Molecular diversity and genetic organization of antibiotic resistance in *Klebsiella* species
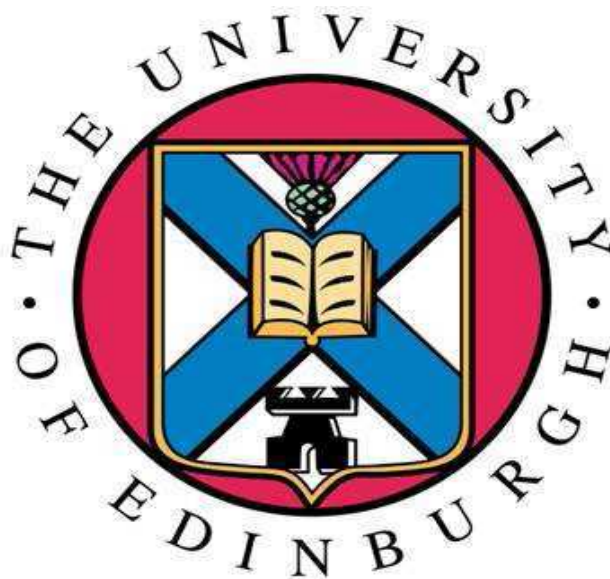
ABDELGAYED METWALY YOUNES

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

2010

ABSTRACT

Klebsiella spp. are opportunistic pathogens that cause hospital and community acquired infections such as pneumonia, urinary tract infection, septicaemia, soft tissue infections, liver abscess, and meningitis. Multidrug-resistant strains possessing extended-spectrum β-lactamases (ESBLs) has become an increasing problem worldwide. The over use and, in some cases, misuse of antibiotics in humans and in animal husbandry has been cited as a responsible factor in the development of drug resistance in all bacterial species. The advancing age; female gender, hospital cross-infection, the food chain trade and human migrations have contributed to increase the risk for community-acquired ESBL.

A total of 223 isolates collected in 2006 and 2007 at Royal Infirmary of Edinburgh, Scotland, 219 *K. pneumoniae*, 2 *K. oxytoca*, 1 *Enterobacter cloacae*, and one isolate *Salmonella enterica* were identified by API 20E and confirmed genotypically with *gyrA* PCR-RFLP method. The antimicrobial susceptibility results showed that 34 (15.2%), 36 (16.1%), 35 (15.7%), 45 (20.2%), 30 (13.5%) and 55 (24.7%) of these strains were found to be resistant to cefotaxime, ceftazidime, ceftriaxone, naladixic acid, ciprofloxacin and cefoxitin. None of the isolates were found resistant to meropenem keeping carbapenems the drug of choice for the treatment of multi-resistant isolates. The overall frequency of ESBL producers observed in this study was 35 (15.7%) most of them 32/35 (91.4%) were from *K. pneumoniae*. The genetic analysis showed that SHV β-lactamases were detected in 32, whereas TEM and CTX-M were detected in 24 and 16 respectively.

From the ESBL-producing isolates, molecular methods identified nine strains possessing ESBL-SHV genes (1 strain bla_{SHV-5}, 1 strain bla_{SHV-80} and 8 strains bla_{SHV-12}), whereas the remaining were from the "non-ESBL" producing strains. Conjugation methods demonstrated that 29/32 isolates harboured transferable bla_{SHV} genes. The large SHV transposon-borne promoters were amplified from only one non-transferable bla_{SHV-11}, 15 isolates produced the small SHV transposon-borne promoters. Furthermore, the IS26 was found 73bp upstream of the bla_{SHV} gene in all

small SHV transposon-borne promoters. A new bla_{LEN} gene was identified from *K. pneumoniae* (KpII) phylogenetic group but remained susceptible to all cephalosporins.

Sixteen (7.3%) of *K. pneumoniae* isolates were found to be producers of the CTX-M-15 ESBL, of which two isolates (12.5%) were reported to be from community-acquired infections. The insertion sequence IS*Ecp1* was detected by sequencing 48 nucleotides upstream of $bla_{CTX-M-15}$ in all isolates but one. Five different clones of CTX-M-15-producing isolates were identified by PFGE.

The findings indicated a higher prevalence of *qnr* genes than in previous studies but still low in general. By PCR, 18 (8%) (11 *qnrB1*, 2 *qnrB6* and 5 *qnrA1*) genes were identified from *K. pneumoniae* isolates. Also, the findings indicated the frequent co-expression of fluoroquinolones and ESBLs resistance in the same isolate.

Two *K. oxytoca* strains were isolated from urine and blood specimens of hospitalized patients. Both strains were positive for the bla_{OXY-2} gene. One strain showed resistance to pencillins, monbactams, cephalosporins including cefotaxime and ceftazidime but was not inhibited by clavulanic acid. It differed by an amino acid substitution Ala237→Thr, which enhances the binding of cefotaxime.

S1-nuclease plasmid profiles were obtained for some isolates. A total of one to two plasmids, ranging in size from approximately 40 to 210 kb, were observed per strain. The plasmids from 24 ESBL *K. pneumoniae* strains were assigned to be IncN or IncFII replicons. Analysis of phylogenetic groups showed that the majority of *K. pneumoniae* isolates were belonged to KpI-type. Both *K. oxytoca* strains were assigned to be KoII phylogenetic group based on *rpoB* and *gyrA* sequencing.

Integrons are capable of capturing and mobilizing genes called gene cassettes which play an important role in the dissemination of antimicrobial resistance through horizontal transmission. In fact, the present study indicated a high frequency of occurrence of class 1 integrons among ESBL-positive *K pneumoniae*. Three isolates

positive for class 1 integrons were found positive for class 2 integrons as well. Class 1 integrons including *dfr*, *aadA* and *ereA2* gene cassettes have been identified by sequencing, which confer resistance to trimethoprim, streptomycin/spectinomycin and erythromycin respectively.

In conclusion, the results from this thesis report the emergence of hospital and community-acquired highly resistant CTX-15 β-lactamase in the Edinburgh, Scotland. The prevalence of ESBL-producing isolates in Scotland is still much lower than in many other European countries. The dissemination of SHV- and TEM- β-lactamase types in this study is more predominate than CTX-M-15.

DECLARATION

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

Abdelgayed Younes

DEDICATION

I dedicate this thesis to:

My parents, the source of love, support and strength

My wife, Hanem, for her love, encouraging and patience

My childrens, Eman and Alaa who have enriched my life

ACKNOWLEDGEMENT

TABLE OF CONTENTS

Contents   page

PUBLICATIONS AND PRESENTATIONS

A. Younes, A. Hamouda, J. Dave, S.G.B. Amyes (2010). Prevalence of transferable $bla_{\text{CTX-M-15}}$ from hospital and community-acquired *Klebsiella pneumoniae* isolates in Scotland. Journal of Antimicrobial Chemotherapy. Accepted 2 November 2010.

A. Younes, A. Hamouda, S.G.B. Amyes (2010). First report of a novel extended-spectrum beta-lactamase KOXY-2 producing *Klebsiella oxytoca* that hydrolysis cefotaxime and ceftazidime. Journal of Chemotherapy, under review.

A. Younes (2005). Use of polymerase chain reaction (PCR) in diagnosis of *Clostridium perfringens* in chickens. (Master thesis), College of Veterinary Medicine, Cairo University, Egypt.

A. Younes, A. Hamouda, J. Dave, A. Gibb, S.G.B. Amyes, T. Schneiders (2008). Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, And QnrS among clinical isolates of *Klebsiella pneumoniae* in Scotland. *In*: 18th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). (Abstract).

A. Younes, A. Hamouda, T. Schneiders, S.G.B. Amyes (2008). Prevalence of transferable CTX-M-15 extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in Scotland. *In*: 48th Microbiology's Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). Oral presentation given by Amyes SG.B.

P.M. Lansdell, J. Boyes, N. Khanna, A. Hamouda, A.M. Younes, S.G. Amyes (2010). Local epidemiology of ESBL and AmpC producing Enterobacteriaeceae in urine samples from Glasgow. *In*: 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). (Abstract).

LIST OF TABLES

LIST OF FIGURES

ABBREVIATION

Ala	Alanine
Asp	Aspartic acid
Asn	Asparagine
Arg	Arginine
BSAC	British society for antimicrobial chemotherapy
bp	Base pair
cfu	Colony forming unit
DNA	Deoxynucleic acid
dNTP	Deoxynucleoside triphosphate
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
Ile	Isoleucine
IC_{50}	Concentration of inhibitor required to inhibit 50% of enzyme
kbp	Kilo base pair
LPS	Lipopolysaccharide
LB	Luria-Bertani
Leu	Leucine
Lys	Lysine
MIC	Minimum inhibitory concentration
MIC50	Minimum inhibitory concentration for 50% of strains
MIC90	Minimum inhibitory concentration for 90% of strains
mA	Milliamp
Met	Methionine
mRNA	Messenger RNA
OD	Optical density
PCR	Polymerase Chain Reaction
Pro	Proline
RNA	Ribonucleic acid
rpm	Round per minute
Ser	Serine

Spp.	Species
Thr	Threonine
Tyr	Tyrosine
UTI	Urinary tract infection
V	Volt
Val	Valine
W	Watt
µl	Micro-litre

CHAPTER-1:

INTRODUCTION

1.1. Antimicrobial Agents

1.1.1 Historical perspective

The initial discovery of antibiotics is generally begins with the Scottish scientist Alexander Fleming in 1928. He observed a mould of *Penicillium notatum* produced a diffusible antibacterial agent, penicillin, which inhibited the growth of *Staphylococcus aureus*. Fleming carried out a number of studies on the filtrate of liquid cultures of *Penicillium notatum*; he determined the antibacterial activity of this antibiotic *in vitro*, as well as its non-toxicity when injected into mice and rabbits. But Fleming failed to stabilise the active antibiotic (Rolinson 1998; Bennett and Chung 2001; Goldsworthy and McFarlane 2002; Geddes 2008). Attempts to obtain purified penicillin in the 1930s were mostly unsuccessful; however, a study carried out by Howard Florey and Ernst Chain in 1940 showed that Penicillin was highly effective against a streptococcal infection in mice. They purified penicillin by freeze-drying sufficient for clinical use and they discovered a new strain of *Penicillium* which was able to produce high yields of penicillin that has led to the development of modern antibiotics (Goldsworthy and McFarlane 2002).

In 1944, the Russian-born biochemist and soil microbiologist Selman Abraham Waksman discovered an antibiotic product from soil bacterium *Streptomyces griseus*. The product, Streptomycin, was the first antibiotic useful for infections caused by Gram-negative bacteria (Kingston 2004).

From this discovery, many new classes of antibiotic had been discovered by the 1960s. The semisynthetic penicillins were introduced by Beecham in early 1960s included methicillin (1960), ampicillin (1961) and cloxacillin (1962). At the same time the cephalosporin C was established by Abraham and Newton (Rolinson 1998).

1.1.2. Mechanism of action of Antimicrobial drugs

Antimicrobial drugs have several mechanisms (Brooks 2007; Amyes 2010) include:

i) Interference with cell wall synthesis such as β-lactam antibiotics now include: penicillinase-resistant, amino-, carboxy-, indanyl-, and ureido-penicillins; first- to fifth-generation cephalosporins; monobactams; and carbapenems.

ii) Interference of protein synthesis: Aminoglycosides, Macrolides, Tetracyclines, Lincomycins, Chloramphenicol, Linezolid, Streptogramins.

iii) Interference with nucleic acid (DNA) synthesis by interfering with DNA gyrase and topoisomerase IV: Quinolones, Metronidazole.

iv) Inhibition of Ribonucleic acid (RNA) synthesis by acting on DNA-directed RNA polymerase: Rifamycins.

v) Inhibition of a metabolic pathway by acting on the synthesis of tetrahydropholic acid: Trimethoprim, Sulfamethoxazole.

vi) Disruption of bacterial membrane structure: Polymyxins.

1.1.3. β-Lactam Antibiotics

1.1.3.1. Structure

The β-lactam antibiotics can be divided into six different groups, the penicillins, cephalosporins, carbapenems, cephamycins, monobactams, and β-lactamase inhibitors (Smet, Martel *et al.* 2008). β-lactam antibiotics contain a β-lactam ring which is a heteroatomic ring structure consisting of three carbon atoms and one nitrogen atom. The β-lactam ring of natural or semisynthetic penicillins is fused with a thiazolidine ring. In cephalosporins, the β-lactam ring is merged with a dihydrothiazine ring. In the carbapenems, the β-lactam ring is combined with a hydroxyethyl side chain, lacking an oxygen or sulphur atom in the bicyclic nucleus. In contrast to the antibiotics, clavulanic acid, a β-lactamase inhibitor, is composed of a β-lactam ring fused with an oxazolidine ring and does not possessed an amide function (Livermore and Williams 1996; Amyes 2010).

In general, modifications of the R and R' groups alter the pharmacokinetic and antibacterial properties of β-lactam antibiotics; for example, substitutions at position 7 of cephalosporins increase the penetration into the periplasmatic space and the stability against β-lactamases, but may reduce antibiotic activity (Donowitz and Mandell 1988).

1.1.3.2. Mechanism of action of β-lactam antibiotics

β-Lactam antibiotics act on bacteria by inhibiting the bacterial enzymes, transpeptidases and carboxypeptidases, located in the cytoplasmic membrane which catalyses synthesis of the cross-linked peptidoglycan. These enzymes are commonly called penicillin-binding-proteins (PBPs) (Spratt 1994).

The cell wall of enterobacteria consists of an inner cytoplasmic membrane and outer layer consisting of lipopolysaccharides (LPS) and lipoproteins. LPS consists of lipid A, core polysaccharide, and O antigen. The periplasm is a space between the inner cytoplasmic membrane and outer lipid membrane. This space contains a loose network of peptidoglycan chains. A large layer of peptidoglycan found in the Gram-positive bacteria; Gram-negative bacteria have a much thinner layer of peptidoglycan surrounded by a lipid bilayer outer membrane. The individual peptidoglycan cell wall units are produced inside the cell, the final cross-linking is catalysed outside the cytoplasmic membrane by a group of membrane-anchored bacterial enzymes known as the cell-wall transpeptidases (Fisher, Meroueh *et al.* 2005; Wilke, Lovering *et al.* 2005).

Peptidoglycan is an essential component of the bacterial cell wall. It protects the organism from osmotic rupture, determines cell shape, and is integral to cell growth and division. The peptidoglycan is composed of a basic repeating unit of an alternating chains of disaccharide *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) linked by β-(1,4)-glycoside units. The carboxyl group of muramic acid is usually replaced by an aminoacid chain composed of four aminoacids. The most common are L-alanine, D-alanine, D-glutamic acid, D-glutamine and L-lysine or

diaminopimelic acid (DAP) (Tipper and Strominger 1965; Livermore and Williams 1996).

In the cross-linking reaction in Gram-negative bacteria, a peptide bond is formed between the D-alanine on one chain and the free amino end of a diamino pimelic acid on the other chain causing the elimination of the terminal D-alanine and designated the transpeptidase reaction. The β-lactam antibiotics are analogues of the terminal amino acid (D-alanyl-D-alanine) residues on the precursor NAM/NAG-peptide subunits of the peptidoglycan layer. In the presence of the β-lactam antibiotics, the transpeptidases and carboxypeptidases react with acyl-D-alanyl-D-alanine to form a lethal serine-ester-linked acyl (penicilloyl, cephalosporoyl) enzyme complex. The β-lactam-enzyme complex is very stable, and blocks the normal transpeptidation reaction. This result disrupts the synthesis of the cell wall and makes the growing bacteria highly susceptible to cell lysis and death (Tipper and Strominger 1965; Ghuysen 1988; Livermore and Williams 1996; Wilke, Lovering *et al.* 2005).

1.1.3.3. Mechanisms of resistance to β-lactam antibiotics

There are four major ways bacteria avoid the bactericidal effect of β-lactam antibiotics; alteration of penicillin binding proteins (PBPs), lack or diminished expression of outer membrane proteins (OMPs), active efflux pumps promoting the transport of the antibiotic from within the cell to the external environment, resulting in an intermediate level of resistance and finally, production of β-lactamases that hydrolyze the β-lactam ring and render the antibiotic inactive before it reaches the penicillin-binding protein (PBP) target.

1.1.3.3.1. PBPs modifications

PBPs are divided into two subgroups: low molecular mass (LMM) and high molecular mass (HMM) enzymes. The HMM enzymes are further subdivided into the bifunctional class A enzymes and the monofunctional transpeptidase class B enzymes (Wilke, Lovering *et al.* 2005). The penicillin-binding module contains three

conserved motifs that form the active cavity. They are the Ser-X-X-Lys (SXXK), the Ser-X-Asn (SXN), and the Lys-Thr/Ser-Gly (KT/SG) motifs (Hakenbeck 1998).

Alterations of PBPs have been described in both Gram-negative and Gram-positive organisms, assuming a more important role in Gram-positive bacteria. There are several PBP-mediated mechanisms of β-lactam resistance, including:

1- Point mutations altering an amino acid. In *E. coli*, at least three different amino acid substitutions within the transpeptidase domain of the PBP3, conferred at least seven-fold resistance to cephalexin but not to other cephalosporins, penicillins or monobactams. Amino acid modifications in one or two enzymes have very little effect on the resistance level (Hedge and Spratt 1985). Mutations in PBPs 1A, 2B, and 2X play an important role in the development of resistance to ß-lactam antibiotics by *S. pneumoniae* (Sanbongi, Ida *et al.* 2004).

2- The acquisition of foreign PBP resistant to β-lactam antibiotics; for example, the acquisition of PBP2a by methicillin-resistant *Staphylococcus aureus* confers resistance to β-lactam antibiotics (Hackbarth and Chambers 1989).

3- Recombination between susceptible PBPs and those of less susceptible species. This hybrid protein, originated in an interspecies homologous recombination, presents slightly less susceptibility to β-lactams (Dougherty 1986).

4- Overexpression of a PBP. When PBP5 is overexpressed, it is responsible for both natural insensitivity and acquired intrinsic resistance to penicillin in enterococci (Fontana, Cerini *et al.* 1983).

1.1.3.3.2. Permeability-based resistance

The outer membrane of Gram-negative bacteria plays an important role serving as a diffusion barrier for penetration of hydrophilic compounds and interacts with the bacterial environment. In Gram-positive bacteria the β-lactam can easily reach the cytoplasmic membrane, whereas in Gram-negative the crossing of the outer membrane is essentially done through protein channels, the porins (Nikaido 1989).

The porins are divided into two classes: specific and non-specific. In *E. coli,* OmpC, and OmpF, represent the non-specific porins that permit the general diffusion of small polar molecules. A loss of either of these porins has been related to antibiotic resistance (Nikaido 1989). The OmpA protein in *E. coli* is a homologue of the major porin of *P. aeruginosa* OprF (Nikaido 2003). In *Klebsiella* spp. OmpK36, OmpK35 and OmpK34 are the homologues of OmpC, OmpF and OmpA (Alberti, Rodriquez-Quinones *et al.* 1995; Domenech-Sanchez, Martinez-Martinez *et al.* 2003). *K. pneumoniae* strains usually express OmpK35 and OmpK36, the ESBL-producing strains commonly express only one of these, normally OmpK36, or no porin at all (Martinez-Martinez, Hernandez-Alles *et al.* 1996; Hernandez-Alles, Conejo *et al.* 2000). In addition to the major porins, minor porins have been described including OmpK37 from *K. pneumoniae*, OmpN from *E. coli* and OmpS2 from *Salmonella typhi*. The percentages of identity and similarity of OmpK37 with the OmpS2 and OmpN porins are 80 and 88% and 77 and 85%, respectively (Domenech-Sanchez, Hernandez-Alles *et al.* 1999). The outer membrane alterations in *K. pneumoniae* are not decisive factors in increasing resistance to antimicrobial agents, but porin loss co-operates with beta-lactamase production to increase resistance to beta-lactams (Hernandez-Alles, Conejo *et al.* 2000).

1.1.3.3.3. Efflux pump

The third mechanism involved in resistance to β-lactam antibiotics is the expression of efflux pumps. These proteins transport the antibiotic from within the cell to the external environment. A characteristic of efflux pumps is the variety of molecules

they may transport, due to poor substrate specificity. Thus, this multidrug efflux system plays an important role in providing resistance to a very wide range of compounds in Gram-negative bacteria (Nikaido 1998; Poole 2004).

Efflux was first described as a mechanism of resistance to tetracycline in *Escherichia coli* (McMurry, Petrucci *et al.* 1980); reviewed by Poole (2005). Bacterial efflux systems capable of accommodating antimicrobials generally fall into five classes, the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) family (Poole 2004; Poole 2005).

One of these multidrug efflux pumps is the resistance nodulation division (RND). Examples of this superfamily include the AcrAB system of *E. coli* and the MexAB-OprM system of *Pseudomonas aeruginosa*. The substrates of this family is diverse and includes antibiotics, dyes, and detergents (Nikaido 1998). However, members of the MFS, MATE and SMR families also show a limited ability to promote resistance to some biocides and antibiotics (Poole 2004).

1.1.3.3.4. Enzyme production

The fourth and most important mechanism of resistance to β-lactam antibiotics is the production of β-lactamase enzymes. The β-lactamases confer significant antibiotic resistance to their bacterial hosts by hydrolysis of the amide bond of the β-lactam ring. Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl (penicilloic or cephalosporoic) enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that utilize at least one active-site zinc ion. These enzymes are especially important in Gram-negative bacteria as they constitute the major defence mechanism against β-lactam-based drugs (Livermore 1995; Wilke, Lovering *et al.* 2005). Beta-lactamases have been designated as ''enzymes hydrolysing amides, amidines and other C-N bonds . . . separated on the basis of the substrate: . . . cyclic amides'' (Bush, Jacoby *et al.* 1995).

Emergence of resistance to β-lactam antibiotics began even before the development of penicillin. The first identification of β-lactamase enzyme was isolated in *E. coli* before the use of penicillin in medical practice (Abraham and Chain 1940). Many genera of Gram-negative bacteria naturally possess chromosomal-mediated β-lactamase. These enzymes and penicillin-binding proteins (PBPs) are thought to have evolved from a common ancestor, which probably assist the bacteria in competition with other naturally producing β-lactams bacteria (Ghuysen 1991). The first plasmid-mediated β-lactamase in Gram-negatives, TEM-1, was described in 1965 (the designation "TEM" came from the patient's name, Temoniera) (Datta and Kontomichalou 1965). At the same time, another plasmid-mediated β-lactamase, known as "SHV-1" (sulfhydryl variable), was found in *K. pneumoniae* and *E. coli*. (Paterson and Bonomo 2005). The genes encoding β-lactamases can be located on the bacterial chromosome, on plasmids, on transposons, or on integrons. These mobile genetic elements enable β-lactamases dissemination to other members of the *Enterobacteriaceae* family, *H. influenzae, N. gonorrhoeae, and P. aeruginosa*, and rise the incidence of multi-drug resistant bacteria with complex resistance patterns (Bradford 2001; Weldhagen 2004; Paterson and Bonomo 2005). The mutations in TEM and SHV β-lactamases gave them expanded spectrum of activity against oxyimino-β-lactams (oxyimino-cephalosporins), and then these enzymes were called extended-spectrum β-lactamases (ESBLs) (Bradford 2001; Gniadkowski 2008).

1.1.4. Extended spectrum β-lactamases

Extended spectrum β-lactamases (ESBLs) are "β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid" (Paterson and Bonomo 2005). ESBLs are located in two subgroups of group 2, namely subgroups 2be (extended spectrum β-lactamases; Ambler's class A enzymes) and 2d (cloxacillin-hydrolyzing β-lactamases; Ambler's

class D ESBLs) according to the β-lactamase functional classification scheme (Ambler, Coulson *et al.* 1991; Bush, Jacoby *et al.* 1995).

The first ESBL was identified by Knothe, Shah *et al.* (1983) in a nosocomial *K. pneumoniae* strain isolated in Germany in 1983; since then, over 500 variants of the clavulanic acid-inhibited form (TEM, SHV, CTX-M, OXA) have been described worldwide (http://www.lahey.org/studies/webt.htm). They are most prevalent in *Klebsiella* spp., and their epidemiology reflects a mixture of mutations, plasmid transfer and or clonal spread (Livermore and Woodford 2006). A single clone has spread among 14 French hospitals, including a serotype K25 *K. pneumoniae* with SHV-4 (Arlet, Rouveau *et al.* 1994).

The most common ESBL phenotypes arise from point mutations in the bla_{TEM}, bla_{SHV}, or bla_{CTX} genes resulting in alterations of the primary amino acid sequence of the enzyme (Bradford 2001; Gniadkowski 2001; Paterson and Bonomo 2005; Paterson 2006). These mutations usually occurred at position 104 (TEM), 146 (SHV), 156 (SHV), 164 (TEM), 167 (CTX-M), 169 (SHV), 179 (SHV and TEM), 205 (TEM), 237 (TEM), 238 (SHV and TEM) and 240 (TEM, SHV and CTX-M) (Gniadkowski 2008). Many of the organisms that harbour ESBLs are also resistant to other classes of antibiotics, such as aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and sulfonamides (Bonnet 2004).

1.1.4.1. Classification and nomenclature of β-lactamases

Several attempts have been developed to classify β-lactamases. The first scheme to classify β-lactamases was proposed in 1968 to group it into penicillinases and cephalosporinases (Sawai, Mitsuhashi *et al.* 1968). In 1970, another classification scheme proposed by Jack and Richmond (1970), which was updated in 1973 by Richmond and Sykes (1973). This scheme classifies the β-lactamases from Gram-negative into five groups based on whether this enzyme hydrolysed penicillin more or less rapidly than cephaloridine and whether its activity was inhibited by cloxacillin and/or *p*-chloromercuribenzoate. In 1976, Sykes and Matthew (1976)

extended this last scheme to include plasmid-mediated β-lactamases differentiated by isoelectric focusing (IEF). After that, Mitsuhashi and Inoue (1981) proposed a scheme where they added the category cefuroxime-hydrolyzing β-lactamase to the penicillinase and cephalosporinase. Molecular structure classification was first proposed by Ambler (1980) with two classes. Class A including PC1 β-lactamase from *S. aureus* and class B metallo-β-lactamase II from *B. cereus*. Furthermore, Jaurin and Grundstrom (1981) described class C cephalosporinases from *E. coli* K-12. The designation of class D β-lactamase were segregated from the other serine β-lactamases after sequencing of PSE-2 and OXA-1 hydrolyzing carbencillin and oxacillin (Ouellette, Bissonnette *et al.* 1987; Huovinen, Huovinen *et al.* 1988).

One of the most used classification schemes is Ambler's (Ambler, Coulson *et al.* 1991) based upon amino acid sequences. He classified the β-lactamases into four molecular classes, A, B, C and D. Moreover, Bush, Jacoby *et al.* (1995) extended his classification scheme of 1989 (Bush 1989a; Bush 1989b; Bush 1989c) attempted to correlate the functional characteristics with the molecular structure recognizes four major β-lactamase classes, one of which (Group 2) is split into eight subgroups. Finally, Bush and Jacoby (2010) updated his classification scheme of 1995 by adding new functional subgroups to the scheme as a result of identification of new major β-lactamase families variants (Table 1.1).

β-lactamases are categorized based on similarity in amino acid sequence (Ambler classes A through D) or on substrate and inhibitor profile (Bush-Jacoby-Medeiros Groups 1 through 4) classification schemes are widely accepted. As such, this work also follows these classification schemes.

Table 1.1: β-lactamase classification schemes modified from (Bush and Jacoby 2010):

Ambler class	Bush-Jacoby group	Distinctive substrates	Inhibited by		Representative enzymes
			CA / TZB	EDTA	
C	1	Cephalosporins	-	-	AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
C	1e	Cephalosporins	-	-	GC-1, CMY-37
A	2a	Pencillins	+	-	PC1
A	2b	Pencillins, early cephalosporins	+	-	TEM-1, TEM-2, SHV-1
A	2be	Extended-spectrum cephalosporins, monobactams	+	-	TEM-3, SHV-2, CTX-Ms, PER, VEB
A	2br	Penicillins	-	-	TEM-30, SHV-10
A	2ber	Extended-spectrum cephalosporins, monobactams	-	-	TEM-50
A	2c	Carbencillin	+	-	PSE-1, CARB-3
A	2ce	Carbencillin, cefepime	+	-	RTG-4
D	2d	Cloxacillin	V	-	OXA-1, OXA-10
D	2de	Extended-spectrum cephalosporins	V	-	OXA-11, OXA-15
D	2df	Carbapenems	V	-	OXA-23, 0XA-48
A	2e	Extended-spectrum cephalosporins	+	-	CEPA
A	2f	Carbapenems	V	-	KPC-2, IMI-1, SME-1
B	3a (B1)	Carbapenems	-	+	IMP-1, VIM-1, IND-1, CcrA
	(B2)				L1, CAU-1, GOB-1, FEZ-1
B	3b (B3)	Carbapenems	-	+	CphA, Sfh-1
Unkown	4	-			

(V), Variable, (+), Yes, (-), No, CA, Clavulanic acid, TZB, Tazobactam

1.1.4.2. Active site

The β-lactamases are divided into two classes; serine and metallo β-lactamases that do not share sequence or structural homology. In classes A, C and D an active site serine and a molecular mass of approximately 29,000 Da, is responsible for the β-lactam hydrolysis (Bradford 2001). The three classes of serine β-lactamases, A, C and D share similarity on the protein structure level, which proves that they are derived a common ancestor (Hall and Barlow 2004). In class B metallo β-lactamases need a bivalent cation, usually zinc, to be able to catalyse the β-lactam ring (Garau, Garcia-Saez *et al.* 2004; Walsh 2005; Bebrone 2007)

1.1.5. The clinically most important β-lactamases

1.1.5.1. Class A β-lactamases

1.1.5.1.1. TEM β-lactamases

The TEM family of ESBLs constitutes the largest and widely disseminated group of these enzymes. Their evolutionary precursors are the TEM-1 and TEM-2 penicillinases (Livermore 1995; Medeiros 1997; Bradford 2001). TEM-1, was first reported in 1965 from an *E. coli* isolate (Datta and Kontomichalou 1965). Plasmid-mediated TEM-1 is the most prevalent β-lactam inactivating enzyme found in enteric bacilli especially in *E. coli* and *K. pneumoniae*, they are also found with increasing frequency in other Gram-negative species (Liu, Gur *et al.* 1992; Bradford 2001). TEM-1, is able to hydrolyse ampicillin more than carbenicillin, oxacillin, or cephalothin, and is inhibited by clavulanic acid. TEM-1 is encoded by a series of gene alleles, bla_{TEM-1A} to bla_{TEM-1F}, which differ from each other by specific silent mutations. Each of these genes could initiate a separate evolutionary lineage of mutant derivatives (Leflon-Guibout 2000).

TEM-2, the first derivative of TEM-1, had a single amino acid substitution at position 39 (Gln39→Lys) from the original TEM-1 β-lactamase. This substitution

changes the isoelectric point from 5.4 in TEM-1 to 5.6 in TEM-2. The *bla*TEM-2 gene possesses a stronger promoter than the promoter of the *bla*TEM-1 gene which confers a higher enzymatic activity when compared to TEM-1 producing strains (Jacoby and Carreras 1990). TEM-1a, TEM-1b and TEM-2 are encoded in transposable elements such as transposons, Tn*3*, Tn*2* and Tn*1*, respectively (Datta and Kontomichalou 1965; Partridge and Hall 2005).

TEM-3, originally reported in 1987, was the first TEM-type β-lactamase that displayed the ESBLs phenotype. Sequencing of TEM-3 revealed that the enzyme was related to TEM-2 and differed by two amino acids from its parent TEM-2 enzyme: Lys→Glu at position 102 and Ser→Gly at position of 236 (Sougakoff, Goussard *et al.* 1988).

One to five point mutations are especially important for producing the ESBL phenotype and extend their hydrolytic spectra; they include amino acid substitutions at positions Asp104, Arg164, Ala237, Gly238 and Glu240 (Bradford 2001; Bonnet 2004). The mutations at position Gly238 in the active site pushes the β-strand out and away from the reactive Ser70 residue, resulting in a slightly expanded active site, which could improve binding and accommodate cephalosporins with bulky side-chains. Ser238 is critical for cefotaxime hydrolysis whereas both Ser238 and Lys240 are needed for strong ceftazidime hydrolysis (Huletsky, Knox *et al.* 1993). Although mutation Glu104→Lys improves the ability of the enzyme to hydrolyse cephalosporins and monobactams, it is not sufficient to confer true resistance and is always found in clinical isolates associated with at least one mutation at another part of the active site. The Glu104→Lys substitution modifies the precise positioning of the SDN loop, which is involved in the binding and catalysis of the substrate (Petit, Maveyraud *et al.* 1995). Moreover, amino acid substitutions at position Arg164 are the most common mutations in TEM enzymes. Substitutions with either serine or histidine remove the ionic bonds that arginine has made with acidic residues and this allows the Ω loop to move to one side thus opening the active site for bulky β-lactam variants and increased catalytic efficiency for cefotaxime, ceftazidime and aztreonam (Sowek, Singer *et al.* 1991). In addition, the substitution Ala237→Thr is found in

TEM-5 and TEM-24 and it improves binding of the enzyme to cefotaxime by facilitating the creation of a hydrogen bond between the Thr237 and the cefotaxime molecule (Knox 1995).

TEM enzymes confer a phenotype of resistance to β-lactamase inhibitors conferring an inhibitor resistant TEM (IRT) phenotype. Currently, more than 28 bla_{TEM} gene variants are resistant to inhibitors like clavulanic acid (http://www.lahey.org/studies/). Amino acid substitutions in TEM at positions Met69, Ser130, Arg244, Arg275 and Asn276 are usually associated with the resistance to β-lactamase inhibitors (Canton, Morosini *et al.* 2008; Drawz and Bonomo 2010).

Although inhibitor-resistant TEM (IRT) enzymes generally confer less resistance to oxyimino-cephalosporins than parental enzymes, a few enzymes have hydrolytic activity against oxyimino-cephalosporins together with inhibitor resistance. These enzymes are referred to as complex mutants of TEM (CMT). A CMT enzyme possesses both of the amino acid substitutions observed in TEM ESBLs and those observed in inhibitor-resistant TEMs (Canton, Morosini *et al.* 2008). First enzyme of this group described in France in 1997, CMT-1 (TEM-50), presented the ESBL substitutions of TEM-15 and the IRT-substitutions of IRT-4 (Sirot, Recule *et al.* 1997). Another example, TEM-125 (CMT-) enzyme combines the amino acid substitutions of TEM-12 (ESBL) and those of inhibitor-resistant TEM-39 (Robin, Delmas *et al.* 2006). Other enzymes presenting amino acid substitutions of ESBL and IRT enzymes were also described in Poland, Portugal and other regions of France (Fiett, Palucha *et al.* 2000; Poirel, Mammeri *et al.* 2004; Machado, Coque *et al.* 2007). Currently, about 9 CMT gene variants are reported (http://www.lahey.org/studies/). CMT-type β-lactamases are a great challenge in detection of ESBLs in clinical laboratories because phenotypic detection methods of ESBLs depend on the inhibition of ESBLs by β-lactamase inhibitors, which is absent in CMT-type β-lactamases (Canton, Morosini *et al.* 2008; Drawz and Bonomo 2010).

1.1.5.1.2. SHV β-lactamases

SHV enzymes are classified in groups 2b and 2be of the Bush-Jacoby-Medeiros classification scheme and in Ambler class A (Ambler, Coulson *et al.* 1991; Bush, Jacoby *et al.* 1995). SHV refers to sulfhydryl variable. "This designation was made because it was thought that SHV hydrolysed cephaloridine but not benzylpencillin in the presence of p-chloromercuribenzoate, inhibitory sulfhydryl binding chemical (Matthew 1979; Tzouvelekis and Bonomo 1999). This activity was never confirmed in later studies with purified enzyme of the SHV-type β-lactamases (Paterson and Bonomo 2005). SHV-1 was first described in 1972 and called Pit-2 from the author's name Pitton (Pitton 1972). SHV-1 confers resistance to ampicillin, amoxicillin, carbenilcillin and ticarcillin (Livermore 1995).

SHV-1 share 84-88.9% and 91.8% amino acid sequence similarity with LEN-1 (a chromosomally encoded β-lactamase and *K. pneumoniae* species-specific penicillinase) and OHIO-1 (a plasmid encoded β-lactamase) respectively. Whereas the amino acid sequence identity between SHV-1 and TEM-1 is 63.7-67%. The SHV-1 substrate binding cavity is 0.7-1.2 Å larger than in TEM-1 (Heritage, M'Zali *et al.* 1999; Kuzin, Nukaga *et al.* 1999; Tzouvelekis and Bonomo 1999; Reynolds, Thomson *et al.* 2006).

A chromosomal copy of bla_{SHV-1} or bla_{SHV-11} or close relatives, encoding non-extended-spectrum enzymes, considered as native to the great majority of *K. pneumoniae* strains (Babini and Livermore 2000; Ford and Avison 2004; Lee, Cho *et al.* 2006). Other reports considered the SHV-1 β-lactamase is most commonly found in *K. pneumoniae* and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species (Tzouvelekis and Bonomo 1999). SHV enzymes were also detected in other *Enterobacteriaceae* as a plasmid-mediated β-lactamases (Matthew 1979; Sabate, Miro *et al.* 2002).

Plasmid-mediated bla_{SHV} genes are possibly mobilized from genome to plasmid mediated by IS*26* (Ford and Avison 2004). IS*26* is widely distributed among

plasmids facilitating the mobilization of chromosomal sequences containing resistance genes. Also, IS*26* was found associated with a class 1 integrons which is considered as a critical step in the evolution of diverse multiresistance plasmids found in clinical enterobacteria (Miriagou, Carattoli *et al.* 2005). IS*26* is an 820-bp long insertion sequence that typically generates 8 bp target duplication upon transposition (Mollet, Iida *et al.* 1983). It is demonstrated for IS*1* that the length of target site duplication sequences may vary according to the sequence of the integrated site (Machida and Machida 1987). It is believed that the IS*26* insertion increases promoter strength through the introduction of a different –35 region (Podbielski, Schonling et al. 1991a). IS*26* insertion was found 2 kbp upstream in the plasmid-mediated SHV-5 enzyme (Gutmann, Ferre *et al.* 1989). In plasmid-mediated SHV-2a, SHV-11 and SHV-12, IS*26* insertion was identified into the *bla*SHV promoter (Podbielski, Schonling et al. 1991b; Nuesch-Inderbinen, Kayser et al. 1997).

The first of these enzymes capable of hydrolyzing the newer beta-lactams, SHV-2, was found in a single strain of *K. ozaenae* isolated in Germany (Knothe, Shah *et al.* 1983). Sequencing of the SHV-2 gene showed that only one amino-acid substitution of Gly238→Ser was differed from SHV-1 (Kliebe, Nies *et al.* 1985). The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a glycine for serine at position 238, glutamic acid for lysine at position 240 (Huletsky, Knox *et al.* 1993; Kurokawa, Yagi *et al.* 2000; Bradford 2001; Hujer, Hujer *et al.* 2002).

The serine residue at position 238 is crucial for effective hydrolysis of ceftazidime. The Gly238→Ser pushes the β-strand out and away from the active site Ser70, which expands the active site even more when compared to TEM, and permits greater substrate versatility against penicillins and cephalosporins. These findings were confirmed by site-directed mutagenesis studies (Huletsky, Knox *et al.* 1993; Hujer, Hujer *et al.* 2002). Furthermore, the lysine residue at position 240 is responsible for hydrolysis of cefotaxime. Resistance to this antibiotic is increased in enzymes such

as SHV-5 and SHV-12, which possess at least both Gly238→Ser and Glu240→Lys substitutions (Huletsky, Knox *et al.* 1993; Nuesch-Inderbinen, Kayser *et al.* 1997).

Also, the residue Asp179 is positioned in the Ω loop of the SHV active site. Amino acid substitution of Asp179→Ala (SHV-6), Asn (SHV-8) and Gly(SHV-24) possibly increase the movement of the Ω loop, expanding the binding site and destabilizing the Glu166 (Knox 1995). This substitution conferred high-level resistance to ceftazidime but not to cefotaxime (Kurokawa, Yagi *et al.* 2000).

Contrary to TEM β-lactamases, there are few SHV that confer resistance to β-lactamase inhibitors. Until now, only few (five) enzymes were detected which present mutations that can confer loss of susceptibility to β-lactamase inhibitors (Drawz and Bonomo 2010). For example, amino acid substitutions of residues Met69→Ile (SHV-49) (Dubois, Poirel *et al.* 2004), Ser130→Gly (SHV-10) (Prinarakis, Miriagou *et al.* 1997) and Ala187→Thr (SHV-26) (Chang, Siu *et al.* 2001) confer an increase of resistance against clavulanic acid associated with the loss of affinity to penicillins and narrow-spectrum cephalosporins. By site-saturation mutagenesis, SHV enzymes in vitro do no benefit proportionally from the simultaneous presence of amino acid substitutions in residues 69, 244 and 276 of naturally occurring in TEM enzymes (Randegger and Hachler 2001).

1.1.5.1.3. CTX-M β-lactamases

In the second half of the 1980s, non-TEM and non-SHV plasmid class A ESBLs enzymes have been reported, among them the CTX-M type β-lactamases (active on Cefotaxime) (Bonnet 2004). CTX-M first detected in Japan in 1986 from a cefotaxime-resistant *E. coli* (the enzyme was named FEC-1) (Matsumoto, Ikeda *et al.* 1988). A few years later in 1989, a similar cefotaxime-resistant clinical *E. coli* strain from Germany was reported to produce β-lactamase enzyme designated CTX-M-1 (Bauernfeind, Grimm *et al.* 1990). In 1992, a new plasmid-mediated cefotaximase, designated CTX-M-2, was described from multidrug-resistant *Salmonella* t*yphimurium* isolated in 1990 in Argentina (Bauernfeind, Casellas *et al.* 1992). At the

same year, the same type of ESBL was reported in clinical *E. coli* strain MEN, isolated at the beginning of 1989 in France and designated MEN-1 (Barthelemy, Peduzzi *et al.* 1992). The CTX-M β-lactamases constitute one of the most rapidly growing ESBL families. Over the last decade CTX-M types have replaced TEM and SHV mutants as the predominant ESBLs in numerous countries of Africa, Europe, South America and Asia. The CTX-M enzymes have been identified worldwide (Baraniak, Fiett *et al.* 2002; Saladin, Cao *et al.* 2002; Livermore and Woodford 2006; Livermore, Canton *et al.* 2007).

The CTX-M family now contains 96 enzymes (http://www.lahey.org/studies/) and subclassified by amino acid similarities into 5 groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 in which the members of each group share 94% identity (Bonnet 2004). In contrast to TEM and SHV families, which were originated by amino acid substitutions of their parent enzymes TEM-1, TEM-2 and SHV-1, the CTX-M ESBLs were acquired by the horizontal gene transfer from other bacteria. The gene sequences encoding the regions surrounding the plasmid-mediated CTX-M enzymes show high identities to those of the regions surrounding *kluyvera georgiana* chromosomal bla_{KLUG} genes or the *K. ascorbata* chromosomal bla_{KLUA} genes suggesting the mobilization of this type of β-lactamase-encoding gene from the chromosome to plasmids. Thus, it is considered that the CTX-M-8 and CTX-M-9 probably originated from *Kluyvera georgiana* bla_{KLUG} (Poirel, Kampfer *et al.* 2002; Olson, Silverman *et al.* 2005), whereas the CTX-M-1 and CTX-M-2 family evolved from *K. ascorbata* chromosomal genes bla_{KLUA} (Humeniuk, Arlet *et al.* 2002; Rodriguez, Power *et al.* 2004).

The CTX-M enzymes confer higher level resistance to cefotaxime, ceftriaxone and aztreonam than to ceftazidime (Dutour, Bonnet *et al.* 2002; Saladin, Cao *et al.* 2002). However, some point mutations produced enzymes with a much stronger activity against ceftazidime. For example, CTXM-15 was derived from CTX-M-3 by a Asp240→Gly substitution, which increased catalytic activity to ceftazidime (Poirel, Gniadkowski *et al.* 2002). The Asp240→Gly substitution was observed in CTX-M-32 and CTX-M-53 (CTX-M group 1) presenting higher catalytic activity against

ceftazidime (Cartelle, del Mar Tomas *et al.* 2004; Doublet, Granier *et al.* 2009). CTX-M-27 and CTX-M-16, differing from CTX-M-9 and CTX-M-14 respectively by the Asp240→Gly mutation, also present higher catalytic efficiencies against ceftazidime (Bonnet, Dutour *et al.* 2001; Bonnet, Recule *et al.* 2003). Asp240→Gly substitution appears to increase the flexibility of B3 β-strand and synchronized motions of the β3 strand with the reactive Ser70 and residues 167 to 170 of the Ω-loop. These modifications allow a deep insertion of ceftazidime in the catalytic pocket and increase activity against ceftazidime (Chen, Delmas *et al.* 2005; Delmas, Chen *et al.* 2008).

Another substitution responsible for higher levels of resistance to ceftazidime, as compared to cefotaxime, is the Pro167→Ser mutation, which differentiates CTX-M-19 from CTX-M-14 (Poirel, Naas *et al.* 2001). The mutation at Pro167 in the Ω-loop modifies the interaction between β-lactams with Ser237 on the β3 strand and Ser130 on the SXN loop sites, which are fairly distant from the mutation site. The hydrogen bond of the C-4 carboxylate of ceftazidime with Ser130 of CTX-M-19 may well correlate with the strong ability of CTX-M-19 to hydrolyze ceftazidime (Kimura, Ishiguro *et al.* 2004).

CTX-M enzymes are susceptibile to β-lactamase inhibitors, although a low-level of resistance to the combination of clavulanic acid with amoxicillin and ticarcillin could be observed (Bonnet 2004). CTX-M-14 was able to hydrolye sulbactam, clavulanate and tazobactam retain their ability to inactivate this enzyme (Ishii, Galleni *et al.* 2007).

The ability of insertion sequences (IS*Ecp1,* IS*CR,* IS*26,* IS*10 and* IS*903*) to mobilize and to promote the expression of β-lactamase genes may explain the current spread of CTX-M-type enzymes worldwide. The insertion sequence IS*Ecp1* was firstly identified upstream of *bla*$_{CMY-4}$ from an *E. coli* isolate from the United Kingdom (Eckert, Gautier *et al.* 2006)The insertion sequences IS*Ecp1* or IS*Ecp1*-like are involved in the mobilization of *bla*$_{CTX-M}$ genes and have repeatedly been observed upstream of ORFs encoding the several *bla*$_{CTX-M}$ genes belonging to the CTX-M-1,

CTXM-2, CTX-M-9, and CTX-M-25 subgroups. Also, the IS*Ecp1* element provides the promoter for expression of bla_{CTX-M} genes (Karim, Poirel *et al.* 2001; Saladin, Cao *et al.* 2002; Poirel, Decousser *et al.* 2003; Eckert, Gautier *et al.* 2006; Poirel, Naas *et al.* 2008). The IS*Ecp1* belongs to the IS*1380* family and is capable of mobilizing the neighboring genes by one-ended transposition mechanism. The mobilization of $bla_{CTX-M-2}$ from *K. ascorbata* to *E. coli* was achievable in the presence of IS*Ecp1* (Lartigue, Poirel *et al.* 2006).

The IS*CR* (IS common regions) (formerly called orf513) was identified as being closely associated with the spread of many antibiotic resistance genes (Canton and Coque 2006; Toleman, Bennett *et al.* 2006; Poirel, Naas *et al.* 2008). The $bla_{CTX-M-2}$ gene was possibly acquired by a plasmid through *Orf513*, the so-called CR (common region) element. The IS*CR1* is strongly associated with the emergence and dissemination of the $bla_{CTX-M-2}$ gene in all $bla_{CTX-M-2}$-containing Gram-negative isolates in Argentina (Arduino, Roy *et al.* 2002; Arduino, Catalano *et al.* 2003). In Europe, $bla_{CTX-M-9}$ is most commonly associated with IS*CR1* elements as part of a complex class 1 integron, *In60* (Toleman, Bennett *et al.* 2006). IS*CR* elements can mobilize large sections of adjacent DNA via a rolling circle replication mechanism. Different bla genes, including bla_{CTX-M}, bla_{CMY}, bla_{DHA-1}, bla_{IMP-1}, bla_{VEB}, bla_{VIM-1} and *qnrA* as well as other resistance genes have been associated with IS*CR1*. IS*CRs* are also associated with *Salmonella enterica* serovar *Typhimurium* pathogenecity genomic islands (SGIs) and on conjugative transposons encoding sulfamethoxazole, trimethoprim and streptomycin resistance genes from *Vibrio cholerae* (Toleman, Bennett *et al.* 2006). IS*CR* elements can be divided into two groups: IS*CRs1* are those that form complex class 1 integrons and IS*CRs-2* to *-13* are those associated with non-class 1 integrons (Bebrone 2007).

The insertion sequence IS*26* was described by Saladin, Cao *et al.* (2002) to be upstream of a $bla_{CTX-M-1}$ gene. The presence of IS*26* in all isolates probably due to the presence of this insertion sequence on known plasmids, such as IncFII, IncN, and IncL/M (Novais, Canton *et al.* 2007). In addition, IS*10* mobile element has been observed upstream of $bla_{CTX-M-8}$ (Bonnet, Sampaio *et al.* 2000), an IS*903*-like

element was observed downstream of the $bla_{CTX-M-14}$ and $bla_{CTX-M-17}$ genes (Pai, Choi *et al.* 2001; Cao, Lambert *et al.* 2002). Furthermore, the presence of a phage-related sequence was identified immediately upstream of $bla_{CTX-M-10}$ in several CTX-M-10-producing isolates in Spain suggesting that bacteriophage might be involved in an acquisition of $bla_{CTX-M-10}$ (Oliver, Coque *et al.* 2005).

Plasmids encoding CTX-M enzymes are often highly transmissible that enable the genes to be transferred from one bacterial species to another and from one genus to another. The genes responsible for CTX-M β-lactamases are encoded by plasmids belonging to the narrow host-range incompatibility types (IncFI, IncFII, IncHI2 and IncI) or the broad host-range incompatibility types (IncN, IncP1, IncL/M and IncA/C) (Canton and Coque 2006; Novais, Canton *et al.* 2007; Coque, Novais *et al.* 2008; Carattoli 2009)

Isolates producing CTX-M enzymes were reported worldwide, especially CTX-M-15, CTX-M-14, CTX-M-3, CTX-M-2, and CTX-M-9. In the United Kingdom the CTX-M-15 has become the most prevalent enzyme among *E. coli* strains during the last couple of years. CTX-M-1 co-existed initially with isolates harbouring CTX-M-9, CTXM- 14, and SHV-12, and to some extent with TEM (Livermore, Canton *et al.* 2007). In Spain the high prevalence of CTX-M-9, CTX-M-14, CTX-M-15 and CTX-M-10 has been reported in several studies of the *E. coli*, *K. pneumoniae* and *Salmonella* spp. isolates (Hernandez, Martinez-Martinez *et al.* 2005; Oliver, Coque *et al.* 2005; Valverde, Coque *et al.* 2008; Oteo, Cuevas *et al.* 2009). A great variety of CTX-M enzymes has been identified in French hospitals including CTX-M-1, CTX-M-2, CTX-M-3, CTX-M-9 CTX-M-14, CTX-M-15, CTX-M-21 and CTX-M-27 ESBLs characterized (Dutour, Bonnet *et al.* 2002; Saladin, Cao *et al.* 2002; Arpin, Dubois *et al.* 2003; Eckert, Gautier *et al.* 2004; Arpin, Quentin *et al.* 2009). In the eastern European countries $bla_{CTX-M-3}$ is the predominant enzyme. CTX-M-15 also identified with a lesser extent (Gniadkowski, Schneider *et al.* 1998; Edelstein, Pimkin *et al.* 2003; Empel, Baraniak *et al.* 2008). Additionally, the CTX-M-15 is the most-reported CTX-M type in Scandinavian countries (Lytsy, Sandegren *et al.* 2008).

In Canada, a similar CTX-M prevalence to the European countries with a degree of diversity among CTX-M enzyme types, with CTX-M-14 and CTX-M-15 being the most abundant, while isolates producing CTX-M-2, -3, -24, 13 and -27 were relatively rare (Mulvey, Bryce *et al.* 2004; Pitout, Church *et al.* 2007). CTX-M ESBLs were first reported in 2002 in five states (Virginia, Idaho, Ohio, Washington, and Texas) in the United States (Moland, Black *et al.* 2003). However, CTX-M-encoding genes including CTX-M-14, CTX-M-15 and CTX-M-3 considered rare in the United States (Castanheira, Mendes *et al.* 2008; Hanson, Moland *et al.* 2008).

In South America, CTX-M-2, was described from multidrug-resistant *Salmonella* ty*phimurium* isolated in 1990 in Argentina (Bauernfeind, Casellas *et al.* 1992). CTX-M-2 is the most common and has been broadly found in Argentina, Uruguay, Paraguay and Brazil. Also, CTX-M-8 and -9 have been found in Brazil (Bonnet 2004). *E. coli* producing CTX-M-15 and CTX-M-9 variants (CTX-M-14 and CTX-M-24) were predominant among community isolates from Bolivia and Peru (Pallecchi, Bartoloni *et al.* 2007).

In the Far East, CTX-M-2 and CTX-M-3 were predominant from Japan (Yamasaki, Komatsu *et al.* 2003), while in China, CTX-M-9, CTX-M-13 and CTX-M-14 enzymes were already the most frequent ESBL in 1997 and 1998 (Chanawong, M'Zali *et al.* 2002). In India, the CTX-M-15 considered as the most successful and widespread CTX-M among others. CTX-M-15 was detected in a two month period of the year 2000 from *E. coli*, *K. pneumoniae* and *Enterobacter aerogenes* (Karim, Poirel *et al.* 2001).

1.1.5.2. Class A carbapenemases

The molecular class A carbapenemases, including the plasmid-mediated serine β-lactamases KPC (for "*K. pneumoniae* carbapenemase") and GES (Guiana extended spectrum) and the chromosomally encoded SME (for "*Serratia marcescens* enzyme) and IMI/NMC (imipenem-hydrolyzing β-lactamase/not metalloenzyme carbapenemase) enzymes. The class A carbapenem enzymes have the ability to

hydrolyze a broad spectrum of antibiotics, including carbapenems, cephalosporins, penicillins, and aztreonam, and all are inhibited by clavulanic acid and tazobactam (Queenan and Bush 2007).

The chromosomal β-lactamases SME and IMI/NMC enzymes have been detected in rare clinical isolates among *Serratia marcescens* and *Enterobacter cloacae* isolates (Queenan and Bush 2007).

Plasmid-mediated KPC was first detected in a *K. pneumoniae* clinical isolate from North Carolina in 1996 (Yigit, Queenan *et al.* 2001). KPC has become common in the east coast of the United States especially after new variants KPC-2 and KPC-3 were reported from isolates of *K. pneumoniae* in Baltimore, MD and New York, NY respectively (Moland, Hanson *et al.* 2003; Woodford, Tierno *et al.* 2004). Out side USA, KPC enzymes were reported from *E. coli* isolates from Israel (Navon-Venezia, Chmelnitsky *et al.* 2006), from *S. marcescens*, *K. pneumoniae*, and *E. coli* in China (Wei, Du *et al.* 2007; Cai, Zhou *et al.* 2008), and from *K. pneumoniae* in Europe (Naas, Nordmann *et al.* 2005; Woodford, Zhang *et al.* 2008; Fontana, Favaro *et al.* 2010; Wendt, Schutt *et al.* 2010), and from *K. pneumoniae* and *Pseudomonas* spp. from Central and South America (Villegas, Lolans *et al.* 2006; Villegas, Lolans *et al.* 2007). So far eleven variants are known (http://www.lahey.org/studies/), distinguished by one or two amino-acid substitutions.

Enzymes of the GES type, also called IBC (integron-borne cephalosporinase), is an infrequently encountered family that was first described in 2000 from *K. pneumoniae* from French Guiana (Poirel, Le Thomas *et al.* 2000) and from an *E. cloacae* isolate in Greece (Giakkoupi, Tzouvelekis *et al.* 2000). GES-1 presents activity against penicillins and extended-spectrum cephalosporins. This enzyme does not hydrolyse aztreonam, cephamycins and carbapenems and inhibited by β-lactamase inhibitors (Naas, Poirel *et al.* 2008). The GES-2 variant was reported from a clinical isolate of *P. aeruginosa* isolated in 2000 in South Africa from blood cultures. GES-2 had a single amino acid substitution Gly170→Asn inside the omega loop of the catalytic site resulting in slow carbapenem hydrolysis, and is less inhibited by clavulanic

(Poirel, Weldhagen *et al.* 2001). Just like GES-2, other variants GES-4, GES-5, and GES-6 and GES-8 have Gly170→Asn substitution associated with imipenem hydrolysis. GES enzymes have been reported worldwide from Greece, France, Portugal, South Africa, French Guiana, Brazil, Argentina, Korea, and Japan (Queenan and Bush 2007; Naas, Poirel *et al.* 2008).

1.1.5.3. Class B Metallo β-lactamases

The molecular class B enzymes 'metallo-β-lactamases' were first to distinguish from serine β-lactamases in 1980 by Ambler (1980). Metallo-β-lactamases (MBLs) required one or two zinc ions for their activity. They hydrolyse penicillin, cephalosporins and carbapenems but not monobactams. The metallo β-lactamases are EDTA-inhibited enzymes and they are resistant to β-lactamase inhibitors. The metallo β-lactamases are subdivided on the basis of sequence alignments into three subclasses B1, B2 and B3. Phylogenetic studies suggest that B1 and B2 descend from a common ancestor and subclass B3 shares only structural similarities with these subclasses. B1 and B3 are able to bind one or two zinc ions, B2 are mono-Zn enzymes that have evolved specificity toward carbapenems. The families of acquired MBLs enzymes have been identified until now are, IMP (active on imipenem), VIM (Verona integron-encoded metallo β-lactamase), GIM (German imipenemase), SPM (São Paulo MBL), and SIM (Seoul imipenemase) (Garau, Garcia-Saez *et al.* 2004; Walsh 2005; Walsh, Toleman *et al.* 2005; Bebrone 2007; Queenan and Bush 2007).

The IMP family comprises 27 types designated 1 to 27 (http://www.lahey.org/studies/). IMP-1, the first MBL conferring carbapenem resistance, was first discovered in 1988 in a *Pseudomonas aeruginosa* strain in Japan (Watanabe, Iyobe *et al.* 1991). IMP-1 constitutes the most common metallo-β-lactamase in Japan, in both enteric Gram-negative organisms and in *Pseudomonas* and *Acinetobacter* species (Rasmussen and Bush 1997). The emergence of IMP-type enzymes have been described, mostly in Japan, China, Taiwan, Australia, USA, Canada, Brazil and Europe (Walsh, Toleman *et al.* 2005). The first member of the

IMP family found in Europe, IMP-2, was reported in an *A. baumannii* isolate from Italy in 1997 (Cornaglia, Riccio *et al.* 1999).

VIM family, a second growing family of carbapenemases, was first discovered in *P. aeruginosa* in Verona, Italy in 1997. As found for bla_{IMP} genes, the bla_{VIM-1} gene was integrated as a gene cassette into a class 1 integron (Lauretti, Riccio *et al.* 1999). Since then, a total of 24 VIM-type MBLs have been reported (http://www.lahey.org/studies/). Although the VIM-types were initially considered as the European counterpart of the IMP-type predominant in Southeast Asia, nowadays VIM-type MBLs have established endemicity not only in Europe but also in Asia, South and North America and Australia. VIM-types also detected in *Pseudomonas putida*, *Pseudomonas fluorescens*, *A. baumannii*, *K. pneumoniae*, *K. oxytoca*, *E. cloacae*, *Proteus mirabilis* and *E. coli* (Walsh, Toleman *et al.* 2005; Maltezou 2009).

SPM was first detected from the clinical *P. aerugenosa* strain in 1997 in São Paulo, Brazil and then designated bla_{SPM-1} (Toleman, Simm *et al.* 2002). Whereas GIM-1 was isolated in Germany in 2002 from the *P. aerugenosa* strain (Castanheira, Toleman *et al.* 2004). The enzyme SIM was first detected in Korea from *A. baumannii* strains. SIM-1 exhibited (64 to 69%) amino acid identity to the IMP family (Lee, Yum *et al.* 2005). Since their initial discoveries, SPM, GIM, and SIM metallo β-lactamases have not spread beyond their countries of origin.

1.1.5.4. Class C AmpC-type β-lactamases

With continuing use of 7-α-methoxy-cephalosporins (cefoxitin and cefotetan) and β-lactamase inhibitor combinations (clavulanate, sulbactam or tazobactam) with (amoxicillin, ticarcillin, ampicillin, or piperacillin) plasmids encoding class C β-lactamases appeared (Philippon, Arlet *et al.* 2002). AmpC-type β-lactamases constitute molecular class C in Ambler's classification scheme. AmpC β-lactamases mediate resistance to most penicillin, broad and extended-spectrum cephalosporins and β-lactamase inhibitor-β-lactam combinations (Hanson 2003; Jacoby 2009).

Cefoxitin resistance used as indicator for AmpC-mediated resistance but it can also be an indication of loss outer membrane permeability (Philippon, Arlet *et al.* 2002).

AmpC is typically encoded on the chromosome of Gram-negative bacteria including; *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, *Hafnia alvei* and *Serratia marcescens*. The chromosomal *ampC* gene expression in these organisms is inducible by β-lactam antibiotics such as cefoxitin and imipenem but poorly induced by the third- or fourth-generation cephalosporins. In *E. coli* and *Shigella* spp., AmpC is usually constitutive, minimal and poorly expressed, while in *Klebsiella* and *Salmonella* species the *ampC* gene is missing from the chromosome. Induction requires the DNA-binding protein AmpR, AmpD and AmpG (Livermore 1995; Jones 1998; Philippon, Arlet *et al.* 2002; Hanson 2003; Schmidtke and Hanson 2006; Jacoby 2009).

