ฤทธิ์ต้านเชื้อแบคทีเรียในหลอดทดลองของ ยาอิมิพีเน็มร่วมกับยาโคลิสติน ต่อเชื้อซูโดโมแนส
แอรูจิโนซา ที่ดื้อต่อยาหลายชนิด

นางสาว ศิรินาถ ภานุวงษ์

IN VITRO ANTIBACTERIAL ACTIVITY OF IMIPENEM IN COMBINATION
WITH COLISTIN AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS
AERUGINOSA*

Miss Sirinat Phanuwong

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title   *IN VITRO* ANTIBACTERIAL ACTIVITY OF IMIPENEM IN COMBINATION WITH COLISTIN AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA*

By   Miss Sirinat Phanuwong

Field of Study   Pharmacology

Thesis Advisor   Associate Professor Siriporn Fungwithaya, M.Sc.

Thesis Co-advisor   Associate Professor Pintip Pongpech, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

....Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pornpen Pramyothin, Ph.D.)

THESIS COMMITTEE

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(Associate Professor Pol.Lt.Col. Somsong Lawanprasert, Ph.D.)

....Thesis Advisor

(Associate Professor Siriporn Fungwithaya, M.Sc.)

....Thesis Co-advisor

(Associate Professor Pintip Pongpech, Ph.D.)

....Member

(Assistant Professor Penphun Naenna, M.Sc.)

....Member

(Santad Chanprapaph, Ph.D.)

ศิรินาถ ภานุวงษ์ : ฤทธิ์ต้านเชื้อแบคทีเรียในหลอดทดลองของยา อิมิพีเน็ม ร่วมกับยา โคลิสติน ต่อเชื้อซูโดโมแนส แอรูจิโนซา ที่ดื้อต่อยาหลายชนิด. (IN VITRO ANTIBACTERIAL ACTIVITY OF IMIPENEM IN COMBINATION WITH COLISTIN AGAINST MULTIDRUG-RESISTANT PSEUDOMONAS AERUGINOSA) อ. ที่ปรึกษา: รศ. ศิริภรณ์ ฟุ้งวิทยา. อ.ที่ปรึกษาร่วม: รศ. ดร. พิณทิพย์ พงษ์เพ็ชร, 109 หน้า.

วัตถุประสงค์ในการวิจัยครั้งนี้เพื่อศึกษาฤทธิ์ต้านเชื้อของยา imipenem ร่วมกับยา colistin ต่อเชื้อ P.aeruginosa จำนวน 30 สายพันธุ์ จากการทดลองพบว่าเชื้อ P.aeruginosa 19 สายพันธุ์ (63.33%) เป็นสายพันธุ์ที่ดื้อต่อยาหลายชนิด (multidrug-resistant strains) และดื้อต่อยา imipenem ทั้งหมด 8 สายพันธุ์ (26.67%) โดยมีค่า MIC อยู่ที่ 16 µg/ml แต่ยังคงมีความไวต่อ colistin ทั้ง 30 สายพันธุ์ (100%) โดยมีค่า MIC อยู่ในช่วง 0.5-4 µg/ml ไม่พบการสร้าง metallo-β-lactamase ในทุกสายพันธุ์ ที่ศึกษา เมื่อทำการประเมินการใช้ imipenem ร่วมกับ colistin โดยวิธี checkerboard method พบการเสริมฤทธิ์กัน (synergy) 20 สายพันธุ์ (66.67%) เสริมฤทธิ์กันบางส่วน (partial synergy) 3 สายพันธุ์ (10%) และอีก 7 สายพันธุ์ (23.33%) ให้ผลเพียงเพิ่ม ฤทธิ์กัน (additive) เมื่อนำสายพันธุ์ที่ดื้อต่อ imipenem ทั้ง 8 สายพันธุ์ มาศึกษาต่อเพื่อประเมินผลในการฆ่าเชื้อโดยวิธี Time kill พบว่า การใช้ยา imipenem เดี่ยวๆ ที่ความเข้มข้น 1xMIC สามารถฆ่าเชื้อได้ 99.9% ในเวลา 2 ชั่วโมง 1 สายพันธุ์ และเพิ่มขึ้นเป็น 5 สายพันธุ์ในชั่วโมงที่ 8 แต่เชื้อกลับเจริญขึ้นได้อีก (regrowth) 6 สายพันธุ์ (75%) ในเวลา 24 ชั่วโมง เมื่อเพิ่มความเข้มข้นของยา imipenem เป็น 2xMIC จำนวนเชื้อที่ถูกฆ่าได้ 99.9% จะเพิ่มขึ้น และสามารถลดจำนวนเชื้อที่กลับเจริญขึ้นอีกในเวลา 24 ชั่วโมง ให้ เหลือเพียง 2 สายพันธุ์ (25%) ส่วนการใช้ colistin เดี่ยวๆ ในความเข้มข้น 0.25xMIC ไม่สามารถฆ่าเชื้อได้ในระดับ 99.9% และ เมื่อเพิ่มยาเป็น 0.5xMIC จะสามารถฆ่าเชื้อได้ในระดับ 99.9% เพียง 1 สายพันธุ์ ในช่วงเวลา 2-8 ชั่วโมง แต่เชื้อทั้ง 8 สายพันธุ์ (100%) จะกลับเจริญขึ้นได้อีก ที่เวลา 24 ชั่วโมง แต่เมื่อใช้ imipenen 1xMIC ร่วมกับ colistin 0.25xMIC หรือ 0.5xMIC พบว่า สามารถฆ่าเชื้อได้ 99.9% ตั้งแต่ 2 ชั่วโมงแรกจำนวน 6 สายพันธุ์ (75%) แต่ใน imipenem 1xMIC กับ colistin 0.25xMIC เชื้อจะ กลับเจริญขึ้นมาอีกที่เวลา 24 ชั่วโมง จำนวน 2 สายพันธุ์ (25%) ขณะที่เมื่อใช้ร่วมกับ colistin ขนาด 0.5xMIC จะไม่มีเชื้อกลับ เจริญได้อีก ส่วนจำนวนเชื้อที่ถูกฆ่าภายใน 24 ชั่วโมง (BA_{24}) ของการใช้ imipenem 1xMIC ร่วมกับ colistin ทั้ง 2 ความเข้มข้น(0.25xMIC และ 0.5xMIC) ไม่แตกต่างกัน (p>0.05) แต่พบว่าค่า BA_{24} ที่เกิดจากการใช้ colistin เดี่ยวในความเข้มข้น 0.25xMIC จะแตกต่างจากเมื่อใช้ 0.5xMIC และแตกต่างจากผลที่ใช้ imipenem เดี่ยวๆ หรือเมื่อใช้ยาทั้ง 2 อย่างร่วมกันอย่างมีนัยสำคัญ (p<0.05) ส่วนการใช้ colistin เดี่ยวๆ ในความเข้มข้น 1xMIC หรือ 2xMIC หรือ 4xMIC หรือ 8xMIC พบว่าที่เวลา 2 ชั่วโมง จำนวน เชื้อที่ถูกฆ่าได้ 99.9% จะแตกต่างกันตามความเข้มข้นที่เพิ่มขึ้น คือฆ่า 2, 4, 6, และ7 สายพันธุ์ ตามลำดับ แต่ที่เวลา 24 ชั่วโมง colistin ในขนาด $\leq$4xMIC มีการกลับเจริญขึ้นของเชื้อ จำนวนเชื้อที่ถูกฆ่าภายใน 24 ชั่วโมง (BA_{24}) หลังจากการได้รับ colistin ใน ขนาด 1xMIC, 2xMIC, 4xMIC และ 8xMIC ไม่ต่างกัน (p>0.05) นอกจากนั้น ผลของการเปลี่ยนแปลงสัณฐานวิทยาของเชื้อเมื่อ เจริญในยาทั้ง 2 ชนิดผสมกัน พบว่าที่ผนังเซลล์ของเชื้อมีการเปลี่ยนแปลงอย่างชัดเจน โดยมีผนังเซลล์ถูกทำลายเมื่อเทียบกับเซลล์ ที่ไม่ได้รับยา ซึ่งจะทำให้เชื้อถูกฆ่าได้ดีขึ้น

จากผลการทดลองนี้แสดงให้เห็นว่า การใช้ imipenem ในขนาด 1 MIC (16 µg/ml) ร่วมกับ colistin ขนาด 0.5 MIC มี ประสิทธิภาพดีที่สุด ในขณะที่การให้ colistin เดี่ยวๆจะต้องใช้ในขนาดถึง 8 MIC จึงจะเห็นผลในการฆ่าเชื้อ 99.9% ตลอด 24 ชม. และไม่มีการกลับเจริญขึ้นอีก ดังนั้น การใช้ imipenem ร่วมกับ colistin จึงอาจเป็นอีกทางเลือกหนึ่งในการรักษาโรคติดเชื้อที่เกิด จาก เชื้อ P.aeruginosa ที่ดื้อต่อยาหลายชนิด และยังอาจจะช่วยลดขนาดของ colistin ที่จะใช้ในการรักษาเพื่อลดอัตราการเกิดพิษ ของ colistin ได้

ภาควิชา ....เภสัชวิทยา.................. ลายมือชื่อนิสิต...

สาขาวิชา ....เภสัชวิทยา.................. ลายมือชื่ออาจารย์ที่ปรึกษา...

ปีการศึกษา ....2550............... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม...

487 66074 33: MAJOR PHARMACOLOGY
KEY WORD: *PSEUDOMONAS AERUGINOSA* / IMIPENEM / COLISTIN / COMBINATION

SIRINAT PHANUWONG: *IN VITRO* ANTIBACTERIAL ACTIVITY OF IMIPENEM IN COMBINATION WITH COLISTIN AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA*. THESIS ADVISOR: ASSOC. PROF. SIRIPORN FUNGWITHAYA, M.Sc., THESIS COADVISOR: ASSOC. PROF. PINTIP PONGPECH, Ph.D., 109 pp.

