BETA-LACTAMASE MEDIATED RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM STATE HOSPITALS IN KWAZULU-NATAL

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2008

Submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy in the School of Pharmacy and Pharmacology, Faculty of Health Sciences, University of KwaZulu-Natal

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.

ACKNOWLEDGEMENTS

I thank God for giving me the strength and courage to undertake this study

This study would not have been possible without the assistance, love and support of many people and my heartfelt thanks goes to:

My supervisor, Prof Sabiha Essack, thanks for the assistance, support, constructive criticism and guidance throughout this study. I really appreciated it.

My co-supervisor, Prof Willem Sturm, for the guidance and constructive criticism

My colleague and fellow student, Sara Govinden for all the help, support, technical assistance and friendship.

My colleagues in the Discipline of Pharmacy, especially Prof Thiru Govender, Les Murugan, Aasha Bagwandeen and Vassie Naidoo, thanks for the love, support, friendship and guidance.

My husband and children thank you for the unconditional love and support you have always given me.

TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION

1.1. β-LACTAM ANTIBIOTICS

β-lactam antibiotics, embracing penicillins with or without β-lactamase inhibitors, cephalosporins, cephamycins, 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-aminocephalosporanic 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 (Günseren *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; Míro *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, cephamycins 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 (gluatamic 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 β-lactamase affinity (lowering the K_m) and increasing V_{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-β-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 β-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 β-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 β-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 β-lactamases are one of the most clinically significant families of plasmid-mediated β-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 β-lactamase with activity against penicillins (Ma *et al.*, 2005). Point mutations of the genes encoding for SHV-type β-lactamases can change their profile of activity to include most

β-lactams with the exception of cephamycins 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-SHV 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-lacatamases 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 bla_{CTX} 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-1enzyme 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 world-wide, 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 cephamycins 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:

- Mocktar C, Govinden U, Sturm AW, Essack SY. (2007). TEM-145 and TEM-146 β-lactamases produced by *Escherichia coli* isolates from state hospitals in KwaZulu-Natal, South Africa. *Afr. J. Biotech.* 6: 493-496.

- Mocktar C, Govinden U, Sturm AW, Essack SY. (2008). CMY-20, a novel AmpC-type β-lactamase from South African clinical *Escherichia coli* isolates. *Diag. Microbiol. Infect. Dis.* 60: 405-408.

- Mocktar C, Govinden U, Sturm AW, Essack SY. (2008). 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. *Afr. J. Biotech.* 7:2547-2550.

African Journal of Biotechnology Vol. 6 (5), pp. 493-496, 5 March, 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

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

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Accepted 4 January, 2007

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 bla_{TEM}. 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 (Blásquez 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).

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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 has begun to emerge. The use of β-lactam-β-lactamase inhibitor combinations has selected mutant derivatives of the TEM and SHV families of class A β-lactamases that have become relatively resistant to inactivation by β-lactamase inactivators and thereby confer resistance to these combinations (Vakulenko et al., 1998). 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.

Primer	Sequence	Purpose of primer	Reference
TEM1(F)	5'-ATGAGTATTCAACATTTCCGTG-3'	Amp	Essack et al., 2001
TEM2(R)	5'-TTACCAATGCTTAATCAGTGAG-3'	Amp/Seq	Essack et al., 2001
TEM3(R)	5'-TTCTGTGACTGGTGAGTACT-3'	Seq	Essack et al., 2001
TEM4(R)	5'-GAGTAAGTAGTTCGCCAGTT-3'	Seq	Essack et al., 2001
TEM5(F)	5'-CTGCAGCAATGGCAACAAC-3'	Amp/Seq	Designed for this study

F, forward primer; R, reverse primer; Amp, amplification; Seq, sequencing.

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 (bioMérieux sa, Lyon, France). *E. coli* ATCC 25922 and *E. coli* NCTC 50192 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-parenteral, cefoxitin, ceftriaxone, cefotaxime, ceftazidime, cefepime, meropenem 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 (Biolab, 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 (Biolab, Johannesburg, South Africa) by the freeze-thaw method (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 solution (0.05% (w/v); Oxoid, Hampshire, England). An isoelectric point marker pI calibration kit (4.7 to 10.6; BDH, England) was the standard used.

