DECLARATION

This study represents the original work of the candidate and has not been submitted in any form to another university.

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ABSTRACT

*Escherichia coli*, one of the most common pathogens causing urinary tract infections, has shown increased resistance to commonly used antibiotics. In this study we analyzed the β-lactamase profiles of 38 inhibitor-resistant *E. coli* isolates obtained from public hospitals at three different levels of healthcare in KwaZulu-Natal, selected on the basis of their resistance profiles to the three antibiotic/inhibitor combinations, viz., amoxicillin/clavulanate, ampicillin/sulbactam and piperacillin/tazobactam. The isolates were subjected to MIC determinations, IEF analysis, plasmid profile analysis, PCR of the different β-lactamase genes and sequencing thereof to detect the possible mechanism/s of resistance. A range of β-lactamases including two novel inhibitor-resistant TEM β-lactamases, TEM-145 and TEM-146 were detected in two isolates whilst a novel plasmid-mediated AmpC-type β-lactamase, CMY-20 was detected in three isolates. Other β-lactamases included OXA-1, TEM-55, SHV-2, CTX-M-1 and TEM-1. Changes were detected in the chromosomal AmpC promoter/attenuator regions in one isolate. Diverse β-lactamase genes and plasmid profiles inferred extensive mobilization of β-lactamase genes causing the concern of limited therapeutic options in the face of increasing resistance.
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CHAPTER ONE

INTRODUCTION
1.1. β-LACTAM ANTIBIOTICS

β-lactam antibiotics, embracing penicillins with or without β-lactamase inhibitors, cephalosporins, cephemycins, oxycephamycins, monobactams, carbapenems, penems and carbacephems, are the most frequently prescribed antimicrobial agents in clinical practice (Canica et al., 1998; Edwards & Betts, 2000). Starting with the discovery of benzylpenicillin in 1928 and its first clinical use in 1940, numerous natural and synthetic compounds have been described based upon the active structure of penicillin, i.e., the β-lactam ring. The penicillins and cephalosporins which are based on 6-aminopenicillanic acid and 7-aminoccephalosporanic acid respectively, are the two classical β-lactam families. In addition, various non-classical β-lactams have been developed, including monocyclics and carbapenems. The success of β-lactams may be attributed to low toxicity and the availability of so many derivatives (Livermore & Williams, 1996).

Antibiotic resistant pathogenic bacteria has become a major problem, especially amongst Gram-negative bacteria causing hospital infections (Silva et al., 1999; Wiener et al., 1999). Resistance has been caused by the widespread use, and sometimes abuse, of broad-spectrum antibiotics not only in medical practice (Günsören et al., 1999; Matagne et al., 1999) but also in animal husbandry (Matagne et al., 1999). The three main mechanisms to antibiotic resistance are:

- Decreased permeability of the drug into the cell by the loss or alteration of outer membrane proteins (Omps) (Heritage et al., 1999; Silva et al., 1999).
- Decreased affinity of the target penicillin binding proteins (Silva et al., 1999; Edwards & Betts, 2000).
• Production of β-lactamases that hydrolyze the β-lactam bond in the antibiotics, thus destroying its functionality (Heritage et al., 1999; Kuzin et al., 1999; Silva et al., 1999; Vakulenko et al., 1998). These are either chromosomal enzymes that can be over-expressed (derepressed) or encoded by plasmids (Edwards & Betts, 2000).

The most common mechanism for resistance in Gram-negative bacteria is the expression of β-lactamases (Pitout et al., 1998; Silva et al., 1999; Vakulenko et al., 1998). Two strategies have been devised to counter the resistance of bacteria, viz., the development of new β-lactam drugs that are inherently less susceptible to β-lactamases and the combination of mechanism based inactivators and a penicillin (Chaibi et al., 1999).

1.2. ESCHERICHIA COLI

Escherichia coli belongs to the family Enterobacteriaceae, which is a large, heterogeneous group of Gram-negative rods. Their natural habitat is the intestinal tract of humans and animals (Brooks et al., 2001). *E. coli*, one of the most frequently isolated nosocomial pathogens (Yagi et al., 1997; Clarke et al., 2003) is also the most common cause of urinary tract infections (Brooks et al., 2001). Other diseases caused include meningitis, septicaemia, wound infections, pneumonia, dysentery and diarrhea (Bhatia & Ichhpujani, 2004). *E. coli* isolates causing diarrhea are classified by the characteristics of their virulence properties into the following classes, viz., enteropathogenic *E. coli* (EPEC) (causes diarrhea in infants especially in developing countries), enterotoxigenic *E. coli* (ETEC) (common cause of ‘travelers’ diarrhea’ and an important cause of diarrhea in infants in developing countries), enterohaemorrhagic *E. coli* (EHEC) (associated with
haemorrhagic colitis, a severe form of diarrhea), enteroinvasive *E. coli* (EIEC) (similar to shigellosis) and enteroaggregative *E. coli* (EAEC) (causes acute and chronic diarrhea) (Brooks *et al.*, 2001).

1.3. β-LACTAMASES

Analysis of known β-lactamase sequences has resulted in them being divided according to their amino acid sequences into four classes, designated A to D (Poirel *et al.*, 1999). Class A β-lactamases are of importance since they exhibit highly variable substrate profiles and are generally encoded by plasmids, which facilitate transfer between cells (Ibuka *et al.*, 1999). The class A enzymes which were originally designated penicillinases because they hydrolyzed penicillins with occasional weak activity towards cephems, include many of the plasmid-mediated enzymes from Gram-negative bacteria and chromosomal enzymes from Gram-positive bacteria. Class B β-lactamases are the metalloenzymes and exhibit a very large spectrum of activity. These plasmid-encoded enzymes are not sensitive to the common β-lactamase inactivators but always hydrolyzed carbapenems, a family of β-lactams that is not susceptible to most class A, C and D enzymes. Class C β-lactamases include the chromosomal cephalosporinases of Gram-negative bacteria and confer resistance not only to classical compounds such as ampicillin and cephalothin but also to third generation cephalosporins (Matagne *et al.*, 1999). Class D β-lactamases are unique enzymes with the ability to hydrolyze oxacillin, a semi-synthetic penicillin stable to many β-lactamases (Kuzin *et al.*, 1999).
Amongst the four β-lactamase classes, the class A enzymes are the most frequently encountered in clinical isolates because of plasmid-mediated selection and transfer in response to the introduction of new β-lactams. “Extended-spectrum” class A β-lactamases are variants of a parental type and have 1-5 amino acid substitutions for better hydrolysis of second and third generation cephalosporins (Rasheed et al., 1997; Gniadkowski et al., 1998; Kuzin et al., 1999).

The following is a synopsis of β-lactamases encountered in E. coli, a species traditionally susceptible to most β-lactam antibiotics:

1.3.1. INHIBITOR-RESISTANT β-LACTAMASES (IRTs)

β-lactamase inhibitors protect the β-lactams from hydrolysis by the β-lactamases when the inhibitors are used in combination with β-lactams. Three β-lactamase inhibitors, clavulanic acid, sulbactam and tazobactam, are available for clinical use in combination with a number of penicillins (Vakulenko et al., 1998; Doi et al., 2004). The inhibitors mainly target Ambler class A β-lactamases and inactivate their active site serines, thus potentiating the actions of β-lactamase-sensitive compounds (Doi et al., 2004).

Amoxicillin/clavulanate is a β-lactam-β-lactamase inhibitor combination that has antimicrobial activity against Gram-positive, Gram-negative and anaerobic organisms, but resistance has emerged with increasing use of this antimicrobial agent. (Kaye et al., 2004). Resistance to amoxicillin/clavulanate appeared first in Escherichia coli isolates, then in other species of enterobacteria (Speldooren et al., 1998) and could be attributed to
the hyperproduction of the chromosomal class C β-lactamase, hyperproduction of TEM-1 β-lactamase, production of oxacillinases, the production of TEM-derived enzymes whose β-lactamases activities are longer inhibited clavulanate (IRTs) and modification of the outer membrane proteins (Henquell et al., 1994; Sirot et al., 1994; Speldooren et al., 1998; Chaibi et al., 1999; Leflon-Guibert et al., 2000; Miro et al., 2002).

IRT β-lactamases have been isolated since the beginning of the 1990s and are mostly produced by strains of *E. coli* but also by clinical isolates of *Klebsiella* spp and *Proteus mirabilis*. These enzymes confer resistance to penicillins and their combinations with β-lactamase inhibitors. IRT variants are derivatives of TEM-1 and TEM-2 β-lactamases and differ from the parental enzymes by various combinations of amino acid substitutions (Goussard & Courvalin, 1999). Mutations at amino acid positions 69, 130, 244, 275 and 276 (according to Ambler’s numbering) that have been shown or postulated to play a role in determining resistance to inhibitors (Alonso et al., 2002; Vakulenko & Golemi, 2002). These substitutions decrease the affinity for β-lactams and alter the way in which the enzymes interact with suicide inactivators (Alonso et al., 2002).

1.3.2. EXTENDED-SPECTRUM β-LACTAMASES (ESBLs)

In Gram-negative pathogens, β-lactamases remain the most common cause of β-lactam resistance, and their increasing prevalence, as well as their extensive evolution, appears to be directly linked to the clinical use of sub-classes of β-lactams (Pitout et al., 2005). The introduction of expanded-spectrum cephalosporins in clinical practice represented a major breakthrough for the treatment of nosocomial infections caused by
Enterobacteriaceae producing broad-spectrum β-lactamases such as the widespread TEM-1, TEM-2 and SHV-1 enzymes (Perilli et al., 2002). However, the extensive use of these drugs was rapidly followed by the emergence of resistance (Perilli et al., 2002; Graffunder et al., 2005). ESBLs are able to hydrolyse broad-spectrum cephalosporins and monobactams which are stable to classic TEM and SHV enzymes (Jacoby & Han, 1996; Yang et al., 1998; Girlich et al., 2001; Lee et al., 2004; Briñas et al., 2005), as well as to the older penicillins and cephalosporins (Jacoby & Han, 1996) while remaining susceptible to β-lactamase inhibitor combinations, cefamycins and carbapenems (Graffunder et al., 2005; Pitout et al., 2005).

The genes encoding extended-spectrum β-lactamases (ESBLs) are usually carried by multi-drug resistance plasmids which are readily transferable and which may carry genes encoding resistance to other antibiotics such as aminoglycosides (Yang et al., 1998; Silva et al., 1999; Mulvey et al., 2004). These plasmids strongly facilitate the spread of enzymes among strains of many species of Gram-negative bacteria but Klebsiella pneumoniae and E. coli remain the most frequently reported producers of ESBLs. Since the first ESBL-expressing isolate was reported in a K. pneumoniae isolate in Germany in 1983 many kinds of ESBLs have been characterized and described. (Gniadkowski et al., 1998; Girlich et al., 2001; Mulvey et al., 2004) and infections caused by ESBL-producing members of the family Enterobacteriaceae have rapidly increased (Girlich et al., 2001).
ESBLs have been described world wide and are a major cause of nosocomial infections associated with high mortality (Gray et al., 2006). They exhibit high degrees of diversity in their structures and activities and several families have been identified on the basis of their evolutionary and/or functional similarities (Gniadkowski et al., 1998). Most ESBLs are derived from the classical TEM-1, TEM-2 and SHV-1 enzymes, by amino acid substitutions in their sequences (Spanu et al., 2002; Briñas et al., 2005; Delmas et al., 2005; Robin et al., 2006), which extend the substrate specificities to extended-spectrum cephalosporins (Delmas et al., 2005).

