The synergistic activity of antibiotic combinations against colistin-resistant *Acinetobacter baumannii* clinical isolates and its mechanisms related to colistin resistance

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การเสริมฤทธิ์ของยาปฏิชีวนะต่อเชื้อ *Acinetobacter baumannii* ที่ดื้อต่อยาโคลิสตินที่แยกได้จากผู้ป่วยและกลไกในการดื้อยาโคลิสติน

นายสุกฤษฎ์ ศรีสกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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สุกฤษฎ์ ศรีสกุล : การเสริมฤทธิ์ของยาปฏิชีวนะต่อเชื้อ *Acinetobacter baumannii* ที่ดื้อต่อยาโคลิสตินที่แยกได้จากผู้ป่วยและกลไกในการดื้อยาโคลิสติน. (The synergistic activity of antibiotic combinations against colistin-resistant *Acinetobacter baumannii* clinical isolates and its mechanisms related to colistin resistance) อ.ที่ปรึกษาหลัก : อ. ดร.ธนิษฐา ฉัตรสุวรรณ

อุบัติการณ์การเพิ่มขึ้นของเชื้อ *Acinetobacter baumannii* ที่ดื้อต่อยาโคลิสตินเป็นปัญหาสำคัญในการรักษาโรคติดเชื้อด้วยยาปฏิชีวนะ เนื่องจากยาปฏิชีวนะที่มีประสิทธิภาพต่อการรักษาเชื้อดื้อยานี้มีอยู่อย่างจำกัด การใช้ยาปฏิชีวนะมากกว่าหนึ่งชนิดร่วมกันจึงเป็นทางเลือกในการรักษาอย่างหนึ่ง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาอุบัติการณ์การดื้อยาโคลิสตินในเชื้อ *A. baumannii* และกลไกที่เกี่ยวข้องในการดื้อยาโคลิสติน รวมทั้งศึกษาผลของการใช้ยาปฏิชีวนะร่วมกันสองชนิดในการกำจัดเชื้อ *A. baumannii* ที่ดื้อยาโคลิสติน ตัวอย่างเชื้อในการศึกษานี้เป็นเชื้อ *A. baumannii* ที่แยกมาจากผู้ป่วย จำนวน 317 ตัวอย่างซึ่งแยกมาจากเชื้อ *Acinetobacter* spp. ที่ดื้อยาคาร์บาพีเนมจำนวน 341 สายพันธุ์ด้วยวิธีการระบุชนิดเชื้อด้วยยีน *gyrB* การดื้อยาโคลิสตินในเชื้อ *A. baumannii* พบอุบัติการณ์ร้อยละ 15.1 ทำการศึกษากลไกการดื้อยาโคลิสตินและการเสริมฤทธิ์กันของยาปฏิชีวนะ จากตัวแทนเชื้อ *A. baumannii* ที่ดื้อยาโคลิสตินจำนวน 30 สายพันธุ์ พบว่า 27 สายพันธุ์ (ร้อยละ 90) มีการดัดแปลงโครงสร้างของ lipid A ด้วย phosphoethanolamine (pEtN) การศึกษาลำดับกรดอะมิโนใน PmrC พบการเปลี่ยนแปลงที่ตำแหน่ง N284D ในเชื้อทุกสายพันธุ์ โดยพบการแทนที่กรดอะมิโนที่เหมือนกัน 48 ตำแหน่งในเชื้อ 22 สายพันธุ์ (ร้อยละ 73.3) และ 8 สายพันธุ์มีการแทนที่กรดอะมิโนที่ต่างกันออกไป ใน PmrA พบว่าเชื้อ 22 สายพันธุ์ (ร้อยละ 73.3) มีการเปลี่ยนแปลงของกรดอะมิโน 4 ตำแหน่ง พบการเปลี่ยนแปลงกรดอะมิโนทั้งหมด 46 ตำแหน่งใน PmrB โดยการเปลี่ยนแปลงที่ตำแหน่ง A227V พบในเชื้อจำนวน 29 สายพันธุ์ (ร้อยละ 96.7) ซึ่งในตำแหน่งนี้มีรายงานว่ามีความสัมพันธุ์กับการดื้อยาโคลิสติน สำหรับเอนไซม์ที่เกี่ยวข้องในการสร้าง lipopolysaccharide พบการเปลี่ยนแปลงกรดอะมิโนที่ตำแหน่ง V3A และ E117K ในเชื้อ 2 (ร้อยละ 6.7) และ 22 (ร้อยละ 93.3) สายพันธุ์ตามลำดับ การเพิ่มการแสดงออกของ efflux pump หรือกลายพันธุ์ในยีนที่เกี่ยวข้องกับการสร้าง lipopolysaccharide เป็นกลไกที่เกี่ยวข้องกับการดื้อยาโคลิสตินและอาจส่งผลต่อการดื้อยาในเชื้อที่นำมาศึกษา ผลการเสริมฤทธิ์กันของยาปฏิชีวนะด้วยวิธี checkerboard ในเชื้อ 30 สายพันธุ์พบว่าคู่ยา colistin/sulbactam, colistin/fosfomycin และ sulbactam/fosfomycin มีการเสริมฤทธิ์กันในเชื้อ *A. baumannii* ร้อยละ 86.7, 33.3 และ 70 ตามลำดับ ผลการศึกษา โดยวิธี Time-kill เพื่อยืนยันผลการเสริมฤทธิ์ของคู่ยา colistin/sulbactam ในวิธี checkerboard ในตัวแทนเชื้อ 6 สายพันธุ์พบว่าคู่ยา colistin/sulbactam มีการเสริมฤทธิ์ในการฆ่าเชื้อ *A. baumannii* ที่ดื้อยาโคลิสตินทั้งหมด โดยสรุปการศึกษานี้พบการเพิ่มขึ้นของอุบัติการณ์การดื้อยาโคลิสตินในเชื้อ *A. baumannii* โดยกลไกหลักในการดื้อยาคือการดัดแปลงโครงสร้างของ lipid A ด้วย pEtN ซึ่งเกี่ยวข้องกับการเปลี่ยนแปลงลำดับกรดอะมิโนในโปรตีน PmrC PmrB และ PmrA นอกจากนี้คู่ยา colistin/sulbactam ที่มีการเสริมฤทธิ์กันได้ดี ยังอาจจะใช้เป็นทางเลือกเพื่อการรักษาการติดเชื้อ *A. baumannii* ที่ดื้อยาโคลิสตินได้

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Sukrit Srisakul : The synergistic activity of antibiotic combinations against colistin-resistant *Acinetobacter baumannii* clinical isolates and its mechanisms related to colistin resistance. Advisor: TANITTHA CHATSUWAN, Ph.D.

The increasing development of colistin resistance in *Acinetobacter baumannii* clinical isolates is a global concern. Due to the limitation of effective antibiotics, the antibiotic combination therapies are the alternative choices. Here, we investigated the prevalence of colistin resistant *A. baumannii* (CoR-AB) clinical isolates and their resistance mechanisms, and examined the *in vitro* synergistic activities of the antibiotic combinations against CoR-AB. Among 341 carbapenem-resistant *Acinetobacter* spp., 317 (92.0%) were identified as *A. baumannii* by *gyrB* multiplex PCR. The rate of colistin resistance was 15.1% in *A. baumannii* isolates with MIC range of 0.125 – 64 mg/L. A total of 30 representative CoR-AB isolates were selected for the studies of colistin resistance mechanisms and synergistic activity of antibiotic combinations. The phosphoethanolamine (pEtN) addition to lipid A was found in 27 of 30 (90%) CoR-AB clinical isolates by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS). In PmrC, The N248D mutation was found in 30 CoR-AB isolates. The 22 of 30 (73.3%) CoR-AB isolates harbored identical substitution in 48 amino acid positions and the other 8 isolates harbored different amino acid substitutions. For PmrA, the 4 amino acid stitutions were found in 22 (73.3%) CoR-AB isolates. Twenty-five amino acid substitutions were found in PmrB. Twenty-nine (96.7%) isolates contained the A227V mutation in PmrB that was previously described as the cause of colistin resistance. Alterations of the lipopolysaccharide production enzyme (LpxD) were identified at V3A and E117K (6.7% and 93.3%, respectively). Other mechanisms including overexpression of efflux pumps or mutations in genes associated with lipopolysaccharide production might be associated with colistin resistance in our isolates. The synergistic activities of colistin/sulbactam, colistin/fosfomycin, and sulbactam/fosfomycin combinations were identified in 86.7%, 33.3%, and 70% of CoR-AB isolates, respectively, by checkerboard assay. The time-kill study confirmed the synergistic effect of colistin/sulbactam with various concentration combinations against 6 representative CoR-AB isolates. In conclusion, this study indicated the increasing prevalence of colistin resistance in *A. baumannii*. The pEtN modification is the major mechanism of colistin resistance, which is contributed to the mutations in PmrC, PmrB, and PmrA proteins. The colistin/sulbactam combination could be used as alternative therapy for CoR-AB infections.

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ABBREVIATIONS

Abc	*Acinetobacter baumannii-calcoaceticus*
µL	microliter
µM	micromole
AMK	Amikacin
Ara4N	4-deoxy-aminoarabinose
ATCC	American type culture collection
bla	Beta-lactamase gene
BLAST	Basic local alignment search tool
bp	Base pairs
CAMHB	Cation-adjusted Mueller-Hinton broth
CAMPs	Cationic antimicrobial peptides
CFU	Colony forming unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
COL	Colistin
CoR-AB	Colistin-resistant *A. baumannii*
DNA	Deoxynucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
et al	Et alii
FICI	Fractional inhibitory concentration index
FOS	Fosfomycin
G-6-P	Glucose-6-phosphate
IMP	Imipenem
IMP	Imipenemase
IS	Insertion sequence
KPC	*Klebsiella pneumoniae* carbapenemase
LB	Lysogeny broth
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide

LVX	Levofloxacin
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
MBLs	Metallo-beta-lactamases
mcr	Mobilized colistin resistance
MDR	Multidrug-resistant
MEM	Meropenem
mg/L	Milligram/liter
mg/mL	Milligram/milliliter
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
mM	millimole
NARST	National antimicrobial resistance surveillance Thailand
NDM	New Delhi metallo-beta-lactamase
°C	Degree Celsius
OXA	Oxacillinases
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDR	Pandrug-resistant
pEtN	Phosphoethanolamine
RND	Resistant-nodulation-division
rpm	Round per minute
SUL	Sulbactam
TBE	Tris-borate EDTA buffer
TCs	Two-component systems
TSB	Tryptic soy broth
VIM	Verona imipenemase
XDR	Extremely drug-resistant

CHAPTER I

INTRODUCTION

Acinetobacter baumannii-calcoaceticus (*Abc*) complex is the group of Gram-negative coccobacilli bacteria in the genus *Acinetobacter* that consists of many members such as *A. baumannii*, *A. pittii*, *A. nosocomialis* (1, 2). The most hazardous pathogen is *A. baumannii* because it has emerged as the most problematic nosocomial pathogen in the global healthcare system and rapid increase in antimicrobial resistance rate (3). It is associated with various infections, including ventilator-associated pneumonia, bloodstream infections, skin and soft tissue infections, and urinary tract infections. The infections caused by *A. baumannii* are related to high mortality and morbidity, especially in critically ill patients (4, 5). *A. baumannii* has rapidly developed antibiotic resistance due to its remarkable abilities to up-regulate and acquired resistance elements. While carbapenems were commonly used against *A. baumannii* infection, multidrug-resistant (MDR) and extensively drug-resistant (XDR) *A. baumannii* isolates (including carbapenem resistance) had been emerged (6). The rising prevalence of MDR- and XDR-*A. baumannii* further limits the antibiotic options and has enforced the use of colistin (7).

Colistin, a member of polymyxin antibiotic, has been re-introduced to use against carbapenem-resistant *A. baumannii* as a "last-resort" antibiotic (8). It can bind to the lipopolysaccharide (LPS) of bacteria, leading to cell death (9). However, colistin-resistant *A. baumannii* (CoR-AB) was first reported in the Czech Republic, and then the prevalence has been increasing across geographic regions (10). The recent study revealed the global rate of colistin resistance in *A. baumannii* was 13%. In Thailand, the data from various tertiary care hospitals showed that 14.3% of CoR-AB clinical isolates were found in MDR-*A. baumannii* (11).

The mechanisms of colistin resistance involve the modification of lipid A (component of LPS), loss of lipid A, and up-regulation of efflux pumps. (12). In *A. baumannii*, the main mechanism of colistin resistance is the mutations in *pmrCAB*

operon that are associated with the phosphoethanolamine (pEtN) addition to lipid A (13, 14). Moreover, the lipid A modification can cause by the addition of galactosamine (15). These two kinds of modification reduce the affinity binding between the colistin and lipid A and cause colistin resistance. The inactivation of LPS biosynthesis related to the mutations in *lpxACD* genes or insertion inactivation were reported as mechanisms of colistin resistance in *A. baumannii* (16, 17). Additionally, the plasmid-mediated colistin resistance (*mcr*) gene has been described to involve in colistin resistance in Enterobacteriaceae (18). To date, the recent study indicated the discovery of *mcr-1* gene and *mcr-4.3* variant in *A. baumannii* clinical samples (19, 20).

The antibiotic combination therapy is one strategy to substitute the new drug development. Different activities of each antibiotic in combination may be enhanced by each other and reduce their toxicities (21, 22). The colistin-based combinations such as colistin plus rifampicin and colistin plus vancomycin were indicated synergistic activity against MDR-AB and CoR-AB (23-26). Sulbactam is the beta-lactamase inhibitor that has bactericidal activity against *A. baumannii* (8). Previous studies were reported the *in vitro* synergy of colistin plus sulbactam or fosfomycin, which may be the treatment options for carbapenem-resistant *A. baumannii* (27, 28). However, the data of effective combination against CoR-AB is limited.

Since colistin is a last-resort antimicrobial agent and the development of colistin resistance in *A. baumannii* during treatment of colistin alone, the antibiotic combinations are considered for therapeutic choices to decrease colistin resistance in *A. baumannii*. The activity of each drug may be enhanced by antibiotic combination and shows synergistic effect against colistin-resistant *A. baumannii.* Also, the combinations can reduce the adverse effects of each antibiotic and prevent the antibiotic-resistant strain during the treatment. However, there were limited data on the antibiotic combinations against CoR-AB. The synergistic activities of antibiotic combinations were investigated in this study including colistin/sulbactam, colistin/fosfomycin, and sulbactam/fosfomycin. Moreover, there were few data on

molecular mechanisms of colistin resistance and relatedness to the activity of antibiotic combinations in *A. baumannii*. Therefore, this study aimed to investigate the mechanisms of colistin resistance and the synergistic activities of antibiotic combinations against colistin-resistant *A. baumannii* clinical isolates from Thailand.

CHAPTER II

OBJECTIVES

1. To determine the prevalence and mechanisms of colistin resistance in colistin-resistant *A. baumannii* clinical isolates
2. To investigate synergistic activities of antibiotic combinations against colistin-resistant *A. baumannii* clinical isolates

CHAPTER III

LITERATURE REVIEW

1. Bacteriology of *Acinetobacter baumannii*

The genus *Acinetobacter* is currently defined as aerobic, glucose-non-fermentative, catalase-positive, oxidase-negative, Gram-negative coccobacillus. This genus is classified in phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, and family Moraxellaceae (3). According to the discovery of Bouvet and Grimont in 1986, the species in the genus *Acinetobacter* are differentiated into 12 DNA groups (genospecies) by DNA-DNA hybridization (29). Currently, the genus *Acinetobacter* comprises more than 50 species. The most clinically relevant organisms are *Abc* complex including *A. baumannii*, *A. pittii* (formerly *Acinetobacter* genospecies 3), *A. nosocomialis* (formerly *Acinetobacter* genospecies 13TU), and one environmental species, *A. calcoaceticus*. Recently, *A. seifertii* and *A. dijkshoonniae*, which recovered from human specimens, are also included in the *Abc* complex (30). However, identification to species level in the *Abc* complex remains complicated because they are closely related and show similar phenotypic and biochemical properties. Various molecular methods are used to identify including DNA-DNA hybridization (gold standard method), 16s rRNA and *rpoB* sequence analyses, amplified ribosomal DNA restriction analysis (ARDRA), multiplex polymerase chain reaction of specific gene (16S-23S rRNA ITS region, *recA* gene, *gyrB* gene, bla_{OXA-51}-like gene), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) with different accuracy rate and reliability. (3)

Among *Abc* complex, *A. baumannii* is the important opportunistic pathogen related to nosocomial infections such as pneumonia (especially ventilator-associated pneumonia), bacteremia, skin and soft tissue infections, urinary tract infections, and central nervous system infections (31). *A. baumannii* commonly infects critically ill patients and promotes increased mortality and morbidity. The abilities of *A. baumannii* to survive under a wide range of environmental conditions and to persist

for extended periods of time on surfaces make it a frequent cause of outbreaks of infection and an endemic, healthcare-associated pathogen (5).

2. *Acinetobacter baumannii*: pathogenesis and virulence factors

2.1 Environmental persistence

The persistence of *A. baumannii* on surfaces can cause colonization on nosocomial surfaces and subsequently transmit to patients. Under the routinely desiccation and disinfection of hospital surfaces, *A. baumannii* can develop resistance to those stresses (32). Capsular polysaccharides prevent loss of water from bacterial cells under desiccation period and maintain bacterial viability (33). Furthermore, the lipooligosaccharides (LOS), composition of outer membrane, conserve water and hydrophilic nutrients in the cell and may involve in desiccation resistance (34). Desiccation-rehydration process can cause damage to DNA including alkylation, oxidation, cross-linking, base removal, and strand breaks. The role of RecA protein, which was required for homologous recombination and DNA repair, was the protection of DNA damage during desiccation period (32). However, the mechanisms of desiccation persistence were not fully investigated and characterized.

In addition, desiccation can also induce oxidative stress. However, *A. baumannii* produces proteins that are involved in the elimination of reactive oxygen species. Indeed, the increased expression of catalase gene, *katG*, by insertion sequence, IS*Aba*1, may be involved in resistance to the level of hydrogen peroxide (35). Disinfectants are extensively used in health care settings to eradicate pathogen on inanimate surfaces. Unfortunately, *A. baumannii* develops resistance to many disinfectants, for example, the presence of *Acinetobacter* chlorhexidine (AceI) efflux that effectively pumps chlorhexidine out of the cells (36).

2.2 Biofilm formation

Microbial biofilms are possibly related to host-bacterial interactions and involve in device-associated infections. *A. baumannii* can form biofilms not only within tissue but also abiotic surfaces such as endotracheal tubes. The two-component (TC) regulatory system, BfmRS, that regulates type I chaperone-usher

pilus (Csu pili) of *A. baumannii* is required for biofilm formation and maintenance on inanimate surface (37). Moreover, some clinical strains of *A. baumannii* carried the *csuA/BABCDE* locus which regulated the protein GacSA TC system which was likely to have a functional role in biofilm formation and promoted by exposure to sub-inhibitory concentrations of antibiotics (38, 39). Interestingly, *A. baumannii* also secretes biofilm-associated proteins (Bap_{Ab}) by type I secretion system (T1SS) which mediates cell-cell adhesion contributing to maturation of biofilm on medical devices. Although other factors in *A. baumannii* including RTX-like domain-containing protein and poly-beta-(1,6)-*N*-acetylglucosamine may have roles in biofilm formation, they need further investigations of their specific molecular mechanisms (40, 41).

2.3 Motility

Even though, "*Acinetobacter*" means "non-motile rod", but researchers found the hyper-motility form of *A. baumannii* in the *Caenoerhabditis elegans* infection model with increased virulence. Some of *A. baumannii* strains show two forms of bacterial locomotion. First, twitching motility is related to functional type IV pili that can retraction and extraction to bring cell forward. The upregulation of genes that encode for biosynthesis of type IV pili during growth in human serum indicates that type IV pili may have a role in bacterial motility (42, 43). Second, Surface-associated motility seems like swarming motility of *Pseudomonas aeruginosa*. However, motility of *A. baumannii* currently demonstrate to depend on 1,3-diaminopropane, quorum sensing, and LOS production, not flagella (44-46).

2.4 Glycoconjugates

Glycoconjugates define as carbohydrate components on bacterial cell wall or cell membrane that interact with surrounding environment. Glycoconjugates express the bacterial defense mechanism to stress, host immunity, and have a role in virulence of *A. baumannii.* Various surface carbohydrates are known to be virulence factors of *A. baumannii* including LPS, capsular polysaccharide, glycosylated protein, and peptidoglycan (47).

In *A. baumannii*, LPS is also named lipooligosaccharide (LOS) because it does not produce an O antigen. LOS is an essential component of the outer cell membrane contributing to cell viability. Host immune response recognizes bacterial LOS via Toll-like receptor 4 (TLR4) resulting in stimulation of inflammatory process. Moreover, lipid A, the lipid anchor of LOS, is the target of cationic polymicrobial peptides (CAMPs) and polymyxin antibiotics. Modification of lipid A or complete loss of LPS involve in increased tolerance to CAMPs and polymyxins (48).

Capsular polysaccharide is the extracellular element of most *A. baumannii* strains. It can protect bacterial cell from host defense mechanisms. Indeed, *A. baumannii* mutants that lack capsular production are attenuated and subsequently killed by the complement system (49). Additionally, sub-inhibitory concentrations of antibiotics may increase capsule synthesis that regulating by the BfmRS TC system (50).

2.5 Micronutrient acquisition systems

Transition metals including iron, zinc, and manganese are essential nutritions not only for host but also bacteria. The micronutrient scavenging systems were required for bacterial virulence that was confirmed by high-throughput transposon screen (47). *A. baumannii* carries the catechol-hydroxymate siderophore, named acinetobactin, which has a high-affinity iron-chelating molecule (51, 52). Other iron-chelating molecules are found in *A. baumannii* such as fimbactin A-F, baumannoferrin A-B (53, 54). Furthermore, The ZnuABC system and ZigA protein, regulated by transcriptional repressor (Zur), are zinc acquisition system in *A. baumannii*. These molecules respond to zinc-limited conditions or the presence of calprotectin, host chelating molecules (55, 56).

2.6 Protein secretion

Protein secretion systems are commonly involved in virulence of most Gram-negative bacteria. The proteins secreted by these systems can interact with environmental and also host immunity. First, *Acinetobacter* trimeric autotransporter identified in *A. baumannii* has a role in adhesion to host extracellular matrices and

membrane components (57). Moreover, most *A. baumannii* strains use a type VI secretion system (T6SS) to defeat other bacteria in polymicrobial infection. Finally, there are many effector proteins secreted by type II secretion system (T2SS) of *A. baumannii* such as lipase (LipA) and metalloprotease (CpaA). These secreted enzymes are required for virulence in the lung infection model (58, 59).

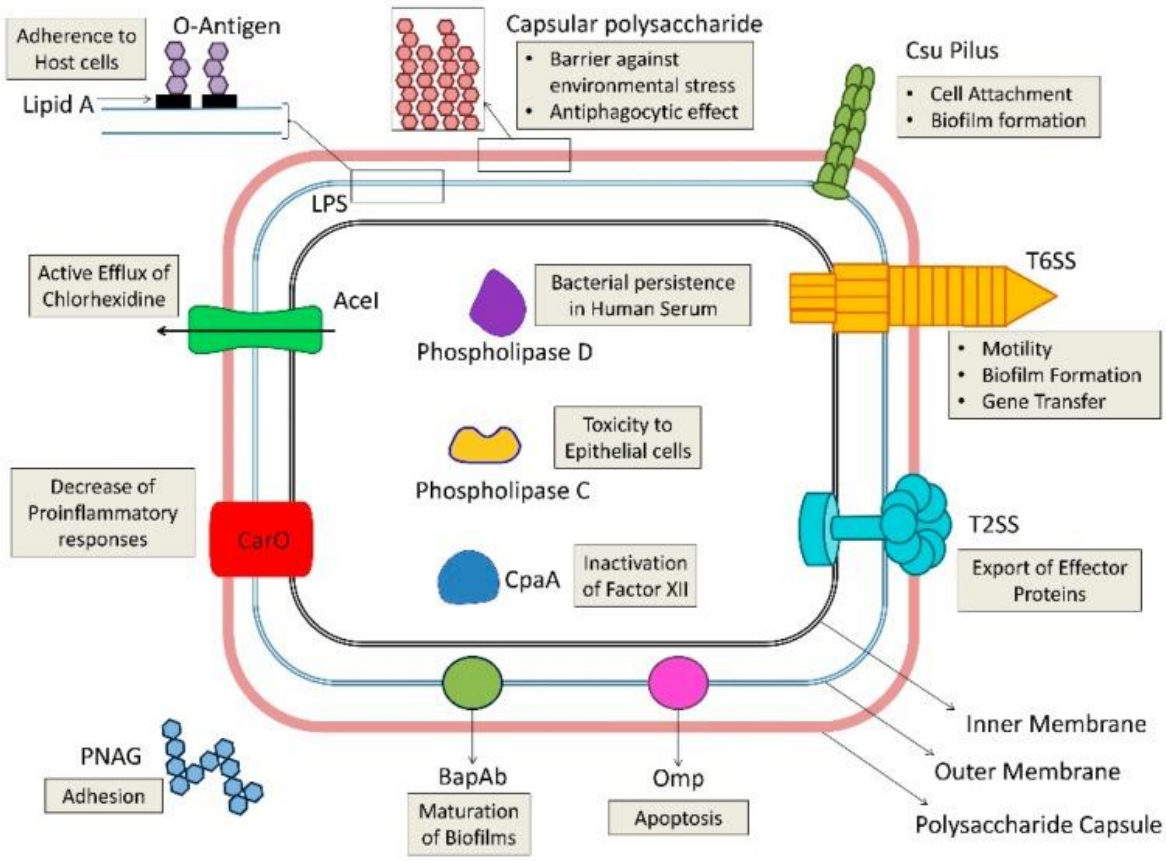


Figure 1. The virulence factors of *A. baumannii* (31)

3. Common antibiotic use in treatment of *A. baumannii* infections

A. baumannii is the causative pathogen that can cause pneumonia, skin infections, cystitis or pyelonephritis, and bloodstream infections. The infections mostly occur in hospital, described in nosocomial infections. *A. baumannii* usually establishes resistance to several antibiotics and spreads through patients in hospital. The antimicrobial therapy against infections due to *A. baumannii* is based on antimicrobial susceptibility testing. As *A. baumannii* has intrinsic resistance to several antibiotics such as penicillins, second- and third-generation cephalosporins, and

macrolides. The antibiotics commonly used to treat *A. baumannii* infections included ceftazidime, cefepime, carbapenems, fluoroquinolones, and aminoglycosides (8).

In general practice, carbapenem antibiotics have been major therapeutic option against *A. baumannii* due to their exquisite bactericidal activity and broad range of spectrum. Meropenem and Imipenem were mostly used both in empirical therapy and specific therapy in patients suspected infection with drug-resistant Gram-negative bacteria (5). Doripenem and biapenem were the newly approved antibiotics in carbapenem groups. These novel antibiotics showed equivalent bactericidal activity against *A. baumannii* when compared with meropenem and imipenem. However, discordance in susceptibilities between the carbapenems had been reported. The selection of antibiotics in this group required local susceptibility data (60, 61).

Sulbactam is the beta-lactamase inhibitor that expresses intrinsic bactericidal activity against multidrug-resistant *A. baumannii* strains. Its activities relate to the affinity for penicillin-binding proteins. Usually, sulbactam has commercially available in combination with ampicillin which does not increase activity of sulbactam. (3, 8). Many studies confirmed significant clinical outcomes of ampicillin-sulbactam in treatment of various *A. baumannii* infections (62-65). Unfortunately, the susceptibility of *A. baumannii* to sulbactam was declined in the period of time, especially in carbapenem-resistant *A. baumannii* isolates (66).

Aminoglycosides are other antibiotics that show *in vitro* activity against *A. baumannii*. Aminoglycosides are not commonly used as a single agent because of their serious renal toxicity. Some studies reported the greater outcome of inhaled aminoglycoside versus intravenous aminoglycosides, both in combination with intravenous beta-lactams, in the treatment of cystic fibrosis by Gram-negative bacteria (67, 68).

Since the appearance of antibiotic-resistant strains during the treatment of single antibiotics and the slow rate of new antibiotic development, the combination of antibiotics should be considered to be the effective therapeutic option for *A.*

baumannii infections (7). The combination of active antibiotics with different mechanisms of action may elicit synergistic activity. Several antibiotic combinations against MDR-*A. baumannii* were investigated including combinations among carbapenems, aminoglycosides, sulbactam, rifampicin, polymyxins, ciprofloxacin, tigecycline, and fosfomycin (27, 28, 69-74). The researches on antibiotic combinations found controversial results in the same combinations, however, the most effective combined antibiotics were reported to be colistin plus carbapenems or rifampin against carbapenem-resistant *A. baumannii* (8).

4. Carbapenem-resistant *A. baumannii*: mechanisms of resistance

A. baumannii becomes the successful pathogen because of its plentiful ability to develop antimicrobial resistance. During treatment of infections by antibiotics, *A. baumannii* rapidly develops antibiotic resistance. When bacteria develop resistance to more than three classes of antibiotics, they are named multidrug-resistant bacteria (MDR). Moreover, *A. baumannii* can develop resistance to all available antibiotics, it named as extremely-drug resistant (XDR) or pan-drug resistant (PDR) isolate (3, 7). One of the most described mechanisms is the resistance to carbapenems, the broad-spectrum antibiotics commonly used in *A. baumannii* infections. The production of beta-lactam inactivating enzymes is the most prevalent resistance mechanism in *A. baumannii*. According to 4 Ambler classes of beta-lactamases, the serine oxacillinases (OXA type) and metallo-beta-lactamases (MBLs) have carbapenemase activities. The OXA-type enzymes are grouped into OXA-23, OXA-24, OXA-51, and OXA-58. The first identified OXA-type enzyme was OXA-23 enzyme from clinical isolate from England in 1985 (75). High prevalence and global dissemination of OXA-23-producing *A. baumannii* has been reported (76). Two other acquired OXA-type gene clusters with carbapenemase activity have been described, including $bla_{\text{oxa-24-like}}$ and $bla_{\text{oxa-58-like}}$ genes (77, 78). These 3 clusters of carbapenemase genes are encoded on plasmid resulting in its widespread distribution (3). The last gene cluster is the $bla_{\text{oxa-51-like}}$ genes that are intrinsically encoded on chromosome of *A. baumannii.* Its role in carbapenem resistance appears to be related to the presence of IS*Aba1*. Insertion of IS*Aba1* in the promoter

sequence of the bla_{OXA-23} gene has been reported to be associated with overexpression of $bla_{OXA-23, -51, -58}$ in *A. baumannii* (79).