The purpose of this study was to determine the *in vitro* activity of imipenem in combination with colistin against 30 strains of *P.aeruginosa*. Nineteen strains (63.33%) were found to be multidrug-resistant strains (MDR) and 8 strains (26.67%) of these organisms were imipenem-resistant with MIC = 16 μg/ml while all strains were susceptible to colistin (MIC range were 0.5-4 μg/ml). Metallo-β-lactamase production could not be detected in all strains. The combination of imipenem and colistin showed synergistic activity against 20 strains (66.67%), partial synergy in 3 strains (10%), and additive in 7 strains (23.33%) by checkerboard method. In the time-kill study, using 8 strains of imipenem-resistant *P.aeruginosa*, it was shown that 99.9% killing were observed at 2nd hour, when exposed to 1xMIC of imipenem, and increased to 5 strains at 8th hour. The regrowth were observed in 6 strains(75%) at the 24th hour. When the concentration of imipenem was increased to 2xMIC, the number of strains which were 99.9% killing were increased and the regrowth were observed in only 2 strains at the 24th hour. The 0.25xMIC of colistin alone showed only bacteriostatic activity (99% killing) but when the concentration of colistin was increased to 0.5xMIC, the 99.9% killing was observed in 1 strain during 2nd-8th hour. However, all strains regrew at the 24th hour.

Whereas, the combination of imipenem 1xMIC with colistin 0.5xMIC could show 99.9% killing in 6 strains (75%) without regrowth while in the combination of 1xMIC of imipenem and 0.25xMIC colistin, bacterial regrowth occurred in 2 strains (25%) at the 24th hour. There were no significantly difference ($p>0.05$) among the BA_{24} of the both combination (1xMIC of imipenem+0.5xMIC of colistin and 1xMIC of imipenem+0.25MIC of colistin), while BA_{24} of the single drug (colistin 0.25xMIC and 0.5xMIC) were significantly difference ($p<0.05$) and also significantly difference from BA_{24} of imipenem alone or BA_{24} of the both combination. Colistin 1xMIC, 2xMIC,4xMIC and 8xMIC showed the 99.9% killing in 2, 4, 6 and 7 strains, respectively. At 24 hours, the regrowth was observed when using colistin $\leq$ 4xMIC. There no significantly difference ($p>0.05$) was observed among the BA_{24} from the different concentrations of colistin. Moreover, the morphology change of *P. aeruginosa* strain no.10 after was exposed to the combination of imipenem and colistin. The roughly spherical surface and bacterial lysis could be clearly seen under the electron microscope.

The results suggested that antibacterial activity of imipenem 1xMIC and colistin 0.5xMIC showed the best activity, whereas colistin monotherapy might need the very high concentration (8xMIC) of drug. Bactericidal activity (99.9% killing) of this combination was observed during the 24th hours of study without any regrowth. Therefore, the combination of imipenem and with lower dose of colistin can be the alternative treatment of infections caused by MDR *P. aeruginosa*. The lower dose of colistin could lead to the decrease in nephrotoxicity.

Department ....Pharmacology...................Student's signature....*Sirinat Phanuwong*....

Field of study ......Pharmacology....................Advisor's signature.*Siriporn Fungwithaya*

Academic year ....2007................................Co-advisor's signature...*Pintip Pongpech*

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LIST OF ABBREVIATIONS

%	=	percent
°C	=	degree of celsius
β	=	beta
μ	=	micro
μg	=	microgram
ATCC	=	American Type Culture Collection
AUBKC	=	Area under the bacterial killing regrowth curves
AUC	=	Area under the curve
BA_{24}	=	Bacteriolytic area of 24 hours
CF	=	Cystic fibrosis
CFU	=	Colony forming unit
CLSI	=	Clinical and Laboratory Standards Institute
cm	=	centimeter
E. coli	=	*Escherichia coli*
EDTA	=	Ethylenediaminetetraacetic acid
et al	=	et alii (and other peoples)
FIC	=	Fractional Inhibitory Concentration
Fig	=	Figure
g	=	gram
hr	=	hour
ICU	=	Intensive care unit
L	=	liter
Log	=	decimal logarithm
MBL	=	Metallo-beta-lactamase
mg	=	milligram
MHA	=	Meuller-Hinton Agar
MHB	=	Meuller-Hinton Broth
MIC	=	Minimum Inhibitory Concentration
min	=	minute
ml	=	milliliter

mm	=	millimeter
mM	=	millimolar
NARST	=	National antimicrobial resistance surveillance center Thailand
NCCLS	=	The Nation Committee for Clinical Laboratory Standards
NNIS	=	National Nosocomial Infections Surveillance System
No.	=	number of isolates
NSS	=	Normal Saline Solution
Opr	=	Outer membrane proteins
P. aeruginosa	=	*Pseudomonas aeruginosa*
PBP	=	Penicillin-binding proteins
TSA	=	Tryptic Soy Agar
UTI	=	Urinary Tract Infections

CHAPTER I

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the major causes of hospital-acquired (nosocomial) infection. This organism has the unique ability to infect all body systems, especially the respiratory tract (Pollack, 1990; Strattoncw, 1983). In addition, it can survive and replicate everywhere within the hospital environment (Whitby and Rampling, 1972; Favevo *et al*., 1971; Burden and Whitby, 1967).

The infection from this organism is commonly found in patients with chronic illness who have to stay in the hospital for a long period of time, particularly, the patients with underlying diseases or with history of intravenous drug abuse, cystic fibrosis and lung cancer. Most of these patients also have defective immune systems which leads to the occurrence of nosocomial infection, a major cause of morbidity and mortality for hospital patients (Pedersen *et al*., 1986). At present, an increase in the incidence of *P. aeruginosa* infections in the hospitals in Thailand as well as in other countries all over the world is an important health problem.

Infection due to *P. aeruginosa* which is commonly found in skin and eye, may lead to folliculitis, soggy dermatitis of the interdigital spaces or otitis externa, burn wounds, blood and cerebrospinal fluid lead to bacteremia. In addition, these infections are commonly found in patients with anatomical abnormalities on the long-term catheterization or ventilation.

Empirical therapy for a patient with a serious infection for which the suspected etiologic agent is *P. aeruginosa* generally consists of an antipseudomonal β-lactam (e.g., carbapenem, ceftazidime, cefepime, piperacillin, or piperacillin-tazobactam), aminoglycoside (gentamicin or amikacin), fluoroquinolone (ciprofloxacin or levofloxacin) as monotherapy or the combination of an antipseudomonal β-lactam with an aminoglycoside or fluoroquinolone. Carbapenems, mainly imipenem and meropenem, are potent agents for the treatment of infections due to multidrug-

resistant pseudomonades. These drugs have considerable β-lactamase stability and overall have the broadest spectrum of activity when compared with other β-lactams. However, the resistance to carbapenems has increased among *P. aeruginosa* isolates. Mechanisms of resistance to carbapenems are associated with the reduction of antimicrobials uptake as a result of the loss in OprD porin combined with the over-expression of an efflux pump system. Even though the high-level resistance to carbapenems is still uncommon in *P. aeruginosa,* but the drug resistance can be caused by the presence of class B *β*-lactamases, the metallo-β-lactamases (MBLs). Thus, the antimicrobial resistance of the *P. aeroginosa* may cause the complication in the treatment of infections and cause the adversely affect clinical outcomes and the treatment costs for patients (Carmeli *et al*., 1999; Harris *et al*., 1999).

New antimicrobial agents with activity against *P. aeruginosa* will not be available in the near future, making ongoing surveillance of the activities of currently available agent critical. Recently, there has been the renewed interest in antimicrobial agents, which had earlier been abandoned because of their serious adverse effects. For example, colistin which has the excellent *in vitro* activity against many species of aerobic gram-negative bacilli was extensively used during the 1960s to the early 1980s. Because of serious adverse effects including toxicity involves the kidney and central nervous system of colistin, the systemic utilization has been discouraged. However, recent studies have reported the safe use of colistin (Garnacho-Montero *et al*., 2003; Markou *et al*., 2003). Data from Tascini *et al*., 2006 suggested that colistin, in combination with rifampin and imipenem, is safe and effective, in promoting healing in diabetic foot infection due to multidrug-resistant *P. aeruginosa*.

All about that, the hypothesis of this study was investigated for the combination of imipenem and colistin produced synergistic antimicrobial effects against MDR *P. aeruginosa*. Therefore, this study will be emphasized on the effects of imipenem and colistin combination on the multidrug-resistant *P. aeruginosa* isolated from Thai patients. In order to obtain the informative conclusions on this aspect, the experimental studies are designed to determine:

1. The antibacterial susceptibility patterns of *P. aeruginosa* against the antimicrobial agents which are commonly used in the treatment of *P. aeruginosa* infection.

2. The metallo-β-lactamases production in imipenem resistance strains.

3. The combination effect of imipenem and colistin against the multidrug-resistant strains of *P. aeruginosa* and the bactericidal activity of this combination.

4. The morphological changes of multidrug-resistant *P. aeruginosa* in the combination between imipenem and colistin.

CHAPTER II

LITERATURE REVIEWS

1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative, aerobic rod, belonging to the bacterial family *Pseudomonadaceae*. It lives primarily in water, soil and vegetation. This bacteria is an important cause of both community-acquired infections and hospital-acquired infections. Community-acquired infections include, but are not limited to, ulcerative keratitis (usually associated with contact lens use), otitis externa (typically in immunocompromised hosts such as those with diabetes mellitus), and skin and soft tissue infections (including diabetic foot infections). Hospitalized patients may be colonized with *P. aeruginosa* on admission or may acquire the organism during their hospital stay. *P. aeruginosa* can be isolated from nearly any conceivable source within hospitals. Nosocomial infections caused by *P.aeruginosa* included pneumonia, urinary tract infections (UTIs), bloodstream infections, surgical site infections and skin infections in the setting of burn injuries. Chronic sinopulmonary colonisation and recurrent infection from *P. aeruginosa* are seen in patients with cystic fibrosis (CF). The infections caused by *P. aeruginosa* are not only common, but they have also been associated with high morbidity and mortality when compared with other bacterial pathogens. Of additional concern is the antibacterial resistance trend that has been noted in large database on nosocomial *P. aeruginosa* isolates (Moet and Jones, 2004; NNIS system report 2004).

Infections by this pathogen are often difficult to treat because of their intrinsic resistance to various antimicrobial agents including many β-bactams, the macrolides, the tetracyclines, the co-trimoxazole (trimethoprim/sulfamethoxazole) and most fluoroquinolone. The increasing frequency of multidrug-resistant *P. aeruginosa* strains is concerning as the limited efficacious antimicrobial options. The definition of multidrug-resistant *P. aeruginosa* has been established as the isolates that are intermediate resistant or resistant to at least three drugs in the following classes: antipseudomonal β-lactams, aminoglycosides, and fluoroquinolones. Multidrug-

resistant (MDR) *P. aeruginosa* has become relatively common in ICUs. Data published by the SENTRY antimicrobial surveillance program revealed that, between 1997 and 2002, 10.4% of ICU bloodstream *P. aeruginosa* isolates were MDR, as defined by resistance to ceftazidime, piperacillin, gentamicin and ciprofloxacin (Biedenbach, Moet and Jones , 2004).

