EFFICACY OF FOSFOMYCIN ALONE AND IN COMBINATION WITH CARBAPENEMS AGAINST CARBAPENEMS-RESISTANT *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

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ABSTRACT

Pseudomonas aeruginosa has been found to be highly infectious in nosocomial infected patients, particularly in immunocompromised patients. The patients infected with this bacterium are mostly difficult to treat due to the resistance development of the bacteria. Carbapenems are β-lactam agents with a high potency for bacterial infection. The efficacy of carbapenems is effective against Gram-positive and Gram-negative bacteria. Currently, the increasing occurrences of carbapenems resistance phenomena have been reported in many countries. The aims of this study were to determine the efficacy of fosfomycin alone and in combination with carbapenems against seventy carbapenems-resistant *P. aeruginosa* isolates. The best characteristics of fosfomycin are less toxicity, the ability to inhibit a broad spectrum of bacteria and the capability to enhance the synergy. Therefore, fosfomycin is an interesting antibiotic to study the efficacy of the combination antibiotic effect with carbapenems.

In this study, seventy carbapenems-resistant *P. aeruginosa* clinical isolates were firstly examined for their on susceptibility to fosfomycin, meropenem, imipenem, and doripenem using the broth microdilution method. The results of this experiment showed that the MIC ranges of fosfomycin, meropenem, imipenem, and doripenem were 8 - >1024, 8 – 256, 2 – 256, and 1 - >256 µg/ml, respectively. It was found that MIC_{90} of fosfomycin were more than 1024 µg/ml and MIC_{90} of all carbapenems were 64 µg/ml. In synergy testing, the combination between fosfomycin and doripenem indicated the maximum percentage of synergistic at about 45.71%, followed by the combination of fosfomycin with meropenem and fosfomycin with imipenem at about 40 and 38.57%, respectively. However, the statistical difference was not significant between three groups of combination antibiotics with a 95% confidence interval. The determination of the time-killing rate at various concentrations demonstrated the concentrations of 0.25xMIC and 0.5xMIC at 8 hours in fosfomycin combined with meropenem and imipenem were synergistic. Fosfomycin combined with doripenem showed a synergistic effect at 0.25xMIC after 8 hours and at 0.5xMIC after 4 hours. The bacterial killing rates of these combinations appeared in a concentration-dependent manner. The strains with positive synergy results possessed carbapenems resistance mechanisms by the overexpression of efflux pumps (MexAB and MexXY) and the loss of OprD protien. The time-kill assay indicated that fosfomycin combined with carbapenems showed synergism at 0.25xMIC within 8 hours. Mutation frequency analysis did show no difference between given alone and in combination with antimicrobials. Clear disruption of the bacterial cell wall was observed by SEM at 0.25xMIC after being treated with antimicrobials for 8 hours. Remarkably, combinations between carbapenems and fosfomycin showed anti-biofilm activity. All of these results supported the combination treatment between fosfomycin and carbapenems had promising effects against carbapenems-resistant *P. aeruginosa* strains. Especially, fosfomycin, combined with doripenem, was an interesting alternative option for carbapenems-resistant *P. aeruginosa* infections.

KEY WORDS: CARBAPENEMS-RESISTANT *PSEUDOMONAS AERUGINOSA* / COMBINATION THERAPY / FOSFOMYCIN

92 pages

ประสิทธิภาพของยาฟอสโฟมัยซินเดี่ยวและเมื่อใช้ร่วมกับยากลุ่มคาร์บาพีเนมส์ต่อซูโดโมแนส แอรูจิโนซา สายพันธุ์ดื้อต่อยากลุ่มคาร์บาพีเนมส์ที่แยกจากผู้ป่วย

EFFICACY OF FOSFOMYCIN ALONE AND IN COMBINATION WITH CARBAPENEMS AGAINST CARBAPENEMS-RESISTANT *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

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บทคัดย่อ

ซูโดโมแนส แอรูจิโนซา ก่อให้เกิดการติดเชื้อในผู้ป่วยที่พักรักษาตัวในโรงพยาบาลค่อนข้างมาก โดยเฉพาะอย่างยิ่งผู้ป่วยที่มีระบบภูมิคุ้มกันบกพร่อง ผู้ป่วยที่ติดเชื้อจากแบคทีเรียชนิดนี้มักจะพบปัญหาในการรักษาเนื่องจากการพัฒนาการดื้อยา ยาคาร์บาพีเนมส์จัดเป็นยาปฏิชีวนะเบต้า-แลคแตมที่มีประสิทธิภาพในการรักษาผู้ป่วยติดเชื้อแบคทีเรีย การออกฤทธิ์นั้นออกฤทธิ์ได้ดีต่อทั้งแบคทีเรียแกรมบวกและแกรมลบ แต่ปัจจุบันพบอุบัติการณ์การดื้อต่อยาคาร์บาพีเนมส์เพิ่มมากขึ้นเรื่อยๆในหลายประเทศ วัตถุประสงค์ของการศึกษาในครั้งนี้เพื่อศึกษาหาประสิทธิภาพของยาฟอสโฟมัยซินเดี่ยว และประสิทธิภาพการใช้ยาร่วมกับยากลุ่มคาร์บาพีเนมส์ต่อเชื้อซูโดโมแนส แอรูจิโนซาที่ดื้อต่อยากลุ่มคาร์บาพีเนมส์ทั้งหมด 70 สายพันธุ์ คุณลักษณะที่โดดเด่นของฟอสโฟมัยซินได้แก่ ความเป็นพิษต่อเซลล์ในระดับต่ำมีประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อได้กว้าง และความมีประสิทธิภาพในการเสริมฤทธิ์ของยาฟอสโฟมัยซิน ด้วยเหตุนี้จึงส่งผลให้ยาฟอสโฟมัยซินมีความน่าสนใจในการนำมาศึกษาปฏิกิริยาการใช้ยาร่วมกับยากลุ่มคาร์บาพีเนมส์

ในการศึกษานี้ ตัวอย่างเชื้อซูโดโมแนส แอรูจิโนซาดื้อต่อยากลุ่มคาร์บาพีเนมส์ทั้งหมด 70 สายพันธุ์ ได้ถูกนำมาทดสอบหาค่าความไวต่อยาฟอสโฟมัยซิน, เมอโรพีเนมส์, อิมิพีเนมส์, และโดริพีเนมส์โดยวิธีการทำการเจือจางในอาหารเหลวระดับไมโคร ซึ่งจากผลการทดลองที่ได้พบว่าช่วงค่าความเข้มข้นของยาในระดับต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อสำหรับยาฟอสโฟมัยซิน, เมอร์โรพีเนมส์, อิมิพีเนมส์, และโดริพีเนมส์มีค่าเท่ากับ 8 - >1024, 8 – 256, 2 – 256, และ 1 - >256 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ โดยพบว่าค่าความเข้มข้นของยาในระดับต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อได้ร้อยละ 90 ของยาฟอสโฟมัยซินอยู่ที่มากกว่า 1024 ไมโครกรัมต่อมิลลิลิตร และสำหรับยาในกลุ่มคาร์บาร์พีเนมส์นั้นมีค่าความเข้มข้นของยาในระดับต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อได้ร้อยละ 90อยู่ที่ 64 ไมโครกรัมต่อมิลลิลิตร ในการทดสอบการใช้ยาปฏิชีวนะร่วมเพื่อหาประสิทธิภาพในการเสริมฤทธิ์กัน พบว่าการทดสอบยาร่วมฟอสโฟมัยซินร่วมกับโดริพีเนมส์มีค่าการเสริมฤทธิ์สูงสุดเท่ากับร้อยละ 45.71 ตามด้วยการใช้ร่วมกับเมอร์โรพีเนมส์และอิมิพีเนมส์ คือร้อยละ 40 และ 38.57 ตามลำดับ อย่างไรก็ตามเมื่อนำค่าการเสริมฤทธิ์ที่ได้มาหาค่าความแตกต่างทางสถิติที่ระดับความเชื่อมั่นร้อยละ 95 พบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญ และเมื่อนำหาค่าอัตราเวลาการเกิดการฆ่าเชื้อแบคทีเรียเพื่อหาเวลาที่สามารฆ่าเชื้อที่ความเข้มข้นต่างๆ พบว่าที่ความเข้มข้น 0.25 และ 0.5 เท่าของค่าความเข้มข้นของยาในระดับต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อ ที่ 8 ชั่วโมง ในการใช้ยาร่วมฟอสโฟมัยซินกับทั้งเมอโรพีเนมส์ และอิมิพีเนมส์ สามารถเกิดการเสริมฤทธิ์กันในการฆ่าเชื้อ ยาร่วมฟอสโฟมัยซินกับโดริพีเนมส์พบว่าสามารถเกิดการเสริมฤทธิ์กันในการฆ่าเชื้อได้ที่ความเข้มข้น 0.25 เท่าของค่าความเข้มข้นของยาในระดับต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อในการฆ่าเชื้อหลังจากสัมผัสกับยา 8 ชั่วโมง และ 0.5 เท่า หลังจากสัมผัสกับยา 4 ชั่วโมง โดยความสามารถในการฆ่าเชื้อของยาร่วมนี้จะขึ้นอยู่กับความเข้มข้นที่นำไปใช้ สายพันธุ์ที่เกิดการประสิทธิภาพการเสริมฤทธิ์กันมีกลไกการดื้อต่อยาคาร์บาพีเนมส์ ซึ่งได้แก่ ปั๊มขับยาปฏิชีวนะที่แสดงออกมากเกิน (เมกซ์เอบี และ เมกซ์เอกซ์วาย) การสูญเสีย โอพีอาร์ดี การหาระยะเวลาที่ยายังคงประสิทธิภาพในการยับยั้งการเจริญเติมโตของเชื้อพบว่าฟอสโฟมัยซินร่วมกับคาร์บาพีเนมส์แสดงการเสริมฤทธิ์กันที่ค่าความเข้มข้น 0.25 เท่าของค่าความเข้มข้นของยาในระดับต่ำสุดที่สามารถยับยั้งการเจริญเติบโตของเชื้อที่ 8 ชั่วโมง การทดสอบหาค่าความถี่ในการเกิดการกลายพันธุ์ของทั้งยาเดี่ยวและยาร่วมค่าที่ได้นั้นไม่แตกต่างกัน ประสิทธิภาพการทำลายผนังเซลล์ถูกวัดด้วยกล้องจุลทรรศน์อิเล็คตรอนแบบส่องกราด ที่ความเข้มข้น 0.25 เท่าของค่าความเข้มข้นของยาในระดับต่ำสุดสามารถยับยั้งการเจริญเติบโตของเชื้อหลังจากสัมผัสกับยาเป็นเวลา 8 ชั่วโมง ยาร่วมระหว่างคาร์บาพีเนมส์และฟอสโฟมัยสินแสดงให้เห็นถึงการทำลายทำลายไบโอฟิล์มได้อย่างสิ้นเชิง จากผลการทดลองทั้งหมดได้สนับสนุนการใช้ยาร่วมระหว่างยาฟอสโฟมัยซินกับยากลุ่มคาร์บาพีเนมส์ถึงการมีประสิทธิภาพในการต่อต้านเชื้อซูโดโมแนส แอรูจิโนซาสายพันธุ์ที่ดื้อต่อยากลุ่มคาร์บาพีเนมส์ โดยเฉพาะอย่างยิ่งยาร่วมฟอสโฟมัยซินกับโดริพีเนมส์นั้นมีความน่าสนใจในการเป็นทางเลือกต่อการติดเชื้อซูโดโมแนส แอรูจิโนซาที่ดื้อต่อยาคาร์บาพีเนมส์ได้

92 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF ABBREVIATION

P. aeruginosa	*Pseudomonas aeruginosa*
PAO1	*Pseudomonas aeruginosa* PAO1
CR-PA	Carbapenbems resistant *Pseudomonas aeruginosa*
MIC	Minimal inhibitory concentration
MIC_{50}	Minimum Inhibitory Concentration required to inhibit the growth of 50% of organisms
MIC_{90}	Minimum Inhibitory Concentration required to inhibit the growth of 90% of organisms
MCBT	Multiple combination bactericidal testing
FICI	Fractional inhibitory concentration index
CFU	Colony forming unit
NO3	Nitrate
CR	Carbapenems resistance
MDR	Multidrug resistance
DHP-1	Dehydropeptidase-1
PBPs	Penicillin-binding protein
MRSA	Methicillin-resistant *Staphylococcus aureus*
OMPs	Outer membrane proteins
CAP	Complicated community-acquired pneumonia
CSSSI	Complicated skin structure infections
CIAI	Complicated intra-abdominal infections
MBL	Mettalobeta-lactamase
GlpT	Glycerol-3 phosphate transporter
UhpT	Hexose phosphate transporter
CLSI guideline	Clinical & Laboratory Standards Institute guideline
TNTC	Too numerous to count
ND	Not detected

LIST OF ABBREVIATION (cont.)

PAE	Post antibiotic effect
SEM	Scanning electron microscopy

CHAPTER I
INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, aerobic bacterium in the family Pseudomonadaceae. This microorganism is an extensive environmental bacterium which is classified as an opportunistic pathogen in both community and hospitals. It expands resistance mechanisms to antimicrobial compounds by the metabolic diversity, transport efficacy, adaptive ecology and degradative antibiotics (1). Generally, infections with this microorganism including ulcerative keratitis especially with contact lens use, otitis media, skin and soft tissue infection, especially lung infection in pneumonia patients have associated with high mobility and mortality rate (2). The percentages of epidemiology with *P. aeruginosa* infection are about 11-13.8% of all nosocomial infections, 14-21% of bacteremia episodes in acute leukemia, 97.5% of cystic fibrosis patients by the age of 3 years (3). Initial empirical antibacterial options for treatment of *P. aeruginosa* infection are piperacillin/tazobactam, cefepime, imipenem/cilastatin, meropenem, aztreonam, fosfomycin, or plus one of the following, gentamicin, tobramycin, amikacin, levofloxacin, ciprofloxacin. Additionally, the other antimicrobial agents are colistin and doripenem (2). The combination therapy is expected for *P. aeruginosa* treatment to prevent cross-resistance and enhances efficacy of antibiotics (4).

The main antimicrobial agent used in this study, fosfomycin (1,2-epoxypropylphosphonic acid), a phosphonic acid antibiotic produced from *Streptomyces fradiae* and other species of Streptomyces, has been reported on low toxicity in humans and less side effect directed to gastrointestinal tract such as nausea, vomiting, or diarrhea. It has been successfully used to treat various bacteria including *P. aeruginosa* by inhibiting the first step in cell wall biosynthesis (5, 6). There have been reports on synergistic effects of fosfomycin in combination with other antimicrobials such as aminoglycosides, colistin, netilmicin, tigecycline and carbapenems against many microorganisms (7-9). However, some researchers have reported *in vitro* fosfomycin resistance in *P. aeruginosa.* (10). Carbapenems are potent

antimicrobial agents against *P. aeruginosa* which act as β-lactam antibiotics by inhibiting cell wall synthesis (11, 12). Recently, the incidence of carbapenems-resistant *P. aeruginosa* in hospitals is highly increased (13). Recommendation criteria for treating patients with lethal infection or drug-resistant is combination therapy (14, 15). Therefore, we hypothesized that fosfomycin may be used in combination with carbapenems in order to provide better efficacy for treating *P. aeruginosa* infection. The methods for synergy study include checkerboard dilution assays, time-kill curve methods, multiple combination bactericidal testing (MCBT), and Epsilometer test (E-test) (16). The microdilution checkerboard method is the assay that has been used to pair two agents usually from different antibiotic classes to estimate potential synergy, The fractional inhibitory concentration (FIC) at which the synergy is defined as 0.25 times of the minimal inhibition concentration decreasing from the MIC of each drug when compared to the MIC in combination (15, 16). Time-kill assay can be used to measure bactericidal activity and studies the dynamics of synergism or antagonism. By this method, the synergism is defined as ≥2logCFU/ml decreased with the combination when compared to the most active agent alone (15-18). This study will focus on efficacy of fosfomycin alone and in combination with carbapenems against carbapenems-resistant *P. aeruginosa* clinical isolates. It is expected that synergism of these drugs can reduce concentrations of drugs used and prevent cross-resistance to the antibiotic of microorganisms.

Objectives

1. To evaluate MIC values of fosfomycin, imipenem, meropenem and doripenem against carbapenems-resistant *P. aeruginosa* clinical isolates.
2. To investigate the synergistic effect of combinations of fosfomycin and carbapenems on carbapenems-resistant *P. aeruginosa* clinical isolates.
3. To investigate the post antibiotic effect of fosfomycin, imipenem, meropenem, and doripenem by individual antimicrobials and combined antimicrobials.

CHAPTER II
LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*

P. aeruginosa is Gram-negative bacteria in Pseudomonadaceae family. It is ubiquitous in moist environment as soil, marsh, sink, drain including plants and animals tissue. It is an opportunistic pathogen because of it rarely infects healthy individuals. In fact, it is a problem in healthcare settings and a major cause of nosocomial infection about 11-13.8% (13-15). Several diseases caused by *P. aeruginosa* are bacteremia in burn patients, urinary tract infection, lung infection in cystic fibrosis, surgical site infection, and pneumonia infection with high mortality rate. In addition, it also has the problem in treatment option because of the intrinsic resistance to several antibiotics (15, 19).

2.1.1 Characteristics

P. aeruginosa is commonly found in moist environment. Aerobic condition is required for the growth of this bacterium; however it can grow on without O_2 condition if there have enough the amount of NO_3 like respiratory electron acceptor. The adaptive nutrition and minimum nutrition requirement is advantageous living for bacteria (1, 20, 21). The optimal temperature for bacterial growth is 37°C and able to grow at high temperature to 42°C (20, 22). The colonies of *P. aeruginosa* are able to produce soluble pigments are fluorescent pigment pyoverdin and blue pigment pyocyanin (20, 23).

2.1.2 Pathogenesis and virulence factors

2.1.2.1 Pathogenesis of *P. aeruginosa*

According to the characteristic of *P. aeruginosa*, it prefers to infect in moist areas such as the perineum, axilla, ear, nasal mucosa and throat. It scarcely colonizes healthy person but has higher rate with hospitalization (24). It is the

common cause of respiratory tract infection and also chronic infection in cystic fibrosis patients because of the adaptive ability to antibiotic resistance. The study of microarray showed a variety of epithelial cell genes that activated responsible to the attachment of *P. aeruginosa*. The pathogenesis of *P. aeruginosa* is quite diversity from many factors and the complexity of genetics. Also, it can survive in many different environments (25).

2.1.2.2 The virulence factors

The virulence factors of its pathogenesis are composed of many factors with complication. The surface interaction of *P. aeruginosa* with pili (type V) is importance for adherence for cell membrane and other surfaces. The expression of extracellular polysaccharide alginate can occur in *P. aeruginosa*. Alginate secretions produce mucoid on culture with *mucA* gene mutant expression (13, 26). This alginate is a barrier for immune system response and important for biofilm formation (13). The four enzymes of type III secretion system are ExoS, ExoT, ExoU and ExoY. The results of these activations are inactive immune system, leading to cell injury and cell death. *P. aeruginosa* with these enzyme systems is known to cause high mortality rate (13, 21). Moreover, many secreted virulence factors contribute the pathogenesis such as exotoxin A, alkaline protease, elastases, and protease IV (13). Lastly, antibiotic resistance mechanisms are major concern on *P. aeruginosa*. All virulence factors of *P. aeruginosa* are demonstrated in Figure 2.1. It shows the important virulence factors that are the aggregation of bacterial biofilms and also the surface factors of *P. aeruginosa* planktonic cells. These surface factors are flagellum, pilus, type III secretion proteins, secreted factors including efflux pumps. The diseases caused by *P. aeruginosa* are showed in Table 2.1.

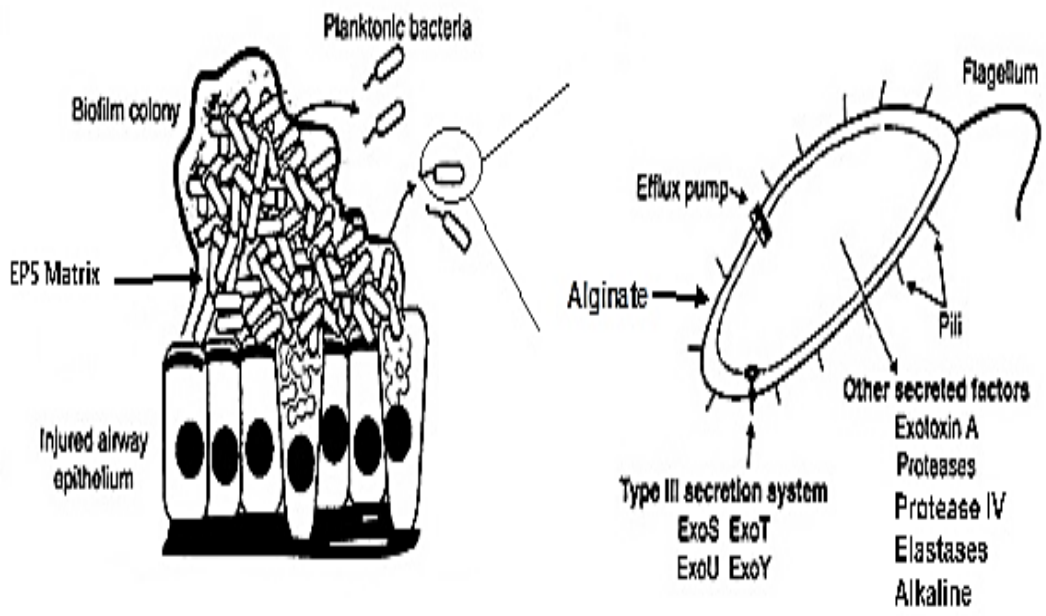


Figure 2.1 Virulence factors of *P. aeruginosa* (13)

Table 2.1 Diseases caused by *P. aeruginosa* (adapted from (27))

Infectious organ	Diseases
Respiratory tract	Acute pneumonia, Chronic lower respiratory tract infection
Urinary tract	Acute infections, Chronic infections
Gastrointestinal tract	Necrotising enterocolitis, Peri-rectal infections
Central nervous system	Meningitis, Brain abscess
Blood	Bacteremia, Septicemia
Eye	Keratitis (corneal ulcer)
Ear	Otitis externa (swimmer's ear), Malignant external otitis
Skin and soft tissue infections	Dermatitis, Wound infections, Burn wound sepsis, Ecthyma gangrenosa, Pyoderma, Folliculitis, Unmanageable forms of acnes vulgaris
Heart	Endocarditis
Bone and joint infections	Stenoarticular pyoarthrosis, Vertebral osteomyelitis, Symphysis pubis infection, Osteochondritis of the foot, Chronic contiguous osteomyetitis

2.1.3 Biofilms

P. aeruginosa biofilm is difficult to treat with antibiotics as *in vitro* susceptibility studies previously showed the resistance of biofilm cells to killing (28, 29). Biofilms are the community of bacterial cells that are incorrigible attachment. It is embedded in a matrix of extracellular polymeric substances as they have produced (29). The biofilm structure is showed in Figure 2.2.

