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ORIGINAL RESEARCH

In vitro Synergistic Activities of Fosfomycin in Combination with Other Antimicrobial Agents Against Carbapenem-Resistant *Escherichia coli* Harboring *bla*$_{NDM-1}$ on the IncN2 Plasmid and a Study of the Genomic Characteristics of These Pathogens

Kalyarat Kaewnirat[1], Surachat Chuaychob[2], Arnon Chukamnerd[1], Rattanaruji Pomwised[3], Komwit Surachat[4,5], May Thet Paing Phoo[3], Chanitnart Phaothong[3], Chanida Sakunrang[4], Kongpop Jeenkeawpiam[4], Thanaporn Hortiwakul[2], Boonsri Charernmak[2], Sarunyou Chusri[1,2]

[1]Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand; [2]Division of Infectious Diseases, Department of Internal Medicine, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand; [3]Division of Biological Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand; [4]Molecular Evolution and Computational Biology Research Unit, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand; [5]Division of Computational Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand

Correspondence: Sarunyou Chusri, Division of Infectious Diseases, Department of Internal Medicine, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand, Tel +66 8 973 40446, Email sarunyouchusri@hotmail.com

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Purpose: The spread of New Delhi metallo-β-lactamase (NDM) encoded by the *bla*$_{NDM}$ gene has been a global health crisis for many years. Most of *bla*$_{NDM}$-harboring bacteria commonly carry various antimicrobial resistance (AMR) genes on their chromosomes or plasmids, leading to limited treatment options. Thus, we aimed to evaluate the synergistic effects of fosfomycin in combination with other antimicrobial agents against *bla*$_{NDM}$-harboring carbapenem-resistant *Escherichia coli* (CREC) and to characterize the whole-genome and plasmid sequences of these pathogens.

Methods: Thirty-eight CREC isolates were collected from patients in the Medicine Ward, Songklanagarind Hospital, Thailand. The activity of fosfomycin in combination with other antimicrobial agents against CREC isolates harboring *bla*$_{NDM}$ on the plasmid was evaluated using the checkerboard method. In this method, the serial dilutions of two antibiotics were mixed with the cultured CREC, the mixtures were incubated, and FICI was calculated to interpret the synergistic activity of the combination. The whole-genome and particular plasmids of these pathogens were sequenced using next-generation sequencing. Sequence analysis, especially on antimicrobial resistance (AMR) genes, mobile-genetic elements (MGEs), and virulence genes was performed using many bioinformatics tools.

Results: Of the *E. coli* 38 isolates, only 3 isolates contained the *bla*$_{NDM-1}$ gene, which is located on the IncN2 plasmid. The combinations of fosfomycin with aminoglycosides, colistin, tigecycline, sitafloxacin, and ciprofloxacin were synergies against *bla*$_{NDM-1}$-harboring CREC isolates. Genomic analysis revealed that these isolates harbored many β-lactam resistance genes and other AMR genes that may confer resistance to aminoglycoside, fluoroquinolone, rifampicin, trimethoprim, sulfonamide, tetracycline, and macrolide. Also, various MGEs, especially the *bla*$_{NDM-1}$-bearing IncN2 plasmid, were present in these isolates.

Conclusion: Our study demonstrated some synergistic effects of antimicrobial combination against CREC isolates harboring *bla*$_{NDM-1}$ on the IncN2 plasmid. Also, our data on the whole-genome and plasmid sequences might be beneficial in the control of the spread of *bla*$_{NDM-1}$-harboring CREC isolates. The linkages between *bla*$_{NDM-1}$-carrying plasmid, patient information, and time of collection will be elucidated to track the horizontal gene transfer in the future.

Keywords: antimicrobial resistance gene, checkerboard method, next-generation sequencing, bioinformatics tool, mobile genetic element

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Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) has been a serious public health concern for many decades. Carbapenems were an important, life-saving mainstay treatment for many years, but due to widespread over- and inappropriate use, in recent years widespread resistance to this antibiotic has developed.[1] Importantly, as CRE can hydrolyze almost all β-lactam antibiotics, the treatments are limited, resulting in a high mortality rate.

CREC resists carbapenem through various mechanisms, especially the production of carbapenemase enzymes.[2] The carbapenemase-encoding genes are usually located on mobile genetic elements (MGEs) that can transfer these particular genes to other pathogenic bacteria. Among them, the bla_{NDM} gene is one of the most prevalent and strong carbapenemase genes with high hydrolysis activity.[3] The outbreak of bla_{NDM} gene has been reported in many parts of the world such as Australia (1.6%), America (11%), Africa (11%), Europe (17%), and Asia (58%) and the evidence therefore confirm the global spread of the bla_{NDM} gene.[4] Moreover, the infection caused by this pathogen seriously threaten the patient with a high mortality rate.[5] The bla_{NDM} gene can be detected on many types of plasmids and transposons (Tn), especially Tn*125*.[6] A previous study reported that *Acinetobacter* spp. acted as the intermediate host to disseminate the bla_{NDM-1} into Enterobacteriaceae.[7] The bla_{NDM-1} gene is mostly found within the bracket of two copies of IS*Aba125* in the form of Tn*125*, which provides a −35 region promoter for the expression of bla_{NDM-1}. However, the genetic content of Tn*125* can be truncated due to the insertion of various insertion sequence (IS) elements.[8] Outbreaks of bla_{NDM} have been reported on almost all continents. Twenty replicon types of bla_{NDM}-bearing plasmids, IncHI2, IncHI3, IncN, IncN2, IncC, IncB/O/K/Z, IncFIA, IncFIB, IncFIC, IncFIII, IncHI1, IncL/M, IncP, IncR, IncT, IncX1, IncX3, IncX4, IncY, and ColE10, have been identified.[3] The co-existence of other antimicrobial resistance (AMR) genes located on these plasmids has led to the current crisis and challenging clinical situation.

