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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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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, Claifornia, 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.

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