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A novel ceftazidime-hydrolysing extended-spectrum β-lactamase, CTX-M-54, with a single amino acid substitution at position 167 in the omega loop

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Objectives: **To characterize a novel ceftazidime-hydrolysing CTX-M mutant, designated CTX-M-54, produced by *Klebsiella pneumoniae* clinical isolate BDK0419 and to investigate its genetic environment.**

Methods: **Antimicrobial susceptibilities were determined by disc diffusion and agar dilution methods, and the double-disc synergy test was carried out. Detection of genes encoding class A β-lactamases was performed by PCR amplification, and the genetic organization of the *bla*$_{CTX-M-54}$ gene was investigated by PCR and sequencing of the regions surrounding this gene. Kinetic parameters were determined from purified CTX-M-54.**

Results: **The strain BDK0419 contained a transferable plasmid with a molecular size of ~21 kbp that carries both *bla*$_{SHV-2a}$ and *bla* $_{CTX-M-54}$ β-lactamase genes, along with two other plasmids. The *bla*$_{CTX-M-54}$ gene was flanked upstream by an IS*Ecp1* insertion sequence and downstream by an IS*903*-like element. CTX-M-54 had a P167Q substitution within the omega loop region of class A β-lactamases compared with the sequence of CTX-M-3. The MIC of ceftazidime for *K. pneumoniae* BDK0419 was 16-fold higher than that of cefotaxime; however, the kinetic parameter of CTX-M-54 against ceftazidime revealed a low catalytic efficiency.**

Conclusions**: This work shows once again that novel CTX-M enzymes with an expanded activity towards ceftazidime through a single amino acid substitution can be identified from clinical isolates. Thus, detection of CTX-M enzymes can no longer be based solely on the resistance phenotypes of clinical isolates towards ceftazidime and cefotaxime.**

Keywords: cefotaximase, IS*Ecp1*, IS*903*

Introduction

CTX-M-type extended-spectrum β-lactamases (ESBLs), the most widespread enzymes among non-TEM and non-SHV plasmid-mediated ESBLs, were initially reported in the second half of the 1980s in Europe.[1] As the designation 'CTX' indicates, these enzymes preferentially hydrolyse cefotaxime but not ceftazidime. However, there have been recent reports of CTX-M mutants exhibiting a significant hydrolytic activity against ceftazidime. Amino acid substitutions at positions 167 and 240 (Ambler's numbering scheme) in CTX-M-type enzymes have been associated with expansion of activity towards ceftazidime. CTX-M-15,

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CTX-M-16 and CTX-M-27 harbour an Asp→Gly substitution at position 240.[2–4] CTX-M-19 (derived from CTX-M-18) and CTX-M-23 (derived from CTX-M-19) harbour a Pro→Ser and a Pro→Thr substitution at position 167 in the omega loop, respectively.[5,6]

This report identifies another ceftazidime-hydrolysing CTX-M mutant, designated CTX-M-54. CTX-M-54 differed from CTX-M-3 only by the substitution Pro-167→Gln and is the third CTX-M enzyme harbouring an amino acid substitution at position 167 after CTX-M-19 and CTX-M-23.

Materials and methods

Bacterial strains

Klebsiella pneumoniae clinical isolate BDK0419 was identified with the API 20E system (bioMérieux, Marcy l'Etoile, France). *Escherichia coli* BL21(DE3) was the host for cloning experiments, and *E. coli* J53 Azide^R was used as a recipient strain for conjugative transfer. *E. coli* ATCC 25933 was used as an MIC reference strain.

Antimicrobial susceptibility testing

Antibiotic-containing discs (BBL, Cockeysville, MD, USA) were used for routine antibiograms by disc diffusion assay according to the recommendations of the CLSI.[7] The double-disc synergy test was carried out on Mueller–Hinton agar (Difco Laboratories, Detroit, MI, USA) with discs of ceftazidime, cefotaxime and aztreonam, each containing 30 μg of the drug, placed at distances of 20 mm (centre to centre) from a disc containing amoxicillin–clavulanic acid (20 μg/10 μg) in the centre of the plate.[8] MICs were determined by the agar dilution method with Mueller–Hinton agar with an inoculum of 10^4 cfu.[9] MICs of β-lactams were determined alone or in combination with a fixed concentration of clavulanic acid (4 mg/L).

