Synergistic Effects of Meropenem in Combination with Aminoglycosides against Carbapenem-Resistant *Escherichia coli* Harboring Extended-Spectrum Beta-Lactamase and Carbapenemase Genes

Pawarisa Terbtothakun

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Microbiology (International Program)

Prince of Songkla University

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ABSTRACT

Infections due to carbapenem-resistant *Escherichia coli* (CREC) are problematic due to limitation in treatment options. Combination therapies of existing antimicrobial agents have become a reliable strategy to control these infections. In this study, the synergistic effects of meropenem in combination with aminoglycosides were assessed by checkerboard and time-kill assays. Of the 35 isolates, 19 isolates (54.3%) were resistant to carbapenems (imipenem and meropenem) with the MIC ranges from 16 to 128 µg/mL. These isolates were resistant to almost all antibiotic classes. Molecular characteristics revealed co-harboring of carbapenemase (*bla*$_{\text{NDM-1}}$, *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{OXA-48}}$) and extended-spectrum β-lactamases (ESBL) genes (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$). The checkerboard assay displayed synergistic effects of meropenem and several aminoglycosides against most CREC isolates. Time-kill assays further demonstrated strong synergistic effects of meropenem in combination with either amikacin, gentamicin, kanamycin, streptomycin, and tobramycin. The results suggested that meropenem in combination with aminoglycoside therapy might be an efficient optional treatment for infections cause by CREC.

Keywords: aminoglycosides, antibiotic synergism, carbapenem-resistant *Escherichia coli*, combination therapy, extended-spectrum β-lactamase genes.

ชื่อวิทยานิพนธ์ ประสิทธิภาพการเสริมฤทธิ์กันของยา meropenem ในการใช้ร่วมกับยากลุ่ม aminoglycosides ต้านเชื้อ carbapenem-resistant *Escherichia coli* ที่มียีน extended-spectrum beta-lactamase และยีน carbapenemase

ผู้เขียน นางสาวปวริศา เติบโตฐากุล

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บทคัดย่อ

การติดเชื้อ carbapenem-resistant *Escherichia coli* (CREC) เป็นปัญหาสำคัญ เนื่องจากมีทางเลือกในการรักษาที่จำกัด การรักษาแบบผสมผสานโดยการใช้ยาที่มีอยู่ในปัจจุบัน จึงเป็นอีกทางเลือกหนึ่งในการควบคลุมการติดเชื้อ การศึกษาครั้งนี้ ผลเสริมฤทธิ์ของยา meropenem ร่วมกับยากลุ่ม aminoglycosides ได้รับการประเมินโดยวิธี checkerboard และ time-kill เชื้อ *Escherichia coli* จำนวน 35 ไอโซเลท พบว่าดื้อต่อยากลุ่ม carbapenems (imipenem และ meropenem) 19 ไอโซเลท (54.3%) ด้วยช่วงของการดื้อยา 16-128 μg/mL นอกจากนี้ ยังพบการดื้อยาปฏิชีวนะในกลุ่มอื่นๆอีกด้วย การศึกษาระดับโมเลกุลชี้ให้เห็นว่ามีการแสดงออกร่วมกันของยีนในกลุ่ม carbapenemase ($bla_{\text{NDM-1}}$, $bla_{\text{NDM-5}}$ และ $bla_{\text{OXA-48}}$) ร่วมกับยีนกลุ่ม extended-spectrum β-lactamases ($bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM}) วิธี checkerboard แสดงผลการเสริมฤทธิ์กันของยา meropenem และยากลุ่ม aminoglycosides ต้านเชื้อ CREC วิธี time-kill สนับสนุนและยืนยันผลการเสริมฤทธิ์ของยา meropenem ร่วมกับยา amikacin, gentamicin, kanamycin, streptomycin, และยา tobramycin ผลการทดสอบชี้ให้เห็นว่าการใช้ยา meropenem รักษาร่วมกับยากลุ่ม aminoglycosides อาจเป็นอีกทางเลือกหนึ่งที่มีประสิทธิภาพในการรักษาการติดเชื้อที่มีสาเหตุมาจากเชื้อ CREC

คำสำคัญ: อะมิโนไกลโคไซด์, การเสริมฤทธิ์กันของยาปฏิชีวนะ, เชื้อ *Escherichia coli* ที่ดื้อต่อยากลุ่มคาร์บาพีเนม, การรักษาแบบผสมผสาน, ยีน extended-spectrum β-lactamase

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Pawarisa Terbtothakun

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LIST OF ABBREVIATIONS

%	percentage
℃	degree Celsius
µl	microliter
AACs	acetyltransferases
ABC	ATP-binding cassette superfamilies
ANTs	nucleotidyltransferase
APHs	phosphotransferases
CRE	carbapenem-resistant *Enterobacteriaceae*
CREC	carbapenem-resistant *Escherichia coli*
CLSI	Clinical Laboratory Standards Institute
DNA	deoxyribonucleic acid
ESBL	extended-spectrum β-lactamase
IMP	imipenemase
KPC	*Klebsiella pneumoniae* carbapenemase
MATE	multidrug and toxic compound extrusion
MDR	multi-drug resistant
MRSA	methicillin-resistant *Staphylococcus aureus*
MFS	major facilitator superfamily
mL	milliliter
NDM	New Delhi metallo-β-lactamases

OXA	oxacillinases
PBPs	penicillin-binding proteins
PCR	polymerase chain reaction
RND	resistance nodulation division
rRNA	ribosomal ribonucleic acid
SMR	small multidrug resistance
VIM	verona integrin-encoded metallo-β-lactamase

LIST OF PUBLICATIONS

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Synergistic Antibacterial Effects of Meropenem in Combination with Aminoglycosides against Carbapenem-Resistant *Escherichia coli* Harboring bla_{NDM-1} and bla_{NDM-5}

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Abstract: Infections due to carbapenem-resistant *Escherichia coli* (CREC) are problematic due to limitation in treatment options. Combination therapies of existing antimicrobial agents have become a reliable strategy to control these infections. In this study, the synergistic effects of meropenem in combination with aminoglycosides were assessed by checkerboard and time-kill assays. Of the 35 isolates, 19 isolates (54.3%) were resistant to carbapenems (imipenem and meropenem) with the MIC ranges from 16 to 128 µg/mL. These isolates were resistant to almost all antibiotic classes. Molecular characteristics revealed co-harboring of carbapenemase (bla_{NDM-1}, bla_{NDM-5} and bla_{OXA-48}) and extended-spectrum β-lactamases (ESBL) genes (bla_{CTX-M}, bla_{SHV} and bla_{TEM}). The checkerboard assay displayed synergistic effects of meropenem and several aminoglycosides against most CREC isolates. Time-kill assays further demonstrated strong synergistic effects of meropenem in combination with either amikacin, gentamicin, kanamycin, streptomycin, and tobramycin. The results suggested that meropenem in combination with aminoglycoside therapy might be an efficient optional treatment for infections cause by CREC.

Keywords: aminoglycosides; antibiotic synergism; carbapenem-resistant *Escherichia coli*; combination therapy; ESBL genes

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1. Introduction

Infections due to carbapenem-resistant *Escherichia coli* (CREC), particularly the New Delhi metallo-β-lactamases (NDM)-producing isolates, are critically problematic to global health care [1]. These infections usually yield unfavorable clinical outcomes, prolonged length of hospitalization and high hospital costs [2]. The national antimicrobial resistance surveillance data reported by the Thailand National Institute of Health (2016–2018), indicated a high prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) (93%) among hospitalized patients in Thailand [3]. In the past, carbapenems were the most reliable antimicrobial agents against hospital-acquired infections caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* [4]. However, extensive usage as both empirical and definitive regimens [5], resulted in the emergence of CRE [4].

Enterobacteriaceae resistance to carbapenems is mainly associated with the production of several kinds of carbapenemases, which are enzymes capable of hydrolyzing carbapenems and other β-lactams [6]. In addition, the lack of porin proteins by alteration in the permeability of the bacterial cell membrane, and overexpression of efflux pumps are additive carbapenem resistance mechanisms [7]. Numerous epidemiological studies have suggested

that the acquisition of carbapenemase-encoding genes might lead to a rapid outbreak mostly in the hospital-setting and sometimes in the community-setting [8–10]. Moreover, the specific class of the carbapenemase should be considered during the development of novel antimicrobial agents as each class possesses a unique mechanism and spectrum of activity [11]. Previous studies have reported that ceftazidime-avibactam binds reversibly to class A, C, and some D β-lactamases [12,13], whereas imipenem-cilastatin-relebactam and meropenem-vaborbactam reversibly and competitively inhibited class A and C β-lactamases [14,15]. However, these antibiotics did not inhibit metallo-β-lactamases such as NDM carbapenemases [12,14,15]. Globally, the predominant carbapenemases include NDM, *Klebsiella pneumoniae* carbapenemase (KPC), Verona integrin-encoded metallo-β-lactamase (VIM), imipenemase (IMP), and oxacillinases (OXA)-type enzymes, which are encoded by bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, and bla_{OXA} genes, respectively [6]. However, bla_{NDM} has gained relevance due to the high-level of resistance to many clinically available β-lactams and ease of horizontal transfer between different isolates. To date, several variants of NDM enzymes have been identified [16] with amino acid substitutions at different positions. NDM-5 differed from NDM-1 by substitutions at positions 88 (Val→Leu) and 154 (Met→Leu), and several studies have showed that $bla_{\mathrm{NDM\text{-}5}}$ is carried by conjugatable IncX3 plasmids responsible for the rapid spread [17–19].

Currently, therapeutic options for the management of infections caused by CREC are limited [20]. Moreover, the development of new antimicrobial agents are costly, time-consuming, and require various stages of toxicological evaluations to ensure safety [11]. Hence, combining existing antimicrobial agents has become a strategy against several kinds of infections caused by multi-drug resistant (MDR) organisms [21]. Previous studies have supported the use of combination therapy as an effective treatment option for infections caused by several MDR Gram-negative bacteria [22–24]. A recent study demonstrated the synergistic effect of meropenem and aminoglycosides against KPC-2 and NDM-1-producing carbapenem-resistant *Klebsiella pneumoniae* [25]. Additionally, the ability of meropenem to potentiate aminoglycoside activity, largely dependent on the MexXY-OprM multidrug efflux system, has been shown [26]. However, data for combinations between meropenem and several aminoglycosides against CREC harboring bla_{NDM} genes is lacking. This study evaluated the effects of meropenem in combination with several commonly used aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin, and tobramycin) on CREC isolates harboring bla_{NDM} genes.

2. Results and Discussion

2.1. Bacterial Isolates

A total of 35 suspected CREC isolates were collected from eight hospitals located in Southern Thailand. The isolates were obtained from various clinical specimens, including blood (n = 11), rectal (n = 19), throat (n = 3) and environment (n = 2). Data of isolates and antimicrobial response to imipenem and meropenem are shown in Supplementary Materials Table S1. The results indicated that 19 isolates were resistant to carbapenems. Demographic information, clinical data and outcomes of the patients infected with CREC are presented in Table S2. Similar to previous reports of risk factors associated with CRE acquisition or infection [27,28], most of patients in this study had previous exposure to various antimicrobial agents, particularly carbapenems. The results support previous observation that exposure to antibiotics including β-lactams such as carbapenems and cephalosporins, as well as fluoroquinolones were associated with CRE [23]. Patient information indicated that most of the patients were admitted in intensive care units (ICU), which are in consonance with observations of a previous study that showed high prevalence of carbapenemase producing Enterobacteriaceae in the ICU [29].

2.2. The Antibiogram of Carbapenem-Resistant E. coli Isolates

The susceptibility profile of CREC isolates was evaluated against 15 conventional antibiotics including carbapenems (imipenem and meropenem), aminoglycosides (amikacin,

gentamicin, kanamycin, streptomycin, and tobramycin), cefoperazone-sulbactam, ceftolozane-tazobactam, colistin, cephalosporins (cefotaxime and ceftazidime), fosfomycin, and glycylcyclines (minocycline and tigecycline). The MICs of antibiotics except carbapenems and aminoglycosides were recorded in Table S3 and summarized in Table 1. The results suggested that three antibiotics including colistin, fosfomycin, and amikacin were effective against CREC isolates, with percentage efficacy of 100%, 89.47% and 73.7%, respectively.

To date, polymyxins, fosfomycin, aminoglycosides, and tigecycline are considered choice drugs for the management of infections caused by carbapenem-resistant Gram-negative bacteria [30]. However, resistance to these antibiotics is increasing rapidly with high chance of toxicity due to the relative high doses required for monotherapy medications. Results of this study revealed that approximately 79% of CREC isolates were resistant to tigecycline, contrary to previous reports of 0.7% and 11.2% [31,32]. In addition, the low plasma levels of tigecycline [33] constitutes a clinical concern for mono-therapeutic administration. Polymyxin on the other hand showed excellent antimicrobial effects against CREC with a 100% susceptibility. However, the nephrotoxicity and poor tissue perfusion of polymyxins [34] are limiting factors hindering extensive therapeutic usage. The rapid acquisition of resistance and sodium overload with intravenous fosfomycin [35] are also of clinical concern.

Table 1. Summary of antimicrobial susceptibility of 19 carbapenem-resistant isolates.

Antibiotics	MIC (μg/mL)			Percentage %		
	Range	MIC_{50}	MIC_{90}	Susceptible	Intermediate	Resistant
			Aminoglycoside			
Amikacin	2–> 1024	4	>1024	73.7	0	26.3
Gentamicin	1–> 1024	64	>1024	21	5.3	73.7
Kanamycin	8–> 1024	128	>1024	21	0	79
Streptomycin	16–1024	512	1024	0	0	100
Tobramycin	1–> 1024	32	>1024	5.3	10.5	84.2
			β-lactam + β-lactamase inhibitor			
Cefoperazone-sulbactam	256–> 1024	512	>1024	5.3	0	94.7
Ceftolozane-tazobactam	1024–> 1024	>1024	>1024	5.3	0	94.7
			Carbapenem			
Imipenem	16–128	64	128	0	0	100
Meropenem	32–128	128	128	0	0	100
			Cephalosporin			
Cefotaxime	256–> 1024	>1024	>1024	0	0	100
Ceftazidime	1024–> 1024	>1024	>1024	0	0	100
			Fluoroquinolone			
Ciprofloxacin	0.5–512	128	256	5.3	10.5	84.2
Levofloxacin	<0.5–64	16	32	26.3	0	73.7
			Glycylcycline			
Minocycline	<2–16	<2	16	68.4	15.8	15.8
Tigecycline	0.0625–4	2	4	21	0	79
			Other			
Colistin	0.25–2	0.5	2	100	0	0
Fosfomycin	16–1024	16	1024	89.5	0	10.5

2.3. Antimicrobial Susceptibility to Carbapenem and Aminoglycosides

The MIC of carbapenems and aminoglycosides on 19 CREC isolates were determined by the broth microdilution method (Table 2) The 19 isolates were resistant to imipenem (MIC_{50} = 64 μg/mL and MIC_{90} = 128 μg/mL), meropenem (MIC_{50} = 128 μg/mL and MIC_{90} = 128 μg/mL), and streptomycin (MIC_{50} = 512 μg/mL and MIC_{90} = 1024 μg/mL). In addition, 16 isolates were resistant to tobramycin (MIC_{50} = 32 μg/mL and MIC_{90} > 1024 μg/mL), while two isolates were intermediate. Furthermore, 14 and 15 isolates displayed resistance against gentamicin (MIC_{50} = 64 μg/mL and MIC_{90} > 1024 μg/mL) and kanamycin (MIC_{50} = 128 μg/mL and MIC_{90} > 1024 μg/mL), respectively. In contrast, amikacin showed high efficacy on 14 isolates.