In the treatment of CREC infections, the widespread use of carbapenems as monotherapy has resulted in increasing resistance to these antibiotics and other β-lactams. A combination of antibiotics therefore could be an optional therapy to reduce further resistance development and/or enhance the antimicrobial activity. A previous study reported that the combination of a carbapenem (e.g., ertapenem) with fosfomycin resulted in a synergistic effect of increased antimicrobial activity. This theory could lead to effective alternative treatments for infections caused by carbapenem-resistant Enterobacteriaceae (CRE).[9]

Although some antibiotics such as fosfomycin, tigecycline, and colistin are considered as a treatment option for the CRE infections, fosfomycin is mostly considered for severe patients due to its broad-spectrum bactericidal activity and less toxicity than other antibiotics of choice. However, many studies suggested that fosfomycin should be combined with other antibiotics to avoid the fosfomycin resistance that might be rapidly developed during the therapy.[10,11] Additionally, there are few studies reporting the fosfomycin-based combination against bla_{NDM-1}-harboring CREC. Thus, the objective of this study was to assess the synergistic activities of fosfomycin in combination with other antimicrobial agents against bla_{NDM-1}-harboring CREC, and to genetically characterize the entire genome of these pathogens.

Materials and Methods

Bacterial Isolation and Identification

The bacterial isolates were obtained from colonized patients in the Medicine Ward, Songklanagarind Hospital, between March and April 2017. One µg/mL of meropenem containing MacConkey agar plates were used to screen and select carbapenem-resistant Gram-negative bacteria.[12] The strain of *Escherichia coli* was identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF).

Antimicrobial Susceptibility Testing (AST) and Detection of the bla_{NDM} Gene

Thirty-eight *E. coli* isolates were confirmed to be carbapenem-resistant isolates by evaluation of the minimum inhibitory concentrations (MICs) of imipenem and meropenem through the broth microdilution method. *E. coli* ATCC 25922 was used as the quality control.[13] The bla_{NDM} gene was detected among these CREC isolates by a PCR method using the NDM-F primer (GGTTTGGCGATCTGGTTTTC) and the NDM-R primer (CGGAATGGCTCATCACGATC).[10,14] The PCR products were then checked using agarose gel electrophoresis and the expected size was 621 bp. In addition, the

MIC values of other 9 antibiotics (fosfomycin, ciprofloxacin, doripenem, levofloxacin, tigecycline, sitafloxacin, colistin, gentamicin, and amikacin) and 1 β-lactamase inhibitor (sulbactam) (Sigma-Aldrich) were evaluated against bla_{NDM}-harboring isolates by the broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2016 or US Food and Drug Administration (FDA) breakpoints for tigecycline or European Committee on Antimicrobial Susceptibility Testing (EUCAST) for colistin.[13,15,16]

Evaluation of Synergistic Effects

All bla_{NDM}-harboring CREC isolates were assessed for the synergistic activity of fosfomycin in combination with imipenem, meropenem, doripenem, gentamicin, amikacin, ciprofloxacin, levofloxacin, sitafloxacin, colistin, tigecycline, and sulbactam (Sigma-Aldrich, USA) using the checkerboard method. As we used fosfomycin, the Clinical and Laboratory Standards Institute (CLSI) recommended adding 25 mg/mL of glucose-6-phosphate (G-6-P) in the 96-well plates containing Mueller-Hinton broth (MHB). The Fractional Inhibitory Concentration Index (FICI) was used to interpret the synergism (FICI $\leq$ 0.5), indifference (0.5 $<$ FICI $\leq$ 4.0), or antagonism (FICI $>$ 4.0).

DNA Extraction and Whole-Genome Sequencing (WGS)

The genomic DNA (gDNA) of all bla_{NDM}-harboring CREC isolates was extracted using a TIANamp Bacterial DNA Kit (Tiangen, Beijing, China), following the manufacturer's instructions. Then, the quality of the gDNA was checked in terms of concentration and purity with a Thermo Scientific™ NanoDrop 2000 and gel electrophoresis, respectively. The qualified DNA samples were then sent to Beijing Genomics Institute (BGI), Beijing, China, for conducting short-read sequencing (WGS) with the use of a BGISEQ-500 sequencer (Beijing Genomics Institute, Beijing, China).

Plasmid Extraction and Sequencing

Plasmid DNA was extracted by the alkaline lysis method, as previously described.[17] Briefly, the bacteria were treated with a base solution (0.2 N NaOH-1% SDS) to denature the double-strand DNA. An acid solution (4M potassium acetate-2M acetic acid) was immediately added to neutralize the mixture, which was then centrifuged at high speed to separate the plasmid DNA from the chromosomal DNA. The purity of the plasmid DNA was visualized by agarose gel electrophoresis. The qualified plasmids were then sequenced using NovaSeq 6000 sequencer (Beijing Headquarters Novogene Co., Ltd. Beijing, China).

Data Analysis of WGS and Plasmids

All sequence reads were *de novo* assembled using Unicycler v0.4.7.[18] The assembled sequences were then annotated using Prokka v1.12.[19] For analysis of these sequence reads, the contigs of all CREC isolates were uploaded to the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/), a free online bioinformatics service allowing scientists to analyze sequencing data in relation to infectious diseases. Here, we used ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/)[20] and PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/),[21] with a set of 95% identity and 80% minimum length for the detection of acquired AMR genes and plasmids, respectively. Meanwhile, VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/)[22] with a set of 90% identity and 60% minimum length and MLST 2.0 (Multi-Locus Sequence Typing) (https://cge.cbs.dtu.dk/services/MLST/)[23] were used to identify virulence genes and sequence types (STs), respectively. In addition to the CGE website, we also used other web-based bioinformatics tools. For other mobile-genetic elements (MGEs), insertion sequences (ISs) and integrons were also investigated using blastn against the IS database and integron_finder, respectively. The bacteriophage genomes were explored using phaster (https://phaster.ca/),[24] while CRISPR-Cas regions were detected using CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index). The bacteriocin-encoding genes were also investigated using BAGEL4 (http://bagel4.molgenrug.nl/index.php).[25]

Pan-Genome and Phylogenetic Analysis

The pan-genome profiles of bla_{NDM}-harboring CREC isolates were compared to published genomes from Africa (n = 11), Austria (n = 39), Germany (n = 120), and Thailand (n = 12), which are available from the National Center for

Biotechnology Information (NCBI).[26–29] The pan-genome analysis was performed using Roary v3.13.0,[30] with minimum blastx identity and core definition threshold at 95% and 99%, respectively. A phylogenetic tree was generated from the pan-genome alignment, based on accessory genes, using a maximum likelihood method (1,000 bootstraps). The interactive visualization of the phylogenetic tree was performed using the Geneious R10.26[31] and Phandango website (https://jameshadfield.github.io/phandango/).[32] Additionally, a pan-genome frequency plot, a pie chart of the pan-genome, and a presence and absence matrix against a phylogenetic tree were also created using roary_plots script (https://github.com/sanger-pathogens/Roary/tree/master/contrib/roary_plots).

