Synergistic activities of antibiotic combinations against carbapenem-resistant *Klebsiella pneumoniae* and mechanisms of carbapenem resistance in *Klebsiella pneumoniae* clinical isolates.

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A Thesis Submitted in Partial Fulfillment of the Requirements
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การเสริมฤทธิ์ของยาต้านจุลชีพต่อเชื้อ *Klebsiella pneumoniae* ที่ดื้อยาในกลุ่มคาร์บาพีเนมและกลไกการดื้อยากลุ่มคาร์บาพีเนมในเชื้อ *Klebsiella pneumoniae* ที่แยกได้จากผู้ป่วย

น.ส.เนตรชนก มู่ฮำหมัดอารี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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เนตรชนก มู่ฮำหมัดอารี : การเสริมฤทธิ์ของยาต้านจุลชีพต่อเชื้อ *Klebsiella pneumoniae* ที่ดื้อยาในกลุ่มคาร์บาพีเนมและกลไกการดื้อยากลุ่มคาร์บาพีเนมในเชื้อ *Klebsiella pneumoniae* ที่แยกได้จากผู้ป่วย. (Synergistic activities of antibiotic combinations against carbapenem-resistant *Klebsiella pneumoniae* and mechanisms of carbapenem resistance in *Klebsiella pneumoniae* clinical isolates.) อ.ที่ปรึกษาหลัก : อ. ดร.ธนิษฐา ฉัตรสุวรรณ

การดื้อยาในกลุ่ม carbapenems ของเชื้อ *Klebsiella pneumoniae* พบเพิ่มสูงขึ้นทั่วโลก วัตถุประสงค์ของการศึกษานี้ เพื่อตรวจหากลไกการดื้อยากลุ่ม carbapenems ในเชื้อ *K. pneumoniae* ที่แยกได้จากผู้ป่วยไทยและศึกษาการเสริมฤทธิ์กันของยาต้านจุลชีพต่อเชื้อ *K. pneumoniae* ที่ดื้อยาในกลุ่ม carbapenems โดยทำการศึกษาในเชื้อ *K. pneumoniae* ที่ดื้อยาในกลุ่ม carbapenems (CRKP) จำนวน 240 ตัวอย่าง ซึ่งแยกได้จากผู้ป่วยที่เข้ารักษาในโรงพยาบาลจุฬาลงกรณ์ ระหว่างเดือนกันยายน พ.ศ. 2559 ถึง พ.ศ. เมษายน 2563 ทำการทดสอบความไวรับต่อยาต้านจุลชีพต่อยา imipenem, meropenem, amikacin, fosfomycin และ ciprofloxacin โดยวิธี agar dilution และวิธี broth microdilution ต่อยา colistin ความชุกในการดื้อต่อยา imipenem, meropenem, ciprofloxacin, fosfomycin, amikacin, และ colistin คือ 90.42, 91.67, 95.84, 41.25, 20, และ 12.08% ตามลำดับ เชื้อ CRKP ส่วนใหญ่ (62.92%) เป็นเชื้อที่ดื้อต่อยาต้านจุลชีพหลายขนาน จากเชื้อ CRKP ทั้งหมดจำนวน 240 ตัวอย่าง พบว่า 236 ตัวอย่าง (98.33%) มียีน carbapenemase อย่างน้อย 1 ชนิด โดยพบ $bla_{\text{NDM-like}}$ ร่วมกับ $bla_{\text{OXA-48-like}}$ มากที่สุด (43.75%) ตามด้วย $bla_{\text{NDM-like}}$ (27.50%), $bla_{\text{OXA-48-like}}$ (25.42%), $bla_{\text{IMP-like}}$ (0.83%), และ $bla_{\text{OXA-48-like}}$ ร่วมกับ $bla_{\text{IMP-like}}$ (0.83%) ตรวจไม่พบ $bla_{\text{KPC-like}}$ และ $bla_{\text{VIM-like}}$ เชื้อ *K. pneumoniae* ที่มียีน carbapenemase ทั้งหมด 236 ตัวอย่าง ให้ผลบวกต่อการทำงานของเอนไซม์ carbapenemase ซึ่งทดสอบโดยวิธี modified carbapenem inactivation method (mCIM) การแสดงออกที่เพิ่มขึ้นของ efflux pump ทดสอบโดยใช้ carbonyl cyanide m-cholorophenyl hydrazone (CCCP) เป็นตัวยับยั้ง ตรวจพบได้ในเชื้อ CRKP 13.33% พบการขาดหายไปของ OmpK35 และ/หรือ OmpK36 โดยใช้วิธี SDS-PAGE ในเชื้อ CRKP 98.33% การสร้างเอนไซม์ carbapenemase ร่วมกับการขาดหายไปของ OmpK เป็นกลไกที่พบมากที่สุดในการดื้อต่อยาในกลุ่ม carbapenems (83.33%) โดยพบค่า MIC ของยา carbapenems ในระดับสูงในตัวอย่างที่มียีน $bla_{\text{NDM-like}}$ ร่วมกับ $bla_{\text{OXA-48-like}}$ มีการขาดหายไปของ OmpK35 และ OmpK36 และมีการแสดงออกที่เพิ่มขึ้นของ efflux pump

การใช้ยาต้านจุลชีพร่วมกัน ได้แก่ colistin ร่วมกับ imipenem, colistin ร่วมกับ meropenem, fosfomycin ร่วมกับ amikacin, และ fosfomycin ร่วมกับ imipenem ถูกนำมาตรวจหาการเสริมฤทธิ์กันโดยวิธี checkerboard ในตัวแทนเชื้อ CRKP จำนวน 31 ตัวอย่าง ผลการศึกษาพบว่าการใช้ยา fosfomycin ร่วมกับ imipenem ให้ผลการเสริมฤทธิ์มากที่สุด (22.58%) ตามด้วย fosfomycin ร่วมกับ amikacin (14.81%) นอกจากนี้พบการเสริมฤทธิ์ของ fosfomycin ร่วมกับ imipenem ในเชื้อ CRKP 3 ตัวอย่างที่ให้ผล additive จากการใช้ยา colistin ร่วมกับ carbapenems เมื่อทำการทดสอบเพื่อยืนยันผลการเสริมฤทธิ์กันของยา โดยวิธี Time-kill ในเชื้อ CRKP 7 ตัวอย่าง พบว่า การทดสอบโดยวิธี Time kill สอดคล้องกับวิธี checkerboard assay และพบความสามารถในการฆ่าเชื้อ (bactericidal activity) ใน CRKP 6 ตัวอย่าง(85.71%) การศึกษานี้สรุปได้ว่าการสร้างเอนไซม์ carbapenemase และการขาดหายไปของ OmpK เป็นกลไกหลักที่พบในเชื้อ *K. pneumoniae* ที่ดื้อยาในกลุ่ม carbapenems ที่แยกได้จากผู้ป่วยไทย และการใช้ยา fosfomycin ร่วมกับ imipenem อาจจะเป็นหนึ่งในทางเลือกที่ใช้ในการรักษาการติดเชื้อ CRKP

สาขาวิชา	จุลชีววิทยาทางการแพทย์	ลายมือชื่อนิสิต .....
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Netchanok Muhummudaree : Synergistic activities of antibiotic combinations against carbapenem-resistant *Klebsiella pneumoniae* and mechanisms of carbapenem resistance in *Klebsiella pneumoniae* clinical isolates.. Advisor: TANITTHA CHATSUWAN, Ph.D.

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has been increasing reported worldwide. The aims of this study were to investigate the mechanisms of carbapenem resistance in *K. pneumoniae* Thai isolates and study the synergistic activities of antibiotic combinations against CRKP isolates. Two-hundred and forty CRKP clinical isolates were obtained from patients at King Chulalongkorn Memorial Hospital between September 2016 and April 2020. The antibiotic susceptibility testing was determined by agar dilution to imipenem, meropenem, amikacin, fosfomycin, ciprofloxacin and by broth microdilution to colistin. The prevalence rates of resistance to imipenem, meropenem, ciprofloxacin, fosfomycin, amikacin, and colistin were 90.42%, 91.67%, 95.84%, 41.25%, 20%, and 12.08%, respectively. The majority of CRKP isolates (62.92%) were multidrug-resistant (MDR). Of the 240 CRKP isolates, 236 (98.33%) harbored at least one carbapenemase gene. The $bla_{\text{NDM-like}}$ plus $bla_{\text{OXA-48-like}}$ were the most common carbapenemase genes (43.75%), followed by $bla_{\text{NDM-like}}$ (27.50%), $bla_{\text{OXA-48-like}}$ (25.42%), $bla_{\text{IMP-like}}$ (0.83%), and $bla_{\text{OXA-48-like}}$ plus $bla_{\text{IMP-like}}$ (0.83%). Neither $bla_{\text{KPC-like}}$ nor $bla_{\text{VIM-like}}$ were detected. All isolates harboring carbapenemase genes were positive for carbapenemase activity by modified carbapenem inactivation method (mCIM). The overexpression of efflux pump was detected in 13.33% of CRKP isolates by using carbonyl cyanide m-cholorophenyl hydrazone (CCCP) inhibitor. The loss of OmpK35 and/or OmpK36 was found in 98.33% of CRKP isolates by SDS-PAGE. The combination of carbapenemase production and loss of OmpK was the most common mechanism of carbapenem resistance (83.33%). High level of carbapenem MIC (≥64 mg/L) was found in isolates harboring $bla_{\text{NDM-like}}$ plus $bla_{\text{OXA-48-like}}$ together with loss of both OmpK35 and OmpK36, and overexpression of efflux pump.

Antibiotic combinations including colistin plus imipenem, colistin plus meropenem, fosfomycin plus amikacin, and fosfomycin plus imipenem were screened for the synergistic activity by checkerboard assay in 31 representative isolates. The most effective combination was fosfomycin plus imipenem which showed synergism in 22.58%, followed by 14.81% in fosfomycin plus amikacin combination. Three CRKP isolates that had additive effect in colistin-based combination showed synergistic effect in fosfomycin plus imipenem combination. Time-kill assay was used to confirm the synergism against 7 CRKP isolates. The results from time-kill assay were consistent with the checkerboard assay. The bactericidal activity was found in 6 isolates (85.71%). In conclusion, this study showed that the carbapenemase production and loss of OmpK were the major mechanisms of carbapenem resistance in CRKP Thai isolates. The combination of fosfomycin plus imipenem may be one of the alternative antibiotic combination for treatment of CRKP infections.

Field of Study:	Medical Microbiology	Student's Signature .....
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Netchanok Muhummudaree

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ABBREVIATIONS

AK	Amikacin
Asp (D)	Aspartic acid
ATCC	American Type Culture Collection
bla	β-lactamase gene
bp	Base pairs
^{0}C	Degree Celsius
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CT	Colistin
DNA	Deoxynucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
et al	Et alii
ETP	Ertapenem
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FICI	Fractional inhibitory concentration index

FOS	Fosfomycin
G6P	Glucose-6-phosphate
G	Glycine
IMP	Imipenem
IS	Insertion sequence
kDa	Kilodalton
MEM	Meropenem
mg	Milligram
MIC	Minimum inhibitory concentration
ml	Milliliter
mm	Millimeter
OMP	Outer membrane protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
T	Threonine
µL	Microliter

CHAPTER I
INTRODUCTION

Klebsiella pneumoniae is a Gram-negative, encapsulated, rod-shaped bacterium, belonging to the family Enterobacteriaceae (1). *K. pneumoniae* is an important opportunistic pathogen in both of nosocomial- and community-acquired infections including pneumonia, urinary tract infection (UTI), bloodstream infection and soft tissue infection, which are mostly found in patients with underlying diseases (1, 2). *K. pneumoniae* can form biofilm on medical devices (*e.g.*, catheters and endotracheal tubes) that provide a significant source of infections in catheterized patients (1).

The first line antibiotics for treatment of *K. pneumoniae* infection are β-lactams group (*e.g.,* cephalosporins, and carbapenems), aminoglycosides (*e.g.,* gentamicin, and amikacin), fluoroquinolones, and tetracycline (3). Nowadays, *K. pneumoniae* has been reported to be resistant to these antibiotics especially carbapenems. The emergence of carbapenem resistance in *K. pneumoniae* has been increasing reported worldwide (4).

Carbapenems are bactericidal β-lactam antibiotics that are effective against many Gram- positive, Gram-negative and anaerobic bacteria (5). However, infections caused by carbapenem-resistant *K. pneumoniae* (CRKP) become the major problem worldwide. The CDC has identified carbapenem-resistant Enterobacterales as an urgent threat to human health (6). In Thailand, the percentages of carbapenem-resistant *K. pneumoniae* reach to 10-11% in 2019, which increases more than 50% when compared with those (2.3–3.4%) in 2015 (7). CRKP is associated with high mortality rates especially in immunocompromised patients (8).

The mechanisms of carbapenem resistance in *K. pneumoniae* are involved by i) carbapenemase production, ii) the deficiency or loss of outer membrane proteins (OMPs), iii) the overexpression of AcrAB-TolC efflux pumps. The major mechanism of

carbapenem resistance is the carbapenemase production. There are three main groups of carbapenemase enzymes including ambler class A (penicillinases) (*e.g.*, KPC (*K. pneumoniae* carbapenemase), class B (metallo-β-lactamases) (*e.g.*, NDM, IMP, VIM, GIM, and SIM), and Class D (oxacillinases) (*e.g.*, OXA-48)

The treatment options of CRKP infections are monotherapy and combination therapy including polymyxins (colistin and polymyxin B), tigecycline, and fosfomycin (9). However, the monotherapy is not effective for treatment of infections caused by CRKP infection. There are reports of high mortality rates of monotherapy treatment (10, 11). The side effects of colistin monotherapy are nephrotoxic (50–60%) and neurotoxic (9). Several studies showed that the combination therapy may be the better way for CRKP infections when compared with monotherapy (9, 12, 13).

The aim of combination therapy is the synergistic activity. Colistin- based combination including colistin plus carbapenems (*e.g.,* doripenem, imipenem and meropenem), tigecycline, and rifampicin showed synergistic activity against CRKP (14, 15). On the other hand, some studies showed antagonistic activity of colistin plus tigecycline combination against CRKP isolates (16). Another antibiotic combination is fosfomycin- based combination. Fosfomycin plus carbapenems or amikacin combination showed synergistic activity and suppressed growth of fosfomycin or amikacin-resistant *K. pneumoniae* (17, 18). However, some studies showed regrowth of CRKP at 24 hrs. in fosfomycin-based combination (19).

Nowadays, *K. pneumoniae* isolates are resistant to carbapenems, the last resort of antibiotics for the treatment of infections. The combination therapies are the treatment options for CRKP infection. However, there are few studies on the mechanisms of carbapenem resistance and synergistic activity of antibiotic combination against CRKP clinical isolates in Thailand. Therefore, this study aims to study the carbapenem resistance mechanisms and synergistic activities of antibiotic combinations against CRKP clinical isolates from King Chulalongkorn Memorial Hospital, Thailand.

CHAPTER II

OBJECTIVES

1. To study the synergistic activities of antibiotic combinations against CRKP Thai isolates.
2. To investigate the mechanisms of carbapenem resistance in *K. pneumoniae* Thai isolates.
3. To study the association between the mechanisms of carbapenem resistance and the synergistic activities of antibiotic combinations against CRKP Thai isolates.

CHAPTER III
LITERATURE REVIEW

1. *K. PNEUMONIAE*

1.1 Features of *K. pneumoniae*

K. pneumoniae is a Gram-negative, encapsulated, rod-shaped bacterium, belonging to the family Enterobacteriaceae (1). *K. pneumoniae* is able to ferment lactose, oxidase-negative, non-motile, and does not produce indole. The *K. pneumoniae* colonies on MacConkey agar are pink and mucoid. The optimum temperature for growth of *K. pneumoniae* is at 37 °C.

K. pneumoniae can colonizes mucosal surfaces such as gastrointestinal tract and oropharynx (1, 2). *K. pneumoniae* is an important pathogen responsible for nosocomial- and community-acquired infections including pneumonia, urinary tract infection (UTI), bloodstream infection and soft tissue infection which are mostly in patients with underlying diseases (1, 2). *K. pneumoniae* can form biofilm on medical devices (*e.g.,* catheters and endotracheal tubes) that provide a significant source of infection in catheterized patients (1).

1.2 Pathogenesis and virulence of *K. pneumoniae*

K. pneumoniae virulence factors can help them to penetrate host cells and escapes them from the host immune response including humoral and cellular innate immunity.

1.2.1 Capsule

Capsule is a polysaccharide matrix that coats the cell. It contains 4 to 6 sugars and uronic acids (as negatively charged components). The capsules can be classified into 78 serological types (20). Acapsular *K. pneumoniae* strains are less virulent than encapsulated strains in mouse models (21). The roles of capsules of *K. pneumoniae* are preventing phagocytosis and opsonophagocytosis from immune cells, hiding the

bactericidal action of antimicrobial peptides (*e.g.,* human beta-defensins 1 to 3, and lactoferrin), blocking complement components (*e.g.*, C3) from interacting with the membrane, and reduction of pro-inflammatory cytokine production (*e.g.*, reactive oxygen species(ROS), IL-8, IL-6, and TNF-α production) (2). The roles of capsule in *K. pneumoniae* virulence are shown in Figure 1.

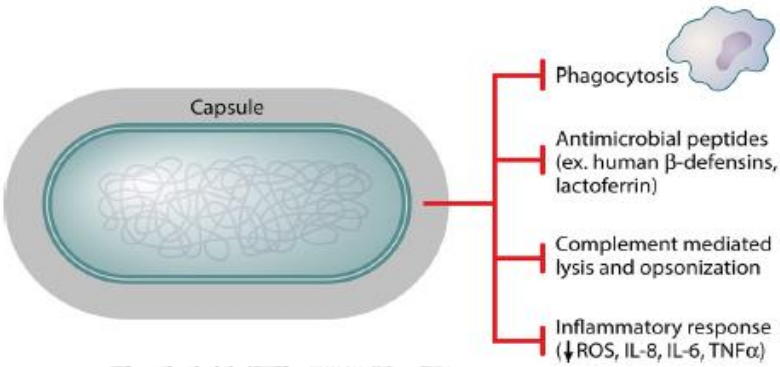


Figure 1. The roles of capsule in *K. pneumoniae* virulence (2).

1.2.2 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is a major component of outer membrane of all Gram-negative bacteria (2). LPS can divided into 3 major subunits including lipid A, an oligosaccharide core, and O antigen. O antigen can be classified into 9 serotypes and O1 is the most common serotype in *K. pneumoniae* isolates (2). The important role of O antigen is protected against humoral defense (*i.e.*, complement). It can inhibit C1q binding and complement activation. The inflammatory response of host can activate by lipid A. In addition, the lipid A can inhibit the bactericidal action of cationic antimicrobial peptides (2). The roles of LPS in *K. pneumoniae* virulence are shown in Figure 2.

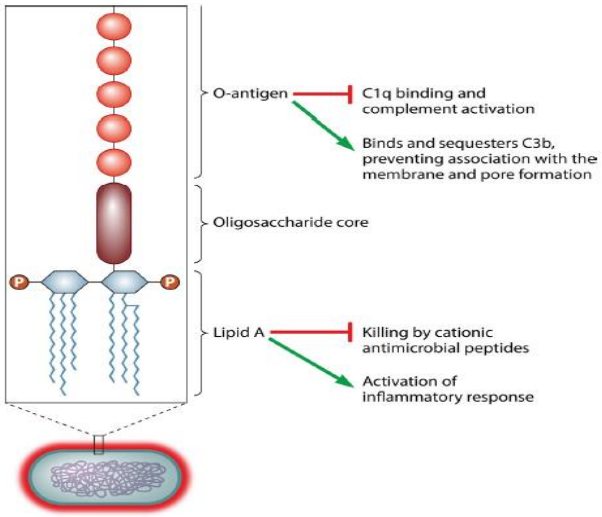


Figure 2. The roles of LPS in *K. pneumoniae* virulence (2).

1.2.3 Types 1 and 3 fimbriae

Types 1 and 3 fimbriae are the major adhesive structures in *K. pneumoniae* that located on the bacterial cell surface. Type 1 fimbriae are thin and filamentous and type 3 fimbriae are helix-like filaments. The role of type 1 fimbriae is involved in an invasion into bladder cell and biofilm formation within the bladder-epithelial cell (22). The role of type 3 fimbriae to mediate *in vitro* the formation of *K. pneumoniae* biofilms on both abiotic and biotic surface (22). They can bind to the surface of medical devices (*e.g.,* urinary catheters), leading to the ability to grow and persist in the patient. The structures of type 1 and 3 fimbriae are shown in Figure 3.

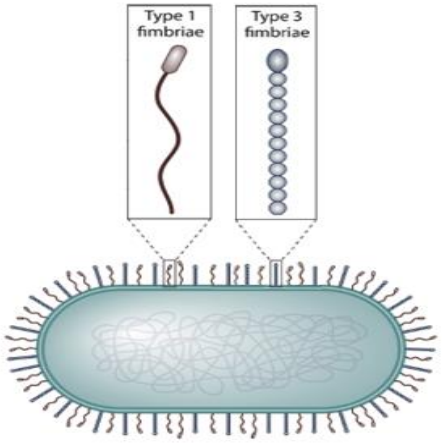


Figure 3. The structures of types 1 and 3 fimbriae (2).

1.2.4 Siderophores

The iron is a limited resource and essential factor in bacterial growth. *K. pneumoniae* must obtain iron from the environment for growth during infection. Therefore, *K. pneumoniae* secrete the siderophores proteins for stealing iron from host iron-chelating proteins or scavenging it from the environment (2). The siderophores were reported in *K. pneumoniae* including enterobactin, salmochelin, yersiniabactin, and aerobactin. The classical *K. pneumoniae* phenotypes can produce only enterobactin, and yersiniabactin. In addition, the hypervirulent *K. pneumoniae* phenotypes can also produce salmochelin, and aerobactin (23). The excess of iron in host's tissue increased bacterial pathogenicity (22).

1.2.5 Urease

Many strains of *K. pneumoniae* can produce the extracellular urease enzyme. The urease can hydrolyze urea to ammonia and carbamate. Therefore, urease is the virulence factor that plays a role in urinary tract infection and the formation of infection stones (22).

2. TREATMENT OF *K. PNEUMONIAE* INFECTIONS

The antibiotics for treatment of *K. pneumoniae* infection are β-lactams (*e.g.,* cephalosporins, and carbapenems), aminoglycosides (*e.g.,* gentamicin, and amikacin), fluoroquinolones, and tetracycline (3). Nowadays, carbapenem resistance in Enterobacteriaceae has been increasing worldwide. The Centers for Disease Control and Prevention (CDC) identified carbapenem-resistant Enterobacteriaceae (CRE) as an urgent threat to human health (6). The treatment of CRKP infection is difficult to treat because fewer antibiotics are effective against them. Polymyxin E (colistin), tigecycline, and fosfomycin have been reported to be effective antibiotics for CRKP infection (3).

3. CARBAPENEMS

Carbapenems are one of the bactericidal β-lactam antibiotics, which is composed of a β-lactam ring in a molecule. Carbapenems have effective activity against many Gram-positive, Gram-negative, and anaerobic bacteria (5, 24). The mode of action of carbapenems is the inhibition of penicillin- binding proteins (PBPs) or transpeptidases (TPases) which are enzymes involved in peptidoglycan biosynthesis, resulting in a weak of cell wall. Finally, bacterial cell is destroyed by osmotic pressure (25).

The most common carbapenems that used for treatment of Gram-negative infections are doripenem, ertapenem, imipenem, meropenem. They contain a carbapenem backbone but different on the side chain at C2 of carbapenem backbone as shown in Figure 4 (26).

Imipenem is the first carbapenem that is used for treatment for severe infections caused by cephalosporin-resistant Gram-negative bacteria (25). Imipenem are degraded by dehydropeptidase-1 (DHP-1) enzyme that is located in renal tubules. So, it requires co-administration of a DHP-1 inhibitor (*e.g.*, cilastatin) for inhibiting the DHP-1 degradation and prolonging its half-life (25). The later carbapenems including ertapenem, meropenem, and doripenem are stable to DHP-1 degradation because they contain a 1-β- methyl constituent on the carbapenem backbone (25).

Carbapenems are antibiotics that are used to treat moderate to severe patients with nosocomial infection. The *in vitro* activity of carbapenems against Gram-positive and Gram-negative bacteria demonstrated that meropenem and doripenem had slightly more active than imipenem against Gram-negative bacteria (26). On the other hand, imipenem had slightly more active against Gram-positive bacteria than meropenem (26). Ertapenem has limitation in treatment of non-fermentative Gram-negative bacteria (*e.g., P. aeruginosa*) infections that are commonly caused by nosocomial pathogens. Therefore, the role of ertapenem in treatment is suitable for mild to moderate patients with community-acquired infection (27, 28).

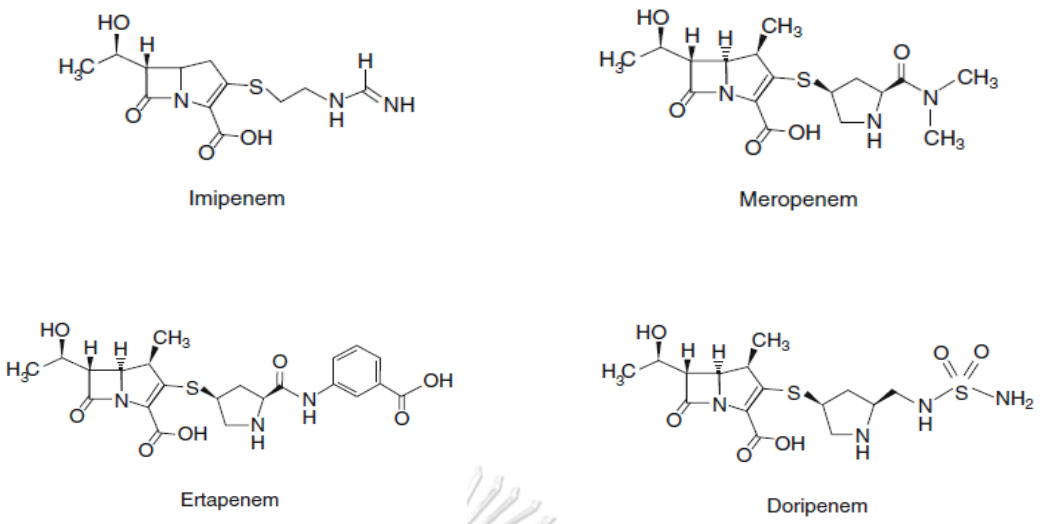


Figure 4. Chemical structures of carbapenems (26).

4. CARBAPENEM RESISTANCE IN *K. PNEUMONIAE*

CDC defined CRE in two criteria including i) non-susceptibility to at least one of carbapenems (*e.g.*, including imipenem, meropenem, ertapenem, or doripenem) of Enterobacteriaceae, and ii) the production of carbapenemase enzymes of Enterobacteriaceae that make them resistant to carbapenems (29). Many organisms of Enterobacterales became to CRE such as *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., and *Serratia* spp. (30). Several studies showed that the prevalence of CRKP is higher than other members of the Enterobacteriaceae family (30-32). Therefore, CRKP becomes a major problem in healthcare systems worldwide.

4.1. The mechanism of carbapenem resistance in *K. pneumoniae.*

The mechanisms of carbapenem resistance in *K. pneumoniae* are carbapenemase productions, the loss of outer membrane proteins and overexpression of efflux pumps (33).

4.1.1 Carbapenemase productions

The production of carbapenemases is the major carbapenem resistance mechanism in *K. pneumoniae* (34). The carbapenemases are bacterial periplasmic enzymes that hydrolyze β-lactam ring structure of antibiotics, preventing the

antibiotics from binding the PBP target (24). These enzymes are divided into 3 classes including Ambler class A, B, and D β-lactamases. (35).

Class A carbapenemases contain serine atom at the active site and are partially inhibited by β-lactamase inhibitors (*e.g.*, clavulanic acid, boronic acid, and tazobactam) (35). The members of this enzyme group are chromosomally encoded-enzyme group (*e.g.,* NmcA (not metalloenzyme carbapenemase A), SME, IMI-1, and SFC-1) and plasmid encoded-enzyme group *(e.g.,* KPC-2 to KPC-13*,* GES-1 to GES-20, IMI-2*)* (36). KPC is the most common class A carbapenemase and was first reported in Spain in 2001 (37). The bla_{KPC} gene is plasmid-encoded and is transported in a Tn*4401* that causes it rapidly transferable between other bacteria (38). The endemic areas of KPC-producers are USA, China, Italy, Greece, and Taiwan (36, 39-41).