Table 2. Antibacterial profile of aminoglycoside and carbapenem resistance in 19 carbapenem-resistant *Escherichia coli* isolates.

Clinical Isolate	Source	*bla* Genotype		MIC (μg/mL)						
				Carbapenem		Aminoglycoside				
		Carbapenemase	ESBL	Imipenem	Meropenem	Amikacin	Gentamicin	Kanamycin	Streptomycin	Tobramycin
CREC 1	Rectal	bla_{NDM-1}	bla_{CTX-M}, bla_{TEM}	64 (R)	64 (R)	2 (S)	64 (R)	16 (S)	1024 (R)	16 (R)
CREC 2	Rectal	bla_{NDM-1}	bla_{CTX-M}, bla_{TEM}	64 (R)	64 (R)	2 (S)	64 (R)	16 (S)	1024 (R)	8 (I)
CREC 3	Rectal	-	bla_{CTX-M}, bla_{SHV}, bla_{TEM}	128 (R)	128 (R)	64 (R)	64 (R)	64 (R)	256 (R)	128 (R)
CREC 4	Throat	bla_{NDM-5}	bla_{CTX-M}, bla_{TEM}	64 (R)	64 (R)	4 (S)	32 (R)	64 (R)	512 (R)	32 (R)
CREC 5	Rectal	bla_{NDM-5}	bla_{CTX-M}, bla_{TEM}	64 (R)	64 (R)	4 (S)	64 (R)	64 (R)	512 (R)	32 (R)
CREC 6	Rectal	bla_{NDM-5}	bla_{CTX-M}, bla_{TEM}	32 (R)	64 (R)	4 (S)	1 (S)	32 (S)	64 (R)	8 (I)
CREC 7	Throat	bla_{NDM-1}	bla_{CTX-M}, bla_{TEM}	128 (R)	128 (R)	8 (S)	128 (R)	256 (R)	512 (R)	64 (R)
CREC 8	Rectal	bla_{NDM-1}	bla_{CTX-M}, bla_{TEM}	128 (R)	128 (R)	8 (S)	128 (R)	128 (R)	16 (R)	64 (R)
CREC 9	Environment	bla_{NDM-1}	bla_{CTX-M}, bla_{TEM}	64 (R)	128 (R)	8 (S)	64 (R)	512 (R)	512 (R)	64 (R)
CREC 10	Rectal	-	bla_{TEM}	64 (R)	128 (R)	4 (S)	0.5 (S)	8 (S)	32 (R)	1 (S)
CREC 11	Blood	-	bla_{CTX-M}, bla_{TEM}	32 (R)	64 (R)	>1024 (R)	>1024 (R)	>1024 (R)	32 (R)	>1024 (R)
CREC 12	Blood	bla_{NDM-5}	bla_{CTX-M}, bla_{TEM}	64 (R)	128 (R)	>1024 (R)	>1024 (R)	>1024 (R)	32 (R)	>1024 (R)
CREC 13	Blood	bla_{NDM-5}	bla_{CTX-M}, bla_{TEM}	32 (R)	128 (R)	8 (S)	1 (S)	128 (R)	32 (R)	32 (R)
CREC 14	Blood	bla_{NDM-5}	bla_{TEM}	16 (R)	32 (R)	>1024 (R)	>1024 (R)	>1024 (R)	1024 (R)	512 (R)
CREC 15	Blood	bla_{NDM-5}	bla_{CTX-M}, bla_{TEM}	64 (R)	64 (R)	4 (S)	1 (S)	128 (R)	512 (R)	16 (R)
CREC 16	Blood	bla_{NDM-5}	bla_{CTX-M}, bla_{TEM}	64 (R)	128 (R)	2 (S)	128 (R)	128 (R)	512 (R)	16 (R)
CREC 17	Blood	bla_{NDM-5}	bla_{TEM}	64 (R)	128 (R)	4 (S)	64 (R)	64 (R)	256 (R)	16 (R)
CREC 18	Blood	bla_{NDM-1}, bla_{OXA-48}	bla_{CTX-M}, bla_{TEM}	64 (R)	128 (R)	4 (S)	64 (R)	128 (R)	256 (R)	32 (R)
CREC 19	Blood	bla_{NDM-1}	bla_{CTX-M}, bla_{TEM}	128 (R)	32 (R)	128 (R)	8 (I)	1024 (R)	512 (R)	128 (R)

R, resistant; S, susceptible; I, intermediate.

Aminoglycosides are an important class of bactericidal antibiotics that are frequently used for the treatment of severe infections caused by Gram-negative bacteria. The major resistance mechanism to aminoglycosides in Gram-negative bacteria is the production of aminoglycoside-modifying enzymes (AMEs) or the modification of ribosome by acquired 16S rRNA methyltransferases (RMTases) [36,37]. AMEs modify select to specific aminoglycosides, hence bacterial isolates show discordant susceptibility among different aminoglycosides.

A previous study demonstrated the co-occurrence of aminoglycoside and β-lactam resistance mechanisms in *E. coli* isolates [38]. In addition, co-harboring of ESBLs, carbapenemases, and 16S rRNA methylase genes within a plasmid have been noted to result in multidrug-resistance in Enterobacteriaceae [39].

2.4. Genotypic Resistance Mechanism in Carbapenem-Resistant E. coli Isolates

The 19 CREC isolates were screened for antimicrobial resistance genes including carbapenemase genes (bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, and bla_{OXA-48}) and ESBL genes (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) using PCR (Table 3). The results for carbapenemase genes, demonstrated high prevalence of bla_{NDM-1} and bla_{NDM-5}. However, bla_{OXA-48} was observed in one of the tested isolates. Furthermore, co-harboring of carbapenemase and ESBL genes were represented in almost all isolates. The results showed that six isolates with bla_{NDM-1} co-harbored bla_{CTX-M} and bla_{TEM} (Table 2). Additionally, CREC 18 carrying bla_{NDM-1} and $bla_{OXA-48,}$ co-harbored ESBL genes (bla_{CTX-M} and bla_{TEM}). bla_{NDM-5} was found in nine isolates co-harboring ESBL genes (bla_{CTX-M} and bla_{TEM}). However, two out of the nine isolates that harbored bla_{NDM-5} had only bla_{TEM}. The results further showed that three of the isolates had no carbapenemase genes but carried ESBL genes. According to the Ambler classification method, carbapenemase-produced by *Enterobacteriaceae* can be classified into three classes including class A, class B, and class D β-lactamases [6]. However, the clinical relevance of Ambler class C is still unknown [40]. The most widely spread carbapenemase in *E. coli* include class A; KPC, class B; NDM-1, NDM-5, NDM-9, and VIM, class D; OXA-48, OXA-181, and OXA-244 [41,42]. Class A, B and D β-lactamases enzymes are plasmid-mediated and are responsible for the high levels of antimicrobial resistance and rapid dissemination by horizontal transfer [43]. Epidemiological studies have revealed the diversity of carbapenemases predominate in several regions and countries [43]. In the United States, Argentina, Columbia, Greece, Israel, and Italy, KPC-producing *Enterobacteriaceae*, are mostly endemic among nosocomial isolates [1]. NDM was reported as the main carbapenemase-mediating resistance in *E. coli* isolates in India, Pakistan, and Sri Lanka, whereas OXA-48 was reported in North Africa, Malta, and Turkey [44]. NDM and OXA-48 were identified in both nosocomial and community-acquired pathogens [43,45]. A recent study done in Thailand reported a high prevalence (99%) of CREC isolates having at least one carbapenemase-producing gene (CP-gene) [3]. The most common CP-gene among CREC isolates in Thailand were bla_{NDM} (94%) and a $bla_{OXA-48-like}$ (18%) gene [3]. In this study, bla_{NDM} was found in 16 isolates, including seven isolates harboring bla_{NDM-1} and nine isolates harboring bla_{NDM-5}. Similar results were reported in a recent study with a high prevalence of NDM-1 in *E. coli* [46]. The increased usage of antibiotics maybe driving the evolution of NDM-1 variants. M154L amino acid substitution in NDM-5 was the most common substitution in all NDMs variants leading to increase carbapenemase activity [47]. However, a previous study reported that the difference in the activity of NDM-5 and NDM-1 is due to variations in the affinity for zinc [48]. Moreover, V88L amino acid substitution in NDM-5 contribute to lower catalytic activity on imipenem and meropenem [49]. Several studies showed that bla_{NDM-5} was carried by IncX3 plasmids which have been shown to be conjugatable and could explain the rapid spread of bla_{NDM-5}-carrying isolates [50]. However, bla_{KPC} which is the most commonly found in the United States [1], was not presented in this study. So far, the prevalence of bla_{KPC} in Thailand has remained very low. A previous report indicated a 0.02% (n = 12,741) prevalence of bla_{KPC-13} among *Enterobacteriaceae* and 1.7% (n = 181) among CRE isolates [51], whereas a separate

report showed that the prevalence rate of bla_{KPC-2} in CRE isolates was 0.13% (n = 2245) [52]. Furthermore, the study illustrated the co-existence of carbapenemase and ESBL genes in CREC isolates. Carbapenems were used as first-line antibiotic for treatment of infection caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. Thus, the co-harboring of multiple antibiotic resistance genes will promote multi-resistance, which might amount to significant therapeutic concerns.

Table 3. Primers used for PCR amplification of carbapenemase and ESBL genes.

Primer Name		Sequence (5′ to 3′)	Amplicon Size (bp)	Reference
		Carbapenemase		
bla_{IMP}	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232	[53]
	IMP-R	GGTTTAAYAAAACAACCACC		
bla_{KPC}	KPC-F	CGTCTAGTTCTGCTGTCTTG	798	
	KPC-R	CTTGTCATCCTTGTTAGGCG		
bla_{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTC	621	
	NDM-R	CGGAATGGCTCATCACGATC		
bla_{OXA-48}	OXA-F	GCGTGGTTAAGGATGAACAC	438	
	OXA-R	CATCAAGTTCAACCCAACCG		
bla_{VIM}	VIM-F	GATGGTGTTTGGTCGCATA	390	
	VIM-R	CGAATGCGCAGCACCAG		
		Extended-spectrum β-lactamase		
bla_{CTX-M}	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	573	[54]
	CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG		
bla_{SHV}	bla-SHV.SE	ATGCGTTATATTCGCCTGTG	747	
	bla-SHV.AS	TGCTTTGTTATTCGGGCCAA		
bla_{TEM}	TEM-164.S	TCGCCGCATACACTATTCTCAGAATGA	445	
	TEM-165.AS	ACGCTCACCGGCTCCAGATTTAT		

2.5. The Combined Effect of Meropenem and Aminoglycosides

The results of antimicrobial combinations against the 19 CREC isolates are shown in Table 4 and summarized in Table S4. Synergistic effects were observed for meropenem plus gentamicin and meropenem plus streptomycin in 16 (84.2%) isolates, followed by meropenem plus kanamycin and meropenem plus tobramycin in 15 (79%) isolates. Furthermore, synergistic activity was observed in 13 (68.4%) isolates for meropenem plus amikacin. The isolate CREC 11 (bla_{CTX-M} and bla_{TEM}), with high resistance to aminoglycosides, was resistant to all combinations, while isolate CREC 12 (bla_{NDM-5}, bla_{CTX-M} and bla_{TEM}) was susceptible to meropenem plus amikacin, or gentamicin, or streptomycin combinations. Combination of meropenem plus gentamicin and meropenem plus tobramycin exhibited synergism against CREC 14 (bla_{NDM-5} and bla_{TEM}). The cross resistance of CREC 11 to all the combinations might be due to the cumulative effects of other resistance mechanisms such as overexpression of efflux pump and/or porin with the β-lactamases leading to high level of resistance. However, the results did not reveal an antagonistic effect for the tested combinations.

The results revealed that addition of aminoglycosides as adjunctive therapy to meropenem could restore meropenem activity against CREC isolate harboring bla_{NDM}. Combination of meropenem and aminoglycosides might promote membrane disruption since aminoglycosides exert disruptive effects on the outer membrane structure by binding with the negatively charged lipopolysaccharides in the outer membrane of Gram-negative bacteria. Thus, the aminoglycoside promotes the permeabilizing effect and enhances the periplasmic target site penetration of other antibiotics such as carbapenems used in combination [55,56]. Meropenem is a safe, well-tolerated, and commonly used as monotherapy or as combination regimens for hospital-acquired infection due to several MDR Gram-negative bacteria [57–59]. Similarly, aminoglycosides are effective against Gram-negative aerobic bacteria including resistant *Enterobacteriaceae* [60]. However, aminoglycosides monothera-

pies can lead to unfavorable clinical outcomes due to rapid emergence of resistance, and nephrotoxicity among patients with prolonged usage of aminoglycosides [61,62].

Table 4. Effects of meropenem and aminoglycosides combinations on 19 carbapenem-resistant *Escherichia coli*.