2. Antibacterial therapy

2.1 Carbapenem

Carbapenems are β-lactams that contain a fused β-lactam ring and a 5-membered ring system that differ from the penicillins in being unsaturated and containing a carbon atom instead of the sulfur atom. This class of antibiotics has a broader spectrum of activity than do most other β-lactam antibiotic.

Carbapenem, mainly imipenem and meropenem, are potent agents for the treatment of infections due to multidrug-resistant *P. aeruginosa.* Imipenem, like other β-lactam antibiotics, binds to penicillin-binding proteins (PBPs), the binding of the β-lactam molecule to PBPs prevents bacteria from completing transpeptidation (cross-linking) of peptidoglycan stand leading to disrupts bacterial cell wall synthesis (thus preventing the synthesis, and causes death of susceptible microorganisms). It is very resistant to hydrolysis by most β-lactamases (Figure 2-1).
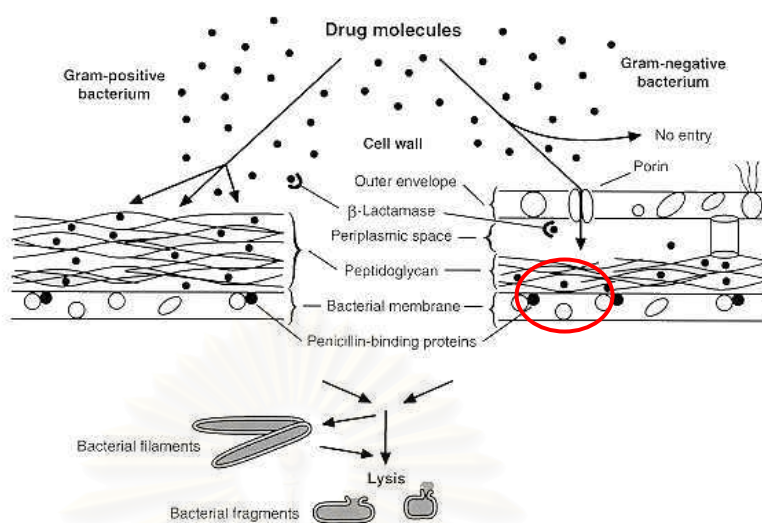
Figure 2-1 Mechanism of action of imipenem to binding to PBPs , leading to death of susceptible microorganisms (picture from webpage http://gsbs.utmb.edu/microbook/ch011.htm)

Imipenem (N-formimidoylthienamycin monohydrate) is a crystalline derivative of thienamycin, which is produced by *Streptomyces cattleya*. The compound thienamycin is unstable, but imipenem, the N-formimidoyl derivative, is stable. Its chemical name is (5 *R*, 6 *S*)-3-[[2-(formimidoylamino) ethyl] thio]-6-[(*R*)-1-hydroxyethyl]-7-oxo-1-azabicyclo [3.2.0] hept-2-ene-2-carboxylic acid monohydrate. It is an off-white, non hygroscopic crystalline compound with a molecular weight of 317.37. It is sparingly soluble in water and slightly soluble in methanol. Its empirical formula is $C_{12} H_{17} N_3 O_4 S \cdot H_2 O$. The structural formula of imipenem is as follow (Figure 2-2):
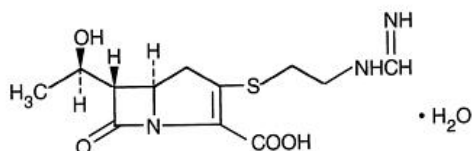
Figure 2-2 Structure of imipenem (picture from webpage www.medsafe.gout.nz/p/Primaxininj.htm)

Imipenem is not absorbed orally. The drug is hydrolyzed rapidly by a dipeptidase found in the brush border of the proximal renal tubule. Because concentrations of active drug in urine were low, an inhibitor of the hydropeptidase was synthesized. This compound is called cilastatin. A preparation has been developed that contains equal amounts of imipenem and cilastatin.

Cilastatin sodium is the sodium salt of a derivatized heptenoic acid. Its chemical name is sodium (*Z*)-7-[[(*R*)-2-amino-2-carboxyethyl]thio]-2-[(*S*)-2,2dimethylcyclo propanecarboxamid] -2- heptenoate. It is an off-white to yellowish-white, hygroscopic, amorphous compound with a molecular weight of 380.43. It is very soluble in water and in methanol. Its empirical formula is $C_{16} H_{25} N_2 O_5 S Na$, and its structural formula is (Figure 2-3):
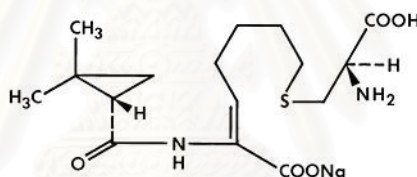
Figure 2-3 Structure of cilastatin (picture from webpage
www.medsafe.gout.nz/p/Primaxininj.htm)

The activity of imipenem is excellent *in vitro* for a wide variety of aerobic and anaerobic microorganisms. Streptococci (including penicillin-resistant *S. pneumoniae*), enterococci (excluding *Enterococcus faecium* and non-β-lactamase-producing penicillin-resistant strains), staphylococci (including penicillinase-producing strains), and listeria are all susceptible. Although some strains of methicillin-resistant staphylococci are susceptible, many strains are not. Activity is excellent against the Enterobacteriaceae, including those organism that are cephalosporin-resistant by virtue of expression of chromosomal or plasmid extended-spectrum β-lactamases. Most strains of *Pseudomonas* spp. and *Acinetobacter* spp. are inhibited.

Imipenem-cilastatin is effective for a wide variety of infections, including urinary tract and lower respiratory infection; intra-abdominal and gynecological infections, bacterial septicemia, endocarditis, and skin, soft-tissue, bone, and joint infections. Dosage for adult normal renal and hepatic function administration by intravenous 500 mg every 6 hours or 1 g every 8 hours. After the intravenous administration of 500 mg imipenem, peak concentration in plasma average 33 µg/ml. Both imipenem and cilastatin have a half-life of about 1 hour. When administered concurrently with cilastatin, approximately 70% of administered imipenem is recovered in the urine as the active drug. Dosage should be modified for patients with renal insufficiency.

Carbapenems Resistance Problems

Currently, resistance to carbapenems is an increasing problem among *P. aeruginosa* isolates. Several reports have shown that imipenem resistance increased (Goossen, 2002; Androde *et al*., 2003; Karlowsky *et al*., 2005). In the United states (Table 2-1), 2001-2003, the susceptibility of *P. aeruginosa* isolates to imipenem decreased by 5.6% (from 84.4% to 78.8%), in addition, data in Europe in 2003 showed that resistance to imipenem were 44.9%. Of additional concern is frequent isolation of *P. aeruginosa* resistant to carbapenems, a class of antibacterials often prescribed when bacterial isolates are resistant to cephalosporins and fluoroquinolones.

Table 2-1 Summary of antimicrobial susceptibility testing results for clinical isolates of *Pseudomonas aeruginosa* collected from hospital microbiology laboratory across the United Stated, 2001-2003

Antimicrobial, year[b]	MIC, μg/mL				MIC interpretation, % of isolates[a]		
	Range	Modal	MIC_{50}	MIC_{90}	Susceptible	Intermediate	Resistant
Cefepime, 2003	0.12 to >32	4	4	16	79.5	11.9	8.6
Ceftazidime							
2001	0.25 to >16	2	4	>16	80.5	5.4	14.0
2002	0.25 to >16	2	4	>16	79.7	5.1	15.2
2003	0.25 to >32	2	4	32	80.6	6.5	12.9
Ciprofloxacin							
2001	0.008 to >4	0.12	0.5	>4	66.5	6.6	26.8
2002	0.004 to >4	0.12	0.25	>4	67.4	5.3	27.3
2003	≤0.002 to >128	0.12	0.25	32	68.8	5.9	25.3
Gatifloxacin, 2003	0.015 to >128	1	2	32	61.2	9.4	29.4
Gentamicin							
2001	≤0.12 to >8	2	4	>8	75.5	11.1	13.4
2002	≤0.12 to >8	2	4	>8	76.7	11.7	11.6
2003	≤0.12 to >8	2	4	>8	72.3	13.7	13.9
Imipenem							
2001	0.06 to >8	1	1	8	84.4	6.8	8.8
2002	≤0.03 to >8	1	2	>8	81.9	5.3	12.8
2003	0.06 to >8	2	2	>8	78.8	5.2	16.0
Levofloxacin							
2001	0.03 to >8	0.5	1	>8	66.0	7.0	27.0
2002	0.008 to >8	0.5	1	>8	67.7	5.9	26.4
2003	0.008 to >128	0.5	1	32	65.3	7.4	27.3
Meropenem, 2003	0.008 to >16	0.5	0.5	8	85.8	4.4	9.8
Moxifloxacin, 2003	0.03 to >128	2	4	128	…[c]	…	…
Piperacillin-tazobactam							
2001	≤0.25 to >64	8	8	>64	85.6	…[d]	14.4
2002	≤0.25 to >64	8	8	>64	85.8	…	14.2
2003	≤0.25 to >64	8	8	>64	87.0	…	13.0

NOTE. Modal MIC, most frequently occurring MIC.
[a] MICs were interpreted, by use of NCCLS break points, as susceptible, intermediate, and resistant [5].
[b] There were 514 isolates from 2001, 998 isolates from 2002, and 882 isolates from 2003. Cefepime, gatifloxacin, meropenem, and moxifloxacin were tested in 2003. The concentration ranges used in testing varied slightly from year to year for some antimicrobial agents.
[c] NCCLS MIC interpretative break points are not defined for moxifloxacin tested against *P. aeruginosa* and other non-*Enterobacteriaceae* species [5].
[d] NCCLS intermediate MIC interpretative break points are not defined for piperacillin-tazobactam tested against *P. aeruginosa* and other non-*Enterobacteriaceae* species [5].

