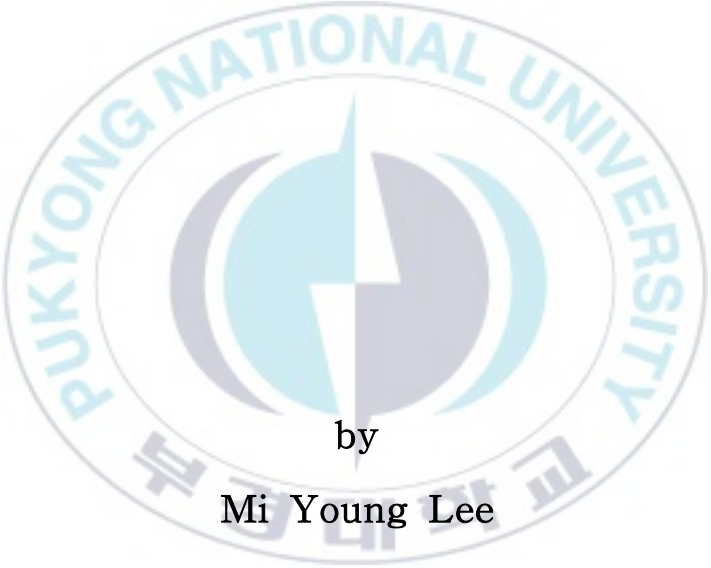
Thesis for the Degree of Doctor of Philosophy

Characterization of KPC-2 carbapenemase producing *Enterobacteriaceae* and test for interspecies transferability



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August 2020

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KPC-2 카바페넴 효소 생성 장내세균의 특성 및 종간 이동성 확인

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Characterization of KPC-2 carbapenemase producing *Enterobacteriaceae* and test for interspecies transferability

A dissertation

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Abbreviations

AMK	amikacin
ARD	aminoglycoside resistance determinants
BHI	brain-heart infusion
CDC	Centers for Disease Control and Prevention
CDS	coding sequences
CG258	clonal group 258
CLSI	Clinical and Laboratory Standards Institute
CPE	carbapenemase-producing *Enterobacteriaceae*
CRE	carbapenem-resistant *Enterobacteriaceae*
CR-KP	carbapenem-resistant *K. pneumoniae*
CTX	cefotaximase
eCIM	EDTA-modified carbapenem inactivation method
EDTA	ethylenediaminetetraacetic acid
ESBL	extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	extraintestinal pathogenic *E.coli*
FEZ-1	*Legionella gormannii*
GEN	gentamicin
GES	Guiana extended-spectrum
IMI	imipenem hydrolyzing enzyme
IMP	imipenemase
IND	*Chryseobacterium indologenes*
IS	insertion sequence
KCDC	Korea Centers for Disease Control and Prevention
KPC	*Klebsiella pneumoniae* carbapenemase
KPC-2	*Klebsiella pneumoniae* producing carbapenemase-2

KPC-Kp	KPC-2-producing *K. pneumoniae*
MBL	metallo-β-lactamase
mCIM	modified carbapenem-inactivation method
MDR	multidrug-resistant
MHA	Muller-Hinton agar
MHT	modified Hodge test
MIC	minimum inhibitory concentration
NDM-1	New Delhi metallo-β-lactamase-1
NMC	not metalloenzyme carbapenemase
OMPs	Outer membrane proteins
ORFs	open reading frames
OXA	oxacillinase
PABL	plasmid-mediated AmpC β-lactamase
PCR	polymerase chain reaction
RND	Resistance-nodulation division
ST	sequence type
SHV	sulfhydryl variant
SME	*Serratia marcescens* enzyme
TEM	Temoneira β-lactamase
TSI	tryptic soy infusion
VIM	Verona integron-encoded metallo-β-lactamases
WGS	Whole-genome sequencing
WHO	World Health Organization
XDR	extensively drug-resistant
16S rDNA	16S ribosomal DNA

KPC-2 카바페넴 효소 생성 장내세균의 특성 및 종간 이동성 확인

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요 약

항생제 내성 박테리아의 출현은 항생제 사용 빈도가 증가한 후 전 세계적으로 심각한 문제가 되었다. 특히 Carbapenem 내성 장내세균(carbapenem-resistant *Enterobacteriaceae*, CRE)의 경우 국내에서는 2010년에 최초로 보고되었으나 의료 환경에서 빠르게 확산하는 경향을 보여 더 큰 위협이 되고 있다.

이에 최근 2년간의 단일기관에서의 카바페넴 효소 생성 장내세균(carbapenemase-producing *Enterobacteriaceae*, CPE) 분포양상과 항생제 내성비율을 조사하였으며 이 중 종간 전파양상을 보인 5균주를 선택하여 플라스미드 및 전체 게놈 서열을 분석 비교하였다. 또한, KPC를 생성하는 장내세균으로부터 KPC 전파 클론의 특성과 플라스미드를 통한 *blaKPC*의 수평 이동에 관해 연구하였다.

2017년 8월부터 2019년 8월까지 국내 단일 종합병원에서 분리한 CRE와 CPE를 조사하였다. 다양한 의료용 시료로부터 40 균주의 CPE가 분리

되었으며, 이들에 대하여 항생제 감수성, β-lactamase 유전자 검출을 위한 PCR, 다좌위 서열 형별분석 (Multi Locus Sequence Typing, MLST), 게놈 염기서열 분석, curing, 접합에 의한 플라스미드 전달 분석이 이루어졌다. 40 균주 모두 다제 내성을 가지고 있었으며, 장내세균의 균주의 75% ciprofloxacin에 내성을 보인 반면 72.5%는 trimethoprim-sulfamethoxazole에 대한 내성을 나타내었다. 접합에 의한 플라스미드 수평 전달은 성공적으로 이루어졌으며 (23/40, 57.5%), 이는 2010년에 보고된 접합 성공률과 비교하면 4.3배 높은 수치로서 카바페넴 저항성 확산의 가능성을 보여 준다. 또한, 트랜스포존 Tn4401의 높은 검출빈도 (4/40, 85%)는 CRE의 빠른 전파와 균 진화의 가능성을 제시한다.

CPE의 서로 다른 세균 종간의 수평적 이동을 동일환자로부터 순차적으로 분리된 고위험 클론 대장균 (CPEc171209, ST410)과 *Klebsiella pneumoniae* (CPKp171210, ST307)를 이용하여 조사하였다. CPEc171209 혈청형 O8:H21이고 항생제 내성 클래이드 C4/H24에 속하며, 5개의 플라스미드를 가지고 있다. CPKp171210는 3개의 플라스미드를 가지고 있었다. 두 균주 모두 다양한 항생제 내성 유전자를 추가적으로 가지고 있다. IncX3 계열의 플라스미드 pECBHS_9_5는 불완전한 형태의 트랜스포존 Tn*4401a* 안에 blaKPC-2를 포함하고 있으며, 중복된 접합인자와 *blaSHV-182*를 포함하고 있다. 이 플라스미드는 *K. pneumoniae* CPKp171210로 분리된 IncX3 계열의 플라스미드 pKPBHS_10_3와 99% 일치하였다. 이 플라스미에는 또한 IncX3 계열의 플라스미드의 접합 전달에 필요한 *tra/trb* 클러스터, type IV pilus.를 암호화하는 *pil* 유전자 등이 존재하여 접합에 의한 KPC-2 유전자의 수평적 전이를 가능하게 한다.

KPC-2를 생산하는 장내세균 세 균주를 동일한 환자로부터 순차적으로

분리하여, 이들의 분자생물학적 특성 및 KPC-2 유전자의 수평적 전달 과정을 조사하였다. *K. pneumoniae* (CPKp1825) ST 307가 최초로 분리되었으며, 그 후 *K. aerogenes* (CPEa1826)와 *Escherichia coli* (CPEc1827)가 차례로 분리되었다. 이들 균주에 대하여 항생제 감수성, β-lactamase 유전자 검출을 위한 PCR, 다좌위 서열 형별분석 (Multi Locus Sequence Typing, MLST), 게놈 염기서열 분석, curing, 접합에 의한 플라스미드 전달 분석이 이루어졌다. 게놈 분석 결과 각 균주 모두 2개씩의 플라스미드를 가지고 있었으며, 이들은 플라스미드에 포함되어있는 제4형 분비 체계, pilus 유전자, *tra* 유전자 등에 의하여 접합을 통하여 전달될 수 있었다. *blaKPC-2* 유전자는 CPKp1825, CPEa1826와 CPEc1827에서 각각 분리된 pKPBHS_25_2, pEABHS_26_2, 그리고 pECBHS_27_1 플라스미드에 위치하는 트랜스포존 Tn4401a에 위치하였다. 특히 플라스미드 pEABHS_26_2와 pECBHS_27_1에 존재하는 트랜스포존 Tn4401a에서는 플라스미드 pKPBHS_25_2과 비교하여 일부 결손이 발견되었으며, 이는 두 개의 플라스미드가 CPKp1825가 가지고 있는 플라스미드 pKPBHS_25_2로부터 수평 전달되었음을 제시한다.

위 연구결과들은 우리나라에 심각한 수준의 항생제 내성을 가지는 균주들이 빠르게 출현하고 있다는 것을 보여 준다. 특히, 본 연구에서 확인되거나 증명된 것과 같이 종이 다른 세균 사이에 접합이나 트랜스포존에 의하여 수평전달이 가능한 플라스미드 상에 *blaKPC-2* 유전자가 존재한다는 것은 이들에 대한 국가 수준에서의 억제 방법 및 지침서의 개발이 시급히 필요하다는 것을 제시한다.

Chapter I

GENERAL INTRODUCTION

1. Enterobacteriaceae infection and antibiotic resistance

1.1 *Main causes of antibiotic resistance*

After 70 years of extensive use of antibiotics, antibiotic resistance is now being recognized as a worldwide crisis in modern medicine [1]. There has been a dramatic increase in infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens belonging to the *Enterobacteriaceae* group. This group is of great concern because its members are common natural inhabitants of our microbiome.

The World Health Organization (WHO) lists the following as major causes for antibiotic resistance in humans: 1) excessive use and prescription of antibiotics, 2) poor antibiotic awareness (if the patient arbitrarily stops taking antibiotics or prefers it unnecessarily), 3) misuse of antibiotics in the fisheries industry, 4) lack of infection control and hygiene in nursing homes, 5) personal hygiene, and 6) lack of new antibiotic development (such as lack of R&D). The annual OECD Health and Medical Report of 2014 showed that antibiotic consumption by country was in the order of Belgium (29.4 DID), Italy (29.3 DID), Korea (28.4 DID), and Luxembourg (27.9 DID) as of 2012, and that the antibiotic consumption in Korea was higher than the average in

other countries (20.6 DID). Acute respiratory disease accounts for most antibiotic prescriptions. further antibiotics are not medically effective against this disease; rather, if one stops taking them, then one risks invasion by resistant bacteria. In addition, according to the US CDC, 50 % of all antibiotic prescriptions for acute respiratory disease are unnecessary [2].

1.2 *Antibiotic resistance in Enterobacteriaceae*

Enterobacteriaceae infections were found to be resistant to beta-lactams (β-lactams) and aminoglycosides. Most of the treatments were possible due to the use of fluoroquinolone antibiotics; however, in the 1990s, extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* were discovered. The rate of resistance of infectious bacteria to various antibiotics including cephalosporin has been steadily increasing [3]. Carbapenem antibiotics, such as imipenem and meropenem, are produced by ESBL-producing *Enterobacteriaceae*. Carbapenems are considered as the last option for the treatment of diseases caused by MDR gram-negative bacteria [4]. However, over a decade ago, species producing carbapenemase, which degrades carbapenem antibiotics, were discovered [5]; and it has been shown that carbapenem resistance is gradually spreading worldwide. In the 2000s, it was found that Klebsiella spp., among other gram-negative bacteria, is MDR and exhibits resistance to various antibiotics. In North America, Africa, and Asia, strains with various resistance patterns were found [6]. Infection by *K. pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* in hospitals has been reported to increase the mortality of infected individuals due to the unavailability of sufficient number of treatments against the antibiotic-resistant bacteria [7]. Most infections

caused by KPC-producing *Enterobacteriaceae* are known to be urinary tract infections resulting from use of infected surgical equipment or catheter [8,9]. Common characteristics of patients having KPC related infections include long-term hospitalization, intensive care unit hospitalization, use of surgical equipment and immunosuppressive drugs, and multidrug treatment before early bacterial culture [9,10]. Recently, it has been reported that patients infected with imipenem-resistant strains have much higher mortality than those infected with imipenem-sensitive strains [8].

1.3 *Carbapenem antibiotic*

Carbapenem is a β-lactam antibiotic with the largest antibacterial spectrum and good antibacterial activity against gram-positive bacteria, gram-negative bacteria, and anaerobic bacteria. The first carbapenem antibiotic, imipenem, was patented in 1975 and approved for medical use in 1985. Imipenem is hydrolyzed in the kidney and the use of imipenem may lead to side effects in the central nervous system. To compensate for this, meropenem was developed in 1996. In addition, ertapenem was developed in 2001 and doripenem in 2007. Clavulanic acid and sulbactam, which irreversibly inhibit β-lactamase, and β-lactam inhibitors such as tazobactam, have little antibacterial activity. However, when they are combined with β-lactam antibiotics that are well hydrolyzed by β-lactamase in combinations such as ampicillin/sulbactam, amoxicillin/clavulanate, and piperacillin/tazobactam, antibacterial activity against β-lactamase-producing bacteria is enhanced [11], (Figure 1).

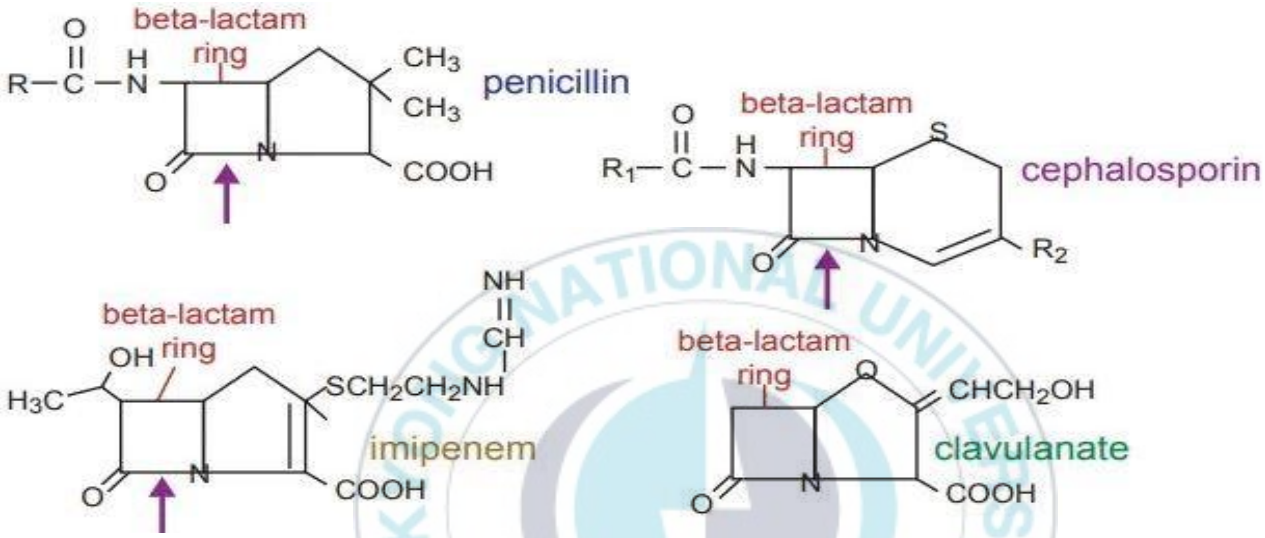

Figure 1. Structures of β-lactam antibiotics.

Molecular structure of penicillin, cephalosporin, imipenem and clavulanate. β-lactam antibiotics are analogues of d-alanyl- d-alanine—the terminal amino acid residues on the precursor NAM/NAG-peptide subunits of the nascent peptidoglycan layer. The structural similarity between β-lactam antibiotics and d-alanyl- d-alanine facilitates their binding to the active site of PBPs.

2. Mechanism of resistance to carbapenem

2.1 *Outer membrane proteins (OMPs)*

The outer membranes of Gram-negative bacteria contain proteins called porins that form channels that allow the passage of many hydrophilic substances, including antibiotics [12]. In *Enterobacteriaceae,* the loss of OMPs, such as OmpK35 and OmpK36, has significantly reduced their sensitivity to β-lactam antibiotics [13,14]. It is known that the loss of OMPs and the production of EBSLs are associated with resistance to carbapenem [14,15]. The loss of OmpK36 reduces the outer membrane permeability of *K. pneumoniae* to carbapenem and reduces the intermediate resistance and sensitivity of *K. pneumoniae* to meropenem and ertapenem, respectively. OmpK36 actively restricts the passage of ertapenem and meropenem; however, it does not act on imipenem [16]. This phenomenon was confirmed by the observation that imipenem can penetrate the bacterial cell wall more quickly than meropenem [17].

