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Molecular Analysis of Carbapenem-Resistant *Enterobacteriaceae* at a South Korean Hospital

Miyoung Lee[1,2] and Tae-Jin Choi[1]*

[1]*Department of Microbiology, Pukyoung National University, Busan 48513, Republic of Korea*
[2]*Department of Laboratory Medicine, BHS Hanseo Hospital, Busan 48253, Republic of Korea*

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The prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) is increasing globally, resulting in high mortality rates. Although CRE is a relatively recent problem in Korea (the first case was not diagnosed until 2010), it is responsible for serious morbidities at an alarming rate. In this study, we carried out a molecular genetic analysis to determine the incidence of CRE and carbapenemase-producing *Enterobacteriaceae* (CPE) at a general hospital in Korea between August 2017 and August 2019. Forty strains of CPE were isolated from various clinical specimens and analyzed via antimicrobial susceptibility testing, polymerase chain reaction to detect β-lactamase genes, deoxyribonucleic acid sequencing, multilocus sequence typing, curing testing, and conjugal transfer of plasmids. The results demonstrated that all 40 isolates were multidrug-resistant. The fluoroquinolone susceptibility test showed that 75% of the *Enterobacteriaceae* isolates were resistant to ciprofloxacin, whereas 72.5% were resistant to trimethoprim-sulfamethoxazole. Further, conjugation accounted for 57.5% of all resistant plasmid transfer events, which is 4.3-fold higher than that observed in 2010 by Frost *et al*. Finally, the high detection rate of transposon Tn4401 was associated with the rapid diffusion and evolution of CPE. Our results highlight the rapid emergence of extensively drug-resistant strains in Korea and emphasize the need for employing urgent control measures and protocols at the national level.

Keywords: **Fluoroquinolone, *blaKPC*, *Escherichia coli* ST410, *Klebsiella pneumoniae* ST307, carbapenem-resistant *Enterobacteriaceae*, carbapenemase-producing *Enterobacteriaceae***

Introduction

Approximately 700,000 people die annually from antibiotic-resistant infections, and this number will expectedly surpass 10 million deaths per year by 2050 [1]. Antibiotic-resistant, gram-negative bacteria pose a serious threat worldwide, and, owing to the lack of available therapeutic options, the Centers for Disease Control and Prevention (CDC) has listed carbapenem-resistant members of the family *Enterobacteriaceae* as an "immediate public health threat that requires urgent and aggressive action" [2]. Two resistant forms of *Enterobacteriaceae* are of particular concern in the hospital setting: carbapenem-resistant *Enterobacteriaceae* (CRE) and carbapenemase-producing *Enterobacteriaceae* (CPE). Both CRE and CPE are associated with high mortality owing to their resistance to all available antibiotics.

In Korea, the first person infected with carbapenem-resistant *Enterobacteriaceae* was reported in December 2010. In 2014, a sudden increase in *Klebsiella pneumoniae* carbapenemase (KPC) cases was observed nationwide, initially occurring as hospital-based outbreaks [3], which subsequently led to inter-hospital or inter-regional spread [4], thereby resulting in the endemic stage of KPC in 2015. Following this outbreak, numerous cases of CRE and CPE were reported in

***Corresponding author**
Tel: +82-51-629-5617, Fax: +82-51-629-5619
E-mail: choitj@pknu.ac.kr

Korea. As the mortality associated with invasive infections caused by CPE is extremely high [5], the spread of CPE is of immense clinical concern.

β-Lactamases are grouped into four classes according to their amino acid sequence: class A includes KPC and Temoneira β-lactamase (TEM); class B consists of metallo-β-lactamases, such as Verona integron-encoded metallo-β-lactamases (VIM), Imipenem (IMP), and New Delhi metallo-β-lactamase-1(NDM-1); class C includes AmpC β-lactamases; and class D consists of oxacillinase (OXA)-type enzymes. Carbapenem resistance in *K. pneumoniae* has been reported in most countries and can be attributed to plasmid-mediated AmpC cephalosporinases. AmpC cephalosporinases are associated with porin modifications [6, 7] and reduced outer membrane permeability owing to porin loss in combination with the production of either extended-spectrum β-lactamases (ESBLs) or β-lactamases, which are capable of hydrolyzing carbapenems (carbapenemases) [8, 9].

