Characterisation of β-Lactamases Implicated in Resistance to β-Lactam Antibiotics in Urinary Tract Infections.

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Submitted to the Department of Medical Microbiology, Faculty of Health Sciences, University of the Free State

In fulfilment of the requirement for the degree
Master of Medical Science (M.Med.Sc.)

November 29, 2005

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ABBREVIATIONS

AMC – Amoxicillin/clavulanic acid

AMP – Ampicillin

AMX – Amoxicillin

ATCC – American Type Culture Collection

ATM - Aztreonam

bp	-	Base pair
CAC	–	Ceftazidime/ clavulanic acid
CAZ	–	Ceftazidime
CFU	–	Colony Forming Unit
cm	–	Centimetre
CRC	–	Ceftriaxone/ clavulanic acid
CRX	–	Ceftriaxone
CTC	–	Cefotaxime/ clavulanic acid
CTN	–	Cephalotin
CTR	–	Ceftriaxone
CTX	–	Cefotaxime
CXC	–	Cefuroxime/ clavulanic acid
CXM	–	Cefuroxime
DNA	–	Deoxyribonucleic acid
EDTA	–	Ethylenediaminetetracetic acid
Etest	–	Epsilomer test
ESBL	–	Extended spectrum ß-lactamase
FOX	–	Cefoxitin
GNB	-	Gram negative bacilli
g	–	Gravitational force
Hz	–	Hertz
I	–	Intermediate
IEF	–	Isoelectric focussing
IPDD	–	Inhibitor potentiated disc diffusion
kb	–	Kilobase
K_m	–	Michaelis Menten constant
lb/in^2	–	pounds per square inch
mA	-	milliamps
MI	–	Michigan
MIC	–	Minimum Inhibitory concentration
MH	–	*Mueller Hinton*
min	–	Minute
MLS	-	Medical Laboratory Science
mm	–	Millimetre
MWM	–	Molecular Weight Marker
n	–	Number
nm	–	Nanometer
NCCLS	-	National Committee for Clinical Laboratory

		Standards
NLF	–	Non-Lactose Fermentor
OMPs	–	Outer membrane proteins
PBPs	–	Penicillin binding proteins
PCR	–	Polymerase Chain Reaction
Pen G	–	Penicillin G
pH	–	Hydrogen ion concentration
pI	–	Isoelectric point
PIP	–	Piperacillin
PIT	–	Piperacillin/ tazobactam
R	–	Resistant
®	-	Registered
rpm	–	Rotation per minute
s	-	Second
S	-	Sensitive
SHV	–	Sulfhydryl variable
TEM	–	Temoniera
™	-	Trademark
UT	–	Urinary tract
UTI	–	Urinary tract infection
UV	–	Ultra violet
V_{max}	-	Maximum velocity
V	–	Volts
W	–	Watts
γ	-	Lambda
µg	-	Microgram
µg/ml	–	Microgram per millimetre
µl	–	Microlitre

Acknowledgements

Father God you are worthy to be praised and adored, for providing us with Dr. M.M.Theron.

Riki, I thank you, from the bottom of my heart, for turning the whole concept into a study that will benefit Lesotho; for helpful suggestions, comments and support, even through the birth of Seboloka; and excellent technical assistance after the study leave had expired. The Lord bless you richly.

One is grateful to the staff (scientific, administrative and support) at the Microbiology Department of the N.H.L.S, Bloemfontein, that were instrumental in providing study and reference isolates and love all these years.

We thank SmithKline Beecham for generous supply of clavulanic acid.

The following persons are recognised for their past and ongoing support: SRK, Tabane, Mokholoane, Seboloka and 'Malobiane:- I thank you for your tolerance during the challenge. Bo 'Me' ba Basotho at UOFS: 'Mapheello, 'Maseoehla, Tjoetso, Mpho, Lebohang, 'Masechaba, Mats'eliso, 'Mats'olo and Teboho – for accommodation and meals. You never indicated that I was a nuisance.

Thank you N.L.C congregation, H.C.F family, M.L.S staff at N.H.T.C, Moafrika staff – for spiritual and moral support.

Nkhono 'Matsietsi and 'Me' 'Marefiloe – thank you for being overseers of Ramainoane's family. Ke bone botsoali.

The study was supported in part by the National Manpower Development Secretariat, Maseru.

Abstract

South Africa is not excluded from the problems encountered world-wide in the treatment of nosocomial urinary tract infections, commonly caused by enzyme-producing *Enterobacteriaceae*. These enzymes include the ß-lactamases and extended-spectrum ß-lactamases (ESBLs) capable of

hydrolysing the ß-lactam agents and in particular the expanded-spectrum cephalosporins frequently used. The study was designed to determine the role of ß-lactamases in resistance development in commonly encountered pathogens implicated in urinary tract infections and to characterise the enzymes involved. Resistance to the ß-lactam agents amoxicillin, ceftriaxone, ceftriaxone, piperacillin and cefoxitin was suspected to involve the presence of one or more β-lactamases in the isolates from Bloemfontein hospitals. Diverse and complex β-lactamases were identified and ESBLs were detected in 80% of the isolates. These β-lactamases were characterised by isoelectric focusing (IEF) and genetic analysis (DNA amplification by PCR) to investigate the presence of possible genes responsible for resistance development. The production of *bla*$_{TEM}$ and *bla*$_{SHV}$ type genes was demonstrated. Isolates harbouring these genes were highly resistant to amoxicillin and piperacillin, with MIC_{90}s of >128µg/ml. Resistance to these antibiotics was shown to be readily transferred between strains and there was an indication that the resistance genes are carried on plasmids and was transferred by conjugation. A plasmid of 9-10 kb was detected in 83% of the isolates and could be one of the mechanisms implicated in the transfer of ESBLs in uropathogenic bacteria. ß-Lactam resistance could be attributed to the presence and action of ß-lactamases such as the TEM and SHV type enzymes and this resistance can be transmitted between bacteria, causing problems specifically in the hospital environment. Further and continuous investigations are required to find a solution for this ever increasing problem.

CHAPTER 1

INTRODUCTION

1.1 Urinary tract infections

Urinary tract infections (UTIs) are among the most common syndromes seen by physicians in the community and hospital setting affecting children and adults. The term urinary tract infection encompasses a broad range of clinical entities that are associated with a common finding of a significant amount of bacteria in urine and a positive urine culture (Dusé & Klugman, 1993). The infection may be community acquired or nosocomial, often as a consequence of urethral catheterization and is more prevalent in women (Neu, 1992; Bannister, Begg & Gillespie, 2000; Hooton, 2001).

Symptoms of UTI depend on whether the infection is in the lower UT (urethritis and cystitis) or in the upper UT (acute non-obstructive pyelonephritis) and are characterized by a rapid onset of dysuria, urgency and frequent micturation. Severe infection may result in loss of renal function and serious long-term sequelae (Mims *et al.,* 1993). Although the majority of infections are acute and short-lived, they may contribute to bacteremia, with consequent morbidity and mortality. A variety of microorganisms have been implicated in UTIs. Most Gram-negative bacilli that cause UTI originate in the colon, contaminate the

urethra, ascend into the bladder and most of the time migrate, to the kidney or prostrate. Although the exact pathogenesis of UTI and host predispositions are not clearly understood (Murray *et al*., 1998), factors potentially contributing to the development of UTI include anatomic abnormalities, metabolic factors, hospitalization, gender and genetic factors in women (Dusé & Klugman, 1993). Populations at risk include the elderly, young adults, children and the newborn (Richmond, 1981; Sanders *et al*., 1988; Neu, 1992).

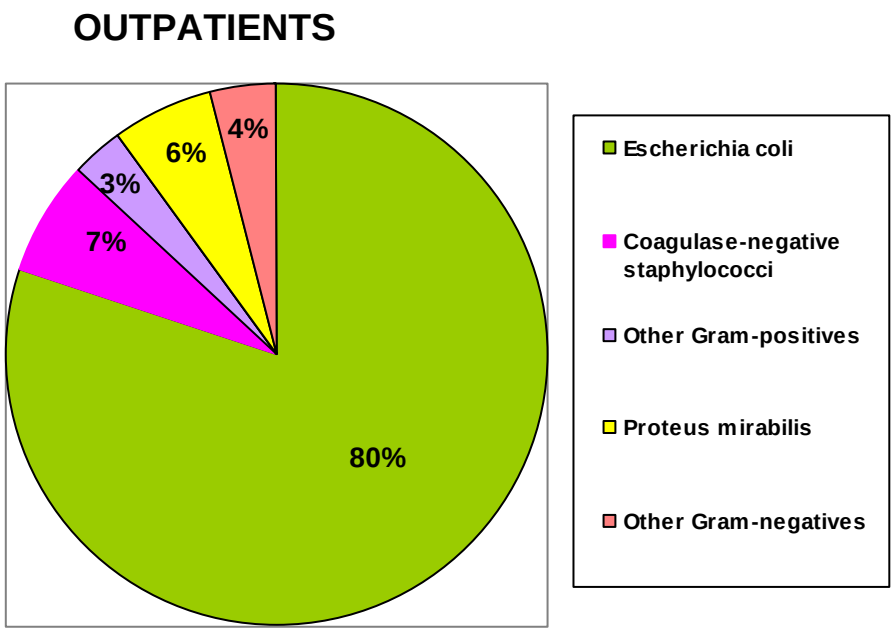
1.1.1 Aetiology of UTIs

Enterobacteriaceae are endogenous to the gastrointestinal tract and are often implicated in ± 80% of all UTIs. In Figure 1.1 it can be seen that *Escherichia coli* causes 50% - 80% of cystitis cases and community acquired UTIs (Sanders *et al*., 1988; Finkelstein *et al*., 1998) while *Klebsiella pneumoniae* is responsible for another 8% to 13%. *Staphylococcus saprophyticus* cause between 5% and 20% (Neu, 1997) and together with group D Streptococci (Enterococci) are the only Gram positive enteric bacteria that commonly cause UTIs (Rebuck *et al*., 2000). In hospitalised patients, the most common causative agents of UTI include opportunistic bacteria *Proteus*, *Serratia* and *Pseudomonas*, and are also known to be resistant to most common antibacterial agents (Burton, *sine annum*; Virella, 1997).

Infrequent causative agents of UTIs include bacteria such as *Staphylococcus aureus*, *Gardnerella vaginalis*, *Corynebacterium* and *Lactobacilli*, yeasts such as *Candida* (in diabetics or patients with indwelling urinary catheters) and viruses such as Adenovirus type 2 (associated with acute haemorraghic cystitis in children) (Dusé & Klugman, 1993). Non-specific urethritis is frequently caused by sepsis of *Chlamydia ureaplasma* and *Mycoplasma*, mainly introduced by sexual contact. Gonococci may also invade the urinary tract as well as the reproductive system, by entering the bladder via the urethra with an interim phase or periurethral and distal urethral colonization (Hooton, 2000).

1.1.2 Clinical manifestations of UTIs

Common symptoms of urethritis are burning or stinging at the meatus, causing marked frequency of micturation, with associated discomfort or pain (Hooton, 2000). Characteristic symptoms of cystitis include suprapubic aching and tenderness (Bannister *et al*., 2000). Acute pyelonephritis is recognised by loin pain with tenderness. Fever and chills are common (Virella, 1997). Many urinary infections can, however, occur without specific symptoms (Sanders *et al*., 1988).



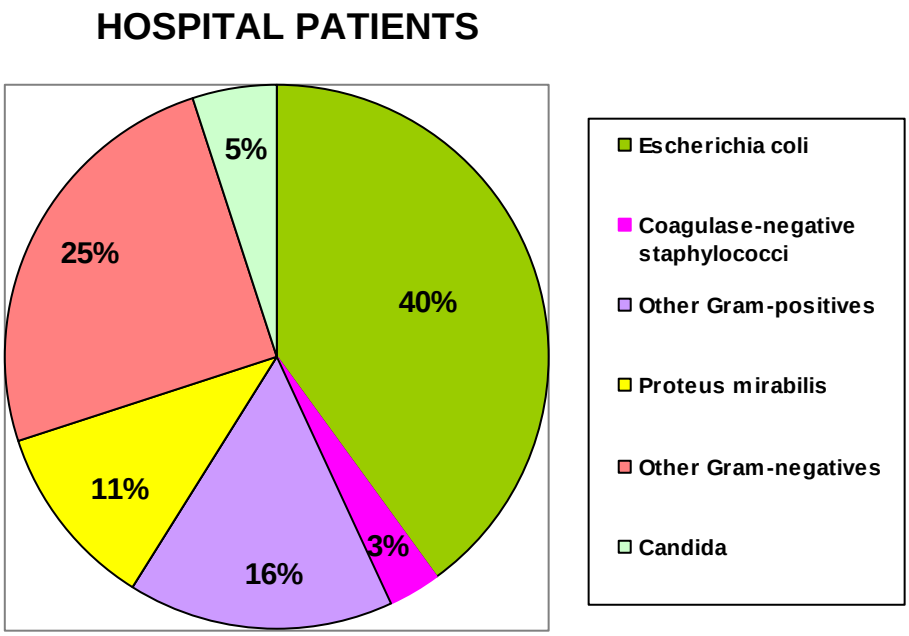


Figure 1.1 Common causes of urinary tract infections. The percentage of infections caused by different bacteria in outpatients compared with hospital inpatients is shown. *Escherichia coli* is by far the most common isolate in both groups of patients.

1.2 Pathogenic bacteria implicated

1.2.1 *Escherichia coli*

E. coli can remain in the urinary tract by adhering to the epithelial receptor cells lining the UT and are able to avoid elimination by the flushing action of voided urine. They possess various virulence factors such as (1) fimbriae or pilli (2) haemolysin (3) aerobactin and (4) exotoxins and other features. Fimbriae or pilli are highly specialized surface glycoprotein projections, that serve as ligands for glycoprotein and glycolipid receptors on uro-epithelial cells in the bladder, urethra and the vagina (Bannister *et al*., 2000). Haemolysin degrades renal tubular red cells (Dusé & Klugman, 1993; McClane & Mietzner, 1999). Aerobactin is a sidophore that enhances iron acquisition and chelation (Dusé & Klugman, 1993; McClane & Mietzner, 1999; Hooton, 2000). Other virulence features include the capsular acid polysaccharide antigen that appear, together with exotoxins, to assist in localization of organisms in the kidney to cause renal damage and are associated with pyelonephritis and inhibition of phagocytosis (Jarlier *et al*., 1988; McClane & Mietzner, 1999). Although most *E. coli* can cause UTIs, the disease is most commonly associated with specific serotypes other than those associated with gastrointestinal tract (Mims *et al*., 1993).

1.2.2 *Klebsiella pneumoniae*

K. pneumoniae are opportunistic pathogens frequently associated with community acquired infections such as primary lobar pneumonia in immunocompromised and other high risk patient groups and 8-13% of cases of UTIs are attributed to infection by *K. pneumoniae* (Murray *et al*., 1998). It is also known to be a major cause of nosocomial infections and infections of wound and soft tissues. (Vaara & Vaara, 1983; Murray *et al*., 1998). Alcoholics, people with compromised pulmonary function, and other patients in high-risk groups (patients with central venous and urinary tract catheters, patients on mechanical ventilation, etc.) are also at risk of being infected with *K. pneumoniae* (Jarlier *et al*., 1988; Weiner *et al*., 1999; Rebuck *et al*., 2000; Lautenbach *et al*., 2001).

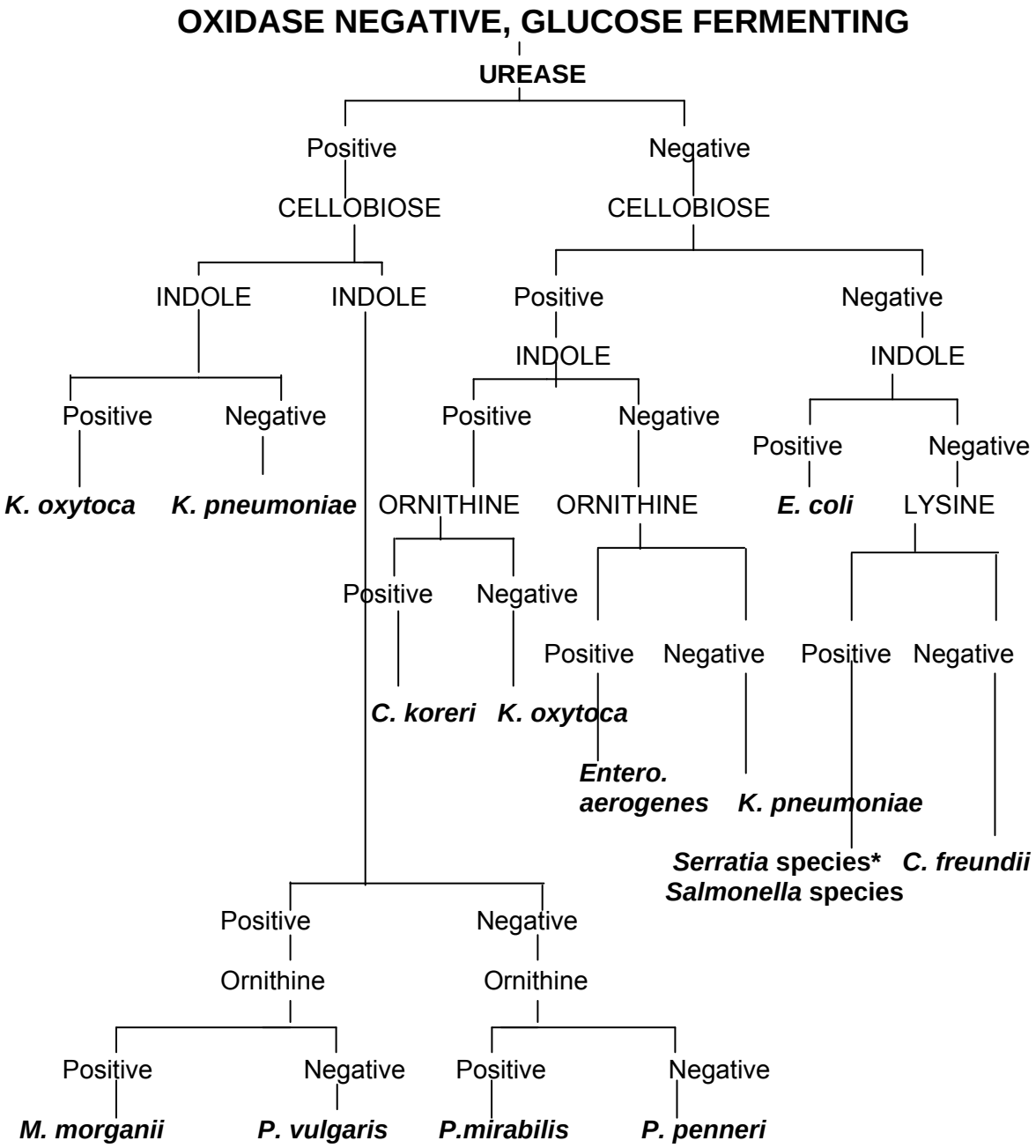
Several factors contribute to the virulence of *K. pneumoniae*: (1) the presence of cell wall receptors enable the organism to attach to host cells and protect the bacteria from phagocytosis and intracellular killing by polymorphonuclear leukocytes, (2) an extensive polysaccharide capsule (K antigen) protects the bacterial cell from phagocytosis and directly suppresses the immune response (Mims *et al*., 1993; Hooton, 2000), (3) production of long chain-O-antigen polysaccharide in the endotoxin of the outer membrane that may contribute to resistance by inhibiting complement-mediated serum killing, and (4) the possession of a large plasmid (180 kb) that encodes aerobactin (a protein involved in iron acquisition and regulation of the mucoid phenotype).

1.3 Isolation and identification of UTIs

The urinary tract as well as the urine secreted in the kidneys and the bladder is bacteriologically sterile. However, the urethral meatus and surrounding perineum are colonized with a mixture of skin and bowel flora so that normal voided urine may contain small numbers of bacteria (McClane & Mietzner, 1999; Bannister *et al*., 2000). A number of screening techniques have been developed for the rapid detection of UTI with mid-stream urine as the most suitable specimen. These techniques involve application of dipsticks to detect glucose, blood, protein, nitrite and leucocyte esterase. Other tests include semi-quantitative bacterial culture, where infection is usually indicated if colony counts $>10^8$/L. The diagnosis of a UTI is confirmed by demonstrating the presence of an etiologic agent, which usually is a bacterium in the urine (Virella, 1997). The purity of the culture is also important as a mixed culture is likely to indicate a contaminated specimen.

For most clinical situations, simple or presumptive identification is sufficient, e.g. a lactose fermenting organism capable of indole production with a characteristic colonial morphology, may be labeled a coliform. Checkerboard matrices may also provide simple presumptive and accurate species identification (Collee *et al*., 1996). Commercial identification systems, semi-automated and automated, produce a species or genus identification by use of a computer database (Bannister *et al*., 2000). An illustration of flow chart method for manual identification of genera and species of *Enterobacteriaceae* is given in Figure 1.2 (Adapted from Collee *et al*., 1996).

