BETA-LACTAM RESISTANCE PROFILES IN URINARY TRACT INFECTION AMONG ESCHERICHIA COLI ISOLATES IN BLOEMFONTEIN

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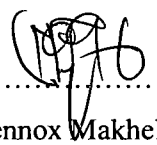

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Lennox Makhelane Maqutu

DEDICATION

To my mother, 'Mè 'Mamarelane and my late father ntate Dumezweni

For the love they have given me

"If I see further, it is by standing on the shoulders of giants" — Isaac Newton

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CHAPTER 1

LITERATURE REVIEW

1.1 URINARY TRACT INFECTIONS

Urinary tract infections represent a serious health problem. Eighty percent of all urinary tract infections in outpatients are caused by uropathogenic *Escherichia coli* (*E. coli*) strains possessing both types 1 and P-fimbriae. The remaining twenty percent of infections are caused by coagulase-negative staphylococci (*Staphylococci saprophyticus*) and other Gram-positive organisms, e.g. *Enterococcus faecalis*. The ability to produce P-fimbriae is correlated with the ability to cause urinary tract infections seemingly by mediating the adherence of the organism to uroepithelial cells. Type 1 mannose-sensitive pili are important in strains colonizing the bladder, while type P pili, encoded for by the *papG* operon, favour colonizing the kidney. Capsular acidic polysaccharide K-antigen is associated with the ability to cause pyelonephritis and enable *E. coli* to inhibit phagocytosis. Haemolysins may act as membrane-damaging toxins ***(1)***.

Escherichia coli strains that cause urinary tract infections belong to a restricted set of lineages that are characterized by certain O: K: H serotypes, and by other virulence factors such as adhesions and toxins that help to overcome the host's immune system and facilitate colonisation and invasion of the host. Although these virulence factors are generally inherited vertically, i.e. chromosomally, in some instances virulence determinants such as the *papG* operon may be transmitted horizontally by plasmids. Three molecular variants of *papG* (I, II and III) are encoded for by allelic genes. These *papG* variants exhibit subtly different receptor-binding preferences, which confer differences in host range and in clinical syndrome capability. Allele III is the predominant *papG* allele among *E. coli* strains in children and women with cystitis. Allele II is predominant among strains from children and women with uncomplicated pyelonephritis, particularly bacteraemia patients ***(1)***. Urinary tract infections (UTIs) remain one of the most common infections in adult women of all ages. Worldwide, an estimated 150 million UTIs occur annually. In the United States, UTIs account for more than six billion dollars in direct health care costs. These infections are a frequent reason for chemotherapy; however, antibiotic use for treating UTIs is problematic because of the very high rate of β-lactam resistance.

Recurrent urinary tract infections are common in young healthy women. Women with these infections have an increased susceptibility to vaginal colonisation with pathogenic organisms. Risk factors for recurrent urinary tract infections include sexual intercourse, use of spermicides and having a first UTI at an early age ***(2)***.

Many other factors that are thought to predispose to recurrent UTIs, including pre- and post-coital voiding patterns, frequency of urination, whereas wiping patterns and douching

have not been proven to be risk factors for UTIs. Contrary to the predominantly behavioural risk factors for young women, mechanical or physiological factors that affect bladder emptying are most strongly associated with recurrent UTIs in healthy post-menopausal women *(3)*.Urinary tract infections are common during pregnancy and all pregnant women should be screened for bacteriuria and subsequently treated with antibiotics when colonisation is present. Pregnant women are at risk because they develop urethral dilation from the beginning of the sixth week to the twenty-fourth week of their gestation period. Increased bladder volume and decreased bladder tone contribute to increased urinary stasis and urethrovesical reflux, consequently 20-30% of pregnant women develop urinary tract infections such as cystitis and pyelonephritis. Pyelonephritis can be a life-threatening disease, with increased risk of prenatal and neonatal morbidity *(4)*. Complications may result from neurogenic bladder dysfunction, of which the most frequent complications are urinary tract infections. Antibiotics should be administered immediately for these infections *(5)*.

Urinary tract infections are also very common in elderly people, although infection may be asymptomatic. In both women and men, the prevalence increases with increasing age. Elderly women who have lower urinary tract symptoms are less likely than younger women to be cured by antibiotics, particularly short courses of therapy, because of the selection pressure exerted by the use of antibiotics over a long period. In the elderly, the prevalence of urinary tract infection is high due to impaired immunity or a coexisting illness. Bacteriuria is uncommon in younger male populations beyond the newborn period (because of the long urethra) and in Jewish people (because of circumcision) as compared to elderly men beyond the age of sixty-five, who often have prostatitic enlargement. Factors that contribute to the development of urinary tract infections in the elderly include the following: loss of the oestrogen effect on the genitourinary mucosa among elderly women who have not been institutionalised, increased residual urine, and genitourinary abnormalities such as cystoceles, rectoceles and bladder diverticula. In addition, some chronic medical conditions e.g. diabetes mellitus occur more frequently in the elderly. Diabetics have a three times greater prevalence of bacteriuria than non-diabetic women *(6)*. Generally 10^5 or more colony forming units (CFUs) / ml of urine is regarded significant bacteriuria, although the patients may be asymptomatic or symptomatic.

1.2. PATHOGENESIS OF URINARY TRACT INFECTIONS

The first step in the establishment of urinary tract infections is the interaction of bacterial adhesive proteins with epithelial cells. This event is often followed by invasion of the epithelial cells. Invasion of host cells is regarded as the means by which bacteria escape the harsh extracellular environment where immunoglobulin, complement, defensin and other

antibacterial substances are in abundance. Uropathogenic *E. coli* (UPEC) are presumed to be the primary causative agents of urinary tract infections. One of the organelles that have been associated with the invasion of epithelial cells by UPEC is the type 1 pilus *(7)*. Type 1 pili are composite structures of the peritrichously-expressed filamentous surface structures. They consist of a 7 nm thick right-handed helical rod made up of repeating FimA sub-units joined to a 3 nm thick distal tip fibrillum containing two adaptor proteins, FimG and FimF, and the adhesin FimH. It requires at least eight genes for type 1 pili to assemble. Within the bladder the precise role of type 1 pili and specifically of FimH in triggering exfoliation of bladder epithelial cells is unclear. One theory consistent with available data is that type 1 pilus-mediated attachment and invasion facilitates the efficient delivery of bacteria-associated lipo-polysaccharide (LPS) to host epithelial cells. Subsequently, LPS interactions with the host receptors activate signalling pathways leading to induction of apoptosis and exfoliation. When UPEC interact with superficial bladder epithelial cells there can be a cascade of reactions. The uptake of UPEC by superficial bladder cells serves to entrap invading bacteria. The superficial bladder cells could be viewed as a vessel, collecting and storing UPEC for later disposal via exfoliation, followed by micturition *(7)*. Subsequently, infected superficial bladder epithelial cells eventually slough off into the urine, carrying with them any internalized bacteria. Therefore, UPEC must have a means of escaping from within dying superficial cells before completion of exfoliation, in order to persist within the bladder. Before exfoliation from infected superficial bladder cells, pathogenic events increase the likelihood that UPEC can successfully evade the exfoliation response and colonise other bladder epithelial cells. In effect, from the point of view of incoming UPEC, the superficial bladder cells may serve as a temporary beachhead, providing a passageway to surrounding superficial cells and to the less accessible underlying bladder epithelium. The underlying epithelial cells may provide a more stable environment for a long-term persistence of UPEC within the bladder, as well as protecting UPEC from potentially detrimental interactions with soluble antimicrobial products within the urine, including antibiotics. UPEC can persist within the bladder tissue even in the presence of antibiotic treatment.

1.3 TREATMENT OF URINARY TRACT INFECTIONS

Urinary tract infections are subdivided into urethritis, cystitis, prostatitis and pyelonephritis according to the localisation of infection. According to the type of infection, they can be divided into symptomatic, asymptomatic, acute (first or single), recurrent, chronic, complicated and uncomplicated infections. Clinical symptoms of acute, uncomplicated infections such as cystitis and leucocyturia in young women are sufficient

reason for the early initiation of a three-day empirical antimicrobial therapy. Urine culture should be carried out prior to the initiation of antimicrobial therapy especially in pregnant women, diabetics, recurrent UTIs and in the case of unsuccessful prior treatment in patients with pyelonephritis. All symptomatic UTIs, as well as asymptomatic bacteriuria in pregnant women, diabetics and, preschool children must be treated. All Patients are given prophylaxis prior to urologic-gynaecologic surgery. In complicated UTIs it is especially important to determine and eliminate, or at least control, the factors that complicate UTIs. Antimicrobial agents suitable for UTI therapy include fluoroquinolones, co-trimoxazole, β-lactam antibiotics, aminoglycosides and nitrofurantoin. Tetracycline, quinolones, nitroimidezole macrolides, and azalides in cases of sexually transmitted infections caused by *Chlamydia trachomatis, Neisseria gonorrhoeae (N. gonorrhoeae), Trichomonas vaginalis (T. vaginalis)* and *Ureaplasma urealyticum.* Cystitis is treated for 1-3 days or 7 days, asymptomatic bacteriuria for 3-7 days, uncomplicated pyelonephritis for 10-14 days and bacterial prostatitis for 2-4 weeks. Recommended duration of therapy for chronic and complicated UTIs is 7-14 days. In some patients, therapy can last for several weeks, even up to six months. Chemoprophylaxis in recurrent, uncomplicated UTIs should be given for at least six months ***(8)***.

Bacteriuria is common in pregnancy. If left untreated, asymptomatic bacteriuria will lead to acute pyelonephritis in 20-30% of cases. This may result in low birth weight infants, premature delivery and occasionally, stillbirth. Therefore, it is a serious threat for the mother and unborn child. Bacteriuria is associated with a 50% increase in the risk of premature delivery, pre-eclampsia, hypertension, anaemia and post-partum endometritis. Effective treatment of asymptomatic bacteriuria significantly reduces the incidence of pyelonephritis, premature deliveries and low birth weight infants. Before agents are prescribed in pregnancy, it is essential to ensure that they are safe for both the foetus and the mother. ß-lactam antibiotics such as nitrofurantoin, ampicillin, amoxicillin and cephalosporins are frequently used. Studies have shown that the pharmacokinetics of some β-lactam antibiotics is altered during pregnancy, resulting in faster renal elimination and lower plasma concentrations of these drugs. Therefore, the dose should be increased in pregnancy for these drugs ***(9)***.

1. 4 BETA-LACTAM RESISTANCE

Beta-lactam antibiotics are the most frequently prescribed antibiotics in the world. Resistance to this important class of antibiotics poses a very complex problem ***(10)***. Many strains of *E. coli* are resistant to a wide range of β-lactam antibiotics. Initially the use of

inhibitors to counteract β-lactam resistance was marginally successful but recently increasing numbers of reports of inhibitor resistance are being published. Resistance to β-lactam antibiotics in Gram-negative bacteria can arise in many different ways:

i. Production of β-lactam inactivating enzymes (β-lactamases). These enzymes may be plasmid- or chromosomally mediated. Ultimately all resistance arises from mutations in chromosomal or plasmid genes *(11, 12)*.
ii. Alterations to the target penicillin binding proteins (PBPs) or enzymes that will prevent inhibition of cell wall synthesis *(13)*.
iii. Reduced permeability (loss of porin proteins) that may prevent the attainment of effective periplasmic β-lactam concentration *(14)*.
iv. The ability to pump β-lactams out of the periplasmic space or multidrug efflux pumps mechanism *(15)*.
v. Derivation of extended-spectrum β-lactamases (ESBLs) by alteration of the configuration of the active sites of TEM, SHV and other β-lactamases, by mutation *(16)*.
vi. ß-Lactam resistance due to β-lactamase inhibition *(16)*.
vii. Combinations of the above.

Genetic recombination and selection can over time result in the accumulation of single step mutations and can thus multiply antimicrobial-resistant strains, particularly in hospitals. This is a consequence of exposing high-density patient populations to high antimicrobial use with the resultant risk of cross-infection *(16)*. Although distinct mechanisms exist for β-lactam resistance, it has now become evident that interplay between two or more resistance mechanisms is frequently responsible for high levels of resistance in clinical isolates of common pathogens such as *E. coli (17)*. Production of high levels of β-lactamase, either constitutively or induced, in addition to alterations in outer membrane proteins (porin), is frequently observed in *Klebsiella pneumoniae* and other Gram-negative bacteria *(18)*.

1.4.1 ß-LACTAMASE-MEDIATED RESISTANCE

The production of β-lactamases is the most prevalent mechanism of β-lactam resistance among clinical isolates of *E. coli*. These enzymes inactivate β-lactam antibiotics by hydrolysing the β-lactam ring of β-lactam compounds. This is a very efficient mechanism of β-lactam resistance to readily hydrolysable drugs, due to enzymes that are found in the periplasmic space between the outer and cytoplasmic membranes of Gram-negative organisms or free in the surrounding environment of Gram-positive organisms. Enhancement

of the level of enzyme production or decrease of drug entry caused by porin alterations may lead to resistance to even slowly hydrolysable drugs ***(18)***.

1.4.1.1 PLASMID-MEDIATED ß-LACTAMASES

Under optimum conditions, resistance transfer factors (RTF) can transfer antimicrobial resistance genes at high frequencies to other bacterial cells. These plasmids encode all necessary functions to transfer antimicrobial resistance markers from cell to cell by conjugation. When a conjugative, plasmid-containing population of donor cells encounters a population of recipient cells that do not contain plasmids, the plasmid will be transferred exponentially from donor cells to recipient cells. This transfer is much more rapid than is possible by mutation followed by selection. Many of the recipient cells, now called transconjugants, will thus become resistant to the antimicrobial for which resistance markers are carried on the donor plasmids ***(19)***.

The discovery of new β-lactam antibiotics with increased activity against Gram-negative bacteria was welcomed because Gram-negative bacteria began to replace staphylococci as the predominant nosocomial pathogens. However, resistance quickly became a problem due to plasmid-mediated β-lactamases. These enzymes were TEM and SHV types that disseminated first among the members of the *Enterobacteriaceae*, then to *Pseudomonas* and finally to members of the genera *Haemophilus* and *Neisseria.* Broad-spectrum β-lactam penicillins such as azlocillin, mezlocillin and piperacillin were soon developed followed by the β-lactamase-resistant cephalosporins, cefotaxime and ceftazidime. Recently, two new classes of β-lactam agents were developed viz monobactams (aztreonam) and carbapenems (imipenem, meropenem and ertapenem). These new agents are resistant to hydrolysis by the recognised types of β-lactamases. Mutants of TEM and SHV enzymes possessing activity against these extended-spectrum β-lactam agents quickly developed. In comparison to their parent enzymes, these mutant extended spectrum β-lactamases (ESBLs) have a few amino acid substitutions, enabling them to hydrolyse the newer penicillins and cephalosporins ***(20)***.

1.4.1.2 CHROMOSOMALLY MEDIATED ß-LACTAMASES

Any bacterium that has genetic information for the production of β-lactamase on its chromosome may mediate resistance to penicillins, cephalosporins, monobactams and carbapenems. These genes are collectively termed *ampC* genes and can be induced to high-level β-lactamase production by β-lactam antibiotics. Induction of *ampC* genes is dependent

upon a second gene, *ampR*. This gene is the transcriptional regulator. In the absence of the inducer (e.g. a β-lactam antibiotic), *ampR* represses the synthesis of β-lactamase whereas in the presence of a β-lactam agent, this gene activates the synthesis of β-lactamase. The activator form of *ampR* is regulated by *ampG*, which senses the disruption of peptidoglycan by a β-lactam agent. The repressor form of *ampR* is then converted to the activated form. When the *ampR* is in its activator form, the expression of *ampC* is greatly stimulated and the result is an increased production of β-lactamase, with resulting hydrolysis of the β-lactam and hence resistance to the β-lactam. In the absence of the inducer (i.e. the β-lactam agent), *ampR* represses the synthesis of β-lactamase. Deletion mutants of *ampR* repress the synthesis of β-lactamase. In the presence of the inducer, this gene activates the synthesis of β-lactamase. Deletion mutants of *ampR* generate a non-inducible phenotype that produces an enzyme constitutively at a low level. This is consistent with its dual role of both repressor and activator. The disruption of peptidoglycan synthesis by amoxycillin induces the synthesis of β-lactamase that depends on the second adjacent *ampR*, which represses the synthesis of β-lactamase. In the presence of the inducer, e.g. clavulanic acid, this gene activates the synthesis of AmpC β-lactamase. The repressor form of *ampR* is regulated by *ampD*, possibly in association with *ampE*. When the inducer is removed, *ampD* reverses *ampR* to its repressor form. This suppresses the production of AmpC and turns off the production of β-lactamase, and high minimum inhibitory concentration (MIC) values for amoxycillin are recorded ***(20)***.

1.4.2 PENICILLIN BINDING PROTEIN-MEDIATED RESISTANCE

ß-lactams exert their antibacterial effect by inactivating high-molecular-weight transpeptidases, carboxypeptidases or endopeptidases that catalyse the final stages of cross-linking reactions during peptidoglycan synthesis ***(21)***. These targets, which are called penicillin binding proteins (PBPs), have high affinities for β-lactam antibiotics and will therefore be inactivated by them, causing a weakened cell wall which leads to arrest of growth and cell death. Alterations to PBPs therefore cause β-lactam PBP-mediated resistance ***(22)***.
The other impediment to PBP-mediated resistance is that penicillin is a substrate analogue, and reduction in affinity needs a subtle restructuring of the active centre of the transpeptidase domain of the high-molecular-weight PBPs, so that they decrease their affinity for penicillin without impairing their ability to recognise the normal substrate. The rarity of PBP-mediated resistance is also because such resistance is difficult to achieve, because β-lactam agents have many killing targets. Reduction in the affinity of these targets for the antibiotic is necessary for the development of high-level resistance. In *N. gonorrhoeae* for example, PBP1 and PBP2

are essential enzymes, and inactivation of either of these is a lethal event, whereas PBP3 is a low-molecular-weight enzyme that is not believed to be a lethal target for β-lactam agents. This is shown by the fact that development of resistance in *Neisseria gonorrhoea* requires a reduction in the affinity of the high-molecular-weight PBP with the highest affinity, PBP2, as inhibition of this enzyme causes the bacterium to be inhibited at the MIC of penicillin for *N. gonorrhoeae (22)*.

1.4.3 ß-LACTAM RESISTANCE DUE TO IMPERMEABILITY

All Gram-negative bacteria are surrounded by an additional membrane layer, the outer membrane, which reduces the accessibility of the β-lactams to their target enzymes (PBPs). The ability of the outer membrane to exclude hydrophobic molecules is an unusual feature among biological membranes and serves to protect *E. coli* from bile salts. Because of its lipid nature, the outer membrane would be expected to exclude hydrophilic molecules as well. However, the outer membrane has special channels consisting of protein molecules called porins, which permit the passive diffusion of low-molecular-weight hydrophilic compounds like sugars, amino acids, certain ions and nutrient molecules. These molecules penetrate the outer membrane relatively slowly, which accounts for the relatively high antibiotic resistance of Gram-negative bacteria. Porins, exemplified by OmpC, D, and F and PhoE of *E. coli,* are trimeric proteins that penetrate both faces of the outer membrane. They form relatively non-specific pores that permit the free diffusion of small hydrophilic molecule across the membrane. Alterations in these porins or mutants that lack some of the porins diminish the amount of β-lactam antibiotics that can enter the cell, and therefore cause β-lactam resistance due to impermeability *(23)*. The outer membrane slows, but does not prevent, the entry of small hydrophilic molecules. Most β-lactam antibiotics penetrate so rapidly that drug level should equalise across the membrane. This happens in much less than one bacterial generation time; the drug is destroyed or removed once it reaches the periplasmic space. Removal is by β-lactamase, but it may also be via the PBPs side reaction by efflux-control mechanism *(24)*.

