Isolation and Characterisation of Extended Spectrum β-Lactamases in South African *Klebsiella pneumonia* Isolates

By

Yashini Naidoo

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Supervisor:
Prof. Sabiha Essack

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To my Mother and my Brother

Abstract

The use of antibiotics and antimicrobial drugs has played a large role in the elimination of many infectious diseases, however the wide spread use of such drugs has given rise to the phenomenon of antimicrobial resistance and has rendered antibiotics ineffective to a broad range of bacteria. The aim of the study was to ascertain the differences if any in the phenotypic and genotypic resistance profiles of *K. pneumoniae* isolated from a single tertiary hospital in two surveillance studies undertaken at different times, viz., 2001 and 2007 with special emphasis on ESBLs. A correlation with antibiotic use was also undertaken.

ESBL positives were identified and phenotypic resistance profiles were generated based on the resistance profiles of individual isolates by means of their MIC data. The molecular detection of ESBLs was carried out using representative isolates and sequencing was based on the phenotypic expression of the most common ESBL genes. The data was summarized using median values and interquartile ranges. Antibiotic use and susceptibility in 2000 was compared to that in 2007 using a Wilcoxon signed rank test for paired data since the same drugs were tested in both years.

Of the isolates that were tested, sequencing revealed that TEM – 1 was identified in all isolates and SHV-1 and SHV-2 were identified in 60 % in the isolates collected in 2000 and 77 % and 11 % respectively in the isolates collected in 2007. SHV – 11 was present in 67% of isolates from 2007 and 55% of those were in combination with SHV – 1. Sequencing also revealed CTXM-15 present in one of the isolates collected in 2007. There was 100% susceptibility to cefoxitin and only one isolate in 2007 showing an intermediate result to imipenem.

No novel β-lactamases were identified in this study; however the decrease in susceptibility over time is proof of bacterial evolution. The variety of β-lactamases and diversity of plasmid profiles in these two small populations provides proof to the claim that dissemination of resistance in *Klebsiella pneumonia* is effortless. Statistical analysis showed an increase in resistance from the year 2000 to 2007 however the correlation between overall antibiotic use and the increase in resistance did not reach statistical significance. It was observed that resistance increased despite only a slight increase in the use of a few antibiotics to which we attributed co-carriage of resistance genes.

DECLARATION 1

The experimental work described in this dissertation was carried out at the School of Health Sciences, University of KwaZulu-Natal, Durban, Westville, under the supervision of Professor Sabiha Essack. These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Signed: Naidoo

On this 21 day of SEPTEMBER 2012

As the candidate's supervisor I have approved this dissertation for submission.

Prof Sabiha Essack

On this _____ day of _____ 2012

DECLARATION 2 - PLAGIARISM

I, YASHINI NAIDOO declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Signed: Naidoo

Table of Contents

List of Figures

gene; attI1, the integration site; qacE, partially deleted gene that encodes quartenary ammonium compound (disinfectants and antiseptics) resistance; sulI, sulphonamide resistance; attC, sequence on the gene cassette recognized by the integrase (Fluit and Schmitz, 2004).

Figure 3.1 Isolate KEH 81 showing a positive result for an ESBL using the Doubledisk synergy test. The extension of the zone of inhibition of ceftazidime toward amoxicillin/clavulanate confirms the positive result. A – AMC (9mm), B – CAZ (12mm), C – CROX (12mm), D – AZT (14mm), E – CTX (12mm). AMC – amoxicillin clavulanate, CAZ – ceftazidime, CROX – cefuroxime, AZT – aztreonam, CTX – cefotaxime.

Figure 3.2 Illustration of the increase in aminoglycosides and fluoroquinolones over the two collection periods indicating multi-drug resistance

List of Tables

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My loved ones who have been the source of my emotional and spiritual well-being ~

> ***Mum****:* You have been and are still an exemplary example in my life, both as a mother and as my friend. You are a major contributor to my success and it is due to your constant love and encouragement and the ability you have to make me feel like "I can do anything". I thank you for everything that you are and I love you.
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> ***Darryl****:* You have been my biggest motivator and my best friend. Thank you for never giving up on me, for giving me the "kick" when I needed it and for making me the luckiest sister in the world. I love you, always, more than rainbows.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Resistance and its impact

Ever since antibiotics and other antimicrobial drugs first became widely used in the World War II era, they have saved countless lives and eliminated serious complications in many diseases and infections (NIAID, 2006). Antimicrobials are said to be modern medicines greatest achievement. Subsequent to the advent of antimicrobials however, many of them have not sustained the power they initially were thought to possess and many of them have become ineffective.

Over time, the widespread use of antibiotics has resulted in extensive evolutionary adaptations in bacteria that enabled them to survive these powerful drugs (NIAID, 2006) Antimicrobial resistance has facilitated the survival of microbes and hence the battle against disease and infection is becoming alarmingly difficult to win. Antibiotic resistance is comparable to survival of the fittest, where the increased prevalence of resistance is as a result of evolution. Infectious organisms have the ability to adapt very rapidly to new environmental conditions and just a single random genetic mutation can facilitate bacterial evolution (Philippon, Arlet and Jacoby, 2002).

It is increasingly evident that resistance has become a problem with all categories of antibiotics and is especially so in the β-lactam antibiotics which include but are not limited to penicillin, penicillin derivatives, cephalosporins, carbapenems, cephamycins, carbecephems and the monobactams. The β-lactam group of antibiotics is by far the largest group of antibacterial agents used in clinical medicine. Their structure is based on the four-membered nitrogen-containing β-lactam ring that gives them the antibacterial activity (Duerden, 1987). The β-lactam antibiotics have structural similarities with the binding sites of bacterial substrates which enable them to attach to and inactivate the transpeptidases involved in bacterial cell wall synthesis (Williams, 1999).

The three primary mechanisms of beta-lactam antibiotic resistance are: i) alterations to the structure of the antibiotic target site, ii) changes that prevent access of an adequate concentration of the antibiotic to the active site, and, most commonly and most importantly, iii) enzymatic inactivation of the antibiotic (Mulvey and Simor, 2009). A change in the structure of the target

may result in the inability of the antibiotic to bind to its target. β-lactam antibiotics bind to penicillin binding proteins (PBPs) and a change in the active site of PBPs can lower the affinity of β-lactam antibiotics therefore increasing resistance to these agents (Drawz and Bonomo, 2010). Preventing the access of an antibiotic to its target is a resistance strategy as a result of a permeability barrier or the presence of an efflux pump mechanism (Mulvey and Simor, 2009). In order to access PBPs on the inner plasma membrane β-lactam antibiotics must either diffuse through or directly cross porin channels on the outside of the bacterial cell wall. Point mutations or insertion sequences in porin-encoding genes can produce proteins with decreased function, either a change in the porin channel size or the regulation of opening and closing of porins which lowers the permeability to β-lactams. Some organisms have evolved to express an active efflux mechanism that exports the antibiotics from the cytoplasm before allowing any binding to occur (Drawz and Bonomo, 2010).

Bacteria may produce enzymes that modify or destroy the chemical structure of an antibiotic rendering it inactive. In the case of β-lactam antibiotics, the most effective way for bacteria to counteract the effect of these antibiotics has been by producing β-lactamases which are enzymes that inactivate the antibiotics by hydrolyzing the β-lactam ring (Kong, Schneper and Mathee, 2010). β-lactamases were thought to have evolved from penicillin binding proteins and the first plasmid mediated β-lactamase, TEM-1 was discovered in the early 1960's (Bradford, 2001). Other β-lactamases were soon discovered which conferred resistance to the extended spectrum cephalosporins hence these new β-lactamases were named extended-spectrum β-lactamases (Paterson and Bonomo, 2005). Extended-spectrum β-lactamases (ESBLs) are those β-lactamases that are capable of conferring bacterial resistance to the penicillins, first-, second- and third-generation cephalosporins and aztreonam and are inhibited by β-lactamase inhibitors such as clavulanic acid (Rawat and Nair, 2010). Many new β-lactam antibiotics have been developed that were specifically designed to be resistant to the hydrolytic action of β-lactamases. However with each new class, new β-lactamases have emerged causing resistance to that class of drug (Bradford, 2001).

More than a thousand unique protein sequences for β-lactamases have been identified and more are discovered yearly (Malloy and Campos, 2011). The classification of β-latamases involve two major approaches: the first one called the Bush-Jacoby-Medeiros classification scheme is based on the similarities in the functional characteristics of the enzyme while the second approach, the Ambler classification scheme is based on the molecular similarities of the enzyme (Kong,

Schneper and Mathee, 2010). The Bush-Jacoby-Medeiros classification scheme is comprised of four groups and subgroups in which the ESBLs belong to group 2be or 2d (Rawat and Nair, 2010) The molecular classification of β-lactamases is based on the nucleotide and the protein sequences in these enzymes. The β-lactamases under this classification are divided into four groups or molecular classes, A, B, C and D. Classes A, C and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine whereas class B β-lactamases make use of an active site zinc ion to facilitate hydrolysis (Bush and Jacoby, 2010).

Antibiotic resistance may either be intrinsic or acquired. Intrinsic resistance is straightforward to treat because intrinsic mechanisms are those specified by naturally occurring genes found on the host's chromosomes (Alekshun and Levy, 2007). Acquired resistance may occur in two ways: by mutation which is a random event or by the transfer of resistance determinants borne on mobile genetic material including but not limited to plasmids, bacteriophages, transposons, integrons, gene cassettes and insertion sequences (Table 1.1). The process of gene transfer that disseminates information is accomplished by transformation, conjugation and transduction. Transformation is the transfer of naked DNA following bacterial cell death into receptive bacterium (Barker, 1999).

Conjugation is horizontal gene transfer by cell to cell contact. Plasmids are mobile genetic elements, which are extra-chromosomal DNA that transfer horizontally within and across bacteria via conjugation. Plasmids can also serve as vehicles for transposons and integrons therefore through plasmid conjugation bacteria are exposed to an extensive number of genes from the mobile gene pool. (Williams and Hergenrother, 2008). Transduction is the transfer of DNA from one cell to another mediated by a bacteriophage. It occurs as a consequence of rare errors in phage reproduction when a small percentage of phage particles that are produced contain DNA from the host cell in place of, or in addition to the normal phage genome (Bennett *et al*., 2004).

Table 1.1 Mobile Genetic Elements (Alekshun and Levy, 2007)

Genetic element	General characteristics	Resistance determinants(s)
Plasmid	Variable size (1 - >100kb), conjugative and mobilizable	R Factor, multiple resistances
Insertion Sequence	Small (2kb), contains terminal inverted repeats, and specifies a transposase	IS1, IS3, IS4 etc.
Composite (compound)	Flanked by insertion sequences and /or inverted repeats	Tn5: Kan, Bleo and Str
Complex transposon	Large (>5kb), flanked by short terminal inverted repeats, and specifies a transposase and recombinase	Tn1 and Tn3: β-lactamase Tn7: Tmp, Str, Spc Tn1546: glycopeptides
Conjugative transposon	Promotes self transfer	Tn916: Tet and Mino
Transposable bacteriophage	A bacterial virus that can insert into the chromosome	Mutation
Integron	Facilitates acquisition and dissemination of gene cassettes, specifies an integrase, attachment sites, and transcriptional elements to drive the expression of	Class 1: Multiple single determinants and MDR Class 2: (Tn7) Class 3: Carbapenems Class 4: Vibrio spp. Super integron

Abbreviations: Bleo, bleomycin; Kan, kanamycin; Mino, minocycline; Spc, spectinomycin; Str, streptomycin; Tet, tetracycline; Tmp, trimethoprim.

1.2 β-Lactam Antibiotics

1.2.1 β-Lactam antibiotic mechanism of action

β-lactams are among the classes of antibiotics that interfere with specific steps in homeostatic cell wall biosynthesis. Successful treatment with a cell wall synthesis inhibitor can result in changes to cell shape and size, induce cellular stress responses, and result in cell lysis (Kohanski, Dwyer and Collins, 2010). Bacteria rely on a heavily cross-linked peptidoglycan layer for the preservation of cell shape and rigidity. This peptidoglycan layer is comprised of a repeating unit of an alternate disaccharide, *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM) (Wilke, Lovering and Strynadka, 2005). A pentapeptide is attached to each NAM unit, and the cross-linking of two D-alanine–D-alanine NAM pentapeptides is catalyzed by PBPs, which act as transpeptidases (Drawz and Bonomo, 2010).

β-lactams inhibit the transpeptidation precisely in the last stage of the synthesis of the cell wall. For β -lactams to act, it is necessary for the bacteria to be in the multiplication phase, which is when the cell wall is synthesized (Mar and Francesc, 2003) The β-lactam ring is stereochemically similar to the D-alanine–Dalanine of the NAM pentapeptide, and PBPs incorrectly use the β -lactam as a "building block" during cell wall synthesis. This results in acylation of the PBP, which renders the enzyme unable to catalyze further transpeptidation reactions. As cell wall synthesis slows to a halt, peptidoglycan autolysis continues (Fig 1.1). This results in weakly cross-linked peptidoglycan, which makes the growing bacteria highly susceptible to cell lysis and death (Drawz and Bonomo, 2010).

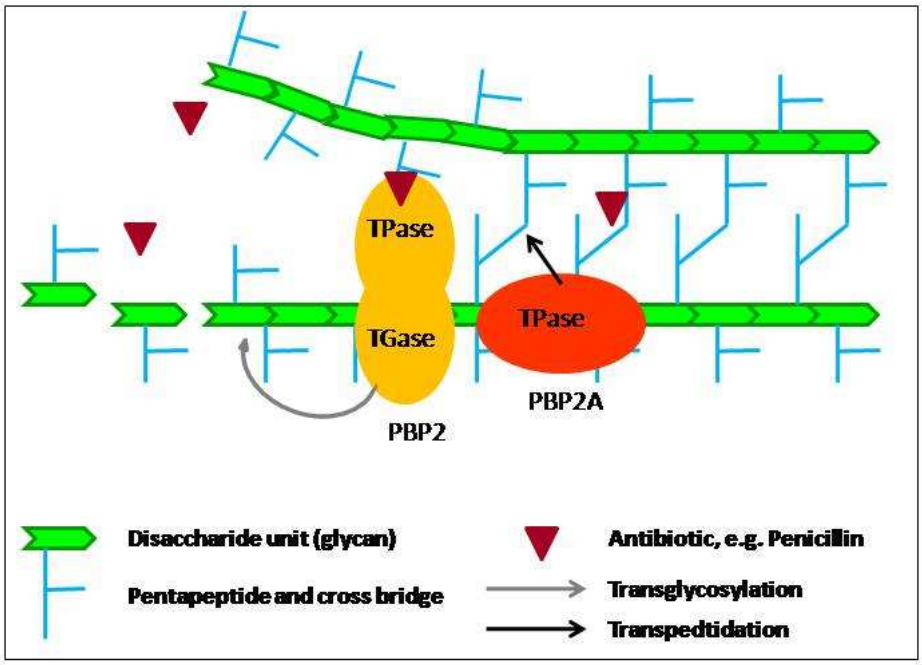

Fig 1.1 Schematic representation of antibiotic mechanism of action illustrating the breaking of the cross-linked peptidoglycan bridge (Depardieu *et al.*, 2007)

After 70 years of clinical use, which began with the administration of penicillin to a patient with staphylococcal sepsis in 1941, β -lactam antibiotics have become frequently prescribed. The continual rise of resistance and the progress in understanding its molecular mechanisms has further augmented interest and has led us to discover the wealth of information on each component of this family of antibiotics (Mar and Francesc, 2003). They all have a β lactam ring, which can be hydrolyzed by β-lactamases. The groups differ from each other by additional rings, e.g. thiazolidine ring for penicillins, cephem nucleus for cephalosporins, none for monobactams and double ring structure for carbapenems (Samaha-Kfoury and Araj, 2003).

1.2.2 Penicillins

In 1921, Alexander Fleming a Scottish bacteriologist, suffering from a common cold, inoculated agar plates with his own nasal secretion to determine the change of his nasal bacteria. No colonies appeared for several days. This suggested the presence of a substance that affected the ability of bacteria to grow. The substance, initially named lysozyme was found to be very effective causing cell wall lysis in many Gram-positive bacteria. (Kong, Schneper and Mathee, 2010). In 1928, Fleming noted that a culture of *Penicillium notatum*, a contaminating mould,

produced a substance that inhibited the growth of *Staphylococcus aureus* and this substance was so named penicillin (Henderson, 1997). Due to the difficulties of production and purification, penicillin was not used in the treatment of infections until 1941 where penicillin was produced in sufficient quantities to conduct clinical trials (Wright, 1999).

Penicillins contain a β-lactam ring and a thiazolidine ring to form 6-aminopenicillanic acid creating the characteristic double ring (Figure 1.2). They also have a side chain, which varies among penicillins, in position 6 of the β-lactam ring and that is what defines their properties (Mar and Franchesc, 2003).The β-lactam nucleus, 6-aminopenicillanic acid 6-APA proved to be the key in penicillin synthesis and modification. Thereafter, semi-synthetic β-lactam compounds have been developed continuously and systematically (Kong, Schneper and Mathee, 2010).