1.3.2.1. TEM

The TEM family of ESBLs constitutes the largest and widely disseminated group of these enzymes in nosocomial isolates of Enterobacteriaceae worldwide and their dissemination continues to compromise antimicrobial chemotherapy (Perilli et al., 2002; Baraniak et al., 2005).

TEM-1 is able to hydrolyze penicillins and cephalosporins such as cephalothin. TEM-2 differs from TEM-1 by substitution of lysine for glutamine at position 37 ((Philippon et al., 1989) but its substrate profile remains very similar to that of TEM-1 (Bradford, 2001). The first outbreak of a TEM-derived ESBL, TEM-3, was reported in 1989 in France (Morris et al., 2003).

The TEM-type ESBLs show a reduced catalytic efficiency towards penicillins and a broadened spectrum towards expanded-spectrum cephalosporins (Perilli et al., 2002). The
amino acid substitutions that occur within the TEM-enzyme occur at a limited number of positions. The combinations of the amino acid changes results in alterations in the phenotype of the enzyme (Bradford, 2001). In TEM-type ESBLs, the most significant amino acid substitutions occur at positions 104 (glutamic acid to lysine), 164 (arginine to serine or histidine), 238 (glycine to serine) and 240 (glutamic acid to lysine) (Bradford, 2001; Baraniak et al., 2005; Ikryannikova et al., 2008). When the glutamic acid at position 104 is substituted by lysine, the long side chain of lysine could interact with the carboxylic acid group of oxyimino-cephalosporins such as ceftazidime. This electrostatic attraction would increase the initial binding, possibly increasing the \( \beta \)-lactamase affinity (lowering the \( K_m \)) and increasing \( V_{\text{max}} \) values, but only in conjunction with other mutations such as Arg-164 (Knox, 1995; Petit et al., 1995). The high catalytic efficiency against oxyimino-\( \beta \)-lactams such as ceftazidime and cefotaxime is linked to the substitution of arginine at position 164 and glycine at position 238 (Maveyraud et al., 1996). The Arg-164-Ser substitution reported in TEM-12 allows this enzyme to hydrolyze ceftazidime more effectively than cefotaxime (Morosini et al., 1995). X-ray structural studies have shown that in the TEM-1 enzyme, at position 164, the arginine is below the binding site of the omega loop (positions 162-179) that contains the catalytic Glu-166 and the guanidium side chain is strongly linked by electrostatic interaction and hydrogen bonds to Asp-179 which lies across the neck of the loop. Substitution of the arginine at position 164 is one of the most common substitutions observed in TEM ESBLs. When the arginine is replaced by either serine or histamine, it results in one less hydrogen bond donor which can weaken the linkage across the neck of the omega loop. This allows for more flexibility in the loop and this creates more space for bulky \( \beta \)-lactam
constituents (Knox, 1995). Site-directed mutagenesis studies showed improved $k_{cat}$/$K_m$ for oxyiminocephalosporins such as ceftazidime and cefotaxime (Sowek et al., 1991). Substitution of the glycine at position 238 with serine extends the substrate spectrum of the TEM-enzyme to include ceftazidime (Maveyraud et al., 1996). The side chain of the amino acid at position 238 is on the inner side of the B3 $\beta$-strand. It lies very close to the side chain of the amino acid at position 69; therefore there is a correlation between the sizes of the side chains of each amino acid. If the side chain of both the amino acids at positions 69 and 238 are large, there is crowding and this may displace the B3 $\beta$-strand outward. This results in the lower portion of the binding site becoming slightly expanded, resulting in cephalosporins with rigid branched acyl-amido substituents being able to form hydrogen bonds with residue 237. This could result in lower $K_m$ values. When the glutamic acid is replaced by lysine at position 240, the side chain of lysine is able to form an electrostatic bond with the carboxylic acid group of the oxyimino cephalosporin. This results in hydrolysis of ceftazidime and aztreonam. TEM-24 has the amino acid at position 104 and 240 substituted with lysine and this enzyme has the highest $V_{max}$ for ceftazidime (Knox, 1995).

1.3.2.2. SHV

SHV-type $\beta$-lactamases are one of the most clinically significant families of plasmid-mediated $\beta$-lactamases (Kim & Lee, 2000) and may be found amongst many members of the family Enterobacteriaceae (Heritage et al., 1999). SHV-1 is a narrow spectrum $\beta$-lactamase with activity against penicillins (Ma et al., 2005). Point mutations of the genes encoding for SHV-type $\beta$-lactamases can change their profile of activity to include most
β-lactams with the exception of cephemycins and carbapenems (Kim & Lee, 2000). The first ESBL, derived from SHV-1 was reported in 1983 and was named SHV-2 (Morris et al., 2003). The serine at amino acid position 238 was replaced by glycine in SHV-1 and this amino acid allowed for more efficient hydrolysis of cefotaxime than ceftazidime (Kim & Lee, 2000; Hujer et al., 2002). The amino acid substitution at position 238 causes displacement of the B3 β-strand in the SHV-enzyme and this causes a slight opening of the active site, thus allowing entrance of the bulkier oxyimino cephalosporins (Randegger et al., 2000). In SHV-5 there is an amino acid change at position from glutamic acid (SHV-2) to lysine (SHV-5) and this mutation was found to increase the hydrolytic activity of this enzyme against ceftazidime and aztreonam rather than cefotaxime (Bradford et al., 1995; Kim & Lee, 2000). Position 240 of the SHV-enzyme is at the bottom of the β-pleated sheet and when the lysine at this position is replaced with a negatively charged amino acid such as glutamic acid, it attracts and binds the terminal carboxyl group on the long oxyimino side chains of ceftazidime and aztreonam (Heritage et al., 1999).

1.3.2.3. CTX-M

The emergence of new enzyme groups that have a typical ESBL resistance phenotype but that are non-TEM and non-SHY derivatives have been reported. CTX-M-type β-lactamases constitute a group of enzymes encoded by transferable plasmids (Eckert et al., 2004). This family of plasmid-mediated ESBLs has been classified in Ambler class A (Bonnet et al., 2000). They are capable of hydrolyzing broad-spectrum cephalosporins (Eckert et al., 2004) and are inhibited by clavulanic acid, sulbactam and tazobactam (Ma
et al., 2002; Eckert et al., 2004). CTX-M β-lactamases have emerged in many countries of the world (Edelstein et al., 2003; Cartelle et al., 2004). Since the first report of CTX-M ESBL from a strain of E. coli in 1989, the CTX-M family of ESBLs has spread in several countries in Europe, Latin America, Africa and Asia (Kiratisin et al., 2007). The first organisms producing β-lactamases of this type were identified both as single and epidemic clinical isolates in distant geographic regions (Germany, France, South America) in the early 1990s (Edelstein et al., 2003).

CTX-M enzymes can be subclassified by amino acid sequence similarities into various clusters, viz., CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25, named after the enzyme first discovered for each lineage (Edelstein et al., 2003; Pagani et al., 2003; Bonnet, 2004; Eckert et al., 2004; Mugnaioli et al., 2006). Within each cluster there is a high degree of blaCTX gene sequence identity (>95%), although members of different subtypes share only 70% to 77% similarity at nucleotide level. The finding of Kluyvera ascorbata species-specific β-lactamases (KluA) which share 98.6 to 100% identity with CTX-M-2 and CTX-M-5 and analysis of DNA sequences adjacent to the KluA- and CTX-M-coding genes provide strong evidence of the direct evolution of the CTX-M-2 cluster from the chromosomal enzyme of K. ascorbata (Baraniak et al., 2002; Edelstein et al., 2003). Likewise, the chromosomal KLUG-1 enzyme of Kluyvera georgiana was found to be the most probable source of the CTX-M-8 that shares 99% identity with KLUG-1 (Edelstein et al., 2003; Pagani et al., 2003). The origin of the other CTX-M clusters are not so evident yet, although a number of class A chromosomal β-lactamases, including KLUC-1 of Kluyvera cryocrescens, FONA of Serratia fonticola, RAHN-1 of
*Rahnella aquatilis*, OXY-1 of *Klebsiella oxytoca* and SED-1 of *Citrobacter sedlakii* display partial (72.9 to 85.9%) homology with different CTX-M enzymes (Bonnet *et al.*, 2000; Edelstein *et al.*, 2003).

In contrast with TEM- and SHV-type ESBLs, most of the CTX-M enzymes are much more active against cefotaxime and ceftriaxone than against ceftazidime displaying significantly higher levels of resistance to cefotaxime compared with ceftazidime (Baraniak *et al.*, 2002; Ma *et al.*, 2002; Edelstein *et al.*, 2003; Woodford *et al.*, 2004).

However, the classical phenotype of resistance conferred by CTX-M β-lactamases is not universal among all CTX-M producers, since many factors, including production of additional β-lactamases or mutations altering the substrate specificity of CTX-M enzymes can mask their presence (Edelstein *et al.*, 2003). ESBLs belonging to this type, named cefotaximases (CTX-M), have caused outbreaks of cefotaxime-resistant enterobacteria mainly in South America, Eastern Europe and Japan (Oliver *et al.*, 2001).

When first reported in literature, ESBL-producing organisms were found in nosocomial isolates from large metropolitan hospitals. Today, ESBLs have been identified worldwide, not only from major teaching hospitals but also from community hospitals and nursing homes. The rapid spread of ESBLs has caused significant threats to the therapy for infections and usage of expanded-spectrum β-lactams (Yang *et al.*, 1998).
1.3.3. AMPC β-LACTAMASES

This pattern of resistance to both cefoxitin and β-lactamase inhibitors coupled with reduced susceptibility to third generation cephalosporins is often referred to as the “AmpC phenotype” (Forward et al., 2001). The emergence of E. coli strains resistant to extended-spectrum cephalosprins and cephemycins is a major concern to clinicians (Clarke et al., 2003). Strains have become resistant by overexpression of chromosomally-encoded AmpC β-lactamases or by acquisition of a transferable ampC gene on a plasmid or other transferable element. The transferable ampC gene products are commonly called plasmid-mediated AmpC β-lactamases (Perez-Perez & Hanson, 2002; Clarke et al., 2003) and the majority is found in nosocomial isolates of E. coli and K. pneumoniae (Perez-Perez & Hanson, 2002). Plasmid-mediated ampC genes are derived from the chromosomal ampC genes of several members of the family Enterobacteriaceae, including Enterobacter cloacae, Citrobacter freundii, Morganella morganii and Hafnia alvei (Forward et al., 2001; Perez-Perez & Hanson, 2002). In E. coli, resistance provided by class C β-lactamases can be plasmid-mediated or due to overexpression of the chromosomal ampC gene (Tracz et al., 2005). Chromosomally encoded and plasmid-encoded AmpC β-lactamases, with the exception of ACC-1, confer resistance to many β-lactams including cephamycins and β-lactam β-lactamase inhibitors (Lee et al., 2004).

