Molecular Characterisation of ESBLs from *Klebsiella pneumoniae* and *Escherichia coli*

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Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

2002

Abstract

The most common mechanism of resistance in clinically significant bacteria to β-lactam antibiotics is the production of Class A β-lactamase enzymes that hydrolyse the β-lactam bond of this family of antibiotics. Extended-spectrum β-lactamases (ESBLs) are produced and expressed by Enterobacteriaceae. They are derived from bla_{TEM-1}, bla_{TEM-2} and bla_{SHV-1} genes. ESBLs are capable of inactivating second and third generation cephalosporins. They have been found mainly in *Klebsiella pneumoniae* and *Escherichia coli*, while more recently in other species of Enterobacteriaceae and Gram-negative bacteria.

In this study, one hundred-and-one unique patient isolates of *K. pneumoniae* (69) and *E.coli* (32) from different patients, flagged as ESBL-positive by the Vitek system (GNS 526 card) were collected. These strains were isolated from a variety of clinical specimens submitted to the clinical bacteriology laboratories of the Royal Infirmary of Edinburgh (RIE). Of the 101 strains tested, 15 *E.coli* were subsequently found to be ESBL-negative by E-test ESBL strips. On re-testing with Vitek using the (GNS 532 card which had superseded the GNS 526 card), 14 of these were found to be ESBL-negative despite originally flagging as ESBL-positive. The remaining 87 ESBL-producing strains were also tested with E-test ESBL strips and additionally subjected to the double disc diffusion (DDD) method for the detection of ESBLs. Of these, one was falsely negative for ESBL-production by E-test ESBL strips, and 7 were false negative by the double disc diffusion method. The 87 isolates were tested for susceptibility to a wide range of

antibiotics by both Vitek microdilution technique (NCCLS) and disc diffusion method (NCCLS).

In addition, MICs of the isolates were obtained by agar dilution to aminoglycosides (gentamicin and tobramycin) and the quinolone, ciprofloxacin, and by E-test strips to cefotaxime and ceftazidime.

To study the epidemiology of ESBL producing strains of *K. pneumoniae* and *E.coli*, all isolates were examined by Pulsed-field gel-electrophoresis (PFGE) analysis using the *Xba*1 restriction enzyme. *K. pneumoniae* demonstrated seven distinct clusters, each containing isolates with similar coefficients, which were identified among 62 isolates. A high level of genetic heterogeneity was found among six other isolates, and one isolate was non-typeable by PFGE. PFGE demonstrated that 5 of the 18 *E.coli* were genetically related, 2 isolates belonged to one group, 8 isolates were genetically distinct and 3 were non-typeable. The presence of bla_{TEM} and bla_{SHV} were detected by dot-blot hybridisation and polymerase chain reaction (PCR) for all 87 isolates. β-lactamase and genetic material from strains demonstrating presumption ESBL production were subjected to further study by a combination of Iso-electric focusing (IEF) and PCR-restriction fragment length polymorphisms (PCR-RFLP) respectively. Four β-lactamase bands with pI values of 5.4, 7.0, 7.6 and 8.2 were identified.

The epidemiology of ciprofloxacin-resistance and its relationship to ESBL producing *K. pneumoniae* was studied for 32 (46.6%) isolates that were found to be resistant to ciprofloxacin.

To investigate the epidemiological relationship between the ciprofloxacin resistant/ESBL-positive strains, PFGE analysis indicated that the clinical isolates belonged to 4 distinct genotypes (A,B,C and D). PCR was used to amplify the *gyr*A and *par*C genes from genomic DNA of the ciprofloxacin-resistant isolates. The amplified product was sent for analysis by automated DNA sequencing and the resulting DNA sequence compared with *gyr*A and *par*C genes of *K. pneumoniae*. The sequencing results demonstrated that alteration of the *gyr*A subunit DNA gyrase at amino acid 83 and/or amino acid 87 was shown to play a central role in conferring high-level quinolone resistance in *K. pneumoniae* possessing ESBLs.

Declaration

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

Ali Dashti

Acknowledgements

I am indebted to many people who have assisted me in various ways throughout my PhD Study:

I would like to thank my supervisors Prof. Sebastian Amyes and Dr. Robert Paton for their excellent supervision, encouragement and support of this work over the past three years.

I am grateful to my parents without whom I would have never have got this far, I would like to thank them for their prayers and encouragement during my study.

I would like thank my brothers, sisters, parents in law and best friends for their moral support and encouragement.

I wish to thank all my colleagues and staff of molecular chemotherapy for their helpful, friendship and support.

I would like to thank Kuwait University for the scholarship and for providing me with the opportunity and financial support.

Finally, a special thank must go to my wife Tasneem for her love, patience and encouragement during my study. If it was not her support, this thesis would not have been completed.

Publications and Presntations

Dashti, A. A., Miles, R. S., Amyes, S. G. B. and Paton, R. (2001). Efficacy of Vitek system for detection Extended-Spectrum β-lactamase in clinical isolates of *Klebsiella pneumoniae* and *E.coli*. 22nd International Congress of Chemotherapy, Amsterdam, Netherlands.

Dashti, A. A., Miles, R. S., Amyes, S. G. B. and Paton, R. (2002). Epidemiology of ciprofloxacin resistance and its relationship to ESBL-producing *Klebsiella pneumoniae*. 12th European Congress of Clinical Microbiology and Infectious Disease, Milan, Italy.

Dashti, A. A., Amyes, S. G. B. and Paton, R. (2002). Characterisation of Extended-Spectrum β-lactamases identified by the Vitek system and their subsequent comparison with other commercial ESBL-testing systems. Journal of Clinical Pathology. (paper submitted for publication).

Bello, H., Dominguez, M., **Dashti, A. A.,** Gonzales-Rocha, G. and Amyes, S. G. B. (2002). SHV-39: a new Extended-Spectrum β-lactamase found throughout Chile. Abstract of the 42 Inter-science Conference on Antimicrobial Agent and Chemotherapy, San Diego, USA.

Abbreviations

7- ACA	7-amino cephalosporanic acid
λ	Wave length of light
*Amp*C	Ampicillin C
bla	beta-lactamase gene
bp	base pairs
DDD	Double Disc Diffusion
DNA	Deoxy ribonucleic acid
dNTP	Deoxy nucleotide triphosphate
ESBL	Extended-spectrum beta-lactamases
E.coli	*Escherichia coli*
E-test	Epsilon test
ISs	Insertion of insertion sequence
IEF	Isoelectric focusing
IRT	Inhibition resistant beta-lactamase
Kb	Kilo base
KDa	Kilo Daltons
K. pneumoniae	*Klebsiella pneumoniae*
L	Litre
MIC	Minimum Inhibitory Concentration
M	Molar
mM	milli molar
ml	milli litre
mg	milli gram
μg	micro gram
μl	micro litre
min	minutes
NCCLS	National Committee for Clinical Laboratory Standard
NCTC	National Collection of Type Cultures
NNISS	National Nosocomial Infection Study System

OMP	Outer membrane protein
PCR	Polymerase Chain Reaction
PBP	Penicillin-Binding-Protein
PFGE	Pulsed-field Gel Electrophoresis
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
P-CMB	P-chloromercuribenzonate
QRDR	Quinolone Resistance Determining Region
RNase	Ribonuclease
RIE	Royal Infirmary of Edinburgh
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium Dodecyl Sulphate
Sec	Seconds
SHV	Sulp-hydryl Variable
S.R61	Streptomyces R61
TAE	Tris-acetate buffer
TBE	Tris-boric buffer
TEM	contraction of Temoneria
TEMED	Tetramethyllethylenediamine
Tris	Tris (hydroxy methyl) methyl amide
UV	Ultraviolet
V	Volts
V/v	Volume per volume
W	Watts
W/v	Weight per volume

The standard single letter and three letter abbreviations are used for the amino acids

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Chapter 1

Introduction

1.1 Prologue

The discovery of antibiotics was one of the greatest advances in the history of medicine, saving more lives than any other clinical armamentarium, increasing average life expectancy by years.

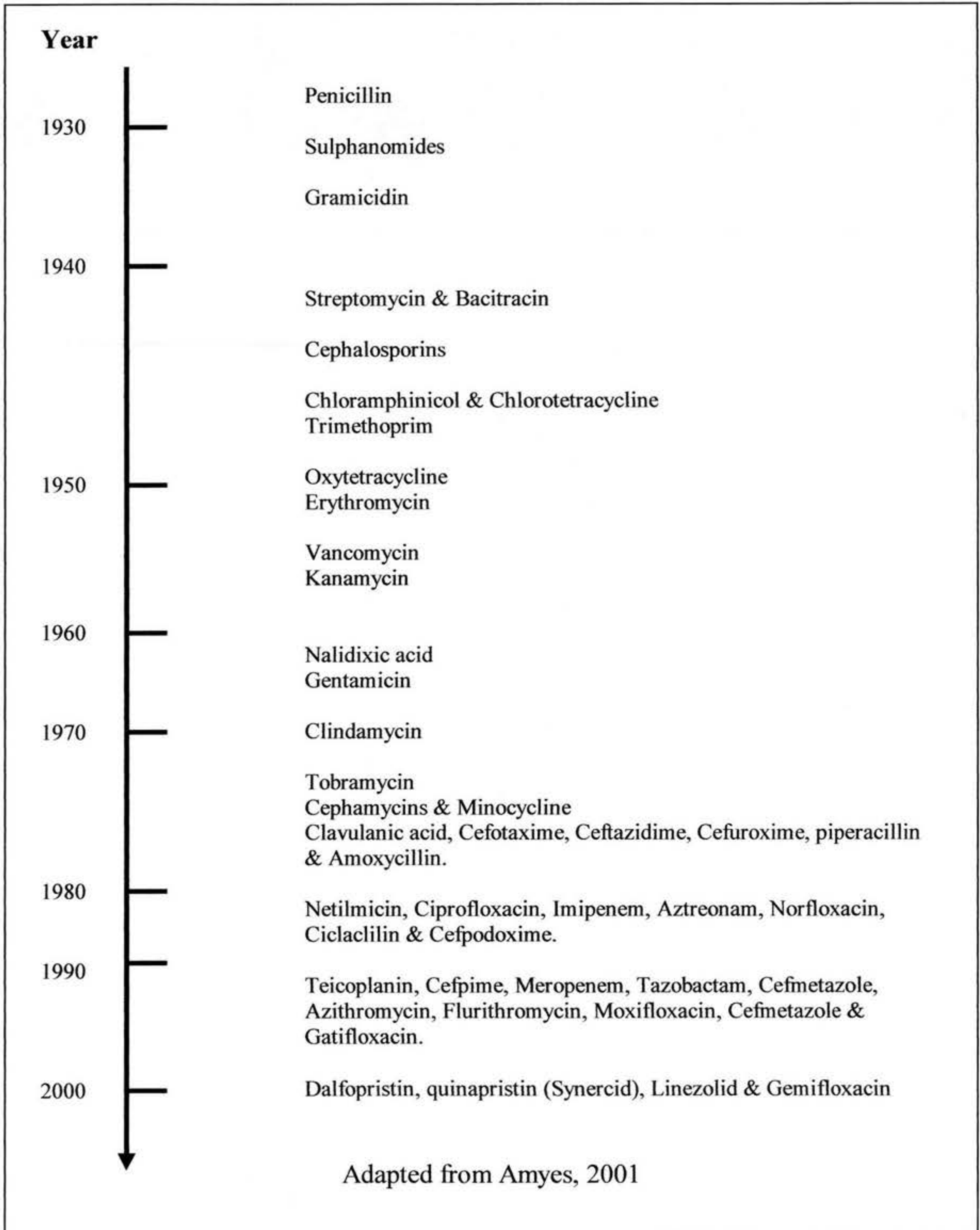
In 1969 an historic statement was made at that time by US Surgeon General testifying to the congress "…it is the time to close the book on infectious diseases"*. This statement was made at an optimistic time when more than 25,000 antibiotic products had been developed and antibiotic resistance was not a major public health issue.

Heavy use of antibiotics in clinical treatment began with the introduction of penicillins in the 1940s and over the next sixty years numerous classes of antibiotics have been introduced (see figure 1.1). However, the widespread use of these antibiotics in medicine and animal husbandry has compromised their success and provided strong selective pressure for the emergence and persistence of bacterial resistance to these antibiotics. Pharmaceutical companies have tried to overcome this dilemma by modification of existing antibiotics and the introduction of newly developed novel drugs into clinical use. However, the genetic versatility of bacteria has enabled them to respond by modifying existing resistance mechanisms or by acquiring resistance genes from other bacteria.

* Quote taken from: report on regional medical programs to the president and the congress

Figure 1.1

Evolution of antibiotics classes over the last sixty years



Adapted from Amyes, 2001

1.2 Introduction

In the 1890s Paul Ehrlich (Ehrlich, 1913) predicted that chemical compounds could be found or synthesized that would specifically inhibit or kill parasitic micro-organisms without damage to the host. Such synthetic compounds have been found. By contrast, penicillin is an antibiotic, a naturally occurring compound synthesized by one organism but capable in low concentration of inhibiting or destroying another (selective toxicity) (Ehrlich, 1913).

The general term antimicrobial agent includes antibiotics and synthetic compounds such as the sulfonamides and trimethoprim. Bacteria are able to develop resistance to antimicrobials, and it is only by a combination of judicious use and ongoing intensive research that antimicrobial chemotherapy continues to be effective in most cases and to be one of the hallmarks of modern medical practice. Nowadays, many different classes of antibiotics are used individually or in combination in clinical treatment regimens but the most commonly used and frequently prescribed group of antimicrobials are the β-lactams. β-lactams comprise a wide selection namely; penicillins, cephalosporins, carbapenems in addition to other related compounds.

1.3 What is an antibiotic ?

Webster's New World Dictionary of the American language defines an antibiotic as " any of certain chemical substances produced by various microorganisms, especially bacteria, fungi and actinomycetes, and having the capacity, in dilute solutions, to inhibit the growth of or to destroy bacteria and other microorganisms" . This definition leaves us in the somewhat awkward position of having to use more cumbersome terms to allow the inclusion of drugs that inhibit the growth or destroy bacteria and other microorganisms but are not produced by microorganisms. Such compounds include the sulfonamides and trimethoprim; man-made modifications of microbial products, such as the semi-synthetic penicillins; and alkylation of aminoglycosides such as amikacin and netilmicin.

Lietman, in 1986, thought it would be more appropriate to redefine an antibiotic as "any of certain chemical substances produced by various microorganisms or by humans and having the capacity, in dilute solutions, to inhibit the growth of or to destroy bacteria and other microorganisms" (Lietman, 1986).

The essence of an antibiotic is its selective toxicity. Selective toxicity refers to the capacity of an antibiotic to adversely affect essential bacterial processes without adversely affecting the host's cells. Paul Ehrlich, is credited with the term "magic bullet" used to describe selective toxicity " a chemical that was selectively toxic to invading microbes but innocuous to humans" (Lietman, 1986).

1.4 The birth of penicillin

The discovery of penicillin ranks as one of the most important in the history of medicine (Fleming, 1929). It was the first, and remains the most important, of the antibiotics. Pneumonia, puerperal fever, bacterial meningitis and staphylococcal septicaemia remain common diseases, however, the development of penicillin revolutionized their treatment.

Penicillin was discovered by Alexander Fleming in 1928 (Hare, 1970). At the end of July in 1928, Fleming left his laboratory, unaware that he was due to make an historic discovery when he returned in September of the same year. One of the dishes that he had inoculated previously and left on the bench, caught his eye. He observed that a contaminating mould was causing apparent lysis of colonies of *Staphylococcus aureus* he had previously inoculated onto the plate. The colonies took on a "ghost" like appearance, indicating, that the mould was causing the bacteria to lyse. The mould was subsequently identified as *Penicillium notatum* (Fleming, 1929).

Fleming was later to receive worldwide acclaim for the discovery and is still generally regarded as the man who gave penicillin to the world. However, while Fleming undoubtedly discovered penicillin, and was first to try and use it to cure infections, his attempts to achieve the all important purification were largely unsuccessful. Nor did he achieve the first cures with penicillin, an accolade which fell to one of his former students, Cecil G. Paine, who did his work independently of Fleming at the Royal Infirmary in Sheffield during 1929-1930 (Hare, 1970; Wainwright, 1990).

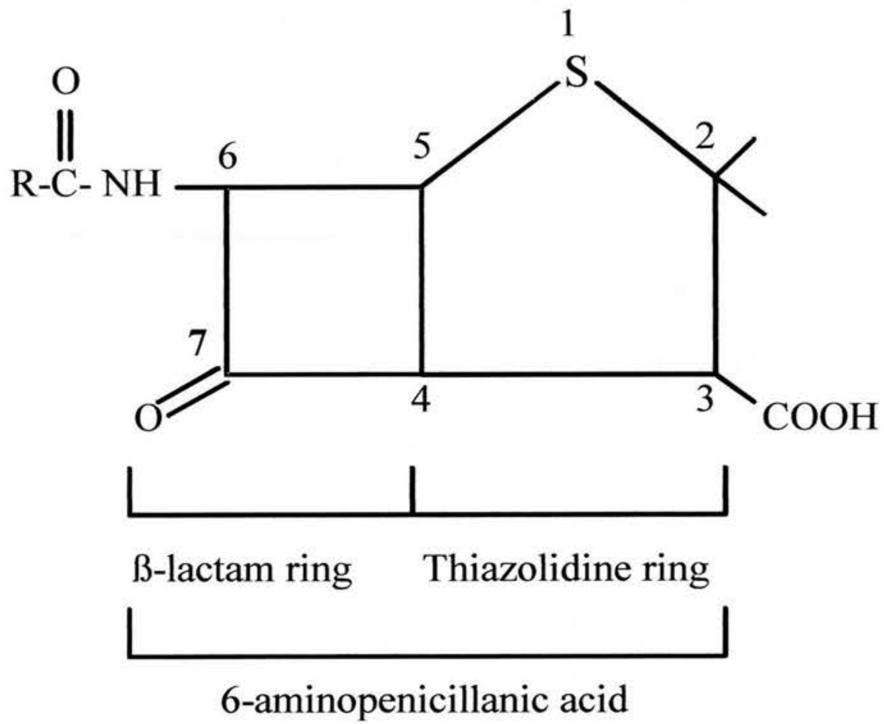
The miraculous therapeutic powers of penicillin were to remain unrecognized, however, until around 1940, when it was purified by a team of Oxford University scientists led by Howard Florey (Wainwright, 1990). The development of penicillin then moved to the USA, where scientists were to be remarkably successful at producing large quantities of cheap penicillin just in time to treat Allied soldiers, from D-day onwards.

The discovery and subsequent development of penicillin is one of the most remarkable stories to emerge from the history of medical and scientific research. its appearance encouraged scientists to search for new antimicrobial agents which then entered the physician's armoury. The chemical structure of penicillins is shown in figure 1.2

1.5 β-lactam antibiotics

β-lactam antibiotics are among the most widely used drugs in clinical practice around the world. This group of antibiotics consists of penicillin, cephalosporins, monobactams and carbapenems. This family can be divided into bi-cyclic penicillins (penams, penams carbapenems, oxapenams), cephalosporins (cephems, cephamycins, oxacephems, carbacephems) and the monocyclic monobactams (see figure 1.3). The possession of an intact β-lactam ring is essential to their antibacterial activity. Differences in side chains of the basic molecules influence pharmacological properties and antibacterial spectra by determining permeability into the bacterial cell, affinity for enzymes involved in cell wall synthesis, and susceptibility or resistance to inactivation by β-lactamase enzymes.

Figure 1.2



Chemical structure of penicillins

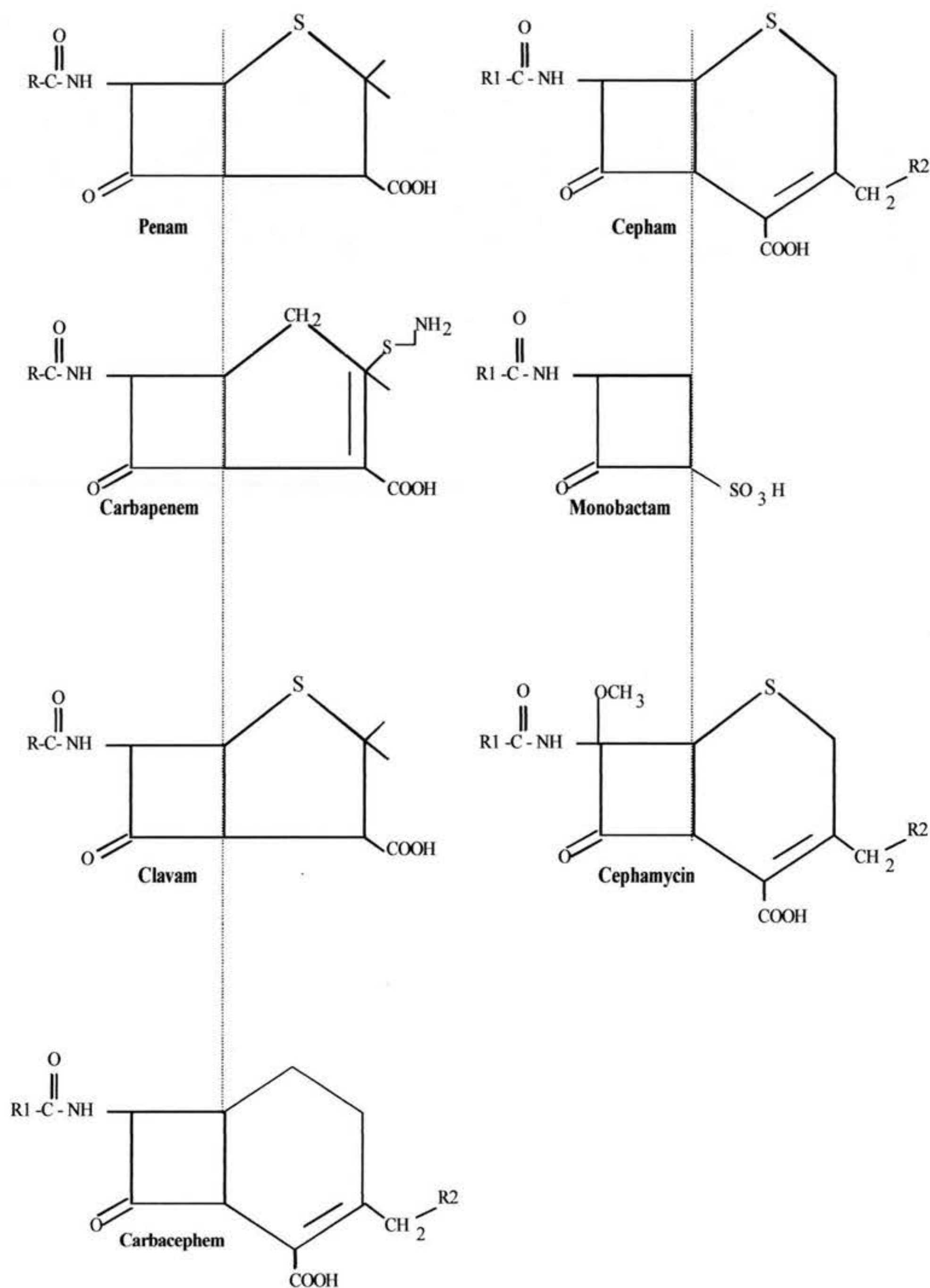
β-lactam antibiotics are usually bactericidal to the growing susceptible bacteria (Neu, 1986).

1.5.1 Mode of action of β-lactam antibiotics

β-lactams inhibit synthesis of the cell wall, a structurally unique feature found in Gram-negative and Gram-positive bacteria but which is absent from mammalian cells. The cell wall which provides bacteria with a physical barrier against substances that may damage the cell. The major component of the cell wall, which plays a vital role in providing rigidity and strength to this structure, is the polymer peptidoglycan. β-lactams act by interfering with the biosynthesis of peptidoglycan, specifically targeting the enzymes involved in the final stages of its assembly (Greenwood, 1995).

Bacteria may have existed more than 3.5 billion years ago (Holland, 1997; Manchester *et al.*, 1995). A primary structural feature of bacteria is the cell wall whose function is to provide support for the maintenance of bacterial morphology (Massova and Mobashery, 1998).

Figure 1.3



Skeletons of ß-lactam antibiotics.
The ß-lactam ring (to the left of the dashed line) is shared by all these compounds.

1.5.2 The structure of the bacterial cell wall

The cell wall of Gram-negative bacteria differs in structure from that of Gram-positive bacteria. Each possess peptidoglycan, but the wall of Gram-positive bacteria is a thicker, less complex structure consisting of a network of cross-linked peptidoglycan interspersed with teichoic acid and teichuronic acid polymers, amounts of which vary between species (Neidhardt *et al.*, 1990a). Gram-negative bacteria have a much thinner peptidoglycan layer, which is covered by a lipid bi-layer outer membrane. This consists of lipopolysaccharide and protein and prevents many hydrophilic and some hydrophobic molecules from penetrating the cell. Nutrients required by the cell are able to gain entry through this barrier via protein channels called porins located within the outer membrane (Neidhardt *et al.*, 1990a). These channels are also utilized by the hydrophilic β-lactam antibiotics to gain access to their target enzymes located within the periplasmic space (Nikaido, 1993). The differential activity of β-lactams is due to their ability to cross the membrane and move through the porin channels, which in turn is determined by their size as well as physicochemical structure (Livermore, 1987).

In general, peptidoglycan is made of linear chains of alternating β1-4 linked N-acetyl glucosamine and N-acetlylmuramic acid residues that are crossed-linked via oligopeptide bridges attached to the muramic acid residues (Neidhardt *et al.*, 1990b). Convention consist of the synthesis of pentapeptide precursors in the cytoplasm that are transported across the cell membrane, after which they are added to the existing peptidoglycan chain. There are a number of enzymes involved in the final cross-linking process (Barnikel *et al.*, 1983). Transpeptidases catalyse the formation of a

peptide bond between the carboxyl of the penultimate D-alanine of one pentapeptide, and the amino group of the middle amino acid of another pentapeptide. The loss of the second pentapeptide's terminal D-alanine, which is catalysed by carboxypeptidases, provide the energy required for this reaction (Barnikel *et al.*, 1983).

1.5.3 Penicillin-binding-proteins (PBPs)

The targets of β-lactam antibiotics are proteins with transpeptidase, caboxypeptidase, and transglycosylase activity (Georgopapadoakou, 1993; Ghuysen, 1991; Ghuysen, 1997; Jamin *et al.*, 1995). They are also known as penicillin-binding–proteins (PBPs) since they are able to bind penicillin efficiently (Massova and Mobashery, 1998). PBPs 1a, 1b, 2 and 3 of Gram-negative bacteria have essential functions in the peptidoglycan synthesis (Ghuysen, 1991). They are numbered in descending order according to their molecular weight. In addition, their numbers vary between species (Ghuysen, 1994; Ghuysen, 1997). The N-terminal domain of PBP 1a and PBP 1b display transglycosylase activity (Ghuysen, 1997). β-lactams exert their effect by their action as substrate analogues for the PBPs. The amide group (O=C-N) of the β-lactam ring is similar in confirmation to the carboxyl terminal of the D-alanyl-D-alanine. PBPs thus effectively recognise the antibiotic as they would their normal substrate, allowing it to interfere with the cell wall formation by commandeering the active-site cavity (Tipper and Strominger, 1965). PBPs share a common feature, in which the C-terminal transpeptidase domain with a conserved active-site serine residue is acylated by the β-lactam resulting in the formation of a stable covalent ester and inactivation of the enzyme (Ghuysen, 1988).

Such interaction depends on the enzyme that is involved. PBPs 1, 2 and 3 are transpeptidase penicillin-binding proteins to which most β-lactams bind (Georgapapadoakou, 1993; Massova and Mobashery, 1998).

1.6 Antibiotic resistance in bacteria

1.6.1 The origin of bacterial resistance

Antibiotic resistance was observed soon after the advent of antimicrobial chemotherapy in clinical practice. The first β-lactamase was identified even before the wide spread use of penicillin (Abraham and Chain, 1940).

1.6.2 Antibiotics and their producers

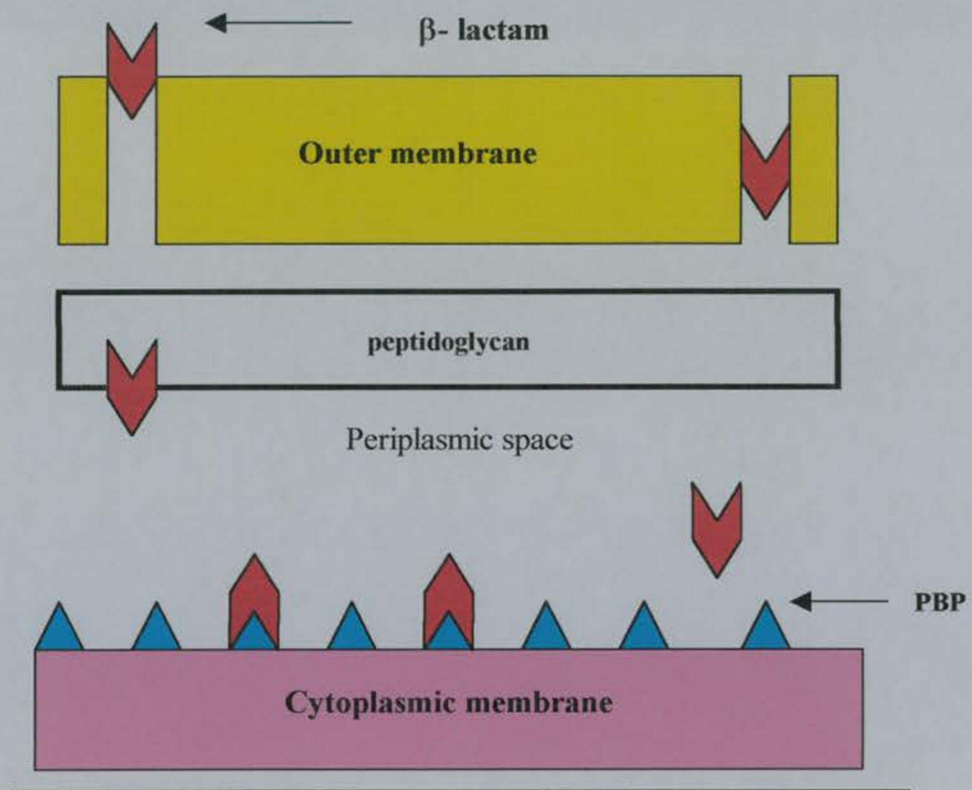
Many theories abound to explain the function of antibiotics in producer microorganisms. The most plausible theory suggests a functional role in the survival of producing organisms, thus offering a competitive advantage to the cell (Brian, 1957). It has even been suggested that antibiotics act as bacterial pheromones in promoting the transfer of plasmids between different bacteria (Mazodier and Davies, 1991). What is clearly established is that antibiotics are secondary metabolites that are produced after the logarithmic growth phase of the organism, and that enzymes involved in antibiotic biosynthesis are repressed during active growth. Demain, in 1974 stated another theory, namely that they have a regulatory role during the transition from vegetative cell to spore in sporulation organisms (Demain, 1974).

1.6.3 Mechanisms of resistance to β-lactams

Many antibiotics in use today were discovered in soil microorganisms. It is hardly surprising, therefore, that bacteria have developed the remarkable ability to acquire resistance to them. There is evidence suggesting that one source of resistance genes found in clinical isolates are soil micro-organisms which produce many of the antibiotics in use today (Thompson and Gary, 1983). There are other potential sources, for instance, the modification of genes encoding essential metabolic enzymes, also known as 'housekeeping' genes (Rather *et al.*, 1993).

β-lactam antibiotics exert their antimicrobial effect by interfering with bacterial cell wall biosynthesis. This is accomplished by the drugs attaching covalently to their targets, PBPs. The PBPs are diverse enzymes involved in cell wall synthesis, and are anchored in the cytoplasmic membrane of the bacterium (Georgopapadoakou, 1993; Pitout *et al.*, 1997). The site at which ß-lactam drugs bind to PBPs is located on the portion of the PBP that extends into the periplasmic space of Gram-negative bacteria (Pitout *et al.*, 1997). Covalent binding to PBPs interferes with the synthesis of cell wall and ultimately leads to cell death. To reach target PBPs, β-lactam antibiotics must penetrate through the outer membrane of Gram-negative bacteria porins, because the hydrophilic nature of these drugs precludes passage through the hydrophobic proteins of the outer membrane (Pitout *et al.*, 1997). (See figure 1.4). In Gram-positive bacteria the outer-membrane is absent and the β-lactam molecule can attach directly to PBPs on the cytoplasmic membrane.

Figure 1.4



In Gram-negative bacteria, β-lactam antibiotics must enter through porins in the outer membrane, traverse the periplasmic space, and attach to their target penicillin-binding proteins (PBPs) located on the outer aspect of the cytoplasmic membrane

Resistance to β-lactam antibiotics may occur at any of the steps involved in the action of the antibiotic. There are essentially 3 main mechanisms described, by which bacteria evade the action of β-lactam antibiotics:

1- Alteration in outer membrane permeability (in Gram –ve bacteria).

2- Alteration in target enzymes PBPs (in both Gram –ve and Gram +ve bacteria).

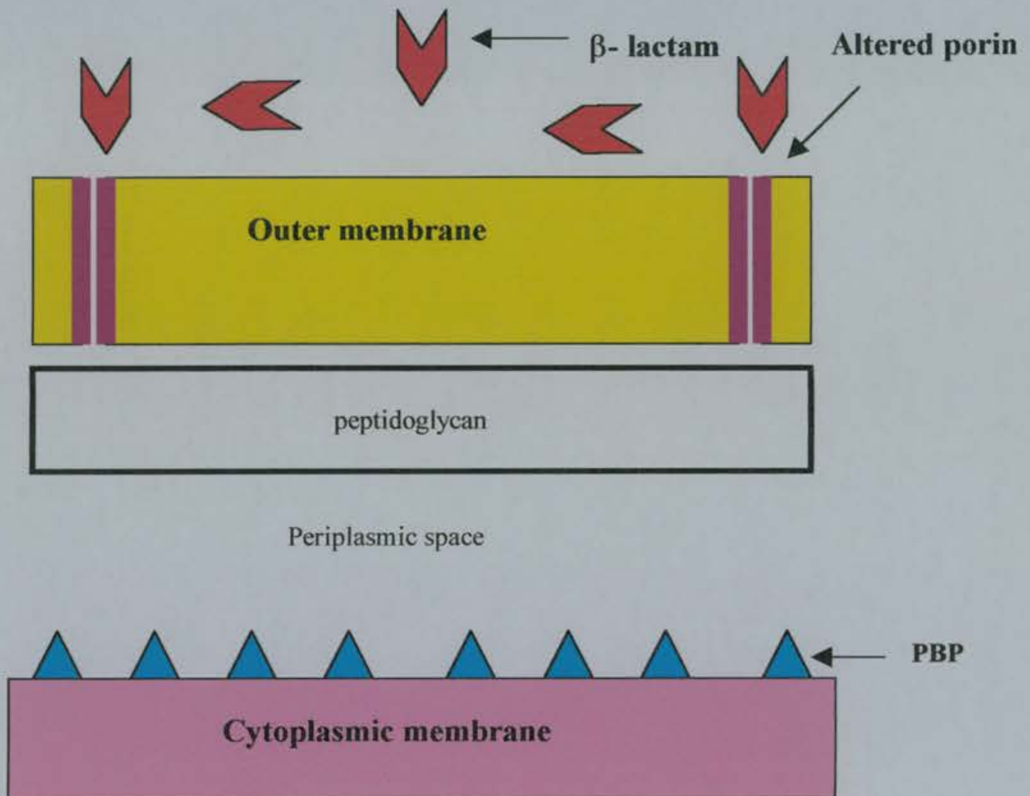
3- Enzymatic degradation of the drug (production of β-lactamases in both Gram –ve and Gram +ve bacteria).

1.6.3.1 Alteration in outer membrane permeability

In Gram-negative bacteria, the presence of porin channels in their outer membrane facilitates the trans-membrane diffusion of hydrophilic molecules, including β-lactams (Nikaido, 1989; Yoshimura and Nikaido, 1985). However, mutations resulting in the loss of a specific porin can result in an increase in resistance to β-lactams. This is evident with *Pseudomonas aeruginosa*, a pathogen renowned for its intrinsic resistance to a wide range of antibiotics (Pitout *et al.*, 1997; Yoshimura and Nikaido, 1985).

Increased resistance to β-lactams has also been associated with a decrease in the number of porins produced by some bacteria (see figure 1.5),(Yamazaki *et al.*, 1989). Loss of porin expression in clinical isolates of *Klebsiella pneumoniae* due to insertion of insertion sequences (ISs) within the porin gene is a mechanism that has been described in isolates obtained from patients under going antibiotic treatment (Hernandez *et al.*, 1999).

Figure 1.5



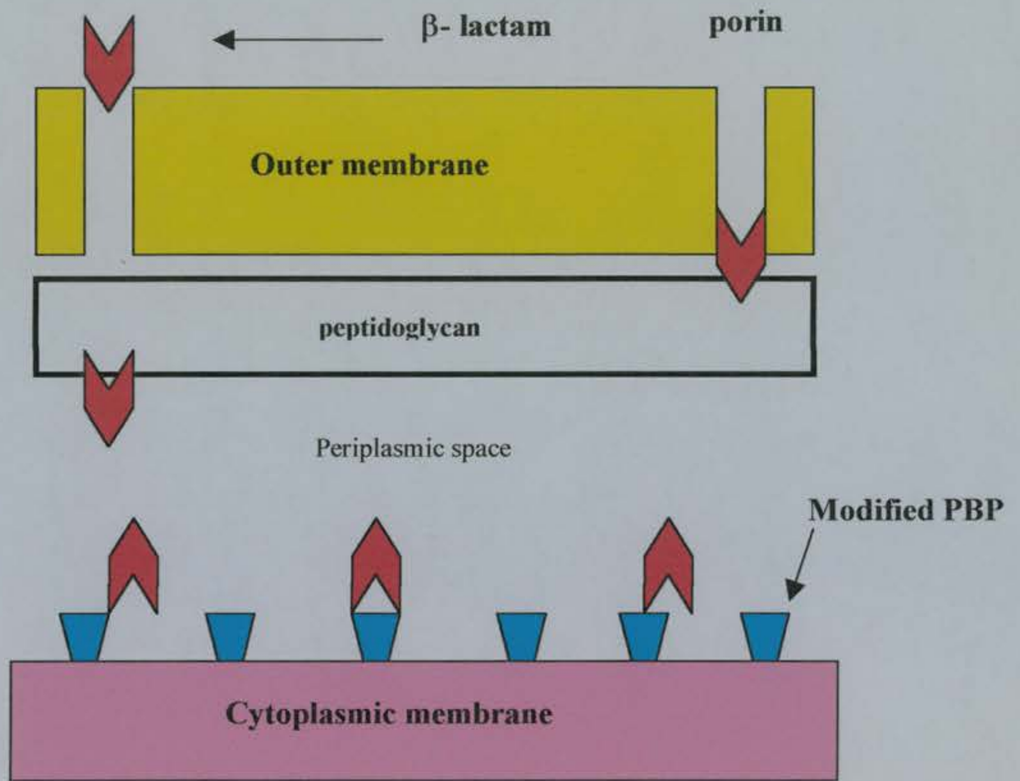
Alteration in porin proteins that impede drug (β-lactam antibiotic) penetration into the bacterial cell

It is usually insufficient for reduced permeability alone to prevent antibiotic resistance, and subsequently, it generally occurs in conjunction with a second mechanism to produce this effect (Nikaido, 1981). In the case of β-lactams, this additional mechanism is usually the production of β-lactamases (Hancock and Bell, 1988).

1.6.3.2 Alteration in target enzymes (PBPs)

Alteration in the binding characteristics of PBPs for β-lactam compounds may result in the development of resistance (Malouin and Bryan, 1986). (See figure 1.6). Alteration in target enzymes is more common in Gram-positive bacteria, and in certain fastidious Gram-negatives (Livermore and Wood, 1990). Clinical resistance to β-lactams in Gram-negative bacteria is not commonly associated with alteration in PBPs (Spratt, 1988). It is generally not regarded as a primary resistance mechanism in other Gram-negative bacteria that have more efficient mechanisms at their disposal. It is likely that in bacteria in which this resistance mechanisms occurs, the development of β-lactam resistance is as a result of multiple amino acid substitutions, each resulting in a relatively small decrease in affinity of the PBP for the β-lactam, and this may potentially alter the stability of the enzyme. Re-stabilising amino acid substitutions may also occur (Hedge and Spratt, 1985).

Figure 1.6



Modification of the target site of (β-lactam antibiotics), the PBPs

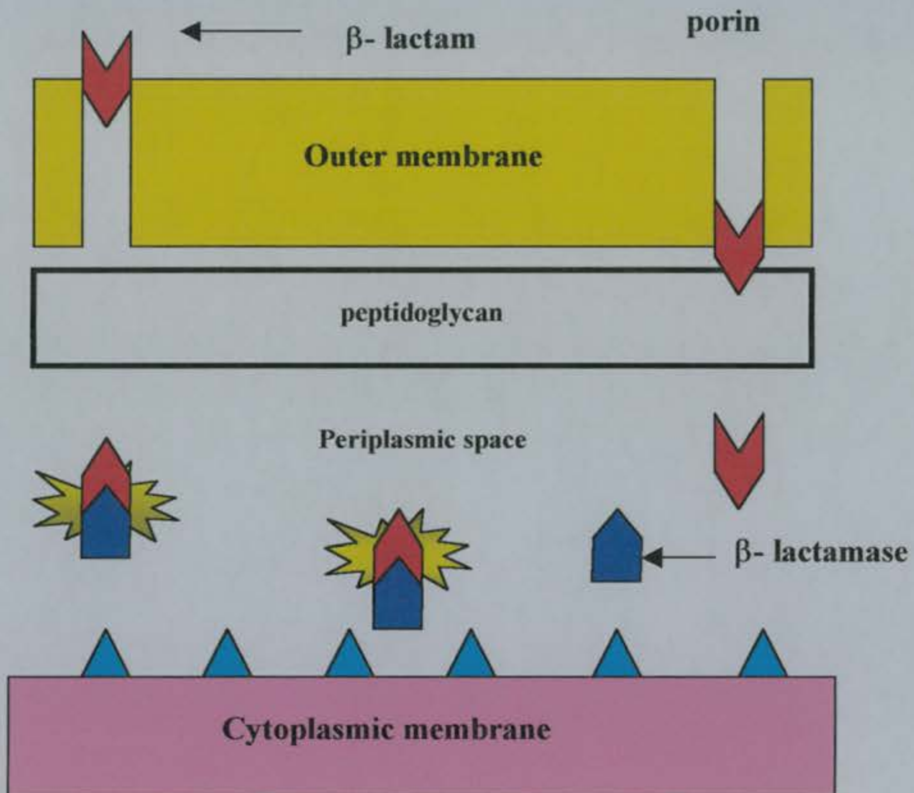
1.6.3.3 Enzymatic inactivation of substrate

Enzymatic inactivation by the production of β-lactamase (see figure 1.7) is by far the most widespread as well as the most common mechanism of resistance to β-lactam antibiotics. β-lactamase enzymes cleave the amide bond of the β-lactam ring thus rendering inactive a vast number of these antibiotics (Dever and Dermody, 1991). β-lactamases are produced by both Gram-negative and Gram-positive bacteria, and their impact on antimicrobial efficacy has been immense.

1.7 The origin and evolution of β-lactamases

It has been conceded that β-lactamases might have evolved from penicillin-sensitive D-anayl-D-alanine-cleaving peptidases (DD-peptidase/PBPs), that are involved in the synthesis of peptidoglycan, and are the target for β-lactam antibiotics. Kelly *et al* (Kelly *et al*, 1986), collated the DD-peptidase from *Streptomyces* R61 with the β-lactamase from *Bacillus licheniformis* 749/C. In spite of the fact that the two strains belonged to different species and lacked close homology in their primary structure, the three dimensional structure of the two enzymes were similar, implying a close relationship between the two. The theory that β-lactamases have evolved from PBPs hypothesis is now generally accepted as a result of sequence-based analysis, and structural and enzymatic information. We now know that β-lactamases and PBPs share several highly conserved amino acid sequences (Massova and Mobashery, 1998).

Figure 1.7



Production of drug-inactivating enzyme, β-lactamase.

Kelly hypothesised that β-lactamases probably evolved in soil bacteria, like *Streptomyces* spp. Significant progress has been made by Huletsky (Huletsky *et al.*, 1990), in explaining how the class A β-lactamases evolved (see 1.7.1 and 1.8.1). They constructed a phylogenetic tree, that split the β-lactamases into Gram-negative and Gram-positive subgroups and demonstrated that β-lactamases from Gram-positive bacteria probably evolved earlier in evolution than those of the Gram-negative bacteria. Kirby, (Kirby, 1992) following on from the work of Huletsky, examined the protein sequences of 18 Class A β-lactamases and 2 Class C β-lactamases and constructed a phylogenetic tree employing the DD-peptidase of S*treptomyces* R61 as an out group. Kirby's work suggested that all the Class A and C β-lactamases were evolved from an actinomycete β-lactamase, that had previously evolved from the DD-peptidase gene.