Class B carbapenemases require one or more zinc atoms at the active site and is inhibited by ethylenediaminetetraacetic acid (EDTA) (35). The most common class B metallo-β-carbapenemase families include NDM-1, IMP, VIM, GIM, and SIM. VIM- and IMP-producers are commonly found in Europe and Asia (36). The endemic areas of NDM-producers are India, Pakistan, and Bangladesh (36, 42, 43). NDM producers have also been reported in USA and south Africa (13, 44-46). NDM and IMP-producing *K. pneumoniae* have been reported in Thailand (47, 48).

Class D carbapenemases contain serine atom at the active site and are poorly inhibited by a β-lactamase inhibitor (*e.g.,* clavulanic acid, boronic acid, and tazobactam) and EDTA (35). They commonly hydrolyze isoxazolylpenicillins (*e.g.*, oxacillin, cloxacillin, and dicloxcillin). They can hydrolyze carbapenems but have very weak activity against extended-spectrum cephalosporins (*e.g*., cefepime, and ceftazidime) (49). The class D oxacillinase families include 12 subgroups (*e. g.,* OXA-23, OXA-24/40, OXA-48, OXA-51, OXA-58, OXA-134a, OXA-143, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235) (36). However, only the OXA-23, OXA-48, OXA-51, and OXA-58 have been reported in *K. pneumoniae* (50). The OXA-48-producers have been reported in Turkey, Egypt, Tunisia, India, Thailand (36).

4.1.2 The deficiency or loss of outer membrane proteins (OMPs)

The outer membrane proteins (OMPs) or porins are the channel-forming membrane proteins that allow the transport of small hydrophilic molecules (*e.g.*, iron, nutrients, and antibiotics into the bacterial cell) (51). Two major outer membrane porins in *K. pneumoniae* are OmpK35 and OmpK36 (52). The pore size of OmpK35 is slightly larger than OmpK36, which makes it easier for large and/or lipophilic β-lactams to pass through the ompK35 pores (52, 53). OmpK36 appears to be more cation-selective than OmpK35 (53).

The porin-related mutations can cause *K. pneumoniae* resistance to β-lactams, fluoroquinolones, and chloramphenicol (54). The deficiency or loss of OmpK35 and/or OmpK36 is associated with the reduced susceptibility of carbapenems in *K. pneumoniae* (51). The altered *ompK35* genes contained insertion sequence disruption (*e.g.,* IS*10* insertion), frameshift mutation *(e.g.,* one bp insertion, or one bp deletion, or one amino acid substitutions), and nonsense point mutation, causing a premature stop codon (55-57).

The altered of *ompK36* genes contain insertion sequence disruption (*e.g.*, IS*5*, IS*1*, IS*4*, IS*903*) (57, 58), causing a disrupting the open reading frame. The nonsense point mutation and frameshift mutation were founded in *ompK36* gene, but these mutations were found less than mutations in *ompK35* gene (56, 57, 59). Moreover, the insertion of two amino acids such as glycine (G) and aspartic acid (D) (GD insertion) at positions 134-135, or aspartic acid (D) and threonine (T) (DT insertion) at position 137-138 were associated with increased doripenem MICs (60), and ertapenem MICs (61), respectively. However, the only loss of outer membrane proteins showed a little reduction of carbapenem susceptibility. Tsai *et al.* (51) demonstrated that the combination of the loss or downregulation of outer membrane proteins with cephalosporinases (*e.g.*, ESBL or AmpC) production can lead to resistance or reduced susceptibility to carbapenems in non-carbapenemase-producing *K. pneumoniae*. Therefore, the loss of outer membrane proteins is not the main carbapenem resistance mechanism.

4.1.3. Overexpression of efflux pumps.

Bacterial efflux pumps are proteins that are localized and imbedded in the cytoplasmic membrane. The function of bacterial efflux pumps is to drive many compounds (*e.g.*, antibiotics) out of cells. The most significant efflux pumps in Gram-negative bacteria are members of resistance nodulation-division (RND) family (62). The RND efflux pump family is the secondary transporter obtaining energy from proton(H^+)(63). AcrAB-TolC, an RND efflux pump family, is associated with carbapenem resistance in *K. pneumoniae* (64). The AcrAB-TolC is divided into 3 parts: inner membrane AcrB protein, the outer membrane protein TolC and the membrane fusion protein AcrA (65).

The expression of *acrAB* operon is controlled by local repressor AcrR and several global transcriptional regulators such as AraC family, RamA, MarA, SoxS, and RarA (66). The regulatory pathways for expression of the AcrAB-TolC efflux pump are shown in Figure 5. The expression of *ramA*, *marA*, and *soxS* genes are repressed by *ramR*, *marR*, and *soxR*, respectively. Therefore, the mutations in these genes might lead to upregulation of the efflux pump (66). Findlay *et al.* (67) demonstrated that the overexpression of efflux pumps due to premature stop codon of *ramR* gene together with other resistance mechanisms play a role in reduced meropenem susceptibility in the patient who received meropenem therapy. The overexpression of efflux pump activity can be detected using efflux pump inhibitors such as cyanide *m*-chlorophenylhydrazone (CCCP) , and phenylalanine-arginine beta-naphthylamide (PAβN). However, the result from this method showed the controversial effects of overexpression of AcrAB and carbapenem resistance in *K. pneumoniae*. Filgona *et al.* (68) found that the ertapenem, and doripenem MICs were decreased four-fold MIC values in the presence of CCCP, efflux pump inhibitor. In contrast, the study by Padilla *et al.* (69) found that no carbapenem MICs were changed in the presence of CCCP.

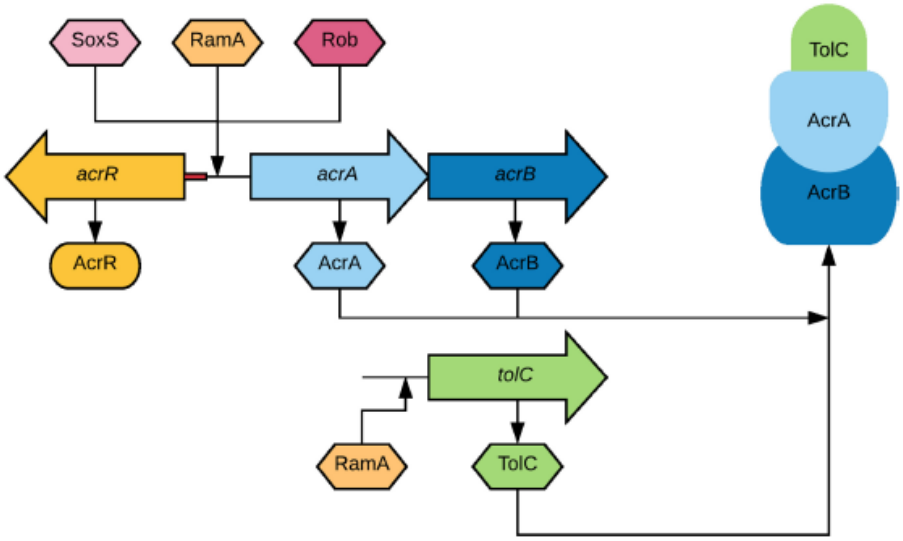


Figure 5. The regulatory pathways for expression of the AcrAB-TolC efflux pump (70).

4.2. The prevalence of carbapenem-resistant *K. pneumoniae*

CRKP was first reported emerged in 1996 in North Carolina, USA (71). The report from National Antimicrobial Resistance Surveillance Center Thailand (NARST) showed that the prevalence of CRKP during 2003-2019 increased from 0.8 % to 12.5 % for ertapenem. Likewise, the prevalence of CRKP was 1% for imipenem and 1.2 % for meropenem in 2000. It dramatically increased to 10.1% for imipenem and 11% for meropenem in 2019 (31). The European Centre for Disease Prevention and Control found that the prevalence of CRKP was 6.1 % in 2016 and increased to 7.5% in 2018 (32, 72). The report of India's Antimicrobial Resistance Surveillance and Research Network (IAMRSN) found that the prevalence of imipenem-non-susceptible *K. pneumoniae* was increased to 54.4 % in 2019 (73). The Surveillance of Multicenter Antimicrobial Resistance in Taiwan (SMART) found 5.2 to 7.2 % of CRKP in 2018 that was higher rate than 2.9 to 5.8 % of CRKP in 2016 (74, 75). On the other hand, some country reported low prevalence of CRKP such as 2.9 % of CRKP in Malaysia in 2017 (76), 1 % of CRKP in Cambodia in 2016 (77), 0.7 % of CRKP in Japan in 2019 (78).

However, the trend of prevalence of CRKP in these countries is higher than in previous years.

The prevalence of major carbapenemases such as KPC, IMP, VIM, NDM, and OXA-48 family is dependent on geographic regions. KPC and its variants are commonly found in USA, European countries, and Asia (*e.g.*, China, Hongkong, and Taiwan) (79). NDM is commonly found in Asia (*e.g.,* India, Bangladesh, and Pakistan) (79). OXA-48 and its variant are commonly found in middle east (*e.g.*, Turkey, Saudi Arabia, Israel) (79). Nowadays, several studies showed the spreading of carbapenemase producer to other regions (44-46, 80, 81). The prevalence of NDM producers were increased and became to the second carbapenemase producer commonly found in China (80, 81). NDM producers have been reported in USA (44-46). However, the NDM producers remained low prevalence in USA (82). In India, there are reports of NDM coproduced with other carbapenemases in CRKP such as NDM and KPC co-producers or NDM and OXA-48 co-producers (83, 84). The prevalence of NDM, OXA-48, IMP, and KPC producers were reported in Thailand (47, 85, 86). These studies showed that the prevalence of carbapenemase-producing *K. pneumoniae* was found at a low level (0.33 to 11.11%) and co-producers of carbapenemases were not present in 2009-2011. Surprisingly, Laolerd *et al.* reported high prevalence of CRKP (71.43%) and NDM and OXA-48 coproducers (32.74%) were detected in 2016 (87). So, the emergence of CRKP has become a significant problem.

5. TREATMENT OF CARBAPENEM-RESISTANT *K. PNEUMONIAE* INFECTION

CRKP isolates frequently resist to carbapenems and other antibiotics. The monotherapy is not effective for treatment of infections caused by these bacteria. Daikos *et al.* (10) reported that the morality rate of the patients with carbapenemase-producing *K. pneumoniae* bloodstream infection was 27.2 % by combination therapy and 44.4 % by monotherapy. Tumbarello *et al.* (11) demonstrated that the mortality rate of patients with KPC-producing *K. pneumoniae* infection was 30.2 % by combination therapy and 38.4 % by monotherapy.

The aim of combination therapy is the synergistic activity of antibiotics against CRKP. Many antibiotic combinations against CRKP were studied. The synergistic activity of antibiotic combinations including colistin, carbapenems (*e.g.*, imipenem, meropenem, ertapenem, doripnem), amikacin, tigecycline, fosfomycin, rifampicin was reported (14, 15, 17).

Several studies showed that the colistin-based combinations are effective against CRKP. The combination of colistin plus carbapenems including imipenem, meropenem, and doripenem showed synergy and bactericidal activity (14, 15, 88, 89). Lim *et al*. (88) reported that the combination of doripenem and polymyxin B was more effective than other carbapenems in polymyxin B-based combination. The combination of colistin and rifampicin were reported to be effective against CRKP (14, 15, 90, 91). The combination of colistin with a new drug such as tigecycline has been investigated. The colistin and tigecycline combination showed synergy and bactericidal activity against ESBL-producing *K. pneumoniae* isolates with carbapenem resistance (92) and carbapenemase-producing *K. pneumoniae* isolates (55, 93). However, some studies showed the antagonism activity of this combination against KPC-producing *K. pneumoniae* in murine thigh infection model (16, 18). The mechanism of colistin and other antibiotic combinations may be the disruption of the outer membrane by colistin and enhancing another antibiotic entry into the cell (89).

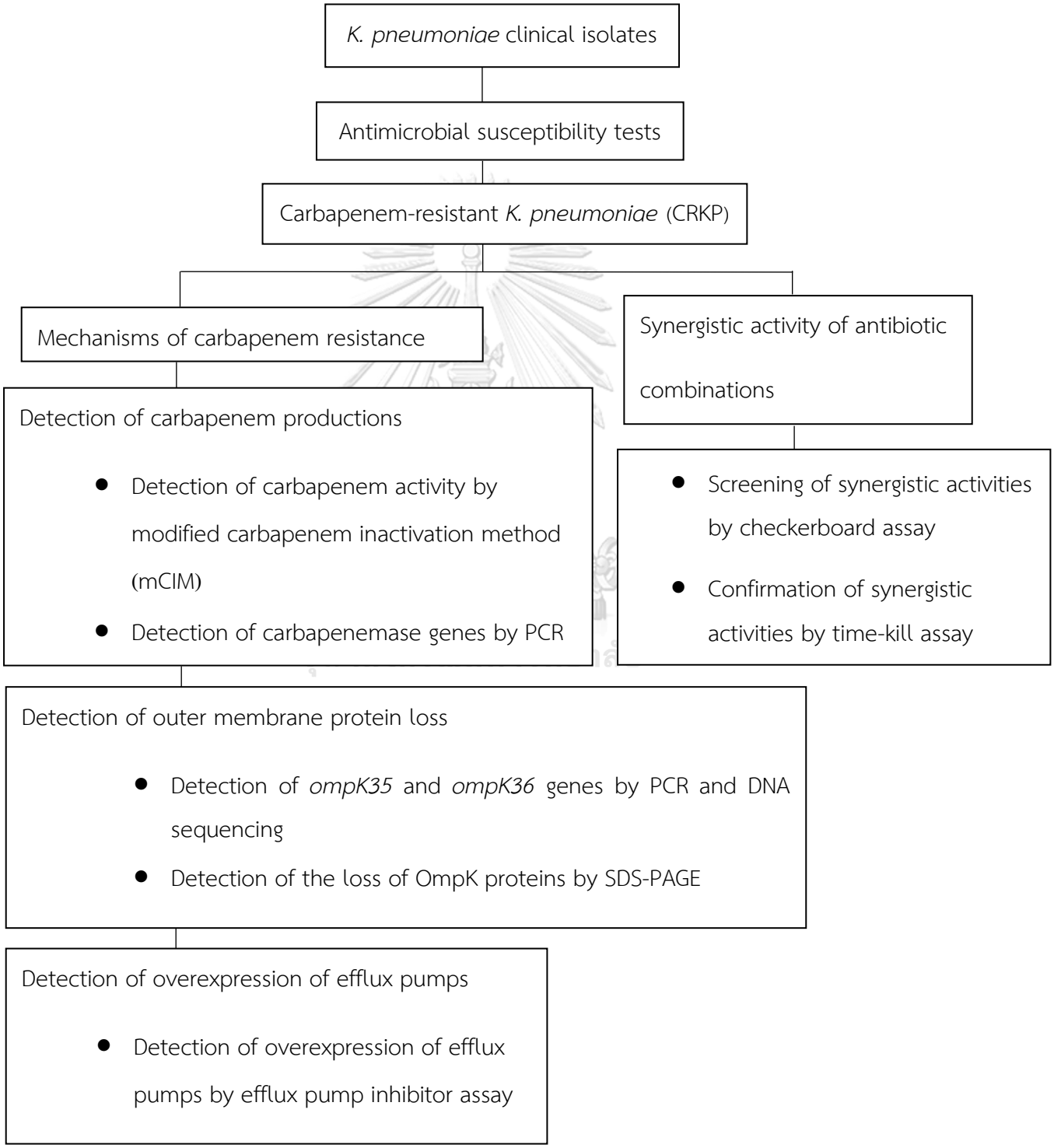
Other effective combinations are fosfomycin-based combination such as fosfomycin plus carbapenems or amikacin. The combination of fosfomycin and carbapenems showed the synergistic activity against CRE and non-fermentative bacteria (17, 94-96). The combination of fosfomycin and amikacin showed the synergistic activity and bactericidal effect against carbapenemase-producing *K. pneumoniae* (17) and multidrug-resistant non-fermentative Gram-negative bacteria (97). Sime *et al.* (18) demonstrated that the combination of fosfomycin and amikacin efficiently suppressed growth of fosfomycin or amikacin-resistant *K. pneumoniae*. However, some studies showed regrowth of CRKP at 24 hr. in fosfomycin-based

combination (19). So, the synergistic activity of this combination against CRKP is controversial. The mechanism of fosfosmycin and other antibiotic combinations may cause by the disruption of the cell wall synthesis by fosfomycin and may increase uptake of another antibiotic into the cell (17).

CHAPTER IV

MATERIALS AND METHODS

Methodology scheme



1. BACTERIAL STRAINS

1.1 Sample size

A collection of *K. pneumoniae* isolates were obtained from different patients at King Chulalongkorn Memorial Hospital, Thailand. The report from Laolerd *et al*. (87) showed that 81.95% of carbapenem-resistant *K. pneumoniae* were carbapenemase producers.

Determination of sample sizes:

$$N = \frac{Z_{\alpha}^{2}\, PQ}{d^{2}}$$

P = Prevalence

Q = 1-P

D = acceptable error = 0.05

$N = 1.96^2 \times 0.8195 \times (1-0.8195) / 0.05^2$

N = 227.3 isolates

A total of 227 carbapenem-resistant *K. pneumoniae* clinical isolates were used in this study.

1.2. *K. pneumoniae* isolates

A total of 240 CRKP isolates were obtained from different patients at King Chulalongkorn Memorial Hospital, Thailand between September 2016 and April 2020. All isolates were identified by conventional method. The biochemical tests included triple sugar iron medium test (TSI), motility test, indole test, citrate utilization test, urease test and growth at 37 ^{0}C. The pure isolates were kept in tryptic soy broth with 20 % glycerol and stored at -80 ^{0}C.

1.3. Quality control strains for MIC determination

Staphylococcus aureus ATCC25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control strains for susceptibility testing.

2. ANTIMICROBIAL SUSCEPTIBILITY TESTING

All 240 CRKP isolates were subjected to the antibiotic susceptibility testing for carbapenems (imipenem and meropenem), aminoglycosides (amikacin), fosfomycin, and ciprofloxacin by agar dilution method. The colistin susceptibility testing was determined by broth microdilution method. The minimum inhibitory concentrations (MICs) of imipenem, meropenem, amikacin, ciprofloxacin, fosfomycin, and colistin were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) (98). *S. aureus* ATCC25923, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were used as control strains.

As for agar dilution method, each antimicrobial agent was adjusted to 2-fold dilution with sterile deionized distilled water and mixed well in Mueller-Hinton agar (MHA) (BBL, BD Diagnostic Systems, Sparks, MD). The final concentration of each antimicrobial agent was 0.007-512 mg/L. As for fosfomycin susceptibility testing, each agar plate was supplemented with 25 mg/L of glucose-6-phosphate (G6P) (Sigma-Aldrich Chemical Co., St. Louis, MO) as recommended by CLSI. The broth microdilution method was performed in 96-well culture plates. The serial 2-fold dilution of colistin was prepared in cation-adjusted MHB (CAMHB) (BBL, BD Diagnostic Systems, Sparks, MD), ranging from 0.03-512 mg/L of colistin concentration.

CRKP isolates, *S. aureus* ATCC25923, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were subcultured on MHA and incubated at 37 ^{o}C for 18-24 hrs. The pure colonies were suspended in 3 ml of sterile normal saline (NSS) and adjusted turbidity to 0.5 McFarland (1.5 x 10^{8} CFU/ml). After that each suspension was diluted 1:10 with NSS and applied onto MHA supplement with antibiotics as final

concentration approximately 10^4 CFU/spot and incubated at 37 °C for 18-24 hrs. For broth microdilution method, the adjusted suspension was diluted 1:100 with NSS and 20 ul of adjusted suspension were applied into CAMHB with colistin as final concentration approximately $1.5x10^5$ CFU/ml and incubated at 37 °C for 18-24 hrs.

Table 1. Standard MICs for interpretation of *K. pneumoniae*

Antimicrobial agent	MIC QC ranges (mg/L)				Reference
	S. aureus ATCC 25923	*E. faecalis* ATCC 29212	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	
Imipenem	0.016-0.06	0.5-2	1-4	-	(98)
Meropenem	0.03-0.12	2-8	0.008-0.06	0.12-1	(98)
Ciprofloxacin	0.12-0.5	0.25-2	0.004-0.016	0.12-1	(98)
Amikacin	1-4	64-256	0.5-4	1-4	(98)
Fosfomycin	0.5-4	32-128	0.5-2	2-8	(98)
Colistin	-	-	0.25-2	0.5-4	(98)

Table 2. Acceptable ranges for quality control strains used for monitoring the accuracy of MICs

Antimicrobial agent	Interpretation MIC (mg/L)			Reference
	Susceptible	Intermediate	Resistant	
Imipenem	≤1	2	≥4	(98)
Meropenem	≤1	2	≥4	(98)
Ciprofloxacin	≤1	2	≥4	(98)
Amikacin	≤16	32	≥64	(98)
Fosfomycin	≤64	128	≥256	(98)
Colistin	-	≤2	>2	(98)

3. DETECTION OF CARBAPENEMASE GENES AND CARBAPENEMASE PRODUCTION

3.1. Detection of carbapenemase activity

A total 240 CRKP isolates were determined for carbapenemase production by modified carbapenem inactivation method (mCIM) as recommended by CLSI (99). CRKP isolates were subcultured on MHA and incubated at 37 ^{o}C for 18-24 hrs. One µl loopful of pure colonies was suspended in 2 ml tryptic soy both (TSB) and vortexed for 10 to 15 seconds. A 10 µg meropenem disk was added to each tube using sterile forceps and incubated at 37 ^{o}C for 4 hrs ± 15 minutes. The pure colony of *E. coli* ATCC 25922 was suspended in 3 ml of NSS and adjusted turbidity to 0.5 McFarland (1.5 x 10^{8} CFU/ml) and inoculated onto MHA plates. The meropenem disk from each TSB-meropenem disk suspension was removed from the tube and then placed on the MHA plate previously inoculated with *E. coli* ATCC 25922. The MHA plates were invested and incubated at 37 ^{o}C for 18-24 hrs. The NDM or KPC-producing *K. pneumoniae* that confirmed carbapenemase genes by multiplex PCR were used for positive control. The *K. pneumoniae* ATCC 13883 was used for negative control.

As for modified carbapenem inactivation method (mCIM) result interpretation, the diameter of the zone of inhibition around each meropenem disk was measured. A zone diameter of 6-15 mm or the presence of pinpoint colonies within a 16-18 mm zone was considered carbapenemase positive (*i.e.,* test isolate produced carbapenemase). A zone diameter of ≥ 19 mm with clear zone was considered carbapenemase negative (*i.e.,* test isolate did not produce carbapenemase). A zone diameter of 16-18 mm or ≥ 19 mm and the presence of pinpoint colonies within the zone was considered carbapenemase indeterminate (*i.e.,* the presence or absence of a carbapenemase cannot be confirmed).

3.2. Detection of carbapenemase genes

A total 240 CRKP isolates were determined for carbapenemase genes including class A (bla_{KPC}), class B (bla_{NDM}, bla_{IMP} and bla_{VIM}), and class D bla_{oxa-48} carbapenemases by multiplex PCR.

3.2.1 DNA preparation

The DNA templates were prepared by heat-lysis method. A single colony of CRKP isolates was suspended in 50 µl of sterile distilled water and boiled at 100 °C for 10 minutes. The boiling suspension was centrifuged at 13,000 rpm for 10 minutes. The supernatant was used as DNA template and stored at -20 °C.

3.2.2 Primers

The carbapenemase genes including class A (bla_{KPC}), class B (bla_{NDM}, bla_{IMP} and bla_{VIM}), and class D bla_{OXA-48} carbapenemases were determined by multiplex PCR. The specific primers for screening carbapenamase genes are listed in Table 3.

3.2.3 Amplification of $bla_{KPC-like}$, $bla_{NDM-like,}$ and $bla_{OXA-48-like}$ genes by multiplex PCR

The specific primers for $bla_{KPC-like}$, $bla_{NDM-like,}$ and $bla_{OXA-48-like}$ genes were previously described by Poirel *et al.* (100). The multiplex PCR was performed in final volume of 25 µl/reaction, containing 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs) (Thermo Fisher Scientific, USA), 0.2 mM OXA-48F and OXA-48R primers, 0.4 mM KPC-F and KPC-R primers, 0.6 mM of NDM-F and NDM-R primers, and 1.25 U *Taq* polymerase (Thermo Fisher Scientific, USA), and 2 µl DNA template. The PCR conditions were contained initial denaturation step (94 °C, 10 min) followed by 35 cycles of denaturation (94 °C, 30 s), annealing (57 °C, 40 s) and extension (72 °C, 50 s), and a single final extension of 5 min at 72°C.

3.2.4 Amplification of $bla_{VIM-like}$, and $bla_{IMP-like}$ genes by multiplex PCR.

The specific primers for $bla_{VIM-like}$, and $bla_{IMP-like}$ genes were previously described by Ellington *et al.* (101). The multiplex PCR was performed in final volume of

25 µl/reaction, 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.4 mM of IMP-F and IMP-R, 0.16 mM VIM-F and VIM-R primers, and 0.625 U *Taq* polymerase and 2 µl DNA template. The PCR conditions were contained initial denaturation step (94 °C, 5 min) followed by 35 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and extension (72 °C, 50 s), and a single final extension of 5 minutes at 72 °C.

3.2.5 Analysis of PCR products

The PCR products were analyzed by electrophoresis on 1.5% agarose gel in 0.5 X Tris-borate-EDTA buffer (TBE; 0.045 M Tris-borate, 0.0001 M EDTA pH 8.3±1) and 1x concentration of StainIN™ GREEN Nucleic Acid Stain was added to the solution before pouring the gel into a casting tray. The 6X loading dye buffer was mixed with PCR products and loaded into the gel in electrophoresis chamber containing of 0.5X TBE. Electrophoresis was run for 35 minutes at 100 volts and visualized using Gel Documentation System (Bio-Rad).

Table 3. Specific primers for amplification of carbapenemase genes

Carbapenemase gene	Primer	Sequence (5′->3′)	Product size (bps)	Reference
bla$_{KPC-like}$	KPC-F	CGTCTAGTTCTGCTGTCTTG	798	(100)
	KPC-R	CTTGTCATCCTTGTTAGGCG		
bla$_{NDM-like}$	NDM-F	GGTTTGGCGATCTGGTTTTC	621	(100)
	NDM-R	CGGAATGGCTCATCACGATC		
bla$_{OXA-48-like}$	OXA48-F	GCGTGGTTAAGGATGAACAC	438	(100)
	OXA48-R	CATCAAGTTCAACCCAACCG		
bla$_{VIM-like}$	VIM-F	GATGGTGTTTGGTCGCATA	390	(101)
	VIM-R	CGAATGCGCAGCACCAG		
bla$_{IMP-like}$	IMP-F	GGAATAGAGTGGCTTAAYTCTC	188	(101)

4. DETECTION OF ESBL AND AMPC GENES

4.1. Detection of ESBL genes

The non-carbapenemase-harboring *K. pneumonaie* isolates were determined for AmpC and ESBL genes by multiplex PCR. The DNA template was performed as described in section 3.2.1.

4.1.1 Primers

The ESBL genes were determined by multiplex PCR. The specific primers for screening ESBL genes are listed in Table 4.

4.1.2. Amplification of *bla*$_{OXA-1}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ genes by multiplex PCR

The specific primers for *bla*$_{OXA-1}$, *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were previously described by Colom *et al*. (102). The specific primers for screening ESBL genes are listed in Table 4. The multiplex PCR was performed in final volume of 25 µl/reaction, 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.06 mM of OXA-1-F and OXA-1-R, 0.04 mM of TEM-C and TEM-H, and 0.08 mM of SHV-F and SHV-R, 0.5 U *Taq* polymerase and 3 µl DNA template. The PCR conditions were contained initial denaturation step (94 °C, 5 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 1 min), and a single final extension of 10 minutes at 72 °C.