PCR detection of bla_{TEM} genes

A suspension of colonies from an overnight culture on Nutrient Agar (Biolab, 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 bla_{TEM} 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 DQ105528 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 7.8 and 6.6 and 8.6 were detected for *E. coli* NWE11 and *E. coli* PMM40, respectively. PCR amplification revealed that both isolates harboured bla_{TEM} 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 pI values of 6.0 and 6.6 could be identified as transcripts of TEM β-lactamase genes and those with pI 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; L221M and R244H, while 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).

The R244H mutation, found in TEM-145 and TEM-146

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

Antibiotic	*E. coli* PMM40	*E. coli* NWE11
AMP	**>32**	**>32**
SAM	**>48**	**>48**
AMC	**>64**	**>64**
PIP	**>512**	**>512**
TZP	**96**	20 (I)
CEF	**>128**	**64**
CXM	8	8
FOX	8	8
CRO	<1	<1
CTX	<0.5	<0.5
CAZ	4	8
FEP	<1	<1
MEM	<0.5	<0.5
ATM	2	4

Abbreviations: AMP, ampicillin; SAM, ampicillin/ sulbactam; AMC, amoxicillin/clavulanate; PIP, piperacillin; TZP, piperacillin/tazobactam; CEF, cephalotin; CXM, cefuroxime-parenteral; FOX, cefoxitin; CRO, ceftriaxone; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; MEM, meropenem; ATM, aztreonam; (I) denotes intermediate resistance, boldface denotes resistance.

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, glycine and leucine (www.lahey.org/studies/temtable.asp, last accessed June 2006). In the TEM-1 enzyme, Arg-244 is located on strand β_4. Its side chain overlaps strand β_3 and thereby takes part in interactions with the substrate in the active site (Bret et al., 1997). When Arg-244 is replaced by an amino acid with a short side chain such as cysteine, serine or histidine, the enzyme-substrate interaction is modified and affinity for the substrate decreases. Moreover, the shorter side chains of these residues would be unable to activate the water molecule involved in the inactivation process of clavulanate (Bret et al., 1997; Chaibi et al., 1999). Site-specific mutations induced at the active site of TEM β-lactamase has confirmed that replacement of arginine by serine, cysteine or threonine leads to clavulanic acid resistance (Bret et al., 1997). Sulbactam and tazobactam are thought to use a different mechanism and are not dependent on the structurally conserved water molecule (Chaibi et al., 1999).

The M182I found in TEM-146 β-lactamase has not been reported before. The other substitution reported at this position is the substitution of a threonine for the methionine. The M182T mutation has been reported for TEM-20, TEM-32 (IRT-3), TEM-43, TEM-52, TEM-63/64, TEM-72, TEM-87, TEM-88, TEM92-94, TEM-106, TEM-107, TEM-113, TEM-124, TEM-126, TEM-131, TEM-135 and TEM-149 (www.lahey.org/studies/temtable.asp, last assessed June 2006). Residue182 which is located just before the α-8 helix is far from the active site (Chaibi et al., 1999) and the M182T mutation has been found in several natural ESBL mutant enzymes. It has been shown by thermodynamic and enzymatic studies that this substitution does not affect β-lactamase activity but acts as a global stabilizer. The M182T substitution restores the stability lost by substitutions near the active site and, hence, is a rescue substitution for TEM ESBL-type mutants (Delmas et al., 2005). The T114P mutation is the first report of a mutation at this position.

IEF analysis showed that the *E. coli* isolates each produced two enzyme bands but each of the isolates possessed three enzymes, i.e., TEM-1, the *E. coli* chromosomal AmpC and either TEM-145 or TEM-146. Also, the reported pI values for most IRTs has been 5.2 to 5.4 (Chaibi et al., 1999). A possible reason for the presence of only two IEF bands instead of three could be the minimal expression of TEM-145/TEM-146, hence two IEF bands. The pI values of 6.0 and 6.6 is not typical of an IRT. This could possibly be attributed to minimal expression of TEM-145 and TEM-146.

Conclusion

The resistance of the isolates to the inhibitor combinations could be attributed to the presence of TEM-145 and TEM-146 β-lactamases 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.

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Available online at www.sciencedirect.com
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Diagnostic Microbiology and Infectious Disease 60 (2008) 405–408
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

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.

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

1. Introduction

The emergence of resistance to the cephalosporins, cephamycins, 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-1 and ACT-1), Central and South American cluster (FOX-1 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* J62-1 was used as the recipient in the conjugation experiments (Livermore and Corkill, 1992).

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doi:10.1016/j.diagmicrobio.2007.11.009

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, cefotaxime, ceftriaxone, ceftazidime, 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 (Biolab, 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 (Biolab) 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 bla_{CMY} gene.