1.3.3.1. CHROMOSOMAL AMPC β-LACTAMASES

The AmpC cephalosporinase gene in E. coli is normally located on the chromosome and is weakly expressed because of a weak promoter and a weak transcriptional attenuator. Wild-type strains produce a basal level of this enzyme which does not result in ampicillin
and cephalosporin resistance. This enzyme can be hyper-produced due to genetic variation. Although AmpC β-lactamases are responsible for antibiograms similar to ESBLs, they are poorly inhibited by β-lactam β-lactamase inhibitor combination (Siu et al., 2003). The production of the AmpC β-lactamase in *E. coli* is not inducible, as are most class C cephalosporinases, but is constitutive because of the absence of the *ampR* regulator gene (Caroff et al., 2000; Corvec et al., 2002) and the level of transcription of the *ampC* gene thus depends mostly on the strength of the *ampC* promoter (Corvec et al., 2002).

Strains carrying the wild-type gene produce a low basal amount of AmpC and are susceptible to ampicillin (Mulvey et al., 2005). Mutations in the promoter region of *ampC* have been described as the mechanism for AmpC hyper-production (Tracz et al., 2005). Numerous mutations have been described in the *ampC* promoter of clinical strains hyperproducing a chromosomal cephalosporinase. The most frequently described *E. coli* *ampC* ‘strong’ promoter harbours mutations at positions -88, -82, -42, -18, -1 and +58 (Caroff et al., 2000). *E. coli* promoters harbour two hexamers of conserved sequences, the -35 region and the -10 one, called the Pribnow box, which play an important role in the level of gene transcription. The -35 consensus sequence TTGACA and the -10 consensus sequence TATAAT have been defined. The closer to the consensus the sequences are, the stronger the promoter. The interbox distance also plays an important role in the transcription rate. Ideally it is 17bp long (Corvec et al., 2002). Attenuator mutations are thought to destabilize the hairpin structure allowing for greater read through (Tracz et al., 2005).
Although poorly expressed in *E. coli*, these enzymes are important to recognize since they provide an even broader spectrum of resistance (Alvarez *et al.*, 2004).

### 1.3.3.2. PLASMID-MEDIATED AMPC β-LACTAMASES

Plasmid-mediated AmpC β-lactamases, first reported in 1988 (Mulvey *et al.*, 2005), have arisen through the transfer of chromosomal genes for the inducible AmpC β-lactamase onto plasmids (Thomson, 2001; Mulvey *et al.*, 2005). These plasmid-mediated genes are of special interest because their mobility allows them to emerge in one genus or species and spread to different organisms (Mulvey *et al.*, 2005). This transfer has resulted in plasmid-mediated AmpC β-lactamases in isolates of *E. coli, K. pneumoniae, Salmonella* spp, *C. freundii, E. aerogenes* and *P. mirabilis*. Most of the plasmid-mediated AmpC β-lactamases have similar substrate profiles to the parent enzymes from which they appear to be derived (Thomson, 2001). Plasmid-mediated AmpC β-lactamases have been derived from chromosomal AmpC genes of *C. freundii* (CMY-2A, LAT-1 and BIL-1) (Odeh *et al.*, 2002; Batchelor *et al.*, 2005), *E. cloacae* (MIR-1) and *P. aeruginosa* (MOX-1 and FOX-1) (Odeh *et al.*, 2002).

### 1.4. MOTIVATION FOR STUDY

Various classes of β-lactamases have been reported throughout the world in *E. coli* including TEM- (Kaye *et al.*, 2004), SHV- (Rasheed *et al.*, 1997), CTX-M- (Woodford *et al.*, 2004) and plasmid-mediated AmpC-type β-lactamases (Philippon *et al.*, 1989; Gazouli *et al.*, 1996; Bou *et al.*, 2000; Girlich *et al.*, 2000; Yan *et al.*, 2006). Resistance
to the β-lactam antibiotics amongst *E. coli* clinical isolates is an increasing problem world-wide. The development of β-lactamase mediated resistance in *E. coli* has several clinical implications (Clarke *et al*., 2003).

This study investigated β-lactamase mediated resistance in *E. coli* isolated in 16 demographically representative public hospitals at three levels of health care in KwaZulu-Natal. A total of 1270 bacterial isolates were collected; of which 300 (23.6%) were *E. coli* isolates. One hundred and seventy-nine of these isolates were resistant to one of the three antibiotic-inhibitor combinations used in this study; 38 isolates were selected on the basis of their resistance profiles to the three antibiotic/inhibitor combinations, viz., amoxicillin/ clavulanate, ampicillin/ sulbactam and piperacillin/ tazobactam.

1.5. AIM

To phenotypically and genotypically characterize β-lactamase mediated resistance in *E. coli* isolated in state hospitals in KwaZulu-Natal.

1.6. OBJECTIVES

1. To identify the bacterial isolates using the API20E identification system.
2. To determine antibiotic susceptibility using Kirby Bauer disc diffusion method following CLSI guidelines against a range of β-lactam antibiotics.
3. To detect the presence of ESBLs using appropriate ESBL E-test strips.
4. To detect the presence of β-lactamases by iso-electric focusing (IEF).
5. To detect the presence of plasmids encoding resistance using the alkaline lysis method.

6. To detect the presence of β-lactamase genes such as TEM-, SHV-, CTX-M- and plasmid-mediated AmpC β-lactamases and changes in chromosomal AmpC of *E. coli* by PCR using appropriate primers.

7. To identify the genes detected by PCR reactions by DNA sequencing.
CHAPTER TWO

SCIENTIFIC PAPERS
2.1. PAPERS PUBLISHED

The following papers were published in journals:


TEM-145 and TEM-146 β-lactamases produced by Escherichia coli isolates from state hospitals in KwaZulu-Natal, South Africa

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Full Length Research Paper

Two Escherichia coli isolates which were isolated from the urine of patients in state hospitals in KwaZulu-Natal, South Africa were investigated to determine the sequence of the TEM β-lactamases responsible for their resistance to β-lactamase inhibitors. The isolates were subjected to MIC determinations, iso-electric focusing analysis, plasmid analysis, polymerase chain reaction (PCR) for the detection of β-lactamase genes and sequencing of the blaTEM. Analysis of the nucleotide sequences revealed the presence of two novel TEM β-lactamases, TEM-145 and TEM-146 which had the R244H mutation. Mutations at position 244 have been previously reported in other inhibitor-resistant TEMs (IRTs).

Key words: Escherichia coli, plasmid-mediated, TEM β-lactamase.

INTRODUCTION

β-lactam antibiotics are the most frequently prescribed antimicrobial agents in clinical practice (Canica et al., 1998; Pitout et al., 2005). In response, bacteria have acquired resistance to these antibiotics. In parallel with molecular modifications introduced to overcome this resistance, bacteria have modified their resistance mechanisms. The most significant mechanism of resistance to the β-lactam antimicrobial agents is the production of β-lactamase enzymes (Blasquez et al., 2000; Morris et al., 2003). In members of the family Enterobacteriaceae, the most prevalent mechanism of resistance to broad-spectrum β-lactams is detoxification of the drugs by plasmid-mediated enzymes that are variants of TEM and SHV penicillinases. The TEM-derived extended-spectrum β-lactamases (ESBLs) or inhibitor-resistant β-lactamases differ from the parental TEM-1 and TEM-2 penicillinases by various combinations of amino acid substitutions (Goussard and Courvalin, 1999).

Amoxicillin/clavulanate is a β-lactam-β-lactamase inhibitor combination that has antimicrobial activity against a wide variety of organisms. Escherichia coli, one of the most common pathogens, is usually susceptible to amoxicillin/clavulanate (Kaye et al., 2004). However, resistance resistance to amoxicillin/clavulanate appeared first in E. coli isolates and then in other species of enterobacteria (Speldooren et al., 1998). Resistance to the combination of amoxicillin and clavulanate in strains of E. coli may be due to a numbers of factors, four of which are enzymatic, viz., hyper-production of class C chromosomal β-lactamase, hyper-production of plasmid-mediated TEM-1 or TEM-2, production of inhibitor-resistant TEM (IRT) and the production of a relatively inhibitor-resistant OXA-type β-lactamase (Sirot et al., 1994; Speldooren et al., 1998). In this study we report the presence of two novel TEM β-lactamases; TEM-145 and TEM-146 which were found in two E. coli clinical isolates.
Table 1. Primers used in PCR studies.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose of primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM1(F)</td>
<td>5'-ATGAGTATCGGATCCGCTG-3'</td>
<td>Amp</td>
<td>Essack et al., 2001</td>
</tr>
<tr>
<td>TEM2(R)</td>
<td>5'-TTTGGCGGATCCGCTGAG-3'</td>
<td>Amp/Seq</td>
<td>Essack et al., 2001</td>
</tr>
<tr>
<td>TEM3(R)</td>
<td>5'-TTTGGCGGATCCGCTGAG-3'</td>
<td>Seq</td>
<td>Essack et al., 2001</td>
</tr>
<tr>
<td>TEM4(R)</td>
<td>5'-AGTAACTGGCAGTCGAG-3'</td>
<td>Seq</td>
<td>Essack et al., 2001</td>
</tr>
<tr>
<td>TEM5(F)</td>
<td>5'-CTGACACGATGGGACACAC-3'</td>
<td>Amp/Seq</td>
<td>Designed for this study</td>
</tr>
</tbody>
</table>

MATERIAL AND METHODS

Bacterial Strains

E. coli NWE11 and E. coli PMM40 were isolated from the urine samples of patients in hospitals in Kwazulu-Natal, South Africa in 2000. The identity of the strains as E. coli was confirmed using the API20E identification system (bioMerieux sa, Lyon, France). E. coli ATCC 25922 and E. coli NCTC 50102 served as the control for determination of minimum inhibitory concentrations (MIC) and plasmid size analysis respectively. E. coli CF204 was used as a TEM-3 positive control.

Antibiotic susceptibility testing

Susceptibility testing was undertaken using the Kirby Bauer disc diffusion method according to CLSI (formerly NCCLS) guidelines. The following antibiotics were used: ampicillin, ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin, piperacillin, piperacillin/tazobactam, cephalotin, cefuroxime-panerental, cefoxitin, ceftriaxone, cefotaxime, ceftazidime, cefepime, imipenem and aztreonam (Mast Diagnostics, Merseyside, UK). MIC values were extrapolated by the BIOMIC automated reading system and software package (Giles Scientific, New York).

