Molecular Mechanisms of Carbapenem Resistance in
Enterobacter cloacae Clinical Isolates from Korea and
Clinical Outcome

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Abstract. We investigated the molecular mechanisms of carbapenem resistance in clinical isolates of Enterobacter cloacae and their clinical characteristics. Nonreplicable E. cloacae isolates were recovered from six cancer patients and one patient with liver cirrhosis at a tertiary-care hospital in Korea between 2002 and 2009. Two patients who were considered to have a true infection caused by these microorganisms have died. All isolates produced AmpC β-lactamases, including ACT-1, ACT-2, MIR-3 and DHA-1, and CTX-M- or SHV-type extended-spectrum β-lactamase. Two isolates produced plasmid-borne VIM-2 carbapenemase. All probes specific for blaAmpC genes hybridized with I-CeuI chromosomal fragments were also recognized by a probe specific for 16S rDNA, suggesting a chromosomal location. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that a major outer membrane protein, OmpF, was absent in all isolates. PFGE of XbaI-digested DNA were considered to be unrelated. The results of our study suggest that the chromosomal AmpC β-lactamase with impermeability in E. cloacae clinical isolates implicated in reduced carbapenem susceptibility. Although carbapenem-resistant E. cloacae isolates were isolated in a few patients in our study, the clinical outcomes were grave. Therefore, the patients colonized or infected by carbapenem-resistant E. cloacae isolates should gain attention of antibiotic therapy.

Introduction

Enterobacter cloacae has recently emerged as an important nosocomial pathogen which harbors inducible chromosomal AmpC β-lactamase. Carbapenems are the drug of choice in the treatment of serious infections caused by producing extended-spectrum β-lactamase (ESBL) E. cloacae. The emergence of carbapenem-resistant E. cloacae is of great concern due to limited antimicrobial treatment options. Moreover, clinical outcomes of the patients infected by carbapenem resistant Enterobacteriaceae are known to be grave [1-3]. Marchaim et al. [2] reported that infections caused by KPC-2-producing-Enterobacter strains were associated with increased mortality (33%). Falcone et al. [4] reported that patents with infections caused by VIM-1-producing E. cloacae had high risk factors such as relapse of infection and prolonged duration of antibiotic therapy.

Carbapenem resistance in E. cloacae isolates has arisen via Ambler classes A or B carbapenem-hydrolyzing β-lactamases. NMC-A and IMI-1 are chromosomally encoded class A carbapenemases, which are first identified from E. cloacae clinical isolates in 1990 and 1984, respectively [5, 6]. KPC-2 or -3 producer E. cloacae clinical isolates were reported in the USA in 2003 [1]. Class B carbapenemase such as VIM-1, VIM-2, and IMP-1 have been identified in E. cloacae clinical isolates since the first report of plasmid-borne VIM-2 in an E. cloacae KU680 from Korea in 2000 [7-10]. Carbapenem resistance in E. cloacae has also been attributed to AmpC enzymes or ESBLs combined with impermeability [11, 12]. Reduction in expression level of outer membrane proteins (OMPs), such as OmpC and OmpF, was involved with impermeability in E. cloacae [12-14].
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age (years)</th>
<th>Depart</th>
<th>Comorbidity</th>
<th>Duration of hospital stay before acquisition (in days)</th>
<th>Charlson comorbidity score</th>
<th>Previous use of carbapenem or cephalosporin (duration in days)</th>
<th>Isolation site(s)</th>
<th>Previous E. cloacae isolation</th>
<th>Clinical illness</th>
<th>Treatment regimens/ outcome</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>M/67</td>
<td>Medical ICU</td>
<td>Non small cell lung cancer, Recent steroid use due to radiation pneumonitis</td>
<td>21</td>
<td>8</td>
<td>IPM (16)</td>
<td>Sputum</td>
<td>No</td>
<td>Pneumonia</td>
<td>CPZ, TZP, CIP, SXT, VA/ Failure</td>
</tr>
<tr>
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<td>M/49</td>
<td>Surgical ward</td>
<td>HTN, CBD cancer s/p Whipple operation</td>
<td>10</td>
<td>6</td>
<td>CEF (10)</td>
<td>Peritoneal fluid</td>
<td>Yes</td>
<td>Intraabdominal infection</td>
<td>CPZ, CEF, ISE / Improve</td>
</tr>
<tr>
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<td>M/56</td>
<td>Surgical ward</td>
<td>DM, s/p liver transplantation due to cirrhosis</td>
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<td>5</td>
<td>CTX (8), ZOX (10), CPZ (15), ETP (2)</td>
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<td>Colonization</td>
<td>LZD, SXT/ ND</td>
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<td>F/59</td>
<td>Surgical ICU</td>
<td>HTN, DM, s/p liver transplantation due to HCC</td>
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<td>7</td>
<td>CRO (83), CPZ (15), ZOX (15), MEM (16)</td>
<td>Bile</td>
<td>Yes</td>
<td>Colonization</td>
<td>AN, LVX, VA/ ND</td>
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<td>5</td>
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<td>Pediatric ward</td>
<td>ALL</td>
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<td>2</td>
<td>No</td>
<td>Urine</td>
<td>No</td>
<td>Colonization</td>
<td>ISE, TEC, SXT/ ND</td>
</tr>
<tr>
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<td>F/1</td>
<td>Pediatric ward</td>
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<td>18</td>
<td>2</td>
<td>CFP (12)</td>
<td>Urine</td>
<td>Yes</td>
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<td>CEF, ISE/ ND</td>
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<td>NHL, Do-not-resuscitate state</td>
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<td>4</td>
<td>CPZ (30)</td>
<td>Throat, sputum, oral</td>
<td>Yes</td>
<td>Oral infection</td>
<td>CPZ, ISE, AN, TEC/ Failure</td>
</tr>
</tbody>
</table>