2.4. β-Lactamase analysis

The β-lactamases of the *E. coli* clinical isolates were extracted from a nutrient broth (Biolab) culture by the freeze–thaw method (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, Basingstoke, UK). An isoelectric point marker pI 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 (Biolab) 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 bla_{TEM} genes were carried out as described previously (Essack et al., 2001). The amplification mixture for the detection of the bla_{CMY} 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 bla_{SHV} (Essack et al., 2000), bla_{CTX} (Edelstein et al., 2003), bla_{MOX} (Pérez-Pérez and Hanson, 2002), bla_{FOX} (Pérez-Pérez and Hanson, 2002), bla_{DHA} (Pérez-Pérez and Hanson, 2002), bla_{ACC} (Pérez-Pérez and Hanson, 2002), and bla_{ACT} (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.

Table 1
Primers used in PCR studies

Primer	Sequence	Purpose of primer	Reference
TEM 1(F)	5′-ATGAGTATTCAACATTTCCGTG-3′	Amp	Essack et al. (2001)
TEM 2(R)	5′-TTACCAATGCTTAATCAGTGAG-3′	Amp/Seq	Essack et al. (2001)
TEM 3(R)	5′-TTCTGTGACTGGTGAGTACT-3′	Seq	Essack et al. (2001)
TEM 4(R)	5′-GAGTAAGTAGTTCGCCAGTT-3′	Seq	Essack et al. (2001)
TEM 5(F)	5′-CTGCAGCAATGGCAACAAC-3′	Amp/Seq	Designed for this study
CMY-F	5′-GACAGCCTCTTTCTCCACA-3′	Amp/Seq	Zhao et al. (2001)
CMY-R	5′-TGGAACGAAGGCTACGTA-3′	Amp/Seq	Zhao et al. (2001)
CMY-F1	5′-GAAAAAATCGTTATGCTGC-3	Amp/Seq	Designed for this study
CMY-R1	5′-CCGTATAGGTGGCTAAGTGC-3′	Amp/Seq	Designed for this study
CMY-F2	5′-GTGAAATCCAGCGTTATTGA-3′	Amp/Seq	Designed for this study
CMY-R2	5′-CTTTTCAAGAATGCGCCAGG-3′	Amp/Seq	Designed for this study

F = forward primer; R = reverse primer; Amp = amplification; Seq = sequencing.

gov/Blast; last accessed June 2006). The nucleotide and amino acid sequences of the CMY-type β-lactamases of the isolates were deposited in GenBank and assigned the following accession numbers: *E. coli* KEH1 AY960293, *E. coli* KEH32 AY936494, and *E. coli* KEH42 AY940316.

3. Results

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-type 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 BIL-1.

Table 2
MIC (μg/mL) profiles of the *E. coli* clinical isolates and the transconjugant of *E. coli* KEH32

Antibiotic	*E. coli* KEH1	*E. coli* KEH32	*E. coli* KEH42	Transconjugant *E. coli* KEH32
AMP	>48	>48	>48	>48
PIP	>512	>512	>512	>512
TIC	>256	>256	>256	>256
CAR	>512	>512	>512	>512
AMC	>64	>64	>64	48
SAM	>48	>48	>48	>48
TZP	>512	>512	>512	48
CEF	>128	>128	>128	96
CXM	96	>128	>128	>128
FEP	4	4	8	4
FOX	>96	>96	>96	>96
CTX	>256	>256	>256	128
CRO	>96	>96	>96	32
CAZ	>128	>128	>128	>128
MEM	1	1	1	<0.5
ATM	>64	>64	>64	48
AMK	>256	>256	>256	6
TMP/SXT	>256	>256	>256	Not done
CHL	32	>64	>64	16

AMP = ampicillin; PIP = piperacillin; TIC = ticarcillin; CAR = carbenicillin; AMC = amoxicillin/clavulanate; SAM = ampicillin/ sulbactam; TZP = piperacillin/ tazobactam; CEF = cefalotin; CXM = cefuroxime–parenteral; FEP = cefepime; FOX = cefoxitin; CTX = cefotaxime; CRO = ceftriaxone; CAZ = ceftazidime; MEM = meropenem; ATM = aztreonam; AMK = amikacin; TMP/SXT = trimethoprim sulfamethoxazole; CHL = chloramphenicol.