1.4.4 RESISTANCE BY EFFLUX-CONTROL MECHANISMS

Multi-drug efflux pumps with unusually broad specificity may create a decreased accumulation of β-lactam and other antibiotics inside the bacterial cell. This may be a consequence of physiological regulation or mutations in the *emrE* gene. Overproduction of EmrE protein through the introduction of multi-copy plasmids that contain the mutant gene

makes *E. coli* more resistant to tetracycline and erythromycin. The other mechanism is by way of multi-drug exporter proteins located in the cytoplasmic membrane, an outer membrane channel and a periplasmic "linker" protein that connects the outer membrane channel and the transporter protein. These pumps excrete drug molecules directly into the medium and because the outer membrane slows down the re-entry of the drugs, they produce significant resistance. Various types of multidrug efflux mechanisms exist e.g. Bmr (Bacillus multidrug resistance), NorA (norfloxacin) and QacA (quaternary ammonium compounds group), which pump out cationic dyes/membrane-permeable cations/fluoroquinolones and quaternary ammonium disinfectants/cationic dyes *(24)*.

1.4.5 THIRD-GENERATION ß-LACTAMS AND EXTENDED-SPECTRUM β-LACTAMASE-PRODUCING *ESCHERICHIA COLI* ISOLATES

Extended-spectrum β-lactamases were recognised because of their ability to hydrolyse cefotaxime, ceftazidime and a range of cephalosporins, monobactams and many other older β-lactam antibiotics except oxyimino-β-lactams. Because of the configuration of their active site they are related to TEM-1 or TEM-2 types, whilst others are of the SHV type. Their importance is that these previously rarely-observed enzymes may be more common in clinical strains than expected. Therefore, not only do they limit therapeutic options but their dissemination can also proliferate. They are also associated with selection pressure in the hospital environment *(25)*.

Resistance to the extended-spectrum β-lactam antibiotics occurs when mutants producing high levels of enzymes are selected during therapy. They are known as extended-spectrum β-lactamases because of their ability to hydrolyse a broader spectrum of β-lactam antibiotics than the parental broad-spectrum β-lactam antibiotics are able to do *(26)*.

1.4.6 ß-LACTAM RESISTANCE DUE TO CIRCUMVENTION OF β-LACTAMASE INHIBITION

Initially it was thought that β-lactamase inhibitors would solve the problem of β-lactam resistance. However, bacteria soon evolved mechanisms to circumvent the inhibitory effect of β-lactamase inhibitors. The mechanism involved is hyper-production of TEM-I β-lactamase, and other factors such as decreased uptake of the antibiotic. TEM-1 β-lactamases found mostly in *E. coli* are inhibitor-resistant, but in recent years a SHV-derived β-lactamase, SHV-10, resistant to clavulanic acid was reported for the first time in clinically significant isolates of *E. coli (27)*.

1.5 A FUNCTIONAL CLASSIFICATION SCHEME FOR β-LACTAMASES

Beta-lactamases may be classified according to their molecular structures, based on their nucleotide or amino acid sequences *(28)*. ß-lactamases may also be divided into groups based on their functional characteristics. The major groups of enzymes are defined by their substrate hydrolysis data and inhibitor profiles according to the classification scheme proposed by Bush *et al.* *(29)*. ß-lactamases may also be classified phenotypically by the Richmond-Sykes classification scheme *(29)*.

i. **Group 1** is a group of cephalosporinases that is not inhibited by clavulanic acid. Representative enzymes in this group are AmpC enzymes from Gram-negative bacteria; e.g. MIR-1.

ii. **Group 2** β-lactamases consist of a number of subclasses because of the diversity of substrates that they utilise. They tend to have a strong affinity for clavulanic acid. They are class A enzymes.

iii. **Group 2a** penicillinases include many of the penicillinases of Gram-positive organisms that are inhibited by active site-directed β-lactamase inhibitors. They are usually chromosomal enzymes, often inducible, generally with molecular weights of ± 30,000 and with basic isoelectric points. They are class C enzymes.

iv. **Group 2b** β-lactamases hydrolyse both penicillins and cephalosporins but are inhibited by clavulanic acid. TEM and SHV-1 enzymes are represented in this group.

v. **Group 2be** enzymes are of the molecular class A. Their preferred substrates are penicillins, narrow-spectrum and extended-spectrum cephalosporins and monobactams. Representative enzymes in this group are TEM-3, TEM-26 and SHV-2 to SHV-6.

vi. **Group 2br** enzymes are of the molecular class A but whose preferred substrates are penicillins only. They may or may not be inhibited by clavulanic acid. Representative enzymes in this group are TEM-30 to TEM-36, and TRC.

vii. **Group 2c** are the penicillinases whose preferred substrates are the penicillins and carbenicillin. These enzymes are inhibited by clavulanic acid. Representative enzymes in this group are PSE-1, PSE-3 and PSE-4. They are of the molecular class A.

viii. **Group 2d** are the penicillinases II and III respectively. Their preferred substrates are penicillins and cloxacillin. Representative enzymes in this group are OXA-1 to OXA-11, PSE-2 and OXA-10. They are of the molecular class D.

ix. **Group 2e** prefer cephalosporins as their substrates. They are inhibited by clavulanic acid. They are inducible cephalosporinases from *Proteus vulgaris* and belong to the molecular class A.

x. **Group 2f** are of molecular class A whose preferred substrates are penicillins, cephalosporins and carbapenems. They are inhibited by clavulanic acid.

xi. **Group 3** are of molecular class B. Their substrates are most β-lactams including carbapenems.

xii. **Group 4** have penicillins as their preferred substrates. Representative enzymes in this group are penicillinases from *Pseudomonas cepacia.*

1.6 EPIDEMIOLOGY

The upper and lower urinary tracts are the sources of approximately 40% of all urinary tract infections. Most nosocomial urinary tract infections occur in patients who have undergone some form of urinary tract procedure or indwelling catheters. Hospitalised women who have had complicated labours are particularly at risk. Indwelling catheters are a major predisposing factor for nosocomial urinary tract infections ***(30)***.

Group 1 AmpC β-lactamases are clinically important because they confer resistance to a wide variety of β-lactam antibiotics, including cefoxitin and β-lactam-β-lactamase-inhibitor combinations ***(31)***.

When using some of the newer β-lactam antibiotics to treat infections due to initially-sensitive strains, variants emerge that exhibit resistance not only to the drug employed, but also to the entire class of β-lactam antibiotics. Such multiple (joint) resistance results from hyper-production of class 1 β-lactamase due to mutation in the chromosomal *ampC* regulatory genes. This gene is primarily a cephalosporinase but results in resistance to all cephalosporins as well as to penicillins when produced in large amounts. This multiple resistance poses serious problems to both patients and clinicians.

Susceptibility tests are usually performed in hospitals or diagnostic laboratories, so that the acquisition of resistance markers is quickly detected. However, the lack of effective treatment for seriously ill patients may have serious consequences and also facilitates the spread of resistant strains. Patients with a specific bacterial infection may become infected by another bacterium resistant to the antibiotic being administered. This has been observed in UTI patients with indwelling catheters ***(32)***.

1.7 AIMS OF THIS STUDY

i. To obtain ampicillin-resistant *E. coli* isolates from hospitalised patients suspected of having urinary tract infections.

ii. To determine β-lactam susceptibility profiles of these isolates by the Kirby-Bauer disk diffusion method.

iii. To determine the prevalence of transferable β-lactamase genetic markers in the different gender and age groups.

iv. To determine minimum inhibitory concentration distributions for β-lactam antibiotics by agar dilution method.

v. To detect extended-spectrum β-lactamase-producing organisms and determine the rates at which they occur in different gender and age groups.

vi. To determine the extent of multiple resistance (joint resistance) among ampicillin-resistant isolates and their transconjugants.

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CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS AND METHODS

2.1.1 MATERIALS

2.1.1.1 BACTERIAL STRAINS

One hundred and twenty ampicillin-resistant (*amp*$^{+}$), nalidixic acid sensitive (*nal*^{r}), lactose-positive (*lac*$^{+}$) urinary tract *E. coli* isolates were obtained from the clinical laboratories at Bloemfontein's Universitas and Pelonomi Hospitals over a one-year period. Duplicate isolates were excluded. These isolates were purified and re-identified as *E. coli* by the Mastascan identification system (Mast Group, Ltd., United Kingdom). Pure cultures were obtained by streaking for single colonies on MacConkey agar containing 50 µg / ml of ampicillin. Single colonies were then picked and inoculated into Mueller-Hinton broth, grown overnight at 37°C and re-streaked for single colonies on MacConkey agar containing 50 µg / ml of ampicillin. Three colonies were picked and stored at -20°C in a glucose-proteose freeze mixture. *E. coli* J62 (*lac*$^{-}$, *nal*^{r}) was used as a recipient strain in conjugation studies. *E. coli* American type culture collection (ATCC 25922) was used as the quality reference strain for susceptibility testing.

2.1.1.2 MACCONKEY AGAR

MacConkey Agar (Oxoid Limited, Hampshire, England) was prepared with 52 g in 1 litre of distilled water and boiled to dissolve. The media was sterilised at 121°C for 15 minutes. The surface of the gel was dried before inoculation. Cultures on this media were grown to differentiate between lactose-negative and lactose-positive organisms.

2.1.1.3 MUELLER-HINTON BROTH

Mueller-Hinton Broth (Difco Laboratories, Detroit, Michigan, USA) was prepared with 21 g of Mueller-Hinton Broth in 1 litre of distilled water and boiled to dissolve. The media was sterilised at 121°C for 15 minutes. The broth was used to grow liquid cultures at 37°C in an orbital shaking incubator. The media complied with the requirements of the National Committee for Clinical Laboratory Standards (NCCLS) *(1)* (now called Clinical Laboratory Standards Institute (CLSI).

2.1.1.4 MUELLER-HINTON AGAR

Mueller-Hinton agar (Difco Laboratories, Detroit, Michigan, and USA) was prepared by suspending 38 g in 1 litre of distilled water and boiled to dissolve. The media was then

sterilised at 121°C-124°C for 15 minutes. The media complied with the requirements of the Clinical Laboratory Standards Institute (CLSI).

2.1.1.5 FREEZE MIXTURE

Freeze medium was prepared by:

i. Dissolving 14 g glucose (Merck, Germany) in 30% glycerol to a final volume of 100 ml.

ii. Dissolving 14 g proteose peptone (Difco Laboratories, Detroit, Michigan, USA) in 30% distilled water to a final volume of 100 ml.

The solutions were autoclaved separately. A working solution was prepared by aseptically adding together equal volumes of the stock glucose and peptone solutions.

2.1.1.6 SENSITIVITY DISKS AND ANTIBIOTIC POWDER STANDARDS

The antibiotics disks and antibiotic powder standards that were used in this study were all obtained from Mast Group, Ltd., Merseyside, United Kingdom:

Piperacillin-tazobactam (78 μg +10 μg), amoxycillin (10 μg), piperacillin (100 μg), augmentin (30 μg), cephazolin (30 μg), cefoxitin (30 μg,) ceftazidime (30 μg), ceftriaxone (30 μg), cefotaxime (30 μg), ciprofloxacin (10 μg), amikacin (10 μg), tobramycin (10 μg).

Antibiotic reference standards from the following sources were used: amoxycillin (Smith-Kline Beecham, Betchworth, UK) and ampicillin (Smith-Kline Beecham, Betchworth, UK), Universitas Hospital Pharmacy, Bloemfontein; augmentin, Smith-Kline Beecham Pharmaceuticals, Sunninghill; piperacillin, Lederle Laboratories, Isando; cefotaxime, Hoechst, Bramley; ceftazidime, Glaxo Pharmaceuticals, Johannesburg; ceftriaxone, Merck Pharmaceuticals, Midrand; cefuroxime, Parke Davis, Tokai; cefepime, Bristol-Myers Squibb, NJ, USA; cephazolin and cefoxitin, Eli Lilly, Isando; imipenem, MSD (Pty) Ltd, Johannesburg.

2.1.2 METHODS

2.1.2.1 IDENTIFICATION OF *E. COLI* ISOLATES

The identification of *E. coli* isolates was carried out according to the general guidelines described in the Standard Operating Procedures (T004V1) of the Department of Medical Microbiology at Universitas Hospital in Bloemfontein. This consisted of Gram staining, plate

colonial morphology and different biochemical and antibiotic disk tests. The identification of isolates was confirmed by using the Mastascan identification system.

2.1.2.2 MASTASCAN IDENTIFICATION

The identification of *E. coli* was done by the Mastascan identification system. This consisted of a range of biochemical test media, specifically designed for use with multipoint inoculation.

The fifteen most discriminatory tests often chosen for use with the Mastascan are as follows: rhamnose fermentation (RHAM), sucrose fermentation (SUC), melibiose fermentation (MEL), sorbitol fermentation (SORB), amygdaline fermentation (AMYG), xylose fermentation (XYL), ornithine decarboxylase fermentation (ODC), phenylalanine deamination (PPA), O-Nitrophenyl-B-D-Galactopyranoside test (ONPG), inositol fermentation (INOS), urease production (UREA), malonate utilisation (MALO), motility (MOT), indole production (IND) and dulcitol fermentation (DUL).

Four additional tests were done in conjunction with the main fifteen:

i. Glucose fermentation (GLUC) for recognition of non-fermenters, e.g. *Pseudomonas* species.
ii. *Pseudomonas*-selective medium (PYO) detects growth of that organism.
iii. Beta-glucoronidase production (BGA), which is characteristic of *Escherichia coli* and *Shigella*. Sugar reactions are compared.
iv. Carbohydrate agar base (CHO) which is preferably used as a control against which all sugar reactions are compared.

2.1.2.3 PREPARATION OF MASTASCAN MEDIA

The media were presented in pre-weighed foil sachets, the contents of which were sufficient to prepare a final volume of 200 ml of medium. The contents of each sachet was suspended in the indicated volume of de-ionised water. This was thoroughly mixed, heated gently and boiled to dissolve completely. Each medium was then autoclaved at 121°C for 15 minutes except xylose, which was autoclaved at 115°C for 10 minutes. Poured plates could be used immediately after drying, or stored in sealed plastic bags at 4°C for up to two weeks before use.

After inoculation and incubation for 18-24 hours, each reaction was scored as positive or negative by reference to the Mastascan interpretation guidelines.

2.2 TRANSFER OF RESISTANCE DETERMINANTS

Plasmid-mediated resistance to β-lactam agents was transferred by conjugation to *E. coli* J62 (*lac*$^-$ *nal*^{r}). Matings were performed in Mueller-Hinton broth (Difco Laboratories, USA). Equal volumes (0.1 ml) of exponentially growing cultures of donor and recipient strains were added to 10 ml pre-warmed Mueller-Hinton broth (Difco Laboratories, USA) and incubated at 37°C overnight. Transconjugants were selected on MacConkey agar supplemented with 50 μg / ml ampicillin and 50 μg / ml nalidixic acid. Lactose-negative colonies resistant to nalidixic acid and the test antibiotic were inoculated into Mueller-Hinton broth, incubated for 6 hours and re-streaked on MacConkey agar containing ampicillin. Lactose-negative colonies were selected and were considered transconjugants.

2.3 MINIMUM INHIBITORY CONCENTRATION (MIC) DETERMINATION

The National Committee for Clinical Laboratory Standards (NCCLS) * agar dilution method *(1)* was used to determine minimum inhibitory concentrations (MICs) of the following 12 β-lactam antibiotics: ampicillin, amoxycillin, augmentin, piperacillin, cefoxitin, cefotaxime, cefepime, ceftazidime, cephazolin, ceftriaxone, cefuroxime and imipenem.

Inocula were prepared by suspension of three colonies from MacConkey agar plates containing 50 μg / ml of ampicillin and cultured in Mueller-Hinton broth. A cell suspension of 5 x 10^5 colony forming units (CFUs), corresponding to a 1 / 20 dilution of a 0.5 McFarland standard, was applied to Mueller-Hinton agar plates containing the above-mentioned antibiotics at different concentrations with a Steers multi-point replicator device to deliver 10^3 to 10^5 CFUs per spot. Plates were incubated aerobically for 18 h at 35°C.

2.4 KIRBY-BAUER DISK DIFFUSION METHOD

Antibiotic susceptibility profiles were determined by the Kirby-Bauer disk diffusion method. This method was also used to determine the correlation between the MIC values and inhibition zone diameters in order to predict β-lactam treatment outcomes.

The susceptibility of isolates were determined and interpreted according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS)* *(1)*, using unsupplemented Mueller-Hinton agar (Difco Laboratories, Detroit, Michigan) that was used to define susceptibility or resistance to the antibiotics listed above.

2.5 EXTENDED-SPECTRUM β-LACTAMASE (ESBL) DETECTION

The double disk technique of Jarlier *et al.* ***(2)***. was used for the detection of ESBL-producing organisms. This test was executed by applying four 30 μg antibiotic disks (cefotaxime, ceftazidime, amoxycillin and ceftriaxone), placed 30 mm (centre to centre) apart around a disk of augmentin (20 μg of amoxycillin plus 10 μg of clavulanate), to a lawn of the test organism (i.e. the clinical isolates). Extension of the inhibition zone towards the disk containing clavulanate suggested the presence of extended-spectrum β-lactamases ***(2)***.

2.6 STATISTICAL ANALYSIS OF β-LACTAM MULTI-RESISTANCE

The extent of joint resistance among β-lactam antibiotics was analysed in 106 isolates of *E. coli.* The extent of multiple resistance in urinary tract isolates was assessed by comparing two agents at a time. The observed prevalence of joint resistance was compared with the rate of double resistance expected if it had been acquired as two independent events. In the analysis the number of independently variable classes was taken as two, i.e. jointly resistant and not jointly resistant. Thus the degrees of freedom (i.e. number of classes less one; notationally, df = k–1) in this analysis was equal to one. Chi-square (χ^2) values were calculated as:

$$\chi^2 = \sum \left[\frac{\left((Observed - Expected) - 1/2 \right)^2}{Expected} \right]$$

The deduction of ½ from the (observed – expected) difference is known as the Yates correction term and adds to the accuracy of the Chi-square determination when the number of either of the "expected" or "observed" classes is small ***(3)***.

For other situations where more classes were found, the homogeneity chi-squared test was performed.

