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Detection of the Carbapenem Resistance Gene in Gram-Negative Rod Bacteria Isolated from Clinical Specimens

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Abstract

Carbapenem-resistant *Enterobacteriaceae* (CRE) pose an increasing public health threat and has limited treatment options with high associated mortality. Genotypes of carbapenemase that threaten public health (bla_{KPC}, bla_{NDM}, bla_{IMP}, and bla_{VIM}) and $bla_{OXA-48-like}$ genes were detected by phenotypic and molecular diagnosis, and related gene distribution patterns were investigated. Phenotypic testing using the modified Hodge test confirmed positivity in all 41 strains examined, and carbapenemase inhibitory testing using meropenem + phenyl boronic acid or meropenem + EDTA confirmed positivity in 18 and 8 strains, respectively. Polymerase chain reaction (PCR) revealed the presence of amplification products in 28 strains of bla_{KPC}, 25 strains of bla_{NDM}, 5 strains of bla_{IMP}, 1 strain of bla_{VIM}, and 13 strains of $bla_{OXA-48-like}$. In addition, 7 strains of bla_{KPC}+ bla_{NDM}, 1 strain of bla_{KPC} + bla_{IMP}, 1 strain of bla_{NDM} + $bla_{OXA-48-like}$, 1 strain of bla_{NDM} + bla_{VIM}, 4 strains of bla_{KPC} + bla_{NDM} + bla_{IMP}, and 4 strains of bla_{KPC} + bla_{NDM} + $bla_{OXA-48-like}$ were identified. Melting curve analysis using real-time PCR was wholly consistent with PCR results. The study shows that genetic identification of highly specific CRE by real-time PCR could be used to provide early diagnoses and infection control, improve surveillance, and prevent the transmission of CRE.

Keywords

bla_{KPC}
bla_{NDM}
bla_{IMP}
bla_{VIM}
$bla_{OXA-48-like}$
Real-time PCR

1. Introduction

Enterobacteriaceae are opportunistic pathogens that cause serious nosocomial infections, with a rapidly increasing prevalence of infections caused by highly antibiotic-resistant Gram-negative strains among hospitalized patients worldwide [1,2]. Carbapenem is a broad-spectrum, potent β-lactam antibiotic used to treat serious infections caused by multidrug-resistant bacteria. They have been traditionally used to treat infections caused by extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae*, and are currently considered the antibiotic of last resort [3].

However, the widespread use of carbapenems

has led to a rapid increase in carbapenem-resistant *Enterobacteriaceae* (CRE), and the spread of carbapenemase-producing *Enterobacteriaceae* (CPE) has become a global threat to public health [4]. Antibiotic resistance is one of the biggest threats today, and if no action is taken, drug-resistant diseases could cause 10 million deaths per year by 2050 [5]. Due to limited antibiotic options and high mortality rates, infections caused by multidrug-resistant (MDR), extensively drug-resistant (XDR), and Gram-negative bacteria (GNB) have become a major challenge for global health organizations [6-10].

The mechanisms of resistance to β-lactam antibiotics include decreased permeability, overexpression of efflux pumps, mutation and modification of antibiotic target structures, and modification of antibiotics by hydrolysis. Among them, decreased expression of a protein called a highly permeable porin (outer membrane protein, Omp) may be intrinsically related to antibiotic resistance by reducing entry into the outer cell wall. For example, *Pseudomonas aeruginosa* is intrinsically resistant to a variety of antibiotics due to reduced expression of the classical high-permeability porin, which is an important example of bacterial resistance to β-lactam antibiotics [11].

Furthermore, β-lactam resistance in *Enterobacteriaceae* is mainly induced by the expression of enzymes that cleave the β-lactam ring. The increase in ESBL-producing bacteria has not only increased the clinical use of carbapenems, but also increased carbapenemase activity. Carbapenemase is a large family of β-lactamases that were first identified in *Enterobacteriaceae* and are characterized by having a serine in common with the target [11]. These β-lactamases are categorized into four main groups (class A, B, C, and D) based on their amino acid sequence. Class A includes serine β-lactamases with an active site, class B includes metallo-β-lactamases, class C includes AmpC β-lactamases, and class D includes oxacillinases, and each class has β-lactamases with varying activity against carbapenem class antibiotics. CPE express an acquired carbapenemase gene that specifically hydrolyzes the carbapenem β-lactam ring and belongs to the amplicon class A, B, and D groups of β-lactamases typically found in acquired plasmids, but can also be present in other transferable genetic elements inserted into chromosomes [12].

Class A includes ESBL and *Klebsiella pneumoniae* carbapenemase (KPC), which are chromosomally encoded [*Serratia marcescens* enzyme (SME), non-metallo-carbapenemase A (NMC-A), *Serratia fonticola* carbapenemase-1 (SFC-1), penicillinase (PenA), *Francisella philomiragia* carbapenemase (FPH-1), sulfhydryl variable-38 (SHV-38)], plasmid-encoded [KPC, Guiana extended spectrum (GES), French imipenemase (FRI-1)], or both (imipenemase, IMP) [13]. The best known of these, the serine carbapenemase KPC, was first identified in *Klebsiella pneumoniae* in 1996, and has since been characterized in most clinical *Enterobacteriaceae* and several species such as *P. aeruginosa* and *Acinetobacter baumannii* [14,15]. The *KPC* gene is plasmid-borne and is associated with a dominant clone of *K. pneumoniae* ST258 found worldwide [16]. This *KPC* gene is often carried by the pKpQIL plasmid or closely related variants, which encode proteins that can be distinguished by single amino acid substitutions, and there are also several variant forms of the *KPC* gene that mostly retain similar activity [17]. In general, class A carbapenemases reduce susceptibility to imipenem-sensitive bacteria and enable hydrolysis of a variety of β-lactams, including carbapenem [18].

Class B β-lactamases are also known as metallo-β-lactamases (MBLs) because they require a divalent cation (typically Zn^{2+}) as a metal cofactor to hydrolyze β-lactams [19,20]. MBLs have a broad substrate range and can inhibit all β-lactam antibiotics except monobactams [21]. MBLs belonging to class B are the most clinically relevant carbapenemases and are divided into three subclasses, B1, B2, and B3, based on structural and functional points [11]. The most clinically relevant MBLs, including the most frequent Verona integron-encoded MBL (VIM), imipenemase

(IMP), and New Delhi MBL (NDM), belong to the B1 subclass. These MBLs are typically located within an integron structure, a genetic device that allows different bacteria to adapt and evolve rapidly through the stockpiling and expression of new genes, usually linked to a mobile plasmid or transposon, a deoxyribonucleic acid (DNA) sequence that can move from one location on a chromosome to another to facilitate the transfer of resistance genes between bacteria [22].