This study also reported a mutation in the same position but with a different amino acid (S309N). This is the 1st report of the R125S and R146T mutations. All the isolates were negative for the presence of the other β-lactamases tested. The isolates did not display any changes in the promoter and attenuator regions of the chromosomal AmpC. The *E. coli* J62-1 transconjugants all showed similar resistance profiles as their donors. PCR showed the presence of a CMY-type β-lactamase.

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 oxyimino 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 ceftazidime 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 mercuric 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 BIL-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 (Bauernfeind 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 cephamycin-resistant Gram-negative bacteria.

Acknowledgments

This study was funded by research grants from the Medical Research Council (MRC), National Research Foundation (NRF), and the University of KwaZulu-Natal.

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African Journal of Biotechnology Vol. 7 (15), pp. 2547-2550, 4 August, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

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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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-lactamases (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 (-35 and -10) 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

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Table 1. Primers used in PCR studies.

Primer	Sequence	Purpose of primer	Reference
TEM 1(F)	5'-ATGAGTATTCAACATTTCCGTG-3'	Amp	Essack et al., 2001
TEM 2(R)	5'-TTACCAATGCTTAATCAGTGAG-3'	Amp/Seq	Essack et al., 2001
TEM 3(R)	5'-TTCTGTGACTGGTGAGTACT-3'	Seq	Essack et al., 2001
TEM 4(R)	5'-GAGTAAGTAGTTCGCCAGTT-3'	Seq	Essack et al., 2001
TEM 5(F)	5'-CTGCAGCAATGGCAACAAC-3'	Amp/Seq	Designed for this study
COL A	5'-ACGACGCTCGCGCCTTA-3'	Amp/Seq	Bret et al., 1998
COL B	5'-AAGAATCT GCCAGGCGGC-3'	Amp/Seq	Bret et al., 1998
AmpC R1	5'-GTTTGCTGCGTGACGGGCTG -3'	Amp/Seq	Designed for this study
AmpC F1	5'-ACCACGCGAT GCACGATCTG-3'	Amp/Seq	Designed for this study
AmpC R2	5'- GATGACA GCAAGGAAAAGCGGAG-3'	Amp/Seq	Designed for this study
AmpC F2	5'-GCCGGTAAATCCTGACACCATC-3'	Amp/Seq	Designed for this study
CTX-MF	5'-TTTGCGATGTGCAGTACCAGTAA-3'	Amp/Seq	Edelstein et al., 2003
CTX-MR	5'-CGATATCGTTGGTGGTGCCATA-3'	Amp/Seq	Edelstein et al., 2003

F, forward primer; R, reverse primer; Amp, amplification; Seq, sequencing.

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 API20E 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, ceftazidime, 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 (Biolab, 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 pI calibration kit (4.7 to 10.6; BDH, England) was used as the standard.

PCR detection of bla_{TEM}, bla_{CTX}, and bla_{AmpC} genes

Bacterial DNA was prepared by suspending few freshly grown colonies from an overnight Nutrient Agar (Biolab, 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 bla_{TEM} was carried out as described by Essack et al. (2001). The amplification mixture for the detection of bla_{CTX} and bla_{AmpC} 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 ethidium 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 pIs of 6.5 and 6.6. PCR and sequencing data showed that the isolate harboured TEM-1 and CTX-M1. Comparison of

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

Isolate	AMP	PIP	AMC	TZP	CEF	CXM	FEP	FOX	CTX	CRO	CAZ	MEM	ATM
NGZ55	6	10	>64	4	>128	8	2	>96	1	2	6	0.5	4
ATCC 25922	4	ND	6	1	24	4	<1	2	<0.5	<1	1	<0.5	<2

AMP, ampicillin; PIP, piperacillin; AMC, amoxicillin/clavulanate; TZP, piperacillin/tazobactam; CEF, cephalotin; 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[a].

Isolate	Mutation
E. coli 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

[a]Compared with *E. coli* K-12 (Caroff et al., 2000).