1.7.1 Structure and mechanisms of the action of β-lactamases

β-lactamases are represented by two broad categories, based on the structure of their active-site. The first category comprises those with a serine amino acid at this site, and can be further subdivided into molecular classes designated A, C and D on the basis of sequence similarity (Ambler, 1980; Bush *et al.*, 1995). The second category comprises the small but rapidly growing group of class B β-lactamases, or metallo-enzymes, so called because they utilise 1 or 2 zinc ions to disrupt the β-lactam ring rather than the serine ester mechanism of serine-based β-lactamases (Carfi *et al.*, 1998).

1.7.2 Structure of the serine β-lactamases

β-lactamase and DD-peptidase similarity is not restricted to 'mechanistic' properties. X-ray studies have highlighted striking structural analogies between the *Streptomyces* R61 (*S*.R61) DD-peptidase and several class A and class C β-lactamases (Kelly *et al*., 1986; Matagne *et al*., 1998). These enzymes are all medium-sized monomeric proteins, that consist of two structural domains (an all-α and an α/β domain) with the active site located in a groove between the two domains. Compared with the class A β-lactamases, both class C enzymes as well as *S*.R61 PBP have additional loops and secondary structure on the surface of the all-α domain, at a distance from the active site. However, the active serine is located at the N-terminus of the first hydrophobic helix of the all- α domain (α-2 helix) (Matagne *et al*., 1998).

In the immediate vicinity of the active-site serine residue of the DD-peptidase and β-lactamase, several conserved elements have been identified, which appear to be directly or indirectly involved in the substrate recognition and catalytic processes. Careful comparisons of the primary and tertiary structures have allowed identification of the same structural and functional elements in all active-site serine penicillin-recognizing enzymes (Joris *et al*., 1991; Sanschagrin *et al*., 1995).

The first element contains the active serine and, one helix-turn downstream, a lysine residue whose side-chain also points into the active site. The proximity of the serine and lysine side-chains, which are hydrogen-bonded, suggested the likely involvement

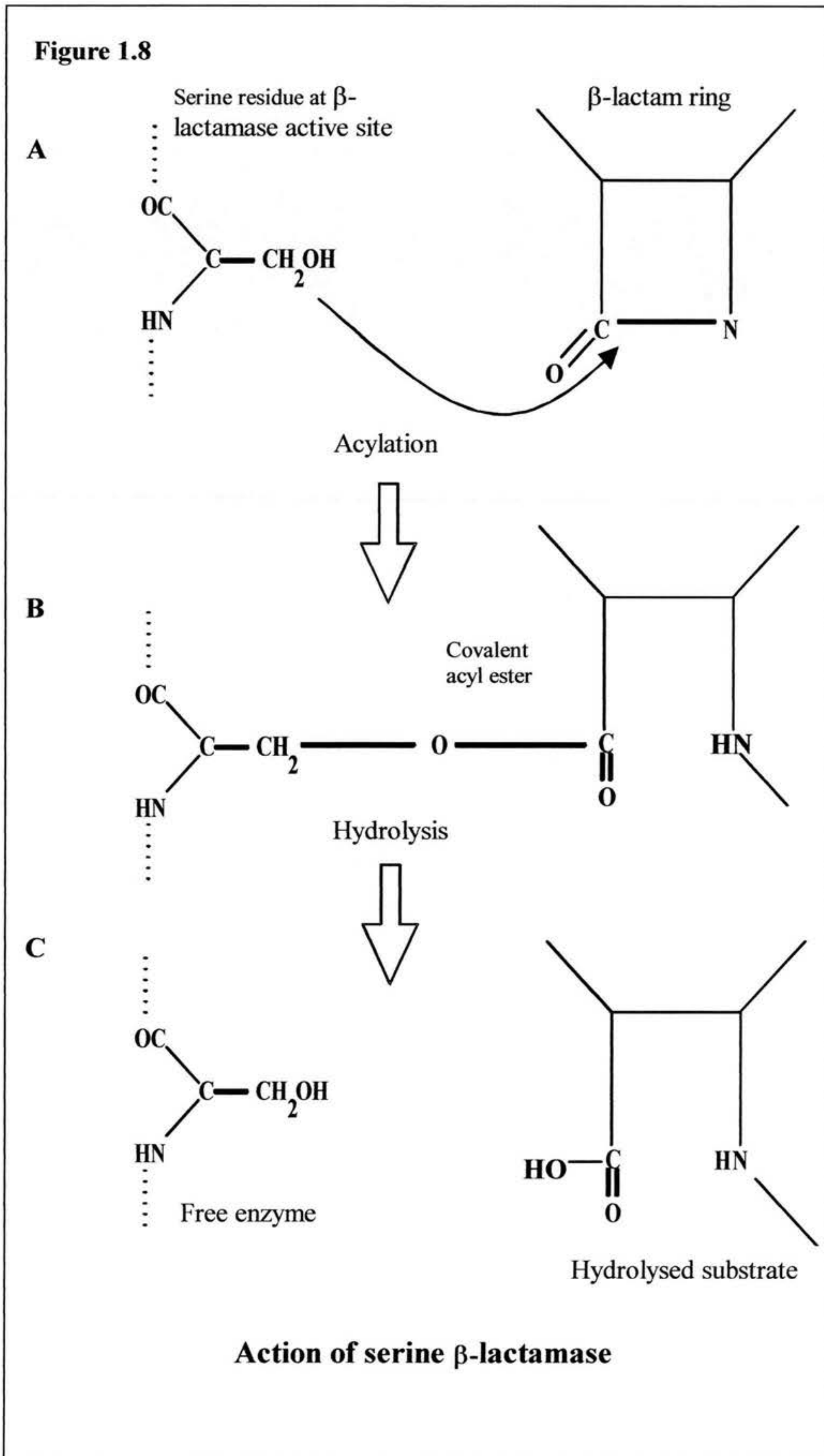
of the lysine side-chain amino group in the catalytic process (Herzberg and Moult, 1987; Strynadka *et al.*, 1992). The second element is located on a short loop in the all-α domain, where it forms one side of the catalytic cavity. It consists of Tyr-Xaa-Asn (β-lactamase of class C and D, some PBPs) or Ser-Xaa-Asn (β-lactamase of class A, most PBPs) sequences (Matagne *et al.*, 1998). The third element is called the KTG triad and is located on the innermost strand of the α-sheet (α/β domain) that forms the opposite wall of the catalytic cavity. It is generally a Lys-Thr-Gly sequence, but Lys is replaced by His or Arg in a few exceptional cases and Thr by Ser in several class A β-lactamases (Matagne *et al.*, 1998).

A fourth element containing a negatively charged residue has been tentatively identified in all enzymes, but it seems to play a catalytic role only in the Class A β -lactamase, found on a 16-19 residue loop [Arg^{161}-Asn^{170} in TEM-1 (Jelsch *et al.*, 1993)] usually referred to as Ω-loop. In most cases, this loop contains the Glu^{166}-Xaa-Glu-Leu-Asn^{170} sequence where the two residues Glutamic acid-166 and Aspartic acid-170 seem essential in positioning the conserved water molecule very close to the active serine (Lamottebrasseur *et al.*, 1991).

1.7.3 Serine ester mechanism of Class A, C and D β-lactamases

The majority of β-lactamases harbour a serine at the active site that forms a non-covalent complex with the β-lactam compound. (See figure 1.8). The carbonyl group of the β-lactam ring is attacked by a free hydroxyl on the side chain of the serine residue (Ser-70 in class A and D, Ser-64 in class C) at the active-site of the

Figure 1.8



Action of serine β-lactamase

enzyme to yield a covalent acyl ester (Livermore, 1993). Apart from the crucial role of the serine active site in the process of β-lactam acylation, additional residues are known to play an important role in the formation of the acyl-enzyme intermediate, and in the regeneration of the free enzyme (Massova and Mobashery, 1998). In TEM-1 the hydroxyl group of Ser-68 binds to the β-lactam molecule. However, Arg-244, Ser/Thr-235 and Ser-130 also contribute to the existence of multiple hydrogen bonding interactions with the substrate carboxylate (Imtiaz *et al.*, 1993; Zafaralla *et al.*, 1992).

1.8 Classification of β-lactamases

In 1968, Sawai discriminated penicillinases from cephalosporinases based on their response to antisera (Sawai *et al.*, 1968). As more information on enzyme function became available and with the discovery of new β-lactamases, other classification schemes have evolved (Bush, 1989a; Bush, 1989b; Bush, 1989c). Ambler was the first to propose a molecular classification scheme (Ambler, 1980), which is still the most widely used scheme.

1.8.1 The Ambler classification scheme

Richard Ambler originally identified two classes of enzyme, class A and class B, based on their molecular structure (Ambler, 1980). There are now four molecular classes to which β-lactamases are assigned based on the similarity of their amino acid sequences (Huovinen and Jacoby, 1991; Ouellet *et al.*, 1987).

1.8.1.1 Class A β-lactamases

All the class A enzymes have a molecular mass of ~30 KDa, share considerable homology and have a serine moiety at their active site. Class A includes TEM-1 and SHV-1, which are two of the most prevalent β-lactamases found in Enterobacteriaceae (Wiedemann *et al.*, 1989). Ambler concluded that their similarity was so great that they must have diverged from a single ancestral gene.

In 1991, Ambler introduced a standard numbering scheme for class A β-lactamases, by aligning 20 class A proteins and attaching numbers to the alignment (Ambler *et al.*, 1991). In this scheme the active site serine residue has been given the number Ambler 70. Many β-lactamases that belong to this class are encoded by genes on plasmids or transposones, and can be produced by both Gram-negative as well as Gram-positive bacteria (Bush *et al.*, 1995).

1.8.1.2 Class B β-lactamases

Class B comprise the metallo-β-lactamases, that require a bivalent metal ion, usually zinc for their activity, and β-lactamases II from *Bacillus cereus* 5/B/6. Class B enzymes show no similarity in sequence to class A enzymes. Sanders (Sanders, 1989), suggested that a new class E, could be created for the L1 β-lactamase found in *Stenotrophomonas maltophilia* (Saino *et al.*, 1982).

1.8.1.3 Class C β-lactamases

This class was first recognised by Jaurin and Grundstrom in 1981 (Jaurin and Grundstrom, 1981). Confirmation of this group was also shown by Knott-Hunziker *et al* (Knott-Hunziker *et al.*, 1982). Succeeding experiments with DNA probes indicated that the genes encoding this class of enzyme are widely spread through the chromosomes of Gram-negative bacteria (Bergstrŏm *et al.,* 1982). These chromosomal cephalsoporinases also function with a serine residue at the active site, but are very different in structure to those of the class A enzymes (Sanders, 1989). In contrast to class A enzymes, class C enzymes are generally not inhibited by clavulanic acid.

1.8.1.4 Class D β-lactamases

This class was proposed by Huovinen *et al.* (Huovinen *et al.*, 1988), who after having deduced the amino acid sequence of the PSE-2 β-lactamase, found that it shared extensive homology with the OXA-2 β-lactamase (Dale *et al.*, 1985). None of these enzymes share structural similarities with TEM or *Amp*C β-lactamases, and were therefore placed in a new class designated class D (Dale *et al.*, 1985).

1.8.2 The Bush classification scheme of β-lactamases

The most recent scheme of Bush attempts to correlate both the phenotypic and the molecular properties of known β-lactamases (Bush *et al.*, 1995). This classification system catalogues a large number of β-lactamases in detail on the basis of their functional characteristics. It refrains from using the location of the gene encoding the

β-lactamases as a primary classification factor, reflecting the ability of a large number of these genes to move between chromosome and plasmid.

There are four main functional groups within this classification scheme of β-lactamases.

1.8.2.1 Bush classification scheme: Group 1

The chromosomal cephalosporinases of this group, often referred to as *Amp*C-type β-lactamases, are intrinsically resistant to β-lactamase inhibitors and are ubiquitous in most enterobacteria. The amount and the mode of expression of these enzymes vary between genera (Sanders and Sanders, 1988). Some of the genes encoding the β-lactamases have migrated onto plasmids in clinical isolates of *K. pneumoniae* and *E. coli* (Sanders and Sanders, 1992).

1.8.2.2 Bush classification scheme: Group 2

β-lactamases of this group belong to Ambler molecular classes A or D. They are generally inhibited by active-site directed inhibitors such as clavulanic acid. The β-lactamases of this group hydrolyse a diverse range of substrates, which is acknowledged by the definition of subgroups for these enzymes. Extended spectrum β-lactamases that are derived from the TEM and SHV enzymes constitute subgroup 2b- and subgroup 2be. Those that have a reduced affinity for β-lactamase inhibitors comprise the 2br group. The main criterion for group 2d is the preferential hydrolysis of cloxacillin or oxacillin (hydrolysis rate of greater than 50% than that for benzylpenicillin) (Bush *et al.*, 1995). β-lactamases that hydrolyse carbenicillin at a

rate of greater than 60% compared with that for benzylpenicillin have been allocated to group-2c. Group 2f comprise the active-site serine carbapenemases. Many of the genes that encode these β-lactamases are found on plasmids.

1.8.2.3 Bush classification scheme: Group 3

This group comprises metallo-β-lactamases that are not inhibited by the classical β-lactamase inhibitors with the exception of EDTA, a metal chelator and p-chloromercuribenzonate (p-CMB), an amino acid modifier.

1.8.2.4 Bush classification scheme: Group 4

This group contains penicillinases that are not inhibited by the suicide inactivator, clavulanic acid. The LCR-1 β-lactamase from *P. aeruginosa* was originally a member of group 4 (Bush, 1989c); however, sequence data have subsequently revealed its homology with Class D OXA enzymes (Couture *et al.*, 1992), with the result that it has been relocated to group 2d (Bush *et al.*, 1995).

In general, Bush and colleagues have endeavoured to produce "workable, and a potentially useful compilation of β-lactamase characteristics". However, as they concede, "No functional classification will ever be completely satisfactory" (Bush *et al.*, 1995). As more sequence data are produced and revealed about β-lactamases, and enzyme variants emerge with altered substrate and inhibitor profiles as a result of point mutations of their genes, it is likely that the composition of the classification group described here will alter accordingly.

1.9 The development of modern cephalosporins

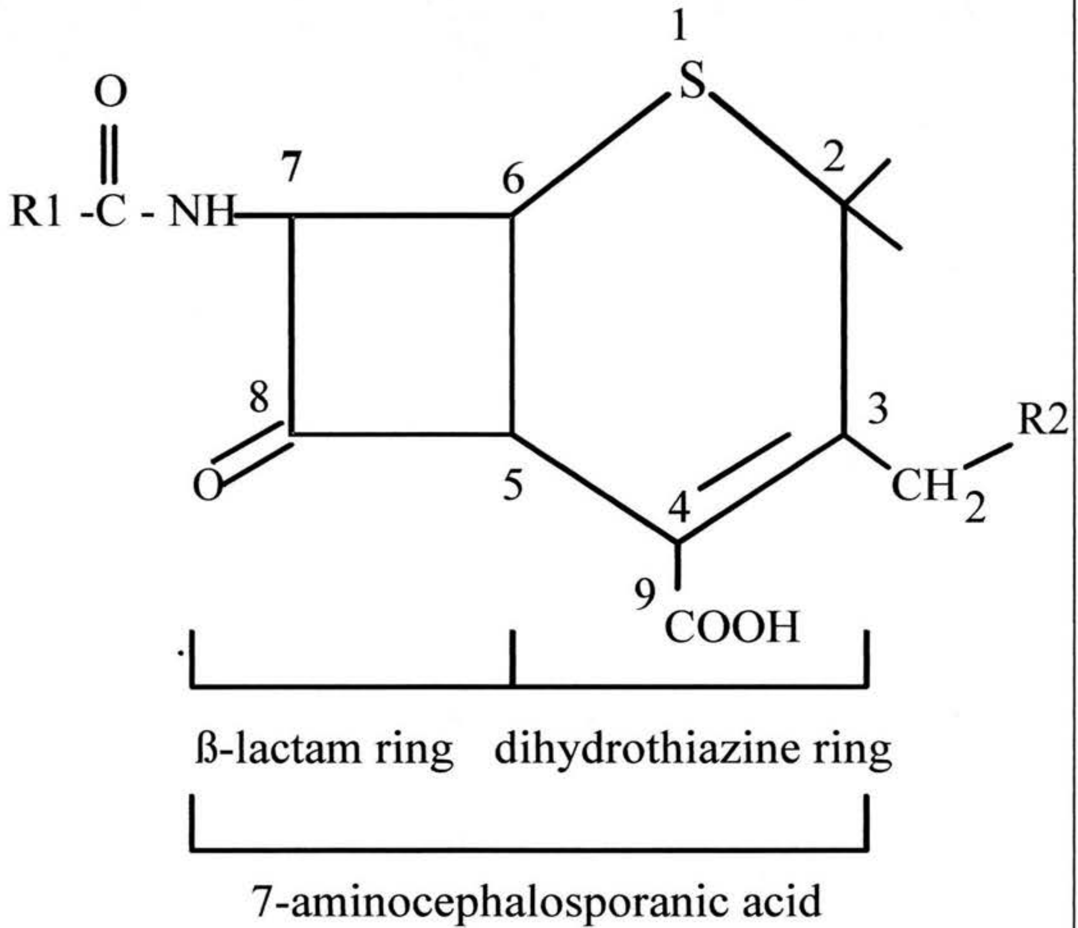
In 1945, a bacteriologist, Giuseppi Brotzu, studied the microbial flora of seawater near a sewage out-fall in Sardinia, and came across a strain of *Cephalosporium acremonium,* which secreted a substance with activity against a number of Gram-positive as well as Gram-negative bacteria. Due to lack of facilities and expertise, he passed a culture to Florey's team in Oxford for further analysis. Their studies revealed a number of antibiotic substances. However, it was not until the mould was grown on a much larger scale that these were identified. The first was named cephalosporin P because it demonstrated activity against certain Gram-positive bacteria (Burton and Abraham, 1951).

Sufficient amounts of cephalosporin P could not be obtained for clinical trials. However, another antibiotic substance, initially designated cephalosporin N, was discovered from the extraction procedure used to obtain cephalosporin P. This was active against Gram-negative bacteria and penicillin-sensitive staphylococci, and was undoubtedly the same substance that Brotzu had originally found. It was discovered that this antibiotic was inactivated by the penicillinase from *Bacillus cereus* (Newton and Abraham, 1954) and was subsequently re-named penicillin N. As a result of the difficulties in successfully isolating penicillin N, this compound was never produced in quantity by the pharmaceutical industry. A small amount of another substance was found during an attempt to establish the molecular structure of penicillin N by chromatography. This was named cephalosporin C, the first and original member of the class of antibiotics known as cephalosporins (Newton and Abraham, 1955). Unlike penicillin N, cephalosporin C was relatively acid-stable and demonstrated a

high resistance to inactivation by *B. cereus* and staphylococcal penicillinases. Although it was used in the treatment of urinary tract infection in children (Fleming, 1963), cephalosporin C did not play a major role in the treatment of infections caused by penicillinase-producing staphylococci, as by this time the semi-synthetic penicillins were very much in the therapeutic spotlight.

The modification of the side chains of the 7-aminocephalosporanic acid (7-ACA) nucleus (see figure 1.9) generated new cephalosporins that were active against bacteria which had shown resistance to the older cephalosporins. As these later cephalosporins, in turn, succumbed to novel β-lactamases, compounds with a still wider spectrum of activity and high stability against β-lactamase degradation were sought (Du Bois *et al.,* 1995). The most recent cephalosporins possess broad spectra of activity against Gram-negative bacteria, including some *P. aeruginosa* strains. At the time of their introduction, these cephalosporins were considered to be an example of the ultimate ability to overcome antimicrobial resistance, especially that mediated by the ubiquitous TEM-1. Amongst these cephalosporins, e.g. cefotaxime, ceftazidime and ceftriaxone, the increase in activity against Gram-negative bacteria is ascribed to a modification of the side-chain attached to the 7-carbon of the 7-ACA nucleus, to one containing a 2-aminothiazole group. More than that, these three cephalosporins also contain an *o*-substituted oxyimino group on the 7-β-acyl side-chain. This group of cephalosporins can be epitomised by ceftazidime, with a carboxylic alkoxyimino group, which shows slow outer membrane penetration but confers a significant degree of anti-pseudomonas activity, and cefotaxime, with a

Figure 1.9



Chemical structure of cephalosporins and 7-aminocephalosporanic acid

methoxyimino side-group, which exhibits faster penetration but has little anti-pseudomonas activity (Du Bois *et al.,* 1995).

1.9.1 Classification of the cephalosporins

The most widely accepted classification of cephalosporins splits them into 7 groups, which belong to four generations (see figure 1.9). Group 1 and 2 (the "early compounds") often referred to as the first generation cephalosporins. Second generation cephalosporins have only group 3 compounds that are resistant to β-lactamases. Groups 4, 5 and 6, have compounds resistant to β-lactamases and generally exhibit enhanced antibacterial activity (Wise, 1997). These are known as third generation cephalosporins. The fourth generation of cephalosporins consist only of group 7 (Wise, 1997). In general, the groups listed below differentiate these compounds based on the route of administration, their degree of antimicrobial activity and their stability to bacterial β-lactamases (Wise, 1997).

1.9.1.1 Group 1 cephalosporins

The parental compounds of this group were developed in response to a need for a more effective antibiotic against staphylococci. They have moderate antimicrobial activity and relative stability to staphylococcal β-lactamases. However, they are hydrolysed by many enzymes produced by the Enterobacteriaceae. The first compounds to be developed in this group were cephalothin and cephaloridine.

1.9.1.2 Group 2 cephalosporins

The compounds within this group are administered orally and demonstrate moderate resistance to some of the enterobacterial β-lactamases. The first of these compounds to be developed was cephalexin, which has a methyl-group at C_3. This was followed by cefaclor, which has chlorine in place of the methyl-group, and cephradine, which has the phenyl group of cephalexin replaced by a acyclohexadiene group.

1.9.1.3 Group 3 cephalosporins

All compounds that belong to this group are parenteral and include the related group, the cephamycins, which share the common feature of a methoxy group attached to the 7-α position of the β-lactam ring conferring stability to the structure. This group displays moderate antimicrobial activity and includes activity against the anaerobe *Bacteroides fragilis*.

1.9.1.4 Group 4 cephalosporins

This group includes parenteral compounds with potent antimicrobial activity and resistance to a wide range of β-lactamases. They are characterised by the presence of an N-acyl side-chain containing a 2-aminothiazole group.

1.9.1.5 Group 5 cephalosporins

The compounds of this group originate from an attempt to improve the intrinsic activity of the previous oral cephalosporins. Although they have improved activity

against many of the enterobacteria, their spectrum against the Gram-positive bacteria is not as good.

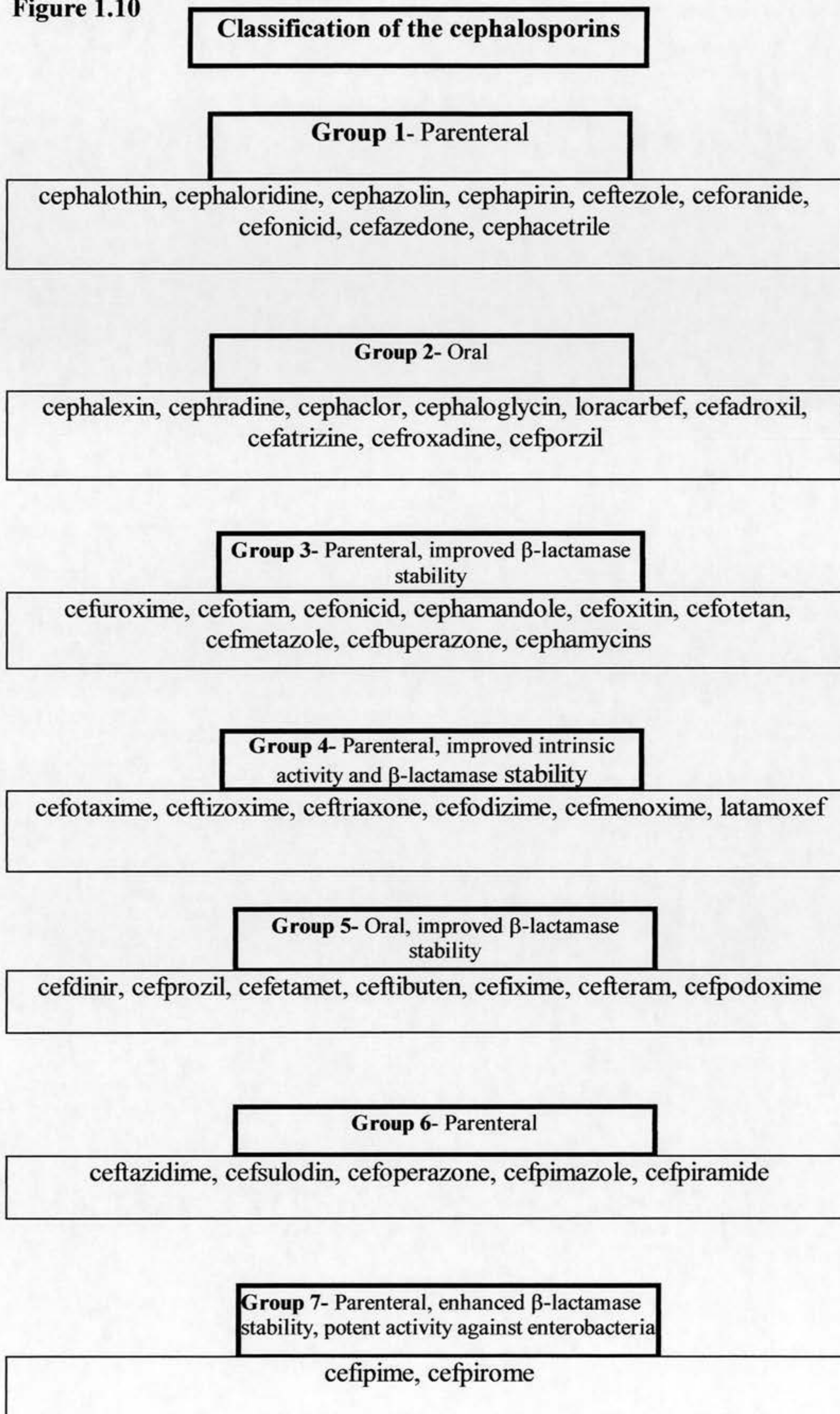
1.9.1.6 Group 6 cephalosporins

These are parenteral compounds that are characterised by their activity against *P. aeruginosa* and resistance to a wide range of β-lactamases.

1.9.1.7 Group 7 cephalosporins

Group 7 includes the parental compounds cefepime and cefpirome. The major properties of this group are similar to those of group 4 agents (Wise, 1997). They should probably be reserved for use in patients with severe infection caused by bacteria with plasmid as well as chromosomally mediated β-lactamases. However, they have potent activity against enterobacteria, enhanced activity against staphylococci and increased penetration and are zwitterionic into the bacterial cell. (See figure 1.10).

Figure 1.10



Adapted from Wise, 1997

1.10 The history of extended-spectrum β-lactamases

The earliest clinical strain known to possess an extended spectrum β-lactamase (ESBL) was isolated in England in 1982 (Du Bois *et al.,* 1995). The strain was identified as *Klebsiella oxytoca* and was responsible for an out break of infection in a neonatal unit in Liverpool. The initial antibiotic profile indicated that the infection was originally due to a strain that was gentamicin-resistant but ceftazidime-sensitive and which produced TEM-1 β-lactamase. Ceftazidime was used in treatment to control it, but subsequent isolates of *K. oxytoca* from the unit showed that the strain had become ceftazidime-resistant. The ceftazidime resistance gene was transferable and carried on a 141-kb plasmid (Payne *et al*., 1990). Subsequent detailed biochemical analysis showed that the ceftazidime resistance was mediated by a new β-lactamase, which was able to slowly hydrolyse ceftazidime (Payne *et al.,* 1990). In spite of the fact that the earliest transferable ESBL to be identified has now been shown to have originated in England, it is on the mainland of Europe where most of these derivative enzymes have been found and where they were first reported (Jacoby and Medeiros, 1991; Payne and Amyes, 1991; Philippon *et al*., 1989a). In 1983, three strains of *K. pneumoniae* and one *Serratia marcescens* isolated in (West) Germany were demonstrated to confer transferable resistance to cefotaxime (Knothe *et al*., 1983). The plasmid-encoded β-lactamase was a modification of the ubiquitous SHV-1 enzyme and was designed SHV-2 (Knothe *et al*., 1983). The nucleotide sequence of the SHV-2 gene showed a single nucleotide (point mutation) difference from the SHV-1 gene, changing the amino acid at position 238 from glycine to serine (Huletsky *et al*., 1990). This observation spawned a succession of reports of

transferable, plasmid-encoded resistance to cephalosporins. So far, almost all the broad-spectrum ß-lactamase enzymes described have been found to be modifications of either TEM or SHV ß-lactamases (Du Bois *et al.,* 1995).

1.10.1 Spread of genes conferring resistance to β-lactam antibiotic

Throughout the 1960s and 1970s there was a relentless rise in reports of resistance to β-lactams as a consequence of the selection of bacteria that produce β-lactamase. This increase in the level of resistance was due, at least in part to the spread of self-transmissible plasmids that encode multiple resistance phenotypes. Furthermore, the first prokaryotic transposon carried a *bla*-$_{TEM}$ gene (Hedegs and Jacob, 1974). Transposons that encode extended-spectrum β-lactamases have also been described (Heritage *et al*., 1992). The occurrence of the *bla*-gene on mobile genetic elements undermines attempts to classify these elements by genetic location as transposons may jump between plasmids and the bacterial chromosome.

1.10.2 Nomenclature of β-lactamases

There has been considerable confusion over the nomenclature of β-lactamases. There is no rational basis for the naming of these enzymes.

The name 'TEM' is a contraction of Temoniera, the name of the patient from whom the first ampicillin-resistant *E.coli* was isolated. In contrast, 'SHV' is a contraction of 'sulphydryl variable' a description of the biochemical properties of this β-lactamase.

Furthermore, β-lactamases may be given one name when first identified, only to have this name changed after subsequent studies have allowed a more complete characterisation of the properties (Heritage *et al.*, 1999). CTX-1 was so called because it conferred resistance to cefotaxime. Nucleotide sequence analysis showed that this enzyme had arisen by the accumulation of a point mutation in the gene encoding TEM β-lactamase. Consequently, CTX-1 is now named TEM-3. Similarly, SHV-1 has also been called PIT-2, as it was first described by Pitton in 1972 (Pitton, 1972).

1.10.3 Classification of extended-spectrum β-lactamases

The classification of β-lactamases has always proved problematic. Several schemes have been proposed for the classification of this large family of enzymes. The first proposal was to divide β-lactamases into the penicillinases that hydrolyse penicillin, and cephalosporinases that attacked cephalosporins. The biochemical activity and substrate profiles of different enzymes formed the basis of other early classification schemes (Jack and Richmond, 1970). Later, the location of the genetic determinants, whether plasmid mediated or chromosomal, became incorporated into classification schemes. Data from isoelectric focusing studies and enzyme kinetics were also considered important and these formed the basis of subsequent classification (Du Bios *et al.*, 1995). These schemes all had major anomalies but, following rapid developments in molecular biology, sequence homology studies were able to resolve difficulties with previous classification schemes (Richmond *et al.,* 1971). It is current practice to classify β-lactamases by their nucleotide sequence and for most enzymes this works well; however, all the extended-spectrum β-lactamases described fall into

class A and D (Ambler, 1980), which encompasses enzymes whose genes share as little as 35% identity but retain the same overall shape. Nevertheless, the lack of direct correlation between sequence and enzyme function is epitomised with the prototype enzymes. Enzymatically, it is impossible to distinguish between TEM-1 and SHV-1 (Du Bios *et al.*, 1995). In contrast, the gene for TEM-5 has only three nucleotide changes from TEM-1 (i.e. >99.5% homology) (Sougakoff *et al.*, 1989), but these enzymes are easily distinguished on the basis of their substrate specificity. Biochemical properties of the TEM-5 β-lactamase and the resistance profiles that it confers are of immense clinical importance. An alternative categorisation scheme (Payne and Amyes, 1991), based on the biochemical properties and resistance profiles that enzymes confer, shows that the mutation produce three major changes in function. Group 1 ESBLs confer decreased susceptibility to ceftazidime and possess an amino acid substitution that increases the ability of the enzyme to hydrolyse ceftazidime faster than cefotaxime. Groups 2 and 3 enzymes are able to confer higher levels of resistance to both ceftazidime and/or cefotaxime. However, group 2 enzymes hydrolyse ceftazidime to a greater extent than cefotaxime, whereas enzymes in group 3 hydrolyse cefotaxime better than ceftazidime. An additional group has also been suggested, (Du Bios *et al.*, 1995) namley Group 0 (zero), which accommodates the in vitro ability to hydrolyse cefotaxime more efficiently than ceftazidime. However, the ability of cefotaxime to rapidly penetrate the outer membrane ensures that this hydrolysis is insufficient to overcome the drug's antibacterial activity (Du Bios *et al.*, 1995).

1.10.4 Relation between structure and function of β-lactamases

The minimum inhibitory concentration (MIC) is the universally recognised indicator of bacterial sensitivity to antibiotics, and is defined as the lowest concentration of the agent that is required to inhibit bacterial multiplication. These values can be used to compare the in vitro manifestations of each mutation with the biochemical properties of the enzyme and the resistance profile that it confers, allowing a structure-function relationship to be deduced and an evolutionary scheme to be proposed (Amyes *et al.*, 1992). The susceptibility profile will to some extent, be dependent on the encoding plasmid and the promoter controlling the β-lactamase gene. However, the important comparison of the relative MICs of cefotaxime and ceftazidime is less dependent on these factors as both MICs will be influenced by them (Du Bios *et al.*, 1995).

1.10.5 Types of ESBLs

Most ESBLs are derivatives form TEM or SHV enzymes (Bush *et al.*, 1995). There are now more than 100 TEM-type β-lactamases and more than 35 SHV- type enzymes (for amino acid sequences for TEM and SHV extended spectrum β-lactamases, see http://www.lahey.org/studies/web.htm). With both of these groups of enzymes, a few point mutations at selected loci within the gene give rise to the extended-spectrum phenotype. TEM- and SHV- type ESBLs are most often found in *E.coli* and *K. pneumoniae*; however, they have also been found in *Proteus* spp., *Providencia* spp., and other genera of Enterobacteriaceae (Bradford, 2001).

1.10.5.1 SHV-derived enzymes

As it has been mentioned previously (section 1.10), the first extended–spectrum SHV enzyme was described in 1983 in clinical isolates of *K. pneumoniae, K. ozaenae* and *Serratia marcescens* (Knothe *et al.*, 1983). Because of its similarity to SHV-1 the new enzyme was named SHV-2 (Kliebe *et al.*, 1985). A single amino acid substitution, in which glycine at position 238 in SHV-1 is replaced by serine in SHV-2 alters the spectrum of activity of the SHV-1 β-lactamase to encompass extended-spectrum cephalosporins (Brathēlēmy *et al.*, 1988).

In 1988, Jarlier *et al* (Jarlier *et al.*, 1988) described SHV-3, a β-lactamase isolated from *K. pneumoniae*. This was isolated from a patient in an intensive therapy unit in a French hospital where SHV-2 had also been reported. Both SHV-2 and SHV-3 have a spectrum of activity that includes extended-spectrum cephalosporins and they share a substrate and inhibitor profile. At the nucleotide sequence level, a point mutation causes the substitution of leucine at amino acid position 205 in SHV-3 for the arginine that is found at this position. Both SHV-1 and SHV-2 also have a serine residue at amino acid position 238. It is thus probable that SHV-3 evolved from SHV-2 by a point mutation.

The isoelectric point (pI) of both SHV-1 and SHV-2 is 7.6 whereas that of SHV-3 is pI 7.0. Soon after the first description of SHV-3, yet another member of this family was described (Burē *et al.*, 1988). The SHV-4 β-lactamase is another enzyme with extended-spectrum cephalosporinase activity. It was first described in a strain of *K. pneumoniae* in 1987 and like SHV-3, it was first seen in a French hospital. The strain

of *K. pneumoniae* that harboured SHV-4 disseminated rapidly and by 1990 had been found in 14 hospitals throughout France (Arlet *et al.*, 1994). The pI of SHV-4 is 7.8. Analysis of amino acid sequence data shows that SHV-4 evolved by a point mutation in the gene that codes for SHV-3. In the case of SHV-4 the amino acid substitution occurs at position 240, where as lysine replaces the glutamic acid found in this position in the other members of the SHV family.

The fifth member of the SHV family was first observed in Chile (Gutmann *et al.*, 1989). This was the first variant not to have been first observed in Europe. SHV-5 was again first described from *K. pneumoniae* and has a pI of 8.2. As with SHV-4, the amino acid found at position 240 in SHV-5 is lysine. It is possible, however, to say from which ancestral gene the SHV-5 determinant evolved, it is most likely to have evolved by a point mutation from SHV-2.

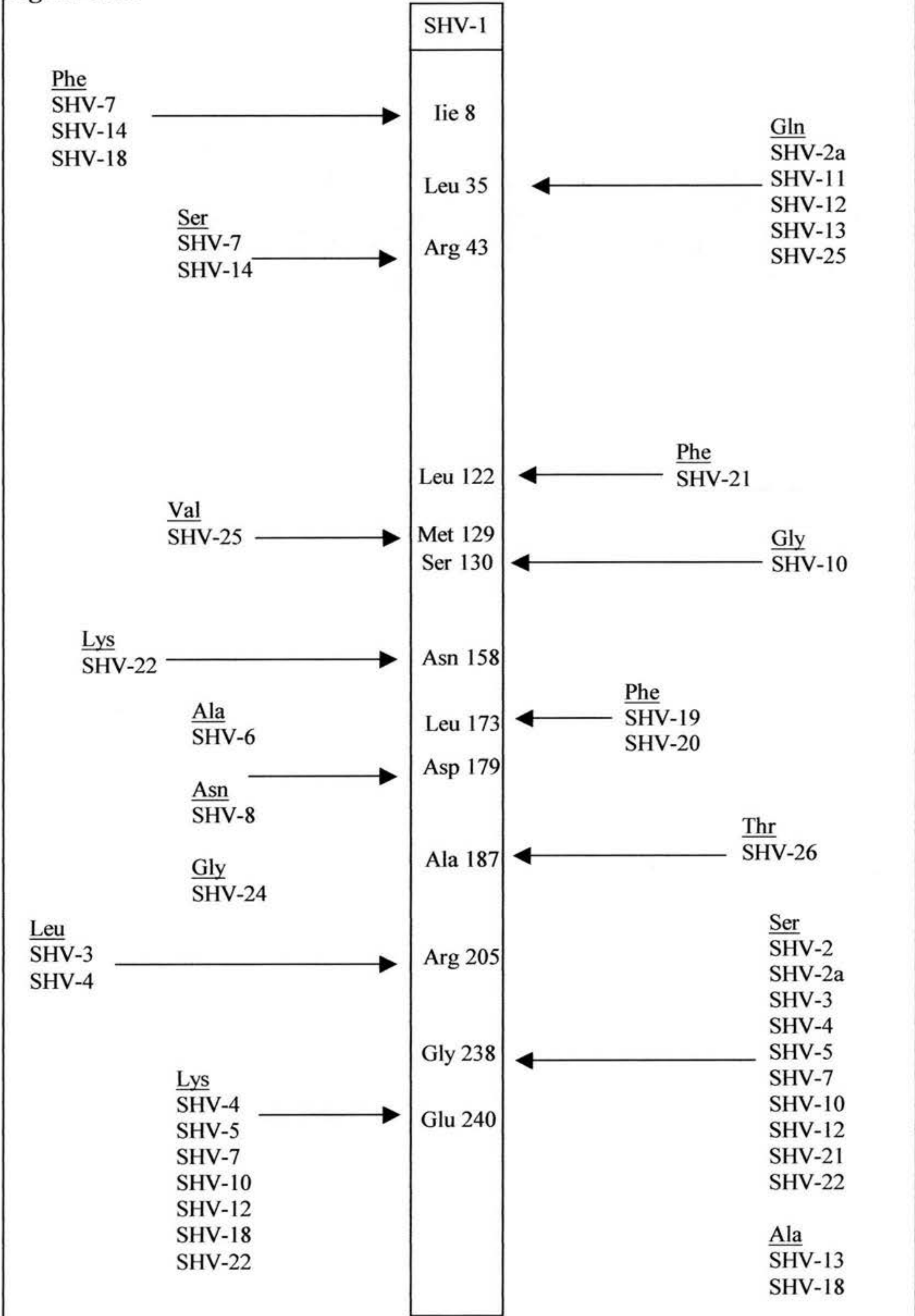
In 1991, the SHV-6 β-lactamase was discovered (Arlet *et al.*, 1991). This is an unusual enzyme in that its spectrum of activity is different from the other ESBLs in this family. SHV-6 is able to hydrolyse ceftazidime but it has no activity against other extended-spectrum cephalosporins such as cefotaxime. It is also inactive against monobactams such as aztreonam, which distinguishes it from other extended spectrum SHV enzymes. SHV-6 was first found in France where it was produced by a strain of *K. pneumoniae* isolated from a patient on a paediatric oncology ward. It is probable that SHV-6 arose by a point mutation from SHV-1 since these two enzymes differ only at amino acid position 179 where the aspartic acid found in SHV-1 has been replaced with alanine in SHV-6 (Arlet *et al.*, 1997). In 1993 a new ESBL SHV

type SHV-7 was described in *E.coli* was isolated in the USA (Bradford *et al.,* 1995). *E.coli* provided the background for the emergence of SHV-8 (Rasheed *et al.,* 1997). This was also first observed in the USA. The SHV-9 (Prinarakis *et al.*, 1996) β-lactamase appears to have evolved from SHV-5. This enzyme was found to be produced by *E.coli, K. pneumoniae* and *S. marcescens* isolated from a Greek hospital in 1995 (Prinarakis *et al.*, 1996). The latest additions to the SHV family were both first described from Switzerland and are found in a number of species in the Enterobacteriae (Nüesch *et al.*, 1997). SHV-11 is a variant of either SHV-1 or SHV-3 and does not possess extended-spectrum cephalosporinase activity and the other SHV is SHV-12, which is an ESBL. The current evolutionary picture is shown in figure 1.11. Table 1.1 summarizes the first report of each of the SHV β-lactamases (Heritage *et al.*, 1999).

1.10.5.2 TEM–derived enzymes

TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E.coli* is as a result of the production of TEM-1 (Livermore, 1995). This enzyme is also responsible for the acquired penicillin or ampicillin resistance that is seen in Gram-negative bacteria such as *Nesseria gonorrhoeae* and *Haemophilus influenzae* (Livermore, 1995). TEM-1 is able to hydrolyse penicillin and early cephalosporins. TEM-2, the first derivative of TEM-1 had a single amino acid substitution from the original β-lactamases (Bradford, 2001).

Figure 1.11



Amino acid substitution in SHV ESBL derivatives. The amino acid numbering is according to the scheme of Ambler *et al* (Ambler *et al.*, 1991). Substitution found in SHV-type ESBL derivatives are shown under amino-acids of SHV-1. SHV-type variants may contain more than one amino acid substitution. Adapted from Bradford, 2001.