The MBL carbapenemases were less identified in *A. baumannii* than OXA-type carbapenemases, but their hydrolytic activities toward carbapenems were more potent. The MBLs have broad spectrum of substrates that can hydrolyze all beta-lactam antibiotics, except monobactam. Several groups of MBLs have been reported in *A. baumannii* including IMP (Imipenemase), VIM (Verona Imipenemase), GIM (German Imipenemase), SIM (Seoul Imipenemase), and NDM (New Delhi metallo-beta-lactamase) (80-82). Unlike the OXA-type enzymes, MBLs were commonly found within integrons. In *A. baumannii*, class 1 integrons contained resistance gene cassettes, resulting in overexpression of multiple resistance genes (83-85). MDR *A. baumannii* frequently carrying integrons and consequently impacted clinical outcome (86).

Efflux pumps are also associated with carbapenem resistance in *A. baumannii*. According to four families of efflux pumps, the resistant-nodulation-division (RND) superfamily is most described to be involved in carbapenem resistance in *A. baumannii* (87). The AdeABC efflux pump, belonging RND family, composes of three components: transmembrane protein (AdeB), inner membrane fusion protein (AdeA), and outer membrane protein (AdeC). The expression of AdeABC is controlled by AdeRS (two-component system). The point mutation or insertion of IS*Aba1* in the *adeS* gene can lead to overexpression of AdeABC (88). The AdeABC pump has a broad range of substrates, including beta-lactams, aminoglycosides, erythromycin, chloramphenicol, tetracyclines, and fluoroquinolones. Thus, the overexpression of AdeABC may result in multidrug resistance (77, 89-92).

The other mechanism that involves in carbapenem resistance is permeability defects. The outer membrane porins are the bacterial structures which allow transport of molecules (including antibiotics) through the bacterial membrane and affect membrane permeability. Reduction of some porins, such as CarO, Omp22-33, Omp33-36, Omp37, Omp43, Omp44, and Omp47 was reported to be associated with imipenem and meropenem resistance in *A. baumannii* (93-98). The loss of CarO porin

influenced by insertion inactivation of *carO* gene was observed in imipenem-resistance *A. baumannii* (98). The combination of the loss of CarO porin and the production of OXA-23 carbapenemases resulted in decreased carbapenem susceptibility (99).

5. Colistin use in multidrug-resistant *A. baumannii* infections

The treatment of carbapenem-resistant *A. baumannii* infections often requires the use of colistin (COL). Colistin belongs to the polymyxin class, the polypeptide antibiotic, that was initially extracted from *Paenibacillus polymyza* (100). The structure of polymyxins is similar to CAMPs, which are the host defense mechanisms against bacterial infections. The mechanism of action of colistin is binding to the outer membrane of Gram-negative bacteria. The positively charged domains of colistin make electrostatic interaction with negatively charged phosphate groups of lipid A. This binding leads to membrane permeability change and cytoplasmic leakage then consequently to cell death (Figure 2) (101, 102). After its discovery, colistin has been widely used for treatment of Gram-negative bacterial infections. However, the important toxicities of colistin are nephrotoxic and neurotoxic causing the restriction of use of this antibiotic (103). After that, it was replaced by others such as beta-lactams (including carbapenems) and aminoglycosides (including ciprofloxacin and levofloxacin), new and less toxic antibiotics.

In the last two decades, colistin has been re-introduced to treatment of drug-resistant *A. baumannii* and other Gram-negative bacteria. Because of the emerging of MDR- and XDR-*A. baumannii*, colistin seems to be the last-resort antibiotic for these infections (104). The use of colistin against MDR-*A. baumannii* are both monotherapy and combination therapies. There are several colistin-based combinations that have been reported *in vivo* and *in vitro* synergistic activity including colistin/rifampicin, colistin/carbapenems, colistin/sulbactam, colistin/tigecycline, and colistin/ daptomycin (71, 73, 105-108). However, resistance to colistin has been reported among *A. baumannii* clinical strains worldwide as a result of the increasing use (109).

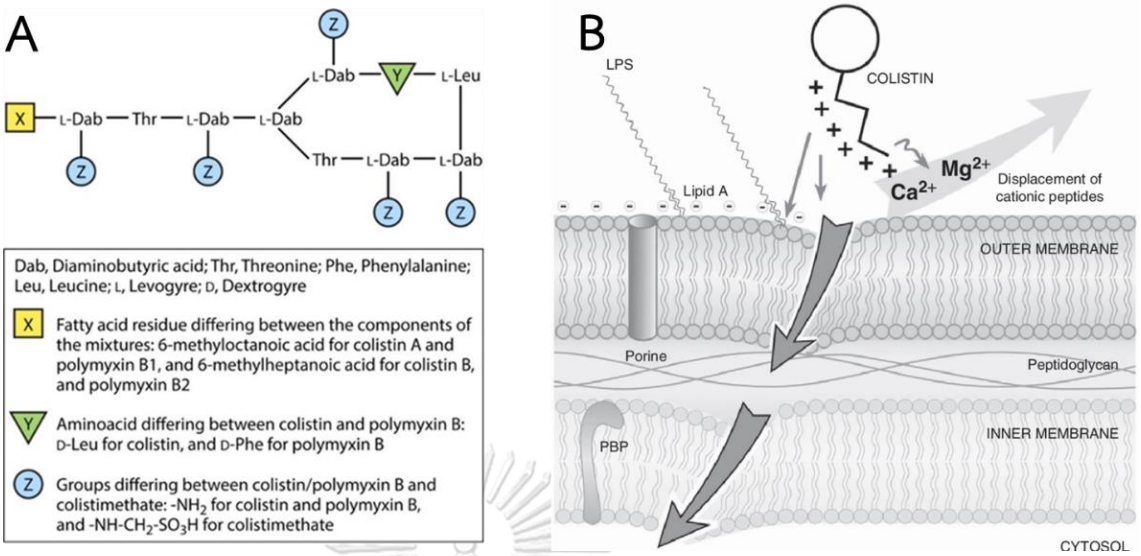


Figure 2. Structure of polymyxin antibiotics (A) (104) and colistin mechanism of action (B) (9)

6. Mechanisms of colistin resistance in *A. baumannii*

Colistin is a cationic amphiphilic agent in polymyxin group that interacts with lipid A component of LPS of bacterial membrane, resulting in membrane destruction and cell death. In the MDR-*A. baumannii* treatment, colistin is a mainstay option with or without other antimicrobials such as carbapenems, sulbactam, or tigecycline. However, the increasing colistin usage brings to the occurrence of colistin resistance in *A. baumannii*. The previous antibiotic treatments are important factors that influence the emergence of colistin resistance rather than person-to-person transmission (104). The colistin resistance in *A. baumannii* is mediated by chromosomal or plasmid genes.

6.1 Chromosomal-mediated colistin resistance

6.1.1 Addition of phosphoethanolamine to lipid A

Because of the ionic binding action of colistin to the phosphate group of lipid A, specific modification of lipid A producing more positively charge is one of mechanism of colistin resistance in Gram-negative bacteria. The common lipid A structure is composed of core amino sugar, lipid side chains, and phosphate groups. However, the acylation patterns are different among species. *A. baumannii* produces four different lipid A species, which the major species are hepta-acylated lipid A (*m/z*

1,910). The minor lipid A species are hexa-acylated lipid A (*m/z* 1,728), penta-acylated lipid A (*m/z* 1,530), and tetra-acylated lipid A (*m/z* 1,404) (Figure 3B). Qureshi, Z.A., *et al.* examined the lipid A of CoR-AB clinical isolates by MALDI-TOF MS. They found that colistin-resistant isolates had an additional ion at *m/z* 2,034 (Figure 3), which corresponded to pEtN modification to hepta-acylated lipid A (109). Moreover, Palletier, M.R., *et al*. revealed that the lipid A modification was caused by the addition of galactosamine (15).

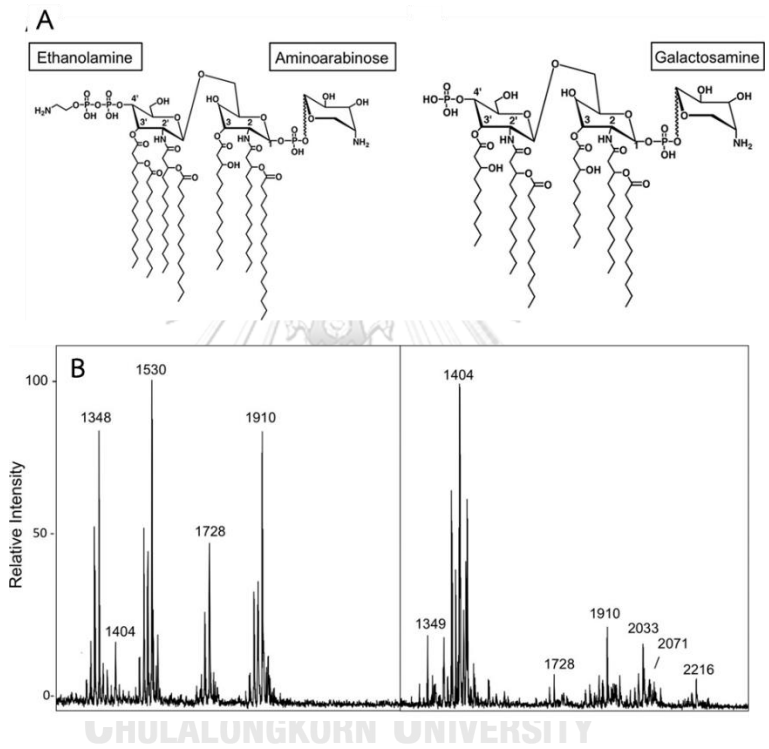


Figure 3. The predicted lipid A structure (A) and mass spectrum (B) of colistin-resistant *A. baumannii* (14)

Two-component systems (TCs), PmrAB and PhoPQ that are associated with the expression of lipid A modification genes were found to be associated with colistin resistance in *Salmonella enterica*, *Escherichia coli*, and *P. aeruginosa* (110-114). The PmrA is the regulator protein that can activate the expression of *ugd, pmrF* operon, and *pmrC* which encode UDP-glucose dehydrogenase, 4-deoxy-aminoarabinose (Ara4N) biosynthetic enzymes, and pEtN transferase, respectively. These kinds of enzymes involve the addition of Ara4N and pEtN to lipid A (115-117). However, Ara4N (and its biosynthesis genes) and PhoPQ have not been reported in *A. baumannii*

(118). Therefore, the hypothesis of colistin resistance mechanism in *A. baumannii* was involved in PmrAB TC and pEtN addition. The PmrAB was first described in 2009 by comparing the nucleotide sequences of *pmrA* and *pmrB* that encoded PmrA and PmrB, respectively, between colistin-susceptible and laboratory-induced CoR-AB strains. They found that colistin resistance in *A. baumannii* was associated with mutations in *pmrA* and *pmrB* genes. Furthermore, the result also showed the increased expression of *pmrA* gene (119). Park Y.K., *et al.* investigated the mutations in *pmrCAB* and the expression level of *pmrA* and *pmrB* in both colistin-resistant clinical and laboratory-induced strains. The results showed that alteration of amino acid sequence in PmrAB may not be important for colistin resistance, but the elevated expression of PmrAB TC is essential for resistance (120). The recent research analyzed the role of PmrCAB in several clinical and laboratory mutants of CoR-AB. They hypothesized that two distinct genetic events, at least one mutation in PmrB and up-regulated expression of *pmrA* and *pmrB*, were required for colistin resistance in *A. baumannii*. Moreover, these genetic events led to the addition of pEtN to hepta-acylated lipid A species, causing the LPS modification that directly conferred the colistin resistance (13, 14).

The most recent study found that the global transcription regulator H-NS played a role in regulating genes involving in colistin resistance. This study indicates that both modification and inactivation of H-NS can increase colistin resistance. The insertion inactivation of H-NS by IS*Aba125* resulted in overexpression of *eptA*, a close *pmrC* homolog, but not increased expression of *pmrCAB* (screening by transcriptomic analysis). The *eptA* gene encodes pEtN transferase. Therefore, the upregulation of *eptA* may cause colistin resistance by the pEtN addition mechanism (121, 122).

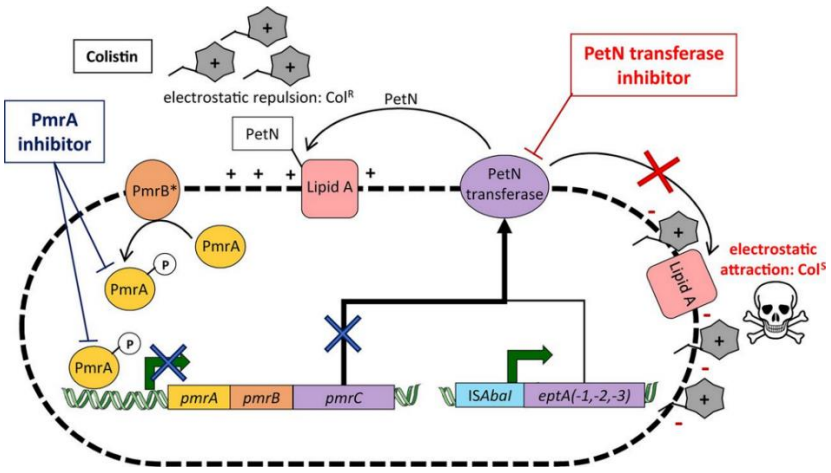


Figure 4. Presentation of colistin resistance mechanisms in *A. baumannii*

6.1.2 Loss of lipopolysaccharide production

The lipid A, part of LPS, is an important structure of cell membrane and virulence factor of Gram-negative bacteria. The studies of *E. coli* LPS production indicated that there were nine enzymatic steps producing hexa-acylated lipid A. The first hypothesis of the loss of LPS was proposed by Moffatt J.H., *et al*. They analyzed the genes involving in lipid A production in both laboratory-derived and clinal colistin-resistant strains. Their results showed the wide range of mutations (single point mutation, amino acid deletion, and frameshift) in LpxA, LpxC, and LpxD because of the nucleotide mutations in *lpxA*, *lpxD*, and *lpxC* genes (16). Furthermore, the insertion inactivation by novel IS element in *lpxD* and IS*Aba11* in either *lpxA* and *lpxC* were observed in these studies. These genetic alterations resulted in complete loss of LPS, leading to reduced colistin susceptibility (123).

The complete loss of LPS affects the susceptibility to colistin, which is associated with *lpxA, lpxC,* and *lpxD* genes. LPS modification can be a result of acylation and deacylation process regulated by *lpxM, pagL,* and *lpxR* gene (124, 125).

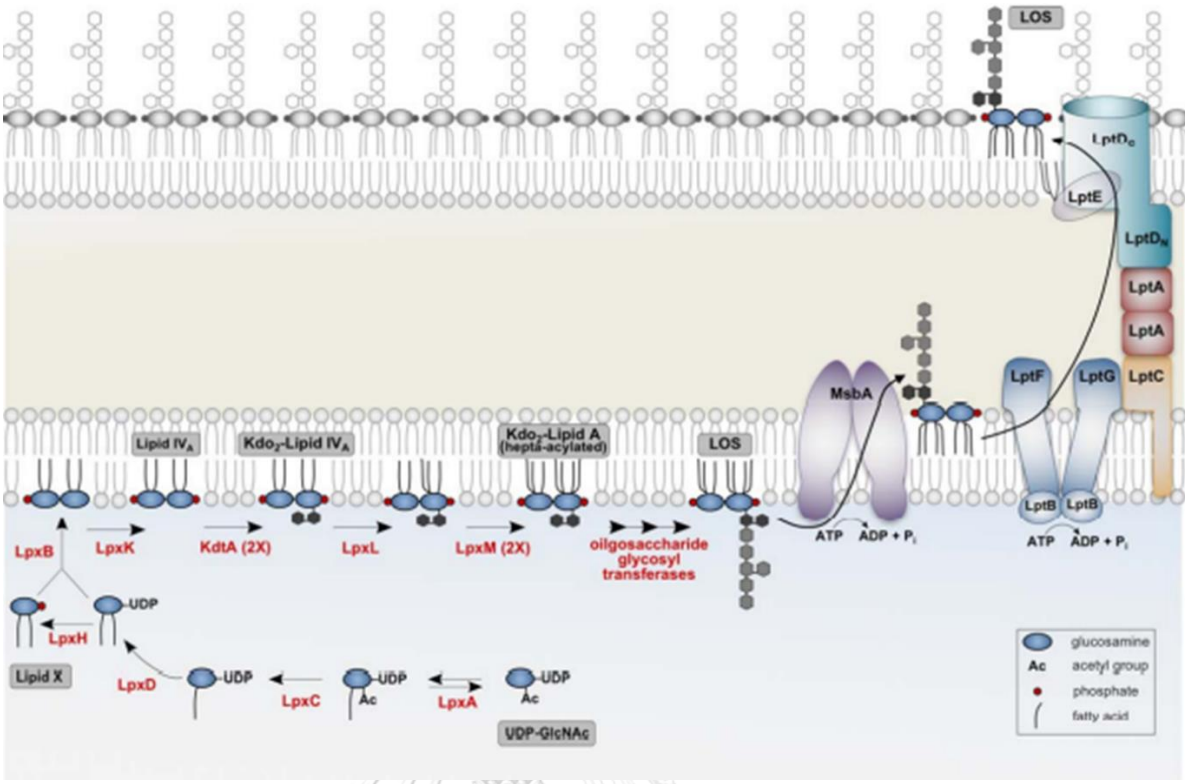


Figure 5. Schematic overview of *A. baumannii* lipooligosacccharides production and transportation (126)

6.2 Plasmid-mediated colistin resistance

Besides the chromosomal-mediated colistin resistance, the plasmid-borne genes (*mcr*) are recently public health concern. These genes are located on transferable plasmids, which can be transferred to other Gram-negative bacteria (18, 127). The *mcr* genes encode the pEtN transferase enzyme, resulting in pEtN addition to lipid A. MCR enzymes generally confer low to moderate colistin resistance (12). The *mcr-1* gene was first identified in commensal *E. coli* isolates from food animals in China during surveillance (18). Consequently, the *mcr*-positive Enterobacteriaceae strains were identified in many countries in Asia, Europe, Africa, and Americas, from both infected patients and environmental (12). The MCR-2 was next uncovered from *E. coli* isolated from livestock in Belgium. The enzyme encoded by *mcr-2* shared 80.65% amino acid identity with the MCR-1 enzyme (127). Subsequently, *mcr-1.2*, *mcr-3* to *mcr-9* and their variants were identified in *E. coli*, *S. enterica*, *Moraxella* sp.,

and *Klebsiella pneumoniae* (128-135). Most recently, the *mcr-10* was described in China(136).

In *A. baumannii*, the *mcr-1* gene was first reported in clinical isolate from Pakistan in 2019 (19). Recently, the *mcr-4.3* variant was discovered in *A. baumannii* isolated from pig in China and imported raw food and clinical sample in Czech Republic. These variants were located on pAb-MCR4.3 and/or pAB18PR065 that cannot be transferred by conjugation, transformation, or electroporation (20, 137).

6.3 Heteroresistance

Heteroresistance is defined as the presence of subpopulations of resistant organisms in an isolate considered to be susceptible by standard testing methodologies. It is a poorly characterized phenomenon wherein a population of bacteria with similar genotypes may exhibit variable phenotypic responses to an antimicrobial agent. Heteroresistance may also be described as heterogeneous resistance, population-wide variation of resistance, and heterogeneity of response to antibiotics. The clinical significance of heteroresistance is still unclear, but the concern remains that the more resistant subpopulations may be selected out during therapy (138). Furthermore, heteroresistance to colistin was first described in 2006 among clinical isolates of MDR-*A. baumannii* that were reported as susceptible to colistin based on the minimum inhibitory concentration (MIC). The investigators raised concerns for rapid development of resistance and therapeutic failure if colistin is used at suboptimal dose or as monotherapy (139). A subsequent study attempted to associate colistin heteroresistance in *A. baumannii* with prior colistin therapy and demonstrated a significantly higher degree of heteroresistance in isolates from patients with prior colistin treatment (140).

7. Prevalence of colistin-resistant *A. baumannii*

The global rate of colistin-resistant *Acinetobacter* spp. has been steadily increasing since the first description of colistin-resistant strains in 1999 (141). In 2005 and 2007, South Korea reported the high colistin resistance rate in *A. baumannii* (142, 143). Then colistin-resistant strains were increasing found worldwide (144, 145). In earlier studies, several reports in Europe presented that colistin resistance rates were

below 7%. (146-148). However, other reports in Bulgaria and Spain showed the higher colistin resistance rates of 16.7% and 19.1%, respectively (74, 149). Then, in 2014, the increasing rate of colistin resistance was reported in Greece (21.1%) (150). In Asia, various studies indicated that the resistance rates were under 12% between 2007 to 2011 (151-154). Another report from Korea showed the highest rate of colistin resistance at 30.6% (143). The report of worldwide SENTRY Antimicrobial Surveillance database summarized that the colistin resistance rate worldwide was 4.1% from 2001 to 2016. For each geographic region, the colistin resistance rates were 2.5% in Asia-Pacific, 6.1% in Europe, 4.6% in North America, and 1.9% in Latin America (10). The recent systematic review collected the data from different 41 studies between 2010-2019 to establish an overall epidemiology analysis of polymyxin resistance rate in *A. baumannii*. This study found that the overall rate of polymyxin resistance was 13%, whereas the higher rate was observed in America (29%), followed by Europe (13%), and Asia (10%). Only two studies from Africa, collected in this study, indicated the resistance rate of 4% (155). On the other hand, colistin heteroresistance in *A. baumannii* was first reported in Korea in 2006 (139) and was then found in other world regions. The rates of colistin heteroresistance ranged from 18.6% to 100% (12, 22).

In Thailand, the data from National Antimicrobial Resistance Surveillance Thailand (NARST) showed that the prevalence of CoR-AB is only 0.3% (156). The CoR-AB in Thailand was increasingly studied in recent years. Two studies reported colistin resistance rates of 9.3% and 3.6% in 2014-2015 (157, 158). In 2020, the CoR-AB was found in 14.3% among 300 *A. baumannii* clinical isolates from 13 tertiary care hospitals in Thailand (11). Moreover, the heteroresistance rate was reported to be 44% in *A. baumannii* isolates in Thailand (159).

8. Therapeutic strategies against colistin-resistance *A. baumannii* infection

Unfortunately, the infections caused by CoR-AB have been reported increasingly worldwide. The inappropriate dosing of colistin may prone to develop colistin-resistant strains. The given dose of colistin should be depended on the pharmacokinetic/ pharmacodynamics profile of colistin and the susceptibility profile

of organisms (22). The combination therapy of various antibiotics becomes the main therapeutic option and can enhance the activity of combined drugs against colistin-resistant strains. The most effective and regularly investigated combination is colistin plus rifampin (7, 22, 160). The combination of colistin and rifampicin showed significantly synergistic activity more than other colistin-based combination against CoR-AB in checkerboard assay (160, 161). Nevertheless, some *in vitro* studies have conflicting results of colistin plus rifampin activity (162). Following *in vitro* studies, clinical trials that investigated the synergistic effect of colistin plus rifampin versus colistin alone demonstrated non-significant results against CoR-AB (70). Furthermore, combinations of carbapenems and colistin are frequently used in treatment against carbapenem-resistant and colistin-resistant *A. baumannii*. The carbapenems including meropenem, imipenem, and doripenem plus colistin showed high rate of synergy in both checkerboard and time-kill assay (107, 161, 163). The combination of colistin with a new drug such as tigecycline has been investigated. The colistin/tigecycline combination showed acceptable results against CoR-AB (107, 164). There are several attempts to combine colistin with various antibiotics such as vancomycin, trimethoprim-sulfamethoxazole, sulbactam, fosfomycin, and sitafloxacin, but the synergistic effects of these combinations remain unclear (22, 162, 164-167) The triple combination against CoR-AB was investigated in some studies. The combination of colistin plus meropenem and ampicillin/sulbactam can eliminate CoR-AB in hollow-fiber infection model better than antibiotic alone and double combinations (168).

CHAPTER IV

MATERIALS AND METHODS

1. Bacterial strains

1.1 *Acinetobacter* spp. clinical isolates

Three hundred and forty-one of *Acinetobacter* spp. isolates were obtained from the Department of Microbiology, King Chulalongkorn Memorial Hospital. The bacteria were isolated and collected from different patients during February to April 2017, February to March 2018, September to November 2018, and February to July 2019. In addition, *A. baumannii* isolate A5 and AJN3B were obtained from previous studies (169).

1.2 *Acinetobacter baumannii* quality control strain

A. baumannii ATCC 19606 was used in analysis of mechanisms of resistance.

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 in antimicrobial susceptibility testing as recommended by Clinical and Laboratory Standard Institute (CLSI) (170).

All bacterial strains in this study were store at -80 °C in tryptic soy broth with 20% glycerol until required.

2. Identification of *A. baumannii*

All *Acinetobacter* spp. were identified as genus *Acinetobacter* by Gram stain and biochemical test. All isolates were separated to species level by the molecular method as described below.

2.1 Species identification by molecular method (presence of bla_{oxa-51} gene)

DNA was extracted from bacterial strains by boiling method. The colonies of *A. baumannii* were suspended in 200 µL of DNase-free water then boiled at 100 °C for 10 minutes and centrifuged at 13,000 rpm for 10 minutes. Supernatant was collected to new collecting tube and used in the detection of interesting genes.

The bla_{OXA-51} gene was detected by PCR. The presence of bla_{OXA-51} gene was one criterion for identification of *A. baumannii* because it was an intrinsic carbapenemase gene (171). However, another species, *A. nosocomialis* (genospecies 13TU) had been reported to carry bla_{OXA-51} gene (172). Moreover, the insertion sequence can also interfere the detection of bla_{OXA-51} gene in *A. baumannii* (173). Thus, all isolates had to be confirmed the species level by the multiplex PCR for *gyrB* gene.

2.1 Multiplex polymerase chain reaction for *gyrB* genes

The differentiation of species in *Abc* complex can be performed by *gyrB* multiplex PCR. The specific primers were designed for different PCR product size of each species because the *gyrB* nucleotide sequences of each species of *Abc* complex was different. The *A. baumannii* shows the PCR products of 490 bp and 294 bp while *A. pittii* and *A. nosocomialis* show the PCR products of 194 bp and 294 bp, respectively. The primers and PCR condition were performed as described in the previous study (2).

Table 1. Primers for multiplex PCR for *gyrB* gene

Primer name	Sequence (5´-3´)	Species	Reference
D14	GACAACAGTTATAAGGTTTCAGGTG	*A. calcoaceticus*	2
D19	CCGCTATCTGTATCCGCAGTA	*A. calcoaceticus*	
D8	CAAAAACGTACAGTTGTACCACTGC	Genospecies 3	
Sp2F	GTTCCTGATCCGAAATTCTCG	*A. baumannii*	
Sp4F	CACGCCGTAAGAGTGCATTA	*A. baumannii* and genospecies 13TU	
Sp4R	AACGGAGCTTGTCAGGGTTA	*A. baumannii* and genospecies 13TU	

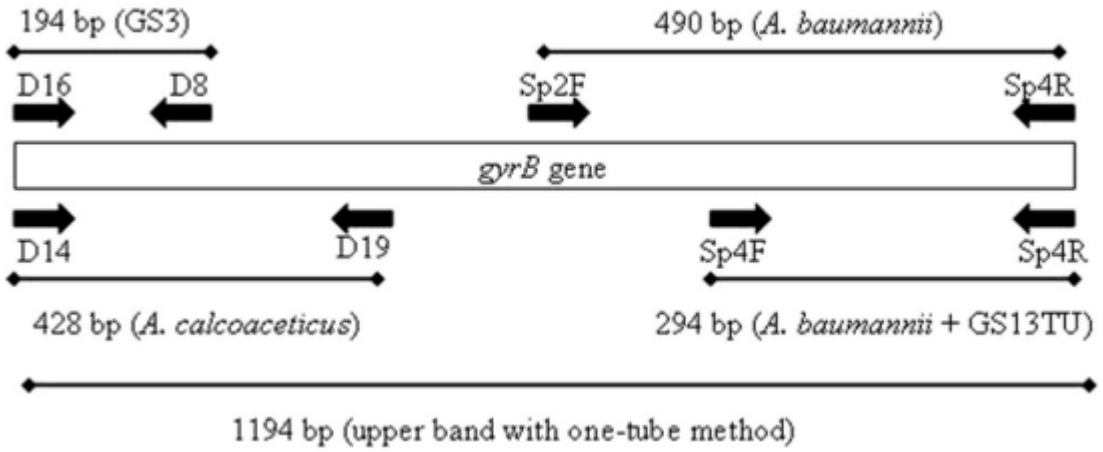


Figure 6. Primer annealing sites on *gyrB* gene for *gyrB* multiplex PCR

3. Antimicrobial susceptibility testing

All *Acinetobacter* spp. isolates were performed antimicrobial susceptibility test for meropenem, imipenem, amikacin, ciprofloxacin, levofloxacin, sulbactam, and fosfomycin by agar dilution method. The broth microdilution method was performed for colistin susceptibility. The MIC was defined as the lowest concentration of antibiotic that can inhibit the growth of microorganisms. The susceptibilities of meropenem, imipenem, amikacin, ciprofloxacin, levofloxacin, and colistin were interpreted according to the CLSI guidelines (170). In addition, the interpretation criteria of ampicillin/sulbactam for *Acinetobacter* spp. was applied for sulbactam susceptibility. The susceptibility of fosfomycin was interpreted according to the criteria for Enterobacteriaceae in CLSI guidelines (Table 2) (170).

3.1 Agar dilution method

Two-fold dilutions of each antibiotic were composed at final concentration 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 mg/L in Mueller-Hinton II agar (MHA) (BBL, BD® Diagnostic Systems, USA). For fosfomycin susceptibility test, each agar plate was supplemented with 25 mg/L of glucose-6-phosphate (G6P) (Sigma-Aldrich, USA) as recommended by CLSI.

Acinetobacter spp. isolates and quality control strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 26853, *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212) were prepared in normal saline to 0.5 McFarland turbidity (~1.5 x 10^8 CFU/mL) and then

diluted 10-fold with normal saline. Each bacterial suspension was applied onto MHA supplemented with antibiotics as final concentration of approximately 10^4 CFU/spot and incubated at 37 °C for 18 - 24 hours.