Plasmid size analysis

Plasmid DNA was extracted from overnight cultures in Nutrient Broth (Biobab, Johannesburg, South Africa) using the alkaline lysis method (Kado and Liu, 1981). Plasmid sizes were calculated using computer software (Syngene-Gene Genius, Bio Imaging Systems, Syngene).

β-Lactamase analysis

The β-lactamases were extracted from pure overnight cultures in Nutrient Broth (Biobab, Johannesburg, South Africa) by the freeze-thaw method (Livermore and Williams, 1996). Analytical isoelectric focusing (IEF) was performed in amphotone polyacrylamide gels (pH 3.5 - 9.5; Amersham Biosciences, Uppsala, Sweden). β-lactamase bands were detected with nitrocefin solution (0.05% (w/v); Oxoid, Hampshire, England). An isoelectric point marker pI calibration kit (4.7 to 10.6; BDH, Poole, England) was the standard used.

PCR detection of blaTEM genes

A suspension of colonies from an overnight culture on Nutrient Agar (Biobab, Johannesburg, South Africa) was made in purified water (50 μl). Bacterial DNA was prepared by heating this suspension at 95°C for 5 min. PCR amplifications were then performed in a Gene Amp PCR System (Applied Biosystems, California, USA). The primers (Inqaba Biotechnology, Pretoria, South Africa) used are described in Table 1. The amplification of blaTEM genes was carried out as described previously (Essack et al., 2001). PCR products (5 μl) were analyzed by gel electrophoresis. Gels were stained with ethidium bromide and photographed using UV illumination. PCR products were purified and sequenced (Spectrumedix SCE2410 genetic analysis system, Spectrumedix, Pennsylvania, USA).

Nucleotide sequence accession number

The sequences obtained were analyzed using the BLAST 2.0 software available on the website of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST; last accessed June 2006). The nucleotide and amino acid sequences of TEM-145 and TEM-146 were deposited in GenBank and assigned the accession numbers DQ105526 and DQ105529, respectively.

RESULTS AND DISCUSSION

The MIC results are shown in Table 2. Both isolates were resistant to amoxicillin/clavulanate and ampicillin/sulbactam. E. coli PMM40 was resistant to piperacillin/tazobactam whilst E. coli NWE11 displayed intermediate resistance to piperacillin/tazobactam. Plasmids of 129.2 kb and 112.2 kb and 112.1 kb were detected in E. coli NWE11 and E. coli PMM40, respectively. IEF analysis revealed two β-lactamase bands for each of the isolates. β-lactamases with iso-electric points of 6.0 and 7.6 and 6.6 and 8.6 were detected for E. coli NWE11 and E. coli PMM40, respectively. PCR amplification revealed that both isolates harboured blaTEM genes and also the E. coli chromosomal AmpC gene (results not shown). Sequencing data identified the TEM genes as follows: E. coli NWE11 carried TEM-1 and TEM-146 (novel) and E. coli PMM40 carried TEM-1 and TEM-145 (novel). All isolates were also tested for the presence of SHV-, CTX- and plasmid-mediated CMY-genes (results not shown) but these were not found. The β-lactamases with pl values of 6.0 and 6.6 could be identified as transcripts of TEM β-lactamase genes and those with pl values of 7.8 and 8.6 as transcripts of the E. coli chromosomal AmpC genes. The TEM-β-lactamase enzymes showed the following changes compared to TEM-1: TEM-145 had two changes; T114P, M182I and R244H. TEM-146 had three changes; T114P, M182I and R244H.

Resistance to the β-lactam-β-lactamase inhibitor combinations and ESBLs in E. coli is an emerging problem (Kaye et al., 2004). The emergence of IRT-producing strains might be related to the frequent use of inhibitor-containing formulations in hospitals and in general practice (Chaibi et al., 1999).
β-lactamases, has been found in only one other TEM β-lactamase, viz., TEM-57 (IRT-15). Other reported mutations of Arg-244 are substitutions with serine, cysteine or threonine leads to clavulanic acid resistance which each had a mutation at position 244.

ACKNOWLEDGEMENTS

This study was funded by research grants from the MRC, NRF and the University of KwaZulu-Natal.

REFERENCES


Table 2. MICs (µg/ml) of the E. coli isolates investigated in this study.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>E. coli PMM40</th>
<th>E. coli NWE11</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>SAM</td>
<td>&gt;48</td>
<td>&gt;48</td>
</tr>
<tr>
<td>AMC</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>PIP</td>
<td>&gt;512</td>
<td>&gt;512</td>
</tr>
<tr>
<td>TZP</td>
<td>96</td>
<td>20 (I)</td>
</tr>
<tr>
<td>CEF</td>
<td>&gt;128</td>
<td>64</td>
</tr>
<tr>
<td>CXM</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>FOX</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>CRO</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CTX</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>CAZ</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>FEP</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>MEM</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>ATM</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Abbreviations: AMP, ampicillin; SAM, ampicillin/ sulbactam; AMC, amoxicillin/clavulanate; PIP, piperacillin; TZP, piperacillin/tazobactam; CEF, cephalothin; CXM, cefuroxime-parenteral; FOX, cefotaxime; CAZ, cefotaxime; FEP, cephexime; MEM, meropenem; ATM, aztreonam; (I) denotes intermediate resistance, boldface denotes resistance.
CMY-20, a novel AmpC-type β-lactamase from South African clinical Escherichia coli isolates

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Received 24 August 2007; accepted 20 November 2007

Abstract

In this study, we report the presence of a novel plasmid-mediated AmpC-type β-lactamase that was isolated from 3 clinical Escherichia coli isolates at a tertiary teaching hospital in Durban, South Africa. The nucleotide sequence of the genes encoding this novel β-lactamase was found to be >94% identical to the nucleotide sequences of the plasmid-mediated AmpC-type β-lactamases originating from Citrobacter freundii. This enzyme differed from CMY-2 by 3 amino acid substitutions and was designated CMY-20.

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Keywords: CMY-20; Escherichia coli; Plasmid-mediated β-lactamase

1. Introduction

The emergence of resistance to the cephalosporins, cephemycins, and β-lactamase inhibitors among members of the family Enterobacteriaceae and, in particular, Escherichia coli clinical isolates is a cause for concern. This broad spectrum of resistance to these antibiotics is a common feature of the AmpC class of enzymes (Bush et al., 1995). Plasmid-mediated cephalosporinases conferring resistance to oxyimino-cephalosporins and cephamycins as well as to β-lactamase inhibitors have been described since 1989. Many of them have amino acid sequences close to those of chromosomal-encoded and inducible AmpC enzymes of the family Enterobacteriaceae (Doi et al., 2002).

These plasmid-mediated cephalosporinases have been classified into clusters corresponding to a typical geographic location such as the North American cluster (MIR-I and ACT-1), Central and South American cluster (FOX-I and FOX-2), Asian cluster (CMY-1 and MOX-1), and Mediterranean Middle East cluster (CMY-2, CMY-2b, LAT-1 and LAT-2) (Verdet et al., 1998). The most widespread plasmid-mediated AmpC β-lactamases are those derived from the chromosomal cephalosporinases of Citrobacter freundii (Miriagou et al., 2004).

In this study, we report the presence of a novel CMY-type AmpC β-lactamase, CMY-20, isolated from 3 E. coli isolates out of a total of 330 from a study investigating β-lactam-mediated resistance to commonly used antibiotics in public sector hospitals in South Africa.

2. Methods and materials

2.1. Bacterial strains

E. coli KEH1 and E. coli KEH42 were isolated from peritoneal fluid and E. coli KEH32 from a pus swab from 3 different patients in a tertiary teaching hospital in Durban, South Africa, in 2000. Identification of the E. coli isolates were confirmed using the API20E identification system (bioMérieux, Lyon, France). E. coli ATCC 25922 was used as the quality control for susceptibility testing, and E. coli NCTC 50912 was the reference strain used to determine plasmid size. E. coli 162-1 was used as the recipient in the conjugation experiments (Livermore and Corkill, 1992).
2.2. Antibiotic susceptibility testing

Susceptibility testing was undertaken using the disc diffusion test according to the NCCLS (2003) guidelines. MIC values were extrapolated by the BIOMIC, an automated reading system and software (Giles Scientific, New York, NY), using the following antibiotics: ampicillin, piperacillin, ticarcillin, carbenicillin, amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam, cefalotin, cefuroxime—parenteral, cefepime, cefoxitin, ceftaxime, ceftriaxone, cefazidime, meropenem, aztreonam, amikacin, trimethoprim/sulfamethoxazole, and chloramphenicol (Mast Diagnostics, Merseyside, UK).

2.3. Plasmid size analysis and resistance transfer

Plasmid DNA was extracted from the E. coli isolates using the alkaline lysis method (Kado and Liu, 1981). Plasmid sizes were calculated using computer software (Syngene-Gene Genius, Bio Imaging Systems, Syngene, Cambridge, UK).

Mating experiments were performed by mixing equal quantities of overnight nutrient broth (Biola, Johannesburg, South Africa) cultures of the clinical isolate as donor and E. coli J62-1 as the recipient, and incubating the mixture for 6 h at 37 °C. The cefoxitin-resistant E. coli J62-1 transconjugants were selected on MacConkey agar (Biola) supplemented with nalidixic acid (50 μg/mL) and cefoxitin (10 μg/mL). MICs (antibiotics listed in Table 2) of the transconjugant were determined, and the transconjugant was also subjected to polymerase chain reaction (PCR) for the detection of the blaCMY gene.

2.4. β-Lactamase analysis

The β-lactamases of the E. coli clinical isolates were extracted from a nutrient broth (Biola) culture by the freeze—thaw method (Livermore and Williams, 1996). Analytical isoelectric focusing (IEF) was performed in amphoteric polyacrylamide gels (pH 3.5—9.5; Amersham Biosciences, Uppsala, Sweden). β-Lactamase bands were detected with nitrocefin (Oxoid, Basingstoke, UK). An isoelectric point marker pl calibration kit (4.7—10.6; BDH, Poole, UK) was used as the standard.

