were also found to be positive by PCR for O25b-ST131. It was revealed in Chapter 9 that genes associated with the international clone O25b-ST131 were found to be present in 70% of *E. coli* which was hospital-associated and 30% which was community-associated.

The clonal spread of resistance genes, especially within the community, poses an increased problem with regards to treatment options. A report by Cagnacci et al., (2008) revealed a correlation between ciprofloxacin resistance and *E. coli* clones O15: K52 H1 and O25b-ST131 in urinary-tract infections. In Chapter 6, it can be seen that 90% of all *Enterobacteriaceae* were resistant to ciprofloxacin whereas 100% of CTX-M-15 *IS*26 *E. coli* were resistant and as mentioned previously all of which were found to be associated with O25b-ST131.

ESBL plasmids often harbour genes for other mechanisms of resistance including aminoglycosides (e.g. gentamicin and amikacin), quinolones and Trimethoprim-sulfamethoxazole (TMP-SMX) thus causing strains to be mutidrug resistant

(Paterson et al., 2000). As demonstrated in Chapter 6, 31% of all isolates were resistant to gentamicin whereas, 11% were resistant to amikacin. 89% of all *Enterobacteriaceae* isolates were found to be resistant to trimethoprim-sulfamethoxazole. With such an increase in CTX-M prevalence especially in the community, the pressure posed on treatment options is greatened further, as the choices are limited. Therefore, serious infections would require the use of carbapenems (Livermore and Hawkey, 2005, Peirano and Pitout, 2010, Dhillon and Clark, 2011). However, the use of carbapenems could alter the selection pressure which could thereby increase the prevalence of the carbapenem resistant bacteria, which would limit antimicrobial treatment to a greater extent (Livermore, 2009). The most recent major public health concern regarding carbapenemases has been the emergence of the New Delhi Metallo-β-lactamase 1 (NDM-1), a metallo-β-lactamase which is associated with a transmissible mobile element. NDM-1 was originally isolated from *K. pneumoniae* in India and has now spread to other *Enterobacteriaceae.* It has been isolated in many countries around the world including the UK. Infections with NDM-1 are problematic to treat, as bacteria harbouring this type of β-lactamase are resistant to all antimicrobial agents except the polymyxins i.e. colistin (Yong et al., 2009, Moellering, 2010, Kumarasamy et al., 2010).

As well as analysing the susceptibility of currently administered antibiotics, the susceptibility of temocillin and tigecycline were also evaluated. Temocillin and tigecycline have been shown to have an improved stability to ESBL and AmpC-producing *Enterobacteriaceae*. In this study, using BSAC MIC breakpoints, temocillin was effective against 98% of all isolates and tigecycline was effective against 89% of all isolates tested. However, despite temocillin being 98% effective, the most active antibiotics tested in this study were the carbapenems. All isolates were 100% susceptible except for ertapenem, for which 1% of isolates were resistant.

With the molecular epidemiology of ESBLs established, the laboratory detection of ESBLs could be evaluated. As well as utilising commercial and automated ESBL and AmpC detection methods, improvements to the current detection system could be suggested. Currently, ESBL detection in the laboratory involves a two-step

procedure whereby *Enterobacteriaceae* are firstly screened for ESBL production and then confirmed as ESBL producers. BSAC recommends that cefpodoxime is used as an indicator of choice (BSAC, 2007, BSAC, 2009) as variable resistance to cefotaxime and ceftazidime are seen (Livermore and Brown, 2001). With regards to ESBL screening, the addition of ceftazidime, as outlined in the HPA QSOP 51 guidelines, would be advisable to achieve a greater sensitivity. As for ESBL confirmation, cefpodoxime is also the indicator cephalosporin of choice stipulated by BSAC. However, in this study cefpodoxime had a sensitivity of 96% for the confirmation of ESBL in molecularly confirmed isolates. The addition of cefotaxime or ceftazidime would increase the sensitivity of the Synergy Test to 98%. A combination of all three cephalosporin discs would achieve a sensitivity of 99%. However, the laboratory detection of ESBL can often be problematic due to the presence of AmpC enzymes. In this study, one *C. freundii* isolate was found to possess TEM-116 and an *ampC* gene. Unfortunately, the isolate was negative to all phenotypic ESBL tests and would have therefore been overlooked in the clinical setting.