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 TAC**A**AT to TAC**C**AT (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 cephamycins 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 oxyiminocephalosporins. 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 16 bp 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 17 bp between the -10 box

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 ceftazidime 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 DNA sequencing studies have shown the presence of only TEM-1 and CTX-M1 in this isolate. This isolate, in 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 TAC**A**AT to TAC**C**AT (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

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

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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

Category: Original Article

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

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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**

47 Introduction

48 The rapid increase of β-lactam resistance amongst members of the family
49 Enterobacteriaceae is a major source of concern.[1] The increased and repeated use of β-
50 lactam antibiotics has led to them becoming ineffective, mainly as a result of the
51 production of β-lactamases such as TEM-1 and SHV-1, which hydrolyze penicillins and
52 narrow spectrum cephalosporins.[2,3,4] E*scherichia coli* which is a common component of
53 the intestinal flora of humans and animals, can cause serious diseases including
54 septicaemia and urinary and wound infections.[5] In clinical isolates of *E. coli,* plasmid-
55 mediated TEM-1 β-lactamase is the most frequently encountered enzyme. This enzyme
56 confers resistance to aminopenicillins and carboxypenicillins without affecting their
57 susceptibility to third generation cephalosporins.[2]
58 In order to overcome this resistance, two types of β-lactams were developed, viz., β-
59 lactams resistant to hydrolysis such as extended spectrum cephalosporins; and β-
60 lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam.[4,6] Although β-
61 lactamase inhibitors have poor antibacterial activities by themselves, they covalently bind
62 to the active sites of class A β-lactamases and inhibit them.[7]
63 However, with increasing use of these antimicrobial agents, resistance again began to
64 emerge. The first TEM-type extended spectrum β-lactamases (ESBLs) were characterized
65 in 1987 and TEM penicillinases resistant to inhibitors were characterized in the 1990s.[8]
66 Inhibitor-resistant TEM β-lactamases (IRTs) have been detected in several members of
67 the Enterobacteriaceae from different countries globally.[9]
68 We investigated β-lactamase mediated resistance in *E. coli* isolates obtained from a
69 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[10] 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.[11] Isoelectric focusing (IEF) was performed on ampholine polyacrylamide gels (pH 3.5- 9.5; Amersham Biosciences, Uppsala, Sweden) using an isoelectric point marker pI 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.[12] Plasmid sizes were calculated using computer software (Syngene-Gene Genius, Bio Imaging Systems, Syngene, Cambridge, United Kingdom).

PCR detection of bla_{TEM}, bla_{SHV}, bla_{CTX}, bla_{CMY}, bla_{OXA} and bla_{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 bla_{TEM} and bla_{SHV} genes,[13] bla_{CMY}[14] and bla_{OXA}[15] were carried out as described previously. The amplification mixture for the detection of bla_{CTX} and bla_{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

115 cycles of 1min at 94°C, 1min at 56°C, 1 min at 72°C and a final extension step of 7 min at
116 72°C. PCR products (5µl) were analyzed by gel electrophoresis. Gels were stained with
117 ethidium bromide and photographed using UV illumination. PCR products were purified
118 and sequenced (Spectrumedix SCE2410 genetic analysis system, Spectrumedix, State
119 College, Pennsylvania, USA). The isolates were also tested for the presence of bla_{FOX},
120 bla_{MOX}, bla_{BIL}, bla_{LAT}, bla_{ACT}, bla_{ACC} and bla_{DHA} (data not shown).
121
122 Results
123 The MIC results are shown in Table 3. From the table it can be seen that 38 (100%), 34
124 (89%) and 26 (68%) of the isolates displayed reduced susceptibility to amoxicillin/
125 clavulanate, ampicillin/sulbactam and piperacillin/tazobactam respectively. All 38
126 isolates also displayed reduced susceptibility to cephalothin. With the exception of *E. coli*
127 NGZ55 and *E. coli* GJC95, all the isolates were resistant to ampicillin, piperacillin,
128 carbenicillin and ticarcillin. The only antibiotic to which all the isolates were sensitive
129 was meropenem. Table 3 summarizes the plasmid profiles and β-lactamase characteristics
130 of the isolates. From the table it can be seen that 1-5 plasmids ranging in size from 13.4kb
131 to 311.1kb were detected in the isolates. No plasmids were detected in isolates ESH31,
132 GJC53, GJC85, GJC95 and ADD66. TEM-1 was found in all isolates, whilst TEM-55
133 was found in isolate MDD39. Isolates PMM40 and NWE11 produced the inhibitor-
134 resistant enzymes, TEM-145 and TEM-146 respectively.[18] A plasmid-mediated AmpC
135 type β-lactamase, CMY-20 was produced by isolates KEH1, KEH32 and KEH42.[14]
136 Isolate GJC33 produced OXA-1 whilst 12 of the isolates produced CTX-M1. SHV-2 was
137 detected in 2 of the isolates.

