กลไกการดื้อยากลุ่ม carbapenems ในเชื้อ *Acinetobacter* species และการเสริมฤทธิ์กันของยาต้านจุลชีพต่อเชื้อ *Acinetobacter* species ที่ดื้อยากลุ่ม carbapenems

นางสาวอุทัยพร สิงห์คำอินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

MECHANISMS OF CARBAPENEM RESISTANCE IN *ACINETOBACTER* SPECIES AND SYNERGISTIC ACTIVITIES OF ANTIBIOTICS AGAINST CARBAPENEM-RESISTANT *ACINETOBACTER* SPECIES

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Microbiology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2016

Thesis Title	MECHANISMS OF CARBAPENEM RESISTANCE IN *ACINETOBACTER* SPECIES AND SYNERGISTIC ACTIVITIES OF ANTIBIOTICS AGAINST CARBAPENEM-RESISTANT *ACINETOBACTER* SPECIES
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อุทัยพร สิงห์คำอินทร์ : กลไกการดื้อยากลุ่ม carbapenems ในเชื้อ *Acinetobacter* species และการเสริมฤทธิ์กันของยาต้านจุลชีพต่อเชื้อ *Acinetobacter* species ที่ดื้อยากลุ่ม carbapenems (MECHANISMS OF CARBAPENEM RESISTANCE IN *ACINETOBACTER* SPECIES AND SYNERGISTIC ACTIVITIES OF ANTIBIOTICS AGAINST CARBAPENEM-RESISTANT *ACINETOBACTER* SPECIES) อ .ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.ธนิษฐา ฉัตรสุวรรณ, 222 หน้า.

Acinetobacter calcoaceticus-Acinetobacter baumannii complex เป็นเชื้อฉวยโอกาสที่สำคัญซึ่งเป็นสาเหตุของการติดเชื้อในโรงพยาบาล การดื้อยากลุ่ม carbapenems ใน *Acinetobacter* spp. มีรายงานเพิ่มขึ้นทั่วโลก วัตถุประสงค์ของการศึกษานี้เพื่อตรวจหาอัตราการดื้อยาและกลไกการดื้อยากลุ่ม carbapenems ใน *Acinetobacter* spp. ที่แยกได้จากผู้ป่วยไทย และตรวจหาการเสริมฤทธิ์กันของยาต้านจุลชีพที่นำมาใช้ร่วมกันต่อเชื้อ *Acinetobacter* spp. ที่ดื้อยากลุ่ม carbapenems อัตราการดื้อยากลุ่ม carbapenems ใน *A. baumannii*, *A. pittii* และ *A. nosocomialis* คือ 86.6%, 26.3% และ 22.7% ตามลำดับ ยีนที่สร้างเอนไซม์ carbapenemases ที่พบมากที่สุดใน *A. baumannii*, *A. pittii* และ *A. nosocomialis* ที่ดื้อยากลุ่ม carbapenems คือ $bla_{\text{OXA-23}}$ ตัวแทนเชื้อที่มียีนที่สร้างเอนไซม์ carbapenemase ประกอบด้วย *A. baumannii* จำนวน 23 สายพันธุ์ *A. pittii* จำนวน 8 สายพันธุ์และ *A. nosocomialis* จำนวน 5 สายพันธุ์ ถูกนำมาทดสอบในขั้นตอนถัดไป การแสดงออกที่ลดลงของ outer membrane proteins พบใน *A. baumannii* จำนวน 9 สายพันธุ์และ *A. pittii* จำนวน 4 สายพันธุ์ แต่ไม่พบใน *A. nosocomialis* พบการแทรกตัวของ insertion sequence ชนิดใหม่ (IS*Aba40*) ในยีน *carO* ของ *A. baumannii* สายพันธุ์ AB58 และ AB97 กลไกการแสดงออกที่เพิ่มขึ้นของ efflux pump ไม่พบใน *A. baumannii* และ *A. nosocomialis* แต่พบใน *A. pittii* 3 สายพันธุ์ การศึกษาความสัมพันธ์ของเชื้อ พบว่าใน *A. baumannii* ส่วนใหญ่เป็น ST195 และ ST542 ใน *A. pittii* ส่วนใหญ่เป็น ST1425 และ ST1178 ใน *A. nosocomialis* เป็นกลุ่มความสัมพันธ์เดียวกัน คือ ST598 สำหรับantibiotic resistance island พบ AbaR4-type จำนวน 4 แบบ โดยแบบที่มียีน $bla_{\text{OXA-23}}$ พบเฉพาะใน *A. baumannii* และ *A. nosocomialis*

การศึกษาผลของยาต้านจุลชีพร่วมกัน ได้แก่ imipenem และ meropenem ร่วมกับ amikacin หรือ colistin หรือ fosfomycin พบว่าการใช้ยา imipenem ร่วมกับยา fosfomycin ให้ผลการเสริมฤทธิ์กันมากที่สุดคือ 65.2%, 62.5% และ 80% ของ *A. baumannii*, *A. pittii* และ *A. nosocomialis* ตามลำดับ การเสริมฤทธิ์กันยืนยันด้วยวิธี time-kill พบว่าในเชื้อทุกตัวอย่างที่ทดสอบให้ผลการเสริมฤทธิ์กันเหมือนวิธี checkerboard ยกเว้น *A. baumannii* AB250 เชื้อทุกตัวอย่างที่ทดสอบดื้อยา fosfomycin การศึกษานี้ไม่พบกลไกการดื้อยา fosfomycin ซึ่งได้แก่ การกลายพันธุ์ของ MurA, การสร้างเอนไซม์ fosfomycin-modifying enzyme, การแสดงออกที่เพิ่มขึ้นของ efflux pump และกลไก cell wall recycling bypass pathway การศึกษา population ของเชื้อพบว่ามี subpopulation ของ *Acinetobacter* spp. ที่ดื้อยา fosfomycin ในระดับสูงกว่าค่า MIC เดิม ในขณะที่ภาวะ tolerance ต่อยา imipenem พบเฉพาะ *A. baumannii* AB250 ผลดังกล่าวแสดงให้เห็นว่าภาวะ tolerance ต่อยา imipenem น่าจะเกี่ยวข้องกับการเสริมฤทธิ์กันของ imipenem กับ fosfomycin จากผลการศึกษานี้แสดงให้เห็นว่ากลไกการดื้อยากลุ่ม carbapenems ที่สำคัญใน *Acinetobacter* spp. คือการสร้างเอนไซม์ carbapenemases การศึกษานี้พบยีน $bla_{\text{OXA-58}}$ ร่วมกับ $bla_{\text{IMP-14a}}$ ใน *A. pittii* ที่ดื้อยากลุ่ม carbapenems เป็นครั้งแรกและพบ IS*Aba40* ชนิดใหม่ซึ่งแทรกอยู่ในยีน *carO* ของ *A. baumannii* ที่ดื้อยากลุ่ม carbapenems การใช้ยา imipenem ร่วมกับ fosfomycin อาจเป็นตัวเลือกหนึ่งของการใช้ยาต้านจุลชีพร่วมกันเพื่อรักษาการติดเชื้อ *Acinetobacter* spp. ที่ดื้อต่อยากลุ่ม carbapenems

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5287847220 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS: ACINETOBACTER SPECIES / CARBAPENEM RESISTANCE / CARBAPENEMASE / PORIN / EFFLUX PUMP / ANTIBIOTIC RESISTANCE ISLAND / ANTIBIOTIC COMBINATION / ANTIBIOTIC SYNERGISM

UTHAIBHORN SINGKHAM-IN: MECHANISMS OF CARBAPENEM RESISTANCE IN *ACINETOBACTER* SPECIES AND SYNERGISTIC ACTIVITIES OF ANTIBIOTICS AGAINST CARBAPENEM-RESISTANT *ACINETOBACTER* SPECIES. ADVISOR: TANITTHA CHATSUWAN, Ph.D., 222 pp.

Acinetobacter calcoaceticus-A. baumannii complex is one of the most important opportunistic pathogens that cause nosocomial infection. Carbapenem resistance in *Acinetobacter* spp. has been increasingly reported worldwide. The objectives of this study were to investigate the prevalence and mechanisms of carbapenem resistance in *Acinetobacter* spp. Thai isolates and to determine the synergistic effects of antibiotic combinations against carbapenem-resistant *Acinetobacter* spp. The prevalences of carbapenem resistance in *A. baumannii*, *A. pittii* and *A. nosocomialis* were 86.6%, 26.3% and 22.7%, respectively. The most common carbapenemase gene in carbapenem-resistant *A. baumannii*, *A. pittii* and *A. nosocomialis* was $bla_{\text{OXA-23}}$. The representative isolates carrying carbapenemase genes including 23 *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis* were further investigated. The reduction of outer membrane proteins was observed in 9 *A. baumannii* and 4 *A. pittii* isolates, but was not detected in *A. nosocomialis*. The *carO* gene of *A. baumannii* AB58 and AB97 was disrupted by a novel insertion sequence, IS*Aba40*. No overexpression of efflux pump was found in *A. baumannii* and *A. nosocomialis*, but was observed in 3 *A. pittii* isolates. The clonal study showed that the major clones of *A. baumannii* were ST195 and ST542, those of *A. pittii* were ST1178 and ST1425. *A. nosocomialis* belonged to a single clone, ST598. Analysis of antibiotic resistance islands demonstrated 4 subtypes of AbaR4 island. AbaR4 subtype carried $bla_{\text{OXA-23-like}}$ was found in *A. baumannii* and *A. nosocomialis*.

The *in vitro* activities of antibiotic combinations including imipenem and meropenem plus amikacin, colistin or fosfomycin were evaluated. The best combination was imipenem plus fosfomycin which showed synergism in 65.2%, 62.5% and 80% of *A. baumannii*, *A. pittii* and *A. nosocomialis*, respectively. The synergism was confirmed by time-kill assay. The results were similar to checkerboard assay except that of *A. baumannii* AB250. All of time-kill tested isolates were resistant to fosfomycin, but the resistance mechanisms including MurA mutation, fosfomycin-modifying enzyme production, overexpression of efflux pump and cell wall recycling bypass pathway were not detected. The population analysis of *Acinetobacter* spp. showed subpopulations that had higher fosfomycin MICs. Tolerance to imipenem was found in *A. baumannii* AB250. The results indicate that imipenem tolerance may be associated with synergism when imipenem is combined with fosfomycin.

The results showed that carbapenemase production is the major mechanism of carbapenem resistance in *Acinetobacter* spp. This is the first report of carbapenem-resistant *A. pittii* carrying $bla_{\text{OXA-58}}$ and $bla_{\text{IMP-14a}}$ and the disruption of the *carO* gene by a novel IS*Aba40* in carbapenem-resistant *A. baumannii*. This study suggests that combination of imipenem and fosfomycin may become the effective combination therapy for treatment of carbapenem-resistant *Acinetobacter* spp. infection.

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

Academic Year: 2016 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following whose gave me the possibility to success my thesis: Tanittha Chatsuwan, Ph.D., my thesis advisor at Department of Microbiology, Faculty of Medicine, Chulalongkorn University for her guidance, kindness, helpful suggestion and encouragement throughout eight year of the course.

Associate Professor Ariya Chindamporn, Ph.D., the chairman of the thesis committee, Department of Microbiology, Faculty of Medicine, Chulalongkorn University for her critisms.

The examiner members of thesis committee, Associate Professor Somying Tumwasorn, Ph.D. and Assistant Professor Kanitha Patarakul, M.D., Ph.D., Department of Microbiology, Faculty of Medicine, Chulalongkorn University and Associate Professor Aroonwadee Chanawong, Ph.D., Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Khon Kaen University for their valuable discussion and kindness.

This work was financially supported by grant from the Chulalongkorn University Graduate Scholarship to Commemorate the 72th Anniversary of His Majesty King Bhumibol Adulyadej, the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksompot Endowment Fund) from Chulalongkorn University Graduate School and the Research Grant for Graduate Studies from the National Research Council of Thailand.

And sincere thanks to all members in inter-department of Medical Microbiology, all staff at Department of Microbiology, Faculty of Medicine, Chulalongkorn University for their kindness and helpful.

Finally, I am deeply appreciate to my parants and my family for their understanding and support during eight year of this study.

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ABBREVIATIONS

AK	amikacin
Ala (A)	alanine
Arg (R)	arginine
Asp (D)	aspartic acid
ATCC	American Type Culture Collection
bla	β-lactamase gene
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAZ	ceftazidime
CCCP	cyanide 3-chlorophenylhydrazone
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CIP	ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CRO	ceftriaxone
CT	colistin
CTX	cefotaxime
Cys (C)	cysteine
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide-tri-phosphate
DR	direct repeat
EDTA	ethylenediamine tetra acetic acid
et al.	et alii
EU	European
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FEP	cefepime
FICI	fractional inhibitory concentration index
FOF	fosfomycin
G6P	glucose-6-phosphate
GN	gentamicin
IPM	imipenem
IRL	inverted repeat left
IRR	inverted repeat right
IS	insertion sequence
kbp	kilo base pair
kDa	kilodalton
Leu (L)	leucine
Lys (K)	lysine

M	molar
MEM	meropenem
mg	milligram
MIC	minimum inhibitory concentration
mL	milliliter
MLST	multilocus sequence typing
mM	millimolar
mm	millimeter
mRNA	messenger ribonucleic acid
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
NCBI	National Center for Biotechnology Information
^{o}C	degree Celsius
OMP	outer membrane protein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
TBE	tris-borate-EDTA
Tn	transposon
U	unit

CHAPTER I
INTRODUCTION

Acinetobacter species are Gram-negative bacteria that are the important opportunistic pathogens associated with nosocomial infections including blood stream infection, respiratory tract infection, urinary tract infection, and wound infection in immunocompromised patients. Nowadays, 72 species of the genus *Acinetobacter* have been identified (1). The group of species, *A.calcoacetius-A. baumannii* (Acb) complex is the most common pathogen in clinical setting especially *A. baumannii*, *A. pittii* and *A. nosocomialis* (2).

Antimicrobial agents for treatment of *Acinetobacter* spp. infection are aminoglycosides, fluoroquinolones, board-spectrum cephalosporins, beta-lactam plus beta-lactamase inhibitor and carbapenems. Recently, *Acinetobacter* spp. are frequently resistant to these antimicrobial agents. The emergence of antibiotic resistance in *Acinetobacter* spp. especially *A. baumannii* has been rising worldwide (3, 4).

Carbapenems, the highest beta-lactam activity, are the drug of choice for treatment. Unfortunately, carbapenem resistance rate in *Acinetobacter* spp. has been high, especially in Asia (4). The resistance rate of more than 50% has been reported (4). The molecular mechanisms of carbapenem resistance are mediated by i) carbapenemase production, ii) reduction of carbapenems into bacterial cell, and iii) increase extrude carbapenems outside the cell. There are two types of carbapenemases found in *Acinetobacter* spp., serine-carbapenemases encoded by $bla_{\text{OXA-51,}}$ $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, and $bla_{\text{OXA-58}}$ (5), and metallo-carbapenemases encoded by bla_{IMP}, bla_{VIM}, and bla_{NDM} (6, 7). The $bla_{\text{OXA-23}}$ has been found in chromosomal antibiotic resistance island (8). Loss or reduced outer membrane proteins, CarO, 33-36 kDa OMP, and OprD in *Acinetobacter* spp. are associated with carbapenem resistance (9-11). Moreover, overexpression of efflux pumps, AdeABC, AdeDE and AdeXYZ can reduce carbapenem MICs in *A. baumannii* and *Acinetobacter* spp. (12). However, the major mechanism of carbapenem resistance is carbapenemase production.

Although colistin and tigecycline are used for the treatment of carbapenem-resistant *Acinetobacter* spp. infection, both of them are usually reserved to be the last resort of antimicrobial agents. Moreover, colistin has neurotoxic and nephrotoxic effects to human (13). Therefore, using those antimicrobial agents is limited and carbapenems have been used in combination therapy (14).

Combination therapy is the most effective strategy to combat multidrug-resistant *Acinetobacter* spp. However, there are no standards of combination therapy for treatment. The aim of antibiotic combinations is synergistic effect. Carbapenems have also been the main antibiotic in the combination. Many *in vitro* studies of carbapenem and colistin combinations showed synergistic effect against various Gram-negative bacteria including *A. baumannii* (15, 16). Moreover, aminoglycosides have been highly effective against multidrug-resistant Gram-negative bacteria when used in combination with carbapenems (17). Fosfomycin is antimicrobial agent that inhibits bacterial cell wall synthesis. Fosfomycin is one of the most frequently used antimicrobials for treatment of infection caused by extended-spectrum beta-lactamase (ESBL)-producing Gram-negative bacteria and multidrug-resistant *Pseudomonas aeruginosa*. Although *Acinetobacter* spp. are intrinsically resistant to fosfomycin, many studies showed synergism when fosfomycin was used in combination with sulbactam or carbapenems (14).

Nowadays, carbapenem-resistant *Acinetobacter* spp. especially *A. baumannii* are the major causes of nosocomial infection worldwide including Thailand. However, there are very few studies of molecular mechanisms of carbapenem resistance and synergy of antibiotic combinations against carbapenem-resistant *Acinetobacter* spp. in Thai isolates (18, 19). Moreover, antibiotic resistance island has not yet been reported in *Acinetobacter* spp. except *A. baumannii* and *A. nosocomialis* Thai isolates (20, 21). Therefore, the purposes of this study were to investigate (i) the carbapenem resistance mechanisms, (ii) the antibiotic resistance islands, and (iii) the *in vitro* effects of carbapenem combination with amikacin, colistin and fosfomycin against carbapenem-resistant *Acinetobacter* spp. with different mechanisms by checkerboard and time-kill assay. Moreover, the possible synergistic mechanism of the best antibiotic combination will be characterized.

CHAPTER II
OBJECTIVES

I. To investigate prevalence and molecular mechanisms of carbapenem resistance in *Acinetobacter* spp. Thai isolates

II. To characterize antibiotic resistance islands in carbapenem-resistant *Acinetobacter* spp. Thai isolates

III. To study synergistic activities of antibiotic combinations against carbapenem-resistant *Acinetobacter* spp. Thai isolates

CHAPTER III
LITERATURE REVIEW

1. BACTERIOLOGY

Acinetobacter species are Gram-negative coccobacilli and non-fermentative bacteria. They can grow on MacConkey agar and produce catalase and are non-motile. *Acinetobacter* species belong to the Phylum Proteobacteria, Class Gammaproteobacteria, Order Pseudomonadales and Family Moraxellaceae. Nowadays, in the genus *Acinetobacter*, there are 66 published species names and 6 tentative species names (1). *A. calcoaceticus-A. baumannii* (Acb) complex is the most organism group of species frequently isolated from human clinical specimens. Acb complex includes *A. baumannii, A. calcoaceticus, A. pittii, A. nosocomialis, A. seifertii* and *A. dijkshoorniae*. Unfortunately, these 6 species cannot be completely differentiated by phenotypic tests (biochemical tests) as shown in Table 1 (22). Although the gold standard for species identification is DNA-DNA hybridization, molecular methods including Random Amplified Polymorphic DNA (RAPD), Amplified rDNA Restriction Analysis (ARDRA), 16S rRNA and *rpoB* sequence analysis, 16S-23S rRNA gene intergenic spacer sequence and Mass Spectrometry (MS) have been used for identification (1, 23-26).

Acb complex is the most common opportunistic pathogen that can cause hospital-acquired respiratory tract infection (especially ventilator-associated pneumonia) and bloodstream infection. Acb complex can disseminate from patients to other patients and medical personnel by respiratory droplets. Moreover, Acb complex can survive in general hospital environment such as medical device (especially ventilator). The diagram of Acb complex dissemination in medical setting are shown in Figure 1 (27).

Table 1. Phenotypic identification of Acb complex (22).

	A. baumannii	*A. calcoaceticus*	*A. nosocomialis*	*A. pittii*	*A. seifertii*	*A. dijkshoorniae*
Growth at 44ºC	+	-	95	10	13	+
Growth at 41ºC	+	9	+	+	94	+
Growth at 37ºC	+	91	+	+	+	+
Growth at 35ºC	+	+	+	+	+	+
Growth at 32ºC	+	+	+	+	+	+
Acidification of D-glucose	+	91	+	95	-	+
Assimilation of						
L-Arginine	+	+	+	+	+	+
Citrate (Simmons)	+	91	+	+	+	+
D-Glucose	-	-	-	-	-	-
Malonate	88	+	20	95	75	+
L-Ornithine	76	+	95	95	81	93

Adapted from Metabolic and physiological characteristics of *Acinetobacter* spp. (22); +, All tested strains positive; -, all tested strains negative; numbers are percentage of tested strains with clearly positive reactions.

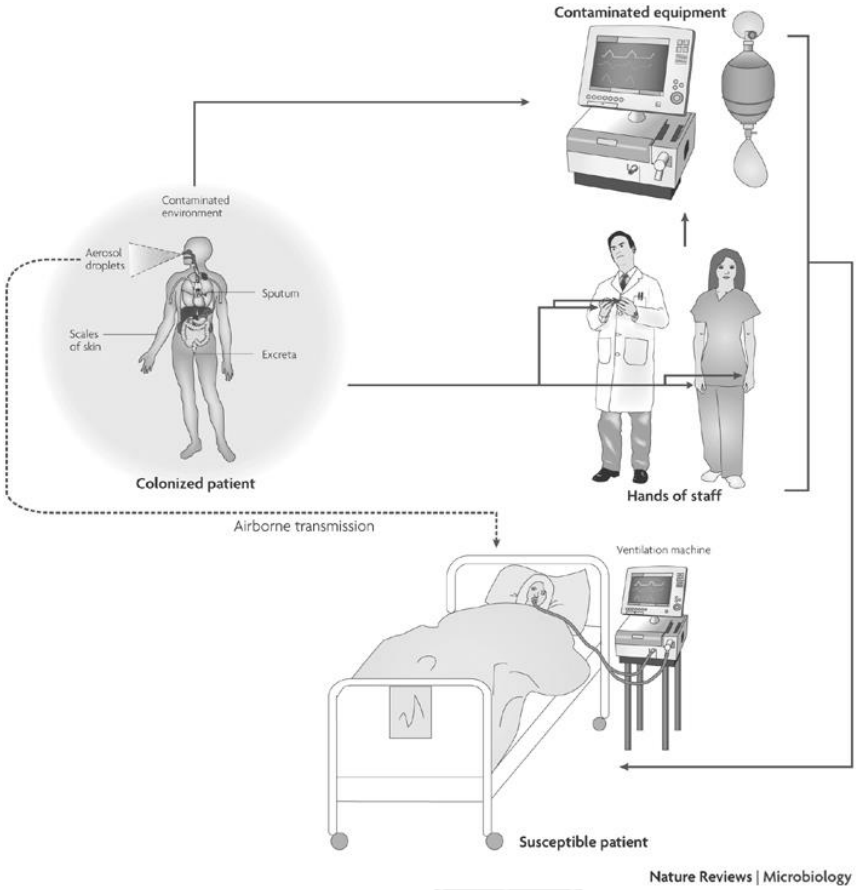


Figure 1. Diagram of Acb complex dissemination in medical setting (27)

2. VIRULENCE FACTORS AND PATHOGENESIS

2.1 Lipopolysaccharide (LPS) and Lipooligosaccharide (LOS)

LPS and LOS are the major components of outer membrane in Gram-negative bacteria including *Acinetobacter* spp. LPS structure is composed of lipidA and polysaccharide chain, called O-antigen, whereas LOS has lipidA and oligosaccharide. LOS has been found in *Acinetobacter* spp. strain that cannot produce O-antigen (lack of *O*-glycosylation enzymes, WaaL ligase and PglL enzyme) (28). Both LPS and LOS in *Acinetobacter* spp. contain endotoxin (lipidA) that can be recognized by Toll-like receptor 4 (TLR4) and can activate host immune response (29). LipidA also involved in virulence and opsonophagocytic killing in mouse model (30). Modification of lipidA resulted in colistin or polymyxin resistance in *Acinetobacter* spp. (31).

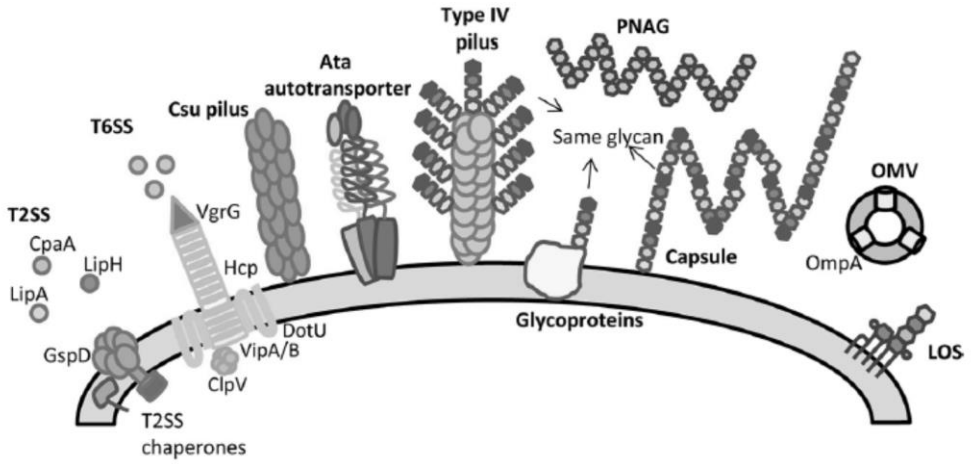


Figure 2. Overview of virulence factors in *Acinetobacter* species (32)

2.2 Glycoproteins (or O-glycosylated membrane proteins)

Glycosylation or O-glycosylation system in *Acinetobacter* species requires WaaL ligase and PglL enzyme which are important in O-antigen biosynthesis. The O-glycosylation system has been found in all species of Acb complex and required for virulence and biofilm formation in mouse model (28).

2.3 Pili

Pili (as shown in Figure 2) are the appendage in the surface of bacteria that involve in bacterial attachment, motility and transfer of genetic materials. Csu pili (CsuA/BABCDE mediated pili) found in *Acinetobacter* spp. played a role in attachment and biofilm formation on abiotic (33) but not on biotic surface (human airway epithelial cells).

2.4 Capsule

An extracellular polysaccharide capsule of *Acinetobacter* spp. can prevent bacterial clearance in body fluid, human serum and tissue of mouse model (34). Antimicrobial agents including chloramphenicol and erythromycin can modulate the capsule production. The capsule production resulted in resistance to killing by complement and immune system (35).

2.5 Protein secretion systems

Protein secretion systems are the multi-protein transporters that are composed of inner membrane protein, periplasmic protein and outer membrane protein. There are

many evidences demonstrating that secretion systems including type II secretion system, type VI secretion system and autotransporter are involved in virulence of *Acinetobacter* spp.

2.5.1 Type II secretion system (T2SS)

T2SS has been found in many species of *Acinetobacter* including *A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. calcoaceticus* and *A.junii* (36). The substrates of T2SS were LipA, LipH lipase and CpaA protease which played a role in virulence in mouse model (36).

2.5.2 Type VI secretion system (T6SS)

T6SS is composed of two main components, Hcp (important membrane protein for protein secretion) and VgrG (effector protein on the tip of T6SS). Although T6SS was found in most strains of *A. baumannii*, only some strains can kill *Escherichia coli* and other nosocomial pathogens via T6SS-dependent killing (37). However, T6SS is required for virulence in *Galleria mellonella* model, but not for biofilm formation (37).

2.6 Outer membrane protein A (OmpA)

The study by Gaddy *et al*. demonstrated that *A. baumannii* produced partial biofilm on abiotic surface and strong biofilm on biotic surface (*Candida* filament and human epithelial cell) and killed yeast and epithelial cell by secreted protein, OmpA (38). OmpA also induced dendritic cell apoptosis and necrosis via reactive oxygen species (ROS) production (39). The OmpA was packed, secreted, and delivered to host cells by outer membrane vesicles (OMVs) and then induced host cell death (40).

2.7 Siderophore synthesis

Almost all bacteria require appropriate concentration of iron for growth. Iron concentration in general environments and human bodies is very low and rarely free form. Therefore, iron acquisition pathways are essential for all bacteria including *Acinetobacter* spp. Siderophore is an iron chelator, which is produced by bacteria for capturing and transporting iron into bacterial cells (41). Acinetobactin is a catechol-hydroxamate siderophore of *A. baumannii* (42). Acinetobactin-mediated iron uptake is a major iron acquisition in *A. baumannii*. Zimbler *et al*. demonstrated that inactivation of *bauA* and *basD*, which encoded acinetobactin transporter and biosynthesis function, respectively, had an effect on the ability of growth in iron-limiting conditions (43). However, when acinetobactin biosynthesis and transport gene were inactivated, *A.*

baumannii was able to use hemin as iron source (43). Intracellular iron is regulated by iron repressor protein, Fur. At high level of intracellular iron, the Fur binds with iron and inhibits the component of the acinetobactin secretion machinery (43).

2.8 Biofilm-associated protein (Bap)

Many proteins or biological substances involved in biofilm formation in *Acinetobacter* spp. including LPS or LOS, membrane glycoproteins, Csu pili and polysaccharide capsule. Bap is large membrane protein related to biofilm formation in various bacteria including *A. baumannii* (44). Bap was required to be essential for biofilm formation and maturation on abiotic surface and involved in human cell adherence in *A. baumannii* (45). Bap was also found in other species in Acb complex. It was found that Bap structure showed immunoglobulin-like complex that required for biofilm formation (46).

3. TREATMENT OF *ACINETOBACTER* INFECTION

The Genus *Acinetobacter* has been appeared in Bergey's Manual of Systematic Bacteriology since 1974, and only *A.calcoacetius* was described (47). At that time, the available antimicrobial agents were sulfonamide, cephalosporins and aminoglycosides, however the resistant strain for these antimicrobial agents was reported (47). *Acinetobacter* showed variable metabolic pathway and phenotypic identification. In 1985, molecular method, DNA-DNA hybridization was used for identification, thus, more species of *Acinetobacter* were described including *A. calcoaceticus-A. baumannii* complex. Then, the available antimicrobial agents were quinolones and carbapenems, unfortunately, the resistant strains were also reported (47).

Nowadays, the problem of treatment of *Acinetobacter* spp. infection is antimicrobial agent resistance including multidrug resistance (resistant to 3 classes of antimicrobial agents), extremely drug resistance (resistant to more than 3 classes of antimicrobial agents but not all classes of them) and pan drug resistance (resistant to all classes of antimicrobial agents) (48). Recommended antimicrobial agents for treatment of multidrug-resistant *Acinetobacter* spp. infection are extended-spectrum cephalosporins plus aminoglycoside (expected of synergism), colistin (if susceptible), ampicillin/sulbactam plus aminoglycoside (some studies showed effective activity of sulbactam against *Acinetobacter* spp.) and tigecycline. However, the limitations of this

recommendation are (i) the clinical microbiology laboratory does not perform synergism of antimicrobial agents, (ii) although colistin shows effective *in vitro* activity against *Acinetobacter* spp., heteroresistance to colistin resulted from previous colistin treatment has been reported (49), (iii) although *in vitro* sulbactam has effective activity, resistance strains and resistance by penicillin-binding protein mutation have been described (50).

4. CARBAPENEMS

Carbapenems are one of beta-lactam antibiotics composing of beta-lactam ring and have the most effective activity against Enterobacteriaceae and non-fermentative bacteria including *P. aeruginosa* and *Acinetobacter* spp. The beta-lactams inhibit bacterial cell wall synthesis by inactivation (permanently acylation) of penicillin-binding proteins (PBPs) or transpeptidase enzymes that cross-link peptidoglycan chains. Then, cell wall synthesis is disrupted and autolysis occurs together. The bacterial cell wall is weakened, leak of substance by abnormal osmotic pressure and finally, it causes bacterial cell death (51).

The most common carbapenems used for treatment of Gram-negative bacteria infection are imipenem, meropenem, ertapenem, biapenem and doripenem. They are composed of carbapenem backbone and different at the side chains on C2 of carbapenem backbone as shown in Figure 3. (52).

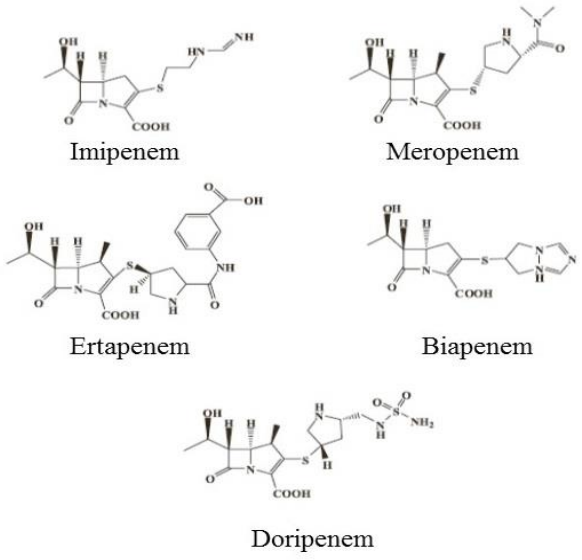


Figure 3. Chemical structures of carbapenems (52)

Imipenem is the first carbapenem used in clinical treatment and is the most stable carbapenem against beta-lactamase and cephalosporinase. However, for clinical use, imipenem has to be combined with cilastatin for prevention of deactivation by dehydropeptidase I (DHP-I) from renal brush border (53).

Meropenem is 1-beta-methyl carbapenem that resists to DHP-I. It has been shown indifferent inhibitory activity with imipenem against *Acinetobacter* spp. However, imipenem has been showed higher bactericidal activity (54).

Ertapenem, doripenem and biapenem are also 1-beta-methyl carbapenem s which are resistant to DHP-I, but they had limited *in vitro* activity against Gram-negative bacteria including *Acinetobacter* spp. (55-57).

In conclusion, imipenem and meropenem are the most active carbapenems against *Acinetobacter* spp., whereas ertapenem, doripenem and biapenem have effects against Enterobacteriaceae and Gram-positive bacteria. Thus, imipenem and meropenem are suitable for empirical treatment of hospital-acquired infection (such as *Acinetobacter* spp. infection) and other carbapenems are suitable for community-acquired infection (58).

5. CARBAPENEM RESISTANCE IN *ACINETOBACTER* SPECIES

Molecular mechanisms of carbapenem resistance in *Acinetobacter* spp. are carbapenemase production, overexpression of efflux pumps and loss or reduced porins as described below.

5.1 Carbapenemase production

5.1.1 OXA-type carbapenemases

The OXA-type carbapenemases in *Acinetobacter* spp. are classified into 6 groups: OXA-51, OXA-23, OXA- 24, OXA-58, OXA-143 and OXA-235 (5, 59-61). OXA-type carbapenemases belong to molecular class D beta-lactamase because they contained a serine-threonine-phenylalanine-lysine tetrad (STFK) at positions 70-73, a serine-serine/threonine-glycine (KSG) at positions 216-218 and a phenylalanine-glycine-asparagine (FGN) at positions 70-73 (5).

The first group is OXA-51 carbapenemase, first found in *A. baumannii* (62). *A. baumannii* has been reported to intrinsically possess chromosomal $bla_{\text{OXA-51}}$ (63, 64). However, plasmid-mediated $bla_{\text{OXA-51}}$ has been reported (65). The presence of OXA-

51 was used to identify *A. baumannii* (66). Moreover, OXA-51 was found in *A. nosocomialis* and other organisms such as *Enterobacter cloacae, E. coli* and *Klebsiella pneumoniae* (61, 67). There are 95 enzymes in OXA-51 family (61). The intrinsic OXA-51 is overexpressed by a strong promoter, upstream IS*Aba1*, leading to carbapenem resistance (68). The outbreak of carbapenem-resistant *A. baumannii* carrying IS*Aba1*-upstream *bla*$_{\text{OXA-51}}$ has been reported from South Korea (69).

The second group is OXA-23 carbapenemase. There are 19 enzymes in this group (61). The OXA-23 carbapenemase was first found in carbapenem-resistant *A. baumannii* from Edinburgh (70). However, the OXA-23 was found in other *Acinetobacter* spp. such as *A. pittii, A. nosocomialis, A. junii, A. radioresistens, A. lwoffii* and *A. baylyi* and other organisms including *Proteus mirabilis* and *K. pneumoniae* (61, 71). The OXA-23 showed higher carbapenemase activity than other group of OXA-type carbapenemases (61). Most of carbapenem-resistant *A. baumannii* isolates from worldwide carried *bla*$_{\text{OXA-23}}$ (72). The *bla*$_{\text{OXA-23}}$ genes have been located on plasmid with transposons such as Tn*2006*, Tn*2007*, Tn*2008*, and Tn*2009* (73, 74). Moreover, the chromosomal *bla*$_{\text{OXA-23}}$ was found in antibiotic resistance islands (75).

The third group is OXA-24 carbapenemase. There are 7 enzymes in this group including OXA-24, OXA-25, OXA-26, OXA-72, OXA-139, OXA-160 and OXA-207 (61). This carbapenemase enzyme was found in many organisms including *A. baumannii, A. pittii, A. haemolyticus, A. calcoaceticus, A. baylyi, P. aeruginosa* and *K. pneumoniae* (61, 76). The carbapenem-resistant *A. baumannii* isolates carrying *bla*$_{\text{OXA-24}}$ have often been isolated from many countries in Europe and America (5, 77, 78). The OXA-24 was reported from carbapenem-resistant *A. pittii* isolates in Italy (79).

The fourth group is OXA-58 carbapenemase. There are 4 enzymes in this group including OXA-58, OXA-96, OXA-97 and OXA-164. These enzymes have been found in *Acinetobacter* spp. including *A. baumannii, A. pittii* and *A. nosocomialis* and other organisms including *E. coli, E. cloacae* and *K. pneumoniae* (61, 80). The carbapenem-resistant *A. baumannii* carrying *bla*$_{\text{OXA-58}}$ has been emerged in Spain, Italy and Croatia (81-83). The high level of carbapenem resistance in *A. baumannii* was associated with upstream structures of *bla*$_{\text{OXA-58}}$, which played a role in the expression of this gene, including IS*Aba3* and hybrid promotor of IS*1008* and IS*Aba3* in carbapenem-resistant *A. baumannii* and hybrid promotor of IS*1006* and IS*Aba3* in *A. nosocomialis* (84, 85).

Moreover, the *bla*$_{\text{OXA-58}}$ has been reported in both carbapenem-resistant and carbapenem-susceptible *A. pittii* isolates and carbapenem-resistant *A. nosocomialis* (79).

The fifth group is OXA-143 carbapenemase. There are 5 enzymes in OXA-143 group. The OXA-143 enzyme was first identified from carbapenem-resistant *A. baumannii* isolate from Brazil (59). It showed 88% of amino acid similarity to OXA-24. Recently, the carbapenem-resistant *A. baumannii* carrying *bla*$_{\text{OXA-143}}$ has been emerged in Latin America especially Brazil (86, 87). Moreover, *bla*$_{\text{OXA-143}}$ has been found in carbapenem-susceptible *A. pittii* isolate (88).

The sixth group is OXA-235 carbapenemase. There are 3 enzymes in this group including OXA-235, OXA-236 and OXA-237 (60). The *bla*$_{\text{OXA-235}}$ was found in carbapenem-resistant *A. baumannii* isolates from the US and Mexico, the *bla*$_{\text{OXA-236}}$ and the *bla*$_{\text{OXA-237}}$ was found in carbapenem-resistant *A. baumannii* isolates from the US (60).

In addition, OXA-48 carbapenemase involved in carbapenem resistance. There are 11 enzymes in this group. The OXA-48 has been first identified from carbapenem-resistant *K. pneumoniae* (89). The emergences of carbapenem-resistant *K. pneumoniae* and *E. coli* have been reported worldwide (90-93). Recently, the *bla*$_{\text{OXA-48}}$-carrying *A. baumannii* has been isolated from faecal specimen (94).

5.1.2 Metallo-carbapenemases

The metallo-carbapenemase enzymes belong to molecular class B beta-lactamase. The most common metallo-carbapenemases reported in *Acinetobacter* spp. are IMP (Imipenemase), VIM (Verona imipenemase), GIM (German imipenemase), SIM (Seoul imipenemase), SPM (Sao Paulo metallo-beta-lactamase), and NDM (New Delhi metallo-beta-lactamase) (95-98). There are 53 enzymes of IMP carbapenemase (IMP-1 to IMP-53) (https://www.lahey.org/Studies/other.asp). IMP-1 was first reported in carbapenem-resistant *Serratia marcescens* clinical isolates from Japan (99). The emergence of IMP-carrying Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* spp. has been reported worldwide (100-102). There are 46 enzymes of VIM carbapenemase (VIM-1 to VIM-46). VIM was first described in a carbapenem-resistant *P. aeruginosa* clinical isolate from Verana, Italy (103). The *bla*$_{\text{VIM}}$ has been found in carbapenem-resistant *A. baumannii* isolates from many countries (104-106).

GIM carbapenemases include GIM-1 and GIM-2 enzymes. GIM-1 was first identified in a carbapenem-resistant *P. aeruginosa* isolate from Dusseldorf, Germany (107). The GIM-1 enzyme showed 43% and 39% amino acid similarity to VIM and IMP, respectively. Moreover, the *bla*$_{GIM-1}$ gene was found in *A. pittii* and Enterobacteriaceae from Germany (108-110). The GIM-2 enzyme, variant of GIM-1 was found in carbapenem-resistant *E. cloacae* from Germany (111).

SIM carbapenemase was first described in carbapenem-resistant *A. baumannii* from South Korea (96). The *bla*$_{SIM}$ was found in carbapenem-resistant *A. nosocomialis* from South Korea (112). The SPM carbapenemase was first reported in *P. aeruginosa* from Sao Paulo, Brazil. The outbreak of *bla*$_{SPM}$-carrying *P. aeruginosa* was reported in Brazil (113, 114). However, the *bla*$_{SPM}$-carrying *P. aeruginosa* has been reported outside Brazil, in Switzerland (115).

NDM carbapenemase was first described in carbapenem-resistant *K. pneumoniae* from New Delhi, India (116). Nowadays, the emergence of NDM-producing *Enterobacteriaceae*, *P. aeruginosa* and *A. baumannii* has increased worldwide including Thailand (117-119). The NDM was also found in non-*baumannii Acinetobacter* (120, 121).

5.2 Overexpression of efflux pumps

The function of bacterial efflux pumps is to drive many compounds including antibiotics outside cells. There are 3 families of efflux pumps in Gram-negative bacteria which involve in antibiotic resistance including RND (Resistance-Nodulation-cell Division) family, ABC (ATP-binding cassette) superfamily and MFS (Major Facilitator Superfamily) (Figure 4) (122).

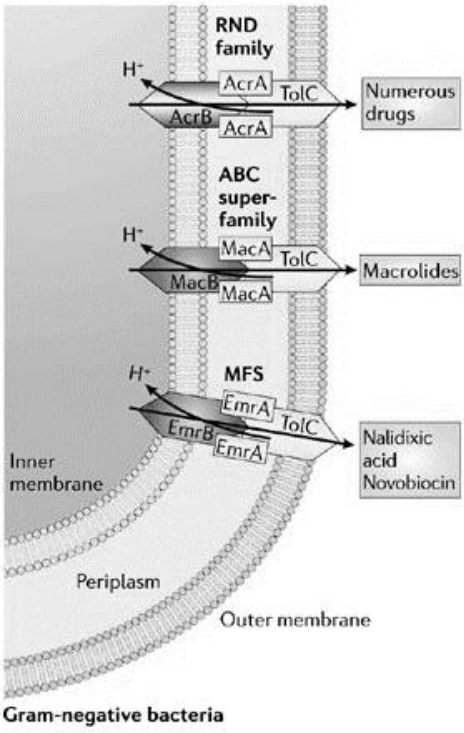


Figure 4. Efflux pump families in Gram-negative bacteria (122)

In *Acinetobacter* spp., the efflux pumps associated with carbapenem resistance belong to RND family including AdeABC, AdeDE and AdeXYZ (123-126). The AdeABC efflux pump is composed of inner membrane protein (AdeB), transmembrane protein (AdeA) and outer membrane protein (AdeC). The AdeABC expression is regulated by two-component regulatory proteins, AdeR and AdeS (127). AdeABC efflux pump has been found in 80% of *A. baumannii* (12). The overexpression of efflux pump activity can be detected by using efflux pump inhibitors such as CCCP (carbonyl cyanide 3-chlorophenylhydrazone), reserpine and 1-(1-naphthylmethyl)-piperazine (NMP). However, the results from this method showed controversial effects of overexpression of AdeABC and carbapenem resistance in *A. baumannii*. Hu *et al*. found that the carbapenem MICs were decreased in the presence of CCCP (124). In contrast, the study by Pournaras *et al*. found that no carbapenem MICs were changed in the presence of CCCP (128). Although AdeABC plays a role in carbapenem resistance, it does not confer high level resistance (129).

The AdeDE efflux pump, first described in *A. pittii*, is composed of inner membrane protein (AdeE) and transmembrane protein (AdeD) (130). When *adeE* was inactivated, the meropenem MIC was decreased. This indicates that AdeDE efflux

pump plays a role in meropenem resistance in *A. pittii*. The AdeDE efflux was found in 70% of *A. pittii* and was rarely found in *A. nosocomialis* (126).

The AdeXYZ efflux pump has been found in *A. pittii* and *A. nosocomialis* (125, 126). The AdeXYZ showed 99% amino acid similarity to AdeIJK efflux in *A. baumannii* (125). However, the role of AdeXYZ efflux pump in carbapenem resistance is still unclear.

5.3 Reduced or loss of outer membrane proteins (OMPs)

OMPs or porins function as influx of antibiotics into cells. The OMPs play a role in carbapenem resistance by reduced or loss of expression. The first OMP reported in carbapenem-resistant *A. baumannii* was CarO (9). The CarO had specific channels for imipenem, but not meropenem (131). Although CarO has imipenem binding site, its role in carbapenem is still controversial. The reduced CarO conferred carbapenem resistance in *A. baumannii* in the presence of carbapenemase production (132). The disruption of *carO* by insertion sequences including IS*Aba1*, IS*Aba10*, IS*Aba15*, IS*Aba125* and IS*Aba825* resulted in loss of CarO (133-136). The *carO* expression level was studied in non-*baumannii Acinetobacter* from South Korea but no reduced *carO* was detected (112).

The 33-36 kDa OMP was first described from imipenem-resistant *A. baumannii* (10). The reduced 33-36 kDa OMP was found with OXA-143 production in carbapenem-resistant *Acinetobacter* spp. clinical isolates from Brazil. The 43 kDa OMP or OprD has been characterized in *A. baumannii* (11). It was homologous to OprD in *P. aeruginosa,* which had specific binding site of basic amino acid and imipenem. This indicated that OprD might play a role in imipenem resistance in *A. baumannii*. The study by Smani *et al*. showed that OprD in *A. baumannii* belonged to subgroup of OprD family, similar to OprD in *P. aeruginosa* and there was no specific channel for imipenem and meropenem (137). The mutation of *oprD* showed no effect on carbapenem resistance in *A. baumannii* (138). Moreover, in non-*baumannii Acinetobacter*, the expression level of *oprD* was not different in carbapenem-resistant and carbapenem-susceptible isolates (112).

5.4 Antibiotic resistance islands in *Acinetobacter* species

Bacterial genomic islands are defined as the horizontal genetic elements on bacterial chromosome containing multiple genes that have the same function such as

pathogenicity island contains multiple virulence genes (139). Antibiotic resistance island is a large (>10 kb) mobile genetic element that contains multiple resistance genes (140).

In *A. baumannii*, the antibiotic resistance island is called AbaR (*A. baumannii* resistance island). The AbaR is integrated into chromosomal *comM* gene. AbaR1, the first AbaR reported in multidrug-resistant *A. baumannii* strain AYE from France (141), is genomic island of 86 kb, containing 45 antibiotic resistance genes such as resistance genes to beta-lactam, aminoglycosides, fluoroquinolones and trimethoprim. The genomic island of *A. baumannii* strain SDF, human body lice strain was 20-kb in length and did not carry antibiotic resistance gene (141). These results suggested that multidrug resistance phenotype might be associated with antibiotic resistance island. AbaR2 was identified from the genome of *A. baumannii* strain ACICU (142). AbaR2 was smaller than AbaR1 and contained only aminoglycoside and beta-lactam resistance genes. At presents, 26 AbaRs (AbaR1-AbaR26) have been reported (74, 75, 141-148).

AbaRs can be classified into two types by using their transposon-backbone structures. The first type, AbaR1-type, has Tn*6019* backbone (141). The AbaR1-type, including AbaR1, AbaR3, AbaR5-AbaR19, AbaR21, and AbaR26, is usually found in *A. baumannii* European clone I (EU clone I) (Figure 5) (146). Tn*6019* contains arsenic resistance operon (*tniA-tniB*-orf2-orf3-*trkA-trxB-arsH-arsB-arsC-uspA-sup*-orf4). The *uspA* gene in Tn*6019* is interrupted by Tn*6018*. Tn*6018* contains cadmium resistance genes (*cadR-cadA-lspA-tnpA*) and multiple resistance genes of resistance island (Figure 6) (8). These resistance islands often carry aminoglycoside, tetracycline, and heavy-metal resistance genes.

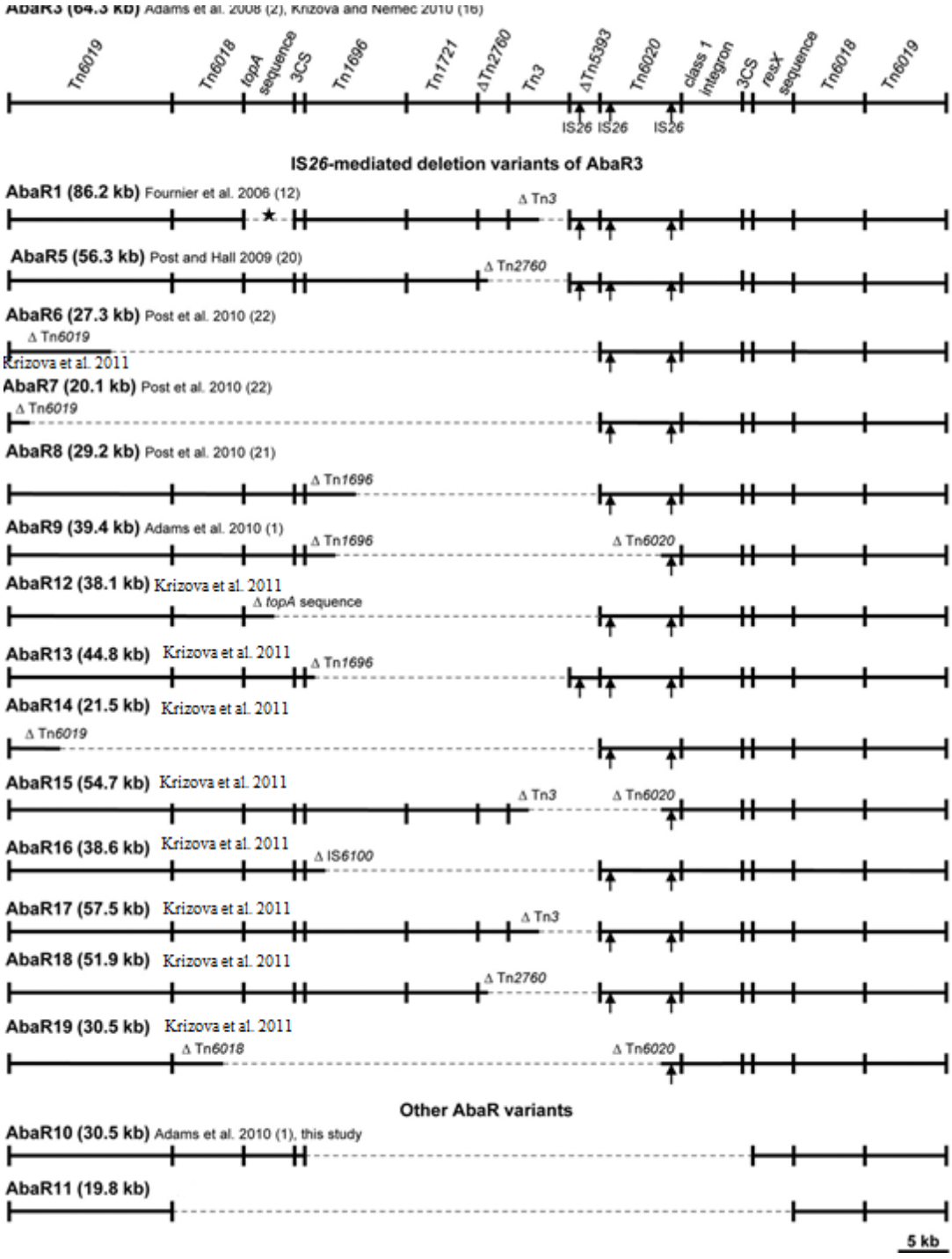


Figure 5. Genetic structures of AbaR1-types (146)

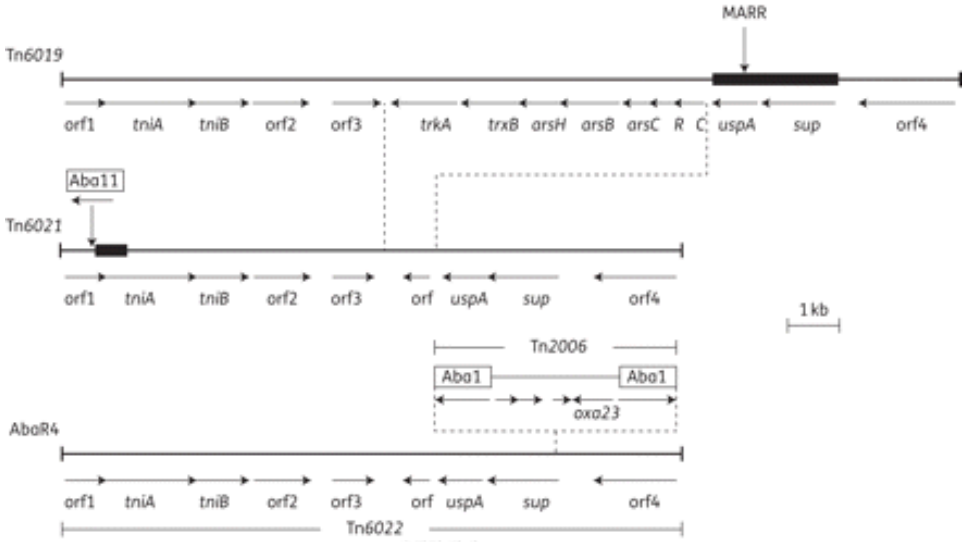


Figure 6. Genetic structures of backbone transposon and AbaR4-type (8)

The second type is AbaR4-type. Most AbaR4 has Tn*6022* backbone (149), containing orf1-*tniA*-*tniB*-orf2-orf3-orf-*uspA*-*sup*-orf4 (Figure 6). The simple AbaR4 island carried Tn*6022* backbone, which *sup* gene was interrupted by Tn*2006* (Figure 6). The AbaR4-type island was found in *A. baumannii* belonging to EU clone II. However, AbaR4 was reported in EU clone I, *A. baumannii* AB0057. After the first AbaR4 has been identified in 2008, AbaR4 subtypes were reported in *A. baumannii* isolates from Korea (149), China (AbaR22) (74), and Latvia (AbaR25) (148). The AbaR4-type islands are shown in Figure 7 (21). Most AbaR4-type islands except AbaR22 carried bla_{OXA23} carbapenemase gene (74). Moreover, AbaR4 was also reported in *A. nosocomialis* (149).

The resistance islands found in *A. baumannii* isolated from Thailand were AbaR25-I and AbaR4-AB210 types (Figure 7). *A. nosocomialis* isolated from Thailand carried AbaR4 island which was similar to AbaR4-D36 type in Figure 7 (20).

According to transposon-backbone structures and ACCGC-direct repeat sequences at both ends of AbaR, AbaRs may be mobilized similar to transposition of transposon (141).