2.1.3.1 The formation of biofilms

The suitable condition for biofilms growth is found in high shear environment such as rapidly flowing surroundings. The planktonic bacterial cells are able to attach to the surface of cells and initiate the growth of biofilms formation in shear forces (29). The exopolysaccharide matrix is formed during the biofilm formation. This condition makes high viscoelastic structure as the rubbery feature. Whereas, the bacterial biofilms have low tensile strength and break easily in the

condition of low shear environment. Biofilms formation under high shear condition is significantly strong and resistance to mechanical breakage (30).

2.1.3.2 The relationship between mechanism of biofilms formation and diseases

Bacterial biofilms of *P. aeruginosa* might increase the violence of disease, especially in cystic fibrosis patients and urinary tract infection patients. Moreover, the biofilms are also critical in immunocompromised patients who cannot eliminate and battle invading of bacterial biofilms. Nowadays, the mechanism of biofilms created involving to the diseases is unclear but the possible process are suggested by four mechanism (28, 29, 31).

(I) Bacterial biofilm cell aggregation: the detachment of planktonic cell from the biofilms is able to grow and still active. Moreover, it can produce the biofilms and cells aggregation within this layer resulting increasing violence infection (28).

(II) Production of endotoxin: the aggregation of bacteria is generating the bacterial biofilm and also secretes endotoxin. The results of many planktonic cell aggregations are related to the level of endotoxin. However, no data of level and kinetic support that endotoxin is generated from bacterial biofilm (29).

(III) Resistance to immune system: Meluleni *et al*. found the efficacy of antibodies in cystic fibrosis patient. It was no function of the bacterial cells elimination. The immune response cannot eradicate the invader within the biofilms. The hypothesis of Yasuda *et al*. is the bacterial biofilms involving to the increasing of the bacterial resistance to polymorphoneuclear leukocytes resulting more readily to cause an infection (31).

(IV) Generation of bacterial resistance: bacteria are able to exchange plasmid via the conjugation mechanism within bacterial biofilms. The aggregation of planktonic cells under the bacterial biofilms is more enhancement of cell conjugation. Ehlers and Bouwere who demonstrated this criterion showed that the rate of *P. aeruginosa* conjugation was significantly higher in bacterial biofilms than planktonic condition (15), (32).

2.1.3.3 Antibiotic resistance

The nature structure of biofilms is attributed to antibiotic resistance. The conventional mechanisms of responding to antibiotic resistance are one or more of following: i) reducing the concentration of penetrated antibiotics through the biofilm matrix, ii) altering the sensitivity structure of its target action and iii) physiological development in other environmental stresses (29). Moreover, *P. aeruginosa* biofilms is more tolerance to antibiotics and components of host immune systems. It can adaptive in the differential physiological and induce the specific tolerance mechanism. The data of planktonic and bacterial biofilms sensitivity to the antibiotics was showed in Table 2.2. *P. aeruginosa* ATCC 27853 showed higher antibiotic concentration (>1,024 µg/ml) to against bacterial biofilms than planktonic cells (1 µg/ml).

Table 2.2 The data of planktonic and bacterial biofilm sensitivity to the antibiotic (adapted from (29))

Organism	antibiotic	MIC or MBC of planktonic sensitivity (µg/ml.)	Concentration effective against biofilm (µg/ml.)
Gram-negative bacteria			
E. coli ATCC 25922	Ampicillin	2(MIC)	512[b]
P. aeruginosa ATCC 27853	Imipenem	1(MIC)	>1,024[b]
P. pseudomallei	Ceftazidime	8(MBC)	800[c]
Gram-positive bacteria			
S. aureus NCTC 8325-4	Vancomycin	2(MBC)	20[a]
Steptococcus sanguis 804	Doxycycline	0.063(MIC)	3,015[d]

[a] Concentration required for 99%.
[b] Minimal biofilm eradication concentration.
[C] Concentration required for ~99% reduction.
[d] Concentration required for >99.9% reduction.

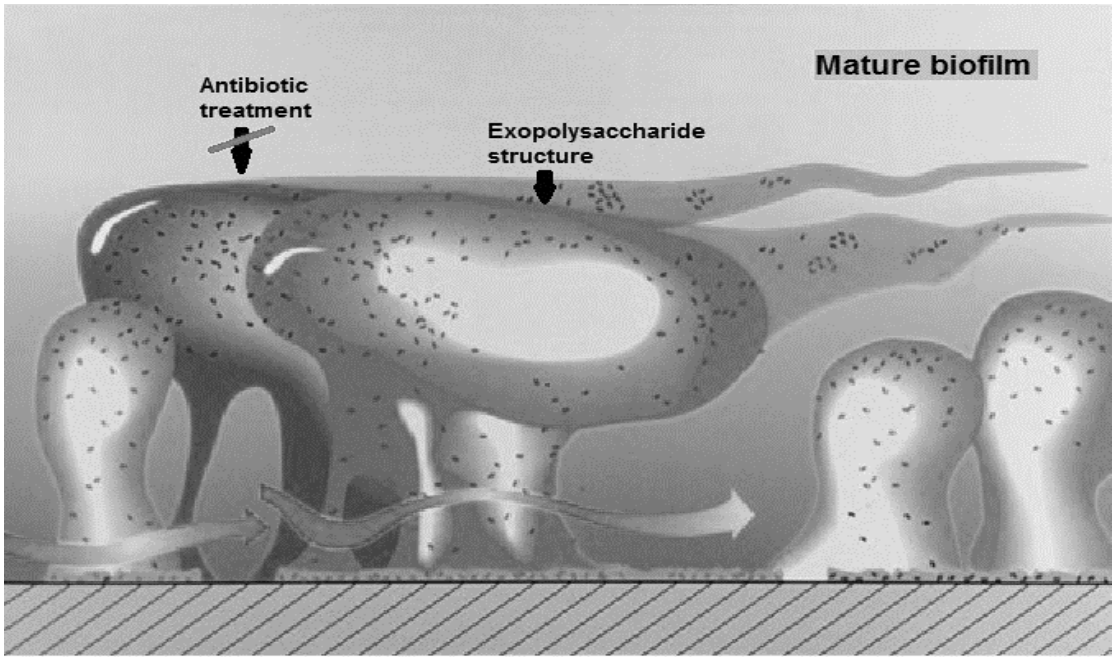


Figure 2.2 Biofilms structure (29)

The bacterial biofilms have many factors that affect to the antibiotic sensitivity such as the limitation of antibiotic diffusion, the antibiotic neutralization by enzyme, the functional heterogeneity of biofilm, the slow growth of persistent cells within biofilms. Moreover, the phenotype of biofilms is most effect to antibiotic resistance. The antibiotic is eliminated by cell membrane alteration properties and efflux pumps (33). One important characteristic for antibiotic resistance of biofilms is lowering penetration antibiotic agents. The antibiotic is diffused through the biofilm matrix for target disruption of the cells. The structure of exopolysaccharides functions as the barrier to protect bacteria from antibiotic diffusion is showed in Figure 2.2. Therefore, the penetration of antibiotic into the bacterial cells hardly occurs and must be at high concentration for killing effect in deep layer (34). Moreover, the bacterial biofilms protect the cells from the elimination of immune system and reduces the potency of antibiotic as the study of Lewis *et al*. (35). They were reported that the extracellular matrix of *P. aeruginosa* had anionic charge which could reduce the efficacy of fluoroquinilone and aminoglycoside when diffused through the matrix layer. Another mechanism of bacterial resistance to antibiotic agents is slow growth rate of planktonic cells within the biofilms.

2.1.4 Epidemiology

Nosocomial infection with *P. aeruginosa* is wide spread around the world. The most frequencies of nosocomial infection with this bacterium are respiratory tract infection, urinary tract infection and wound infection (24). In USA, *P. aeruginosa* was ranked in the second among nosocomial infection, the third among nosocomial urinary tract infection and the seventh among nosocomial bacteremia (36). *P. aeruginosa* most commonly cause septicemia in immunodeficiency patients such as in acute leukemia patients about 14-21% (37). *P. aeruginosa* is serious pathogen in cystic fibrosis patients. It has been reported up to 97.5% of children with CF which were infected with this bacterium in the age of 3 years (13). The rates of infection were found increasing in high prevalence rate as epidemiology of global trend (38). The reports of National Nosocomial Infections Surveillance System (NNIS) with *P. aeruginosa* infected surgical sites in ICUs were 9.5% during 1986-2003 (13). The rate of nosocomial infection from the US Centers for Disease Control and Prevention including NNIS was about 17% (39). In Europe, it is the third bacterial isolates from nosocomial infected patients in intensive care unit (ICU) (40). In Thailand, according to National Antimicrobial Resistance Surveillance Center, Thailand (NARST), *P. aeruginosa* was ranked only after *Escherichia coli* and *Klebsiella pneumonia*e detected from nosocomial infected specimens during 2006-2008 (4). Especially, burns patients have been reported to be associated with *P. aeruginosa.* It is important pathogen that can colonize during the first week of hospitalization. (13). Moreover, the frequency of *P. aeruginosa* antibiotic resistance has increasing during decade. The prevalence of carbapenems resistance was concerned after the emerging of antibiotic resistance in *P. aeruginosa*. The rate of imipenem resistance between 1998 to 2004 was showed about 19.1% (41).

2.1.5 Therapeutic options for *P. aeruginosa* infection

P. aeruginosa is intrinsically resistance to many antibiotic agents. Therefore, the essential control of the infection is in concern. The American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) provide the guidelines for *P. aeruginosa* infected patient management (19, 39) as showed in Figure 2.3.

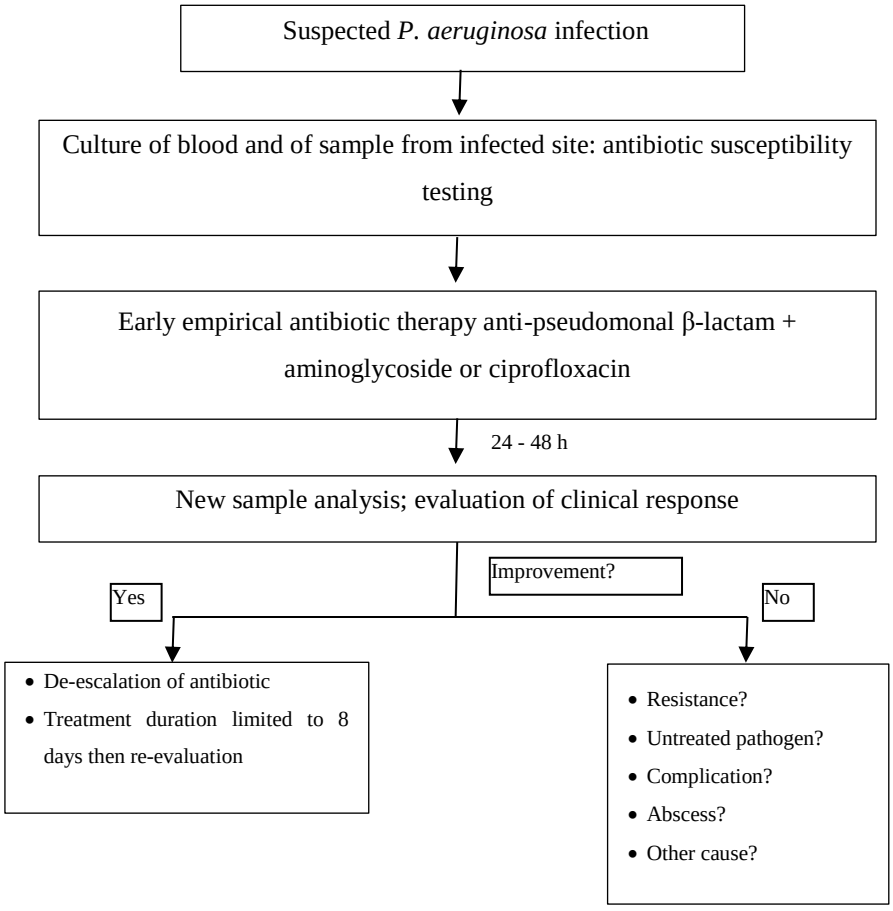


Figure 2.3 An algorithm of *P. aeruginosa* infected clinical managements (19)

2.1.5.1 Antibiotic therapy

The decision of antibiotic selection is depended on the patient's symptoms and risk factors. The initial antibiotic combination treatment usually starts with of β-lactam antibiotic such as penicillins, cephalosporins or carbapenems with either of aminoglycosides or fluoroquinolones, especially ciprofloxacin (13, 19) as showed in Table 2.3. However, the monitoring of patient's condition is also important for prevention of antibiotic resistance and adverse effect of treatment (27).

Table 2.3 The antibiotics for *P. aeruginosa* infected treatment (adapted from (42))

Class	Antibiotic	Advantage	Disadvantage
Penicillins	Ticarcillin, Carbenicillin, Piperacillin/Tazobactam	Synergy with aminoglycoside	May induce β-lactamase
Cephalosporins	Ceftazidime, Cefoperazone		May induce β-lactamase
Aminoglycosides	Gentamycin, Tobramycin, Amikacin	Oral administration, single agent used, synergy with β-lactam	Narrow therapeutic/ toxic, low active in cerebrospinal fluid
Quinolones	Ciprofloxacin		toxic
Polymyxins	Colistin	Active, rarely resistance	toxic
Carbapenems	Imipenem, Meropenem, Doripenem	Broad spectrum	May induce β-lactamase, rapid development of resistance

The extent of antibiotic resistance and antibiotic susceptibility profile may help the decision of antibiotic usage and appropriate dose. The determination of risk-factor for MDR *P. aeruginosa* before antibiotic treatment may concern the efficacy of the antibiotic. Finally, monitoring the trend of antibiotic resistance and the susceptibility should do over time (8).

2.1.5.2 Single antibiotic agent versus combination therapy

Previous studies on monotherapy and combinations were debated yet unclear on the efficacy. Combination cases have been reported at least two antibiotics in combination could enhance the activity. However, in the study of β-lactam monotherapy and β-lactam combined with aminoglycoside for sepsis did not show difference in all of cause in mortality (43). Importantly, the susceptible isolate is known before treatment in combination therapy (13). The introduction of aminoglycosides in *P. aeruginosa* treatment recommended use in combination with alternative active agent (39, 43).

2.2 Antibiotic resistance mechanism

P. aeruginosa possess intrinsic antibiotic resistance as the component structure of various efflux systems, antibiotic inactivation enzymes and antibiotic resistance genes (44). The infection of resistant gene is a big problem concerned in public healthcare treatment due to its higher mortality rate compared with other nosocomial infections (45).

Table 2.4 The mechanisms of antibiotic resistance in *P. aeruginosa* (adapted from (15, 24))

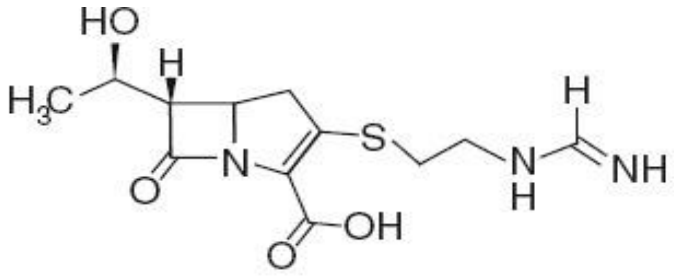
Class	Antibiotic	Resistance mechanism
Penicillins	Ticarcillin, Carbenicillin, Piperacilin	AmpC depression, Up-regulation of MexAB-OprM, β-lactamase expression
Cephalosporins	Ceftazidime, Cefoperazone, Cefepime, Cefpirome	AmpC depression, Up-regulation of MexAB-OprM, β-lactamase expression (especially, with cephasporins cefeprime and cefpirome the mechanism can found overexpression of MexCD-OprJ
Aminoglycosides	Gentamicin, Tobramycin, Amikacin,	Up-regulation of MexXY in impermeability type-resistance, expression of aminoglycoside-modifying enzyme
Quinilones	Ciprofloxacin	GyrA topoisomerase subunit mutant, overexpression of multidrug efflux pump
Polymyxins	Colistin	Membrane changes
Carbapenems	Imipenem, Meropenem	OprD loss, upregulation of MexEF-OprN (for meropenem overexpression of the MexAB-OprM.

Table 2.4 showed the resistance mechanisms of *P. aeruginosa*. Indeed, the antibiotic resistance is involved to the uptake of efflux plump system and enzyme β-lactamase. The low permeability of outer membrane and multidrug efflux system will decrease the susceptibility to antibiotic (48). Several antibiotic resistances are related to MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXT-OprM. Two efflux systems of MexAB-OprM and MexXY-OprM have been extensively studied about antibiotic resistance (15).

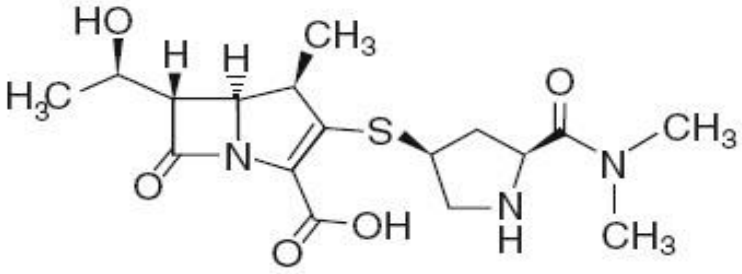
MexAB-OprM system is the prototype resistance-nodulation-division (RND) system with OprM as the cytoplasmid pump protein. The mutation of all these expressions will increase the susceptibility to several antibiotics that are quinolones, sulfamethoxazole, chloramphenicol, tetracycline, trimethoprim and some β-lactam (46). MexXY-OprM is function similar to MexAB-OprM, which is the major mechanism of resistance to aminoglycoside and erythromycin (15). The exposure of β-lactam antibiotic is induced the production of AmpC chromosomal β-lactamase, which inhibit β-lactam antibiotic (47).

2.3 Carbapenems

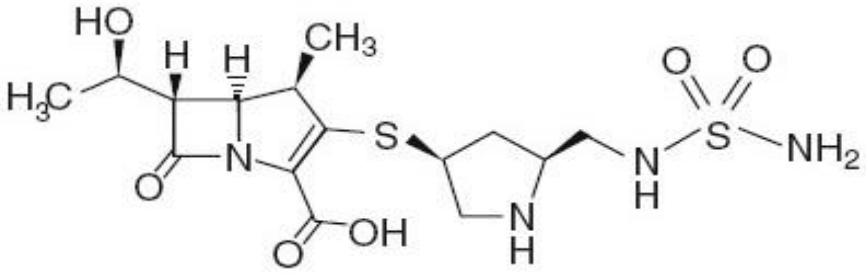
Carbapenems is β-lactam antibiotic with broad spectrum against Gram-positive, Gram-negative, aerobic and anaerobic bacteria including *P. aeruginosa*. It has been used more than seventy years ago. Thienamycin is parental of these drugs group, which produced from *Streptomyces cattleya* (48). Its molecule is unstable; therefore the next N-formimidoyl derivative called imipenem was discovered. Imipenem is sensitive and degraded by dehydropeptidase-1(DHP-1) in renal tubules. Hence, DHP-1 inhibitor as cilastatin is added when administration. Later, meropenem, ertapenem and the newest carbapenems as doripenem were introduced (8). The current status of carbapenems was showed in Table 2.5 that indicated the approval in the US country. Bacterial cell wall synthesis is inhibited by the action of carbapenems that bind to penicillin-binding protein (PBPs) (49, 50). Carbapenems are stable to most β-lactamase enzymes including AmpC-β-lactamase and extended-spectrum-β-lactamase (ESBL). Carbapenems as imipenem, meropenem, doripenem, panipenem/betamipron and biapenem are broad spectrum excluded to *Enterococcus feacalium*, MRSA, and *Stenotrophomona maltopilla*. However, *P. aeruginosa* resisted to ertapenem, because of the lack of anti-pseudomonal activity. Recently, carbapenems resistance in *P. aeruginosa* is become problem due to the high prevalence resistance rate in global including Thailand (4, 51-53) .The chemical structures of carbapenems in potent group to *P. aeruginosa* are showed in Figure 2.4.