The class D carbapenemases produced by *Enterobacteriaceae* include oxacillinase-48-like β-lactamases [3]. OXA-type β-lactamases are a group of enzymes found in *A. baumannii* and *K. pneumoniae* strains, and OXA-48 and related variants are clinically relevant because they make infections difficult to treat [11,23]. OXA-48 and similar carbapenemases induce relatively weak hydrolysis of penicillins and carbapenems, but not cephalosporins [24]. High levels of carbapenem resistance can occur when these enzymes are found in combination with other β-lactamases, such as ESBL, or with porin changes that lead to permeability defects [3].

Currently, KPC is endemic in the United States, Israel, South America, and some countries in Europe and Asia, with class B New Delhi metallo-β-lactamases predominant in Asia, and class D OXA-48-like carbapenemases predominant in North Africa and Europe [25].

Currently, the main threat of antibiotic-resistant bacteria is MDR Gram-negative strains that have developed resistance to carbapenems in particular, and along with CRE, carbapenem-resistant *A. baumannii* (CRAB), and carbapenem-resistant *P. aeruginosa* (CRPA) are at the top of the World Health Organization's list of antibiotic-resistant "priority pathogens" [26].

The production of carbapenemase is the main mechanism underlying carbapenem resistance worldwide, which is a public health concern for all countries and therefore requires close monitoring as antimicrobial resistance continues to grow. One of the major challenges in controlling the spread of CRE is the fact that patients with CRE infection identified by routine clinical cultures represent only a small fraction of patients harboring CRE. Colonization of the gastrointestinal tract is asymptomatic, and colonized patients serve as reservoirs for transmission. These carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (CP-CRE) can be rapidly transmitted within and between institutions when introduced into the healthcare system by colonized patients [27]. Rapid identification of patients colonized with CP-CRE allows for the implementation of infection control precautions to prevent transmission, and such testing has the potential to halt the spread of these highly resistant organisms at local, regional, and national levels. Given the critical relationship between identification of CP-CRE, timely initiation of effective antimicrobial therapy and infection control interventions, and patient outcomes, there is a clear need for rapid tests for CP-CRE detection. Rapid diagnosis has the potential to improve surveillance, diagnosis, and treatment of CRE, which pose a significant public health threat with limited treatment options and high mortality rates [25].

In order to identify and rapidly and accurately detect CPEs that pose a threat to public health, this study detected bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and $bla_{OXA\text{-}48\text{-}like}$ genes among the genotypes of carbapenemase by phenotyping and molecular diagnosis using carbapenem-resistant Gram-negative bacilli collected from October 2019 to June 2020, and identified the distribution patterns of related genes.

2. Materials and methods

2.1. Assay strain

The strains used for the analysis were 41 Gram-negative *Staphylococcus aureus* isolates with confirmed drug resistance to imipenem, meropenem, and ertapenem using VITEK 2 automated instrument identification (ID) system (bioMérieux, Marcy l'Etoile,

France) antimicrobial susceptibility testing (AST) cards collected from October 2019 to June 2020 at Gyeongnam Regional General Hospital.

2.2. Identification of strains

The 41 isolates used in the assay were bacterially identified with a Gram-negative (GN) kit (bioMérieux, Marcyl 'Etoile, France) using a VITEK 2 automated instrument ID system (bioMérieux, Marcy l'Etoile, France). Single colonies grown on trypticase soy agar (TSA) were taken and the turbidity of the suspension was allowed to reach McFarland 0.5. After filling the GN kit, the cassette was placed in the loader at the bottom right of the VITEK device when the door was opened. The strain and accuracy of the identification were then checked in the VITEK program.

2.3. Phenotypic carbapenemase screening tests

The 41 strains used in the assay were screened for carbapenemase by disk diffusion. Test strain suspensions were adjusted to McFarland 0.5 and inoculated evenly by swabbing onto Mueller-Hinton medium. Then, imipenem, meropenem, and ertapenem (10 μg, BBL, Cockeysville, MD, USA) were placed in the center of the medium and incubated for 18 to 24 hours in a 37°C incubator, and the antimicrobial-specific disk inhibition zone diameters were measured and read according to the criteria of the Clinical and Laboratory Standards Institute (CLSI) [28]. Carbapenemase phenotype detection was performed using the modified Hodge test (MHT). The standard strain *E. coli* ATCC 25922 was suspended in McFarland 0.5 and the suspension was diluted 1:10 in saline. The suspension was then evenly spread on MacConkey medium (BD Difco, New Jersey, USA). Generally, the MHT test is performed in Muller-Hinton medium, but it is sometimes modified to MacConkey medium to improve the sensitivity of CPE detection because the release of β-lactamase from cells is enhanced in the presence of bile compounds in MacConkey medium [29].

A disk of imipenem, meropenem, and ertapenem (10 μg each) was placed in the center of the petri dish, and the test strain was taken with a metal loop and streaked from the disk to the edge of the petri dish. After 18 to 24 hours of incubation in an incubator, the isolate was considered positive if it grew into a clover leaf-like indentation around the streaked strain [28]. The isolate was then screened for the presence of class A carbapenemase (KPC) and class B carbapenemase (MBL) production using the carbapenemase inhibition test (CIT). The isolates were spread on Muller-Hinton medium after making a strain suspension with McFarland 0.5. After placing three meropenem disks at appropriate intervals, 10 μL of phenylboronic acid (PBA) for the detection of class A carbapenemase (KPC) and ethylenediaminetetraacetic acid (EDTA) reagent for the detection of class B carbapenemase (MBL) on one of the disks. After 16 to 20 hours of incubation in a 37°C incubator, the respective disk inhibition zones were measured, and the test method and result interpretation of CIT were read according to the CPE diagnostic method guidelines, and the integrated interpretation was made based on the test results of MHT and CIT [30].

