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Emergence of CTX-M-12 extended-spectrum β-lactamase-producing *Escherichia coli* in Korea

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***Objectives*: To characterize CTX-M-12 extended-spectrum β-lactamase (ESBL) produced by clinical *Escherichia coli* isolates 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 environments of the $bla_{CTX\text{-}M\text{-}12}$ genes were investigated by PCR and sequencing of the regions surrounding the genes. Kinetic parameters were determined from purified CTX-M-12.**

***Results*: Sequence data for the CTX-M-1 cluster from three clinical *E. coli* isolates indicated the presence of CTX-M-12. An IS*Ecp1* insertion sequence was located 49 bp upstream of $bla_{CTX\text{-}M\text{-}12}$ in all three *E. coli* isolates. CTX-M-12 had a more potent hydrolytic activity against cefotaxime than against ceftazidime and was encoded on a self-transferable ∼18 kbp plasmid.**

***Conclusions*: This work shows that CTX-M-12, which confers high-level resistance to cefotaxime but not to ceftazidime, has emerged in Korea. The $bla_{CTX\text{-}M\text{-}12}$ gene was associated with an upstream IS*Ecp1* insertion sequence.**

Keywords: IS*Ecp1*, horizontal transfer, ERIC-PCR

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] At present, the CTX-M family comprises more than 50 enzymes that have greater hydrolytic activity against cefotaxime than ceftazidime. In Korea, CTX-M-15 and CTX-M-3 were reported to be the most prevalent ESBLs in clinical *Escherichia coli* isolates in a survey of 12 Korean hospitals in 2003.[2]

CTX-M-12 ESBL was first detected from *Klebsiella pneumoniae* isolates from an outbreak among six newborn babies in Kenya in 2001.[3] And then, the ESBL was also detected from a clinical *K. pneumoniae* isolate from Colombia and from a clinical *E. coli* isolate from China.[4,5] CTX-M-12 differs from CTX-M-3, the nearest CTX-M neighbour, by three amino acid substitutions along with five silent point changes.

The IS*Ecp1* element is able to achieve the transfer of the downstream DNA sequence by a one-ended transposition process.[1] This element has repeatedly been observed upstream

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of ORFs encoding the CTX-M enzymes. In the present study, we report the first isolation of CTX-M-12 ESBL from three clinical *E. coli* isolates from Korea. Additionally, we have characterized the genetic environment of the $bla_{CTX-M-12}$ genes and have measured kinetic parameters of CTX-M-12.

Materials and methods

Bacterial strains

Clinical isolates of *E. coli* were identified with the VITEK system (bioMérieux, Marcy l'Etoile, France). *E. coli* BL21(DE3) was the host for cloning experiments. *E. coli* J53 Azide^R and *E. coli* ATCC 25933 were used as a recipient strain for conjugative transfer and an MIC reference strain, respectively.

Antimicrobial susceptibility testing and mating-out assays

Antimicrobial susceptibilities were determined by disc diffusion and agar dilution methods according to the recommendations of the CLSI.[6,7] MICs of β-lactams were determined alone or in combination with a fixed concentration of clavulanic acid (4 mg/L). ESBL-production was detected by the double-disc synergy (DDS) test.[8] Conjugation experiments were carried out as described previously.[2]

PCR experiments

Searches for genes coding for class A ESBLs were performed by PCR amplification.[2] The templates for PCR amplification in clinical isolates were a whole-cell lysate. The PCR products were subjected to direct sequencing. Both strands of each PCR product were sequenced twice with an automatic sequencer (model 373A; Applied Biosystems, Weiterstadt, Germany). The genetic organization of the $bla_{CTX-M-12}$ gene was investigated by PCR and sequencing of the regions surrounding this gene.