AmpC enzymes have been named according to the resistance produced to cephamycins (CMY, 53 variants), cefoxitin (FOX, 7 variants), moxalactam (MOX, 8 variants) or latamoxef (LAT, variants), according to the type of enzyme, such as Ambler class C (ACC , 4 variants) or AmpC type (ACT, 8 variants), according to the site of discovery, such as the Miriam Hospital in Providence (MIR, 5 variants) or the Dhahran Hospital in Saudi Arabia (DHA, 4 variants) and according the patient name Bilal (BIL) (Philippon, Arlet *et al.* 2002).

AmpC enzymes were considered as exclusively chromosomal until 1989, when MIR-1, the first plasmid-encoded AmpC enzyme, was discovered (Papanicolaou, Medeiros *et al.* 1990). A plasmid-encoded β-lactamase (CMY-1) conferring extended broad spectrum resistance including cephamycins was identified in a *K. pneumoniae* strain isolated from a patient's wound. The new cephamycinase (CMY-1) was more strongly inhibited by sulbactam in the majority of combinations than by clavulanic acid or tazobactam (Bauernfeind, Chong *et al.* 1989). Infections by plasmid-encoded AmpC producing enterobacterial isolates resistant to extended-spectrum cephalosporins or aztreonam have become a serious problem worldwide in multihospital (Winokur, Canton *et al.* 2001; Philippon, Arlet *et al.* 2002; Alvarez,

Tran *et al.* 2004; Song, Kim *et al.* 2005). CMY-2 enzyme is the most common plasmid-mediated AmpC β-lactamase worldwide (Jacoby 2009). The association of CMY-encoding genes with mobile elements, such as IS*Ecp1,* IS*CR1* or IS*26*, might be the reason involved in mobilization of AmpC enzymes worldwide (Hossain, Reisbig *et al.* 2004; Miriagou, Tzouvelekis *et al.* 2004; Hopkins, Deheer-Graham *et al.* 2006; Toleman, Bennett *et al.* 2006).

Failure to detect these β-lactamases has contributed to their uncontrolled spread and occasional therapeutic failures. (Perez-Perez and Hanson 2002) developed a multiplex PCR assay that proved useful as a rapid screening tool for the detection of plasmid-encoded *ampC* genes.

1.1.5.5. Class D OXA β-lactamases

The OXA β-lactamases differ from the TEM and SHV enzymes and they belong to class D (2d) according to Ambler classification (Ambler, Coulson *et al.* 1991). The OXA group mainly occur in *Acinetobacter* and *Pseudomonas* species. The OXA β-lactamases attack the oxyimino-cephalosporins and have a high hydrolytic activity against oxacillin, methicillin and cloxacillin more than benzylpenicillin, inhibited less efficiently by clavulanate and their activity is inhibited by NaCl (Heritier, Poirel *et al.* 2005; Poirel and Nordmann 2006; Walther-Rasmussen and Hoiby 2006). Currently, over than 180 different variants of OXA enzymes have been identified on the protein level (http://www.lahey.org/studies/).

Whereas most of the genes encoding class D oxacillinases have commonly been found on plasmids incorporated as gene cassettes in integrons, several chromosomal encoded oxacillinases have been reported (Heritier, Poirel *et al.* 2005; Walther-Rasmussen and Hoiby 2006).

Most OXA-type β-lactamases are not regarded as ESBLs, because they do not hydrolyse the extended-spectrum cephalosporins. OXA-11 a variant of OXA-10 (previously known as PSE-2) is the first extended-spectrum from *P. aeruginosa*

ABD in October 1991 from blood cultures of a burn patient in Turkey (Hall, Livermore *et al.* 1993). Substitutions of an asparagine for serine at position 73, or an aspartate for glycine at position 157 are probably required for the ESBL phenotype (Bradford 2001). Most OXA-type ESBLs derive from OXA-10 (OXA-11, OXA-13, OXA- 14, OXA-16, OXA-17 OXA-19 and OXA-28), or to a lesser extent from OXA-2 (OXA-15 and OXA-32), or others unrelated to any recognised broad-spectrum OXA enzymes (OXA-18 and OXA-45) (Naas, Poirel *et al.* 2008).

The first OXA β-lactamase ARI-1 (for "*Acinetobacter* resistant to imipenem"), later identified as OXA-23, with carbapenemase activity was described by Paton *et al.* in 1993. The strain was isolated in 1985 from a patient in the Royal Infirmary of Edinburgh, Scotland (Paton, Miles *et al.* 1993). The hydrolysis of carbapenems by the class D oxacillinase in *A. baumannii* was classified into four subgroups of eight clusters and they have been designated OXA-23-like; OXA-40-like; OXA-51-like and OXA-58-like (Brown and Amyes 2006), whereas Walther-Rasmussen and Hoiby (2006) subclassified carbapenem-hydrolysing OXA enzymes into eight distinct branches or subgroups including OXA-23, OXA-24, OXA-48, OXA-50, OXA-51, OXA-55, OXA-58 and OXA-60. Another subgroup carbapenem-hydrolyzing oxacillinase OXA-62 was identified in *Pandoraea pnomenusa* from cystic fibrosis patients (Schneider, Queenan *et al.* 2006). OXA-51-like enzymes is intrinsic and naturally found in all *A. baumannii* strains tested (Heritier, Poirel *et al.* 2005).

1.1.6. Plasmid-mediated quinolone resistance; *QnrA, QnrB and QnrS*

After introduction of fluroquinolones, resistance to these agents by *Enterobacteriacae* has become common and wide spread (Tran, Jacoby *et al.* 2005). Quinolones act by inhibiting the action of topoisomerases II (DNA gyrase) for Gram-negative bacteria and topoisomerase IV in the Gram-positive bacteria. Quinolones act by binding to gyrase/topoisomerase IV–DNA and forming quinolone-gyrase/topoisomerase IV-DNA complex which is responsible for the inhibition of DNA replication (Nordmann and Poirel 2005; Tran, Jacoby *et al.* 2005).

The quinolone resistance results from mutations in the chromosomally-encoded type II topoisomerases, and via the upregulation of efflux pumps, or porin-related genes (Drlica and Zhao 1997; Tran, Jacoby *et al.* 2005). Recent studies demonstrate that the plasmid-mediated *qnr* genes (*qnrA, B and S*) play an emerging role in the dissemination of fluoroquinolone resistance. *qnr,* the gene product, is a member of the pentapeptide repeat family of proteins and has to block the action of ciprofloxacin on purified DNA gyrase and topoisomerase IV (Tran, Jacoby *et al.* 2005). Plasmid mediated resistance to quinolone was first reported in 1998 in a *K. pneumoniae* clinical strains isolated in 1994 in Birmingham, Alabama, USA (Martinez-Martinez, Pascual *et al.* 1998), and then in Canada, Asia, Australia, Turkey and Europe (Poirel, Leviandier *et al.* 2006). Another *qnr* gene, *qnrS*, has also been found in a plasmid from a strain of *Shigella flexnri* isolated in Japan (Hata, Suzuki *et al.* 2005). Recently, *qnrB*-like determinants were found in *Citrobacter koseri, E. coli, Enterobacter cloacae,* and *K. pneumoniae* from the United States and India (Jacoby, Walsh *et al.* 2006).

Two other genes, *qnrC* and *qnrD*, were more recently reported from China. The *qnrC* was described in a clinical strain of *Proteus mirabilis* from Shanghai, China, coding for a 221-amino-acid protein. QnrC shared 64%, 41%, 59%, and 43% amino acid identities with QnrA1, QnrB1, QnrS1, and QnrD respectively (Wang, Guo *et al.* 2009). The *qnrD* gene was described from four *Salmonella enterica* isolates obtained from humans in the Henan Province of China. QnrD encodes a 214-amino-acid pentapeptide repeat protein and showed 48% similarity to *qnrA1*, 61% similarity to *qnrB1*, and 32% similarity to *qnrS1* (Cavaco, Hasman *et al.* 2009).

The *qnr* genes have been detected in a series of enterobacterial species, but mostly from *E. coli*, *Enterobacter* spp., *Klebsiella* spp. and *Salmonella* spp. (Mammeri, Van De Loo *et al.* 2005; Nordmann and Poirel 2005; Rodriguez-Martinez, Poirel *et al.* 2006; Poirel, Cattoir *et al.* 2008).

The *qnrA* gene is thought to have originated in *Shewanella algae,* an environmental species from marine and fresh water (Poirel, Rodriguez-Martinez *et al.* 2005), Qnr-

like proteins have been detected from water-borne *Vibrionaceae* bacterial isolates and also proposed to be a possible origin of the clinically more important QnrA, QnrS, and QnrB determinants (Cattoir, Poirel *et al.* 2007b).

qnrA and *qnrS* determinants share 59% amino acid identity, whereas both determinants share 40% and 37% amino acid identity with *qnrB* respectively (Jacoby, Walsh *et al.* 2006; Poirel, Leviandier *et al.* 2006). The presence of the *qnr* gene increased the MICs to nalidixic acid and fluoroquinolones by four- to eightfold (Martinez-Martinez, Pascual *et al.* 1998; Tran and Jacoby 2002; Wang, Sahm *et al.* 2004; Mammeri, Van De Loo *et al.* 2005).

qnrA genes has been identified in complex In*4* family class 1 integrons, known as complex *sul1*-type integrons that may act as a recombinase for mobilization of CTX-M, and ampC. These genetic structures possess one 5`-conserved segment (5`-CS) which contains *intI1* gene encoding for the integrase enzyme and duplicated 3`-conserved segments (3`-CS) each of them contains *qacEΔ1* and *sul1* genes. The two 3`-CS surround a common region (CR) which contains the IS*CR1* (*orf513*) and a unique region. Usually, a single copy of IS*CR1* is found downstream from *qnrA1*, but in some cases is bracketed by two copies of IS*CR1* (Mammeri, Van De Loo *et al.* 2005; Nordmann and Poirel 2005; Robicsek, Jacoby *et al.* 2006; Toleman, Bennett *et al.* 2006).

The *qnrB1* located in a multi resistance plasmid in an integron-like structure near *orf513* (*orf1005*) gene (Jacoby, Walsh *et al.* 2006). Also, the *qnrB2* and the *qnrB6* determinants were reported to be located in a complex *sul1*-type integrons. The genetic structure contained duplicate copies of IS*CR1* surround the gene (Garnier, Raked *et al.* 2006).

Unlike *qnrA* and *qnrB*, *qnrS* genes have been reported that it is not part of a *sul1*-type integron and not as a form of a gene cassette in a common class 1 integrons. The *qnrS1* has been found to associate with Tn*3-like bla*~TEM-1~-containing transposon. Other reports found a novel Ambler class A β-lactamase gene, now named *bla*~LAP~,

instead of the Tn*3* element (Hata, Suzuki *et al.* 2005; Nordmann and Poirel 2005; Poirel, Leviandier *et al.* 2006; Robicsek, Jacoby *et al.* 2006; Lavilla, Gonzalez-Lopez *et al.* 2008).

The co-presence of *qnr genes* and ESBL genes on same isolates has been regularly reported (Nordmann and Poirel 2005; Jacoby, Walsh *et al.* 2006; Robicsek, Jacoby *et al.* 2006; Lavilla, Gonzalez-Lopez *et al.* 2008).

1.2. The genus *Klebsiella*

1.2.1. General characteristics

The genus *Klebsiella* is non-motile, non-sporulating, lactose-fermenting, oxidase-negative, and Gram-negative with a prominent polysaccharide capsule of considerable thickness which gives the colonies their glistening and mucoid appearance on agar plates. *Klebsiella* is rod shape 0.3-1 µm in diameter and 0.6-6 µm in length arranged singly, in pairs or in short chains. *Klebsiella* is facultative bacteria and the colonies appear large, mucoid, and red with diffusing red pigment on MacConkey agar indicating fermentation of glucose and acid production. *Klebsiella* are normal inhabitant of the intestinal tract of human and animal, soil, water and botanical environment (Ørskov 1984; Podschun and Ullmann 1998; Brisse, Grimont *et al.* 2006; Brooks 2007).

1.2.2. TAXONOMY

Klebsiella pneumoniae is a member of the *Enterobacteriaceae* family, recognised over 100 years ago as a cause of community-acquired pneumonia. The organism was named to honour Edwin Klebs, a 19th century German microbiologist (Brisse, Grimont *et al.* 2006; Keynan and Rubinstein 2007).

The genus *Klebsiella* was originally divided into 3 main species based on biochemical reactions and medical importance into three species corresponding to the diseases they caused: *K. pneumoniae*, *K. ozaenae*, and *K. rhinoscleromatis*. Based on DNA-DNA hybridization data *K. ozaenae* and *K. rhinoscleromatis*, taxonomically, are regarded as subspecies of *K. pneumoniae* (Podschun and Ullmann 1998; Drancourt, Bollet *et al.* 2001; Hansen, Aucken *et al.* 2004).

The *K. oxytoca* was originally isolated from old milk. It was further considered as a distinct group from *K. pneumoniae* (Jain, Radsak *et al.* 1974). Other four new species: *K. planticola, K. terrigena, K. trevisanii,* and *K. ornithinolytica* were

identified in 1980s by (Bagley, Seidler *et al.* 1981; Izard, Ferragut *et al.* 1981; Ferragut, Izard *et al.* 1983; Sakazaki, Tamura *et al.* 1989) respectively. The *K. planticola* and *K. trevisanii* were subsequently combined and consider as *K. planticola* on the basis of DNA-DNA hybridization (Gavini, Izard *et al.* 1986). *Enterobacter aerogenes* and *Calymmatobacterium granulomatis*, due to their close relationships to *Klebsiella* species, were regarded as an eighth and ninth member of the genus *Klebsiella* and named *Klebsiella mobilis* and *Klebsiella granulomatis*, respectively (Carter, Bowden *et al.* 1999; Drancourt, Bollet *et al.* 2001). The three species: *K. terrigena*, *K. planticola*, and *K. ornithinolytica* have recently been transferred to the new genus *Raoultella* (Drancourt, Bollet *et al.* 2001).

Clinical isolates of *K. pneumoniae* fall into four phylogenetic groups based on nucleotide variations of the *gyrA*, *parC*, and *rpoB* genes, named KpI, KpII-A, KpII-B, and KpIII, with the newly described species *K. variicola* appearing to correspond to KpIII (Alves, Dias *et al.* 2006). There are five sequence clusters, KoI, KoII, KoIII, KoIV and KoVI were found within *K. oxytoca* (Fevre, Jbel *et al.* 2005).

1.2.3. Pathogenesis and clinical importance

K. pneumoniae is the most medically important species of the group. *K oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens (Podschun and Ullmann 1998). In recent years, *Klebsiellae* have become important pathogens in nosocomial infections (Alves, Dias *et al.* 2006). *K. pneumoniae* is also a potential community-acquired pathogen (Ko, Paterson *et al.* 2002).

Extended-spectrum β-lactamases resistant Gram-negative bacteria have been associated with increased mortality, length of hospitalization and hospital costs (Schwaber, Navon-Venezia *et al.* 2006). In humans, *Klebsiella* species may colonize the skin, pharynx, or gastrointestinal tract. They may also colonize sterile wounds, urine and may be regarded as normal flora in many parts of the colon, intestinal and biliary tract (Podschun and Ullmann 1998; Brisse, Grimont *et al.* 2006). Transmission of *Klebsiella* from patient to patient results from contaminated medical

equipments and contaminated hands of medical personnel and blood products, whereas, the portals of *Klebsiella* infections are surgical wounds, peritoneum, catheter entrance sites, urinary, respiratory, and biliary tracts (Goetz, Rihs *et al.* 1995; Podschun and Ullmann 1998).

K. pneumoniae is an opportunistic and major hospital-acquired pathogen, causing urinary tract infections, nosocomial pneumonia, bacteraemia and septicemia (Ko, Paterson *et al.* 2002). Community-acquired pneumonia is a very severe fatal illness with a rapid onset, high fever, and haemoptysis (currant jelly sputum), bulging interlobar fissure and cavitary abscesses observed by chest radiographic. The observed mortality rates range from about 25 to 50% (Feldman, Ross *et al.* 1995). Mortality rates are as high as 50% and approach 100% in hospitalized, immunocompromised patient with underlying diseases such as diabetes mellitus (Sahly and Podschun 1997; Ko, Paterson *et al.* 2002).

The *Klebsiellae* species are second only to *E. coli* causing bacteraemia representing 3-8% of all nosocomial bacterial infection (Podschun and Ullmann 1998). Community acquired bacteraemia usually caused by urinary tract infection, vascular catheter infection, and cholangitis (Ko, Paterson *et al.* 2002). In addition, the *Klebsiellae* species especially *K. pneumoniae* has been shown to cause intra-abdominal infections mediated by heat stable and heat labile enterotoxins (Rennie, Anderson *et al.* 1990).

Since 1981, a distinctive syndrome of community-acquired *K. pneumoniae* septicaemia with liver abscess has been reported in Taiwan. This syndrome is characterized by high mortality (10 to 40%), and some cases have been complicated by meningitis or endophthalmitis (Fang, Sandler *et al.* 2005). The disease has also been reported in North America and Europe (Keynan and Rubinstein 2007). The symptoms of the disease characterized by fatigue, anorexia, nausea, diffuse abdominal discomfort, pleuritic chest pain, jaundice and fever (Lederman and Crum 2005). The K1 capsular serotype is the predominant serotype of *K. pneumoniae* strains causing liver abscess (Struve, Bojer *et al.* 2005). In both Taiwan and Korea,

K1 capsular serotype is accounted for about 60% of *K. pneumoniae* strains causing liver abscess in these countries ((Fung, Chang *et al.* 2002; Chung, Lee *et al.* 2007). The frequency of the *rmpA* gene (mucoviscosity-associated gene A) is associated with the hypermucoviscosity phenotype of *K. pneumoniae* strains that cause liver abscess in Taiwan (Fang, Chuang *et al.* 2004; Yu, Ko *et al.* 2006). Rahimian and his colleagues (Rahimian, Wilson *et al.* 2004) elucidated that the liver abscess usually occur in patients were of Asian ethnicity, indicating a possible genetic linkage to disease susceptibility. Lederman and Crum (2005) reported a case series of pyogenic liver abscess caused by *Klebsiella*, 67% of their patients were Filipinos compared with only 29% non Filipinos among those patients with liver abscesses caused by different bacterial pathogens. The presence of variable underlying diseases or conditions such as diabetes mellitus, heavy alcohol drinking, biliary tract diseases, malignancy, liver cirrhosis, end-stage renal disease, intra-abdominal infections, history of abdominopelvic surgery, history of steroid use, and history of previous antibiotic use considered as risk factors for the K1 serotype *K. pneumoniae* liver abscess (Kim, Chung *et al.* 2009).

K. pneumoniae has also become a common cause of community acquired bacterial meningitis in adults in Taiwan. The proportion of cases of bacterial meningitis due to *K. pneumoniae* in one Taiwanese hospital increased from 8% during 1981 through 1986 to 18% during 1987 through 1995 (Tang, Chen *et al.* 1997). Outside Taiwan, cases of *K. pneumoniae* meningitis have occurred, predominantly in other parts of Asia (Yanagawa, Nakamura *et al.* 1989; Li, Shing *et al.* 2001; Ohmori, Shiraki *et al.* 2002), Europe and North America (Holder and Halkias 1988; Giobbia, Scotton *et al.* 2003; Bouadma, Schortgen *et al.* 2006; Braiteh and Golden 2007). The rarity of these cases outside Asia raises the possibility of ethnicity or country of origin predisposing individuals to invasive disease (Ko, Paterson *et al.* 2002).

K. pneumoniae consider as the most common pathogen in bacterial endogenous endophthalmitis associated with liver abscesses in Asia. About 13% of *K. pneumoniae* endogenous endophthalmitis are complication of liver abscesses with the predisposing factors of endogenous *K. pneumoniae* endophthalmitis complication

are diabetic, and impaired immune function in diabetic's patients (Han 1995; Wong, Chan *et al.* 2000; Seale, Lee *et al.* 2007). The disease also has been reported in countries of non- Asian origin (Scott, Matharoo *et al.* 2004; Lederman and Crum 2005). In addition, the *K. pneumoniae* genotype K1 is capable of causing catastrophic septic ocular or central nervous system as complications from pyogenic liver abscess especially in underlying patients (Fang, Lai *et al.* 2007).

K. pneumoniae has been implicated in the development of ankylosing spondylitis because of the high incidence of *Klebsiella* in the bowel flora of patients whose disease is in an active state (Ogasawara, Kono *et al.* 1986; Sahly and Podschun 1997). Schelenz, Bramham *et al.* (2007) reported two cases of acute septic arthritis due to extended-spectrum beta-lactamase (ESBL) producing *K. pneumoniae* in renal transplant adult patients following a hospital acquired bacteraemia. Also, *K. pneumoniae* was associated with chronic diarrhoea in HIV-infected persons (Nguyen Thi, Yassibanda *et al.* 2003).

Klebsiella strains are responsible for severe infections in animals, including metritis in mares following transmission from an infected stud especially capsular serotype K1, K2, K5 and K7 (Platt, Atherton *et al.* 1976). As well, *K. pneumoniae* can cause bovine mastitis (Braman, Eberhart *et al.* 1973). *Klebsiella* strains causes serious infection in other animals including dogs, monkeys, guinea pigs, muskrats, birds and Fox (Brisse, Grimont *et al.* 2006).

Moreover, *K. oxytoca* is among the top 4 pathogens that cause infection in patients in neonatal intensive care units. It is the second most frequent cause of Gram-negative neonatal bacteraemia (Podschun and Ullmann 1998). *Klebsiella oxytoca* considered as an opportunistic infections in laboratory rodents (Bleich, Kirsch *et al.* 2008).

K. pneumoniae subsp. *rhinoscleromatis* is associated with rhinoscleroma which was first *Klebsiella* species ever described in 1882 by von Frisch and characterized by a chronic inflammatory process of the nasopharynx. This infection is distributed around the world and become an endemic in certain areas in Eastern Europe, Latin

America, central Africa, and southern Asia (Sahly and Podschun 1997; Hart and Rao 2000).

Ozena, a chronic atrophic rhinitis caused by *K. pneumoniae* subsp. *ozaenae* characterized by necrosis of the mucosa and mucopurulent nasal discharge (Strampfer, Schoch *et al.* 1987). *K. pneumoniae* subsp. *ozaenae* may cause invasive infections, especially in immunosuppressed hosts such as bacteraemia with or without meningitis, otitis, mastoiditis, urinary tract infections, wound infections, corneal ulcers, pneumonia, or brain abscess (Brisse, Grimont *et al.* 2006).

1.2.4. PATHOGENCITY FACTORS

1.2.4.1. Capsular Antigens

Capsule polysaccharide (CPS) is recognized as one of the most important virulence factors of *Klebsiella* spp. The capsular repeating subunits are consisting of four to six sugars (glucose, galactose, mannose, fucose, and rhamnose) and, very often, uronic acids (as negatively charged components) (Podschun and Ullmann 1998; Brisse, Grimont *et al.* 2006). The *K. pneumoniae* K2 capsular polysaccharide has been reported to contain glucose, mannose, and N-acetyl-glucuronic acid (Arakawa, Ohta *et al.* 1991). The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers to protect the bacterium from phagocytosis by macrophage, and prevents killing of the bacteria by bactericidal serum factors (Simoons-Smit, Verweij-van Vught *et al.* 1986; Kabha, Nissimov *et al.* 1995; Cortes, Borrell *et al.* 2002; Lin, Chang *et al.* 2004; Brisse, Grimont *et al.* 2006).

In vitro studies have shown that the presence of CPS inhibits the deposition of the complement component C3 onto the bacterium, and reduces adhesion and phagocytosis of the bacterium by 12 macrophages and epithelial cells (Oelschlaeger and Tall 1997; Alvarez, Merino *et al.* 2000; Cortes, Borrell *et al.* 2002). The CPS-mediated virulence involves high resistance of K1 or K2 capsules to phagocytosis by

macrophage. In contrast, non-K1/K2 isolates are usually not resistant to phagocytosis and less virulent than K1/K2 strains (Simoons-Smit, Verweij-van Vught *et al.* 1986; Kabha, Nissimov *et al.* 1995; Cortes, Borrell *et al.* 2002; Lin, Chang *et al.* 2004).

The macrophages can recognize bacterial surface with mannose content of the CPS (Keisari, Kabha *et al.* 1997). *K. pneumoniae* with loose, thin capsule is reported to be a virulent (Wacharotayankun, Arakawa *et al.* 1993). Rapidly cleared K21a strain was shown to be less virulent, and K2 strain that is viable in blood is highly virulent. The binding of K21a to macrophage is responsible for its clearance, and the binding is inhibited by yeast mannan (Kabha, Nissimov *et al.* 1995). Macrophages with the mannose-a-2/3-mannose-specific lectin or mannose receptor recognize, ingest, and subsequently kill *Klebsiella* serotypes containing the capsular types with low virulence, such as the K7 or K21a antigen, repeating sequences mannose-a-2/3-mannose or L-rhamnose-a-2/3-L-rhamnose by process called lectinophagocytosis which defined as nonopsonic phagocytosis that is based on recognition between surface lectins on one cell and surface carbohydrates on the opposing cell. Lectinophagocytosis may be mediated either by bacterial surface lectins such as fimbriae or by phagocyte lectins that act as receptors. In contrast, strains that lack these repeating sequences are not recognized by macrophages and hence phagocytosis does not take place (Ofek, Goldhar *et al.* 1995; Keisari, Kabha *et al.* 1997). Wu *et al.* (2008) concluded that the possession of fucose might contribute to *Klebsiella* virulence by avoiding phagocytosis because fucose on bacteria had been implicated in immune evasion.

The study done by Arakawa, Wacharotayankun *et al.* (1995) determined the 24,329-bp nucleotide sequence of the *cps* region that is responsible for capsular polysaccharide synthesis in a K2 *K. pneumoniae* strain. Nineteen possible open reading frames (ORFs) were identified in sequenced area. Nassif *et al.* demonstrated that the mucoid phenotype is determined by a gene, designated "*rmpA*" (regulator of mucoid phenotype) located on a 180-kb plasmid, which also carries the aerobactin gene. Hypothetically, *rmpA* is a regulatory gene which controls the production of a shield (of unknown composition) surrounding the bacteria and protecting them from

interaction with anticapsule-specific antibodies. The 50% lethal dose for mice was increased by 1000-fold after a mutation in *rmpA*, although the mutated strain still expresses its capsular specificity (Nassif, Fournier *et al.* 1989; Nassif, Honore *et al.* 1989).

Capsular serotypes K1 and K2 are considered to be the predominant virulent strains of *K. pneumoniae*. Capsular type K1 is most frequently associated with acute pneumonia, but K2, K3, K4, K5, and K6 can also be involved (Brisse, Grimont *et al.* 2006). K1 has been further investigated as the most common serotype isolated from patients with *K. pneumoniae* liver abscess and endophthalmitis (Chuang, Fang *et al.* 2006).

Many reports linked between virulence determinants of *K. pneumoniae* capsule with liver abscess formation and serotype-specific genes like the *magA* gene for the K1 serotype and the *k2A* gene for the K2 serotype (Arakawa, Wacharotayankun *et al.* 1995; Kabha, Nissimov *et al.* 1995; Cortes, Borrell *et al.* 2002; Fang, Chuang *et al.* 2004; Lin, Chang *et al.* 2004; Struve, Bojer *et al.* 2005; Chuang, Fang *et al.* 2006; Yu, Ko *et al.* 2006).

Moreover, *K. pneumoniae* surface bound CPS may act as a protective shield on the bacterial surface against antimicrobial peptides (APs). Sub-lethal concentrations of APs induce an increase in the transcription of the *cps* operon which correlates with an increase in the amount of surface-bound CPS (Campos, Vargas *et al.* 2004). In addition, Llobet, Tomas *et al.* (2008) demonstrated that the decoy action of CPSs contributes significantly to the resistance against APs for pathogens expressing anionic CPS. The protective action of CPS will also allow the pathogen to activate other countermeasures against APs such as alteration of surface charge, which is a time-consuming process. The released CPS traps APs thereby blocking their bactericidal activity through the concentrations of defensins present in tissues which are close to the MIC for several bacteria. Recently, Moranta and his colleagues (Moranta, Regueiro *et al.* 2010) started to study whether *K. pneumoniae* expresses mechanisms of resistance against APs. Concentrations of APs present in infected

tissues, for example those found in the surface liquid lining the airway epithelium, could be rather high due to the increased production of APs after recognition of the pathogen. Finally, Evrard, Balestrino *et al.* (2010) suggested that *K. pneumoniae* CPS, by hampering bacterial binding and internalization, induces a defective immunological host response, including maturation of dendritic cells (DCs) and pro-Th1 cytokine production, whereas the LPS O antigen seems to be involved essentially in DCs activation.

1.2.4.2. Adhesion

Adhesion to mucosal and epithelial cell surfaces is often the first step in the development of colonization and infection. Adhesins are often also haemagglutinins and may be located on fimbriae (pili) that protude on the surface of the bacterial cells. More than 80% of clinical isolates of *K. pneumoniae*, but few *K. oxytoca* strains, express type-1 fimbriae (Podschun and Sahly 1991). Strains of *K. pneumoniae* commonly express three types of pili known as types 1 (common pili), 3 and 6. Pili are nonflagellar, filamentous projections on the bacterial surface. These structures are up to 10 mm long and have a diameter of 1 to 11 nm; they consist of polymeric globular protein subunits (pilin) with a molecular mass of 15 to 26 kDa (Podschun and Ullmann 1998).

Type 1 fimbriae are well characterized and found in the most of enterobacterial species. Type-1 fimbriae were expressed in more than 80% of clinical isolates of *K. pneumoniae*, but few *K. oxytoca* strains. They mediate adhesion to mannose-containing structures present on host cells or in the extracellular matrix and cause agglutination of guinea pig erythrocytes. A number of studies have shown that type 1 fimbriae are an important virulence factor in *K. pneumoniae* urinary tract infection (Podschun and Sahly 1991; Podschun and Ullmann 1998; Brisse, Grimont *et al.* 2006; Struve, Bojer *et al.* 2008; Struve, Bojer *et al.* 2009).

Type 3 fimbriae are characterized by their ability to agglutinate erythrocytes treated with tannic acid. It is called mannose-resistant, *Klebsiella*-like hemagglutination

(MR/K-HA) because it was thought this fimbrial type is synthesized only by *Klebsiella.* In addition to *Klebsiella* species, type 3 fimbriae become common in *Enterobacter*, *Serratia*, *Proteus*, and *Providencia* isolates (Struve, Bojer *et al.* 2009). Type 3 fimbriae are encoded by the *mrk* gene cluster which comprises the major fimbrial subunit *mrkA* gene and the *mrkD* fimbrial adhesin responsible for mannose resistant *Klebsiella*-like hemagglutination. In vitro studies have revealed that type 3 fimbriae mediate binding to different structures in human; human endothelial cells, epithelia of the respiratory tract, uroepithelial cells, and type V collagen structures (Allen, Gerlach *et al.* 1991; Brisse, Grimont *et al.* 2006; Struve, Bojer *et al.* 2009).

Furthermore, type 3 fimbriae are also believed to contribute to the formation of extended extracellular structures known as biofilms. Biofilms serve as structural anchors, barriers to contact with host defences and as impediments to antibiotics (Brisse, Issenhuth-Jeanjean *et al.* 2004; Bortz, Jackson *et al.* 2008).

1.2.4.3. Lipopolysaccharide

The lipopolysaccharide (LPS) molecule is composed of three distinct sections; lipid A, a core polysaccharide and a side chain O-antigen (O-Ag) polysaccharide. Nine O-antigen types are distinguished in *K. pneumoniae*, O1 being the most frequent. The most important role of the O-antigen is to protect *K. pneumoniae* from complement mediated killing (Podschun and Ullmann 1998; Hansen, Mestre *et al.* 1999; Brisse, Grimont *et al.* 2006).

The lipid A anchors the LPS molecule into the outer membrane and is also an endotoxin, stimulating the immune system through agonism of Toll-like receptor 4 (TLR4) which is present on macrophages, dendritic cells and other cell types inducing NF-kB mediated production of cytokines (Alexander and Rietschel 2001). The core polysaccharide links the O-Ag onto the lipid A molecule and usually is negatively charged due to phosphate substitutions. The O-Ag forms a polysaccharide layer that extends up to 30 nm into the surrounding media (Kastowsky, Gutberlet *et al.* 1992).

1.2.4.4. Other Factors

There are many other factors contributing in pathogenicity of *Klebsiella* spp. such as siderophores, cytotoxins, enterotoxins and haemolysin.

Iron is essential for bacterial growth. Bacteria secure their supply of iron in the host by secreting high-affinity, low-molecular-weight iron chelators, called siderophores that are capable of solubilizing and importing the required iron bound to host proteins. Two different groups of siderophores are mostly produced in the genus *Klebsiella*, phenolates (enterobactin) (also known as enterochelin) and hydroxamates (aerobactin). All strains were found to produce enterochelin, only a few could produce aerobactin. The *Klebsiella* spp. produced third siderophore called "yersiniabactin," which is encoded by the *Yersinia* high-pathogenicity island, but without known role in prevalence and pathogenesis (Podschun and Ullmann 1998; Brisse, Grimont *et al.* 2006).

1.2.5. TYPING

The important of typing methods is to obtain information about endemic and epidemic nosocomial outbreaks of *Klebsiella* infections and to determine the clonality of the strains. There are two methods have been used in *Klebsiella* typing.

1.2.5.1. Phenotypic typing

1.2.5.1.1 Biotyping

Biotyping is based on biochemical reactions and environmental tolerance. It is suitable method of typing for smaller not optimally equipped laboratories. It is useful in assessing outbreaks of *Klebsiella* but it is considered to be of little use in epidemiological studies. Biotyping can be carried out by using API 20E system with supplementary tests. *Klebsiella* strains are often currently identified by using

automated instruments based on classical biochemical tests, such as the Vitek and API systems. Laboratories might use macrotube tests alone or by combining API 20E with additional macrotube tests (Podschun and Ullmann 1998). However, identification to the species level is often difficult, because some of the species share similar biochemical profiles (Monnet and Freney 1994; Wang, Cao *et al.* 2008).

Hansen *et al.*, (Hansen, Aucken *et al.* 2004) evaluated a test panel consisting of 18 biochemical tests on 242 strains preliminarily identified as belonging to the genus *Klebsiella* comprising all *Klebsiella* species and subspecies from three countries. This panel was used to differentiate any *Klebsiella* species, except *Klebsiella rhinoscleromatis*, from its closest relative as shown in Table 1.2.

Table 1.2: Differentiation of *Klebsiella* species modified from Hansen *et al.* (2004).

Biochemical test	Result for species								
	K. pneumoniae	*K. ozaenae*	*K. rhinoscleromatis*	*K. oxytoca*	*K. terrigena*	*K. planticola*	*K. ornithinolytica*	*E. aerogenes*	*P. agglomerans*
Lysine decarboxylase	+	V	-	+	+	+	+	+	-
Ornithine decarboxylase	-	-	-	-	-	-	+	+	-
Indole	-	-	-	+	-	V	+	-	-
Methyl red	V	V	+	V	+	+	+	-	+
Voges-Proskauer	+	-	-	+	+	+	V	+	+
Growth at 10°C	-	-	-	V	+	+	+	V	+
Gas from lactose at 44.5°C	V	-	-	-	-	-	-	-	-
Malonate	+	-	+	+	+	+	+	+	+
Fermentation of:									
D-Arabinose	V	V	V	+	-	V	V	-	-
_-Gentiobiose	+	+	-	+	+	+	+	+	V
D-Melizitose	-	-	-	V	+	-	-	-	-
2-Deoxy-D-ribose	V	-	V	V	+	+	+	V	+
L-Sorbose	V	V	V	+	+	+	+	-	-
D-Tagatose	V	-	V	+	V	-	V	-	-
Utilization of:									
m-Hydroxy-benzoate	-	-	-	+	V	-	-	V	-
Histamine	-	-	-	-	+	+	+	V	-
Hydroxy-L-proline	V	-	V	-	V	+	+	+	-
Degradation of pectate	-	-	-	+	-	-	-	-	-

(+): positive, (-): negative, (V): variable

1.2.5.1.2. Serotyping

Serotyping is a reaction of specific antiserum to surface-exposed antigen determinant. Serotyping is currently the most widely used technique for typing *Klebsiella* species. The immunodeterminant of *Klebsiella* is the capsule. Klebsiellae usually have well developed polysaccharide capsules, which give their colonies their characteristic mucoid appearance. There are 77 distinct capsular antigens of 82 described recognized an internationally. Klebsiellae also possesses 12 different O-antigen types, but they are difficult to classify because they are covered by the heat-stable capsules (Ørskov 1984; Hansen, Skov *et al.* 2002; Brisse, Issenhuth-Jeanjean *et al.* 2004).

The techniques used in Serotyping are, countercurrent imunoelectrophoresis and immnofluroescence. The Quellung test depends on the adsorption of capsular antibodies onto the CPS, which results in a change in the refractive index. But disadvantages of serotyping are (i) the occurrence of large number of serological cross-reactions among the 77 capsule types, (ii) the weak reaction due to a weak antigen which affects interpretation, (iii) time consuming methodology, (iv) lack of commercially available anti-capsule antisera, and (v) occurrence of non-typable isolates (Podschun and Ullmann 1998; Brisse, Issenhuth-Jeanjean *et al.* 2004; Paterson and Bonomo 2005; Brisse, Grimont *et al.* 2006)

To avoid these drawbacks, Brisse, Issenhuth-Jeanjean *et al.* (2004) developed a molecular method that would enable determination of the capsular serotypes of *Klebsiella* isolates without the use of antiserum. PCR amplification of the capsular antigen gene cluster (cps) was followed by digestion with the restriction enzyme *HincII* (cps PCR-RFLP analysis).

1.2.5.1.3. Phage Typing

Bacteriophage typing is based on the susceptibility of bacterial strains to a panel of bacteriophages. Phage typing of *Klebsiella* was first developed in the 1964 by Milch

and Deak (1964). Phage typing has never become widespread because it shows poor typing rate of 19 to 67%; the lack of standardization and inoculum concentration; the limited availability of bacteriophages; the stability of bacteriophages must be evaluated and maintained over time. For these reasons, bacteriophage typing is useful mainly as a secondary method in combination with serologic testing but not used as an alternative to capsule typing (Podschun and Ullmann 1998; Brisse, Grimont *et al.* 2006).

1.2.5.1.4. Bacteriocin Typing

Bacteriocins are bactericidal substances, usually proteins, produced by bacteria to inhibit the growth of other bacteria through inhibition of protein and nucleic acid synthesis and uncouple electron transport from active transport of thiomethyl-β-D-galactoside and potassium. Bacteriocins usually inhibit the growth of other bacteria members of the same species. Bacteriocins technique has become the method of choice for typing of organisms belonging to *Klebsiella* genus because only 34% of all *Klebsiella* isolates produce bacteriocins (Podschun and Ullmann 1998; Brisse, Grimont *et al.* 2006).

1.2.5.2. Molecular Typing Methods

Molecular typing methods, as applied to the genus *Klebsiella*, have increasingly become an integral part of both clinical and research microbiology laboratories. Microbial genotyping techniques consider valuable tools to distinguish bacterial strains or clones. Genotyping methods are divided into two methods; protein based methods and nucleic acid based methods.

1.2.5.2.1. Protein based methods

1.2.5.2.1.1. SDS-PAGE

SDS-PAGE method has been used to subtype *Klebsiella* by (Costas, Holmes *et al.* 1990). Who did compared it with capsular serotyping and concluded that SDS-PAGE protein profiles could be used as an effective method.

1.2.5.2.2. Nucleic acid based methods

1.2.5.2.2.1. PCR amplification and sequencing

Klebsiella oxytoca can be differentiated from other *Klebsiella* species by a specific PCR targeting the *pehX* gene involved in pectin degradation (Kovtunovych, Lytvynenko *et al.* 2003).

1.2.5.2.2.2. Pulsed-field Gel Electrophoresis (PFGE)

PFGE may be used for genotyping or genetic fingerprinting. It is commonly considered a gold standard in epidemiological studies of pathogenic organisms. PFGE analysis is recommended to address fine-scale epidemiological studies and can detect chromosomal rearrangements which by mobile elements with rapid evolutionary rates (Hansen, Skov *et al.* 2002; Vimont, Mnif *et al.* 2008).

1.2.5.2.2.3. Randomly amplified polymorphic DNA (RAPD)

The first use of RAPD was in 1996. This technique utilizes low-stringency PCR amplification with single primers of arbitrary sequence to generate strain-specific arrays of anonymous DNA fragments. RAPD technique may be used to determine taxonomic identity, assess kinship relationships, analyze mixed genome samples, and create specific probes.

1.2.5.2.2.4. Restriction fragment length polymorphism (RFLP)

Identification of the isolates were confirmed by *gyrA* PCR-RFLP using restriction enzymes *Hinc*II, *Taq*I and *Hae*III of the 441-bp fragment of the *gyrA* gene, and the 940-bp fragment of the RNA polymerase beta subunit gene (*rpoB*) allowing for the identification of *K. pneumoniae* species and *K. oxytoca* genetic groups (Brisse and Verhoef 2001; Brisse, van Himbergen *et al.* 2004).

1.2.5.2.2.5. Multilocus sequence typing (MLST)

A MLST method was previously developed for *K. pneumoniae*. MLST is a nucleotide sequence-based method used for characterizing the genetic relationships among bacterial isolates. It provides computerized data that allow multi-user international databases available. MLST is more appropriate for strain phylogeny and large-scale epidemiology. Analysis of nosocomial isolates showed that MLST can discriminate among epidemiologically unrelated isolates (Diancourt, Passet *et al.* 2005; Vimont, Mnif *et al.* 2008).

1.2.5.2.2.6. Repetitive sequence-based PCR (rep-PCR)

The rep-PCR is a rapid method for strain typing and delineation of bacteria. It uses primers targeting noncoding repetitive elements interspersed throughout the bacterial genome. Standardized, commercially available kits containing primers and PCR master mix reagents are marketed as DiversiLab systems which automated the detection and analysis by using microfluidics for rapid detection in a single day, and digitized the corresponding information in a software package. The standardized, digitized gel images are stored for comparison between runs and between laboratories (Pitout, Campbell *et al.* 2009).

Aims of this study:

- To establish the prevalence, variants and mechanisms of ESBLs (TEM, SHV, CTX-M, OXY) among *K. pneumoniae* and *K. oxytoca* isolates from Scotland.

- To investigate the mechanisms of emergence of CTX-M enzyme within hospital and community.

- To investigate the prevalence of plasmid-mediated quinolone resistance, *qnrA, S* and *B* in ciprofloxacin sensitive and resistant *Klebsiella pneumoniae* isolates.

CHAPTER-2:

MATERIALS AND METHODS

2.1. Bacterial strains

A total of two hundred and twenty three clinical isolates consecutively collected at Royal Infirmary of Edinburgh, Scotland. From these, seven isolates *K. pneumoniae* were collected during March, 2006. A year later, another 216 isolates were isolated from August to October 2007. From the relevant information available, the majority of the isolates 181 (81.1%) were collected from urine samples, 16 (7.2%) strains were collected from blood samples. The remainder 26 (11.7%) of the total strains were from assorted samples, including sputum, tip dialysis line, nephrostomy swabs and arthritis swabs.

2.2. The phenotypic identification method

All isolates were inoculated onto MacConky agar (Oxoid) plates and incubated overnight at 37°C. An isolated colony was used for identification by the phenotypic API 20E method according to the manufacturer's instructions (BioMerieux, Marcy L'Etoile, France). Also, isolates were routinely identified in hospital laboratory by Vitek2 system.

2.3. The genotypic identification (AFLP)

Identification of the isolates were confirmed by *gyrA* PCR-RFLP using restriction enzymes HincII, TaqI and HaeIII of the 441-bp fragment of the *gyrA* gene, and the 940-bp fragment of the RNA polymerase beta subunit gene *rpoB* allowing for the identification of *K. pneumoniae* species and *K. oxytoca* genetic groups as previously described (Brisse, van Himbergen et al. 2004; Brisse and Duijkeren 2005).

2.4. Standard laboratory strains for susceptibility test

Three strains, *Staphylococcus aureus* NCTC 6571, *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662 were used as MIC controls. Isolates SHV-1, SHV-3, TEM-1 and TEM-2 from lab collections were used as IEF controls.

2.5. Storage of isolates

Isolates were inoculated onto MacConky agar (Oxoid, Basingstoke) plates and incubated overnight at 37°C. A single colony was added to the Cryobank System beads (Mast Diagnostic, UK) to each vial for long-term storage according to the manufacturer's instructions at -70°C.

2.6. Growth media

All growth media were sterilized by autoclaving for 15 minutes at 121°C and cooled prior to bacterial inoculation. Normal saline was prepared with 0.85% NaCl and then sterilized before use.

2.7. Chemicals, buffers and media

All chemicals were purchased from Sigma-Aldrich Company (Poole, UK) Ltd unless otherwise stated. All media were purchased in powder from Oxoid (Basingstoke, UK) and then prepared with distilled water before sterilization according to manufacturer's instructions.

2.8. Preparation of electrocompetent bacteria

A single clone of *E. coli* DH10B or DH5α from lab collections was grown overnight in 50 ml of LB broth at 37°C. 10 ml of the overnight culture were inoculated into 1 L of LB broth and incubated at 37°C until it reaches 0.5 at an OD_{600}nm. The culture was rapidly chilled on ice for 30 minutes. The bacteria were transferred into centrifugation containers and centrifuged for 10 minutes at 6000 g (600 rotor centrifuge) at 4°C. The supernatants were discarded and the bacteria pellets were resuspended in equal volume of sterilised 10% glycerol. The bacteria were centrifuged for 10 minutes at 6000 g. The pellets were resuspended in 250 ml of 10% glycerol followed by a centrifugation at 6000 g for 10 minutes. Then, the supernatants were discarded and the pellets resuspended in 125 ml of 10% glycerol

and centrifuged at 6000 g for 10 minutes. Finally, the cells were resuspended in 10% glycerol to give a final volume of 2-5 ml and were aliquoted to 100 µl into 1.5ml Eppendorff tubes. The cells were frozen and stored at -70°C until used.

2.9. Antimicrobial susceptibility testing

2.9.1. Antimicrobial agents

Antimicrobial agents used in this study were: Imipenem (Merck Sharpe Dohme, Rahway, NJ, USA), meropenem (ASTRA Zeneca, Loughborough, UK), potassium clavulanate (Sigma), cefotaxime (Sigma), ceftazidime (Sigma), ceftriaxone (Sigma), aztreonam (Sigma), cefepime (Sigma), ampicillin (Sigma), co-amoxiclav (Augmentin) (Sigma), nalidixic acid (Sigma), ciprofloxacin (Bayer AG, Germany), gentamicin (Sigma) and amikacin (Sigma), rifampicin (Sigma).

2.9.2. Minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations (MICs) are considered the `gold standard' for determining the antimicrobials susceptibility of organisms. Isolates were tested for antimicrobial susceptibility by agar double dilution method (MIC) following the BSAC guidelines (BSAC 2006). Minimum inhibitory concentrations were performed on IsoSensitest (IST) agar containing the appropriate concentrations of antimicrobial agents. The bacterial inocula were adjusted to be at 0.5 McFarland standards. Bacterial cultures were diluted to 10^4 cfu in physiological saline before use. The inoculation was done using a multipoint inoculator (Denley; Surrey, UK) to deliver 1 µl of suspension (10^4 cfu/spot) on to the surface of the agar. Inoculated plates were incubated overnight at 37º C. Standard strains *Staphylococcus aureus* NCTC 6571, *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662 were used to validate the experiment.

Isolates found resistant to cefotaxime, ceftriaxone or ceftazidime, were considered as potential ESBL producers and were subsequently subjected to confirmatory tests.

2.9.3. Confirmation of ESBLs production

2.9.3.1. Disc diffusion method

The antimicrobial susceptibility of isolates was tested by the disc diffusion test. Briefly, an overnight culture in IST broth was diluted in sterile 0.85% saline; spread the inoculum over the entire surface of the plate by three dimensional ways. A selective of discs has been applied to the surface of the agar within 15 minutes of inoculation.

2.9.3.2. Double disc synergy method

Synergy between cefotaxime and clavulanate was detected by placing a disc of Augmentin (20 µg of amoxicillin plus 10 µg of clavulanate) and a disc of cefotaxime 30 mm apart (centre to centre). A clear-cut extension of the edge of the cefotaxime inhibition zone toward the disc containing clavulanate (as shown in figure 1) was interpreted as synergy. The double-disc synergy test was considered positive when decreased susceptibility to cefotaxime was combined with synergy between cefotaxime and Augmentin (Jarlier, Nicolas et al. 1988).

2.10. Analytic isoelectrical focusing (IEF)

This experiment was performed with polyacrylamide gels containing ampholines with a pH range of 3.5 to 10.0 as previously described (Mathew, Harris et al. 1975). The pI values of the β-lactamases were determined by comparison with the pI values of known β-lactamases, TEM-1 (pI 5.4), TEM-2 (pI 5.6), SHV-1 (pI 7.6) and SHV-3 (pI 7.0).

2.10.1. Preparation of β-lactamases

The bacterial strains were grown in 5 ml of sterile nutrient broth overnight at 37°C with shaking (200osc/min). Cells were harvested by centrifugation at 3000 g

(H1000B rotor, Sorvall RT 6000D, Du pont) for 15 minutes at 4°C. The pellet was resuspended in 2 ml of 50mM sodium phosphate buffer, pH7.0 and the cell suspensions were transferred into bijou bottles. Cells were subjected to ultrasonication (MSE Soniprep 150, MSE instruments, Crawley) for 3 x 30 seconds at amplitude of 8 microns with 1 minute cooling period between each sonication. The cell lysate was cleared by centrifugation at 16000 g for 10 minutes at 4°C. The β-lactamase extracts were stored at -20°C until required.

2.10.2. Detection of β-lactamase activity by nitrocephin spot assay

A 30 µl volume of β-lactamase preparation was added to 100 µl of nitrocephin (50 mg/L) in a microtitre plate. The β-lactamase activity was equal to the time in seconds for the colour change of the solution from yellow to red, and the time in seconds for this change to take place was recorded.

2.10.3. Gel casting

The casting chamber was formed from two glass plates separated by rubber tubing placed along their perimeters and clamped together into which the gel solution was poured prior to assembly. One of the glass plates was coated with a binding solution (0.55 w/v gelatine, 0.5% w/v chromium potassium sulphate dodecahydrate), and dried at 55 C to promote adhesion of the gel to the glass. The other plate was siliconised to permit easy separation of the polymerized gel. The gel solution has been poured between the glass plates and was left to polymerise for at least 4 hours. The components of the gel in the order they were added shown in Table 2.1.

2.10.4. Loading IEF gel

The volume (µl) of β-lactamase preparation that was equivalent to its nitrocephin spot test time was applied to the gel surface close to the anode. A maximum of 15 µl of preparation was added per lane. β-lactamase preparations of known isoelectrical points were also applied to the gel in order to quantify the gradient.

Table 2.1: composition of an IEF gel

Material	Volume added	Final concentration
Distilled water	25	-
40% (w/v) ampholines pH 3.5-10	2.0	2% (w/v)
100 g acrylamide plus 2.7 g methylene bisacrylamide (BDH) in 300 ml distilled water	9.0	Acrylamide 75 g/L Bisacrylamide 2 g/L
Riboflavin (20mg/L)	4.0	2 mg/L
5% (v/v) TEMED*	0.2	0.005% (v/v)

2.10.5. Running conditions

Isoelectric focusing was performed at 500 V (limiting), 1 W (constant) and 20 mA (limiting) for 18 hours at 4ºC.

2.10.6. Visualisation of β-lactamases after electrophoresis

The β-lactamase activity was detected by soaking a piece of filter paper (Whatman No. 1, Whatman International Ltd, Maidstone) in nitrocephin solution (50 mg/L) and overlaying the surface of the gel. The focused β-lactamases appeared as red bands on a yellow background. Stained gels were photographed with a Polaroid camera using a Tiffen green filter.

2.10.7. Inhibitor overlays

The β-lactamase inhibition was detected by soaking a filter paper in the required concentrations of antimicrobial inhibitor solutions and laid over the gel surface for 10 minutes at 37°C. The filter paper was removed and the gel was stained with nitrocephin as described in section (2.10.6).

2.11. Conjugation studies by broth mating method

Broth mating experiment was performed with *E. coli* J62-2 (Rif^R). Cultures of each donor and the recipient strain were grown in LB broth (Sigma) and then mixed in the ratio of 1:9 and incubated overnight at 37°C. The mating mixture was serially diluted in 0.9% saline. A 100 µl aliquot of each dilution was spread onto MacConkey agar plates containing (50 mg/L amoxicillin) or (2 mg/L cefotaxime) or (8 mg/L naladixic acid) and (150 mg/L rifampicin). Controls of each strain were prepared by inoculating equal volumes of each broth onto separate agar plates containing the same antibiotic concentrations. Both control plates and conjugated plates were incubated at 37°C overnight or until colonies were visible.

2.12. Transformation

For transformation, plasmid DNA was transferred by electroporation into *E. coli* DH5α cells. Transformants were plated on LB agar supplemented with appropriate antimicrobial agent concentrations.

2.13. Electroporation

The DNA was mixed with the electrocompetent *E. coli* bacteria in 2 mm gap cuvette. The cuvette containing the sample was placed on the shocking chamber of the gene pulser (Electroporation 32 System) and the mixture was electroporated at 2.5 volt. Cells were recovered by adding 500 µl LB medium and incubating at 37°C for 1hour whilst shaking.

2.14. Preparation of DNA

2.14.1. Extraction of DNA from the bacterial isolates

Bacterial culture was grown in 5 ml IST broth and then 1.5 ml of the culture was centrifuged at 16000 g for 1 minute. The pellet was resuspended in 100 µl of distilled

water by repeated pipetting and boiling for 10 minutes followed by centrifuge at 10000 g for 1 minute. The supernatant was transferred to another tube and kept at -20ºC until used. 1 µl was used as DNA template.

Genomic DNA was extracted using the Puregene DNA isolation Kit (Qiagen, UK) according to the manufacturer's instructions.

2.14.2. PCR reagents

All PCR reagent were performed with Promega reagents (Promega, Southampton, UK), Go Taq Flexi DNA Polymerase (5u/µl) in 5x green or colourless buffer, $MgCl_2$ solution (25mM) and deoxynucleoside triphosphates (dNTP) / PCR nucleotide mix (10mM each). Sterilized distilled water was used as the diluents.

2.14.3. PCR reaction

All PCR reactions unless otherwise indicated were performed in 50µl volumes in PCR tubes. The reaction mixture consisted of 2.5mM $MgCl_2$, 0.1mM dNTPs, 5X reaction buffers, 0.05mM each primer, 0.2 units of *Taq*, 1µl extracted DNA template and the volume of the reaction mixture was completed to 50µl using sterilized distilled water. The thermal cycler was adjusted according to the type of the reaction. All amplification experiments included a negative control blank which contained all reagents with the exception of target DNA.

2.14.4. Primers used

Primers were designed either with perlprimer v1.1 software (http://perlprimer.sourceforge.net/), or the sequences were taken from previously published work. Table 2.2 shows primers used for PCR amplifications in this study. All primers were synthesised by (Eurofins MWG Operon, UK).

Table 2.2: Oligonucleotides used for PCR amplification and Sequencing

Primer	Sequence	Reference
mQnrAm-F	AGA GGA TTT CTC ACG CCA GG	(Cattoir, Poirel et al. 2007a)
mQnrAm-R	TGC CAG GCA CAG ATC TTG AC	,,
mQnrBm-F	GGM ATH GAA ATT CGC CAC TG	,,
mQnrBm-R	TTT GCY GYY CGC CAG TCG AA	,,
mQnrSm-F	GCA AGT TCA TTG AAC AGG GT	,,
mQnrSm-R	TCT AAA CCG TCG AGT TCG CG	,,
QnrA-A	GGG TAT GGA TAT TAT TGA TAA AG	(Cattoir, Weill et al. 2007)
QnrA-B	CTA ATC CGG CAG CAC TAT TA	,,
QnrS-A2	AGT GAT CTC ACC TTC ACC GC	,,
QnrS-B2	CAG GCT GCA ATT TTG ATA CC	,,
qnrS –F	TGG AAA CCT ACA ATC ATA CAT ATC G	(Hopkins, Wootton et al. 2007)
qnrs –R	TTA GTC AGG ATA AAC AAC AAT ACC C	,,
qnrB1-F	ACC TGA GCG GCA CTG AAT TTA T	(Park, Kang et al. 2009)
qnrB1-R	TCG CAA TGT GTG AAG TTT GC	,,
qnrB4-F	GAT GAC TCT GGC GTT AGT TGG	,,
qnrB4-R	CCA TGA CAG CGA TAC CAA GA	,,
CTX-M_R	TTT GCG ATG TGC AGT ACC AGT AA	(Edelstein, Pimkin et al. 2003)
CTX-M_F	CGA TAT CGT TGG TGG TGC CAT A	,,
CTX-M1_A	CTT CCA GAA TAA GGA ATC	(Dutour, Bonnet et al. 2002)
CTX-M1_B	CCG TTT CCG CTA TTA CAA	,,
ISEcp1A	GCA GGT CTT TTT CTG CTC C	(Poirel, Decousser et al. 2003)
ISEcp1b	TTT CCG CAG CAC CGT TTG C	,,
PROM+	TGC TCT GTG GAT AAC TTG C	,,
ISF	GCG GTA AAT CGT GGA GTG AT	(Woodford, Ward et al. 2004)
ISR	ATT CGG CAA GTT TTT GCT GT	,,
Kp-TEM_for	CCC CTA TTT GTT TAT TTT TC	(Mabilat and Goussard 1993)
Kp-TEM_rev	GAC AGT TAC CAA TGC TTA ATC A	,,
TEM-C_F	GGG CAA GAG CAA CTC GG	(Arpin, Dubois et al. 2003)
TEM-D_F	CAG CAA TGG CAA CAA CGT TG	,,
TEM-F_R	CAA CGT TGT TGC CAT TGC TGC AG	,,
TEM-G_R	ACC GAG TTG CTC TTG CCC	,,
Kp-SHV_for	GCC GGG TTA TTC TTA TTT GTC GC	(Mabilat and Goussard 1993)
Kp-SHV_rev	TCT TTC CGA TGC CGC CGC CAG TCA	,,
SHV1_F	GGA CTA CTC GCC GGT CAG C	(Arpin, Dubois et al. 2003)
SHV2_R	GCT GAC CGG CGA GTA GTC C	,,
SHV3_F	GAT TGT CGC CCT GCT TTG G	,,
SHV4_R	CCA AGC AGG GCG ACA ATC	,,

IS26-FCJ	CAT TTC AAA AAC TCT GCT TAC	(Diestra, Juan et al. 2009)
SHV-12-F-I	TAC ACA GGC GAA TAT AAC GC	,,
SHV-12-R-I	GAT CGA GCA CTG GCA ACG	,,
DEOR-R	TGA TTC CAA TAG AAC GAC ATC	,,
RECF-F	TCC CGC ACT CGC GAT AC	,,
OXY-F	ATG ATA AAA AGT TCG TGG C	This study
OXY-R	TTA AAG CCC TTC GGT CAC	This study
A1	GAA CAT AGC GGC TCC TTA T	This study
VIC-2	GGT TAC AAC TTC GAA GAC TC	(Brisse and Duijkeren 2005)
VIC-3	GGC GAA ATG GCW GAG AAC CA	,,
gyrA-A	CGC GTA CTA TAC GCC ATG AAC GTA	(Brisse and Verhoef 2001)
gyrA-C	ACC GTT GAT CAC TTC GGT CAG G	,,
P-F 5	GGG GAC AAG TTT GTA CAA AAA AGC AGG	This study
	CTC CGC CGG AAT TCC ATC ATC AAT A	
attB2-F	GGG GAC AAG TTT GTA CAA AAA AGC AGG	This study
	CTC CGC CAT GAT AAA AAG TTC GTG G	
attB2-R	GGG GAC CAC TTT GTA CAA GAA AGC TGG	This study
	GTT TAA AGC CCT TCG GTC AC	
Intl1-F	CCT TCG AAT GCT GTA ACC GC	(Murinda, Ebner et al. 2005)
Intl1-R	ACG CCC TTG AGC GGA AGT ATC	,,
qacED1-F	GAG GGC TTT ACT AAG CTT GC	,,
qacED1-R	ATA CCT ACA AAG CCC CAC GC	,,
sul1-F	TCA GAC GTC GTG GAT GTC G	,,
sul1-R	CGA AGA ACC GCA CAA TCT CG	,,
Intl2-F	CAC GGA TAT GCG ACA AAA AGG T	(Machado, Canton et al. 2005)
Intl2-R	GTA GCA AAC GAG TGA CGA AAT G	,,
FIA FW	CCA TGC TGG TTC TAG AGA AGG TG	(Carattoli, Bertini et al. 2005)
FIA RV	GTA TAT CCT TAC TGG CTT CCG CAG	,,
FIB FW	GGA GTT CTG ACA CAC GAT TTT CTG	,,
FIB RV	CTC CCG TCG CTT CAG GGC ATT	,,
A/C FW	GAG AAC CAA AGA CAA AGA CCT GGA	,,
A/C RV	ACG ACA AAC CTG AAT TGC CTC CTT	,,
N FW	GTC TAA CGA GCT TAC CGA AG	,,
N RV	GTT TCA ACT CTG CCA AGT TC	,,
FII-F	CTG TCG TAA GCT GAT GGC	,,
FII-R	CTC TGC CAC AAA CTT CAG C	,,
L/M FW	GGA TGA AAA CTA TCA GCA TCT GAA G	,,
L/M RV	CTG CAG GGG CGA TTC TTT AGG	,,
I1 FW	CGA AAG CCG GAC GGC AGA A	,,

I1 RV	TCG TCG TTC CGC CAA GTT CGT	,,
FIC FW	GTG AAC TGG CAG ATG AGG AAG G	,,
FIC RV	TTC TCC TCG TCG CCA AAC TAG AT	,,
5-CS	GGC ATC CAA GCA CAA GC	(Zhao, White et al. 2001)
3-CS	AAG CAG ACT TGA CTG AT	,,

2.14.5. Screening of PCR products

Agarose (1gm) was added to 100 ml TAE buffer (40mM Tris acetate, 20mM Acetic acid, 1mM EDTA, pH7.6). The agarose was solubilized by heating in a microwave oven for 10 minutes and then left to cool to room temperature. Five µl of each of the PCR product samples were applied to the gel along with 5 µl molecular weight marker after mixing each with 1 µl loading buffer on a piece of parafilm. Each mixture was applied to a slot using 10 µl micropipette. The electrophoresis cell was covered and the power supply was switched on and adjusted at 10 Volt/cm. The gel was immersed in ethidium bromide solution (0.5 mg/L) for between 10-30 minutes depending on the concentration of DNA being analysed. The gel was taken out from the cell and examined under gel documentation.