A commercial AmpC and ESBL detection kit (Mast® AmpC and ESBL Detection Set) was shown to be 98% sensitive overall and proved to be an adequate detection system for AmpC with a sensitivity of 100%. However, an increased number of ESBL and AmpC producing *Enterobacteriaceae* would need to be evaluated. As for the use of automated systems to detect ESBL in *E. coli* and *Klebsiella* spp., the BD Phoenix™ Automated System was found to have 99% sensitivity.

Another aim in this study was to investigate the use of the BD Phoenix automated system and Bruker Daltonics MALDI Biotyper for bacterial identification. In order to improve patient care, the need for a rapid and accurate identification of bacteria is essential (Carbonnelle et al., 2011, Seng et al., 2009). Typically within the clinical laboratory, bacteria are identified using conventional phenotypic methods such as growth on various media, colony morphology, Gram stain and other biochemical tests. Using these methods, bacterial identification can take between 24 and 36 hours (Cherkaoui et al., 2010) therefore the correct treatment of patients by empirical antibiotic therapy can be delayed (Seng et al., 2009). Using these two systems, a

concordance of 97% was noted. However, the length of time to identify bacteria using the BD Phoenix automated system is significantly longer than the Bruker Daltonics MALDI Biotyper. The Bruker Daltonics MALDI Biotyper is a rapid identification tool capable of identifying a bacterial isolate in minutes (90 minutes to 2 hours to complete a 96 well plate) as opposed to approximately 16 hours. However, the BD Phoenix automated system has the added capability of producing MIC information, which allows the BD Xpert system to deduce ESBL production.

The final aim in this study was to investigate whether ESBL-producing *E. coli* isolates had a greater likelihood of forming biofilms. Bacterial conjugation is a mechanism by which genetic information can spread and gene transfers often occur in biofilms, as their complex network of communities is ideally suited to the role (Hausner and Wuertz, 1999). Wang *et al.*, (2004) identified a gene locus in *E. coli* known as *pgaABCD*. This locus, which can be horizontally transferred, was found to be associated with the promotion of surface binding and intercellular adhesion through the production of poly-β-1, 6-N-acetyl-D-glucosamine (PGA) (Wang et al., 2004). As shown in Chapter 8, all ESBL and AmpC-producing *E. coli* isolates possessed the *pgaABCD* gene locus but not all produced a phenotypic biofilm. Aside from growth conditions and surface determinants, another explanation to postulate why isolates possess the *pgaABCD* locus but not produce a phenotypic biofilm could be due to a link between *pgaABCD* and CsrA. *pgaABCD* expression can be repressed by CsrA (carbon storage regulator) (Wang et al., 2005). CsrA is a RNA binding protein that negatively regulates several metabolic pathways e.g. glycogen biosynthesis, gluconeogenesis, and glycogen catabolism (Liu and Romeo, 1997). CsrA also serves as an activator of biofilm dispersal under certain conditions (Jackson et al., 2002).

In summary, this study contributes to an understanding of the molecular epidemiology and prevalence of ESBLs in *Enterobacteriaceae* isolated within the ABM Bro Morgannwg University Health Board. The results were comparable to the current UK epidemiology whereby CTX-M group 1, particularly CTX-M-15 were the most prevalent. Clonal relationships were observed between *E. coli* isolates in this study, UK epidemic *E. coli* strain A and subsequently the international clone, O25b-ST131. With changes in molecular epidemiology and the emergence of new

enzyme types, it is vital that continuous monitoring of current detection methods and antibiotics sensitivity is undertaken to avoid the misidentification of ESBLs and therefore, the use of ineffective agents to treat patients.