Clinical Isolate	Meropenem + Amikacin		Meropenem + Gentamicin		Meropenem + Kanamycin		Meropenem + Streptomycin		Meropenem + Tobramycin	
	MIC [a]	ΣFICI	MIC [a]	ΣFICI	MIC [a]	ΣFICI	MIC [a]	ΣFICI	MIC [a]	ΣFICI
CREC 1	8/0.5	0.38 (S)	2/8	0.16 (S)	8/4	0.38 (S)	1/256	0.27 (S)	4/2	0.19 (S)
CREC 2	16/0.125	0.31 (S)	2/8	0.16 (S)	16/4	0.50 (S)	1/256	0.27 (S)	4/2	0.31 (S)
CREC 3	8/16	0.31 (S)	2/16	0.27 (S)	32/8	0.38 (S)	32/32	0.38 (S)	4/8	0.09 (S)
CREC 4	4/1	0.31 (S)	8/8	0.38 (S)	8/16	0.38 (S)	8/64	0.25 (S)	8/4	0.25 (S)
CREC 5	16/0.25	0.31 (S)	2/8	0.16 (S)	4/16	0.31 (S)	8/64	0.25 (S)	8/4	0.25 (S)
CREC 6	8/2	0.63 (I)	8/0.125	0.25 (S)	4/8	0.31 (S)	8/8	0.25 (S)	2/2	0.28 (S)
CREC 7	8/2	0.31 (S)	8/8	0.13 (S)	8/32	0.19 (S)	4/128	0.28 (S)	8/8	0.19 (S)
CREC 8	2/2	0.27 (S)	2/16	0.14 (S)	32/32	0.50 (S)	128/1	1.02 (S)	8/8	0.19 (S)
CREC 9	4/2	0.28 (S)	16/8	0.25 (S)	8/32	0.19 (S)	8/128	0.31 (S)	16/8	0.25 (S)
CREC 10	4/2	0.53 (I)	4/0.125	0.28 (S)	16/2	0.38 (S)	16/8	0.38 (S)	32/0.5	0.75 (I)
CREC 11	32/32	0.53 (I)	16/512	0.75 (I)	64/8	1.01 (I)	64/8	1.25 (I)	64/1024	2.00 (I)
CREC 12	32/256	0.50 (S)	8/128	0.19 (S)	64/8	0.51 (I)	32/8	0.50 (S)	64/8	0.51 (I)
CREC 13	2/2	0.27 (S)	1/0.5	0.51 (I)	2/32	0.27 (S)	4/16	0.53 (S)	2/8	0.27 (S)
CREC 14	16/32	0.53 (I)	0.5/128	0.14 (S)	16/512	1.00 (I)	1/128	0.16 (I)	4/64	0.25 (S)
CREC 15	16/1	0.38 (S)	8/0.25	0.31 (S)	2/32	0.27 (S)	32/1	0.25 (S)	8/4	0.31 (S)
CREC 16	64/0.25	0.63 (I)	4/16	0.16 (S)	4/32	0.28 (S)	8/128	0.31 (I)	16/2	0.25 (S)
CREC 17	32/2	0.75 (I)	4/8	0.16 (S)	16/16	0.38 (S)	8/64	0.31 (S)	8/8	0.56 (I)
CREC 18	32/1	0.50 (S)	2/16	0.27 (S)	16/32	0.38 (S)	8/64	0.31 (S)	4/8	0.28 (S)
CREC 19	2/32	0.31 (S)	16/2	0.75 (I)	4/512	0.63 (I)	4/128	0.38 (S)	8/32	0.50 (S)

S, synergy; I, indifferent. [a] minimum inhibitory concentration of combination of meropenem/aminoglycoside. The FICI results for each combination were interpreted as follows: FICI $\leq$ 0.5, synergism; 0.5 < FICI $\leq$ 4, indifference; and FICI > 4, antagonism.

2.6. Time-Kill Assay

The time-kill effects of meropenem combined with either amikacin, gentamicin, kanamycin, streptomycin, or tobramycin were evaluated on CREC 12 (Figure 1). The results revealed a synergistic bactericidal effect at 1/4 meropenem plus 1/4 amikacin at 4 h. (Figure 1A) and 1/4 meropenem plus 1/4 gentamicin at 2 h. (Figure 1B) with a $\geq$3 $\log_{10}$ CFU/mL reduction in cell growth when compared to the MIC of individual antibiotics. Furthermore, an indifferent effect was revealed at 1/4 meropenem plus 1/4 kanamycin (Figure 1C). At 12 h, combination between 1/4 meropenem plus 1/4 streptomycin (Figure 1D) presented a synergistic bactericidal effect, while combination of 1/4 meropenem plus 1/4 Tobramycin revealed a synergistic effect (Figure 1E).

For CREC 18 at 8 h, 1/4 meropenem plus 1/4 amikacin showed a synergistic bactericidal effect (Figure 2A). Similar results were observed at 4 h with 1/4 meropenem plus 1/4 gentamicin (Figure 2B), at 8 h for 1/4 meropenem plus 1/4 kanamycin (Figure 2C), or 1/4 streptomycin (Figure 2D), and at 2 h for 1/4 meropenem plus 1/4 tobramycin against isolate CREC 18 (Figure 2E). However, a regrowth was observed at 8 h for meropenem and tobramycin combination, and at 12 h for meropenem and amikacin or gentamicin combination. Our results showed inconsistencies between the FICI, and time kill methods. Similar findings have been reported by previous studies [63,64].

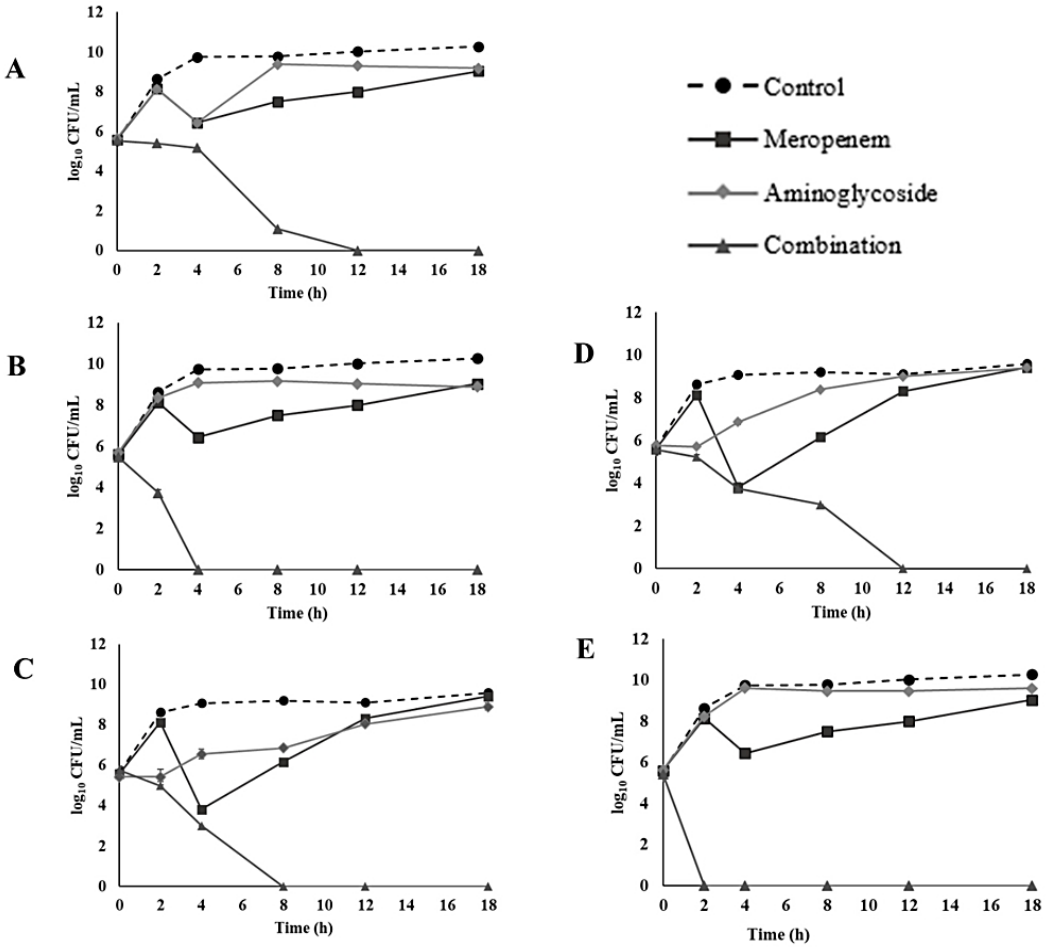

Figure 1. Time-kill curves of 1/4 MIC (32 μg/mL) meropenem and 1/4 MIC aminoglycosides combination against CREC 12: (**A**) amikacin (256 μg/mL), (**B**) gentamicin (256 μg/mL), (**C**) kanamycin (256 μg/mL), (**D**) streptomycin (8 μg/mL), and (**E**) tobramycin (256 μg/mL).

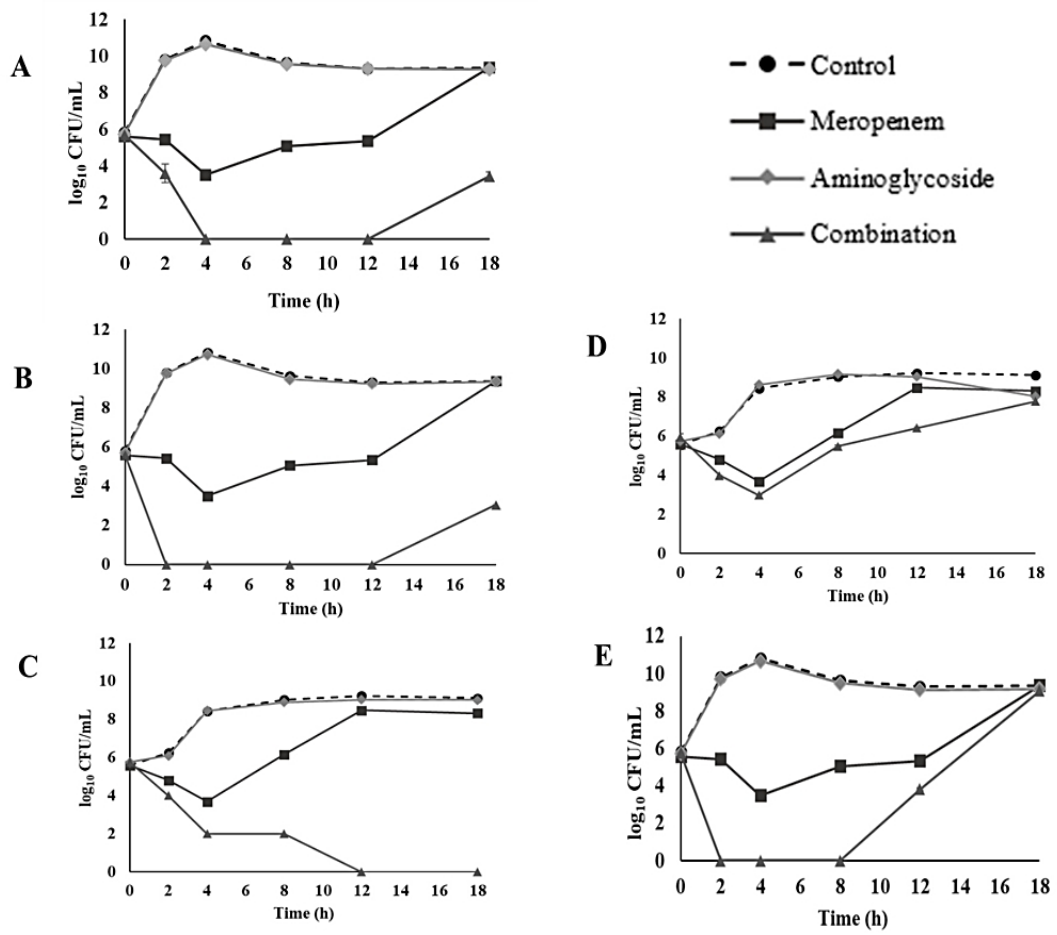

Figure 2. Time-kill curves of 1/4 MIC (32 μg/mL) meropenem and 1/4 MIC aminoglycosides combination against CREC 18: (**A**) amikacin (1 μg/mL), (**B**) gentamicin (16 μg/mL), (**C**) kanamycin (32 μg/mL), (**D**) streptomycin (64 μg/mL), and (**E**) tobramycin (8 μg/mL).

3. Materials and Methods

3.1. Chemical and Media

All culture media were purchased from Becton Dickinson & Co. Difco TM (Franklin Lakes, NJ, USA). Colistin sulfate, minocycline hydrochloride, and tobramycin were obtained from Sigma-Aldrich, (Saint Louis, MO, USA). Amikacin, ciprofloxacin, cefotaxime, gentamicin, kanamycin, levofloxacin, and streptomycin were purchased from Siam Bheasach Co, Ltd. (Bangkok, Thailand). Tigecycline was purchased from Pfizer Inc. (Philadelphia, PA, USA). Ceftazidime was obtained from Reyoung Pharmaceutical Co., Ltd. (Shandong, China). Imipenem was obtained from Merck Sharp & Dohme Corp. (Elkton, VA, USA). Meropenem was obtained from M&H Manufacturing Co. Ltd. (Samutprakarn, Thailand). Cefoperazone/sulbactam was obtained from L.B.S. Laboratory Ltd. (Bangkok, Thailand). Ceftolozane/tazobactam was obtained from Steri-Pharma, LLC (Syracuse, NY, USA). Fosfomycin was obtained from Meiji Seika Kaisha, Ltd. (Tokyo, Japan).

3.2. Bacterial Collection and Identification

A total of 35 suspected CREC isolates were collected from eight hospitals located in Southern Thailand. The isolates grew on MacConkey agar supplemented with imipenem at 6 μg/mL. All isolates were identified to species level using standard biochemical tests

and MALDI-TOF-MS. *E. coli* ATCC 25922 was used as quality control. The samples were kept in tryptic soy broth supplemented with 20% glycerol and stored at −80 °C.

3.3. Screening for Carbapenem Resistance

Resistance of the 35 suspected CREC isolates was assessed by the broth microdilution method according to the Clinical and Laboratory Standards Institute [65]. Briefly, the isolates were grown in cation-adjusted Mueller–Hinton broth (CAMHB). Bacterial cultures were adjusted with sterile 0.85% NaCl to McFarland 0.5 turbidity standard. Aliquot of 100 µL diluted bacterial suspension (1×10^6 CFU/mL) was mixed with 100 µL antibiotic in a 96-well plate and incubated at 37 °C for 18 h. The minimum inhibitory concentration (MIC) was expressed as the lowest concentration of the antibiotic that inhibits visible growth after incubation as indicated by the resazurin test.

3.4. Antibiogram of Carbapenem-Resistant Isolates

Confirmed CREC isolates were exposed to 17 conventional antibiotics including carbapenem (imipenem and meropenem), aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin, and tobramycin), cefoperazone-sulbactam, ceftolozane-tazobactam, cephalosporins (cefotaxime and ceftazidime), colistin, fluoroquinolone (ciprofloxacin and levofloxacin), fosfomycin, glycylcyclines (minocycline and tigecycline). The MICs of the antibiotics were determined using the broth microdilution method as previously detailed. The MIC for fosfomycin, was determined by the agar dilution method. Briefly, cation-adjusted Mueller–Hinton agar (CAMHA) was supplemented with 25 mg/L glucose-6-phosphate (G6P) as recommended by CLSI guidelines [65]. The bacterial suspension (approximately 1×10^4 CFU/mL) was spotted at 10 microliters on the surface of each agar plate containing the antibiotic.