FIGURE 1.2: Flow chart showing the LOGIC system for identification of Gram negative bacilli isolated from urine (Adapted from Collee *et al*., 1996).



The LOGIC scheme includes tests for lysine and ornithine decarboxylation, indole production, glucose and cellobiose fermentation and urease production.

(*) Salmonella and Serratia species are distinguished by additional biochemical and serological tests.

1.4 Recommended treatment regimens of UTIs

Treatment of uncomplicated UTIs have in the past included aminopenicillins (amoxicillin, ampicillin), first generation cephalosporins (cephalexin), second generation cephalosporins (ceftibuten), third generation cephalosporins, sulfamethaxazole-trimethoprim combinations (cotrimoxazole), amoxicillin/ clavulanic acid (augmentin), and recently, the flouroquinolones such as ciprofloxacin (Dusé & Klugman, 1993; Hindler *et al*., 1994; Abdel-Rahman & Kearns, 1998; Bannister *et al*., 2000).

1.4.1 ß-Lactam antibiotics

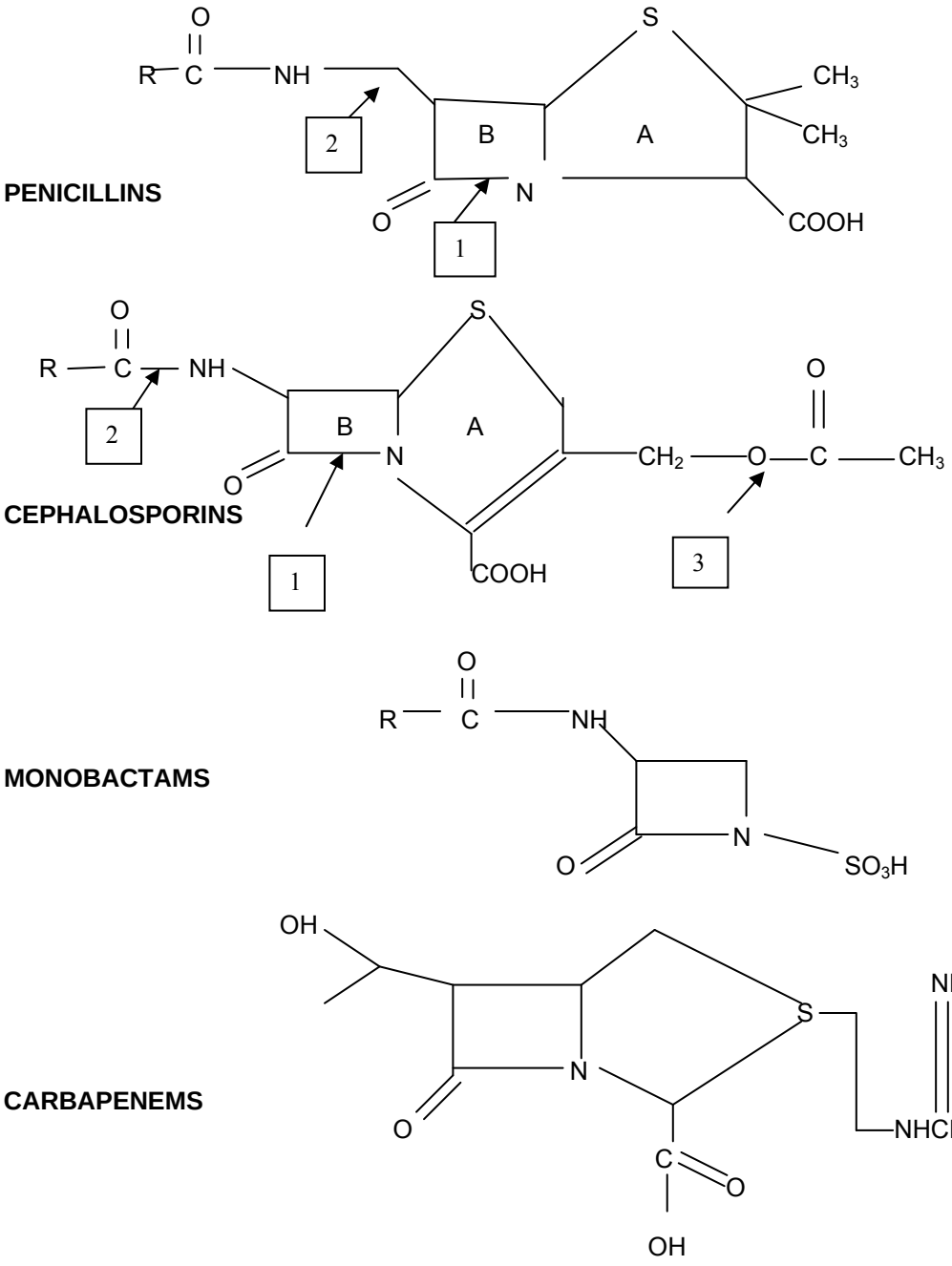
β-Lactam antibiotics form a large group of different compounds containing a β-lactam nucleus. Different groups within the family are distinguished by the structure of the ring and the side chain attached to the β-lactam nucleus (Figure 1.3). Penicillins differ from cephalosporins in that penicillins contain a 5-membered thiazolidine ring complex and cephalosporins contain a 6-membered dihydrothiazine ß-lactam ring complex (Dever & Dermody, 1991) (Figure 1.3). Since the discovery of β-lactam antibiotics in 1928 by Alexander Fleming (Rolinson, 1998), developments have led to the synthesis of semi-synthetic compounds that can be divided into bicyclic penicillins, cephalosporins, monocyclic monobactams, and β-lactamase inhibitor combinations (Hamilton-Miller, 1999) (Figure 1.3). β-Lactam antibiotics are commonly prescribed and

have a wide spectrum clinical use because of low toxicity and strong bactericidal activity (Gruneberg, 1994; Petrosino *et al*., 1998). They can be used in higher dosages for treatment of more severe infections and UTIs (Sanders & Sanders, 1992).

The primary targets for β-lactam antibiotics are the penicillin-binding proteins (PBPs), consisting of transpeptidases and carboxypeptidases responsible for creating cross-linkages between peptide chains. Following penetration of the bacterial cell surface, β-lactam antibiotics attach to the PBPs, to form a β-lactam-PBP complex. Catalytic activity is lost and cell wall synthesis and division interrupted (Richmond, 1981; Essack, Alexander & Pillay, 1994; Prober, 1998; Wright, 1999).

The anti-bacterial effect of all β-lactam antibiotics depends on the capacity of the antibiotic to diffuse through the cell membrane of the bacterial cell, the affinity of the antibiotic for its target proteins (the PBPs anchored in the cytoplasmic membrane of the bacterium) and the stability of the antibiotic against bacterial degradation complex system (Dever & Dermody, 1991; Pitout *et al*., 1997). β-Lactam antibiotics inhibit the cross linking (final stage) of peptidoglycan or murein synthesis of actively dividing bacterial cells (Figure 1.4) (Gould & Mackenzie, 1997).

FIGURE 1.3: Structure of β-lactam antibiotics with sites for enzymatic degradation of penicillins/cephalosporins.

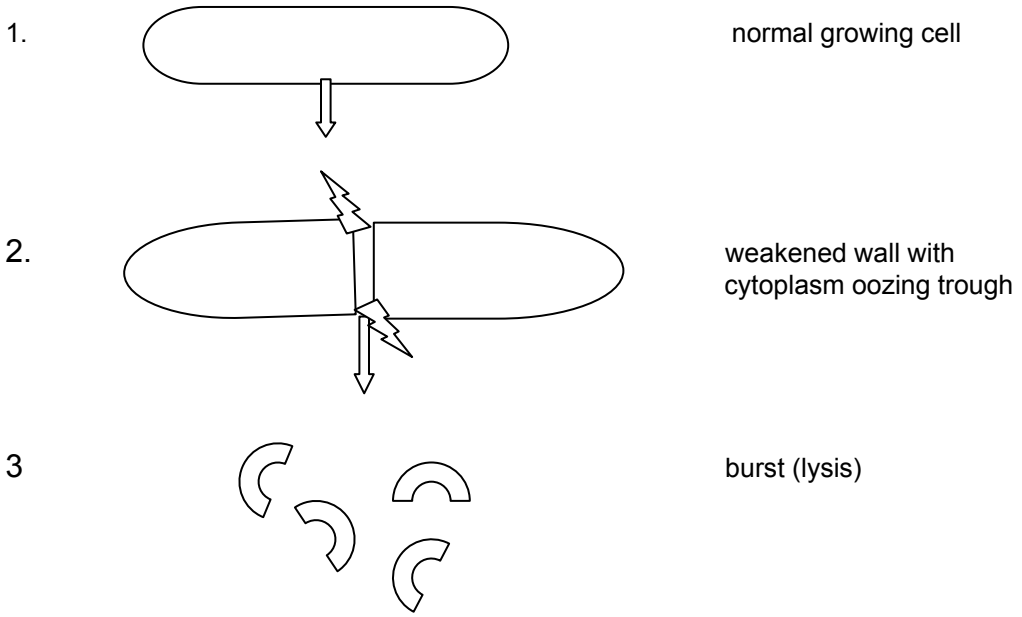


A = Classical penam and cepham ring respectively [also known as thiazolidine ring in basic structure], B = Four-membered β-lactam ring [azetidinone], shared by all compounds; it remains unmodified (Hamilton-Miller, 1999). R = Acyl side chain, varies according to specific agent..
1 = β-lactamases, 2 = acylases, 3 =3 = esterases.

Three PBPs of *E. coli* have been shown to be targeted by ß-lactam agents: PBP-1, PBP-2 and PBP-3. Inhibition of PBP-1A and 1B is associated with rapid killing and lysis of bacterial cell, inhibition of PBP-2 results in spherical cell wall deficient non-growing cells, and inhibition of PBP-3 will cause lysis as well as filamentous changes (Bryan & Godfrey, 1991; Georgepapadakou, 1993; Katsanis *et al*., 1994; Gould & Mackenzie, 1997).

β-Lactam antibiotics can also inhibit bacterial growth by mechanisms that do not solely involve the inhibition of cell wall synthesis. Inhibition of the formation of cell wall precursor by these agents can result in autolysis through the unsuppressed activity of murein hydrolases (Dever & Dermody, 1991). Murein hydrolases are autolytic enzymes that cause nicks in the cell wall to provide sites for new peptidoglycan synthesis during cell wall enlargement. The inhibition of cell wall synthesis by β-lactam antibiotics does not alter the activity of these enzymes, therefore, bacterial autolysis can result from the effects of osmotic pressure on the cell wall damaged by murein hydrolases (Wright, 1999).

FIGURE 1.4: The consequences of the interruption of peptidoglycan synthesis, such is caused by penicillins and cephalosporins (Richmond, 1981).



1. Normal rod-shaped bacterium.
2. Inhibition of peptidoglycan biosynthesis leads to a weakening of the cell walls. This is often first seen at the point where the next division furrow would become apparent were growth to continue normally.
3. The cell bursts.

Introduced in the 1960s cephalosporins are widely used routinely in many pre-operative procedures, because of their broad-spectrum effectiveness and low toxicity (SAML supplement). Cephalosporins are classified according to the route of administration and their *in vitro* anti-bacterial spectra (Bannister *et al*., 2000). Currently there are four generations of agents, each succeeding a generation possessing a greater spectrum of activity. The antibacterial activity of cephalosporins depends on their ability to penetrate the bacterial cell wall, resist inactivation by β-lactamases and bind to and inactivate PBPs. Resistance can, however, develop at each of these steps (Prober, 1998).

Expanded-spectrum cephalosporins were specifically designed and introduced into clinical practice in the early 1980s, being resistant to hydrolysis by the older broad-spectum β-lactamases commonly encountered at that time, i.e. β-lactamases such as TEM-1, TEM-2 and SHV-1 (Pitout *et al*., 1998). Some of the well-known agents include cefotaxime, ceftazidime and ceftriaxone (Heritage *et al*., 1999).

1.5 Antibiotic resistance

Resistance to antibiotics has evolved due to misuse in clinical treatment (Nandivada & Amyes, 1990; Chaibi *et al*., 1999; Essack *et al*., 2001). This resistance now represents a serious threat to effective treatment. Resistance may be defined as the ability of a micro- organism to resist the action of antimicrobial agents at concentrations achievable in the body after normal dosage (Mims *et al*, 1993).

Resistance to β-lactam antibiotics in Gram-negative bacteria can arise in different ways:

(i) Production of β-lactam antibiotic inactivating enzymes excreted in the periplasmic space (Silva *et al*., 1999; Wright, 1999),

(ii) Modifications/alterations in the normal target PBPs or enzymes, preventing or reducing inhibition of cell wall synthesis or metabolic pathways (Baker, 1999; Silva *et al*., 1999),

(iii) Reduced permeability (loss of certain porin proteins) through alterations in the bacterial wall pores that may prevent the attainment of effective periplasmic β-lactam antibiotic concentration (Hindler, Howard & Keiser, 1994), (iv) ability to pump out β-lactam antibiotics (Dever & Dermody, 1991; Baker, 1999; Menashe *et al*., 2001),

(v) Tolerance (Turnridge, 1998),

(vi) Combination of two or more of these mechanisms (Hindler, Howard & Keiser, 1994; Sougakoff & Jarlier, 2000).

1.5.1. β-Lactamases

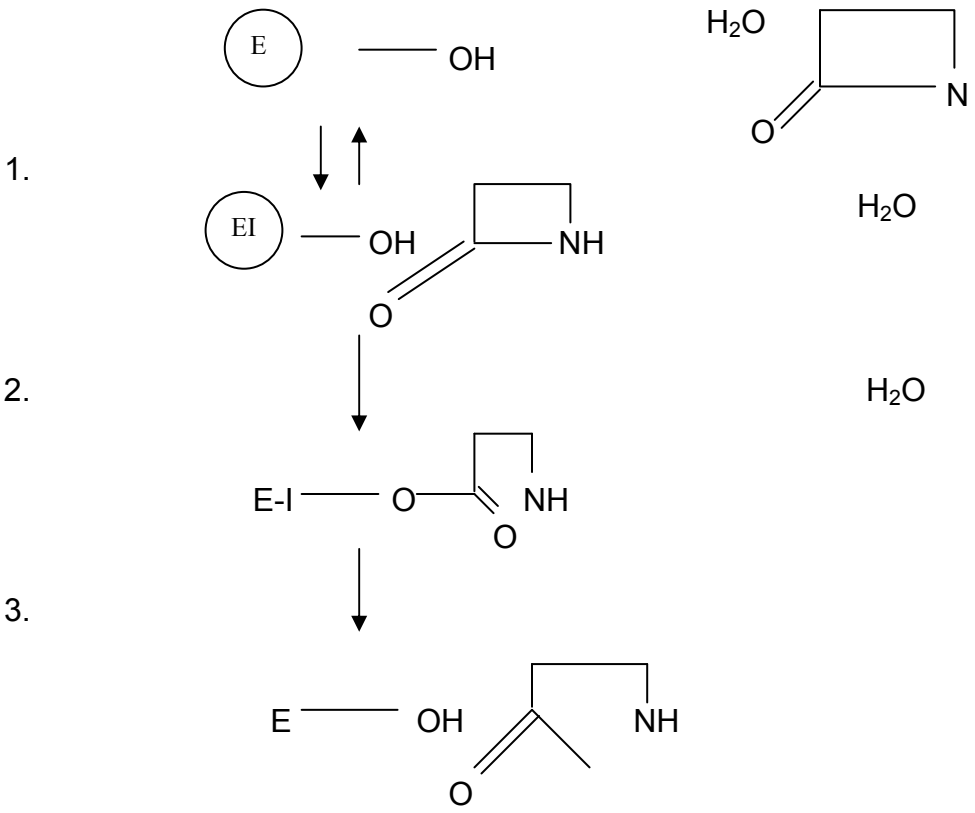
The most important and widespread mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria is due to enzyme mediated antibiotic degradation (Neu, 1992). Three classes of enzymes that can hydrolyse β-lactam antibiotics are (1), β-lactamases (2) acylases, and (3) esterases (Dever & Dermody, 1991). The enzyme hydrolyses the β-lactam antibiotic to acidic derivatives without antibacterial properties (Ho *et al.,* 1998).

β-Lactamase production is mediated by genes carried on a plasmid or on the chromosome and more than one type may be produced by the same species at the same or different times (Hindler, Howard & Keiser, 1994). Chromosomal β-lactamases can be constantly produced (constitutive β-lactamases of *E. coli*, *Shigella* species, *Proteus mirabilis*) or only in the presence of β-lactam antibiotics (inducible β-lactamases of *Pseudomonas aeruginosa*, *Enterobacter* species, *Citrobacter* species, *Serratia* species, *Morganella* species and *Providencia rettgeri*). Cefoxitin, imipemen and first generation cephalosporins are potent inducers of chromosomal β-lactamases (Gorbach *et al*., 1997; Prober, 1998). The expression of chromosomally mediated β-lactamases is usually not constitutive, but can be induced or derepressed by exposure to β-lactam antibiotics (Dever & Dermody, 1991).

(i) Structure and regulation of β-lactamases

The first of these ß-lactamases was recognised by Abraham and Chain in 1940 (Bush, 1989). Several different schemes have been proposed to classify this large family of enzymes (Medeiros, 1984; Bonafede & Rice, 1997; Heritage *et al*., 1999) and a review by Bush (2001) reported that more than 190 unique enzymes have been described. Most of the β-lactamases function via serine ester hydrolysis mechanisms, as illustrated in Figure 1.5 (Livermore, 1993; Livermore 1995; Livermore 1998). The –OH group shown in the enzyme structure is on the side chain of the active serine. Phases of reaction are (1) reversible non-covalent binding of the β-lactamase to the β-lactam ring; (2) rupture of the β-lactam ring, which becomes covalently acylated on to the active site serine; and (3) hydrolysis of the acyl enzyme to reactivate the β-lactamase and liberate the inactivated drug molecule. This results in the loss of antibacterial activity of β-lactam agents. β-Lactamases are grouped into four molecular classes, based on their primary sequence homology. Three of these classes (A, C, and D) are serine active-site enzymes and one class (class B) is comprised of zinc-dependant (Ethylene Diamine Tetra Acetic acid inhibited) enzymes, better known as metallo-ß-lactamases (Philippon *et al.*, 1989; Livermore, 1996; Bush, 2001).

FIGURE 1.5: Action of a Serine β-lactamases.



1. Reversible non-covalent binding of the β-lactamase to the β-lactam ring,
2. Rupture of the β-lactam ring, which becomes covalently acylated on to the active site serine,
3. Hydrolysis of the acyl enzyme to reactivate the β-lactamase and liberate the inactivated drug molecule.

Stability to hydrolysis arises if the presence of bulky side chain prevents the β-lactam approaching the active serine or if a side chain displaces the water molecule from the active site. Inhibitors may simply yield acyl enzymes that fail to hydrolyse or may fragment after attachment to the active serine. Class B β-lactamases function differently, using a zinc ion to attack the β-lactam ring.

(ii) β-Lactamases implicated in resistance to β-lactam antibiotics used in urinary tract infections

TEM-1, TEM-2 and SHV-1 are widespread enzymes that attack the narrow-spectrum cephalosporins, cefamandole and cefoperazone and most of the penicillins. The name "TEM" is a contraction of Temoniera, the name of the patient from whom resistant bacteria were isolated, whereas SHV is a contraction of sulfhydryl variable; a description of the biochemical properties of this β-lactamase (Heritage *et al*., 1999). Epidemiological studies have shown the plasmid-mediated enzymes TEM-1, TEM-2 and SHV-1 to be the most commonly encountered with TEM-1 predominant and responsible for 90% of ampicillin resistance in *E. coli* (Baker, 1999). The degree of resistance depends on the amount of TEM and SHV enzymes, which can vary 150-fold among isolates, reflecting gene dosage and promoter efficiency (Reguera *et al*., 1991; Heritage *et al*., 1999). These enzymes can be distinguished by biochemical criteria (substrate profiles, kinetic properties and reaction with inhibitors), physical properties (molecular size and isoelectric point), or by genetic criteria, such as

inducibility and location of their genes on plasmids or on the chromosome (Philippon *et al*., 1989; Jacoby, 1994; Livermore & Williams, 1996).

Within the bacterial cell, β-lactamases contribute to antibiotic resistance in several ways. In *E. coli* (TEM-1 producers) the β-lactamases remain localised in the periplasmic space and can destroy antibiotic molecules as they make their way through the outer membrane. Consequently, high level of resistance occur within a single bacterial cell (Medeiros, 1984; Zhou *et al*., 1994). Several parameters contribute to the level of antibiotic resistance mediated by a particular β-Lactamase in a population of bacteria. These include rate of hydrolysis (V_{max}), affinity for the antibiotic (K_m), and the amount of β-lactamase produced (Pitout *et al*., 1998).