3.2 Broth microdilution method

According to CLSI, the susceptibility test of colistin was performed by broth dilution method. A two-fold serial dilution of colistin was prepared in cation-adjusted Mueller-Hinton broth (CAMHB) (BBL, BD® Diagnostic Systems, USA) in flat-bottomed 96-well plate at final concentration between 0.125 to 128 mg/L. Fresh culture colonies of *Acinetobacter* spp. and quality control strains (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 26853) were suspended in normal saline to the turbidity of 0.5 McFarland standard then diluted 1:100 with normal saline. Bacterial suspension was added into broth prepared with 2-fold serial dilution of colistin as final concentration approximately 10^5 CFU/mL and incubated at 37 °C for 18 - 24 hours.

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

Antimicrobial agent	Interpretation MIC (mg/L)			Reference
	Susceptible	Intermediate	Resistant	
Meropenem	≤ 2	4	≥ 8	170
Imipenem	≤ 2	4	≥ 8	
Amikacin	≤ 16	32	≥ 64	
Ciprofloxacin	≤ 1	2	≥ 4	
Levofloxacin	≤ 2	4	≥ 8	
Colistin	-	≤ 2	≥ 4	
Fosfomycin	-	≤ 128	≥ 256	
Sulbactam	≤ 8	16	≥ 32	

Abbreviation: MIC, minimum inhibitory concentration

4. Screening of carbapenemase-encoding genes

4.1 Detection of OXA-type carbapenemase genes

DNA extract of each *Acinetobacter* spp. strain from boiling method was used to detect OXA-type carbapenemase genes. The multiplex PCR was performed to detect OXA-type carbapenemase genes including $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$, and $bla_{\text{OXA-58}}$. The specific primers were described in the previous study (174) and shown in Table 3. The PCR reaction was performed in 0.2 ml PCR tube with the 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, 0.625 U of *Taq* DNA polymerase, and 2 µL of boiled DNA. The amplification was performed in thermal cycler (ProFlex® PCR system, Applied Biosystems. USA) with an initial of 94 °C for 5 minutes, followed by 30 cycles of 94 °C for 25 seconds, 52 °C for 40 seconds, and 72 °C for 50 seconds and a final extension at 72 °C for 6 minutes. The PCR products were separated on 1.5% agarose gel containing non-carcinogen dye (StainIN™ GREEN nucleic acid stain, highQu GmbH, Germany) in 0.5x TBE buffer by using electrophoresis at 100 volts for 30 minutes. The agarose gel was visualized under UV light and 100 bp plus DNA ladder (Thermo Fisher® Scientific, USA) was used as approximate DNA size.

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

Primer name	Gene	Sequence (5´-3´)	Product size (bp)	reference
OXA23-F	*bla*$_{\text{OXA-23-like}}$	GATCGGATTGGAGAACCAGA	501	174
OXA23-R		ATTTCTGACCGCATTTCCAT		
OXA24-F	*bla*$_{\text{OXA-24-like}}$	GGTTAGTTGGCCCCCTTAAA	249	
OXA24-R		AGTTGAGCGAAAAGGGGATT		
OXA51-F	*bla*$_{\text{OXA-51-like}}$	TAATGCTTTGATCGGCCTTG	353	
OXA51-R		TGGATTGCACTTCATCTTGG		
OXA58-F	*bla*$_{\text{OXA-58-like}}$	AAGTATTGGGGCTTGTGCTG	599	
OXA58-R		CCCCTCTGCGCTCTACATAC		

Abbreviation: bp, base pairs

4.2 Detection of metallo-carbapenemase genes

The multiplex PCR was performed to detect metallo-carbapenemase genes including *bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM}}$. The specific primers were described in the previous study (175) and shown in Table 4. The PCR reaction was performed in 0.2 ml PCR tube with the 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 IMP-F and IMP-R, 0.16 µM of VIM-F and VIM-R, 0.625 U of *Taq* DNA polymerase, and 2 µL of boiled DNA. The amplification was performed in thermal cycler (ProFlex® PCR system, Applied Biosystems. USA) with an initial of 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 50 seconds and a final extension at 72 °C for 6 minutes. The PCR products were detected as previously described in the method for detection of OXA-type carbapenemase genes detection.

Table 4. Primers for detection of metallo-carbapenemase genes

Primer name	Gene	Sequence (5´-3´)	Product size (bp)	reference
IMP-F	*bla*$_{\text{IMP-like}}$	GGAATAGAGTGGCTTAAYTCT	188	175
IMP-R		CCAAACYACTASGTTATCT		
VIM-F	*bla*$_{\text{VIM-like}}$	GATGGTGTTTGGTCGCATA	390	
VIM-R		CGAATGCGCAGCACCAG		

Abbreviation: bp, base pairs

4.3 Detection of *bla*$_{\text{KPC}}$, *bla*$_{\text{OXA-48}}$, and *bla*$_{\text{NDM}}$

The multiplex PCR was performed to detect other carbapenemase genes including *bla*$_{\text{KPC}}$, *bla*$_{\text{OXA-48}}$, and *bla*$_{\text{NDM}}$. The specific primers were described in the previous study (176) and shown in Table 5. The PCR reaction was performed in 0.2 ml PCR tube with the 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, 1.25 U of *Taq* DNA polymerase, and 2 µL of boiled DNA. The amplification was performed in thermal cycler (ProFlex® PCR system, Applied Biosystems. USA) with an initial of 94 °C for 10 minutes, followed by 35 cycles of 94 °C for 30 seconds, 52 °C for 40 seconds, and 72 °C for 50 seconds and a final extension at 72 °C for 5 minutes. The PCR products were detected as previously described in the method for detection of OXA-type carbapenemase genes.

Table 5. Primers for detection of *bla*$_{\text{NDM-like}}$, *bla*$_{\text{OXA-48-like}}$, and *bla*$_{\text{KPC-like}}$ genes

Primer name	Gene	Sequence (5´-3´)	Product size (bp)	reference
NDM-F	*bla*$_{\text{NDM-like}}$	GGTTTGGCGATCTGGTTTTC	621	176
NDM-R		CGGAATGGCTCATCACGATC		
OXA-48-F	*bla*$_{\text{OXA-48-like}}$	GCGTGGTTAAGGATGAACAC	438	
OXA-48-R		CATCAAGTTCAACCCAACCG		
KPC-F	*bla*$_{\text{KPC-like}}$	CGTCTAGTTCTGCTGTCTTG	798	
KPC-R		CTTGTCATCCTTGTTAGGCG		

Abbreviation: bp, base pairs

5. Nucleotide sequencing of gene involved in mechanisms of colistin resistance

The genomic DNA of CoR-AB isolates was extracted by commercial DNA extraction kit (Purelink® genomic DNA mini kit, Invitrogen, USA) following the manufacturer's recommendation. The entire *pmrCAB* operon was amplified by PCR using primers in Table 6. The PCR reaction was performed in 0.2 ml PCR tube with the total volume of 50 µL reaction by adding the following components: 1x *Taq* buffer, 2 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each primer, 1.25 U of *Taq* DNA polymerase, and 2 µL of template DNA. The amplification was performed in thermal cycler (ProFlex® PCR system, Applied Biosystems. USA) with an initial of 94 °C for 7 minutes, followed by 30 cycles of 94 °C for 30 seconds, 52 °C for 15 seconds, and 72 °C for 4 minutes and a final extension at 72 °C for 10 minutes. For *lpxACD* genes, each gene was amplified by PCR using primers in Table 6. The PCR reaction was performed in 0.2 ml PCR tube with the total volume of 50 µL reaction by adding the following components: 1x *Taq* buffer, 2 mM $MgCl_2$, 200 µM dNTP, 0.4 µM of each primer, 1.25 U of *Taq* DNA polymerase, and 2 µL of template DNA. The amplification was performed in thermal cycler (ProFlex® PCR system, Applied Biosystems. USA) with an initial of 94 °C for 10 minutes, followed by 35 cycles of 94 °C for 30 seconds,

52 °C for 15 seconds, and 72 °C for 45 seconds and a final extension at 72 °C for 10 minutes. The PCR products were detected as previously described in the method for detection of OXA-type carbapenemase genes.

The amplicons were purified by Hiyield® Gel/PCR DNA mini kit (Real Biotech Corporation, Taiwan) and TIANquick® Midi Purification kit (TIANGEN, China). The purified amplicons were sequenced by using the Bigdye Terminator V3.1 Cycler sequencing kit by 1st base DNA sequencing service, Singapore. The nucleotide sequences were translated to amino acid sequences by the ExPASy translate tool (http://web.expasy.org/translate/). The amino acid sequences were analyzed by Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The mutations in these nucleotide and amino acid sequences were carried out in multiple sequence alignment by Florence Corpet (http://multalin.toulhouse.inra.fr/mutalin/multalin.html) and compared with reference nucleotide sequences of *A. baumannii* ATCC 19606 (GenBank accession number CP045110.1) and reference amino acid sequences of PmrC (GenBank accession number QFQ06528.1), PmrA (GenBank accession number QFQ06527.1), PmrB (GenBank accession number QFQ06526.1), LpxA (GenBank accession number QFQ05735.1), LpxC (GenBank accession number QFQ07043.1), and LpxD (GenBank accession number QFQ05737.1).

Table 6. Primers for amplification of entire *pmrCAB* operon and *lpxACD* genes

Primer name	Gene	Sequence (5´-3´)	Product size (bp)	reference
FullpmrCAB-F	Entire *pmrCAB*	GCATCATAAAAAGATTGTAGTCAC	3,699	14
FullpmrCAB-R		GCGATTTGTATTCATCGTTTTGAG		
pmrC-F		ATGTTTAATCTCATTATAGCCA		
pmrC-R		TTAGTTTACATGGGCACAA		
$pmrC_2$-F		GGTTGTTATTGAAGAAAGTAT		
$pmrC_2$-R		TCAATCCAAGTCACTTGGTAAC		
pmrA-F		ATGACAAAAATCTTGATGATTGAAGAT		
pmrA-R		TTATGATTGCCCCAAACGGTAG		
pmrB-F		GTGCATTATTCATTAAAAAAAC		
pmrB-R		TCACGCTCTTGTTTCATGTA		
$pmrB_2$-F		GGTTCGTGAAGCTTTCG		
$pmrB_2$-R		CCTAAATCGATTTCTTTTTG		
Dcap-F2		AAACACCGACCACTGCAAAT		This study
pmrC-R2		GCGTATGGTGCTCAGTTCTCT		This study
lpxC-F	*lpxC*	TGAAGATGACGTTCCTGCAA	1,501	16
lpxC-R		TGGTGAAAATCAGGCAATGA		
lpxA-F	*lpxA*	TGAAGCATTAGCTCAAGTTT	1,178	
lpxA-R		GTCAGCAAATCAATACAAGA		
lpxD-F	*lpxD*	CAAAGTATGAATACAACTTTTGAG	1,143	
lpxD-R		GTCAATGGCACATCTGCTAAT		

Abbreviation: bp, base pairs

6. Screening of plasmid-mediated colistin resistance (*mcr*) genes

DNA extract of 30 CoR-AB isolated from boiling method was used to detect *mcr* genes by multiplex PCR. The specific primers were described in the previous studies (131, 177) and shown in Table 7. The 2 multiplex PCR were performed to detection of *mcr-1* to *mcr-5* and *mcr-6* to *mcr-9*. The PCR reaction was performed in 0.2 ml PCR tube with the total volume of 25 µL reaction by adding the following components: 1x *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM of each primer, 1.25 U of *Taq* DNA polymerase, and 2 µL of boiled DNA. The amplification was performed in thermal cycler (ProFlex® PCR system, Applied Biosystems. USA) with an initial of 94 °C for 15 minutes, followed by 30 cycles of 94 °C for 50 seconds, 52 °C for 90 seconds, and 72 °C for 60 seconds and a final extension at 72 °C for 10 minutes. The PCR products were separated on 1.5% agarose gel containing non-carcinogen dye (StainIN™ GREEN nucleic acid stain, highQu GmbH, Germany) in 0.5x TBE buffer by using electrophoresis at 100 volts for 30 minutes. The agarose gel was visualized under UV light and 100 bp plus DNA ladder (Thermo Fisher® Scientific, USA) was used as approximate DNA size.

Table 7. Primers for amplification of *mcr-1* to *mcr-9* genes

Primer name	Gene	Sequence (5´-3´)	Product size (bp)	reference
mcr-1-F	*mcr-1*	AAAGACGCGGTACAAGCAAC	213	177
mcr-1-R		GCTGAACATGCACGGCACAG		
mcr-2-F	*mcr-2*	CGACCAAGCCGAGTCTAAGG	92	
mcr-2-R		CAACTGCGACCAACACACTT		
mcr-3-F	*mcr-3*	ACCTCCAGCGTGAGATTGTTCCA	169	
mcr-3-R		GCGGTTTCACCAACGACCAGAA		
mcr-4-F	*mcr-4*	AGAATGCCACTCGTAACCCG	230	
mcr-4-R		GCGAGGATCATAGTCTGCCC		
mcr-5-F	*mcr-5*	CTGTGGCCAGTCATGGATGT	98	
mcr-5-R		CGAATGCCCGAGATGACGTA		
mcr-6-F	*mcr-6*	AGCTATGTCAATCCCGTGAT	252	131
mcr-6-R		ATTGGCTAGGTTGTCAATC		
mcr-7-F	*mcr-7*	GCCCTTCTTTTCGTTGTT	551	
mcr-7-R		GGTTGGTCTCTTTCTCGT		
mcr-8-F	*mcr-8*	TCAACAATTCTACAAAGCGTG	856	
mcr-8-R		AATGCTGCGCGAATGAAG		
mcr-9-F	*mcr-9*	TTCCCTTTGTTCTGGTTG	1011	
mcr-9-R		GCAGGTAATAAGTCGGTC		

Abbreviation: bp, base pairs

7. Analysis of lipid A structure by matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF MS)

7.1 Extraction of lipid A from colistin-resistant *A. baumannii* clinical isolates

Lipid A was extracted and purified by an ammonium hydroxide-isobutyric acid method. The isolates were grown in lysogeny broth (LB) (BBL, BD® Diagnostic Systems, USA) at 37 °C with shaking overnight. Bacterial suspension was centrifuged at 5,000 X g for 20 minutes to collect the cell pellet. The bacterial pellet was resuspended with appropriate volume of PBS then lyophilized. Twenty milligrams of lyophilized cells were resuspended in 800 µL isobutyric acid:1 M ammonium hydroxide (5:3, vol/vol) (Sigma-Aldrich®, Germany) and incubated in a 1.5-ml tube at 100 °C for 1 – 2 hours with occasional vortex. Samples were then cooled on ice and centrifuged at 2,000 X g for 15 minutes. Supernatants were transferred to new tubes and added with equal volumes of water, then lyophilized. To purify lipid A, these lyophilized samples were washed twice with 400 µL of methanol and centrifuged (2,000 X g for 15 minutes) to pellet the insoluble lipid A. Subsequently, the insoluble lipid A was solubilized in 100 to 200 µL chloroform:methanol:water (12:6:1, vol/vol/vol), then immediately analyzed by MALDI-TOF MS

7.2 Analysis of lipid A structure

Analysis of lipid A was performed by JMS-S3000 SpiralTOF™-plus MALDI-TOF MS (JOEL Ltd., Japan) in negative spiral mode. Each spectrum was collected with an enough laser shot with 45% laser intensity. Norharmane (Sigma-Aldrich®, Germany) was used as a matrix. A 1 µL of each sample was deposited on the halide-targeted plate and covered with the same amount of the matrix suspended at 10 mg/ml in the same solvent of sample. An ESI tuning mix was used to calibrate MALDI-TOF MS. Further calibration was performed using lipid A extracted from *E. coli* ATCC 25922 grown in LB at 37 °C.

8. Screening of synergistic activities of antimicrobial combinations by checkerboard assay

The synergistic activity of colistin/sulbactam, colistin/fosfomycin, and sulbactam/fosfomycin against CoR-AB isolates were tested by checkerboard assay.

Combinations of antimicrobials were made in 96-well microtiter plate by adding two-fold serial dilution of first drug in a row mixed with two-fold serial dilution of second drug in a column. Eight-fold MIC of each antibiotic was prepared. The stock solution of first antibiotic was added in column 12 (A12 – H12) then serially diluted with 50 µL to column 2 (A2 – H2). For the panel of second antibiotic, the 2-fold serial dilutions (4x MIC to 1/16x MIC) were prepared from 8x MIC stock solution in sterile tubes with 5 mL volume. Fifty microliters of each dilution of second antibiotic were added to 96-well plate, following Figure 7. After that, the volume of all wells was adjusted to be 180 µL with CAMHB (BBL, BD® Diagnostic Systems, USA). Every combination was prepared in duplicate. The bacterial inoculum was prepared by incubating a few colonies of bacteria in CAMHB at 37 °C for 2 hours with shaking. The bacterial suspension was adjusted to 0.5 McFarland turbidity standard and then diluted to 1:100 with CAMHB. Twenty µL of prepared inoculum was added to all wells of antibiotic combination panel (except H12). The plates were incubated at 37 °C for 18 – 24 hours. Synergistic activity determined by fractional inhibitory concentration index (FICI), calculated by sum of the FICs for each drug; the FIC was defined as the MIC of each drug in combination divided by the MIC of drug when used alone (the equation is shown below). Synergism is defined as a FIC index of ≤ 0.5, partial synergism as a FIC index between 0.5 – 1, indifference as a FIC index of > 0.5 but ≤ 4, and antagonism as a FIC index of > 4 (160, 166).

$$FICI = \frac{MIC_{drug\ A\ in\ combination}}{MIC_{drug\ A\ alone}} + \frac{MIC_{drug\ B\ in\ combination}}{MIC_{drug\ B\ alone}}$$

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											

Figure 7. Checkerboard panel of antibiotic A plus antibiotic B

9. Antimicrobial combination kinetic test by time-killing assay

After screening of antibiotic combination activity by checkerboard assay, the best effective combination was confirmed for synergism by time-killing assay. In this study, the best combination against CoR-AB was colistin plus sulbactam. Thus, the activity of colistin plus sulbactam (at concentrations of 0.5x and 0.25x MIC) was investigated against CoR-AB clinical isolates with different colistin susceptibility levels.

Conditions of antibiotic alone and combination were prepared as follow 1) no antibiotics 2) 0.5x MIC of colistin 3) 0.5x MIC of sulbactam 4) 0.25x MIC of colistin 5) 0.25x MIC of sulbactam 6) 0.5x MIC of colistin plus 0.5x MIC of sulbactam 7) 0.5x MIC of colistin plus 0.25x MIC of sulbactam 8) 0.25x MIC of colistin plus 0.5x MIC of sulbactam 9) 0.25x MIC of colistin plus 0.25x MIC of sulbactam. All conditions were prepared in 125 ml Erlenmeyer's flasks with 9.9 mL total volume.

The bacterial inoculum was prepared by incubating a few colonies of bacteria in CAMHB at 37 °C for 2 hours with shaking. The bacterial suspension was adjusted to 0.5 McFarland turbidity standard. One hundred microliters of prepared inoculum were added to each antibiotic condition flask. After that, the flasks were incubated at 37 °C with shaking. At time 0, 2, 4, 6, 8, 10, 12, and 24 hours after incubation, the 20 µL of each condition was collected for viable cell count. This sampling was then diluted in normal saline from 10^{-1} to 10^{-8}. Ten microliters of each dilution were spotted on MHA with 10 spots. The MHA plates were incubated at 37 °C for 18 – 24 hours and then colony was counted and calculated for CFU/mL. All these experiments were performed in triplicate.

The mean and standard deviation of the viable bacterial cells in each condition were plotted on a semi-log graph. Synergy of the antimicrobial combination was defined as a ≥ 2 log decrease in CFU/mL as compared with the most single active antibiotic. The bactericidal effect was considered if the viable cells were decreased $\geq$ 3 log CFU/mL when compared with start cells. (28, 107, 160)

CHAPTER V

RESULTS

1. Bacterial strains and identification

Three hundred and forty-one of carbapenem-resistant *Acinetobacter* spp. clinical isolates were obtained from different patients at King Chulalongkorn Memorial Hospital. Among the genus *Acinetobacter*, the *Abc* complex, including *A. baumannii*, *A. calcoaceticus*, *A. pittii*, and *A. nosocomialis*, are difficult to differentiate to species level by conventional methods. Therefore, the *gyrB* multiplex PCR and the presence of intrinsic bla_{OXA-51} gene were implicated in this study. The species identification of *Acinetobacter* spp. by *gyrB* multiplex PCR showed that 317 (92.96%), 8 (2.35%), and 2 (0.59%) were identified as *A. baumannii*, *A. pittii*, and *A. nosocomialis*, respectively. Fourteen (4.11%) isolates were not identified by *gyrB* multiplex PCR and designated as non-*Abc* complex. The identification of *A. baumannii* by detection for the presence of the intrinsic bla_{OXA-51} gene showed that 307 (96.84%) of 317 *A. baumannii* isolates, 2 (25%) of 8 *A. pittii*, 1 (50%) of 2 *A. nosocomialis*, and 5 (35.71%) of 14 non-*Abc* complex harbored the bla_{OXA-51} gene. The results demonstrated that the bla_{OXA-51} gene was detected not only in *A. baumannii* but also in *Acinetobacter* spp.

Table 8. 341 *Acinetobacter* spp. were isolated from different specimens

Type of specimen		No. of isolate (isolates)	Isolate (%)
Sterile sites = 127 isolates (37.24%)	Endotracheal aspirate	82	24.05
	Body fluid	16	4.69
	Blood	15	4.40
	Tissue biopsy	7	2.05
	Bronchoalveolar lavage	5	1.47
	Cerebrospinal fluid	1	0.29
	Nasal cavity	1	0.29
Non-sterile site = 214 isolates (62.76%)	Sputum	172	50.44
	Urine	27	7.92
	Pus	15	4.40
	Total	341	100

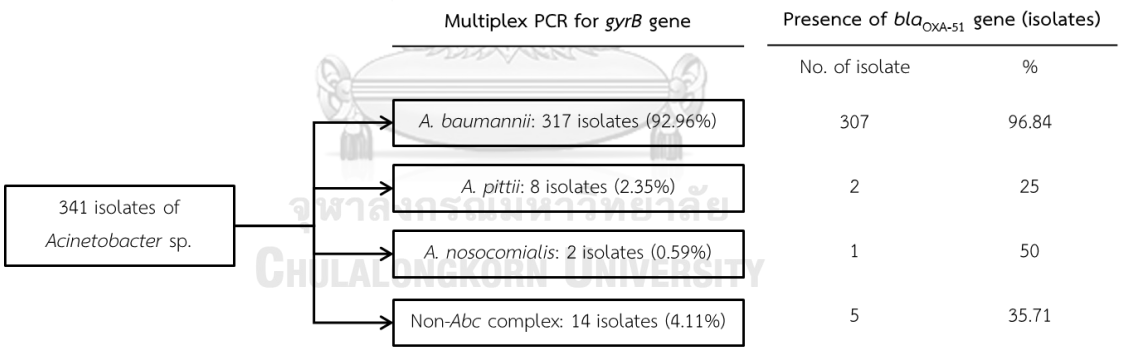
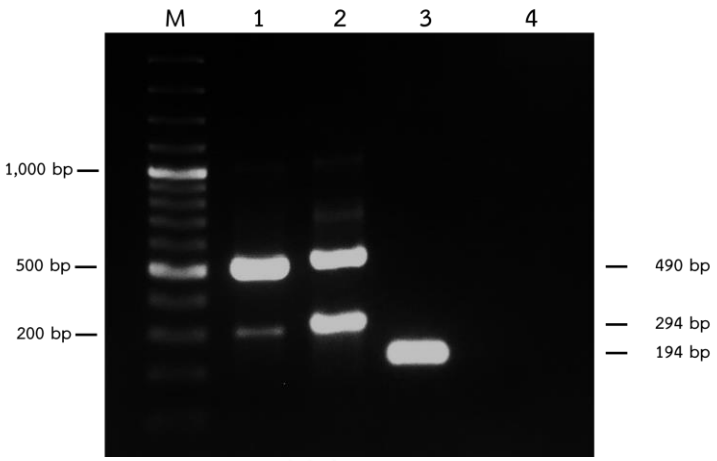


Figure 8. Species identification of 341 *Acinetobacter* spp. clinical isolates by *gyrB* multiplex PCR and presence of bla_{OXA-51} gene



(Lanes: M, 100-bp marker; 1, *A. baumannii* ATCC 19606; 2, *A. baumannii*; 3, *A. pittii*; 4, non-*Abc* complex)

Figure 9. *gyrB* multiplex PCR for species identification of *Abc* complex

2. Antimicrobial susceptibility testing

The antimicrobial susceptibility of 341 *Acinetobacter* spp. clinical isolates are shown in Table 9. All *Acinetobacter* spp. isolates were resistant to meropenem and imipenem with the MIC range of 8 to >256 mg/L. Fifty-three of all *Acinetobacter* spp. isolates (15.54%) were resistant to colistin. Of the 53 isolates, 48 (90.57%) were *A. baumannii.* The MIC of colistin ranged from 0.125 to >256 mg/L with the MIC_{50} and MIC_{90} of 1 and 4 mg/L, respectively. Of all isolates, 281 (82.40%), 334 (97.95%), 322 (94.43%), 273 (80.06%), and 328 (96.19%) were resistant to amikacin, ciprofloxacin, levofloxacin, fosfomycin, and sulbactam, respectively.

Table 9. Antimicrobial susceptibility of 341 *Acinetobacter* spp. clinical isolates

Antimicrobial agent	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	No. of resistance (isolates)	% resistance
COL	0.125 - >256	1	4	53	15.54
MEM	8 - >256	64	256	341	100
IMP	8 - >256	128	256	341	100
AMK	1 - >256	>256	>256	281	82.40
CIP	0.5 - >256	64	256	334	97.95
LVX	0.5 - >256	16	32	322	94.43
FOS[a]	64 - >256	256	>256	273	80.06
SUL[b]	1 - >256	32	128	328	96.19

[a]The interpretation criteria for Enterobacteriaceae by the CLSI was used for fosfomycin susceptibility.

[b]The interpretation criteria of ampicillin/sulbactam for *Acinetobacter* spp. by the CLSI was used for sulbactam susceptibility.

Abbreviations: AMK, amikacin; CIP, ciprofloxacin; COL, colistin; FOS, fosfomycin; IMP, imipenem; LVX, levofloxacin; MEM, meropenem; MIC, minimum inhibitory concentration; SUL, sulbactam

Table 10. Antimicrobial susceptibility of *A. baumannii*, *A. pittii*, *A. nosocomialis*, and non-*Abc* complex

Antimicro-bial agents	MIC of (mg/L)															
	A. baumannii (317 isolates)				*A. pittii* (8 isolates)				*A. noosocomialis* (2 isolates)				non-*Abc* complex (14 isolates)			
	MIC range	MIC_{50}	MIC_{90}	% resistance	MIC range	MIC_{50}	MIC_{90}	% resistance	MIC range	MIC_{50}	MIC_{90}	% resistance	MIC range	MIC_{50}	MIC_{90}	% resistance
COL	0.125-64	1	4	15.14%	0.125-2	0.5	2	0	0.25-0.5	-	-	0	0.125->256	1	64	35.71%
MEM	32->256	64	128	100%	32->256	128	256	100%	64-128	-	-	100%	8->256	128	256	100%
IMP	16->256	128	256	100%	32->256	128	256	100%	64-128	-	-	100%	8->256	128	>256	100%
AMK	1->256	>256	>256	81.7%	2->256	64	>256	87.50%	128->256	-	-	100%	2->256	>256	>256	92.86%
CIP	0.5->256	64	256	98.74%	1-64	32	64	87.50%	64	-	-	100%	1-256	64	256	85.71%
LVX	0.5-128	16	32	94.95%	4-32	8	32	87.50%	16	-	-	100%	2-32	16	32	85.71%
FOS[a]	64->256	256	>256	80.44%	128->256	128	256	50%	256	-	-	100%	128->256	256	>256	85.71%
SUL[b]	1->256	32	64	85.89%	16->256	64	128	100%	32	-	-	100%	16->256	32	>256	100%

[a]The interpretation criteria for Enterobacteriaceae by the CLSI was used for fosfomycin susceptibility.

[b]The interpretation criteria of ampicillin/sulbactam for *Acinetobacter* spp. by the CLSI was used for sulbactam susceptibility.

Abbreviations: AMK, amikacin; CIP, ciprofloxacin; COL, colistin; FOS, fosfomycin; IMP, imipenem; LVX, levofloxacin; MEM, meropenem; MIC, minimum inhibitory concentration; SUL, sulbactam

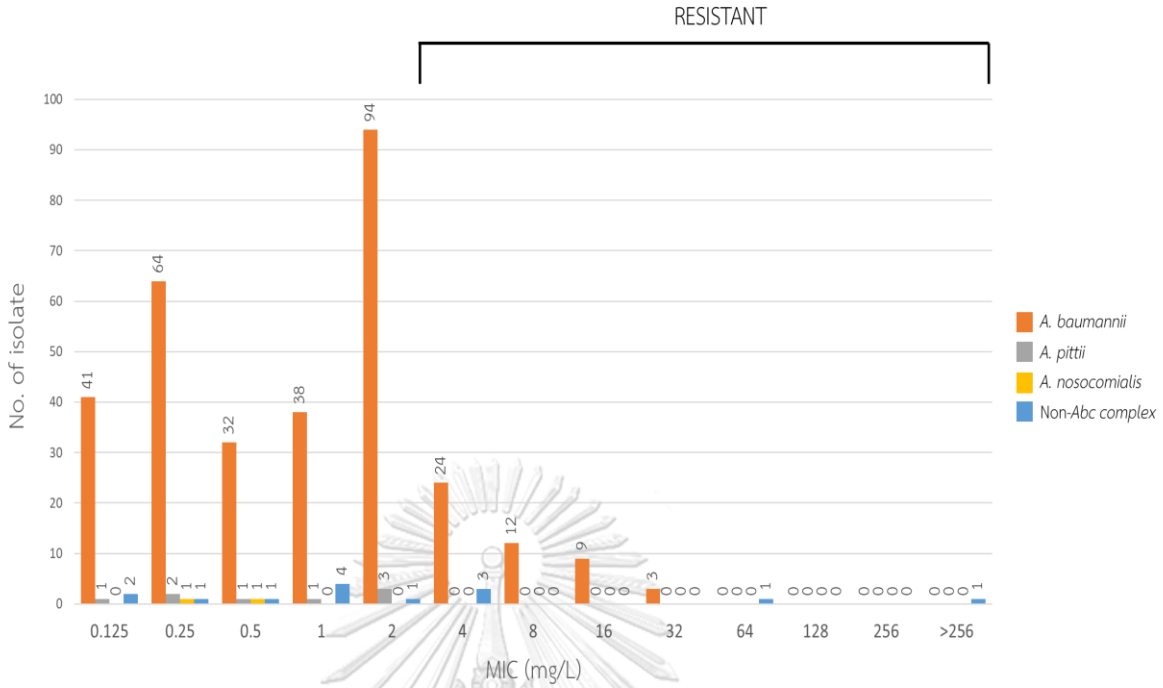


Figure 10. Distribution of colistin MIC against 341 *Acinetobacter* spp.