3.5. Genotypic Determination of Carbapenemase and ESBL

Genomic DNA from *E. coli* was prepared using Presto™ Mini gDNA Bacteria Kit. Quantification of the extracted DNA was determined by spectroscopy at 260 nm. Antimicrobial resistance genes, including carbapenemase (bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$, and bla_{VIM}) and ESBL ($bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM}) were detected by PCR using the primers shown in Table 3. The amplification conditions for detecting IMP, KPC, and OXA-48 genes were initial denaturation at 94 °C for 10 m, 36 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 50 s, and final elongation at 72 °C for 5 m. The amplification condition for NDM and VIM genes were initial denaturation at 94 °C for 10 m, 36 cycles of 94 °C for 30 s, 56 °C for 40 s, and 72 °C for 50 s, and final elongation at 72 °C for 5 m. The amplification conditions for detecting ESBL genes included CTX-M, SHV, and TEM genes were initial denaturation at 95 °C for 15 m, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 m, and final elongation at 72 °C for 10 m.

3.6. Checkerboard Technique

The synergistic activities of meropenem combined with five aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin, and tobramycin) on CREC were determined by the checkerboard technique. Briefly, 100 µL of 1×10^6 CFU/mL bacterial suspension was added to wells containing 50 µL of each subinhibitory concentrations of meropenem and aminoglycosides. The plates were incubated for 18 h at 37 °C. Inhibitory concentrations were determined as concentrations without bacterial growth as indicated by the resazurin test. The experiments were performed in triplicate for three independent repeats. The activity of the antimicrobial combinations was defined by the fractional inhibitory concentration index (FICI), as follows:

$$\text{FICI} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}} \quad (1)$$

FICI results for each combination were interpreted as follows: FICI $\leq$ 0.5, synergism; 0.5 < FICI $\leq$ 4, indifference; and FICI > 4, antagonism. *E. coli* ATCC 25922 was used as standard control strains for the assays [66].

3.7. Time-Kill Assay

The activity of meropenem and aminoglycosides combinations were confirmed by the time-kill assay. Antibiotics were tested alone and in combination at 1/4 MIC. An inoculum size of 1×10^6 CFU/mL was added and incubated at 37 °C. Bacterial growth controls were maintained throughout the experiment. Bacterial growth was assessed at 0, 2, 4, 8, 12 and 18 h by plating 10-fold serially diluted suspensions on Mueller–Hinton agar plates. Plates were incubated overnight at 37 °C, and the number of colonies were counted. The experiments were performed in triplicate and recorded as mean averages. Bactericidal activity was defined as a $\geq$3 $\log_{10}$ CFU/mL reduction when compare the number of viable cells at time zero (0 h). Antibiotic combination synergism was defined as a $\geq$2 $\log_{10}$ CFU/mL at 18 h for the antimicrobial combination, compared with the most active agent. Indifferent was defined as <2 $\log_{10}$ CFU/mL increase or decrease at 18 h for the drug combination when compare with the most active drug and antagonism was defined as $\geq$2 $\log_{10}$ CFU/mL increase between the combination and the most active single drug [67].

4. Conclusions

Combination therapies have been highlighted as a possible treatment option for the management of infections caused by drug resistant bacterial isolates. This study demonstrated that combinations of meropenem with aminoglycoside might still be an efficient therapeutic option for the treatment of CREC harboring $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-5}}$. However, due to indifferent results observed with the FICI, it is important to consider other mechanisms of aminoglycoside and carbapenem co-resistance. In addition, further studies on toxicology, pharmacokinetics and pharmacodynamics of these combination regimens are required prior to clinical trials.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10081023/s1, Table S1: Screening for carbapenem resistance in 35 suspected carbapenem-resistant *Escherichia coli* isolates, Table S2: Clinical information and outcome of patients in 19 carbapenem-resistant *Escherichia coli* (CREC) isolates, Table S3: Minimum inhibitory concentrations of antimicrobial agents against the 19 carbapenem-resistant *Escherichia coli* isolates, Table S4: Summary of the synergistic effects of meropenem in combination with aminoglycosides against 19 carbapenem-resistant *Escherichia coli*.

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Institutional Review Board Statement: This retrospective study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Prince of Songkla University, Thailand (EC: 54-080-14-1-2). The researchers were granted permission to extract the data from the database with a waiver of consent because of the observational nature of the study. All data were fully anonymized before being accessed and analyzed.

Data Availability Statement: Data is contained within the article or Supplementary Material.

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CHAPTER 1

INTRODUCTION

Background

Infections due to carbapenem-resistant *Escherichia coli* (CREC), particularly the New Delhi metallo-β-lactamases (NDM)-producing isolates, are critically problematic to global health care (1). These infections usually yield unfavourable clinical outcomes, prolonged length of hospitalization and high hospital costs (2). The national antimicrobial resistance surveillance data reported by the Thailand National Institute of Health (2016-2018), indicated a high prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) (93%) among hospitalized patients in Thailand (3). In the past, carbapenems were the most reliable antimicrobial agents against hospital-acquired infections caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* (4). However, extensive usage as both empirical and definitive regimens (5), resulted in the emergence of CRE (4).

Enterobacteriaceae resistance to carbapenems is mainly associated with the production of several kinds of carbapenemases, which are enzymes capable of hydrolyzing carbapenems and other β-lactams (6). In addition, the lack of porin proteins by alteration in the permeability of the bacterial cell membrane, and overexpression of efflux pumps are additive carbapenem resistance mechanisms

(7). Numerous epidemiological studies have suggested that the acquisition of carbapenemase-encoding genes might lead to a rapid outbreak mostly in the hospital setting and sometimes in the community setting (8-10). Moreover, the specific class of the carbapenemase should be considered during the development of novel antimicrobial agents as each class possesses a unique mechanism and spectrum of activity (11). Previous studies have reported that ceftazidime-avibactam binds reversibly to class A, C, and some D β-lactamases (12, 13), whereas imipenem-cilastatin-relebactam and meropenem-vaborbactam reversibly and competitively inhibited class A and C β-lactamases (14, 15). However, these antibiotics did not inhibit metallo-β-lactamases such as NDM carbapenemases (12, 14, 15). Globally, the predominant carbapenemases include NDM, *Klebsiella pneumoniae* carbapenemase (KPC), Verona integrin-encoded metallo-β-lactamase (VIM), imipenemase (IMP), and oxacillinases (OXA)-type enzymes, which are encoded by *bla*$_{\text{NDM}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{IMP}}$, and *bla*$_{\text{OXA}}$ genes, respectively (6). However, *bla*$_{\text{NDM}}$ has gained relevance due to the high level of resistance to many clinically available β-lactams and ease of horizontal transfer between different isolates. To date, several variants of NDM enzymes have been identified (16) with amino acid substitutions at different positions. NDM-5 differed from NDM-1 by substitutions at positions 88 (Val → Leu) and 154 (Met → Leu), and several studies have shown that *bla*$_{\text{NDM-5}}$ is carried by conjugatable IncX3 plasmids responsible for the rapid spread (17-19).

Currently, therapeutic options for the management of infections caused by CREC are limited (20). Moreover, the development of new antimicrobial agents is costly, time-consuming, and require various stages of toxicological evaluations

to ensure safety (11). Hence, combining existing antimicrobial agents has become a strategy against several kinds of infections caused by multi-drug resistant (MDR) organisms (21). Previous studies have supported the use of combination therapy as an effective treatment option for infections caused by several MDR Gram-negative bacteria (22-24). A recent study demonstrated the synergistic effect of meropenem and aminoglycosides against KPC-2 and NDM-1-producing carbapenem-resistant *Klebsiella pneumoniae* (25). Additionally, the ability of meropenem to potentiate aminoglycoside activity, largely dependent on the MexXY-OprM multidrug efflux system, has been shown (26). However, data for combinations between meropenem and several aminoglycosides against CREC harbouring bla_{NDM} genes is lacking. This study evaluated the effects of meropenem in combination with several commonly used aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin, and tobramycin) on CREC isolates harbouring bla_{NDM} genes.

Review of literature

1. Antibiotics

Antibiotics can be divided into two types based on the type of action, including bactericidal and bacteriostatic. Several antibiotics were generated from soil microbes such as bacterial genus: *Streptomyces* spp., *Actinomyces* spp., *Bacillus* spp. and Fungi: *Penicillium* spp. and *Cephalosporium* spp. (27) Furthermore, semisynthetic and chemotherapeutic drugs were grouped by the process of synthesis. Semisynthetic drugs are synthesized by modifying the chemical structure of natural compounds, in order to increase their properties such as reducing toxicity and improving stability (28). Another type is chemotherapeutic drugs, which are chemically synthesized from laboratories (29). The spectrum activities of antibiotics consist of broad-spectrum activity and narrow-spectrum activity (30). The commonly used antibiotics were summarized in **Table 1.**

Table 1. Antibiotic classification (31).

Class of antibiotics				**Examples**	**Mode of action**
β-lactams	β-lactamase inhibitors			avibactam, clavulanic acid, sulbactam, tazobactam	cell wall synthesis inhibitors
	penicillins	pennicillinase sensible	aminopenicillins	amoxicillin, ampicillin	
			natural penicillin	penicillin G, penicillin VK	
		penicillinase resistant		dicloxacillin, nafcillin, oxacillin	
		anti-pseudomonal	carboxypenicillins	carbenicillin, ticarcillin	
			ureidopenicillin	azlocillin, mezlocillin, piperacillin	
	cephalosporins	1st generation		cefadroxil, cefazolin, cephalexine, cephradrine	
		2nd generation		cefaclor, cefoxitin, cefprozil, cefuroxime	
		3rd generation		cefoperazone, ceftriaxone, cefotaxime, ceftazidime	
		4th generation		cefepime, cefpirome	
		5th generation		ceftaroline, ceftolozane	
	carbapenems			doripenem, ertapenem, imipenem, meropenem	
	monobactams			aztreonam	
no lactams	glycopeptides			dalbavancin, oritavancin, telavancin, vancomycin	
	other			colistin, daptomycin, isoniazid, polymixin B	

Class of antibiotics	Examples	Mode of action
aminoglycosides	amikacin, gentamicin, kanamycin, streptomycin, tobramycin	protein synthesis inhibitors
tetracyclines	democlocyclin, doxycyclin, minocycline, tetracyclin tigecyclin	
oxazolidonones	linezolid, tidezolid	
streptogramins	quinupristin	
chloramphenicol		
macrolides	erythromycin, clarithromycin, azithromycin	
lincosamides	clindamycin, lincomycin	
fluoroquinolones	ciprofloxacin, levofloxacin, norfloxacin, sparfloxacin	DNA topoisomerases inhibitors
quinolones	nalidixic acid	
sulfonamides	Sulfamethoxazole, sulfasalazine, sulfisoxazole	folic acid synthesis inhibitor
DHFR inhibitors	pyrimethamine, trimethoprim	
nitroimidazoles	metronidazole, tinidazole	DNA damage
rifampin		mRNA synthesis

Mode of action of antibiotics

antibiotics were sorted into 5 groups following the mechanism of action of antibiotic (32).

1) Inhibitor of cell wall synthesis

Cell wall structure of bacteria differs from other organisms by the presence of peptidoglycan that surrounds bacterial cells, which is not found in a eukaryote. Hence, the bacterial cell wall is target site of various antibiotics due to selective toxicity that is effective only for bacteria not specific for humans and animals. The functions of bacterial cell wall are to maintain the bacterial cell shape and protect bacteria from lysis due to high osmotic pressure (33, 34). The main component in the bacterial cell wall is peptidoglycan, which is consist of N-acetylglucosamine and N-acetylmuramic acid and was cross-linked with short peptides by the reaction of transpeptidase and carboxypeptidase, known as penicillin-binding proteins (PBPs) (35). Antibiotics that function as an inhibitor of cell wall synthesis are β-lactam drugs (penicillin, cephalosporins, carbapenems, and monobactams), glycopeptide (vancomycin and teicoplanin), and fosfomycin.

2) Inhibitor of protein synthesis

Protein synthesis comprises transcription and translation, which have four main steps: initiation, elongation, termination, and recycling (36). The main structure of protein synthesis is the ribosome, which transforms the genetic information encoded in the messenger RNA (mRNA) into the polypeptide sequence. Bacterial 70S ribosomes consist of two subunits, including 30S subunit and 50S subunit. Inhibition

of protein synthesis stops or slows the growth of bacterial cells (37)**.** Antibiotics that inhibit protein synthesis such as aminoglycosides (amikacin, gentamicin, kanamycin, tobramycin, and streptomycin), tetracycline (doxycycline, minocycline, tetracycline, and tigecycline), macrolide (azithromycin, clarithromycin, and erythromycin).

3) Inhibitor of nucleic acid synthesis

Bacterial DNA synthesis requires a group of enzymes, which is involved in DNA replication, known as topoisomerases. Lacking these enzymes will result in abnormal DNA formation (38, 39). Quinolones are a group of antibiotics that interrupt nucleic acid synthesis by inhibiting topoisomerase (most frequently type II topoisomerase), which involve DNA replication. Antibiotics in quinolone group such as ciprofloxacin, norfloxacin, ofloxacin, and levofloxacin (40).

4) Inhibitor of essential metabolite synthesis

Antibiotics that inhibit essential metabolite synthesis are synthetic drugs. In this type of inhibition, an enzyme is inhibited in the process of tetrahydrofolate production, which was used in nucleic synthesis reactions. For examples of antibiotics in this group are sulfonamide and trimethoprim (41).

5) Inhibitor of cell membrane function

The primary function of bacterial cell wall is to protect the cell from internal pressure. The peptidoglycan was considered as a permeability barrier except for small substrates (42). Polymyxins, which are positively charged molecule antibiotics, were generated from *Paenibacillus polymyxa* (*Bacillus polymyxa*) (43).

The function of polymyxins is to inhibit bacterial membrane by interacting with lipid A of an outer membrane and disrupting the phospholipids. These actions lead to osmotic imbalance causing cell death (44). Polymyxins have 5 types (A-E types) but only 2 types were used, polymyxin B and polymyxin E (colistin). Daptomycin is a cyclic lipopeptide that was generated from *Streptomyces roseosporus* (45). The main structure is the peptide core which is attached to the fatty acid chain. Normally, Daptomycin was used in the treatment of Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus aureus* (46).

Mechanism of antibiotic resistance in bacteria

Mechanisms of antibiotic resistance are classified into intrinsic resistance and acquired resistance. For intrinsic resistance, some bacterial species have a unique structural characteristic that provides resistance to certain antibiotics (42)**.** On the contrary, acquired resistance is the naturally susceptible bacteria develop resistance against antibiotics by acquiring genes from other bacterial strains. The main mechanisms of antibiotic resistance in Gram-negative bacteria consist of three mechanisms (47), including enzymatic degradation, structural modification of porins, and overexpression of efflux pump.

1) Enzymatic degradation

β-lactams operate by binding with PBPs interrupting the cross-linked of the glycan chains in bacterial cell walls. β-lactamase enzymes (**Table 2**) are common mechanisms in bacterial resistance to antibiotics, which hydrolyze the amide bond of

the β-lactam ring resulting in the inactivation of the antibiotics**.** In *Enterobacteriaceae* family, β-lactamase enzyme locates in the periplasmic space inhibiting antibiotics before it reads to the PBPs. TEM and SHV are two types of β-lactamases in *Enterobacteriaceae* which are encoded in plasmid or transposon (48).