Natural penicillins were the first agents in the penicillin family to be introduced for clinical use. Benzylpenicillin, or Penicillin G are the parent drugs of this family and have been used extensively. The natural penicillins proved to be initially very successful and it was this success that led to the development of the penicillinase-resistance penicillins; methicillin, oxacillin, nafcillin, dicloxacillin, cloxacillin and flucloxacillin (Wright, 1999). Methicillin was the first semi-synthetic penicillin to be introduced into clinical use then followed the replacement of methicillin by nafcillin and oxacillin as it was found that these two penicillins were similar in their effectiveness (Wright, 1999).

Further manipulation of the basic penicillin structure introduced penicillins which had an extended activity against gram-negative bacilli. These are the aminopenicillins, carboxypenicillins and ureidopenicillins. Aminopenicillins are water soluble and pass through porin channels in the cell walls of some gram negative organisms (Gallagher and MacDougall, 2011). This group of penicillins has a broader antibacterial spectrum because they have a better capacity to penetrate the porins of gram negative bacteria (Mascaretti, 2003). Extended spectrum penicillins were developed with the goal of treating infections caused by *Pseudomonas aeruginosa*, and include the carboxypenicillins and the ureidopenicillins (Chevez-Bueno and Stull, 2009).

The carboxypenicillins consist of carbenicillin and ticarcillin; the substitution of a carboxyl group for the amino group on ampicillin produces carbenicillinand the subsequent substitution on the carbenicillin produces ticarcillin (Wright, 1999). Unlike ampicillin, carbenicillin and ticarcillin

are not very active against enterococci because of poor binding to their PBPs (Chevez-Bueno and Stull, 2009). The ureidopenicillins are semisynthetic derived from the ampicillin molecule with acyl side chain adaptations. They have enhanced aerobic gram-negative activity including anti-pseudomonal activity. Included in this group are mezlocillin, azlocillin and piperacillin (Chevez-Bueno and Stull, 2009). Their enhanced activity over other penicillins is attributed to their structure which allows greater penetration of the cell wall and the increased affinity to PBPs (Wright, 1999). Due to the structure of penicillins and the ability to adapt their side chains, it has been possible to extend the antibacterial spectrum of these antibiotics.

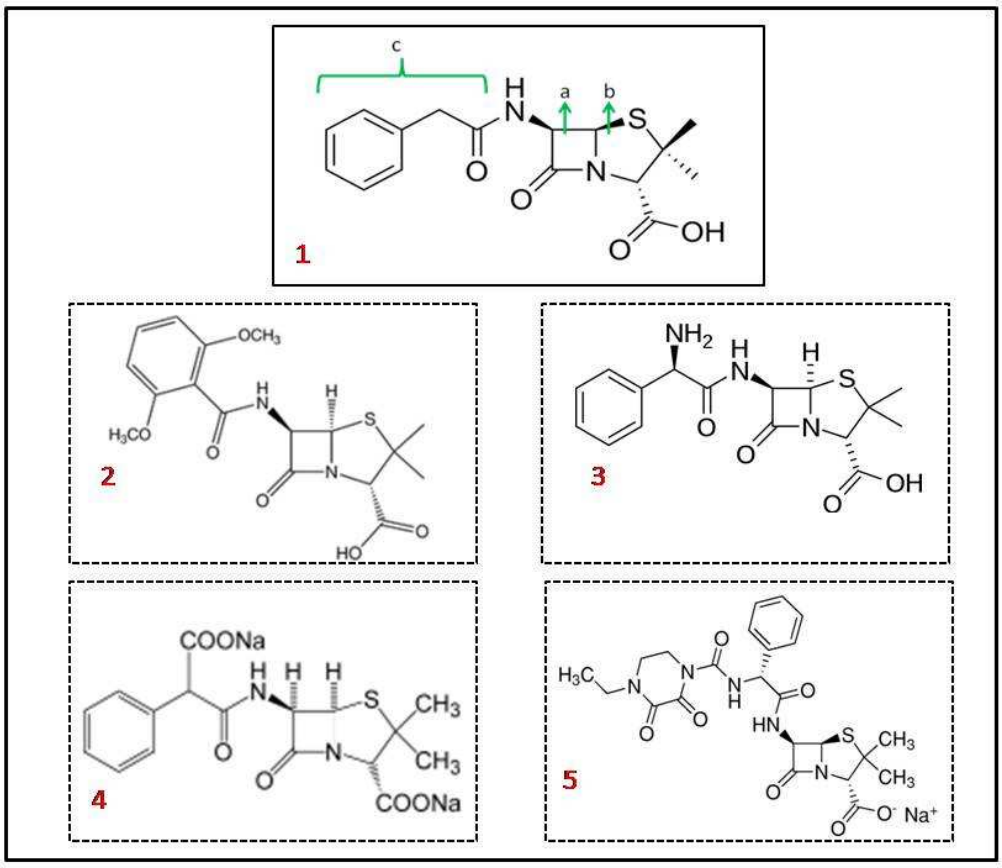

Figure 1.2 Chemical structure of the various penicillins found in this family. 1 – Benzyl penicillin (a – β-lactam ring, b – Thiazolidine ring, c – variable side chain), 2- Methicillin, 3- Ampicillin, 4 – Carbenicillin and 5 – Piperacillin. (Drawz and Bonomo, 2010).

1.2.3 Cephalosporins

The cephalosporin ring structure is derived from 7-aminocephalosporanic acid. Cephalosporins contain the basic beta-lactam ring but the cephalosporin structure allows for more gram negative activity than the penicillins (Duerden, 1987). Penicillins and cephalosporins have the same four-member beta-lactam ring, but cephalosporins have an additional atom in the side ring. (Harrison and Bratcher, 2008). The cephalosporin basic structure consists of a cephem nucleus, which consists of a dihydrothiazine ring instead of the thiazolidine ring characteristic of penicillins. Specific modifications in the acyl side chains result in different cephalosporins (Figure 1.3.2), (Mar and Franchesc, 2003). Most cephalosporins are broad-spectrum compounds, affecting both Gram positive and Gram negative bacteria; however major differences in their spectra exist. (Livermore and Williams, 1996).

Cephalosporins are classified by generation. Lower-generation cephalosporins have more gram-positive activity and higher-generation cephalosporins have more gram-negative activity. The fourth generation drug cefepime is the exception to the rule due to the gram-positive activity being equivalent to first-generation and gram-negative activity equivalent to third-generation cephalosporins. Individual cephalosporins in the higher generations have differentiating properties that may warrant specific indications (Harrison and Bratcher, 2008).

The first generation cephalosporins are moderate spectrum agents, with a spectrum of activity that includes penicillinase-producing, methicillin-susceptible staphylococci and streptococci. The first two cephalosporins to be introduced into clinical practice were cephalothin and cephaloridine (Duerden, 1987). The intrinsic activity of cephalosporins in the first generation against Gram-positive cocci is similar to that of benzylpenicillin and is sustained against producers of β-lactamases (Livermore and Williams, 1996).

The second generation cephalosporins have a greater Gram-negative spectrum while retaining some activity against Gram-positive cocci. Because of greater stability against beta-lactamases, enhanced activity occurs among second-generation cephalosporins (Harrison and Bratcher, 2008). The second-generation cephalosporins include but are not limited to cefamandole, cefonicid, ceforanide, and cefuroxime. Although cefoxitin, cefotetan, and cefmetazole are also included in this class, these agents are technically considered cephamycins because of their methoxy side chain at C7.

These antibiotics are usually active against the same organisms as the first-generation cephalosporins, but they have more activity against certain aerobic Gram-negative bacteria and *Haemophilus influenzae* (Kalman and Barriere, 1990)

Third generation cephalosporins have a broad spectrum of activity and further increased activity against Gram-negative organisms. Some members of this group have decreased activity against Gram-positive organisms (Mandell, 1990). Two of the oral third generation cephalosporins, cefixime and ceftibuten have an advantage over the other oral cephalosporins because of their greater β-lactamase stability and their Gram negative coverage. Cefexime however has poor activity against staphylococci and is only marginally active against pneumococci (Harrison and Bratcher, 2008). The parenteral 3rd-generation cephalosporins are generally less active against staphylococci and susceptible to first generation cephalosporins, but they have an expanded spectrum of activity against Gram negative bacteria (Kalman, 1990).

Fourth generation cephalosporins are extended-spectrum agents with similar activity against Gram-positive organisms as first-generation cephalosporins. They have a greater resistance to beta-lactamases than the third generation cephalosporins. They are also used against *Pseudomonas aeruginosa* (Kalman, 1990).

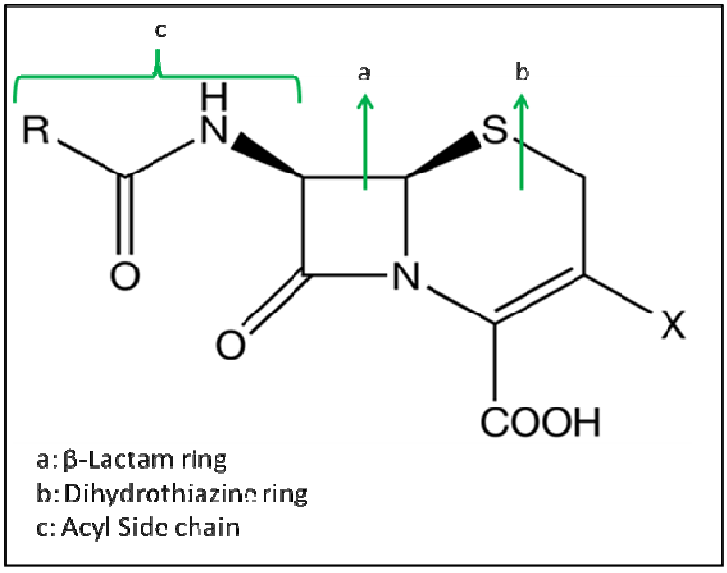

Fig 1.3. Representative structure of Cephalosporin illustrating the common β-lactam ring, a feature shared with the Penicillins, the Dihydrothiazine ring and the Acyl side chain which may be altered to create the different generations (Drawz and Bonomo, 2010).

1.2.4 Carbapenems

The carbapenems are β-lactam antimicrobial agents with an exceptionally broad spectrum of activity. Carbapenems are stable to most β-lactamases including AmpC beta-lactamases and the extended-spectrum beta-lactamases. These drugs continue to be one of the most active classes of antibiotics against many resistant pathogens. (Zhanel *et al*., 2007). Carbapenems are related to penicillin (penam) and cephalosporin (cephem). They differ from the penams by the presence of a carbon at position 1 instead of a sulphur, and unsaturation in the 5-membered ring (Devasahayam *et al.*, 2011). Among the many different structurally distinct classes of β-lactams, the carbapenem class is considered to be the most potent and to have the widest spectrum of antimicrobial activity (Shah, 2008).

Carbapenems are capable of passing through porins in outer wall of Gram negative bacteria and have a high affinity to PBPs. This class of antibiotics consist of imipenem, meropenem ertapenem and doripenem whose broad spectrum of activity includes activity against many Gram-positive, Gram-negative and anaerobic bacteria (Zhanel *et al*., 2007). Imipenem (Fig 1.4) was licensed in 1984 in Germany. It was more than 10 years before a second carbapenem, meropenem (Fig 1.4), was licensed in 1995, while ertapenem (Fig 1.4) was licensed by the European Community in 2002 (Shah, 2008). Doripenem, the newest addition to this family of antibiotics is a synthetic antibiotic that is structurally related to β-lactam antibiotics. This compound is stable in the presence of β-lactamases and is resistant to inactivation by renal dehydropeptidases. Doripenem, like the other carbapenems, inhibits bacterial cell wall synthesis by inactivating essential PBPs, ultimately causing cell death (Greer, 2008). It is probable that due to the activity of these drugs additional carbapenems will be developed to add to this class of antibiotics.

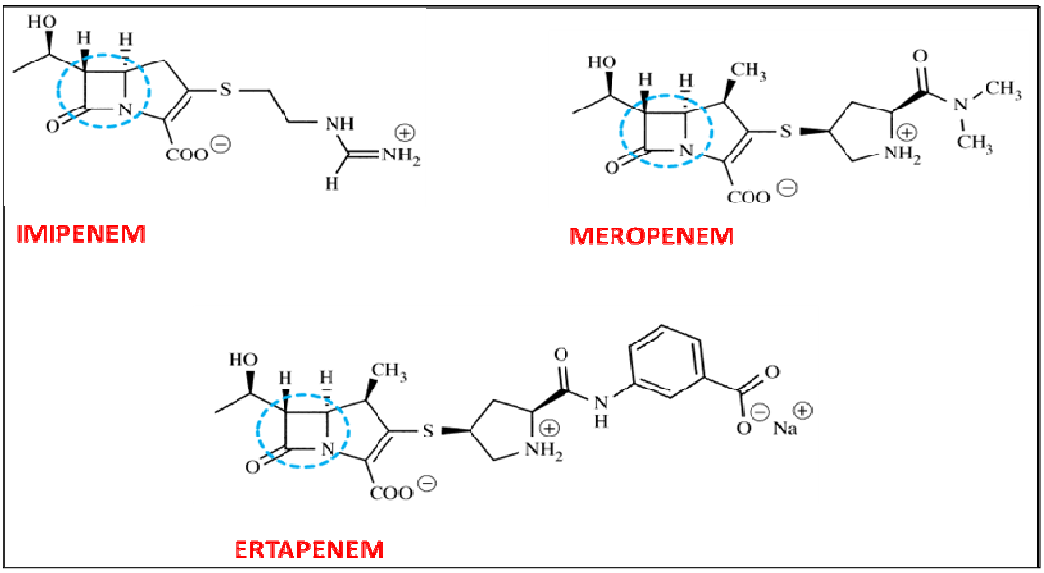

Fig 1.4 Carbepenems: Imipenem, Meropenem and Ertapenem illustrating the characteristic β-lactam ring (circled) and the carbon in the five-membered ring in place of the sulphur in the cephalosporins and the penicillins (Shah and Isaacs, 2003).

1.3 β-Lactamase inhibitors

β-Lactamase inhibitors are structurally related to penicillin, retaining the amide bond of the β-lactam group of the parent compound, but with a modified side chain (Figure 1.5). The three Class A β-lactamase inhibitors clinically used; are clavulanic acid, sulbactam and tazobactam (Babic, Hujer and Bonomo, 2006). These three beta-lactamase inhibitors, acting like β-lactams, bind to the active site of β-lactamases but fail to be efficiently hydrolyzed. Instead, they undergo "inactivation kinetics" in which the acyl-enzyme complex is either hydrolyzed very slowly and produce chemical changes that allow irreversible binding to the enzyme (Perez-Llarena and Bou, 2009). Carbapenems, monobactams and cephalosporins have shown inhibition activity against class C β-lactamases and even selectively class A β-lactamases, however their selection for inhibition coupled with increasing resistance among them do not technically render these β-lactamases inhibitors (Perez-Llarena and Bou, 2009).

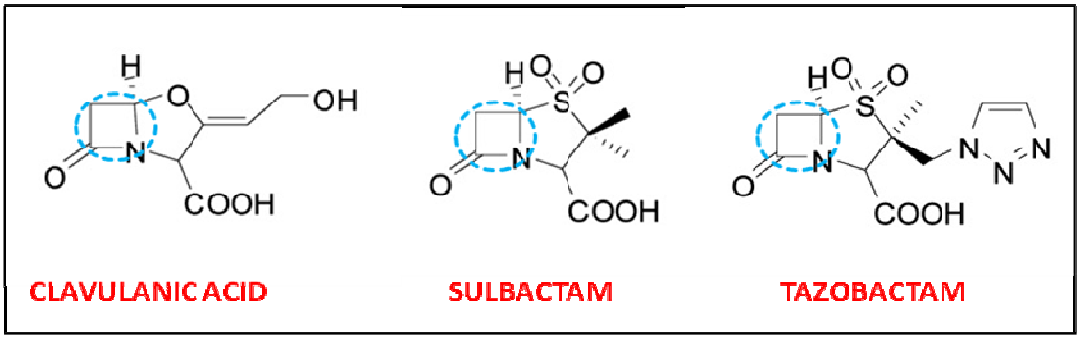

Fig 1.5 Chemical structure of Class A β-Lactamase inhibitors. All members have the characteristic β-Lactam ring with modified side chains (Perez-Llana and Bou, 2009).

1.3.1 Clavulanic acid

Clavulanic acid inhibits many molecular class A β-lactamases, including staphylococcal penicillinases, the most prevalent plasmid-mediated β-lactamases of Gram-negative rods, and the chromosomal enzymes from *Bacteroides fragilis*, *Proteus vulgaris*, and *Citrobacter diversus* (Livermore and Williams, 1996). Clavulanic acid was the first β-lactamase inhibitor introduced into clinical medicine and was isolated from *Streptomyces clavuligerus* in the 1970s. Clavulanate showed little antimicrobial activity alone, but when combined with amoxicillin, clavulanate significantly lowered the amoxicillin MICs against *Staphylococcus aureus*, *K. pneumoniae*, *Proteus mirabilis*, and *Escherichia coli* (Drawz and Bonomo, 2010).