4.1.3 Amplification of *bla*$_{CTX-M}$ and *bla*$_{VEB}$ genes by multiplex PCR

The specific primers for *bla*$_{CTX}$ and *bla*$_{VEB}$ genes were previously described by Bonnet *et al*. (103) and Udomsantisuk *et al*. (104). The multiplex PCR was performed in final volume of 25 µl/reaction, 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.1 mM of CTX-MA and CTX-MB, 0.05 mM of VEB-A and VEB-B, 0.5 U *Taq* polymerase and 3 µl DNA template. The PCR conditions were contained initial denaturation step (94 °C, 5 min) followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 1 min), and a single final extension of 10 minutes at 72 °C.

4.1.4 Analysis of PCR products

The PCR products were analyzed as described in section 3.2.5

Table 4. Specific primers for amplification of ESBL genes

ESBL genes	Primer	Sequence (5′->3′)	Product size (bps)	Reference
bla_{VEB}	VEB-A	CCTTTTGCCTAAAACGTGGA	216	(104)
	VEB-A	TGCATTTGTTCTTCGTTTGC		
bla_{SHV}	SHV-F	AGGATTGACTGCCTTTTTG	392	(102)
	SHV-R	ATTTGCTGATTTCGCTCG		
bla_{TEM}	TEM-C	ATCAGCAATAAACCAGC	516	(102)
	TEM-H	CCCCGAAGAACGTTTTC		
bla_{CTX-M}	CTX-MA	CGCTTTGCGATGTGCAG	550	(103)
	CTX-MB	ACCGCGATATCGTTGGT		
bla_{OXA-1}	OXA-F	ATATCTCTACTGTTGCATCTCC	619	(102)
	OXA-R	AAACCCTTCAAACCATCC		

4.2. Amplification of AmpC genes by multiplex PCR

4.2.1 Primers

The AmpC genes in *K. pneumoniae* isolates were screened for bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC} and bla_{FOX} by multiplex PCR. The specific primers of AmpC genes are listed in Table 5.

4.2.2 Amplification of bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC} and bla_{FOX} by multiplex PCR

The specific primers for bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC} and bla_{FOX} were reported previously by Perez *et al.* (105). The multiplex PCR was performed in final volume of 25 µl/reaction, 1X buffer, 2mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.8 mM of MOXMF, MOXMR, FOXMF and FOXMR primers, 0.6 mM of DHAMF and DHAMR primers, 0.5 mM of ACCMF and ACCMR primers, 0.4 mM of CITMF and CITMR primers, 0.3 mM of EBCMF and EBCMR primers and 1.25 U *Taq* polymerase and 2 µl DNA template. The PCR conditions were contained denaturation step (94°C, 3 min) followed by 25 cycles

of denaturation (94°C, 30 s), annealing (62°C, 30 s) and extension (72°C, 1 min) and a single final extension of 10 min at 72°C.

4.2.4 Analysis of PCR products

The PCR products were analyzed as described in section 3.2.5

Table 5. Specific primers for amplification of AmpC genes

AmpC genes	Primers	Sequence (5´->3´)	Product size (bps)	Reference
MOX	MOXMF	GCTGCTCAAGGAGCACAGGAT	520	(105)
	MOXMR	CACATTGACATAGGTGTGGTGC		
CIT	CITMF	TGGCCAGAACTGACAGGCAAA	462	(105)
	CITMR	TTTCTCCTGAACGTGGCTGGC		
DHA	DHAMF	AACTTTCACAGGTGTGCTGGGT	405	(105)
	DHAMR	CCGTACGCATACTGGCTTTGC		
ACC	ACCMF	AACAGCCTCAGCAGCCGGTTA	346	(105)
	ACCMR	CCCCGAAGAACGTTTTC		
EBC	EBCMF	TCGGTAAAGCCGATGTTGCGG	392	(105)
	EBCMR	CTTCCACTGCGGCTGCCAGTT		
FOX	FOXMF	AACATGGGGTATCAGGGAGATG	190	(105)
	FOXMR	CAAAGCGCGTAACCGGATTGG		

5. DETECTION OF ESBL AND AMPC ACTIVITY

5.1. Detection of ESBL activity by combination disk method

CRKP isolates, which had no carbapenemase genes were further investigated of the presence of ESBL by combination disk method (106). The pure colonies were suspended in 3 ml of NSS and adjusted turbidity to 0.5 McFarland (1.5×10^8 CFU/ml). Disks containing 30 ug CTX or CAZ disks with and without 10 ug clavulanic acid were placed on MHA and incubated at 37 °C for 18-24 hrs. An increased zone size of ≥5 mm in the presence of clavulanic acid was considered as ESBL production.

5.2. Detection of AmpC acitivity by modified Hodge test (MHT)

CRKP isolates, which had no carbapenemase genes, were further investigated for the presence of AmpC acitivity by modified Hodge test (MHT). The pure colony of *E. coli* ATCC 25922 was suspended in 3 ml of NSS and adjusted turbidity to 0.5 McFarland (1.5 x 10^8 CFU/ml) and inoculated onto MHA plates. The cefoxitin disk (30 µg) was placed at the center of the MHA plate. The single cultured *K. pneumoniae* colony was picked and drowned from the edge of cefoxitin disk approximate 3-5 mm to the margin of MHA plate. After incubation at 35 °C for 18-24 hrs, the presence of distorted inhibition zone was interpreted as positive for modified Hodge test. (107).

6. DETECTION OF *OMPK35* AND *OMPK36* GENES AND OUTER MEMBRANE PROTEINS

6.1. Detection of *ompK35* and *ompK36* genes

A total 240 CRKP isolates were determined for o*mpK35* and o*mpK36* genes by PCR. The DNA template was performed as described in section 3.2.1.

6.1.1 Primers

The *ompK* genes including o*mpK35* and o*mpK36* genes were determined by PCR. The specific primers for screening o*mpK35* and o*mpK36* genes are listed in Table 6. The PCR products of *ompK35* and *ompK36* genes were 320 bps and 393 bps, respectively.

6.1.2 Amplification of *ompK35 and ompK36* genes by PCR

The specific primers for *ompK35* and *ompK36* genes were previously described by Hamzaoui *et al.* (108). The PCR was performed in final volume of 25 µl/reaction, 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.2 mM of OmpK35-F and OmpK35-R primers or 0.2 mM of OmpK36-F and OmpK36-R primers, and 0.625 U *Taq* polymerase and 2 µl DNA template. The PCR conditions were contained initial denaturation step (95 °C, 3 min) followed by 30 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 30 s) and extension (72 °C, 30 s), and a single final extension of 5 minutes at 72 °C.

6.1.3 Analysis of PCR products

The PCR products were analyzed as described in section 3.2.5

6.2 Amplification of entire *ompK35* and *ompK36* genes

The entire genes of *ompK35* and *ompK36* were amplified by simplex PCR using the specific primers in Table 5. The PCR products of entire *ompK35* and *ompK36* genes were 1938 bps and 1609 bps, respectively. The PCR was performed in final volume of 25 µl/reaction, 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.2 mM of OmpK35-out F and OmpK35- out R primers or 0.2 mM of OmpK36- out F and OmpK36- out R primers, and 0.625 U *Taq* polymerase and 2 µl DNA template. The PCR conditions were contained initial denaturation step (95 ℃, 3 min) followed by 30 cycles of denaturation (95 ℃, 15 s), annealing (60 ℃, 30 s) and extension (72 ℃, 30 s), and a single final extension of 5 min at 72 ℃. The PCR products were analyzed as described in section 3.2.5

The PCR product was purified by using HiYield™ Gel/PCR DNA Mini kit. The purified PCR products were sequenced by using BIgDye Terminator V3.1 Cycler sequencing kit by 1st Base DNA sequencing service, Malaysia. The nucleotide sequence of entire *ompK* gene was converted to amino acid sequence by using ExPASy translates tool (https://web.expasy.org/translate/). The amino acid sequence was analyzed by using Basic Local Alignment Search Tool (BLAST) from National Center for Biology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 6. Specific primers for amplification of *ompK* genes

***ompK* genes**	**Primer**	**Sequence (5′->3′)**	**Product size (bps)**	**Reference**
ompK35	OmpK35-out F	AAGACTACTGGTGGTTATCGCGACCT	1938	(108)
	OmpK35-out R	CGACAAAAAGCGCGAAGGTTT		
	OmpK35-F2	GTCGAAGCGGCAACCGATTATG	320	(108)
	OmpK35-R2	GCTTCGGCTTTGTCGCCATT		
ompK36	OmpK36-out F	CCATTAATCGAGGCTCCTCTTACCA	1609	(108)
	OmpK36-out R	CCGGTTGAAATAGGGGTAAACAGAC		
	OmpK36-F2	GAGTTGCGTTGTAGGTCTGG	393	(108)
	OmpK36-R2	GGCGACACCTACGGTTCTGACAA		

6.3. Detection of outer membrane proteins

6.3.1 Outer membrane protein extraction

The outer membrane proteins of *K. pneumoniae* were extracted by using ultracentrifugation as described by Barwa *et al.* (109). A pure colony of each CRKP isolate was grown in nutrient broth (NB) pH 7.00 ± 0.2 at 37 ℃ for 18-24 hrs. with shaking. Then, 20 ml of bacterial overnight cultures were added to 80 ml of nutrient broth (NB) pH 7.00 ± 0.2 at 37 ℃ for 4 hrs. with shaking. Logarithmic phase cultures were collected by centrifugation at 5,000 rpm for 30 minutes at 4 ℃, resuspended with 10 ml of cool phosphate saline buffer (PBS) and sonicated by using sonicator at amplitude 35-40% for 5 minutes (with 30 seconds of pulse and 30 seconds of off) on ice. Then, the cell debris was pelleted at 5,000 rpm for 30 minutes at 4 ℃ and the supernatant was centrifuged at 100,000 g for 1 hr. The pellets were incubated with 1 % sodium *N*-lauryl sarcosine for 30 min at 25 °C and collected by ultracentrifugation at 100,000 g for 1 hr. and resuspended with PBS. The OMPs were stored at 4 ℃.

6.3.2 Study of OMP profiles by SDS-PAGE

The protein concentration was determined by using Bio-Rad assay. Ten µg protein of OMPs of each bacterial isolate were mixed with 6X loading buffer and incubated at 95 °C for 15 minutes. The OMP sample was performed using 10% polyacrylamide of stacking gel and 12% acrylamide of separate gel by electrophoresis at 80 voltage for 2 hrs. The separate gel with OMPs was stained with coomassie brilliant blue and destained with distilled water. The OMPs of *K. pneumoniae* ATCC 13883 were used as OMP control as described by Chen *et al.* (110). The mass size of OmpK35 and OmpK36 were 37 and 36 kDa., respectively (111).

7. DETECTION OF OVEREXPRESSION OF EFFLUX PUMPS

7.1 Detection of overexpression of efflux pump by efflux pump inhibitor assay

The efflux pump activity was detected by using efflux pump inhibitor, carbonyl cyanide m-chlorophenylhydrazone (CCCP). The MHA was supplemented with imipenem or meropenem at two-fold dilution of concentration (0.007 to 1024 mg/L)

and other set of MHA was supplemented with imipenem or meropenem and 20 mg/L of CCCP. The bacterial pure colonies were suspended in 3 ml of sterile NSS and adjusted turbidity to 0.5 McFarland (1.5 x 10^8 CFU/ml). After that, each suspension was diluted 1:10 with sterile NSS and applied onto MHA supplement with antibiotics with CCCP or without CCCP as final concentration approximately 10^4 CFU/spot and incubated at 37 ^{o}C for 18-24 hrs. A decrease at least 4 folds of imipenem or meropenem MIC with CCCP was positive for efflux pump activity (68).

8. STUDY SYNERGISTIC ACTIVITIES OF ANTIBIOTIC COMBINATION

8.1 Screening of synergistic activities of antibiotic combinations against CRKP by checkerboard assay

The checkerboard assay was performed in 96-well plate. The stock solution of each antibiotic was prepared at 8X MIC concentration. The checkerboard panel of antibiotic A was prepared as follows (Figure 6). Firstly, 50 µl of CAMBH were added to all wells of 96-well plate except column 1 and H12. Secondly, 50 µl of 8X MIC concentration of antibiotic A stock solution was added to all wells of column 12 except H12 and then serial dilution with 50 µl from column 12 column to 2 except H12.

The checkerboard panel of antibiotic B was prepared as follows (Figure 7). Firstly, 100 µl of CAMHB were added to all wells of 96-well plate except row 1 and H12. Secondly, 100 µl of 8X MIC concentration of antibiotic B stock solution was added to all wells of row H except H12 and then serial dilution with 100 µl from row H to row B

After that, 50 µl of diluted antibiotic B were transferred to the same wells of checkerboard panel of antibiotic A as follows (Figure 8). The volume of all wells was adjusted to 180 µl with CAMHB. To prepare of bacterial inoculum, the bacterial pure colonies were suspended in 3 ml of sterile NSS and adjusted turbidity to 0.5 McFarland (1.5 x 10^8 CFU/ml). The suspension was then diluted 1:100 with sterile NSS and applied 20 µl into all wells (except H12: added 20 µl of CAMHB) and incubated at 37 ^{o}C for 18-24 hrs. All these experiments were evaluated in duplicate.

50 µl of antibiotic A

stock solution (8X MIC)

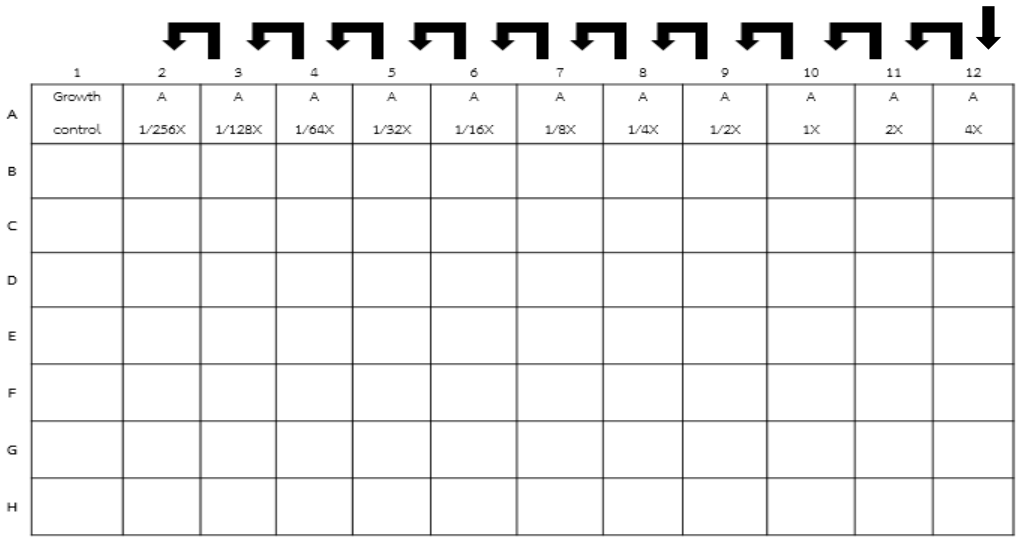


Figure 6. Preparation of checkerboard panel of antibiotic A

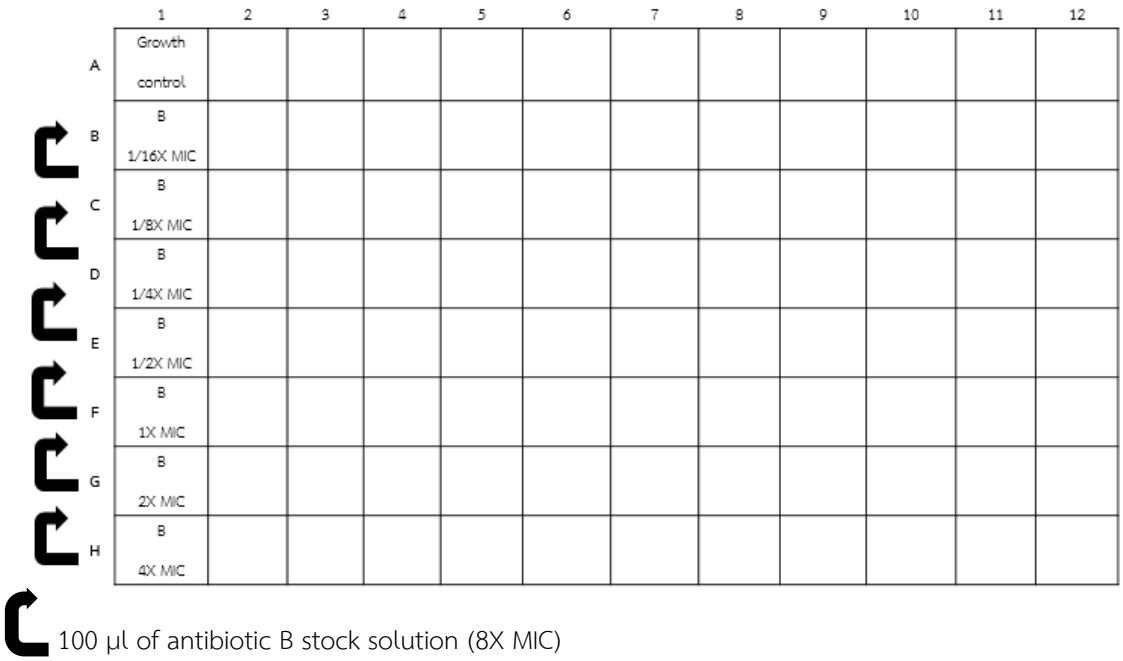


Figure 7. Preparation of checkerboard panel of antibiotic B

	1	2	3	4	5	6	7	8	9	10	11	12
A	Growth control	A 1/256X	A 1/128X	A 1/64X	A 1/32X	A 1/16X	A 1/8X	A 1/4X	A 1/2X	A 1X	A 2X	A 4X
B	B 1/16X MIC	A 1/256X +B 1/16X	A 1/128X +B 1/16X	A 1/64X +B 1/16X	A 1/32X +B 1/16X	A 1/16X +B 1/16X	A 1/8X +B 1/16X	A 1/4X +B 1/16X	A 1/2X +B 1/16X	A 1X +B 1/16X	A 2X +B 1/16X	A 4X +B 1/16X
C	B 1/8X MIC	A 1/256X +B 1/8X	A 1/128X +B 1/8X	A 1/64X +B 1/8X	A 1/32X +B 1/8X	A 1/16X +B 1/8X	A 1/8X +B 1/8X	A 1/4X +B 1/8X	A 1/2X +B 1/8X	A 1X +B 1/8X	A 2X +B 1/8X	A 4X +B 1/8X
D	B 1/4X MIC	A 1/256X +B 1/4X	A 1/128X +B 1/4X	A 1/64X +B 1/4X	A 1/32X +B 1/4X	A 1/16X +B 1/4X	A 1/8X +B 1/4X	A 1/4X +B 1/4X	A 1/2X +B 1/4X	A 1X +B 1/4X	A 2X +B 1/4X	A 4X +B 1/4X
E	B 1/2X MIC	A 1/256X +B 1/2X	A 1/128X +B 1/2X	A 1/64X +B 1/2X	A 1/32X +B 1/2X	A 1/16X +B 1/2X	A 1/8X +B 1/2X	A 1/4X +B 1/2X	A 1/2X +B 1/2X	A 1X +B 1/2X	A 2X +B 1/2X	A 4X +B 1/2X
F	B 1X MIC	A 1/256X +B 1X	A 1/128X +B 1X	A 1/64X +B 1X	A 1/32X +B 1X	A 1/16X +B 1X	A 1/8X +B 1X	A 1/4X +B 1X	A 1/2X +B 1X	A 1X +B 1X	A 2X +B 1X	A 4X +B 1X
G	B 2X MIC	A 1/256X +B 2X	A 1/128X +B 2X	A 1/64X +B 2X	A 1/32X +B 2X	A 1/16X +B 2X	A 1/8X +B 2X	A 1/4X +B 2X	A 1/2X +B 2X	A 1X +B 2X	A 2X +B 2X	A 4X +B 2X
H	B 4X MIC	A 1/256X +B 4X	A 1/128X +B 4X	A 1/64X +B 4X	A 1/32X +B 4X	A 1/16X +B 4X	A 1/8X +B 4X	A 1/4X +B 4X	A 1/2X +B 4X	A 1X +B 4X	A 2X +B 4X	Sterile control

Figure 8. Preparation of checkerboard panel of antibiotic A plus antibiotic B

The interpretation of the result, the fractional inhibitory concentration (FIC) is calculated for each antibiotic at a given concentration combination by the following formula (96).

$$\text{FIC index} = \frac{\text{MIC (drug A in combination)}}{\text{MIC (drug A alone)}} + \frac{\text{MIC (drug B in combination)}}{\text{MIC (drug B alone)}}$$

The synergistic activities are interpreted as follow (112):

- Synergy: FIC index ≤ 0.5
- Additive: 0.5 > FIC index ≤ 1
- Indifference: 1 > FIC index ≤ 4
- Antagonism: FIC index > 4

8.2 Confirmation of synergistic activities by time-kill assay

CRKP isolates that showed the synergistic activities by checkerboard assay were confirmed by using time-kill assay. Each CRKP isolate was tested in 9 growth conditions including no antibiotic (growth control), 1X MIC of antibiotic A, 1X MIC of antibiotic B, 0.5X MIC of antibiotic A, 0.5X MIC of antibiotic B, 1X MIC of antibiotic A and 1X MIC of antibiotic B, 0.5X MIC of antibiotic A and 0.5X MIC of antibiotic B, 1X MIC of antibiotic A and 0.5X MIC of antibiotic B, 0.5X MIC of antibiotic A and 1X MIC of antibiotic B. All growth conditions were performed in 10 ml volume. The volume of CAMHB, antibiotic and 0.5 McFarland of bacterial suspension in each growth condition are shown in Table 7.

The flasks were incubated at 37 °C with shaking (120 rpm). The viable bacterial cells are collected at 0, 2, 4, 6, 8, 12, and 24 hrs. At each condition time, 20 ul of growth conditions were removed and diluted in NSS from 10^{-1} to 10^{-7} and spotted each dilution on MHA plate with 5 spots (10 ul/spot) in duplicate. The MHA plates were incubated at 37 °C for 18-24 hrs. The colonies were counted and calculated to CFU/ml. The numbers of viable bacterial cell (CFU/ml) in each growth condition were plotted on a semi-log graph.

As for interpretation of time kill assay, the synergism was defined as a ≥ $2\log_{10}$ (CFU/ml)-fold decrease in combination compared with the single antibiotic. Indifference was defined as a <2 $\log_{10}$ CFU/ml fold increase or decrease in combination compared with the single antibiotic. The antagonism was defined as a ≥ $2\log_{10}$ (CFU/ml)-fold increase in combination compared with the single antibiotic. The bactericidal activity was defined as a ≥ $3\log_{10}$ (CFU/ml)-fold decrease when compared to the number of viable cells at initial time point. The lower limit of detection was $2\log_{10}$ (CFU/ml). Time-kill assay was performed at least 3 times for confirmation of the result.

Table 7. Growth conditions used in time-kill assay.

Growth condition	Volume of CAMHB (ml)	Volume of Antibiotic A (ml)	Volume of Antibiotic B (ml)	Volume of 0.5 McFarland of bacterial suspension (ml)
No antibiotic (growth control)	9.9	-	-	0.1
1X MIC of antibiotic A	9.8	0.1	-	0.1
1X MIC of antibiotic B	9.8	-	0.1	0.1
0.5X MIC of antibiotic A	9.8	0.1	-	0.1
0.5X MIC of antibiotic B	9.8	-	0.1	0.1
1X MIC of antibiotic A and 1X MIC of antibiotic B	9.7	0.1	0.1	0.1
0.5X MIC of antibiotic A and 0.5X MIC of antibiotic B	9.7	0.1	0.1	0.1
1X MIC of antibiotic A and 0.5X MIC of antibiotic B	9.7	0.1	0.1	0.1
0.5X MIC of antibiotic A and 1X MIC of antibiotic B	9.7	0.1	0.1	0.1

8.3 Statistical Analysis

Statistical analysis was performed using the SPSS software (Version 22). Chi-square test was used to compare between mechanisms of carbapenem resistance and synergistic activity of antibiotic combinations. *P*-values of less than 0.05 were considered to be statistically significant.

CHAPTER V

RESULTS

1. BACTERIAL STRAINS

A total of 240 non-duplicate CRKP isolates were obtained from clinical samples between September 2016 and April 2020 from King Chulalongkorn Memorial Hospital. All isolates were resistant to ertapenem and/or imipenem and/or meropenem. CRKP isolates were isolated from 141 males (58.75 %) and 99 females (41.25 %). The numbers of CRKP isolates were collected, 29 isolates in 2016, 27 isolates in 2017, 42 isolates in 2018, 135 isolates in 2019, and 7 isolates in 2020. The majority of CRKP isolates (72.92 %) were obtained from nonsterile sites, including 102 isolates (42.5%) from urine, followed by 57 isolates (23.75%) from sputum, and 16 isolates (6.66%) from pus. Sixty-five (27.08 %) CRKP isolates were isolated from sterile sites, including 19 isolates (7.92%) from body fluid, followed by 18 isolates (7.5%) from endotracheal aspiration, 17 isolates (7.08%) from blood, 4 isolates (1.67%) from tissue biopsy, 4 isolates (1.67%) from bronchoalveolar lavage (BAL), and 3 isolates (1.25%) from tip-catheter. The types of clinical specimens among the 240 CRKP are shown in Table 8.

2. ANTIMICROBIAL SUSCEPTIBILITY TESTING

The susceptibility of 6 antibiotics including carbapenems (imipenem and meropenem), aminoglycosides (amikacin), fosfomycin, ciprofloxacin, and colistin was determined against the 240 CRKP isolates. The minimum inhibitory concentration (MIC) range, MIC_{50}, MIC_{90} and interpretation of these 6 antibiotics against the 240 CRKP isolates are shown in Table 9. The CRKP isolates showed very high resistance rates to ciprofloxacin (95.84%), followed by meropenem (91.67%), imipenem (90.42%), fosfomycin (41.25%), amikacin (20%), and colistin (12.08%), respectively. The increasing prevalence of colistin resistance was observed in this study. High levels of MIC_{50} and/or MIC_{90} were observed in all antibiotics tested except colistin.

Table 8. Types of clinical specimens among the 240 CRKP isolates

Source	Specimen types	No. of isolates	Total (%)
Sterile sites			
(27.08%, 65/240)	Body fluid	19	7.92
	Endotracheal aspiration	18	7.5
	Blood	17	7.08
	Tissue biopsy	4	1.67
	Bronchoalveolar lavage (BAL)	4	1.67
	Tip-catheter	3	1.25
Non-sterile sites			
(72.92%, 175/240)	Urine	102	42.5
	Sputum	57	23.75
	Pus	16	6.66
		240	100

Table 9. The antibiotic susceptibility among the 240 CRKP isolates

Antimicrobial agents	MICs (mg/L)			Interpretation		
	Range	MIC_{50}	MIC_{90}	Susceptible (%)	Intermediate (%)	Resistant (%)
Imipenem	0.25 - 1024	64	128	14 (5.83)	9 (3.75)	217 (90.42)
Meropenem	0.25 - 512	128	256	9 (3.75)	11 (4.58)	220 (91.67)
Amikacin	1 - >512	16	>512	154 (64.17)	38 (15.83)	48 (20)
Ciprofloxacin	0.015 ->512	128	512	8 (3.33)	2 (0.83)	230 (95.84)
Fosfomycin	2 - >512	128	>512	117 (48.75)	24 (10.00)	99 (41.25)
Colistin	<0.03 ->512	1	16	-	211 (87.92)	29 (12.08)

The MIC distribution of antibiotics including imipenem, meropenem, amikacin, fosfomycin, ciprofloxacin, and colistin are shown in Figures 9 to 14. The imipenem and meropenem MIC ranges were 0.25 to 1024 and 0.25 to 512 mg/L, respectively. The majority of CRKP isolates showed high levels of carbapenem MICs (≥64 mg/L) including imipenem (55.84%) and meropenem (70%). The ciprofloxacin MIC ranged from 0.015 to >512 mg/L and 87.5% of these isolates exhibited high levels of ciprofloxacin resistance (≥64 mg/L). The amikacin resistance in CRKP isolates (29.17%) showed high level of fosfomycin MIC (>512 mg/L). The colistin-resistant CRKP isolates (11.13%) had a colistin MIC range of 16 to 64 mg/L.