Table 1.1

Occurrence of some β-lactamases of the SHV family with the geographical distribution of the mutant variants

β-lactamases	Country of origin	Year	Other countries from which the β-lactamases has been observed
SHV-1	Switzerland	1974	
SHV-2	Germany	1983	China, Denmark, France, Spain, South Africa, Switzerland, Tunisia, UK
SHV-2a	Germany	1986	Korea, Switzerland
SHV-3	France	1986	UK, USA
SHV-4	France	1987	Belgium, Portugal, UK, USA
SHV-5	Chile	1987	Australia, Austria, Denmark, Egypt, France, Greece, Italy, Poland, South Africa, Taiwan, UK, USA
SHV-6	France	1991	
SHV-7	USA	1993	
SHV-8	USA	1990	
SHV-9	Greece	1996	
SHV-10	Greece	1997	
SHV-11	Switzerland	1997	
SHV-12	Switzerland	1997	Algeria, Korea

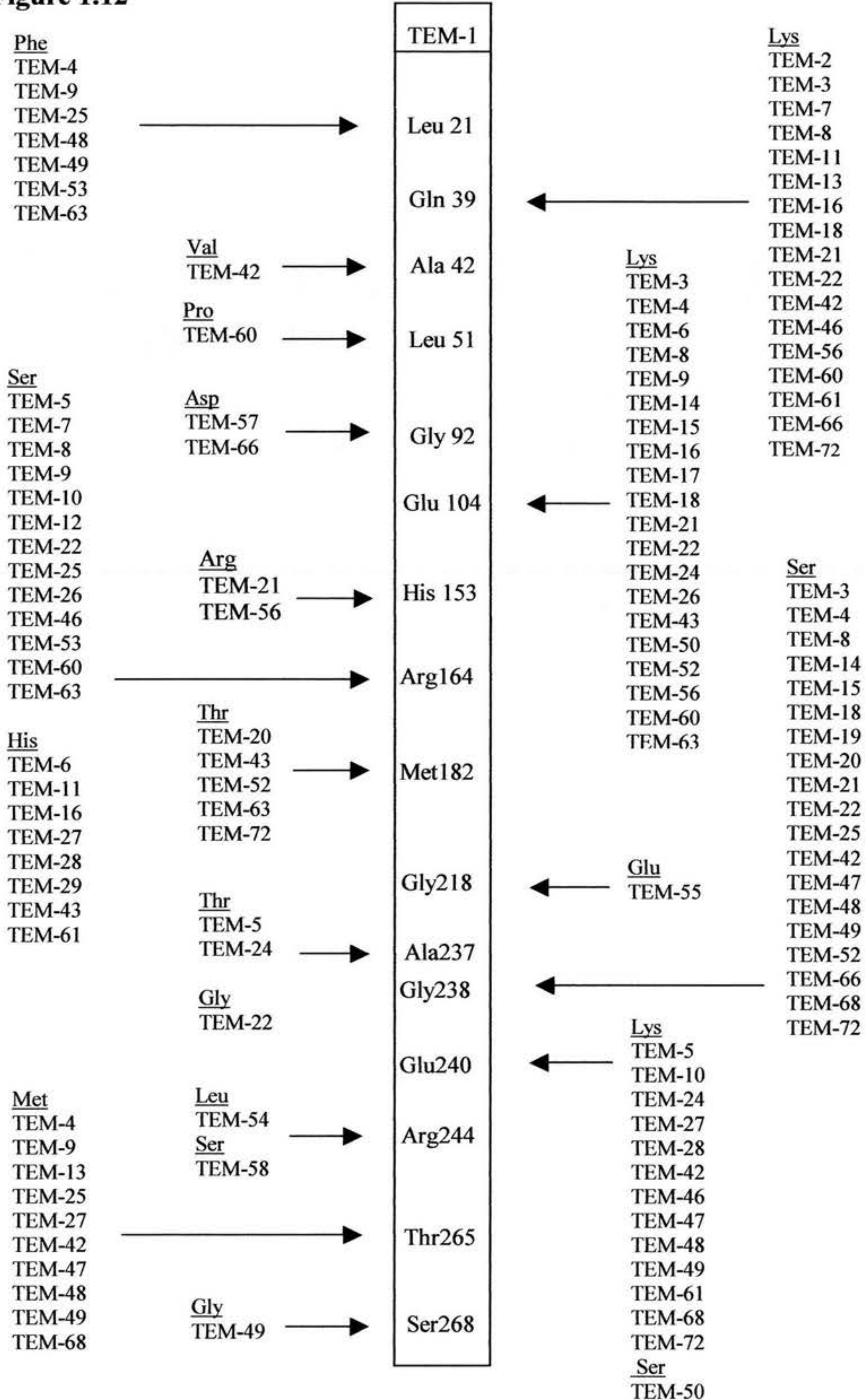
Adapted from Heritage *et al.*, 1999

TEM-1/2 β-lactamases were detected among resistant Gram-negative organisms in the 1960s shortly after the introduction of ampicillin to the clinical armamentarium (Datta and Knotomichalou, 1965).

The mutational relationship is well known and defined amongst this group of enzymes (Bradford, 2001). However, they are much more complicated than those in SHV enzymes. It can be assumed that the mutation between TEM-1 and TEM-2, namely, the change from glutamine to lysine at position 39 occurred before later enzymes emerged. As shown in (figure 1.12), the amino acid substitution that occur within the TEM enzyme occur at a limited number of positions. Therefore, two clearly separate TEM-derived enzyme groups exist in the TEM-derived enzymes; these are TEM-1 derived and TEM-2 derived.

The analysis of a three-dimensional structure of TEM-1 β-lactamase (Jelsch *et al.*, 1992, 1993) places the sites of amino acid variation adjacent to the proposed position of the respective cephalosporin 7-substitutions when the β-lactam molecule is bound at the active site. The amino acid mutation alters the shape of the active-site, directly or indirectly, to reduce hindrance of β-lactamase-cephalosporin binding. In some cases, changes may promote binding through additional charge-charge interaction with drug moieties. The first alteration from TEM-1 leading to the enzymes TEM-5 or TEM-9 is likely to have been the alteration of arginine-164 to serine.

Figure 1.12



Amino acid substitution in TEM ESBL derivatives. The amino acid numbering is according to the scheme of Ambler *et al* (Ambler *et al.*, 1991). Substitution found in TEM-type ESBL derivatives are shown under amino acids of TEM-1. TEM-type variants may contain more than one amino acid substitution. Only amino acid substitution that are common to TEM-type ESBL are shown in this figure. Adapted from Bradford, 2001.

The resultant β-lactamase, TEM-12, has a limited capability to hydrolyze both ceftazidime and cefotaxime and is classified as group 1 (Du Bios *et al.*, 1995). The production of this enzyme decreases ceftazidime susceptibility, but hardly affects sensitivity to cefotaxime. The first emergence of ESBL TEM enzymes in the UK was in response to ceftazidime treatment (Payne *et al.*, 1990). The same substitution can take place with the TEM-2 β-lactamase to give TEM-7, which has the same properties as TEM-12. The increased capability to hydrolyze 7-oxyimino cephalosporins does not arise from the acquisition of serine at position 164 but rather from the loss of arginine. The loss of this amino acid breaks two salt bridges, with asparagine-179 and glutamic acid-171, which maintains the Ω-loop (Jelsch *et al.*, 1992; Jelsch *et al.*, 1993). Substitution of arginine-164 by histidine in TEM-2 also gives an enzyme "TEM-11" with the same properties.

Interestingly, although substitution of arginine at position 164 is common in the TEM enzymes, this substitution has not been found amongst the SHV group. We suspect that the usually short α-7 helix of the TEM-1 enzyme (Jelsch *et al.*, 1992), compared with other Class A β-lactamases, may explain the lack of substitution at this point among SHV enzymes.

The development of TEM-9 can be accomplished by two distinct routes. The TEM-17 mutation, substituting glutamic acid with lysine at position 104 leads to TEM-12. Synergy results from the combination of the lysine-104 and serine-164 substitution (TEM-26), increasing the MIC of ceftazidime out of proportion to the contribution of each separate mutation. In contrast the MIC of cefotaxime conferred by TEM-26 is

the same as that conferred by TEM-17. The TEM-26 to TEM-9 transition (threonine-265 to methionine) increases the MIC of cefotaxime to a greater extent than the MIC of ceftazidime. Mutation of TEM-12 at position 240 to change the amino acid from glutamic acid to lysine creates TEM-10, which has greatly increased resistance to ceftazidime. Further substitution of threonine for alanine at position-237 (TEM-5) considerably increases resistance to cefotaxime but not to ceftazidime.

In the years since that first report, over 90 additional TEM derivatives have been described (for amino acid sequence for TEM and SHV extended spectrum β-lactamases see http://www.lahey.org/studies/temtable.htm). Some of these β-lactamases are inhibitor-resistant β-lactamases (IRT), but the majority of the new derivatives are ESBLs.

This variety represents a unique example of protein evolution in "real time" (Petrosino *et al.*, 1998). Such diversification was probably a consequence of an equivalent diversification of elective challenge resulting from the introduction of multiple β-lactam antibiotic molecules designed to resist hydrolysis by TEM-1, in particular broad-spectrum cephalosporins such as cefotaxime and ceftazidime as well as β-lactam inhibitors (Negri *et al.*, 2000).

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1.10.6 The evolution of *Amp*C genes

The evolution of TEM and SHV enzymes has not been the only challenge to the use of cephalosporins and β-lactam/β-lactamase inhibitor combinations in the last decade. The migration of Ambler class C chromosomal β-lactamases on to plasmids and their subsequent dissemination into clinically relevant species has produced isolates resistant to virtually all cephalosporins and β-lactamase inhibitors (Horii *et al*., 1993; Leiza *et al*., 1994). Increasing resistance to β-lactams means that in some cases, particularly intensive care units, we are reliant on the carbapenems as the only remaining effective β-lactam agents. However, as the use of these compounds increases so will the selective pressure for resistance to develop. Alternatively it may be that the biggest challenge to the carbapenems comes from the rapid dissemination of mobile resistance genes into clinically relevant species. The dissemination of the IMP-1 gene throughout Japan in *P. aeruginosa*, and its detection in *K. pneumoniae*, demonstrates the potential for this to happen (Senda *et al*., 1996).

1.11 The Quinolones

One of the most exciting advances in antimicrobial chemotherapy was the discovery and development of the quinolone group (see figure 1.13). The quinolones drugs are synthetic compounds which do not resemble any compounds found in living organisms. The first quinolone to be synthesized was nalidixic acid which was introduced into clinical use in 1962 (Lesher *et al.*, 1962). Since then, there has been a small, but significant, increase in the number of resistant clinical isolates of species previously susceptible to these agents (King *et al.*, 2000).

In the 1970s, several other antimicrobial agents that were chemically related to nalidixic acid, such as oxolinic acid and cinoxacin, were introduced. However, the most significant discovery in the development of quinolone antibacterial agents came when it was discovered that the insertion of a fluorine at position 6 in the basic nucleus enhanced and broadened the antimicrobial activity. (See figure 1.14). These newer quinolones, such as ciprofloxacin, moxifloxacin, clinafloxacin, gatifloxacin, and gemifloxacin exhibited antimicrobial activity approximately a thousand times that of nalidixic acid (Wolfson and Hooper, 1985).

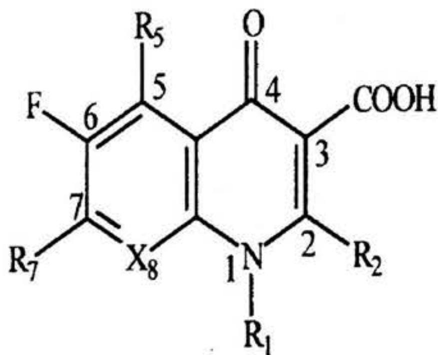
1.11.1 Binding of quinolones to DNA

Since quinolone resistance mutation occurred in the *gyr*A gene, it was believed that quinolone drug bound directly to the A subunit of DNA gyrase (Gellet *et al.*, 1977; Sugino *et al.*, 1977). However, the occurrence of quinolone resistance mutations in

Figure 1.13

Quinolones chemical structure

Quinolone pharmacore



Nalidixic acid

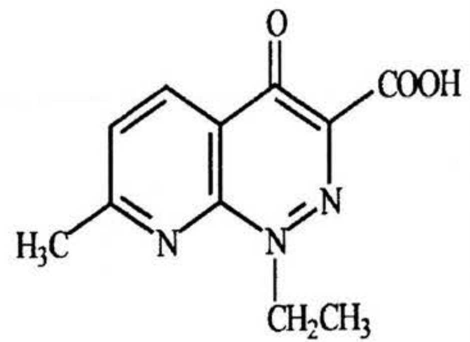
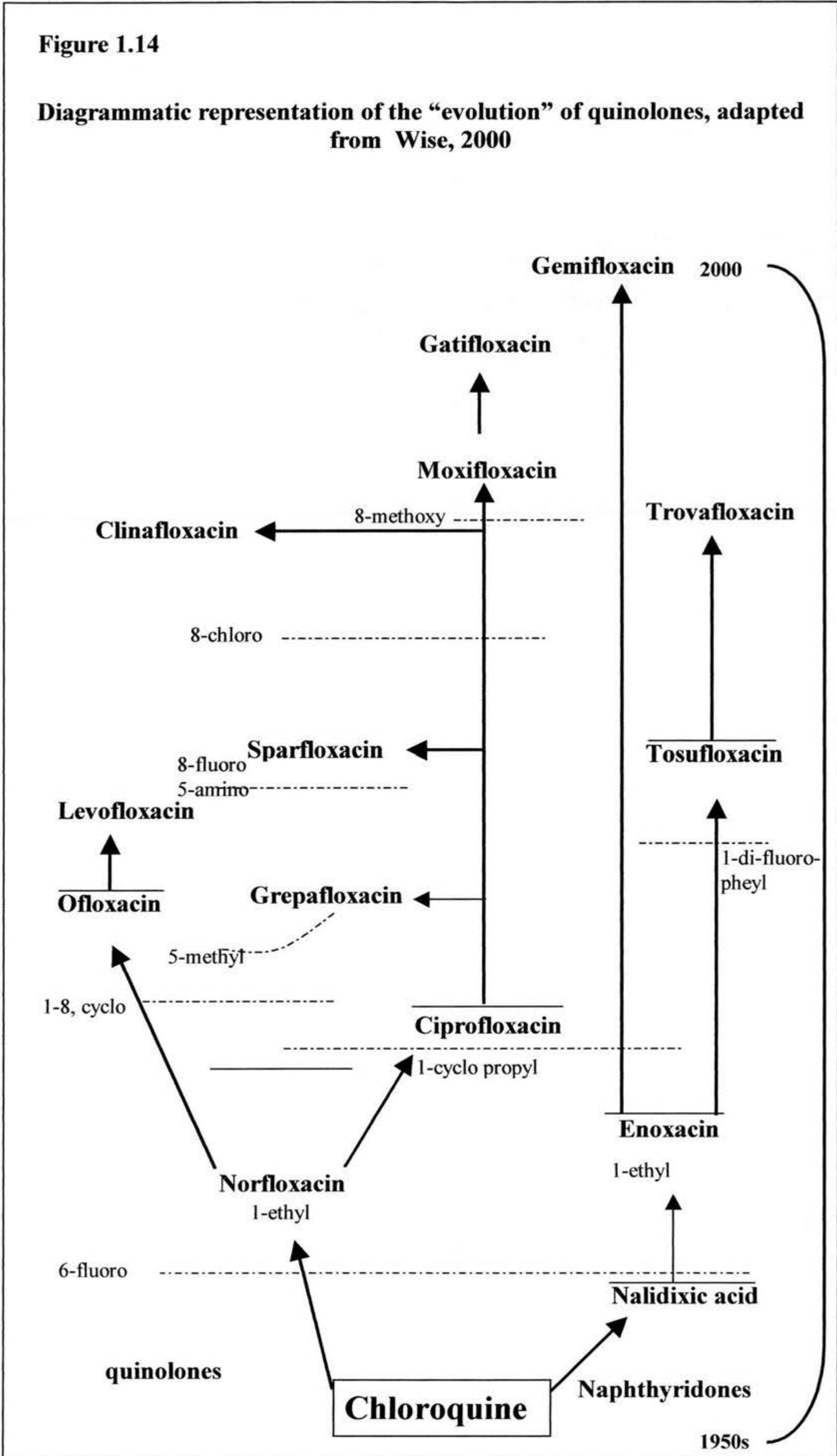


Figure 1.14

Diagrammatic representation of the "evolution" of quinolones, adapted from Wise, 2000



*gyr*B suggested that the quinolone binding site might encompass both subunit A and B (Yamagishi *et al.*,1986). Binding studies undertaken by Shen and Pernet suggested that quinolones bound to DNA but not to gyrase (Shen and Pernet, 1985). In 1988 Toranletti and Pedeini, observed that the binding of quinolones to DNA causes the DNA to unwind to a small extent (Tornaletti and Pedrini, 1988). It has been suggested that quinolones bind preferentially and cooperatively to single-stranded DNA (Shen *et al.*, 1989a) and this led to the idea that they interact with a single stranded pocket of DNA formed during the strand passage reaction catalyzed by gyrase. This hypothesis was modified slightly by Shen *et al* (Shen *et al.*, 1989b, 1989c), who later proposed a cooperative drug DNA binding model for the binding quinolones to the gyrase-DNA complex.

Quinolones are thought to interact with the bases of DNA through hydrogen bonds that occur between the 3-carboxy and 4-oxo groups, which almost all quinolones possess. At least four quinolone molecules are believed to bind per DNA binding site and ring stacking and hydrophobic interactions contribute to this co-operativity. Gyrase interactions with the C7 group position of the drug was postulated by Shen *et al* (Shen *et al.*, 1989c) while Maxwell later pointed out that the C7 group is rather variable (Maxwell, 1992). (See figure 1.13). A new angle has been added to this drug binding hypothesis with the discovery that mutation in *gyr*A where tyrosine-122 is replaced with either phenylalanine or serine (which can not cleave DNA) still can bind quinolone drugs (Critchlow and Maxwell, 1996).

1.11.2 Mechanisms of quinolone resistance

Quinolone resistance has been mapped to a mutation within a specific region DNA gyrase termed the quinolone resistance determining region (QRDR) (Wiedemann and Heisig, 1994). Initial studies on the development of quinolone resistance demonstrated that there are two basic strategies that bacteria could adopt to circumvent the action of quinolones. First, alteration of DNA gyrase, which is the target site of quinolones, and secondly, mutation that lead to reduced access of quinolone to DNA gyrase, that could occur by either efflux systems found in both Gram-negative and Gram-positive bacteria, or could occur by alteration in the outer membrane of Gram-negative bacteria (Thomson, 1999). Alteration in the structure of DNA gyrase has been commonly identified in resistant clinical isolates (Ball, 1994; Maxwell, 1997; Tillotson *et al.*, 1997). DNA gyrase is composed of two subunits, A and B, and most changes have been found in the QRDR region of the A subunit. (Tillotson *et al.*, 1997). This region is highly conserved within bacterial species and alteration within it has been documented in different variety of species e.g. *E.coli*, *Salmonella* spp., *Acinetobacter* spp., in addition to others (Brown *et al.*, 1996; Deguchi *et al.*, 1995; Vila *et al.*, 1995; Yoshida *et al.*, 1988). Nowadays, quinolone usage accounts for 11% of all antibiotic prescriptions (Thomson, 1999) and thus, it is not surprising to note that the first instance of plasmid-mediated fluoroquinolone resistance in a clinical isolate of *K. pneumoniae* has been identified (Martinez *et al.,* 1998), although plasmid-mediated transferable resistance to quinolones in clinical isolates is not yet thought to be a major clinical concern (Tillotson *et al.*, 1997).

However, a recent report documented that a multi-resistance plasmid (conferring resistance to ceftazidime, cefotaxime, gentamicin, streptomycin, tobramycin, trimethoprim and chloramphenicol) also conferred reduced susceptibility to quinolones (Martinez *et al.,* 1998).

1.12 *Klebsiella* spp A nosocomial pathogen

Klebsiella spp. are Gram-negative non-sporing, non-motile bacilli, which tend to be short and thick, e.g. 1-2 x 0.8μm. Virtually all freshly isolated strains form a well defined polysaccharide capsule.

Klebsiella is well known to most clinicians as a cause of community-acquired bacterial pneumonia, occurring particularly in chronic alcoholics (Carpenter, 1990) and showing characteristic radiographic abnormalities on chest X-rays due to severe pyogenic infection with a high fatality rate if untreated. The vast majority of *Klebsiella* infections, however, are associated with hospitalization. As an opportunistic pathogen, *K. pneumoniae* primarily attacks immuno-compromised individuals who are hospitalized and suffering from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction. Nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, medically the most important species of the genus. To a much lesser degree *K. oxytoca* has been isolated from human clinical specimens. It is estimated that *Klebsiella* spp. cause up to 7 % of all nosocomial bacterial infection in the United States and in Europe. No great geographical variations in frequency have been noted (Podschun and Ullmann, 1998). In the United States, *Klebsiella* spp. account for 3 to 7% of all nosocomial

bacterial infection, placing them among the eight most important infectious pathogens in hospitals (Podschun and Ullmann, 1998; Schaberg *et al.*, 1991). Data collected from the United Kingdom (Bergogne-Neberezin, 1995) and from Germany (Podschun and Ullmann, 1998) show remarkable similarities to those reported by the centers for Disease Control and Prevention, Atlanta, USA.

The urinary tract is the most common site of infection. *Klebsiella* account for 6 to 17% of all nosocomial urinary tract infections (UTI) and shows an even higher incidence in specific groups of patients at risk, e.g., patients with neuropathic bladder or with diabetes mellitus (Lye *et al.*, 1992). As a cause of nosocomial Gram-negative infection, *K. pneumoniae* is second only to *E. coli* (Duggan *et al.*, 1985; Pittet and Wenzel, 1993; Yinnon *et al.*, 1996), (see table 1.2).

In paediatric wards, nosocomial *Klebsiella* infections are especially troublesome- particularly in premature infants and intensive care units. *Klebsiella* spp. are often the pathogen involved in neonatal sepsis, in both early manifestation and late manifestation infections (Gotoff, 1992).

As a result of the extensive spread of antibiotic-resistant strains, particularly ESBL-producing strains, there has been renewed interest in *Klebsiella* infection.

Table 1.2

Ranking of *Klebsiella* compared to all other bacterial infection

Infection	% of infections caused by *K. pneumoniae*	Rank
UTI	6-17	5-7
Pneumonia	7-14	2-4
Septicaemia	4-15	3-8
Wound infections	2-4	6-11
Nosocomial infection in ICU	4-17	4-9
Neonatal septicaemia	3-20	2-8

Adapted from (Podschum and Ullmann, 1998).

1.12.1 Epidemiology of *Klebsiella*

K. pneumoniae is ubiquitous in nature. It probably has two common habitats, the environment, where they are found in surface water, sewage, soil, plants (Brown and Seider, 1973), and the mucosal surfaces of mammals such as humans, horses, or swine, which they colonize. In this respect, the genus *Klebsiella* is similar to *Enterobacter* and *Citrobacter* but unlike *Shigella* spp or *E.coli*, which are common in humans but not in the environment (Brown and Seider, 1973). In humans *K. pneumoniae* is present as a saprophyte in the nasopharynx and in the intestinal tract. The carrier rate differs considerably from study to study. The detection rate in stool samples ranges from 5 to 38%, while rates in the nasopharynx range from 1 to 6% (Davis and Matsen, 1974). Gram-negative bacteria do not find good growth conditions on the human skin, unlike Gram-positive bacteria. *K. pneumoniae* are rarely found there and are regarded simply as transient members of skin flora (Kloos and Musselwhite, 1975). These carrier rates change dramatically in the hospital environment, where colonization rates increase in direct proportion to the length of stay. Even hospital personal have elevated rates of *Klebsiella* carriage (Podschum and Ullmann, 1998). Reported carrier rates in samples from hospitalised patients are 77% in the stool, 19% in the pharynx, and 42% on the hands of patients (Davis and Matsen, 1974). The high rate of nosocomial *K. pneumoniae* colonisation appears to be associated with the use of antibiotics rather than with factors connected with delivery of care in hospitals (Podschum and Ullmann, 1998). Previous antibiotic therapy is significantly associated with acquisition of *Klebsiella* by the patient. In one study 2 weeks after admission to the hospital, a two-to fourfold increase in the colonization rates with *Klebsiella* was observed; this increase occurred primarily in

patients receiving antibiotics, especially in patients receiving broad-spectrum or multiple antibiotics. In the hospital setting, the local antibiotic policy is a major determinant of the colonization pattern. The significance of increased colonization was illustrated by the observation that the nosocomial infection rate with *K. pneumoniae* in patients carrying hospital-acquired intestinal *Klebsiella* was four times higher than that seen in non carriers (Podschum and Ullmann, 1998). Furthermore, widespread use of antimicrobial therapy has often been held responsible for the occurrence of multiply- resistant *K. pneumoniae* strains in hospitals (Tullus *et al.*, 1988). These undesired effects may be reversed by strict control of antibiotic use, demands for strategies to avoid the overuse of antibiotics in prophylaxis and empirical therapy.

1.12.2 The incidence of ESBL- producing *Klebsiella pneumoniae*

The incidence of ESBL-producing *K. pneumoniae* isolated in the United States has been reported to be 5% of the *K. pneumoniae* strains tested in the National Nosocomial Infection Study system (NNISS) (Jacoby, 1996). In Europe, the frequency of such strains seems to be even higher. Fourteen to 16% of *K. pneumoniae* clinical isolates have been found to harbour ESBLs in France and England (Sirot, 1995). In particular regions, or hospitals, the incidence rate can reach 25 to 40% (Burwen *et al.*, 1994). However, the percentage of ceftazidime-resistant strains may be much higher, because the conventional disc diffusion criteria used in the routine laboratory underestimates the incidence of these isolates (Jacoby and Han, 1996).

1.13 *Escherichia coli*

The genus *Escherichia* was formerly subdivided into a number of species by differences in sugar fermentation reactions, but nowadays only one species, *E.coli,* is recognized (Pattison *et al.*, 1995) although it is subdivided into serotypes and biotypes. *E.coli* are Gram-negative bacilli most stains (~80%) are motile, though motility is often feeble on primary isolation, and most strains (~80%) are fimbriate. *E.coli* is the most common nosocomial Gram-negative bacterium followed by *K. pneumoniae* (Podschum and Ullmann, 1998). The intestinal commensal strains of *E.coli* commonly cause opportunistic infection in other parts of the body where there is some abnormality or impairment of defences. They are a common cause of urinary tract infection (cystitis, pyelititis and pyelonephritis) and are commonly present in appendix abscesses, peritonitis, cholecystitis, septic wounds and bedsores. These microorganisms are able to infect the lower respiratory passages or cause bacteriaemia and endotoxic shock.

1.13.1 The incidence of ESBL-producing *E.coli*

Ampicillin resistance among these members of the Enterobacteriaceae is due primarily to the production of Bush group2 β-lactamase (Bush *et al.*, 1995; Sanders and Sanders, 1992). For *E.coli*, this is usually due to the presence of TEM-1 β-lactamase. *E.coli* strains expressing a high level of ESBL enzyme will also be resistant to expanded-spectrum cephalosporins and aztreonam. Prevalence of resistance to ampicillin and older cephalosporins is on the increase in these species due primarily to the dissemination of plasmids encoding TEM-1 or SHV-1 β-

lactamases (Pitout *et al*., 1997). In many geographic locations, resistance to ampicillin among clinical isolates of *E.coli* exceeds 50% (Bush *et al*., 1995). After the introduction of expanded-spectrum cephalosporins, strains of *E.coli* that were resistant to these drugs and aztreonam began appearing in certain areas in Europe (Thomson *et al*., 1996). Subsequent studies showed that this resistance was due to the presence of new forms of TEM-1, TEM-2, and SHV-1 enzymes, which were capable of hydrolyzing the expanded-spectrum cephalosporins. These ESBLs have been recovered from isolates of *K. pneumoniae* and *E.coli* worldwide. ESBLs have also been recovered from other species of Enterobacteriaceae, although they are much less common (Pitout *et al*., 1997).

1.14 The aim of this study

1. To determine susceptibility of ESBL producing *K. pneumoniae* and *E.coli* isolated from Edinburgh to various antimicrobial agents.
2. To detect the presence of extended-spectrum β-lactamases (ESBLs) by use of various methods.
3. To characterize the relationship between the ESBL-producing *K. pneumoniae* and *E.coli* by genotypic (PCR, DNA-hybridization and PFGE) and phenotypic (OMPs, antibiogram and IEF) methods.
4. To explore the relationship between the production of extended-spectrum β-lactamases (ESBLs) and resistance to ciprofloxacin in *K. pneumoniae* isolates.
5. To identify and characterize ESBLs in *K. pneumoniae* strains isolated from Chile.

1.15 Significance of this study

The results of this study provide information on the presence of ESBLs in *K. pneumoniae* and *E.coli* isolated from patients samples from Edinburgh and the surrounding districts. It enriches our knowledge of the mechanisms of resistance to third generation cephalosporins and ciprofloxacin in addition to other antimicrobial agents. In addition, the study provides useful epidemiological and surveillance information on the susceptibility of these particularly organisms at a local level. This will hopefully provide clinicians with future guidance for empirical therapy for such strains.

The study also demonstrates the identification and characterization of a novel SHV enzyme from strains of *K. pneumoniae* isolated from hospitalized patients throughout Chile.

Chapter 2

Materials And Methods

2.1 Bacterial Isolates

A total of 87 putative ESBL-producing clinical bacterial isolates were collected from patients in the Edinburgh Royal Infirmary (ERI) and surrounding district between May 1999 to March 2000. The isolates were from various clinical samples and comprised 69 *K. pneumoniae* and 18 *E. coli.* All were single isolates from individual patients. They were chosen on the basis of their putative resistance to any of the second or third generation cephalosporins, as determined using the Vitek system. Table (2.1) lists the isolates and their clinical sources. The 87 isolates were speciated by both the automated Vitek system and by the API 20E system.

2.2 Storage of strains

Bacterial strains were inoculated onto MacConkey agar plates and incubated at 37°C overnight to check their purity. All strains were stored at -70°C in cryovials (Alpha Laboratories, East Leigh, Hampshire) until required. Working stock was stored on nutrient agar slopes at room temperature.

2.3 Culture media

Unless otherwise stated, all growth media and components were sterilised by autoclaving for15 minutes at 121°C.

2.3.1 Solid media

Nutrient agar, Iso-Sensitest (IST) agar, MacConkey agar, and Mueller-Hinton agar were all obtained from Oxoid (Basingstoke, UK). They were prepared according to the manufacturer's instructions and sterilised as described above. The agar was

Table 2.1

Demographics of the 87 ESBL-producing clinical strains

Strain	Sex	Date of birth	Species	Clinical source	Date of sample collection	Ward or GP
AD313	F	1911	*K. pneumoniae*	Urine	28.6.1999	W22
AD314	F	1930	*K. pneumoniae*	Swab	20.7.1999	W22
AD315	M	1941	*K. pneumoniae*	Respiratory	23.8.1999	W15
AD316	F	1904	*K. pneumoniae*	Urine	15.11.1999	W15
AD317	F	1913	*K. pneumoniae*	Urine	16.8.1999	GP
AD318	M	1960	*K. pneumoniae*	Respiratory	21.9.1999	W15
AD319	F	1903	*K. pneumoniae*	Urine	26.7.1999	W15
AD320	F	1924	*K. pneumoniae*	Swab	23.8.1999	W15
AD321	M	1960	*K. pneumoniae*	Respiratory	23.9.1999	W17
AD322	M	1941	*K. pneumoniae*	Swab	24.8.1999	W15
AD323	M	1926	*K. pneumoniae*	Urine	8.10.1999	W15
AD324	M	1933	*K. pneumoniae*	Respiratory	22.7.1999	W15
AD325	F	1909	*K. pneumoniae*	Swab	12.11.1999	W37
AD326	F	1920	*K. pneumoniae*	Urine	8.10.1999	W15
AD327	M	1918	*K. pneumoniae*	Urine	11.10.1999	W15
AD328	F	1927	*K. pneumoniae*	Urine	13.11.1999	W47
AD329	F	1939	*K. pneumoniae*	Urine	13.11.1999	W38
AD330	F	1939	*K. pneumoniae*	Urine	18.11.1999	W22
AD331	F	1921	*K. pneumoniae*	Respiratory	24.11.1999	W33
AD332	M	1930	*E.coli*	Urine	29.11.1999	GP
AD333	M	1935	*K. pneumoniae*	Swab	10.1.2000	W5
AD334	F	1938	*K. pneumoniae*	Respiratory	10.1.2000	W14
AD335	F	1936	*K. pneumoniae*	Urine	16.12.1999	W14
AD336	M	1937	*E.coli*	Urine	16.4.2000	GP
AD337	F	1915	*K. pneumoniae*	Urine	21.2.2000	W14
AD338	F	1979	*K. pneumoniae*	Urine	2.6.1999	W15
AD339	M	1920	*K. pneumoniae*	Urine	3.3.2000	W37
AD340	M	1911	*K. pneumoniae*	Urine	16.2.2000	W5
AD341	F	1933	*K. pneumoniae*	Urine	28.2.2000	W11
AD342	M	1911	*K. pneumoniae*	Urine	6.2.2000	W5
AD343	M	1932	*K. pneumoniae*	Urine	29.2.2000	W22
AD344	M	1932	*K. pneumoniae*	Urine	22.2.2000	W22
AD345	F	1917	*K. pneumoniae*	Urine	27.2.2000	W33
AD346	M	1939	*K. pneumoniae*	Urine	16.2.2000	W15
AD348	F	1915	*K. pneumoniae*	Urine	11.3.2000	W15

Table 2.1 (cont.)

AD349	F	1956	*E.coli*	Urine	16.3.2000	GP
AD350	F	1962	*K. pneumoniae*	Urine	28.3.2000	W5
AD351	F	1983	*K. pneumoniae*	Respiratory	27.3.2000	W15
AD352	M	1920	*K. pneumoniae*	Urine	24.3.2000	GP
AD353	M	1935	*K. pneumoniae*	Urine	28.3.2000	W5
AD354	M	1923	*K. pneumoniae*	Urine	31.3.2000	W13
AD355	M	1942	*K. pneumoniae*	Urine	11.4.2000	W15
AD356	M	1935	*K. pneumoniae*	Urine	20.6.2000	W43
AD357	F	1932	*E.coli*	Urine	10.4.2000	GP-2
AD358	M	1963	*K. pneumoniae*	Respiratory	14.4.2000	W15
AD359	M	1916	*K. pneumoniae*	Respiratory	21.4.2000	GP
AD360	F	1961	*E.coli*	Urine	19.4.2000	GP-1
AD362	F	1952	*K. pneumoniae*	Respiratory	18.4.2000	W15
AD363	F	1918	*K. pneumoniae*	Respiratory	27.7.2000	W15
AD364	F	1918	*K. pneumoniae*	Urine	26.7.2000	W15
AD365	F	1949	*K. pneumoniae*	Respiratory	27.7.2000	W11
AD366	M	1920	*K. pneumoniae*	Urine	28.7.2000	W33
AD367	F	1928	*K. pneumoniae*	Swab	4.7.2000	W8
AD368	F	1911	*K. pneumoniae*	Urine	26.4.2000	GP
AD369	F	1959	*E.coli*	Urine	25.4.2000	GP-1
AD370	M	1961	*K. pneumoniae*	Urine	7.4.2000	GP
AD371	F	1920	*E.coli*	Urine	7.4.2000	GP
AD374	M	1924	*K. pneumoniae*	Blood	19.6.2000	W15
AD375	F	1928	*K. pneumoniae*	Urine	25.6.2000	W33
AD377	F	1946	*K. pneumoniae*	Respiratory	3.7.2000	W15
AD378	M	1928	*K. pneumoniae*	Respiratory	13.7.2000	W15
AD380	M	1943	*K. pneumoniae*	Respiratory	17.7.2000	W15
AD382	F	1935	*K. pneumoniae*	Respiratory	28.6.2000	W15
AD384	M	1924	*K. pneumoniae*	Swab	3.7.2000	W15
AD385	F	1957	*E.coli*	Urine	28.6.2000	GP
AD386	M	1947	*E.coli*	Urine	1.7.2000	GP
AD387	F	1957	*E.coli*	Urine	12.7.2000	GP-1
AD388	F	1908	*E.coli*	Urine	28.6.2000	GP
AD389	F	1930	*K. pneumoniae*	Swab	19.6.2000	W15
AD391	F	1928	*K. pneumoniae*	Urine	27.6.2000	W3
AD392	M	1926	*K. pneumoniae*	Urine	3.7.2000	W29
AD393	F	1954	*E.coli*	Urine	28.6.2000	GP-1
AD395	F	1926	*K. pneumoniae*	Swab	17.6.2000	W5
AD399	F	1938	*E.coli*	Urine	19.6.2000	GP
AD400	F	1952	*E.coli*	Swab	8.6.2000	GP
AD401	F	1920	*K. pneumoniae*	Urine	9.6.2000	W37
AD402	M	1938	*K. pneumoniae*	Urine	22.5.2000	W8
AD403	F	1962	*E.coli*	Urine	3.5.2000	GP

Table 2.1 (cont.)

AD404	F	1946	*K. pneumoniae*	Urine	29.3.2000	W15
AD405	F	1922	*E.coli*	Urine	8.5.2000	GP-2
AD407	F	1930	*K. pneumoniae*	Urine	7.4.2000	W25
AD408	M	1955	*K. pneumoniae*	Urine	29.5.2000	W42
AD409	M	1960	*K. pneumoniae*	Urine	29.3.2000	W42
AD410	M	1920	*K. pneumoniae*	Urine	16.6.2000	W32
AD411	F	1926	*K. pneumoniae*	Urine	14.6.2000	W32
AD412	M	1915	*E.coli*	Urine	9.6.2000	GP
AD413	F	1964	*E.coli*	Urine	18.6.2000	GP-1

All ward within Edinburgh Royal Infirmary (ERI).

W3: Surgical ward.

W5: Surgical ward.

W8: Surgical ward.

W11: Surgical ward.

W13: Surgical ward.

W17: Cardio thoracic ward.

W22: Medical ward.

W25: Medical ward.

W32: Medical ward.

W33: Medical ward.

W37: Medical ward.

W38: Medical ward.

W42: Renal ward.

W43: Renal ward.

GP-1: Eastfield medical center.

GP-2: West Linton health center.

GP: Other isolates were from single GPs.

allowed to cool to 50°C before being poured into sterile petri dishes (Sterilin Ltd, Stone, Staffordshire) and allowed to set. The plates were then stored at 4°C.

2.3.2 Liquid media

Nutrient broth and brain heart infusion broth (BHIB), were obtained from Oxoid and Luria Bertani (LB) was obtained from GIBCO (BRL, Life Technologies, UK). Cells were dissolved in distilled water and sterilised as described before.

2.4 Chemical reagents

All chemical reagents used were supplied by Sigma chemicals (Dorset, UK) unless otherwise stated.

2.5 Antimicrobial susceptibility testing

2.5.1 Antimicrobial discs

All antimicrobial discs and their concentration for disc diffusion susceptibility testing method are listed in table 2.5.1. Antimicrobial discs were supplied by Becton Dickinson (Cockeysville, USA).

Table 2.5.1 Antimicrobial agents discs and concentrations

No	Antimicrobial	Concentration
1	Ampicillin	30 µg
2	Augmentin	10 µg
3	Cephalexin	30 µg
4	Cephalothin	2.5 µg
5	Cefotaxime	50 µg
6	Ceftazidime	10 µg
7	Tazobactam	10 µg
8	Meropenem	10 µg
9	Ciprofloxacin	1 µg
10	Gentamicin	10 µg
11	Tobramycin	10 µg
12	Amikacin	30 µg
13	Chloramphenicol	30 µg
14	Trimethoprim	30 µg
15	Nitrofurantoin	30 µg

2.5.1.1 Disc-diffusion method

Isolates were diluted in normal saline to a turbidity equivalent to 0.5 MacFarland standard 10^{-8}colony-forming units per ml (cfu/ml). The diluted culture was used to flood seed Mueller-Hinton agar plates. Excess fluid was removed and the plates allowed to dry. Antibiotic discs were then applied and plates were incubated at 35°C for 18-24 hours in air. Control strains used were *E.coli* NCTC 10418 and *E.coli* β-lactamase NCTC 11560. Results were reported as sensitive, resistant or intermediate by comparing the diameter of the zone of inhibition around the antibiotic discs with published values provided by the National Committee for Clinical Laboratory standards (NCCLS).

2.5.2 Antimicrobial powders

The following antimicrobial agents were used for MIC determinations by agar dilution. Gentamicin and tobramycin were purchased from David Bull Laboratories, (Warwick, UK). Ciprofloxacin from Bayer (England). All compounds were dissolved in sterile distilled water as recommended.

2.5.2.1 Minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) were determined according to the guidelines of the Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy (1991). Bacterial strains were grown overnight at 37°C in IST broth and diluted in 0.9% saline to give 10^7 cfu/ml. A denley multipoint inoculator (Denley, Surrey) was used to inoculate 1 μl of the diluted suspension onto agar plates containing serially diluted antibiotics to give a

final inoculum of 10^4 cfu/spot. Recommended NCTC strains were included as controls (Working Party on Antibiotic Sensitivity Testing, 1991).

2.5.2.2 Vitek system

The Vitek system (bio-Mérieux) was used for identification or/and susceptibility testing. Each identification card (GNI+) contains 30 biochemical tests, which require no addition of reagents. It is able to identify more than 300 species. The Vitek system is able to test a wide range of antimicrobial agents. Interpretation of susceptibility results are pre- programmed into the system, the breakpoint values being based on NCCLS guidelines.

2.5.2.3 Epsilon test (E-test)

The E-test (AB Biodisk, Cambridge, UK) employs a more stringent and convenient method of determining susceptibility of bacterial isolates. It comprises a plastic strip embedded with an antibiotic gradient. An overnight broth culture was used to flood-seed Mueller Hinton agar plates. The plastic strips were placed on the surface of the agar plates and the antibiotic is released into the agar, to yield " pear-shaped" zones of inhibition. The plates were incubated overnight at 37°C in air and MIC of the antibiotic is indicated by the point at which the zone of inhibition intersects with the strip.

2.6 Methods for ESBL detection

2.6.1 Vitek system

The Vitek system is able to detect ESBL production by an automated version of the synergy test, which is based on detection of the inhibitory effect of clavulanic acid on ESBL in the presence of either cefotaxime or ceftazidime. The test is interpreted by measuring the difference in bacterial growth with or without inhibitor.

2.6.2 E-test ESBL strips

E-test ESBL strips (AB Biodisk, Cambridge) employ a more stringent method of detecting the presence of ESBLs. The strip is comprised of a plastic double ended strip with two shorter antibiotic and antibiotic/inhibitor gradients aligned in opposing directions from the center. Two strips are currently available containing either cefotaxime and cefotaxime/clavulanic acid, or ceftazidime and ceftazidime/clavulanic acid. These strips yield both the MIC of the cephalosporins and the MIC ratio of the antibiotic relative to the antibiotic/inhibitor combination, which indicates the presence or absence of an ESBL. Interpretation of the results was as recommended by the manufacturer. A positive reaction is indicated by the ratio of the antibiotic and antibiotic/inhibitor. A value of $\geq$ 8mg/l indicates ESBL activity, and a value of < 8mg/l indicates no ESBL activity.

2.6.3 Double disc diffusion (DDD) test

Antimicrobial double discs (cefotaxime 30 μg, cefotaxime 30 μg + clavulanic acid 10 μg, ceftazidime 30 μg, ceftazidime 30 μg + clavulanic acid 10 μg and cefpodoxime 10 μg, cepodoxime 1 μg + clavulanic acid 10 μg) were supplied by Oxoid Ltd.

The DDD test was performed according to NCCLS guidelines. A broth culture was adjusted to a 0.5 MacFarland and swabbed onto an Mueller-Hinton agar plate. The combination and corresponding cephalosporin discs were placed on the agar plates using a disc dispenser (Oxoid). The plates were incubated at 35°C for 18-24hours before the zone size was recorded. A positive result (the production of an ESBL) was indicated by a zone size difference of ≥ 5mm between the combination disc and the corresponding standard antibiotic disc. Interpretation of the results was as for manufacturer's instructions.

2.7 Plasmid DNA preparation

Cells from 1.5 ml of an overnight incubation at 37°C LB culture were harvested by centrifugation at 13000 rpm (MSE microcentrifuge) for 1 minute and re-suspended in 100μl of solution 1 (5mM sucrose, 10mM EDTA and 25mM Tris-pH 8.0) and re-suspended by vortexing. Two hundred μl of solution 2 (0.2N NaOH and 1% (w/v) SDS) was added and mixed by inversion till the cells were lysed. The tubes were allowed to stand for 3 minutes, before solution 3 (3M sodium acetate, pH 4.8) was added and mixed by inversion. The tubes were incubated on ice for at least 30

minutes, after which, the tubes were centrifuged for 5 minutes at 13000 rpm, the supernatant was transferred to a new microfuge tube, 1000 μl of 95% ethanol was added and the tubes were centrifuged for 15 minutes at 13000 rpm. After centrifugation, the supernatant was discarded and the white pellet washed with 500μl of 80% ethanol for 5 minutes. The DNA was air dried and re-suspended in 200μl of sterile TE buffer and stored at -20°C until required.

2.8 DNA- Labelling

The DNA fragment probe was labelled by DIG-dUTP (alkali-labile) in the polymerase chain reaction by the PCR DIG probe synthesis kit (Roche diagnostic GmbH, Germany).

2.8.1 DNA-DNA Hybridisation (Dot-Blot)

Hybond™-N^+ nylon membrane (Amersham pharmacia Biotech UK Limited) was spotted with 2.5 μl of prepared DNA from the 87 strains. The membrane was dried in air and the DNA was fixed onto the membrane by baking the membrane at 80°C for 2 hours (Bio-Rad Gel dryer model 583). The membrane then was incubated in pre-hybridisation solution (50% formamide, deionized, 5XSSC, 0.1%(w/v) N-lauroylsarcosine, 0.02%(w/v) SDS, and 2% of blocking reagent (1/5 volume of blocking solution, 10Xconcentration) for 2 hours at 68°C. After pre-hybridisation a denatured labelled probe was added to 5 ml of pre-hybridisation fluid and the membrane was incubated overnight at 68°C in a hybridization incubator (Techne Hybridiser HB-ID, Techne Ltd, Cambridge). Following hybridisation, the membrane

was washed twice with 250 ml of wash solution 1 (2x SSC, 0.1%(w/v) SDS) at room temperature with gentle agitation (MK V Orbital Shaker, LH Engineering) for 10 minutes with each. This was followed by another wash with wash solution 2 (0.1%x SSC, 0.1% (w/v) SDS) twice at 68°C for 15 minutes each.