Clavulanic acid thus has inconsequential intrinsic antimicrobial activity, despite sharing the β-lactam ring that is characteristic of beta-lactam antibiotics. Clavulanic acid lacks the side chain bound to the C-6 atom that is characteristic of β-lactam antibiotics, and it also has an exocyclic substituent attached to the C-2 atom (Perez-Llana and Bou, 2009). The similarity in chemical structure, however, allows the molecule to act as a competitive inhibitor of beta-lactamases secreted by certain bacteria to confer resistance to beta-lactam antibiotics. This inhibition restores the antimicrobial activity of beta-lactam antibiotics against β-lactamase-secreting resistant bacteria (James and Gurk-Turner, 2001).

1.3.2 Sulbactam

In 1978, Pfizer laboratories synthesised the penicillanic acid sulphone known as sulbactam. This represents an important difference with respect to clavulanic acid, as the former was obtained by semi-synthesis. This compound shows very good beta-lactamase-inhibitory activity, mainly against class A beta-lactamases (Perez-Llana and Bou, 2009). Sulbactam is an irreversible inhibitor of beta-lactamase; it binds the enzyme and does not allow it to interact with the antibiotic. Sulbactam is able to inhibit the most common forms of beta-lactamase but is not able to interact with the ampC cephalosporinase. Thus, it confers little protection against bacteria such as *Pseudomonas aeruginosa*, *Citrobacter*, *Enterobacter*, and *Serratia*, which often express this gene (James and Gurk-Turner, 2001).

1.3.3 Tazobactam

Tazobactam was reported in 1987, it was also obtained via semi-synthesis as an inhibitor with good affinity to most class A and some class D beta-lactamases and with moderate affinity to class C β-lactamases (Perez-Llana and Bou, 2009). Its mechanism of action is very similar to that of clavulanic acid and sulbactam which can act either reversibly or transiently (Drawz and Bonomo, 2010). It is a compound which inhibits the action of bacterial β-lactamases. It broadens the spectrum of piperacillin by making it effective against organisms that express β-lactamase and would normally degrade piperacillin. Tazobactam sodium is a derivative of the penicillin nucleus and is also a penicillanic acid sulfone (James and Gurk-Turner, 2001).

1.4 β-Lactamases

β-lactamases are among the most heterogeneous group of resistance enzymes. The number of unique protein sequences for β-lactamases have exceeded 1000 (K. Bush and G. Jacoby, http://www.lahey.org/Studies/ , 2012. These globular proteins are composed of alpha–helices and beta–pleated sheets. Two systems are commonly used to classify β -lactamases: the Ambler scheme and the Bush- Jacoby-Medeiros scheme as summarized in Table 1.2. Both of these classification schemes are used interchangeably in the literature. (Perez *et al*., 2007).

As previously mentioned extended spectrum beta lactamases or ESBLs are those β-lactamases conferring resistance to a wide array of β-lactam antibiotics. ESBL-producing organisms pose

unique challenges to researchers and clinicians since these ESBLs are enzymes that are capable of hydrolyzing penicillins, broad-spectrum cephalosporins and monobactams (Rupp, 2003). Class A and C β-lactamases are the most common and have a serine residue at the active site as do class D β-lactamases. Class B compromises the metallo β-lactamases (Jacoby, 2005). Classes A, C and D share a similar fold (Figure 1.6) and all have a mechanism that involves creation of a serine nucleophile by de-protonation of an active site serine with a general base, nucleophilic attack of the β-lactam ring to form an acyl-enzyme intermediate, and hydrolysis of the intermediate using a general base activated water molecule. (Wilke, Lovering and Strynadka, 2005).

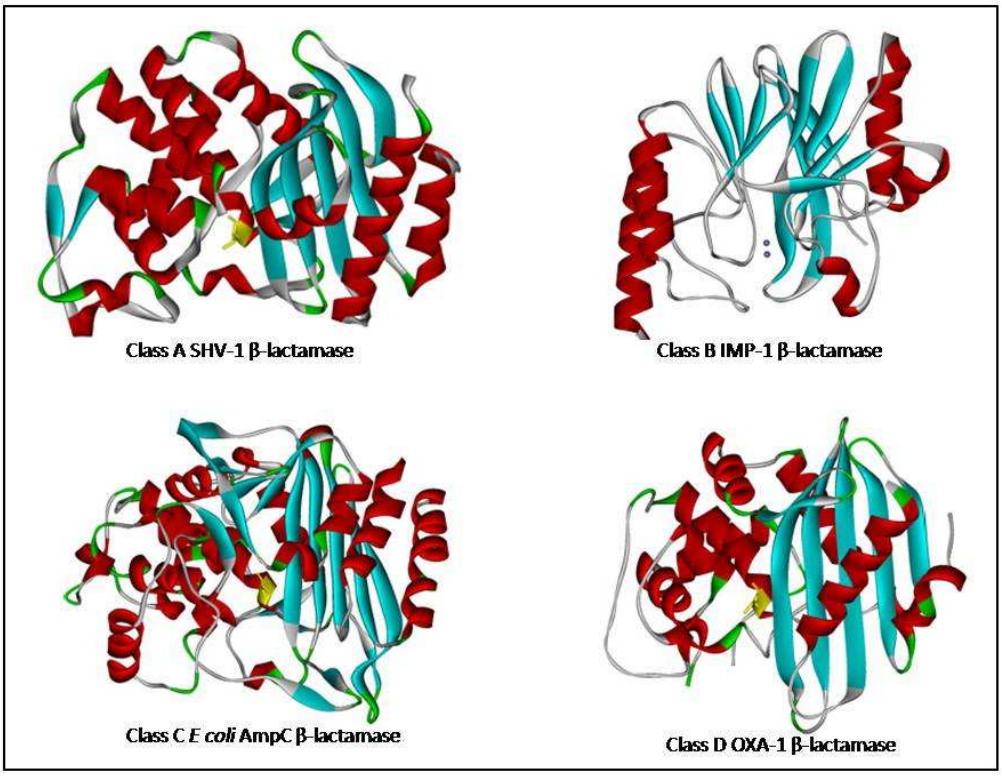

Fig 1.6 Schematic representation of Class A, B, C and D β-lactamases illustrating the fold structures of these β-lactamases (Drawz and Bonomo, 2010).

Ambler Class A enzymes (Bush Group 2) are penicillinases that are susceptible to beta-lactamase inhibitors. The TEM-1 and SHV-1, the extended spectrum beta-lactamase precursors in group 2b, are the beta-lactamases usually found in *Escherichia coli* and *K. pneumoniae* that confer resistance to penicillins, ampicillin and piperacillin (Babic, Hujer and Bonomo, 2006).

Table 1.2 Revised Classification System of beta-lactamases including the Molecular class scheme (Bush and Jacoby, 2010).

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB[a]	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI[b]	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

[a] CA, clavulanic acid; TZB, tazobactam.
[b] NI, not included.

The first plasmid-mediated β-lactamase in Gram-negative bacteria, TEM-1 was described in the early 1960s. It was so designated because it was isolated from the blood culture of a patient named Temoniera in Greece (Rawat and Nair, 2010). The SHV, β-lactamase found primarily in *K. pneumoniae*, was named from the term "sulf*h*ydryl reagent *v*ariable", was discovered shortly after (Drawz and Bonomo, 2010). Subgroup 2b β-lactamases readily hydrolyze penicillins and early cephalosporins, such as cephaloridine and cephalothin, and are strongly inhibited by clavulanic acid and tazobactam. They include the TEM-1, TEM-2 and SHV-1 enzymes (Bush and Jacoby, 2010). Subgroup 2 be comprises ESBLs, 'e' denoting extended (Table 1.2). These broad-spectrum enzymes retain the activity against penicillins and cephalosporins of subgroup 2b β-lactamases and in addition hydrolyze one or more oxyimino- β-lactams, such as cefotaxime, ceftazidime, and aztreonam. The first and largest subset of subgroup 2be was derived by amino acid substitutions in TEM-1, TEM-2, and SHV-1 that broadened their substrate spectrum (Bush and Jacoby, 2010). As with TEM, SHV-type ESBLs have one or more amino acid substitutions around the active site (Jacoby, 2005). More than 150 varieties of SHV are currently recognized on the basis of unique combinations of amino acid replacements.

The third largest group of ESBLs after the TEM and SHV groups is the CTX – M enzymes. Based on sequence homology, these are divided into five subgroups with around 40 members. Most of these subgroups have evolved as a result of the chromosomal β-lactamase genes escaping from *Kluvera spp*. Having migrated to mobile DNA, the CTX-M β-lactamases may evolve further (Rawat and Nair, 2010). Unlike most TEM and SHV-derived ESBLs, CTX-M beta lactamases hydrolyze cefotaxime and ceftriaxone better than they do ceftazidime. It also appears that CTX-M enzymes are more readily inhibited by tazobactam than they are by clavulanic acid (Perez, *et al.,* 2007). According to studies conducted by Rawat and Nair (2010), it has been suggested that the CTX-M-type ESBLs may now actually be the most frequent ESBL type worldwide.

K. pneumoniae carbapenemase (KPC) is an Ambler class A β-lactamase which confers resistance to all β-lactam agents, including carbapenems (Kitchel, Sundin and Patel, 2009). The first detection of a bla_{KPC}-positive *K. pneumoniae* (KPC-Kp) isolate was in 1996 in North Carolina. This klebsiella isolate was resistant to all β lactams, but carbapenem minimum inhibitory concentrations (MICs) were slightly decreased after addition of one of the β lactamase inhibitors, clavulanic acid. The discovery of this plasmid encoded β-lactamase, KPC-1, was followed by several publications of another single amino acid variant, KPC-2, in hospitalised patients from the east coast of the USA (Nordmann, Cuzon and Naas, 2009). KPC β-lactamases have been identified in many Enterobacteriaceae and *Pseudomonas* spp.;

however, *K. pneumoniae* still remains the most common species possessing these enzymes (Endimiani *et al.,* 2009). Since its emergence in North Carolina, KPC-Kp has caused serious outbreaks in several countries including Israel and China.

Ambler class B (Bush Group 3) enzymes are metallo-beta-lactamases (MBLs) that use one of two zinc (Zn2+) atoms for inactivating penicillins and cephalosporins suggesting that the MBLs are likely to have evolved separately from the other Ambler classes, which have serine at their active site (Drawz and Bonomo, 2010). MBLs were first formally categorized from serine β-lactamases in 1980 in the classification scheme proposed by Ambler. In 1989, Bush further classified MBLs into a separate group (group 3) according to their functional properties and remains the recommended referencing system for β-lactamases. This scheme was primarily based on substrate profiles, their sensitivity to EDTA, and their lack of inhibition by serine β-lactamase inhibitors (Walsh *et al.,* 2005).

In bacteria, MBLs confer resistance to carbapenems, cephalosporins and penicillins. The class B β-lactamases are completely distinct from the serine β-lactamases in terms of sequence, fold and mechanism. There are three subclasses of class B MBLs (B1 to B3). Classes B1 and B3 are able to bind one or two zinc ions, whereas the class B2 enzymes appear to be mono-nuclear (Wilke *et al.,* 2005). In the bi-nuclear MBLs, the zinc ions are proximal to each other and are separated by a bridging hydroxide that has been proposed to be the attacking nucleophile in β-lactam hydrolysis. The class B1 and B3 MBLs can also function as mono-nuclear enzymes, in which a single zinc ion (that occupies the Zn1 site) coordinates the nucleophilic hydroxide; it has been suggested that this mechanism predominates in the presence of substrate under physiological conditions (Wilke *et al.,* 2005). The MBLs have poor affinity or hydrolytic capability for monobactams and are not inhibited by clavulanic acid or tazobactam. Instead, they are inhibited by metal ion chelators such as EDTA (Bush and Jacoby, 2010).

Ambler class C enzymes (Bush Group 1) include the chromosomally encoded AmpC type beta-lactamses found in *Citrobacter freundii*, *Enterobacter aerogenes* and *Enterobacter cloacae* (Babic, Hujer and Bonomo, 2006). Characteristically, AmpC β-lactamases provides resistance to cephamycins as well as to oxyimino-β-lactams and are resistant to inhibition by clavulanic acid (Jacoby and Munoz-Price, 2005). The sequence of the *amp*C gene from *E. coli* was reported in 1981. It differed from the sequence of penicillinase-type β-lactamases such as TEM-1 but, like them, had serine at its active site (Jacoby, 2009). The chromosomally encoded β-lactamases have been a subject of intense study since the 1980s. Most of these enzymes are cephalosporinases that are capable of hydrolyzing third generation

cephalosporins. The chromosomal β-lactam hydrolyzing gene, *amp*C, of *Escherichia coli* K-12 was first to be cloned and sequenced (Kong *et al*, 2010). Plasmid-mediated AmpC β-lactamases have been found worldwide but are less common than extended-spectrum β-lactamases, and in *E. coli*, they appear to be less often a cause of cefoxitin resistance than an increased production of chromosomal AmpC β-lactamase (Jacoby, 2009). AmpC enzymes encoded by both chromosomal and plasmid genes are evolving to hydrolyze broad-spectrum cephalosporins more efficiently and according to much of the literature there needs to be improvements in clinical detection of these enzymes lest the intensity of their resistance mechanisms be underestimated (Jacoby, 2009).

OXA, an abbreviation for oxacillin-hydrolyzing β-lactamases, represented one of the most prevalent plasmid-encoded β-lactamase families in the late 1970s and early 1980s (Queenan and Bush, 2007). Ambler class D (Bush group 2f), are serine β-lactamases that are able to hydrolyze oxacillin. The OXA enzymes of *Acinetobacter baumannii* and *P. aeruginosa* represent the most structurally diverse and rapidly growing group of beta-lactamases. Depending on the OXA enzyme, these beta-lactamases confer resistance to penicillins, cephalosporins, extended-spectrum cephalosporins (OXA-type ESBLs) or carbapenems (OXA-type carbapenemases). OXA enzymes are relatively resistant to clavulanic acid inactivation, but are inhibited by sodium chloride (Babic, Hujer and Bonomo, 2006). Some confer resistance predominantly to ceftazidime (Jacoby, 2005).

1.5 ESBLs in South Africa and Africa

ESBLs are now a problem worldwide but the phenomenon began in western Europe most likely because that is where ESBLs were first used clinically (Bradford, 2001). The epidemiology of ESBLs vary among institutions, Meyer *et al.,* (2007), reported SHV and TEM type ESBLs in Pretoria Academic Hospital. Essack, Hall and Livermore, (2004) reported the presence of five β-lactamase genes in a single South African *K. pneumonia* isolate. Several outbreaks of infections with ESBL-producing *Klebsiella* have been reported from South Africa previously. In 2006, carbapenem-resistant, ESBL producing *K. Pneumonia* were detected in Cape Town and this has led to researchers in other institutions investigate the prevalence of these enzymes (Meyer *et al.,* 2007).

In 2008, Govinden *et al.,* reported TEM-63 in *Salmonella spp*. which was first reported by Essack *et al.,* (2001) in *K. pneumonia* isolates. The same study included the presence of TEM – 131 and TEM-116, the latter, reported for the first time in South Africa in *Salmonella spp*. Those multi-drug resistant strains of *Salmonella spp*. also harbored SHV, CTX-M, OXA and

CMY type β-lactamases in unique combinations which was a rare occurrence as well as the discovery of CTX-M-37 being reported for the first time in South Africa (Govinden *et al.*, 2008). Another study investigating the β-lactamases in *E. coli* in state hospitals in Kwa-Zulu Natal in 2009 also revealed a great deal of diversity of β-lactamase genes reporting CMY, SHV, OXA, CTXM, TEM as well as two novel inhibitor resistant TEM β- lactamases, TEM-145 and TEM 146 (Mocktar *et al.*, 2009).

ESBLs have also been documented in Israel, Saudi Arabia, and a variety of North African countries. Outbreaks of *Klebsiella* infections with strains resistant to third-generation cephalosporins have been reported in Nigeria and Kenya without definitive identification of the ESBL (Paterson, 2005). Okesola and Makanjuola, (2009) reported a notable increase in resistance in *Klebsiella spp*., with69 % of the isolates tested showing resistance to third generation cephalosporins. A novel CTX-M enzyme (CTX-M-12) has been found in Kenya. Characterization of ESBLs from South Africa has revealed TEM and SHV types (especially SHV-2 and SHV-5). (Paterson, 2005)

1.6 Dissemination of Resistance Determinants

1.6.1 Transduction

Transduction occurs when viral particles, known as bacteriophages, pick up chromosomal DNA from the donor bacteria and transfer it to recipient cells by infection (Figure 1.7), (Lartigue *et al.*, 2007). Transduction is responsible for some of the transfer of bacterial drug resistance genes among clinical strains of *Staphylococcus aureus.* Fragments of bacterial genomes or small bacterial plasmids can be moved from one bacterial cell to another in this way. Survival of the DNA in the new cell requires either 'rescue' by recombination or the transferred DNA must be able to replicate independently in its new host. If the DNA is a linear fragment of a larger replicon, such as a fragment of the chromosome of the cell in which the phage reproduced, then any genes on it will only survive if they are rescued by recombination (Bennett *et al.*, 2004). Homologous recombination is a DNA metabolic process that provides high fidelity, template dependent repair or tolerance of complex DNA damages. It plays a prominent role in authentically duplicating the genome by providing critical support for DNA replication (Li and Heyer, 2008).