2.5. PCR detection and molecular characterization of β-lactamases

Bacterial DNA was prepared by suspending few freshly grown colonies from an overnight Nutrient Agar (Biola) culture of E. coli in 50 μL of purified water and heating the cells at 95 °C for 5 min. PCR amplifications were then performed in a Gene Amp PCR System (Applied Biosystems, Foster City, CA). PCR conditions for the amplification of blaTEM genes were carried out as described previously (Essack et al., 2001). The amplification mixture for the detection of the blaCMY gene was made up to 50 μL and contained 25 μL AmpliTaq Gold PCR Master Mix (Applied Biosystems), purified water, 10 pmol of each primer (Inqaba Biotechnology, Pretoria, South Africa; refer to Table 1), and 2 μL of the template DNA. PCR conditions were as follows: an initial denaturation for 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and a final extension step of 7 min at 72 °C. PCR products (5 μL) were analyzed by gel electrophoresis. Gels were stained with ethidium bromide and photographed using UV illumination. PCR products were purified and sequenced (Spectrumedix SCE2410 genetic analysis system; Spectrumedix, State College, PA). The isolates were also tested for the presence of blaSHV (Essack et al., 2000), blaCTX (Edelstein et al., 2003), blaDOROXY (Pérez-Pérez and Hanson, 2002), blaDOROXY (Pérez-Pérez and Hanson, 2002), blaCMY (Pérez-Pérez and Hanson, 2002), blaACC (Pérez-Pérez and Hanson, 2002), and blaACT (Pérez-Pérez and Hanson, 2002) genes by PCR. In addition, the promoter and attenuator regions of the chromosomal AmpC of E. coli were also investigated to determine whether there were any mutations in these regions.

2.6. Nucleotide sequence accession number

The sequences obtained were analyzed using the BLAST 2.0 software available on the Web site of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).
The MIC results for the *E. coli* clinical isolates investigated in this study are shown in Table 2. These isolates were resistant to all the cephalosporins tested as well as the β-lactamase inhibitor combinations, but susceptibility to cefepime and meropenem was retained. Each of the 3 *E. coli* isolates harbored a single 148.5-kb plasmid. IEF analysis revealed the presence of β-lactamases with pIs of 6.0, 5.9, and 6.6 in *E. coli* KEH1, *E. coli* KEH32, and *E. coli* KEH42, respectively, and sequencing data showed that these isolates harbored TEM-1. IEF also revealed the presence of β-lactamases with pI values of 7.8, 7.7, and 8.0, respectively. These values are consistent with the production of AmpC-β-lactamases. Sequencing data showed that the 3 isolates harbored a novel plasmid-mediated AmpC-type β-lactamase. This novel cephalosporinase, assigned CMY-20, is a member of the *C. freundii* group and differs from CMY-2 by the following substitutions, viz., R125S, R146T, and S309N. The S309T substitution has been reported for B1L-1.

### 4. Discussion

Strains with plasmid-mediated AmpC enzymes are generally resistant to aminopenicillins, carboxypenicillins, and ureidopenicillins. The enzymes also provide resistance to cephalosporins in the oximino and the 7-α-methoxy groups. In addition, these enzymes are also active against aztreonam, although some strains are susceptible to this antibiotic. With regard to the β-lactamase inhibitors, *E. coli* derivatives with plasmid-mediated AmpC enzymes are generally resistant to inhibitor combinations, with the possible exception of tazobactam (Philippon et al., 2002).

In a previous study (Verdet et al., 1998), Proteus mirabilis harboring CMY-4 was susceptible cefazolin and piperacillin/tazobactam and displayed intermediate resistance to cefoxitin, whereas in another study (Decré et al., 2002), the isolates harboring CMY-4, CMY-12, and CMY-2 were susceptible to piperacillin/tazobactam and, with the exception of the *Klebsiella pneumoniae* isolate harboring CMY-2, were susceptible to aztreonam. Furthermore, plasmids encoding AmpC enzymes often carry multiple resistance genes, conferring resistance to aminoglycosides, chloramphenicol, sulfonamides, tetracycline, trimethoprim, or mercucir ion (Stapleton et al., 1999). In this study, the 3 clinical *E. coli* isolates investigated were also found to be multiresistant, conferring resistance to amikacin, trimethoprim-sulfamethoxazole, chloramphenicol, and gentamicin, in addition to the β-lactam antibiotics described above.

The amino acid sequence of CMY-20 showed that this enzyme had the β-lactamase active site SVSK at residues 64 to 67, the conserved KTG triad at residues 315 to 317, and the typical class C YXN motif (Decré et al., 2002; Philippon et al., 2002), thus, indicating that the enzyme was an AmpC-type β-lactamase. Analysis of the sequence of CMY-20 using Blast 2.0 revealed a high degree of homology with other plasmid-encoded AmpC-β-lactamases such as B1L-1, LAT-1, CMY-2, and LAT-2, which are closely related to the chromosomal *ampC* gene of *C. freundii*, suggesting mobilization of the chromosomal *ampC* gene from *C. freundii* onto plasmids (Bauerfeind et al., 1996; Koeck et al., 1997).

The appearance of plasmid-borne AmpC-β-lactamases should be a cause for concern. Genes for the AmpC enzymes have been located on plasmids of varying sizes (Horii et al., 1994; Stapleton et al., 1999). These plasmids, although not
all are self-transmissible, are transferable by transformation (Stapleton et al., 1999) or mobilization onto integrons and gene cassettes (Gazouli et al., 1997).

Plasmid-mediated class C β-lactamases have been found worldwide, most frequently in K. pneumoniae and, to a lesser extent, in E. coli, P. mirabilis, and the salmonellae. They have been reported in Africa (Algeria and Tunisia), Asia, Europe, the Middle East, North America, and South and Central America. CMY-2 is the most prevalent of the plasmid-mediated AmpC enzymes and the most widely distributed geographically (Decré et al., 2002; Philippon et al., 2002). Although the plasmid-mediated AmpC-type β-lactamases have been found throughout the world, their occurrence on the African continent is relatively rare, and there have been few documented reports (Philippon et al., 2002). The only report of CMY-type enzymes in Africa, thus far, has been the characterization of a plasmid-mediated CMY-2 β-lactamase from an Algerian isolate of Salmonella senftenberg (Koeck et al., 1997), and the other report was of a CMY-4 plasmid-mediated β-lactamase from a Tunisian P. mirabilis clinical isolate (Verdet et al., 1998). Thus far, there have been no documented reports of plasmid-mediated CMY-2-type β-lactamases in E. coli in Southern Africa, and to the best of our knowledge, this is the 1st report of a Southern African E. coli clinical isolate harboring a CMY-2 type β-lactamase.

5. Conclusion

Plasmid-mediated AmpC enzymes can confer resistance to all β-lactams other than 4th-generation cephalosporins and carbapenems and hence pose a major therapeutic challenge. It is thus imperative to institute surveillance systems to monitor the further dissemination of these emerging cephapmycin-resistant Gram-negative bacteria.

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References


Full Length Research Paper

The effect of mutations in the AmpC promoter region on β-lactam resistance from an Escherichia coli clinical isolate in a public sector hospital in KwaZulu-Natal, South Africa

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2Department of Medical Microbiology, Nelson R Mandela Medical School, University of KwaZulu-Natal, South Africa.

Accepted 16 June, 2008

The ampC promoter and attenuator regions of an Escherichia coli clinical isolate from a public hospital in KwaZulu-Natal was investigated to detect the presence of mutations in these regions. The isolate was subjected to MIC determinations, IEF analysis, PCR for the presence of β-lactamases and sequencing of the ampC gene. Analysis of the ampC promoter and attenuator regions of the isolate showed that the isolate had mutations in the promoter region and this included insertions of nucleotides in the spacer region between the -10 and -35 Pribnow boxes. The insertion of an extra nucleotide in the spacer region between the -10 and -35 boxes affects the resistance of bacteria to β-lactam antibiotics.

Key words: Escherichia coli, promoter, pribnow box.

INTRODUCTION

All strains of Escherichia coli possess a gene that encodes an AmpC β-lactamase (Clark et al., 2003). The AmpC gene in E. coli is normally located on the chromosome and is weakly expressed because of a weak promoter and a weak transcriptional attenuator (Siu et al., 2003; Mulvey et al., 2005). Most E. coli strains do not produce clinically relevant levels of the chromosomally encoded AmpC β-lactamase (Martinez-Martinez et al., 2000). Wild-type strains produce a basal level of this enzyme which does not result in ampicillin and cephalosporin resistance (Siu et al., 2003; Mulvey et al., 2005). Gene amplification or mutations at either the promoter and/or attenuator regions of the structural β-lactamase gene can result in AmpC hyper-production (Martinez-Martinez et al., 2000). Such hyper-production of AmpC β-lactamase contributes to resistance to ampicillin, extended-spectrum cephalosporins and β-lactam-β-lactamase inhibitor combinations (Martinez-Martinez et al., 2000; Siu et al., 2003). AmpC β-lactamases display similar MIC values to extended-spectrum β-lactamas- ses (ESBLs). However, in contrast to ESBLs, AmpC β-lactamases are poorly inhibited by β-lactam-β-lactamase inhibitor combinations (Siu et al., 2003).

Unlike other members of the family Enterobacteriaceae, in E. coli, ampC is not inducible since there is no ampR regulatory gene (Caroff et al., 2000) and consequently, in E. coli the level of transcription of the ampC gene depends mostly on the strength of the ampC promoter. E. coli harbours two hexamers of conserved sequences, the -35 region and the -10 one, called the Pribnow box, which play an important role in gene transcription. The -35 consensus sequence TTGACA and the -10 consensus sequence TATAAT have been described (Corvec et al., 2002). Genetically, promoter changes, an optimized distance (17 bp) in the Pribnow box and the presence of more than one copy of ampC, have been described as crucial factors for AmpC hyper-production (Nelson and Gay Elisha, 1999; Siu et al., 2003). Mutations in the promoter region of ampC have been described as a mechanism for AmpC hyper-production. The mutations are thought to generate
promoters that more closely resemble the E. coli consensus, which leads to over-expression of the normally low level constitutively expressed ampC (Mulvey et al., 2005; Tracz et al., 2005). The most frequently described E. coli ampC 'strong' promoter harbours mutations at positions -88, -82, -42, -18, -1 and +58 (Caroff et al., 2000). Attenuator mutations are thought to destabilise the hairpin structure allowing greater read-through (Mulvey et al., 2005; Tracz et al., 2005). In this study we examined the DNA sequence upstream of the ampC gene in an E. coli isolate from a patient in a hospital in KwaZulu-Natal, South Africa with increased resistance to cefoxitin to determine mutations occurring in the promoter and attenuator regions.

MATERIALS AND METHODS

Bacterial strains

E. coli NGZ55 was isolated from a patient in a public sector hospital in KwaZulu-Natal, South Africa in 2000. The identity of the isolate as E. coli was confirmed using the AP120E identification system (bioMérieux sa, Lyon, France). E. coli ATCC 25922 was used as the MIC control.

Antibiotic susceptibility testing

Susceptibility testing was undertaken using the disc diffusion test according to CLSI guidelines (2005). MICs values were extrapolated by the BIOMIC an automated reading system and software (Giles Scientific, New York), using the following antibiotics: ampicillin, ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin, piperacillin, piperacillin/tazobactam, cephalotin, cefuroxime, cefoxitin, ceftriaxone, cefotaxime, cefazidime, cefepime, meropenem and aztreonam (Mast Diagnostics. Merseyside, UK).