Data Availability

The assembled genomes of the $bla_{\text{NDM-1}}$-harboring CREC isolates from this study have been submitted to the NCBI GenBank under BioProject number PRJNA780210 with BioSample numbers SAMN23132882, SAMN23132883, and SAMN23132884.

Results

Bacterial Isolates and Clinical Data

A total of 38 *E. coli* isolates were collected from the patients and were classified the carbapenem resistance. Due to a screening method can be used to collect the isolates that provide non-susceptible to the carbapenem (intermediate resistance and resistance), only 6 (15.8%) out of 38 isolates were then identified as CREC by phenotypic method and a half of the CREC isolates (n = 3) were positive for the bla_{NDM} gene. These 3 bla_{NDM}-harboring isolates were obtained from the rectums of 2 female and 1 male patients, who had cardiac arrest or pulmonary edema as common underlying disease. Importantly, all 3 patients have previously received carbapenem and other antibiotics, which might be the reason for carbapenem-resistance isolates.

Antimicrobial Susceptibilities in CREC Isolates

The MIC values of the 11 tested antibiotics against the 3 isolates of $bla_{\text{NDM-1}}$-carrying CREC are shown in Table 1. The 3 isolates were resistant to imipenem, meropenem, and doripenem with high MIC values ranging from 16 to 64 µg/mL. These isolates were resistant to all tested fluoroquinolones, ciprofloxacin (32 µg/mL), and levofloxacin (64 and 128 µg/mL), while sitafloxacin resistance (8 µg/mL) was found in 2 isolates. The low level of colistin resistance was also observed in all isolates. In addition, resistance to gentamicin and fosfomycin was detected in 2 (16 and 32 µg/mL) and 1 (256 µg/mL) isolates, respectively. However, all isolates were still susceptible to tigecycline and amikacin.

Synergistic Effects Against CREC Isolates

The synergy study showed that synergistic effects of fosfomycin plus gentamicin, amikacin, ciprofloxacin, sitafloxacin, colistin, or tigecycline were observed in 1 isolate, while there was no difference in the combination of fosfomycin with imipenem, meropenem, doripenem, and levofloxacin in any isolates (Table 2). The antagonistic effect of fosfomycin in combination with sulbactam was seen in 1 isolate (Table 2).

Table 1 Minimum Inhibitory Concentration (MIC) Values of 11 Antibiotics and 1 β-Lactamase Inhibitor Against $bla_{\text{NDM-1}}$-Harboring CREC Isolates

Isolate Code	MIC Value (µg/mL)											
	FOS	IPM	MEM	DOR	CIP	LVX	TIG	STFX	CT	GN	AMK	SUL
CREC003	0.25 (S)	32 (R)	64 (R)	16 (R)	128 (R)	32 (R)	0.5 (S)	8 (R)	4 (R)	32 (R)	2 (S)	128
CREC004	0.5 (S)	16 (R)	32 (R)	32 (R)	64 (R)	32 (R)	0.5 (S)	4 (I)	4 (R)	16 (R)	2 (S)	128
CREC038	256 (R)	32 (R)	64 (R)	32 (R)	128 (R)	32 (R)	1 (S)	8 (R)	4 (R)	1 (S)	2 (S)	128

Abbreviations: FOS, fosfomycin; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; DOR, doripenem; LVX, levofloxacin; SUL, sulbactam; TIG, tigecycline; STFX, sitafloxacin; CT, colistin; GN, gentamicin; AMP, amikacin; R, resistance; I, intermediate resistance; S, susceptible.

Table 2 The Synergistic Effects of Fosfomycin in Combination with Other Antimicrobial Agents Against *bla*$_{NDM-1}$-Harboring CREC Isolates

Combination	No. of Isolates		
	Synergy	Indifference	Antagonistic
Fosfomycin + Imipenem	0	3	0
Fosfomycin + Meropenem	0	3	0
Fosfomycin + Doripenem	0	3	0
Fosfomycin + Gentamicin	1	2	0
Fosfomycin + Amikacin	1	2	0
Fosfomycin + Ciprofloxacin	1	2	0
Fosfomycin + Levofloxacin	0	3	0
Fosfomycin + Sitafloxacin	1	2	0
Fosfomycin + Sulbactam	0	2	1
Fosfomycin + Colistin	1	2	0
Fosfomycin + Tigecycline	1	2	0

Sequence Types (STs) and Antimicrobial Resistance (AMR) Genes in CREC Isolates

In the MLST analysis, CREC003 and CREC004 were identified in ST448, while CREC048 was identified in ST131, as shown in Table S1. The study found that the *bla*$_{NDM-1}$ gene was present in the 3 isolates noted previously. Other β-lactam resistance genes, *bla*$_{TEM-1C}$, *bla*$_{TEM-57}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, and *bla*$_{CMY-2}$, were also found in these CREC isolates. Additionally, the CREC isolates carried other AMR genes, conferring resistance to aminoglycosides (*aac(3)-IId, aph (3")-Ib, aph(6)-Id, aadA5, aadA16*, and *aac(6')-Ib-cr*), fluoroquinolone (*qnrB6*), rifampicin (*ARR-3*), trimethoprim (*dfrA17* and *dfrA27*), sulfonamide (*sul1* and *sul2*), tetracycline (*tet*(A)), and macrolide (*mph*(A)). The distribution of AMR genes is illustrated in Figure 1 and Table S2.