2.8.2 Detection

After hybridisation and stringency washes, the nylon membrane was washed for 5 minutes in wash buffer (0.1M Maleic acid + 0.3(v/v) Tween® 20) and incubated for 30 minutes in 100 ml 1x blocking solution (by diluting the stock solution 1:10 in maleic acid) with gentle agitation. The nylon membrane was then incubated for 30 minutes in 20 ml of diluted antibody-conjugate solution (diluted anti-DIG-AP conjugate (vial3) to 75mu/ml (vial2)) with gentle agitation. The unbound antibody-conjugate solution was removed by washing twice for 15 minutes each in 100 ml of washing buffer (0.1M Maleic acid + 0.3(v/v) Tween® 20). The nylon membrane was equilibrated for 2-5 minutes in 20 ml of detection buffer (0.1M Tris-HCL, 0.1M NaoH, pH 9.5). Finally the membrane was treated with 1:100 diluted CSPD (Boehringer Mannheim, Germany) detection buffer and exposed to X-ray film (Polaroid black/white) for 20 minutes at room temperature.

2.9 Isolation of chromosomal DNA

Genomic DNA was extracted for PCR by using a Puregene DNA isolation kit (Gentra system kit, USA) according to the manufacturer's instructions.

2.10 Polymerase chain reaction (PCR) primer design

Primers were either designed with primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), or the sequence was taken from previously published work. All primers were synthesized by the Oswell DNA service (Southampton University, Southampton, UK). Primers used for each PCR experiment are shown in table 2.10.1

Table 2.10.1

Oligonucleotide primers used for PCR amplification

Primer pair	Sequence (5' to 3')	Reference
SHV SHV	CGC CGG GTT ATT CTT ATT TGT CGC TCT TTC CGA TGC CGC CGC CAG TAC	(Nüesch *et al.*, 1996)
SHV -1 SHV -1	CTG GGA AAC GGA ACT GAA TG GGG GTA TCC CGC AGA TAA AT	(Hanson *et al.*, 1999)
TEM -1 TEM -1	ATG AGT ATT CAA CAT TTC CG CCA ATG CTT ATT CAG TGA GG	(Arlet *et al.*, 1995)
*gyr*A *gyr*A	TGC GAG AGA AAT TAC ACC AAT ATG TTC CAT CAG CCC	Primer design
*par*C *par*C	CTG AAT GCC AGC GCC AAA TT TGC GGT GGA ATA TCG GTC GC	Primer design

2.10.1 PCR reaction mixture

Each PCR reaction was performed in a total volume of 100 μl (table 2.10.2) prepared in a 0.5 polypropylene microcentrifuge tube (Alpha Laboratories, UK) with Cyclogene thermocycycler (Techne, Cambridge, UK) with a heated lid, thereby negating the need for the use of a sterile oil overlay. Each reaction mixture was subjected to 30 cycles, with each three steps: denaturation, annealing and extension. Different cycling protocols were used to amplify the different bla_{SHV}, bla_{TEM} genes *par*C and *gyr*A gene.

Table 2.10.2

PCR reaction components

Component	Stock concentration	Volume added /μl	Final concentration
10x Taq Buffer*	100 mM Tris-HCl/500 mM KCl	10	10 mM/ 50 mM
$MgCl_2$	25 mM	8	2.5 mM
dNTPs**	2 mM	10	0.2 mM
Primer1	10 pmol/ μl	1	10 pmol
Primer2	10 pmol/ μl	1	10 pmol
MilliQ water	-	-	make volume up to 100 μl
Taq DNA	5 U/μl	5 μl of 1:10 dilution	2.5 U

* Supplied by Promega UK; ** 2 mM 4dNTPs stock- 2 mM of each dNTP (Boehringer Mannheim, Sussex) in TE buffer pH 7.5. Stored at -20°C in 0.5 ml aliquots

2.10.2 PCR cycles

The cycling parameters for the different genes are shown in table 2.10.3. To validate the PCR reaction, negative as well as positive controls were incorporated and used.

2.10.3 PCR product size

All expected PCR product sizes are detailed and listed in table 2.10.4

Table 2.10.3

PCR heating cycle protocol for different PCR reactions

TEM-1 Protocol			
Segment	**Temperature**	**Time**	**Cycles**
1	95°C	5 minutes	1
2-a	94°C	30 seconds	30
2-b	55°C	1 minute	
2-c	70°C	1 minute	
3	75°C	10 minutes	1
SHV - Protocol			
Segment	**Temperature**	**Time**	**Cycles**
1	95°C	5 minutes	1
2-a	94°C	1 minute	30
2-b	56°C	1 minute	
2-c	70°C	1 minute	
3	72°C	10 minutes	1
***gyr*A Protocol**			
Segment	**Temperature**	**Time**	**Cycles**
1	95°C	5 minutes	1
2-a	94°C	1 minute	30
2-b	54°C	1 minute	
2-c	70°C	1 minute	
3	72°C	7 minutes	1

Table 2.10.3 (cont.)

*par*C Protocol			
Segment	**Temperature**	**Time**	**Cycles**
1	95°C	5 minutes	1
2-a	94°C	1 minute	30
2-b	55.5°C	1 minute	
2-c	70°C	1 minute	
3	72°C	7 minutes	1
SHV-1 Protocol			
Segment	**Temperature**	**Time**	**Cycles**
1	95°C	5 minutes	1
2-a	94°C	1 minute	32
2-b	57°C	1 minute	
2-c	70°C	1 minute	
3	72°C	10 minutes	1

Table 2.10.4

Expected product sizes for PCR

PCR Reaction	Expected product size
bla $_{SHV}$ primers	1017 bp
bla $_{SHV-1}$ primers	383 bp
bla $_{TEM-1}$ primers	858 bp
DNA *gyr*A primers	626 bp
DNA *par*C primers	346 bp

2.10.4 Analysis of PCR products

PCR products were separated at neutral pH vales in 1% w/v agarose (GIBCO BRL, Life Technologies, UK) gels in TAE buffer (40 mM Tris-acetate pH 7.6, 1 mM EDTA). Under these conditions, double stranded DNA is negatively charged hence loaded near the cathode and migrates towards the anode with the application of an electric field (Aaji and Borst, 1972). Gel electrophoresis was carried out in a mini-subcell GT (Bio Rad, UK) under the constant voltage of 100V (Powerpac 300, Bio Rad, UK) for 40 minutes, depending on the migration of the bands. For larger gels subcell GT (Biorad, UK) gel tanks were used. A DNA ladder (Generuler™, MBI, Fermentas) was run alongside the PCR products verification of the sample's molecular size. Each DNA sample was mixed with 2µl of loading buffer (30%w/v glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol) before loading the wells.

2.10.5 Staining and visualization of DNA

The electrophoresed fragments were visualized after electrophoresis by staining for one hour in a 50 µg/ml ethidium bromide solution. If necessary, de-staining was carried out with gentle agitation in distilled water to remove excess ethidium bromide and reduce background fluorescence. DNA was viewed by placing the gels on a UV transilluminator (UV products, Cambridge, UK) and photographed.

2.10.6 Purification of DNA

PCR products were purified by using the Qiaquik PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. The final volume for elution

of the purified products was reduced to 30µl to concentrate the DNA. The concentration of the DNA from the PCR product was approximated by comparing the intensity of the bands after electrophoresis of λ DNA digested with *Hind*III (Sigma, UK) known to contain 0.5µg per micro liter.

2.10.7 Automated DNA sequencing

The PCR primers had been used as sequencing primers at a concentration of 3.2 Pico mol (10^{-12}) per reaction. The DNA was purified as described before prior to sequencing to ensure that the required concentration of 30 to 90 ng was achieved. The DNA sequence was determined by the chain termination method developed by Sanger (1977). Individual fragments of PCR were set up in the ready reaction format for fluorescence based on dideoxy cycle sequencing (PE Applied System, UK). All sequences were determined in the Department of Hematology, Royal Infirmary of Edinburgh, Scotland. Sequences were then analysed by the BLAST online search engine (http://www.ncbi.nih.gov/cgi-bin/BLAST) with the susceptible strains sequence in the database.

2.11 Isoelectric focusing

For any protein there is a characteristic pH, called the isoelectric point (pI), at which the protein has no net charge and therefore will not move in an electrical field. In isoelectric focusing (IEF), proteins are electrophoresed in a narrow tube of polyacrylamide gel in which a pH gradient is established by a mixture of special buffers. Each protein moves to its pI point in the gradient.

2.11.1 Purification of β-lactamase

Each of the 87 isolates was streaked onto a Mueller-Hinton agar slopes and incubated overnight at 37°C. The cell growth was washed off the surface of the Mueller-Hinton agar slope with 2.0 ml of 50mM sodium phosphate buffer, pH 7.0 then, transferred to a fresh sterile bottle and cooled in an ice cold container. Bacterial cells were cooled with ice and disrupted by sonication (MSE Soniprep 150. MSE instruments, Crawley, Sussex) with two 15 second pulses of 6-8 μm of amplitude separated by a 15 second cooling period. The lysate was then cleared of cell debris by centrifugation at 4°C in an MSE microcentrifuge at 13000 rpm for 10 minutes. The cell free supernatant was stored at -20°C until required.

2.11.2 Assessment of β-lactamase activity in the enzyme preparation

The reaction time for a 30 μl volume of the β-lactamase preparation to change the colour of 100 μl of nitrocephin solution (50mg/ml) from yellow to red was taken as an indication of the β-lactamase activity of the enzyme preparation.

2.11.3 Analytical isoelectric focusing

β-lactamases were identified by analytical IEF as described by Matthew *et al.*, (1975). The β-lactamase preparation was focused on thin layer polyacrylamide gels containing broad range (pH 3.5-10) or a mixture of broad range (pH 3.5-10) and narrow range (pH 6-8) carrier ampholytes (1:1). Thin layers of polyacrylamide gel, 200mm x 150mm x 1mm were prepared by the polymerisation of the gel solution described in table 2.11.3, catalysed by riboflavin in the presence of UV light between

two glass plates 1mm apart. To promote adhesion of the polyacrylamide gel to one of the 2 glass plates, it was necessary to coat one glass with binding solution. The remaining glass plate was siliconised, to prevent adhesion.

Preparation of binding solution was performed by dissolving 0.5% (w/v) gelatine (ca.225.bloom from calf skin, Aldrich Chemical co. Ltd., Gillingham, Dorset) and distilled water. Glass plates (210mm x 160mm) were submersed in the coating solution for 10 minutes, before removal from the solution and drying in the incubator at 55°C for 20 minutes prior to use.

Samples of β-lactamase preparation were loaded close to the anode on the gel surface (volume in μl equivalent to spot test time). For all gels, β-lactamases with known pIs were focused alongside novel β-lactamase enzymes.

2.11.4 Running conditions

The running condition of the analytical IEF gels was at constant power of 1 watt overnight at 4°C (power setting: voltage = 500V, current = 20mA, power = 1.0W).

2.11.5 Visualisation of β-lactamases after electrophoresis

The focused β-lactamase bands were visualised by overlaying the polyacrylamide gel with sheets of filter paper (Whatman® No.1, Whatman International Ltd, Maidstone) soaked in 1mM nitrocephin. pIs of unknown or novel enzymes were estimated from

the focused bands of enzymes of known pI. Stained gels were photographed with a Polaroid camera using a Tiffen green filter.

2.11.6 Pre-cast IEF gel

Pre-cast IEF gels (Ampholine® PAGplate, Pharmacia Biotech, Uppsala, Sweden) are polyacrylamide gel cast on plastic support films. The dimensions of the gel (110 mm x 245 mm x 1mm) allow the screening of large number of samples. The Pharmacia Ampholine PAG plate (pH range 3.5 to 9.5) was placed on a LKB 2217 Ultraphor Electophoresing apparatus (Pharmacia Biotech) according to the manufacturer's instructions, and run at temperature of 4°C overnight. Strips of filter paper are coated with 1M of H_3PO_4 and 1M of NaOH. These strips are then placed on the anode and cathode respectively. The running conditions and staining methods are described in the analytical isoelectric focusing section.

2.12 Restriction fragment length polymorphism (RFLP)

PCR–restriction fragment length polymerase (PCR-RFLP) is known as a fast and easy genetic approach used to distinguish between the sensitive and resistant *gyr*A and *par*C by the loss of single nucleotide.

2.12.1 Restriction of products from *bla*$_{SHV}$ PCR

SHV- specific PCR products were used directly in an *Nhe*I restriction endonuclease assay (Promega, Ltd). The full–length PCR product which was refractory to

restriction by *Nhe*I doesn't represent SHV-1, while, the cleavage product would represent SHV-2 and/or SHV-5. The IEF values and the sequence of the cleavage SHV could confirm its type. SHV PCR product was restricted with *Nhe*I (10u/μl), 5μl of restriction buffer (10 mM Tris-HCl, pH7.5, 60 mM NaCl, 7mM $MgCl_2$), 1 μl of bovine serum albumin (BSA) (0.1mg/L), 4μl of sterile MilliQ water and 40 μl of amplified PCR product. Digestion was carried out for a maximum four hours at 37°C. Fragments were resolved by agarose gel electrophoresis using 2% agarose in 1xTAE buffer, then stained with ethidium bromide, and visualized with a UV light.

2.12.2 Restriction of products from *bla*$_{TEM}$ PCR

The endonuclease digestion of TEM-specific PCR product was performed directly using 10μl of PCR products without purification, according to the recommendation of the restriction endonuclease suppliers *Mse*I (New England, Biolabs, England) and *Sau3 AI* (promega, Ltd). Using the following amounts 5μl of restriction buffer (10 mM Tris-HCl, pH7.5, 60 mM NaCl, 7mM $MgCl_2$), 1 μl of BSA (0.1mg/L), 4μl of sterile MilliQ water. Restriction patterns of PCR products were analysed by agarose gel electrophoresis using 2% agarose in 1xTAE buffer, then stained with ethidium bromide, and visualized with a UV light.

Table 2.11.3

Composition of analytical IEF gels

Ingredient Stock Solution	Volume (ml)	Final concentration
Sterile distilled water	25	-
40% (w/v) ampholines	2.0	2% (w/v)
100 g acrylamide pluse 2.7 g methylene bisacrylamise (BDH) in 300 ml sterile distilled water	9.0	acrylamide-75 g/L bisacrylamise- 2 g/L
5% (v/v) TEMED*	0.2	0.005% (v/v)
Riboflavin (20 mg/L)	4.0	2 mg/L

* Tetramethyl-ethylenediamine

2.12.3 Restriction of products from topoisomerase PCR

RFLP was used to screen for the *gyr*A specific PCR product as used directly in an *Hinf*I restriction endonuclease (Promega, Ltd). Conferring quinolone resistance, for example resulting in the substitution of amino-acid 83 threonine in the quinolone resistant determining region (QRDR) of *gyr*A region results in the loss of the *Hinf*I G/ANTC restriction site. *Hinf*I was therefore used to screen for the presence of this mutation in the clinical samples. QRDR were restricted with (10u/μl), 5μl of restriction buffer (10 mM Tris-HCl, pH7.5, 60 mM NaCl, 7mM $MgCl_2$), 1 μl of BSA (0.1mg/L), 4μl of sterile MilliQ water and 40 μl of amplified PCR product. Digestion was carried out for a maximum three hours at 37°C. Fragments were resolved by agarose gel electrophoresis using 2% agarose (MAST Gel™ BB mast diagnostic Mast group Ltd. Merseyside, UK) in 1xTAE buffer, then stained with ethidium bromide, and visualized with a UV light.

2.13 Pulse field gel electrophoresis (PFGE)

Restricted genomic DNA fragments were separated on a Contour-clamped Homogenous Electric Field (CHEF) electrophoresis system (CHEF-II, Bio Rad) according to the manufacturer's instructions

2.13.1 Preparation of genomic DNA in plugs

Strains were inoculated into 10 ml of nutrient broth (Oxoid) and incubated at 37°C overnight. The cells were harvested by centrifugation at 3000 rpm for 30 minutes (

SORVALL R77 pulse) at 4°C. The supernatant was discarded, and the cells were washed 2X in 10 ml of cell suspension buffer CSB (Tris-HCl, EDTA, NaCl, pH 8.0). After the final wash, the cells were re-suspended in 5ml of CSB and kept at room temperature.

Low melting point agarose (2%) (Bio-Rad) was dissolved in TE buffer (10 mM Tris-HCl, 10 mM Na_2 EDTA, pH 7.5) using a microwave oven and placed at 56°C until required. Equal volumes of CSB and 2% low melting point agarose (500 µl from each) were pipetted into sterile eppendorf tubes. The tubes were mixed well by inverting several times and kept molten at 56°C. Plug moulds (Bio-Rad) were wiped with ethanol and sealed with tape at the bottom. Ninety µl from each culture/agarose mixture was carefully pipetted into each area of the mould to give a total of 5 plugs per strain. The moulds were then allowed to set at room temperature for approximately 20 minutes, after which time the tap was removed from the bottom and plugs were carefully transferred into sterile bijou bottles by pushing them through from the moulds with a sterile pipette tip.

2.13.2 Lysis of DNA in agarose plugs

A 3 ml volume of lysis buffer (1% N- lauroylsarcosine, 50 mM Na_2 EDTA, pH 9.5) containing 0.5 mg/ml proteinase K (Sigma) was added to each bijou bottle and plugs were incubated at 56°C for 3 days in a waterbath (GallenKamp). After incubation, the lysis buffer was removed and then, the plugs were washed in 2 ml of TE buffer for 30 minutes at room temperature. The wash stage was repeated a further 2 times with fresh TE buffer used each time. The plugs were subsequently stored in TE

buffer at 4°C until ready for use (Plugs were stored under these condition for several months with no DNA degradation if the TE buffer was replaced approximately every 4 weeks).

2.13.3 Digestion of DNA in agarose plugs

DNA plugs were transferred into a sterile petri dish where an appropriate portion (equivalent to the size of the well formed) was cut using a sterile scalpel blade. Each plug piece was washed with sterile distilled water for 10 minutes, and then allowed to equilibrate in 1X restriction buffer at 37°C for 30 minutes before the digestion. The DNA plugs were transferred to a new sterile eppendorf to which the following components were added in this order: (I) 87μL of MilliQ water, (ii) 9 μl of 10X enzyme buffer (Promega, Ltd.), (iii) 2 μl of 1 μg/μl BSA (Promega, Ltd.), (iv) 4 μl of 10u/μl *Xba*I restriction endonuclease (Promega, Ltd.) and (v) 87 μl of sterile MilliQ water.

The contents were mixed well by gently tapping the side of the eppendorf several times. The DNA was subsequently digested overnight at 37°C, after which the restriction mixture was removed and the plug piece carefully transferred to sterile 20 ml universal containers. A 10 ml volume of sterile MilliQ water was added to each universal to wash the DNA plugs. The universals were kept for 30 minutes at room temperature. Plugs were stored at 4°C until the gel was ready to be loaded.

2.13.4 Preparation of agarose gel

The gel casting tray was wiped with absolute alcohol and assembled according to the manufacturer's instructions (Bio-Rad). Tris-Borate-EDTA (TBE) buffer was diluted 1:10 to give a final concentration of X 0.5 in a 2 litre volume. A 100 ml volume of this dilution was aliquoted into a sterile 250 ml Duran bottle (Schott), into which PFGE-grade agarose (Bio-Rad) was added. The agarose in the TBE buffer was melted in a microwave oven and then kept at 55°C to cool. The cooled agarose was carefully poured into the assembled casting tray (leaving approximately 3 ml in the bottle at 55°C) and allowed to set for at least 45 minutes. The rest of the diluted buffer was added to the electrophoresis cell (Bio-Rad), and allowed to circulate with cooling until it reached the required temperature.

2.13.5 Loading of PFGE gel

Once the comb had been carefully removed from the gel, the plugs containing restricted DNA were inserted into the appropriate wells using a sterile spatula, ensuring that no air bubbles were introduced. DNA size standards (λ ladder range from 48.5 to 970 Kbp, Bio-Rad) were also loaded into a well approximately half way along the gel. The wells were filled with the rest of the molten agarose and the gel was kept at 4°C until the agarose had set.

2.13.6 Running conditions

The gel was removed from the casting tray and placed in the electrophoresis cell (CHEF-II, Bio Rad) Contour-clamped Homogenous Electric Field. Gel running condition were as follows:

Initial pulse time 5 seconds	Voltage 200 volts (6v/cm)
Final pulse time 45 seconds	Run temperature 14°C
Run time 22 hours	Gel concentration 1%

2.13.7 Gel staining and visualization of PFGE patterns

The gel was carefully removed from the electrophoresis cell and transferred to a container to which 200 ml of ethidium bromide solution (0.5 mg/L) had been added. Staining was carried out for 45 minutes, followed by 2x15 minute washes in distilled water.

PFGE patterns were visualised on a UV transilluminator and photographs were taken by a diversity database camera.

2.13.8 Analysis of PFGE patterns

The data obtained for PFGE patterns on the gels were analysed by Diversity database finger printing software (Bio-Rad). In which, the presence/ absence of shared fragments used to estimate the relatedness of the PFGE patterns was presented in a binary data matrix. The relatedness were compared by RAPdistance package version 1.04, (http//:www.life.anu.edu.au.molecular/software/rapid.html). The RAPdistance program calculates the genetic distance by the Nei distance method (Nei, 1972). Patterns were also analysed manually using the criteria of Tenover (Tenover *et al.*, 1995).

2.14 Outer membrane protein (OMPs) preparation

Bacterial strains were inoculated into 100 ml of BHIB (Oxoid) and incubated at 37°C overnight with gentle agitation (200 osc/min). The cells were harvested by centrifugation at 6000 g (Sorvall® RC-5B Refrigerated Super-speed centrifuge) at 4°C. After the supernatant had been discarded, the pellet was washed in 10 ml of 50 mM sodium phosphate, pH 7.0, the cells were centrifuged again at 3000 g , and the pellet was re-suspended in 3 ml of 50 mM sodium phosphate, pH 7.0.

Cells were subjected to ultrasonication (MSE Soniprep 150, MSE instrument, Crawley) for 3 x 30 seconds at an amplitude of 8 microns with constant cooling and with a 1 minute cooling period between each sonication. The cell lysate was cleared (unbroken cells removed) by centrifugation (MSE Microcentaur centrifuge) at high speed 13000g for 10 minutes at 4°C. The supernatant containing OMP's was transferred to a centrifuge tube. Cell membrane was sedimented from the supernatant fluid by centrifugation at 15600 g for 30 minutes at 4°C. The pellet was re-suspended in 3 ml of 2% (w/v) N-laurylsarcosine and incubated for 30 minutes at room temperature. Then sarcosyl-insoluble OMP's were sedimented by ultracentrifugation (40000 g for 60 minutes at 4°C). The clear OMP's pellet was re-suspended in 1 ml of sterile MilliQ water and stored at -20°C until required.

2.14.1 Dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of OMPs

OMPs for each isolate were mixed with an equal volume of protein sample buffer (stock= 4 ml distilled water, 1 ml 0.5 M Tris-HCl, pH 6.8, 800 μl glycerol, 1.6 ml 10% (w/v) SDS, 400 μl 2-mercaptoethanol, 200 μl 1% bromophenol blue), heated at 100°C for 5 minutes in a waterbath, and kept on ice until required for gel loading. A 30 μl volume of each sample was loaded into the wells of a ready-made gel (Bio Rad). Low range molecular weight marker (Bio Rad) was added to a well on one side of the gel. The gel was inserted into a mini-gel electrophoresis apparatus (Miniprotean® II, Bio Rad) then electrode buffer (0.025M Tris, 0.19M glycine and 0.1% SDS, pH 8.3) was added and electrophoresis was carried out at 200 V for 45 minutes or until the dye in the sample buffer had reached the end of the gel.

2.14.2 Staining (SDS-PAGE) of OMPs

SDS- PAGE gels were stained in Coomassie Blue (40% v/v methanol, 10%v/v acetic acid, 0.25% w/v Coomassie blue R-250) with gentle shaking for a minimum of one hour. The gel was transferred to a destaining solution (40% v/v methanol, 10% v/v acetic acid in distilled water) and destained with gentle shaking for approximately 4-5 hours, or until the background became clear. Destaining solution was replaced with fresh solution several time during this period. As this procedure results in shrinking, the gel was subsequently immersed in 20% (v/v) glycerol until it resumed its original length and shape.

Chapter 3

Characterisation of Extended Spectrum β-lactamases Identified By The Vitek System And Their Subsequent Comparison With Other Commercial ESBL Testing Systems

3.1 Introduction

Since the introduction of third-generation cephalosporins into clinical practice there has been excessive use of extended-spectrum cephalosporins in hospitals (Du Bois *et al.*, 1995; Heritage *et al.*, 1999). The effectiveness of these β-lactam antibiotics has been diminished by *Klebsiella* spp. that are resistant to their mode of action. This resistance has spread to strains of *E.coli* and to other Gram-negative bacteria (Sanders et al., 1996). Investigations into the mechanism responsible for this resistance have revealed the presence of extended spectrum β-lactamase (ESBL) enzymes derived from the ubiquitous TEM-1/2 and SHV-1 family. To date, there are over 100 derivatives of TEM β-lactamases and more than 35 derivatives of SHV β-lactamases (Bush *et al.*, 1995; Bradford *et al.*, 1995; Bradford *et al.*, 1996; Bradford, 2001; Jacoby and Medeiros, 1991; Philippon *et al.*, 1989b; Sanders *et al.*, 1996). These enzymes are usually less efficient at hydrolysis of penicillins than their parent enzymes, but show hydrolytic activity against cephalosporins. Their detection is difficult to demonstrate in currently used routine susceptibility tests. Therefore, ESBL-producing *Klebsiella* spp. and *E.coli* may appear falsely susceptible to newer cephalosporins (Katsanis *et al.*, 1994; Philippon *et al.*, 1989b; Sanders *et al.*, 1993). There is clearly a requirement to detect the resistance mechanism itself rather than to rely on *in vitro* susceptibility testing. The Vitek system (bioMérieux, Ltd., Basingstoke, England) has an ESBL detection test as an integral part of the routine susceptibility test card. There have been several positive studies on the efficacy on the Vitek system with known ESBL producing strains (Gibb and Crichton, 2000; Sanders *et al.*, 1996).

This study was performed to assess the behaviour of ESBLs in clinical strains detected by this system in comparison with two other commercial ESBL detection methods.

3.2 Materials and Methods

3.2.1 Bacterial isolates

One hundred and one clinical isolates of *Klebsiella pneumoniae* (69) and *E.coli* (32) were obtained from various clinical samples sent to the clinical bacteriology laboratories of the Royal Infirmary of Edinburgh (RIE). The 101 isolates had previously been identified and reported as ESBL-positive by the Vitek system. Working isolates were stored on nutrient agar slopes at room temperature until required.

3.3 Results

Presence of ESBL activity was initially detected by the Vitek system. The test is based on the inhibitory effect of clavulanic acid on the ESBL in the presence of either cefotaxime or ceftazidime and in the presence of the antibiotic on its own. The test is interpreted by measuring the difference in bacterial growth with or without inhibitor. Based on the automated version of the synergy test in the Vitek system, 87 isolates were ESBL positive. ESBL activity in all those isolates was re-confirmed by the E-

test ESBLs strips (see 3.3.1). Of the 101 strains originally tested, 15 were negative for ESBL production when tested with both cefotaxime and ceftazidime E-test strips. These 15 E-test negative strains, (all *E. coli*) were re-tested with Vitek (GNS-532 card) and 14 of these strains were subsequently found to be ESBL negative. One strain still was identified as ESBL positive by Vitek. The re-tested 14 ESBL negative strains were not subjected to any further studies.

3.3.1 E-test ESBL Strips

3.3.1.1 Cefotaxime

The cefotaxime ESBL strip detected the presence of ESBL activity in 83 (95.4%) of the tested isolates (16 *E.coli* and 67 *K. pneumoniae*) (see table 3.1). Of these strains, 19 (22%) demonstrated "phantom phenomena" as described in the manufacturer's interpretation guide*. Four strains were negative for ESBL activity with the cefotaxime ESBL strip, 2 strains of *E.coli* (A332, A336) and 2 strains of *K. pneumoniae* (A359, A338).

* A rounded phantom inhibition zone that occurs below CT or TZ strips is indicative of ESBL production.

3.3.1.2 Ceftazidime

With ceftazidime E-test strips, ESBL activity was detected in 47 (54%) of the study isolates, (11 *E. coli* and 36 *K. pneumoniae*) (see table 3.1). Of these, 1 *E.coli* and 2 *K. pneumoniae* were positive by the demonstration of "phantom phenomena".

Only 40 of ESBL-positive strains gave a positive ESBL reaction with both strips, the other 47 were only positive with one or other of the strips used. The strains that were deemed ESBL-positive by the production of phantom phenomena with the cefotaxime strip were all positive with ceftazidime with the exception of one strain. All ESBL E-test results are presented in appendix I.

The use of the combination of both cefotaxime and ceftazidime E-test ESBL strips detected ESBLs in 86 of the 87 test isolates (98.8%) see figure (3.1). This data illustrates the use of both strips is essential for optimal detection of ESBL activity. Only one strain of *K. pneumoniae* that demonstrated positive ESBL activity with the Vitek system was not detected by E-test ESBL strips despite repeat testing. This strain remained positive when re-tested with Vitek.

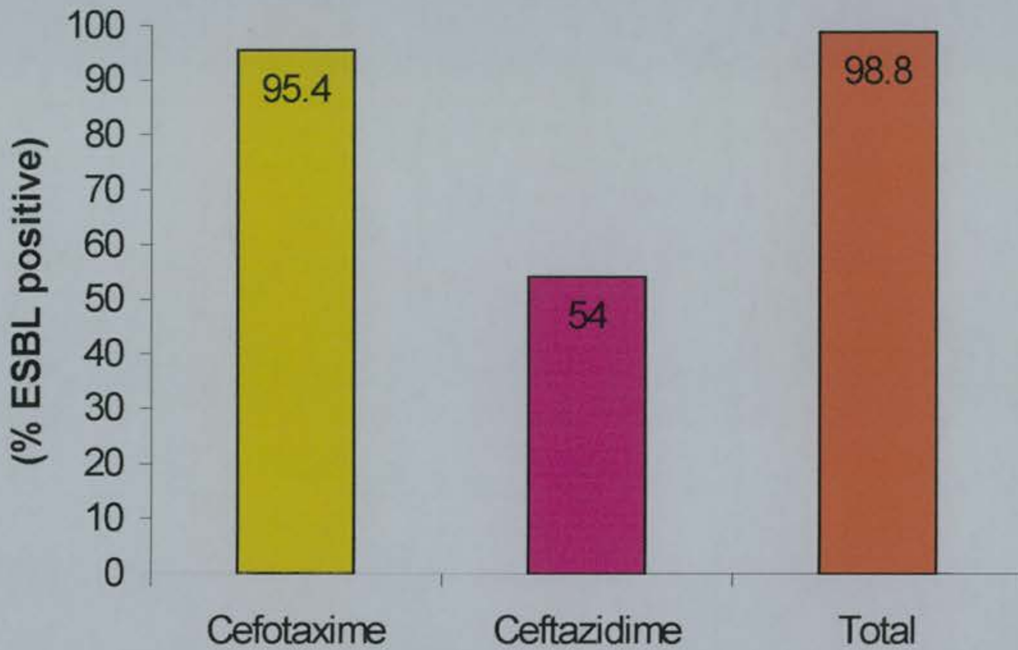
Table 3.1

Results of ESBL E-test strips with the 87 test isolates

E-test ESBL positive	*E. coli*	*K. pneumoniae*	Total
Cefotaxime	16	67	83
Ceftazidime	11	36	47

Figure 3.1

Percentage of the 87 test strains positive by either cefotaxime or ceftazidime ESBL strips



Figures 3.2 and 3.3 show representative plates with E-test strips indicating ESBL positive and negative strains respectively.

Figure 3.2

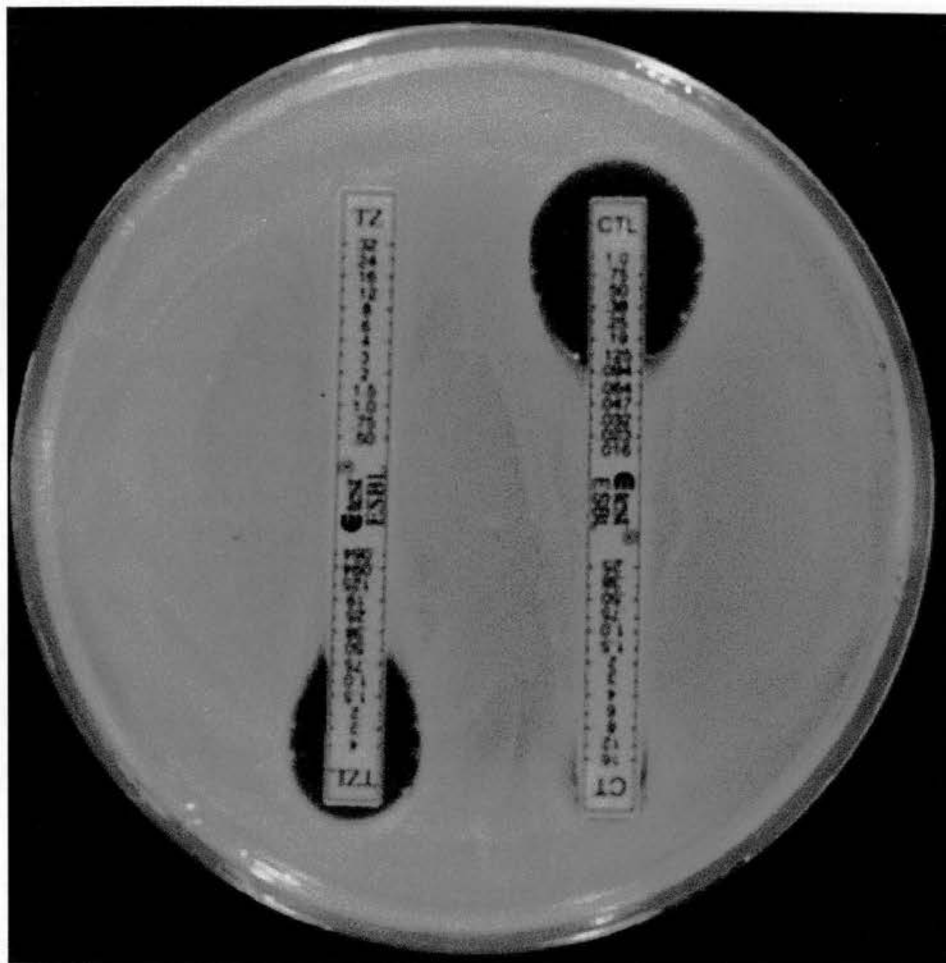


Plate showing positive ESBL reaction with E-test strips containing cefotaxime + cefotaxime/clavulanic acid (CT/CTL) and ceftazidime + ceftazidime/clavulanic acid (TZ/TZL). The zone of inhibition is read on the two halves of each strip where the inhibition ellipse intercepts the strip.

Cefotaxime ESBL strip
CTL= 0.125mg/l
CT/CTL= 16/0.125 Ratio = 128 indicating +ve ESBL
CT=16mg/l

Ceftazidime ESBL strip
TZ= 32.0mg/l
TZL= 0.75mg/l
TZ/TZL = 32/0.75 Ratio = 42.6 indicating +ve ESBL

Figure 3.3

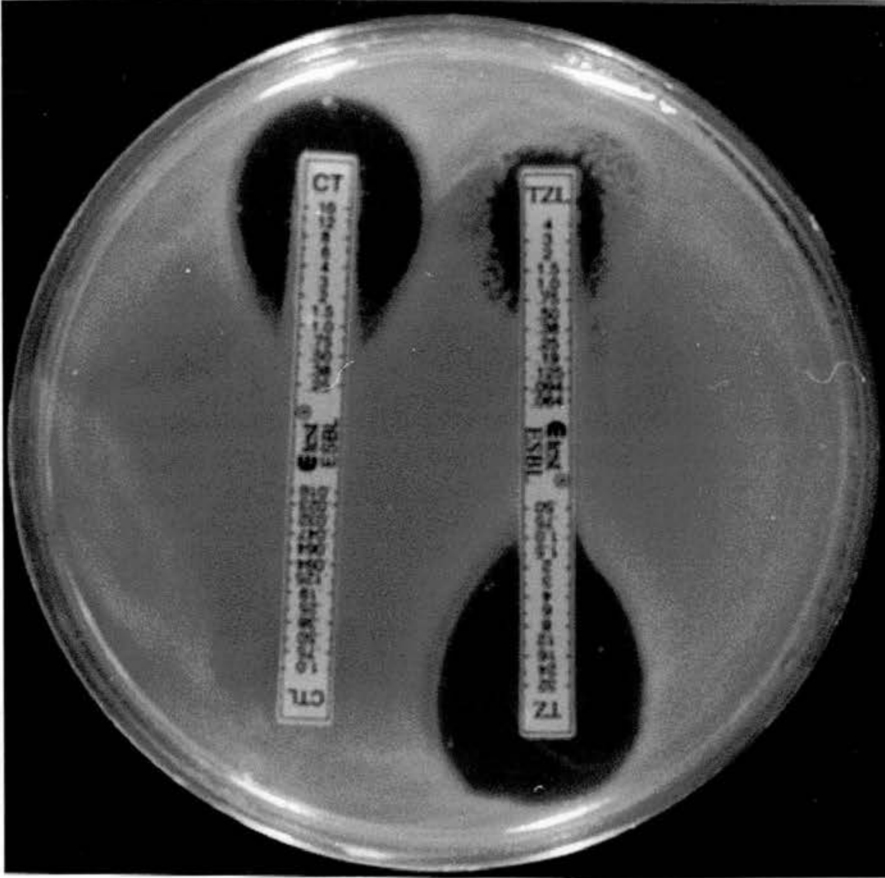


Plate showing negative ESBL reaction with E-test strips containing cefotaxime + cefotaxime/clavulanic acid (CT/CTL) and ceftazidime + ceftazidime/clavulanic acid (TZ/TZL).

Ratio value <8 for both strips.

3.3.2 Double disc diffusion (DDD)

Testing was performed according to NCCLS guidelines (NCCLS, 1999). A positive result was indicated by a zone size difference of $\geq$ 5mm diameter between the combination disc and the corresponding standard antibiotic disc (see figures 3.4 and 3.5). Results of the DDD method are summarized in table 3.2. The results of the DDD for individual isolates are presented in appendix II.

Using the DDD method, the combination disc containing cefotaxime detected the presence of ESBL activity in 74 (85%) (64 *K. pneumoniae* and 10 *E.coli*) strains whilst the cefpodoxime combination disc detected ESBL activity in 64 (73.5%) strains (54 *K. pneumoniae* and 10 *E.coli*). The ceftazidime combination disc had the poorest sensitivity, detecting the presence of ESBL activity in only 40 (46%) strains (31 *K. pneumoniae* and 9 *E.coli*). The strain of *K. pneumoniae* that demonstrated no ESBL activity by E-test was ESBL positive with the DDD method. When the results of all three cephalosporin combination discs were taken into consideration, 80 (92%) of the 87 ESBL-positive strains were detected (table 3.2).

A comparison of results of the DDD test and those obtained with the E-test ESBL strips (table 3.3) showed that E-test strips were more sensitive at detecting ESBL production than the DDD test. The DDD test demonstrated ESBL activity in 80 (92%) of strains whilst E-test strips detected ESBL production in 86 (98.8%) of the test strains.

Figure 3.4

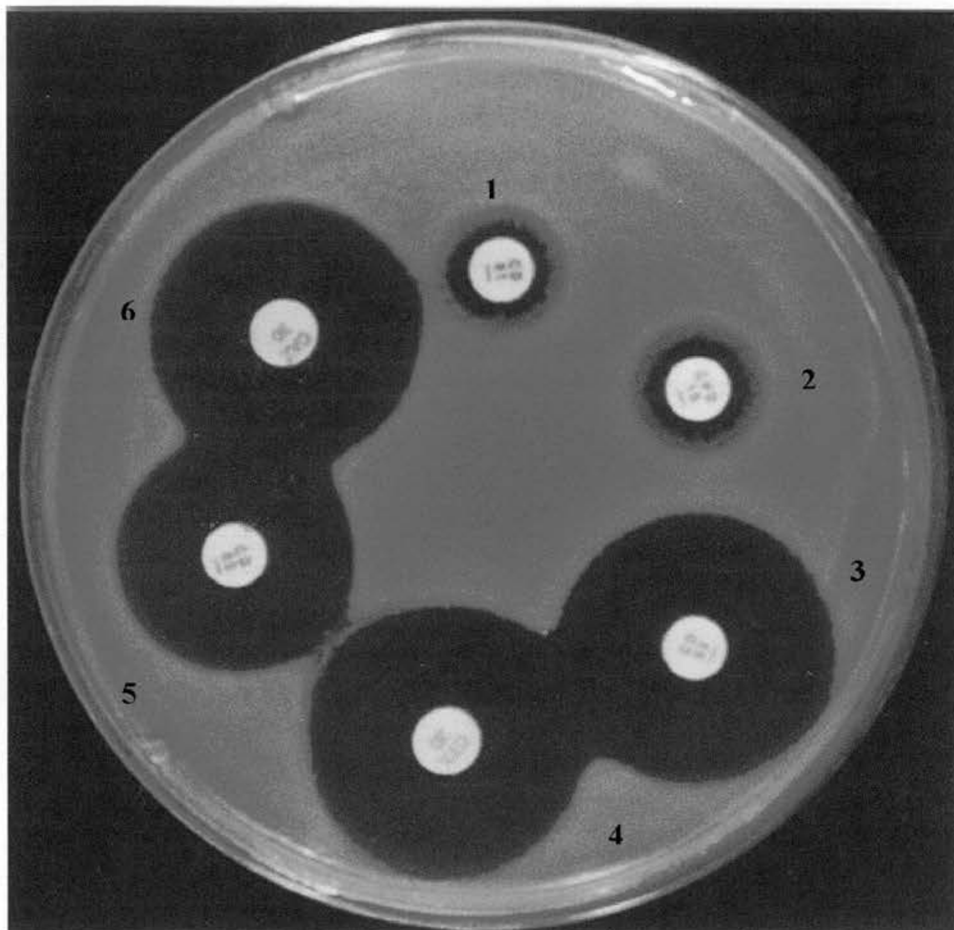


Figure showing a negative ESBL reaction with the DDD test indicated by a zone difference of < 5mm diameter between the 3 combination discs and the corresponding standard

1- Cefotaxime disc.

2- Combination disc of cefotaxime + clavulanic acid.

3- Cefpodoxime disc.

4- Combination disc of cefpodoxime + clavulanic acid.

5- Ceftazidime disc.

6- Combination disc of ceftazidime + clavulanic acid.

Figure 3.5

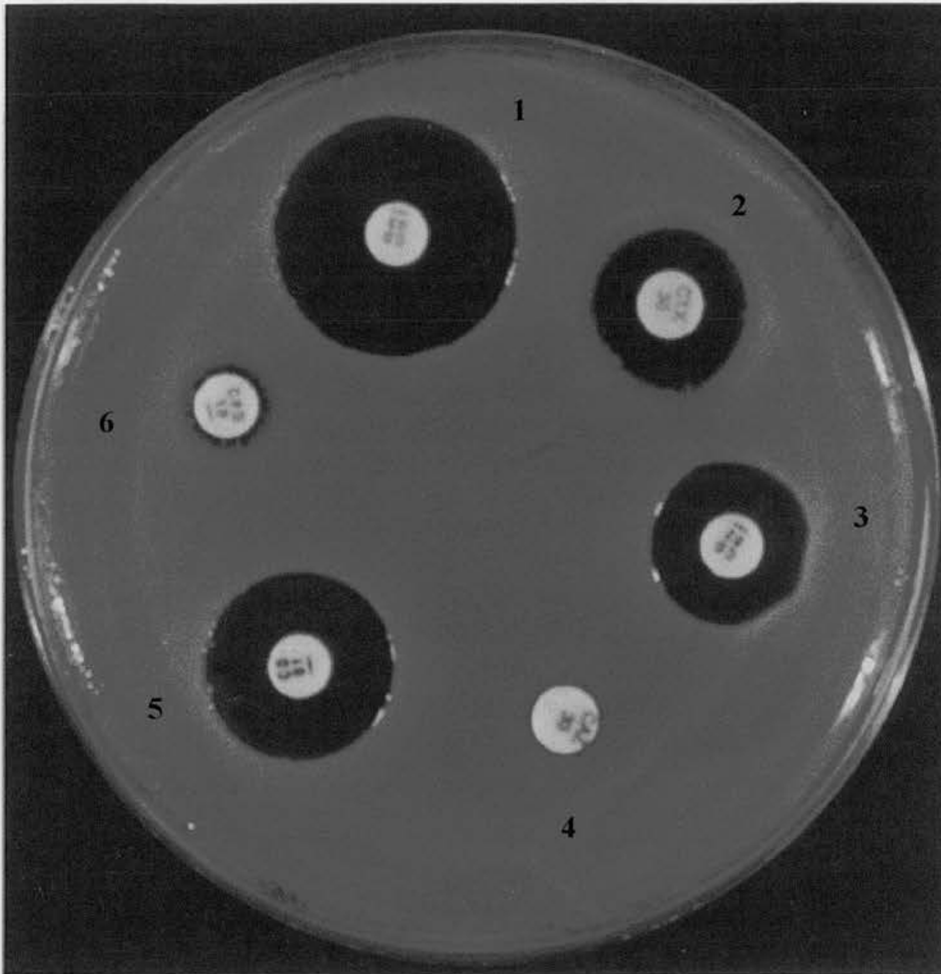


Figure showing a positive reaction with the DDD test indicated by a zone difference of ≥ 5mm diameter between the 3 combination discs and the corresponding standard antibiotic discs.