By mid-1980s, resistance to expanded-spectrum cephalosporins had appeared in clinically significant Gram-negative bacteria, caused by the production of β-lactamases. Among the first of the extended-spectrum β-lactamases to cause significant clinical problems, were mutants derived from SHV-1 or TEM-1 β-lactamases. The genes encoding these mutants are present on mobile genetic elements, facilitating their spread in nosocomial pathogens (Heritage *et al*., 1999).

(a) TEM-1

TEM-1 is the most common plasmid encoded β-lactamase and was first recognized in *E. coli* in 1965 (Bryan & Godfrey, 1991; Ho *et al*., 1998). It has since spread to 20 – 60% of isolates of *Enterobacteriaceae*, with varying frequency in species and location. Expression of TEM-1 is constitutive but the amount varies amongst strains. Low level TEM-1 causes resistance to ampicillin, amoxicillin and ticarcillin, while higher levels can result in resistance to piperacillin, mezlocillin, cephalothin, cefamandole and cefoperazone (Livermore, 1993).

(b) SHV-1

SHV-1 is a narrow spectrum β-lactamase with activity against penicillins. It was first described as a chromosomally encoded β-lactamase in *Klebsiella* (Heritage, 1999). It is classified in the Bush group 2b enzymes (Sanders & Sanders, 1992) and is encoded by bla_{SHV-1} gene, commonly encountered in clinical isolates on self-transmissible plasmids.

The amount of enzyme produced and expressed vary more than one hundred fold amongst strains, depending on the number of plasmid copies per organism, the efficiency of the promoter and the degree of gene amplification (Jacoby & Carreras, 1990; Abdel-Rahman & Kearns, 1998; Petrosino *et al.*, 1998).

(c) Extended Spectrum β-Lactamases (ESBLs)

ESBLs are a diverse group of enzymes that have the common property of causing resistance to expanded spectrum cephalosporins. The first type to be discovered, and the clinically most significant, is derived from common plasmid-mediated β-lactamases by one or more amino acid substitutions that expand the spectrum of the enzyme. The second type are plasmid-mediated enzymes conferring resistance to α-methoxy cephalosporins such as cefoxitin and cefotetan and to the oxyimino β-lactam antibiotics. A third type provides resistance to carbapenems such as imipenem and meropenem as well as the oxyimino and α-methoxy cephalosporins (Jacoby, 1994).

Infection by ESBL producing organisms is associated with several identifiable risk factors such as hospitalisation in an intensive care unit, intra-abdominal sepsis, prior antibiotic therapy, urinary and central venous catheter insertion, mechanical ventilation, surgery and prolonged hospital stay (Menashe *et al*., 2001). ESBL producing organisms were first found in nosocomial isolates from large metropolitan hospitals. Since then, ESBLs have been identified from major teaching hospitals, community hospitals and nursing homes (Yang *et al*., 1998; Weiner *et al*., 1999). There is a considerable geographical spread of these β-lactamases and they are becoming a global problem in treating infections with expanded spectrum β-lactam antibiotics (Jacoby & Medeiros, 1991; Thomson & Amyes, 1992; Heritage *et al*., 1999; Pai *et al*., 1999). Because they are encoded on plasmids, these enzymes are easily transmissible from one organism to

another and may carry genes encoding resistance to other antibiotics such as aminoglycosides (Yang *et al*., 1998; Menashe *et al.,* 2001; Winokur *et al.,* 2001).

Plasmid mediated ESBLs have been reported from Europe, Africa and Asia (Quinn *et al*., 1989; Nandivada & Amyes, 1990; Bush *et al*., 1995; Kesah *et al*., 1996; Liu *et al*., 1998). Although members of the family *Enterobacteriaceae* that produce ESBLs have been recovered from most medical centers in South Africa since the late 1980s limited information exists regarding different types of ESBLs produced by *E. coli* and *K. pneumoniae* (Essack, Alexander & Pillay, 1994; Pitout *et al*., 1998; Essack *et al*., 2001). Frequency of ESBL producing bacteria appears to correlate with the extent of prior use of expanded spectrum cephalosporins (Essack, Alexander & Pillay, 1994; Lee *et al*., 2001).

Laboratory detection of ESBLs remains difficult because of the significantly different susceptibility patterns of third generation cephalosporins (Waterer & Wunderick, 2001). Unfortunately these ESBLs appear to be resistant to newer cephalosporins or aztreonam. Some ESBLs confer high level resistance to all oxyimino β-lactams, but resistance levels are only slightly increased or selectively increased for other β-lactam agents. This creates a problem in the clinical laboratory since organisms producing less active ESBLs can fail to reach current resistance breakpoints and are probably more prevalent than is currently recognized (Katsanis *et al*., 1994; Jacoby & Han, 1996). The clinical laboratory is therefore faced with the challenge of specifically detecting ESBL producing *Enterobacteriaceae.*

ESBLs are often resistant to currently available β-lactam-inhibitor combinations. Although these enzymes may be highly susceptible to clavulanic acid, they may reach such high levels in the periplasmic space of the bacterial cell that they cannot be effectively inactivated by the inhibitor (Sanders *et al.,* 1988; M'Zali *et al.,* 1997).

1.5.2 Penicillin–binding proteins

Resistance may be achieved by either reduced affinity of the PBPs for the antimicrobial agent, modification of the PBP structure or the appearance of a new PBP that shows little or no binding of the β-lactam antibiotic (Hindler, Howard & Keiser, 1994).

1.5.3 Reduced permeability

Gram-negative bacteria have a unique outer membrane outside the peptidoglycan that acts as an intrinsic permeability barrier against external influences, protecting them against host factors such as lysozyme. The porins in the outer membrane may also prevent or reduce the penetration of antibiotics (Pitout *et al*., 1997). The porin proteins form non-specific trans-membrane diffusion channels that allow exchange of nutrients and other substances such as

antibiotics between the extra-cellular environment and the periplasmic space (Hancock, 1987; Hernandez-Alles *et al*., 1999). β-Lactam antibiotics have to penetrate porin channels in the outer membrane and transverse the periplasmic space in order to bind to the PBPs (Hindler & Howard, 1994; Pitout *et al*., 1997). Changes in outer membrane permeability may consequently decrease binding to PBPs located on the inner membrane as a result of the limited influx of antibiotics (Dever & Dermody, 1991).

Reduced permeability in Gram-negative bacteria is mainly mediated by the loss or modification of the outer membrane proteins of 35-50 kD (Moosdeen, 1997), although the number and size of porins vary among different Gram-negative organisms (Prober, 1998). Two outer membrane proteins (Omps) of mutant *E. coli* K-12 have been identified and characterized as OmpF and OmpC (Reguera *et al*., 1991). Porin deficient mutants are known as Omp R mutants, and there is a possibility that decreased porin content could be caused by some R plasmids (Nikaido, 1989).

In *K. pneumoniae*, resistance to cephamycins, such as cefoxitin, can be attributed to a loss of one or both of the two major outer membrane porins OmpK35 or OmpK36 produced by these organisms. It is also known that most ESBL-producing *K. pneumoniae* strains lack OmpK35, which may result in the selection of additional mechanisms of resistance, including the loss of OmpK36 or efflux (Doménech-Sanchez *et al.*, 2003).

1.5.4 Efflux

Many different bacteria are able to pump out antibiotics. In "pumping" out the antibiotic from the cell, the organism can prevent it from reaching the concentration necessary for effective action. This can cause resistance not only to the prescribed antibiotic but also to multiple other antibiotics, even different classes of antibiotics (Waterer & Wunderink, 2001). Physiologically these pumps appear to be part of the natural defense mechanisms of bacteria against toxic compounds that exist in the environment (Nikaido, 1998). The sequence of proven and putative multi-drug efflux pumps from complete genome of *E. coli* have been analysed recently (Nikaido, 1998).

1.6 Role of plasmids in resistance

Acquired resistance may occur as a result of spontaneous chromosomal mutations or by acquisition of extra-chromosomal elements (called plasmids) through conjugation (Pitout *et al*., 1996; Abdel-Rahman & Kearns, 1998). Plasmid-mediated resistance to β-lactam antibiotics in *E. coli* is of major concern in hospitals. In South Africa this has resulted in widespread resistance of *E. coli* to ampicillin and co-trimoxazole, preventing their usage in urinary tract infections. (Klugman, 1993). This was also found in a recent study in Israel (Finkelstein *et al*., 1998). Studies show that for both nosocomial and community acquired infections (such as UTIs), the mortality and morbidity in prolonged hospitalisation,

are twice as high in patients infected with antibiotic-resistant strains than with susceptible strains (Pitout *et al.,* 1997; Silva *et al*., 1999; Lautenbach *et al*., 2001). Resistance consequently requires the use of more toxic or expensive antibiotics (Hindler, Howard & Keiser, 1994).

Plasmids are self-replicating circular DNA, smaller and separate from the bacterial genome, that can be transferred (some are self-transmissible) into another bacterial strain or species (Baker, 1999). They encode multiple resistance phenotypes and carry genetic information that may provide selective advantage to the bacteria (Mims *et al*., 1993). Bacterial plasmids that encode proteins responsible for antibiotic resistance are referred to as resistance (R) factors (Dever & Dermody, 1991; Hindler *et al.*, 1994). Plasmid-mediated resistance can be passed to distantly related bacterial species by conjugation, and the expression of these enzymes is usually constitutive. Bacteria can possess plasmids that can code for more than one β-lactamase in addition to their expression of chromosomal enzyme. Due to carriage of plasmids and promiscuous exchange of such material between bacteria, these resistance genes have spread widely and are also subject to mutation (Lee, Yuen & Kumana, 2001). Plasmid mediated β-lactamases were first recognized in Gram-negative bacteria in the early 1960s, shortly after the introduction of ampicillin (Livermore, 1993).

Transposons are genetic elements capable of transfer among a wide-variety of plasmids and of jumping between plasmids and bacterial chromosomes (Heritage

et al., 1999). Three of the TEM-like β-lactamases are encoded by transposons. TEM-1 is determined by Tn*3* and TEM-2 by Tn*1*, while SHV-1 is encoded by a transposon unrelated to Tn*1* (Medeiros, 1984). The occurrence of bla_{TEM} genes on mobile genetic elements undermines attempts to classify these elements by genetic location as transposons may jump between plasmids and the bacterial chromosome (Heritage *et al*., 1999). Transposons that encode ESBL activity have also been described (Heritage *et al*., 1999).

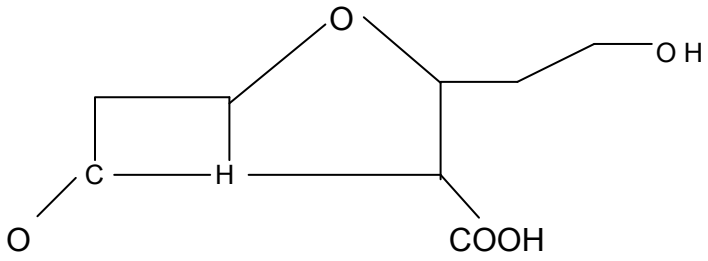
1.7 β-Lactam-β-lactamase Inhibitor Combinations

In an effort to overcome the hydrolytic action of β-lactamases, different therapeutic approaches such as the use of (a) β-lactamase stable β-lactam antibiotics, (b) metal ion chelators such as EDTA, (c) amino acid modifiers such as boronic acid, and (d) active-site-directed irreversible inhibitors such as clavulanic acid, sulbactam and tazobactam were attempted (Bush & Sykes, 1986; Sougakoff & Jarlier, 2000). It was found that the combination of a β-lactamase inhibitor such as clavulanic acid with a β-lactam antibiotic offered not only stability against inactivating β-lactamases, but also expanded the spectrum of activity of the primary antibiotic (Chaibi *et al*., 1999).

Clavulanic acid is produced by a strain of *Streptomyces clavuligerous* and is a potent inhibitor of plasmid mediated β-lactamases produced by both Gram-positive and Gram-negative bacteria. It is effective against broad spectrum β-

lactamases but not enzymes that are primarily cephalosporinases (Bryan & Godfrey, 1991; Livermore & Williams, 1996; Gorbach *et al*., 1997). Clavulanic acid is structurally similar to penicillins and cephalosporins in that it contains a β-lactam ring and is able to fit into the catalytic centre of the β-lactamase enzyme (Rolinson, 1991), although the β-lactam ring is fused to an oxazolidine ring (Figure 1.6). Clavulanic acid can penetrate the bacterial cell the same way as a β-lactam antibiotic and bind to the catalytic site of intra- and extra-cellular β-lactamases, including those that are plasmid encoded (Richmond-Sykes classes III and V) and the chromosomally encoded β-lactamases (Richmond-Sykes classes II and IV) of Gram-negative and Gram-positive bacteria (Todd & Benfield, 1990; Dever & Dermody, 1991). This binding is a complex physiochemical process (Figure 1.5). The end result of this binding is the prevention of inactivation of the accompanying β-lactam antibiotic (Lee *et al*., 2001).

FIGURE 1.6: Structure of Clavulanic acid (Chaibi *et al*., 1999).



The discovery of clavulanic acid, and its introduction into clinical practice led to the discovery of other compounds that could function as β-lactamase inhibitors (Chaibi *et al*., 1999). These include derivatives of penicillanic acid, β-lactam sulfones such as sulbactam and tazobactam (Moellering, 1993). Enzyme inhibitors may function via a number of mechanisms, including competitive (reversible), non-competitive and terminal (suicide) inhibition (Abdul-Rahman & Kearns, 1998).

The overall antibacterial spectrum of these combinations depends on microbiological factors such as (i) the effectiveness of the enzyme inactivation, (ii) the amount of β-lactamase produced (iii) the intrinsic properties of the β-lactam in the combination, (v) the permeability and intrinsic susceptibility of bacteria to the inhibitor, (v) the physiochemical conditions such as pH, and (vi) the characteristics of the inhibitor (Sanders *et al*., 1988; Livermore, 1993; Ho *et al*., 1998).

Although many investigators feel that the combination of amoxicillin and clavulanic acid should be tested at a fixed concentration of clavulanic acid (Thompson, Miles & Amyes, 1995), it has not yet been specifically decided whether a β-lactamase inhibitor should be used in a fixed concentration or titrated in a fixed ratio with the antibiotic (Greenwood, 1996). For effective inhibition β-lactamase inhibitors must penetrate to the same extent as the β-lactam antibiotic and be present for a long enough period. Since a fixed combinations of inhibitor and β-lactam antibiotic is used in clinical practice, it is important that both agents

are perfectly matched. The ratio of β-lactam to inhibitor normally ranges from 1:1 to 30:1 in terms of weight per dose (Lee *et al*., 2001).

1.7.1 Inhibitor Resistant β-Lactamases

Until 1989 all plasmid-mediated β-lactamases identified were susceptible to clavulanic acid and could theoretically be controlled by the use of β-lactam-inhibitor therapy (Essack, Alexander & Pillay, 1994). However, from the mid-1980s to early 1990s, inhibitor resistant isolates of members of the family *Enterobacteriaceae* were noted (Thompson *et al*., 1990; Thompson & Sanders, 1992; Zhou *et al*., 1994; French, Shannon & Simmons, 1996). Such resistant isolates have since been increasingly noted in UTIs (Nicolas-Chainone, 1997).

Failure to effectively inhibit these β-lactamases may be attributed to (1) type of β-lactamase involved (Thompson *et al*., 1990), (2) modification of the kinetic properties of the TEM β-lactamase due to amino acid substitutions (Bouthors, Jarlier & Sougakoff, 1998; Therrien & Levesque, 2000), (3) decreased permeability to β-lactams, (4) level at which a β-lactamase is produced (Brun *et al*., 1994), (5) decreased uptake of the antibiotic due to modification of the outer membrane protein of Gram-negative organisms (Espinasse *et al*., 1997; Nicolas-Chanione, 1997; Simpson *et al*., 1998), (6) production of β-lactamase not readily inhibited by suicide inhibitors like most chromosomal class C inducible β-lactamases (Tenover *et al*., 1999), (7) production of OXA-type enzymes and/or hyperproduction of cephalosporinases that are less sensitive than TEM to

inhibition by clavulanic acid (Stapleton *et al*., 1995), and (8) a combination of overproduction and decreased uptake (Stapleton *et al*., 1995; Espinasse *et al*., 1997).

Inhibitor resistant β-lactamases have been described in *E. coli*, *K. pneumoniae*, *Proteus mirabilis* and *Citrobacter freundii* (Todd & Benfield, 1990; Sanders & Sanders, 1992; Nordman, 1998). About 6% of urinary tract pathogens in the UK are resistant to amoxicillin/clavulanic acid. In other European countries, resistance rates are much higher: 10-25% of *E. coli* isolates in France, and ~ 25% isolates from urinary tract infections in Italy (Nicolas-Chanione, 1997). Data from Southern Africa are scarce, but the majority of inhibitor resistant isolates are isolated from urine specimens (Therrien & Levesque, 2000).

1.8 Detection of β-lactamase mediated resistance in the laboratory

Routine antimicrobial susceptibility testing of significant isolates are performed to make reasonable predictions of the treatment outcome and to facilitate selection of an appropriate antibiotic (Greenwood, 1996; Olsson-Liljequist & Forsgren, 1997; Jorgensen & Ferraro, 1998; Walker & Thornsberry, 1998; Gould, 2000). Factors that should be considered in susceptibility testing include (1) predictability of susceptibility to drug(s) of choice, (2) body sites from where the organism was

isolated, (3) quantity of organisms present (quantitative cultures), (4) presence of other organisms and quantitation of each, and (5) presence of any unique host factors (Hindler *et al*., 1994).

1.8.1 Phenotypic tests of antimicrobial susceptibility

Laboratory tests measure directly or indirectly, and under controlled conditions, the inhibitory or killing effect of the antimicrobial on the pathogen isolated from the patient. The most popular methods are based on filter-paper discs containing specific antibiotic concentrations placed on agar plates containing the test isolate (Bannister, Begg & Gillespie, 2000). The agar disc diffusion method measures zone sizes and organisms are designated as sensitive [S], intermediate [I]/moderately sensitive, or resistant [R] (Gould, 2000). An infection with a sensitive organism should clinically respond to standard doses of the agent reported, while that caused by resistant organisms should not. Infection with an organism with intermediate (or moderate) sensitivity may or may not respond to standard doses but will probably respond if the agent is concentrated at the site of infection or if the dosage is increased (Phillips, 1991; NCCLS, 1997; Ringertz *et al*., 1997; Bannister *et al*., 2000).

Revised diameters of the zone of inhibition for cefpidoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone have recently been implemented by the NCCLS to be used as a screening tool for ESBL production (NCCLS, 1997). In

1999, the NCCLS added confirmation tests for ESBL-producing strains and recommended that the interpretation of test results with expanded spectrum cephalosporins and aztreonam be changed to resistance for ESBL-positive strains (Tenover *et al*., 1999). In laboratories that use broth dilution methods, ESBL production should be suspected when MICs of cefpidoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone are $> 1\mu g/ml$ (Paterson & Yu, 1999).

Because of the risk of failing to detect ESBL production, various tests were proposed to enhance ESBL detection. The clavulanic acid double-disc potentiation test was first described by Jarlier *et al.* in 1988 (Sanders *et al*., 1988; Moland & Thompson, 1994; Sirot, 1995). This test has, however, some inherent problems and may fail to detect some ESBL producing strains (Tenover *et al*., 1999), because of (1) differences in optimal or precise disc spacing (Thompson & Sanders, 1992; Smith & Chamber, 1995; Sirot, 1995; Ho *et al*., 1998), (2) disc potency (Moland & Thompson, 1994; Sirot, 1995), (3) false negative results (Ho *et al*., 1998; Tenover *et al*., 1999), (4) appropriate control tests (including innoculum density, composition of test medium, agar depth, temperature, atmosphere and end points during incubation) (Hindler *et al*., 1994; Phillips, 1991; NCCLS, 1997; Olsson-Liljeequist & Forsgren, 1997).

In the clavulanic acid double disc potentiation test, some investigators have suggested ceftazidime resistance to be a suitable marker for ESBL production because the antibiotic is a substrate for most ESBLs and should be an appropriate indicator antibiotic (Ho *et al*., 1998; Paterson & Yu, 1999). This has,

however been shown to be unreliable and it is suggested that the test also be carried out with aztreonam as well (Katsanis *et al*., 1994; Paterson & Yu, 1999).

Another agar diffusion method for performing antimicrobial susceptibility testing is the Epsilometer or E-test which is based on reduction of ceftazidime MIC in the presence of a fixed concentration (2μg/ml) of clavulanic acid (Cormican *et al.*, 1996; White *et al*., 1996). This method employs diffusion into agar from a thin strip impregnated with varying concentrations of an antibiotic along a gradient (Baker *et al*., 1999).