Mobile Genetic Elements (MGEs) in CREC Isolates

Various mobile genetic elements (MGEs) were identified in the *bla*$_{NDM-1}$-harboring CREC isolates, as shown in Figure 2 and Tables S3–S5. For plasmids, we found IncFIA, IncFII(K), and IncN2 plasmids in all isolates. The IncA/C2, IncFIB (AP001918), IncFII (pAMA1167-NDM-5), IncQ1, and IncX4 plasmids were identified in only the ST448 isolates, while the IncFII (pRSB107) plasmid was only observed in the ST131 isolate.

Many insertion sequences (ISs), namely IS*102*, IS*1222*, IS*2*, IS*200F*, IS*4*, IS*5*, IS*903*, IS*Ec31*, IS*Kpn26*, IS*Lad1*, IS*Sen4*, and IS*Yen3,* were also detected in all CREC isolates. IS*Ec17*, IS*Ec27*, IS*Ec44*, IS*Ech5*, and IS*Kpn43* were only

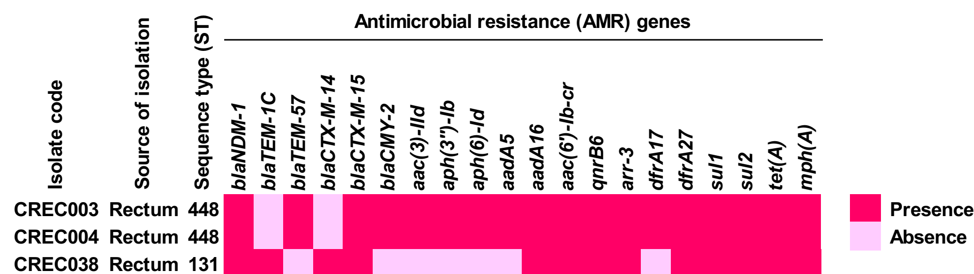

Figure 1 Distribution of antimicrobial resistance (AMR) genes in *bla*$_{NDM-1}$-harboring CREC isolates.

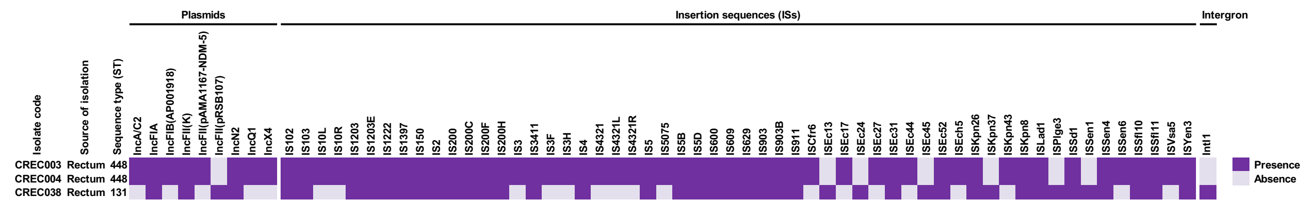

Figure 2 Distribution of mobile genetic elements (MGEs) in *bla*$_{NDM-1}$-harboring CREC isolates.

found in the ST448 isolates, while IS*Ec13*, IS*Ec24*, IS*Ec45*, IS*Kpn37*, IS*Plge3*, and IS*Sen1* were only identified in the ST131 isolate. For integrons, a class 1 integron with the carriage of *aac(6)-Ib* gene responsible for aminoglycoside resistance was detected in the ST131 isolate.

Virulence-Associated Genes in CREC Isolates

Besides the identification of AMR genes, we also looked for virulence-associated genes and the results are shown in Figure 3 and Table S6. We found the *fyuA, gad, irp2, terC*, and *traT* genes in all 3 *bla*$_{NDM-1}$-harboring CREC isolates, while the *afaA, afaC, afaD, lpfA*, and *nfaE* genes were only detected in the ST448 isolates, and the *chuA, yfcV, iss, iha, ompT, iucC, iutA, sitA, kpsE, kpsMII_K5, papA_F43, sat*, and *usp* genes were only found in the ST131 isolate.

CRISPR-Cas Region in CREC Isolates

In the analysis of the CRISPR-Cas system, 5 to 7 CRISPR positions were found in 3 isolates of *bla*$_{NDM-1}$-harboring CREC. The CRISPR positions contained 1 to 18 spacers. Additionally, 2 positions of cas locus was also identified in all ST448 isolates, which were further classified as CRISPR-Cas type-IF and type-IE. The details of the CRISPR-Cas region are provided in Tables 3 and S7.

Bacteriophage Genome in CREC Isolates

We found bacteriophage genomes in these isolates, as shown in Figure 4 and Table S8. PHAGE_Entero_mEp460 genome was found in all CREC isolates. Two bacteriophage genomes, PHAGE_Entero_cdtI and PHAGE_Klebsi_4LV2017, were only detected in the ST448 isolates, while 4 bacteriophage genomes, PHAGE_Escher_TL_2011b, PHAGE_Entero_BP_4795, PHAGE_Entero_P88, and PHAGE_Pectob_ZF40, were only found in the ST131 isolate.

Bacteriocin Class in CREC Isolates

The results of bacteriocins are shown in Figure 5 and Table S9. We found that 3 genes, encoding bottromycin, sactipeptides, and colicin, were detected in the ST131 isolate, while only bottromycin was identified in the ST448 isolates.

bla$_{NDM-1}$-Bearing IncN Plasmid in CREC Isolates

All 3 *E. coli* isolates carried the *bla*$_{NDM-1}$ gene on a plasmid (pSK20-NDM1, pSK21-NDM1, and pSK22-NDM1) belonging to the IncN2 incompatibility group. The structure of the *bla*$_{NDM-1}$-bearing IncN2 plasmid is shown in Figure 6.

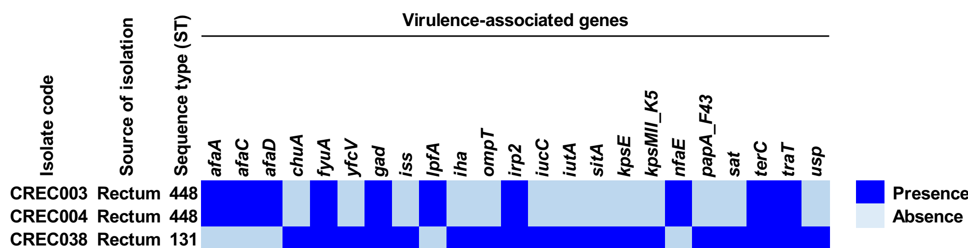

Figure 3 Distribution of virulence-associated genes in *bla*$_{NDM-1}$-harboring CREC isolates.