138 Discussion

139 Inhibitor resistance in *E. coli* may be attributed to: hyperproduction of class C
140 chromosomal β-lactamase (cephalosporinase), hyperproduction of plasmid-mediated
141 TEM-1 or TEM-2, production of an IRT or the production of a relatively inhibitor-
142 resistant OXA-type β-lactamase.[19,20]

143 The first amoxicillin/clavulanate-resistant *E. coli* isolate was noted in 1987 in France.[21]
144 Amoxicillin/clavulanate is a β-lactam β-lactamase inhibitor combination that has
145 antibacterial activity against gram positive, gram negative and anaerobic microorganisms.
146 *E. coli*, one of the most common pathogens in community acquired and nosocomial
147 infections, is usually susceptible to amoxicillin/clavulanate. [15] However, with increasing
148 use, resistance to amoxicillin/clavulanic acid appeared, first in *E. coli* isolates, then in
149 other species of enterobacteria.[19]

150 A study by Henquell et al.[2] showed that 40% of *E. coli* isolates from hospitals and 18% of
151 isolates from private laboratories were resistant to amoxicillin/clavulanate. Other studies
152 carried out in the United States[15] and France[21] showed that 3% and 5% of the *E. coli*
153 isolates were resistant to amoxicillin/clavulanate respectively. A recent study in
154 Pakistan[22] which investigated the prevalence and antibiotic resistance of urinary tract
155 pathogens showed that 58% of the *E. coli* isolates were resistant to amoxicillin/
156 clavulanate whilst a study in Tanzania[23] which conducted a surveillance of antimicrobial
157 resistance in a tertiary hospital revealed that 28% of the *E. coli* isolates were resistant to
158 amoxicillin/clavulanate. Two studies conducted in South Africa[26,27] using bacterial
159 cultures obtained from 7 private pathology practices in all the private hospitals in
160 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.[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.[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.[18] Residue 182 is located just before the α-helix and this is far from the binding site.[3] This substitution does not affect the β-lactamase activity but rather acts as a global stabilizer.[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.[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.[7,27]

184 Isolates PMM40 and NWE11 displayed resistance to amoxicillin/clavulanate and
185 ampicillin/sulbactam whilst isolate PMM40 was resistant and isolate NWE11 displayed
186 intermediate resistance to piperacillin/tazobactam.
187 A plasmid-mediated AmpC-type β-lactamase, viz., CMY-20, was found in 3 of the
188 isolates. These isolates, viz., KEH1, KEH32 and KEH42 were resistant to all the β-
189 lactamases tested with the exception of meropenem and cefepime. In addition, these 3
190 isolates were also resistant to other antibiotics such as chloramphenicol, gentamicin and
191 trimethoprim/sulfametoxazole (data not shown). CMY-20 belongs to the *Citrobacter*
192 *freundii* group and has the following substitutions compared to CMY-2: R125S, R146T
193 and S309N.[14] Bacteria with plasmid-mediated AmpC enzymes are generally resistant to
194 inhibitor combinations, with the possible exception of tazobactam.[28] All 3 isolates were
195 resistant to all the antibiotic-inhibitor combinations tested.
196 GJC-33 displayed reduced susceptibility to all three inhibitor combinations. This isolate
197 in addition to harbouring TEM-1, also harboured OXA-1. OXA-type β-lactamases confer
198 resistance to ampicillin and cephalosporin and they are poorly inhibited by clavulanic
199 acid[29] and this could have contributed to the inhibitor resistance of this isolate.
200 Isolate NGZ55 was resistant to cefoxitin but only TEM-1 and CTX-M1 was found in this
201 isolate. Analysis (data not shown) of the chromosomal AmpC promoter region of this
202 isolate revealed that there were changes in the promoter region.[30] This isolate had an
203 extra base pair between the -35 and -10 region and this created an optimal distance of 17
204 base pairs in this region. A distance of 16 base pairs leads to low level of transcription of
205 this gene whilst an optimal distance of 17 base pairs plays a role in promoter strength.[31]
206

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

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

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

2. Poster presentation: Novel inhibitor-resistant β-lactamases TEM-145 and TEM-146, from two *Escherichia coli* clinical isolates. Antibiotic Resistance November 2007, Kololi, The Gambia