Independence of variables was tested in cases where it was necessary to compare one set of observations with another set taken under different conditions, using the following formula:

$$\chi^2 = \frac{\left(|ad - bc| - 1/2\, N \right)^2 N}{(a+b)(a+c)(c+d)(b+d)}$$

2.7 REFERENCES

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*Note that the name "**National Committee for Clinical Laboratory Standards**" (NCCLS) has been changed to "**Clinical Laboratory Standards Institute**" (CLSI)

CHAPTER 3

TRANSFER OF BETA-LACTAM RESISTANCE DETERMINANTS

3.1 ABSTRACT

One hundred and twenty ampicillin-resistant *E. coli* urinary tract isolates of patients from Pelonomi and Universitas Hospitals were mated with *E. coli* J62 (*lac*$^-$ *nal*^{r}) in Mueller-Hinton broth. Transfer of ampicillin resistance was demonstrated for 54 out of 120 isolates (45%). Of these transconjugants, 33 were selected at random for a more detailed analysis of resistance markers. Of the selected transconjugants all 33 (100 %) were jointly resistant to ampicillin and amoxycillin, 32 (96.9%) to piperacillin, and 16 (48.5%) to augmentin Cephazolin resistance was demonstrated in 5 (15.2%) of the transconjugants. Cefoxitin and ceftazidime resistance was demonstrated in 4 (12.2%) transconjugants, whilst for ceftriaxone and cefotaxime 2 (6.1%) of the transconjugants demonstrated resistance. The distribution of ampicillin-resistant isolates showed that 90 out of 120 (75%) were isolated from female patients. The highest number of ampicillin-resistant strains was isolated from the 21-30 age groups in both male and female patients. Ampicillin resistance was found to be significantly higher in females than in males in all age groups. A similar tendency was observed for transconjugants where 20 out of 31 (64.5%) were from female patients. More than 97.5% of isolates were inhibited by cefotaxime, more than 95.6% were inhibited by ceftazidime, more than 90% were inhibited by ceftriaxone, more than 91.1% were inhibited by cefepime and more than 90.2% were inhibited by cefoxitin.

3.2 INTRODUCTION

Conjugation is a process by which genetic material is transferred between related bacteria by the transfer functions of self-transmissible autonomous DNA molecules, called plasmids. This mechanism represents genetic functions by which antibiotic-resistance genes spread through bacterial populations. Plasmids encoding for antibiotic resistance are called resistance transfer factors (RTFs) to distinguish them from plasmids not mediating antibiotic resistance. Genetic functions required for transfer are encoded for by *tra* genes that are carried by self-transmissible plasmids or conjugative plasmids. Plasmids lacking *tra* genes are not self-transmissible and are non-conjugative. Transfer of antibiotic-resistance genes by conjugation involves direct cellular contact between donor and recipient bacteria.

Ampicillin-resistant urinary tract *E. coli* isolates were chosen to determine the transfer frequency of ampicillin resistance. The co-transfer frequency of other β-lactam-resistance determinants was also determined.

Beta-lactam antibiotics inhibit peptidoglycan synthesis by binding to penicillin binding proteins (PBPs), carboxy-peptidases and transpeptidases that are responsible for the final

stages of peptidoglycan synthesis. Cell death or lysis results from inactivation or incomplete cell wall synthesis.

Although PBPs have highly diverse amino acid sequences, they also have a number of similar features, such as a C-terminal transpeptidase domain with a conserved active-site serine residue. Most bacterial resistance to β-lactams is due to β-lactamases that inactivate the antibiotics.

Another determinant of β-lactam resistance is enzyme-substrate affinity. Once the enzyme- substrate complex is recognised, inhibition of the β-lactams takes place. Therefore, susceptibility tests can be used in order to characterise bacteria that are more likely to spread resistance genes. The transfer of plasmid-mediated resistance requires that the use of β-lactams be restricted in order to prevent the development of increasing resistance in Bloemfontein. The donor plasmids carry the *tra* gene. The oral agents that are most often recommended for treating UTIs are usually expensive and generally unavailable in rural areas or peripheral hospitals. Therefore there is an urgent need to make these agents available, particularly for patients with upper urinary tract infections, for whom the consequences of prescribing antibiotics where the isolate is resistant could be severe or even fatal. The emergence of transferable enzymatic resistance to a wide range of β-lactams in *E. coli*, and its spread to other bacteria of potential pathogenicity, such as J62, indicates a possible risk of recurrence of nosocomial outbreaks caused by these species. Dissemination of such resistance could compromise the future use of β-lactams in urinary tract infections.

3.3 AIMS OF THIS CHAPTER

i. To determine if β-lactam resistance is transferable to an *E. coli* recipient.
ii. To determine the prevalence of transferable β-lactamase genetic markers in different gender and age groups.
iii. To determine the cumulative MIC distribution frequencies registered for β-lactam antibiotics co-transferred with ampicillin.

3. 4 RESULTS AND DISCUSSION

From 120 consecutive urine samples containing ampicillin-resistant *E. coli* strains, 54 (45%) ampicillin-resistant transconjugants were isolated. A random selection of 33 transconjugants was tested for resistance to β-lactam antibiotics. All thirty-three transconjugants carried resistance to ampicillin and amoxycillin. Resistance was defined by zone diameters according to the National Committee for Clinical Laboratory Standards

(NCCLS)* *(1)*. Many β-lactam resistant strains from the urinary tract could not be inhibited by β-lactam concentrations achievable in the serum with normal dosage, because β-lactam compounds reach very high concentrations in the urine and selection for antibiotic resistance will consequently also be for isolates resistant to very high concentrations of antibiotics.

Studies have shown that the basis of β-lactam resistance in *E. coli* isolates was probably multi-factorial, i.e. over-expression of TEM or Amp C β-lactamase *(2)*.

TEM-1 is the most widespread β-lactamase worldwide, and the amino acid substitutions are not unusual and are frequently found in clinical variants that are also able to hydrolyse third-generation cephalosporins and monobactams *(3)*.

The isolated *E. coli* strains demonstrated resistance to nine β-lactam agents in various combinations. In addition to ampicillin, variable numbers of isolates were also resistant to amoxycillin, augmentin, piperacillin, cephazolin, cefoxitin, ceftazidime, ceftriaxone and cefotaxime. However, in the light of earlier data by Pitout *et al.*, this relatively high prevalence of β-lactam resistance represented a long-standing trend. The level of resistance transferred for amoxycillin, augmentin and piperacillin was higher than for the cephalosporins. However, this has shown that the extended-spectrum β-lactamases have properties similar to the chromosomal AmpC β-lactamases *(4)*.

Chromosomal AmpC cephalosporinases were expressed at low levels. Mutations leading to hyperactive production of AmpC β-lactamases probably rendered the bacteria resistant to some of the cephalosporins. They, however, needed to be induced to express resistance. Most plasmids are transferred efficiently for only a short period of time during the growth cycle. The *tra* (transfer) genes are repressed during much of the growth cycle and conjugation can only take place during the short period when the cells are competent. The synthesis of pili and other *tra* functions do not take place when cells are not competent. The repression is relieved occasionally in some cells, allowing a small percentage of cells to transfer their plasmid at any given time *(5)*.

Bacterial plasmids and the resistance determinants they carry represent a serious threat to the clinical utility of β-lactam antibiotics ***(Table 3.1)***. The discovery of β-lactamase inhibitors was thought to have solved the problem of β-lactam resistance. Unfortunately, bacteria have quickly evolved new mechanisms to overcome the inhibitory effect of β-lactamase inhibitors.

The distribution of patients according to age and gender is shown. Bacteriuria is uncommon in young male populations beyond the new-born period. Factors that probably contribute to the development of urinary tract infections in the elderly include the following:

the effect of oestrogen loss on the genitourinary mucosa among non-institutionalised elderly women, increased residual urine and genitourinary abnormalities. Some chronic medical conditions e.g. diabetes occur more frequently in the elderly. In females, diabetics have a three times greater prevalence of bacteriuria than non-diabetic women *(6)*.

Table 3.1 The number of transconjugants resistant to various β-lactam antibiotics

	Amp	Amx	Pip	Aug	Cfz	Fox	Caz	Ctr	Ctx
Breakpoints* (mm)	≤16	≤16	≤20	≤17	≤ 17	≤17	≤17	≤22	≤20
Transconjugants	33	33	32	16	5	4	4	2	2
% Resistant	100	100	96.9	48.5	15.6	12.1	12.1	6.1	6.1

The National Committee for Clinical Laboratory Standards (NCCLS)* *breakpoints (zone diameter in mm)*

Amp: *Ampicillin* **Amx**: *Amoxycillin* **Pip**: *Piperacillin* **Aug**: *Augmentin*
Cfz: *Cefazolin* **Fox**: *Cefoxitin* **Caz**: *Ceftazidime* **Ctr**: *Ceftriaxone* **Ctx**: *Cefotaxime*

Reasons why urinary tract *E. coli* are notoriously resistant to antibiotics include the following: presence of mixed infections in which only the sensitive pathogens are eliminated, use of incorrect antibiotic, or administration of an antibiotic in inadequate dosage for rather longer period for selective pressure period; inability of an antibiotic to achieve bactericidal levels within the renal parenchyma, or poor local and humoral defence mechanisms in the kidney, when compared to other loci *(7)*.

In *E. coli,* conjugative transposons of some ESBLs confer high-level resistance to all oxyimino β-lactams, but for some β-lactams resistance is only slightly increased or even decreased. Multiple β-lactam resistance determinants have been discovered integrated into plasmids or transposons at specific sites called intergrons *(8)*. A conjugative transposon can be present in bacterial strains that have extra-chromosomal genes or plasmids too small to encode the necessary transfer genes *(9)*.

Urinary tract infections are common during pregnancy and all pregnant women with asymptomatic bacteriuria should be treated with antibiotics. Pregnant women are at risk because they develop urethral dilation from the beginning of the sixth week to the twenty-fourth week of their gestation period. Increased bladder volume and decreased bladder tone contribute to increased urinary stasis and urethrovesical reflux; consequently, 20-30% of pregnant women develop urinary tract infections such as cystitis and pyelonephritis.

Table 3.2 Distribution of ampicillin resistant isolates in patients according to age and gender

Age (yrs)		Females	Males	df	χ^2
1 – 10	**Observed**	**9**	**5**		
	Expected	7	7		
	Obs – Exp	2	-2		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.5714	0.1250	1	0.6964
11 – 20	**Observed**	**9**	**2**		
	Expected	5.5	5.5		
	Obs – Exp	3.5	-3.5		
	$\chi^2 = (Obs - Exp)^2 / Exp$	2.227	2.227	1	4.4545
21 – 30	**Observed**	**27**	**8**		
	Expected	17.5	17.5		
	Obs – Exp	9.5	-9.5		
	$\chi^2 = (Obs - Exp)^2 / Exp$	90.25	90.25	1	180.5
31 – 40	**Observed**	**12**	**2**		
	Expected	7	7		
	Obs – Exp	5	-5		
	$\chi^2 = (Obs - Exp)^2 / Exp$	3.57	3.57	1	7.14
41 – 50	**Observed**	**8**	**2**		
	Expected	5	5		
	Obs – Exp	3	-3		
	$\chi^2 = (Obs - Exp)^2 / Exp$	1.8	1.8	1	3.6
51 – 60	**Observed**	**9**	**5**		
	Expected	7	7		
	Obs – Exp	2	-2		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.5714	0.5714	1	1.1428
61 – 70	**Observed**	**10**	**4**		
	Expected	7	7		
	Obs – Exp	3	-3		
	$\chi^2 = (Obs - Exp)^2 / Exp$	1.285	1.285	1	2.5714
71 – 90	**Observed**	**6**	**2**		
	Expected	4	4		
	Obs – Exp	2	-2		
	$\chi^2 = (Obs - Exp)^2 / Exp$	1	1	1	2
Summed data	**Observed**	**90**	**30**	**8**	**202.1051**
	Expected	60	60		
	Obs – Exp	30	30		
	χ^2	15	15		**30**

The homogeneity chi-squared value of 202.1051 – 30 = 172.1051 exceeds the value of 15.51 at the 95 % level of significance for 8 degrees of freedom. The hypothesis that the classes are homogeneous is rejected. Therefore there is a difference in the number of male and female patients, with many more female than male patients.

Pyelonephritis can be a life-threatening disease, with increased risk of prenatal and neonatal morbidity.

Complications may result from neurogenic bladder dysfunction, of which the most frequent complications are urinary tract infections. Antibiotics should be administered immediately for these infections ***(10)***.

Augmentin resistance among *E. coli* isolates is determined by the interaction between β-lactamase activity and clavulanic-acid inhibition. Possible variables include the degree of enzyme induction and the exact chemistry of the enzyme-inhibitor interaction. Both amoxycillin and clavulanic acid can induce *E. coli* penicillinases ***(11)***. The results show that *E. coli* isolates transferred augmentin resistance markers to J62. The mechanism of resistance is the production of TEM-derived β-lactamases with reduced affinity for clavulanic acid.

Table 3.3 Transferable β-lactamase genetic markers in different gender and age groups

Age (yrs)		Females	Males	df	χ^2
1 – 20	**Observed**	**6**	**2**		
	Expected	4	4		
	Obs – Exp	2	-2		
	$\chi^2 = (Obs - Exp)^2 / Exp$	1	1	1	2.0
21 – 35	**Observed**	**8**	**4**		
	Expected	6	6		
	Obs – Exp	2	-2		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.6667	0.6667	1	1.3334
36 – 50	**Observed**	**0**	**1**		
	Expected	0.5	0.5		
	Obs – Exp	-0.5	0.5		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.5	0.5	1	1.0
> 50	**Observed**	**7**	**3**		
	Expected	5	5		
	Obs – Exp	2	-2		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.8	0.8	1	1.6
Summed data	**Observed**	**21**	**10**	**4**	**5.9334**
	Expected	15.5	15.5		
	Obs – Exp	5.5	5.5		
	χ^2	1.9516	1.9516		**3.9032**

The homogeneity chi-squared value of 5.9334 – 3.9032 = 2.0302 does not exceed the value of 9.49 at the 95 % level of significance for 4 degrees of freedom. The homogeneity chi-squared value is well within the accepted limits. The hypothesis that the classes are homogeneous is accepted. Therefore there is no difference in the number of transconjugants (ratio of transconjugants to isolates) between male and female patients.

For piperacillin, some donor resistance was transferred to the transconjugants. This showed that bacteria can display multiple resistance when exposed to one drug. It is almost as if bacteria strategically anticipate the confrontation of the other drugs when they resist one such as amoxycillin. If resistance genes were present on the plasmid, the surviving bacteria recruited these genes, thereby acquiring immediate resistance.

Table 3.4 Cumulative distribution frequencies of isolates inhibited at specified MICs

Breakpoints µg / ml	2*	8*	36*	8 / 2*	20*	40*	62*	40*	30*	60*	24*
	PENICILLINS				CEPHALOSPORINS						
MICs µg / ml	Amp	Amx	Pip	Aug	Ctx	Caz	Ctr	Crx	Cef	Cfz	Fox
0.0078	NT	NT	NT	NT	NT	NT	NT	NT	0	NT	NT
0.015	NT	NT	NT	NT	0	0	0	NT	7.6	NT	NT
0.03	NT	NT	NT	NT	1.2	11	10	NT	8.9	NT	NT
0.06	NT	NT	NT	NT	37.5	60.4	40	NT	10.1	NT	NT
0.125	NT	NT	NT	NT	66.2	90.1	40	0	43.1	NT	NT
0.25	NT	NT	NT	NT	85	93.4	40	0	44.3	NT	NT
0.5	NT	NT	NT	0	87.5	93.4	40	0	55.7	0	0
1	NT	NT	0	5	90	93.4	40	1.1	65.8	6.6	1
2	0	0	8.9	14.6	90	94.5	40	11.1	91.1	36.3	16.7
4	2.6	0	15.6	15.7	97.5	95.6	40	46.7	91.1	68.1	64.7
8	2.6	6.7	16.7	25.8	NT	NT	NT	82.2	NT	76.9	90.2
16	2.6	15.6	17.8	69.7	NT	NT	NT	90	NT	82.4	91.2
32	2.6	16.7	21.1	91.1	NT	NT	NT	90	NT	86.8	93.1
64	6.5	16.7	27.8	100	NT	NT	NT	NT	NT	87.9	99.1
128	7.8	16.7	27.8	100	NT	NT	NT	NT	NT	93.4	100
256	9.1	16.7	61.1	NT	NT	NT	NT	NT	NT	97.8	NT
512	18.2	16.7	75.6	NT	NT	NT	NT	NT	NT	NT	NT
1024	42.9	16.7	94.4	NT	NT	NT	NT	NT	NT	NT	NT
2048	67.5	20.0	100	NT	NT	NT	NT	NT	NT	NT	NT

* *Serum minimum inhibitory concentrations*

NT: *Not tested*

Amp: *Ampicillin* **Amx**: *Amoxycillin* **Pip**: *Piperacillin* **Aug**: *Augmentin*

Ctx: *Cefotaxime* **Caz**: *Ceftazidime* **Ctr**: *Ceftriaxone* **Crx**: *Cefuroxime*

Cef: Cefepime **Cfz**: *Cephazolin* **Fox**: *Cefoxitin*

In summary, conjugation may be viewed as a form of replication, because the single-stranded DNA that remained behind in the donor and the single-stranded copy that entered the recipient was made into double-stranded DNA before integration occurred. This may also be seen as a form of recombination. Conjugation appears to be particularly common in bacteria that are naturally conjugative. This has been particularly important in the development of resistance to some newer β-lactam antibiotics, and in the generation of antigenic diversity.

Table 3.4 shows that some β-lactamases confer high-level resistance to oxyimino-β-lactams, but for other β-lactamases resistance is only slightly increased or selectively increased for particular β-lactams. This creates a problem for the clinical laboratory, since organisms producing less active ESBLs can fail to reach current National Committee for Clinical Laboratory Standards (NCCLS)* breakpoints for resistance yet can cause significant disease. For example, for cefuroxime 90% of the isolates were inhibited at 16 μg / ml, yet the breakpoint is 32 μg / ml. For cefoxitin 90.2% of the isolates were inhibited at 8 μg / ml, but the breakpoint for cefoxitin resistance is 32 μg / ml. For both cefotaxime and ceftazidime 90% of the isolates were inhibited at low drug concentrations of 1.0 and 0.125 μg / ml respectively. In contrast to the general situation with the cephalosporins, relatively few isolates of the penicillins, viz ampicillin and amoxycillin were inhibited at drug concentrations of 512 μg / ml and higher. Many isolates were rather insensitive to clavulanic acid inhibition as was indicated by the fact that the 90% level of inhibition was only reached at 32 μg / ml. For piperacillin, 94.4% of *E. coli* isolates were inhibited at 1024 μg / ml. This indicated that the isolates were highly resistant to piperacillin inhibition. The isolates were all resistant to the penicillins at MICs beyond that achievable *in vivo*.