2.4. Detection of carbapenem resistance genes by polymerase chain reaction

Nucleic acids were extracted from 41 strains used in the analysis and subjected to polymerase chain reaction (PCR). Nucleic acid extraction was performed by the Gram-negative (GN) method using the Wizard Genomic DNA purification kit (Promega, Wisconsin, USA). Genetic testing for five of the carbapenemases (KPC, NDM, IMP, VIM, and OXA-48-like) was then performed (**Table 1**) [31-37]. A total of 20 μL of reaction solution was prepared by mixing 1 μL of 5 pmol of primers, 2 μL of DNA, and distilled water in AccuPower PCR PreMix (Bioneer, Daejeon, Korea). A total of 35 cycles of amplification reactions were performed using a dual block PCR C1000 Thermal

Table 1. Primers for the detection of carbapenemase-producing bacteria

Gene	Amplicon size (bp)	Primer sequences	GeneBank accession number	Reference
bla_{KPC}	785	5'-TCGCTAAACTCGAACAGG-3' 5'-TTACTGCCCGTTGACGCCCAATCC-3'	EU784136	[31]
bla_{NDM}	621	5'-GGTTTGGCGATCTGGTTTTC-3' 5'-CGGAATGGCTCATCACGATC-3'	FN396876.1	[32]
bla_{IMP}	587	5'-GAAGGCGTTTATGTTCATAC-3' 5'-GTACGTTTCAAGAGTGATGC-3'	AF244145.1	[33]
bla_{VIM}	389	5'-GTTTGGTCGCATATCGCAAC-3' 5'-AATGCGCAGCACCAGGATAG-3'	AF191564.1	[33]
$bla_{OXA\text{-}48\text{-}like}$	438	5'-GCGTGGTTAAGGATGAACAC-3' 5'-CATCAAGTTCAACCCAACCG-3'	ON651448.1	[33]
qbla_{KPC}	106	5'-TTGTTGATTGGCTAAAGGG-3' 5'-CCATACACTCCGCAGGTT-3'	EU244644	[34]
qbla_{NDM}	128	5'-GATCCTCAACTGGATCAAGC-3' 5'-GATCCTCAACTGGATCAAGC-3'	JQ060896.1	[35]
qbla_{IMP}	172	5'-TTGACACTCCATTTACTGCTA-3' 5'-TCATTTGTTAATTCAGATGCATA-3'	KF723585	[36]
qbla_{VIM}	247	5'-GAGTTGCTTTTGATTGATACAG-3' 5'-TCGATGAGAGTCCTTCTAGA-3'	GQ288396	[36]
q$bla_{OXA\text{-}48\text{-}like}$	100	5'-GTAGCAAAGGAATGGCAA-3' 5'-CCTTGCTGCTTATTCTCA-3'	ON586156.1	[37]

Cycler (Bio-Rad Laboratories, Inc., California, USA) at 95°C for 5 minutes, followed by 45 seconds at 95°C, 45 seconds at 60°C, and 1 minute at 72°C, and an extension reaction at 72°C for 5 minutes. The amplified PCR products were confirmed by electrophoresis on 2% agarose gel containing ethidium bromide (EtBr) at 100 volts for 40 minutes. The amplified PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequences were analyzed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Massachusetts, USA) and ABI 3730XL (Applied Biosystems, Massachusetts, USA), and the determined sequences were subjected to comparative analysis using the blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) available on the website of the National Center for Biotechnology Information (NCBI).

2.5. Diagnosis of carbapenemase genes using melting curve analysis of real-time PCR

For genotyping by melting curve analysis of real-time polymerase chain reaction (real-time PCR), iQ™ SYBR® Green supermix (Bio-Rad Laboratories, Inc., California, USA) was used. The reaction solution was prepared by adding 10 μL of iQ ™ SYBR® Green supermix, 1 μL of primer mix, 1 μL of DNA, and 8 μL of distilled water, to make a total of 20 μL. Real-time PCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., California, USA), and strains were analyzed in duplicate. The genes of KPC and NDM were subjected to a 5-minute reaction at 95℃ followed by 10 seconds at 95℃, 30 seconds at 60℃, and 10 seconds at 72℃ for a total of 40 cycles, and the genes of IMP, VIM, and OXA-48-like were subjected to a 5-minute reaction at 95℃ followed by 10 seconds at 95℃, 37 seconds at 55℃, and 10 seconds at 72℃ for a total of 40 cycles. All genes analyzed were subjected to a melting curve by increasing the temperature at a rate of 0.5°C per second from 65°C to 95°C after the end of the last PCR reaction. Determination of the limit of detection (LOD) was analyzed using a standard curve. A no-template control (NTC) was performed to confirm the generation of non-specific amplification products in SYBR green, and the reaction composition of the NTC was the

remaining reaction solution except for the mold, and the reaction conditions were the same as the analysis process.

3. Result

3.1. Sample characteristics

This study was conducted on 41 strains collected from Gyeongnam Regional General Hospital, and the strains were identified using the VITEK 2 automated instrument ID system (bioMérieux, Marcy l'Etoile, France). 9 strains of *Acinetobacter baumannii*, 9 strains of *Pseudomonas aeruginosa*, 8 strains of *Klebsiella pneumoniae*, 5 strains of *Escherichia coli*, 3 strains of *Enterobacter cloacae*, 2 strains of *Enterobacter aerogenes*, 1 strain of *Acinetobacter pittii*, 1 strain of *Citrobacter freundii*, 1 strain of *Pseudomonas putida*, 1 strain of *Pseudomonas rhodesiae*, and 1 strain of *Serratia marcescens*. The frequency of isolated specimens was 12 (29.3%) in urine, 9 (22%) in blood, 8 (19.5%) in sputum, 4 (9.8%) in tracheal aspirates, 3 (7.3%) in bronchial aspirates, 1 (2.4%) in ascitic fluid, 1 (2.4%) in pus, and 3 (7.3%) in other (**Figure 1**).

3.2. Phenotypic carbapenemase screening methods

Disk diffusion method was performed using imipenem, meropenem, and ertapenem for all strains identified in the clinical specimens, and antibiotic susceptibility testing was based on the CLSI guidelines for determining antibiotic resistance [28]. Then, MHT, a screening test, was performed to read the results, and MHT with ertapenem confirmed positive results in all 41 strains (**Table 2**).

Furthermore, CIT test was performed as a screening test, and the analysis confirmed positive results in 18 out of 41 strains in meropenem + PBA, and the strains were 7 *K. pneumoniae*, 4 *P. aeruginosa*, 4 *E. coli*, 2 *E. aerogenes*, and 1 *C. freundii*. The results of meropenem + EDTA showed that 8 out of 41 strains were positive, and the strains were 3 *E. cloacae*, 2 *A. baumannii*, 1 *K. pneumoniae*, 1 *E. coli*, and 1 *P. aeruginosa* (**Table 3**).

Based on the results of MHT and CIT, integrated analysis was performed, and 18 strains of KPC, 8 strains of class B carbapenemase, and 15 strains of class D carbapenemase were identified (**Table 2**).