Imipenem



Meropenem



Doripenem

Figure 2.4 The chemical structure of imipenem, meropenem, and doripenem (50)

Table 2.5 The status of carbapenems (adapted from (50))

Carbapenems	The data of approval
Imipenem	US approval 1987
Meropenem	US approval 1996
Ertapenem	US approval 2001
Doripenem	US approval 2007
Panipenem/betamipron	Japanese approval 1993
Biapenem	Japanese approval 2001

2.3.1 Mechanism of action

Carbapenems are act as a class of β-lactam antibiotic, which act by binding to penicillin-binding proteins (PBPs). The antibiotic is uptaked through the bacterial cells via outer membrane proteins (OMPs) as porins. The enzyme PBPs is involving to cell wall biosynthesis of peptidoglycan. It composes of transglycolases, transpeptidase, and carboxypeptidase, which it bond to carbapenems binding site. For this reason, the bacterium is loss of osmotic pressure due to defective peptidoglycan. Eventually, the bacteria are destroyed because of cell bursts (8).

2.3.2 Carbapenems activity

The activity of imipenem is potent against Gram-negative bacteria more than meropenem, while doripenem is potent to Gram-positive and Gram-negative bacteria. The limitation of ertapenem is not active to *P. aeruginosa* (8, 54, 55). The data of *in vitro* activity of carbapenems against *P. aeruginosa* was demonstrated in Table 2.6. The activity of carbapenems is remarkable due to many factors. First, the size of molecule is smaller than caphalosporins. They have positive and negative charges inside the molecule. Therefore, these charges enhance the excavation via outer membrane (56). Moreover, carbapenems can combine with other antibiotic agents against several bacteria and prevent upcoming multidrug resistance (8). The examples of combination carbapenems to other antibiotic agents against *P. aeruginosa* are demonstrated in Table 2.7.

Table 2.6 ***In vitro*** **activity data of carbapenems against 829 strains** ***P. aeruginosa*** **(adapted from (57))**

Carbapenems	The minimal inhibition concentration (μg/ml)	
	MIC_{50}	MIC_{90}
Imipenem	1	>8
Meropenem	0.5	16
Ertapenem	8	>8
Doripenem	0.5	8

Table 2.7 Example of combination carbapenems with other antibiotics against ***P. aeruginosa*** **(adapted from (8, 42))**

Carbapenems	Other antibiotics
All in carbapenems	Fluoroquinolone, Aminoglycoside
Imipenem	Tachyplesin
Meropenem	Polymyxin
Meropenem and imipenem Doripenem	Colistin

2.3.3 Role of therapy

Carbapenems are used for Gram-negative, Gram-positive bacterial infection including Enterobacteriaceae. Its abilities are broad spectrum to several bacteria and stable to β-lactamase enzymes (50). The patients with complicated infection as complicated community-acquired pneumonia (CAP), complicated skin structure infections (CSSSI) and complicated intra-abdominal infections (CIAI) recommended the antibiotic treatment with carbapenems (32, 58). Carbapenems are primary recommendation for highly mortality rate of *P. aeruginosa* infection patients (50).

2.3.4 Mechanism of carbapenems resistance in *P. aeruginosa*

Carbapenems resistance in *P. aeruginosa* involved to the production of mettalobeta-lactamase (MBL) and also the ability of hydrolyze all β-lactam but not effected to aztreonam (51). They have many mechanisms involving to carbapenems resistance including over expression of several efflux pumps, reduction of drug permeability such as the inactivation of OprD porin, and β-lactamase enzyme production. The resistant mechanisms of *P. aeruginosa* are acquired resistant gene and intrinsic carbapenems resistance mechanisms (32, 53).

2.3.4.1 AmpC β-lactamase

The occurrence of carbapenems resistance is found most frequency as other β-lactam antibiotics. The unique structure of carbapenems tolerates many β-lactamase. Recently, *P. aeruginosa* develops many mechanisms of antibiotic resistance involving to over-production of AmpC β-lactamase (59). AmpC β-lactamase is one of β-lactamase enzymes, which it divided from Ambler's structural classification in class C. It carries serine in active site center. Serine acts to β-lactam ring of carbapenems by cleavage amine bond of β-lactam ring, which it brings to inactivating antibiotic. Class C β-lactamase is not classification in carbapenemase, which it is not much effected to carbapenems resistance. However, this enzyme is effected to carbapenems resistance when combine with over-expression of efflux system or reduction of outer membrane permeability (32).

2.3.4.2 Loss of OprD protein

The inactivation of OprD porin is major related to reduction of susceptibility of carbapenems due to the outer membrane protein OprD allows entry with carbapenems (53). The mutation of the oprD gene is leading to carbapenems resistance (60). The porin OprD reduction combined with AmpC β-lactamase is inducing imipenem resistance (61).

2.3.4.3 Over-expression of efflux pumps

MexAB-OprM system is elimination efflux pump for several antibiotics. The substrates for this system are fluoroquinolones, tetracycline, chloramphenicol, carbenicillin, piperacillin, ceftazidime, cefepime and aztreonam. The property of hydrophobic side chain of meropenem is also effected to this system (53, 62). Meropenem is one of substrate for MexCD-OprJ system; however this system is

not expression in normal condition (63). MexXY-OprM may induce the resistance with meropenem but not imipenem (64). However, meropenem and doripenem have been found the inactivation of OprD affected to *P. aeruginosa* resistance to these carbapenems, especially when AmpC β-lactamase or MexAB-OprM is overexpression together (65).

2.4 Fosfomycin

The prevalence of antibiotic resistance in *P. aeruginosa* is increasing related to the shortage of new antibiotic (17). Fosfomycin is phosphonic acid derivative with broad antibacterial spectrum and also effective against *P. aeruginosa* (66). It was discovered more than 40 years ago from *Streptomyces* species (67). It has three formulas; fosfomycin tromethamine is a soluble sult, fosfomycin calcium for oral use, and fosfomycin disodium for intravenous use. Its chemical structure is unrelated to any available antibiotics (68) as showed in Figure 2.5. The target of fosfomycin is inhibiting mucopeptide synthesis by inactivation phosphoenolpyruvate transferase as the first enzyme in peptidoglycan synthesis. Therefore, it can be used in combination with other antibiotic agent such as β-lactam and no development of cross-resistant to other class when using in combination (66).

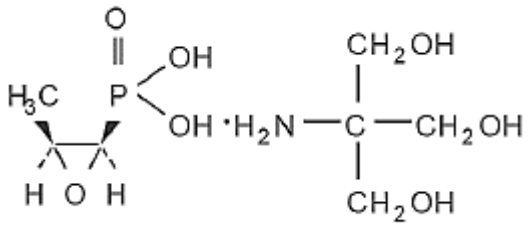


Figure 2.5 The chemical structure of fosfomycin (66)

2.4.1 Form of fosfomycin

Phosphonic antibiotics in use are fosfomycin, fosmidomycin and alafosfalin. The first initially is fosfomycin-disodium, which it used for patient with serious infections and used for many years in many countries (69). Fosfomycin tromethamine is oral from with monobasic hydrosoluble. It occasionally using in some countries of Europe with sepsis and soft tissue infection (70).

2.4.2 Mechanism of action

Fosfomycin is acting to initial stage of cell wall biosynthesis, which inhibiting the step of peptidoglycan synthesis by blocking the formation of *N*-acetylmuramic acid (71). It is uptake to the bacterial cells via both of nutrient transport systems that are glycerol-3 phosphate (GlpT) and hexose phosphate transporter (UhpT). However, it has been proven that *P. aeruginosa* lack a specific transport system for glucose-6-phosphate sugar, therefore fosfomycin is only uptake to *P. aeruginosa* cell via GlpT transport system (72, 73). In Figure 2.6 is the inactivation by fosfomycin. Fosfomycin inactivates the covalent of UDP-N-acetylglucosamine-enolpyruvyl transferase enzyme of MurA. This enzyme is catalyzing enzyme in peptidoglycan synthesis (71).

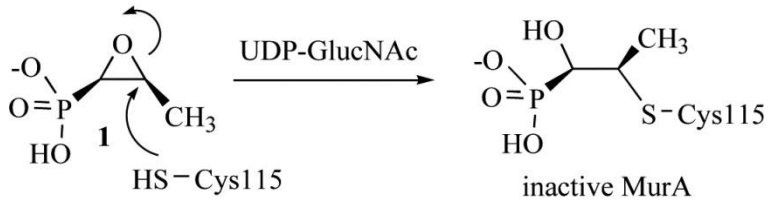


Figure 2.6 The mechanism of inactivation of MurA by fosfomycin (74)

2.4.3 Antibiotic activity

Fosfomycin shows activity against many Gram-positive cocci such as *Staphylococcus aureus*, *Streptococcus pneumonia*, *Enterococcus* species and also against Gram-negative bacteria such as *E. coli*, *Proteus mirabilis*, *K. pneumonia*e. It also found effective against *P. aeruginosa* when using in combination with β-lactams antibiotic or aminoglycosides (66). Fosfomycin is time-dependent killing manner against Gram-positive and Gram-negative bacteria (75). Combination effects of fosfomycin with other antibiotics have been shown in Table 2.8.

2.4.4 The mechanism of resistance to fosfomycin

The several mechanisms of fosfomycin resistance are reduction of drug uptake, the differentiation of target site and the inactivation of antibiotic. The chromosome mutation is resulting to low intracellular level of antibiotic due to the abnormity of fosfomycin membrane transport system. Fosfomycin can uptake into the

cell via two nutrient transport systems that are GlpT and UhpT. Some mutation of genes responsible for their system may induce of fosfomycin resistance. It also occure when the alteration in biological system that altered the transporters. Moreover, the resistance to fosfomycin can often associated with the presence of inactivated enzyme to the antibiotic (71).

Table 2.8 Percentage of synergy with other antibiotic agent against *P. aeruginosa* from several studies. (adapted from (76))

Antibiotic	Percentage of synergy
Amikacin	46.38%
Aztreonam	67.5%
Cefepime	76.37%
Ceftazidime	41.62%
Ciprofloxacin	48.26%
Gentamicin	48.63%
Imipenem	36.13%
Levofloxacin	93.45%

2.4.5 Adverse effect

Fosfomycin has very low adverse effect, generally are rash, headache, nausea, rhinitis, vaginitis, etc. Fosfomycin showed less toxicity data about 1-10% that have been reported in patient treatment (70, 75).

2.5 Combination therapy

The alternative treatment for multidrug resistant Gram-negative bacteria is the antibiotic combination therapy. In the patient infected with organisms more commonly acquired in hospital setting including *P. aeruginosa* that suggests the antibiotic combination more potent than monotherapy. The antibiotic combinations generally use the potent antibiotic such β-lactam and aminoglycoside or fluoroquinolone combined with other antibiotics. The initiation of combination therapy using is often justified by at least one of three as following.

(i) To enhancement the activity by two difference spectra of antibiotic and ensure that the pathogen is adequately covered by at least one of them.

(ii) To ensure that the synergy activity observed *in vitro* of two antibiotics compared to one. The results of them can be used to estimate the activity in clinical outcome.

(iii) To prevent the delay of antibiotic resistance during antibiotic combination therapy (77).

2.5.1 The advantage of combination therapy

2.5.1.1 Extension the activity of antibiotic spectrum

The first expectation outcome from the combination therapeutic is providing a broad spectrum to kill the bacterial cell. The synergistic of bactericidal activity is the potential that should be occurred during the treatment (78). In case of serious infections such as acute bacterial endocarditis, septic shock, or neonatal meningitis are not responsible to drugs of choice, the combination antibiotic will considered as the alternative treatment.

2.5.1.2 Reduction of toxicity

In generally, the combination drug treatment is using the dosage of antibiotic lower than monotherapy. The high dose of independent antibiotic may cause the toxicity whereas the combined antibiotic will decrease their effect. Cai *et al*. have showed that fosfomycin combined with aminoglycosides was able to reduce the dosage against *P. aeruginosa*. The reduction of dosage using was decreasing the potential nephrotoxicity and cytotoxicity caused by aminoglycosides (6). Moreover, the studies in clinical outcome of patients with Gram-negative infection

between monotherapy and combination therapy were showed that monotherapy as effective as combination therapy with lower dose of each antibiotics. Also, it showed the improvement of survival rate in patients with septic shock (78). On the other hand, some studies found the increase in nephrotoxicity after threatment with the combination therapy of aminoglycoside combined with other antibiotics (77). Table 2.9 was showed the comparative adverse effect in clinical studies between monotherapy compared with combination antibiotic against MDR *P. aeruginosa*. The treatment of colistin alone compared with colistin plus aztreonam, piperacillin, ceftrazidime, imipenem or ciprofloxacin were not difference in response rate as well as nephrotoxicity no increased.

Table 2.9 The comparative of adverse effects in clinical studies between monotherapy and combination studies against *P. aeruginosa* (adapted from (77)).

Bacterial infection	Antibiotic combination	Results of adverse effect
P. aeruginosa in cystic fibrosis patients	Colistin VS colistin plus aztreonam, piperacillin, ceftazidime, imipenem or ciprofloxacin	No difference in response rates; nephrotoxicity increased with combination therapy
MDR *P. aeruginosa*	Colistin VS colistin plus amikacin or antipseudomonal β-lactam	No difference in response rates
MDR *P. aeruginosa* in patients with diabetic foot infection	Colistin VS colistin plus rifampin or imipenem	No difference in response and nephrotoxicity rates

2.1.5.3 Minimization of antibiotic resistance

Nowadays, the evidence of antibiotic resistance in *P. aeruginosa* is increasing (13, 17, 62, 79). The combined antibiotic can reduce the occasion of antibiotic resistance during treatment. Several studies on the combination therapy in *P. aeruginosa* showed the potential to inhibit growth of bacterial cells. *In vitro* studies by Lister PD *et al* showed that the combination between levofloxacin and imipenem caused delay of antibiotic resistance (80, 81), especially in *P. aeruginosa*. Anyway, the combination therapy is not superior to monotherapy until the development of resistance to β-lactam antibiotic. Thus, the combination may benefit to the patient with severe infections and critically ill patients (82).

2.6 Synergy testing

Antibiotic combinations are used for optional seriously ill patients or who may have septicemia. It is used for preventing of emergence antibiotic resistance, to extend the antibiotic spectrum coverage than using with single agent for treatment and reduced dosage (12, 83).

2.6.1 Synergy definition

The synergy in antibiotic combinations are demonstrated when the efficacy in bactericidal or inhibition of combination is greater than the activity of individual agents (78). The synergy between β-lactam antibiotics and aminoglycosides against both of Gram-positive and Gram- negative bacteria has been reported (84), (12).

2.6.2 Synergy study methods

The determination of synergy is performed *in vitro* before dispensation of combination antibiotics. Despite the difference in each technique, the expectation of the results in synergism and antagonism are similar. Synergy is the positive result of interaction effect that is the enhancement of activity to inhibited bacteria in each antibiotic. So that, the combination is significant effective greater than used individual antibiotic. Antagonism is the negative results of interaction effect, which is the

confutation of activity to inhibited bacteria. It is significant effect less than used individual. Additive or indifference is summing interaction effect no difference to individual antibiotic (12), (75). The techniques for synergy study are demonstrated in Table 2.10.

Table 2.10 The techniques for synergy study (adapted from (12))

Techniques	Antibiotic effect	Advantage/disadvantage
Checkerboard	Bacteriostatic	Many different combinations and concentrations can be tested, does not assess killing rates
Killing curve	Bacteriostatic	Few combinations can be tested, but killing effect over time can be determined
Disk diffusion	Bacteriostatic	Gradient produced by drug diffusion does not necessarily have clear relationship with achievable concentrations, qualitative information may be useful for screening
Paper strip diffusion	Bacteriostatic	Traditional paper strip method produces only qualitative results, difficult to relate to achievable concentrations

CHAPTER III
MATERIALS AND METHODS

3.1 Antibiotics and chemical reagents

Fosfomycin	Sigma-Aldrich, USA
Meropenem	366 Pharma (Nanjing), China
Imipenem	366 Pharma (Nanjing), China
Doripenem	Shionogi & Co., Japan
Glutaraldehyde solution	Calbiochem, Germany
Osmium tetroxide	Sigma-Aldrich, USA
Ethyl alcohol	Lab-Scan, Bangkok, Thailand
Sodium chloride	Merck, Germany
Sulfuric acid	Merck, Germany
Barium chloride	Merck, Germany

3.2 Culture media

Agar	CONDA, USA
Tryptic soy broth	Becton, Dickinson and Company, USA
Mueller Hinton broth	Becton, Dickinson and Company, USA

3.3 Equipments

Autoclave MLS-302,	Sanyo Electric, Japan
Incubator BE 600,	Memmert, Germany
Laminar air flow hood	Holten, Thermo Scientific, USA

pH-meter	Ultra Basic UB-10, Denver Instrument, USA
Shaking incubator	22 NW, Memmert, Germany
Spectrophotometer	Thermo Fisher Scientific, USA
Refrigerated centrifuge	Dynamica, South Korea
Vortex mixer	Vortex-2 Genie, Scientific
Water bath incubator	WNB22, Memmert, Germany
96-well microtiter plates	Thermo scientific Nunc, China
Nunc-Immuno TSP 96 Pins in Lid	Thermo scientific Nunc, China
Hot air oven	UL50, Memert
Multichannel pipette	Rainin, USA
Micropipette	Gilson, France
Scanning electron microscope	Hitachi S-510, Japan
Rocking table	MR-1, Biosan
Ultrasonic bath	model 250HT, VWR Scientific

3.4 Bacterial strains

3.4.1 *P. aeruginosa* clinical isolated strains

Seventy clinical carbapenems-resistant *P. aeruginosa* isolates were obtained from nine hospitals all around regions of Thailand as described in Table 3.1. The bacteria were performed by broth microdilution technique to determine the antibiotic susceptibility with carbapenems. The susceptibility breakpoint was defined according to the Clinical and Laboratory Standards Institute (CLSI) guideline 2012 (85). Carbapenems resistant *P. aeruginosa* (CR-PA) was defined as the strain resisted to at least one of meropenem, impenem, and doripenem (4). The protocol was approval the ethical submission by Mahidol University Institutional Review Board (MU-IRB) (4, 52) and reference to ethical no. MU-IRB 2009/013.0303.

Table 3.1 Sources of seventy carbapenems-resistant *P. aeruginosa* clinical isolated strains

Region of Thailand	Number strains of selection
Central	16
North	10
Northeast	15
South	11
East	18

3.5 Determination of antibiotic susceptibility

The seventy CR-PA clinical isolates were determined for the susceptibility to fosfomycin, meropenem, imipenem, and doripenem by broth microdilution method, according to CLSI guideline (85). Briefly, *P. aeruginosa* colony on agar plate were cultured in cation-adjusted Mueller-Hinton broth (CA-MHB) at 37°C for overnight before adjusted to the 0.5 McFarland turbidity standard approximately $1.5x10^8$ CFU/ml. The antibiotics solution were proposed in appropriate solvent in Table 3.2. This method was tested by using 96-well microtiter plates. The soluble antibiotic was loaded into the well and was then diluted in the serial of two-fold dilution. After that, the inocula of bacteria (about $1.5x10^5$ CFU/well) was added and before incubated at 37ºC for 18 hours. The maximum concentration of fosfomycin was limited at 1,024 µg/ml and carbapenems at 256 µg/ml. The lower concentrations which the invisible growth of bacteria was observed report as the minimal inhibition concentration (MIC). The results of MIC were interpreted the susceptibility followed CLSI breakpoint.

Table 3.2 Appropriate solvents for antibiotics preparation (85)**.**

Antibiotics	**Solvents**
Fosfomycin	Water
Meropenem	Water
Imipenem	Phosphate buffer, pH 7.2, 0.01 mol/L
Doripenem	0.85% physiological saline

3.6 Determination of broth microdilution checkerboard assay

The broth microdilution checkerboard method was used for determination of synergistic effect between fosfomycin and carbapenems (meropenem, imipenem, and doripenem). This method was performed according to Clinical Microbiology Procedures Handbook (86).

3.6.1 Antibiotics preparation

Antibiotics preparations were performed in 96 wells microtiter plates. The volume of 50 ml of Mueller-Hilton broth was added into each well of plate. The concentrations of fosfomycin and carbapenems were two times the final panel concentration. The limitation of MIC concentration of fosfomycin and carbapenems did not exceed 1,024 µg/ml and 256 µg/ml, respectively. The working stock solution of fosfomycin was prepared over 8x MIC concentration and carbapenems were prepared working stock at the concentration starting of 0.25x to 4x MIC. The volume of 50 ml working stock of fosfomycin was placed in last rows of 96 wells microtiter plate and diluted by serial two-fold dilution. The volume of 50 ml of carbapenems stock solution was distributed among column by added each concentration into each column and added 50 ml of the inoculum into each well. Thus, each well was containing the combination concentrations of two antibiotics with both 2x MIC at the final panel. The pattern of antibiotics concentration was shown in Table 3.3.

Table 3.3 The concentrations pattern in the panel of checkerboard assay

	Fos 0	**Fos 0.125xMIC**	**Fos 0.25xMIC**	**Fos 0.5xMIC**	**Fos 1xMIC**	**Fos 2xMIC**
CR 0					MIC	
CR 0.125xMIC				IND		
CR 0.25xMIC			SYN			
CR 0.5xMIC		IND				
CR 1xMIC	MIC					
CR 2xMIC						

Fos = fosfomycin

CR = carbapenems

MIC = minimal inhibition concentration

IND = indifference

SYN = synergy

3.6.2 Inoculation

The colonies of *P. aeruginosa* were inoculated into 5 ml CA-MHB and incubated overnight at 37ºC. After incubation period, the bacterial suspensions were adjusted the turbidity to 0.5 McFarland turbidity Standard (approximately $1.5x10^8$ CFU/ml) and ten-fold diluted to 10^5 CFU/ml. 96-well plate was incubated at 37ºC for 18 hours.