2.14.6. Purification of PCR product

PCR products were purified either directly or by separation of the DNA fragment from 1% TAE agarose gel electrophoresis. The DNA band of the correct size was cut out from the gel. The purification of DNA from the direct PCR and gel purification were done using (Qiagen purification kit) according to the manufacturer's instructions.

2.14.7. Determination of DNA concentration

The concentration and purity of the purified DNA was determined by measuring the UV absorbance at 260 and 280 nm. The DNA concentration was calculated with the OD260nm (1 OD260nm = 50 μg/ml dsDNA or 33 μg/ml ssDNA). The purity was

estimated with the $OD_{260}nm/OD_{280}nm$ ratio, with a ratio of ca. 1.8 indicating a low degree of protein contamination.

2.14.8. Sequencing of the genes

The resulting PCR products were sequenced by the Sanger method using an ABI 373A DNA sequencer (PE Applied Biosystems, Warrington, UK). The BLAST and FASTA programs of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nhi.gov) were used to search databases for similar nucleotide and amino acid sequences. The amino acid sequences were translated using the EXPASY translate website (http:www.expasy.ch/tools/dna.html). Multi-alignment of DNA or amino acid sequences was performed with the online website Multalin (http://www.toulouse.inra.fr/multalin.html) or Clustal W tools (http://www.ebi.ac.uk/Tools/clustalw2/).

The sequencing of the bla_{OXY-2} genes was done commercially by GATC Biotech (Konstanz, Germany), using pDONR207 vector forward and reverse primers.

2.14.9. Restriction endonuclease digestion

Restriction endonuclease reactions were performed according to the manufacturer's recommendations. In general, 1.5 μg DNA was digested for 2 hours at the appropriate temperature with 10-20U enzyme. Efficacy of the cleavage reaction was determined by 0.7% agarose gel electrophoresis stained with ethidium bromide.

2.15. Pulsed Field Gel Electrophorasis (PFGE)

2.15.1. Preparation and digestion of DNA in plugs

PFGE was applied to assess the clonality of bacterial isolates. Without shaking overnight cultures from 5ml of Brain Heart Infusion (BHI) were collected by centrifuged at 4000 g for 20 minutes. The supernatant was removed and the pellets

were re-suspended in 2ml of PIV buffer (1.0 M Tris base, 1.0 M NaCl, pH 7.6) and allowed to equilibrate to 50ºC for an hour. An equal volume of 1.6% low-melting-point agarose (Boehringer Mannheim, Indianapolis, USA) prepared in PIV buffer was added to each suspended cell and the mixture pipetted into Bio-Rad (Richmond, California, USA) plug moulds and refrigerated at 4ºC for 30 minutes.

The protocol then followed that described by (Miranda, Kelly et al. 1996). The cells embedded in agarose plugs were incubated in 5ml of lysis buffer (1.0 M Tris, 1.0 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% Na lauroyl sarcosine) supplemented with 50mg/ml RNase and 1mg/ml lysozyme overnight at 37ºC. The lysis buffer was replaced by ESP buffer (0.4 M EDTA, 1% sodium lauroyl sarcosine, 0.5mg/ml proteinase K, pH 9.0) and incubated at 50ºC for 24 hours prior to washing once with 1X TE at 37ºC (5 mM Tris HCl, 5 mM EDTA, pH7.5) containing 1mM phenylmethylsulfonyl fluoride and three times wash with 0.1X TE for at least 30 minutes each wash. Plugs could be stored at this stage at 4ºC in 0.1X TE buffer.

For PFGE digestion, a slice of plug was equilibrated in 200μl of the 1X restriction endonuclease buffer for 2 hours at room temperature. After this, the slice was incubated with 30 U of *Xba1* restriction endonuclease (Promega, UK) in a fresh 100 μl 1 X reaction buffer containing 1μl BSA in water bath at 37ºC for 12 hours. The reaction was stopped by adding 300 µl of 0.5X TBE buffer (0.089M Tris base, 0.089 Boric acid, 2.5 mM disodium EDTA) (ICN Biomedical, Aurora, Ohio, USA). Plug slices were then loaded into wells of 1% PFGE agarose gel preapared in 0.5 TBE buffer. Electrophoresis was performed in a CHEF-DRII system (Bio-Rad Laboratories, Ltd). The gel was run for 24 hours at 200V (6v/cm) at 14ºC with 5-45 initial and final pulse times. Lambda ladder PFGE marker (New England, Biolabs) was used as the size standard marker. Gels were stained for 30 min in ethidium bromide (0.5mg/ml), destained twice in distilled water for 30 minutes and visualized by UV transillumination.

2.15.2. Interpretation of PFGE

All isolates were analysed using BioNumerics software, version 4. Isolates which clustered together with a similarity of >85% were considered to belong to the same PFGE type. Results were obtained in phylogenetic trees.

2.16. Southern Blotting Protocol

2.16.1. Transferring of DNA

DNA quantities were assessed by running 1µl of DNA with 9µl of gel loading buffer on a 1.2% gel for 45 minutes, 38mv, and 70V. The DNA was digested with an appropriate restriction endonuclease for 2 hours at optimal temperature and subjected to electrophoresis on a 1% agarose gel. The gel was stained for 15 mins in ethidium bromide (125 µl in 250 ml dH$_2$O). The top right hand corner of the gel was cut for orientation. Transfer of restricted DNA was carried out by soaking the gel for 15 minutes in 750 ml acid wash. Then the gel was soaked in 750 ml alkaline wash for 60 minutes. Finally, the gel was soaked twice for 20 minutes in 750 ml neutral wash. The gel was rinsed briefly in distilled water between each wash. The neutral wash was poured off, and the gel was flipped over and placed on the centre (uncovered) part of a wick of 3 sheets of blotting paper sandwiched together. The dated nylon membrane was soaked in the 10 X SSC and then placed on top of the gel. Individually three pieces of blotting paper was soaked in 10 X SSC and lied carefully over the membrane. The stack of paper towels, a glass plate, and a bottle of around 500 g were placed on top of this. Each time air bubbles were removed. The station was left for 8-24 hours to allow capillary transfer to occur.

2.16.2. Preparation of DIG labelled probe

The PCR reaction for amplification of labelled probe was carried out using PCR DIG probe synthesis kit (Roche) as described below:

	DIG labelled probe	Unlabelled control
dH$_2$O	33.25	30.25
PCR buffer + MgCl$_2$ (Vial 3)	5.0	5.0
PCR DIG labelling mix (Vial 2)	2.5	-
dNTP stock (Vial 4)	2.5	5.0
Primer A	2.0	2.0
Primer B	2.0	2.0
Enzyme Mix (Vial 1)	0.75	0.75
DNA template	2.0	5.0

The PCR programme was as follow: 2 minutes at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 50°C, and 30 sec at 72°C, and final extension of 7 min at 72°C. To confirm the probe has been produced, 5μl PCR product mixed with 5μl loading dye was run against the unlabelled control PCR on 1.2% Gel at 70 V for 45 minutes.

2.16.3. Hybridisation procedure

Hybridisations with labelled probes were performed using DIG easy-hybridisation kit (Roche, Germany). The nylon membrane was placed on a piece of saran wrap/cling film and U.V. cross-linked for two minutes on both sides. The hybridisation incubator (Techne Hybridiser, Cambridge) was adjusted to 50°C. The membrane was pre-hybridised in the hybridisation oven with 20 ml DIG easy hyb for 30 minutes. The labelled probe was boiled for 5 minutes and rapidly cold on ice. For hybridisation, appropriate concentration (5-20 μl) of the labelled probe was added to 20 ml DIG easy hyb., and incubated overnight at the appropriate temperature for the probe.

2.16.4. Post-hybridisation washes

2 X 5 minutes in Wash 1 at room temperature.
2X 15 minutes in Wash 2 at 68°C.

2.16.5. Immunological detection

All steps were done at room temperature with agitation

-1-5 minutes in 100 ml 1 X washing Buffer.

- 30 minutes in 100 ml 1 X blocking solution.

- 30 minutes in antibody solution (10 µl antibody in 200 ml 1 x blocking solution).

- 2 X 15 minutes in 100 ml 1 X washing Buffer.

-3 minutes in 20 ml 1 X detection buffer.

- 20-25 drops CDP* ready-to-use was added to the DNA side of the membrane in a hybridisation bag or saran wrap and was incubated for 5 minutes at 37ºC. Autoradiography film (Hyperfilm^{TM}, Amersham Life Science) was exposed initially for 30 minutes and extended up to 5 hours depending on the strength of the signal after the initial period.

2.16.6. Stripping and reprobing of DNA blots

Membranes were saturated in 2X SSC and stored at -20ºC in a hybridisation bag/saran wrap. Reprobing the membrane was carried out by rinsing the membrane briefly in distilled water followed by rinsing the membrane in the stripping solution (0.1% (w/v) SDS) which had been brought to the boil at 5ml/cm^2 membrane for 15 minutes at 37ºC to remove the DIG labelled probe. This step was repeated again and the membrane was rinsed thoroughly in 2 x SSC (5-6 rinses) and pre-hybridised and hybridised with second probe. If reprobing was not to be carried immediately, the membranes were wrapped wet in SaranWrap and stored at 4ºC.

Table 2.3: Solutions used in southern blot hybridisation

Solution	Working concn.	Store concn.	Dilution (ml/100 ml)	Storage and Stability
Wash 1	2 X SSC 0.1 % SDS	20X 20%	10 0.5	Room temp. / can be made in advance
Wash 2	0.1 X SSC 0.1% SDS	20X 20%	0.5 0.5	Room temp. / can be made in advance
*Washing Buffer	1X	10X	10 ml in 90 ml dH$_2$O	Room temp.
*Maleic acid Buffer	1X	10X	10 ml in 90 ml dH$_2$O	Room temp.
*Blocking Solution	1X	10X	10ml/90ml 1X maleic acid	Prepare fresh
*Detection Buffer	1X	10X	10 ml in 90 ml dH$_2$O	Room temp. pH9.5
Pre-hybridisation solution	5 X SSC 0.1% N-laurylsarcosine (w/v) 0.02% SDS (w/v) *1% blocking solution (v/v)	20X 20% 10X	25 0.1 g 0.02 g 1 ml	Prepare fresh
Acid wash	0.25 M HCl	35-38%	2.5 (add acid to water)	Room temp.
Alkali wash	1.5 M NaCl 0.5 M NaOH	5 M 1 M	30 50	Room temp./can be made in advance
Neutral wash	0.5 M Tris HCl (pH8.0) 1.5 M NaCl 0.001 M EDTA (pH8.0)	1 M 5 M 0.5 M	50 30 0.2	Room temp./can be made in advance
20 X SSC	3M NaCl 0.3 M NaCitrate	Solid Solid	175.3 g/1000 ml 88.0 g/1000 ml	Room temp./can be made in advance pH7.0

* These reagents are part of the DIG wash and block buffer set

2.17. Plasmid analysis

2.17.1. Extraction of plasmid DNA

Several different plasmid extraction protocols were used in this study. The extraction methods employed included the mini and midi kit (Qiagen), and miniprep rapid alkaline lysis method described by (Birnboim and Doly 1979) with some modifications. Briefly, a single bacterial colony was selected from the LB plates and

was transferred to 5 ml of LB broth and incubated overnight at 37°C with constant shaking. The cells were harvested by centrifugation at 3000 g for 10 minutes at 4°C. The supernatant was discarded and the bacterial pellet was washed in 250 μl of TE (10mM Tris, 1mM EDTA, pH8). The bacterial pellet was resuspended in 300 μl of ice-cold solution I. For the alkaline lysis of the bacteria, 300 μl of solution II were added and mixed properly and left on ice for 10 minutes. Three hundred μl of ice-cold solution III were added and were stored again on ice for 10 minutes. The mixture was centrifuged at 16000 g for 10 minutes. The protein and genomic DNA was removed from the supernatant with phenol: chloroform: isoamyl alcohol (25:24:1) mixture followed by centrifugation at 16000 g for 10 minutes. Subsequently, the plasmid DNA was precipitated by double volume of absolute ethanol and washed with 0.5 ml 70% ethanol followed by a centrifugation at 13000 rpm for 10 minutes in each step. Finally, the DNA was dissolved in 50 μl of nuclease-free water.

Solutions for the small scale preparation of the plasmid DNA extraction

Solution I: 50 mM glucose

25 mM Tris Cl pH 8.0

10 mM EDTA pH 8.0

Solution II: 0.2 N NaOH

1% SDS

Solution III: 3 M potassium acetate adjusted to pH5 with glacial acetic acid and then adjusted to the final volume with distilled water.

2.17.2. Determination of plasmid profile by Nuclease S1

This procedure converts the circular plasmid DNA to the linear form to determine the accurate molecular sizes of plasmids. The closed circular supercoiled form moves very slowly, the naked plasmids remain trapped in the sample wells. The protocol was modified from (Barton, Harding et al. 1995), briefly the plugs of PFGE containing DNA was equilibrated into 200 μl S1 buffer (Promega) for 20 minutes at

room temperature. The buffer was replaced with 100 µl of fresh buffer with 8 U of nuclease S1 (160 U/ µl) (Promega) and incubated for 45 minutes at 37ºC. The reaction was stopped by adding 0.5M EDTA (pH8). Then the gel was running as for Xba1 PFGE electrophoresis. The size of plasmids was determined by comparison to Lambda ladder PFGE markers (New England Biolabs).

2.18. DNA cloning of OXY-2 beta-lactamase

2.18.1. Primers design and PCR amplification

The cloning of OXY-2 beta-lactamases was done with Gateway® Technology with Clonase™ (Invitrogen, UK) as described by manufacturer's instructions. Primers were designed using perlprimer v1.1 software (http://perlprimer.sourceforge.net/) and adjusted the annealing temperature to be approximately 55ºC. Two primer pairs were designed for nested PCR. The first primer-pair consisted of gene specific primers containing the internal parts of the attB1 and attB2 recombination sites. The second pair of primers contains the external parts of the attB1 and attB2 recombination site in addition to a 12 nucleotide overlap with the internal forward and reverse primers (Table 2.4).

Table 2.4: Primer construction for nested polymerase chain reaction (PCR)

1	attB1	internal for	**AAAAAGCAGGCT<u>CCGCC</u>***ATGXXXXXXXXXXXXX*
2	attB1	internal rev	**AGAAAGCTGGGT***XXXXXXXXXXXXXXXXXXXX*
3	attB2	external for	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**
4	attB2	external rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**

Bold letters: attB1/attB2 sequence, underlined letters: Kozak sequence, italic letters: forward and reverse sequence of bla_{OXY-2} gene.

The two PCR reactions were performed with Expand Long Template PCR System (Roche) according to the descriptions below, with 10 µl of the first reaction being used as a template for the second PCR reaction.

1st PCR:

10x buffer I	5 µl
Internal Forward Primer 10 pmol/µl	2 µl
Internal Reversed Primer 10 pmol/µl	2 µl
Template 10ng/µl	2 µl
dNTP Mix 10mM	1 µl
Polymerase 1.5U	0.3 µl
Distilled water to final volume	50 µl

2nd PCR:

10x buffer I	5 µl
External Forward Primer 10 pmol/µl	1 µl
External Reversed Primer 10 pmol/µl	1 µl
Template (1st PCR Reaction)	10 µl
dNTP Mix 10mM	1 µl
Polymerase 1.5U	0.3 µl
Distilled water to final volume	50 µl

PCR conditions for both PCRs were as follows: 2 minutes at 94°C, 15 cycles of 10 sec at 94°C, 30 sec at 55°C, and 2 minutes at 68°C, and final extension of 7 min at 68°C. The PCR products were used in cloning vectors *pDONR207, pGBKT7 and pGADT7* which were isolated by midiprep alkaline lyses according to manufacturer's instructions (Qiagen, UK) in reactions described below.

2.18.2. The BP reaction

PCR product	2-7 µl
pDONR 207(150 ng/ µl)	1 µl
BP Reaction buffer (5x)	1.5 µl
BP Clonase	1 µl

The reaction was incubated at 37°C overnight. After incubation, 1 µl of proteinase K was added and incubated for 10 minutes at 37°C. 1 µl of the reaction was transformed into 50 µl of electrocompetent DH10B bacteria. Cells were recovered by adding 500 µl LB medium and incubating at 37°C for 1h whilst shaking. The whole volume of transformation reaction was spread on LB plates containing 15 µg/ml gentamicin and incubated overnight. Plasmid DNA was isolated from single colony by mini prep alkaline lyses (Qiagen Kit). The plasmids were digested with the restriction endonuclease BanII to determine the correct size of the inserted fragment and sent to sequencing to assure no presence of frame shift. This plasmid was used in LR reaction.

2.18.3. The LR reaction

Purified Entry Clone (150 ng/µl)	1 µl
pGBKT7	1 µl
pGADT7	1 µl
Distilled water	1 µl
LR Clonase	1 µl

Two µl of the reaction mixture were transformed into 50 µl of electrocompetent DH10B bacteria. Cells were recovered by adding 500 µl LB medium and incubating at 37°C for 1h whilst shaking. The transformation reaction was divided in two parts. One was spread on LB plates containing 50 µg/ml kanamycin for pGBKT7 (bait vector), the other on ampicillin plates (50 µg/ml) for pGADT7 (prey vector). The plasmids were digested with the restriction endonucleases *EcoRI* and *BamHI* to determine the correct size of the inserted fragment.

2.19. Ribonucleic acid analysis

2.19.1. RNA extraction

Total RNA was extracted from bacterial cultures grown to an optical density of 0.6-1.0 at OD_{600} with RiboPure^{TM}-Bacteria Kit (Ambion, UK). Sterilization of contaminating RNAases from the benches and equipments was done by RNAZAP (Ambion, UK). RNA was electrophoresed on denaturing (1% formaldehyde) agarose gel electrophoresis. The resulting RNA was treated with DNase 1 at 37°C for 30 minutes for removal of the trace amounts of genomic DNA from the eluted RNA.

2.19.2. Reverse transcription

RNA was reverse transcribed to cDNA and PCR amplification of a specific target RNA from total RNA using one-tube, two enzyme system (Access RT-PCR System Kit, Promega). The reverse transcription was done according to the manufacturer's instructions. The PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide. The image was examined using UV Gel Transillumination.

2.20. Determination of outer membrane proteins by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.20.1. Protein extraction

The outer membrane protein was extracted according to the method described by (Bossi and Figueroa-Bossi 2007). Briefly, bacterial cells were grown in 5 ml of LB to OD_{600} of 0.35 and collected by centrifugation at 3000 g (Sorvall RT 6000D) for 15 minutes at 4°C. The pellet was re-suspended in 1 ml of 10 mM Tris pH 8.0 and sonicated with cooling, using three pulses of 20 s with 30 s resting intervals (MSE Soniprep 150, MSE instruments, Crawley). Lysate was centrifuged at 7000 g for

5 minutes. Then the supernatant recovered and centrifuged at 13 000 rpm for 45 minutes at 20°C. The pellet was re-suspended in 0.5 ml of freshly made 10 mM Tris-HCl pH 8.0, 10 mM MgCl$_2$, 2% (v/v) Triton X-100. Furthermore, the suspension was incubated at 37°C for 30 minutes, then centrifuged at 16000 g for 45 minutes at 20°C. Finally, the pellet was re-suspended in 50–100 µl of 100 mM Tris-HCl pH 8.0, 2% SDS. The suspension was kept at –20°C until use.

2.20.2. Polyacrylamide gel electrophoresis

The separating and stacking gels were calculated from the website (http://www.changbioscience.com/calculator/sdspc.htm) as shown below. Ammonium persulfate was the last components to be added just before pouring and mixed gently to avoid the addition of air which affects the gel matrix. The separating solution was poured into the preassembled gel apparatus. Two ml ethanol was added on the surface of separating gel to get even surface and the gel was left to polymerise for approximately 30 minutes. The stacking gel was poured followed by insertion of a 10 well comb and left to polymerise for at least 45 minutes.

Stacking gel 4%		**Separating gel 15%**	
Distilled water (mL)	3.65	Distilled water (mL)	3.55
40% Polyacrylamide (mL)	0.625	40% Polyacrylamide (mL)	3.75
1M Tris(pH6.8)(mL	0.625	1.5M Tris(pH8.8)(mL)	2.5
10% SDS (mL)	0.05	10% SDS (mL)	0.1
10% Ammonium persulfate (µL)	12	10% Ammonium persulfate (µL)	22
TEMED (µL)	12	TEMED (µL)	22
Total volume (mL)	5	Total volume (mL)	10

Samples were mixed with an equal volume of SDS-PAGE loading dye (950 µl, 50 µl β-mercaptoethanol), heated at 100°C for 5 minutes and kept on ice until required for gel loading. About 20 µl volume of each sample was loaded into the wells. Molecular weight markers (Bio Rad) were added to a well to accurate size. The gel

73

was inserted into a gel electrophoresis apparatus (Protean II, Bio Rad). The electrophoresis was run at 200 V for approximately 60 minutes or until the dye had reached the end of the gel.

2.20.3. Protein detection

The membrane was stained in (0.1% w/v Coomassie blue R-250, 50% v/v methanol, 1% v/v acetic acid in distilled water) for 30 minutes. Destaining of the membrane was carried out in destaining solution (50% v/v methanol, 1% v/v acetic acid in distilled water) for 30 minutes, followed by another 30 minutes in distilled water.

RESULTS

CHAPTER - 3:

Identification and Susceptibility Testing

3.1. ABSTRACT

Objectives: This study was planned to investigate the prevalence of ESBL-producing *Klebsiella* spp. from Scotland.

Results: From 223 isolates collected from Royal Infirmary of Edinburgh during 2006 and 2007, 219 *K. pneuminae*, 2 *K. oxytoca*, 1 *E. cloacae*, and one isolate *Salmonella. enterica* were identified by API 20E. Out of 223 isolates tested by MICs, 34 (15.2%) of these strains were resistant to cefotaxime, 36 (16.1%) to ceftazidime, 35 (15.7%) to ceftriaxone, 45 (20.2%) to nalidixic acid, 30 (13.5%) to ciprofloxacin, 55 (24.7%) to cefoxitin and 0 (0%) to meropenem. The overall frequency of ESBL producers observed in this study was 35/223 (15.7%), most of them 32/35 (91.4%) were from *K. pneumoniae*. SHV β-lactamases were detected in 32 (91.4%), TEM and CTX-M were detected in 24 (68.6%) and 16 (45.7%) respectively of ESBLs-positive isolates, two OXY-2 enzyme were detected in *K. oxytoca* isolates. *Qnr* genes were expressed from 18 *K. pneumoniae* isolates.

Conclusion: This work reports phenotypic, genotypic analysis and antimicrobial susceptibility profiles of clinical isolates of *K. pneumoniae* of both community and nosocomial origin recovered from Edinburgh, Scotland.

3.2. INTRODUCTION

Klebsiella spp. are opportunistic human pathogens that can be isolated from various animal and human clinical specimens. *Klebsiella* spp. are responsible for 1% to 5% of all cases of community-acquired pneumonia and between 0% to 23% of those acquired in the hospital and its frequency is greater in alcoholic patients (Podschun and Ullmann 1998). *K. pneumoniae* is a Gram-negative bacterium, which causes hospital and community acquired infections such as pneumonia, urinary tract infection, septicaemia, soft tissue infections. *K. pneumoniae* is responsible for 75–80% of *Klebsiella* infections, particularly which associated with ESBLs (Sarno, McGillivary *et al.* 2002; Brisse, van Himbergen *et al.* 2004). Since 1981 a distinctive syndrome of community-acquired *Klebsiella* bacteraemia with primary liver abscess

has been observed (Ko, Paterson *et al.* 2002; Fang, Sandler *et al.* 2005). *K. pneumoniae* has also become a common cause of community acquired bacterial meningitis in adults in Taiwan (Lederman and Crum 2005).

Extended-spectrum β-lactamases producing Enterobacteriacae are emerging as one of the major public health threats. The wide use, and in some cases, misuse of antibiotics over the past several decades has been cited as a responsible factor in the development of drug resistance in all bacterial species (Arpin, Dubois *et al.* 2003). Recently, the isolation of multidrug-resistant strains of ESBL-producing *K. pneumoniae* has become increasingly common, especially in intensive care units and other high-risk hospital areas (Tumbarello, Spanu *et al.* 2006).

The identification of *Klebsiella* to the species or DNA group level phenotypically is a difficult problem due to significant phenotypic overlapping between strains which are genotypically closely related (Monnet and Freney 1994; Wang, Cao *et al.* 2008). Laboratories might use macrotube tests alone or by combining API 20E with additional macrotube tests (Podschun and Ullmann 1998). The API 20E system is a useful 1st stage for the identification of Gram-negative bacteria.

A simple and rapid genotypic method for the identification of *Klebsiella* using *gyrA* PCR-RFLP profiles using restriction enzymes *Hinc*II, *Taq*I and *Hae*III has been published previously (Brisse, van Himbergen *et al.* 2004; Brisse and Duijkeren 2005).

3.3. RESULTS

3.3.1. Phenotypic identification

Results of phenotypic identification using Vitek2 system and API 20E revealed that 219 isolates were identified as *K. pneumoniae*, 2 isolates *K. oxytoca*, 1 isolates *Enterobacter cloacae*, and one isolate *Salmonella enterica*.

3.3.2. Genotypic identification

Restriction analysis of the *gyrA* gene (*gyrA* PCR-RFLP) using primers gyrA-A and gyrA-C (annealing temperature 50º C) as described by Brisse and Verhoef (2001) was used to assign the 38 (34 ESBLs-positive and 4 ESBLs-negative) isolates to *Klebsiella* species and phylogenetic groups. The isolates were identified as *K. pneumoniae* phylogenetic type KpI (29 isolates), *K. pneumoniae* KpIII (7 isolates), *K. oxytoca* (two isolates). None of the *K. pneumoniae* KpII, *K. planticola* and *K. terrigena* was identified in this study.

Because *gyrA* PCR-RFLP does not distinguish between the two phylogenetic groups of *K. oxytoca*, the *rpoB* gene sequence was determined for the two *K. oxytoca* isolates. The sequence of a 940 bp portion of the *rpoB* gene was determined using primers VIC-2 (GGT TAC AAC TTC GAA GAC TC) and VIC-3 (GGC GAA ATG GCW GAG AAC CA) for PCR and sequencing. The PCR annealing temperature was 50° C. Sequencing was performed using ABI Big Dye Terminator. Both isolates were shown to belong to *K. oxytoca* group II (KoII).

As described by (Brisse, van Himbergen *et al.* 2004; Brisse and Duijkeren 2005), *Hin*cII enzyme did not cut the *gyrA* PCR products from *K. planticola* and *K. terrigena*, whereas it generated profile *Hin*cII-B (298 bp and 143 bp) for all *K. pneumoniae* isolates and profile C (196 bp, 168 bp and 77 bp) for all isolates of *K. oxytoca*. In addition, the use of *Taq*I and *Hae*III separately would distinguish between KpI (profiles *Taq*I-B, *Hae*III-C), KpII (*Taq*I-E, *Hae*III-C or *Hae*III-D) and KpIII (*Taq*I-B and *Hae*III-B). Profile TaqI-B consists of the expected 197-bp, 142-bp, 93-bp and 9-bp fragments, and profile TaqI-E of the expected 197-bp, 151-bp and 93-bp fragments. Profile HaeIII-B consists of the expected 175-bp, 174-bp and 92-bp fragments, profile HaeIII-C of the expected 175-bp, 129-bp, 92-bp and 45-bp fragments, and profile D of the expected 267-bp, 129-bp and 45-bp fragments.
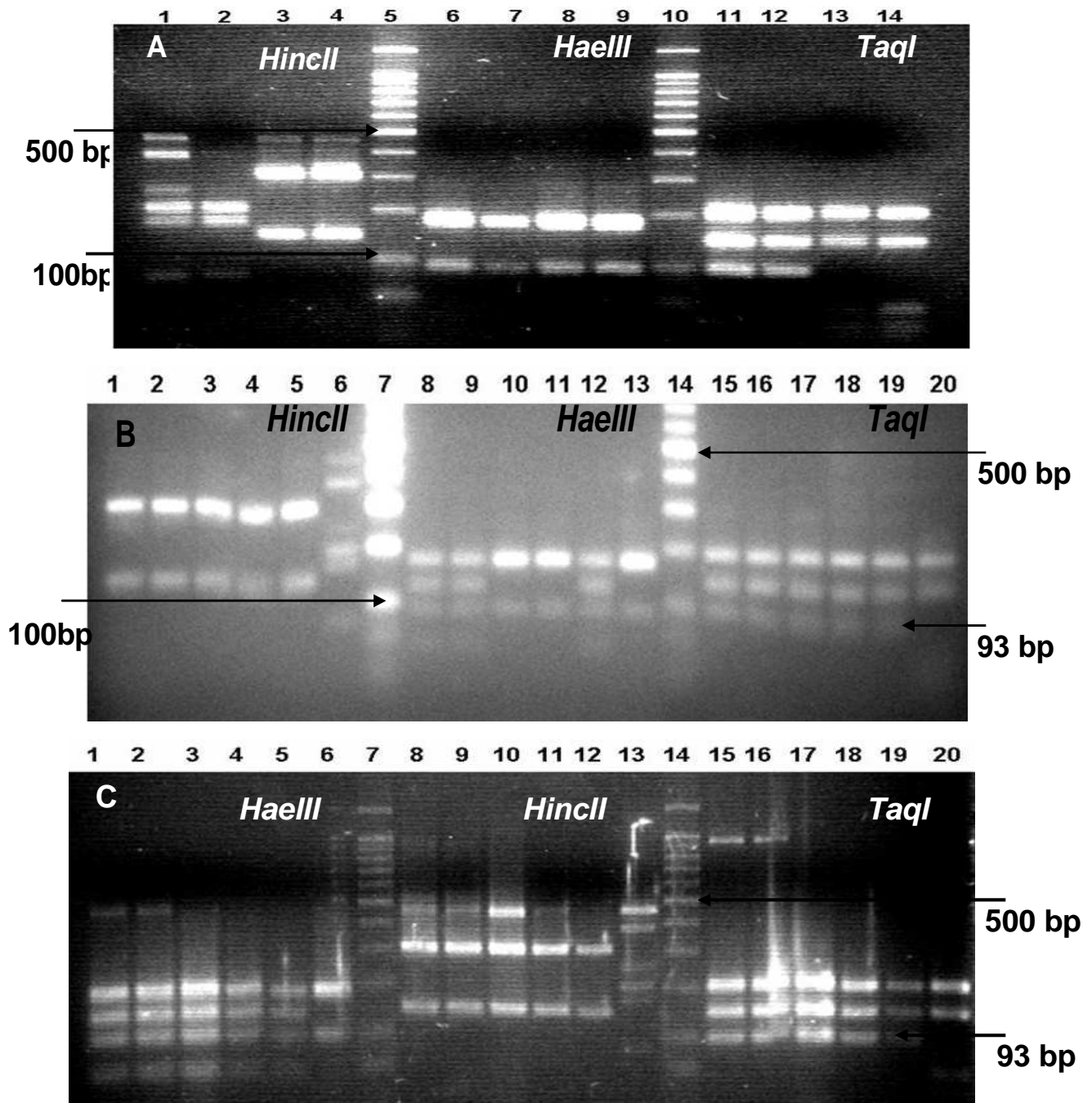
Fig 3.1: Gel electrophoresis of *HincII*, *HaeIII* and *TaqI* restriction endonuclease for some *Klebsiella* isolates. Picture A; lanes 1, 6, 13: *K. oxytoca* isolate MU946294N and. Lanes 2, 7, 14: *K. oxytoca* isolate MB193997E. Lanes 3, 8, 11: isolate 106; Lanes 4, 9, 12: isolate 135. Picture B; isolates numbers 187, 195, 206, 172, 214, and MU946294N. Picture C: isolates numbers MB, 32, 175, 197, 215, and MU946294N respectively. Lane 5, 10 (picture A), lanes 7 and 14 (picture B and C): 100 bp molecular markers.

3.3.3. Antimicrobial susceptibility

The MIC values of a range of antibiotics tested against these isolates are shown in Table 3.1. Out of 223 isolates tested by MICs, the highest percentage of resistance 24.7% (n= 55) was found to cefoxitin, followed by nalidixic acid 20.2% (n = 45). In addition, 34 (15.2%) of these strains were resistant to cefotaxime, 36 (16.1%) to ceftazidime, 35 (15.7%) to ceftriaxone, 30 (13.5%) to ciprofloxacin. Meropenem was the most effective antibiotic tested, with none of isolates resistant.

As shown in Table 3.1 and Fig 3.2, there was a wide range of MICs between the lower and higher level of resistance and also between the MIC_{50} and MIC_{90}. The results of MIC_{50} indicated that more than half of collected isolates were sensitive for all antibiotics used. None of the isolates tested was resistant to meropenem making the meropenem the drug of choice in treatment of ESBL-producing *K. pneumoniae* isolates in Edinburgh.
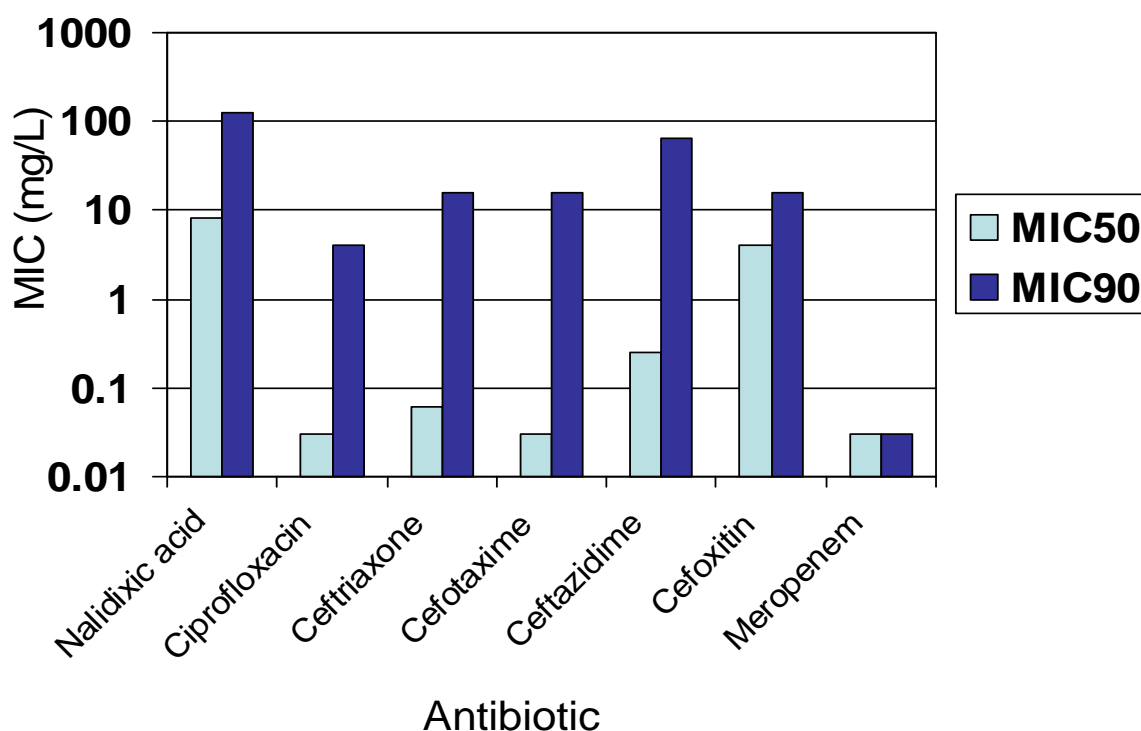
Fig 3.2: Graph representation of MIC_{50} and MIC_{90} values of 219 *K. pneumoniae* isolates.

Table 3.1: Difference of MIC values among 219 *K. pneumoniae* isolates tested.

Antibiotics	MIC range (mg/L)	MIC$_{50}$ (mg/L)	MIC$_{90}$ (mg/L)	% of resistance	BSAC breakpoint*
Nalidixic acid	2 - >128	8	>128	19.6	16
Ciprofloxacin	0.008 - >128	0.03	4	13.2	1
Ceftriaxone	0.008 - >128	0.06	16	14.6	1
Cefotaxime	0.008 - >128	0.03	16	14.6	1
Ceftazidime	0.06 - >128	0.25	64	15.5	2
Cefoxitin	1 - >128	4	16	24.2	8
Meropenem	0.015 - 2	0.03	0.03	0.0	4

(*) Breakpoints as listed in BSAC guidline 2006.

3.3.4. Confirmation of ESBLs production

All isolates that expressed antimicrobial resistance to cefotaxime, ceftazidime or ceftriaxone were subjected to confirmatory methods by double and combination disc diffusion methods as described before in section 2.9.3.1 and 2.9.3.2 (Materials and Methods) and shown in Figs 3.3 and 3.4. The *K. pneumoniae* isolate Mu930798E was found to be ESBLs-negative by confirmatory tests. However, it was resistant to ceftazidime (MIC= 2 mg/L).

Surprisingly, 12 (5.4%) isolates were found to be resistant to meropenem by disc diffusion method. These isolates were sensitive to meropenem by MICs.
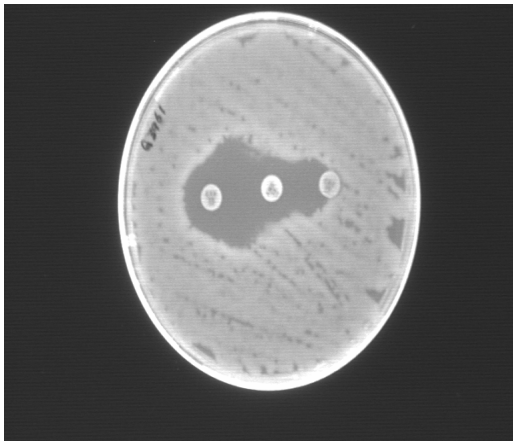
Fig 3.3: Combination disc method showing synergy between cefotaxime, ceftazidime and amoxicillin-clavulanate (amoxiclav). The right disc is cefotaxime, the left is ceftazidime. Amoxiclav disc is in middle.
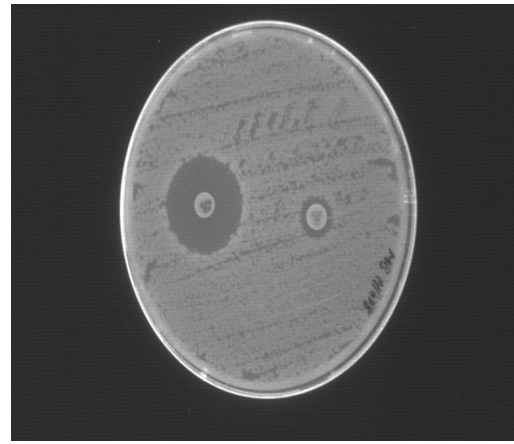
Fig 3.4: Confirmation of ESBLs production by double disc diffusion method. The plate shows that the inhibition zone around cefotaxime-clavulanate (left disc) is more than 5 mm of cefotaxime (right disc).

3.3.5. Prevalence and types of ESBLs

The presence of ESBL-producing isolates was inferred by double and combination disc diffusion methods. The overall frequency of ESBL producers observed in this study was 35/223 (15.7%) and distributed as follows,

The production of ESBL-producing *K. pneumoniae* isolates was 32 /219 (14.61 %) isolates. The types of ESBLs were determined by PCR. SHV β-lactamases were detected in 32 (100 %), whereas TEM and CTX-M were detected in 24 (75 %) and 16 (50 %) respectively of ESBLs-positive isolates. The SHV primer also amplified negative-ESBLs isolates that could explain the presence of high prevalence of SHV β-lactamases that may or may not be ESBLs.

ESBL-producing *K. oxytoca* isolates were 2/2 (100%) and constitute 0.9% of total isolates. These two isolates, MU946294N and MB193997E, were obtained from urine and blood samples obtained respectively from two hospitalized patients during September, 2007. These two isolates were found to be K1 (bla_{OXY-2}) after amplifying and sequencing using specific primers. None of the other ESBL genes tested in this study such as those encoding TEM, SHV and CTX-M β-lactamases were identified by PCR in these two isolates. Additionally, both isolates were found negative by PCR for *qnr* genes. These results were confirmed by IEF analysis as well.

The *Enterobacter cloacae* isolate was recovered from urine of hospitalized patient and tested for ESBL-production. Results show that this isolate expressed ESBL phenotype by MIC but none of bla_{TEM}, bla_{SHV}, bla_{CTX-M} and *qnr* genes were identified by PCR.

The *S. enterica* isolate was tested with MICs for antimicrobial susceptibility. It was found negative for all antibiotic used in this study.

For these reasons, *E. cloacae* and *S. enterica* were excluded from this study.

3.3.6. The source of ESBL-producing isolates

As indicated in Table 3.2, the number of ESBLs-positive isolates recovered from urine was 24 (68.6%) of total ESBLs-positive isolates. Moreover, ESBLs isolates collected from blood and assorted samples were 6 (17.1%) and 5 (14.3%) respectively.

From the relevant information available, the majority 24/35 (68.6%) of isolates that expressed ESBL phenotypes were recovered from hospitalized patients. Eleven (31.4%) isolates, all of them from *K. pneumoniae*, were collected from community-acquired infection as seen in Table 3.2.

Table 3.2: Source and community versus hospitalized of ESBL-producing isolates included in this study

| Micro-organisms | Total isolates | | | | | | ESBL-producing isolates | | | | | Community ESBLs | | Hospitalized ESBLs | |
| | Urine | | Blood | | Assorted | | Urine | Blood | Assorted | Total | | | | | |
	No.	%	No.	%	No.	%	No.	No.	No.	No.	%	No.	%	No.	%
K. pneumoniae	178	79.8	15	6.7	26	11.7	22	5	5	32	14.35	11	34.4	21	65.6
K. oxytoca	1	0.45	1	0.45	0	0	1	1	0	2	0.9	0	0	2	100
E. cloacae	1	0.45	0	0	0	0	1	0	0	1	0.45	0	0	1	100
S. enterica	1	0.45	0	0	0	0	0	0	0	0	0	-	-	-	-
Total	181	81.15	16	7.15	26	11.7	24	6	5	35	15.7	11	31.4	24	68.6

3.3.7. Detection of AmpC

Cefoxitin resistance was found in 55 (24.7%) of total isolates included in this study. Cefoxitin considered as presumptive indication for AmpC production. Confirmation of AmpC β-lactamases production was confirmed with inhibitor-based methods as described by (Coudron 2005). Briefly, 120 mg of phenylboronic acid (benzeneboronic acid (Sigma-Aldrich, UK) was dissolved in 3 ml of dimethyl sulfoxide and another 3 ml of distilled water was added to this solution. Twenty microliters of the stock solution was dispensed onto discs containing 30 µg of cefotetan or blank discs and left to dry for 30 min. Two discs, one disc containing 30 µg of cefotetan and another disc containing 30 µg of cefotetan and 400 µg of boronic acid, were placed on the IST agar similar to the standard disc diffusion method. Inoculated plates were incubated overnight at 35°C. An organism that demonstrated a zone diameter around the disc containing cefotetan and boronic acid that was 5 mm or greater than the zone diameter around the disc containing cefotetan was considered an AmpC producer.

I tried to identify types of bla_{AmpC} genes with multiplex PCR with primers targeting FOX, DHA, BIL, CIT, MOX, ACC and EBC as described by (Perez-Perez and Hanson 2002), but I failed to identify any of these genes even with several PCR condition modifications.

So, identification of bla_{AmpC} genes was not included further in this study.

3.4. DISCUSSION

3.4.1. Phenotypic identification

K. pneumoniae strains are found worldwide associated to pneumonia and urinary tract infections in nosocomial and community environments. *Klebsiella* spp. consider

the second important *Enterobacteriaceae* in bloodstream infections causing 4.7% to 6.0% of bacteraemia (Ko, Paterson *et al.* 2002; Livermore, Hope *et al.* 2008).

Biotyping is useful in assessing outbreaks of *Klebsiella* but it considered of little use in epidemiological studies (Podschun and Ullmann 1998). Identification *Klebsiella* to the species level is often difficult, because some of the species share similar biochemical profiles (Monnet and Freney 1994; Wang, Cao *et al.* 2008). API 20 E was used as a presumptive identification of the genomic species. 219 isolates were identified *K. pneumoniae*, 2 isolates *K. oxytoca*, 1 isolates *E. cloacae*, and one isolate *S. enterica* were identified by this method.

There was a problem in differentiation between *K. pneumoniae* and *K. oxytoca* by API 20E because the two *K. oxytoca* isolates were diagnosed by API 20E to be *K. pneumoniae*, whereas these isolates were identified *K. oxytoca* by Vitek 2 system and results were confirmed using *gyrA* PCR-RFLP method. These results were in agreement with previous finding that the Vitek 2 system is able to identify the *Klebsiella* spp more accurately than API 20E system. That is because biochemical tests like API 20E may fail to differentiate between *K. pneumoniae* and *K. oxytoca* (Kovtunovych, Lytvynenko *et al.* 2003).

3.4.2. Genotypic identification

The *gyrA* PCR-RFLP method was developed recently as a rapid method to identify *Klebsiella* species and phylogenetic groups (Brisse, van Himbergen *et al.* 2004). The present results agree with others which confirm that this method is reliable, faster and cheaper than gene sequencing (Brisse and Duijkeren 2005).

In this study I used restriction analysis of the *gyrA* gene with *gyrA* PCR-RFLP method in assigning the 38 isolates to *Klebsiella* species. The *K. pneumoniae* phylogenetic group 1, KpI, was dominant (29 isolates), followed by *K. pneumoniae* KpIII (7 isolates), *K. oxytoca* KoII (two isolates).

My finding is similar to previous investigations in which the majority of isolates belonged to KpI group. Analysis of 420 clinical isolates from 26 hospitals showed that KpI comprised 80.3% of 305 isolates from blood and 82.2-97.2% of isolates from other clinical sources. KpIII was never found among isolates from urinary tract infections (Brisse, van Himbergen *et al.* 2004). In another study, analysis of phylogenetic groups of representative isolates showed that most of them belonged to KpI-type (74.6%), less to the KpIII-type (20.9%), and only 3% belonged to KpII-type (Valverde, Coque *et al.* 2008). Further report indicated that KpI (78 isolates), KpII (five isolates), KpIII (eight isolates), *K. oxytoca* (three isolates), *K. planticola* (four isolates) and *K. terrigena* (two isolates) were identified from 100 isolates with *gyrA* PCR-RFLP profile (Brisse and Duijkeren 2005).

The *rpoB* gene sequence distinguished the two *K. oxytoca* isolates to belong to phylogenetic group KoII. The both isolates were found to carry bla_{OXY-2} by PCR and DNA sequencing. The previous findings indicated that the chromosomal bla_{OXY} gene of *K. oxytoca* is able, like housekeeping genes, to classify the *K. oxytoca* phylogenetic groups. Also, strains harbouring bla_{OXY-2} gene fall into a clearly distinct phylogenetic group (Granier, Leflon-Guibout *et al.* 2003; Fevre, Jbel *et al.* 2005).

3.4.3. Antimicrobial susceptibility

The natural, semisynthetic and synthetic β-lactam antibiotics can be subdivided into 6 different structural subtypes:

(i) penams (e.g. benzylpenicillin, ampicillin)

(ii) cephems which include 2nd and 3rd generation cephalosporins, (e.g. cefuroxime, cefotaxime, ceftazidime)

(iii) cephamycins as 7-α-methoxy cephalosporins (e.g. cefoxitin)

(iv) monobactams as monocyclic molecules (e.g. aztreonam)

(v) penems with a 2,3-double bond in the fused thiazoline ring (e.g. faropenem)

(vi) carbapenems with an unsaturated fused 5-membered ring (e.g. imipenem).

The resistance to 3rd generation cephalosporins among *K. pneumoniae* varies between different geographic locations and in several countries the resistance to 3rd generation cephalosporins has increased since the 1990s (Potz, Hope *et al.* 2006; Livermore, Canton *et al.* 2007). In UK, emergence of this resistance mechanism compared with other European countries may result from differences in cephalosporin usage, methods of detecting ESBL producers, prevalence of resistant serovars, or the import of resistant strains through travel (Yates and Amyes 2005).

In this study, antimicrobial susceptibility showed a moderate-level of resistance in all isolates against cefoxitin and nalidixic acid with 24.7% (n= 55) and 20.2% (n = 45) respectively. The low-level of resistance 34 (15.2%), 36 (16.1%), 35 (15.7%) and 30 (13.5%) of these strains was found toward cefotaxime, ceftazidime, ceftriaxone and ciprofloxacin respectively. Compared with other antimicrobial agents tested, none of the isolates were found resistant to meropenem keeping these the drug of choice in treatment of multi-resistant isolates.

Interestingly, 12 isolates were found border-line resistance to meropenem by disc diffusion method but not with MICs. The most likely explanation is these isolates might produce either class A carbapenemases (KPC, GES) or MBLs (IMP, VIM) with low-level of resistance. Further work is needed to investigate the exact mechanism(s) of resistance.

These results concatenated with other findings that carbapenems were active against all ESBL-positive enterobacteria in different studies from Italy (Luzzaro, Mezzatesta *et al.* 2006; Mugnaioli, Luzzaro *et al.* 2006). Other reports confirmed that meropenem and imipenem had greatest activity against ESBL-producing *E. coli* and *Klebsiella* spp. in both Europe (96.9–100.0%) and the United States (100.0%) (Goossens and Grabein 2005). Furthermore, imipenem remained >99% active against *K. pneumoniae* isolates collected from Latin America, Asia/Pacific Rim, Europe, and North America (Reinert, Low *et al.* 2007). So, this study agrees with the conclusion drawn that the continued efficacy of carbapenems such as meropenem confirms that these remain first-line agents for treatment of nosocomial infections caused by

Enterobacteriaceae-producing ESBL or AmpC β-lactamases (Goossens and Grabein 2005).

Production of extended-spectrum β-lactamases (ESBLs) in particularly by *E. coli* and *Klebsiella* spp. is the most important means of resistant to third and fourth generation cephalosporins such as ceftazidime and cefepime and occurrence of antimicrobial resistance in the hospital setting (Goossens and Grabein 2005). The prevalence and genotype of ESBLs from clinical isolates vary between countries and even from different hospitals and locations at which they are isolated from (Bradford 2001).

In this study, ESBL production was detected in 35/223 (15.7%) of the strains by MICs and confirmed by combination and synergy double disc methods.

This result is quite similar to the survey in Portugal, whereas (14%) of isolates reported to be ESBLs (Mendonca, Ferreira *et al.* 2009). Moreover, in the Asia-Western Pacific region the ESBL rate of *Klebsiella* spp. was (17.8%) (Turnidge, Bell *et al.* 2002).

But results presented here show a lower prevalence when it compared with the higher prevalence reported in other literatures in which ESBL was reported in 1999 in paediatrics centres in France to be (32%) (Raymond, Nordmann *et al.* 2007). Additionally, in South America, rates of up to 45% are reported for *K. pneumoniae* strains, with a comparable rate of 8% in Europe (Winokur, Canton *et al.* 2001). In a survey of intensive care units in Europe undertaken in 1994, it was shown that 24% of third-generation resistant isolates of *Klebsiella* in France carried the ESBL phenotype, and in Turkey 59% and in Portugal 49%. This is in marked contrast to the findings in the UK and Spain, where 0 and 1% isolates carried the ESBL phenotype (Livermore and Yuan 1996). In Italy it was (20%) (Luzzaro, Mezzatesta *et al.* 2006). In a recent study based on the Tigecycline Evaluation and Surveillance Trial (TEST) global surveillance database, the rate of ESBL production was highest among the *K. pneumoniae* isolates collected in Latin America, followed by Asia/Pacific Rim,

Europe, and North America with 44.0%, 22.4%, 13.3% and 7.5%, respectively (Reinert, Low *et al.* 2007).

On the other hand, the lower prevalence were found in previous reports show that 0.1% of *E. coli* and 0.3% of *K. pneumoniae* isolates clinical isolates recovered from 196 medical institutions during January 1997 to January 1998 in Japan were ESBLs (Yagi, Kurokawa *et al.* 2000). Moreover, ESBLs are prevalent in 3.9% and 7.7% of E. coli species and 8.6% and 28.3% of *Klebsiella* samples from North America and the rest of the world, respectively (Sader, Hsiung *et al.* 2007). In another report, *Klebsiella* spp. strains with ESBL phenotype were detected in an overall frequency of 4% in the United States and 12% in Europe in the last 6 to 8 years (Goossens and Grabein 2005). Whereas in Austria an overall frequency of 1% of *Klebsiella* spp. were found to carry ESBL phenotype (Eisner, Fagan *et al.* 2006).

Concurrent with these findings, Mulvey, Bryce *et al.* (2004) demonstrated that the most prevalent enzymes in Canada were SHV derived, and that TEM-type ESBLs were rarely found. In another study, SHV-type ESBL was the most common ESBL, occurring in 67.1% (49 of 73) of isolates. In addition, TEM-type ESBL was found in just 16.4% (12 of 73) of isolates. CTX-M-type ESBLs ($bla_{CTX-M-2}$ type and $bla_{CTX-M-3}$ type) were found in 23.3% (17 of 73) of isolates (Paterson, Hujer *et al.* 2003).

Of the strains tested, 16/223 (7%) expressed CTX-M enzymes. This confirms the emergence of this family of β-lactamases in *K. pneumoniae* in Scotland as in other countries. A recent dramatic increase in ESBL-producing organisms is being observed both in hospitals and in the community, mainly caused by the CTX-M-15 enzyme (Canton and Coque 2006; Livermore, Canton *et al.* 2007). CTX-M-15 first reported in the UK in 2003, co-existed with CTX-M-9, CTX-M-14, SHV-12 and to a lesser extent with TEM derivatives both in the hospital and in the community. It has now become the most prevalent enzyme in both settings (Yates, Brown *et al.* 2004; Livermore, Canton *et al.* 2007). In Italy, in 2003, only 12% of *K. pneumoniae* strains presented CTX-M enzymes (Mugnaioli, Luzzaro *et al.* 2006), and between 1998 and

2003, the SENTRY program in the Asia-Pacific region, reported that 25% of strains possessed CTX-M enzymes (Bell, Chitsaz *et al.* 2007).

Overall, of the 223 strains studied, 24 possessed non-ESBL-TEM-1b genes. TEM β-lactamase is one of the most widely distributed β-lactamases, which hydrolyse the ampicillin and related antimicrobials such as piperacillin, carbenacillin. The survey of *E. coli* collected from faeces of patients presenting for elective surgery in West London in 1968, was found 17% carried plasmid-mediated ampicillin resistance (Datta 1969). Additionally, from all isolates tested only 18 (8%) of the strains presented resistance to fluoroquinolones. A multi-continental study reported similar resistance to fluoroquinolones (8%) (Deshpande, Jones *et al.* 2006). The association of beta-lactamase production and transferable quinolone resistance genes, *qnr*, was reported (6.7%) in Enterobacteriaceae strains (Castanheira, Mendes *et al.* 2008).

In this study, 24 of total 35 ESBL-producing strains (68.6%) of our isolates from patients with urinary tract infections were ESBL-producing strains, whereas the ESBLs isolates collected from blood were 6 (17.1%) isolates.

Consistent with this study, many reports indicated the high proportions of ESBLs – producing *Enterobacteriaceae* especially *E. coli* and *Klebsiella* spp. isolated from urinary tract infection patients (Rodriguez-Bano, Navarro *et al.* 2004). The corresponding Figures are 27%, 2% for Latin America and United States respectively (Turnidge, Bell *et al.* 2002). Furthermore, most (63%) of CTX-M producers were recovered from patients with urinary tract infections from Austria (Eisner, Fagan *et al.* 2006). A Canadian study showed that total of 146 patients (93%) had ESBL-producing *E. coli* identified from urine cultures (Pitout, Hanson *et al.* 2004). Additionally, ESBL strains were isolated from 45 urine cultures and from 6 blood cultures (2 strains were isolated from cultures of both urine and blood) of 49 community acquired patients infected with *E. coli* (Rodriguez-Bano, Navarro *et al.* 2004).

On the other hand, these results were in contrast to findings reported by Tumbarello, Spanu *et al.* (2006), when he found about 30% of ESBL-producing *K. pneumoniae* isolates that examined were isolated from blood.

The ESBL-producing *Enterobacteriaceae* have increased among community-acquired strains (Rodriguez-Bano, Navarro *et al.* 2004; Woodford, Ward *et al.* 2004; Pitout, Nordmann *et al.* 2005). This study illustrated that the majority 24/35 (68.6%) of isolates expressed ESBLs phenotypes were recovered from hospitalized patients. Eleven (31.4%), all of them from *K. pneumoniae*, isolates were collected from community-acquired infection. But this information has some limitations. We do not have enough information about how long patients been in hospital, the ambulatory patients who visited hospitals regularly. Also, the information concerning previous hospital admissions, underlying diseases (diabetes mellitus, recurrent UTI), and antibiotics use was not available.

As reviewed by (Pitout, Nordmann *et al.* 2005), the first community-acquired ESBL strain was found in Ireland in 1998. A population-based surveillance CTX-M-producing *E. coli* study in Canada showed that ESBL-producing *E. coli* is predominantly a community-onset pathogen. Of 157 patients, 111 (71%) had community-onset infections and 46 (29%) had nosocomial infections (Pitout, Hanson *et al.* 2004). In another Canadian study, 177 (72%) of patients were community acquired, whereas 70 (28%) were healthcare associated (Laupland, Church *et al.* 2008). A French study demonstrated that the ESBL-producing *Enterobacteriaceae* (including *E. coli*, *K. pneumoniae* and *Proteus mirabilis*) was identified in 39 isolates (1.5%) that produced various types of ESBLs (TEM-3, -19, SHV-4 and CTX-M-1). ESBL producers were isolated from 38 patients, including 33 residents of 11 clinics or nursing homes (acquired infections) and 5 from nosocomial acquisitions (Arpin, Dubois *et al.* 2003). Another Spanish study cited by (Rodriguez-Bano, Navarro *et al.* 2004) showed that 93% of ESBL-producing *K. pneumoniae* strains were isolated from inpatients, 51% of ESBL-producing *E. coli* strains were isolated from outpatients. Additionally, more than 70% of all ESBL-producing isolates of community origin screened for the presence of CTX-M enzymes were found to be

positive, compared with 21% of all ESBL producers of hospital origin (Eisner, Fagan *et al.* 2006). In the UK, investigators reported that Of 291 CTX-M-producing *E. coli* isolates studied from 42 UK centres, 70 (24%) were reportedly from community patients, many of whom had only limited recent hospital contact (Woodford, Ward *et al.* 2004).

The increase in community-acquired ESBL infections in the United Kingdom has not yet been explained. A Canadian study was published confirmed that advancing age; female gender and the presence of other sever medical conditions increase the risk for community-acquired ESBLs (Laupland, Church *et al.* 2008). Also, the human immigrants play a major role in transferring the community-acquired ESBLs (Pitout, Nordmann *et al.* 2005; Livermore, Canton *et al.* 2007; Laupland, Church *et al.* 2008). Another review stated that antibiotic overuse in humans and animals, hospital cross-infection, the food chain, trade and human migration seem to have contributed to the recent dissemination of ESBLs outside hospitals, although the role of these factors is variable and linked to particular epidemiological situations (Coque, Novais *et al.* 2008).

3.5. CONCLUSION

These findings report on distribution of ESBLs among the clinical isolates collected from Edinburgh district, Scotland. The β-lactamases found in this study represented a low percentage of resistance than other previous findings worldwide. This is first to report in emergence of bla_{CTX-M} from Edinburgh district with CTX-M-15 is the only CTX-M-type described in this study. However, the dissemination of TEM- and SHV-type ESBLs in this study is more predominate than CTX-M-15. Finally, the community-acquired ESBLs infections still lower than the hospitalized infections and the majority of ESBLs isolates were collected from urinary tract infection patients.

CHAPTER-4:

Prevalence of transferable $bla_{CTX-M-15}$ from hospital and community-acquired *Klebsiella pneumoniae* isolates in Scotland

4.1. ABSTRACT

***Objectives*:** This study was carried out to investigate the prevalence and genetic characteristics of transferable $bla_{CTX-M-15}$ from hospital and community-acquired *Klebsiella pneumoniae* isolates in Scotland.

***Methods*:** A total of 219 clinical isolates of *K. pneumoniae* was collected in 2006 and 2007 at Royal Infirmary of Edinburgh, Scotland. The isolates were tested for antimicrobial susceptibility by the agar double dilution method. PCR and sequencing were used to detect bla_{CTX-M}, bla_{TEM}, bla_{SHV} and *qnr* genes. The genetic environment was analysed using various set of PCR primers. Clonality of the isolates was assessed by PFGE.

***Results*:** Sixteen (7.3%) isolates were found to be producers of CTX-M-15 ESBLs, of which two isolates (12.5%) were reported to be from community-acquired infections. The insertion sequence IS*Ecp1* was detected by sequencing 48 nucleotides upstream of $bla_{CTX-M-15}$ in all isolates except one. One to two plasmids, ranging in size from approximately 40 to 210 kb, were observed per strain. By PCR-based replicon typing method, the plasmids carrying the $bla_{CTX-M-15}$ were assigned to IncFII or with IncN types. Sequencing and PCR analysis revealed the presence of complex Class 1 integrons in all isolates except one. Two isolates positive for class 1 integrons were positive for class 2 integrons as well. Five different clones of CTX-M-15-producing isolates were identified by PFGE.

***Conclusions*:** This work reports the emergence of hospital and community-acquired CTX-M-type enzymes in the Edinburgh area, Scotland.

4.2. INTRODUCTION

The CTX-M β-lactamases constitute one of the most rapidly growing ESBL families. Outbreaks of CTX-M enzyme have been described in bacteria from numerous countries of Africa, Europe, South America and Asia (Baraniak, Fiett *et al.* 2002; Livermore and Woodford 2006; Livermore, Canton *et al.* 2007). The CTX-M family is subclassified into 5 groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 in which the members of each group share 94% identity (Bonnet 2004).