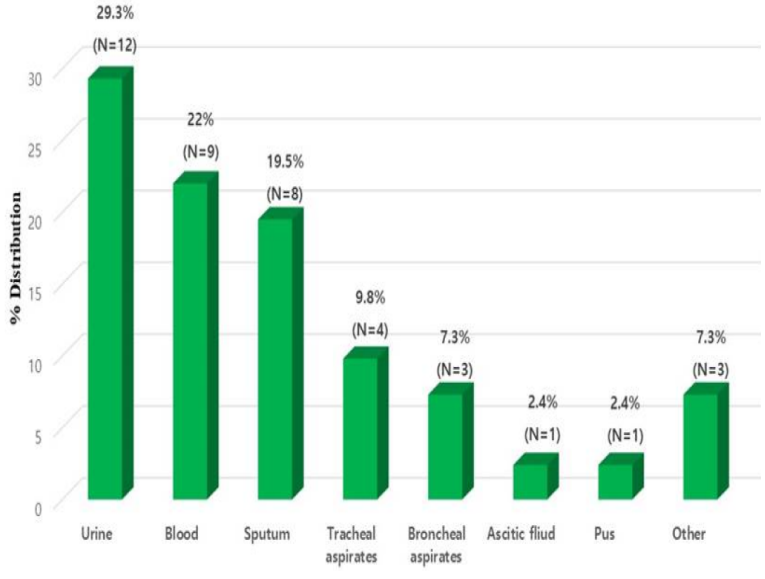

Figure 1. Frequency of detection in specimens

Table 2. Carbapenem resistance Gram-negative rod bacteria analyzed in screening test

No.	ID	Strain	Disk diffusion			Modified Hodge test	Carbepenemase inhibition test		Interpretation
			IMP	MEM	ETP		MEM + EDTA	MEM + PBA	
1	KBN12P06891	*Enterobacter aerogenes*	R	R	R	Positive	Negative	Positive	KPC
2	KBN12P06956	*Klebsiella pneumoniae*	R	R	R	Positive	Negative	Positive	KPC
3	KBN12P07156	*Klebsiella pneumoniae*	R	R	R	Positive	Positive	Negative	Class B carbapenemase
4	KBN12P07157	*Klebsiella pneumoniae*	R	R	R	Positive	Negative	Positive	KPC
5	KBN12P06899	*Klebsiella pneumoniae*	R	R	R	Positive	Negative	Positive	KPC
6	KBN12P06900	*Klebsiella pneumoniae*	R	R	R	Positive	Negative	Positive	KPC
7	KBN12P06901	*Klebsiella pneumoniae*	R	R	R	Positive	Negative	Positive	KPC
8	KBN12P06957	*Klebsiella pneumoniae*	R	R	R	Positive	Negative	Positive	KPC
9	KBN12P06958	*Klebsiella pneumoniae*	R	R	R	Positive	Negative	Positive	KPC
10	KBN12P06847	*Pseudomonas aeruginosa*	R	R	R	Positive	Negative	Positive	KPC
11	KBN12P06890	*Enterobacter aerogenes*	R	R	R	Positive	Negative	Positive	KPC
12	KBN12P06954	*Escherichia coli*	R	R	R	Positive	Negative	Positive	KPC
13	KBN12P06953	*Escherichia coli*	R	R	R	Positive	Positive	Negative	Class B carbapenemase
14	KBN12P06896	*Escherichia coli*	R	R	R	Positive	Negative	Positive	KPC
15	KBN12P07135	*Citrobacter freundii*	R	R	R	Positive	Negative	Positive	KPC
16	KBN12P06893	*Enterobacter cloacae*	R	R	R	Positive	Positive	Negative	Class B carbapenemase
17	KBN12P06894	*Escherichia coli*	R	R	R	Positive	Negative	Positive	KPC
18	KBN12P06895	*Escherichia coli*	R	R	R	Positive	Negative	Positive	KPC
19	KBN12P06952	*Enterobacter cloacae*	R	R	R	Positive	Positive	Negative	Class B carbapenemase
20	KBN12P06951	*Enterobacter cloacae*	R	R	I	Positive	Positive	Negative	Class B carbapenemase
21	KBN12P06844	*Acinetobacter baumannii*	R	R	R	Positive	Positive	Negative	Class B carbapenemase
22	KBN12P06845	*Acinetobacter baumannii*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
23	KBN12P06846	*Acinetobacter baumannii*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
24	KBN12P06863	*Acinetobacter baumannii*	R	R	I	Positive	Negative	Negative	Class D carbapenemase
25	KBN12P06864	*Acinetobacter baumannii*	R	R	R	Positive	Positive	Negative	Class B carbapenemase
26	KBN12P06865	*Acinetobacter baumannii*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
27	KBN12P07178	*Acinetobacter baumannii*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
28	KBN12P07179	*Acinetobacter baumannii*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
29	KBN12P07180	*Acinetobacter baumannii*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
30	KBN12P07083	*Acinetobacter pittii*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
31	KBN12P06831	*Pseudomonas rhodesiae*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
32	KBN12P07166	*Serratia marcescens*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
33	KBN12P06862	*Pseudomonas putida*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
34	KBN12P07183	*Pseudomonas aeruginosa*	R	R	R	Positive	Negative	Positive	KPC
35	KBN12P07181	*Pseudomonas aeruginosa*	R	R	R	Positive	Negative	Positive	KPC
36	KBN12P06866	*Pseudomonas aeruginosa*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
37	KBN12P06867	*Pseudomonas aeruginosa*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
38	KBN12P07182	*Pseudomonas aeruginosa*	R	R	R	Positive	Negative	Positive	KPC
39	KBN12P06868	*Pseudomonas aeruginosa*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
40	KBN12P06848	*Pseudomonas aeruginosa*	R	R	R	Positive	Negative	Negative	Class D carbapenemase
41	KBN12P06849	*Pseudomonas aeruginosa*	R	R	R	Positive	Positive	Negative	Class B carbapenemase

Abbreviations: R, resistant; I, intermedius; IMP, imipenem; MEM, meropenem; ETP, ertapenem; KPC, *Klebsiella pneumoniae* carbapenemases; EDTA, ethylenediaminetetraacetic acid; PBA, phenylboric acid

Table 3. Gram-negative rod bacteria showing positive results in modified Hodge test and carbapenemase inhibition test (MHT, N = 41; Meropenem + PBA, N = 18; Meropenem + EDTA, N = 8)