The dissemination of the *blaKPC* gene, encoding β-lactamase, occurs by the clonal spread of bacterial hosts harboring the gene or by horizontal transfer of transposons and plasmids carrying the gene [10, 11]. Plasmids are transmissible by conjugation [12–14]. Smillie et al. reported that approximately 14% of full-sequenced plasmids were predicted to be conjugative [15]. Hence, conjugation is one of the most effective mechanisms used to spread genetic elements among bacteria [16]. The highly mobile 10-kb Tn3-type transposon Tn*4401* was considered the primary cause of the successful dissemination of *blaKPC* [17].

Carbapenems are β-lactam antibiotics employed as the last line of treatment during antibiotic therapy. There are few treatment options for patients infected with carbapenem-resistant CRE and CPE. Therefore, it is of great clinical significance to effectively slow down the rate of resistance transfer until a new antibiotic is available. Taking this into consideration, it is possible to slow down the rate of infection through active monitoring of CPE and by strengthening clinical management programs. Management in the form of a coping protocol to identify the antibiotic resistance risk, guidelines for the effective use of antibiotics, and the identification of trends in carbapenem-resistant enterobacteria are required for effective prevention. Moreover, these measures could play essential roles in understanding and controlling the spread of carbapenem-resistant, gram-negative pathogens.

In this study, we evaluated the incidence of CRE and CPE in a single general hospital in South Korea and characterized the β-lactamases in CRE clinical isolates.

Materials and Methods

Patient description

The study was performed at a general hospital in Busan, South Korea, between August 2017 and August 2019. During the study period, all non-duplicated clinical isolates of *Enterobacteriaceae* obtained from inpatients that exhibited a reduced sensitivity to carbapenems were characterized. This prospective study was performed in accordance with the ethical standards given in the Declaration of Helsinki and was approved by the Institutional Review Board of the BHS Hanseo Hospital, Busan, Korea (approval number CTS-19-004).

Bacterial isolates and antimicrobial susceptibility testing

Clinical isolates were identified by standard microbiological procedures and VITEK-2 (bioMérieux Vitek, USA). The identification of all isolates was confirmed by 16S ribosomal DNA (16S rDNA) sequencing [18]. Antimicrobial susceptibilities were determined using VITEK 2 AST N224 cards (bioMérieux Vitek) and disk diffusion tests on Mueller-Hinton agar (Becton Dickinson, USA) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. Susceptibility testing was performed with the following antibiotics: ampicillin, aztreonam, cefotaxime, ceftazidime, cefoxitin, ciprofloxacin, amikacin, gentamicin, imipenem, meropenem, ertapenem, and colistin. The minimum inhibitory concentration (MICs) for colistin were assessed via the broth microdilution method using cation-adjusted Mueller-Hinton broth (Becton Dickinson) following the Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. The susceptibility test results for tigecycline were confirmed by the E-test (bioMérieux, France).

For the modified carbapenem inactivation method test, organisms were incubated with a meropenem disk in tryptic soy broth (Difco Laboratories, USA). For the ethylene diamine tetra acetic acid (EDTA)-modified carbapenem inactivation method test, EDTA was added to the broth to chelate metal ions necessary for the metallo-

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β-lactamase function. Following incubation, the disks were fished out and placed on a lawn of susceptible *Escherichia coli* (ATCC 25922) to determine whether the test organisms degraded meropenem. Zone diameters were measured and interpreted following the CLSI guidelines [19].

Genotyping of β-lactamases and the outer membrane protein

The β-lactamase-encoding gene was evaluated by polymerase chain reaction (PCR). PCR was also performed to detect genes encoding carbapenemases (IMP-1-type, VIM-2-type, NDM, KPC, Guiana-extended-spectrum β-lactamase, and OXA-48-like) and ESBLs (CTX (cefotaximase) -M-1-, CTX-M-9-, TEM-, and sulfhydryl variant (SHV)-type) [20]. CPE isolates were also examined for the presence of plasmid-mediated AmpCs (*blaACT*, *blaACC*, *blaCMY*, and *blaDHA*), aminoglycoside resistance determinants (ARD, including armA, rmtA, rmtB, and rmtD) [21, 22], and fluoroquinolone resistance determinants (including qepA, qnrA, qnrB, and qnrS) as previously described [23]. PCR was performed to detect the genes encoding the outer membrane protein (OmpK35, OmpK36) [24].

Isotyping the Tn4401 transposon

To determine the isotypes of Tn4401, isotype-specific forward primers (a–e) for the five most common isotypes [17] were newly designed (Table S1), and PCR was carried out with a universal reverse primer targeting *blaKPC*. The amplicons were sequenced for verification [25].