3.5 CONCLUSIONS

Fifty-four out of 120 (45%) ampicillin-resistant isolates transferred β-lactam resistance determinants to an *E. coli* recipient by conjugation. Seventy-five percent (90 / 120) of ampicillin resistant isolates were from female patients, indicating that urinary tract infections are more prevalent in females than in males. Bacteria infecting females are thus more likely to be exposed to antimicrobial agents than bacteria infecting males. The result was that more transferable β-lactamase genetic determinants originated from females (21 / 31 or 67.7%) than from males (10 / 31 or 32.2%). Two of the transconjugants originally selected were lost, resulting in only 31 being subjected to antibiotic resistance determinant analysis. A higher percentage of isolates from females than from males showed ampicillin resistance. However, as shown in ***Table 3.3***, no significant difference was observed in the rates at which resistance

determinants transferred for female or male isolates. Significantly more isolates transferred penicillin resistance than cephalosporin resistance. Both ampicillin and amoxycillin markers were present in the original 33 selected transconjugants. Piperacillin and augmentin resistance determinants were present in 32 (96.9%) and 16 (48.5%) of the transconjugants respectively. Cephalosporin resistance was transferred at much lower rates than penicillin resistance, with cephazolin at 15.2%, cefoxitin and ceftazidime at 12.1%, and ceftriaxone and cefotaxime both at 6.1%.

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*Note that the name **"National Committee for Clinical Laboratory Standards"** (NCCLS) has been changed to **"Clinical Laboratory Standards Institute"** (CLSI)

CHAPTER 4

MINIMUM INHIBITORY CONCENTRATION DISTRIBUTION FOR BETA-LACTAM ANTIBIOTICS

4.1 ABSTRACT

Minimum inhibitory concentrations (MICs) for 12 β-lactam agents were determined using the National Committee for Clinical Laboratory Standards*(2) agar dilution method. Inocula were prepared by suspending three colonies from MacConkey agar plates in Mueller-Hinton broth and incubating for 18 hours. Plates containing antibiotic dilutions were inoculated with 5 x 10^6 colony forming units, equivalent to a 1 / 20 dilution of a 0.5 McFarland standard using a multipoint inoculator. MICs were read after 18 hours of incubation at 35°C. MICs showed considerable resistance for some isolates. Resistance levels were high for penicillins while the cephalosporins showed low levels of resistance. The addition of clavulanic acid to amoxycillin raised the activity of the combination drug augmentin to far beyond that of amoxycillin. Resistance levels were lower for imipenem.

The results supported the hypothesis that although distinct mechanisms exist for β-lactam resistance, interplay between two or more resistance mechanisms is frequently responsible for high levels of resistance in clinical isolates of common pathogens such as urinary tract *E. coli.*

4.2 INTRODUCTION

The minimum inhibitory concentration is defined as the lowest concentration of an antibiotic inhibiting visible growth after 18 to 24 hours *(1)*. Minimum inhibitory concentrations can be determined for any organism responsible for an infection requiring antimicrobial chemotherapy. Minimum inhibitory concentration determinations are necessary because it is difficult to predict the antibiotic susceptibility of the organism from its identity alone. Minimum inhibitory concentration determinations can also be of value in epidemiological studies of resistance to new antibiotics *(2)*.

Some antibiotics may be associated with the emergence of resistance during prolonged chemotherapy. Isolates that were initially susceptible may become resistant within three to four days of therapy. This occurs most frequently in *Enterobacter*, *Citrobacter*, and *Serratia* species *(3)*.

Extended-spectrum β-lactamases (ESBLs) are enzymes that inactivate third-generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime, the monobactam aztreonam, related oxyimino-β-lactams as well as some of the older penicillins. These developed as a result of mutation in genes mediating the production of TEM and SHV enzymes. The mutations alter the configuration of the enzymes near their active sites. The affinity and hydrolytic ability of these β-lactamases for oxyimino compounds are increased while at the

same time overall enzyme efficiency is decreased. Some ESBLs confer high-level resistance to all oxyimino-β-lactams, but for other β-lactams resistance is increased only slightly. This creates a problem for the clinical laboratory, since organisms producing less-active ESBLs can fail to reach current National Committee for Clinical Laboratory Standards* MIC breakpoints for resistance but they may cause considerable disease *(4)*.

The importance of using MICs to screen for resistance that might otherwise be missed by routine laboratory testing cannot be overemphasized. One of the disadvantages of MIC determination is that the result is read at only one point in time. A more dynamic estimate of bacterial susceptibility can be gained by measuring the decrease in the number of survivors in a bacterial population over time. Killing curves can be determined for this purpose. However, as with MIC tests, it is not feasible to determine killing curves manually for every isolate, but killing curves can provide useful information for difficult treatment problems. An alternative would be to use antibiotic combinations. This could have the following advantages: a synergistic effect can be obtained, emergence of persistent organisms can be prevented or delayed, polymicrobial infections can be effectively treated, and serious infections can be treated before the infecting organism is identified. Thus MIC determination could help to avoid prolonging hospital stays and so reduce health care costs, by identifying the appropriate concentration and antibiotic to be used.

4. 3 AIM OF THIS CHAPTER

To determine minimum inhibitory concentration frequency distributions for urinary tract *E. coli* isolates.

4.4 RESULTS AND DISCUSSION

4.4.1 SUSCEPTIBILITY TO THE PENICILLINS

Ampicillin is a broad-spectrum antimicrobial agent that is sensitive to β-lactamase. The percentage of resistant organisms decreased from 97.4 % at 32 μg / ml to 32.5% at 2048 μg / ml for donors and from 81.3% to 6.3% at the same concentrations for transconjugants ***(Figure 4.1).*** The resistance levels of transconjugants were consistently lower than the levels for donors. This phenomenon cannot be explained with the data obtained from this study. It is conceivable that the wild-type donor isolates could have carried more than one RTF or chromosomal β-lactamase gene, resulting in slightly higher resistance levels for donor strains. Transconjugants, on the other hand, had J62 as host cytoplasm without additional chromosomal β-lactamase genes or impermeability barriers.

Amoxycillin, like ampicillin, is a broad-spectrum antibiotic that is sensitive to β-lactamase. Resistance decreased from 83.3% at 32 μg / ml to 80.0% at 2048 μg / ml for donors and from 96.9% to 53.1% at the same concentrations for transconjugants ***(Figure 4.2).*** Compared to ampicillin, higher levels of resistance were observed for amoxycillin at concentrations ≥ 256 μg / ml. It is clear from ***Figure 4.1*** and ***Figure 4.2*** that these antibiotics can reach exceptionally high resistance levels in urinary tract isolates. Breakpoints for urinary tract *E. coli* isolates ***(Table 4.1)*** should therefore always be taken into consideration when amoxycillin or ampicillin is administered in urinary tract antibiotic therapy.

Table 4.1 CLSI Susceptibility Guidelines for MICs of ß-Lactam antibiotics for organisms isolated from the urinary tract

ß-Lactam agents	Zone diameter (mm)	MIC [μg / ml]
Ampicillin	≤13	≥ 32
Amoxycillin	≤13	≥ 32
Piperacillin	≤17	≥128
Augmentin	≤13	≥32 /16
Ceftriaxone	≤13	≥64
Ceftazidime	≤14	≥32
Cefotaxime	≤14	≥64
Cefepime	≤14	≥16
Cefoxitin	≤14	≥32
Cephazolin	≤14	≥32
Cefuroxime	≤14	≥32
Imipenem	≤13	≥16

Piperacillin has a higher molecular weight than ampicillin. It is assumed that β-lactam compounds with higher molecular weights show more specificity for β-lactamases. This is the case with higher-molecular-weight PBPs ***(5)***. It seems logical that at identical antibiotic concentrations, the susceptibility to piperacillin would be higher than to ampicillin. The MIC distribution for piperacillin ***(Figure 4.3)*** shows that at 32 μg / ml, 21.1% of donors and 59.4% of the transconjugants were susceptible to piperacillin. At 2048 μg / ml, 95.6% of donors and 100% of transconjugants were susceptible. This indicates that piperacillin is considerably more active than ampicillin in the strains tested. The pronounced difference of susceptibility between donors and transconjugants is again noted but unexplained.

Fig 4.1 MIC DISTRIBUTION FOR AMPICILLIN

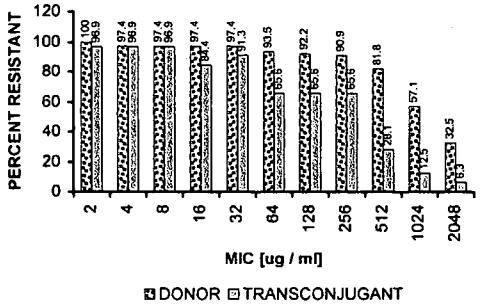


Fig 4.2 MIC DISTRIBUTION FOR AMOXYCILLIN

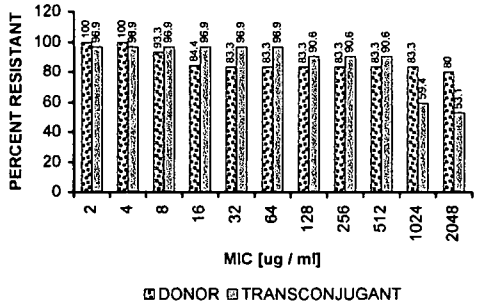


Fig 4.3 MIC DISTRIBUTION FOR PIPERACILLIN

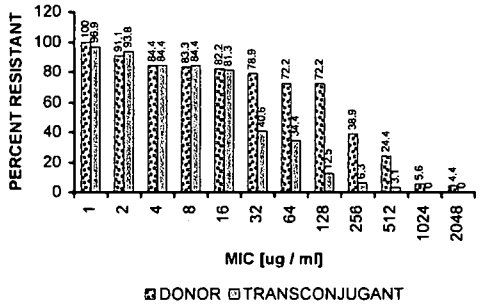


Fig 4.4 MIC DISTRIBUTION FOR AUGMENTIN

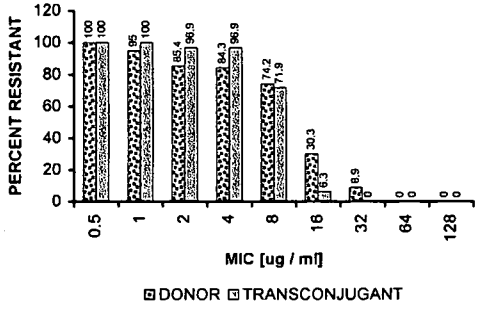


Fig 4.5 MIC DISTRIBUTION FOR CEFTAZIDIME

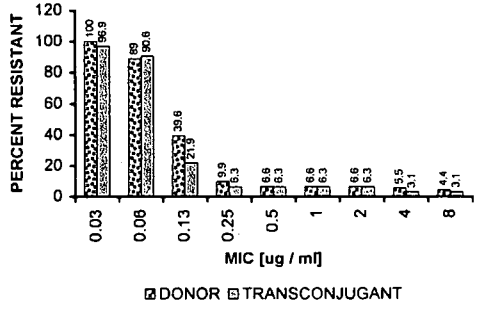


Fig 4.6 MIC DISTRIBUTION FOR CEFOTAXIME

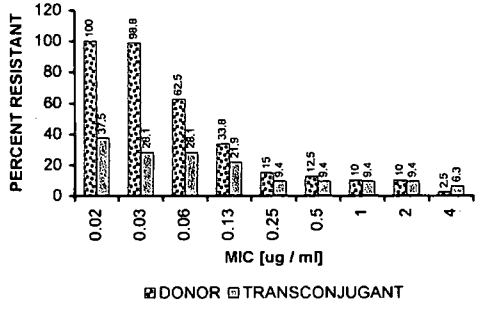


Fig 4.7 MIC DISTRIBUTION FOR CEFEPIME

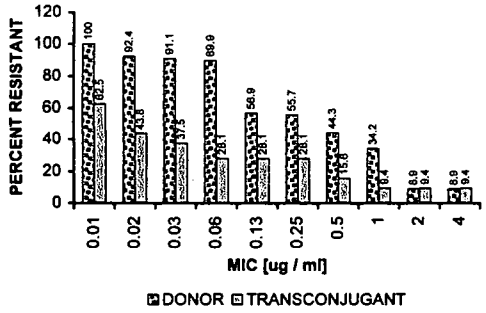


Fig 4.8 MIC DISTRIBUTION FOR CEFOXITIN

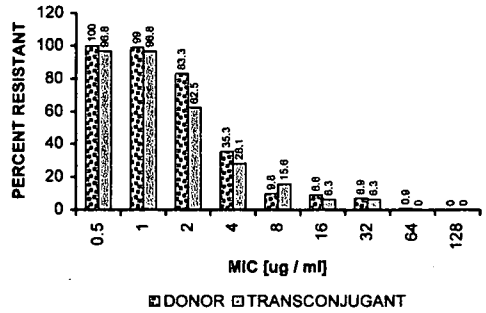


Fig 4.9 MIC DISTRIBUTION FOR CEPHAZOLIN

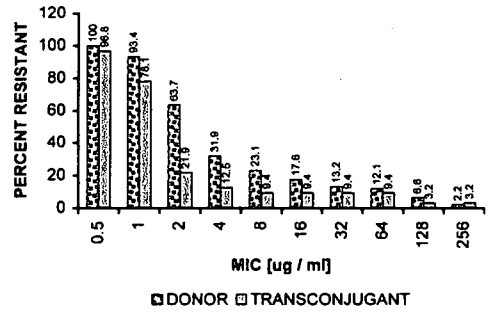


Fig 4.10 MIC DISTRIBUTION FOR CEFUROXIME

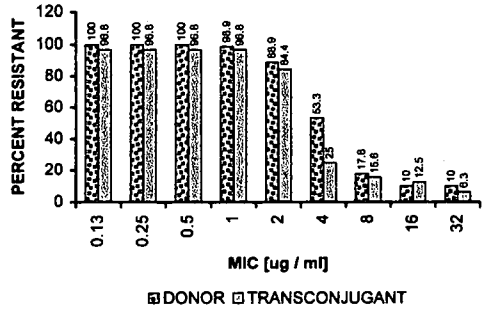
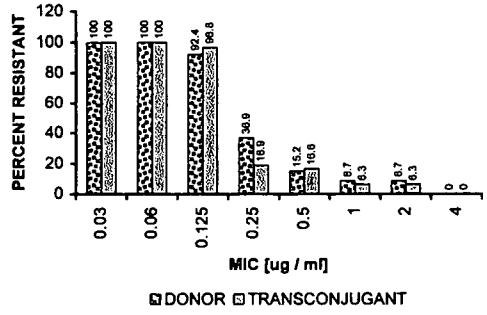


Fig 4.11 MIC DISTRIBUTION FOR IMIPENEM



The inhibitory effect of clavulanic acid is demonstrated in ***Figure 4.4***. At 16μg / ml, 84.4% of donors and 96.9% of transconjugants were resistant to amoxycillin ***(Figure 4.2)***. At 16 μg / ml of augmentin the corresponding resistance percentages were 30.3% and 6.3%. Adding clavulanic acid to amoxycillin dramatically increased its effectiveness. At 2 μg / ml, 14.6% of donors were susceptible whilst they were all resistant to amoxycillin at the same concentration. It is probable that the resistance to augmentin was not entirely mediated by extended-spectrum β-lactamases, but rather by an over-expression of TEM or AmpC β-lactamase. Over-expression of TEM is known to increase the MIC of some β-lactams ***(6)***.

4.4.2 SUSCEPTIBILITY TO THE CEPHALOSPORINS

ESBLs confer high-level resistance to all oxyimino-β-lactams, but for other ESBLs, resistance is only slightly increased or increased selectively for certain β-lactams ***(7)***. This is a problem for the clinical laboratory, since organisms producing less-active ESBLs can fail to reach current Committee for Clinical Laboratory Standards* breakpoints for resistance yet they can cause considerable disease. Extended-spectrum β-lactamases are the consequence of the use of third-generation cephalosporins; they were unknown before the introduction of these antibiotics in the early 1980`s ***(8)***.

Virtually no resistance was recorded for ceftriaxone. Both J62 (the recipient for all transconjugants) and ATCC 25922 (the *E. coli* control strain) recorded MICs of <0.015 μg / ml. Seven donors and 2 out of 31 transconjugants showed resistance to ceftriaxone at 4 μg / ml.

Figure 4.5 shows that the susceptibility to ceftazidime was 11% at 0.06 μg / ml and 60.4% at 0.13 μg / ml. ***Figure 4.6*** shows that cefotaxime, another cephalosporin with a MIC breakpoint of ≥64 μg / ml, was slightly more active than ceftazidime with susceptibilities of 37.5% at 0.06 μg / ml and 66.2% at 0.13 μg / ml. Cefepime ***(Figure 4.7)***, a fourth-generation cephalosporin with a MIC breakpoint of ≥16 μg / ml, is even less active than ceftazidime and shows susceptibilities of 10.1% at 0.06 μg / ml and 43.1% at 0.13μg / ml.

Cefoxitin ***(Figure 4.8)***, a second-generation cephalosporin with a MIC breakpoint of ≥32 μg / ml, has significant resistance with 6.9% of strains resistant at 32 μg / ml. Cefoxitin shows higher MIC resistance profiles than the mono-anionic counterpart compounds such as cephazolin ***(Figure 4.9)***. Cefuroxime, with a MIC breakpoint of 32 μg / ml, is modestly active against *E. coli* strains ***(Figure 4.10)***. Only 90.0% of the strains were susceptible at the breakpoint of 32 μg / ml.

Imipenem, a broad-spectrum thienamycin β-lactam antibiotic active against many organisms, is partly inactivated by a dipeptidase in the kidney. This may be overcome by administering imipenem in combination with cilastatin, a specific renal dipeptidase inhibitor. This results in prolonged plasma concentrations of imipenem. It may not be reliable to only look at ***Figure 4.11*** and assume that one could administer imipenem in urinary tract infections because it showed a high susceptibility rate *in vitro*.

4. 5 CONCLUSIONS

i. Resistance to extended-spectrum cephalosporins was very low. This probably indicates that extended spectrum β-lactamases are still relatively uncommon in urinary tract isolates in Bloemfontein.

ii. The MIC frequency distributions for penicillins show that elevated doses of β-lactams should be administered in order to maximize the antibiotic concentration at the site of infection, or that a second antibiotic agent or inhibitor should be used in combination.

iii. All isolates were fully sensitive to imipenem ***(Figure 4.11)***.

iv. Antibiotics such as ampicillin, amoxycillin, piperacillin and augmentin require higher doses to be effective against β-lactamase-producing strains.

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*Note that the name "**National Committee for Clinical Laboratory Standards**" (NCCLS) has been changed to "**Clinical Laboratory Standards Institute**" (CLSI)

CHAPTER 5

DETERMINATION OF BETA-LACTAM SUSCEPTIBILITY PROFILES FOR E.COLI BY THE KIRBY-BAUER DISK DIFFUSION METHOD

5.1 ABSTRACT

The β-lactam resistance profiles of urinary tract *E. coli* isolates from Bloemfontein hospitals were studied by Kirby-Bauer disk diffusion antimicrobial susceptibility testing method. Amoxycillin and piperacillin resistances were found to be high (89% and 69% respectively). The resistance rate of piperacillin-tazobactam was relatively low (3% resistant). However, 30% resistance was observed for augmentin. Of the cephalosporins, ceftazidime had the highest resistance rate (17%), followed by cefotaxime (16%), cephazolin (12%) and cefoxitin (6%).