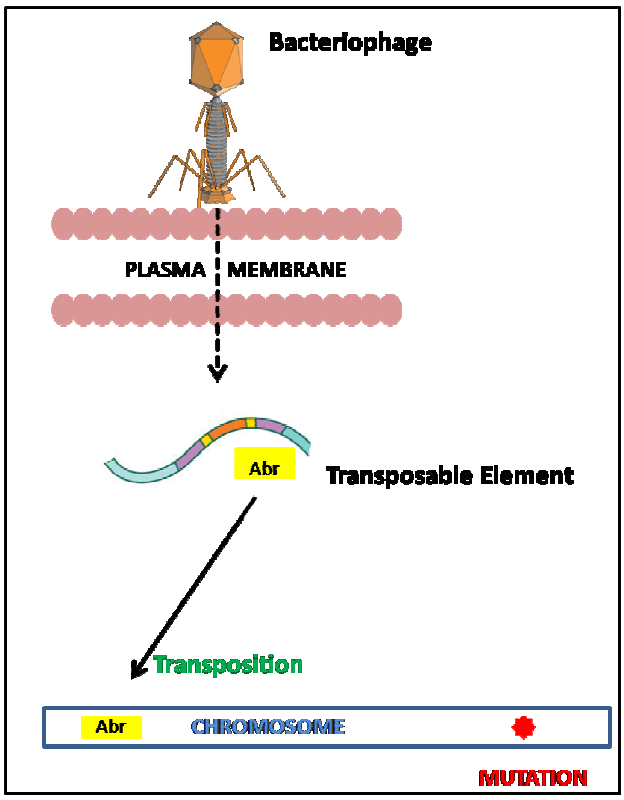

Fig 1.7 Illustration of the process of transduction where bacteria may become antibiotic resistant (Abr) when they acquire the target gene which may have a mutation (Alekshun and Levy, 2007).

1.6.2 Transformation

Transformation refers to the process of bacteria taking up 'naked' or 'free' DNA (Figure 1.8). This DNA is generally recombined into the recipient chromosomes to form genetic recombinants (Lartigue *et al.*, 2008). This mechanism involves only DNA and requires the release of DNA from the donor cell, by cell lysis after which the gene must be rescued by the recipient cell so to prevent degradation (Bennett *et al.*, 2004). Simply, a whole bacterial genome or fragments of the genome from one species is transformed into another bacterial species, which results in new cells that have the genotype and the phenotype of the input genome (Lartigue *et al.*, 2008). The antibiotic resistance may therefore be a result of the bacteria acquiring foreign genetic material and incorporating free DNA segments into their chromosome (Alekshun and Levy, 2007).

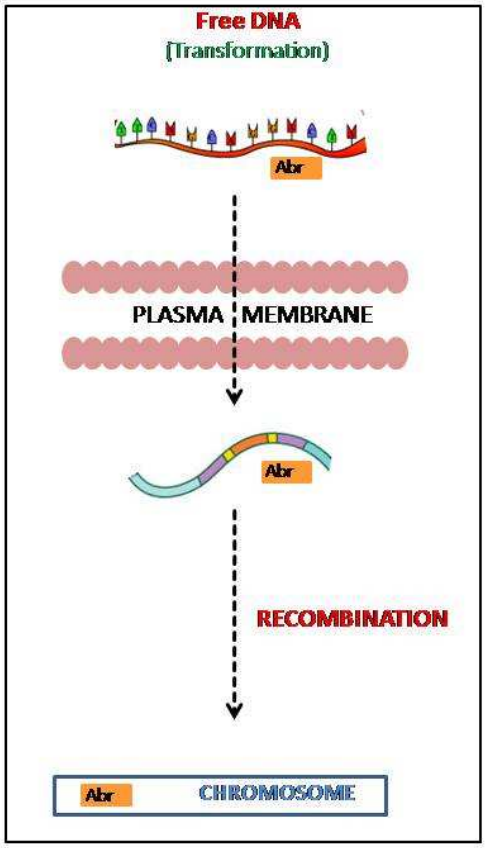

Fig 1.8 Illustration of the process of transformation where bacteria may acquire resistance genes by incorporating segments into their chromosome (Alekshun and Levy, 2007).

1.6.3 Conjugation

Conjugation (Figure 1.9) is a key mechanism for horizontal gene transfer in bacteria, this allows bacteria to acquire genes for antibiotic resistance as well as other attributes (Babic et al., 2008). In the case of transduction and transformation, these two mechanisms depend on the recipient to participate in the process via homologous recombination; conjugation requires the participation of either a conjugative plasmid or a conjugative transposon, each of which encodes a DNA transfer system that has evolved to specifically mediate this horizontal transfer (Bennett et al., 2004,). An important feature of conjugation is that a single donor cell can convert a population of recipient cells to donor cells especially in the spread of an outbreak (Frost and Koraimann, 2010). In addition to functions involved in their replication and transfer, plasmids have been known to encode resistance to one or many antibiotics. Some plasmids can transfer between different species whereas others are confined to one genus or species. Therefore a plasmid does not need to reside only in a particular host in order to contribute to the spread of resistance (van Hoek et al., 2011).

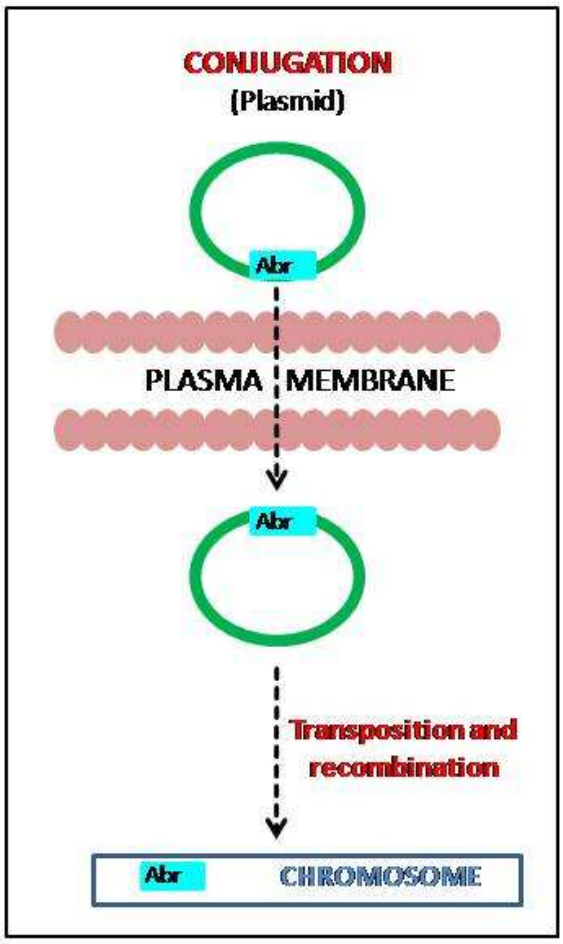

Fig 1.9 Illustration of bacterial conjugation involving the transfer of genetic material vis plasmids or conjugative transposons (Alekshun and Levy, 2007).

1.6.4 Mobile genetic elements

These plasmids and transposons are referred to as mobile genetic elements. Mobile genetic elements fall into two types: elements that can move from one cell to another such as plasmids and conjugative transposons and elements that can move from one genetic localization to another in the same cell such as transposons, gene cassettes and insertion sequences (IS) (Bennett, 2009).

Plasmids have been described as small, auxiliary, dispensable chromosomes. Conjugative plasmids are plasmids that are able to promote their own transfer and the transfer of other plasmids from one bacterial cell to another In general; they exist separately from and are replicated independently of the main bacterial chromosome (Bennett, 2009). Transposons are essentially 'jumping gene' systems that incorporate a resistance gene within the element.

They come in many forms, recognized by structure, genetic relatedness and mechanism of transposition and can carry a variety of resistance genes (Bennett *et al.*, 2004). Transposons belong to the set of mobile elements called transposable elements that encompass small cryptic elements called insertion sequences (Bennett, 2009). Insertion sequence elements are small and may vary in size between 0.8 and 2.5 kb and they encode very specific functions required for their transposition. IS elements may be present in one or several copies and contained on plasmids, or chromosomes and they must reside on the conjugative plasmid for intercellular transfer (Depardieu *et al.*, 2007).

Integrons are elements that are capable of insertion, excision and expression of gene cassettes (Grape *et al.*, 2005). They comprise a site-specific recombination system that recognizes and captures mobile gene cassettes. An integron includes a gene for an integrase (*int*) and for an adjacent recombination site (*attI*) (Fluit and Schmitz, 2004). A gene cassette is a small autonomous, non-replicating, double-stranded, circular DNA molecule found on integrons (Bennett, 2009). Integrons can be divided into two groups: Resistance Integrons (RIs) and Super Integrons (SIs). RIs carry gene cassettes that encode resistance against antibiotics and are primarily located on the chromosome or the plasmid. The SI group is large and chromosomally-located with gene cassettes which contain a variety of functions (Fluit and Schmitz, 2004). The insertion of a gene cassette is strictly oriented so that the start of the gene carried by the cassette is the nearest *int*. Gene cassettes can be inserted one after the other into the integron insertion site, *attI*, therefore producing impressive resistance gene arrays (Bennett, 2009).

The four classes of integrons so far identified are class 1, 2, 3 and 4. They are distinguished by their respective integrase (*int*) genes. Class 4 is a distinctive class of integrons located in the *Vibrio cholerae* genome and is not known to be associated with antibiotic resistance (White, McIver and Rawlinson, 2001). Most RI conforms to a structure known as a class 1 integron (Figure 1.10). Class 1 integrons have been reported in many gram negative bacteria including *K. pneumonia* (Fluit and Schmitz, 2004).

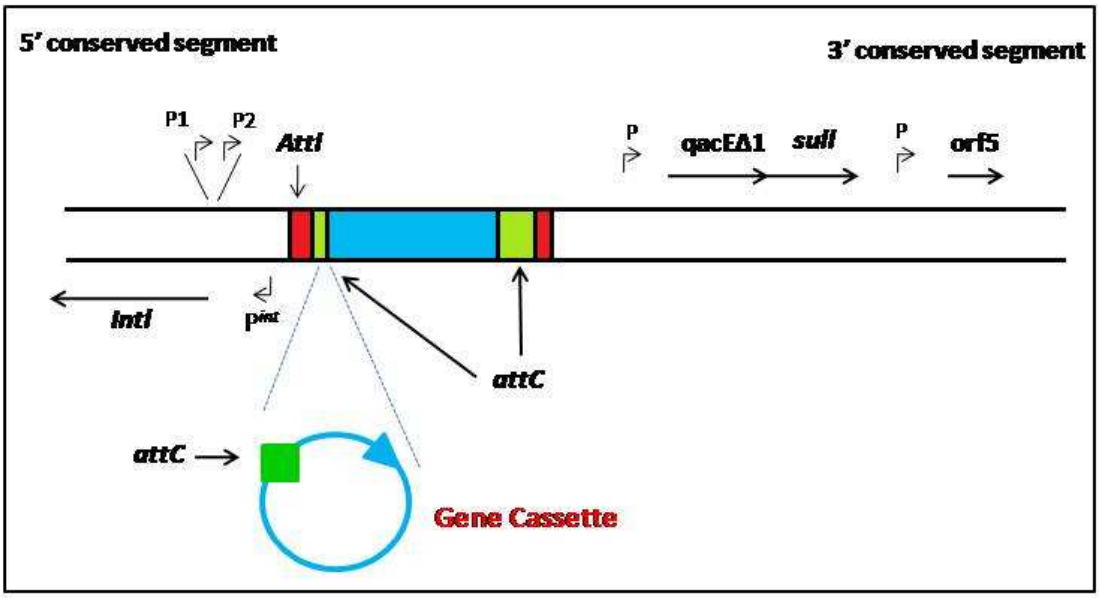

Fig 1.10 A schematic representation of a class 1 integron with a gene cassette. P1, promoter for the transcription of gene cassettes; P2, second promoter that is usually inactive; int, integrase gene; attI1, the integration site; qacE, partially deleted gene that encodes quartenary ammonium compound (disinfectants and antiseptics) resistance; sulI, sulphonamide resistance; attC, sequence on the gene cassette recognized by the integrase (Fluit and Schmitz, 2004).

Broad spectrum β-lactam antibiotics were introduced in 1981 and shortly after their release, ESBL producing organisms were discovered in Germany in 1983. To date the rise of these infections has become significant enough for it to be recognized and in recent years the phenomenon of multi drug resistance (MDR) has been raised (Hyle et al., 2005). MDR can be defined as the resistance to multiple antibiotics and or other antibiotic classes in addition to the β-lactam antibiotics (Zhao et al., 2010). As previously mentioned, class 1 integrons make up the majority of the integrons found in clinical isolates and they have been associated with MDR in hospital settings (Leverstein van Hall, et al., 2003). Much of the literature has associated class 1 integrons with MDR due to the gene cassettes expressing the qacE and the sulI genes. MDR has been described particularly in K. pneumonia isolates by Zhao et al., (2010). They discovered that more than 27 plasmids in K. pneumonia isolates were found to carry genetic determinants for MDR. Another study found metallo β-lactamases embedded in gene cassettes on a class 1 integron specifically in a K. pneumonia isolate that showed MDR previously (Zhao et al., 2010)

1.7 *K. pneumonia*

K. pneumonia has been a known human pathogen since the late nineteenth century when it was first isolated by Edwin Klebs. It is often called Friedlander's pneumonia, in honor of the first man who identified it as a significant respiratory pathogen back in 1882. Although *Klebsiella* is most often thought of as a nosocomial pathogen, it can also be a cause of serious infection acquired outside the hospital (Prince *et al.,* 1997). *Klebsiella* is a Gram-negative, non-motile and rod-shaped bacterium, which can live in water, soil and plants and is pathogenic to humans and animals (Zhao *et al.*, 2010). *Klebsiellae* are protected from drying by their capsule and so survive better than other *Enterobacteriaceae* on skin and fomites, facilitating cross-infection (Yuan *et al.,* 1998). It is a common hospital-acquired pathogen, causing urinary tract infections, nosocomial pneumonia and intra-abdominal infections (Ko *et al.,* 2002). A geographic difference in this organism has been recognized and several outbreaks of various infections have been documented. Extensive use of broad spectrum antibiotics in hospitalized patients has led to both increased carriage of *Klebsiella*, and subsequently, the development of MDR strains that produce ESBLs (Zhao *et al.,* 2010).

Until the mid-1980s, resistance to broad spectrum β-lactam antibiotics was known to be limited to organisms with inducible chromosomal β-lactamase genes; this form of resistance is not transmissible (Jain *et al.,* 2003)**.** It was a surprise when a species of *Klebsiella* with plasmid-mediated resistance to extended-spectrum cephalosporins was isolated in Germany in 1983, and in the following year, similar resistance in *Klebsiella* was reported in France (Jain *et al.*, 2003). Being encoded on plasmids enables these enzymes to be easily transmissible from one organism to another. *K. pneumoniae* is by far the most common species in which ESBL has been recognized, accounting for 75% of ESBL-producing isolates. Plasmid-mediated ESBL production had been detected in 14–16% of *K. pneumoniae* isolates in Europe in 2001 (Menashe *et al.*, 2001).

It is important to note though that cross-infection is not the only explanation for dissemination of ESBLs, plasmid transfer is also an important consideration. In the first major outbreak of ESBL producers, Sirot, (1995), documented transmission of plasmids encoding TEM-3 β-lactamase between different *Klebsiellae* and to other *Enterobacteriaceae* (Yuan *et al.*, 1998). Several studies have shown that ESBL-producing *Klebsiella* strains have an increased ability to adhere to human epithelial cells, probably due to plasmid-coded production of fimbrial or nonfimbrial adhesins (Sahly *et al.,* 2004). Resistance appeared initially in organisms such as *Enterobacter cloacae* that could, by mutation overproduce their chromosomal AmpC. Later, however, resistance appeared in bacterial species that lack an inducible AmpC enzyme, such

as *K. pneumonia* and this resistance was found to be mediated by plasmids encoding ESBLs (Philippon, Arlet and Jacoby, 2002). Plasmid-mediated AmpC beta-lactamases have arisen through the transfer of chromosomal genes for the inducible AmpC beta-lactamase onto plasmids. This transfer has resulted in plasmid-mediated AmpC beta-lactamases in isolates of *Escherichia coli, K. pneumoniae, Salmonella* spp., *Citrobacter freundii, Enterobacter aerogenes,* and *Proteus mirabilis* Plasmid-mediated AmpCs differ from chromosomal AmpCs in that they are uninducible (Thomson, 2001).

Like their counterpart on the chromosome, such enzymes provided a broader spectrum of resistance of ESBLs and were not blocked by commercially available inhibitors. Furthermore, in a strain with decreased outer membrane permeability such enzymes can provide resistance to carbapenems as well, as has been observed with clinical isolates of *K. pneumoniae* during an outbreak in New York (Philippon, Arlet and Jacoby, 2002).

It is anticipated that *Klebsiella* strains will become more virulent and resistant to antibiotics in the future, an eventuality that could contribute to the spread of certain *Klebsiella* clones by virtue of their greater resistance to antibiotics and better adherence to host tissues (Sahly *et al.,* 2004).