**Abbreviations:** M, male; F, female; HTN, hypertension; ALL, acute lymphoblastic leukemia; DM, diabetes mellitus; HCC, hepatocellular carcinoma; URI, upper respiratory infection; IPM, imipenem; ETP, ertapenem; MEM, meropenem; CTX, Cefotaxime; ZOX, ceftizoxime; CPZ, cefoperazone-sulbactam; CRO, ceftiraxone; CFP, cefpiran; TZP, piperacillin-tazobactam; CIP, ciprofloxacin; SXT, Trimethoprim-sulfamethoxazole; VA, vancomycin; CEF, cefpiramide; ISE, iespamicin; LZD, linezolid; AN, amikacin; LVX, levofloxacin; TEC, teicoplanin; ND, not detected.
The aims of this study were to investigate the carbapenem resistant mechanisms in clinical isolates of *E. cloacae* from a tertiary-care hospital in Korea and present the clinical characteristics of patients infected or colonized by these organisms.

**Materials and Methods**

**Patients and bacterial isolates.** Clinical isolates of carbapenem resistant *E. cloacae* were recovered from seven inpatients of a tertiary-care hospital in Korea between 2002 and 2009. The isolates were identified using conventional biochemical methods and the Vitek 2 system (bioMérieux, Marcy l’Etoile, France). Clinical features of seven patients were reviewed through electronic medical records. *E. cloacae* ATCC13047 was used as a reference strain.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibilities were determined by disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines [15]. The modified Hodge test and the imipenem and EDTA-sodium mercaptoacetic acid double-disk synergy (IEDDS) test were performed on MacConkey agar plates as previously described for the screening of carbapenemases and metallo-β-lactamases (MBLs), respectively [16]. MICs of imipenem and meropenem were determined using the E-test (Becton Dickinson, Sparks, MD) on Mueller-Hinton agar plates with or without a fixed concentration of clavulanic acid (CA; 4 mg/L; Sigma-Aldrich Corporation, St. Louis, MO) as an inhibitor of ESBL, aminophenyl boronic acid (APB; 300 mg/L; Sigma-Aldrich Corporation) as an inhibitor of AmpC β-lactamase, or Phe-Arg-β-naphthylamide (PAβN; 40 mg/L; Sigma-Aldrich Corporation) as an efflux pump inhibitor.

**PCR experiments for β-lactamase genes.** Detection of genes coding for AmpC β-lactamases, ESBLs, and classes A and B carbapenemases was performed by PCR amplification with primers described previously [17, 18], PCR products were subjected to direct sequencing with an automatic DNA sequencer (model 3730xl; Applied Biosystems, Weiterstadt, Germany).

**SDS-PAGE for OMPs.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to investigate alterations in OMPs as previously described [19]. Briefly, bacterial cells were disrupted by ultrasonic disintegration and the supernatants were treated with 0.1 M sodium carbonate. After incubation on ice for 1 hr, OMPs were collected by centrifugation at 115,000 g for 2 hr at 4°C and were analyzed by SDS-PAGE on a Mini-PROTEAN 3 Cell apparatus (Bio-Rad, Hercules, CA). The 10% (wt/vol) polyacryl gels were stained with Coomassie Brilliant Blue.