CTX-M types evolved through the escape of chromosomal genes from *Kluyvera ascorbata* and *Kluyvera Georgiana* (Humeniuk, Arlet *et al.* 2002; Poirel, Kampfer *et al.* 2002; Rodriguez, Power *et al.* 2004; Olson, Silverman *et al.* 2005). The ability of insertion sequences IS*Ecp1,* IS*CR1*, phage-related elements and plasmids to mobilize and to promote the expression of β-lactamase genes may explain the current spread of CTX-M-type enzymes worldwide (Poirel, Decousser *et al.* 2003; Bonnet 2004; Oliver, Coque *et al.* 2005; Canton and Coque 2006).

The CTX-M-15 β-lactamase was first detected in isolates from India (Karim, Poirel *et al.* 2001). It is derived from CTX-M-3 by an Asp240→Gly substitution, which increased catalytic activity to ceftazidime (Poirel, Gniadkowski *et al.* 2002). Since 2003, *E. coli* isolates with the CTX-M-15 ESBL have become widely distributed throughout the UK. Five major related *E. coli* strains, A to E, were identified by PFGE. They all belong to sequence type ST131 and serotype O25:H4. Strain A is the most distributed lineage in the UK (Woodford, Ward *et al.* 2004; Lau, Kaufmann *et al.* 2008).

Little is known about the population structure of CTX-M-15-producing *K. pneumoniae* causing outbreaks in UK when compared to far better identified CTX-M-15-producing *E. coli*.

4.3. RESULTS

4.3.1. Characterization of β-lactamases

The overall frequency of ESBL-producing *K. pneumoniae* observed in this study was 32/219 (14.61 %). The presence of CTX-M genes was screened by PCR, initially with universal primers, CTX-M_F and CTX-M_R, which were able to detect the CTX-M genes belonging to different groups (Edelstein, Pimkin *et al.* 2003), and then with primers specific for $bla_{CTX-M-1}$ group (Dutour, Bonnet *et al.* 2002). PCR conditions were as follows: 5 min at 94°C, 35 cycles of 30 seconds at 95°C, 1 min at 52°C, and 1 min at 72°C, and final extension of 7 min at 72°C. The resulting PCR

products were sent for sequencing. Results revealed that CTX-M genes were detected in 16 (7.3%) isolates of *K. pneumoniae* representing 16/32 (50%) of all ESBL *K. pneumoniae* producing isolates. All isolates were identified as CTX-M-15 by results of sequence analysis of the deduced amino acid sequences.

4.3.2. The β-lactam susceptibility profile

The pattern of resistance to antimicrobial agents in all 16 cefotaxime-resistant *K. pneumoniae* strains is shown in Table 4.1. The antimicrobial susceptibility patterns showed that all strains (100%) harboured CTX-M-15 ESBLs were characterized by high resistance to all cephalosporins tested including cefotaxime, ceftazidime, ceftriaxone, cefuroxime and cephalexin. In addition, all isolates were found resistant to aztreonam (a monobactam). Among the CTX-M-15 producing isolates, 93.8% (n = 15) were resistant to fluoroquinolones (ciprofloxacin) and nalidixic acid. Resistance to cefoxitin was observed in 81.3% (n = 13) of the isolates. Overall, none of the isolates tested was resistant to meropenem.

CTX-M-15 ESBLs confer high-level resistance to ceftazidime and cefotaxime with (MIC range: 64->128 mg/L). Sequence analysis of all 16 isolates revealed there was glycine at position 240. This substitution has already been reported in CTX-M-15, CTX-M-16, CTX-M-32 and CTX-M-27 and is known to confer high-level resistance to ceftazidime.

4.3.3. Association of *bla*CTX-M-15 with resistance genes

The association between $bla_{CTX-M-15}$ and the genes coding bla_{TEM}, bla_{SHV}, *qnrA*, *qnrB* and *qnrS* was identified by amplification by PCR from genomic DNA using oligonucleotide primers as described before (Mabilat and Goussard 1993; Cattoir, Poirel *et al.* 2007). As shown in Table 4.2, all isolates harbouring the CTX-M enzyme were found to carry either SHV-1 or SHV-11 or SHV-12 or SHV-5 or SHV-80 β-lactamases by PCR. Moreover, all isolates but one was found to express TEM-1b. Additionally, in this study, ten of *qnrB1* genes were associated with CTX-M-15.

Table 4.1: Antimicrobial susceptibility of CTX-M-producing *K. pneumoniae* isolates (MIC: mg/ L).

Given No.	I D	Amoxicillin	Piperacillin	Nalidixic acid	Ciprofloxacin	Ceftriaxone	Cefotaxime	Ceftazidime	Aztreonam	Cefuroxime	Cephalexin	Meropenem	Cefoxitin
32	Mu 932 491 P	>256	64	>128	16	>128	>128	64	64	>128	>128	0.03	8
33	Mu 932 330	>256	>256	>128	16	>128	>128	>128	64	>128	>128	0.03	>128
69	Mu 936 327 N	>256	>256	>128	>128	>128	128	64	128	>128	>128	0.03	8
73	Mu 936 619 A	>256	>256	>128	>128	>128	128	64	128	>128	>128	0.03	8
91	Mu 939 312 V	>256	>256	>128	16	>128	>128	64	128	>128	>128	0.03	4
113	Mu 940 148 Z	>256	>256	>128	16	>128	>128	64	128	>128	>128	0.03	4
115	Mu 940 460 H	>256	>256	>128	32	>128	>128	64	128	>128	>128	0.03	8
175	Mg 768 689 P	>256	>256	>128	>128	>128	128	128	128	>128	>128	0.03	16
187	Mu 949847 W	>256	>256	>128	>128	>128	>128	128	128	>128	>128	2	64
195	Mr 148167 R	>256	>256	8	0.25	>128	>128	128	128	>128	>128	0.06	4
197	Mu 950618 L	>256	>256	>128	>128	>128	>128	128	128	>128	>128	0.06	64
213	Mu 960 478	>256	>256	>128	>128	>128	128	64	128	>128	>128	0.06	8
215	Mu 966 491 T	>256	>256	>128	>128	>128	>128	128	128	>128	>128	0.25	64
MB	Mb141078	>256	>256	>128	>128	64	64	>128	128	>128	>128	0.06	16
B79	B143279	>256	>256	>128	>128	128	64	128	128	>128	>128	0.06	16
BV	B14124V	>256	>256	>128	>128	128	64	>128	64	>128	>128	0.03	16

Table 4.2: Characterization of CTX-M-producing *K. pneumoniae* isolates.

Given No.	I D	IncFII	IncN	upstream	*ISEcp1*	Sul1	Int1	qacE	Int2	CS (kb)	transconjugation	PFGE Clone	Plasmid profile	Plasmid(s) size kb	TEM	SHV	Qnr
69	Mu 936 327 N	+	+	+	+	+	+			1	+	1	1	210	+	-11	B1
73	Mu 936 619 A	+	+	+	+	+	+	+		1	+	1	1	210	+	-11	B1
175	Mg 768 689 P	+	+	+	+	+	+			1	+	1	1	210	+	-12	B1
187	Mu 949847 W	+	+	+	+	+	+			1	+	1	1	210	+	-12	B1
197	Mu 950618 L	+	+	+	+	+	+			1	+	1	1	210	+	-5	B1
213	Mu 960 478	+	+	+	+	+	+			1	+	1	2	195	+	-11	B1
215	Mu 966 491 T	+	+	+	+	+	+			1	+	1	2	195	+	-11	B1
MB	Mb141078	+	+	+	+	+	+			1	+	2	3	135, 180	+	-11	B1
B79	B143279	+	+	+	+	+	+			1	+	2	3	135, 180	+	ND	B1
BV	B14124V	+	+	+	+	+	+			1	+	2	3	135, 180	+	-11	B1
32	Mu 932 491 P	-	+	+	+	-	+			2	+	3	4	60, 120	+	-	-
33	Mu 932 330	-	+	+	+	-	+			2	+	3	4	60, 120	+	-	-
91	Mu 939 312 V	-	+	+	+	-	+			2	+	3	4	60, 120	+	-11	-
113	Mu 940 148 Z	-	+	+	+	-	+			2	+	3	4	60, 120	+	ND	-
115	Mu 940 460 H	+	+	-	-	+	-			-	+	4	5	40, 60	-	-11	-
195	Mr 148167 R	+	+	+	+	+	+	+		1	+	5	6	75	+	-11	-

(+) positive result, (-) negative result, (ND) not determined, (CS) conserved sequence of class 1 integrons, (kp) kilo base pair.

Table 4.3: Source and isolation date of CTX-M producing *K. pneumoniae* isolates

Given ID	I D	Sample	Date of collection	Location
32	Mu 932 491 P	Urine	9/8/07	RIE
33	Mu 932 330	Urine	8/8/07	RIE
69	Mu 936 327 N	Urine	17/8/07	Milton practice (Mount castle) (G.P)
73	Mu 936 619 A	Urine	17/8/07	RIE, Immediate care centre
91	Mu 939 312 V	Urine	13/8/07	RIE, renal transplant
113	Mu 940 148 Z	Urine	27/8/07	RIE, renal transplant
115	Mu 940 460 H	Urine	27/8/07	Charles Bell Pavilion 2 (Morningside hospital)
175	Mg 768 689 P	N. swab	12/9/07	WGH
187	Mu 949847 W	Urine	18/9/07	Liberton hospital
195	Mr 148167 R	Sputum	18/9/07	RIE
197	Mu 950618 L	Urine	19/9/07	Linlithgow group medical practice (G.P)
213	Mu 960 478	Urine	10/10/07	WGH
215	Mu 966 491 T	Urine	23/10/07	WGH
	Mb141078	Blood	13/3/06	WGH
	B143279	Blood	13/3/06	WGH
	B14124V	Blood	13/3/06	WGH

RIE: Royal Infirmary of Edinburgh, WGH: Western General Hospital, N. swab: nephrostomy swab.

4.3.4. Phylogenetic type

Identification of the phylogenetic types of strains using *gyrA* PCR-RFLP, all 16 isolates producing CTX-M-15 were assigned to be *K. pneumoniae* phylogenetic group I (KpI).

4.3.5. Hospital versus community specimen

As listed in Table 4.3, among the 16 CTX-15-producing isolates for which relevant information was available, two isolates (12.5%) were reported to be derived from community-acquired infections. This was a significantly lower proportion of the community-acquired CTX-M-producing *K. pneumoniae* than had been found in the hospital isolates. The two isolates were collected from urine specimens, the remaining isolates collected from hospitalized patients. The majority of the CTX-M-producing isolates were associated with urinary tract infections (11 strains) representing (69%) of all isolates. Three (19%), 1 (6%), and 1 (6%) were isolated from blood, sputum, and nephrostomy swab respectively. As expected, most (82%) of the urine samples were from patients categorised as having hospital-acquired infections.

4.3.6. Plasmid and replicon type determination

S1-nuclease plasmid profiles were obtained for all CTX-M-producing isolates after plasmid DNA PFGE electrophoresis was performed in 1% agarose. A total of one to two plasmids ranging in size from approximately 40 to 210 kb were observed per strain as shown in Table 4.2 and Figs 4.1, 4.2. A total of 8 isolates produced a single plasmid, two plasmids were identified from 8 isolates as well. The putative high-molecular-weight plasmids of approximately 210 kb were obtained and confirmed after several gel electrophoresis runs.

Fingerprint analysis of extracted $bla_{CTX-M-15}$-carrying plasmids revealed 6 different restriction profiles using *EcoR1* and *Pst1* endonucleases.

Plasmids were classified according to their incompatibility group with the PCR-based replicon-typing. The primer pairs targeting FIA, FIB, FIC, I2, I1, L/M, N, A/C, and FII replicons were used in separate PCR reactions. The primers were used as described before (Carattoli, Bertini *et al.* 2005) and stated in Table 2.2 (Materials and Methods). PCR conditions were as follows: 5 min at 94°C, 30 cycles of 30 seconds at 95°C, 1 min at 60°C, and 1 min at 72°C, and final extension of 7 min at 72°C except for FII primers, the annealing temperature was 52°C. Results indicated that the IncN plasmid was identified from all isolates, the IncFII plasmid was obtained from 12 isolates only.

In this study the IncN plasmid by PCR is always present when the $bla_{CTX-M-15}$ gene was identified even in the absence of IncFII. As the IncN plasmid is the only type present, these results are the first to indicate the role of IncN replicon types in the dissemination of the $bla_{CTX-M-15}$ genes.
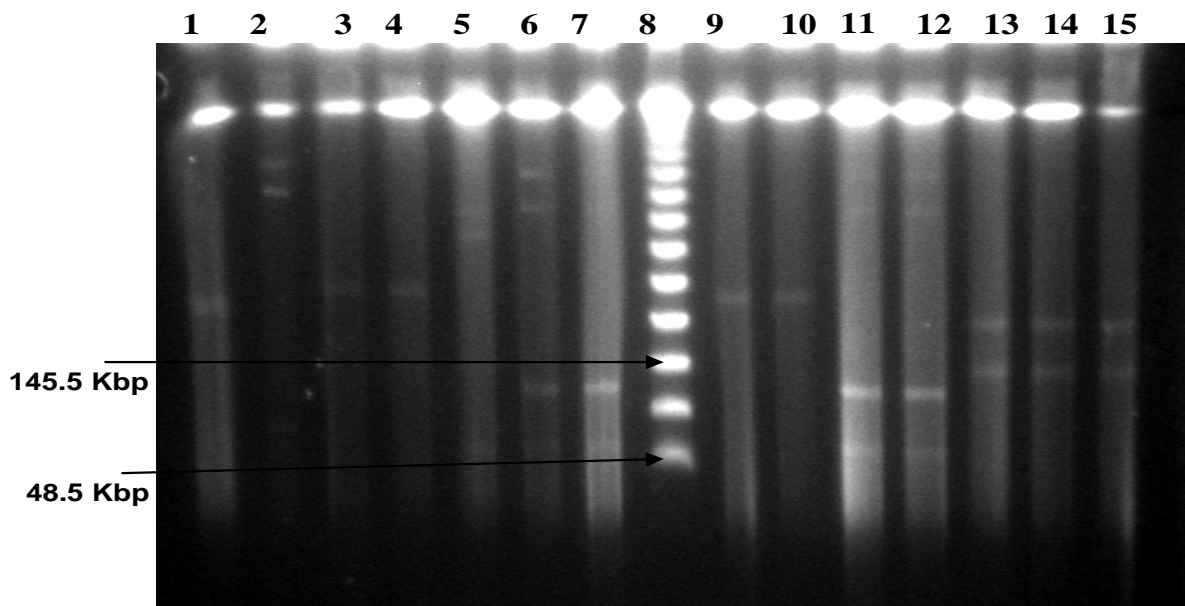
Fig 4.1: Pulsed field gel electrophoresis of S1-nuclease plasmid profiles for all CTX-M-producing isolates. Lane 1: isolate no 215. Lane 2: isolate no 195. Lane 3: isolate no 197. Lane 4: isolate no 187. Lane 5: isolate no 115. Lane 6: isolate no 113. Lane 7: isolate no 91. Lane 8: Lambda ladder PFGE marker standard size. Lane 9: isolate no 69. Lane 10: isolate no 73. Lane 11: isolate no 33. Lane 12: isolate no 32. Lane 13: isolate no MB. Lane 14: isolate no BV. Lane 15: isolate no. B79.
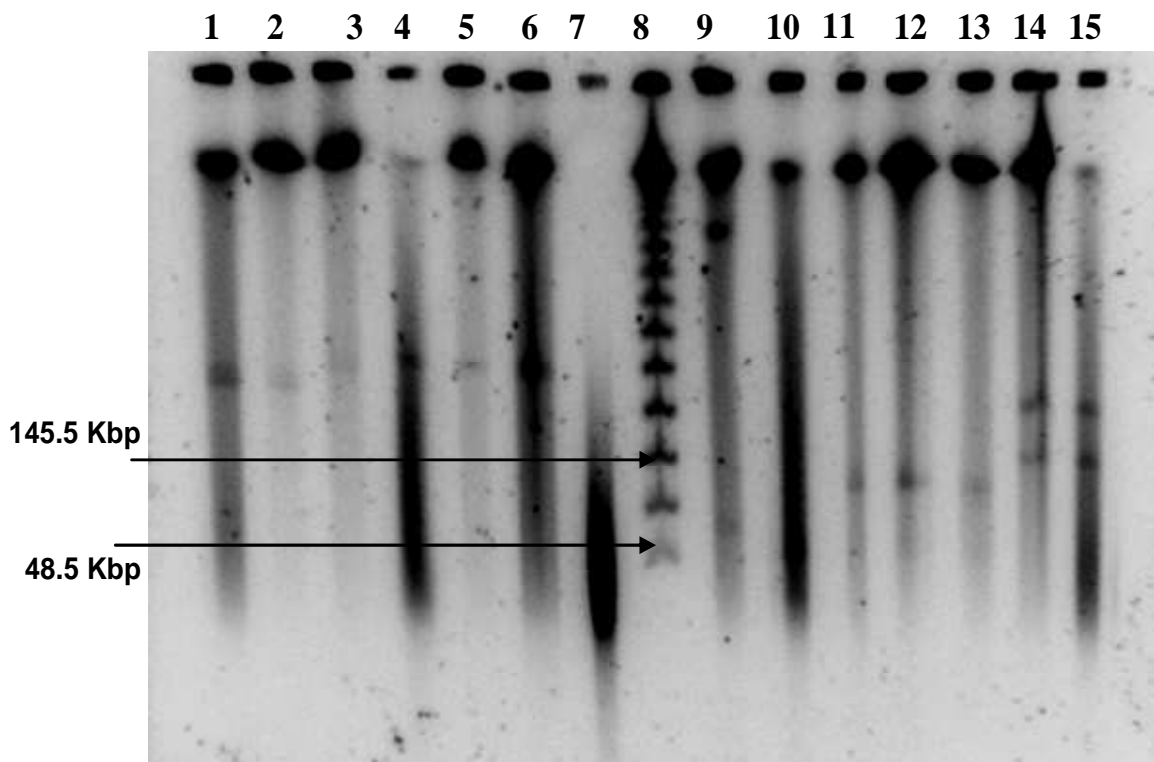
Fig 4.2: Pulsed field gel electrophoresis of S1-nuclease plasmid profiles for CTX-M-producing isolates. Lanes 1 to 15: isolates numbers 213, 215, 197, 187, 175, 73, 69, Lambda ladder PFGE marker, 195, 115, 113, 33, 32, BV, MB respectively.

4.3.7. Identification of plasmids by probe hybridization

PCR amplification of $bla_{CTX-M-15}$ from template DNA with universal primers, CTX-M_F and CTX-M_R, to amplify 550 bp fragment within the gene. The PCR product was analysed by gel electrophoresis to confirm the correct size of the product. The plasmid DNA from the strains was restricted with *Apa1* endonuclease which does not cut within CTX-M gene and transferred to a piece of nylon membrane. The hybridization study did not give accurate information about the plasmids carrying CTX-M-15. As shown in Fig 4.3, the intra-genic gene probe was hybridized strongly with isolates 113 and 195, a weak signal was obtained from isolate 115. However, the probe was hybridized with the chromosomal DNA from these strains and the signals were very strong. In addition, attempts to probe the CTX-M genes from S1 nuclease PFGE failed in this study, even after several trials.

Chromosomal DNA
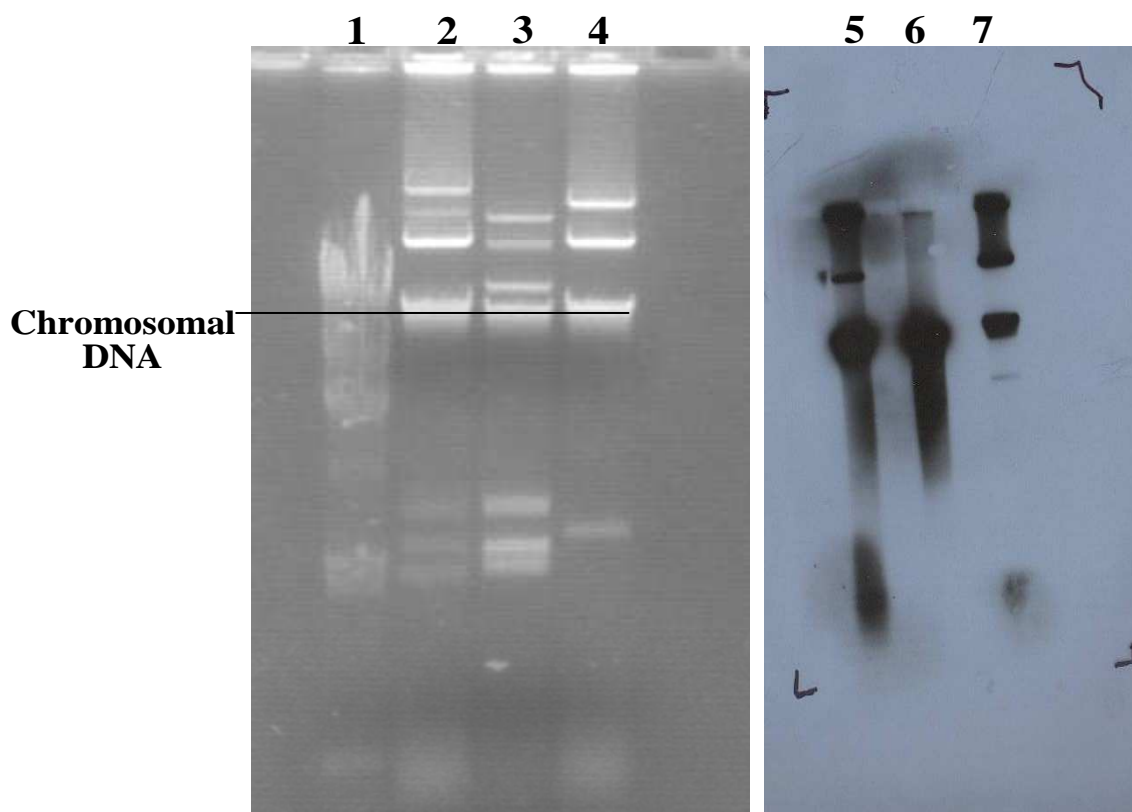
Fig 4.3: Probe hybridization of CTX-M-15. Lane 1: Lambda DNA/Hind111 molecular marker. Lane 2: isolate no. 113. Lane 3: isolate no. 115. Lane 4: isolate no. 195. Lane 5: hybridized isolate no. 195. Lane 6: hybridized isolate no. 115. Lane 7: hybridized isolate no. 113.

4.3.8. Exploration of the regions surrounding $bla_{CTX-M-15}$ genes

The genetic organization of the bla_{CTX-M} genes was investigated by PCR and sequencing of the regions surrounding these genes. Detection of upstream IS*Ecp1* insertion sequence was investigated using CTX-M1_R reverse and Prom+ primers (Poirel, Decousser *et al.* 2003). The internal IS*26* was determined using primers (ISF and ISR) as described before (Woodford, Ward *et al.* 2004). The annealing temperatures were 55ºC and 52ºC for IS*Ecp1* and IS*26* insertion sequences respectively. PCR identified the insertion sequence IS*Ecp1* upstream of the $bla_{CTX-M-15}$ gene in all strains (Table 4.2) except one strain (strain 115). IS*Ecp1* was detected by sequencing 48 nucleotides upstream of $bla_{CTX-M-15}$.

IS*26* insertion sequence was identified upstream in all isolates and found about 200 bp integrated in IS*Ecp1* insertion sequence.

4.3.9. Incidence of class 1 and class 2 integrons

To characterize the presence of inserted gene cassettes within the variable region, the primer pair 5_ CS and 3_ CS for class 1 integrons was used (Zhao, White *et al.* 2001). The results identified class 1 integrons in all but one of the CTX-M-producing *K. pneumoniae* isolates (Table 4.2). PCR identified two different sizes of class 1 integrons, the largest one was ~ 2 kb and the second was ~1.0 kb in size. After sequencing of representative samples the three different gene cassette arrangements were as follows *dfrA12 + aadA2*, *aadA1, aadA2*.

K. pneumoniae isolates were screened for the presence of *Intl1*, *Sul1*, and *qacEΔ1* which represent class 1 integrons, and for *intI2* integrase encoded on class 2 integrons (Machado, Canton *et al.* 2005). The 15 integrons-positive isolates did contain the conserved *qacEΔ1-sul1* region. All isolates were tested for the presence of class 2 integrons using *Intl2* primers. Only two isolates were positive for class 2 integrons. These two isolates were positive for class 1 integrons as well.

Four of these isolates were negative for integrase (*intI1*) gene. Interestingly, the isolates which was negative for 3′-conserved segments, gave a band with integrase (*intI1*) gene (Fig 4.4).
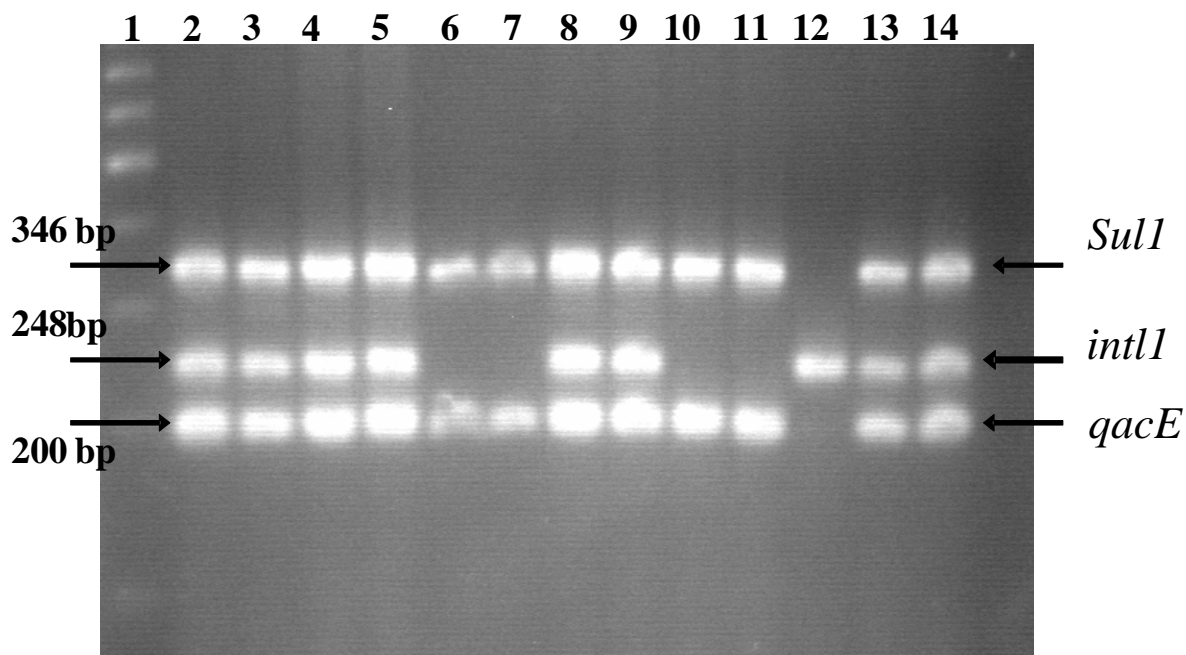
Fig 4.4: Gel electrophoresis of *intI1*, *qacEΔ1*, and *sulI1* multiplex PCR for 13 CTX-M-producing isolates. Lane 1: molecular marker of 100 bp. Lane 2: isolate no MB. Lane 3: isolate no BV. Lane 4: isolate no 69. Lane 5: isolate no 73. Lane 6: isolate no 32. Lane 7: isolate no 33. Lane 8: isolate no 175. Lane 9: isolate no 187. Lane 10: isolate no 91. Lane 11: isolate no 113. Lane 12: isolate no 115. Lane 13: isolate no 195. Lane 14: isolate no 215.

4.3.10. Transfer of resistance

Cefotaxime resistance transferred by transconjugation and transformation was obtained for all CTX-M-producing *K. pneumoniae* isolates.

4.3.11. Epidemiological analysis

PFGE analysis was used to analyze the molecular epidemiology of the 16 CTX-M-15-producing clinical isolates. As indicated from the result of PFGE in Figs 4.5 and 4.6, there were 5 different clusters of CTX-M-15-producing isolates. The first large cluster comprised seven strains which appeared to have near identical patterns (Figure 4.5; Lane 8, 9, 10, 11 and 12). Four strains also appear to have the same pattern (Figure 4.5; Lanes 3, 4, 5 and 6) and constitute the second cluster. Similarly a

third clone comprising three further strains (Figure 4.5 Lanes 13 and 14 respectively) appear to have the same patterns. The two remaining clusters each comprised only one strain, strain 195 (Figure 4.5 Lanes 1) and strain 115 (Figure 4.5 Lane 2) respectively.
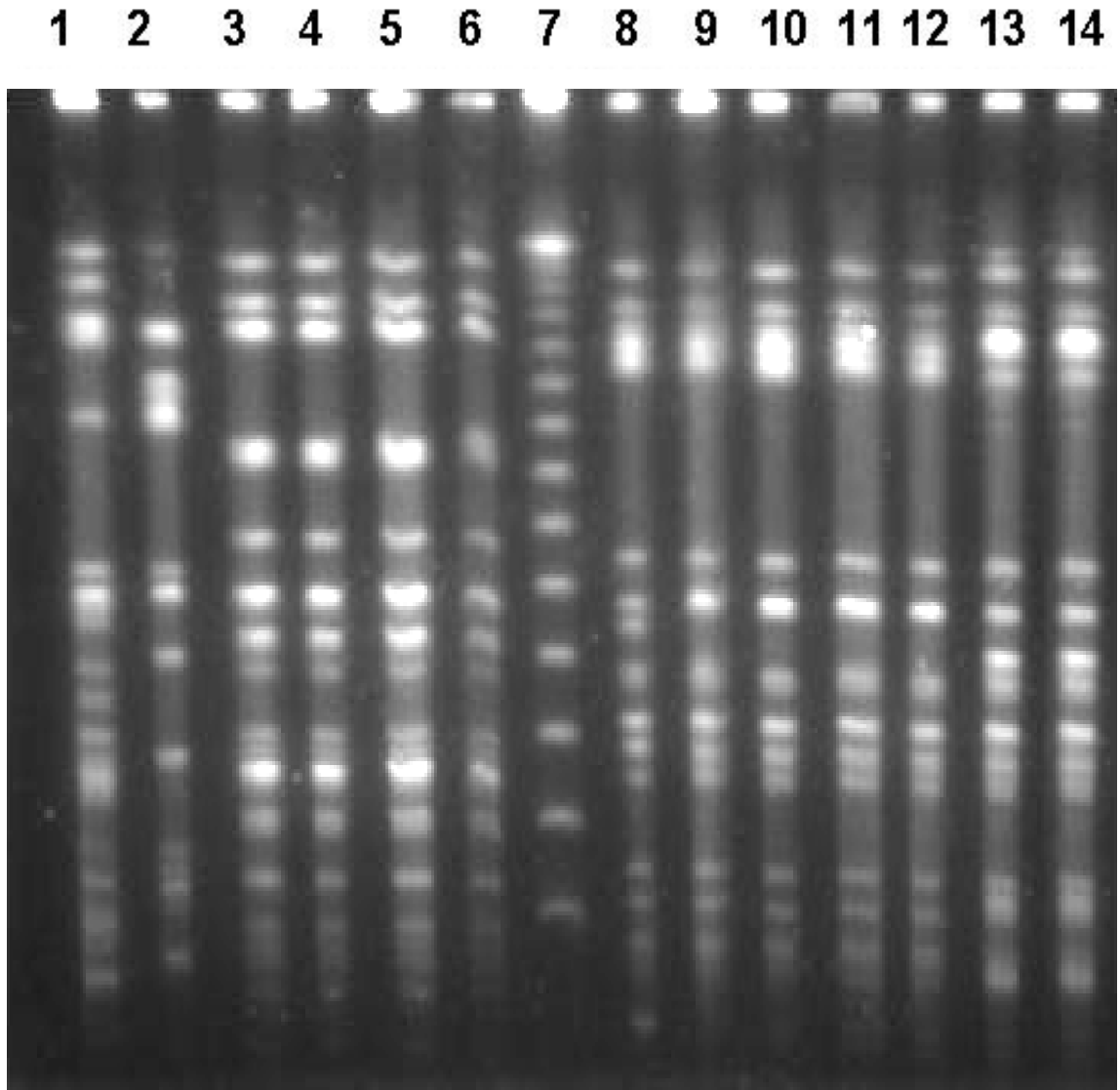
Fig 4.5: PFGE patterns of CTX-M-producing *K. pneumoniae* isolates. Lane 1: isolate no 195. Lane 2: isolate no 115. Lane 3: isolate no 32. Lane 4: isolate no 33. Lane 5: isolate no 91. Lane 6: isolate no 113. Lane M: Lambda ladder PFG marker standard size. Lane 7: isolate no 215. Lane 8: isolate no 69. Lane 9: isolate no 73. Lane 10: isolate no 175. Lane 11: isolate no 187. Lane 12: isolate no MB. Lane 13: isolate no BV.

xba1   **xba1**

ID	Site	Year
213	urine	2007
215	urine	2007
175	nephrost.	2007
187	urine	2007
197	urine	2007
69	urine	2007
73	urine	2007
BV	blood	2006
MB	blood	2006
113	urine	2007
32	urine	2007
33	urine	2007
91	urine	2007
115	urine	2007
195	sputum	2007
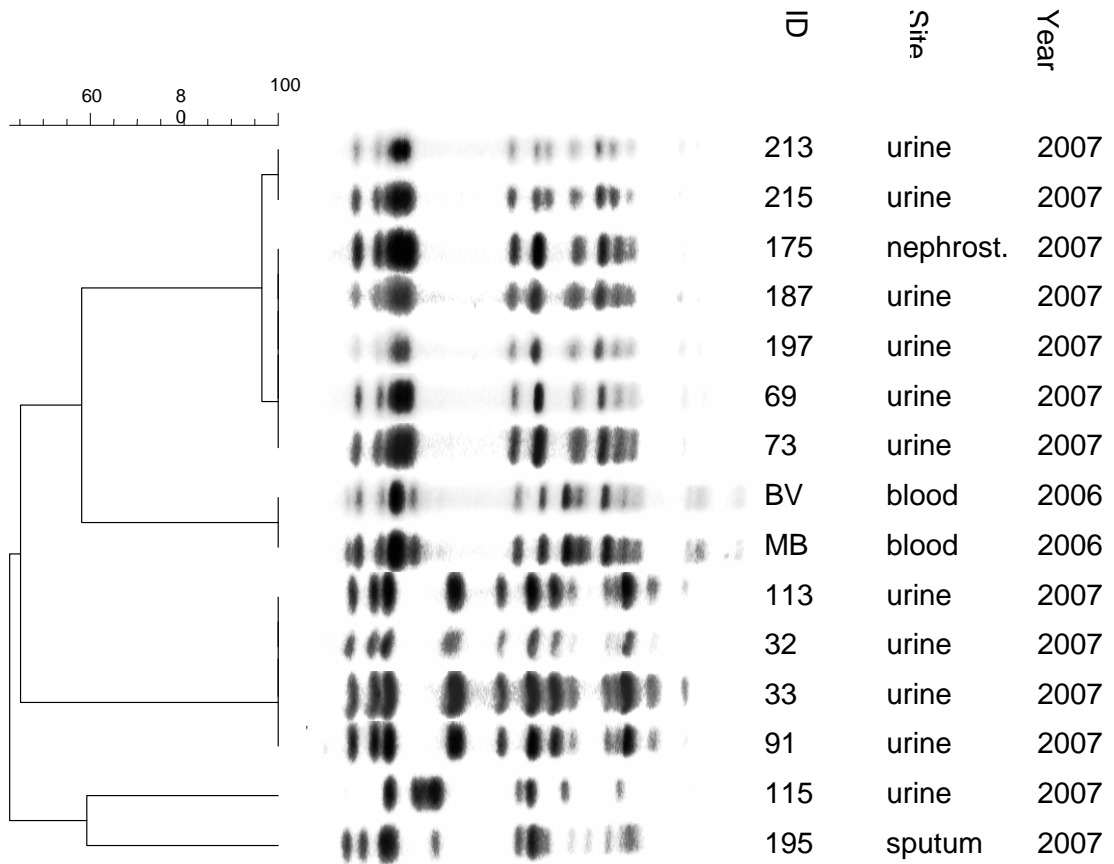
Fig 4.6: PFGE dendogram analysis of CTX-M-producing *K. pneumoniae* isolates. Dendrogram showing the relationship between isolates of *K. pneumoniae* obtained after *Xba1*-digested the chromosomal DNA of 16 clinical isolates.

4.4. DISCUSSION

CTX-M first detected in Japan in 1986 from a cefotaxime-resistant *E. coli* (the enzyme was named FEC-1) (Matsumoto, Ikeda *et al.* 1988). A few years later in 1989, a similar cefotaxime-resistant clinical *E. coli* strain from Germany was reported to produce β-lactamase enzyme designated CTX-M-1 (Bauernfeind, Grimm *et al.* 1990). CTX-M β-lactamases constitute a novel and rapidly growing family of plasmid-mediated ESBLs (Baraniak, Fiett *et al.* 2002). The rise in cephalosporin resistance may be attributable to the spread of extended-spectrum β-lactamases, particularly CTX-M types (Livermore, Canton *et al.* 2007). Outbreaks of CTX-M-15-producing *K. pneumoniae* isolates have been recently described in some European countries (Lytsy, Sandegren *et al.* 2008; Mesko Meglic, Koren *et al.* 2009).

The overall frequency of *K. pneumoniae* ESBL producers observed in this study was 32 /219 (14.61 %). By PCR CTX-M genes were detected in 16 (7.3%) isolates of *K. pneumoniae.* CTX-M-15 was the only CTX-M enzyme identified by sequence analysis of the deduced amino acid sequences. The expression of pI (8.6) band by IEF was the confirmation of presence of CTX-M-15 β-lactamase.

In this study, I identified CTX-M-15 enzymes, which is the most common CTX-M type in UK. The first CTX-M ESBL in the UK was found in 2000, in one isolate of *K. oxytoca* (Alobwede, M'Zali *et al.* 2003). During June 2001, the first hospital outbreak, 36 *K. pneumoniae* isolates from 33 patients from 15 different wards and departments around Birmingham described as CTX-M-25-like (Brenwald, Jevons *et al.* 2003). In 2001, a survey examined over 900 *E. coli* from 28 hospitals in the UK and Ireland and recorded 4 isolates with CTX-M-15 enzyme (Mushtaq, Woodford *et al.* 2003). In 2004, Woodford, Ward *et al.* (2004) identified an epidemic clone of *E. coli* producing CTX-M-15 that had become widely distributed throughout the UK.

In Scotland, CTX-M was reported in two clinical *salmonellae* isolates harbouring different CTX-M-type enzymes without mention for their types (Yates and Amyes 2005).

CTX-M-15 β-lactamase was previously reported among isolates of *K. pneumoniae* in European countries. In a multi-centric study from Russia, CTX-M gene was reported in 35.9 % of *E. coli* and 34.9% of *K. pneumoniae* ESBL strains (Edelstein, Pimkin *et al.* 2003). In a nationwide survey in Italy, CTX-M producing strains were reported by 10 of the 11 participating centres, with remarkably variable rates among the centres (1.2 to 49.5% of the ESBL producers) (Mugnaioli, Luzzaro *et al.* 2006). In Another study, Among 149 ESBL-producing *Enterobacteriaceae* isolates collected from patients in Austria from 1998 to 2004, 49 (38 *E. coli* and 11 *Klebsiella* spp.) isolates were CTX-M producers. The majority of CTX-M producers (38/49; 78%) had CTX-M-15 enzymes; the remainder had group 9 enzymes (Eisner, Fagan *et al.* 2006). Additionally, $bla_{CTX-M-15}$ enzymes were detected by sequencing in 60/177 (34%) of ESBL-producing *K. pneumoniae* isolates collected from eight Slovenian hospitals during 2005 and 2006 (Mesko Meglic, Koren *et al.* 2009). As reviewed, the spread of a *K. pneumoniae* clone with CTX-M-15 has recently been reported in Hungary (Livermore, Canton *et al.* 2007). The high prevalence (100%) was found in Spain where all 162 isolates were identified as ESBL-producers, and the $bla_{CTX-M-15}$ gene was detected in all isolates (Oteo, Cuevas *et al.* 2009). Furthermore, the first major ESBL outbreak in Scandinavia was caused by a clonal spread of 64 multiresistant *K. pneumoniae* producing CTX-M-15 (Lytsy, Sandegren *et al.* 2008).

Worldwide, CTX-M types especially the most widely distributed CTX-M-15 have replaced TEM and SHV mutants as the predominant ESBLs in numerous countries of Africa, Europe, South America and Asia (Baraniak, Fiett *et al.* 2002; Saladin, Cao *et al.* 2002; Livermore and Woodford 2006; Livermore, Canton *et al.* 2007). CTX-M-15 was first detected in *E. coli* isolated from India during 2001 and considered as the most successful and widespread CTX-M among others (Karim, Poirel *et al.* 2001). In Canada, CTX-M-15 was identified from *E. coli* and *K. pneumoniae* (Boyd, Tyler *et al.* 2004; Mulvey, Bryce *et al.* 2004; Pitout, Church *et al.* 2007). Also, CTX-M-15 was reported from United States (Castanheira, Mendes *et al.* 2008).

As shown in Table 4.2, all isolates harbouring the CTX-M enzyme were found to carry either SHV-1 or SHV-11 or SHV-12 or SHV-5 β-lactamases by PCR. Moreover, all isolates but one were found to express TEM-1. This association is frequent and has already been described (Boyd, Tyler *et al.* 2004; Eckert, Gautier *et al.* 2004). In this study, ten of *qnrB1* genes were associated with CTX-M-15. The co-presence of *qnrB* and ESBL genes on same plasmids has been regularly reported (Jacoby, Walsh *et al.* 2006).

The pattern of resistance to antimicrobial agents in all 16 cefotaxime-resistant *K. pneumoniae* strains showed that the strains harboured CTX-M-15 ESBLs characterized by high resistance to all cephalosporins tested in this study. Resistance of CTX-M-producing isolates to cefotaxime, ceftazidime, ceftriaxone, cefuroxime, cephalexin, nalidixic acid, ciprofloxacin, cefoxitin and meropenem was found to be 100, 100, 100, 100, 100, 93.8, 93.8, 81.3 and 0% respectively. This high resistance to most antibiotics is explained by the finding that the bla_{CTX-M} genes are commonly found on plasmids often carrying genes that confer resistance to multiple antibiotics, including aminoglycosides, chloramphenicol, sulfonamide, trimethoprim, and tetracycline (Bonnet 2004).

The CTX-M enzymes confer higher level resistance to cefotaxime, cefotriaxone and aztreonam than to ceftazidime (Dutour, Bonnet *et al.* 2002; Saladin, Cao *et al.* 2002). CTXM-15 was derived from CTX-M-3 by (Asp240Gly) substitution, which increased catalytic activity to ceftazidime (Poirel, Gniadkowski *et al.* 2002). These findings indicated that CTX-M-15 ESBLs confer high-level resistance to ceftazidime (MIC range: 64- to >128 mg/L). Sequence analysis revealed an Asp240→Gly substitution. This substitution has already been reported in CTX-M-15, CTX-M-16, CTX-M-32 and CTX-M-27 and is known to confer high-level resistance to ceftazidime (Cartelle, del Mar Tomas *et al.* 2004; Doublet, Granier *et al.* 2009; Poirel, Gniadkowski *et al.* 2002)

Another substitution responsible for higher levels of resistance to ceftazidime, as compared to cefotaxime, is the Pro167Ser mutation, which differentiates CTX-M-19

from CTX-M-14 (Poirel, Naas *et al.* 2001). But this substitution not found in these results.

Concerning "non β-lactam" susceptibility of CTX-M producers, all strains but one were resistant to ciprofloxacin. High rates of ciprofloxacin resistance in CTX-M producing isolates have also been reported in previous findings (Pitout, Nordmann *et al.* 2005; Eisner, Fagan *et al.* 2006).

Identification of the phylogenetic types of CTX-M-15-producing strains using *gyrA* PCR-RFLP, all isolates were assigned to be *K. pneumoniae* phylogenetic group I (KpI). In contrast to these findings, there was a clear relationship between the KpIII group and production of CTX-M-10 enzymes (44.5% of CTX-M isolates) (Valverde, Coque *et al.* 2008).

The majority of the CTX-M-producing isolates were associated with urinary tract infections (11 strains) representing (69%) of all isolates. Three (19%), 1 (6%), and 1 (6%) were isolated from blood, sputum, and nerphrostomy swab respectively. As expected, most (82%) of the urine samples were from patients categorised as having hospital-acquired infections. Among the 16 CTX-15-producing isolates for which relevant information was available, two isolates (12.5%) were reported to be from community-acquired infections collected from urine specimens, the remaining isolates collected from hospitalized patients. These findings show a significantly lower proportion of the community-acquired CTX-M-producing *K. pneumoniae* if it compared with other studies in UK, where 70 (24%) of 291 CTX-M-producing *E. coli* isolates studied from 42 UK centres were reported from community patients, many of whom had only limited recent hospital contact (Woodford, Ward *et al.* 2004). In another study, 22 ESBL-producing Enterobacteriaceae (9 *E. coli*, 7 *E. cloacae*, 4 *C. freundii*, 1 *Klebsiella* spp. and 1 *Salmonella* spp.) were detected among 565 faecal specimens from York. The authors identified eight ESBL-producing *E. coli* and one ESBL-producing *Salmonella* spp. were from community patients, the remaining 13 isolates were collected from hospital patients. The β-lactamases identified were 5 *bla*CTX-M-15, 3 *bla*CTX-M-14 and 9 *bla*CTX-M-9 (Munday, Whitehead *et*

al. 2004). Moreover, Most (63%) CTX-M producers were recovered from patients with urinary tract infections. In total, more than 70% of all ESBL-producing isolates of community origin screened for the presence of CTX-M enzymes were found to be positive, compared with 21% of all ESBL producers of hospital origin (Eisner, Fagan *et al.* 2006).

For all CTX-M-producing isolates, a total of one to two plasmids ranging in size from approximately 40 to 210 kb were observed per strain with S1-nuclease plasmid profiles as shown in Table 4.2. Similar to these results, $bla_{CTX-M-32}$ and $bla_{CTX-M-1}$ (CTX-M group-1) were carried in a single 40-kb IncN plasmid or with an IncL/M plasmid of 50 kb from different *E. coli* strains in Spain since 2000 (Novais, Canton *et al.* 2007). In another study, $bla_{CTX-M-15}$ alone or with *aac(6')-Ib* and/or *aac(6')-Ib-cr* co-localised on IncR, IncFII, IncFIIk replicons on different plasmids size of ca. 30 kb, 60 kb, 75 kb, 80 kb, 85 kb, 90 kb, 190 kb, 290-kb and 340-kb (Coelho, Gonzalez-Lopez *et al.* 2010). In a Spanish study, all CTX-M-15 clones studied were harboured the same plasmid (pRYCE34) of 180 kb isolated from 2002 to 2004 (Valverde, Coque *et al.* 2008). Hybridization studies showed that $bla_{CTX-M-15}$ was located on ~150-kb plasmids *K. pneumoniae* strains from Slovenia (Mesko Meglic, Koren *et al.* 2009).

In this study the IncN plasmid determined by PCR is always present when $bla_{CTX-M-15}$ gene was identified even in the absence of IncFII. As the IncN plasmid is the only type present, these results are the first to indicate the role of IncN replicon types in the dissemination of the $bla_{CTX-M-15}$ genes in *K. pneumoniae*, although IncN plasmid has been found on one occasion to carry the CTX-M-15 gene in *E. coli* (Marcadé, Deschamps *et al.* 2009). It was confirmed that mobilization and spread of CTX-M-15 seems to be related to IncFII plasmids (Coque, Novais *et al.* 2008; Carattoli 2009). The $bla_{CTX-M-15}$ was also detected in IncFI or IncL/M types (Lavollay, Mamlouk *et al.* 2006; Oteo, Cuevas *et al.* 2009). Additionally, $bla_{CTX-M-32}$ and $bla_{CTX-M-1}$ (CTX-M group-1) were located in a single 40-kb IncN plasmid or with an IncL/M plasmid of 50 kb from different *E. coli* strains in Spain since 2000 (Novais, Canton *et al.* 2007). Additionally, plasmids encoding CTX-M-15 carried three distinct replicons, IncFII,

IncR and IncFIIk, were reported among 37 *K. pneumoniae* strains isolated in the Barcelona metropolitan area (Coelho, Gonzalez-Lopez *et al.* 2010).

There should be great concern about the dissemination of the gene on broad-host-range IncN plasmids, because it maximises the further spread of CTX-M-1-like enzymes among other members of *Enterobacteriaceae* (Novais, Canton *et al.* 2007).

PCR identified the insertion sequence IS*Ecp1* upstream of the $bla_{CTX-M-15}$ gene in all strains (Table 4.2) except one strain (strain 115). IS*Ecp1* was detected by sequencing 48 nucleotides upstream of $bla_{CTX-M-15}$. This result was consistent with other studies, a 48bp sequence, previously described for $bla_{CTX-M-15}$ from India and previously named as the W sequence (Karim, Poirel *et al.* 2001; Poirel, Decousser *et al.* 2003; Eckert, Gautier *et al.* 2006). The insertion sequences IS*Ecp1* or IS*Ecp1*-like were repeatedly identified upstream of many bla_{CTX-M} genes, and played an important role in the mobilization and expression of these genes (Cao, Lambert *et al.* 2002; Saladin, Cao *et al.* 2002; Poirel, Decousser *et al.* 2003; Eckert, Gautier *et al.* 2006), suggesting a similar origin and organisation from those already described previously.

The presence of IS*26* was found upstream of the $bla_{CTX-M-15}$ gene in all strains tested by PCR. The insertion sequence IS*26* was described by Saladin *et al.* (Saladin, Cao *et al.* 2002) to be upstream of a $bla_{CTX-M-1}$ gene. The presence of IS*26* in all isolates probably due to the presence of this insertion sequence on known plasmids, such as IncFII, IncN, and IncL/M (Novais, Canton *et al.* 2007).

Class 1 integrons are widespread genetic elements that allow promoterless bacteria to capture and express gene cassettes. These integrons contribute to the dissemination of antibiotic resistance genes between bacteria of the same or of different species (Jove, Da Re *et al.* 2010). Results identified class 1 integrons in all but one of CTX-M-producing *K. pneumoniae* isolates (Table 4.2). PCR identified two different sizes of class 1 integrons, the largest one was ~ 2 kb and the second was ~1.0 kb in size. After sequencing of representative samples the three different gene cassette arrangements were *dfrA12 + aadA2, aadA1, aadA2*. Similar to my study, class 1

integrons including *dfr* and *aadA* gene cassettes have been the most prevalent type of integrons (Vinue, Saenz *et al.* 2008).

The conserved *qacEΔ1-sul1* region was identified by PCR from all the 15 integrons-positive isolates. These results were in agreement with findings that the 3′-conserved segments of the class 1 integrons usually contain the genetic determinants *qacEΔ1* and *sul1* (Carattoli 2001).

In this study I identified four isolates beard class 1 integrons were negative for integrase (*intI1*) gene. (Jove, Da Re *et al.* 2010) reported that there are currently 13 Pc variants corresponding to 10 variants of the class 1 integrase *Intl1*. The Pc promoter in class 1 integrons is located within the *intI1* coding sequence. The Pc polymorphism affects the amino acid sequence of *IntI1* and the effect of this feature on the integrase recombination activity.

Cefotaxime resistance transferred by transconjugation and transformation was obtained for all CTX-M-producing *K. pneumoniae* isolates. These results indicating the highly transconjugated IncN plasmid carry CTX-M-15. The corresponding Figures indicated that 60 isolates comprised plasmids encoding CTX-M-15 enzyme were highly transmissible as well (Mesko Meglic, Koren *et al.* 2009).

PFGE analysis was used to analyze the molecular epidemiology of the 16 CTX-M-15-producing clinical isolates. As indicated from the result of PFGE, there were 5 different clones of CTX-M-15-producing isolates. In Spain, one epidemic clone characterized by PFGE from a single institution in Madrid (Valverde, Coque *et al.* 2008). In another study, (Oteo, Cuevas *et al.* 2009) identified seven PFGE clusters corresponded to seven different *K. pneumoniae* STs collected from five Spanish hospitals. Mesko Meglic, Koren *et al.* (2009) identified 11 pulsed-field gel electrophoresis-defined strains, with several clusters of closely related isolates among 60 *K. pneumoniae* from eight Slovenian hospitals.

4.5. CONCLUSION

Despite the prevalence of CTX-M-15 in UK, less work has been done on *Klebsiella* spp. with CTX-M enzymes. This work confirms the emergence of hospital and community-acquired CTX-M-type enzymes and their spread in the Edinburgh area, Scotland. Additionally, this study showed some alarmingly high rates (50%) of CTX-M-15 among ESBL-producing *K. pneumoniae* isolates. By PCR all isolates were associated with IncFII and/or with the broad host range IncN plasmid, in future would accelerate the spread of these genes. I identified five PFGE clusters of *K. pneumoniae* isolates conferring high level resistance to most antibiotics tested.

CHAPTER-5:

Prevalence of *bla*$_{SHV}$ genes from *K. pneumoniae* clinical isolates in Scotland

5.1. ABSTRACT

All 219 *K. pneumoniae* strains isolated during 2006 and 2007 were collected from the Royal Infirmary of Edinburgh. The total number of amplified bla_{SHV} genes from 60 (32 ESBLs-positive and 28 "non-ESBL" producing) isolates was 54 (90%) isolates. From the ESBL-producing isolates, molecular methods identified ten strains possessing ESBL-SHV genes (1 strain $bla_{SHV-5,}$ 1 strain bla_{SHV-80} and 8 strains bla_{SHV-12}) and 19 isolates producing narrow-spectrum SHV β-lactamases (17 strain carry bla_{SHV-11} and 2 isolates with bla_{SHV-1}). Conjugation methods demonstrated that 29/32 isolates harbour transferable bla_{SHV}. All bla_{SHV-12} but one, bla_{SHV-80} and bla_{SHV-5} were successfully transconjugated. The large SHV transposon-borne promoter (IS26-lacY-recF-SHV) was amplified from only one isolate harbouring non-transferable bla_{SHV-11}, 15 isolates were produced the small SHV transposon-borne promoters (IS26-SHV). The genetic relatedness of the isolates was studied by PFGE analysis. The strains were diverse, and 17 clusters were defined, the largest including 7 strains mostly isolated from urine and expressed SHV and CTX-M-15.

5.2. INTRODUCTION

SHV enzymes are classified in groups 2b and 2be of the Bush-Jacoby-Medeiros classification scheme and in Ambler class A (Ambler, Coulson *et al.* 1991; Bush, Jacoby *et al.* 1995). SHV-1 was first described in 1972 and called Pit-2 from the author's name Pitton (Pitton 1972). The chromosomal SHV-1 confers resistance to ampicillin, amoxicillin, carbenicillin and ticarcillin and considered to be ubiquitous amongst the great majority of *K. pneumoniae* strains (Livermore 1995; Babini and Livermore 2000). SHV enzymes were also detected in other Enterobacteriaceae as plasmid-mediated β-lactamases (Matthew 1979; Sabate, Miro *et al.* 2002). IS*26* insertion sequence mediates mobilization of plasmid-mediated bla_{SHV} genes from genome to plasmid and increases promoter strength through the introduction of a different –35 region (Podbielski, Schonling *et al.* 1991a). The majority of SHV variants possess an ESBL phenotype characterized by the substitution of a glycine

for serine at position 238 and/or glutamic acid for lysine at position 240 (Huletsky, Knox *et al.* 1993; Kurokawa, Yagi *et al.* 2000; Bradford 2001; Hujer, Hujer *et al.* 2002).

5.3. RESULTS

5.3.1. Prevalence of SHV ESBLs

SHV β-lactamases were detected in 32/32 (100%) of ESBLs-positive isolates (all from *K. pneumoniae*) using primers (Kp-SHV_for and Kp-SHV_rev) as described earlier and shown in Table 2.2 (Materials and Methods). PCR conditions were as follows: 5 min at 94°C, 35 cycles of 40 seconds at 95°C, 1 min at 65°C, and 1 min at 72°C, and final extension of 7 min at 72°C. The bla_{SHV-11} was detected after sequencing in 21 ESBLs-positive strains (17 isolates alone and 4 isolates associated with bla_{SHV-12} and bla_{SHV-80}). Other previously described narrow-spectrum β-lactamases genes detected here were bla_{SHV-1} (2 strains, 1 with bla_{TEM-1}, and 1 together with $bla_{CTX-M-15}$). The extended-spectrum SHV β-lactamases genes detected were; bla_{SHV-12} (8 strains, 1 alone, 1 together with *qnrA1*, 3 together with $bla_{CTX-M-15}$ and bla_{TEM-1} genes and 3 together with bla_{TEM-1}), bla_{SHV-80} (1 strain co-expressed $bla_{CTX-M-15}$ and bla_{TEM-1}) and bla_{SHV-5} (1 strain together with $bla_{CTX-M-15}$, bla_{TEM-1} and *qnrB1*). In addition, 3 strains carry an unidentified bla_{SHV} (Table 5.1).

The isolate 33 that carries bla_{SHV-80} showed an unusual resistance phenotype toward meropenem antibiotic. It showed once increase in MIC (8 mg/L) and then back to (0.03 mg/L).

5.3.2. Prevalence of *bla*$_{SHV}$ from "non-ESBL-producing" isolates

Amplification of bla_{SHV} from 28 "non-ESBL-producing" strains revealed that 22 isolates were positive for bla_{SHV}, 6 isolates were negative. The bla_{SHV-11} was detected from one representative isolate with sequencing. This isolate was found to carry bla_{SHV-11} which coexisted with *qnrB6* gene.

The total number of amplified bla_{SHV} genes from 60 (32 ESBL-positive and 28 ESBL-negative) *K. pneumoniae* isolates was 54 (90%), whereas 6 (10%) isolates were negative for bla_{SHV} gene.

5.3.3. Presence of more than one copy of bla_{SHV} genes in the same isolate

Four isolates (numbers 32, 84, 208 and 33) all were identified as SHV-11 with DNA sequencing. But after the sequencing repeated each isolate was found to express another SHV enzyme. The new enzymes were identified as bla_{SHV-12} from strains (32, 84, 208), the strain number 33 was found to harbour bla_{SHV-80}.

5.3.4. Association of bla_{SHV} with resistance genes

The bla_{TEM} was detected in 24 strains associated with bla_{SHV}; no TEM-derived ESBLs were found by representative sequenced samples, 16 isolates co-expressed $bla_{CTX-M-15}$. 18 isolates were found to be positive by PCR for the *qnr* genes. Of these *qnr*-positive isolates, 16 isolates were ESBL-positive (11 *qnrB1* gene and 5 *qnrA1* gene) and 2 isolates were ESBLs-negative, both of them harboured the *qnrB6* gene (Table 5.1).

5.3.5. Phylogenetic groups

Analysis of phylogenetic groups of 32 ESBLs SHV-producing isolates tested showed that most of them, 26 isolates (81.25%), were belonged to KpI-type. In addition, 6 isolates were belonged to the KpIII-type (18.75%). All isolates containing CTX-M-15 belonged to the KpI group. Interestingly, there was a clear relationship between the KpIII group and production of *qnrA* genes in which 4 of 5 isolates were found to harbour the *qnrA1* gene (Table 5.1).

Table 5.1: Characterization of SHV-producing isolates.

Strain	ID	SHV	SHV conjugation	IS26-SHV bp	IncFII	IncN	Intl 1	Sul1	qac	Intl 2	TEM	Qnr	CTX-M	KP phylogentic type	CS class 1 bp
32	Mu 932 491 P	SHV-11/12	+	1500	-	+	-	+	+		+	-	+	I	2k
33	Mu 932 330	SHV-11/80	+	1500	-	+	-	+	+		+	-	+	I	2k
69	Mu 936 327 N	SHV-11	+	1500	+	+	+	+	+		+	B1	+	I	1k
73	Mu 936 619 A	SHV-11	+	1500	+	+	+	+	+	+	+	B1	+	I	1k
84	Mu 939 776 Z	SHV-11/12	+	800	ND	ND	+	+	+		+	-	-	III	1.5- 2k
85	Mu 939 772	SHV-1*	+	-	-	-	-	-	-		+	-	-	I	-
91	Mu 939 312 V	SHV-11	+	1500	-	+	-	+	+		+	-	+	I	2k
106	Mu 939 649 W	SHV-11	+	-	+	+	+	+	+		+	A1	-	III	1.5- 2k
113	Mu 940 148 Z	SHV-11	+	1500	-	+	-	+	+		+	-	+	I	2k
114	Mu 940 595 G	SHV-12	+	800	+	+	+	+	+		-	-	-	I	ND
115	Mu 940 460 H	SHV-1 *	+	800	+	+	+	-	-		-	-	+	I	-
125	Mu 941 508 P	ND	+	-	+	-	-	-	-		-	-	-	III	1.5- 2k
135	Mu 943 702 T	SHV-11	+	800	+	+	+	+	+		+	A1	-	III	1.5-2k
175	Mg 768 689 P	SHV-12	+	800	+	+	+	+	+		+	B1	+	I	1k
187	Mu 949847 W	SHV-12	+	800	+	+	+	+	+		+	B1	+	I	1k
195	Mr 148167 R	SHV-11	-	3kb	+	+	+	+	+	+	+	-	+	I	1k
197	Mu 950618 L	SHV-5	+	-	+	+	+	+	+		+	B1	+	I	1k

CS: Conserved sequence of class 1 integrons

(-): Negative

(+): Positive

ND: Not determined

Continued

Table 5.1: Characterization of SHV-producing isolates.