1- Combination disc of cefotaxime + clavulanic acid.

2- Cefotaxime disc.

3- Combination disc of ceftazidime + clavulanic acid.

4- Ceftazidime disc.

5- Combination disc of cefpodoxime + clavulanic acid.

6- Cefpodoxime disc.

Table 3.2

ESBL reactions with the DDD method for the 87 test isolates

Antibiotic disc used + clavulanate	***E. coli*** **No. (%)**	***K. pneumoniae*** **No. (%)**	**Total No. (%)**
Cefotaxime	10 (55)	64 (92.7)	74 (85)
Ceftazidime	7 (39)	39 (56)	46 (53)
Cefpodoxime	10 (55)	54 (78)	64 (73.5)
All three antibiotics	13 (72)	67 (97)	80 (92)

Table 3.3

Comparison of the DDD test and E-test ESBL strips in detecting ESBL production in the 87 test strains

Combination disc/strip	**Double Disc Diffusion Method. No. ESBL positive (%)**	**E-test ESBL strip No. ESBL positive (%)**
Cefotaxime	74 (85)	84 (96.5)
Ceftazidime	40 (46)	47 (54)
Cefpodoxime	64 (73.5)	-
Total (%) ESBL +ve	80 (92)	86 (98.8)

Chapter 4

Antimicrobial Resistance Amongst ESBL-Producing *Klebsiella pneumoniae* and *E.coli*

4.1 Introduction

Since penicillin was introduced into clinical practice in the 1940s, the evolution of bacteria clearly indicates that antimicrobial resistance will develop given sufficient time and use of a particular agent or class of agents. β-lactam resistance in clinically important pathogens has increasingly limited their usefulness. To treat infections due to β-lactamase-producing bacteria that were resistant to penicillin and early cephalosporin derivatives, new generations of relatively enzyme-stable and broad-spectrum cephalosporin derivatives were introduced in the late 1970s and early 1980s. However, over the past decade, antibiotic-resistant mutants producing ESBLs have emerged among Gram-negative bacteria, predominantly *E.coli* and *K. pneumoniae* (Livermore, 1995).

The emergence of ESBL-producing isolates has important clinical and therapeutic implications. Firstly, in most bacterial isolates, resistance determinants for ESBL production are carried on plasmids that can be easily spread from organism to organism (Bush, 1996). Secondly, the spread of resistance toward extended-spectrum cephalosporins further limits the use of the β-lactam class and may lead to increased prescribing of more broad-spectrum and expensive drugs such as imipenem. In addition, these resistant isolates may escape detection with routine susceptibility testing performed by a clinical microbiology laboratory, which can result in adverse therapeutic outcomes (Tenover *et al.*, 1999).

More importantly, antibiotic selection for treatment of serious infections due to ESBL-producing *E.coli* and *K. pneumoniae* is a clinical challenge because of the

complex nature of in *vitro* susceptibility testing and in *vivo* correlation (Wong-Beringer, 2001). The biggest challenge lies in overcoming widespread unawareness among clinicians regarding these resistant organisms which results from under-reporting by microbiology laboratories and lack of an obvious marker to indicate production of an ESBL (Paterson and Yu, 1999).

Some ESBLs confer high-level of resistance to all oxyimino-β-lactams, but some are selective for particular β-lactams. This creates a problem for clinical laboratories, since organisms producing less active ESBLs can fail to reach current NCCLS break point values for resistance yet can cause significant disease (Katasanis *et al.*, 1994).

4.2 Results of the antimicrobial susceptibility tests

4.2.1 Disc diffusion (NCCLS)

All 87 isolates were tested for susceptibility to 15 different antibiotics by disc diffusion following the NCCLS method (NCCLS, 1999). Results of the susceptibility patterns are summarized in table 4.1. Results for each isolate are presented in appendix III.

All strains were susceptible to meropenem and resistant to ampicillin. In addition, 98.8% (86) and 97.7% (85) of them were resistant to amoxicillin and cephalothin respectively. Similarly 95% (83) were resistant to cefuroxime, 86% (75) to

trimethoprim, 78% (68) to ciprofloxacin, 72% (63) to tazobactam, 71% (62) to chloramphenicol, 62% (54) to amikacin, 51.7% (45) to nitrofurantoin and tobramycin, 46% (40) to cefotaxime and 39% (34) were resistant to ceftazidime and gentamicin.

Table 4.1

Results in No. of antimicrobial susceptibility testing by the NCCLS disc diffusion method to various antimicrobials for the 87 ESBL positive strains

Antibiotic Disc	Resistant	Intermediate	Sensitive
Ampicillin	87	-	-
Amoxicillin	86	-	1
Trimethoprim	75	-	12
Cephalothin	85	-	2
Cefuroxime	83	2	2
Cefotaxime	40	34	13
Ceftazidime	34	7	46
Gentamicin	34	1	52
Ciprofloxacin	68	1	18
Tobramycin	45	-	42
Tazobactam	63	-	25
Meropenem	-	-	87
Chloramphenicol	62	4	21
Nitrofurantoin	45	19	23
Amikacin	54	24	9

4.2.2 Vitek System

All 87 isolates were tested for susceptibility to the same antimicrobial agents as those used for the NCCLS disc diffusion test. The results are summarized in table 4.2 and antibiograms for each isolate detailed in appendix IV.

All isolates were susceptible to meropenem and amikacin. 98.8% (86) were resistant to ampicillin and cephalothin. 93% (81) were resistant to cefuroxime, 89.6% (78) to ceftazidime, 86.2% (75) to cefotaxime, 82.7% (72) to amoxicillin, 74.7% (65) to trimethoprim, 65.5% (57) to chloramphenicol, 49.5% (43) to ciprofloxacin, 41.4% (36) to tobramycin and finally, 40% (35) to both nitrofurantoin and gentamicin. A few 14.9% (13), of the isolates showed intermediate resistance to amoxicillin following NCCLS guidelines.

4.2.3 Comparison of susceptibility determined by disc diffusion (NCCLS) and the Vitek system.

Results of both methods were in complete agreement for meropenem. Amoxicillin, ampicillin, cephalothin and gentamicin all had one discrepancy, while two differences were found with the cefuroxime results. There was more variation and disagreement for the results for amikacin. All strains were reported sensitive by the Vitek system while the disc diffusion method indicated that 62% were resistant and 27.5% were intermediate. Results for ceftazidime were even more discrepant in which 39% of the isolates were resistant by the disc diffusion method while the Vitek reported 89% resistance to this drug.

Table 4.2

Results of susceptibility tests in No. by the Vitek system to various antimicrobial of the 87 ESBL positive strains.

Antibiotic Disc	Resistant	Intermediate	Sensitive
Ampicillin	86	-	1
Amoxicillin	85	-	2
Trimethoprim	65	-	22
Cephalothin	86	-	1
Cefuroxime	81	-	6
Cefotaxime	75	-	12
Ceftazidime	78	-	9
Gentamicin	35	-	52
Ciprofloxacin	43	-	44
Tobramycin	36	-	51
Tazobactam	85	-	2
Meropenem	-	-	87
Chloramphenicol	57	-	20
Nitrofurantoin	35	-	52
Amikacin	-	-	87

Forty percent of strains were resistant to nitrofurantoin by the Vitek system, while 57% were reported resistant by disc diffusion. Sixty-five percent of isolates were reported to be resistant to chloramphenicol with Vitek compared to 71% by the disc diffusion method. For tazobactam, 79% of the isolates were resistant by Vitek, and 72% by the disc diffusion method. For trimethoprim, 74% were resistant by the Vitek system, and 86.2% by the disc diffusion method, while for ciprofloxacin, 72%, were resistance by disc diffusion and 49% by Vitek. For cefotaxime, there was only one discrepancy between the results obtained by the Vitek system and the disc diffusion method.

4.2.4 Determination of minimum inhibitory concentration (MIC) by E-test

MICs were determined for both third generation cephalosporins; cefotaxime and ceftazidime by the use of E-test strips. Results of the E-tests are summarized in table 4.3, and results for each isolate detailed in appendix V.

The MIC for ceftazidime to the test strains as determined by E-test strips ranged from a low of 0.5 mg/l to a high of >32mg/l. The MIC results were using NCCLS breakpoint values for cefotaxime and ceftazidime, and the results interpreted as either sensitive, intermediate or resistant. Forty-seven percent (No.=41) of the test strains were resistant to ceftazidime with MIC values of 32.0 mg/l or higher. The remainder were susceptible to ceftazidime, with only one strain showing intermediate

Table 4.3

Determination of the MIC of the 87 test isolates by Etest strips

Antibiotic	**MIC**	***E.coli***	***K. pneumoniae***	**Total**
Cefotaxime	> 16 mg/l	1	19	20
	16 mg/l	9	38	47
Total resistant to cefotaxime				67
Antibiotic	**MIC**	***E.coli***	***K. pneumoniae***	**Total**
Ceftazidime	> 32 mg/l	1	2	3
	32 mg/l	7	31	38
Total resistant to ceftazidime				41

resistance. With cefotaxime, MICs ranged from 0.25 mg/l to 16.0 mg/l. Twenty-three percent (No.=20) of strains were resistant to cefotaxime with MIC values ≥16.0 mg/l. Fifty-four percent (47) were of intermediate resistance. Twenty-three percent (No. =20) were sensitive.

For the majority of strains, E-test results were easy to interpret. However, in a few isolates, results were not clear-cut and there was a double zone of inhibition. Furthermore, For some strains, there was a growth of single colonies within the zone of inhibition, these resistant colonies were subbed for purity and repeated. The same results were obtained indicating that they were not true mutants.

4.2.5 MICs of the 87 test strains to gentamicin, tobramycin and ciprofloxacin by serial agar dilution

The MICs for gentamicin, tobramycin and ciprofloxacin were determined by the agar dilution method. The results are presented in table 4.4.

MICs for gentamicin ranged from 0.25-> 32.0 mg/l. Thirty five (40%) of the isolates were resistant to gentamicin, 32 (6 *E.coli* and 26 *K. pneumoniae*) had MIC values of ≥ 32 mg/l., and 3 *K. pneumoniae* isolates had MIC values of 16 mg/l. One *K. pneumoniae* isolate demonstrated intermediate resistance to gentamicin with an MIC value of 8 mg/l.

MICs of tobramycin ranged from 0.25-> 32.0 mg/l. Forty (46%) of the isolates were resistance to tobramycin. Twenty strains (1 *E.coli* and 19 *K. pneumoniae*) had MICs

values of ≥ 32 mg/l., whereas 20 (5 *E.coli* and 15 *K. pneumoniae*) had MIC values of 16 mg/l. Only 4 isolates (1 *E.coli* and 3 *K. pneumoniae*) demonstrated an MIC value of 8mg/l, indicating intermediate resistance.

MICs of ciprofloxacin ranged from 0.25- >32 mg/l. Forty (46%) of the isolates were resistant to ciprofloxacin. Thirty-eight of these strains (6 *E.coli* and 32 *K. pneumoniae*) had MIC values ≥ 32 mg/l. Two *K. pneumoniae* had MIC values of 32.0 mg/l. Eleven (12.6%) of *K. pneumoniae* demonstrated intermediate resistance with MIC values of >2 mg/l. but <32mg/l.

4.2.6 Comparison of MICs obtained by the agar dilution method and the Vitek system

For gentamicin, both methods gave similar results, for which 40% of the isolates were resistant to gentamicin. The tobramycin results were similar, with the exception of four isolates that were resistant by the agar dilution method but considered as sensitive by the Vitek system. A similar observation was found in the case of ciprofloxacin, in which only 3 isolates showing resistance by the Vitek system were reported as intermediate by the agar dilution method.

4.2.7 Comparison of MIC values for cefotaxime and ceftazidime obtained by the Vitek system and E-test strips

E-test indicated that 23% of the isolates were resistant to cefotaxime and 54% reported as intermediate. MICs obtained with the Vitek system showed that 88.5% of

Table 4.4

MICs of the 87 test strains to gentamicin, tobramycin and ciprofloxacin by serial agar dilution

Antibiotic	MIC	*E.coli*	*K. pneumoniae*	Total
Gentamicin	≥ 32 mg/l	6	26	32
	16 mg/l	-	3	3
Total resistant to gentamicin				**35**
Antibiotic	**MIC**	*E.coli*	*K. pneumoniae*	**Total**
Tobramycin	≥ 32 mg/l	1	19	20
	16 mg/l	5	15	20
Total resistant to tobramycin				**40**
Antibiotic	**MIC**	*E.coli*	*K. pneumoniae*	**Total**
Ciprofloxacin	≥ 32 mg/l	6	32	38
	32	-	2	2
Total resistant to ciprofloxacin				**40**

the 87 isolates were resistant to cefotaxime, eight of these isolates were reported as intermediate by E-test strips.

There was less agreement with MICs for ceftazidime. Forty eight percent of the test strains were resistant to ceftazidime by E-test strips, whereas by Vitek 89.6% of the isolates were reported resistant.

4.2.8 Comparison of susceptibility results as determined by disc-diffusion (DD), Vitek system and E-test to both cefotaxime and ceftazidime for the 87 ESBL positive strains

The results are summarized in table 4.5. With cefotaxime, 20 (23%) of the isolates were resistant by E-test, 40 (46%) by disc diffusion and 75 (86.2%) by the Vitek system. With ceftazidime, 41 (47%) of the isolates were resistant by E-test, 34 (39%) by disc diffusion and 78 (89.6%) by the Vitek system. The numbers of strains classified as intermediate resistance for cefotaxime were 47 (54%) by E-test, 34 (39%) by disc diffusion. None of the isolates were classified as intermediate by the Vitek system. This is because we opted not to have an intermediate category for cefotaxime and ceftazidime (i.e. we used, user defined breakpoints*) and any isolate which would have been classified as intermediate resistance was termed as resistant. For ceftazidime, one (1.15%) strain was intermediate by E-test, seven (8%) by disc diffusion and none by the Vitek system.

Table 4.5

Comparison of susceptibility results as determined by Disc-Diffusion (DD), Vitek system and E-test to both cefotaxime and ceftazidime for the 87 ESBL positive strains

E-Test					
Cefotaxime (87 strains)			Ceftazidime (87 strains)		
Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
20	47	19	45	1	41
Disc-Diffusion					
Cefotaxime (87 strains)			Ceftazidime (87 strains)		
Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
13	34	40	46	7	34
Vitek System *					
Cefotaxime (87 strains)			Ceftazidime (87 strains)		
Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
12	-	75	9	-	78

* Break point for ceftazidime (8 sensitive and 16-32 mg/l resistant), and for cefotaxime (4 sensitive and 8-64 mg/l resistant).

Chapter 5

Molecular Characterization and Epidemiology of ESBLs from *K. pneumoniae* and *E.coli* isolated from patients from the RIE and surrounding district

5.1 Introduction

ESBL enzymes, derived from bla_{TEM-1}, bla_{TEM-2} and bla_{SHV-1} genes, confer resistance to the β-lactams and monobactams (D'Agata *et al.*, 1998). ESBL production has been found mainly in *K. pneumoniae* but has also been described in other Enterobacteriaceae, including *E.coli*, *Proteus mirabilis*, *Salmonella* spp., *Enterobacter* spp., *Citrobacter* spp. and *Serratia* spp. (De Champs *et al.*, 1991; Sirot *et al.*, 1992; Hammami *et al.*, 1991; Mariotte *et al.*, 1994; Pitit *et al.*, 1990).

Outbreaks of nosocomial infections due to ESBL-producing strains are being increasingly reported in intensive care units (ICUs) and chronic care facilities (Bingen *et al.*, 1993; Bauernfeind *et al.*, 1993; Naumovski *et al.*, 1992; Rice *et al.*, 1990). The reservoir for these strains appear to be the gastrointestinal tract of patients (De Champs *et al.*, 1989), with patient-to-patient contamination occurring via the hands of health care providers (Casewell and Philip, 1977).

Hospital colonisation by these ESBL-producing strains usually is a complex phenomenon involving different mechanisms of dissemination of several epidemic strains (Gouby *et al.*, 1994; Johnson *et al.*, 1992; Weller *et al.*, 1997), dissemination of plasmids and resistance genes (Bingen *et al.*, 1993; Kitzis *et al.*, 1988; Naumovski *et al.*, 1992), or concurrent dissemination of plasmids, genes and strains (De Champs *et al.*, 1991). Moreover, identical ESBLs have evolved independently in different places at different times (Hibbert *et al.*, 1994) and, occasionally, single isolates have carried multiple ESBLs (Liu *et al.*, 1998).

5.2 Results

5.2.1 Results of isoelectric focusing (IEF)

Assessment of β-lactamase activity was performed using the nitrocephin spot test. The time in seconds for nitrocephin to change from yellow to red is proportional to the volume in μl of β-lactamase preparation added to the isoelectric focusing gel (Paton, 1994). Most strains demonstrated positive activity within 10 seconds. Three isolates took up to 30 minutes. Four strains failed to show any β-lactamase activity by the spot test.

5.2.1.1 Analytical isoelectric focusing

Sonicated extracts of the 87 ESBL producing isolates were examined by analytical IEF. Crude β-lactamase extracts of the 87 clinical isolates were applied to polyacrylamide gel containing broad - range ampholines (pH 3.5 – 10.0).

Control β-lactamases of known pI were added to analytical IEF gels in order to establish the isoelectric point of the unknown enzymes. Six isolates exhibited β-lactamases bands characteristic of TEM derived enzymes with pI values of 5.4. Thirty-two isolates exhibited β-lactamase bands at pI 7.6, which is identical to the pI of SHV2/SHV-1. Eight isolates exhibited β-lactamase bands at pI 7.0, which is identical to that of SHV-3. Some isolates exhibited more than one enzyme, with differing pI values. Thirty-five isolates exhibited two enzymes with pI values of 5.2

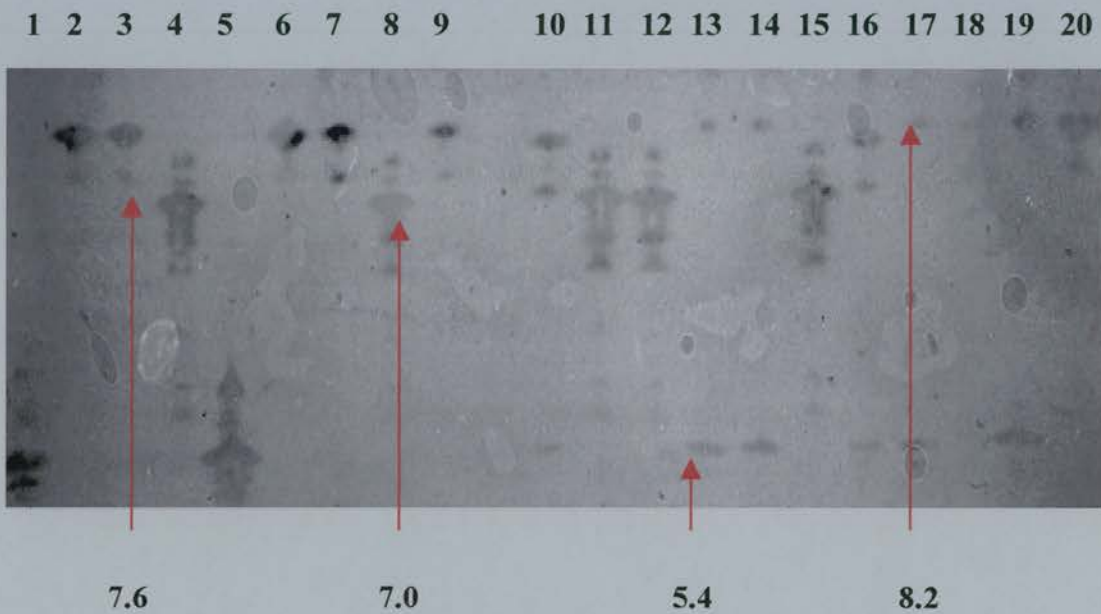
and 7.6 identical to that of TEM-1 and SHV1. Two isolates had pIs of 8.2 indicating that they were probably derived from SHV. Four other isolates (AD338, AD346, AD382 and AD389) exhibited β-lactamase bands with pI values of 5.2 (TEM derived), 7.6 and 8.2. These are identical to pIs of the SHV1 and SHV-5. β-lactamases. (See figure 5.1, tables 5.1. and 5.2).

5.2.2 Results of PCR using SHV and TEM primers on the 87 ESBL positive clinical isolates of *E. coli* and *K. pneumoniae*

Plasmid DNA from all the 87 studied isolates was extracted and analysed for the presence of the two most common ESBL genes bla_{TEM}, bla_{SHV} derivatives by PCR. Primers specific for each of the ESBL (bla_{TEM} and bla_{SHV}) were used. The results are summarised in table 5.3.

The amplified products obtained with primers specific for the bla_{TEM} were 858 bp (see figure 5.2), which is the expected product size of the amplified gene with the set of primers used. The bla_{TEM} ESBL was detected in 42 isolates consisting of 33 *K. pneumoniae* and 9 *E.coli* strains. The bla_{SHV} ESBL was detected in 81 out of the 87 tested isolates (see figure 5.3), consisting of 66 *K. pneumoniae* and 15 *E.coli*. The size of the amplified product was 383 bp which is the expected size of amplified product with the primers used. From the 87 tested isolates, 36 of them were positive for both ESBL genes (bla_{TEM} and bla_{SHV}), consisting of 30 *K. pneumoniae* and 6 *E.coli*. In total, PCR was successful in detecting either bla_{TEM} and/or bla_{SHV} genes in all tested 87 isolates.

Figure 5.1



Analytical IEF of β-lactamases produced by *K. pneumoniae* and *E.coli* isolates from clinical specimens.

Lane 1: TEM-1 control strain.
Lane 2: SHV-1 control strain.
Lane 4,8,11,12,13 and 15: strains with pI value 7.0
Lane 3,6,7,9,18 and 20: strains with pI value 7.6
Lane 5 : strain with pI value of 5.4
Lane 13, 14, 16, 17: strains with pI values of 5.2, 7.6 and 8.2
Lane 10 and 19: strains with pI values of 5.4 and 7.6

Table 5.1

pI values of β-lactamase activity of the 69 isolates of *Klebsiella pneumoniae*

IEF pI Values	Strain tested
7.0	AD333 AD374 AD375
7.6	AD313 AD314 AD317 AD319 AD325 AD328 AD329 AD330 AD331 AD334 AD335 AD336 AD337 AD339 AD340 AD342 AD343 AD344 AD350 AD352 AD353 AD354 AD355 AD366 AD370 AD391 AD392 AD401 AD410 AD411
5.4 and 7.6	AD315 AD316 AD318 AD320 AD321 AD322 AD323 AD324 AD326 AD327 AD341 AD345 AD348 AD351 AD356 AD358 AD362 AD363 AD364 AD365 AD367 AD368 AD377 AD378 AD380 AD384 AD395 AD402 AD404 AD407 AD408 AD409
5.4, 7.6 and 8.2	AD338 AD346 AD382 AD389

Table 5.2

pI values of β-lactamase activity of the 18 isolates of *E.coli*

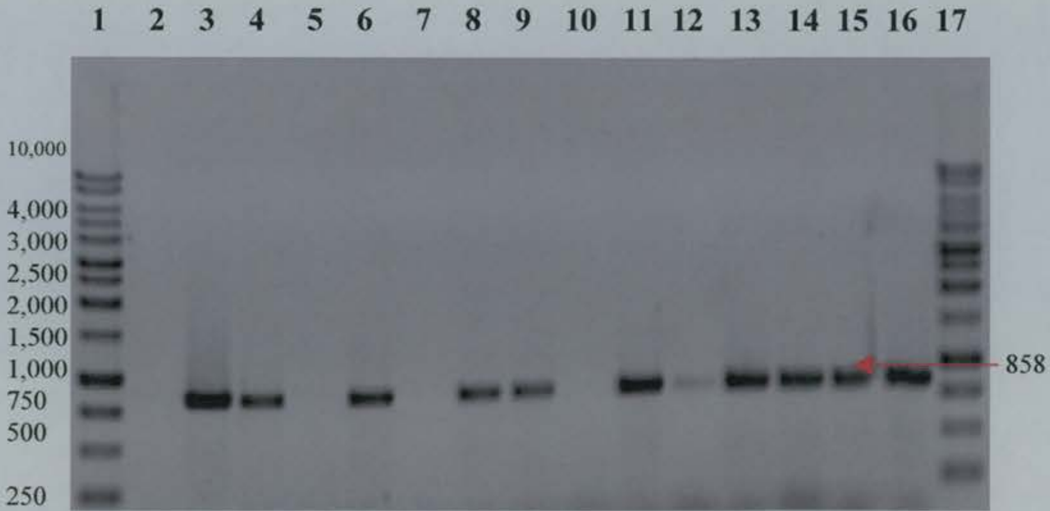
IEF pI Values	Strains tested
5.4	AD332 AD357 AD385 AD399 AD400 AD403
7.0	AD369 AD371 AD386 AD387 AD388 AD393
7.6	AD336 AD349
5.4 and 7.6	AD405 AD412
8.2	AD360 AD413

Table 5.3

PCR results

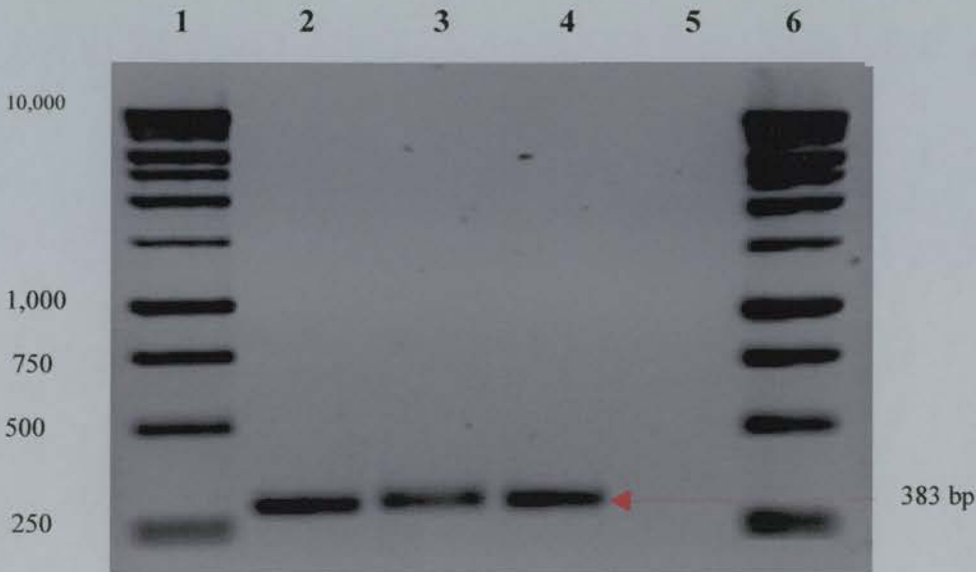
Detection	***E. coli***	***K. pneumoniae***	**Total number of isolates**
SHV-only	**9**	**36**	**45**
TEM-only	**3**	**3**	**6**
SHV+TEM	**6**	**30**	**36**
Total detected	**18**	**69**	**87**

Figure 5.2



Ethidium bromide-stained agarose gel showing PCR products with molecular size of 858 bp of *bla*$_{TEM}$. Lanes 1 and 17 : shows 1Kb molecular size marker, Lanes 2 and 3 are negative and positive control strains respectively

Figure 5.3



PCR showing a *bla*$_{SHV}$ product of molecular size 383bp, Lanes 1 and 6: shows 1Kb molecular size marker. Lanes 5 and 4 are negative and positive control strains respectively

5.2.3 Results of Dot-Blot (DNA-DNA) Hybridisation

The results of Dot-Blot hybridisation are listed in Appendix (VI). A bla_{SHV} DNA probe of molecular size 1017bp was used for the identification of bla_{SHV} gene, and DNA probe of molecular size 838bp was used for the identification of bla_{TEM} gene. The labelled SHV probe hybridised with the DNA of 77 isolates, 63 from *K. pneumoniae* and 14 from *E.coli*. The TEM probe hybridised with DNA of 62 isolates, 51 from *K. pneumoniae* and 11 from *E.coli*. See figures 5.4 A and 5.4 B.

5.2.3.1 Hybridisation of PCR products against labelled DNA probe

To confirm that the PCR products contained DNA specific for ESBL enzymes, the PCR products for SHV and TEM were transferred to Hybond-N+ membrane by southern blot after agarose gel electrophoresis and probed with the labelled DNA probes used in the Dot-Blot experiment. This was performed with probes for bla_{SHV} SHV and bla_{TEM}. The probe hybridised with the expected corresponding PCR product.

5.2.3.2 Comparison of Dot-Blot and PCR for the detection of bla_{SHV} and bla_{TEM}

A comparison of the results obtained by both methods is presented in table 5.4. The two common bla_{SHV} and bla_{TEM} enzymes were detected by PCR and dot-blot hybridisation experiments.

Table 5.4

Summary of ESBL detected by Dot-Blot and PCR

Gene	PCR	Dot-Blot
SHV	81	77
TEM	42	62
SHV Only	45	25
TEM Only	6	10
SHV and TEM	36	52

PCR was more effacious at detecting *bla*$_{SHV}$ with 81 positive reactions compared to 77 for dot blot. Dot-blot however appeared to be more efficient at detection of *bla*$_{TEM}$ with 62 positive reactions compared to 42 for PCR. *bla*$_{SHV}$ was detected in 65 *K. pneumoniae* and 16 *E.coli* by PCR, while it was detected in 63 *K. pneumoniae* and 14 *E.coli* by dot-blot.

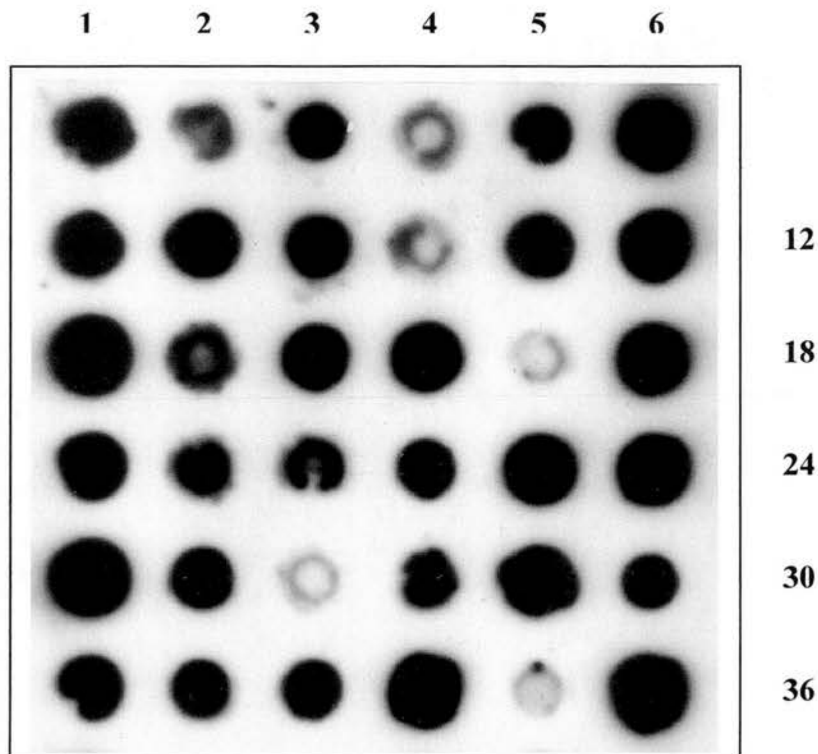
PCR detected the *bla*$_{TEM}$ in 42 isolates, 34 *K. pneumoniae* and 8 *E.coli,* while dot-blot detected TEM in 62 isolates, 51 *K. pneumoniae* and 11 *E.coli.* One isolate (AD392) was detected by dot blot but not by PCR. However, it was confirmed as ESBL positive by the DDD, Etest ESBL strips and the Vitek system in addition to dot blot.

5.2.4 Pulsed field Gel-electrophoresis (PFGE) of 69 *K. pneumoniae* isolates

The PFGE data obtained for all restriction fragments on the gels was analysed by diversity database finger printing software (Bio-Rad). Data obtained was transformed into a binary data matrix in which each fragment was scored as 1 (present) or 0 (absent). Thus, the data from PFGE experiments in which enzyme *Xba*I was used yielded a matrix of 68 *K. pneumoniae* X 29 characters (one isolate failed to type). Their relatedness was compared by the Rapiddistance package version 1.04 which also generated a dendogram for the distinct genotypes. PFGE analysis of these *K. pneumoniae* isolates with *Xba*I revealed 13 distinct genotypes. The results are summarised in table 5.6 and, the patterns obtained with *XbaI* are shown in figures 5.5 and 5.6.

Figure 5.4 (A)

Dot-blot



Representative DNA-DNA hybridisation using a labelled PCR-product to detect bla_{SHV} in the clinical isolates showing positive and negative reactions:

No. 36: positive control

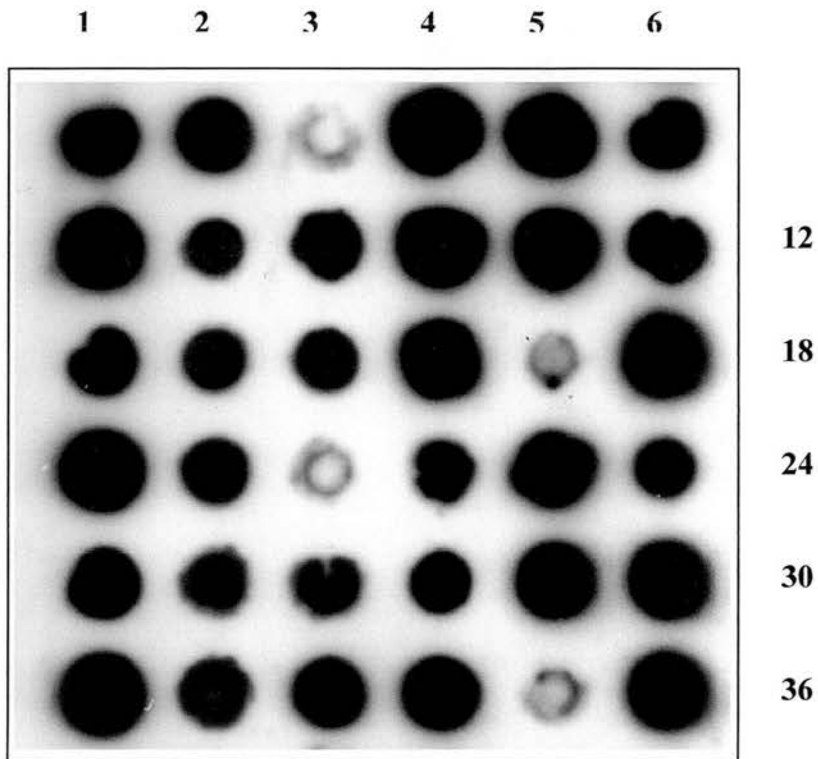
No. 35: negative control

Nos. 4,10,17 and 27: show a negative reaction.

Nos.1, 2, 3, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, 34: show a positive reaction.

Figure 5.4 (B)

Dot-blot



Representative DNA-DNA hybridisation using a labelled PCR-product to detect bla_{TEM} in the clinical isolates showing positive and negative reactions:

No. 36: positive control

No. 35: negative control

Nos. 3,17 and 21: show a negative reaction.

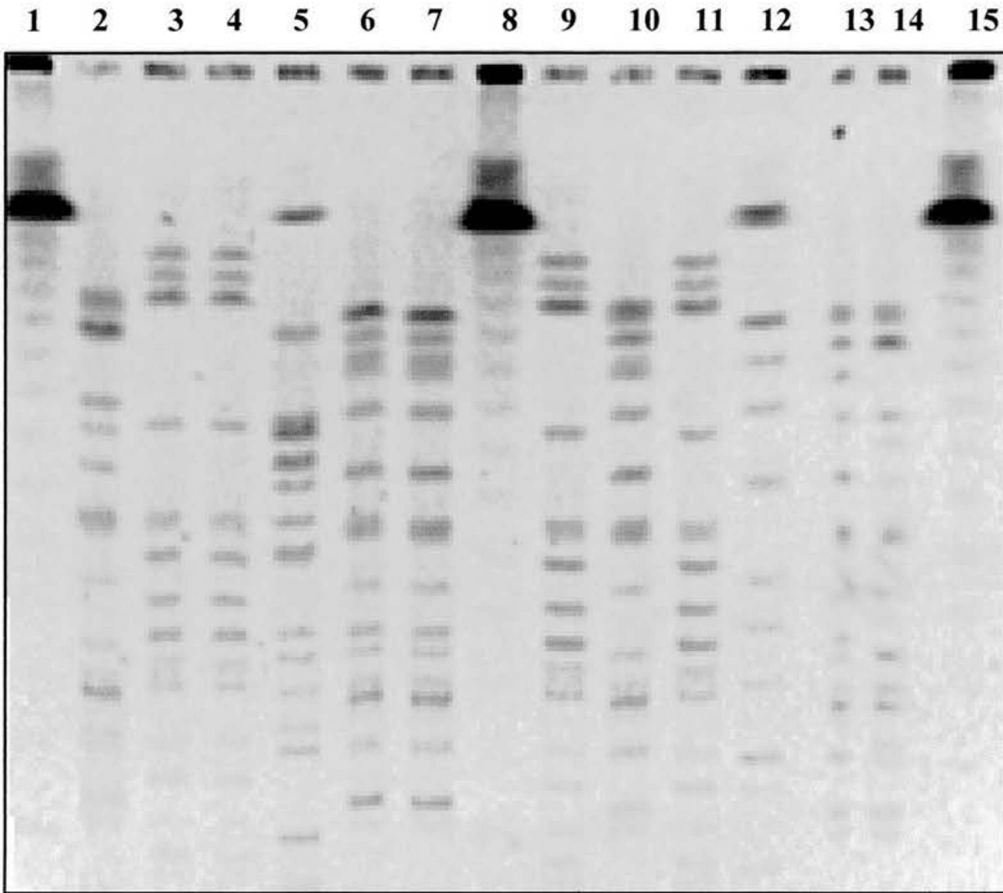
Nos.1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20, 22, 23, 24, 25, 26, 28, 27, 29, 30, 31, 32, 33, 34: show a positive reaction.

Table 5.5

Distribution of ESBL-producing *K. pneumoniae* into PFGE groups

PFGE group	Isolate	Total No.
group A	AD315 AD316 AD320 AD322 AD326 AD327 AD345 AD346 AD351 AD356 AD362 AD364 AD338 AD348 AD363 AD367 AD377 AD378 AD382 AD384 AD389 AD395 AD402 AD404 AD408	25
group B	AD313 AD314 AD325 AD328 AD329 AD330 AD339 AD343 AD344 AD401 AD410 AD411	12
group C	AD340 AD342 AD354 AD355	4
group D	AD318 AD319 AD321 AD324 AD331 AD334 AD335 AD337 AD350 AD353	10
group E	AD317 AD323 AD358 AD365 AD370	5
group F	AD380 AD391 AD409	3
group G	AD375 AD366 AD374	3
group H	AD407	1
group I	AD368	1
group J	AD333	1
group K	AD359	1
group L	AD352	1
group M	AD341	1

Figure 5.5



PFGE profile of *Xba*I macrorestriction fragments of ESBL producing *K. pneumoniae*.

Lanes 1, 8 and 15: Lambda DNA ladder marker.

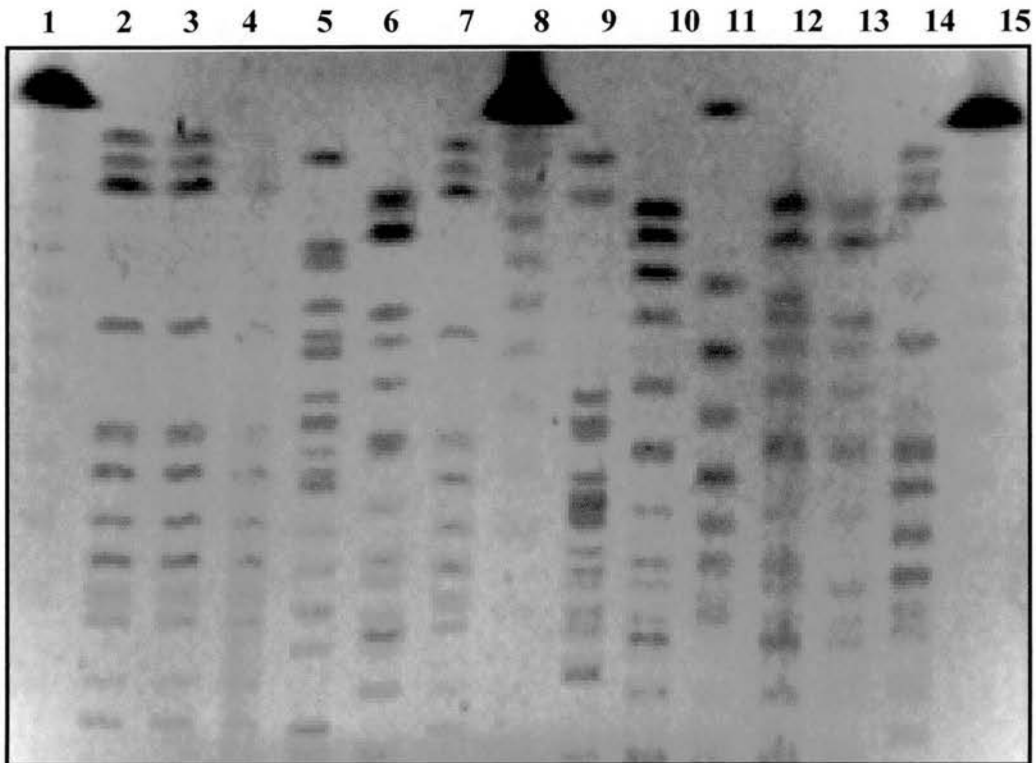
Lanes 2 and 14: isolates belonging to PFGE group B

Lanes 3, 4, 9, and 11: isolates belonged to PFGE group A.

Lanes 5 and 12: isolates belonged to PFGE group E

Lanes 6, 7,10 and 13: isolates belonged to PFGE group D.

Figure 5.6



PFGE profile of *Xba*I macrorestriction fragments of ESBL producing *K. pneumoniae*.

Lanes 1, 8 and 15: Lambda DNA ladder marker.

Lanes 2, 3, 7 and 14: PFGE group A.