Purification of CTX-M-12 β-lactamase

The PCR product obtained with the primers CTX-M-12 pcrF (5′-GG GAA TTC CAT ATG GTT AAA AAA TCA CTG CG-3′; *Nde*I site underlined) and CTX-M-12 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 pET30a (Novagen, Milan, Italy). The resultant pET30a-CTX-M-12 expression construct was transformed into *E. coli* BL21(DE3). LB broth (1 L) supplemented with kanamycin (50 mg/L) was cultured at 37°C. Isopropyl-β-D-thiogalactopyranoside (final concentration 0.4 mM) was added when the culture reached an A_{600} of 0.6, and the culture was incubated overnight at 20°C. The cells were harvested by centrifugation and resuspended in 50 mL of buffer A [50 mM Tris (pH 7.0), 500 mM NaCl, 10 mM imidazole]. Cells were disrupted in a microfluidizer (at 15 000 psi) and the lysate centrifuged at 15 000 **g** for 40 min. The supernatant was loaded onto a Ni-NTA column (XK16, Amersham-Pharmacia-Biosciences, Milan, Italy) pre-equilibrated with buffer A, and washed with buffer B [50 mM Tris (pH 7.0), 500 mM NaCl, 25 mM imidazole]. The β-lactamase was eluted with a linear gradient of imidazole (25–300 mM in 1 h). The fractions containing nitrocefin-hydrolysing activity (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 3 mg/mL (purity $\gg$98%).

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 a 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.[2] 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). Extinction coefficients of each antibiotic substrate used in the spectrophotometric assays were the same as described previously.[9] The steady-state kinetic parameters (K_m and k_{cat}) were determined under initial-rate conditions using Lineweaver–Burk plot.

Enterobacterial repetitive consensus (ERIC)-PCR

ERIC-PCRs were performed in 50 μL volumes containing 10 ng of genomic DNA from three clinical *E. coli* isolates containing the $bla_{CTX-M-12}$ gene, 4 mM $MgCl_2$, 50 pM of each primer [ERIC-1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′)], 1.25 U of TaKaRa Ex *Taq* polymerase (TaKaRa, Otsa, Shiga, Japan), 0.2 mM each of dATP, dCTP, dGTP and dTTP in 25 mM TAPS [*N*-Tris(hydroxy)methyl-3-amino-propane sulphonic acid, pH 9.3], 50 mM KCl and 1 mM 2-mercaptoethanol. Amplification was carried out as described previously[10] and amplicons were analysed by gel electrophoresis.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession numbers DQ658220, DQ658221 and DQ658222.

Results and discussion

E. coli SME3, SSE5 and SCE4 were isolated from urine specimens of three patients hospitalized at three different hospitals in Seoul and Gumi, Korea, in 2004. The isolates were resistant to cefotaxime, but susceptible to ceftazidime and all exhibited positive results in the DDS test, indicating ESBL production.

PCR amplifications using primers specific for ESBL-encoding genes revealed that all three *E. coli* isolates possessed both bla_{TEM} and $bla_{CTX-M-1}$-type genes. Sequences of the bla_{TEM} PCR amplicons were 100% identical to the bla_{TEM-1} sequence. Sequence data from the amplicons of the CTX-M-1 cluster indicated the presence of CTX-M-12 (GenBank accession no. AF305837). An IS*Ecp1* insertion sequence, which may play a role in the mobilization of the bla_{CTX-M} genes by a transcriptional mechanism by recognizing a variety of DNA sequences as right inverted repeats (IRs),[11] was located 49 bp upstream of $bla_{CTX-M-12}$ in all three *E. coli* isolates. IS*Ecp1* possessed two imperfect IRs, the left IR (CCTAGATTCTACGTCAGT) and the right IR (ACACACGTGGAATTTAGG), made of 18 bp with 14 of these 18 bp being complementary. A putative promoter

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Table 1. Kinetic parameters of CTX-M-12 β-lactamase against substrates

Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Benzylpenicillin	32.90	669.30	20.343
Cefaloridine	268.70	198.40	0.738
Cefalotin	806.00	146.40	0.182
Cefoxitin	25.30	0.02	0.001
Ceftazidime	464.50	1.80	0.004
Cefotaxime	99.70	312.10	3.130
Aztreonam	177.40	17.10	0.096
Imipenem	ND	0.01	ND

ND, not determined.