Strain	ID	SHV	SHV conjugation	IS26-SHV bp	IncFII	IncN	Intl 1	Sul1	qac	Intl 2	TEM	Qnr	CTX-M	KP phylogentic type	CS class 1 bp
203	Mg 769750	ND	-	800	-	+	+	+	+		-	-	-	I	2k
204	Mu 953545 C	SHV-11	+	800	+	+	+	+	+		+	A1	-	III	1.5-2k
205	Mu 953096 R	SHV-11	+	800	-	+	+	+	+		-	-	-	I	2k
206	Mu 956107 Q	SHV-11	+	800	-	+	+	+	+		+	A1	-	III	1.5-2k
208	Mg 771610 V	SHV-11/12	-	800	+	+	+	+	+		+	-	-	I	2k
210	Mu 956764 R	SHV-11	+	800	-	-	+	+	+		+	B1	-	I	2k
213	Mu 960 478	SHV-11	+	1500	+	+	+	+	+		+	B1	+	I	1k
214	Mb 195 491 Y	SHV-12	+	800	-	+	+	+	+	+	-	A1	-	I	ND
215	Mu 966 491 T	SHV-11	+	1500	+	+	+	+	+		+	B1	+	I	1k
MB	Mb141078	SHV-11	+	1500	+	+	+	+	+		+	B1	+	I	1k
B79	B143279	SHV-11	+	1500	+	+	+	+	+		+	B1	+	I	1k
B31	Mb143231	SHV-11	+	-	-	-	-	-	-		-	-	-	I	ND
U54	Mu724454	SHV-12	+	800	ND	ND	+	+	+		+	-	-	I	2k
BV	B14124V	SHV-11	+	1500	+	-	+	+	+		+	B1	+	I	1k
MR	Mr112663	ND	+	800	-	-	-	-	-		-	-	-	I	ND
172	Mu 948 095 C	LEN- new	+	ND	-	+	+	+	+		-	B6	-	III	1.5-2k

5.3.6. Conjugation

Conjugation and genotyping of 32 *K. pneumoniae* isolates producing SHV-type ESBLs demonstrated that 29 isolates harbour transferable bla_{SHV}. From these transferable bla_{SHV} isolates: 50% (n=16) carry bla_{SHV-11}, 21.9% (n=7) carry bla_{SHV-12}, 6.25% (n=2) carry bla_{SHV-1}, 6.25% (n=2) carry unidentified SHV genes, 3.1% (n=1) carry bla_{SHV-5}, and 3.1% (n=1) carry bla_{SHV-80}. Additionally, 3 (9.4%) isolates harbour non-transferable bla_{SHV}: one isolate produce chromosomal bla_{SHV-11}, one isolate harboured bla_{SHV-12}, and one isolate not sequenced.

Attempts to transfer ampicillin resistance TEM from the study strains to *E. coli* by conjugation showed that about 80% isolates harbouring bla_{TEM} failed to donate their plasmids to the recipient strain. Moreover all CTX-M strains were successfully transconjugated.

5.3.7. Genetic environment

Results shown in Table 5.1 indicated that the large SHV transposon-borne promoters (IS26-lacY-recF-SHV) were amplified from only one large SHV transposon-containing isolate harboured non-transferable bla_{SHV-11} using primers IS26-FCJ and SHV-12-F-1 as in Table 2.2 (Materials and Methods). The amplified PCR product was sequenced from both ends, which enabled sequencing of the region in both directions. The sequence was quite similar to accession number AJ245670 [GeneBank]. The sequence product had about 500 bp which is corresponding to *kdp*C gene and was missing in the AJ245670 GeneBank.

The promoter sequence from large SHV transposon-containing isolate was (AAAAAT) instead of (ACAAAT) in the -10 region. This promoter has (C→A) mutation at second nucleotide in the -10 region.

A total of 15 isolates produced a PCR product of about 800bp. DNA sequencing of representative samples were found to harbour the small SHV transposon-borne

promoter (IS26-SHV). The representative DNA samples were sequenced and compared with GenBank EF370423 [GenBank]) sequence. The results revealed that IS*26* was found 73 bp upstream of the *bla*$_{SHV}$ genes as shown in Fig 5.1.

Eleven isolates produced a band of ~1.5 Kbp but it proved impossible to sequence these isolates. The remaining isolates were negative by PCR for IS*26*.

The last two groups, negative group and ~1.5 kbp producing group, were produced a PCR product of about 800bp after several repeats of the PCR reactions. The most likely explanation is these isolates each harbour two or more different *bla*$_{SHV}$ genes.

In addition, the RecF protein and DEOR transcriptional regulator were identified by PCR upstream and downstream from all isolates using primers SHV-12-F-1, SHV-12-R-1, DEOR-R and RECF-F as in Table 2.2 (Materials and Methods). The sequencing of representative samples revealed that the putative DEOR transcriptional regulator was identified 21 bp downstream of all *bla*$_{SHV}$ strains tested by PCR when the genes was compared with GenBank EF370423 [GenBank] sequence as shown in Fig 5.2.

Fig 5.1: Alignment of IS*26-bla*SHV from strain 84 and the GeneBank EF370423

```
84              TCGGTGTTCAACGTCAGACGGGCACCGGCCTTCGCGTTTGAGCAGAGCAAGCGCGCGACC 240
EF370423                        GGCACCGGCCTTCGCGTTTGAGCAGAGCAAGCGCGCGACC 40
                                ****************************************
84              ATAGGCGGGCGCTTTATCCGTGTTGATGAATCGCGGGATCTGCCACTTCTTCACGTTGTT 300
EF370423        ATAGGCGGGCGCTTTATCCGTGTTGATGAATCGCGGGATCTGCCACTTCTTCACGTTGTT 100
                ************************************************************
84              GAGGATTTTACCCAGAAACCGGTATGCAGCTTTGCTGTTACGACGGGAGGAGAGATAAAA 360
EF370423        GAGGATTTTACCCAGAAACCGGTATGCAGCTTTGCTGTTACGACGGGAGGAGAGATAAAA 160
                ************************************************************
84              ATCGACAGTGCGGCCCCGGCTGTCGACGGCCCGGTACAGATACGCCCAGCGGCCATTGAC 420
EF370423        ATCGACAGTGCGGCCCCGGCTGTCGACGGCCCGGTACAGATACGCCCAGCGGCCATTGAC 220
                ************************************************************
84              CTTCACGTAGGTTTCATCCATGTGCCACGGGCAAAGATCGGAAGGGTTACGCCAGTACCA 480
EF370423        CTTCACGTAGGTTTCATCCATGTGCCACGGGCAAAGATCGGAAGGGTTACGCCAGTACCA 280
                ************************************************************
84              GCGCAGCCGTTTTTCCATTTCAGGCGCATAACGCTGAACCCAGCGGTAAATCGTGGAGTG 540
EF370423        GCGCAGCCGTTTTTCCATTTCAGGCGCATAACGCTGAACCCAGCGGTAAATCGTGGAGTG 340
                ************************************************************
84              ATCGACATTCACTCCGCGTTCAGCCAGCATCTCCTGCAGCTCACGGTAACTGATGCCGTA 600
EF370423        ATCGACATTCACTCCGCGTTCAGCCAGCATCTCCTGCAGCTCACGGTAACTGATGCCGTA 400
                ************************************************************
84              TTTGCAGTACCAGCGTACGGCCCACAGAATGATGTCACGCTGAAAATGCCGGCCTTTGAA 660
EF370423        TTTGCAGTACCAGCGTACGGCCCACAGAATGATGTCACGCTGAAAATGCCGGCCTTTGAA 460
                                                                          --
84              TGGGTTCATGTGCAGCTCCATCAGCAAAAGGGGATGATAAGTTTATCACCACCGACTATt 720
EF370423        TGGGTTCATGTGCAGCTCCATCAGCAAAAGGGGATGATAAGTTTATCACCACCGACTATt 520
                ************************************************************
                ------------
84              tgcaACAGTGCCAACGCCGGGTtattctTATTTGTCGCTTCTTTACTCGCCTTTATCGGC 780
EF370423        tgcaACAGTGCCAACGCCGGGTtattctTATTTGTCGCTTCTTTACTCGCCTTTATCGGC 580
                ************************************************************

84              CTTCACTCAAGGATGTATTGTGGTTATGCGTTATATTCGCCTGTGTATTATCTCCCTGTT 840
EF370423        CTTCACTCAAGGATGTATTGTGGTTATGCGTTATATTCGCCTGTGTATTATCTCCCTGTT 640
                ************************************************************
84              AGCCACCCTGCCGCTGGCGGTACACGCCAGCCCGCAGCCGCTTGAG              886
EF370423        AGCCACCCTGCCGCTGGCGGTACACGCCAGCCCGCAGCCGCTTGAGCAAATTAAACAAAG 700
                **********************************************
```

(Bold **ATG**) Start codon for *bla*SHV (positon 606)

(Small case) Promoter of SHV at position 520-525 (-35 signal), 543-548 (-10 signal)

(-) Iverted repeat sequence of IS*26* (position 519-532)

(*) Indicate identity

Fig 5.2: Alignment of DEOR transcriptional regulator gene from isolate 84 with the GeneBank EF370423

```
84                                          AATCAGCAAATCGCCGGGATCGGCGCGG  28
EF370423   TGCGGGATACGCCGGCGAGCATGGCCGAGCGAAATCAGCAAATCGCCGGGATCGGCGCGG 1440
                                            ****************************

                                                                    ---
84         CGCTGATCGAGCACTGGCAACGCtaaGCCGGCGGTGGCCGCGCGCGTTATCCGGCCCGCA  88
EF370423   CGCTGATCGAGCACTGGCAACGCtaaGCCGGCGGTGGCCGCGCGCGTTATCCGGCCCGCA 1500
           ************************************************************

84         GCACCTTGCAGGCGTGCCGGGCGATATGACTGGCGGCGGCATCGGAAAGATGCCGGTCGG 148
EF370423   GCACCTCGCAGGCGTGCCGGGCGATATGACTGGCGGCGGCATCGGAAAGATGCCGGTCGG 1560
           ****** *****************************************************

84         TAATGATAGTGGTGAACCGGGTCAAAGGTAACGCCATAAACGTGGCCACCTGATTGTATT 208
EF370423   TAATGATGGTGGTGAACCGGGTCAAAGGTAACGCCATAAACGTGGCCACCTGATTGTATT 1620
           ******* ****************************************************

84         TCGAACTGTCGCACAGCAGGATGCTTCTCGCGCTGACCTGGCTGACGGTCTCCTTGACGG 268
EF370423   TCGAACTGTCGCACAGCAGGATGCTTCTCGCGCTGACCTGGCTGACGGTCTCCTTGACGG 1680
           ************************************************************

84         TAACCTTGTTCTCATCAGGGGTGAATATCCCGTGACTGTCCCAGCCGCTGGCGGAGATAA 328
EF370423   TAACCTTGTTCTCATCAGGGGTGAATATCCCGCGACTGTCCCAGCCGCTGGCGGAGATAA 1740
           ******************************** ***************************

84         AGGCCGTATCGATAGCCAGGTGGCGTAACGTACGCGCCGCCGATTCGCCCACGCAGGAGC 388
EF370423   AGGCCGTATCGATAGCCAGGTGGCGTAACGTACGCGCCGCCGATTCGCCCACGCAGGAGC 1800
           ************************************************************

84         GGTTCTCCCGGCACAGAGTGCCGCCGGTGTGGATCACGCCGCACTGGCTGGCATCGATCA 448
EF370423   GGTTCTCCCGGCACAGAGTGCCGCCGGTGTGGATCACGCCGCACTGGCTGGCATCGATCA 1860
           ************************************************************

84         GCAGCTGGGTTATCTCAAAATCATTGGTGACCACCTGGAGATCGTTCCGGTCGAGGATCG 508
EF370423   GCAGCTGGGTTATCTCAAAATCATTGGTGACCACCTGGAGATCGTTCCGGTCGAGGATCG 1920
           ************************************************************

84         CCCGCGCCAGCGCCAGGGTGGTAGTCCCGGCATCCAGATAGATGCAACTGTTTTTAGCGA 568
EF370423   CCCGCGCCAGCGCCAGGGTGGTAGTCCCGGCATCCAGATAGATGCAACTGTTTTTAGCGA 1980
           ************************************************************

84         TATGACTCGCCGCCAGCGCGCCGATCGCCTGTTTCTCCTCACTCTGCAGCGTGCTTTTCA 628
EF370423   TATGACTCGCCGCCAGCGCGCCGATCGCCTGTTTCTCCTCACTCTGCAGCGTGCTTTTCA 2040
           ************************************************************

84         CCAGATGACTGGGTT                                              687
EF370423   CCAGATGACTGGGTTCCGCGGCCAGCCGGCTGACGGCGCGTACGCCGCCCGAGACGCTGA 2100
           **************
```

(Small case): taa, stop codon of *bla*SHV

(-) **TAT** (position 1487): stop codon of DEOR transcriptional regulator.

5.3.8. Genetic environment of bla_{SHV-12} and bla_{SHV-5}

The environment of bla_{SHV-12} genes identified in this study confirmed that IS26 was found 73 bp upstream of the bla_{SHV-12} gene in all cases but one which produced a band of 1.5 kbp but it could not be sequenced. Furthermore, in all SHV-12-producing strains, the putative DEOR transcriptional regulator (GenBank EF370423 [GenBank]) was identified 21 bp downstream of the bla_{SHV-12} gene.

The environment of bla_{SHV-5} gene was surrounded by a gene encoding a putative RecF protein (GenBank AY532647 [GenBank]) upstream and the gene coding the putative DEOR transcriptional regulator downstream (Figure 5.3). The upstream IS26 of bla_{SHV-5} was negative by PCR.
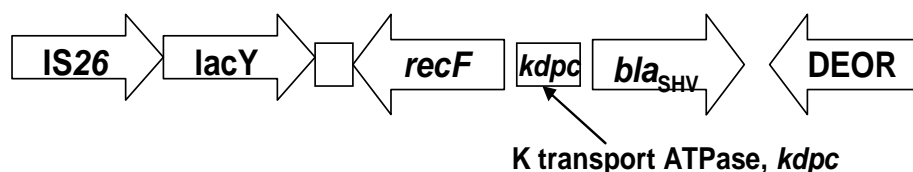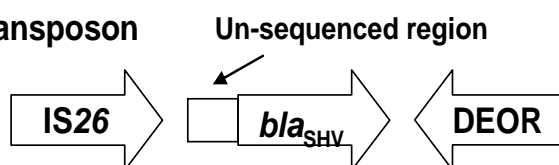
Fig 5.3: Large and small SHV transposons. The large SHV transposon map is based upon the sequence of *Escherichia coli* plasmid p1658/97 and the small transposon map is based upon the sequence of *Enterobacter cloacae* plasmid pEC-IMPQ. The large SHV transposon typically harbours non-transferable a bla_{SHV} encoding the non-ESBL SHV-11. The small SHV transposon typically harbours a bla_{SHV} encoding the non-ESBL SHV-11, SHV-1 or the ESBL derivative SHV-12.

5.3.9. Reverse transcriptase PCR Results

bla_{SHV} mRNA levels were measured using reverse transcriptase PCR, and the difference in the cycle number between the two amplification reactions (or ΔCt values) which provides a measure of relative gene dosages of each allele were calculated as follows: ΔCt = Ct (bla_{SHV} cDNA) – Ct (16S cDNA). Three isolates carry different transposon (Large, Small, and un-sequenced) were analysed (Table 5.2) and (Fig. 5.4, 5.5, 5.6). The ΔCt for the large SHV transposon-containing isolates was 110.1 cycles, which is significantly more than the value for the small SHV transposon isolates (63.3 cycles). Thus, the presence of the large SHV transposon increases bla_{SHV} mRNA by ~2x. This confirms that the mutated promoter has enhanced the activity.

Table 5.2: The ΔCt value of reverse transcriptase of bla_{SHV} mRNA from the different transposons

Isolates	SHV Ct value	16S RNA Ct value	ΔCt values
187, small transposon	71.2	7.9	63.3
195, large transposon	123	12.9	110.1
215, un-sequenced	90.6	9.11	81.4
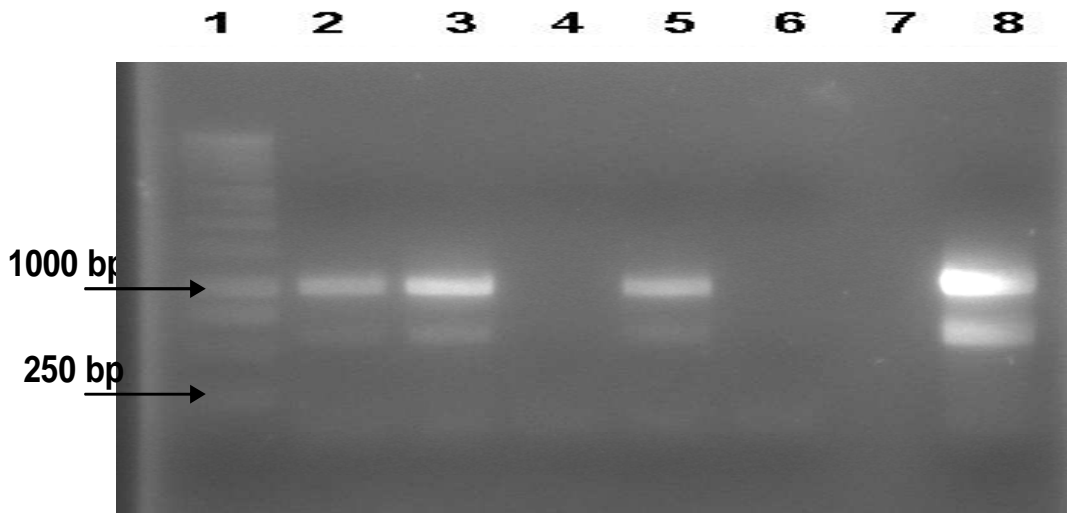
Fig 5.4: Gel electrophoresis of reverse transcriptase amplification of SHV enzyme. Lane 1: 1Kbp molecular marker. Lane 2: isolate no. 187. Lane 3: isolate no. 195. Lane 5: isolate no. 215. Lane 8: genomic DNA.
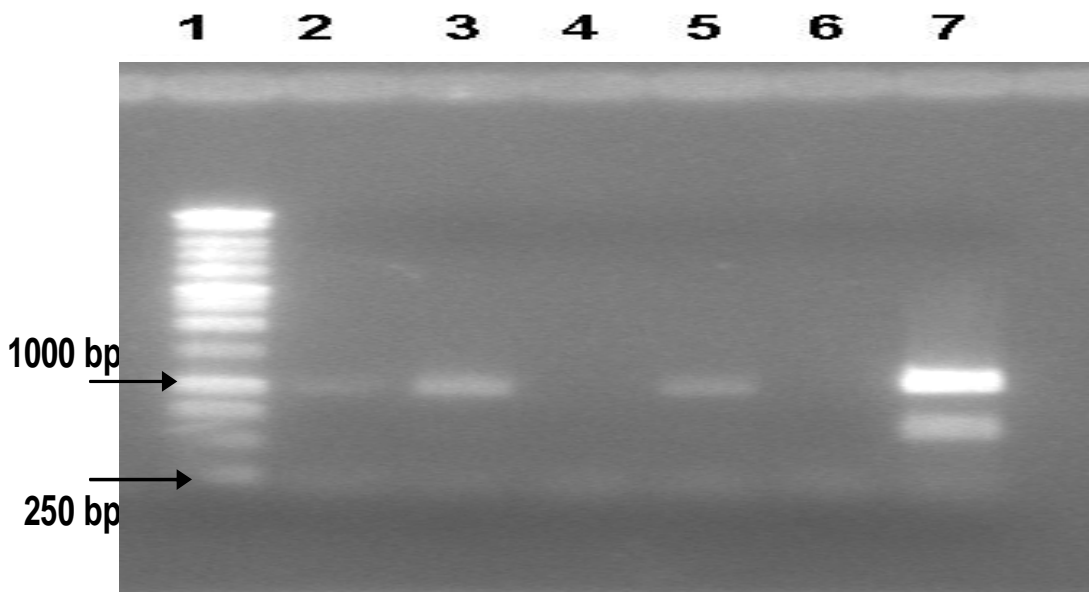
Fig 5.5: Gel electrophoresis showing amplification of 16s ribosomal RNA. Lane 1: 1Kbp molecular marker. Lane 2: isolate no. 187. Lane 3: isolate no. 195. Lane 5: isolate no. 215. Lane 7: genomic DNA.
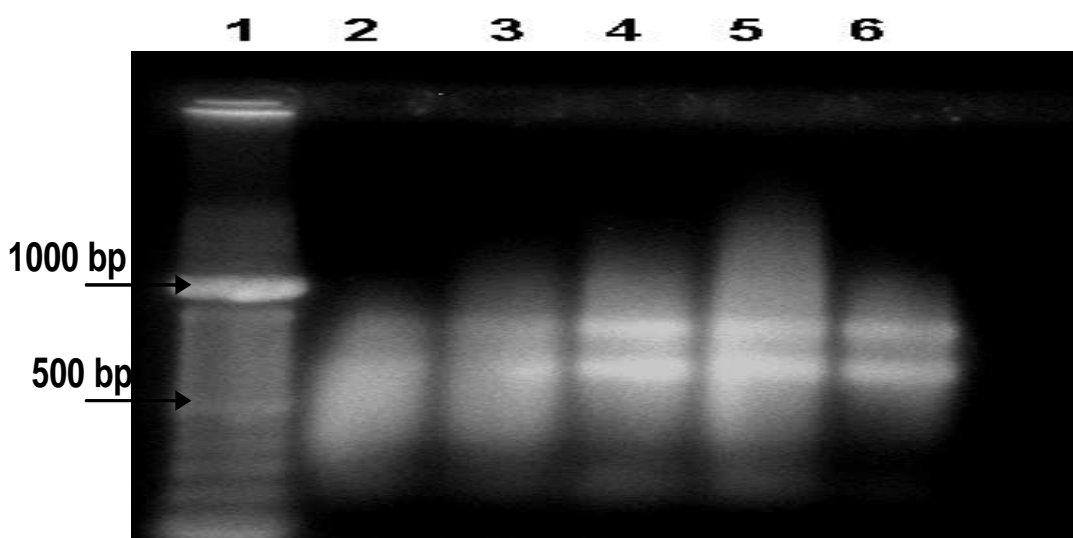
Fig 5.6: Gel electrophoresis showing extraction of mRNA. Lane 1: 100 bp molecular marker. Lane 2: isolate no. 197 (excluded due to degraded mRNA). Lane 3: isolate no. B31 (excluded due to degraded mRNA). Lane 4: isolate no. 187. Lane 5: isolate no. 195. Lane 7: isolate no. 215.

5.3.10. Incidence of class 1 and class 2 integrons

Results of class 1 integrons tested from 32 ESBLs-positive strains by PCR revealed that 9 isolates produce (~2kbp band), 11 isolates produce a 1kbp band and 6 isolates produce 2 bands (1.5kbp and 2kbp), 2 isolates were negative. Four isolates did not run by PCR. The sequencing of representative samples from each group show that the first group (~ 2 kb in size) were found to harbour two gene cassettes; dihydrofolate reductase and aminoglycoside adenyltransferase genes, *dfrA12* and *aadA2*, which confers resistance to trimethoprim and, streptomycin/spectinomycin; the second group (1.0 kb in size) was identified either with the *aadA2* or *aadA1* gene cassette; the third group (1.5kbp and 2.1 kbp in size) found to be dihydrofolate reductase gene, *dfrA12* and erythromycin esterase *ereA2* genes.

Class II integrons were identified in three isolates. These isolates were positive for class I integrons as well but none of these isolates were sent for sequencing.

The *intl1*, *sul1*, and *qacEΔ1* genes representing the class1 integrons were identified by PCR as shown in Table 5.1. Five isolates were negative for all of these genes, 4 isolates lacked *intl1* and harboured *sul1*, and *qacEΔ1* genes. One isolate carried *intl1* only while it was negative for class1 integrons primer (5-CS and 3-CS).

5.3.11. Isoelectric focusing (IEF) analysis

IEF of crude extracts from isolates revealed β-lactamases with pIs of 8.2 (SHV-12 and SHV-5) and 7.6 (SHV-1, SHV-11 and SHV-80). Twenty-four isolates produced enzymes with a pI of 5.4, which matched the TEM-1 β-lactamase. In addition, 16 isolates were found to express pI of 8.6 matching the CTX-M-15 β-lactamase. Some isolates produced other pIs bands.

5.3.12. Plasmid analysis

The plasmids analysis from ESBL *K. pneumoniae* strains carrying *bla*_SHV_ genes were assigned to be IncN and/or IncFII replicons. The IncN type was identified by PCR from 24 isolates, the IncFII type was amplified from 18 isolates from which 16 isolates were found to carry both IncFII and IncN replicons. Two isolates did not determine. In addition, 4 isolates were negative for any replicons type identified using the primers pairs targeting FIA, FIB, FIC, I2, I1, L/M, N, A/C, and FII replicons.

Both IncFII and IncN replicons types were identified by PCR from all strains carrying *bla*_SHV-12_ and *bla*_SHV-5_ genes except one isolate producing *bla*_SHV-12_ was assigned to be IncN plasmids only. Additionally, the strain carrying *bla*_SHV-80_ was found to harbour IncN plasmid replicon only.
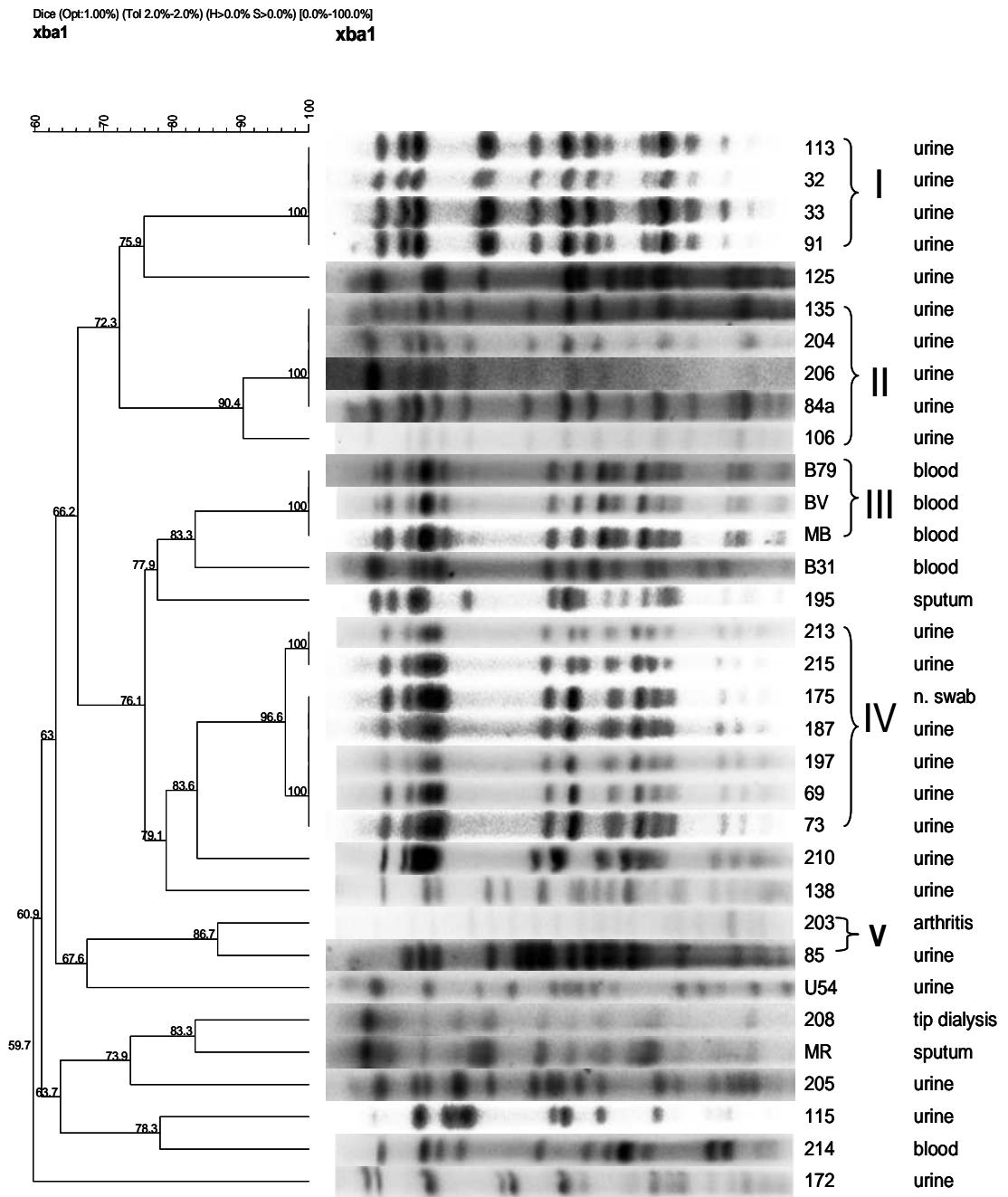
Fig 5.7: PFGE dendrogram showing the relationship between SHV-producing *K. pneumoniae* isolates obtained after *Xba1*-digested the chromosomal DNA. A band position tolerance of 2.0% was used in PFGE pattern analysis with the Dice band-based similarity coefficient. Strain numbers and source are shown on the right. Clones in clusters are highlighted.

5.3.13. PFGE

PFGE analysis was used to assess the clonality of 33 of the *K. pneumoniae* strains producing SHV and LEN β-lactamases. Results of PFGE revealed that 17 PFGE (> 85 similarity) profile types were identified. Five profile types, defined clusters I to V, included two or more isolates that were genetically related (with >85% similarity) (Fig 5.7). The largest cluster IV consisted of 7 strains producing mainly SHV associated with CTX-M-15, TEM and *qnrB1*. The second largest cluster II consisted of five strains, all expressed SHV, TEM and most (n = 4) co-expressed *qnrA1* genes. Clusters I and III contained 4 and 3 isolates obtained from urine and blood respectively, all of them characterized by production of CT-M-15 co-expressed with SHV. On the other hand, the isolate number 172 which carries *bla*$_{LEN}$ was unrelated to other isolates tested and identified in separate cluster. Moreover, the remaining isolates had PFGE profiles with a similarity of < 85% and were considered to be unrelated.

5.4. DISCUSSION

Detection of ESBL-producing *K. pneumoniae* can be difficult. The reasons include: the hyperproduction of non-ESBL β-lactamases, SHV-1 or SHV-11, due to high gene copy number or a single base pair change in promoter sequence, or modifications in outer membrane proteins co-existing with TEM-1, SHV-1 and SHV-11. These resulted in increased MICs and confer an ESBL similar phenotype causing false positive results (Podbielski, Schonling *et al.* 1991a; Rice, Carias *et al.* 2000; Wu, Siu *et al.* 2001).

Klebsiella pneumoniae produces species-specific class A chromosomal β-lactamases that confer resistance to ampicillin, amoxicillin, carbenicillin and ticarcillin. Three families of chromosomal β-lactamases, including SHV, LEN and OKP, have been identified in clinical *K. pneumoniae* isolates (Lee, Cho *et al.* 2006). The SHV-type

ESBLs were successful and widely distributed in *K. pneumoniae*. A survey from seven countries (South Africa, Argentina, Australia, Turkey, USA, Taiwan and Belgium) shown that SHV-type ESBLs were occurred in 49/73 of the isolates collected in 1996/7 from all of the countries, SHV-5 being the most common genotype (Paterson, Hujer *et al.* 2003).

K. pneumoniae isolates are expected to present an intrinsic resistance to ampicillin (Heritage, M'Zali *et al.* 1999), but only 90% (54/60) of tested strains in this study were positive by PCR for SHV genes. Similar resistance findings reported that 95% (178/187) of *K. pneumoniae* isolates collected from Portugal showed reduced susceptibility to ampicillin (Mendonca, Ferreira *et al.* 2009). In an Australian study, the bla_{SHV} was identified from all 21 *Klebsiella pneumoniae* isolates obtained between 1991 and 1995. Of these 13 strains were ESBL-positive (6 carried bla_{SHV-2a} gene and 7 carried the bla_{SHV-12} gene) and 8 strains were ESBL-negative (7 strains carried bla_{SHV-11} and one strain carried bla_{SHV-1} gene) (Howard, van Daal *et al.* 2002).

Consistent with previous results by Lee, Cho *et al.* (2006), the bla_{SHV-11} was the most prevalent gene described in this study in 21 isolates (17 isolates alone plus 4 isolates associated with bla_{SHV-12} and bla_{SHV-80}). The bla_{SHV-11} has been described most often in *K. pneumoniae* (Nuesch-Inderbinen, Kayser *et al.* 1997), and may be the ancestor of bla_{SHV-2a} and bla_{SHV-12} (Kim, Shin *et al.* 2002; Ford and Avison 2004). The non-ESBL phenotype conferred by SHV-11 shows that the Leu35Gln substitution between SHV-11 and SHV-1 has little or no significance with respect to hydrolysis of expanded-spectrum cephalosporins, and therefore its appearance is likely to be due to drift rather than antibiotic selection (Howard, van Daal *et al.* 2002).

Using direct sequencing, 3 isolates producing SHV-type β-lactamases showed mixed sequences from bla_{SHV-11} (Leu35→Gln), and bla_{SHV-12} (Leu35→Gln, Gly238→Ser, Glu240→Lys) and one isolate showed bla_{SHV-11} (Leu35→Gln) and bla_{SHV-80} (Leu35→Gln, Ala146→Thr). The possibility of multiple alleles in a single isolate has previously been reported previously. Lee, Cho *et al.* (2006) showed that *K. pneumoniae* strains carrying bla_{SHV-11} on the chromosome abundantly express

plasmid-derived SHV-12, concluding that bla_{SHV-11} was transferred from the chromosome by an IS26 originating from a bla_{SHV-12} element which showed a direct relationship between IS26 and bla_{SHV}. Furthermore, 4 isolates from 13 ESBL-positive strains were carried copies of a non-ESBL-encoding gene either bla_{SHV-1} or bla_{SHV-11} in addition to the bla_{SHV-2a} or bla_{SHV12} gene (Howard, van Daal et al. 2002).

The bla_{SHV-80} has substitution Ala146→Thr and showed unusual resistance to meropenem. The substitution of Ala146→Val was observed before in bla_{SHV-38} which considered the first example of an SHV-1 derivative that had reduced susceptibility to several expanded-spectrum cephalosporins and imipenem. The author indicated that the Ala146Val substitution is located at the N terminus of an alpha helix, neither in the β-lactam binding site nor in the catalytic site. It is parallel to the region from positions 161 to 164 of the omega loop structure known to play a key role in β-lactam hydrolysis at a distance of 3.1 Å. The amino acid Ala146 is oriented toward the solvent and may significantly disturb the conformation of the N-terminal part of the helix (Poirel, Heritier et al. 2003). On the other hand, the authors whose first described bla_{SHV-80} did not mention any resistance toward imipenem or meropenem (Mendonca, Ferreira et al. 2009).

Almost all SHV-derived ESBLs have a G→A mutation which specifies a glycine-to-serine substitution at amino acid 238. In this study the bla_{SHV-12} was detected in 8 strains (1 alone, 1 together with $qnrA1$, 3 together with $bla_{CTX-M-15}$ and bla_{TEM-1} genes and 3 together with bla_{TEM-1}). SHV-12 was first identified in 1997 in Switzerland (Nuesch-Inderbinen, Kayser et al. 1997). The bla_{SHV-12} evolved from the branch of bla_{SHV-2a} by evolutionary analyses (Ford and Avison 2004). The bla_{SHV-12} later reported from various continents and there has been a predominance of SHV-12 in south-east Asia (Lee, Cho et al. 2006), Spain (Diestra, Juan et al. 2009) and Italy (Carattoli, Miriagou et al. 2006).

The plasmid-encoded SHV-5 was first identified in *K. pneumoniae* in 1989. SHV-5 tends to hydrolyse most penicillins, narrow-spectrum and extended-spectrum cephalosporins, less on aztreonam and inhibited by β-lactamase inhibitors (clavulanic

acid, sulbactam, and tazobactam) (Gutmann, Ferre *et al.* 1989). In this study the SHV-5 was identified in one strain only. This result is in contrast to the previous studies until the late 1990s, European surveys of extended-spectrum β-lactamases (ESBLs) almost exclusively found TEM and SHV enzymes especially SHV-2 and SHV-5 which largely found in *Klebsiella* spp. (Livermore, Canton *et al.* 2007). Also, previous studies have demonstrated that SHV-5 is the most frequent ESBL in several enterobacterial species recovered from hospitalized patients in Mexico (Garza-Ramos, Davila *et al.* 2009).

My results are quite different from that was observed before in Scotland, in which SHV-2 and SHV-5 were detected in clinical isolates of *K. pneumoniae* collected at the Royal Infirmary of Edinburgh between May 1999 and March 2000 (Dashti, Paton *et al.* 2006). Moreover, 38 patients colonized with multiply-resistant ESBL bla_{SHV-2} producing *K. pneumoniae*, were discharged from hospital to 22 nursing or residential homes during a hospital-based outbreak, in the Grampian region, Aberdeen, Scotland (Bird, Browning *et al.* 1998). Another study linked between the *qnrA1* gene and bla_{SHV-5} from *K. pneumoniae* isolates collected in 2006 (Hamouda, Vali *et al.* 2008).

These findings are consistent with the English study where, the sequence analysis identified TEM-1, SHV-1/28, SHV-11, CTX-M-15 or CTX-M-33 variants among the *K. pneumoniae* isolates, and TEM-1, SHV-12 or KPC-4 in the *Enterobacter* spp. isolates from among clinical reference submissions received between April 2006 and March 2007 (Doumith, Ellington *et al.* 2009). In another study, 11 of 15 *qnrA*-positive isolates, including single isolates of *E. coli* and *C. freundii*, 4 *K. pneumoniae* and 9 *E. cloacae* recovered during 2003-2005 in Liverpool were carried class 1 integrons and expressed the ESBL bla_{SHV-12} (Corkill, Anson *et al.* 2005).

On the other hand, the results show dissimilarity with the another English study, where SHV-2 (n=4), SHV-5 (n=3), and SHV-4 associated with TEM-15, and TEM-26 (n=1) were identified from 10 distinct ESBL-producing strains of *K. pneumoniae* that caused hospital outbreaks in England from 1991 to 1994 (Shannon, Stapleton *et al.* 1998).

It is well-known that SHV ESBLs are plasmid associated in *K. pneumoniae* because an ESBL-conferring mutation in the poorly expressed chromosomal gene will not confer a phenotype (Hammond, Harris *et al.* 2008; Turner, Andersson *et al.* 2009). The conjugation results showed that the majority (29/32) of *K. pneumoniae* isolates harbour transferable bla_{SHV}. These results highlight that most of these genes were plasmids associated and characterized by high transferability. These findings enhance the possibility of mutational changes and production of ESBL phenotypes. In addition, the strain 195 which carry bla_{SHV-11} and contain the large SHV transposon was failed to conjugate with the recipient strain. This result indicates a chromosomal location which agrees with other findings that the large SHV transposon is chromosomal location (Turner, Andersson *et al.* 2009). Furthermore, *K. pneumoniae* strain 208 possessed bla_{SHV-12} could not be transferred by conjugation indicating a nonconjugative plasmid location.

Dissemination of SHV ESBLs may be largely mediated by plasmids or insertion sequences. The prevalence of *K. pneumoniae* isolates with more than one β-lactamase has increased in recent years because high mobilization of ESBLs genes from genome to plasmid mediated by insertion sequences especially IS*26* (Ford and Avison 2004). The close association of insertion sequences with antibiotic resistance genes strongly suggests an active role for these sequences in the evolution or dissemination of antibiotic resistance genes (Kim, Shin *et al.* 2002). IS*26* was found associated with a class 1 integrons which considered as a critical step in the evolution of diverse multiresistance plasmids found in clinical enterobacteria (Miriagou, Carattoli *et al.* 2005). IS*26* generates 8 bp target duplication upon transposition (Mollet, Iida *et al.* 1983). IS*26* has been associated with several antibiotic resistance genes, including *aphA1* in Tn*2680*, a *blaT–aac5* operon in plasmid pUZ3644, an IAB operon in plasmid pBWH77, *dhfrVIII* in plasmid pLMO226 and *dfr13*, *aadA4*, bla_{TEM-1} and *sul2* gene in plasmid pUK2381 (Kim, Shin *et al.* 2002).

All isolates but one were found to carry the small SHV transposon. IS*26* insertion in this study was identified in the bla_{SHV} promoter with 14bp target duplication. It is

known that the small SHV transposon has a strong promoter that was generated by insertion of IS*26* into the *bla*SHV promoter region through the introduction of a different –35 region (Kim, Shin *et al.* 2002; Turner, Andersson *et al.* 2009). The previous findings reported that the IS*26* sequence was inserted immediately upstream of *bla*SHV-11, *bla*SHV-2a and *bla*SHV-12 genes generates the hybrid promoter consisting of the –35 region derived from IS*26* and the –10 region from the *bla*SHV promoter itself. The IS*26*-insertion generated a hybrid promoter in which the TTGTGA –35 region of the *bla*SHV-12 promoter was replaced by the –35 sequence TTGCAA provided by the left inverted repeat of IS*26*. This hybrid produced an efficient promoter and increased β-lactam resistance (Podbielski, Schonling *et al.* 1991).

In this study by PCR the insertion sequence IS*26* couldn't amplify associated with *bla*SHV-5 gene. The similar findings indicated that copies of IS*26* are present, but not immediately adjacent to the region of homology with pACM1 carrying *bla*SHV-5 gene (Preston, Venezia *et al.* 2004). In contrast to this result, IS*26* insertion was found 2 kbp upstream in the plasmid-mediated SHV-5 enzyme (Gutmann, Ferre *et al.* 1989).

The large SHV transposon-borne promoters (IS26-lacY-recF-SHV) were amplified from only one isolate harboured non-transferable *bla*SHV. The sequence from the large SHV transposon-containing isolate was identical to the *bla*SHV promoter sequence reported by (Rice, Carias *et al.* 2000). This promoter has enhanced activity, due to a C→A mutation in the –10 region (Turner, Andersson *et al.* 2009).

Seven of the 8 *bla*SHV-12 genes were located in IncN and IncFII plasmids but one isolate was assigned to be IncN plasmids. On the other hand, it was not possible to determine the incompatibility group of the other one with the set of primers used. These results are in agreement with another study that correlated *bla*SHV-12 with a diversity of IncI1, IncK, IncFII and IncH12 (Diestra, Juan *et al.* 2009). Whereas other study linked *bla*SHV-12 with IncFII, IncI1 and IncA/C plasmids (Carattoli, Miriagou *et al.* 2006).

The plasmids carrying bla_{SHV-5} gene were assigned to be IncFII and IncN replicons types. Similar to these results, the bla_{SHV-5} gene was located in IncFII plasmid and its environment was surrounded by a gene encoding a putative RecF protein upstream and the gene coding the putative DEOR transcriptional regulator downstream (Diestra, Juan *et al.* 2009). Moreover, the plasmid p1658/97 carrying bla_{SHV-5} gene was found to bears two replication systems, IncFII and IncFIB (Zienkiewicz, Kern-Zdanowicz *et al.* 2007). In 2004, bla_{SHV-5} was identified on a compound transposon flanked by IS*26* insertion sequences in the pACM1 plasmid from *K. oxytoca* obtained from an outbreak in 1993 in USA (Preston, Venezia *et al.* 2004). Subsequently, the compound transposon was found on *Salmonella enterica* pSEM plasmid isolated from October 2000 to February 2001 from infants with gastroenteritis in Romania (Miriagou, Filip *et al.* 2002). Also, bla_{SHV-5} was identified on a compound transposon in p1658/97 plasmid from a clonal *E. coli* outbreak in a Warsaw, Poland, hospital in 1997 (Zienkiewicz, Kern-Zdanowicz *et al.* 2007).

Integrons play an important role in the dissemination of antimicrobial resistance through horizontal transmission. The integrons platform is composed of a gene (*intI*) encoding an integrase (that catalyzes the gene cassette movement by site-specific recombination), a recombination site (*attI1*), and a functional promoter (Pc) that directs transcription of the inserted gene cassettes. Gene cassettes are small mobile units composed of one coding sequence and a recombination site, *attC*. Integrons exchange gene cassettes through integrase-catalyzed site-specific recombination between *attI* and *attC* sites, resulting in the insertion of the gene cassette at the *attI* site, or between two *attC* sites, leading to the excision of the gene cassette(s) from the gene cassette array. To date, more than 130 gene cassettes have been described, conferring resistance to almost all antibiotic classes. Integrons are divided into two major groups: the resistant integrons (RI) and the super-integrons (SI). At present, five classes of resistant integrons are known to have a role in the dissemination of antibiotic-resistance genes. Class 1 integrons is the commonest; this is followed by class 2, whereas class 3 is rare. Class 4 and class 5, have been identified through their involvement in the development of trimethoprim resistance in *Vibrio* species. The superintegrons have been identified in a distinctive class of integrons in the

Vibrionaceae and in a branch of the pseudomonads genome and is not known to be associated with antibiotic resistance (Machado, Canton *et al.* 2005; Jove, Da Re *et al.* 2010).

In the present study I observed a high frequency of occurrence of integrons among ESBL-positive *K pneumoniae*. Results of class 1 integrons tested from 28 ESBLs-positive strains by PCR revealed that 26/28 (93%) were positive, 2 (7%) isolates were negative. Three isolates (11%) were found positive for class 2 integrons; these isolates were found positive for class 1 integrons as well.

The corresponding findings reported that Class 1 integrons were more frequently found (67%) among *E. coli* producing-ESBL collected over 12 years in a single hospital in Madrid, Spain (Machado, Canton *et al.* 2005). Moreover, the presence of integrons was demonstrated in 29/100 (29%) of *E. coli* isolates from healthy humans in Spain. The *intI1* gene was identified in 26 of these isolates, the *intI2* gene in 1 isolate and both the *intI1* and *intI2* genes in 2 additional isolates (Vinue, Saenz *et al.* 2008). In another study, class 1 integrons were present in 78 isolates (34.2%) in clinical *K. pneumoniae* isolates collected from Taiwan (Chang, Fang *et al.* 2009).

The *dfrA12 + aadA2*, *aadA2*, *aadA1* and *dfrA12 + ereA2* gene cassettes have been identified by sequencing within the variable region of class 1 integrons in *K. pneumoniae* isolates collected, which confer resistance to trimethoprim, streptomycin/spectinomycin and erythromycin respectively.

To our knowledge, this the first to report the presence of arrangement of *dfrA12 - ereA2* gene cassette identified within *K. pneumoniae* isolates.

Previous reports identified *dfrA1 + aadA1*, *aadA*, *dfrA17 + aadA5*, *dfrA7*, *dfrA5*, *dfrA1* and *dfrA12 + orfF + aadA2* gene cassettes. Most of them corresponded to different variants of *dfrA* and *aadA* genes (43% and 54%, respectively). The author also concluded that *dfrA1 + aadA1* was the combination most frequently detected not only in his study but also in *E. coli* isolates recovered from healthy and sick humans,

animals and foods in other studies (Vinue, Saenz *et al.* 2008). Furthermore, (*aadA1*), (*dfrA12-orfF-aadA2*), (*dfrA1-aadA1*), (*dfrA16-aadA2*), (*aadA2*) were identified in another spanish investigations (Machado, Canton *et al.* 2005). In addition, 26 different gene cassettes were identified from a study in Taiwan including resistance genes to trimethoprim (*dfr1*, *5*, *7*, *12*, *17*), erythromycin (*ereA2*), chloramphenicol (*cmlA*, *catB3*, *catB8*), ampicillin (*bla*$_{OXA-30}$), carbenicillin (*bla*$_{CARB-8}$), rifampin (*arr3*), and aminoglycosides (*aadA1*, *2*, *5* for streptomycin, *aadB* for gentamicin, and *aac(6')-Ib*, *aac(6')-Ib-cr*, *aac(6')-Il*, *aac(6')-II*, *aac(6')-IIc* for aminoglycoside-6'-*N*-acetyltransferase) with different arrangements. A class 1 integron carrying the *dfr12–orfF–aadA2* cassette array was the most frequently found (Chang, Fang *et al.* 2009).

5.5. CONCLUSION

The data, showing the emergence and spread of new SHV variants in *K. pneumoniae*, is at variance with what has been found before in Scotland. The *bla*$_{SHV-80}$ detected in this study may be considered of particular interest. In agreement with others, the presence of the IS*26* element upstream of *bla*$_{SHV}$ genes described in this study strongly suggests an active role for these sequences in the evolution and dissemination of antibiotic resistance genes. Moreover, population structure analysis has revealed the presence of highly transfer broad-host-range plasmids, IncN, harbouring the ESBL β-lactamases genes which able to disseminate within different clones. Finally, class 1 integrons are highly frequent among ESBL-producing *K. pneumoniae* clinical isolates. However, they encode resistances that currently affect older antibiotics thus they do not have a significant contribution towards ESBL dissemination.

CHAPTER- 6:

Evolution and spread of parental SHV-1 and SHV-11 enzymes

6.1. ABSTRACT

Antimicrobial resistance genes in pathogenic bacteria are considered the most rapidly evolving DNA sequences. The diversity of bla_{SHV-1} and bla_{SHV-11} genes based on the presence or absence of the non-synonymous mutation T92A was investigated. A total of 88 bla_{SHV-1} and bla_{SHV-11} genes was used in this study. Among the bla_{SHV} genes studied, 47 were identified as bla_{SHV-11} and 41 were bla_{SHV-11} gene. Synonymous nucleotide mutations A402G, G705A, C786G and C324T were the most frequent. A new nucleotide substitution G846A was observed within 3 isolates sequenced in this study.

6.2. INTRODUCTION

The SHV enzymes are distributed worldwide. SHV-1 is the most prevalent β-lactamase in *K. pneumoniae*, but it is also present as a plasmid-borne gene in various other species (Livermore 1995; Bradford 2001). Non-synonymous nucleotide substitutions of the parental bla_{SHV-1} gene led to the emergence of ESBL enzymes, which are responsible for higher levels of resistance to extended-spectrum cephalosporins (Paterson and Bonomo 2005). The bla_{SHV-1} and bla_{SHV-11} genes differ by one synonymous nucleotide substitutions T92A coding for non-synonymous mutation corresponding to the Leu35Gln substitution. This substitution are found in many bla_{SHV} genes (Nuesch-Inderbinen, Kayser *et al.* 1997). In the SHV family, ESBL activity is most frequently associated with a Gly238Ser substitution, and activity is frequently increased by a Glu240Lys substitution. The TEM situation is much more complex and less frequently involves Gly238 (Bradford 2001; Hammond, Harris *et al.* 2008). Until now, 119 SHV enzymes have been identified but several of them do not code for ESBL enzymes (http://www.lahey.org/studies/).

The aim of this study was to analyse the diversity of nucleotide sequences of 88 SHV-encoding genes. The analysis of this diversity allowed predicting the emergence of new resistant variants.

6.3. RESULTS AND DISCUSSION

β-lactamases offer one of the best examples of protein diversification and evolution as a mechanism of rapid adaptation of bacterial populations to diverse environments (Gniadkowski 2008). Nucleotide sequence analysis showed that CTX-1 enzyme (conferred resistance to cefotaxime) had arisen by the accumulation of point mutations in the gene encoding a TEM β-lactamase and consequently, CTX-1 is now named TEM-3. Similarly, SHV-1 has also been called PIT-2 (Heritage, M'Zali *et al.* 1999).

Here I didn't study evolution of all bla_{SHV} family but described the mutations only within SHV-1 and SHV-11 variants. A total of 88 bla_{SHV} genes was used in this study were 14 (12 SHV-11 and 2 SHV-1) sequenced from our collection and 74 downloaded manually from NCBI GenBank database (Table 6.1) which designated either SHV-1 or SHV-11. All these isolates do not confer resistance to broad-spectrum cephalosporins. The bla_{SHV-1} gene sequence, as found in the GenBank database (accession number AF148850), was used as reference for the development of the framework classification. A nucleotide sequence of 861 bp from each bla_{SHV} gene analysed in this study (beginning at nucleotide 1 of the coding region, according to standard nucleotide numbering. Nucleotides sequences of whole gene were found in all isolates but 4 isolates. All sequences were aligned without insertion-deletion events, and there were no missing or ambiguous data with Multi-alignment online website Multalin (http://www.toulouse.inra.fr/multalin.html). The nucleotide substitutions were calculated manually.

The previous reports classified the bla_{SHV} gene family based on the following principle: for gene $bla_{SHV-1-X-1v}$ or $_{-11v}$, X is the gene number that has already been attributed to the bla_{SHV} gene and corresponding enzyme; 1 or 11 indicates that this gene derived from either bla_{SHV-1} or bla_{SHV-11} parental genes; the number attributed to "v" indicates the framework variant according to the order of appearance in GenBank and literature. Ford & Avison (2004), named different gene sequence frameworks "v1" to "v4" following the name of the bla_{SHV} gene (ex: $bla_{SHV-1v4}$).

Mendonca, Nicolas-Chanoine *et al.* (2009) identified 83 different *bla*SHV gene sequence frameworks from (1 to 83). These frameworks resulted from the combination of both synonymous nucleotide mutations at 39 different nucleotide positions and the T92A non-synonymous mutation.

Among the 88 *bla*SHV gene sequences tested, 47 (53.4%) were *bla*SHV-1 gene, characterized by a T residue at position 92 resulting in amino acid Leu at position 35, 41 (46.6%) were *bla*SHV-11 gene with the non-synonymous mutation T92A encoding amino acid substitution Leu35Gln. Similar to these results, Mendonca, Nicolas-Chanoine *et al.* (2009) identified 45 *bla*SHV gene sequence frameworks were based on the parental gene *bla*SHV-1, 38 were based on the *bla*SHV-11 gene. On the other hand, (Lee, Cho *et al.* 2006) reported that 62% *bla*SHV-11 genes and 35% *bla*SHV-1 among 142 *bla*SHV genes studied.

Alignment of all nucleotide sequences obtained showed 19 polymorphic sites dose not implied amino acid changes. One nucleotide substitution observed occurred at sites G846A among 3 *K. pneumoniae* isolates sequenced in this study. Synonymous nucleotide mutations A402G (91%), G705A (57%), C786G (62.5%) and C324T (40%) were the most frequent observed in this study. The corresponding figures indicated that the appearance of these mutations were A402G (90%), G705A (60%) and C786G (66%) (Mendonca, Nicolas-Chanoine *et al.* 2009). In addition, (Nuesch-Inderbinen, Kayser *et al.* 1997) reported that 59% of 34 *bla*SHV genes had synonymous mutation A402G and 9% G705A.

6.4. CONCLUSION

The study of genes evolution and evolutionary forces acting on the genetic diversification can provide the ability to predict the emergence of new resistant variants. They also provide the opportunity for continuing basic scientific research to a future understanding of the evolution of *bla*SHV family.

Accession number	Nucleotide number																		
	31	92	156	324	357	402	505	516	615	633	705	720	741	759	762	765	786	795	846
AF148850	C	T	G	C	C	A	C	C	G	G	G	G	G	C	T	G	C	C	G
Nucleotide changed	T	A	C	T	T	G	T	T	A	A	A	C	A	G	C	A	G	T	A
Time of change	1	47	1	35	22	80	1	3	1	8	50	10	1	4	18	1	55	16	3
Percent of change	1.1%	53.4%	1.1%	40%	25%	91%	1.1%	3.4%	1.1%	9%	57%	11.4%	1.1%	4.5%	20.5%	1.1%	62.5%	18%	3.4%

Table 6.1: Polymorphic nucleotides site mutations and percentage of these mutations within parental bla_{SHV-1} and $bla_{SHV-11.}$ The accession numbers used in this study are: AF148850, AF124984, FJ668818, FJ668813, FJ668802, DQ478720, GU083598, GQ389703, DQ219472, AY826416.1, FJ668814, FJ668811, FJ668810, FJ668809, GQ389701, EU418908, X98099, EU280315, X98100, Y18299, FJ483937, DQ219477, X98098, GU083599, AY528717, EF035561, EF035557, GQ389706, EU418909, DQ219473, AF462396,GQ387358, DQ219475, DQ219476, DQ219474, EF035567, HM363638, EU376965, AY293069, EF035562, EF035560, EF035563, FJ668804, EF035564, FJ668800, FJ668799, EF035558, GQ463148, GQ463147, DQ166780, DQ166779, GQ389707, GQ389705,DQ166782, GQ389704, GQ389702, GU211012, EF035565, DQ219478, GU197545, AF187732, AF117743, GU197549, GU197543, GU197542, GU197544, GU197546, GU197548, GU197547, HM590432, GQ470430, GQ470428. The isolates numbers B31, 206, 91, 205, 204, 215, 69, 210, 213, BV, 73, 195, MB. 115, 138, 85 were sequenced and used from this study.

CHAPTER - 7:

A new LEN β-lactamase produced by a clinical *K. pneumoniae* strain

7.1. ABSTRACT

A new bla_{LEN-25} from the *K. pneumoniae* (KpIII) phylogenetic group was identified in this study. This gene was located in a transferable plasmid bearing class 1 integrons.

7.2. INTRODUCTION

LEN-1 is a chromosomally encoded β-lactamase found in many strains of *K. pneumoniae*. Nucleotide sequence analysis shows that the gene encoding LEN-1 is very closely related to the nucleotide sequence of the bla_{SHV-1} and OHIO-1 genes (Heritage, M'Zali *et al.* 1999). *K. pneumoniae* strains have been classified into KpI, KpII and KpIII clusters according to the intrinsic β-lactamases they express. More than 80% of strains belong to group KpI, which express SHV enzymes, groups KpII and KpIII each account for 10% of the population and express OKP and LEN enzymes, respectively (Brisse and Verhoef 2001; Haeggman, Lofdahl *et al.* 2004). LEN-1 may confer resistance to ampicillin, amoxicillin, carbenicillin, and ticarcillin, but not to extended-spectrum β-lactams. In addition, LEN enzymes have been detected worldwide, but only in sporadic cases (Haeggman, Lofdahl *et al.* 2004).

7.3. RESULTS

7.3.1. PCR and DNA sequencing of β-lactamase gene

The bla_{LEN} gene was identified and sequenced with bla_{SHV} primers (Kp-SHV_for and Kp-SHV_rev) allowing the complete sequence of the bla_{LEN} gene to be determined, as described earlier in section 5.3.1 (Chapter-5).

A new bla_{LEN} variant was detected in ESBL-negative strain 172. This β-lactamase contained 286 amino acids and shared 99.3% identity with LEN-2 (GenBank

AY037780). It showed 6 nucleotides coding for 3 amino acid differences in comparison to bla_{LEN-2} (Val84→Leu, Thr114→Ala and Ile257→Leu), according to the standard numbering scheme of (Ambler, Coulson *et al.* 1991). The substitutions Thr114→Ala was firstly described in this study. Threonine in position 114 does not shown significant influence to the resistance to third-generation cephalosporins. The deduced enzyme was designated LEN-25 according the nomenclature proposed in http://www.pasteur.fr/recherche/genopole/PF8/betalact_en.html.

None of other transferable class A β-lactamases, such as the TEM and CTX-M genes, were identified from this strain. This is similar to other reports where individual isolates did not co-express SHV and LEN enzymes (Haeggman, Lofdahl *et al.* 2004).

7.3.2. Susceptibility testing

As shown in Table 9.1 (Page 173), the antimicrobial susceptibility of the strain harbouring LEN-25 presented lower resistance values to cefotaxime (0.03 mg/L), ceftazidime (0.25 mg/L) and ceftriaxone (0.03 mg/L) keeping this type of β-lactamases remained susceptible to all cephalosporins. These characteristics are typical of LEN-β-lactamases (Bush, Jacoby *et al.* 1995). No synergy with ceftazidime and the β-lactamase inhibitor was detected, indicating that this strain was a non-ESBL producer. Additionally, this isolate was sensitive for meropenem (MIC = 0.015 mg/L) and cefoxitin (MIC = 2 mg/L).

Interestingly, high MICs of ciprofloxacin (4 mg/L) and nalidixic acid (32 mg/L) to the LEN-25 β-lactamase isolate were observed. By PCR this isolate was found to carry *qnrB6* gene. The presence of the *qnr* gene increased the MICs of nalidixic acid and fluoroquinolones by four- to eightfold (Martinez-Martinez, Pascual *et al.* 1998; Mammeri, Van De Loo *et al.* 2005). Other Quinolone resistance mechanisms such as loss of outer membrane permeability, mutations of *gyrA* and *parC* genes, efflux pumps were not investigated in this study.

7.3.3. Gene transfer

It is known that LEN-type β-lactamases are chromosome-encoded enzymes. In comparison with chromosome-encoded β-lactamases, plasmid-encoded β-lactamases are highly reproducible and can transfer resistance more easily. Transference of β-lactam resistance gene was tested as previously described in the Material and Methods chapter. The bla_{LEN-25} carrying isolate was successfully transconjugated to the recipient strain, which implies that the gene encoding for this β-lactamase is located on a transferable plasmid. Consistent with my result Chen, Zhang *et al.* (2005) identified the first LEN-5 encoded by a plasmid of more than 48 kbp co-expressed with $bla_{CTX-M-14}$ gene in a clinical isolate of *K. pneumoniae* from China.

7.3.4. Characterization of *bla*$_{LEN}$

The strain positive for chromosomal bla_{LEN} gene was belonged to the KpIII-type. This result was in agreement with the previous findings that strains belongiong to the KpIII type harbour and express the LEN enzymes (Brisse and Verhoef 2001; Haeggman, Lofdahl *et al.* 2004).

The plasmid carrying bla_{LEN-25} gene was designated to the IncN replicons type. Unfortunately the size of plasmid was not determined in this work. Furthermore, this isolate was positive for class 1 integrons and found to carry *Sul1* and *qacEΔ1* genes in the 3 conserved sequence.

Using PFGE, this isolate was unrelated to other isolates tested and identified in separate cluster (Fig 5.7).

7.3.5. Nucleotide sequence accession number

The new bla_{LEN-25} sequence was submitted to the GeneBank Sequence Database under the accession number HQ709169.