Data from China during 1996 to 2002 (Table 2-2) was showed the decrease susceptibility of imipenem to 20% and increase the MDR strain from 11.5% to 20.5% (Wang and Chen, 2005)

Table 2-2 MDR prevalence of *P. aeruginosa* and *A. baumannii* from 1996-2002 in China (Wang and Chen 2005)

Organism	MDR[a] (%)					
	1996	1998	1999	2000	2001	2002
P. aeruginosa	11.5	11.5	11.7	16.3	14.9	20.5
A. baumannii	21.5	17.6	20.0	39.5	32.1	23.6

[a] MDR: Isolates resistant to 3 or 4 antimicrobials among ceftazidime, ciprofloxacin, amikacin and imipenem

In Thailand, the study at Songklanagarind hospital showed that 55% of *P .aeruginosa* isolates were low level imipenem resistance (MIC 8-32 μg/ml) and 45% were high level resistance (MIC > 32 μg/ml) (Tunyapanit *et al*., 2007). Data from National Antimicrobial Resistance Surveillance center Thailand (NARST), 2000-2005 showed that imipenem resistance has been increased by 5% (Table 2-3).

Table 2-3 *Pseudomonas aeruginosa* versus imipenem during January 2000 to December 2005), data from NARST

Year	%Resistant	%Intermediate	%Susceptible
2000	1619 (12%)	155 (1%)	11004 (86%)
2001	2241 (13%)	243 (1%)	13593 (84%)
2002	1619 (12%)	155 (1%)	11004 (86%)
2003	1730 (13%)	211 (1%)	10737 (84%)
2004	2263 (15%)	247 (1%)	12522 (83%)
2005	2828 (17%)	321 (1%)	13473 (81%)

Mechanisms of Antimicrobial Resistance

General mechanism of antimicrobial resistance include blocked of drug entry, active efflux from the cell, enzymatic degradation and target structured alteration (Figure 2-4). *P. aeruginosa* is capable of effecting any of these mechanisms in the development of resistance.
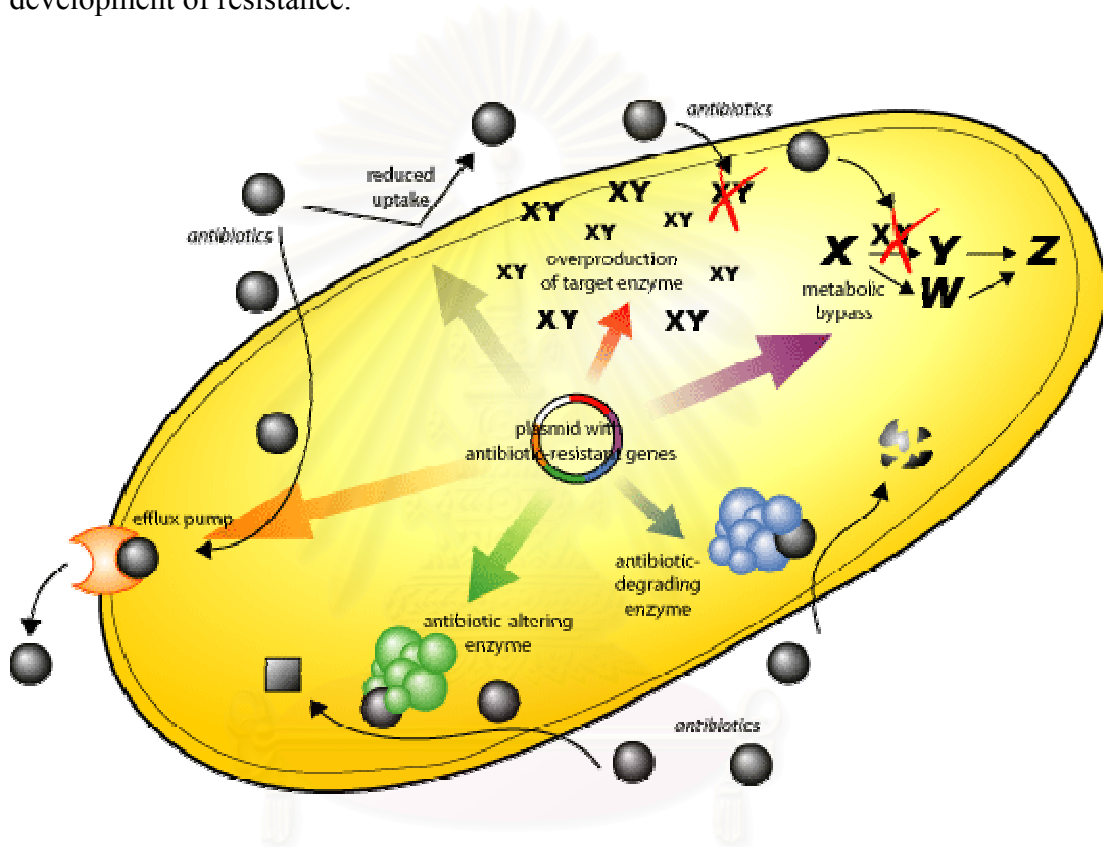
Figure 2-4 Antibacterial resistance mechanism (picture from webpage www.scq.ubc.ca/?p=405)

Mechanisms of low-level resistance to carbapenems (MIC 8–32 mg/L) are associated with reduced uptake as a result of loss of the OprD porin (is a carbapenem-specific outer membrane porin) combined with carbapenem-hydrolyzing- β-lactamase or by the overexpression of an efflux pump system. Decreased or absent expression of OprD has been shown to be a primary mechanism of carbapenem resistance in both clinical and laboratory isolates of *P. aeruginosa*.

Antibacterials may be extruded from within *P. aeruginosa* via multidrug efflux pumps. These multidrug efflux pumps are named for their protein components such as MaxA-MaxB-OprM (Figure 2-5). The genome of *P. aeruginosa* contains at

last 10 distinct efflux pump system operons. Overexpression of a multidrug efflux pump raises the mean inhibitory concentration of any drug susceptible to the pump, and each pump is able to handle multiple antibacterial substrates. Expression of several of these efflux pumps in *P. aeruginosa* has now been unequivocally linked to clinically relevant MDR phenotypes, though only MexAB-OprM is responsible for intrinsic MDR.
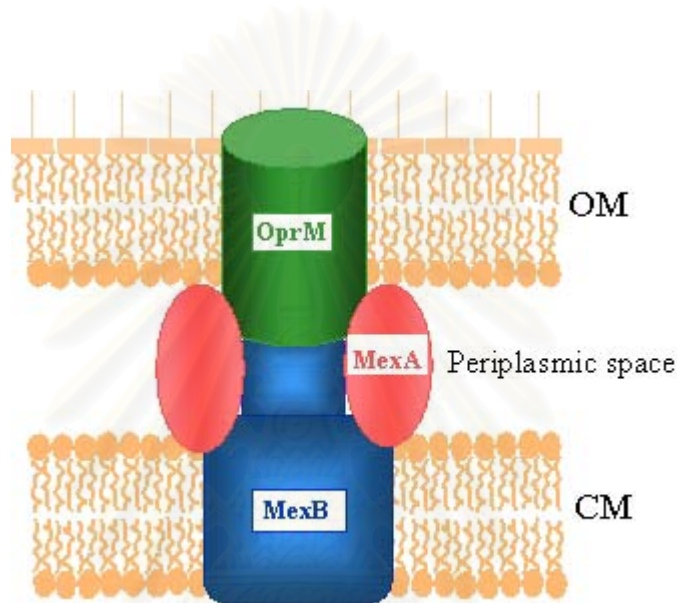
Figure 2-5 MexA-MexB-OprM pump in *Pseudomonas aeruginosa* (picture from webpage www.phar.cam.ac.uk/ri/venter.html)

Whereas, high-level resistance to carbapenems (MIC > 32 mg/L) is still uncommon in *P. aeruginosa,* but can be caused by the presence of carbapenemase (Table 2-4). These include Class B metallo- β-lactamase belonging to the IMP, VIM and SPM groups, Class A enzymes belonging to the SME, NMC/ IMI and KPC groups and several Class D (OXA) enzymes. However, overuse of imipenem has been associated with the isolation of *P. aeruginosa* strains producing class B enzymes (Carbapenemases; IMP and VIM), that are able to hydrolyse all broad spectrum β-lactams except monobactams leading to imipenem resistance strain. At least three major groups of plasmid-mediated MBLs (the IMP, VIM, and SPM types) have been recognized worldwide, and their genetic determinants are often associated with integrons. IMP-1-producing *Serratia marcescens* was initially identified in Japan in

1991.   IMP-1-producing *P. aeruginosa* was the first MBL identified in carbapenem-resistant *P. aeruginosa,* and its countrywide spread in Gram-negative bacilli was reported in Japan.   Many novel MBLs were identified during past few years, including VIM-1 from *P. aeruginosa* in Italy, VIM-2 from *P. aeruginosa* in France, and SPM-1 from *P. aeruginosa* in Brazil.   Moreover, genes for these MBLs are usually located in integrons that successfully accumulate many antibiotic-resistant gene cassettes as a gene cluster.   Integron-located resistance genes provide them with a wide potential for expression and dissemination.   At least nine genetically different integrons have been identified in various bacterial species, and class 1, class 2, and class 3 integrons are often found in pathogenic Gram negative bacilli, including *P. aeruginosa*, *P. putida*, and *Acinetobacter* species.   Among these integrons, those in class 1 and class 3 have been reported to carry genetic determinants for MBLs.   The cassette-associated recombination sites, known as 59-base elements, are located downstream of inserted genes and are of variable length.   The organization of a class 1 integron that carries the gene for VIM-2 type MBLs was characterized for *P. aeruginosa.* Class 1 integrons, which are most commonly isolated from antibiotic-resistant clinical strains, possess two conserved segments.   OXA-type β-lactamase are resistance determinants of increasing clinical important, due to there potential activity on oxyimino-cephalosporins and carbapenems, their overall poor susceptibility to β-lactamase inactivaters.   A novel OXA-type enzyme, named OXA-46 was found to be encoded by gene cassette inserted into a class 1 integron from a multidrug-resistant clinical isolates of *P. aeruginosa* (Giuliani *et al*., 2005).

Table 2-4 Selected β-lactamases of Gram-negative bacteria (Modified from Jacoby and Munos-Price, 2005).