Treatment	No.	Name of organism	Number of isolates
MHT	1	*Acinetobacter baumannii*	9 (22%)
	2	*Pseudomonas aeruginosa*	9 (22%)
	3	*Klebsiella pneumoniae*	8 (19.6%)
	4	*Escherichia coli*	5 (12.2%)
	5	*Enterobacter cloacae*	3 (7.3%)
	6	*Enterobacter aerogenes*	2 (4.9%)
	7	*Acinetobacter pittii*	1 (2.4%)
	8	*Citrobacter freundii*	1 (2.4%)
	9	*Pseudomonas putida*	1 (2.4%)
	10	*Pseudomonas rhodesiae*	1 (2.4%)
	11	*Serratia marcescens*	1 (2.4%)
		Total	41 (100%)
MEM + PBA	1	*Klebsiella pneumoniae*	7 (38.9%)
	2	*Pseudomonas aeruginosa*	4 (22.2%)
	3	*Escherichia coli*	4 (22.2%)
	4	*Enterobacter aerogenes*	2 (11.1%)
	5	*Citrobacter freundii*	1 (5.6%)
		Total	18 (100%)
MEM + EDTA	1	*Enterobacter cloacae*	3 (37.5%)
	2	*Acinetobacter baumannii*	2 (25%)
	3	*Klebsiella pneumoniae*	1 (12.5%)
	4	*Escherichia coli*	1 (12.5%)
	5	*Pseudomonas aeruginosa*	1 (12.5%)
		Total	8 (100%)

Abbreviations: MHT, modified Hodge test; MEM, meropenem; EDTA, ethylenediaminetetraacetic acid; PBA, phenylboric acid

3.3. Carbapenemase gene detection using molecular biological methods

Genetic testing for five of the carbapenemases (KPC, NDM, IMP, VIM, and OXA-48-like) was performed on all strains analyzed (**Figure 2**). Among the 41 strains, 28 were positive for the bla_{KPC} gene, 25 for the bla_{NDM} gene, 5 for the bla_{IMP} gene, 1 for the bla_{VIM} gene, and 13 for the $bla_{OXA\text{-}48\text{-}like}$ gene (**Table 4**).

Analysis of the 28 amplified bla_{KPC} strains showed that they were 8 strains of *P. aeruginosa*, 6 strains of *A. baumannii*, 5 strains of *K. pneumoniae*, 4 strains of *E. coli*, 2 strains of *E. aerogenes*, 1 strain of *A. pittii*, 1 strain of *C. freundii*, and 1 strain of *S. marcescens*. Analysis of 25 amplified bla_{NDM} strains showed 2 strains of *K. pneumoniae*, 3 strains of *E. coli*, 9 strains of *P. aeruginosa*, 4 strains of *A. baumannii*, 3 strains of *E. cloacae*, 1 strain of *A. pittii*, 1 strain of *C. freundii*, 1 strain of *P. putida*, and 1 strain of *P. rhodesiae*. Analysis of five amplified bla_{IMP} strains revealed three *P. aeruginosa*, one *A. pittii*, and one *S. marcescens*. One amplified bla_{VIM} strain was *P. putida*, and 13 amplified $bla_{OXA\text{-}48\text{-}like}$ strains were identified as 4 strains of *E. coli*, 1 strain of *P. aeruginosa*, 3 strains of *A. baumannii*, 3 strains of *E. cloacae*, 1 strain of *E. aerogenes*, and 1 strain of *C. freundii*. In the result of PCR test, 7 strains of bla_{KPC} + bla_{NDM}, 1 strain of bla_{KPC} + bla_{IMP}, 1 strain of bla_{NDM} + $bla_{OXA\text{-}48\text{-}like}$, 1 strain of bla_{NDM} + bla_{VIM}, 4 strains of bla_{KPC} + bla_{NDM} + bla_{IMP}, and 4 strains of bla_{KPC} + bla_{NDM} + $bla_{OXA\text{-}48\text{-}like}$ were identified as having two or more genes.

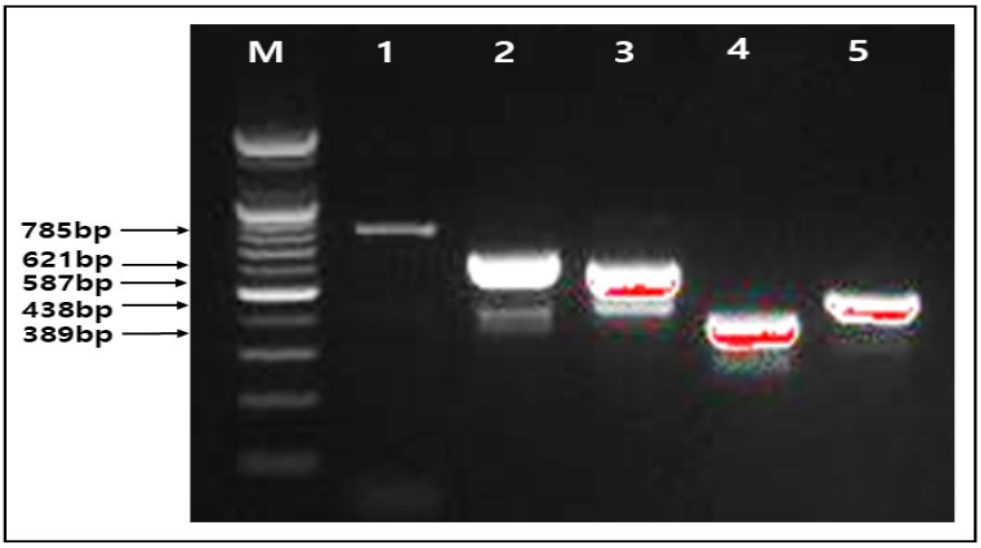

Figure 2. PCR amplification profile of the carbapenemase gene from the Gram-negative rod bacteria isolates. M, 100 bp DNA ladder marker; 1: bla_{KPC} gene, size of 785 bp; 2: bla_{NDM} gene, size of 621 bp; 3: bla_{IMP} gene, size of 587 bp; 4: bla_{VIM} gene, size of 389 bp; 5: $bla_{OXA-48-like}$ gene, size of 438 bp

Table 4. Analysis of carbapenemase-producing Gram-negative rod bacteria and carbapenemase types in PCR result