Fig 7.1: Amino acid difference between *bla*LEN-2 and isolate 172 (LEN-25)
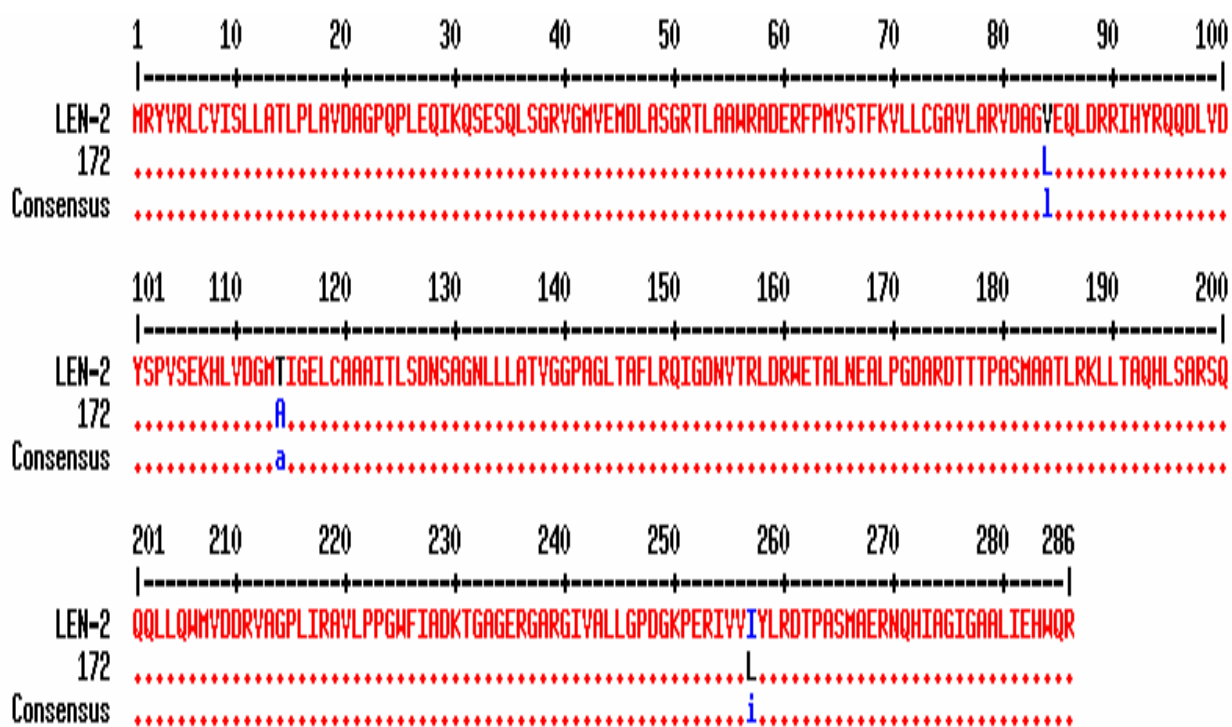
7.4. CONCLUSION

The new *bla*LEN-25 enzyme showed 3 amino acid differences in comparison to LEN-2, neither of these changed the enzyme to be ESBL. This result is the second to report that *bla*LEN gene was carried in transferable plasmid suggesting more complications of resistance in future.

CHAPTER- 8:

First report of a novel extended-spectrum beta-lactamase KOXY-2 producing *Klebsiella oxytoca* that hydrolysis cefotaxime and ceftazidime

8.1. ABSTRACT

Objectives: The aim of this study was to study the broad-spectrum resistance of the bla_{OXY-2} producing *Klebsiella oxytoca*.

Methods: *K. oxytoca* strains MU946294N and MB193997E were isolated from urine and blood specimens from patients in Scotland. The isolates were tested for antimicrobial susceptibility by the agar double dilution method. The identification of the strains was by the nucleotide sequences of the *rpoB* and *gyrA* genes. The strains were genotyped by pulsed-field gel electrophoresis (PFGE) to determine their clonal relationship.

Results: Strain MU946294N was resistant to pencillins, monobactams, cephalosporins including cefotaxime and ceftazidime but was not inhibited by clavulanic acid. Isolate MB193997E displayed a β-lactam resistance phenotype consistent with chromosomal β-lactamase overproduction. No common transferable β-lactamase genes, for TEM, SHV, and CTX-M, could be amplified in either strain. However, positive amplification by PCR was found with primers for the bla_{OXY-2} gene. Sequencing of this β-lactamase gene in MU946294N revealed it differed by one mutation from the all other bla_{OXY} genes previously reported, with an amino acid substitution Ala237→Thr, which enhances the binding of cefotaxime. Strain MB193997E showed mutations at positions 255 and 283, neither of which affect the function. Based on *rpoB* and *gyrA* characterization, both strains were assigned to be KoII phylogenic group. However, the two isolates were completely dissimilar from each other by PFGE.

Conclusions: The present study is the first to report the substitution of alanine to threonine at position 237 in the OXY-2 β-lactamase and this enhances resistance to the extended-spectrum β-lactam cefotaxime.

8.2. INTRODUCTION

Klebsiella oxytoca is an important opportunistic pathogen causing serious infections especially among neonates and intensive care units (Podschun and Ullmann 1998; Fevre, Jbel *et al.* 2005). The K1 β-lactamase, first identified from a sick child

infected with *Klebsiella aerogenes* (*Klebsiella pneumoniae*) 1082E in Glasgow, Scotland. In 1986, this strain was renamed *Klebsiella oxytoca* SC10,436 based on changes in *Klebsiella* nomenclature (Arakawa, Ohta *et al.* 1989; Granier, Leflon-Guibout *et al.* 2002).

The chromosome of wild-type *K. oxytoca* carries a β-lactamase gene, which is constitutively expressed at low levels and usually confers low-level resistance to amino- and carboxy-penicillins (Livermore 1995; Fournier and Roy 1997). In addition, the overproduction of the chromosomally-encoded β-lactamase is due to up-mutations in the promoter sequence of the genes, *K. oxytoca* isolates have been found to be resistant to broad-spectrum cephalosporins (cefotaxime and ceftriaxone) and monobactams (Fournier, Arlet *et al.* 1994; Fournier, Lu *et al.* 1995; Wu, Dornbusch *et al.* 1999). The *in vivo* selection of *K. oxytoca* strain conferring resistance to ceftazidime due to Proline167Serine substitution was recently reported (Mammeri, Poirel *et al.* 2003).

The DNA sequence of bla_{OXY} of *K. oxytoca* differs from those of bla_{TEM} and bla_{SHV} by more than 50% and is completely unrelated to bla_{AmpC} (Arakawa, Ohta *et al.* 1989). (Fournier, Roy *et al.* 1996) divided the β-lactamases of *K. oxytoca* into two main groups: bla_{OXY-1} and bla_{OXY-2}. These two genes share 87% nucleotide sequence identity. Four other groups of *K. oxytoca* genes have recently been reported and named; bla_{OXY-3}, bla_{OXY-4} (Granier, Leflon-Guibout *et al.* 2003), bla_{OXY-5} and bla_{OXY-6} (Fevre, Jbel *et al.* 2005). These β-lactamases have been placed in functional group 2be in Bush's scheme and in class A of Ambler's classification (Ambler, Coulson *et al.* 1991; Bush, Jacoby *et al.* 1995).

8.3. RESULTS

8.3.1. Bacterial strains

Strains MU946294N and MB193997E, identified by routine testing (API System; bioMerieux, France) as *K. oxytoca* were obtained, both in 2007, from two patients

hospitalized in the Royal Infirmary of Edinburgh, Scotland. Strain MU946294N was obtained from a patient with a urinary tract infection and strain MB193997E was isolated from blood of encephalopathy patient who had been treated with meropenem for 7 days and then improved after the medication was changed to ciprofloxacin.

8.3.2. Antimicrobial susceptibility

As shown in Table 8.1, the strain MU946294N showed an unusual resistance phenotype characterized by resistance to cefotaxime, and ceftazidime. This strain also expressed high levels of resistance to amoxicillin, piperacillin, cephalexin, cefuroxime, and naladixic acid, moderate resistance to aztreonam, ceftriaxone and cefoxitin and was susceptible to meropenem.

On the other hand, isolate MB193997E was resistant to amoxicillin, piperacillin, cephalexin, cefuroxime, ceftriaxone, sensitive to nalidixic acid, ceftazidime, meropenem and was border-line resistant to cefotaxime (0.5 mg/L) by MIC, and (28 ± 1 mm) by disc diffusion test (BSAC guideline: 20 mm). Both strains were previously reported resistant to cefotaxime by the Vitek 2 system.

Results obtained from cloned strains without promoters shown that the two strains revealed identical phenotypic resistance to amoxicillin, piperacillin, ciprofloxacin, ceftriaxone, aztreonam and cefoxitin. The strain MU946294N conferred ceftazidime insuceptibility 2-fold higher than strain MB193997E, cefotaxime resistance was found 4 to 16 fold higher in strain MU946294N than that observed in strain MB193997E.

The mutation in the promoter confers at least a 4 to 9 fold increase in MIC (Fournier, Gravel *et al.* 1999); however, it proved impossible to clone the gene with its promoter.

The synergy between cefotaxime, ceftazidime and clavulanate was observed in both strains as seen in Fig. 8.1.

8.3.3. PCR and Nucleotide sequencing

DNA amplification with a crude extract of *K. oxytoca* as the template was performed by PCR with consensus primers OXY-F (ATG ATA AAA AGT TCG TGG C) and OXY-R (TTA AAG CCC TTC GGT CAC) which amplified the entire bla_{OXY-2} gene based on the alignment of previously published nucleotide sequence of the bla_{OXY-2} genes. PCR conditions were as follows: 5 min at 94°C, 30 cycles of 30 sec at 95°C, 45 sec at 50°C, and 1 min at 72°C, and final extension of 7 min at 72°C. The sequence of entire coding region were amplified by PCR using the combination of primers OXY-R primer and A1- (GAA CAT AGC GGC TCC TTA T) at position (145 to 163) according the sequence previously published (Fournier, Roy *et al.* 1996).

As indicated in Fig. 8.2, the nucleotide sequencing of *K. oxytoca* strain MU946294N revealed a bla_{OXY-2} gene which differed by one mutation from the other all bla_{OXY} genes reported previously, with an amino acid substitution Alanine, Serine or Glycine to Threonine at position 237 following the numbering scheme for class A β-lactamases. This mutation has produced the first cefotaxime-resistant OXY-2 β-lactamase producing *K. oxytoca* enzyme.

There were two mutations in strain MB193997E; Aspartic acid to Asparagine at position 255, and Alanine to Threonine at position 283. Neither mutation appeared to affect the phenotype resistance of the strain.

The deduced enzymes were designated OXY-2-9 form isolate MU946294N and OXY-2-10 from isolate MB193997E according the nomenclature proposed in http://www.pasteur.fr/recherche/genopole/PF8/betalact_en.html.

8.3.4. Association with other β-lactamases

None of transferable β-lactamases (CTX-M, TEM, SHV) enzymes and plasmid-mediated quinolone (*qnr*) genes were amplified in either strain.

Table 8.1: Antimicrobial susceptibility of parent and cloned *K. oxytoca* strains

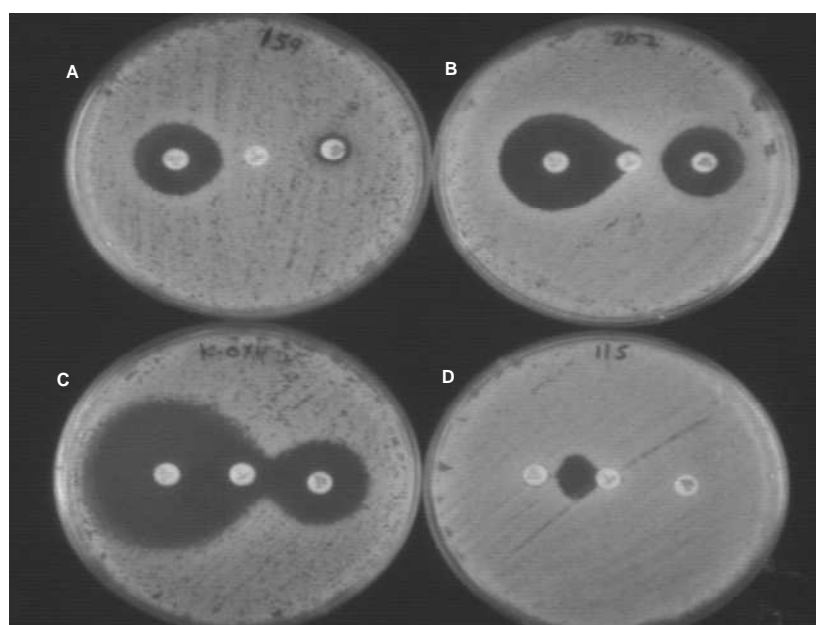| β-lactam	MIC (mg/L)			
	$bla_{OXY-2-9}$	$bla_{OXY-2-10}$	Cloned $bla_{OXY-2-9}$ without promoter	Cloned $bla_{OXY-2-10}$ without promoter
Amoxicillin	>256	>256	>128	>128
Piperacillin	>256	>256	>128	>128
Nalidixic acid	>128	4	-	-
Ciprofloxacin	0.5	0.015	0.03	0.03
Ceftriaxone	64	4	0.12 – 0.25	0.12- 0.25
Cefotaxime	8	0.5	0.12 - 1	0.03 – 0.06
Ceftazidime	4	0.25	0.5 - 1	≤ 0.5
Aztreonam	32	128	0.5 - 1	0.5 - 1
Cefuroxime	>128	>128	-	-
Cephalexin	>128	32	-	-
Meropenem	0.03	0.03	-	-
Cefoxitin	16	2	4	4

Fig 8.1: synergy disc diffusion test. A; isolate no. MU946294N. B: isolate no. MB193997E. C: *K. oxytoca* standard sensitive strain. D: *K. pneumoniae* resistant strain no. 115. Ceftazidime, amoxiclav and cefotaxime antibiotic discs are arranged from left to right respectively in all plates.

```
MU946294  MIKSSWRKIAMLAA-VPLLLASGALWASTDAIHQKLTDLEKRSGGRLGVALINTADNSQILYRGDERFAMCST
MB193997  --------------A-------------------------------------------------------
SG77      ----------------------------------------------------------------------
KH11      ----------------------------------------------------------------------
SG43      ----------------------------------------------------------------------
SB136     --------------A-------------------------------------------------------
SB175T    --------------A-------------------------------------------------------
SL911     --------------A-------------------------------------------------------
SG176     --------------A-------S-----------------------------------------------
SC103436  --------------A-------S-----------------------------------------------
Strain B  --------------A-------------------------------------------------------
                  80                                                        140
```

```
MU946294  SKVMAAAAVLKQSESNKEVVNKRLEINAADLVVWSPITEKHLQSGMTLAELSAATLQYSDNTAMNLIIGYLGG
MB193997  ----------------------------------------------------------------------
SG77      ----------------------------------------------------------------------
KH11      ----------------------------------------------------------------------
SG43      ----------------------------------------------------------------------
SB136     ----------------------------------------------------------------------
SB175T    ----------------------------------------------------------------------
SL911     ----------------------------------------------------------------------
SG176     ----------------------------------------------------------------------
SC103436  ----------------------------------------------------------------------
Strain B  ----------------------------------------------------------------------
                                          180
```

```
MU946294  PEKVTAFARSIGDATFRLDRTEPTLNTAIPGDERDTSTPLAMAESLRKLTLGDALGEQQRAQLVTWLKGNTTG
MB193997  ----------------------------------------------------------------------
SG77      ----------------------------------------------------------------------
KH11      ----------------------------------------------------------------------
SG43      ----------------------------------------------------------------------
SB136     --------H-------------------------------------------------------------
SB175T    ----------------------------------------------------------------------
SL911     ----------------------------------------------------------------------
SG176     ----------------------------------------------------------------------
SC103436  --------------------------------------------------------N-------------
Strain B  ----------------------S----------------------------------------------
                  237                                                  280
```

```
MU946294N GQSIRAGLPESWVVGDKTGTGDYGTTNDIAVIWPEDHAPLVLVTYFTQPQQDAKNRKEVLAAAAKIVTEGL
MB193997E -------------------A-------------------N-------------------------T------
SG77      -------------------G----------------------------------------------------
KH11      -------------------A----------------------------------------------------
SG43      -------------------A----------------------------------------------------
SB136     -------------------A----------------------------------------------------
SB175T    -------------------A-------------------I--------------------------------
SL911     -------------------A----------------------------------------------------
SG176     -------------------A----------------------------------------------------
SC103436  -------------------A-------------N--------------------------------------
Strain B  -----------------S------------------------------------------------------
```

Fig 8.2: Alignments of the amino acid sequencing of strains MU946294N and MB193997E with those of OXY-2-1, OXY-2-2, OXY-2-3, OXY-2-4, OXY-2-5, OXY-2-6, OXY-2-7, OXY-2-8 and strain B of *K. oxytoca*. The amino acids are numbered according to (Ambler, Coulson *et al.* 1991).

8.3.5. Isoelectric focusing

By isoelectric focusing, one band of pI 5.6 was observed for the strain MU946294N (OXY-2-9); the band for strain MB193997E (OXY-2-10) was of pI 5.8 as shown in Fig. 8.3.

The IEF gel was overlaid with varying concentration of clavulanic acid. The IC_{50} of the β-lactamase inhibitor clavulanate was at least 200-fold higher in both strains MU946294N and MB193997E than those for the inhibitor susceptible strain.
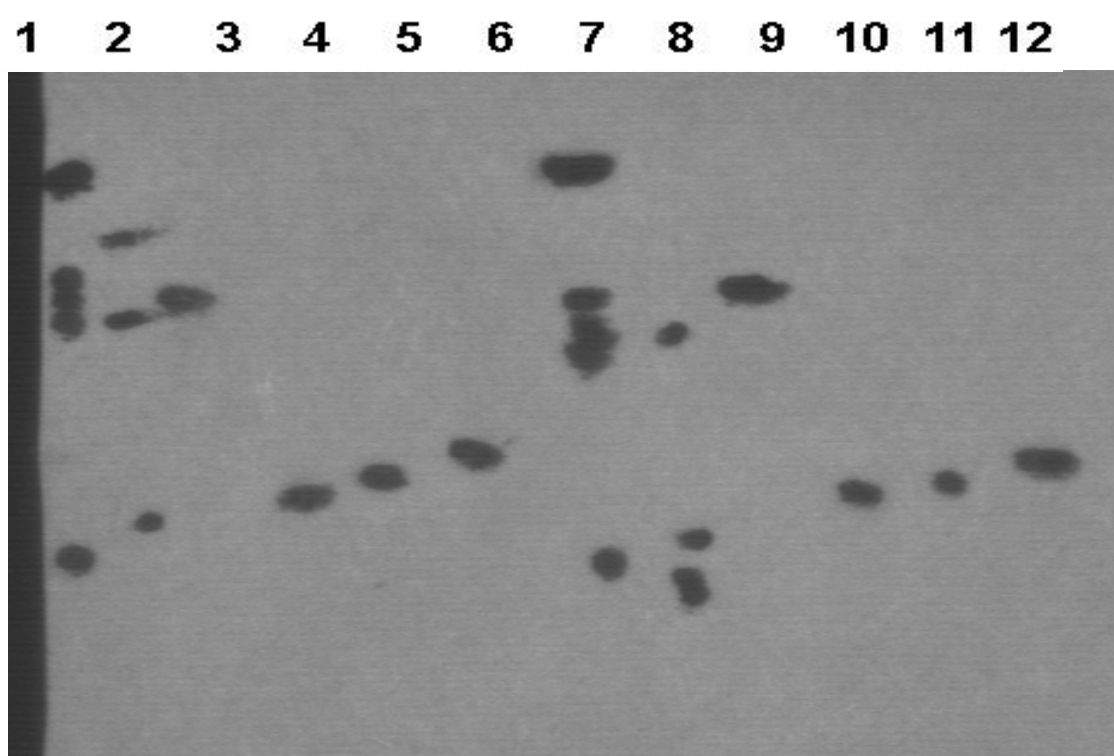

Fig 8.3: IEF polyacrylamide gel of OXY-2 β-lactamases of strains MU946294N, MB193997E. Lane 1, 7: standard marker. Lane 2, 8: isolate no. MB. Lane 3, 9: bla_{SHV-1} standard strain. Lane 4, 10: bla_{TEM-1} standard strain. Lane 5, 11 isolate MU946294N. Lane 6, 12: isolate MB193997E.

8.3.6. The *bla*_{OXY-2} promoter

The promoter regions for the β-lactamase genes from the two isolates were sequenced and compared with the promoter sequence of wild-type *bla*_{OXY-2} (TTGTCA for -35 and GATAGT for -10), the *bla*_{OXY-2} promoter of both strains MB193997E and MU946294N had a substitution (G→A) of the fifth base in the -10 consensus sequence (GATAAT).

8.3.7. KOXY β-lactamase sequence comparison and phylogenetic tree

The amino acid sequences of the OXY-2 β-lactamases of strains MU946294N (OXY-2-9), MB193997E (OXY-2-10), strain B (accession number AY303807) and strains (SB136, SB175, SG77, KH11, SG43, SG176, SL911, and SC10,436) previously published that corresponding to OXY-2 β-lactamases (OXY-2-1, OXY-2-2, OXY-2-3, OXY-2-4, OXY-2-5, OXY-2-6, OXY-2-7, OXY-2-8) according to (Fevre, Jbel *et al*. 2005) were aligned with ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2/).

The nucleotides sequences of the *rpoB* and *gyrA* previously published and those of the strains MU946294N and MB193997E were aligned by using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/). The phylogenetic tree was built with Geneious software (http://www.geneious.com/). As shown in Fig. 8.4, both strains were assigned to be KoII based on *rpoB* and *gyrA* characterization. The 940-bp fragment of the *rpoB* gene of the strain MU946294N showed 100% of identity with strain SB136 (KoII) and 99% with strains SB175T, ATC13182 (2 nucleotides) and SB23833 (3 nucleotides). Strain MB193997E was 99% identical to all previous strains and the difference was in 1 to 4 nucleotides.

The identity between strain MU946294N and strain MB193997E of the *rpoB* gene showed 1 nucleotide change with a percentage of 99%.

The two strains differed by only two nucleotides based on *gyrA* characterization, and by one to two nucleotides changes with the KoII *K. oxytoca* strains previously published.
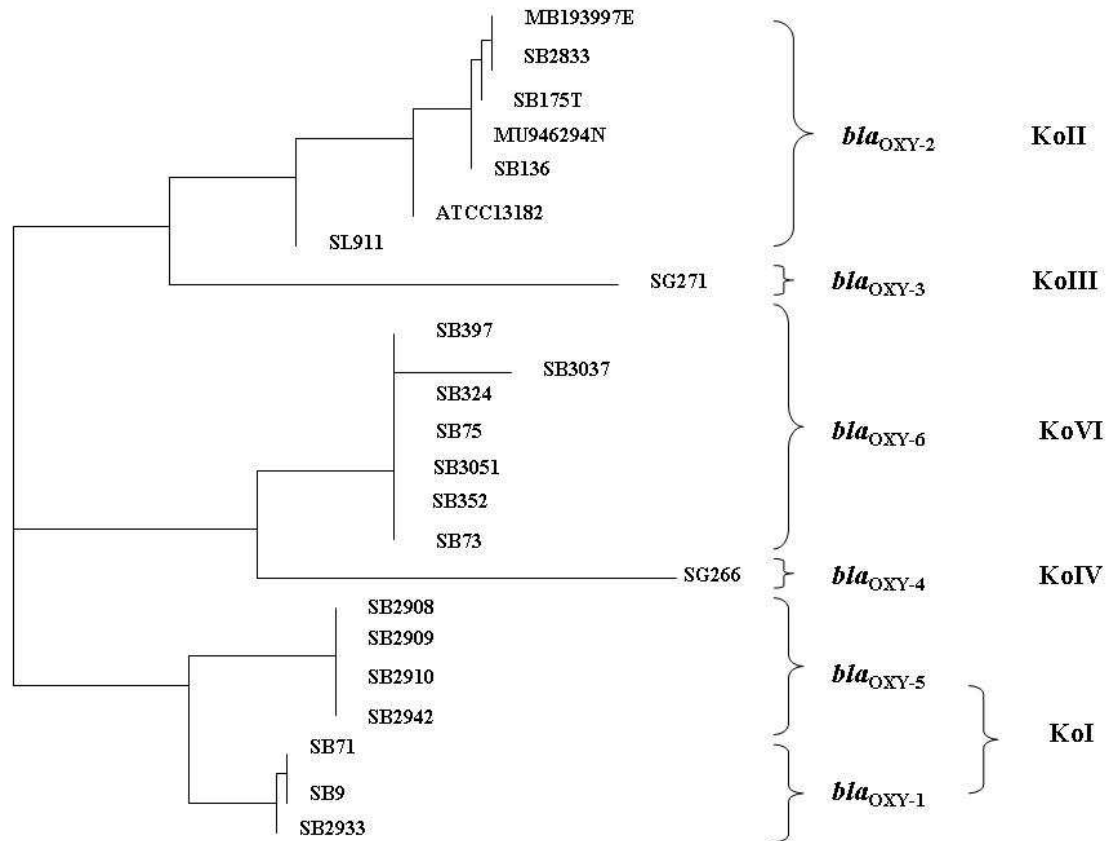
Fig. 8.4: Phylogeny of the *K. oxytoca rpoB* genes sequences. The neighbour-joining tree was rooted using no outgroup. Seven *rpoB* genes belong to each KoI, KoVI and KoII groups. One *rpoB* gene represents KIII and KIV groups.

8.3.8. PFGE

The clonal relationship between the two isolates was studied by pulsed-field gel electrophoresis (PFGE). Plugs were digested with 30U of *Apa1* (Promega, UK). As indicated from the result of PFGE in Fig 8.5, the two isolates were completely different from each other, showing considerably more than three band changes.
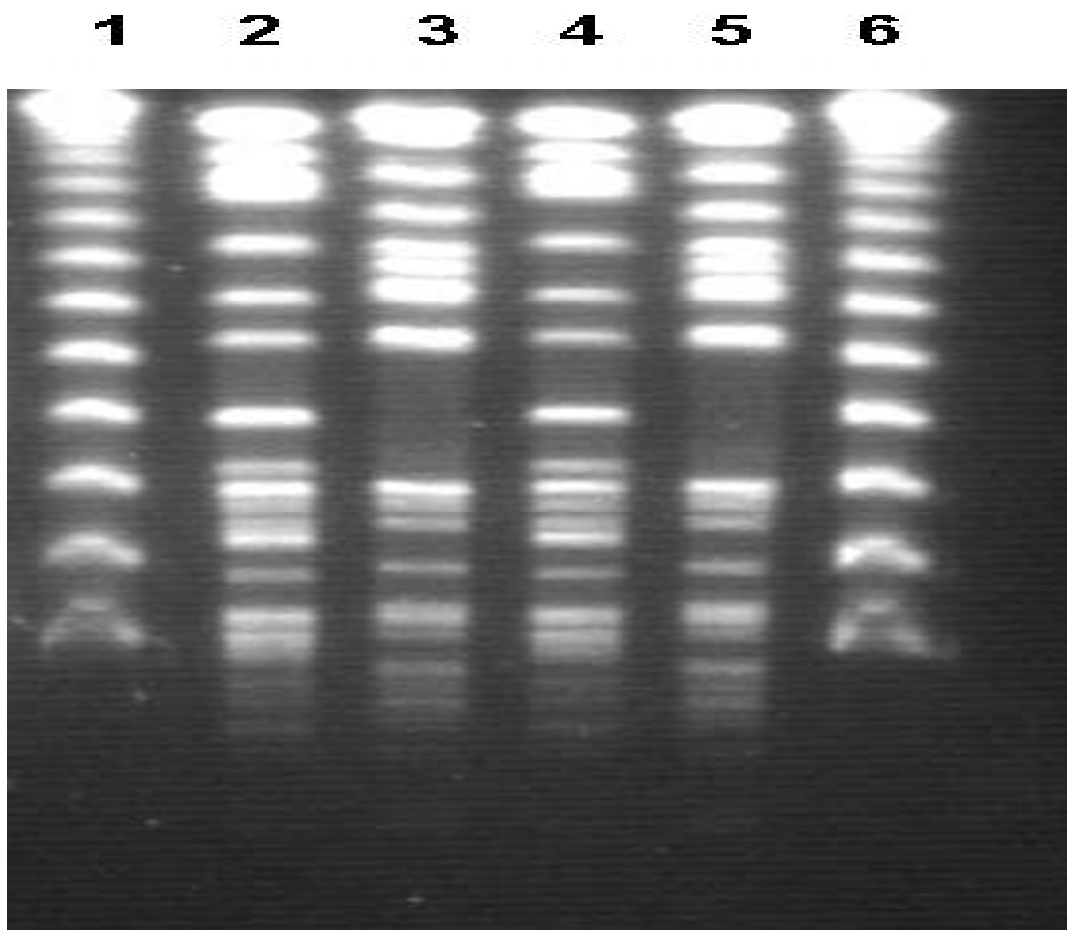
Fig. 8.5: PFGE patterns of *K. oxytoca* strains MU946294N and MB193997E. Lane 1 and 6: Lambda DNA marker; lanes 2 and 4: repeated strain MU946294N; lanes 3 and 5: repeated strain MB193997E.

8.3.9. Outer membrane proteins

The outer membrane proteins of the isolates were studied as detailed in Material and methods section and described by Bossi and Figueroa-Bossi (2007). The results shown in Fig 8.6 and highlighted by arrows revealed that the outer membrane profiles were identical in two *K. oxytoca* isolates studied, except for the MU946294N strain, which exhibited reduced expression of the 36-kDa outer membrane protein on a sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). This loss might explain cefoxitin-resistance. In addition, there was the loss of another minor band.
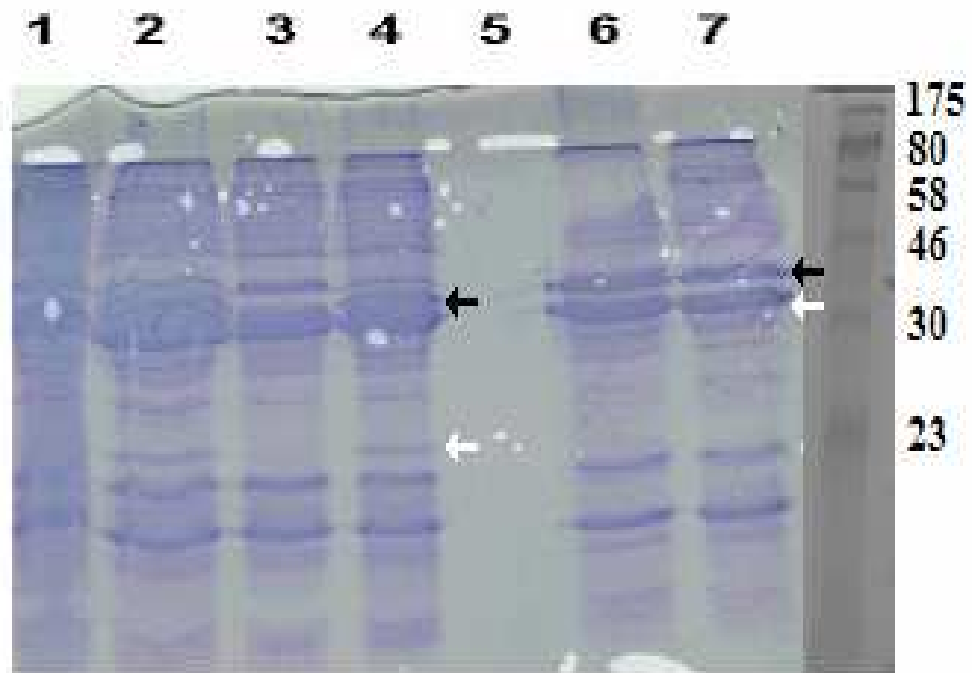
Fig 8.6: SDS-PAGE of outer membrane protein. Lane 1 and 3: isolate MU946294N. Lane 2 and 4: isolate MB193997E. Lane 6: *K. pneumoniae* isolate 175. Lane 7: *K. pneumoniae* isolate 187. The right lane is protein marker (range 23 to 175 kDa). The white arrow indicates OpmK34, the black arrow indicates OmpK35/36, the lower white arrow indicates loss of minor band.

8.3.10. GenBank accession numbers

The *bla*$_{OXY-2-9}$, *bla*$_{OXY-2-10}$ genes and the *rpoB* gene partial sequences of strains MU946294N and MB193997E have been registered in GenBank database under accession numbers FJ785625, FJ785626, FJ785627 and FJ785628 respectively.

8.4. DISCUSSION

Chromosomal β-lactamases are overproduced from bacteria by several mechanisms: by increased gene copy numbers, by promoter mutations or acquisition of insertion sequences, or by mutation in a regulator gene (Fournier, Gravel *et al.* 1999). The OXY-2 β-lactamase group hydrolyzes several β-lactams including carbenicillin, cephalothin, ceftriaxone, and aztreonam better than the OXY-1 β-lactamase group (Fournier and Roy 1997). The ability of the *K. oxytoca* β-lactamases to hydrolyze extended-spectrum β-lactams is a result of over-production of the enzyme (Fournier, Arlet *et al.* 1994).

Strain MU946294N, described here, is the first OXY-2 producing *K. oxytoca* that confers resistance to both cefotaxime (8 mg/L) and ceftazidime (4 mg/L) when compared with other OXY β-lactamases previously published.

An interesting result is a replacement of Alanine, Serine or Glycine at position 237 in the strain MU946294N by Threonine. The substitution Threonine 237 that occurred in the signal sequence affects the catalytic properties of the enzyme. Mutagenesis experiments have shown that Asparagine as well as Threonine substitutions increase catalytic efficiency on cephems over penems (Healey, Labgold *et al.* 1989). Residue 237 belongs to the oxyanion pocket. This pocket polarizes the β-lactam's carbonyl group, which is strongly attracted by the hydrogen bonding of backbone NH amide groups of amino acids at positions 70 and 237. The side chain at residue Alanine237 is on the outer, exposed side of the B3 β-strand that forms the right edge of the binding site. Cefotaxime, with its branched oximino substituent, is unable to form a hydrogen bond to the Alanine at position 237, but replacement of Alanine237 with a hydrogen bond acceptor such as Threonine enhances the binding of cefotaxime (Knox 1995; Fournier and Roy 1997). On the other hand, the substitution located at position 237 could be also responsible for the reduced susceptibility to ceftazidime. This is the case for some TEM-, SHV- and CTX-M-type ESBLs (Decre, Burghoffer *et al.* 2004).

The substitution of Threonine at position 237 was observed in plasmid-mediated extended-spectrum β-lactamases, TEM-5, TEM-24, TEM-86, TEM-114, TEM-121, TEM-130, TEM-131 and TEM-136 (http://www.lahey.org/Studies/). All these β-lactamases had higher levels of resistance to ceftazidime ranging from (>32 to 512 mg/l). The cefotaxime MICs were clearly increased in TEM-5, TEM-24, TEM-86, TEM-131 (4 to 64 mg/l) (Petit, Sirot *et al.* 1988; Chanal, Sirot *et al.* 1989; Kruger, Szabo *et al.* 2004; Baraniak, Fiett *et al.* 2005). However, TEM-121 has lost hydrolysis activity against cefotaxime and ceftriaxone due to a loss of its affinity for these substrates (Poirel, Mammeri *et al.* 2004).

The *bla*OXY-2 promoter of strains MB193997E and MU946294N had a substitution (G→A) of the fifth base in the -10 consensus sequence. The mutation (G→A) led to a four- to nine fold increase over the wild type promoter strength (Fournier, Gravel *et al.* 1999). The resistance phenotype of the strain MB193997E displayed a β-lactam resistance pattern identical to that displayed by *K. oxytoca* strains overproducing β-lactamase. The nucleotide substitution Asp255→Asn acid has been previously published in isolates SC10,436 and *K. oxytoca* strain KER (Sirot, Labia *et al.* 1998; Granier, Leflon-Guibout *et al.* 2002). The Ala283→Thr mutation has not been previously published in any of OXY, TEM, SHV, and CTX-M β- lactamases.

In this study I reported on the resistance of *K. oxytoca* strains MU946294N and MB193997E to amoxicillin-clavulanate. This resistance pattern is similar to that observed when an inhibitor-resistant TEM (IRT) enzyme is produced. However, there were no TEM derivatives in strains MU946294N and MB193997E, as identified by PCR and further confirmed by pI value, pI 5.6 and pI 5.8 respectively. These investigations were consistent with previous literatures in which, (Sirot, Labia *et al.* 1998) reported the first *K. oxytoca* strain conferred resistance to inhibitors and was due to the amino acid substitution Ser130→Gly. The resistance had been due to the use of amoxicillin-clavulanate (3g/day) for 10 days.

The substitution of Alanine to Glycine or Serine to Alanine at position 237 modifies susceptibility to clavulanic acid, but not to tazobactam. Also, the presence of

Threonine at this position could be responsible for the relatively high inhibitor concentration of clavulanic acid required to give 50% inhibition (IC_{50}s) observed for the *K. oxytoca* β-lactamases (Fournier and Roy 1997; Mammeri, Poirel *et al.* 2003).

The neighbour-joining trees obtained based on the sequence of the 940-bp fragment of the *rpoB* gene and 441-bp fragment of the *gyrA* gene were very similar. The nucleotide variation at these two housekeeping genes was in very close similarity with the phylogeny for the OXY-2 β-lactamase genes. These results were in agreement with the previous findings, in which the chromosomal *bla* gene of *K. oxytoca* is able, like housekeeping genes, to classify the *K. oxytoca* groups. Also, strains harbouring *bla*$_{OXY-2}$ gene fall into a clearly distinct phylogenetic group (Granier, Leflon-Guibout *et al.* 2003; Fevre, Jbel *et al.* 2005).

The PFGE was used to measure strain similarity, this method has been used for epidemiological studies and is one of the most discriminative genotyping methods for typing various bacterial genera including *Klebsiella* spp (Hansen, Skov *et al.* 2002). Results obtained from neighbour-joining trees based on *rpoB* and *gyrA* were different from PFGE results, this might be due to the fact that PFGE can detect chromosomal rearrangements, which may be caused by mobile elements with rapid evolutionary rates (Vimont, Mnif *et al.* 2008).

8.5. CONCLUSION

This is the first report of an Ala→Thre substitution at Ambler position 237 in an OXY-types β-lactamases. The present study is the first to report that the *bla*$_{OXY-2}$ gene family can confer resistance to extended-spectrum β-lactams (cefotaxime, ceftazidime). Beside ESBL resistance, the same isolate confer resistance to amoxicillin-clavuanate. This could represent emergence of CMO (Complex Mutant OXY) capable of conferring ESBL plus IRO (inhibitor resistance OXY) phenotype.

CHAPTER - 9:

Distribution and genetic characterization of plasmid-mediated quinolone resistance (*Qnr*) genes among *K. pneumoniae* in Scotland

9.1. ABSTRACT

Objectives: This study aimed to determine the prevalence of *qnr* genes and their association with the presence of ESBLs in clinical *K. pneumoniae* isolates from Scotland.

Results: Of the 219 isolates tested, 18 (8.2%) were found to be positive by PCR for the *qnr* genes. These positive isolates were further assessed by PCR where 11 possessed the *qnrB1* gene, 2 harboured the *qnrB6* gene and 5 contained the *qnrA1* gene. None of *qnrS* genes were identified. All of the 18 *qnr* positive isolates were found to be resistant to nalidixic acid and ciprofloxacin. Ten of the *qnrB1* positive isolates were associated with CTX-M, SHV and TEM, one isolate with TEM and SHV-types β-lactamases. All *qnrA1* positive isolates were associated with TEM and SHV. All isolates were found to carry IncN and/or IncFII plasmids bearing class 1 integrons possess *intI1* gene encoding for the integrase enzyme and *qacEΔ1* and *sul1* genes.

Conclusion: These findings indicate the higher prevalence of *qnr* genes than in previous studies but still low in general. Also, these findings indicate the co-expression of fluoroquinolone and extended-spectrum β-lactam resistance among *Klebsiella pneumoniae* isolates in Scotland.

9.2. INTRODUCTION

Quinolone resistance results from mutations in the chromosomally-encoded type II topoisomerases, and via the upregulation of efflux pumps, or porin-related genes (Drlica and Zhao 1997; Tran, Jacoby *et al.* 2005). The plasmid-mediated *qnr* genes (*qnrA, B, S, C* and *D*) play an emerging role in the dissemination of fluoroquinolone resistance. The first plasmid-mediated quinolone resistance protein Qnr was identified in a *Klebsiella pneumoniae* isolate in 1994 (Martinez-Martinez, Pascual *et al.* 1998), where the presence of the *qnr* gene increased the MICs of nalidixic acid and fluoroquinolones by four- to eight-fold (Martinez-Martinez, Pascual *et al.* 1998; Tran and Jacoby 2002; Wang, Sahm *et al.* 2004; Mammeri, Van De Loo *et al.* 2005).

The *qnrA* gene has been identified in complex In*4* family class 1 integrons, known as complex sul1-type integrons that may act as a recombinase for mobilisation of the antibiotic resistance genes located nearby (e.g., *qnr*, *bla*$_{CTX-M}$ and *amp*C) (Mammeri, Van De Loo *et al.* 2005; Nordmann and Poirel 2005; Robicsek, Jacoby *et al.* 2006). The *qnrB1* located in a multi resistance plasmid in an integron-like structure near *orf513* (*orf1005*) gene (Jacoby, Walsh *et al.* 2006). Unlike *qnrA* and *qnrB*, *qnrS* genes have been reported that it is not part of a sul1-type integron and not as a form of a gene cassette in a common class 1 integrons (Hata, Suzuki *et al.* 2005). Recently, two other plasmid-mediated quinolone resistance genes, namely, *qnrC* and *qnrD*, have been identified from China (Cavaco, Hasman *et al.* 2009; Wang, Guo *et al.* 2009). In addition, an association of quinolone resistance with the production of ESBLs has been reported. This study aimed to determine the prevalence of *qnr* genes and its association with the presence of ESBLs in clinical *K. pneumoniae* isolates from Scotland (Nordmann and Poirel 2005).

9.3. RESULTS

9.3.1. Initial PCR results

In this study, different sets of already published primers as shown in Table 2.2 (Materials and Methods) were used in multiplex PCR for detection of *qnr* genes (Cattoir, Poirel *et al.* 2007; Cattoir, Weill *et al.* 2007; Hopkins, Wootton *et al.* 2007; Park, Kang *et al.* 2009). The number of *qnr* genes identified by PCR was 47/219 with a percentage of (21.5 %). These results are considered as a high percentage of prevalence. This result was confirmed by several repeats of the PCR amplification. After sequencing of these amplifications, the most of them came up as hypothetical proteins (false positive results). The actual number of *qnr* genes confirmed after sequencing was 18 (5 *qnrA* and 13 *qnrB*) genes. My results indicate that there is a need to set up specific primers to identify *qnr* genes.

9.3.2. Prevalence of quinolone resistance

Of all *K. pneumoniae* isolates tested, 18/219 (8.2%) were found to be positive by PCR and DNA sequencing for the *qnr* genes. From these isolates, 16/32 (50%) *qnr* genes were found in ESBL-producing isolates. The 18 *qnr* positive isolates were further assessed by PCR, where 13 possessed the *qnrB* gene, 5 contained the *qnrA* gene, none of isolates were found to harbour the *qnrS* genes. Out of 13 *qnrB* positive isolates sent for sequencing, 11 of them were assigned to be *qnrB1* and two isolates were *qnrB6*. All *qnrA*-positive isolates sent for sequencing were confirmed to be *qnrA1* (Tables 9.1 and 9.2).

None of these genes (*qnrB*, *qnrA* and *qnrS*) were identified from *K. oxytoca* isolates.

Resistance to nalidixic acid was found in all 18 *qnr* positive isolates with MIC ranges from 32 to >128 mg/L. As well, all isolates were resistant to ciprofloxacin but one (border-line resistance) with MICs range (0.5 - >128 mg/L) as shown in Table 9.1.

9.3.3. Association between *qnr* genes and extended-spectrum β-lactamase

As shown in Table 9.1, twelve of 13 *qnrB* genes were associated with SHV-types β-lactamases. Only one isolate carrying *qnrB6* was associated with new LEN-type β-lactamase. All of the *qnrB1* positive isolates were found to carry the TEM-1 β-lactamase, both the *qnrB6* positive isolates were negative for the TEM-1 β-lactamase. Furthermore, 10 isolates were found to be associated with the CTX-M-15 β-lactamase.

Four of *qnrA* positive isolates were found to be associated with the TEM-1 β-lactamase, all *qnrA* positive isolates were associated with either SHV-11 or SHV-12 β-lactamases. However of the isolates those were tested none were found to harbour bla_{CTX-M} and the *qnrA* genes.

Table 9.1: antimicrobial susceptibility of *qnr*-producing *K. pneumoniae* isolates

	I D	Qnr gene	NA	CIP	CFR	CTX	CAZ	MEP	CFX	β-lactamases
69	Mu 936 327 N	*qnrB1*	>128	>128	>128	128	64	0.03	8	TEM-1, SHV-11, CTX-M-15
73	Mu 936 619 A	*qnrB1*	>128	>128	>128	128	64	0.03	8	TEM-1, SHV-11, CTX-M-15
175	Mg 768 689 P	*qnrB1*	>128	>128	>128	128	128	0.03	16	TEM-1, SHV-12, CTX-M-15
187	Mu 949847 W	*qnrB1*	>128	>128	>128	>128	128	2	64	TEM-1, SHV-12, CTX-M-15
197	Mu 950618 L	*qnrB1*	>128	>128	>128	>128	128	0.06	64	TEM-1, SHV-5, CTX-M-15
210	Mu 956764 R	*qnrB1*	128	>128	>128	128	128	0.03	8	TEM-1, SHV-11
213	Mu 960 478	*qnrB1*	>128	>128	>128	128	64	0.06	8	TEM-1, SHV-11, CTX-M-15
215	Mu 966 491 T	*qnrB1*	>128	>128	>128	>128	128	0.25	64	TEM-1, SHV-11, CTX-M-15
MB	Mb141078	*qnrB1*	>128	>128	64	64	>128	0.06	16	TEM-1, SHV-11, CTX-M-15
B7	B143279	*qnrB1*	>128	>128	128	64	128	0.06	16	TEM-1, SHV-11, CTX-M-15
BV	B14124V	*qnrB1*	>128	>128	128	64	>128	0.03	16	TEM-1, SHV-11, CTX-M-15
138	Mu 943 892 B	*qnrB6*	32	2	0.06	0.06	0.12	0.03	2	SHV-11
172	Mu 948 095 C	*qnrB6*	32	4	0.03	0.03	0.25	0.015	2	LEN-25
106	Mu 939 649 W	*qnrA1*	32	0.5	16	8	64	0.03	8	TEM-1, SHV-11
135	Mu 943 702 T	*qnrA1*	>128	8	16	16	128	0.03	8	TEM-1, SHV-11
204	Mu 953545 C	*qnrA1*	>128	8	8	8	128	0.03	4	TEM-1, SHV-11
206	Mu 956107 Q	*qnrA1*	128	4	8	8	128	0.015	4	TEM-1, SHV-11
214	Mb 195 491 Y	*qnrA1*	>128	32	128	16	128	0.03	8	SHV-12

Table 9.2: Characterization of *qnr*-producing *K. pneumoniae* isolates

|	ID	Qnr	conjugation	IncFII	IncN	*IntI*	*SulI*	*qacEΔ1*	*IntI2*	Kp type	CS class 1 integrons
69	Mu 936 327 N	*qnrB1*	+	+	+	+	+	+		I	1k
73	Mu 936 619 A	*qnrB1*	+	+	+	+	+	+	+	I	1k
175	Mg 768 689 P	*qnrB1*	-	+	+	+	+	+		I	1k
187	Mu 949847 W	*qnrB1*	+	+	+	+	+	+		I	1k
197	Mu 950618 L	*qnrB1*	+	+	+	+	+	+		I	1k
210	Mu 956764 R	*qnrB1*	-	-	-	+	+	+		I	2k
213	Mu 960 478	*qnrB1*	+	+	+	+	+	+		I	1k
215	Mu 966 491 T	*qnrB1*	+	+	+	+	+	+		I	1k
MB	Mb141078	*qnrB1*	-	+	+	+	+	+		I	1k
B7	B143279	*qnrB1*	+	+	+	+	+	+		I	1k
BV	B14124V	*qnrB1*	+	+	+	+	+	+		I	1k
138	Mu 943 892 B	*qnrB6*	+	-	+	+	+	+		I	-ve
172	Mu 948 095 C	*qnrB6*	+	-	+	+	+	+		III	1.5-2k
106	Mu 939 649 W	*qnrA1*	+	+	+	+	+	+		III	1.5-2k
135	Mu 943 702 T	*qnrA1*	+	+	+	+	+	+		III	1.5-2k
204	Mu 953545 C	*qnrA1*	+	+	+	+	+	+		III	1.5-2k
206	Mu 956107 Q	*qnrA1*	-	-	+	+	+	+		III	1.5-2k
214	Mb 195 491 Y	*qnrA1*	+	-	+	+	+	+	+	I	1.5-2k
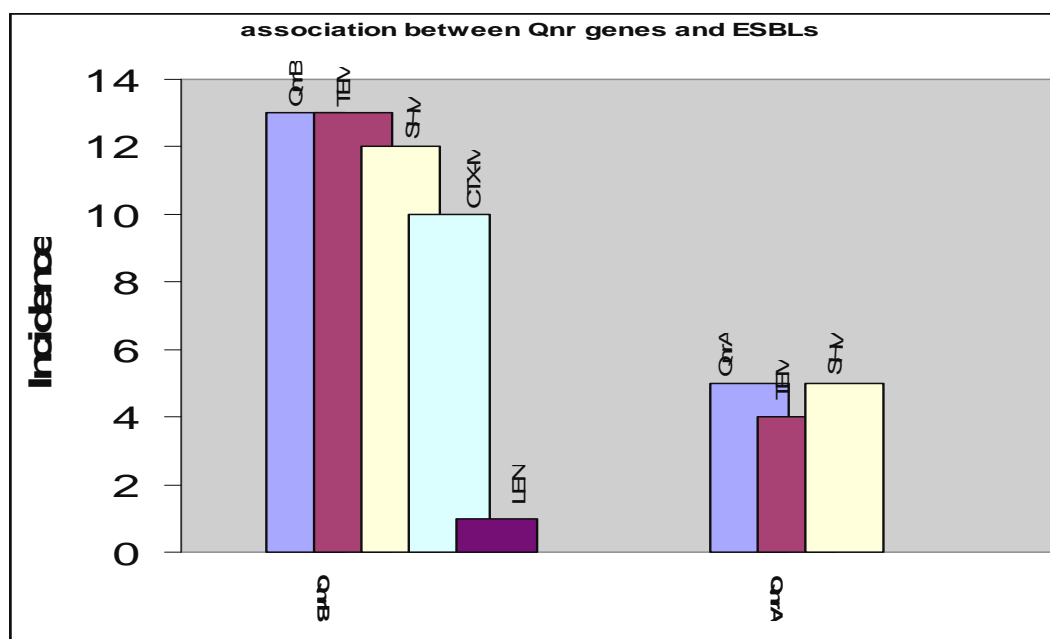
Fig 9.1: Association between *qnr* genes and ESBL enzymes. All of the *qnrB1*-positive isolates were co-expressed TEM, SHV/LEN β-lactamases, whereas 10 isolates were found to be associated with the CTX-M-15 β-lactamase. In contrast, none of *qnrA*-producing isolates were found to harbour bla_{CTX-M}

9.3.4. Phylogenetic typing

The phylogenetic types of the isolates were performed using *gyrA* restriction endonuclease as indicated in Table 9.2. All isolates belong to *qnrB* was assigned to phylogenetic type I (KpI) except one isolate which carry *qnrB6* and bla_{LEN} was assigned to be KpIII. In contrast to *qnrB*, four of *qnrA1* positive isolates were assigned to be KpIII except one isolate was found to be KpI phylogenetic type.

9.3.5. Incidence of class 1 and class 2 integrons

As shown in Table 9.2, all *qnr*-producing isolates were found located in complex *sul1*-type integrons and were positive for *intl1*, *sul1*, and *qacEΔ1* genes which confer resistance to sulphonamide and quaternary ammonium compounds. Additionally, all isolates but one was positive for conserved sequence carrying gene cassettes.

Interestingly, all *qnrA*-positive isolates were found to harbour the same gene cassette *dfrA5-ereA2* genes, whereas *qnrB*-producing isolates were not.

9.3.6. Plasmid profile

Analysing the plasmid electrophoretic profiles of *qnr*-positive isolates, all but one *qnrA1*-positive isolates showed identical plasmid profile (Fig 9.2). The plasmid patterns of *qnrB1* and *qnrB6*-positive isolates were different.

The plasmid typing was performed using primers as described by (Carattoli, Bertini *et al.* 2005). As shown in Table 9.2, all *qnrB1*-positive isolates, except isolate 210, were assigned to be IncFII plasmid type. *qnrB6*-positive isolates were negative for IncFII plasmid, all but one *qnrA1*-positive isolates were IncFII negative plasmid type. IncN plasmid type was amplified by PCR from all isolates except two isolates harbouring *qnrB1* gene.

Using S1 nuclease digestion followed by PFGE for some isolates showed that all *qnr*-positive clinical isolates possessed 1 to 2 plasmids of varying sizes.

9.3.7. Conjugation and transformation

Transfer of *qnrA* gene to *E. coli* J62-2 isolate confirmed that four of five *qnrA1*-positive isolates were successfully transconjugated. Ten of 13 *qnrB*-positive transconjugant isolates were obtained (Fig 9.3). This is indication of highly transferable plasmids carrying *qnr* genes. *bla*$_{CTX-M-15}$, *bla*$_{SHV-}$ and some of *bla*$_{TEM}$ were found to be cotransferred with *qnr* genes.

9.3.8. Clonal relationships among isolates

Finally, the clonal relationship among the isolates in this study was assessed by studying the PFGE genomic DNA profiles. As shown in Fig 9.4, *qnrB1*-positive samples were grouped in two major clonally related clusters. However, the isolates producers for *qnrB6* were separated in individual clones. In addition, four of five *qnrA1*-positive isolates were all clonally related.
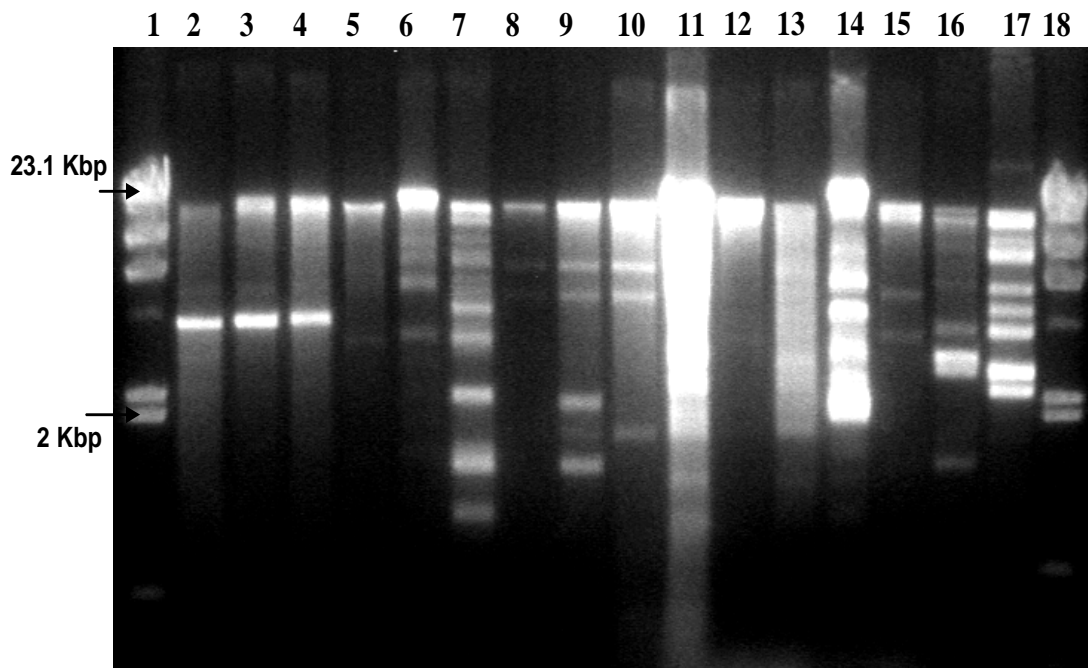
Fig. 9.2: Gel electrophoresis of *Pst1* restriction endonuclease. Lane 1, 18: Lambda DNA/HindIII Markers. Lanes 2, 3, 4 and 15: *qnrA1*-producing isolates 135, 204, 206 and 214. Lanes 5 and 6: *qnrB6*-producing isolates 138 and 172 respectively. Lanes 7, 8, 9, 10: *qnrB1*-producing isolates. Lane 11: isolate 32. Lane 12: isolate 84. Lane 13: isolate 114. Lane 14: isolate 208. Lane 16: isolate U54. Lane 17: isolate 115.
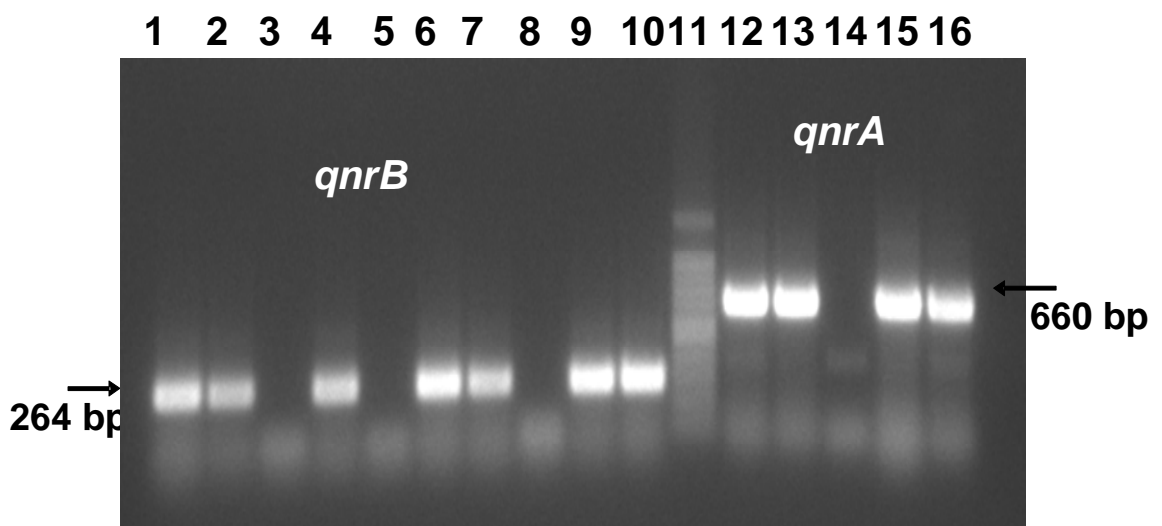
Fig 9.3: Gel electrophoresis shown PCR amplification of *qnrA* and *qnrB* genes from transconjugated isolates. Lanes (1 to 10): *qnrB* gene from isolates 69, 73, 175, 187, 210, 197, 213, MB, 215 and BV respectively. Lane 11: molecular marker of 100 bp. Lanes (12 to 16) *qnrA1*: isolates no. 106, 135, 206, 204 and 214 respectively.

Fig 9.4: PFGE dendrogram showing the clonality of 18 *qnr* genes harboured *K. pneumoniae* isolates. A band position tolerance of 2.0% was used in PFGE pattern analysis. Strain numbers and clusters are shown on the right.

9.4. DISCUSSION

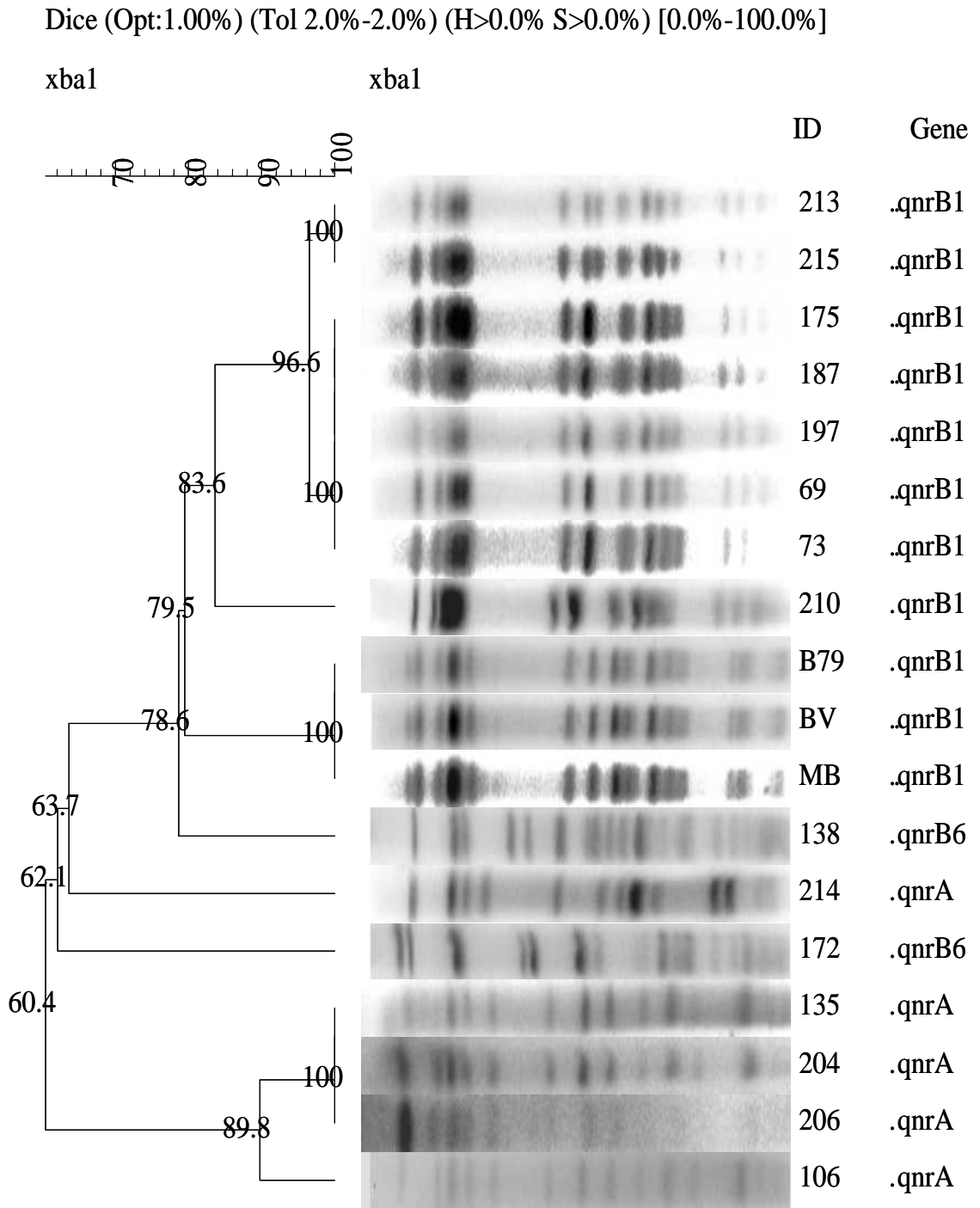Resistance to fluoroquinolones by family *Enterobacteriacae* became common and widespread shortly after the introduction of these agents (Tran, Jacoby *et al.* 2005). Until now there are many mechanisms identified responsible for quinolone resistance. These mechanisms include i) the Qnr proteins (QnrA, QnrB, QnrS, QnrC, and QnrD) which protect the DNA gyrase and topoisomerase IV from inhibition by quinolones, ii) the aminoglycoside acetyltransferase variant, *aac(6′)-Ib-cr*, capable of acetylating and reducing the activity of norfloxacin and ciprofloxacin, iii) the recently described fluoroquinolone-specific efflux pump protein, QepA, involved in pumping fluoroquinolones out of bacterial cells (Poirel, Cattoir *et al.* 2008; Cavaco, Hasman *et al.* 2009).