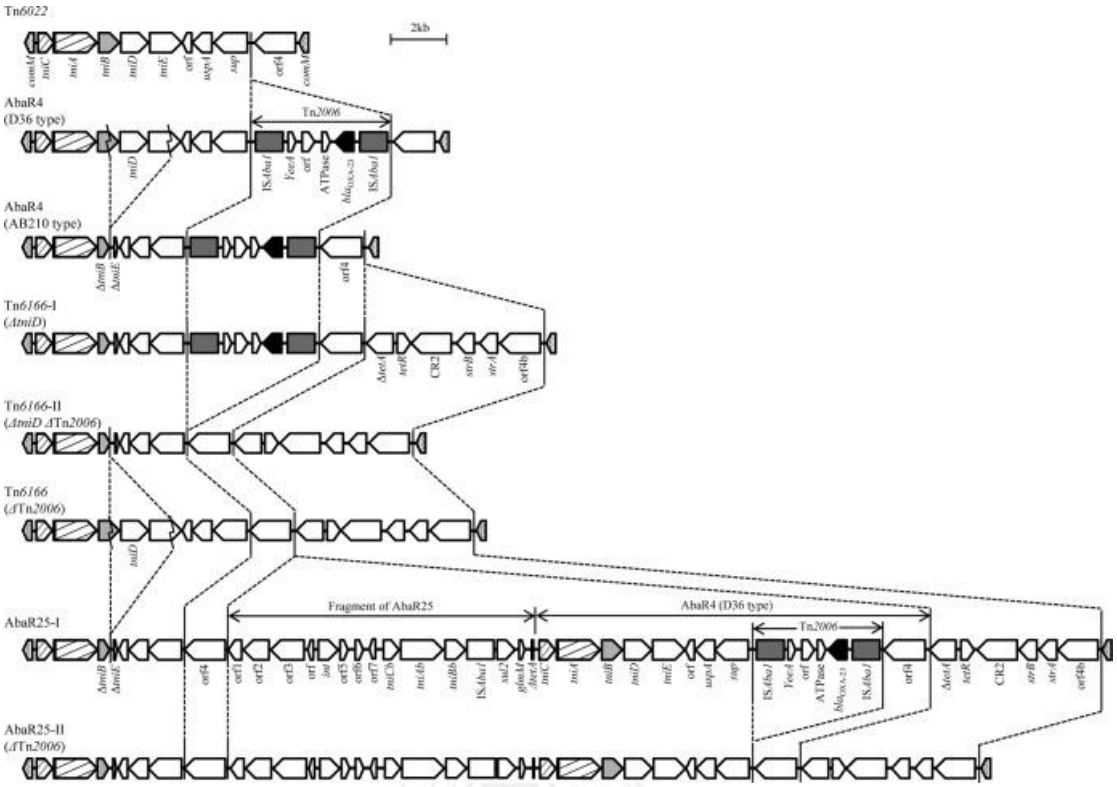


Figure 7. Genetic structures of AbaR4-type islands (21)

6. COMBINATION THERAPY

The objectives of antibiotic combination therapy include enhancement activity of antibiotics, reduction dose and toxicity of antibiotics, reduction of resistance strains and synergistic activities against multidrug-resistant bacteria. An effective treatment of carbapenem-resistant *Acinetobacter* spp. is antibiotic combination therapy. However, no standard guideline in clinical laboratory for *in vitro* testing of antibiotic combination against *Acinetobacter* spp. The synergistic effect of antibiotic combination has been performed only in research laboratory. Many antibiotic combinations have been studied against Gram-negative bacteria as described below.

The combination of beta-lactams and aminoglycosides showed the *in vitro* synergism of penicillin or cephalosporins plus aminoglycosides against Enterobacteriaceae. These included cefazolin plus amikacin, cephalothin plus amikacin, cefotaxime plus amikacin and azlocillin plus amikacin (150). The combination of carbapenems and aminoglycoside showed little synergistic effect against carbapenem-susceptible and carbapenem-resistant *P. aeruginosa.* Weiss *et al*.

(151) reported that synergism of imipenem and tobramycin against antibiotic-resistant *P. aeruginosa* strains was found only 19%. In contrast, Dundar *et al*. reported that synergy of imipenem plus tobramycin was not detected against imipenem- and tobramycin-resistant strains and the synergism was detected only 15% of imipenem- and tobramycin-susceptible strains (152).

Aminoglycosides inhibited bacterial protein synthesis by binding to bacterial ribosome. The mechanisms of aminoglycoside resistance in *Acinetobacter* spp. include the production of aminoglycoside-modifying enzymes, overexpression of efflux pumps and reduced influx of aminoglycosides (153). The purpose mechanisms of synergism of carbapenems and aminoglycosides are that cell wall synthesis is inhibited by beta-lactam and aminoglycosides uptake is enhanced to kill bacteria (17).

Fluoroquinolones inhibit bacterial DNA replication by inhibiting topoisomerase enzyme. The combination of beta-lactams and fluoroquinolones such as penicillin or cephalosporins plus fluoroquinolones did not show synergism against Enterobacteriaceae (154). The effects of cephalosporins plus fluoroquinolones were variable against non-fermentative bacteria. Thus, combination of beta-lactam and fluoroquinolone does not seem to have any benefit against non-fermentative bacteria. Fluoroquinolones were demonstrated to be mutagenic in bacteria and enhanced carbapenem resistance mutation rate in *P. aeruginosa* (155).

The combination of fluoroquinolones and aminoglycosides such as ciprofloxacin plus gentamicin, netilmicin, amikacin or tobramycin were reported for synergism against some species of Enterobacteriaceae and non-fermentative bacteria (156). However, no synergism of levofloxacin or ciprofloxacin plus amikacin was found against *A. baumannii* (157).

Colistin is polypeptide antibiotic that interferes lipopolysaccharide in bacterial cell membrane. It is effective last-resort antibiotic against *Acinetobacter* spp. However, it has neurotoxic and nephrotoxic effects. Therefore, colistin is commonly used in combination with other antibiotics. However, the controversial activity was reported in non-fermentative bacteria including *P. aeruginosa* and *A. baumannii* (18, 158-160). Colistin plus imipenem showed synergistic activity against 42%-100% and 75%-100% of carbapenem-resistant *Acinetobacter* spp. by checkerboard and time-kill assay, respectively (161).

Fosfomycin is an old antibiotic that inhibits cell wall synthesis as shown in Figure 8 (162). The target of fosfomycin is MurA enzyme that adds phosphoenolpyruvate (PEP) to UDP-N-acetylglucosamine and gets the peptidoglycan precursor (enolpyruvyl-UDP-GlcNAc) as shown in Figure 9-10. Fosfomycin acts as PEP analog which inhibits MurA activity. Many mechanisms of fosfomycin resistance have been reported including the mutation of MurA, fosfomycin-modifying enzyme production and overexpression of efflux pump (163, 164). Recently, the cell wall recycling bypass pathway has been reported in *E. coli*, *P. aeruginosa* and *Pseudomonas putida.* It was believed to be an intrinsic mechanism of fosfomycin resistance (Figure 10) (165). *P. aeruginosa* and *P. putida* can recycle anhydromuropeptide to UDP-MurNAc, thus, MurA is neglected in cell wall synthesis.

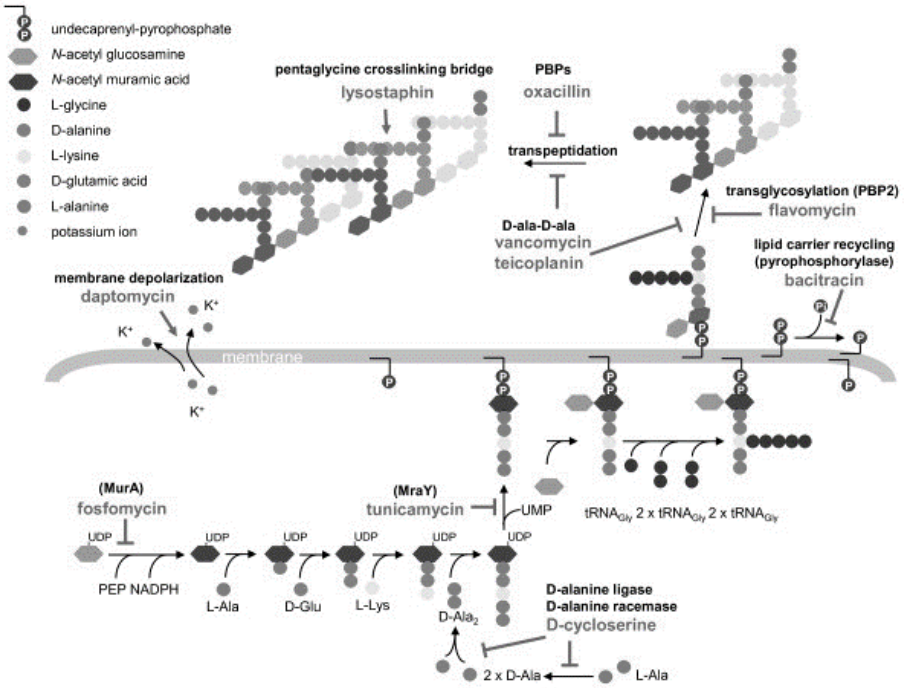


Figure 8. Mechanisms of antibiotics by inhibition cell wall synthesis (162)

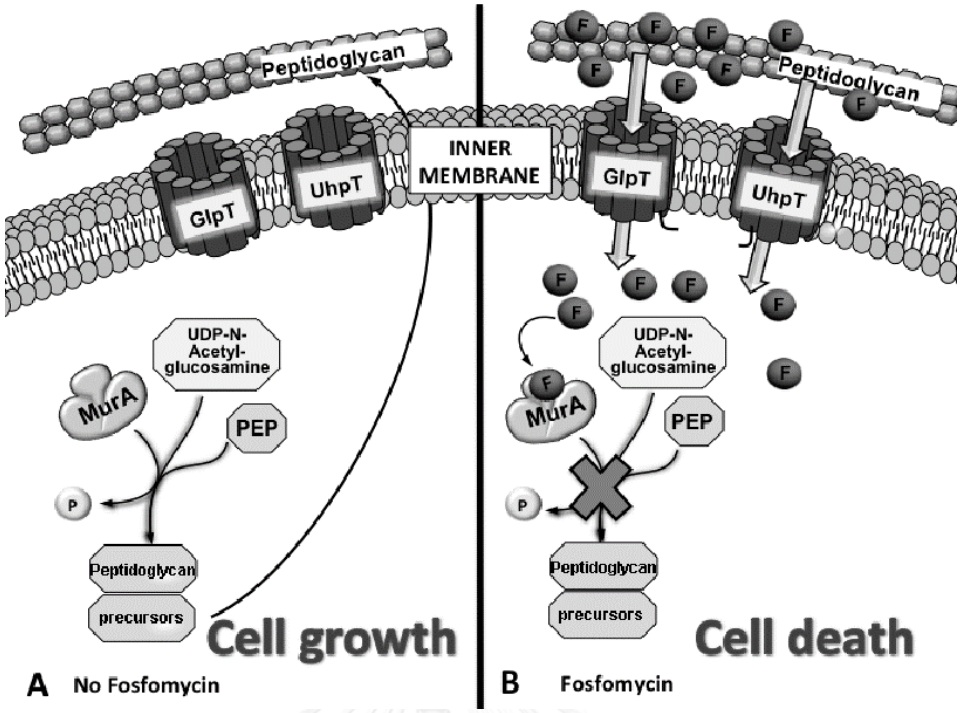


Figure 9. Inhibition of peptidoglycan synthesis by fosfomycin (164)

Fosfomycin is one of the most frequently used antibiotic for ESBL-producing Gram-negative bacteria and multidrug-resistant *P. aeruginosa*. Although *A. baumannii* showed intrinsic resistance to fosfomycin (166), synergistic activity of fosfomycin plus sulbactam or colistin have been reported (18, 167).

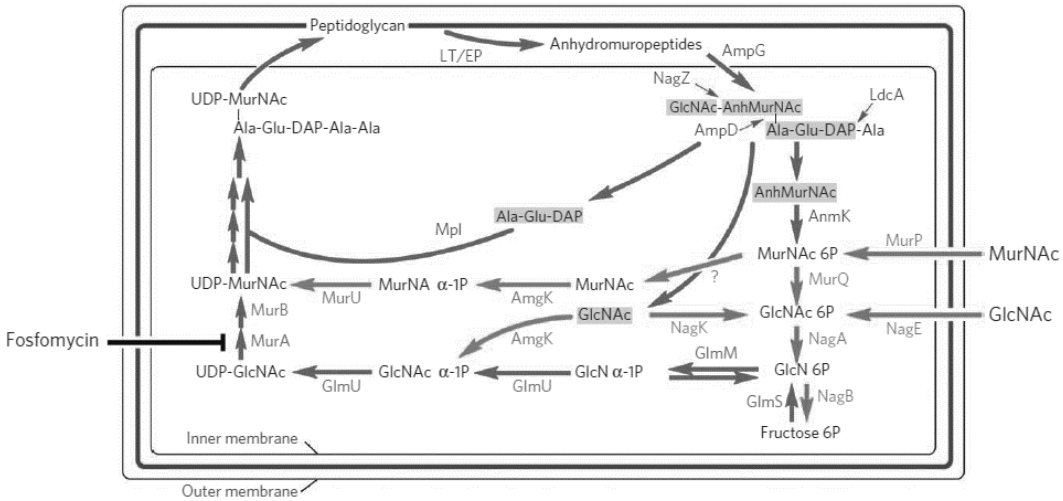


Figure 10. Cell wall recycling bypass pathway in *Pseudomonas* spp. and *E.coli* (165)

Nowadays, carbapenem-resistant *Acinetobacter* spp. especially *A. baumannii* are the major causes of nosocomial infection worldwide including Thailand. However, there are very few studies of molecular mechanisms of carbapenem resistance and synergy of antibiotic combinations against carbapenem-resistant *Acinetobacter* spp. in Thai isolates. Moreover, antibiotic resistance island has not yet been reported in *Acinetobacter* spp. except *A. baumannii* and *A. nosocomialis* Thai isolates. Therefore, this study aimed to investigate (i) the carbapenem resistance mechanisms, (ii) the antibiotic resistance islands, and (iii) synergistic activities of antibiotic combinations against carbapenem-resistant *Acinetobacter* spp. Thai isolates. Moreover, synergistic mechanism of the best effective antimicrobial agent combination will be characterized.

CHAPTER IV
MATERIALS AND METHODS

1. BACTERIAL STRAINS

1.1 *Acinetobacter* spp. isolates

Three hundred and forty-six of *Acinetobacter* spp. were isolated and collected from different patients at King Chulalongkorn Memorial Hospital in Thailand between January 2010 and December 2011. The $bla_{\text{OXA-24}}$-carrying *A. baumannii* isolate A10 was obtained from previous study (168). In addition, another 6 *A. baumannii* were collected from different patients at King Chulalongkorn Memorial Hospital between May 2015 and July 2016.

1.2 Quality control strains for study mechanism of resistance

A. baumannii ATCC 19606, a carbapenem-susceptible strain, was used as quality control for OMP study and overexpression of efflux pump gene study.

1.3 Quality control strains for antimicrobial susceptibility testing

E. coli ATCC 25922, *P. aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains for antimicrobial susceptibility test.

All bacterial strains in this study were stored at -80^{o}C in tryptic soy broth with 20% glycerol until required.

2. BACTERIAL IDENTIFICATION

All *Acinetobacter* spp. were identified as genus *Acinetobacter* by gram stain and biochemical test. All isolates were identified for species level by molecular method as described below.

2.1 Gram stain

Acinetobacter spp. are Gram-negative coccobacilli or cocci. *Acinetobacter* spp. are 1.0-1.5 μm in diameter and 1.5-2.5 μm in length.

2.2 Biochemical tests

The biochemical tests used for identification of genus *Acinetobacter* are as described before and summarized in Table 1 (22). An isolated colony of each bacterial isolates was picked and inoculated onto biochemical tests.

2.1.1 Oxidase test

The filter paper that applied N, N, N′, N′-tetramethylethylenediamine (substrate of cytochrome oxidase) was streaked with each *Acinetobacter* spp. colonies. All of *Acinetobacter* spp. cannot produce cytochrome oxidase, thus, the color on filter paper is not changed.

2.1.2 Triple Sugar Iron (TSI) test

TSI contains 1:10:10 of glucose, lactose and sucrose. A single colony of *Acinetobacter* spp. was streaked on and stabbed into the medium and incubated at 37^{o}C for 24 hours. *Acinetobacter* spp. showed K/N (alkaline slant/ neutral butt) because they cannot ferment glucose, lactose and sucrose.

2.1.3 Acidification from D-glucose

Oxidation or fermentation of dextrose test (OF test) was used for detection of acidification from D-glucose. An opened tube and closed tube (overlay with sterile melted petrolatum) were inoculated with colony of *Acinetobacter* spp. and incubated at 37^{o}C for 24 hours. *Acinetobacter* spp. can oxidize D-glucose thus, color of opened tube is changed to yellow because of acidification.

2.1.4 Citrate utilization test

Simmons citrate medium contains citrate and was inoculated with colony of *Acinetobacter* spp. and incubated at 37^{o}C for 24 hours. Organisms that can utilize citrate

as carbon source, alkaline products will change the pH of medium and color of medium will be changed to blue. Most of Acb complex can utilize citrate.

2.1.5 Urease test

Urea medium contains urea and was inoculated with colony of *Acinetobacter* spp. and incubated at 37ºC for 24 hours. Organisms that can produce urease enzyme, acid products will change the pH of medium and change color to pink. Most of Acb complex cannot produce urease.

2.1.6 Nitrate reduction test

The purpose of nitrate reduction test is to detect whether organisms can reduce nitrate to nitrite or nitrogen gas. Nitrate broth contains nitrate and was inoculated with *Acinetobacter* spp. and incubated at 37ºC for 24 hours and then reagent A (alpha-naphthylamine) and reagent B (sulfanilic acid) were added. If nitrate was reduced to nitrite, color of broth will be changed to red. However, if color of broth is not changed, zine oxide was added. If nitrate was reduced to be nitrogen gas, after zinc oxide adding the color of broth will be changed to red. *Acinetobacter* spp. cannot reduce nitrate to nitrite or nitrogen gas.

2.3 Molecular method for species identification

After all *Acinetobacter* spp. was identified by Gram stain and biochemical tests, all isolates were identified the species by molecular method as described below.

2.3.1 The presence of $bla_{\text{OXA-51}}$ gene

A. baumannii carried intrinsic carbapenemase gene, $bla_{\text{OXA-51}}$ (66). The presence of this gene was used to be one criteria for *A. baumannii* identification. The $bla_{\text{OXA-51}}$ gene was detected by PCR (multiplex PCR for OXA-type carbapenemase genes detection). However, the $bla_{\text{OXA-51}}$ has also been reported in *Acinetobacter* genospecies 13TU (*A. nosocomialis*) (67). Thus, all of isolates had to be confirmed species level by other method, multiplex PCR for *gyrB*.

2.3.2 Multiplex PCR for *gyrB* gene

Species of Acb complex can be differentiated by multiplex PCR for *gyrB* gene (169). The nucleotide sequences of *gyrB* gene of each species in Acb complex are different. Therefore, the specific primers were designed for different PCR product size of each species. The primers and PCR condition of *gyrB* multiplex PCR were performed as described by Higgins *et al*. (169).

3. EXTRACTION OF BACTERIAL DNA

3.1 Extraction of DNA by boiling method

DNA extraction by boiling method was used for detection of OXA-type carbapenemase genes, metallo-carbapenemase genes, KPC, OXA-48 and NDM carbapenemase genes. Moreover, boiled DNA was used for screening resistance genes such as OMP genes, efflux pump genes and fosfomycin resistance genes.

Suspension of 3-4 colonies of *Acinetobacter* spp. in 200 µl of DNase-free water was boiled at 100^{o}C for 10 minutes and centrifuged at 13,000 rpm for 5 minutes. Supernatant containing DNA was collected to a new tube and stored at -20^{o}C.

3.2 Extraction of plasmid DNA by commercial kit

Plasmid DNA from commercial extraction kit was used to amplify entire gene of *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-58}}$ and *bla*$_{\text{IMP}}$. The extraction of plasmid DNA was performed by HiYield™ Plasmid Mini Kit. The plasmid DNA samples were stored at -20^{o}C.

3.3 Extraction of genomic DNA by commercial kit

Genomic DNA from commercial extraction kit was used for antibiotic resistance island study including PCR mapping and DNA sequencing. The genomic DNA was performed by PureLink® Genomic DNA. The genomic DNA samples were stored at -20^{o}C.

4. RNA EXTRACTION AND CDNA AMPLIFICATION

4.1 Bacterial culture conditions

For mechanisms of carbapenem resistance study, mRNA expression level of carbapenemase genes and efflux pump genes of *Acinetobacter* spp. were evaluated. One ml of *Acinetobacter* spp. in Luria-Bertani (LB) broth was transferred to new 99 ml of LB broth. Then, the flask of LB was incubated at 37^{o}C for 2-3 hours with shaking. The bacterial cell pellet was used for RNA extraction.

For mechanisms of fosfomycin resistance study, mRNA expression level of *murA, abaF* (efflux pump), *ampG* and *murU* were evaluated. One ml of *Acinetobacter* spp. in LB broth was transferred to new 99 ml of cation adjusted Mueller Hinton broth (MHB) and MHB supplemented with fosfomycin at final concentration to 0.25X, 0.5X and 1X of fosfomycin MIC of each isolates. Then, the flask of LB was incubated at 37^{o}C for 2-3 hours with shaking. The bacterial cell pellet was used for RNA extraction.

4.2 RNA extraction

The bacterial cell pellet was collected by centrifugation at 12,000 rpm for 5 minutes, resuspended with 1 ml of Invitrogen™ TRIzol™ Reagent and incubated at room temperature for 5 minutes. Then, 200 µl of chloroform was added, mixed for 15 seconds and incubated at room temperature for 2-3 minutes. After centrifugation at 12,000 g at 4^{o}C for 15 minutes, aqueous phase was removed to a new 1.5 ml centrifuge tube, the 500 µl of isopropanol was added, mixed gently and incubated at room temperature for 10 minutes. After centrifugation at 12,000 g at 4^{o}C for 10 minutes, the supernatant was discarded and RNA pellet was suspended with 1 ml of 70% ethanol and centrifuged at 7,500 g at 4^{o}C for 5 minutes. Then, the supernatant was discarded and RNA pellet was air dried for 5-10 minutes. RNA pellet was suspended with RNase-free water and incubated at 65^{o}C for 10-15 minutes. RNA concentration was evaluated by NanoDrop ND-1000. The amount of total RNA about 1.5 µg of each samples was converted to cDNA.

4.3 amplification of cDNA

The total RNA (1.5 µg) of each sample was prepared to be cDNA by RT-PCR using SuperScript® III First-Strand Synthesis System. The total RNA (1.5 µg) was incubated with random hexamer primer and dNTP at 65ºC for 10 minutes. Then, 10 µl of cDNA synthesis mixture (including 1X RT buffer, 100 mM of $MgCl_2$, 0.2 M DTT, 40U of RNaseOUT and 200U of SuperScript® III RT) was added to prepared total RNA. The cDNA was synthesized by using Applied Biosystems Veriti Thermal Cycler at 25ºC for 10 minutes, 50ºC for 50 minutes and 85ºC for 5 minutes. After that the tube was chilled on ice, 1µl of RNaseH was added and incubated at 37ºC for 20 minutes. The cDNA sample was stored at -20ºC or used for PCR immediately.

5. ANTIMICROBIAL SUSCEPTIBILITY TEST

All 353 *Acinetobacter* spp. isolates were performed antimicrobial susceptibility test for imipenem, meropenem, amikacin, gentamicin, ceftazidime, ceftriaxone, cefotaxime, cefepime, ciprofloxacin and colistin by agar dilution method. The 23 *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis* representative isolates were performed susceptibility test for fosfomycin. The minimal inhibitory concentration (MIC) is defined as the lowest concentration of antibiotic that can inhibit the growth of organism. The MICs of each antibiotic were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) (170) except that of fosfomycin is interpreted according to criteria for *E. coli* from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (171).

Mueller Hinton agar was supplemented with 2-fold dilution of each antibiotic including imipenem, meropenem, amikacin, colistin and fosfomycin at final concentrations of 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 mg/L. For fosfomycin susceptibility test, each agar plate was supplemented with glucose-6-phosphate (G6P) as recommended by CLSI.

Acinetobacter spp. isolates, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were prepared in normal saline to turbidity of 0.5 McFarland (~1.5×10^8 CFU/ml) and then diluted 1:10 with normal saline. Each bacterial suspension was applied onto Mueller Hinton agar supplemented with antibiotics as final concentration of approximately 10^4 CFU/spot and incubated at 37^{o}C for 18-24 hours.

Table 2. MIC interpretation standards for *Acinetobacter* spp.

Antimicrobial agent	Interpretation MIC (mg/L)			Reference
	Susceptible	Intermediate	Resistant	
Imipenem	≤ 2	4	≥ 8	(170)
Meropenem	≤ 2	4	≥ 8	(170)
Amikacin	≤ 16	32	≥ 64	(170)
Gentamicin	≤ 4	8	≥ 16	(170)
Cefotaxime	≤ 8	16 – 32	≥ 64	(170)
Ceftriaxone	≤ 8	16 – 32	≥ 64	(170)
Ceftazidime	≤ 8	16	≥ 32	(170)
Cefepime	≤ 8	16	≥ 32	(170)
Ciprofloxacin	≤ 1	2	≥ 4	(170)
Colistin	≤ 2	-	≥ 4	(170)
Fosfomycin	≤ 32	-	> 32	(171)

6. DETECTION OF CARBAPENEMASE ACTIVITY

6.1 Carbapenemase activity

All 353 *Acinetobacter* spp. isolates were detected for carbapenemase production by modified Hodge test. *E. coli* ATCC 25922 suspension was adjusted to the turbidity of 0.5 McFarland with normal saline and spreaded onto Mueller Hinton agar. An imipenem disc (10 µg) was applied at center of the Mueller Hinton agar plate. Each of *Acinetobacter* spp. tested isolates was streaked from the edge of imipenem disc to the edge of Mueller Hinton agar plate then the plate was incubated at 37ºC for 18-24 hours. If *Acinetobacter* spp. produce carbapenemases, the distorted inhibition zone of *E. coli* will be present because imipenem is hydrolyzed by carbapenemases.

6.2 Metallo-carbapenemase activity

All 353 *Acinetobacter* spp. isolates were detected for metallo-carbapenemase production by EDTA-disc synergy test. *Acinetobacter* spp. were adjusted to the turbidity of 0.5 McFarland with normal saline and spreaded onto Mueller Hinton agar. An imipenem disc (10 µg) and a blank disc were applied onto the plate (distance from each edge of disc 10 mm) then 10 µl of 0.5M of EDTA were added on the blank disc. The plate was incubated at 37ºC for 18-24 hours. If *Acinetobacter* spp. produce metallo-carbapenemases, the enhancement of inhibition zone between each disc will be present because metallo-carbapenemases are inhibited by EDTA.

7. DETECTION OF CARBAPENEMASE GENES AND MRNA EXPRESSION

7.1 Detection of OXA-type carbapenemase genes

OXA-type carbapenemase genes including *bla*$_{\text{OXA-51}}$, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-143}}$, and *bla*$_{\text{OXA-235}}$ were detected by multiplex PCR as described by Woodford *et al*. (172) and Higgins *et al*. (60, 173). The specific primers are shown in Table 3. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.1 µM of each primer except 0.05 µM of OXA-58-F and OXA-58-R, 0.625U of *Taq* DNA polymerase and 2 µl of boiled DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial cycle of 94^{o}C for 5 minutes, followed by 30 cycles of 94^{o}C for 25 seconds, 52^{o}C for 40 seconds and 72^{o}C for 50 seconds and a final extension at 72^{o}C for 6 minutes. The PCR products were separated on 1% agarose gels containing 0.5 µg/ml of ethidium bromide in 0.5X TBE buffer by using electrophoresis at 100 volts for 50 minutes and visualized under UV light and 100 bp plus DNA ladder was used as approximate DNA size.

Table 3. Primers for amplification of OXA-type carbapenemase genes

primer name	gene	sequence (5'-3')	product size (bp)	reference
OXA23-F	*bla*$_{OXA-23}$ like	GATCGGATTGGAGAACCAGA	501	(172)
OXA23-R		ATTTCTGACCGCATTTCCAT		
OXA24-F	*bla*$_{OXA-24}$ like	GGTTAGTTGGCCCCCTTAAA	249	(172)
OXA24-R		AGTTGAGCGAAAAGGGGATT		
OXA51-F	*bla*$_{OXA-51}$ like	TAATGCTTTGATCGGCCTTG	353	(172)
OXA51-R		TGGATTGCACTTCATCTTGG		
OXA58-F	*bla*$_{OXA-58}$ like	AAGTATTGGGGCTTGTGCTG	599	(172)
OXA58-R		CCCCTCTGCGCTCTACATAC		
OXA143-F	*bla*$_{OXA-143}$ like	TGGCACTTTCAGCAGTTCCT	149	(173)
OXA143-R		TAATCTTGAGGGGGCCAACC		
OXA235-F	*bla*$_{OXA-235}$ like	TTGTTGCCTTTACTTAGTTGC	768	(60)
OXA235-R		CAAAATTTTAAGACGGATCG		
ISAba1-F	IS*Aba1*	CACGAATGCAGAAGTTG	549	(174)
ISAba1-R		CGACGAATACTATGACAC		
ISAba2-F	IS*Aba2*	CATCATAGTGACAGAGGTGTGC	268	(168)
ISAba2-R		AAGGTGACACATAACCTAGTGC		
ISAba3-F	IS*Aba3*	CAATCAAATGTCCAACCTGC	403	(175)
ISAba3-R		CGTTTACCCCAAACATAAGC		
ISAba4-F	IS*Aba4*	ATTTGAACCCATCTATTGGC	612	(176)
ISAba4-R		ACTCTCATATTTTTTCTTGG		

7.2 Detection of metallo-carbapenemase genes

Metallo-carbapenemase genes including *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, and *bla*$_{SPM}$ were detected by multiplex PCR as described by Ellington *et al*. (177) and Samarnthai (168). The specific primers are shown in Table 4. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.05 µM of each primer except 0.4 µM of IMP-F and IMP-R, 0.16 µM of VIM-F and VIM-R, 0.625U of *Taq* DNA polymerase and 2 µl of boiled DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial cycle of 94^{o}C for 5 minutes, followed by 35 cycles of 94^{o}C for 30 seconds, 52^{o}C for 30 seconds and 72^{o}C for 50 seconds and a final extension at 72^{o}C for 6 minutes. The PCR products were detected as previously described in the method of OXA-type carbapenemase genes detection.

Table 4. Primers for amplification of metallo-carbapenemase genes

primer name	gene	sequence (5'-3')	product size (bp)	reference
IMP-F	*bla*$_{IMP}$ like	GGAATAGAGTGGCTTAAYTCT	188	(177)
IMP-R		CCAAACYACTASGTTATCT		
VIM-F	*bla*$_{VIM}$ like	GATGGTGTTTGGTCGCATA	390	(177)
VIM-R		CGAATGCGCAGCACCAG		
GIM-F	*bla*$_{GIM}$like	TCGACACACCTTGGTCTGAA	477	(177)
GIM-R		AACTTCCAACTTTGCCATGC		
SIM-F	*bla*$_{SIM}$like	TACAAGGGATTCGGCATCG	304	(168)
SIM-R		CCAACCAAAAGCTCTCTTTATC		
SPM-F	*bla*$_{SPM}$ like	AAAATCTGGGTACGCAAACG	271	(177)
SPM-R		ACATTATCCGCTGGAACAGG		

7.3 Detection of *bla*$_{KPC}$, *bla*$_{OXA-48}$ and *bla*$_{NDM}$

The other carbapenemase genes including *bla*$_{KPC}$, *bla*$_{OXA-48}$ and *bla*$_{NDM}$ were detected by multiplex PCR as described by Poirel *et al*.(178) and the specific primers are shown in Table 5. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each primer, 1.25U of *Taq* DNA polymerase and 2 µl of boiled DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94ºC for 10 minutes, followed by 35 cycles of 94ºC for 30 seconds, 52ºC for 40 seconds and 72ºC for 50 seconds and a final extension at 72ºC for 5 minutes. The PCR products were detected as previously described in the method of OXA-type carbapenemase genes detection.

Table 5. Primers for amplification of KPC, OXA-48 and NDM carbapenemase genes

primer name	**gene**	**sequence (5'-3')**	**product size (bp)**	**reference**
NDM-F	*bla*$_{NDM}$ like	GGTTTGGCGATCTGGTTTTC	621	(178)
NDM-R		CGGAATGGCTCATCACGATC		
OXA-48-F	*bla*$_{OXA-48}$ like	GCGTGGTTAAGGATGAACAC	438	(178)
OXA-48-R		CATCAAGTTCAACCCAACCG		
KPC-F	*bla*$_{KPC}$like	CGTCTAGTTCTGCTGTCTTG	798	(178)
KPC-R		CTTGTCATCCTTGTTAGGCG		

7.4 Amplification of entire carbapenemase genes

The entire genes of *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-58}$ and *bla*$_{IMP}$ were amplified by simplex PCR using specific primers in Table 6. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of plasmid DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94ºC for 10 minutes, followed by 30 cycles of 94ºC for 45 seconds, 52ºC for 45 seconds and 72ºC for 45 seconds and a final extension at 72ºC for 5 minutes. The PCR products were detected as previously described in the method of OXA-type carbapenemase genes detection.

The PCR product was purified by using HiYield™ Gel/PCR DNA Mini Kit. The purified PCR products were sequenced by using the BigDye Terminator V3.1 Cycler sequencing kit by 1st Base DNA sequencing service, Malaysia. The nucleotide sequence of entire carbapenemase gene was converted to amino acid sequence by using ExPASy translate tool (http://web.expasy.org/translate/). The amino acid sequence was analyzed by using Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Multiple sequence alignment by Florence Corpet (http://multalin.toulouse.inra.fr/ multalin/ multalin.html).

Table 6. Primers for amplification of entire carbapenemase genes and entire *murA* gene

primer name	gene	sequence (5'-3')	product size (bp)	reference
ENOXA51-F	entire bla_{OXA-51}	TACGCCAATCCATACAGCAA	1416	(179)
ENOXA51-R		GCTTGACGCTGCTTTTTACC		
ENOXA23-F	entire bla_{OXA-23}	GATGTGTCATAGTATTCGTCG	1065	(179)
ENOXA23-R		TCACAACAACTAAAAGCACTG		
ENOXA58-F	entire bla_{OXA-58}	TTATCAAAATCCAATCGGC	933	(180)
ENOXA58-R		TAACCTCAAACTTCTAATTC		
ENOXA24-F	entire bla_{OXA-24}	GTACTAATCAAAGTTGTGAA	582	(181)
ENOXA24-R		TTCCCCTAACATGAATTTGT		
IMP-F38	entire bla_{IMP}	GCAACATTGCAGTTGCAGAA	704	this study
IMP-R741		TTAGTCGCTTGGCTGTGATG		
IMP-R588M		CTTTGGCCAAGCTTCTAAA		
ENmurA-F	entire *murA*	GAGGCGGAAGTAGTGGTGAG	1764	this study
ENmurA-R		GAGCGCCATGTTCCATAAGT		

7.5 Detection of carbapenemase mRNA expression

The mRNA expression level of carbapenemase genes including *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-58}$ and *bla*$_{IMP}$ were evaluated by RT-PCR. The synthesized cDNA was amplified by monoplex PCR using specific primers are shown in Table 3-4. The PCR reaction was performed in a thin-walled PCR tube with total 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of synthesized cDNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94^{o}C for 10 minutes, followed by 25 cycles of 94^{o}C for 45 seconds, 52^{o}C for 45 seconds and 72^{o}C for 45 seconds and a final extension at 72^{o}C for 5 minutes. The PCR products were separated on 1% agarose gels containing 0.5 µg/ml of ethidium bromide in 0.5X TBE buffer by using electrophoresis at 100 volts for 50 minutes. The separated PCR products were visualized under UV light and 100 bp plus DNA ladder was used as approximate DNA size. The relative intensity of DNA bands was calculated by comparing with 16S RNA and using ImageJ. All these experiments were evaluated in triplicate and the results showed as mean and standard deviation.

Table 7. Primers for RT-PCR of carbapenemase genes, efflux pump genes, and fosfomycin resistance genes

primer name	gene	sequence (5'-3')	product size (bp)	reference
OXA-23-F	*bla*$_{OXA-23}$	GATCGGATTGGAGAACCAGA	501	(172)
OXA-23-R		ATTTCTGACCGCATTTCCAT		
OXA-24-F	*bla*$_{OXA-24}$	GGTTAGTTGGCCCCCTTAAA	249	(172)
OXA-24-R		AGTTGAGCGAAAAGGGGATT		
OXA-58-F	*bla*$_{OXA-58}$	AAGTATTGGGGCTTGTGCTG	599	(172)
OXA-58-R		CCCCTCTGCGCTCTACATAC		
IMP-F	*bla*$_{IMP}$	GGAATAGAGTGGCTTAAYTCT	188	(177)
IMP-R		CCAAACYACTASGTTATCT		
adeB-F	*adeB*	TTAACGATAGCGTTGTAACC	541	(182)
adeB-R		TGAGCAGACAATGGAATAGT		
adeE-F	*adeE*	GTAGTAGTTCGGCAGGACAA	376	(126)
adeE-R		GCGGTTCTAACATCTGATGG		
adeY-F	*adeY*	CAATCTGCAACTGCGCTT	587	(126)
adeY-R		TCAACAGCTTCTGCGGTA		
murA-F	*murA*	TACGGAAATGCCAAGGTTTC	232	this study
murA-R		TGGTTACACCATCTGCCAAA		
ampG-F	*ampG*	ACAGGCGCAACTCAAGAT	459	(183)
ampG-R		CCCAATAAAGCAGCAACA		
murU-F	*murU*	ACGCTTTTGTACACCCAACC	191	this study
murU-R		GTGCCCCATTTCCTCAACTA		
16S RNA-F	16S *RNA*	GGAGGAAGGTGGGGATGACG	241	(182)
16S RNA-R		ATGGTGTGACGGGCGGTGTG		
abaF-F	*abaF*	ATCGGGATCCAGCAAAATTTGCACACTGTC	732	(163)
abaF-R		ATCGGGATCCTTGCAAAGAACCTATTAATCTAAAT		

8. DETERMINATION OF LOSS OR REDUCED OUTER MEMBRANE PROTEINS

8.1 Outer membrane protein extraction

Outer membrane proteins of *Acinetobacter* spp. were extracted by using ultracentrifugation as described by Zander *et al*. (184) with a slight modification. A single colony of *Acinetobacter* spp. on Tryptic Soy Agar (TSA) was inoculated in 10 ml LB broth and incubated at 37^{o}C overnight with shaking. Then, 2 ml of bacterial overnight culture was added to 180 ml of LB broth and incubated at 37^{o}C for 2-3 hours with shaking. Bacterial cells were collected by centrifugation at 4,600 rpm for 30 minutes at 4^{o}C, resuspended with 10 ml of phosphate buffer saline (PBS) and broken by using sonicator at amplitude 40-45% for 5 minutes (with 30 seconds of pulse and 30 seconds of off) on ice. The membrane fractions were collected by ultracentrifugation at 100,000g for 1 hour at 4^{o}C. Then, OMPs were extracted by using 2% sodium *N*-lauroylsarcosine, collected by ultracentrifugation and resuspended with PBS.

8.2 Study of OMPs profiles by SDS-PAGE

The protein concentration was determined by using Bio-Rad protein assay. Ten µg of OMP of each bacterial isolate was mixed with 6X loading buffer and incubated at 100^{o}C for 10 minutes. The OMP samples were separated in 4% polyacrylamide of stacking gel and 12% polyacrylamide of separate gel by electrophoresis at constant currant (30 mA) for 80 minutes. The OMP of *A. baumannii* ATCC 19606 was used to be OMP control as described by Zander *et al*. (184). Then, separate gels with OMP were stained with Coomassie Brilliant Blue and destained with distilled water. After that, the gels were air dried on cellophanes for OMPs analysis by using imageJ and for long-term storage. All these experiments were evaluated in triplicate and the results were shown as mean and standard deviation.

8.3 OMPs analysis by using ImageJ

Dried polyacrylamide gels were scanned to be photo files and these photos were analyzed by using ImageJ. The intensity of protein bands including 29 kDa, 33-36 kDa and 43 kDa bands were calculated and compared with OMPs of *A. baumannii* ATCC 19606.

8.4 Detection of *carO*, 33-36 kDa OMP and *oprD* genes

The genes encoding CarO, 33-36 kDa OMP and OprD were amplified in a thin-walled PCR tube with total 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.1 µM of each primer (Table 8), 0.625U of *Taq* DNA polymerase and 2 µl of boiled DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with initial of 94^{o}C for 5 minutes, followed by 30 cycles of 94^{o}C for 45 seconds, 52^{o}C for 45 seconds and 72^{o}C for 45 seconds and a final extension at 72^{o}C for 6 minutes. The PCR products were detected as previously described in the method of OXA-type carbapenemase genes detection.

Moreover, entire *carO* gene was amplified by the same PCR condition and the PCR products were purified by using HiYield™ Gel/PCR DNA Mini Kit. The purified PCR products were sequenced by using the BigDye Terminator V3.1 Cycler sequencing kit by 1st Base DNA sequencing service, Singapore.

Table 8. Primers for amplification of *carO*, 33-36 kDa OMP and *oprD* genes

primer name	**gene**	**sequence (5'-3')**	**product size (bp)**	**reference**
33-36 OMP-F	*33-36 kDa* like	AAGGTGAGGCATACGTTCCA	502	(184)
33-36 OMP-R		TTTACGTTACCACCCCAAGC		
43 OMP-F	*oprD* like	ATGCTAAAAGCACAAAAACTTAC	1327	this study
43 OMP-R		TTAGAATAATTTCACAGGAATATC		
carO-F	*carO* like	GATGAAGCTGTTGTTCAT	678	this study
carO-R		TTACCAGAAGAAGTTCAC		
entire carO-F	entire *carO*	TCAACACCACATGGATTGCT	1328	this study
entire carO-R		TTCAACAGCTTGGCGAATTT		

9. DETERMINATION OF EFFLUX PUMP OVEREXPRESSION

9.1 Detection of efflux pump activity

The activity of efflux pumps was detected by using efflux pump inhibitors. The susceptibility of imipenem and meropenem was performed as described in Part 5. Antimicrobial susceptibility test. The Mueller Hinton agar was supplemented with imipenem or meropenem at two-fold dilution of concentration (0.015 to 256 mg/L) and another set of Mueller Hinton agar was supplemented with imipenem or meropenem and 100 µM CCCP or 25 mg/L of reserpine. Bacteria was inoculated at final concentration approximately 10^4 CFU/spot and incubated at 37ºC for 18-24 hours. The overexpression of efflux pump activity was defined as the decrease of imipenem or meropenem MIC at least 4-fold compared with no efflux pump inhibitors.

9.2 Detection of efflux pump mRNA expression

The mRNA expression level of efflux pump genes including *adeB* (*A. baumannii*), *adeE* (non-*A. baumannii*) and *adeY* (non-*A. baumannii*) was evaluated by RT-PCR. The synthesized cDNA was amplified by monoplex PCR using specific primers as shown in Table 7. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of synthesized cDNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94ºC for 10 minutes, followed by 25 cycles of 94ºC for 45 seconds, 52ºC for 45 seconds and 72ºC for 45 seconds and a final extension at 72ºC for 5 minutes. The PCR products and the relative intensity of DNA bands were evaluated as previously described in the method of the detection of carbapenemase mRNA expression.

10. MULTILOCUS SEQUENCE TYPING (MLST)

The clonal of *Acinetobacter* spp. was determined by multilocus sequence typing (MLST) using Oxford scheme. This scheme uses nucleotide fragments of 7 housekeeping genes including citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), glucose dehydrogenase B (*gdhB*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*), glucose-6-phosphate isomerase (*gpi*), RNA polymerase sigma factor (*rpoD*) and the specific primers are shown in Table 9.

The monoplex PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of plasmid DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94^{o}C for 3 minutes, followed by 30 cycles of 94^{o}C for 45 seconds, 52^{o}C for 45 seconds and 72^{o}C for 45 seconds and a final extension at 72^{o}C for 6 minutes. The PCR products were detected as previously described in the method of OXA-type carbapenemase genes detection.

The PCR product was prepared and sequenced as previously described in the method of amplification of entire carbapenemase genes. The nucleotide sequences of 7 housekeeping genes were analyzed for the MLST profiles using *Acinetobacter baumannii* MLST (Oxford) database (http://pubmlst.org /abaumannii/). Moreover, the novel allelic number and novel sequence type (ST) from this study were submitted to *Acinetobacter baumannii* MLST (Oxford) database.

Table 9. Primers for amplification and sequencing of 7 housekeeping genes of MLST Oxford scheme

primer name	gene	sequence (5'-3')	product size (bp)	reference
Citrato-F1	*gltA*	AATTTACAGTGGCACATTAGGTCCC	722	(185)
Citrato-R12		GCAGAGATACCAGCAGAGATACACG		
gyrB-F	*gyrB*	TGAAGGCGGCTTATCTGAGT	594	(185)
gyrB-R		GCTGGGTCTTTTTCCTGACA		
GDHB-1F		GCTACTTTTATGCAACAGAGCC		
GDH SEC-F	*gdhB*	ACCACATGCTTTGTTATG	774	(185)
GDHB 755R		GTTGAGTTGGCGTATGTTGTGC		
GDH SEC R		GTTGGCGTATGTTGTGC		
RA1	*recA*	CCTGAATCTTCYGGTAAAAC	425	(185)
RA2		GTTTCTGGGCTGCCAAACATTAC		
cpn60_F	*cpn60*	GGTGCTCAACTTGTTCGTGA	640	(185)
cpn60_R		CACCGAAACCAGGAGCTTTA		
gpi_F	*gpi*	GAAATTTCCGGAGCTCACAA	456	(185)
gpi_R		TCAGGAGCAATACCCCACTC		
rpoD_F	*rpoD*	ACCCGTGAAGGTGAAATCAG	672	(185)
rpoD_R		TTCAGCTGGAGCTTTAGCAAT		

11. DETERMINATION OF ANTIBIOTIC RESISTANCE ISLANDS

Antibiotic resistance islands in *Acinetobacter* spp. were investigated by PCR mapping and DNA sequencing. The first step was the amplification of intact *comM* by PCR. The second step was PCR for junction of *comM* and antibiotic resistance island (J3 and J5) and the third step was PCR mapping of antibiotic resistance island as shown in Figure 11. The monoplex PCR reaction was performed in a thin-walled PCR tube with a total volume of 50 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers (Table 10), 1.25U of *Taq* DNA polymerase and 4 µl of genomic DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94^{o}C for 5 minutes, followed by 30 cycles of 94^{o}C for 1 minute, 55^{o}C for 1 minute and 72^{o}C for 1 minute and a final extension at 72^{o}C for 6 minutes. The amplicons were detected as previously described in the method of OXA-type carbapenemase genes detection. The PCR products were prepared and sequenced as previously described in the method of amplification of entire carbapenemase genes.

Table 10. Primers for antibiotic resistance island study

primer name	gene	sequence (5'-3')	product size (bp)	reference
comM-F	*comM*	TGCTGCAATGAGCTGAAAGT	982	(145)
comM-R		GCCTCTCATTGAGGTTGAGG		
J3-F	Junction	TATCAGCAGCAAAACGATGG	846	(186)
J3-R	at 3'	AATCGATGCGGTCGAGTAAC		
J5-F	Junction	TTTGGGAAGCAATCAATAGTC	310	(146)
J5-R	at 5'	ATTGGCCTCACGCCTGCCT		
comMtniB-F	*comM*	TGCTGCAATGAGCTGAAAGT	3120	(145)
comMtniB-R	to *tniB*	GCGATTCAAAATATCGGTCAA		
comMgap-F		GTTTGAATCGACCCTTGAGC	1942	this study
comMgap-R		TGTACCTGCTGTCGTCTTCG		
tniBtniE-F	*tniB* to	GCGATAGTGAACGGATTGAGA	560 or	(8)
tniBtniE-R	*tniE*	TTGCCCATTAAGCACAACAG	3410	
tnigap-F		TGCGAAATCATTATAGGGAGGT	1682	this study
tnigap-R		TCGCATAAATCAGCACAACC		
tniEuspA-F	*tniE* to	TGGGTCAGAAATGGGCTTAG	1552	this study
tniEuspA-R	*uspA*	CTCTTGGGCAAAAGAGCAAC		
uspAISAba1gap-F		CCAGAAGTGTGCCTGACTGA	1448	this study
uspAISAba1gap-R		AAGAAGCATGGACGACTCTCA		
orf-orf4-F	orf to	TTGCTCAAGTGTACCCCTTTG	8244 or	this study
orf-orf4-R	orf4	GGGCGAAAATTATTGTTCTTA	3430	
ISAba1-OXA23-F	IS*Aba1* to	CGACGAATACTATGACAC	2760	(174)
ISAba1-OXA23-R	$bla_{\text{OXA-23}}$	GATCGGATTGGAGAACCAGA		(172)
OXA23-orf4-F	$bla_{\text{OXA-23}}$	TGCTGAACCGTACAACCAGA	1455	this study
OXA23-orf4-R	to orf4	TCCACCCTAGTTTATTTACCCATC		
ISAba1-5comM-F	IS*Aba1*	CGACGAATACTATGACAC	3390	(174)
ISAba1-5comM-R	to 5'*comM*	TGCTCCTGCAGATTTGCCCA		(146)

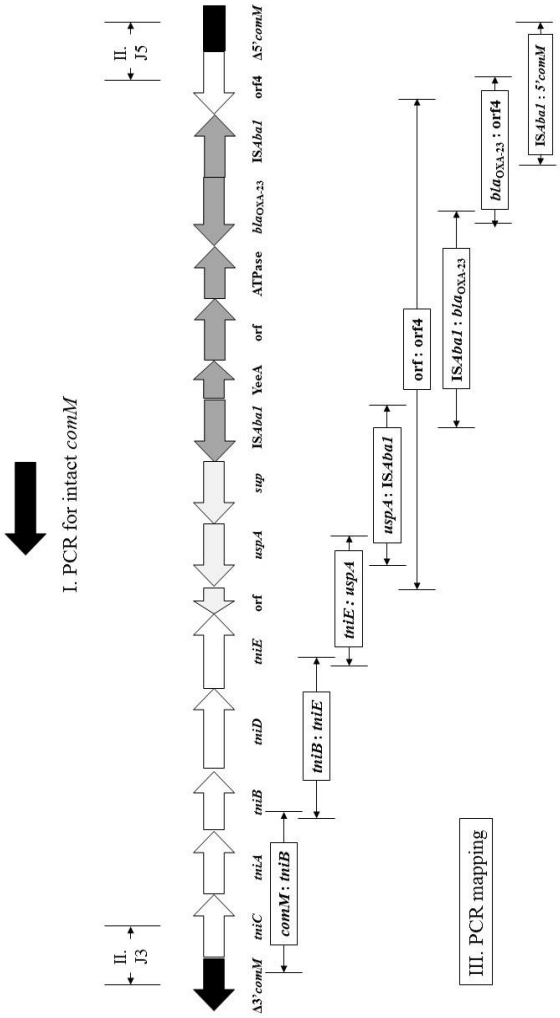


Figure 11. Scheme of PCR mapping for antibiotic resistance island study

12. SCREENING OF SYNERGISM OF ANTIBIOTIC COMBINATIONS BY CHECKERBOARD ASSAY

12.1 Preparation of antibiotic microdilution checkerboard plate

In this study, imipenem plus amikacin, imipenem plus colistin, imipenem plus fosfomycin, meropenem plus amikacin, meropenem plus colistin and meropenem plus fosfomycin were evaluated against carbapenem-resistant *Acinetobacter* spp. by using checkerboard assay.

Checkerboard assay was performed in 96-well culture plates. Each antibiotic stock solution was prepared at 8X MIC concentration. Checkerboard panel of antibiotic A was prepared as follows (Figure 12) (i) added 50 µl of MHB to all wells of 96-well plate (except A1, B1, C1, D1, E1, F1, G1, H1 and H12), (ii) added 50 µl of antibiotic A stock solution to A12, B12, C12, D12, E12, F12 and G12, (iii) then serial diluted with 50 µl from the twelfth column to second column (except H12).

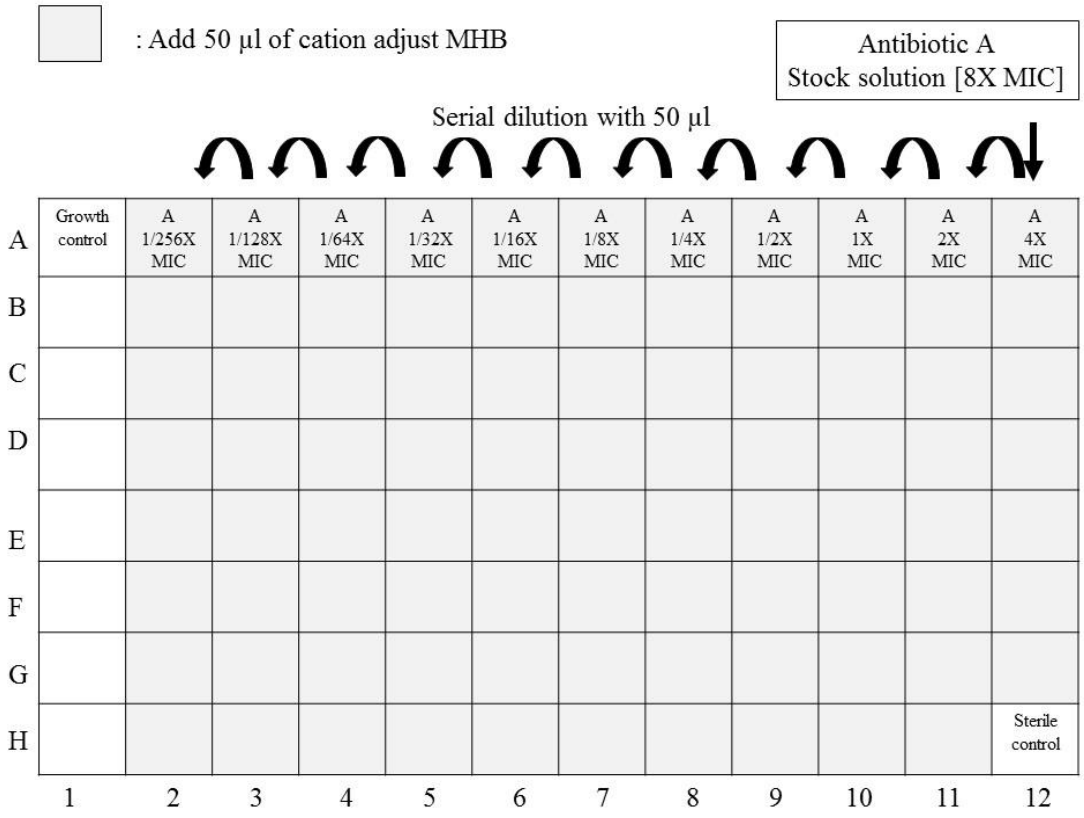


Figure 12. Preparation of checkerboard panel of antibiotic A

Checkerboard panel of antibiotic B was prepared in another 96-well culture plate as follows (Figure 13) (i) added 100 µl of MHB to all wells (except A1-A12 and H12), (ii) added 100 µl of antibiotic B stock solution to H1-H11, (iii) serial diluted with 100 µl from row H to row B (except H12).