Multilocus sequence typing (MLST)

PCR and sequencing (Table S2) were carried out for seven housekeeping genes: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* for *E. coli* [26]; *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* for *K. pneumoniae* [27]; and *aspC*, *clpX*, *fadD*, *mdh*, *arcA*, *dnaG*, and *lysP* for *Citrobacter freundii*. Nucleotide sequences were compared with those in the MLST database (http://bigsdb. web.pasteur.fr/klebsiella for *K. pneumoniae* and http://mlst.warwick.ac.uk/mlst/dbs/Ecoli for *E. coli*) to identify allelic types and STs.

Bacterial conjugation

For the standard agar mating method, 40 CPE strains were used as the donor, and sodium azide-resistant *E. coli* J53 was used as the recipient [28]. Following overnight mating at 37℃ on brain-heart infusion (BHI) agar (MB cell, USA), transconjugants were selected on BHI addition agar with 100 μg/ml sodium azide and 0.5 μg/ml IMP. The conjugated strains were identified via PCR, β-lactamase-encoding, ESBLs, plasmid-mediated AmpCs, ARD, and sensitivity to fluoroquinolones.

Curing test

KPC-carrying plasmid curing was performed via the temperature-mediated plasmid emission method. Briefly, CRE strains were subjected to elevated temperature-mediated plasmid elimination by sequential passages in BHI broth (MB cell) at 42℃ twice daily for 2 weeks. After 2 weeks, cultures were diluted and plated onto tryptic soy infusion (TSI; Difco Laboratories Inc.) medium to obtain single colonies. Suspected cured colonies were identified by selecting colonies and replating them onto TSI medium containing 5 μg/ml IMP (Sigma-Aldrich, USA) and a control TSI medium. Colonies that failed to grow in the presence of IMP were suspected to be cured and were further analyzed for *blaKPC* loss by PCR as well as antibiotic susceptibility testing [29].

Results

Patient characteristics

Carbapenemases were responsible for resistance in 40 isolates (66.7% of all 60 ertapenem-resistant *Enterobacteriaceae*). The mean age of patients with CPE was 81.4 years (range, 57–92 years), and 27 patients (67.5%) were females (Fig. 1A). The 40 isolates comprised 27 *K. pneumoniae* isolates (67.5%), 9 *E. coli* isolates (22.5%), 2 *C. freundii* isolates (5%), 1 *Klebsiella oxytoca* isolate (2.5%), and 1 *Klebsiella aerogenes* isolate (2.5%) (Fig. 1B).

Antimicrobial susceptibilities

All isolated strains showed a multidrug-resistant (MDR) phenotype. According to the antibiotic susceptibility profiles, all 40 isolates were resistant to ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefazolin, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, ertapenem, and IMP. The results of fluoroquinolone susceptibility testing showed that 32 isolates (80%) were resistant to ciprofloxacin, whereas the tri-

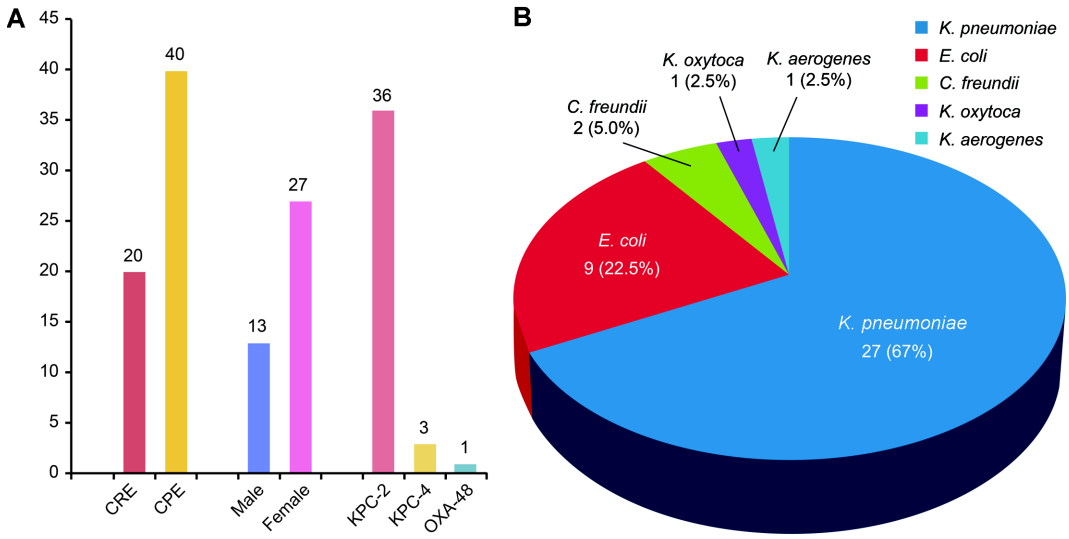

Fig. 1. Distribution of species in carbapenemase-producing *Enterobacteriaceae* (CPE) isolates. (A) Distribution of carbapenemase genotypes in carbapenem resistant *Enterobacteriaceae* isolates (n = 60), (B) Distribution of genotypes of CPE isolates (n = 40)