**Pulsed-field gel electrophoresis (PFGE).** Plugs containing whole genomic DNA of the isolates were digested with *Xba*I, *I-Ceu*I or *S1* nuclease. DNA fragments were separated using PFGE with a CHEF-DRII device (Bio-Rad). The PFGE conditions of *Xba*I-macrorestriction analysis were 6 V/cm for 20 hr with pulse times ranging from 0.5 to 60 seconds at 14°C. The pulse times for *I-Ceu*I and *S1* nuclease restriction analysis were 9 to 90 seconds. The lambda ladder (Bio-Rad) was used as a DNA size marker. Dendrograms were generated by the unweighted pair group method with arithmetic average method, and DNA relatedness was calculated based on the criteria suggested by Tenover et al. [20].

**Southern blotting.** The gels with *I-Ceu*I-digested chromosomal DNA and *S1* nuclease-treated linearized plasmids were blotted onto nylon membranes (Bio-Rad) and hybridized with probes specific for the *bla*<sub>ACT</sub>, *bla*<sub>MIR-3</sub>, *bla*<sub>AmpC</sub> and 16S rDNA. The probes were obtained via PCR experiments as described above. Probe labeling, hybridization, and detection were performed with the DIG DNA Labeling and Detection kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer’s protocols.

**Results**

**Description of the patients.** Clinical characteristics and outcomes of seven patients were presented in Table 1. The median age of the patients was 56 years (range, 1-67), and three patients were male. Four patients had chronic or malignant liver diseases, and three patients had hematologic malignancies. The median Charlson comorbidity score was 5 (range, 2-8). Five patients had been previously colonized with carbapenem-susceptible *E. cloacae*. They were exposed to the oxyimino-cephalosporins during their prolonged hospital stay (median duration, 64 days; range, 10-160)
before carbapenem-resistant *E. cloacae* strains were isolated. Three cases were considered to be true infection, while remaining four cases to be simple colonization, judging from CDC/NHSN surveillance definition of health care-associated infection. Only one patient with intra-abdominal infection improved with antimicrobial treatment, while two patients with pneumonia or oral infection died within 30 days of infection.

**Antimicrobial susceptibilities.** All seven isolates were nonsusceptible to piperacillin, amoxicillin-clavulanic acid, ceftoxitin, cefotaxime, aztreonam, cefoperazone-sulbactam, and trimethoprim-sulfamethoxazole using disk diffusion assay. They exhibited reduced susceptibilities to imipenem or meropenem (MIC range, 0.75-12 mg/L) using E-test. APB lowered imipenem and meropenem MICs 2- to 21-fold and 2- to 32-fold, respectively, while CA and PAßN did not (Table 2). One isolate (YMC 03/4/397) showed positive results with the modified Hodge and the IEDDS tests, suggesting MBL production.

**Identification of β-lactamase genes.** PCR and sequencing experiments detected AmpC β-lactamase genes such as *bla*<sub>ACT-1</sub>, *bla*<sub>ACT-2</sub>, *bla*<sub>MIR-3</sub> and *bla*<sub>DHA-1</sub> genes in four isolates. Both *bla*<sub>ACT-2</sub> and *bla*<sub>DHA-1</sub> genes were detected in one isolate (YMC 02/9/932). AmpC β-lactamase genes identical to the sequence of *E. cloacae* TR211 strain (GenBank accession number: DQ478700) were detected in three isolates. The ESBL genes such as *bla*<sub>SHV-12</sub>, *bla*<sub>CTX-M-57</sub>, *bla*<sub>CTX-M-3</sub>, *bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-57</sub> genes were detected. Two isolates carried both *bla*<sub>CTX-M-57</sub> and *bla*<sub>SHV-12</sub> genes. Two isolates (YMC 03/4/397, YMC 03/4/397) were found to produce VIM-2 MBL. The *bla* genes encoding class A carbapenemase including KPC, IMI, GES and SME were not detected in any of the isolates.

**Locations of β-lactamase genes.** In Southern blot assay, probes specific for the *bla*<sub>ACT-1</sub> and the *bla*<sub>ACT-2</sub> hybridized with 450-kb I-CeuI chromosomal fragments from YMC 08/12/3793 and 600-kb from YMC 09/5/165 isolate, respectively. Probes for the *bla*<sub>MIR-3</sub> and the *bla*<sub>DHA-1</sub> were hybridized with 550-kb and 250-kb I-CeuI chromosomal fragments from YMC 04/8/2304 and YMC 02/9/932 isolates, respectively. The unnamed AmpC β-lactamase genes probe hybridized with approximately 350 kb I-CeuI chromosomal fragments in