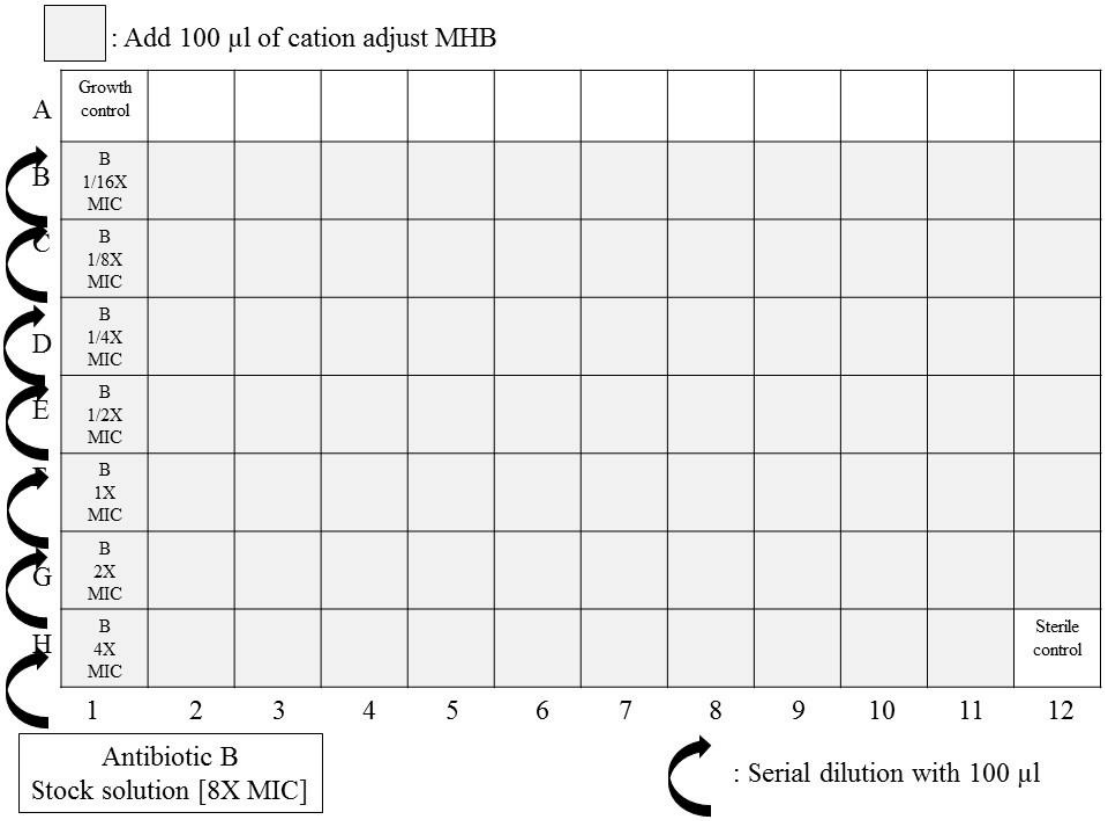


Figure 13. Preparation of checkerboard panel of antibiotic B

After that, 50 µl of diluted antibiotic B in each well were transferred to the same wells of checkerboard panel of antibiotic A (Figure 14). The volume of all wells was adjusted to be 180 µl with MHB.

Growth control	A 1/256X MIC	A 1/128X MIC	A 1/64X MIC	A 1/32X MIC	A 1/16X MIC	A 1/8X MIC	A 1/4X MIC	A 1/2X MIC	A 1X MIC	A 2X MIC	A 4X MIC
B 1/16X MIC											
B 1/8X MIC											
B 1/4X MIC											
B 1/2X MIC											
B 1X MIC											
B 2X MIC											
B 4X MIC											Sterile control

Figure 14. Checkerboard panel of antibiotic A plus antibiotic B

12.2 Preparation of bacterial inoculum

A few colonies of *Acinetobacter* spp. were inoculated to MHB and then were incubated at 37ºC for 2 hours with shaking. After that, the turbidity was adjusted to 0.5 McFarland standard and diluted to 1:100 with MHB. Twenty µl of prepared inoculum was added to all wells of antibiotic combination panel (except H12: added 20 µl of MHB). The plates were incubated at 37ºC for 18-24 hours. All these experiments were evaluated in duplicate.

12.3 Interpretation

The MIC_A, MIC_{A+B}, MIC_B and MIC_{B+A} were read by naked eyes, and calculated for fractional inhibitory concentration index (FIC index or FICI). The interpretation are as follows:

$$\text{FIC index} = \frac{MIC_{A+B}}{MIC_A} + \frac{MIC_{B+A}}{MIC_B}$$

Synergism is FICI ≤ 0.5
Indifference is $0.5 <$ FICI ≤ 4
Antagonism is FICI > 4

13. CONFIRMATION OF SYNERGISM OF ANTIBIOTIC COMBINATION BY TIME-KILL ASSAY

After activities of antibiotic combination were screened by checkerboard assay, the best effective combination was confirmed for synergism by time-kill assay. In this study, the best combination against carbapenem-resistant *Acinetobacter* spp. was imipenem plus fosfomycin. Thus, activity of imipenem plus fosfomycin was investigated against carbapenem-resistant *Acinetobacter* spp. with different resistance mechanisms. The several concentrations of imipenem and fosfomycin were tested against *Acinetobacter* spp. including 0.25X, 0.5X, 1X and 2X the MICs. The appropriate concentrations were the concentration which could not kill *Acinetobacter* spp. in alone but could kill the bacteria in the combination. In this study, the appropriate concentrations of imipenem and fosfomycin were 0.5X and 1X the MICs.

Each *Acinetobacter* spp. isolate was tested in 9 growth conditions including no antibiotic (growth control), 1X MIC of imipenem, 0.5X MIC of imipenem, 1X MIC of fosfomycin, 0.5X MIC of fosfomycin, 1X MIC of imipenem plus 1X MIC of fosfomycin, 1X MIC of imipenem plus 0.5X MIC of fosfomycin, 0.5X MIC of imipenem plus 1X MIC of fosfomycin and 0.5X MIC of imipenem plus 0.5X MIC of fosfomycin. All conditions were performed in 125 ml flasks, containing 29.7 ml MHB supplemented with imipenem or/and fosfomycin (for fosfomycin, 25 mg/L of G6P was supplemented)

A few colonies of *Acinetobacter* spp. were inoculated to MHB and incubated at 37ºC for 2 hours with shaking. After that, the turbidity was adjusted to 0.5 McFarland standard. Three hundred µl of prepared inoculum was added to each growth condition flasks. The flasks were incubated at 37ºC with shaking for 24 hours. At 0, 2, 4, 6, 12 and 24 hours of incubation, the 20 µl of growth conditions were removed and diluted in normal saline from 10^{-1} to 10^{-7}. Each dilution was spotted on MHA plate with 5 spots (10 µl/spot) in duplicate. The MHA plates were incubated at 37ºC for 18-24 hours and then colony was counted and calculated for CFU/ml. All these experiments were evaluated in triplicate. The mean and standard deviation of the numbers of viable bacterial cell (CFU/ml) in each growth conditions were plotted on a semi-log graph.

Interpretations of time-kill assay were synergism if the viable cells decreased $\geq 2\log_{10}$ CFU/ml when compared with the most single active antibiotic and bactericidal

effect if the viable cells were decreased $\geq 3\log_{10}$ CFU/ml when compared with start cell (187).

14. DETERMINATION OF FOSFOMYCIN RESISTANCE MECHANISMS

14.1 Detection of *murA* gene

14.1.1 Detection of MurA mutation

The entire *murA* gene was amplified by monoplex PCR using specific primers in Table 5. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of plasmid DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94ºC for 10 minutes, followed by 30 cycles of 94ºC for 45 seconds, 52ºC for 45 seconds and 72ºC for 45 seconds and a final extension at 72ºC for 5 minutes. The amplicons were detected as previously described in the method of OXA-type carbapenemase genes detection. The PCR products were prepared and sequenced as previously described in the method of amplification of entire carbapenemase genes. The mutation of MurA active site, Cys116 and ligand interaction including Lys22, Arg120 and Arg398 were evaluated.

14.1.2 Detection of *murA* mRNA expression

The mRNA expression level of *murA* gene was evaluated by RT-PCR. The synthesized cDNA was amplified by monoplex PCR using specific primers are shown in Table 4. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of synthesized cDNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94ºC for 10 minutes, followed by 25 cycles of 94ºC for 45 seconds, 52ºC for 45 seconds and 72ºC for 45 seconds and a final extension at 72ºC for 5 minutes. The PCR products and the relative intensity of DNA bands were evaluated as previously described in the method of the detection of carbapenemase mRNA expression.

14.2 Detection of fosfomycin-modifying enzymes

Many bacteria resist to fosfomycin by producing fosfomycin-modifying enzymes, including FosA, FosB, FosC and FosX (164). In this study, the fosfomycin-modifying enzyme genes were amplified by monoplex PCR using specific primers in Table 11. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of plasmid DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94ºC for 10 minutes, followed by 30 cycles of 94ºC for 45 seconds, 52ºC for 45 seconds and 72ºC for 45 seconds and a final extension at 72ºC for 5 minutes. The PCR products were detected as previously described in the method of OXA-type carbapenemase genes detection.

Table 11. Primers for fosfomycin-modifying enzyme gene detection

primer name	**gene**	**sequence (5'-3')**	**product size (bp)**	**reference**
fosA-F	*fosA3*	CGGAGCCTATCTCTCCTGTG	219	this study
fosA-R		CCGTCAGGGTCGAGAAAATA		
fosB-F	*fosB*	AGGTGAGACCTCGGCCTATT	302	this study
fosB-R		GAGGTTTAGCCTCTTTATAATAACTCA		
fosC-F	*fosC2*	GGGCATATCTGAGCTTGGAG	212	this study
fosC-R		CAATTTATGGCCGTCAGGAT		
fosX-F	*fosX*	GTTGCGTTTAAGGCAGGAAG	425	this study
fosX-R		GGCTCCATTTGTTGGACAGT		

14.3 Detection of overexpression of efflux pump

14.3.1 Detection of efflux pump activity

The activity of efflux pumps was detected by using efflux pump inhibitors. The susceptibility of fosfomycin was perform as described in Part 5. Antimicrobial susceptibility test. The Mueller Hinton agar was supplemented with fosfomycin at two-fold dilution of concentration (0.015 to 256 mg/L) and another set of Mueller Hinton agar was supplemented with fosfomycin and 100 µM CCCP or 25 mg/L of reserpine. All of MHA plates were supplemented with 25 mg/L of G6P. Bacteria was inoculated at final concentration approximately 10^4 CFU/spot and incubated at 37ºC for 18-24 hours. The overexpression of efflux pump activity was defined as the decrease of

imipenem or meropenem MIC at least 4-fold compared with no efflux pump inhibitors (124).

14.3.2 Detection of efflux pump mRNA expression

The mRNA expression level of efflux pump gene, *abaF*, that has been reported to be in only *A. baumannii* was evaluated by RT-PCR (163). The synthesized cDNA was amplified by monoplex PCR using specific primers are shown in Table 7. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction that adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of synthesized cDNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94^{o}C for 10 minutes, followed by 25 cycles of 94^{o}C for 45 seconds, 52^{o}C for 45 seconds and 72^{o}C for 45 seconds and a final extension at 72^{o}C for 5 minutes. The PCR products and the relative intensity of DNA bands were evaluated as previously described in the method of the detection of carbapenemase mRNA expression.

14.4 Detection of cell wall recycling bypass pathway

14.4.1 Detection of gene in cell wall recycling bypass pathway

The important transporter gene (*ampG*), beta-N-acetyl glucosaminidase gene (*nagZ*), AnhMurNAc kinase gene (*anmK*), MurNAc or GlcNAc kinase gene (*amgK*) and MurNAc alpha-1-phosphate-uridylyl transferase gene (*murU*) were amplified by monoplex PCR using primer in Table 12 (165). The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primer, 1.25U of *Taq* DNA polymerase and 2 µl of plasmid DNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94^{o}C for 10 minutes, followed by 30 cycles of 94^{o}C for 45 seconds, 52^{o}C for 45 seconds and 72^{o}C for 45 seconds and a final extension at 72^{o}C for 5 minutes. The PCR products were detected as previously described in the method of OXA-type carbapenemase genes detection.

Table 12. Primers for cell wall recycling bypass pathway genes

primer name	gene	sequence (5'-3')	product size (bp)	reference
ampG-F	*ampG*	ACAGGCGCAACTCAAGAT	459	(183)
ampG-R		CCCAATAAAGCAGCAACA		
nagZ-F	*nagZ*	TTTGATTGCTGTCGACCAAG	240	this study
nagZ-R		AAGCCACGGTCACCAATTAC		
anmK-F	*anmK*	ATTCGAACCCGAGTTACGTG	216	this study
anmK-R		AGCGTAAAACCATGCTCTGG		
amgK-F	*amgK*	CTTTGGTGATGTGCTGCTGT	210	this study
amgK-R		TTTTGCTCAGCTGTTGGATG		
murU-F	*murU*	ACGCTTTTGTACACCCAACC	191	this study
murU-R		GTGCCCCATTTCCTCAACTA		

14.4.2 Detection of *ampG* and *murU* mRNA expression

The mRNA expression level of transporter gene, *ampG*, and *murU* gene were evaluated by RT-PCR. The synthesized cDNA was amplified by monoplex PCR using specific primers as shown in Table 12. The PCR reaction was performed in a thin-walled PCR tube with a total volume of 25 µl reaction by adding the following components: 1X *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each forward and reverse primers, 1.25U of *Taq* DNA polymerase and 2 µl of synthesized cDNA. The amplification was performed in Applied Biosystems Veriti Thermal Cycler with an initial of 94ºC for 10 minutes, followed by 25 cycles of 94ºC for 45 seconds, 52ºC for 45 seconds and 72ºC for 45 seconds and a final extension at 72ºC for 5 minutes. The PCR products and the relative intensity of DNA bands were evaluated as previously described in the method of the detection of carbapenemase mRNA expression.

15. DETERMINATION OF POPULATION ANALYSIS

15.1 Population analysis of imipenem susceptibility

Overnight culture of *Acinetobacter* spp. in MHB was diluted as serial dilution from 10^{-1} to 10^{-7} in normal saline. Diluted bacterial sample was plated on MHA supplemented with imipenem at final concentration 0, 8, 16, 32, 64, 128, 256, 512 and 1024 mg/L. The plates were incubated at 37ºC for 24 hours and visible colonies were counted. The numbers of CFU/ml of bacteria that grew at each imipenem concentration were plotted on a semi-log graph.

15.2 Population analysis of fosfomycin susceptibility

An overnight culture of *Acinetobacter* spp. in MHB was diluted as serial dilution from 10^{-1} to 10^{-7} in normal saline. Diluted bacterial sample was plated on MHA supplemented with fosfomycin at final concentration 0, 8, 16, 32, 64, 128, 256, 512 and 1024 mg/L and 25 mg/L of G6P. The plates were incubated at 37ºC for 24 hours and visible colonies were counted. The numbers of CFU/ml of bacteria that grew at each fosfomycin concentration were plotted on a semi-log graph.

CHAPTER V
RESULTS

1. BACTERIAL STRAINS

A total of 353 *Acinetobacter* spp. isolates used in this study. The 346 *Acinetobacter* spp. were isolated and collected between January 2010 and December 2011. These isolates were used to study the prevalence of carbapenem resistance and the presence of carbapenemase genes. Of the 346 isolates, 29 isolates (16 *A. baumannii* isolates, 8 *A. pittii* isolates and 5 *A. nosocomialis* isolates) with different carbapenem MICs and different carbapenemase genes were randomly selected to study the reduction of OMPs, overexpression of efflux pumps and antibiotic combination activity. Moreover, one *A. baumannii* isolate collected in 2008 (isolate A10) and 6 *A. baumannii* isolates collected between May 2015 and July 2016 (isolate A4 to A9) were used to study carbapenem resistance mechanisms and antibiotic combination activity.

Of the 346 *Acinetobacter* spp. isolates, 76 (22.0%) were isolated from specimens collected from patients in intensive care units (ICUs) and 270 (78.0%) were isolated from specimens collected from patients in non-ICUs. The isolates included 1 isolate (1.2%) from blood, 24 isolates (7.0%) from body fluids, 141 isolates (40.8%) from sputum, 6 isolates (1.7%) from respiratory aspirate, 17 isolates (4.9%) from respiratory tract secretion and washing, 71 isolates (20.5%) from pus, 52 isolates (15.0%) from urine, 16 isolates (4.6%) from catheter and 15 isolates (4.3%) from other specimens.

A. baumannii isolates A4 to A9 were isolated from body fluid and *A. baumannii* isolate A10 was isolated from pus. All these 7 isolates were isolated from specimens collected from patients in non-ICUs.

2. BACTERIAL IDENTIFICATION

1. Gram stain and biochemical tests

All 353 *Acinetobacter* spp. were Gram-negative coccobacilli and showed biochemical test results of genus *Acinetobacter* including oxidase negative, alkaline slant/neutral butt on TSI, oxidized glucose (dextrose), citrate utilization positive, urease negative and reduced nitrate to nitrite or nitrogen gas negative. In conclusion, all isolates in this study, were characterized to genus of *Acinetobacter* by biochemical tests.

2. Identification of *A. baumannii* by the presence of $bla_{\text{OXA-51-like}}$

The presence of $bla_{\text{OXA-51-like}}$ was detected by multiplex PCR for OXA-type carbapenemase genes. Of the 353 *Acinetobacter* spp. isolates, 311 (88.1%) carried $bla_{\text{OXA-51-like}}$ and 42 (11.9%) did not contain $bla_{\text{OXA-51-like}}$. Therefore, 311 isolates carrying $bla_{\text{OXA-51-like}}$ were hypothesized to be *A. baumannii*. All of them were identified for species by multiplex PCR for *gyrB* gene.

3. Species identification by multiplex PCR for *gyrB*

Of the 353 *Acinetobacter* spp. isolates, 312 (88.4%) were identified as *A. baumannii*, 22 (6.2%) were *A. pittii* and 19 (5.4%) were *A. nosocomialis*. One isolate (isolate AB182) did not harbored $bla_{\text{OXA-51-like}}$ but was identified as *A. baumannii* by multiplex PCR for *gyrB*. This isolate was confirmed the absence of $bla_{\text{OXA-51-like}}$ by amplification of entire $bla_{\text{OXA-51}}$ gene.

Normally, the PCR product of entire $bla_{\text{OXA-51}}$ (Table 6) was 1416 bp but the PCR product from isolate AB182 was 2725 bp. This result indicated that there were some elements interrupted $bla_{\text{OXA-51}}$. The nucleotide sequencing results showed the insertion sequence, IS*Aba19* interrupted at 379th nucleotide of $bla_{\text{OXA-78}}$, which is $bla_{\text{OXA-51}}$ variant. IS*Aba19* was 1309 bp long, contained 903-bp open reading frame (ORF) encoding transposase *insA* and *insB* and was bound by 27-bp inverted repeat sequence (TGAACCGTACCGGGTTTGTCGGAGAGT) with a 3-bp duplication site (CTT) (Figure 15). Therefore, isolate AB182 had incomplete $bla_{\text{OXA-51}}$ variant, $bla_{\text{OXA-78}}$.

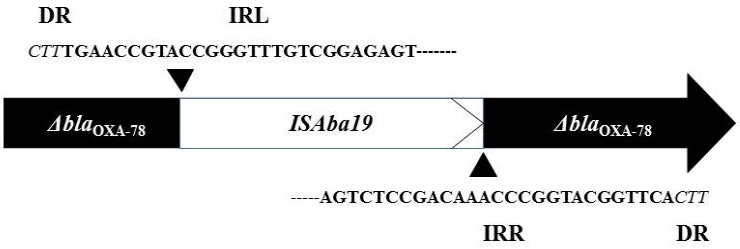


Figure 15. Genetic scheme of disrupted *bla*$_{\text{OXA-78}}$ by IS*Aba19* in *A. baumannii* isolate AB182

In conclusion, the results showed that there were 312 *A. baumannii* isolates (88.4%), 22 *A. pittii* isolates (6.2%) and 19 *A. nosocomialis* isolates (5.4%) in this study.

3. ANTIMICROBIAL SUSCEPTIBILITY TEST

The susceptibility of 10 antibiotics (imipenem, meropenem, amikacin, gentamicin, ceftriaxone, ceftazidime, cefotaxime, cefepime, ciprofloxacin and colistin) against *Acinetobacter* spp. was determined by agar dilution method. The minimum inhibitory concentrations (MICs) of these 10 antibiotics against 353 *Acinetobacter* spp. were evaluated and shown in Appendix D.

In *A. baumannii*, the antimicrobial susceptibility and interpretation are shown in Table 13. The prevalence of imipenem and meropenem resistance were 86.6% and 86.6%, respectively. The imipenem and meropenem MIC ranges were 0.125 to 256 mg/L with imipenem MIC_{50} and MIC_{90} of 64 and 128 mg/L, respectively and meropenem MIC_{50} and MIC_{90} of 32 and 64 mg/L, respectively. The prevalence of aminoglycoside resistance was 75.4% and 79.1% to amikacin and gentamicin, respectively. The extended-spectrum cephalosporin resistance rates were 84.6%, 84.6%, 85.2% and 87.5% for cefotaxime, ceftriaxone, ceftazidime and cefepime, respectively. The prevalence of ciprofloxacin resistance was 84.9%. All of *A. baumannii* were susceptible to colistin with MIC range of 0.5 to 2 mg/L and MIC_{50} and MIC_{90} of 1 mg/L and 2 mg/L, respectively.

Table 13. The antibiotic susceptibilities among the 305 *A. baumannii* isolates

Antimicrobial agents	MICs (mg/L)			Interpretation		
	Range	MIC_{50}	MIC_{90}	Resistance (%)	Intermediate (%)	Susceptible (%)
Imipenem	0.125-256	64	128	264 (86.6)	0	41 (13.4)
Meropenem	0.125-256	32	64	264 (86.6)	0	41 (13.4)
Amikacin	1->256	>256	>256	230 (75.4)	7 (2.3)	68 (22.3)
Gentamicin	0.5->256	>256	>256	241 (79.1)	5 (1.6)	59 (19.3)
Cefotaxime	4->256	>256	>256	258 (84.6)	23 (7.5)	24 (7.9)
Ceftriaxone	4->256	>256	>256	258 (84.6)	38 (12.5)	9 (2.9)
Ceftazidime	2->256	>256	>256	260 (85.2)	14 (4.6)	31 (10.2)
Cefepime	1->256	256	>256	267 (87.5)	4 (1.3)	34 (11.2)
Ciprofloxacin	0.06->256	64	128	259 (84.9)	0	46 (15.1)
Colistin	0.5-2	1	2	0	-	305 (100)

For the *A. pittii* isolates, the antimicrobial susceptibility and interpretation are shown in Table 14. The prevalence of imipenem and meropenem resistance were 22.7% and 18.2%, respectively. The MIC ranges of imipenem and meropenem were 0.25 to 32 mg/L and 0.125 to 32 mg/L, respectively. The MIC_{50} and MIC_{90} of imipenem were 0.5 mg/L and 32 mg/L, respectively. The MIC_{50} and MIC_{90} of meropenem were 1 mg/L and 32 mg/L, respectively. The aminoglycoside resistance rates were 18.2% and 36.4% for amikacin and gentamicin, respectively. The resistance rates of cefotaxime, ceftriaxone, ceftazidime and cefepime were 27.3%, 22.7%, 36.4% and 40.9%, respectively. The prevalence of ciprofloxacin resistance rate was 31.8% and the MIC range was 0.03 to 256 mg/L. All of *A. pittii* was susceptible to colistin. The MIC range was 0.25 to 1 mg/L. The MIC_{50} and MIC_{90} were 1 mg/L.

Table 14. The antibiotic susceptibilities among the 22 *A. pittii* isolates

Antimicrobial agents	MICs (mg/L)			Interpretation		
	Range	MIC_{50}	MIC_{90}	Resistance (%)	Intermediate (%)	Susceptible (%)
Imipenem	0.25-32	0.5	32	5 (22.7)	2 (9.1)	15 (68.2)
Meropenem	0.125-32	1	32	5 (22.7)	1 (4.6)	16 (72.7)
Amikacin	1->256	4	>256	4 (18.2)	2 (9.1)	16 (72.7)
Gentamicin	0.25->256	2	>256	8 (36.4)	0	14 (63.6)
Cefotaxime	4->256	16	>256	6 (27.3)	11 (50.0)	5 (22.7)
Ceftriaxone	8->256	16	>256	5 (22.7)	12 (54.6)	5 (22.7)
Ceftazidime	4->256	16	>256	8 (36.4)	6 (27.3)	8 (36.4)
Cefepime	2->256	8	>256	9 (40.9)	0	13 (59.1)
Ciprofloxacin	0.03-256	0.125	64	7 (31.8)	0	15 (68.2)
Colistin	0.25-1	1	1	0	-	22 (100)

A. nosocomialis showed the lowest of resistant prevalence to all antimicrobial agents (Table 15). The prevalences of imipenem and meropenem resistance were 26.3%. The imipenem and meropenem MIC range was 0.125 to 32 mg/L and 0.5 to 64 mg/L. The MIC_{50} and MIC_{90} of imipenem were 0.5 mg/L and 16 mg/L, respectively. The MIC_{50} and MIC_{90} of meropenem were 0.5 mg/L and 32 mg/L, respectively. There was no isolate resistant to aminoglycosides. No cefotaxime resistance isolate was found. The resistance rates of ceftriaxone, ceftazidime and cefepime were 5.3%, 21.1% and 31.6%, respectively. The prevalence of ciprofloxacin resistance was 5.3% and the MIC range was 0.03 to 64 mg/L. There was no colistin-resistant *A. nosocomialis* in this study. The colistin MIC range was 0.25 to 2 mg/L. The MIC_{50} and MIC_{90} of colistin were 1 mg/L and 2 mg/L, respectively.

Table 15. The antibiotic susceptibilities among the 19 *A. nosocomialis* isolates

Antimicrobial agents	MICs (mg/L)			Interpretation		
	Range	MIC_{50}	MIC_{90}	Resistance (%)	Intermediate (%)	Susceptible (%)
Imipenem	0.125-32	0.5	16	5 (26.3)	1 (5.3)	13 (68.4)
Meropenem	0.5-64	0.5	32	5 (26.3)	0	14 (73.7)
Amikacin	1-8	2	4	0	0	19 (100)
Gentamicin	0.5-2	0.5	2	0	0	19 (100)
Cefotaxime	8-32	16	32	0	14 (73.7)	5 (26.3)
Ceftriaxone	16-256	16	32	1 (5.3)	18 (94.7)	0
Ceftazidime	4-32	16	32	4 (21.1)	10 (52.3)	5 (26.3)
Cefepime	4-64	8	64	6 (31.6)	0	13 (68.4)
Ciprofloxacin	0.03-64	0.125	1	1 (5.3)	0	18 (94.7)
Colistin	0.25-2	1	2	0	-	19 (100)

The resistance rates of 10 antimicrobial agents of *Acinetobacter* spp. are shown in Figure 16. The resistance rate of imipenem, meropenem, amikacin, gentamicin, cefotaxime, ceftazidime, ceftriaxone, cefepime and ciprofloxacin in *A. baumannii* showed significantly higher than *A. pittii* and *A. nosocomialis* (**$p < 0.01$). *A. pittii* also had higher resistance rate to gentamicin (**$p < 0.01$), cefotaxime (*$p < 0.05$) and ciprofloxacin (*$p < 0.05$) than *A. nosocomialis*. The resistance rates of amikacin, ceftriaxone, ceftazidime and cefepime of *A. pittii* were higher than *A. nosocomialis*. However, the carbapenem resistance rates in *A. nosocomialis* were higher than *A. pittii*.

The resistance breakpoint of imipenem MIC is ≥ 8 mg/L and the MIC distribution of imipenem is shown in Figure 17. The peaks of imipenem resistance in *A. baumannii*, *A. pittii* and *A. nosocomialis* were 64, 32 and 16 mg/L, respectively. The peaks of imipenem susceptible in *A. baumannii* and *A. nosocomialis* were 0.5 mg/L and 0.25 mg/L, respectively. The peaks of imipenem susceptible in *A. pittii* were 0.25 mg/L and 0.5 mg/L. The resistance breakpoint of meropenem MIC is ≥ 8 mg/L and the MIC distribution of imipenem is shown in Figure 18. The peaks of meropenem resistance in *A. baumannii*, *A. pittii* and *A. nosocomialis* were 64, 32 and 32 mg/L, respectively. The peaks of meropenem susceptible were 0.25, 1 and 0.5 mg/L, respectively.

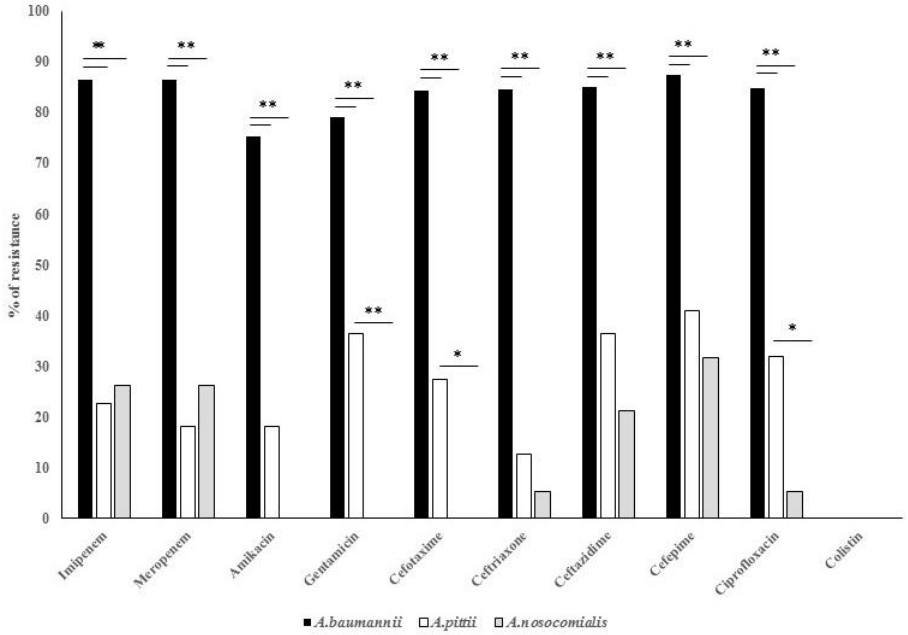


Figure 16. Prevalences of antibiotic resistance in *Acinetobacter* spp. isolates

*: $p < 0.05$; **: $p < 0.01$ (statistical analysis by using Chi-square test)

For amikacin, the MIC breakpoint is 64 mg/L and the MIC distribution is shown in Figure 19. The peaks of amikacin resistance in *A. baumannii* and *A. pittii* were >256 mg/L. There was no amikacin-resistant *A. nosocomialis* isolate. The peaks of amikacin susceptible in *A. baumannii*, *A. pittii* and *A. nosocomialis* were 4, 2 and 2 mg/L, respectively. The gentamicin breakpoint is 16 mg/L and the MIC distribution of gentamicin is shown in Figure 20. The peaks of gentamicin resistance in *A. baumannii* and *A. pittii* were >256 mg/L. No gentamicin-resistant *A. nosocomialis* was found. Peaks of gentamicin susceptible in *A. baumannii*, *A. pittii* and *A. nosocomialis* were1, 1 and 0.5 mg/L, respectively.

The resistance breakpoint of cefotaxime MIC is 64 mg/L and the MIC distribution is shown in Figure 21. The peaks of cefotaxime resistance in *A. baumannii* and *A. pittii* were >256 mg/L. No cefotaxime-resistant *A. nosocomialis* was found. The peaks of cefotaxime susceptible in *A. baumannii*, *A. pittii* and *A. nosocomialis* were 8 mg/L. The resistance breakpoint of ceftriaxone MIC is 64 mg/L and the MIC distribution is shown in Figure 22. The peaks of ceftriaxone resistance in *A. baumannii*, *A. pittii* and *A. nosocomialis* were >256, >256 and 256 mg/L. The peaks of ceftriaxone

susceptible in *A. baumannii* and *A. pittii* were 8 mg/L. No ceftriaxone-susceptible *A. nosocomialis* was found. Most of *A. nosocomialis* isolate were intermediate resistance to ceftriaxone (94.7%, 18/19). The resistance breakpoint of ceftazidime is 32 mg/L and the MIC distribution is shown in Figure 23. The peaks of ceftazidime resistance in *A. baumannii*, *A. pittii* and *A. nosocomialis* were >256, >256 and 32 mg/L, respectively. The peaks of ceftazidime susceptible were 8, 8 and 4 mg/L, respectively. For cefepime, the resistance breakpoint is 32 mg/L and the MIC distribution is shown in Figure 24. The peaks of cefepime resistance in *A. baumannii* and *A. nosocomialis* were 256 mg/L and 32 mg/L, respectively. The peaks of cefepime resistance in *A. pittii* were 128 and >256 mg/L. The peaks of cefepime susceptible in *A. baumannii*, *A. pittii* and *A. nosocomialis* were 4, 4 and 8 mg/L, respectively.

For ciprofloxacin, the resistance breakpoint is 4 mg/L and the MIC distribution is shown in Figure 25. The peaks of ciprofloxacin resistance in *A. baumannii* and *A. nosocomialis* were 64 mg/L. The peaks of ciprofloxacin resistance in *A. pittii* were 32 mg/L and 64 mg/L. The peaks of ciprofloxacin susceptible in *A. baumannii*, *A. pittii* and *A. nosocomialis* were 0.125 mg/L.

The resistance breakpoint of colistin is 4 mg/L and the MIC distribution is shown in Figure 26. No colistin-resistant *Acinetobacter* spp. was found in this study. The colistin MICs of the most *A. baumannii, A. pittii* and *A. nosocomialis* were 1 mg/L.

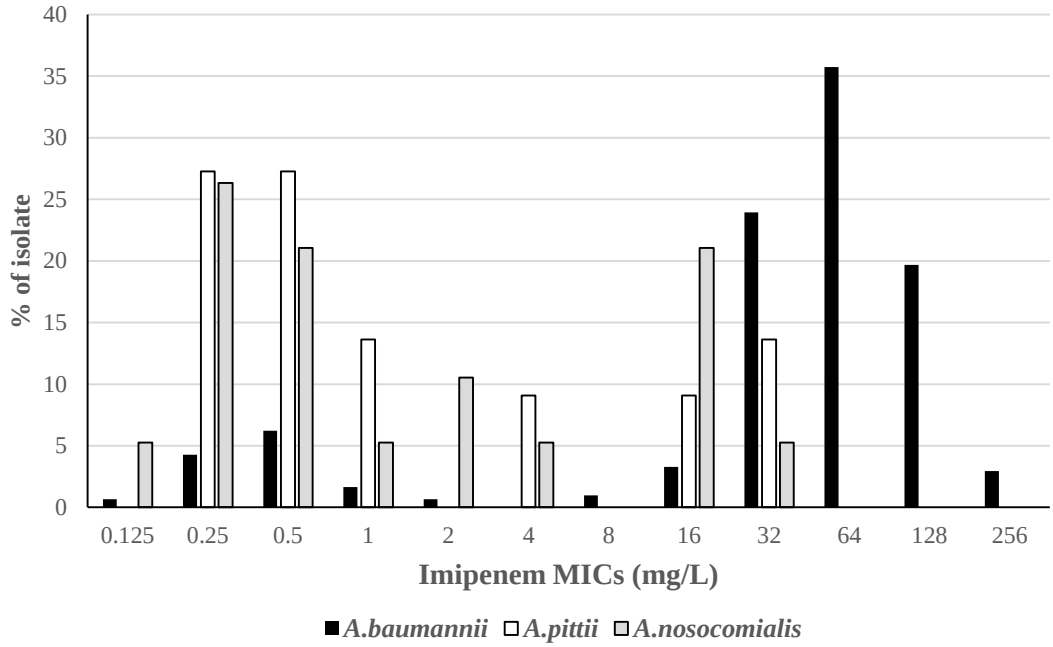


Figure 17. Distribution of imipenem MICs among the *Acinetobacter* spp. isolates

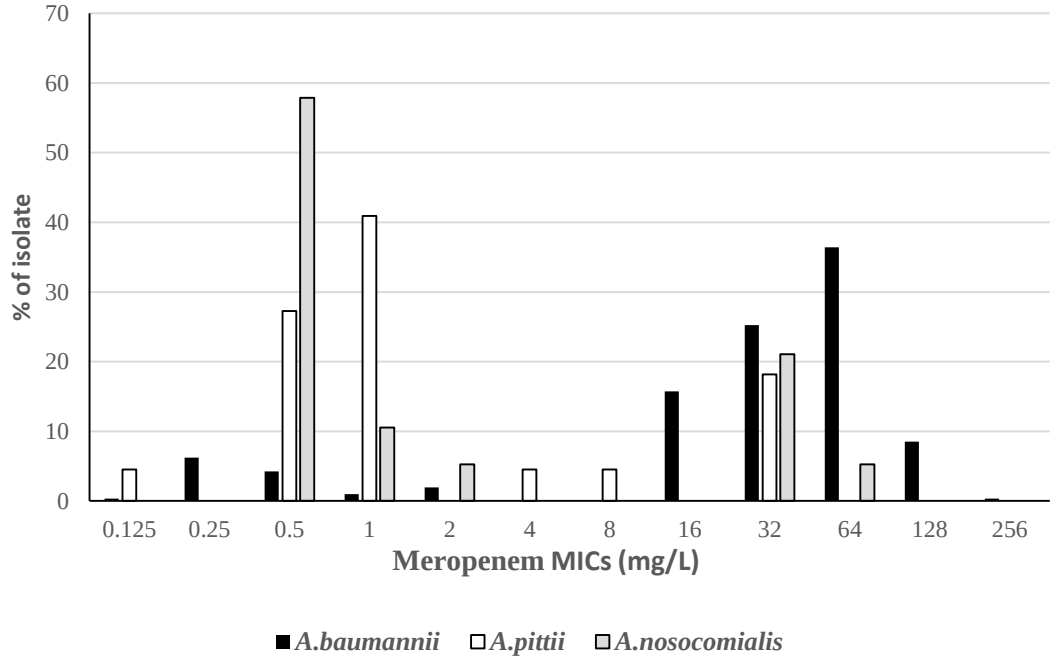


Figure 18. Distribution of meropenem MICs among the *Acinetobacter* spp. isolates

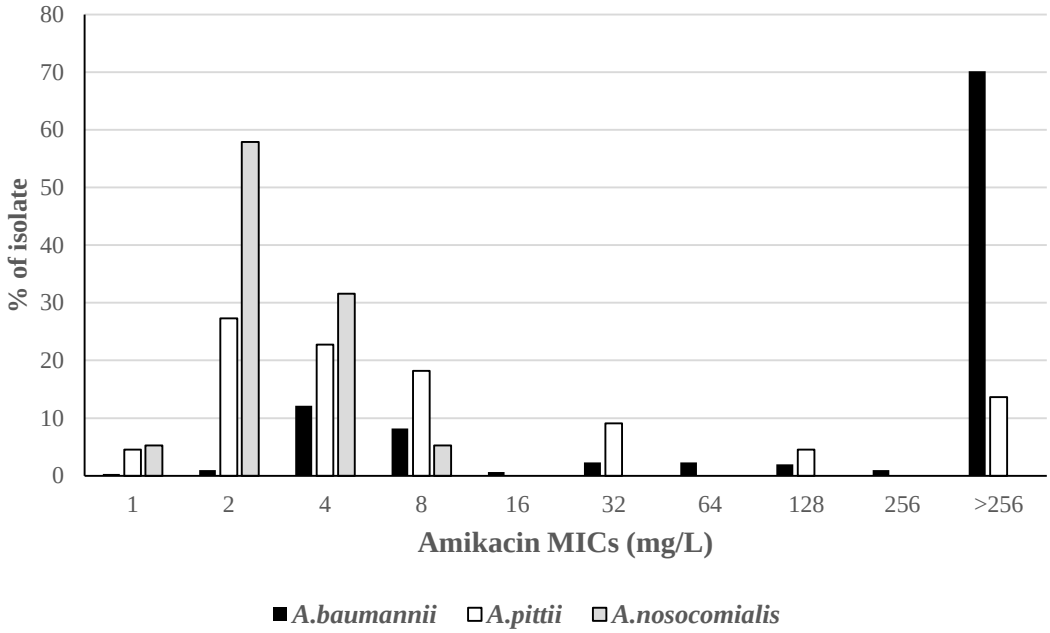


Figure 19. Distribution of amikacin MICs among the *Acinetobacter* spp. isolates

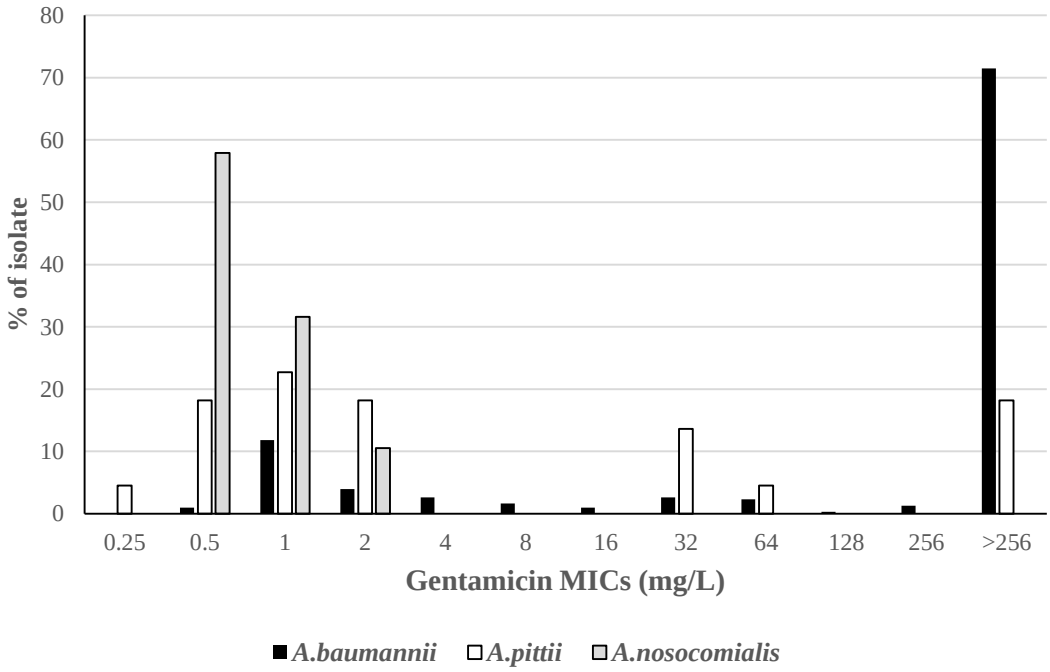


Figure 20. Distribution of gentamicin MICs among the *Acinetobacter* spp. isolates

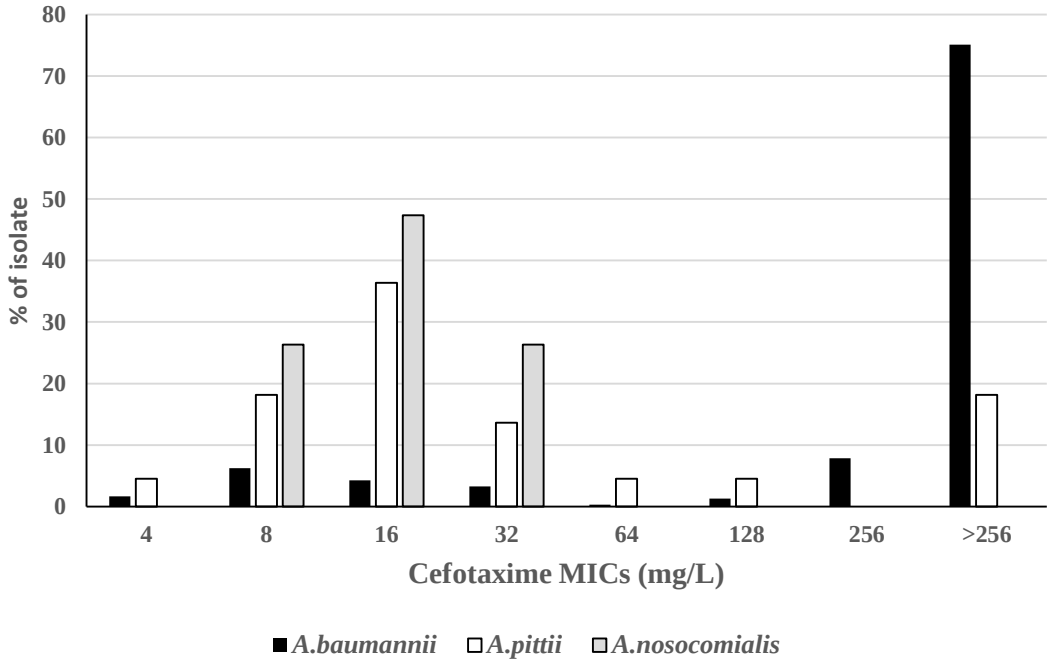


Figure 21. Distribution of cefotaxime MICs among the *Acinetobacter* isolates

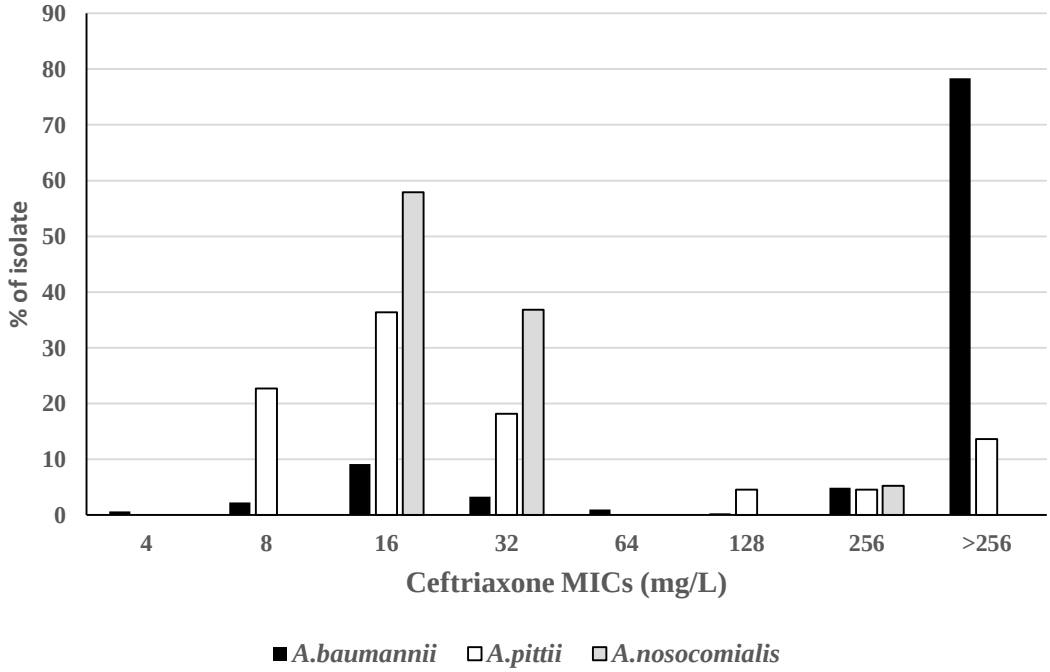


Figure 22. Distribution of ceftriaxone MICs among the *Acinetobacter* spp. isolates

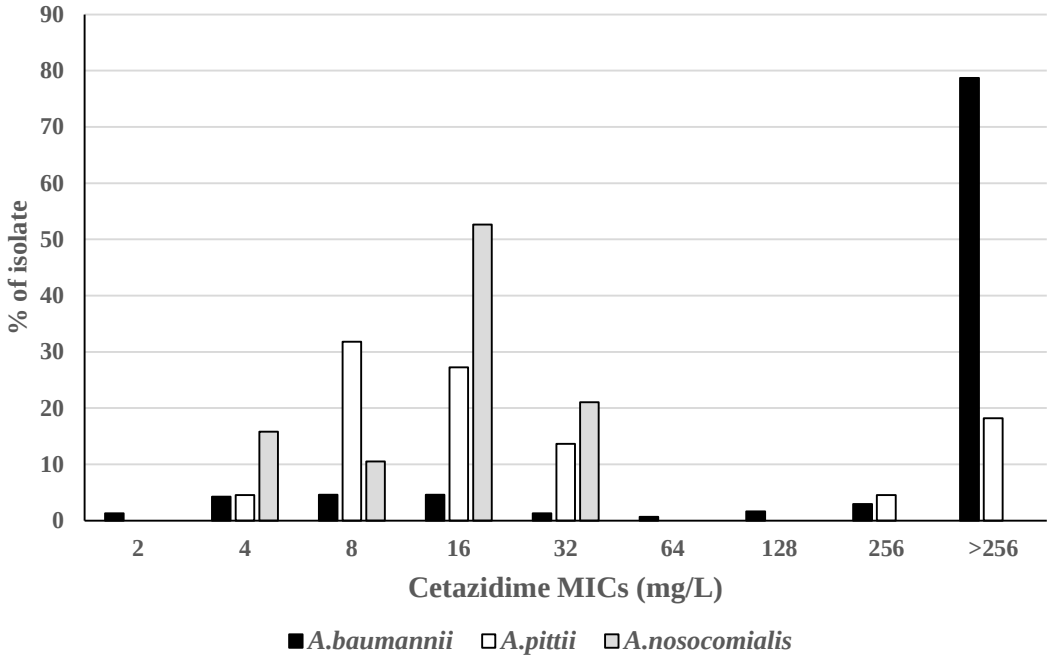


Figure 23. Distribution of ceftazidime MICs among the *Acinetobacter* spp. isolates

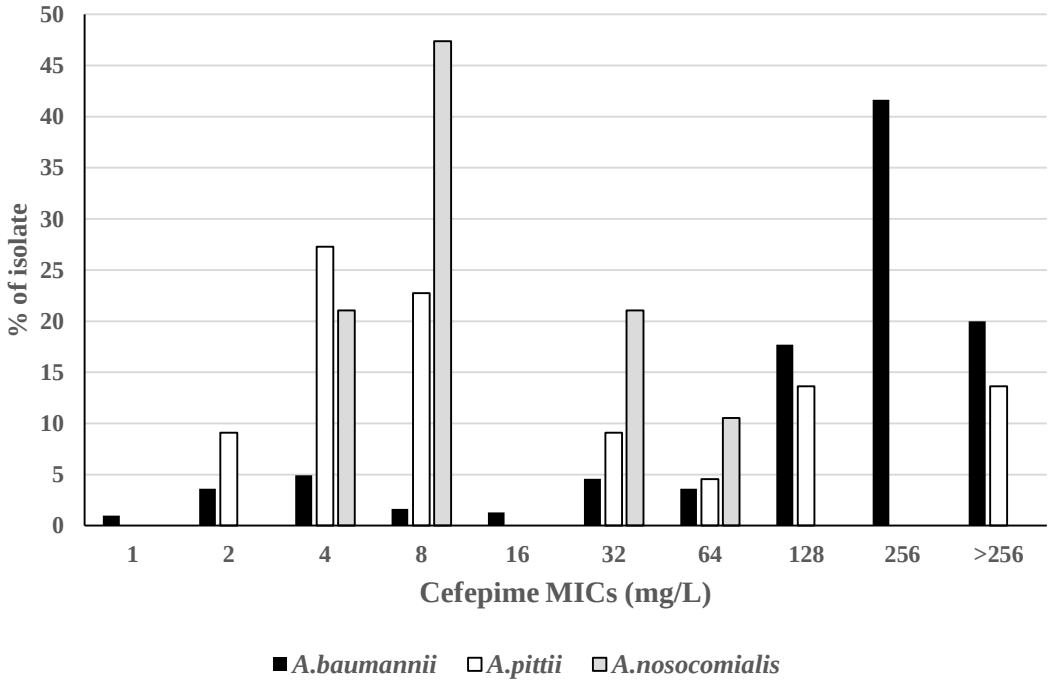


Figure 24. Distribution of cefepime MICs among the *Acinetobacter* spp. isolates

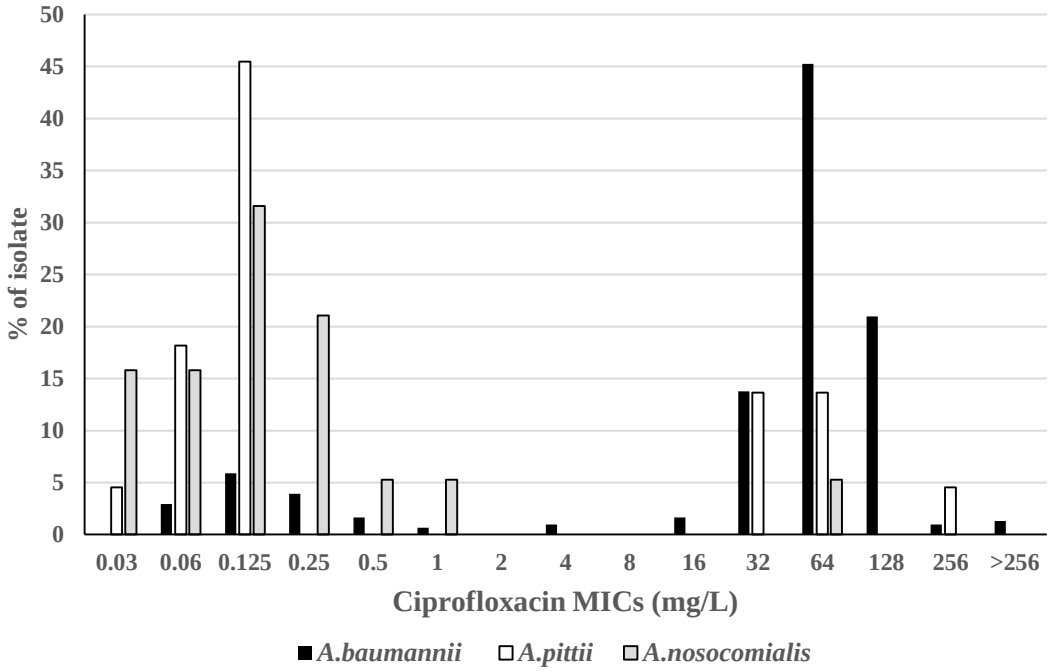


Figure 25. Distribution of ciprofloxacin MICs among the *Acinetobacter* spp. isolates

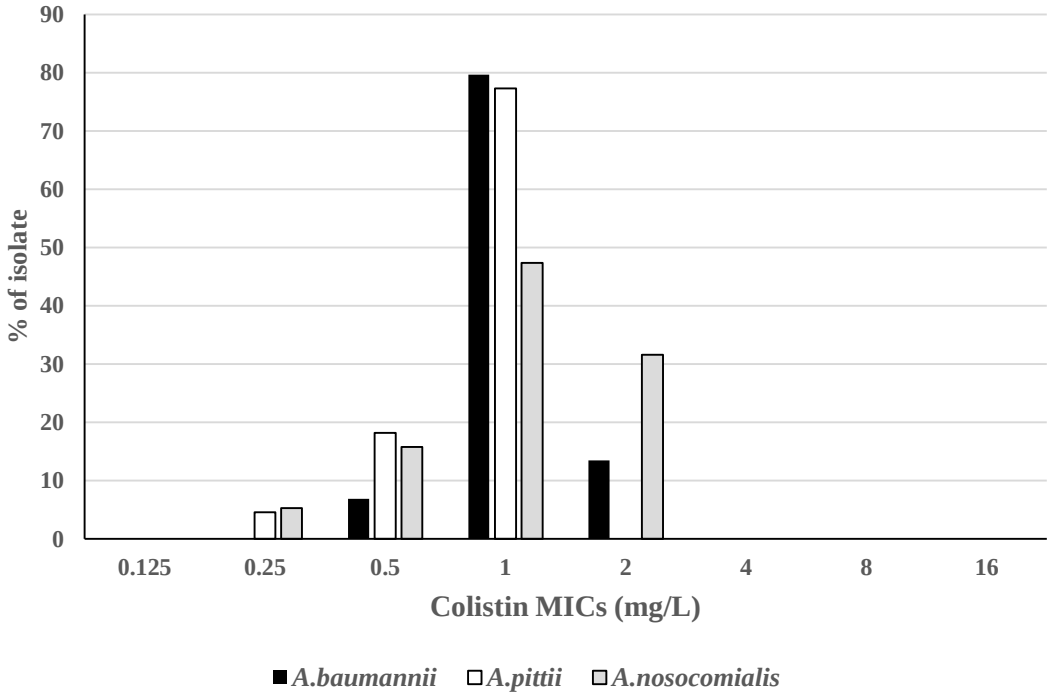


Figure 26. Distribution of colistin MICs among the *Acinetobacter* spp. isolates

4. DETECTION OF CARBAPENEMASE ACTIVITY

1. Detection of carbapenemase activity

The carbapenemase activity was detected by modified Hodge test. Of the 305 *A. baumannii*, 264 (86.6%) were positive for carbapenemase activity and imipenem and meropenem MICs ranges were 8 to 256 mg/L and 8 to 256 mg/L, respectively. Of 22 *A. pittii*, 5 (22.7%) were positive for carbapenemase activity and imipenem and meropenem MICs ranges were 16 to 32 mg/L and 8 to 32 mg/L, respectively. Of 19 *A. nosocomialis*, 5 (26.3%) were positive for carbapenemase activity and imipenem and meropenem MICs ranges were 16 to 32 mg/L and 32 to 64 mg/L, respectively (Figure 27-28).