β-lactamase analysis

The β-lactamases of the E. coli clinical isolate was extracted from a Nutrient Broth (Biobal, South Africa) culture as described previously (Livermore and Williams, 1996). Analytical isoelectric focusing (IEF) was performed in ampholine polyacrylamide gels (pH 3.5 - 9.5; Amersham Biosciences, Uppsala, Sweden). β-lactamase bands were detected with nitrocefin (Oxoid). An isoelectric point marker pl calibration kit (4.7 to 10.6; BDH, England) was used as the standard.

PCR detection of blaTEM, blaCTX, and blaAmpC genes

Bacterial DNA was prepared by suspending few freshly grown colonies from an overnight Nutrient Agar (Biobal, South Africa) culture of E. coli in 50 μl of purified water and heating the cells at 95°C for 5 min. PCR amplifications were then performed in a Gene Amp PCR System (Applied Biosystems, USA). The primers used are described in Table 1. PCR conditions for the amplification of blaTEM was carried out as described by Essack et al. (2001). The amplification mixture for the detection of blaCTX and blaAmpC genes were each made up to 50 μl and contained 25 μl AmpliTaq Gold PCR Master Mix (Applied Biosystems, California, USA), purified water, 10 pmol of each primer (Inqaba Biotechnology, Pretoria, South Africa) and 2 μl of the template DNA. PCR conditions were as follows: AmpC: an initial denaturation for 30 s at 94°C, 30 cycles of 30 s at 94°C, 30 s at 57°C, 1 min at 72°C and a final extension step of 7 min at 72°C; and CTX: an initial denaturation for 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C and a final extension step of 7 min at 72°C. PCR products (5 μl) were analyzed by gel electrophoresis. Gels were stained with ethidiun bromide and photographed using UV illumination. PCR products were purified and sequenced (Spectrumedix SCE2410 genetic analysis system, Spectrumedix, Pennsylvania, USA).

RESULTS

The MIC results obtained for E. coli NGZ55 and the control strain, E. coli ATCC 25922 are shown in Table 2. E. coli NGZ55 was resistant to amoxicillin/ clavulanate, ampicillin, cephalotin, cefoxitin and cefuroxime. IEF analysis revealed the presence of β-lactamases with pl of 6.5 and 6.6. PCR and sequencing data showed that the isolate harboured TEM-1 and CTX-M1. Comparison of

Table 1. Primers used in PCR studies.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose of primer</th>
<th>Reference</th>
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<tbody>
<tr>
<td>TEM 1(F)</td>
<td>5'-ATGAGTATTCACATTGTCGTG-3'</td>
<td>Amp</td>
<td>Essack et al., 2001</td>
</tr>
<tr>
<td>TEM 2(R)</td>
<td>5'-TTACCATCTGTNGTGTAAGT-3'</td>
<td>Amp/Seq</td>
<td>Essack et al., 2001</td>
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<td>Seq</td>
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<td>Essack et al., 2001</td>
</tr>
<tr>
<td>TEM 5(F)</td>
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<td>Amp/Seq</td>
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<tr>
<td>COL A</td>
<td>5'-ACGACGCTGCGGCTTTA-3'</td>
<td>Amp/Seq</td>
<td>Bret et al., 1998</td>
</tr>
<tr>
<td>COL B</td>
<td>5'-AAAGATCTGCAGGCGC-3'</td>
<td>Amp/Seq</td>
<td>Bret et al., 1998</td>
</tr>
<tr>
<td>AmpC R1</td>
<td>5'-GGTGAGCTGTCATCGGGCT-3'</td>
<td>Amp/Seq</td>
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</tr>
<tr>
<td>AmpC F1</td>
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<td>Amp/Seq</td>
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<tr>
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<td>Designed for this study</td>
</tr>
<tr>
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<td>5'-GCCGATTAAATCTGACACATC-3'</td>
<td>Amp/Seq</td>
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<td>CTX-MF</td>
<td>5'-TTTGCGGATGTGAGTACCCGAT-3'</td>
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<td>Designed for this study</td>
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<td>Amp/Seq</td>
<td>Designed for this study</td>
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<td>Amp/Seq</td>
<td>Bret et al., 1998</td>
</tr>
<tr>
<td>COLA 5'</td>
<td>5'-ACGACGCTGCGGCTTTA-3'</td>
<td>Amp/Seq</td>
<td>Bret et al., 1998</td>
</tr>
<tr>
<td>AmpC R2</td>
<td>5'-GATGACAGCAAGAGGAAAGCGAG-3'</td>
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<tr>
<td>COL B</td>
<td>5'-AAAGATCTGCAGGCGC-3'</td>
<td>Amp/Seq</td>
<td>Bret et al., 1998</td>
</tr>
<tr>
<td>AmpC R1</td>
<td>5'-GGTGAGCTGTCATCGGGCT-3'</td>
<td>Amp/Seq</td>
<td>Designed for this study</td>
</tr>
<tr>
<td>AmpC F1</td>
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<td>Amp/Seq</td>
<td>Designed for this study</td>
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<td>AmpC F2</td>
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<td>Amp/Seq</td>
<td>Designed for this study</td>
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<tr>
<td>AmpC C2</td>
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<td>Amp/Seq</td>
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<tr>
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<td>Amp/Seq</td>
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<tr>
<td>CTX-MR</td>
<td>5'-TGGATATCGTTGAGTTGGGATGAT-3'</td>
<td>Amp/Seq</td>
<td>Designed for this study</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer; Amp, amplification; Seq, sequencing.
the AmpC sequences using Blast 2.0 (http://www.ncbi.nlm.nih.gov/Blast; last accessed June 2006) showed that the sequences corresponded to the chromosomal AmpC sequences of *E. coli*.

The *E. coli* isolates showed changes in the AmpC promoter region compared to that of *E. coli* K-12. The changes observed in the promoter and attenuator regions are shown in Table 3. The optimal distance between the -10 and -35 Pribnow boxes is 17bp. *E. coli* NGZ55 had an additional nucleotide, G, between positions -26 and -25. Mutations in the -10 box included changes at position -10 and this changed the -10 box from TACAAAT to TACCAT (changes in boldface). Changes upstream of the -35 box included A(-82)C, T(-47)G, A(-43)C, G(-40)C, A(-39)C and A(-37)T.

Isolate *E. coli* NGZ55 in addition showed a further nucleotide addition, A, between positions +8 and +9. Changes between the attenuator region and the start codon included the following: T(+41)G, T(+43)A and T(+45)G. *E. coli* NGZ55 also had the following extra nucleotides: an additional C between positions +39 and +38 and an additional C between positions +54 and +53.

No SHV-, OXA- and plasmid-mediated AmpC type-β-lactamases were detected (data not shown).

**DISCUSSION**

*E. coli* is an important pathogen that commonly causes serious infections (Forward et al., 2001) and therefore, the emergence of *E. coli* strains resistant to the cephalosporins should be a cause for concern to clinicians managing infections in both the community and institutional setting. Some of these strains have become resistant by virtue of their hyperproduction of chromosomally encoded AmpC β-lactamases whilst others have acquired plasmidic β-lactamases; most often those derived from *Citrobacter freundii* (Clark et al., 2003).

The chromosomal cephalosporinase gene, *ampC*, of *E. coli* is regulated by a weak promoter and a transcriptional attenuator. Strains carrying the wild-type gene produce low basal amounts of AmpC and are inherently susceptible to ampicillin. Occasionally, this enzyme is overproduced in *E. coli* and these strains are resistant to cefoxitin and have reduced susceptibilities to the newer β-lactams such as the oxyzimecephalosporins. Some hyperproducers also contain more than one copy of *ampC*, while others contain mutations in the regulatory and/or attenuator regions of *ampC*, resulting in more efficient transcription of the structural gene (Nelson and Gay Elisha, 1999).

AmpC β-lactamase production in *E. coli* is controlled by a typical *E. coli* promoter. There is no repressor gene, as is seen in *Enterobacter cloacae* and *C. freundii*. An analysis of the promoter region in *E. coli* revealed the presence of two conserved regions, the -35 box and the -10 box, also called the Pribnow box. The sequence of the -35 and -10 hexamers and the interbox distance are crucial for efficient binding of RNA polymerase, thereby influencing the level of transcription of the gene (Caroff et al., 2000; Forward et al., 2001; Corvec et al., 2002). An interbox distance of 16bp and the presence of a hairpin attenuator structure contribute to low-level transcription of the gene (Corvec et al., 2002). For most promoters the degree of homology to the -35 box consensus sequence, TTGACA, and the -10 box consensus sequence, TATAAT, is directly related to promoter strength. Variations in the promoter and attenuator regions of *ampC* are a mechanism of hyperproduction of the AmpC protein that results in resistance or reduced susceptibility to cefoxitin (Mulvey et al., 2005). Susceptible strains of *E. coli* have an inefficient *ampC* promoter that differs by several nucleotides from the strong promoter sequence. Consequently, very little AmpC β-lactamase is produced (Forward et al., 2001).

In this study, isolate *E. coli* NGZ55 had an extra nucleotide, G, between positions +26 and +25. This created an optimal distance of 17bp between the -10 box

---

**Table 2. MIC (µg/ml) profiles of *E. coli* NGZ55 and *E. coli* ATCC 25922 (control strain).**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>AMP</th>
<th>PIP</th>
<th>AMC</th>
<th>TZP</th>
<th>CEF</th>
<th>CXM</th>
<th>FEP</th>
<th>FOX</th>
<th>CTX</th>
<th>CRO</th>
<th>CAZ</th>
<th>MEM</th>
<th>ATM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGZ55</td>
<td>6</td>
<td>10</td>
<td>&gt;64</td>
<td>4</td>
<td>&gt;128</td>
<td>8</td>
<td>2</td>
<td>&gt;96</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>ATCC 25922</td>
<td>4</td>
<td>ND</td>
<td>6</td>
<td>4</td>
<td>24</td>
<td>4</td>
<td>&lt;1</td>
<td>&lt;0.5</td>
<td>&lt;1</td>
<td>1</td>
<td>&lt;0.5</td>
<td>&lt;2</td>
<td></td>
</tr>
</tbody>
</table>

AMP, ampicillin; PIP, piperacillin; AMC, amoxicillin/clavulanate; TZP, piperacillin/tazobactam; CEF, cefalotin; CXM, cefuroxime; FEP, cefepime; FOX, cefoxitin; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; MEM, meropenem; ATM, aztreonam.

ND = not determined.