### 10.2 Future work

In order to further to support and further the findings of this thesis a number of experiements could be carried out. As illustrated in Chapter 3, a number of isolates which were phenotypically ESBL positive were found to be molecularly ESBL negative using TEM, SHV and CTX-M primers. This finding was attributed to the possibility of rarer/atypical or even novel ESBL genes being present. In order to investigate this further, primers specific for other ESBL genotypes could be used. Additionally, the use of isoelectric focusing (IEF) could be used to provide information on isoelectric point values and inhibitor profiles in order to characterise the β-lactamase enzyme present (Wiegand et al., 2007).

A second collection of *Enterobacteriaceae* isolates could be be collected and screened for ESBL and carbapenemase production in order for the molecular epidemiology at two different timepoints to be compared. The presence of ST131 and clonal relatedness could also be investigated. This would be a relevant study as the National electronic Library for Medicines (NeLM) announced in February 2012 that the Department of Health was making £500,000 available for further research into ESBLs (http://www.nelm.nhs.uk/en/NeLM-Area/News/2012---February/09/Department-of-Health-announces-funding-for-new-research-into-antibiotic-resistance-bacteria-/).

Another theme that could be investigated further would be the potential of using MALDI-TOF/MS to deduce the clonal relatedness of O25b-ST131 isolates. At the time of writing, only one study investigating this could be found. Using MALDI biotyper software, Kmet et al. (2011) identified that ST131 positive *E. coli* isolates obtained from humans were unrelated to 2 related ST131 positive *E. coli* isolates originating from poultry (n=1) and calf (n=1). The use of MALDI-TOF/MS would detect the relatedness of epidemiologically relevant rapidly.

In this thesis, biofilm formation in ESBL-producing *E. coli* was investigated. *E. coli* obtained from cow endometrium was used as a non-human control population. A

large number of these isolates were found to be positive for the *pgaABCD* locus and a number also expressed a phenotypic biofilm in vitro. However, from the box-plot featured in Chapter 8 Figure 8.7, it can be noted that a range of absorbance readings were obtained. The isolates could be ranked into groups based on absorbance readings so that a correlation could be investigated between biofilm production and severity of disease to determine how severe the original disease was. This would provide information on whether the more adherent isolates i.e. those with a higher absorbance reading were associated with more severe infection in the cow endometrium. Additionally, epithelial cells could be grown and *E. coli* (with a range of different absorbance readings) could be applied to each cell culture to investigate invasiveness.

Experiments to investigate the virulence of the bovine isolates would also be an interesting future work perspective. Isolates could be exposed to monocytes and levels of cytokine production could be measured. Karlsson et al. (2004) stimulated monocytes with *E. coli* isolates with or without human recombinant gamma interferon (rIFNγ). Cytokines were determined by ELISA. It was revealed that increased levels of interleukin (IL)-12p70, IL-10, IL-6 and tumour necrosis factor (TNF) was measured in response to *E. coli* (Karlsson et al., 2004). Another study by Agace et al. (1993) exposed a urinary tract model and monocytes to *E. coli*. The urinary tract model expressed increased levels of IL-6, IL-1α and IL-8, whereas, the monocytes expressed increased levels of IL-1α, IL-1β, IL-6, IL-8 and TNFα (Agace et al., 1993).

In addition, the isolates could be also be exposed to neutrophils in order to study their ability to resist killing. Aarestrup et al. (1994) carried out this experiment in *S. aureus* isolates, which had been isolated from cows with mastitis. In their study, bacteria were grown overnight and cells were washed and resuspended to give a concentration of $10^8$ CFU before being opsonised and exposed to neutrophils. Bacteria killing by neutrophils was measured by a colorimetric assay (Aarestrup et al., 1994). However, the comparision of CFU before and after exposure to neutrophils could be a simpler approach.

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