PCR experiments

Plasmid DNA of *K. pneumoniae* BDK0419 was extracted as described previously,[10] and this DNA was used as a template in PCR experiments with a series of primers designed for the detection of class A β-lactamase genes and their extended-spectrum derivatives: bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{PER\text{-}1}$, $bla_{VEB\text{-}1}$, $bla_{IBC/GES}$ and bla_{TLA} (Table 1). The PCR products were subjected to direct sequencing by the dideoxynucleotide chain-termination method with an automatic DNA sequencer (ABI 3700, Perkin-Elmer, Foster City, CA, USA). Both strands of the PCR products were sequenced twice.

The genetic organization of the $bla_{CTX\text{-}M\text{-}54}$ gene was investigated by PCR and sequencing of the regions surrounding this gene. The internal IS*Ecp1* forward primers (TN1-F and BTN-F) and CTX-M-1 reverse primers (CTX-M-1R and FM-1R) were used to investigate the promoter regions of the $bla_{CTX\text{-}M\text{-}54}$ gene. PCR primers corresponding to sequences upstream (*pemK* and IS*Ecp1*) and downstream (IS*903*) of the $bla_{CTX\text{-}M\text{-}54}$ gene were also used (Table 1).

Table 1. Sequences of the primers used in the study

PCR target	Primer name	Primer sequence
$bla_{CTX\text{-}M}$ (CTX-M-1 cluster)	CTX-M-1F	5′-CCGTCACGCTGTTGTTAGG-3′
	CTX-M-1R	5′-GACGATTTTAGCCGCCGAC-3′
	BM-1F	5′-ACTATGGCACCACCAACGAT-3′
	FM-1R	5′-TTCGGTTCGCTTTCACTTTT-3′
$bla_{CTX\text{-}M}$ (CTX-M-2 cluster)	CTX-M-2F	5′-CGG TGC TTA AAC AGA GCG AG-3′
	CTX-M-2R	5′-CCA TGA ATA AGC AGC TGA TTG CCC-3′
$bla_{CTX\text{-}M}$ (CTX-M-8 cluster)	CTX-M-8F	5′-ACG CTC AAC ACC GCG ATC-3′
	CTX-M-8R	5′-CGT GGG TTC TCG GGG ATA A-3′
$bla_{CTX\text{-}M}$ (CTX-M-9 cluster)	CTX-M-9F	5′-GAT TGA CCG TAT TGG GAG TTT-3′
	CTX-M-9R	5′-CGG CTG GGT AAA ATA GGT CA-3′
$bla_{PER\text{-}1}$	PER-1F	5′-GTT AAT TTG GGC TTA GGG CAG-3′
	PER-1R	5′-CAG CGC AAT CCC CAC TGT-3′
bla_{VEB}	VEB-F	5′-ACC AGA TAG GAG TAC AGA CAT ATG A-3′
	VEB-R	5′-TTC ATC ACC GCG ATA AAG CAC-3′
bla_{GES} and bla_{IBC}	GES/IBC-F	5′-GTT AGA CGG GCG TAC AAA GAT AAT-3′
	GES/IBC-R	5′-TGT CCG TGC TCA GGA TGA GT-3′
bla_{TLA}	TLA-F	5′-CGC GAA AAT TCT GAA ATG AC-3′
	TLA-R	5′-AGG AAA TTG TAC CGA GAC CCT-3′
pemK	PK-F	5′-AGGGGAAATCTGGCTTGTCT-3′
	PK-R	5′-TCTGTCGAAACAATGGGTCA-3′
IS*Ecp1*	TN1-F	5′-TCTGCTCCTTGAGAATGCAA-3′
	TN1-R	5′-TCGCCCAAAATGACTTTAGC-3′
	BTN-F	5′-CGGTGGGTCATCTCTTGCTA-3′
	FTN-R	5′-TAATTCGTCGCAAAATGCAA-3′
IS*903*	IS9-F	5′-TGGCCCACCTACAATAAAGC-3′
	IS9-R	5′-GGCATACCTGCTTTCGTCAT-3′
	FIS9-R	5′-TGCCTCGTGAAGAAGGTGTT-3′

Mating-out assays

Conjugation experiments were carried out between *K. pneumoniae* BDK0419 (donor) and azide-resistant recipient strain *E. coli* J53 on Mueller–Hinton agar plates.[11] Transconjugants were selected on Mueller–Hinton agar plates supplemented with ceftazidime (2 mg/L) and sodium azide (100 mg/L).