The results demonstrated that all carbapenem-resistant *A. baumannii*, *A. pittii*, and *A. nosocomialis* had carbapenemase activities.

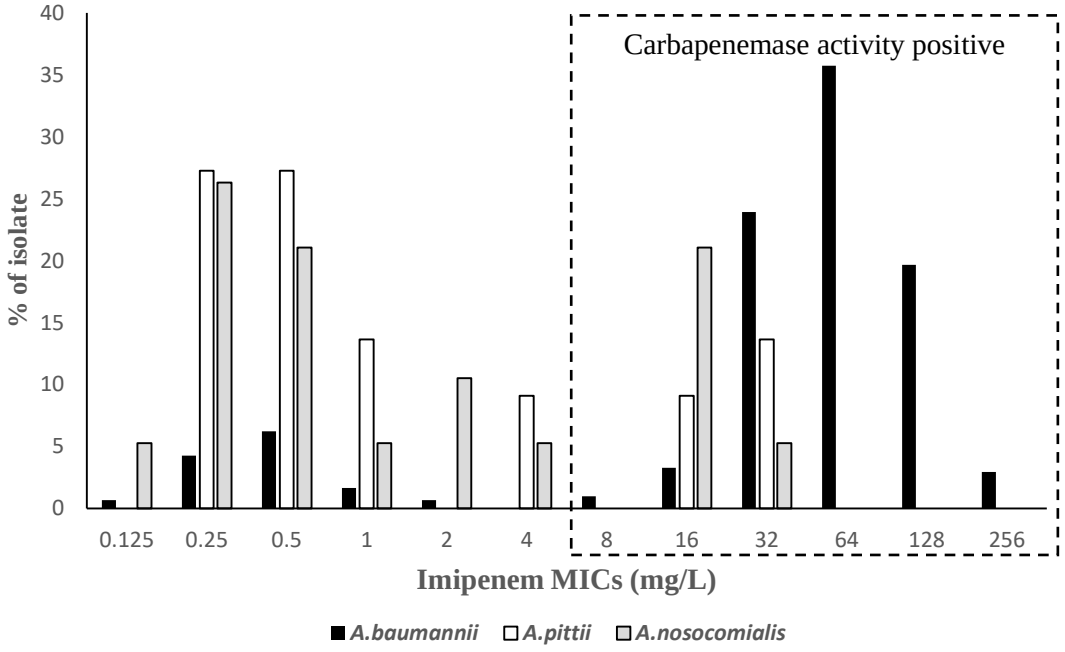


Figure 27. Distribution of imipenem MICs and carbapenemase activity in *Acinetobacter* spp.

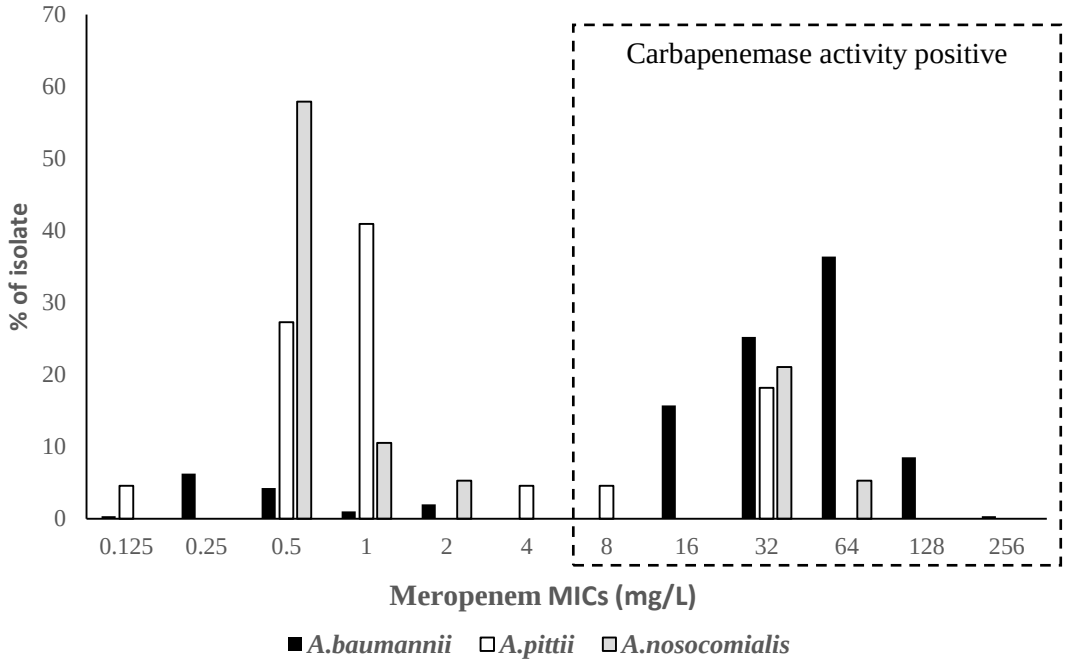


Figure 28. Distribution of meropenem MICs and carbapenemase activity in *Acinetobacter* spp.

2. Metallo-carbapenemase activity

The metallo-carbapenemase activity was detected by EDTA-disc synergy test. There was no metallo-carbapenemase activity in all *A. baumannii* and *A. nosocomialis* isolates. However, metallo-carbapenemase activity was found in one carbapenem-resistant *A. pittii* isolate (isolate AP1). Isolate AP1 showed 32 mg/L MIC of imipenem and meropenem and also had metallo-carbapenemase activity.

5. DETECTION OF CARBAPENEMASE GENES AND MRNA EXPRESSION

1. Detection of OXA-type carbapenemase genes

OXA-type carbapenemase genes including *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-143-like}}$ and *bla*$_{\text{OXA-235-like}}$ were detected by multiplex PCR and the results of all 346 *Acinetobacter* spp. are shown in Appendix D. The presence of OXA-type carbapenemase genes of all 305 *A. baumannii* isolates are shown in Table 16. All *A. baumannii* isolates carried intrinsic carbapenemase gene, *bla*$_{\text{OXA-51-like}}$. Forty-one (13.5%) isolates carried only *bla*$_{\text{OXA-51-like}}$. These 41 isolates did not have carbapenemase activities and were susceptible to imipenem and meropenem. Their imipenem and meropenem MIC ranges were 0.125 to 2 mg/L. Only carbapenemase activity-positive *A. baumannii* isolates had acquired OXA-type carbapenemase genes including *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-58-like}}$ and *bla*$_{\text{OXA-24-like}}$. The major carbapenemase genes in *A. baumannii* was *bla*$_{\text{OXA-23-like}}$. The presence of *bla*$_{\text{OXA-51-like}}$ with *bla*$_{\text{OXA-23-like}}$ was found in 262 *A. baumannii* isolates (85.9%). The MIC ranges of imipenem and meropenem were 8 to 256 mg/L and 16 to 256 mg/L, respectively. One isolate (0.3%) carried *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{OXA-24-like}}$ with 16 mg/L of imipenem and meropenem MICs. One isolate (0.3%) carried *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{OXA-58-like}}$ with 8 mg/L and 16 mg/L of imipenem and meropenem MICs, respectively. The *bla*$_{\text{OXA-143-like}}$ and *bla*$_{\text{OXA-235-like}}$ were not detected in all *A. baumannii* isolates.

Table 16. OXA-type carbapenemase genes in the 305 *A. baumannii* isolates

Carbapenemase activity	OXA-carbapenemase gene	No of isolates (%)	Imipenem MIC range (mg/L)	Meropenem MIC range (mg/L)
Negative	*bla*$_{\text{OXA-51-like}}$	41 (13.5)	0.125 - 2	0.125 - 2
Positive	*bla*$_{\text{OXA-51-like}}$ + *bla*$_{\text{OXA-23-like}}$	262 (85.9)	8 - 256	16 - 256
Positive	*bla*$_{\text{OXA-51-like}}$ + *bla*$_{\text{OXA-24-like}}$	1 (0.3)	16	16
Positive	*bla*$_{\text{OXA-51-like}}$ + *bla*$_{\text{OXA-58-like}}$	1 (0.3)	16	8

The presence of carbapenemase genes in all 22 *A. pittii* isolates are summarized in Table 17. The *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-143-like}}$ and *bla*$_{\text{OXA-235-like}}$ were not found in all *A. pittii* isolates. Among the 22 *A. pittii* isolates, 14 isolates (63.6%) with no carbapenemase activity did not carry any carbapenemase gene and were susceptible to carbapenems. Their imipenem and meropenem MIC ranges were 0.25 to 4 mg/L and 0.125 to 4 mg/L, respectively. However, 3 carbapenem-susceptible isolates (13.6%) carried *bla*$_{\text{OXA-58-like}}$. They were negative for carbapenemase activity and their imipenem and meropenem MIC ranges were 0.5 to 1 mg/L and 1 to 1 mg/L, respectively. The 2 isolates (9.2%) of carbapenem-resistant *A. pittii* with carbapenemase activity carried *bla*$_{\text{OXA-58-like}}$. Their imipenem and meropenem MIC ranges were 16 to 32 mg/L and 8 to 32 mg/L, respectively. Three carbapenem-resistant *A. pittii* (13.6%), positive for carbapenemase activity, harbored *bla*$_{\text{OXA-23-like}}$. Their MIC ranges of imipenem and meropenem were 16 to 32 mg/L and 32 to 32 mg/L, respectively.

Table 17. OXA-type carbapenemase genes in the 22 *A. pittii* isolates

Carbapenemase activity	OXA-carbapenemase gene	No of isolates (%)	Imipenem MIC range (mg/L)	Meropenem MIC range (mg/L)
Negative	No	14 (63.6)	0.25 - 4	0.125 - 4
Negative	*bla*$_{\text{OXA-58-like}}$	3 (13.6)	0.5 - 1	1 - 1
Positive	*bla*$_{\text{OXA-58-like}}$	2 (9.2)	16 - 32	8 - 32
Positive	*bla*$_{\text{OXA-23-like}}$	3 (13.6)	16 - 32	32 - 32

The presence of carbapenemase genes in all 19 *A. nosocomialis* isolates are summarized in Table 18. The *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-143-like}}$ and *bla*$_{\text{OXA-235-like}}$ were not found in all *A. nosocomialis* isolates. Fourteen isolates of *A. nosocomialis* (73.7%) with no carbapenemase activity did not carry any carbapenemase genes. They were susceptible to carbapenems with imipenem and meropenem MIC ranges of 0.125 to 4 mg/L and 0.5 to 2 mg/L, respectively. All 5 carbapenem-resistant *A. nosocomialis* positive for carbapenemase activity carried *bla*$_{\text{OXA-23-like}}$. Their imipenem and meropenem MIC ranges were 16 to 32 mg/L and 32 to 64 mg/L, respectively.

Table 18. OXA-type carbapenemase genes in the 19 *A. nosocomialis* isolates

Carbapenemase activity	OXA-carbapenemase gene	No of isolates (%)	Imipenem MIC range (mg/L)	Meropenem MIC range (mg/L)
Negative	No	14 (73.7)	0.125 - 4	0.5 - 2
Positive	*bla*$_{\text{OXA-23-like}}$	5 (26.7)	16 - 32	32 - 64

2. Detection of metallo-carbapenemase genes

No metallo-carbapenemase genes including *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{GIM}}$, *bla*$_{\text{SIM}}$, and *bla*$_{\text{SPM}}$ were found in any *A. baumannii* and *A. nosocomialis* isolates. Among the 22 *A. pittii*, one isolate, AP1 carried *bla*$_{\text{IMP-like}}$ gene. Isolate AP1 was positive for metallo-carbapenemase activity. The isolate AP1 co-harbored *bla*$_{\text{IMP-like}}$ and *bla*$_{\text{OXA-58-like}}$ and its imipenem and meropenem MICs were 32 mg/L (Table 19).

3. Detection of *bla*$_{\text{KPC-like}}$, *bla*$_{\text{OXA-48-like}}$ and *bla*$_{\text{NDM-like}}$ genes

The *bla*$_{\text{KPC-like}}$, *bla*$_{\text{OXA-48-like}}$ and *bla*$_{\text{NDM-like}}$ genes were detected by multiplex PCR. No *bla*$_{\text{KPC-like}}$, *bla*$_{\text{OXA-48-like}}$ and *bla*$_{\text{NDM-like}}$ genes were found in any *A. baumannii*, *A. pittii* and *A. nosocomialis* isolates.

4. Amplification and DNA sequencing of entire carbapenemase genes

Sixteen *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis* isolates that carried different carbapenemase genes and exhibited different levels of resistance to imipenem and meropenem were selected as representative isolates for entire carbapenemase gene study. The entire intrinsic carbapenemase gene (*bla*$_{\text{OXA-51}}$) and acquired carbapenemase genes (*bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-24}}$ and *bla*$_{\text{IMP}}$) were amplified, sequenced and analyzed. The results are shown in Table 19. All 16 representative *A. baumannii* isolates carried *bla*$_{\text{OXA-51-like}}$. The major intrinsic genes were *bla*$_{\text{OXA-66}}$ (56.3%, 9/16) and *bla*$_{\text{OXA-67}}$ (25.0%, 4/16). Another 3 isolates carried *bla*$_{\text{OXA-70}}$ (AB3), *bla*$_{\text{OXA-68}}$ (AB227) or *bla*$_{\text{OXA-95}}$ (AB250). All 14 representative *A. baumannii* that were positive for partial *bla*$_{\text{OXA-23-like}}$, carried *bla*$_{\text{OXA-23}}$. Isolate AB227 and isolate AB250 carried *bla*$_{\text{OXA-58}}$ and *bla*$_{\text{OXA-24,}}$ respectively.

Of the 8 representative *A. pittii*, one isolate (AP1) carried two acquired carbapenemase genes including *bla*$_{\text{IMP-14a}}$ and *bla*$_{\text{OXA-58}}$. Four isolates carried *bla*$_{\text{OXA-58}}$

and 3 isolates carried *bla*$_{\text{OXA-23}}$ gene. All 5 representative *A. nosocomialis* carried acquired *bla*$_{\text{OXA-23}}$.

Table 19. Sequence analysis of intrinsic and acquired carbapenemase genes in *Acinetobacter* spp.

Isolate	MIC (mg/L)		Intrinsic carbapenemase gene		Acquired carbapenemase gene	
	IPM	MEM	Partial	Entire	Partial	Entire
AB1	32	32	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB2	64	64	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB3	16	32	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-70}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB4	64	64	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB5	64	128	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB6	32	32	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB9	16	32	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB29	32	64	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-67}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB58	32	64	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-67}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB97	32	64	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-67}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB227	16	8	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-68}}$	*bla*$_{\text{OXA-58-like}}$	*bla*$_{\text{OXA-58}}$
AB250	16	16	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-95}}$	*bla*$_{\text{OXA-24-like}}$	*bla*$_{\text{OXA-24}}$
AB13	32	64	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-67}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB35	32	64	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB55	32	64	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AB354	64	128	*bla*$_{\text{OXA-51-like}}$	*bla*$_{\text{OXA-66}}$	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AP1	32	32	-	-	*bla*$_{\text{OXA-58-like}}$ *bla*$_{\text{IMP-like}}$	*bla*$_{\text{OXA-58}}$ *bla*$_{\text{IMP-14a}}$
AP4	32	32	-	-	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AP7	0.5	1	-	-	*bla*$_{\text{OXA-58-like}}$	*bla*$_{\text{OXA-58}}$
AP8	0.5	1	-	-	*bla*$_{\text{OXA-58-like}}$	*bla*$_{\text{OXA-58}}$
AP14	16	8	-	-	*bla*$_{\text{OXA-58-like}}$	*bla*$_{\text{OXA-58}}$
AP16	32	32	-	-	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AP17	1	1	-	-	*bla*$_{\text{OXA-58-like}}$	*bla*$_{\text{OXA-58}}$
AP23	16	32	-	-	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AN1	16	32	-	-	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AN4	16	32	-	-	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AN12	32	64	-	-	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AN15	16	32	-	-	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$
AN20	16	32	-	-	*bla*$_{\text{OXA-23-like}}$	*bla*$_{\text{OXA-23}}$

AB, *A. baumannii*; AP, *A. pittii*; AN, *A. nosocomialis*; -, Not found

In addition, the upstream and downstream elements of carbapenemase genes were analyzed by PCR mapping using primer for IS*Aba* as shown in Table 3. All OXA-23-producing *Acinetobacter* spp. (262 *A. baumannii*, 3 *A. pittii* and 5 *A. nosocomialis* isolates) contained IS*Aba1* upstream and downstream of $bla_{\text{OXA-23-like}}$. Two *A. baumannii* and 5 *A. pittii* carrying $bla_{\text{OXA-58}}$ had IS*Aba3* upstream and downstream of $bla_{\text{OXA-58.}}$ No insertion sequences (IS*Aba1*, IS*Aba2*, IS*Aba3* and IS*Aba4*) were found upstream or downstream of $bla_{\text{OXA-24}}$ in both *A. baumannii* isolates.

6. DETERMINATION OF LOSS OR REDUCED OUTER MEMBRANE PROTEINS

The outer membrane proteins of all representative isolates (23 *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis* isolates) were extracted by using 2% sodium *N*-lauroylsarcosine and ultracentrifugation. The OMP profiles of all representative *Acinetobacter* spp. were studied by SDS-PAGE. The results of OMP profiles by SDS-PAGE are shown in Appendix E. The relative intensities of CarO, 33-36 kDa OMP and OprD bands in SDS-PAGE were calculated and compared with *A. baumannii* ATCC 19606, which is susceptible to carbapenems and these results are shown in Table 20-21.

The relative intensities of OMPs of *A. baumannii* are shown in Table 20. In this study, the reduced OMPs were defined as relative intensity that was lower than 0.7 (p<0.01). Of all 23 *A. baumannii*, reduced OMPs was found in 10 isolates (43.5%). Among those 10 isolates, 5 isolates showed reduced expression of 1 OMP, 3 isolates showed reduced expression of 2 OMPs and 2 isolates showed reduced expression of 3 OMPs.

The results of OMP expressions and carbapenem resistance in *A. baumannii* are summarized in Table 22. Among the 19 isolates carrying $bla_{\text{OXA-23-like}}$, 12 isolates (63.2%) had no reduced OMP expression. Their imipenem and meropenem MIC ranges were 16 to 64 mg/L and 32 to 128 mg/L. One isolate carrying $bla_{\text{OXA-23-like}}$ (AB1) had reduced CarO and its imipenem and meropenem MICs were 32 mg/L. Three isolates carrying $bla_{\text{OXA-23-like}}$ (AB2, AB9 and AB13) had reduced 33-36 kDa OMP and their imipenem and meropenem MIC ranges were 16 to 64 mg/L and 32 to 64 mg/L. Isolate AB97 carrying $bla_{\text{OXA-23-like}}$ with reduced CarO and 33-36 kDa OMP had the similar level of carbapenem MICs (32 mg/L and 64 mg/L for imipenem and meropenem) to isolate AB29 with reduced 33-36 kDa OMP and OprD and isolate AB58 with reduced CarO, 33-36 kDa OMP and OprD.

In $bla_{\text{OXA-58-}}$like-carrying *A. baumannii*, isolate A6 had no reduction of OMP. Its imipenem and meropenem MICs were 64 mg/L. In contrast, isolate AB227 with reduced 33-36 kDa OMP had lower level of carbapenem MICs (16 mg/L and 8 mg/L for imipenem and meropenem, respectively).

Isolate A10 carrying *bla*$_{\text{OXA-24-like}}$ showed reduced 33-36 kDa OMP and OprD and its imipenem and meropenem MICs were 128 mg/L and 256 mg/L, respectively. In contrast, isolate AB250 showed reduction of all 3 OMPs but showed lower imipenem and meropenem MICs (16 mg/L).

Table 20. Relative intensities of OMPs in the 23 representative *A. baumannii*

Isolate	MIC (mg/L)		Carbapenemase	Relative intensity of OMPs		
	IPM	MEM		CarO	33-36 kDa OMP	OprD
AB1	32	32	OXA-23	**0.56**	1.23	1.15
AB2	64	64	OXA-23	1.35	**0.43**	0.73
AB3	16	32	OXA-23	1.78	0.75	0.95
AB4	64	64	OXA-23	2.03	0.75	0.83
AB5	64	128	OXA-23	3.20	1.19	0.93
AB6	32	32	OXA-23	3.72	2.08	1.28
AB9	16	32	OXA-23	1.61	**0.61**	0.75
AB29	32	64	OXA-23	1.37	**0.46**	**0.57**
AB58	32	64	OXA-23	**0.14**	**0.29**	**0.51**
AB97	32	64	OXA-23	**0.30**	**0.51**	1.04
AB227	16	8	OXA-58	0.90	**0.33**	0.94
AB250	16	16	OXA-24	**0.18**	**0.32**	**0.43**
AB13	32	64	OXA-23	1.33	**0.61**	0.95
AB35	32	64	OXA-23	2.14	1.15	1.66
AB55	32	64	OXA-23	1.80	1.28	1.75
AB354	64	128	OXA-23	2.05	0.87	1.18
A4	64	64	OXA-23	2.24	1.24	1.70
A5	32	64	OXA-23	1.66	1.01	1.64
A6	64	64	OXA-58	1.00	1.00	0.79
A7	64	64	OXA-23	3.44	2.29	1.95
A8	64	128	OXA-23	1.54	0.93	2.38
A9	64	128	OXA-23	1.05	1.45	3.22
A10	128	256	OXA-24	0.80	**0.55**	**0.43**

Table 21. Relative intensities of OMPs in the 8 *A. pittii* and 5 *A. nosocomialis* representative isolates

Isolate	MIC (mg/L)		Carbapenemase	Relative intensity of OMPs		
	IPM	MEM		CarO	33-36 kDa OMP	OprD
AP1	32	32	IMP14a, OXA-58	1.00	1.17	1.13
AP4	32	32	OXA-23	1.03	1.17	1.18
AP7	0.5	1	OXA-58	**0.58**	0.72	0.72
AP8	0.5	1	OXA-58	0.71	0.95	0.87
AP14	16	8	OXA-58	**0.33**	**0.49**	**0.56**
AP16	32	32	OXA-23	**0.52**	**0.50**	0.81
AP17	1	1	OXA-58	**0.56**	**0.60**	**0.57**
AP23	16	32	OXA-23	0.78	1.11	0.82
AN1	16	32	OXA-23	1.06	1.11	0.92
AN4	16	32	OXA-23	1.07	1.08	0.86
AN12	32	64	OXA-23	0.97	0.95	0.82
AN15	16	32	OXA-23	0.98	0.98	1.19
AN20	16	32	OXA-23	1.23	1.35	1.92

The relative intensities of OMPs of *A. pittii* are shown in Table 21. Among the 8 representative *A. pittii*, the reduction of OMPs was found in 4 isolates (50%). One isolate (AP7) had reduced CarO. Another isolate (AP16) showed reduced CarO and 33-36 kDa OMP. Two isolates (AP14 and AP17) showed reduction of all 3 OMPs including CarO, 33-36 kDa OMP and OprD.

The results of reduction of OMPs and carbapenem resistance in *A. pittii* are summarized in Table 22. Among 3 *A. pittii* carrying $bla_{\text{OXA-23-like}}$, 2 isolates (AP4 and AP23) had no reduction of OMP and their imipenem and meropenem MIC ranges were 16 to 32 mg/L and 32 to 32 mg/L, respectively. AP16 with reduced CarO and 33-36 kDa OMP showed the same level of carbapenem resistance.

Isolate AP1, co-harboring $bla_{\text{OXA-58-like}}$ and $bla_{\text{IMP-like}}$, with no reduction of OMP showed the same level carbapenem resistance to $bla_{\text{OXA-23-like}}$-carrying isolates with and without reduced OMPs (AP16, AP4 and AP23). Isolate AP8 that carrying $bla_{\text{OXA-58-like}}$, with no reduced OMP was susceptible to carbapenems. This was similar to AP7 that carried $bla_{\text{OXA-58-like}}$ and had reduced CarO. AP7 was susceptible to carbapenem. Another couple isolates which carried $bla_{\text{OXA-58-like}}$ and had reduction of 3 OMPs

showed different carbapenem susceptibilities. Isolate AP14 was resistant while isolate AP17 was susceptible to carbapenems.

The relative intensities of OMPs of *A. nosocomialis* are shown in Table 21. Among the 5 carbapenem-resistant *A. nosocomialis*, no isolate showed the reduction of OMPs.

Table 22. Reduced OMPs and carbapenem resistance in *A. baumannii*

No. of isolate	Reduced OMP	Carbapenemase	MIC range (mg/L)	
			IPM	MEM
12	NF	OXA-23	16 - 64	32 - 128
1	CarO	OXA-23	32	32
3	33-36 kDa	OXA-23	16 - 64	32 - 64
1	CarO and 33-36 kDa	OXA-23	32	64
1	33-36 kDa and OprD	OXA-23	32	64
1	all 3 OMPs	OXA-23	32	64
1	NF	OXA-58	64	64
1	33-36 kDa	OXA-58	16	8
1	33-36 kDa and OprD	OXA-24	128	256
1	all 3 OMPs	OXA-24	16	16

NF, Not found

Table 23. Reduced OMPs and carbapenem resistance in *A. pittii*

No of isolate	Reduced OMP	Carbapenemase	MIC range (mg/L)	
			IPM	MEM
2	NF	OXA-23	16 - 32	32
1	CarO and 33-36 kDa	OXA-23	32	32
1	NF	OXA-58	0.5	1
1	NF	OXA-58, IMP14a	32	32
1	CarO	OXA-58	0.5	1
2	all 3 OMPs	OXA-58	1 - 16	1 - 8

NF, Not found

In addition, the entire *carO*, 33-36 kDa OMP gene and *oprD* gene of all 23, 8 and 5 representative *A. baumannii*, *A. pittii* and *A. nosocomialis* were amplified and sequenced. No mutation of *carO*, 33-36 kDa OMP gene and *oprD* nucleotide sequences in all isolates except *A. baumannii* isolate AB58 and AB97.

Normally, the expected PCR product of primer carO-F and carO-R was 678 bp but the PCR products from both of them were about 1700 bp. After entire *carO* amplification and sequencing, the results revealed that *carO* gene was interrupted by

novel insertion sequence. This insertion sequence was submitted to ISFinder database (https://www-is.biotoul.fr/) and named IS*Aba40*. IS*Aba40* was 1039 bp long, containing 933-bp open reading frame (ORF) encoding a putative transposase and was bound by 16-bp inverted repeat sequences (GGCTTTGTTGCACAAA) with a 9-bp duplication site (GGTAAAAAC) (Figure 29). The ORF of IS*Aba40* showed 96% amino acid similarity to IS*Aha1*, an insertion sequence found in *A. haemolyticus*. This was the first report of interrupted *carO* by novel insertion sequence, IS*Aba40*. The nucleotide sequences of IS*Aba40* interrupted *carO* in isolate AB58 and 97 were submitted to the GenBank database under accession number KX987115 and KX987116, respectively.

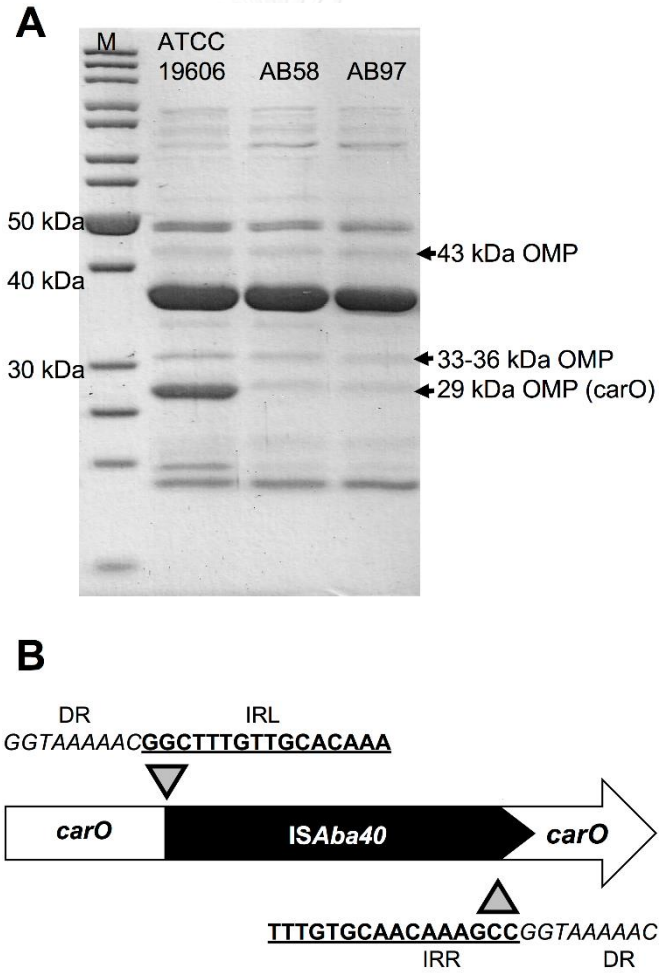


Figure 29. OMP profiles and genetic scheme of disrupted *carO* by IS*Aba40* in *A. baumannii* isolate AB58 or AB97

7. DETERMINATION OF EFFLUX PUMP OVEREXPRESSION

1. Detection of efflux pump activity

The overexpression of efflux pump activity in *Acinetobacter* spp. was detected by using efflux pump inhibitors, CCCP and reserpine. The overexpression is defined as the reduction of carbapenem MICs at least 4-fold in the presence of inhibitors. All 271 carbapenem-resistant *A. baumannii*, 8 *A. pittii* carrying carbapenemase genes and 5 carbapenem-resistant *A. nosocomialis* were determined for overexpression of efflux pump. The carbapenem MICs of all tested *Acinetobacter* spp. isolates in the presence and absence of inhibitors are shown in Appendix D.

The effects of inhibitors on carbapenem MIC among *Acinetobacter* spp. are summarized in Table 23. Most of *A. baumannii* isolates did not change the imipenem MICs in the presence of CCCP (88.9%, 241/271) and reserpine (89.3%, 242/271). The imipenem MICs were 2-fold reduced in 30 isolates (11.1%) and 29 isolates (10.7%) of *A. baumannii* in the presence of CCCP and reserpine, respectively. The meropenem MICs did not change in 257 *A. baumannii* isolates (94.3%) in the presence of CCCP and reserpine, whereas the reduction of 2-fold meropenem MIC were found in 14 *A. baumannii* isolates (5.7%) in the presence of CCCP and reserpine.

The imipenem MICs of all 8 *A. pittii* isolates did not change in the presence of CCCP. The imipenem MICs of most *A. pittii* isolates did not change in the presence of reserpine, whereas one isolate showed 2-fold reduction of the MIC. All *A. pittii* isolates showed no change of meropenem MICs in the presence of CCCP and reserpine.

In *A. nosocomialis*, all 5 isolates showed no change of imipenem and meropenem MIC in the presence of CCCP and reserpine.

In this study, overexpression of efflux pumps was not detected by using efflux pump inhibitors in all carbapenem-resistant *Acinetobacter* spp. isolates. The expression of mRNA of efflux pump genes were evaluated in next experiment.

Table 24. Efflux pump activities in *Acinetobacter* spp.

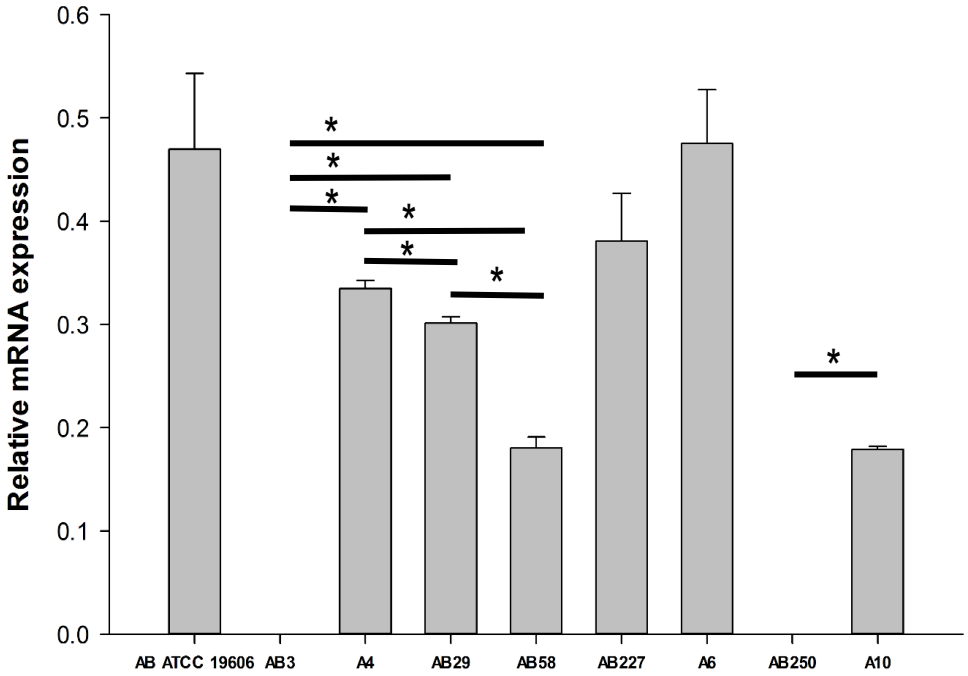
Species	IPM and CCCP				IPM and reserpine				MEM and CCCP				MEM and reserpine			
	Fold of MIC decrease	No. of isolate (%)	MIC ragne (mg/L)		Fold of MIC decrease	No. of isolate (%)	MIC ragne (mg/L)		Fold of MIC decrease	No. of isolate (%)	MIC range (mg/L)		Fold of MIC decrease	No. of isolate (%)	MIC range (mg/L)	
			IPM	IPM + CCCP			IPM	IPM + reserpine			MEM	MEM + CCCP			MEM	MEM + reserpine
A.baumannii	2	30 (11.1)	32-256	16-128	2	29 (10.7)	32-256	16-128	2	14 (5.7)	16-128	8-64	2	14 (5.7)	16-128	8-64
	-	241 (88.9)	16-256	16-256	-	242 (89.3)	16-256	16-256	-	257 (94.3)	8-256	8-256	-	257 (94.3)	8-256	8-256
A.pitii	2	0	-	-	2	1 (12.5)	32	16	2	0	-	-	2	0	-	-
	-	8 (100)	0.5-32	0.5-32	-	7 (87.5)	0.5-32	0.5-32	-	8 (100)	0.5-32	0.5-32	-	8 (100)	1-32	1-32
A.nosocomialis	2	0	-	-	2	0	-	-	2	0	-	-	2	1 (16.7)	64	32
	-	5 (100)	16-32	16-23	-	5 (100)	16-32	16-23	-	5 (100)	16-32	16-23	-	4 (83.3)	32-32	32-32

2. Detection of efflux pump mRNA expression

The carbapenem efflux pumps that have been reported in *A. baumannii* were AdeABC. The mRNA expression level of efflux pump genes including *adeB* in *A. baumannii*, *adeE* and *adeY* in *A. pittii* were evaluated by RT-PCR. The results are shown in Figures 30, 34 and 37. The *adeE* and *adeY* efflux pump genes were not found in *A. nosocomialis* isolate in this study. Therefore, this experiment was evaluated in *A. baumannii* and *A. pittii*.

Of the 8 carbapenem-resistant *A. baumannii*, there was no isolate that showed overexpression of *adeB* when compared with that of *A. baumannii* ATCC 19606. The $bla_{\text{OXA-23}}$-carrying *A. baumannii* without both reduced OMPs and overexpression of *adeB* (AB3) showed carbapenem resistance phenotype with carbapenem MICs of 16-32 mg/L. The overexpression of *adeB* (when compared with AB3) was observed in A4. High level of carbapenem MICs (64 mg/L) was found. However, the $bla_{\text{OXA-23}}$ carrying isolates with reduced OMPs (AB29 and AB58) had only 2-fold increase in imipenem MIC (32 mg/L). AB29 and AB58 isolates showed the same level of carbapenem resistance (both carbapenem MICs and $bla_{\text{OXA-23}}$ mRNA expression as shown in Figure 31. AB29 had higher efflux pump expression and AB58 had reduced CarO expression. These results indicate that OXA-23 can confer carbapenem resistance and reduced OMPs and overexpression of efflux pump lead to increase carbapenem MICs in *A. baumannii*.

Of the 2 $bla_{\text{OXA-58}}$-carrying *A. baumannii*, AB227 had lower carbapenem MICs than that of A6, even though AB227 showed higher $bla_{\text{OXA-58}}$ expression level than that of A6 (Figure 32). Efflux pump expression in A6 was higher than AB227, whereas AB227 also had reduced 33-36 kDa OMP. These results suggest that co-expression of OXA-58 and efflux pump rather than reduced OMP are involved in high level carbapenem resistance in *A. baumannii*.



IPM MIC (mg/L)	**0.5**	**16**	**64**	**32**	**32**	**16**	**64**	**16**	**128**
MEM MIC (mg/L)	**2**	**32**	**64**	**64**	**64**	**8**	**64**	**16**	**256**
Carbapenemase	-	**OXA 23**	**OXA 23**	**OXA 23**	**OXA 23**	**OXA 58**	**OXA 58**	**OXA 24**	**OXA 24**
Reduced OMPs (kDa OMPs)	-	-	-	**33-36, 43**	**29, 33-36, 43**	**33-36**	-	**29, 33-36, 43**	**33-36, 43**

Figure 30. Expression of *adeB* efflux pump mRNA in *A. baumannii*

**: $p < 0.01$ (statistical analysis by using ANOVA)

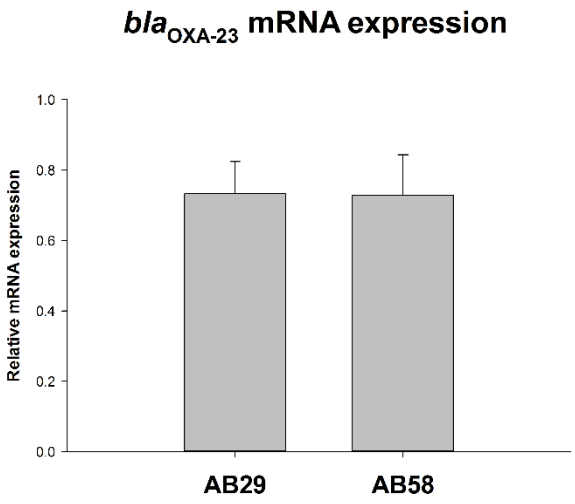


Figure 31. Expression of $bla_{\text{OXA-23}}$ in *A. baumannii* isolate AB29 and AB58

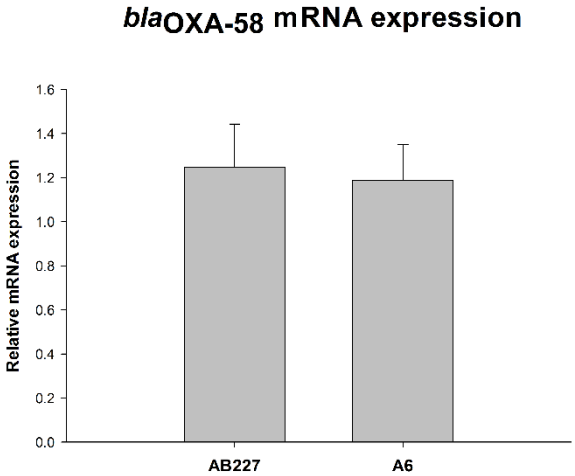


Figure 32. Expression of *bla*$_{OXA-58}$ in *A. baumannii* isolate AB227 and A6

In *bla*$_{OXA-24}$-carrying *A. baumannii*, isolate A10 and AB250 had similar levels of *bla*$_{OXA-24}$ expression (Figure 33). While isolate AB250 had reduction of all 3 OMPs, A10 had 2 reduced OMP expression. However, A10 had higher carbapenem MICs (128 – 256 mg/L) than those of A250 (MICs = 16 mg/L). The isolate A10 had *adeB* expression level higher than that of AB250 (Figure 30). These results suggest that co-expression of OXA-24 and efflux pump rather than reduced OMPs are associated with high level carbapenem resistance in *A. baumannii*.

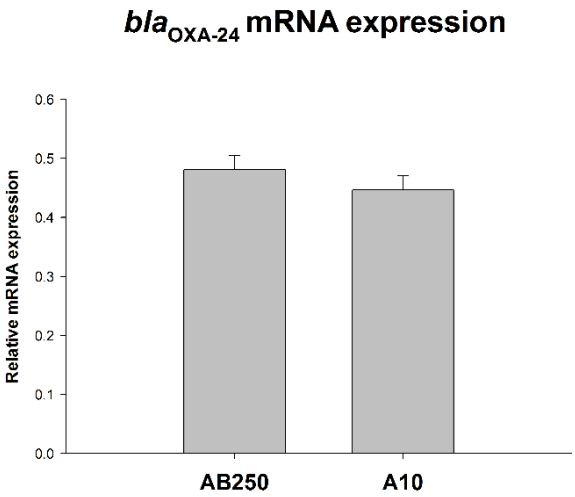
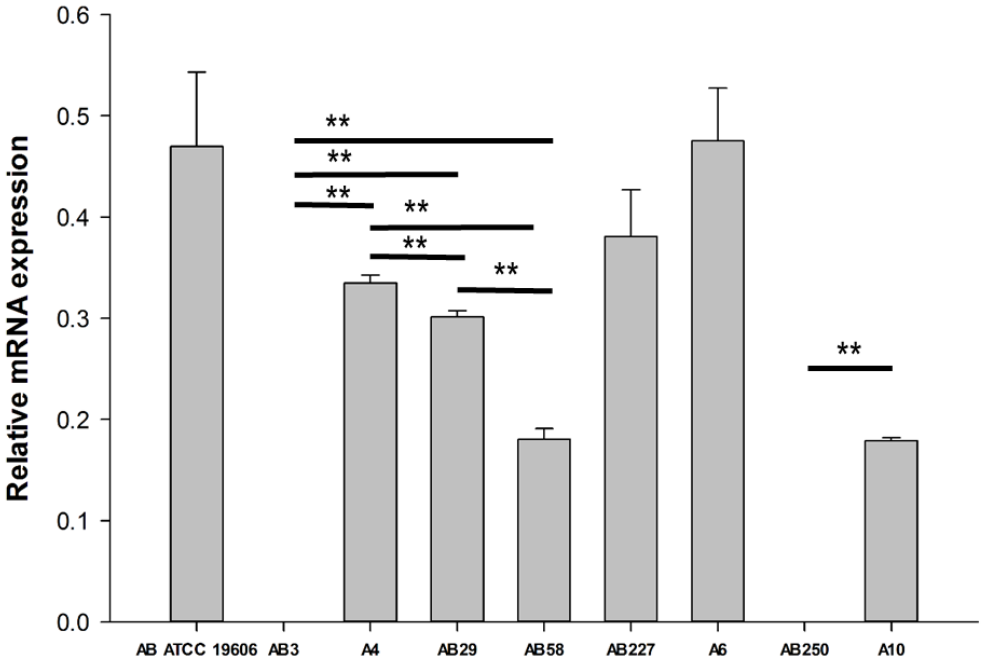


Figure 33. Expression of *bla*$_{OXA-24}$ in *A. baumannii* isolate AB250 and A10



IPM MIC (mg/L)	**0.5**	**16**	**64**	**32**	**32**	**16**	**64**	**16**	**128**
MEM MIC (mg/L)	**2**	**32**	**64**	**64**	**64**	**8**	**64**	**16**	**256**
Carbapenemase	-	**OXA 23**	**OXA 23**	**OXA 23**	**OXA 23**	**OXA 58**	**OXA 58**	**OXA 24**	**OXA 24**
Reduced OMPs (kDa OMPs)	-	-	-	**33-36, 43**	**29, 33-36, 43**	**33-36**	-	**29, 33-36, 43**	**33-36, 43**

Figure 34. Expression of *adeE* efflux pump mRNA in *A. pittii*

**: $p < 0.01$ (statistical analysis by using ANOVA)

Overexpression of *adeE* was found in 3 carbapenem-resistant *A. pittii* (AP14, AP1 and AP16) when compared with carbapenem-susceptible AP3 (Figure 34). Although *A. pittii* isolate AP7 had the reduction of CarO, it showed the lowest imipenem MIC (0.5 mg/L). This isolate had the lowest $bla_{\text{OXA-58}}$ mRNA expression (Figure 35). Surprisingly, AP17 had low imipenem MIC (1 mg/L), although it showed the highest OXA-58 expression and reduction of all OMPs. This isolate had the lowest expression of *adeE* efflux pump gene. AP1 co-harboring OXA-58 and IMP-14a carbapenemases showed higher carbapenem MICs than those of AP14. Moreover, $bla_{\text{OXA-58}}$ expression in *A. baumannii* was higher than that of all *A. pittii* isolates (Figure 32 and 35). These results suggest that carbapenem resistance in $bla_{\text{OXA-58}}$-carrying *A.*

pittii was strongly associated with the expression of *adeE*. However, co-expression of OXA-58 and efflux pump or reduction of OMPs can confer resistance to carbapenems.

The *bla*$_{\text{OXA-23}}$-carrying *A. pittii*, AP16, which was positive for *adeE* overexpression and loss of 29 and 33-36 kDa OMPs showed higher imipenem MIC than that of AP23. This indicates that co-expression of OXA-23 and overexpression of efflux pump or reduced OMPs lead to higher level of carbapenem MICs in *A. pittii*.

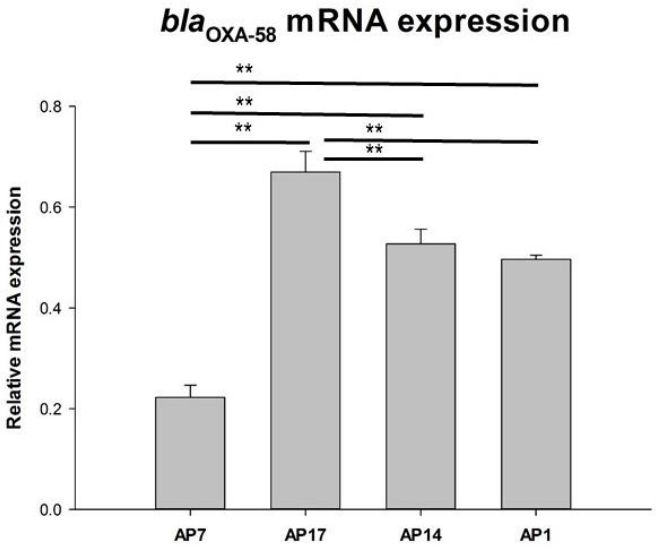


Figure 35. Expression of *bla*$_{\text{OXA-58}}$ in *A. pittii* isolate AP7, AP17, AP14 and AP1
**: $p < 0.01$ (statistical analysis by using ANOVA)

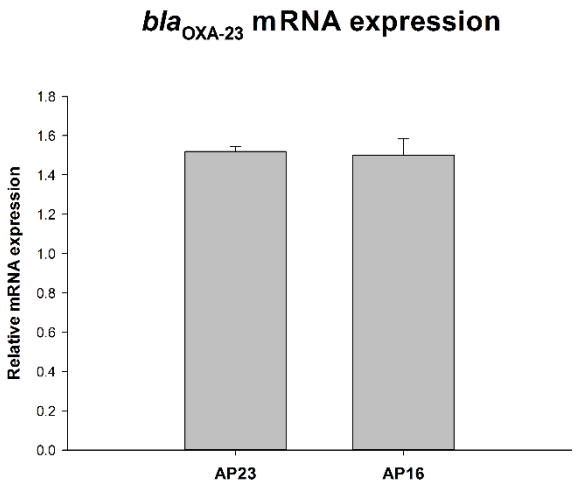
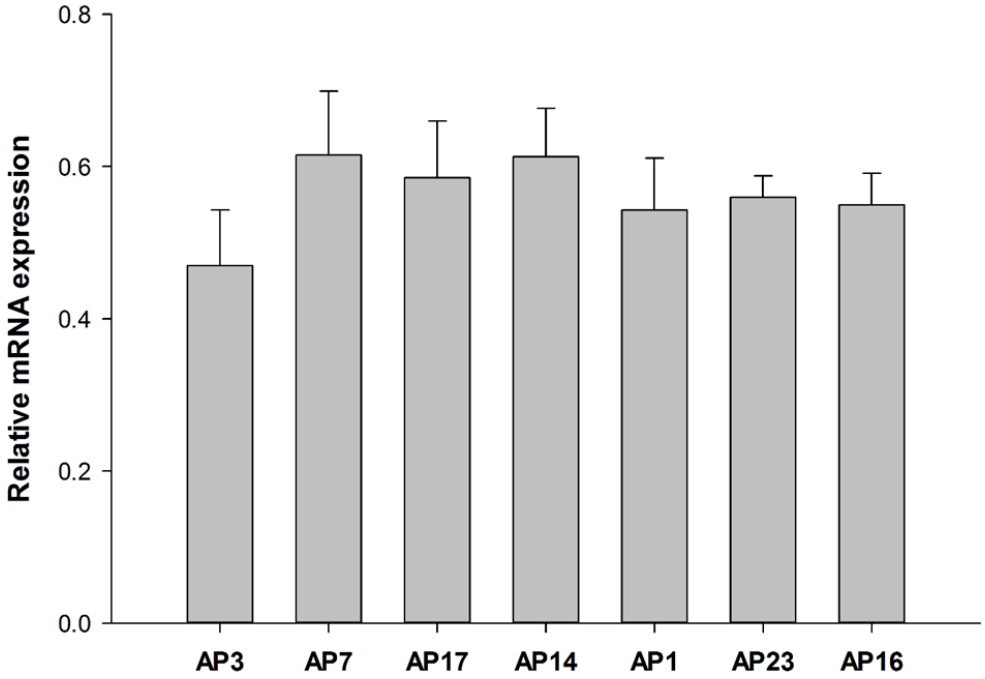


Figure 36. Expression of *bla*$_{\text{OXA-23}}$ in *A. pittii* isolate AP23 and AP16



IPM MIC (mg/L)	0.5	0.5	1	16	32	16	32
MEM MIC (mg/L)	0.5	1	1	8	32	32	32
carbapenemase	-	OXA 58	OXA 58	OXA 58	OXA58 IMP14a	OXA 23	OXA 23
Reduced OMPs (kDa OMPs)	-	29	29, 33-36, 43	29, 33-36, 43	-	-	29, 33-36

Figure 37. Expression of *adeY* efflux pump mRNA in *A. pittii*

The expression of *adeY* efflux pump in *A. pittii* are shown in Figure 37. The result showed that *adeY* overexpression was not detected in *A. pittii* isolates when compared with carbapenem-susceptible *A. pittii* (AP3). The *adeY* expression level was indifferent among *A. pittii* isolates.

In conclusions, carbapenemase production is the main resistance mechanism in *A. baumannii*, *A. nosocomialis* and *A. pittii*. The overexpression of efflux pumps and reduction of OMPs are the additional mechanisms for the increase of carbapenem MICs in *Acinetobacter* spp.

8. MULTILOCUS SEQUENCE TYPING (MLST)

The allelic numbers and sequence type of *A. baumannii* are shown in Table 24. All of 23 *A. baumannii* belonged to 15 STs. The most common STs were ST195 and ST542. They were identified in 4 isolates each. In this study, one novel *gyrB* allelic number and 6 novel STs (including ST1415, ST1416, ST1417, ST1418, ST1423 and ST1426) were found. The novel *gyrB* allelic number of isolate AB4 was named number 159 and novel ST type, ST 1423 was assigned. Although no new allelic number was found in 6 isolates (AB3, AB250, AB35, A4, A8 and A10), all of them were considered belonging to 5 novel STs (ST1415, ST1416, ST1417, ST1418 and ST1426).

A. baumannii isolates carrying *bla*$_{\text{OXA-23}}$ belonged to various STs (Table 25). The major clone, ST195 and ST542 consisted of *bla*$_{\text{OXA-23}}$-positive isolates, whereas OXA-58- and OXA-24-positive isolates belonged to different clones. The reduction of OMPs were seem the clonal specific mechanisms (except isolate AB2 belonging to ST195) because all isolates that belonging to ST542 found reduced OMPs especially 33-36 kDa OMP. Moreover, reduced OMPs also found in strains belonging to ST806, ST514, ST208, ST1416 and ST1426.

Table 25. Allelic numbers and sequence types (STs) of 23 *A. baumannii*

Isolate	Allelic number							Sequence type (ST)
	gltA	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*	
AB1	21	15	3	2	35	202	4	806
AB2	1	3	3	2	2	96	3	195
AB3	1	52	29	28	18	103	7	1415[b]
AB4	1	159[a]	3	2	2	142	3	1423[b]
AB5	1	3	3	2	2	96	3	195
AB6	1	3	3	2	2	96	3	195
AB9	1	52	29	28	18	114	7	514
AB29	1	15	12	6	28	153	40	542
AB58	1	15	12	6	28	153	40	542
AB97	1	15	12	6	28	153	40	542
AB227	1	3	3	2	2	97	3	208
AB250	1	12	56	1	1	177	26	1416[b]
AB13	1	15	12	6	28	153	40	542
AB35	1	3	3	102	2	96	3	1417[b]
AB55	1	3	3	2	4	106	2	1166
AB354	1	159[a]	3	2	2	142	3	1423[b]
A4	1	3	3	102	2	96	3	1417[b]
A5	1	15	2	28	1	107	32	229
A6	1	81	11	48	18	224	43	1001
A7	1	3	3	2	2	96	3	195
A8	21	48	58	42	36	114	4	1418[b]
A9	18	15	3	2	1	106	4	551
A10	1	15	13	12	4	163	5	1426[b]

[a]: novel allelic number and [b]: novel sequence type

Table 26.Sequence types (STs) and carbapenem resistance mechanisms in *A. baumannii*

Isolate	Sequence type (ST)	MICs (mg/L)		Carbapenem resistance mechanism		
		IPM	MEM	Carbapenemase	reduced OMP (kDa)	over-expression of efflux pump
AB2	195	64	64	OXA-23	33-36	NF
AB5		64	128	OXA-23	NF	NF
AB6		32	32	OXA-23	NF	NF
A7		64	64	OXA-23	NF	NF
AB29	542	32	64	OXA-23	33-36, 43	NF
AB58		32	64	OXA-23	29,33-36, 43	NF
AB97		32	64	OXA-23	29, 33-36	NF
AB13		32	64	OXA-23	33-36	NF
AB35	1417	32	64	OXA-23	NF	NF
A4		64	64	OXA-23	NF	NF
AB4	1423	64	64	OXA-23	NF	NF
AB354		64	128	OXA-23	NF	NF
AB1	806	32	32	OXA-23	29	NF
AB3	1415	16	32	OXA-23	NF	NF
AB9	514	16	32	OXA-23	33-36	NF
AB55	1166	32	64	OXA-23	NF	NF
A5	229	32	64	OXA-23	NF	NF
A8	1418	64	128	OXA-23	NF	NF
A9	551	64	128	OXA-23	NF	NF
AB227	208	16	8	OXA-58	33-36	NF
A6	1001	64	64	OXA-58	NF	NF
AB250	1416	16	16	OXA-24	29,33-36,43	NF
A10	1426	128	256	OXA-24	33-36, 43	NF

NF, Not found

The allelic numbers and sequence type of *A. pittii* and *A. nosocomialis* are shown in Table 26. *A. pittii* isolates belonged to various STs (6 STs). Many novel allelic numbers were found including 1 *gltA*, 2 *gyrB*, 3 *gdhB*, 2 *cpn60* and 2 *rpoD* allelic numbers. There were novel STs including ST1419, ST1420, ST1424, ST1425 and ST1427. ST1178 and ST1425 consisted of two isolates each. All *A. nosocomialis* belonged to the same clone, ST958.