β-Lactamase	Examples	Substrates	Inhibition by Clavulanic Acid*	Molecular Class
Broad-spectrum	TEM-1, TEM-2, SHV-1	Benzylpenicillin (penicillin G), aminopenicillins (amoxicillin and ampicillin), carboxypenicillins (carbenicillin and ticarcillin), ureidopenicillin (piperacillin), narrow-spectrum cephalosporins (cefazolin, cephalothin, cefamandole, cefuroxime, and others)	+++	A
	OXA family	Substrates of the broad-spectrum group plus cloxacillin, methicillin, and oxacillin	+	D
Expanded-spectrum	TEM family and SHV family	Substrates of the broad-spectrum group plus oxyimino-cephalosporins (cefotaxime, cefpodoxime, ceftazidime, and ceftriaxone) and monobactam (aztreonam)	++++	A
	Others (BES-1, GES/IBC family, PER-1, PER-2, SFO-1, TLA-1, VEB-1, and VEB-2)	Same as for TEM family and SHV family	++++	A
	CTX-M family	Substrates of the expanded-spectrum group plus, for some enzymes, cefepime	++++	A
	OXA family	Same as for CTX-M family	+	D
AmpC	ACC-1, ACT-1, CFE-1, CMY family, DHA-1, DHA-2, FOX family, LAT family, MIR-1, MOX-1, and MOX-2	Substrates of expanded-spectrum group plus cephamycins (cefotetan, cefoxitin, and others)	0	C
Carbapenemase	IMP family, VIM family, GIM-1, and SPM-1	Substrates of the expanded-spectrum group plus cephamycins and carbapenems (ertapenem, imipenem, and meropenem)	0	B
	KPC-1, KPC-2, and KPC-3	Same as for IMP family, VIM family, GIM-1, and SPM-1	+++	A
	OXA-23, OXA-24, OXA-25, OXA-26, OXA-27, OXA-40, and OXA-48	Same as for IMP family, VIM family, GIM-1, and SPM-1	+	D

* Plus signs denote relative sensitivity to inhibition.

Risk factors for multidrug-resistant *P. aeruginosa* infection included prolonged hospitalization, exposure to antimicrobial therapy, and immunocompromised states such as human immunodeficiency virus infection. Patients with severe multidrug-resistance *P. aeruginosa* infections should be treated with combination therapy, consisting of an antipseudomonal β-lactam with an aminoglycoside or fluoroquinolone rather than aminoglycoside and fluoroquinolone

combinations, to provide adequate therapy and improve patient outcomes. The reasons for use combination therapy are produce synergistic/additive antimicrobial effects, to prevent emergence of resistant bacterial and to expand the spectrum of antibacterial activity beyond that of the individual antibiotic alone, moreover to permit use of lower dose of one of the antimicrobial. However, the major disadvantages to combination therapy are the added cost and the possibility of toxicity (Brody et al., 1994).

2.2 Colistin

The increasing problem of multidrug-resistant gram-negative bacteria causing severe infection and the shortage of new antibiotics to combat them has led to the re-evaluation of polymyxin, this class of antibiotic consists of fine chemically different compounds, polymyxin A, B, C, D and E (colistin). Only polymyxin B and E have been used in clinical practice. Colistin consists of cyclic haptapeptide and a tripeptide side chain acylated at the amino terminus by fatty acid. The amino acid components in the molecule of colistin are D-leucine, L-threonine, and L-α-γ diamino butyric acid (Figure 2-6).

Figure 2-6 structure of colistin (picture from
www.infektionsnetz.at/view.php?datID=1053)

Colistin, a polypeptide antibiotic was first isolated in Japan from *Bacillus colistinus* in 1947. It has been available since 1959 for the treatment of infection caused by gram-negative bacteria including *P. aeruginosa* and *A. baumannii*, but was replaced in 1970 by antibiotic agents became available considered less toxic, such as aminoglycoside and other antipseudomonal.

Colistin is bactericidal to gram-negative bacteria by detergent-like mechanism interfering with the structure and function of the outer and cytoplasmic membranes of bacteria. This mechanism involves interaction with lipopolysaccharides and phospholipids of the outer membrane and electrostatic interference with the outer membrane by competitively displacing divalent cations (calcium and magnesium) from the negatively charged phosphate groups of membrane lipids (Hancock *et al*., 1999). The resultant damage to the osmotic barrier leads to leakage of intracellular contents (Figure 2-7).
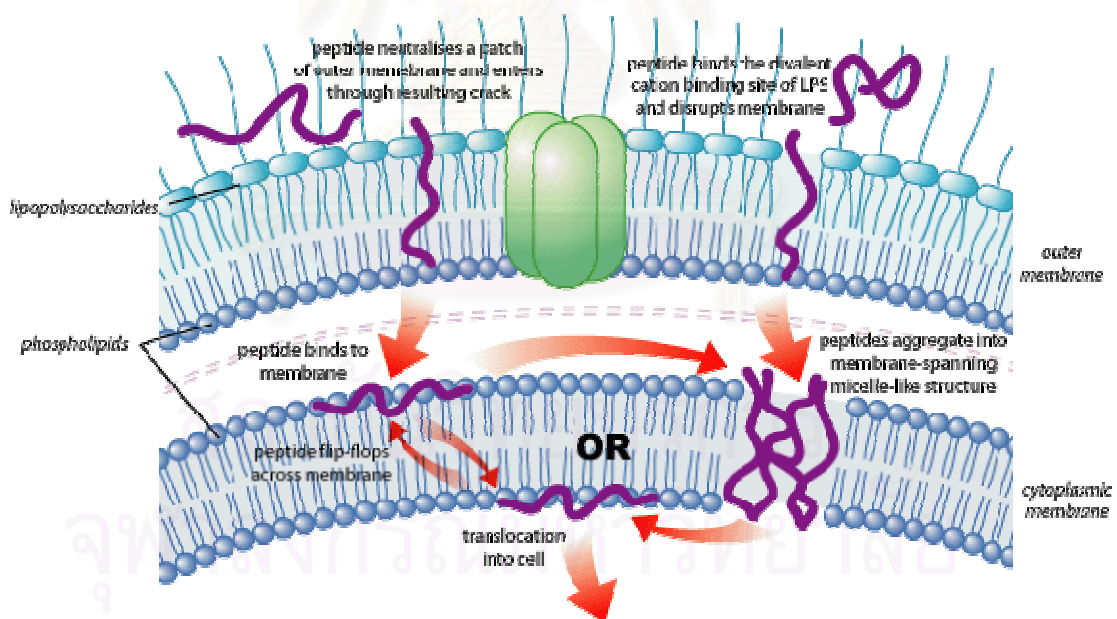
Figure 2-7 The proposed mechanism of action for cationic peptides (picture from webpage www.scq.ubc.ca/?p=405)

Intravenous colistin is usually prescribed at 2.5-5 mg/kg per day divided into two or three doses (in US). In a study of 12 patients with cystic fibrosis who received intravenous colistimesthate sodium at 160 mg (2 million IU) every 8 hours (for patients with body weights >50 kg) or 80 mg (1 million IU) every 8 hours (for patients with body weights <50 kg), the mean ($\pm$SD) half-life of colisthemesthate was 124$\pm$52 min, and the mean ($\pm$SD) half-life of colistin sulfate was 251$\pm$79 min. Mean ($\pm$SD) total body clearance and mean ($\pm$SD) volume of distribution of colistimethate sodium were 2.0$\pm$0.5 ml/min/kg and 340$\pm$95 ml/kg, respectively (Li, 2003). Toxicity involves the kidney and central nervous system, and because of serious adverse effects colistin systemic utilization has been discouraged. Report of early administration of polymyxin was associated with renal and neurological effect in considerably large number of patients. Nephrotoxicity is an important side effect of colistin. Recent studies of the use of intravenous colistin have reported rate of nephrotoxicity ranging from 8% to 14.3% (Lenden *et al*., 2003; Garnacho-Montero *et al*., 2003; Falagas *et al.,* 2005). The proposed mechanism by which colistin induces it nephrotoxic effects including acute tubular necrosis, is closely related to its mechanism of action against gram-negative bacteria. Specifically, colistin increases the tubular epithelial cell membrane permeability, resulting in an increased influx of cations, anions and water, leading to cell swelling and lysis (Falagas *et al.,* 2005).

However, several report published during the period 1999 to 2003 revealed that colistin were effective and safe for treatment of patients infected with multidrug-resistant *P. aeruginosa* and *A. baumannii* (Levin *et al*., 1999; Stein, Raoult, 2002; Koomanochai *et al*., 2007). Recently, the use of systemic colistin has shown efficacy against multidrug-resitant *P. aeruginosa* and Acinetobacter species (Levin *et al*., 1999; Slein, Raoult, 2002; Reina *et al*., 2005). Data from Falagas *et al*. (2005) showed that IV colistimethate sodium (CMS) did not lead to nephrotoxicity in large proportion of patients in prospective study.

There are limited data on mechanism of resistance to colistin. Studies on *P. aeruginosa* suggest a role for OprH (or H1) an outer membrane protein which is overexpressed in low Mg^{2+} environments resulting in resistance to polymyxin B and

gentamicin.   The enterobacteriaceae, changes in negatively-charged surface lipopolysaccharides induced by the regulatory loci *pmrA* and *phoP*, generate resistance to polymyxin (Li *et al*, 2005). Colistin resistance is best documented in *P. aeruginosa* (Denton *et al*., 2002). A survey of cystic fibrosis patients in United Kingdom reported that 3.1% of *P. aeruginosa* isolated were resistance to colistin (Pitt *et al*., 2003). Colistin is rare resistance because the compound self-promotes its penetration of the cell envelope and cause irreversible disruption of cytoplasmic membrane.

There are few experimental and clinical studies in the literature regarding synergistic activity of colistin with other antimicrobial agent against MDR Gram-negative bacteria. Timurkaynak *et al*., (2006) reported that the combination between colistin and rifampin produced the synergy effect 2 of 5 strains of MDR *P. aeruginosa* by checkerboard method. In addition, synergistic activity of colistin with ceftazidime was also note in an *in vitro* study of 2 MDR *P.aeruginosa* strains (Gunderson *et al*., 2003). The combination of colistin, rifampin, and amikacin was synergistic in vitro and led to treatment success in an immunosuppressed patient with multiple abscesses of the lungs, perineum, and gluteus due to MDR *P. aeruginosa*. Morever, data from Tascini *et al*., (2006) found that the combination between colistin, imipenem and rifampin to treatment of diabetic foot infection with osteomylitis due to MDR *P. aeruginosa* infection was efficiency and safety.

CHAPTER III

MATERIALS & METHODS

MATERIALS

1. Microorganisms

The bacterial isolates used throughout this study were 30 strains of *Pseudomonas aeruginosa* which were clinically isolated from the patients at Siriraj Hospital between January and December 2006. All the strains were kindly provided by Assistant Professor Chanwit Tribuddharat, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University. *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as the control strains. All isolates were stored at -20º C in Tryptic Soy Broth: glycerol (85:15) and subcultured twice before use.