Aminoglycosides can bind to the 30S subunit ribosome inhibiting bacterial protein synthesis. Modifying enzymes are causing bacterial resistance in aminoglycosides. There are 3 main chemical modifications, including acetyltransferases (AACs), nucleotidyltransferase (ANTs), or phosphotransferases (APHs) (49).

Table 2. β-lactamase enzyme classification (50)

β-lactamase	**Classification**		**Enzyme**	**Example**
	Bush Jacoby	**Ambler**		
Serine β-lactamase	1	Class C	cephalosporinase	AmpC, ACT-1, CMY-2, DHA-1, FOX-1
	2a	Class A	penicillinase	PC1
	2b		penicillinase	TEM-1, TEM-2, SHV-1, TEM-3, SHV-2
	2be		ESBL	CTX-M-15, TEM-30, SHV-10
	2br		inhibitor-resistant TEM (IRT)	
	2d	Class D	oxacillinase	OXA-1, OXA-10,
	2de		OXA-ESBL	OXA-11, OXA-15
	2df		OXA carbapenemase	OXA-23, OXA-48
Metallo β-lactamase	3a	Class B1	carbapenemase	IMP, VIIM, NDM
	3b	Class B2		CphA, imiS
	3c	Class B3		L1

2) Structural modification of porins

The reduction of antibiotic influx can restrict intracellular access to an antibiotic. Porin proteins generally control an influxion of substrates, which are able to form to be open channels. These proteins allow the passive transportation of molecules across lipid bilayer membranes. Thus, porins can be considered as potential targets for bactericidal agents. The modification in porin structures result in an alteration of membrane permeability and it is a mechanism of bacteria to escape from antibiotics (51).

3) Overexpression of efflux pump.

Bacterial efflux systems consist of five different families of transporters, including the resistance nodulation division (RND) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR), the multidrug and toxic compound extrusion (MATE) families, and the ATP-binding cassette (ABC) superfamilies (Figure 1) (52). ABC transporters apply ATP hydrolysis as the energy source, but the others are dependent on proton motive force (31). The efflux systems that associate with *E. coli* were summarized in **Table 3.**

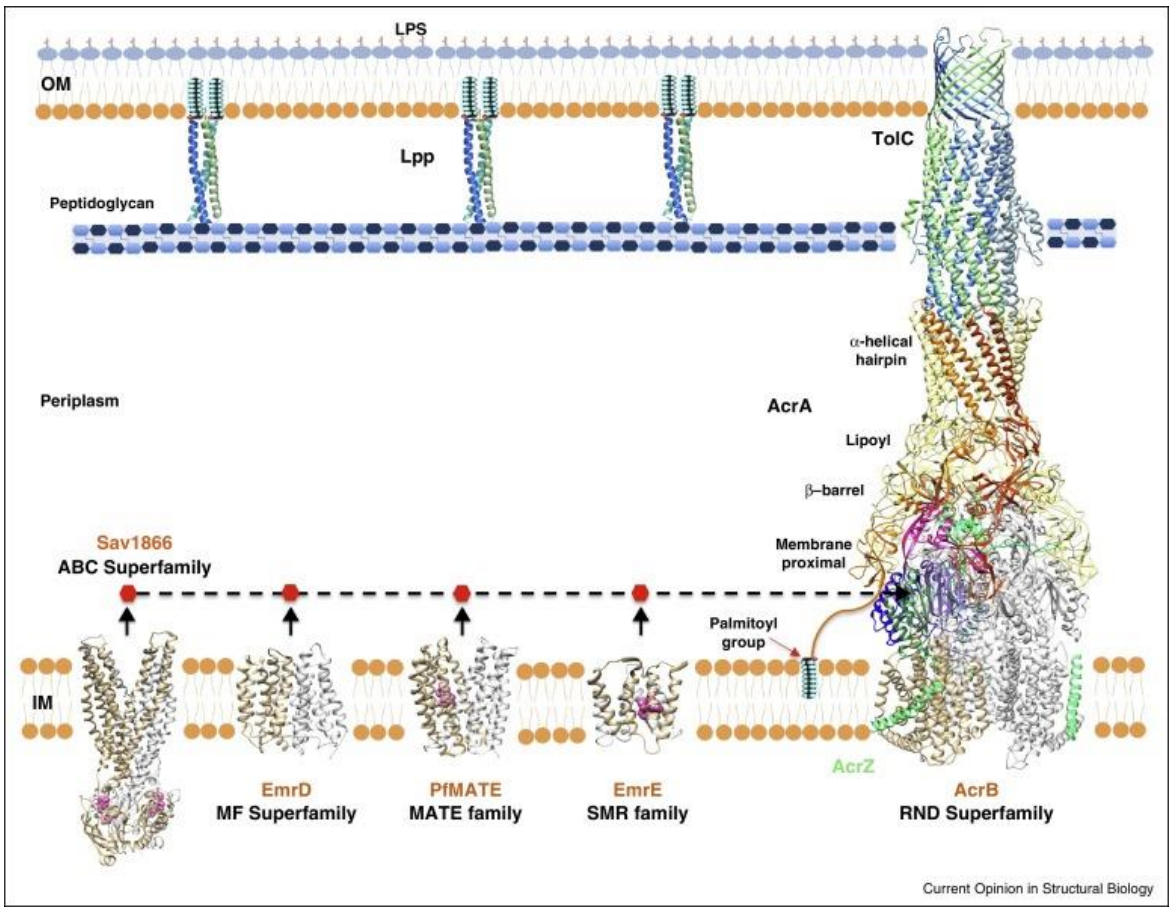

Figure 1. The structure of five efflux pumps superfamilies (52).

Table 3. Examples of efflux pumps belong to major efflux pump families in *Escherichia coli* strain.

Efflux pump family	**Example**	**Substrate**	**References**
ABC	*MacAB-TolC*	macrolides	(53)
MFS	*MdfA*	chloramphenicol, doxycycline, norfloxacin, tetracycline	(54)
	QepA/QepA2	fluoroquinolones	(55)
RND	*AcrAB-TolC*	β-lactam, chloramphenicol, fluoroquinolones, macrolides, novobiocin, rifampicin, tetracycline, tigecycline	(56),(57)
	OqxAB	chloramphenicol, fluoroquinolones	(58)
SMR	*EmrE*	acriflavine, ethidium bromide, quaternary ammonium compounds	(59), (60)

2. *Escherichia coli*

E. coli is a bacterium in the member of *Enterobacteriaceae* family. The most prevalent in gastrointestinal tracts of humans and warm-blood animals. Generally, *E. coli* is a normal flora microorganism and lives in a mutually beneficial association with hosts. Moreover, it was considered as an opportunistic pathogen causing the prominence of urinary tract infection, meningitis, neonatal, and septicaemia (61).

Resistance mechanisms of *Escherichia coli*

Prolonged and extensive usage of antibiotics over time resulted in an increased incidence of resistance in *E. coli* Multidrug-resistant strains are rising worldwide due to the spread of genes, which are located on mobile genetic elements, including plasmids, integrons and transposons.

E.coli is intrinsically resistant to penicillin G, which is the first β-lactam in clinical practice, due to the outer membrane barrier (62). Furthermore, β-lactamase production is an important factor that causes broad-spectrum resistance to β-lactam. β-lactamases, the wide classes of enzymes, are regularly produced by *Enterobacteriaceae* and frequently encoded on plasmids (63). Several different types of β-lactamases were described in (**Table 2**). ESBLs provide resistance to various antibiotics including third and fourth generation cephalosporins and monobactams.

Currently, carbapenem resistance in *Enterobacteriaceae* was classified to be an urgent threat level by CDC. The prevalence problems in nosocomial infection are primarily caused by plasmid-encoded carbapenemases (6). Fluoroquinolone resistance genes are frequently observed in combination with ESBL gene. Fluoroquinolone resistance *qnr* and *aac(6') Ib-cr* genes are frequently associated with β-lactam resistance genes, mainly $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ (64, 65). For aminoglycoside resistance, alteration of the 16S rRNA site by methyltransferase enzymes has emerged as a serious threat. Moreover, the 16S rRNA methyltransferase *armA* gene is often accompanied by the carbapenemase genes on the same mobile genetic element lead to pan drug-resistant mechanisms in bacteria (66, 67).

3. Combination therapy

Since the emergence of a widespread of MDR, combination therapy has been used in critically ill patient treatments.

The advantages of using combination therapy such as (68)

1) Toxicity of monotherapy may decrease when each of the antibiotics is selected to combine by using at a lower concentration.

2) An increasing of features to cover all bacterial pathogens when combination therapy is used by using more than one antibiotic.

3) The synergistic action of antibiotics combination of two or more antibiotics being greater than individual activity

4) The opportunity of the emergence of resistance against two drugs are lower than a single drug.

Objectives

1. To evaluate the occurrence of ESBL- and carbapenemase genes among carbapenem-resistant *E. coli*

2. To study the antibiotic susceptibility pattern in carbapenem-resistant *E. coli* isolated from the patients

3. To investigate the synergistic effect of meropenem in combination with aminoglycosides against carbapenem-resistant *E. coli*

CHAPTER 2

MATERIALS AND METHODS

1. Chemical and Media

All culture media were purchased from Becton Dickinson & Co. Difco TM (Franklin Lakes, NJ, USA). Colistin sulfate, minocycline hydrochloride, and tobramycin were obtained from Sigma-Aldrich, (Saint Louis, MO, USA). Amikacin, ciprofloxacin, cefotaxime, gentamicin, kanamycin, levofloxacin, and streptomycin were purchased from Siam Bheasach Co, Ltd. (Bangkok, Thailand). Tigecycline was purchased from Pfizer Inc. (Philadelphia, PA, USA). Ceftazidime was obtained from Reyoung Pharmaceutical Co., Ltd. (Shandong, China). Imipenem was obtained from Merck Sharp & Dohme Corp. (Elkton, VA, USA). Meropenem was obtained from M&H Manufacturing Co. Ltd. (Samutprakarn, Thailand).Cefoperazone/sulbactam was obtained from L.B.S. Laboratory Ltd. (Bangkok, Thailand). Ceftolozane/tazobactam was obtained from Steri-Pharma, LLC (Syracuse, NY, USA). Fosfomycin was obtained from Meiji Seika Kaisha, Ltd. (Tokyo, Japan).

2. Bacterial Collection and Identification

A total of 35 suspected CREC isolates were collected from eight hospitals located in Southern Thailand. The isolates grew on MacConkey agar supplemented with imipenem at 6 µg/mL. All isolates were identified to species level using standard biochemical tests and MALDI-TOF-MS. *E. coli* ATCC 25922 was used as quality control. The samples were kept in tryptic soy broth supplemented with 20% glycerol and stored at -80 °C.

3. Screening for Carbapenem Resistance

Resistance of the 35 suspected CREC isolates was assessed by the broth microdilution method according to the Clinical and Laboratory Standards Institute (69). Briefly, the isolates were grown in cation-adjusted Mueller-Hinton broth (CAMHB). Bacterial cultures were adjusted with sterile 0.85% NaCl to McFarland 0.5 turbidity standard. An aliquot of 100 µL diluted bacterial suspension ($1x10^6$ CFU/mL) was mixed with 100 µL antibiotic in a 96-well plate and incubated at 37 °C for 18 h. The minimum inhibitory concentration (MIC) was expressed as the lowest concentration of the antibiotic that inhibits visible growth after incubation as indicated by the resazurin test.

4. Antibiogram of Carbapenem-Resistant Isolates

Confirmed CREC isolates were exposed to 17 conventional antibiotics including carbapenem (imipenem and meropenem), aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin, and tobramycin), cefoperazone-sulbactam, ceftolozane-tazobactam, cephalosporins (cefotaxime and ceftazidime), colistin, fluoroquinolone (ciprofloxacin and levofloxacin), fosfomycin, glycylcyclines (minocycline and tigecycline). The MICs of the antibiotics were determined using the broth microdilution method as previously detailed. The MIC for fosfomycin, was determined by the agar dilution method. Briefly, cation-adjusted Mueller-Hinton agar (CAMHA) was supplemented with 25mg/L glucose-6-phosphate (G6P) as recommended by CLSI guidelines (69). The bacterial suspension (approximately $1x10^4$ CFU/mL) was spotted at 10 µL on the surface of each agar plate containing the antibiotic.

5. Genotypic Determination of Carbapenemase and ESBL

Genomic DNA from *E. coli* was prepared using Presto™ Mini gDNA Bacteria Kit. Quantification of the extracted DNA was determined by spectroscopy at 260 nm. Antimicrobial resistance genes, including carbapenemase (*bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, and *bla*$_{VIM}$) and ESBL (*bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$) were detected by PCR using the primers shown in **Table 4.** The amplification conditions for detecting IMP, KPC, and OXA-48 genes were initial denaturation at 94 °C for 10 m, 36 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 50 s, and final elongation at 72 °C for 5 m. The amplification condition for NDM and VIM genes were initial denaturation

at 94 °C for 10 m, 36 cycles of 94 °C for 30 s, 56 °C for 40 s, and 72 °C for 50 s, and final elongation at 72 °C for 5 m. The amplification conditions for detecting ESBL genes included CTX-M, SHV, and TEM genes were initial denaturation at 95 °C for 15 m, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 m, and final elongation at 72 °C for 10 m.

Table 4. Primers used for PCR amplification of carbapenemase and ESBL genes.

Primer name		Sequence (5' to 3')	Amplicon size (bp)	Reference
		Carbapenemase		
bla$_{IMP}$	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232	(70)
	IMP-R	GGTTTAAYAAAACAACCACC		
bla$_{KPC}$	KPC-F	CGTCTAGTTCTGCTGTCTTG	798	
	KPC-R	CTTGTCATCCTTGTTAGGCG		
bla$_{NDM}$	NDM-F	GGTTTGGCGATCTGGTTTTC	621	
	NDM-R	CGGAATGGCTCATCACGATC		
bla$_{OXA-48}$	OXA-F	GCGTGGTTAAGGATGAACAC	438	
	OXA-R	CATCAAGTTCAACCCAACCG		
bla$_{VIM}$	VIM-F	GATGGTGTTTGGTCGCATA	390	
	VIM-R	CGAATGCGCAGCACCAG		
		Extended-spectrum β-lactamase		
bla$_{CTX-M}$	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	573	(71)
	CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG		
bla$_{SHV}$	bla-SHV.SE	ATGCGTTATATTCGCCTGTG	747	
	bla-SHV.AS	TGCTTTGTTATTCGGGCCAA		
bla$_{TEM}$	TEM-164.S	TCGCCGCATACACTATTCTCAGAATGA	445	
	TEM-165.AS	ACGCTCACCGGCTCCAGATTTAT		

6. Checkerboard Technique

The synergistic activities of meropenem combined with five aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin, and tobramycin) on CREC were determined by the checkerboard technique. Briefly, 100 µL of $1x10^6$ CFU/mL bacterial suspension was added to wells containing 50 µL of each subinhibitory concentration of meropenem and aminoglycosides. The plates were incubated for 18 h at 37 °C. Inhibitory concentrations were determined as concentrations without bacterial growth as indicated by the resazurin test. The experiments were performed in triplicate for three independent repeats. The activity of the antimicrobial combinations were defined by the fractional inhibitory concentration index (FICI), as follows:

$$\mathbf{FICI} = \frac{\textbf{MIC of drug A in combination}}{\textbf{MIC of drug A alone}} + \frac{\textbf{MIC of drug B in combination}}{\textbf{MIC of drug B alone}}$$

FICI results for each combination were interpreted as follows: FICI ≤ 0.5, synergism; $0.5 < \text{FICI} \leq 4$, indifference; and FICI > 4, antagonism. *E. coli* ATCC 25922 was used as standard control strains for the assays (72).