methoprim-sulfamethoxazole resistance rates were 72.5%. The aminoglycoside test results showed that 19 strains (47.5%) were resistant to gentamicin and two strains (5%) were intermediately resistant to amikacin. Tigecycline and colistin resistance was confirmed in two strains (5%). The combination of β-lactamases relative to the susceptibility test results is shown in Table 1.

Distribution of molecular typing

Among the 40 CPE isolates, 36 (90%), 3 (7.5%), and 1 (2.5%) were KPC-2, KPC-4, and OXA-48 producers, respectively (Fig. 1A). We also observed a high prevalence of ESBLs. Other β-lactamases were observed as well, including CTX-M-1 group (57.5%), CTX-M-4 group (15%), SHV-like (50%), and TEM-like (32.5%) β-lactamase isolates. Plasmid-mediated AmpC was rarely co-produced (4/40, 10%); in KPC-2 producers, co-production was observed with DHA (n = 2) and ACC (n = 1), whereas in KPC-4 producers, co-production was only observed with CIT (n = 1). Concerning the analysis of fluoroquinolone resistance determinants for the 40 carbapenemase strains, 67.5% of the 27 *Enterobacteriaceae* isolates carried a fluoroquinolone resistance determinant. The 40 carbapenemase isolates comprised 22 *qnrB*

Table 1. Antimicrobial susceptibilities of CPE isolates and phenotypes.

CPE Isolates (%)	Antimicrobial susceptibility										Porin loss (%)		Conjugation
	No. (%) of isolates												
	FOX	FEP	ETP	IMP	AK	GN	CIP	SXT	TIG	CST	OmpK35	OmpK36	
CPE strains (n = 40)	0	0	1 (2.5)	1 (2.5)	39 (97.5)	31 (77.5)	8 (20)	11 (27.5)	38 (95)	38 (95)	18 (45)	17 (42.5)	23 (57.5)
KPC-2	0	0	0	0	31 (100)	17 (54.8)	6 (19.4)	8 (25.8)	26 (83.9)	25 (80.6)	15 (48.4)	16 (51.6)	20 (64.6)
ΔTn*4401a* (n=26)	0	0	0	0	26 (100)	13 (50)	6 (23.1)	6 (23.1)	26 (100)	25 (3.85)	14 (53.8)	13 (50)	17 (65.4)
ΔTn*4401b* (n = 5)	0	0	0	0	5 (100)	4 (80)	0	2 (40)	5 (100)	5 (100)	1 (20)	3 (60)	3 (60)
KPC-4	0	0	0	0	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	3 (100)	3 (100)	0	0	1 (33.3)
Δ*Tn4401b* (n = 3)	0	0	0	0	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	3 (100)	3 (100)	0	0	1 (33.3)
Total (n = 34)	0	0	0	0	33 (97.1)	19 (55.9)	8 (23.53)	10 (29.42)	29 (85.3)	28 (82.4)	15 (44.1)	16 (47.1)	21 (61.8)
Transconjugant strains (n = 23)	0	0	0	0	23 (100)	14 (60.9)	6 (26.1)	7 (30.4)	22 (95.7)	23 (100)	-	-	-
Cured strains (n = 2)	1 (50)	1 (50)	2 (100)	1 (50)	2 (100)	2 (100)	1 (50)	1 (50)	2 (100)	2 (100)	-	-	-

Abbreviations: CPE, carbapenemase-producing *Enterobacteriaceae*; FOX, cefoxitin; FEP, cefepime; ETP, ertapenem; IMP, imipenem; AK, amikacin; GN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; TIG, tigecycline; CST, colistin.

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Table 2. Identification of resistance determinants among the 40 CPE isolates by PCR.