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

Isolate	Source	Plasmid Size(s)	pI value(s)	β-lactamase (s) found
NGZ55	Pus swab	129.21 84.61	6.51 6.6	TEM-1 CTX-M1
GP6	Urine	112.05	6.13 7.93	TEM-1
GP34	Urine	112.05	5.94 7.45	TEM-1
GP45	Pus swab	84.55 28.39	5.94 7.17	TEM-1 SHV-2
GP54	Pus swab- sub cutaneous	129.00 36.20	5.08 7.22	TEM-1 SHV-2
GP68	Urine	112.05 54.06	7.75	TEM-1
NWE11	Urine	129.21 112.2	6.00 7.75	TEM-1 TEM-146
ADD66	Urine	ND	6.92	TEM-1
PS19	Pus swab- septic wound	129.21	6.32 7.26	TEM-1 CTX-M1
PS26	Pus swab- septic abdominal wound	171.36 97.43	5.57 6.2	TEM-1
PS68	Pus swab- cellulitis and blisters	148.8	6.23 8.11	TEM-1 CTX-M1
GT27	Urine	197.33	6.69 8.68	TEM-1 CTX-M1
GT44	Urine	197.33 97.33 21.48	7.75	TEM-1
ESH18	Urine	148.8 15.47	5.55 7.93 8.12	TEM-1 CTX-M1
ESH31	Urine	ND	5.83 6.01 8.48	TEM-1
ESH90	Pus swab	137.77	6.19 8.21	TEM-1
KEH1	Peritoneal fluid	148.5	6.00 8.54	TEM-1 CMY-20
KEH32	Pus swab	148.5	5.85 8.75	TEM-1 CMY-20
KEH42	Peritoneal fluid	148.5	6.60 8.39	TEM-1 CMY-20
PMM40	Urine	112.05	6.6	TEM-1

			8.3	TEM-145
PMM86	Blood	226.56 97.34 58.34	5.92 8.21	TEM-1
MDD38	Urine	109.36	6.65	TEM-1
MDD39	Urine	127.57 32.32	6.88 7.75	TEM-1 TEM-55
EGU4	Pus swab- scrotal abscess	171.36	6.41 8.3	TEM-1
EGU5	Urine	171.36	6.01 7.75	TEM-1
EGU68	Urine	171.36 97.43	6.6 8.3	TEM-1
GJC5	Pus swab- right ear	211.76	6.51 8.2	TEM-1
GJC31	Urine	197.33	7.11 7.38 7.66 8.39	TEM-1
GJC33	Urine	158.82	6.13 6.7	TEM-1 OXA-1
GJC35	Urine	311.01 108.18 41.71 19.14 13.43	6.03 6.98	TEM-1 CTX-M1
GJC53	Urine	ND	6.04 7.55	TEM-1
GJC65	Urine	108.18 70.91 33.72 22.05	6.04 7.55	TEM-1
GJC78	Urine	151.82 43.73	6.79 8.68	TEM-1 CTX-M1
GJC79	Urine	345.65	6.41 8.49	TEM-1 CTX-M1
GJC85	Urine	ND	6.79 8.35	TEM-1 CTX-M1
GJC87	Urine	139.42	6.32 8.68	TEM-1 CTX-M1
GJC91	Urine	127.39 31.42	6.60 8.68	TEM-1 CTX-M1
GJC95	unknown	ND	6.51	TEM-1

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371 ND- not detected

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373 Table 2: Primers used in PCR studies
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Primer	Primer Sequence	Purpose of primer	Reference
COL A	5'-ACGACGCTCGCGCCTTA-3'	Amp/Seq	17
COL B	5'-AAGAATCT GCCAGGCGGC-3'	Amp/Seq	17
AmpC R1	5'-GTTTGCTGCGTGACGGGCTG -3'	Amp/Seq	Designed for this study
AmpC F1	5'-ACCACGCGAT GCACGATCTG-3'	Amp/Seq	Designed for this study
AmpC R2	5'- GATGACA GCAAGGAAAAGCGGAG-3'	Amp/Seq	Designed for this study
AmpC F2	5'-GCCGGTAAATCCTGACACCATC-3'	Amp/Seq	Designed for this study
CTX-MF	5'-TTTGCGATGTGCAGTACCAGTAA-3'	Amp/Seq	16
CTX-MR	5'-CGATATCGTTGGTGGTGCCATA-3'	Amp/Seq	16