Table 3 CRISPR-Cas System in bla_{NDM-1}-Harboring CREC Isolates

Isolate Code	Source of Isolation	Sequence Type (ST)	CRISPR Region			Cas Region		
			CRISPR Position	No. of Direct Repeat (DR)	No. of Spacer	Cas Position	*cas* Locus	Cas Type
CREC003	Rectum	448	Position 1	2	1	Position 1	*cas6_csy3_csy2_csy1_cas3-cas2_cas1*	Cas type-IF
			Position 2	19	18	Position 2	*cas2_cas1_cas6_cas5_cas7_cse2_cse1_cas3*	Cas type-IE
			Position 3	14	13			
			Position 4	2	1			
			Position 5	12	11			
			Position 6	10	9			
			Position 7	2	1			
CREC004	Rectum	448	Position 1	14	13	Position 1	*cas1_cas3-cas2_csy1_csy2_csy3_cas6*	Cas type-IF
			Position 2	19	18	Position 2	*cas2_cas1_cas6_cas5_cas7_cse2_cse1_cas3*	Cas type-IE
			Position 3	2	1			
			Position 4	12	11			
			Position 5	10	9			
			Position 6	2	1			
			Position 7	2	1			
CREC038	Rectum	131	Position 1	2	1	-	-	-
			Position 2	2	1			
			Position 3	5	4			
			Position 4	7	6			
			Position 5	8	7			

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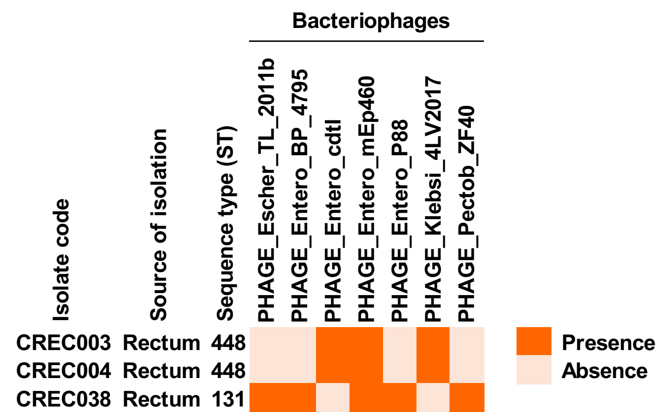

Figure 4 Presence of bacteriophage genomes in *bla*$_{NDM-1}$-harboring CREC isolates.

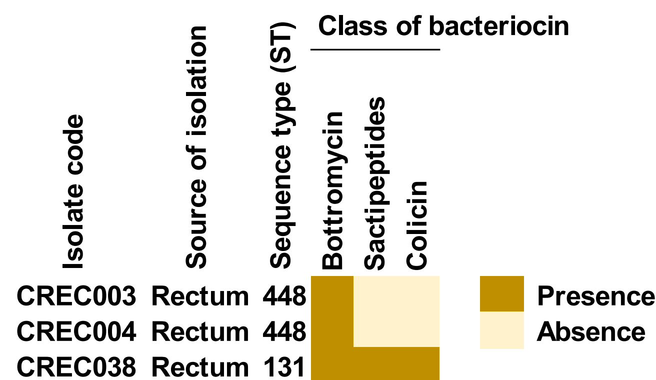

Figure 5 Presence of bacteriocin-encoding genes in *bla*$_{NDM-1}$-harboring CREC isolates.

The pSK20-NDM1, pSK21-NDM1, and pSK22-NDM1 plasmids were closely similar, all having a length of 41,190 bp. All of the IncN2 plasmids had the same backbone, consisting of genes encoding for plasmid stability (*stbA, stbB*, and *stbC*), replication (*repA*), and conjugal transfer (*tra*). Only one antibiotic resistance gene was found, *bla*$_{NDM-1}$. The genetic environment of *bla*$_{NDM-1}$ shares a single conserved region, which was a Tn*3*-like structure, IS*Aba125*, IS*Ec33, bla*$_{NDM-1}$, *bla*$_{MBL}$, ΔtnpF, IS*Sen4*, and Tn*5403*. The *bla*$_{NDM-1}$ gene was bracketed by two insertion sequences (IS), IS*Ec33* and IS*Sen4*, which belong to the IS*630* and IS*3* families, respectively. The upstream IS*Aba125* was interrupted by the insertion of IS*Ec33* but not the part of the promoter involved in the expression of *bla*$_{NDM-1}$. The sequence analysis revealed that the accessory module was inserted into the IncN2 plasmid between the *fipA* and *nuc* genes.

Pan-Genome and Phylogenetic Tree of CREC Isolates

The results of the pan-genome analysis are shown in Figure 7, while the phylogenetic tree is visualized in Figure S1. From 26,093 genes recorded in our study, 23,331 (89.41%) and 2,762 (10.59%) genes were identified as accessory and core genes, respectively. In comparison to previously published genomes, the CREC003 and CREC004 isolates (ST448) could be grouped in the same clade, while the ST131 isolate (CREC038) was located in a different clade (Figures 7 and S1). The ST448 isolates contained the *ymfD, ymfE*, and *nfaA* genes encoding the e14 prophage with SAM-dependent methyltransferase, the e14 prophage with inner membrane protein, and the non-fimbrial adhesin 1, respectively. The *flhC_1, mntH_2, alba, moaA_2*, and *csoR* genes encoding the flagellar transcriptional regulator FlhC, the divalent metal cation transporter MntH, the antilisterial bacteriocin subtilosin biosynthesis protein AlbA, the GTP 3',8-cyclase, and the copper-sensing transcriptional repressor CsoR were present as important features in ST131 isolate.