3.6.3 Interpretation of synergy

The invisible growth of bacteria at the lowest concentration of each individual antibiotic was recorded as the MIC. Each combination was determined the growth of bacteria and recorded the result. These results were calculated as the fractional inhibitory concentration (FIC) for synergistic interpretation. The FIC in each combination reaction was derived from the MIC of antibiotic (ΣFICI) in each combination. The results of summation FIC value was examine and interpreted as followed (2).

$$\text{FIC of fosfomycin} = \frac{\text{MIC of fosfomycin in combination}}{\text{MIC of fosfomycin alone}}$$

$$\text{FIC of each carbapenems} = \frac{\text{MIC of each carbapenems in combination}}{\text{MIC of each carbapenems alone}}$$

$$\Sigma\text{FICI} = \text{FIC of fosfomycin} + \text{FIC of each carbapenems}$$

Synergy	:	$\Sigma FICI \leq 0.5$
Indifference or additive	:	$0.5 < \Sigma FICI < 4$
Antagonism	:	$\Sigma FICI > 4$

3.7 Tests of statistical significance

Statistical difference was used to determine synergistic distinction between three groups of combination. The chi-square test was chosen for analysis these data at statistically significant consideration of *P*-value ≤ 0.05.

3.8 Determination the rate of killing by antibiotic agents when used in combination

3.8.1 Time-kill assay for individual antibiotic agent

This method was performed with *P. aeruginosa* PAO1 as the protocol according to NCCLS guideline (87). The inoculum preparation performed as previously described. Time-kill for individual antibiotic determinations were examined the concentration of fosfomycin, imipenem, meropenem, and doripenem at 0.125xMIC, 0.25xMIC, 0.5xMIC, 1xMIC, and 2xMIC. The primary of each reaction tubes were composed of the bacterial cell approximately 10^5 CFU/ml. The defined antibiotics and CA-MHB were adjusted the volume to 10 ml. The primary reaction tubes were incubated at 37ºC in shaking water bath incubator. The samples of each antibiotic reaction were collected at 0, 2, 4, 8, and 16 hours of incubation period. The aliquots of samples were serially diluted with 0.9% sodium chloride by ten-fold serial dilution. Bacterial count was determined on each of serial dilution by colony plate

counting after incubating at 37ºC overnight. The growth control of *P. aeruginosa* PAO1 strain was performed as similar. The numbers of viable bacteria derived in CFU/ml were converted to log CFU/ml and plotted the time-kill curve.

3.8.2 Time-kill assay for antibiotic combination

The method of time-kill assay for determining synergy was applied according to Clinical Microbiology Procedures Handbook (88). The concentrations in time-kill for combination antibiotic between fosfomycin with carbapenems were 0.125xMIC, 0.25xMIC, and 0.5xMIC. The inoculation, antibiotic preparation, and sample collection were prepared as the same of procedure in time-kill assay for individual antibiotic agent.

The values of log CFU/ml of individual antibiotic agents were compared to the growth control at sampling times. The agent that could to decrease log CFU/ml from growth control was the most active agent. Synergy was determined as ≥2log CFU/ml decrease of the most active antibiotic compared with combination at sample time. Antagonism was defined as ≥2log CFU/ml increase of the most active antibiotic compared with combination at sampling time.

3.9 Determination of relationship between synergy effects, carbapenems susceptibility with carbapenems resistance mechanisms

The results of carbapenems resistance mechanism of overexpression of MexAB-OprM, MexXY-OprM, inactivation of OprD protein, and AmpC β-lactamase overexpression were derived from previous study (52, 85). The synergistic stains and carbapenems susceptibility were determined the relationship with these mechanisms to observe the percentage of positive in carbapenems resistance mechanisms detection.

3.10 Study of the frequency resistance

This study was applied to determine the phenotypic spontaneous mutation after single exposure by using the single step mutation method. The concentration selected to study was 2xMIC of individual and combined antibiotics. The numbers of bacterial selection for this determination were 5 strains. The first one was *P. aeruginosa* PAO1 as the standard wild-type strain. The second one was MTCN-CM06 strain on which synergistic effect of all combinations were observed. The third was MTCB-SI19 strain, on which synergistic effect of fosfomycin and meropenem was observed. The fourth MTCE-CH35, whichsynergistic effect of fosfomycin and imipenem was observed. The last one was MTCN-UB 45, on which synergistic effect of fosfomycin and doripenem was observed. The bacterial inoculum was cultured until the cells approximately 10^9-10^{10} CFU/ml. The inoculum suspensions were spreaded on cation-Mueller Hinton agar plate containing 2xMIC of each individual and combined antibiotics before incubated at 37ºC. After 48 hours of incubation, the viable bacteria were calculated by dividing the number of bacteria growing at defined antibiotic concentration by number of bacteria in inoculum (18, 89).

$$\text{Frequency of mutation} = \frac{\text{Number of bacterial growth in antibiotic}}{\text{Number of bacteria in inoculum}}$$

3.11 Determination of post antibiotic effect (PAE)

The post antibiotic effect (PAE) was performed to determine the pharmacodynamics of suppressed bacterial growth after short time exposure to the antibiotic. The period using of examination was 2 hours. The antibiotic concentration was examined at 2xMIC of each individual and combined antibiotics. The bacterial inoculum of *P. aeruginosa* PAO1 was cultured overnight and adjusted the turbidity to 10^6 CFU/ml in the reaction tube. The reaction tubes were contained 2xMIC of antibiotic, bacterial suspension, and adjusted the volume to 5 ml. The culture was incubated in shaking water bath incubator at 37ºC for 2 hours. After that, the suspension was centrifuged at 5000g for 5 min. The precipitates of bacterial cell were washed two times with 0.9% sodium chloride. The bacterial cells were resuspended with 5ml fresh pre-warm MHB. After resuspended immediately, the samples were

collected at 0 hour for colony plate counting. The samples were reincubated which sampling at 1, 2, 3, 4, 5, 6, 7, and 8 hours of incubation periods. No antibiotic was determined as the control with the same procedure. Bacterial count was determined at specific time intervals (18, 90, 91).

$$PAE = T_A - T_C$$

T_A = the time necessary for the number of viable organism in test culture to increase by log CFU/ml above the number observed immediately after removal of antibiotic.

T_C = the time necessary for the number of viable organism in the untreated culture to increase by log CFU/ml above the number observed immediately after centrifugation.

3.12 Morphological study by scanning electron microscopy (SEM)

This method compared the disruption of bacterial cell wall after treated with individual and combined antibiotics. The antibiotics in this study were fosfomycin, doripenem, and combined both of them.

3.12.1 Sample preparation

The antibiotic reactions were prepared at 0.25xMIC as the synergy concentration in the reaction tubes. The bacterial inoculum of *P. aeruginosa* PAO1 was added to the reaction tubes that provided the final viable cell count approximately 10^6 CFU/ml. The reaction tubes were incubated at 37ºC for 8 hours in shaking water bath incubator. After that, the reactions were centrifuged and washed with 0.9% sodium chloride. The bacterial cells were fixed by 2.5% glutaraldehyde at room temperature overnight. After pre-fixation, the cell precipitates were washed three times 15 minute each, with 0.1M phosphate buffer. The post-fixation was performed by using 1% OsO_4 for 2 hours at room temperature. The steps of sample dehydration were performed by using ethanol series. The samples were dried by critical point dryer

and mounted on aluminum sample stubs. The last step was coating of the stubs with gold (92, 93).

3.12.2 Observation of morphological changes

The sample stubs were observed by Hitachi s-510 scanning electron microscope at original magnification of x15000. The model of this SEM was shown in Figure 3.1.

Figure 3.1 Hitachi s-510 scanning electron microscope

3.13 Bacterial biofilms study

3.13.1 Biofilms formation

The strain selected for biofilm susceptibility testing was *P. aeruginosa* PAO1. The biofilm formation assay was adapted as describes previously (94-96). The inoculum was incubated overnight before adjusted the turbidity to 0.5 McFarland turbidity standard (approximately 10^8 CFU/ml). Biofilms formation was performed in 96 wells microtiter plate with 96 pegs on lid cover plate (Nunc TSP, Transferable Solid Phase Screening system; Nalgene Nunc International) as demonstrated in Figure 3.2. The 100 µl of the inoculum were added into each well of the 96 wells microtiter plate and covered with lid containing 96 pegs. After that, the plates were incubated for 24 hours on rocking table with the speed of 30 rpm at temperature of 37 ºC. Biofilms were held on pegs when complete of incubation period was compleated.

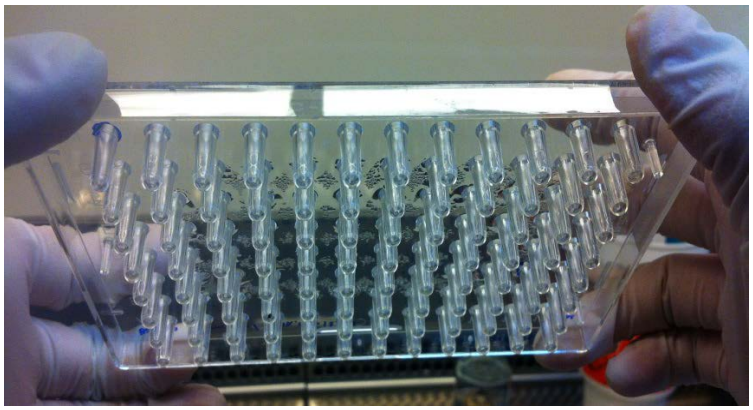


Figure 3.2 Transferable solid phase screening system

3.13.2 Determination of biofilms susceptibility with individual antibiotics and combination.

Fosfomycin and carbapenems were determined for the minimal biofilm eradication concentration (MBEC) as the minimal concentration of antibiotic for killing the bacterial biofilm. The individual antibiotic susceptibility was performed following to MBEC technique as described previously (94-96).

The biofilms on pegs lid were replaced in a new 96 wells microtiter plate containing 0.01 M of potassium phosphate buffer (pH7.5) to remove non-binding cells. The new 96 wells micro-titer plate containing antibiotic concentrations was covered with same pegs lid. Fosfomycin, imipenem, meropenem and doripenem were tested at the concentrations of 0.125xMIC, 0.5xMIC, 1xMIC and 2xMIC. No antibiotic added was tested in parallel as the positive control. The condition of incubation used as same as that of biofilm formation. The pegs lid was rinsed 2 times with 0.01 M of potassium phosphate buffer after 24 hours of incubation. The existing bacterial biofilms was determined after sonication for 5 minutes in the new 96 wells microtiter plate containing new medium. The viable bacterial biofilm was determined by plate-counting on TSA. The colony derived was calculated logarithmic colony forming unit decrease and evaluated the differentiation of log CFU/ml from positive control with log CFU/ml from antibiotic treatment.

The combination antibiotics were examined three pairs of fosfomycin combined with imipenem, meropenem and doripenem. They were performed as the same of individual antibiotic including the interpretation of MBEC.

CHAPTER IV
RESULTS

4.1 Determination of antibiotic susceptibility

Seventy clinically isolated carbapenems-resistant *P. aeruginosa* strains from various regions of Thailand were examined for antibiotic susceptibility by disc diffusion method from previous study (52). Carbapenems resistance (CR) was defined by being resistance to at least one in three carbapenems antibiotics (2, 53). The susceptibility results of all CR-PA strains and the standard strain, *P. aeruginosa* PAO1 were shown in appendix 1. As the criteria, the susceptibility showed that almost all clinical isolated strains from various hospitals resisted to at least one drug of carbapenems. The sensitivity of clinical isolated strain MTCS-SR24 was found intermediate to all of carbapenems. However, eight clinical isolated strains were found susceptible to only doripenem.

Since the CLSI guideline was not defined the MIC breakpoint of fosfomycin, the results of fosfomycin were reported only MIC values. The summary of MIC values and susceptibility were shown in Table 4.1.

Table 4.1 The susceptibility summary of seventy CR-PA clinical isolates

Antibiotic	MIC range	MIC_{50}	MIC_{90}	Percentage of susceptible (n)		
				susceptible	intermediate	resistant
Fosfomycin	8 - >1,024	256	>1,024	-	-	-
Carbapenems						
meropenem	8 – 256	32	64	0% (0)	1.43% (1)	98.57% (69)
imipenem	1 - >256	16	64	2.86% (2)	7.14% (5)	90% (63)
doripenem	2 - 256	4	64	11.43% (8)	42.86% (30)	45.71% (32)

Values are presented in µg/ml

The MIC range of fosfomycin to CR-PA was 8 - >1024 µg/ml. The MIC_{50} and MIC_{90} of fosfomycin were 256 and >1024 µg/ml, suggesting that the MIC values were distributed in broad interval. For the results of carbapenems, MIC ranges for meropenem, imipenem, and doripenem were 8-256, 1->256, and 2-256 µg/ml, respectively. The percentage of carbapenems resistance was shown in Table 4.1. Meropenem showed the highest resistance rate about 98.57%. Imipenem and doripenem had the resistance percentage about 90% and 85.71 %, respectively.

4.2 Synergy testing

Combinations between fosfomycin and carbapenems were examined for alternative treatment of CR-PA infection. The experiments were tested with seventy carbapenems-resistant *P. aeruginosa* isolates by broth microdilution checkerboard. The FIC values of all strains were demonstrated in Table 4.3. The results were concluded for synergistic effect percentage in Table 4.2.

The summary of synergistic effect showed the highest percentage at 45.71% when fosfomycin combined with doripenem followed by fosfomycin combined with meropenem (40%), and fosfomycin combined with imipenem (38.57%). As the results of indifference, fosfomycin combined with doripenem did have the lowest percentage of indifference at 54.28% which fosfomycin combined with meropenem was 61.43%, and fosfomycin combined with imipenem was 61.43%. Interestingly, there was no antagonism presented in any combinations.

The statistical method used to analyze the variation for three combinations was chi-square test. It was used to determine qualitative data for association between two variables. It was found that the percentages of synergism in all three combinations indicated no significant difference with 95% confidence interval.

Table 4.2 The percentage of synergistic effects with seventy carbapenems-resistant isolates

Antibiotic combination	n	Percentage of synergistic effect (n)		
		Synergy	Indifference	Antagonist
fosfomycin + meropenem	70	40% (28)	60% (42)	0% (0)
fosfomycin + imipenem	70	38.57% (27)	61.43% (43)	0% (0)
fosfomycin + doripenem	70	45.71% (32)	54.29% (38)	0% (0)

4.3 Time-kill assay for individual antibiotic agent

This study examined the rate of killing bacteria for individual antibiotic agent (fosfomycin, meropenem, imipenem, and doripenem) with *P. aeruginosa* PAO1. The results of log CFU/ml were plotted with sampling time to determine the correlation between the antibiotic and time of killing. Bacteria were taken at 0, 2, 4, 8, and 16 hours of culture treatment and the initial bacterial cells of approximately 5 log CFU/ml were used in this study. The growth control showed that during the first of 2 hours, the lag phase of growth curve was shown. After that, it was into the acceleration phase. At 4 hours, it showed the growth phase. The last of sampling time was found the viable cells about 17 log CFU/ml at 16 hours.

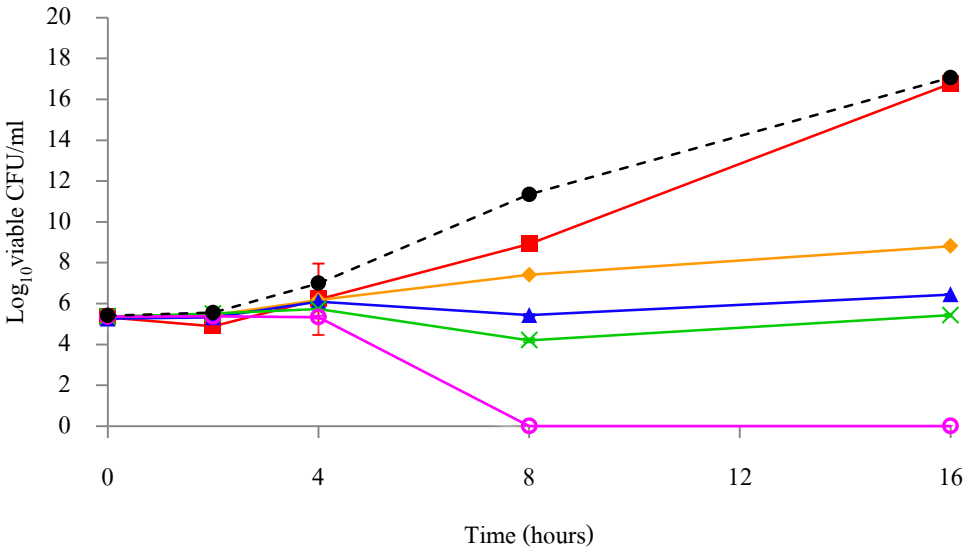


Figure 4.1 Time-kill curve of *P. aeruginosa* PAO1: untreated (-•-), treated with 0.125xMIC fosfomycin (-■-), 0.25xMIC fosfomycin (-◆-), 0.5xMIC fosfomycin (-▲-), 1xMIC fosfomycin (-×-), and 2xMIC fosfomycin (-○-)

The potential of fosfomycin to kill bacterial cells was examined (Figure 4.1). The bactericidal effect was defined at 3 log CFU/ml viable bacterial cell count decrease when compared to the control (87). The results showed that fosfomycin at 0.125xMIC did not show bactericidal activity. After 8 hours of cultivation, 0.25x, 05x, 1x, and 2xMIC of fosfomycin showed some antibacterial effect. However, only cells treated with 2xMIC of fosfomycin were completely killed after 8 hours.

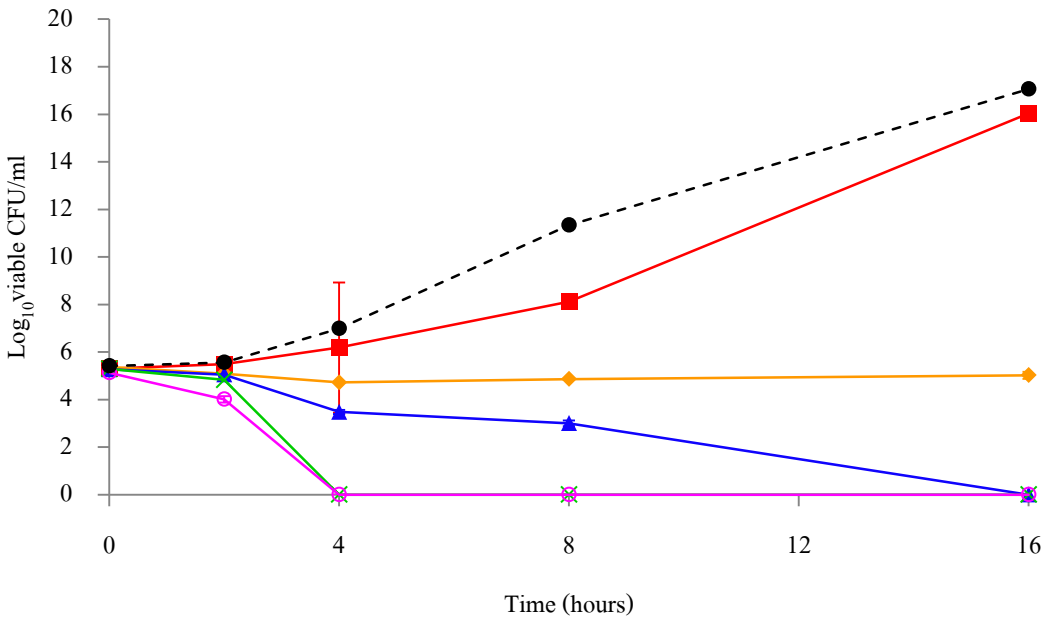


Figure 4.2 Time-kill curve of *P. aeruginosa* PAO1: untreated (-•-), treated with 0.125xMIC meropenem (-■-), 0.25xMIC meropenem (-◆-), 0.5xMIC meropenem (-▲-),1xMIC meropenem (-✳-), and 2xMIC meropenem (-○-)

The potential of meropenem against *P. aeruginosa* PAO1 was shown in Figure 4.2. The result showed that meropenem at 0.125xMIC was found less bactericidal effect at 8 hours of culture and recovered the growth after this time. The bactericidal effect of meropenem at 0.25x, 0.5x, 1x, and 2xMIC were found at 4 hours of incubation. Meropenem at 0.25xMIC was found the bacterial cell recovery growth after 4 hours. The recovery growth was found except meropenem at 0.5x, 1x, and 2xMIC. Meropenem at 0.5xMIC was completely killed bacterial cells after 16 hours while meropenem at 1x, 2xMIC were found at 4 hours.