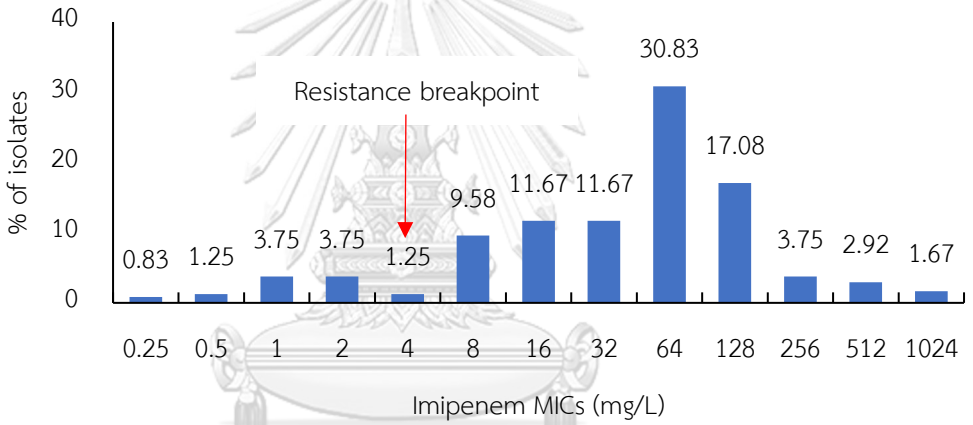


Figure 9. The distribution of imipenem MICs among the 240 CRKP isolates

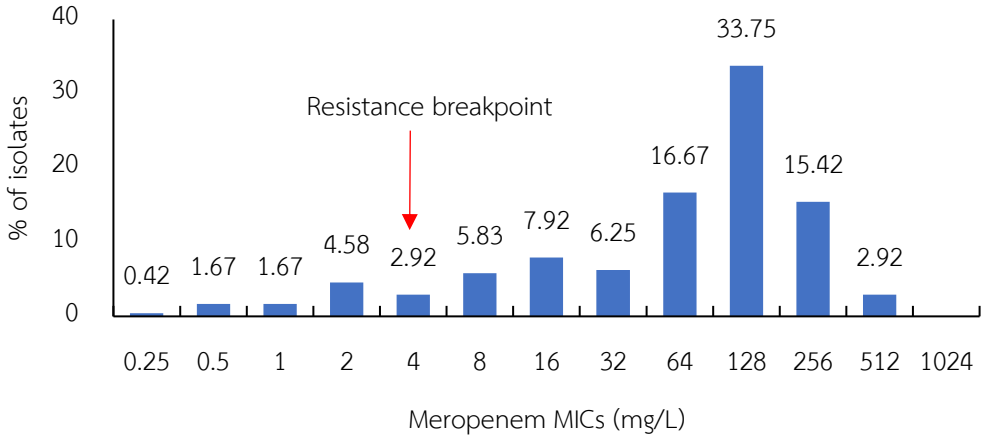


Figure 10. The distribution of meropenem MICs among the 240 CRKP isolates

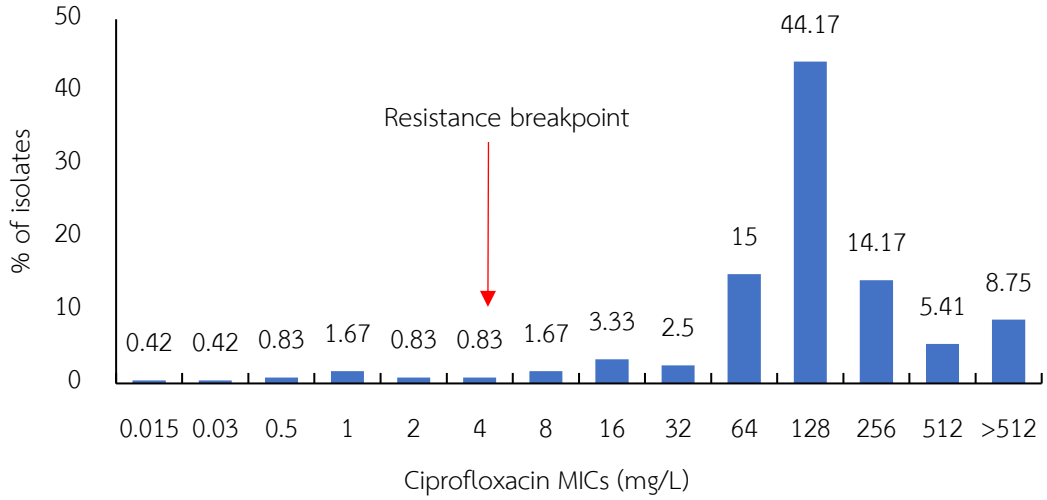


Figure 11. The distribution of ciprofloxacin MICs among the 240 CRKP isolates

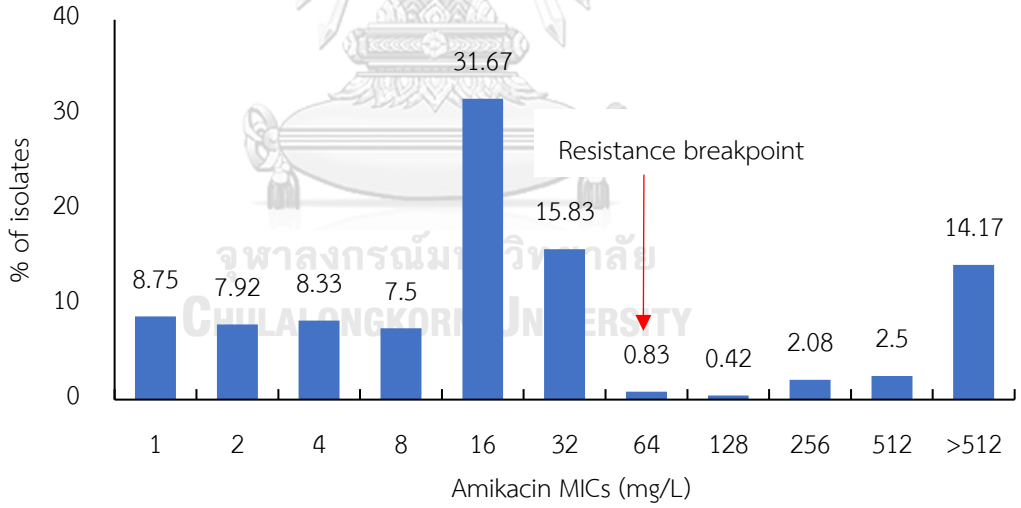


Figure 12. The distribution of amikacin MICs among the 240 CRKP isolates

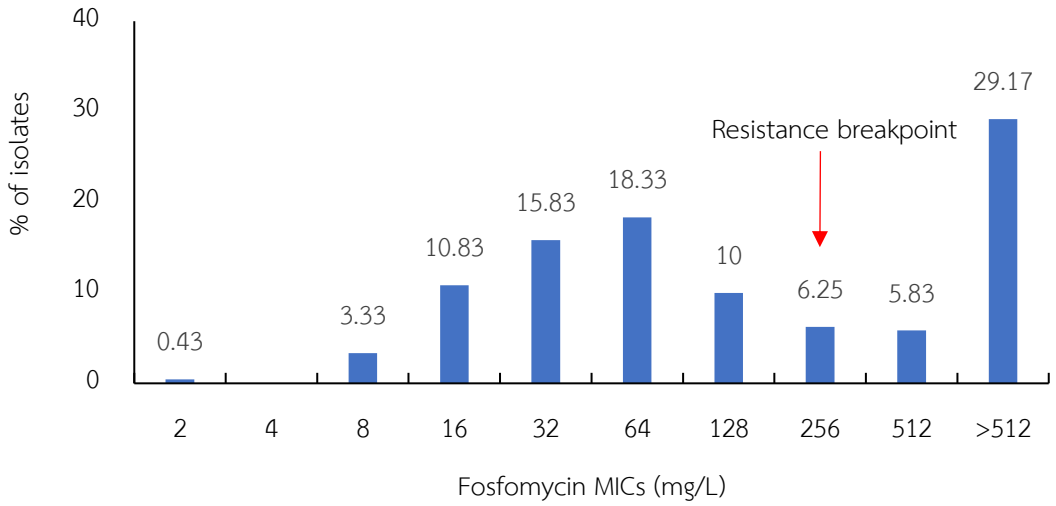


Figure 13. The distribution of fosfomycin MICs among the 240 CRKP isolates

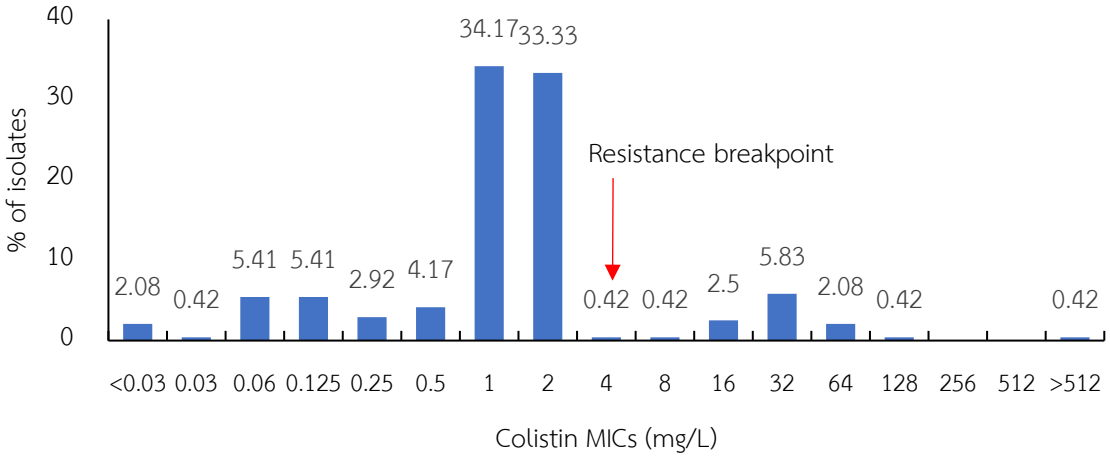


Figure 14. The distribution of colistin MICs among the 240 CRKP isolates

The antibiotic resistance patterns of the 240 CRKP isolates showed that the majority of isolates (62.92%, 151/240) were classified as multidrug-resistant (MDR) *K. pneumoniae* isolates, which were non-susceptible to at least one agent in three or more antibiotic categories (Figure 15). In this study, MDR isolates were classified by using intermediate and resistance breakpoint for 5 antibiotics including imipenem, meropenem, ciprofloxacin, fosfomycin, and amikacin while colistin used resistance breakpoint. The most common antibiotic resistance pattern in MDR *K. pneumoniae*

was the pattern of carbapenems, ciprofloxacin and fosfomycin resistance (29.80%, 45/151), followed by carbapenems, amikacin, ciprofloxacin, and fosfomycin resistance (27.81%, 42/151). Sixteen isolates (10.60%, 16/151) of MDR isolates were resistant to all antibiotics tested.

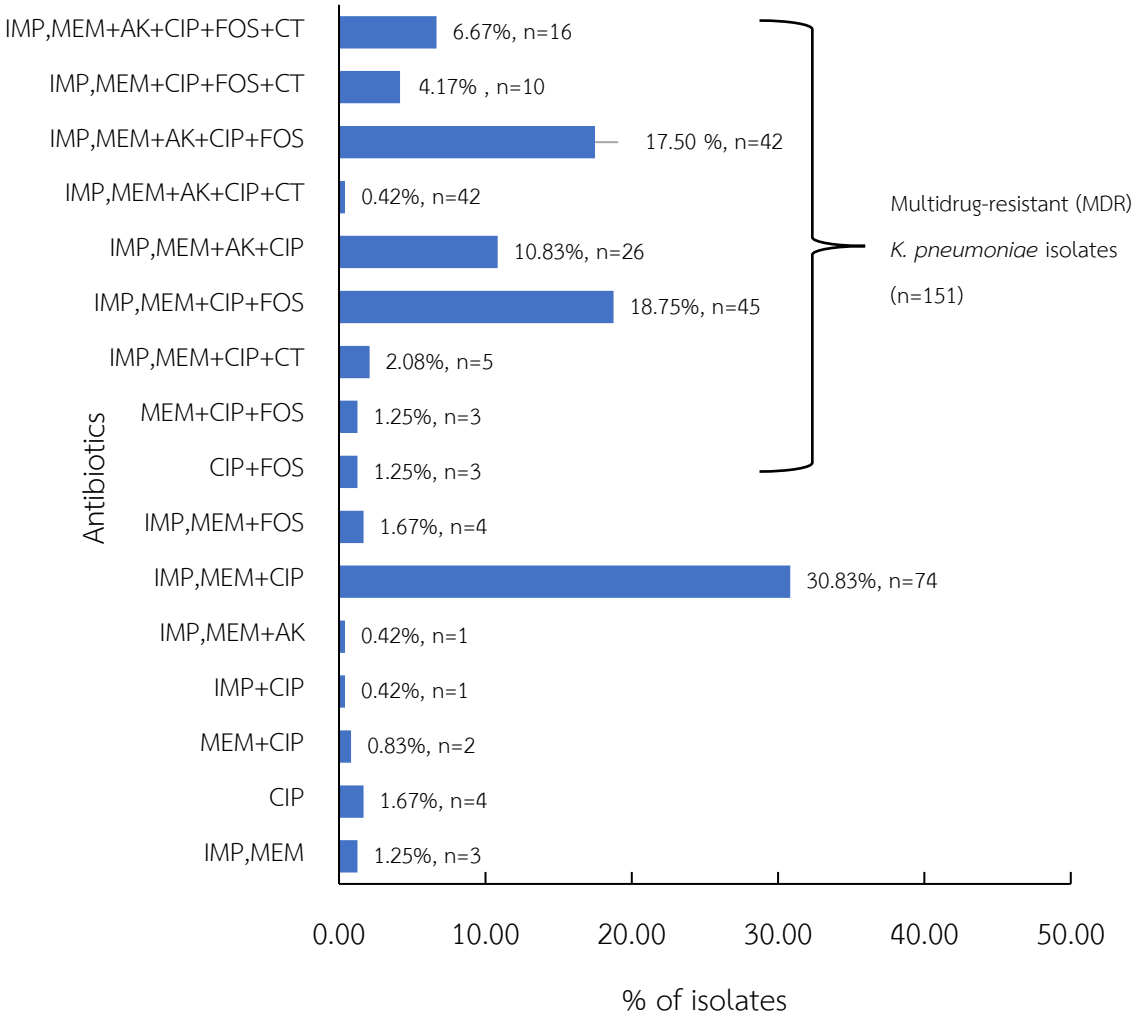


Figure 15. The antibiotic resistance patterns among the 240 CRKP isolates

Antibiotics: IMP, imipenem; MEM, meropenem; CIP, ciprofloxacin; AK, amikacin; FOS, fosfomycin; and CT, colistin

3. DETECTION OF CARBAPENEMASE PRODUCTION

3.1. Detection of carbapenemase genes

The carbapenemase genes including $bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$, $bla_{\text{KPC-like}}$, $bla_{\text{IMP-like}}$, and $bla_{\text{VIM-like}}$ were detected by multiplex PCR. The distribution of carbapenemase genes among the 240 CRKP isolates are shown in Table 10. CRKP isolates carrying carbapenemase genes were found in 98.33 %. The $bla_{\text{NDM-like}}$ plus $bla_{\text{OXA-48-like}}$ were the most common carbapenemase genes (43.75 %), followed by $bla_{\text{NDM-like}}$ (27.50%), $bla_{\text{OXA-48-like}}$ (25.42%), $bla_{\text{IMP-like}}$ (0.83%), and $bla_{\text{OXA-48-like}}$ plus $bla_{\text{IMP-like}}$ (0.83%). The $bla_{\text{KPC-like}}$ and $bla_{\text{VIM-like}}$ were not found in this study.

Carbapenemase genes were not detected in 4 CRKP isolates (1.67%). These isolates were further screened for ESBL and AmpC genes. The results showed that they harbored at least one ESBL gene including $bla_{\text{OXA-1-like}}$ plus $bla_{\text{CTX-M}}$ (isolate no. 93), bla_{TEM} plus $bla_{\text{CTX-M}}$ (isolate no. 102), bla_{SHV} (isolate no. 147), and $bla_{\text{OXA-1-like}}$, bla_{SHV} plus $bla_{\text{CTX-M}}$ (isolate no. 158). The imipenem and meropenem MIC ranges were 1 to 2 and 4 to 8 mg/L, respectively.

Table 10. The distribution of carbapenemase genes, carbapenem MIC ranges in the 240 CRKP isolates

Carbapenemase gene	Total no. of isolates (%)	MIC range (mg/L)	
		Imipenem	Meropenem
$bla_{\text{IMP-like}}$	2 (0.83)	16-32	4-8
$bla_{\text{OXA-48-like}}$ + $bla_{\text{IMP-like}}$	2 (0.83)	2-8	8
$bla_{\text{OXA-48-like}}$	61(25.42)	0.25-256	0.25-256
$bla_{\text{NDM-like}}$	66 (27.5)	1-1024	4-512
$bla_{\text{NDM-like}}$ + $bla_{\text{OXA-48-like}}$	105 (43.75)	2-1024	2-512
No carbapenemase genes detected	4 (1.67)	1-2	4-8

The distribution of each carbapenemase gene and carbapenem MICs among the 236 CRKP isolates are shown in Figures 16 to 20. The imipenem and meropenem MIC ranges of isolates carrying only $bla_{\text{NDM-like}}$ were 1 to 1024 mg/L, and 4 to 512 mg/L, respectively. High level of carbapenem MICs (≥64 mg/L) were observed in 43.94% and 46.97% of imipenem MIC and meropenem MIC, respectively. The imipenem and meropenem MICs for isolates carrying only $bla_{\text{OXA-48-like}}$ were found at low to high levels of carbapenem MICs (0.25 to 256 mg/L). High levels of carbapenem MICs (≥ 64 mg/L) were observed in 14.75% of isolates for imipenem and 54.10% for meropenem. The imipenem and meropenem MIC ranges of isolates co-carrying $bla_{\text{NDM-like}}$ and $bla_{\text{OXA-48-like}}$ were 2 to 1024 and 2 to 512 mg/L, respectively. Most of them (>92%) had high levels of carbapenem MICs (≥ 64 mg/L) when compared with those carrying one carbapenemase gene, especially in isolates carrying $bla_{\text{OXA-48-like}}$. Low prevalence of $bla_{\text{IMP-like}}$ gene was found in 0.83% (2/240). The MIC ranges of imipenem and meropenem in $bla_{\text{IMP-like}}$-harboring CRKP isolates were 16 to 32 mg/L, and 4 to 8 mg/L, respectively. The $bla_{\text{OXA-48-like}}$ plus $bla_{\text{IMP-like}}$ co-harboring *K. pneumoniae* isolates were found in 2 isolates (0.83%). Their imipenem and meropenem MIC ranges were 2 to 8 mg/L for imipenem and 4 to 8 mg/L for meropenem, respectively.

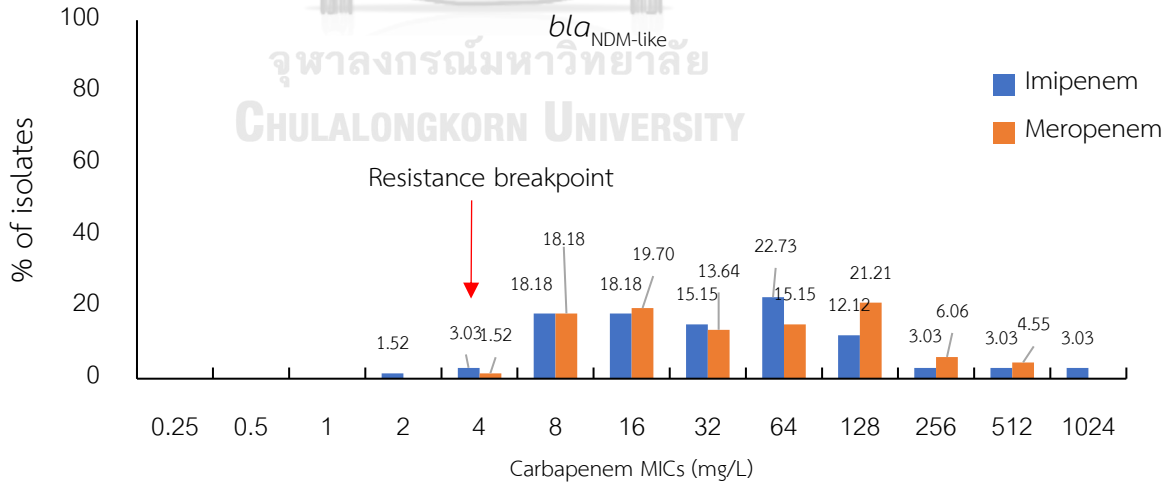


Figure 16. The distribution of $bla_{\text{NDM-like}}$ gene and carbapenem MICs among 66 CRKP isolates

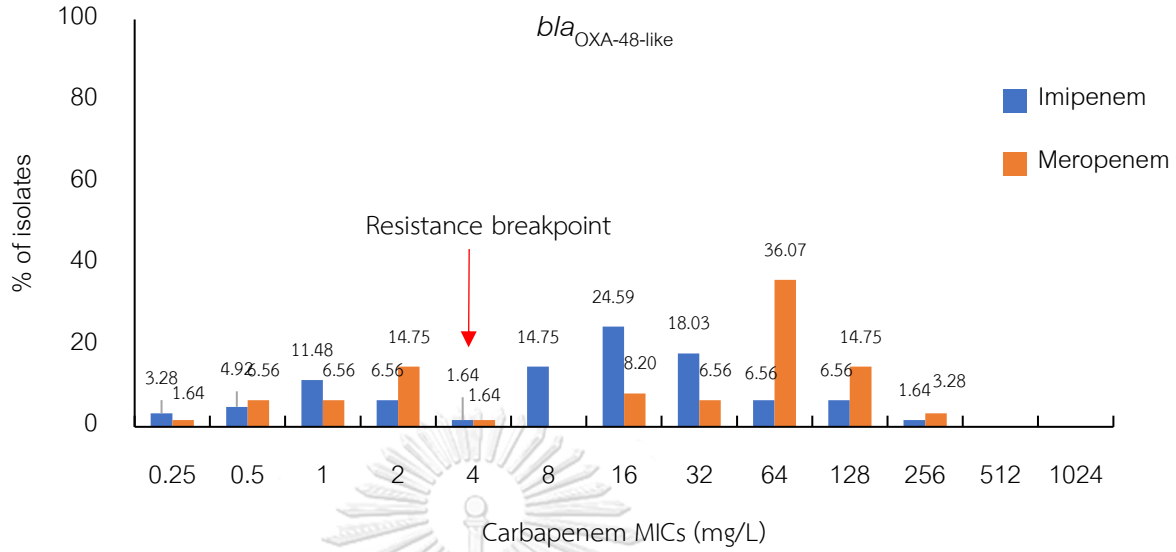


Figure 17. The distribution of $bla_{OXA-48-like}$ gene and carbapenem MICs among 61 CRKP isolates

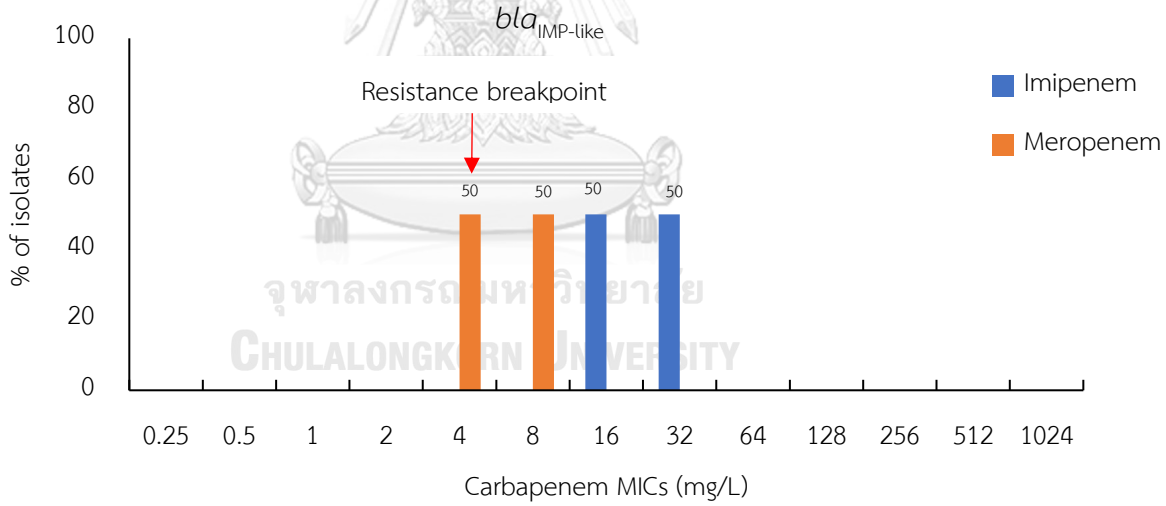


Figure 18. The distribution of $bla_{IMP-like}$ gene and carbapenem MICs among 2 CRKP isolates

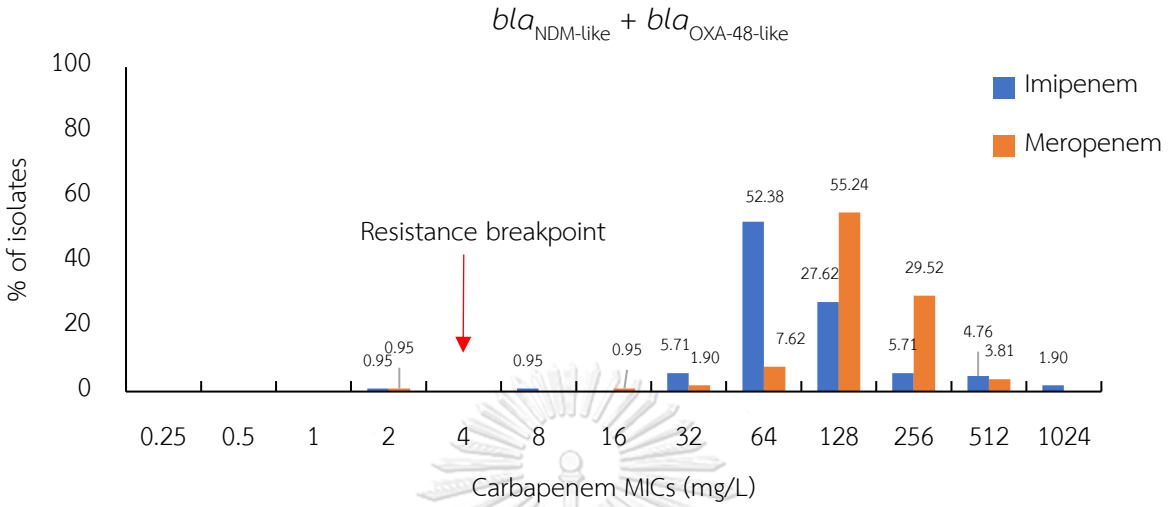


Figure 19. The distribution of *bla*$_{\text{NDM-like}}$ plus *bla*$_{\text{OXA-48-like}}$ genes and carbapenem MICs among 105 CRKP isolates

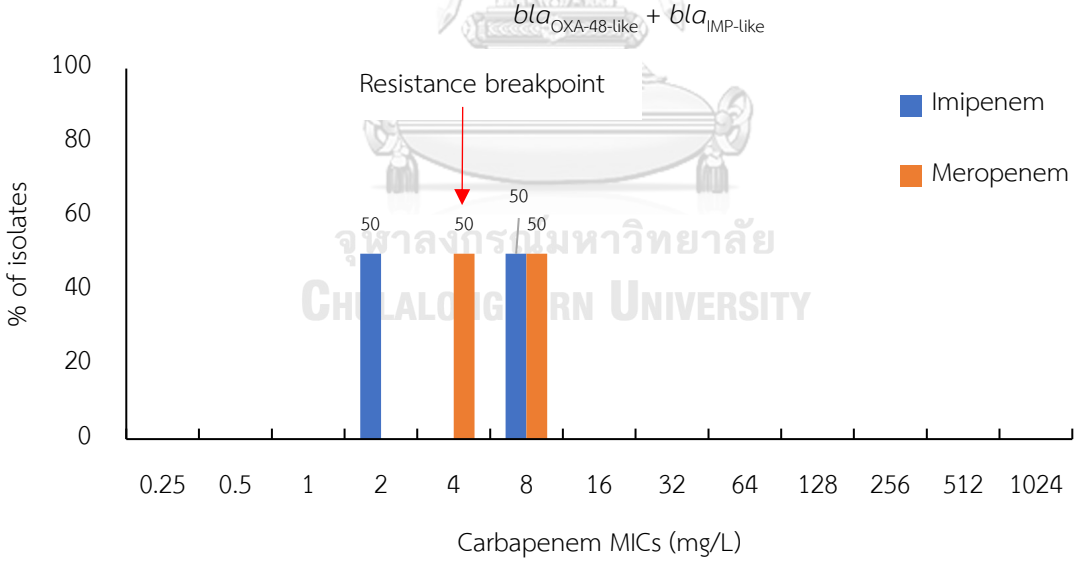
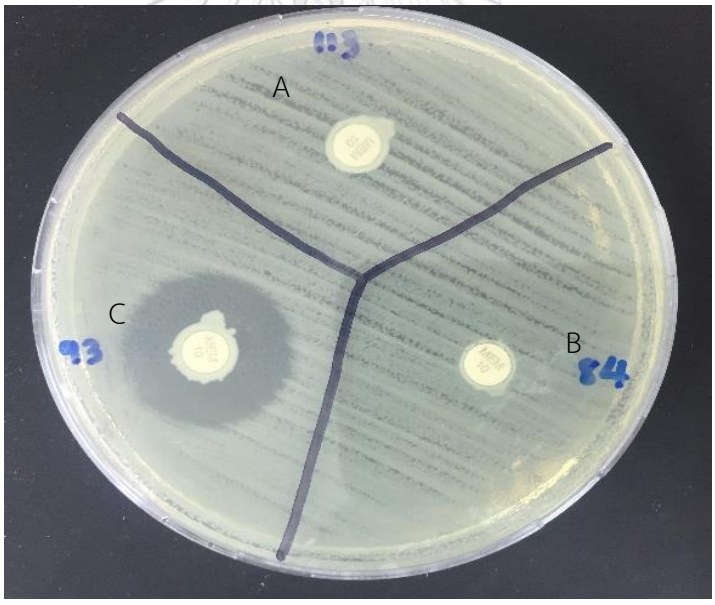


Figure 20. The distribution of *bla*$_{\text{OXA-48-like}}$ plus *bla*$_{\text{IMP-like}}$ genes and carbapenem MICs among 2 CRKP isolates

3.2. Detection of carbapenemase activity

A total 240 CRKP isolates were determined for carbapenemase production by mCIM. The result showed that the carbapenemase activity was found in all 236 isolates carrying carbapenemase genes including $bla_{\text{NDM-like}}$ and $bla_{\text{OXA-48-like}}$, $bla_{\text{OXA-48-like}}$, $bla_{\text{NDM-like}}$, $bla_{\text{IMP-like}}$, and $bla_{\text{OXA-48-like}}$ and $bla_{\text{IMP-like}}$. Two $bla_{\text{OXA-48-like}}$ -harboring CRKP isolates with low level of carbapanem MICs (0.25 to 1 mg/L) had carbapenemase activity by mCIM. Four *K. pneumoniae* isolates that were negative for carbapenemase genes showed negative result for carbapenemase activity. Therefore, the detection of carbapenemase activity by mCIM showed no false-negative and false-positive results in CRKP carrying carbapenemase genes. The representative results of carbapenemase activity by mCIM are shown in Figure 21.