2. Chemicals

Standard powders

Two standard powders were used: colistin (potency = 437.8 µg/mg) was kindly provided from Atlantic Pharmaceutical Co., Ltd, Thailand and working standard solutions were prepared immediately prior to use, as specified by the manufacturers. Imipenem and cilastatin for injection (Biopharm, Thailand) was used as working standard of imipenem (potency = 463 mg of imipenem/ 463 mg of cilastatin). The potency of working standard was obtained by assay against standard powder of imipenem according to USP 24, 2000.

Susceptibility disks

Ten antimicrobial disks which were used to determine susceptibility pattern of bacterial strains were cefepime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), rifampin (5 µg), piperacillin-tazobactam (100/10 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), imipenem (10 µg) and colistin (10 µg). All of the disks, which were used to determine inhibition zone, were purchased from BBL chemicals (Beckton Dickinson, USA).

E-test strips

E-test strips containing cefepime (0.016-256μg/ml), amikacin (0.016-256μg/ml), piperacillin-tazobactam (0.016-256μg/ml), ciprofloxacin (0.002-32μg/ml) and imipenem (0.002-32μg/ml) were used for the determination the minimum inhibitory concentration (MIC) against all bacterial strains. All of these were purchased from AB BIODISK Solna, Sweden.

3. Media and Reagents

Muller-Hinton Agar (MHA) and Muller-Hinton Broth (MHB) which were purchased from BBL chemicals (Beckton Dickinson, USA) were used as the test medium for all bacterial strains.

Tryptic Soy Agar (TSA) which were purchased from BBL chemicals (Beckton Dickinson, USA) were used as the culture media for *P. aeruginosa, P. aeruginosa* ATCC 27853 and *E.coli* ATCC 25922.

Sterile water was used as the solvent for the chemical powders to develop the working solution.

Sterile normal saline solution (NSS) was chosen as the diluent of the inoculum in the turbidity adjusting process to quantity the precise numbers of bacteria. This NSS was also applied as the diluent of the specimens in colony counting procedures in the time kill method.

METHODS

1. Disk diffusion test was performed to determine the susceptibility patterns of bacterial strains to antibiotics.

2. E-test method was performed to determine the minimum inhibitory concentration (MIC) of the tested agents and to detect the multidrug-resistant strains.

3. Determination of metallo-β-lactamase production in imipenem resistant strains was done by the double disk diffusion method.

4. Broth macrodilution method was performed to determine the minimum inhibitory concentration (MIC) of the test agents.

5. Checkerboard microdilution panel method was done to determine the synergism between imipenem and colistin.

6. Time kill method was done to determine the combined bactericidal activity of imipenem and colistin.

7. Scanning electron microscope was used to observe the morphological cell change of *P.aeruginosa* after exposure to imipenem, colistin and the combination of imipenem and colistin.

1. Disk diffusion test to determine the susceptibility pattern of bacterial strains to antibiotics.

Kirby-Bauer Disk susceptible test was performed according to the Disk diffusion method by NCCLS, 2004. All isolates including the control strain were tested to determine susceptibility pattern of the organism against 10 antimicrobial agents.

1.1 Preparation of media

1.1.1 Mueller-Hinton agar (MHA) was prepared from a commercially available dehydrated base according to the manufacturer's instructions.

1.1.2 Immediately after autoclaving, the media was allowed to cool in a 45°C to 50°C water bath.

1.1.3 The freshly prepared and cooled medium was poured into glass, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of media approximately 4 mm. This corresponds to 25 ml of media for plates with a diameter of 90 mm.

1.1.4 The agar medium should be allowed to cool at room temperature and all prepared plates must be examined for the sterility by incubating at 37°C for 24 hours.

1.1.5 Unless the plates were used the same day, there were stored in a refrigerator (2°C to 8°C) and should be used within 7 days after preparation.

1.2 Preparation of inoculum

1.2.1 The well-isolates colony of each 18 hours *P. aeruginosa* from clinical specimen, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were selected from Tryptic Soy Agar (TSA) plates and transferred to a tube containing 7 ml normal saline solution (NSS).

1.2.2 The suspension was adjusted to match the turbidity of the 0.5 McFarland standard solution. A suspension containing approximately 1 to 2x10^{8} CFU/ml of bacteria was obtained.

1.3 Inoculation of the test plates

1.3.1 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab should be rotated several time and pressed firmly on the inside wall of the tube above the fluid leveling order to remove excess inoculum from the swab.

1.3.2 The dried surface of an agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculums.

1.3.3 The lid was left agar for 5 minutes to allow for any excess surface moisture to be absorbed before applying the antibiotic disks.

1.4 Application of disks to inoculated agar plates

1.4.1 The antibiotic disks were applied to the surface of the medium with sterile forceps. Each disk was pressed down to ensure complete contact with the agar surface. They was distributed evenly so that they were no closer than 24 mm from center to center

1.4.2 The plate were inverted and incubated at 37°C for 24 hours before measuring the zones of inhibition.

1.5 Reading plates and interpreting results

1.5.1 After 24 hours of incubation, each plate was examined. The diameters of zones of inhibition, including the diameter of the disk were measured with digital sliding venier caliper.

1.5.2 The size of the inhibition zone was interpreted by referring to the NCCLS, 2004 and the organisms were reported as either susceptible, intermediate, or resistant to the agents that have been tested (Tables3-1).

Table 3-1 Zone diameter interpretive standards breakpoints for *P. aeruginosa,* *P.aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 to 10 antimicrobial agents (NCCLS, 2004).

Drug	Disk content (μg)	Zone diameter (mm)				
		P. aeruginosa			*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922
		R[a]	I[b]	S[c]		
amikacin	30	≤14	15-16	≥17	18-26	19-26
cefepime	30	≤14	15-17	≥18	24-30	29-35
ceftazidime	30	≤14	15-17	≥18	22-29	25-32
ciprofloxacin	5	≤15	16-20	≥21	25-33	30-40
colistin	10	≤8	9-10	≥11	-	11-15
gentamicin	10	≤12	13-14	≥15	16-21	19-26
imipenem	10	≤13	14-15	≥16	20-28	26-32
piperacillin/tazobactam	100/10	≤17	-	≥18	25-33	24-30
rifampin	5	≤16	17-19	≥20	-	8-10
tobramycin	10	≤12	13-14	≥15	19-25	18-26

R[a]= Resistant, I[b]= intermediate resistant, S[c]= susceptible

2. E-test method to determine minimum inhibitory concentration (MICs)

E-test method was performed according to CLSI, 2006. (*P. aeruginosa* ATCC 27853 was also includes in this study as the control strains). The minimum inhibitory concentrations (MICs) of 5 broad-spectrum antibiotics (cefepime, amikacin, ciprofloxacin, imipenem and piperacillin-tazobactam) against all 30 isolates of *P. aeruginosa* were determined in order to screen for the multidrug-resistant strain. The method was briefly described as followed:

2.1 Preparation of media

2.1.1   Mueller-Hinton Agar (MHA) was prepared from a commercially available dehydrated base according to the manufacturer's instructions.

2.1.2   Immediately after autoclaving, it was allowed to cool in a 45°C to 50°C water bath.

2.1.3   The freshly prepared and cooled medium was poured into glass, flat-bottomed. petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponded to 25 to 30 ml of media per plate with a diameter of 90 mm.

2.1.4 The agar medium was allowed to cool at room temperature and all prepares plates were examined for the sterility by incubating at 37° C for 24 hours.

2.1.5 Unless the plates were used the same day, they were stored in a refrigerator (2°C to 8°C) and were used within 7 days after preparation.

2.2 Preparation of inoculum

2.2.1 The well-isolates colony of each 18 hours *P. aeruginosa* from clinical specimen and *P. aeruginosa* ATCC 27853 were selected from Tryptic soy agar (TSA) plates and transferred to a tube containing 7 ml normal saline solution (NSS).

2.2.2 The suspension was adjusted to match the turbidity of the 0.5 McFarland standard solution. This resulted in a suspension containing approximately 1 to $2x10^{8}$ CFU/ml.

2.3 Inoculation test plates

2.3.1 Optimally, within 15 minutes after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the inoculum suspension and excess fluid was removed by pressing the swab against the inside wall of the test tube. The entire agar surface was steaked three times by rotating the plate approximately 60 degree each time to evenly distribute the inoculum.

2.3.2 Excess moisture was allowed to be absorbed for about 10 minutes so that the surface was completely dried before applying the E-test strips.

2.4 Application of E-test strips to inoculated agar plates

2.4.1 The E-test strips were applied to the surface of the medium at the center of the plate with sterile forceps. Once applied, the strip had not been moved because of instantaneous release of antibiotic into the agar.

2.4.2 The plate were inverted and incubated at 37°C for 18 hours.

2.5 Reading plates and interpreting results

2.5.1 The MICs were recorded as the lowest concentrations of antimicrobial agents that could completely inhibited the growth of the tested organisms. The MIC value was read where the edge of the inhibition ellipse intersects the side of the strip. When growth occurred along the entire strip i.e. no inhibition ellipse was seen, the MIC was reported as greater than (>) the higher value on the scale. When the inhibition ellipse was below the strip i.e. it did not intersect the strip, the MIC was reported as less than (<) the lowest value on the scale.

2.5.2 The MICs were interpreted by referring to the CLSI, 2006 and organisms were reported as either susceptible or resistant to the agents that have been tested (Tables 3-2).

Table3-2 MICs interpretive standard breakpoints (µg/ml) by E-test (CLSI, 2006)

Antibiotic	Minimum Inhibitory Concentrations [MICs] (µg/ml)				
	P. aeruginosa			*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922
	S[a]	I[b]	R[c]		
amikacin	≤16	32	≥64	1.0-4.0	0.5-4.0
cefepime	≤8	16	≥32	1.0-4.0	0.016-0.064
ciprofloxacin	≤1	2	≥4	0.125-0.5	0.004-0.015
imipenem	≤4	8	≥16	1.0-4.0	0.064-0.25
piperacillin-tazobactam	≤64	-	≥128	1.0-8.0	1.0-4.0

S[a]= Susceptible, I[b]= Intermediate resistant, R[c]= Resistant

2.6 Screening for the multidrug-resistant strains

An isolate was considered to be the multidrug-resistant strain when it was resistant to three or more of the following broad-spectrum agents: cefepime, amikacin, ciprofloxacin, piperacillin-tazobactam and imipenem according to the MIC values from the previous test.

3. Determination of metallo-β-lactamase production in imipenem resistant strains by double disk diffusion method (NCCLS, 2004)

A double disk diffusion test was constructed for the detection of metallo-β-lactamase-producing gram-negative bacteria. The 30 μg ceftazidime disks and a filter disk containing EDTA which was a metallo-β-lactamase inhibitor were used in this test. The EDTA disk was used in this study because the agent has been reported to be able to block metallo-β-lactamase (Payne *et al.,* 1994). When the bacteria produce this enzyme, a distinct growth inhibitory zone appears between the ceftazidime disk and the filter disk containing EDTA.