1.9 Objectives

South Africa is considered to be one of the countries where β-lactam antibiotics are widely used by doctors in the community (Klugman, 1993) and this heavy usage has selected for resistance, which is often caused by β-lactamases (Thompson, Sanders & Sanders, 1994). South Africa can now also be counted under the list of countries where ESBLs are posing a serious problem for antimicrobial therapy.

Estimates of relative incidences and types of different ESBLs from a variety of biological sources have been obtained from different teaching hospitals around the country (Pitout *et al*., 1998; Essack *et al*., 2001). However there is no detailed

knowledge of resistance patterns from the Universitas and Pelonomi hospitals, Bloemfontein, especially in urinary tract infections.

Since β-lactamase expression is the major resistance mechanism of bacteria to β-lactam antibiotics, the study was designed to determine if resistance in *E. coli* and *K. pneumoniae*, two of the most frequently encountered urinary pathogens was related to β-lactamase production and identify and characterise the principal β-lactamases involved.

This study was done to determine:

(i) The antibiotic susceptibility patterns of the isolates collected against ß-lactam agents in the treatment of UTIs.
(ii) The prevalence of ß-lactam resistance in clinically important Gram negative bacteria isolated from urinary tract infections in the hospital.
(iii) Possible mechanisms of antibiotic resistance development to ß lactam-agents.
(iv) Possible resistance genes present in resistant strains.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Isolates

A total of one hundred and twenty-three clinical isolates of *E. coli* (114) and *K. pneumoniae* (nine) isolated from patients with urinary tract infections at the Universitas and Pelonomi hospitals in Bloemfontein were collected from September 1999 to March 2000. These isolates were identified by routine laboratory techniques that are was based on colonial morphology appearance and lactose fermentation on MacConkey agar. Isolates were screened for ampicillin/amoxicillin resistance by routine disc susceptibility and those reported to be resistant to ampicillin and/or amoxicillin were stored at –70°C in a solution containing 7% glucose, 7% peptone and 30% glycerol. Consecutive sub-culturing was done on MacConkey agar containing ampicillin.

2.2 Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) of 8 antimicrobial agents were determined by the National Committee for Clinical Laboratory Standards (NCCLS) agar dilution method (NCCLS, 1997). Antimicrobial agents included aztreonam, cefotaxime, ceftriaxone, ceftazidime, piperacillin (Lederle, NY, USA), piperacillin-tazobactam, amoxicillin (SmithKline Beecham Laboratories, UK), cefoxitin and cefuroxime (Lederle, NY, USA).

The inoculum was prepared from overnight cultures in Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, MI, USA). From these cultures, a 0.5 McFarland suspension was prepared in saline (0.85% NaCl). From this solution a 1/10 dilution was made in saline which would contain 10^7 CFU (Colle *et al.*, 1996). The cell suspensions were inoculated onto the surface of Mueller-Hinton (MH) agar containing doubling dilutions of antimicrobial agents using a multipoint inoculator (Mast Laboratories, Merseyside, UK) to deliver 1 x 10^5 CFU/spot. MICs were read after 24 h incubation at 37°C. The MIC was recorded as the lowest concentration of antibiotic that inhibited growth, disregarding one or two colonies or a trailing haze of growth. Control strain *Escherichia coli* ATCC 25922 was included in each series. Approved and tentative NCCLS susceptible breakpoints or preliminary breakpoints as suggested by respective manufacturers were used.

2.3 Transfer of resistance

2.3.1 Conjugation

To determine if the genes are located on the chromosome or on a plasmid, conjugation was be done by using selected resistant strains as donors and *Escherichia coli* 711 as selective recipient in all conjugation studies.

Donor strains (ampicillin resistant [Amp^r, nalidixic acid susceptible [Nal^s and lactose fermenting [LF]) and recipient strain (ampicillin susceptible [Amp^s], nalidixic acid resistant [Nal^r] and non-lactose fermenting [NLF]) were inoculated into nutrient broth separately and incubated at 37°C until the cells reached logarithmic growth phase. Two hundred μl of recipient and 50μl of donor were then added to five ml of fresh nutrient broth and incubated overnight at 37°C. A loop-ful of the mixture was then spread on the surface of MacConkey agar containing ampicillin (25 μg/ml) plus nalidixic acid (30μg/ml) as selective antibiotics. The plates were incubated overnight at 37°C. Colonies of *E. coli* 711 that grew on the nalidixic acid/ampicillin selective plates and again on subculture on ampicillin plates were regarded as transconjugants, and confirmed by checking their growth requirements (phenotype NLF, Nal^r, and Amp^r).

2.3.2 Plasmid studies

Plasmid extraction was performed using a High Pure Plasmid Isolation Kit (Roche Diagnostic, Germany) according to manufacturer's specifications.
A quarter of plate of confluent growth from a MacConkey agar plate was suspended in 250 μl of Buffer + RNase suspension solution. Lysis buffer solution was added and the tubes inverted until the suspension had cleared. Binding buffer solution was then added and the tubes inverted until the suspension had become cloudy and flocculent precipitate formed. The lysate was then centrifuged for 10 min at 14 000 rpm. The supernatant was poured into a High Pure filter tube and centrifuged at maximum speed. The step was repeated and supernatant washed in two runs of Buffer I and Buffer II. Plasmid DNA was eluted by addition of 100 μl elution buffer to a microcentrifuge tube and collected by centrifugation for 30 s at 14 000 rpm. The microcentrifuge contained the eluted plasmid DNA.

The plasmid preparations (18 μl, mixed with 2 μl of 10 x TAE buffer) were separated on 1.5% agarose gels (NuSieve, FMC BioProducts, Rockland, USA) using 1 x TAE buffer for 1 h at 85 V and sized approximately employing a supercoiled DNA ladder, range 2-16.2 kb (Promega, Madison, USA) and a known 39 kb tetracycline-resistance plasmid of *Neisseria gonorrhoeae*.

2.4 Possible resistance mechanisms

2.4.1 Production of ß-lactamases

A Screening isolates for ß-lactamases

Selected isolates were be tested for the production of ß-lactamases with:

(1) The iodometric tube method, using Pen G as the substrate (Livermore & Williams, 1996). Crude extracts of the ß-lactamases were obtained by sonication and centrifugation of overnight cultures of the test isolates. Twenty μl of starch indicator, (containing 1% soluble starch), 20 μl iodine reagent (containing 2% iodine in 53% potassium iodide) and 100μl benzyl penicillin were pipetted into a glass test tube. Crude enzyme (100μl) was added and the mixture vigorously shaken at room temperature. When β-lactamase was present, the blue-black colour of the mixture disappeared and the solution became milky white within 5 min.

(2) The Nitrocefin method where cells were mixed directly onto the moist nitrocefin on the filter paper and left up to 1 h for any colour reaction to occur. A change from yellow to red indicated the production of ß-lactamase.

B Isoelectric point values (pI's)

Isoelectric point values (pIs) were determined by isoelectric focusing in agarose gels (Pharmacia) containing Pharmalyte (pH range: 3 to 10; Pharmacia). Gels were run for 3h at a maximum potential difference of 400V and stained with nitrocefin 500mg/ml. ß-Lactamases with known pI's were used for comparison.

Agarose gels were prepared by melting 1 g of agarose (Agarose IEF; Pharmacia Fine Chemicals, Uppsala, Sweden), 5 g sorbitol (Merck, Darmstadt, Germany), 40ml of 25% glycerol and 20ml distilled H_2O in a boiling water bath, and all components dissolved. The solution was cooled to 50°C and 1ml of 40% ampholyte mixture (pH: 3-10, Sigma, Louis, USA) added. Agarose gels were cast by pouring the mixture onto preheated glass plates (124mm x 100mmx 0.15mm) fitted with a sheet of GelBond film (FMC Corp., Maine, USA). After solidifying, the gels were covered with Parafilm® plastic film and allowed to "age" in a humid chamber at 4°C overnight.

After "aging", excess liquid was removed by blotting with filter paper for 5 to 10 min. Thin-layer agarose gel IEF was performed with a cooled (10°C) electrophoresis stage (BIOPHORESIS™, Horizontal electrophoresis Cell). The coolant model was MgW Lauda, K4R, 220 V, 1500 W, 50Hz; (Western Germany). Samples (5µl) were applied to the surface of the gel 2, cm from the anode.

Electrode wicks (9.5cm) soaked in appropriate solutions were placed onto corresponding electrode poles. The anode solution used was 0.5M acetic acid (Merck, Midrand, South Africa), and 0.5M NaOH (BDH, Halfway House, South Africa) was used as the cathode. Focusing was done for 3 hours with a BIORAD Model 3000Xi electrophoresis power supply set at 3 W constant power, 226 V limiting and 13 mA limiting.

Enzyme activities were detected by overlaying the gel with filter paper (124mm x 100mm) saturated with 0.05% nitrocefin solution. The gels were then placed in a fixative solution for 15 minutes, immersed in 95% ethanol to ensure clear background, dried and bands detected by staining in Coommassie brilliant blue, destained and air dried.

Strains producing TEM-1, -3, -4 and SHV-2, -3, -4 (pIs 5.4, 6.3, 5.9 and 7.6, 7.0, 7.75 respectively) were used as standards. A broad range IEF standard (pH 4.6-9.6) BIO-RAD, California, USA was also included in each run.

2.4.2 ESBL production

Production of ESBLs was studied by the double disc synergy and inhibition potentiated disc diffusion tests as described by Jarlier *et al.,* (1988). Isolates were inoculated on MH-agar plates. Discs containing respectively ceftazidime (30µg), cefazolin (30µg), cefoxitin (30µg) and aztreonam were placed 25mm (centre to centre of the discs), from a disc containing ampicillin/ clavulanic acid (20µg). After overnight incubation at 37°C, the diameters of inhibition zones around the antibiotic discs were measured using a Venier Caliper. A clear extension of the edges of the inhibition zone of any of the antibiotics towards the disc containing clavulanic acid, was regarded as a phenotypic confirmation of the presence of ESBL (Jarlier *et al.,* 1988; Acar & Goldstein, 1996; Collee *et al.,* 1996).

Isolates positive for ESBL production were subjected to polymerase chain reaction (PCR) amplification using primers designed for the detection of bla_{TEM} and bla_{SHV} genes.

2.4.3 Inhibition

Inhibitory effect of clavulanic acid on the efficacy of the different antimicrobial agents was tested by using:

(1) Microbiological assays, where MH agar was seeded with amoxicillin-sensitive *E. coli* 711. Enzyme extract (as described in section 2.5) of the organism was added to wells made in the agar.

(2) The agar dilution method (as described in section 2.2) was done, but a fixed concentration of inhibitors such as clavulanic acid or tazobactam were added to all dilutions of each antimicrobial agent (as described in section 2.6).

2.5 Crude enzyme extraction

Overnight cultures in nutrient broth were centrifuged at 15 000**g** for 30 min at 4°C. The pellet was washed with 0,1M phosphate buffer (pH7) and resuspended in the same buffer. Cells were disrupted by French Pressure Cell Press (American Instrument company, Maryland, USA) at 12 000lb/in^2, and the cell debris removed by centrifugation (40 000**g**, 30 min, 4°C) [Beckman, model L3-50 ultracentrifuge, USA]. The supernatant was used as the crude extract containing the β-lactamase.

2.5.1 Concentration of β-lactamase preparations

Samples of 5ml crude β-lactamase extract were freeze-dried overnight on a Virtis Freezemobile (model 12SL, Virtis Company, New York, USA). These lyophilised preparations were resuspended in 0.5ml of 0.1M phosphate buffer to provide 10-fold concentrations of the crude enzyme extracts.

2.6 Effect of clavulanic acid

To investigate the influence of clavulanic acid on a mixture of enzymes, dilutions of crude extracts were preincubated for 10min at 4°C in the presence of 0, 1, 2, 4 and 8μg/ml clavulanic acid. Subsequently the lysates were added to the agar processed as described above. Lithium clavulanic acid was reconstituted as for checkerboard studies. Since each well could take up 40μl enzyme extract, 10μl clavulanic acid and 30μl enzyme were mixed together. The whole plate was also preincubated for one hour at 4^0C then transferred to 37^0C. The clavulanic acid concentration was further diluted from 0, 1.6, 3.2, 6.4, and 1.28μg/well.

An amoxicillin sensitive *E. coli* 711 was cultured in 100ml nutrient broth for 24h at 37°C and suspended to 0.5 McFarland to produce semi-confluent growth. Before pouring the 90mm petri dishes, 100μl of this *E. coli* suspension plus 32μl of 1 000μg amoxicillin (to give a final concentration of 16μg amoxicillin) were added to 20ml of MH-agar kept at 50°C, and allowed to set at room temperature. Wells

of 6mm diameter were made in the agar. Each well could accommodate 40μl of the crude enzyme preparation. Plates were incubated overnight at 37°C.

2.7 Resistance gene analysis

2.7.1 Cell lysate preparation

Isolates were inoculated onto MH-agar and incubated overnight at 37°C. Half plate of cells from overnight culture were harvested by suspending in 250 μl of TE buffer (50 mM Tris hydrochloride, 20 mM EDTA, pH 7.5) plus 1,25 μl of 10mg/ml lysozyme. The suspension was incubated at room temperature for 20 min. Lysis was enhanced by the addition of Triton X–100 in 50mM Tris (pH 7.5) and 1,25 μl proteinase K (5mg/ml) and incubation continued for 30 min at 37°C. This lysate was used as DNA substrates in the PCR amplification reaction.

2.7.2 PCR amplification

Amplification was done using primers and methods as described by Pitout *et al.* (1998). Primers used were SHV-A: 5' CAC TCA AGG ATG TAT TGT G and SHV-B: 5' TTA GCG TTG CCA GTG CTC G, TEM-A: 5' TCG GGG AAA TGT GCG CG and TEM-B: 5' TGC TTA ATC AGT GAG GCA CC. These primers anneal to nucleotide numbers 103-121 and 988-970

respectively, on the *bla*$_{SHV}$ gene. Amplification was performed in a final volume of 25 µl, the reaction mixture comprising 1.5 µl lysate, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP and 25 pmols of each primer. Amplification was performed in a thermocycler (Perkin Elmer, GeneAmp PCR System 9600, Connecticut, USA). The PCR program consisted of an initial denaturation step at 95°C for 180s before 1µl of Taq DNA polymerase (Roche, Indianapolis, USA) was added at 55°C. Amplification was continued as follows: 30 cycles of DNA denaturation at 94°C for 60s, primer annealing at 67°C for 60s, and primer extension at 72^0C for 1min. SHV primers amplified at 885-bp fragment, while TEM primers amplified at 971-bp fragment (Pitout *et al.,* 1998).

The primers specific for SHV and TEM genes were synthesised using Oligo 1000M DNA Synthesiser, version 4,2 (University of Cape Town, Cape Town, South Africa).

2.7.3 Agarose gel electrophoresis

PCR products (25 µl) were mixed with 2-8 µl of 10 x TAE buffer and 1-4 µl of bromophenol blue (0.25%). PCR mixtures were applied to 1-1.5% agarose gels (NuSieve; FMC BioProducts, Rockland, USA) and products separated by electrophoresis for 2-4 h at 90 V using 1 x TAE running buffer (4.84 g/l Tris, 0.37 g/l EDTA, pH 8). The products were visualised by staining with

ethidium bromide and photographed under UV illumination (λ 312 nm). A 100 bp DNA ladder (*ABgene*, Surrey, UK) was included in each run. The developed gel was photographed with a Polaroid MP-4 camera (Massachusetts, USA) using a Polaroid Studio B/W film (UK).

2.7.4 DNA extraction

PCR products (100 µl) were separated by agarose gel electrophoresis (section 2.4.2) on 1% gels and bands of predicted sizes cut from the gels with minimum exposure to UV illumination. The DNA was extracted from the gel slices using a Nucleon GX extraction kit (Amersham, Buckinghamshire, England) by incubation in sodium perchlorate (for extracting fragments <500 bp) or sodium iodide (for fragments ≥500 bp) at 55°C for 5 min. Resin (15 µl) was then added and the samples incubated at room temperature for 1 min. After centrifugation in a microcentrifuge at top speed (16 000 x *g*) for 30 sec, the pellet was resuspended in 1 ml Washing Solution, followed by a 1:1 dilution with 99% ethanol. The sample was again centrifuged at top speed for 30 sec and the supernatant discarded. The pellet was incubated at 55°C (tube with open lid) until all the alcohol had evaporated. Sterile distilled H_2O (10 µl) was added to the pellet after 1 min incubation at room temperature. After centrifugation for 30 s the supernatant containing the DNA was carefully removed to a clean tube. The final step was repeated to yield approximately 20 µl of DNA sample.

2.8 Protein concentration determination

Protein concentration was determined by the Biuret method. A range of bovine serum albumin concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0ml) of 10mg/ml was prepared. To 500µl of the standard protein dilution and extract preparations, 4 ml Biuret reagent was added and samples incubated for 20 min at 37°C. Absorbance (540 nm) was measured on a Klett-Summerson colorimeter. A linear plot of the OD_{540} of the protein standards was constructed and unknown concentrations of the test samples determined.

CHAPTER 3

COMPARATIVE ACTIVITIES OF ANTIMICROBIAL AGENTS

3.1 INTRODUCTION

Although the proportion of Gram-negative organisms causing nosocomial infections decreased during the 1980s and the 1990s, they remain an ever-present threat to patients. After diagnosing a UTI appropriate antimicrobial agents must be selected (Dusé & Klugman, 1993) and administered as soon as possible to improve the outcome of therapy (Waterick & Wunderick, 2001).

Different countries use different antibiotic panels for treatment of the same site of infection. Treatment of UTIs in the United Kingdom include ciprofloxacin, nitrofurantoin, cotrimoxazole, trimethoprim, nalidixic acid, first generation cephalosporins, a sulphonamide, tetracycline and amoxicillin (Gruneberg, 1994). In South African hospitals, including the Universitas and Pelonomi hospitals in Bloemfontein, these infections are treated with ampicillin, cotrimoxazole, gentamicin, nitrofurantoin, amox-clav, cefoxitin, cefotaxime, ceftazidime and/or ceftriaxone. In intensive care units, ciprofloxacin, piperacillin-tazobactam,

cefepime, meropenem, imipenem, tobramycin and amikacin are given as secondary agents (Personal communication, Anne McLeod).

One of the major objectives of a clinical laboratory is guidance in rational therapy by susceptibility testing of isolates from clinically significant specimens (Amsterdam, 1996; Walker & Thornsberry, 1998). Susceptibility tests performed on bacteria suspected of causing an infection (Hindler, Howard & Keiser, 1994) have been designed to allow the best choice of antibiotic with the narrowest spectrum and highest effectiveness against isolated bacteria (Jarlier *et al*., 1988).

Determining of minimum inhibitory concentration (MIC) is done in most large surveys of susceptibility tests where results from different studies may be compared (Martinez & Baquero, 1990). MIC determination is considered a reliable indication of emerging antibiotic resistance among several important bacterial pathogens (Hindler, Howard & Keiser, 1994; Phillips, 1998; Jorgensen & Ferraro, 2002).

3.2 RESULTS AND DISCUSSION

One hundred and twenty-three clinical strains isolated from urine samples were identified as either *E. coli* or *K. pneumoniae* and selected from the diagnostic laboratories as being resistant to amoxicillin. Specimens were collected from both male and female patients with UTIs, some of these patients were immunocompromised. Clinical diagnosis mostly indicated recurrent UTI and lower abdominal pain, intra-uterine growth, post forsa tumour, spina bifida and acute renal failure. The ages of patients ranged from four days to ninety-one years.

All the isolates (including the control strains) were resistant to amoxicillin (MICs ≥8μg/ml), while only one isolate (Ec80) was susceptible for piperacillin (MIC 8μg/ml) (Table 3.1). The MICs for amoxicillin ranged from 8 to >128μg/ml and for piperacillin from <0.25 to >128μg/ml. However, high levels of susceptibility were still noted in all the isolates for most of the third generation cephalosporins and cefoxitin. The MICs for cefotaxime (76% susceptible), cefuroxime (74% susceptible), ceftriaxone (84% susceptible) and ceftazidime (81% susceptible) ranged from <0.25 to >128μg/ml, while the range for cefoxitin was 1-128μg/ml. Susceptibility to these expanded spectrum β-lactams was restored in all of the isolates when 2μg/ml of clavulanic acid and 4μg/ml of tazobactam were added to amoxicillin and piperacillin respectively. MIC_{50} and MIC_{90} of the β-lactam agents, alone and in combination with clavulanic acid and tazobactam against all of the isolates are shown in Table 3.3.