A and B indicated positive for carbapenemase activity by mCIM,
C indicated negative for carbapenemase activity by mCIM.

Figure 21. The modified carbapenem inactivation method (mCIM)

4. DETECTION OF OVEREXPRESSION OF EFFLUX PUMPS

The overexpression of efflux pump activity in CRKP was performed by phenotypic method using efflux pump inhibitor, CCCP. At least 4-fold MIC reduction in the presence of inhibitor indicated possible overexpression of the efflux pump. The role of CCCP in reduction of MIC of carbapenems on the 240 CRKP isolates is summarized in Table 11.

At least 4 -fold (4 to 8-fold) MIC reduction was observed in imipenem with CCCP (12.08, %, 29/240) and meropenem with CCCP (5.42%, 13/240) (Table 4). A 4-fold MIC reduction in imipenem with CCCP and meropenem with CCCP was observed in 9.17 % (n=22/240) and 5. 42% (n=13/240). An 8-fold MIC reduction was observed in imipenem with CCCP (2.92%, 7/240) but not in meropenem with CCCP.

At least 4-fold-reduction of either imipenem with CCCP or meropenem with CCCP were interpreted to be positive for overexpression of efflux pump. Therefore, 32 CRKP isolates (13.33%) were positive for overexpression of efflux pump, that were in the reduction imipenem MIC with CCCP (19 isolates), meropenem with CCCP (3 isolates) and both imipenem and meropenem with CCCP (10 isolates).

Table 11. The role of CCCP in reduction of carbapenem MICs for the 240 CRKP isolates

Fold decrease in MIC	Imipenem and CCCP			Meropenem and CCCP		
	No. of isolates (%)	MIC range (mg/L)		No. of isolates (%)	MIC range (mg/L)	
		IMP	IMP +CCCP		MEM	MEM +CCCP
No change in MIC	149 (62.08)	0.25-1024	0.25-1024	158 (65.83)	0.25-512	0.25-512
2	62 (25.83)	2- 512	1- 256	69 (28.75)	0.5-512	0.25-256
4	22 (9.17)	4-1024	1-256	13 (5.42)	16-512	4-128
8	7 (2.92)	8-128	1-16	0 (0)	-	-

5. DETECTION OF LOSS OF OUTER MEMBRANE PROTEINS

5.1 Detection of the loss of outer membrane proteins by SDS-PAGE

The outer membrane proteins of all 240 CRKP isolates were extracted by using 1 % sodium *N*-lauryl sarcosine and performed by SDS-PAGE. The major outer membrane proteins of *K. pneumoniae* were OmpK35 and OmpK36 proteins. The result showed that most of the CRKP isolates (98.33%, 236/240) showed the loss of OmpK35 and/or OmpK36, especially OmpK35. Two-hundred twenty CRKP isolates (91.67%) lost only OmpK35. Sixteen isolates (6.66%) lost both of OmpK35 and OmpK36. Four CRKP isolates (1.66%) had both OmpK35 and OmpK36 (Figure 22). The sizes of OmpK35 and OmpK36 proteins were ~37 and ~36 kDa., respectively (Figure 23). The presence of OmpK35 proteins was confirmed by growing the isolates in Luria-Bertani (LB) broth. LB broth has the high osmolarity medium which induces the production of OmpK36 but inhibits the expression of *ompK35* gene (52). The result showed that these 4 isolates (isolate No. 1073, 203, 221 and 234) had OmpK35 when growing in NB broth but not in LB broth (Figure 24). The loss of only OmpK36 was not found in this study.

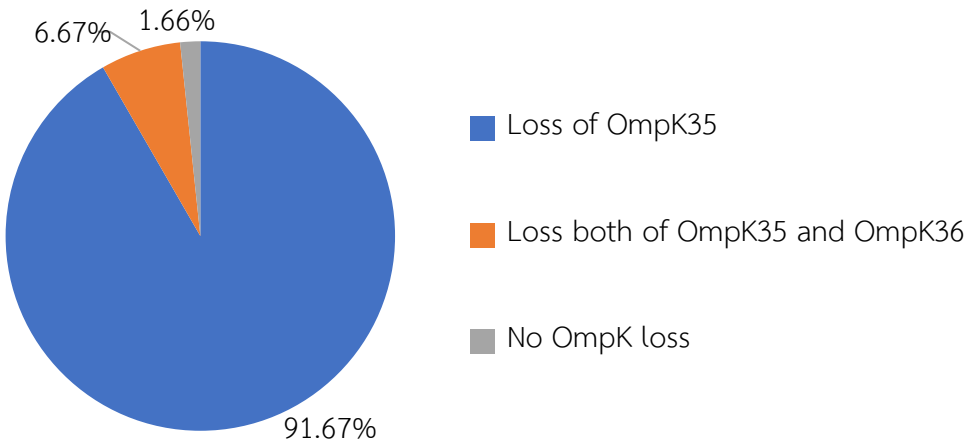
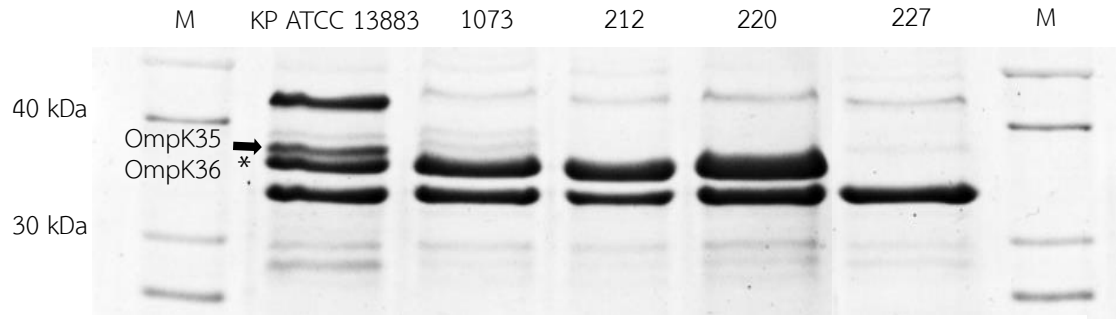
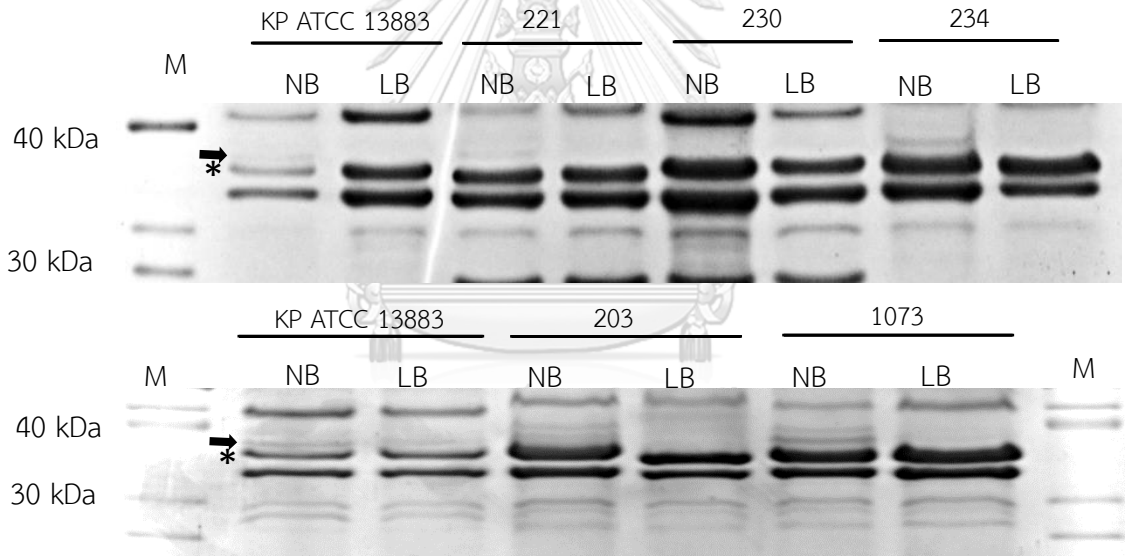


Figure 22. Characteristics of loss of outer membrane proteins in 240 CRKP isolates



Lane M: Protein molecular weight marker (kDa); Lane C: KP ATCC 13883 is control; 1073-227: CRKP isolates; Asterisk indicates OmpK36; Black arrow indicates OmpK35.

Figure 23. OMP Profiles of CRKP isolates on 12 % sodium dodecyl sulphate-polyacrylamide gel



Lane M: Protein molecular weight marker (kDa); Lane KP ATCC 13883 is control; 221-1073: CRKP isolates; Asterisk indicates OmpK36; Black arrow indicates OmpK35.

Figure 24. OMP Profiles of CRKP isolates that grew in nutrient broth (NB) or Luria-Bertani (LB) broth

5.2 Detection of *ompK35* and *ompK36* genes by PCR and DNA sequencing

All 240 CRKP isolates were screened for the *ompK35* and *ompK36* genes by PCR. Twelve CRKP isolates with different carbapenem MIC levels were selected to be representative isolates for *ompK35* sequencing and 7 representative isolates CRKP were selected for *ompK36* gene sequencing.

The amino acid of OmpK35 and OmpK36 sequences of 12 CRKP isolates with loss of OmpK35 protein and 7 CRKP isolates with loss OmpK36 protein were compared with those of *K. pneumoniae* KCTC 2242 (GenBank accession no. CP002910.1). Total amino acid of OmpK35 and OmpK36 were 359 and 368 aa, respectively. The results of *ompK35* sequencing showed that all isolates had mutations, leading to stop codon and truncated OmpK35 protein. The early stop codon in OmpK35 was commonly found at amino acid position 145 (33.33%, 4/12) and 273 (33.33%, 4/12). The early stop codon at amino acid position 273 in OmpK35 (isolate no. 251, 197, 1184, 1186) was occurred by nucleotide C substitution to T at nucleotide position 817 (C817T), leading to stop codon (TAG). The stop codon at amino acid position 145 (isolate no. 80, 241, 263, 1157) was occurred by nucleotide G deletion at position 391. Stop codon was also found at amino acid 89 (isolate no. 78), 91 (isolate no. 175), and 66 (isolate no. 1190). These mutations caused early terminates of OmpK35 protein (Figure 25 and Table 12).

The mutations in *ompK36* gene were more variable than those of *ompK35* gene (Figure 26 and Table 13). Stop codons at different amino acid positions including 79, 125, 156, 315 were detected in OmpK36. OmpK36 from 2 CRKP isolates (isolates no. 1153 and 260) had no early stop codon but they had deletion, insertion, and substitutions of the amino acid at various positions. There were no the insertions of glycine (G) - aspartic acid (D) at amino acid position 134 -135 and insertion of aspartic acid (D) - threonine(T) at amino acid position 137 -138, which were in highly conserved domain of loop3. The loop3 domain of OmpK36 was at amino acid position 115 to 148. These mutations in loop 3 have been reported to be associated with increased carbapenem MICs (60, 61, 113).

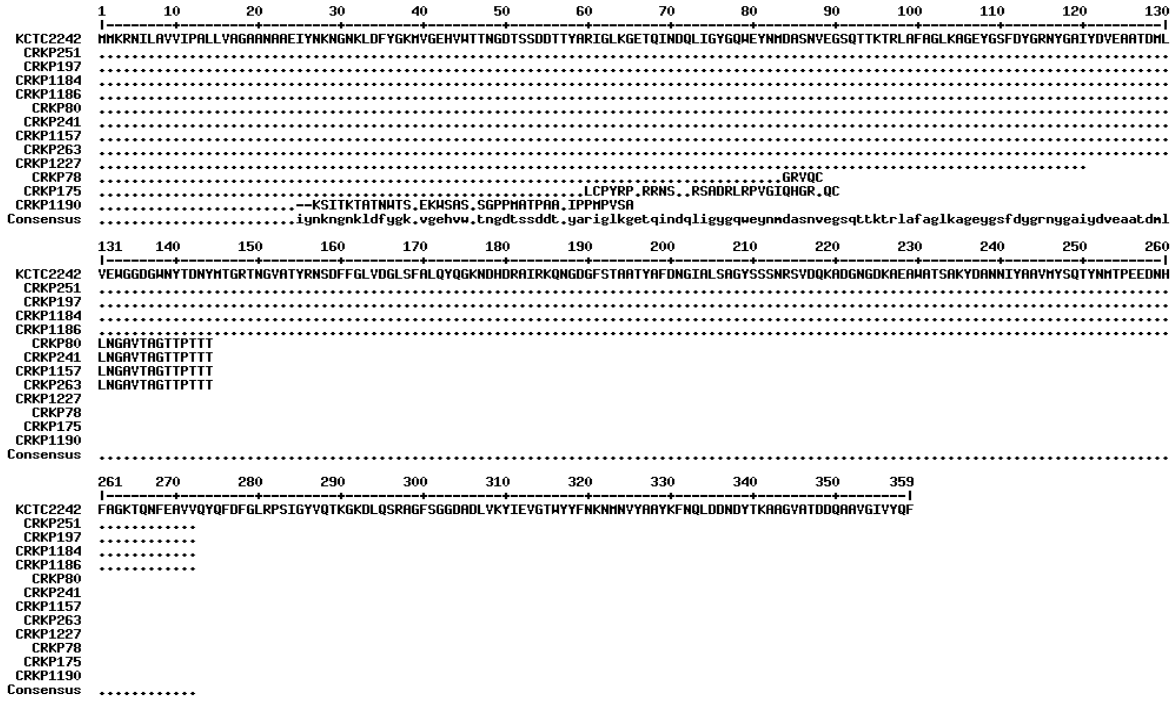


Figure 25. Alignment of amino acid sequences of OmpK35 from 12 representative CRKP isolates and those of *K. pneumoniae* KCTC 2242 (GenBank accession no. CP002910.1). Dots represent identical amino acids. Dashes represent deleted amino acids.

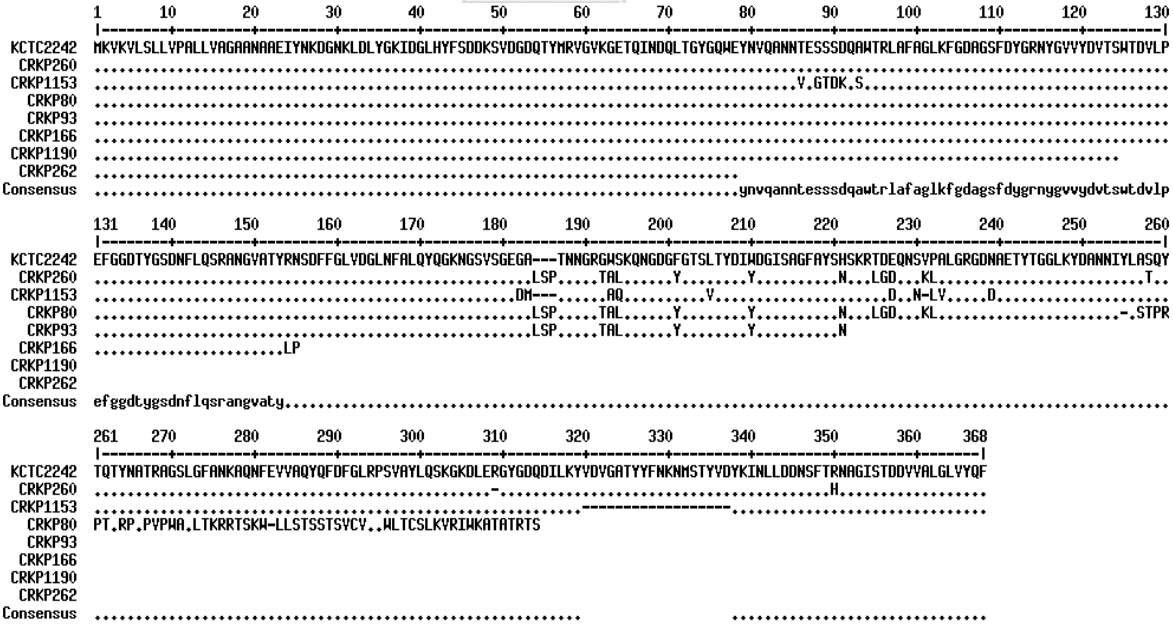


Figure 26. Alignment of amino acid sequences of OmpK36 from 7 representative CRKP isolates and those of *K. pneumoniae* KCTC 2242 (GenBank accession no. CP002910.1). Dots represent identical amino acids. Dashes represent deleted amino acids.

Table 12. The summary of amino acid sequence analysis of OmpK35 from 12 representative CRKP isolates

Isolate no.	Carbapenemase gene	MIC (mg/L)		SDS-PAGE result		Amino acid
		IMP	MEM	OmpK35	OmpK36	
78	*bla*$_{\text{NDM-like}}$	8	8	-	+	5 aa substitutions[a], Stop codon aa 89
175	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	8	16	-	+	27 aa substitutions[b], Stop codon aa 91
197	*bla*$_{\text{OXA-48-like}}$	8	16	-	+	Stop codon aa 273
263	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	32	32	-	+	Stop codon aa 145
1157	*bla*$_{\text{IMP-like}}$	32	8	-	+	Stop codon aa 145
1184	*bla*$_{\text{OXA-48-like}}$	64	128	-	+	Stop codon aa 273
241	*bla*$_{\text{OXA-48-like}}$	128	64	-	-	Stop codon aa 145
1227	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	256	256	-	+	Stop codon aa 121
251	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	256	256	-	+	Stop codon aa 273
1190	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	512	128	-	-	2 aa deletions[C], 36 aa substitutions[C], Stop codon aa 66
1186	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	1024	256	-	-	Stop codon aa 273
80	*bla*$_{\text{NDM-like}}$	1024	512	-	-	Stop codon aa 145

[a]Isolate no. 78, aa substitutions: D84G, A85R, S86V, N87Q, V88C

[b]Isolate no. 175, aa substitutions: R60L, I61C, G62P, L32Y, K64R, G65P, T67R, Q68R, I69N, N70S, Q73R, I74S, G75A, Y76D, G77R, Q78L, W79R, E80P, Y81V, N82G, M83I, D84Q, A85H, S86G, N87R, E89Q, G90C

[C]Isolate no. 1190, 2 aa deletion at position 25 and 26, aa substitution: N27K, K28S, N29I, G30T, N31K, K32T, L33A, D34T, F35N, Y36W, G37T, K38S, V40E, G41K, E42W, H43S, V44A, W45S, T47S, N48G, G49P, D50P, T51M, S52A, S53T, D54P, D55A, T56A, Y58I, A59P, R60P,I61M, G62P, L63V, K64S, G65A

Table 13. The summary of amino acid sequence analysis of OmpK36 from 7 representative CRKP isolates

Isolate no.	Carbapenemase gene	MIC (mg/L)		SDS-PAGE result		amino acid
		IMP	MEM	OmpK35	OmpK36	
93	*bla*$_{\text{NDM-like}}$	2	4	-	-	3 aa insertion[a], 5 aa substitutions[a], Stop codon aa 222
1153	*bla*$_{\text{NDM-like}}$	4	8	-	-	21 aa deletions[b], 14 aa substitutions[b]
166	*bla*$_{\text{OXA-48-like}}$	32	64	-	-	2 aa substitutions[C], Stop codon aa 156
260	*bla*$_{\text{OXA-48-like}}$	32	64	-	-	1 aa deletion[d], 3 aa insertions[d], 20 aa substitutions[d]
262	*bla*$_{\text{OXA-48-like}}$	128	128	-	-	Stop codon aa 79
1190	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	512	128	-	-	Stop codon aa 125
80	*bla*$_{\text{NDM-like}}$	1024	512	-	-	2 aa deletion[e], 3 aa insertions[e], 63 aa substitutions[e], Stop codon aa 315

[a]Isolate no. 93, 2 aa insertion at position 184, 185 and 186, aa substitution: G192T, W193A, S194L, W210Y, H221N

[b]Isolate no. 1153, 21 aa deletion at position 229, 317-334, aa substitution: T86V, S88G, S89T, S90D, D91K, A93S, G182D, A183M, W190A, S191Q, L202V, E224D, S227N, V228L, A230V, N236D

[C]Isolate no. 166, aa substitution: R154L, N155P

[d]Isolate no. 260, 1 aa deletion at position 309, 3 aa insertion at position 184-186, 11 aa substitution: G192T, W193A, S194L, F201Y, W210Y, H221N, T225L, D226G, E227D, V231K, P232L, S258T, R350H

[e]Isolate no. 80, 2 aa deletion at position 255, 282, 3 aa insertion at position 184-186, 61 aa substitution: G192T, W193A, S194L, F201Y, W210Y, H221N, T225L, D226G, E227D, V231K, P232L, A257S, S258T, Q259P, Y260R, T261P, Q262T, Y264R, N265P, T267P, R268V, A269P, G270W, S271A, G273L, F274T, A275K, N276R, K277R, A278T, Q279S, N280K, F281W, V283L, V284L, A285S, Q286T, Y287S, Q288S, F289T, D290S, F291V, G292C, L293V, S296W, V297L, A298T, Y299C, L300S, Q301L, S302K, K303V, G305R, K305I, D306W, L307K, E308A, R309T, G310A, Y311T, G312R, D313T, Q314S

All mechanisms of carbapenem resistance are summarized in Table 14. The results showed that the combination of carbapenemase production and loss of OmpK were the most prevalent mechanism (83.33%, 200/240), followed by the combination of carbapenemase production, loss of OmpK, and overexpression of efflux pump (13.33%, 32/240), carbapenemase production (1.67%, 4/240), and loss of OmpK (1.67%, 4/240). Most of the isolates (58%, 116/200) that had carbapenemase production and loss of OmpK showed higher levels of carbapenem MICs (≥64 mg/L) than those exhibited only carbapenemase production (0.5 to 16 mg/L) or loss of OmpK (2 to 4 mg/L). Most of the isolates (59%, 19/32) that harbored 3 mechanisms of resistance including carbapenemase production, loss of OmpK, and overexpression of the efflux pump showed high-level carbapenem MICs (≥64 mg/L). Four CRKP isolates that lacked only OmpK were investigated for the presence of ESBL and AmpC genes. The result showed that they harbored at least one of ESBL genes. So, the production of ESBL together with loss of OmpK showed reduced susceptibility to carbapenems (1 to 2 mg/L for imipenem MIC and 2 to 4 mg/L for meropenem MIC).

Table 14. Summary of mechanisms of carbapenem resistance in the 240 CRKP isolates

Mechanism	No. of isolates (%)	Imipenem (mg/L)			Meropenem (mg/L)		
		MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
Carbapenemase production	4 (1.67)	0.5-16	0.5	2	0.5-8	1	4
Loss of OmpK	4 (1.67)	1-2	1	2	2-4	4	4
Carbapenemase production and loss of OmpK	200 (83.33)	0.25-1024	64	128	0.25-512	128	256
Carbapenemase production and loss of OmpK, and overexpression of efflux pump	32 (13.33)	4-1024	64	128	2-512	64	256

6. SCREENING OF SYNERGISTIC ACTIVITY OF ANTIBIOTIC COMBINATION BY CHECKERBOARD ASSAY

The synergistic activities of colistin plus imipenem and colistin plus meropenem combination were investiagated against CRKP by using checkerboard assay. Seventeen of CRKP isolates with different carbapenem MIC levels and mechanisms of cabapenem resistance were selected to be representative isolates. The summary of checkerboard results is shown in Tables 15 and 16. The additive activity was observed more frequently for colisitn plus meropenem (76.47%, 13/17) than colistin plus imipenem (64.70%, 11/17). Synergism was not observed in this combination. The synergistic activity (FICI <0.5) was found in combination of fosfomycin plus imipenem (22.58%, 7/31) with FICI values ranging from 0.1289 to 0.5 (Table 17). The synergistic activty was found in combination of fosfomycin plus amikacin against 4 CRKP isolates (14.81%, 4/27) with FICI values ranging from 0.265 to 0.5 (Table 17). The additive activity was detected on most CRKP representative isolates in both fosfomycin plus amikacin (77.78%) and fosfomycin plus imipenem (70.97%). No antagonism was found in this study. The FICI values of all antibiotic combinations against representative CRKP isolates are shown in appendex D, Table 3.