7. Time-Kill Assay

The activity of meropenem and aminoglycosides combinations were confirmed by the time-kill assay. Antibiotics were tested alone and in combination at 1/4 MIC. An inoculum size of $1x10^6$ CFU/mL was added and incubated at 37 °C. Bacterial growth controls were maintained throughout the experiment. Bacterial growth was assessed at 0, 2, 4, 8, 12, and 18 h by plating 10-fold serially diluted suspensions

on Muller-Hinton agar plates. Plates were incubated overnight at 37 °C, and the number of colonies were counted. The experiments were performed in triplicate and recorded as mean averages. Bactericidal activity was defined as a ≥ 3 $\log_{10}$ CFU/mL reduction when compare the number of viable cells at time zero (0 h). Antibiotic combination synergism was defined as a ≥ 2 $\log_{10}$ CFU/mL at 18 h for the antimicrobial combination, compared with the most active agent. Indifferent was defined as < 2 $\log_{10}$ CFU/mL increase or decrease at 18 h for the drug combination when compare with the most active drug and antagonism was defined as ≥ 2 $\log_{10}$ CFU/mL increase between the combination and the most active single drug (73).

CHAPTER 3

RESULTS AND DISCUSSION

1. Bacterial Isolates

A total of 35 suspected CREC isolates were collected from eight hospitals located in Southern Thailand. The isolates were obtained from various clinical specimens, including blood (n = 11), rectal (n = 19), throat (n = 3) and environment (n = 2). Data of isolates and antimicrobial response to imipenem and meropenem are shown in **Table 5.** The results indicated that 19 isolates were resistant to carbapenems. Demographic information, clinical data and outcomes of the patients infected with CREC are presented in **Table 6.** Similar to previous reports of risk factors associated with CRE acquisition or infection (74, 75), most patients in this study had previous exposure to various antimicrobial agents, particularly carbapenems. The results support previous observation that exposure to antibiotics including β-lactams such as carbapenems and cephalosporins, as well as fluoroquinolones were associated with CRE (23). Patient information indicated that most of the patients were admitted to intensive care units (ICU), which are in consonance with observations of a previous study that showed a high prevalence of carbapenemase-producing Enterobacteriaceae in the ICU (76).

Table 5. Screening for carbapenem resistance in 35 suspected carbapenem-resistant *Escherichia coli* isolates

Clinical isolate	Code	Source of isolation	MIC (µg/ml)	
			Imipenem	Meropenem
EC1	1PSUsep1R/2	Rectal	0.25 (S)	0.25 (S)
EC2	1PSU6R/2	Rectal	0.25 (S)	0.0156 (S)
EC3	2PSU6R/1	Rectal	64 (R)	64 (R)
EC4	2PSU6R/2	Rectal	64 (R)	64 (R)
EC5	1HY4R/2	Rectal	0.25 (S)	0.0156 (S)
EC6	1HY8R	Rectal	128 (R)	128 (R)
EC7	1HY13Th/1	Throat	64 (R)	64 (R)
EC8	1HY13R/1	Rectal	64 (R)	64 (R)
EC9	1SK1R/1	Rectal	0.25 (S)	0.0156 (S)
EC10	2ST1R/1	Rectal	0.25 (S)	0.0156 (S)
EC11	2ST4R/2	Rectal	0.25 (S)	0.0156 (S)
EC12	2ST7R/1	Rectal	0.25 (S)	0.003 (S)
EC13	2ST7R/2	Rectal	0.25 (S)	0.25 (S)
EC14	1PT5R/1	Rectal	32 (R)	64 (R)
EC15	1PA5Th/1	Throat	0.25 (S)	0.0156 (S)
EC16	1PA5E	Environment	0.25 (S)	0.0156 (S)
EC17	1PA21Th/1	Throat	128 (R)	128 (R)
EC18	1PA21R	Rectal	128 (R)	128 (R)
EC19	1PA21E	Environment	64 (R)	128 (R)
EC20	2PA3R/1	Rectal	0.25 (S)	0.0156 (S)
EC21	2PA3R/2	Rectal	0.25 (S)	0.0156 (S)
EC22	2PA7R/1	Rectal	0.5 (S)	0.0156 (S)
EC23	2PA9R/1	Rectal	0.25 (S)	0.0156 (S)
EC24	2PA21R/1	Rectal	64 (R)	128 (R)
EC25	SK018	Blood	32 (R)	64 (R)
EC26	SK019	Blood	0.5 (S)	0.0156 (S)
EC27	SK020	Blood	0.25 (S)	0.0156 (S)
EC28	SK021	Blood	64 (R)	128 (R)
EC29	TR003	Blood	32 (R)	128 (R)
EC30	PT024	Blood	16 (R)	32 (R)
EC31	PT033	Blood	64 (R)	128 (R)
EC32	PT048	Blood	64 (R)	128 (R)
EC33	PT051	Blood	64 (R)	128 (R)
EC34	NT002	Blood	64 (R)	128 (R)
EC35	NT004	Blood	128 (R)	32 (R)

R, resistant; S, susceptible

Table 6. Clinical information of patients in 19 carbapenem-resistant *Escherichia coli* (CREC) isolates.

Isolate	Code	Hospital	Source of isolation	Sex	Age	Initial ward	Underlying disease	Previous use of antibiotics
CREC1	2PSU6R/1	Songklanagarind	Rectal	M	73	ICU medicine	DM, HTN, DLD, CVA, CAD, CKD	CRO, IMP
CREC2	2PSU6R/2							
CREC3	1HY8R	Hatyai	Rectal	M	63	ICU medicine	HTN, CKD	CRO, MEM
CREC4	1HY13Th/1	Hatyai	Throat	F	59	ICU surgery	DM, CVA, CAD	CRO, ETP
CREC5	1HY13R/1	Hatyai	Rectal					
CREC6	1PT5R/1	Phatthalung	Rectal	M	48	ICU medicine	HTN, DLD, CKD	CRO, CAZ, PIP/TAZ. IMP
CREC7	1PA21Th/1	Pattani	Throat	M	84	General medicine	DLD, CVA, CAD	CAZ, MEM
CREC8	1PA21R	Pattani	Rectal					
CREC9	1PA21E	Pattani	Environment					
CREC10	2PA21R/1	Pattani	Rectal	M	47	General medicine	CAD	CRO, PIP/TAZ
CREC11	SK018	Songkhla	Blood	M	61	General medicine	COPD	CRO, AZM, PIP/TAZ
CREC12	SK021	Songkhla	Blood					
CREC13	TR003	Trang	Blood	F	46	General surgery	HTN, CVA, CKD	CRO, LVX, ETP
CREC14	PT024	Pattani	Blood	F	52	ICU surgery	CKD, COPD	CRO, IMP

Isolate	Code	Hospital	Source of isolation	Sex	Age	Initial ward	Underlying disease	Previous use of antibiotics
CREC15	PT033	Pattani	Blood	M	36	General medicine	HTN, CKD	CAZ, LVX, MEM
CREC16	PT048	Pattani	Blood	M	37	ICU medicine	DM, CKD	CRO, PIP/TAZ, IMP
CREC17	PT051	Pattani	Blood	M	41	ICU surgery	CAD, CKD	LVX, IMP
CREC18	NT002	Naradhiwas Rajanagarindra	Blood	F	65	General surgery	DM	CRO, LVX, IMP
CREC19	NT004	Naradhiwas Rajanagarindra	Blood	F	49	General medicine	HTN, CKD	CRO, MEM

AZM, azithromycin; CAD, coronary artery disease; CAZ, ceftazidime; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CRO, ceftriaxone; CVA, cerebrovascular disease; DM, diabetes mellitus; DLD, dyslipidemia; ETP, ertapenem; HTN, hypertension; IMP, imipenem; LVX, levofloxacin; MEM, meropenem; PIP/TAZ, piperacillin/tazobact

2. The Antibiogram of Carbapenem-Resistant *E. coli* Isolates

The susceptibility profile of CREC isolates was evaluated against 15 conventional antibiotics including carbapenems (imipenem and meropenem), aminoglycosides (amikacin, gentamicin, kanamycin, streptomycin, and tobramycin), cefoperazone-sulbactam, ceftolozane-tazobactam, colistin, cephalosporins (cefotaxime and ceftazidime), fosfomycin, and glycylcyclines (minocycline and tigecycline). The MICs of antibiotics except carbapenems and aminoglycosides were recorded in **Table 7** and summarized in **Table 8**. The results suggested that three antibiotics including colistin, fosfomycin, and amikacin were effective against CREC isolates, with percentage efficacy of 100% , 89.5% and 73.7%, respectively. To date, polymyxins, fosfomycin, aminoglycosides, and tigecycline are considered choice drugs for the management of infections caused by carbapenem- resistant Gram-negative bacteria (77). However, resistance to these antibiotics is increasing rapidly with a high chance of toxicity due to the relatively high doses required for monotherapy medications. Results of this study revealed that approximately 79% of CREC isolates were resistant to tigecycline, contrary to previous reports of 0.7% and 11.2% (78, 79). In addition, the low plasma levels of tigecycline (80) constitute a clinical concern for mono-therapeutic administration. Polymyxin on the other hand showed excellent antimicrobial effects against CREC with a 100% susceptibility. However, the nephrotoxicity and poor tissue perfusion of polymyxins (81) are limiting factors hindering extensive therapeutic usage. The rapid acquisition of resistance and sodium overload with intravenous fosfomycin (82) are also of clinical concern.

Table 7. Minimum inhibitory concentrations of antimicrobial agents against the 19 carbapenem-resistant *Escherichia coli* isolates

Clinical isolate	Minimum inhibitory concentration (μg/ml)									
	β-lactam + β-lactamase inhibitor		Cephalosporins		Fluoroquinolone		Glycylcyclines		Other	
	cefoperazone + sulbactam	ceftolozane + tazobactam	cefotaxime	ceftazidime	ciprofloxacin	levofloxacin	tigecycline	minocycline	colistin	fosfomycin
CREC1	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	64 (R)	32 (R)	1 (R)	<2 (S)	0.5 (S)	16 (S)
CREC2	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	128 (R)	32 (R)	1 (R)	<2 (S)	2 (S)	16 (S)
CREC3	32 (S)	4 (S)	256 (R)	1024 (R)	4 (R)	<0.5 (S)	2 (R)	<2 (S)	1 (S)	64 (S)
CREC4	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	128 (R)	16 (R)	0.5 (S)	16 (R)	0.5 (S)	16 (S)
CREC5	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	128 (R)	16 (R)	1 (R)	<2 (S)	0.5 (S)	16 (S)
CREC6	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	0.5 (S)	<0.5 (S)	0.5 (S)	<2 (S)	0.5 (S)	16 (S)
CREC7	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	2 (I)	2 (S)	2 (R)	8 (I)	0.5 (S)	16 (S)
CREC8	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	4 (R)	1 (S)	2 (R)	4 (S)	2 (S)	16 (S)
CREC9	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	2 (I)	1 (S)	2 (R)	8 (I)	0.5 (S)	16 (S)
CREC10	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	64 (R)	32 (R)	1 (R)	4 (S)	1 (S)	16 (S)
CREC11	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	128 (R)	16 (R)	4 (R)	<2 (S)	0.25 (S)	16 (S)
CREC12	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	64 (R)	8 (R)	2 (R)	<2 (S)	0.25 (S)	16 (S)

Clinical isolate	Minimum inhibitory concentration (μg/ml)									
	β-lactam + β-lactamase inhibitor		Cephalosporins		Fluoroquinolone		Glycylcyclines		Other	
	cefoperazone + sulbactam	ceftolozane + tazobactam	cefotaxime	ceftazidime	ciprofloxacin	levofloxacin	tigecycline	minocycline	colistin	fosfomycin
CREC13	>1024 (R)	>1024 (R)	256 (R)	>1024 (R)	128 (R)	8 (R)	2 (R)	<2 (S)	0.5 (S)	16 (S)
CREC14	>1024 (R)	>1024 (R)	>1024 (R)	>1024 (R)	16 (R)	8 (R)	0.06 (S)	8 (I)	1 (S)	16 (S)
CREC15	256 (R)	>1024 (R)	>1024 (R)	>1024 (R)	128 (R)	8 (R)	0.25 (S)	<2 (S)	1 (S)	16 (S)
CREC16	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	256 (R)	32 (R)	2 (R)	16 (R)	1 (S)	32 (S)
CREC17	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	256 (R)	32 (R)	2 (R)	16 (R)	0.5 (S)	16 (S)
CREC18	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	512 (R)	64 (R)	2 (R)	<2 (S)	0.5 (S)	1024 (R)
CREC19	512 (R)	>1024 (R)	>1024 (R)	>1024 (R)	256 (R)	16 (R)	4 (R)	<2 (S)	1 (S)	1024 (R)

R, resistant; I, intermediate; S, susceptible

Table 8. Summary of antimicrobial susceptibility of 19 carbapenem-resistant *Escherichia coli* isolates.