Resistance to amoxicillin and piperacillin were the highest for all the isolates. When clavulanic acid was added, the MICs for amoxicillin decreased 2-3 fold, while inhibition to piperacillin-tazobactam was not detected or was 3- fold. Increased resistance to amoxicillin (MIC $>$128μg/ml) was found in all of the isolates tested (Table 3.1 and 3.3). On comparing the results, the amoxicillin resistance was 90% higher than the 45% reported from European countries (Wesley, 1996) and the 58% to 68% reported for Spain and Israel (Stapleton *et al*., 1995). This could possibly be due to the over-use of amoxicillin as first choice treatment for urinary tract infections in South African hospitals. This increased resistance was also reported from one hospital in Athens, Greece, where 100% of *E. coli* isolated from patients between November 1987 and January 1988 were resistant to ampicillin (Sanders & Sanders, 1992).

TABLE 3.1: Antimicrobial susceptibility profiles of isolates resistant to amoxicillin and piperacillin (n=122).

	MIC (µg/ml)												
	AMX	**AMC**	**CTX**	**CTC**	**CRX**	**CRC**	**CXM**	**CXC**	**CAZ**	**CAC**	**PIP**	**PIT**	**FOX**
Ec1	8	8	1	0.5	≤0.25	≤0.25	8	8	≤0.25	≤0.25	32	16	4
Ec2	>128	8	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	2
Ec3	>128	8	1	0.5	≤0.25	≤0.25	8	8	≤0.25	≤0.25	>128	16	4
Ec4	>128	128	2	2	≤0.25	≤0.25	8	8	≤0.25	≤0.25	>128	16	>128
Ec5	>128	16	1	0.5	≤0.25	≤0.25	2	2	0.5	0.5	>128	16	2
Ec6	>128	2	0.5	≤0.25	≤0.25	≤0.25	2	2	≤0.25	≤0.25	>128	8	2
Ec7	>128	2	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	128	16	4
Ec8	>128	16	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	16	8
Ec9	>128	16	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec10	>128	8	1	0.5	≤0.25	≤0.25	4	4	1	≤0.25	>128	16	4
Ec11	>128	32	2	1	≤0.25	≤0.25	8	8	0.5	0.5	>128	16	8
Ec12	>128	8	1	1	≤0.25	≤0.25	8	8	≤0.25	≤0.25	>128	8	8
Ec13	>128	16	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec14	>128	128	32	64	1	1	>128	128	0.5	0.5	64	64	>128
Ec15	>128	8	0.5	≤0.25	≤0.25	≤0.25	2	4	≤0.25	≤0.25	>128	8	2
Ec16	>128	16	2	1	≤0.25	≤0.25	8	8	≤0.25	≤0.25	>128	16	4
Ec17	64	64	>128	64	16	32	>128	64	64	32	>128	>128	>128
Ec18	>128	128	>128	64	32	64	>128	64	64	32	>128	>128	>128
Ec19	>128	8	128	1	32	≤0.25	32	4	128	1	>128	32	4
Ec20	>128	8	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	8
Ec21	>128	8	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	16	4
Ec22	>128	16	2	1	≤0.25	≤0.25	4	8	≤0.25	≤0.25	>128	8	4
Ec23	>128	16	1	1	≤0.25	≤0.25	8	8	≤0.25	≤0.25	>128	16	8
Ec24	>128	8	1	0.5	≤0.25	≤0.25	4	4	0.5	0.5	>128	32	4
Ec25	>128	8	1	0.5	≤0.25	≤0.25	8	8	≤0.25	≤0.25	>128	16	4
Ec26	>128	16	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	16	8
Ec27	>128	8	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec28	>128	128	>128	128	128	64	64	64	128	64	>128	128	128
Ec29	>128	8	8	4	≤0.25	≤0.25	16	16	1	0.5	>128	>128	64
Ec30	>128	8	1	0.5	≤0.25	≤0.25	8	8	≤0.25	≤0.25	>128	16	4
Ec31	>128	8	1	0.5	≤0.25	≤0.25	4	8	≤0.25	≤0.25	>128	16	4

	MIC (µg/ml)												
	AMX	**AMC**	**CTX**	**CTC**	**CRX**	**CRC**	**CXM**	**CXC**	**CAZ**	**CAC**	**PIP**	**PIT**	**FOX**
Ec32	>128	8	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec33	>128	8	8	4	≤0.25	≤0.25	64	16	1	1	>128	>128	8
Ec34	>128	128	>128	64	64	64	>128	128	128	64	>128	>128	>128
Ec35	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec36	>128	16	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec37	>128	16	1	1	≤0.25	≤0.25	4	4	0.5	≤0.25	>128	16	4
Ec38	>128	16	0.5	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	8
Ec39	>128	8	0.5	0.5	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	4
Ec40	>128	16	0.5	0.5	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	4
Ec41	>128	16	0.5	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec42	>128	16	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec43	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec44	>128	64	>128	32	16	16	>128	32	64	32	>128	>128	>128
Ec45	>128	16	0.5	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	16	4
Ec46	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec47	>128	8	0.5	0.5	1	≤0.25	4	2	≤0.25	≤0.25	128	16	4
Ec48	>128	16	0.5	0.5	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	8
Ec49	>128	16	0.5	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec50	>128	8	0.5	0.5	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	4
Ec51	>128	16	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	8
Ec52	>128	8	0.5	0.5	≤0.25	≤0.25	4	2	≤0.25	≤0.25	128	4	4
Ec53	>128	64	16	64	0.5	2	>128	64	2	4	>128	64	>128
Ec54	>128	16	1	1	1	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec55	>128	64	32	32	2	32	>128	64	2	32	>128	64	>128
Ec56	>128	64	16	64	2	2	>128	64	2	2	>128	64	>128
Ec57	>128	64	>128	64	32	32	>128	64	128	32	>128	128	128
Ec58	>128	16	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	8
Ec59	>128	64	>128	64	128	32	>128	64	128	32	>128	>128	>128
Ec60	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec61	>128	128	16	32	2	2	>128	64	2	2	>128	64	>128
Ec62	>128	64	128	32	8	8	>128	32	>128	16	>128	>128	>128
Ec63	>128	64	32	4	2	≤0.25	32	32	>128	4	>128	64	>128
Ec64	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec65	>128	8	0.5	0.5	≤0.25	≤0.25	2	2	≤0.25	≤0.25	>128	4	4

	MIC (µg/ml)												
	AMX	**AMC**	**CTX**	**CTC**	**CRX**	**CRC**	**CXM**	**CXC**	**CAZ**	**CAC**	**PIP**	**PIT**	**FOX**
Ec66	>128	64	>128	64	32	32	>128	64	64	32	>128	>128	>128
Ec67	>128	32	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec68	>128	64	>128	64	128	32	>128	64	64	32	>128	>128	>128
Ec69	>128	8	≤0.25	≤0.25	≤0.25	≤0.25	1	1	≤0.25	≤0.25	>128	4	4
Ec70	>128	16	1	1	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	32	8
Ec71	>128	16	1	1	≤0.25	≤0.25	8	4	≤0.25	≤0.25	128	8	8
Ec72	>128	32	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	8
Ec73	>128	16	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	8
Ec74	64	4	≤0.25	≤0.25	≤0.25	≤0.25	2	1	≤0.25	≤0.25	32	4	4
Ec75	>128	32	0.5	≤0.25	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	4
Ec76	>128	16	0.5	≤0.25	≤0.25	≤0.25	4	2	≤0.25	≤0.25	128	4	8
Ec77	>128	16	0.5	≤0.25	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	4
Ec78	>128	16	32	1	2	0.5	8	2	32	0.5	>128	8	4
Ec79	>128	64	>128	64	32	32	>128	64	64	32	>128	>128	>128
Ec81	>128	32	2	2	≤0.25	≤0.25	8	4	1	8	>128	16	8
Ec82	>128	16	>128	>128	32	2	>128	>128	>128	128	>128	>128	128
Ec83	>128	16	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	8
Ec84	128	4	0.5	≤0.25	≤0.25	≤0.25	2	2	≤0.25	≤0.25	32	8	4
Ec85	>128	32	1	1	≤0.25	≤0.25	8	4	≤0.25	≤0.25	>128	8	16
Ec86	>128	64	16	2	0.5	≤0.25	16	8	8	0.5	>128	8	8
Ec87	>128	16	0.5	≤0.25	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	4
Ec88	>128	32	0.5	≤0.25	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	8
Ec89	>128	16	0.5	≤0.25	≤0.25	≤0.25	2	2	≤0.25	≤0.25	>128	8	4
Ec90	>128	16	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	8
Ec91	>128	64	32	64	1	32	>128	64	2	4	>128	>128	>128
Ec92	>128	64	2	2	≤0.25	≤0.25	2	2	≤0.25	≤0.25	>128	16	>128
Ec93	>128	32	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	32	8
Ec94	>128	16	0.5	≤0.25	≤0.25	≤0.25	4	2	0.5	≤0.25	>128	32	4
Ec95	>128	16	≤0.25	≤0.25	≤0.25	≤0.25	2	2	≤0.25	≤0.25	>128	4	8
Ec96	>128	32	0.5	≤0.25	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	4
Ec97	>128	32	0.5	≤0.25	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	8	4
Ec98	>128	64	>128	64	32	32	>128	64	128	32	>128	>128	>128
Ec99	>128	64	>128	64	32	64	>128	64	32	32	>128	>128	>128

	MIC (µg/ml)												
	AMX	**AMC**	**CTX**	**CTC**	**CRX**	**CRC**	**CXM**	**CXC**	**CAZ**	**CAC**	**PIP**	**PIT**	**FOX**
Ec100	>128	16	0.5	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4
Ec101	>128	16	0.5	0.5	≤0.25	≤0.25	2	2	≤0.25	≤0.25	≤0.25	8	4
Ec102	>128	16	0.5	0.5	≤0.25	≤0.25	2	2	≤0.25	≤0.25	>128	4	4
Ec103	>128	32	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	16	8
Ec104	>128	64	>128	64	32	32	>128	64	128	32	>128	>128	>128
Ec105	>128	64	1	1	≤0.25	≤0.25	4	4	≤0.25	0.5	>128	16	>128
Ec106	>128	16	≤0.25	≤0.25	≤0.25	≤0.25	2	1	≤0.25	≤0.25	>128	4	2
Ec107	>128	32	1	1	≤0.25	≤0.25	4	4	0.5	1	>128	16	16
Ec108	>128	64	>128	>128	128	32	>128	64	64	32	>128	>128	>128
Ec109	>128	16	1	0.5	≤0.25	≤0.25	2	2	≤0.25	≤0.25	64	16	16
Ec110	>128	64	16	64	8	32	>128	64	128	32	>128	128	>128
Ec111	>128	8	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	64	≤0.25	1
Ec112	>128	32	1	0.5	≤0.25	≤0.25	2	2	≤0.25	≤0.25	>128	4	4
Ec113	>128	32	1	0.5	≤0.25	≤0.25	4	2	≤0.25	≤0.25	>128	16	4
Ec114	>128	64	>128	64	32	32	>128	64	128	32	>128	>128	>128
Kp115	>128	16	0.5	0.5	≤0.25	≤0.25	2	2	≤0.25	≤0.25	128	16	4
Kp116	>128	32	0.5	0.5	≤0.25	≤0.25	2	1	0.5	0.5	>128	16	2
Kp117	>128	64	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	16	>128
Kp118	>128	16	1	2	≤0.25	≤0.25	8	8	2	0.5	>128	>128	16
Kp119	>128	16	0.5	0.5	≤0.25	≤0.25	4	2	0.5	≤0.25	>128	16	4
Kp120	>128	128	8	32	1	≤0.25	128	64	0.5	≤0.25	32	32	>128
Kp121	>128	64	32	32	8	8	>128	32	>128	16	>128	>128	>128
Kp122	>128	16	0.5	0.5	≤0.25	≤0.25	4	2	≤0.25	≤0.25	128	16	4
Kp123	>128	8	1	0.5	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	8	4

NCCLS susceptibility breakpoints (µg/ml):
AMX (amoxicillin) ≤8; AMC (amoxicillin/clavulanic acid) ≤4; CTX (cefotaxime) ≤8; CTC (cefotaxime/clavulanic acid) ≤4; CRX (ceftriaxone) ≤8; CRC (ceftriaxone/clavulanic acid) ≤4; CXM (cefuroxime) ≤8; CXC (cefuroxime/clavulanic acid) ≤4; CAZ (ceftazidime) ≤8; CAC (ceftazidime/ clavulanic acid) ≤4; PIP (piperacillin) ≤16; PIT (piperacillin/tazobactam) ≤8; FOX (cefoxitin) ≤8.

TABLE 3.2: Antimicrobial susceptibility profiles of isolates with extended resistance profiles (n=37).

	MIC (µg/ml)												
	AMX	**AMC**	**CTX**	**CTC**	**CRX**	**CRC**	**CXM**	**CXC**	**CAZ**	**CAC**	**PIP**	**PIT**	**FOX**
Ec4	>128	128	2	2	≤0.25	≤0.25	8	8	≤0.25	≤0.25	>128	16	>128
Ec14	>128	128	32	64	1	1	>128	128	0.5	0.5	64	64	>128
Ec17	64	64	>128	64	16	32	>128	64	64	32	>128	>128	>128
Ec18	>128	128	>128	64	32	64	>128	64	64	32	>128	>128	>128
Ec28	>128	128	>128	128	128	64	64	64	128	64	>128	128	128
Ec29	>128	8	8	4	≤0.25	≤0.25	16	16	1	0.5	>128	>128	64
Ec34	>128	128	>128	64	64	64	>128	128	128	64	>128	>128	>128
Ec35	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec43	>128	64	>128	64	32	32	128	64	128	32	>128	128	>128
Ec44	>128	64	>128	32	16	16	>128	32	64	32	>128	>128	>128
Ec46	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec53	>128	64	16	64	0.5	2	>128	64	2	4	>128	64	>128
Ec55	>128	64	32	32	2	32	>128	64	2	32	>128	64	>128
Ec56	>128	64	16	64	2	2	>128	64	2	2	>128	64	>128
Ec57	>128	64	>128	64	32	32	>128	64	128	32	>128	128	128
Ec59	>128	64	>128	64	128	32	>128	64	128	32	>128	>128	>128
Ec60	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec61	>128	128	16	32	2	2	>128	64	2	2	>128	64	>128
Ec62	>128	64	128	32	8	8	>128	32	>128	16	>128	>128	>128
Ec63	>128	64	32	4	2	≤0.25	32	32	>128	4	>128	64	>128
Ec64	>128	64	>128	64	32	32	>128	64	128	32	>128	128	>128
Ec66	>128	64	>128	64	32	32	>128	64	64	32	>128	>128	>128
Ec68	>128	64	>128	64	128	32	>128	64	64	32	>128	>128	>128
Ec79	>128	64	>128	64	32	32	>128	64	64	32	>128	>128	>128
Ec82	>128	16	>128	>128	32	2	>128	>128	>128	128	>128	>128	128
Ec91	>128	64	32	64	1	32	>128	64	2	4	>128	>128	>128
Ec92	>128	64	2	2	≤0.25	≤0.25	2	2	≤0.25	≤0.25	>128	16	>128
Ec98	>128	64	>128	64	32	32	>128	64	128	32	>128	>128	>128
Ec99	>128	64	>128	64	32	64	>128	64	32	32	>128	>128	>128

	MIC (µg/ml)												
	AMX	**AMC**	**CTX**	**CTC**	**CRX**	**CRC**	**CXM**	**CXC**	**CAZ**	**CAC**	**PIP**	**PIT**	**FOX**
Ec104	>128	64	>128	64	32	32	128	64	128	32	>128	>128	>128
Ec105	>128	64	1	1	≤0.25	≤0.25	4	4	≤0.25	0.5	>128	16	>128
Ec108	>128	64	>128	>128	128	32	>128	64	64	32	>128	>128	>128
Ec110	>128	64	16	64	8	32	>128	64	128	32	>128	128	>128
Ec114	>128	64	>128	64	32	32	>128	64	128	32	>128	>128	>128
Kp117	>128	64	1	1	≤0.25	≤0.25	4	4	≤0.25	≤0.25	>128	16	>128
Kp120	>128	128	8	32	1	≤0.25	128	64	0.5	≤0.25	32	32	>128
Kp121	>128	64	32	32	8	8	>128	32	>128	16	>128	>128	>128

NCCLS susceptibility breakpoints (µg/ml):
AMX (amoxicillin) ≤8; AMC (amoxicillin/clavulanic acid) ≤4; CTX (cefotaxime) ≤8; CTC (cefotaxime/clavulanic acid) ≤4; CRX (ceftriaxone) ≤8; CRC (ceftriaxone/clavulanic acid) ≤4; CXM (cefuroxime) ≤8; CXC (cefuroxime/clavulanic acid) ≤4; CAZ (ceftazidime) ≤8; CAC (ceftazidime/clavulanic acid) ≤4; PIP (piperacillin) ≤16; PIT (piperacillin/tazobactam) ≤8; FOX (cefoxitin) ≤8.

TABLE 3.3: MIC_{50} and MIC_{90} of the β-lactam agents alone and in combination with an inhibitor for all the clinical isolates (n=123).

Antimicrobial agent	Susceptibility breakpoint	% Susceptible at breakpoint	MIC (µg/ml)		
			MIC_{50}	MIC_{90}	Range
Amoxicillin	≤8	0.8	>128	>128	8->128
Amox/clav	≤4	24	16	64	2->128
Cefotaxime	≤8	76	1	>128	≤0.25->128
Cefotax/clav	≤4	76	1	64	≤0.25->128
Ceftriaxone	≤8	84	≤0.25	32	≤0.25-128
Ceftriax/clav	≤4	80	≤0.25	32	≤0.25-64
Cefuroxime	≤8	74	4	>128	≤0.25->128
Cefurox/clav	≤4	63	4	64	≤0.25->128
Ceftazidime	≤8	81	≤0.25	128	≤0.25->128
Ceftaz/clav	≤4	79	≤0.25	32	≤0.25-128
Piperacillin	≤16	1.6	>128	>128	≤0.25->128
Pip/tazobactam	≤8	71	16	>128	≤0.25->128
Cefoxitin	≤8	67	8	>128	1-128

Amoxicillin in combination with clavulanic acid (2μg/ml) was effective against 23% of all isolates (Table 3.2). Approximately 23% of the isolates became susceptible on addition of clavulanic acid, 36% showed intermediate resistance, and 59% still remained resistant. Resistance to the combination of amoxicillin and clavulanic acid was first described in 1989 in clinical isolates of *E. coli* isolated from urine samples in France (Therrien & Levesque, 2000). More than a decade since then, the results of this study identified a similar problem. In France, the mechanism of resistance was found to be the production of TEM-derived β-lactamases showing reduced affinity for clavulanic acid (Therrien & Levesque, 2000). It would be interesting to identify the β-lactamase responsible for resistance in South African urinary isolates since the resistance values from this study are much higher. The challenge is to find values applicable to South Africa, as it has been noted that difficulties encountered in measuring susceptibility to amoxicillin-clavulanic acid can cause disagreement between laboratories and that evaluation of increased resistance is specific for each laboratory.

Piperacillin exhibited a slightly better activity against all the isolates (Table 3.2). In contrast to Livermore (1995) who summarised that 30% of hospital *E. coli* isolates are resistant to piperacillin (Livermore, 1995), this study found that 93.4% of the isolates were resistant to piperacillin. This value is slightly higher than the 80% resistance found by Thompson *et al*. (1990), where, among 74 strains that included a wide variety of types and levels of β-lactamase, only 20% were

susceptible to piperacillin. Babini & Livermore (2000) reported that all putative ESBL producers were resistant to piperacillin at 16μg/ml.

Addition of 4μg/ml tazobactam enhanced the activity of piperacillin, with 70.7% of piperacillin resistant β-lactamase producing clinical isolates now being susceptible, with the MIC_{50} at 16μg/ml. Livermore & Yuan (1996) found that piperacillin/tazobactam inhibited 70% of putative ESBL producers at 16+4μg/ml. In reducing the MIC of piperacillin from resistant (>128μg/ml) to susceptible (16μg/ml) among enteric isolates, our findings are also similar to those obtained by Kuck *et al.* (1989).

It has been noted that a great majority of inhibitor-resistant TEM-producing *E. coli* isolates remained susceptible to the piperacillin-tazobactam combinations, but not to a combination of amoxicillin with clavulanic acid. This difference may be due to the greater inherent activity of piperacillin alone against inhibitor-resistant TEM-producing *E. coli* isolates (Nicholas-Chanoine, 1997). There was no difference in the number of isolates with intermediate resistance, while the number of resistant isolates also decreased by more than 50%.

Seventy percent of the isolates were susceptible to cefotaxime. This was a similar result to one from South India in 1990, where sensitivity to cefotaxime was 96% (Nandivada & Amyes, 1990). Addition of clavulanic acid to cefotaxime reduced the MIC_{50} and MIC_{90} by one dilution only. This reduction is insufficient to render any of the isolates susceptible to the combination (Chaibi *et al*., 1999) as

ESBL production is defined as a three fold or more dilution in the presence of clavulanic acid (Babini & Livermore, 2000).