The fosfomycin plus imipenem combination showed synergistic activity against 4 $bla_{\text{NDM-like}}$-harboring *K. pneumoniae* isolates with loss of OmpK35 (1 isolate) and loss of OmpK35 with overexpression of efflux pump (3 isolates). The synergistic activity of this combination was found in 2 $bla_{\text{OXA-48-like}}$-harboring *K. pneumoniae* isolates with loss of OmpK35 (isolate no. 1184) and loss of both OmpK35 and OmpK36 (isolate no. 241). The synergistic activity of fosfomycin plus imipenem was also found in 1 CRKP coharboring $bla_{\text{OXA-48-like}}$ and $bla_{\text{IMP-like}}$ with loss of OmpK35. The synergistic activity of fosfomycin plus amikacin was observed in 2 CRKP harboring $bla_{\text{NDM-like}}$ with loss of OmpK35 and overexpression of efflux pump. Synergistic activity was observed in 2 $bla_{\text{OXA-48-like}}$ -harboring *K. pneumoniae* isolates with loss of OmpK35 (isolate no. 1184)

and loss of both OmpK35 and OmpK36 with overexprssion of efflux pump (isolate no. 262). The results are shown in Table 15.

The association of synergistic activity of fosfomycin plus amikacin or imipenem combination and mechanisms of carbapenem resistance was analyzed by chi-square test. *P*-values of less than 0.05 were considered to be statistically significant. There was no statistically significant difference between mechanisms of carbapenem resistance and synergy of fosfomycin plus amikacin (*P*=0.23) or fosfomycin plus imipenem (*P*=0.80) (Table 18). Similarly, the association of carbapenemase which was the major resistance mechanism, and the fosfomycin-based combination was also not statistically significant, with *P*=0.48 for fosfomycin plus amikacin and *P*=0.35 for fosfomycin plus imipenem (Table 19).

In conclusion, fosfomycin plus imipenem is the best combination against CRKP isolates. The antagonism activity was not found in all combinations. However, the synergistic activity of antibiotic combination was not found associated with mechanisms of carbapenem resistance. Additionally, the synergistic activity of fosfomycin plus imipenem combination was confirmed by time-kill assay.

Table 15. Summary results of mechanisms of carbapenem resistance and the synergistic activities of colistin plus imipenem and colistin plus meropenem against 17 CRKP isolates

Mechanisms			No. of isolates (%)	CT+IMP			CT+MEM		
Carbapenemase production	Loss of OmpK	Over-expression of efflux pump		Synergy n (%)	Additive n (%)	Indifference n (%)	Synergy n (%)	Additive n (%)	Indifference n (%)
+	+	+	2 (11.76)	0	2 (100)	0	0	2 (100)	0
+	+	-	14 (82.35)	0	8 (57.14)	6 (35.29)	0	10 (71.43)	4 (28.57)
+	-	-	1 (5.88)	0	1 (100)	0	0	1 (100)	0
Total			17 (100)	0	11 (64.70)	6 (35.29)	0	13(76.47)	4 (23.53)

Antibiotics: CT, colistin; IMP, imipenem; and MEM, meropenem.

Table 16. Summary results of mechanisms of carbapenem resistance and the synergistic activities of fosfomycin plus amikacin and fosfomycin plus imipenem against 27 and 31 CRKP

Mechanisms			No. of isolates (%)	FOS+AK			No. of isolates (%)	FOS+IMP		
Carbapenemase production	Loss of OmpK	Over-expression of efflux pump		Synergy n (%)	Additive n (%)	Indifference n (%)		Synergy n (%)	Additive n (%)	Indifference n (%)
+	+	+	10 (37.04)	3 (30)	7 (70)	0	11 (35.49)	3 (27.27)	7 (63.64)	1 (9.09)
+	+	-	16 (59.26)	1 (6.25)	14 (87.5)	1 (6.25)	19 (61.29)	4 (21.05)	14 (73.69)	1 (5.26)
+	-	-	1 (3.70)	0	0	1 (100)	1 (3.22)	0	1 (100)	0
Total			27 (100)	4 (14.81)	21 (77.78)	2 (7.40)	31 (100)	7 (22.58)	22 (70.97)	2 (6.45)

Antibiotics: FOS, fosfomycin; AK, amikacin; and IMP, imipenem

Table 17. Synergistic activity of antibiotic combination against CRKP isolates using the checkerboard assay

Antibiotic combination	Isolate.	Mechanisms of carbapenem resistance			MIC (mg/L)					FICI[a]
		Carbapenemase gene	Loss of porin	Over-expression of efflux pump	IMP	MEM	AK	FOS	CT	
FOS + IMP (n=7/31, 22.58%)	1150	*bla*$_{\text{NDM-like}}$	OmpK35	-	64	128	16	256	32	0.3125
	1175	*bla*$_{\text{NDM-like}}$	OmpK35	+	8	8	4	512	0.25	0.325
	78	*bla*$_{\text{NDM-like}}$	OmpK35	+	8	8	8	8	0.5	0.5
	267	*bla*$_{\text{NDM-like}}$	OmpK35	+	64	128	4	>512	1	0.5
	1184	*bla*$_{\text{OXA-48-like}}$	OmpK35	-	64	128	32	128	1	0.5
	241	*bla*$_{\text{OXA-48-like}}$	OmpK35, OmpK36	-	128	64	4	128	0.06	0.5
	1172	*bla*$_{\text{OXA-48-like}}$, *bla*$_{\text{IMP-like}}$	OmpK35	-	2	4	16	256	0.25	0.1289
FOS + AK (n=4/27, 14.81%,)	78	*bla*$_{\text{NDM-like}}$	OmpK35	+	8	8	8	8	0.5	0.375
	1175	*bla*$_{\text{NDM-like}}$	OmpK35	+	8	8	4	512	0.25	0.265
	1184	*bla*$_{\text{OXA-48-like}}$	OmpK35	-	64	128	32	128	1	0.3125
	262	*bla*$_{\text{OXA-48-like}}$	OmpK35, OmpK36	+	128	128	256	>512	0.06	0.5

[a]FICI is fractional inhibitory concentration index. FICI ≤ 0.5 indicates synergistic activity, 0.5 - 1 indicates additive activity, 1-4 indicates indifference, and FICI > 4 indicates antagonism.

Antibiotics: CT, colistin; IMP, imipenem; MEM, meropenem; FOS, fosfomycin; and AK, amikacin

Table 18. Association of mechanisms of carbapenem resistance and antibiotic combination against CRKP isolates

Mechanism	FOS + AK (n=27)				FOS + IMP (n=31)			
	No. of isolates	Synergy n (%)	No synergy n (%)	*P* - value[a]	No. of isolates	Synergy n (%)	No synergy n (%)	*P* - value[a]
Carbapenemase production	1	0 (0)	1 (100)		1	0(0)	1 (100)	
Carbapenemase production + loss of OmpK	16	1 (6.25)	15 (93.75)		19	4 (21.05)	15 (78.95)	
Carbapenemase production + loss of OmpK +Overexpression of efflux pump	10	3 (30)	7 (70)	0.23	11	3(27.27)	8 (72.73)	0.80
Total	27	4 (14.81)	23 (85.19)		31	7 (22.58)	24 (77.42)	

[a]*P*-values of less than 0.05 were considered as significant

Antibiotics: FOS, fosfomycin; AK, amikacin; and IMP, imipenem

Table 19. Association of carbapenemase genes and antibiotic combination against CRKP isolates

Carbapenemase gene	No. of isolates	FOS + AK (n=27)			No. of isolates	FOS + IMP (n=31)		
		Synergy n (%)	No synergy n (%)	*P*-value[a]		Synergy n (%)	No synergy n (%)	*P* – value[a]
$bla_{\text{IMP -like}}$	2	0 (0)	2 (100)		2	0(0)	2 (100)	
$bla_{\text{NDM-like}}$	11	2 (18.18)	9 (81.82)		11	4 (36.36)	7 (63.64)	
$bla_{\text{OXA-48-like}}$	6	2 (33.33)	4 (66.67)		10	2 (20)	8 (80)	
$bla_{\text{IMP-like}}$+ $bla_{\text{OXA-48-like}}$	2	0 (0)	2 (100)	0.48	2	1 (50)	1 (50)	0.35
$bla_{\text{NDM-like}}$ + $bla_{\text{OXA-48-like}}$	6	0 (0)	6 (100)		6	0(0)	6(100)	
Total	27	4 (14.81)	23 (85.19)		31	7 (22.58)	24 (77.42)	

[a]*P*-values of less than 0.05 were considered as significant

Antibiotics: FOS, fosfomycin; AK, amikacin; and IMP, imipenem

7. CONFIRMATION OF SYNERGISTIC ACTIVITIES BY TIME-KILL ASSAY

The fosfomycin plus imipenem showed the most effective combination against CRKP isolates by checkerboard assay. Therefore, this combination was confirmed synergistic activity by time-kill assay.

Time-kill curve results of each single agent and combination against the 7 CRKP isolates are shown in Figures 27 to 29. The concentrations of fosfomycin and imipenem at 1x MIC and 1/2x MIC for each alone, and both 1x MIC and 1/2x MIC for any two agents were tested against all CRKP isolates. The time-kill results showed that both fosfomycin or imipenem alone could not inhibit bacterial growth during 24 hrs. However, the result showed that all the fosfomycin plus imipenem combination exhibited synergistic activity at 24 hrs. (>2 $\log_{10}$ reduction in the CFU/ml of combination compared with the most active single agent alone) in all 7 CRKP isolates. The synergistic activity also exhibited bactericidal activity (>3 $\log_{10}$ reduction in the CFU/ml of combination compared with the number of viable cells at initial time point) against all CRKP isolates except isolate no. 78.

In conclusion, the synergistic activity results of time-kill were correlated with checkerboard assay. So, the best effective combination against carbapenem-resistant *K. pneumoniae* isolates was fosfomycin plus imipenem. Time-kill curves exhibited synergistic activity and bactericidal activity of fosfomycin plus imipenem combination in isolates with different mechanisms of carbapenem resistance. Therefore, the mechanisms of carbapenem resistance were not associated with activity of antibiotic combinations.

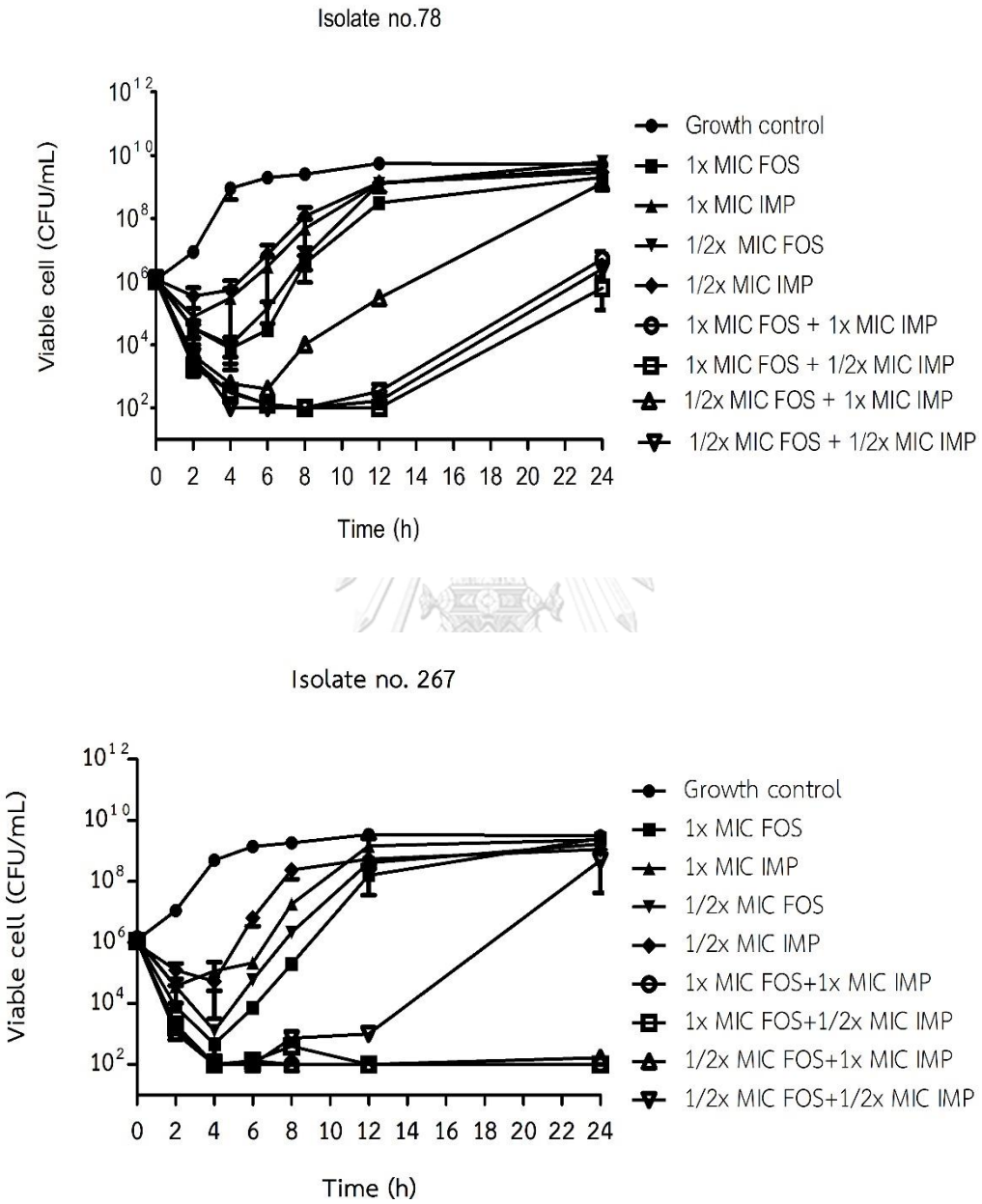


Figure 27. Time-kill curves of fosfomycin (FOS) and imipenem (IMP) against 4 *bla*$_{\text{NDM-like}}$-harboring *K. pneumoniae*

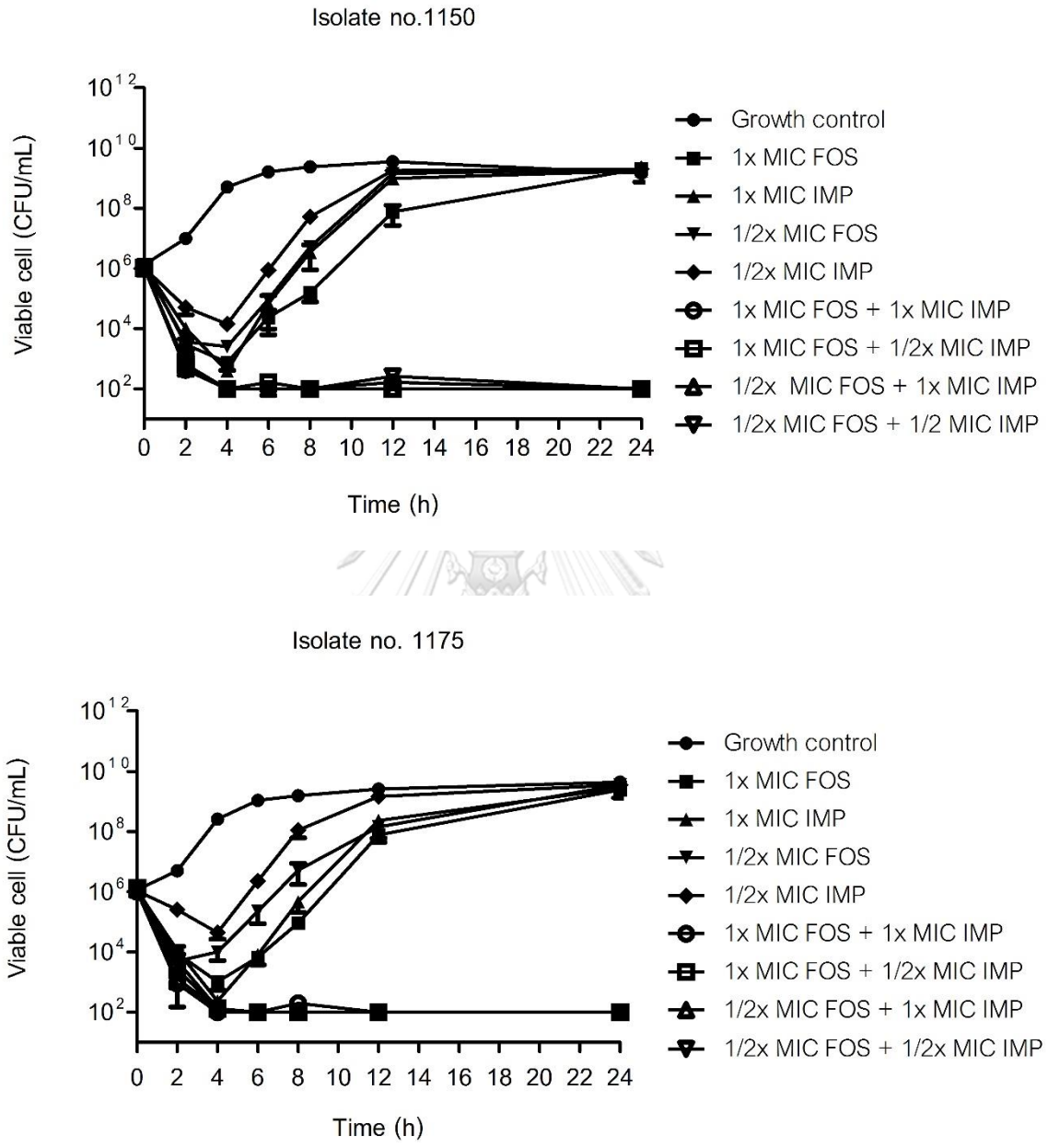


Figure 27. Time-kill curves of fosfomycin (FOS) and imipenem (IMP) against 4 *bla*$_{\text{NDM-like}}$-harboring *K. pneumoniae* (continuous)

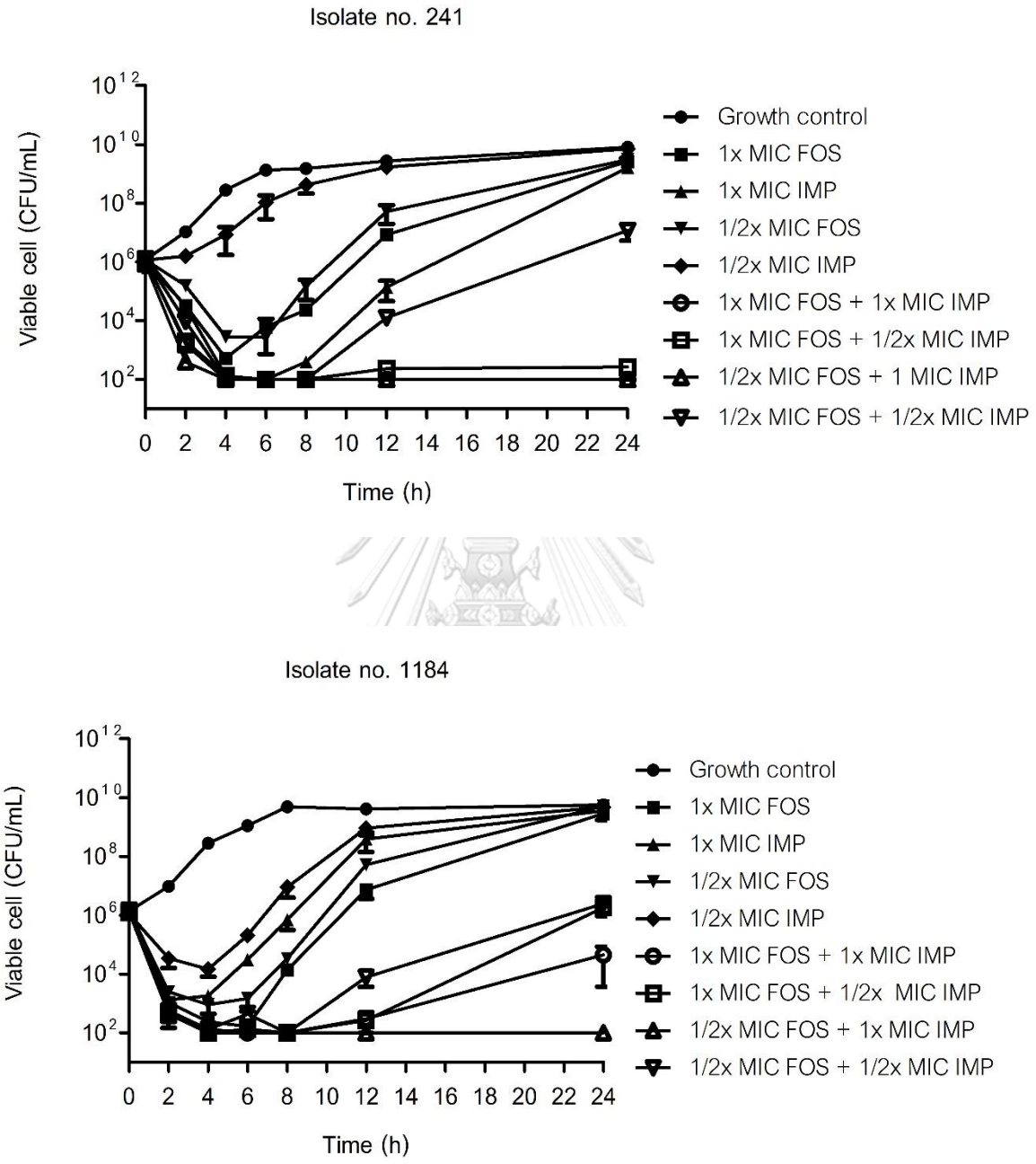


Figure 28. Time-kill curves of fosfomycin (FOS) and imipenem (IMP) against 2 $bla_{\text{OXA-48-like}}$-harboring *K. pneumoniae*

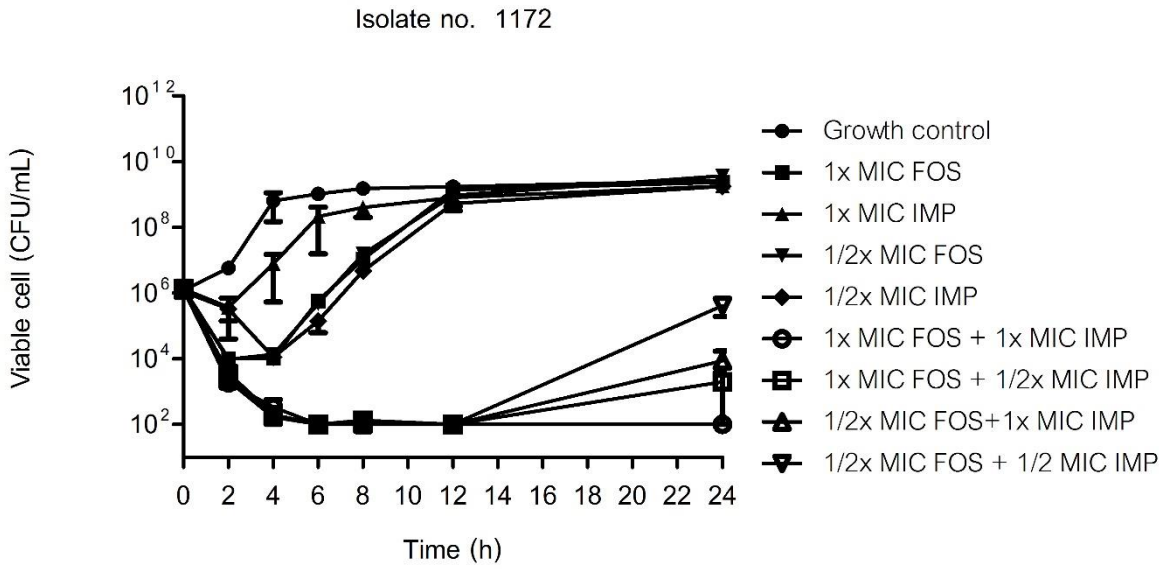


Figure 29. Time-kill curves of fosfomycin (FOS) and imipenem (IMP) against 1 *bla*$_{\text{OXA-48-like}}$ and *bla*$_{\text{IMP-like}}$-coharboring *K. pneumoniae*

CHAPTER VI
DISCUSSION

The carbapenem-resistant *K. pneumoniae* (CRKP) spreads rapidly and increases in many countries (31, 32, 73). This study collected 240 CRKP isolates from patients at King Chulalongkorn Memorial Hospital between September 2016 and April 2020. CRKP was commonly found in an elderly person, whose average age in males and females was 64 and 67 years old, respectively. The CRKP were mostly isolated from non-sterile sites (72.92%), often found in urine (42.5%), followed by sputum (23.75%) and pus (6.66%). Several studies demonstrated that the CRKP was commonly isolated from urine culture, and was an important cause of urinary tract infection (114, 115).

The result of antimicrobial susceptibility testing demonstrated that amikacin was found to be the second most effective antibiotic after colistin. The results showed that 64.17% of CRKP isolates were susceptible to amikacin. The report from Uttaradit Hospital demonstrated that the prevalence of amikacin-susceptible CRKP isolates was 87.8% (114), similar to that reported from china (69.7 %) (116). In Thailand, the data from NARST showed that the prevalence of amikacin-susceptible in *K. pneumoniae* isolates was very high (94.7%) in 2019 (31). So, amikacin seem to be effective antibiotic for CRKP treatment. This study found that most CRKP isolates (96%) were also resistance to ciprofloxacin with a high resistance rate which was similar to previous studies (31, 85, 117, 118). This finding suggests that ciprofloxacin seems to be not effective for using in treatment CKPP infection.

The majority of CRKP were multidrug-resistant (MDR) isolates (62.92%). The most common pattern was carbapenem, ciprofloxacin and fosfomycin non-susceptibility. Surprisingly, colistin resistance rate was high (12.08 %). The prevalence of colistin and carbapenem-resistant *K. pneumoniae* has been reported in several studies (10.6 to 50%) (119-122). The data from NARST which showed that colistin resistance rate in *K. pneumoniae* had trend to be increasing , which 2.4 % in 2018 and 3.6 % in 2019 (31, 123). The high prevalence of colistin resistance in CRKP isolates in

this study may be due to the dissemination of same clonal of these CRKP isolates within the hospital. Therefore, the clonal relatedness of colistin- and carbapenem-resistant *K. pneumoniae* isolates should be further investigated.

This study investigated 3 mechanisms of carbapenem resistance in *K. pneumoniae* including carbapenemase production, loss of outer membrane proteins, and the overexpression of efflux pumps. The first mechanism of carbapenem resistance is the carbapenemase production. Two-hundred thirty-six CRKP isolates (98.33%) harbored at least one carbapenemase gene and were positive for carbapenemase activity. Four CRKP isolates were negative for carbapenemase activity and carbapenemase genes. Other mechanisms of resistance must be involved in these isolates. This study showed high prevalence of the coexistence of $bla_{\text{NDM-like}}$ and $bla_{\text{OXA-48-like}}$ (43.75%) which was higher than those reported from previously studies in Thailand (0 to 32.74%) (48, 118, 124), China (0.14%) (125), Singapore (3.17%) (126), and India (9.3%) (84). In Thailand, the prevalence of carbapenemase-producing *K. pneumoniae* was low (3.85 to 11.11 %) and carbapenemase -coharboring *K. pneumoniae* isolates were not detected during 2009 to 2011 (47, 85, 86). However, Laolerd *et al.* demonstated that CRKP isolates co-harboring $bla_{\text{NDM-like}}$ was detected in 32.74% at Ramathibodi Hospital in 2016 (87). The prevalence of bla_{KCP} in Thailand was very low (0 to 0.13%) (85, 86, 127) and was not detected in this study. The high prevalence of bla_{KCP}-harboring *K. pneumoniae* has been reported in Greece (80%) (40), China (58.33%) (39),and USA (47.9 %) (128). Most $bla_{\text{OXA-48-like}}$ plus bla_{NDM} -coharboring *K. pneumoniae* isolates showed high-level carbapenem MICs (64 mg/L for imipenem MIC_{50} and 128 mg/L for meropenem MIC_{50}) when compared to $bla_{\text{OXA-48-like}}$ (16 mg/L for imipenem MIC_{50} and 64 mg/L for meropenem MIC_{50}) or bla_{NDM} (64 mg/L for imipenem MIC_{50} and 128 mg/L for meropenem MIC_{50}). In this study, the result showed that 14 % of CRKP isolates harboring $bla_{\text{OXA-48-like}}$ had high-level carbapenem MICs (≥64 mg/L). The high-level carbapenem MICs in these OXA-48 producers may be associated

with other mechanisms including loss of OmpK and co-production of ESBL and/or AmpC (113, 129, 130).