1.8 Resistance containment Strategies

Various strategies have been proposed to improve use of antibiotics and prevent the emergence of antibiotic resistance. Antibiotic practice guidelines or protocols have emerged as a potentially effective means of both avoiding unnecessary antibiotic administration and increasing the effectiveness of prescribed antibiotics. Restricting specific antibiotics or antibiotic classes from the hospital formulary has been adopted as a strategy to reduce the occurrence of antibiotic resistance and antimicrobial costs. Such an approach has been shown to reduce pharmacy expenses and adverse drug reactions from the restricted drugs (Okesola and Makanjuola, 2009). Another proposed strategy to curtail the development of antimicrobial resistance, in addition to the judicious overall use of antibiotics, is to use drugs with a narrow antimicrobial spectrum or "older" antibiotics. Several investigations suggest that some infections, such as community-acquired pneumonia and urinary tract infections, can usually be successfully treated with narrow-spectrum antibiotic agents, especially if the infections are not life threatening (Kollef and Fraser, 2001).

It is evident that *K. pneumonia* has a significant predilection to resistance mechanisms of various β-lactam antibiotics therefore making it an excellent representative organism for our investigation.

Aim

To elucidate the molecular epidemiology of β-lactamase mediated resistance, in selected *K. pneumonia* isolates from a tertiary hospital in KwaZulu-Natal, South Africa and to ascertain correlation between antibiotic use and susceptibility (if any).

Objectives:

1. To determine the production of ESBLs by using the double disk synergy test (DDST) for ESBL detection.
2. To undertake preliminary typing of β-lactamases on the basis of MICs.
3. To undertake DNA sequencing to definitively identify the ESBL genes and to correlate genotypes with phenotypes.
4. To investigate genetic determinants of resistance via plasmid banding patterns
5. To ascertain the correlation between resistance and susceptibility to antimicrobial consumption

CHAPTER TWO

METHODOLOGY

2.1 Bacterial Isolates

The 24 putative ESBL positive *K pneumonia* isolates investigated in this study were collected from a tertiary hospital in KwaZulu-Natal, South Africa. Of the 13 *K. pneumonia* isolates collected in the year 2000, 5 were tested as ESBL positive. These were KEH 3, 6, 21, 81,100. These isolates formed part of a previous study by Essack *et al.,* which was undertaken to evaluate the appropriateness of nationally devised standard treatment guidelines (STGs) within the public healthcare system in KwaZulu-Natal. The study highlighted the importance of surveillance-based treatment guidelines for infections over the guidelines set by expert committees as the former takes into considerations many factors such as, microbial etiology of the disease, antibiotic use and antibiotic resistance (Essack *et al.,* 2005).

The remaining isolates were collected in 2007 and formed part of an active, disease-based surveillance study also ascertaining the appropriateness of treatment guidelines, but on a disease basis. The isolates were collected from three hospitals, one district, one regional and one tertiary. *K. pneumoniae* featured as a causative organism in nosocomial infections and *K. pneumoniae* isolated in the tertiary hospital were included in this study.

Of these, 14 were subjected to full laboratory investigations as a result of cost constraints.

2.1.1 Ethical Considerations

This study as well as the study by Essack *et al.,* (2005) had ethical clearance from the University of KwaZulu-Natal.

2.2 Bacterial Identification

The isolates were identified using the API 20E system (bioMerieux, Lyons, France) as per the manufacturer's guidelines.

Table 2.1 A list of the Bacterial Test Isolates used in the study and the year of collection

Bacterial Isolate	Year of Isolation	Bacterial Isolate	Year of Isolation
KEH100	2000	KEH3328	2007
KEH3	2000	KEH3409	2007
KEH21	2000	KEH2786	2007
KEH6	2000	KEH3434	2007
KEH81	2000	KEH3281	2007
KEH1506	2007	KEH2605	2007
KEH2093	2007	KEH2787	2007
KEH1883	2007	KEH2924	2007
KEH7123	2007	KEH893	2007
KEH3217	2007	KEH2007	2007
KEH7689	2007	KEH6740	2007
KEH3322	2007	KEH3262	2007

2.3 Susceptibility Testing

2.3.1 Agar Dilution

The microbiological parameter used since the beginning of antibiotic therapy is the minimum inhibitory concentration (MIC). The MIC is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after overnight incubation (Andrews, 2001). MICs are used to confirm unusual resistance, to give definitive answers when borderline results are obtained or when disk diffusion methods give false negative results (Dalhoff, Ambrose and Mouton, 2009).

Agar dilution and broth dilution are the most commonly used methods to determine MICs where bacterial cell suspensions are dotted directly onto the agar plates that have integrated different antibiotic concentrations. Broth dilution uses a liquid growth medium containing geometrically increasing concentrations of the antibiotic which is inoculated with a defined number of cells (Wiegand, Hilpert and Hancock, 2008).

The advantage of both the broth and agar dilution methods is that both techniques generate very good quantitative results. The disadvantage of using the broth dilution method is that the manual task of preparing the antibiotic solutions is tedious and the possibility of errors in the

preparation is very high. The biggest concern however is the interpretation of results. Micro broth dilution has been widely used which uses an automated system to interpret results however, this technique requires a large amount of reagents and is not a very cost effective method (Jorgensen and Ferraro, 2009).

The agar dilution technique has shown accuracy in determination of MICs specifically when a full dilution range of the antibiotic is used. This technique also enables the testing of many organisms against a succession of dilutions of just a single antibiotic concurrently as well as the possibility of extending the concentrations of antibiotics as far as is required (Varela *et al.*, 2008). MICs are a good phenotypic indicator to determine the resistance or susceptibility of an isolate to antibiotics; however, it is inconclusive with respect to the mechanism of resistance to the antimicrobial agent. (Wiegand *et al*., 2008). Phenotypic measures of the level of susceptibility of bacterial isolates to antimicrobial agents are nevertheless largely relevant due to mutations, gene acquisitions, and expression mechanisms in multi-drug resistance which renders effortless detection via molecular techniques difficult (Jorgensen and Ferraro, 2009).

Materials:

- Test Isolates
- Control Isolate *E. coli* 25922
- Antibiotic stock solutions (Sigma-Aldrich®)
- Mueller Hinton Agar (*Biochemika, Fluka* Sigma-Aldrich®)
- Mueller Hinton broth (*Biochemika, Fluka* Sigma-Aldrich®)
- Sterile distilled Water
- 0.5 M McFarland standard

Apparatus:

- Automated 48-pin replicator for inoculation (Boekel Scientific). This apparatus enables the pins to dip into the micro-titre plate containing the inoculum and then dot the inoculum onto the surface of the agar. It is efficient and prevents cross contamination.
- 96 well micro-titre plate

Procedure:

- The antibiotic stock solutions were prepared using the solvents and diluents for preparation of stock solutions of antimicrobial agents as per the guidelines contained in the CLSI manual(CLSI M100-S18, 2008) using the following formula:

$$W = \frac{(C \times V)}{P}$$

Where P = the potency given by the manufacturer (µg/mg)
V = the volume of antibiotic is required. The desired volume was 20ml
C = the final concentration required. The desired concentration was 10 mg/L
W = the weight of the antibiotic required to be dissolved in V. (Wiegand *et al.*, 2008)

- The antibiotic dilution range depending on the CLSI break points for resistance and susceptibility for each antibiotic was then determined and prepared as shown in table 2.2.

Table 2.2 Antibiotic dilution chart for agar dilution method.

Antimicrobial concentration (mg/L)	Volume of antibiotic stock solution (ml)	Final concentration when adding 25 ml agar
10	640	256
10	320	128
10	160	64
10	40	16
1	200	8
1	100	4
1	50	2
0.1	250	1
0.1	125	0.5
0.1	62.5	0.25
0.1	31.25	0.125

- The Mueller Hinton agar plates were prepared in sterile 30 ml labeled bottles and autoclaved. The agar was cooled down to 50 °C and then the antibiotic dilutions were added into respective bottles and plated accordingly.

- ❖ The inoculum was prepared using Mueller Hinton broth. Individual colonies were transferred into the broth and incubated at 37°C for 4-6 hours until the turbidity resembled the 0.5 M McFarland standard. The isolates were adjusted to match the McFarland with sterile distilled water if necessary and the inoculum was used within 30 minutes
- ❖ The inoculum was pipetted using a micro pipette into the sterilized 96 well micro-titre plate along with the positive and negative controls.
- ❖ The agar plates were aligned on the base and the automated pin replicator transferred small volumes of inoculum from the micro-titre plate onto the Mueller Hinton Agar plates.
- ❖ The plates were incubated overnight at 37°C.
- ❖ The MIC was determined as the lowest concentration that showed no growth after overnight incubation.

2.3.2 Tests for the detection of ESBLs

Agar dilution and disk diffusion screening methods have been recommended by the CLSI. There are criteria and interpretive standards to assist in the phenotypic screening for ESBL positive isolates. Based on the MIC breakpoints provided by the CLSI to ascertain the expression of ESBLs, disk diffusion tests are used for confirmation, however, many studies use both agar dilution and disk diffusion for confirmation of ESBL production as disk diffusion alone has shown false positives with errors in the detection of ESBL-mediated resistance encountered with both automated and manual disk diffusion methods (Perez *et al.*, 2007). Using disk diffusion methods for antibiotic susceptibility testing can screen for ESBL production by noting specific zone diameters which indicate a high probability of ESBL production (Paterson and Bonomo, 2005).If the zone diameters indicate a suspicion for ESBL production then further phenotypic tests must be carried out for confirmation of ESBLs. Inaccurate identification of ESBL producers has clinical implications as well as for antibiotic use and it is therefore imperative that detection guidelines are adhered to.

Since the 1980's specific phenotypic tests have been developed for ESBL detection, whether it be disk diffusion on agar, MIC determination or automated systems (Drieux, *et al.*, 2008). There has been extensive research into these screening methods with specific attention to their accuracy and rapidity. The double disk synergy test, the three dimensional test, the E Test and the Vitek test, including their modifications have been introduced as screening methods for ESBL detection (Essack, 2000).

The ESBL E test has been developed to measure the interaction between extended spectrum cephalosporins and clavulanate. The E tests are plastic, two sided strips containing gradients of cefotaxime, ceftazidime or cefepime either alone on one side of the strip or combined with clavulanate on the other end (Drieux, *et al.,* 2008). A positive test result is the reduction of the test drugs MIC by a ratio of ≥8 in the presence of clavulanate. A positive result can also be confirmed by the presence of a rounded or a phantom zone below the lowest concentration of the test drug and a deformation of the inhibition ellipse at the tapered end of the test drug (Drieux, *et al.,* 2008). The E- test may fail if the MIC values for the cephalosporins fall outside the range of those present on the test strip (Linscott and Brown, 2005) and the reading of the inhibition zones may very well influence results negatively as these zones have been frequently reported as complex due to the presence of mutants along the zone border. It is therefore imperative that researchers and technicians be well trained to recognize the subtle ellipse deformations and phantom zones (Leverstein-van Hall, *et al.,* 2003).

The VITEK 2 test, a relatively new modification and is an improvement on the VITEK 1 because of its increased specificity, is an automated ESBL test which uses the antimicrobial susceptibility data generated to suggest the phenotype of the tested isolate and thereby determine the susceptibility and resistance to antibiotics not tested (Schwaber, *et al.,* 2005) This test assesses the antibacterial activity of cefepime, cefotaxime and ceftazidime either alone or in the presence of clavulanate while making use of card wells containing the test drugs in various concentrations either alone or in combination with clavulanate. After inoculation the cards are introduced into the VITEK 2 machine where the turbidity is measured for each antibiotic tested and the proportional reduction of growth in the wells containing cephalosporin with clavulanate is compared to the cephalosporin alone and is then interpreted ESBL positive or negative (Drieux, *et al.,* 2008). The VITEK test is not as cost effective as manual methods and has rendered false positives. This test has shown a low specificity with numerous false positives reported in many studies (Wiegand *et al.,* 2008) and it has been reported that the VITEK test should not be used as the sole method of detection until further studies prove otherwise (Schwaber *et al.,* 2006).

The three dimensional test proposed by Thomson *et al.,* (1984), is a modification of disk diffusion tests and it demonstrates substrate specificities of bacterial β-lactamases present. The test involves inoculating the surface of the agar with a 0.5 McFarland of the test organism and a higher density of inoculum is dispensed into a circular slit in the agar just 3mm from the antibiotic disks. The test as described by Thomson *et al.,* (1984), inoculates a control strain on the inner surface before the slit and the test organism inoculated on the outer surface after the slit and could test up to 8 antibiotics at a time. Inactivation of the antibiotic, as it diffuses

through the slit, results in a distortion or a discontinuity in the circular inhibition zone (Drieux, *et al.,* 2008). Since its discovery there have been many modifications to the three dimensional test, specifically to test the presence of AmpC β-lactamases. It has been reported that the technique proved to be difficult in terms of the circular slit and some modifications involved linear slits but having to fill the slits with the enzyme extracts proved to be tedious (Manchanda and Singh, 2003). The technique does have its advantages as it requires less training and minimal reagents and has not had very high reports of false positives with high detection rates (Drieux, *et al.,* 2008; Manchanda and Singh, 2003).

2.3.3 Double Disk Synergy Test (DDST)

The DDST is a very cost-effective technique has been used in a wide range of *Enterobactericeae,* it is regarded as a reliable test but sometimes it is necessary to adjust the spacing between the disks to increase its reliability (Drieux, *et al.,* 2008). The technique is relatively simple however the interpretation of the results is subjective. There have been instances where false-negative results have been reported with isolates harboring the SHV-2, SHV-3 or TEM-12 ESBLs. In addition to that, sensitivity may be reduced when ESBL expression is very low, leading to wide zones of inhibition (Rawat and Nair, 2010). According to CLSI guidelines, isolates which have a positive phenotypic confirmatory test should be reported as resistant to all cephalosporins (except the cephamycins,) and aztreonam. β-lactam/β-lactamase inhibitor combinations such as ticarcillin-clavulanate or piperacillin-tazobactam must be reported as susceptible if MICs or zone diameters are within the appropriate range (Rawat and Nair, 2010).

2.3.4 Tests for the detection of AmpC β-lactamases

The detection of AmpC's has proven to be a challenge for many laboratories. There has been methods devised based on disk diffusion methodology by many laboratories to detect AmpC's. Nasim *et al.,* (2004), studied the use of a cefoxitin agar medium (CAM) in comparison to a modified three dimensional AmpC assay (M3D). The CAM assay which is time consuming and labor intensive gave the authors a similar result to the M3D, however like many studies gave false positives. A study by Coudron, Hanson and Climo (2003), reported on the inconsistency in their phenotypic testing of AmpC's as the disk diffusion showed 15% AmpC positives but that number was further reduced by the three dimensional test and isoelectric focusing. The detection of plasmid mediated AmpC's prove to be very technically demanding with the use of methods that were time consuming and although a few tests were easier to perform many of them seemed difficult to interpret. In this study we chose

to perform an AmpC disk test based according to Black, Moland and Thomson (2005), which is based on the use of TRIS-EDTA which permeabilizes the bacterial cell wall and releases β-lactamases into the external environment. The test allows for six isolates to be tested at a time and the interpretation was straightforward. The authors reported a very high number of positive isolates in their study (95%) indicating the accuracy, simplicity and convenience of using such an assay. They did report however that their study did not include plasmid mediated AmpC's however additional studies showed positive results in other enterobacteriaceae. Genetic testing will however be necessary as a follow up on such isolates.

In this study both the agar dilution as well as the double disk synergy test was used to confirm ESBL producers. The TRIS-EDTA disk test was used to confirm AmpC producers. The disk diffusion test was carried out according to CLSI guidelines and zone diameters were measured.

Materials:

- Test Isolates
- *E coli* positive Control 25922
- ESBL - producing isolate from a previous study (Essack, Connolly and Sturm, 2005))
- Mueller Hinton Agar (*Biochemika, Fluka* Sigma-Aldrich®)
- Mueller Hinton Broth(*Biochemika, Fluka* Sigma-Aldrich®)
- Antibiotic Disks (Oxoid)
    - Amoxicillin-clavulanate (AMC)
    - Ceftazidime (CAZ)
    - Cefuroxime (CROX)
    - Cefotaxime (CTX)
    - Aztreonam (AZT)
- 0.5 M McFarland Standard

Procedure:

- A few colonies were picked from overnight growth on Nutrient Agar and inoculated into 5 ml of Mueller Hinton Broth
- The broths were incubated at 37°C overnight until turbid
- The turbidity was matched to the 0.5 M McFarland standard (Appendix 1) with sterile distilled water
- A sterile cotton swab was dipped into the broth and pressed firmly above the fluid level to drain off excess broth.

- ❖ The swab was then streaked over the entire agar surface several times until the inoculum was evenly distributed
- ❖ The plates were left to dry for 2-5 minutes but not more than 10 minutes
- ❖ The following disks were placed onto the agar: Aztreonam (AZT), Ceftazidime (CAZ), Cefuroxime (CROX), Cefotaxime (CTX) and Amoxicillin/Clavulanate (AMC). The AMC disks were placed at the centre and the rest were placed around the centre not less than 25 mm apart from each other as well as from the AMC disk.
- ❖ Plates were incubated at 37°C overnight.
- ❖ An enhancement of the zone nearest to the AMC disk indicated a positive test for ESBLs.