Purification of CTX-M-54 β-lactamase

The PCR product obtained with primers CTX-M-54 pcrF (5′-GG GAA TTC CAT ATG GTT AAA AAA TCA CTG CG-3′; *Nde*I site underlined) and CTX-M-54 pcrR (5′-CCG CTC GAG CAA ACC GTC GGT GAC GAT TTT-3′; *Xho*I site underlined) was purified with a QIAquick column (Qiagen, Courtaboeuf, France) and ligated in the *Nde*I and *Xho*I sites of plasmid pET30a (Novagen, Milan, Italy). It was then subjected to confirmatory sequencing. The expression vector constructed, named pET30a-CTX-M-54, was introduced into *E. coli* BL21(DE3) competent cells. *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-54 was cultured at 37°C in 1 L of LB broth supplemented with kanamycin (50 mg/L). Isopropyl-β-D-thiogalactopyranoside (final concentration, 0.4 mM) was added when the culture reached an OD_{600} of 0.6, and the culture was incubated overnight at 20°C. The cells were harvested by centrifugation and were resuspended in 50 mL of buffer A [50 mM Tris (pH 7.0), 500 mM NaCl, 10 mM imidazole] and were then disrupted by microfluidizer at 15 000 psi. The cell lysate was finally centrifuged at 15 000 **g** for 40 min to remove cell debris. The cleared supernatant was loaded onto an Ni-NTA column (XK16, Amersham-Pharmacia-Biosciences, Milan, Italy) pre-equilibrated with buffer A. After loading, buffer B [50 mM Tris (pH 7.0), 500 mM NaCl, 25 mM imidazole] was used to wash the column. The β-lactamase was eluted with a linear gradient of imidazole (25–300 mM in 1 h). The fractions containing nitrocefin-hydrolysing activity and purity-confirmed by SDS–PAGE were pooled and dialysed with buffer C [50 mM Tris (pH 7.0), 300 mM NaCl without imidazole]. The final protein concentration was 0.315 mg/mL (purity >>90%).

Isoelectric focusing (IEF)

To determine the isoelectric point (pI), 5 μL of the condensed supernatant containing β-lactamase was loaded onto a Novex IEF Gel (pH 3-10; Invitrogen, Carlsbad, CA, USA) with an Xcell surelock Mini-Cell system (Invitrogen). Running conditions were 100 V constant for 1 h, 200 V constant for 1 h and 500 V for 30 min.[11] The pI of the β-lactamase was measured by staining the gel with a 0.05% solution of nitrocefin (Oxoid, Basingstoke, UK).

Kinetic measurements

Purified β-lactamase was used for kinetic measurements performed at 30°C with 100 mM sodium phosphate buffer (pH 7.0) with a Cary 300 Bio UV-visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). Wavelengths of 235 nm for benzylpenicillin (Sigma, St Louis, MO, USA); 236 nm for ampicillin (Sigma); 318 nm for aztreonam (Sigma); 482 nm for nitrocefin (Oxoid); 260 nm for ceftazidime (Sigma), cefotaxime and cefuroxime (Sigma); 265 nm for cefalothin (Sigma); and 260 nm for cefaloridine (Sigma) were used. Extinction coefficients of each antibiotic substrate used in the spectrophotometric assays were same as described previously.[4] The steady-state kinetic parameters (K_m and k_{cat}) were determined under initial-rate conditions using Lineweaver–Burk plot.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession number DQ303459.