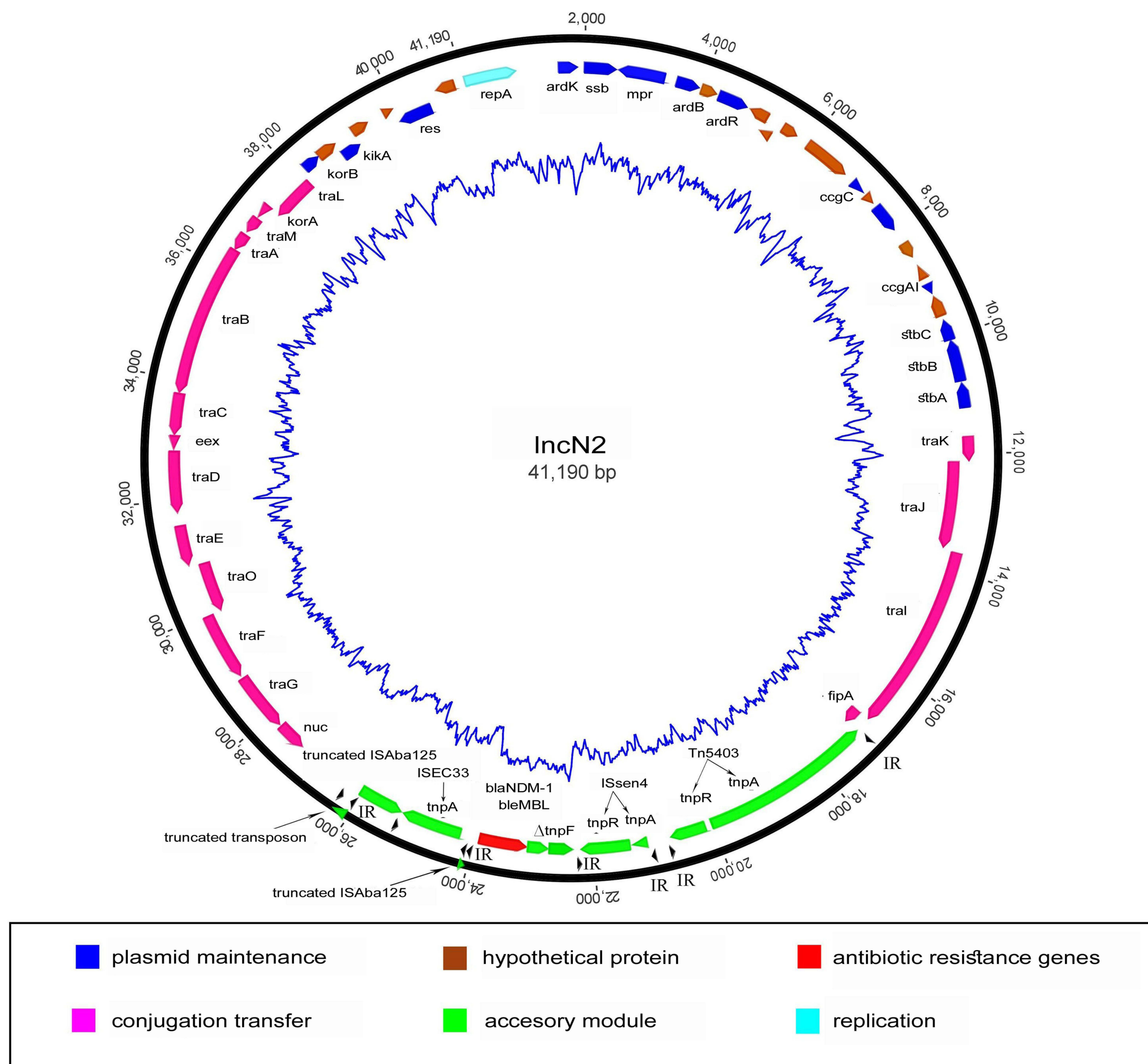

Figure 6 Structure of IncN2 plasmid in *bla*$_{NDM-1}$-harboring CREC isolates.

Discussion

In 2019, the Centers for Disease Control and Prevention (CDC) considered the infection caused by CRE as an urgent threat, due to the high level of estimated cases in hospitalized patients, mortality rates, and attributable health-care costs. CRE, particularly CREC, developed resistance mechanisms for many antibiotic classes such as carbapenems, aminoglycosides, and fluoroquinolones.[33,34] Here, we report on the synergistic effects of fosfomycin in combination with other antimicrobial agents against *bla*$_{NDM-1}$-harboring CREC isolates as well as the genomic characteristics of these pathogens. According to the MIC results, we found that all 3 *bla*$_{NDM-1}$-harboring CREC isolates were resistant to more than 3 antibiotic classes, indicating multidrug resistance in these isolates. Although they were susceptible to tigecycline and amikacin, the use of these antibiotics as monotherapy still needs to be of concern since the target bacteria can develop resistance mechanisms during therapy.[35] We then evaluated the synergistic activity of antibiotic combination for combating these pathogens. In the synergism study, we found that the use of fosfomycin combined with aminoglycosides, colistin, tigecycline, and sitafloxacin provided synergistic effects against the ST131 isolate of the *bla*$_{NDM-1}$-harboring CREC. Similar to previous study, Cebrero-Cangueiro et al (2021) found that fosfomycin plus gentamicin showed a synergistic effect against carbapenemase-producing Enterobacteriaceae