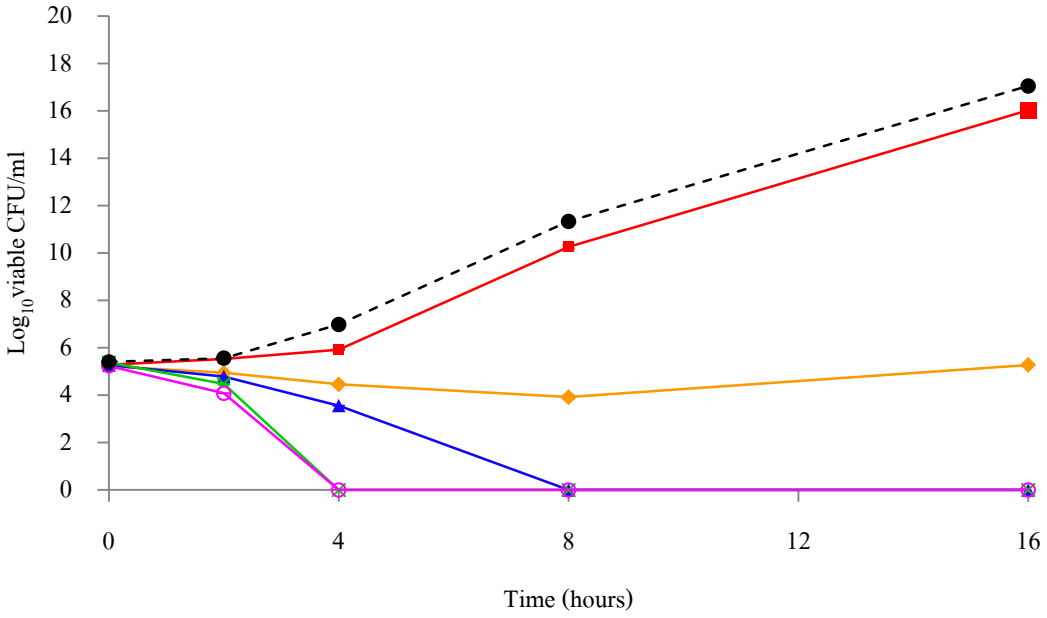


Figure 4.3 Time-kill curve of *P. aeruginosa* PAO1: untreated (-•-), treated with 0.125xMIC imipenem (-■-), 0.25xMIC imipenem (-◆-), 0.5xMIC imipenem (-▲-), 1xMIC imipenem (-✳-), and 2xMIC imipenem (-○-)

The potential of imipenem against *P. aeruginosa* PAO1 was shown in Figure 4.3. The result showed that imipenem at 0.125xMIC no bactericidal activity. The bactericidal effect of imipenem at 0.25xMIC was found at 8 hours of incubation and recovered growth after 8 hours. Imipenem at 0.5x, 1x, and 2xMIC were found the bactericidal activity after 4 hours of incubation. Imipenem at 0.5xMIC was completely killed bacterial cell after 8 hours while imipenem at 1x, and 2xMIC were detected at 4 hours.

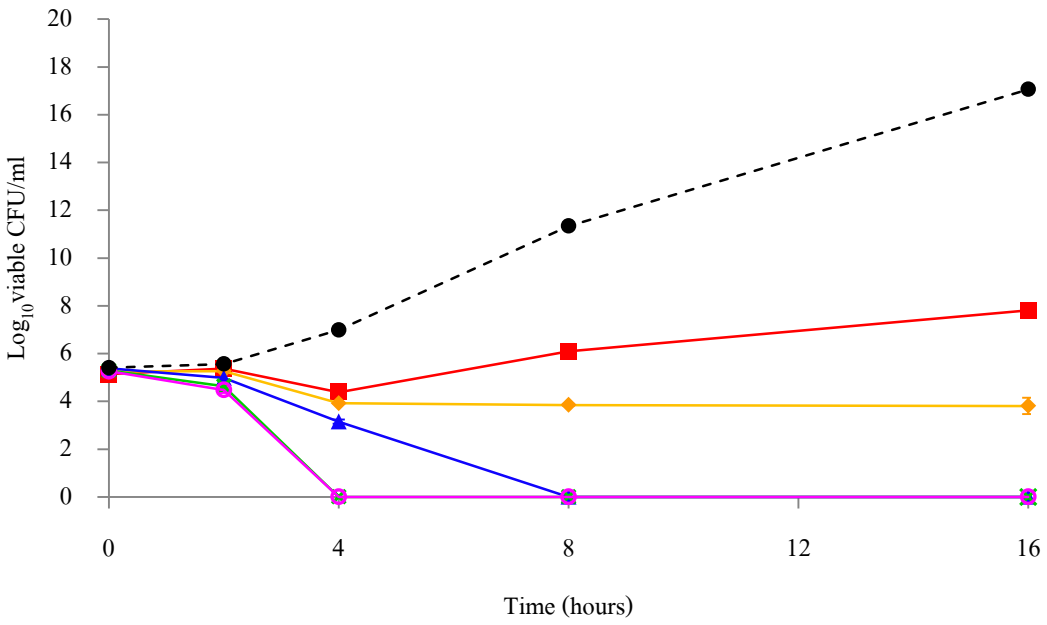


Figure 4.4 Time-kill curve of *P. aeruginosa* PAO1: untreated (-•-), treated with 0.125xMIC doripenem (-■-), 0.25xMIC doripenem (-◆-), 0.5xMIC doripenem (-▲-), 1xMIC doripenem (-✳-), and 2xMIC doripenem (-○-)

The potential of doripenem against *P. aeruginosa* PAO1 was shown in Figure 4.4. The result showed that doripenem at 0.125xMIC was found less bactericidal effect at 8 hours and recovered growth after 8 hours. The bactericidal effect of doripenem at 0.25x, 0.5x, 1x, and 2xMIC were found at 4 hours of incubation. Doripenem at 0.25xMIC was found less bactericidal effect at 4 hours of incubation. However, doripenem at 0.5xMIC was completely killed bacterial cell after 8 hours while doripenem at 1x, and 2xMIC were found at 4 hours.

4.4 Time-kill assay for antibiotic combination

The study of time-kill assay was examined for consideration time of synergy between fosfomycin and carbapenems. This experiment was performed at the antibiotic concentrations of 0.125x, 0.25x, and 0.5xMIC against *P. aeruginosa* PAO1. The result of synergy was defined as the bacterial viable cell reduction at ≥2log CFU/ml compared to the most active antibiotic (88).

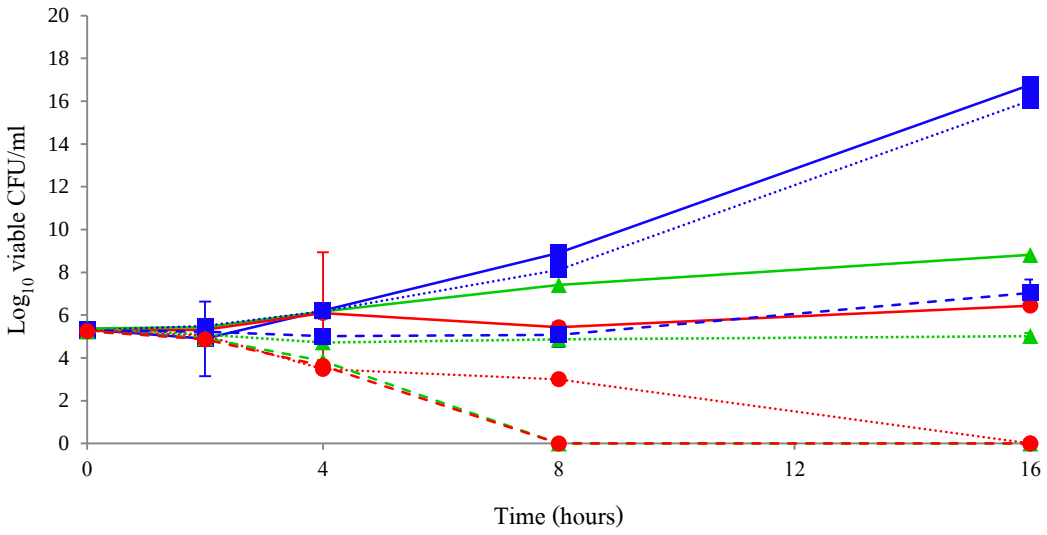


Figure 4.5 Time-kill curve of 0.125xMIC fosfomycin (—■—), 0.25xMIC fosfomycin (—▲—), 0.5xMIC fosfomycin (—●—), meropenem 0.125xMIC (···■···), meropenem 0.25xMIC (···▲···), meropenem 0.5xMIC (···●···), 0.125xMIC fosfomycin + 0.125xMIC meropenem (- ■ -), 0.25xMIC fosfomycin + 0.25xMIC meropenem (- ▲ -), and 0.5xMIC fosfomycin + 0.5xMIC meropenem (- ● -) with *P. aeruginosa* PAO1

Time kill assay between fosfomycin and meropenem was indicated results in Figure 4.5. The results showed bacterial killing in individual and combination antibiotics. In all concentration of combinations, the results were synergistic. Fosfomycin combined with meropenem at 0.125xMIC showed synergy at 8 hours. It reduced the viable bacterial cells about 3.04 log CFU/ml. As the same with 0.25 and 0.5xMIC, these concentration occurred synergy at 8 hours by reducing 4.86 and 3 log CFU/ml of viable bacterial cells, respectively.

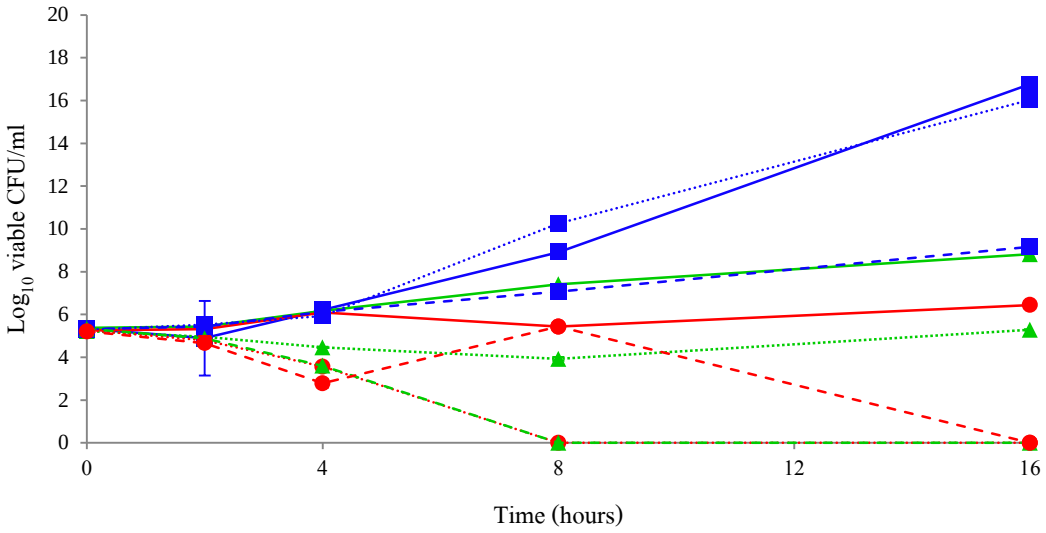


Figure 4.6 Time-kill curve of 0.125xMIC fosfomycin (—■—), 0.25xMIC fosfomycin (—▲—), 0.5xMIC fosfomycin (—●—), 0.125xMIC imipenem (···■···), 0.25xMIC imipenem (···▲···), 0.5xMIC imipenem (···●···), 0.125xMIC fosfomycin + 0.125xMIC imipenem (- ■ -), 0.25xMIC fosfomycin + 0.25xMIC imipenem (- ▲ -), and 0.5xMIC fosfomycin + 0.5xMIC imipenem (- ● -) with *P. aeruginosa* PAO1

Time kill assay of fosfomycin and imipenem was indicated in Figure 4.6. The results showed bacterial killing of individual and combined antibiotics. Fosfomycin combined with imipenem at 0.125xMIC showed synergistic effect at 16 hours. It reduced viable bacterial cells at about 6.87 log CFU/ml. At the concentration of 0.25xMIC of both antibiotics, synergistic effect occurred at 8 hours by reducing 3.92 log CFU/ml. There were no difference when treated with 0.5xMIC between single and combination treatment. However, combination treatment showed more effective than fosfomycin alone by 5.43 log CFU/ml reduction.

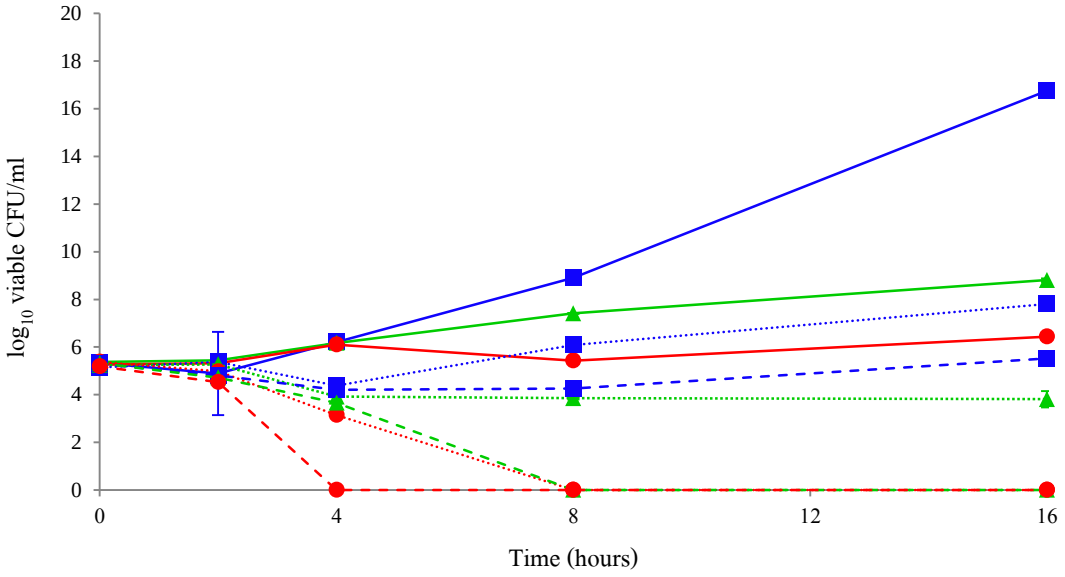


Figure 4.7 Time-kill curve of 0.125xMIC fosfomycin (—■—), 0.25xMIC fosfomycin (—▲—), 0.5xMIC fosfomycin (—●—),0.125xMIC doripenem (···■···), 0.25xMIC doripenem (···▲···), 0.5xMIC doripenem (···●···), 0.125xMIC fosfomycin + 0.125xMIC doripenem (- -■- -), 0.25xMIC fosfomycin + 0.25xMIC doripenem (- -▲- -), and 0.5xMIC fosfomycin + 0.5xMIC doripenem (- -●- -) with *P. aeruginosa* PAO1

Time kill assay of fosfomycin and doripenem was indicated in Figure 4.7. The results showed bacterial killing of individual and combined antibiotics. In all concentrations of combinations, the results showed that fosfomycin combined with doripenem at 0.125xMIC occurred synergy at 16 hours. It reduced the viable bacterial cell about 2.29 log CFU/ml. At concentration of 0.25xMIC, synergy occured at 8 hours by 3.85 log CFU/ml reduction. Moreover, at 0.5xMIC, synergy occurred at 4 hours by 3.15 log CFU/ml reduction.

4.5 Determination of correlation between synergistic effects, carbapenems susceptibility and carbapenems resistance mechanisms

Previous information of carbapenems resistance mechanisms of seventy CR-PA clinical isolates was analyzed to study the relationship of synergism, carbapenems susceptibility and resistance mechanisms. The mechanisms of resistance were over expression of MexAB-OprM, MexXY-OprM, loss of OprD porin, and AmpC-β-lactamase overproduction. The overexpression of multidrug efflux pump induces the resistance to many antibiotics. MexAB-OprM and MexXY-OprM overexpression have been reported to involve antibiotic resistance to meropenem and some effects to doripenem. Also, the overproduction of AmpC-β-lactamase involved to carbapenems resistance. Moreover, the loss of OprD porin increase the minimal inhibition concentration value of carbapenems.

Table 4.3 The relationship between synergistic effect of fosfomycin combined with carbapenems and percentage of positive in carbapenems-resistant mechanisms detection with seventy CR-PA clinical isolates

Combination drugs	Strains of synergy : n	Percentage of positive in carbapenems-resistant mechanisms detection			
		Mex AB (n)	Mex XY (n)	Opr D (n)	Amp C (n)
fosfomycin+ meropenem	28	82.14 % (23)	57.14 % (16)	82.14 % (23)	0 % (0)
fosfomycin + imipenem	27	96.30 % (26)	70.37 % (19)	88.89 % (24)	0 % (0)
fosfomycin + doripenem	32	87.50 % (28)	65.63 (21)	81.25 % (26)	0 % (0)

The strains of CR-PA clinical isolates with synergy in each combination were determined the relationship between resistance mechanisms and synergistic effect. The strains which showed synergy when fosfomycin combined with meropenem exhibited the positive result in MexAB-OprM overexpression resistance

mechanism about 71.88%, MexXY-OprM overexpression about 57.14%, and loss of OprD porin about 82.14%. While, the synergistic strains with fosfomycin combined with imipenem exhibited the positive result in MexAB-OprM overexpression resistance mechanism at about 96.30%, MexXY-OprM overexpression about 70.37%, and loss of OprD porin about 88.89%. Lastly, fosfomycin combined with doripenem showed the positive result in MexAB-OprM overexpression resistance mechanism about 87.50%, MexXY-OprM overexpression about 65.63% and loss of OprD porin about 81.26%. However, the previous result of Khuntayaporn P *et. al*. study did not detect the positive result in overproduction of AmpC-β-lactamase resistance mechanism.

Table 4.4 The relationship between carbapenems susceptibility and percentage of positive in carbapenems-resistant mechanisms detection with seventy CR-PA clinical isolates

Antibiotic resistance	Strains of resistance : n	Percentage of positive in carbapenems-resistant mechanisms detection			
		MexAB (n)	MexXY (n)	OprD (n)	Amp C (n)
meropenem resistance	5	100% (5)	100% (5)	100% (5)	0% (0)
imipenem resistance	0	0% (0)	0% (0)	0% (0)	0% (0)
doripenem resistance	0	0% (0)	0% (0)	0% (0)	0% (0)
meropenem and imipenem resistance	32	71.88% (23)	56.25% (18)	78.13% (25)	0% (0)
Meropenem and doripenem resistance	1	100% (1)	100% (1)	100% (1)	0% (0)
imipenem and doripenem resistance	0	0% (0)	0% (0)	0% (0)	0% (0)
all resistance	31	90.32% (28)	54.84% (17)	83.87% (26)	0% (0)

The strains of CR-PA clinical isolates were determined the relationship between resistance mechanisms and carbapenems susceptibility. The strains which showed only meropenem resistance that exhibited the positive result in MexAB-OprM, MexXY-OprM overexpression and loss of OprD resistance mechanism 100%. This result showed similarly to the result of determined the relationship between resistance mechanisms with both of meropenem and doripenem resistance. The strains

which showed both of meropenem and imipenem resistance exhibited the positive result in overexpression of MexAB-OprM about 71.88%, MexXY-OprM about 56.25% and loss of OprD resistance mechanism about 78.13%. Moreover, the strains with all carbapenems resistance exhibited the positive result in overexpression of MexAB-OprM about 90.32%, MexXY-OprM about 54.84% and loss of OprD about 83.87%. In this study did not found the strain with only imipenem or doripenem resistance and also both of imipenem and doripenem resistance strain.

4.6 Study on the frequency of resistance

Spontaneous mutation by single-step mutant method was performed to determine phenotypic mutants. The frequencies of resistance were examined at 2xMIC and 4xMIC with *P. aeruginosa* PAO1 and four strains of CR-PA clinical isolates. The results were demonstrated in Table 4.6 and 4.7.

The frequencies of resistance at 2xMIC with *P. aeruginosa* PAO1 were detected on agar plates, similarly to the MTCE-CM06, MTCB-SI19, MTCE-CH35, and MTCN-UB45 strains (Table 4.6). Individual fosfomycin did have the range of frequency of resistance between $3.15x10^{-9}$ to $2.72x10^{-11}$, while all strains showed no growth after treated with meropenem. The frequencies of resistance with imipenem of *P. aeruginosa* PAO1, MTCE-CH35, and MTCN-UB45 could not be found, while MTCB-SI19 showed the frequencies of resistance about 2.42x10-9. For doripenem, the frequencies of resistance were found only with MTCB-SI19 about $2.42x10^{-9}$. In case of the combination, fosfomycin combined with meropenem did have the frequencies in the range of $5.76x10^{-8}$ to $2.42x10^{-9}$. Although, *P. aeruginosa* PAO1 and MTCE-CH35 showed no growth on agar plate. The range of frequencies resistance of fosfomycin combined with imipenem was $1.92x10^{-8}$ to $4.41x10^{-10}$ and some bacterial growth were detected with MICB-SI19. Lastly, fosfomycin combined with doripenem was determined the range of resistance frequencies about $1.92x10^{-8}$ to $7.95x10^{-9}$ and no growth of *P. aeruginosa* PAO1, MTCE-CH35.

The frequencies of resistance at 4xMIC were found only with fosfomycin individual examination in the range of $2.71x10^{-8}$ to $7.09x10^{-9}$ (Table 4.7), while the rest did not grow on the plate.