Strain	No.	Class A		Class B		Class D	Non-carbapene mase	bla_{KPC} + bla_{NDM}	bla_{KPC} + bla_{IMP}	bla_{NDM} + $bla_{OXA-48-like}$	bla_{NDM} + bla_{VIM}	bla_{KPC} + bla_{NDM} + bla_{IMP}	bla_{KPC} + bla_{NDM} + $bla_{OXA-48-like}$
		bla_{KPC}	bla_{NDM}	bla_{IMP}	bla_{VIM}	$bla_{OXA-48-like}$							
Acinetobacter baumannii	9	6	4	-	-	3	2	2	-	-	-	-	1
Pseudomonas aeruginosa	9	8	9	3	-	1	-	5	-	1	-	3	-
Klebsiella pneumoniae	8	5	2	-	-	-	1	-	-	-	-	-	-
Escherichia coli	5	4	3	-	-	4	-	-	-	-	-	-	2
Enterobacter cloacae	3	-	3	-	-	3	-	-	-	-	-	-	-
Enterobacter aerogenes	2	2	-	-	-	1	-	-	-	-	-	-	-
Acinetobacter pittii	1	1	1	1	-	-	-	-	-	-	-	1	-
Citrobacter freundii	1	1	1	-	-	1	-	-	-	-	-	-	1
Pseudomonas putida	1	-	1	-	1	-	-	-	-	-	1	-	-
Pseudomonas rhodesiae	1	-	1	-	-	-	-	-	-	-	-	-	-
Serratia marcescens	1	1	-	1	-	-	-	-	1	-	-	-	-
Total	41	28 (68.3%)	25 (61%)	5 (12.2%)	1 (2.4%)	13 (31.7%)	3 (7.3%)	7 (17.1%)	1 (2.4%)	1 (2.4%)	1 (2.4%)	4 (9.8%)	4 (9.8%)

Of the seven bla_{KPC} + bla_{NDM} strains, five were *P. aeruginosa*, two were *A. baumannii*, one bla_{KPC} + bla_{IMP} was *S. marcescens*, one bla_{NDM} + $bla_{OXA-48-like}$ was *A. baumannii*, and one bla_{NDM} + bla_{VIM} was *P. putida*. Four strains of bla_{KPC} + bla_{NDM} + bla_{IMP} were identified as three strains of *P. aeruginosa*, and one strain of *A. pittii*. Four strains of bla_{KPC} + bla_{NDM} + $bla_{OXA-48-like}$ were identified as two strains of *E. coli*, one strain of *A. baumannii*, and one strain of *C. freundii*.

The genes used in the subsequent analysis were all genotyped by sequence analysis, and the sequences were compared using the blast program of NCBI. For the bla_{KPC} gene, Genebank No. CP094994.1, with an average homology of 99% and an average of 3 bases missing from the original sequence (Gap 1%). For the bla_{NDM} gene, Genebank No. CP095662.1, with an average homology of 100% and no bases missing (Gap 0%). For the bla_{IMP} gene, Genebank No. AP022367.1, with homology average of 99%, an average of 2 bases missing from the original sequence (Gap 1%). For the bla_{VIM} gene, Genebank No. MN256633.1, with an average homology of 99%, and an average of 3 bases missing from the original sequence (Gap 1%). For the $bla_{OXA-48-like}$ gene, Genebank No. MN654419.1, with an average homology of 99%, and an average of 1 base missing from the original sequence (Gap 1%).

3.4. Detection of carbapenemase gene using melting curve analysis

All strains used in the analysis were run in two replicates, and the NTC assay confirmed that there were no amplification products other than the sample. In the assay, a sample was considered positive if it exceeded the threshold before the crossing point (Cp) of 30 cycles, and negative if the Cp was greater than 30. The real-time PCR analysis identified 28 positive strains for the bla_{KPC} gene, 25 for the bla_{NDM} gene, 5 for the bla_{IMP} gene, 1 for the bla_{VIM} gene, and 13 for the $bla_{OXA-48-like}$ gene among 41 strains, which were 100% consistent with the PCR results. The average cycle threshold (Ct) values of the

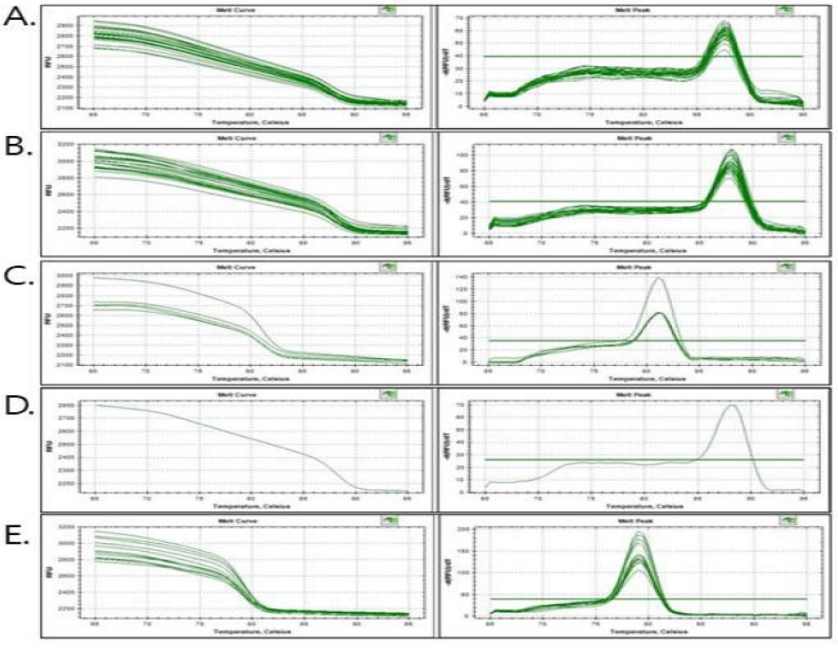

Figure 3. Melting curve analysis of carbapenemase gene. (A) Melting curve analysis of bla_{KPC} amplicon was generated that showed the fragment melting temperature (Tm) of 87.5°C. (B) Melting curve analysis of bla_{NDM} amplicon was generated that showed the fragment Tm of 88°C. (C) Melting curve analysis of bla_{IMP} amplicon was generated that showed the fragment Tm of 81°C. (D) Melting curve analysis of bla_{VIM} amplicon was generated that showed the fragment Tm of 88.5°C. (E) Melting curve analysis of $bla_{OXA-48-like}$ amplicon was generated that showed the fragment Tm of 79°C.

amplified genes were 17.23 ± 1.65 for bla_{KPC}, 21.44 ± 3.85 for bla_{NDM}, 17.42 ± 4.02 for bla_{IMP}, 19.22 ± 0.21 for bla_{VIM}, and 24.01 ± 4.79 for $bla_{OXA-48-like}$, and there were no amplification products in the negative strains. Melting curve analysis of the amplification products showed that bla_{KPC} had a melting temperature (Tm) of 87.5℃, bla_{NDM} had a Tm of 88℃, bla_{IMP} had a Tm of 81℃, bla_{VIM} had a Tm of 88.5℃, and $bla_{OXA-48-like}$ had a Tm of 79℃ (**Figure 3**). Standard curve analysis was performed using the amplified samples, and the limit of detection (LOD) was confirmed to be 21.48 ng/mL for the bla_{KPC} gene, 31.38 ng/mL for the bla_{NDM} gene, 31.38 ng/mL for the bla_{IMP} gene, 19.77 ng/mL for the bla_{VIM} gene, and 36.39 ng/mL for the $bla_{OXA-48-like}$ gene.