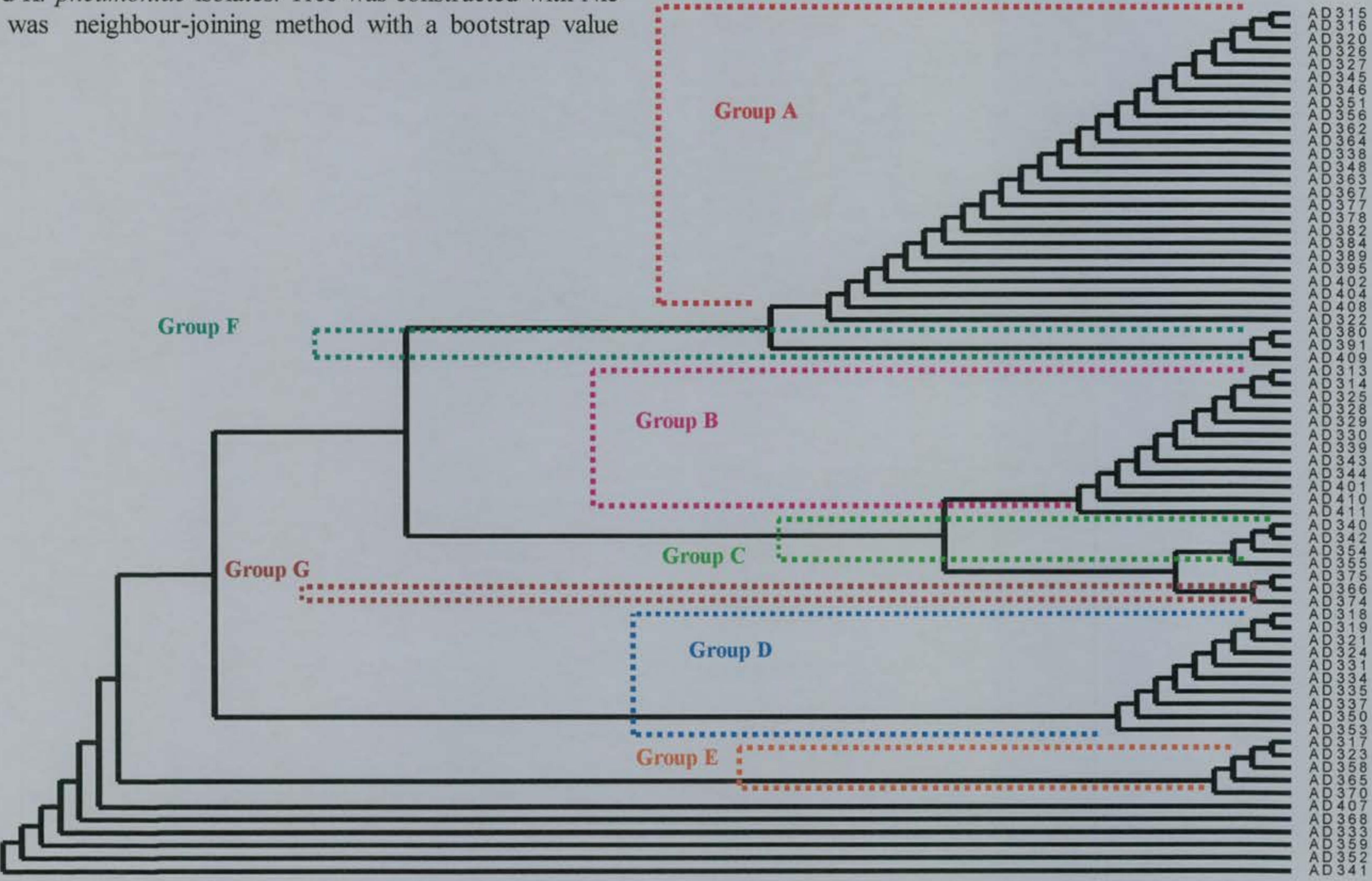
Lane 9: PFGE group I.

Lane 5: PFGE group E.

Lane 10: PFGE group H.

Lanes 6, 12 and 13: PFGE group G.

Figure 5.7
Computer-generated rectangular dendogram of 68 ESBL produced *K. pneumoniae* isolates. Tree was constructed with Nie method was neighbour-joining method with a bootstrap value 1,000.



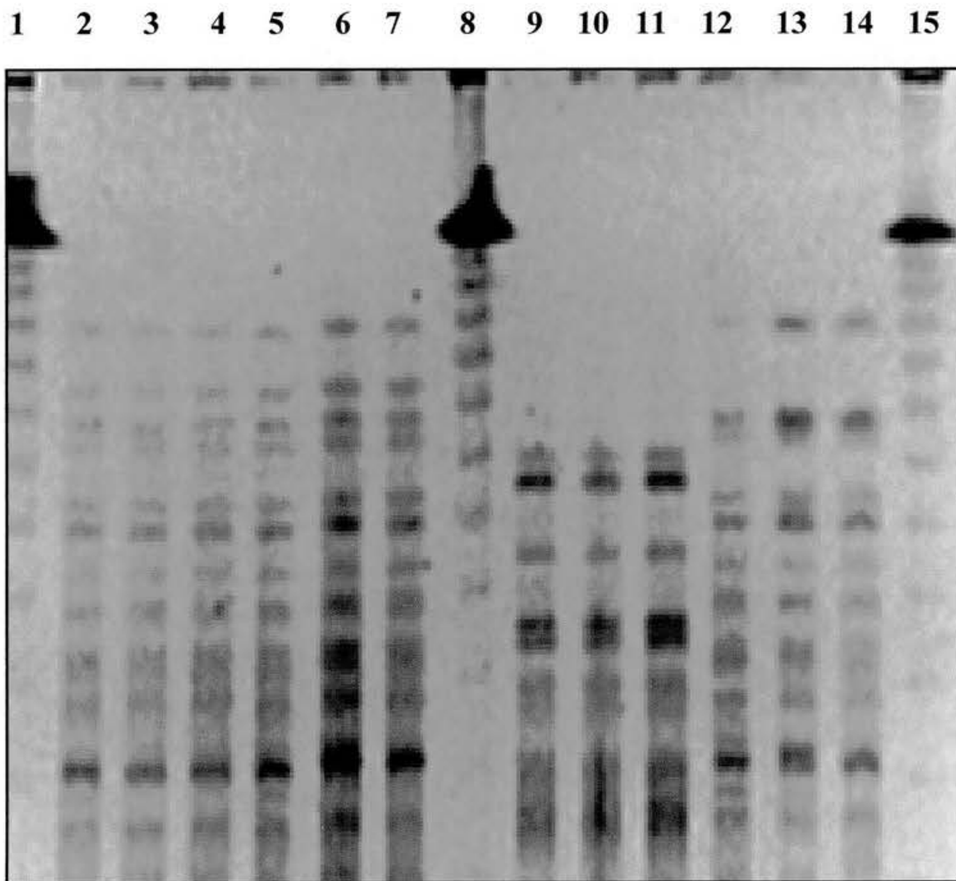
Computer-generated dendogram analysis of the PFGE of the 68 strains of *K. pneumoniae*

Seven clusters each containing isolates with a similar coefficient were identified among 62 isolates of *K. pneumoniae* . A high level of genetic heterogeneity was found among the remaining 6 isolates and between each cluster. One isolate (AD392) was not typed by PFGE.

Of all the restriction endonuclease enzymes tested, only X*ba*1 was found to generate a PFGE profile that could be interpreted clearly. However, in support of these results, PFGE groups A, B and D had very similar band patterns when the *Spe*1 restriction endonuclease was used. (See figure 5.8).

The isolates examined in this study have been shown to belong to several PFGE groups (A, B, C, D, E, F and G). However, PFGE group A was by far the most common, having been found mainly within Ward 15 of the RIE but also in other wards around the hospital and from a single isolate from a GP. Our results mirror that of previous studies indicating that the dissemination of strains producing SHV-β-lactamases especially SHV-5, in hospitals is as a result of clonal spread (Decre *et al.*, 1998).

Figure 5.8



DNA profile produced by pulsed-field gel electrophoresis of *Spe*I macrorestriction fragments of ESBL producing *K. pneumoniae*.

Lanes 1, 8 and 15: Lambda DNA ladder marker.

Lanes 2-7: isolates from PFGE group A

Lanes 9-1: isolates from PFGE group B.

Lanes 12 -14: isolates from PFGE group D.

Table 5.6

shows the distribution of ESBL-producing *K. pneumoniae* in their PFGE groups including the clinical sources of the isolates.

PFGE group	Sex		Source of isolate		
Group A (25 isolates)	**Male**	**Female**	**Urine**	**Swab**	**Sputum**
	9	16	12	6	7
PFGE group	**Sex**		**Source of isolate**		
Group B (12 isolates)	**Male**	**Female**	**Urine**	**Swab**	**Sputum**
	4	8	10	2	-
PFGE group	**Sex**		**Source of isolate**		
Group C (4 isolates)	**Male**	**Female**	**Urine**	**Swab**	**Sputum**
	4	-	4	-	-
PFGE group	**Sex**		**Source of isolate**		
Group D (10 isolates)	**Male**	**Female**	**Urine**	**Swab**	**Sputum**
	4	6	5	-	5

Table 5.6 (Cont.)

PFGE group	Sex		Source of isolate		
Group E (5 isolates)	Male	Female	Urine	Swab	Sputum
	3	2	3	-	2
PFGE group	Sex		Source of isolate		
Group F (3 isolates)	Male	Female	Urine	Swab	Sputum
	2	1	2	-	1
PFGE group	Sex		Source of isolate		
Group G (3 isolates)	Male	Female	Urine	Blood	Sputum
	2	1	2	1	-

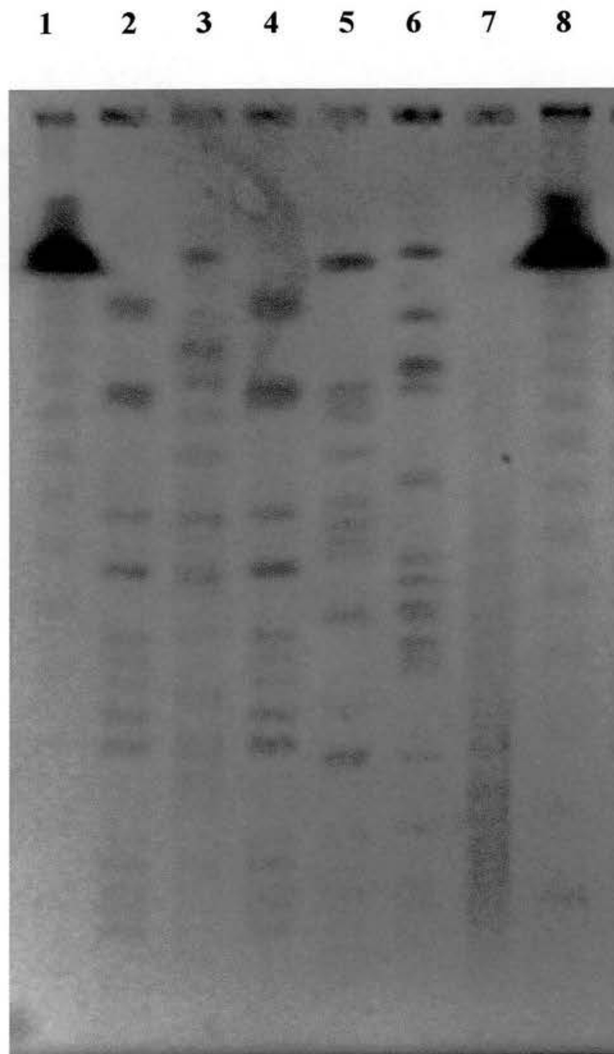
The other PFGE groups showed no genetic relationship with any other strain (H, I, J, K, L and M). These were collected from various GPs and from a variety of differing clinical specimens (See, table 2.1 Materials and Methods).

Twenty-six strains were isolated from single patients admitted to Ward 15 (general intensive care unit). All these isolates belonged to PFGE groups that showed a genetic relationship to each other manually using the criteria of Tenover (Tenover *et al.*, 1995). These were termed PFGE A-G. Eighteen of these 26 strains belonged to PFGE A. All the 26 isolates collected from Ward 15 produced TEM-1 and SHV-2, while four also produced SHV-5 β-lactamase.

5.2.4.1 PFGE analysis for the 18 ESBL-producing *E.coli* isolates

Eight of the ESBL-producing *E.coil* isolates demonstrated diverse PFGE patterns indicating no genetic relationship between any other isolate. These were collected from different GPs in Edinburgh. Five isolates demonstrated identical PFGE patterns (PFGE X). These were collected from the same GP source. PCR products from representative isolates of ESBL-producing *E. coli* were subjected to automated DNA sequencing, the extracted β-lactamases from these isolates were also subjected to IEF analysis. The results demonstrated that, two of the five harboured β-lactamases with pI values 5.4 (TEM-1) and 7.6 (SHV-2), two with pI value of 7.6 (SHV-2), and one harboured ß-lactamases with pI value of 8.2 (SHV-5). Two isolates belonged to PFGE group Z and harboured a β-lactamase with a pI value of 5.4 (TEM-33).Three isolates failed to type by PFGE (See figure 5.9).

Figure 5.9



Representative PFGE profile of *Xba*I macrorestriction fragments of ESBL producing *E.coli*.

Lanes 1 and 8: Lambda DNA ladder marker.

Lanes 2 and 4: isolates from PFGE group X.

Lanes 3, 5 and 6: represent different PFGE patterns that belonged to different groups.

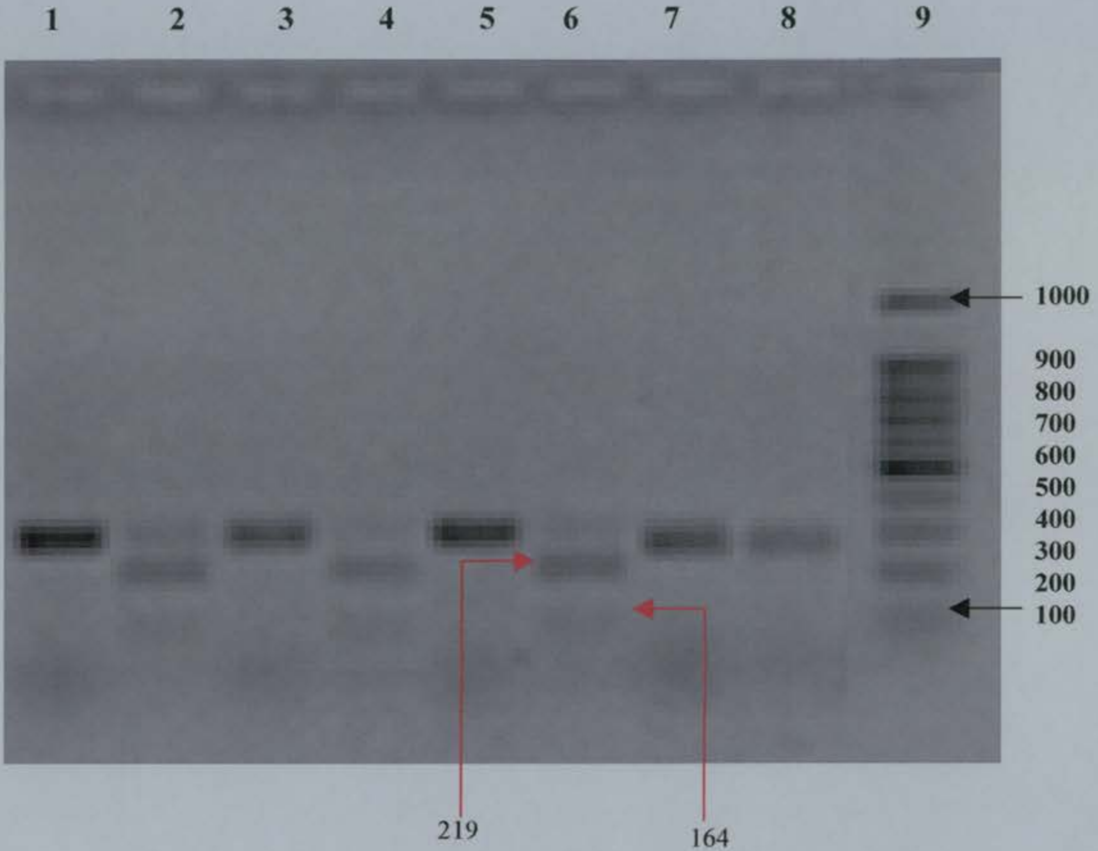
Lane 7: non-typeable isolate with PFGE.

5.2.5 Restriction fragment length polymorphism (RFLP)

An *Nhe*1 restriction endonuclease was used to cleave the SHV-specific PCR product for 66 strains harbouring a ß-lactamase with a pI value of 7.6 (see table 5.1 and 5.2) Successful restriction of all 66 strains indicated that this enzyme was SHV-2 derived (figure 5.10). The PCR product was then sent for automated sequencing and the presence of the bla_{SHV-2} was confirmed. *Nhe*1 was also used to cleave the PCR SHV products for the three isolates with a pI of 7.0 thus suggesting that these isolates harboured bla_{SHV-3} .

*Mse*I and *Sau3A*I restriction endonucleases were used to restrict the TEM PCR products to differentiate between TEM-1, 19 and 20. All these enzymes have identical pI values of 5.4. (Arlet *et al.,* 1995). Results of restriction patterns are shown in figures 5.11 and 5.12. The use of these enzymes did not distinguish between TEM derivatives. All TEM enzymes demonstrated a pI value of 5.4. Automated DNA sequencing was performed on a representative TEM PCR product from four representative strains from PFGE group A, two from PFGE B-G and X, one from PFGE group that contained only a single isolate. The results show that the majority were TEM-1 β-lactamase, however, few isolates (6 *E.coli*) produced TEM-33 which has a point mutation at amino acid 69 in which methionine (M) changed to leucine (L).

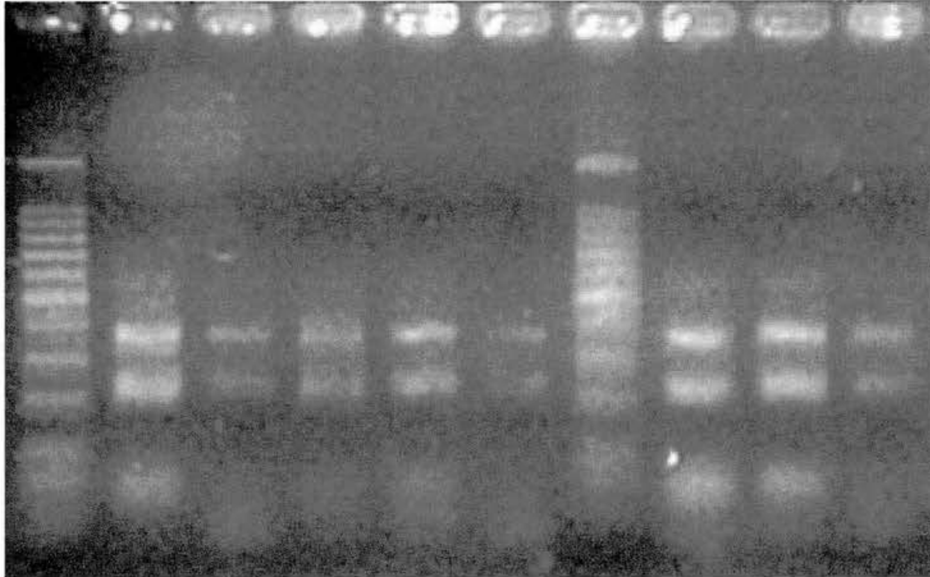
Figure 5.10



Agarose gel showing RFLP analysis of SHV-specific PCR product restricted with *Nhe*1 restriction endonuclease. The enzyme cleaved the SHV PCR product bands resulting in two bands with molecular size 219 bp and 164 bp, indicating a mutation from bla_{SHV-1} to bla_{SHV-2}.

Figure 5.11

Digestion of TEM PCR product with *Mes*I endonuclease.

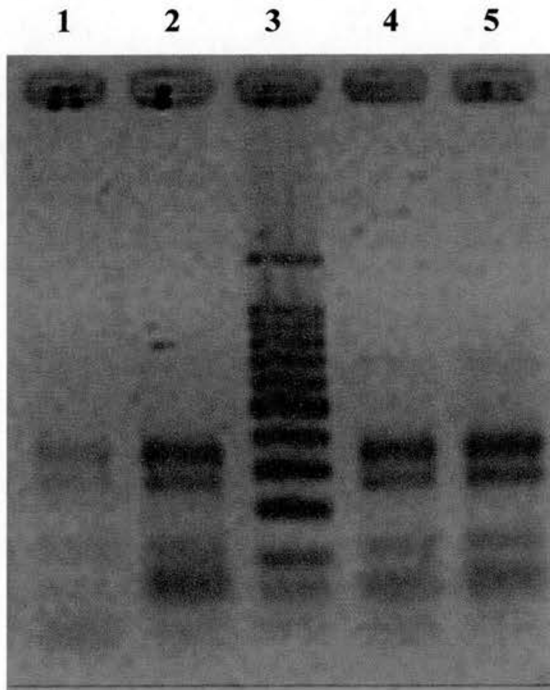


1 2 3 4 5 6 7 8 9 10

Lanes 1 and 7: DNA marker (100 bp)
Lane 2: TEM-1.
Lanes 3, 4,5,6,8,9 and 10: TEM-PCR product digested with *Mes*I

Figure 5.12

Digestion of TEM PCR product with *Sau3A*I endonuclease



Lane 3: DNA marker (100 bp)
Lane 1: TEM-1 control.
Lanes 2, 4 and 5: TEM-PCR product digested with *Sau3A*I

5.2.6 Plasmid Isolation

Two different plasmids were found, with sizes of 70 and 141 kb. The results seems to indicate that the *bla*$_{SHV}$ gene was carried on the larger plasmid with molecular size 141 Kb and the smaller plasmid of 70 Kb carried the *bla*$_{TEM}$ gene. Further plasmid restriction studies would be necessary to determine if the plasmids were related, (see table 5.7).

Table 5.7

Results of plasmid isolation of the 68 ESBL-producing *K. pneumoniae*

PFGE	No. of stains	β-lactamase	Plasmid No.	Plasmid size
A	21	TEM-1, SHV-2	2	70 & 141 kb
A	4	TEM-1, SHV-2 & SHV-5	2	70 & 141 kb
B	12	SHV-2	1	141 kb
C	4	TEM-1 & SHV-2	2	70 & 141 kb
D	10	SHV-2	1	141 kb
E	5	TEM-1 & SHV-2	2	70 & 141 kb
F	3	SHV-2	1	141 kb
G	3	SHV-2	1	141 kb
H	1	SHV-2	1	141 kb
I	1	SHV-2	1	141 kb
J	1	SHV-2	1	141 kb
K	1	SHV-2	1	141 kb
L	1	SHV-2	1	141 kb
M	1	SHV-2	1	141 kb

Chapter 6

The Epidemiology of Ciprofloxacin-Resistance and its Relationship to ESBLs Production in *Klebsiella pneumoniae*

6.1 Introduction

When ciprofloxacin was first introduced, resistance in *K. pneumoniae* and other Enterobacteriaceae, such as *E. coli*, was virtually unknown. However, in the last 10 years, cases of bacteraemia with ciprofloxacin-resistant *E.coli* have increased in number, together with upward trends in the use of quinolones in the community and in hospital (Pena *et al.*, 1995). The occurrence of ciprofloxacin resistance in *K. pneumoniae* is now well known and exceeds 5% in many centres in North America, Europe, and Asia (Blondeau *et al.*, 1999; Jones *et al.*, 1996; Thomson, 1999; Turnidge, 1995).

At the same time resistance to ciprofloxacin emerged, resistance to β-lactams antibiotics became prominent. This resistance was as a result of ESBLs which mediate resistance to newer β-lactams agents possessing an oxyamino group, such as ceftazidime, ceftriaxone, cefotaxime, and aztreonam. In addition, plasmids that carry and contain genes encoding ESBLs also harbour genes that encode mechanisms of resistance to other classes of antimicrobials. In many regions of the world where antibiotic use is high, ESBLs are present in ~25% of all *K. pneumoniae* from intensive care units, and patient-to-patient transfer of resistant organisms frequently occurs (Paterson *et al.*, 2000).

Some years ago, (Hobson *et al.*, 1996), there was a major out break of ESBL in *Klebsiella spp* in Aberdeen. Until recently, it was not thought that ESBL infiltration had been a problem in Edinburgh. However, the introduction of the Vitek system

with its integrated ESBL detection system demonstrated that *K. pneumoniae* harbouring ESBLs were present within the hospital environment. Until this finding, other studies from the same area had shown no evidence of ESBL-producing *K. pneumoniae* (Paton, 1994). Many of these strains showed concomitant resistance to ciprofloxacin.

This study was performed to determine the relationship between ESBL production and ciprofloxacin resistance in *K. pneumoniae* isolated from the Royal Infirmary of Edinburgh and associated GP practices.

6.2 Results

MIC values according to NCCLS criteria (NCCLS, 1999) demonstrated that 32 (46.4%) of the 69 ESBL-producing *K. pneumoniae* were resistant to ciprofloxacin; 11 (16%) were intermediate and 26 (37.6%) were sensitive. All ciprofloxacin-resistant *K. pneumoniae* were reported by the laboratory as resistant to the third generation cephalosporins cefotaxime and ceftazidime. Thirty (93.8%) were resistant to tobramycin and 24 (75.2%) were resistant to gentamicin. (Table 6.1).

6.2.1 Isoelectric focusing (IEF)

The β-lactamase complement of the 32 ciprofloxacin-resistant ESBL-positive isolates was investigated. All isolates harboured a β-lactamase with a pI of 7.6, which was probably an SHV-derived β-lactamase. Twenty-five of the 32 strains also

demonstrated a TEM-derivative β-lactamase with a pI of 5.4. Four of these 25 isolates also harboured another β-lactamase with a pI value of 8.2. These β-lactamases have subsequently been identified by DNA sequencing as SHV-2 (pI 7.6), SHV-5 (pI 8.2) and TEM-1 β-lactamase (pI 5.4) (see table 6.1).

6.2.2 Pulsed field gel-electrophoresis (PFGE)

PFGE analysis of the 32 ciprofloxacin-resistant ESBL-producing *K. pneumoniae* isolates indicated that they belonged to four different genotype cluster groups, (see figure 6.1) designated A, B, C and D. Group A consisted of 25 isolates, group B, 3 isolates. group C and D, 2 isolates each. (see table 6.1).

6.2.3 DNA-sequencing

Representative PCR products were taken from each of the PFGE cluster groups; four from group A, and one from the other groups, and analysed by automated sequencing for mutations in the *gyr*A and/or *par*C gene regions. The sequencing results confirmed mutations in both genes.

To confirm the mutation in the *gyr*A region, RFLP was performed and confirmed a mutation at Ser 83 .

For the *par*C mutation, all the representative isolates demonstrated a point mutation at position 80 from AGC (serine), to ATC (isoleucine). Figure 6.2 demonstrates the *par*C amplified PCR product.

Table 6.1

Summery of results for the 32 ciprofloxacin-resistant ESBL-producing strains of *K. pneumoniae.*

PFGE group	Antibiogram (Resistance)	β-lactamase complement	No. of isolates
A	CAZ, CTX, CIP, GM & TC	TEM-1 and SHV-2	21
A	CAZ, CTX, CIP, GM & TC	TEM-1 , SHV-2 and SHV-5	2
A	CAZ, CTX, CIP & TC	TEM-1 , SHV-2 and SHV-5	2
B	CAZ, CTX & CIP	SHV-2	1
B	CAZ, CTX, CIP & TC	SHV-2	2
C	CAZ, CTX, CIP, GM & TC	SHV-2	2
D	CAZ, CTX, CIP & TC	SHV-2	2

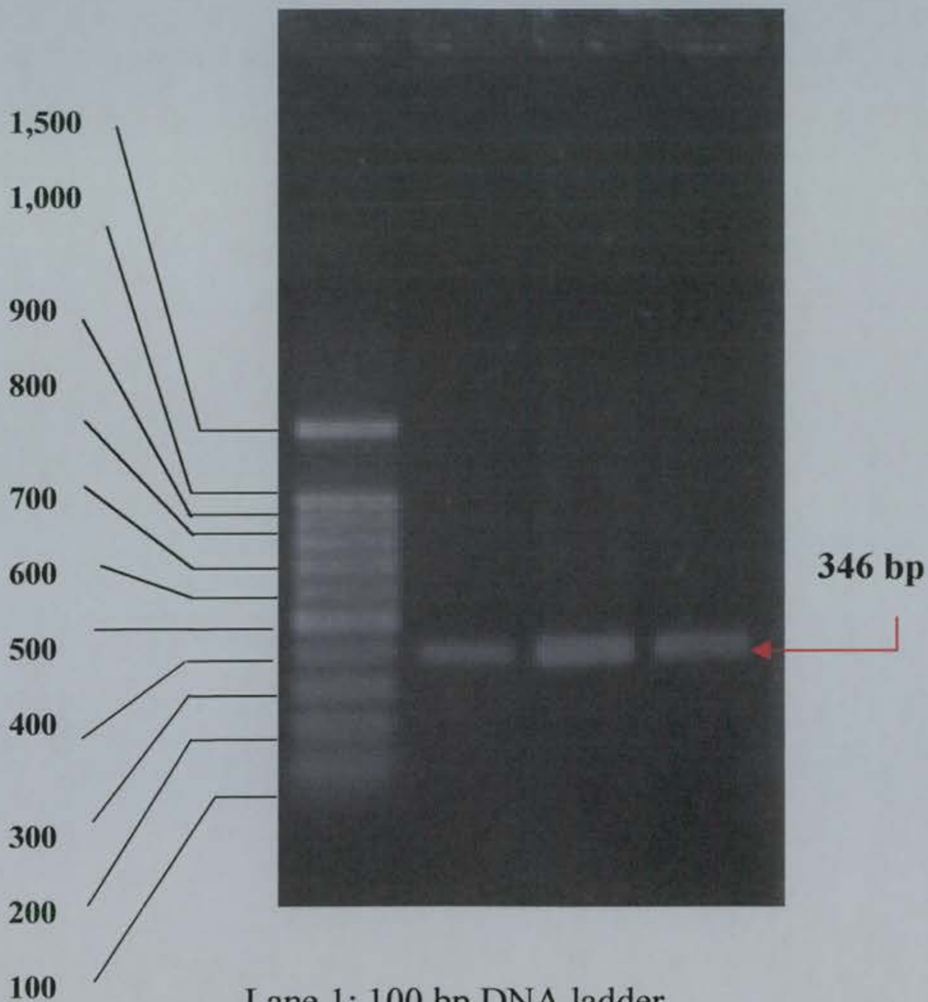
CAZ = ceftazidime, CTX = cefotaxime, CIP = ciprofloxacin, GM = gentamicin, TC = tobramycin.

Figure 6.1

Dendogram of the PFGE analysis of the 32 ciprofloxacin resistant strains *of K. pneumoniae*

Figure 6.2

Agarose gel electrophoresis of *par*C PCR products from ciprofloxacin-resistant *K. pneumoniae*



Lane 1: 100 bp DNA ladder

Lane 2: *par*C PCR product of AD409

Lane 3: *par*C PCR product of AD327

Lane 4: *par*C PCR product of AD409

In the *gyr*A region of the type strain *E.coli* NCTC 10418 and ciprofloxacin-sensitive ESBL-positive *K. pneumoniae*, the codons at amino acid position 83 were TCC (serine) and ACT (threonine) respectively, while at amino acid position 87 both control strains expressed GAC (aspartic acid). Figure 6.3 shows the DNA sequence of the *gyr*A gene of *K. pneumoniae* as found in GENE bank. Figure 6.4 shows the sequence for the *gyr*A PCR products from all 4 strains sequenced from PFGE group A.

Figure 6.5 Shows the DNA sequence of the *gyr*A gene of *K. pneumoniae* strain as found in GENE bank and Dimre & Das,1990. Figure 6.6 Shows the automated sequencing results for the *gyr*A PCR products from PFGE groups B, C and D.

Figure 6.7 demonstrates *Par*C QRDR sequence of *K. pneumoniae* as listed in GENE bank. Figure 6.8 Shows a representative automated sequencing result for *Par*C gene of the ciprofloxacin resistant *K. pneumoniae* strains.

Twenty-five of the 32 ciprofloxacin-resistant isolates, were PFGE group A. Four representative strains of this group were sequenced and showed a mutation from TCC (serine), to TTC (phenylanlanine) at codon 83 and a change from GAC (aspartic acid) to TAC (tyrosine) at codon 87. The representative PCR products for the other seven clinical isolates demonstrated a single point mutation at codon 83, ACT (threonine) to ATT (isoleucine).

Figure 6.3

GyrA QRDR sequence of *K. pneumoniae*, ciprofloxacin-susceptible, as listed in GENBANK (AF055258)

66	67	68	69	70	71	72	73	74	75
tca	gcc	cgt	gtc	gtt	ggt	gac	gta	atc	ggt
S	A	R	V	V	G	D	V	I	G
76	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**	**85**
aaa	tac	cac	ccg	cac	ggc	gac	tcc	gcg	gta
K	Y	H	P	H	G	D	S	A	V
86	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**
tac	gac	acc	atc	gtg	cgt	atg	gcg	cag	ccg
Y	D	T	I	V	R	M	A	Q	P
96	**97**	**98**	**99**	**100**	**101**	**102**	**103**	**104**	**105**
ttc	tcg	ctg	cgt	tac	atg	ctg	gtg	gac	ggc
F	S	L	R	Y	M	L	V	D	G
106	**107**	**108**	**109**	**110**	**111**	**112**	**113**	**114**	**115**
cag	ggt	aac	ttt	ggt	ttc	atc	gac	ggc	gac
Q	G	N	F	G	S	I	D	G	D
116	**117**	**118**	**119**	**120**	**121**	**122**	**123**	**124**	**125**
tcc	gcc	gcg	gcg	atg	cgt	tat	acc	gaa	att
S	A	A	A	M	R	Y	T	E	I
126	**127**	**128**	**129**	**130**	**131**	**132**	**133**	**134**	**135**
cgt	ctg	gcg	aaa	atc	gct	cat	gag	ctg	atg
R	L	A	K	I	A	H	E	L	M

136	137	138	139	140	141	142	143	144	145
gcc	gat	ctt	gaa	aaa	gag	acg	gtc	gat	ttc
A	D	L	E	K	E	T	V	D	F
146	**147**	**148**	**149**	**150**	**151**	**152**	**153**	**154**	**155**
gtc	gac	aac	tat	gac	ggt	acg	gag	cgt	att
V	D	N	Y	D	G	T	E	R	I
156	**157**	**158**	**159**	**160**	**161**	**162**	**163**	**164**	**165**
ccg	gsc	gtc	atg	ccg	acc	aaa	att	cct	aac
P	D	V	M	P	T	K	I	P	N
166	**167**	**168**	**169**	**170**	**171**	**172**			
ctg	ctg	gtg	aac	ggc	gcc	tcc			
L	L	V	N	G	A	S			

Single-letter amino-acid codon:

A alanine, **C** cysteine, **D** aspartic acid, **E** glutamic acid, **F** phenylalanine, **G** glycine, **H** histidine, **I** isoleucine, **K** lysine, **L** leucine, **M** methionine, **N** aspargine, **P** proline, **Q** glutamine, **R** arginine, **S** serine, **T** threonine, **V** valine, **Y** tyrosine.

Figure 6.4

***Gyr*A QRDR sequence of ciprofloxacin-resistant (MIC ≥ 32 mg/l) *K. pneumoniae* from PFGE group A**

66	67	68	69	70	71	72	73	74	75
tca	gcc	cgt	gtc	gtt	ggt	gac	gta	atc	ggt
S	A	R	V	V	G	D	V	I	G
76	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**	**85**
aaa	tac	cac	ccg	cac	ggc	gac	TTC	gcg	gta
K	Y	H	P	H	G	D	F	A	V
86	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**
tac	TAC	acc	atc	gtg	cgt	atg	gcg	cag	ccg
Y	Y	T	I	V	R	M	A	Q	P

Two point mutations were observed: at amino acid 83, TCC (serine) to TTC (phenylalanine) and at amino acid 87, GAC (aspartic acid) to TAC (tyrosine).

Figure 6.5

***GyrA* QRDR sequence of ciprofloxacin-susceptible *K. pneumoniae*, as listed in GENBANK (X16817.KPGYRA) and Dimre & Das,1990**

66	67	68	69	70	71	72	73	74	75
tct	gcc	cgt	gtc	gtt	ggt	gac	gta	atc	ggt
S	**A**	**R**	**V**	**V**	**G**	**D**	**V**	**I**	**G**
76	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**	**85**
aaa	tac	cac	cct	cat	ggt	gat	act	gcc	gtg
K	**Y**	**H**	**P**	**H**	**G**	**D**	**T**	**A**	**V**
86	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**
tat	gac	acc	att	gta	cgt	atg	gcg	cag	cca
Y	**D**	**T**	**I**	**V**	**R**	**M**	**A**	**Q**	**P**
96	**97**	**98**	**99**	**100**	**101**	**102**	**103**	**104**	**105**
ttc	tcc	ctg	cgt	tac	atg	ctg	gta	gat	ggc
F	**S**	**L**	**R**	**Y**	**M**	**L**	**V**	**D**	**G**
106	**107**	**108**	**109**	**110**	**111**	**112**	**113**	**114**	**115**
cag	ggt	aac	ttc	ggt	tct	atc	gac	ggc	gac
Q	**G**	**N**	**F**	**G**	**S**	**I**	**D**	**G**	**D**
116	**117**	**118**	**119**	**120**	**121**	**122**	**123**	**124**	**125**
tcc	gcc	gca	gca	atg	cgt	tat	acg	gaa	atc
S	**A**	**A**	**A**	**M**	**R**	**Y**	**T**	**E**	**I**

Figure 6.6

***GyrA* QRDR sequence of ciprofloxacin-resistant (MIC = 32 mg/l) *K. pneumoniae* strains from PFGE groups B, C and D**

66	67	68	69	70	71	72	73	74	75
tca	gcc	cgt	gtc	gtt	ggt	gac	gta	atc	ggt
S	A	R	V	V	G	D	V	I	G
76	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**	**85**
aaa	tac	cac	ccg	cac	ggc	gac	ATT	gcg	gta
K	Y	H	P	H	G	D	I	A	V
86	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**
tac	gac	acc	atc	gtg	cgt	atg	gcg	cag	ccg
Y	D	T	I	V	R	M	A	Q	P

Only one point mutation was observed at amino acid 83, ACT (threonine) to ATT (isoleucine).

Figure 6.7

Par**C QRDR sequence of *K. pneumoniae* ciprofloxacin-susceptible strain, as listed in GENEBANK (AF303646)**

76	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**	**85**
ccg	cac	ggc	gac	agc	gcc	tgc	tat	gaa	gcg
P	**H**	**G**	**D**	**S**	**A**	**C**	**Y**	**E**	**A**
86	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**
atg	gtg	ctg	atg	gcg	cag	ccg	ttc	tct	tac
M	**V**	**L**	**M**	**A**	**Q**	**P**	**F**	**S**	**Y**
96	**97**	**98**	**99**	**100**	**101**	**102**	**103**		
cgc	tat	ccg	ctg	gtg	gat	ggt	cag		
R	**Y**	**P**	**L**	**V**	**D**	**G**	**Q**		

Single-letter amino-acid codons:

A alanine, **C** cysteine, **D** aspartic acid, **E** glutamic acid, **F** phenylalanine, **G** glycine, **H** histidine, **I** isoleucine, **K** lysine, **L** leucine, **M** methionine, **N** aspargine, **P** proline, **Q** glutamine, **R** arginine, **S** serine, **T** threonine, **V** valine, **Y** tyrosine.

Figure 6.8

Representative *par*C QRDR sequence of *K. pneumoniae* strains (MIC ≥ 32 mg/l), AD 313, AD 314, AD 315, AD 409 and AD 380

76	77	78	79	80	81	82	83	84	85
ccg	cac	ggc	gac	ATC	gcc	tgc	tat	gaa	gcg
P	H	G	D	I	A	C	Y	E	A
86	**87**	**88**	**89**	**90**	**91**	**92**	**93**	**94**	**95**
atg	gtg	ctg	atg	gcg	cag	ccg	ttc	tct	tac
M	V	L	M	A	Q	P	F	S	Y
96	**97**	**98**	**99**	**100**	**101**	**102**	**103**		
cgc	tat	ccg	ctg	gtg	gat	ggt	cag		
R	Y	P	L	V	D	G	Q		

Only one point of mutation was observed at amino acid 80, AGC (serine) to ATC (isoleucine)

6.2.3 Relationship between antibiotic resistance and PFGE groups

Twenty-three of the 25 isolates that belonged to PFGE group A were resistant to ceftazidime, cefotaxime, gentamicin and tobramycin; however, the remaining two isolates from this group were resistant to all antimicrobial agents tested with the exception of gentamicin.

Of the 3 PFGE group B isolates, two were resistant to ceftazidime, cefotaxime, tobramycin, and sensitive to gentamicin, however, the third isolate was sensitive to both gentamicin and tobramycin.

Both isolates that belonged to PFGE group C were resistant to all the antibiotics tested, while isolates from PFGE group D were sensitive to gentamicin only. See table 6.2.

Isolates belonging to PFGE group A all harboured both the SHV-2 (pI 7.6) and TEM-1 (pI 5.4) β-lactamase. In addition, four of the 25 strains also harboured SHV-5 (pI 8.2).

The isolates belonging to group B,C and D harboured only a SHV-2 β-lactamase. Table 6.3 shows the distribution of the β-lactamase complement in the four different PFGE groups.

Table 6.2

Susceptibility results of the PFGE groups of *K. pneumoniae* to various antibiotics

PFGE group	CAZ	CTX	CIP	GM	TC	Total
A	R	R	R	R	R	23
A	R	R	R	S	R	2
B	R	R	R	S	R	2
B	R	R	R	S	S	1
C	R	R	R	R	R	2
D	R	R	R	S	R	1
D	R	R	R	S	S	1
Total R	32	32	32	25	30	**32**

CAZ = ceftazidime, CTX = cefotaxime, CIP = ciprofloxacin, GM = gentamicin, TC = tobramycin. R indicates resistance, S indicates sensitive.

Table 6.3

Results of IEFs and PFGE on the 32 ciprofloxacin resistant strains of *K. pneumoniae*

PFGE group	pI values	ESBL	Total No.
A	5.4 and 7.6	TEM-1 and SHV-2	21
A	5.4, 7.6 and 8.2	TEM-1 , SHV-2 and SHV-5	4
B	7.6	SHV-2	3
C	7.6	SHV-2	2
D	7.6	SHV-2	2

6.2.4 Outer membrane protein (OMP) analysis

SDS-PAGE analysis of the OMPs showed that, seven of the clinical isolates expressed two major OMPs of about approximately 35KDa and 36 KDa. Twenty five of the isolates belonging to PFGE group A were deficient in OMP 36KDa, and harboured TEM-1 , SHV-2 and SHV-5 β-lactamase.

Isolates lacking the 36KDa porin (with a concomitant expression of SHV-5), were resistant to all antimicrobial agents tested, and this porin loss may be partially responsible for this.

The 36KDa porin was absent from isolates for which MICs of cefotaxime, ceftazidime, gentamicin, tobramycin and ciprofloxacin were greater than 32mg/l. However, the expression of porin, as visualised by SDS-PAGE (figure 6.9), was clearly seen in isolates that were sensitive to ceftazidime. On the other hand, another porin of size ~ 41 KDa was absent in isolates belonging to group B,C and D, and these isolates harboured the SHV-2 ESBL only, however, sufficient expression of 36KDa was found in their OMPs. The common or major porin that appeared to be expressed in high quantity by all the clinical isolates was shown to be 35KDa.

Figure 6.9

Outer membrane protein

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis-profile of outer membrane proteins of *K. pneumoniae* isolates, with three major outer membrane proteins with approximate molecular size of 41, 36 and 35 KDa.

Lanes 1 & 10: Molecular size marker with molecular sizes given in kilo-Dalton.
Lane 2: *K. pneumoniae* ESBL positive, ciprofloxacin sensitive used as control.
Lane 3: AD 315 (loss of 41 KDa. band)
Lane 4: AD 320 (loss of 41 KDa. band)
Lane 5: AD 327 (loss of 41 KDa. band)
Lane 6: AD 313 (loss of 36 KDa. band)
Lane 7: AD 339 (loss of 36 KDa. band)
Lane 8: AD 380 (loss of 36 KDa. band)
Lane 9: AD 409 (lost of 36 KDa. band)

Chapter 7

SHV-39: A novel extended-spectrum β-lactamase (ESBL) found in *Klebsiella pneumoniae* strains isolated from Chile

7.1 Introduction

SHV-1 β-lactamase is found most commonly in *K. pneumoniae* and it is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species (Tzouvelekis and Bonomo, 1999). bla_{SHV} or related genes is integrated in many strains of *K. pneumoniae* through their chromosomal DNA (Livermore, 1995). Although it has been hypothesized that the gene encoding SHV-1 may exist as part of a transposable element, it has never been proven (Jacoby and Sutton, 1991). There are relatively few derivatives of SHV-1 especially when compared with TEM-type β-lactamases. Furthermore, changes in the bla_{SHV} structural gene have been shown to occur in fewer position compared with $bla_{TEM.}$ (See figure 1.10).

The majority of SHV-variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at position 238. A number of variants related to SHV-5 also have a substitution of lysine for glutamate at position 240. Interestingly, both the Gly238Ser and Glu240Lys amino acid substitutions mirror those seen in TEM-types ESBLs (Bradford, 2001).

The serine residue at position 238 is critical for the efficient hydrolysis of ceftazidime, and the lysine residue is critical for the efficient hydrolysis of cefotaxime (Huletsky *et al.*, 1993).

7.2 Materials

The seven strains used in this study were collected from hospitalised patients in Santiago, Valparaiso, Concepcion and Puerto Montt in Chile. (See table 7.1 and figure 7.1).

7.3 Results

7.3.1 Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) were determined by serial agar dilution. The results demonstrated that all seven strains were resistant to both ceftazidime and cefotaxime with MICs of >256 mg/l and 16-32 mg/ml respectively.