Regression analyses were done in order to determine the correlation between MIC values and inhibition zone diameters. It was found that there was good correlation for the penicillins and penicillin-inhibitors. No correlation was found among the cephalosporins. Seventy-five percent (90 / 120) of isolates originated from female patients, but the resistant-to-susceptible ratio did not differ significantly between genders. The numbers of isolates in different age groups approximated the distribution of age groups in the general population.

5.2 INTRODUCTION

A diverse group of *E. coli* serotypes are capable of causing UTIs and other serious infections. In 1990 purchase of anti-infective agents by pharmacies in the United States totalled $4.6 billion. This amount increased to $8.2 billion in 1994. The cost of β-lactam antibiotics alone, prescribed by medical practitioners in 1994, amounted to $4.5 billion. There has been an increase in resistance to antimicrobial agents in recent years ***(1)***. From current perspectives on β-lactam resistance a number of predictions can be made. Firstly, that antimicrobial resistance will continue to increase as long as currently available antimicrobial agents are used and misused ***(2)***. Secondly, that bacteria will continue to evolve and acquire new mechanisms of resistance to antimicrobial agents. Third, that the policies now in place for dealing with increasing resistance will not be effective ***(3)***. Lastly, that in order to contain antimicrobial resistance, surveillance of β-lactam agents is necessary at local, national and international levels.

5.3 AIMS OF THIS CHAPTER

i. To obtain ampicillin-resistant *E. coli* isolates from hospitalised patients of different gender and age groups with urinary tract infections.

ii. To determine antibiotic sensitivity profiles by using the Kirby-Bauer disk diffusion antimicrobial susceptibility method.

iii. To determine the correlation between inhibition zone diameters and MIC values.

5.4 RESULTS AND DISCUSSION

In vitro antimicrobial susceptibility data can be good predictors of the response of urinary tract infections to antimicrobial therapy ***(4)***. It has been shown that standard *in vitro* susceptibility testing may be reliable for predicting the outcome of chemotherapy ***(5)***. In one clinical trial with 545 urinary tract infections it was found that 66% of infections with susceptible organisms were cured ***(6)***. One study using standard *in vitro* susceptibility testing has also reported similar cure rates for susceptible isolates ***(7)***. In a study of 3-day therapy of cystitis in women, susceptible organisms were frequently eradicated ***(8)***. In the past, longer duration therapy has been used to treat persistent infections. Males who are infected with *E. coli* and who have minimal structural abnormalities often enjoy a better prognosis ***(8)***.

The recommended zone diameters and MIC values for the susceptible and resistant categories, as recommended by the Committee for Clinical Laboratory Standards* for β-lactam agents potentially useful in the treatment of urinary tract infections, were used in this study. These recommendations are applicable to both disk and broth susceptibility testing and do not require alternative testing materials for urinary isolates, e.g. higher-concentration antibiotic disks.

The advantage of the Kirby-Bauer disk diffusion susceptibility testing method in urinary tract infections over other susceptibility testing methods is that it provides *in vitro* information to assist medical practitioners in selecting antimicrobial agents effective in inhibiting growth of organism in the urine and genital tract tissues.

Urine specimens with multiple Gram-negative organism counts of $\geq 10^5$ colony forming units (CFUs) may be due to contamination at collection or improper handling that subsequently allowed multiplication of organisms at room temperature. Sub-inhibitory concentrations of antimicrobials have been demonstrated to have important influences on organisms. In some cases, these sub-inhibitory concentrations have been shown to be bacteriostatic and not bactericidal ***(9)***.

Dissemination of resistant strains in hospitals occurs mainly by person-to-person transmission that may result from ineffective application of basic infection control techniques by hospital personnel.

One hundred and six isolates randomly selected from 120 ampicillin-resistant isolates were subjected to Kirby-Bauer disk diffusion susceptibility testing. The gender of the patients from which these isolates originated was recorded. These isolates were subjected to a contingency Chi-square test to determine if the numbers of isolates originating from male and female patients differed significantly.

In ***Tables 5.1*** and ***5.2*** the survey results and calculations are given. A cursory inspection seemed to indicate that much fewer isolates were from male patients, and that there appeared to be fewer cephalosporin-resistant and more penicillin-resistant isolates. Analysis of the data showed these assumptions to be invalid.

Table 5.1 Number of amoxycillin-resistant isolates from female and male patients

Profile	Females	Males	Total
Resistant	55	23	78
Susceptible	23	5	28
Total	**78**	**28**	**106**

From ***Table 5.1***:

$$\chi^2 = \frac{\left(|ad - bc| - \frac{1}{2}N\right)^2 N}{(a+b)(a+c)(c+d)(b+d)}$$

= [(| 55 x 5 – 23 x 23 | - 0.5 x 106)^2 x 106] / (55 + 23) (55 + 23) (23 + 5) (23 + 5)

= 0.89783, 1 df

The chi-squared value is small enough, probably between 0.5 and 0.3, to show statistical independence. Therefore accept the hypothesis that there was no difference between the ratio of amoxycillin-resistant isolates to the total number of isolates in the male and female patient groups.

Table 5.2 Number of cephalosporin-resistant isolates from female and male patients

Profile	Females	Males	Total
Resistant	11	4	15
Susceptible	67	24	91
Total	**78**	**28**	**106**

From ***Table 5.2***:

$$\chi^2 = \frac{\left(|ad - bc| - \frac{1}{2}N\right)^2 N}{(a+b)(a+c)(c+d)(b+d)}$$

= [(| 11 x 24 – 4 x 67 | - 0.5 x 106)^2 x 106] / (11 + 24) (11 + 67) (67 + 24) (4 + 24)

= 0.03659, 1 df

Therefore accept the hypothesis that there was no difference between the ratio of cephalosporin-resistant isolates to the total number of isolates in the male and female patient groups.

Women with recurrent lower urinary tract infections are frequently given amoxycillin as prophylaxis ***(10)***. The major therapeutic limitation for amoxycillin is the high frequency of resistant β-lactamase-producing *E. coli*. This could be due to the extensive use of amoxycillin for a variety of infections in the past. Urinary tract infections are often treated empirically and susceptibility tests are often carried out only when the patient has not responded to one or more courses of antibiotics.

Amoxycillin and ampicillin are often used as oral therapy for Gram-negative UTIs, but the high rate of *in vitro* resistance demonstrated in this study and others suggests that these antibiotics should be used only after susceptibility has been verified ***(10)***.

Urine analysis of isolates would generally not be appropriate for asymptomatic women because that would indicate that there was contamination at collection of the specimens ***(11)***.

Third generation cephalosporins have become associated with a rise in the occurrence of antibiotic-associated diarrhoea due to *Clostridium difficile* as well as an increase in the prevalence of antibiotic-resistant organisms such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci,* and extended-spectrum β-lactamase-producing Gram-negative bacilli. Although the rate of resistance to ceftriaxone is very low at present, it should be used with caution for *E. coli* UTIs due to the undesirable side effects associated with this antibiotic ***(11)***.

Figure 5.1 shows a high percentage of piperacillin resistance in isolates from males and females. Piperacillin poses an increasingly complex problem for clinicians because of its increasing usage. Infections in males rarely occur spontaneously, except during the neonatal period, but rather tend to follow catheterisation of the urinary tract. The infection will most often persist, be difficult to treat, and be difficult to eradicate if the prostrate becomes colonised or if the patient has urinary calculi or stones, obstruction to urine flow or structural abnormalities. Infections caused by resistant pathogens have higher rates of morbidity and mortality associated with them than do infections caused by susceptible strains. The use of piperacillin is expensive, ampicillin or amoxycillin are cheaper alternatives ***(12)***.

Although antibiotics effective against Gram-positive and Gram-negative bacteria do not generally constitute reliable therapy against extended-spectrum β-lactamase producers, their substitution in place of cephalosporins appears to reduce the emergence of ESBL-resistant pathogens ***(13)***.

Because of this phenomenon, additional testing for suspected organisms is necessary. Expanded testing will be of assistance to clinicians in optimizing the clinical care of patients with Gram- negative infections ***(14)***.

In Western Australia, greater use of third-generation cephalosporins was shown to correlate with more *Clostridium difficile*-associated diarrhoea in a large teaching hospital during the 1980s. During the 1990s the use of third-generation cephalosporins in this hospital remained high and at the end of 1998 a policy was introduced to prevent the use of ceftriaxone (the only third generation cephalosporin in use) without prior approval. This resulted in a decline in the use of third-generation cephalosporins and a 50% reduction in the incidence of *Clostridium difficile*-associated diarrhoea during 1999 and 2000 ***(14)***.

The detection, prevalence, and clinical implications of infection by ESBL-producing *E. coli* are of considerable concern. These enzymes hydrolyse β-lactams including the cephalosporins ceftazidime, ceftriaxone and cefotaxime, thus resulting in treatment failures ***(15)***. Standard *in vitro* testing may report these isolates as susceptible when in fact they are resistant *in vivo* because they fail to reach current Committee for Clinical Laboratory Standards* breakpoints for resistance.

Augmentin can theoretically be more effective in the treatment of women than in the treatment of men. The urine of females is more alkaline than that of males, which would allow increased activity of amoxycillin ***(15)***.

The use of β-lactamase inhibitors in combination with β-lactam antibiotics is currently the most successful strategy to combat specific resistance mechanisms. Their broad spectrum of activity originates from the ability of inhibitors to inactivate a wide range of β-lactamases produced by plasmid-mediated TEM β-lactamases. The combination of amoxycillin and clavulanic acid is very effective to overcome resistance due to β-lactamases. Augmentin is useful when the susceptibility of the organism to amoxycillin is unknown, or for the treatment of amoxycillin-resistant strains. The negative effect of a large inoculum of bacteria against augmentin is cause for concern.

Despite the attractiveness of augmentin for the treatment of urinary tract infections, it is more expensive than most other penicillins. When an organism is found to be susceptible to one of the older β-lactams, that antibiotic should be used first. Selection of a β-lactam should be based on a report of lower MICs. Resistance to the combination of amoxycillin and clavulanate was first described in 1989 in clinical isolates of *E. coli* recovered from blood, urine and semen specimens of individuals admitted to Cochin Hospital in France ***(16)***. The mechanism of resistance was found to be the production of TEM-derived β-lactamases that showed reduced affinity for clavulanic acid ***(16)***. Clavulanic acid is excreted via the kidneys. Thus, the high concentration of this agent found in urine may have selected inhibitor-resistant (IRT) variants of TEM β-lactamase in urinary tract pathogens. Inhibitor-resistant TEM

β-lactamase producing bacteria were reported mainly in France, the UK and Greece. Bacterial strains producing IRT enzymes are also present in other countries, but have not been reported extensively because of the inherent difficulty of detecting the IRT phenotype ***(16)***.

Regarding the difference between genders, it appears that penicillin-resistant strains consistently are found at slightly higher levels in males ***(Figure 5.1)***. Females carry more cephalosporin-resistant strains, the difference being much more pronounced. Cefoxitin is a notable exception, where almost 90 percent of males carried resistant strains compared to about 10 percent in females.

There was a pronounced difference in resistance levels between penicillins and cephalosporins ***(Figures 5.2*** and ***5.3)***. Among the penicillins, high levels of resistance to amoxycillin and piperacillin were recorded. With the inhibitor-antibiotic combinations (augmentin and piperacillin-tazobactam), resistance was dramatically reduced, with piperacillin-tazobactam showing almost no resistance.

Age does not appear to be a significant factor influencing the relative rates of resistance in all antibiotic types, except in the case of cephalosporins in the 11 to 20 year group.

Amino acid substitution was proposed to be responsible for the IRT phenotype ***(17)***. TEM-1 is the most widespread β-lactamase worldwide, and amino acid substitutions in this enzyme are not unusual and are frequently found in clinical variants able to hydrolyse third-generation cephalosporins ***(18)***.

The bacterial penicillin binding proteins (PBPs) and TEM-1 β-lactamases from *E. coli* have evolved, leading to higher levels of resistance to both β-lactams and inhibitors ***(19)***. The clinically useful inhibitors clavulanic acid, sulbactam and tazobactam are not active against all β-lactamases ***(Figure 5.1)***.

Resistance to piperacillin was 70.5% for females and 82.1% for males ***(Figure 5.1)***. This was one of the highest resistance rates in both males and females. Combining tazobactam with piperacillin decreased resistance to 3.84% for females and to 10.78% resistance for males.

Cephazolin may be a useful agent for the treatment of urinary tract infections, due to the general susceptibility of organisms ***(Figure 5.1)***.

Cefoxitin was originally developed because of its activity against organisms such as *E. coli*. Although cefoxitin showed activity against *E. coli* ***(Figure 5.1)***, it was less active in males and therefore should not be used in preference to cephazolin.

The true prevalence of organisms producing cephalosporinase is currently unknown. Patients at risk of acquiring an infection by an organism producing a cephalosporinase include those with previous antibiotic exposure, undergoing surgery or instrumentation, prolonged

hospital stays, admission to an intensive care unit and admission to a nursing home. Many *E. coli* isolates that produce extended-spectrum β-lactamases do not appear to be resistant to third-generation cephalosporins on conventional disk susceptibility testing ***(20)***.

These enzymes require an appropriate test for their detection. Such tests include the double disk synergy test, or the more sophisticated three-dimensional test described by Thomson and Sanders ***(21)***.In addition, ESBL-producing bacteria are frequently resistant to many classes of non-β-lactam antibiotics, resulting in difficult-to-treat infections ***(21)***.

If the double disk synergy test is not applied, problems with bacteria that produce extended-spectrum β-lactamases may only be apparent when therapeutic failures occur. Ceftazidime should not be administered based on the results of ***Figure 5.1*** because of probable ESBL production. The enzyme TEM-26 was first reported in 1992 in a paediatric oncology ward in Stanford, California, where ceftazidime was used to treat neutropaenic children ***(22)***.These enzymes were responsible for an episode of ceftazidime resistance in a chronic care unit in Massachusetts in 1988 ***(23)***.

In 1989, the extended-spectrum cephalosporin ceftazidime was extensively used to treat patients with suspected Gram-negative septicaemia in the paediatric oncology ward at St James's University Hospital, Leeds. In November 1989, two patients presented with septicaemia caused by ceftazidime-resistant Gram-negative bacilli. These isolates were shown to be ESBL-producing *Enterobacteriaceae* by the double disk synergy test described by Philippon *et al* ***(24)***.

The reason why the types of commonly-found ESBL *E. coli* enzymes vary at different times and locations remains to be determined. If the ribotyping profiles of clinical isolates show similar patterns, there would probably be clonal spread between hospitals. Therefore, plasmids or other transposable elements might have spread among the clinical isolates through the patients, and the selection pressure by similar antimicrobial use in each hospital might have selected organisms that contained similar plasmids.

Figures 5.2 and ***5.3*** show a low degree of resistance to all extended-spectrum β-lactam antibiotics in all age groups. ESBL-producing urinary tract *E. coli* strains are a growing concern. Organisms that produce these enzymes are mostly *K. pneumoniae* and *E. coli.* These enzymes are highly efficient at inactivating the newer third-generation cephalosporins e.g. cefotaxime, ceftazidime and ceftriaxone ***(Figure 5.3)***. The emergence and dissemination of ESBL-producing urinary tract *E. coli* in hospitalised patients may be the consequence of clonal dissemination of a few epidemic strains along with horizontal transmission of resistance by plasmids among urinary tract *E. coli* isolates in Bloemfontein hospitals.

Since 38.3% of patients in hospitals are of the age group 41-100, ceftriaxone has become the antibiotic of choice for the treatment of infections such as pneumonia in many hospitals in recent years. This extensive use of ceftriaxone resulted in resistance due to strong selection pressure.

A second type of transmissible resistance to oxyimino-β-lactams arises from plasmid acquisition of a normal chromosomal *ampC* gene. In addition to oxyimino-β-lactam resistance, AmpC β-lactamase also provides resistance to cephamycins such as cefoxitin. *E. coli* and *Klebsiella* isolates with this type of resistance are currently thought to be rare ***(25)***.

The inhibition zone diameters for individual antibiotics can be interpreted as belonging to susceptible, intermediate or resistant categories by referring to an interpretative chart. The rationale behind these interpretative charts is that the relationship between the inhibition zone diameter and the MIC is known and that the limits for the categorisation of susceptibility (breakpoints) take into account the distribution of strains as to susceptibility ranges and the levels of the antibiotics achievable *in vivo*.

The outer limit of the inhibition zone contains an antibiotic concentration similar to the MIC of that antibiotic for a specific organism. The MIC is expressed in logarithmic form and plotted against the inhibition zone diameter produced by any given disk. The linear regression lines resulting from such analyses were obtained via a standardised method employing Mueller-Hinton agar and a Steers replicator. The regression line is calculated from the formula of least squares and consists of the $\log_2$ MIC value as the independent variable and the zone diameter as the dependent variable. MICs are plotted on the y-axis as the dependent variable. This latter presentation more readily adapts for the approximation of the MIC from a given zone size produced by a fixed amount of antibiotic. The breakpoint values for MIC and inhibition zone values are represented in ***Table 5.3*** for standard antibiotic susceptibility disks.