Antibiotics	MIC (µg/mL)			Percentage %		
	Range	MIC_{50}	MIC_{90}	Susceptible	Intermediate	Resistant
Aminoglycoside						
Amikacin	2 - > 1024	4	> 1024	73.7	0	26.3
Gentamicin	1 - > 1024	64	> 1024	21	5.3	73.7
Kanamycin	8 - > 1024	128	> 1024	21	0	79
Streptomycin	16 - 1024	512	1024	0	0	100
Tobramycin	1 - > 1024	32	> 1024	5.3	10.5	84.2
β-lactam + β-lactamase inhibitor						
Cefoperazone-sulbactam	256 - > 1024	512	> 1024	5.3	0	94.7
Ceftolozane-tazobactam	1024 - > 1024	> 1024	> 1024	5.3	0	94.7
Carbapenem						
Imipenem	16 - 128	64	128	0	0	100
Meropenem	32 - 128	128	128	0	0	100
Cephalosporin						
Cefotaxime	256 - > 1024	> 1024	> 1024	0	0	100
Ceftazidime	1024 - > 1024	> 1024	> 1024	0	0	100
Fluoroquinolone						
Ciprofloxacin	0.5 - 512	128	256	5.3	10.5	84.2
Levofloxacin	< 0.5 - 64	16	32	26.3	0	73.7
Glycylcycline						
Minocycline	< 2 - 16	< 2	16	68.4	15.8	15.8
Tigecycline	0.0625 - 4	2	4	21	0	79
Other						
Colistin	0.25 - 2	0.5	2	100	0	0
Fosfomycin	16 - 1024	16	1024	89.5	0	10.5

3. Antimicrobial Susceptibility to Carbapenem and Aminoglycosides

The MIC of carbapenems and aminoglycosides on 19 CREC isolates were determined by the broth microdilution method (**Table 9**). The 19 isolates were resistant to imipenem (MIC50 = 64 µg/mL and MIC90 = 128 µg/mL), meropenem (MIC50 = 128 µg/mL and MIC90 = 128 µg/mL), and streptomycin (MIC50 = 512 µg/mL and MIC90 = 1024 µg/mL). In addition, 16 isolates were resistant to tobramycin (MIC50 = 32 µg/mL and MIC90 > 1024 µg/mL), while two isolates were intermediate. Furthermore, 14 and 15 isolates displayed resistance against gentamicin (MIC50 = 64 µg/mL and MIC90 > 1024 µg/mL) and kanamycin (MIC50 = 128 µg/mL and MIC90 > 1024 µg/mL), respectively. In contrast, amikacin showed high efficacy on 14 isolates.

Aminoglycosides are an important class of bactericidal antibiotics that are frequently used for the treatment of severe infections caused by Gram-negative bacteria. The major resistance mechanism to aminoglycosides in Gram-negative bacteria is the production of aminoglycoside-modifying enzymes (AMEs) or the modification of the ribosome by acquired 16S rRNA methyltransferases (RMTases) (49, 83). AMEs modify select to specific aminoglycosides, hence bacterial isolates show discordant susceptibility among different aminoglycosides. A previous study demonstrated the co-occurrence of aminoglycoside and β-lactam resistance mechanisms in *E. coli* isolates (84). In addition, co-harbouring of ESBLs, carbapenemases, and 16S rRNA methylase genes within a plasmid has been noted to result in multidrug resistance in *Enterobacteriaceae* (85).

Table 9. Antibacterial profile of aminoglycoside and carbapenem resistance in 19 carbapenem-resistant *Escherichia coli* isolates.

Clinical isolate	Source	*bla* genotype		MIC (µg/mL)						
				carbapenem		aminoglycoside				
		carbapenemase	ESBL	imipenem	meropenem	amikacin	gentamicin	kanamycin	streptomycin	tobramycin
CREC 1	Rectal	$bla_{\text{NDM-1}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	64 (R)	64 (R)	2 (S)	64 (R)	16 (S)	1024 (R)	16 (R)
CREC 2	Rectal	$bla_{\text{NDM-1}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	64 (R)	64 (R)	2 (S)	64 (R)	16 (S)	1024 (R)	8 (I)
CREC 3	Rectal	-	$bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}	128 (R)	128 (R)	64 (R)	64 (R)	64 (R)	256 (R)	128 (R)
CREC 4	Throat	$bla_{\text{NDM-5}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	64 (R)	64 (R)	4 (S)	32 (R)	64 (R)	512 (R)	32 (R)
CREC 5	Rectal	$bla_{\text{NDM-5}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	64 (R)	64 (R)	4 (S)	64 (R)	64 (R)	512 (R)	32 (R)
CREC 6	Rectal	$bla_{\text{NDM-5}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	32 (R)	64 (R)	4 (S)	1 (S)	32 (S)	64 (R)	8 (I)
CREC 7	Throat	$bla_{\text{NDM-1}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	128 (R)	128 (R)	8 (S)	128 (R)	256 (R)	512 (R)	64 (R)
CREC 8	Rectal	$bla_{\text{NDM-1}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	128 (R)	128 (R)	8 (S)	128 (R)	128 (R)	16 (R)	64 (R)
CREC 9	Environment	$bla_{\text{NDM-1}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	64 (R)	128 (R)	8 (S)	64 (R)	512 (R)	512 (R)	64 (R)
CREC 10	Rectal	-	bla_{TEM}	64 (R)	128 (R)	4 (S)	0.5 (S)	8 (S)	32 (R)	1 (S)
CREC 11	Blood	-	$bla_{\text{CTX-M}}$, bla_{TEM}	32 (R)	64 (R)	> 1024 (R)	> 1024 (R)	> 1024 (R)	32 (R)	> 1024 (R)
CREC 12	Blood	$bla_{\text{NDM-5}}$	$bla_{\text{CTX-M}}$, bla_{TEM}	64 (R)	128 (R)	> 1024 (R)	> 1024 (R)	> 1024 (R)	32 (R)	> 1024 (R)

Clinical isolate	Source	*bla* genotype		MIC (μg/mL)						
				carbapenem		aminoglycoside				
		carbapenemase	ESBL	imipenem	meropenem	amikacin	gentamicin	kanamycin	streptomycin	tobramycin
CREC 13	Blood	*bla*$_{NDM-5}$	*bla*$_{CTX-M}$, *bla*$_{TEM}$	32 (R)	128 (R)	8 (S)	1 (S)	128 (R)	32 (R)	32 (R)
CREC 14	Blood	*bla*$_{NDM-5}$	*bla*$_{TEM}$	16 (R)	32 (R)	> 1024 (R)	> 1024 (R)	> 1024 (R)	1024 (R)	512 (R)
CREC 15	Blood	*bla*$_{NDM-5}$	*bla*$_{CTX-M}$, *bla*$_{TEM}$	64 (R)	64 (R)	4 (S)	1 (S)	128 (R)	512 (R)	16 (R)
CREC 16	Blood	*bla*$_{NDM-5}$	*bla*$_{CTX-M}$, *bla*$_{TEM}$	64 (R)	128 (R)	2 (S)	128 (R)	128 (R)	512 (R)	16 (R)
CREC 17	Blood	*bla*$_{NDM-5}$	*bla*$_{TEM}$	64 (R)	128 (R)	4 (S)	64 (R)	64 (R)	256 (R)	16 (R)
CREC 18	Blood	*bla*$_{NDM-1}$, *bla*$_{OXA-48}$	*bla*$_{CTX-M}$, *bla*$_{TEM}$	64 (R)	128 (R)	4 (S)	64 (R)	128 (R)	256 (R)	32 (R)
CREC 19	Blood	*bla*$_{NDM-1}$	*bla*$_{CTX-M}$, *bla*$_{TEM}$	128 (R)	32 (R)	128 (R)	8 (I)	1024 (R)	512 (R)	128 (R)

R, resistant; S, susceptible; I, intermediate

4. Genotypic Resistance Mechanism in Carbapenem-Resistant *E. coli* Isolates

The 19 CREC isolates were screened for antimicrobial resistance genes including carbapenemase genes (*bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{OXA-48}$) and ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$) using PCR (**Table 10**). The results for carbapenemase genes demonstrated high prevalence of *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$. However, *bla*$_{OXA-48}$ was observed in one of the tested isolates. Furthermore, co-harbouring of carbapenemase and ESBL genes were represented in almost all isolates. The results showed that six isolates with *bla*$_{NDM-1}$ co-harboured *bla*$_{CTX-M}$ and *bla*$_{TEM}$ (**Table 9**). Additionally, CREC 18 carrying *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$, co-harboured ESBL genes (*bla*$_{CTX-M}$ and *bla*$_{TEM}$). *bla*$_{NDM-5}$ was found in nine isolates co-harboring ESBL genes (*bla*$_{CTX-M}$ and *bla*$_{TEM}$). However, two out of the nine isolates that harboured *bla*$_{NDM-5}$ had only *bla*$_{TEM}$. The results further showed that three of the isolates had no carbapenemase genes but carried ESBL genes. According to the Ambler classification method, carbapenemase-produced by *Enterobacteriaceae* can be classified into three classes including class A, class B, and class D β-lactamases (6). However, the clinical relevance of Ambler class C is still unknown (86). The most widely spread carbapenemase in *E. coli* include class A; KPC, class B; NDM-1, NDM-5, NDM-9, and VIM, class D; OXA-48, OXA-181, and OXA-244 (87, 88). Class A, B and D β-lactamases enzymes are plasmid-mediated and are responsible for the high levels of antimicrobial resistance and rapid dissemination by horizontal transfer

(89). Epidemiological studies have revealed the diversity of carbapenemases predominate in several regions and countries (89). In the United States, Argentina, Columbia, Greece, Israel, and Italy, KPC-producing *Enterobacteriaceae*, are mostly endemic among nosocomial isolates (1). NDM was reported as the main carbapenemase-mediating resistance in *E. coli* isolates in India, Pakistan, and Sri Lanka, whereas OXA-48 was reported in North Africa, Malta, and Turkey (90). NDM and OXA-48 were identified in both nosocomial and community-acquired pathogens (89, 91). A recent study done in Thailand reported a high prevalence (99%) of CREC isolates having at least one carbapenemase-producing gene (CP-gene) (3). The most common CP-gene among CREC isolates in Thailand were bla_{NDM} (94%) and a $bla_{\text{OXA-48-like}}$ (18%) gene (3). In this study, bla_{NDM} was found in 16 isolates, including seven isolates harbouring $bla_{\text{NDM-1}}$ and nine isolates harbouring $bla_{\text{NDM-5}}$. Similar results were reported in a recent study with a high prevalence of NDM-1 in *E. coli* (92). The increased usage of antibiotics may be driving the evolution of NDM-1 variants. M154L amino acid substitution in NDM-5 was the most common substitution in all NDMs variants leading to increase carbapenemase activity (93). However, a previous study reported that the difference in the activity of NDM-5 and NDM-1 is due to variations in the affinity for zinc (94). Moreover, V88L amino acid substitution in NDM-5 contribute to lower catalytic activity on imipenem and meropenem (95). Several studies showed that $bla_{\text{NDM-5}}$ was carried by IncX3 plasmids which have been

shown to be conjugatable and could explain the rapid spread of *bla*$_{\text{NDM-5}}$ carrying isolates (96). However, *bla*$_{\text{KPC}}$ which is the most commonly found in the United States (1), was not presented in this study. So far, the prevalence of *bla*$_{\text{KPC}}$ in Thailand has remained very low. A previous report indicated a 0.02% (n = 12,741) prevalence of *bla*$_{\text{KPC-13}}$ among *Enterobacteriaceae* and 1.7% (n = 181) among CRE isolates (97), whereas a separate report showed that the prevalence rate of *bla*$_{\text{KPC-2}}$ in CRE isolates was 0.13% (n = 2245) (98). Furthermore, the study illustrated the co-existence of carbapenemase and ESBL genes in CREC isolates. Carbapenems were used as first-line antibiotics for the treatment of infection caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. Thus, the co-harboring of multiple antibiotic resistance genes will promote multi-resistance, which might amount to significant therapeutic concerns.

Table 10. Effects of meropenem and aminoglycosides combinations on 19 carbapenem-resistant *Escherichia coli*.

Clinical Isolate	meropenem+amikacin		meropenem+gentamicin		meropene+kanamycin		meropenem+streptomycin		meropenem+tobramycin	
	MIC[a]	ΣFICI	MIC[a]	ΣFICI	MIC[a]	ΣFICI	MIC[a]	ΣFICI	MIC[a]	ΣFICI
CREC 1	8/0.5	0.38 (S)	2/8	0.16 (S)	8/4	0.38 (S)	1/256	0.27 (S)	4/2	0.19 (S)
CREC 2	16/0.125	0.31 (S)	2/8	0.16 (S)	16/4	0.50 (S)	1/256	0.27 (S)	4/2	0.31 (S)
CREC 3	8/16	0.31 (S)	2/16	0.27 (S)	32/8	0.38 (S)	32/32	0.38 (S)	4/8	0.09 (S)
CREC 4	4/1	0.31 (S)	8/8	0.38 (S)	8/16	0.38 (S)	8/64	0.25 (S)	8/4	0.25 (S)
CREC 5	16/0.25	0.31 (S)	2/8	0.16 (S)	4/16	0.31 (S)	8/64	0.25 (S)	8/4	0.25 (S)
CREC 6	8/2	0.63 (I)	8/0.125	0.25 (S)	4/8	0.31 (S)	8/8	0.25 (S)	2/2	0.28 (S)
CREC 7	8/2	0.31 (S)	8/8	0.13 (S)	8/32	0.19 (S)	4/128	0.28 (S)	8/8	0.19 (S)
CREC 8	2/2	0.27 (S)	2/16	0.14 (S)	32/32	0.50 (S)	128/1	1.02 (S)	8/8	0.19 (S)
CREC 9	4/2	0.28 (S)	16/8	0.25 (S)	8/32	0.19 (S)	8/128	0.31 (S)	16/8	0.25 (S)
CREC 10	4/2	0.53 (I)	4/0.125	0.28 (S)	16/2	0.38 (S)	16/8	0.38 (S)	32/0.5	0.75 (I)
CREC 11	32/32	0.53 (I)	16/512	0.75 (I)	64/8	1.01 (I)	64/8	1.25 (I)	64/1024	2.00 (I)
CREC 12	32/256	0.50 (S)	8/128	0.19 (S)	64/8	0.51 (I)	32/8	0.50 (S)	64/8	0.51 (I)
CREC 13	2/2	0.27 (S)	1/0.5	0.51 (I)	2/32	0.27 (S)	4/16	0.53 (S)	2/8	0.27 (S)
CREC 14	16/32	0.53 (I)	0.5/128	0.14 (S)	16/512	1.00 (I)	1/128	0.16 (I)	4/64	0.25 (S)
CREC 15	16/1	0.38 (S)	8/0.25	0.31 (S)	2/32	0.27 (S)	32/1	0.25 (S)	8/4	0.31 (S)
CREC 16	64/0.25	0.63 (I)	4/16	0.16 (S)	4/32	0.28 (S)	8/128	0.31 (I)	16/2	0.25 (S)
CREC 17	32/2	0.75 (I)	4/8	0.16 (S)	16/16	0.38 (S)	8/64	0.31 (S)	8/8	0.56 (I)

Clinical Isolate	meropenem+amikacin		meropenem+gentamicin		meropene+kanamycin		meropenem+streptomycin		meropenem+tobramycin	
	MIC[a]	**ΣFICI**	**MIC[a]**	**ΣFICI**	**MIC[a]**	**ΣFICI**	**MIC[a]**	**ΣFICI**	**MIC[a]**	**ΣFICI**
CREC 18	32/1	0.50 (S)	2/16	0.27 (S)	16/32	0.38 (S)	8/64	0.31 (S)	4/8	0.28 (S)
CREC 19	2/32	0.31 (S)	16/2	0.75 (I)	4/512	0.63 (I)	4/128	0.38 (S)	8/32	0.50 (S)

S, synergy; I, indifferent. [a] minimum inhibitory concentration of combination of meropenem/aminoglycoside. The FICI results for each combination were interpreted as follows: FICI ≤ 0.5, synergism; 0.5 < FICI ≤ 4, indifference; and FICI > 4, antagonism.