<table>
<thead>
<tr>
<th>Strains</th>
<th>MICs (mg/L)</th>
<th>β-lactamase gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without inhibitors</td>
<td>APB</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------</td>
<td>-----</td>
</tr>
<tr>
<td>IPM</td>
<td>MER</td>
<td>IPM</td>
</tr>
<tr>
<td>YMC 09/5/165</td>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>YMC 08/12/3793</td>
<td>4</td>
<td>0.75</td>
</tr>
<tr>
<td>YMC 06/7/204</td>
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<tr>
<td>YMC 05/10/530</td>
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<td>YMC 04/8/2304</td>
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<td>YMC 03/4/397</td>
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<tr>
<td>YMC 02/9/932</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>1</sup>The *bla* genes with sequence identical to that of *ampC* gene of the TR211 strain (Genbank accession number: DQ478700).

**Abbreviations:** APB, aminophenylboronic acid (300 mg/L); CA, clavulanic acid (4 mg/L); PAßN, Phe-Arg-ß-naphthylamide (40 mg/L); IPM, imipenem; MEM, meropenem; ESBL, extended-spectrum β-lactamase; MBL, metallo-β-lactamase; ND, not detected; NG, no growth.

**Table 2.** Carbapenems MICs with or without inhibitors and β-lactamase genes for clinical isolates of *E. cloacae*
Carbapenem-resistant E. cloacae

three isolates. However, all were hybridized with S1 nuclease-treated linearized plasmids. The 16S rDNA probes were hybridized with the same chromosomal fragments hybridized with the probes specific for all AmpC β-lactamase genes detected. These findings suggest the presence of all AmpC β-lactamase genes detected in our isolates on a chromosome rather than on a plasmid (data not shown). However, the blaVIM-2 probe hybridized with 400 kb S1 nuclease-treated plasmids not with I-I-CeuI chromosomal fragments, which suggested plasmid location of that gene.

SDS-PAGE for OMPs. SDS-PAGE analysis of OMPs revealed that all isolates showed lacked or diminished expression of OmpF porins compared to the reference strain E. cloacae ATCC 13047. Two isolates (YMC 09/5/165 and YMC 04/8/2304) lacked both OmpF and OmpC porins. All the isolates retained expression of OmpA porins compared to the control strain (Figure 1).

Clonal relatedness. XbaI-digested DNA from all isolates showed genetic similarity of 65% to 85%, and were considered to be unrelated.

Discussion

Over an eight-year period, seven cases of clinical isolates of E. cloacae exhibiting carbapenem resistance were identified in a tertiary-care hospital in Seoul, Korea. All patients were in immunosuppressive states, undergoing chemotherapy or post-operation. Most of these patients acquired those bacteria during a prolonged hospital stay, and were exposed to the oxyimino-cephalosporins in hospital. Three cases were considered to be true infections while four cases simple colonization. Of these, two of three patients died in spite of appropriate antibiotic treatment. Although carbapenem-resistant E. cloacae were isolated in a few patients in our study, the clinical outcomes were grave. Therefore, the patients colonized or infected by carbapenem-resistant E. cloacae isolates should gain attention of antibiotic therapy.

Acquired carbapenem resistance is still uncommon among E. cloacae but can arise in the presence of classes A or B carbapenemase and AmpC or ESBLs enzymes combined with impermeability [12, 14]. In our study, carbapenem-resistant E. cloacae clinical isolates were comediated by chromosomally encoded AmpC enzymes and OMPs loss. The expression of OmpF porin of all isolates was greatly decreased based on SDS-PAGE experiments, while that of OmpC porin was decreased in two isolates. The expression of OmpA porins in two isolates lacking both OmpC and OmpF porins was relatively stronger compared to that of the other OmpA porins. It supported that minor porins may express stronger to acquire nutrients for their fitness, when

Figure 1. SDS-PAGE analysis of outer membrane proteins (OMPs) of E. cloacae isolates. Lane M, protein molecular weight size markers of 25, 37 and 50 kDa; lanes 1 to 7, seven experimental isolates; lane C, the control strain E. cloacae ATCC 13047.
major porins were greatly diminished [21]. Synergy test with PAβN did not indicate efflux as a major component in carbapenem resistance, although it has been reported to have contributed to ertapenem resistance in clinical isolates of E. cloacae [12]. VIM-2 carrying E. cloacae clinical isolate was rare in our study, and was the second report in the world.

In conclusion, the reduced carbapenem susceptibility in clinical isolates of E. cloacae was associated with porin loss combined with chromosomal AmpC β-lactamase. Although the prevalence of carbapenem-resistant E. cloacae and patients infected with these microorganisms are still low, their occurrence needs to be continuously monitored.

Acknowledgements

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References