Table 5.3 Breakpoint values for inhibition zone diameters and MIC values*

Antibiotic	Resistant	Intermediate	Susceptible	Control	MIC breakpoints µg / ml
Augmentin	≤ 13 mm	14-16 mm	≥18 mm	16-22 mm	2 – 8
Piperacillin	≤ 17 mm	18-20 mm	≥21 mm	24-30 mm	1 – 8
Cephazolin	≤ 14 mm	15-17 mm	≥18 mm	23-29 mm	1 – 4
Cefoxitin	≤ 14 mm	15-17 mm	≥18 mm	23-29 mm	1 – 4

Fig 5.1 KIRBY-BAUER DISK RESISTANCE PROFILES BY GENDER

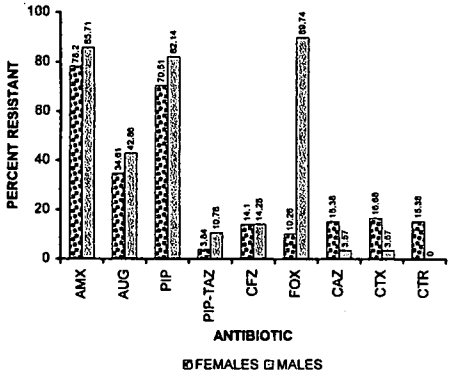


Fig 5.2 KIRBY-BAUER DISK RESISTANCE PROFILES BY AGE (PENICILLINS)

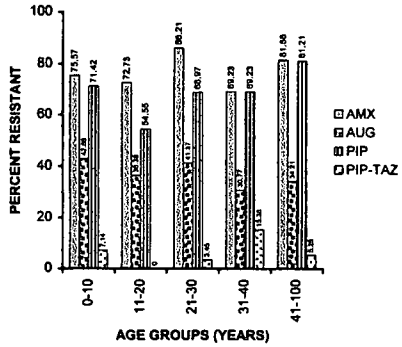


Fig 5.3 KIRBY-BAUER DISK RESISTANCE PROFILES BY AGE (CEPHALOSPORINS)

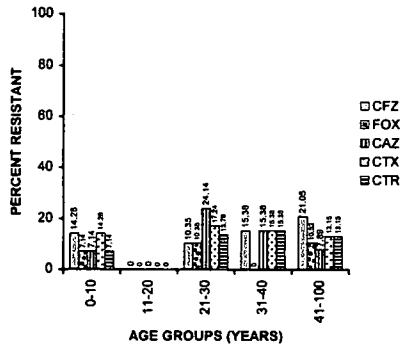


Fig 5.4 REGRESSION LINE OF AUGMENTIN MICs AGAINST ZONE DIAMETER

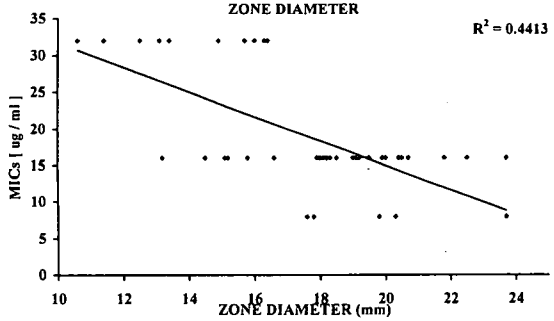


Fig 5.5 REGRESSION LINE OF PIPERACILLIN MICs AGAINST ZONE DIAMETER

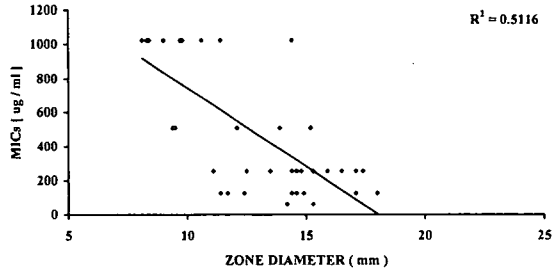


Fig 5.6 REGRESSION LINE OF CEPHAZOLIN MICs AGAINST ZONE DIAMETER

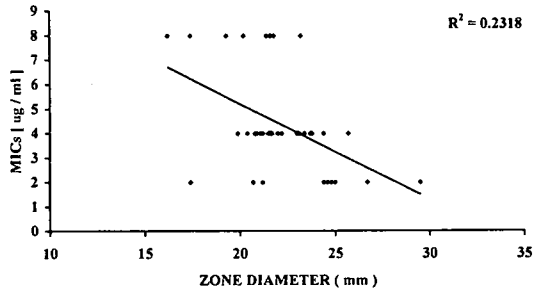
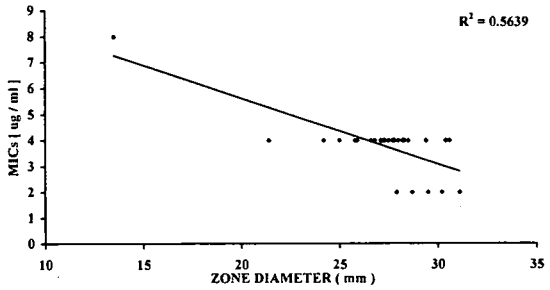


Fig 5.7 REGRESSION LINE OF CEFOXITIN MICs AGAINST ZONE DIAMETER



5. 5 CONCLUSIONS

i. The prevalence of hospital-acquired urinary tract infections caused by β-lactam-resistant *E. coli* isolates is a cause for concern as it can lead to an increase in morbidity and mortality.

ii. The preponderance of isolates originating from female patients may be indicative of a higher urinary tract infection rate for females and the fact that women get UTIs in their reproductive age.

iii. Age and gender do not have significant effects on either resistance profiles or the ratios of resistant to susceptible strains in patients.

iv. The Kirby-Bauer disk susceptibility testing technique alone or in combination with multi-point replicator technology is effective for providing fast, reliable and affordable susceptibility data for clinical use.

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*Note that the name "**National Committee for Clinical Laboratory Standards**" (NCCLS) has been changed to "**Clinical Laboratory Standards Institute**" (CLSI)

CHAPTER 6

DETECTION OF EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING ESCHERICHIA COLI ISOLATES

6.1 ABSTRACT

Many ESBL-producing isolates did not appear to be resistant to ceftazidime, ceftriaxone and cefotaxime when using the Kirby-Bauer disk diffusion technique or when assessed by MIC values. The double disk (DD) technique described by Jarlier *et al.* proved to be more sensitive for detecting extended-spectrum β-lactamases than the Kirby-Bauer disk diffusion technique. Of one hundred and six *E. coli* isolates from urinary tract infections, fifty-two (49.1%) were found to produce ESBLs by the Jarlier double disk (D) method. In contrast, only twelve (23 %) were initially detected by determining MICs and Kirby-Bauer inhibition zone diameters for amoxycillin, piperacillin, augmentin, cephazolin, cefoxitin, ceftazidime, ceftriaxone and cefotaxime. Each of the 52 ESBL producing strains were categorised according to specimen type. Two strains were isolates from urine bags, 16 from urine catheters, 10 from midstream urine specimens, 2 from supra-pubic aspiration and 22 from unspecified samples.

ESBLs prevalence was not correlated with specimen type or length of hospital stay.Class A extended-spectrum β-lactamases hydrolysed extended-spectrum β-lactams and were inhibited by clavulanic acid and tazobactam. These β-lactamases are divided into two groups: TEM and SHV derivatives, and non-TEM and non-SHV extended-spectrum β-lactamases. The plasmid-mediated cephalosporinases also hydrolysed extended-spectrum cephalosporins and cefoxitin and were not inhibited by clavulanic acid.

6.2 INTRODUCTION

Extended-spectrum β-lactamase-producing organisms can erroneously be reported as susceptible to third-generation cephalosporins. In South America and Eastern Europe, for example, ESBL-producing organisms resistant to ceftazidime often appear to be susceptible. In order to improve the accuracy of reporting of ESBL detection, the new CLSI guidelines for screening and confirmatory testing of ESBL production by *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *E. coli* should be used. For laboratories using the disk diffusion test as the initial method of antibiotic susceptibility testing, the revised inhibition zone diameters for all antibiotics should also be used ***(1)***.

Many methods are available for detecting ESBLs. All *Enterobacteriaceae* with either decreased zone diameter of $\leq$ 30 mm or MICs $\geq$ 2 μg / ml to the extended-spectrum cephalosporins (ceftazidime, cefotaxime or ceftriaxone) and aztreonam should be subjected to the Jarlier double disk test for detection of ESBL production ***(2)***.

It is of concern that some of the strains producing ESBLs appear sensitive with routine susceptibility testing while they are in fact resistant. It is important that these organisms be monitored and policies for antibiotic use and infection control be put in place to stop their spread. Rising β-lactam resistance rates among bacterial pathogens have resulted in increased morbidity and mortality from nosocomial infections. Widespread use of certain β-lactam antibiotics has been shown to foster development of β-lactamases in previously susceptible bacterial populations. Reduction in the use of these agents and concomitant increase in the use of β-lactam antibiotics in combination therapy with aminoglycosides (e.g. ampicillin and gentamicin) have greatly restored bacterial susceptibility. Microbiologists should explicitly report the presence of ESBLs to clinicians, because ESBLs have important implications for antibiotic therapy in patients with serious infections by ESBL-producing organisms *(1)*.

Many hospital-acquired outbreaks of ESBL-producing isolates associated with prolonged hospital stays, surgery, previous antibiotic exposure, and instrumentation, admission to intensive care and admission to nursing homes have been reported. The extensive use of ceftazidime in particular has been implicated in hospital-acquired outbreaks in the United States. In each outbreak the numbers of ESBL-producing isolates at the institutions were markedly reduced when restrictions were placed on the use of ceftazidime as well as employing rigorous hand washing techniques, barrier nursing and isolation of infected and colonized patients *(3)*.

6. 3 AIMS OF THIS CHAPTER

i. To determine the prevalence of ESBL-producing and conjugative *E. coli* isolates.
ii. To determine the prevalence of ESBL-producing isolates in different specimen types.
iii. To determine the relationship between length of hospital stay and colonisation by ESBL-producing *E. coli* isolates.

6. 4 RESULTS AND DISCUSSION

AmpC β-lactamases mediate resistance to cephamycins, e.g. cefoxitin, and to oxyimino β-lactams. *Klebsiella pneumoniae* and *E. coli* isolates with this type of resistance are currently thought to be rare *(3)*. ***Tables 6.1*** and ***6.2*** show that these enzymes are group 2 penicillinases inhibited by the active, site-directed β-lactamase inhibitors, clavulanate and tazobactam. ESBL-producing organisms do not appear to be resistant to the newer cephalosporins with routine susceptibility testing *(1)*. Because they often occur in clinical isolates that are resistant to other useful agents, therapeutic options can be limited *(4)*. These inhibitor-resistant

penicillinases are TEM derivatives (except for SHV-10) and plasmid-mediated, and are mainly from *E. coli* isolates *(5)*. There is a second type of transmissible resistance to oxyimino-β-lactam that arises when plasmids acquire chromosomal *ampC* genes.

Table 6.2 shows that there are plasmid-mediated penicillinases and broad-spectrum β-lactamases that are inhibited by β-lactamase inhibitors. All isolates with MIC values above 4μg / ml are considered resistant to piperacillin and broad-spectrum β-lactams such as amoxycillin. These enzymes are inhibited by the active site-directed β-lactamase inhibitors: tazobactam and clavulanic acid. They are of the molecular class A and their preferred substrates are the penicillins *(5)*. Extended-spectrum cephalosporins have been specifically designed to resist hydrolysis by older broad-spectrum β-lactamases such as TEM-1, TEM-2, and SHV-1 ***(Table 6.2)***. The response of non-inducible members of the family *Enterobacteriaceae* to the extended-spectrum cephalosporins has been to produce mutant forms of the older β-lactamases called extended-spectrum β-lactamases *(6)*. All extended-spectrum cephalosporins (ceftazidime, ceftriaxone and cefotaxime) resist degradation by broad-spectrum β-lactamases ***(Tables 6.1*** and ***6.2)***.

The first report to identify the different types of ESBLs produced by members of the family *Enterobacteriaceae* isolated from major medical centres in South Africa was published in 1998 *(6)*. There are Ambler class A penicillinases that possess an extended hydrolysis spectrum directed toward third-generation cephalosporin inhibitors such as clavulanic acid and tazobactam inhibit the activity of these ESBLs *(7)*.

Many extended-spectrum penicillinase genes have been sequenced. These enzymes are mainly derivatives of TEM-1, TEM-2 and SHV-1 restricted-spectrum β-lactamases. Unlike their progenitors, SHV-2 to SHV-9 and TEM-3 to TEM-29, TEM-42, TEM-43 and TEM-51 have point mutations along the 280 amino-acid length of the protein. Limited amino-acid changes are contained within the catalytic sites and may be the basis for the extended hydrolytic properties of these enzymes *(7)*.

ESBLs have also been detected and isolated from patients colonised under improved environmental hygienic standards. The spread of ESBLs can therefore be attributed primarily to patient-to-patient transfer, rather than to direct selection of point-mutation derivatives *(7)*.

The extended-spectrum class A β-lactamases that are not TEM and SHV derivatives are PER-1 enzymes. Each gives resistance to all the cephalosporins, aztreonam, and penicillins but not to carbapenems and cephamycins *(8)*.

Table 6.1: Detection of ESBLs by MIC Resistance to third Generation Cephalosporins

Isolates	Pip-taz	Amx (mm)	Amx [Mic]	Pip (mm)	Pip [Mic]	Aug (mm)	Aug [Mic]	Cfz (mm)	Cfz [Mic]	Fox (mm)	Fox [Mic]	Caz (mm).	Caz[Mi]	Ctr (mm)	Ctr[Mic]	Ctx(mm)	Ctx [Mic]
MB1539	25	0	≥2048	11.4	1024	14.9	32	21.3	256	27.8	64	30	≥8	33.6	>4	35.3	≥4
MB1977	23.6	0	≥2048	13.3	16	22.5	16	14.4	≥256	26.4	128	33	0.125	26.3	0.03	34.4	≥4
MB2396	22.7	0	2048	9	2048	13.4	32	11.5	64	34.4	8	28	4	35.6	0.06	36.1	0.125
MB2516	19.9	23.9	8	19.7	8	17.9	0.25	8.9	256	26.3	64	28	≥8	27.6	≥4	0	0.008
MB2730	31.1	0	≥2048	19.5	128	19.13	16	23.8	4	27.9	8	31	0.25	36.8	0.03	36.7	≥4
MB2871	27.1	0	2048	27.2	64	23.2	16	32.1	≥256	23.7	32	24	≥8	14.2	≥4	15.8	≥4
MB3056	23.7	0	≥2048	11.4	128	16.4	32	20	256	25.4	32	29	≥8	31.7	0.008	33.1	0.06
MB3087	29.2	10.85	≥2048	9.7	1024	19.1	16	19.2	128	27.4	64	25	0.125	30.9	0.03	32.5	>4
MB3328	22.7	0	≥2048	8.3	1024	19.5	16	21.9	32	25.8	4	27	0.5	28.6	≥4	31.3	0.125
MB3580	24.7	0	2048	10.5	64	17.5	16	25	2	21.4	64	32	0.125	30.6	≥4	33.5	≥4
MB3727	22.7	0	≥2048	14.4	256	15.2	16	18.2	256	17.4	64	28	≥8	30.1	≥4	30.4	≥4
MB3729	22.7	0	≥2048	14.1	1024	16.5	16	18.2	128	25.8	64	29	0.125	30.7	≥4	29.6	≥4

Resistant isolates

Zone Diameter (mm)

Minimum inhibitory concentration (mic)

Piperacillin/tazobactam (Pip-taz, Amoxycillin (Amx), Piperacillin (Pip), Augmentin (Aug)

Cephazolin (Cfz), Cefoxitin (Fox), Ceftazidime (Caz), Ceftriaxone (Ctr), Cefotaxime (Ctx)

Table 6.2: Detection of ESBLs by the Jarlier`s Double Disk Method

Isolates	Pip-taz	Amx (mm)	Amx [Mic]	Pip (mm)	Pip [Mic]	Aug (mm)	Aug [Mic]	Cfz (mm)	Cfz [Mic]	Fox (mm)	Fox [Mic]	Caz (mm).	Caz[Mi]	Ctr (mm)	Ctr[Mic]	Ctx(mm)	Ctx [Mic]
MB1477	27.5	0	>2048	16	64	20.4	16	22	4	27	8	29	0.3	0.3	0.3	0.3	0.3
MB1508	28.7	0	16	13	2	19.7	1	24	1	27	4	29	0.1	0.1	0.1	0.1	0.1
MB1527	26.5	0	>2048	10	512	16.3	32	23	8	26	2	29	0.1	0.1	0.1	0.1	0.1
MB1534	27.6	0	>2048	10	1024	16.3	64	20	16	30	2	31	0.5	0.5	0.5	0.5	0.5
MB1536	25.9	0	>2048	17	256	19.5	16	23	4	26	4	28	0.1	0.1	0.1	0.1	0.1
MB1539	25	0	>2048	11	1024	14.9	32	21	256	28	64	30	>8	>8	>8	>8	>8
MB1553	26.6	0	>2048	9	512	18.9	32	22	8	28	4	31	0.1	0.1	0.1	0.1	0.1
MB1928	26.8	0	>2048	11	256	19.2	16	22	4	27	4	29	0.1	0.1	0.1	0.1	0.1
MB1931	28	0	>2048	17	256	19.5	16	22	4	27	4	29	0.3	0.3	0.3	0.3	0.3
MB1942	23.9	0	>2048	8	>2048	13.7	64	15	32	31	4	30	0.3	0.3	0.3	0.3	0.3
MB1977	23.6	0	>2048	13	16	22.5	16	14	>256	26	128	33	0.1	0.1	0.1	0.1	0.1
MB2065	31	0	>2048	12	128	20.5	16	23	4	28	8	31	0.5	0.5	0.5	0.5	0.5
MB2066	27.1	0	>2048	15	64	19.9	32	22	4	29	8	31	0.3	0.3	0.3	0.3	0.3
MB2072	23.6	0	>2048	9	1024	16	32	20	8	29	4	30	0.3	0.3	0.3	0.3	0.3
MB2767	28	0	>2048	15	512	19.9	16	22	4	29	4	31	0.3	0.3	0.3	0.3	0.3
MB2396	22.7	0	2048	9	2048	13.4	32	12	64	34	8	28	4	4	4	4	4
MB2404	27.1	0	>2048	17	128	18.5	16	24	2	30	4	30	0.1	0.1	0.1	0.1	0.1
MB2474	25.9	0	>2048	11	1024	10.6	32	19	16	26	4	29	0.1	0.1	0.1	0.1	0.1
MB2481	27.5	0	>2048	12	512	19.8	32	21	8	30	2	32	0.1	0.1	0.1	0.1	0.1
MB2509	25.9	0	>2048	18	128	14.5	16	25	2	26	8	29	0.3	0.3	0.3	0.3	0.3
MB2684	26.7	0	>2048	16	256	17.7	16	23	2	31	2	29	0.1	0.1	0.1	0.1	0.1
MB2697	29.3	0	2048	15	256	17.6	8	20	2	23	2	19	0.1	0.1	0.1	0.1	0.1
MB2703	27.3	0	>2048	15	256	16.6	16	16	8	24	8	29	0.3	0.3	0.3	0.3	0.3
MB2726	29.5	0	>2048	18	512	19.9	16	24	2	20	2	30	0.1	0.1	0.1	0.1	0.1
MB2731	29.3	0	>2048	18	512	19.8	16	26	4	31	4	32	0.1	0.1	0.1	0.1	0.1
MB2881	25.3	0	>2048	17	256	17.9	16	23	4	27	8	29	0.3	0.3	0.3	0.3	0.3
MB2936	28.2	0	>2048	18	512	19.9	32	24	4	28	4	30	0.3	0.3	0.3	0.3	0.3
MB2951	25.5	0	>2048	15	256	15.8	16	21	4	25	8	28	0.3	0.3	0.3	0.3	0.3