Antibiotic class/ Resistance gene	CPE strains N = 40 (%)	Transconjugants strains N = 23 (%)	Cured strains N = 2 (%)
Carbapenems			
KPC-2	36 (90)	16 (69.6)	0
KPC-4	3 (7.5)	3 (13.0)	0
OXA-48	1 (2.5)	1 (4.3)	0
Cephalosporins			
SHV	20 (50)	11 (47.8)	0
TEM	13 (32.5)	2 (8.7)	0
CTX-M-1	23 (57.5)	7 (30.4)	1 (50)
CTX-M-9	6 (15)	1 (4.3)	0
AmpC			
DHA	2 (5)	0	0
CIT	1 (2.5)	0	0
ACC	1 (2.5)	0	0
Aminoglycoside			
rmtA	1 (2.5)	0	0
rmtB	11 (27.5)	3 (13.0)	0
rmtC	2 (5)	0	0
rmtD	4 (10)	0	0
armA	2 (5)	2 (8.7)	0
Fluoroquinolone			
qnrA	8 (20)	0	0
qnrB	17 (42.5)	12 (52.2)	2 (100)
qnrS	8 (20)	7 (30.4)	0
qepA	6 (15)	1 (4.3)	0

Abbreviations: CPE, carbapenemase-producing *Enterobacteriaceae*; KPC, *Klebsiella pneumonia* carbapenemase; OXA, oxacillinase; SHV, sulfhydryl variant; TEM, Temoneira β-lactamase; CTX-M, cefotaximase.

(55%), 8 *qnrA* (20%), 8 *qnrS* (20%), and 6 qepA (15%) isolates. The analysis of aminoglycoside resistance in these strains showed that 18 strains (45%) displayed resistance: 11 *rmtB* (27.5%), 4 *rmtD* (10%), 2 *rmtC* (5%), 2 *armA* (5%), and 1 *rmtA* (2.5%) isolates (Table 2). In addition to the production of ESBLs or AmpC enzymes, the deletion or mutation of porins, such as OmpK35 and OmpK36, was reportedly correlated with increased carbapenem MICs [30]. A multi-agency survey conducted in 2013 confirmed that porins, including OmpK35 and OmpK36, were observed in 27 isolates (67.5%) [31]. In our study, the porins OmpK35 and OmpK36 were observed in 18 (45%) and 17 (42.5%) isolates, respectively. OmpK35 and OmpK36 were both deleted or mutated at the same rate (20%) (Table 3).

MLST

K. pneumoniae was analyzed by MLST based on the analysis of the seven housekeeping genes from the 27 clinical isolates of KPC-producing *K. pneumoniae*. Using this approach, we observed five sequence types (STs). Using the *K. pneumoniae* MLST database, we detected the clones ST307 (18/27, 66.7%) and ST11 (6/27, 22.2%).

Furthermore, three types were identified: ST429 (1/27, 3.7%), ST2521 (1/27, 3.7%), and ST3660 (1/27, 3.7%) (Table 4). *E. coli* MLST demonstrated that the STs ST410 and ST3520 were the most common among the carbapenemase-producing strains (2/9, 22.2%), followed by ST117 (1/9, 11.1%), ST617 (1/9, 11.1%), ST720 (1/9, 11.1%), ST457 (1/9, 11.1%), and ST52 (1/9, 11.1%).

Isotyping the Tn*4401* transposon

The Tn*4401* isotype was detected in 34 of the 40 analyzed strains (34/40, 85%). The major isotypes associated with Tn*4401* bracketing the *blaKPC* gene were Tn*4401a* (26/40, 65%) and Tn*4401b* (8/40, 20%). Tn*4401b* was identified in all three strains of *blaKPC*-4, which is consistent with the results of Jeong *et al.* [25]. For the six non-isotypable transposons (15%) by the Tn*4401*-isotyping PCR, amplification between the 3′-end of the upstream ISKpn7 and the *blaKPC* gene was performed, and no amplicons were produced. Regarding strains carrying Tn*4401*, 20 strains were identified (20/34, 59%), which increases the risk of carbapenem resistance transmission. The transposon isolates are listed in Table 4.

Conjugation

Carbapenem resistance was successfully transferred from CPE strains to *E. coli* J53 by conjugation (23/40, 57.5%). All transconjugants exhibited significantly reduced carbapenem susceptibility, with ertapenem and imipenem MICs of 4–8 mg/l and 8–16 mg/l, respectively. In addition, the transconjugants exhibited MDR phenotypes similar to those of the clinical CPE isolate donors. The transconjugants were also resistant to cephalosporins but were susceptible to aminoglycosides (Table 1). Importantly, the transconjugant assays enabled the simultaneous transfer of *blaKPC*-2, *blaKPC*-4, blaOXA-

Table 3. Possible genes and outbreaks identified in the CPE collection.