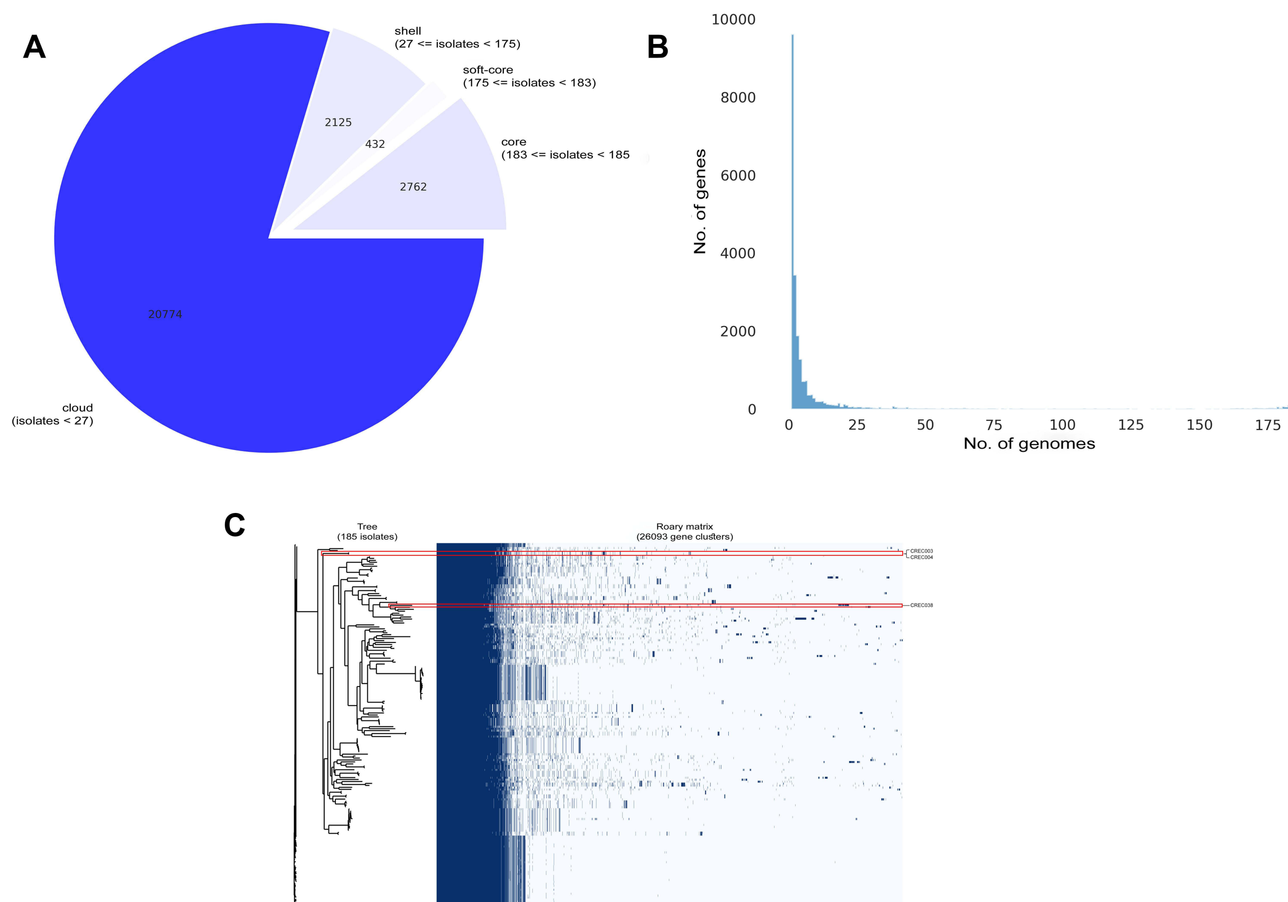

Figure 7 Genomic diversity among *bla*$_{NDM-1}$-harboring CREC isolates compared to previously published genomes.
Notes: (**A**) A pie chart of the pan-genome, (**B**) a pan-genome frequency plot, and (**C**) a presence and absence matrix against a phylogenetic tree.

(CPE).[36] The synergistic effects of fosfomycin plus colistin and tigecycline have been also reported in approximately 20% of the carbapenem-resistant *K. pneumoniae* (CRKP).[10] The combination of fosfomycin with ciprofloxacin showed the synergistic effect against only 1 isolate of the ST448 in our study. The benefit of combination therapy is to suppress the emerging resistance mechanisms that probably occur during the treatment.[37]

In WGS analysis, 2 and 1 isolates of *bla*$_{NDM-1}$-harboring CREC were identified in ST448 and ST131, respectively. These STs have been previously identified as an internationally spread clone.[38,39] The *E. coli* ST131 clone has been reported as the major clone causing human infections and is predominantly found in Southeast Asia.[40] The previous study has been demonstrated that the majority of this strain carried the *bla*$_{KPC}$ gene which commonly produces *Klebsiella pneumoniae* carbapenemase (KPC) and this gene is easily transferred to another bacteria through the MGEs. Thus, it raises the concern about the increase of carbapenem-resistant strains.[41] In AMR detection, we found that the CREC isolates carried many AMR genes conferring resistance to carbapenems, third-generation cephalosporins, other β-lactams, aminoglycosides, fluoroquinolones, rifampicin, trimethoprim, sulfisoxazole, tetracycline, and macrolides. These isolates have also been classified as multidrug-resistant (MDR) isolates, which was in concordance with our MIC results. In Germany, Welker et al (2020) reported that *E. coli* ST131 carried both carbapenem and other β-lactam resistance genes.[42] In South Korea, Shin et al (2021) also demonstrated that 23 AMR genes, especially the *bla*$_{NDM}$ gene, were present in *E. coli* isolates.[43] Here, we also assessed the correlation among the antimicrobial susceptibility, synergistic activity of fosfomycin-based combination, and WGS results. The combinations of fosfomycin with aminoglycosides were synergy in the ST131 isolate having low MIC values of gentamicin and amikacin, which carried a lower number of aminoglycoside resistance genes than the ST448 isolates having a high MIC value of gentamicin (Tables 1 and 2, Figure 1).