The carbapenemase activity was detected by modified carbapenem inactivation method (mCIM). There were no false-positive and false-negative results when compared with the presence of carbapenemase genes. The result found that this method can detect carbapenemase activity in $bla_{\text{OXA-48-like}}$ -harboring *K. pneumoniae* with low-level carbapenem MICs (0.25 to 2 mg/L). This finding was similar to the study by Yamada *et al.* (131), who showed that the mCIM could detect the carbapenemase activity in carbapenem-producing *K. pneumoniae* with low -level carbapenem MICs. Kuchibiro *et al.* (132) showed 100 % sensitivity and specificity of mCIM when compared to other methods such as the modified Hodge test (MHT) and Carba NP test. Pierce *et al.* (133) demonstrated that the mCIM could improve the detection of carbapenemase with weaker hydrolytic activity (e.g., OXA-type carbapenemases) , low level of carbapenemase gene expression, or metallo-β-lactamases that required divalent cations for activity.

The second mechanism of carbapenem resistance that was investigated in this study was the overexpression of efflux pumps. The AcrAB-TolC efflux pump is associated with carbapenem resistance in *K. pneumoniae* (64). This study detected the overexpression of efflux pump by phenotypic method, using efflux pump inhibitor, CCCP. The result showed that most of the CRKP isolates (86.67%) were negative for efflux pump activity. The overexpression of efflux pump activity was found in only 13.33%. This finding showed that the efflux pump overexpression was not the main mechanism of carbapenem resistance in these CRKP isolates. Osei *et al.*(134) found that only 4.17% of CRKP isolates were positive for overexpression of efflux pump in meropenem with CCCP. Filgona *et al.* demonstrated that carbapenem susceptibility of CRKP isolates (37.3%) was increased in the presence of CCCP (2 to ≥ 4-fold) (68). Some studied showed that there was no change of carbapenem MICs with the addition of CCCP in all isolates (135, 136). Chetri *et al.* (137) observed that the *acrAB* gene was

able to increase expression when carbapenem concentrations were increased. Some studies showed that the overexpression of efflux pumps reduced susceptibility to carbapenems but did not confer high-level resistance to carbapenems (137-139).

The loss of outer membrane proteins (OMPs) that uptake carbapenems in to the bacterial cells was the third mechanism of carbapenem resistance investigated in this study. The main OMPs in *K. pneumoniae* are OmpK35 and OmpK36 (51). The loss of OMPs were detected by SDS-PAGE. Most CRKP isolates (98.83%) lost either OmpK35 and/or OmpK36. Some studies showed that the loss of OmpK35 was commonly found in CRKP (73.5 to 100 %) (56, 59). Most of the CRKP isolates (70%) harboring carbapenemase genes with loss OmpK35 and/or OmpK36 tended to have increased carbapenem MICs (≥ 32 mg/L) when compared to isolates that harbored only carbapenemase genes (0.5 to 16 mg/L of carbapenem MIC ranges). The study of Wong et al. (113) demonstrated that the combination of carbapenemase production with loss OmpK35 and/ OmpK36 lead to increasing of carbapenem MICs (64 to 128-fold) when compared to isolates that harbored only carbapenemase gene (8-fold). Four ESBL-producing CRKP with loss of OmpK35 and/or OmpK36 had similar carbapenem MICs with isolates that had carbapenemase production. This finding showed that the loss of OmpK35 and/or OmpK36 together with ESBL production can cause carbapenem resistance without carbapenemase production. Several studies demonstrated that the loss of OmpK 35 and/or OmpK36 together with ESBL and/or AmpC β-lactamase production showed slight increase in carbapenem MICs by 2- to 8-fold, leading to carbapenem non-susceptibility or resistance (51, 139-141).

In this study, the porin-related mutations in *ompK35* gene led to stop codon which was commonly found in CRKP isolates (100%). The results were similar to the study by Wu *et al.* (57). Stop codon was commonly found in OmpK35 at various amino acid positions such as 67, 77, and 272. Moreover, stop codon in OmpK 35 has been reported at amino acid positions 26, 42, 63, 108, 89, 173, and 230 (92, 108, 142-144). The study of Stein *et al.* (55) found that the frameshift insertion was commonly found

in their CRKP isolates, followed by frameshift deletion and one amino acid substitutions, respectively. Shen *et al.* (56) showed that 15 ST11 CRKP isolates had A deletion at position 86, resulting in early frameshift and premature stop codon. Our 5 from 7 representative CRKP isolates were found stop codon in OmpK36 while various amino acid mutations were observed in OmpK36 among 2 CRKP isolates. IS (insertion sequence) was not detected in both *ompK35* and *ompK36* in this study. IS*1* and IS*10* were reported to be present in *ompK35* in CRKP isolates (143). Several studies reported IS including IS*1,* IS*2,* IS5 family (e.g., IS*903* and IS*Ec68*), IS*6*, IS*26,* IS*1380* in *ompK36* gene (143-146). Moreover, some studies reported that GD insertion at position 134-135 or DT insertion at position 137-138 in L3 loop of OmpK36 was associated with increased carbapenem MICs (60, 61, 113). The limitation in this study is that the functions of the OmpK in the presence of OmpK35 and/or OmpK36 isolates have not been investigated. So, mutations in loop3 of OmpK36 were not detected in this study. This study demonstrated that our CRKP isolates were resistant to carbapenems by various mechanisms including carbapenemase production, and/or loss of outer membrane proteins, and/or overexpression of efflux pumps.

The representative CRKP isolates were selected by different carbapenem MIC level and resistance mechanisms including carbapenemase production, loss of outer membrane proteins, and overexpression of efflux pump. Colistin plus carbapenem combinations were used as alternative antibiotics to treat CRKP infections (10, 147). Therefore, a combination of colistin and carbapenems were selected to study synergistic activity. However, the results observed that the colistin plus carbapenems combinations did not have synergistic activity in 17 representative CRKP isolates. This finding was unlike some studies that showed synergistic activity of colistin plus carbapenem combinations against CRKP isolates (38.5 to 65 %) (14, 15, 148, 149). Oliva *et al.* showed that the synergistic activity of colistin plus meropenem combination was up to 46.1 % (14). The study of Souli *et al.* found that colistin plus imipenem combination showed synergistic activity against 50 % of bla_{VIM-1}-harboring *K. pneumoniae* isolate but this combination was antagonism against 55.6 % of non-

colistin -susceptible CRKP isolates (150). So, the synergistic activity of colistin-based combination against CRKP isolates is still controversial. However, this study suggests that colistin-based combination may not be good antibiotic combination against our CRKP isolates because none of CRKP isolates showed synergistic activity.

The combination of colistin and tigecycline was used for the treatment of CRKP infections (151). Di Carlo P *et al.* (151) demonstrated that the combination of colistin and tigecycline showed successful treatment for 2 KPC-producing *K. pneumoniae* infection in patients who had intra-abdominal abscesses. Dundar *et al.* (152) showed that colistin plus tigecycline combination showed synergistic activity against 70% of carbapenem-resistant *Klebsiella* spp. isolates. The study of Stein *et al.* (55) showed that colistin plus tigecycline combination showed synergistic activity against 65% of CRKP isolates. Similarly, to the study of Jonathan *et al.* (153) found that 60% of CRKP isolates showed synergistic activity of this combination against CRKP isolates. However, there are no studies on the colistin and tigecycline combination against CRKP clinical isolates in Thailand. The combination of colistin and tigecycline should be further investigated for synergistic activity against CRKP Thai isolates.

The synergistic activity of fosfomycin and amikacin or imipenem combination were further investigated in the representative isolates. In this study, the most effective antibiotic combination was fosfomycin plus imipenem combination. Seven isolates (22.58%) showed synergistic activity in fosfomycin plus imipenem combination while 4 isolates (14.81%) in fosfomycin plus amikacin combination. Seven CRKP isolates that had synergistic activity in fosfomycin plus imipenem combination were resistant to fosfomycin (fosfomycin MICs of 128 to >512 mg/L). Similar to the study of Evren *et al*. (95), 42% of isolates showed synergistic activity of this combination against fosfomycin- and carbapenem-resistant *K. pneumoniae* isolates. Moreover, this combination showed synergistic activity against MDR- *E. coli* isolates, which were also resistant to fosfomycin (154). This study demonstrated that the synergistic activity of fosfomycin plus imipenem combination was commonly found in 57.14% of the $bla_{\text{NDM-like}}$ harboring-*K. pneumoniae* isolates, followed by 28.57% of $bla_{\text{OXA-48-like}}$ isolates, and 14.29% of the $bla_{\text{IMP-like}}$ plus $bla_{\text{OXA-48-like}}$ isolates. Few studies demonstrated that

fosfomycin plus imipenem combination was the effective combination against OXA-48-producing- *K. pneumoniae* (42 to 74%) (94, 95). The sample size in each carbapenem resistance mechanisms was limited in this study.

The association between mechanisms of carbapenem resistance and fosfomycin plus imipenem combination was not statistically significant. Interesting in the result showed that 3 CRKP isolates that did not have synergistic activity in colistin-based combinations showed synergistic activity in fosfomycin-based combination. Therefore, this finding suggests that the fosfomycin plus imipenem combination may be an alternative treatment against these CRKP isolates. Both fosfomycin and carbapenems inhibit bacterial cell wall synthesis but they have different mechanisms of action. Fosfomycin inhibits the Mur A, an essential enzyme for the formation of UDP-GlcNac-3-O-enol pyruvate during the first step in peptidoglycan synthesis while carbapenems inhibit cross-linking of cell wall synthesis by inhibition of PBPs, resulting in lysis of bacterial cell and death (24, 155).

However, this study suggests that fosfomycin plus amikacin combination was not the good alternative antibiotic combination due to only 14.81 % (n=4/27) of CRKP isolates showed synergistic activity. The study by Erturk *et al.* (156) showed that synergistic activity of this combination was found in OXA-48 and/ or NDM producing *K. pneumoniae* (29 %). The synergistic activity was most commonly found in OXA-48, and OXA-48 plus NDM – coproducing *K. pneumoniae* isolates. Some studied showed synergistic activity of fosfomycin plus amikacin combination against KPC-producing *K.* pneumoniae (5.1%) (17). The synergistic activity of this combination has been reported in other organisms such as MDR-*A. baumannii* and *E. coli* (157, 158). The mechanism of fosfomycin and amikacin combination may be the inhibition of the bacterial cell wall synthesis by fosfomycin and the increased uptake of amikacin into the cell, leading to inhibition of protein synthesis by amikacin (17).

In this study, the best antibiotic combination against CRKP isolates was fosfomycin plus imipenem combination, which was confirmed by time-killing assay. All seven representative CRKP isolates with different mechanisms of carbapenem resistance showed synergistic activity and bactericidal effect of fosfomycin plus imipenem combination. The results from time-killing assay were consistent with the checkerboard assay. Previous studies investigated the synergistic activity of fosfomycin plus imipenem combination against CRKP isolates by only checkerboard assay (94, 95). They did not confirm the synergistic activity and bactericidal activity of this combination by time-kill assay. Therefore, the bactericidal activity of fosfomycin plus imipenem combination was confirmed in this study.

CHAPTER VII
CONCLUSION

Carbapenem-resistant *K. pneumoniae* (CRKP) is one of the most common pathogens of nosocomial infections. This study showed that the prevalence rates of resistance to imipenem, meropenem, ciprofloxacin, fosfomycin, amikacin, and colistin was 90.42%, 91.67%, 95.84%, 41.25%, 20%, and 12.08%, respectively. The majority of CRKP isolates (62.92%) were multidrug-resistant. Most CRKP isolates (98.33%) harbored at least one carbapenemase gene. The $bla_{\text{NDM-like}}$ plus $bla_{\text{OXA-48-like}}$ were the most common carbapenemase genes. The overexpression of efflux pump was detected in 13.33% by using CCCP. There were 98.33% of CRKP isolates showed loss of OmpK35 and/or OmpK36. The combination of carbapenemase production and loss of OmpK were the most prevalent mechanism (83.33%). Additionally, the high level of carbapenem MIC was found in isolates harboring $bla_{\text{NDM-like}}$ plus $bla_{\text{OXA-48-like}}$ together with loss of both OmpK35 and OmpK36, and overexpression of efflux pump.

The effective alternative treatment of CRKP infection is combination therapy. Antibiotic combinations including colistin plus imipenem, colistin plus meropenem, fosfomycin plus amikacin, and fosfomycin plus imipenem were screened for the synergistic activity by checkerboard assay in 31 representative isolates. The colistin-based combination including colistin plus imipenem, or meropenem combination did not show the synergistic activity against all representative CRKP isolates. The most effective combination was fosfomycin plus imipenem which showed synergism in 22.58%. Time-kill assay was used to confirm the synergism against 7 CRKP isolates. The results from time-kill assay were consistent with the checkerboard assay. The association between mechanisms of carbapenem resistance and antibiotic combinations was not found in this study.

In conclusion, this study showed that the combination of carbapenemase production and loss of OmpK were the major mechanisms of carbapenem resistance in CRKP Thai isolates. The combination of fosfomycin plus imipenem may be one of the alternative antibiotic combination for treatment of CRKP infection.

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APPENDIX A

REAGENTS AND INSTRUMENTS

Reagent

40 % Acrylamide/Bis solution (29:1)	(Bio-Rad, USA)
Agarose	(Amresco, USA)
Amikacin sulphate	(Hi-media, India)
Ammonium per sulfate	(Amresco, USA)
Bio-Rad protein assay	(Bio-Rad, USA)
Boric acid	(Sigma, USA)
Bromphenol blue	(Bio-Rad, USA)
CCCP	(Sigma, USA)
Ciprofloxacin	(Sigma, USA)
Colistin	(Sigma, USA)
Coomassie brilliant blue	(Bio-Rad, USA)
DNA gel loading Dye (6X)	(Thermo Fisher Scientific, USA)
dNTP	(Thermo Fisher Scientific, USA)
EDTA	(Amresco, USA)
Fosfomycin sodium	(Meji, Japan)
GeneRuler 100 bp plus DNA ladder	(Thermo Fisher Scientific, USA)
Glacial acetic acid	(Merck, Germany)
Glucose-6-phosphate	(Sigma, USA)
Glycerol	(Merck, Germany)
Glycine	(Research Organic, USA)
HiYield Gel/PCR DNA mini kit	(RBCBioscience, Taiwan)
Hydrochloric acid	(Merck, Germany)
Imipenem	(Apollo, England)
LB broth	(BBL, USA)
MacConkey agar	(Oxoid, USA)
Meropenem	(Wako, Japan)
Mueller-Hinton II agar	(BBL, USA)

Mueller-Hinton II broth (cation-adjusted)	(BBL, USA)
N-lauroylsarcosine sodium salt	(Merck, Germany)
Nutrient Broth (NB)	(BBL, USA)
Phosphate buffer saline	(Sigma, USA)
Sodium chloride	(Amresco, USA)
Sodium dodecyl sulfate (SDS)	(BioBasic, Canada)
Sodium hydroxide	(Merck, Germany)
Taq DNA polymerase	(Thermo Fisher Scientific, USA)
TEMED	(Amresco, USA)
Tris	(Amresco, USA)
Tryptic soy broth (TSB)	(BBL, USA)
β-mercaptoethanol	(Merck, Germany)

Instruments

Incubator	(Thermo Fisher Scientific, USA)
Incubator shaker	(Thermo Fisher Scientific, USA)
Microcentrifuge	(Eppendrof, Germany)
Ultracentrifuge	(Backman Coulter, USA)
UV/Visible spectrophotometer	(Bio-Rad, USA)
Vibra-cell processor	(Sonics, USA)
Thermal Cycler	(Thermo Fisher Scientific, USA)
NanoDrop 1000 spectrophotometer	(Thermo Fisher Scientific, USA)
UV transilluminator	(Montreal Biotech, Canada)

APPENDIX B

MEDIA AND ANTIBIOTIC SOLUTION PREPARATION

1. LB broth (BBL, USA)

Suspend 25 g of the dehydrated LB broth in 1000 ml of distilled water and mixed homogeneously and sterilized by autoclaving at 121 °C for 15 minutes. The LB broth were stored at 4 °C

2. MacConkey agar (Oxoid, USA)

Suspend 51.5 g of the dehydrated MacConkey agar in 1000 ml of distilled water and mixed homogeneously and sterilized by autoclaving at 121 °C for 15 minutes. The MacConkey agar plates were stored at 4 °C

3. Mueller-Hinton II agar (BBL, USA)

Suspend 38 g of the dehydrated Mueller-Hinton II agar in 1000 ml of distilled water and mixed homogeneously and sterilized by autoclaving at 121 °C for 15 minutes. The Mueller-Hinton II agar plates were stored at 4 °C

4. Mueller-Hinton II broth (cation-adjusted) (BBL, USA)

Suspend 22 g of the dehydrated Mueller-Hinton II broth (cation-adjusted) in 1000 ml of distilled water and mixed homogeneously and sterilized by autoclaving at 121 °C for 15 minutes. The Mueller-Hinton II broth (cation-adjusted) were stored at 4 °C

5. Nutrient broth (BBL, USA)

Suspend 8 g of the dehydrated Nutrient broth in 1000 ml of distilled water and mixed homogeneously and sterilized by autoclaving at 121 °C for 15 minutes. The Nutrient broth were stored at 4 °C

6. Typtic soy broth (TSB) (BBL, USA)

Suspend 30 g of the dehydrated Tryptic soy broth in 1000 ml of distilled water and mixed homogeneously and sterilized by autoclaving at 121 °C for 15 minutes. The Tryptic soy broth was stored at 4 °C

7. Sterile 0.85% NaCl

Suspend 8.5 g of NaCl in 1000 ml of distilled water and mixed homogeneously and sterilized by autoclaving at 121 °C for 15 minutes. The sterilize 0.85% NaCl was stored at 4 °C

8. Antibiotic stock solution

8.1 Imipenem, stock concentration 10240 mg/L

Preparation of stock solution, 0.0512 g of imipenem was dissolved by 5 ml of sterilized distilled water.

8.2 Meropenem, stock concentration 10240 mg/L

Preparation of stock solution, 0.0512 g of meropenem was dissolved by 5 ml of sterilized distilled water.

8.3 Amikacin, stock concentration 5120 mg/L

Preparation of stock solution, 0.0256 g of Amikacin was dissolved by 5 ml of sterilized distilled water.

8.4 Ciprofloxacin, stock concentration 5120 mg/L

Preparation of stock solution, 0.0256 g of Ciprofloxacin was dissolved by 0.5 ml of 0.05% and then, was dissolved by 0.45 ml of sterilized distilled water.

8.5 Fosfomycin, stock concentration 5120 mg/L

Preparation of stock solution, 0.0256 g of Fosfomycin was dissolved by 5 ml of sterilized distilled water.

8.6 Colistin, stock concentration 5120 mg/L

Preparation of stock solution, 0.0256 g of colistin was dissolved by 5 ml of sterilized distilled water.

APPENDIX C

REAGENT PREPATION

1. 0.5 M EDTA (pH 8.0)

Preparation of 0.5 M EDTA, 186.1 g of EDTA was dissolved in 800 ml of distilled water. Adjust the pH to 8.0 and the volume to 1000 ml. This reagent was stored at room temperature.

2. 10X Tris-Borate buffer (TBE)

Preparation of 10X Tris-Borate buffer (TBE), 108 g of Tris base, 55 g of Boric acid, and 40 ml of 0.5 M EDTA (pH 8.0) were mixed and dissolved in 1000 ml of distilled water. The TBE buffer was sterilized by autoclaving at 121°C for 15 min. This reagent was stored at room temperature.

3. 1.5 M Tris-HCl (pH 8.8)

Preparation of 1.5 M Tris-HCl (pH 8.8), 18.17 g of Tris base was suspended and completely dissolved in 60 ml of distilled water. Adjust the pH to 8.8 by 1 N HCl and the volume to be 100 ml. This reagent was stored at 4°C

4. 0.5 M Tris-HCl (pH 6.8)

Preparation of 0.5 M Tris-HCl (pH 6.8), 6.06 g of Tris base was suspended and completely dissolved in 60 ml of distilled water. Adjust the pH to 6.8 by 1 N HCl and the volume to be 100 ml. This reagent was stored at 4°C.

5. 1.5% Agarose gel

Preparation of 1.5% agarose gel, 1.5 g of agarose was suspended and dissolved by heating in 100 ml of 0.5X TBE buffer

6. 10% SDS

Preparation of 10% of SDS, 10 of SDS was was dissolved in 100 ml of distilled water. This reagent was stored at room temperature.

7. 6X Protein sample buffer

4X Tris HCl/SDS pH 8.8	7 ml
Glycerol	3 ml
SDS	1 g
DTT	0.93 g
Bromophenol blue	1.2 mg

Dissolve the solution and adjust volume to 10 ml. This reagent was stored at -20 °C.

8. 10x Protein running buffer (pH 8.3)

Preparation of 10x protein running buffer (pH 8.3), 30.3 g of Tris base, 144 of glycine and 10 of SDS were mixed and dissolved in 1000 ml of distilled water. This reagent was stored at 4 °C.

9. 10% APS

Preparation of 10% APS, 10 g of APS was dissolved by 100 ml of distilled water. This reagent was stored at -20°C.

10. 12 % Polyacrylamide gel (separate gel)

Distilled water	4.4 ml
40% Acrylamide/Bis	3 ml
1.5 M Tris buffer (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10 % APS	50 µL
TEMED	5 µL

11. 10 % Polyacrylamide gel (stacking gel)

Distilled water	2.89 ml
30% Acrylamide/Bis	0.79 ml
0.5 M Tris buffer (pH 6.8)	1.25 ml
10% SDS	50 ml
10 % APS	34 µL
TEMED	14 µL

12. Phosphate buffer saline (pH 7.4)

Preparation of phosphate buffer saline (pH 7.4), 1 pouch of phosphate buffer saline powder was dissolved in 1000 ml of distilled water. The TBE buffer was sterilized by autoclaving at 121°C for 15 min. This reagent was stored at room temperature.

13. 1% Sodium *N*-lauroylsarcosine

Preparation of 1% sodium *N*-lauroylsarcosine, 1 g of *N*-lauroylsarcosine was dissolved in 100 ml of distilled water. This reagent was stored at room temperature.

14. Coomassie Blue R-250 staining reagent

Preparation of coomassie blue R-250 staining reagent, dissolve 0.25g dye in 90 ml of methanol and deionized water (1:1) and 10 ml of acetic acid, then filter the solution through a Whatman No.1 filter to remove any particulate matter. This reagent was stored at room temperature.

APPENDIX D

ADDITIONAL RESULTS

Table 1. Results of MICs of 6 antibiotic against 240 CRKP isolates.

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
917	Sputum	64	128	16	128	16	2
918	Pus	64	128	16	64	32	2
919	blood	64	128	16	128	32	2
921	Bal	128	256	16	128	>512	32
924	Pus	128	128	16	256	>512	32
926	Sputum	128	128	16	128	>512	32
927	Sputum	64	128	16	128	16	0.125
932	Tissue (right leg)	64	128	32	128	64	0.25
933	Endo	16	64	256	128	>512	2
934	Sputum	64	256	16	128	32	2
936	Urine	64	128	32	128	>512	2
937	Urine	32	64	8	128	8	2
942	Urine	64	128	8	128	>512	2
944	Endo	8	64	>512	64	>512	16
946	Body fluid	64	256	16	128	>512	64
947	Sputum	256	512	32	256	>512	32
952	Blood	64	128	16	128	>512	2
971	Urine	64	128	8	128	32	2
975	Blood	128	128	32	128	16	2
1058	End	16	32	512	128	>512	2
1059	Blood	128	256	256	1024	64	2
1061	Urine	64	256	32	128	64	0.06
1063	Pus	128	128	16	128	64	2
1064	Urine	16	16	16	32	8	0.5
1066	Urine	64	64	32	128	64	2
1071	Urine	128	64	256	1024	128	2
1073	Blood	16	4	1	1	8	1

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
1074	Urine	128	256	512	>512	256	2
1079	Urine	64	128	32	128	64	2
1104	Urine	64	128	8	256	>512	2
1108	Urine	16	16	>512	256	64	0.06
1207	Sputum	64	128	32	256	64	0.125
1208	Sputum	64	128	1	>512	16	2
1211	Sputum	64	128	16	128	256	2
1212	Sputum	32	64	32	256	64	2
1213	Endo	64	128	16	256	>512	0.125
1214	Sputum	64	128	16	256	>512	0.125
1215	Pus	16	128	>512	64	>512	1
1216	Sputum	64	128	32	128	32	2
1217	Urine	64	64	16	1024	32	2
1218	Bal	32	64	2	128	32	1
1120	Sputum	64	256	16	128	>512	2
1121	Urine	64	256	1	128	>512	2
1224	Urine	64	128	32	128	128	32
1225	Pus	256	256	32	1024	>1024	32
1226	Urine	64	128	32	512	1024	2
1227	Urine	256	256	16	1024	32	2
1228	Sputum	32	64	32	256	128	1
1146	Sputum	8	16	>1024	64	>1024	2
1149	Blood	8	16	512	256	>512	2
1150	Urine	64	128	16	512	256	32
1152	Urine	64	128	32	>512	32	2
1153	Urine	4	8	1	0.5	128	2
1154	Plural fluid	128	256	16	512	32	32
1156	Urine	8	16	16	128	>1024	2
1157	Urine	32	8	1	0.03	128	0.03
1158	Pus	8	16	4	128	128	2
1159	Urine	32	128	16	128	16	2

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
1169	Urine	128	32	32	>1024	64	2
1170	Urine	16	32	32	128	8	2
1172	Urine	2	4	16	128	256	0.25
1174	Sputum	64	64	32	128	64	2
1175	Urine	8	8	4	128	512	0.25
1180	Sputum	64	128	32	128	512	2
1183	Sputum	64	128	16	1024	32	2
1184	Urine	64	128	32	64	128	1
1186	Sputum	1024	512	64	128	>1024	2
1187	Urine	32	64	2	128	32	2
1189	Sputum	64	64	16	128	>1024	32
1190	Tip-catheter	512	128	2	256	128	2
1192	Sputum	512	64	2	256	128	2
1194	Sputum	128	128	>1024	1024	256	16
1195	Sputum	8	4	16	64	32	1
1197	Tip-catheter	8	8	8	256	16	2
77	Urine	128	128	8	256	32	1
78	Urine	8	8	8	64	8	0.5
79	Urine	2	2	32	32	32	1
80	Sputum	1024	512	32	32	128	16
81	Pus	64	128	32	64	64	1
82	Blood	64	128	16	256	64	1
83	Urine	64	128	>512	256	>512	1
84	Urine	32	64	2	256	256	2
85	Pus	32	64	32	128	64	1
86	ENT	16	16	16	64	64	1
87	Sputum	512	256	>512	128	64	32
89	Urine	16	8	16	512	>512	0.25
90	Urine	8	8	1	>512	256	0.5

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
91	Urine	64	64	8	64	>512	2
92	Sputum	8	8	16	64	32	1
93	Urine	2	4	32	128	512	1
95	Urine	8	8	16	256	32	2
96	Sputum	64	128	16	128	64	2
98	Urine	64	128	16	128	512	1
99	Urine	2	2	1	0.015	32	1
100	Sputum	1024	512	32	>512	256	0.25
101	Urine	64	64	16	128	64	1
102	Body fluid bile	2	4	2	128	64	1
103	Sputum	128	16	1	32	128	1
104	Blood	64	128	16	256	16	32
105	Sputum	64	128	1	128	32	2
106	Sputum	64	128	16	128	32	2
107	Blood	64	128	16	128	16	2
108	Body fluid	64	128	2	64	>512	1
109	Urine	8	32	16	128	32	2
111	Body fluid	128	256	16	128	64	128
112	Sputum	8	16	32	128	16	2
113	Urine	64	128	1	128	16	2
114	Urine	128	128	16	64	16	64
115	Tissue	128	256	16	128	32	2
116	Urine	64	128	4	64	8	1
117	Urine	64	64	>512	128	256	1
118	Bal	128	256	16	128	64	2
119	Endo	64	128	16	128	64	32
120	Blood	64	32	4	512	512	1