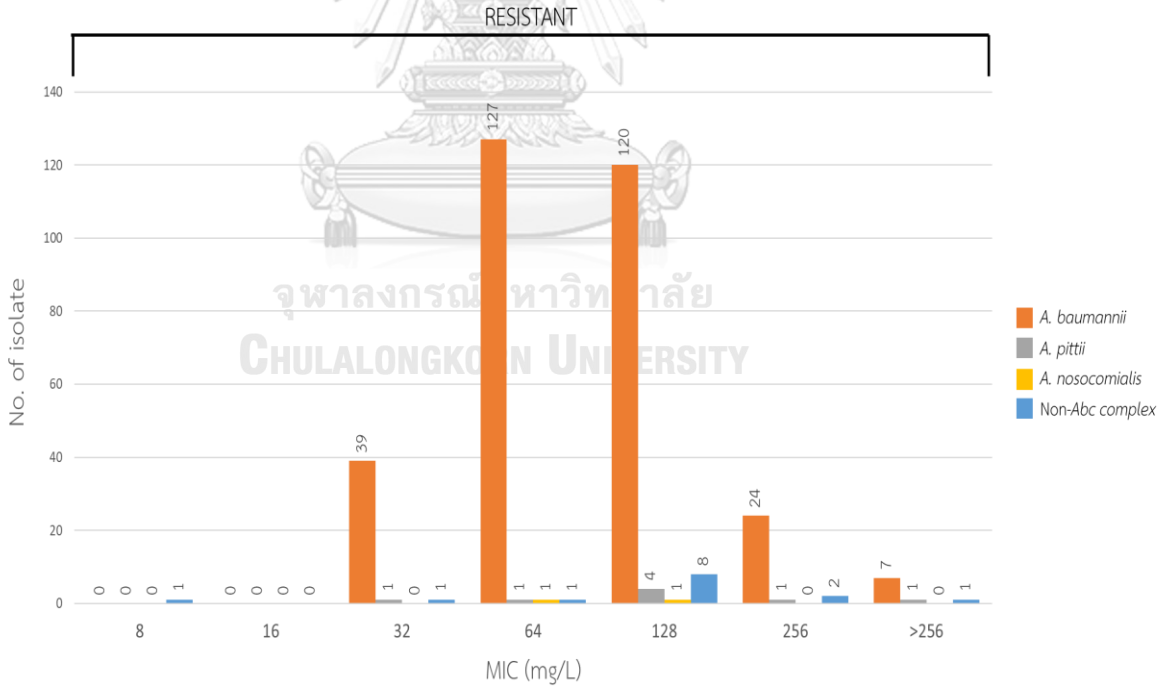


Figure 11. Distribution of meropenem MIC against 341 *Acinetobacter* spp.

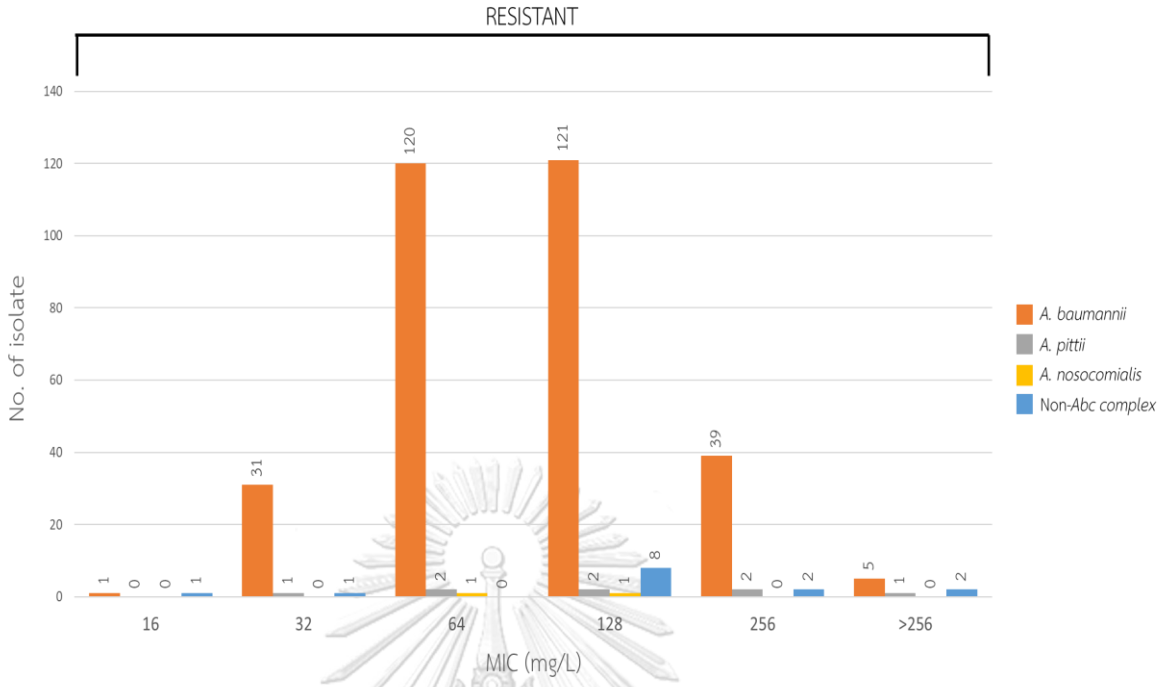


Figure 12. Distribution of imipenem MIC against 341 *Acinetobacter* spp.

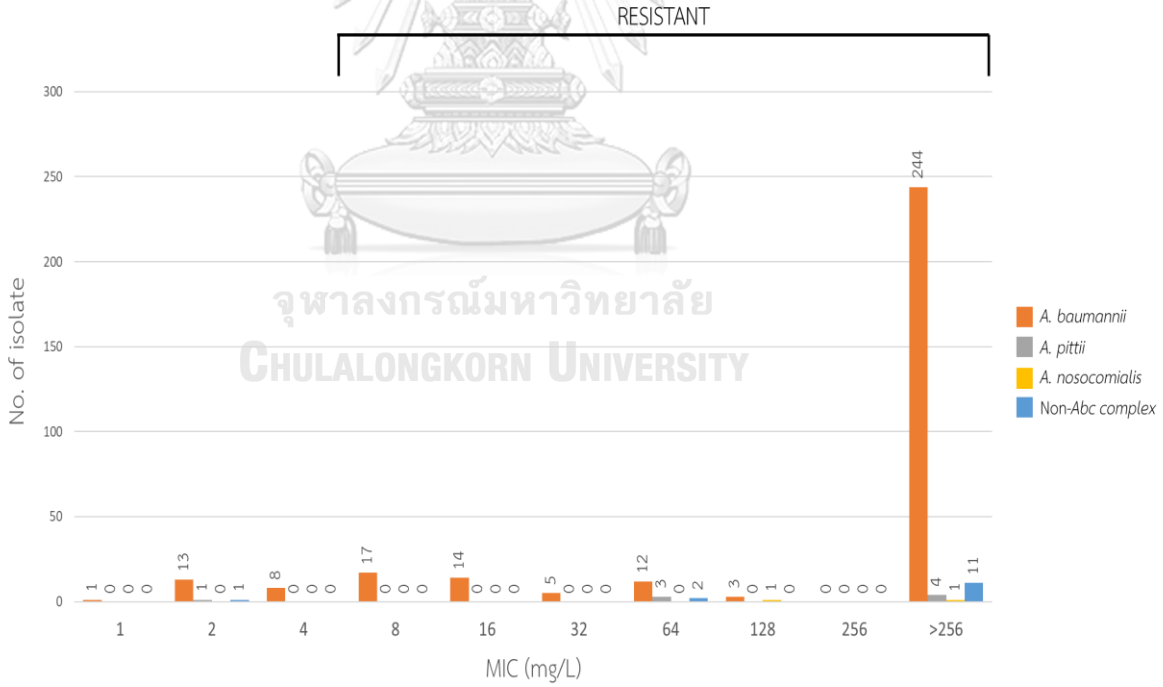


Figure 13. Distribution of amikacin MIC against 341 *Acinetobacter* spp.

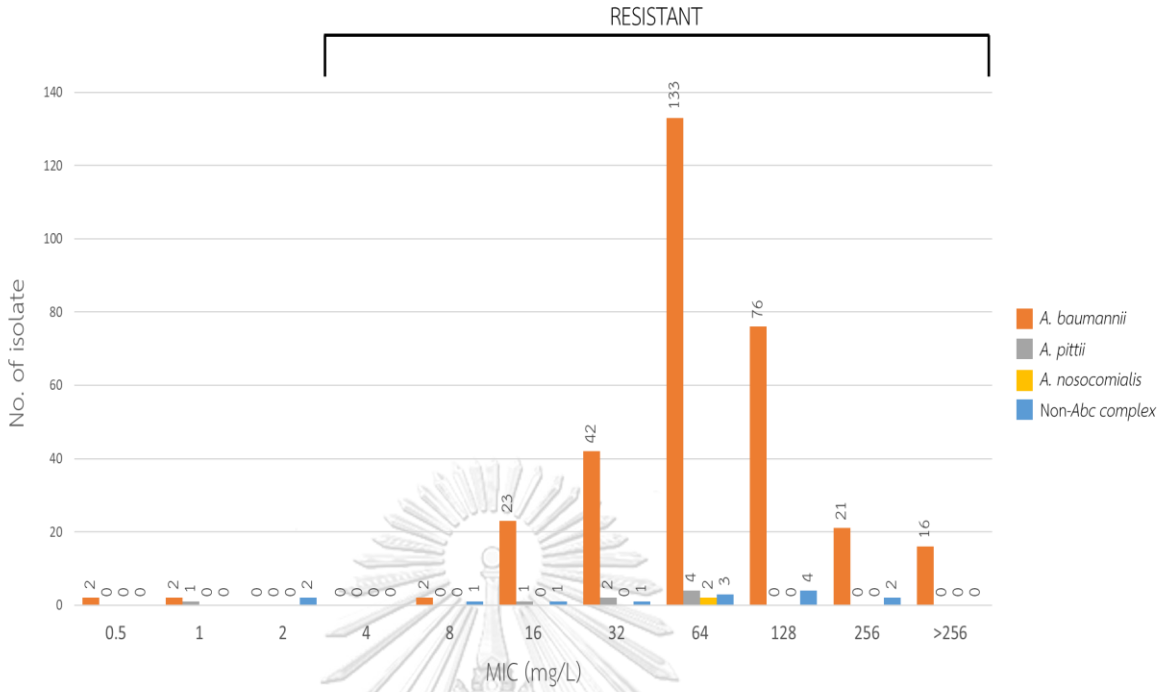


Figure 14. Distribution of ciprofloxacin MIC against 341 *Acinetobacter* spp.

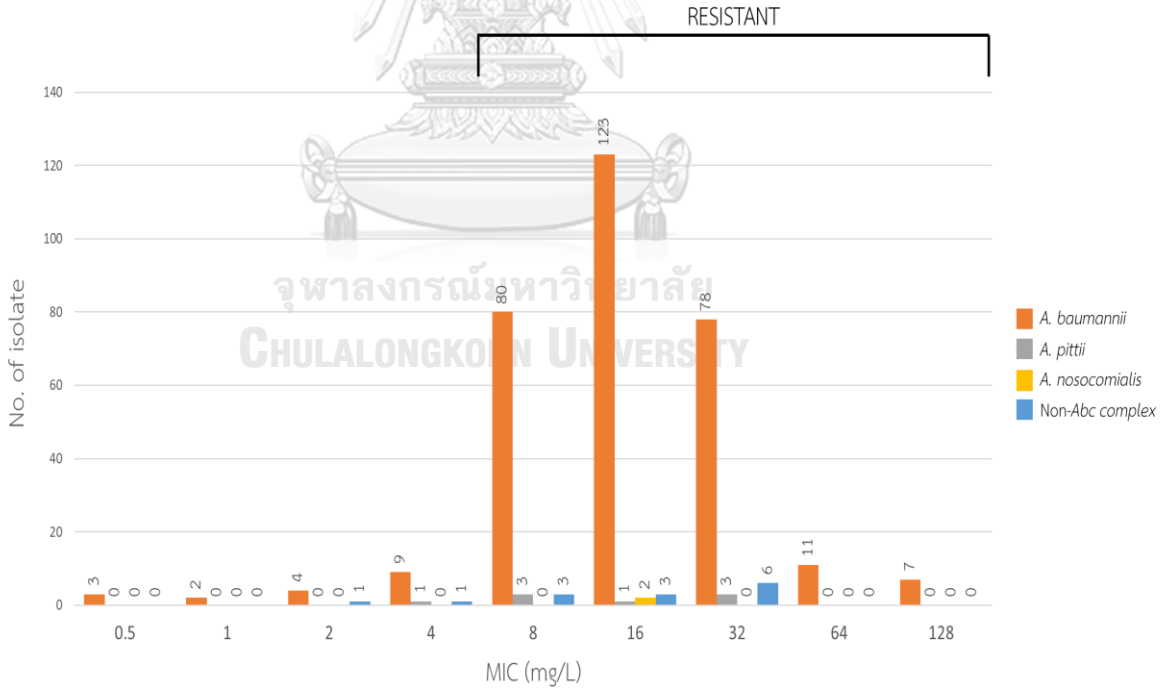


Figure 15. Distribution of levofloxacin MIC against 341 *Acinetobacter* spp.

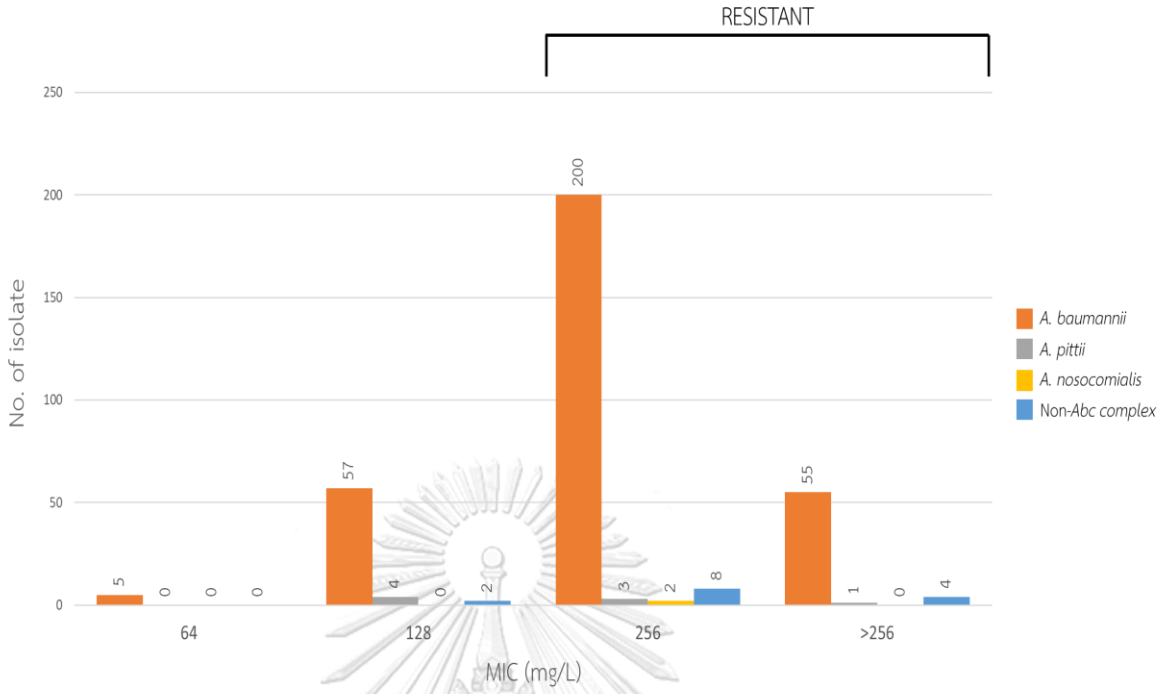


Figure 16. Distribution of fosfomycin MIC against 341 *Acinetobacter* spp.

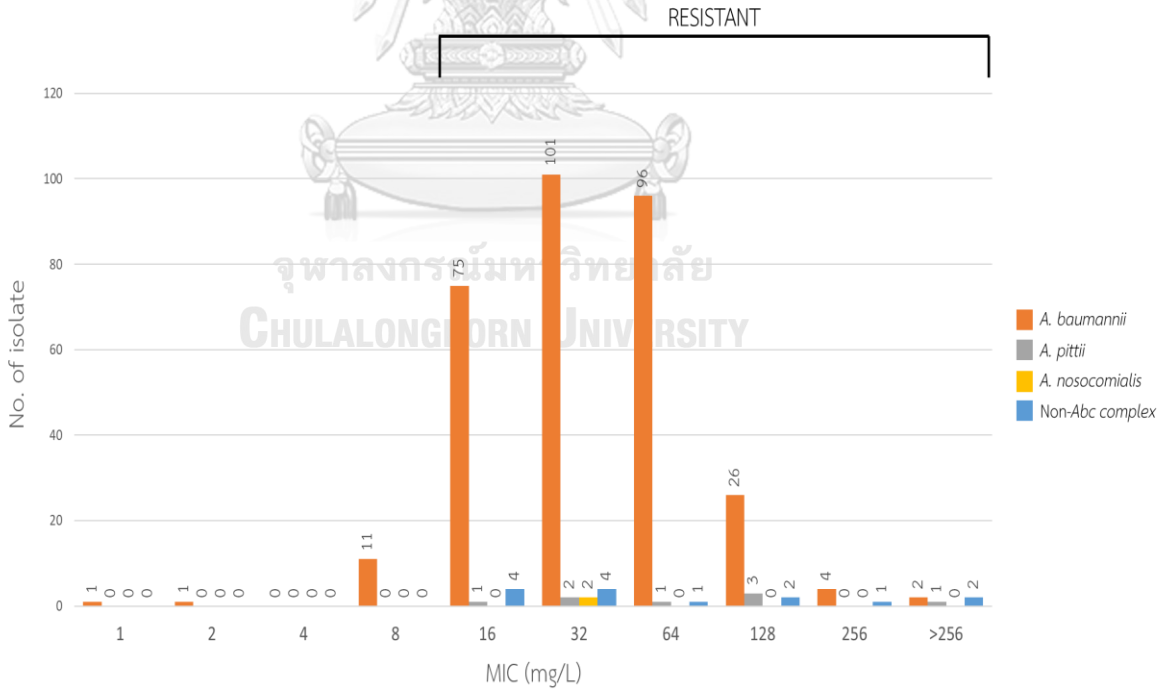


Figure 17. Distribution of sulbactam MIC against 341 *Acinetobacter* spp.

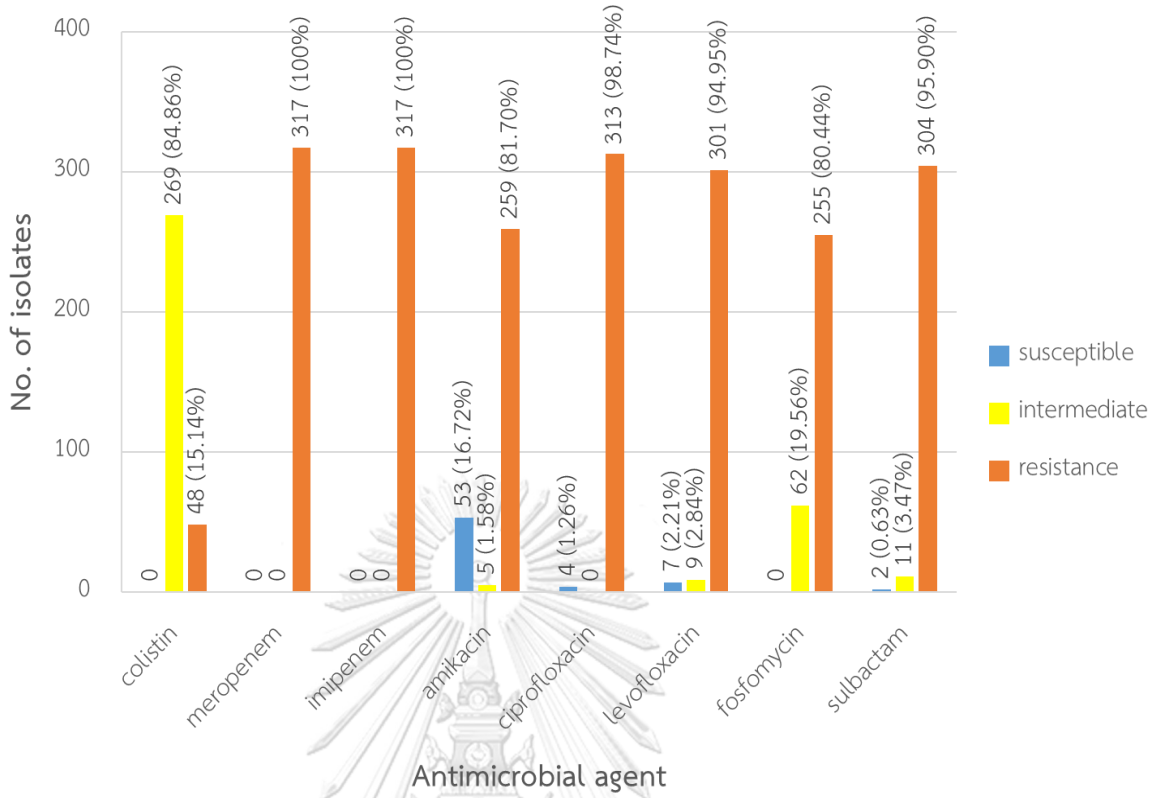


Figure 18. Antimicrobial susceptibility of 317 *A. baumannii* clinical isolates

3. Screening of carbapenemase-encoding genes in *Acinetobacter* spp. clinical isolates

The presence of carbapenemase-encoding genes in *Acinetobacter* spp. clinical isolates are shown in Table 11. For acquired OXA-type carbapenemase genes, the most prevalent gene was the combination of $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-51-like}}$ which was found in 297 (87.10%) *Acinetobacter* spp. isolates. Of these 297 isolates, there were 289 (97.31%) *A. baumannii*, 2 (0.67%) *A. pittii*, 1 (0.34%) *A. nosocomialis*, and 5 (1.68%) non-*Abc* complex. The $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-51-like}}$, and $bla_{\text{OXA-58-like}}$ combination was found in 4 (1.14%) *A. baumannii* isolates. The combination of $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-24-like}}$ was found in 2 (0.57%) isolates and the coexistence of $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-58-like}}$ was found in 1 (0.28%) *A. baumannii* isolate. Of the 8 *A. pittii* isolates, 1 isolate harbored only the $bla_{\text{OXA-23-like}}$ gene and another carried only the $bla_{\text{OXA-58-like}}$ gene. The $bla_{\text{OXA-48-like}}$ gene was harbored in 1 non-*Abc* isolate.

The other carbapenemase genes were found. Of the 8 *A. pittii* isolates, 2 were presented only the $bla_{\text{NDM-like}}$ gene and 1 contained the $bla_{\text{IMP-like}}$ plus $bla_{\text{OXA-58-like}}$ gene. Furthermore, the $bla_{\text{IMP-like}}$ gene was found only in 1 isolate of non-*Abc* complex. The $bla_{\text{VIM-like}}$ and $bla_{\text{KPC-like}}$ genes were not detected in any isolates in this study.

Table 11. carbapenemase-encoding genes in 341 *Acinetobacter* spp. clinical isolates

Carbapenemase-encoding genes	MIC range (mg/L)		*A. baumannii* (317 isolates)		*A. pittii* (8 isolates)		*A. nosocomialis* (2 isolates)		Non-*Abc* (14 isolates)	
	meropenem	imipenem	No. of isolate (isolates)	%	No. of isolate (isolates)	%	No. of isolate (isolates)	%	No. of isolate (isolates)	%
$bla_{\text{OXA-23-like}}$ + $bla_{\text{OXA-51-like}}$	32->256	32->256	289	91.17	2	25	1	50	5	35.71
$bla_{\text{OXA-51-like}}$	32-128	64-256	11	3.47	0	0	0	0	0	0
$bla_{\text{IMP-like}}$	32->256	32->256	0		0	0	0	0	1	7.14
$bla_{\text{OXA-23-like}}$ + $bla_{\text{OXA-51-like}}$ + $bla_{\text{OXA-58-like}}$	32-128	32-128	4	1.26	0	0	0	0	0	0
$bla_{\text{OXA-58-like}}$	64-256	64-256	1	0.32	1	12.5	1	50	1	7.14
$bla_{\text{NDM-like}}$	128->256	64->256	0	0	2	25	0	0	0	0
$bla_{\text{OXA-24-like}}$ + $bla_{\text{OXA-51-like}}$	32	16-32	2	0.63	0	0	0	0	0	0
$bla_{\text{IMP-like}}$ + $bla_{\text{OXA-58-like}}$	32	32	0	0	1	12.5	0	0	0	0
$bla_{\text{OXA-23-like}}$	64	64	0	0	1	12.5	0	0	0	0
$bla_{\text{OXA-48-like}}$	128	128	0	0	0	0	0	0	1	7.14
$bla_{\text{OXA-51-like}}$ + $bla_{\text{OXA-58-like}}$	256	256	1	0.32	0	0	0	0	0	0

4. Mutations in gene encoding lipid A phosphoethanolamine transferase (*pmrCAB* operon)

The two-component system proteins, PmrA (response regulator), PmrB (sensor kinase), and PmrC (lipid A phosphoethanolamine transferase) are implicated in colistin resistance in *A. baumannii*. Thirty CoR-AB isolates with different MICs were selected for DNA sequencing of the *pmrCAB* operon. The complete coding region of *pmrCAB* operon of colistin-resistant isolates was compared with that of *A. baumannii* ATCC 19606 (Genbank accession number CP045110.1) and that of *A. baumannii* ATCC 17978 (Genbank accession number CP000521.1). The active domain sites were predicted by searching in NCBI domain predictor (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The major amino acid substitutions were found in 22 (73.3%) CoR-AB isolates with MICs range of 4 - 32 mg/L. These isolates harbored 48 substitutions in PmrC (Figure 19.), 4 substitutions in PmrA (Figure 20.), and 20 substitutions in PmrB (Figure 21.). In PmrC, all isolates had N284D mutation in the sulfatase domain. Of 8 isolates (176, 213, 216, 1060, 1126, 1129, 1139, and A5), 13 different amino acid substitutions were detected at V42I, R109H, I155V, V135A, F150L, V203M, R214Q, D282G, V321I, A354S, V470I, K498N, and K515T (Figure 19). Twenty-two isolates were found I18T and T44N mutations in response regulator receiver domain of PmrA. Isolate 176, 213, 216, 1060, 1126, 1129, 1139, and A5 were not found any mutation in PmrA (Figure 20). In PmrB, 29 CoR-AB isolates were found mutations at V227A of the histidine kinase domain of PmrB. Seven different amino acid substitutions were found in 8 isolates (176, 213, 216, 1060, 1126, 1129, 1139, and A5) (Figure 21). The S14P, A138T, R165P, H440N, and A444V substitutions were detected in transmembrane domains and G260D and L274F substitutions were found in the histidine kinase domain of PmrB.