2.2 *Efflux pumps*

To increase the removal of antibiotics from the intracellular compartment (or the intermembrane space in Gram-negative bacteria), some bacteria contain membrane proteins that function as exporters, called efflux pumps, for certain antimicrobial agents. These pumps expel the drug from the cell at a high rate, meaning that the drug concentrations are never sufficiently high to elicit an antibacterial effect. Most efflux pumps are multidrug transporters that efficiently pump a wide range of antibiotics, contributing to MDR [18]. The exact mechanism of active efflux pumps in release of antibiotics from bacterial cells is not known. However, the upregulation of *ramA*, a transcriptional activator, and the presence of *acrA*, an resistance-nodulation division (RND) family efflux pump gene, indicate that these gene products are responsible for the high minimum inhibitory concentration (MIC) against ertapenem. These efflux systems and antibiotic permeation mechanisms are known to confer high tolerance to ertapenem in Enterobacteria cloacae [19]. Among efflux pumps, RND family is known to reduce the sensitivity of ertapenem and meropenem [19-21]. In *Pseudomonas aeruginosa* as well as in intestinal bacteria, overexpression of efflux pumps, along with loss of porin and overexpression of AmpC, leads to carbapenem resistance [20,22]

2.3 *Carbapenemase*

As cephalosporin antibiotic resistance in gram-negative bacteria, including *enterobacteria*, has increased worldwide, carbapenems have been used as therapeutic agents for treatment of infections caused by AmpC β-lactamase- and ESBL-producing bacteria [23]. Carbapenem is a β-lactam antibiotic with the largest antibacterial spectrum and good antibacterial activity against gram-positive bacteria, gram-negative bacteria, and anaerobic bacteria.

As the amount of carbapenem used increased, various carbapenem-degrading enzymes emerged. β-lactamases have been divided into classes A, B, C, and D by Ambler (Table 1) based on homology of amino acid sequences [24-26].

Among the carbapenemases belonging to Class A, genes encoding the *Serratia marcescens* enzyme (SME), not metallo enzyme carbapenemase (NMC), and imipenem hydrolyzing enzyme (IMI) are located in chromosome, while genes for Guiana extended-spectrum (GES) and KPC are located in plasmid. In addition, genes for Verona integron-encoded metallo-β-lactamases (VIM), Imipenemase (IMP), and New Delhi metallo-β-lactamase (NDM), which are Class B metallo-β-lactamases required for zinc cation on active sites, are all located in the plasmid, whereas

Chryseobacterium indologenes (IND) and *Legionella gormannii* (FEZ-1) are located in the chromosome. Among the AmpC β-lactamases belonging to Class C, are chromosomally encoded in some gram-negative bacilli and plasmid-mediated AmpC β-lactamase(PABL) is transferred from chromosomes to plasmid [24-26].

The gene of OXA type enzyme, a serine β-lactamase belonging to Class D, is located in either a plasmid or the chromosome. Chromosomal OXA gene has been reported to be congenital in some bacteria in the natural environment [24-26].

Table 1. β-lactamase classification schemes

Molecular classification (Ambler)	Common Name	β-lactam resistance	Representative examples
A	Carbapenemase, penicillins, cephalosporins	Aztreonam, β-lactam/β-lactamase inhibitor combinations, carbapenems, variable inhibition by β-lactamase inhibitor	*NMC-A, IMI-1, SME-1, SFC, BIC-1, GES-2, KPC*
B	Metallo-β-lactamase	All β-lactams except aztreonam, not inhibited by β-lactamase inhibitors	*IMP-1, VIM-1, SPM-1, GIM-1, SIM-1, NDM-1*
C	Cephalosporinase	Penicillins, cephalosporins, not inhibited by β-lactamase inhibitors	*AmpC, CMY-2, ACT-1, DHA-1, ACC-1, Fox-1*
D	Carbapenemase	Penicillins, cephalosporins, aztreonam, β-lactam/β-lactamase inhibitor, variable	*OXA-23, OXA-24, OXA-40, OXA-48, OXA-51, OXA-66*
	Oxacillinase	Penicillins, variable inhibition by β-lactamase inhibitor	*OXA-1, OXA-10, OXA-15*

2.3.1 Molecular class A carbapenemase

Class A carbapenemase includes NMC/IMI, SME, KPC, and requires serine in active-site. Class A carbapenemase can be divided into two categories depending on the location of the gene. Carbapenemase genes located in the chromosome include SME, NMC, and IMI. SME was first found in S. marcescens and spread to other bacteria with various genetic variations [27,28].

The amino acid sequences of IMI and NMC-A exhibit 97% homology. These enzymes exhibit resistance to carbapenems, penicillins, and aztreonam, but exhibit sensitivity to extended-spectrum cephalosporins. IMI and NMC-A are inhibited by clavulanic acid, but not SME. The Class A carbapenem gene located in chromosome is known to have a limitation on its spread [27-29].

The second category of Class A carbapenemases includes carbapenemase located in plasmid, such as GES and KPC. To date, 13 variants of KPC and 17 genetic mutations of GES have been reported [30,31]. GES was isolated from French Guiana in 1998, while KPC was isolated from *K. pneumoniae* and is known to be located in a genetically movable structure called integron in the plasmid [27-29]. GES exhibits the ability to decompose penicillins, cephalosporins, and carbapenems, while it is inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam [27,29,32].

2.3.2 Molecular Class B Metallo-β-lactamase

Molecular Class B Metallo-β-lactamase (MBL) is an enzyme that requires metal ions (Zn^{2+}) on active sites to degrade β-lactam. Unlike serine β-lactamase, MBL is not inhibited by clavulanic acid, but is inhibited by metal chelating reagents such as EDTA. Most MBLs can break down almost all β-lactams except aztreonam. The early MBLs were found in *Bacillus cereus*, *Aeromonas spp.*, and in the chromosome of *Stenotrophomonas maltophilia*. Additionally, they are also located in the integron in the form of gene cassette. It has been reported that it is possible for MBLs to move in association with a transposon [33]. To date, more than 30 variants have been reported for IMP and VIM [34]. Both enzymes are found mainly in *P. aeruginosa*.

Recently, many of the bacteria belonging to the *Acinetobacter spp.* and intestinal bacteria have been found to possess the class B Metallo-β-lactamase [29,31,33]. New Delhi Metallo-β-lactamase (NDM) was first discovered in *K. pneumoniae* as a new molecular class B enzyme in 2009 and was isolated [26,31]. NDMs from NDM-1 to NDM-7 have been detected worldwide in patients or travelers treated and operated in India and elsewhere [31]. NDM-1 has been detected in *K. pneumoniae*, *K. oxytoca, E. coli, Citrobacter freundii, Morganella morganii, Providencia spp., Proteus spp.*, *E. cloacae*, and *A. baumanii* [23].

2.3.3 Molecular Class D OXA carbapenemase

OXA (oxacillinase) enzyme is a class D serine β-lactamase, which is used as a catalyst for isozazolylpenicillin oxacillin. When it was first discovered, cloxacillin was named oxacillinase because it was more effective than penicillin, and now it has been renamed as Class D OXA carbapenemase [35]. Currently, 200 OXA enzymes have been identified and most of them are known to be unable to break down extended-spectrum cephalosporins [30,31] and there are about 40 types of carbapenem which are hydrolyzed [36]. OXA type carbapenemase was found in Scotland in 1983 and was reported to produce OXA-23 [37].

Later, enzymes that degrade carbapenem, such as OXA-48 and OXA-181, were found. OXA-48/181 was first discovered in Turkey in 2004 from the intestinal bacteria. Subsequently, it has been found in *A. baumanii*, and many of them have been found in *K. pneumoniae*. OXA-48/181 has spread rapidly to Europe and the Middle East [38].

3. *Klebsiella pneumoniae* carbapenemase (KPC)

3.1 *KPC characteristics*

KPC is an Ambler Class A β-lactamase, which corresponds to 2f of the Bush functional group [35]. KPC was found to be a novel enzyme of 2f group, including SME, NMC-A, and IMI-1. In particular, KPC can more effectively break down oxyimino-cephalosporin and break down penicillins, cephalosporins, and aztreonam, unlike other 2f group enzymes such as SME, NMC-A, and IMI-1. In addition, KPC showed a decrease in hydrolysis effect by clavulanic acid and tazobactam, beta-lactamase inhibitors [16]. KPC-producing bacteria exhibit resistance to carbapenem antibiotics such as meropenem, imipenem, ertapenem, and doripenem. Moreover, other antibiotic resistance mechanisms, such as loss of OMPs, have resulted in the development of highly resistant strains [8,22,39]. Till date, 13 KPC subtypes based on amino acid variations have been reported since KPC-1 was discovered [17,20,21,40]. KPC enzymes have been reported in all Gram-negative members of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa.* and *Enterobacter*) pathogens [41]. Although actual prevalence and epidemiological status varies markedly with countries, ranging

from sporadic to endemic, they occur worldwide [42,43]. These pathogens represent a new paradigm in resistance and pathogenesis and are responsible for life-threatening diseases affecting hospitalized and immune-compromised patients [44,45]. More than 50% of vulnerable patients with hospital-acquired pneumonia die if their infection is caused by a strain producing KPC-2 [46,47].

KPC-2 has an overall structure similar to those of other class A enzymes. In addition, β-lactamase has only 50% protein sequence conservation compared to CTX-M-1, 39% compared to SHV-1, and 35% compared to TEM-1. KPC-2 is more like other class A carbapenemases, having 55% identity to NmcA and Imi-1, 63% identity to Sfc-1, and 57% identity to Sme-1. The KPC-2 β-lactamase possesses a large and shallow active site, allowing it to accommodate “bulkier” β-lactams [48]. As a result of these structural characteristics, KPC-2 is regarded as a versatile β-lactamase [49]; thus, it is a penicillinase, carbapenemase, and cephamycinase and an extended-spectrum β-lactamase [50,51].

3.2 *Discovery and spread of KPC*

KPC-2 was first reported in the United States in 2001 [16], and continued to be reported until 2005, but the geographical distribution of KPC found in enterobacteria including *K .pneumoniae* was limited to the eastern United States [16,52]. Since then, the carbapenem-resistant *K. pneumoniae* (CRKP) has spread at a rapid rate throughout the United States. Especially, the diffusion of sequence type (ST) 258 monoclonals has been a serious problem [53]. KPC was also found in other *Enterobacteriaceae*, including *E. coli, Enterobacter spp., Salmonella enterica, Proteus mirabilis,* and *Citrobacter freundii* in addition to *K. pneumoniae* [26,35,36]. In addition to *Enterobacteriaceae,* it was first discovered in *P. aeruginosa* in 2007 [37]. KPC genes are known to exist in plasmids, but in *P. aeruginosa*, they have been reported to be located in chromosome [31-33].

KPC- producing *Enterobacteriaceae* have spread rapidly around the world, and they are not limited to specific regions, in stitutions, and organizations.

In Israel, when KPC-3 producing *K. pneumoniae* was discovered, the characteristics of the strain were investigated to confirm whether it was the same clone as in the United States.

This showed that *K. pneumoniae* could move between continents [54]. In the United States, France, and Israel, the KPC-producing *Enterobacteriaceae* were first discovered in 2005 [55]. In South America, KPC-producing *K. pneumoniae* was first discovered in Colombia in 2006 [56]. Since then, it has also been reported in Brazil and Argentina [57,58]. In China, KPCs were found from various intestinal bacterial samples and they are spreading gradually [59], and KPC-producing *K. pneumoniae* has been reported in almost all countries in Europe [60]. According to 2018 data from the Korea Centers for Disease Control and Prevention, 8618 strains of carbapenemase-producing *Enterobacteriaceae* bacteria possess KPC-2 (4316, 72.8%), including *K. pneumoniae* (5619, 65.2%) and *E. coli* (1482, 17.2%). Various intestinal bacteria were also reported to possess KPCs [61].

3.3 *MLST of KPC and plasmid type*

In order to confirm the spread pattern of KPC gene, multilocus sequence typing (MLST) was performed on KPC-producing *K. pneumoniae* strains, which were isolated from 18 U.S. states, India, and Israel from 1996 to 2008, and most of them were ST258. ST14 was also found in the strains, but ST258 was more common [62-64]. According to the data reported in Korea in 2016, *K. pneumoniae* ST 307 and *E. coli* ST 410 were the most common sequence types [65].

Among diverse subtypes of KPC genes, the *blaKPC-2* gene is carried by IncFIA-, IncFIIk-, IncN-, and IncA/C-type plasmids in the USA; IncN-, IncA/C- and IncF-type plasmids in China; an IncFIIA-type plasmid in France; an IncFIIk-type plasmid in Greece; and IncFIIs-, IncN-, and IncHI2- plasmids in Israel. Conversely, the blaKPC-3 gene is associated with IncFIA-, IncFIIk-, IncN-, and IncA/C-type plasmids in the USA; an Inc-FIIk-type plasmid in Italy; IncFII-, IncA/C-, and ColE-type plasmids in Spain; and IncFIIs-, IncN-, and IncHI2-plasmids in Israel [66,67]. Although an IncX3-type plasmid is prevalent in *K. pneumoniae* carrying the blaKPC gene [68], it has rarely been reported in *E. coli.*

3.4 *Structure of KPC gene*

KPC is a plasmid-mediated enzyme, which is located in a transposon consisting of various insertion sequences in various sizes of plasmids, and can be moved between different species and different plasmids [39,52]. KPC genes are found to be present in Tn4401, a transposon of Tn3-type [8]. Tn4401 is a 'conjugative transposon' because it has a resistant gene and crosses between bacteria freely. It is also called 'jumping gene'. The structure of transposon Tn4401 includes transposase gene (tnpA), resolvase gene (tnpR), two insertion sequences (ISKpn7 and ISKpn6), and blaKPC [10]. Transposon Tn4401 can be divided into isoforms from a to e, which are classified according to the disappearance of the upstream part of the KPC gene. Tn4401a and Tn4401c have 99 bp and 215 bp deletions, respectively, in this region, in contrast to Tn4401b that does not have any deletion. The isoforms d and e have 68 bp and 255 bp deletions, respectively, in the upstream region of the KPC gene. However, all of the above isoforms have the same arrangement in the downstream region of the KPC gene [69,70].

3.5 *Purpose of the study*

To our knowledge, the ability to readily hydrolyze clavulanic acid, sulbactam, and tazobactam is very uncommon in class A enzymes [71]. Moreover, KPC-2 β-lactamases are becoming geographically widespread. The purposes of this study were to: 1) survey the prevalence of KPC- CPE strains in Korea by analyzing the KPC- CPE strains isolated in a local hospital for two years from 2017 to 2019, 2) characterize the molecular genetic structure of the isolated KPC- CPE strains by whole genomic sequencing, and 3) confirm the horizontal interspecies transmission of KPC-CPE characteristics among *Enterobacteriaceae.*

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Chapter Ⅱ

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

1. Abstract

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 Frost LS et al. Finally, the high detection rate of transposon Tn*4401* 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.

2. 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 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), Imipenemase (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 Tn*3*-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.

3. Materials and Methods

3.1 *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 B Hospital, Busan, Korea (approval number CTS-19-004).

3.2 *Bacterial isolates and antimicrobial susceptibility testing*

Clinical isolates were identified by standard microbiological procedures and VITEK-2 (bioMérieux Vitek, Hazelwood, MO, 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) and disk diffusion tests on Mueller-Hinton agar (MH; Becton Dickinson, Franklin Lakes, NJ, 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 [19,20]. The susceptibility test results for tigecycline were confirmed by the E-test (bioMérieux, Marcy-l'Etoile, France).

For the modified carbapenem inactivation method test, organisms were incubated with a meropenem disk in tryptic soy broth (TSI; Difco Laboratories, Detroit, MI, 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-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].

3.3 *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, VIM, NDM, KPC, KPC-2, Guiana-extended-spectrum β-lactamase, and OXA-48-like) and ESBLs (CTX (cefotaximase) -M-1-, CTX-M-9-, TEM-, and sulfhydryl variant (SHV)-type) [21]. 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) [22,23], and fluoroquinolone resistance determinants (including qepA, qnrA, qnrB, and qnrS) as previously described [24]. PCR was performed to detect the genes encoding the outer membrane protein (OmpK35, OmpK36) [25](Table 1).

3.4 *Isotyping the Tn4401 transposon*

To determine the isotypes of Tn*4401*, isotype-specific forward primers (a - e) for the five most common isotypes [17] were newly designed (Table 2), and PCR was carried out with a universal reverse primer targeting *blaKPC*. The amplicons were sequenced for verification [26].

Table 1. Primer for PCR identification of resistance determinants.