3.1 Preparation of media

3.1.1   Mueller-Hinton agar (MHA) was prepared from a commercially available dehydrated base according to the manufacturer's instructions.

3.1.2   Immediately after autoclaving, it was allowed to cool in a 45^oC to 50^oC water bath.

3.1.3   The freshly prepared and cooled medium was poured into glass, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of media approximately 4 mm. This corresponded to 25 to 30 ml of media per plate with a diameter of 90 mm.

3.1.4   The agar medium should be allowed to cool to room temperature and all prepared plates was examined for the sterility by incubating at 37^oC for 24 hours.

3.1.5   Unless the plates were used in the same day, they were stored in a refrigerator (2^oC to 8^oC) and were used within 7 days after preparation.

3.2 Preparation of inoculums

3.2.1   The well-isolates colony of each 18 hours imipenem resistant *P. aeruginosa* strains were selected from Tryptic Soy Agar (TSA) plates and transferred to a tube containing 7 ml normal saline solution (NSS).

3.2.2 The suspension was adjusted to match the turbidity of the 0.5 McFarland standard solution. This resulted in a bacterial suspension containing approximately 1 to 2×10^8 CFU/ml.

3.3 Inoculation test plates

3.3.1 Optimally, within 15 minutes after the turbidity of the inoculums suspension was adjusted, a sterile cotton swab was dipped into the inoculum suspension and excess fluid was removed by pressing the swab against the inside wall of the test tube. The entire agar surface was carefully streaked three times, rotating the plate approximately 60 degree each time to evenly distribute the inoculum.

3.3.2 Excess moisture was allowed to be absorbed for about 10 minutes so that the surface was completely dried before applying the ceftazidime disk and EDTA disk.

3.4 Application of disks to inoculated agar plates

3.4.1 The two commercially supplied Kirby-Beuer disks, each containing 30μg of ceftazidime were then placed on the plates. The distance between the two ceftazideme disks was kept at about 4 to 5 cm, and filter disk containing 5 μl of 600 mM EDTA was placed near one of the ceftazidime disks within a center-to-center distance of 1.0 to 2.5 cm.

3.4.2 The plate were inverted and incubated at 37°C for 18 hours.

3.5 Reading plates and interpreting results

A positive result showed the growth inhibitory zone between the two disks expanded as shown in Figure 3-1, while no change was evident around the two double disks containing ceftazidime with or without EDTA for negative result.
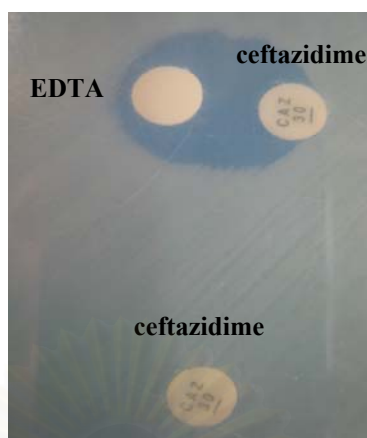
Figure 3-1 Assessment of positive metallo-β-lactamase production with double disks technique.

4. Broth macrodilution method to determine minimum inhibitory concentration (MICs), NCCLS, 2004

Broth macrodilution method was performed according to NCCLS, 2004 in order to determine minimal inhibitory concentrations (MICs) of imipenem and colistin against all tested isolates.

4.1 Preparation of antimicrobial solution

4.1.1 The two-fold dilution of imipenem solution (0.03-256 µg/ml) and colistin solution (0.03-256 µg/ml) were prepared by diluting the drug in MHB. The antimicrobial concentrations used in initial solutions were prepared to be 2-fold higher than the desired final concentration (0.06-512 µg/ml).

4.1.2 One ml of each antimicrobial dilution in broth was transferred to the sterile tube except for the last tube (no antimicrobial agent) which was served as a growth control.

4.2 Preparation of inoculum

4.2.1 The well-isolates colony of each 18 hours *P. aeruginosa* from clinical specimen and *P. aeruginosa* ATCC 27853 were selected from Tryptic Soy Agar (TSA) plates and transferred to a tube containing 7 ml normal saline solution (NSS).

4.2.2   The suspension was adjusted to match the turbidity of the 0.5 McFarland standard solution.   This resulted in a bacterial suspension contained approximately 1 to 2 x 10^8 CFU/ml.

4.2.3   The suspension was diluted 1:100 in MHB to give final inoculum density of 1 x 10^6 CFU/ml.

4.2.4   One ml aliquot of test organism was added to one set of tubes and 1 ml of control organism to the other. The contents were mixed thoroughly. Thus, the final inoculum in broth contained approximately 5 x 10^5 CFU/ml.

4.2.5 Two uninoculated tubes of antibiotic-free broth (the first tube was use as a control tube for the adequacy of the broth to support the growth of the organism which the second was a check of sterility) were also included. The set of tubes were incubated at at 37^oC for 24 hours.

4.3 Test result interpretation

4.3.1   The lowest concentration of antimicrobial agent that resulted in complete inhibition of visible growth represented the MIC. A very faint haziness or a small bottom of possible growth was generally disregarded.

4.3.2   The MICs were interpreted by referring to the NCCLS, 2004 and the organisms were reported as either susceptible, intermediate, or resistant to the agents that have been tested (Table 3-3).

Table 3-3 MICs interpretive standard breakpoints (µg/ml) by broth macrodilution (NCCLS, 2004)

| Drug | Minimum Inhibitory Concentrations [MICs] (µg/ml) | | | |
| | P. aeruginosa | | | P. aeruginosa |
	S^a	I^b	R^c	ATCC2785
imipenem	≤ 4	8	≥16	0.06-0.25
colistin [d]	≤4	-	≥8	0.25-1

[a] =susceptible, [b] =intermediate, [c] =resistant, [d] =recommended by British Society of Antimicrobial Chemotherapy (BSAC)

5. Checkerboard microdilution panel method to determine synergism between imipenem and colistin

Checkerboard method was performed according to NCCLS, 2004 and to Moody, 2004. All isolates were included in the test to determine the combination effect of imipenem and colistin. The concentrations tested for colistin were 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32µg/ml while these for imipenem were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64µg/ml, respectively.

5.1 Preparing test broth

5.1.1 Mueller-Hinton broth (MHB) was prepared from a commercially available dehydrated base according to the manufacturer's instructions.

5.1.2 The medium concentrations used in the initial solutions were prepared to be four-fold higher than the desired final concentration.

5.2 Preparing diluted antimicrobial agents

5.2.1 The two-fold dilutions of drugs were prepared volumetrically in the broth.

5.2.2 The antimicrobial concentrations used in the initial solutions were prepared to be four-fold higher than the desired final concentration and the concentrations tested for each antimicrobial agents typically ranged from 5 dilutions below the MIC to double the MIC or higher.

5.3 Broth dilution testing

A standardized inoculum for the microdilution broth method may be prepared by suspending colonies directly to obtain the turbidity of the 0.5 McFarland standard.

5.3.1 The adjusted inoculum suspension was diluted in broth within 15 minutes after the inoculation, each tube contained approximately 5×10^5 CFU/ml.

5.3.2 The final volume of 200 µl in each well consisted of 50 µl of MHB, 50 µl of imipenem, 50 µl of colistin and 50 µl of bacterial suspension was obtained.

5.3.3 A series of antimicrobials containing four time the desired final concentrations were taken to produce the desired range of drug concentration by adding an aliquot of those solution to each well in the appropriate row or column as shown in Figure 3-2.

Imipenem											
64	64/0.03	64/0.06	64/0.12	64/0.25	64/0.5	64/1	64/2	64/4	64/8	64/16	64/32
32	32/0.03	32/0.06	32/0.12	32/0.25	32/0.5	32/1	32/2	32/4	32/8	32/16	32/32
16	16/0.03	16/0.06	16/0.12	16/0.25	16/0.5	16/1	16/2	16/4	16/8	16/16	16/32
8	8/0.03	8/0.06	8/0.12	8/0.25	8/0.5	8/1	8/2	8/4	8/8	8/16	8/32
4	4/0.03	4/0.06	4/0.12	4/0.25	04/0.5	4/1	4/2	4/4	4/8	4/16	4/32
2	2/0.03	2/0.06	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32
1	1/0.03	1/0.06	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32
0	0.0.3	0.06	0.12	0.25	0.5	1	2	4	8	16	32

Colistin

Figure 3-2 Checkerboard technique, serial dilution of imipenem and colistin are performed using drugs proportional to MICs of the drugs being tested (Modified from Eliopoulos and Moellering, 1996).

5.4 Reading plates and interpreting results

5.4.1 After 16-24 hours, each panel was examined to determine MIC, the MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the panel as detected by the unaided eye. The amount of growth in the tubes containing the antibiotic was compared with the amount of growth in the positive-control well (no antibiotics) and the negative-control well (no organism) used in each set of tests when determining the growth end points.

5.4.2 The interpretation of the antimicrobial combination interaction were done by reading the first clear well in each row of panel with both agents.

5.4.3   Based on this reading, fractional inhibitory concentrations (FICs) were calculated for each antimicrobial alone and in combination.   The following formulars were used to calculate the FIC.

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

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

5.4.4 The fractional inhibitory concentration index (FICI) or $\sum$FIC for this combinations was calculated according to the following formula.

$$\text{FIC index } (\textstyle\sum\text{FIC}) = \text{FIC of imipenem} + \text{FIC of colistin}$$

5.4.5   FIC index results for each combination were defined as :

Synergy: decrease in the MIC of each agent was $\geq$ 4-fold ($\sum$FIC $\leq$ 0.5).

Partial synergy: decrease in MIC of 1 agent was $\geq$ 4-fold and decrease in the MIC of the other agent was 2-fold ($\sum$FIC > 0.5 and <1).

Additive: decrease in the MIC of both agents was 2-fold ($\sum$FIC = 1).

Indifference: interactions did not meet the above criteria and were not antagonist ($\sum$FIC >1 and < 4).

Antagonist: increase in the MIC of both agents was $\geq$ 4-fold ($\sum$FIC $\geq$ 4).

The smallest FIC value was used to establish the antimicrobial combination interaction for each specific strain, except for antagonist, which was preferably reported.   Results were expressed as percentage of isolates with synergy, partial synergy, additive, indifference and antagonist.