Table 4.5 Results of frequency resistance by single-step mutants at 2xMIC

Antibiotic	*P. aeruginosa* PAO1	MTCN-CM06	MTCB-SI19	MTCE-CH35	MTCN-UB45
fosfomycin	TNTC	$3.15x10^{-9}$	$2.06x10^{-10}$	$2.72x10^{-11}$	$3.2x10^{-9}$
meropenem	ND	ND	ND	ND	ND
impenem	ND	TNTC	TNTC	ND	ND
doripenem	ND	ND	$2.42x10^{-9}$	ND	ND
fosfomycin + meropenem	ND	$6.8x10^{-8}$	$2.42x10^{-9}$	ND	$5.76x10^{-8}$
fosfomycin + imipenem	$7.24x10^{-9}$	$8.22x10^{-9}$	TNTC	$4.41x10^{-10}$	$1.92x10^{-8}$
fosfomycin + doripenem	ND	$7.95x10^{-9}$	$3.64x10^{-9}$	ND	$1.92x10^{-8}$

TNTC = too numerous to count (The colony were counted >300 colony)

ND = not detected (No colony grew on agar plate)

Table 4.6 Results of frequency resistance by single-step mutants at 4xMIC

Antibiotic	*P. aeruginosa* PAO1	MTCN-CM06	MTCB-SI19	MTCE-CH35	MTCN-UB45
fosfomycin	$2.97x10^{-9}$	$7.09x10^{-9}$	$1.75x10^{-9}$	$2.71x10^{-8}$	$6.47x10^{-9}$
meropenem	ND	ND	ND	ND	ND
imipenem	ND	ND	ND	ND	ND
doripenem	ND	ND	ND	ND	ND
fosfomycin + meropenem	ND	ND	ND	ND	ND
fosfomycin + imipenem	ND	ND	ND	ND	ND
fosfomycin + doripenem	ND	ND	ND	ND	ND

ND = not detected (No colony grew on agar plate)

4.7 Determination of post antibiotic effect (PAE)

The determinations of suppression in bacterial recovery growth after exposure to antibiotic were performed by post antibiotic effect method. The study was examined at the concentration of 2xMIC with *P. aeruginosa* PAO1. The bacteria were treated for 2 hours of culture treatment which PAE in these conditions were shown in Table 4.8.

Table 4.7 Post antibiotic effect of fosfomycin and carbapenems with 2XMIC of individual and in combination concentration against *P. aeruginosa* PAO1.

Antibiotic	MIC (µg/ml)	PAE (h)
fosfomycin	128	1
meropenem	4	2
imipenem	2	2
doripenem	2	2
fosfomycin+meropenem	32/0.5	2
fosfomycin+imipenem	16/0.5	2
fosfomycin+doripenem	16/0.5	2

At 2xMIC concentration, fosfomycin showed the efficacy to suppress the recovery growth of bacterial cell about 1 hour. Carbapenems showed that PAE were 2 hours the same as PAE fosfomycin when combined with carbapenems.

4.8 Morphological study by scanning electron microscopy (SEM)

Morphological study from antibiotic disruption was observed by SEM. This study used *P. aeruginosa* PAO1 treated with synergistic concentration of fosfomycin and carbapenems. Doripenem was the representative in carbapenems drug group.

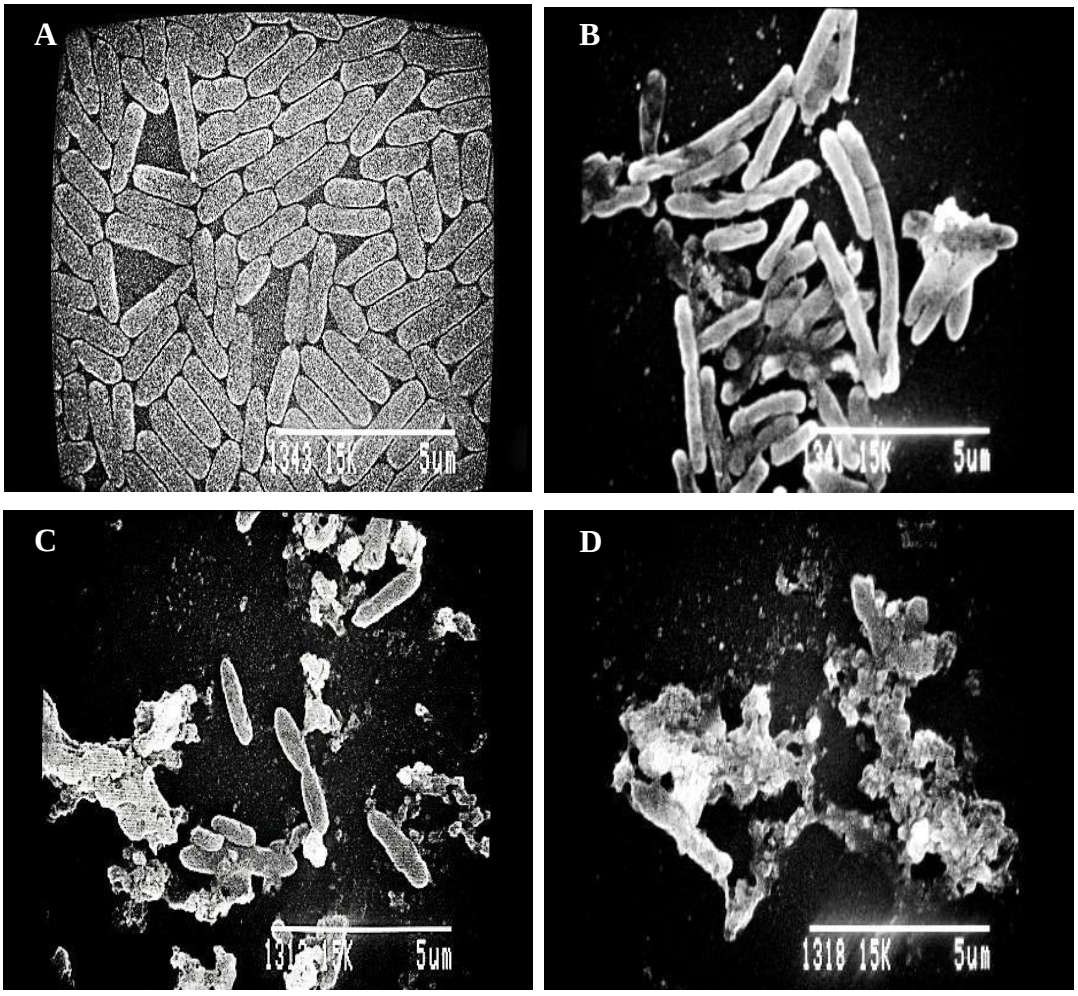


Figure 4.8 The photograph of *P. aeruginosa* PAO1 (A) treated with 0.25x MIC of fosfomycin (B), 0.25x MIC of doripenem (C), and 0.25x MIC of fosfomycin combined with 0.25x MIC of doripenem (D) after 8 hours. (Magnification of x15000)

Figure 4.8A exhibited rod shape of normal *P. aeruginosa* PAO1 cell when no antibiotic treatment. As showed in Figure 4.8B, Cells treated with fosfomycin were deformed of pore incidence and curly bacterial cell walls. These effects were noticeable observation in Figure 4.8C when treated with doripenem. Cells were destroyed as broken cells and some indicated pore of cell wall. Interestingly, the combination of drug treatment were extremely destroyed the cell. The bacterial cell walls were damage as indicated by blast cells (Figure 4.8D).

4.9 Biofilms study

4.9.1 Efficacy of antibiotics for bacterial biofilm removal

Effects of individual antibiotics to bacterial biofilms were indicated in Table 4.9. Individual antibiotics which were fosfomycin, meropenem, imipenem and doripenem were able to eliminate the viable bacterial biofilms depending upon the concentration. At the concentrations of 0.25xMIC, 0.5xMIC and 1xMIC of fosfomycin, logarithmic decrease in colony forming units were less than 1 log CFU/ml, following by 2xMIC that about 1.46 log CFU/ml. The greatest value in fosfomycin reaction was derived from bacterial biofilms treated with 0.125x MIC. In biofilms treated with meropenem, the logarithmic decrease in colony forming units were less than 1 logCFU/ml after applying the concentrations of 0.125xMIC, 0.25xMIC and 0.5x MIC. The highest value of logarithmic decrease was showed at 2xMIC as 3.59 logCFU/ml followed by 1xMIC at 1.87 logCFU/ml. Imipenem showed the best logarithmic decrease in colony forming unit at 1xMIC followed by 0.25xMIC, 2xMIC. Lastly, doripenem showed the highest activity in biofilm removal when compared to other antibiotics. The logarithmic decrease in colony forming unit of doripenem at 1xMIC, 0.5xMIC, 2xMIC, 0.25xMIC and 0.125xMIC were 7.78 log CFU/ml, 7.55 logCFU/ml, 7.16 logCFU/ml, 3 logCFU/ml and less than 1 logCFU/ml, respectively.

Table 4.8 The bacterial biofilms logarithmic decrease showed in colony forming unit after exposed individual antibiotics.

Antibiotic	Logarithmic decrease in colony-forming unit				
	0.125xMIC	0.25xMIC	0.5xMIC	1xMIC	2xMIC
fosfomycin	2.69	<1	<1	<1	1.46
meropenem	<1	<1	<1	1.87	3.59
imipenem	<1	1.69	<1	5.31	1.47
doripenem	<1	3.00	7.55	7.78	7.16

It could be summarized that doripenem showed the best efficacy at 1xMIC when compared to other concentration. Similarly, imipenem showed the best activity at 1xMIC but the efficacy was less than doripenem. Meropenem and fosfomycin showed less effective of bacterial biofilm eradication at the same concentration (Figure 4.9).

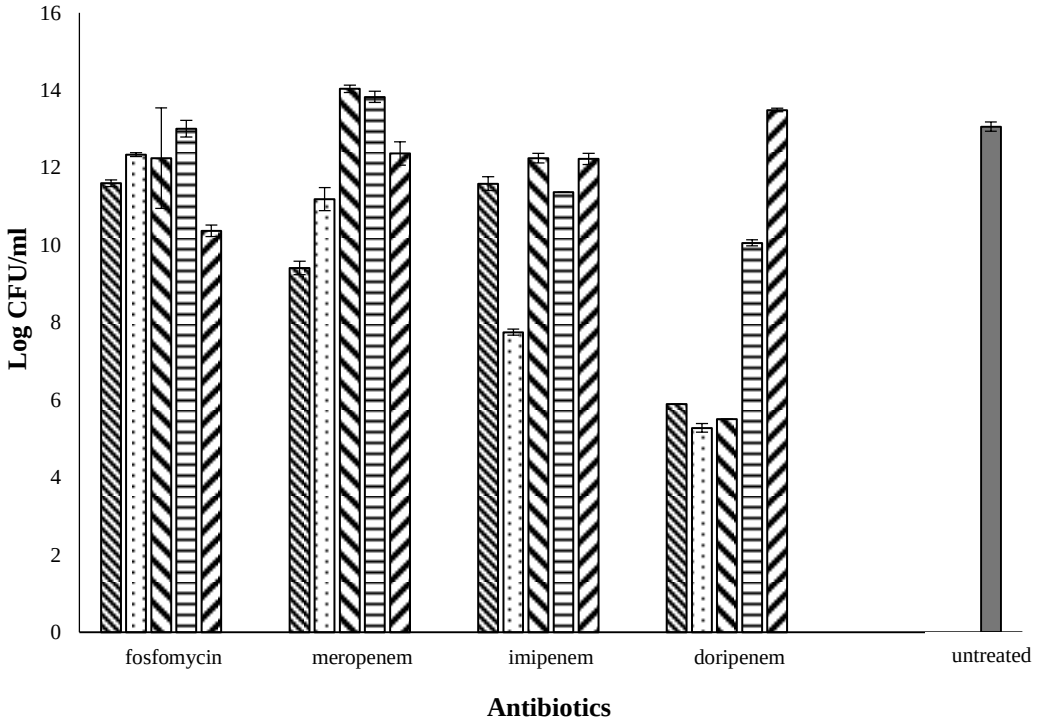


Figure 4.9 The efficacy of each antibiotic at 2xMIC (▨), 1xMIC (⊡), 0.5xMIC (▧), 0.25xMIC (▤), 0.125xMIC (▨), and untreated (■) to bacterial biofilms of *P. aeruginosa* PAO1.

4.9.2 Biofilms eradication with antibiotic combination treatment.

Effect of combined antibiotics to the bacterial biofilms were indicated in Tabal 4.10. Fosfomycin combined with carbapenems depending upon the concentration. The combiantion between fosfomycin and meropenem was able to decrease about 2.61 log CFU/ml, less than one log CFU/ml, 5.68 log CFU/ml bacterial biofilms at 0.125x, 0.25x, and 0.5x MIC, respectively. This combination showed the same value of decreasing logarithmic decrease in colony forming unit at 1x MIC and 2x MIC about 13.06 log CFU/ml. Fosfmycin combined with imipenem was able to

decrease about 3.69 log CFU/ml, 8.86 log CFU/ml, 13.06 log CFU/ml bacterial biofilms at 0.5x, 1x, and 2x MIC while, the concentrations of 0.125x and 0.25x MIC slightly exhibited the decreasing of bacterial biofilms with less than one logCFU/ml. Lastly, fosfomycin combined with doripenem was able to decrease about 2.51 log CFU/ml, 1.38 log CFU/ml, 8.76 log CFU/ml, 8.54 log CFU/ml, and 9.12 lo CFU/ml bacterial biofilms at 0.125x, 0.25x, 0.5x, 1x, and 2x MIC, respectively.

Table 4.9 The bacterial biofilms logarithmic decrease showed in colony forming unit after exposed combined antibiotics.

Antibiotic	Logarithmic decrease in colony-forming unit				
	0.125xMIC	**0.25xMIC**	**0.5xMIC**	**1xMIC**	**2xMIC**
Fosfomycin + Meropenem	2.61	<1	5.68	13.06	13.06
Fosfomycin + Imipenem	<1	<1	3.69	8.86	13.06
Fosfomycin + Doripenem	2.51	1.38	8.76	8.54	9.12

Figure 4.10 showed the result of activity of antibiotic combinations to bacterial biofilm. The greatest value of activity to eradicate bacterial biofilms was fosfomycin combined with meropenem at 1x and 2x MIC. This result was the same to fosfomycin combined with imipenem at 2x MIC. At the same concentration of fosfomycin combined with doripenem, the logarithmic decrease was less than meropenem and imipenem combinations. Moreover, fosfomycin combined doripenem at 0.5xMIC showed the greatest efficacy than both combinations at the same concentration.

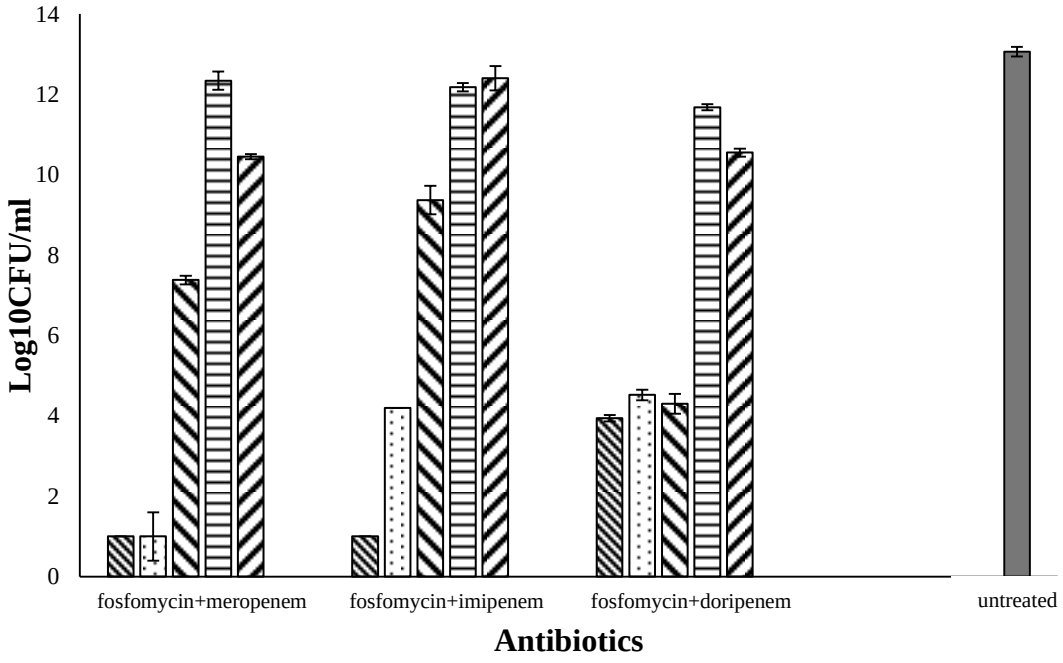


Figure 4.10 The efficacy of combination antibiotics at 2xMIC (▨), 1xMIC (⊡), 0.5xMIC (▧), 0.25xMIC (⊟), 0.125xMIC (▨), and untreated (■) to bacterial biofilms of *P. aeruginosa* PAO1.

CHAPTER V
DISCUSSION

The emergence of antibiotic resistant *P. aeruginosa* is being global problem in healthcare including Thailand (4, 10, 52, 53, 97). Carbapenems are group of β-lactam antibiotics that show potential against Gram-positive, Gram-negative bacteria including aerobes and anaerobes. These drugs are used for antipseudomonal infection. Patients with complicated infection and risk to mortality are recommended to antibiotic treatment with carbapenems (50, 58, 98). Recently, carbapenems resistance was discovered in patients with *P. aeruginosa* nosocomial infection (32, 42, 53, 62, 65). Therefore, the alternative treatment in carbapenems-resistant *P.aeruginosa* is being new options. The combination of antibiotic therapy is taken to consideration for using in antibiotic resistance of *P. aeruginosa* that involve carbapenems-resistant *P. aeruginosa* (39, 99).

This study focused on fosfomycin combined with carbapenems to enhance the inhibitory activity on the growth carbapenem-resistant *P. aeruginosa* clinical isolates from different regions of Thailand (4, 52). Fosfomycin is a unique structure of phosphonic acid derivative antibiotic. It is broad spectrum against most of Gram-positive bacteria and Gram-negative bacteria such as *P. aeruginosa* and less profile of toxicity (66). Interestingly, it has been reported the effective against *P. aeruginosa* in combination with β-lactam antibiotics (66, 76). This study focused on fosfomycin combined with carbapenems against seventy CR-PA clinical isolates. Most of CR-PA clinical isolates showed carbapenems resistance more than 80% of MIC value according to the susceptibility breakpoint of CLSI guideline (85). Most CR-PA showed high MIC value of fosfomycin at MIC_{90} ≥1,024 μg/ml. The susceptibility breakpoint of fosfomycin was defined the resistance at ≥256 μg/ml (100). However, British Society for Antimicrobial defined the resistance to fosfomycin at >128 mg/ml (101), and >32 mg/ml with European committee on antibiotic susceptibility testing (102). Therefore, fosfomycin was recommended to use in combination therapy for

P. aeruginosa infection treatment. The combination could reduce the MIC value of fosfomycin and enhance the efficacy of bactericidal activity (103, 104).

For this reason, the combination between fosfomycin and carbapenems was of interest to study for the synergy against CR-PA. The determinations of synergy were performed by broth microdiltion checkerboard as the standard evaluation of antibiotic combination in laboratory (12). The results percentage synergistic effect showed that fosfomycin combined with doripenem did have the highest percentage of synergy in 45.71% of the isolates when compared to the other combinations tested. The FIC values of fosfomycin combined with meropenem and imipenem were 40% and 38.57%, respectively. All combination showed no statistically difference. Kastoris AC *et al*. studied the combination between fosfomycin and imipenem against multidrug resistant *P. aeruginosa* clinical isolated strains and showed the percentage of synergy about 36.13% (76). Similarly, the combination of fosfomycin and meropenem in the study by Okazaki M *et al*. (104) showed synergistic effect between fosfomycin and meropenem. Doripenem is the new antibiotic in carbapenems. It was approved in 2007 (57). Therefore, the data of synergistic effect between fosfomycin combined with doripenem still have not enough information. However, the study of fosfomycin with other antibiotics did show several reports of synergistic effect against *P. aeruginosa* (76). The data have been reported synergistic effects with fosfomycin and other antimicrobials for Gram-positive and Gram-negative strains including *P. aeruginosa*. Cai Y et al. (6) showed fosfomycin synergistic effects with other treatments against *P. aeruginosa*.

Fosfomycin combined with carbapenems against *P. aeruginosa* PA01 were demonstrated the rate of bacterial killing by time-kill assay and also tested in individual antibiotic. After 8 hours of treatment with fosfomycin at 0.25x MIC, 0.5x MIC, 1x MIC and 2x MIC occurred the bactericidal activity. Only fosfomycin at 2x MIC was completely killed the bacterial cells. Accordingly, fosfomycin was indicated time dependent killing manner (75). Similarly, carbapenems were antibiotics which act in time dependent fashion (56, 98). However, the results of combination between fosfomycin and carbapenems showed the bactericidal activity with concentration-dependent fashion. Previous studies have been reported the bacterial activity of fosfomycin combined with other antibiotics by concentration-dependent fashion (5,

18, 105). Macleod *et al*. examined fosfomycin combined with tobramycin against *P. aeruginosa* and *S. aureus* (18) and showed the rate of bacterial killing in concentration-dependent fashion. Moreover, Corvec S *et al*. studies showed that fosfomycin combined with tigecycline and colistin did have the killing in concentration-dependent fashion (105).