The majority of the isolates were susceptible to ceftazidime (81.3%). These findings were similar to those reported from South India in 1990, where 133 out of 138 *E. coli* isolates tested were susceptible (Nandivada & Amyes, 1990). The MIC range for transconjugants was notably reduced to susceptible levels (2μg/ml) in the presence of clavulanic acid (0.25 - 2μg/ml). There were no isolates with intermediate resistance. The percentage of resistant isolates (18.75%) remained the same even in the presence of clavulanic acid. In two comparative studies carried out in the United States in 1990/1992 and 1986/1994, the ceftazidime resistance rate was 55% and 43% respectively (Weiner *et al*., 1999; Nordmann, 1998). Many ESBL-producing clinical isolates have demonstrated frank resistance to ceftazidime (and aztreonam), and resistance to the agents can serve markers to identify ESBL-producing strains (Swenson *et al*., 1999)

Screening *E. coli* and *Klebsiella* isolates against cefuroxime is desirable in detecting organisms producing ESBLs as the hyper-producers of β-lactamases are resistant to cefuroxime (Leung *et al*., 1997). Eighty percent of the isolates were resistant to cefuroxime. Low rate of transferable β-lactam resistance (12.6%) were reported for faecal isolates of *Klebsiella* species from South Africa where 5 out of 80 isolates (6.25%) transferred β-lactam resistance of *E. coli* and none of *K. oxytoca* (0/22) (Leung *et al*., 1997). The findings are also similar to

those obtained from Danish intensive care units where less than five percent of *K. pneumoniae* isolates decreased susceptibility to cefuroxime (Schumacher *et al*., 2000). The challenge from these results is to investigate further if the β-lactamase likely to be responsible for low susceptibility is chromosomal or plasmid-borne.

Sixty seven percent of the isolates showed susceptibility to cefoxitin. This was also the case in a previous study carried out in South Africa by Essack *et al*., 1994, where the MIC results revealed a resistance to cefoxitin, even in the presence of 5μg/ml clavulanic acid (Essack *et al*., 1994). *E. coli* may become resistant to cephamycines and oxyimino cephalosporins as a results of promotor and attenuator mutations or the acquisition of ß-lactamases (Forward *et al.*, 2001). Resistance as a result of the loss of a porin has also been rorted for both *E. coli* and *K. pneumoniae* (Ananthan and Subha, 2005).

Current knowledge about antibiotic resistance in urinary tract isolates in South Africa is limited, although resistance due to ESBL production is a global problem. Studies attempting to investigate the production of ESBL in South Africa never included urine samples, except for one sample that was part of a wide variety of samples tested (Essack *et al*., 2001), yet multiple antibiotic resistant *Klebsiella* and *E. coli* are recovered from urine specimens (Weiner *et al*., 1999).

Results from the present study indicated that for treatment of urinary tract infections, cephalosporins are still useful (percentage susceptible at breakpoints

ranged from 74% to 84%) followed by piperacillin-tazobactam (71%), cefoxitin (65%), and amoxicillin-clavulanic acid (24%). Amoxicillin and piperacillin alone were not effective.

Recommended treatment of suspected infection with ESBL-producing organisms should include cefotaxime, ceftazidime, ceftriaxone, and cefoxitin. Laboratory reporting of cephalosporins susceptibility, however, has to be done with caution because ESBL producers are notoriously prone to be reported as susceptible as MICs often remain below the NCCLS breakpoints of 8-16μg/ml. Animal and clinical data indicate that even low level MIC (1-2μg/ml) ESBL mediated resistance is associated with clinical failure (Livermore & Yuan, 1996).

As 75% of the isolates appeared to be resistant to the inhibitor clavulanic acid, treatment with amoxicillin-clavulanic acid is not indicated. Based on high resistance percentages, it may be speculated that these strains are widespread in the country, but have not been reported. To maintain effective therapy against urinary isolates, it is imperative to monitor susceptibility data as antimicrobial resistance is constantly emerging.

CHAPTER 4

MECHANISMS OF RESISTANCE

4.1 INTRODUCTION

Antibiotic resistant *Enterobacteriaceae* cause major clinical problems in animal and human healthcare. This resistance is related to increasing consumption of antimicrobial drugs (Feira *et al*., 2002). ß-Lactam agents such as penicillins and cephalosporins are the most widely used antibiotics and the production of β-lactamases are the greatest cause of resistance to them. The impact of β-lactamases has already been huge, and their potential to challenge antimicrobial chemotherapy remains un-exhausted (Livermore, 1995). Definitive identification of these enzymes is possible by gene sequencing, but simple β-lactamase detection and typing tests can be valuable in the clinical laboratory (Livermore & Brown, 2001).

4.1.1 ESBLs

The genes generally responsible for β-lactamase mediated ampicillin resistance in *E. coli* and *Klebsiella* species can undergo single point mutations that result in the production of novel ß-lactamases capable of hydrolyzing extended spectrum cephalosporins, aztreonam, as well as older β-lactam agents. These enzymes, known as extended spectrum ß-lactamases (ESBLs) have been noted in several Gram negative species and are also associated with a variety of antimicrobial resistance profiles (Murray, 1999). Clinical isolates that produce ESBLs are frequently associated with nosocomial outbreaks.

ESBL activity in *Escherichia coli*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* can be detected by using a standard disc diffusion susceptibility test method with cefotaxime (30µg), ceftriaxone (30µg), ceftazidime (30µg), cefpodoxime (10µg), or aztreonam (30µg) discs. The most practical test is simply to screen with ceftazidime since virtually all ESBLs cause resistance to this compound. When the isolates show reduced susceptibility to ceftazidime, double disc tests can be used to screen for synergy between this compound and clavulanic acid, which is most conveniently available in amoxicillin-clavulanic acid (20µg:10µg) disc. When the ceftazidime zone is expanded by an MIC ratio of eight or more in the presence of clavulanic acid, production of an ESBL is inferred (Livermore, 1995; Watt *et al*., 2000).

Detection of organisms producing ESBLs can be difficult because their presence in a bacterial cell does not always produce a resistance phenotype when using traditional MIC of disc diffusion interpretative criteria published by the NCCLS (Tenover *et al*., 1999). Early detection of ESBLs can allow important infection control decisions to be made. Of importance in this study are direct tests for β-lactamase activity in Gram negative species and tests for ESBLs whereby a positive reaction indicates that the isolate is resistant to the β-lactam agent in question. There are thee direct β lactamase assays, the acidimetric, iodometric and chromogenic methods. In this study the iodometric method on crude extracts, employing phosphate-buffered penicillin and starch as substrates, was used. β-Lactamase activity on electrophoresis gels was detected by the chromogenic nitrocefin test.

4.1.2 Inhibitor potentiated disc diffusion (IPDD)

Putative ESBL producers can be identified on the basis of a positive result in the double disc synergy test, and the IPDD with or without inhibitor are used to confirm the double disc synergy test. IPDD is very sensitive and has the advantage that three substrates can be tested on one agar plate (Ho *et al*., 1998; Liu *et al*., 1998). The only drawback is that it is unable to detect inhibitor resistant groups of ESBLs. IPDD is more convenient than the double disc synergy test and much less expensive than E-test ESBL screening.

4.2 RESULTS AND DISCUSSION

All the strains of the family *Enterobacteriaceae* that were found to be intermediate or resistant to amoxicillin were suspected of harbouring one or more β-lactamases, as they all gave positive results with the iodometric test. Susceptibility patterns of 20 selected isolates of *E. coli* and *K. pneumoniae* suspected to harbour ESBLs to six β-lactams and two β-lactam- β-lactamase inhibitor combinations are shown in Table 4.1. All of the isolates were resistant to cefoxitin, piperacillin-tazobactam, cefuroxime, and had decreased susceptibilities to piperacillin, cefotaxime and amoxicillin compared with the MICs for the ESBL negative *E. coli* strain ATCC 25922. All the strains were susceptible to cefotaxime and ceftazidime.

4.2.1 Double disc diffusion

To provide clinically useful information and definitive positive reaction to confirm if the enzyme detected was not just any form of protein, activity was detected biologically by demonstrating the ineffectiveness of a β-lactam against a susceptible *E. coli* 711 in the double disc synergy method. Disc approximation that has served as the reference method for detecting ESBL producing strains for a number of years was used in this study (Tenover *et al*., 1999). Results for the selected isolates are given in Table 4.2.

Sixteen of the twenty selected isolates (80%) indicated the production of ESBL with double disc diffusion, using four discs. This finding is similar to that obtained by Gulay *et al*. (2000) who found that ESBL production was positive in 84% of the isolates as determined by the double disc synergy test. The proportion of ESBL producing selected strains with diminished inhibition zones, signifying a reduced susceptibility, was 80% for ceftazidime, 75% for aztreonam and cefoxitin, 60% for cephazolin. The results found for ceftazidime were similar to those reported by Ho *et al.* (1998), indicating that ceftazidime is effective in detecting ESBL producing isolates, using NCCLS breakpoints (Jacoby & Han, 1996; Murray, 1999; Tenover *et al*., 1999). In comparison, the percentage ESBL-producing strains found by using aztreonam and cefoxitin, was lower. The prevalence rate is, however, quite high compared with those of other reports (Stapleton *et al*., 1995; Liu *et al.,* 1998). The results furthermore show a wide range of frequencies of resistance and disagreements between laboratories, indicating that evaluation of increased resistance is specific for each laboratory.

Although cephalothin is not included in the NCCLS list of substrates for extended spectrum ESBLs (NCCLS, 1997), it was included, because at the time of the study it as used in the treatment of UTIs in local hospitals However, this cephalosporin showed similar ESBL detection when compared to the other antimicrobial agents used in this study (Table 4.2). Clavulanic acid and tazobactam had the same effect on all isolates, enhancing the action of amoxicillin and piperacillin by 80% (16/20). This supports the statement by Lister

et al. (1997) that the potency of tazobactam, against plasmid-mediated β-lactamases, is generally comparable to that of clavulanic acid.

TABLE 4.1: Antimicrobial susceptibility of uropathogenic *E. coli/K. pneumoniae* transconjugated isolates (n=20).

	MIC (µg/ml)							
Isolate	**AMX**	**CTX**	**CTR**	**CXM**	**CAZ**	**FOX**	**PIP**	**PIT**
Ec 14	>1024	2	≤0.25	4	1	8	>1024	16
Ec 17	64	0.5	≤0.25	4	≤0.25	8	32	16
Ec 28	64	1	≤0.25	4	≤0.25	8	64	16
Ec 33	32	0.5	≤0.25	4	≤0.25	8	16	16
Ec 34	32	1	≤0.25	4	≤0.25	8	16	16
Ec 35	>1024	0.25	≤0.25	4	≤0.25	8	32	32
Ec 46	64	0.5	≤0.25	4	≤0.25	8	32	16
Ec 51	64	0.5	≤0.25	4	≤0.25	8	32	16
Ec 56	>1024	1	≤0.25	4	≤0.25	8	>1024	16
Ec 60	>1024	8	0.5	4	>1024	8	>1024	16
Ec 66	>1024	1	≤0.25	4	≤0.25	8	>1024	16
Ec 75	64	0.5	≤0.25	4	≤0.25	8	32	16
Ec 79	>1024	1	≤0.25	4	1	8	128	32
Ec 98	>1024	0.5	≤0.25	4	≤0.25	8	>1024	16
Ec 99	>1024	1	≤0.25	4	≤0.25	8	>1024	16
Ec 108	128	1	≤0.25	4	≤0.25	32	32	16
Ec 119	>1024	2	≤0.25	8	1	32	64	32
Kp 78	>1024	2	≤0.25	16	0.5	16	>1024	32
Kp 82	>1024	4	0.5	4	>1024	8	>1024	16
Kp 94	>1024	0.5	≤0.25	4	≤0.25	16	>1024	16
***E. coli* 711**	>1024	0.5	≤0.25	≤0.25	≤0.25	4	>1024	8

AMX= amoxicillin, CTX= cefotaxime, CTR= ceftriaxone, CXM= cefuroxime, CAZ = ceftazidime, FOX= , cefoxitin, PIP= piperacillin, PIT= piperacillin-tazobactam.

TABLE 4.2: Disc diffusion test results of selected uropathogenic *E. coli/K. pneumoniae* transconjugated isolates (n=20).

Isolate	Zone diameter (mm)							
	AMX	**AMC**	**PIP**	**PIT**	**ATM**	**FOX**	**CTN**	**CAZ**
Ec 14	10	12	14.7	24.9	26	16	18	26.6
Ec 17	0	19	8	24.7	32.5	28.6	18.8	26
Ec 28	0	11	9.6	25	35	31.4	19.7	31
Ec 33	0	16.8	13.5	26	35	19.5	20	31.4
Ec 34	0	12.5	14.4	20.9	33.5	20.5	23.5	34
Ec 35	0	19.4	18.6	29	36	30.2	22.2	28
Ec 46	0	8	15.5	30	12.7	30	12.5	13.5
Ec 51	24.6	16	28.5	31	36	27.5	28	34
Ec 56	23	12	29.9	30.8	37	30	26.5	32
Ec 60	0	15.7	0	24	9	26	16.6	0
Ec 66	0	21	8.3	24.5	29	19.6	23.8	35.4
Ec 75	22	22	24.7	22	32.2	29.9	21	29.9
Ec 79	22.7	18.7	29.4	27.9	36.9	31.3	26.6	32.2
Ec 98	0	20	7.9	24.4	32	30.5	16.6	28
Ec 99	0	19.4	9.9	26	33.6	33.6	16.6	30.5
Ec 108	21	25	27	28.8	35	33.8	27	28.8
Ec 119	10	11.9	22.5	23	26	18	16.6	27
Kp 78	19.6	21	27.7	29	37.5	32	28	33.5
Kp 82	0	9.2	14.7	24	27.7	16	18	23.6
Kp 94	0	21.6	10	27.4	33	30.3	21.6	31.1
***E. coli* 711**	29.9	22	29.5	31.4	37.3	27.3	27	26.8

ATM= aztreonam; FOX= cefoxitin; CTN= cephalotin; CAZ= ceftazidime; AMC= amoxicillin/ clavulanic acid.

Susceptibility breakpoints: ATM ≤28; FOX ≤23; CTN ≤19; CAZ ≤25; (P.L. Ho *et al.*, 1998).

4.2.2 Effect of an inhibitor

Inhibition of ß-lactamases by 16 µg/ml clavulanic acid is shown in Table 4.3. The MICs of 5/20 isolates (Ec35, Ec46, Ec66, Ec79 and Ec108) were unaffected by clavulanic acid. Usually cefotaxime and ceftazidime are tested with and without clavulanic acid (4 µg/ml). Some literature suggests that in order to avoid false negative results due to substrate specificity, a minimum of two agents should be tested by the inhibitor potentiated disc diffusion method (Ho *et al*., 1998). Amoxicillin was used to demonstrate the effect of an inhibitor. Resistance was also passed from donor isolates to transconjugants, yet most of the transconjugants were sensitive to the presence of clavulanic acid.

4.2.3 Isoelectric focusing (IEF)

In Table 4.4 the isoelectric point (pI) values for ß-lactamases produced by 37 isolates with extended resistance profiles, selected from Chapter 3, are given. These include the 20 selected isolates from Table 4.1 & 4.2. Control strains carrying plasmids encoding TEM-1-4 and SHV-1-2, SHV-14 were co-focused in parallel with the extracts and served as standards (Table 4.5). It is possible to detect the properties of a β-lactamase if its isoelectric focusing pattern is identical with that of an enzyme that has already been characterized (Matthew *et al*., 1975). Isoelectric focusing indicated the production of β-lactamases in 32/37 isolates (pIs ranging from 3.4 to 9.1). Approximately 25% of these isolates

produced β-lactamases with two different pI values, while in the remaining 75% a single pI value was detected. This finding is similar to that reported by Feira *et al.* (2002), where 27% of isolates produced two or more enzymes, and 73% produced a single enzyme.

Table 4.6 shows the β-lactamases detected by isoelectric focusing in the 20 selected isolates that were moderately susceptible or resistant to amoxicillin-clavulanic acid. Eight isoelectric focusing profiles were found. Fifty percent of these isolates appeared to produce both TEM and SHV enzymes, as electro-focusing bands were seen in the regions typical of both enzyme families. Multiple TEM β-lactamases have previously been found in single isolates, and combinations of TEM and SHV enzymes have also been reported in studies done in South Africa (Essack *et al.*, 2001).

TABLE 4.3: Inhibitor potentiation, using amoxicillin + 16µg/ml clavulanic acid.

Isolate	MIC (µg/ml)	
	Amoxicillin	**Amoxicillin + Clavulanic acid**
Ec14	>1024	4
Ec17	>1024	4
Ec28	>1024	1
Ec33	>1024	4
Ec34	>1024	2
Ec35	>1024	2
Ec46	>1024	>1024
Ec51	>1024	2
Ec56	>1024	>1024
Ec60	>1024	4
Ec66	>1024	4
Ec75	>1024	2
Ec79	>1024	2
Ec98	>1024	4
Ec99	>1024	8
Ec108	512	4
Ec119	>1024	>1024
Ec78	1024	>1024
Ec82	>1024	1
Kp94	>1024	>1024

Table 4.4: Susceptibility profiles and Isoelectric point values of ß-lactamases.

Isolate	MIC (µg/ml)							pI
	AMX	CTX	CRX	CXM	CAZ	PIP	FOX	
Ec14	>128	32	1	>128	0.5	64	>128	5.7
Ec17	64	>128	16	>128	64	>128	>128	4.8
Ec18	>128	>128	32	>128	64	>128	>128	6.8; 7.2
Ec19	>128	128	32	32	128	>128	4	4.6
Ec28	>128	>128	128	64	128	>128	128	6.9; 8.4
Ec33	>128	8	≤0.25	64	1	>128	>128	5.2
Ec34	>128	>128	64	>128	128	>128	>128	8.6; 8.4
Ec35	>128	>128	32	>128	128	>128	>128	4.6
Ec43	>128	>128	32	>128	128	>128	>128	5.8
Ec44	>128	>128	16	>128	64	>128	>128	8.0
Ec46	>128	>128	32	>128	128	>128	>128	5.3; 7.9
Ec47	>128	0.5	1	4	≤0.25	>128	4	6.8
Ec51	>128	1	≤0.25	4	≤0.25	>128	8	4.6
Ec53	>128	16	0.5	>128	2	>128	>128	8.3
Ec56	>128	16	2	>128	2	>128	>128	no band
Ec57	>128	>128	32	>128	128	>128	128	7.5
Ec59	>128	>128	128	>128	128	>128	>128	8.8
Ec60	>128	>128	32	>128	128	>128	>128	5.0
Ec61	>128	16	2	>128	2	>128	>128	8.9; 8.6
Ec62	>128	128	8	>128	>128	>128	>128	no band
Ec63	>128	32	2	32	>128	>128	>128	7.7; 5.5
Ec66	>128	>128	32	>128	64	>128	>128	5.5
Ec68	>128	>128	128	>128	64	>128	>128	7.0
Ec75	>128	0.5	≤0.25	4	≤0.25	>128	4	8.2; 3.4
Ec78	>128	32	2	8	32	>128	4	6.5
Ec79	>128	>128	32	>128	64	>128	128	8.9
Ec82	>128	>128	32	>128	>128	>128	>128	5.4
Ec86	>128	16	0.5	16	8	>128	8	9.0; 7.8
Ec98	>128	>128	32	>128	128	>128	>128	no band
Ec99	>128	>128	32	>128	32	>128	>128	no band
Ec104	>128	>128	32	>128	128	>128	>128	9.0; 8.0
Ec108	>128	>128	128	>128	64	>128	>128	no band
Ec113	>128	1	≤0.25	4	≤0.25	32	4	8.4; 4.8
Ec119	>128	0.5	≤0.25	4	32	>128	4	5.0
Ec120	>128	8	1	128	0.5	>128	>128	7.7
Kp94	>128	0.5	≤0.25	4	0.5	>128	4	6.6
Kp118	>128	1	≤0.25	8	2	>128	16	6.3
Kp121	>128	32	8	>128	>128	>128	>128	8.1; 4.6

NCCLS susceptibility breakpoints (µg/ml): AMX (amoxicillin) ≤8; CTX (cefotaxime) ≤8; CRX (ceftriaxone) ≤8; CXM (cefuroxime) ≤8; CAZ (ceftazidime) ≤8; PIP (piperacillin) ≤16; FOX (cefoxitin) ≤8

Table 4.5: Isoelectric point values of standard strains.