Gene	Primer	Sequence	PCR size(bp)
β-lactamase	VIM_F	ATTGGTCTATTTGACCGCGTC	780
	VIM_R	TGCTACTCAACGACTGAGCG	
	IMP_F	CATGGTTTGGTGGTTCTTGT	488
	IMP_R	ATAATTTGGCGGACTTTGGC	
	NDM_F	TGGAATTGCCCAATATTATGC	813
	NDM_R	TCAGCGCAGCTTGTCGGCCATGC	
	OXA-48_F	TTGGTGGCATCGATTATCGG	743
	OXA-48_R	GAGCACTTCTTTTGTGATGGC	
	GES_F	ATGCGCTTCATTCACGCAC	863
	GES_R	CTATTTGTCCGTGCTCAGGA	
	KPC-2_F	ATGTCACTGTATCGCCGTCT	893
	KPC-2_R	TTTTCAGAGCCTTACTGCCC	
	Kpc-_F	CTGTCTTGTCTCTCATGGCC	795
	Kpc_R	CCTCGCTGTGCTTGTCATC	
ESBLs	TEM_F	ATGAGTATTCAACATTTCCGT	861
	TEM_R	TTACCAATGCTTAATCAGTGA	
	SHV_F	CCGGGTTATTCTTATTTGTCGCT	831
	SHV_R	TAGCGTTGCCAGTGCTCG	
	CTX-M-1_F	ACCGTCACGCTGTTGTTAGG	819
	CTX-M-1_R	CAAGGTGACGATTTTAGCCG	
	CTX-M-9_F	GTGCAACGGATGATGTTCG	844
	CTX-M-9_R	ATGATTCTCGCCGCTGAAG	
AmpC	ACT_F	TCGGTAAAGCCGATGTTGCGG	302
	ACT_R	CTTCCACTGCGGCTGCCAGTT	
	ACC_F	AACAGCCTCAGCAGCCGGTTA	346
	ACC_R	TTCGCCGCAATCATCCCTAGC	
	CMY_F	TGGCCAGAACTGACAGGCAAA	462
	CMY_R	TTTCTCCTGAACGTGGCTGGC	
	DHA_F	AACTTTCACAGGTGTGCTGGGT	405
	DHA_R	CCGTACGCATACTGGCTTTGC	

aminoglycoside	rmtA_F	CCCCATCGTGTGTTGGATAT	635
	rmtA_R	GCACGCCCATACAGATGTTA	
	rmtB_F	CGCATCCTGACTGAGG	584
	rmtB_R	GGTGCGTGCAGCCTCT	
	rmtD_F	CACAGCAAATCGCCCA	532
	rmtD_R	CGGTCAGTGCGTAAAC	
	armA_F	TGAAAGAGTCGCAACATTAAATGA	590
	armA_R	CCATTGGTATAAAGCTAATGGATTG	
fluoroquinolone	qnrA_F	AGAGGATTTCTCACGCCAGG	580
	qnrA_R	TGCCAGGCACAGATCTTGAC	
	qnrB_F	GGAATTGAAATTCGCCACTG	264
	qnrB_R	TTTGCCGCCCGCCAGTCGAA	
	qnrS_F	GCAAGTTCATTGAACAGGGT	428
	qnrS_R	TCTAAACCGTCGAGTTCGGCG	
	qepA_F	CCGACAGGCCCACGACGAGGATGC	549
	qepA_F	TCGGCGGCGTGTTGCTGGAGTTCT	
outer membrane protein	ompK35_F	AACTTATTGACGGCAGTGGC	1054
	ompK35_R	TTGGTAAACGATACCCACGG	
	ompK36_F	GCAGTGGCATAATAAAAGGCA	1080
	ompK36_R	ACTGGTAAACCAGGCCCAG	

Table 2. Primers used for isotyping of the Tn4401 transposon.

Name	Sequence (5′-3′)	Purpose	Specific for Tn4401 isotype
For blaKPC subtyping			
blaKPC-F	ATGTCACTGTATCGCCGTCT	PCR/Sequencing	-
blaKPC-R	TTTTCAGAGCCTTACTGCCC	PCR/Sequencing	-
For Tn4401 isotyping			
abcde-396-377_F_495	ACCCGAATGATCCAGGTGGG	PCR	a / b / c / d / e
abcd_-275-256_F_374	CTTGGCCAGGACTTCCTGAG	PCR	a / b / c / d
_b__-266-147_F_265	GGTGCCAGGGACTTACCAAC	PCR	b
_b_d_-114-95_F_213	GTTACAGCCTCTGGAGAGGG	PCR	b / d
ab_d_-69-50_F_168	CCAGCTGTAGCGGCCTGATT	PCR	a / b / d
For Tn4401 isotyping			
kpc_uni-80-99_R	GAATGGTTCCGCGACGAGGT	PCR/Sequencing	a / b / c / d / e

Abbreviations: PCR, polymerase chain reaction.

3.5 *Multilocus sequence typing (MLST)*

PCR and sequencing (Table 3) were carried out for seven housekeeping genes: *adk, fumC, gyrB, icd, mdh, purA,* and *recA* for *E. coli*[27]; *gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB* for *K. pneumoniae* [28]; 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.

Table 3. Primers used in MLST.

Species	Gene	Name	Sequence (5′-3′)	Tm
Klebsiella pneumoniae	rpoB	Vic3_F	GGCGAAATGGCWGAGAACCA	50°C
		Vic2_R	GAGTCTTCGAAGTTGTAACC	
	gapA	gapA173_F	TGAAATATGACTCCACTCACGG	60°C
		gapA181_R	CTTCAGAAGCGGCTTTGATGGCTT	
	mdh	mdh130_F	CCCAACTCGCTTCAGGTTCAG	50°C
		mdh867_R	CCGTTTTTCCCCAGCAGCAG	
	pgi	pgi1F_F	GAGAAAAACCTGCCTGTACTGCTGGC	50°C
		pgi1R_R	CGCGCCACGCTTTATAGCGGTTAAT	
	phoE	phoE604.1_F	ACCTACCGCAACACCGACTTCTTCGG	50°C
		phoE604.2_R	TGATCAGAACTGGTAGGTGAT	
	infB	infB1F_F	CTCGCTGCTGGACTATATTCG	50°C
		infB1R_R	CGCTTTCAGCTCAAGAACTTC	
	tonB	tonB1F_F	CTTTATACCTCGGTACATCAGGTT	45°C
		tonB2R_R	ATTCGCCGGCTGRGCRGAGAG	
Escherichia coli	adk	adk_F	GCAATGCGTATCATTCTGCT	52°C
		adk_R	CAGATCAGCGCGAACTTCAG	
	fumC	fumC_F	CCACCTCACTGATTCATGCG	
		fumC_R	CGGTGCACAGGTAATGACTG	
	gyrB	gyrB_F	CGGGTCACTGTAAAGAAATTATCG	
		gyrB_R	GTCCATGTAGGCGTTCAGGG	

	icd	icd_F	TACATTGAAGGTGATGGAATCG	
		icd_R	GTCTTTAAACGCTCCTTCGG	
	mdh	mdh_F	TCTGAGCCATATCCCTACTG	
		mdh_R	CGATAGATTTACGCTCTTCCA	
	purA	purA_F	CTGCTGTCTGAAGCATGTCC	
		purA_R	CAGTTTAGTCAGGCAGAAGC	
	recA	recA_F	AGCGTGAAGGTAAAACCTGTG	
		recA_R	ACCTTTGTAGCTGTACCACG	
Citrobacter freundii	aspC	aspC-F	GTTTCGTGCCGATGAACGTC	50°C
		aspC-R	AAACCCTGGTAAGCGAAGTC	
	clpX	clpX-F	CTGGCGGTCGCGGTATACAA	
		clpX-R	GACAACCGGCAGACGACCAA	
	fadD	fadD-F	GCTGCCGCTGTATCACATTT	
		fadD-R	GCGCAGGAATCCTTCTTCAT	
	mdh	mdh-F	GTCGATCTGAGCCATATCCCTAC	
		mdh-R	TACTGACCGTCGCCTTCAAC	
	arcA	arcA-F	GACAGATGGCGCGGAAATGC	
		arcA-R	TCCGGCGTAGATTCGAAATG	
	dnaG	dnaG-F	ACCGCCGATCACATACAACT	
		dnaG-R	TGCACCAGCAACCCTATAAG	
	lysP	lysP-F	GCTACGTCGTGAACTGAAGG	
		lysP-R	TGTCCCCTGGAAGGAGAAGC	

Abbreviations: MLST, multilocus sequence typing; Tm,

3.6 *Bacterial conjugation*

Conjugation was employed to confirm the possibility of plasmid delivery including KPC-2 gene. The 40 K. pneumonia strains were used as donor strains and sodium azide-resistant E. coli J53 was used as the recipient [29]. For resistance transfer, the test bacteria and E. coli J53 were cultured in brain-heart infusion (BHI) medium (MB cell, Los Angeles, CA, USA) at 37°C for 18 h. E. coli J53 was cultured continuously, while test bacteria was diluted in the ratio 1:9 with 5 mL of BHI.

The culture of E. coli J53 and each test bacterium was mixed in the ratio of 1:4 after cultivating the strains under the same conditions for 4 h, following which the mixture was incubated for 30 min. The mixture (100 μL) was then inoculated on a BHI agar plate and the plate was desiccated. The dried BHI agar plate was incubated at 37°C for 18 h. Following incubation, transconjugants were selected using BHI addition agar supplemented with 100 μg/mL sodium azide and 0.5 μg/mL imipenem. The conjugated strains were identified via PCR, β-lactamase-encoding, extended-spectrum β-lactamases, plasmid-mediated AmpCs, 16S ribosomal methyl transferases, and sensitivity to quinolones.

3.7 *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, Los Angeles, CA, USA) 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, St. Louis, MO, 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 [30].

4. Results

4.1 *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 (Figure 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%) (Figure 1b).

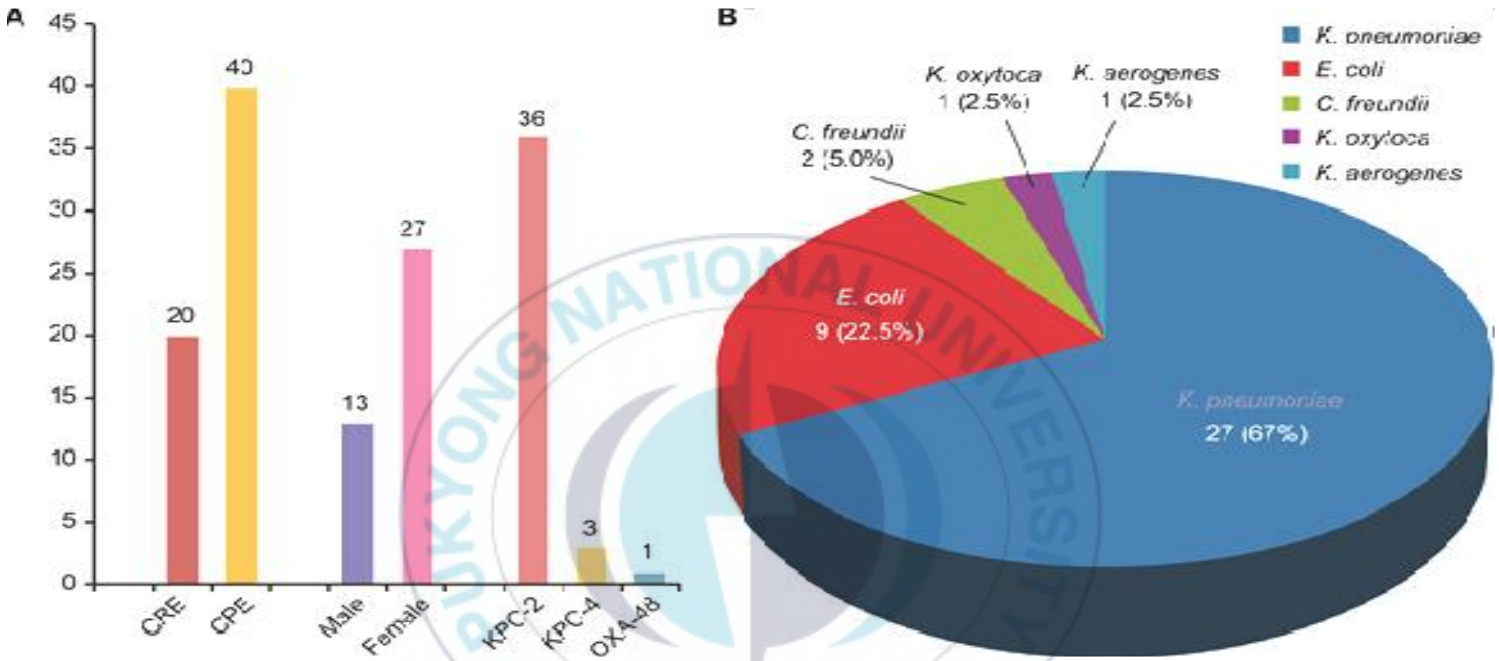

Figure 1. Distribution of species in carbapenemase-producing *Enterobacteriaceae* (CPE) isolates.

A. Distribution of carbapenemase genotypes in carbapenem-resistant *Enterobacteriaceae* isolates (n=60)

B. Distribution of genotypes of CPE isolates (n=40)

4.2 *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 trimethoprim-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 4.

Table 4. Antimicrobial susceptibilities of CPE isolates and phenotypes.

CPE Isolates (%)	Antibiotic 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)
Δ*Tn4401a* (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)
Δ*Tn4401b* (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.

4.3 *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 (Figure 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* (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 5). 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 [31]. A multi-agency survey conducted in 2013

confirmed that porins, including *OmpK35* and *OmpK36,* were observed in 27 isolates (67.5%) [32]. 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 6).

Table 5. 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 pneumoniae* carbapenemase; OXA, oxacillinase; SHV, sulfhydryl variant; TEM, Temoneira β-lactamase; CTX-M, cefotaximase.

Table 6. 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 pneumoniae*; ECO, *Escherichia coli*; CFR, *Citrobacter freundii*; KOX, *Klebsiella oxytoca*; KAE, *Klebsiella aerogenes*; ST, sequence type; IMP, imipenem.

4.4 *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 7). *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%).

4.5 *Isotyping the Tn4401 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. [26]. For the six non-isotypable transposons (15%) by the Tn*4401*-isotyping PCR, amplification between the 3′-end of the upstream IS*Kpn7* 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 7.

Table 7. *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	-	-
bla*KPC-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)
bla*KPC-2 Tn*4401b	1 (2.5)	2 (5)	-	-	-	-	-	-	1 (2.5)	1 (2.5)	-	-	-	-
bla*KPC-4 Tn*4401b	-	-	-	-	1 (2.5)	-	-	-	-	-	1 (2.5)	-	1 (2.5)	-

Abbreviations: KPC, *Klebsiella pneumoniae* carbapenemase.

4.6 *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 4). Importantly, the transconjugant assays enabled the simultaneous transfer of *blaKPC-2*, *blaKPC-4*, *blaOXA-48*, ESBL, and AmpC, as well as aminoglycoside and fluoroquinolone resistance-determining genes (Table 5).

4.7 *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 4.

5. Discussion

In the 1940s, the first antibiotics were derived from mold, and since then antibiotic development has progressed toward their 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 [33]. 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 [34]. In veterinary medicine, fluoroquinolones have served as effective therapeutics for treating enteric infections and respiratory diseases in food-producing and companion animals [35]. 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 [36,37]. Since the late 1980s, fluoroquinolones used in human medicine have differed from those used in veterinary medicine [38,39]. 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 [40,41]. 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 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 [42]. 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 [43,44]; 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 [26]. 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* [45]. Furthermore, clones co-producing CPE and *CTX-M-15* were recently identified in Italy [46] and Denmark [47]. The high distribution of *CTX-M-1* reported in this study highlights the risk of emergence and spread of high-risk clones (Table 6). 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 [48]. 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 [32]. 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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Chapter Ⅲ

Transfer of KPC-2 Carbapenemase gene from a high risk clone of *Escherichia coli* ST410 to *Klebsiella pneumoniae*

1. Abstract

Sequence type 410 (ST410) of *Escherichia coli* is an extraintestinal pathogen associated with multi-drug resistance. In this study, we aimed to investigate the horizontal propagation pathway of a high-risk clone of *E. coli* and *Klebsiella pneumoniae* carbapenemase (KPC). *blaKPC*-encoding *E. coli* and *K. pneumoniae* isolates were evaluated, and complete sequencing and comparative analysis of *blaKPC*-encoding plasmids from *E. coli* and *K. pneumoniae*, antimicrobial susceptibility tests, polymerase chain reaction, multilocus sequence typing, and conjugal transfer of plasmids were performed. Whole-genome sequencing was performed for plasmids mediating *KPC-2* production in *E. coli* and *K. pneumoniae* clinical isolates. Strains *E. coli* CPEc171209 and *K. pneumoniae* CPKp171210 were identified as ST410 and ST307, respectively. CPEc171209 harbored five plasmids belonging to serotype O8:H21, which is in the antimicrobial-resistant clade C4/H24. The CPKp171210 isolate harbored three plasmids. Both strains harbored various additional antimicrobial resistance genes. The IncX3 plasmid pECBHS_9_5 harbored *blaKPC-2* within a truncated Tn*4401a* transposon, which also contains *blaSHV-182* with duplicated conjugative elements. This plasmid displayed 100% identity with the IncX3 plasmid

pKPBHS_10_3 from the *K. pneumoniae* CPKp171210 ST307 strain. The genes responsible for the conjugal transfer of the IncX3 plasmid included *tra/trb* clusters and pil genes coding the type IV pilus. ST410 can be transmitted between patients, posing an elevated risk in clinical settings. The emergence of a KPC-producing *E. coli* strain (ST410) is concerning because the *blaKPC-2*-bearing plasmids may carry treatment resistance across species barriers. Transgenic translocation occurs among carbapenem-resistant bacteria and these bacteria, which may spread rapidly via horizontal migration.