Results

Description of the clinical isolate

K. pneumoniae BDK0419 was isolated from a sputum specimen of a female patient hospitalized at an intensive care unit in a tertiary-care hospital in Sungnam, Korea, in May 2004, for hypertensive intracranial haemorrhage and pulmonary oedema. Strain BDK0419 exhibited resistance to ampicillin, ampicillin–sulbactam, piperacillin, ceftazidime, amikacin, gentamicin and tobramycin and susceptibility to piperacillin-tazobactam, cefoxitin, cefotaxime, cefepime, aztreonam, imipenem, ciprofloxacin and trimethoprim/sulfamethoxazole. The strain exhibited a positive double-disc synergy test, thus indicating the production of ESBLs.

PCR and sequencing of the β-lactamase genes

PCR amplifications using primers specific for ESBL-encoding genes revealed that *K. pneumoniae* BDK0419 possessed both bla_{SHV} and $bla_{CTX\text{-}M\text{-}1}$-type genes. Sequence of the bla_{SHV} PCR amplicon with *K. pneumoniae* BDK0419 was 100% identical to the $bla_{SHV\text{-}2a}$ sequence. Sequence data of the $bla_{CTX\text{-}M\text{-}1}$-type gene indicate an open reading frame of 873 bp, corresponding to a putative protein of 291 amino acids. The $bla_{CTX\text{-}M\text{-}1}$-type gene differed from $bla_{CTX\text{-}M\text{-}3}$, the nearest CTX-M neighbour, by only a Pro (CCG) to Gln (CAG) substitution at Ambler position 167. As this substitution has not previously been described in the CTX-M-type β-lactamases, the enzyme from *K. pneumoniae* BDK0419 appears to be a novel β-lactamase and has been designated CTX-M-54.

Genetic environment of $bla_{CTX\text{-}M\text{-}54}$

An IS*Ecp1* insertion sequence, comprising an intact *tnpA* gene, was located 128 bp upstream of $bla_{CTX\text{-}M\text{-}54}$. IS*Ecp1* possesses two imperfect inverted repeats (IRs), the left IR (IRL, CCTAGATTCTACGTCAGT) and the right IR (IRR, ACACACGTGGAATTTAGG), made of 18 bp with 14 of these 18 bp being complementary. A putative promoter consisting of the –10 (TACAAT) and –35 (TTGAA) regions, which drives $bla_{CTX\text{-}M\text{-}54}$ transcription, was observed within the 3′ non-coding sequence of IS*Ecp1*. To identify further DNA sequences surrounding the $bla_{CTX\text{-}M\text{-}54}$ gene, several long-range PCR

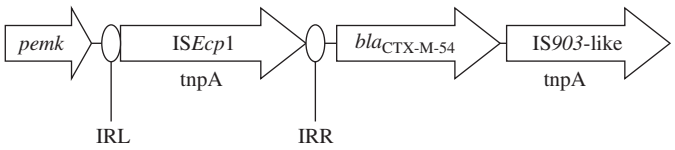

Figure 1. Schematic map of a 4063 bp DNA fragment that contains *pemK*, IS*Ecp1*, IS*903*-like and the $bla_{CTX\text{-}M\text{-}54}$ genes from the *K. pneumoniae* BDK0419 clinical isolate. Inserted genes and their transcriptional orientation are indicated by arrows. Two imperfect inverted repeats (IRs), the left IR (IRL) and the right IR (IRR), of the IS*Ecp1* insertion sequence are represented by ovals.

Table 2. MIC values of antimicrobial agents for *K. pneumoniae* BDK0419, *E. coli* trcBDK0419 and *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-54

	MIC (mg/L)		
Antibiotic[a]	*K. pneumoniae* BDK0419	*E. coli* trcBDK0419	*E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-54
Ampicillin	>256	>256	>256
Ampicillin + CLA	>256	>256	>256
Cefoxitin	4	4	4
Aztreonam	16	2	4
Ceftazidime	128	128	128
Ceftazidime + CLA	16	16	16
Cefotaxime	8	8	8
Cefotaxime + CLA	1	1	1
Cefepime	1	1	1
Cefepime + CLA	0.5	0.25	0.5
Imipenem	0.25	0.25	0.25

[a]CLA, clavulanic acid at a fixed concentration of 4 mg/L.

experiments were performed using a series of primers. The upstream region of the IS*Ecp1* insertion sequence contained the *pemK* gene. The pemK protein, the *pemK* gene product, is supposed to inhibit the growth of host cells that have lost the *pem*$^+$ plasmid.[12] One-hundred and twenty-four nucleotides from the stop codon, the $bla_{\text{CTX-M-54}}$ gene was flanked by a IS*903*-like element (Figure 1).