Strain	pI
E.coli Atcc 25922	8.4
SHV-1	No band
SHV-2	8.2; 5.8
SHV-4	9.1; 8.4
TEM-1	5.7
TEM-2	No band
TEM-3	5.2
TEM-4	5.5

Seventy percent of the selected isolates (14/20) produced enzymes that co-focused with an SHV related enzyme (pI range 7.0-8.9) and were suspected to be SHV derived (Chanal *et al*., 1989; Liu *et al*., 1998; Siu *et al*., 1999). The second most common pI was in the range 5.4-6.3 (enzymes co-focusing with TEM derived enzymes [Essack *et al*., 2001]) which were detected in 25% (5/20) of the isolates. Two of these strains expressed SHV related β-lactamases in addition to the TEM-related β-lactamases. These results are contrary to those obtained by Stapleton *et al.* (1995), who found the majority of isolates (67%) to produce enzymes that co-focused with TEM related enzymes (pI 5.4). Four isolates produced β-lactamases with a pI of 6.5 to 6.9.

Diverse and complex β-lactamases have been found in the Bloemfontein isolates and this is disconcerting. However, these isolates appear to produce β-lactamases that represent major types found worldwide. There is also need to

pursue β-lactamases with pIs between 6.5-6.9 since a similar result was obtained by Essack *et al*. (2001), from Durban isolates, but without any follow-up. Pitout *et al*. (1998) found that *Proteus mirabilis* and *E. coli* isolates from various parts of South Africa produced TEM-26-type, SHV-2 and SHV-5 ESBLs. They found an Amp C-related enzyme with a pI of 8.0, that conferred resistance to cefoxitin as well as the expanded spectrum cephalosporins in a *K. pneumoniae* strain. In the current study, 25% of the isolates grouped together, showing pI>8. Nine out of 10 of these isolates were actually resistant to cefoxitin, which included eight *E. coli* and one *K. pneumoniae* strain (Kp121). Resistance in this *K. pneumoniae* may be due to the loss of OmpK35 and OmpK36, as well as the presence of an AmpC type enzyme.

Findings further identified the organisms as producing different ESBLs. These enzymes pose a serious problem for antimicrobial therapy as some of these enzymes can also be encoded by transposons, which are genetic elements capable of transfer among a wide variety of plasmids and chromosomes. Not all *Klebsiellae* strains resistant to cephalosporins are ESBL-producers and the detection of isolates with other potent β-lactamases presents further challenges. Some *Klebsiellae* strains have acquired plasmids (that have been found in *E. coli* as well) encoding Amp C enzymes that have "escaped" from the chromosomes of other *Enterobacteriaceae*. Organisms inferred to have ESBLs are reported be resistant to all extended spectrum cephalosporins irrespective of the results of routine susceptibility tests (Pitout, 1998).

CHAPTER 5

TRANSFER OF RESISTANCE

5.1 INTRODUCTION

The ability of plasmids to transfer genes from one cell to another was first discovered in the 1950s by Joshua Lederberg and Edward Tatum in studies on a particular *E. coli* plasmid (Atlas *et al*., 1998). Large plasmids such as resistance transfer factor (80kb) can often mediate their own transfer from one cell to another by a process called conjugation. Conjugative plasmids that encode resistance to one or more antibiotics are of particular significance for understanding the genetic basis of antibiotic resistance (Murray *et al.*, 1998). Strains of *K. pneumoniae* and *E. coli* resistant to newer ß-lactam antibiotics where mechanism of resistance involved is due to a new transferable, plasmid mediated ß-lactamases, have been described (Jarlier *et al.*, 1988; Leung *et al.*, 1997). Antimicrobial susceptibility is routinely determined by disk diffusion comparative method at the routine diagnostic laboratory and in the last decade resistance to antibiotics used to treat UTIs has increased, prompting investigations into the spread of such resistance.

In this study *E. coli* and *K. pneumoniae* isolated from patients suspected of suffering from UTI, that showed unusual pattern of resistance to most ß-lactam antibiotics, including broad spectrum cephalosporins, were included to determine if they harboured resistance determinants (genes) carried on plasmids and to determine the transmissibility of resistance determinants. Antimicrobial resistance in the hospital environment is of great concern to clinical practice since production of plasmid-mediated ß-lactamases seems to be the major mechanism of resistance to amoxicillin in *E. coli* and many other Enterobacteria (Brun *et al.,* 1994). In this study the susceptibility of the wild type strains and their transconjugants were determined for ß-lactam agents on their own as well as in the presence of an inhibitor, clavulanic acid, in order to determine the transferability of resistance mediated by ESBL. Leung *et al.* (1997) found transferable ß-lactamases and carried out Southern blotting/hybridisation studies to detect ß-lactamase genes on non-transferable plasmids. In this study the presence of plasmids was investigated and whether or not its resistance characteristics could be transferred by conjugation.

5.2 RESULTS AND DISCUSSION

Non-lactose fermenting colonies of the recipient *E. coli* strain (Ec711) and *K. pneumoniae* that grew on MacConkey agar containing nalidixic acid and ampicillin were isolated as presumed transconjugants. From all of the 123 isolates subjected to conjugation, 26 transconjugant strains were isolated. Susceptibility profiles are illustrated in Table 5.1-5.3. The MIC of amoxicillin for all the transconjugants ranged from 32->128 µg/ml, a value two-fold higher than the susceptibility breakpoint (≤8µg/ml). The MIC range of piperacillin for the transconjugants was 16->128 µg/ml, which also indicated that resistance was transferred (Table 5.1-5.3). As all 26 of the transconjugants were resistant to amoxicillin and piperacillin, it may be an indication that resistance to these antibiotics is carried on the plasmid and was transferred by conjugation. All the donors were resistant to all the antibiotics tested, except to ceftriaxone that showed reduced susceptibility patterns. However, it has been reported that ß-lactamases are able to reduce susceptibility without raising MICs above the breakpoint (Livermore, 1995). As it was demonstrated that resistance to the oxyiminocephs and cefoxitin was not readily transferred to the *E. coli* recipient, it may be suggested that the gene(s) responsible for resistance is not carried on a self-transmissible plasmid.

TABLE 5.1: Comparative MICs of donors and transconjugants against ß-lactam agents (n=25).

	MIC (µg/ml)													
	AMX		CTX		CRX		CXM		CAZ		PIP		FOX	
Isolate	D	T	D	T	D	T	D	T	D	T	D	T	D	T
Ec14	>128	>128	32	2	1	≤0.2	>128	4	0.5	1	64	>128	>128	8
Ec17	64	64	>128	0.5	16	≤0.2	>128	4	64	≤0.2	>128	32	>128	8
Ec28	>128	64	>128	1	128	≤0.2	64	4	128	≤0.2	>128	64	128	8
Ec33	>128	32	8	0.5	≤0.2	≤0.2	64	4	1	≤0.2	>128	16	8	8
Ec34	>128	>128	>128	1	64	≤0.2	>128	4	128	≤0.2	>128	16	>128	8
Ec35	>128	>128	>128	≤0.2	32	≤0.2	>128	4	128	≤0.2	>128	32	>128	8
Ec46	>128	64	>128	0.5	32	≤0.2	>128	≤0.2	128	≤0.2	>128	32	>128	8
Ec51	>128	64	>128	0.5	32	≤0.2	>128	4	128	≤0.2	>128	32	>128	8
Ec53	>128	64	16	0.5	0.5	≤0.2	>128	4	2	≤0.2	>128	32	>128	16
Ec56	>128	>128	>128	1	2	≤0.2	>128	4	2	≤0.2	>128	>128	>128	8
Ec57	>128	>128	>128	0.5	32	≤0.2	>128	4	128	≤0.2	>128	>128	>128	16
Ec60	>128	>128	>128	8	32	0.5	>128	4	128	>128	>128	>128	>128	8
Ec61	>128	64	16	0.5	2	≤0.2	>128	4	2	≤0.2	>128	16	>128	8
Ec66	>128	>128	>128	1	32	≤0.2	>128	4	64	≤0.2	>128	>128	>128	8
Ec75	>128	64	0.5	0.5	0.5	0.5	4	4	32	0.5	>128	32	4	8
Ec78	>128	>128	32	2	2	≤0.2	8	16	32	0.5	>128	>128	4	16
Ec79	>128	>128	>128	1	32	≤0.2	>128	4	64	1	>128	128	>128	8
Ec82	>128	>128	>128	4	32	0.5	>128	4	>128	>128	>128	>128	128	8
Ec91	>128	64	32	0.5	1	≤0.2	>128	4	2	≤0.2	>128	16	>128	8
Ec94	>128	>128	0.5	0.5	≤0.2	≤0.2	4	4	0.5	≤0.2	>128	>128	4	16
Ec98	>128	>128	>128	0.5	32	≤0.2	>128	4	128	≤0.2	>128	>128	>128	8
Ec99	>128	>128	>128	1	32	≤0.2	>128	4	32	≤0.2	>128	>128	>128	8
Ec108	>128	128	>128	1	128	≤0.2	>128	4	64	≤0.2	>128	32	>128	32
Ec113	>128	64	0.5	0.5	≤0.2	≤0.2	2	4	≤0.2	≤0.2	128	64	4	8
Kp119	>128	>128	0.5	2	≤0.2	≤0.2	4	8	0.5	1	>128	64	4	32

AMX = amoxicillin; CTX = cefotaxime; CRX = ceftriaxone; CXM = cefuroxime; CAZ = ceftazidime; PIP = piperacillin; FOX = cefoxitin.
D = donor strain.
T = transconjugant.

TABLE 5.2: Percentage susceptibility, MIC_{50} and MIC_{90} of the ß-lactam agents for selected donors.

ANTIMICROBIAL AGENT	SUSC BREAKPOINT (µg/ml)	% SUSC AT BREAKPOINT	MIC (µg/ml)		
			MIC_{50}	MIC_{90}	RANGE
Amoxicillin	≤8	0	>128	>128	64->128
Amoxicillin/clav acid	≤4	10	64	128	8->128
Cefotaxime	≤8	20	>128	>128	0.5->128
Cefotaxime/clav acid	≤4	28	64	64	≤0.25->128
Ceftriaxone	≤8	44	16	64	≤0.25->128
Ceftriaxone/clav acid	≤4	48	16	32	≤0.25->128
Cefuroxime	≤8	20	>128	>128	2->128
Cefuroxime/clav acid	≤4	24	64	64	2->128
Ceftazidime	≤8	36	64	128	≤0.25->128
Ceftazidime/clav acid	≤4	44	32	32	≤0.25->128
Piperacillin	≤16	0	>128	>128	32->128
Piperacillin/tazobactam	≤4	0	128	>128	8->128
Cefoxitin	≤8	25	>128	>128	4->128

TABLE 5.3: Percentage susceptibility, MIC_{50} and MIC_{90} of the ß-lactam agents for the transconjugants isolated.

ANTIMICROBIAL AGENT	SUSC BREAKPOINT (µg/ml)	% SUSC AT BREAKPOINT	MIC (µg/ml)		
			MIC_{50}	MIC_{90}	RANGE
Amoxicillin	≤8	0	>128	>128	32->128
Amoxicillin/clav acid	≤4	0	32	64	32-128
Cefotaxime	≤8	100	0.5	2	≤0.25-8
Cefotaxime/clav acid	≤4	100	0.5	1	≤0.25-2
Ceftriaxone	≤8	100	≤0.25	≤0.25	≤0.25-0.5
Ceftriaxone/clav acid	≤4	100	≤0.25	≤0.25	≤0.25-0.25
Cefuroxime	≤8	96	4	4	4-16
Cefuroxime/clav acid	≤4	96	4	4	4-8
Ceftazidime	≤8	92	≤0.25	1	≤0.25->128
Ceftazidime/clav acid	≤4	100	≤0.25	1	≤0.25-2
Piperacillin	≤16	16	64	>128	16->128
Piperacillin/tazobactam	≤4	84	16	32	16-32
Cefoxitin	≤8	76	8	16	8-32

All the selected transconjugants were susceptible to cefotaxime, ceftriaxone and cefoxitin. Two isolates (Ec60 and Ec82) indicated the transfer of high-level resistance to ceftazidime (MIC>128µg/ml) (Table 5.1). Eighty % of the selected donors were resistant to cefuroxime, while only one of the corresponding transconjugants (Ec78T) had an MIC (16 µg/ml) that would indicate it to be resistant. In previous studies on faecal isolates of *Klebsiella* spp., low rates of transferable β-lactam resistance (12.6%) were reported in South Africa where 5/80 isolates (6.25%) transferred β-lactam resistance of *E. coli* and none of *K. oxytoca* (0/22) (Leung *et al*., 1997). The challenge from these results is to investigate further if the β-lactamase likely to be responsible for low transfer is chromosomal or plasmid-borne. Sixty seven % of the isolates and 76% of the selected donors were susceptibile to cefoxitin.

As 75% of the isolates were resistant to the inhibitor clavulanic acid, treatment with amoxicillin/clavulanic acid is not indicated (Table 5.5). It may be possible that these highly resistant are widely encountered, but have not been reported. To maintain effective therapy against urinary tract infections, it is imperative to monitor susceptibility data as antimicrobial resistance is constantly emerging.

TABLE 5.4: Representation of transferability of ß-lactam resistance in donors and transconjugants with high MIC values for amoxicillin and piperacillin.

	MIC (µg/ml)						
Isolate	**AMX**	**CTX**	**CRX**	**CXM**	**CAZ**	**PIP**	**FOX**
Ec46 D	>128	>128	32	>128	128	>128	>128
Ec46 T	64	0.5	≤0.25	≤0.25	≤0.25	32	8
Ec57 D	>128	>128	32	>128	128	>128	>128
Ec57 T	>128	0.5	≤0.25	4	≤0.25	>128	16
Ec66 D	>128	>128	32	>128	64	>128	>128
Ec66 T	>128	1	≤0.25	4	≤0.25	>128	8
Ec98 D	>128	>128	32	>128	128	>128	>128
Ec98 T	>128	0.5	≤0.25	4	≤0.25	>128	8
Ec99 D	>128	>128	32	>128	32	>128	>128
Ec99 T	>128	1	≤0.25	4	≤0.25	>128	8
Ec108 D	>128	>128	128	>128	64	>128	>128
Ec108 T	128	1	≤0.25	4	≤0.25	32	32

AMX = amoxicillin; CTX = cefotaxime; CRX = ceftriaxone; CXM = cefuroxime; CAZ = ceftazidime; PIP = piperacillin; FOX = cefoxitin.
D = donor strain.
T = transconjugant.

The MICs of amoxicillin for both donors and resistant transconjugants were notably reduced to susceptible levels in the presence of 16µg/ml clavulanic acid (Table 5.5).

TABLE 5.5: Inhibitor potentiation against amoxicillin tested at 16µg/ml clavulanic acid

	DONOR MIC (µg/ml)		TRANSCONJUGANT MIC (µg/ml)	
Isolate	**AMX**	**AMC**	**AMX**	**AMC**
Ec14	>1024	>1024	16	2
Ec17	>1024	>1024	16	2
Ec28	>1024	2	>1024	1
Ec33	>1024	>1024	16	4
Ec34	>1024	>1024	8	2
Ec35	>1024	>1024	>1024	2
Ec46	>1024	4	512	>1024
Ec51	>1024	>1024	8	2
Ec56	>1024	>1024	>1024	0
Ec60	>1024	>1024	>1024	4
Ec66	>1024	>1024	>1024	4
Ec75	>1024	>1024	8	2
Ec79	>1024	>1024	>1024	2
Ec98	>1024	4	>1024	4
Ec99	>1024	8	>1024	8
Ec108	512	>1024	8	4
Ec119	>1024	>1024	>1024	>1024
Ec78	1024	>1024	1024	>1024
Ec82	>1024	>1024	>1024	1
Kp94	>1024	>1024	>1024	0

AMX = amoxicillin; AMC = amoxicillin/clavulanic acid.

Resistance to ß-lactam antibiotics found in many Enterobacteriaceae is thought to be due to production of plasmid-mediated ß-lactamases (Brun *et al.,* 1994). The large size of the plasmids harboured by micro-organisms, may enable them to mediate their own transfer between bacterial cells during conjugation. Eighty-three % (25/30) of the donor strains indicated the presence of one or two plasmids, with molecular sizes ranging from 10-13 kb (Figure 5.1, gels A-D). Plasmids were detected in only 40% (12/30) of the transconjugants, with sizes ranging from 2-10 kb. The presence of these plasmids in the wild type strains and the corresponding transconjugants is illustrated in Table 5.6. A plasmid of 9-10 kb was detected in most clinical isolates (20/25) and transconjugants (10/25). Four of the donor strains and three of the transconjugants isolates had more than one plasmid band. A similar situation was found in a study done by Essack *et al.* (2001) in Durban, Kwa Zulu Natal, South Africa. Further investigation would be necessary to determine if these plasmids could be the source of different enzymes and the cause of multiple resistance to spread.

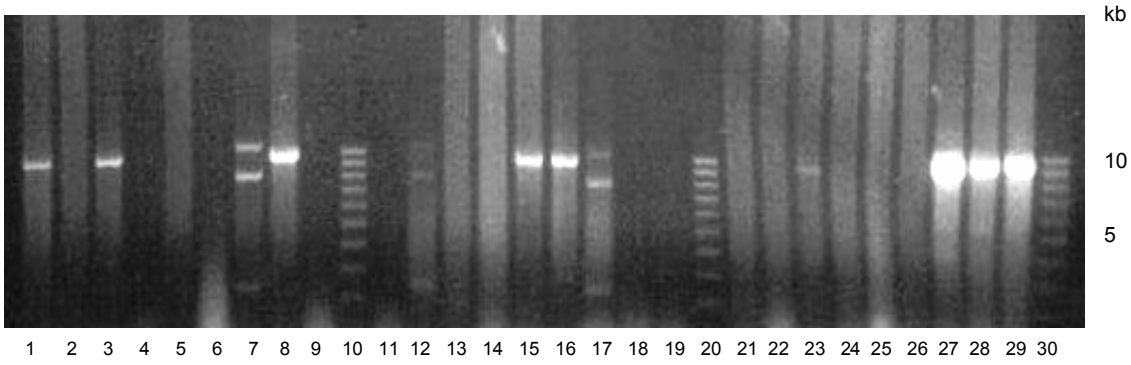


FIGURE 5.1: Plasmid profiles of the selected transconjugants. Lanes: **1**, Ec66 ; **2**, Ec35; **3**, Ec68; **4**, Ec14; **5**, Ec17; **6**, Ec82; **7**, Ec75; **8**, Ec78; **9**, Ec79; **10**, Plasmid size marker; **11**, Ec113; **12**, Ec46; **13**, Ec51; **14**, Ec53; **15**, Ec56; **16**, Ec57; **17**, Ec108; **18**, Ec60; **19**, Ec61; **20**, Plasmid size marker; **21**, Ec28; **22**, Ec59; **23**, Ec33; **24**, Ec34; **25**, Ec91; **26**, Kp119; **27**, Ec94; **28**, Ec98; **29**, Ec99; **30**, Plasmid size marker.

TABLE 5.6: Estimated plasmid sizes of isolates and their transconjugants (kb).

ISOLATE	DONOR	TRANSCONJUGANT
Ec14	10	-
Ec17	10	-
Ec28	10	-
Ec33	10	-
Ec34	10	8
Ec35	10	-
Ec46	10	2, 8, 10
Ec51	10	-
Ec53	10	-
Ec56	-	9
Ec57	10	9
Ec60	-	-
Ec61	9	-
Ec66	9.5	9
Ec75	-	5.5, 8, 10.5
Ec78	9	9
Ec79	9	-
Ec82	9	-
Ec91	10	-
Ec94	-	9-10
Ec98	10	9-10
Ec99	10	9-10
Ec108	3, 11, 13	7, 9
Ec113	9, 11	-
Kp119	10	-

CHAPTER 6

MOLECULAR ANALYSIS OF RESISTANCE

6.1 INTRODUCTION

The incidence of nosocomial infection caused by members of the family Enterobacteriaceae that produce ESBLs and other enzymes capable of hydrolysing cefotaxime, ceftriaxone, ceftazidime and aztreonam are increasing worldwide (Tenover & Rasheed, 1999). In the past, antimicrobial resistance used to be assessed using conventional susceptibility testing methods; however, recently genetic susceptibility test methods have become available. These methods have the potential to provide a more rapid and reliable assessment when compared to phenotypic testing methods. A specific example for which genetic methods have been demonstrated to be more accurate than conventional phenotypic methods include assessment of extended spectrum β-lactamase resistance in Gram negative facultative anaerobic bacteria such as *E. coli* and *K. pneumoniae* (Cockerill, 1998).