2. Introduction

The gram-negative bacteria family *Enterobacteriaceae* includes pathogens that are responsible for a large number of infections and deaths worldwide each year. Furthermore, the continuously increase in the prevalence of antibiotic resistance in this bacteria family poses a serious problem [1]. Carbapenemases that endow bacteria with carbapenem resistance often jeopardize allopathic treatment for infectious diseases caused by common nosocomial pathogens. Currently, *Klebsiella pneumoniae* carbapenemases (KPCs), belonging to the Ambler class A carbapenemases, are the most clinically concerning enzymes because of their global presence and capability of using a broad spectrum of substrates, including most β-lactam drugs, except cephamycins [2,3]. Molecular epidemiological studies on multilocus sequence typing (MLST) for KPC-producing *Escherichia coli* have revealed 131 more widespread strains than the ST410, ST69, ST93, ST167, ST354, and ST3948 strains based on their sequence type (ST) [4-7]. *E. coli* ST 410 has been reported worldwide as an extraintestinal pathogen and associated with resistance to fluoroquinolones, third-generation cephalosporins, and carbapenems. Recent studyies on ten extended-spectrum β-lactamase (ESBL)-producing *E. coli* ST410 isolates from Germany indicated that this lineage includes a new successful clone with

cross-sectorial transmission among wildlife, humans, pets, and animals used for commercial purposes through various environmental modes [8,9]. The accumulation of multidrug resistance in *E. coli* ST410 over the past two decades, along with its potential for transmission between patients, indicates its high risk in clinical settings [10]. Previous studies on carbapenemase-producing *Enterobacteriaceae* (CPE) among inpatients demonstrated the clonal spreading of CPE between patients [11,12]. The transposon Tn*4401*, which is based on highly mobile Tn*3*, facilitates the dissemination of *blaKPC* [13], potentially resulting in horizontal gene transfer to other bacterial species. As the mortality rate associated with invasive infections caused by CPE is high [14], the increasing incidence of carbapenem-resistant bacteria, which are resistant to nearly all antibiotics, is of great concern [15]. This study was performed to investigate the horizontal propagation pathway of the KPC-producing high-risk clone of *E. coli* ST410 in one patient. The mechanism and KPC delivery pathways based on the acquisition of multidrug resistance, including resistance to carbapenem, were investigated using whole-genome sequencing. This study elucidated the risk of this *E. coli* clone to spread genes involved in antibiotic resistance across species, providing clues that can be integrated in the management of high-risk clones in South Korea.

3. Materials and Methods

3.1 *Patient description*

A 79-year-old man with chronic kidney disease was admitted to a general hospital (Busan, Korea) on December 12, 2017, owing to kidney disease. Upon admission, a KPC-2 *E. coli* isolate (CPEc171209) was detected in rectal swabs, and on December 21, 2017, i.e., the 9th day of hospitalization, KPC-2 *K. pneumoniae* (CPKp171210) was isolated. This prospective study was conducted under the approval of the Institutional Review Board of the BHS Hanseo Hospital, Busan, Korea (approval number CTS-19-002).

3.2 *Bacterial isolates and antimicrobial susceptibility testing*

We evaluated the clinical strains of *E. coli* (CPEc171209) and *K. pneumoniae* (CPKp171210) isolated from the rectal swabs harvested from the patient. Bacteria were identified through standard microbiological procedures and VITEK-2 (bioMérieux Vitek, Hazelwood, MO, USA). The identification of all isolates was confirmed through 16S rDNA sequencing [16]. VITEK 2 AST N224 cards (bioMérieux) and disk diffusion tests on Mueller-Hinton agar (MH; Becton Dickinson, Franklin Lakes, NJ, USA) were used to determine antimicrobial susceptibilities in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. The susceptibility test was conducted with ampicillin, aztreonam, cefotaxime, ceftazidime, cefoxitin, ciprofloxacin, amikacin, gentamicin, imipenem, meropenem, ertapenem, and colistin. The minimum inhibitory concentration (MIC) for colistin were determined using the broth microdilution method with Mueller-Hinton broth (Becton Dickinson) in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [17,18]. The susceptibility results for tigecycline were confirmed through the E-test (bioMérieux, Marcy-l'Etoile, France).

For the modified carbapenem inactivation method (mCIM) test,

organisms were incubated with a meropenem disk in tryptic soy broth (TSI; Difco Laboratories, Detroit, MI, USA). For the ethylene diamine tetra acetic acid (EDTA)-mCIM (eCIM) test, EDTA was added to the broth to chelate the metal ions necessary for metallo-β-lactamase function. After incubation, the disks were removed and placed on a lawn of susceptible *E. coli* to determine whether the test organisms degrade meropenem. Clearance zones were measured and interpreted in accordance with the CLSI guidelines [17].

3.3 *Genotyping of β-lactamases and outer membrane proteins*

The β-lactamase-encoding gene was selected through polymerase chain reaction (PCR). Genes encoding carbapenemases (GES, IMP-1-type, KPC, KPC-2, NDM, OXA-48-like, and VIM-2-type) and extended-spectrum β-lactamases (CTX-M-1-, CTX-M-9-, SHV-, and TEM-type) were assessed by PCR as previously described [19]. CPE isolates were examined for the presence of 16S ribosomal methyltransferases (armA, rmtA, rmtB, and rmtD) [20,21] and quinolones (qepA, qnrA, qnrB, and qnrS), as previously described [22], and of genes encoding outer membrane proteins (ompK35 and ompK36) [23].

3.4 *MLST*

PCR and sequencing of the amplified DNA fragments were performed for seven housekeeping genes: *adk, fumC, gyrB, icd, mdh, purA,* and *recA* of *E. coli* [24] and *gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB* of *K. pneumoniae* [25]. Nucleotide sequences were compared to those available in the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) to identify allelic numbers and STs.

3.5 *Bacterial conjugation*

Bacterial conjugation was conducted using the *E. coli* CPEc171209 and *K. pneumoniae* CPKp171210 strains as donors and sodium azide-resistant *E. coli* J53 strain as the recipient, following a standard agar mating method [26]. After overnight incubation at 37°C on brain-heart infusion agar (MB Cell, Los Angeles, CA, USA), transconjugants were selected on brain-heart infusion agar supplemented with 100 μg/mL sodium azide and 0.5 μg/mL imipenem.

3.6 *Whole-genome sequencing*

Whole-genome sequencing of CPEc171209 and CPKp171210 was performed. Single-molecule real-time sequencing was performed using a PacBio RSII instrument (Pacific Biosciences, Menlo Park, CA, USA). Schematic diagrams of the multiple alignments of plasmids were generated manually by realigning the linear plasmid maps generated using the SnapGene Viewer software (http://www.snapgene.com/products/snapgene viewer/). Prokka 1.11 (http://www.vicbioinformatics.com/software.prokka.shtml) was used for sequence annotation. ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), IS-Finder (https://isfinder.biotoul.fr/), Plasmid Finder (https://cge.cbs.dtu.dk/services/PlasmidFinder/), Virulence Factor Database (http://www.mgc.ac.cn/VFs/), Restriction-Modification Finder (https://cge.cbs.dtu.dk/services/Restriction-ModificationFinder/), Serotype Finder (https://cge.cbs.dtu.dk/services/SerotypeFinder/), Fim Typer (https://cge.cbs.dtu.dk/services/FimTyper/), CH Typer (https://cge.cbs.dtu.dk/services/CHTyper/), and TA Finder 1.0 (http://202.120.12.133/TAfinder/index.php) were used to identify resistance genes, insertion elements, replication origins, virulent elements, and toxin and antitoxin systems, respectively.

3.7 *Data availability*

GenBank accession numbers for the two sequenced genomes are WMHS01000001-WMHR01000006 (CPEc171209) and WMHR01000001-WMHR01000004 (CPKp171210).

4. Results

4.1 *Antimicrobial susceptibilities and molecular typing*

The antimicrobial susceptibility profiles of the *E. coli* and *K. pneumoniae* strains are presented in Table 1. According to the antibiotic susceptibility profiles, both isolates were resistant to ampicillin, aztreonam, cefotaxime, ceftazidime, cefoxitin, ciprofloxacin, gentamicin, imipenem, meropenem, and ertapenem but susceptible to amikacin, tigecycline, and colistin. Carbapenemase susceptibility tests revealed that both isolates were resistant (Table 2). Furthermore, transconjugant strains displayed multidrug resistance (MDR) patterns during drug susceptibility assays, albeit with reduced cephalosporin and aminoglycoside resistance (Table 3). Differentiation tests for phenotypic carbapenemase were positive for KPC production in both isolates. PCR of β-lactamase genes and sequence analysis of the resulting products supported the presence of *blaKPC-2* and *blaSHV-like* in both isolates. Also, ompK36 was obliterated from CPKp171210.

Table 1. Antimicrobial susceptibilities and epidemiological properties of KPC-producing *Enterobacteriaceae* isolates[a]

Isolate ID	Specimen	Date	MLST	*bla*KPC		Susceptible antibiotics[b]						Carbapenemase differentiation test[c]		Porin loss
						MIC (μg/mL)				Zone diameter (mm)				
			ST	Subtype	Bracketed by	AK	GN	CST	TIG	AK	CST	mCIM	eCIM	
CPEc171209	Rectal	Dec 12, 2017	410	*blaKPC-2*	*ΔTn4401a*	4	-	0.25	0.075	20	12	+	-	-
CPKp171210	Rectal	Dec 21, 2017	307	*blaKPC-2*	*ΔTn4401a*	≤2	≤1	0.25	0.25	20	14	+	-	ompK36

[a]The breakpoints were applied according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Tigecycline susceptibility was confirmed by E-test (bioMérieux), and colistin susceptibility was confirmed by broth microdilution.

[b]Disk diffusion test results were interpreted according to the CLSI guidelines. The results for colistin are not shown because of the lack of suggested breakpoints.

[c]The eCIM is only interpreted when the mCIM result is positive. In contrast to mCIM, when the eCIM result is interpreted, pinpoint colonies within the zone of growth inhibition around the meropenem disk incubated in the presence of EDTA should be ignored. An indeterminate mCIM result occurs when the zone size is 16 - 18 mm, when the zone size is ≥19 mm with pinpoint colonies in the zone of growth inhibition, or when carbapenemase production cannot be confirmed. An eCIM zone size of 16 - 18 mm with pinpoint colonies in the zone of growth inhibition is also considered a positive mCIM result.

Abbreviations: AK, amikacin; GN, gentamicin; CST, colistin; TIG, tigecycline; mCIM, modified carbapenem inactivation method.; eCIM, EDTA-modified carbapenem inactivation method.

Table 2. Carbapenem susceptibility profiles of KPC-producing *Enterobacteriaceae* isolates[a]

Antibiotics	MIC (μg/mL)				Zone diameter (mm)				Interpretation
	CPEc 171209	CPKp 171210	TCPEc 171209	TCPKp 171210	CPEc 171209	CPKp 171210	TCPEc 171209	TCPKp 171210	
IMP	≥16	≥16	≥16	≥16	0	0	0	0	R
MEM	-	-	-	-	0	0	0	0	R
ETP	≥8	≥8	≥8	4	0	0	0	0	R

[a] CPEc171209 and CPKp171210 are pre-conjugation strains, and TCPEc171209 and TCPKp171210 are the conjugated strains.
Abbreviations: IMP, imipenem; MEM, meropenem; ETP, ertapenem.

Table 3. Antibiotic susceptibility profiles of KPC-producing *Enterobacteriaceae* isolates[a]

Isolate ID[b]	MIC (μg/mL)																
	AMP	AMOX-CLA	TZP	CFZ	FOX	CTX	CAZ	FEP	ATM	ETP	IMP	AK	GN	CIP	SXT	TIG	CST
CPEc171209	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	≥16	4	≥16	≥4	≤0.5	0.075	0.25
CPKp171210	≥32	≥32	≥128	≥64	16	8	16	2	≥64	≥8	≥16	≤2	≤1	≥4	2	0.25	0.25
TCPEc171209	≥32	≥32	≥128	≥64	≥64	≥64	≥64	≥64	≥64	≥8	8	4	≥16	≥4	≤0.5	0.075	0.25
TCPKp171210	≥32	≥32	≥128	≥64	32	8	16	2	≥64	4	≥16	≤2	≤1	≥4	≤0.5	0.25	0.25

[a]The breakpoints were applied according to the Clinical and Laboratory Standards Institute guidelines.

[b]CPEc171209 and CPKp171210 are pre-conjugation strains, and TCPEc171209 and TCPKp171210 are the conjugated strains.

Abbreviations: AMP, ampicillin; AMOX-CLA, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; CFZ, cefazolin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; ETP, ertapenem; IMP, imipenem; AK, amikacin; GN, gentamicin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; TIG, tigecycline; CST, colistin; MIC, minimum inhibitory concentration.

4.2 *Sequencing and annotation of CPEc171209*

The *E. coli* strain CPEc171209 genome comprised 5,293,485 bp with a 4,787,633-bp chromosome and five plasmids. They harbored the virulence factors gad and lpfA as well as S83L and D87N substitutions in *gyrA*, an S80I substitution in *parC*, an S458A substitution in *parE*, and unknown mutations in *parC*, *pmrA, 23S, 16S_rsC, pmrB, 16S_rsH,* and *16S_rsB*. The type II restriction enzyme *M.Eco*JA03PDcm and *M.Eco*GVI genes were detected. CPEc171209 belonged to ST410, the serotype belonged to STO8, and H21 and its subtype belonged to fum C4 and fim H24, respectively. Furthermore, outer membrane proteins F and C were detected. Chromosomes included *mph (A)* for macrolide resistance.

4.2.1 pECBHS_9_1 plasmid composition

The pECBHS_9_1 plasmid consisted of a 188,153-bp circular DNA molecule with an average G+C content of 51.1% and 126 annotated open reading frames (ORFs).

The IncA/C2 plasmid (pECBHS_9_1) carried *blaCTX-M-14* and *blaTEM-1C* for β-lactam resistance, *aadA5*, and *armA* for aminoglycoside resistance, *mph (A)* for macrolide resistance, *catA1* for phenicol resistance, *sul1* and *sul2* for sulfonamide resistance, and *dfrA17* for trimethoprim resistance. The plasmid harbored class 1 integrons with a truncated Tn*3* transposon with *blaTEM-1C* (Figure 1; Table 4).

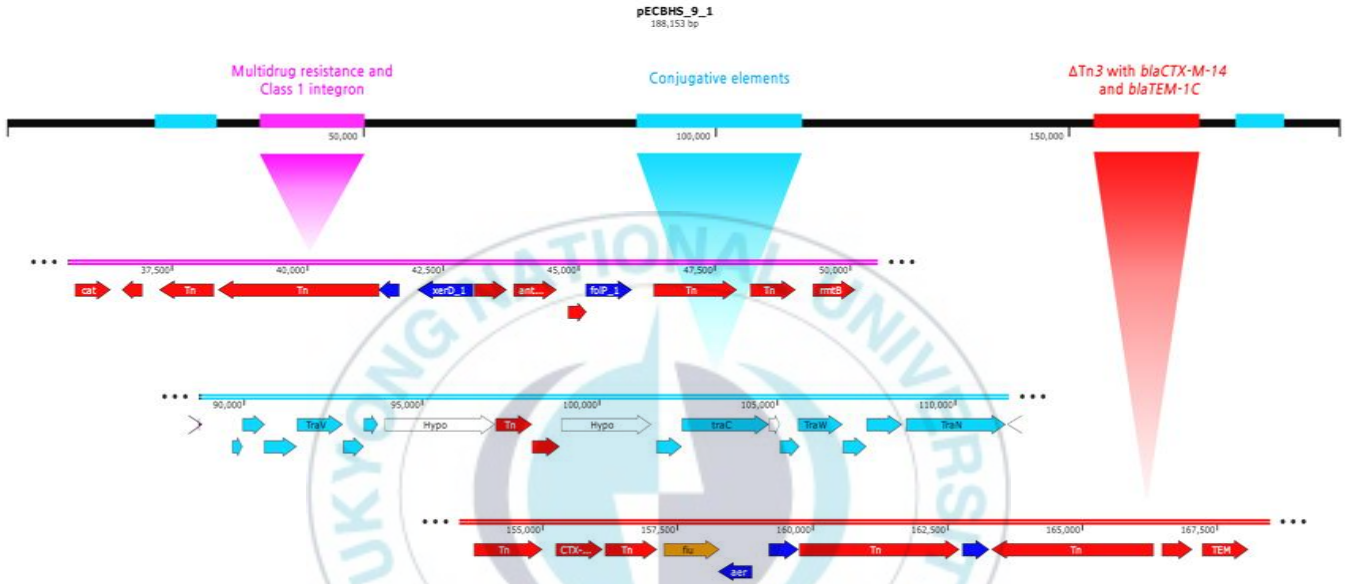

Figure 1. Linear map of pECBHS_9_1. The IncA/C2 plasmid (pECBHS_9_1) harbored *blaCTX-M-14* and *blaTEM-1C* for β-lactam resistance, *aadA5* and *armA* for aminoglycoside resistance, *mph (A)* for macrolide resistance, *catA1* for phenicol resistance, *sul1* and *sul2* for sulfonamide resistance, and *dfrA17* for trimethoprim resistance. The plasmid harbored class 1 integrons containing a truncated Tn*3* transposon with *blaTEM-1C*.

Key: Pink line, multidrug resistance and class 1 integron; red line, Tn*3* transposon; sky blue line, conjugative elements.