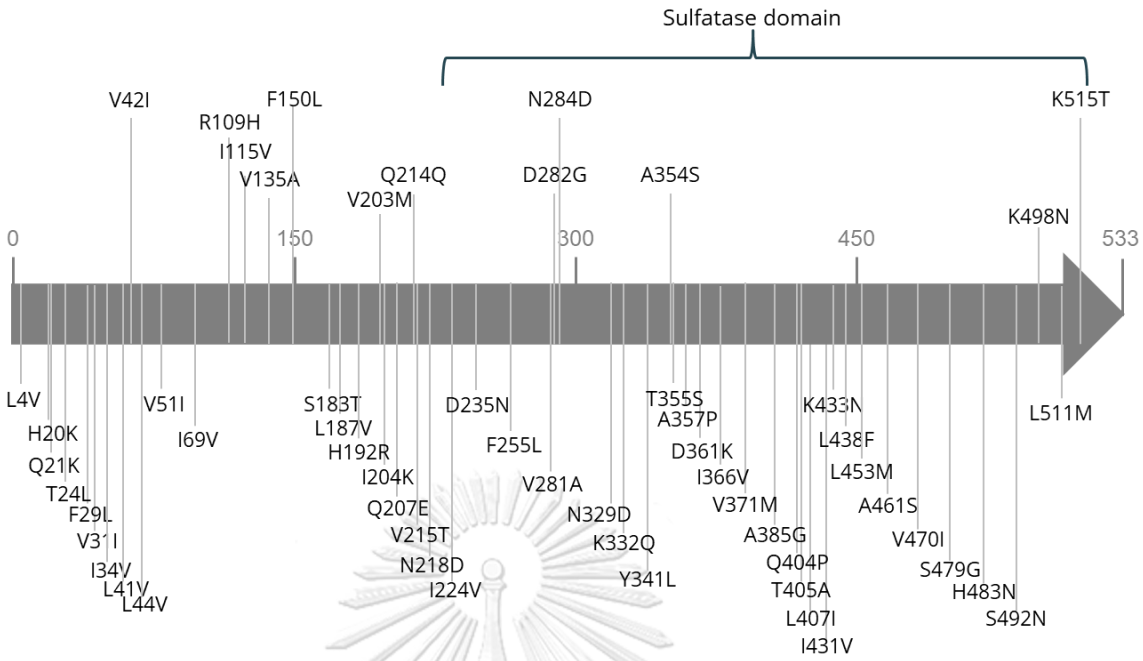


Figure 19. Amino acid changes in PmrC protein of colistin-resistant *A. baumannii* clinical isolates

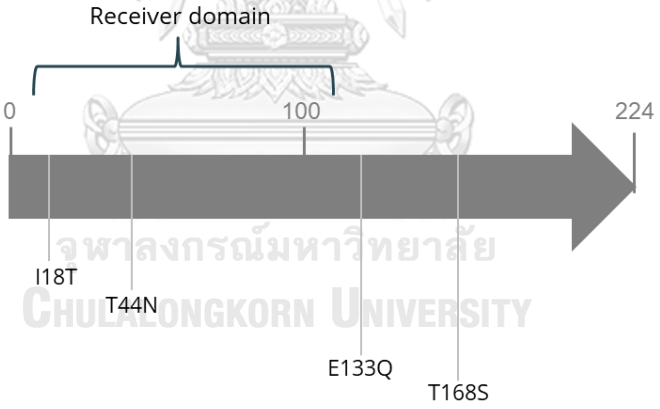
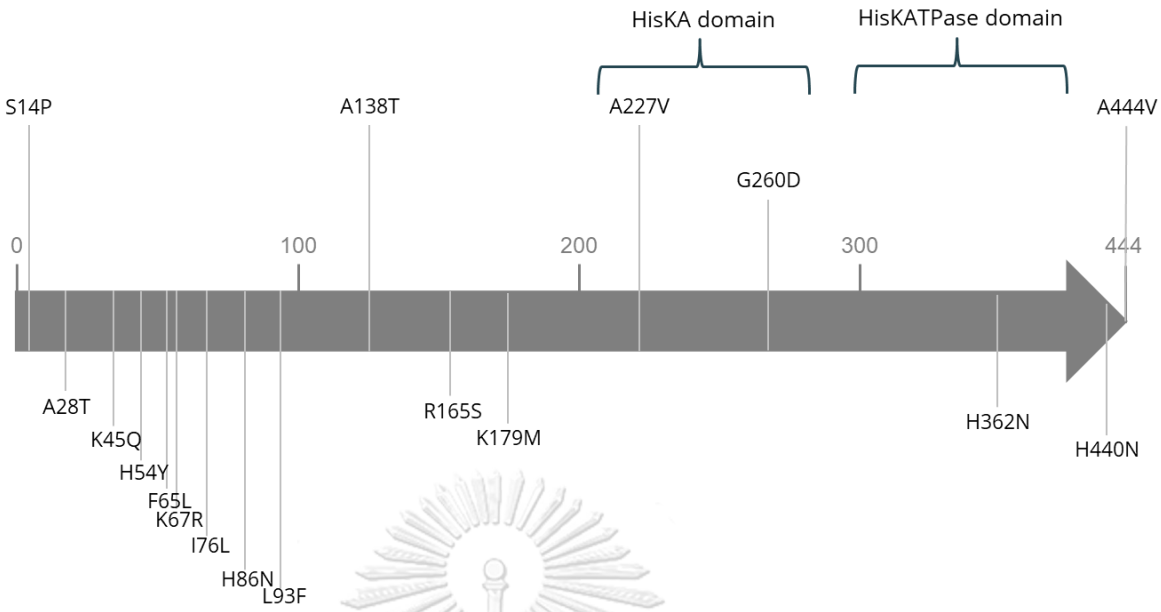


Figure 20. Amino acid changes in PmrA protein of colistin-resistant *A. baumannii* clinical isolates



(Abbreviations: HisKA, histidine kinase A; HisKATPase, histidine kinase-like adenosine tri-phosphatase)

Figure 21. Amino acid changes in PmrB protein of colistin-resistant *A. baumannii* clinical isolates

5. Mutations in gene involved in lipid A production (*lpxACD*)

The lipid A biosynthesis is an important step for the complete growth of Gram-negative bacteria. In *E.coli*, nine enzymatic steps are involved in lipid A production (178). However, a previous study showed that only three genes, *lpxACD*, were important in lipid A production in *A. baumannii*. This study investigated the mutations in the *lpxACD* gene involved in colistin resistance in *A. baumannii* clinical isolates. From sequence analysis, 28 isolates had amino acid substitution at E117K of the LpxD protein. The other two isolates had mutation at V3A. Mutations in LpxA and LpxC were not detected in all CoR-AB isolates in this study.

6. Screening of plasmid-mediated colistin resistance (*mcr*) genes

The *mcr* genes encode the pEtN transferase that modifying the LPS and resulting in the reduced susceptibility to colistin (12). The two sets of multiplex PCR

were performed to the detection of *mcr-1* to *mcr-5* and *mcr-6* to *mcr-9*. The 30 representative CoR-AB isolates were not detected in any *mcr* genes.

7. Analysis of lipid A structure in colistin-resistant *A. baumannii* clinical isolates

Lipid A modification by phosphoethanolamine (pEtN) is known to be involved in the mechanism of colistin resistance in *A. baumannii*. We analyzed the lipid A moieties from 30 CoR-AB clinical isolates with different MICs by MALDI-TOF MS compared with lipid A spectrum of *A. baumannii* ATCC 19606. In previous studies, disaccharide backbone of lipid A of *A. baumannii* is composed of two beta-(1,6)-linked 2-amino-2-deoxyglucose with phosphorylation at positions 1 and 4′. The lipid A spectrum of *A. baumannii* ATCC 19606 showed 4 major peaks which were consistent with a deprotonated *bis*-phosphoryl hepta-acylated lipid A (*m/z* 1,910), deprotonated *bis*-phosphoryl hexa-acylated lipid A (*m/z* 1,728), deprotonated *bis*-phosphoryl penta-acylated lipid A (*m/z* 1,530), and deprotonated *bis*-phosphoryl tetra-acylated lipid A (*m/z* 1,404). In the 30 colistin-resistant isolates, all isolates showed the predominant peaks at *m/z* 1,404 and 1,910. Most isolates, except isolate 176, 216, and 1126, were detected additional peak at *m/z* 2,034, corresponding to the addition of pEtN (predicted *m/z* 124) to the *bis*-phosphoryl hepta-acylated lipid A. Other peaks that were detected in most isolates (isolate 176, 1049, 1129, 1251, 1341, 1344, 1353, 1364, 1374, 1505, 1512, 1521, 1539, 1536, A5, and AJN3B) were *m/z* 1,895 and 2,018. These peaks may be consistent with deoxygenation at fatty acid residues of hepta-acylated lipid A and pEtN-hepta-acylated lipid A, respectively. Furthermore, isolate 1060, 1512, and A5 were found that the intensity of *m/z* 1,910 was relatively decreased when compared to other isolates.

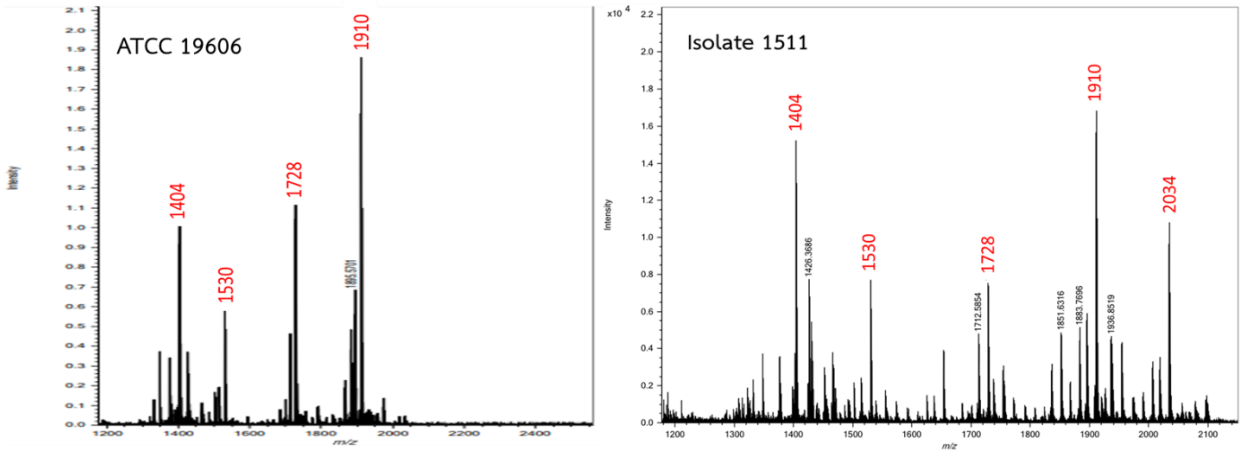


Figure 22. Lipid A spectrum of colistin-resistant *A. baumannii* clinical isolate 1511 compared with that of *A. baumannii* ATCC 19606 by MALDI-TOF MS

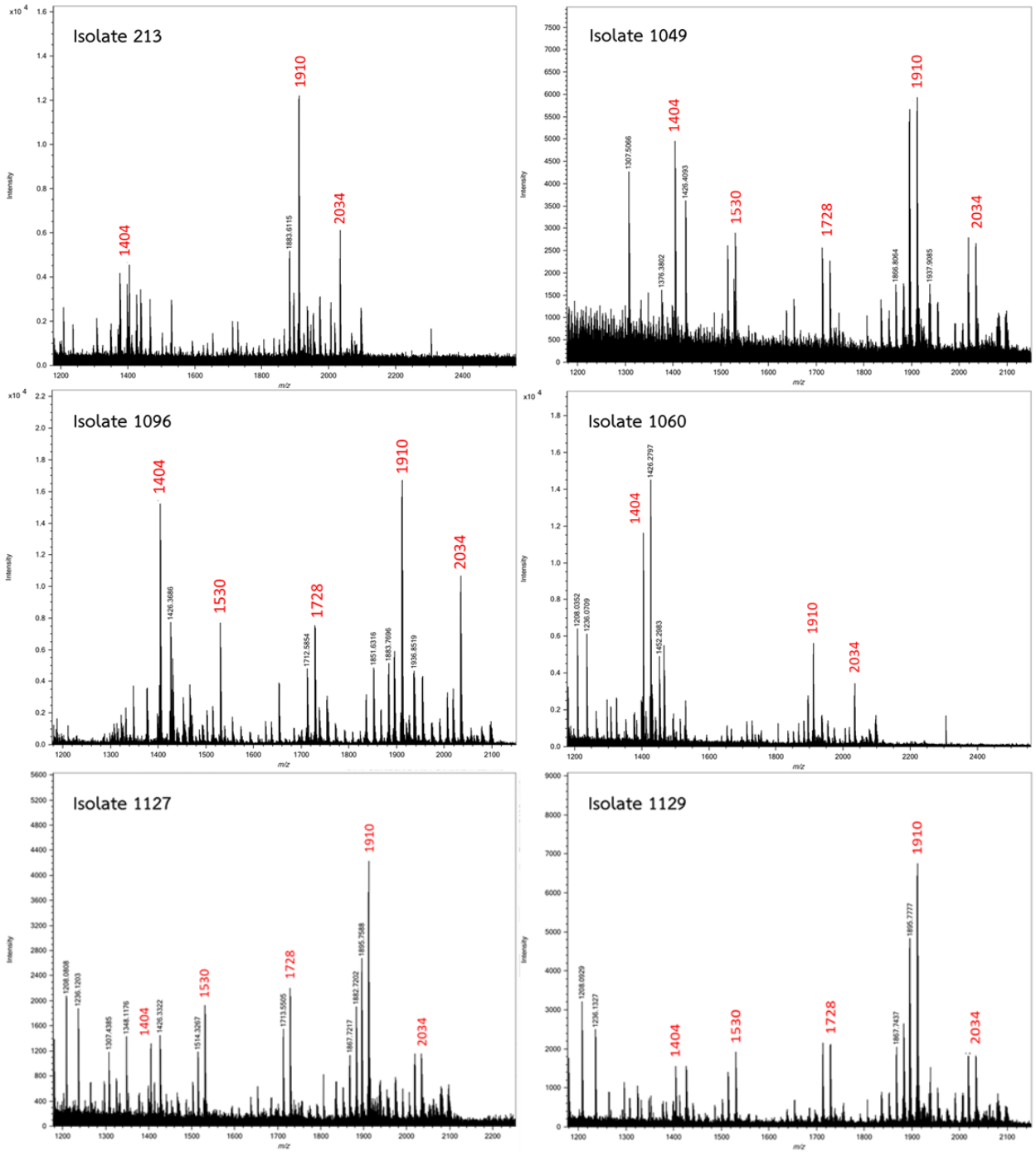


Figure 23. Phosphoethanolamine addition in 27 colistin-resistant *A. baumannii* clinical isolates

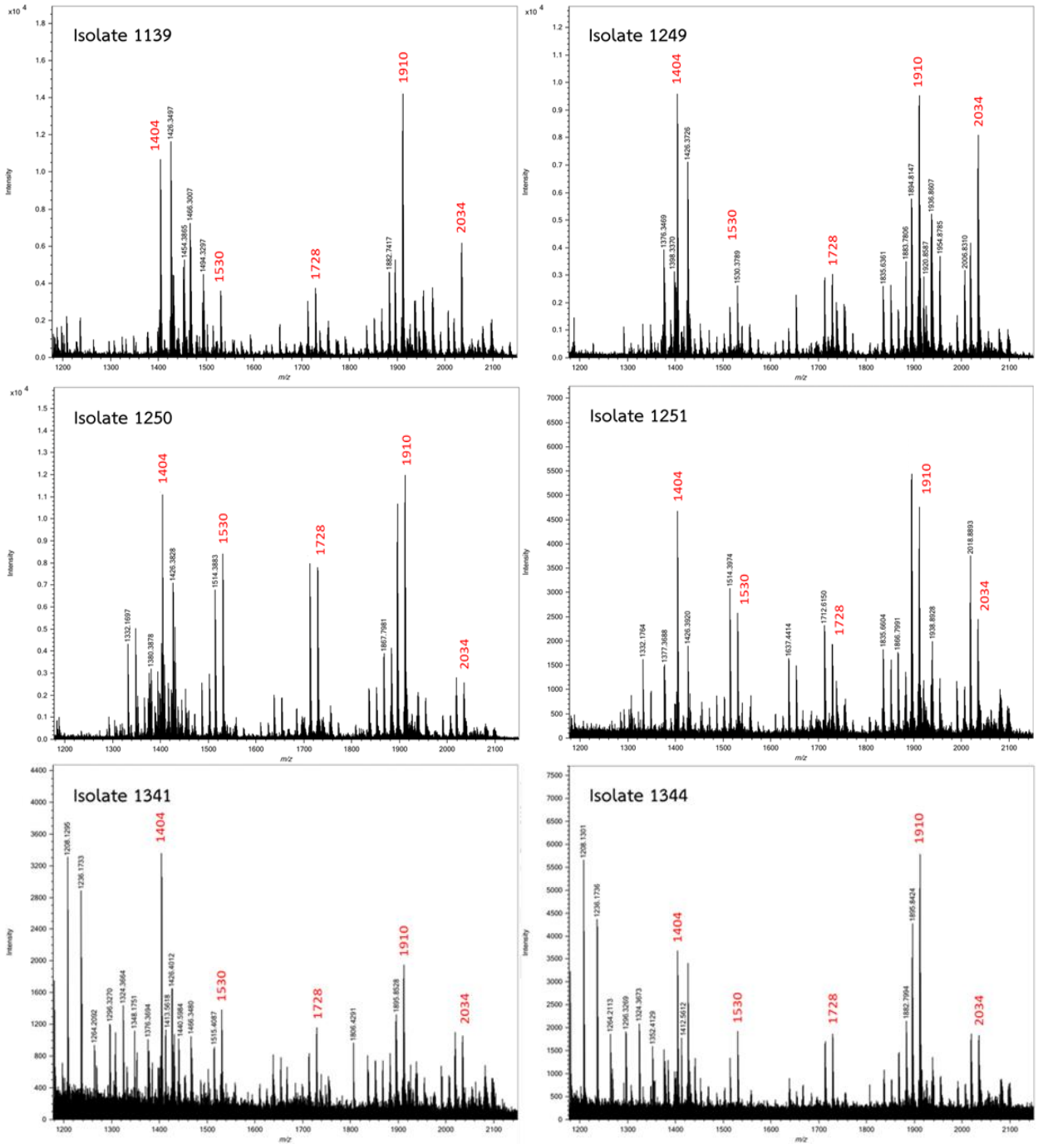


Figure 22. (cont.) Phosphoethanolamine addition in 27 colistin-resistant *A. baumannii* clinical isolates

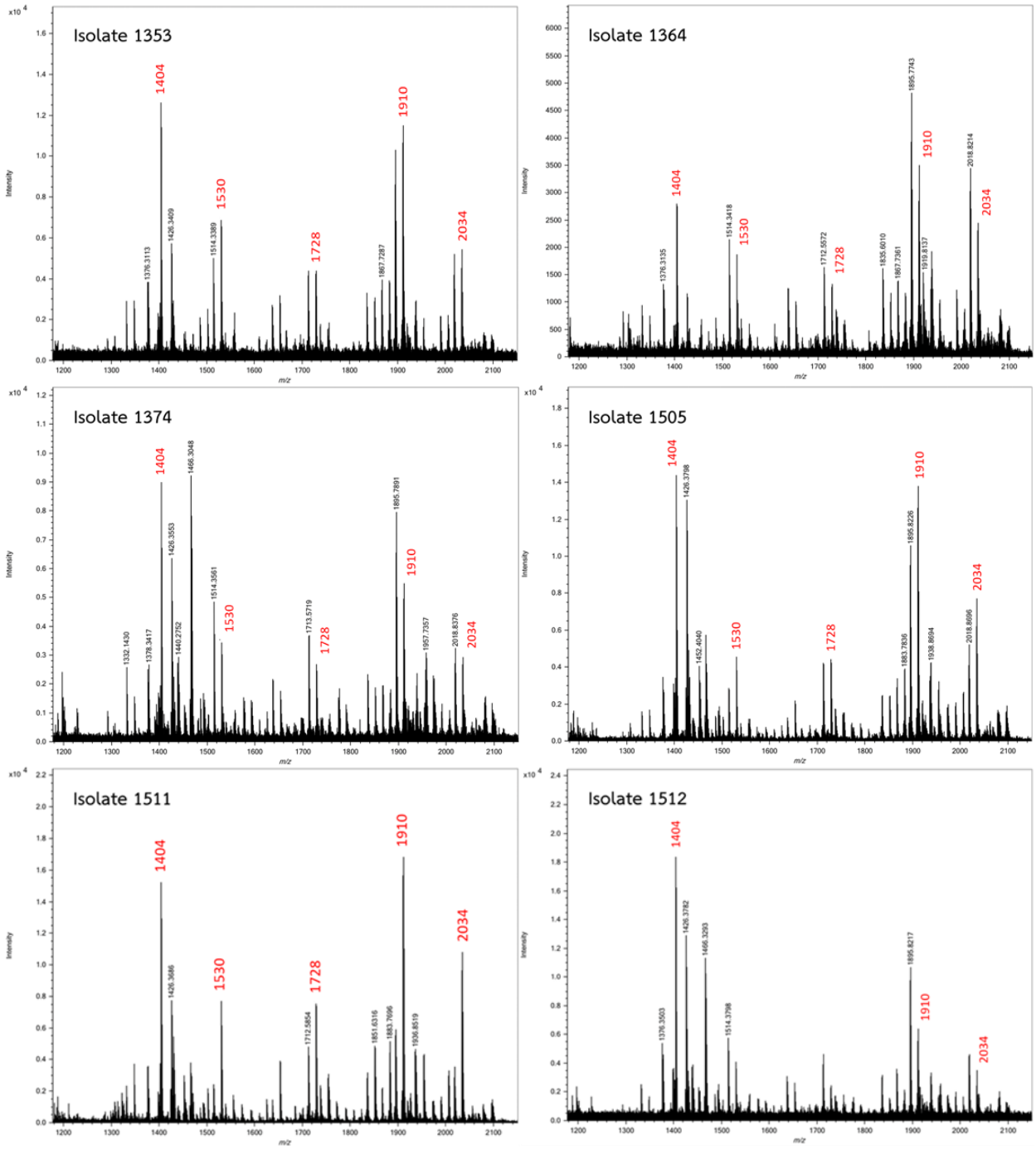


Figure 22. (cont.) Phosphoethanolamine addition in 27 colistin-resistant *A. baumannii* clinical isolates

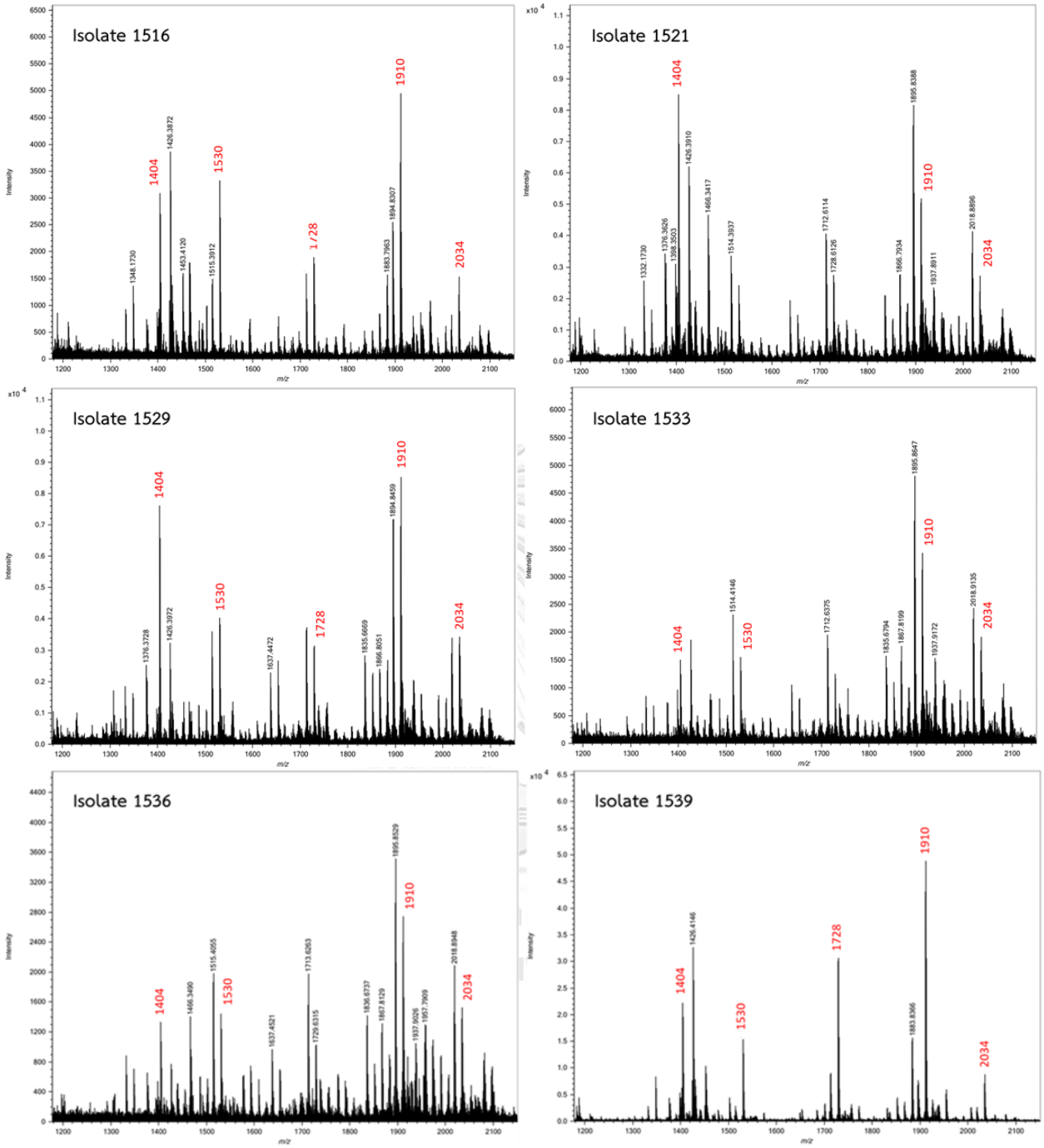


Figure 22. (cont.) Phosphoethanolamine addition in 27 colistin-resistant *A. baumannii* clinical isolates

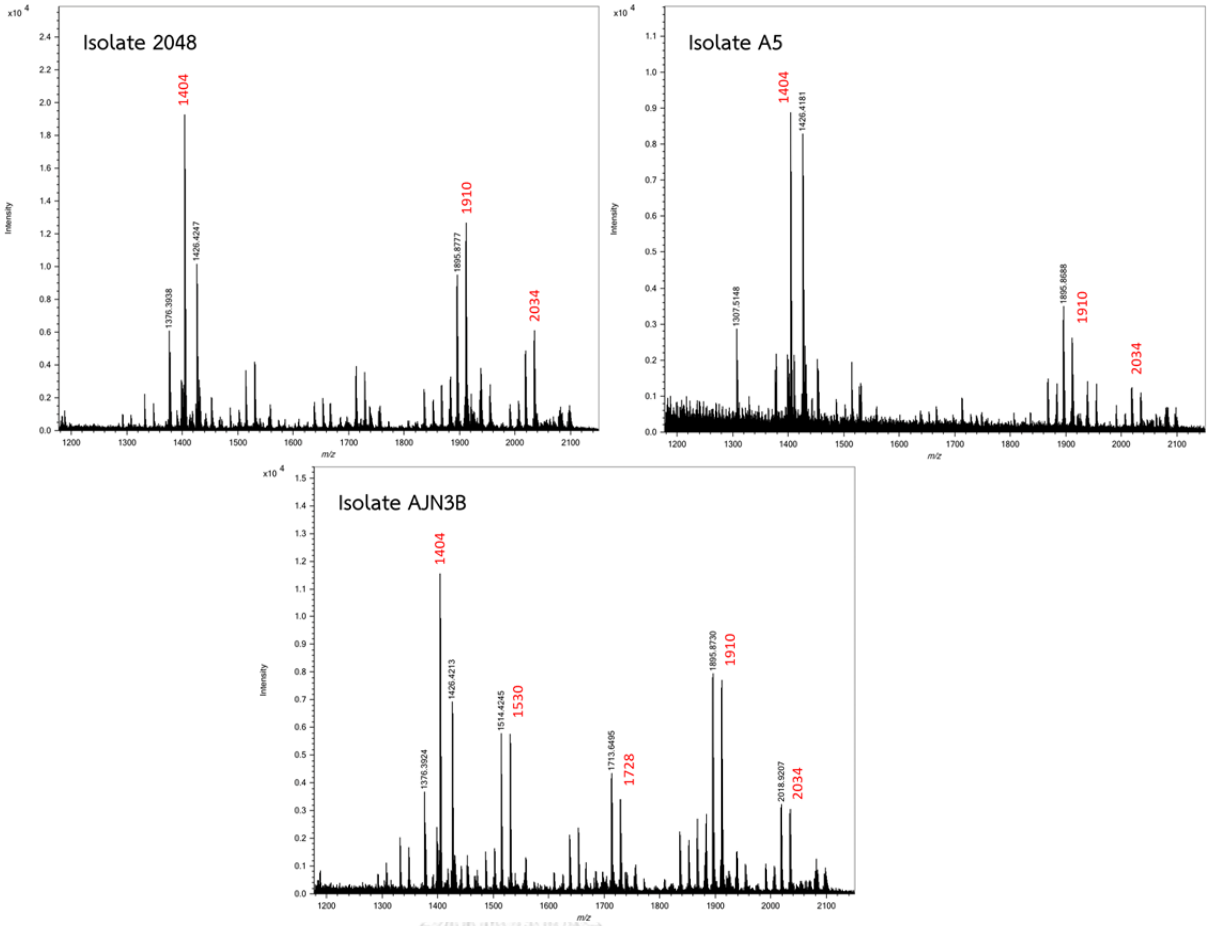


Figure 22. (cont.) Phosphoethanolamine addition in 27 colistin-resistant *A. baumannii* clinical isolates

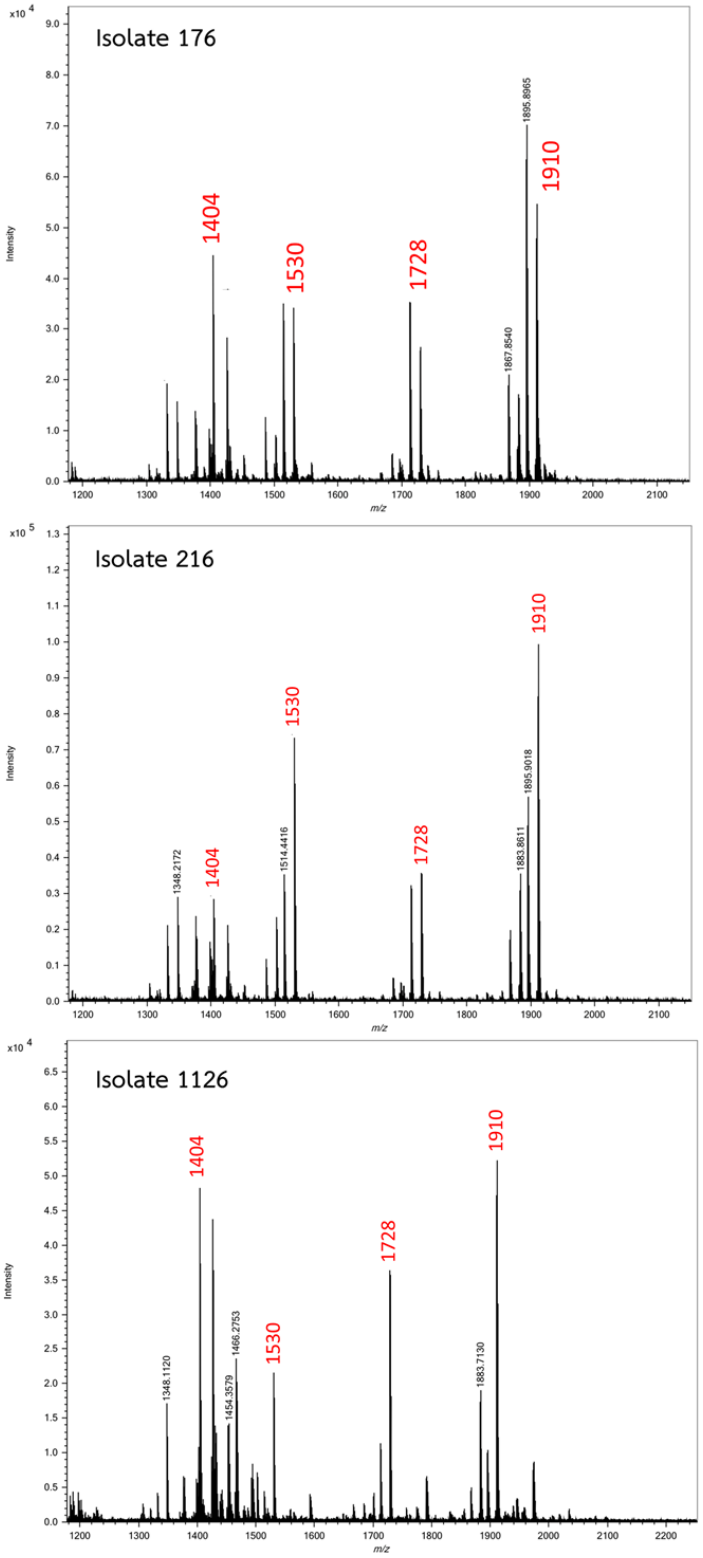


Figure 24. Lipid A spectra with no phosphoethanolamine addition of colistin-resistant *A. baumannii* clinical isolates by MALDI-TOF MS

The association between mechanisms of colistin resistance and colistin MICs is shown in Table 12. This study focused on mutation in LpxACD and pEtN addition that related to mutations in *pmrCAB* operon. Of 30 representative CoR-AB isolates, 26 (86.7%) carried both mechanisms with the MIC range of 4 – 64 mg/L and MIC_{50}/MIC_{90} of 8 and 32 mg/L, respectively. Two (6.7%) isolates were found only mutation at E117K in LpxD with MICs of 4 and 16 mg/L. One isolate was found only pEtN modification and MIC was 4 mg/L. One isolate with the MIC of 4 mg/L did not harbor any mutations in LpxD and no pEtN addition.