Most genotypic methods include a step in which the “target” nucleic acid is amplified. The process is usually accomplished with the use of polymerase chain reaction (PCR). The PCR is recognised as being one of the most useful, compelling and exciting advancement accelerating the pace of life science research. Due to its powerful utility, breadth of application and reliability, it can detect and amplify DNA

sequences from just a few molecules of “target” nucleic acid (SIGMA Catalogue, 2001). PCR based methods have proved extremely effective in detecting antimicrobial resistance genes of complex mechanisms found in Gram negative and Gram positive bacteria, including among others, those encoding β-lactam resistance in Enterobacteriaceae (*bla*$_{TEM}$, *bla*$_{SHV}$)(Pfaller *et al*., 2001). It has the advantage of being speedy, sensitive and specific (Mims *et al*., 1998; Roche Magazine 40, *sine annum*; Schwarts, 2002), and can produce extremely large number of relevant genes without having to clone the said DNA (Eglinton & Hall, 1996). The PCR method was employed in this study to generate genomic amplification products of *bla*$_{TEM}$ and or *bla*$_{SHV}$ genes in selected *E. coli* and *K. pneumoniae* isolates. Since TEM-type and SHV-type ß-lactamases are reported to be ESBLs responsible for resistance to ß-lactam antibiotics (Pfaller *et al.,* 2001), PCR amplification of the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes was carried out with the use of *bla*$_{TEM}$ and *bla*$_{SHV}$ specific primers on selected amoxicillin resistant strains (Chapter 3), and their corresponding transconjugant strains.

6.2 RESULTS AND DISCUSSION

PCR amplification results are demonstrated in Figures 6.1-6.4 and summarised Tables 6.1-6.2. The *bla*$_{TEM}$ gene was detected in 23/26 of the donor strains and 7/26 of the transconjugants. Four of these transconjugants demonstrated the presence of a plasmid (Chapter 5), which could imply that the gene and of course the resistance was carried on the plasmid and consequently transferred by conjugation (Table 6.1). The *bla*$_{SHV}$ gene was detected in 14/26 of the donor strains, but was absent from any of the transconjugants (Table 6.2). Some strains of *K. pneumoniae* have been reported to carry non-transferrable SHV-encoding plasmids of 120kb (Leung *et al.,* 1997). One of these donors (Ec60) also did not indicate the presence of the *bla*$_{TEM}$ gene. The other 13 all produced positive amplification products for both the *bla*$_{TEM}$ and the *bla*$_{SHV}$ genes (Table 6.1 & 6.2). Simultaneous production of multiple SHV ESBLs by single isolates has rarely been reported previously in South Africa, but a combination of TEM and SHV enzymes had previously been found in single isolates (Essack *et al.*, 2001). The *bla*$_{TEM}$ gene was demonstrated in two of the transconjugants (Ec53 and Ec57), yet a plasmid could not be detected in either of these isolates.

The PCR amplification products may have been positive as a result of contamination, or the gene may have been transferred by another mechanism, which will require further studies. In two other transconjugants (Ec68 and Ec108) one or two plasmids plasmids were demonstrated, yet no *bla*$_{TEM}$ or *bla*$_{SHV}$ gene were found after PCR amplification. The transconjugant of Ec108 was resistant to amoxicillin

and piperacillin, but not to the third generation cephalosporins of cefoxitin, as was the case for the wild type donor strain. Could it be possible that resistance in the transconjugant strain was expressed only to some agents? This is a very interesting observation and should be further investigated.

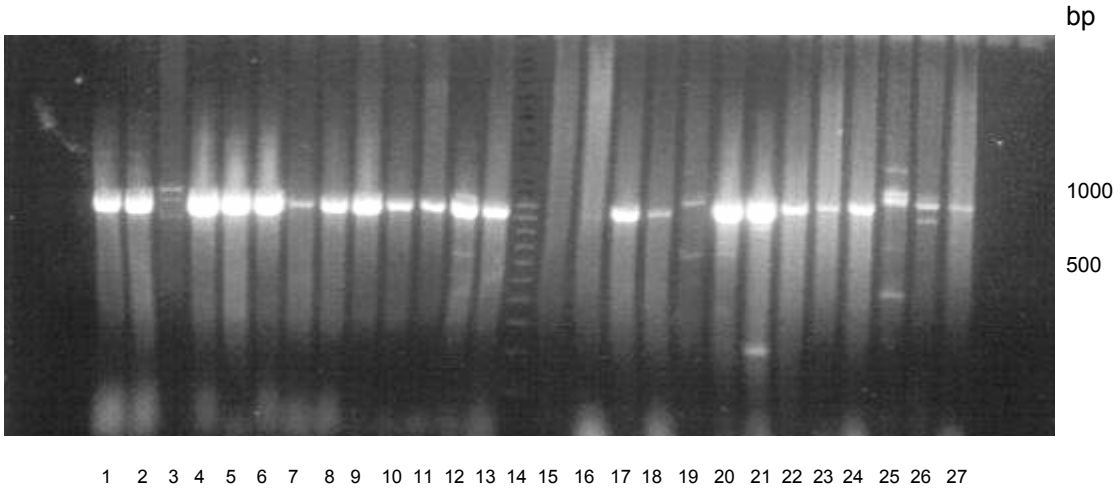


Figure 6.1: PCR products (*bla*$_{TEM}$ gene ±971 bp) of 26 donor strains obtained after amplification. Lanes **1**, Ec14; **2**, Ec17; **3**, Ec18; **4**, Ec28; **5**, Ec33; **6**, Ec34; **7**, Ec35; **8**, Ec43; **9**, Ec44; **10**, Ec46; **11**, Ec51; **12**, Ec53; **13**, Ec57; **14**, DNA MWM; **15**, Ec59; **16**, Ec60; **17**, Ec61; **18**, Ec66; **19**, Ec68; **20**, Ec75; **21**, Ec79; **22**, Ec82; **23**, Ec98; **24**, Ec99; **25**, Ec108; **26**, Ec113; **27**, Ec119

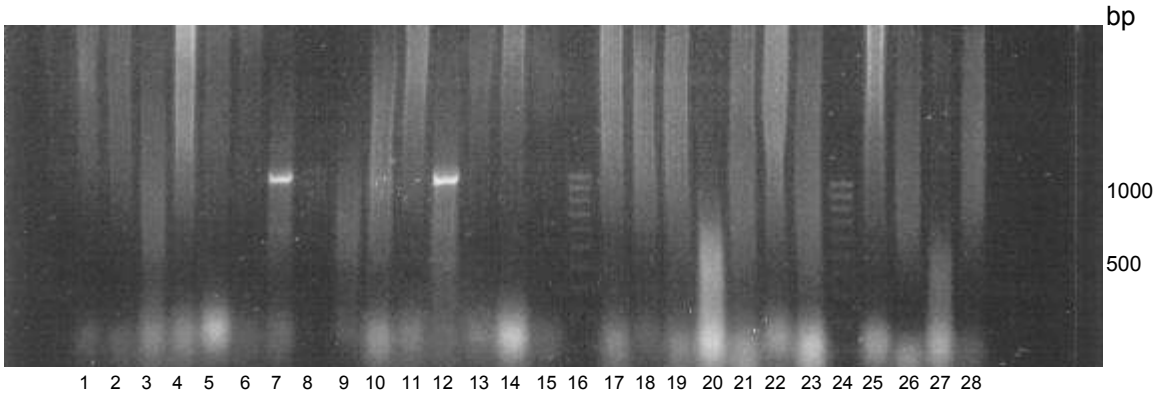


Figure 6.2: PCR products (*bla*$_{TEM}$ gene ±971 bp) of 26 transcon-jugants obtained after amplification. Lanes **1**, Ec14; **2**, Ec17; **3**, Ec18; **4**, Ec28; **5**, Ec33; **6**, Ec34; **7**, Ec35; **8**, Ec43; **9**, Ec44; **10**, Ec46; **11**, Ec51; **12**, Ec53; **13**, Ec57; **14**,Ec59; **15**, Ec60; **16**, DNA MWM; **17**, Ec61; **18**, Ec66; **19**, Ec68; **20**, Ec75; **21**, Ec79; **22**, Ec82; **23**, Ec98; **24**, DNA MWM; **25**, Ec99; **26**, Ec108; **27**, Ec113; **28**, Ec119

Table 6.1: PCR products after amplification with the TEM primers.

Isolate	*bla*$_{TEM}$ gene		Plasmid in
	Donor	Transconjugant	Transconjugant (Ch 5)
Ec14	+	-	-
Ec17	+	-	-
Ec18	+	-	-
Ec28	+	-	-
Ec33	+	-	-
Ec34	+	-	-
Ec35	+	+	-
Ec43	+	-	-
Ec44	+	-	-
Ec46	+	+	+
Ec51	+	-	-
Ec53	+	+	-
Ec57	+	+	-
Ec59	-	-	-
Ec60	-	-	-
Ec61	+	-	-
Ec66	+	+	+
Ec68	-	-	+
Ec75	+	+	+
Ec79	+	-	-
Ec82	+	-	-
Ec98	+	-	+
Ec99	+	+	+
Ec108	-	-	+
Ec113	+	-	-
Ec119	+	-	-

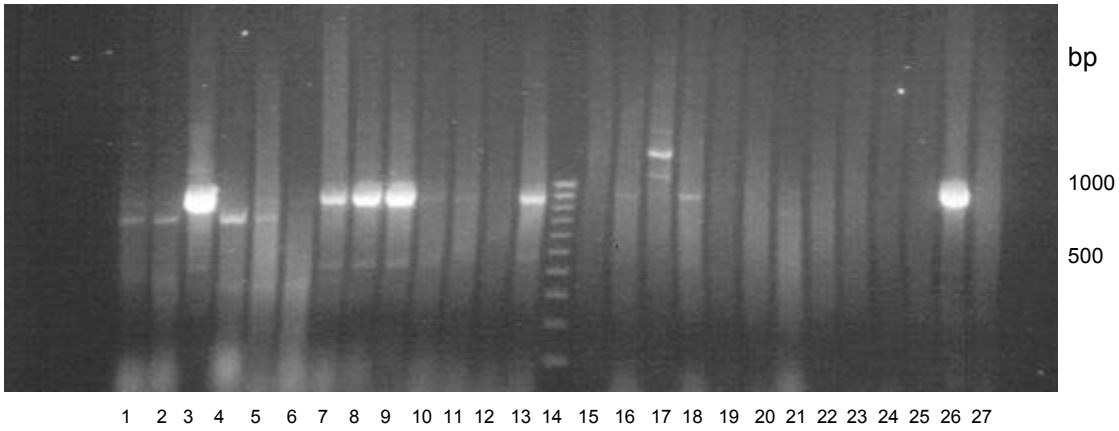


Figure 6.3: PCR products (*bla*$_{SHV}$ gene ±885 bp) of 26 donor strains obtained after amplification. Lanes **1**, Ec14; **2**, Ec17; **3**, Ec18; **4**, Ec28; **5**, Ec33; **6**, Ec34; **7**, Ec35; **8**, Ec43; **9**, Ec44; **10**, Ec46; **11**, Ec51; **12**, Ec53; **13**, Ec57; **14**, DNA MWM; **15**, Ec59; **16**, Ec60; **17**, Ec61; **18**, Ec66; **19**, Ec68; **20**, Ec75; **21**, Ec79; **22**, Ec82; **23**, Ec98; **24**, Ec99; **25**, Ec108; **26**, Ec113; **27**, Ec119

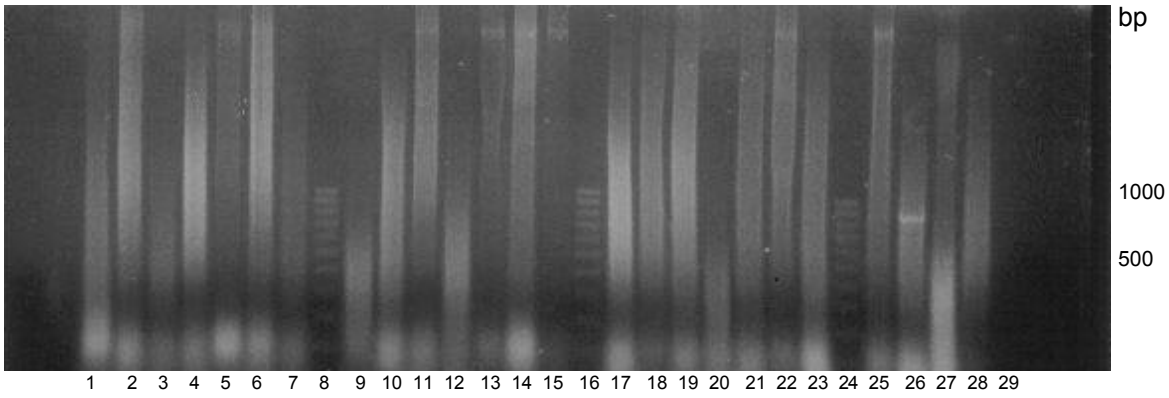


Figure 6.4: PCR products (*bla*$_{SHV}$ gene ±885 bp) of 26 transconjugants obtained after amplification. Lanes **1**, Ec14; **2**, Ec17; **3**, Ec18; **4**, Ec28; **5**, Ec33; **6**, Ec34; **7**, DNA MWM; **8**, Ec35; **9**, Ec43; **10**, Ec44; **11**, Ec46; **12**, Ec51; **13**, Ec53; **14**, Ec57; **15**, Ec59; **16**, DNA MWM; **17**, Ec60; **18**, Ec61; **19**, Ec66; **20**, Ec68; **21**, Ec75; **22**, Ec79; **23**, Ec82; **24**, DNA MWM; **25**, Ec98; **26**, Ec99; **27**, Ec108; **28**, Ec113; **29**, Ec119.

Table 6.2: PCR products after amplification with the SHV primers.

Isolate	*bla*$_{SHV}$ gene		Plasmid in
	Donor	Transconjugant	Transconjugant (Ch 5)
Ec14	+	-	-
Ec17	+	-	-
Ec18	+	-	-
Ec28	-	-	-
Ec33	-	-	+
Ec34	+	-	-
Ec35	+	-	-
Ec43	+	-	-
Ec44	+	-	-
Ec46	+	-	+
Ec51	+	-	-
Ec53	-	-	-
Ec57	+	-	-
Ec59	-	-	-
Ec60	+	-	-
Ec61	+	-	-
Ec66	+	-	+
Ec68	-	-	+
Ec75	-	-	+
Ec79	-	-	-
Ec82	-	-	-
Ec98	-	-	+
Ec99	-	-	+
Ec108	-	-	+
Ec113	+	-	-
Ec119	-	-	-

Multidrug resistance was more evident in isolates containing *bla*$_{SHV}$ related genes (Table 6.3). The presence of multiple gene copies is thought to increase the likelyhood of enzyme hyper-production, thus potentially increasing resistance to weak substrates and ß-lactamase inhibitor combinations (Essack *et al.*, 2001). Three isolates (Ec59, Ec68 and Ec108) showed no evidence of either *bla*$_{TEM}$ or *bla*$_{SHV}$ genes and antimicrobial resistance was also not detected in the transconjugant after conjugation. It was indicated that ß-lactam resistance could be attributed to the presence and action of ß-lactamases such as the TEM and SHV type enzymes. It was also indicated that this resistance, as well as the genes implicated, can be transmitted between bacteria, in so doing causing problems specifically in the hospital environment.

Table 6.3: Multi-drug resistance in strains harbouring *bla*$_{SHV}$ genes.

	MIC (µg/ml)						
Isolate	**AMX**	**CTX**	**CRX**	**CXM**	**CAZ**	**PIP**	**FOX**
Ec14	>128	32	1	>128	0.5	64	>128
Ec17	64	>128	16	>128	64	>128	>128
Ec18	>128	>128	32	>128	64	>128	>128
Ec34	>128	>128	64	>128	128	>128	>128
Ec35	>128	>128	32	>128	128	>128	>128
Ec43	>128	>128	32	>128	128	>128	>128
Ec44	>128	>128	16	>128	64	>128	>128
Ec46	>128	>128	32	>128	128	>128	>128
Ec51	>128	1	≤0.25	4	≤0.25	>128	8
Ec57	>128	>128	32	>128	128	>128	128
Ec60	>128	>128	32	>128	128	>128	>128
Ec61	>128	16	2	>128	2	>128	>128
Ec66	>128	>128	32	>128	64	>128	>128
Ec113	>128	1	≤0.25	4	≤0.25	>128	4

Susceptibility breakpoints:

AMX = amoxicillin (≤8µg/ml); CTX = cefotaxime (≤8µg/ml);
CRX = ceftriaxone; (≤8µg/ml); CXM = cefuroxime (≤8µg/ml);
CAZ = ceftazidime (≤8µg/ml); PIP = piperacillin (≤16µg/ml);
FOX = cefoxitin (≤8µg/ml).

CONCLUSIONS

Resistance to antibiotics and in particular the ß-lactam antibiotics are continuously causing increasing concern in hospitals worldwide. The most important and widespread mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria is due to enzyme mediated antibiotic degradation. β-Lactamase production is mediated by genes carried on a plasmid or on the chromosome. The genes encoding β-lactamases can reside on bacterial chromosomal DNA or on plasmids The TEM-type and SHV-type are widespread enzymes that attack the narrow-spectrum cephalosporins, cefamandole and cefoperazone and most of the penicillins. Resistance to expanded-spectrum cephalosporins had appeared in clinically significant Gram-negative bacteria, also as a result of the production of β-lactamases. Among the first of the extended-spectrum β-lactamases to cause significant clinical problems, were mutants derived from narrow-spectrum SHV-1 or TEM-1 β-lactamases. Low level TEM-1 causes resistance to ampicillin, amoxicillin and ticarcillin, while higher levels can result in resistance to piperacillin, mezlocillin, cephalothin, cefamandole and cefoperazone. SHV-1 is a narrow spectrum β-lactamase with activity against penicillins. ESBLs are a diverse group of enzymes that have the common property of causing resistance to expanded spectrum cephalosporins. There is a

considerable geographical spread of these β-lactamases and they have become a global problem in treating infections. They have been recognised to be encoded on plasmids and are therefore easily transmissible from one organism to another. The incidence of nosocomial infection caused by members of the family Enterobacteriaceae that produce ESBLs and other enzymes capable of hydrolysing cefotaxime, ceftriaxone, ceftazidime and aztreonam are increasing worldwide In light of this dilemma, the study was designed to determine if resistance in *E. coli* and *K. pneumoniae*, two of the most frequently encountered urinary pathogens was related to β-lactamase production and identify and characterise the principal β-lactamases involved.

One hundred and twenty-three clinical strains isolated from urine samples were identified as *E. coli* and *K. pneumoniae* and selected from the diagnostic laboratories as being resistant to amoxicillin. Antimicrobial susceptibility profiles revealed high-level resistance to ß-lactam agents such as amoxicillin, cefotaxime, cefuroxime, piperacillin and cefoxitin. The addition of an inhibitor was only effective in ±40% of the resistant isolates and 75% of the isolates was also resistant to clavulanic acid. ESBL production was indicated in 80% of the isolates by the double-disc diffusion method, the method routinely used in susceptibility testing in most diagnostic laboratories. To determine the properties of the ß-lactamases detected, their isoelectric focusing patterns were determined. This indicated the production of β-lactamases in 32/37 isolates with pI values ranging from 3.4 to 9.1, with ± 25% of these isolates producing β-lactamases with two different pI values.

Seventy percent of the selected isolates produced enzymes that co-focused with an SHV related enzyme (pI range 7.0-8.9) and were suspected to be SHV derived. The second most common pI was in the range 5.4-6.3 (enzymes co-focusing with TEM derived enzymes), detected in 25% of the isolates. Two of these strains expressed SHV related β-lactamases in addition to the TEM-related β-lactamases. The appearance of strong bands at pI 9.0 or above could be characteristic of an ampC enzyme and it may be necessary to carry out $bla_{TEMAMP\text{-}C}$ hybridization and subject the isolates to PCR amplification. Diverse and complex β-lactamases have been found in the Bloemfontein isolates and this is disconcerting. However, these isolates appear to produce β-lactamases that represent major types found worldwide.

The presence of plasmids were investigated and whether or not its significant characteristics could be transferred by conjugation. Twenty-six transconjugant strains were isolated, with their MICs for amoxicillin ranging from 32->128 µg/ml, a value two-fold higher than the susceptibility breakpoint (≤8µg/ml) and for piperacillin ranging from 16->128 µg/ml, also indicating resistance transfer. As all 26 of the transconjugants were resistant to amoxicillin and piperacillin, it may be an indication that resistance to these antibiotics is carried on the plasmid and was transferred by conjugation. Eighty-three % of the donor strains indicated the presence of one or two plasmids and plasmids were found in 40% of the transconjugants. A plasmid of 9-10 kb that was detected in most clinical isolates and transconjugants may be responsible for the production of ESBLs in these

strains. Four of the donor strains and three of the transconjugants isolates had more than one plasmid band.

The *bla*$_{TEM}$ gene was detected in 23/26 of the donor strains and 7/26 of the transconjugants of which four of the latter also carried a plasmid, implying possible resistance transfer by conjugation. The *bla*$_{SHV}$ gene was detected in 14/26 of the donor strains, but not in any the transconjugants and multi-drug resistance was also more evident in isolates containing *bla*$_{SHV}$ related genes. Both of the genes were detected in 13/26 of the donor strains. ß-Lactam resistance could be attributed to the presence and action of ß-lactamases such as the TEM and SHV type enzymes and this resistance can be transmitted between bacteria, causing problems specifically in the hospital environment. Further and continuous investigations are required to find a solution for this ever increasing problem.

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