Transfer of resistance to ceftazidime

K. pneumoniae BDK0419 contained three plasmids with molecular sizes of ~21 (pBDK0419), 3.5 and 3 kbp. The strain transferred pBDK0419 containing both $bla_{\text{SHV-2a}}$ and $bla_{\text{CTX-M-54}}$ genes to the *E. coli* J53 Azide^R recipient by mating experiments.

β-Lactam susceptibility

Agar dilution MIC testing confirmed that the *K. pneumoniae* BDK0419 was resistant to ampicillin and ceftazidime, intermediate to aztreonam, and susceptible to cefoxitin, cefotaxime, cefepime and imipenem (Table 2). Notably, the MIC of ceftazidime for this strain was 16-fold higher than that of cefotaxime. The β-lactam resistance phenotypes of the transconjugant (*E. coli* trcBDK0419) and *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-54 were almost identical to those of the *K. pneumoniae* BDK0419 (i.e. the MICs of ceftazidime were 16-fold higher than those of cefotaxime). Clavulanic acid restored the activities of ceftazidime in all three strains.

IEF analysis

IEF of the sonic extract of *E. coli* trcBDK0419 showed two bands with pI values of 7.6 and 8.0. IEF of the partially purified β-lactamase of *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-54 revealed a band with a pI value of 8.0. The relative molecular mass of CTX-M-54, determined by SDS–PAGE analysis, was ~28 kDa (data not shown).

Table 3. Kinetic parameters of CTX-M-54 β-lactamase against substrates

Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Benzylpenicillin	19	22	1.2
Cefalothin	325	11	0.03
Cefaloridine	215	145	0.7
Ampicillin	33	4	0.1
Cefotaxime	182	34	0.2
Cefuroxime	95	61	0.6
Ceftazidime	48	0.13	0.003
Nitrocefin	29	147	5.1
Aztreonam	244	9	0.04

Kinetic studies

The kinetic parameters for the CTX-M-54 β-lactamase showed that it had activity against most β-lactams including benzylpenicillin, cefaloridine, cefuroxime and cefotaxime (Table 3). There was a discrepancy between MIC results and the kinetic parameters of the enzyme against ceftazidime and cefotaxime. The catalytic efficiency (k_{cat}/K_m) of CTX-M-54 against cefotaxime (0.2 $\mu M^{-1} s^{-1}$) was higher than that against ceftazidime (0.003 $\mu M^{-1} s^{-1}$).

Discussion

The strain BDK0419 contained a transferable plasmid with a molecular size of ~21 kbp (pBDK0419) that carries both $bla_{\text{SHV-2a}}$ and $bla_{\text{CTX-M-54}}$ β-lactamase genes, along with two other plasmids. CTX-M-54 had a P167Q substitution within the omega loop region of class A β-lactamases compared with the sequence of CTX-M-3. Amino acid substitutions at this

position of CTX-M-type β-lactamases have been associated with expansion of activity towards ceftazidime.[13]

MICs of ceftazidime for all *K. pneumoniae* BDK0419, the transconjugant (*E. coli* trcBDK0419) and *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-54 were 16-fold higher than those of cefotaxime. These results indicate that CTX-M-54 preferentially hydrolyses ceftazidime but not cefotaxime. However, the kinetic parameters of CTX-M-54 against ceftazidime revealed a low catalytic efficiency. A similar discrepancy between MIC results and kinetic data has been reported in CTX-M-19, ascribed to a rapid loss of the activity of the enzyme caused by instability of the protein.[5]