Table 2. MICs of β-lactams for CTX-M-12 ESBL-producing clinical *E. coli* isolates, *E. coli* trcSCE4 and *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-12

	MICs (mg/L)				
Antibiotic[a]	*E. coli* SME3[b]	*E. coli* SSE5[b]	*E. coli* SCE4[b]	*E. coli* trcSCE4[c]	*E. coli* trfSCE4[d]
Ampicillin	>256	>256	>256	>256	>256
Cefoxitin	64	16	16	16	2
Aztreonam	16	32	32	128	0.25
Ceftazidime	4	2	4	16	0.06
Ceftazidime + CLA	2	1	1	8	0.06
Cefotaxime	32	64	64	256	32
Cefotaxime + CLA	8	32	32	128	0.06
Cefepime	16	32	32	128	4
Cefepime + CLA	4	16	8	64	0.06
Imipenem	0.12	0.12	0.12	0.25	0.25

[a]Clavulanic acid (CLA) at a fixed concentration of 4 mg/L.
[b]Clinical *E. coli* isolates containing the $bla_{CTX\text{-}M\text{-}12}$ gene.
[c]*E. coli* trcSCE4 (transconjugant), *E. coli* J53 Azide[R] carrying plasmid pSCE4.
[d]*E. coli* trfSCE4 (transformant), *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-12.

consisting of the –10 (TACAAT) and –35 (TTGAAA) regions was observed within the 3′ non-coding sequence of IS*Ecp1*.

IEF of the partially purified β-lactamase of *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-12 revealed a band with a pI value of 9.0. The kinetic parameters for the CTX-M-12 β-lactamase showed that it had activity against benzylpenicillin, cefaloridine and cefotaxime (Table 1). The catalytic efficiency (k_{cat}/K_m) of CTX-M-12 against cefotaxime (3.130 μM^{-1} s^{-1}) was much higher than that against ceftazidime (0.004 μM^{-1} s^{-1}).

All three *E. coli* isolates contained a plasmid with molecular sizes of ∼18 kbp containing $bla_{CTX\text{-}M\text{-}12}$ and $bla_{TEM\text{-}1}$ genes. But ERIC-PCR of the three *E. coli* isolates proved that these strains were not clonal (data not shown), indicating that horizontal transfer of the $bla_{CTX\text{-}M\text{-}12}$ gene had occurred and suggesting the possibility of further spread of this gene in the future. Despite repeated attempts, only one (SCE4) among three *E. coli* isolates transferred the plasmid (pSCE4) to the *E. coli* J53 Azide[R] recipient by mating experiments.

Agar dilution MIC testing confirmed that all three *E. coli* isolates were highly resistant to ampicillin, intermediate or resistant to aztreonam, cefoxitin, cefotaxime and cefepime, and susceptible to ceftazidime and imipenem (Table 2). The β-lactam resistance phenotypes of the transconjugant (*E. coli* trcSCE4) were almost identical to those of the parent strain. In the Kenya study, the CTX-M-12-producing *K. pneumoniae* isolates were resistant to cefotaxime (MIC 24 mg/L by Etest), but the presence of clavulanic acid lowered the MIC of the drug 750 times to 0.032 mg/L.[3] In our cases, however, clavulanic acid restored the activities of cefotaxime in *E. coli* BL21(DE3) carrying plasmid pET30a-CTX-M-12 only, but not in both the clinical *E. coli* isolates and the *E. coli* transconjugant.

In summary, this work shows that CTX-M-12 has now emerged in Korea, in addition to its recent description in China.[5] Our kinetic characterizations show that CTX-M-12 was more active against cefotaxime than against ceftazidime, and we have also demonstrated the association of the $bla_{CTX\text{-}M\text{-}12}$ with an upstream IS*Ecp1* element.

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Transparency declarations

None to declare.

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