Table 6.2: Detection of ESBLs by the Jarlier`s Double Disk Method continued

Isolates	Pip-taz	Amx (mm)	Amx [Mic]	Pip (mm)	Pip [Mic]	Aug (mm)	Aug [Mic]	Cfz (mm)	Cfz [Mic]	Fox (mm)	Fox [Mic]	Caz (mm).	Caz[Mi]	Ctr (mm)	Ctr[Mic]	Ctx(mm)	Ctx [Mic]
MB2967	26.2	0	>2048	12	128	18.3	16	24	4	28	4	29	0.1	0.1	0.1	0.1	0.1
MB2975	26.7	0	>2048	14	1024	12.5	32	16	8	28	8	30	0.1	0.1	0.1	0.1	0.1
MB2881	24.6	0	>2048	15	128	17.9	32	21	4	27	8	29	0.1	0.1	0.1	0.1	0.1
MB2986	26.3	0	>2048	15	128	20.7	16	21	4	26	8	28	0.1	0.1	0.1	0.1	0.1
MB3010	25.2	0	>2048	14	128	18.1	16	21	2	28	4	30	0.1	0.1	0.1	0.1	0.1
MB3037	23.2	0	>2048	0	1024	20	32	17	16	27	4	29	0.3	0.3	0.3	0.3	0.3
MB3045	26.3	0	>2048	17	1024	19.8	16	22	8	28	4	28	0.1	0.1	0.1	0.1	0.1
MB3050	21.4	0	>2048	11	1024	19	16	19	8	25	4	28	0.1	0.1	0.1	0.1	0.1
MB3056	23.7	0	>2048	11	128	16.4	32	20	256	25	32	29	>8	>8	>8	>8	>8
MB3080	20.1	8.3	>2048	0	>2048	0	64	18	32	19	8	27	0.3	0.3	0.3	0.3	0.3
MB3096	28.1	12.5	>2048	12	128	21.9	16	21	4	24	8	28	0.1	0.1	0.1	0.1	0.1
MB3190	26.6	0	>2048	14	256	15.1	16	17	8	0	8	0	0.3	0.3	0.3	0.3	0.3
MB3328	22.7	0	>2048	8	1024	19.5	16	22	32	26	4	27	0.5	0.5	0.5	0.5	0.5
MB3580	24.7	0	2048	11	64	17.5	16	25	2	21	64	32	0.1	0.1	0.1	0.1	0.1
MB3595	27.8	0	2048	10	64	23.7	16	25	2	29	2	27	0.1	0.1	0.1	0.1	0.1
MB3601	21.5	0	>2048	14	64	19.4	16	17	2	19	16	30	0.1	0.1	0.1	0.1	0.1
MB3695	25.4	0	>2048	14	128	21.5	16	21	2	28	4	29	0.1	0.1	0.1	0.1	0.1
MB3727	22.7	0	>2048	14	256	15.2	16	18	256	17	64	28	>8	>8	>8	>8	>8
MB3729	22.7	0	>2048	14	1024	16.5	16	18	128	26	64	29	0.1	0.1	0.1	0.1	0.1
MB3731	28.5	0	>2048	18	512	18.2	16	21	4	21	4	29	0.1	0.1	0.1	0.1	0.1
MB3878	26.5	0	>2048	10	256	19.7	16	23	4	28	2	28	0.1	0.1	0.1	0.1	0.1
MB3879	25.5	0	>2048	19	128	21.4	8	30	2	25	4	28	0.1	0.1	0.1	0.1	0.1
MB3938	30.7	0	>2048	17	256	21.5	16	25	2	27	4	31	0.1	0.1	0.1	0.1	0.1
MB3962	25.9	0	1024	15	64	20.6	16	24	2	28	4	30	0.1	0.1	0.1	0.1	0.1
Breakpoint (mm)	20	16		20		17		17		17		17		20		22	
Mic resistance				>32		>128		>16		>32		>32		>32		>64	

Fifty two out of 106 *E. coli* strains from 5 different specimen types produced ESBLs ***(Table 6.3)***. Two (100%) out of 2 strains from urine bag specimens demonstrated ESBL production. Twenty two (45.8%) out of 48 strains from unspecified specimens produced ESBLs. This underscored the importance of proper specimen labelling.

Table 6.3 Numbers of ESBL-producing *E. coli* isolates from different specimen types

		ESBL $^-$	ESBL $^+$	df	χ^2
		Exp = 54 / 106 x Obs	*Exp = 52 / 106 x Obs*		
Urine bag	**Observed**	**0**	**2**		
	Expected	1.01887	0.98113		
	Obs – Exp	-1.01887	1.01887		
	$\chi^2 = (Obs - Exp)^2 / Exp$	1.01887	1.05806	1	2.07693
Catheter	**Observed**	**12**	**16**		
	Expected	14.26415	13.73585		
	Obs – Exp	-2.26415	2.26415		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.35939	0.37321	1	0.73260
Mid-stream	**Observed**	**15**	**10**		
	Expected	12.73585	12.26415		
	Obs – Exp	2.26415	-2.26415		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.40252	0.41800	1	0.82052
Supra-pubic	**Observed**	**1**	**2**		
	Expected	1.52830	1.47170		
	Obs – Exp	-0.52830	0.52830		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.18262	0.18965	1	0.37227
Unspecified	**Observed**	**26**	**22**		
	Expected	24.45283	23.54717		
	Obs – Exp	1.54717	-1.54717		
	$\chi^2 = (Obs - Exp)^2 / Exp$	0.09789	0.10166	1	0.19955
Summed data	**Observed**	**54**	**52**	**5**	**4.20187**
	Expected	54.0	52.0		
	Obs – Exp	0.0	0.0		
	χ^2	0.0	0.0		**0.0**

The homogeneity chi-squared value of 4.20 – 0.0 = 4.20 does not exceed the value of 11.07 at the 95 % level of significance for 5 degrees of freedom. The hypothesis that the classes are homogeneous is therefore accepted. Chi-squared values for the individual specimen classes are small enough in all cases to show statistical independence of the particular experimental conditions. Therefore there is no difference between the prevalence of ESBL-producing isolates in different specimen types.

Infection with ESBLs producing strains was observed in urinary tract *E. coli* isolates among hospitalised and not-hospitalised patients in Bloemfontein ***(Table 6.4)***. Patients whose specimens were taken more than 48 hours after admission were considered hospitalised.

Those whose specimens were taken before 48 hours of admission were considered non-hospitalised. Of the 33 isolates from hospitalised patients, 17 (51.5 %) produced ESBLs. For non-hospitalised patients, 53 (47.9 %) of 73 isolates produced ESBLs.

Table 6.4 Numbers of ESBL-producing *E. coli* isolates from different patient groups

ESBL	Hospitalised	Not Hospitalised	Total
Negative	16	38	54
Positive	17	35	52
Total	**33**	**73**	**106**

From ***Table 6.4***:

$$\chi^2 = \frac{\left(|ad - bc| - \frac{1}{2}N\right)^2 N}{(a+b)(a+c)(c+d)(b+d)}$$

= [(| 16 x 35 – 17 x 38 | - 0.5 x 106)^2 x 106] / (16 + 38)(16 + 17)(17 + 35)(38 + 35)

= 0.0171, 1 df

This chi-squared value is small enough to show statistical independence with a probability between 0.9 and 0.7. Therefore there is no difference between the prevalence of ESBL-producing isolates in hospitalised and non-hospitalised patients.

The ratio of ESBL producers to non-producers in hospitalised and non-hospitalised patients is approximately the same. The finding that there was no difference between the prevalence of ESBL-producing isolates in hospitalised and non-hospitalised patients could be influenced by changing the cut-off from 48 hours to a later point.

6.5 CONCLUSIONS

Some ESBLs confer high-level resistance to all oxyimino β-lactams, but for other ESBLs, resistance is only slightly increased or decreased selectively for β-lactams. This creates a problem for clinical laboratories since organisms producing less-active ESBLs can fail to reach current Committee for Clinical Laboratory Standards* breakpoints for resistance yet can cause significant disease.

ESBL prevalence is not correlated with specimen type or with the length of hospital stay. The 48-hour cut-off point used in this study was probably too short to differentiate between the two patient categories. In this study, there were more outpatients than hospitalised patients with infection by extended-spectrum β-lactamases.

As antimicrobial resistance becomes more prevalent, clinicians are relying increasingly on broad-spectrum agents for prophylaxis and treatment of infections of critically ill patients. While selection pressure and antibiotic use and abuse have been important factors in the rapid emergence of resistance, the inconsistent application of basic infection control techniques by hospital personnel may account for the dissemination of resistant strains in hospitals.

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*Note that the name **"National Committee for Clinical Laboratory Standards"** (NCCLS) has been changed to **"Clinical Laboratory Standards Institute"** (CLSI)

CHAPTER 7

BETA-LACTAM MULTI-RESISTANCE IN ESCHERICHIA COLI ISOLATES

7.1 ABSTRACT

Multiple resistances among *E. coli* isolates was assessed by calculating the extent of cross resistance using chi-square analysis. Higher levels of multiple resistances were found among penicillin resistant bacteria than among cephalosporin resistant bacteria. The prevalence of penicillin resistant donors was higher among the donors than the transconjugants. The probability of an *E. coli* isolate to pairs of antibiotics were calculated. It was found that the probability of dual resistance among penicillins was very high. However, the probability of dual resistance among cephalosporins and penicillins were very low.

7.2 INTRODUCTION

It is known that when treating infections due to sensitive strains of several important bacterial species with extended-spectrum cephalosporins, variants emerge that exhibit resistance not only to the agent employed but also to an entire class of β-lactam antibiotics. Such multiple resistance results from mutational hyper-production of class 1 β-lactamase. Multiple resistance poses serious problems to the patient and to the hospital environment and mandates hospital-wide antibiotic resistance surveillance to detect the impact of usage of the newer broad-spectrum agents ***(1)***.

The extent of β-lactam multi-resistance among *E. coli* isolates was assessed by comparing the numbers of isolates resistant to two β-lactam agents at a time. The observed incidence of joint resistance between antibiotic pairs was compared to the rate of joint resistance expected if it had been acquired as two independent events.

Joint resistance among cephalosporins and penicillins was evident in both *E. coli* isolates and transconjugants. *E. coli* belongs to the inducible class of organisms because of its innate capacity to produce chromosomally-encoded class 1 cephalosporinase at high levels in response to a β-lactam-inducer drug. Joint resistance to class 1 cephalosporinase is the result of a mutation in the resistor's regulatory genes leading to hyper-production of this enzyme.

7. 3 AIMS OF THIS CHAPTER

i. To determine the extent of joint resistance among β-lactam antibiotics.

ii. To determine the probability of an organism being resistant to a second β-lactam agent if it is already resistant to a first.

7.4 RESULTS AND DISCUSSION

The extent of joint resistance among β-lactam antibiotics was analysed in isolates of *E. coli*. Expected cross-resistance was calculated as shown below. Expected single-agent resistance rates were determined from the MIC values of isolates and transconjugants for individual antibiotics. The observed numbers of jointly resistant isolates were determined by inspection of their respective MIC values ***(Tables 7.1*** and ***7.2)***.

Calculation of joint resistance rates in *E. coli* isolates

Single-Agent Resistance Rates

Amoxycillin: 90 / 90 resistantAugmentin: 76 / 90 resistant

Joint Resistance to Both Amoxycillin and Augmentin

N_e = (a x b) / n, where N_e = Expected no. of isolates jointly resistant to both antibiotics

a = Number of isolates resistant to antibiotic 1

b = Number of isolates resistant to antibiotic 2

Expected number of jointly resistant isolates = (76 x 90) / 90 = 76

Observed number of jointly resistant isolates = 76 / 90

To test if joint resistance is a result of independent events, χ^2 values are calculated for each of the 12 β-lactam combinations. These values are determined by the following equation:

χ^2 = (Observed no. resistant – Expected no. resistant – 0.5)^2 / (Expected no. resistant)

= (76 – 76 – 0.5)^2/ 76 = 0.003289, 1 df

Biological cross-resistance may be the result of either identical or different resistance mechanisms. These resistance mechanisms may be mediated by genes on chromosomes or on plasmids, and they inhibit the activity of the affected antibiotics proportionally. Genes for the AmpC enzymes have begun to appear on plasmids. Different mechanisms of resistance are generally mediated by plasmids carrying multiple genes, each mediating a different β-lactamase with activities that sometimes overlap. Joint resistance due to identical mechanisms occurs far more frequently between chemically-related than between unrelated antibiotics.

Chessboards were constructed by counting the number of isolates co-resistant for antibiotic pairs. For example, among donors (Table 7.1) the number of isolates that were resistant to both ampicillin and piperacillin was 75. Chessboards studies were read after 16 to 20 hours of incubation at 35°C.

Table 7.1 Individual and Joint Resistance in Donors of *E. coli*

	Amp	Amx	Aug	Pip	Cxm	Taz	Ctr	Crx	Cef	Cfz	Fox	Imp
Amp	90	90.000	76.000	75.000	12.000	6.000	7.000	9.000	7.000	21.000	9.000	8.000
		90	76	75	12	6	7	9	7	21	9	8
Amx		90	76.000	75.000	12.000	6.000	7.000	9.000	7.000	21.000	9.000	8.000
			76	75	12	6	7	9	7	21	9	8
Aug			76	63.330	10.130	5.060	5.910	7.600	5.910	17.730	7.600	6.750
				75	10	5	6	8	7	20	8	7
Pip				75	1.770	5.000	5.830	7.500	5.830	17.500	7.500	6.600
					*10	5	6	8	7	20	8	7
Cxm					12	0.800	0.930	1.200	0.930	2.800	*1.200*	*1.060*
						3	*5	*7	*7	6	*7	*6
Taz						6	0.466	0.600	0.466	1.400	0.600	0.530
							*4	*5	*3	*6	*5	*5
Ctr							7	0.700	0.540	1.630	0.700	0.620
								*6	*5	*6	*6	*6
Crx								9	0.700	2.100	0.900	1.380
									*7	*8	*9	*8
Cef									7	1.630	0.700	0.620
										*6	*7	*6
Cfz										21	2.100	1.860
											*8	*7
Fox											9	0.800
												*8
Imp												8

Table 7.2 Individual and Joint Resistance in Transconjugants of *E. coli*

	Amp	Amx	Aug	Pip	Cxm	Taz	Ctr	Crx	Cef	Cfz	Fox	Imp
Amp	33	33.000	33.000	27.000	3.000	2.000	4.000	4.000	3.000	3.000	3.000	2.000
		33	33	27	3	2	4	4	3	3	3	2
Amx		33	33.000	27.000	3.000	2.000	4.000	4.000	3.000	3.000	3.000	2.000
			33	27	3	2	4	4	3	3	3	2
Aug			33	27.000	3.000	2.000	4.000	4.000	3.000	3.000	3.000	2.000
				27	3	2	4	4	3	3	3	2
Pip				27	2.455	1.636	3.273	3.273	2.455	2.455	2.455	1.636
					3	2	3	3	3	3	3	2
Cxm					3	0.182	0.364	0.364	0.273	0.273	0.273	0.182
						*2	*3	2	*3	*3	*3	*2
Taz						2	0.242	0.242	0.182	0.182	0.182	0.121
							*2	1	*2	*2	*2	1
Ctr							4	0.485	0.364	0.364	0.364	0.242
								*4	*3	*3	*3	*2
Crx								4	0.364	0.364	0.364	0.242
									2	2	2	*2
Cef									3	0.273	0.273	0.091
										*3	*3	*2
Cfz										3	0.273	0.091
											*3	*2
Fox											3	0.091
												*2
Imp												2

Number resistant to individual antibiotics

Number expected to be resistant to both antibiotics in the pair

Number found to be resistant to both antibiotics in the pair

*** The chi-squared value is significant at the 95 % level (1 df) and resistance arose independe**

Ampicillin (Amp), Amoxycillin (Amx), Augmentin (Aug), Piperacillin (Pip), Cefotaxime (Ctx), Ceftazidime (Taz), Ceftriaxone (Ctr), Cefepime (Cef), Cephazolin (Cfz), Cefuroxime(Crx) Cefoxitin (Fox), Imipenem (Imp)

The correlation between ampicillin and amoxycillin resistance is shown in ***Figure 7.1.*** Correlation is a measure of the degree to which variables vary together. The correlation coefficient, r is an unbiased estimate of the population correlation coefficient. The *coefficient of determination,* R^2 is the square of the correlation coefficient r.

Joint resistance between these two antibiotics is inferred from the correlation between their MIC values. A perfect correlation is indicated by a coefficient of determination of R^2 = 1.0, which indicates complete joint resistance. As R^2 values decrease, progressively less joint resistance is demonstrated until no joint resistance is found when $R^2 = 0$. The concentration of ampicillin required to confer resistance on *E. coli* strains was consistently below that required to cause resistance to amoxycillin. Even though the activities of amoxycillin and ampicillin are biologically similar, they have different hydrolytic activities. Therefore, strains inhibited by low concentrations of ampicillin will be inhibited by low concentrations of amoxycillin, and similarly for high concentrations.

In ***Figure 7.2*** the correlation coefficient of determination is: $R^2 = 0.7443$, which is indicative of a very high degree of cross-resistance between ampicillin and amoxycillin for transconjugants. However, other types of mutations that would not have been shown by the transconjugants belong to the chromosomally encoded "inducible" class, so named because of their capacity to produce class I cephalosporinase in response to a wide range of β-lactam antibiotics. The cross-resistance pattern is likely due to the resistor's regulatory genes sometimes referred to as "mosaic genes". Cross-resistance is also found in isolates. Joint resistance was evident also among transconjugants. The emergence of transferable enzymatic resistance to newer β-lactams in *E. coli* strains indicates that ESBL-mediating genes are on conjugative plasmids and that this type of resistance can now spread to sensitive strains at faster rates than in the past. Nosocomial outbreaks of infections by ESBL-producing organisms do occur.

Low levels of joint resistance between cephalosporins and ampicillin is evident in *E. coli* isolates ***(Figures 7.3*** and ***7.4).*** The role of mutations in emerging β-lactam resistance concerns changes in β-lactamases that extends their spectrum of activity. Until recently, these enzymes were not able to hydrolyse newer extended-spectrum cephalosporins such as cefotaxime and ceftazidime. However, in 1982, mutant forms of β-lactamases were reported that were capable of inactivating the extended-spectrum cephalosporins. When the amino acid sequences of the first ESBLs were examined, only three differences were found between these ESBLs and wild-type β-lactamases. These amino acid changes were point mutations in the coding sequence of the β-lactamase gene. Under selection pressure by high cephalosporin usage in hospital wards, organisms with mutations that extend the spectrum of activity of β-

lactamases can emerge and disseminate *(2)*. The relatively low level of resistance of these strains to third-generation cephalosporins may cause problems in the interpretation of disk-diffusion tests, because inhibition zones may approximate the range of sensitive strains *(3)*. The development and spread of ceftazidime-resistant *Klebsiella pneumoniae* is a good example of this phenomenon. These organisms, which contain variations of common TEM or SHV β-lactamases, caused a number of outbreaks in the United States. Reduced membrane permeability limiting a drug's access into the cell as well as changes in a drugs target site can also be a result of mutation *(2)*.***Figure 7.5*** shows that the concentration of augmentin required to confer resistance on *E. coli* strains was consistently below that required to cause resistance to amoxycillin on its own. The explanation is that the clavulanate in augmentin inhibits β-lactamase, thus preventing the enzyme from destroying the amoxycillin with the result that the isolate is resistant to amoxycillin. Therefore, strains inhibited by low or high concentrations of amoxycillin tend to be inhibited by correspondingly low or high levels of augmentin, despite the higher activity of augmentin. Since breakpoints are important to the definition of resistance, it may be helpful to give an elaboration. Biological resistance does not necessarily imply clinical resistance and the same proviso applies to joint resistance. ***Figure 7.6*** shows that no correlation exists between the MICs for amoxycillin and augmentin in *E. coli* transconjugants. This is because J62, the recipient strain, does not produce as much β-lactamase as the wild type isolates. Wild type strains probably produce β-lactamase from both their plasmids and their chromosomes. It is known that lactamase genes on chromosomes can often be induced whereas plasmid genes cannot.