Table 12. Summary of mechanisms of colistin resistance in 30 CoR-AB isolates

Mechanisms of colistin resistance		No. of isolates (%)	MIC of colistin (mg/L)		
Mutation at E117K in LpxD	pEtN addition		Range	MIC_{50}	MIC_{90}
+	+	26 (86.7%)	4 - 64	8	32
+	-	2 (6.7%)	4 – 16	4	16
-	+	1 (3.3%)	16	16	16
-	-	1 (3.3%)	4	4	4

Abbreviation: MIC, minimum inhibitory concentration; pEtN, phosphoethanolamine

Table 13. The unique amino acid substitutions in PmrC and PmrB, and the mutation at E117K in LpxD in the 8 CoR-AB isolates

Isolate	Colistin MIC (mg/L)	Mutations in PmrC													Mutations in PmrB						Mutation at E117K in LpxD
		V42I	R109H	I115V	V135A	F150L	V203M	R214Q	D282G	N284D	V321I	A354S	V470I	K498N	K515T	S14T	A138T	A227V	G260D	L274F	A444V	
176	4						■			■	■	■	■					■				
216	4			■					■	■								■				■
213	8	■		■	■			■		■		■					■	■	■		■	■
1060	16	■	■			■				■		■			■						■	■
A5	16									■				■				■				
1129	8	■				■				■		■			■	■	■	■		■	■	■
1126	32	■				■				■		■			■	■	■	■		■	■	■
1139	64	■				■				■		■			■	■	■	■		■	■	■

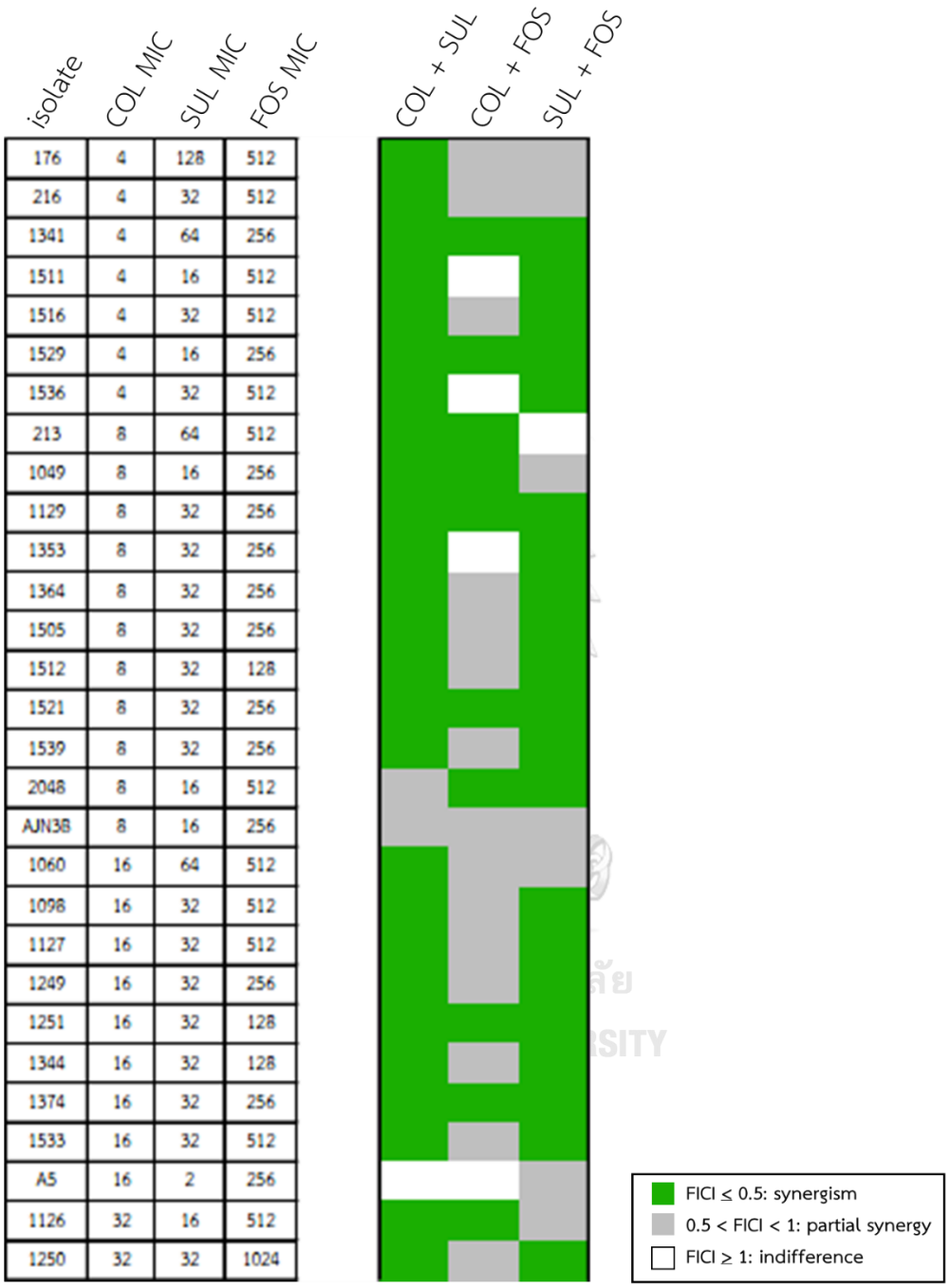
Abbreviation: MIC, minimum inhibitory concententration

8. Checkerboard assay of colistin plus sulbactam or fosfomycin and sulbactam plus fosfomycin against colistin-resistant *A. baumannii* clinical isolates

In this study, checkerboard analysis was performed with three antibiotic combinations against 30 CoR-AB clinical isolates. The results are shown in Table 14 and Figure 25. Among 3 combinations, the most effective combination was colistin plus sulbactam (COL/SUL) (synergy against 86.7% of isolates), followed by sulbactam plus fosfomycin (SUL/FOS) (synergy against 70% of isolates), and colistin plus fosfomycin (COL/FOS) (synergy against 33.3% of isolates). The partial synergy ($0.5 < FICI < 1$) was mostly observed in combination of COL/FOS (53.3%), followed by SUL/FOS (26.7%) and COL/SUL (10%). Four isolates showed indifferent effect ($1 \leq FICI < 4$) in COL/FOS combination. Isolate 213 and A5 were found indifferent effect in combinations of SUL/FOS and COL/SUL, respectively. None of isolates showed antagonism ($FICI \geq 4$).

Table 14. Result of checkerboard synergy test of three antibiotic combinations against 30 colistin-resistant *A. baumannii* clinical isolates

Antibiotic combination	Isolate(s) with the indicated test result (isolates/%)		
	Synergy ($FICI \leq 0.5$)	Partially synergy ($0.5 < FICI < 1$)	Indifferent ($1 \leq FICI < 4$)
Colistin + Sulbactam	26/86.7%	3/10%	1/3.3%
Colistin + Fosfomycin	10/33.33%	16/53.3%	4/13.3%
Sulbactam + Fosfomycin	21/70%	8/26.7%	1/3.3%



isolate	COL MIC	SUL MIC	FOS MIC
176	4	128	512
216	4	32	512
1341	4	64	256
1511	4	16	512
1516	4	32	512
1529	4	16	256
1536	4	32	512
213	8	64	512
1049	8	16	256
1129	8	32	256
1353	8	32	256
1364	8	32	256
1505	8	32	256
1512	8	32	128
1521	8	32	256
1539	8	32	256
2048	8	16	512
AJN38	8	16	256
1060	16	64	512
1098	16	32	512
1127	16	32	512
1249	16	32	256
1251	16	32	128
1344	16	32	128
1374	16	32	256
1533	16	32	512
A5	16	2	256
1126	32	16	512
1250	32	32	1024

(Abbreviations: COL, colistin; FICI, fraction inhibitory concentration index; FOS, fosfomycin, MIC, minimum inhibitory concentration, SUL, sulbactam)

Figure 25. Heat map of synergistic activities screened by checkerboard assay against 30 colistin-resistant *A. baumannii* clinical isolates

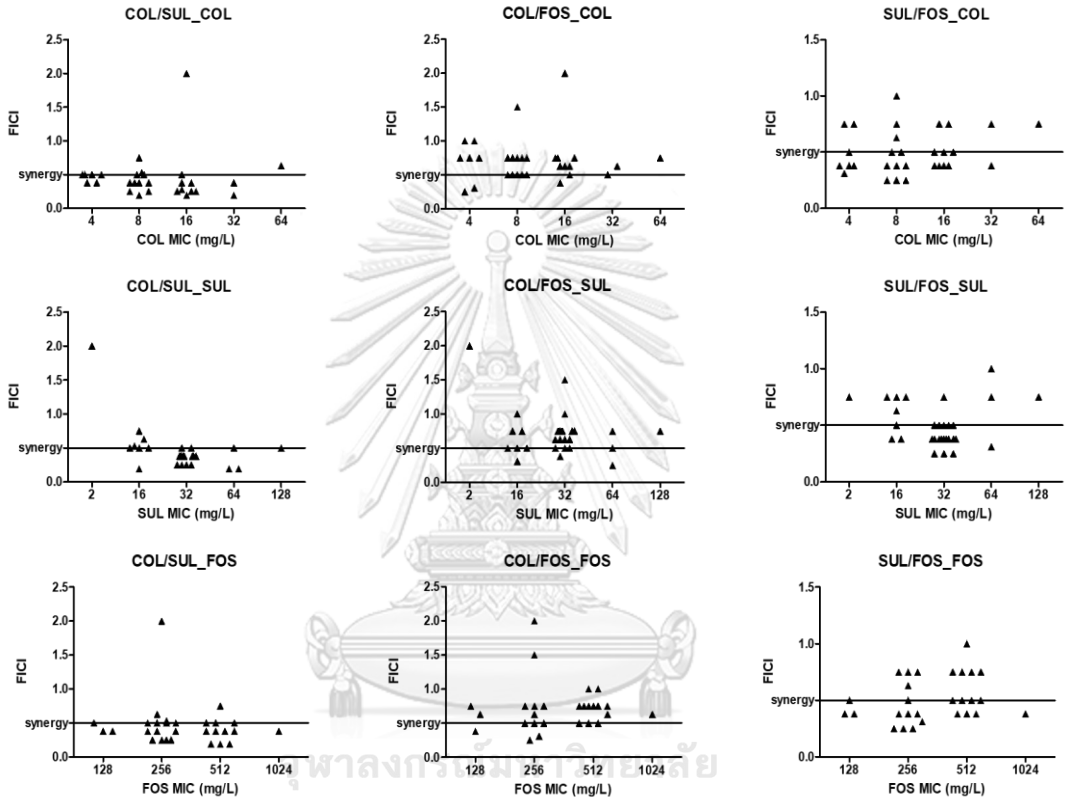
The association between mechanisms of colistin resistance with synergistic activities of antibiotic combinations is calculated by Chi-square test and shown in Table 15. Of the 26 isolates harboring mutation in LpxD and pEtN addition, the COL/SUL, COL/FOS, and SUL/FOS combinations showed synergistic effect in 23 (88.5%), 9 (34.6%), and 21 (80.8%), respectively. The synergy was found in the combination of COL/SUL in 2 isolates carrying only mutation in LpxD, COL/FOS in one isolate. The SUL/FOS combination showed partial synergy in both isolates. Moreover, an isolate that had only pEtN addition showed no synergistic effect in all combinations. The COL/SUL combination showed synergy in an isolate that did not contain those of colistin resistance mechanisms, but the COL/FOS and SUL/FOS combinations did not show synergistic activity. The combination of SUL/FOS exhibited significant association with mechanisms of resistance (*P*-value = 0.005). The mechanisms of colistin resistance did not demonstrate significant association with synergistic activity of COL/SUL (*P*-value = 0.071) and COL/FOS (*P*-value = 0.736) combinations.

Table 15. Summary result of synergistic activities of antibiotic combinations with different mechanisms of colistin resistance

Mechanisms		No. of isolates (%)	COL/SUL		*P*-value	COL/FOS		*P*-value	SUL/FOS		*P*-value
Mutation at E117K in LpxD	pEtN additions		Synergy	No synergy		Synergy	No synergy		Synergy	No synergy	
+	+	26 (86.7%)	23 (88.5%)	3 (11.5%)	0.071	9 (34.6%)	17 (65.4%)	0.736	21 (80.8%)	5 (19.2%)	0.005*
+	-	2 (6.7%)	2 (100%)	0		1 (50%)	1 (50%)		0	2 (100%)	0.83
-	+	1 (3.3%)	0	1 (100%)		0	1 (100%)		0	1 (100%)	0.3
-	-	1 (3.3%)	1 (100%)	0		0	1 (100%)		0	1 (100%)	0.120

Abbreviations: COL, colistin; FOS, fosfomycin; pEtN, phosphoethanolamine; SUL, sulbactam

The FICIs of each combination are plotted in graph related to colistin, sulbactam, and fosfomycin MICs and shown in Figure 26. The association between antibiotic MICs of each isolate and their synergistic activities of antibiotic combinations was calculated by the Chi-square test. None of combinations demonstrated significant association with MICs of each antibiotic.

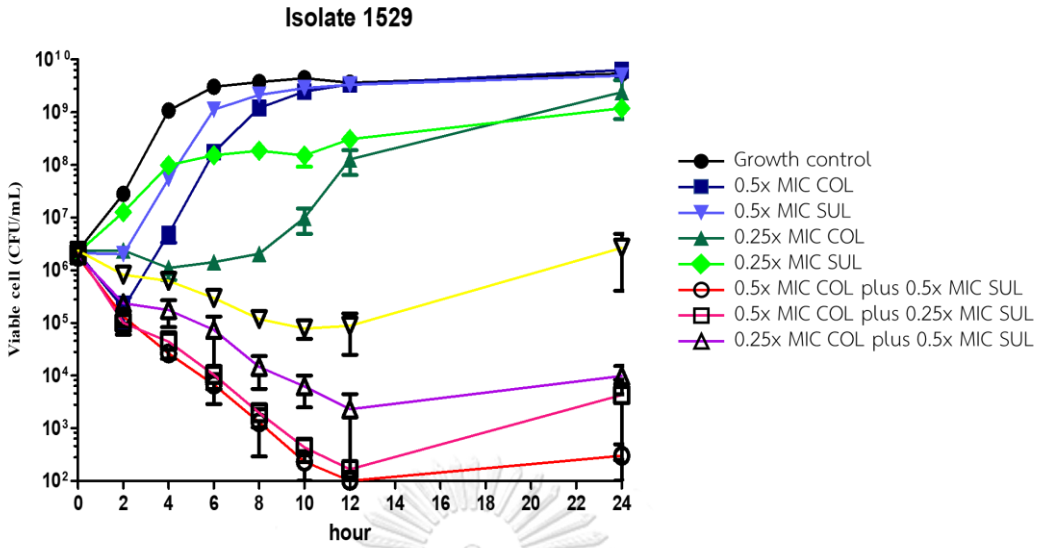


(Abbreviations: COL, colistin; FICI, fraction inhibitory concentration index; FOS, fosfomycin; MIC, minimum inhibitory concentration; SUL, sulbactam)

Figure 26. Synergistic activities of 3 antibiotic combinations with different MICs

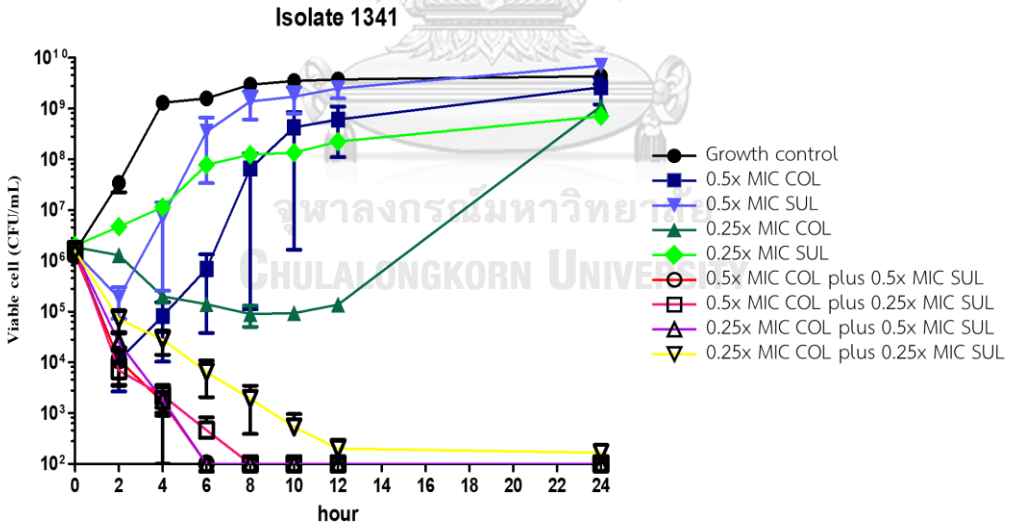
9. *in vitro* synergistic activity testing of colistin plus sulbactam against colistin-resistant *A. baumannii* clinical isolates

To confirm the synergistic activities of antibiotic combinations, the time-kill assay of the most effective combination (COL/SUL) from checkerboard assay was determined against six representative isolates of CoR-AB with different colistin susceptibility. The combinations of 0.5x MIC of colistin with 0.5x MIC or 0.25x MIC of sulbactam and 0.25x MIC of colistin with 0.5x MIC or 0.25x MIC of sulbactam were tested. This assay was performed in triplicate on 3 separate days for each isolate then the means and standard deviations of CFU/mL in each time point were plotted in graph. All of combinations demonstrated synergistic activity between colistin plus sulbactam against isolate 1341 (COL MIC: 4 mg/L), 1529 (COL MIC: 4 mg/L), 1129 (COL MIC: 8 mg/L), 1521 (COL MIC: 8 mg/L), 1251 (COL MIC: 16 mg/L), and 1374 (COL MIC: 16 mg/L). Every combination expressed excellent bacterial growth suppression (no viable cell) at 10 hours when tested with isolate 1251, 1374, and 1521. For isolate 1129, the combination of 0.25x MIC of colistin plus 0.25x MIC of sulbactam showed re-growth of bacteria at 24 hours and had no bactericidal activity. The re-growth of isolate 1529 was observed when tested with combination of 0.25x MIC of colistin plus 0.5x MIC of sulbactam. Moreover, the combination of 0.25x MIC of colistin plus 0.5x or 0.25x MIC of sulbactam showed no bactericidal activity against isolate 1529.



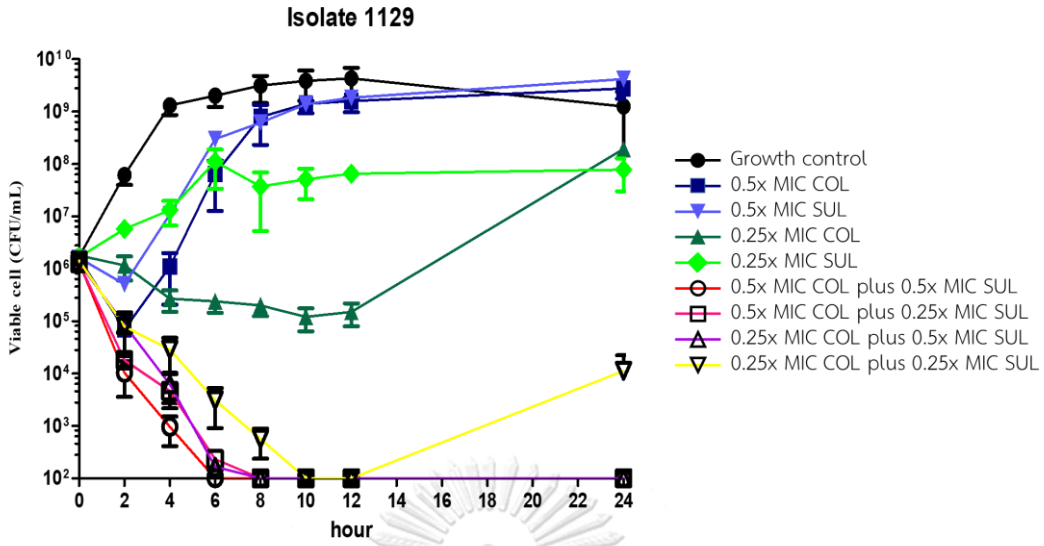
(Abbreviations: COL, colistin; FOS, fosfomycin; MIC, minimum inhibitory concentration; SUL, sulbactam)

Figure 27. *in vitro* time-kill assay of colistin plus sulbactam against colistin-resistant *A. baumannii* isolate 1529 (colistin MIC of 4 mg/L)



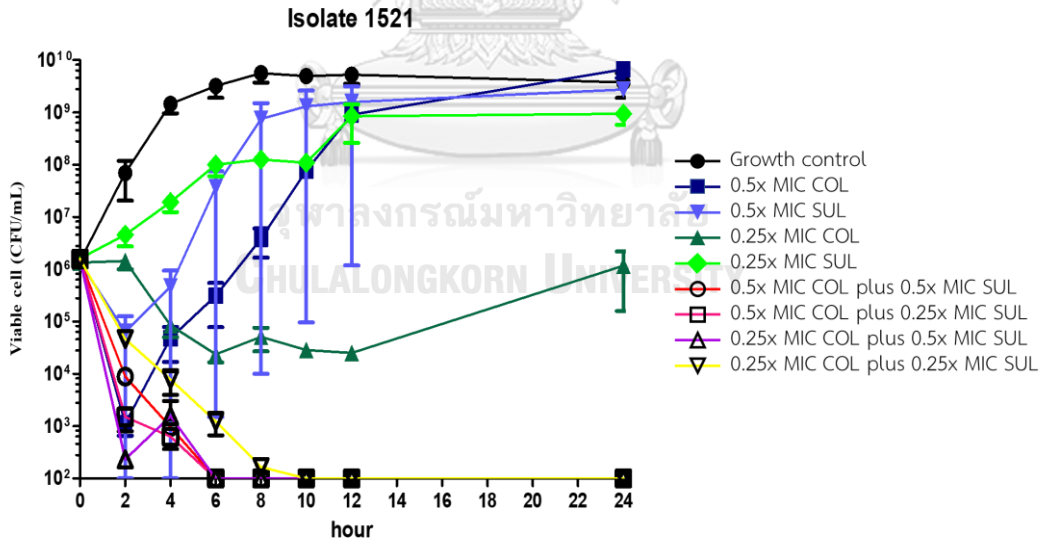
(Abbreviations: COL, colistin; FOS, fosfomycin; MIC, minimum inhibitory concentration; SUL, sulbactam)

Figure 28. *in vitro* time-kill assay of colistin plus sulbactam against colistin-resistant *A. baumannii* isolate 1341 (colistin MIC of 4 mg/L)



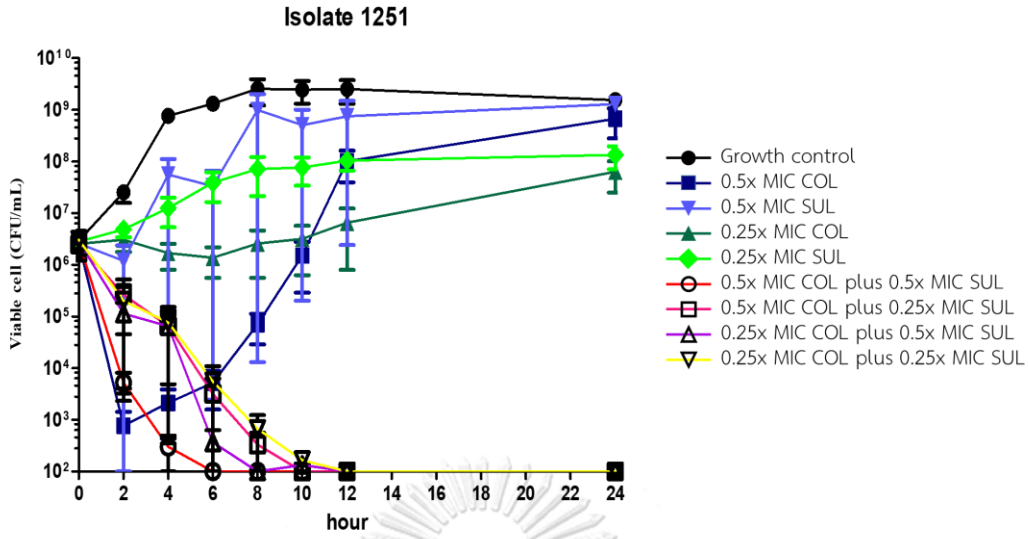
(Abbreviations: COL, colistin; FOS, fosfomycin; MIC, minimum inhibitory concentration; SUL, sulbactam)

Figure 29. *in vitro* time-kill assay of colistin plus sulbactam against colistin-resistant *A. baumannii* isolate 1129 (colistin MIC of 8 mg/L)



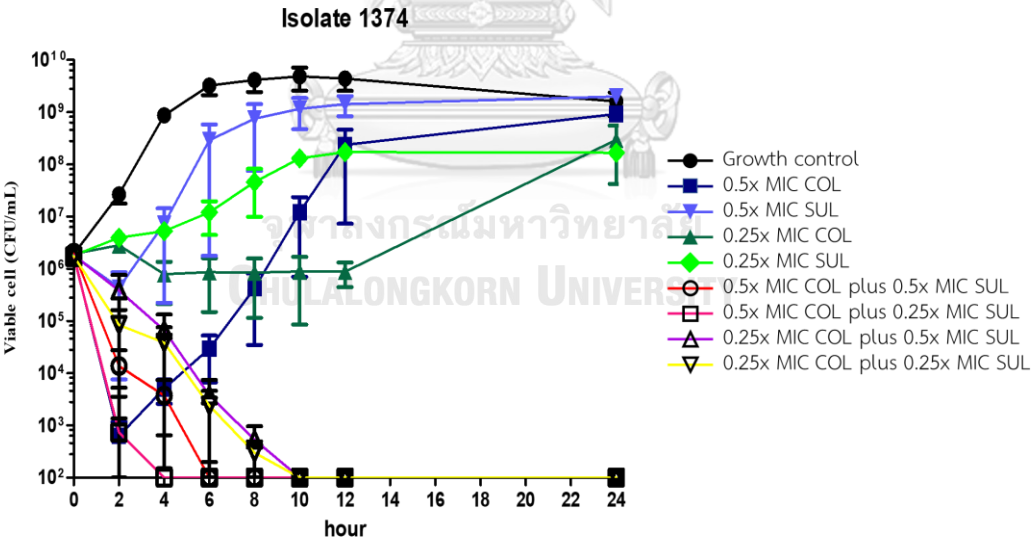
(Abbreviations: COL, colistin; FOS, fosfomycin; MIC, minimum inhibitory concentration; SUL, sulbactam)

Figure 30. *in vitro* time-kill assay of colistin plus sulbactam against colistin-resistant *A. baumannii* isolate 1521 (colistin MIC of 8 mg/L)



(Abbreviations: COL, colistin; FOS, fosfomycin; MIC, minimum inhibitory concentration; SUL, sulbactam)

Figure 31. *in vitro* time-kill assay of colistin plus sulbactam against colistin-resistant *A. baumannii* isolate 1251 (colistin MIC of 16 mg/L)



(Abbreviations: COL, colistin; FOS, fosfomycin; MIC, minimum inhibitory concentration; SUL, sulbactam)

Figure 32. *in vitro* time-kill assay of colistin plus sulbactam against colistin-resistant *A. baumannii* isolate 1374 (colistin MIC of 16 mg/L)

CHAPTER VI

DISCUSSION

Over the past few decades, *A. baumannii* has become an important pathogen that causes many nosocomial infections. Broad-spectrum antibiotics such as carbapenems are commonly used for treatment of *A. baumannii* infections (7). The rapid development of antimicrobial resistance in *A. baumannii* leads to increased infection caused by MDR-, XDR-, and PDR-strains (including carbapenem-resistant strains) (31). Nowadays, the antibiotics of choice in *A. baumannii* treatment are limited. Colistin and polymyxin B, the previously restricted antibiotics, are considered to be re-emerged as the last-resort treatment options for MDR-, and XDR-*A. baumannii* infections. However, increasing colistin resistance in *A. baumannii* has been reported across geographic regions (104). This study investigated the prevalence of colistin resistance in carbapenem-resistant *A. baumannii* clinical isolates, the mechanisms of colistin resistance, and effective antibiotic combinations to eliminate these strains.

Due to the difficulty in the differentiation among species members in *Acinetobacter baumannii-calcoaceticus* complex by conventional method (3), we identified to the species level by the *gyrB* multiplex PCR. The results observed that 92.96%, 2.35%, and 0.59% of isolates were identified as *A. baumannii*, *A. pittii*, and *A. nosocomialis*, respectively. The detection for the presence of the $bla_{\text{oxa-51-like}}$ gene by PCR was a simple method used to identify *A. baumannii*. However, $bla_{\text{oxa-51-like}}$ gene was absent in some *A. baumannii* (173, 179) and was also found in other *Acinetobacter* spp. including *A. nosocomialis* (180). In this study, the absence of $bla_{\text{oxa-51}}$ gene was found in 10 *A. baumannii* isolates, which may result from the disruption of $bla_{\text{oxa-51-like}}$ by insertion sequence elements (173). *A. baumannii* was the major species isolated from clinical samples. Similar to previous studies, *A. baumannii* has been major reported in the infection by *Abc* complex. The most accurate method for *Abc* complex identification is *rpoB* sequencing. However, the

gyrB multiplex PCR is fast and reliable method. This multiplex PCR can yield a result in less than 2 hours with robust, reproducible, and cost-effective. The accurate rates of this method were found to be 98.2%, 93.4%, and 77.2% for *rpoB* gene sequencing, 16S rRNA gene sequencing, and *gyrB* multiplex PCR, respectively, in identification of *Abc* complex at the species level (181). Moreover, the *gyrB* multiplex PCR exhibited 100% concordance in the identification of the clinically relevant species in *Abc* complex (2).