Table 27. Allelic numbers and sequence types (STs) of 8 *A. pittii* and 5 *A. nosocomialis*

Isolate	Allelic number							Sequence type (ST)
	gltA	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*	
AP1	56	104	137	7	25	153	74	1419[b]
AP4	91	160[a]	167[a]	7	99[a]	153	120[a]	1424[b]
AP7	56	104	168[a]	67	52	153	75	1425[b]
AP8	56	104	168[a]	67	52	153	75	1425[b]
AP14	56	129	137	7	51	153	74	1178
AP16	102[a]	161[a]	169[a]	87	100[a]	153	121[a]	1427[b]
AP17	56	129	137	7	51	153	74	1178
AP23	56	104	137	7	51	153	74	1420[b]
AN1	39	65	142	30	25	114	28	958
AN4	39	65	142	30	25	114	28	958
AN12	39	65	142	30	25	114	28	958
AN15	39	65	142	30	25	114	28	958
AN20	39	65	142	30	25	114	28	958

[a]: novel allelic number and [b]: novel sequence type

A. pittii carrying $bla_{\text{OXA-58}}$ or $bla_{\text{OXA-23}}$ belonged to various different clones (Table 27). The reduction of OMPs in *A. pittii* were found only in isolates that belonged to ST1425, ST1178 and ST1427. This mechanism was clonal specific except isolate AP7. The overexpression of *adeE* was found in different clones (ST1178, ST1419 and ST1427).

In this study, carbapenem-resistant *A. nosocomialis* isolates were homogenous. All of them were resistant to carbapenems by OXA-23 carbapenemase production and belonged to the same clone, ST958 (Table 27).

Table 28. Sequence types (STs) and carbapenem resistance mechanisms in *A. pittii* and *A. nosocomialis*

Isolate	Sequence type (ST)	MIC (mg/L)		Carbapenem resistance mechanism		
		IPM	MEM	Carbapenemase	Reduced OMP (kDa)	over-expression of efflux pump
AP7	1425	0.5	1	OXA-58	29	NF
AP8		0.5	1	OXA-58	NF	NF
AP14	1178	16	8	OXA-58	29,33-36,43	*adeE*
AP17		1	1	OXA-58	29,33-36,43	NF
AP1	1419	32	32	OXA-58, IPM-14a	NF	*adeE*
AP4	1424	32	32	OXA-23	NF	NF
AP16	1427	32	32	OXA-23	29,33-36	*adeE*
AP23	1420	16	32	OXA-23	NF	NF
AN1	958	16	32	OXA-23	NF	NF
AN4	958	16	32	OXA-23	NF	NF
AN12	958	32	64	OXA-23	NF	NF
AN15	958	16	32	OXA-23	NF	NF
AN20	958	16	32	OXA-23	NF	NF

NF, Not found

In conclusion, carbapenem-resistant *A. baumannii* and -*A. pittii* isolates were heterogenous while *A. nosocomialis* isolates were homogeneous. Carbapenemase production and reduction of OMPs mechanisms appeared to be clonal specific. However, some strains and some clones did not have these mechanisms. The overexpression of efflux pump was found only in different clone of *A. pittii*.

9. DETERMINATION OF ANTIBIOTIC RESISTANCE ISLANDS

Antibiotic resistance islands (AbaRs) in 12 *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis* isolates were investigated by PCR and DNA sequencing. The first step, intact *comM* were amplified (negative for intact *comM* indicated the presence of AbaR). Then, amplification of genes in antibiotic resistance islands were performed by PCR using primer as described in Figure 11. The results of PCR mapping in *Acinetobacter* spp. are shown in Table 28 and the schemes of AbaRs are shown in Figure 38.

The results showed that only *A. baumannii*, *A. pittii* and *A. nosocomialis* carrying $bla_{\text{OXA-23}}$ failed to amplify intact *comM*. This indicated that only $bla_{\text{OXA-23}}$-carrying isolates found AbaR in interrupted *comM*. In this study, 4 different AbaR types were found and all of them were AbaR4 subtypes. The first subtype was AbaR4 with loss of *tniD* and Tn*2006* and was found in isolate AB6 (ST195). The backbone transposon of this AbaR subtype was Tn*6022* which was composed of *tniC-tniA-tniB-tniE*-orf-*uspA-sup*-orf4 (lack of *tniD*).

The second subtype was AbaR4 with loss of Tn*2006*. This subtype was only composed of complete Tn*6022* backbone and was the most common AbaR in this study. They were found in 6 *A. baumannii*, 3 *A. pittii* and 4 *A. nosocomialis* isolates. All of *A. baumannii* belonging to ST542 (AB9, AB29, AB58 and AB97) and most *A. nosocomialis* belonging to ST958 (AN1, AN4, AN12 and AN20) carried this AbaR4 subtype.

The third subtype was AbaR4 with loss only *tniD*. This subtype was the AbaR that carried $bla_{\text{OXA-23}}$ within Tn*2006*. The Tn*2006* interrupted in *sup* of Tn*6022*. While Tn*6022* in this subtype loss of *tniD* gene. There were 3 *A. baumannii* isolates carried this AbaR4 subtype belonging to ST806, ST195 and ST1423 (AB1, AB5 and AB4, respectively).

The last AbaR4 subtype was the complete AbaR4 that was composed of complete Tn*6022* integrated by complete Tn*2006*. This island was found in *A. nosocomialis* AN15 belonging to ST958. After that, the third and fourth AbaR4 subtypes that were found in *A. baumannii* AB4 and *A. nosocomialis* AN15 were amplified and sequenced for the whole island structure.

DNA sequencing results showed that AbaR4 subtype in *A. baumannii* isolate AB4 (AbaR4-AB4) was 13998-bp in size. It integrated in *comM* gene at ACCGC direct

repeat (DR) site (Figure 39). The AbaR4-AB4 was flanked by both of 26-bp inverted repeat sequence, however the backbone transposon (Tn*6022*) lacked the *tniD* gene. The *sup* gene in AbaR-AB4 was interrupted by Tn*2006* (4804-bp length) which carried *bla*$_{\text{OXA-23}}$ gene. The Tn*2006* integrated at CCCGCGAAT duplication site on *sup* gene and was flanked by both of 39-bp inverted repeat sequence.

The AbaR4 subtype island in *A. nosocomialis* isolate AN15 (AbaR4-AN15) was 16711-bp in length. The integration site on *comM* was GCCGC, this site was different with *comM* direct repeat of AbaR4-AB4 (ACCGC). However, AbaR4-AN15 was flanked by the same 26-bp inverted repeat sequence. The Tn*6022* backbone in AbaR4-AN15 composed of *tniC*, *tniA*, *tniB*, *tniD*, *tniE*, orf, *uspA*, *sup* and orf4. The nucleotide sequences of *tniC*, *tniD*, orf, *uspA*, *sup* and orf4 showed 100% similarity to AbaR4-AB4, however *tniA*, *tniB* and *tniE* showed 99% similarity. The Tn*2006* carrying *bla*$_{\text{OXA-23}}$ integrated at the same position on *sup* as AbaR4-AB4 (the same nucleotide sequences of direct repeat on *sup* and inverted repeat on IS*Aba1*).

All these results indicate that *comM* is the hotspot for integration of Tn*6022* although the sequences of *comM* and Tn*6022* are different between species. Tn*2006* carrying *bla*$_{\text{OXA-23}}$ on island may disseminate among different species and clones of *Acinetobacter*.

Table 29. PCR mapping of antibiotic resistance island study in *Acinetobacter* spp.

Isolate	Intact *comM*	J3/J5	PCR mapping							
			comM: *tniB*	*tniB*: *tniE* (bp)	*tniE*: *uspA*	*uspA*: IS*Aba1*	orf: orf4	IS*Aba1*: bla_{OXA-23}	bla_{OXA-23}: orf4	IS*Aba1*:5*comM*
AB1	-	+/+	+	560	+	+	-	+	+	+
AB2	-	+/+	+	3410	+	+	+	+	-	-
AB3	-	+/+	+	3410	+	+	+	+	+	-
AB4	-	+/+	+	560	+	+	-	+	+	+
AB5	-	+/+	+	560	+	+	-	+	+	+
AB6	-	+/+	+	560	+	-	+	+	-	-
AB9	-	+/+	+	3410	+	-	+	+	-	-
AB29	-	+/+	+	3410	+	-	+	+	+	-
AB58	-	+/+	+	3410	+	-	+	+	+	-
AB97	-	+/+	+	3410	+	-	+	+	+	-
AB227	+	-	ND	ND	ND	ND	ND	ND	ND	ND
AB250	+	-	ND	ND	ND	ND	ND	ND	ND	ND
AP1	+	-	ND	ND	ND	ND	ND	ND	ND	ND
AP4	-	+/+	+	3410	+	-	+	+	+	-
AP7	+	-	ND	ND	ND	ND	ND	ND	ND	ND
AP8	+	-	ND	ND	ND	ND	ND	ND	ND	ND
AP14	+	-	ND	ND	ND	ND	ND	ND	ND	ND
AP16	-	+/+	+	3410	+	-	+	+	+	-
AP17	+	-	ND	ND	ND	ND	ND	ND	ND	ND
AP23	-	+/+	+	3410	+	-	+	-	+	-
AN1	-	+/+	+	3410	+	-	+	+	+	-
AN4	-	+/+	+	3410	+	-	+	+	+	-
AN12	-	+/+	+	3410	+	-	+	+	+	-
AN15	-	+/+	+	3410	+	-	+	+	+	+
AN20	-	+/+	+	3410	+	-	+	+	+	-

+, Positive; -, Negative; ND, Not determined

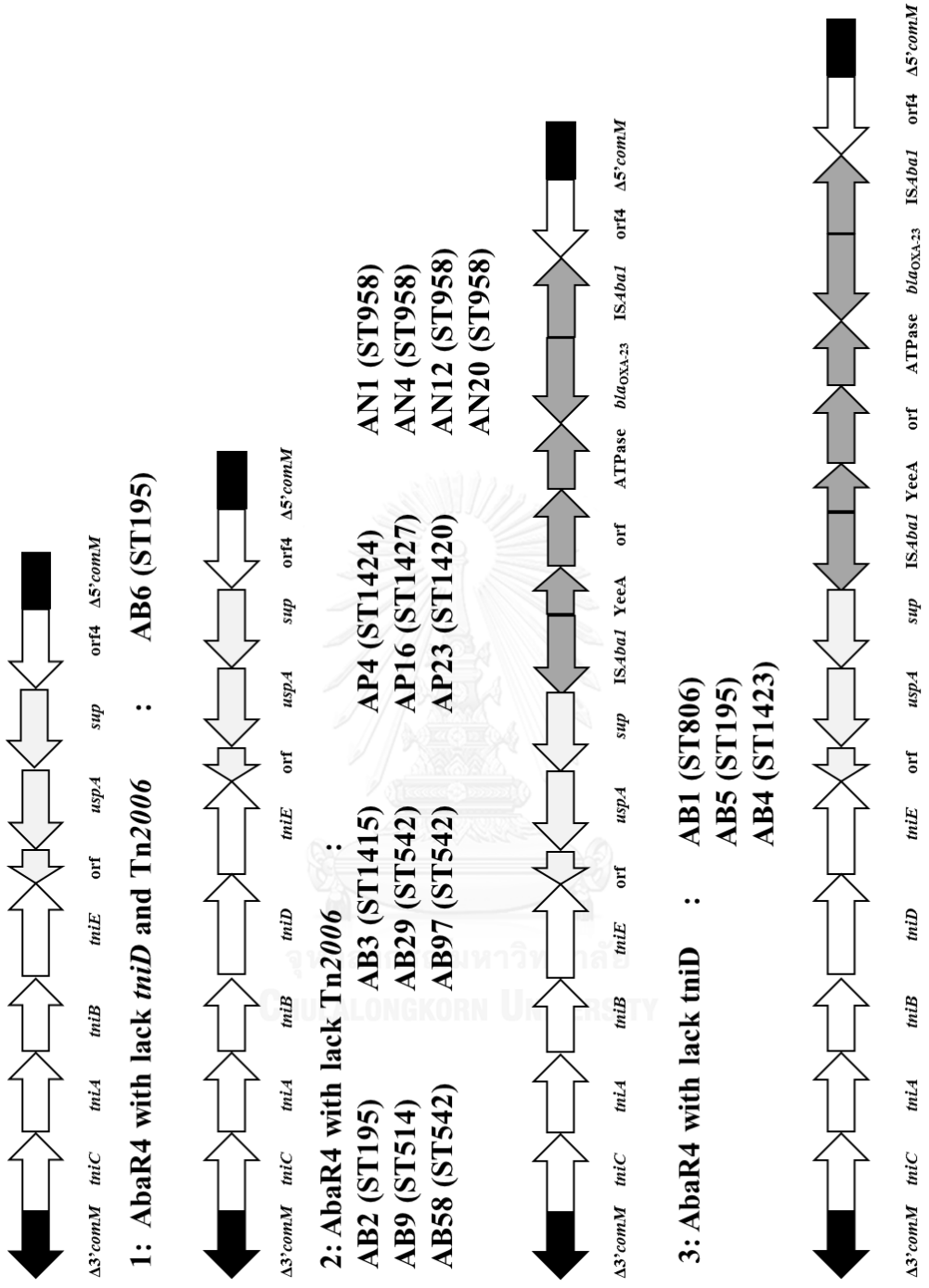


Figure 38. Schemes of antibiotic resistance island structures by PCR mapping in this study

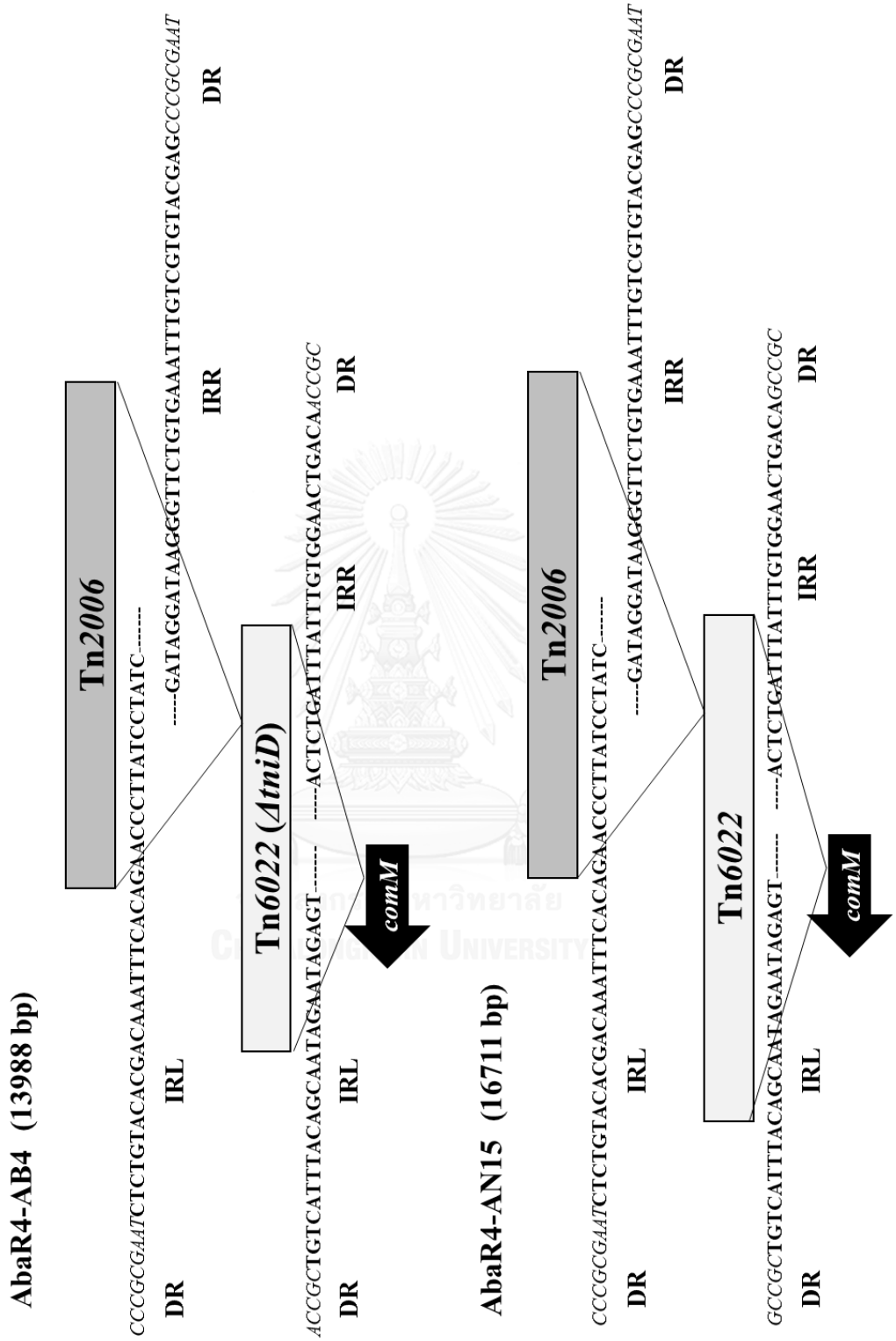


Figure 39. Schemes of integration sites of AbaR4-AB4 and AbaR4-AN15

10. SCREENING OF SYNERGISM OF ANTIBIOTIC COMBINATIONS BY CHECKERBOARD ASSAY

Activities of antibiotic combinations against carbapenem-resistant *Acinetobacter* spp. were evaluated by using checkerboard method as described in material and methods. The results of antimicrobial susceptibility test are shown in Table 29 and the results of antibiotic combinations are shown in Table 30 for *A. baumannii* and Table 31 for *A. pittii* and *A. nosocomialis*.

Synergistic effects were found in 65.2%, 46.2%, 30.8% and 17.4% for imipenem plus fosfomycin, meropenem plus amikacin, imipenem plus amikacin and imipenem plus colistin, respectively against carbapenem-resistant *A. baumannii*. No synergism was found in meropenem plus colistin and meropenem plus fosfomycin. No antagonism was found in any combination against *A. baumannii* isolates. All combinations showed synergism against isolates belonging to various clones and different resistance mechanisms. However, most of ST542 isolates displayed synergisms of imipenem plus fosfomycin and meropenem plus amikacin.

In *A. pittii*, synergisms were found in 62.5%, 50%, 33.3% and 25% for imipenem plus fosfomycin, imipenem plus amikacin, meropenem plus amikacin and meropenem plus fosfomycin, respectively. No synergism was found in imipenem plus colistin and meropenem plus colistin. No antagonism was found in all combinations against *A. pittii* isolates.

The best combination was imipenem plus fosfomycin and imipenem plus amikacin. Four isolates were highly resistant to amikacin (MICs > 256 mg/L). Therefore, imipenem plus amikacin was not studied in these isolates. Most synergistic isolates in imipenem plus fosfomycin carried $bla_{\text{OXA-23}}$ (AP4, AP16 and AP23) except AP1 that carried $bla_{\text{OXA-58}}$ and bla_{IMP14a}. While synergistic effects of imipenem plus amikacin, meropenem plus amikacin and meropenem plus fosfomycin were found in different clones and resistance mechanisms.

Table 30. Carbapenem resistance mechanisms and antimicrobial susceptibilities of *Acinetobacter* spp. isolates

Isolate	Carbapenemase	Reduced OMP (size of OMP)	overexpression of efflux pump	Sequence type	MIC (mg/L)				
					Imipenem	Meropenem	Colistin	Amikacin	Fosfomycin
AB2	OXA23	33-36	-	195	64	64	2	>256	128
AB5	OXA23	NF	-	195	64	128	1	>256	128
AB6	OXA23	NF	-	195	32	32	1	>256	128
A7	OXA23	NF	-	195	64	64	1	>256	256
AB29	OXA23	33-36, 43	-	542	32	64	1	>256	128
AB58	OXA23	29,33-36, 43	-	542	32	64	1	32	256
AB97	OXA23	29, 33-36	-	542	32	64	1	16	128
AB13	OXA23	33-36	-	542	32	64	1	64	128
AB35	OXA23	NF	-	1417	32	64	1	8	256
A4	OXA23	NF	-	1417	64	64	1	>256	256
AB4	OXA23	NF	-	1423	64	64	1	>256	256
AB354	OXA23	NF	-	1423	64	128	1	>256	256
AB1	OXA23	29	-	806	32	32	2	>256	128
AB3	OXA23	NF	-	1415	16	32	2	0.5	128
AB9	OXA23	33-36	-	514	16	32	2	0.5	128
AB55	OXA23	NF	-	1166	32	64	1	>256	256
A5	OXA23	NF	-	229	32	64	1	32	256
A8	OXA23	NF	-	1418	64	128	1	1	256
A9	OXA23	NF	-	551	64	128	1	>256	256
AB227	OXA58	33-36	-	208	16	8	1	32	128
A6	OXA58	NF	-	1001	64	64	1	32	256
AB250	OXA24	29,33-36,43	-	1416	16	16	1	4	128
A10	OXA24	33-36, 43	-	1426	128	256	2	2	256
AP7	OXA58	29	-	1425	0.5	1	1	>256	128
AP8	OXA58	NF	-	1425	0.5	1	1	>256	128
AP14	OXA58	29,33-36,43	+	1178	16	8	0.5	2	256
AP17	OXA58	29,33-36,43	-	1178	1	1	1	128	256
AP1	OXA58 IMP14a	NF	+	1419	32	32	0.5	2	256
AP4	OXA23	NF	-	1424	32	32	1	>256	128
AP16	OXA23	29,33-36	+	1427	32	32	1	>256	128
AP23	OXA23	NF	-	1420	16	32	1	2	128
AN1	OXA23	NF	-	958	16	32	1	2	128
AN4	OXA23	NF	-	958	16	32	2	2	256
AN12	OXA23	NF	-	958	32	64	2	2	128
AN15	OXA23	NF	-	958	16	32	1	2	256
AN20	OXA23	NF	-	958	16	32	2	2	256

NF, Not found; +, Positive for overexpression of efflux pump; -, Negative for overexpression of efflux pump

Table 31. Antimicrobial agent combinations against carbapenem-resistant *A. baumannii* different resistance mechanisms

Isolate	Carbapenemase	Reduced OMP (size of OMP)	Sequence type	FIC index					
				IPM: AK	IPM: CT	IPM: FOF	MEM: AK	MEM: CT	MEM: FOF
AB2	OXA23	33-36	195	ND	0.63	**0.31**	ND	0.75	0.75
AB5	OXA23	NF	195	ND	2.00	0.56	ND	1.00	1.00
AB6	OXA23	NF	195	ND	2.00	**0.50**	ND	0.63	1.00
A7	OXA23	NF	195	ND	0.63	1.00	ND	0.75	2.00
AB29	OXA23	33-36, 43	542	**0.31**	2.00	**0.31**	**0.38**	0.75	0.53
AB58	OXA23	29,33-36, 43	542	0.75	2.00	**0.38**	**0.38**	0.63	2.00
AB97	OXA23	29, 33-36	542	**0.50**	2.00	**0.31**	**0.50**	0.63	1.00
AB13	OXA23	33-36	542	0.75	0.75	0.75	**0.38**	1.00	0.75
AB35	OXA23	NF	1417	0.75	**0.50**	**0.50**	**0.50**	0.56	0.75
A4	OXA23	NF	1417	ND	0.75	**0.50**	ND	1.00	1.00
AB4	OXA23	NF	1423	ND	2.00	**0.50**	ND	1.00	1.00
AB354	OXA23	NF	1423	ND	**0.50**	0.53	ND	0.75	0.75
AB1	OXA23	29	806	ND	0.63	0.56	ND	0.75	0.75
AB3	OXA23	NF	1415	0.75	0.63	**0.31**	**0.50**	2.00	0.75
AB9	OXA23	33-36	514	0.75	0.63	**0.31**	1.00	0.75	0.53
AB55	OXA23	NF	1166	ND	**0.50**	1.00	ND	0.63	0.75
A5	OXA23	NF	229	0.63	0.75	2.00	0.63	1.00	1.00
A8	OXA23	NF	1418	**0.50**	0.75	0.75	0.75	2.00	2.00
A9	OXA23	NF	551	ND	**0.50**	**0.50**	ND	0.75	0.56
AB227	OXA58	33-36	208	0.75	0.75	**0.31**	1.00	0.63	0.75
A6	OXA58	NF	1001	0.75	0.75	**0.31**	1.00	0.63	0.75
AB250	OXA24	29,33-36,43	1416	**0.38**	1.00	**0.50**	0.75	0.75	0.75
A10	OXA24	33-36, 43	1426	0.75	2.00	**0.50**	1.00	0.63	1.00

NF, Not found; ND, Not determined

Table 32. Antimicrobial agent combinations against carbapenem-resistant *A. pittii* and *A. nosocomialis* different resistance mechanisms

Isolate	Carbapenemase	Reduced OMP (size of OMP)	overexpression of efflux pump	Sequence type	FIC index					
					IPM: AK	IPM: CT	IPM: FOF	MEM: AK	MEM: CT	MEM: FOF
AP7	OXA58	29	-	1425	ND	2.00	2.00	ND	0.75	**0.50**
AP8	OXA58	NF	-	1425	ND	0.75	2.00	ND	0.75	0.75
AP14	OXA58	29,33-36,43	+	1178	**0.38**	0.75	**0.27**	0.56	0.75	0.75
AP17	OXA58	29,33-36,43	-	1178	2.00	2.00	1.00	**0.50**	2.00	0.52
AP1	OXA58 IMP14a	NF	+	1419	**0.50**	1.00	**0.38**	1.00	0.56	**0.50**
AP4	OXA23	NF	-	1424	ND	1.00	**0.50**	ND	1.00	1.00
AP16	OXA23	29, 33-36	+	1427	ND	1.00	**0.25**	ND	0.75	0.75
AP23	OXA23	NF	-	1420	0.75	1.00	**0.50**	0.63	1.00	1.00
AN1	OXA23	NF	-	958	0.75	0.63	0.63	0.52	1.00	1.00
AN4	OXA23	NF	-	958	0.63	2.00	**0.50**	0.75	0.75	**0.50**
AN12	OXA23	NF	-	958	2.00	0.62	**0.38**	0.52	1.00	1.00
AN15	OXA23	NF	-	958	**0.38**	2.00	**0.50**	0.63	1.00	**0.50**
AN20	OXA23	NF	-	958	**0.38**	1.00	**0.50**	0.75	2.00	**0.50**

NF, Not found; ND, Not determined; +, Positive for overexpression of efflux pump; -, Negative for overexpression of efflux pump

In *A. nosocomialis*, the synergisms were found in 80% (4/5 of isolates), 60% (3/5 of isolates) and 40% (2/5 of isolates) of imipenem plus fosfomycin, meropenem plus fosfomycin and imipenem plus amikacin, respectively. The most effective combination was imipenem plus fosfomycin. No synergism was found in imipenem plus colistin, meropenem plus amikacin and meropenem plus colistin. No antagonism was shown against all *A. nosocomialis* isolates.

The limitation of this study was that only OXA-23 carbapenemase-producing isolates were found in *A. nosocomialis* and all of them belonged to the same ST clone. However, each combination had synergistic effect against only some isolates. Thus, this indicate that synergism of antibiotic combination is not specific with clones and resistance mechanism of *A. nosocomialis*.

In conclusion, imipenem plus fosfomycin is the best combination against *A. baumannii*, *A. pittii* and *A. nosocomialis*. No antagonism of any antibiotic combination was found in this study. The effect of antibiotic combinations is not associated with clonal and carbapenem resistance mechanism of *Acinetobacter* spp. isolates. Next, the effect of imipenem plus fosfomycin was confirmed by *in vitro* time-kill assay.

11. CONFIRMATION OF SYNERGISM OF ANTIBIOTIC COMBINATION BY TIME-KILL ASSAY

The results from checkerboard assay showed that imipenem plus fosfomycin was the most effective combination against carbapenem-resistant *Acinetobacter* spp. isolates. Therefore, this combination was confirmed for the *in vitro* synergism by time-kill assay.

Imipenem plus fosfomycin was tested against 8 *A. baumannii*, 4 *A. pittii* and 2 *A. nosocomialis* isolates (all of them showed synergism by checkerboard assay). The concentrations of imipenem and fosfomycin at 1X MIC and 0.5X MIC were used alone and in combination against all *Acinetobacter* spp. except that 0.5X and 0.25X MIC of imipenem and 1X and 0.5X MIC of fosfomycin were tested against *A. baumannii* isolate A6.

Time-kill curves of imipenem plus fosfomycin against $bla_{\text{OXA-23}}$-carrying *A. baumannii* (AB3, AB29, AB58 and A4) are shown in Figure 40. Imipenem and meropenem alone could not inhibit bacterial growth during 24 hours but antibiotic combinations showed effective killing. Isolates AB3 and A4, resistant to carbapenems by only OXA-23 production showed synergism at 1X MIC IPM plus 1X MIC FOF while A4 also showed synergy of 1X MIC IPM plus 0.5X MIC FOF. Isolate AB29 with loss of 33-36 kDa OMP was found synergy of all combination except 0.5X MIC IPM plus 0.5X MIC FOF. Isolate AB58 with reduced 29 and 33-36 kDa OMPs was found synergy only of 1X IPM plus 1X MIC FOF. All synergism also showed bactericidal effect against all isolates.

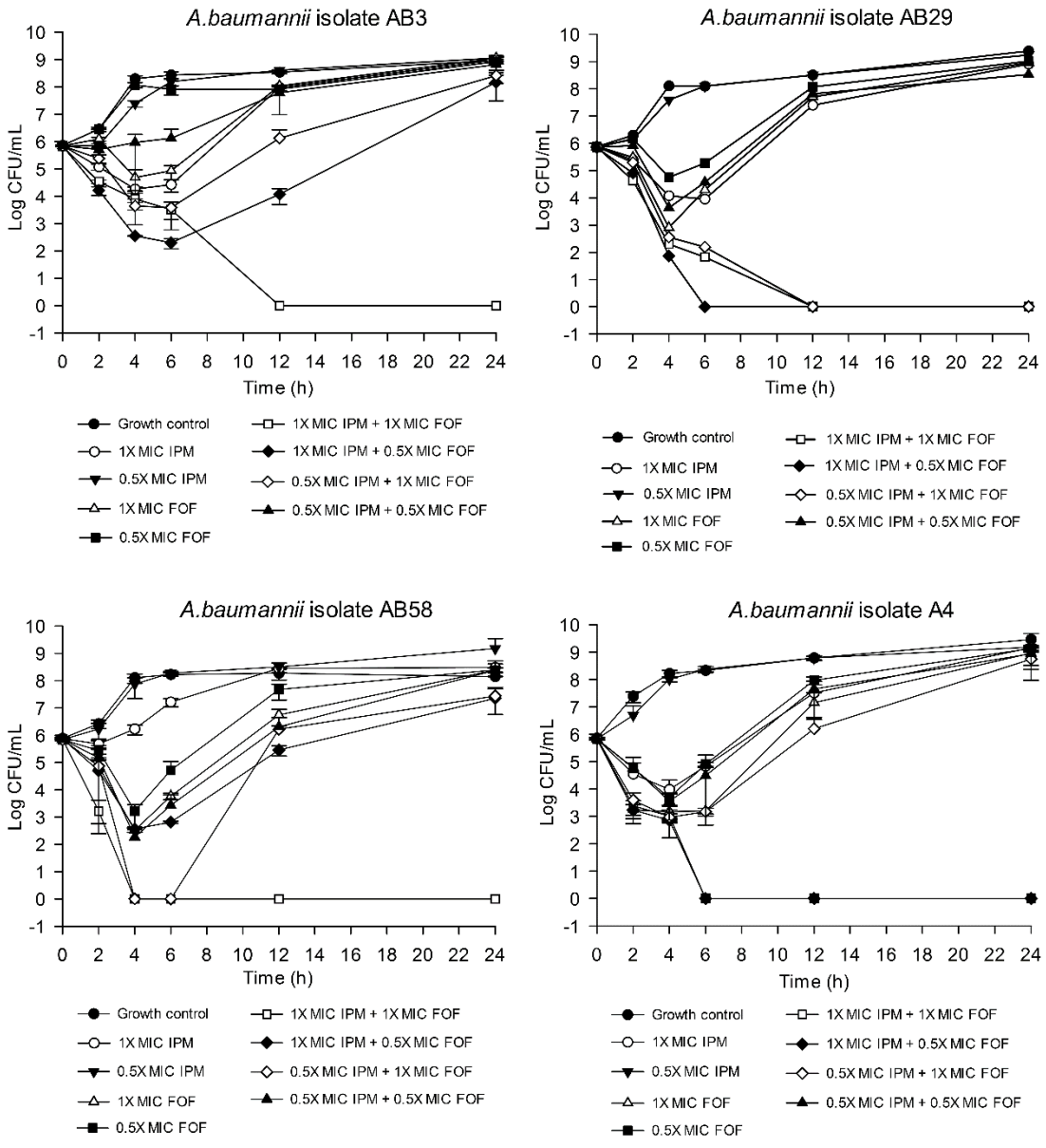


Figure 40. Time-kill curves of imipenem plus fosfomycin against *bla*$_{\text{OXA-23}}$-carrying *A. baumannii*

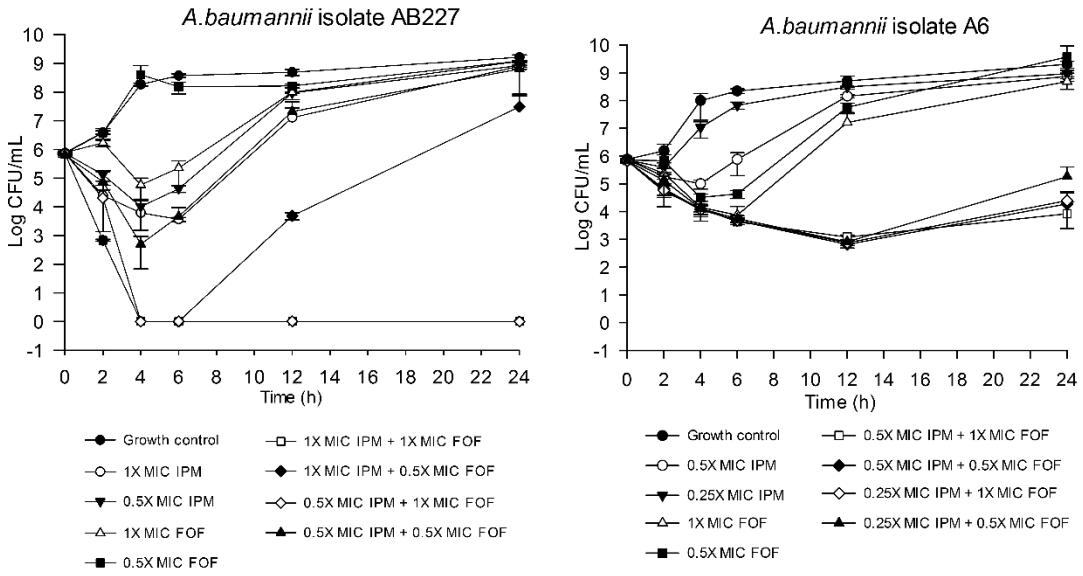


Figure 41. Time-kill curves of imipenem plus fosfomycin against *bla*$_{\text{OXA-58}}$-carrying *A. baumannii*

OXA-58-producing isolates with loss of 33-36 kDa OMP (AB227) and without reduced OMP (A6) showed different killing curves of antibiotic combination (Figure 41). At the concentration of 1X MIC IPM plus 0.5X MIC FOF and 0.5X MIC IPM plus 0.5X MIC FOF could inhibit growth of AB227 but also showed regrowth at 12 and 6 hours, respectively. However, at 1X MIC IPM plus 1X MIC FOF and 0.5X MIC IPM and 1X MIC FOF showed effective killing against AB227 within 4 hours and no regrowth was found during 24 hours. All combinations showed synergism against A6 although regrowth was detected at 24 hours (viable cells were lower than antibiotic alone at least 2log$_{10}$ CFU/ml).

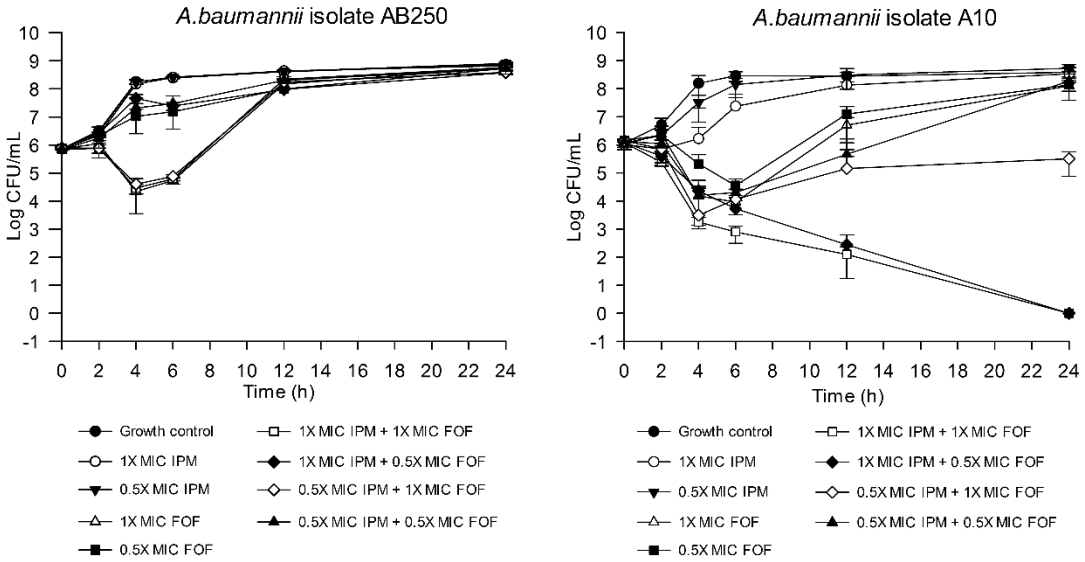


Figure 42. Time-kill curves of imipenem plus fosfomycin against *bla*$_{OXA-24}$-carrying *A. baumannii*

Time-kill curves of imipenem plus fosfomycin of OXA-24 carbapenemase-producing isolate with loss of all 3 OMPs (AB250) and with reduced 33-36 kDa OMP and OprD (A10) are shown in Figure 42. Imipenem and fosfomycin alone showed no effect against AB250 except 1X MIC FOF that could kill within 4 hours but regrowth showed at 6 hours. When used in combination, 1X MIC IPM plus 0.5X MIC FOF and 0.5X MIC IPM plus 0.5X MIC FOF showed no killing. The 1X MIC IPM plus 1X MIC FOF and 0.5X MIC IPM plus 1X MIC FOF showed killing within 4 hours but after that showed regrowth. Thus, no synergism was found in any combination against AB250. In the case of A10, all combinations killed bacteria within first 6 hours then 0.5X MIC IPM plus 1X MIC FOF and 0.5X MIC IPM plus 0.5X MIC FOF showed regrowth at 12 hours. Only 1X MIC IPM plus 1X MIC FOF and 1X MIC IPM plus 0.5X MIC FOF showed synergistic effect with bactericidal effect at 24 hours.

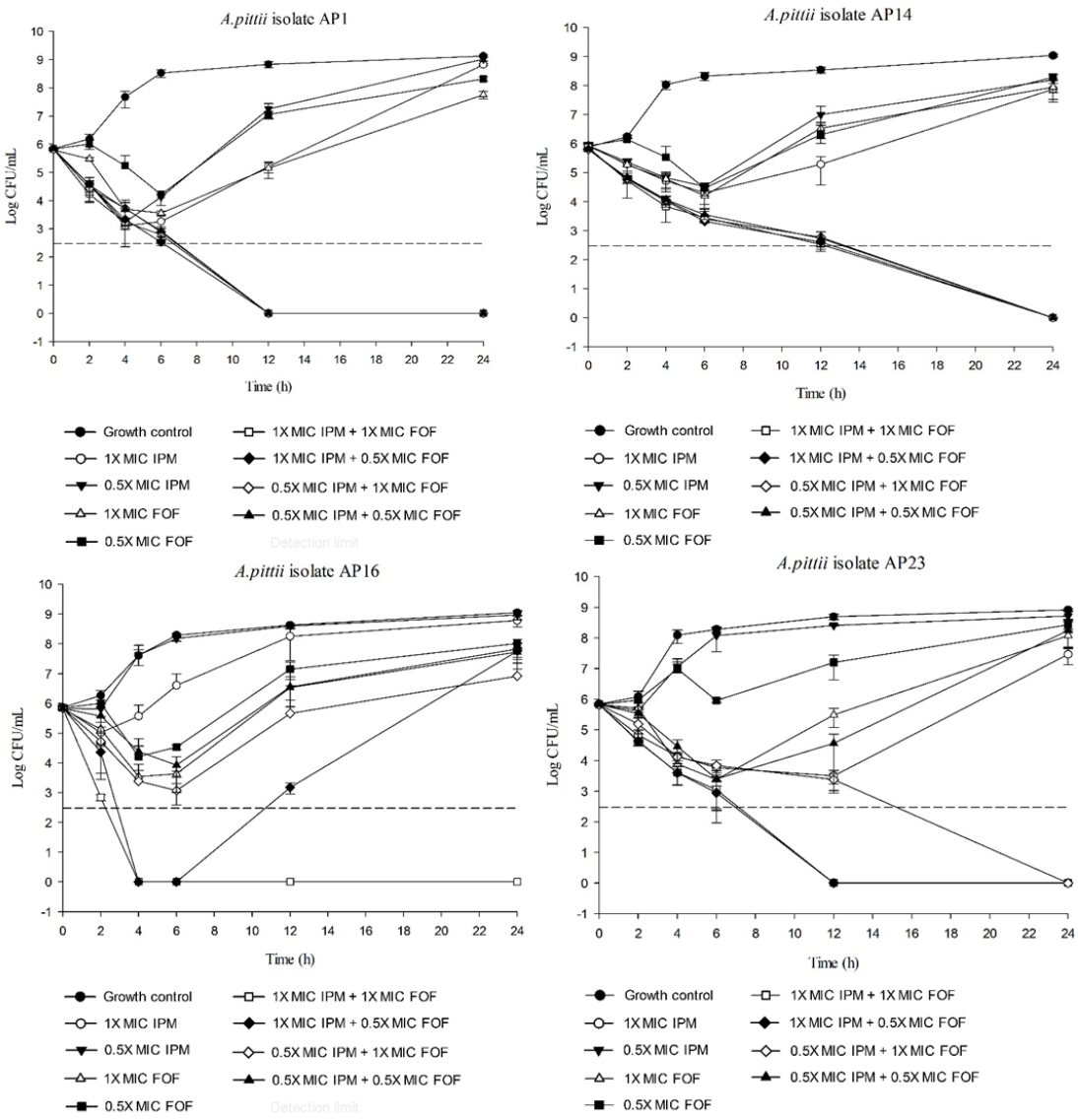


Figure 43. Time-kill curves of imipenem plus fosfomycin against *A. pittii*

The results of time-kill assay against *A. pittii* are shown in Figure 43. The OXA-58- and IMP14a-co-producing isolate with overexpression of efflux pump (AP1) and OXA-58 producing isolate with reduced all 3 OMPs (AP14) were compared for the killing curve of imipenem plus fosfomycin. Each of imipenem and fosfomycin alone inhibited growth of these isolates at first 6 hours and then the regrowth was detected. All combination of imipenem plus fosfomycin showed effective killing against them until displayed for synergism at 24 hours. All synergism also showed bactericidal effect against both of them.

For *bla*$_{\text{OXA-23}}$-carrying *A. pittii*, isolate AP16 with reduced 29 and 33-36 kDa OMPs and isolate AP23 without reduced OMP were studied. The results showed that antibiotic alone could not inhibit growth of them. Imipenem plus fosfomycin in some concentrations had effective killing and showed synergism against them. In isolate AP16 only 1X MIC IPM plus 1X MIC FOF showed rapid killing within 4 hours and reach synergism at 24 hours. All combinations displayed for synergistic effect at 24 hours except 0.5X MIC IPM plus 0.5X MIC FOF in isolate AP23.

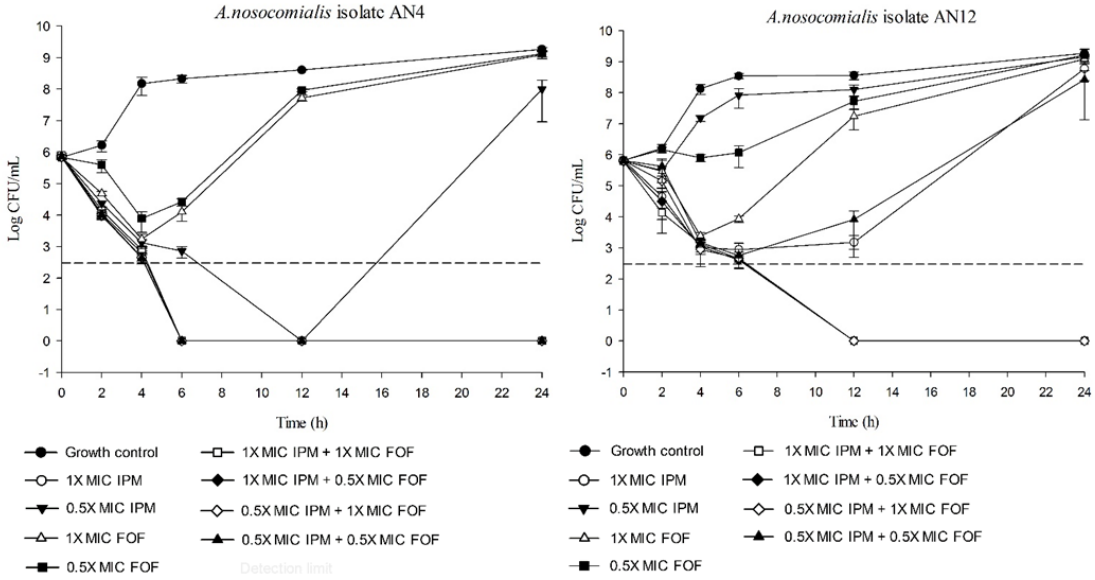


Figure 44. Time-kill curves of imipenem plus fosfomycin against *A. nosocomialis*

The *A. nosocomialis* isolates, AN4 and AN12, were selected for time-kill assay and the killing curves are shown in Figure 44. Each of imipenem or fosfomycin alone could not inhibit growth after 24 hours except 1X MIC IPM that could inhibit growth of AN4. Thus, synergism in AN4 was found in only 0.5X MIC IPM plus 1X MIC FOF and 0.5X MIC IPM plus 0.5X MIC FOF. Isolate AN12 found synergism at all combinations except 0.5X MIC IPM and 0.5X MIC FOF. All synergism also showed bactericidal effect against all isolates.

In conclusion, the synergistic results of checkerboard were correlated with time-kill result except isolate AB250. Therefore, imipenem plus fosfomycin was the best effective combination against carbapenem-resistant *Acinetobacter* spp. isolates. Time-kill curves showed synergism and bactericidal activity of imipenem plus fosfomycin in different mechanisms of carbapenem resistance. Thus, mechanisms of resistance were not associated with activity of antibiotic combinations.

12. DETERMINATION OF FOSFOMYCIN RESISTANCE MECHANISMS

1. Detection of *murA* gene

The intrinsic resistance to fosfomycin is drug target mutation. The target of fosfomycin is UDP-N-acetylglucosamine enolpyruvyl transferase or MurA enzyme that adds phosphoenolpyruvate (PEP) to UDP-N-acetylglucosamine and removes phosphate group to get product enolpyruvyl-UDP-GlcNAc.

1.1 Detection of MurA mutation

The entire *murA* of *Acinetobacter* species is 1257 bp in length, encoding for 418 amino acids MurA and shows 59% amino acid similarity to *E. coli* MurA (*E. coli* murA is 419 amino acid length). The active site of *A. baumannii* MurA containing 59 amino acids were identified by CASTp (Computed Atlas of Surface Topography of proteins) (188). Mutation that involved fosfomycin resistance has not been reported in *A. baumannii*. However, the mutations at catalytic active site, Cys115 and ligand binding site including Lys22, Arg120 and Arg397 in *E. coli* (this positions in *A. baumannii* are Cys116, Lys22, Arg121 and Arg398) were found in fosfomycin-resistant isolates. Moreover, mutations at Asp369 and Leu370 in *E. coli* (at Asp370 and Leu371 in *A. baumannii* but not been reported yet) also related to fosfomycin resistance (the square in Figure 45).

All 23 *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis* (AN4 and AN12) were selected for study of MurA mutation. The entire *murA* gene was amplified, sequenced and translated to amino acid sequence. No mutations at Cys116, Lys22, Arg121, Arg398, Asp370 and Leu370 were found in all *Acinetobacter* spp. isolates (Table 32). The amino acid sequences of MurA of representative isolates (*A. baumannii*, AB250 and A10; *A. pittii*, AP1 and AP23; *A. nosocomialis*, AN4 and AN12) are shown in Figure 45.

A. baumannii AB250 and A10 with fosfomycin MIC 128 and 256 mg/L, respectively showed identical amino acid sequences of MurA which were 59% similarity to MurA of *E. coli*. While *A. pittii* AP1 and AP23 with fosfomycin MIC of 256 and 128 mg/L, respectively also showed identical amino acid sequences of MurA which were 58% similarity to MurA of *E. coli*. In *A. nosocomialis* AN4 and AN12 with fosfomycin MIC 256 and 128 mg/L, respectively showed 99% similarity to each other

(Lysine or Glutamine at position 205). However, they were 58% similarity to MurA of *E. coli*.

MurA of *A. baumannii*, *A. pittii* and *A. nosocomialis* were different at the positions 258 to 262 (the circle in Figure 45). There were Asp-Leu-Leu-Glu-Ala in *A. baumannii*, Ala-Leu-Leu-Glu-Asp in *A. pittii* and Ala-Leu-Leu-Glu-Asp in *A. nosocomialis*. However, these positions have not been reported to be related to fosfomycin resistance in *Acinetobacter* spp. or other organisms.

These results suggest that active site of *Acinetobacter* spp. MurA is available for production of peptidoglycan precursor. Therefore, the next experiment was to detect the expression level of *murA* mRNA.

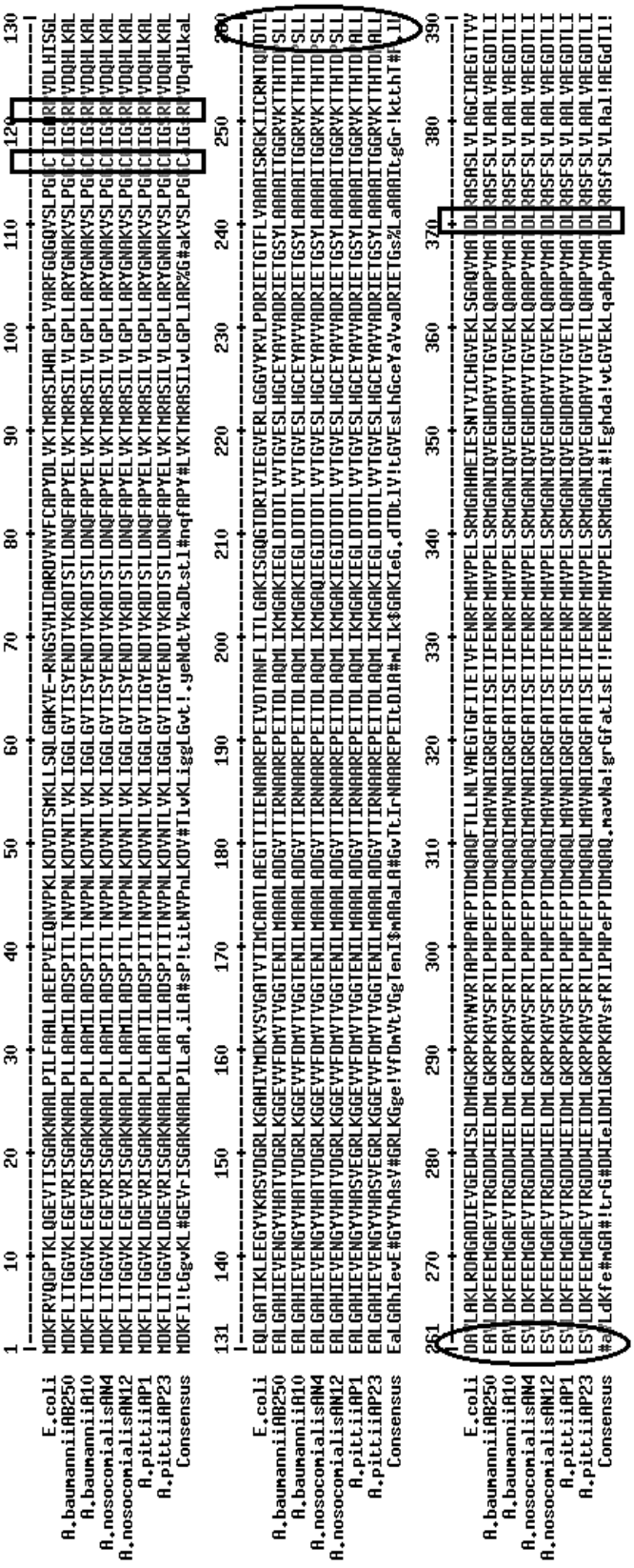


Figure 45. Alignments of amino acid sequences of MurA of *Acinetobacter* spp.

Table 33. Fosfomycin resistance mechanisms and antimicrobial susceptibilities of *Acinetobacter* spp. isolates

Isolate	FOF MIC (mg/L)			Overexpression efflux activity	MurA mutation	FOF modifying enzyme	Cell wall recycling gene				
	FOF	FOF + CCCP	FOF + reserpine				*ampG*	*nagZ*	*amnK*	*amgK*	*murU*
AB2	128	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AB5	128	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AB6	128	128	128	NF	NF	NF	ND	ND	ND	ND	ND
A7	256	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AB29	128	128	128	NF	NF	NF	+	+	+	+	+
AB58	256	128	128	NF	NF	NF	+	+	+	+	+
AB97	128	64	128	NF	NF	NF	ND	ND	ND	ND	ND
AB13	128	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AB35	256	128	128	NF	NF	NF	ND	ND	ND	ND	ND
A4	256	128	128	NF	NF	NF	+	+	+	+	+
AB4	256	256	256	NF	NF	NF	ND	ND	ND	ND	ND
AB354	256	256	256	NF	NF	NF	ND	ND	ND	ND	ND
AB1	128	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AB3	128	128	128	NF	NF	NF	+	+	+	+	+
AB9	128	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AB55	256	128	128	NF	NF	NF	ND	ND	ND	ND	ND
A5	256	128	128	NF	NF	NF	ND	ND	ND	ND	ND
A8	256	256	256	NF	NF	NF	ND	ND	ND	ND	ND
A9	256	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AB227	128	64	128	NF	NF	NF	+	+	+	+	+
A6	256	128	128	NF	NF	NF	+	+	+	+	+
AB250	128	64	128	NF	NF	NF	+	+	+	+	+
A10	256	128	128	NF	NF	NF	+	+	+	+	+
AP7	128	128	128	NF	NF	NF	+	+	+	-	-
AP8	128	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AP14	256	256	128	NF	NF	NF	+	+	+	-	-
AP17	256	256	128	NF	NF	NF	+	+	+	-	-
AP1	256	256	128	NF	NF	NF	+	+	+	-	+
AP4	128	128	64	NF	NF	NF	ND	ND	ND	ND	ND
AP16	128	128	128	NF	NF	NF	-	+	+	-	-
AP23	128	128	128	NF	NF	NF	+	+	+	-	+
AN1	128	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AN4	256	128	128	NF	NF	NF	+	+	+	+	+
AN12	128	128	128	NF	NF	NF	+	+	+	+	+
AN15	256	128	128	NF	NF	NF	ND	ND	ND	ND	ND
AN20	256	256	256	NF	NF	NF	ND	ND	ND	ND	ND

NF, Not found; ND, Not determined; +, Positive; -, Negative

1.2 Detection of *murA* mRNA expression

The expression level of *murA* mRNA was detected in 2 representative *A. baumannii* (AB250 and A10), 2 *A. pittii* (AP1 and AP23) and 2 *A. nosocomialis* (AN4 and AN12) without fosfomycin and with 0.25X, 0.5X and 1X MIC of fosfomycin. The expression of *murA* of *A. baumannii* AB250 and A10 that showed synergism and no synergism of imipenem plus fosfomycin, respectively were indifferent and did not change in the presence of fosfomycin. In *A. pittii* AP1 and AP23, *murA* expressed at the same level and did not change in the presence of fosfomycin. Although the *murA* mRNA expression level of *A. baumannii* was higher than that of *A. pittii*. They had the same level of fosfomycin MIC (128-256 mg/L).