**Table 3. Mutations in promoter and attenuator regions.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> NGZ55</td>
<td>Extra G between -26 and -25; A(-10)C; extra A between +8 and +9; extra C between +39 and +38; extra C between +54 and +53</td>
</tr>
</tbody>
</table>

*a*Compared with *E. coli* K-12 (Caroff et al., 2000).
and the -35 box and this has been shown in previous studies to play a role in promoter strength (Forward et al., 2001; Siu et al., 2003; Mulvey et al., 2005). This isolate was resistant to cefoxitin with reduced susceptibility to cefazidime and aztreonam. However, this isolate was susceptible to piperacillin/tazobactam and ampicillin/sulbactam (results not shown) and this MIC profile is suggestive of an ESBL-producer. However, PCR and addition to the extra nucleotide between positions -26 and -25, also had a mutation at position -10 to give a new -10 box which changed it from TACAAT to TACCAT (change shown in boldface). It also had nucleotide insertions in the regions just outside the attenuator region and this included an extra A between positions +8 and +9 and an extra C between positions +39 and +38. Sequencing data have also shown an extra nucleotide, C, between positions +54 and +53 which are situated close to the ATG start codon. Previous studies (Forward et al., 2001) have shown that mutations in the attenuator region result in increased transcription of the ampC gene.

### Conclusion

While chromosomal-mediated resistance might spread more slowly than mobilized β-lactamases, it is likely that with intense antibiotic pressure seen in today's medical practice that chromosomal-mediated resistance will become an increasing problem and may further limit our antibiotic choices (Forward et al., 2001). Routine surveillance of sensitivity to cefoxitin in E. coli is thus advocated.

### ACKNOWLEDGEMENTS

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### REFERENCES


2.2. SUBMITTED PAPER

The following article was submitted to The Southern African Journal of Epidemiology and Infections and am awaiting response (Ref. No.: 122-417-2-SM-DOC)

Chunderika Mocktar, Usha Govinden, Adrian W Sturm and Sabiha Essack.

Complexity and diversity of beta-lactamase expression in inhibitor resistant *Escherichia coli* from public hospitals in KwaZulu-Natal, South Africa
Complexity and diversity of beta-lactamase expression in inhibitor resistant *Escherichia coli* from public hospitals in KwaZulu-Natal, South Africa

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²Department of Medical Microbiology, Nelson R Mandela Medical School, University of KwaZulu-Natal

Running Title: Diversity of beta-lactamases in South African *E. coli*

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Word Count Abstract: 125

Word Count Text: 1706
Abstract

β-lactamase profiles of 38 inhibitor-resistant *Escherichia coli* isolates obtained from public hospitals in KwaZulu-Natal, selected on the basis of their resistance profiles to one/more of amoxicillin/clavulanate, ampicillin/ sulbactam and piperacillin/tazobactam were analyzed. Isolates were subjected to iso-electric focusing, plasmid profile determination, PCR of the different β-lactamase genes and sequencing thereof to detect the possible mechanism/s of inhibitor-resistance. A range of β-lactamases including two inhibitor-resistant TEM β-lactamases (TEM-145 and TEM-146), a plasmid-mediated AmpC-type β-lactamase (CMY-20), OXA-1, TEM-55, SHV-2, CTX-M1 and TEM-1 were detected. Diverse β-lactamase genes and/or enzyme combinations, and plasmid profiles inferred extensive mobilization of resistance genes. Inhibitor resistance could be attributed to a range of mechanisms including but not limited to inhibitor-resistant TEM β-lactamases, hyper-production of TEM-1, hyper-production of chromosomal AmpC and OXA β-lactamases.

Keywords: *Escherichia coli*, β-lactamases, inhibitor-resistance
Introduction

The rapid increase of β-lactam resistance amongst members of the family Enterobacteriaceae is a major source of concern. The increased and repeated use of β-lactam antibiotics has led to them becoming ineffective, mainly as a result of the production of β-lactamases such as TEM-1 and SHV-1, which hydrolyze penicillins and narrow spectrum cephalosporins. Escherichia coli which is a common component of the intestinal flora of humans and animals, can cause serious diseases including septicaemia and urinary and wound infections. In clinical isolates of E. coli, plasmid-mediated TEM-1 β-lactamase is the most frequently encountered enzyme. This enzyme confers resistance to aminopenicillins and carboxypenicillins without affecting their susceptibility to third generation cephalosporins.

In order to overcome this resistance, two types of β-lactams were developed, viz., β-lactams resistant to hydrolysis such as extended spectrum cephalosporins; and β-lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam. Although β-lactamase inhibitors have poor antibacterial activities by themselves, they covalently bind to the active sites of class A β-lactamases and inhibit them. However, with increasing use of these antimicrobial agents, resistance again began to emerge. The first TEM-type extended spectrum β-lactamas (ESBLs) were characterized in 1987 and TEM penicillinases resistant to inhibitors were characterized in the 1990s. Inhibitor-resistant TEM β-lactamases (IRTs) have been detected in several members of the Enterobacteriaceae from different countries globally. We investigated β-lactamase mediated resistance in E. coli isolates obtained from a surveillance study in 16 demographically representative public hospitals at three levels of
health care in KwaZulu-Natal. A total of 1270 bacterial isolates were collected; of which
300 (23.6%) were *E. coli* isolates. One hundred and seventy-nine of these isolates were
resistant to one of the three antibiotic-inhibitor combinations used in this study; 38
isolates were chosen for this study as a representative of particular antibiograms from
specific hospitals.

Materials and Methods

Bacterial Strains

Thirty-eight *E. coli* isolates from patients seeking care in public hospitals in KwaZulu-
Natal were included in this study (Table 1). Isolates were identified using the API20E
identification system (bioMérieux sa, Lyon, France). *E. coli* ATCC 25922 and *E. coli*
NCTC 50192 served as the quality control for susceptibility testing and plasmid size
analysis respectively.

Antibiotic Susceptibility Testing

Susceptibility testing was undertaken by a modification of the Kirby-Bauer disk diffusion
method following CLSI\(^1\) guidelines using the following antibiotics: ampicillin,
piperacillin, ticarcillin, carbenicillin, amoxicillin/clavulanate, ampicillin/sulbactam,
piperacillin/tazobactam, cephalotin, cefuroxime, cefepime, cefoxitin, cefotaxime,
ceftriaxone, ceftazidime, meropenem and aztreonam (Mast Diagnostics, Merseyside,
UK).
β-lactamase analysis

The β-lactamases were extracted from overnight Nutrient Broth (Biolab, Johannesburg, South Africa) cultures by the freeze-thaw method.\textsuperscript{11} Isoelectric focusing (IEF) was performed on ampholine polyacrylamide gels (pH 3.5-9.5; Amersham Biosciences, Uppsala, Sweden) using an isoelectric point marker pl calibration kit (4.7 to 10.6; BDH, Poole, England) as the standard. β-lactamase bands were detected with nitrocefin (Oxoid, Basingstoke, England).

Plasmid profile analysis

Plasmid DNA was extracted from overnight Nutrient Broth (Biolab) cultures using the method as described previously.\textsuperscript{12} Plasmid sizes were calculated using computer software (Syngene-Gene Genius, Bio Imaging Systems, Syngene, Cambridge, United Kingdom).

PCR detection of \textit{bla}\textsubscript{TEM}, \textit{bla}\textsubscript{SHV}, \textit{bla}\textsubscript{CTX}, \textit{bla}\textsubscript{CMY}, \textit{bla}\textsubscript{OXA} and \textit{bla}\textsubscript{AmpC} genes

Bacterial DNA was prepared by suspending overnight Nutrient Agar (Biolab) in distilled water and heating at 95°C for 5 min. PCR amplifications were then performed in a Gene Amp PCR System (Applied Biosystems, Foster City, California, USA). PCR conditions for the amplification of \textit{bla}\textsubscript{TEM} and \textit{bla}\textsubscript{SHV} genes,\textsuperscript{13} \textit{bla}\textsubscript{CMY}\textsuperscript{14} and \textit{bla}\textsubscript{OXA}\textsuperscript{15} were carried out as described previously. The amplification mixture for the detection of \textit{bla}\textsubscript{CTX} and \textit{bla}\textsubscript{AmpC} genes were each made up to 50µl and contained 25µl AmpliTaq Gold PCR Master Mix (Applied Biosystems, California, USA), purified water, 10pmol of each primer (Inqaba Biotechnology, Pretoria, South Africa; Table 2) and 2µl of the template DNA. PCR conditions were as follows: CTX: an initial denaturation for 5 min at 94°C, 35
cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C and a final extension step of 7 min at 72°C. PCR products (5 µl) were analyzed by gel electrophoresis. Gels were stained with ethidium bromide and photographed using UV illumination. PCR products were purified and sequenced (Spectrumedix SCE2410 genetic analysis system, Spectrumedix, State College, Pennsylvania, USA). The isolates were also tested for the presence of \( \text{bla}_{\text{FOX}} \), \( \text{bla}_{\text{Mox}} \), \( \text{bla}_{\text{BIL}} \), \( \text{bla}_{\text{LAT}} \), \( \text{bla}_{\text{ACT}} \), \( \text{bla}_{\text{ACC}} \) and \( \text{bla}_{\text{DHA}} \) (data not shown).

Results

The MIC results are shown in Table 3. From the table it can be seen that 38 (100%), 34 (89%) and 26 (68%) of the isolates displayed reduced susceptibility to amoxicillin/clavulanate, ampicillin/sulbactam and piperacillin/tazobactam respectively. All 38 isolates also displayed reduced susceptibility to cephalothin. With the exception of \( \text{E. coli} \) NGZ55 and \( \text{E. coli} \) GJC95, all the isolates were resistant to ampicillin, piperacillin, carbenicillin and ticarcillin. The only antibiotic to which all the isolates were sensitive was meropenem. Table 3 summarizes the plasmid profiles and β-lactamase characteristics of the isolates. From the table it can be seen that 1-5 plasmids ranging in size from 13.4 kb to 311.1 kb were detected in the isolates. No plasmids were detected in isolates ESH31, GJC53, GJC85, GJC95 and ADD66. TEM-1 was found in all isolates, whilst TEM-55 was found in isolate MDD39. Isolates PMM40 and NWE11 produced the inhibitor-resistant enzymes, TEM-145 and TEM-146 respectively. A plasmid-mediated AmpC type β-lactamase, CMY-20 was produced by isolates KEH1, KEH32 and KEH42. Isolate GJC33 produced OXA-1 whilst 12 of the isolates produced CTX-M1. SHV-2 was detected in 2 of the isolates.
Inhibitor resistance in *E. coli* may be attributed to: hyperproduction of class C chromosomal β-lactamase (cephalosporinase), hyperproduction of plasmid-mediated TEM-1 or TEM-2, production of an IRT or the production of a relatively inhibitor-resistant OXA-type β-lactamase.19,20

The first amoxicillin/clavulanate-resistant *E. coli* isolate was noted in 1987 in France.21

Amoxicillin/clavulanate is a β-lactam β-lactamase inhibitor combination that has antibacterial activity against gram positive, gram negative and anaerobic microorganisms. *E. coli*, one of the most common pathogens in community acquired and nosocomial infections, is usually susceptible to amoxicillin/clavulanate.15 However, with increasing use, resistance to amoxicillin/clavulanic acid appeared, first in *E. coli* isolates, then in other species of enterobacteria.19

A study by Henquell et al.2 showed that 40% of *E. coli* isolates from hospitals and 18% of isolates from private laboratories were resistant to amoxicillin/clavulanate. Other studies carried out in the United States15 and France21 showed that 3% and 5% of the *E. coli* isolates were resistant to amoxicillin/clavulanate respectively. A recent study in Pakistan22 which investigated the prevalence and antibiotic resistance of urinary tract pathogens showed that 58% of the *E. coli* isolates were resistant to amoxicillin/clavulanate whilst a study in Tanzania23 which conducted a surveillance of antimicrobial resistance in a tertiary hospital revealed that 28% of the *E. coli* isolates were resistant to amoxicillin/clavulanate. Two studies conducted in South Africa26,27 using bacterial cultures obtained from 7 private pathology practices in all the private hospitals in Johannesburg, Cape Town, Durban, Bloemfontein and Pretoria during 2006 and 2007.
showed that 37% and 39% of the *E. coli* isolates were resistant to amoxicillin/clavulanate respectively. In this study, 59.6% of the *E. coli* isolates were resistant to amoxicillin/clavulanate. The study by Brink et al.\textsuperscript{24} showed significant regional differences in antibiotic susceptibility in resistance patterns in South Africa and the *E. coli* isolates from the private hospitals in Durban showed 42% resistance to amoxicillin/clavulanate. There have been no documented reports of amoxicillin/clavulanate resistance amongst *E. coli* isolates from public sector institutions in South Africa. The South African, Tanzanian and Pakistani studies only reported amoxicillin/clavulanate resistance but did not investigate mechanisms of resistance.