The TRIS-EDTA test was performed with modifications as follows:

Materials

- Test Isolates
- *E coli* positive Control 25922
- Putative AmpC producers from a previous study (Essack, Connolly and Sturm, 2005)
- 10X TRIS-EDTA (appendix one)
- Mueller Hinton Agar (*Biochemika, Fluka* Sigma-Aldrich®)
- Mueller Hinton Broth(*Biochemika, Fluka* Sigma-Aldrich®)
- Cefoxitin Antibiotic Disks (Oxoid)
- Sterile filter paper disks
- Saline
- 0.5 McFarland Standard

Procedure:

- ❖ A few colonies were picked from overnight growth on Nutrient Agar and inoculated into 5 ml of Mueller Hinton Broth
- ❖ The broths were incubated at 37°C overnight until turbid
- ❖ The turbidity was matched to the 0.5 M McFarland standard (Appendix 1) with sterile distilled water
- ❖ Sterile filter paper disks (AmpC disks) were prepared by applying a 1:1mixture of saline and 10X TRIS-EDTA and were then stored at 8 °C
- ❖ A sterile cotton swab was dipped into the broth containing the *E. Coli* 25922 and pressed firmly above the fluid level to drain off excess broth.

- ❖ The swab was then streaked over the entire agar surface several times until the inoculum was evenly distributed
- ❖ Three cefoxitin disks were placed 25mm apart on the inoculated surface of the agar.
- ❖ The prepared AmpC disks were inoculated with 20µl of each test isolate prior to use and placed on either side of the cefoxitin disk making sure the AmpC disks touch the cefoxitin disks.
- ❖ A total of 6 isolates were tested each time

2.4 Plasmid Isolation

Many β-lactamase genes have been found on plasmids in *K. pneumonia* including those encoding ESBLs (Wei *et al.*, 2005). Plasmid profiling is important to ascertain the diverse genetic determinants on which resistance genes reside. Studies have shown that members of a single epidemic strain may carry different plasmids and genotypically non-related strains can produce the same ESBL due to plasmid transfer between species. Independent evolution due to antibiotic pressure plays a role as well as it has been found that different plasmids have the ability to mediate the same ESBL (Paterson and Bonomo, 2005).

In most bacteria, plasmids are the major vector of horizontal gene transfer. Plasmids are capable of self transfer between strains and species which is responsible for the capture of different resistance genes which gives rise to the multi drug resistant (MDR) phenotype (Hawkey and Jones, 2009). A study by Karisik, *et al.*, (2006) reported multi drug resistant strains of E. coli with plasmids exhibiting genetic linkage of three β-lactamase genes, $bla_{\text{CTXM-15}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1.}}$ Plasmids carrying the platform for MDR may spread across different bacterial species therefore strategies need to be developed to control dissemination of antimicrobial drug resistance (Hopkins, *et al.*, 2005; Coque, *et al.*, 2002).

Plasmid isolation and gel electrophoresis was performed according to Kado and Liu 1981.

Materials:

- Test isolates
- Positive control *E coli* 50192
- SDS buffer (Appendix 1)
- Tris solution (Appendix 1)
- 3%SDS/50mM Tris (Appendix 1)
- Phenol/Chlorform (50:50)

- 95% Ethanol
- Agarose (TopVision™ Fermentas)
- TE Buffer (Appendix 1)
- TAE Buffer (Appendix 1)
- Bromophenol blue
- Ethidium Bromide (Sigma-Aldrich®)
- Electrophoresis apparatus
- UV Gel spectrophotometer (Gel Doc XR, BioRad)
- 1kb Molecular weight marker (MWM) (Gene ruler ™ Fermentas)

Isolation:

- ❖ Cultures were grown on nutrient agar plates overnight and inoculated into 20 ml of nutrient broth and incubated overnight at 37 °C.
- ❖ 2 ml of broth was pippetted into a labelled eppendorf tube and centrifuged at high speed for 5 minutes to pellet the inoculums. The supernatant was discarded.
- ❖ The pellet was re-suspended in 1 ml of 3%SDS/50mM Tris and incubated at 65 °C for 1 hour
- ❖ 1 ml of phenol/chloroform was added and allowed to stand at room temperature for 15 minutes
- ❖ The tubes were centrifuged at high speed for two minutes and removed carefully to not disturb the contents of the tube
- ❖ The smallest micropipette was used to extract the upper aqueous phase without touching the intermediate phase. This was transferred this into a clean labelled tube.
- ❖ It was centrifuged at a high speed for 5 minutes and the supernatant was discarded.
- ❖ The pellet was washed in ice cold 95% ethanol and centrifuge again for 3 minutes
- ❖ the supernatant discarded very carefully making sure not to lose the pellet and dried in a sterile laminar flow bench.
- ❖ 50 µl of sterile distilled water was added and isolates were stored at 4°C overnight before electrophoresis

Electrophoresis:

- ❖ A 0.8% gel using the agarose and TE buffer was prepared. The solution was heated over a Bunsen burner stirring constantly.
- ❖ After cooling ethidium bromide was added to the cooled agarose solution at a final concentration of 0.5µg/ml.

- The solution was then poured into the gel casting mould and allowed to harden for 50 to 60 minutes.
- Once the gel had hardened, the comb was removed ensuring there was no rupture of the gel at the bottom of the wells
- The gel still in the gel plate was put into the electrophoresis chamber and the chamber was filled to the marked line with TAE buffer.
- 5 µl of the plasmid DNA isolates were mixed with 2µl of Bromphenol Blue on a sheet of parafilm and the 7µl was loaded into the wells starting with the MWM and ending with the *E. coli* 50192 positive control.
- The lid and power leads were placed onto the apparatus and electrophoresis was carried out at 12V/cm for 1.5 to 2 hours.
- After adequate migration the gel was placed into the gel doc system to view the bands.
- The gel doc program incorporated the MWM sizes and estimated the size of the plasmid bands which were also compared with the positive control.

2.5 PCR and Sequencing to detect β-lactamase genes

PCR and sequencing are the most accurate methods used to definitively identify and characterise ESBL genes and these methods are able to work with a large number of isolates simultaneously as well as having the ability to detect mutants (Pitout and Laupland, 2008). The limiting factor about PCR is that it is not cost-effective and it is also confounded when isolates contain multiple genes from the same ESBL family. Genetic determination of resistance has the potential to provide a more reliable assessment of antimicrobial resistance. Genetic methods assess the genotype of an organism whereas conventional susceptibility methods assess the phenotype which in some cases is insufficient due to gene expression (Cockerill, 1999).

Genetic methods have been very successful in detecting the presence of bla_{KPC} enzymes as the detection of carbapenem-resistant organisms by automated or semi-automated methods have been problematic because of the low levels of resistance (Hindiyeh, *et al.,* 2008). PCR and sequencing was done on a select number of isolates. Funding was limited hence we were unable to sequence every isolate. The objective was to sequence those genes commonly found in *K. pneumonia* on the basis of the MIC results obtained. Subsequent to PCR, isolates were purified and sequencing was outsourced to Inqaba Biotech.

Materials:

- DNA from test isolates
- Positive control isolates: *E.coli* J53 Expressing SHV-1, *E.coli* J53 Expressing TEM-1 and *E.coli* J53 Expressing TEM-2
- Putative AmpC producers from a previous study (Essack, Connolly and Sturm, 2005).
- PCR primers (Table 2.3)
- 2 x PCR Master mix (Inqaba Biotech)
- Thermocycler (GeneAmp® 9700, ABI)
- Absolute Ethanol
- TE buffer
- Biowave Life Science DNA spectrophotometer (Biochrom)

PCR and sequencing to detect bla_{SHV} and bla_{TEM} genes was undertaken as previously described by Essack *et al*, (2001). PCR and sequencing to detect bla_{CTX-M}, bla_{CMY}, bla_{OXA} and bla_{DHA} genes was undertaken as previously described by Govinden *et al*, (2008); Kaye, (2004); Perez-Perez and Hanson, (2002) and Zhao *et al*, (2001). PCR and sequencing to detect bla_{KPC} genes was done as previously described by Yigit *et al*, (2001). A list of all the primers used follows.

Table 2.3 PCR primers used for the detection of ESBL genes.

Enzyme	Primers	Reference
TEM	5' ATG AGT ATT CAA CAT TTC CG 3'	Essack *et al*, (2001).
	5' CCA ATG CTT AAT CAG TGA GG 3'	Essack *et al*, (2001).
SHV	5' ATG CGT TAT ATT CGC CTG TG 3'	Essack *et al*, (2001).
	5' GTT AGC GTT GCC AGT GCT CG 3'	Essack *et al*, (2001).
CTX-M-3A	5' GGTTAAAAAATCACTGCG 3'	Govinden *et al*, (2008)
CTX-M-1B	5' CCGTTTCCGCTATTACAA 3'	Govinden *et al*, (2008)
CTX-M (F)	5' TTTGCGATGTGCAGTACCAGTAA 3'	Govinden *et al*, (2008)
CTX-M (R)	5' CGATATCGTTGGTGGTGCCATA 3'	Govinden *et al*, (2008)
DHA-1	5' GGGGAGATAACGTCTGACCA 3'	Zhao *et al*, (2001)
	5' TAGCCAGATCCAGCAATGTG 3'	Zhao *et al*, (2001)
CMY-1	5' TCACATCGGCTTCACAGAGC 3'	Kaye, (2004)
	5' CCATGGTGATGCTGTCAAAGA 3'	Kaye, (2004)
CMY-2	5'CAACACGGTGCAAATCAAAC 3'	Kaye, (2004)
	5'CATGGGATTTTCCTTGCTGT 3'	Kaye, (2004)
OXA-1F	5' ACACAATACATATCAACTTCGC 3'	Perez-Perez and Hanson, (2002)
OXA-1R	5'AGTGTGTTTAGAATGGTGATC 3'	Perez-Perez and Hanson, (2002)
KPC -1F	5' CTGGAGGACTATGCACTT 3'	Yigit *et al*, (2001)
KPC -1R	5' ATACCACCCTGACAGCCG 3'	Yigit *et al*, (2001)

Procedure:

- The DNA from test isolates was prepared by growing bacteria overnight on Mueller Hinton Agar. Individual colonies were suspended in distilled water. The suspensions were heated at 95°C for 3 minutes and the supernatant was used for the PCR
- 1 µl of each primer pair was mixed with 28µl of the pre-mixed master mix and topped up to 99µl with sterile distilled water. The last addition was 1µl of the template.

- PCR was run on the GeneAmp® 9700 and the conditions for bla_{TEM} and bla_{SHV} were set at the following:

	95°C for 3 minutes
30 cycles of	95°C for 1 minute
	55°C for 1 minute
	72°C for 1 minute

- The DNA from test isolates from bla_{CTX-M} , bla_{OXA}, bla_{CMY}, bla_{DHA} and bla_{KPC} was prepared by growing bacteria overnight on Mueller Hinton Agar. Individual colonies were suspended in distilled water they were heat-shocked for 10 minutes at 100℃ and briefly centrifuged and the supernatant was extracted using a QIAamp DNA Mini Kit (Qiagen)

- Conditions for bla_{CTX-M} was set at the following:

	94°C for 3 minutes
25 cycles	94°C for 30 seconds
	54°C for 1 minute
	72°C for 2 minutes
	72°C for 7 minutes

- Conditions for bla_{OXA} was set at the following:

	95°C for 5 minutes
30cycles	94°C for 30 seconds
	62°C for 30 seconds
	72°C for 30 seconds
	72°C for 10 minutes

- Conditions for bla_{CMY} was set at the following:

	94°C for 10 minutes
30 cycles	94°C for 1 minute
	54°C for 1 minute
	72°C for 2 minutes
	72°C for 10 minutes

- Conditions for bla_{DHA} was set at the following:

	94°C for 3 minutes
25 cycles	94°C for 30 seconds
	64°C for 30 seconds
	72°C for 1 minute
	72°C for 7 minutes

- Conditions for bla_{KPC} was set at the following:

  30 cycles of amplification

  94°C for 1 minute

  55°C for 1 minute

  72°C for 1 minute

- The PCR product was then purified for sequencing
- Purification was as follows:

  90 µl of PCR product

  10µl of sodium acetate solution

  250 µl of absolute ethanol

- All of the above were added into individual sterile Eppendorf tubes and the tubes were incubated at -20°C for 30 minutes.
- They were centrifuged for 5 minutes at a high speed and the supernatant was discarded
- They were washed further with 70 % Ethanol and centrifuged at high speed for 5 minutes
- The supernatant was discarded and the tubes were left to dry.
- The pellet was dissolved in 90 µl of TE buffer and was analyzed on the Biowave Life Science DNA spectrophotometer to check purity and concentration
- The remaining 10µl of PCR product was run on an agarose gel to ensure each reaction was successful.
- The purified PCR products were then outsourced to Inqaba Biotech for sequencing.
- Sequences were analyzed using BLAST 2.0 (Basic Local Alignment Search Tool) software available on the website of National Center for Biotechnology information (http://www.ncbi.nhlm.nih.gov/blast /BLAST.egi.).

2.6 Antibiotic Use Data

The daily defined dose (DDD) is the most common method to quantify antibiotic use in hospitals (Liem *et al.*, 2010). The DDD, recommended by the World Health Organisation (WHO) is a technical unit of measurement and is based on an assumed average maintenance dose per day, for the main indication of the drug, in adults (Filius *et al.*, 2005). Antibiotic use data has in many studies proven to be vital in identifying the reason for the decrease in susceptibility for many strains of bacteria. It has been widely documented that cautionary use of antibiotics may assist in reducing the resistance that has escalated over a number of years. Based on previous studies reporting an decrease in susceptibility correlating positively in the increase of antibiotic use we wanted to compare the resistance profiles we had with the antibiotic use data.

Antibiotic use data was calculated according to Essack *et al.*, (2005). The data was analysed for both time frames and were obtained from the hospital pharmacy records. It was expressed as usage density rate. The total number of grams of each antibiotic used was divided by the number of grams per daily dose for the specific antibiotic, then divided by the patient-days, and multiplied by 1 000 to give the number of DDDs per 1 000 patient-days on the assumption that all beds within the hospital were 100% occupied at any given time. King Edward Hospital (KEH) converted from a tertiary hospital to a district/regional hospital in 2007, therefore the number of beds in KEH decreased.

2.7 Statistical Analysis

The frequency distribution for utilization and susceptibility was not normally distributed. The data was summarized using median values and interquartile ranges. Antibiotic use and susceptibility in 2000 was compared to that in 2007 using a Wilcoxon signed rank test for paired data since the same drugs were tested in both years. The drugs tested in both years are as follows: ampicillin, piperacillin sodium, piperacillin/tazobactam, amoxicillin-clavulanate, cefuroxime sodium, cefotaxime, ceftazidime, amikacin, gentamicin, nalidixic acid, ceftriaxone and ciprofloxacin. The drugs were divided into two groups those that showed a decrease in susceptibility and those that did not. The change in utilization between the years 2000 and 2007 was then calculated for each group in order to determine if changes in utilization differed in the two groups.

CHAPTER THREE

RESULTS AND DISCUSSION

The aims of this study was to ascertain the differences if any in the phenotypic and genotypic resistance profiles of *K. pneumoniae* isolated from a single tertiary hospital in two surveillance studies undertaken at different times, viz., 2001 and 2007 with special emphasis on ESBLs. A correlation with antibiotic use was also undertaken.

3.1 Detection and characterisation of ESBLs

The ESBL –producing isolates were confirmed using the double disk synergy test and the agar dilution test according to the parameters set by CLSI guidelines to rule out any false positive isolates, the data can be seen in tables 3.1 and 3.2. Figure 3.1 shows a positive result for ESBLs by the extension of the ceftazidime (CAZ) zone of inhibition towards the amoxicillin/clavulanate (AMC) disk. The AmpC disk test yielded no positives in these isolates

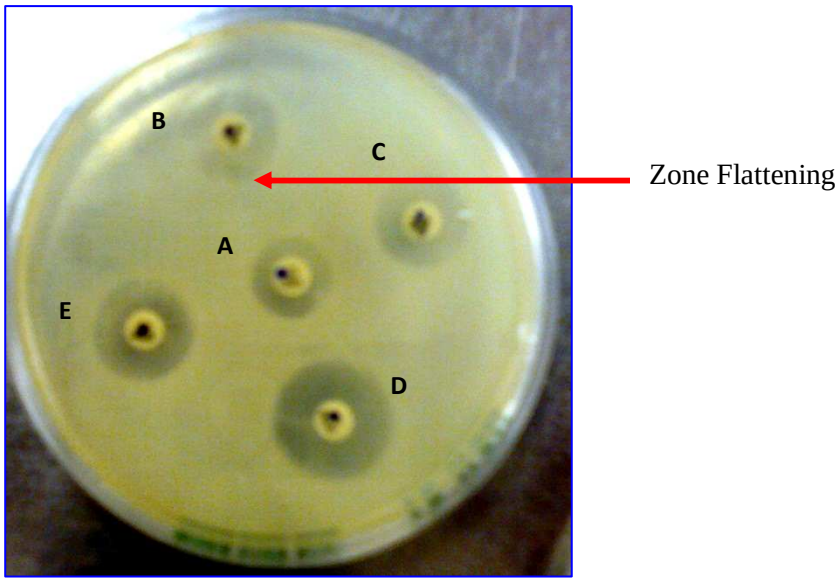

Figure 3.1 Isolate KEH 81 showing a positive result for an ESBL using the Doubledisk synergy test. The extension of the zone of inhibition of ceftazidime toward amoxicillin/clavulanate confirms the positive result. A – AMC (9mm), B – CAZ (12mm), C – CROX (12mm), D – AZT (14mm), E – CTX (12mm). AMC – amoxicillin clavulanate, CAZ – ceftazidime, CROX – cefuroxime, AZT – aztreonam, CTX – cefotaxime.