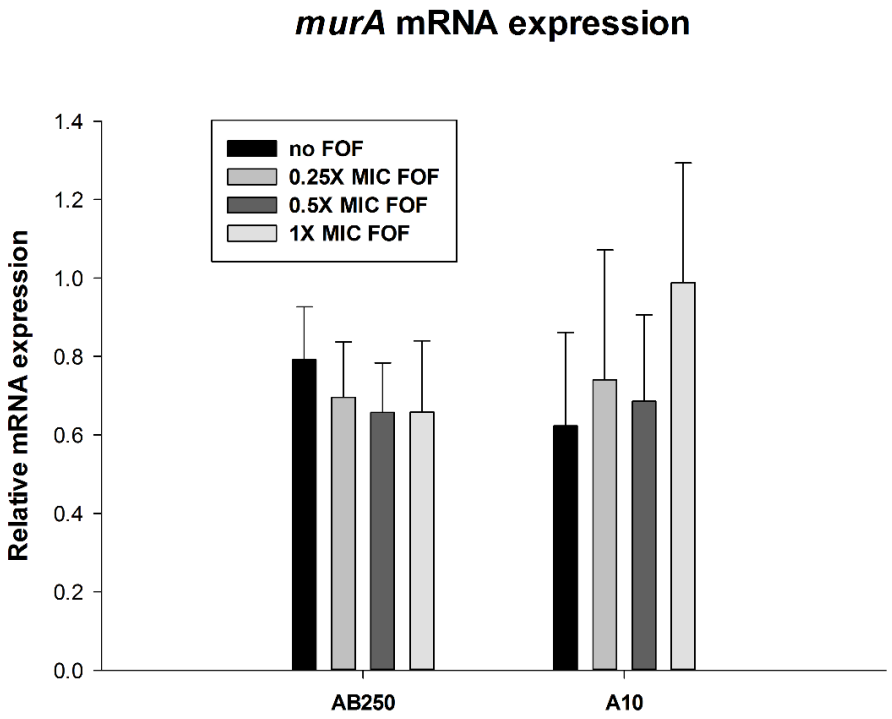


Figure 46. Expression of *murA* mRNA in *A. baumannii* isolate AB250 and A10

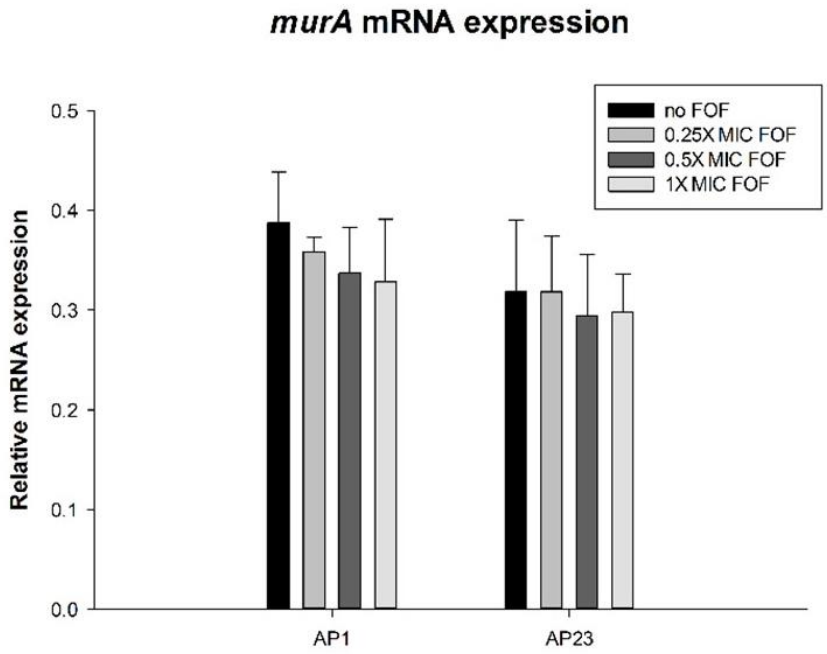


Figure 47. Expression of *murA* mRNA in *A. pittii* isolate AP1 and AP23

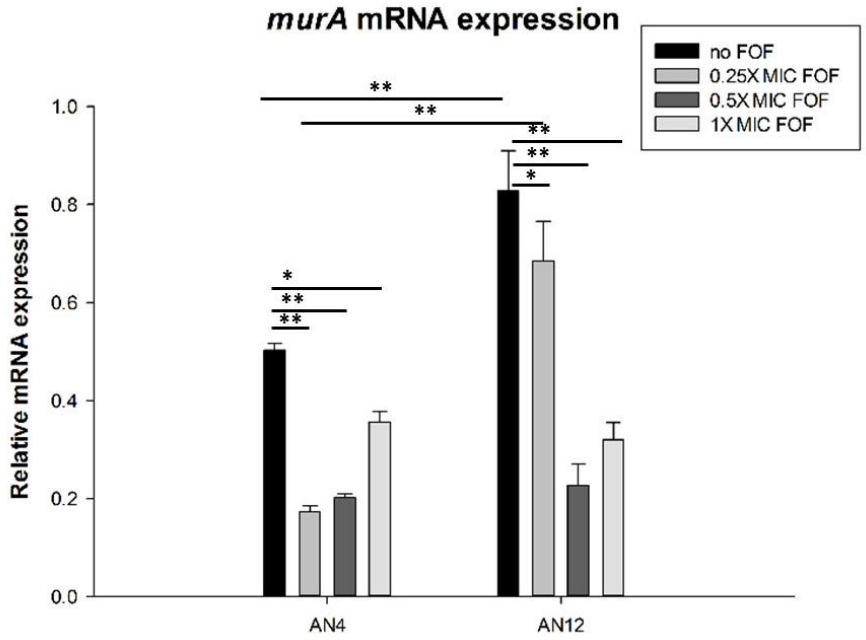


Figure 48. Expression of *murA* mRNA in *A. nosocomialis* isolate AN4 and AN12
*: $p < 0.05$; **: $p < 0.01$ (statistical analysis by using ANOVA)

Surprisingly, the expression level of *murA* of *A. nosocomialis* AN4 with the fosfomycin MIC of 256 mg/L was significantly lower than that of AN12 (fosfomycin MIC=128 mg/L) (Figure 48). Both isolates showed decreased *murA* expression in the presence of fosfomycin.

These results suggest that there may be other mechanisms of peptidoglycan synthesis in *A. baumannii* and *A. pittii* because they showed normal expression level of *murA* in the presence of fosfomycin. MurA was important for peptidoglycan synthesis in *A. nosocomialis*, because it was suppressed by fosfomycin.

2. Detection of fosfomycin-modifying enzymes

All 23 *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis* were detected for gene encoding fosfomycin-modifying enzymes including FosA, FosB, FosC and FosX, by PCR. No *fosA*, *fosB*, *fosC* and *fosX* was detected in all *A. baumannii*, *A. pittii* and *A. nosocomialis* isolates (Table 32).

3. Detection of overexpression of efflux pump

Only AbaF (*A. baumannii* Fosfomycin efflux) efflux pump has been reported to be related to fosfomycin resistance in *A. baumannii*. Therefore, efflux pump activity and mRNA of *abaF* expression were investigated.

3.1 Detection of efflux pump activity

The activity of fosfomycin efflux pump of 23 *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis* was detected by using efflux pump inhibitors, CCCP and reserpine. Overexpression of efflux pump activity is defined as reduction of carbapenem MICs at least 4-fold in the presence of inhibitor. The fosfomycin MICs are shown in Table 32.

In the presence of CCCP or reserpine, there was no fosfomycin MICs decreased 4-fold in all tested isolates. Therefore, overexpression of fosfomycin efflux pumps was not detected by using inhibitors in all tested *Acinetobacter* spp. isolates. The expression of mRNA of *abaF* gene was evaluated in the next experiment.

3.2 Detection of efflux pump mRNA expression

The AbaF efflux pump has been reported only in *A. baumannii*. In this study, the *abaF* gene was screened in 23 *A. baumannii*, 8 *A. pittii* and 5 *A. nosocomialis*. The results showed that only *A. baumannii* carried *abaF* gene. The expression of *abaF* in *A. baumannii* AB250 and A10 were determined in the absence and presence of fosfomycin at 0.25X, 0.5X and 1X MIC. The results are shown in Figure 49.

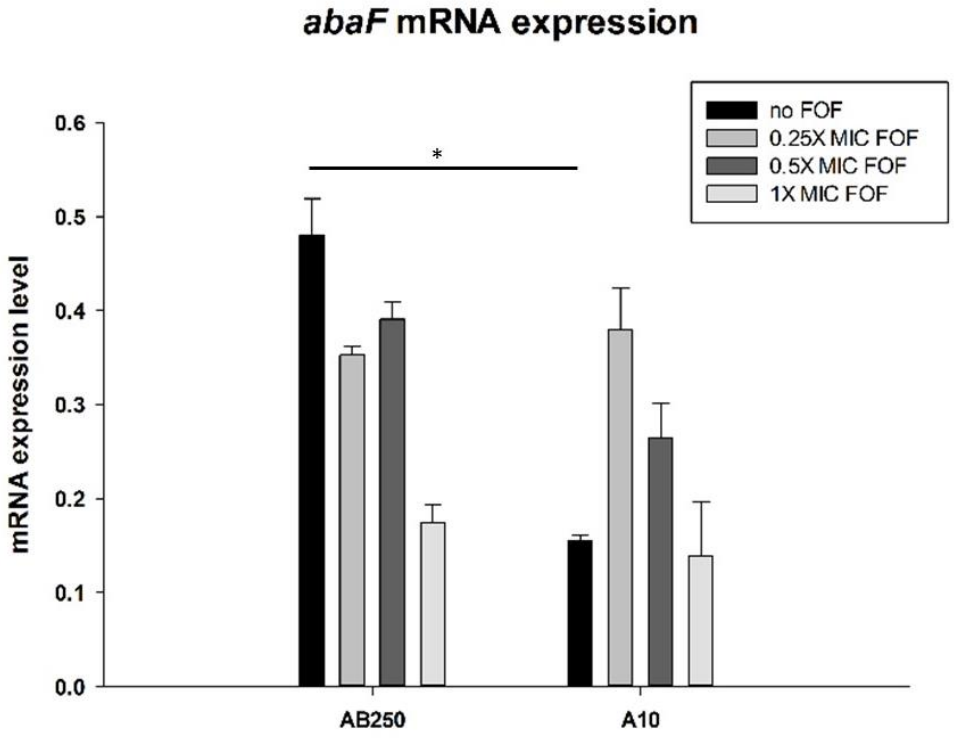


Figure 49. Expression of *abaF* mRNA in *A. baumannii* AB250 and A10
*: $p < 0.05$ (statistical analysis by using ANOVA)

At normal condition (no fosfomycin), AB250 (fosfomycin MIC = 128 mg/L) showed higher expression of *abaF* than that of A10 (fosfomycin MIC = 256 mg/L). However, in the presence of fosfomycin (0.25X and 0.5X of MIC), expression of efflux pump was increased in A10, but not AB250. The result suggests that AbaF efflux pump may be associated with fosfomycin susceptibility. However, there must be other mechanisms of resistance involved.

4. Detection of cell wall recycling bypass pathway

Recently, cell wall recycling bypass was reported to be the new possibility for intrinsic fosfomycin resistance in Gram-negative bacteria including *E. coli*, *P. aeruginosa* and *P. putida* (165). Therefore, in this study, cell wall recycling bypass pathway was determined in *Acinetobacter* spp. isolates.

4.1 Detection of genes in cell wall recycling bypass pathway

Genes involved in cell wall recycling bypass pathway including *ampG*, *nagZ*, *amnK*, *amgK* and *murU* were detected by PCR in 8 *A. baumannii*, 6 *A. pittii* and 2 *A. nosocomialis*. The results are shown in Table 32. All these genes were found in all tested *A. baumannii* and *A. nosocomialis* isolates. No tested *A. pittii* isolate carried all of these genes. AP1 and AP23 isolates lacked *amgK*, while AP7, AP14 and AP17 lacked *amgK* and *murU*. AP16 carried only *nagZ* and *amnK*. According to cell wall recycling bypass pathway in *P. putida*, *ampG* encoding anhydromuropeptide transporter and *murU* encoding uridylyl transferase which convert MurNAc alpha-1-phosphate to uridine diphosphate (UDP)-MurNAc. They were the first transporter and the last enzyme that important in cell wall recycling bypass pathway. Thus, *Acinetobacter* spp. that carried *ampG* and *murU* (AB250, A10, AP1, AP23, AN4 and AN12) were studied for expression level of these genes in the presence of fosfomycin.

4.2 Detection of *ampG* and *murU* mRNA expression

The level of *ampG* and *murU* expression without fosfomycin and with 0.25X, 0.5X or 1X MIC fosfomycin were detected by RT-PCR. Both AB250 and A10 showed decreased expression of *ampG* and *murU* in dose-dependent manner (Figure 50). In the presence of 0.25X MIC fosfomycin, *ampG* and *murU* expression were increased in isolate A10 and then expression level was decrease.

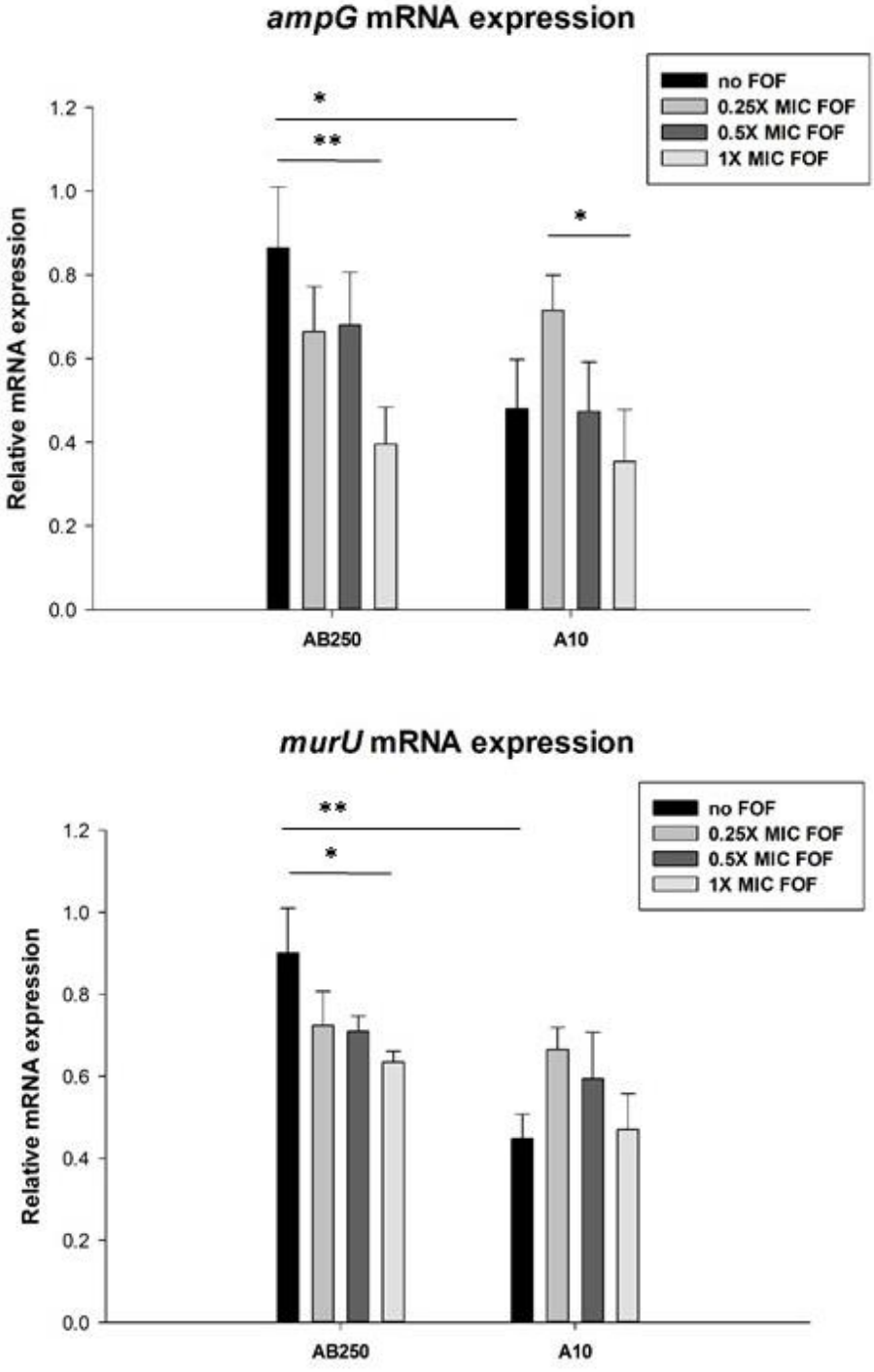


Figure 50. Expression of *ampG* and *murU* mRNA in *A. baumannii* AB250 and A10
*: $p < 0.05$; **: $p < 0.01$ (statistical analysis by using ANOVA)

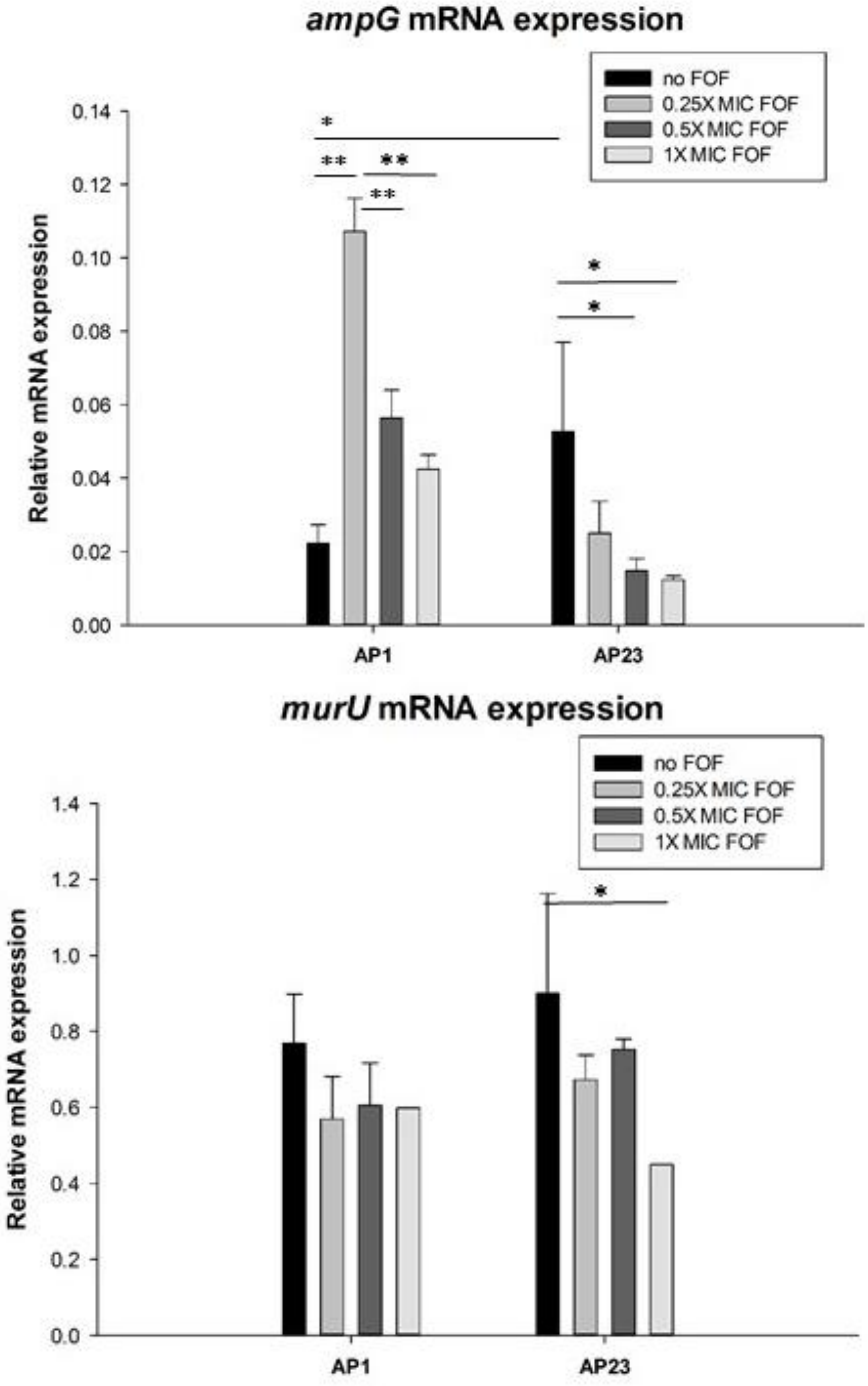


Figure 51. Expression of *ampG* and *murU* mRNA in *A. pittii* AP1 and AP23
*: $p < 0.05$; **: $p < 0.01$ (statistical analysis by using ANOVA)

In both *A. pittii*, expression levels of *ampG* were lower than *A. baumannii* and were decrease as dose-dependent manner (Figure 50-51). Moreover, isolate AP1 showed more increase expression of *ampG* in presence of 0.25X MIC fosfomycin. However, expression of *murU* showed a few decrease levels in the presence of fosfomycin (Figure 51).

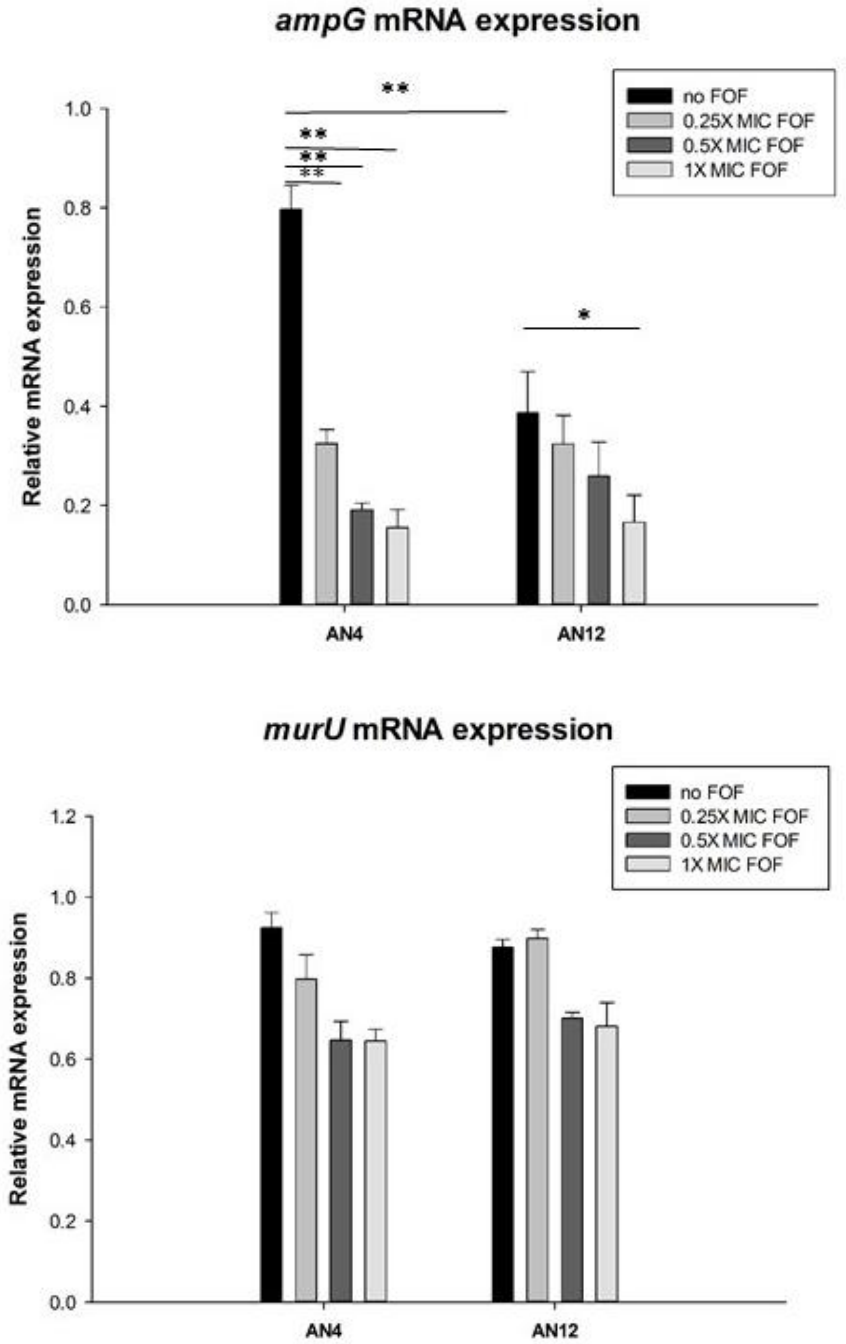


Figure 52. Expression of *ampG* and *murU* mRNA in *A. nosocomialis* AN4 and AN12
*: $p < 0.05$; **: $p < 0.01$ (statistical analysis by using ANOVA)

In *A. nosocomialis*, expression level of *ampG* and *murU* were decreased in the presence of fosfomycin (Figure 52).

The results indicate that the cell wall recycling pathway may occur in *Acinetobacter* spp., although some genes that involved in this bypass pathway could not be detected in some isolates. Therefore, this bypass pathway in *Acinetobacter* spp. should be further characterized.

13. DETERMINATION OF POPULATION ANALYSIS

1. Population analysis of fosfomycin susceptibility

For now, the intrinsic fosfomycin resistance mechanism in *Acinetobacter* spp. is still unclear. The results of time-kill by fosfomycin alone against all *Acinetobacter* spp. showed rapid killing at 4 hours and then the regrowth was observed. These regrowth populations may be some mutants or persisters. Thus, population analysis against fosfomycin was studied to figure out this regrowth population in 2 representative *A. baumannii* (AB250 and A10), 2 *A. pittii* (AP1 and AP23) and 2 *A. nosocomialis* (AN4 and AN12).

The population analysis of *A. baumannii* AB250 showed that about 10^3 of 10^9 CFU/mL of population persisted on MHA supplemented with fosfomycin above the MIC (MIC = 128 mg/L) (Figure 53). This result suggests that AB250 consists of heterogeneous resistance population. Similar to *A. baumannii* A10, 10^5 and 10^3 of 10^9 CFU/mL of A10 population survived on MHA supplemented with fosfomycin at the MIC (256 mg/L) and above the MIC (>256 mg/L), respectively (Figure 53). Although fosfomycin showed bactericidal effect on both AB250 and A10 (viable cells decrease $\geq 3\log_{10}$ CFU/mL on MHA supplemented with MIC of fosfomycin compared with no fosfomycin), the highly resistant subpopulations (about 10^3 CFU/mL) were observed.

In *A. pittii*, the population analysis showed similar profiles to *A. baumannii*. About 10^5 of 10^9 CFU/ml of AP1 and AP23 population persisted on MHA supplemented with fosfomycin at the MICs (256 and 128 mg/L, respectively) (Figure 54). This indicates that fosfomycin has bactericidal effect on *A. pittii*. However, fosfomycin above the MIC could not inhibit 10^3 CFU/mL subpopulation of both AP1 and AP23 (Figure 54).

In *A. nosocomialis* AN4 and AN12, heterogeneous resistance population to fosfomycin were found. About 10^4 of 10^9 CFU/mL of AN4 and AN12 population could grow on MHA supplemented with imipenem at the MICs (256 mg/L and 128 mg/L, respectively) and above the MIC (>256 and >128 mg/L, respectively) (Figure 55). The presence of subpopulation of AN4 and AN12 survivors indicate that they are heterogeneous resistance population to fosfomycin.

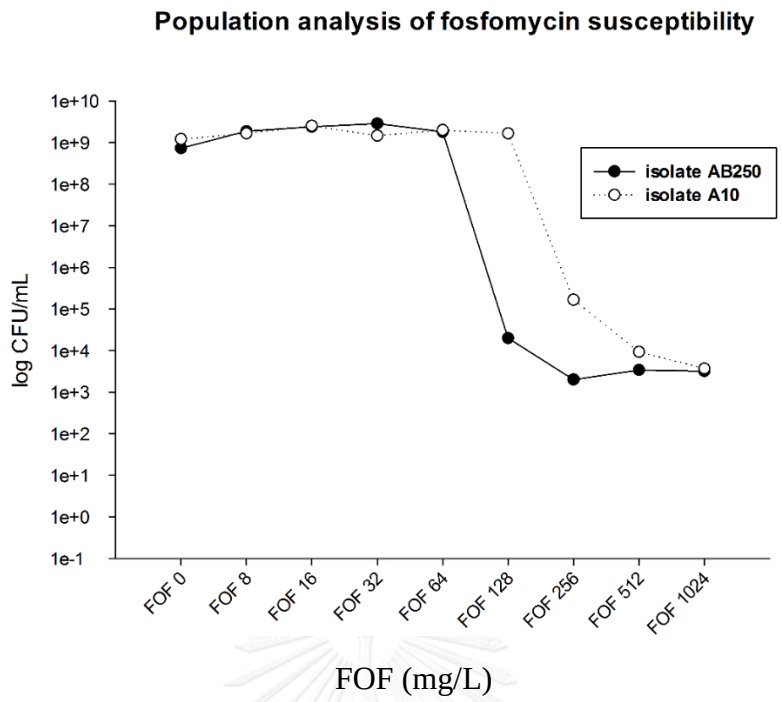


Figure 53. Population analysis of fosfomycin susceptibility in *A. baumannii* AB250 and A10

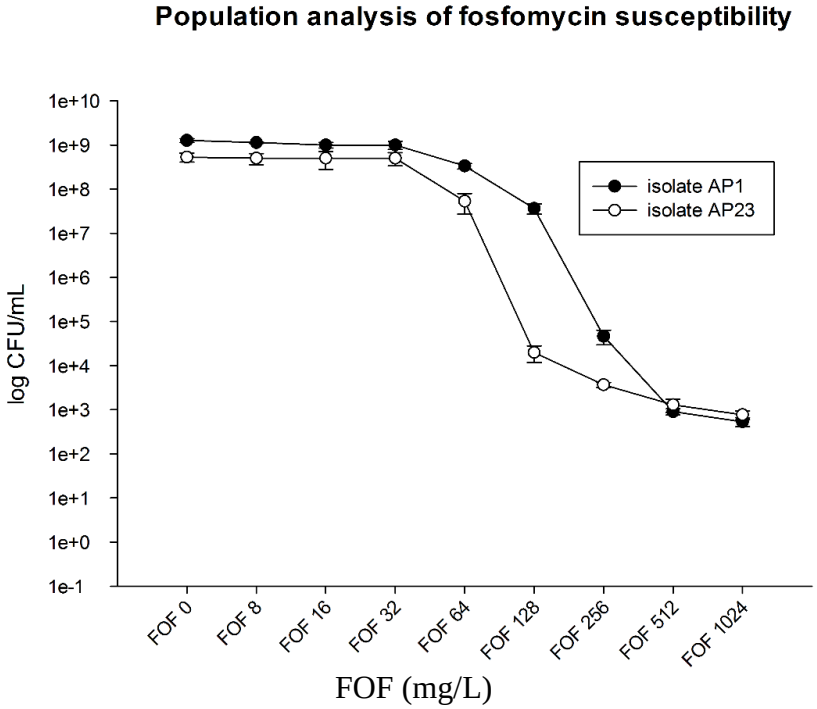


Figure 54. Population analysis of fosfomycin susceptibility in *A. pittii* AP1 and AP23

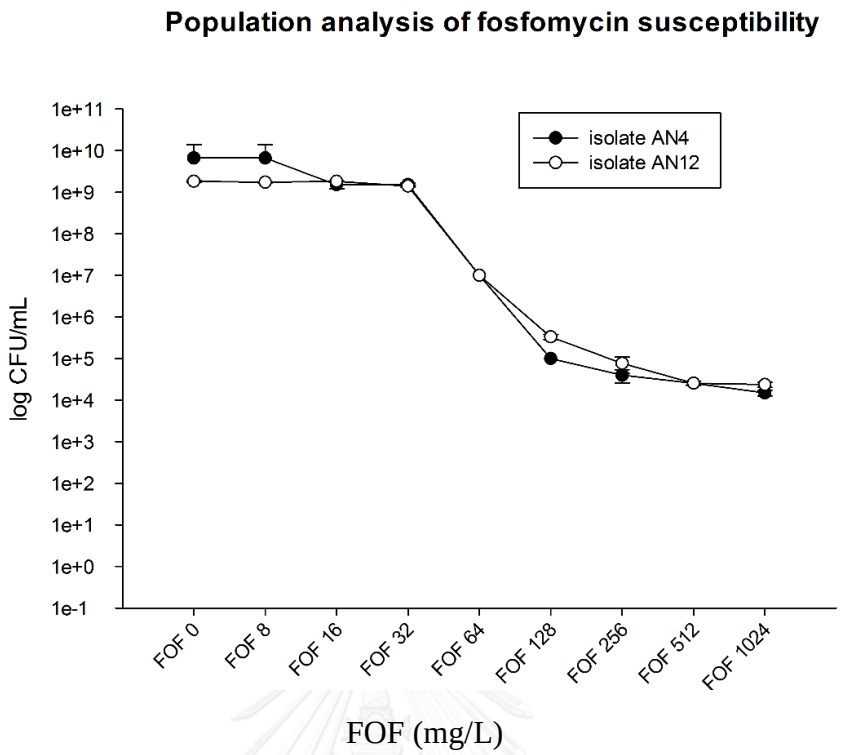


Figure 55. Population analysis of fosfomycin susceptibility in *A. nosocomialis* AN4 and AN12

These results from population study indicate that fosfomycin has bactericidal effect against *Acinetobacter* spp. isolates, although heterogeneous resistance population are present in all representative *Acinetobacter* spp. isolates. The frequent of persisted subpopulation to fosfomycin was about 10^{-6}.

These subpopulations were isolated and determined for fosfomycin susceptibility and resistance mechanisms. Two independent colonies on MHA supplemented with 512 mg/L of fosfomycin from AB250 and A10 were determined for fosfomycin susceptibility and characterized for resistance mechanisms. Fosfomycin MIC of subpopulation of AB250 and A10 were increased 2-fold from MIC of whole population (128 to 256 mg/L of AB250 and 256 to 512 mg/L of A10) indicating the subpopulation persistence to fosfomycin. Fosfomycin resistance mechanism including MurA mutation, fosfomycin-modifying enzymes, overexpression of efflux pump and cell wall recycling, were determined in these subpopulations. No resistance mechanism was found in these subpopulations.

All results from fosfomycin resistance studies conclude that *Acinetobacter* spp. are intrinsic resistance to fosfomycin and have population persistence to fosfomycin. However, the fosfomycin resistance mechanism in *Acinetobacter* spp. is still unclear. The possible mechanisms may be lack of transporter of fosfomycin, have other efflux pumps, other fosfomycin-modifying enzymes and different cell wall synthesis bypass mechanism which were not tested in this study.

2. Population analysis of imipenem susceptibility

According to the time-kill assay results, at 1X MIC of imipenem could not kill or inhibit growth of *Acinetobacter* spp. while at 1X MIC of fosfomycin could inhibit growth at first 4 hours. When 1X MIC of imipenem and 1X MIC of fosfomycin were combined, the synergism was present (except *A. baumannii* AB250). Imipenem had high activity in combination with fosfomycin, and it is possible that synergism occur by imipenem killing of fosfomycin persistence subpopulations. Thus, population analysis of imipenem was explored the magnitude of killing of imipenem against *Acinetobacter* spp. population. The 2 representative *A. baumannii* (AB250 and A10), 2 *A. pittii* (AP1 and AP23) and 2 *A. nosocomialis* (AN4 and AN12) were studied for population analysis profile of imipenem.

The population analysis of imipenem of *A. baumannii* A10 showed that about 10^5 of 10^9 CFU/mL of population could grow on MHA supplemented with imipenem at the MIC (16 mg/L) but no growth was seen at above MIC (>16 mg/L) (Figure 56). This result indicates that isolate A10 is homogeneous resistant population to imipenem and imipenem shows bactericidal effect on it. In contrast, *A. baumannii* AB250, about 10^6 and 10^3 of 10^9 CFU/mL of population could grow on MHA supplemented with imipenem at the MIC (16 mg/L) and 2X MIC (32 mg/L), respectively (Figure 56). Although imipenem showed bactericidal effect and AB250 showed homogeneous population, there are 10^3 CFU/mL that persist on MHA supplemented with 2X MIC of imipenem.

Two individual colonies of survivors isolate AB250 on 2X MIC imipenem were isolated and determined whether it is tolerance or persistence. The survivor of AB250 showed similar imipenem MIC to whole population (16 mg/L) indicated that it was tolerance.

A. pittii AP1 and AP23 showed that about 10^3 and 10^4 of 10^9 CFU/mL of population could survive on MHA supplemented with imipenem at the MIC (32 and 16 mg/L, respectively) (Figure 57). Both AP1 and AP23 did not grow on MHA supplemented with imipenem above the MIC (>32 and >16 mg/L, respectively) (Figure 57). These results suggest that imipenem has bactericidal effect and AP1 and AP23 were homogeneous resistance population to imipenem.

In *A. nosocomialis* AN4, imipenem MIC of 16 mg/L could completely kill AN4 in time-kill assay. Its population analysis profile showed that no growth of AN4 was seen on MHA supplemented with 16 mg/L of imipenem. While in isolate AN12, 10^3 CFU/mL of population could grow on MHA supplemented with imipenem at the MIC (32 mg/L) and no growth was detected in the presence of imipenem above the MIC (>32 mg/L). These results suggest that AN4 and AN12 were homogeneous resistance to bactericidal agent, imipenem.

These results showed that imipenem had bactericidal effect against carbapenem-resistant *Acinetobacter* spp. and carbapenem-resistant *Acinetobacter* spp. produced homogeneous resistance population to imipenem. However, only isolate AB250 showed tolerance to imipenem and no synergism of imipenem plus fosfomycin. Therefore, imipenem tolerance may be related with synergy of imipenem plus fosfomycin because imipenem plays an important role in this combination.

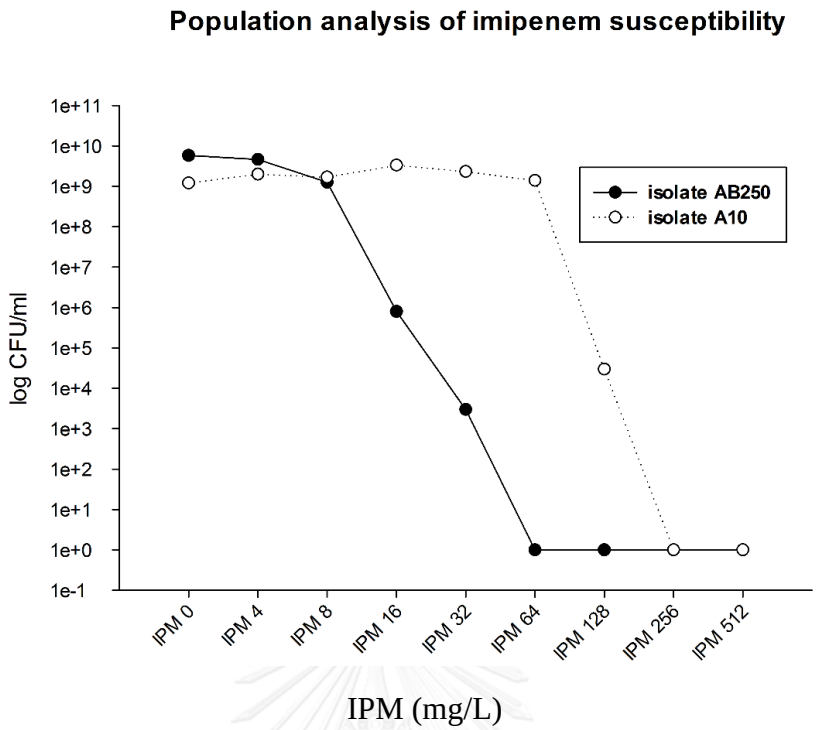


Figure 56. Population analysis of imipenem susceptibility in *A. baumannii* AB250 and A10

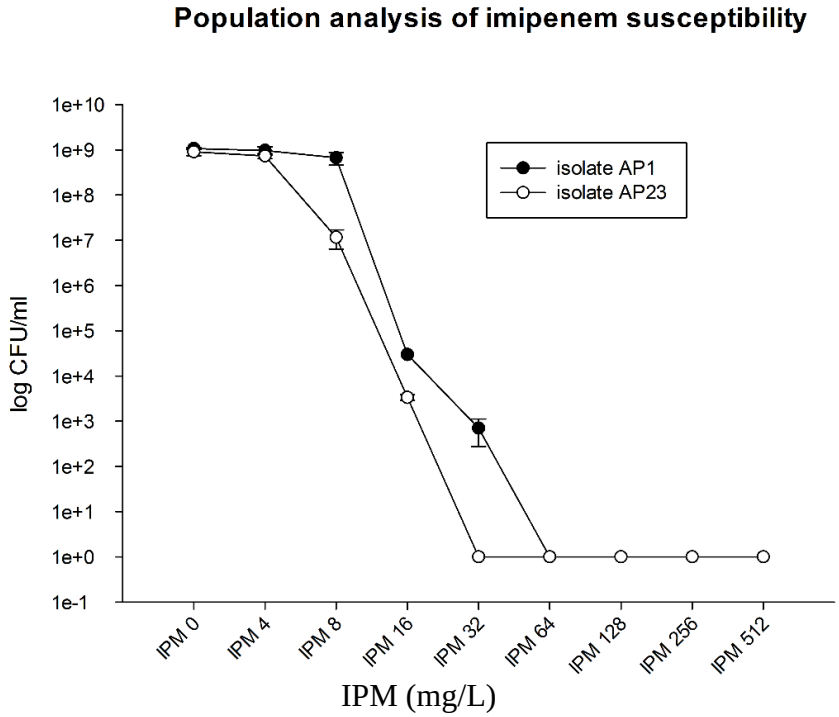


Figure 57. Population analysis of imipenem susceptibility in *A. pittii* AP1 and AP23

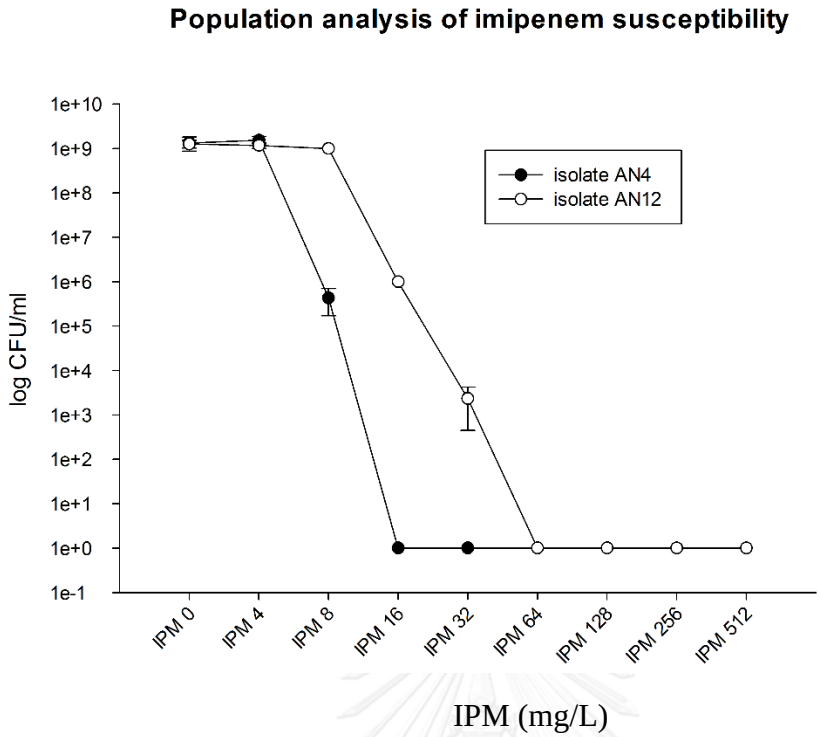


Figure 58. Population analysis of imipenem susceptibility in *A. nosocomialis* AN4 and AN12

The summarized results of carbapenem and fosfomycin susceptibilities, clonal study, carbapenem resistance mechanisms, activities of antibiotic combination by checkerboard and time-kill assay and population analyis of 23, 8 and 5 representative *A. baumannii*, *A. pittii* and *A. nosocomialis*, respectively are shown in Table 34-35.

Table 34. Summarized results of 23 representative *A. baumannii*

Isolate	IPM MIC	MEM MIC	FOF MIC	ST	carbapenemase	reduced OMP	efflux pump	CK	TK	FOF resistance subpopulation	IPM tolerance
AB1	32	32	128	806	OXA-66, OXA-23	CarO	-	I	ND	ND	ND
AB2	64	64	128	195	OXA-66, OXA-23	33-36 kDa	-	S	ND	ND	ND
AB3	16	32	128	1415	OXA-70 OXA-23	-	-	S	S	+	-
AB4	64	64	256	1423	OXA-66, OXA-23	-	-	S	ND	ND	ND
AB5	64	128	128	195	OXA-66, OXA-23	-	-	I	ND	ND	ND
AB6	32	32	128	195	OXA-66, OXA-23	-	-	S	ND	ND	ND
AB9	16	32	128	514	OXA-66, OXA-23	33-36 kDa	-	S	ND	ND	ND
AB29	32	64	128	542	OXA-67, OXA-23	33-36 kDa, OprD	-	S	S	+	-
AB58	32	64	256	542	OXA-67, OXA-23	CarO, 33-36 kDa, OprD	-	S	S	+	-
AB97	32	64	128	542	OXA-67 OXA-23	CarO, 33-36 kDa	-	S	ND	ND	ND
AB22	16	8	128	208	OXA-68, OXA-58	33-36 kDa	-	S	S	+	-
AB25	16	16	128	1416	OXA-95, OXA-24	CarO, 33-36 kDa, OprD	-	S	No	+	+
AB13	32	64	128	542	OXA-67, OXA-23	33-36 kDa	-	I	ND	ND	ND
AB35	32	64	256	1417	OXA-66, OXA-23	-	-	S	ND	ND	ND
AB55	32	64	256	1166	OXA-66, OXA-23	-	-	I	ND	ND	ND
AB35	64	128	256	1423	OXA-66, OXA-23	-	-	I	ND	ND	ND
A4	64	64	256	1417	OXA-51-like, OXA-23	-	-	S	S	+	-
A5	32	64	256	229	OXA-51-like, OXA-23	-	-	I	ND	ND	ND
A6	64	64	256	1001	OXA-51-like, OXA-58	-	-	S	S	+	-
A7	64	64	256	195	OXA-51-like, OXA-23	-	-	I	ND	ND	ND
A8	64	128	256	1418	OXA-51-like, OXA-23	-	-	I	ND	ND	ND
A9	64	128	256	551	OXA-51-like, OXA-23	-	-	S	ND	ND	ND
A10	128	256	256	1426	OXA-51-like, OXA-24	33-36 kDa, OprD	-	S	S	+	+

-, Negative; +, Positive; I, Indifferent; S, Synergism; No, No synergism; ND, Not determined

Table 35. Summarized results of 8 and 5 representative *A. pittii* and *A. nosocomialis*, respectively

Isolate	IPM MIC	MEM MIC	FOF MIC	ST	carbapenemase	reduced OMP	efflux pump	CK	TK	FOF resistance subpopulation	IPM tolerance
AP1	32	32	256	1419	OXA-58, IMP-14a	-	+	S	S	+	-
AP4	32	32	128	1424	OXA-23	-	-	S	ND	ND	ND
AP7	0.5	1	128	1425	OXA-58	CarO	-	I	ND	ND	ND
AP8	0.5	1	128	1425	OXA-58	-	-	I	ND	ND	ND
AP14	16	8	256	1178	OXA-58	CarO, 33-36 kDa, OprD	+	S	S	ND	ND
AP16	32	32	128	1427	OXA-23	CarO, 33-36 kDa	+	S	S	ND	ND
AP17	1	1	256	1178	OXA-58	CarO, 33-36 kDa, OprD	-	I	ND	ND	ND
AP23	16	32	128	1420	OXA-23	-	-	S	S	+	-
AN1	16	32	128	958	OXA-23	-	-	I	ND	ND	ND
AN4	16	32	256	958	OXA-23	-	-	S	S	+	-
AN12	32	64	128	958	OXA-23	-	-	S	S	+	-
AN15	16	32	256	958	OXA-23	-	-	S	ND	ND	ND
AN20	16	32	256	958	OXA-23	-	-	S	ND	ND	ND

-, Negative; +, Positive; I, Indifferent; S, Synergism; No, No synergism; ND, Not determined

CHAPTER VI
DISCUSSION

Acb complex is one of the most common pathogens of nosocomial infection. Identification of species in Acb complex by using biochemical tests is unreliable (1). Although *A. baumannii* are the most important pathogen in this genus (189), *A. pittii* and *A. nosocomialis* infection have been reported in many studies (2, 3, 190, 191). In this study, among 346 *Acinetobacter* spp. from many types of specimen during 2010 to 2011, *A. baumannii*, *A. pittii* and *A. nosocomialis* were found in 88.2%, 6.3% and 5.5%, respectively. In Taiwan, among 135 *Acinetobacter* spp. from bacteremia patients during 2007 to 2009, *A. baumannii*, *A. pittii* and *A. nosocomialis* were found in 64.4%, 8.9% and 26.7%, respectively by using 16S-23S ribosomal RNA intergenic spacer sequencing identification (2). In U.S., among 147 *Acinetobacter* spp. from bacteremia patients during 2005 to 2012, *A. baumannii*, *A. pittii* and *A. nosocomialis* were found in 78.9%, 19.0% and 2.0%, respectively by using *rpoB* sequencing identification. All these 3 studies showed similar result that *A. baumannii* is the major pathogen in Acb complex, although they were identified by different methods.

In the present study, *Acinetobacter* spp. isolated from patients in non-ICUs were higher than ICU patients. This may be because at that time there were greater specimen from non-ICUs. On the other hand, this suggests that *Acinetobacter* spp. are successful pathogen in both ICUs and non-ICUs. In this study, most *Acinetobacter* spp. were isolated from sputum (40.8%). The data from the National Antimicrobial Resistance Surveillance Center, Thailand (NARST) showed that during 2016 from 55 hospitals, 58.9% of Acb complex were isolated from sputum (192).

Acinetobacter spp. were identified for species by three methods including biochemical tests, the presence of $bla_{\text{OXA-51}}$ and multiplex PCR for *gyrB*. The biochemical tests could not differentiate species of Acb complex, as described by Necmec (1). The detection of the presence of $bla_{\text{OXA-51}}$ was suggested for *A. baumannii* identification by Turton *et al.* (66). However, the disrupted $bla_{\text{OXA-51}}$ by IS*Aba16* (193) and IS*Aba19* (194) have been reported. The interrupted $bla_{\text{OXA-78}}$ by IS*Aba19* resulted in larger amplicon of $bla_{\text{OXA-51}}$ by multiplex PCR for OXA-type carbapenemases (194).

In this study, the same interruption was found in *A. baumannii* AB182, but the result was negative for *bla*$_{\text{OXA-51}}$ amplicon by multiplex PCR. Moreover, *bla*$_{\text{OXA-51}}$ was found in non-*A. baumannii* including *A. nosocomialis* (67) and *A. seifertii* (195). The *bla*$_{\text{OXA-51}}$ was not found in *A. pittii* and *A. nosocomialis* in this study. These results suggest that only the presence of *bla*$_{\text{OXA-51}}$ is not reliable for *A. baumannii* identification. The multiplex PCR for *gyrB* was developed for identification species of Acb complex by Higgins *et al*. (169). In this study, all *bla*$_{\text{OXA-51}}$-carrying isolates were identified as *A. baumannii* by *gyrB* multiplex PCR.

The prevalence of carbapenem resistance in *Acinetobacter* spp.

In this study, carbapenem resistance rate in *A. baumannii* (86.6%) was higher than *A. nosocomialis* (26.3%) and *A. pittii* (22.7%), respectively. Similar to the study from Taiwan, the imipenem susceptible rates were 48.8% in *A. baumannii*, 66.7% in *A. pittii* and 94.3% in *A. nosocomialis* (2). However, the resistance rates of all 3 species in present study were higher than in Taiwan. The data from NARST showed that imipenem and meropenem susceptible rates in Acb complex during 2016 were 29.4% and 29.3%, respectively (192). The imipenem susceptible rate in *A. baumannii* isolated from a university hospital in Thailand was 23.3% (196). In the case of *A. baumannii*, carbapenem resistance rate in this study was higher than other studies in Thailand. This may be because King Chulalongkorn Memorial Hospital is a referral hospital that provides for other hospital transferred patients. Moreover, amikacin and ciprofloxacin resistance rate in *A. baumannii* of this study (75.4% and 84.9%) was similar to Songklanagarind Hospital, Thailand (81.2% and 81.7%) and Taiwan (72.1% and 82.6%). However, carbapenem resistance rate of *A. baumannii* in this study was lower than that in Vietnam which carbapenem resistance was up to 100% (197). The emergence of carbapenem resistance in *A. baumannii* has been reported worldwide, but not in Japan. The prevalence of carbapenem resistance was only 1.84% in Japan (198). No colistin-resistant *A. baumannii* was found in this study, Songklanagarind Hospital and Taiwan. However, NARST reported that colistin resistance in Acb complex was 0.1% in 2016. Colistin-resistant *A. baumannii* was reported to be 5% in Vietnam in 2011 to 2013 (197).

In Thailand, *A. pittii* and *A. nosocomialis* have been reported from Songklanagarind Hospital (199). All of 18 *A. nosocomialis* and 7 *A. pittii*, isolated from

patients with hospital-acquired infection, were susceptible to imipenem and meropenem (199). Therefore, the present study is the first report of carbapenem resistance in *A. pittii* and *A. nosocomialis* in Thailand.

In Latin America, of the 118 *Acinetobacter* spp., 5 isolates were identified as *A. pittii* and one of them (20%) was resistant to carbapenems. In addition, six isolates were identified as *A. nosocomialis* and 3 of them (50%) were resistant to carbapenems (200). In Taiwan, of the 135 *Acinetobacter* spp., 12 isolates were identified as *A. pittii* and 33.3% showed resistance to carbapenems. There were 36 isolates identified as *A. nosocomialis* and 5.7% showed resistance to carbapenems (2). In South Korea, of the 287 *Acinetobacter* spp., 15 isolates were identified as *A. pittii* and 8 of them (53.3%) were resistant to carbapenems. However, 98 isolates were identified as *A. nosocomialis* and 4 of them (4.1%) were resistant to carbapenems (112). Thus, in the present study, prevalence of carbapenem resistance in *A. pittii* was higher than reports from Latin America but was lower than those reported from Taiwan and South Korea. The prevalence of carbapenem-resistant *A. nosocomialis* in this study was higher than those in Taiwan and South Korea but lower than in Latin America. The amikacin resistance rate in *A. pittii* (18.2%) and *A. nosocomialis* (0%) in this study were lower than the report from Taiwan (33.3% and 19.4%, respectively) (2). Ciprofloxacin resistance rate in *A. pittii* (31.8%) was higher and in *A. nosocomialis* (5.3%) was lower than the report from Taiwan (2). No colistin-resistant *A. pittii* and *A. nosocomialis* was found in this study and in Taiwan. However, 2 isolates of colistin-resistant *A. nosocomialis* were found in South Korea.

This study showed that antibiotic resistance rates especially carbapenem resistance in *A. baumannii* were extremely higher than *A. pittii* and *A. nosocomialis*. Therefore, correct species identification is very important for treatment and prevalence of antibiotic resistance study.

The mechanisms of carbapenem resistance in *Acinetobacter* spp.