5. The Combined Effect of Meropenem and Aminoglycosides

The results of antimicrobial combinations against the 19 CREC isolates are shown in **Table 4** and summarized in **Table 11**. Synergistic effects were observed for meropenem plus gentamicin and meropenem plus streptomycin in 16 (84.2%) isolates, followed by meropenem plus kanamycin and meropenem plus tobramycin in 15 (79%) isolates. Furthermore, synergistic activity was observed in 13 (68.4%) isolates for meropenem plus amikacin. The isolate CREC 11 (*bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$), with high resistance to aminoglycosides, was resistant to all combinations, while isolate CREC 12 (*bla*$_{\text{NDM-5}}$, *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$) was susceptible to meropenem plus amikacin, or gentamicin, or streptomycin combinations. The combination of meropenem plus gentamicin and meropenem plus tobramycin exhibited synergism against CREC 14 (*bla*$_{\text{NDM-5}}$ and *bla*$_{\text{TEM}}$). The cross-resistance of CREC 11 to all the combinations might be due to the cumulative effects of other resistance mechanisms such as overexpression of efflux pump and/or porin with the β- lactamases leading to high level of resistance. However, the results did not reveal an antagonistic effect for the tested combinations.

The results revealed that the addition of aminoglycosides as adjunctive therapy to meropenem could restore meropenem activity against CREC isolate harbouring *bla*$_{\text{NDM}}$. Combination of meropenem and aminoglycosides might promote membrane disruption since aminoglycosides exert disruptive effects on the outer membrane structure by binding with the negatively charged lipopolysaccharides in the outer membrane of Gram- negative bacteria. Thus, the aminoglycoside promotes the permeabilizing effect and enhances the periplasmic target site penetration of other

antibiotics such as carbapenems used in combination (99, 100). Meropenem is a safe, well-tolerated, and commonly used as monotherapy or as combination regimens for hospital-acquired infection due to several MDR Gram-negative bacteria (101, 102). Similarly, aminoglycosides are effective against Gram-negative aerobic bacteria including resistant *Enterobacteriaceae* (103). However, aminoglycosides monotherapies can lead to unfavourable clinical outcomes due to rapid emergence of resistance, and nephrotoxicity among patients with prolonged usage of aminoglycosides (104, 105).

Table 11. Summary of the synergistic effects of meropenem in combination with aminoglycosides against 19 carbapenem-resistant *Escherichia coli*.

Combination	**Outcomes**	
	Synergism (%)	**Indifference** (%)
meropenem + amikacin	13 (68.4)	6 (31.6)
meropenem + gentamicin	16 (84.2)	3 (15.8)
meropenem + kanamycin	15 (78.9)	4 (21.1)
meropenem + streptomycin	16 (84.2)	3 (15.8)
meropenem + tobramycin	15 (78.9)	4 (21.1)

6. Time-Kill Assay

The time-kill effects of meropenem combined with either amikacin, gentamicin, kanamycin, streptomycin, or tobramycin were evaluated on CREC 12 (Figure 2). The results revealed a synergistic bactericidal effect at 1/4 meropenem plus 1/4 amikacin at 4 h. (Figure 2A) and 1/4 meropenem plus 1/4 gentamicin at 2 h. (Figure 2B) with

a ≥ 3 $\log_{10}$ CFU/mL reduction in cell growth when compared to the MIC of individual antibiotics. Furthermore, an indifferent effect was revealed at 1/4 meropenem plus 1/4 kanamycin (Figure 2C). At 12 h, the combination between 1/4 meropenem plus 1/4 streptomycin (Figure 2D) presented a synergistic bactericidal effect, while the combination of 1/4 meropenem plus 1/4 Tobramycin revealed a synergistic effect (Figure 2E). For CREC 18 at 8 h, 1/4 meropenem plus 1/4 amikacin showed a synergistic bactericidal effect (Figure 3A). Similar results were observed at 4 h with 1/4 meropenem plus 1/4 gentamicin (Figure 3B), at 8 h for 1/4 meropenem plus 1/4 kanamycin (Figure 3C), or 1/4 streptomycin (Figure 3D), and at 2 h for 1/4 meropenem plus 1/4 tobramycin against isolate CREC 18 (Figure 3E). However, a regrowth was observed at 8 h for meropenem and tobramycin combination, and at 12 h for meropenem and amikacin or gentamicin combination. Our results showed inconsistencies between the FICI and time-kill methods. Similar findings have been reported by previous studies (106, 107).

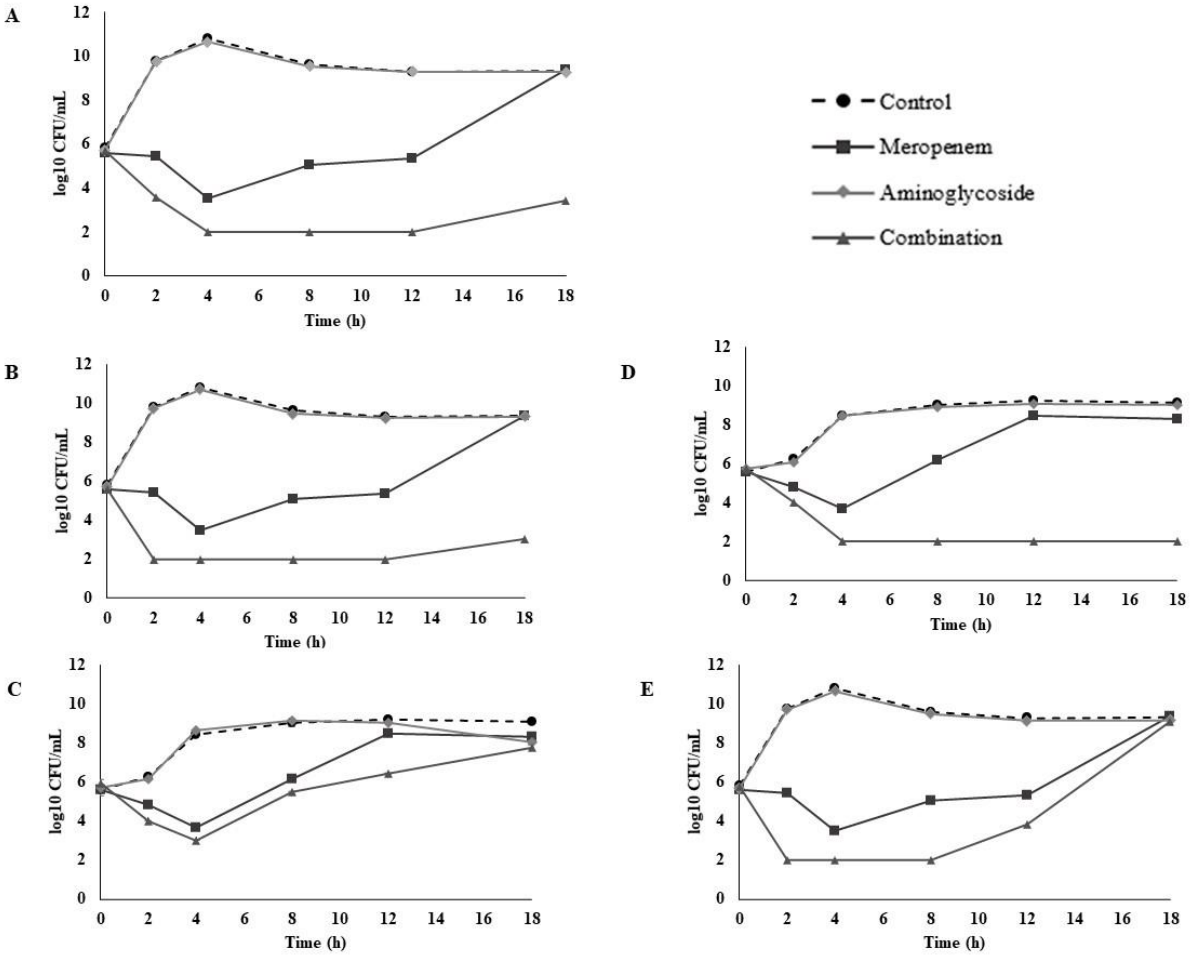

Figure 2. Time-kill curves of 1/4 MIC (32 µg/mL) meropenem and 1/4 MIC aminoglycosides combination against CREC 12. (A) Amikacin (256 µg/mL), (B) Gentamicin (256 µg/mL), (C) Kanamycin (256 µg/mL), (D) Streptomycin (8 µg/mL), and (E) Tobramycin (256 µg/mL).

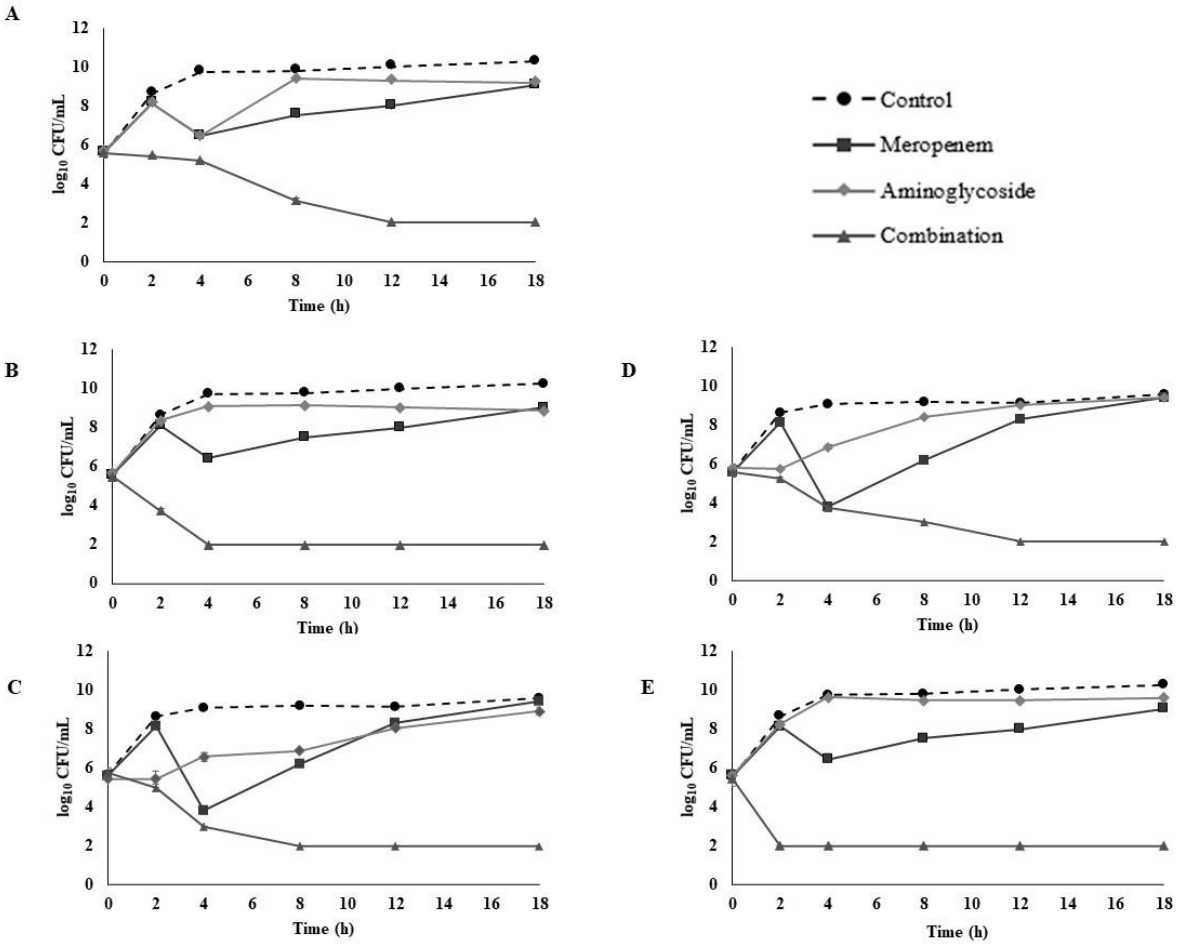

Figure 3. Time-kill curves of 1/4 MIC (32 µg/mL) meropenem and 1/4 MIC aminoglycosides combination against CREC 18. (A) Amikacin (1 µg/mL), (B) Gentamicin (16 µg/mL), (C) Kanamycin (32 µg/mL), (D) Streptomycin (64 µg/mL), and (E) Tobramycin (8 µg/mL).

CHAPTER 4

CONCLUSION

Combination therapies have been highlighted as a possible treatment option for the management of infections caused by drug-resistant bacterial isolates. This study demonstrated that combinations of meropenem with aminoglycoside might still be an efficient therapeutic option for the treatment of CREC harbouring *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{NDM-5}}$. However, due to indifferent results observed with the FICI, it is important to consider other mechanisms of aminoglycoside and carbapenem co- resistance. In addition, further studies on toxicology, pharmacokinetics and pharmacodynamics of these combination regimens are required prior to clinical trials.

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APPENDIX

Culture Media

1. Cation-adjusted Mueller-Hinton broth

Approximate Formula *Per Liter

Beef Extract	3.0	g
Acid Hydrolysate of Casein	17.5	g
Starch	1.5	g

Suspend 22 g of the power in 1 L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 116 to 121°C for 10 minutes. Do not overheat at any time. Test samples of the finished product for performance using stable, typical control cultures.

2. Tryptic Soy Agar

Approximate Formula *Per Liter

Pancreatic Digest of Casein	15.0	g
Papic Digest of Soybean	5.0	g
Sodium Chloride	5.0	g
Agar	15.0	g

Suspend 40.0 g of the powder in 1 L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes.

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Publication in Journal

1. **Terbtothakun P,** Nwabor OF, Siriyong T, Voravuthikunchai SP, Chusri S. Synergistic antibacterial effects of meropenem in combination with aminoglycosides against carbapenem-resistant *Escherichia coli* harboring $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-5}}$. Antibiotics (Basel, Switzerland). 2021:10(8):1023.

2. Nwabor OF, **Terbtothakun P**, Voravuthikunchai SP, Chusri S. Evaluation of the synergistic antibacterial effects of Fosfomycin in combination with selected antibiotics against carbapenem-resistant *Acinetobacter baumannii*. Pharmaceuticals. 2021;14(3):185.

3. Nwabor OF, **Terbtothakun P**, Voravuthikunchai SP, Chusri S. A bibliometric meta-analysis of colistin resistance in *Klebsiella pneumoniae*. Diseases. 2021;9(2):44.

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