4. Discussion

Multidrug-resistant strains are microorganisms that are resistant to multiple classes of antibiotics, some of which are responsible for the majority of healthcare-associated infections and can evade the action of antimicrobial agents. The prevalence of serious infections caused by multidrug-resistant strains has been continuously increasing over the years, and international travel, migration, and movement of patients from one country to another have increased the risk of spreading these infections, which are a major cause of increasing morbidity and mortality worldwide [38]. It is difficult to prevent the transmission of some resistance determinants found in mobile elements such as ESBL-encoding genes and carbapenemase-encoding genes. CPEs carrying plasmid-encoded resistance genes transmit their resistance genes horizontally among various Gram-negative rod bacteria. In particular, carbapenemase produced by *Enterobacteriaceae* is of greatest interest from a public health perspective. The continuous increase in the proportion of CPEs other than *K. pneumoniae* or *E. coli* is concerning because it may be due to genetic recombination and acquisition of new resistance genetic determinants by other bacteria found in the intestinal tract, which is a repository of enteric bacteria [39]. Furthermore, nosocomial transmission and inter-hospital spread of CPEs are more frequent within countries than between countries, thus it is necessary to implement surveillance programs for patients to reduce the spread of healthcare-associated infections such as CPEs [39,40].

Therefore, in this study, for the identification and rapid and accurate detection of CPE, 41 strains of Gram-negative bacilli confirmed to be resistant to carbapenem agents were used to detect the carbapenemase gene by phenotypic examination and molecular diagnosis, and to identify the distribution pattern of the related genes.

For genetic diagnosis, all strains used in the analysis were tested for bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and $bla_{OXA-48-like}$ genes, and positive strains were genotyped by sequencing, resulting in an average homology of 99% for all genes used in the analysis.

After comparing the results of the integrated analysis of the phenotypic test and the PCR results, 18 strains of KPC-producing strains were positive in the integrated analysis and 28 strains were positive in the PCR test, and 10 strains (35.7%) were false negatives in the phenotypic test. In the integrated analysis, 8 strains of presumptive NDM were identified, 25 strains were positive by PCR test, and 17 strains (68%) were false negative by phenotypic test. In the integrated analysis, 15 strains were found to be class D carbapenemase (OXA-48-like) producers, 13 strains were positive by PCR, and 2 strains (13.3%) were false positives by phenotypic testing.

MHT, a commonly used phenotypic test, was the earliest to be applied to detect potential KPC-producing strains of CRE, but showed low sensitivity in detecting NDM and OXA-48-like CPE, and gave false-positive results in CRE due to porin alterations combined with ESBL and/or AmpC β-lactamases overproduction [41]. In addition, boronate-based tests for CIT using inhibitors show high sensitivity for the detection of KPC-producing strains, but specificity problems may occur in isolates with reduced susceptibility to carbapenem

due to high expression of AmpC-type β-lactamases and porin deficiency [42].

Recently, the Carba NP test and its modifications, which compensate for the shortcomings of MHT and CIT, are suitable for the detection of various carbapenemases of CRE and CR-*P. aeruginosa*, and have been applied in the clinical microbiology field because they are convenient biochemical tests that provide results within 15 to 30 minutes. In addition, the modified carbapenem inactivation method (mCIM) and EDTA-modified CIM (eCIM) tests can be utilized to reliably distinguish MBL-producing CRE strains from serine class carbapenemase-producing strains. Nevertheless, the mCIM/eCIM test takes approximately 6–12 hours to obtain results, is subjective to interpretation, and also performed slightly better than CPE in detecting VIM-producing *P. aeruginosa* and OXA-producing *A. baumannii* isolates [41].

The MHT and CIT tests, which were utilized as early phenotyping methods, are difficult to identify accurate antibiotic resistance genotypes due to multiple readout errors and low detection efficiency depending on the enzyme type. Carba NP, mCIM, and eCIM, which are complementary tests, may reflect subjective views in test interpretation, and non-nucleic acid-based detection methods have problems with simultaneous detection of multiple genes rather than one gene.

In the result of PCR and real-time PCR test analysis, 28 strains of bla_{KPC} (68.3%), 25 strains of bla_{NDM} (61%), 5 strains of bla_{IMP} (12.2%), 1 strain of bla_{VIM} (2.4%), and 13 strains of $bla_{OXA\text{-}48\text{-}like}$ (31.7%) were identified among 41 strains.

In Korean studies, Choi and Lee's study in 2022 [40] found a detection rate of 68.9% for bla_{KPC}, 25.2% for bla_{NDM}, and 1.5% for $bla_{OXA\text{-}48\text{-}like}$, and Yang and Park's study in 2021 [42] found a detection rate of 71.4% for bla_{KPC} and 22.9% for bla_{NDM}. In addition, Korean national data from 2018–2019 showed 70.0% for bla_{KPC} and 24.0% for bla_{NDM}, but a global survey showed that bla_{KPC} was the most common gene at 53.18%, followed by $bla_{OXA\text{-}48\text{-}like}$ at 20.09%, and bla_{NDM} at 19.42% [40]. Other than that, Han *et al.* [4] found that $bla_{KPC\text{-}2}$ (51.6%) and bla_{NDM} (35.7%) were the most common carbapenemase genes among CREs, and confirmed that the appearance of $bla_{OXA\text{-}232}$, bla_{IMP}, and various other carbapenemase genes has been increasing in recent years. The results of this study also confirmed the emergence of various carbapenemase genes in addition to the existing genes, especially the increased detection of class B carbapenemase genes.