Of the 305 *A. baumannii* isolates, 41 carbapenem-susceptible isolates were negative for carbapenemase activity and did not carry carbapenemase genes. Of all 264 carbapenem-resistant *A. baumannii*, *bla*$_{\text{OXA-23-like}}$ was found in 262 isolates (99.2%), *bla*$_{\text{OXA-58-like}}$ in 1 isolate (0.4%) and *bla*$_{\text{OXA-24-like}}$ in 1 isolate (0.4%). The *bla*$_{\text{OXA-23}}$ was the major carbapenemase in carbapenem-resistant *A. baumannii* in this study. The report from Siriraj Hospital showed that all 37 randomly selected carbapenem-resistant *A. baumannii* carried *bla*$_{\text{OXA-23-like}}$ (201). The data from regional hospital in the north of Thailand also showed that all 13 carbapenem-resistant *A. baumannii* carried *bla*$_{\text{OXA-23-like}}$ (202). The data from King Chulalongkorn Memorial Hospital during January 2004 to August 2007 showed that of the 413 carbapenem-resistant *A. baumannii*, 86.2% carried *bla*$_{\text{OXA-23-like}}$, 1.0% carried *bla*$_{\text{OXA-24-like}}$, 1.4% carried *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-24-like}}$, 1.7% carried *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-58-like}}$, 5.8% carried *bla*$_{\text{OXA-24-like}}$ and *bla*$_{\text{OXA-58-like}}$ and 3.9% carried *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24-like}}$ and *bla*$_{\text{OXA-58-like}}$ (203). Later, during 2008 to 2010, of the 402 carbapenem-resistant *A. baumannii*, 397 isolates carried *bla*$_{\text{OXA-23-like}}$, 1 isolate carried *bla*$_{\text{OXA-24-like}}$ and 4 isolates carried *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-58-like}}$ (204). In this study, the majority of carbapenem-resistant *A. baumannii* carried *bla*$_{\text{OXA-23-like}}$, 1 isolate carried *bla*$_{\text{OXA-24-like}}$ and 1 isolate carried *bla*$_{\text{OXA-58-like}}$. This indicates that *bla*$_{\text{OXA-58-like}}$ and *bla*$_{\text{OXA-24-like}}$ in *A. baumannii* were replaced by *bla*$_{\text{OXA-23-like}}$. The transition of carbapenemase genes in *A. baumannii* has been reported in Southern China (205). In Southern China, during 2002 to 2009, the *bla*$_{\text{OXA-58-like}}$-carrying *A. baumannii* isolates were replaced by *bla*$_{\text{OXA-23-like}}$-carrying isolates (205), whereas in Italy, during 2005 to 2009, *bla*$_{\text{OXA-23-like}}$-carrying *A. baumannii* isolates were displaced by *bla*$_{\text{OXA-58-like}}$-carrying isolates (206). Nowadays, *bla*$_{\text{OXA-23}}$-carrying *A. baumannii* has been emerged worldwide. In Japan, the prevalence of carbapenem-resistant *A. baumannii* was 1.84%. All carbapenem-resistant isolates haboured *bla*$_{\text{OXA-23-like}}$ (198).

In this study, the prevalence of *bla*$_{\text{OXA-58-like}}$ in *A. baumannii* was 0.4%. This prevalence was as low as other countries in Asia including India (4.2%) (207), Iran (3.2%) (208) and Vietnam (0.35%) (209). In contrast, the prevalence of *bla*$_{\text{OXA-58-like}}$ was high in Europe including Greece (100%) (210), Italy (22.8%) (206) and Turkey (79%) (211). The data from worldwide and this study suggest that emergence of *bla*$_{\text{OXA-}}$

$_{58}$-isolates are replaced by *bla*$_{OXA-23}$-carrying isolates. This may be because *bla*$_{OXA-58}$-carrying isolates conferred lower carbapenem resistance (205).

The *bla*$_{OXA-24}$ is the carbapenemase that was firstly reported in Spain (123). After that, prevalence of *bla*$_{OXA-24}$-carrying *A. baumannii* especially *bla*$_{OXA-72}$ variant has been reported with lower prevalence rate than other carbapenemase genes in worldwide including Turkey (4.7%) (212), Romania (a case report from chronic leg ulcer culture) (213), Brazil (a few case reports from wound, tracheal aspirate and urine culture) (214, 215) and China (1.5%) (216). In Thailand, the first report of *bla*$_{OXA-72}$-carrying *A. baumannii* was in 2004 (unpublished of GenBank number AY739646.1). In the study from Siriraj Hospital, *bla*$_{OXA-24}$ was not found in *A. baumannii* (201). The trend of prevalence of *bla*$_{OXA-24}$-carrying *A. baumannii* in King Chulalongkorn Memorial Hospital was decreased from 6.3% in 2004 to 2007 (203), to 0.2% in 2008 to 2010 (204) and 0.4% in this study.

This study is the first report that characterized the prevalence and mechanism of carbapenem resistance in Acb complex including *A. pittii* and *A. nosocomialis* in Thailand. The prevalence of carbapenem resistance in *A. pittii* was 22.7%. From all 5 carbapenem-resistant isolates, 1 carried *bla*$_{OXA-58}$ with *bla*$_{IMP-14a}$, 1 carried *bla*$_{OXA-58}$ and 3 carried *bla*$_{OXA-23}$. An *A. pittii* isolate co-harboring *bla*$_{OXA-58}$ and *bla*$_{IMP-4}$ variant has been isolated from wound infection of an Australian patient (217), whereas carbapenem-resistant *A. pittii* in Taiwan carried *bla*$_{OXA-58}$ and *bla*$_{IMP-1}$ (218). One carbapenem-resistant *A. pittii* carrying only *bla*$_{OXA-58}$ was found in China, however it was susceptible to carbapenems (219). Similar to the present study, 3 isolates of carbapenem-susceptible *A. pittii* also carried *bla*$_{OXA-58}$. In China, *bla*$_{OXA-58}$-carrying *A. pittii* was susceptible to carbapenems although there were IS*Aba3* or truncated of its upstream *bla*$_{OXA-58}$ (220). This result was similar to the report from Ireland that *A. pittii* carrying *bla*$_{OXA-58}$ with upstream and downstream by IS*Aba3* was susceptible to carbapenems (221). In the present study, carbapenem-resistant *A. baumannii* carrying *bla*$_{OXA-58}$ showed relative mRNA expression about 1.2 but both resistant and susceptible *A. pittii* isolates showed expression level about 0.2 to 0.6. This suggests that the expression level of *bla*$_{OXA-58}$ does not confer carbapenem resistance in *A. pittii*. However, *A. pittii* carrying *bla*$_{OXA-58}$, AP17 (susceptible isolate), had higher expression level than that of AP14 (resistance isolate) but AP14 showed higher *adeE* efflux

expression. This concludes that *bla*$_{OXA-58}$ alone is inadequate to confer resistance but its combination with overexpression of efflux pump results in carbapenem resistance. All of *A. pittii* carrying *bla*$_{OXA-23}$ without reduction of OMPs and with or without overexpression of efflux pumps were resistant to carbapenems. These results indicate that *bla*$_{OXA-23}$ is adequate for carbapenem resistance, according to high level of expression (relative mRNA expression about 1.4). However, other mehanisms may involve in carbapenem resistance. The results are similar to *A. pittii* isolates from Ireland, Colombia and South Korea (79). However, the report from China, Hong Kong, Taiwan, Italy, Germany and Ireland, carbapenem-susceptible *A. pittii* also carried *bla*$_{OXA-23}$ (79, 221). These suggest the requirement of other mechanisms to confor carbapenem resistance.

In this study, carbapenem-resistant *A. nosocomialis* showed the same clone with the same OXA-carbapenemase gene, *bla*$_{OXA-23}$. This is similar to carbapenem-resistant *A. nosocomialis* reported from Brazil (200) and South Africa (79), that harbored *bla*$_{OXA-23}$. However, carbapenem-susceptible *A. nosocomialis* carrying *bla*$_{OXA-23}$ was also reported in Brazil (200). The emergence of carbapenem-non-susceptible *A. nosocomialis* carrying *bla*$_{SIM}$ (112) and *bla*$_{OXA-182}$ (variant of *bla*$_{OXA-143}$) (222) were observed in South Korea.

Interestingly, carbapenem-susceptible *A. pittii* and *A. nosocomialis* which carried carbapenemase genes including *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ have been reported in many studies (79, 200, 221). The results suggest that *Acinetobacter* spp. are harboring sources of carbapenemase genes.

The second mechanism of carbapenem resistance is reduction or loss of porins, outer membrane proteins that uptake carbapenems into bacterial cell. The first OMP reported in carbapenem-resistant *A. baumannii* is CarO (9). The conformation of CarO showed the channel formation but no specific site for imipenem was found (223). However, this result is controversial with the study by Catel-Ferreira *et al*. which showed that the primary structure of CarO was specific for imipenem, but not meropenem and reduced *carO* expression was related to carbapenem resistance level (131). After that, many studies have been reported that reduction or loss of CarO was present in carbapenem-resistant *A. baumannii* clinical isolates (132, 224). In the present study, reduction of CarO was found in 4 *A. baumannii*, 3 of which carried *bla*$_{OXA-23}$ and

had imipenem MIC of 32 mg/L. The other isolate carried *bla*$_{\text{OXA-24}}$ and had imipenem MIC of 16 mg/L. Two of them (AB58 and AB97) showed interrupted *carO* gene by a novel insertion sequence, IS*Aba40*. This study is the first report of IS*Aba40* and the first report of interrupted *carO* by IS*Aba40*. CarO interrupted by insertion sequences was reported in many studies including IS*Aba1* (225), IS*Aba125* (135), IS*Aba825* (135), IS*Aba10* (134) and IS*Aba15* (226). In this study, interrupted *carO A. baumannii* AB58 and AB97 belonged to the same clone (ST542) as AB29 carrying intact *carO*. However, all of these 3 isolates showed the same carbapenem MICs. Similar to the study by Kim *et al*., *A. baumannii* carrying *bla*$_{\text{OXA-23}}$ with interrupted *carO* by IS*Aba15* and intact *carO*, belonging to the same clone (ST92) showed similar level of carbapenem resistance (226). Moreover, the report from Taiwan showed that *A. baumannii* carrying *bla*$_{\text{OXA-72}}$ with interrupted *carO* by IS*Aba1* had imipenem MIC between 64 to 128 mg/L, whereas isolates with intact CarO had carbapenem MIC of 32 to >128 mg/L (225). This indicates that interrupted *carO* does not play an important role in carbapenem resistance mechanism in *A. baumannii*.

In this study, reduction of CarO was detected in 4 *A. pittii* isolates with imipenem MIC of 0.5 to 32 mg/L and meropenem MIC of 1 to 32 mg/L, whereas normal CarO expression isolates had imipenem MIC of 0.5 to 32 mg/L. Three of them carried *bla*$_{\text{OXA-58}}$ and another isolate carried *bla*$_{\text{OXA-23}}$. All of 4 *A. pittii* isolates with reduced CarO harbored intact *carO*. A study from South Korea showed that all 8 carbapenem-resistant *A. pittii* did not carried *carO* gene (112). The results indicate that *carO* gene was not present in all *A. pittii* isolates and reduction of expression is not important for high-level resistance to carbapenems. Another report from Canada found that *A. pittii* with or without reduced *carO* expression was still susceptible to carbapenems (227). This suggests that only reduced *carO* expression is inadequate to confer carbapenem resistance. All *A. nosocomialis* isolates in this study had normal CarO expression. In Canada, reduction of *carO* expression was observed in all 4 *A. nosocomialis*, but all of them were susceptible to carbapenems (227). This confirms that reduced CarO is not sufficient to confer carbapenem resistance in *A. nosocomialis*.

The reduction of 33-36 kDa OMP was first described in imipenem-resistant *A. baumannii* by Clark *et al*. (10). The reduced expression of 33-36 kDa OMP was reported to be associated with carbapenem resistance (228). In this study, the reduced

33-36 kDa OMP was found in 9 *A. baumannii* isolates with the imipenem MICs of 16 to 128 mg/L. Three of them also had reduction of other OMPs and showed imipenem MICs of 16-128 mg/L. Whereas, the isolates with reduced 33-36 kDa OMP only had imipenem MICs of 16-64 mg/L. This indicates that reductions of 33-36 kDa OMP or other OMPs do not have a major effect on carbapenem MIC. In *A. pittii*, there were reduced 33-36 kDa OMP in 3 isolates with imipenem MICs of 1-16 mg/L. All of them also showed reduction of other OMPs. However, the imipenem MIC was not different from that of isolates with normal expression of all 3 OMPs (0.5-32 mg/L). This is the first report of reduced 33-36 kDa OMP in *A. pittii*. However, it is not the major mechanism of carbapenem resistance.

The 43 kDa OMP was described as homologue OMP to OprD in *P. aeruginosa* which is an imipenem transporter (11). This OMP had specific channel for imipenem uptake (137). In this study, reduced OprD was found in 4 *A. baumannii* isolates with imipenem MICs of 16-128 mg/L, whereas no reduced OMP isolates had MIC of 16-64 mg/L. Similar to the study by Smani *et al*. (138) imipenem MICs of these isolates were indifferent. The *oprD* gene of *A. baumannii* ATCC 17978 (susceptible to carbapenems) was knocked out, but its carbapenem MIC was not changed. Many studies including the present study suggest that the 43 kDa OMP may not involve in carbapenem resistance in *A. baumannii*.

In this study, reduced OprD was found in both carbapenem-susceptible (AP17) and carbapenem-resistant *A. pittii* (AP14) carrying $bla_{\text{OXA-58}}$ isolates. This is similar to *A. pittii* isolates from South Korea that expression level of *oprD* in resistant and susceptible isolates was indifferent (112). No reduced OprD was observed in any *A. nosocomialis* isolates. Carbapenem-susceptible *A. nosocomialis* isolates showed similar level of *oprD* expression to resistant isolates (112). The results suggest that reduction of OprD is not associated with carbapenem resistance in *A. nosocomialis*.

Another mechanism of carbapenem resistance is overexpression of efflux pumps that extrude carbapenems outside bacterial cells. The overexpression of efflux activity was detected by using efflux pump inhibitors. Positive result for overexpression of efflux pump activity was defined as the decrease at least 4-fold of carbapenem MICs in the presence of inhibitors. However, the result of efflux pump activity by using inhibitor against carbapenem-resistant *A. baumannii* clinical isolates from many studies

are still controversial (182, 229-232). In this study, two inhibitors including CCCP and reserpine were used for the detection of efflux pump activity. The CCCP acts as strong uncoupling agent that interrupts electrochemical gradient of bacterial cell, thus, efflux pumps which act by proton motive force will be inhibited. The reserpine acts as the blocker of substrate channel of efflux pumps. In this study, no overexpression of efflux pump activity was detected by both inhibitors. This is similar to the studies of carbapenem resistance mechanism in *A. baumannii* isolates from Spain and the US (123, 233). However, many studies from Taiwan and China showed about 2 to 32-fold reduction of carbapenem MICs in the presence of inhibitors (234-236).

Detection of efflux pump activity in *A. pittii* and *A. nosocomialis* has been evaluated by Park *et al*. by using efflux pump inhibitor, phenyl-arginine-β-naphthylamide (PAβN) (112). They found overexpression of efflux pump in one *A. nosocomialis* but this strain carried $bla_{\text{SIM-1}}$ gene with low level of resistance to imipenem and meropenem (MIC 8 mg/L and 16 mg/L, respectively). Thus, efflux pumps did not confer high-level resistance to carbapenems in this *A. nosocomialis* isolate. In this study, there was no overexpression of efflux pump activity in carbapenem-resistant *A. baumannii*, *A. pittii* and *A. nosocomialis*. This may be because using inhibitor has less sensitivity for detection. This method requires at least 4-fold decrease of carbapenem MIC for overexpression. Thus, it is possible that overexpression of efflux pump is insufficient to confer high level carbapenem resistance. Another limitation of this method is the specificity of inhibitors. The CCCP interferes proton gradient of bacterial cell, thus effects on all proton motive force efflux pumps in bacteria (237). Whereas the reserpine blocks the substrate channel of efflux pumps, thus, it can block many efflux pumps (237).

As there is a limitation of overexpression of efflux pump detected by using inhibitors. The expression levels of mRNA of efflux pumps genes were evaluated. In *A. baumannii*, the efflux pump that has been reported involving carbapenem resistance is AdeABC efflux pump. The AdeABC pump is resistance-nodulation-division (RND) system that is composed of outer membrane protein (AdeC), major infusion protein (AdeA) and transporter protein (AdeB). Thus, the AdeB is an important part of this efflux pump. The first report of this efflux pumps involved in aminoglycoside resistance in *A. baumannii* was the study of Magnet *el al*. (238). Later, many studies showed that

the mRNA expression of *adeB* gene were significantly higher in carbapenem-resistant than carbapenem-susceptible *A. baumannii* isolates (129, 239, 240). Therefore, in the present study, the expression level of *adeB* mRNA was evaluated in carbapenem-resistant *Acinetobacter* spp. compared to carbapenem-susceptible strains.

No carbapenem-resistant *A. baumannii* (AB3, A4, AB29, AB58, AB227, A6, AB250 and A10) showed higher expression level of *adeB* than *A. baumannii* ATCC 19606 (carbapenem-susceptible strain). Although no overexpression of *adeB* was detected in any isolates, $bla_{\text{OXA-23}}$-carrying isolate AB4 showed higher expression of *adeB* than AB3 and also had higher imipenem and meropenem MICs. This is similar to $bla_{\text{OXA-58}}$- and $bla_{\text{OXA-24}}$-carrying *A. baumannii* isolates. *A. baumannii* A6 had higher expression of *adeB* and higher carbapenem MICs than AB227, although both of them carried $bla_{\text{OXA-24}}$. *A. baumannii* A10 had higher *adeB* expression and higher carbapenem MICs than AB250 although both of them carried $bla_{\text{OXA-58}}$. These results were similar to study of Cardoso *et al*. that among $bla_{\text{OXA-23}}$-carrying *A. baumannii* overexpression of *adeB* involved in high carbapenem resistance (241). The results indicate that although expression of efflux pump is not the major mechanism to confer carbapenem resistance, the combination of efflux pump with carbapenemase production can confer high-level carbapenem resistance in *A. baumannii*.

In *A. pittii*, efflux pumps that involved in carbapenem resistance are AdeDE and AdeXYZ (126, 130). Both of them belong to RND system. The AdeDE is composed of inner membrane protein (AdeZ), major infusion protein (AdeX) and transporter protein (AdeY). The AdeY is an important part of this efflux pump. The AdeDE efflux pump include AdeD, major infusion protein and AdeE, transporter protein without specific outer membrane protein. Thus, AdeE is an important part of this efflux pump. The *adeE* and *adeY* genes have been found in 1% of *A. pittii* isolates (126). In the present study, both of these genes were found in all 7 tested isolates. The overexpression of *adeE* and *adeY* mRNA in carbapenem-resistant *A. pittii* was compared to that of carbapenem-susceptible isolate, AP3. Three isolates (AP14, AP1 and AP16) with overexpression of *adeE* had high imipenem MICs (16 to 32 mg/L) and meropenem MICs (8 to 32 mg/L), whereas isolates with no overexpression of *adeE* (AP7, AP17 and AP23) had lower imipenem MIC (0.5 to 16 mg/L) and meropenem MIC (1 to 32 mg/L). In $bla_{\text{OXA-58}}$-carrying isolates, AP1 with overexpression of *adeE* and co-harboring $bla_{\text{IMP-14a}}$ had

higher carbapenem MIC (32 mg/L of imipenem and meropenem) than those of AP14 harboring only *bla*$_{\text{OXA-58}}$ and reduced 3 OMPs. AP14 carrying OXA-58 with reduced OMPs and overexpression of *adeE* had higher carbapenem MICs (8 to 16 mg/L) than AP17 (1 mg/L) with no overexpression of *adeE*. The *bla*$_{\text{OXA-58}}$-carrying isolates AP7 and AP17 with no overexpression of *adeE* had susceptible phenotype to carbapenems. In *bla*$_{\text{OXA-23}}$-carrying *A. pittii*, isolate AP16 showed higher imipenem MIC (2-fold) than AP23 with no overexpression of *adeE*. No *A. pittii* isolate showed overexpression of *adeY*. There was no study of *adeE* and *adeY* expression in carbapenem-resistant *A. pittii* isolate. Thus, this is the first study that characterized overexpression of efflux pump by both activity and mRNA level. These results indicate that carbapenemase production with overexpression of efflux pump confer high-level carbapenem resistance in *A. pittii*.

In *A. nosocomialis*, the prevalences of *adeE* and *adeY* gene were 84% and 96%, respectively in South Korea (126). In the present study, neither *adeE* nor *adeY* genes were found in the 5 carbapenem-resistant *A. nosocomialis*. There was no study on overexpression of efflux pump in *A. nosocomialis* isolates, but previous studies suggest that this mechanism may involve in carbapenem resistance because they carried carbapenemase gene, but had susceptible phenotype (112, 200). The present study showed that expression of *bla*$_{\text{OXA-23}}$ is adequate to confer high-level resistance to carbapenems.

Multilocus sequence typing (MLST) of carbapenem-resistant *Acinetobacter* spp.

The clonality of 23 carbapenem-resistant *A. baumannii* with different resistance mechanisms was performed by using MLST. All 7 allelic numbers of 7 housekeeping genes and allelic profiles of sequence types were obtained from the PubMLST database. A novel allelic number of *gyrB* was found in AB4 and AB354 and was numbered as *gyrB* 159. Among 23 *A. baumannii* isolates, they belonged to 15 STs. This indicates that carbapenem-resistant *A. baumannii* in this study are various clones. Of the 15 STs, there were 6 novel STs including ST1415, ST1416, ST1417, ST1418, ST1423 and ST1426. This indicates unique clones of carbapenem-resistant *A. baumannii* in this study. The major clones were ST195 and ST542. *A. baumannii* ST195 has been reported in outbreak of carbapenem resistance in Malaysia (242-244), China (245-247) and Saudi Arabia (248). In this study, all *A. baumannii* ST195 carried *bla*$_{\text{OXA-23}}$, similar to the previous outbreak reports from Malaysia and China. This suggests the clonal

outbreak of *bla*$_{OXA-23}$-carrying *A. baumannii*. All of *A. baumannii* ST542 carried *bla*$_{OXA-23}$. This study is the first report of clonal spread of *bla*$_{OXA-23}$-carrying *A. baumannii* ST542. Interestingly, all *bla*$_{OXA-24}$-carrying *A. baumannii* belonged to novel clones, ST1416 and ST1426 which are different from other studies. The *bla*$_{OXA-58}$-carrying isolates belonged to ST1001 and ST208. *A. baumannii* ST208 has been reported as outbreak of *bla*$_{OXA-23}$-carrying isolates in China (246) and U.S. (249) and no-acquired carbapenemase gene-carrying isolates in Japan (250). *A. baumannii* ST1001 has not been reported. Our study suggests that outbreak isolates are *bla*$_{OXA-23}$-carrying *A. baumannii* isolates belonging to ST195 and ST542. However, there are various clones of *A. baumannii*. This may be because King Chulalongkorn Memorial Hospital is a referral hospital where the patients are referred from small hospitals.

A. pittii showed a variety of clones as *A. baumannii*. Among 8 carbapenemase gene-carrying *A. pittii*, they belonged to 6 STs. The novel allelic numbers were found in all loci except *recA* and *gpi*. They were 1 of *gltA* (*gltA* 102), 2 of *gyrB* (*gyrB* 160 and *gyrB* 161), 3 of *gdhB* (*gdhB* 167, *gdhB* 168 and *gdhB* 169), 2 of *cpn60* (*cpn60* 99 and *cpn60* 100) and 2 of *rpoD* (*rpoD* 120 and *rpoD* 121). Among 6 STs, there were 5 novel STs (ST1419, ST1424, ST1425, ST1427 and ST1420), indicating that *A. pittii* in this study are unique. Major clones of *A. pittii* were ST1425 and ST1178. *A. pittii* belonging to these clones carried *bla*$_{OXA-58}$. *A. pittii* carrying *bla*$_{OXA-58}$ showed carbapenem resistance phenotype if they had overexpression of efflux pump, reduced OMPs or other carbapenemase gene. Therefore, the spread of *A. pittii* should be aware because some susceptible isolates carry carbapenemase genes.

All carbapenem-resistant *A. nosocomialis* belonged to ST958 and carried *bla*$_{OXA-23}$. *A. nosocomialis* ST958 from Czech was submitted to PubMLST in 2015. The present study showed that carbapenem-resistant *A. nosocomialis* isolates were the clonal spread of ST958. These 5 carbapenem-resistant *A. nosocomialis* isolates resulted in carbapenem resistance rate of 26% (5 of 19 *A. nosocomialis* isolates). On the other hand, they were the same clone and the exactly carbapenem resistance rate may be only 3% (1 of 19 *A. nosocomialis* isolates).

The antibiotic resistance island in *Acinetobacter* spp.

In this study, interrupted *comM* was found in bla_{OXA-23}-carrying *A. baumannii*, *A. pittii* and *A. nosocomialis*, indicating the presence of antibiotic resistance island. This is similar to the fact that antibiotic resistance island was not found in bla_{OXA-24} or bla_{OXA-58} carrying *Acinetobacter* spp. isolates. This may be because of the occurring of resistance island in *Acinetobacter* spp. is clonal specific. Other than *A. baumannii*, the antibiotic resistance islands have been reported in *A. nosocomialis* and *A. seifertii* (20, 251). Previous study showed that all *A. pittii* isolates had intact *comM* gene (251). Therefore, this study is the first report of antibiotic resistance island in *A. pittii* although they carried AbaR4 subtype that lack of bla_{OXA23}.

According to our PCR mapping results, there were 4 subtypes of antibiotic resistance island in *Acinetobacter* spp. All 4 subtypes were found in both carbapenem-susceptible and resistant *A. baumannii* from South Korea (149). The first subtype was AbaR4 with lack of *tniD* in Tn*6022* backbone and Tn*2006*. It was found in *A. baumannii* AB6 (ST195). This subtype was reported in *A. baumannii*, *A. nosocomialis* and *A. seifertii* (252). The Tn*6022* lacking *tniD* has been found in backbone of islands in many studies (242, 253).

The majority of *A. baumannii*, *A. pittii* and *A. nosocomialis* in the present study harbored AbaR4 type which lacked Tn*2006*. The carbapenemase gene, bla_{OXA-23}, was reported to be on Tn*2006*. Similar to our study, Kim *et al*. demonstrated that the majority of tested *A. baumannii* isolates from South Korea carried this AbaR4 subtype (149, 252). Moreover, it was also found in one *A. baumannii* isolate from Taiwan (21). This AbaR4 subtype had full transposon backbone, Tn*6022* and carried intact *sup* gene which was the hotspot for Tn*2006* integration.

The third subtype island was AbaR4 type with Tn*2006* lacking *tniD* in Tn*6022*. This subtype has been firstly reported by Kim *et al*. in *A. baumannii* from South Korea and also found in isolates from India, Malaysia and Thailand (21, 149).

The fourth subtype island was full AbaR4 island carrying bla_{OXA-23}. In this study, this subtype was found in only one *A. nosocomialis* AN15 (ST958) but not in any *A. baumannii* isolate. This subtype was reported in *A. nosocomialis* from South Korea (251). However, the majority of *Acinetobacter* spp. carrying this subtype island was *A. baumannii*, according to many studies from Asia (21, 149).

The nucleotide sequences of AbaR4 lacking *tniD* in *A. baumannii* AB4 showed 13998-bp in length. The integration in *comM* was ACCGC which is the same position of both AbaR3 and AbaR4 type islands (141, 145, 254). This suggests that ACCGC in *comM* of *A. baumannii* is the hotspot for integration of transposon backbone of antibiotic resistance island. In *A. baumannii* AB4, the backbone was Tn*6022* that lacked *tniD*. Tn*6022*, belonging to Tn*7* transposon, was composed of *tniC*, *tniA*, *tniB*, *tniD* and *tniE* which were homologous to *tnsA*, *tnsB*, *tnsC*, *tnsD* and *tnsE* of Tn7, respectively(255). In Tn7 transposon, TnsABC are important for transposition with target site specific protein TnsD or TnsE. This suggests that Tn*6022* that lack *tniD* may be loss ability to transposition. AbaR4-AB4 carried complete Tn*2006* harboring $bla_{\text{OXA-23}}$. The Tn*2006* transposon integrated at CCCGCGAAT site on *sup* gene and was flanked by double inverted repeats, indicating the transposition of Tn*2006* onto *sup* gene. Therefore, AbaR4-AB4 may be an untransposable antibiotic resistance island but Tn2006 is able to transposition.

The AbaR4-AN15 consisted of complete Tn*6022* backbone including *tniC*, *tniA*, *tniB*, *tniD* and *tniE* and complete Tn*2006* carrying $bla_{\text{OXA-23}}$. But the AbaR4-AN15 showed different integration site on *comM* (direct repeat GCCGC) and different inverted repeat sequence (TGTCATTTACAGC AATAGAATAGAGT) with AbaR4-AB4. These indicate the different target of backbone transposon of these two antibiotic resistance islands which was confirmed by nucleotide sequences of Tn*6022*. The nucleotide sequences of *tniC* and *tniD* were 100% similarity to those of isolate AB4 but *tniA*, *tniB* and *tniE* showed 99% similarity to those of isolate AB4. This may be because different TniE selects different target of transposition.

The patterns of resistance islands in *A. baumannii* in this study are similar to those from the study from South Korea by Kim *et al*. (149). The major clones of *A. baumannii* in this study were ST195 and ST542. This indicates the transfer of antibiotic resistance island between clones of *A. baumannii* by transposon Tn*6022*. Two isolates of *A. baumannii* ST195 from Malaysia had larger AbaR4-type than those of isolates in this study (242). One isolate carried AbaR4-type island with Tn*6022* backbone which was integrated by Tn*2006* and carried tetracycline and streptomycin resistance genes in the next. The other isolate harbored AbaR4 type island with Tn*6022* that carried Tn*2006* and tetracycline and streptomycin resistance genes in the next, respectively.

These suggest that AbaR4 subtype is the simplest resistance island carrying the hotspot for integration of other mobile genetic elements.

The synergism of antibiotic combinations against carbapenem resistance *Acinetobacter* spp.

Checkerboard assay

In this study, the activity of antibiotic combinations was evaluated against 29 carbapenem-resistant *A. baumannii* with different resistance mechanisms including OXA-23, OXA-24 or OXA-58 production with reduced or non-reduced OMPs. The most effective combination was imipenem plus fosfomycin. This combination showed synergism against all OXA-24- or OXA-58-carrying isolates, 50% of OXA-23-carrying with non-reduced OMP isolates and 71.4% of OXA-23-carrying with reduced OMPs. These results suggest that synergism does not specific with carbapenem resistance mechanism. No synergism was found against carbapenem-resistant *A. baumannii* by Leite *et al*. (256). However, combination of imipenem plus fosfomycin was highly effective against methicillin-resistant *S. aureus* (MRSA) by inhibiting the production of penicillin binding proteins (PBPs) (257, 258). In this study, carbapenem-resistant *A. baumannii* belonged to various clones. However, synergism was found against 50% of isolates belonging to ST195 and 75% of isolates belonging to ST542. Thus, synergism is not clonal specific.

Meropenem plus amikacin, imipenem plus colistin and imipenem plus amikacin also showed effective activity against carbapenem-resistant *A. baumannii*. The synergism of meropenem plus amikacin was observed against all isolates belonging to ST542. Isolates belonging to ST195 were not determined because amikacin MICs were higher than 256 mg/L. Although no synergism of this combination found against colistin-susceptible and colistin-resistant *A. baumannii* (256), synergism has been reported against 33% of OXA-48-producing *K. pneumoniae* (259). This combination showed successful treatment against brain abscesses by ESBL-producing *K. pneumoniae* (260). The combination of imipenem and amikacin showed lower synergy effect (30.8%) than meropenem (46.2%) against *A. baumannii* in this study. This suggests that for combination of carbapenems and amikacin, meropenem may be more effective than imipenem. However, when combined with fosfomycin or colistin, imipenem provides greater effect than meropenem.

There were various carbapenem resistance mechanisms in *A. pittii* found in this study including OXA-58 production (AP8), OXA-58 production with reduced OMPs (AP7 and AP17), OXA-58 and IMP-14a production with overexpression of efflux pump (AP1), OXA-58 production with reduced OMPs and overexpression of efflux pump (AP14), OXA-23 production (AP4 and AP23) and OXA-23 production with reduced OMPs and overexpression of efflux pump (AP16). The limitation of this study is low sample numbers of each carbapenem resistance mechanism. Among all 8 *A. pittii* isolates, the best combination was imipenem plus fosfomycin (62.5%), imipenem plus amikacin (50%), meropenem plus amikacin (33.3%) and meropenem plus fosfomycin (25%), respectively. There is no study of fosfomycin in combination with carbapenems against *A. pittii*. The present study is the first report of synergism of imipenem plus fosfomycin against carbapenemase gene-carrying *A. pittii*. Although the synergism was found against all OXA-23-producing isolates and 40% of OXA-58-producing isolates, all of them were carbapenem-resistant *A. pittii* (AP14, AP1, AP4, AP16 and AP23). The major clone was ST1425 and ST1178 but only isolate AP14 belonging to ST1178 showed synergism. Therefore, the synergism may not be carbapenem resistance mechanism specific and clonal specific.

The effect of imipenem plus amikacin was found in 2 of 3 tested *A. pittii* isolates in this study but Sheng *et al*. (161) showed that synergism was found in all 2 carbapenem-resistant *A. pittii*. They also found synergism of imipenem plus colistin against all 2 carbapenem-resistant *A. pittii*. The synergisms of meropenem plus fosfomycin and meropenem plus amikacin were detected against 2 and 1 of tested isolates, respectively. The synergism of imipenem plus fosfomycin seems to be related with OXA-23 production in *A. pittii*. However, there were only 3 isolates in this study.

In *A. nosocomialis*, the best combination was imipenem plus fosfomycin, the synergism was found in 4 of 5 carbapenem-resistant isolates. The synergisms of meropenem plus colistin and imipenem plus amikacin were found in 3 and 2 isolates, respectively. The synergism of imipenem plus fosfomycin seems to be associated with OXA-23 production in *A. nosocomialis*. However, the limitation of this study was only the same clone of OXA-23-producing isolates included.

There are a few studies of antibiotic combination against non-*baumannii Acinetobacter* (161, 261). This may be because antibiotic resistance rates of them were

lower than those of *A. baumannii*. The best combination was imipenem plus fosfomycin, although all 3 species of *Acinetobacter* were intrinsically resistant to fosfomycin as described previously (166). The resistance mechanisms of carbapenems especially OXA-23 production were related with synergism of imipenem plus fosfomycin. This synergism may not be assumed in other clinical isolates because of limitation of sample numbers in this study. However, the majority of all 3 species were resistant to carbapenems by OXA-23 production. The synergism of imipenem plus fosfomycin was demonstrated against 100% of *A. pittii*, 80% of *A. nosocomialis* and 58% of *A. baumannii* that produced OXA-23. Therefore, imipenem plus fosfomycin may be a new choice of antibiotic combination to combat carbapenem-resistant *Acinetobacter* spp. infection.

Time-kill assay

Imipenem plus fosfomycin is the best combination against 3 species of *Acinetobacter* in this study. Thus, synergism was confirmed by time-kill assay. In *A. baumannii* isolates with OXA-23 production (AB3 and A4), OXA-23 production with reduced 33-36 kDa OMP and OprD (AB29) and OXA-23 production with reduced all 3 OMPs (AB58) were studied by time-kill assay. All of them showed synergism and bactericidal effect of this combination. Of the 2 isolates producing OXA-58, both of them showed synergism but only one isolate (AB227) showed bactericidal effect. Of the 2 isolates producing OXA-24, only one isolate A10 showed synergism and bactericidal effect.

Among 4 *A. pittii* isolates, all of them including AP1 (OXA-58 and IMP-14a production with overexpression of efflux pump), AP14 (OXA-58 production with reduced all 3 OMPs and overexpression of efflux pump), AP16 (OXA-23 production with reduced OMPs and overexpression of efflux pump) and AP23 (OXA-23 production) showed synergism and bactericidal effect of imipenem plus fosfomycin. All 2 *A. nosocomialis* isolates producing OXA-23 showed synergism and bactericidal effect of imipenem plus fosfomycin.

The results of checkerboard were correlated with time-kill assay in 7 of 8 *A. baumannii* isolates (87.5%), all 4 *A. pittii* (100%) and all 2 *A. nosocomialis* (100%) isolates. This is similar to the study by White *et al*. which showed that the results from time-kill was in agreement with the results from checkerboard about 44 to 88% (262,

263). The synergism of imipenem plus fosfomycin has been found against *P. aeruginosa* strain PAO1 and the inhibition of cell wall recycling bypass pathway enhanced the synergism of imipenem plus fosfomycin (264).

Fosfomycin resistance mechanism in *Acinetobacter* spp.

Fosfomycin is an antimicrobial agent that inhibit bacterial cell wall synthesis and its target is MurA that adds phosphoenolpyruvate (PEP) to UDP-N-acetylglucosamine and removes phosphate group to get enolpyruvyl-UDP-GlcNAc product. Although *Acinetobacter* spp. were intrinsically resistant to fosfomycin, only the study of Sharma *et al*. that described the efflux pump of fosfomycin in *A. baumannii* (163). Thus, fosfomycin resistance mechanisms that have been reported in other organisms were studied in *Acinetobacter* spp. in the present study.

The first mechanism is MurA mutation. The essential amino acid at active site of MurA in *Acinetobacter* spp. is Cys116 and ligand biding site are Lys22, Arg121 and Arg398. Moreover, mutation of MurA at Asp370 and Leu371 have been reported in fosfomycin-resistant *E. coli* (265, 266). The mutations of these amino acids have been reported to be associated with fosfomycin resistance in *E. coli* (164). In this study, no MurA mutation was found in all 3 species of *Acinetobacter* isolates. This result is similar to the study of Kaur *et al*. that indicated the MurA as a novel drug target in *A. baumannii* (188). The expression level of *murA* in the presence of fosfomycin was not increased in all 3 species of *Acinetobacter*. Fosfomycin has been reported as a competitive inhibitor of MurA and MurA was inactivated by concentration dependent manner (267). This suggests that there may be other peptidoglycan synthesis pathways that does not need MurA.

The next mechanism is fosfomycin-modifying enzyme production. No gene encoding for fosfomycin-modifying enzyme was found in all 3 species of *Acinetobacter*. These genes were *fosA, fosB, fosC* and *fosX*. The *fosA* gene has been reported in fosfomycin-resistant Enterobacteriaceae including *E. coli*, *Salmonella*, *K. pneumoniae* and *P. aeruginosa* (268-270), but it has not been reported in *Acinetobacter* spp. Lately, 5 subtypes of *fosA* have been found including *fosA, fosA2, fosA3, fosA4* and *fosA5*(271). The *E. coli* J53 transconjugant of *fosA*-carrying plasmid showed various level of fosfomycin MIC (from 1 mg/L to 512 - 1024 mg/L) (271, 272). The *fosB* gene was commonly found in Gram-positive bacteria including *Staphylococci* and

Enterococci. There were 6 subtypes of *fosB* (*fosB1* to *fosB6*) (273, 274). *S. aureus* ATCC 25923 transconjugant of *fosB*-carrying plasmid showed increased fosfomycin from 2 mg/L to 64 to 128 mg/L (274). The *fosC* has been reported in *Pseudomonas syringae* and *E. coli* (275, 276). The CSH-2 conjugants of *fosC*-carrying plasmid showed increased fosfomycin MIC from 2 mg/L to >256 mg/L. There were two subtypes of *fosX* genes including *fosX* and $fosX^{CC}$. The *fosX* was reported in *Listeria monocytogenes* (277). The $fosX^{CC}$ was found in *Campylobacter* spp. and fosfomycin MIC of *E. coli* DH5α transconjugant was increased from 0.5 mg/L to 256 mg/L (278). These data suggested that the producing of fosfomycin-modifying enzyme can confer high-level resistance to fosfomycin in Gram-negative and Gram-positive bacteria.

The third mechanism is overexpression of efflux pump. The fosfomycin pump in *A. baumannii* was only AbaF reported by Sharma *et al*. (163). They found that *A. baumannii* clinical isolates showed higher expression level of *abaF* in the presence of fosfomycin and overexpression of AbaF activity was detected by using inhibitor, CCCP. Although *abaF* was found in *A. baumannii* in this study, no overexpression was detected by RT-PCR and by using CCCP. Therefore, AbaF may not be the main mechanism of resistance. The results were similar to the study by Sharma *et al*. which showed that the *abaF* mutant strain had decreased fosfomycin MIC from 256 mg/L to 32 mg/L. This indicates that *A. baumannii* may have intrinsic resistance to fosfomycin at 32 mg/L.

Lately, the novel possible intrinsic mechanism of fosfomycin resistance called cell wall recycling bypass pathway has been reported in *P. putida*, *P. aeruginosa* and *E. coli* (165, 279, 280). This mechanism may be present in *Acinetobacter* spp. because they have fosfomycin intrinsic resistance. The resistance mechanism has not been reported except AbaF efflux pump. Thus, in this study, the presence of essential genes in this pathway including *ampG* (transport of anhydromuropeptides), *nagZ* (hydrolyze anhydromuropeptides to GlcNAc and anhydroMurNAc-Ala-Glu-DAP-Ala), *anmK* (convert anhydromurNAc to MurNAc-6P), *amgK* (convert MurNac to MurNA α-1P) and *murU* (convert MurNA α-1P to UDP-MurNAc) were detected in 3 species of *Acinetobacter*. All of these genes were found in all tested *A. baumannii* and *A. nosocomialis*. Among 6 *A. pittii* isolates, 3 carried *ampG*, *nagZ* and *anmK*, 2 carried *ampG*, *nagZ*, *anmK* and *murU*, and the other isolate carried *nagZ* and *anmK*. The results

suggest that this pathway may exist in *A. baumannii* and *A. nosocomialis*, but may be absent in *A. pittii*.

After that, the expression level of *ampG* and *murU* were determined after 2 hours exposure of fosfomycin at 0.25X, 0.5X and 1X MIC because fosfomycin is effective at exponential phase of growth (cell wall synthesis occur). If the cell wall recycling bypass pathway involves in fosfomycin resistance, the expression level of *ampG* and *murU* should be increased in the presence of fosfomycin for uptaking truncated peptidoglycan and recycling. No overexpression of *ampG* and *murU* was detected in *A. baumannii* AB250 and A10, *A. pittii* AP23 and *A. nosocomialis* AN4 and AN12. In *A. pittii* AP1, only overexpression of *ampG* was detected. These results were not enough to be concluded about cell wall recycling bypass pathway in *Acinetobacter* spp. because of the limitation of this method. To confirm that this pathway involves in fosfomycin resistance, the mutant strains of all genes in this pathway must be constructed, tested for susceptibility, detected by products of each step and complemented all genes back. Thus, this pathway should be further confirmed and characterized. However, these data suggest that *Acinetobacter* spp. have cell wall recycling bypass pathway, but the whole pathway need to be further characterized and the association with fosfomycin resistance is still unclear.

In *E. coli*, mutations in fosfomycin transporters, GlpT and UhpT, resulted in overexpression of these transporters associated with fosfomycin resistance (164). In *P. aeruginosa*, only GlpT was found and mutation in this transporter conferred fosfomycin resistance (281). These transporters have not been reported in *A. baumannii* until the amino acid sequences of glycerol-3-phosphate permease of *A. baumannii* from Morocco (SCY65138.1) and glycerol-3-phosphate transporter (GlpT) of *A. pittii* (WP_068600482.1) have been directly submitted to GenBank. Their amino acid sequences showed 100% similarity to GlpT in *P. aeruginosa* and *E. coli*, respectively, but they were not similar to those of any *Acinetobacter* spp. in GenBank. In this study, these genes were determined in *Acinetobacter* spp., but no PCR product was found. The report of these 2 transporters may be less reliable data.

Therefore, in this study, fosfomycin resistance mechanism is still unclear. The possible mechanisms include other fosfomycin-modifying enzyme production, other

efflux pump, other cell wall recycling bypass pathways and other transporters or the lack of fosfomycin transporters.

The possible mechanism of synergism of imipenem plus fosfomycin against carbapenem-resistant *Acinetobacter* spp.

Although synergism was found in almost all of OXA-23-producing *A. pittii* and *A. nosocomialis*, about half of OXA-23-producing *A. baumannii* did not show synergism. Moreover, *Acinetobacter* spp. belonging to the same clone showed different effect of imipenem plus fosfomycin. Thus, synergism of this combination is not either associated with carbapenem resistance mechanism or clonal specific. The intrinsic resistance mechanism of fosfomycin is still unknown. Of all results, the possible synergy mechanism of imipenem plus fosfomycin is still unclear.

According to time-kill curve of fosfomycin alone, the rapid killing occurred at the first 4 hours and then the regrowth was observed in all tested isolates. This pattern is similar to other studies on fosfomycin killing rate against *P. aeruginosa* and *A. baumannii* (18, 282). This can be hypothesized that *Acinetobacter* spp. synthesize peptidoglycan by *de novo* pathway through MurA. After that, they switch to other bypass pathways. Other bypass pathways were not characterized in this study. Another hypothesis was that there were the heterogeneous population to fosfomycin resistance. To evaluate this hypothesis, the population analysis was used to detect the heterogeneity of *Acinetobacter* spp. population. *A. baumannii* AB250 and A10 and *A. pittii* AP1 and AP23 showed heterogeneous population and subpopulation (1 per 10^6 CFU/ml) that persisted in the presence of fosfomycin above the MIC. The heterogeneous population and subpopulation (1 per 10^5 CFU/ml) of *A. nosocomialis* AN4 and AN12 were found in the presence of fosfomycin above the MIC. These subpopulations were persistence to fosfomycin. The heterogeneous population to fosfomycin has been reported in *P. aeruginosa* (264). The subpopulation of *P. aeruginosa* showed highly resistant to fosfomycin and had the mutation in GlpT. Therefore, our subpopulations of *Acinetobacter* spp. were tested for fosfomycin susceptibility and mechanisms of resistance were characterized. The results showed that subpopulation of all *Acinetobacter* spp. had higher fosfomycin MIC level at least 2-fold, but mechanism of resistance could not be detected (data not shown). The heteroresistance to fosfomycin was reported in *Streptococcus pneumoniae* by mutation of MurA (283). The

heteroresistance to cefepime has been reported in *A. baumannii* (284). These heteroresistance isolates had resistance subpopulation in the susceptible whole-population. In this study, *Acinetobacter* spp. were resistant to fosfomycin with persistence subpopulation to fosfomycin. Therefore, these subpopulations in *Acinetobacter* spp. were called persistent to fosfomycin or persisters.

According to rapid killing by fosfomycin alone at 4 hours, in *Acinetobacter* spp. isolates that showed synergism, imipenem could inhibit the regrowth and provided synergism. This indicates that imipenem may have high impact on synergy of imipenem plus fosfomycin. Thus, population analysis of imipenem susceptibility was evaluated for the killing efficiency of imipenem against *Acinetobacter* spp. population. All of 6 tested *Acinetobacter* spp. showed homogeneous population of imipenem resistance. All synergism isolates including *A. baumannii* A10, *A. pittii* AP1 and AP23 and *A. nosocomialis* AN4 and AN12 were completely killed by imipenem at equal or above MIC. Whereas no synergism isolate, *A. baumannii* AB250, was tolerant to 2X MIC of imipenem. The antibiotic tolerance was defined as population of bacteria that can grow in the presence of antibiotic at the MIC (285). The ampicillin tolerance in *E. coli* could developed to be resistance (286). Therefore, the tolerance to imipenem may be associated with synergism of imipenem plus fosfomycin. The results indicate that only data of MIC and susceptibility to antibiotic may be inadequate to predict synergism of imipenem plus fosfomycin against *Acinetobacter* spp.

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APPENDIES

APPENDIX A
REAGENTS AND INSTRUMENTS

Reagents

Absolute ethanol	(Merck, Germany)
Acrylamide	(Merck, Germany)
Agarose	(Amresco, USA)
Amikacin	(Sigma, USA)
Amonium per sulfate	(Amresco, USA)
Bio-Rad protein assay	(Biorad, USA)
Boric acid	(Sigma, USA)
Bromphenol blue	(Biorad, USA)
CCCP	(Sigma, USA)
Cefepime	(Bristol Myers Squibb, India)
Cefotaxime	(Sigma, USA)
Ceftazidime	(Sigma, USA)
Ceftriaxone	(Sigma, USA)
Chloroform	(Merck, Germany)
Ciprofloxacin	(Sigma, USA)
Colistin	(Sigma, USA)
Coomassie brilliant blue	(Biorad, USA)
DNA Gel Loading Dye (6X)	(Thermo Fisher Scientific, USA)
Dimethylsulfoxide	(Bio Basic Inc., Canada)
dNTPs	(Thermo Fisher Scientific, USA)
EDTA	(Amresco, USA)
Ethidium bromide	(Amresco, USA)
Fosfomycin	(Wako, Japan)
GeneRuler 100 bp DNA ladder	(Thermo Fisher Scientific, USA)
GeneRuler 100 bp plus DNA ladder	(Thermo Fisher Scientific, USA)
Gentamicin	(Sigma, USA)
Glacial acetic acid	(Merck, Germany)
Glucose-6-phosphate	(Sigma, USA)
Glycerol	(Merck, Germany)
Glycine	(OmniPur, USA)
HiYield Gel/PCR DNA mini kit	(RBCBioscience, Taiwan)
HiYield Plasmid mini kit	(RBCBioscience, Taiwan)
Hydrochloric acid	(Merck, Germany)
Imipenem	(MSD, USA)
Isopropanol	(Amresco, USA)

LB broth	(BBL, USA)
MacConkey Agar	(Oxoid, USA)
Meropenem	(AstraZeneca, UK)
Mueller Hinton II Agar	(BBL, USA)
Mueller Hinton II Broth (Cation-Adjusted)	(BBL, USA)
N-lauroylsarcosine sodium salt	(Merck, Germany)
Phosphate buffer saline	(Sigma, USA)
PureLink Genomic DNA kit	(Invitrogen, USA)
Reserpine	(Himedia, India)
Sodium chloride	(Amresco, USA)
Sodium dodecyl sulfate	(Merck, Germany)
Sodium hydroxide	(Merck, Germany)
SuperScript III First-Strand Synthesis System	(Thermo Fisher Scientific, USA)
Taq DNA polymerase	(Thermo Fisher Scientific, USA)
TEMED	(Amresco, USA)
Tris	(Amresco, USA)
Trizol reagent	(Thermo Fisher Scientific, USA)
Tryptic soy broth	(BBL, USA)
Trypticase Soy Agar	(BBL, USA)
β-mercaptoethanol	(Merck, Germany)

Instruments

Automatic pipette	(Gilson, France)
Incubator	(Genlab, UK)
Forma Orbital shaker incubator	(Thermo Fisher Scientific, USA)
Microcentrifuge	(Eppendrof, Germany)
Ultracentrifuge	(Backman Coulter, USA)
UV/Visible spectrophotometer	(Biorad, USA)
Vibra-Cell processors	(Sonics, USA)
High speed refrigerator centrifuge	(Hitachi, Japan)
Benchtop centrifuge	(Hettich, Germany)
Veriti Thermal Cycler	(Thermo Fisher Scientific, USA)
Cooling dry bath incubator	(Major Science, USA)
NanoDrop 1000 spectrophotometer	(Thermo Fisher Scientific, USA)
UV transilluminator	(Montreal Biotech, Canada)
Mini-PROTEAN Tetra cell electrophoresis	(Biorad, USA)

APPENDIX B

MEDIA AND ANTIBIOTIC SOLUTION PREPARATION

1. Mueller-Hinton II agar (Difo, USA)

For preparation of Mueller-Hinton II agar 1,000 mL, 38 g of dehydrated medium was suspended in distilled water, mixed homogeneously, dissolved completely by heating and was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The Mueller-Hinton II agar plates were stored at 4 ^{o}C.

2. Trypticase Soy Agar (Difo, USA)

For preparation of Trypticase Soy agar 1,000 mL, 40 g of dehydrated medium was suspended in distilled water, mixed homogeneously, dissolved completely by heating and was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The Trypticase Soy agar plates were stored at 4 ^{o}C.

3. MacConkey agar (Oxoid, USA)

For preparation of MacConkey agar 1,000 mL, 51.5 g of dehydrated medium was suspended in distilled water, mixed homogeneously, dissolved completely by heating and was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The MacConkey agar plates were stored at 4 ^{o}C.

4. Mueller Hinton II broth (Cation-Adjusted) (BBL, USA)

For preparation of Mueller Hinton II broth 1,000 mL, 22 g of dehydrated medium was suspended in distilled water, mixed homogeneously, dissolved completely by heating and was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The Mueller Hinton II broth was stored at 4 ^{o}C.

5. Tryptic Soy broth (BBL, USA)

For preparation of Tryptic Soy broth 1,000 mL, 30 g of dehydrated medium was suspended in distilled water, mixed homogeneously, dissolved completely by heating and was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The Tryptic Soy broth was stored at 4 ^{o}C.

6. LB broth (BBL, USA)

For preparation of Tryptic Soy broth 1,000 mL, 25 g of dehydrated medium was suspended in distilled water, mixed homogeneously, dissolved completely by heating and was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The Tryptic Soy broth was stored at 4 ^{o}C.

7. Sterile 0.9% NaCl

For preparation of 0.9% NaCl 1,000 mL, 9 g of NaCl was dissolved completely in distilled water and was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The sterilized 0.9% NaCl was stored at room temperature.

8. Antibiotic stock solution

Imipenem, stock concentration 5,120 mg/L

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

Meropenem, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of meropenem was dissolved by 5 mL of sterilized distilled water.

Amikacin, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of amikacin was dissolved by 5 mL of sterilized distilled water.

Gentamicin, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of gentamicin was dissolved by 5 mL of sterilized distilled water.

Ceftazidime, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of ceftazidime was dissolved by 0.5 mL of NaOH and then, was dissolved by 4.5 mL of sterilized distilled water.

Ceftriaxone, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of ceftriaxone was dissolved by 5 mL of sterilized distilled water.

Cefepime, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of cefepime was dissolved by 5 mL of sterilized distilled water.

Cefotaxime, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of cefotaxime was dissolved by 5 mL of sterilized distilled water.

Ciprofloxacin, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of ciprofloxacin was dissolved by 0.5 mL of HCl and then, was dissolved by 4.5 mL of sterilized distilled water.

Colistin, stock concentration 5,210 mg/L

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

Fosfomycin, stock concentration 5,210 mg/L

Preparation of stock solution, 0.0256 g of fosfomycin was dissolved by 5 mL of sterilized distilled water.

APPENDIX C

REAGENTS PREPARATION

1. 10X Tris Boric EDTA (TBE) buffer

For preparation of 10X TBE buffer, 108 g of Tris base, 55 g of boric acid and 40 mL of 0.5M EDTA were mixed and dissolved in 800 mL of distilled water. Adjust the volume to 1000 mL. The TBE buffer was sterilized by autoclaving at 121 ºC for 15 minutes and stored at room temperature.

2. 0.5M EDTA (pH 8.0)

For preparation of 0.5M EDTA, 186.1g 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.

3. 1% Agarose gel

For preparation of 1% Agarose gel, 1 g of agarose was suspended and dissolved by heating in 100 mL of 1X TBE buffer.

4. 10% SDS

For preparation of 10% SDS, 10 g of SDS was suspended and completely dissolved in 90 mL distilled water. Adjust the volume to 100 mL. This reagent was stored at room temperature.

5. 1.5M Tris-HCl (pH 8.8)

For preparation of 1.5M Tris-HCl (pH 8.8), 27.23 g of Tris base was suspended and completely dissolved in 80 mL distilled water. Adjust the pH by using HCl and the volume to be 150 mL. This reagent was stored at 4 ºC.

6. 0.5M Tris-HCl (pH 6.8)

For preparation of 1.5M Tris-HCl (pH 8.8), 6 g of Tris base was suspended and completely dissolved in 60 mL distilled water. Adjust the pH by using HCl and the volume to be 100 mL. This reagent was stored at 4 ºC.

7. 6X protein sample buffer

For preparation of 6X protein sample buffer, 15 mL of glycerol, 0.6mL of 0.5M EDTA, 28 mL of 0.5M Tris HCl (pH 6.8), 5 g of SDS, 0.4626 of DTT and 6 mg of bromphenol blue were mixed and dissolved together. Adjust the volume to 50 mL. This reagent was stored at 4 ºC.