Table 4. Antibiotic resistance genes and integrative conjugative elements in KPC-producing *Enterobacteriaceae* isolates

Strain[a]	Plasmid		Antimicrobial resistance gene								Conjugants
	pKPC	Replicon	β-lactam	Aminoglycoside	Tetracycline	Macrolide	Phenicol	Trimethoprim	Sulphonamide	Quinolone	
CPEc171209	*blaKPC-2*		*blaTEM-1, blaCTX-M-1, blaCTX-M-9, blaSHV*	*armA*							+
TCPEc171209*	*blaKPC-2*		*blaTEM-1, blaCTX-M-1, blaCTX-M-9, blaSHV*	*armA*							
pECBHS_9_1		IncA/C2	*blaCTX-M-14, blaTEM-1C*	*aadA5, armA*		*mph(A)*	*catA1*	*dfrA17*	*sul1, sul2*		
pECBHS_9_2		IncFIA, IncFIB, IncFII	*blaCTX-M-14*	*aac(3)-IIa*	*tet(A)*						
pECBHS_9_5		IncX3	*blaKPC-2, blaSHV-182*								
CPKp171210	*blaKPC-2*		*blaTEM-1, blaCTX-M-1, blaSHV*	*rmtC*						*qnrB*	+
TCPKp171210*	*blaKPC-2*		*blaSHV*							*qnrB*	
pKPBHS_10_1		IncFIB(K)	*blaOXA-1*	*aac(6')-Ib-cr*	*tet(A)*		*catB3*	*dfrA14*		*qnrB1*	
pKPBHS_10_3		IncX3	*blaKPC-2, blaSHV-182*								

[a]CPEc171209 and CPKp171210 are pre-conjugation strains, and TCPEc171209* and TCPKp171210* are the conjugated strains. DNA sequencing was performed for strains that are underlined. Whole-genome sequencing was performed for the indicated plasmids.

4.2.2 pECBHS_9_2 plasmid composition

The incompatible pECBHS_9_2 plasmid contained three origins of replication for IncFIA, IncFIB, and IncFII groups and conjugal transfer (*tra* and *trb*). It also contained *aac (3)-IIa* for aminoglycoside resistance, *blaCTX-M-14* for β-lactam resistance, and *tet (a)* for tetracycline resistance. Along with drug resistance determinants, pECBHS_9_2 contained three toxin/antitoxin systems, including *iucA, iucB, iucC_1, iucC_2, iucD,* and *iutA*, associated with hydroxamate siderophore aerobactin synthesis (Figure 2).

4.2.3 pECBHS_9_3 plasmid and pECBHS_9_4 composition

The 90,979-bp plasmid (pECBHS_9_3) and 85,870-bp plasmid (pECBHS_9_4) did not contain any acquired antimicrobial resistance factors.

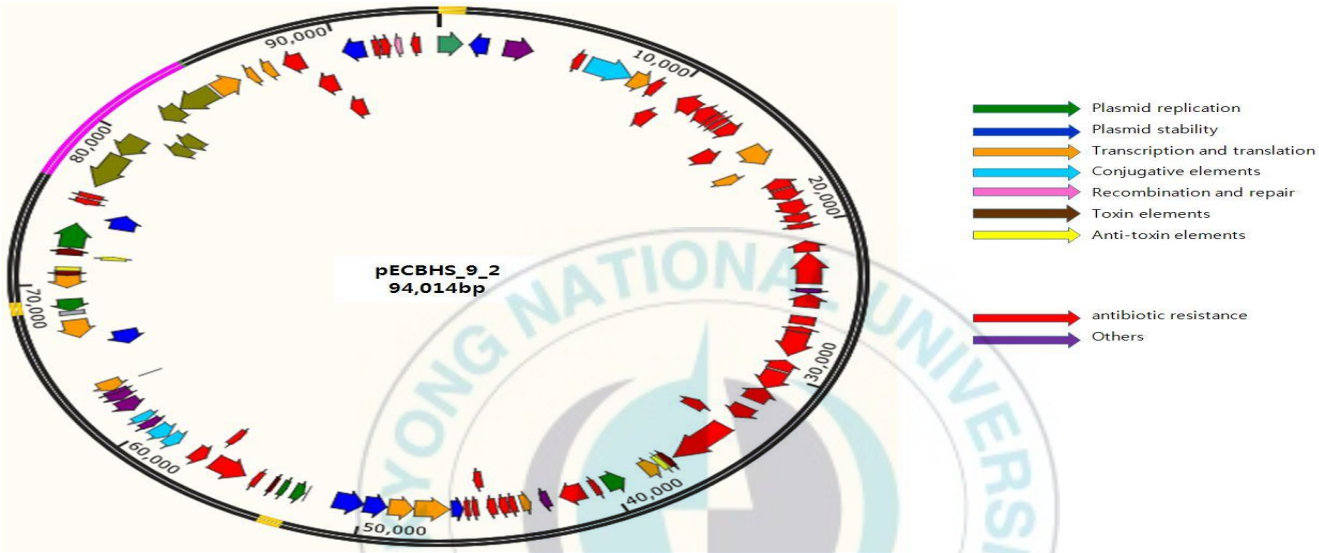

Figure 2. Circular map of pECBHS_9_2.

Circular map of pECBHS_9_2's three replication origins for IncFIA, IncFIB, and IncFII groups and conjugal transfer.

Key: Red line, Tn3 transposon; Blue line, Class 1 integron; Red line, Virulent elements; Yellow line, replicon; Violet line, ΔTn4401a with *blaKPC-2*; Sky blue line, conjugative elements.

4.2.4 pECBHS_9_5 plasmid composition

The 46,836-bp IncX3 plasmid (pECBHS_9_5) belonged to an incompatibility group and harbored *blaKPC-2* and *blaSHV-182* for β-lactam resistance. The circular DNA had a G+C content average of 48.0% and harbored 59 coding sequences and 25 annotated ORFs (Figure 3A). *blaKPC-2* was located on a Tn*4401* variant designated as "isoform a," harboring a 99-bp deletion between *blaKPC* and *istB* [27]. DNA regions for plasmid replication (*repB*) and stability (*parAB* and *stpA*), origin of transcription and translation (*rfaH* and *yjoB*), and conjugal transfer (*tra* and *trb*) were observed, and the plasmid harbored *blaSHV-182* encoding a broad-spectrum β-lactamase (Figure 3A).

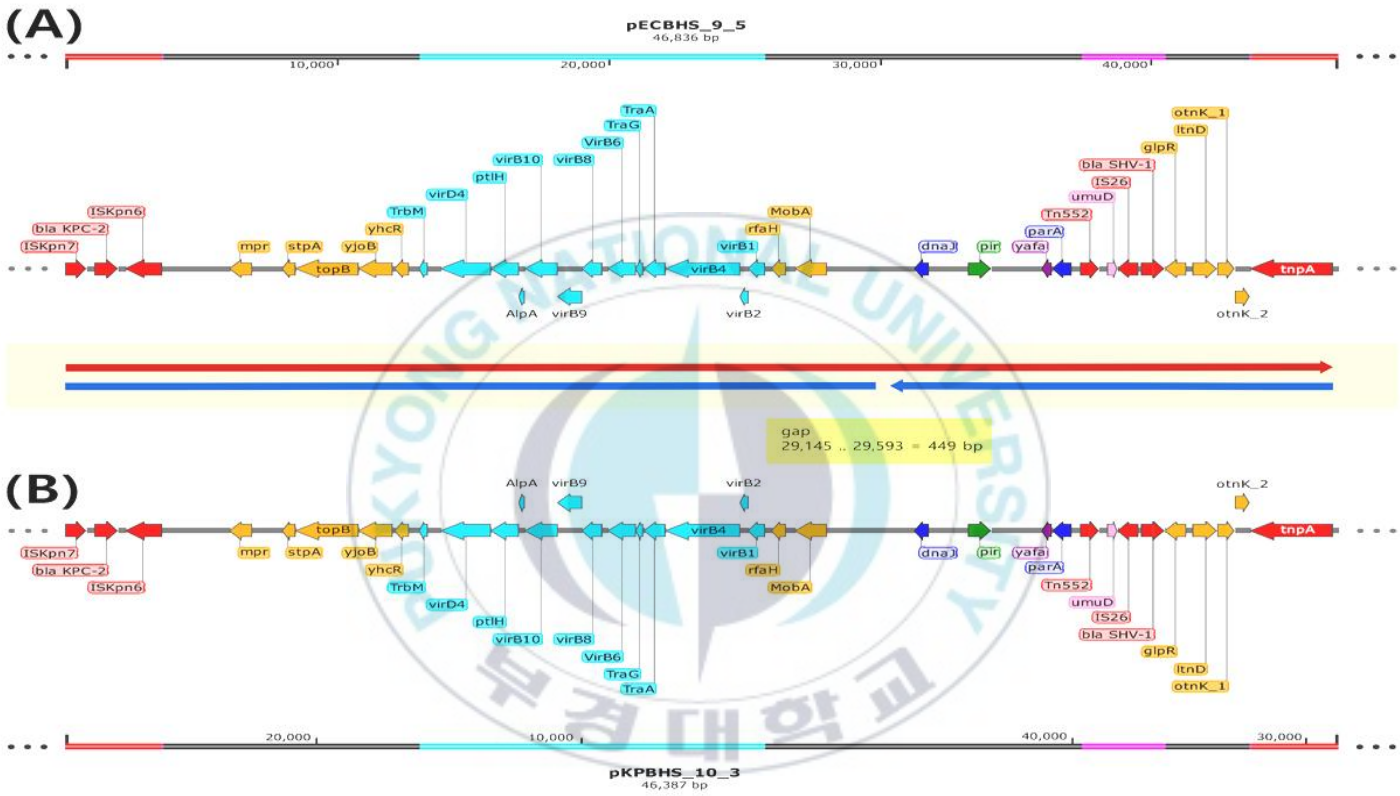

Figure 3. Comparison of the *blaKPC-2* regions of pECBHS_9_5 and pKPBHS_10_3.

A. Linear map of pECBHS_9_5 harboring ΔTn*4401a* with *blaKPC-2*, *blaSHV-182*, and conjugative elements. **B.** Linear map of pKPBHS_10_3 harboring ΔTn*4401a* with *blaKPC-2*, *blaSHV-182*, and conjugative elements. The colored box indicates the gene. Most genes were well preserved but inverted. Genes are denoted by arrow colors based on the following gene function classification: green, plasmid replication; blue, plasmid stability; yellow, transcription and translation; sky blue, conjugative elements; pink, recombination and repair; red, antibiotic resistance; purple, other genes.

Key: Pink line, *blaSHV-182*; red line, ΔTn*4401a* with *blaKPC-2*; sky blue line, conjugative elements.

4.3 *Sequencing and annotation of CPKp171210*

The *K. pneumoniae* strain CPKp171210 genome comprised 5,760,457 bp, with a 5,478,640-bp chromosome and three plasmids. The G+C content average was 57.3%. The 5,018 coding sequences comprised 86 tRNAs, 25 rRNAs, and 173 annotated ORFs. Unknown mutations in *ompK37, ompK36, acrR,* and *ramR* were observed. The chromosome encoded *M.Kpn*34618Dcm and type II restriction enzymes. CPKp171210 belonged to ST307, and the chromosome (CPKp171210) included *fosA* for fosfomycin resistance, *blaSHV-28* for β-lactam resistance, and *oqxA* and *B* for quinolone resistance.

4.3.1 pKPBHS_10_1 plasmid composition

The pKPBHS_10_1 plasmid comprised a 134,995-bp circular DNA with a G+C content average of 52.0% on an average and 56 annotated ORFs. Plasmid pKPBHS_10_1 contained *M.Eco*RII and *Eco*128I type II restriction enzymes. pKPBHS_10_1 is an IncFIB(K) plasmid which harbored *blaOXA-1* for β-lactam resistance, *aac(6´)-Ib-cr* for aminoglycoside resistance, *qnrB1* for fluoroquinolone resistance, *catB3* for phenicol resistance, *tet(A)* for tetracycline resistance, and *dfrA14* for trimethoprim resistance. Plasmid pKPBHS_10_1 harbored CusCFBA genes for mediating resistance to copper and silver(Figure 4).

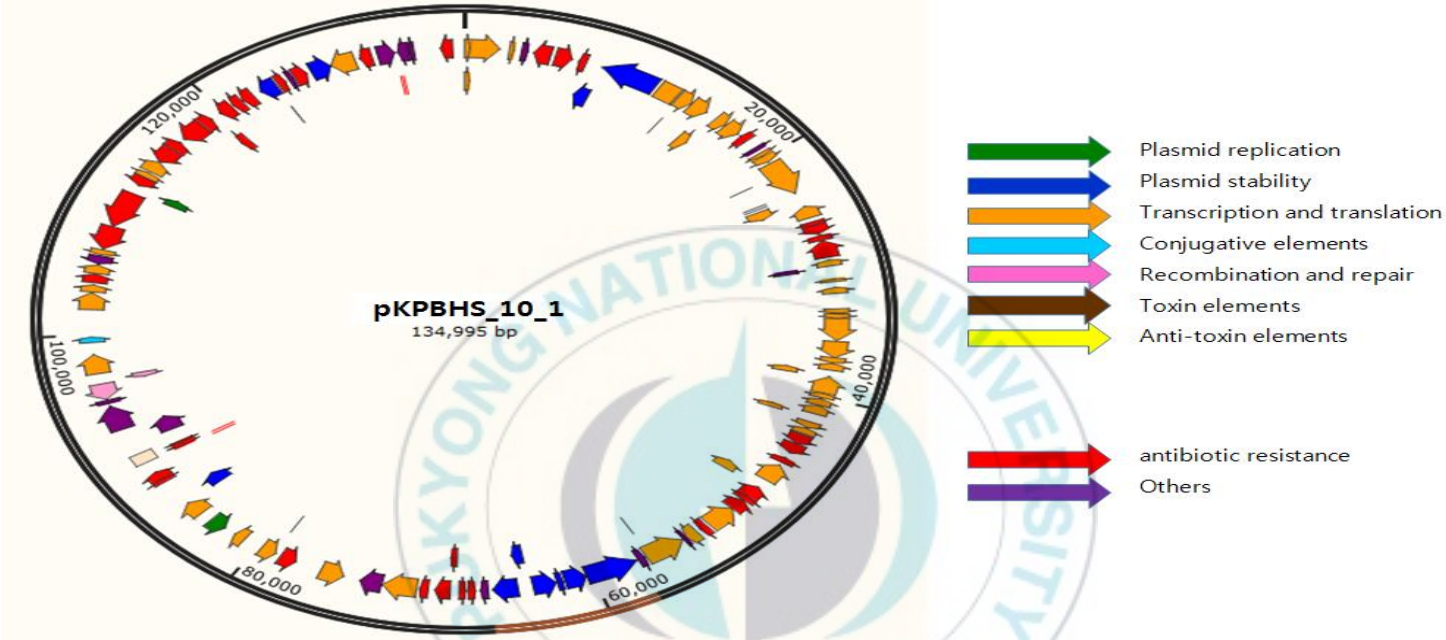

Figure 4. Circular map of pECBHS_10_1.

Circular map of pKPBHS_10_1 harbored CusCFBA genes for mediating resistance to copper and silver.

4.3.2 pKPBHS_10_2 plasmid composition

The plasmid pKPBHS_10_2 contained restriction sites for type I restriction enzymes, *S.Kpn*1420II, and *M.Kpn*928I but it did not contain any acquired antimicrobial resistance factors.

4.3.3 pKPBHS_10_3 plasmid composition

The 46,387-bp incompatible IncX3 plasmid (pKPBHS_10_3) harbored *blaKPC-2* and *blaSHV-182* for β-lactam resistance. This plasmid had a G+C content average of 48.0% and harbored 56 coding sequences and 38 annotated ORFs (Figure 3B). *blaKPC-2* was located on a variant of Tn*4401*, called “isoform a,” that contained a 99-bp deletion between *blaKPC* and *istB*. DNA regions for plasmid replication (*repB*) and stability (*parAB* and *stpA*), origin of transcription and translation (*rfaH* and *yjoB*), and conjugal transfer (*tra* and *trb*) were detected, along with *blaSHV-182* encoding a broad-spectrum β-lactamase (Figure 3B).

4.4 *Comparison of the composition of pECBHS_9_5 and pKPBHS_10_3*

Plasmid pECBHS_9_5 was 449 bp longer than plasmid pKPBHS_10_3. These two plasmids had 99% sequence similarity, except for the extra 449 bp in pECBHS_9_5. Alignment analyses between plasmids pKPBHS_10_3 and pECBHS_9_5 revealed 100% identity to the blaKPC-2 allele (Figure 3). Both plasmids were IncX3 incompatible plasmids harboring *blaKPC-2* and *blaSHV-182* β-lactam resistance genes. These plasmids had an average G+C content of 48.0% and could be transferred to the recipient *E. coli* J53 strain through surface conjugation (Table 3). *blaKPC-2* was located on the Tn*4401* "isoform a" variant (Figure 5).