Strain	Species	Carbapenems	Cephalosporins	AmpC	Aminoglycoside	Fluoroquinolone	ST	Porin loss	Tn*4401* variant	Conjugants	Curing
K1	*KPN*	*KPC-2*	*CTX-M-1*		*rmtB, rmtD*	*qnrB*	307	OMPK36			
K2	*KPN*	*KPC-2*	*CTX-M-1*		*rmtB*	*qnrB*	307	OMPK36	b	+	
K3	*ECO*	*KPC-2*	*TEM1, CTX-M-9*		*armA*		410	OMPK35, OMPK36	*a*	+	
K4	*KPN*	*KPC-2*	*TEM1,CTX-M-1, SHV*	*ACC*	*rmtD*	*qnrB, qepA*	11	OMPK35, OMPK36	b		
K5	*CFR*	*KPC-2*	*TEM1*	*DHA*		*qnrA*	18		*a*		
K6	*KPN*	*KPC-2*	*TEM1, CTX-M-1, SHV*		*rmtC*	*qnrB, qepA*	11	OMPK36	*a*	+	
K7	*KPN*	*KPC-2*	*TEM1, CTX-M-1, SHV*			*qnrB*	11	OMPK35, OMPK36	*a*	+	+ (IMP:I)
K8	*KPN*	*KPC-2*	*TEM1, CTX-M-1, SHV*			*qnrB*	11	OMPK36	b	+	
K9	*KPN*	*KPC-2*	*TEM1, CTX-M-1, SHV*		*rmtC*	*qnrB, qnrS, qepA*	307	OMPK36	*a*	+	
K10	*ECO*	*KPC-2*	*TEM1, CTX-M-1, CTX-M-9, SHV*		*armA*		410		*a*	+	
K11	*KPN*	*KPC-2*	*TEM1, CTX-M-1, SHV*		*rmtB*		307	OMPK36	*a*	+	
K12	*KPN*	*KPC-2*	*TEM1, CTX-M-1, CTX-M-9*			*qnrB*	307	OMPK36	*a*	+	
K13	*KOX*	*KPC-4*							b		
K14	*ECO*	*OXA48*	*TEM1, CTX-M-1*			*qnrS*	617			+	
K15	*KPN*	*KPC-2*	*TEM1, CTX-M-1*		*rmtD*		11	OMPK36	*a*		
K24	*KPN*	*KPC-2*	*CTX-M-1, SHV*				2521		*a*	+	
K25	*KPN*	*KPC-2*	*CTX-M-1, SHV*		*rmtB*	*qnrB*	307	OMPK35, OMPK36	*a*	+	
K26	*KAE*	*KPC-2*	*SHV*					OMPK36	*a*	+	
K27	*ECO*	*KPC-2*	*SHV*				720	OMPK35	*a*	+	
K28	*KPN*	*KPC-2*	*SHV*		*rmtB, rmtD*		307	OMPK35, OMPK36	*a*		
K29	*KPN*	*KPC-2*			*rmtB*	*qnrB, qepA*	307	OMPK35, OMPK36	*a*		
K102	*KPN*	*KPC-2*	*SHV, CTX-M-1*		*rmtB*	*qnrS, qepA*	307	OMPK35	*a*	+	
K103	*KPN*	*KPC-2*	*SHV, CTX-M-1*		*rmtB*	*qnrB, qnrS*	307	OMPK35	*a*	+	
K104	*ECO*	*KPC-2*	*SHV*				3520		*a*		
K105	*ECO*	*KPC-2*	*SHV*				3520		*a*	+	+
K106	*KPN*	*KPC-2*	*SHV*			*qnrB, qnrS*	11		*a*		
K107	*ECO*	*KPC-2*	*SHV, CTX-M-1*			*qnrA, qnrB*	457		b	+	
K108	*KPN*	*KPC-2*	*CTX-M-1, CTX-M-9*			*qnrB, qnrS*	307	OMPK35	*a*		
K109	*KPN*	*KPC-2*				*qnrB, qnrS*	307	OMPK35	*a*		
K110	*KPN*	*KPC-2*	*SHV, CTX-M-1*			*qnrB, qnrS*	307	OMPK35	*a*		
K111	*ECO*	*KPC-2*				*qnrA*	52			+	
K112	*KPN*	*KPC-4*					3660		b	+	
K113	*CFR*	*KPC-4*	*CTX-M-1*	*CIT*		*qnrA*	124		b		
K114	*KPN*	*KPC-2*	*TEM1*			*qnrA, qnrB, qepA*	307	OMPK35, OMPK36	*a*		
K115	*KPN*	*KPC-2*	*CTX-M-1*			*qnrA, qnrB*	307	OMPK35			
K116	*KPN*	*KPC-2*	*CTX-M-1*			*qnrA, qnrB*	307	OMPK35			
K117	*KPN*	*KPC-2*	*SHV, CTX-M-9*		*rmtB*	*qnrA, qnrB*	429	OMPK35, OMPK36	*a*	+	
K118	*ECO*	*KPC-2*	*CTX-M-1*		*rmtA*		117		b		
K119	*KPN*	*KPC-2*			*rmtB*	*qnrB*	307	OMPK35	*a*	+	
K120	*KPN*	*KPC-2*		*DHA*	*rmtB*	*qnrB*	307	OMPK35		+	