In our study, MGEs were also investigated to assess the dissemination mechanisms of the AMR genes. A previous study reported the presence of 16 replicon types of bla_{NDM-1}-bearing plasmids in Enterobacteriaceae, namely ColE, IncA/C, IncN2, IncFIA, IncFIB, IncFII, IncHI1, IncHI3, IncHIB, IncL/M, IncP, IncR, IncT, IncX1, IncX3, and IncY.[3] In this study, IncFIA, IncF(II)K, and IncN2 plasmids were detected in all CREC isolates, while the bla_{NDM-1} gene was identified in the IncN2 plasmid. The bla_{NDM-1}-bearing IncN2 plasmid shared >99% nucleotide identity and 100% query coverage with pJN24NDM1 (GenBank accession no. MK368725), pC2972-5-NDM (GenBank accession no. CP039806), pTR3 (GenBank accession no. JQ349086), and pNDM-ECS01 (GenBank accession no. KJ413946), according to the BLAST search. The pJN24NDM1 is an IncN2 plasmid first identified in an *E. coli* ST405 isolate from the abdominal fluid of a patient in China,[44] while the pC2972-5-NDM and pTR3 plasmids have been reported in *K. pneumoniae* isolates from China[45] and Singapore,[46] respectively. These highly similar plasmids detected from different geographic areas and bacterial species can be assumed to spread by lateral dissemination.[47] The first bla_{NDM-1}-bearing IncN2 was identified in NDM-1-producing *E. coli*, which was isolated from a patient hospitalized in Bangladesh.[48] In 2014, bla_{NDM-1}-bearing IncN2 (pNDM-ECS01) was also detected in *E. coli* ST131 from Central Thailand,[49] similar to our study. This could imply that the *E. coli* ST131 strain might be widely disseminated throughout Thailand. The IncN2 plasmid has the potential to horizontally transfer the bla_{NDM} gene to other pathogenic bacteria, potential worldwide dissemination of the bla_{NDM} gene. In addition, a previous study demonstrated that the bla_{NDM-1} gene located in transposon Tn*125* was responsible for the bla_{NDM-1} spread among *Acinetobacter* spp.[7] The structure of the Tn*125* prototype includes IS*Aba125,* bla_{NDM-1}, ble_{MBL}, Δ*trpF, dsbC, cutA*, Δ*groES, groEL*, IS*CR27*, and IS*Aba125*. In a comparative analysis with the Tn*125* prototype, the Tn*125*-like element in plasmids from this study lacked *dsbC, cutA*, Δ*groES, groEL*, IS*CR27*, and downstream IS*Aba125*.[50] The sequences revealed that the accessory module was located between the *fipA* and *nuc* genes. We hypothesize that these two regions (between *fipA* and *nuc* genes) probably act as the main integration sites for the insertion of transposable elements within IncN plasmids. Same site insertion for the accessory module has also been reported in other IncN plasmids, pKT58A (GenBank accession no. JX065631) and pRSB206 (GenBank accession no. JN102344).[51,52] Thus, this site might act as a significant integration site in IncN2 plasmids. Currently, IncN plasmids have been found all over the world with the highest prevalences in China and the United States.[44]

Furthermore, many ISs were present in these isolates, which are components of transposons providing a cut-and-paste mechanism.[53] As shown in Figure 6, IS*Ec33* and IS*Sen4* bracketed the bla_{NDM-1} on IncN2 plasmid. This structure was the same as the previously reported p271A plasmid, which was isolated from *E. coli* ST101.[54] Notably, we found a class 1 integron in the ST131 isolate, which also provides the ability to drive AMR genes to other bacteria.

To check the pathogenicity of these CREC isolates, the gene encoding siderophore receptor (*fyuA*), glutamate decarboxylase (*gad*), high molecular weight protein 2 non-ribosomal peptide synthetase (*irp2*), tellurium ion resistance protein (*terC*), and outer membrane protein complement resistance (*traT*) were identified in all CREC isolates. Most virulence genes can also be horizontally transferred to other bacteria through MGEs, similar to AMR genes.[55]

In the study of bacterial adaptive immunity system against foreign genetic elements, we found CRISPR-Cas systems in all CREC isolates. These CRISPR-Cas systems contained many spacers, indicating that these isolates may have been previously infected with bacteriophages or invaded by foreign DNA fragments. Bacteriophage genomes were also detected in all CREC isolates. The partial genome of *Escherichia* phage was identified in the ST131 isolate, while 4 partial genomes of *Enterobacteria* phage were present in the ST131 and/or ST448 isolates. Notably, we found the genomes of the *Klebsiella* phage in the ST448 isolates and *Pectobacterium* phage in the ST131 isolate. This may indicate that these CREC isolates had been priorly infected by bacteriophages with the lysogenic life cycle.[56] Also, we hypothesize that these partial phage genomes might carry the AMR genes, as described in a previous study.[57] To prove this phenomenon, long-read WGS is needed to elucidated further.

In the investigation of gene encoding bacteriocins, bottromycin was found in all isolates, while sactipeptides and colicin were only detected in the ST131 isolate. Bottromycin has been previously detected in *E. coli* isolated from the intestine of humans.[58] It commonly provides a natural antimicrobial activity, particularly the modified bottromycin A2 which provides activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).[59]

In our study of the genomic diversity of our bla_{NDM-1}-harboring CREC isolates compared to other published genomes, we found that the specific features of the ST448 and ST131 isolates were different from various STs. In ST488 isolates, the prophage-encoding proteins may be associated with bacterial adaptation to the bacteriophages,[60,61] while non-fimbrial adhesin 1 is probably associated with cell attachment to abiotic surfaces and/or host cells, and the biofilm formation.[62] For the ST131 isolate, the FlhC, MntH, AlbA, and CsoR proteins are probably related to the production of flagellar, the transportation of divalent metal cation, the production of antilisterial bacteriocin subtilosin, and the expression of a copper-sensing repressor, which might be beneficial for bacterial evolution and pathogenicity.[63–66]

Conclusion

The combinations of fosfomycin with aminoglycosides, colistin, tigecycline, and sitafloxacin might be considered as a treatment of infections caused by ST131 carrying the bla_{NDM-1} gene. Our study of the whole-genome and plasmid sequences of the bla_{NDM-1}-harboring CREC isolates provides significant information, especially the presence of AMR genes, MGEs, virulence genes, genes involving bacterial defense mechanisms, and the structure of bla_{NDM-1}-bearing IncN2 plasmid. These particular isolates contained specific features compared to some published genomes, which are probably associated with bacterial adaptation. The findings can be expected to be useful in the development of appropriate treatments and the control of these infections in the future.

Ethical Approval

This study was approved by the Human Research Ethics Committee (HREC), Faculty of Medicine, Prince of Songkla University (Reference Number: 59-352-14-1). Also, the researchers were granted permission to extract the data from the database with waiver of consent because of the observational nature of the study. All data were fully anonymized before the researcher accessed and analyzed.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest.

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