This study showed the immediate-to-high levels of carbapenem MICs in carbapenem-resistant *A. baumannii* clinical isolates. Our isolates demonstrated higher MIC levels of carbapenems than those in the global surveillance report. The data from SENTRY antimicrobial resistance program (1997-2016) showed that the $MIC_{50/90}$ of meropenem and imipenem were >8/>8 mg/L while this study showed the $MIC_{50/90}$ of meropenem and imipenem were 64/128 and 128/256 mg/L, respectively. The SENTRY data also reported 2.6% of PDR-*A. baumannii* (10). Among the 317 carbapenem-resistant strains, 13.6% were resistant to all tested antibiotics, including colistin, which indicated the increasing prevalence of PDR-*A. baumannii*. Our *A. baumannii* isolates had high rate of resistance to amikacin (81.7%), ciprofloxacin (98.74%), and levofloxacin (94.95%). This data demonstrated higher rates of resistance than those in the surveillance data in Asia-Pacific region (1997-2016) that reported 54.8% and 55.2% of amikacin and levofloxacin resistance, respectively. The susceptibility data from Taiwan revealed 15.4%, 44.1%, and 30.9% resistance to amikacin, ciprofloxacin, and levofloxacin, respectively, in *A. baumannii* clinical strains in 2018 (182). The prevalence of sulbactam and fosfomycin resistance were rarely reported. The previous study reported 83.33% resistance to sulbactam in XDR-AB from China in 2015 (167). However, the studies from Thailand showed that the rates of sulbactam resistance were 46.67% and 41.17% in *A. baumannii* isolates in 2015 and 2016, respectively (27, 166). In this study, the increasing rate of sulbactam resistance was found in 85.89%. The prevalence of fosfomycin resistance was

reported to be 100% from recent studies in 2018 (27, 28), according to the susceptibility criteria (R ≥ 32 mg/L) from European Committee on Antimicrobial Susceptibility Testing 2017 (EUCAST). However, this study applied the criteria from CLSI (2020) that has higher cut-off level of resistance (R ≥ 256 mg/L) and showed the lower rate of fosfomycin resistance (80.44%) than previous studies.

In *A. baumannii*, the intrinsic carbapenemase gene, $bla_{\text{OXA-51-like}}$, combined with $bla_{\text{OXA-23-like}}$ gene were found in most isolates (91.2%) in this study, similar to previous studies in Thailand and worldwide (6, 183). The $bla_{\text{OXA-51-like}}$ confers low-level carbapenem MICs. In this study, the $bla_{\text{OXA-51-like}}$-containing isolates showed high MIC levels to carbapenems (meropenem MICs of 32 – 128 mg/L and imipenem MICs of 64 – 256 mg/L). Chen, T., *et al.* found that the increase of carbapenem MICs were associated with the insertion of IS*Aba1* in $bla_{\text{OXA-51-like}}$ upstream region (184). The high carbapenem MICs in our isolates might be contributed to the overexpression of the $bla_{\text{OXA-51-like}}$ by insertion sequences. However, the genetic environments of $bla_{\text{OXA-51-like}}$ gene were not investigated in this study. The $bla_{\text{OXA-23-like}}$ gene is related to higher resistance level to meropenem and imipenem. Moreover, some isolates (1.26%) harbored the $bla_{\text{OXA-58-like}}$ combination with $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23-like}}$. These isolates showed high MIC levels (32 - 128 mg/L) to carbapenems. The lower MIC levels (16-32 mg/L) to carbapenems were observed in some isolates (0.63%) carrying $bla_{\text{OXA-24-like}}$ plus $bla_{\text{OXA-51-like}}$ genes.

In this study, the colistin resistance rate of *A. baumannii* isolates was 15.14%. The colistin resistance rate was lower in early reports from Thailand in 2014-2015 (3.6-9.3%) (157, 158). The recent study showed similar colistin resistance rate (14.3%) in *A. baumannii* isolates collected from many tertiary care hospitals in Thailand from 2016 to 2017 (11). The global antimicrobial surveillance data demonstrated that the worldwide colistin resistance rate was 4.1% in 2001-2016 (10). In some countries, the colistin resistance rate was higher than worldwide data including Spain (19.1% in 2006), Korea (30.6% in 2007), Bulgaria (16.7% in 2009, and Greece (21.1% in 2015)

(143, 144, 149, 150). The recent systematic review integrated from different 41 studies between 2010-2019 found that the rate of polymyxin resistance was 13% (155). This is the rapid increase of colistin resistance prevalence in *A. baumannii* in Thailand and worldwide since previous few years. Five isolates of non-*Abc* complex isolates (35.7%) exhibited colistin resistance with wide MIC range of 4 - >256 mg/L. However, none of *A. pittii* and *A. nosocomialis* were resistant to colistin. The epidemiology research in Taiwan demonstrated the high prevalence of *A. pittii* and *A. nosocomialis* in *Acinetobacter* spp. infections and *A. nosocomialis* showed higher colistin resistance rate than *A. baumannii* and *A. pittii* (185). The small number of *A. pittii* and *A. nosocomialis* in this study cannot be referred as the prevalence of colistin resistance in these species.

The PmrAB two-component system is the outer membrane proteins that respond to the environmental cation changes and cationic antimicrobial peptides (CAMPs) including colistin (186). It is involved in colistin resistance by lipid A modifications through the activation expression of *pmrC* gene that encoded a pEtN transferase (14). After the autophosphorylation reaction of PmrB, the phosphate group is transferred to the conserved aspartate residue in the response regulator receiver domain of PmrA, which enhances DNA binding affinity and affects gene transcription (186). PmrA binds to DNA at the promoter region of *pmrCAB* operon then pEtN transferase is translated from *pmrC* gene. The overexpression of *pmrC* is important for colistin resistance mechanism, which is associated with mutations in PmrA or PmrB (119, 120). The majority of PmrCAB amino acid substitutions were detected in 22 (73.3%) CoR-AB isolates which carried 48 substitutions in PmrC, 4 substitutions in PmrA, and 20 substitutions in PmrB when compared with those of reference strains (*A. baumannii* ATCC 19606 and ATCC 17987). Gerson, S., *et al*. found similar results of mutations in PmrCAB in 2 isolates with MICs of $\geq$ 256 mg/L from different countries. Their amino acid alignment results showed that amino acid sequences of PmrCAB of both isolates demonstrated low identity with PmrCAB

originating from other *Acinetobacter* spp. and Gram-negative pathogens. Moreover, the *pmrCAB* sequences showed high similarity with *pmrCAB* sequences in different clonal lineage (187). This finding suggested that the substitutions in those PmrCAB were possibly resulted from homologous recombination of *pmrCAB* operon across different clonal lineages and *A. baumannii* used this recombination to acquire mutations in the development of colistin resistance. However, our isolates showed lower MIC level (4 – 32 mg/L) than the study of Gerson, S., *et al*. This wide range of MICs might be requiring other mechanisms of resistance including the overexpression of efflux pumps and mutations of other LPS-producing genes (188-190). Further study would be required for investigate the responsibility of each amino acid substitutions in development of colistin resistance.

For PmrB, the previous studies clarified that the P233S and A227V amino acid changes on the histidine kinase domain of PmrB were related to colistin resistance in *A. baumannii* (191, 192). This domain includes the site of histidine autophosphorylation and phosphate transfer reactions. The activation of PmrB results in the phosphorylation of response regulator PmrA protein then regulates expression of *pmrC* (186). In this study, the A227V mutation was observed in most colistin-resistant isolates (96.67%) except isolate 1060. However, none of isolates were found P233S mutation in PmrB. The N440H and A444V substitutions were found in 100% and 16.67% of isolates, respectively. Unfortunately, these mutations have been previously reported in both colistin-susceptible and colistin-resistant strains and were not implicated in colistin resistance (193). In transmembrane parts, the S14P and A138T mutations were observed in some isolates (10% and 16.67%, respectively). The mutation at S14 position was previously reported in colistin-resistant clinical isolates (14). The A138T substitution was also described in colistin-susceptible, colistin-intermediate, and colistin-resistant strains (192). However, Lunha, *et al.* defined that this substitution was predicted as deleterious position through the functional effect of amino acid substitution program and may express high level of

colistin resistance with additional mutations (194). It is suggested that amino acid substitutions in transmembrane domains of PmrB are associated with sensing external stimuli and protein signaling that may be involved in colistin resistance (187, 195).

Few mutations in PmrA including E8D, A12I, I13M, G54E, A80V, and P102H in the receiver domain have been previously reported (150, 191). The I13M and P102R mutations showed 8 to 16-fold increase in colistin MIC and decreased negative charge of cells in site-directed mutation strains (188). The G54E mutation was related to overexpression of *pmrC* gene and resulted in high colistin susceptibility (187). The change at A80V was predicted as deleterious mutations with low colistin MIC (194). However, Gerson, S., *et al*. reported the amino acid differences in PmrA (I18T, T44N, E133Q, S134G, T168S) that resulted from homologous recombination of *pmrCAB* operon which may relate to elevated colistin MICs (187). Similar to previous studies, our results revealed that 22 (73.3%) CoR-AB isolates with wide range of colistin MICs (4 – 32 mg/L) had different amino acid changes at I18T and T44N (on receiver domain), E133Q, and T168S of PmrA.

The mutations in PmrC have rarely been reported to be related to colistin resistance. The previous study showed R125P substitution in PmrC related to colistin resistance at the MICs of 4 – 32 mg/L. Furthermore, higher colistin MICs (more than 64 mg/L) were observed in strains containing PmrB and PmrC substitutions (187). However, none of the isolates contained the R125P mutation in this study. Some studies demonstrated the polymorphism of *pmrC* gene. Lesho E., *et al*. identified the novel allele *pmrC1* in several CoR-AB isolates and their PmrC proteins were more closely identical to that of *A. nosocomialis* homologs than to that of *A. baumannii* (145). Other study indicated that additional copies of *pmrC* in *A. baumannii* genome and intragenic recombination of *pmrC* were contributed to colistin resistance (196). In this study, it was mentioned that there were as many as 56 amino acid changes in PmrC. These differences in amino acid changes may be the effect of DNA

recombination, however, we cannot clarify this genetic event and thus further study would be required.

The relationship between different amino acid substitutions in PmrCAB with colistin MICs was compared in 8 CoR-AB isolates. Three isolates harbored identical substitutions in PmrC (V42I, F150L, N284D, A354S, and K515T) and PmrB (S14P, A138T, A227V, L274F, A444V) with MICs of 8, 32, and 64 mg/L, respectively. The other 5 isolates harbored different substitutions with MICs range of 4 – 16 mg/L. Mutations in reaction domains of PmrB were not found in isolate 1060 (MIC of 16 mg/L). These results suggested that the reduced colistin susceptibility in these isolates required other resistance mechanisms. The combination of mutations was previously reported to be the cause of elevated colistin MICs which were higher than single mutations in any PmrC or PmrB (187). Moreover, the mutations in PmrB were associated with the expression of the *pmrCAB* operon (14, 120). The overexpression of *pmrC* was related with reduced colistin susceptibility in previous study (192). Our study requires further investigations of the expression level of *pmrCAB* operon and other colistin resistance mechanisms to explain the different susceptibility results of our isolates.

The production of LPS in *A. baumannii* was related to the series of enzyme encoded by *lpxA*, *lpxC*, and *lpxD* genes. The mechanisms of colistin resistance in *A. baumannii* are mediated by the loss of LPS due to amino acid substitutions and the insertion inactivation of *lpxACD* genes were confirmed in previous studies (16, 123). This study found the E117K substitution located on LpxD in most isolates (93.33%). However, this substitution was previously observed in both colistin-susceptible and colistin-resistant strains that did not seem to be related to colistin resistance (150, 192). In addition, some isolates (6.67%) harbored the V3A substitution on LpxD that was not previously reported. The insertions of IS element on surrounding genetic environment of these genes were not investigated in this study. The role of V3A substitution and insertion inactivation mediated to colistin resistance in our isolates would be required for further study. The insertion inactivation in *lpxA* and *lpxC* was

previous reported by IS*Aba11* and IS*Aba125* which is associated with high resistance to colistin (MIC > 128 mg/L) (123, 197). In contrast, our results observed synonymous mutations in *lpxA* and *lpxD* and none of insertion sequences were not detected.

The pEtN transferase can be encoded by the *mcr* genes, located on a plasmid. These genes were mostly discovered in Enterobacteriaceae (12, 198). Unlike *pmrCAB* operon, the *mcr* gene can horizontally transfer from species to species. The recent reports firstly described the *mcr-1* and *mcr-4.3* variants in *A. baumannii* isolated from pig, raw food, and clinical samples (19, 20, 137). However, 30 CoR-AB isolates in this study were not detected the *mcr-1* to *mcr-9*.

According to the mechanisms of colistin resistance, LPS modifications by pEtN result in alteration of electrostatic affinity between colistin and lipid A then mediates colistin resistance in *A. baumannii* (22). The lipid A structure analysis by MALDI-TOF MS provided insights into the mechanism of colistin resistance. The addition of pEtN at position 1′ and/or 4′ of *bis*-phosphoryl hepta-acylated lipid A structure has been implied as a major mechanism of colistin resistance in *A. baumannii* (109). The colistin-resistant strains had major $[M-H]^-$ ions at *m/z* 1,910 and additional peak at m/*z* 2,034 (predicted pEtN ion mass: 124) in MS spectra which were corresponded to pEtN addition to hepta-acylated lipid A (15). Our investigation observed this modification in most colistin-resistant isolates (27 of 30 isolates) with wide range colistin MICs of 4 - 32 mg/L. Unfortunately, the peak at *m/z* 2,034 and predicted peak of pEtN addition to minor lipid A species were not observed in three colistin-resistant isolates. These isolates had low-level colistin MIC (4 mg/L), suggesting that other mechanisms, such as the overexpression of efflux pumps (AdeABC or EmrAB) (198), must be involved in colistin resistance in these isolates. The colistin resistance in *A. baumannii* may be involved in the complete loss of LPS, however, all colistin-resistant isolates in this study were able to identify the major lipid A species in MS spectra. Moreover, some isolates were observed predominant $[M-H]^-$ ion peak at *m/z* 1,895 which was predicted to be consistent with loss of -OH molecules from *bis*-

phosphoryl hepta-acylated lipid A. Further study would be required for the investigation of the acylation and structure of lipid A.

During the period of last resort colistin antibiotic treatment, several studies reported the rapidly increasing colistin resistance prevalence in *A. baumannii* (10, 12, 150, 155). The antibiotic combination therapy is suitable treatment against colistin-resistant strains, replacing the lack of newly approved antibiotics. Few studies reported the effective combinations against CoR-AB. Colistin plus rifampin and colistin plus vancomycin were the most investigated and effective antibiotic combinations for *in vitro* testing (25, 161, 164, 165, 199, 200). However, colistin plus rifampicin combination was not showed significant clinical cure rate, even it showed microbiological cure, in the randomize-controlled clinical study (23). The previous studies revealed the *in vitro* synergistic effect of many combinations including colistin plus carbapenems or tigecycline or teicoplanin or sitafloxacin, doripenem plus vancomycin or tigecycline, and tigecycline plus rifampicin (11, 107, 160, 163). In addition, the combination of colistin plus imipenem, colistin plus meropenem, and triple combination of sulbactam plus tigecycline plus colistin showed clinical improvement when treated in patients with colistin-resistant *A. baumannii* infection (166, 201, 202). In the current study, we investigated the *in vitro* activities of COL/SUL, COL/FOS, and SUL/FOS combinations against CoR-AB clinical isolates. The screening of synergistic effect demonstrated high rate of synergy in COL/SUL (86.7%) and SUL/FOS (70%) combinations. The COL/SUL combination was previously reported but the synergistic rate was lower than colistin plus vancomycin (50% vs. 90%) (25). However, this study showed high synergistic activity (86.7%) of COL/SUL combination and similar to COL/VAN combination (90%) against CoR-AB isolates in previous study (25). The COL/SUL combination was also effective against carbapenem-resistant *A. baumannii* and clinically used to treat MDR-*A. baumannii* infections (27, 71, 203). The association between mechanisms of colistin resistance and synergistic activities of antibiotic combinations revealed that the isolates carrying

both E117K in LpxD and pEtN addition showed significantly related to the synergy of SUL/FOS combination. However, the sample sizes of isolates carrying only one mechanism or the absence of resistance mechanisms are limited in this study.

The most effective COL/SUL combination by checkerboard assay was selected for time-kill study against 6 representative colistin-resistant isolates with different MICs. The time-kill data of COL/SUL combination revealed synergistic activity in all tested isolates even in low concentration combination (0.25xMIC plus 0.25xMIC). There was no previous report of *in vitro* time-kill assay of COL/SUL combination against CoR-AB isolate. This present data indicated that the dosage regimen of colistin and sulbactam therapy may be reduced which affects the reduction of drug toxicity. However, the role of sulbactam in reduction of colistin MIC and additional *in vivo* and clinical research of this combination are needed to confirm the benefit in the treatment of CoR-AB infections.

There is no previous report on the synergy of the COL/FOS and SUL/FOS combinations against CoR-AB. In the present study, the COL/FOS combination showed the lowest rate of synergy at 33.3%. However, The COL/FOS combination was previously investigated against carbapenem-resistant *A. baumannii* with controversial results of synergistic effect. Santimaleeworagun *et al*. showed 73.3% rate of synergy of COL/FOS combination which differed from 50% and 33.3% in recent studies (27, 204, 205). The SUL/FOS combination presented lower percentage of synergistic effect than that of COL/SUL combination in this study. Although COL/SUL combination seems to be the therapeutic option, further studies were required to confirm the synergy and advantage in CoR-AB treatment.

CHAPTER VII

CONCLUSION

This study investigated the colistin resistance prevalence and mechanisms of colistin resistance in carbapenem-resistant *A. baumannii* clinical isolates. Our result showed the increasing prevalence of colistin resistance (15.14%) in *A. baumannii* clinical isolates with wide range of MICs. The mechanism of colistin resistance was predominantly caused by the addition of pEtN to lipid A. The mutations in PmrCAB could be involved in the addition of pEtN. We found the common amino acid mutation at A227V in PmrB which was related to colistin resistance. The several amino acid substitutions in active domain of PmrC and PmrA were reported in this study.

The combination of COL/SUL demonstrated most effective synergistic activity against CoR-AB clinical isolates, while the SUL/FOS combination showed slightly lower activity. The results of COL/FOS combination exhibited partial synergy against most CoR-AB isolates. Moreover, in time-kill studies, the COL/SUL combination demonstrated excellent bactericidal and synergistic activities in various concentration combinations against CoR-AB isolates with different colistin MICs. These results indicated the beneficial effect of COL/SUL combination in treatment of CoR-AB infections.

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extensively drug-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemase. J Chemother. 2016;28(3):159-63.

APPENDIX A

REAGENT AND INSTRUMENTS

Reagent	Manufacturer
1,4-dihybroxybutyric acid	Sigma-Aldrich, USA
Agarose	Amresco, USA
Amikacin sulphate	Hi-media, India
Ammonium hydroxide	Sigma-Aldrich, USA
Boric acid	Sigma-Aldrich, USA
Chloroform	Sigma-Aldrich, USA
Ciprofloxacin	Sigma-Aldrich, USA
Colistin sulphate	Sigma-Aldrich, USA
DNA gel loading dye	Thermo fisher scientific, USA
dNTP	Thermo fisher scientific, USA
Fosfomycin sodium	Meiji, Japan
Generuler 100 bp plus DNA ladder	Thermo fisher scientific, USA
Glucose-6-phosphate	Sigma-Aldrich, USA
Glycerol	Merck, Germany
Hiyield® Gel/PCR DNA mini kit	RBCBioscience, Taiwan
Hydrochloric acid	Merck, Germany
Imipenem	Wako, Japan
Isobutyric acid	Sigma-Aldrich, USA
LB broth	BBL, USA
Levofloxacin	Sigma-Aldrich, USA
Meropenem	Wako, Japan
Methanol	Sigma-Aldrich, USA
Mueller-Hinton II agar	BBL, USA
Mueller-Hinton II broth (cation-adjusted)	BBL, USA
Norharmane	Sigma-Aldrich, USA
Phosphate buffer saline	Sigma-Aldrich, USA
Sodium chloride	Amresco, USA

Sodium Hydroxide	Merck, Germany
Sulbactam	Wako, USA
Taq DNA polymerase	Thermo fisher scientific, USA
Tris	Amresco, USA
Tryptic soy broth (TSB)	BBL, USA

Instrument	**Manufacturer**
Incubator	Thermo fisher scientific, USA
Incubator shaker	Thermo fisher scientific, USA
Microcentrifuge	Eppendorf, Germany
UV/Visible spectrometer	Bio-rad, Germany
Thermal cycler	Applied Bioscience, USA
Nanodrop 1000 spectrometer	Thermo fisher scientific, USA
UV transilluminator	Montreal Biotech, Canada
Lyophilizer	
JMS-S3000 SpiralTOF^{TM}-plus MALDI-TOF MS	JOEL, Japan

APPENDIX B

MEDIA AND ANTIBIOTIC SOLUTION PREPARATION

1. LB broth (BBL, USA)

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

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

Suspend 38 g of the dehydrated Mueller-Hinton II agar in 1000 mL of distilled water and mixed homogenously then sterilized by autoclaving at 121 °C for 15 minutes. The Mueller-Hinton II agar was stored at 4 °C.

3. Cation- adjusted Mueller-Hinton II broth (BBL, USA)

Suspend 22 g of the dehydrated cation- adjusted Mueller-Hinton II broth in 1000 mL of distilled water and mixed homogenously then sterilized by autoclaving at 121 °C for 15 minutes. The cation- adjusted Mueller-Hinton II broth was stored at 4 °C.

4. Tryptic soy broth (TSB) (BBL, USA)

Suspend 30 g of the dehydrated TSB in 1000 mL of distilled water and mixed homogenously then sterilized by autoclaving at 121 °C for 15 minutes. The TSB was stored at 4 °C.

5. Sterile normal saline

Suspend 8.5 g of the dehydrated sodium chloride in 1000 mL of distilled water and mixed homogenously then sterilized by autoclaving at 121 °C for 15 minutes. The sterile normal saline was stored at 4 °C.

6. Antibiotic stock solution

6.1. Imipenem, stock concentration 1,024 mg/L

Preparation of stock, 0.0102 g of imipenem was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

6.2. Meropenem, stock concentration 1,024 mg/L

Preparation of stock, 0.0102 g of meropenem was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

6.3. Amikacin, stock concentration 1,024 mg/L

Preparation of stock, 0.0102 g of amikacin was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

6.4. Ciprofloxacin, stock concentration 1,024 mg/L

Preparation of stock, 0.0102 g of ciprofloxacin was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

6.5. Levofloxacin, stock concentration 1,024 mg/L

Preparation of stock, 0.0102 g of levofloxacin was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

6.6. Colistin, stock concentration 1,024 mg/L

Preparation of stock, 0.0102 g of colistin was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

6.7. Sulbactam, stock concentration 1,024 mg/L

Preparation of stock, 0.0102 g of sulbactam was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

6.8. Fosfomycin, stock concentration 1,024 mg/L

Preparation of stock, 0.0102 g of fosfomycin was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

6.9. Glucose-6-phosphate

Preparation of stock, 0.0102 g of glucose-6-phosphate was weighed then dissolved by 10 ml of 5 ml of sterile distilled water.

APPENDIX C

REAGENT PREPARATION

1. 0.5 M EDTA (pH 8.0)

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

2. 10X Tris-Borate buffer (TBE)

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

3. 0.5% agarose gel

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

4. Phosphate buffer saline (pH 7.4)

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

5. Isobyturic acid:1 M ammonium hydroxide (5:3, vol/vol)

Preparation of 1 M ammonium hydroxide, 1.752 mL of ammonium hydroxide solution (28.0 – 30.0% NH_3 basis) then added distilled water and adjusted volume to 50 mL.

Preparation of Isobyturic acid:1 M ammonium hydroxide (5:3, vol/vol), 5 mL of isobyturic acid (99%) and 3 mL of 1 M ammonium hydroxide were mixed homogenously. This reagent was immediately prepared before use.

6. Chloroform:methanol:water (12:6:1, vol/vol/vol)

Preparation of chloroform:methanol:water (12:6:1, vol/vol/vol), 3 mL of chloroform, 1.5 mL of methanol, and 0.25 mL of distilled water were mixed homogenously. This reagent was immediately prepared before use.

7. Norharmane

Preparation of 10 mg/mL of norharmane, 1 mg of norharmane was weighed and dissolved in 0.1 mL of chloroform:methanol:water (12:6:1 vol/vol/vol). This reagent was immediately prepared before use.

APPENDIX D

ADDITIONAL RESULT

Table 16. Result of multiplex *gyrB* PCR, MICs of 8 antibiotic and carbapenemase-encoding genes of 341 *Acinetobacter* spp.