We identified 18 strains (43.9%) with two or more genes among the 41 strains, and the analysis showed that *Pseudomonas* spp. accounted for the majority with 10 strains (55.6%) among 18 strains, followed by *Acinetobacter* spp. with 4 strains (22.2%).

Previous studies have shown that in Egypt, where NDM and OXA-48-like carbapenemase are widely disseminated, about 90% of CREs collected between October 2016 and September 2017 had at least one carbapenemase-encoding gene [43]. In addition, in China, where KPC-2, NDM, and OXA-48-like carbapenemase were predominantly detected in CREs, 9 out of 935 carbapenemase-producing strains were found to produce multiple carbapenemases [4]. In a study by Tălăpan and Rafila in 2022 [39], it was possible to identify MDR/XDR *P. aeruginosa* and *A. baumannii* with increased multi-carbapenemase, with MBL + KPC 0.65%, OXA-48 + MBL 4.56%, OXA-48 + KPC + MBL 0.97% in the form of multigene, and *K. pneumoniae* was the most prevalent strain.

Since the discovery of penicillinase in 1940, the modification of antibiotics by hydrolysis is a major mechanism of antibiotic resistance. The characteristic of these enzymes is that they have the ability to inactivate a wide range of β-lactams, including carbapenem and extended-spectrum cephalosporin. Most recently, β-lactamases have broadened their activity to the mannose-binding lectin (MBL) and other carbapenemases [11].

In general, β-lactamases are found in relatively small amounts in Gram-negative bacteria, but they are strategically located for maximization in

microorganisms because they are located in the plasma membrane space between the inner and outer cell membranes, and in cell wall synthesis, β-lactamases are on the outer surface of the inner membrane. In Gram-negative bacteria, β-lactamases are encoded on the chromosome or plasmid and can hydrolyze a variety of β-lactam antibiotics. Most bacteria produce one form of the enzyme, but different microorganisms elaborate many distinct β-lactamases [11].

Carbapenemase classes A, C, and D are all characterized by having a serine in common with the target [11], while class B includes MBLs that do not form such intermediates. *P. aeruginosa* expresses an acquired carbapenemase gene that specifically hydrolyzes the carbapenem β-lactam ring and belongs to the ambiguous class A, B, and D groups of β-lactamases typically found in acquired plasmids, but can also be present in other transferable genetic elements inserted into chromosomes [12].

In recent years, the rate of carbapenem resistance in *P. aeruginosa* has increased globally, which has become a major concern because it significantly limits the treatment options for patients [44].

Overexpression of efflux genes, the basis of resistance in Gram-negative bacteria, is regulated by mutational mechanisms, and carbapenem resistance in *P. aeruginosa* is caused by chromosomal substitutions resulting in the loss of porin and altered membrane permeability through efflux pump overexpression, inhibition of intrinsic β-lactamases, and acquisition of carbapenemase genes. These carbapenem-resistant *P. aeruginosa* have spread rapidly through the transfer of genetic elements and the contribution of high-risk replication. To date, the carbapenemases identified in *P. aeruginosa* are class A, B, and D, of which class B MBL is the most common type of carbapenemase produced by clinically isolated *P. aeruginosa* [20].

Although *A. baumannii* is considered a less virulent pathogen compared to *K. pneumoniae* and *P. aeruginosa*, it has been recognized that it plays an important role in the spread of widespread resistance genes to other Gram-negative strains [13,25]. *A. baumannii* can rapidly acquire carbapenem resistance genes such as MBLs, and *A. baumannii* strains producing MBLs have been frequently reported in Iran. *A. baumannii* appears to use several mechanisms to resist β-lactam antibiotics, and more research is needed on its drug resistance patterns by applying both phenotypic and genotypic analysis [36].

Since CRE was first reported in Korea in 2010, the emergence of carbapenemase genes has been steadily confirmed [40,42], and in this study, we were able to confirm the increased detection of class B carbapenemase genes in particular. We also confirmed the detection of strains with multiple carbapenemase genes that were not reported in previous Korean studies. This is concerning because the continuous increase in the proportion of CPE other than *K. pneumoniae* or *E. coli* may be due to genetic recombination and acquisition of new resistance genetic determinants by enteric bacteria [39].

Infections caused by antibiotic-resistant strains such as CRE, third-generation cephalosporin-resistant *Enterobacteriaceae* (3GC-R), multidrug-resistant *P. aeruginosa* (MRPA), multidrug-resistant *Acinetobacter* species (MRAS), etc., have very limited treatment options for patients, and appropriate antibiotic treatment within 48 to 72 hours between the onset and transmission of infection is critical, but often the infection is not recognized before reporting and culturing [43]. Production of carbapenemases, including KPC, NDM, and OXA-48-like, is the most common resistance mechanism among clinically isolated CREs [45], and more knowledge is needed to identify resistance genes, resistant organisms, and control their transmission [36]. Numerous nucleic acid- and non-nucleic acid-based methods are currently in use or under development for the rapid detection of these CREs, and it can be expected that future tests will employ rapid methods for both molecular detection of common carbapenemases and non-nucleic acids to determine the overall antimicrobial susceptibility of

isolates [25].

Currently, phenotypic testing according to CLSI guidelines is a non-nucleic acid-based detection method, which takes as long as 24 to 48 hours from isolation to the first result of a suspected carbapenemase-producing strain, and has the disadvantages of subjective interpretation and difficulty in distinguishing between types in cases of multiple carbapenemase production. However, the real-time PCR assay using SYBR Green, which is widely used because it does not require the preparation of separate probes for each gene, requires less time and has lower contamination than conventional PCR methods, and has been shown to provide the advantage of shortened turnaround time in previous studies [46]. It has also been shown to have higher specificity and sensitivity than other diagnostic methods, and to rapidly and accurately detect strains with resistance genes [43].

Since the mechanism of spreading CPE is transposon and plasmid-mediated, the transmission rate is superior to that of non-CPE, and CPE-induced resistance actually accounts for the majority of CRE. Therefore, considering the possibility of rapid horizontal and vertical transmission of genes, accurate and timely identification of these resistance genes will be an important tool to aid infection control measures and guide the selection of appropriate antimicrobial therapy, hence surveillance studies of drug-resistant pathogens are essential [20].

5. Conclusion

In conclusion, active surveillance with rapid and highly specific tests such as real-time PCR and genetic identification for early diagnosis of CRE can improve surveillance, diagnosis, and treatment of CRE, which poses a high public health threat with limited treatment options and high mortality rates. Plus, it will enable effective antimicrobial therapy and timely infection control to prevent transmission.

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Disclosure statement

The authors declare no conflict of interest.

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