In previous study of carbapenems multidrug resistance (4, 52) that showed *P. aeruginosa* with carbapenems resistance did have high percentage of loss OprD porin. Loss of OprD was the major mechanism to carbapenems resistance. OprD transport porin is responsible for basic amino acid uptake into the bacterial cell. It also was the porin for carbapenems diffusion into *P. aeruginosa* cells (8, 49, 50, 54). In this study indicated that fosfomycin enhanced the efficacy of carbapenems transportation into the bacterial cell. The results of greatest percentage of positive MexAB-OprM detection in fosfomycin combined with imipenem synergistic strains indicated that MexAB-OprM did not more affect to imipenem pump out of the bacterial cell. Efflux pumps are the systems that involve in carbapenems extrude via outer membrane protein. The presence of these efflux systems induced the carbapenems resistance, especially meropenem that was the substance for MexAB-OprM efflux pump (13). However, high percentage of positive efflux system was found synergistic effect of fosfomycin combined with carbapenems. Generally, AmpC β-lactamase was less effect to carbapenems resistance. Carbapenems was the potent antibiotic to inhibit β-lactamase enzyme. However, this study did not fine the over-expression of AmpC-β-lactamase. Moreover, results of relationship between strains of carbapenems resistance and positive results of carbapenems resistance mechanism indicated that MexAB-OprM greater affected to the carbapenems resistance, especially meropenem and then followed with loss of OprD and overexpression MexXY-OprM. As the previous studies explained that the efflux pump MexAB more affected to β-lactam antibiotics and meropenem, except imipenem but not for imipenem hyperproducing mutants (62).

The antibiotic resistance frequencies of combination were demonstrated by spontaneous resistance of individual antibiotic. This experiment measured the phenotypic mutation all of mutants present as phenotypic mutation rate. The results of frequencies resistance was the several different genotypic mutation events (89). Treatment high concentration of combination did not show the antibiotic mutation.

Similarly, the individual carbapenems did not show antibiotic mutation. After treated with 2x MIC of carbapenems, low level of frequency resistance was detected. Moreover, after treated with fosfomycin, high rates of frequency resistance at 2x MIC and 4xMIC were detected. Previous studies have showed rapid fosfomycin resistance at high concentration antibiotics. However, *in vivo* study did not show the evidence of fosfomycin development (18, 89). Rojas AR *et al*. reported the frequency resistance of fosfomycin, imipenem, and meropenem in combination and individual against *P. aeruginosa* PAO1 strain at low rate and including against mutant strain that showed the rate frequency resistance at low (7).

The post antibiotic effect method measured antibiotic suppression of microbial growth after treated with the antibiotic at lower MIC concentration. It was an advantage to the determination of pharmacodynamics in combination and application for dosing regimens (106). The results showed PAE values of fosfomycin at 1 hour and other tests at 2 hours. The PAE value of combinations unaltered at the lower concentration. This result was corresponded to the results of time-kill kinetics. It showed slow regrowth of bacterial cells at the concentration below level of inhibition.

The combination of fosfomycin and carbapenems did not show the development of resistance after treat with high concentration. As Nabin K *et al.* report, fosfomycin unique acts at a target site that was not affected to other antibiotic. Therefore, it exhibited a little of other antibiotic cross-resistance (104).

This study used doripenem for testing morphological change to *P. aeruginosa* PAO1 by scanning electron microscope (SEM) (93). Fosfomycin at 0.25x MIC was found less affinity to destroy *P. aeruginosa* PAO1 cells. As previous data, that showed the efficacy of fosfomycin to destroy *P. aeruginosa* cells in high concentration (66, 76). The results of PAO1 morphology after treated with 0.25xMIC of doripenem showed that some bacterial cells were destroyed and appeared as deformed shape of cells. Moreover, the combination of fosfomycin and doripenem at the same concentration was exhibited highly disruption of bacterial cells. This demonstration was compatible to the killing curve of combination between fosfomycin and doripenem at 0.25xMIC after treated 8 hour. The disruption of bacterial cells was occurred by the action of both antibiotics. The combination acted directly on the bacterial cell wall. Fosfomycin activated by UDP-GlcNAc enolpyruvyl transferase

(MurA) inactivation resulting in the inhibition of cell wall synthesis (71). Moreover, it also was a unique target of action that prevented antibiotic cross-resistance to doripenem (107). Doripenem was activated by inhibition of peptidase domain of PBPs protein. It was potent to most β-lactamases (108). The results of morphological change supported the efficacy of fosfomycin combined with doripenem against *P.aeruginosa*.

One of important antibiotic resistant virulence factors was bacterial biofilm. It was encapsulated structure of microorganism that formed as extracellular polymeric substance (EPS) structure (29). This study focused on the activity of combination antibiotics between fosfomycin and carbapenems against carbapenems-resistant *P. aeruginosa* which showed some effects on planktonic cells more than treated with fosfomycin or carbapenems alone. Similarly, the activity to bacterial biofilms was shown in the combinations more than antibiotic alone. Also, several studies have showed that the individual antibiotic had less effective to eradicate the bacterial biofilm (92, 95, 109-113). Koichi M. *et al*. reported fosfomycin combined with ofloxacin were clearly effect *P. aeruginosa* OP14-210 biofilm cells (92). Likewise, the study of Gregory F. *et al*. showed fosfomycin combined with tobramycin could inhibit the activity of initial biofilm formation at 64 mg/L (110). Therefore, fosfomycin combined with carbapenems might be effective to treat *P. aeruginosa* infection. Also, it could reduce the concentration and side effect in treatment.

In conclusion, our study suggested that fosfomycin combined with carbapenems did have more effective than individual carbapenems to treat *P. aeruginosa*. Moreover, this combination effected to enhance the synergistic effect against carbapenems-resistant *P. aeruginosa* strains. Especially, doripenem combined with fosfomycin could effect the resistant strains. The advantage of this efficiency was reduction of drug concentration in conventional treatment. It could be alternative antibiotic against *P. aeruginosa* and also of carbapenems-resistant strains. In addition, the benefit of combination was able to reduce the cost of antibiotic treatment. The best characteristic of fosfomycin is mild adverse effect (66, 69, 70), which is an advantage to be used in combination with carbapenems for *P. aeruginosa* infection treatment.

CHAPTER VI
CONCLUSION

P. aeruginosa is one of Gram-negative pathogen with intrinsic resistance to many antibiotics. Carbapenems is the group of β-lactam with potency to inhibit Gram-negative and Gram-positive bacteria including *P. aeruginosa*. Carbapenems is empirical antibiotics for serious *P. aeruginosa* infection with high mortality. The emergence of carbapenems resistance in *P. aeruginosa* becomes the major problem of clinical treatment setting in public healthcare treatment. Therefore, the alternative treatment is being interesting in the state of shortest development for new antibiotic. Fosfomycin is grouped in phosphonic acid derivatives that has been revived the attention against *P. aeruginosa*. The efficiency of fosfomycin has been reported on the combination with other antibiotics including β-lactam drug to inhibit *P. aeruginosa* growth. The distinctive attributes of fosfomycin are unique structure, prevent cross-resistant to other antibiotic, and also less adverse effect of using in human. Therefore, our study was to determine the efficacy of fosfomycin combined with carbapenems against seventy carbapenems-resistant *P. aeruginosa* clinical isolates from the hospitals in all regions of Thailand.

The susceptibility test was performed to examine the MIC value of each strain by broth microdilution method. The results demonstrated the percentage of resistance about 98.57% with meropenem, 98.57% with imipenem, and 85.71 % with doripenem. Fosfomycin did have high MIC_{90} value more than 1,024 μg/ml. The experiment of synergy test between fosfomycin combined with carbapenems were found synergism of fosfomycin combined with doripenem about 40%, fosfomycin combined with meropenem about 40%, and fosfomycin combined with imipenem about 38.57%. The results did not show the antagonism in all combinations. Therefore, the combination of fosfomycin and carbapenems did have the capability to inhibit carbapenems-resistant *P. aeruginosa*.

P. aeruginosa PAO1 was selected to examine individual and combination of kinetic and rate killing against bacterial cells. The results showed synergistic effect in all combinations. The combination testing indicated the concentration-dependent manner of bacterial killing at 8 hours. The strains with positive synergy results possessed loss of OprD porin more than 80%. This indicated that all resistant strains lacked the specific carbapenems uptake porin. Furthermore, the major mechanism of carbapenems efflux system, MexAB-OprM overexpression, was found more than 70%. Also, the minor mechanism MexXY-OprM, was found the over-expression more than 50%. Therefore, the strains that showed synergistic effect did have carbapenems resistance mechanisms. It exhibited the potential of fosfomycin to enhance carbapenems activity.

The study of frequency resistance with individual and combination did not show the antibiotic resistance at 4xMIC, exceptionally with individual fosfomycin. At the concentration of 2xMIC, the frequency resistance result of combinations showed similarity to the result of individual fosfomycin. The results of PAE value with *P. aeruginosa* PAO1 in combination had no difference to the results of individual carbapenems. The results showed PAE value at 2 hours in combination and individual carbapenems whereas individual fosfomycin showed 1 hour. Therefore, fosfomycin did not affect the combination treatment in terms of resistance and suppression duration.

Treatment of fosfomycin combined with doripenem at 0.25xMIC exhibited clear cell disruption when compared with individual antibiotic. Taken together, all results indicate the useful and potential of fosfomycin in combination with carbapenems. These combinations were able to enhance the activity of synergistic effect against CR-PA. Moreover, after examined the activities of susceptibility to bacterial biofilms that combination treatment were found the decrease in the number of bacterial growth. At 0.5xMIC of combinations, more effect on viable bacterial cells was detected. The results of antimicrobial activity to bacterial biofilms showed bacterial biofilm eradication of fosfomycin combined with carbapenems. Therefore, our study suggested that fosfomycin and carbapenems could be able to use in carbapenems-resistant *P. aeruginosa* infection. However, further studies are needed to examine the mechanisms of fosfomycin which enhance carbapenems transport into

P. aeruginosa cells, the compatibility of dosage regimen, the distribution of combination in tissue organs and blood, including the efficacy on *P. aeruginosa* biofilms synthesis strain which is one of resistance factors to antibiotics.

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APPENDICES

1. The susceptibility of all isolated strains and *P. aeruginosa* PAO1 to carbapenems as determined by broth microdilution method.

PA isolated strains	Susceptibility		
	Meropenem	**Imipenem**	**Doripenem**
PA O1	S	I	S
MTCE-CH 08	R	R	I
MTCE-CH 11	R	R	R
MTCE-CH 28	R	I	R
MTCE-CH 32	R	R	R
MTCE-CH 35	R	R	R
MTCE-CH 41	R	R	R
MTCE-CH46	R	R	I
MTCE-CH 48	R	R	I
MTCN-CM 03.1	R	R	R
MTCN-CM 06	R	R	R
MTCN-CM 10.2	R	I	I
MTCN-CM 11	R	R	I
MTCN-CM 12	R	R	R
MTCN-CM 15.2	R	R	R
MTCN-CM 25	R	I	I
MTCN-CM 27.2	R	R	R
MTCN-CM 28.2	R	R	R
MTCN-CM 41	R	R	I
MTCN-KK04	R	R	S
MTCN-KK16	R	R	R
MTCN-KK 19	R	R	R
MTCN-KK 20	R	R	R
MTCN-KK26	R	R	R
MTCN-KK29	R	R	R
MTCN-KK 50	R	R	R
MTCB-RA 05	R	R	I
MTCB-RA 15	R	R	S
MTCB-RA 22	R	R	I
MTCB-BRA 23	R	R	I
MTCB-RA 30	R	R	R
MTCB-RA 37	R	R	R
MTCB-RA 41	R	R	I
MTCB-RA 42	R	R	I
MTCE-RY 03	R	S	S
MTCE-RY 05	R	R	I
MTCE-RY 08	R	R	I
MTCE-RY 12	R	I	S
MTCE-RY 13	R	R	R
MTCE-RY 14	R	S	I

1. The susceptibility of all isolated strains and *P. aeruginosa* PAO1 to carbapenems as determined by broth microdilution method (cont).

PA isolated strains	Susceptibility		
	Meropenem	**Imipenem**	**Doripenem**
MTCE-RY 15	R	R	R
MTCE-RY 31	R	R	I
MTCE-RY 33	R	R	I
MTCE-RY 37	R	R	S
MTCB-SI 04	R	R	I
MTCB-SI 05	R	R	R
MTCB-SI 14	R	R	I
MTCB-SI 19	R	R	I
MTCB-SI 22	R	R	I
MTCB-SI 26	R	R	S
MTCB-SI 27	R	R	I
MTCB-SI 29	R	R	I
MTCS-SK 2.01	R	R	S
MTCS-SK 14	R	R	R
MTCS-SK 20	R	R	I
MTCS-SK 24	R	R	R
MTCS-SK 38	R	R	I
MTCS-SK 48	R	R	I
MTCS-SR 2.12	R	R	R
MTCS-SR 2.19	R	R	I
MTCS-SR 2.32	R	R	R
MTCS-SR 24	I	I	I
MTCS-SR 27	R	R	S
MTCN-UB 01	R	R	I
MTCN-UB 07	R	R	I
MTCN-UB 12	R	R	R
MTCN-UB 26	R	R	R
MTCN-UB 30	R	R	R
MTCN-UB 40	R	R	R
MTCN-UB 45	R	R	R
MTCN-UB 46	R	R	R

CLSI breakpoint, 2012 for susceptible and resistant to doripenem ≤ 2 and ≥8 μg/ml; imipenem ≤ 2 and ≥8 μg/ml, and meropenem ≤ 2 and ≥8 μg/ml, respectively (85).

2. The FIC values with seventy carbapenems-resistant isolates and *P. aeruginosa* PAO1 strain.

PA isolated Strains	fosfomycin + meropenem		fosfomycin + imipenem		fosfomycin + doripenem	
	FIC index	result	FIC index	result	FIC index	result
PAO1	0.5	SYN	0.25	SYN	0.38	SYN
MTCE-CH 08	1.03	IND	0.56	IND	1.03	IND
MTCE-CH 11	0.63	IND	0.63	IND	0.25	SYN
MTCE-CH 28	1.03	IND	1.13	IND	0.53	IND
MTCE-CH 32	1.03	IND	0.56	IND	0.56	IND
MTCE-CH 35	0.75	IND	0.5	SYN	0.75	IND
MTCE-CH 41	1.03	IND	0.75	IND	1.03	IND
MTCE-CH 46	0.5	SYN	0.75	IND	0.5	SYN
MTCE-CH 48	0.38	SYN	0.63	IND	0.38	SYN
MTCN-CM 03.1	1.25	IND	0.63	IND	0.75	IND
MTCN-CM 06	0.38	SYN	0.5	SYN	0.31	SYN
MTCN-CM 10.2	0.56	IND	0.63	IND	0.1875	SYN
MTCN-CM 11	0.5	SYN	0.5	SYN	0.63	IND
MTCN-CM 12	1	IND	0.75	IND	0.75	IND
MTCN-CM 15.2	1.13	IND	1.13	IND	0.75	IND
MTCN-CM 25	0.63	IND	1.03	IND	0.63	IND
MTCN-CM 27.2	0.5	SYN	0.75	IND	0.75	IND
MTCN-CM 28.2	0.56	IND	0.5	SYN	0.5	SYN
MTCN-CM 41	0.38	SYN	0.38	SYN	0.38	SYN
MTCN-KK 04	0.5	SYN	0.63	IND	0.75	IND
MTCN-KK16	0.38	SYN	0.5	SYN	0.63	IND
MTCN-KK 19	0.56	IND	0.63	IND	0.75	IND
MTCN-KK 20	0.5	SYN	0.5	SYN	0.75	IND
MTCN-KK26	0.75	IND	0.75	IND	1	IND
MTCN-KK29	0.38	SYN	0.28	SYN	0.17	SYN
MTCN-KK 50	0.5	SYN	0.75	IND	0.38	SYN
MTCB-RA 05	1.03	IND	1.03	IND	1.03	IND
MTCB-RA 15	0.13	SYN	0.31	SYN	0.19	SYN
MTCB-RA 23	0.75	IND	1	IND	0.75	IND

2. The FIC values with seventy carbapenems-resistant isolates and *P. aeruginosa* PAO1 strain (cont).

PA isolated Strains	fosfomycin + meropenem		fosfomycin + imipenem		fosfomycin + doripenem	
	FIC index	result	FIC index	result	FIC index	result
MTCB-RA 30	0.53	IND	0.38	SYN	0.38	SYN
MTCB-RA 37	0.38	SYN	0.75	IND	0.38	SYN
MTCB-RA 41	0.5	SYN	0.5	SYN	0.5	SYN
MTCB-RA 42	0.56	IND	1.03	IND	1.03	IND
MTCE-RY 03	1	IND	0.5	SYN	0.5	SYN
MTCE-RY 05	0.75	IND	0.5	SYN	0.5	SYN
MTCE-RY 08	0.75	IND	0.75	IND	0.75	IND
MTCE-RY 12	1.03	IND	0.63	IND	1.03	IND
MTCE-RY 13	0.38	SYN	0.38	SYN	0.5	SYN
MTCE-RY 14	1.03	IND	1.03	IND	1.03	IND
MTCE-RY 15	0.31	SYN	0.375	SYN	0.5	SYN
MTCE-RY 31	1	IND	1	IND	0.75	IND
MTCE-RY 33	1.03	IND	1	IND	1.03	IND
MTCE-RY 37	1.03	IND	1.03	IND	1.03	IND
MTCB-SI 04	0.38	SYN	0.63	IND	0.25	SYN
MTCB-SI 05	0.5	SYN	0.5	SYN	0.38	SYN
MTCB-SI 14	0.75	IND	1.13	IND	0.75	IND
MTCB-SI 19	0.5	SYN	0.75	IND	0.75	IND
MTCB-SI 22	0.5	SYN	0.5	SYN	0.63	IND
MTCB-SI 26	0.38	SYN	0.5	SYN	0.38	SYN
MTCB-SI 27	1	IND	1	IND	1	IND
MTCB-SI 29	0.38	SYN	0.75	IND	0.5	SYN
MTCS-SK 2.01	0.31	SYN	0.38	SYN	0.5	SYN
MTCS-SK 14	0.56	IND	0.31	SYN	0.28	SYN
MTCS-SK 20	1.03	IND	1	IND	1.03	IND
MTCS-SK 24	0.38	SYN	0.28	SYN	0.09	SYN
MTCS-SK 38	1.03	IND	1.03	IND	1.03	IND
MTCS-SK 48	1.03	IND	1.03	IND	1.03	IND
MTCS-SR 2.12	0.5	SYN	0.38	SYN	0.38	SYN

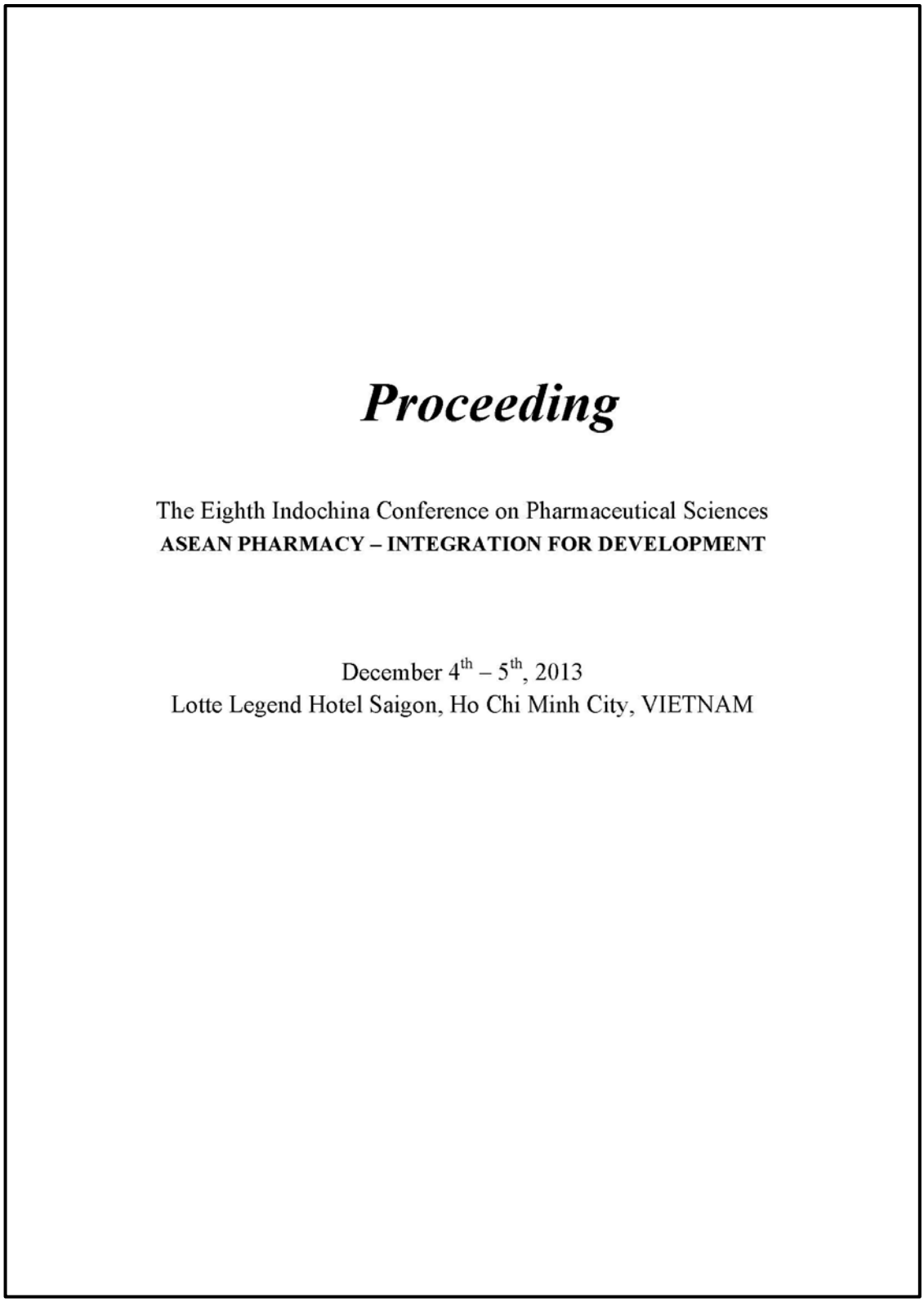
2. The FIC values with seventy carbapenems-resistant isolates and *P. aeruginosa* PAO1 strain (cont).