Abbreviations: CPE, carbapenemase-producing *Enterobacteriaceae*; KPN, *Klebsiella pneumonia*; ECO, *Escherichia coli*; CFR, *Citrobacter freundii*; KOX, *Klebsiella oxytoca*; KAE, *Klebsiella aerogenes*; ST, sequence type; IMP, imipenem.

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Table 4. *bla*$_{KPC}$ mobile elements carried by KPC producers.

Strain	*Klebsiella pneumoniae*					*Escherichia coli*					*Citrobacter freundii*		*Klebsiella oxytoca*	*Klebsiella aerogenes*
Sequence type	307	11	429	2521	3660	410	720	3520	457	117	124	18	-	-
blaKPC-2 Tn*4401a*	13 (32.5)	4 (10)	1 (2.5)	1 (2.5)	-	2 (5)	1 (2.5)	2 (5)	-	-	-	1 (2.5)	-	1 (2.5)
blaKPC-2 Tn*4401b*	1 (2.5)	2 (5)	-	-	-	-	-	-	1 (2.5)	1 (2.5)	-	-	-	-
blaKPC-4 Tn*4401b*	-	-	-	-	1 (2.5)	-	-	-	-	-	1 (2.5)	-	1 (2.5)	-

Abbreviations: KPC, *Klebsiella pneumoniae* carbapenemase.

48, ESBL, and AmpC, as well as aminoglycoside and fluoroquinolone resistance-determining genes (Table 2).

Curing test

A curing test was performed to artificially remove the plasmid containing the KPC-2 gene. However, curing was successfully performed in only two strains, K7 and K105 (2/40, 5%). In each of these strains, curing significantly reduced carbapenem resistance, with imipenem and ertapenem MICs of ≤ 0.5 mg/l and 0.5–2 mg/l, respectively. Other antibiotics that showed similar effects on the clinical CPE isolate donors are also shown in Table 1.

Discussion

In the 1940s, the first antibiotics were derived from mold; however, since then, advances in antibiotic development has relied on chemical synthesis. However, with the emergence of multidrug resistance, the end of the antibiotic era may be near. The World Health Organization warns about a post-antibiotic era, in which no antibiotics will be able to kill bacteria because of their widespread resistance. Currently, hospitals face emerging resistance to third-generation cephalosporins and fluoroquinolones by *Staphylococcus aureus* and *Enterococcus*. Recently, there has been growing speculation about the impossibility of treating CRE in a post-antibiotic era. To the best of our knowledge, this is the first study to conduct molecular genetic analysis of CPE at a single hospital in Korea since the conduction of the CRE survey by the Korea CDC in June 2017. Here, we described the distribution of major β-lactamases in a group of extensively drug-resistant (XDR) *Enterobacteriaceae* isolates from a single hospital, presenting several important results.

Antibiotic resistance is increasing, and, therefore, a new classification termed "XDR" has been created. Fluoroquinolones are important therapeutics used to treat both human and animal infections [32]. The introduction of fluoroquinolones has offered clinicians the ability to treat human cases of complicated urinary tract, gastrointestinal, and respiratory tract infections as well as sexually transmitted diseases [33]. In veterinary medicine, fluoroquinolones have served as effective therapeutics for treating enteric infections and respiratory diseases in food-producing and companion animals [34]. Owing to their antimicrobial activity against a broad spectrum of pathogenic bacteria, advantageous pharmacokinetic characteristics, and low toxicity, fluoroquinolones have become attractive for use in farm animals [35, 36]. Since the late 1980s, fluoroquinolones used in human medicine have differed from those used in veterinary medicine [37, 38]. Nonetheless, a public health concern remains, i.e. the use of fluoroquinolones in livestock may result in bacterial resistance that can be transmitted through the food chain. This can be explained by cross-resistance between fluoroquinolones and enrofloxacin, a commonly used agent in farm animals, which is partially metabolized to ciprofloxacin within animals [39, 40]. Thus, resistance to antibiotics has continuously remained in the food chain and may be permanent; therefore, close management and restrictions are required in the future. Additionally, there is also an emerging resistance to colistin and tigecycline, last-resort antibiotics for CRE treatment.