This study reported on the distribution of the *qnrA, qnrB* and *qnrS* among a collection of 219 *K. pneumoniae* isolates tested by PCR. The prevalence of *qnrA, B and S* genes were 5 (2.28%), 13 (5.9%) and 0 (0%) respectively with an overall distribution of 8.2%.

None of these genes were identified from *K. oxytoca* or from *E. cloacae*.

The previous study concerning the prevalence of *qnr* genes in Scotland reported that only 2 *qnrA* gene were found to be associated with bla_{SHV-5} from *K. pneumoniae* isolates collected in Edinburgh in 2006 (Hamouda, Vali *et al.* 2008).

In the UK, the prevalence of *qnr* genes was completely different from what found in this study. Where (Corkill, Anson *et al.* 2005) found a high prevalence of *qnrA* (32%) in multidrug resistant Enterobacteriacae recovered during 2003-2005 in Liverpool. In another study, only six *S. enterica* strains isolated in the UK belonging to four serotypes (Stanley, Typhimurium, Virchow and Virginia) were positive for *qnrS1* (Hopkins, Wootton *et al.* 2007).

These investigations were consistent with previous literatures worldwide in which, the *qnrA* was estimated to be 7.7% in ciprofloxacin-resistant *E. coli* isolates collected from 2000 to 2002 in Shanghai, China (Wang, Tran *et al.* 2003). Additionally, in China 29 (8.0%) of 362 phenotypic ESBL-producing isolates were positive for *qnr* genes, with *qnrA-*, *qnrB-* and *qnrS*-type alleles detected in 5 (1.9%), 4 (1.5%) and 5 (1.9%) *E. coli* isolates, and in 8 (8.1%), 4 (4.0%) and 4 (4.0%) *K. pneumoniae* isolates respectively (Jiang, Zhou *et al.* 2008)**.**

The high prevalence of *qnr* genes was found among 106 *K. pneumoniae* isolates in U.S.A, whereas *qnrA* and *qnrB* were detected in 15 (14%) and 6 (6%) respectively (Robicsek, Strahilevitz *et al.* 2006).

In contrast, the low incidence of the *qnr* gene was detected in 1 of 297 nalidixic-acid resistant *E. coli* strains (0.3%) in France (Mammeri, Van De Loo *et al.* 2005). In another study, *qnrA*-positive *Enterobacter* spp. and *Citrobacter freundii* isolates were detected in four patients in two intensive care units among 703 cephalosporin-resistant or fluoroquinolone-resistant Enterobacteriaceae which were tested from 34 German intensive care units from 2000 to 2003 (Jonas, Biehler *et al.* 2005). Moreover, the prevalence of *qnr* among enterobacterial clinical isolates carrying ESBLs between 2003 and 2004 in Barcelona was 4.9%. The *qnrA1* was the most prevalent, whereas only one *qnrS* and no *qnrB* were detected (Lavilla, Gonzalez-Lopez *et al.* 2008).

All *qnr*-positive isolates reported in this study were resistant to ciprofloxacin (MIC range 0.5 to >128 g/L) and naldixic acid with (MIC range 32 to >128 g/L). The corresponding finding from Scotland showed that 32 of the 69 ESBL-producing *K. pneumoniae* (46.4%) were found to be resistant to ciprofloxacin, 11 (15.9%) were intermediate and 26 (37.7%) were sensitive (Dashti, Paton *et al.* 2006).

Previous reports demonstrating that *qnr* alone did not confer resistance to fluoroquinolones; however, its presence promotes the selection of additional chromosomally encoded quinolone resistance mechanisms. Also, *qnr* may facilitate

further selection from low-level to high-level resistance with the usage of quinolones (Mammeri, Van De Loo *et al.* 2005; Robicsek, Jacoby *et al.* 2006; Robicsek, Strahilevitz *et al.* 2006; Poirel, Cattoir *et al.* 2008). Moreover, the expression of *qnrA1* considerably increased the mutant prevention concentration compared to strains without this gene (Rodriguez-Martinez, Velasco *et al.* 2007a). Additionally, the detection of *qnr* genes among nalidixic acid and fluroquinolones susceptible isolates has also been described in other studies (Cattoir, Poirel *et al.* 2007; Hopkins, Wootton *et al.* 2007).

From this point, the fluoroquinolone resistance in our strains was probably due to mutations in genes coding for chromosomally encoded type II topoisomerases, efflux pumps (QepA), aminoglycoside acetyltransferase variants such as aac(6′)-Ib-c2 or porin-related proteins; determination of the exact mechanism of resistance was beyond the scope when I started this project.

In the present study, *qnr* genes were found in 18/32 (56.25%) of the ESBL-producing isolates. The prevalence was higher in our ESBL collection. The results indicated that all *qnr*-positive isolates, but 3, carried bla_{TEM}. These 3 isolates were associated with either SHV-11 or SHV-12 or LEN- β-lactamases. Additionally, in 10 isolates, the *qnr* genes co-existed with $bla_{CTX-M-15}$. The association of *qnr*-positive genes, ESBLs and plasmid-mediated cephalosporinases (AmpC) have been described elsewhere (Wang, Sahm *et al.* 2004; Corkill, Anson *et al.* 2005; Mammeri, Van De Loo *et al.* 2005; Nordmann and Poirel 2005; Jacoby, Walsh *et al.* 2006). Moreover, the significant association rate of *qnr* genes with ESBL genes was reported before (Robicsek, Jacoby *et al.* 2006).

To explain co-expression of *qnr* genes and ESBLs, (Rodriguez-Martinez, Velasco *et al.* 2007b) indicated that *qnrA1* and bla_{FOX-5} genes would be located in different structures (4.1 kb and 3.0 kb fragments) in the same conjugative multi-resistance plasmid and located in an integron structure upstream of *qacEΔ1* and *sul1*.

In this study all isolates were found located in complex *sul1*-type integrons and were positive for *intl1*, *sul1*, and *qacEΔ1* which confer resistance to sulphonamide and quaternary ammonium compounds. Consistent with the previous reports in the finding that *qnrA* and *qnrB* genes has been identified in complex In*4* family class 1 integrons, known as complex *sul1*-type integrons. These genetic structures possess one 5`-conserved segment (5`-CS) which contains *intI1* gene encoding for the integrase enzyme and duplicated 3`-conserved segments (3`-CS) each of them contains *qacEΔ1* and *sul1* genes. The two 3`-CS surround a common region (CR) which contains the IS*CR1* (*orf513*) and a unique region. Usually, a single or double copy of IS*CR1* is found downstream from *qnrA1* (Mammeri, Van De Loo *et al.* 2005; Nordmann and Poirel 2005; Garnier, Raked *et al.* 2006; Jacoby, Walsh *et al.* 2006; Robicsek, Jacoby *et al.* 2006; Toleman, Bennett *et al.* 2006; Rodriguez-Martinez, Velasco *et al.* 2007b).

In my work, four of five *qnrA1*-positive isolates were successfully transconjugated. Ten of 13 *qnrB*-positive transconjugant isolates were obtained. This is indication of highly transferable plasmids carrying *qnr* genes.

The plasmid typing was done using primers as described by Caratrolle. All qnr-positive isolates but one were assigned to be IncN. In contrast, IncFII was described from 14 isolates. Interestingly, *qnrB6*-positive isolates were negative for IncFII plasmid. The IncN plasmids has been shown to carry *qnrS* in *S. enterica* isolates from UK (Hopkins, Wootton *et al.* 2007).

One isolate was not typed with the set of primers we used and this may indicate that either this isolate not correspond to any of replicon types or lack of primers we used. In this respect, plasmids isolated from *S. enterica* from the UK as well as the pAH0376 plasmid from *Shigella flexneri* from China were also negative for all these 18 replicons (Hopkins, Wootton *et al.* 2007). Additionally, *bla*$_{CTX-M-15}$ and *aac(6')-Ib* and/or *aac(6')-Ib-cr* located on a 340-kb plasmid that was negative for all the incompatibility groups tested (Coelho, Gonzalez-Lopez *et al.* 2010).

9.5. CONCLUSION

These findings indicate the prevalence rate of *qnr* genes is higher than previous studies but still low in general. In contrast to what has been reported before, all isolates that expressed *qnr* gene were found to be resistant to ciprofloxacin and naladixic acid. These findings indicated that there is a need to ascertain specific primers to identify *qnr* genes. In fact, the results showed higher-prevalence and co-expression of fluoroquinolone resistance among ESBL-producing *K. pneumoniae* isolates in Scotland than elsewhere. Furthermore, *qnrB* genes were more frequent among the isolates collected and are more often associated with ESBL-encoding genes than *qnrA*. Additionally, all isolates were located in complex *sul1*-type class 1 integrons. Finally, association of the *qnrB* and *qnrA* gene with IncN and IncFII plasmids could help disseminate these resistance determinants.

CHAPTER -10

CONCLUDING REMARKS

The β-lactam antibiotics are most widely used to treat infections caused by Gram-negative bacteria. The β-lactamases are, by far, the most important mechanism of resistance to β-lactam antibiotics, whereas production of ESBLs in particularly by *E. coli* and *Klebsiella* spp. confers resistance to third and fourth generation cephalosporins (Paterson and Bonomo 2005; Livermore, Canton *et al*. 2007). Until 2003, *Klebsiella* spp. are the most common ESBL-positive bacteria with mutant forms of *bla*$_{SHV}$ and *bla*$_{TEM}$ genes (Potz, Hope *et al*. 2006). Hospital outbreaks and the inter-hospital dissemination of resistant strains of *K. pneumoniae* are frequently reported (Diancourt, Passet *et al*. 2005).

The results presented in this thesis answer several important questions, such as the distribution of resistance to extended-spectrum cephalosporins and fluoroquinolones? Which variants promote the resistance to β-lactamases? What is the role of amino acid substitutions and their function in the resistance mechanism? How the CTX-M family is disseminated in clinical isolates of *K. pneumoniae* in community and nosocomial environments?

At the end of each chapter, the results are discussed and conclusions are drawn; however, an overall conclusion of the different chapters is needed to put this thesis in context.

This study is the first general surveillance in Scotland because the previous studies had only entered ESBLs isolates in their surveillance. ESBL production was detected in 35/223 (15.7%) of the strains determined by MICs and confirmed by combination and synergy double disc methods with cefotaxime, cetriaxone and ceftazidime as indicator for ESBLs production. The β-lactamases found in this study represented a low percentage of resistance if compared with higher prevalence worldwide.

Carbapenems, such as imipenem and meropenem, have a very broad spectrum of activity and resist hydrolysis by ESBLs and derepressed chromosomal AmpC β-lactamases (Walther-Rasmussen and Hoiby 2006). Compared with other antimicrobial agents tested in this study, all ESBL-producing strains were found

susceptible to meropenem, keeping this the drug of choice in treatment of multi-resistant isolates. These results are in agreement with different studies worldwide that carbapenems were active against all ESBL-positive enterobacteria (Goossens and Grabein 2005; Luzzaro, Mezzatesta et al. 2006).

My findings of this thesis indicate that ESBL-producing *K. pneumoniae* were seen in greater proportions in urine than blood. This was in consistent with many reports from USA, Canada, and Spain. On the other hand, this was in contrast to what found previously in London and south-east England where, ESBL-producing *Enterobacteriaceae* were seen in greater proportions in blood than urine (Potz, Hope *et al.* 2006).

Until 2003, the most ESBL-producing *Klebsiella* spp. were almost exclusively nosocomial, often from specialist units (Potz, Hope *et al.* 2006). The ESBL-producing *Enterobacteriaceae* have increased among community-acquired strains. Community-acquired bacteraemia was defined as a positive blood culture taken on or within 48 hours of admission into hospital (Ko, Paterson *et al.* 2002). The community-acquired findings, with its limitations, shown here were occurred in significant numbers 11 (31.4%) among *K. pneumoniae* isolates submitted by GPs. The similar figure from 42 UK centres is 70 (24%) Of 291 CTX-M-producing *E. coli* isolates studied were reported from community patients (Woodford, Ward *et al.* 2004).

Also, this study has confirmed that identification of *Klebsiella* using *gyrA* PCR-RFLP is a simple, reliable, cheaper and rapid genotypic method to identify *Klebsiella* species and phylogenetic groups. Moreover, VITEK2 had the highest sensitivity, specificity and accuracy to identify the *Klebsiella* spp than the other phenotypic methods. That is because biochemical tests like API 20E may fail to differentiate between *K. pneumoniae* and *K. oxytoca* (Kovtunovych, Lytvynenko *et al.* 2003).

Another main issue studied in chapter 4 was the production of CTX-M enzymes by *K. pneumoniae* isolates. The CTX-M β-lactamases constitute one of the most rapidly

growing ESBL families. Over the last decade CTX-M types have replaced TEM and SHV mutants as the predominant ESBLs in numerous countries. They have evolved from an entirely different route from the SHV/TEM ESBLs in that they derived from the movement of a chromosomal β-lactamases of *Kluyvera* spp. through conjugation into *Klebsiella* and *E. coli* plasmids (Bonnet 2004).

In this study, I identified 16 isolates carrying CTX-M enzymes and this is considered to be the first report of the emergence of this family of β-lactamases in *K. pneumoniae* in Scotland. Only CTX-M-15 enzyme was described in this study associated with five different clones, in contrast to the UK situation where, CTX-M-15 first reported from *E. coli* in the UK in 2003, co-existed with CTX-M-9, CTX-M-14, they all belong to an epidemic clone of *E. coli* sequence type ST131 and serotype O25:H4 (Woodford, Ward *et al*. 2004; Livermore, Canton *et al*. 2007; Lau, Kaufmann *et al*. 2008).

Overall, this surveillance detected 50% of CTX-M-15 enzymes among 32 ESBL-producing *K. pneumoniae* isolates which is similar to the moderate rates showed by other surveillance studies in Europe (Edelstein, Pimkin *et al*. 2003; Mugnaioli, Luzzaro *et al*. 2006); (Eisner, Fagan *et al*. 2006); (Mesko Meglic, Koren *et al*. 2009). However, higher rates, such as 100% were reported in Spain and Scandinavia (Oteo, Cuevas *et al*. 2009; Lytsy, Sandegren *et al*. 2008).

The CTX-M genes are frequently located downstream of an insertion sequence IS*Ecp1*, which played an important role in the mobilization and expression of these genes (Karim, Poirel *et al*. 2001; Cao, Lambert *et al*. 2002). The IS*Ecp1* was identified 48 nucleotides upstream in all strains but one. This result was consistent with a 48bp sequence previously described for $bla_{CTX-M-15}$ from India and previously named as the W sequence (Karim, Poirel *et al*. 2001; Poirel, Decousser *et al*. 2003; Eckert, Gautier *et al*. 2006).

CTX-M enzymes are borne on plasmids that enable intra- and inter-species horizontal spread. These plasmids may carry other resistance determinants (Bonnet,

2004). In this study the IncN plasmid by PCR is always present when $bla_{CTX-M-15}$ gene was identified even in the absence of IncFII. As the IncN plasmid is the only type present, these results are the first to indicate the role of IncN replicon types in the dissemination of the $bla_{CTX-M-15}$ genes in *K. pneumoniae*. The plasmids carrying CTX-M-15 genes had previously been identified to incompatibility groups IncFII, IncFI, IncR and IncFIIk and IncL/M (Lavollay, Mamlouk *et al*. 2006; Coque, Novais *et al*. 2008; Carattoli 2009; Oteo, Cuevas *et al*. 2009; Coelho, Gonzalez-Lopez *et al*. 2010).

In chapter 5, only 90% (54/60) strains were positive by PCR for bla_{SHV} genes. In *K. pneumoniae*, the SHV-type ESBLs through the 1990s were successful and widely distributed worldwide. *K. pneumoniae* isolates are expected to present an intrinsic resistance to ampicillin (Heritage, M'Zali *et al*. 1999).

Although SHV ESBLs have been reported in Scotland, only two SHV variants, SHV-2 and SHV-5, were more common. These data showed the emergence and spread of new SHV variants in *K. pneumoniae*. Among the new SHV enzymes identified in the country SHV-1, SHV-11, SHV-5, SHV-12 and SHV-80. The SHV-12 ESBL, not uncommon in Scotland, was found, but more surprising was the discovery that 3 of the 8 isolates carried CTX-M-type β-lactamases suggests a prominent potential for this determinant to spread among different plasmid replicons. The bla_{SHV-12} has been reported to the predominant SHV enzyme in south-east Asia (Lee, Cho *et al*. 2006), Spain (Diestra, Juan *et al*. 2009) and Italy (Carattoli, Miriagou *et al*. 2006). SHV-80 possessed an amino acid substitution Ala146→Thr and expressed unusual resistance phenotype to meropenem and co-expressed CTX-M. An amino acid substitution, Ala146→Val, described before in SHV-38 which had carbapenem hydrolytic activity (Poirel, Heritier *et al*. 2003).

During the course of this study, it was demonstrated that a number of strains carried both non-ESBL encoding gene (SHV-11) and ESBL (SHV-12 or SHV-80) genes. The prevalence of *K. pneumoniae* isolates with more than one β-lactamase has been described before, especially between SHV-11 and SHV-12 (Lee, Cho *et al*. 2006).

In agreement with others, the presence of the IS*26* element upstream of bla_{SHV} genes described in this study strongly suggests an active role for these sequences in the evolution and dissemination of antibiotic resistance genes (Ford and Avison 2004). All isolates but one were found to carry the small SHV transposon. IS*26* insertion was identified into the bla_{SHV} promoter with 14bp target duplication which provides a strong promoter. The large SHV transposon-borne promoters (IS26-lacY-recF-SHV) were amplified from only one isolate harboured non-transferable bla_{SHV}. This promoter has enhanced activity, due to a C→A mutation in the –10 region (Turner, Andersson *et al.* 2009).

Additionally, class 1 integrons are highly frequent 26/28 (93%) among ESBL-producing *K. pneumoniae* clinical isolates. Three isolates (11%) were found positive for class 2 integrons; these isolates were found positive for class 1 integrons as well. The *dfrA12 + aadA2*, *aadA2*, *aadA1* and *dfrA12 + ereA2* gene cassettes have been identified by sequencing within the variable region of class 1 integrons. They encoded resistances currently correspond to old antibiotics, keeping them without significant contribution of ESBL dissemination.

Furthermore, in chapter 6 I focussed on the differences within SHV-1 and SHV-11. Among 88 SHV enzymes tested, the synonymous nucleotide mutations A402G, G705A, C786G and C324T were the most frequent. A new nucleotide substitution G846A was observed within 3 isolates sequenced from our collection. This study could help in future understanding of the evolution processes of SHV enzymes.

LEN-1 is a chromosomally encoded β-lactamase found in many strains of *K. pneumoniae* (Heritage, M'Zali *et al.* 1999). Until now, a reduced number of studies based on LEN β-lactamases have been published. In chapter 7, a new bla_{LEN} from the ESBL-negative *K. pneumoniae* (KpIII) phylogenetic group was identified in this thesis. It showed 3 amino acid differences in comparison to LEN-2 (Val84→Leu, Thr114→Ala and Ile257→Leu), the substitutions Thr114→Ala was firstly described in this study but does not shown significant influence to the resistance to third-

generation cephalosporins. This result was only the second after (Chen, Zhang et al. 2005) to report the carriage of the bla_{LEN} gene on a transferable plasmid suggesting further complications of resistance in future.

K. oxytoca is an important opportunistic pathogen causing serious infections especially among neonates and intensive care units (Podschun and Ullmann 1998; Fevre, Jbel *et al*. 2005). The chromosome of *K. oxytoca* carries a bla_{OXY} gene, which is constitutively expressed at low levels and usually confers low-level resistance to amino- and carboxy-penicillins (Livermore 1995; Fournier and Roy 1997). The bla_{OXY} first identified from a sick child infected with *K. pneumoniae* in Glasgow, Scotland (Arakawa, Ohta *et al*. 1989; Granier, Leflon-Guibout *et al*. 2002).

In chapter 8, I studied two *K. oxytoca* isolates. One isolate showed resistance to cefotaxime and ceftazidime and has Ala237→Thr substitution. The present study is the first to report the substitution of Ala237→Thr in the bla_{OXY-2} and this enhances resistance to cefotaxime, and ceftazidime. This mutation has produced the first cefotaxime-resistant OXY-2 β-lactamase producing *K. oxytoca* enzyme. Also, the same isolate confer resistance to amoxicillin-clavuanate. This could represent emergence of CMO (Complex Mutant OXY) capable of conferring ESBL plus IRO (inhibitor resistance OXY) phenotype. The massive use of β-lactam plus β-lactamase inhibitor combinations in patients from the community and hospital environments also facilitates the appearance of these type of enzymes (Canton, Morosini *et al*. 2008).

The substitution of Thr237 was clearly increased cefotaxime MICs in TEM-5, TEM-24, TEM-86, TEM-131 (4 to 64 mg/l) (Petit, Sirot *et al*. 1988; Chanal, Sirot *et al*. 1989; Kruger, Szabo *et al*. 2004; Baraniak, Fiett *et al*. 2005). Also, the presence of Threonine at this position could be responsible for the relatively high inhibitor concentration of clavulanic acid (Fournier and Roy 1997; Mammeri, Poirel *et al*. 2003). Recently, Pro167→Ser substitution of *K. oxytoca* strain conferring resistance to ceftazidime was reported (Mammeri, Poirel *et al*. 2003). The first *K. oxytoca* strain

conferred resistance to inhibitors was due to the amino acid substitution Ser130Gly (Sirot, Labia *et al.* 1998).

In recent years, the plasmid-mediated *qnr* genes (QnrA, QnrB, QnrS, QnrC, and QnrD) which protect the DNA gyrase and topoisomerase IV from inhibition by quinolones have become a concern because of its frequent association with ESBL CTX-M and CMY-type enzymes (Lavilla, Gonzalez-Lopez et al. 2008); (Poirel, Cattoir et al. 2008; Cavaco, Hasman et al. 2009). Total of 45 (20.2%) and 30 (13.5%) isolates in all isolates were resistant to nalidixic acid and to ciprofloxacin. The high prevalence of ciprofloxacin-resistant strains found must be taken into account when designing treatment of an ESBL-producing *E. coli* urinary tract infection.

In chapter 9, I reported on the distribution of the *qnrA, qnrB* and *qnrS* among a collection of 219 *K. pneumoniae* isolates. To our knowledge, this study constitutes the second to report on distribution of *qnr* genes in Scotland. The prevalence of *qnrA and B* genes were 5 (2.28%) and 13 (5.9%) respectively with an overall distribution of 8.2%. The *qnr* genes were expressed in 18/32 (56.25%) of total ESBL-producing isolates. In contrast, this finding indicated high-prevalence and different distribution than what reported previously in Scotland (Hamouda, Vali *et al.* 2008). Even though other resistance mechanisms such as efflux pumps (QepA), aminoglycoside acetyltransferase aac(6′)-Ib-cr variants or porin-related proteins are likely to be present.

Furthermore, consistent with the previous findings, all isolates were found in complex *sul1*-type integrons and were positive for *intI1*, *sul1*, and *qacEΔ1* genes. Finally, all qnr-positive isolates but one were assigned to be IncN. In contrast, IncFII was described from 14 isolates. The IncN plasmids has been shown to carry *qnrS* in *S. enterica* isolates from UK (Hopkins, Wootton *et al.* 2007). The association of the *qnrB* and *qnrA* gene with IncN and IncFII plasmids could help disseminate these resistance determinants among other *Enterobacteriaceae*.

Overall, during recent years, attention has been focused on the increment of *E. coli* in ESBL-production particularly in the community setting, whereas little work has been observed in previous studies in *K. pneumoniae* isolates. This study has confirmed that the maintenance of ESBL-producing *K. pneumoniae* isolates within hospitals and in the community. Also, the emergence and spread of new ESBL variants have detected. Moreover, the presence of high prevalence of CTX-M enzymes among ESBLs *K. pneumoniae* isolates as described for *E. coli* in UK.

Finally, the issues studied in this thesis are of particular importance in understanding the spread of β-lactamases and their contribution to resistance in Edinburgh, Scotland.

BIBLIOGRAPHY

Abraham, E. P. & Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. Nature 146: 837.

Alberti, S., Rodriquez-Quinones, F., Schirmer, T., Rummel, G., Tomas, J. M., Rosenbusch, J. P. & Benedi, V. J. (1995). A porin from *K. pneumoniae*: sequence homology, three-dimensional model, and complement binding. Infect Immun 63: 903-910.

Alexander, C. & Rietschel, E. T. (2001). Bacterial lipopolysaccharides and innate immunity. J Endotoxin Res 7: 167-202.

Allen, B. L., Gerlach, G. F. & Clegg, S. (1991). Nucleotide sequence and functions of mrk determinants necessary for expression of type 3 fimbriae in *K. pneumoniae*. J Bacteriol 173: 916-920.

Alobwede, I., M'Zali, F. H., Livermore, D. M., Heritage, J., Todd, N. & Hawkey, P. M. (2003). CTX-M extended-spectrum β-lactamase arrives in the UK. J Antimicrob Chemother 51: 470-471.

Alvarez, D., Merino, S., Tomas, J. M., Benedi, V. J. & Alberti, S. (2000). Capsular polysaccharide is a major complement resistance factor in lipopolysaccharide O side chain-deficient *K. pneumoniae* clinical isolates. Infect Immun 68: 953-955.

Alvarez, M., Tran, J. H., Chow, N. & Jacoby, G. A. (2004). Epidemiology of conjugative plasmid-mediated AmpC β-lactamases in the United States. Antimicrob Agents Chemother 48: 533-537.

Alves, M. S., Dias, R. C., de Castro, A. C., Riley, L. W. & Moreira, B. M. (2006). Identification of clinical isolates of indole-positive and indole-negative *Klebsiella* spp. J Clin Microbiol 44: 3640-3646.

Ambler, R. P. (1980). The structure of β-lactamases. Philos Trans R Soc Lond B Biol Sci 289: 321-331.

Ambler, R. P., Coulson, A. F., Frere, J. M., Ghuysen, J. M., Joris, B., Forsman, M., *et al.* (1991). A standard numbering scheme for the class A β-lactamases. Biochem J 276: 269-270.

Amyes, S. G. B. (2010). Antibacterial Chemotherapy. 1st Ed. Oxford university press. Oxford, UK.

Arakawa, Y., Ohta, M., Kido, N., Mori, M., Ito, H., Komatsu, T., *et al.* (1989). Chromosomal β-lactamase of *K. oxytoca*, a new class A enzyme that hydrolyzes broad-spectrum β-lactam antibiotics. Antimicrob Agents Chemother 33: 63-70.

Arakawa, Y., Ohta, M., Wacharotayankun, R., Mori, M., Kido, N., Ito, H., *et al.* (1991). Biosynthesis of *Klebsiella* K2 capsular polysaccharide in *E. coli* HB101 requires the functions of *rmpA* and the chromosomal *cps* gene cluster of the virulent strain *K. pneumoniae* Chedid (O1:K2). Infect Immun 59: 2043-2050.

Arakawa, Y., Wacharotayankun, R., Nagatsuka, T., Ito, H., Kato, N. & Ohta, M. (1995). Genomic organization of the *K. pneumoniae cps* region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. J Bacteriol 177: 1788-1796.

Arduino, S. M., Catalano, M., Orman, B. E., Roy, P. H. & Centron, D. (2003). Molecular epidemiology of *orf513*-bearing class 1 integrons in multiresistant clinical isolates from Argentinean hospitals. Antimicrob Agents Chemother 47: 3945-3949.

Arduino, S. M., Roy, P. H., Jacoby, G. A., Orman, B. E., Pineiro, S. A. & Centron, D. (2002). *bla*~CTX-M-2~ is located in an unusual class 1 integron (In*35*) which includes *Orf513*. Antimicrob Agents Chemother 46: 2303-2306.

Arlet, G., Rouveau, M., Casin, I., Bouvet, P. J., Lagrange, P. H. & Philippon, A. (1994). Molecular epidemiology of *K. pneumoniae* strains that produce SHV-4 β-lactamase and which were isolated in 14 French hospitals. J Clin Microbiol 32: 2553-2558.

Arpin, C., Dubois, V., Coulange, L., Andre, C., Fischer, I., Noury, P., *et al.* (2003). Extended-spectrum β-lactamase-producing Enterobacteriaceae in community and private health care centers. Antimicrob Agents Chemother 47: 3506-3514.

Arpin, C., Quentin, C., Grobost, F., Cambau, E., Robert, J., Dubois, V., *et al.* (2009). Nationwide survey of extended-spectrum β-lactamase-producing Enterobacteriaceae in the French community setting. J Antimicrob Chemother 63: 1205-1214.

Babini, G. S. & Livermore, D. M. (2000). Are SHV β-lactamases universal in *K. pneumoniae*? Antimicrob Agents Chemother 44: 2230.

Bagley, S., Seidler, R. J. & Brenner, D. J. (1981). *K. planticola* sp. nov.: a new species of Enterobacteriaceae found primarily in nonclinical environments. Curr Microbiol 6: 105-109.

Baraniak, A., Fiett, J., Hryniewicz, W., Nordmann, P. & Gniadkowski, M. (2002). Ceftazidime-hydrolysing CTX-M-15 extended-spectrum β-lactamase (ESBL) in Poland. J Antimicrob Chemother 50: 393-396.

Baraniak, A., Fiett, J., Mrowka, A., Walory, J., Hryniewicz, W. & Gniadkowski, M. (2005). Evolution of TEM-type extended-spectrum β-lactamases in clinical Enterobacteriaceae strains in Poland. Antimicrob Agents Chemother 49: 1872-1880.

Barthelemy, M., Peduzzi, J., Bernard, H., Tancrede, C. & Labia, R. (1992). Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally encoded enzymes of *K. oxytoca*. Biochim Biophys Acta 1122: 15-22.

Barton, B. M., Harding, G. P. & Zuccarelli, A. J. (1995). A general method for detecting and sizing large plasmids. Anal Biochem 226: 235-240.

Bauernfeind, A., Casellas, J. M., Goldberg, M., Holley, M., Jungwirth, R., Mangold, P., *et al.* (1992). A new plasmidic cefotaximase from patients infected with Salmonella typhimurium. Infection 20: 158-163.

Bauernfeind, A., Chong, Y. & Schweighart, S. (1989). Extended broad spectrum β-lactamase in *K. pneumoniae* including resistance to cephamycins. Infection 17: 316-321.

Bauernfeind, A., Grimm, H. & Schweighart, S. (1990). A new plasmidic cefotaximase in a clinical isolate of *E. coli*. Infection 18: 294-298.

Bebrone, C. (2007). Metallo-β-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily. Biochem Pharmacol 74: 1686-1701.

Bell, J., Chitsaz, M., Turnidge, J., Barton, M., Walters, L., & Jones, R. (2007). Prevalence and significance of a negative extended-spectrum beta-lactamase (ESBL) confirmation test result after a positive ESBL screening test result for isolates of *Escherichia coli* and *Klebsiella pneumoniae*: results from the SENTRY Asia-Pacific Surveillance Program. J Clin Microbiol **45**: 1478-82.

Bennett, J. W. & Chung, K. T. (2001). Alexander Fleming and the discovery of penicillin. Adv Appl Microbiol 49: 163-184.

Bird, J., Browning, R., Hobson, R. P., MacKenzie, F. M., Brand, J. & Gould, I. M. (1998). Multiply-resistant *K. pneumoniae*: failure of spread in community-based elderly care facilities. J Hosp Infect 40: 243-247.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513-1523.

Bleich, A., Kirsch, P., Sahly, H., Fahey, J., Smoczek, A., Hedrich, H. J. & Sundberg, J. P. (2008). *K. oxytoca*: opportunistic infections in laboratory rodents. Lab Anim 42: 369-375.

Bonnet, R. (2004). Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother 48: 1-14.

Bonnet, R., Dutour, C., Sampaio, J. L., Chanal, C., Sirot, D., Labia, R., *et al.* (2001). Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp240→Gly. Antimicrob Agents Chemother 45: 2269-2275.

Bonnet, R., Recule, C., Baraduc, R., Chanal, C., Sirot, D., De Champs, C. & Sirot, J. (2003). Effect of D240G substitution in a novel ESBL CTX-M-27. J Antimicrob Chemother 52: 29-35.

Bonnet, R., Sampaio, J. L., Labia, R., De Champs, C., Sirot, D., Chanal, C. & Sirot, J. (2000). A novel CTX-M β-lactamase (CTX-M-8) in cefotaxime-resistant Enterobacteriaceae isolated in Brazil. Antimicrob Agents Chemother 44: 1936-1942.

Bortz, D., Jackson, T., Taylor, K., Thompson, A. & Younger J. (2008). *Klebsiella pneumoniae* flocculation dynamics. Bull Math Biol 70: 745-68.

Bossi, L. & Figueroa-Bossi, N. (2007). A small RNA downregulates LamB maltoporin in *Salmonella*. Mol Microbiol 65: 799-810.

Bouadma, L., Schortgen, F., Thomas, R., Wutke, S., Lellouche, F., Regnier, B. & Wolff, M. (2006). Adults with spontaneous aerobic Gram-negative bacillary meningitis admitted to the intensive care unit. Clin Microbiol Infect 12: 287-290.

Boyd, D. A., Tyler, S., Christianson, S., McGeer, A., Muller, M. P., Willey, B. M., *et al.* (2004). Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum β-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. Antimicrob Agents Chemother 48: 3758-3764.

Bradford, P. A. (2001). Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev 14: 933-951.

Braiteh, F. & Golden, M. P. (2007). Cryptogenic invasive *K. pneumoniae* liver abscess syndrome. Int J Infect Dis 11: 16-22.

Braman, S. K., Eberhart, R. J., Asbury, M. A. & Hermann, G. J. (1973). Capsular types of *K. pneumoniae* associated with bovine mastitis. J Am Vet Med Assoc 162: 109-111.

Brenwald, N. P., Jevons, G., Andrews, J. M., Xiong, J. H., Hawkey, P. M. & Wise, R. (2003). An outbreak of a CTX-M-type β-lactamase-producing *K. pneumoniae*: the importance of using cefpodoxime to detect extended-spectrum β-lactamases. J Antimicrob Chemother 51: 195-196.

Brisse, S. & Duijkeren, E. (2005). Identification and antimicrobial susceptibility of 100 *Klebsiella* animal clinical isolates. Vet Microbiol 105: 307-312.

Brisse, S., Grimont, F. & Grimont, P. (2006). The Genus *Klebsiella*. Prokaryotes 6: 159-196.

Brisse, S., Issenhuth-Jeanjean, S. & Grimont, P. A. (2004). Molecular serotyping of *Klebsiella* species isolates by restriction of the amplified capsular antigen gene cluster. J Clin Microbiol 42: 3388-3398.

Brisse, S., van Himbergen, T., Kusters, K. & Verhoef, J. (2004). Development of a rapid identification method for *K. pneumoniae* phylogenetic groups and analysis of 420 clinical isolates. Clin Microbiol Infect 10: 942-945.

Brisse, S. & Verhoef, J. (2001). Phylogenetic diversity of *K. pneumoniae* and *K. oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, *gyrA* and *parC* genes sequencing and automated ribotyping. Int J Syst Evol Microbiol 51: 915-924.

Brooks, G., Butel, S., Morse, S. (2007). Enteric Gram-negative rods (Enterobacteriacae). In: Jawetz, Melnick & Adelberg's Medical Microbiology. 24th Ed, McGraw-Hill Medical. New York, USA.

Brown, S. & Amyes, S. (2006). OXA (beta)-lactamases in *Acinetobacter*: the story so far. J Antimicrob Chemother 57: 1-3.

BSAC (2006). British Society for Antimicrobial Chemotherapy: Methods for Antimicrobial Susceptibility Testing. Version 5.

Bush, K. (1989a). Classification of β-lactamases: groups 1, 2a, 2b, and 2b'. Antimicrob Agents Chemother 33: 264-270.

Bush, K. (1989b). Classification of β-lactamases: groups 2c, 2d, 2e, 3, and 4. Antimicrob Agents Chemother 33: 271-276.

Bush, K. (1989c). Characterization of β-lactamases. Antimicrob Agents Chemother 33: 259-263.

Bush, K. & Jacoby, G. A. (2010). Updated functional classification of β-lactamases. Antimicrob Agents Chemother 54: 969-976.

Bush, K., Jacoby, G. A. & Medeiros, A. A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 39: 1211-1233.

Cai, J. C., Zhou, H. W., Zhang, R. & Chen, G. X. (2008). Emergence of *Serratia marcescens*, *K. pneumoniae*, and *E. coli* isolates possessing the plasmid-mediated carbapenem-hydrolyzing β-lactamase KPC-2 in intensive care units of a Chinese hospital. Antimicrob Agents Chemother 52: 2014-2018.

Campos, M. A., Vargas, M. A., Regueiro, V., Llompart, C. M., Alberti, S. & Bengoechea, J. A. (2004). Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun 72: 7107-7114.

Canton, R. & Coque, T. M. (2006). The CTX-M β-lactamase pandemic. Curr Opin Microbiol 9: 466-475.

Canton, R., Morosini, M. I., de la Maza, O. M. & de la Pedrosa, E. G. (2008). IRT and CMT β-lactamases and inhibitor resistance. Clin Microbiol Infect 14: 53-62.

Cao, V., Lambert, T. & Courvalin, P. (2002). ColE1-like plasmid pIP843 of *K. pneumoniae* encoding extended-spectrum β-lactamase CTX-M-17. Antimicrob Agents Chemother 46: 1212-1217.

Carattoli, A. (2001). Importance of integrons in the diffusion of resistance. Vet Res 32: 243-259.

Carattoli, A. (2009). Resistance plasmid families in Enterobacteriaceae. Antimicrob Agents Chemother 53: 2227-2238.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L. & Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 63: 219-228.

Carattoli, A., Miriagou, V., Bertini, A., Loli, A., Colinon, C., Villa, L., *et al.* **(2006).** Replicon typing of plasmids encoding resistance to newer β-lactams. Emerg Infect Dis 12: 1145-1148.

Cartelle, M., del Mar Tomas, M., Molina, F., Moure, R., Villanueva, R. & Bou, G. (2004). High-level resistance to ceftazidime conferred by a novel enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240→Gly substitution. Antimicrob Agents Chemother 48: 2308-2313.

Carter, J. S., Bowden, F. J., Bastian, I., Myers, G. M., Sriprakash, K. S. & Kemp, D. J. (1999). Phylogenetic evidence for reclassification of *Calymmatobacterium granulomatis* as *Klebsiella granulomatis* comb. nov. Int J Syst Bacteriol 49: 1695-1700.

Castanheira, M., Mendes, R. E., Rhomberg, P. R. & Jones, R. N. (2008). Rapid emergence of *bla*CTX-M among Enterobacteriaceae in U.S. Medical Centers: molecular evaluation from the MYSTIC Program (2007). Microb Drug Resist 14: 211-216.

Castanheira, M., Toleman, M. A., Jones, R. N., Schmidt, F. J. & Walsh, T. R. (2004). Molecular characterization of a β-lactamase gene, *bla*GIM-1, encoding a new subclass of metallo-β-lactamase. Antimicrob Agents Chemother 48: 4654-4661.

Cattoir, V., Poirel, L., Mazel, D., Soussy, C. J. & Nordmann, P. (2007a). *Vibrio splendidus* as the source of plasmid-mediated *QnrS*-like quinolone resistance determinants. Antimicrob Agents Chemother 51: 2650-2651.

Cattoir, V., Poirel, L., Rotimi, V., Soussy, C. J. & Nordmann, P. (2007b). Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. J Antimicrob Chemother 60: 394-397.

Cattoir, V., Weill, F. X., Poirel, L., Fabre, L., Soussy, C. J. & Nordmann, P. (2007). Prevalence of *qnr* genes in *Salmonella* in France. J Antimicrob Chemother 59: 751-754.

Cavaco, L. M., Hasman, H., Xia, S. & Aarestrup, F. M. (2009). *qnrD*, a novel gene conferring transferable quinolone resistance in *Salmonella enterica* serovar *Kentucky* and *Bovismorbificans* strains of human origin. Antimicrob Agents Chemother 53: 603-608.

Chanal, C. M., Sirot, D. L., Petit, A., Labia, R., Morand, A., Sirot, J. L. & Cluzel, R. A. (1989). Multiplicity of TEM-derived β-lactamases from *K. pneumoniae* strains isolated at the same hospital and relationships between the responsible plasmids. Antimicrob Agents Chemother 33: 1915-1920.

Chanawong, A., M'Zali, F. H., Heritage, J., Xiong, J. H. & Hawkey, P. M. (2002). Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among Enterobacteriaceae in the People's Republic of China. Antimicrob Agents Chemother 46: 630-637.

Chang, C. Y., Fang, Y. T., Tsai, S. M., Chang, L. L. & Yu, W. L. (2009). Characterization of class 1 integrons and gene cassettes in clinical isolates of *K. pneumoniae* from Taiwan. Diagn Microbiol Infect Dis 65: 214-216.

Chang, F. Y., Siu, L. K., Fung, C. P., Huang, M. H. & Ho, M. (2001). Diversity of SHV and TEM β-lactamases in *K. pneumoniae*: gene evolution in Northern Taiwan and two novel β-lactamases, SHV-25 and SHV-26. Antimicrob Agents Chemother 45: 2407-2413.

Chen, S. W., Zhang, R. Z., Lu, Y. M., He, L., Wang, S. Y. & Mu, X. K. (2005). A novel LEN-derived β-lactamase from *K. pneumoniae*. Chin Med J (Engl) 118: 1380-1383.

Chen, Y., Delmas, J., Sirot, J., Shoichet, B. & Bonnet, R. (2005). Atomic resolution structures of CTX-M β-lactamases: extended spectrum activities from increased mobility and decreased stability. J Mol Biol 348: 349-362.

Chuang, Y. P., Fang, C. T., Lai, S. Y., Chang, S. C. & Wang, J. T. (2006). Genetic determinants of capsular serotype K1 of *K. pneumoniae* causing primary pyogenic liver abscess. J Infect Dis 193: 645-654.

Chung, D. R., Lee, S. S., Lee, H. R., Kim, H. B., Choi, H. J., Eom, J. S., *et al.* **(2007).** Emerging invasive liver abscess caused by K1 serotype *K. pneumoniae* in Korea. J Infect 54: 578-583.

Coelho, A., Gonzalez-Lopez, J. J., Miro, E., Alonso-Tarres, C., Mirelis, B., Larrosa, M. N., *et al.* **(2010).** Characterisation of the CTX-M-15-encoding gene in *K. pneumoniae* strains from the Barcelona metropolitan area: plasmid diversity and chromosomal integration. Int J Antimicrob Agents 36: 73-78.

Coque, T. M., Novais, A., Carattoli, A., Poirel, L., Pitout, J., Peixe, L., *et al.* **(2008).** Dissemination of clonally related *E. coli* strains expressing extended-spectrum β-lactamase CTX-M-15. Emerg Infect Dis 14: 195-200.

Corkill, J. E., Anson, J. J. & Hart, C. A. (2005). High prevalence of the plasmid-mediated quinolone resistance determinant *qnrA* in multidrug-resistant Enterobacteriaceae from blood cultures in Liverpool, UK. J Antimicrob Chemother 56: 1115-1117.

Cornaglia, G., Riccio, M. L., Mazzariol, A., Lauretti, L., Fontana, R. & Rossolini, G. M. (1999). Appearance of IMP-1 metallo-β-lactamase in Europe. Lancet 353: 899-900.

Cortes, G., Borrell, N., de Astorza, B., Gomez, C., Sauleda, J. & Alberti, S. (2002). Molecular analysis of the contribution of the capsular polysaccharide and the lipopolysaccharide O side chain to the virulence of *K. pneumoniae* in a murine model of pneumonia. Infect Immun 70: 2583-2590.

Costas, M., Holmes, B. & Sloss, L. L. (1990). Comparison of SDS-PAGE protein patterns with other typing methods for investigating the epidemiology of '*Klebsiella aerogenes*'. Epidemiol Infect 104: 455-465.

Coudron, P. E. (2005). Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in *Klebsiella* spp., *E. coli*, and *P. mirabilis*. J Clin Microbiol 43: 4163-4167.

Dashti, A. A., Paton, R. & Amyes, S. G. (2006). Linkage of ciprofloxacin resistance with a single genotypic cluster of *K. pneumoniae*. Int J Antimicrob Agents 27: 73-76.

Datta, N. (1969). Drug resistance and R factors in the bowel bacteria of London patients before and after admission to hospital. Br Med J 2: 407-411.

Datta, N. & Kontomichalou, P. (1965). Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature 208: 239-241.

Decre, D., Burghoffer, B., Gautier, V., Petit, J. C. & Arlet, G. (2004). Outbreak of multi-resistant *K. oxytoca* involving strains with extended-spectrum β-lactamases and

strains with extended-spectrum activity of the chromosomal β-lactamase. J Antimicrob Chemother 54: 881-888.

Delmas, J., Chen, Y., Prati, F., Robin, F., Shoichet, B. K. & Bonnet, R. (2008). Structure and dynamics of CTX-M enzymes reveal insights into substrate accommodation by extended-spectrum β-lactamases. J Mol Biol 375: 192-201.

Deshpande, L. M., Jones, R. N., Fritsche, T. R. & Sader, H. S. (2006). Occurrence and characterization of carbapenemase-producing Enterobacteriaceae: report from the SENTRY Antimicrobial Surveillance Program (2000-2004). Microb Drug Resist 12: 223-230.

Diancourt, L., Passet, V., Verhoef, J., Grimont, P. A. & Brisse, S. (2005). Multilocus sequence typing of *K. pneumoniae* nosocomial isolates. J Clin Microbiol 43: 4178-4182.

Diestra, K., Juan, C., Curiao, T., Moya, B., Miro, E., Oteo, J., *et al.* (2009). Characterization of plasmids encoding bla_{ESBL} and surrounding genes in Spanish clinical isolates of *E. coli* and *K. pneumoniae*. J Antimicrob Chemother 63: 60-66.

Domenech-Sanchez, A., Hernandez-Alles, S., Martinez-Martinez, L., Benedi, V. J. & Alberti, S. (1999). Identification and characterization of a new porin gene of *K. pneumoniae*: its role in β-lactam antibiotic resistance. J Bacteriol 181: 2726-2732.

Domenech-Sanchez, A., Martinez-Martinez, L., Hernandez-Alles, S., del Carmen Conejo, M., Pascual, A., Tomas, J. M., *et al.* (2003). Role of *K. pneumoniae* OmpK35 porin in antimicrobial resistance. Antimicrob Agents Chemother 47: 3332-3335.

Donowitz, G. R. & Mandell, G. L. (1988). β-Lactam antibiotics (1). N Engl J Med 318: 419-426.

Doublet, B., Granier, S. A., Robin, F., Bonnet, R., Fabre, L., Brisabois, A., *et al.* (2009). Novel plasmid-encoded ceftazidime-hydrolyzing CTX-M-53 extended-spectrum β-lactamase from *Salmonella enterica* serotypes *Westhampton* and *Senftenberg*. Antimicrob Agents Chemother 53: 1944-1951.

Dougherty, T. J. (1986). Genetic analysis and penicillin-binding protein alterations in *Neisseria gonorrhoeae* with chromosomally mediated resistance. Antimicrob Agents Chemother 30: 649-652.

Doumith, M., Ellington, M. J., Livermore, D. M. & Woodford, N. (2009). Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. J Antimicrob Chemother 63: 659-667.

Drancourt, M., Bollet, C., Carta, A. & Rousselier, P. (2001). Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with

description of *R. ornithinolytica* comb. nov., *R. terrigena* comb. nov. and *R. planticola* comb. nov. Int J Syst Evol Microbiol 51: 925-932.

Drawz, S. M. & Bonomo, R. A. (2010). Three decades of β-lactamase inhibitors. Clin Microbiol Rev 23: 160-201.

Drlica, K. & Zhao, X. (1997). DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev 61: 377-392.

Dubois, V., Poirel, L., Arpin, C., Coulange, L., Bebear, C., Nordmann, P. *et al.* **(2004).** SHV-49, a novel inhibitor-resistant β-lactamase in a clinical isolate of *K. pneumoniae*. Antimicrob Agents Chemother 48: 4466-4469.

Dutour, C., Bonnet, R., Marchandin, H., Boyer, M., Chanal, C., Sirot, D. & Sirot, J. (2002). CTX-M-1, CTX-M-3, and CTX-M-14 β-lactamases from Enterobacteriaceae isolated in France. Antimicrob Agents Chemother 46: 534-537.

Eckert, C., Gautier, V. & Arlet, G. (2006). DNA sequence analysis of the genetic environment of various bla_{CTX-M} genes. J Antimicrob Chemother 57: 14-23.

Eckert, C., Gautier, V., Saladin-Allard, M., Hidri, N., Verdet, C., Ould-Hocine, Z., Barnaud, G., *et al.* **(2004).** Dissemination of CTX-M-type β-lactamases among clinical isolates of Enterobacteriaceae in Paris, France. Antimicrob Agents Chemother 48: 1249-1255.

Edelstein, M., Pimkin, M., Palagin, I., Edelstein, I. & Stratchounski, L. (2003). Prevalence and molecular epidemiology of CTX-M extended-spectrum β-lactamase-producing *E. coli* and *K. pneumoniae* in Russian hospitals. Antimicrob Agents Chemother 47: 3724-3732.

Eisner, A., Fagan, E. J., Feierl, G., Kessler, H. H., Marth, E., Livermore, D. M. & Woodford, N. (2006). Emergence of Enterobacteriaceae isolates producing CTX-M extended-spectrum β-lactamase in Austria. Antimicrob Agents Chemother 50: 785-787.

Empel, J., Baraniak, A., Literacka, E., Mrowka, A., Fiett, J., Sadowy, E., *et al.* **(2008).** Molecular survey of β-lactamases conferring resistance to newer β-lactams in Enterobacteriaceae isolates from Polish hospitals. Antimicrob Agents Chemother 52: 2449-2454.

Evrard, B., Balestrino, D., Dosgilbert, A., Bouya-Gachancard, J. L., Charbonnel, N., Forestier, C. & Tridon, A. (2010). Roles of capsule and lipopolysaccharide O antigen in interactions of human monocyte-derived dendritic cells and *K. pneumoniae*. Infect Immun 78: 210-219.

Fang, C. T., Chuang, Y. P., Shun, C. T., Chang, S. C. & Wang, J. T. (2004). A novel virulence gene in *K. pneumoniae* strains causing primary liver abscess and septic metastatic complications. J Exp Med 199: 697-705.

Fang, C. T., Lai, S. Y., Yi, W. C., Hsueh, P. R., Liu, K. L. & Chang, S. C. (2007). *K. pneumoniae* genotype K1: an emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. Clin Infect Dis 45: 284-293.

Fang, F. C., Sandler, N. & Libby, S. J. (2005). Liver abscess caused by magA+ *K. pneumoniae* in North America. J Clin Microbiol 43: 991-992.

Feldman, C., Ross, S., Mahomed, A. G., Omar, J. & Smith, C. (1995). The aetiology of severe community-acquired pneumonia and its impact on initial, empiric, antimicrobial chemotherapy. Respir Med 89: 187-192.

Ferragut, C., Izard, D., Gavini, F., Kersters, K., De Ley, J. & Leclerc, H. (1983). *Klebsiella trevisanii* : a new species from water and soil. Int J Syst Bacteriol 33: 133-142.

Fevre, C., Jbel, M., Passet, V., Weill, F. X., Grimont, P. A. & Brisse, S. (2005). Six groups of the OXY β-Lactamase evolved over millions of years in *K. oxytoca*. Antimicrob Agents Chemother 49: 3453-3462.

Fiett, J., Palucha, A., Miaczynska, B., Stankiewicz, M., Przondo-Mordarska, H., Hryniewicz, W. & Gniadkowski, M. (2000). A novel complex mutant β-lactamase, TEM-68, identified in a *K. pneumoniae* isolate from an outbreak of extended-spectrum β-lactamase-producing *Klebsiella*e. Antimicrob Agents Chemother 44: 1499-1505.

Fisher, J. F., Meroueh, S. O. & Mobashery, S. (2005). Bacterial resistance to β-lactam antibiotics: compelling opportunism, compelling opportunity. Chem Rev 105: 395-424.

Fontana, C., Favaro, M., Sarmati, L., Natoli, S., Altieri, A., Bossa, M. C., Minelli, S., *et al.* (2010). Emergence of KPC-producing *K. pneumoniae* in Italy. BMC Res Notes 3: 40.

Fontana, R., Cerini, R., Longoni, P., Grossato, A. & Canepari, P. (1983). Identification of a streptococcal penicillin-binding protein that reacts very slowly with penicillin. J Bacteriol 155: 1343-1350.

Ford, P. J. & Avison, M. B. (2004). Evolutionary mapping of the SHV β-lactamase and evidence for two separate IS*26*-dependent *bla*$_{SHV}$ mobilization events from the *K. pneumoniae* chromosome. J Antimicrob Chemother 54: 69-75.

Fournier, B., Arlet, G., Lagrange, P. H. & Philippon, A. (1994). *K. oxytoca*: resistance to aztreonam by overproduction of the chromosomally encoded β-lactamase. FEMS Microbiol Lett 116: 31-36.

Fournier, B., Gravel, A., Hooper, D. C. & Roy, P. H. (1999). Strength and regulation of the different promoters for chromosomal β-lactamases of *K. oxytoca*. Antimicrob Agents Chemother 43: 850-855.

Fournier, B., Lu, C. Y., Lagrange, P. H., Krishnamoorthy, R. & Philippon, A. (1995). Point mutation in the pribnow box, the molecular basis of β-lactamase overproduction in *K. oxytoca*. Antimicrob Agents Chemother 39: 1365-1368.

Fournier, B. & Roy, P. H. (1997). Variability of chromosomally encoded β-lactamases from *K. oxytoca*. Antimicrob Agents Chemother 41: 1641-1648.

Fournier, B., Roy, P. H., Lagrange, P. H. & Philippon, A. (1996). Chromosomal β-lactamase genes of *K. oxytoca* are divided into two main groups, *bla*$_{OXY-1}$ and *bla*$_{OXY-2}$. Antimicrob Agents Chemother 40: 454-459.

Fung, C. P., Chang, F. Y., Lee, S. C., Hu, B. S., Kuo, B. I., Liu, C. Y., *et al.* (2002). A global emerging disease of *K. pneumoniae* liver abscess: is serotype K1 an important factor for complicated endophthalmitis? Gut 50: 420-424.

Garau, G., Garcia-Saez, I., Bebrone, C., Anne, C., Mercuri, P., Galleni, M., Frere, J. M. & Dideberg, O. (2004). Update of the standard numbering scheme for class B β-lactamases. Antimicrob Agents Chemother 48: 2347-2349.

Garnier, F., Raked, N., Gassama, A., Denis, F. & Ploy, M. C. (2006). Genetic environment of quinolone resistance gene *qnrB2* in a complex *sul1*-type integron in the newly described *S. enterica* serovar *Keurmassar*. Antimicrob Agents Chemother 50: 3200-3202.

Garza-Ramos, U., Davila, G., Gonzalez, V., Alpuche-Aranda, C., Lopez-Collada, V. R., Alcantar-Curiel, D., *et al.* (2009). The *bla*$_{SHV-5}$ gene is encoded in a compound transposon duplicated in tandem in *Enterobacter cloacae*. Clin Microbiol Infect 15: 878-880.

Gavini, F., Izard, D., Grimont, P. A. D., Beji, A., Ageron, E. & Leclerc, H. (1986). Priority of *K. planticola* Bagley, Seidler, and Brenner 1982 over *K. trevisanii* Ferragut, Izard, Gavini, Kersters, DeLey, and Leclerc 1983. Int J Syst Bacteriol 36: 486-488.

Geddes, A. (2008). 80th Anniversary of the discovery of penicillin: An appreciation of Sir Alexander Fleming. Int J Antimicrob Agents 32: 373.

Ghuysen, J. M. (1988). Bacterial active-site serine penicillin-interactive proteins and domains: mechanism, structure, and evolution. Rev Infect Dis 10: 726-732.

Ghuysen, J. M. (1991). Serine β-lactamases and penicillin-binding proteins. Annu Rev Microbiol 45: 37-67.

Giakkoupi, P., Tzouvelekis, L. S., Tsakris, A., Loukova, V., Sofianou, D. & Tzelepi, E. (2000). IBC-1, a novel integron-associated class A β-lactamase with extended-spectrum properties produced by an *E. cloacae* clinical strain. Antimicrob Agents Chemother 44: 2247-2253.

Giobbia, M., Scotton, P. G., Carniato, A., Cruciani, M., Farnia, A., Daniotti, E., *et al.* (2003). Community-acquired *K. pneumoniae* bacteremia with meningitis and endophthalmitis in Italy. Int J Infect Dis 7: 234-235.

Gniadkowski, M. (2001). Evolution and epidemiology of extended-spectrum β-lactamases (ESBLs) and ESBL-producing microorganisms. Clin Microbiol Infect 7: 597-608.

Gniadkowski, M. (2008). Evolution of extended-spectrum β-lactamases by mutation. Clin Microbiol Infect 14: 11-32.

Gniadkowski, M., Schneider, I., Palucha, A., Jungwirth, R., Mikiewicz, B. & Bauernfeind, A. (1998). Cefotaxime-resistant Enterobacteriaceae isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing β-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. Antimicrob Agents Chemother 42: 827-832.

Goetz, A. M., Rihs, J. D., Chow, J. W., Singh, N. & Muder, R. R. (1995). An outbreak of infusion-related *K. pneumoniae* bacteremia in a liver transplantation unit. Clin Infect Dis 21: 1501-1503.

Goldsworthy, P. D. & McFarlane, A. C. (2002). Howard Florey, Alexander Fleming and the fairy tale of penicillin. Med J 176: 176-178.

Goossens, H. & Grabein, B. (2005). Prevalence and antimicrobial susceptibility data for extended-spectrum β-lactamase- and AmpC-producing Enterobacteriaceae from the MYSTIC Program in Europe and the United States (1997-2004). Diagn Microbiol Infect Dis 53: 257-264.

Granier, S. A., Leflon-Guibout, V., Goldstein, F. W. & Nicolas-Chanoine, M. H. (2003). New *K. oxytoca* β-lactamase genes bla_{OXY-3} and bla_{OXY-4} and a third genetic group of *K. oxytoca* based on bla_{OXY-3}. Antimicrob Agents Chemother 47: 2922-2928.

Granier, S. A., Leflon-Guibout, V., Nicolas-Chanoine, M. H., Bush, K. & Goldstein, F. W. (2002). The extended-spectrum K1 β-lactamase from *K. oxytoca* SC 10,436 is a member of the bla_{OXY-2} family of chromosomal *Klebsiella* enzymes. Antimicrob Agents Chemother 46: 2056-2057.

Gutmann, L., Ferre, B., Goldstein, F. W., Rizk, N., Pinto-Schuster, E., Acar, J. F. & Collatz, E. (1989). SHV-5, a novel SHV-type β-lactamase that hydrolyzes broad-spectrum cephalosporins and monobactams. Antimicrob Agents Chemother 33: 951-956.

Hackbarth, C. J. & Chambers, H. F. (1989). Methicillin-resistant *staphylococci*: genetics and mechanisms of resistance. Antimicrob Agents Chemother 33: 991-994.

Haeggman, S., Lofdahl, S., Paauw, A., Verhoef, J. & Brisse, S. (2004). Diversity and evolution of the class A chromosomal β-lactamase gene in *K. pneumoniae*. Antimicrob Agents Chemother 48: 2400-2408.

Hakenbeck, R. (1998). Mosaic genes and their role in penicillin-resistant *Streptococcus pneumoniae*. Electrophoresis 19: 597-601.

Hall, B. G. & Barlow, M. (2004). Evolution of the serine β-lactamases: past, present and future. Drug Resist Updat 7: 111-123.

Hall, L. M., Livermore, D. M., Gur, D., Akova, M. & Akalin, H. E. (1993). OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) β-lactamase from *P. aeruginosa*. Antimicrob Agents Chemother 37: 1637-1644.

Hammond, D. S., Harris, T., Bell, J., Turnidge, J. & Giffard, P. M. (2008). Selection of SHV extended-spectrum-β-lactamase-dependent cefotaxime and ceftazidime resistance in *K. pneumoniae* requires a plasmid-borne bla_{SHV} gene. Antimicrob Agents Chemother 52: 441-445.

Hamouda, A., Vali, L., Alsultan, A., Dancer, S. & Amyes, S. G. (2008). First report of ciprofloxacin resistance among *K. pneumoniae* harbouring the *qnrA1* gene and producing SHV-5 extended-spectrum β-lactamase in Scotland. J Chemother 20: 753-755.

Han, S. H. (1995). Review of hepatic abscess from *K. pneumoniae*. An association with diabetes mellitus and septic endophthalmitis. West J Med 162: 220-224.

Hansen, D. S., Aucken, H. M., Abiola, T. & Podschun, R. (2004). Recommended test panel for differentiation of *Klebsiella* species on the basis of a trilateral interlaboratory evaluation of 18 biochemical tests. J Clin Microbiol 42: 3665-3669.