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376 F, forward primer; R, reverse primer; Amp, amplification; Seq, sequencing
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405 Table 3: MICs (μg/ml) of the *E. coli* clinical isolates
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Isolate	AMP	PIP	TIC	CAR	AMC	SAM	TZP	CEF	CXM	FEP	FOX	CTX	CRO	CAZ	MEM	ATM
NGZ55	6	10	4	16	>64	8	4	>128	48	4	>96	1	2	6	0.5	4
GP6	>48	>512	>256	>512	>64	48	6	48	10	<1	8	<0.5	2	6	0.5	6
GP34	>48	512	256	>512	>64	20	>512	24	8	<1	4	<0.5	2	4	0.5	2
GP45	>48	512	>256	>512	>64	12	6	>128	10	1	4	1	4	4	0.5	4
GP54	>48	>512	>256	>512	>64	16	6	>128	10	2	6	4	8	8	<0.5	4
GP68	>48	>512	>256	>512	>64	>48	48	64	10	2	6	<0.5	2	12	0.5	4
NWE11	>48	>512	>256	256	>64	>48	20	64	6	2	6	<0.5	<1	4	<0.5	4
ADD66	>48	512	>256	>512	>64	20	4	64	8	2	12	1	<1	4	<0.5	6
PS19	>48	>512	>256	>512	>64	>48	256	>128	10	2	8	1	<1	6	<0.5	4
PS26	>48	>512	>256	>512	>64	>48	48	64	4	1	6	0.5	<1	4	<0.5	<2
PS68	>48	256	>256	>512	>64	10	4	64	6	2	4	0.5	<1	4	<0.5	<2
GT27	>48	>512	>256	>512	>64	>48	48	64	8	4	6	1	<1	4	<0.5	4
GT44	>48	>512	>256	>512	>64	>48	20	48	12	<1	8	1	<1	20	<0.5	>64

ESH18	>48	>512	>256	>512	>64	>48	10	16	6	<1	6	0.5	<1	4	<0.5	<2
ESH31	>48	>512	>256	>512	>64	>48	10	64	6	<1	6	0.5	<1	4	<0.5	<2
ESH90	>48	>512	>256	>512	>64	>48	32	64	6	2	8	1	2	6	<0.5	4
KEH1	>48	>512	>256	>512	>64	>48	>512	>128	> 96	4	>96	>256	>128	>128	1	>64
KEH32	>48	>512	>256	>512	>64	>48	>512	>128	> 96	4	>96	>256	>128	>128	1	>64
KEH42	>48	>512	>256	>512	>64	>48	>512	>128	> 96	8	>96	>256	>128	>128	1	>64
PMM 40	>48	>512	>256	>512	>64	>48	96	>128	6	2	6	0.5	<1	6	<0.5	2
PMM 86	>48	>512	>256	>512	>64	>48	96	64	4	<1	4	1	<1	4	<0.5	4
MDD 38	>48	>512	>256	>512	>64	>48	48	64	8	<1	6	1	<1	4	<0.5	<2
MDD 39	>48	512	>256	>512	>64	>48	>512	64	6	4	6	<0.5	<1	4	<0.5	<2
EGU4	>48	>512	>256	>512	>64	>48	16	64	6	4	8	1	<1	4	<0.5	4
EGU5	>48	>512	>256	>512	>64	>48	32	64	8	1	8	1	<1	4	<0.5	2
EGU68	>48	512	>256	>512	>64	32	8	64	6	1	6	2	<1	4	<0.5	4
GJC5	>48	>512	>256	32	>64	>48	64	32	10	8	6	2	8	>128	1	>64
GJC31	>48	256	>256	>512	>64	48	64	32	12	2	16	96	32	4	0.5	6

GJC33	>48	>512	>256	>512	>64	32	48	24	10	4	4	1	<1	4	<0.5	4
GJC35	>48	512	>256	>512	>64	>48	64	>128	8	<1	6	<0.5	2	4	<0.5	6
GJC53	>48	> 48	>256	>512	>64	8	4	>128	12	1	8	32	6	6	0.5	6
GJC65	>48	>512	>256	>512	>64	32	>512	48	8	1	6	<0.5	<1	4	<0.5	2
GJC78	>48	>512	>256	512	>64	>48	96	48	10	>32	8	<0.5	4	>128	1	>64
GJC79	>48	>512	>256	>512	>64	>48	48	96	10	6	8	<0.5	4	64	0.5	>64
GJC85	>48	>512	>256	>512	>64	>48	>512	64	10	<1	6	<0.5	2	6	0.5	6
GJC87	>48	>512	>256	>512	>64	>48	20	64	8	1	8	<0.5	<1	6	0.5	4
GJC91	>48	>512	>256	>512	>64	32	20	32	6	<1	6	<0.5	<1	4	<0.5	4
GJC95	2	10	6	12	>64	2	20	1	6	1	6	<0.5	<1	4	<0.5	<2

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.

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