PA isolated Strains	fosfomycin + meropenem		fosfomycin + imipenem		fosfomycin + doripenem	
	FIC index	result	FIC index	result	FIC index	result
MTCS-SR 2.19	0.75	IND	0.53	IND	1	IND
MTCS-SR 2.32	0.75	IND	0.5	SYN	0.38	SYN
MTCS-SR 24	0.38	SYN	1.0	IND	0.63	IND
MTCS-SR 27	0.5	SYN	0.5	SYN	0.5	SYN
MTCN-UB 01	0.63	IND	0.5	IND	0.75	IND
MTCN-UB 07	0.75	IND	1.13	IND	0.75	IND
MTCN-UB 12	0.56	IND	0.75	IND	0.75	IND
MTCN-UB 26	1.63	IND	0.31	SYN	0.5	SYN
MTCN-UB 30	0.75	IND	0.75	IND	1	IND
MTCN-UB 40	0.75	IND	0.75	IND	0.5	SYN
MTCN-UB 45	0.75	IND	0.63	IND	0.38	SYN
MTCN-UB 46	0.63	IND	0.38	SYN	0.38	SYN
MTCB-RA 22	0.38	SYN	0.31	SYN	0.38	SYN

IND = indifference

SYN = synergy

3. Proceeding : Characterization of fosfomycin resistance among clinically isolated carbapenems-resistant *Pseudomonas aeruginosa* in Thailand



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CHARACTERIZATION OF FOSFOMYCIN RESISTANCE AMONG CLINICALLY ISOLATED CARBAPENEMS-RESISTANT *PSEUDOMONAS AERUGINOSA* IN THAILAND

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Abstract

The carbapenems, β-lactam antibiotics with broad spectrum activity on Gram-positive and Gram-negative bacteria including anaerobes, have been used extensively in severe infection treatment. Unfortunately, the emergence of carbapenems resistance phenomenon of *Pseudomonas aeruginosa* becomeshighly concerned problem in public healthcare. Renewal of fosfomycin usage has been of interest due to its unique mechanism of action by interfering the first step of cell wall biosynthesis and good side effect profile. Therefore, this study was aimed to investigate the resistance rate of fosfomycin in Thailand to explore the possible treatment option for drug resistant pathogens. Seventy strains of carbapenems-resistant *P. aeruginosa* clinical isolates were collected from various hospitals across Thailand and evaluated for MICs by broth dilution method. The result showed that MIC range of fosfomycin was varied between 8 to over 1,024 μg/ml and the average MIC_{50} was about 265-512 μg/ml. It is noteworthy that significantly high fosfomycin resistance rate was detected at about 90% of carbapenems-resistant strains. The resistance rates were at about 81.25, 80, 100, 100 and 86.67% in the Central, Northern, Eastern, Southern and Northeastern region of Thailand, respectively. The time-kill assay was further performed to examine the killing rate at 0.5, 1, 2 and 4 times of MIC compared with the wild-type strain, *P. aeruginosa* PAO1. Time-kill kinetic revealed that fosfomycin could inhibit the growth of bacteria at the concentration of 1x MIC at 8 hour. The single step mutation was used to investigate the frequency of spontaneous mutation of clinically isolated strains. The frequency of fosfomycin resistance occurred in clinical strains was less than PAO1. Our data demonstrated that fosfomycin had highly frequency of spontaneous mutation rate. This study showed that carbapenems-resistant *P. aeruginosa* in Thailand was highly resistant to fosfomycin. Combination study of fosfomycin is still of interest and under investigation as an optional treatment of carbapenems resistant *P. aeruginosa.*

Keywords: fosfomycin; carbapenems-resistant *P. aeruginosa*; minimum inhibition concentration

Introduction

The carbapenems are antimicrobials in a class of β-lactamantibiotic. It is one of the last resources for bacterial infectiontreatment, especially *P. aeruginosa.* The phenomenons of bacterial resistance to carbapenems have been reported on increasing resistance incidence. One of them is*P. aeruginosa,* whichis a gram-negative bacterium classified asseriously opportunistic pathogen of nosocomial infection.*P. aeruginosa*is intrinsic resistance to numerous antimicrobial agents.The bacterium is able to infect a lot of system; such septicemia, urinary tract infections, pneumonia, chronic lung infections, osteochondritis, dermatitis, andendocarditis. The emergence and dissemination of carbapenems-resistant *P. aeruginosa* are problems in public health worldwide treatment. In Thailand, the strains of carbapenems-resistance have beenincreasing in *P. aeruginosa*infected patients.

Fosfomycin is a phosphonic acid derivative (cis–1,2-epoxypropylphosphonic acid). Itwas initially discoveredmore than 40 years from *Streptomyces* species. The efficacy of fosfomycin has a broad spectrum to inhibitthe growth of gram-positive and gram-negative bacteria. In various countries, fosfomycin has been used to treat patients infected with multidrug resistance. The mode of action of fosfomycin is inhibiting the first step of peptidoglycan biosynthesis bybinding to enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA). The result of inactive MurA enzyme is leading to inhibiting N-acetylmuramic acidsynthesis. Therefore,the bacterial disruption by N-acetylglucosamineand phosphoenolpyruvate is missing in peptidoglycan. The adverse effects of fosfomycin have been reported in low rate; generally associated withmild gastrointestinal disturbances, nausea, neutropenia, local phlebitis, pain at injection site, and eosinophil count changes.However, previous studies have been apprised about spontaneous resistant mutation. For the reason, the aim of study wastoinvestigate the activity of fosfomycin against carbapenems-resistant *P. aeruginosa* in Thailand.

Materials and Methods

Bacterial strains and antibiotics

The seventy strains of carbapenems-resistant *P. aeruginosa*clinically isolated strains were collected from various hospitals in Thailand. *P.aeruginosa* PAO1 wild-type strain was used as the standard strain. The antibioticswerepurchased from Sigma-Aldrich (St Louis, MO, USA).

Determination of minimal inhibition concentration (MICs)

The susceptibility testing was broth-microdilution method as described inCLSI (Clinical and Laboratory Standards Institute 2012).The maximum concentration value of fosfomycin was limited at1,024µg/ml. This method was prepared by serial two-fold dilution with cation-adjusted Mueller-Hinton broth (CA-MHB) (Becton, Dickinson and Company, USA) in 96-well microtiter- plates (Thermo scientific Nunc, China). The bacterial inoculum were cultured for 16-18 hour at 37°C and adjusted to 5 x 10^5 CFU/ml in wells. The MICs were defined as the lowest concentration as invisible growth of bacterial cell.

Time-kill assay

Time-kill interactions were performed in test-tubes contains various concentrations of fosfomycin. The concentrations of fosfomycin were examined at 0.5, 1, 2, and 4 times of MIC. Thereaction tubes were incubated at 37ºC in a shaking water-bath with approximately 5x 10^5CFU/ml of bacterial inoculum.The aliquots (100 µl)of sample were diluted with 0.9%NaCl and colony counting was spreaded on Mueller-Hinton agar plates. These samples were collected to investigate at 0, 2, 4, 8, and 16 hour of incubation, similarly to growth control.

Single-step mutation method

Spontaneous mutation by antibiotic simulated was observed as the resistant antibiotic with the single step mutation. The inoculums approximately 10^9-10^{10} CFU/ml were spreaded on to Mueller-Hinton agar plates which containing 2 times of MIC of individual and combined antibiotics. After incubation at 37ºC for 48 hours, the frequencies of mutationwere calculated from the number of bacteria grew up on each antibiotic platedividedby the number of bacteria in inoculum.

Results and Discussion

Antimicrobial Susceptibility Test

The rising rates of resistance to carbapenems in *P. aeruginosa*have been emerged [1]. Fosfomycin is one of antipseudomonal, which is revival for treatment of *P. aeruginosa* infected patients, due to its good characteristic of fosfomycin as good safety profile [2]. The total susceptibility of seventy strains of carbapenems-resistant *P. aeruginosa*clinical isolatesto fosfomycin wasdemonstrated in Table 1. The MIC ranges of the Central and Northern regions were 32 to more than 1,024 µg/ml.In the Eastern, Southern, and Northeastern regions were 64

- >1024, 128 - >1024, and 8 - >1024µg/mlrespectively. Mostly, MIC_{50} were found more than 256 µg/ml and MIC_{90}were found at more than 1024 µg/ml in all of regions. The MICsin each region were showed high percentage to fosfomycin resistance.The EUCAST standard was usedas a criterion of susceptible interpretation.Therefore,these results demonstrated that Thailand did have high phenomenon of fosfomycin resistance.

Table1: The minimal inhibition concentration values of fosfomycin with seventy strains of carbapenems-resistant *P. aeruginosa* clinical isolates in Thailand

Regions	Number of strains	Minimal inhibition concentration values (µg/ml)			Percentage of resistant strains (%)[a]
		MIC range	MIC_{50}	MIC_{90}	
Central	16	32 - >1024	256	>1024	81.25
Northern	10	32 - >1024	256	>1024	80
Eastern	18	64 - >1024	512	>1024	100
Southern	11	128 - >1024	512	>1024	100
Northeastern	15	8 - >1024	256	>1024	86.67

[a]EUCAST for susceptible breakpoints; susceptible is < 32, and resistance is ≥ 32

Time-kill assay

P. aeruginosa PAO1was killed at 1xMIC of fosfomycin after treating for 8 hours.There was considered, when fosfomycin reduced the number of inoculum ≥ $3\log_{10}$ CFU/ml.The bacteria were able to revive after 8 hours at 0.5, 1xMIC of treatment. Previous studies reported that the fosfomycin killed in time-dependent manner and fairy effective to inhibit *P. aeruginosa*[3].

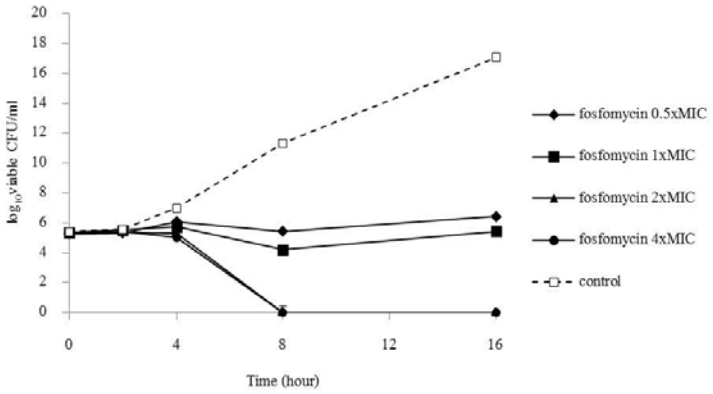

Figure1: Time-kill curve of *P. aeruginosa* PAO1 with fosfomycin at various concentrations.

Single-step mutation method

Table 2 showed the results of frequency mutation rate of *P. aeruginosa* exposed to 2xMIC of fosfomycin. These results demonstratedcarbapenems-resistant *P.aeruginosa* clinical isolates (CR-PA CI) did have the frequency mutation rates less than PAO1 wild-type strain.PAO1 strain wasexhibited highly resistance to fosfomycin. Similarly to previous reviews, *P. aeruginosa* developed mutation frequency for fosfomycin when estimated*in vitro* study but

difference to *in vivo* [4,5]. In clinical trial data, the resistance of fosfomycin was slightly reported with *P. aeruginosa*.

Table 2: The results of frequency mutation by single-step mutation method with carbapenems-resistant *P.aeruginosa* clinical isolates at 2 times of MIC.

Strains of *P. aeruginosa*	Frequency mutation
PAO1	TNTC
CR-PA CI1	3.15×10^{-9}
CR-PA CI2	2.06×10^{-10}
CR-PA CI3	2.72×10^{-11}
CR-PA CI4	3.2×10^{-9}

Conclusion

In Thailand, most carbapenems-resistant *P. aeruginosa* isolates are resistant to fosfomycin. Therefore, antibiotic development or improvement is important for clinical treatment of*P. aeruginosa* infected patients.Although *P. aeruginosa* clinical isolates in Thailand showed resistance,fosfomycin is still the one of interesting drugs because the excellent characteristic of fosfomycin is less toxicity and rarely resistant *in vivo* testing. For this reason, it should be further study onsupplementary efficacy of action as the combination testing with other active substances.

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4. Publication : Synergistic effects of fosfomycin and carbapenems against carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates

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None.

Competing interests

AJvW is co-owner of LabOral Diagnostics (Houten, The Netherlands), a laboratory providing clinical microbiology for dental professionals. All other authors declare no competing interests.

Ethical approval

Not required.

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Synergistic effects of fosfomycin and carbapenems against carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates

Sir,

Carbapenems are potent β-lactam antimicrobials with broad-spectrum antibacterial activity, including against *Pseudomonas aeruginosa* [1]. Nowadays, carbapenem-resistant *P. aeruginosa* (CR-PA) is becoming a critical problem in the clinical treatment of infectious diseases owing to high mortality rates [2,3]. Investigation of novel drugs and improvement of existing antimicrobials are of interest for prevention of this high incidence. One possible treatment option is empirical combination therapy. This study aimed to evaluate the efficacy of fosfomycin in combination with carbapenems against CR-PA.

In total, 70 clinical strains of CR-PA were collected from various hospitals across Thailand during the period 2007–2009. Minimum inhibitory concentrations (MICs) of various antimicrobial agents were determined by the broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [4]. CR-PA was defined as a *P. aeruginosa* isolate that was resistant to one or more antibiotics in the carbapenem drug group. Synergistic studies were performed by the chequerboard technique. For each combination, the fractional inhibitory concentration (FIC) of each antimicrobial was calculated as the MIC of the antimicrobial in combination divided by the MIC of the antimicrobial alone. Fractional inhibitory concentration indices (FICIs) were derived from summation of individual FICs. The efficacy of the combination effect was interpreted from the FICI as follows: synergism was defined as an FICI ≤ 0.5; indifference as $0.5 < \text{FICI} \leq 4$; and antagonism as FICI > 4. Results from the chequerboard synergy assay were analysed statistically by the χ^2 test.

The results of synergy testing, post-antibiotic effect (PAE) and resistance mechanisms for fosfomycin and carbapenems alone and in combination for the 70 clinical CR-PA are shown in Table 1. This study demonstrated that doripenem showed promising activity when combined with fosfomycin (45.7% synergy), followed by meropenem (40.0%) and imipenem (38.6%). Considering all antimicrobials in combination with fosfomycin, there were no statistically significant differences between all three antibiotics in the carbapenem group. Strains with positive synergy results possessed carbapenem resistance mechanisms including efflux pumps (MexAB and MexXY) and overexpression of OprD [5]. In the case of combinations, fosfomycin plus a carbapenem dramatically decreased biofilm formation. Combinations of fosfomycin and meropenem at 1× MIC and 2× MIC demonstrated the best efficacy, with a 13.06 log CFU/mL decrease, whilst fosfomycin combined with doripenem had the highest reduction effect at 0.5× MIC.

A time–kill assay was used to determine the rate of bacterial killing when antimicrobials were applied alone or in combination. Drug synergy occurred at a concentration of 0.25× MIC after 8 h of incubation when fosfomycin and carbapenems were combined. When given alone, fosfomycin, imipenem, meropenem and doripenem showed weaker bacterial inhibitory activity compared with combination regimens. Carbapenems alone demonstrated a PAE after 2 h of exposure to bacteria, whilst fosfomycin showed the same effect within 1 h. At the synergistic concentration, all combinations exhibited a PAE at 2 h after exposure.

The spontaneous antimicrobial mutation assay was performed on selected clinical CR-PA strains (CM06, SI19, CH35 and UB45) and the standard strain PA01 by measuring mutation frequencies after a single exposure to antimicrobials. The frequency of mutations among CR-PA was ca. 10^{-9} to 10^{-11} for fosfomycin and ca. 10^{-8} to 10^{-10} for carbapenems. Fosfomycin combined with carbapenems resulted in a mutation frequency of ca. 10^{-8} to 10^{-9}. There was no significant difference after treatment with an antimicrobial combination compared with antimicrobials alone (Table 1).

Treatment of *P. aeruginosa* with doripenem alone resulted in cell disruption and abnormal bacterial cell shape as determined by scanning electron microscopy. Treatment with fosfomycin alone showed less activity in bacterial cell destruction. When fosfomycin was combined with doripenem at 0.25× MIC, extreme disruption of the bacterial cell membrane and morphological changes occurred. This effect was obviously stronger than following treatment with a single antibiotic. In conclusion, the combination of fosfomycin plus a carbapenem could be of interest as an alternative therapeutic for

Table 1
Minimum inhibitory concentrations (MICs) of fosfomycin and various carbapenems, duration of the post-antibiotic effect (PAE), frequency of mutation, results of synergy testing, and frequency of various resistance mechanisms for fosfomycin and carbapenems alone and in combination for 70 clinical carbapenem-resistant *Pseudomonas aeruginosa* (CR-PA).

Antibiotic(s)	MIC (μg/mL)	PAE (h)	Mutation frequency[a]					Percentage of isolates showing synergy (*n*)	Percentage of isolates positive for carbapenem resistance mechanism (*n*)[b]			
			PA01	CM06	SI19	CH35	UB45		MexAB	MexXY	OprD	AmpC
FOS	128	1	TNTC	3.1×10^{-9}	2.1×10^{-10}	2.7×10^{-11}	3.2×10^{-9}					
Carbapenems												
MEM	4	2	$<2.9\times10^{-9}$	$<7.3\times10^{-9}$	$<3.3\times10^{-9}$	$<1.4\times10^{-10}$	3.8×10^{-8}					
IPM	2	2	$<2.9\times10^{-9}$	TNTC	TNTC	$<1.4\times10^{-10}$	$<5.2\times10^{-9}$					
DOR	2	2	$<2.9\times10^{-9}$	$<7.3\times10^{-9}$	2.4×10^{-9}	$<1.4\times10^{-10}$	$<5.2\times10^{-9}$					
Combinations												
FOS + MEM	32/0.5	2	$<2.9\times10^{-9}$	6.8×10^{-8}	2.4×10^{-9}	$<1.4\times10^{-10}$	5.8×10^{-8}	40.0 (28)	82.1 (23)	57.1 (16)	82.1 (23)	0 (0)
FOS + IPM	16/0.5	2	7.2×10^{-9}	8.2×10^{-9}	TNTC	4.4×10^{-10}	1.9×10^{-8}	38.6 (27)	96.3 (26)	70.4 (19)	88.9 (24)	0 (0)
FOS + DOR	16/0.5	2	$<2.9\times10^{-9}$	7.9×10^{-9}	3.6×10^{-9}	$<1.4\times10^{-10}$	1.9×10^{-8}	45.7 (32)	87.5 (28)	65.6 (21)	81.3 (26)	0 (0)

FOS, fosfomycin; MEM, meropenem; IPM, imipenem; DOR, doripenem; TNTC, too numerous to count.
[a] For selected clinical CR-PA strains (CM06, SI19, CH35 and UB45) and the standard strain PA01.
[b] Among isolates showing synergy.

CR-PA infection. Further in vivo studies should be performed on combination efficacy and pharmacokinetic aspects.

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Trimethoprim/sulfamethoxazole resistance in clinical isolates of *Burkholderia pseudomallei* from Thailand

Sir,

Burkholderia pseudomallei is the cause of melioidosis, a serious infection associated with a mortality rate of 14–43% [1]. Recommended antimicrobial therapy is ≥10 days of parenteral ceftazidime or a carbapenem, followed by oral trimethoprim/sulfamethoxazole (SXT; co-trimoxazole) to complete up to 20 weeks of therapy [2]. A previous evaluation of 1976 clinical *B. pseudomallei* isolated from patients in northeast Thailand between 1992 and 2003 reported that SXT resistance was detected in 13% of isolates [3]. Subsequent studies have reported much lower rates of SXT resistance for isolates from Laos (0.8%), Australia (0.4%) and Cambodia (0%) [4]. Here we report the results of a re-evaluation of SXT resistance in Thailand. Second-line oral treatment in patients infected with SXT-resistant *B. pseudomallei* or in whom SXT is contraindicated is amoxicillin/clavulanic acid (AMC) [1], thus we also evaluated the susceptibility of SXT-resistant isolates to AMC and doxycycline (DOX), which is used less frequently as an alternative to SXT.

Susceptibility to SXT was determined by Etest (bioMérieux, Marcy-l'Étoile, France) [3], with reading of the minimum inhibitory concentration (MIC) at the 80% inhibition point. Interpretative standards for the Etest were based on Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution, which classifies SXT MICs of ≤2/38 mg/L as susceptible and ≥4/76 mg/L as resistant [5]. *Escherichia coli* ATCC 25922 was used as the control. For SXT-resistant isolates, the Etest was used to define susceptibility to trimethoprim (TMP) alone and sulfamethoxazole (SMX) alone. Susceptibility testing to AMC and DOX was also performed using the Etest, in which the MIC was read at the point of no visible growth. *Escherichia coli* ATCC 35218 was used as a control for AMC, and *E. coli* ATCC 25922 was used as a control for TMP, SMX and DOX.

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