Hansen, D. S., Mestre, F., Alberti, S., Hernandez-Alles, S., Alvarez, D., Domenech-Sanchez, A., et al. (1999). *K. pneumoniae* lipopolysaccharide O typing: revision of prototype strains and O-group distribution among clinical isolates from different sources and countries. J Clin Microbiol 37: 56-62.

Hansen, D. S., Skov, R., Benedi, J. V., Sperling, V. & Kolmos, H. J. (2002). *Klebsiella* typing: pulsed-field gel electrophoresis (PFGE) in comparison with O:K-serotyping. Clin Microbiol Infect 8: 397-404.

Hanson, N. D. (2003). AmpC β-lactamases: what do we need to know for the future? J Antimicrob Chemother 52: 2-4.

Hanson, N. D., Moland, E. S., Hong, S. G., Propst, K., Novak, D. J. & Cavalieri, S. J. (2008). Surveillance of community-based reservoirs reveals the presence of CTX-M, imported AmpC, and OXA-30 β-lactamases in urine isolates of *K. pneumoniae* and *E. coli* in a U.S. community. Antimicrob Agents Chemother 52: 3814-3816.

Hart, C. A. & Rao, S. K. (2000). Rhinoscleroma. J Med Microbiol 49: 395-396.

Hata, M., Suzuki, M., Matsumoto, M., Takahashi, M., Sato, K., Ibe, S. & Sakae, K. (2005). Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob Agents Chemother 49: 801-803.

Healey, W. J., Labgold, M. R. & Richards, J. H. (1989). Substrate specificities in class A β-lactamases: preference for penams vs. cephems. The role of residue 237. Proteins 6: 275-283.

Hedge, P. J. & Spratt, B. G. (1985). Amino acid substitutions that reduce the affinity of penicillin-binding protein 3 of *E. coli* for cephalexin. Eur J Biochem 151: 111-121.

Heritage, J., M'Zali, F. H., Gascoyne-Binzi, D. & Hawkey, P. M. (1999). Evolution and spread of SHV extended-spectrum β-lactamases in Gram-negative bacteria. J Antimicrob Chemother 44: 309-318.

Heritier, C., Poirel, L., Fournier, P. E., Claverie, J. M., Raoult, D. & Nordmann, P. (2005). Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. Antimicrob Agents Chemother 49: 4174-4179.

Hernandez, J. R., Martinez-Martinez, L., Canton, R., Coque, T. M. & Pascual, A. (2005). Nationwide study of *E. coli* and *K. pneumoniae* producing extended-spectrum β-lactamases in Spain. Antimicrob Agents Chemother 49: 2122-2125.

Hernandez-Alles, S., Conejo, M., Pascual, A., Tomas, J. M., Benedi, V. J. & Martinez-Martinez, L. (2000). Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of *K. pneumoniae*. J Antimicrob Chemother 46: 273-277.

Holder, C. D. & Halkias, D. (1988). Relapsing, bacteremic *K. pneumoniae* meningitis in an AIDS patient. Am J Med Sci 295: 55-59.

Hopkins, K. L., Deheer-Graham, A., Karisik, E., Batchelor, M. J., Liebana, E. & Threlfall, E. J. (2006). New plasmid-mediated AmpC β-lactamase (CMY-21) in *E. coli* isolated in the UK. Int J Antimicrob Agents 28: 80-82.

Hopkins, K. L., Wootton, L., Day, M. R. & Threlfall, E. J. (2007). Plasmid-mediated quinolone resistance determinant *qnrS1* found in Salmonella enterica strains isolated in the UK. J Antimicrob Chemother 59: 1071-1075.

Hossain, A., Reisbig, M. D. & Hanson, N. D. (2004). Plasmid-encoded functions compensate for the biological cost of AmpC overexpression in a clinical isolate of *Salmonella typhimurium*. J Antimicrob Chemother 53: 964-970.

Howard, C., van Daal, A., Kelly, G., Schooneveldt, J., Nimmo, G. & Giffard, P. M. (2002). Identification and minisequencing-based discrimination of SHV β-lactamases in nosocomial infection-associated *K. pneumoniae* in Brisbane, Australia. Antimicrob Agents Chemother 46: 659-664.

Hujer, A. M., Hujer, K. M., Helfand, M. S., Anderson, V. E. & Bonomo, R. A. (2002). Amino acid substitutions at Ambler position Gly238 in the SHV-1 β-lactamase: exploring sequence requirements for resistance to penicillins and cephalosporins. Antimicrob Agents Chemother 46: 3971-3977.

Huletsky, A., Knox, J. R. & Levesque, R. C. (1993). Role of Ser-238 and Lys-240 in the hydrolysis of third-generation cephalosporins by SHV-type β-lactamases probed by site-directed mutagenesis and three-dimensional modeling. J Biol Chem 268: 3690-3697.

Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labia, R. & Philippon, A. (2002). β-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob Agents Chemother 46: 3045-3049.

Huovinen, P., Huovinen, S. & Jacoby, G. A. (1988). Sequence of PSE-2 β-lactamase. Antimicrob Agents Chemother 32: 134-136.

Ishii, Y., Galleni, M., Ma, L., Frere, J. M. & Yamaguchi, K. (2007). Biochemical characterisation of the CTX-M-14 β-lactamase. Int J Antimicrob Agents 29: 159-164.

Izard, D., Ferragut, C., Gavini, F., Kersters, K., De Ley, J. & Leclerc, H. (1981). *Klebsiella terrigena*, a new species from soil and water. Int J Syst Bacteriol 31: 116-127.

Jack, G. W. & Richmond, M. H. (1970). A comparative study of eight distinct β-lactamases synthesized by Gram-negative bacteria. J Gen Microbiol 61: 43-61.

Jacoby, G. A. (2009). AmpC β-lactamases. Clin Microbiol Rev 22: 161-182.

Jacoby, G. A. & Carreras, I. (1990). Activities of β-lactam antibiotics against *E. coli* strains producing extended-spectrum β-lactamases. Antimicrob Agents Chemother 34: 858-862.

Jacoby, G. A., Walsh, K. E., Mills, D. M., Walker, V. J., Oh, H., Robicsek, A. & Hooper, D. C. (2006). *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob Agents Chemother 50: 1178-1182.

Jain, K., Radsak, K. & Mannheim, W. (1974). Differentiation of the Oxytocum group from *Klebsiella* by deoxyribonucleic acid hybridization. Int J Syst Bacteriol 24: 402-407.

Jarlier, V., Nicolas, M. H., Fournier, G. & Philippon, A. (1988). Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev Infect Dis 10: 867-878.

Jaurin, B. & Grundstrom, T. (1981). AmpC cephalosporinase of *E. coli* K-12 has a different evolutionary origin from that of β-lactamases of the penicillinase type. Proc Natl Acad Sci U S A 78: 4897-4901.

Jiang, Y., Zhou, Z., Qian, Y., Wei, Z., Yu, Y., Hu, S. & Li, L. (2008). Plasmid-mediated quinolone resistance determinants *qnr* and aac(6')-Ib-cr in extended-spectrum β-lactamase-producing *E. coli* and *K. pneumoniae* in China. J Antimicrob Chemother 61: 1003-1006.

Jonas, D., Biehler, K., Hartung, D., Spitzmuller, B. & Daschner, F. D. (2005). Plasmid-mediated quinolone resistance in isolates obtained in german intensive care units. Antimicrob Agents Chemother 49: 773-775.

Jones, R. N. (1998). Important and emerging β-lactamase-mediated resistances in hospital-based pathogens: the AmpC enzymes. Diagn Microbiol Infect Dis 31: 461-466.

Jove, T., Da Re, S., Denis, F., Mazel, D. & Ploy, M. C. (2010). Inverse correlation between promoter strength and excision activity in class 1 integrons. PLoS Genet 6: e1000793.

Kabha, K., Nissimov, L., Athamna, A., Keisari, Y., Parolis, H., Parolis, L. A., *et al.* (1995). Relationships among capsular structure, phagocytosis, and mouse virulence in *K. pneumoniae*. Infect Immun 63: 847-852.

Karim, A., Poirel, L., Nagarajan, S. & Nordmann, P. (2001). Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence IS*Ecp1*. FEMS Microbiol Lett 201: 237-241.

Kastowsky, M., Gutberlet, T. & Bradaczek, H. (1992). Molecular modelling of the three-dimensional structure and conformational flexibility of bacterial lipopolysaccharide. J Bacteriol 174: 4798-4806.

Keisari, Y., Kabha, K., Nissimov, L., Schlepper-Schafer, J. & Ofek, I. (1997). Phagocyte-bacteria interactions. Adv Dent Res 11: 43-49.

Keynan, Y. & Rubinstein, E. (2007). The changing face of *K. pneumoniae* infections in the community. Int J Antimicrob Agents 30: 385-389.

Kim, J., Shin, H. S., Seol, S. Y. & Cho, D. T. (2002). Relationship between bla_{SHV-12} and bla_{SHV-2a} in Korea. J Antimicrob Chemother 49: 261-267.

Kim, J. K., Chung, D. R., Wie, S. H., Yoo, J. H. & Park, S. W. (2009). Risk factor analysis of invasive liver abscess caused by the K1 serotype *K. pneumoniae*. Eur J Clin Microbiol Infect Dis 28: 109-111.

Kimura, S., Ishiguro, M., Ishii, Y., Alba, J. & Yamaguchi, K. (2004). Role of a mutation at position 167 of CTX-M-19 in ceftazidime hydrolysis. Antimicrob Agents Chemother 48: 1454-1460.

Kingston, W. (2004). Streptomycin, Schatz v. Waksman, and the balance of credit for discovery. J Hist Med Allied Sci 59: 441-462.

Kliebe, C., Nies, B. A., Meyer, J. F., Tolxdorff-Neutzling, R. M. & Wiedemann, B. (1985). Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrob Agents Chemother 28: 302-307.

Knothe, H., Shah, P., Krcmery, V., Antal, M. & Mitsuhashi, S. (1983). Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *K. pneumoniae* and *Serratia marcescens*. Infection 11: 315-317.

Knox, J. R. (1995). Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: mutations, specificity, and three-dimensional structure. Antimicrob Agents Chemother 39: 2593-2601.

Ko, W. C., Paterson, D. L., Sagnimeni, A. J., Hansen, D. S., Von Gottberg, A., Mohapatra, S., Casellas, J. M., *et al.* (2002). Community-acquired *K. pneumoniae* bacteremia: global differences in clinical patterns. Emerg Infect Dis 8: 160-166.

Kovtunovych, G., Lytvynenko, T., Negrutska, V., Lar, O., Brisse, S. & Kozyrovska, N. (2003). Identification of *K. oxytoca* using a specific PCR assay targeting the polygalacturonase *pehX* gene. Res Microbiol 154: 587-592.

Kruger, T., Szabo, D., Keddy, K. H., Deeley, K., Marsh, J. W., Hujer, A. M., *et al.* (2004). Infections with nontyphoidal *Salmonella* species producing TEM-63 or a novel TEM enzyme, TEM-131, in South Africa. Antimicrob Agents Chemother 48: 4263-4270.

Kurokawa, H., Yagi, T., Shibata, N., Shibayama, K., Kamachi, K. & Arakawa, Y. (2000). A new SHV-derived extended-spectrum β-lactamase (SHV-24) that hydrolyzes ceftazidime through a single-amino-acid substitution (D179G) in the Ω -loop. Antimicrob Agents Chemother 44: 1725-1727.

Kuzin, A. P., Nukaga, M., Nukaga, Y., Hujer, A. M., Bonomo, R. A. & Knox, J. R. (1999). Structure of the SHV-1 β-lactamase. Biochemistry 38: 5720-5727.

Lartigue, M. F., Poirel, L., Aubert, D. & Nordmann, P. (2006). In vitro analysis of IS*Ecp1B*-mediated mobilization of naturally occurring β-lactamase gene bla_{CTX-M} of *Kluyvera ascorbata*. Antimicrob Agents Chemother 50: 1282-1286.

Lau, S. H., Kaufmann, M. E., Livermore, D. M., Woodford, N., Willshaw, G. A., Cheasty, T., *et al.* **(2008).** UK epidemic *E. coli* strains A-E, with CTX-M-15 β-lactamase, all belong to the international O25:H4-ST131 clone. J Antimicrob Chemother 62: 1241-1244.

Laupland, K. B., Church, D. L., Vidakovich, J., Mucenski, M. & Pitout, J. D. (2008). Community-onset extended-spectrum β-lactamase (ESBL) producing *E. coli*: importance of international travel. J Infect 57: 441-448.

Lauretti, L., Riccio, M. L., Mazzariol, A., Cornaglia, G., Amicosante, G., Fontana, R. & Rossolini, G. M. (1999). Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob Agents Chemother 43: 1584-1590.

Lavilla, S., Gonzalez-Lopez, J. J., Sabate, M., Garcia-Fernandez, A., Larrosa, M. N., Bartolome, R. M., *et al.* **(2008).** Prevalence of *qnr* genes among extended-spectrum β-lactamase-producing enterobacterial isolates in Barcelona, Spain. J Antimicrob Chemother 61: 291-295.

Lavollay, M., Mamlouk, K., Frank, T., Akpabie, A., Burghoffer, B., Ben Redjeb, S., *et al.* **(2006).** Clonal dissemination of a CTX-M-15 β-lactamase-producing *E. coli* strain in the Paris area, Tunis, and Bangui. Antimicrob Agents Chemother 50: 2433-2438.

Lederman, E. R. & Crum, N. F. (2005). Pyogenic liver abscess with a focus on *K. pneumoniae* as a primary pathogen: an emerging disease with unique clinical characteristics. Am J Gastroenterol 100: 322-331.

Lee, K., Yum, J. H., Yong, D., Lee, H. M., Kim, H. D., Docquier, J. D., *et al.* **(2005).** Novel acquired metallo-β-lactamase gene, $bla_{(SIM-1)}$, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob Agents Chemother 49: 4485-4491.

Lee, Y. H., Cho, B., Bae, I. K., Chang, C. L. & Jeong, S. H. (2006). *K. pneumoniae* strains carrying the chromosomal SHV-11 β-lactamase gene produce the plasmid-mediated SHV-12 extended-spectrum β-lactamase more frequently than those carrying the chromosomal SHV-1 β-lactamase gene. J Antimicrob Chemother 57: 1259-1261.

Leflon-Guibout, V., Heym, B. & Nicolas-Chanoine, M. (2000). Updated sequence information and proposed nomenclature for $bla_{(TEM)}$ genes and their promoters. Antimicrob Agents Chemother 44: 3232-3234.

Li, C. K., Shing, M. M., Chik, K. W., Lee, V. & Yuen, P. M. (2001). *K. pneumoniae* meningitis in thalassemia major patients. Pediatr Hematol Oncol 18: 229-232.

Lin, J. C., Chang, F. Y., Fung, C. P., Xu, J. Z., Cheng, H. P., Wang, J. J., Huang, L. Y. & Siu, L. K. (2004). High prevalence of phagocytic-resistant capsular serotypes of *K. pneumoniae* in liver abscess. Microbes Infect 6: 1191-1198.

Liu, P. Y., Gur, D., Hall, L. M. & Livermore, D. M. (1992). Survey of the prevalence of β-lactamases amongst 1000 Gram-negative bacilli isolated consecutively at the Royal London Hospital. J Antimicrob Chemother 30: 429-447.

Livermore, D. M. (1995). β-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev 8: 557-584.

Livermore, D. M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G. M., Arlet, G., *et al.* **(2007).** CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother 59: 165-174.

Livermore, D. M., Hope, R., Brick, G., Lillie, M. & Reynolds, R. (2008). Non-susceptibility trends among Enterobacteriaceae from bacteraemias in the UK and Ireland, 2001-06. J Antimicrob Chemother 62: ii41-54.

Livermore, D. M. & Williams, J. D. (1996). Mode of action and mechanisms of bacterial resistance. In V. Lorian (Ed.), Antibiotics in Laboratory Medicine, 4th Ed. New York, Williams and Wilkins, Baltimore 502-578.

Livermore, D. M. & Woodford, N. (2006). The β-lactamase threat in Enterobacteriaceae, *Pseudomonas* and *Acinetobacter*. Trends Microbiol 14: 413-420.

Livermore, D. M. & Yuan, M. (1996). Antibiotic resistance and production of extended-spectrum β-lactamases amongst *Klebsiella* spp. from intensive care units in Europe. J Antimicrob Chemother 38: 409-424.

Llobet, E., Tomas, J. M. & Bengoechea, J. A. (2008). Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology 154: 3877-3886.

Luzzaro, F., Mezzatesta, M., Mugnaioli, C., Perilli, M., Stefani, S., Amicosante, G., *et al.* **(2006).** Trends in production of extended-spectrum β-lactamases among enterobacteria of medical interest: report of the second Italian nationwide survey. J Clin Microbiol 44: 1659-1664.

Lytsy, B., Sandegren, L., Tano, E., Torell, E., Andersson, D. I. & Melhus, A. (2008). The first major extended-spectrum β-lactamase outbreak in Scandinavia was caused by clonal spread of a multiresistant *K. pneumoniae* producing CTX-M-15. Apmis 116: 302-308.

Mabilat, C. & Goussard, S. (1993). PCR detection and identification of genes for extended-spectrum β-lactamases, p.553-559. In D.H. persing, T.F. Smith, F.C. Teneover and T.J. White (Ed.), Diagnostic molecular microbiology: principles and applications.American Society for Microbiology, Washington, D.C.

Machado, E., Canton, R., Baquero, F., Galan, J. C., Rollan, A., Peixe, L. *et al.* (2005). Integron content of extended-spectrum-β-lactamase-producing *E. coli* strains over 12 years in a single hospital in Madrid, Spain. Antimicrob Agents Chemother 49: 1823-1829.

Machado, E., Coque, T. M., Canton, R., Novais, A., Sousa, J. C., Baquero, F. & Peixe, L. (2007). High diversity of extended-spectrum β-lactamases among clinical isolates of Enterobacteriaceae from Portugal. J Antimicrob Chemother 60: 1370-1374.

Machida, C. & Machida, Y. (1987). Base substitutions in transposable element IS*1* cause DNA duplication of variable length at the target site for plasmid co-integration. Embo J 6: 1799-1803.

Maltezou, H. C. (2009). Metallo-β-lactamases in Gram-negative bacteria: introducing the era of pan-resistance? Int J Antimicrob Agents 33: 405 e401-407.

Mammeri, H., Poirel, L. & Nordmann, P. (2003). In vivo selection of a chromosomally encoded β-lactamase variant conferring ceftazidime resistance in *K. oxytoca*. Antimicrob Agents Chemother 47: 3739-3742.

Mammeri, H., Van De Loo, M., Poirel, L., Martinez-Martinez, L. & Nordmann, P. (2005). Emergence of plasmid-mediated quinolone resistance in *E. coli* in Europe. Antimicrob Agents Chemother 49: 71-76.

Marcade, G., Deschamps, C., Boyd, A., Gautier, V., Picard, B., Branger, C., Denamur, E. & Arlet, G. (2009). Replicon typing of plasmids in *E. coli* producing extended-spectrum β-lactamases. J Antimicrob Chemother 63: 67-71.

Martinez-Martinez, L., Hernandez-Alles, S., Alberti, S., Tomas, J. M., Benedi, V. J. & Jacoby, G. A. (1996). In vivo selection of porin-deficient mutants of *K. pneumoniae* with increased resistance to cefoxitin and expanded-spectrum-cephalosporins. Antimicrob Agents Chemother 40: 342-348.

Martinez-Martinez, L., Pascual, A. & Jacoby, G. A. (1998). Quinolone resistance from a transferable plasmid. Lancet 351: 797-799.

Mathew, A., Harris, A. M. & Marshall, M. J. (1975). The use of analytical isoelectric focusing for detection and identification of β-lactamases. J Gen Microbiol 88: 169-178.

Matsumoto, Y., Ikeda, F., Kamimura, T., Yokota, Y. & Mine, Y. (1988). Novel plasmid-mediated β-lactamase from *E. coli* that inactivates oxyimino-cephalosporins. Antimicrob Agents Chemother 32: 1243-1246.

Matthew, M. (1979). Plasmid-mediated β-lactamases of Gram-negative bacteria: properties and distribution. J Antimicrob Chemother 5: 349-358.

McMurry, L., Petrucci, R. E., Jr. & Levy, S. B. (1980). Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *E. coli.* Proc Natl Acad Sci USA 77: 3974-3977.

Medeiros, A. A. (1997). Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin Infect Dis 24: S19-45.

Mendonca, N., Ferreira, E., Louro, D. & Canica, M. (2009). Molecular epidemiology and antimicrobial susceptibility of extended- and broad-spectrum β-lactamase-producing *K. pneumoniae* isolated in Portugal. Int J Antimicrob Agents 34: 29-37.

Mendonca, N., Nicolas-Chanoine, M. H. & Canica, M. (2009). Diversity of the *bla*(SHV) genes. Diagn Microbiol Infect Dis 65: 439-446.

Mesko Meglic, K., Koren, S., Palepou, M. F., Karisik, E., Livermore, D. M., Pike, R., *et al.* **(2009).** Nationwide survey of CTX-M-type extended-spectrum β-lactamases among *K. pneumoniae* isolates in Slovenian hospitals. Antimicrob Agents Chemother 53: 287-291.

Milch, H. & Deak, S. (1964). Studies on *Klebsiella* infections by phage detection and phage typing. Acta Microbiol Acad Sci Hung 11: 251-261.

Miranda, G., Kelly, C., Solorzano, F., Leanos, B., Coria, R. & Patterson, J. E. (1996). Use of pulsed-field gel electrophoresis typing to study an outbreak of infection due to *Serratia marcescens* in a neonatal intensive care unit. J Clin Microbiol 34: 3138-3141.

Miriagou, V., Carattoli, A., Tzelepi, E., Villa, L. & Tzouvelekis, L. S. (2005). IS*26*-associated *In4*-type integrons forming multiresistance loci in enterobacterial plasmids. Antimicrob Agents Chemother 49: 3541-3543.

Miriagou, V., Filip, R., Coman, G. & Tzouvelekis, L. S. (2002). Expanded-spectrum cephalosporin-resistant *salmonella* strains in Romania. J Clin Microbiol 40: 4334-4336.

Miriagou, V., Tzouvelekis, L. S., Villa, L., Lebessi, E., Vatopoulos, A. C., Carattoli, A. & Tzelepi, E. (2004). CMY-13, a novel inducible cephalosporinase encoded by an *E. coli* plasmid. Antimicrob Agents Chemother 48: 3172-3174.

Mitsuhashi, S. & Inoue, M. (1981). Mechanisms of resistance to β-lactam antibiotics, pp 41–56. In S. Mitsuhashi (ed.), β-lactam antibiotics. Springer-Verlag, New York, NY.

Moland, E. S., Black, J. A., Hossain, A., Hanson, N. D., Thomson, K. S. & Pottumarthy, S. (2003). Discovery of CTX-M-like extended-spectrum β-lactamases in *E. coli* isolates from five US States. Antimicrob Agents Chemother 47: 2382-2383.

Moland, E. S., Hanson, N. D., Herrera, V. L., Black, J. A., Lockhart, T. J., Hossain, A., *et al.* (2003). Plasmid-mediated, carbapenem-hydrolysing β-lactamase, KPC-2, in *K. pneumoniae* isolates. J Antimicrob Chemother 51: 711-714.

Mollet, B., Iida, S., Shepherd, J. & Arber, W. (1983). Nucleotide sequence of IS*26*: a new prokaryotic mobile genetic element. Nucleic Acids Res 11: 6319-6330.

Monnet, D. & Freney, J. (1994). Method for differentiating *K. planticola* and *K. terrigena* from other *Klebsiella* species. J Clin Microbiol 32: 1121-1122.

Moranta, D., Regueiro, V., March, C., Llobet, E., Margareto, J., Larrate, E., *et al.* (2010). *K. pneumoniae* capsule polysaccharide impedes the expression of β-defensins by airway epithelial cells. Infect Immun 78: 1135-1146.

Mugnaioli, C., Luzzaro, F., De Luca, F., Brigante, G., Perilli, M., Amicosante, G., *et al.* (2006). CTX-M-type extended-spectrum β-lactamases in Italy: molecular epidemiology of an emerging countrywide problem. Antimicrob Agents Chemother 50: 2700-2706.

Mulvey, M. R., Bryce, E., Boyd, D., Ofner-Agostini, M., Christianson, S., Simor, A. E. & Paton, S. (2004). Ambler class A extended-spectrum β-lactamase-producing *E. coli* and *Klebsiella* spp. in Canadian hospitals. Antimicrob Agents Chemother 48: 1204-1214.

Munday, C. J., Whitehead, G. M., Todd, N. J., Campbell, M. & Hawkey, P. M. (2004). Predominance and genetic diversity of community- and hospital-acquired CTX-M extended-spectrum β-lactamases in York, UK. J Antimicrob Chemother 54: 628-633.

Murinda, S. E., Ebner, P. D., Nguyen, L. T., Mathew, A. G. & Oliver, S. P. (2005). Antimicrobial resistance and class 1 integrons in pathogenic *E. coli* from dairy farms. Foodborne Pathog Dis 2: 348-352.

Mushtaq, S., Woodford, N., Potz, N. & Livermore, D. M. (2003). Detection of CTX-M-15 extended-spectrum β-lactamase in the United Kingdom. J Antimicrob Chemother 52: 528-529.

Naas, T., Nordmann, P., Vedel, G. & Poyart, C. (2005). Plasmid-mediated carbapenem-hydrolyzing β-lactamase KPC in a *K. pneumoniae* isolate from France. Antimicrob Agents Chemother 49: 4423-4424.

Naas, T., Poirel, L. & Nordmann, P. (2008). Minor extended-spectrum β-lactamases. Clin Microbiol Infect 14: 42-52.

Nassif, X., Fournier, J. M., Arondel, J. & Sansonetti, P. J. (1989). Mucoid phenotype of *K. pneumoniae* is a plasmid-encoded virulence factor. Infect Immun 57: 546-552.

Nassif, X., Honore, N., Vasselon, T., Cole, S. T. & Sansonetti, P. J. (1989). Positive control of colanic acid synthesis in *E. coli* by *rmpA* and *rmpB*, two virulence-plasmid genes of *K. pneumoniae*. Mol Microbiol 3: 1349-1359.

Navon-Venezia, S., Chmelnitsky, I., Leavitt, A., Schwaber, M. J., Schwartz, D. & Carmeli, Y. (2006). Plasmid-mediated imipenem-hydrolyzing enzyme KPC-2 among multiple carbapenem-resistant *E. coli* clones in Israel. Antimicrob Agents Chemother 50: 3098-3101.

Nguyen Thi, P. L., Yassibanda, S., Aidara, A., Le Bouguenec, C. & Germani, Y. (2003). Enteropathogenic *K. pneumoniae* HIV-infected adults, Africa. Emerg Infect Dis 9: 135-137.

Nikaido, H. (1989). Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob Agents Chemother 33: 1831-1836.

Nikaido, H. (1998). Multiple antibiotic resistance and efflux. Curr Opin Microbiol 1: 516-523.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67: 593-656.

Nordmann, P. & Poirel, L. (2005). Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae. J Antimicrob Chemother 56: 463-469.

Novais, A., Canton, R., Moreira, R., Peixe, L., Baquero, F. & Coque, T. M. (2007). Emergence and dissemination of Enterobacteriaceae isolates producing CTX-M-1-like enzymes in Spain are associated with IncFII (CTX-M-15) and broad-host-range (CTX-M-1, -3, and -32) plasmids. Antimicrob Agents Chemother 51: 796-799.

Nuesch-Inderbinen, M. T., Kayser, F. H. & Hachler, H. (1997). Survey and molecular genetics of SHV β-lactamases in Enterobacteriaceae in Switzerland: two novel enzymes, SHV-11 and SHV-12. Antimicrob Agents Chemother 41: 943-949.

Oelschlaeger, T. A. & Tall, B. D. (1997). Invasion of cultured human epithelial cells by *K. pneumoniae* isolated from the urinary tract. Infect Immun 65: 2950-2958.

Ofek, I., Goldhar, J., Keisari, Y. & Sharon, N. (1995). Nonopsonic phagocytosis of microorganisms. Annu Rev Microbiol 49: 239-276.

Ogasawara, M., Kono, D. H. & Yu, D. T. (1986). Mimicry of human histocompatibility HLA-B27 antigens by *K. pneumoniae*. Infect Immun 51: 901-908.

Ohmori, S., Shiraki, K., Ito, K., Inoue, H., Ito, T., Sakai, T., Takase, K. & Nakano, T. (2002). Septic endophthalmitis and meningitis associated with *K. pneumoniae* liver abscess. Hepatol Res 22: 307-312.

Oliver, A., Coque, T. M., Alonso, D., Valverde, A., Baquero, F. & Canton, R. (2005). CTX-M-10 linked to a phage-related element is widely disseminated among Enterobacteriaceae in a Spanish hospital. Antimicrob Agents Chemother 49: 1567-1571.

Olson, A. B., Silverman, M., Boyd, D. A., McGeer, A., Willey, B. M., Pong-Porter, V., *et al.* (2005). Identification of a progenitor of the CTX-M-9 group of extended-spectrum β-lactamases from *Kluyvera georgiana* isolated in Guyana. Antimicrob Agents Chemother 49: 2112-2115.

Ørskov, I. (1984). Genus *Klebsiella Trevisan* 1885. In Bergey's Manual of Systematic Bacteriology, vol. 1, pp. 461-465. Krieg N. R., Holt J. G., ed. Baltimore: Williams & Wilkins.

Oteo, J., Cuevas, O., Lopez-Rodriguez, I., Banderas-Florido, A., Vindel, A., Perez-Vazquez, M., *et al.* (2009). Emergence of CTX-M-15-producing *K. pneumoniae* of multilocus sequence types 1, 11, 14, 17, 20, 35 and 36 as pathogens and colonizers in newborns and adults. J Antimicrob Chemother 64: 524-528.

Ouellette, M., Bissonnette, L. & Roy, P. H. (1987). Precise insertion of antibiotic resistance determinants into *Tn21*-like transposons: nucleotide sequence of the OXA-1 β-lactamase gene. Proc Natl Acad Sci USA 84: 7378-7382.

Pai, H., Choi, E. H., Lee, H. J., Hong, J. Y. & Jacoby, G. A. (2001). Identification of CTX-M-14 extended-spectrum β-lactamase in clinical isolates of *Shigella sonnei*, *E. coli*, and *K. pneumoniae* in Korea. J Clin Microbiol 39: 3747-3749.

Pallecchi, L., Bartoloni, A., Fiorelli, C., Mantella, A., Di Maggio, T., Gamboa, H., *et al.* (2007). Rapid dissemination and diversity of CTX-M extended-spectrum β-lactamase genes in commensal *E. coli* isolates from healthy children from low-resource settings in Latin America. Antimicrob Agents Chemother 51: 2720-2725.

Papanicolaou, G. A., Medeiros, A. A. & Jacoby, G. A. (1990). Novel plasmid-mediated β-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy β-lactams in clinical isolates of *K. pneumoniae*. Antimicrob Agents Chemother 34: 2200-2209.

Park, Y., Kang, H. K., Bae, I. K., Kim, J., Kim, J. S., Uh, Y., Jeong, S. H. & Lee, K. (2009). Prevalence of the extended-spectrum β-lactamase and *qnr* genes in clinical isolates of *E. coli*. Korean J Lab Med 29: 218-223.

Partridge, S. R. & Hall, R. M. (2005). Evolution of transposons containing *bla*~TEM~ genes. Antimicrob Agents Chemother 49: 1267-1268.

Paterson, D. L. (2006). Resistance in Gram-negative bacteria: Enterobacteriaceae. Am J Infect Control 34: S20-28.

Paterson, D. L. & Bonomo, R. A. (2005). Extended-spectrum β-lactamases: a clinical update. Clin Microbiol Rev 18: 657-686.

Paterson, D. L., Hujer, K. M., Hujer, A. M., Yeiser, B., Bonomo, M. D., Rice, L. B. & Bonomo, R. A. (2003). Extended-spectrum β-lactamases in *K. pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type β-lactamases. Antimicrob Agents Chemother 47: 3554-3560.

Paton, R., Miles, R. S., Hood, J., Amyes, S. G., Miles, R. S. & Amyes, S. G. (1993). ARI 1: β-lactamase-mediated imipenem resistance in *Acinetobacter baumannii*. Int J Antimicrob Agents 2: 81-87.

Perez-Perez, F. J. & Hanson, N. D. (2002). Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 40: 2153-2162.

Petit, A., Maveyraud, L., Lenfant, F., Samama, J. P., Labia, R. & Masson, J. M. (1995). Multiple substitutions at position 104 of β-lactamase TEM-1: assessing the role of this residue in substrate specificity. Biochem J 305: 33-40.

Petit, A., Sirot, D. L., Chanal, C. M., Sirot, J. L., Labia, R., Gerbaud, G. & Cluzel, R. A. (1988). Novel plasmid-mediated β-lactamase in clinical isolates of *K. pneumoniae* more resistant to ceftazidime than to other broad-spectrum cephalosporins. Antimicrob Agents Chemother 32: 626-630.

Philippon, A., Arlet, G. & Jacoby, G. A. (2002). Plasmid-determined AmpC-type β-lactamases. Antimicrob Agents Chemother 46: 1-11.

Pitout, J. D., Campbell, L., Church, D. L., Wang, P. W., Guttman, D. S. & Gregson, D. B. (2009). Using a commercial DiversiLab semiautomated repetitive sequence-based PCR typing technique for identification of *E. coli* clone ST131 producing CTX-M-15. J Clin Microbiol 47: 1212-1215.

Pitout, J. D., Church, D. L., Gregson, D. B., Chow, B. L., McCracken, M., Mulvey, M. R. & Laupland, K. B. (2007). Molecular epidemiology of CTX-M-producing *E. coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob Agents Chemother 51: 1281-1286.

Pitout, J. D., Hanson, N. D., Church, D. L. & Laupland, K. B. (2004). Population-based laboratory surveillance for *E. coli*-producing extended-spectrum β-lactamases: importance of community isolates with *bla*CTX-M genes. Clin Infect Dis 38: 1736-1741.

Pitout, J. D., Nordmann, P., Laupland, K. B. & Poirel, L. (2005). Emergence of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) in the community. J Antimicrob Chemother 56: 52-59.

Pitton, J. S. (1972). Mechanisms of bacterial resistance to antibiotics. Ergeb Physiol 65: 15-93.

Platt, H., Atherton, J. G. & Orskov, I. (1976). *Klebsiella* and *Enterobacter* organisms isolated from horses. J Hyg (Lond) 77: 401-408.

Podbielski, A., Schonling, J., Melzer, B. & Haase, G. (1991a). Different promoters of SHV-2 and SHV-2a β-lactamase lead to diverse levels of cefotaxime resistance in their bacterial producers. J Gen Microbiol 137: 1667-1675.

Podbielski, A., Schonling, J., Melzer, B., Warnatz, K. & Leusch, H. G. (1991b). Molecular characterization of a new plasmid-encoded SHV-type β-lactamase (SHV-2 variant) conferring high-level cefotaxime resistance upon *K. pneumoniae*. J Gen Microbiol 137: 569-578.

Podschun, R. & Sahly, H. (1991). Hemagglutinins of *K. pneumoniae* and *K. oxytoca* isolated from different sources. Zentralbl Hyg Umweltmed 191: 46-52.

Podschun, R. & Ullmann, U. (1998). *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11: 589-603.

Poirel, L., Cattoir, V. & Nordmann, P. (2008). Is plasmid-mediated quinolone resistance a clinically significant problem? Clin Microbiol Infect 14: 295-297.

Poirel, L., Decousser, J. W. & Nordmann, P. (2003). Insertion sequence IS*Ecp1B* is involved in expression and mobilization of a *bla*(CTX-M) β-lactamase gene. Antimicrob Agents Chemother 47: 2938-2945.

Poirel, L., Gniadkowski, M. & Nordmann, P. (2002). Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum β-lactamase CTX-M-15 and of its structurally related β-lactamase CTX-M-3. J Antimicrob Chemother 50: 1031-1034.

Poirel, L., Heritier, C., Podglajen, I., Sougakoff, W., Gutmann, L. & Nordmann, P. (2003). Emergence in *K. pneumoniae* of a chromosome-encoded SHV β-lactamase that compromises the efficacy of imipenem. Antimicrob Agents Chemother 47: 755-758.

Poirel, L., Kampfer, P. & Nordmann, P. (2002). Chromosome-encoded Ambler class A β-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum β-lactamases. Antimicrob Agents Chemother 46: 4038-4040.

Poirel, L., Le Thomas, I., Naas, T., Karim, A. & Nordmann, P. (2000). Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In*52* from *K. pneumoniae*. Antimicrob Agents Chemother 44: 622-632.

Poirel, L., Leviandier, C. & Nordmann, P. (2006). Prevalence and genetic analysis of plasmid-mediated quinolone resistance determinants *QnrA* and *QnrS* in Enterobacteriaceae isolates from a French university hospital. Antimicrob Agents Chemother 50: 3992-3997.

Poirel, L., Mammeri, H. & Nordmann, P. (2004). TEM-121, a novel complex mutant of TEM-type β-lactamase from *Enterobacter aerogenes*. Antimicrob Agents Chemother 48: 4528-4531.

Poirel, L., Naas, T., Le Thomas, I., Karim, A., Bingen, E. & Nordmann, P. (2001). CTX-M-type extended-spectrum β-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob Agents Chemother 45: 3355-3361.

Poirel, L., Naas, T. & Nordmann, P. (2008). Genetic support of extended-spectrum β-lactamases. Clin Microbiol Infect 14: 75-81.

Poirel, L. & Nordmann, P. (2006). Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. Clin Microbiol Infect 12: 826-836.

Poirel, L., Rodriguez-Martinez, J. M., Mammeri, H., Liard, A. & Nordmann, P. (2005). Origin of plasmid-mediated quinolone resistance determinant *QnrA*. Antimicrob Agents Chemother 49: 3523-3525.

Poirel, L., Weldhagen, G. F., Naas, T., De Champs, C., Dove, M. G. & Nordmann, P. (2001). GES-2, a class A β-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. Antimicrob Agents Chemother 45: 2598-2603.

Poole, K. (2004). Efflux-mediated multiresistance in Gram-negative bacteria. Clin Microbiol Infect 10: 12-26.

Poole, K. (2005). Efflux-mediated antimicrobial resistance. J Antimicrob Chemother 56: 20-51.

Potz, N. A., Hope, R., Warner, M., Johnson, A. P. & Livermore, D. M. (2006). Prevalence and mechanisms of cephalosporin resistance in Enterobacteriaceae in London and South-East England. J Antimicrob Chemother 58: 320-326.

Preston, K. E., Venezia, R. A. & Stellrecht, K. A. (2004). The SHV-5 extended-spectrum β-lactamase gene of pACM1 is located on the remnant of a compound transposon. Plasmid 51: 48-53.

Prinarakis, E. E., Miriagou, V., Tzelepi, E., Gazouli, M. & Tzouvelekis, L. S. (1997). Emergence of an inhibitor-resistant β-lactamase (SHV-10) derived from an SHV-5 variant. Antimicrob Agents Chemother 41: 838-840.

Queenan, A. M. & Bush, K. (2007). Carbapenemases: the versatile β-lactamases. Clin Microbiol Rev 20: 440-458.

Rahimian, J., Wilson, T., Oram, V. & Holzman, R. S. (2004). Pyogenic liver abscess: recent trends in etiology and mortality. Clin Infect Dis 39: 1654-1659.

Randegger, C. C. & Hachler, H. (2001). Amino acid substitutions causing inhibitor resistance in TEM β-lactamases compromise the extended-spectrum phenotype in SHV extended-spectrum β-lactamases. J Antimicrob Chemother 47: 547-554.

Rasmussen, B. A. & Bush, K. (1997). Carbapenem-hydrolyzing β-lactamases. Antimicrob Agents Chemother 41: 223-232.

Raymond, J., Nordmann, P., Doit, C., Vu Thien, H., Guibert, M., Ferroni, A. & Aujard, Y. (2007). Multidrug-resistant bacteria in hospitalized children: a 5-year multicenter study. Pediatrics 119: e798-803.

Reinert, R. R., Low, D. E., Rossi, F., Zhang, X., Wattal, C. & Dowzicky, M. J. (2007). Antimicrobial susceptibility among organisms from the Asia/Pacific Rim, Europe and Latin and North America collected as part of TEST and the in vitro activity of tigecycline. J Antimicrob Chemother 60: 1018-1029.

Rennie, R. P., Anderson, C. M., Wensley, B. G., Albritton, W. L. & Mahony, D. E. (1990). *K. pneumoniae* gastroenteritis masked by *Clostridium perfringens*. J Clin Microbiol 28: 216-219.

Reynolds, K. A., Thomson, J. M., Corbett, K. D., Bethel, C. R., Berger, J. M., Kirsch, J. F., *et al.* (2006). Structural and computational characterization of the SHV-1 β-lactamase-β-lactamase inhibitor protein interface. J Biol Chem 281: 26745-26753.

Rice, L. B., Carias, L. L., Hujer, A. M., Bonafede, M., Hutton, R., Hoyen, C. & Bonomo, R. A. (2000). High-level expression of chromosomally encoded SHV-1 β-lactamase and an outer membrane protein change confer resistance to ceftazidime and piperacillin-tazobactam in a clinical isolate of *K. pneumoniae*. Antimicrob Agents Chemother 44: 362-367.

Richmond, M. H. & Sykes, R. B. (1973). The β-lactamases of Gram-negative bacteria and their possible physiological role. Adv Microb Physiol 9: 31-88.

Robicsek, A., Jacoby, G. A. & Hooper, D. C. (2006). The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect Dis 6: 629-640.

Robicsek, A., Strahilevitz, J., Sahm, D. F., Jacoby, G. A. & Hooper, D. C. (2006). *qnr* prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. Antimicrob Agents Chemother 50: 2872-2874.

Robin, F., Delmas, J., Archambaud, M., Schweitzer, C., Chanal, C. & Bonnet, R. (2006). CMT-type β-lactamase TEM-125, an emerging problem for extended-spectrum β-lactamase detection. Antimicrob Agents Chemother 50: 2403-2408.

Rodriguez, M. M., Power, P., Radice, M., Vay, C., Famiglietti, A., Galleni, M., Ayala, J. A. & Gutkind, G. (2004). Chromosome-encoded CTX-M-3 from *Kluyvera ascorbata*: a possible origin of plasmid-borne CTX-M-1-derived cefotaximases. Antimicrob Agents Chemother 48: 4895-4897.

Rodriguez-Bano, J., Navarro, M. D., Romero, L., Martinez-Martinez, L., Muniain, M. A., Perea, E. J., *et al.* (2004). Epidemiology and clinical features of infections caused by extended-spectrum β-lactamase-producing *E. coli* in nonhospitalized patients. J Clin Microbiol 42: 1089-1094.

Rodriguez-Martinez, J. M., Poirel, L., Pascual, A. & Nordmann, P. (2006). Plasmid-mediated quinolone resistance in Australia. Microb Drug Resist 12: 99-102.

Rodriguez-Martinez, J. M., Velasco, C., Garcia, I., Cano, M. E., Martinez-Martinez, L. & Pascual, A. (2007a). Mutant prevention concentrations of fluoroquinolones for Enterobacteriaceae expressing the plasmid-carried quinolone resistance determinant *qnrA1*. Antimicrob Agents Chemother 51: 2236-2239.

Rodriguez-Martinez, J. M., Velasco, C., Garcia, I., Cano, M. E., Martinez-Martinez, L. & Pascual, A. (2007b). Characterisation of integrons containing the plasmid-mediated quinolone resistance gene *qnrA1* in *K. pneumoniae*. Int J Antimicrob Agents 29: 705-709.

Rolinson, G. N. (1998). Forty years of β-lactam research. J Antimicrob Chemother 41: 589-603.

Sabate, M., Miro, E., Navarro, F., Verges, C., Aliaga, R., Mirelis, B. & Prats, G. (2002). β-lactamases involved in resistance to broad-spectrum cephalosporins in *E. coli* and *Klebsiella* spp. clinical isolates collected between 1994 and 1996, in Barcelona, Spain. J Antimicrob Chemother 49: 989-997.

Sader, H. S., Hsiung, A., Fritsche, T. R. & Jones, R. N. (2007). Comparative activities of cefepime and piperacillin/tazobactam tested against a global collection of *E. coli* and *Klebsiella* spp. with an ESBL phenotype. Diagn Microbiol Infect Dis 57: 341-344.

Sahly, H. & Podschun, R. (1997). Clinical, bacteriological, and serological aspects of *Klebsiella* infections and their spondylarthropathic sequelae. Clin Diagn Lab Immunol 4: 393-399.

Sakazaki, R., Tamura, K., Kosako, Y. & Yoshizaki, E. (1989). *Klebsiella ornithinolytica* sp. nov., formerly known as ornithinepositive *K. oxytoca*. Curr Microbiol 18: 201-206.

Saladin, M., Cao, V. T., Lambert, T., Donay, J. L., Herrmann, J. L., Ould-Hocine, Z., *et al.* (2002). Diversity of CTX-M β-lactamases and their promoter regions from Enterobacteriaceae isolated in three Parisian hospitals. FEMS Microbiol Lett 209: 161-168.

Sanbongi, Y., Ida, T., Ishikawa, M., Osaki, Y., Kataoka, H., Suzuki, T., *et al.* (2004). Complete sequences of six penicillin-binding protein genes from 40 *Streptococcus pneumoniae* clinical isolates collected in Japan. Antimicrob Agents Chemother 48: 2244-2250.

Sarno, R., McGillivary, G., Sherratt, D. J., Actis, L. A. & Tolmasky, M. E. (2002). Complete nucleotide sequence of *K. pneumoniae* multiresistance plasmid pJHCMW1. Antimicrob Agents Chemother 46: 3422-3427.

Sawai, T., Mitsuhashi, S. & Yamagishi, S. (1968). Drug resistance of enteric bacteria. XIV. Comparison of β-lactamases in Gram-negative rod bacteria resistant to alpha-aminobenzylpenicillin. Jpn J Microbiol 12: 423-434.

Schelenz, S., Bramham, K. & Goldsmith, D. (2007). Septic arthritis due to extended spectrum beta lactamase producing *K. pneumoniae*. Joint Bone Spine 74: 275-278.

Schmidtke, A. J. & Hanson, N. D. (2006). Model system to evaluate the effect of *ampD* mutations on AmpC-mediated β-lactam resistance. Antimicrob Agents Chemother 50: 2030-2037.

Schneider, I., Queenan, A. M. & Bauernfeind, A. (2006). Novel carbapenem-hydrolyzing oxacillinase OXA-62 from *Pandoraea pnomenusa*. Antimicrob Agents Chemother 50: 1330-1335.

Schwaber, M. J., Navon-Venezia, S., Kaye, K. S., Ben-Ami, R., Schwartz, D. & Carmeli, Y. (2006). Clinical and economic impact of bacteremia with extended-spectrum-β-lactamase-producing Enterobacteriaceae. Antimicrob Agents Chemother 50: 1257-1262.

Scott, I. U., Matharoo, N., Flynn, H. W., Jr. & Miller, D. (2004). Endophthalmitis caused by *Klebsiella* species. Am J Ophthalmol 138: 662-663.

Seale, M., Lee, W. K., Daffy, J., Tan, Y. & Trost, N. (2007). Fulminant endogenous *K. pneumoniae* endophthalmitis: imaging findings. Emerg Radiol 13: 209-212.

Shannon, K., Stapleton, P., Xiang, X., Johnson, A., Beattie, H., El Bakri, F., *et al.* (1998). Extended-spectrum β-lactamase-producing *K. pneumoniae* strains causing nosocomial outbreaks of infection in the United Kingdom. J Clin Microbiol 36: 3105-3110.

Simoons-Smit, A. M., Verweij-van Vught, A. M. & MacLaren, D. M. (1986). The role of K antigens as virulence factors in *Klebsiella*. J Med Microbiol 21: 133-137.

Sirot, D., Labia, R., Pouedras, P., Chanal-Claris, C., Cerceau, C. & Sirot, J. (1998). Inhibitor-resistant OXY-2-derived β-lactamase produced by *K. oxytoca*. Antimicrob Agents Chemother 42: 2184-2187.

Sirot, D., Recule, C., Chaibi, E. B., Bret, L., Croize, J., Chanal-Claris, C., Labia, R. & Sirot, J. (1997). A complex mutant of TEM-1 β-lactamase with mutations encountered in both IRT-4 and extended-spectrum TEM-15, produced by an *E. coli* clinical isolate. Antimicrob Agents Chemother 41: 1322-1325.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Catry, B., Herman, L., Haesebrouck, F. & Butaye, P. (2008). Diversity of extended-spectrum β-lactamases and class C β-lactamases among cloacal *E. coli* Isolates in Belgian broiler farms. Antimicrob Agents Chemother 52: 1238-1243.

Song, W., Kim, J. S., Kim, H. S., Park, M. J. & Lee, K. M. (2005). Appearance of *Salmonella enterica* isolates producing plasmid-mediated AmpC β-lactamase, CMY-2, in South Korea. Diagn Microbiol Infect Dis 52: 281-284.

Sougakoff, W., Goussard, S., Gerbaud, G. & Courvalin, P. (1988). Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes. Rev Infect Dis 10: 879-884.

Sowek, J. A., Singer, S. B., Ohringer, S., Malley, M. F., Dougherty, T. J., Gougoutas, J. Z. & Bush, K. (1991). Substitution of lysine at position 104 or 240 of TEM-1pTZ18R β-lactamase enhances the effect of serine-164 substitution on hydrolysis or affinity for cephalosporins and the monobactam aztreonam. Biochemistry 30(13): 3179-3188.

Spratt, B. G. (1994). Resistance to antibiotics mediated by target alterations. Science 264: 388-393.

Strampfer, M. J., Schoch, P. E. & Cunha, B. A. (1987). Cerebral abscess caused by *Klebsiella ozaenae*. J Clin Microbiol 25: 1553-1554.

Struve, C., Bojer, M. & Krogfelt, K. A. (2008). Characterization of *K. pneumoniae* type 1 fimbriae by detection of phase variation during colonization and infection and impact on virulence. Infect Immun 76: 4055-4065.

Struve, C., Bojer, M. & Krogfelt, K. A. (2009). Identification of a conserved chromosomal region encoding *K. pneumoniae* type 1 and type 3 fimbriae and assessment of the role of fimbriae in pathogenicity. Infect Immun 77: 5016-5024.

Struve, C., Bojer, M., Nielsen, E. M., Hansen, D. S. & Krogfelt, K. A. (2005). Investigation of the putative virulence gene *magA* in a worldwide collection of 495 *Klebsiella* isolates: *magA* is restricted to the gene cluster of *K. pneumoniae* capsule serotype K1. J Med Microbiol 54: 1111-1113.

Sykes, R. B. & Matthew, M. (1976). The β-lactamases of Gram-negative bacteria and their role in resistance to β-lactam antibiotics. J Antimicrob Chemother 2: 115-157.

Tang, L. M., Chen, S. T., Hsu, W. C. & Chen, C. M. (1997). *Klebsiella* meningitis in Taiwan: an overview. Epidemiol Infect 119: 135-142.

Tipper, D. J. & Strominger, J. L. (1965). Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci USA 54: 1133-1141.

Toleman, M. A., Bennett, P. M. & Walsh, T. R. (2006). IS*CR* elements: novel gene-capturing systems of the 21st century? Microbiol Mol Biol Rev 70: 296-316.

Toleman, M. A., Simm, A. M., Murphy, T. A., Gales, A. C., Biedenbach, D. J., Jones, R. N. & Walsh, T. R. (2002). Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. J Antimicrob Chemother 50: 673-679.

Tran, J. H. & Jacoby, G. A. (2002). Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci USA 99: 5638-5642.

Tran, J. H., Jacoby, G. A. & Hooper, D. C. (2005). Interaction of the plasmid-encoded quinolone resistance protein *Qnr* with *E. coli* DNA gyrase. Antimicrob Agents Chemother 49: 118-125.

Tumbarello, M., Spanu, T., Sanguinetti, M., Citton, R., Montuori, E., Leone, F., Fadda, G. & Cauda, R. (2006). Bloodstream infections caused by extended-spectrum-β-lactamase-producing *K. pneumoniae*: risk factors, molecular epidemiology, and clinical outcome. Antimicrob Agents Chemother 50: 498-504.

Turner, M. S., Andersson, P., Bell, J. M., Turnidge, J. D., Harris, T. & Giffard, P. M. (2009). Plasmid-borne *bla*SHV genes in *K. pneumoniae* are associated with strong promoters. J Antimicrob Chemother 64: 960-964.

Turnidge, J., Bell, J., Biedenbach, D. J. & Jones, R. N. (2002). Pathogen occurrence and antimicrobial resistance trends among urinary tract infection isolates in the Asia-Western Pacific Region: report from the SENTRY Antimicrobial Surveillance Program, 1998-1999. Int J Antimicrob Agents 20: 10-17.

Tzouvelekis, L. S. & Bonomo, R. A. (1999). SHV-type β-lactamases. Curr Pharm Des 5: 847-864.

Valverde, A., Coque, T. M., Garcia-San Miguel, L., Baquero, F. & Canton, R. (2008). Complex molecular epidemiology of extended-spectrum β-lactamases in *K. pneumoniae*: a long-term perspective from a single institution in Madrid. J Antimicrob Chemother 61: 64-72.

Villegas, M. V., Lolans, K., Correa, A., Kattan, J. N., Lopez, J. A. & Quinn, J. P. (2007). First identification of *Pseudomonas aeruginosa* isolates producing a KPC-type carbapenem-hydrolyzing β-lactamase. Antimicrob Agents Chemother 51: 1553-1555.

Villegas, M. V., Lolans, K., Correa, A., Suarez, C. J., Lopez, J. A., Vallejo, M. & Quinn, J. P. (2006). First detection of the plasmid-mediated class A carbapenemase KPC-2 in clinical isolates of *K. pneumoniae* from South America. Antimicrob Agents Chemother 50: 2880-2882.

Vimont, S., Mnif, B., Fevre, C. & Brisse, S. (2008). Comparison of PFGE and multilocus sequence typing for analysis of *K. pneumoniae* isolates. J Med Microbiol 57: 1308-1310.

Vinue, L., Saenz, Y., Somalo, S., Escudero, E., Moreno, M. A., Ruiz-Larrea, F. & Torres, C. (2008). Prevalence and diversity of integrons and associated resistance genes in faecal *E. coli* isolates of healthy humans in Spain. J Antimicrob Chemother 62: 934-937.

Wacharotayankun, R., Arakawa, Y., Ohta, M., Tanaka, K., Akashi, T., Mori, M. & Kato, N. (1993). Enhancement of extracapsular polysaccharide synthesis in *K. pneumoniae* by RmpA2, which shows homology to NtrC and FixJ. Infect Immun 61: 3164-3174.

Walsh, T. R. (2005). The emergence and implications of metallo-β-lactamases in Gram-negative bacteria. Clin Microbiol Infect 11: 2-9.

Walsh, T. R., Toleman, M. A., Poirel, L. & Nordmann, P. (2005). Metallo-β-lactamases: the quiet before the storm? Clin Microbiol Rev 18: 306-325.

Walther-Rasmussen, J. & Hoiby, N. (2006). OXA-type carbapenemases. J Antimicrob Chemother 57: 373-383.

Wang, M., Cao, B., Yu, Q., Liu, L., Gao, Q., Wang, L. & Feng, L. (2008). Analysis of the 16S-23S rRNA gene internal transcribed spacer region in *Klebsiella* species. J Clin Microbiol 46: 3555-3563.

Wang, M., Guo, Q., Xu, X., Wang, X., Ye, X., Wu, S. & Hooper, D. C. (2009). New plasmid-mediated quinolone resistance gene, *qnrC*, found in a clinical isolate of *Proteus mirabilis*. Antimicrob Agents Chemother 53: 1892-1897.

Wang, M., Sahm, D. F., Jacoby, G. A., Zhang, Y. & Hooper, D. C. (2004). Activities of newer quinolones against *E. coli* and *K. pneumoniae* containing the plasmid-mediated quinolone resistance determinant *qnr*. Antimicrob Agents Chemother 48: 1400-1401.

Wang, M., Tran, J. H., Jacoby, G. A., Zhang, Y., Wang, F. & Hooper, D. C. (2003). Plasmid-mediated quinolone resistance in clinical isolates of *E. coli* from Shanghai, China. Antimicrob Agents Chemother 47: 2242-2248.

Watanabe, M., Iyobe, S., Inoue, M. & Mitsuhashi, S. (1991). Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 35: 147-151.

Wei, Z. Q., Du, X. X., Yu, Y. S., Shen, P., Chen, Y. G. & Li, L. J. (2007). Plasmid-mediated KPC-2 in a *K. pneumoniae* isolate from China. Antimicrob Agents Chemother 51: 763-765.

Weldhagen, G. F. (2004). Integrons and β-lactamases: a novel perspective on resistance. Int J Antimicrob Agents 23: 556-562.

Wendt, C., Schutt, S., Dalpke, A. H., Konrad, M., Mieth, M., Trierweiler-Hauke, B., *et al.* (2010). First outbreak of *K. pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* in Germany. Eur J Clin Microbiol Infect Dis 29: 563-570.

Wilke, M. S., Lovering, A. L. & Strynadka, N. C. (2005). β-lactam antibiotic resistance: a current structural perspective. Curr Opin Microbiol 8: 525-533.

Winokur, P. L., Canton, R., Casellas, J. M. & Legakis, N. (2001). Variations in the prevalence of strains expressing an extended-spectrum β-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. Clin Infect Dis 32: S94-103.

Wong, J. S., Chan, T. K., Lee, H. M. & Chee, S. P. (2000). Endogenous bacterial endophthalmitis: an East Asian experience and a reappraisal of a severe ocular affliction. Ophthalmology 107: 1483-1491.

Woodford, N., Tierno, P. M., Jr., Young, K., Tysall, L., Palepou, M. F., Ward, E., *et al.* (2004). Outbreak of *K. pneumoniae* producing a new carbapenem-hydrolyzing class A β-lactamase, KPC-3, in a New York Medical Center. Antimicrob Agents Chemother 48: 4793-4799.

Woodford, N., Ward, M. E., Kaufmann, M. E., Turton, J., Fagan, E. J., James, D., et al. (2004). Community and hospital spread of *E. coli* producing CTX-M extended-spectrum β-lactamases in the UK. J Antimicrob Chemother 54: 735-743.

Woodford, N., Zhang, J., Warner, M., Kaufmann, M. E., Matos, J., Macdonald, A., et al. (2008). Arrival of *K. pneumoniae* producing KPC carbapenemase in the United Kingdom. J Antimicrob Chemother 62: 1261-1264.

Wu, J. H., Wu, A. M., Tsai, C. G., Chang, X. Y., Tsai, S. F. & Wu, T. S. (2008). Contribution of fucose-containing capsules in *K. pneumoniae* to bacterial virulence in mice. Exp Biol Med (Maywood) 233: 64-70.

Wu, S. W., Dornbusch, K. & Kronvall, G. (1999). Genetic characterization of resistance to extended-spectrum β-lactams in *K. oxytoca* isolates recovered from patients with septicemia at hospitals in the Stockholm area. Antimicrob Agents Chemother 43: 1294-1297.

Wu, T. L., Siu, L. K., Su, L. H., Lauderdale, T. L., Lin, F. M., Leu, H. S., Lin, T. Y. & Ho, M. (2001). Outer membrane protein change combined with co-existing TEM-1 and SHV-1 β-lactamases lead to false identification of ESBL-producing *K. pneumoniae*. J Antimicrob Chemother 47: 755-761.

Yagi, T., Kurokawa, H., Shibata, N., Shibayama, K. & Arakawa, Y. (2000). A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *K. pneumoniae* and *E. coli* in Japan. FEMS Microbiol Lett 184: 53-56.

Yamasaki, K., Komatsu, M., Yamashita, T., Shimakawa, K., Ura, T., Nishio, H., et al. (2003). Production of CTX-M-3 extended-spectrum β-lactamase and IMP-1 metallo β-lactamase by five Gram-negative bacilli: survey of clinical isolates from seven laboratories collected in 1998 and 2000, in the Kinki region of Japan. J Antimicrob Chemother 51: 631-638.

Yanagawa, T., Nakamura, H., Takei, I., Maruyama, H., Kataoka, K., Saruta, T. & Kobayashi, Y. (1989). *K. pneumoniae* meningitis associated with liver abscess: a case report. Jpn J Antibiot 42: 2135-2140.

Yates, C. & Amyes, S. (2005). Extended-spectrum β-lactamases in non-typhoidal *Salmonella* spp. isolated in the UK are now a reality: why the late arrival? J Antimicrob Chemother 56: 262-264.

Yates, C. M., Brown, D. J., Edwards, G. F. & Amyes, S. G. (2004). Detection of TEM-52 in *Salmonella enterica* serovar *Enteritidis* isolated in Scotland. J Antimicrob Chemother 53: 407-408.

Yigit, H., Queenan, A. M., Anderson, G. J., Domenech-Sanchez, A., Biddle, J. W., Steward, C. D., et al. (2001). Novel carbapenem-hydrolyzing β-lactamase,

KPC-1, from a carbapenem-resistant strain of *K. pneumoniae*. Antimicrob Agents Chemother 45: 1151-1161.

Yu, W. L., Ko, W. C., Cheng, K. C., Lee, H. C., Ke, D. S., Lee, C. C., *et al.* (2006). Association between *rmpA* and *magA* genes and clinical syndromes caused by *K. pneumoniae* in Taiwan. Clin Infect Dis 42: 1351-1358.

Zhao, S., White, D. G., Ge, B., Ayers, S., Friedman, S., English, L., *et al.* (2001). Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *E. coli* isolates. Appl Environ Microbiol 67: 1558-1564.

Zienkiewicz, M., Kern-Zdanowicz, I., Golebiewski, M., Zylinska, J., Mieczkowski, P., Gniadkowski, M., *et al.* (2007). Mosaic structure of p1658/97, a 125-kilobase plasmid harboring an active amplicon with the extended-spectrum β-lactamase gene *bla*$_{SHV-5}$. Antimicrob Agents Chemother 51: 1164-1171.