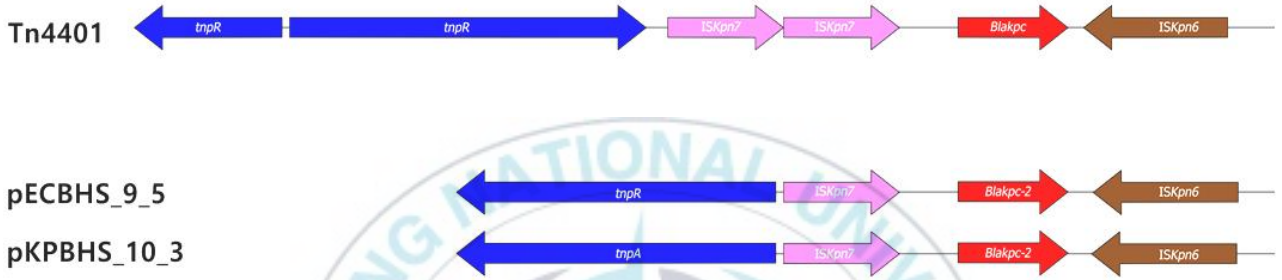

Figure 5. Comparison of wild type Tn*4401* and truncated forms in plasmid pECBHS_9_5 and pKPBHS_10_3.

Transposon Tn*4401* is presented with direct repeats in each plasmid. Arrows in pink box, IS*Kpn7*; arrows in brown box, IS*Kpn6*; blue arrows, transposase; red arrows, *blaKPC* gene.

5. Discussion

In this study, two clinical isolates, KPC-2 *E. coli* and KPC-2 *K. pneumoniae*, were obtained from rectal swabs. Recent studies on *E. coli* ST410 have suggested this strain as another successful pandemic extraintestinal pathogenic *E. coli* strain, with a lineage similar to that of ST131 [8]. Our results are largely concurrent with those of Roer et al. [10], with the *E. coli* (CPEc171209) ST410 strain belonging to the serotype O8:H21 containing antibacterial clade C4/H24 [10]. Roer et al. [10] reported that the *E. coli* ST410 lineage persists and causes recrudescent infections, such as hematological infections, in humans. ST410 has high worldwide prevalence and is widely distributed in the bloodstream, indicating that it can be transmitted between patients and cause hospital outbreaks [10]. *K. pneumoniae* (CPKp171210) ST307 harbors unknown mutations in *ompK36* and *ompK37*. In whole-genome sequencing analysis, these mutations were considered deletion mutations in DNA sequences. Isolates harboring *blaKPC* and expressing *ompK36* reportedly have lower carbapenem MICs. Furthermore, alterations in outer membrane proteins are reportedly associated with increased MICs of carbapenems [23]. Herein, both strains exhibited high or intermediate resistance to all tested antimicrobial agents, except

for amikacin, colistin, and tigecycline. Resistance to a particular antibiotic can affect resistance to another antibiotic. For example, cross-resistance, such as those between cephalosporin and β-lactam, quinolone and methicillin-resistant *Staphylococcus aureus*, and macrolide and pneumococcal resistance, have been reported. MDR *E. coli* CPEc171209 and *K. pneumoniae* CPKp171210 also cause cross-resistance to various antibiotics (Table 1).

The genes responsible for conjugal transfer of the IncX3 plasmid include *tra/trb* clusters and pil genes coding the type IV pilus. The IncX3 type is a predominant plasmid associated with *blaNDM-1* [28] and occasionally harbors *blaKPC* [29], often accompanying a second plasmid including IncFIIκ and ColE types [30]. Plasmid pECBHS_9_5 of CPEc171209 harbored *blaKPC-2* bracketed by the Tn*3*-type transposon Tn*4401* to pKPBHS_10_3 of CPKp171210 (Figure 3). The *blaKPC-2* gene was located within a truncated Tn*4401* transposon; Tn*4401* harbors the *tnpA* gene encoding a transposase, the *tnpR* gene encoding a resolvase, and two insertion sequence elements, IS*Kpn7* and IS*Kpn6*, located at each end of the *blaKPC* gene [31]. Furthermore, both CPEc171209 and CPKp171210 harbored transconjugation factors and the Tn*3*-type transposon, potentially resulting in cross-infection and horizontal migration. Therefore, the risk of interspecies mobility and infection is high. Plasmid pECBHS_9_2

harbors genes encoding virulence factors and toxin/antitoxin systems, which results in the enhancement of bacterial fitness in human hosts and prolonged persistence [32]. KPC-2 *E. coli* and KPC-2 *K. pneumoniae* were continuously monitored by rectal swab detection over the course of 3 months until the patient died. Plasmids pKPBHS_10_1 and pKPBHS_10_2 harbor various genes related to the resistance against diverse kinds of antimicrobial agents and defend themselves through type I and II restriction enzymes. The pKPBHS_10_1 plasmid also harbors CusCFBA proteins mediating resistance to copper and silver through cation efflux. Gram-negative bacteria, including *E. coli*, frequently use tripartite efflux complexes of the resistance-nodulation cell division superfamily transporters to pump out diverse toxic compounds [33,34]. The efflux system CusCFBA is liable for removing biocidal Cu (I) and Ag (I) ions [35,36], and the Cus determinant of gram-negative bacteria encodes CusCFBA proteins. CusA and CusB are essential for copper resistance, while CusC and CusF are required for overall resistance.

This study investigated a case of persistent infection in a patient because of interspecies migration of carbapenem-resistant *Enterobacteriaceae* caused by an *E. coli* ST410 strain. Molecular genetic evidence obtained herein reveals the nature of this occurrence and indicates the risk of ST410 *E. coli* infections. The

emergence of KPC-producing *E. coli* is concerning because the *blaKPC-2*-bearing plasmid may result in interspecies propagation of resistance. *E. coli* ST410 represents a globally distributed lineage and is responsible for diverse antimicrobial resistance determinants, including extended-spectrum β-lactamases, pAmpCs, carbapenemases, and colistin resistance genes. Furthermore, the present results suggest that ST410 is a high-risk bacterium when it infects the host. Common sequences of virulence factors were observed in plasmids in ST410 CPEc171209 and in another high-risk group, ST307 CPKp1210, indicating the potential for cross-transmission, thus limiting treatment alternatives and maintaining long-term pathogenicity.

Carbapenem-resistant bacteria can undergo transgenic translocation and may spread rapidly via horizontal migration in patients. Together with its demonstrated high trainability among patients, *E. coli* ST410 poses an elevated risk in clinical settings; therefore, it should be considered as a lineage with emerging "high-risk" clones and be closely monitored. Although the number of patients analyzed in this study was limited (1 patient), this study clearly elucidates the propagation pattern of KPC-producing *E. coli* (ST410) strains, which frequently occur in South Korea. Further investigation of the molecular genetics of KPC-producing *E. coli* (ST410) are needed.

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Chapter Ⅳ

Antibiotic resistance and KPC-2-encoding promiscuous plasmids from *Klebsiella pneumoniae* ST307 clone

1. Abstract

Recent epidemiological evidence indicates that *Klebsiella pneumoniae* producing carbapenemase-2 (KPC-2), sequence type (ST) 307, a new lineage, is now emerging. Therefore, this study aimed to analyze the complete sequence of plasmids from KPC-2-producing *K. pneumoniae* (KPC-Kp) ST307 strains. We performed antimicrobial susceptibility testing, PCR, multilocus sequence typing, curing test, and whole-genome sequencing to characterize the plasmid-mediated KPC-2-producing *Enterobacteriaceae* clinical isolates. Sequence analysis of KPC-Kp strain ST307 revealed novel plasmid-located virulence factors, including a gene cluster for glycogen synthesis. Three genetic strains were identified in one patient: *K. pneumoniae* (CPKp1825), *Klebsiella aerogenes* (CPEa1826), and *Escherichia coli* (CPEc1827). The *blaKPC*-2 gene transported through *K. pneumoniae* ST307 underwent horizontal transmission between strains. Further, they were able to be transferred through conjugation because they contained the type IV secretion system, pilus genes, and tra genes for conjugal transfer. The *blaKPC*-2 gene was located within a truncated Tn*4401* transposon. Plasmids containing the *blaKPC*-2 gene could not be artificially removed, and thus the three strains could not be cured. The ease of

horizontal transmission of KPC-Kp ST307 carbapenem resistance observed in this study has serious public health and epidemiological implications. This study provided a better understanding of genetic characteristics that can contribute to the success and spread of the KPC-Kp ST307 clone and evaluated its association with antimicrobial resistance genes.

2. Introduction

The worldwide spread of carbapenemase-producing *Klebsiella pneumoniae* has become a major threat to healthcare facilities and human existence [1]. Approximately 700,000 people die annually from antibiotic-resistant infections, and this number is projected to surpass 10 million per year by 2050 [2]. This global phenomenon has been mainly associated with the dissemination of high-risk clones. The well-known KPC-producing clone is the Kp Sequence Type (ST) 258 clone [3] and its related variants, which belong to clonal group 258 (CG258) [4]. However, in recent years, new drug-resistant lineages have emerged internationally [5]. Among them, KPC-Kp ST307 is becoming one of the most clinically relevant clone because its emergence has been recognized in several countries in the last five years [6,7]. The results of analysis of non-CG258 clones of carbapenem-resistant *K. pneumoniae* (CR-KP) strains spanning two years in Colombia showed that 62.2% of the strains were derived from ST307. Patients infected with KPC-Kp ST307 presented high mortality (over 50%) and had longer hospital stays than patients infected with other clones, indicating that this lineage encodes additional factors contributing to its virulence [8]. In addition, the ST307

genome encodes various pathogenic genetic features. In this study, whole-genome sequence analysis of KPC-Kp ST307 and other bacterial strains isolated from a single patient was performed. Further, the horizontal transmission of plasmids containing the promiscuous *blaKPC* gene was investigated across the intestinal bacteria of a patient. Interestingly, KPC-Kp ST307 has been shown to undergo interspecies horizontal gene transfer of the *blaKPC*-2 gene. We also compared the whole-genome sequence and performed minimum inhibitory concentration (MIC) analysis, emphasizing the need for careful use of amikacin (AMK) in infectious disease patients. Hence, we attempted to understand the genetic characteristics that contribute to the growth and spread of the KPC-Kp ST307 clone and evaluated its association with antimicrobial resistance genes. Because Kp ST307 is a relatively new clone, there is currently no sufficient evidence on the genetic characteristics that facilitate its spread or its association with antimicrobial resistance. Therefore, this study will provide novel insights and a better understanding of Kp ST307 for effective therapy.

3. Materials and Methods

3.1 *Sources of KPC-producing Enterobacteriaceae isolates*

The Institutional Review Board of B Hospital, Busan, Korea, approved (approval number CTS-19-003) this prospective study. An elderly woman of 87 years with a history of primary knee arthropathy in both knees was admitted to a general hospital in Busan, South Korea, in August 2018 for arthritis treatment. Carbapenem-resistant *Enterobacteriaceae* was not detected in the rectal swab at first admission. However, after one month, a urine culture test was performed owing to fever, and KPC-2-producing Kp (CPKp1825) was confirmed in the urine culture. Additionally, *Klebsiella aerogenes* (CPEa1826) was detected on the 23rd day and *Escherichia coli* (CPEc1827) on the 26th day in rectal swabs. Interestingly, after the isolation of Kp (CPKp1825), vancomycin-resistant *Enterococcus faecium* was continuously isolated from the urinary culture test, and the stool also tested positive for *Clostridioides difficile* toxin.

3.2 *Bacterial isolates and antimicrobial susceptibility testing*

Clinical isolates were identified using standard microbiological procedures and VITEK-2 (bioMérieux Vitek Inc., Hazelwood, MO, USA). The identification of all isolates was confirmed by 16S rDNA sequencing [9]. Antimicrobial susceptibilities were determined using VITEK 2 AST N224 cards (bioMérieux Vitek Inc., Hazelwood, MO, USA); susceptibility against 12 antibiotics (ampicillin, aztreonam, cefotaxime, ceftazidime, cefoxitin, ciprofloxacin, amikacin, gentamicin, imipenem, meropenem, ertapenem, and colistin) was evaluated by the disk diffusion method on Mueller-Hinton (MH; Becton Dickinson, Franklin Lakes, NJ, USA) agar, following the Clinical and Laboratory Standards Institute (CLSI) guidelines [10]. Susceptibility against colistin was determined by the broth microdilution method using freshly prepared cation-adjusted MH broth, following the Clinical and Laboratory Standards Institute (CLSI) guidelines [10,11,12]. Tigecycline susceptibility was confirmed by E-test (bioMérieux, Marcy-l'Etoile, France). For the modified carbapenem inactivation method (mCIM) test, the organisms were incubated with a meropenem disk in tryptic soy infusion (TSI; Difco Laboratories Inc, Detroit, MI, USA) broth. For the EDTA-modified carbapenem

inactivation method (eCIM) test, EDTA was added to the broth to chelate metal ions necessary for the metallo-β-lactamase function. After incubation, the disks were removed and placed on a lawn of susceptible *E. coli* to determine whether the test organisms hydrolyze the meropenem. Zone diameters were measured and interpreted following CLSI guidelines [10].

3.3 *Detection of resistance genes*

Kp strains were screened for genes encoding extended-spectrum β-lactamases (ESBLs) (blaCTX-M-1-, blaCTX-M-9-, blaTEM-, and blaSHV-type), carbapenemases (blaIMP-1-type, blaVIM-2-type, blaNDM, blaKPC, blaKPC-2, blaGES, and blaOXA-48-like) [13], and outer membrane proteins (ompK35 and ompK36) using PCR (model Verity 96-Well; Applied Biosystems, Eatate, Singapore) [14]. The PCR products were sequenced using an automatic sequencer (model 3730xl; Applied Biosystems, Weiterstadt, Germany), and the nucleotide sequences were compared using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4 *Multilocus sequence typing*

Multilocus Sequence Typing (MLST) PCR and sequencing were carried out for seven housekeeping genes (*gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB*) for Kp [15] and *adk, fumC, gyrB, icd, mdh, purA,* and *recA* for *E. coli* [16]. The nucleotide sequences obtained for both DNA strands were compared with sequences in the MLST database for each species (http://bigsdb.web.pasteur.fr/klebsiella for Kp and http://mlst.warwick.ac.uk/mlst/dbs/Ecoli for *E. coli*), to determine allelic numbers and STs.

3.5 *Bacterial conjugation*

Plasmids carrying the *blaKPC* gene in the CPKp1825, CPEa1826, and CPEc1827 strains were used as donors, and sodium azide-resistant *E. coli* J53 was used as the recipient [17]. For the standard agar mating method, equal amounts of overnight donor and recipient cultures were mixed and immediately spread on brain heart infusion (BHI; MBcell, Los Angeles, CA, USA) agar. After overnight mating at 37 °C, bacterial cells were recovered and plated on MacConkey agar containing sodium azide (100 μg/mL) and imipenem (0.5 μg/mL) to select trans conjugants.

3.6 *Curing test*

KPC-carrying plasmid curing was performed by the temperature-mediated plasmid emission method. Carbapenem-resistant *Enterobacteriaceae* strains were subjected to elevated temperature-mediated plasmid elimination by sequential passages in BHI (BHI; MBcell, Los Angeles, CA, USA) broth, twice a day for a period of two weeks. After two weeks, the cultures were diluted and plated on tryptic soy infusion (TSI; Difco Laboratories Inc) medium to obtain single colonies. Suspected cured colonies were identified by picking and plating onto TSI medium containing 5 μg/mL of imipenem and on a control TSI medium. Colonies that failed to grow in the presence of imipenem were suspected to be cured and were further analyzed for *blaKPC* loss by PCR and antibiotic susceptibility testing [18].

3.7 *Whole-genome sequencing*

Whole-genome sequencing (WGS) of CPKp1825, CPEa1826, and CPEc1827 isolates was performed. Single-Molecule Real-Time sequencing was conducted on a PacBio RSII instrument (Pacific Biosciences, Menlo Park, CA, USA). Schematic diagrams of multiple alignments of plasmids were prepared by manually realigning the linear plasmid maps drawn using the software SnapGene Viewer (http://www.snapgene.com/products/snapgene viewer/). Genomes were compared using Mauve comparison tools (http://darlinglab.org/mauve/). Resistance genes, insertion sequence (IS) elements, replication origins, virulent elements, and toxin and anti-toxin systems were identified. Identification was performed using the following databases:

ResFinder (https://cge.cbs.dtu.dk//services/ResFinder/), ISfinder (https://www-is.biotoul.fr/), plasmid finder (https://cge.cbs.dtu.dk//services/PlasmidFinder/), Restriction-Modification finder (https://cge.cbs.dtu.dk/services/Restriction-ModificationFinder//), Serotype finder (https://cge.cbs.dtu.dk/services/SerotypeFinder/), Fim Typer (https://cge.cbs.dtu.dk/services/FimTyper/), CH Typer (https://cge.cbs.dtu.dk/services/CHTyper/).

3.8 *GenBank accession numbers*

Nucleotide sequence data for CPKp1825, CPEa1826, and CPEc1827 are available under GenBank accession numbers WMHT01000001-WMHT01000003, WMHU01000001-WMHU01000003, and WMHV01000001-WMHV01000003, respectively.

4. Results

4.1 *Antimicrobial susceptibilities and molecular typing*

The Kp (CPKp1825) isolates showed a multidrug resistance (MDR) phenotype. The isolates exhibited resistance to most antibiotics tested, including ampicillin, aztreonam, cefotaxime, ceftazidime, cefoxitin, ciprofloxacin, gentamicin, tigecycline, trimethoprim/sulfamethoxazole, imipenem, meropenem, and ertapenem, but were susceptible to amikacin and colistin. According to the antibiotic susceptibility profiles, *K. aerogenes* (CPEa1826) and *E. coli* (CPEc1827) isolates were found to be resistant to ampicillin, aztreonam, cefotaxime, ceftazidime, cefoxitin, imipenem, meropenem, and ertapenem, but were susceptible to amikacin, gentamicin, tigecycline, ciprofloxacin, trimethoprim/sulfamethoxazole, and colistin. The combination of β-lactamases relative to the site of isolation and susceptibility test results are shown in Table 1.