From ***Table 7.3***, the probability of an isolate that is resistant to one antibiotic also being resistant to one or more other antibiotics can be determined; e.g. if a urinary tract *E. coli* isolate is resistant to piperacillin there is a 22.5 % probability that it will also be resistant to cephazolin resistant.

Shortly after the introduction of third-generation cephalosporins, new mechanisms of resistance appeared. Strains can acquire new mechanisms of resistance by plasmid-borne resistance determinants. ***Table 7.4*** shows the probability of association of plasmid-borne resistance determinants. Comparing ***Tables 7.3*** and ***7.4***, the probabilities of joint resistance in transconjugants and isolates are seen to differ. One explanation is that all the transconjugants are present in the same host (J62). Therefore no outer-membrane, cytoplasmic or chromosomal differences can exist between different transconjugants. However, isolates may have additional non-transferable lactamase genes on their chromosomes and their outer membranes may differ.

Fig 7.1 CORRELATION OF AMPICILLIN AND AMOXYCILLIN MICs (DONORS)

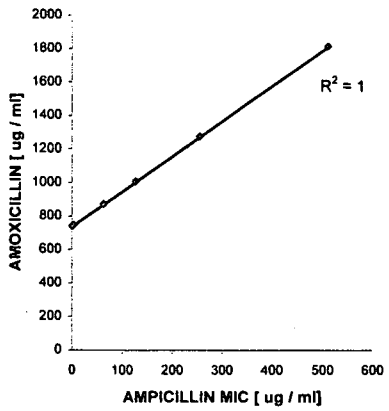

Fig 7.2 CORRELATION OF AMPICILLIN AND AMOXYCILLIN MICs (TRANSCONJUGANTS)

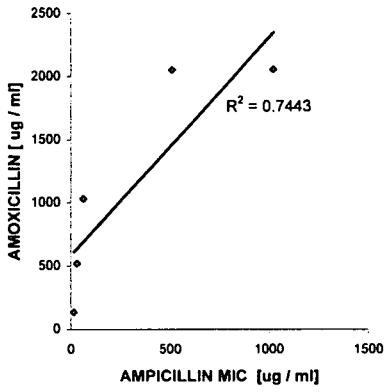

Fig 7.3 CORRELATION OF AMPICILLIN AND CEFTAZIDIME MICs (DONORS)

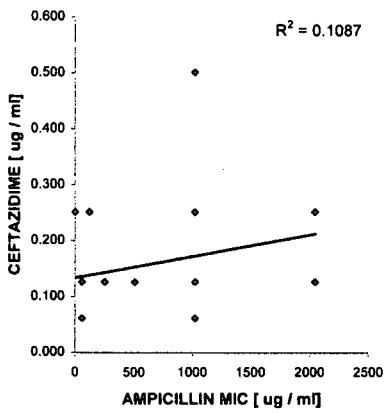

Fig 7.4 CORRELATION OF AMPICILLIN AND CEFOTAXIME MICs (DONORS)

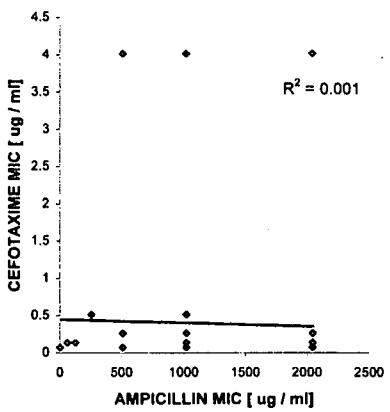

Fig 7.5 CORRELATION OF AMOXICILLIN AND AUGMENTIN MICs (DONORS)

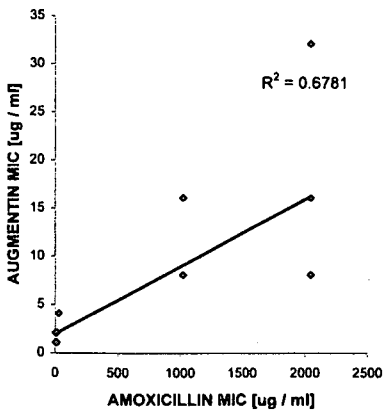

Fig 7.6 CORRELATION OF AMOXICILLIN AND AUGMENTIN MICs (TRANSCONJUGANTS)

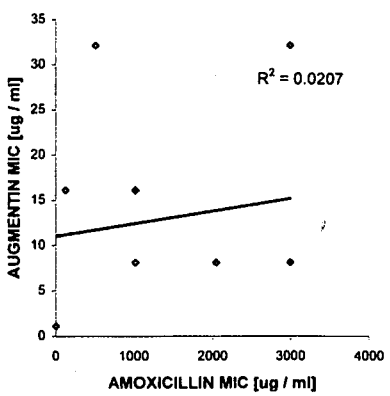

Table 7.3 Probability percentage of dual resistance in a randomly-chosen UTI *E. coli* isolate (90 donors)

		Amp	Amx	Aug	Pip	Cxm	Taz	Ctr	Crx	Cef	Cfz	Fox	Imp
		100	100	84.4	96.6	13.3	6.6	7.7	10	7.7	23.3	10	10.8
Amp	100		100.00	84.400	96.600	13.300	6.600	7.700	10.000	7.700	23.300	10.000	10.800
Amx	100			84.400	96.600	13.300	6.600	7.700	10.000	7.700	23.300	10.000	10.800
Aug	84.4				81.530	0.063	5.597	6.499	8.440	6.499	19.665	8.440	9.115
Pip	96.6					12.848	6.376	7.438	9.660	7.438	22.508	9.660	10.433
Cxm	13.3						0.878	1.024	1.330	1.024	3.099	1.330	1.436
Taz	6.6							0.508	0.660	0.508	1.538	0.660	0.713
Ctr	7.7								0.770	0.593	1.794	0.770	0.832
Crx	10									0.770	2.330	1.000	1.080
Cef	7.7										1.794	0.770	0.832
Cfz	23.3											2.330	2.516
Fox	10												1.080
Imp	10.8												

Table 7.4 Probability percentage of dual resistance in a randomly-chosen UTI *E. coli* transconjugant (33 transconjugants)

		Amp	Amx	Aug	Pip	Cxm	Taz	Ctr	Crx	Cef	Cfz	Fox	Imp
		100	100	100	81.8	9	6.1	12.1	12.1	9.1	9.1	9.1	6.1
Amp	100		100.00	100.00	81.800	9.000	6.100	12.100	12.100	9.100	9.100	9.100	6.100
Amx	100			100.00	81.800	9.000	6.100	12.100	12.100	9.100	9.100	9.100	6.100
Aug	100				81.800	9.000	6.100	12.100	12.100	9.100	9.100	9.100	6.100
Pip	81.8					7.362	4.990	9.898	9.898	7.444	7.444	7.444	4.990
Cxm	9						0.549	1.089	1.089	0.819	0.819	0.819	0.549
Taz	6.1							0.738	0.738	0.555	0.555	0.555	0.372
Ctr	12.1								1.464	1.101	1.101	1.101	0.738
Crx	12.1									1.101	1.101	1.101	0.738
Cef	9.1										0.828	0.828	0.555
Cfz	9.1											0.828	0.555
Fox	9.1												0.555
Imp	6.1												

Resistance percentage to individual antibiotics
Probability percentage of dual resistance between beta-lactam antibiotics in urinary tract *E. coli*

Thus it can be assumed that a β-lactamase-mediating plasmid, when transferred to J62, may show a different level of resistance to different β-lactam antibiotics than in the donor cytoplasm. In general both tables show that cephalosporins are good alternative therapeutic choices if organisms are resistant to ampicillin, amoxycillin and augmentin. Care should be taken not to select antibiotics that are unsuitable for the specific infection.

Chromosomal mutations have in the past played a dominant role in the relatively frequent development of resistance during treatment with broad-spectrum agents. Although *in vitro* activity cannot always be equated to *in vivo* response, *in vitro* resistance is a basis for predicting the usefulness of an antibiotic. Among the β-lactams imipenem represents one of the few alternatives remaining for patients with serious diseases due to the widespread occurrence of multi-resistant urinary tract *E. coli* isolates.

7. 5 CONCLUSIONS

Joint resistance between ampicillin and amoxycillin is inferred from the high correlation between their MIC values (R^2 = 1.0). The concentration of ampicillin required to confer resistance on *E. coli* strains is consistently below that required to cause resistance to amoxycillin. The correlation coefficient of determination is R^2 = 0.7443 for ampicillin and amoxycillin for transconjugants. This is slightly lower than for clinical isolates. Lactamase genes located on the chromosomes cannot be transferred; hence transconjugants are expected to show somewhat higher levels of resistance than their donors.

Poor correlation exists between MIC values recorded for cephalosporins and penicillins respectively. Nevertheless a few transconjugants exhibit high MIC values for cefotaxime. Examination of ***Tables 7.1*** and *7.2* reveals that several transconjugants show high levels of resistance to third and fourth generation cephalosporins. The emergence of transferable enzymatic resistance to newer β-lactams in *E. coli* strains indicates that ESBL-mediating genes are on conjugative plasmids and that this type of resistance can now spread to sensitive strains at faster rates than in the past. Nosocomial outbreaks of infections by ESBL-producing organisms do occur.

7. 6 REFERENCES

1. **Tierno PM**, (1988): Surveillance studies of multiresistance. *Proceedings of a Symposium* (California, October 22, 1988).
2. **Lawrence B**, *et al.* (1999): Impact of use of multiple antimicrobials on changes in susceptibility of gram-negative aerobes. *Clinical Infectious Diseases* **28**: 1017-24.
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CHAPTER 8

SUMMARY

This study was designed to elucidate the epidemiology, nature and extent of β-lactam resistance in urinary tract infections caused by *Escherichia coli* isolates in Bloemfontein hospitals. To reach this goal it was necessary to phenotypically characterise and re-identify the *E. coli* isolates by the Mastascan identification system. Pure cultures were obtained by streaking single colonies onto MacConkey agar containing 50 μg / ml ampicillin. Single colonies were then picked off and inoculated into Mueller-Hinton broth, grown overnight at 37°C and re-streaked onto MacConkey agar containing 50 μg / ml ampicillin. Three colonies were then picked and stored at -20°C in a freeze mixture of 10 % proteose and 10 % glycerol.

E. coli isolates were mated to a universal recipient strain (J62) in pre-warmed Mueller-Hinton broth. Transconjugants were selected on MacConkey agar supplemented with 50 μg / ml ampicillin and 50 μg / ml nalidixic acid. Lactose-negative colonies resistant to nalidixic acid and ampicillin were inoculated into Mueller-Hinton broth, incubated for six hours and re-streaked onto MacConkey agar containing ampicillin. Lactose-negative colonies were picked and considered to be transconjugants. It was found that fifty four (45 %) out of 120 ampicillin-resistant isolates transferred ampicillin-resistance determinants to an *E. coli* recipient (J62) by conjugation. Seventy-five percent of ampicillin-resistant isolates were from female patients, indicating that urinary tract infections are more prevalent in females than in males.

The National Committee for Clinical Laboratory Standards* agar dilution method was used to determine minimum inhibitory concentration (MIC) distributions of 12 β-lactam antimicrobial agents (ampicillin, amoxycillin, piperacillin, augmentin, cefoxitin, cefotaxime, cefepime, ceftazidime, cephazolin, ceftriaxone, cefuroxime and imipenem). Strains found to be resistant had MICs that overlapped the range where susceptibility was normally assumed. This was due to inducible β-lactamase producer strains, which go undetected by the Kirby-Bauer disk diffusion method but can be identified by using the Jarlier double-disk method. MIC frequency distributions for the penicillins showed that elevated doses should be administered in order to maximise antibiotic concentration at the site of infection or that a second antibiotic agent or inhibitor should be used in combination therapy.

Beta-lactam susceptibility profiles were determined by the Kirby-Bauer disk diffusion method. This method was also used to determine the correlation of MIC values with the

inhibition zone diameters in order to predict treatment outcomes from inhibition zone diameters.

The Jarlier double-disk technique was used to detect extended-spectrum β-lactamase-producing organisms and the frequencies at which they occurred. There was no difference between the ratios of ESBL-producers in hospitalised and non-hospitalised patients, although the absolute numbers were different. This was probably due to the 48 hour cut-off point used to define hospitalisation. Samples taken before 48 hours were considered to be non-hospitalised. There were many more female than male patients with urinary tract infections in the Bloemfontein hospitals during the period of the study.

The extent of joint resistance to β-lactam antibiotics among *E. coli* isolates was assessed by comparing two agents at a time. The observed incidence of joint resistance was compared to the rate of double resistance expected if it had been acquired as two independent events.

It was found that even amongst two antibiotics that are biologically cross-resistant (ampicillin and augmentin) a close correlation exists between the concentrations of the two agents required to inhibit individual *E. coli* strains.

Key-words

i. **ß-Lactam antibiotics**: The penicillins and the cephalosporins are two classic β-lactam families.

ii. **Epidemiology**: The study of the distribution and determinants of health-related conditions and events in populations, and the application of this study to the control of health problems.

iii. **Resistant**: A category of strains that are not inhibited by the usually achievable systemic concentrations of an agent with normal dosage schedules.

iv. **Sensitive**: When bacteria are inhibited or killed by an antimicrobial agent at a concentration that can be achieved *in vivo*. Highly sensitive organisms have low minimum inhibitory concentrations (MICs); less sensitive ones have higher MICs.

v. **Donor**: The bacterial strain that is the source of the transferred DNA in a bacterial cross. For example, in a transduction cross, it is the strain in which the phage was previously propagated. In conjugation, it is the strain harbouring the self-transmissible plasmid.

vi. **Transconjugant**: A recipient cell that has received DNA from another cell by conjugation.

vii. **Minimum inhibitory concentrations**: The lowest concentration of an antibiotic inhibiting visible growth after 18 to 24 hours.

viii. **Extended-spectrum β-lactamases**: Enzymes that arise by mutations in β-lactamase genes and confer resistance to aztreonam, cefotaxime, ceftazidime and related oxyimino-β-lactams as well as to older penicillins and cephalosporins.

ix. **Cross-resistance**: This refers to the property demonstrated by an organism that, after *in vivo* or *in vitro* exposure to an antibiotic, loses its susceptibility not only to that agent but also to others antibiotics, which may be of the same or different chemical class as the initial drug.

x. **Coefficient of determination**: The *coefficient of determination,* R^2 is the square of the correlation coefficient. R^2 is the proportion of a total sum of squares that is attributable to another source of variation, the independent variable.

HOOFSTUK 9

OPSOMMING

Hierdie studie is ontwerp om die epidemiologie, aard en omvang te ondersoek van β-laktam weerstandigheid in *Escherichia coli* isolate wat urienweginfeksies in Bloemfonteinse hospitale veroorsaak. Om in hierdie doel te slaag was dit nodig om die *E. coli* isolate fenotipies te karakteriseer en te her-identifiseer d.m.v. die Mastascan identifikasie stelsel. Suiwer kulture is verkry deur uitstreping van enkel kolonies op MacConkey agar wat 50 µg / ml ampisillien bevat het. Enkel kolonies is hiervandaan afgeneem en in Mueller-Hinton sop geïnokuleer. Na oornag-inkubasie by 37°C is die kulture weer op MacConkey agar wat 50 µg / ml ampicillin bevat, uitgestreep. Drie kolonies is hiervandaan afgeneem en geberg by -20°C in 'n vriesmengsel bestaande uit 10 % proteose en 10 % gliserol.

E. coli isolate is in voorafverwarmde Mueller-Hinton sop met 'n universele ontvanger-stam (J62) gepaar. Transkonjugante is vanaf MacConkey agar wat aangevul is met 50 µg / ml ampicillin en 50 µg / ml nalidiksiese suur, geselekteer. Laktose-negatiewe kolonies wat weerstandig is teen ampisillien en nalidiksiese suur is afgeneem en geïnokuleer in Mueller-Hinton sop. Na 6 uur se inkubasie is die kulture weer uitgestreep op ampisillienbevattende MacConkey agar. Laktose-negatiewe kolonies is afgeneem en as transkonjugante geklassifiseer. Vier en vytig (45 %) transkonjugante uit 120 ampisillien-weerstandige isolate is geselekteer in die *E. coli* J62 ontvanger. Vyf en sewentig persent van die ampisillien-weerstandige isolate was vanaf vroulike pasiënte afkomstig, wat daarop dui dat urienweginfeksies meer algemeen in vroue as in mans is.

Die "National Committee for Clinical Laboratory Standards" (NCCLS)* se agar-verdunnings metode is gebruik om die minimum inhibitoriese konsentrasie (MIK) verspreidings van 12 β-laktam antibiotiese verbindings (ampisillien, amoksisillien, piperasillien, augmentin, kefoksitien, kefotaksiem, kefepiem, keftasidiem, kefasolien, keftriaksoon, kefuroksiem en imipenem) te bepaal. Weerstandige stamme se MIKs mag die bestek oorvleuel waar gevoeligheid gewoonlik verwag word. Dit is die gevolg van induseerbare β-laktam produserende stamme wat nie deur die Kirby-Bauer skyfie diffusie-metode waargeneem word nie. Hierdie stamme kan egter deur Jarlier se dubbelskyfie-tegniek aangetoon word. MIK frekwensie-verspreidings vir die penisilliene wys dat verhoogde dosisse gebruik moet word om die antibiotikum-konsentrasie by die infeksiepunt te verhoog, of dat 'n tweede antibiotikum of 'n inhibeerder in kombinasie gebruik moet word.

Beta-laktam gevoeligheidsprofiele is d.m.v. die Kirby-Bauer skyfie diffusie-metode bepaal. Hierdie metode is ook gebruik om die korrelasie met MIK-waardes te bepaal sodat behandelingsuitkomste vanaf inhibisiesone-deursnitte voorspel kan word.

Die Jarlier dubbelskyfie-tegniek is gebruik om verlengde-spektrum β-laktamase-produserende (VSBL) organismes en hul voorkomsfrekwensie te bepaal. Geen verskil tussen die verhoudings van VSBL produseerders in hospitaal- en nie-hospitaal pasiënte kon aangetoon word nie, hoewel die absolute getalle verskillend was. Dit is moontlik as gevolg van die 48 uur afsnypunt wat gebruik is om hospitalisasie te definieer. Monsters wat voor 48 uur geneem is, is beskou as afkomstig van nie-hospitaal pasiënte. Daar was tydens die duur van die ondersoek baie meer vroulike as manlike pasiënte met urienweginfeksies in die Bloemfonteinse hospitale.

Die omvang van kruisweerstandigheid teen β-laktam antibiotika by *E. coli*-isolate is bepaal deur twee middels op 'n slag met mekaar te vergelyk. Die waargenome voorkoms van kruisweerstandigheid is vergelyk met dit wat verwag word indien dit onafhanklik sou onstaan.

Selfs tussen twee antibiotika wat biologies kruis-weerstandig is (ampisillien en augmentin) is 'n noue verwantskap gevind tussen die konsentrasies wat die twee middels benodig om individuele *E.coli*-stamme te inhibeer.