8. 10X protein running buffer (pH 8.3)

For preparation of 10X protein running buffer, 30.3 g of Tris base, 144 g of glycine and 10 g of SDS were mixed and dissolved in 800 mL. Adjust the volume to 1000 mL. This reagent was stored at 4 ^{o}C.

9. 10% APS

For preparation of 10% APS, 1 g of SDS was suspended and completely dissolved in 10 mL distilled water. Adjust the volume to 100 mL. This reagent was stored at -20 ^{o}C.

10. 12% polyacrylamide gel (separate gel)

Distilled water	3.4 mL
40% Acrylamide/Bis	4.0 mL
1.5M Tris buffer, pH 8.0	2.5 mL
10% SDS	0.1 mL
add the following reagent when the casting chambers were ready	
10% APS	50 µL
TEMED	5 µL

11. 4% polyacrylamide gel (stacking gel)

Distilled water	6.1 mL
40% Acrylamide/Bis	1.3 mL
0.5M Tris buffer, pH 6.8	2.5 mL
10% SDS	0.1 mL
add the following reagent when the casting chambers were ready	
10% APS	50 µL
TEMED	10 µL

12. Phosphate buffer saline (pH 7.4)

For preparation of phosphate buffer saline, 1 pouch of phosphate buffered saline powder was dissolved in distilled water. Adjust the volume to 1000 mL. The PBS was sterilized by autoclaving at 121 ^{o}C for 15 minutes and stored at room temperature.

15. 2% sodium N-lauroylsarcosine

For preparation of 2% sodium *N*-lauroylsarcosine, 2 g of *N*-lauryl sarcosine was suspended and completely dissolved in 90 mL distilled water. Adjust the volume to 100 mL. This reagent was stored at room temperature.

APPENDIX D

ADDITIONAL RESULTS

Results of MICs of 10 antibiotics against 312 *A. baumannii* isolates

isolate	specimen	MICs (mg/L)									
		IPM	MEM	AK	GN	CTX	CRO	CAZ	FEP	CIP	CT
AB1	Sputum	32	32	>256	>256	256	>256	256	64	32	2
AB2	Sputum	64	64	>256	>256	>256	>256	>256	>256	64	2
AB3	Aspirate	16	32	0.5	2	32	64	16	64	0.5	2
AB4	Sputum	64	64	>256	>256	>256	>256	>256	>256	128	1
AB5	Aspirate	64	128	>256	>256	>256	>256	>256	256	64	1
AB6	Sputum	32	32	>256	>256	>256	>256	>256	128	128	1
AB8	Sputum	64	64	>256	>256	>256	>256	>256	256	64	1
AB9	Tracheal secretion	16	32	0.5	2	32	32	16	128	1	2
AB11	Sputum	32	16	>256	>256	256	>256	128	32	32	2
AB13	Blood	32	64	64	32	128	256	>256	256	32	1
AB15	Sputum	0.5	0.25	4	1	16	16	8	4	4	2
AB16	Sputum	64	16	>256	>256	>256	>256	>256	256	128	1
AB17	Sputum	0.5	0.25	64	256	8	16	16	4	16	1
AB19	Sputum	128	64	>256	>256	>256	>256	>256	>256	128	1
AB20	Sputum	32	16	>256	>256	>256	>256	>256	256	64	1
AB21	Urine	32	16	>256	>256	>256	>256	256	256	128	1
AB23	Biopsy	0.5	0.5	4	1	16	16	4	4	0.125	1
AB24	Milk	0.125	0.125	4	1	4	4	2	1	0.125	1
AB25	Pus	128	16	>256	>256	>256	>256	>256	256	128	1
AB26	Pus	128	64	>256	>256	>256	>256	>256	256	128	2
AB27	Sputum	128	32	>256	>256	>256	>256	>256	>256	64	1
AB28	Sputum	0.5	0.25	4	1	256	>256	8	4	0.5	1
AB29	Sputum	32	64	>256	256	256	256	>256	256	64	1
AB32	Sputum	64	16	>256	>256	>256	>256	>256	256	128	1
AB34	Sputum	128	32	>256	>256	>256	>256	>256	>256	128	1
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AB59	Sputum	64	32	>256	>256	256	256	128	64	64	1
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AB62	Sputum	32	32	>256	>256	>256	>256	>256	128	64	1
AB63	Pus	64	16	>256	>256	>256	>256	>256	128	64	1
AB64	Sputum	128	32	8	1	32	32	16	64	0.5	1
AB68	Sputum	0.5	0.25	4	1	8	16	8	4	0.125	1
AB69	Catheter	0.5	0.25	8	1	8	8	4	2	0.125	1
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AB78	Sputum	64	64	>256	>256	>256	>256	>256	>256	256	1
AB80	Urine	128	64	>256	>256	>256	>256	>256	256	128	1
AB81	Urine	1	2	>256	>256	>256	>256	>256	>256	128	1
AB83	Biopsy	64	32	>256	>256	>256	>256	>256	256	256	1
AB84	Pus	64	16	8	4	>256	>256	>256	256	128	1
AB85	Sputum	32	16	>256	>256	>256	>256	>256	256	64	1
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AB87	Tracheal secretion	128	64	>256	>256	>256	>256	>256	>256	64	1
AB88	Biopsy	128	32	>256	>256	>256	>256	>256	256	128	1
AB89	Pus	64	64	>256	>256	>256	>256	>256	>256	128	1
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AB106	Pus	64	64	>256	>256	>256	>256	>256	128	64	1
AB107	Pus	32	16	>256	>256	>256	>256	>256	256	64	1
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AB111	Sputum	128	32	>256	>256	256	256	64	32	64	1
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AB119	Sputum	32	16	8	1	16	16	4	32	0.125	1
AB122	Sputum	0.5	0.25	4	1	256	16	2	2	0.06	1
AB123	Pus	128	64	>256	>256	>256	>256	>256	>256	64	1
AB124	Tracheal secretion	256	64	>256	>256	>256	>256	>256	>256	64	1
AB125	Sputum	64	16	8	1	16	16	4	32	0.125	1
AB127	Pus	32	32	>256	>256	>256	>256	>256	256	32	1
AB128	Sputum	64	16	>256	>256	>256	>256	>256	>256	32	1
AB132	Sputum	128	32	>256	>256	>256	>256	>256	256	32	1
AB133	Sputum	64	32	8	1	32	32	8	32	0.25	1
AB138	Tracheal secretion	64	32	>256	>256	>256	>256	>256	128	>256	2
AB142	Sputum	256	64	>256	>256	>256	>256	>256	256	128	1
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AB145	Sputum	64	32	>256	>256	>256	>256	>256	256	64	2
AB147	Tracheal secretion	0.5	0.5	4	1	8	16	4	2	0.125	2
AB148	Sputum	64	16	>256	>256	256	>256	128	64	64	2
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AB151	Catheter	128	32	>256	>256	>256	>256	>256	256	64	2
AB153	Sputum	128	64	>256	>256	>256	>256	>256	>256	>256	2
AB156	Catheter	0.5	0.25	8	2	4	8	2	1	0.125	2
AB158	Tracheal secretion	128	32	>256	>256	>256	>256	>256	256	64	1
AB160	Sputum	0.5	0.25	4	1	32	32	8	16	0.125	1
AB161	Sputum	128	32	>256	>256	>256	>256	>256	256	64	1
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AB165	Sputum	1	2	4	4	>256	>256	>256	32	32	1
AB167	Pus	64	64	>256	>256	>256	>256	>256	256	64	2
AB168	Pus	64	64	>256	>256	>256	>256	>256	256	64	2
AB170	Tracheal secretion	1	1	8	2	4	8	4	1	0.06	1
AB171	Sputum	32	32	>256	>256	>256	>256	>256	128	64	1
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AB176	Pus	64	64	>256	>256	>256	>256	>256	128	64	1
AB177	Sputum	32	32	64	32	256	256	>256	128	32	1
AB179	Catheter	1	2	4	4	>256	>256	>256	32	32	1
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AB181	Catheter	64	64	>256	>256	>256	>256	>256	128	64	2

AB182	Pus	0.25	1	4	1	16	16	4	4	0.25	1
AB183	Pus	64	64	>256	>256	>256	>256	>256	256	64	2
AB185	Sputum	64	64	>256	>256	>256	>256	>256	256	64	2
AB187	Sputum	32	16	4	4	>256	>256	>256	256	128	1
AB188	Sputum	0.5	0.25	8	1	8	8	4	2	0.25	1
AB189	Sputum	32	16	>256	>256	256	>256	128	256	128	2
AB190	Biopsy	16	32	>256	256	>256	>256	>256	128	64	1
AB191	Pus	64	64	>256	>256	>256	>256	>256	>256	64	1
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AB198	Pus	0.25	0.25	4	1	8	8	4	2	0.06	2
AB201	Trachial secretion	32	32	>256	>256	>256	>256	>256	256	64	1
AB202	Sputum	0.25	0.25	4	2	16	16	4	4	32	2
AB203	Sputum	32	32	>256	>256	>256	>256	>256	128	64	1
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AB209	Pus	32	16	32	>256	>256	>256	256	128	64	1
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AB215	Urine	0.25	0.25	4	1	8	16	8	4	0.125	2
AB217	Urine	32	16	>256	>256	>256	>256	>256	32	32	1
AB219	Pus	32	16	>256	>256	>256	>256	>256	128	64	1
AB220	Catheter	64	64	>256	>256	>256	>256	>256	>256	64	1
AB221	Pus	16	16	256	>256	>256	>256	>256	64	4	1
AB222	Eye swab	0.25	0.5	4	1	8	16	8	2	0.25	1
AB223	Pus	64	64	>256	>256	>256	>256	>256	>256	64	1
AB224	Vaginal swab	64	64	>256	>256	>256	>256	>256	>256	64	1
AB225	Urine	16	16	>256	>256	>256	>256	>256	256	64	1
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AB230	Bronchial washing	64	32	>256	>256	>256	>256	>256	>256	64	1
AB234	Sputum	0.5	1	16	32	>256	>256	>256	>256	64	1
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AB236	Body Fluid	32	32	4	1	16	16	8	32	0.125	1
AB237	Body Fluid	64	64	>256	>256	>256	>256	>256	256	64	1
AB238	Urine	64	64	>256	>256	>256	>256	>256	>256	64	1
AB239	Ulcer at Genitalia	0.25	0.25	4	1	8	8	4	2	0.06	0.5
AB240	Pus	32	32	>256	>256	>256	>256	>256	256	64	1
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AB247	BAL	128	64	>256	>256	>256	>256	>256	256	32	1
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AB250	Sputum	16	16	4	1	8	16	8	4	0.06	1
AB254	Sputum	0.25	0.5	4	1	32	32	16	8	0.25	1
AB255	Aspirate	64	64	>256	>256	>256	>256	>256	>256	64	1
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AB259	Urine	64	16	>256	>256	>256	>256	>256	128	64	1
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AB262	Bile	128	32	>256	>256	>256	>256	>256	128	64	1
AB264	Pus	1	0.5	2	1	8	16	8	2	0.06	1
AB265	Urine	32	16	>256	>256	>256	>256	>256	128	64	1
AB266	Biopsy	128	32	>256	>256	>256	>256	>256	256	64	1
AB267	Urine	256	64	>256	>256	>256	>256	>256	>256	32	1
AB268	Pus	32	16	8	8	>256	>256	>256	256	64	1
AB269	Pus	0.5	0.25	2	32	16	16	16	4	0.125	2
AB270	Pus	128	32	>256	>256	>256	>256	>256	256	64	1
AB271	Sputum	32	16	>256	>256	>256	>256	>256	256	64	1
AB273	Sputum	0.5	0.5	4	1	16	16	8	4	0.06	1

AB276	Body Fluid	256	64	>256	>256	>256	>256	>256	256	32	2
AB277	Pus	0.5	0.25	4	1	8	16	8	2	0.06	1
AB278	Urine	64	32	>256	>256	>256	>256	>256	256	32	1
AB279	Urine	32	16	>256	>256	>256	>256	>256	128	64	1
AB282	Urine	64	32	>256	>256	>256	>256	>256	256	32	1
AB284	Pus	0.5	0.5	8	1	16	32	16	4	16	0.5
AB286	Sputum	128	32	1	2	>256	>256	>256	128	128	1
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AB294	Urine	128	64	>256	>256	>256	>256	>256	256	64	2
AB295	Pus	32	32	32	64	>256	>256	256	64	32	1
AB297	Pus	128	32	>256	>256	>256	>256	>256	256	64	1
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AB302	Urine	64	32	>256	>256	>256	>256	>256	256	64	1
AB303	Urine	32	16	4	2	>256	>256	>256	256	64	1
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AB309	Pus	64	16	>256	>256	>256	>256	>256	128	64	1
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AB312	Tracheal secretion	64	16	>256	>256	>256	>256	>256	128	64	2
AB314	Sputum	128	32	>256	>256	>256	>256	>256	256	128	2
AB315	Catheter	64	16	>256	>256	256	>256	256	64	128	1
AB316	Pus	128	64	>256	>256	>256	>256	>256	256	64	1
AB317	Urine	128	64	16	16	>256	>256	>256	>256	>256	2
AB318	Urine	64	16	>256	>256	>256	>256	>256	128	64	1
AB320	Bile	128	64	>256	>256	>256	>256	>256	256	32	1
AB324	Urine	0.5	0.5	4	1	8	16	4	8	0.125	1
AB325	Urine	32	64	>256	>256	>256	>256	256	128	128	1
AB326	Tracheal secretion	32	64	>256	>256	>256	>256	>256	256	64	1
AB328	Biopsy	64	64	>256	>256	>256	>256	>256	256	64	1
AB329	Bile	64	64	>256	>256	>256	>256	>256	256	16	1
AB330	Sputum	16	32	4	1	16	16	16	32	0.06	1
AB331	Pus	2	2	2	1	4	4	2	2	0.25	1
AB332	Pus	16	32	4	0.5	8	16	16	16	4	1
AB333	Pus	64	64	>256	>256	>256	>256	>256	256	128	1
AB334	Catheter	0.5	0.5	4	1	8	16	32	32	0.125	1
AB336	Sputum	64	64	>256	>256	>256	>256	>256	256	64	1
AB337	Catheter	0.25	0.25	4	1	8	16	4	2	0.125	0.5
AB338	Eye swab	0.125	0.25	4	1	8	8	8	8	0.125	2
AB339	Body Fluid	64	128	>256	>256	>256	>256	>256	256	128	1
AB342	Body Fluid	0.25	0.5	4	0.5	8	16	16	16	0.125	1
AB343	Pus	64	64	>256	>256	>256	>256	>256	>256	64	1
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AB345	Pus	64	64	>256	>256	>256	>256	>256	256	64	1
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AB349	Urine	32	64	>256	>256	>256	>256	>256	>256	64	1
AB351	Gastric content	128	128	>256	>256	>256	>256	>256	256	128	0.5
AB353	Urine	64	64	>256	>256	>256	>256	>256	256	64	1
AB354	Ascetic Fluid	64	128	>256	>256	>256	>256	>256	256	128	1
AB355	Urine	64	64	>256	>256	>256	>256	>256	256	64	1
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AB367	Urine	64	64	>256	>256	>256	>256	>256	256	64	1
AB368	Urine	64	64	>256	>256	>256	>256	>256	>256	128	0.5

AB370	Sputum	64	64	>256	>256	>256	>256	>256	>256	64	1
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AB374	Bile	64	64	4	2	32	32	>256	128	0.25	1
AB375	ปลายสาย	32	64	8	2	16	16	32	32	0.25	1
AB376	Pus	32	32	>256	>256	>256	>256	>256	>256	64	1
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AB381	Sputum	32	64	>256	>256	>256	>256	>256	256	64	1
AB382	Pus	0.25	0.5	4	2	8	16	16	8	0.25	1
AB383	Pus	32	32	>256	>256	>256	>256	>256	128	64	1
AB384	Bile	0.25	0.5	4	2	32	32	64	8	0.25	1
AB385	Body Fluid	128	128	>256	>256	>256	>256	>256	256	128	1
AB386	Urine	32	64	128	64	>256	>256	>256	256	128	1
AB387	Sputum	32	64	>256	>256	>256	>256	>256	>256	64	0.5
AB388	Sputum	32	64	>256	>256	>256	>256	>256	128	128	1
AB389	Tracheal secretion	32	64	>256	>256	>256	>256	>256	256	128	1
AB391	Pus	64	64	>256	>256	>256	>256	>256	256	128	2
AB392	Pus	32	64	32	32	128	256	>256	256	32	2
AB393	Urine	32	32	>256	>256	>256	>256	>256	>256	32	1
AB394	Urine	32	32	>256	>256	>256	>256	>256	128	64	1
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AB398	Pus	64	64	>256	>256	>256	>256	>256	>256	64	0.5
AB400	Sputum	32	64	>256	>256	>256	>256	>256	>256	64	1
AB401	Sputum	32	64	128	64	>256	256	>256	256	64	2
AB402	Gastric wash	0.25	0.5	8	1	4	16	16	4	0.25	2
AB404	Body Fluid	64	64	>256	>256	>256	>256	>256	>256	64	0.5
AB405	Body Fluid	32	32	>256	>256	>256	>256	>256	>256	32	2
AB406	ปลายสาย	32	64	>256	>256	>256	>256	>256	256	64	1
AB407	Urine	32	64	>256	>256	>256	>256	>256	128	64	0.5
AB412	Pus	32	64	>256	>256	>256	>256	>256	256	64	1
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AB418	Pus	32	32	>256	>256	>256	>256	>256	256	32	2
AB419	Pus	32	16	>256	>256	>256	>256	>256	64	128	1
AB423	Sputum	64	128	>256	>256	>256	>256	>256	256	128	1
AB424	Sputum	32	64	>256	>256	>256	>256	>256	256	128	0.5
AB425	Sputum	64	128	>256	>256	>256	>256	>256	>256	64	1
AB426	Sputum	0.25	0.25	8	1	32	64	32	4	0.25	1
AB427	Sputum	64	128	>256	>256	>256	>256	>256	256	64	0.5
AB428	Sputum	64	128	>256	>256	>256	>256	>256	256	128	1
AB429	Sputum	128	128	>256	>256	>256	>256	>256	>256	64	1
AB430	Urine	128	128	>256	>256	>256	>256	>256	>256	128	0.5
AB431	Pus	32	32	>256	>256	>256	>256	>256	256	64	1
AB432	ปลายสาย	32	32	>256	>256	>256	>256	>256	128	64	1
AB433	Pus	32	64	>256	>256	>256	>256	>256	128	64	2
AB436	Ascetic Fluid	32	64	>256	>256	>256	>256	>256	128	128	0.5
AB437	Urine	64	64	>256	>256	>256	>256	>256	256	64	1
AB438	Urine	32	64	>256	>256	>256	>256	>256	128	64	1
AB439	Pus	64	64	>256	>256	>256	>256	>256	256	128	1
AB440	Urine	2	2	8	8	256	256	>256	16	64	1
AB441	Sputum	128	128	>256	>256	>256	>256	>256	256	64	0.5
AB444	Pus	32	64	4	0.5	256	>256	>256	256	16	1
AB446	Biopsy	32	32	8	8	>256	>256	>256	256	64	0.5

AB447	Sputum	64	64	>256	>256	>256	>256	>256	256	64	1
AB448	Sputum	64	128	4	4	>256	>256	>256	256	64	1
AB449	Sputum	128	256	>256	>256	>256	>256	>256	>256	64	1
AB451	Sputum	128	128	>256	>256	>256	>256	>256	>256	64	0.5
AB454	Sputum	32	64	64	16	256	256	256	256	32	0.5
AB455	Sputum	64	64	256	256	>256	>256	>256	128	64	1
AB456	Sputum	128	128	>256	>256	>256	>256	>256	256	64	1
AB457	Sputum	128	128	>256	>256	>256	>256	>256	256	128	0.5
AB458	Urine	128	128	>256	>256	>256	>256	>256	256	64	1
AB459	Pus	16	32	8	2	32	32	32	32	1	0.5
AB460	Urine	32	32	>256	>256	>256	>256	>256	>256	64	0.5
AB463	Sputum	64	64	>256	>256	>256	>256	>256	256	64	1
AB465	Sputum	64	64	>256	>256	>256	>256	>256	128	32	0.5
A4	Peritoneal dialysis	64	64	>256	>256	256	>256	>256	256	128	1
A5	pleural fluid	32	64	32	>256	32	32	16	32	128	1
A6	Body Fluid	64	64	32	>256	>256	>256	>256	256	128	1
A7	Body Fluid	64	64	>256	>256	>256	>256	>256	256	128	1
A8	bile	64	128	1	32	>256	>256	>256	128	16	1
A9	bile	64	128	>256	>256	>256	>256	128	256	128	1
A10	pus	128	256	2	ND	ND	ND	ND	ND	ND	2

Results of MICs of 10 antibiotics against 22 *A. pittii* isolates

isolate	specimen	MICs (mg/L)									
		IPM	MEM	AK	GN	CTX	CRO	CAZ	FEP	CIP	CT
AP1	Bile	32	32	2	32	32	32	16	8	0.125	0.5
AP3	Pus	0.5	0.5	4	2	16	16	4	4	0.125	1
AP4	Pus	32	32	>256	>256	16	8	8	128	256	1
AP5	Body Fluid	0.5	0.5	4	2	8	8	8	2	0.06	1
AP6	Aspirate	0.5	0.5	4	1	8	16	8	2	0.125	1
AP7	Urine	0.5	1	>256	>256	64	256	>256	128	32	1
AP8	Urine	0.5	1	32	64	128	128	256	128	32	1
AP9	Eye swab	4	1	4	2	8	8	8	4	0.125	1
AP10	Pus	0.5	0.5	4	0.5	16	16	8	4	0.125	1
AP11	Sputum	0.25	0.5	8	1	32	32	16	32	0.125	1
AP12	Blood	0.25	1	8	2	16	16	16	4	0.125	1
AP13	Aspirate	1	1	8	1	16	16	32	8	0.125	0.5
AP14	Tracheal secretion	16	4	8	32	>256	32	32	32	0.125	0.25
AP15	Catheter	1	1	2	1	16	16	16	4	0.06	0.5
AP16	Urine	32	32	>256	>256	>256	>256	>256	>256	64	1
AP17	Sputum	1	1	128	>256	>256	>256	>256	>256	64	1
AP18	Urine	0.25	0.125	2	0.5	4	8	8	4	0.06	1
AP20	Nose	0.25	0.5	2	0.5	8	8	8	8	0.03	1
AP21	Urine	4	4	32	32	>256	>256	>256	>256	32	0.5
AP22	Pus	0.25	1	1	0.25	16	16	16	8	0.125	1
AP23	Pus	16	32	2	0.5	32	32	32	64	64	1
AP24	Throat Swab	0.25	1	2	1	16	16	16	8	0.06	1

Results of MICs of 10 antibiotics against 19 *A. nosocomialis* isolates

isolate	specimen	MICs (mg/L)									
		IPM	MEM	AK	GN	CTX	CRO	CAZ	FEP	CIP	CT
AN1	Sputum	16	32	2	1	32	256	4	32	1	1
AN2	Pancreatic Fluid	0.5	0.5	8	2	16	32	8	4	0.5	2
AN3	Sputum	0.5	0.5	4	2	8	16	4	4	0.06	1
AN4	Sputum	16	32	2	1	16	16	4	32	0.06	2
AN5	Pleural fluid	0.5	0.5	4	1	16	32	16	4	0.25	1
AN6	Pus	0.5	0.5	4	0.5	16	32	8	4	0.125	2
AN7	Pus	0.25	0.5	2	0.5	8	16	16	8	0.125	0.5
AN8	Sputum	0.25	0.5	2	0.5	16	16	16	8	0.125	0.5
AN9	Sputum	2	1	4	0.5	32	32	32	8	0.25	1
AN10	Bronchial washing	4	0.5	2	1	16	16	16	8	0.25	0.5
AN11	Sputum	2	1	4	1	16	16	16	8	0.125	1
AN12	Sputum	32	64	2	0.5	16	32	32	64	0.06	2
AN13	Sputum	0.25	0.5	1	0.5	8	16	16	8	0.03	1
AN14	Body Fluid	1	0.5	2	0.5	32	16	16	8	0.25	1
AN15	Sputum	16	32	2	0.5	8	32	32	32	0.03	1
AN16	Pus	0.25	0.5	4	0.5	8	16	16	8	0.125	1
AN17	Sputum	0.25	2	2	0.5	32	16	16	8	0.125	0.25
AN19	Sputum	0.125	0.5	2	1	16	16	16	32	0.03	2
AN20	Urine	16	32	2	0.5	32	32	32	64	64	2

Carbapenem resistance mechanisms in *A. baumannii* isolates

Isolate	MIC (mg/L)		Carbapenemase genes					MIC (mg/L)		MIC (mg/L)	
	IPM	MEM	*bla* OXA-51-like	*bla* OXA-23-like	*bla* OXA-24-like	*bla* OXA-58-like	*bla* IMP-like	IPM + CCCP	IPM + reserpine	MEM + CCCP	MEM + reserpine
AB1	32	32	+	+	-	-	-	32	32	32	32
AB2	64	64	+	+	-	-	-	64	64	64	64
AB3	16	32	+	+	-	-	-	16	16	32	32
AB4	64	64	+	+	-	-	-	64	64	128	128
AB5	64	128	+	+	-	-	-	64	64	128	64
AB6	32	32	+	+	-	-	-	32	32	64	32
AB8	64	64	+	+	-	-	-	64	64	32	32
AB9	16	32	+	+	-	-	-	16	16	32	32
AB11	32	16	+	+	-	-	-	32	32	16	16
AB13	32	64	+	+	-	-	-	32	64	64	32
AB15	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB16	64	16	+	+	-	-	-	64	64	16	16
AB17	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB19	128	64	+	+	-	-	-	128	128	64	64
AB20	32	16	+	+	-	-	-	32	32	8	16
AB21	32	16	+	+	-	-	-	32	32	16	16
AB23	0.5	0.5	+	+	-	-	-	ND	ND	ND	ND
AB24	0.125	0.125	+	+	-	-	-	ND	ND	ND	ND
AB25	128	16	+	+	-	-	-	64	64	16	16
AB26	128	64	+	+	-	-	-	64	64	64	64
AB27	128	32	+	+	-	-	-	128	128	32	32
AB28	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB29	32	64	+	+	-	-	-	32	32	64	64
AB32	64	16	+	+	-	-	-	64	64	8	16
AB34	128	32	+	+	-	-	-	128	128	32	32
AB35	32	64	+	+	-	-	-	32	32	64	32
AB44	128	64	+	+	-	-	-	64	64	64	64
AB45	64	32	+	+	-	-	-	32	32	16	16
AB46	32	16	+	+	-	-	-	32	32	16	16
AB51	128	32	+	+	-	-	-	128	128	32	32
AB52	128	32	+	+	-	-	-	128	128	32	32
AB53	64	32	+	+	-	-	-	64	64	32	32
AB54	32	16	+	+	-	-	-	16	32	16	16
AB55	32	64	+	+	-	-	-	32	64	64	64
AB56	32	16	+	+	-	-	-	32	32	16	16
AB57	128	64	+	+	-	-	-	128	128	64	64
AB58	32	64	+	+	-	-	-	32	32	64	64
AB59	64	32	+	+	-	-	-	64	64	32	32
AB60	64	16	+	+	-	-	-	64	64	16	16
AB62	32	32	+	+	-	-	-	16	16	32	32
AB63	64	16	+	+	-	-	-	32	32	16	16
AB64	128	32	+	+	-	-	-	128	128	32	32
AB68	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB69	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB71	64	32	+	+	-	-	-	64	64	32	32
AB75	256	64	+	+	-	-	-	256	256	64	64
AB77	32	32	+	+	-	-	-	32	32	32	32
AB78	64	64	+	+	-	-	-	64	64	64	64
AB80	128	64	+	+	-	-	-	128	128	64	64
AB81	1	2	+	+	-	-	-	ND	ND	ND	ND
AB83	64	32	+	+	-	-	-	64	64	32	32
AB84	64	16	+	+	-	-	-	64	64	16	16
AB85	32	16	+	+	-	-	-	32	32	16	16
AB89	64	64	+	+	-	-	-	64	64	64	64
AB90	128	64	+	+	-	-	-	128	128	64	64
AB91	64	64	+	+	-	-	-	64	64	64	64
AB93	64	128	+	+	-	-	-	64	64	64	64
AB96	64	32	+	+	-	-	-	64	64	16	16
AB97	32	64	+	+	-	-	-	32	32	32	64

AB98	16	16	+	+	-	-	-	16	16	16	16
AB99	64	64	+	+	-	-	-	64	64	64	64
AB101	16	16	+	+	-	-	-	16	16	8	16
AB102	64	32	+	+	-	-	-	64	64	32	32
AB104	64	64	+	+	-	-	-	64	64	64	64
AB105	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB106	64	64	+	+	-	-	-	32	32	64	64
AB107	32	16	+	+	-	-	-	32	32	16	16
AB108	256	64	+	+	-	-	-	128	128	64	64
AB109	64	32	+	+	-	-	-	64	64	32	32
AB111	128	32	+	+	-	-	-	128	128	32	32
AB112	128	32	+	+	-	-	-	128	128	32	32
AB113	32	16	+	+	-	-	-	32	32	16	16
AB114	128	32	+	+	-	-	-	128	128	32	32
AB115	64	32	+	+	-	-	-	64	64	32	32
AB116	128	64	+	+	-	-	-	128	128	64	64
AB117	32	16	+	+	-	-	-	32	32	16	16
AB119	32	16	+	+	-	-	-	32	32	16	16
AB122	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB123	128	64	+	+	-	-	-	128	128	64	64
AB124	256	64	+	+	-	-	-	256	256	64	64
AB125	64	16	+	+	-	-	-	64	64	16	16
AB127	32	32	+	+	-	-	-	16	16	32	32
AB128	64	16	+	+	-	-	-	64	64	16	16
AB132	128	32	+	+	-	-	-	128	128	32	32
AB133	64	32	+	+	-	-	-	64	64	32	32
AB138	64	32	+	+	-	-	-	64	64	32	32
AB142	256	64	+	+	-	-	-	256	256	64	64
AB143	128	64	+	+	-	-	-	128	128	64	64
AB145	64	32	+	+	-	-	-	64	64	32	32
AB147	0.5	0.5	+	+	-	-	-	ND	ND	ND	ND
AB148	64	16	+	+	-	-	-	32	32	8	8
AB150	128	64	+	+	-	-	-	64	64	64	64
AB151	128	32	+	+	-	-	-	128	128	32	32
AB153	128	64	+	+	-	-	-	128	128	64	64
AB156	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB158	128	32	+	+	-	-	-	128	128	32	32
AB160	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB161	128	32	+	+	-	-	-	128	128	32	32
AB163	64	64	+	+	-	-	-	64	64	64	64
AB165	1	2	+	+	-	-	-	ND	ND	ND	ND
AB167	64	64	+	+	-	-	-	32	32	64	64
AB168	64	64	+	+	-	-	-	64	64	64	64
AB170	1	1	+	+	-	-	-	ND	ND	ND	ND
AB171	32	32	+	+	-	-	-	32	32	32	32
AB172	64	64	+	+	-	-	-	32	32	64	64
AB174	64	64	+	+	-	-	-	64	64	64	64
AB175	32	64	+	+	-	-	-	32	32	64	64
AB176	64	64	+	+	-	-	-	32	32	64	64
AB177	32	32	+	+	-	-	-	16	32	32	32
AB179	1	2	+	+	-	-	-	ND	ND	ND	ND
AB180	64	32	+	+	-	-	-	64	64	32	32
AB181	64	64	+	+	-	-	-	64	64	64	64
AB182	0.25	1	+	+	-	-	-	ND	ND	ND	ND
AB183	64	64	+	+	-	-	-	64	64	64	64
AB185	64	64	+	+	-	-	-	64	64	64	64
AB187	32	16	+	+	-	-	-	32	32	16	16
AB188	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB189	32	16	+	+	-	-	-	16	16	8	8
AB190	16	32	+	+	-	-	-	16	16	32	32
AB191	64	64	+	+	-	-	-	64	64	64	64
AB192	32	32	+	+	-	-	-	32	32	32	32
AB193	32	32	+	+	-	-	-	32	32	32	32

AB195	64	64	+	+	-	-	-	64	64	64	64
AB196	32	32	+	+	-	-	-	16	16	32	32
AB197	64	64	+	+	-	-	-	32	32	64	64
AB198	0.25	0.25	+	+	-	-	-	ND	ND	ND	ND
AB201	32	32	+	+	-	-	-	32	32	32	32
AB202	0.25	0.25	+	+	-	-	-	ND	ND	ND	ND
AB203	32	32	+	+	-	-	-	16	16	32	32
AB204	32	64	+	+	-	-	-	32	32	64	64
AB205	16	32	+	+	-	-	-	16	16	32	32
AB209	32	16	+	+	-	-	-	32	32	16	16
AB211	16	16	+	+	-	-	-	16	16	16	16
AB215	0.25	0.25	+	+	-	-	-	ND	ND	ND	ND
AB217	32	16	+	+	-	-	-	32	32	16	16
AB219	32	16	+	+	-	-	-	32	32	16	16
AB220	64	64	+	+	-	-	-	64	64	32	32
AB221	16	16	+	+	-	-	-	16	16	16	16
AB222	0.25	0.5	+	+	-	-	-	ND	ND	ND	ND
AB223	64	64	+	+	-	-	-	64	64	64	64
AB224	64	64	+	+	-	-	-	64	64	64	64
AB225	16	16	+	+	-	-	-	16	16	16	16
AB227	16	8	+	-	-	+	-	16	16	8	8
AB230	64	32	+	+	-	-	-	64	64	32	32
AB234	0.5	1	+	+	-	-	-	ND	ND	ND	ND
AB235	64	64	+	+	-	-	-	64	64	64	64
AB236	32	32	+	+	-	-	-	32	32	32	32
AB237	64	64	+	+	-	-	-	32	32	64	64
AB238	64	64	+	+	-	-	-	32	32	64	64
AB239	0.25	0.25	+	+	-	-	-	ND	ND	ND	ND
AB240	32	32	+	+	-	-	-	32	32	16	16
AB242	64	32	+	+	-	-	-	64	64	32	32
AB247	128	64	+	+	-	-	-	128	128	64	64
AB249	128	64	+	+	-	-	-	128	128	64	64
AB250	16	16	+	-	+	-	-	16	16	16	16
AB254	0.25	0.5	+	+	-	-	-	ND	ND	ND	ND
AB255	64	64	+	+	-	-	-	64	64	64	64
AB256	32	16	+	+	-	-	-	32	32	16	16
AB257	128	64	+	+	-	-	-	128	128	64	64
AB259	64	16	+	+	-	-	-	64	64	16	16
AB260	128	32	+	+	-	-	-	64	64	32	32
AB262	128	32	+	+	-	-	-	64	64	32	32
AB264	1	0.5	+	+	-	-	-	ND	ND	ND	ND
AB265	32	16	+	+	-	-	-	32	32	16	16
AB266	128	32	+	+	-	-	-	128	128	32	32
AB267	256	64	+	+	-	-	-	256	256	64	64
AB268	32	16	+	+	-	-	-	32	32	16	16
AB269	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB270	128	32	+	+	-	-	-	128	128	32	32
AB271	32	16	+	+	-	-	-	32	32	16	16
AB273	0.5	0.5	+	+	-	-	-	ND	ND	ND	ND
AB276	256	64	+	+	-	-	-	256	256	64	64
AB277	0.5	0.25	+	+	-	-	-	ND	ND	ND	ND
AB278	64	32	+	+	-	-	-	64	64	32	32
AB279	32	16	+	+	-	-	-	32	32	16	16
AB282	64	32	+	+	-	-	-	64	64	32	32
AB284	0.5	0.5	+	+	-	-	-	ND	ND	ND	ND
AB286	128	32	+	+	-	-	-	64	64	32	32
AB287	64	32	+	+	-	-	-	64	64	32	32
AB289	64	16	+	+	-	-	-	64	64	16	16
AB293	256	128	+	+	-	-	-	256	256	128	128
AB294	128	64	+	+	-	-	-	128	128	64	64
AB295	32	32	+	+	-	-	-	32	32	32	32

AB297	128	32	+	+	-	-	-	128	128	32	32
AB298	128	32	+	+	-	-	-	128	128	32	32
AB301	256	64	+	+	-	-	-	256	256	64	64
AB302	64	32	+	+	-	-	-	64	64	32	32
AB303	32	16	+	+	-	-	-	32	32	16	16
AB306	256	64	+	+	-	-	-	256	256	64	64
AB307	64	32	+	+	-	-	-	64	64	32	32
AB309	64	16	+	+	-	-	-	64	64	16	16
AB311	128	16	+	+	-	-	-	128	128	16	16
AB312	64	16	+	+	-	-	-	32	32	16	16
AB314	128	32	+	+	-	-	-	128	128	32	32
AB315	64	16	+	+	-	-	-	32	32	16	16
AB316	128	64	+	+	-	-	-	128	128	64	64
AB317	128	64	+	+	-	-	-	128	128	64	64
AB318	64	16	+	+	-	-	-	64	64	16	16
AB320	128	64	+	+	-	-	-	128	128	64	64
AB324	0.5	0.5	+	+	-	-	-	ND	ND	ND	ND
AB325	32	64	+	+	-	-	-	32	32	64	64
AB326	32	64	+	+	-	-	-	32	32	64	64
AB328	64	64	+	+	-	-	-	64	64	64	64
AB329	64	64	+	+	-	-	-	64	64	64	64
AB330	16	32	+	+	-	-	-	16	16	32	32
AB331	2	2	+	+	-	-	-	16	16	32	32
AB332	16	32	+	+	-	-	-	64	64	64	64
AB333	64	64	+	+	-	-	-	32	32	64	64
AB334	0.5	0.5	+	+	-	-	-	ND	ND	ND	ND
AB336	64	64	+	+	-	-	-	64	64	64	64
AB337	0.25	0.25	+	+	-	-	-	ND	ND	ND	ND
AB338	0.125	0.25	+	+	-	-	-	ND	ND	ND	ND
AB339	64	128	+	+	-	-	-	64	64	128	128
AB342	0.25	0.5	+	+	-	-	-	ND	ND	ND	ND
AB343	64	64	+	+	-	-	-	64	64	64	64
AB344	64	64	+	+	-	-	-	64	64	64	64
AB345	64	64	+	+	-	-	-	64	64	64	64
AB346	32	32	+	+	-	-	-	16	16	32	32
AB347	64	128	+	+	-	-	-	64	64	128	128
AB349	32	64	+	+	-	-	-	32	32	64	64
AB351	128	128	+	+	-	-	-	128	128	128	128
AB353	64	64	+	+	-	-	-	64	64	64	64
AB354	64	128	+	+	-	-	-	64	64	128	64
AB355	64	64	+	+	-	-	-	64	64	64	64
AB358	64	64	+	+	-	-	-	64	64	64	64
AB360	64	64	+	+	-	-	-	64	64	64	64
AB362	64	64	+	+	-	-	-	64	64	64	64
AB363	64	128	+	+	-	-	-	64	64	128	128
AB364	64	128	+	+	-	-	-	64	64	128	128
AB365	128	128	+	+	-	-	-	128	128	128	128
AB366	128	128	+	+	-	-	-	128	128	128	128
AB367	64	64	+	+	-	-	-	64	64	64	64
AB368	64	64	+	+	-	-	-	64	64	64	64
AB370	64	64	+	+	-	-	-	64	64	64	64
AB371	64	64	+	+	-	-	-	64	64	64	64
AB373	64	64	+	+	-	-	-	64	64	64	64
AB374	64	64	+	+	-	-	-	64	64	64	64
AB375	32	64	+	+	-	-	-	32	32	64	64
AB376	32	32	+	+	-	-	-	32	32	32	32
AB377	128	64	+	+	-	-	-	128	128	64	64
AB378	64	64	+	+	-	-	-	64	64	64	64
AB380	64	64	+	+	-	-	-	64	64	64	64
AB381	32	64	+	+	-	-	-	32	32	64	64
AB382	0.25	0.5	+	+	-	-	-	ND	ND	ND	ND
AB383	32	32	+	+	-	-	-	32	32	32	32

AB384	0.25	0.5	+	+	-	-	-	ND	ND	ND	ND
AB385	128	128	+	+	-	-	-	128	128	128	128
AB386	32	64	+	+	-	-	-	32	32	64	64
AB387	32	64	+	+	-	-	-	32	32	64	64
AB388	32	64	+	+	-	-	-	32	32	64	64
AB389	32	64	+	+	-	-	-	32	32	64	64
AB391	64	64	+	+	-	-	-	64	64	64	64
AB392	32	64	+	+	-	-	-	32	32	64	64
AB393	32	32	+	+	-	-	-	32	32	32	32
AB394	32	32	+	+	-	-	-	32	32	32	32
AB395	64	128	+	+	-	-	-	64	64	128	128
AB396	64	128	+	+	-	-	-	64	64	128	128
AB398	64	64	+	+	-	-	-	64	64	64	64
AB400	32	64	+	+	-	-	-	32	32	64	64
AB401	32	64	+	+	-	-	-	32	32	64	64
AB402	0.25	0.5	+	+	-	-	-	ND	ND	ND	ND
AB404	64	64	+	+	-	-	-	64	64	64	64
AB405	32	32	+	+	-	-	-	32	32	32	32
AB406	32	64	+	+	-	-	-	32	32	64	64
AB407	32	64	+	+	-	-	-	32	32	64	64
AB412	32	64	+	+	-	-	-	32	32	64	64
AB414	32	32	+	+	-	-	-	32	32	32	32
AB417	128	128	+	+	-	-	-	128	128	128	128
AB418	32	32	+	+	-	-	-	32	32	32	32
AB419	32	16	+	+	-	-	-	32	32	16	16
AB423	64	128	+	+	-	-	-	64	64	64	64
AB424	32	64	+	+	-	-	-	32	32	64	64
AB425	64	128	+	+	-	-	-	64	64	128	128
AB426	0.25	0.25	+	+	-	-	-	ND	ND	ND	ND
AB427	64	128	+	+	-	-	-	64	64	128	128
AB428	64	128	+	+	-	-	-	64	64	128	128
AB429	128	128	+	+	-	-	-	64	64	128	128
AB430	128	128	+	+	-	-	-	128	128	128	128
AB431	32	32	+	+	-	-	-	32	32	32	32
AB432	32	32	+	+	-	-	-	32	32	32	32
AB433	32	64	+	+	-	-	-	32	32	64	64
AB436	32	64	+	+	-	-	-	32	32	64	64
AB437	64	64	+	+	-	-	-	64	64	64	64
AB438	32	64	+	+	-	-	-	32	32	64	64
AB439	64	64	+	+	-	-	-	64	64	64	64
AB440	2	2	+	+	-	-	-	ND	ND	ND	ND
AB441	128	128	+	+	-	-	-	128	128	128	128
AB444	32	64	+	+	-	-	-	32	32	64	64
AB446	32	32	+	+	-	-	-	32	32	32	32
AB447	64	64	+	+	-	-	-	64	64	64	64
AB448	64	128	+	+	-	-	-	64	64	128	128
AB449	128	256	+	+	-	-	-	128	128	256	256
AB451	128	128	+	+	-	-	-	128	128	128	128
AB454	32	64	+	+	-	-	-	32	32	64	64
AB455	64	64	+	+	-	-	-	64	64	64	64
AB456	128	128	+	+	-	-	-	128	128	128	128
AB457	128	128	+	+	-	-	-	128	128	128	128
AB458	128	128	+	+	-	-	-	128	128	128	128
AB459	16	32	+	+	-	-	-	16	16	32	32
AB460	32	32	+	+	-	-	-	32	32	32	32

AB463	64	64	+	+	-	-	-	64	64	64	64
AB465	64	64	+	+	-	-	-	64	64	64	64
A4	64	64	+	+	-	-	-	64	64	128	64
A5	32	64	+	+	-	-	-	32	32	64	64
A6	64	64	+	-	-	+	-	64	64	64	64
A7	64	64	+	+	-	-	-	64	32	64	64
A8	64	128	+	+	-	-	-	64	64	128	64
A9	64	128	+	+	-	-	-	64	64	128	128
A10	128	256	+	-	+	-	-	128	128	256	256

Carbapenem resistance mechanisms in *A. pittii* isolates

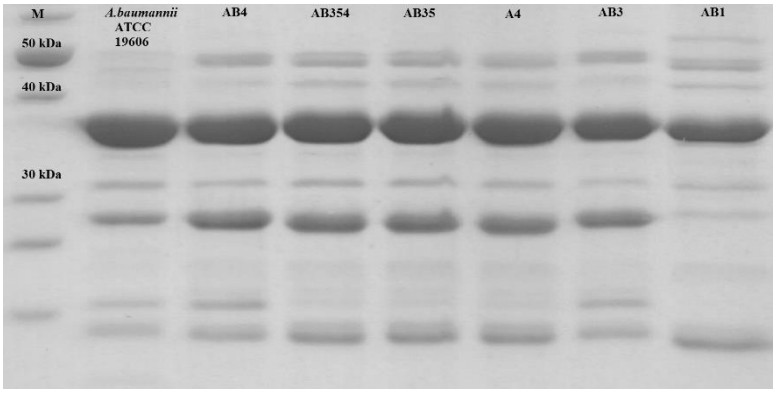
Isolate	MIC (mg/L)		Carbapenemase genes					MIC (mg/L)		MIC (mg/L)		over-expression by RT-PCR
	IPM	MEM	*bla* OXA-51-like	*bla* OXA-23-like	*bla* OXA-24-like	*bla* OXA-58-like	*bla* IMP-like	IPM + CCCP	IPM + reserpine	MEM + CCCP	MEM + reserpine	
AP1	32	32	-	-	-	+	+	32	32	64	32	+
AP3	0.5	0.5	-	-	-	-	-	ND	ND	ND	ND	ND
AP4	32	32	-	+	-	-	-	32	32	32	32	-
AP5	0.5	0.5	-	-	-	-	-	ND	ND	ND	ND	ND
AP6	0.5	0.5	-	-	-	-	-	ND	ND	ND	ND	ND
AP7	0.5	1	-	-	-	+	-	0.5	0.5	1	1	-
AP8	0.5	1	-	-	-	+	-	0.5	0.5	1	1	-
AP9	4	1	-	-	-	-	-	ND	ND	ND	ND	ND
AP10	0.5	0.5	-	-	-	-	-	ND	ND	ND	ND	ND
AP11	0.25	0.5	-	-	-	-	-	ND	ND	ND	ND	ND
AP12	0.25	1	-	-	-	-	-	ND	ND	ND	ND	ND
AP13	1	1	-	-	-	-	-	ND	ND	ND	ND	ND
AP14	16	4	-	-	-	+	-	16	16	4	4	+
AP15	1	1	-	-	-	-	-	ND	ND	ND	ND	ND
AP16	32	32	-	+	-	-	-	32	16	32	32	+
AP17	1	1	-	-	-	+	-	1	1	1	1	-
AP18	0.25	0.125	-	-	-	-	-	ND	ND	ND	ND	ND
AP20	0.25	0.5	-	-	-	-	-	ND	ND	ND	ND	ND
AP21	4	4	-	-	-	-	-	ND	ND	ND	ND	ND
AP22	0.25	1	-	-	-	-	-	ND	ND	ND	ND	ND
AP23	16	32	-	+	-	-	-	16	16	32	32	-
AP24	0.25	1	-	-	-	-	-	ND	ND	ND	ND	ND

Carbapenem resistance mechanisms in *A. nosocomialis* isolates

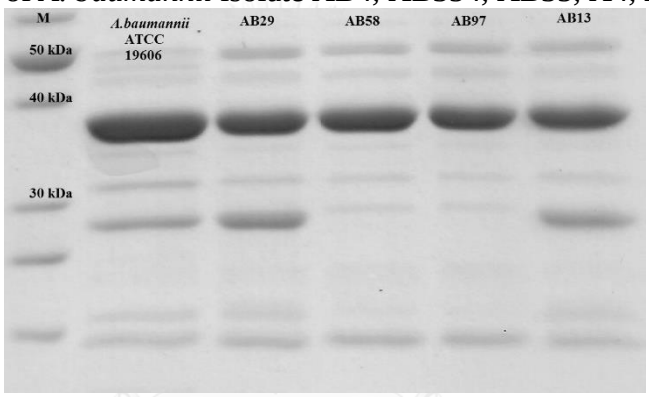
Isolate	MIC (mg/L)		Carbapenemase genes					MIC (mg/L)		MIC (mg/L)	
	IPM	MEM	*bla* OXA-51-like	*bla* OXA-23-like	*bla* OXA-24-like	*bla* OXA-58-like	*bla* IMP-like	IPM + CCCP	IPM + reserpine	MEM + CCCP	MEM + reserpine
AN1	16	32	-	+	-	-	-	16	16	32	32
AN2	0.5	0.5	-	-	-	-	-	ND	ND	ND	ND
AN3	0.5	0.5	-	-	-	-	-	ND	ND	ND	ND
AN4	16	32	-	+	-	-	-	16	16	32	32
AN5	0.5	0.5	-	-	-	-	-	ND	ND	ND	ND
AN6	0.5	0.5	-	-	-	-	-	ND	ND	ND	ND
AN7	0.25	0.5	-	-	-	-	-	ND	ND	ND	ND
AN8	0.25	0.5	-	-	-	-	-	ND	ND	ND	ND
AN9	2	1	-	-	-	-	-	ND	ND	ND	ND
AN10	4	0.5	-	-	-	-	-	ND	ND	ND	ND
AN11	2	1	-	-	-	-	-	ND	ND	ND	ND
AN12	32	64	-	+	-	-	-	32	32	64	32
AN13	0.25	0.5	-	-	-	-	-	ND	ND	ND	ND
AN14	1	0.5	-	-	-	-	-	ND	ND	ND	ND
AN15	16	32	-	+	-	-	-	16	16	32	32
AN16	0.25	0.5	-	-	-	-	-	ND	ND	ND	ND
AN17	0.25	2	-	-	-	-	-	ND	ND	ND	ND
AN19	0.125	0.5	-	-	-	-	-	ND	ND	ND	ND
AN20	16	32	-	+	-	-	-	16	16	32	32

APPENDIX E

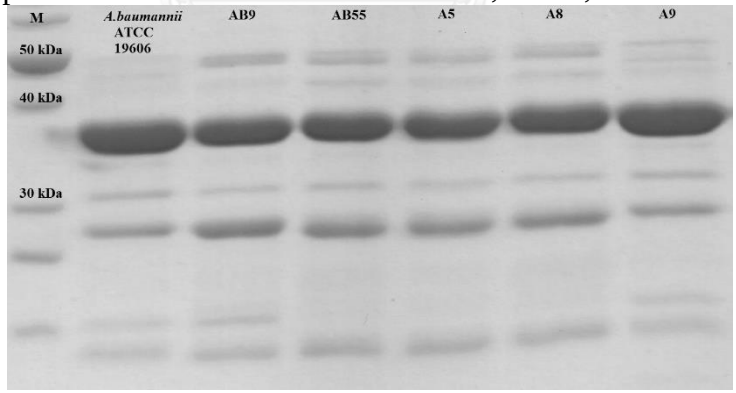
SDS-PAGE OF OMP PROFILE RESULTS



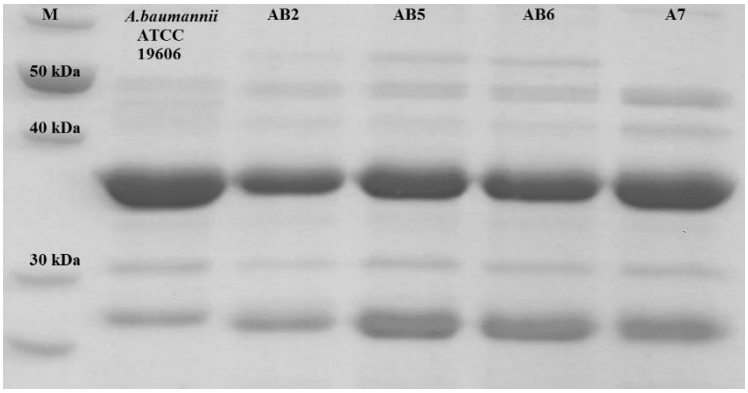
OMP profiles of *A. baumannii* isolate AB4, AB354, AB35, A4, AB3 and AB1



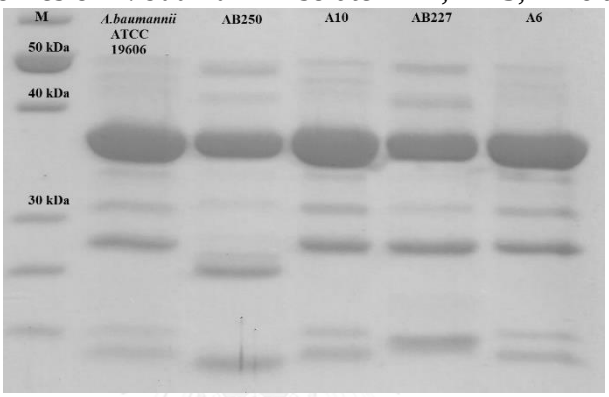
OMP profiles of *A. baumannii* isolate AB29, AB58, AB97 and AB13



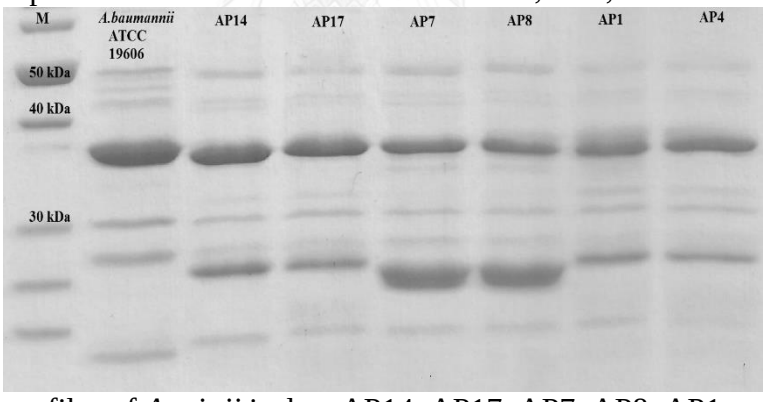
OMP profiles of *A. baumannii* isolate AB9, AB55, A5, A8, AB9



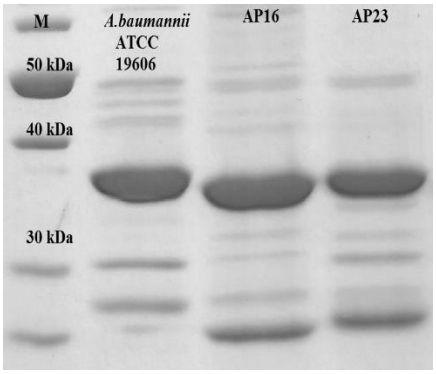
OMP profiles of *A. baumannii* isolate AB2, AB5, AB6 and AB7



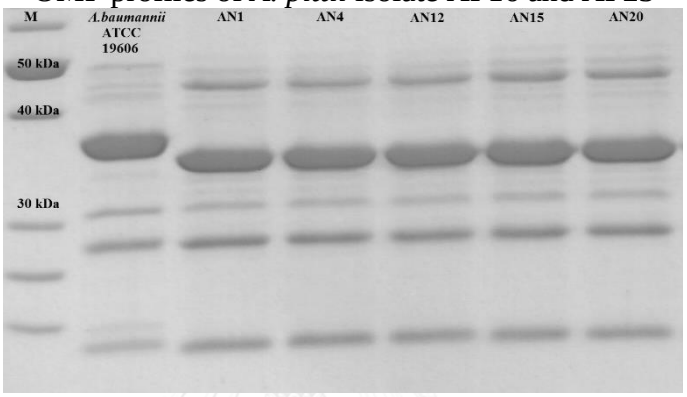
OMP profiles of *A. baumannii* isolate AB250, A10, AB227 and A6



OMP profiles of *A. pittii* isolate AP14, AP17, AP7, AP8, AP1 and AP4



OMP profiles of *A. pittii* isolate AP16 and AP23



OMP profiles of *A. nosocomialis* isolate AN1, AN4, AN12, AN15 and AN20

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