Table 3.1 MICs (µg/ml), plasmid profiles (Kb) and the genes found in the Isolates collected in 2000.

2000	AMC	CARB	P/T	CTHIN	CRO	CTX	CAZ	CROX	AMP	IMI	AZT	Plasmid Profiles (Kb)	Gene/s
KEH6	**32**	**256**	**32**	**256**	16	16	4	**16**	**128**	0.125	**16**	42; 24	TEM1, SHV1, SHV2
KEH3	8	**256**	4	**256**	**128**	**32**	4	**16**	**128**	0.125	**16**	207; 59.6; 27	TEM 1, SHV 2
KEH100	8	**256**	8	**256**	16	8	8	**16**	**128**	0.125	4	207; 56; 5	TEM1, SHV1
KEH21	**32**	**256**	16	**256**	0.25	4	4	8	**128**	0.125	1	207; 154; 59.6; 24; 10; 3; 1.3	TEM 1, SHV 2
KEH81	16	**256**	16	**256**	16	**64**	8	**16**	**128**	0.125	4	207	TEM 1, SHV1

AMC – Amoxicillin/Clavulanate, Carb – Carbenicillin, P/T – Piperacillin/Tazobactam, CTHIN – Cephalothin, CRO – Ceftriaxone, CTX – Cefotaxime, CAZ – Ceftazidime, CROX – Cefuroxime, AMP – Ampicillin, IMI – Imipenem, AZT – Aztreonam.

Table 3.2 MICs (µg/ml), Plasmid Profiles (Kb) and Genes found in representative isolates collected in 2007

2007	AMC	CARB	P/T	CTHIN	CRO	CTX	CAZ	CROX	AMP	IMI	AZT	Plasmid Profiles (Kb)	Gene/s
KEH3409	0.125	**256**	0.125	**256**	**128**	**256**	**16**	**64**	**128**	0.5	**16**	154; 24; 3	TEM 1, SHV 1, SHV 11
KEH1506	**32**	**256**	16	**256**	**128**	**128**	**32**	**128**	**128**	0.25	**32**	207	TEM 1, SHV 1
KEH2924	16	**256**	16	**256**	**64**	**32**	**32**	**32**	**128**	0.5	**16**	207; 27	TEM 1, SHV1
KEH3217	16	**256**	16	**256**	**128**	**256**	**128**	**256**	**128**	0.5	**64**	207; 59.6; 27; 7	TEM 1, SHV 1, SHV 11
KEH893	16	**256**	16	**256**	16	**64**	**16**	**32**	**128**	0.125	8	110; 7	TEM 1, SHV 2
KEH2786	**32**	**256**	**32**	**256**	**128**	**128**	8	**128**	**128**	0.125	**16**	207; 24; 10; 4; 1.7	TEM 1, SHV 11, CTX-M-15
KEH3281	16	**256**	16	**256**	**128**	**256**	**128**	**256**	**128**	8	**64**	207; 154; 24	TEM 1, SHV 1, SHV 11
KEH2605	2	**256**	0.125	**256**	**128**	**128**	**16**	**128**	**128**	0.125	**32**	207; 36	TEM 1, SHV 1, SHV 11
KEH2787	16	**256**	16	**256**	**128**	**128**	8	**128**	**128**	0.125	**16**	207	TEM 1, SHV 1, SHV 11

AMC – Amoxicillin/Clavulanate, Carb – Carbenicillin, P/T – Piperacillin/Tazobactam, CTHIN – Cephalothin, CRO – Ceftriaxone, CTX – Cefotaxime, CAZ – Ceftazidime, CROX – Cefuroxime, AMP – Ampicillin, IMI – Imipenem, AZT – Aztreonam

Due to financial constraints, a comprehensive analysis of a wide range of enzymes was not possible therefore molecular detection of ESBLs were carried out using representative isolates from groups exhibiting similar phenotypic resistance profiles. This was done by grouping isolates according to the antibiotics they showed resistance to using the MIC values. Sequencing was based largely on the phenotypic expression of the most common ESBL genes, however we included the less common bla_{OXA} and bla_{DHA} as MICs shown in tables 3.1 and 3.2, gave indication of the presence of these genes. The OXA-type β-lactamases confer resistance to ampicillin and cephalothin and are poorly inhibited by clavulanic acid (Bradford, 2001). PCR yielded no positives for bla_{OXA}, even though susceptibility results postulated their presence. This may be the result of the bla_{OXA} genes being absent (Poirel, Naas and Nordmann, 2010). Phenotypic tests for DHA β-lactamases were negative; however PCR for DHA was undertaken based on a study done by Roh, *et al.,* (2008) who reported multi drug resistant *K. pneumonia* isolates harboring both SHV and DHA type ESBLs.PCR and sequencing yielded no positives for DHA β-lactamases.

All isolates sequenced carried the genes encoding both the TEM and SHV β-lactamases. TEM – 1 was identified in all isolates and SHV-1 and SHV-2 were identified in 60 % in the isolates collected in 2000 and 77 % and 11 % respectively in the isolates collected in 2007. These results lead us to believe that there could be an anomaly with the strains in both collection periods as we did not have 100 % SHV-1 present. Since every isolate did express an SHV gene, it was postulated that the SHV-1 gene may have been masked by another SHV gene that was preferentially amplified. The reduced susceptibility to amoxicillin-clavulanate and narrow-spectrum cephalosporins in many of the isolates from 2000, as explained by Dubois *et al.,* (2008), could be due to the acquisition of plasmid-mediated broad-spectrum β-lactamase or overproduction of the chromosomal enzyme of SHV-1.

It has been well documented that bla_{SHV} is present on the chromosome of nearly all *K. pneumonia* isolates (Turner *et al.,* 2009)with the great majority possessing a chromosomal copy of either $bla_{SHV\text{-}1}$ or $bla_{SHV\text{-}11}$ or close relatives (Hammond *et al.,* 2007). There has been strong evidence to suggest that bla_{SHV} originated from the chromosome of *K. pneumoniae* and an IS26 element played a role in the mobilization of bla_{SHV} from genome to plasmid (Harada *et al.,* 2008)SHV – 11 was present in 67% of isolates from 2007 and 55% of those were in combination with SHV – 1. Based on the phenotypic profiles we see in this study we postulate that there are B-lactamases present that sequencing did not reveal.

According to the MICs in tables 3.1 and 3.2 respectively we hypothesize that isolates KEH6 and KEH21 from the 2000 isolates and KEH 2786 from 2007 follow the phenotypic profile of TEM-50. Studies by Sirot, *et al.,* 1997 and Stapleton, Shannon and French, 1999 revealed that the phenotypic profile of TEM -50 displays resistance to Amoxicillin-Clavulanate (AMC) and piperacillin-tazobactam (P/T) and shows susceptibility to Cefotaxime (CTX), Ceftazidime (CAZ) and Aztreonam (AZT). It is possible that TEM-1 found in these isolates could have been preferentially amplified by PCR hence the TEM-50 could not be picked up.

SHV-2 was found in 3 isolates in 2000 and 1 isolate in 2007. The phenotypic profiles of all these isolates however did not match each other accurately. A study by Coque, *et al.,* 2002 revealed a number of different resistance profiles for SHV-2. Many of the isolates were sensitive to cefotaxime, ceftazidime and aztreonam while others showed resistance for the same drugs. Due to this difference in resistance profiles, the authors suggest that plasmid dissemination may have facilitated the spread of the $bla_{\text{SHV-2}}$ gene. The selection pressure from treatment of these antibiotics could have facilitated the evolution of $bla_{\text{SHV-1}}$ via mutation therefore producing a derivative that simultaneously harbors $bla_{\text{SHV-1}}$ and $bla_{\text{SHV-2}}$ (Coque, *et al.,* 2002). Sequencing revealed that isolate KEH2786 possesses CTXM-15; the resistance profile however showed an intermediate result to ceftazidime which is uncharacteristic for CTXM-15 as it usually confers high levels of resistance to ceftazidime. A study by Poirel, Gniadkowski and Nordmann, (2002), revealed that CTXM-15 has a single amino acid change compared to CTXM-3 and analysis of their resistance profiles showed that CTXM-3 confers lower or intermediate levels of resistance to ceftazidime. This suggests that KEH 2786 could very well possess a CTXM-3 enzyme as is indicative by both its intermediate resistance to ceftazidime and the higher susceptibility to inhibition by tazobactam compared with clavulanic acid (Poirel, Gniadkowski and Nordmann, 2002).

Isolate KEH3281 was the only isolate showing an intermediate result to imipenem, sequencing however did not reveal any KPC enzymes. Susceptibility to imipenem is a positive finding in this population as the recognition of carbapenemase expression is the key to appropriate treatment of carbapenem resistant enterobacteriacae. However, due to heterogeneous expression of resistance, it creates difficulties in ascertaining carbapenem resistance (Brink *et al.,* 2012). All of the isolates tested in 2000 and 2007 showed high levels of resistance to carbenicillin which is indicative of the CARB-3 enzyme. At the time of sequencing, however CARB-3 was not included in the list of enzymes but based on the resistance profiles we may hypothesize that CARB-3 is present in this population.

A study by Okesola and Makanjuola, (2009) revealed that poor susceptibility to amoxicillin/clavulanate of the isolates in that environment demonstrates a high probability of the production of new β-lactamases. Inhibitor resistant TEM β-lactamases (IRTs) have been reported globally in many different organisms. IRT-producing isolates remain susceptible to cephalosporins, cephamycins, carbapenems, and in most cases are intermediate or resistant to amoxicillin-clavulanate combinations (Martín *et al.,* 2010). IRTs have been reported by Martín *et al.,* (2010) in *E. coli* isolates with a high degree of IRT diversity. A study done by Mocktar *et al.,* (2009) revealed the presence of two IRT enzymes, viz. TEM 145 and TEM 146 in *E. coli* isolates. It was suggested that single or multiple mutations in the structural genes of β-lactamases may have facilitated the emergence of inhibitor resistant enzymes (Mocktar *et al.,* 2009).

In this study, sequencing did not reveal the presence of IRTs. The resistance profiles however do give us an indication of TEM-50 but this phenotypic approach to the detection of IRTS may have limitations due to the inconsistency of the IRT phenotype (Martín *et al.,* 2010). The presence of other resistance mechanisms such as the TEM-1 and the combination of multiple β-lactamases can often render strains resistant to the β-lactam-β-lactamase inhibitor combinations (Bradford *et al.,* 2004). The IRT phenotype can be masked by the preferential amplification and subsequent sequencing of one enzyme, in this case TEM-1. The absence of chromatograms also creates difficulty in ascertaining the presence of these enzymes. Substitutions in the promoter region of the IRT gene coding region also may affect the expression of IRT enzymes thus affecting the selection of IRT producing isolates (Canton *et al.,* 2008). The poor susceptibility to amoxycillin/clavulanate, 6% in 2000 and 33% in 2007 in these two populations could very well indicate the presence of IRTs, however further studies would provide further information in support of that hypothesis.

Plasmids have been important contributors to the rapid acquisition of antibiotic resistance by pathogenic bacteria through their ability to acquire resistance genetic determinants and to transfer them among bacteria belonging to the same or different genera and species (Bistue *et al.,* 2008). These resistance genes which are usually located inside transposable elements, integrons and insertion sequences (IS) help to facilitate their mobility. The plasmid profiles of the isolates in this study showed extensive diversity and the plasmid banding patterns did not match the resistance profiles of isolates within the same group. Studies have shown that members of a single epidemic strain may carry different plasmids and genotypically non-related strains can produce the same ESBL due to plasmid transfer between species (Paterson and Bonomo, 2005; Bauerfiend *et al.,* 1996 and Fiett *et al.,* 2000). Genes for antibiotic resistance and pathogenicity are usually plasmid-borne and this poses a threat when such

plasmids are conjugative as it allows pathogenicity and resistance genes to disseminate across strains, species and even genera (Yi *et al.,* 2010). Independent evolution due to antibiotic pressure plays a role as well as it has been evident that different plasmids have the ability to mediate the same ESBL (Paterson and Bonomo, 2005; Bradford, 2001). Resistance has increased from 2000 to 2007 with the isolates tested in this study; the banding patterns from 2007 shows a slightly higher genetic diversity which could be the reason for the higher resistance in this population. Zhao *et al.,* (2010) reported an increase in multi-drug resistance in isolates collected over two time frames, the isolates collected from the later time showed a remarkable increase in MDR with a high genetic diversity in the plasmids of the *K pneumonia* strains collected. The authors suggest that bacteria strengthen their antibiotic resistance by frequently exchanging multi-resistance plasmids and by having elevated mutation rates in their plasmids. A high copy number of antibiotic resistance genes have also been attributed to a high level of MDR; integrons have the power to assemble resistance genes in a correct orientation to supply a strong promoter for the expression of these genes (Zhao *et al.,* 2010; Nikaido, 2009). The high mutation rate in plasmids as well as the widespread gene duplication or loss, or lateral gene transfer in the plasmids is an important factor to consider in multi-drug resistance (Zhao *et al.,* 2010).

Vaidya, (2011) reported several cases of multi drug resistant *E. coli* and *K. pneumonia* (MDR-EK) in various studies that showed no link between MDR and previous exposure to antibiotics. Rodrigues *et al.,* (2005) reported a case of 100 healthy executives receiving a routine check-up in Mumbai showed the presence of 11% of ESBL producing *E. coli* and *K. pneumonia* with none of them having been admitted in hospital or having received any antibiotic therapy in the last six months before testing. This is indicative of the role that plasmids play in antibiotic resistance. When the selection pressure of antibiotics is exerted, bacteria already have a large population of resistance genes available to them and this creates an ongoing spread of antibiotic resistance (Vaidya, 2011).

3.2 Antibiotic use and resistance

The introduction of antibiotics into modern medicine has been a very important stepping stone in attempting to circumvent the rate of mortality due to nosocomial infections; however the intensive use of antibiotics has only served to increase the frequency of resistance among many pathogens to date (Andersson and Hughes, 2010). Several studies, as reported by Hawkey, (2008) and Andersson and Hughes, (2010) have investigated the effect of the correlation between the reduction in antibiotic use and the increase in susceptibility. Many

studies demonstrated the effectiveness of antibiotic restriction and an increase in susceptibility however such investigations were carried over 12 or more years.

Table 3.3 The change in overall antibiotic use and susceptibility between 2000 and 2007

Antibiotic Use				Susceptibility		
Antibiotic	**2000**	**2007**	**change**	**2000**	**2007**	**change**
Ampicillin	0.009	0.002	-0.07	0	0	0
Piperacillin sodium injection	0.001	0.001	0.0001	0	0	0
Pipracillin/tazobactam injection	0.097	0	-0.097	80	89	9
Amoxicillin-clavulanate	0.973	0.071	-0.902	40	26	-14
Cefuroxime Sodium Injection	0.043	0.024	-0.019	20	0	-20
Cefotaxime Injection	0.006	0.0059	-0.0001	60	0	-60
Ceftazidime Injection	0	0.0002	0.0002	100	11	-89
Amikacin Injection	0.009	0.013	0.004	100	95	-5
Gentamicin injection	0.001	0	-0.001	0	0	0
Nalidixic acid tablets	0.008	0.001	-0.007	60	11	-49
Ceftriaxone	0.016	0.064	0.048	20	0	-20
Ciprofloxacin	0.032	0.026	-0.006	80	11	-69

Table 3.3 shows the change in susceptibility for 2000 and 2007 as well as the change in antibiotic use. Susceptibility decreased between 2000 and 2007 in eight of the drugs that we tested. Three of the drugs tested, viz. ampicillin, piperacillin sodium injection and gentamicin injection showed no susceptibility in both years. There was only one drug, piperacillin/tazobactam injection where susceptibility increased. The median susceptibility decreased significantly between the two years from 0.5 in the year 2000 to 0.055 in the year 2007, p =0.01 (Table 3.4)

Increase in antibiotic resistance has been largely attributed to the overuse of antimicrobials as well as the transfer of resistance genes via plasmids. The results in table 3.3 indicates that the overall antibiotic use has decreased in 2007 however the susceptibility has decreased. There are other factors to consider in the decrease in susceptibility; firstly, infection control; there are strict prevention and infection control guidelines that are to be implemented in hospital and health care settings. The guidelines strictly promote hand hygiene with health care workers when dealing with patients and their environments as well as implementing patient contact precautions for infected patients (Sydnor and Perl, 2011).

In such settings the compliance to adhere to such measures may be poor which undermines the usefulness of such measures. Owens and Rice, (2006) described various studies which report failure to adhere to infection control policies and the ramifications of such non-compliance which ultimately lead to the dissemination of resistant pathogens.