7.3.2 Pulsed field gel-electrophoresis (PFGE)

Strains were genotyped by PFGE using *Xba*1 restriction enzyme, although there were some variation in genotypes, within each hospital there was consistency of banding pattern (PFGE) indicating a genetic relationship. However, there was no relationship between strains isolated from different cities, indicating diversity i.e. different clones.

7.3.3 Analytical iso-electric focusing (IEF)

Sonicated extracts of the seven ESBL-producing *K. pneumoniae* were examined by Analytical iso-electric focusing. (See table 7.2).

Table 7.1

The source of the isolates

Isolate No.	Hospital	City
34	G.Grant-Benavente	Concepcion
100	Catholic	Santiago
129	Gustavo Fricke	Valparasio
130	Gustavo Fricke	Valparasio
139	Catholic	Santiago
150	Catholic	Santiago
329	A hospital in Puerto Montt	Puerto Montt

Figure 7.1

Map of the origin of the 7 ESBL–producing *K. pneumoniae* isolated from Chile

Examination of the IEF gels showed a β-lactamases with pI value of 5.4, present in all strains. A β-lactamases of pI 7.6 was present in 5 strains while β-lactamases of pI 7.0 was present in 3 strains and β-lactamases with pI > 8.0 was found in 5 strains. See table 7.2.

7.3.4 Polymerase chain reaction (PCR)

Plasmid DNA from all seven isolates was extracted (see section 2.7) and were analysed for the presence of bla_{SHV} gene by PCR. The size of the amplified product was 383bp which is the expected size of amplified product with the primers used.

7.3.5 DNA sequencing

PCR products were sent to the Department of Hematology, Royal Infirmary of Edinburgh, Scotland for automated sequencing. The automated analysis of the sequence showed that, all the seven isolates had a Gly238Ser mutation that is characteristic of the SHV-2 ESBL. However, another mutation at amino acid 267 was found, in which ACC (threonine) had changed to TCC (serine). See figure 7.2

Table 7.2

Results of IEF

Isolate No	pI values
34	5.4, 7.6
100	5.4, 7.6 and >8.0
130	5.4, 7.6 and >8.0
150	5.4, 7.0
129	5.4, 7.0 and >8.0
139	5.4, 7.0 , 7.6 and >8.0
329	5.4, 7.6 and >8.0

Figure 7.2

DNA sequencing results that show the new mutation Thr267Ser.

GCT	GGC	GAG	CGG	GGT	GCG	CGC	GGG	ATT	GTC
A	G	E	R	G	A	R	G	I	V
	238	240							
GCT	AGC	AAG	CGG	GGT	GCG	CGC	GGG	ATT	GTC
A	S	K	R	G	A	R	G	I	V
GCC	CTG	CTT	GGC	CCG	AAT	AAC	AAA	GCA	GAG
A	L	L	G	P	N	N	K	A	E
		250							
GCC	CTG	CTT	GGC	CCG	AAT	AAC	AAA	GCA	GAG
A	L	L	G	P	N	N	K	A	E
CGC	ATT	GTG	GTG	ATT	TAT	CTG	CGG	GAT	ACC
R	I	V	V	I	Y	L	R	D	T
		260							267
CGC	ATT	GTG	GTG	ATT	TAT	CTG	CGG	GAT	TCC
R	I	V	V	I	Y	L	R	D	S

Chapter 8

Discussion

8.1 Prologue

Over the last 15 years, ESBLs have gone from being an interesting scientific observation to a phenomenon of great medical importance. The introduction of the oxyimino-β-lactam antibiotics has given rise to the emergence of new β-lactamases. Some of these new β-lactamases, like the TEM- and SHV- type ESBLs, result from simple point mutations in existing β-lactamase genes that lead to a changed substrate profile. Other new β-lactamases, such as the CTX-M type enzymes, have been 'borrowed' from the chromosomally encoded β-lactamases that occur naturally in other species of Enterobacteriaceae (Bradford, 2001). The development and spread of ESBLs have most likely been caused by the overuse of expanded-spectrum cephalosporins in the hospital setting.

Numerous methods have been proposed for detection of ESBLs in clinical isolates. However, it is important to note that none of the methods that rely on phenotypic expression of the β-lactamase will detect every ESBL-producing isolate. Nevertheless, increased awareness of the need to detect ESBL-producing strains among clinical microbiology laboratory and infection control personnel will help in the interpretation of these tests.

Current therapy for strains of Enterobacteriaceae that express ESBLs is limited to such broad-spectrum agents as imipenem. However, there have already been reports of therapeutic failures of this drug with strains that produce multiple β-lactamases (Ahmad *et al.*, 1999) There are limited therapeutic options left for some of these

organisms. Strains expressing extended-spectrum β-lactamases will present a major challenge for clinical microbiologists and clinicians alike as we head into the 21st century.

The majority of clinical laboratories do not have the resources to identify ESBL producing strains by molecular methods, therefore it is important to have an easy to perform methodology that can be used in the routine laboratory.

This study shows the importance of identification of Enterobacteriaceae to species level and the usefulness of the Vitek system for routine detection of ESBLs if we are to report accurate and consistent results to clinicians. In a recent survey of detection of ESBLs in clinical isolates, Tenover *et al.* (Tenover *et al.*, 1999), found that only 18% of laboratories correctly identified challenge organisms as potential ESBL-producers using susceptibility to one or more expanded- spectrum ß-lactam antibiotics as the method of detection. Furthermore, a survey in Europe found that 37% of ESBL producing organisms were mistakenly reported as being susceptible to expanded–spectrum cephalosporins (Livermore and Yuan, 1996). Another recent report described the retrospective detection of ESBLs in five of 20 strains of *K. pneumoniae* isolated from blood cultures. In some of these cases patients were treated with an inappropriate third-generation cephalosporin. Treatment in these patients was changed after clinical failure (Crowley, 2001). If an ESBL detection test was available at the original time of susceptibility testing then appropriate results would have reported to the clinician.

8.2 ESBL detection

There is currently a great need for reliable and efficient tests to detect ESBLs in clinical isolates of Enterobacteriaceae. Conventional susceptibility testing methods, on their own, fail to present reliable susceptibility results for β-lactam antibiotics when testing those species that harbour ESBLs.

8.2.1 Vitek system

Currently, most UK clinical laboratories do not use a standard method for the detection of ESBLs and many clinical laboratories do not routinely identify Enterobacteriaceae to genus and species level. The Vitek system addresses these issues. It will only validate a susceptibility result once the organism has been identified to species level. If the system detects the presence of an ESBL resistance mechanism in strains of *Klebsiella* spp. and *E. coli*, the system then utilises its 'expert' software and applies it to the final susceptibility results. β-lactams susceptible to ESBL activity are then flagged as resistant whether or not the *in vitro* test indicates susceptibility.

This study has shown that the Vitek system, in our hands, whilst easy to perform and without any subjective interpretative of results, reported false positive detection of ESBL activity with fourteen strains of *E. coli*. On re-testing these strains with Vitek (GNS-532 card) they were correctly reported as ESBL negative. All these strains were initially tested with the same batch number of Vitek (GNS-526 card). When re-tested, the GNS-532 card was used; this card has superseded the GNS-526 with accompanying software upgrades. Although internal quality control (QC) is

performed on these cards, an ESBL-producing *E.coli* is not used as part of the QC battery. It was initially thought that the 14 false-positive ESBL *E. coli* might have been as a result of a faulty batch of GNS-526 cards. However, recent work in this laboratory, with ten recently isolated 'Vitek' ESBL-positive *E. coli* showed two of these ten strains to be ESBL-negative by E-test. On re-testing with the same batch of Vitek GNS-532, these two strains were subsequently reported as ESBL negative. Although the percentage false-positive *E. coli* ESBL strains fell from approximately 50% to 20% with the new card it was still a concern that these strains were misreported as ESBL-producers with subsequent 'expert' rules for antibiotic susceptibility to β-lactam antibiotics applied. There appears to be no obvious reason for these results. It is unlikely to be technical error as the Vitek is a highly standardised system. These strains have been forwarded to bioMérieux for further studies. Tzelepi *et al.* have reported that the Vitek ESBL detection test failed to detect the majority of ESBL-producing strains of Enterobacteriaceae (Tzelepi *et al.*, 2000). In a study of *Klebsiella* spp. and *E.coli* expressing well-characterised ß-lactamases, Sanders *et al.* showed that the Vitek ESBL test was 99% sensitive and specific for the detection of ESBLs (Sanders *et al.*, 1996). However a recent study in Brazil showed the Vitek system to have only 90.2% sensitivity (Hsiung *et al.*, 2002). Up-dated computer algorithms in the new Vitek2 system have been shown to categorise the phenotype of susceptibility patterns with various β-lactam antibiotics (Sanders *et al.*, 2000).

It should be noted, that in our hands, some *E. coli* were reported as falsely ESBL-positive. For ESBL positive *K. pneumoniae* in this study the Vitek test was accurate

but it should be noted that this study only included Vitek ESBL-positive strains and was therefore not an evaluation of the Vitek ESBL test itself.

8.2.2 Double disc diffusion

The DDD test requires careful spacing of discs for accurate results and careful interpretation of zone sizes. It is therefore technically demanding. In previous studies, the DDD test was able to detect 82% and 88% of ESBL-positive strains respectively (Gibb and Crichton, 2000; Thomson and Sanders, 1992). The limitations of this test have been described elsewhere (Bush, 1996; M'Zail *et al*., 2000; Thomson and Sanders, 1992). A recent study reported that cefpodoxime achieved a 100% sensitivity rate in detecting ESBLs in tested isolates; the sensitivity of cefotaxime was 92% and that of ceftazidime was 82% (Appleton and Hall, 2000). In contrast, this study showed that cefotaxime was the most efficient cephalosporin for the detection of ESBLs with a sensitivity rate of 85%, with rates of 73.5% for cefpodoxime and 46% for ceftazidime. This may reflect the type of ESBLs in our isolates, but it emphasises the importance of testing more than one cephalosporin.

8.2.3 E-Test ESBL

The commercially available ESBL E-test strip is a quantitative technique and is widely regarded as the 'gold standard' for detection of ESBL production in routine clinical laboratories (M'Zail *et al*., 2000). This test was shown to be more sensitive than the double disc diffusion test in detecting ESBL's in clinical isolates (Cormican *et al.,* 1996). In our hands it detected 98.8% of the test isolates but only if both cefotaxime and ceftazidime strips were used in conjunction. In a study by

Vercauteren *et al.* (Vercauteren *et al.*, 1997), only using the ceftazidime E-test ESBL detected 81% of ESBL-producing strains isolated in their laboratory, compared to 97% detected by the double disk test. This technique is expensive and most clinical laboratories would use it only for confirmation rather than as a routine test.

In conclusion, none of the detection tests that are based on phenotype of the ß-lactamase produced are 100% sensitive or specific for the accurate detection of ESBL's among clinical isolates of Gram-negative bacteria. The need for improved detection of ESBLs in clinical isolates is well recognised (Paterson and Yu, 1999).

The Vitek ESBL test was cost effective as an ESBL screen in so much as the ESBL test is an integral part of the susceptibility card and performed simultaneously with the susceptibility tests. In addition, the Vitek test is interpreted by the system, which removes any errors of subjectivity. No additional outlay of resources is required. We would recommend strains of *E.coli* be confirmed as ESBL positive by an additional method such as E-test.

8.3 Antimicrobial susceptibility profile

8.3.2 Susceptibility and identification by the Vitek system

Identification of Gram-negative bacteria was performed using the GNI card. This card contains 29 different biochemical reaction wells, and one negative control well.

Incubation times varied from two to 15 hours, depending on the growth rate of the organism (Paton, 2000).

The GNI card now has been superceded by the GNI+, which claims identification of more species and a faster identification time (between two to 12 hours) for both Gram-negative and Gram-positive bacteria (Bourbeau and Heiter, 1998).

The Vitek system successfully identified all the test isolates as *K. pneumoniae.* Susceptibility tests were performed on GNS cards, which also have an integrated ESBL detection test. If the instrument flags up an organism as positive for ESBL production the expert software system automatically flags up all cephalosporins as resistant, regardless of the actual test result to the antibiotic. It has been stated elsewhere that if organisms are known to harbour ESBLs then all cephalosporins should be reported as resistant (Livermore *et al.*, 2001). Expert systems such as this are invaluable as an aid for detection of resistance mechanisms. The results from the Vitek show the susceptibility data before it was interpreted by the expert software system, in each case the expert software would have recognised the presence of an ESBL and advised that the susceptibility results for cephalosporins be reported as resistant regardless of the instrument result. The Vitek is now due to be superceded by the Vitek 2 that has an improved Advance Expert system (AES). This system has recently been evaluated by Livermore *et al* (Livermore *et al.*, 2002).

8.3.2 Susceptibility studies by the disc diffusion method

The disc diffusion method is by far the most popular technique used throughout the world and is largely suitable for testing most rapidly growing bacteria. It provides relative data concerning the susceptibility of particular microorganisms against the antibiotic tested.

Disturbingly, the disc diffusion method repeatedly failed to confirm resistance to third generation cephalosporins in ESBL-positive strains. This is not a new finding and has been reported elsewhere (Jacoby and Han, 1996). Interestingly, in our study we found that false susceptibility was most prevalent with ceftazidime which is in contrast to the study by Jacoby and Han (1996) where higher false-susceptibility rates were seen with cefotaxime. This may however, possibly reflect the different populations of organisms tested rather than the antibiotic concerned.

In a study by Babini and Livermore (Babini and Livermore, 2000), it was found that up to 40% of ESBL-producers had been reported as susceptible to cefotaxime and /or ceftazidime. It is disturbing that even armed with the scientific knowledge and methodologies to detect ESBL producers, many laboratories simply do not use this information to good effect and still rely on the disc diffusion method, which has clearly be shown to be fraught with frailty for use with these antibiotics/organism combinations. It is of great concern that patients may receive inappropriate treatment with the obvious repercussions involved.

Aminoglycoside resistance was found to be concomitant with ESBL production in many of the test strains with resistance rates of between 39% and 62% dependant on the aminoglycoside concerned.

8.3.3 MICs determined by agar dilution

The determination of the MIC is the 'gold standard' standard quantitative technique for testing the antimicrobial susceptibility of bacteria (Brown and Brown, 1991). The MIC is determined by serial dilutions of the appropriate antibiotic in either agar or in broth. The most common use of dilution methods is in the evaluation of new antimicrobial agents and in the establishment of criteria for interpretation of disc diffusion methods. However, the dilution methods are not useful for organisms that grow slowly or with organisms where diffusion tests have doubtful reliability (Brown and Brown, 1991).

In this study the MICs of tobramycin, gentamicin and ciprofloxacin for the test organisms were determined by the traditional agar dilution method. Resistance rates for ciprofloxacin, gentamicin and tobramycin were 46%, 40%, and 46% respectively. However, it should be noted that these prevalence rates of resistance are in a highly biased study population and on their own are of little value. Several other studies also report resistance to aminoglycosides as being common among ESBL-producers (Babini and Livermore, 2000; Palucha *et al.*, 1999).

Interestingly, resistance rates for ciprofloxacin in this study were greater than those previously reported (Babini and Livermore, 2000).

8.3.4 MICs determined by E-test strips

MICs to the test strains for cefotaxime and ceftazidime were determined by E-test strips. This test uses plastic strips containing a preformed gradient of antibiotic. This is placed on the agar medium after the plate has been inoculated, the antibiotic diffuses from the plastic strip into the agar medium. In this study for the most part, the endpoints were clear except in a few cases where interpretation of the end point was difficult as a result of the presence of double zones of inhibition. This is known problem with Etest strips and the manufacturer has issued guidelines for the interpretation of the test.

With E-test strips for cefotaxime, 77% of the isolates were resistant/intermediate to cefotaxime. MICs of ceftazidime indicated that only 47% of strains were resistant to this drug.

Treatment failures due to undetected ESBL producing *K. pneumoniae* have already been reported (Karas *et al.*, 1996). No clear connection can be made between ß-lactamase production and the in vitro resistance level of the host (Magdalena *et al.*, 1997). Other factors must therefore be taken into consideration , e.g. permeability changes or the influence of the specific host environment (Magdalena *et al.*, 1997). The MICs of cefotaxime were significantly higher than those of ceftazidime for all

isolates producing ESBL. Similar results were reported in a study by Palucha *et al.* in 1999 (Palucha *et al.*, 1999).

In general, the stability of the antimicrobial gradient produced by E-test is only marginally effected by inoculum, pre-inoculation and pre-diffusion, which generally have a marked effect on the disc diffusion test (Brown and Brown, 1991). In addition, E-test may prove a more reliable alternative for conventional diffusion or dilution test for fastidious or slow growing organisms.

8.4 Molecular detection of bla_{SHV} and bla_{TEM} genes

In this study, the ESBL genes were detected by dot-blot hybridisation and by the amplification of DNA sequence coding bla_{TEM} and bla_{SHV} ESBLs by PCR.

The results obtained from dot-blot for bla_{SHV} were comparable with those obtained by PCR analysis and the results generally agreed. The two techniques showed marked differences for the detection of the bla_{TEM} gene. bla_{TEM} gene was detected by PCR in 42 stains, while it was detected in 62 strains by dot-blot hybridisation. The high incidence of positive bla_{TEM} gene results by dot-blot could be as a result of cross-hybridisation with the bla_{SHV} gene as they have been shown to share more than 68% homology (Heritage *et al.*, 1999), but this is unlikely as the controls were always negative. Therefore these results should be treated with caution and are invariably inconclusive.

PCR has been used extensively in the detection and analysis of resistance genes in bacterial isolates (Brown *et al.*, 1996; Vila *et al.*, 1995), and in the detection of specific micro-organisms directly in clinical and environmental specimens (Brakstad *et al.*, 1992; Persing, 1993; Shirai *et al.*, 1991). It is a very useful technique for the early detection and specific diagnosis of infectious disease. Our data shows that PCR was successful in detecting the presence of bla_{SHV} and/or bla_{TEM} genes in the test isolates.

8.5 The β-lactamases of ESBL-producing *K. pneumoniae* and *E.coli*

The IEF results suggested that four β-lactamases with pI values of 5.4, 7.0, 7.6 and 8.2 were identified. The determination of the isoelectric point on its own is useful for the indication of SHV-derived enzymes but is clearly insufficient for the TEM derived β-lactamases where at least 14 enzymes share the same pI of 5.2 and more than 10 share pI 5.4. Further characterisation requires genetic analysis by sequencing or oligotyping.

Some ESBL-SHV enzymes with pI of 7.6 or 8.3 contain a glycine to serine amino acid substitution at position 238 as a result of a mutation, which creates a new endonuclease restriction site, *Nhe*I (Nüesch *et al.*, 1996). PCR-RFLP with *Nhe*I endonuclease can therefore be used to distinguish bla_{SHV-2} and bla_{SHV-3} from bla_{SHV-1} (Chanawong *et al.*, 2000). The amino acid sequence of bla_{SHV-2} differs from that of bla_{SHV-3} by a change at position 205, where leucine in bla_{SHV-2} is substitution by

arginine in bla_{SHV-3} (Garbargchenon *et al.*, 1990; Peduzzi *et al.*, 1989). This mutation can be detected by DNA-sequencing or by *BcefI* restriction endonuclease, however, currently there are no suppliers of this enzyme (Chanawong *et al.*, 2000). Nevertheless, in organisms that encode multiple SHV-type β-lactamases genes, the combination of IEF and RFLP analysis of SHV-specific PCR products can aid in the identification of multiple SHV-type β-lactamases encoded by one isolate (Hanson *et al.*, 1999). Isoelectric point determination could differentiate these two β-lactamases, SHV-3 has a pI of 7.0 and SHV-2 has a pI 7.6 (Chanawong *et al.*, 2000). Using this information we can therefore deduce that regarding our findings in this study, we have isolates harbouring either SHV-2 or SHV-3 ESBLs.

In conclusion, both PCR-RFLP and IEF are useful aids for more accurate molecular characterisation of β-lactamases and provide a sensitive technique when used in tandem for the identification of SHV-ESBL family genes other than employing direct molecular sequencing. The situation is different with the TEM-ESBL family for which the combination of PCR-RFLP and IEF was not able to identify the TEM-derived enzymes in this study, which subsequently required full DNA sequencing. DNA-sequencing results indicated that enzymes with a pI value of 5.4 were TEM-1 in *K. pneumoniae* isolates and were TEM-33 (IRT) in our *E.coli* isolates.

8.6 Molecular and epidemiological studies of ESBL-producing *K. pneumoniae* and *E.coli*

PFGE is often considered as the "gold standard" of molecular typing techniques (Olive and Bean, 1999). However, molecular typing methods, as applied to the genus *Klebsiella*, are still in their infancy. Preliminary work has been published on plasmid profiles (Hartstein *et al.*, 1993; Podschun and Ullmann, 1998), ribotyping, multilocus enzyme analysis, and applications of pulse-field gel electrophoresis (Bingen *et al.*, 1994). The procedures vary from laboratory to laboratory but lack of standardisation makes it difficult to compare them.

In recent years, *K. pneumoniae* isolates harbouring ESBLs have produced significant outbreaks in hospitals world-wide (Eisen *et al.*, 1995; M'Zali *et al.*, 1996; Rice *et al.*, 1990; Rice *et al.*, 1996; Sirot *et al.*, 1988). Our data, according to the results of PFGE analysis of genomic DNA showed strains isolated throughout 14 wards within the RIE (see table 2.1).

The results of epidemiological studies confirm that the ESBLs-producing *K. pneumoniae* from Ward 15 are as a result of the dissemination of a resistance plasmid (see table 5.7) or mutations in existing plasmid-mediated β-lactamases probably as a result of selective pressure produced by the overuse of third generation cephalosporins. This mutation may also explain why some strains had one SHV-gene variant whereas others had a different variant. Most SHV-differ from one another by only one amino acid (M'Zali *et al.*, 1996), so a single point mutation might modify

the enzyme type. The SHV-5 ESBL present in group A PFGE has probably evolved from an SHV-2 β-lactamase.

The other major PFGE groups B and D consisted of 12 and 10 isolates respectively, producing SHV-2 and TEM-1 or just SHV-2 alone. PFGE group C consisted of 4 isolates producing SHV-2, PFGE group F consisted of 3 isolates, 2 produced TEM-1 and SHV-2 and one produced SHV-2 only. PFGE group E consisted of 5 isolates, one strain produced SHV-2 while the others produced both TEM-1 and SHV-2. PFGE group G consisted of 3 isolates, 2 produced SHV-3 and one SHV-2. The other six individual PFGE groups (H, I, J, K, L, and M) consisted of a single isolate each and were unrelated to the other PFGE groups. Table 2.1 and Table 5.7 shows the demographics of these isolates.

Interestingly isolate AD404 and isolate AD410 had PFGE patterns placing them in PFGE groups A and B respectively. These strains were collected from samples sent to the laboratory by GPs. This would indicate that the dissemination of these particular strains is not limited to the hospital only but occur in the community as well. It is not known whether these patients had been hospitalized prior to isolation of these strains.

In general, the majority of *K. pneumoniae* isolates (95%) harboured an SHV-derived β-lactamase, this was SHV-2 which is in accordance with the situation reported in other studies (Juteršek *et al.*, 2002). From this work, we can suggest that PFGE when combined with IEF analysis is an effective tool for investigating the epidemiology of

ESBL-producing *K. pneumoniae*, and negates the requirement for expensive and time consuming molecular sequencing.

The *E.coli* strains harbouring ESBLs were all collected from GPs, and all of them were isolated from urine samples of patients with UTI infection with the exception of one strain that was isolated from a sputum sample. It is well known that *E.coli* is the main cause of UTI.

Interestingly 5 isolates that belonged to PFGE group X were collected from the same GP. Although these strains were shown not to all harbour the same β-lactamase enzymes (see table 5.3) it indicates the spread of a promiscuous strain of *E. coli* among these patients and further epidemiological investigations are required on the spread of this particular strain.

8.7 Epidemiological findings

Previous epidemiological studies of ESBL-producing strains indicated a combination of epidemic modes of dissemination including strains, self-transferable plasmids and gene transposition (Decre *et al.*, 1998; De Champs *et al.*, 1991; Kitzis *et al.*, 1988; Petit *et al.*, 1990; Sirot *et al.*, 1988).

The presence of this ESBL-harbouring *K. pneumoniae* in these wards was probably initially as a result of heavy use of third generation cephalosporins within the ITU setting and horizontal dissemination has occurred allowing the spread of this strain to other patients in different wards. *Klebsiella* spp. are protected from drying by their

capsule and can survive better than other Enterobacteriaceae on skin and medical equipment facilitating cross-infections (Casewell and Desai, 1983).

Interestingly, an isolate of each PFGE group (A-G) was found from Ward 15. This might also indicate that cross-infection by medical stuff including nurses, physiotherapy stuff or medical students (the RIE is a teaching hospital) may have played a role in the dissemination of these resistant isolates between the different wards within the hospital.

This study clearly indicates the need for on-going careful epidemiological investigations and shows that certainly in this case cross infection within the RIE has become a major problem, not only within the hospital itself but particularly within the ITU. Only with the enforcement of strict infection control measures and strict antibiotic guidelines can we hope to control the appearance and spread of these organisms within the hospital but in the community as well.

8.8 Quinolone resistance and ESBL-producing *K. pneumoniae*

Initial studies on the development of quinolone resistance demonstrated two basic strategies that bacteria could adopt to circumvent the action of quinolones. Alteration in DNA gyrase (the target of the quinolones) and mutation leading to reduced access of quinolone to DNA gyrase (either efflux or alteration in the outer membrane of Gram-negative bacteria) (Wiedemann and Heisig, 1994). Alteration in the structure

of DNA gyrase has been the most commonly identified resistance mechanism in clinical isolates (Ball, 1994; Maxwell, 1997; Wiedemann and Heisig, 1994).

This study clearly shows ciprofloxacin resistance in *K. pneumoniae* is closely associated with ESBL production. This association is of grave concern in as much as ESBL producing *K. pneumoniae* are often resistant to other classes of antimicrobials. Therefore, ciprofloxacin resistance in these strains severely limits already restricted treatment options. It might have been interesting to have investigated ciprofloxacin resistance in ESBL-producing *K. pneumoniae* but this was not performed in this study.

This molecular epidemiological study of ciprofloxacin-resistant ESBL producing *K. pneumoniae* showed genotypically identical isolates within one group consisting of 25 strains. The patterns obtained by Rapiddist were similar to those analysed manually using the criteria of Tenover and Arbeit (Tenover *et al.*, 1995). All the ESBL-positive, ciprofloxacin resistant isolates showed four DNA bands common to all isolates.

8.8.1 The role of *gyr*A and *par*C in quinolone resistance

Alteration of the *gyr*A subunit of DNA gyrase has a central role in conferring high-level quinolone resistance in Gram-negative bacteria (Deguchi *et al.*, 1997a). Ciprofloxacin resistance in *K. pneumoniae* is predominantly due to a chromosomal mutation in the *gyr*A gene (Thomson,1999). RFLP, using a *Hinf*I restriction endonuclease assay, demonstrated a mutation at serine 83 in the QRDR of the *gyr*A

region. This mutation is known to confer quinolone resistance and results in the loss of the *Hin*fI G/ANTC restriction site. RFLP using *Hin*fI can also be useful to distinguish between those *K. pneumoniae* that possess serine at position 83 but not *K. pneumoniae* possessing threonine at position 83. DNA sequencing also showed a point mutation at codon 83 located in the QRDR region. Amino acid position 83 was changed from ACT to ATT (threonine to isoleucine) in seven of the isolates. The other 25 isolates belonging to PFGE group A. In *par*C, one single base change leading to a single amino acid change was identified in all tested isolates. This alteration was present on codon 80, the same alteration in *par*C has been observed in previous studies (Deguchi *et al*., 1997b). Nevertheless, in this study, no strains had alteration in *par*C without the concomitant presence of alteration in *gyr*A. These finding are consistent with other studies indicating that in *K. pneumoniae*, DNA gyrase is a primary target of quinolones, and only a single amino acid change at position 83 or two amino acid changes at position 83 and 87 in *Gyr*A are sufficient to generate high-level resistance to ciprofloxacin. In addition, the accumulation of alteration in *Gyr*A and simultaneous presence of alteration in *Par*C play a complementary role in developing high-level quinolone resistance. (See table 8.1).

Table 8.1

Alteration in GyrA and ParC in quinolone resistant clinical isolates of *K. pneumoniae*

Strain	Amino acid (codon) at the indicated position in				MIC	PFGE group
	GyrA 83	GyrA 87	ParC 80	ParC 84		
E.coli - 10418	TCC (serine)	GAC (aspartic acid)	AGC (serine)	GAA (glutamic acid)	< 0.025 mg/l	–
*K. pneumoniae**	ACT (theronine)	GAC (aspartic acid)	AGC (serine)	GAA (glutamic acid)	<0.025 mg/l	–
AD315	TTC (phenylalanine)	TAC (tyrosine)	ATC (isoleucine)	GAA (glutamic acid)	>32 mg/l	A
AD313	ATT (isoleucine)	GAC (aspartic acid)	ATC (isoleucine)	GAA (glutamic acid)	32 mg/l	B
AD331	ATT (isoleucine)	GAC (aspartic acid)	ATC (isoleucine)	GAA (glutamic acid)	32 mg/l	C
AD409	ATT (isoleucine)	GAC (aspartic acid)	ATC (isoleucine)	GAA (glutamic acid)	32 mg/l	D

* AD317 *K .pneumoniae* ESBL positive, ciprofloxacin sensitive

8.8.2 The role of OMPs in quinolone resistance

Porins are OMPs that allow the non-specific diffusion of small molecules into the bacterial cell. Most of the studies on OMPs have been carried out with *E.coli*, in which two major porins (OMP C and OMP F) have been characterised (Ardanuy *et al.*, 1998). In *K. pneumoniae*, two main porins have been characterised; OMP K35 (the homology of OMP F) and OMP K36 (the homology of OMP C) (Alberti *et al.*, 1995). Recently, loss of the OMP K36 porin has been associated with both cefoxitin resistance and increase in cephalosporin and quinolone MICs (Bradford *et al.*, 1997).

There are several reports on the association of loss of porins and increased resistance to antimicrobial agents, particularly with *E.coli* and *Salmonella typhimurium* (Martinez *et al.*, 1996). For other Gram-negative bacteria, the association of loss of OMP with various size ranges has been reported (Ardanuy *et al.*, 1998; Deguchi *et al.*, 1997b; Martinez *et al.*, 1996), and resistance to antimicrobial agents has been considered evidence of the role of these OMPs as porins (Ardanuy *et al.*, 1998; Martinez *et al.*, 1996). The OMP results in this study demonstrated that some of the isolates had lost one porin that might be 41 KDa. A similar conclusion was found in a study by Deguchi et al. in 1997 (Deguchi *et al.*, 1997b).

Other isolates that belong to PFGE group A showed loss of a porin that might be porin K(36 KDa), similar findings have been reported in other studies (Ardanuy *et al.*, 1998; Martinez *et al.*, 1996). However, in many cases, the exact nature of the protein has not been established, and the assumption that the involved proteins were porins were largely based on their molecular masses (Martinez *et al.*, 1996).

Furthermore, the lack of a good definition of *K. pneumoniae* porins has resulted in their having an inferred, rather than demonstrated, role in antimicrobial resistance in this species (Pangon *et al.*, 1989).

In this study, the diminished activity of ciprofloxacin appears to be related to mutation in gyrase encoding amino acid 83 and the pleiotropic effect of the loss of porin, either 41 or 36 KDa from their OMPs. There are also other resistance mechanisms in this species to be considered. The first study of plasmid-mediated ciprofloxacin resistance has recently been reported (Martinez *et al.*, 1998). Other potential explanations for the association between resistance to 3rd generation cephalosporins and quinolones include active efflux (Paterson *et al.*, 2000).

In general, results from this study confirm previous observations (Martinez *et al.*, 1996; 1999), indicating that resistance to extended-spectrum cephalosporins increases in *K. pneumoniae* strains that produce SHV-type ESBL that lack the two major porins of their species.

As a general conclusion, treatment of infection caused by *K. pneumoniae* producing ESBLs is difficult not only because of their resistance to expanded-spectrum cephalosporins, but also because of their often concomitant resistance to other antimicrobial agents encoded by the same or different plasmid. Ciprofloxacin resistance and ESBL production in members of Enterobacteriaceae are geographically widespread (Paterson *et al.*, 2000). Continued world-wide

surveillance of *K. pneumoniae* isolates is necessary to provide information on the dissemination of these important pathogens.

8.9 The discovery of SHV-39 in *K. pneumoniae* strains isolated from Chilean hospitals

To date, the majority of SHV-type derivatives possess the ESBL phenotype. However, one variant, SHV-10, is reported to have an inhibitor-resistant phenotype. This enzyme appears to be derived from SHV-5 and contains one additional amino acid substitution of glycine for serine 130 (Prinarakis *et al.*, 1997). It is interesting that the inhibitor-resistant phenotype conferred by the Ser140Gly mutation seems to override the strong ESBL phenotype usually seen in enzymes containing the Gly238Ser and the Glu240Lys mutations seen in other SHV-5 type enzymes (Bradford, 2001). The majority of SHV-type ESBLs are found in strains of *K. pneumoniae*.

K. pneumoniae is a widespread nosocomial pathogen within Chilean hospitals. It has largely been treated with ceftriaxone and ceftazidime and resistance has increased markedly. The fifth member of the SHV family (SHV-5) was first observed in Chile (Gutmann *et al.*, 1989). This was the first variant not to have been first observed in Europe. SHV-5 again was first described from *K. pneumoniae* and has a pI of 8.2. As with SHV-4, the amino acid found at position 240 in SHV-5 is lysine. It is possible to speculate from which ancestral gene the SHV-5 determinant evolved as it is most likely to be evolved by a point mutation from SHV-2.

K. pneumoniae strains used in this study were isolated from hospitalised patients from the Catholic University hospital in Santiago, the Gustavo Fricke hospital in Valparaiso, which lies 100 miles to the east, the G.Grant-Benavente hospital in Concepcion which lies 300 miles to the south, and a hospital in Puerto Montt which lies 800 miles to the south, (see figure 7.1). Our results demonstrated that although there were some variations in genotype, within each hospital there was consistency of PFGE banding patterns. However, there was no similarity between strains isolated from different cities showing they were different clones. Examination of the β-lactamases showed that TEM-1 was always present; however, the cephalosporin resistance was associated with an SHV-derived β-lactamase with a pI value greater than 7.0. PCR amplification and sequencing revealed a hitherto undescribed enzyme with not just a Gly238Ser mutation, characteristic of SHV-2, but also a Thr267Ser mutation. This ESBL conferred MICs of ceftazidime of >256 mg/l and MICs between 16-32mg/l for cefotaxime. This β-lactamase has not previously been reported. The amino acid sequence has been deposited with (www.lahey.org/studies/webt.htm) and the enzyme has been designated SHV-39. This enzymes seems to be widely disseminated in Chile both geographically and between different strains of *K. pneumoniae*.

Appendices

Appendix I Results of E-Test ESBL strips

Strains	CT/CTL	Comments	TZ/TZL	Comments
AD 313	256	+Ve ESBL	2.67	-Ve ESBL
AD 314	680	+Ve ESBL	24	+Ve ESBL
AD 315	Phantom	+Ve ESBL	84	+Ve ESBL
AD 316	680	+Ve ESBL	128	+Ve ESBL
AD 317	Phantom	+Ve ESBL	21	+Ve ESBL
AD 318	340	+Ve ESBL	2	-Ve ESBL
AD 319	106	+Ve ESBL	2	-Ve ESBL
AD 320	Phantom	+Ve ESBL	2.64	-Ve ESBL
AD 321	61	+Ve ESBL	340	+Ve ESBL
AD 322	Phantom	+Ve ESBL	128	+Ve ESBL
AD 323	15	+Ve ESBL	2.64	-Ve ESBL
AD 324	340	+Ve ESBL	2.67	-Ve ESBL
AD 325	340	+Ve ESBL	2	-Ve ESBL
AD 326	Phantom	+Ve ESBL	168	+Ve ESBL
AD 327	Phantom	+Ve ESBL	375	+Ve ESBL
AD 328	32	+Ve ESBL	2	-Ve ESBL
AD 329	340	+Ve ESBL	2.67	-Ve ESBL
AD 330	127	+Ve ESBL	2	-Ve ESBL
AD 331	32	+Ve ESBL	2	-Ve ESBL
AD 332	2	-Ve ESBL	2.64	-Ve ESBL
AD 333	680	+Ve ESBL	2	-Ve ESBL
AD 334	16	+Ve ESBL	1.97	-Ve ESBL
AD 335	12	+Ve ESBL	5.3	-Ve ESBL
AD 336	0.27	-Ve ESBL	21	+Ve ESBL
AD 337	24	+Ve ESBL	1	-Ve ESBL
AD 338	Phantom	+Ve ESBL	Phantom	+Ve ESBL
AD 339	21.3	+Ve ESBL	1.5	-Ve ESBL
AD 340	10.40	+Ve ESBL	1.5	-Ve ESBL
AD 341	86.95	+Ve ESBL	0.5	-Ve ESBL
AD 342	32	+Ve ESBL	1.5	-Ve ESBL
AD 343	85.1	+Ve ESBL	1.5	-Ve ESBL
AD 344	42.3	+Ve ESBL	2	-Ve ESBL
AD 345	Phantom	+Ve ESBL	32	+Ve ESBL

AD 346	Phantom	+Ve ESBL	32	+Ve ESBL
AD 348	Phantom	+Ve ESBL	Phantom	+Ve ESBL
AD 349	Phantom	+Ve ESBL	256	+Ve ESBL
AD 350	187.5	+Ve ESBL	4	-Ve ESBL
AD 351	Phantom	+Ve ESBL	168.5	+Ve ESBL
AD 352	680	+Ve ESBL	4	-Ve ESBL
AD 353	170	+Ve ESBL	3.9	-Ve ESBL
AD 354	15.60	+Ve ESBL	2	-Ve ESBL
AD 355	62.5	+Ve ESBL	3.9	-Ve ESBL
AD 356	1000	+Ve ESBL	128	+Ve ESBL
AD 357	34	+Ve ESBL	168.5	+Ve ESBL
AD 358	Phantom	+Ve ESBL	256	+Ve ESBL
AD 359	2.6	-Ve ESBL	2	-Ve ESBL
AD 360	500	+Ve ESBL	15.8	+Ve ESBL
AD 362	phantom	+Ve ESBL	128	+Ve ESBL
AD 363	340	+Ve ESBL	84.5	+Ve ESBL
AD 364	16	+Ve ESBL	32	+Ve ESBL
AD 365	1000	+Ve ESBL	84	+Ve ESBL
AD 366	340	+Ve ESBL	8	+Ve ESBL
AD 367	340	+Ve ESBL	128	+Ve ESBL
AD 368	21	+Ve ESBL	2.6	-Ve ESBL
AD 369	15	+Ve ESBL	7.8	-Ve ESBL
AD 370	10.2	+Ve ESBL	2.6	-Ve ESBL
AD 371	128	+Ve ESBL	21.5	+Ve ESBL
AD 374	Phantom	+Ve ESBL	84	+Ve ESBL
AD 375	250	+Ve ESBL	4	-Ve ESBL
AD 377	695	+Ve ESBL	128	+Ve ESBL
AD 378	250	+Ve ESBL	64	+Ve ESBL
AD 380	500	+Ve ESBL	168	+Ve ESBL
AD 382	1000	+Ve ESBL	340	+Ve ESBL
AD 384	phantom	+Ve ESBL	256	+Ve ESBL
AD 385	16	+Ve ESBL	8	+Ve ESBL
AD 386	16	+Ve ESBL	6	-Ve ESBL
AD 387	170	+Ve ESBL	6	-Ve ESBL
AD 388	0.25	-Ve ESBL	8	+Ve ESBL
AD 389	25	+Ve ESBL	64	+Ve ESBL
AD 391	phantom	+Ve ESBL	84	+Ve ESBL

AD 392	695	+Ve ESBL	2	-Ve ESBL
AD 393	128	+Ve ESBL	84	+Ve ESBL
AD 395	695	+Ve ESBL	500	+Ve ESBL
AD 399	16	+Ve ESBL	1.97	-Ve ESBL
AD 400	21	+Ve ESBL	84.5	+Ve ESBL
AD 401	42	+Ve ESBL	2.7	-Ve ESBL
AD 402	phantom	+Ve ESBL	340	+Ve ESBL
AD 403	15.6	+Ve ESBL	Phantom	+Ve ESBL
AD 404	Phantom	+Ve ESBL	84	+Ve ESBL
AD 405	64	+Ve ESBL	64	+Ve ESBL
AD 407	64	+Ve ESBL	42.5	+Ve ESBL
AD 408	250	+Ve ESBL	128	+Ve ESBL
AD 409	500	+Ve ESBL	500	+Ve ESBL
AD 410	84.5	+Ve ESBL	5.3	-Ve ESBL
AD 411	128	+Ve ESBL	4	-Ve ESBL
AD 412	84.5	+Ve ESBL	2.6	-Ve ESBL
AD 413	170	+Ve ESBL	32	+Ve ESBL

TZ = Ceftazidime **TZL** = Ceftazidime + Clavulanic acid.
CT = Cefotaxime **CTL** = Cefotaxime + Clavulanic acid.

Interpretation :

MIC for CT/CTL or/and TZ/TZL < 8 = -Ve ESBL.
MIC for CT/CTL or/and TZ/TZL ≥ 8 = +Ve ESBL.