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
151	AB	1	64	64	>256	32	2	128	32	+	-	+	-	-	-	-	-	-
152	AB	2	128	128	>256	128	32	128	64	+	-	+	-	-	-	-	-	-
153	AB	2	64	64	>256	128	16	128	8	-	-	-	-	-	-	-	-	-
154	AB	2	128	128	>256	128	32	>256	64	+	-	+	-	-	-	-	-	-
155	AB	2	128	128	>256	64	4	128	64	+	-	+	-	-	-	-	-	-
156	AB	2	256	128	>256	128	32	256	64	+	-	+	-	-	-	-	-	-
157	AB	2	64	128	>256	64	4	>256	64	+	-	+	-	-	-	-	-	-
158	AB	2	128	64	>256	64	8	256	16	-	-	-	-	-	-	-	-	-
159	AB	2	64	128	>256	64	2	128	32	+	-	+	-	-	-	-	-	-
160	AB	2	128	64	>256	64	4	256	16	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
161	AB	1	128	128	>256	64	16	128	64	+	-	+	-	-	-	-	-	-
162	non-AB	1	128	128	>256	32	8	256	64	+	-	+	-	-	-	-	-	-
163	AB	2	128	128	>256	64	8	>256	64	+	-	+	-	-	-	-	-	-
164	AB	2	128	128	>256	128	16	256	64	+	-	+	-	-	-	-	-	-
165	non-ABC	1	128	128	>256	64	4	128	32	+	-	+	-	-	-	-	-	-
166	non-AB	1	128	128	>256	256	16	>256	32	+	-	+	-	-	-	-	-	-
167	AB	1	128	128	>256	64	2	128	64	+	-	+	-	-	-	-	-	-
168	AB	1	>256	128	>256	64	4	256	64	+	-	+	-	-	-	-	-	-
169	AB	1	>256	>256	>256	128	32	128	>256	+	-	+	-	-	-	-	-	-
170	AB	2	>256	128	>256	64	8	256	128	+	-	+	-	-	-	-	-	-
171	AB	1	32	32	2	16	1	128	16	+	-	+	-	-	-	-	-	-
172	AB	0.5	64	64	>256	>256	128	256	64	+	-	+	-	-	-	-	-	-
173	AB	2	64	64	2	32	8	256	32	+	-	+	-	-	-	-	-	-
174	AB	2	32	32	2	64	8	>256	16	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
175	AB	2	32	32	2	32	8	128	16	+	-	+	-	-	-	-	-	-
176	AB	4	32	32	4	8	1	>256	256	+	-	+	-	-	-	-	-	-
177	AB	2	64	64	4	64	16	256	64	+	-	+	-	-	-	-	-	-
178	AB	1	128	128	>256	64	4	256	128	+	-	+	-	-	-	-	-	-
179	AB	2	128	128	>256	128	16	256	128	+	-	+	-	-	-	-	-	-
180	AB	2	128	128	>256	128	16	>256	64	+	-	+	-	-	-	-	-	-
181	AB	1	64	64	>256	256	16	256	32	+	-	+	-	-	-	-	-	-
182	AB	0.5	128	128	>256	64	8	>256	64	+	-	+	-	-	-	-	-	-
183	AB	1	>256	>256	>256	>256	32	>256	>256	+	-	+	-	-	-	-	-	-
184	GS3	1	128	128	64	32	4	>256	128	+	-	+	-	-	-	-	-	-
185	AB	2	128	256	>256	64	8	>256	64	+	-	+	-	-	-	-	-	-
186	non-AB	2	32	32	2	128	16	>256	16	+	-	+	-	-	-	-	-	-
187	AB	2	128	128	>256	128	8	256	64	+	-	+	-	-	-	-	-	-
188	AB	2	64	32	64	32	2	256	64	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
189	AB	1	128	128	>256	128	32	256	128	+	-	+	-	-	-	-	-	-
190	non-AB	4	128	128	>256	128	32	256	32	+	-	+	-	-	-	-	-	-
191	AB	2	128	128	>256	128	16	256	128	+	-	+	-	-	-	-	-	-
192	AB	2	64	64	8	256	64	>256	64	+	-	+	-	-	-	-	-	-
193	AB	2	64	64	>256	128	32	>256	65	+	-	+	-	-	-	-	-	-
194	AB	4	128	128	>256	128	16	>256	128	+	-	+	-	-	-	-	-	-
195	AB	2	128	64	>256	128	16	256	128	+	-	+	-	-	-	-	-	-
196	AB	4	128	64	>256	128	16	>256	64	+	-	+	-	-	-	-	-	-
197	AB	4	128	128	>256	64	16	256	64	+	-	+	-	-	-	-	-	-
199	AB	4	128	128	>256	64	16	>256	128	+	-	+	+	-	-	-	-	-
200	AB	2	64	64	>256	64	16	256	64	+	-	+	+	-	-	-	-	-
201	AB	2	64	128	>256	>256	32	256	64	+	-	+	+	-	-	-	-	-
202	AB	2	64	128	>256	128	16	>256	64	+	-	+	+	-	-	-	-	-
204	AB	2	64	64	>256	>256	32	256	64	+	-	+	+	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
205	AB	2	128	128	>256	64	8	256	128	+	-	+	+	-	-	-	-	-
206	AB	2	64	128	>256	>256	32	>256	64	+	-	+	+	-	-	-	-	-
207	AB	4	128	128	>256	64	8	>256	128	+	-	+	+	-	-	-	-	-
208	AB	2	64	64	>256	32	8	256	64	+	-	+	+	-	-	-	-	-
209	AB	4	32	64	>256	256	32	256	64	+	-	+	+	-	-	-	-	-
210	AB	2	64	64	>256	256	32	>256	64	+	-	+	+	-	-	-	-	-
211	GS3	2	128	128	>256	64	32	128	32	+	-	+	+	-	-	-	-	-
212	AB	2	128	64	>256	64	8	>256	64	+	-	+	+	-	-	-	-	-
213	AB	4	128	64	>256	256	16	>256	64	+	-	+	+	-	-	-	-	-
214	AB	2	64	64	>256	64	8	>256	256	+	-	+	+	-	-	-	-	-
215	AB	4	32	32	8	16	8	>256	8	+	-	+	+	-	-	-	-	-
216	AB	4	256	256	>256	256	16	256	64	+	-	+	+	-	-	-	-	-
217	AB	2	128	128	8	128	8	256	32	+	-	+	-	-	-	-	-	-
218	AB	2	256	256	>256	256	16	256	64	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
219	AB	2	128	256	>256	64	8	256	32	+	-	+	-	-	-	-	-	-
220	AB	2	256	256	>256	256	32	256	32	+	-	+	-	-	-	-	-	-
221	AB	2	256	256	>256	64	16	256	64	+	-	+	-	-	-	-	-	-
222	AB	2	128	256	>256	64	8	256	32	+	-	+	-	-	-	-	-	-
223	AB	2	256	256	>256	64	16	256	64	+	-	+	-	-	-	-	-	-
224	AB	2	256	256	>256	128	16	256	128	+	-	+	-	-	-	-	-	-
225	AB	2	256	256	>256	64	8	128	64	+	-	+	-	-	-	-	-	-
226	AB	2	64	256	4	128	32	128	32	+	-	+	-	-	-	-	-	-
227	AB	2	128	256	>256	256	32	128	64	+	-	+	-	-	-	-	-	-
228	AB	4	256	256	>256	64	8	256	128	+	-	+	-	-	-	-	-	-
229	AB	2	256	256	>256	64	8	256	128	+	-	+	-	-	-	-	-	-
230	AB	2	64	128	>256	>256	128	128	32	+	-	+	-	-	-	-	-	-
661	AB	0.25	128	128	>256	64	16	256	32	-	-	+	-	-	-	-	-	-
662	AB	2	64	128	>256	256	16	>256	64	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
663	AB	0.5	64	64	8	256	16	256	64	+	-	+	-	-	-	-	-	-
664	AB	0.5	128	128	>256	64	16	256	64	+	-	+	-	-	-	-	-	-
665	AB	1	128	128	>256	128	16	256	32	+	-	+	-	-	-	-	-	-
666	AB	0.5	64	64	>256	64	16	256	16	+	-	+	-	-	-	-	-	-
667	AB	1	128	128	>256	64	16	256	128	+	-	+	-	-	-	-	-	-
668	AB	0.5	32	64	>256	64	16	256	16	+	-	+	-	-	-	-	-	-
669	AB	0.5	32	32	2	128	16	256	16	+	-	+	-	-	-	-	-	-
670	AB	0.5	64	64	>256	256	16	>256	64	+	-	+	-	-	-	-	-	-
671	AB	1	64	64	>256	128	16	>256	64	+	-	+	-	-	-	-	-	-
672	AB	0.5	128	64	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
673	AB	0.5	32	32	16	32	16	256	32	+	-	+	-	-	-	-	-	-
674	AB	0.5	32	32	8	16	16	256	16	+	-	+	-	-	-	-	-	-
675	AB	0.5	64	32	>256	64	16	>256	64	-	-	+	-	-	-	-	-	-
676	AB	0.25	64	64	>256	64	16	256	32	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
677	AB	0.25	64	64	>256	32	16	256	16	+	-	+	-	-	-	-	-	-
678	AB	0.125	32	64	16	64	16	128	32	+	-	+	-	-	-	-	-	-
679	non-AB	0.125	128	128	>256	64	16	256	16	-	-	-	-	-	-	-	-	-
680	AB	0.25	64	64	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
681	AB	0.25	128	128	>256	64	16	256	16	+	-	+	-	-	-	-	-	-
682	AB	0.25	64	128	>256	128	16	256	32	+	-	+	-	-	-	-	-	-
683	AB	0.125	256	256	32	128	16	>256	32	+	-	+	-	-	-	-	-	-
684	AB	0.5	64	64	>256	64	16	256	16	+	-	+	-	-	-	-	-	-
685	AB	0.125	128	128	>256	64	16	128	32	+	-	+	-	-	-	-	-	-
686	AB	1	64	128	>256	64	16	>256	32	+	-	+	-	-	-	-	-	-
687	AB	0.125	128	256	2	128	16	128	16	+	-	+	-	-	-	-	-	-
688	AB	0.25	128	128	>256	64	16	256	32	-	-	+	-	-	-	-	-	-
689	AB	0.25	64	128	>256	64	16	256	16	+	-	+	-	-	-	-	-	-
690	AB	0.125	128	128	>256	64	16	>256	16	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
691	AB	0.25	64	64	>256	128	16	256	32	+	-	+	-	-	-	-	-	-
692	AB	0.125	256	256	>256	128	16	256	64	-	-	+	+	-	-	-	-	-
693	AB	0.5	128	128	64	64	16	>256	32	+	-	+	-	-	-	-	-	-
694	AB	0.25	128	128	>256	64	16	256	16	+	-	+	-	-	-	-	-	-
695	AB	0.125	256	256	>256	128	16	256	16	+	-	+	-	-	-	-	-	-
696	AB	0.25	64	128	>256	32	16	128	32	-	-	-	-	-	-	-	-	-
697	GS13TU	0.25	64	64	>256	64	16	256	32	-	-	-	+	-	-	-	-	-
698	AB	0.125	64	32	>256	64	16	256	16	-	-	+	-	-	-	-	-	-
699	GS13TU	0.5	128	128	128	64	16	256	32	+	-	+	-	-	-	-	-	-
1021	AB	1	64	64	>256	128	8	256	16	+	-	+	-	-	-	-	-	-
1022	AB	1	64	64	>256	32	4	256	8	+	-	+	-	-	-	-	-	-
1024	AB	2	64	64	>256	128	0.5	256	64	+	-	+	-	-	-	-	-	-
1029	AB	2	32	64	8	0.5	16	256	32	-	-	-	-	-	-	-	-	-
1030	AB	1	256	256	>256	>256	64	>256	64	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1031	AB	1	64	64	>256	>256	32	256	64	-	-	+	-	-	-	-	-	+
1032	AB	1	128	128	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
1033	AB	2	128	64	>256	64	8	128	64	+	-	+	-	-	-	-	-	-
1034	AB	2	128	256	>256	32	4	256	128	+	-	+	-	-	-	-	-	-
1035	AB	2	32	32	>256	32	8	256	64	+	-	+	-	-	-	-	-	-
1036	non-AB	4	128	128	64	2	2	256	16	-	-	-	-	-	-	-	-	+
1037	AB	1	64	64	>256	128	16	256	256	+	-	+	-	-	-	-	-	-
1038	AB	2	32	64	>256	64	16	256	64	+	-	+	-	-	-	-	-	-
1039	AB	2	128	128	>256	128	32	256	64	+	-	+	-	-	-	-	-	-
1040	AB	1	128	128	>256	64	16	128	64	+	-	+	-	-	-	-	-	-
1041	AB	2	128	256	>256	16	4	>256	8	-	-	+	-	-	-	-	-	-
1042	AB	2	128	128	16	128	32	256	64	+	-	+	-	-	-	-	-	-
1043	GS3	2	>256	>256	>256	64	8	256	32	-	-	-	-	-	-	+	-	-
1044	AB	0.25	64	128	>256	>256	128	256	64	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1045	AB	2	128	128	>256	64	8	256	32	+	-	+	-	-	-	-	-	-
1046	AB	1	64	64	>256	32	8	128	32	+	-	+	-	-	-	-	-	-
1047	AB	2	64	64	>256	64	16	256	64	-	-	-	-	-	-	-	-	-
1048	GS3	0.5	128	64	2	64	8	256	16	-	-	-	-	-	-	+	-	-
1049	AB	16	128	128	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
1050	AB	8	128	256	>256	128	32	128	128	+	-	+	-	-	-	-	-	-
1051	AB	1	32	64	>256	64	8	256	16	+	-	+	-	-	-	-	-	-
1052	AB	4	128	128	>256	256	32	128	64	+	-	+	-	-	-	-	-	-
1053	AB	4	32	64	1	64	8	128	32	-	-	+	-	-	-	-	-	-
1054	AB	1	64	64	8	64	8	256	16	+	-	+	-	-	-	-	-	-
1055	AB	1	128	128	>256	128	16	256	128	+	-	+	-	-	-	-	-	-
1056	AB	1	128	256	>256	128	16	256	64	-	-	+	-	-	-	-	-	-
1057	AB	1	64	128	>256	128	16	128	64	+	-	+	-	-	-	-	-	-
1058	AB	1	32	64	>256	256	16	>256	16	-	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1059	AB	1	32	64	>256	64	8	256	32	-	-	+	-	-	-	-	-	-
1060	AB	4	64	64	>256	128	8	256	64	+	-	+	-	-	-	-	-	-
1061	AB	2	128	64	64	128	8	256	64	+	-	+	-	-	-	-	-	-
1062	GS3	2	128	256	64	32	8	128	128	-	-	-	+	-	-	-	-	-
1063	AB	2	64	64	>256	128	8	128	64	+	-	+	-	-	-	-	-	-
1064	AB	1	64	64	2	32	8	256	32	+	-	+	-	-	-	-	-	-
1065	AB	1	32	32	16	128	8	256	16	+	-	+	-	-	-	-	-	-
1066	AB	2	64	64	>256	64	8	256	32	+	-	+	-	-	-	-	-	-
1067	AB	2	64	64	>256	64	8	256	16	+	-	+	-	-	-	-	-	-
1070	AB	4	256	256	64	1	8	64	128	-	-	-	+	-	-	-	-	-
1071	AB	2	64	64	>256	128	8	256	32	+	-	+	-	-	-	-	-	-
1072	AB	4	128	128	>256	128	8	256	64	+	-	+	-	-	-	-	-	-
1074	AB	2	128	64	>256	64	8	128	64	+	-	+	-	-	-	-	-	-
1075	AB	2	128	256	>256	256	8	256	64	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1076	AB	2	32	32	>256	64	8	256	16	+	-	+	-	-	-	-	-	-
1077	AB	2	64	128	>256	>256	8	256	32	+	-	+	-	-	-	-	-	-
1078	AB	2	128	128	>256	128	8	256	64	+	-	+	-	-	-	-	-	-
1079	AB	4	64	64	>256	64	8	>256	16	+	-	+	-	-	-	-	-	-
1080	AB	2	32	64	>256	64	8	256	16	+	-	+	-	-	-	-	-	-
1081	non-AB	4	128	>256	64	2	8	128	>256	-	-	-	-	-	-	-	-	-
1082	AB	4	32	128	>256	64	8	256	16	+	-	+	-	-	-	-	-	-
1084	AB	2	256	256	16	128	8	256	128	+	-	+	-	-	-	-	-	-
1085	AB	2	64	128	>256	>256	8	256	64	+	-	+	-	-	-	-	-	-
1086	AB	2	128	128	>256	64	8	256	32	+	-	+	-	-	-	-	-	-
1087	AB	2	64	128	>256	64	8	128	8	+	-	+	-	-	-	-	-	-
1088	AB	4	256	256	>256	>256	8	256	64	+	-	+	-	-	-	-	-	-
1090	AB	2	128	128	>256	64	8	256	64	+	-	+	-	-	-	-	-	-
1092	AB	2	128	256	>256	128	8	128	16	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1093	AB	2	128	128	>256	64	8	128	32	+	-	+	-	-	-	-	-	-
1094	AB	2	64	128	>256	64	8	128	64	+	-	+	-	-	-	-	-	-
1095	AB	2	128	128	>256	256	8	128	16	+	-	+	-	-	-	-	-	-
1096	non-AB	>256	>256	>256	>256	256	8	>256	>256	-	-	-	-	+	-	-	-	-
1097	AB	1	64	64	2	64	8	>256	16	+	-	+	-	-	-	-	-	-
1098	AB	4	64	64	>256	64	8	>256	16	+	-	+	-	-	-	-	-	-
1099	AB	4	128	128	>256	64	8	256	128	+	-	+	-	-	-	-	-	-
1100	AB	2	128	64	>256	256	8	128	32	+	-	+	-	-	-	-	-	-
1101	AB	1	64	64	>256	256	8	256	32	+	-	+	-	-	-	-	-	-
1102	AB	2	64	64	>256	128	8	128	128	+	-	+	-	-	-	-	-	-
1103	AB	2	128	128	4	64	8	>256	128	+	-	+	-	-	-	-	-	-
1104	AB	2	32	64	>256	64	8	128	16	+	-	+	-	-	-	-	-	-
1105	AB	2	32	64	>256	64	8	256	16	+	-	+	-	-	-	-	-	-
1106	AB	2	64	64	4	64	8	128	32	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1107	AB	2	128	128	>256	128	8	128	64	+	-	+	-	-	-	-	-	-
1108	AB	1	128	128	>256	>256	8	256	32	+	-	+	-	-	-	-	-	-
1109	AB	2	64	128	8	128	8	128	32	+	-	+	+	-	-	-	-	-
1110	AB	1	128	64	16	256	8	256	16	+	-	+	-	-	-	-	-	-
1111	AB	2	64	128	8	128	8	128	32	+	-	+	-	-	-	-	-	-
1112	AB	2	64	64	8	128	8	128	16	+	-	+	-	-	-	-	-	-
1113	AB	1	32	16	2	0.5	8	256	1	-	+	+	-	-	-	-	-	-
1115	AB	2	128	256	>256	256	8	128	64	+	-	+	-	-	-	-	-	-
1118	AB	0.125	32	32	8	16	8	>256	8	+	-	+	-	-	-	-	-	-
1119	AB	0.125	64	32	16	32	8	256	64	+	-	+	-	-	-	-	-	-
1120	AB	0.25	64	128	>256	64	8	128	64	+	-	+	-	-	-	-	-	-
1121	AB	0.25	128	128	>256	64	16	256	64	+	-	+	-	-	-	-	-	-
1122	AB	0.125	32	32	2	128	16	256	64	+	-	+	-	-	-	-	-	-
1123	AB	0.25	64	64	>256	128	16	256	16	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1124	AB	0.125	32	32	4	64	16	256	16	+	-	+	-	-	-	-	-	-
1125	AB	0.125	64	64	4	64	16	>256	16	+	-	+	-	-	-	-	-	-
1126	AB	32	128	128	>256	64	16	>256	16	+	-	+	-	-	-	-	-	-
1127	AB	16	64	128	>256	1228	16	>256	32	+	-	+	-	-	-	-	-	-
1128	AB	0.5	128	256	64	64	16	256	32	+	-	+	-	-	-	-	-	-
1129	AB	8	64	64	>256	128	16	>256	64	+	-	+	-	-	-	-	-	-
1130	AB	0.5	64	128	>256	>256	16	256	64	+	-	+	-	-	-	-	-	-
1131	AB	2	128	128	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
1132	AB	0.25	64	256	32	1	16	256	32	+	-	+	-	-	-	-	-	-
1133	AB	0.25	128	128	>256	128	16	256	64	+	-	+	-	-	-	-	-	-
1134	AB	0.125	256	256	64	64	16	64	8	+	-	+	-	-	-	-	-	-
1135	AB	0.125	128	128	8	64	16	256	32	+	-	+	-	-	-	-	-	-
1136	AB	0.125	128	128	64	128	16	>256	32	+	-	+	-	-	-	-	-	-
1137	AB	0.125	128	128	16	64	16	256	16	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1138	AB	0.25	256	128	>256	32	16	256	8	+	-	+	-	-	-	-	-	-
1139	AB	32	64	64	>256	64	16	256	16	+	-	+	-	-	-	-	-	-
1220	GS3	0.25	64	64	>256	64	32	256	128	+	-	-	-	-	-	-	-	-
1221	AB	0.125	32	32	16	64	32	128	16	+	-	+	-	-	-	-	-	-
1222	AB	0.25	128	128	>256	64	32	256	32	+	-	+	-	-	-	-	-	-
1223	AB	0.25	128	128	>256	32	32	256	64	+	-	+	-	-	-	-	-	-
1224	AB	0.5	64	128	8	32	32	256	32	+	-	+	-	-	-	-	-	-
1225	AB	0.25	64	64	>256	16	32	>256	32	+	-	+	-	-	-	-	-	-
1226	AB	0.5	64	64	>256	16	32	256	64	+	-	+	-	-	-	-	-	-
1227	AB	0.125	32	32	64	16	32	128	16	+	-	+	+	-	-	-	-	-
1228	AB	0.25	32	32	>256	16	32	256	16	-	+	+	-	-	-	-	-	-
1229	AB	2	256	256	>256	128	32	256	128	-	-	-	-	-	-	-	-	-
1230	AB	0.25	64	64	>256	32	32	256	256	-	-	-	-	-	-	-	-	-
1231	non-AB	0.5	64	128	>256	16	32	>256	32	-	-	-	+	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1232	AB	0.25	128	128	32	64	32	256	64	+	-	+	-	-	-	-	-	-
1233	AB	0.5	128	128	>256	32	32	256	64	+	-	+	-	-	-	-	-	-
1234	AB	0.25	64	64	>256	16	32	256	32	+	-	+	-	-	-	-	-	-
1235	non-AB	0.25	256	256	>256	128	32	256	128	-	-	-	-	-	-	-	-	-
1236	AB	0.125	32	32	32	16	32	128	16	+	-	+	-	-	-	-	-	-
1237	AB	0.25	128	128	>256	64	32	256	64	+	-	+	-	-	-	-	-	-
1238	AB	0.125	32	32	>256	8	32	128	16	+	-	+	-	-	-	-	-	-
1239	AB	0.25	32	32	>256	32	32	64	16	+	-	+	-	-	-	-	-	-
1240	AB	0.125	64	64	>256	64	32	256	16	+	-	+	-	-	-	-	-	-
1241	non-AB	1	256	256	>256	128	32	256	256	-	-	-	-	-	-	-	-	-
1242	AB	0.125	64	64	8	64	32	128	32	+	-	+	-	-	-	-	-	-
1243	AB	0.25	128	128	16	16	32	256	64	+	-	+	-	-	-	-	-	-
1244	AB	0.5	64	64	>256	16	32	256	32	+	-	+	-	-	-	-	-	-
1245	AB	0.25	64	64	>256	16	32	256	128	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1246	AB	0.25	128	128	>256	32	32	256	32	+	-	+	-	-	-	-	-	-
1247	AB	0.125	128	64	>256	32	32	128	32	+	-	+	-	-	-	-	-	-
1248	AB	0.25	64	64	>256	16	64	256	16	+	-	+	-	-	-	-	-	-
1249	AB	16	128	256	128	64	64	256	32	+	-	+	-	-	-	-	-	-
1250	AB	32	128	128	>256	128	64	>256	32	+	-	+	-	-	-	-	-	-
1251	AB	16	>256	256	>256	128	64	128	32	+	-	+	-	-	-	-	-	-
1252	AB	0.25	64	64	>256	16	64	256	16	+	-	+	-	-	-	-	-	-
1253	AB	0.125	64	64	64	16	64	128	64	+	-	+	-	-	-	-	-	-
1254	AB	0.25	128	128	>256	16	64	256	32	+	-	+	-	-	-	-	-	-
1255	AB	0.25	64	64	>256	32	64	256	16	+	-	+	-	-	-	-	-	-
1256	AB	0.125	64	64	>256	16	64	256	16	+	-	+	-	-	-	-	-	-
1257	AB	0.25	128	128	>256	64	128	256	128	+	-	+	-	-	-	-	-	-
1258	AB	0.25	32	32	>256	16	128	256	64	+	-	+	-	-	-	-	-	-
1259	AB	0.125	64	64	>256	16	128	128	32	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1340	AB	2	128	128	>256	128	16	256	32	+	-	+	-	-	-	-	-	-
1341	AB	8	>256	>256	>256	256	16	256	64	+	-	+	-	-	-	-	-	-
1343	AB	0.5	64	64	>256	128	16	256	32	+	-	+	-	-	-	-	-	-
1344	AB	16	128	128	>256	64	16	128	32	+	-	+	-	-	-	-	-	-
1345	AB	0.5	128	128	16	128	16	>256	16	+	-	+	-	-	-	-	-	-
1346	AB	0.25	64	64	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
1347	AB	0.25	64	32	>256	128	16	256	16	+	-	+	-	-	-	-	-	-
1348	AB	0.5	64	64	8	64	16	128	16	+	-	+	-	-	-	-	-	-
1349	AB	0.25	32	32	8	64	16	256	32	+	-	+	-	-	-	-	-	-
1350	AB	0.5	64	64	>256	>256	16	256	16	+	-	+	-	-	-	-	-	-
1351	AB	0.25	64	64	>256	128	16	256	64	-	-	-	-	-	-	-	-	-
1352	AB	0.25	128	64	>256	32	16	256	16	+	-	+	-	-	-	-	-	-
1353	AB	8	128	128	>256	32	16	256	32	+	-	+	-	-	-	-	-	-
1354	AB	0.5	128	64	64	64	16	256	32	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1355	AB	0.25	128	128	128	128	16	64	64	+	-	+	-	-	-	-	-	-
1356	GS3	0.25	256	256	>256	1	16	128	64	-	-	-	-	-	-	-	-	-
1357	AB	0.5	64	64	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
1358	AB	0.25	128	128	>256	64	16	256	16	-	-	-	-	-	-	-	-	-
1359	AB	0.5	128	128	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
1360	AB	0.5	64	128	>256	32	16	256	32	+	-	+	-	-	-	-	-	-
1361	AB	0.125	64	64	2	64	16	256	8	+	-	+	+	-	-	-	-	-
1362	AB	0.25	64	64	>256	64	16	256	16	+	-	+	-	-	-	-	-	-
1363	AB	0.125	32	32	2	64	16	256	32	+	-	+	-	-	-	-	-	-
1364	AB	16	128	128	>256	128	16	256	32	+	-	+	-	-	-	-	-	-
1365	AB	1	64	32	>256	128	16	256	64	+	-	+	-	-	-	-	-	-
1366	AB	0.25	64	64	>256	32	16	256	32	+	-	+	-	-	-	-	-	-
1367	AB	0.125	64	64	16	128	16	256	32	+	-	+	-	-	-	-	-	-
1368	AB	0.25	128	128	>256	128	16	128	32	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1369	non-AB	0.5	64	128	>256	64	16	128	64	-	-	-	-	+	-	-	-	-
1370	AB	0.25	64	64	>256	32	16	256	32	+	-	+	-	-	-	-	-	-
1373	AB	0.25	64	64	>256	32	16	256	16	+	-	+	-	-	-	-	-	-
1374	AB	16	128	128	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
1375	AB	0.25	128	128	64	128	16	256	32	+	-	+	-	-	-	-	-	-
1376	AB	0.5	64	64	>256	64	16	>256	16	+	-	+	-	-	-	-	-	-
1377	AB	0.25	64	64	>256	32	16	256	32	+	-	+	-	-	-	-	-	-
1378	AB	0.125	64	64	>256	64	16	256	32	+	-	+	-	-	-	-	-	-
1379	AB	0.5	32	32	>256	16	4	64	16	+	-	+	-	-	-	-	-	-
1500	AB	0.125	64	64	16	32	16	256	32	+	-	+	-	-	-	-	-	-
1501	AB	0.125	64	64	16	64	16	256	32	+	-	+	-	-	-	-	-	-
1502	AB	0.25	128	128	>256	64	32	>256	16	+	-	+	-	-	-	-	-	-
1503	AB	0.25	64	64	>256	16	32	>256	32	+	-	+	-	-	-	-	-	-
1504	AB	0.125	128	64	>256	32	32	256	16	+	-	+	+	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1505	AB	16	256	256	>256	32	32	256	32	+	-	+	-	-	-	-	-	-
1506	AB	0.25	128	128	>256	64	32	256	16	-	-	+	-	-	-	-	-	-
1507	AB	0.25	64	64	>256	64	32	128	16	+	-	+	-	-	-	-	-	-
1508	AB	1	128	128	>256	64	32	256	32	+	-	+	-	-	-	-	-	-
1509	AB	0.125	64	64	>256	32	32	256	16	+	-	+	-	-	-	-	-	-
1510	non-AB	64	8	16	>256	8	32	256	128	-	-	-	-	-	-	-	-	-
1511	AB	8	256	128	>256	128	32	>256	16	+	-	+	-	-	-	-	-	-
1512	AB	4	128	128	128	128	32	128	32	+	-	+	-	-	-	-	-	-
1513	AB	0.25	128	64	>256	64	32	256	16	+	-	+	-	-	-	-	-	-
1514	AB	0.125	64	128	4	32	32	256	64	+	-	+	-	-	-	-	-	-
1515	AB	0.125	128	128	>256	128	32	256	128	+	-	+	-	-	-	-	-	-
1516	AB	8	256	128	>256	64	32	>256	32	+	-	+	-	-	-	-	-	-
1517	AB	0.125	64	64	>256	64	32	256	32	+	-	+	-	-	-	-	-	-
1518	AB	0.125	64	64	2	64	32	256	16	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1519	AB	0.25	64	64	>256	32	32	256	8	+	-	+	-	-	-	-	-	-
1520	AB	0.5	128	128	>256	64	32	256	32	+	-	+	-	-	-	-	-	-
1521	AB	8	128	256	>256	128	32	256	32	+	-	+	-	-	-	-	-	-
1522	AB	0.25	32	32	8	32	32	256	16	+	-	+	-	-	-	-	-	-
1523	AB	0.25	64	64	>256	64	32	>256	32	+	-	+	-	-	-	-	-	-
1524	non-AB	0.125	128	128	>256	64	32	256	16	-	-	-	-	-	-	-	+	-
1526	AB	0.5	64	64	64	64	32	256	32	+	-	+	-	-	-	-	-	-
1527	AB	0.25	64	64	>256	64	32	256	64	+	-	+	-	-	-	-	-	-
1528	AB	0.25	128	128	>256	64	32	256	64	+	-	+	-	-	-	-	-	-
1529	AB	8	128	128	>256	32	32	256	16	+	-	+	-	-	-	-	-	-
1530	AB	0.25	64	128	>256	64	32	256	32	+	-	+	-	-	-	-	-	-
1531	GS3	0.125	32	32	64	16	32	128	>256	-	-	-	+	+	-	-	-	-
1532	AB	0.25	64	64	>256	32	32	256	32	+	-	+	-	-	-	-	-	-
1533	AB	8	64	64	>256	64	32	>256	32	+	-	+	-	-	-	-	-	-

isolate	species[a]	MIC (mg/L)								Carbapenemase -encoding genes								
		COL	MEM	IMP	AMK	CIP	LVX	FOS	SUL	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-58-like}$	*bla*$_{IMP-like}$	*bla*$_{VIM-like}$	*bla*$_{NDM-like}$	*bla*$_{OXA-48-like}$	*bla*$_{KPC-like}$
1534	AB	0.25	64	128	>256	64	32	256	64	+	-	+	-	-	-	-	-	-
1535	AB	0.125	256	256	>256	128	32	256	64	+	-	+	-	-	-	-	-	-
1536	AB	4	128	128	>256	64	32	>256	32	+	-	+	-	-	-	-	-	-
1537	AB	0.125	64	64	>256	32	32	256	16	+	-	+	-	-	-	-	-	-
1538	AB	0.25	64	64	>256	32	32	256	64	+	-	+	-	-	-	-	-	-
1539	AB	8	128	128	32	64	32	256	32	+	-	+	-	-	-	-	-	-
1089E	AB	4	64	>256	>256	128	8	256	32	+	-	+	-	-	-	-	-	-
2048/2	AB	8	128	256	>256	64	128	128	16	+	-	+	-	-	-	-	-	-
A5	AB	8	128	128	>256	32	>256	256	8	+	-	+	-	-	-	-	-	-
AJN3B	AB	16	>256	>256	>256	64	32	>256	2	+	-	+	-	-	-	-	-	-

[a]GS3: *A. pittii*, GS13TU: *A. nosocomialis*: non-AB: non-*Abc* complex

Table 17. Result of checkerboard synergy test of three antibiotic combinations against 30 colistin-resistant *A. baumannii* clinical isolates.

Isolate	MIC (mg/L)			FICI[a]		
	COL	SUL	FOS	COL + SUL	COL + FOS	SUL + FOS
1529	4	16	256	0.5	0.31	0.38
1341	4	64	256	0.5	0.25	0.31
1511	4	16	512	0.5	1	0.38
216	4	32	512	0.5	0.75	0.75
1516	4	32	512	0.38	0.75	0.38
1536	4	32	512	0.38	1	0.5
176	4	128	512	0.5	0.75	0.75
1512	8	32	128	0.38	0.75	0.38
1049	8	16	256	0.5	0.5	0.63
AJN3B	8	16	256	0.53	0.75	0.75
1129	8	32	256	0.38	0.5	0.25
1353	8	32	256	0.25	1.5	0.38
1364	8	32	256	0.38	0.75	0.5
1505	8	32	256	0.25	0.75	0.25
1521	8	32	256	0.5	0.5	0.38
1539	8	32	256	0.38	0.75	0.25
2048	8	16	512	0.75	0.5	0.5
213	8	64	512	0.19	0.5	1
1251	16	32	128	0.25	0.38	0.5
1344	16	32	128	0.38	0.63	0.38
A5	16	2	256	2	2	0.75
1249	16	32	256	0.25	0.63	0.38
1374	16	32	256	0.25	0.5	0.38
1098	16	32	512	0.38	0.75	0.38
1127	16	32	512	0.38	0.63	0.5
1533	16	32	512	0.38	0.75	0.5
1060	16	64	512	0.19	0.75	0.75
1126	32	16	512	0.19	0.5	0.75
1250	32	32	1024	0.38	0.63	0.38
1139	64	16	256	0.63	0.75	0.75

[a]Interpretation of FICI: FICI $\leq$ 0.5: synergy, 0.5 < FICI < 1: partial synergy, FICI $\geq$ 1: indifference.

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