Such infection control strategies apply to clinical laboratories as well where protocols consistent with CLSI guidelines should be followed with respect to the detection of infectious organisms. Hospitalized patients often undergo certain procedures and invasive manipulations which contribute to colonization and infection with ESBL producing organisms. Pfaller and Segreti, (2006), report a case-control study designed to identify risk factors in acquiring ESBL producing K. pneumonia. They reported that a tracheostomy and the insertion of a Foley catheter, endotracheal tube, nasogastric tube and a central venous catheter were all associated with infection with ESBL-producing *K. pneumonia*. The association was made based on the assumption that patients requiring such invasive manipulations require a longer stay in hospital which renders them susceptible to resistant pathogens. Pena *et al.,* (2001) also reported a case of ICU patients where the infection of ESBL *K pneumonia* was much higher than non-ESBL *K. pneumonia* (96% vs. 46%) due to ICU patients undergoing a larger number of invasive procedures therefore the risk of inappropriate catheter manipulation being greater. Another important factor to consider is surveillance with respect to supply and rational use. In many countries where healthcare is not easily accessible or affordable, many patients seek the assistance of medicine dispensaries whereby self medication without prescription is very common. Surveillance measures need to be put into place for the rational use and availability of antimicrobials.

Table 3.4 Wilcoxon signed rank test for overall Antibiotic use and Susceptibility in 2000 and 2007

	2000			**2007**			
	n	**median**	**IQR**	**n**	**median**	**IQR**	**p value***
Antibiotic Use	12	0.009	(0.0035 - 0.0375)	12	0.004	(0.0006 - 0.0225)	0.12
Susceptibility	12	0.5	(0.1 - 0.8)	12	0.055	(0 - 0.185)	0.01

* using wilcoxon signed rank test

Antibiotic use increased for 4 drugs, viz. amikacin , ceftriaxone, ceftazidime and piperacillin sodium and decreased for the remaining 8 drugs The median use in the year 2000 was slightly higher (median = 0.009) when compared to the year 2007 (median = 0.004) but the difference did not reach statistical significance, p = 0.12.

Many studies could not conclusively provide evidence for an increase in susceptibility despite restricted use of antibiotics and showed still a decrease in susceptibility. With respect to the isolates that were tested in this study, we see a decrease in susceptibility. This can be attributed to the horizontal gene transfer mechanisms mentioned earlier, which microbes make use of to acquire resistance as well as the selective pressure of the antibiotics being used in the time frame between the collection dates of the isolates. Further investigation into this may provide additional evidence to prove the hypothesis with respect to populations in Kwa-Zulu Natal state hospitals.

Table 3.5 The change in grouped Antibiotic Use and Susceptibility for 2000 and 2007

	Antibiotic Use			**Susceptibility**		
Antibiotic	**2000**	**2007**	**change**	**2000**	**2007**	**change**
Decrease in susceptibility						
Increased Antibiotic Use						
Amikacin Injection	0.009	0.013	0.004	100	95	5
Ceftriaxone	0.016	0.064	0.048	20	0	20
Ceftazidime Injection	0	0.0002	0.0002	100	11	89
Decreased Antibiotic Use						
Amoxicillin-clavulanate	0.973	0.071	-0.9021	40	26	14
Nalidixic acid tablets	0.008	0.001	-0.0072	60	11	49
Ciprofloxacin	0.032	0.026	-0.006	80	11	69
Cefuroxime Sodium Injection	0.043	0.024	-0.0189	20	0	20
Cefotaxime Injection	0.006	0.006	-0.0001	60	0	60
No decrease in susceptibility						
Increased Antibiotic Use						
Piperacillin sodium injection	0.001	0.0011	0.0001	0	0	0
Decreased Antibiotic Use						
Piperacillin/tazobactam injection	0.097	0	-0.097	80	89	-9
Ampicillin	0.009	0.002	-0.0069	0	0	0
Gentamicin injection	0.001	0	-0.0006	0	0	0

Table 3.5 shows the comparison of antibiotic use versus susceptibility in groups. The drugs were divided into two groups, those that showed a decrease in susceptibility (n = 8) and those that did not (n = 4). The change in antibiotic use between the years 2000 and 2007 was then calculated for each group in order to determine if change in use differed in the two groups. The change in use for drugs showing no change in susceptibility (median =0.0038) was similar to the change in use for drugs showing a decrease in susceptibility, (median = 0.0035), p = 0.2 (Table 3.6). This lead us to believe that there is co-carriage of resistance genes in the isolates that were tested.

Table 3.6 Wilcoxon signed rank test for the grouped antibiotic use and susceptibility in 2000 and 2007.

Susceptibility	**n**	**median**	**IQR**	**p value***
No change	4	0.0038	(0.003-0.052)	
Decrease	8	0.0035	(-0.0006 - 0.0151)	0.23
Total	12	0.0038	(-0.001 - 0.0151)	

* using wilcoxon signed rank test

For more than two decades, there have been reports on outbreaks caused by gram negative bacilli containing ESBLs and their resistance to cephalosporins as well as other β-lactam antibiotics (Medeiros, 1997). A study by Wiener *et al.,* 1999 uncovered an outbreak in American nursing homes of ceftazidime resistant *K. pneumonia* and *E. coli*. The authors reported that infection with those strains were most often not linked with ceftazidime use and it was possible that the use of trimethoprim-sulfamethoxazole may have selected for ceftazidime-resistant strains because of plasmid linkage of the resistance determinants for these drugs. A later study by Sahm *et al.,* (2001) also reported fluoroquinolone resistance with considerably high levels of ceftazidime resistance in the same isolates.

It has been evident that quinolone resistance was the result of mutations in chromosomal genes coding for targets of quinolone action. However, frequent co-existence of ESBL production and quinolone resistance had been noted (Paterson *et al.,* 2004). Table 3.3 shows evidence of this with the increase in resistance to ceftazidime as well as to the fluoroquinolones with an increase in ceftazidime use but a decrease in fluoroquinolones. Hawkey and Jones, (2009), reported that it is possible that low level plasmid-encoded fluoroquinolone resistance has provided a selective advantage for bacteria exposed to fluoroquinolones to allow the easier selection of high-level resistance mutations in the Gyrase subunit *gyrA*, thus explaining the association of chromosomal quinolone resistance with plasmid-encoded ESBL genes.

An organism that is resistant to multiple drugs is more susceptible to selection by the exposure to even one of those drugs as is evident in our study. Tumbarello *et al.,* (2006) showed significant evidence of this in their population where 50% of the isolates were resistant to fluoroquinolones and were from patients who had been previously undergone antimicrobial treatment. A study by Schwaber *et al.,* (2005), tested four of the most common *Enterobacteriaceae* against ciprofloxacin, gentamicin, amikacin, piperacillin/tazobactam, trimethoprim-sulfamethoxazole and imipenem to compare antimicrobial co-resistance between ESBL producing and non-ESBL producing isolates. High levels of co-resistance were observed (≥ 40%), to all agents with the exception of amikacin and imipenem, in the ESBL producers. The authors suggested that this co-resistance could be a result of transferable elements that confer resistance to antimicrobials other than β-lactams and travel on or alongside ESBL-containing plasmids yielding multi-drug resistant bacteria. Table 3.7 shows the MIC values of the aminoglycosides and fluoroquinolones tested in this study. Many of the isolates collected in 2007 have shown decreased susceptibility to aminoglycosides and fluoroquinolones.

With respect to the resistance to aminoglycosides and fluoroquinolones as well as the resistance to 3rd generation cephalosporins, this study has confirmed the presence of multi-drug resistance in these ESBL- producing strains of *K pneumonia*.

Table 3.7 MIC values (µg/ml) for aminoglycosides and fluoroquinolones tested for both collection dates.

2000	Amikacin	Ciprofloxacin	Gentamicin	Nalidixic Acid
KEH6	0.25	0.125	**64**	8
KEH3	0.5	0.06	**64**	8
KEH100	0.25	0.125	**64**	**128**
KEH21	0.25	0.03	**64**	8
KEH81	0.5	**4**	**64**	**128**
2007	Amikacin	Ciprofloxacin	Gentamicin	Nalidixic Acid
KEH1506	2	**16**	**64**	**128**
KEH893	2	**4**	**64**	16
KEH2605	4	**16**	**64**	**128**
KEH2787	4	**16**	**64**	**128**
KEH2786	2	**16**	**64**	**128**
KEH2924	1	0.25	**64**	**128**
KEH3217	8	**32**	**64**	**128**
KEH3281	**32**	**32**	**64**	**32**
KEH3409	16	**4**	**64**	**128**

Figure 3.2 is an illustrative comparison of the increase in resistance to aminoglycosides and fluoroquinolones between the two collection periods. Due to resistance being carried on a variety of resistance determinants that may be integrated on plasmids of different sizes, this creates difficulty in curbing the spread of resistance by just controlling the use of antimicrobials.

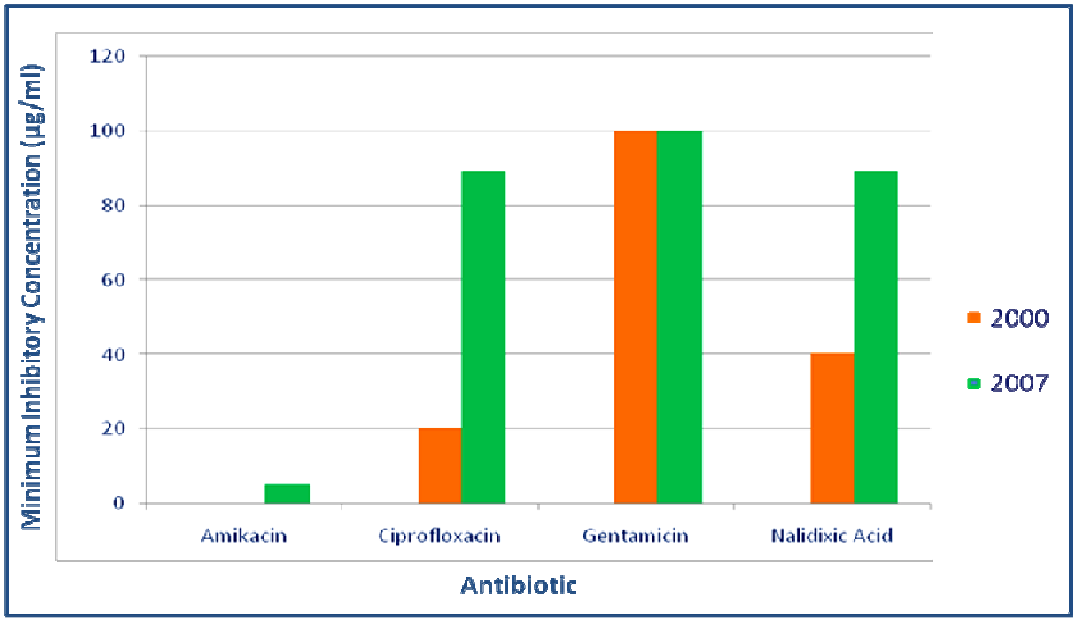

Figure 3.2 Illustration of the increase in resistance to aminoglycosides and fluoroquinolones over the two collection periods

Various modes of dissemination of MDR has been described and attributed to plasmid spread and gene transfer via integrons however it is very likely that dissemination does occur as a result of antibiotic selection pressure (Wener *et al.*, 2010). There have been reports from a few studies which suggest a positive association between antimicrobial consumption and bacterial resistance, however there are many studies that claim that these associations are not evident. The association between antibiotic consumption and resistance is frequently seen at a patient level in case control studies, and also at a hospital and community level, the same relationship can be seen with susceptibility where the reduced use of one agent is associated with increased use of another (Gould, 2009). A study by Bergman *et al.*, (2009) investigated the association between antimicrobial resistance and consumption. There was no direct correlation between resistance and overall consumption but they did report a few significant relationships between resistance and certain antimicrobials such as nitrofurantoin use and nitrofurantoin resistance as well as amoxicillin use and fluoroquinolone resistance. There was however no correlation between fluoroquinolone use and fluoroquinolone resistance. A study by van de Sande-Bruinsman *et al.*, (2008), however did find a strong correlation between fluoroquinolone use and fluoroquinolone resistance which they attribute to mutations in the chromosomal genes resulting in a modification of the molecular target which eventually leads to complete resistance.

A study undertaken by Hsu *et al.,* (2010) reported no significant correlation between antimicrobial use and resistance. One of the reasons suggested by the author was the limitation of using the DDD for antibiotic use data. Resistance selection pressure occurs at the individual level and the DDD does not take into account the individual exposure to antimicrobial treatment as it will vary from patient to patient. It is mainly patients who are susceptible to drug resistant pathogens that receive broad spectrum antibiotics therefore the DDD presents inherent biases based on the factors taken into account to calculate DDD (Hsu *et al.,* 2010). A study by Lai *et al.,* (2011) reported non-uniform relationships with the usage and resistance in their bacterial isolates which was monitored in various hospitals over a period of ten years. The suggested reasons had been the limitation of using the DDD as well as the co-resistance between different classes of antibiotics that were not taken into account.

In light of both arguments however, emphasis must be placed on the rational and sensible use of all antimicrobial agents. It is imperative that antibiotic consumption and the relative rate of antimicrobial resistance are monitored. Infection control measures in hospitals and in the community need to be monitored and strict compliance should be implemented in order to reduce the dissemination of these resistance strains. There needs to be specific attention paid to the accurate detection of ESBL producers, their treatment strategies and infection control policies. These concerns are of the utmost importance in helping reduce this growing epidemic.

CHAPTER FOUR

CONCLUSION, LIMITATIONS AND FURTHER STUDIES

4.1 Conclusion

Many researchers have stressed that bacteria are thought to have evolved 3500 billion years ago and the use of antimicrobials have only been put into effect for about 60 years now, thus leading to the argument that bacteria such as *K. pneumonia* have had more time to adapt to the various conditions in their environment. It has been said by Spellburg *et al*, (2008) that their genetic plasticity and the rapid rate of their replication has given bacteria an immeasurable advantage over even modern medicines constant attempts to curtail the problem of increasing resistance.

The two small bacterial populations in this investigation along with numerous studies done over many years provide proof to this claim. There has been a decrease in susceptibility over the two time frames and although there were no novel β-lactamases identified in this study, we have identified a trend with a decrease in susceptibility over time further proving bacterial evolution. This study presented us with a variety of β-lactamases and a diversity of plasmid profiles and by extension genetic determinants in which they appear to reside has demonstrated the ease of dissemination.

Improvements in prescribing β-lactams has been recommended as a way to reduce the high levels of resistance over the years, however, in this study it was observed that resistance increased despite only a slight increase in the use of a few antibiotics to which we attributed co-carriage of resistance genes. It is certainly advantageous to use antibiotics only when suitable, to try to limit the selective pressure that increases the frequency of resistance. On the other hand, a distinction between an increase in antibiotic usage causing the increase in bacterial resistance or the rate at which resistance spreads (Andersson and Hughes, 2010; Spellburg *et al*, 2008) must be recognized if a solution is to be created to solve the problem of antibiotic resistance.

4.2 Limitations

The full gamut of potential genetic determinants of resistance such as integrons, gene cassettes and insertion sequences were not investigated.
Definitive identification of ESBLs were restricted to a few classes because of financial constraints and the full range of putative ESBLs were thus not investigated.
The acquisition of control strains was precluded by financial constraints

4.3 Further Studies

PCR and sequencing have proven to very complex and expensive in routine genotypic detection of ESBL genes. High Resolution Melt Analysis (HRMA), a fairly recent technique is a simple solution for genotyping, mutation scanning as well sequence matching (Reed *et al*, 2007). HRMA is being increasingly applied to identify resistance mutations in various organisms with a wide range of antimicrobials (Hidalgo-Grass and Strahilevitz, 2010). The use of such a tool will prove very useful to detect resistance easily in the clinical laboratory

APPENDIX ONE

1. **1M Tris Solution pH 7.0**

   To make 1 litre:

   - 121.1 g Tris base
   - 800 ml sterile distilled water
   - Dissolve Tris in sterile distilled water and adjust the pH with concentrated HCl:
   - Bring to 1 litre with sterile distilled water

2. **Sodium Dodecyl Sulphate (SDS) solution**

   A 6% SDS solution was prepared as follows:

   - 6g of Sodium dodecyl sulphate (Sigma Aldrich)
   - 100ml Sterile distilled water

3. **10X TE (Tris-EDTA) Buffer**

   To make 1 litre:

   - 100 ml 1 M Tris-HCl pH 7.5
   - 20 ml 500 mM EDTA pH 8.0 (Fluka)
   - 880 ml sterile distilled water

4. **10X TAE Buffer**

   To make 1 litre:

   - 48.4 g of Tris base [tris(hydroxymethyl)aminomethane]
   - 11.4 mL of glacial acetic acid (17.4 M)
   - 3.7 g of EDTA
   - Sterile distilled water

5. **50 mM Tris: 3% SDS solution**

   - Equal volumes of 50mMtris(hydroxymethyl)aminomethane solution and sodium dodecyl sulphate solution were mixed to yield a final solution of 50 mM tris (pH:8): 3% SDS. (1:1) solution

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