Appendix II Results of the DDD method

Strains	Cefotaxime combination disc	Ceftazidime combination disc	Cefpodoxime combination disc	Predicted ESBL by D.D.
AD 313	+	-	-	CTX
AD 314	+	-	+	CTX & CPD
AD 315	+	+	+	CTX, CAZ & CPD
AD 316	+	+	+	CTX, CAZ & CPD
AD 317	+	+	+	CTX, CAZ & CPD
AD 318	-	-	+	CPD
AD 319	+	-	+	CTX & CPD
AD 320	+	+	+	CTX, CAZ & CPD
AD 321	+	-	+	CTX & CPD
AD 322	+	+	+	CTX, CAZ & CPD
AD 323	+	-	-	CTX
AD 324	+	-	+	CTX & CPD
AD 325	-	-	+	CPD
AD 326	+	+	+	CTX, CAZ & CPD
AD 327	+	+	+	CTX, CAZ & CPD
AD 328	+	-	+	CTX & CPD
AD 329	+	-	-	CTX
AD 330	+	-	+	CTX & CPD
AD 331	+	-	-	CTX
AD 332	+	-	-	CTX
AD 333	+	-	-	CTX
AD 334	+	-	+	CTX & CPD
AD 335	+	-	+	CTX & CPD
AD 336	-	-	-	NONE
AD 337	+	-	+	CTX & CPD
AD 338	+	+	+	CTX, CAZ & CPD
AD 339	+	-	+	CTX & CPD
AD 340	+	-	+	CTX & CPD
AD 341	+	-	-	CTX
AD 342	+	-	-	CTX
AD 343	+	-	-	CTX
AD 344	+	-	-	CTX
AD 345	+	+	+	CTX, CAZ & CPD
AD 346	+	+	+	CTX, CAZ & CPD

AD 348	+	+	+	CTX, CAZ & CPD
AD 349	+	+	+	CTX, CAZ & CPD
AD 350	+	-	-	CTX
AD 351	+	+	+	CTX, CAZ & CPD
AD 352	+	-	+	CTX & CPD
AD 353	+	-	-	CTX
AD 354	+	-	-	CTX
AD 355	+	-	+	CTX & CPD
AD 356	+	+	+	CTX, CAZ & CPD
AD 357	+	+	+	CTX, CAZ & CPD
AD 358	+	+	+	CTX, CAZ & CPD
AD 359	-	-	-	NONE
AD 360	+	+	+	CTX, CAZ & CPD
AD 362	+	+	+	CTX, CAZ & CPD
AD 363	+	+	+	CTX, CAZ & CPD
AD 364	+	+	+	CTX, CAZ & CPD
AD 365	+	+	+	CTX, CAZ & CPD
AD 366	+	-	-	CTX
AD 367	+	+	+	CTX, CAZ & CPD
AD 368	+	-	-	CTX
AD 369	-	-	-	NONE
AD 370	-	-	-	NONE
AD 371	+	-	+	CTX & CPD
AD 374	+	+	+	CTX, CAZ & CPD
AD 375	+	-	+	CTX & CPD
AD 377	+	+	+	CTX, CAZ & CPD
AD 378	+	+	+	CTX, CAZ & CPD
AD 380	+	+	+	CTX, CAZ & CPD
AD 382	+	+	+	CTX, CAZ & CPD
AD 384	+	+	+	CTX, CAZ & CPD
AD 385	-	-	-	NONE
AD 386	-	-	-	NONE
AD 387	+	+	+	CTX, CAZ & CPD
AD 388	-	-	+	CPD
AD 389	+	-	+	CTX & CPD
AD 391	+	+	+	CTX, CAZ & CPD
AD 392	+	-	+	CTX & CPD

AD 393	+	+	+	CTX, CAZ & CPD
AD 395	+	+	+	CTX, CAZ & CPD
AD 399	-	-	-	NONE
AD 400	-	+	+	CAZ & CPD
AD 401	-	-	+	CPD
AD 402	+	+	+	CTX, CAZ & CPD
AD 403	-	+	+	CAZ & CPD
AD 404	+	+	+	CTX, CAZ & CPD
AD 405	+	+	+	CTX, CAZ & CPD
AD 407	+	-	+	CTX & CPD
AD 408	+	+	+	CTX, CAZ & CPD
AD 409	+	+	+	CTX, CAZ & CPD
AD 410	+	-	+	CTX & CPD
AD 411	+	-	+	CTX & CPD
AD 412	+	-	-	CTX
AD 413	+	+	+	CTX, CAZ & CPD

CTX: cefotaxime
CAZ: ceftazidime
CPD: cefpodoxime

Appendix III Determination of Sensitivity by Disc- Diffusion Method

Isolates	Ampicillin	Amoxycillin	Trimethoprim	Cephalothin	Cefuroxime	Cefotaxime	Ceftazidime	Gentamicin	Ciprofloxacin	Tobramycin	Tazobactam	Meropenem	Chloramphenicol	Nitrofurantion	Amikacin
AD 313	R	R	R	R	R	I	S	S	R	S	R	S	R	S	R
AD 314	R	R	R	R	R	I	I	I	I	S	R	S	R	S	I
AD 315	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 316	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 317	R	R	S	R	R	R	I	S	S	S	S	S	S	R	R
AD 318	R	R	R	R	R	R	S	R	R	R	R	S	R	I	R
AD 319	R	R	R	R	R	I	S	S	R	S	R	S	S	I	R
AD 320	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 321	R	R	R	R	R	I	S	R	R	R	R	S	R	I	R
AD 322	R	R	R	R	R	I	R	R	R	R	R	S	R	R	R

AD 323	R	R	R	R	R	R	S	S	S	S	S	S	R	R	R
AD 324	R	R	R	R	R	I	S	S	R	S	R	S	R	I	R
AD 325	R	R	R	R	R	I	S	S	R	S	R	S	R	S	I
AD 326	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 327	R	R	R	R	R	R	I	S	R	R	S	S	R	R	R
AD 328	R	R	R	R	R	I	S	S	R	S	R	S	R	S	I
AD 329	R	R	R	R	R	I	S	S	R	S	R	S	R	S	S
AD 330	R	R	R	R	R	I	S	S	R	S	R	S	R	S	S
AD 331	R	R	S	R	R	I	S	S	R	S	R	S	S	I	I
AD 332	R	R	S	R	I	S	S	S	S	S	R	S	I	I	I
AD 333	R	R	R	R	R	I	S	S	R	S	R	S	S	I	I
AD 334	R	R	R	R	R	I	S	R	R	R	R	S	R	S	I
AD 335	R	R	R	R	R	I	S	S	R	S	R	S	R	S	R
AD 336	R	R	S	R	S	S	S	S	S	S	R	S	S	S	S
AD 337	R	R	R	R	R	I	S	S	R	S	R	S	R	S	S
AD 338	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 339	R	R	R	R	R	I	S	S	R	S	R	S	R	S	I

AD 340	R	R	R	R	R	I	S	S	R	S	R	S	R	S	S
AD 341	R	R	S	R	R	I	S	S	S	S	R	S	S	I	I
AD 342	R	R	R	R	R	I	S	S	R	S	R	S	R	S	I
AD 343	R	R	R	R	R	I	S	S	R	S	R	S	R	S	I
AD 344	R	R	R	R	R	I	I	S	R	S	R	S	R	S	I
AD 345	R	R	R	R	R	R	R	S	R	R	S	S	R	R	R
AD 346	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R
AD 348	R	R	R	R	R	I	R	S	R	R	S	S	R	R	R
AD 349	R	R	R	R	R	R	R	S	R	R	S	S	R	R	R
AD 350	R	R	R	R	R	R	S	S	R	S	R	S	R	S	I
AD 351	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 352	R	R	R	R	R	I	S	S	R	S	R	S	R	R	I
AD 353	R	R	R	R	R	I	S	S	R	S	R	S	S	S	S
AD 354	R	R	S	R	R	S	S	S	S	S	R	S	S	S	S
AD 355	R	R	R	R	R	R	S	S	S	S	R	S	S	R	I
AD 356	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 357	R	R	R	R	R	R	R	R	S	R	S	S	S	R	R

AD 358	R	R	S	R	R	I	R	S	S	R	S	S	S	I	R
AD 359	R	R	R	R	R	S	S	S	R	S	S	S	R	R	R
AD 360	R	R	R	R	R	R	I	R	R	R	S	S	R	R	R
AD 362	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 363	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 364	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 365	R	R	R	R	R	R	R	S	S	R	S	S	S	R	R
AD 366	R	R	R	R	R	I	S	S	R	S	R	S	R	R	I
AD 367	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 368	R	R	R	R	R	I	S	S	R	S	R	S	R	I	I
AD 369	R	R	R	S	I	S	S	S	S	S	S	S	S	S	I
AD 370	R	R	S	R	R	S	I	S	S	S	S	S	S	S	R
AD 371	R	R	R	R	R	I	S	S	S	R	R	S	R	R	R
AD 374	R	R	R	R	R	I	R	S	R	S	R	S	R	R	I
AD 375	R	R	R	R	R	I	S	S	R	S	R	S	R	S	S
AD 377	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 378	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R

AD 380	R	R	R	R	R	I	R	R	R	R	R	S	R	R	R
AD 382	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 384	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 385	R	R	R	R	R	S	S	S	R	S	S	S	R	I	R
AD 386	R	R	S	R	R	S	S	S	S	S	S	S	S	I	I
AD 387	R	R	R	R	R	R	S	R	R	R	S	S	R	S	R
AD 388	R	R	R	R	R	S	S	S	S	S	S	S	I	R	S
AD 389	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 391	R	R	S	R	R	R	R	S	R	R	S	S	R	R	R
AD 392	R	R	R	R	R	R	S	S	R	S	R	S	S	R	R
AD 393	R	R	R	R	R	R	I	R	R	R	S	S	R	R	R
AD 395	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 399	R	R	S	R	R	S	S	S	S	S	S	S	S	I	I
AD 400	R	R	R	R	R	S	S	S	R	R	S	S	I	I	R
AD 401	R	R	R	R	R	I	S	S	R	S	R	S	R	I	I
AD 402	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 403	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S

AD 404	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 405	R	R	R	R	R	R	R	R	S	R	R	S	S	R	R
AD 407	R	R	R	R	R	S	S	S	R	R	S	S	R	R	I
AD 408	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 409	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 410	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
AD 411	R	R	R	R	R	I	S	S	R	S	R	R	R	I	I
AD 412	R	R	R	R	R	I	S	S	R	R	R	S	I	S	R
AD 413	R	R	R	R	R	R	S	R	R	R	S	S	R	I	R

Appendix IV Determination of sensitivity by Vitek System

Isolates	Ampicillin	Amoxycillin	Trimethoprim	Cephalothin	Cefuroxime	Cefotaxime	Ceftazidime	Gentamicin	Ciprofloxacin	Tobramycin	Tazobactam	Meropenem	Chloramphenicol	Nitrofurantion	Amikacin
AD 313	R	R	R	R	R	S	S	S	S	S	R	S	R	S	S
AD 314	R	R	S	R	R	S	S	S	S	S	R	S	R	S	S
AD 315	R	R	R	R	S	S	R	R	R	R	R	S	R	R	S
AD 316	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 317	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S
AD 318	R	R	R	R	R	S	S	R	S	R	R	S	R	S	S
AD 319	R	R	R	R	R	S	S	S	S	S	R	S	S	S	S
AD 320	R	R	R	R	S	S	R	R	R	R	R	S	R	R	S
AD 321	R	R	S	R	R	S	S	R	S	R	R	S	R	S	S
AD 322	R	R	R	R	S	S	R	R	R	R	R	S	R	R	S
AD 323	R	R	R	R	R	R	S	S	S	S	R	S	R	R	S

AD 324	R	R	R	R	R	S	S	S	S	S	R	S	R	S	S
AD 325	R	R	S	R	R	R	R	S	S	S	R	S	R	S	S
AD 326	R	R	R	R	S	S	R	R	R	R	R	S	R	R	S
AD 327	R	R	R	R	R	R	R	S	R	R	R	S	R	R	S
AD 328	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 329	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 330	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 331	R	R	S	R	R	R	R	S	R	S	R	S	S	S	S
AD 332	R	R	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 333	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 334	R	R	R	R	R	R	R	R	S	R	R	S	R	S	S
AD 335	R	R	S	R	R	R	R	S	S	S	R	S	R	S	S
AD 336	R	I	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 337	R	R	R	R	R	R	R	S	R	S	R	S	R	S	S
AD 338	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 339	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 340	R	R	R	R	R	R	R	S	R	S	R	S	R	S	S

AD 341	R	R	S	R	R	R	S	S	S	S	R	S	S	S	S
AD 342	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 343	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 344	R	R	S	R	R	R	R	S	S	S	R	S	R	S	S
AD 345	R	I	R	R	R	R	R	S	R	R	R	S	R	R	S
AD 346	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 348	R	I	R	R	R	R	R	S	R	R	R	S	R	R	I
AD 349	R	S	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 350	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 351	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 352	R	R	R	R	R	R	R	S	S	S	R	S	R	R	S
AD 353	R	R	R	R	R	R	R	S	R	S	R	S	R	S	S
AD 354	R	R	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 355	R	R	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 356	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 357	R	R	R	R	R	R	R	R	R	S	R	S	S	R	S
AD 358	R	R	S	R	R	R	R	S	S	R	R	S	S	S	S

AD 359	R	I	S	R	R	R	R	R	S	S	R	S	R	R	S
AD 360	R	I	R	R	R	R	R	R	R	S	R	S	R	S	S
AD 362	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 363	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 364	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 365	R	R	R	R	R	R	R	R	S	R	R	S	R	R	S
AD 366	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 367	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 368	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 369	R	I	R	R	R	R	R	S	S	S	R	S	S	R	S
AD 370	R	R	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 371	R	R	R	R	R	R	R	S	S	S	R	S	R	R	S
AD 374	R	I	R	R	R	R	R	S	S	S	R	S	S	S	S
AD 375	R	R	R	R	R	R	R	S	R	S	R	S	R	S	S
AD 377	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 378	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 380	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S

AD 382	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 384	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 385	R	I	R	R	R	R	R	S	R	S	R	S	R	S	S
AD 386	R	R	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 387	R	I	R	R	R	R	R	R	R	S	R	S	R	S	S
AD 388	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 389	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 391	R	I	S	R	R	R	R	S	R	R	R	S	R	R	S
AD 392	R	R	S	R	R	R	R	S	R	S	R	S	S	S	S
AD 393	R	I	R	R	R	R	R	R	R	S	R	S	R	S	S
AD 395	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 399	R	R	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 400	R	I	R	R	R	R	R	S	R	R	R	S	S	S	S
AD 401	R	R	S	R	R	R	R	S	S	S	R	S	R	S	S
AD 402	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 403	R	R	S	R	R	R	R	S	S	S	R	S	S	S	S
AD 404	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S

AD 405	R	R	R	R	R	R	R	R	S	R	R	S	S	S	S
AD 407	R	R	R	R	R	R	R	S	S	S	R	S	R	S	S
AD 408	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 409	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S
AD 410	R	R	R	R	R	R	R	S	R	S	R	S	R	S	S
AD 411	R	R	S	R	R	R	R	S	S	S	R	S	R	S	S
AD 412	R	R	R	R	R	R	R	S	R	R	R	S	R	S	S
AD 413	R	I	R	R	R	R	R	R	R	S	R	S	R	S	S

Appendix V -Determination of MIC by agar dilution

Isolate	Ciprofloxacin		Gentamicin		Tobramycin	
	MIC	Comment	MIC	Comment	MIC	Comment
AD 313	32	R	1	S	16	R
AD 314	32	R	1	S	1	S
AD 315	32	R	16	R	16	R
AD 316	32	R	16	R	16	R
AD 317	0.125	S	1	S	1	S
AD 318	0.5	S	16	R	16	R
AD 319	1	S	1	S	2	S
AD 320	32	R	16	R	16	R
AD 321	0.5	S	16	R	16	R
AD 322	32	R	16	R	16	R
AD 323	0.125	S	16	R	16	R
AD 324	0.5	S	16	R	8	I
AD 325	2	I	1	S	1	S
AD 326	32	R	16	R	16	R
AD 327	32	R	1	S	16	R
AD 328	2	I	1	S	1	S
AD 329	2	I	1	S	1	S
AD 330	2	I	1	S	1	S
AD 331	32	R	1	S	1	S
AD 332	0.125	S	1	S	1	S
AD 333	0.5	S	1	S	1	S
AD 334	0.5	S	16	R	16	R
AD 335	0.5	S	1	S	2	S
AD 336	0.25	S	1	S	2	S
AD 337	2	I	1	S	1	S
AD 338	32	R	16	R	16	R
AD 339	2	I	1	S	2	S

AD 340	2	I	1	S	2	S
AD 341	0.125	S	1	S	1	S
AD 342	0.5	S	1	S	1	S
AD 343	0.5	S	1	S	1	S
AD 344	0.5	S	1	S	1	S
AD 345	32	R	1	S	16	R
AD 346	32	R	16	R	16	R
AD 348	32	R	1	S	16	R
AD 349	32	R	2	S	16	R
AD 350	0.5	S	1	S	1	S
AD 351	32	R	16	R	16	R
AD 352	0.5	S	1	S	1	S
AD 353	2	I	1	S	1	S
AD 354	0.125	S	1	S	1	S
AD 355	0.125	S	1	S	1	S
AD 356	32	R	16	R	16	R
AD 357	0.125	S	4	S	16	R
AD 358	0.125	S	1	S	16	R
AD 359	0.25	S	8	I	2	S
AD 360	32	R	16	R	8	I
AD 362	32	R	16	R	16	R
AD 363	32	R	16	R	16	R
AD 364	32	R	16	R	16	R
AD 365	0.25	S	4	S	8	I
AD 366	0.5	S	1	S	1	S
AD 367	32	R	16	R	16	R
AD 368	1	S	1	S	1	S
AD 369	0.125	S	1	S	1	S
AD 370	0.125	S	1	S	1	S
AD 371	0.5	S	1	S	1	S
AD 374	0.5	S	1	S	1	S

AD 375	0.125	S	1	S	1	S
AD 377	32	R	16	R	16	R
AD 378	32	R	16	R	16	R
AD 380	32	R	16	R	16	R
AD 382	32	R	16	R	16	R
AD 384	32	R	16	R	16	R
AD 385	32	R	16	R	16	R
AD 386	0.125	S	1	S	1	S
AD 387	32	R	16	R	4	S
AD 388	0.125	S	1	S	1	S
AD 389	32	R	16	R	16	R
AD 391	32	R	1	S	8	I
AD 392	4	R	1	S	1	S
AD 393	32	R	16	R	4	S
AD 395	32	R	16	R	16	R
AD 399	1	S	1	S	2	S
AD 400	32	R	1	S	16	R
AD 401	32	R	1	S	16	R
AD 402	32	R	16	R	16	R
AD 403	0.125	S	1	S	1	S
AD 404	32	R	16	R	16	R
AD 405	0.125	S	16	R	16	R
AD 407	2	I	1	S	1	S
AD 408	32	R	16	R	16	R
AD 409	32	R	16	R	16	R
AD 410	2	I	1	S	1	S
AD 411	2	I	1	S	1	S
AD 412	32	R	1	S	16	R
AD 413	32	R	16	R	4	S

R = resistant, S = sensitive and I = intermediate

Appendix VI PCR and Dot-Blot results

No	Isolate	PCR		Dot-blot	
		SHV	TEM	SHV	TEM
1.	AD 313	+	-	+	+
2.	AD 314	+	-	+	-
3.	AD 315	+	+	+	+
4.	AD 316	+	+	+	+
5.	AD 317	+	-	+	+
6.	AD 318	+	-	+	+
7.	AD 319	+	-	+	+
8.	AD 320	+	+	+	+
9.	AD 321	+	-	+	-
10.	AD 322	+	+	+	+
11.	AD 323	+	-	+	+
12.	AD 324	+	-	-	+
13.	AD 325	+	-	-	+
14.	AD 326	+	+	+	+
15.	AD 327	+	+	+	+
16.	AD 328	+	-	-	+
17.	AD 329	+	-	+	-
18.	AD 330	+	-	-	+
19.	AD 331	+	-	-	+
20.	AD 332	+	+	-	+
21.	AD 333	+	-	+	+
22.	AD 334	+	-	+	-
23.	AD 335	+	-	+	+
24.	AD 336	+	-	+	-
25.	AD 337	+	-	+	-
26.	AD 338	+	+	+	+
27.	AD 339	+	-	+	+

28.	AD 340	+	-	+	+
29.	AD 341	-	+	+	+
30.	AD 342	+	-	+	+
31.	AD 343	+	-	+	+
32.	AD 344	+	-	+	-
33.	AD 345	+	+	+	+
34.	AD 346	+	+	+	+
35.	AD 348	+	+	+	-
36.	AD 349	+	+	+	+
37.	AD 350	+	-	+	-
38.	AD 351	+	+	+	+
39.	AD 352	+	-	+	-
40.	AD 353	+	-	+	+
41.	AD 354	+	-	+	+
42.	AD 355	-	+	+	-
43.	AD 356	+	+	+	+
44.	AD 357	+	+	+	+
45.	AD 358	-	+	+	+
46.	AD 359	-	+	+	+
47.	AD 360	+	-	+	-
48.	AD 362	+	+	+	+
49.	AD 363	+	+	+	+
50.	AD 364	+	+	+	+
51.	AD 365	+	+	+	-
52.	AD 366	+	-	+	-
53.	AD 367	+	+	+	+
54.	AD 368	+	-	+	-
55.	AD 369	+	-	-	+
56.	AD 370	+	-	+	+
57.	AD 371	+	-	+	-

58.	AD 374	+	-	+	-
59.	AD 375	+	-	+	+
60.	AD 377	+	+	+	+
61.	AD 378	+	+	+	+
62.	AD 380	+	+	+	-
63.	AD 382	+	+	+	+
64.	AD 384	+	+	+	+
65.	AD 385	-	+	+	+
66.	AD 386	+	-	-	+
67.	AD 387	+	-	+	-
68.	AD 388	+	-	+	-
69.	AD 389	+	+	+	-
70.	AD 391	+	+	+	+
71.	AD 392	+	-	-	-
72.	AD 393	+	-	+	-
73.	AD 395	+	+	+	+
74.	AD 399	-	+	+	+
75.	AD 400	+	+	+	+
76.	AD 401	+	-	+	+
77.	AD 402	+	+	+	+
78.	AD 403	+	+	+	+
79.	AD 404	+	+	+	+
80.	AD 405	+	+	+	+
81.	AD 407	+	+	+	+
82.	AD 408	+	+	+	+
83.	AD 409	+	+	+	+
84.	AD 410	+	-	+	-
85.	AD 411	+	-	+	+
86.	AD 412	+	+	-	+
87.	AD 413	+	-	+	-

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Presentations

Detection of extended spectrum β-lactamases by the Vitek system

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Abstract

One hundred and one unique patient isolates of *Klebsiella pneumoniae* (69) and *Escherichia coli* (32), flagged as extended spectrum β-lactamase (ESBL) positive by the Vitek system (GNS-526 card), were collected. These strains were isolated from a variety of clinical specimens submitted to the clinical bacteriology laboratories of the Royal Infirmary of Edinburgh (RIE). Of the 101 strains tested, 15 *E. coli* were found to be ESBL-negative by Etest ESBL strips. On re-testing the 15 *E. coli* with Vitek GNS-532 card which had superseded the GNS-526 card, 14 of these were found to be ESBL negative despite originally flagging as ESBL positive. The remaining 87 ESBL producing strains were also subjected to the double disc diffusion (DDD) technique for the detection of ESBLs. Of these, one was falsely negative by Etest ESBL test strips (using both cefotaxime and ceftazidime strips) and 7 were false negatives by the double disc diffusion method. The Etest false negative ESBL producing strain of *K. pneumoniae* was positive by DDD. Technically, the Vitek method was the least demanding method to perform as it is an integral part of the routine susceptibility test card. Etest strips were reliable but the most expensive of all techniques used. The DDD test, while relatively inexpensive was technically subjective and, in our hands, 7 of the ESBL positive strains that were confirmed by the other 2 techniques, were not detected. Despite the false positive ESBL producing *E. coli*, the Vitek susceptibility card with its integral ESBL test offers the clinical laboratory a valuable and affordable option to screen for ESBL producing *Klebsiella* spp. and *E. coli* as part of the routine laboratory methodology.

Introduction

Since the introduction of third generation cephalosporins into clinical practice there has been excessive use of extended-spectrum cephalosporins in hospitals [1,2]. The effectiveness of these β-lactam antibiotics has been diminished by *Klebsiella* spp. that are resistant to their mode of action. This resistance has spread to strains of *E.coli* and to other Gram negative bacteria as well [3]. Investigations into the mechanism responsible for this resistance have revealed the presence of extended spectrum β-lactamase (ESBL) enzymes derived from the ubiquitous TEM-1/2 and SHV-1 family. To date, there are over 80 derivatives of TEM β- lactamases and more than 26 derivatives of SHV β-lactamases [4,5]. These enzymes are usually less efficient at hydrolysis than their parent enzymes and subsequently their detection is difficult to demonstrate in currently used routine susceptibility tests. Therefore, ESBL producing *Klebsiella* spp. and *E.coli* may appear falsely susceptible to newer cephalosporins [6-10]. There is clearly a requirement to detect the resistance mechanism itself rather than to rely on *in vitro* susceptibility testing. The Vitek system (bioMérieux, Ltd., Basingstoke, England) has an ESBL detection test as an integral part of the routine susceptibility test card and this study was performed to test the efficacy of this system in comparison to two other commercial detection methods.

Materials and Methods

Bacterial isolates

One hundred and one clinical isolates of *Klebsiella pneumoniae* (69) and *E.coli* (32) were obtained from various clinical samples sent to the clinical bacteriology laboratories of the Royal Infirmary of Edinburgh (RIE). The 101 isolates had previously been identified and reported as ESBL positive by the Vitek system. Isolates were stored on nutrient agar slopes at room temperature until required.

ESBL detection methods

Vitek system

The Vitek is an automated system for identification or/and susceptibility testing. Vitek susceptibility test results are expressed as MIC values, and interpreted as susceptible, intermediate or resistant by referral to an NCCLS breakpoint[11]. This system is able to detect ESBL production by an automated version of the synergy test, which is based on detection of the inhibitory effect of clavulanic acid on ESBL in the presence of either cefotaxime or ceftazidime. The test is interpreted by measuring the difference in bacterial growth with or without inhibitor. All isolates were originally tested on GNS-526 cards. Only Etest negative strains were re-tested with GNS-532 cards.

E-Test ESBL strips

Etest ESBL strips (AB Biodisk, Solna, Sweden) are double-ended strips with antibiotic and antibiotic/inhibitor gradients. These strips yield the minimum inhibitory concentration as well as the MIC ratio, which determines the presence of an ESBL. The strips used in this study were ceftazidime + clavulanic acid (TZ/TZL) and cefotaxime + clavulanic acid (CT/CTL) where the recommended ratio value indicates the presence of an ESBL. Interpretation of the result was as recommended by the manufacturer.

Double Disc-Diffusion method

This method was used to test the 87 strains that were ESBL positive by Etest and/or Vitek GNS-532 card. The DDD method was 6 discs, 3 containing ceftazidime (30μg), cefotaxime (30μg), or cefpodoxime (10μg), and 3 with a combination of the same antibiotics with the addition of clavulanate with concentrations of 10μg, 10μg and 1μg respectively (Oxoid Ltd., Basingstoke, England). A broth culture of the test organism was adjusted to a 0.5 McFarland standard and inoculated onto Mueller-Hinton agar (Oxoid). The combination discs and the corresponding standard cephalosporins disc were placed the recommended distance from each other on the plate. The plates were incubated at 37°C for 18 hours in air before the zone sizes were recorded. A positive result was indicated by a zone size difference of ≥ 5mm diameter between the combination disc and the corresponding standard antibiotic disc.

For all ESBL detection methods, known ESBL producing *E.coli* (TEM SA1636 and SHV SA1652) were used as positive controls. *E. coli* NCTC 10418 was used as a negative control.

Results

Of the 101 strains tested, 15 were negative for ESBL production by both cefotaxime and ceftazidime Etest strips. These 15 Etest negative strains, (all *E. coli*) were re-tested with Vitek (GNS-532 card) and 14 of these strains were subsequently found to be ESBL negative. One strain still flagged ESBL positive by Vitek. The re-tested 14 ESBL negative, strains were not subjected to any further studies.

The cefotaxime ESBL strip detected the presence of ESBL activity in 84 (96.5%) of the tested isolates. Of these, 19 (22%) demonstrated phantom phenomena ESBL positive as described in the manufacturer's interpretation guide. Four strains (2 *E. coli* and 2 *K. pneumoniae*) were negative for ESBL activity with cefotaxime ESBL strips.

With ceftazidime Etest strips, ESBL activity was detected in 47 (54%) of the study isolates. Of these, 3 (3.5%) were positive by demonstration of phantom phenomena. The use of the combination of both cefotaxime and ceftazidime E-test ESBL strips detected ESBLs in 86 of the test isolates (98.8%). From this data it appears that the use of both strips is essential for optimal detection of ESBL activity. Only one strain of *K. pneumoniae* that demonstrated positive ESBL activity with the Vitek system was not detected by Etest ESBL strips. This strain remained positive when re-tested with Vitek.

Using the DDD method, the combination disc containing cefotaxime detected the presence of ESBL activity in 74 (85%) (64 *K. pneumoniae* and 10 *E.coli*) strains whilst the cefpodoxime combination disc detected ESBL activity in 64 (73.5%) strains (54 *K. pneumoniae* and 10 *E.coli*). The ceftazidime combination disc was the least efficacious, detecting the presence of ESBL activity in only 40 (46%) strains (31 *Klebsiella pneumoniae* and 9 *E.coli*). The strain of *Klebsiella pneumoniae* that demonstrated no ESBL activity by Etest was ESBL positive with the DDD method. When the results of all 3 cephalosporins combination discs were taken into consideration, 80 (92%) of the 87 ESBL positive strains were detected (Table).

Discussion

To date there is currently a great need for reliable and efficient tests to detect ESBLs in clinical isolates of Enterobacteriaceae. Conventional susceptibility testing methods, on their own, fail to present reliable susceptibility results for β-lactam antibiotics when testing those species that harbour ESBLs.

Currently, most UK clinical laboratories do not use a standard method for the detection of ESBLs and many clinical laboratories do not routinely identify enterobacteriaceae to genus and species level. The Vitek system addresses this issue. It will only validate a susceptibility result once the organism has been identified to species level. If the system detects the presence of an ESBL resistance mechanism in strains of *Klebsiella* spp. and *E. coli*, the system then utilises its 'expert' software and applies it to the final susceptibility results. β-lactams susceptible to ESBL activity are then flagged as resistant whether or not the *in vitro* test indicates susceptibility.

This study has shown that the Vitek system, in our hands, whilst easy to perform and without any subjective interpretative of results, reported false positive detection of ESBL activity with fourteen strains of *E. coli*. On re-testing these strains with Vitek (GNS-532 card) they were correctly reported as ESBL negative. All these strains were initially tested with the same batch number of Vitek (GNS-526 card). When re-tested, the GNS-532 card was used, this card has superseded the GNS-526 with accompanying software upgrades. Although internal quality control (QC) is performed on these cards an ESBL producing *E.coli* is not used as part of the QC battery. It was initially thought that the 14 false positive ESBL *E. coli* might have been as a result of a faulty batch of GNS-526 cards. However, recent work in this laboratory, with ten recently isolated 'Vitek' ESBL positive *E. coli* showed two of these ten strains to be ESBL negative by Etest. On retesting with the same batch of Vitek GNS-532, these two strains were subsequently reported as ESBL negative. Although the percentage false positive *E. coli* ESBL strains fell from approximately 50% to 20% with the new card it was still a concern that these strains were misreported as ESBL producers with subsequent 'expert' rules for antibiotic susceptibility to β-lactam antibiotics applied. There appears to be no obvious reason for these results. It is unlikely to be technical error as the Vitek is a highly standardised system. These strains have been forwarded to bioMérieux for further studies.

The DDD test requires careful spacing of discs for accurate results and careful interpretation of zone sizes. It is therefore technically demanding. In previous studies, the DDD test was able to detect 82% and 88% of ESBL positive strains respectively[12,13]. The limitations of this test have been described elsewhere [12,14,15]. A recent study reported that cepodoxime achieved a 100% sensitivity rate in detecting ESBLs in tested isolates, cefotaxime was 92% and ceftazidime 82%[16]. In contrast, this study showed that cefotaxime was the most efficacious cephalosporin for the detection of ESBLs with a sensitivity rate of 85%, 73.5% for cepodoxime and 46% for ceftazidime.

The commercially available ESBL Etest strip is a quantitative technique and is widely regarded as the 'gold standard' for detection of ESBL production in clinical laboratories[14]. In our hands it detected 98.8% of the test isolates but only if both cefotaxime and ceftazidime strips were used in conjunction. This technique is expensive and most clinical laboratories would use it only for confirmation rather than as a routine test. All 87 strains in this study were confirmed ESBL positive by molecular investigations (data not shown). Most clinical laboratories do not have the resources to identify ESBL producing strains by molecular methods, therefore it is important to have an easy to perform methodology that can be used in the routine laboratory. It should be noted, that in our hands, some *E. coli* were reported as falsely positive ESBL production and we would confirm the Vitek ESBL results by Etest for these strains.

This study shows the importance of identification of Enterobacteriaceae to species level and the usefulness of the Vitek system for routine detection of ESBLs if we are to report accurate and consistent results to clinicians. This was clearly demonstrated in a recent report that described the retrospective detection of ESBLs in five of 20 strains of *K. pneumoniae* isolated from blood cultures. In some of these cases patients were treated with an inappropriate third-generation cephalosporin. Treatment in these patients was changed after clinical failure[17]. If an ESBL detection test was available at the original time of susceptibility testing then appropriate results would have reported to the clinician.

In conclusion, the Vitek test was cost effective in so much as the ESBL test is an integral part of the susceptibility card and performed simultaneously with the susceptibility tests. In addition, the test is interpreted by the system, which removes any errors of subjectivity. No further outlay of resources is required.

Table.
Results of Etest ESBL strips and the Double Disc Diffusion method for detecting the presence of ESBLs in 87 strains of *E. coli* and *K. pneumoniae*

[illegible]	Double Disc Diffusion Method No ESBL positive (%)	Etest ESBL strip No ESBL positive (%)
Cefotaxime	74 (85)	84 (96.5)
Ceftazidime	40 (46)	47 (54)
Cefpodoxime	64 (73.5)	
Total % ESBL positive	80 (92)	86 (98.8)

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Epidemiology of ciprofloxacin-resistance and it's relationship to ESBL producing *Klebsiella pneumoniae*

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Abstract

Objective: To examine the epidemiology of ciprofloxacin resistant ESBL-producing *Klebsiella pneumoniae* strains

Methods: Sixty nine unique patient isolates of *Klebsiella pneumoniae* isolated from a variety of clinical specimens submitted to the clinical bacteriology laboratories of The Royal Infirmary of Edinburgh and associated GP practices were identified and susceptibility testing performed with the Vitek system. Strains that flagged as extended spectrum ß-lactamase (ESBL) positive by the Vitek system were subjected to isoelectric focusing.

Results: The results suggested that all 69 isolates harboured at least one ESBL, this was later confirmed by PCR with bla_{SHV} and/or bla_{TEM} primers. The purified PCR product was subjected to automated sequencing and the results compared with the BLAST online search engine. Of the 69 isolates, 32 (46.6%) were found to be resistant to ciprofloxacin, 11 (16%) were intermediate and 26 (37.6%) were sensitive. To investigate the epidemiological relationship between the ciprofloxacin resistant/ESBL positive strains, pulse-field gel electrophoresis (PFGE) was performed. Rapiddest® software was used to calculate the genetic distance by the Nei distance method. PFGE analysis indicated that the clinical isolates belonged to 4 distinct genotype cluster groups (A, B, C and D), each group/ or cluster was homogenous or compact with respect to certain characteristics. Group A consisted of 25 isolates, group B 3 isolates, Groups C and D, 2 isolates each. These results indicate that the spread of resistance is largely as a result of the dissemination of a single clonal strain. PCR was used to amplify the *gyrA* and *parC* genes from genomic DNA of the ciprofloxacin resistant isolates. The amplified product was sent for analysis by automated DNA sequencing and the resulting DNA sequences compared with the *gyrA* gene of *K. pneumoniae*.

Conclusions: These results indicate that the spread of fluoroquinolone resistance is largely as a results of the dissemination of a single clonal strain. The sequencing results demonstrated that alteration of the *GyrA* subunit DNA gyrase at amino acid 83 and/or amino acid 87 was shown to play a central role in conferring high-level quinolone resistance in *K. pneumoniae* possessing ESBLs.

Introduction:

Initially, when ciprofloxacin was first introduced, resistance in *Klebsiella pneumoniae* and other Enertobacteriacese, such as *Escherichia coli*, was virtually unknown. However, in the last 10 years, cases of bactersemia with ciprofloxacin-resistant *E.coli* have increased in number, together with upward trends in the use of quinolones in the community and in hospital[1]. Although the occurrence of ciprofloxacin resistance in *K. pneumoniae* is now well known and indeed, exceeds 5% in many centres in North America, Europe, and Asia [2,3]. At the same time resistance to ciprofloxacin emerged, resistance to ß-lactams antibiotics became prominent. This resistance was as a result of extended-spectrum ß-lactamases (ESBLs), which mediate resistance to newer ß-lactams agents possessing an oxyamino group, such as ceftazidime, ceftriaxone, cefotaxime, and aztreonam. In addition, plasmids that carry and contain genes encoding ESBLs also harbour genes that encode mechanisms of resistance to other classes of antimicrobials. In many regions of the world where antibiotic use is high, ESBLs are present in ~25% of all *K. pneumoniae* from intensive care units, and patient-to-patient transfer of resistant organisms frequently occurs [4].

To our knowledge, the epidemiology of infection with ciprofloxacin-resistant has not previously been described in Edinburgh. This study was performed to determine the relationship between ESBL production and ciprofloxacin resistance in *K. pneumoniae* isolated from the Royal Infirmary of Edinburgh and associated GP practices.

Materials and methods

Sixty nine single patient clinical isolates of *K. pneumoniae* were collected from various clinical samples submitted to the clinical bacteriology laboratories of the Royal Infirmary of Edinburgh and surrounding district. These were stored on nutrient agar slopes until required.

Identification and susceptibility testing

The identity of all 69 isolates was confirmed with Vitek GNI+ cards (BioMérieux Ltd., Basingstoke, England) and ESBL production was screened for with the integrated ESBL screen on the Vitek GNS-532 card. ESBL production further confirmed with cefotaxime and ceftazidime Etest ESBL strips (AB-Biodisk, Solna, Sweden).

Minimum inhibitory concentrations (MICs) for the strains of *K. pneumoniae* to ciprofloxacin was confirmed by the agar dilution method following NCCLS guidelines [5]. Ciprofloxacin powder was provided by Bayer AG, Newbury, UK). Resistance to ciprofloxacin resistance was defined as MIC ≥ 4 µg/ml, and susceptibility as MIC ≤ 1 µg/ml [7].

Isoelectric Focusing

Isoelectric focusing was performed by the method of Matthews et al [8]

Polymerase chain reaction (PCR)

Primers used to amplify the *gyrA* gene had the following nucleotide sequence; *KpnGyrA1* 5'- AAT ATG TTC CAT CAG CCC-3' and *KpnGyrA2* 5'- TGC GAG AGA AAT TAC ACC-3'., and primers used to amplify the *ParC* gene had the following nucleotide sequence *ParC1* 5'-CTG AAT GCC AGC GCC AAA TT -3' and *ParC2* 5'-TGC GGT GGA ATA TCG GTC GC-3'

Restriction fragment length polymorphism (RFLP)

RFLP, using a *HinfI* restriction endonuclease assay (Promega Ltd.) was used to screen for the specific mutation resulting in the substitution of amino-acid threonine 83 in the QRDR of the gyrA region.

DNA sequencing

Amplified PCR product for gyrA was sent for analyse by an automated DNA sequencing system. The resulting DNA sequences were compared with Klebsiella gyrA gene from the Gene Bank of http://www.ncbi.nlm.nih.gov/blast.

Pulse field gel electrophoresis (PFGE)

PFGE was performed using a Bio-Rad CHEF-DR®II apparatus (Bio-Rad, California, USA). *Xba1* (Promega, Ltd) was used as the restriction enzyme.

Outer membrane proteins (OMPs)

Electrophoretic analysis of OMPs by SDS-PAGE was performed in 11% acrylamide-0.35% bisacrylamide-0.1% SDS using Laemmli buffer.

Results:

Of the 69 ESBL producing *K. pneumoniae*, 32 (46.4%) were found to be resistant to ciprofloxacin, 11 (16%) were intermediate and 26 (37.6%) were sensitive.

Thirty two (46.4%) of the ESBL producing *K. pneumonia* were also ciprofloxacin resistant. PFGE analysis indicated that the clinical isolates belonged to 4 distinct genotype cluster groups (A, B, C and D), each group or cluster was homogenous or compact with respect to each other. Group A consisted of 25 isolates, group B 3 isolates, Group C and D 2 isolates. Table (1) shows the results of ß-lactamases analysis and the different PFGE groups for the 32 isolates. Figure (1) shows the PFGE of a representative strain from each of the four PFGE groups.

Table (1) shows the results of IEF and PFGE

PFGE group	pI values	ESBL	Total No.
A	5.4 and 7.6	TEM-1 and SHV-2	21
A	5.4, 7.6 and 8.2	TEM-1 , SHV-2 and SHV-5	4
B	7.6	SHV-2	3
C	7.6	SHV-2	2
D	7.6	SHV-2	2

Figure (1) : Pulsed-field gel electrophoresis of *XbaI* digested genomic DNA from *K. pneumoniae* isolates from Edinburgh.

Lane 1 :AD315 (PFGE group A)
Lane 2 :AD313 (PFGE group B)
Lane 3 :AD380 (PFGE group C)
Lane 4 :AD409 (PFGE group D)
Lane 5 : Lambda DNA ladder marker

Automated analysis of the sequences results for both *GyrA* and *ParC* PCR products are shown in table (2).

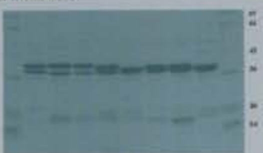
Table (2) Alteration in GyrA and ParC in quinolone resistant clinical isolates of *K. pneumoniae*

[illegible]	[illegible]				[illegible]	[illegible]
	GyrA		ParC			
	83	87	80	84		
[illegible]	TCC ([illegible])	GAC ([illegible])	AGC ([illegible])	GAA ([illegible])	[illegible]	-
*K. pneumoniae**	ACT ([illegible])	GAC ([illegible])	AGC ([illegible])	No change	[illegible]	-
AD315	TTC ([illegible])	TAC ([illegible])	ATC ([illegible])	No change	[illegible]	A
AD313	ATT ([illegible])	No change	ATC ([illegible])	No change	[illegible]	B
AD380	ATT ([illegible])	No change	ATC ([illegible])	No change	[illegible]	C
AD409	ATT ([illegible])	No change	ATC ([illegible])	No change	[illegible]	[illegible]

* [illegible]

SDS-PAGE analysis of the OMPs showed that, the majority of the clinical isolates expressed two major OMPs of about 35KDa and 36KDa. However, some of the isolates were deficient in the 36KDa OMP, these mainly belonged to group A PFGE and harboured TEM-1 , SHV-2 and SHV-5. Figure (2).

Figure (2) Outer membrane protein Sodium SDS page electrophoresis-profile of outer membrane proteins of *K. pneumoniae* isolates, shows three major outer membrane proteins with approximate molecular sizes of 41, 36 and 35 KDa.



Lane 1 & 10 Lambda molecular size marker in Kda.
Lane 2 : *K. pneumoniae* ESBL positive, ciprofloxacin sensitive strain (control).
Lane 3 :AD 315 (lose of 41 KDa. OMP)
Lane 4 :AD 320 (lose of 41 KDa. OMP)
Lane 5 :AD 327 (lose of 41 KDa. OMP)
Lane 6 :AD 313 (lose of 36 KDa. OMP)
Lane 7 :AD 339 (lose of 36 KDa. OMP)
Lane 8 :AD 380 (lose of 36 KDa. OMP)
Lane 9 :AD 409 (lose of 36 KDa. OMP)

Discussion

This work clearly demonstrates that ESBL production in *K. pneumoniae* is closely associated with ciprofloxacin resistance. ESBL-producing *K. pneumoniae* are often resistant to other classes of antimicrobials. This molecular epidemiological study of ciprofloxacin-resistant ESBLs-producing *K. pneumoniae* showed genotypically identical isolates within one large group of 25 strains (78%). The patterns obtained by Rapiddist was similar to those analysed manually using the criteria of Tenover and Arbeit [9].

Ciprofloxacin resistance in *K. pneumoniae* is predominantly due to a chromosomal mutation in the gene *gyrA*, which codes for the target of quinolone activity [10]. RFLP, using a HinfI restriction endonuclease assay was used to screen for specific mutation in the substitution of amino-acid serine 83 in the QRDR of the gyrA region. This mutation is known to confer quinolone resistance and results in the loss of the HinfI G/ANTC restriction site. RFLP using HinfI, can be useful to distinguish between *K. pneumoniae* serine 83, and *K. pneumoniae* threonine 83, which cuts only the gyrA product of the first species (*K. pneumoniae* serine 83). DNA sequencing demonstrated a point mutation at codon 83, located in the QRDR region. In *K. pneumoniae*, two main porins have been characterised ; OMP K35 (the homology of OMP F) and OMP K36 (the homology of OMP C) [11]. Recently, loss of the OMP K36 porin has been associated with both cefoxitin resistance and increase in cephalosporins and quinolone MICs [12].

The OMP results in this study demonstrated that, some of the isolates had lost one porin that might be 41 KDa. The same conclusion was reported in a study by Takash *et al.* in 1997 [13]. Nevertheless, other isolates that belonged to PFGE group A showed the loss of one porin (K36 KDa). The same finding has been suggested in another study [14]. However, the exact nature of the protein has not been established, and the assumption that the involved proteins were porins were largely based on their molecular masses[15].
Results from this study confirm previous observations 16indicating that resistance to extended-spectrum cephalosporins increases in *K. pneumoniae* strains that produce SHV-type ESBLs that lack the two major porins of their species.

Treatment of infection caused by *K. pneumonia* producing ESBL is difficult not only because of their resistance to expanded-spectrum cephalosporins, but also because of their often concomitant resistance to other antimicrobial agents encoded by the same or different plasmid. Ciprofloxacin resistance and ESBLs production in members of Enterobacteriaceae are geographically widespread [4]. Continued world-wide surveillance of *K. pneumonia* isolates is necessary to provide information on the dissemination of these important pathogens.

Reference List

Abstract of the 42 Interscience conferences on Antimicrobial Agent and Chemotherapy (ICAAC), San Diego, USA (2002)

SHV-39: a new extended –spectrum β-lactamase found throughout Chile

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Klebsiella pneumoniae is a widespread nosocomial pathogen within Chilean hospitals. It has largely been treated with ceftriaxone and ceftazidime and resistance has increased markedly.

K. pneumoniae strains were isolated from hospitalised patients from Catholic University hospital in Santiago, the Gustavo Fricke hospital in Valparaiso (100 miles to the east) and the G. Grant-Benavente hospital in Concepcion (300 miles to the south). The minimum inhibitory concentration of antibiotics was determined by agar dilution and all strains were genotyped by pulsed-field gel electrophoreses (PFGE).

The number of β-lactamase was determined by Iso-electric focusing. Although there were some variations in genotype, within each hospital there was consistency of banding pattern (PFGE). However, there was no similarity between strains isolated in the different cities showing they were different clones. Examination of the β-Lactamase showed that TEM-1 was always present; however, the cephalosporin resistance was associated with an SHV-derived β-lactamase with pI greater than 7.0. PCR amplification and sequencing revealed a hitherto undescribed enzyme with not just a **Gly238Ser** mutation, characteristic of SHV-2, but also a **Thr267Ser** mutation. This extended-spectrum β-lactamase (ESBL) conferred MICs of ceftazidime up to greater than 256 mg/L and of cefotaxime between 16 and 32 mg/L.

These results show that there is a new ESBL, now called SHV-39 that is widely disseminated in Chile both geographically and between different strains of *K. pneumoniae*.