PCR and sequencing for β-lactamase genes demonstrated the presence of *blaKPC*-2 in all isolates. *SHV-1-like* broad-spectrum penicillinase, which is intrinsic, was identified by PCR in both isolates (CPKp1825 and CPEa1826). Genes for the plasmid-mediated *CTX-M*-type ESBLs were found in CPKp1825 isolates. In addition, we confirmed that *OmpK35* and 36 were missing from CPKp1825 isolates, *OmpK36* was missing from CPEa1826 isolates, and *OmpK35* was missing from CPEc1827 isolates. CPKp1825, CPEa1826, and CPEc1827 could all be transferred to *E. coli* J53. The conjugated strains had the resistance gene that the pre-junction strain had (Table 2). A curing test was performed to remove the plasmid containing the KPC-2 gene. However, all three strains were not successfully cured (Table 1).

Table 1. Antimicrobial susceptibilities and epidemiological properties of isolated KPC-producing *Enterobacteriaceae*[a]

Isolate ID	Specimen	Date	MLST	blaKPC		Plasmid		Susceptible antibiotics [b]									Carbapenemase differentiation test		Porin loss	Curing test
								MIC (μg/mL)						Zone diameter (mm)			mCIM	eCIM		
			ST	Subtype	Bracketed by	pKPC	Replicon	AMK	GEN	CIP	TIG	CST	TMP/SMX	AMK	GEN	CIP				
CPKp1825	Urine	27-Sep	307	blaKPC-2	ΔTn4401a	pKPBHS25-2	IncX3	16				0.25		22			+	-	ompK35, ompK36	-
CCPKp1825				blaKPC-2				≤2				0.25		22						
CPEa1826	Rectal	21-Oct	-	blaKPC-2	ΔTn4401a	pKPBHS26-2	IncX3	≤2	<=1	<=0.25	0.38	0.25	≤20	23	22	30	+	-	ompK36	-
CCPEa1826				blaKPC-2				≤2	≤1	≤0.25	0.38	0.25	≤20	25	22	30				
CPEc1827	Rectal	23-Oct	720	blaKPC-2	ΔTn4401a	pKPBHS27-1	IncX3	≤2	≤1	<=0.25	0.125	0.25	≤20	20	22	30	+	-	ompK35	-
CCPEc1827				blaKPC-2				≤2	≤1	≤0.25	0.75	0.25	≤20	23	22	30				

[a]Breakpoints were applied according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Tigecycline susceptibility was confirmed by E-test (bioMérieux) and colistin susceptibility was confirmed by broth microdilution.

[b]Disk diffusion test results were interpreted according to the CLSI guidelines.

AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; TIG, tigecycline; SXT, trimethoprim-sulfamethoxazole; CST, colistin; eCIM, EDTA-modified carbapenem inactivation method; mCIM, modified carbapenem inactivation method. The underlined type is the conjugated strain.

Table 2. Resistome and integrative conjugative elements.

Strain	Plasmid	Antimicrobial resistance gene								Conjugants
	Replicon	β-lactam	Aminoglycoside	Tetracycline	Macrolide	Phenicol	Trimethoprim	Sulphonamide	Fluoroquinolone	
CPKp1825		*blaKPC-2, blaCTX-M-1, blaSHV,blaTEM-1*	*rmtB*							+
CCPKp1825		*blaKPC-2, blaCTX-M-1, blaSHV,blaTEM-1*	*rmtB*							
pKPBHS_25_1	IncFIB(K), IncFII(K)	*blaOXA-1*	*aac(6′)-Ib-cr*			*catB3*				
pKPBHS_25_2	IncX3	*blaKPC-2, blaCTX-M-15, blaSHV-182, blaTEM-1B*	*aac(3)-Iia, aph(3″)-Ib, aph(6)-Id*				*dfrA14*	*sul2*		
CPEa1826		*blaKPC-2,blaSHV*								+
CCPEa1826		*blaKPC-2,blaSHV*								
pEABHS_26_1	IncP-1									
pEABHS_26_2	IncX3	*blaKPC-2, blaSHV-182*								
CPEc1827		*blaKPC-2*								+
CCPEc1827		*blaKPC-2*								
pECBHS_27_1	IncX3	*blaKPC-2*								
pECBHS_27_2	IncFIB(K)									

CPKp1825, CPEa1826, and CPEc1827 are pre-conjugation strains, and CCPKp1825, CCPEa1826, and CCPEc1827 are the conjugated strains, which were analyzed using DNA sequencing.
Whole-genome sequencing was performed for the indicated plasmids.

4.2 *Sequencing and annotation of CPKp1825*

We found that Kp strain CPKp1825 had a 5,812,656-bp genome. It contained a single chromosome and two plasmids. The CPKp1825 chromosome consisted of a 5,541,320-bp circular DNA molecule with an average G+C content of 57.2%, harboring 5,143 annotated open reading frames (ORFs). Unknown mutations in the genomes included P161R, G164A, F172S, R173G, L195V, F197I, and K201M amino acid substitutions in AcrR. L188Q, K194* substitution in RamR, *OmpK36*, and *OmpK37* were also confirmed. The type II restriction enzyme, *M.Kpn34618Dcm*, was found. Further, CPKp1825 was identified as an ST307 strain. The chromosome of CPKp1825 isolates included *blaSHV*-28 and *blaSHV*-106 for β-lactam resistance, *oqxA*, *B* for quinolone resistance, and *fosA* for fosfomycin resistance.

The pKPBHS_25_1 plasmid consisted of a 198,487-bp circular DNA molecule with an average G+C content of 52.1%, harboring 56 annotated ORFs (Figure 1A). The pKPBHS_25_1 plasmid is a Multi-replicon [IncFIB(K), IncFII(K)], and included *aac(6')-Ib-cr* for fluoroquinolone and aminoglycoside resistance, *blaOXA*-1 for β-lactam resistance, and *catB3* for phenicol resistance. Further, pKPBHS_25_1 included CusCFBA proteins that mediated resistance to copper and silver by cation efflux and carried five

putative virulence clusters: the lacZYI operon, the Fec-like iron (III) dicitrate and glutathione ABC-transport systems, the urea transport system, and the cluster for glycogen synthesis. A 35-kb region on pKPBHS_25_1 contained a Type IV secretion system, the pilus genes, and tra genes for conjugal transfer (Figure 1C).

The other plasmid, pKPBHS_25_2 (72,849-bp), an incompatibility group X type 3 (IncX3) plasmid, belonged to an incompatibility group and harbored *blaKPC*-2, *blaCTX-M*-15, and *blaSHV*-182 genes for β-lactam resistance. It contained a circular DNA molecule with an average G+C content of 49.5% and harbored 89 coding sequences (CDS) and 43 annotated ORFs (Figure 1B). Genes encoding type II restriction enzymes, *Eco*128I, and *M.Eco*RII were identified. Plasmid pKPBHS_25_2 included *aac(3)-IIa*, *aph(3'')-Ib*, and *aph(6)-Id* for aminoglycoside resistance, *blaCTX-M*-15, *blaKPC*-2, *blaSHV*-182, and *blaTEM*-1B for β-lactam resistance, *sul2* for sulfonamide resistance, and *dfrA14* for trimethoprim resistance. The *blaKPC*-2 gene was located within a truncated Tn*4401* transposon. Further, ΔIS*Kpn*7-*blaKPC*-2-IS*Kpn*6 had a 99-bp deletion between IS*Kpn*7 and bla*KPC*, indicating that it was a Tn*4401a* isoform. The plasmid carried the Tn*3* transposon containing *blaTEM*-1B. A 13-kb region on pKPBHS25-2 contained a type IV secretion system, the pilus genes, and *tra* genes for conjugal transfer.

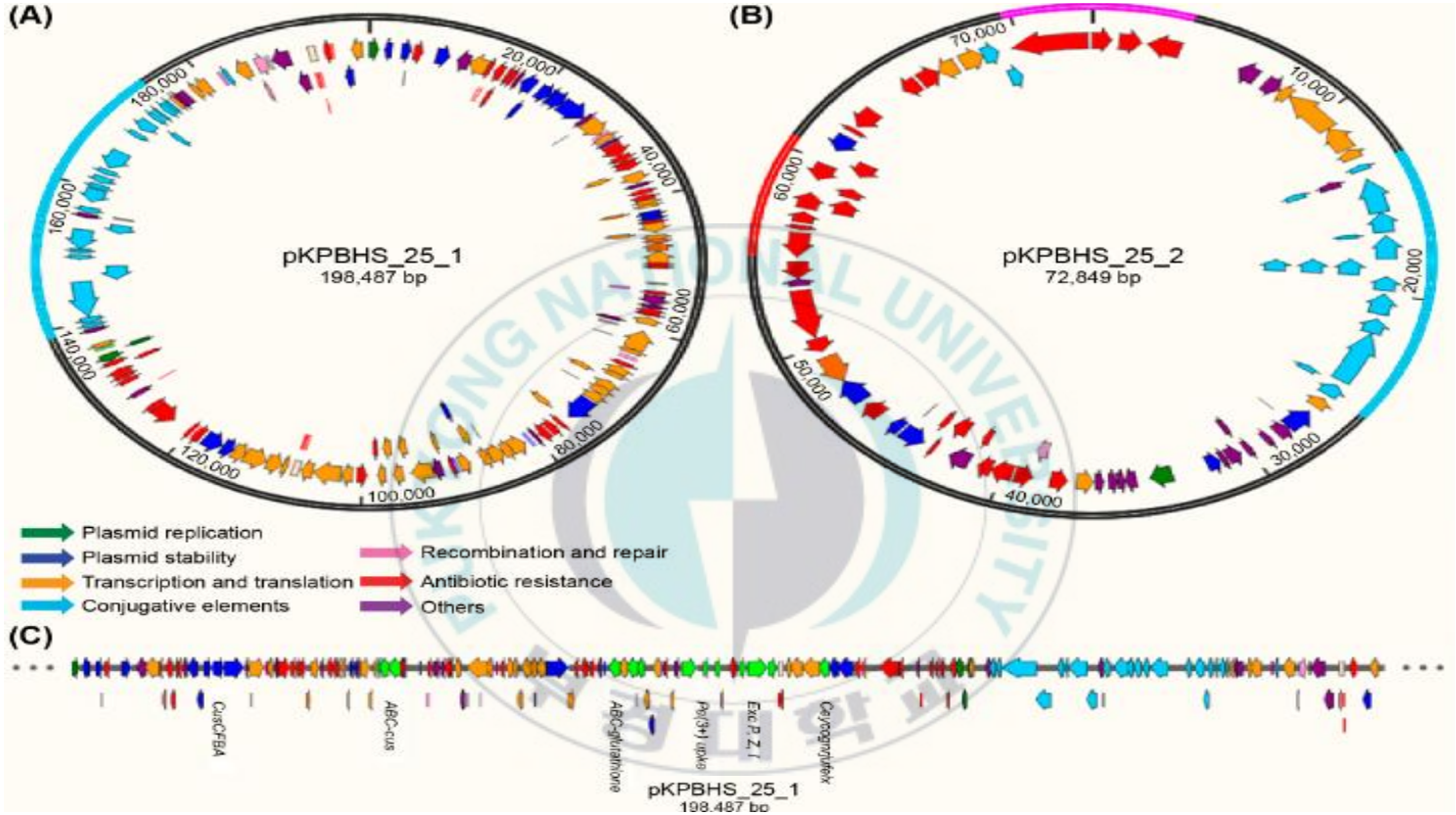

Figure 1. Genetic organization of plasmids associated with blaKPC

A. Circular map of pKPBHS_25_1 with two replication origins for IncFIB(K) and IncFII(K) groups, containing copper-transporting efflux system (CusCFBA) and five putative virulence clusters.

B. Circular map of pECBHS_25_2 containing ΔTn*4401a* with $bla_{CTX-M-15}$, bla_{KPC-2}, $bla_{SHV-182}$, and bla_{TEM-1B} and conjugative elements.

C. Variant pKPBHS_25_1 plasmids identified in ST307. Each arrow indicates plasmid scaffold genes and their direction of transcription. The locus Tra is indicated by a square sky blue arrow with capital letters, indicating the respective *tra* genes (i.e., *traG*, G; *traF*, F; *traO*, O, etc.). Related genes (*tnpA*, *tnpR*, *tnpM*), resistance genes, and insertion sequences are indicated by red arrows. Other genes are indicated by colored arrows as follows: yellow, transcription and translation genes; blue, CusCFBA; green, clusters encoding putative virulence determinants. Legends: redline, Tn*3* transposon; pink line, ΔTn*4401a* with bla_{KPC-2}; sky blue line, conjugative elements.

4.3 *Sequencing and annotation of CPEa1826*

WGS revealed that *K. aerogenes* strain CPEa1826 had a 5,254,026-bp genome with two plasmids (pEABHS_26_1 and pEABHS_26_2). The chromosome consisted of a 5,151,404-bp circular DNA molecule with an average G+C content of 55.0%. It had 4,733 CDS, consisting of 87 tRNAs and 25 rRNAs and harbored 165 annotated ORFs. The nucleotide sequence variations of unknown mutations in 23S, 16S rsH, B, C, and amino acid change of rpoB were confirmed. M.SenAnal, the methyltransferase type I restriction enzyme, was also found. Chromosome (CPEa1826) did not contain any acquired antimicrobial resistance determinants. Plasmid pEABHS_26_1 belonged to the IncP-1 group, and the 55,786-bp circular DNA molecule had an average G+C content of 46.5% and harbored 62 annotated ORFs. It did not contain any acquired antimicrobial resistance gene and consisted of duplicated type IV secretion systems and conjugative elements. The 46,836-bp plasmid pEABHS_26_2 carrying the *blaKPC*-2 and *blaSHV*-182 genes belonged to the IncX3 group. pEABHS_26_2 had an average G+C content of 48.0% and harbored 65 annotated ORFs (Figure 2A). The *blaKPC*-2 gene was located within a truncated Tn*4401* transposon.

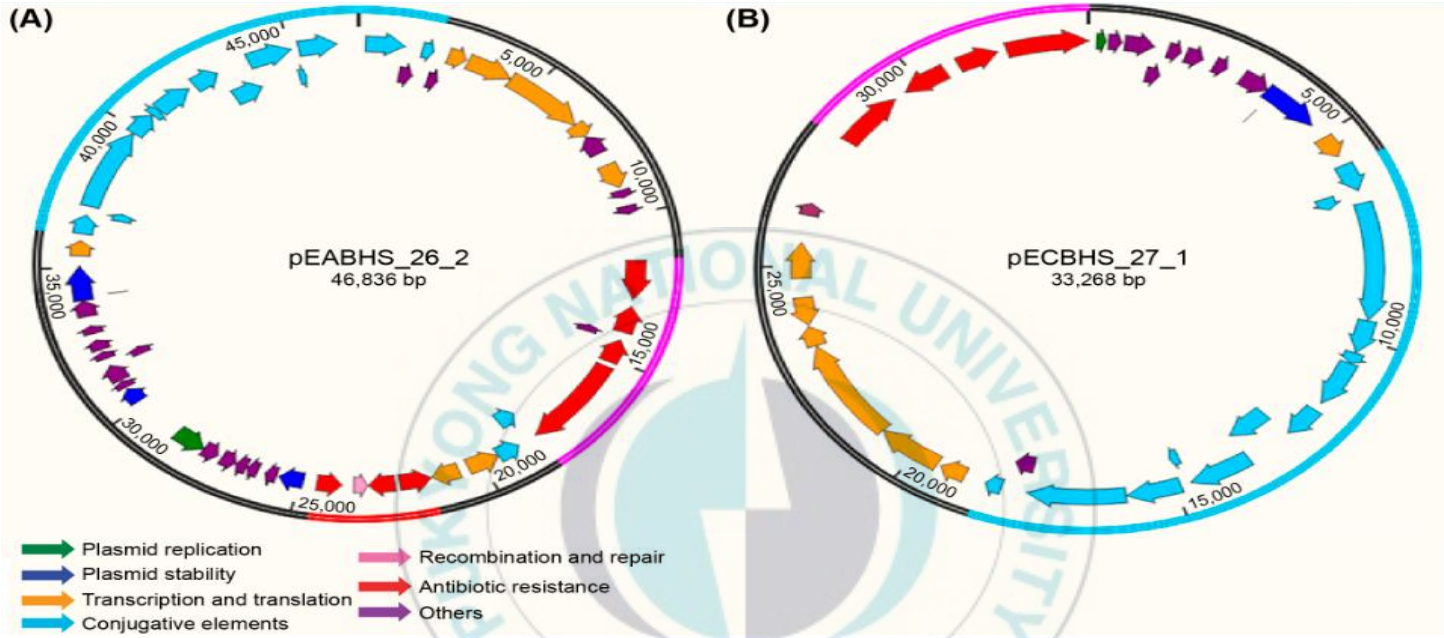

Figure 2. Circular map of pEABHS_26_2 and pECBHS_27_1

A. Circular map of pEABHS_26_2 containing ΔTn*4401a* with bla_{KPC-2} and $bla_{SHV-182}$ genes. B. Circular map of pECBHS_27_1 containing ΔTn*4401a* with bla_{KPC-2} and conjugaltransfer.