In this study PCR and sequencing results showed that all the isolates carried TEM-type β-lactamases. This is to be expected as one of the mechanisms of β-lactam-β-lactamase mediated resistance in *E. coli* is TEM hyper-production.\textsuperscript{2}

Two inhibitor-resistant TEM β-lactamases, viz., TEM-145 and TEM-146 were found in isolates PMM40 and NWE11 respectively. TEM-145 had 2 substitutions compared with the sequence of TEM-1, i.e., L221M and R244H whilst TEM-146 had 3 substitutions compared to TEM-1, i.e., T114P, M182I and R244H.\textsuperscript{18} Residue 182 is located just before the α-helix and this is far from the binding site.\textsuperscript{3} This substitution does not affect the β-lactamase activity but rather acts as a global stabilizer.\textsuperscript{26}

It has been shown that many inhibitor resistant clinical isolates and laboratory mutants may have emerged as a result of single or multiple mutations in the structural genes of their β-lactamases.\textsuperscript{6} Substitution of critical residues at positions 69, 130, 244 and 276 have been shown to produce the IRT phenotype by themselves or in combination with other placements.\textsuperscript{7,27}
Isolates PMM40 and NWE11 displayed resistance to amoxicillin/clavulanate and ampicillin/sulbactam whilst isolate PMM40 was resistant and isolate NWE11 displayed intermediate resistance to piperacillin/tazobactam.

A plasmid-mediated AmpC-type β-lactamase, viz., CMY-20, was found in 3 of the isolates. These isolates, viz., KEH1, KEH32 and KEH42 were resistant to all the β-lactamases tested with the exception of meropenem and cefepime. In addition, these 3 isolates were also resistant to other antibiotics such as chloramphenicol, gentamicin and trimethoprim/sulfamethoxazole (data not shown). CMY-20 belongs to the *Citrobacter freundii* group and has the following substitutions compared to CMY-2: R125S, R146T and S309N. Bacteria with plasmid-mediated AmpC enzymes are generally resistant to inhibitor combinations, with the possible exception of tazobactam. All 3 isolates were resistant to all the antibiotic-inhibitor combinations tested.

GJC-33 displayed reduced susceptibility to all three inhibitor combinations. This isolate in addition to harbouring TEM-1, also harboured OXA-1. OXA-type β-lactamases confer resistance to ampicillin and cephalosporin and they are poorly inhibited by clavulanic acid and this could have contributed to the inhibitor resistance of this isolate.

Isolate NGZ55 was resistant to cefoxitin but only TEM-1 and CTX-M1 was found in this isolate. Analysis (data not shown) of the chromosomal AmpC promoter region of this isolate revealed that there were changes in the promoter region. This isolate had an extra base pair between the -35 and -10 region and this created an optimal distance of 17 base pairs in this region. A distance of 16 base pairs leads to low level of transcription of this gene whilst an optimal distance of 17 base pairs plays a role in promoter strength.
Conclusion

The diversity of enzymes and the high incidence of resistance of *E. coli* clinical isolates from public hospitals in KwaZulu-Natal to inhibitor combinations are of great concern and use of these drugs needs to be carefully monitored.

Funding

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Ethical Clearance

Granted by UKZN Ethical Clearance Committee (Ethical Clearance No. 0092A)

Conflict of interest

None

Conference Presentations

1. Poster presentation: Novel inhibitor-resistant β-lactamases TEM-145 and TEM-146, from two *Escherichia coli* clinical isolates. Federation of the Infectious Disease Society of South Africa (FIDSSA) October, 2007, Cape Town, South Africa
References


Table 1: Epidemiology, phenotypes and β-lactamase genes of *E. coli* isolates collected in 2000 from public hospitals in KwaZulu-Natal

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ND- not detected

096835
Table 2: Primers used in PCR studies

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F, forward primer; R, reverse primer; Amp, amplification; Seq, sequencing
Table 3: MICs (µg/ml) of the E. coli clinical isolates

<p>| Isolate | AMP | PIP | TIC | CAR | AMC | SAM | TZP | CEF | CXM | FEP | FOX | CTX | CRO | CAZ | MEM | ATM |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| NGZ55   | 6   | 10  | 4   | 16  | &gt;64 | 8   | 4   | &gt;128| 48  | &gt;96 | 1   | 2   | 6   | 0.5 | 4   |
| GP6     | &gt;48 | &gt;512| &gt;256| &gt;512| &gt;64 | 48  | 6   | 48  | 10  | &lt;1  | 8   | &lt;0.5| 2   | 6   | 0.5 | 6   |
| GP34    | &gt;48 | 512 | 256 | &gt;512| &gt;64 | 20  | &gt;512| 24  | 8   | &lt;1  | 4   | &lt;0.5| 2   | 4   | 0.5 | 2   |
| GP45    | &gt;48 | 512 | &gt;256| &gt;512| &gt;64 | 12  | 6   | &gt;128| 10  | 1   | 4   | 1   | 4   | 4   | 0.5 | 4   |
| GP54    | &gt;48 | &gt;512| &gt;256| &gt;512| &gt;64 | 16  | 6   | &gt;128| 10  | 2   | 6   | 4   | 8   | 8   | &lt;0.5| 4   |
| GP68    | &gt;48 | &gt;512| &gt;256| &gt;512| &gt;64 | &gt;48 | 48  | 64  | 10  | 2   | 6   | &lt;0.5| 2   | 12  | 0.5 | 4   |
| NWE11   | &gt;48 | &gt;512| &gt;256| &gt;256| &gt;512| &gt;48 | 20  | 64  | 6   | 2   | 6   | &lt;0.5| 1   | 4   | &lt;0.5| 4   |
| ADD66   | &gt;48 | 512 | &gt;256| &gt;512| &gt;64 | 20  | 4   | 64  | 8   | 2   | 12  | 1   | &lt;1  | 4   | &lt;0.5| 6   |
| PS19    | &gt;48 | &gt;512| &gt;256| &gt;512| &gt;64 | &gt;48 | 256 | &gt;128| 10  | 2   | 8   | 1   | &lt;1  | 6   | &lt;0.5| 4   |
| PS26    | &gt;48 | &gt;512| &gt;256| &gt;512| &gt;64 | &gt;48 | 48  | 64  | 4   | 1   | 6   | 0.5 | &lt;1  | 4   | &lt;0.5| &lt;2  |
| PS68    | &gt;48 | 256 | &gt;256| &gt;512| &gt;64 | 10  | 4   | 64  | 6   | 2   | 4   | 0.5 | &lt;1  | 4   | &lt;0.5| &lt;2  |
| GT27    | &gt;48 | &gt;512| &gt;256| &gt;512| &gt;64 | &gt;48 | 48  | 64  | 8   | 4   | 6   | 1   | &lt;1  | 4   | &lt;0.5| 4   |
| GT44    | &gt;48 | &gt;512| &gt;256| &gt;512| &gt;64 | &gt;48 | 20  | 48  | 12  | &lt;1  | 8   | 1   | &lt;1  | 20  | &lt;0.5| &gt;64 |</p>
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Abbreviations: AMP, ampicillin; PIP, piperacillin; TIC, ticarcillin; CAR, carbenicillin; AMC, amoxicillin/clavulanate; SAM, ampicillin/ sulbactam; TZP, piperacillin/ tazobactam; CEF, cephalotin; CXM, cefuroxime; FEP, cefepime; FOX, cefoxitin; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; MEM, meropenem; ATM, aztreonam
CHAPTER THREE

CONCLUSIONS AND RECOMMENDATIONS
3.1. CONCLUSIONS

The following are significant findings:

- Two novel TEM-derived IRTs, TEM-145 and TEM-146, were detected in isolates PMM40 and NWE11 respectively.
  TEM-145 differed from TEM-1 by the L221M and R244H substitutions.
  TEM-146 differed from TEM-1 by T114P, M182I and R244H substitutions.

- A novel plasmid-mediated AmpC-type β-lactamase CMY-20, was detected in 3 isolates, viz., KEH1, KEH32 and KEH42.
  This novel differed from CMY-2 by the R125S, R146T and S309N substitutions.

- Changes were detected in the AmpC promoter/attenuator regions of isolate NGZ55:
  Extra G between -26 and -25; A(-10)C; extra A between +8 and +9; extra C between +39 and +38; extra C between +54 and +53

- OXA-1 was detected in 1 isolate, GJC33.

- The ESBL, CTX-M-1 was detected in 11 isolates, viz., NGZ55, PS19, PS68, GT27, ESH18, GJC33, GJC78, GJC79, GJC85, GJC87 and GJC91.

- An ESBL derived TEM β-lactamase, TEM-55 was detected in 1 organism, MDD-39.
• The ESBL, SHV-2, was detected in 2 isolates, GP45 and GP54.

• The universal presence of TEM-1 was evident in all *E. coli* isolates studied.

• β-lactamase genes were present in diverse combinations and in isolates with diverse plasmid profiles indicating the extensive mobilization of β-lactamase genes.
3.2. RECOMMENDATIONS

The following are recommended for further investigation:

- The novel TEM-145, TEM-146 and CMY-20 genes should be cloned into a recipient so that their unique resistance phenotypes and kinetic characteristics may be established.

- The identity of undetermined β-lactamases evident by IEF and not verified by PCR should be established.

- The outer membrane protein profiles of the isolates should be established to ascertain the contribution of permeability (if any) to the resistance profiles observed.
REFERENCES


type β-lactamases among clinical isolates of Enterobacteriaceae in Paris, France.