Interestingly, our study showed that CRE is more susceptible to amikacin, an antibiotic of the aminoglycoside family. The EUCAST(European Committee on Antimicrobial Susceptibility Testing) guidelines state that the amikacin susceptibility status must be revised to "intermediate" should a member of the *Enterobacteriaceae* test

as tobramycin intermediate or resistant, and gentamicin and amikacin susceptible [41].

Therefore, we recommend the sparingly use of amikacin to treat intestinal bacterial infections resistant to other aminoglycosides.

The most predominant KPC-producing clone reported globally is *K. pneumoniae* ST258 [42, 43]; however, this was not the case in South Korea. Rather, the predominant South Korean KPC producer was the *K. pneumoniae* ST307 clone. ST11 was also observed in Korea at a significantly lower prevalence than ST307. According to a study by Jeong *et al*., the four *K. pneumoniae* subclones ST307/Tn*4401a*[*blaKPC*-2], ST307/Tn*4401b*[*blaKPC*-4], ST392/Tn*4401b*[*blaKPC*-4], and ST11/ND[*blaKPC*-2] triggered inter-regional dissemination of KPC producers in Korea [25]. In this study, various *K. pneumoniae* subclones and *E. coli* subclones were isolated; the primary *K. pneumoniae* subclones observed were ST307/Tn*4401a*[*blaKPC*-2], ST307/Tn*4401b*[*blaKPC*-4], ST11/Tn*4401a*[*blaKPC*-2], and Tn*4401b*[*blaKPC*-2], while the observed *E. coli* clones were ST410/Tn*4401a*[*blaKPC*-2] and ST3520/Tn*4401a*[*blaKPC*-2]. Importantly, the Tn*4401* isotypes differed in the promoter of the *blaKPC* gene, which was closely associated with the level of gene expression and caused variation in carbapenem susceptibility [12]; however, there was no significant difference in susceptibility in this study.

K. pneumoniae ST307 and *E. coli* ST410 have garnered attention as potentially high-risk CPE producers and are associated with a notorious clone, CTX-M-15 [44]. Furthermore, clones co-producing CPE and CTX-M-15 were recently identified in Italy [45] and Denmark [46]. The high distribution of CTX-M-1 reported in this study highlights the risk of emergence and spread of high-risk clones (Table 1). High-risk clones are globally distributed and associated with various antimicrobial resistance determinants, such as ease of transmission, persistence in hosts, and effective transmission between hosts. These high-risk clones possess enhanced pathogenicity and are more prone to cause severe and recurrent infections. Thus, considering the clonal expansion over the past decades and increased antimicrobial resistance, ST307 and ST410 should be prospectively monitored in South Korea.

The CPE incidence and molecular genetic traits observed at this hospital revealed trends similar to those reported in the Disease Control Headquarters survey conducted in 2017 [47]. However, the CPE incidence rate increased by 25% and that of CRE increased by 5% in 2018 relative to those in 2017. While the previous CRE occurrence in Korea was characterized by ESBL, AmpC, or porin loss, the current study reveals that carbapenemase-producing bacteria currently represent the greatest risk to public health [31]. Transfer by conjugation accounted for 57.5% of resistant isolates, which is 4.3-fold higher than that found in the 2010 study [14]. Furthermore, the high detection rate found for transposon Tn*4401* revealed the rapid spread and evolution of CPE.

In summary, our study highlights that carbapenem resistance can be transferred among strains through transconjugation processes, which results in a higher number of strains with reduced carbapenem susceptibility. We found that the causes of carbapenem resistance spread were plasmids and transposons; therefore, more efforts are needed to control the spread of CPE in hospitals. The treatment of CRE infections often involves a combination therapy, including carbapenem, aminoglycosides, and fosfomycin; however, this therapeutic option is not yet available in South Korea. Thus, medical institutions should monitor whether carbapenem-resistant enterobacteria-infected patients are isolated, and if so, these institutions should prevent the spread through infection control measures, such as patient contact tracing, thorough use of personal protective equipment, and contact inspection. Systematic monitoring and regulation alone will enable better control and prevent the spread of KPC producers.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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