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
121	Sputum	64	32	4	256	32	2
122	Urine	64	128	16	128	512	>512
123	Endo	16	32	2	128	>512	0.06
124	Sputum	1	2	16	2	8	2
125	Endo	64	128	1	128	64	0.5
126	Sputum	64	128	16	128	64	2
127	Bal	128	256	>512	512	>512	1
129	Discharge fluid	64	32	1	8	16	1
132	Pus	32	64	>512	128	128	0.06
133	Sputum	256	256	16	128	64	1
134	Urine	128	256	16	256	32	1
135	Sputum	64	128	16	128	64	1
136	Pus	64	16	16	64	16	1
137	Blood	256	256	16	128	32	1
138	Urine	2	1	2	128	16	2
139	Urine	1024	512	32	128	256	0.125
140	Urine	64	64	8	512	16	1
141	Endo	16	16	8	64	2	1
143	Endo	256	256	16	128	64	2
144	Urine	128	256	1	128	64	1
146	Urine	64	128	2	256	64	0.5
147	Endo	1	4	1	4	256	1
148	Penis	128	256	16	128	64	1
149	Urine	1	2	4	64	>512	1
150	Sputum	1	0.5	512	>512	>512	1
151	Sputum	128	256	>512	>512	512	64
152	Urine	128	128	256	256	16	1
153	Urine	32	32	2	1	16	2
154	Urine	32	16	4	64	>512	2
156	Tissue	8	128	512	512	>512	1

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
157	Urine	64	128	8	128	>512	0.5
158	Endo	1	2	4	1	>512	1
159	Body fluid bile	128	256	2	256	32	1
160	Urine	64	128	1	64	16	1
161	Urine	0.5	0.5	4	512	32	1
162	Urine	16	4	4	128	512	1
163	Urine	32	256	8	128	32	1
164	Urine	16	8	16	64	>512	2
166	Blood	32	64	2	1	256	2
167	Sputum	1	0.25	1	4	16	1
168	Urine	64	256	16	>512	64	1
169	Urine	16	64	16	64	128	0.5
170	Urine	8	32	1	16	>512	1
171	Urine	16	32	4	64	16	1
172	Sputum	32	32	4	64	32	1
173	Urine	0.25	1	2	512	32	1
175	Urine	8	16	32	128	8	2
176	Blood	64	256	16	128	64	2
179	Sputum	8	32	2	128	16	1
180	Urine	8	8	16	64	16	1
181	Sputum	0.25	0.5	1	>512	>512	1
183	Urine	64	128	4	128	64	1
184	Urine	16	16	32	128	64	2
185	Urine	64	128	16	128	128	1
186	Sputum	8	32	>512	128	128	2
188	Urine	8	2	8	16	32	1
190	Urine	64	128	16	>512	32	1

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
191	Blood	64	64	16	16	>512	1
192	Urine	2	2	8	8	32	1
193	Body fluid	1	2	4	64	>512	1
194	Sputum	128	128	8	128	128	2
195	Sputum	16	64	>512	128	256	2
196	Urine	512	256	>512	128	128	2
197	Urine	8	16	32	64	128	0.125
199	Sputum	128	128	16	128	>512	1
200	Urine	256	512	8	64	512	1
201	Body fluid	128	256	2	128	128	2
202	Blood	32	128	32	128	>512	16
203	Sputum	0.5	1	16	128	128	1
204	Sputum	128	256	16	128	256	0.5
205	Urine	64	128	16	128	128	1
206	Urine	64	128	32	128	>512	1
208	Urine	32	128	1	64	64	0.06
210	Urine	128	128	2	128	64	1
213	Sputum	16	64	2	512	>256	1
214	Urine	64	256	16	256	>256	64
216	Urine	128	128	8	256	64	1
217	Urine	128	128	1	64	>256	1
218	Urine	16	16	4	8	16	0.125
219	Body fluid	32	64	>512	16	>256	0.06
221	Sputum	0.5	0.5	4	128	32	0.125
222	Urine	64	128	16	128	64	0.06
223	Blood	16	64	16	128	64	0.06
224	Endo	16	64	16	128	256	1
226	Body fluid	32	64	>512	16	>256	1

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
228	Urine	16	8	32	128	16	2
229	Endo	2	2	>512	256	>256	1
230	Urine	128	256	16	128	>256	1
231	Urine	32	64	>512	64	>256	2
232	Sputum	128	256	128	128	64	2
233	Endo	128	256	16	128	64	1
234	Body fluid	2	8	>512	0.5	32	2
235	Blood	32	128	>512	16	>256	0.5
236	Body fluid	32	64	>512	32	>512	1
237	Sputum	32	8	4	128	16	2
238	Endo	8	64	>512	16	128	2
239	Sputum	16	64	16	128	64	1
240	Endo	128	128	>512	256	32	1
241	Urine	128	64	4	64	128	0.06
242	Tip	128	128	32	128	64	1
243	Pus	512	256	>512	8	>512	32
244	Body fluid	32	64	>512	64	>512	0.25
245	Sputum	1	1	8	2	512	0.125
246	Urine	128	128	16	128	16	0.125
247	Endo	1	2	16	16	32	0.125
248	Endo	16	64	>512	128	128	64
249	Pus	32	16	32	64	32	0.125
251	Sputum	256	256	16	128	>512	8
252	Urine	16	64	>512	256	512	1
253	Urine	64	128	64	64	512	0.5
255	Body fluid	512	512	32	256	>512	4
256	Pus	512	256	1	64	>512	32
257	Body fluid (Bile)	128	128	>512	256	>512	0.25

Strains	Type of specimen	MIC (mg/L)					
		IMP	MEM	AK	CIP	FOS	CT
258	Urine	16	64	>512	256	>512	0.125
259	Body fluid	256	128	>512	256	>512	<0.03
260	Sputum	32	64	4	>512	512	<0.03
261	Body fluid (Bile)	32	128	>512	256	>512	0.06
262	Urine	128	128	256	>512	>512	0.06
263	Urine	32	32	>512	>512	>512	0.06
264	Sputum	128	128	>512	128	>512	16
265	Pus (Wound)	16	16	16	512	512	<0.03
266	Urine	4	2	2	32	64	0.06
267	Urine	64	128	4	512	>512	1
268	Urine	16	64	>512	128	>512	<0.03
269	Body fluid (Bile)	64	128	>512	128	>512	<0.03
270	Pus (Esophagostomy wound)	4	16	32	64	>512	16

Table 2. Mechanisms of carbapenem resistance among 240 CRKP isolates

Strains	Carbapenemase gene	SDS-PAGE		MIC (mg/L)		Fold decrease in MIC	MIC (mg/L)		Fold decrease in MIC
		OmpK35 Protein	OmpK36 Protein	IMP	IMP +CCCP		MEM	MEM +CCCP	
917	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	16	4	128	128	1
918	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	16	4	128	64	2
919	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	64	2
921	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	32	4	256	64	4
924	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	32	4	128	64	2
926	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	32	4	128	64	2
927	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
932	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
933	$bla_{\text{OXA-48-like}}$	-	+	16	4	4	64	64	1
934	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	256	128	2
936	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
937	$bla_{\text{NDM-like}}$	-	+	32	8	4	64	16	4
942	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
944	$bla_{\text{OXA-48-like}}$	-	+	8	4	2	64	64	1
946	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	16	4	256	256	1
947	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	-	256	256	1	512	128	4
952	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
971	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
975	$bla_{\text{NDM-like}}$	-	+	128	16	8	128	128	1
1058	$bla_{\text{OXA-48-like}}$	-	+	16	8	2	32	32	1
1059	$bla_{\text{OXA-48-like}}$	-	+	128	64	2	256	128	2
1061	$bla_{\text{OXA-48-like}}$	-	+	64	64	1	256	128	2
1063	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	128	1	128	128	1
1064	$bla_{\text{NDM-like}}$	-	+	16	16	1	16	8	2
1066	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	64	16	4
1071	$bla_{\text{NDM-like}}$	-	+	128	128	1	64	64	1
1073	$bla_{\text{IMP-like}}$	+	+	16	16	1	4	2	2
1074	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	64	2	256	128	2
1079	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	64	2

Strains	Carbapenemase gene	SDS-PAGE		MIC (mg/L)		Fold decrease in MIC	MIC (mg/L)		Fold decrease in MIC
		OmpK35 Protein	OmpK36 Protein	IMP	IMP +CCCP		MEM	MEM +CCCP	
1104	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	8	8	128	128	1
1108	$bla_{\text{OXA-48-like}}$	-	+	16	16	1	16	16	1
1207	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
1208	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
1211	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	64	2
1212	$bla_{\text{NDM-like}}$	-	+	32	32	1	64	64	1
1213	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	64	2
1214	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
1215	$bla_{\text{OXA-48-like}}$	-	+	16	16	1	128	128	1
1216	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
1217	$bla_{\text{NDM-like}}$	-	+	64	8	8	64	64	1
1218	$bla_{\text{NDM-like}}$	-	+	32	32	1	64	64	1
1120	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	256	128	2
1121	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	256	256	1
1224	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
1225	$bla_{\text{NDM-like}}$	-	+	256	128	2	256	128	2
1226	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
1227	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	256	256	1	256	128	2
1228	$bla_{\text{NDM-like}}$	-	+	32	32	1	64	64	1
1146	$bla_{\text{OXA-48-like}}$	-	+	8	8	1	16	16	1
1149	$bla_{\text{OXA-48-like}}$	-	+	8	4	2	16	16	1
1150	$bla_{\text{NDM-like}}$	-	+	64	64	1	128	128	1
1152	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
1153	$bla_{\text{NDM-like}}$	-	-	4	4	1	8	8	1
1154	$bla_{\text{NDM-like}}$	-	+	128	128	1	256	128	2
1156	$bla_{\text{NDM-like}}$	-	+	8	4	2	16	8	2
1157	$bla_{\text{IMP-like}}$	-	+	32	32	1	8	4	2
1158	$bla_{\text{NDM-like}}$	-	+	8	1	8	16	8	2
1159	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	32	8	4	128	64	2

Strains	Carbapenemase gene	SDS-PAGE		MIC (mg/L)		Fold decrease in MIC	MIC (mg/L)		Fold decrease in MIC
		OmpK35 Protein	OmpK36 Protein	IMP	IMP +CCCP		MEM	MEM +CCCP	
1169	$bla_{\text{NDM-like}}$	-	+	128	64	2	32	32	1
1170	$bla_{\text{NDM-like}}$	-	+	16	2	8	32	8	4
1172	$bla_{\text{OXA-48-like,}}$ $bla_{\text{IMP-like}}$	-	+	2	1	2	4	4	1
1174	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	16	4	64	64	1
1175	$bla_{\text{NDM-like}}$	-	+	8	2	4	8	4	2
1180	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	64	2
1183	$bla_{\text{NDM-like}}$	-	+	64	16	4	128	64	2
1184	$bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	64	2
1186	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	-	1024	1024	1	512	256	2
1187	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	32	16	2	64	64	1
1189	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	64	64	1
1190	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	-	512	256	2	128	128	1
1192	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	512	512	1	64	64	1
1194	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	128	1	128	64	2
1195	$bla_{\text{NDM-like}}$	-	+	8	8	1	4	4	1
1197	$bla_{\text{NDM-like}}$	-	+	8	8	1	8	4	2
77	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	64	2	128	128	1
78	$bla_{\text{NDM-like}}$	-	+	8	1	8	8	4	2
79	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	2	1	2	2	2	1
80	$bla_{\text{NDM-like}}$	-	-	1024	256	4	512	128	4
81	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	64	2
82	$bla_{\text{NDM-like}}$	-	+	64	32	2	128	64	2
83	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	64	2
84	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	32	32	1	64	64	1
85	$bla_{\text{NDM-like}}$	-	+	32	16	2	64	32	2
86	$bla_{\text{NDM-like}}$	-	+	16	4	4	16	4	4
87	$bla_{\text{NDM-like}}$	-	+	512	256	2	256	128	2
89	$bla_{\text{NDM-like}}$	-	+	16	16	1	8	4	2
90	$bla_{\text{OXA-48-like,}}$ $bla_{\text{IMP-like}}$	-	+	8	8	1	8	8	1
91	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	64	64	1
92	$bla_{\textit{NDM-like}}$	-	+	8	8	1	8	4	2
93	No carbapenemase gene detected	-	-	2	1	2	4	4	1

Strains	Carbapenemase gene	SDS-PAGE		MIC (mg/L)		Fold decrease in MIC	MIC (mg/L)		Fold decrease in MIC
		OmpK35 Protein	OmpK36 Protein	IMP	IMP +CCCP		MEM	MEM +CCCP	
95	*bla*$_{NDM-like}$	-	+	8	8	1	8	4	2
96	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	16	4	128	64	2
98	*bla*$_{NDM-like}$	-	+	64	32	2	128	64	2
99	*bla*$_{OXA-48-like}$	-	+	2	1	2	2	1	2
100	*bla*$_{NDM-like}$	-	-	1024	10124	1	512	256	2
101	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	8	8	64	32	2
102	No carbapenemase gene detected	-	-	2	1	2	4	4	1
103	*bla*$_{NDM-like}$	-	-	128	128	1	16	8	2
104	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
105	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
106	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
107	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
108	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
109	*bla*$_{NDM-like}$	-	+	8	8	1	32	32	1
111	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	128	128	1	256	128	2
112	*bla*$_{NDM-like}$	-	+	8	4	2	16	8	2
113	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
114	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	128	64	2	128	128	1
115	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	128	128	1	256	128	2
116	*bla*$_{NDM-like}$	-	+	64	64	1	128	128	1
117	*bla*$_{NDM-like}$	-	+	64	64	1	64	64	1
118	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	128	128	1	256	128	2
119	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
120	*bla*$_{NDM-like}$	-	+	64	64	1	32	32	1
121	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	32	32	1
122	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
123	*bla*$_{OXA-48-like}$	-	+	16	16	1	32	32	1
124	*bla*$_{OXA-48-like}$	-	+	1	1	1	2	2	1
125	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
126	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	64	64	1	128	128	1
127	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	-	+	128	64	2	256	128	2

Strains	Carbapenemase gene	SDS-PAGE		MIC (mg/L)		Fold decrease in MIC	MIC (mg/L)		Fold decrease in MIC
		OmpK35 Protein	OmpK36 Protein	IMP	IMP +CCCP		MEM	MEM +CCCP	
129	$bla_{\text{NDM-like}}$	-	+	64	64	1	32	32	1
132	$bla_{\text{OXA-48-like}}$	-	+	32	16	2	64	64	1
133	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	256	128	2	256	256	1
134	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	128	1	256	256	1
135	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
136	$bla_{\text{NDM-like}}$	-	+	64	64	1	16	16	1
137	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	256	128	2	256	256	1
138	$bla_{\text{OXA-48-like}}$	-	+	2	2	1	1	0.5	2
139	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	1024	1024	1	512	512	1
140	$bla_{\text{NDM-like}}$	-	+	64	64	1	64	64	1
141	$bla_{\text{NDM-like}}$	-	+	16	16	1	16	16	1
143	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	256	128	2	256	256	1
144	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	64	2	256	256	1
146	$bla_{\text{NDM-like}}$	-	+	64	64	1	128	128	1
147	No carbapenemase gene detected	-	+	1	1	1	4	4	1
148	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	128	1	256	256	1
149	$bla_{\text{OXA-48-like}}$	-	+	1	1	1	2	2	1
150	$bla_{\text{OXA-48-like}}$	-	+	1	1	1	0.5	0.5	2
151	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	128	1	256	256	1
152	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	128	1	128	128	1
153	$bla_{\text{NDM-like}}$	-	+	32	32	1	32	64	1
154	$bla_{\text{OXA-48-like}}$	-	+	32	64	1	16	16	1
156	$bla_{\text{OXA-48-like}}$	-	+	8	8	1	128	128	1
157	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
158	No carbapenemase gene detected	-	+	1	1	1	2	2	1
159	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	128	1	256	256	1
160	$bla_{\text{NDM-like}}$	-	+	64	64	1	128	128	1
161	$bla_{\text{OXA-48-like}}$	-	+	0.5	0.5	1	0.5	0.5	1
162	$bla_{\text{OXA-48-like}}$	-	-	16	16	1	4	4	1
163	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	32	32	1	256	128	2

Strains	Carbapenemase gene	SDS-PAGE		MIC (mg/L)		Fold decrease in MIC	MIC (mg/L)		Fold decrease in MIC
		OmpK35 Protein	OmpK36 Protein	IMP	IMP +CCCP		MEM	MEM +CCCP	
164	$bla_{\text{NDM-like}}$	-	+	16	16	1	8	16	1
166	$bla_{\text{OXA-48-like}}$	-	-	32	32	1	64	64	1
167	$bla_{\text{OXA-48-like}}$	-	+	1	1	1	0.25	0.25	1
168	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	256	128	2
169	$bla_{\text{OXA-48-like}}$	-	+	16	16	1	64	64	1
170	$bla_{\text{OXA-48-like}}$	-	+	8	8	1	32	32	1
171	$bla_{\text{NDM-like}}$	-	+	16	16	1	32	16	2
172	$bla_{\text{NDM-like}}$	-	+	32	32	1	32	32	1
173	$bla_{\text{OXA-48-like}}$	-	+	0.25	0.25	1	1	1	1
175	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	8	8	1	16	16	1
176	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	256	256	1
179	$bla_{\text{NDM-like}}$	-	+	8	4	2	32	32	1
180	$bla_{\text{NDM-like}}$	-	+	8	8	1	8	8	1
181	$bla_{\text{OXA-48-like}}$	-	+	0.25	0.25	1	0.5	0.25	2
183	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	32	2	128	128	1
184	$bla_{\text{NDM-like}}$	-	+	16	16	1	16	16	1
185	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
186	$bla_{\text{OXA-48-like}}$	-	+	8	16	1	32	64	1
188	$bla_{\text{OXA-48-like}}$	-	+	8	8	1	2	2	1
190	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
191	$bla_{\text{OXA-48-like}}$	-	+	64	64	1	64	64	1
192	$bla_{\text{OXA-48-like}}$	-	+	2	2	1	2	2	1
193	$bla_{\text{OXA-48-like}}$	-	+	1	1	1	2	2	1
194	$bla_{\text{NDM-like}}$	-	+	128	128	1	128	128	1
195	$bla_{\text{OXA-48-like}}$	-	+	16	16	1	64	32	2
196	$bla_{\text{NDM-like}}$	-	-	512	512	1	256	256	1
197	$bla_{\text{OXA-48-like}}$	-	+	8	8	1	16	16	1
199	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	64	2	128	128	1
200	$bla_{\text{NDM-like}}$	-	+	256	256	1	512	512	1
201	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	-	+	128	128	1	256	128	2

Strains	Carbapenemase gene	SDS-PAGE		MIC (mg/L)		Fold decrease in MIC	MIC (mg/L)		Fold decrease in MIC
		OmpK35 Protein	OmpK36 Protein	IMP	IMP +CCCP		MEM	MEM +CCCP	
202	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	128	128	1
203	*bla*$_{\text{OXA-48-like}}$	+	+	0.5	0.5	1	1	0.5	2
204	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	128	1	256	256	1
205	*bla*$_{\text{NDM-like}}$	-	+	64	64	1	128	128	1
206	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
208	*bla*$_{\text{NDM-like}}$	-	+	32	32	1	128	64	2
210	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	64	2	128	128	1
213	*bla*$_{\text{OXA-48-like}}$	-	+	16	32	1	64	64	1
214	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	64	128	1	256	128	2
216	*bla*$_{\text{NDM-like}}$	-	+	128	64	2	128	128	1
217	*bla*$_{\text{NDM-like}}$	-	+	128	128	1	128	128	1
218	*bla*$_{\text{NDM-like}}$	-	+	16	16	1	16	8	2
219	*bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	64	64	1
221	*bla*$_{\text{OXA-48-like}}$	+	+	0.5	0.5	1	0.5	0.5	1
222	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
223	*bla*$_{\text{OXA-48-like}}$	-	+	16	16	1	64	64	1
224	*bla*$_{\text{OXA-48-like}}$	-	+	16	16	1	64	64	1
226	*bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	64	64	1
228	*bla*$_{\text{NDM-like}}$	-	+	16	16	1	8	8	1
229	*bla*$_{\text{OXA-48-like}}$	-	+	2	2	1	2	1	2
230	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	64	2	256	256	1
231	*bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	64	64	1
232	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	128	1	256	256	1
233	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	128	1	256	256	1
234	*bla*$_{\text{NDM-like}}$	+	+	2	2	1	8	4	2
235	*bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	128	64	2
236	*bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	64	64	1
237	*bla*$_{\text{NDM-like}}$	-	+	32	16	2	8	8	1
238	*bla*$_{\text{OXA-48-like}}$	-	+	8	8	1	64	64	1
239	*bla*$_{\text{OXA-48-like}}$	-	+	16	16	1	64	64	1

Strains	Carbapenemase gene	SDS-PAGE		MIC (mg/L)		Fold decrease in MIC	MIC (mg/L)		Fold decrease in MIC
		OmpK35 Protein	OmpK36 Protein	IMP	IMP +CCCP		MEM	MEM +CCCP	
240	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	128	1	128	128	1
241	*bla*$_{\text{OXA-48-like}}$	-	-	128	128	1	64	64	1
242	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	128	1	128	128	1
243	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	512	512	1	256	256	1
244	*bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	64	64	1
245	*bla*$_{\text{OXA-48-like}}$	-	+	1	1	1	1	1	1
246	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	128	1	128	128	1
247	*bla*$_{\text{OXA-48-like}}$	-	+	1	1	1	2	2	1
248	*bla*$_{\text{OXA-48-like}}$	-	+	16	16	1	64	64	1
249	*bla*$_{\text{NDM-like}}$	-	+	32	16	2	16	16	1
251	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	256	128	2	256	256	1
252	*bla*$_{\text{OXA-48-like}}$	-	+	16	16	1	64	64	1
253	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	64	64	1	128	128	1
255	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	-	512	512	1	512	256	2
256	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	512	256	2	256	256	1
257	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	128	64	2	128	64	2
258	*bla*$_{\text{NDM-like}}$	-	+	16	4	4	64	16	4
259	*bla*$_{\text{OXA-48-like}}$	-	+	256	128	2	128	64	2
260	*bla*$_{\text{OXA-48-like}}$	-	-	32	32	1	64	16	4
261	*bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	128	64	2
262	*bla*$_{\text{OXA-48-like}}$	-	-	128	32	4	128	128	1
263	*bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$	-	+	32	32	1	32	32	1
264	*bla*$_{\text{OXA-48-like}}$	-	+	128	128	1	128	64	2
265	*bla*$_{\text{NDM-like}}$	-	+	16	4	4	16	4	4
266	*bla*$_{\text{OXA-48-like}}$	-	+	4	1	4	2	2	1
267	*bla*$_{\text{NDM-like}}$	-	+	64	16	4	128	32	4
268	*bla*$_{\text{OXA-48-like}}$	-	+	16	16	1	64	32	2
269	*bla*$_{\text{OXA-48-like}}$	-	+	64	16	4	128	32	4
270	*bla*$_{\text{NDM-like}}$	-	+	4	1	4	16	4	4

Table 3. Antibiotic combinations against CRKP isolates with different resistance

Isolate.	Mechanism of carbapenem resistance			MIC (mg/L)					FICI			
	Carbapenemase gene	Loss of porin	Over-expression of efflux pump	IMP	MEM	AK	FOS	CT	CT+ IMP	CT + MEM	FOS+ AK	FOS+ IMP
1073	$bla_{\text{IMP-like}}$	No loss of porin	-	16	4	1	8	1	1	1	2	0.531
1157	$bla_{\text{IMP-like}}$	OmpK35	-	32	8	1	128	0.03	2	1	1	1
1175	$bla_{\text{NDM-like}}$	OmpK35	+	8	8	4	512	0.25	0.625	0.75	0.265	0.325
1064	$bla_{\text{NDM-like}}$	OmpK35	-	16	16	16	8	0.5	1	1	1	0.5625
249	$bla_{\text{NDM-like}}$	OmpK35	-	32	16	32	32	0.125	2	2	0.75	0.75
1225	$bla_{\text{NDM-like}}$	OmpK35	-	256	256	32	>1024	32	1	0.75	ND	ND
100	$bla_{\text{NDM-like}}$	OmpK35 , OmpK36	-	1024	512	32	256	0.25	1	2	1	0.5625
944	$bla_{\text{OXA-48-like}}$	OmpK35	-	8	64	>512	2048	16	2	0.75	ND	0.625
123	$bla_{\text{OXA-48-like}}$	OmpK35	-	16	32	2	>512	0.06	1	2	2	0.625
132	$bla_{\text{OXA-48-like}}$	OmpK35	-	32	64	>512	128	0.06	1	1	ND	1
1061	$bla_{\text{OXA-48-like}}$	OmpK35	-	64	256	32	64	0.06	1	0.75	1	0.75
1184	$bla_{\text{OXA-48-like}}$	OmpK35	-	64	128	32	128	1	1	1	0.3125	0.5
241	$bla_{\text{OXA-48-like}}$	OmpK35, OmpK36	-	128	64	4	128	0.06	1	1	0.75	0.5
204	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	OmpK35	-	128	256	16	256	0.5	2	1	0.625	0.75
251	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	OmpK35	-	256	256	16	>512	8	2	0.75	ND	ND
947	$bla_{\text{NDM-like}}$, $bla_{\text{OXA-48-like}}$	OmpK35, OmpK36	+	256	512	32	>512	32	0.75	0.625	ND	ND
90	$bla_{\text{OXA-48-like}}$, $bla_{\text{IMP-like}}$	OmpK35	-	8	8	1	256	0.5	2	2	1	0.75

ND, not determined

Antibiotics: CT, colistin; IMP, imipenem; MEM, meropenem; FOS, fosfomycin; and AK, amikacin

Isolate.	Mechanism of carbapenem resistance			MIC (mg/L)					FICI			
	Carbapenemase gene	Loss of porin	Over-expression of efflux pump	IMP	MEM	AK	FOS	CT	CT+ IMP	CT + MEM	FOS+ AK	FOS + IMP
129	*bla*$_{NDM-like}$	OmpK35	-	64	32	1	16	1	ND	ND	1	0.75
1150	*bla*$_{NDM-like}$	OmpK35	-	64	128	16	256	32	ND	ND	0.515	0.3125
78	*bla*$_{NDM-like}$	OmpK35	+	8	8	8	8	0.5	ND	ND	0.375	0.5
1158	*bla*$_{NDM-like}$	OmpK35	+	8	16	4	128	2	ND	ND	1	2
86	*bla*$_{NDM-like}$	OmpK35	+	16	16	16	64	1	ND	ND	1	1
267	*bla*$_{NDM-like}$	OmpK35	+	64	128	4	>512	1	ND	ND	0.625	0.5
80	*bla*$_{NDM-like}$	OmpK35, OmpK36	+	1024	512	32	128	16	ND	ND	0.75	0.5625
197	*bla*$_{OXA-48-like}$	OmpK35	-	8	16	32	128	0.125	ND	ND	0.75	0.75
248	*bla*$_{OXA-48-like}$	OmpK35	-	16	64	>512	128	64	ND	ND	ND	0.75
269	*bla*$_{OXA-48-like}$	OmpK35	+	32	128	>512	>512	<0.03	ND	ND	ND	1
262	*bla*$_{OXA-48-like}$	OmpK35, OmpK36	+	128	128	256	>512	0.06	ND	ND	0.5	0.75
932	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	OmpK35	-	64	128	32	64	0.25	ND	ND	1	2
139	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	OmpK35	-	1024	512	32	256	0.125	ND	ND	0.75	0.625
1159	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	OmpK35	+	32	128	16	16	2	ND	ND	0.625	1
1066	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	OmpK35	+	64	64	32	64	2	ND	ND	0.625	0.75
101	*bla*$_{NDM-like}$, *bla*$_{OXA-48-like}$	OmpK35	+	64	64	16	64	1	ND	ND	1	0.75
1172	*bla*$_{OXA-48-like}$, bla$_{IMP-like}$	OmpK35	-	2	4	16	256	0.25	ND	ND	1	0.1289

ND, not determined

Antibiotics: CT, colistin; IMP, imipenem; MEM, meropenem; FOS, fosfomycin; and AK, amikacin

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