Legend: red line, Tn*3* transposon; pink line, ΔTn*4401a* with bla_{KPC-2}; sky blue line, conjugative elements.

4.4 *Sequencing and annotation of CPEc1827*

E. coli strain CPEc1827 had a 5,033,400-bp genome. It had a single chromosome and one plasmid. The chromosome consisted of a 4,908,776-bp circular DNA molecule with an average G+C content of 50.5%. It had 4,463 CDS, consisting of 90 tRNAs, 22 rRNAs, and harbored 178 annotated ORFs. The genomes had S57T amino acid substitutions in ParC. In addition, the nucleotide sequence variations of unknown mutations of 16S_rrsB, H, C, 23S, and amino acid change of pmrB and parC were confirmed as well as the 23S, 16S rsH, B, C, and amino acid change of rpoB. Type II restriction enzymes, *M.Eco*E455Dcm and *M.Eco*GVI, were also found. CPEc1827 was identified as ST720, and it belonged to the serotypes O17/O77, H41, with subtyping as fum C3 and fim H65. Chromosomes included *mdf(A)* for macrolide, lincosamide, and streptogramin B resistance. The 91,356-bp contig did not contain any acquired antimicrobial resistance determinants. The 33,268-bp IncX3 plasmid (pECBHS_27_1) carried *blaKPC*-2 for β-lactam resistance (Figure 2B). The *blaKPC*-2 gene was located within a truncated Tn*4401* transposon and the conjugal transfer was located at 12,738bp (*tra*, *trb*, etc.).

4.5 *Comparison of pKPBHS_25_2, pEABHS_26_2, and pECBHS_27_1*

The plasmids, pKPBHS_25_2 from *K. pneumoniae*, pEABHS_26_2 from *K. aerogenes*, and pECBHS_27_1 from *E. coli* were 72,849-bp, 46,836-bp, and 33,268-bp in size, respectively. They are incompatible plasmids belonging to the IncX3 plasmid group (Table 1) These plasmids harbored *blaKPC*-2 gene for β-lactam resistance (Figure 3). The *blaKPC*-2 gene was located on the Tn*4401* 'isoforma' variant, which contained a 99-bp deletion between *blaKPC* and *istB*.

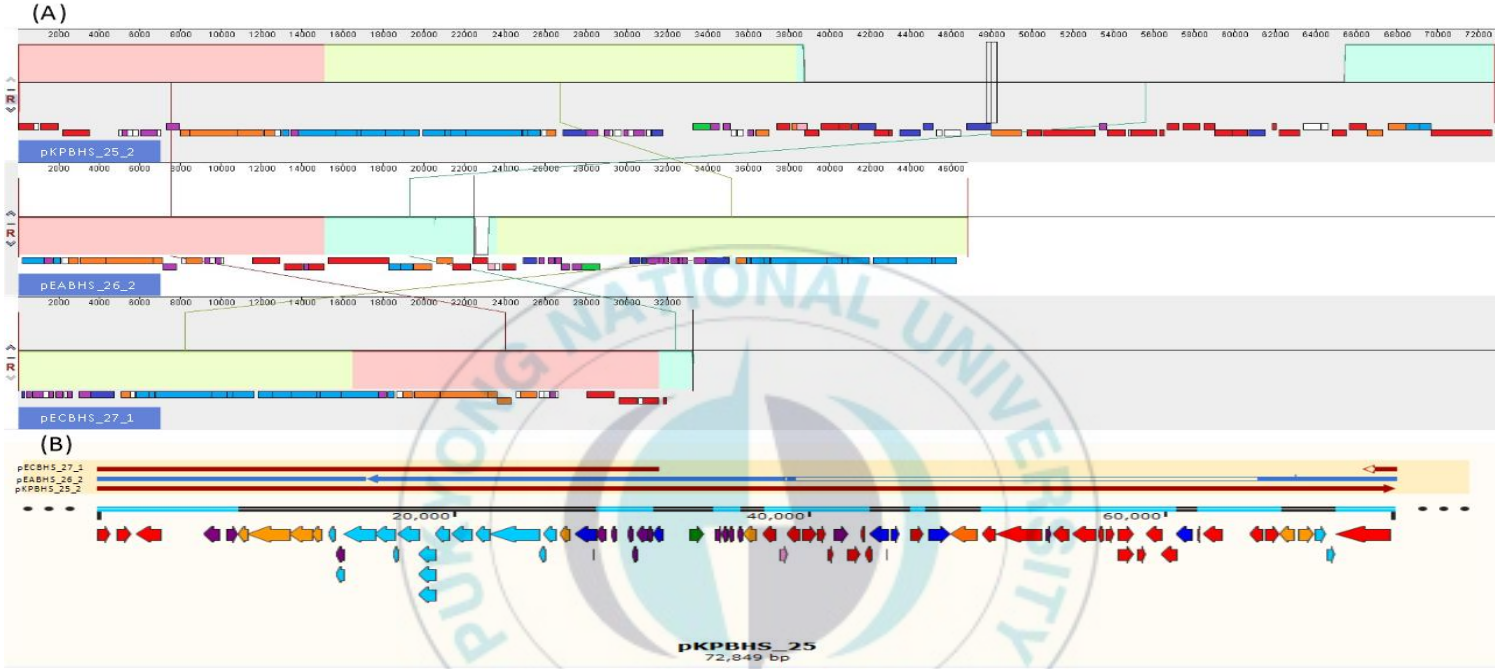

Figure 3. Linear comparison of the genetic elements in plasmid pKPBHS_25_2 from *K. pneumoniae*, pEABHS_26_2 from *K. aerogenes*, and pECBHS_27_1 from *E. coli* surroundings of *blaKPC*-2

A. One fragment unit showing yellow, red, blue, and green on the contig. The colored box under the contig indicates the A. One fragment unit showing yellow, red, blue, and green on the contig. The colored box under the contig indicates the gene. Gene contents are similar along the synteny on the contig. Fragment is well preserved, but inversion exists. Most of the genes are well preserved.

B. pKPBHS_25_2, pEABHS_26_2, and pECBHS_27_1 genes are denoted by arrows and colored based on gene function classification. Other genes are indicated by colored arrows as follows: green, plasmid replication; blue, plasmid stability; yellow, transcription and translation; sky blue, conjugative elements; pink, recombination and repair; red, antibiotic resistance; purple, other genes.

5. Discussion

KPC-Kp ST307 is a novel lineage that has the potential to become an epidemic or 'high-risk' clone. The well-known KPC-producing clone on a global level is *K. pneumoniae* ST258 [3,4]. However, according to the literature, it was not prevalent in South Korea. The foremost clone in South Korea was KPC-Kp ST307, and the clone ST11 accounted for 50% of the prevalence of ST307 [19]. Several reports indicated local dissemination of ST307 harboring *blaKPC* genes, *blaKPC*-2 (Columbia, USA, SouthKorea), and *blaKPC*-3 (Italy) [8,20-21], whereas an analysis of 1700 ESBL-producing Kp from a hospital network in Texas, USA found a high prevalence of *blaCTX-M*-15-positive ST307 strains, with one-third of the strains also carrying *blaKPC*-2 genes and only three carrying *blaKPC*-3 [22].

Other reports showed that *blaCTX-M*-15 was common in ST307 strains [8,22,23]. According to reports by Wyres KL et al. in 2018 [24], ParC 80I and GyrA 83I fluoroquinolone resistance-associated mutations were conserved in all genomes. The *blaCTX-M*-15 ESBL gene was found in 89 (93.7%) genomes, and 81(85.3%) were harbored in combination with *sul2, dfrA14,* and *strAB* with/without *aac(3)-IIa*, which were all linked

to an MDR plasmid. These results are consistent with the current study. In addition to *blaCTX-M*-15, *blaSHV*-182 and *blaTEM*-1B were continuously detected during the 38-day hospitalization period of the patient, causing continuous infection. It can be suggested that plasmid-mediated glycogen synthesis may help ST307 isolates to survive under limited nutrient availability and that the urea transport system may facilitate colonization of the urinary tract by this clone. Urinary tract colonization may also be sustained by the unusual p-fimbria identified in all of our KPC-Kp ST307 genomes [23].

Type II restriction enzymes were also observed in CPKp1825 and CPEc1827, which are enzymes originally present in bacterial cells. The function of a type II restriction enzyme is to protect its own DNA by removing extracellular foreign DNA that enter the cell to maintain the pathological state. The presence of different promoters leading to various levels of expression may be responsible for the variability in resistance level, but may not be sufficient to explain the high level of resistance, as previously suggested [25].

All three strains were found to have lost their outer membrane proteins. The strain lacking *OmpK36* alone became resistant to cefazolin, cephalothin, and cefoxitin, as measured by antimicrobial

susceptibility testing, showing that the disruption of *ompK36* resulted in greater drug resistance than did the *ompK35* mutant. The additional loss of *OmpK35* further increased the MICs, demonstrating that the double-deletion strain was highly resistant to the antibiotics [26]. All these genetic functions worked in tandem to make KP ST307 more resistant and more difficult to treat in patients. The MIC analysis of amikacin in this study should be interpreted cautiously [27]. The sensitivity was confirmed in MIC and antibiotic disk tests, but the whole-genome sequence analysis showed that *aac(3)-lla, aph(6)-ld, aph(3'')-lb*, etc. were identified as resistance genes. Only 3% (2/65) and 11% (7/65) of the isolates were non-susceptible to AMK using the CLSI and EUCAST breakpoints, respectively [10,28].

These low rates were observed despite the fact that 73% (16/22) of aminoglycoside-modifying enzyme (AME)+ isolates possessed *aac(6′)-Ib*, which is reported to confer AMK resistance. In fact, AMK MICs below the susceptibility breakpoints are commonly observed among *Enterobacter* and other *Enterobacteriaceae* that possess *aac(6′)-Ib* [27,29]. The clinical significance of susceptible MICs against bacteria carrying AMEs that are known to cause resistance is not established, and it is unclear whether MICs or molecular markers are more relevant to treatment responses [30].

Indeed, the EUCAST states that should a member of the *Enterobacteriaceae* test as tobramycin (TOB) intermediate or resistant and gentamicin (GEN) and AMK susceptible, its AMK susceptibility status must be revised to “intermediate” [28]. Until the clinical significance of these issues is resolved, our study recommends that AMK be used with caution in the treatment of *Enterobacter* infections that are resistant to other aminoglycosides.

Our analysis revealed that the *blaKPC-2* gene that was transported through KPC-Kp ST307 could be horizontally transmitted between strains. KPC-Kp ST307 was encoded within a conjugable plasmid, which can be speculated as a possible dissemination mechanism within other species of gram-negative bacilli. Plasmids from all three strains could be transmitted through conjugation. It was also possible to cross the species with Tn*4401*, a transposable element, i.e., a gene that can move its position within the genome. A curing test was performed to artificially remove plasmids containing the KPC-2 gene. However, all three strains were not successfully cured. This shows the limitation of symptomatic treatment. Concurrently, it shows the risk of KPC-Kp ST307.

This study had two limitations. First, only the strains at the

time of carbapenemase-producing *Enterobacteriaceae* were analyzed, and no molecular microbiological evidence was given to the carbapenemase-resistance *Enterobacteriaceae* that occurred later, which limited the molecular genetics approach. Second, no epidemiological study of the KPC-Kp isolates was conducted on the origin of the occurrence. The ease of horizontal transmission of KPC-Kp ST307 observed in this study has serious public health and epidemiological implications. MDR Kp ST307 should be monitored alongside other well-known clones to detect emerging MDR threats. These findings indicate an urgent need for enhanced surveillance of KPC-Kp ST307. Especially, its occurrence in South Korea, with high frequency, suggests a health threat. Controlling the spread and limiting the impact of KPC-Kp ST307 isolates in South Korea hospitals will require intensive efforts in both public and private healthcare centers.

6. Conclusion

In this thesis, we focus on the main features of Carbapenemase-producing Carbapenem-resistant *Enterobacteriaceae* (CP-CRE) that illustrate its position as an important antibiotic-resistant pathogen. We provide an overview of all antibiotic resistance gene (ARG). We also evaluate the genetic background of CRE leading to the evolution of multidrug-resistant (MDR) bacteria to extensively drug-resistant (XDR) clones and use these insights to suggest directions for antibiotic use. Along with the resistome, we present the characteristics of five fully sequenced antibiotic resistance plasmids of CP-CRE that enable the inter- and intra-species transfer of resistance via horizontal transfer.

The DNA analysis and antibiotic resistance evaluation of the 40 CP-CRE bacterial isolates collected over two years in our research and the rapid identification of CP-CRE may help in rapid diagnosis and infection management in medical institutions, to prevent secondary hospital infections. Although our data were obtained from the molecular genetic analysis of CPE in a single hospital, they show a similar pattern to that of the distribution of carbapenemase-producing *Enterobacteriaceae* bacterial types, and carbapenemase-producing *Enterobacteriaceae* types and sequence

types of data obtained from the Korea Centers for Disease Control and Prevention (KCDC) in 2018 [31]. Although infectious diseases have strong genetic and geographical factors, CPE infections in this report did not show geographical characteristics, but were similar to the data obtained from the CDC. It is also necessary to analyze the trends likely to affect regional infections in the future. Characterization of previous CRE occurrences in Korea by ESBL, AmpC, or porin loss indicated that the carbapenemase-producing bacteria currently represent the greatest community health risk. To combat these bacteria, a combined effort at the hospital, community, and governmental level is required. CRE-infected patients have high mortality rates, and the range of therapeutic agents available to treat their condition is limited. Thus, the treatment of CRE infection often involves a combination therapy, including carbapenem, aminoglycosides, and fosfomycin. Monotherapies of colistin or tigecycline are the last line of treatment. However, in Korea, the use of multiple antibiotics has cost limitations owing to insurance cuts, and thus, the government needs to improve its health insurance premiums. In addition, efforts are required to develop new antibiotics. However, if an infection-control infrastructure for the proper use of antibiotics is not adequately constructed and implemented, there is a risk that new drugs will rapidly become ineffective.

Therefore, strict antibiotic and infection control systems should be implemented.

These data may serve an important role in understanding and controlling the spread of carbapenem-resistant Gram-negative pathogens.

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Characterization of KPC-2 carbapenemase producing *Enterobacteriaceae* and test for interspecies transferability

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ABSTRACT

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.

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 test, PCR detection of the β-lactamase genes, multilocus sequence typing, genomic sequencing, curing, and conjugational transfer of plasmids. All 40 isolates were

multidrug-resistant and 75% of the *Enterobacteriaceae* isolates were resistant to ciprofloxacin whereas 72.5% were resistant to trimethoprim-sulfamethoxazole. Conjugation accounted for 57.5% of all resistant plasmid transfer events, which is 4.3-fold higher than that observed in 2010. The high detection rate of transposon Tn*4401* (34/40, 85%) was associated with the rapid diffusion and evolution of CPE.

The horizontal propagation pathway CPE between different bacterial species was studied by using high-risk clone of *E. coli* (CPEc171209, ST410) and *Klebsiella pneumoniae* (CPKp171210, ST307) sequentially isolated from one patient. CPEc171209 harbored five plasmids belonging to serotype O8:H21, which is in the antimicrobial-resistant clade C4/H24. The CPKp171210 isolate harbored three plasmids. Both strains harbored various additional antimicrobial resistance genes. The IncX3 plasmid pECBHS_9_5 harbored *blaKPC-2* within a truncated Tn*4401a* transposon, which also contains *blaSHV-182* with duplicated conjugative elements. This plasmid displayed 99% identity with the IncX3 plasmid pKPBHS_10_3 from the *K. pneumoniae* CPKp171210. The genes responsible for the conjugal transfer of the IncX3 plasmid included *tra/trb* clusters and pil genes coding the type IV pilus.

Three KPC-2 producing *Enterobacteriaceae* were sequentially

isolated from one patient and characterized. *K. pneumoniae* (CPKp1825) ST 307 was isolated first followed by *Klebsiella aerogenes* (CPEa1826) and *Escherichia coli* (CPEc1827) within a month. These stains were analyzed by antimicrobial susceptibility, PCR detection of the β-lactamase genes, multilocus sequence typing, curing, and whole-genome sequencing. Sequence analysis revealed that all three strains contain two plasmids per each, which were able to be transferred through conjugation by using the type IV secretion system, pilus genes, and tra genes encoded by the plasmid. The *blaKPC*-2 gene was located in the transposon Tn4401a on incurable IncX3 plasmids pKPBHS_25_2, pEABHS_26_2 and pECBHS_27_1 of CPKp1825, CPEa1826 and CPEc1827, respectively. However, partial deletion was observed in the Tn4401a on pEABHS_26_2 and pECBHS_27_1, which indicated the horizontal transfer from pKPBHS_25_2 in CPKp1825,

Our results highlight the rapid emergence of extensively drug-resistant strains in Korea. Furthermore, the presence of the *blaKPC-2* on plasmid that can be horizontally transferred among bacterial species by conjugation as probed or observed in this study emphasize the need for employing urgent control measures and protocols at the national level.

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많은 분들께 감사를 드립니다.

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