The $bla_{\text{CTX-M-54}}$ gene was flanked upstream by an IS*Ecp1* insertion sequence and downstream by an IS*903*-like element. The $bla_{\text{CTX-M}}$ genes belonging to the CTX-M-1, CTX-M-2 and CTX-M-9 clusters are associated with IS*Ecp1*-like insertion sequences.[14] The $bla_{\text{CTX-M-17}}$ and $bla_{\text{CTX-M-19}}$ genes are also associated with IS*903*-like elements.[14,15] IS*Ecp1*-like insertion sequences may play a role in the mobilization of the $bla_{\text{CTX-M}}$ genes by a transcriptional mechanism by recognizing a variety of DNA sequences as right IRs. However, the role of IS*903*-like elements in the mobilization process of the $bla_{\text{CTX-M}}$ genes has not yet been demonstrated.

The present work shows once again that novel CTX-M enzymes with an expanded activity towards ceftazidime through a single amino acid substitution can be identified from clinical isolates. Thus, detection of CTX-M enzymes can no longer be based solely on the resistance phenotypes of clinical isolates towards ceftazidime and cefotaxime.

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Transparency declarations

None to declare.

References

1. Bonnet R. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother* 2004; **48**: 1–14.

2. Poirel L, Gniadkowski M, Nordmann P. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum β-lactamase CTX-M-15 and of its structurally related β-lactamase CTX-M-3. *J Antimicrob Chemother* 2002; **50**: 1031–4.

3. Bonnet R, Dutour C, Sampaio JL *et al*. Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp-240→Gly. *Antimicrob Agents Chemother* 2001; **45**: 2269–75.

4. Bonnet R, Recule C, Baraduc R *et al*. Effect of D240G substitution in a novel ESBL CTX-M-27. *J Antimicrob Chemother* 2003; **52**: 29–35.

5. Poirel L, Naas T, Le Thomas I *et al*. CTX-M-type extended-spectrum β-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. *Antimicrob Agents Chemother* 2001; **45**: 3355–61.

6. Sturenburg E, Kuhn A, Mack D *et al*. A novel extended-spectrum β-lactamase CTX-M-23 with a P167T substitution in the active-site omega loop associated with ceftazidime resistance. *J Antimicrob Chemother* 2004; **54**: 406–9.

7. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk Susceptibility Tests—Ninth Edition: Approved Standard M2-A9*. CLSI, Wayne, PA, USA, 2006.

8. Jarlier V, Nicolas MH, Fournier G *et al*. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. *Rev Infect Dis* 1988; **10**: 867–78.

9. Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Seventh Edition: Approved Standard M7-A7*. CLSI, Wayne, PA, USA, 2006.

10. Jeong SH, Lee K, Chong Y *et al*. Characterization of a new integron containing VIM-2, a metallo-β-lactamase gene cassette, in a clinical isolate of *Enterobacter cloacae*. *J Antimicrob Chemother* 2003; **51**: 397–400.

11. Ryoo NH, Kim E-C, Hong SG *et al*. Dissemination of SHV-12 and CTX-M-type extended-spectrum β-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* and emergence of GES-3 in Korea. *J Antimicrob Chemother* 2005; **56**: 698–702.

12. Tsuchimoto S, Nishimura Y, Ohtsubo E. The stable maintenance system *pem* of plasmid R100: degradation of pemI protein may allow pemK protein to inhibit cell growth. *J Bacteriol* 1992; **174**: 4205–11.

13. Kimura S, Ishiguro M, Ishii Y *et al*. Role of a mutation at position 167 of CTX-M-19 in ceftazidime hydrolysis. *Antimicrob Agents Chemother* 2004; **48**: 1454–60.

14. Poirel L, Lartigue M-F, Decousser J-W *et al*. IS*Ecp1B*-mediated transposition of $bla_{\text{CTX-M}}$ in *Escherichia coli*. *Antimicrob Agents Chemother* 2005; **49**: 447–50.

15. Cao V, Lambert T, Courvalin P. ColE1-like plasmid pIP843 of *Klebsiella pneumoniae* encoding extended-spectrum β-lactamase CTX-M-17. *Antimicrob Agents Chemother* 2002; **46**: 1212–7.