6. Time kill method was done to determine the bactericidal activity of the colistin alone and the combination between imipenem and colistin.

The antibacterial activity of the combination was performed according to the time kill method (NCCLS, 1999). Eight isolates, were imipenem-resistant strains, were tested to determine the bactericidal activity of colistin alone and of the combination of imipenem plus colistin against multidrug-resistant *P. aeruginosa*. The selected drugs and bacteria in time kill method must be correlated with checkerboard method to define MICs as describe previously.

6.1 Determination of bactericidal activity of the combination between imipenem and colistin

6.1.1 Imipenem concentration was prepared to 1xMIC and 2xMIC, colistin was prepared to 0.5xMIC and 0.25xMIC. Antimicrobial concentrations used in initial (stock) solutions were prepared to be ten fold higher than the desired final concentrations.

6.1.2 A 1 ml of each drug was pipetted into Mueller Hinton broth (MHB) for the preparation of the working media before adding the standardized inoculum (final volume of working media = 9 ml). By doing so, there were 7 groups including control (no antimicrobial agents), 1xMIC of imipenem, 2xMIC of imipenem, 0.5xMIC of colistin, 0.25xMIC of colistin, the combination between 1xMIC of imipenem and 0.5xMIC of colistin, and the combination between 1xMIC of imipenem and 0.25xMIC of colistin.

6.1.3 Inoculum which was adjusted to match the turbidity of the 0.5 McFarland standard solution, contained approximately 1 to $2x10^8$ CFU/ml was then diluted ten fold to make 1 to $2x10^7$ CFU/ml of the bacterial inoculum.

6.1.4 A 1 ml of inoculum was pipetted to the 9 ml working media 9 ml and incubated at 37^oC in a shaking water bath.

6.1.5 The samples were collected for culture at the time 0, 2, 4, 6, 8 and 24 hours after the microorganisms were exposed to each group of the antimicrobials including the control group.

6.1.6 A 0.5 ml of the collected sample was diluted ten fold in 4.5 ml NSS and 20 µl of each dilution was dropped to the surface of TSA plates which were then incubated at 37°C for 18 hours.

6.1.7 The quantity of survival bacteria in each group was calculated to obtain the killing curves data. The quantity of survival bacteria in each group was calculated to obtain the killing curves data.

6.1.8 Killing curves were constructed by Microsoft Excel 2007 at each time interval. The log change of the viable cell counts compared to the starting inoculums was determined.

6.1.8.1 The results were analyzed by determining the number of strains which the yield changes in the log number of CFU/ml of -1, -2 and -3 at 2, 4, 6, 8 and 24 hours compared to the counts at 0 hours. A given concentration of antimicrobial alone or in combination was considered bactericidal if it reduced the original inoculums size by ≥3 log CFU/ml (≥99.9% killing) at each of the time periods or bacteriostatic if the inoculums size was reduced by 0-3 log CFU/ml. The regrowth was defined as an increase of ≥2 log CFU/ml after 6 hours (Amterdam, 1996; Pankuch, Jacobs and Appelbaum, 1994).

6.1.8.2 The quantitative evaluation of antimicrobial effect was calculates as in the published article (Firsov *et al*., 1997).
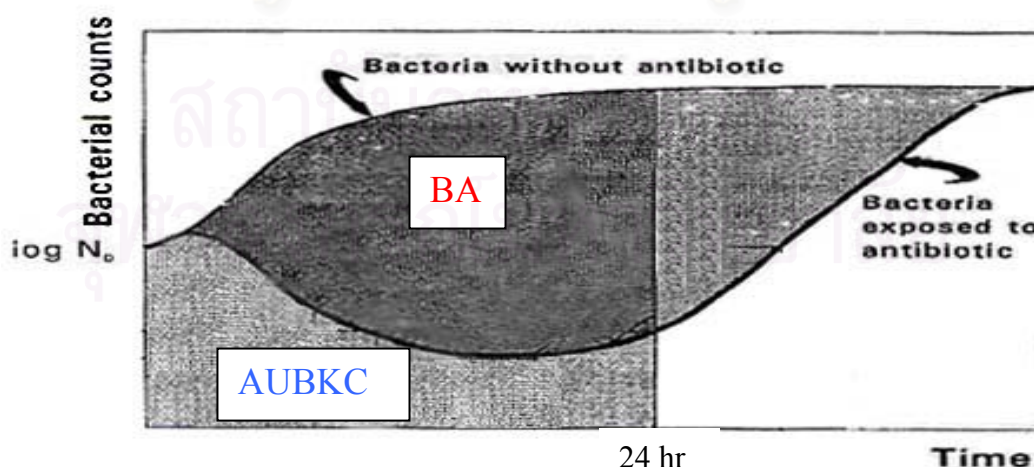
Figure 3-3 Parameters for quantifying bacterial killing, regrowth curve and the antimicrobial effect (Modified from Firsov *et al.,* 1997).

The following parameters were calculated by various methodologies as followed:

$AUBKC_{0-24}$ = Area under the bacterial killing and regrowth curves that were calculated by the trapezoidal rule for 24 hours.

Bacteriolytic area for 24 hours (BA_{24}) = the area between control growth curve and the bacterial killing and regrowth curves ($AUBKC_{0-24}$ of the control growth curve subtracted by $AUBKC_{0-24}$ of the bacterial killing and regrowth curves)

6.2 Determination of bactericidal activity of colistin

6.2.1 Colistin concentrations were prepared to 1xMIC, 2xMIC, 4xMIC and 8xMIC. Antimicrobial concentrations used in initial (stock) solutions were prepared to be ten fold higher than the desired final concentrations.

6.2.2 A 1 ml of each drug was pipetted into 8 ml Mueller Hinton broth (MHB) for the working media preparation before adding the standardized inoculum (final volume of working media = 9 ml). By doing so, there were 5 groups including the control (no antimicrobial agents), 1xMIC of colistin, 2xMIC of colistin, 4xMIC of colistin and 8xMIC of colistin.

6.2.3 Inoculum which was adjusted to match the turbidity of the 0.5 McFarland standard solution, contained approximately 1 to $2x10^8$ CFU/ml was then diluted ten fold to make 1 to $2x10^7$ CFU/ml of the bacterial inoculum.

6.2.4 A 1 ml of inoculum was pipetted to the working media and was incubated at 37°C in a shaking water bath.

6.2.5 The samples were collected for culture at the time 0, 2, 4, 6, 8 and 24 hours after the microorganism were exposed to each group of the antimicrobials including the control group.

6.2.6 A 0.5 ml of the collected sample was diluted ten fold in 4.5 ml NSS and 20 μl of each dilution was dropped on the surface of TSA plates which were then incubated at 37°C for 18 hours.

6.2.7 The quantity of the survival bacteria in each group was calculated to obtain the killing curves data.

6.2.8 Killing curves were constructed by Microsoft Excel 2007 at each time interval. The log change of the viable cell counts compared to the starting inoculum was determined.

6.2.8.1 The results were analyzed by determining the number of strains which yield changes in the log number of CFU/ml of -1, -2 and -3 at 2, 4, 6, 8 and 24 hours as compared to the counts at 0 hours. A given concentration of antimicrobial alone or in combination was considered bactericidal if it reduced the original inoculums size by ≥ 3 log CFU/ml ($\geq 99.9\%$ killing) at each of the time periods or bacteriostatic if the inoculums size was reduced by 0-3 log CFU/ml. The regrowth was defined as an increase of ≥ 2 log CFU/ml after 6 hours (Amterdam, 1996; Pankuch, Jacobs and Appelbaum, 1994).

6.2.8.2 The quantitative evaluation of antimicrobial effect ($AUBKC_{0-24}$, BA24) was calculated as in page 36.

Statistic analysis

One-way ANOVA was used to compare the Log change of viable cell counts, BA_{24}, which were expressed in their mean value ($\pm$ SD) values. Any value of $p < 0.05$ was considered as significant difference.

7. Determination of the morphological cell structure change of *P. aeruginosa* after exposure to imipenem, colistin and the combination of the drugs (Modified from Kobayashi *et al.,* 2004).

The scanning electron microscopy was chosen to examine the morphological changes in *P. aeruginosa* when exposed to imipenem, colistin and the combination after 2 hours. The selected concentration of drugs and bacterial strains in this study was correlated to those in the broth dilution test and time kill study.

7.1 Imipenem concentration was prepared to 1xMIC while colistin concentration was to 0.5xMIC. Antimicrobial concentrations used in

initial (stock) solutions were prepared ten fold greater than the desired final concentration.

7.2   One ml of each drug was pipetted into 8 ml Mueller Hinton broth (MHB) for the working media preparation before adding the standardized inoculums (final volume of working media = 9 ml). By doing so, there were 4 groups including the control (no antimicrobial agents), 1xMIC of imipenem 1xMIC, 0.5xMIC of colistin and the combination between imipenem and colistin 0.5xMIC.

7.3   Inoculum was adjusted to match the turbidity of the 0.5 McFarland standard solution, contained approximately 1 to 2 x 10^8 CFU/ml was then diluted ten fold to make 1 to 2 x 10^7 CFU/ml of the bacterial inoculum.

7.4   One ml of inoculum was pipetted to the working media and was then incubated at 37^oC in a shaking water bath.

7.5   The specimens were collected after 2 hours of exposure in order to detect the morphological changes.

7.6   The specimens were centrifuged at low speed centrifugation (3000 x g) for 10 minutes to change suspending bacterial cells to be sedimented (this procedure conducted at 4^oC to keep bacteriostatic condition).

7.7   The specimens was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.2 for 2 hours then they were rinsed twice in phosphate buffer for 5 min/each and once in distilled water for 10 minutes.

7.8   After that the specimens were dehydrated with a graded series of ethanol (30%, 50%, 70%, 90% 5 min/each and absolute ethanol 3 times, 5 min/time).

7.9   The specimens were critical point dried (Critical Point Dryer, Balzer model CPD 020), mounted and coated with gold (Sputter Coater, Balzers model SCD 040).

7.10   The specimens were observe under a scanning electron microscope (JEOL, model JSM-5410LV) and were photographed.

CHAPTER IV

RESULTS

Disk diffusion test

The disk diffusion test according to NCCLS (2004) was performed. The antimicrobial susceptibility patterns (antibiograms) against 10 antimicrobial agents were shown in Table 4.1. There were 8 different susceptibility patterns according to the types and numbers of the susceptible agents. Four strains (13.33%) were susceptible to 8 antimicrobial agents and were classified as pattern 1. One isolate (3.33%) which was susceptible to 5 antimicrobial agents, was classified in pattern 2. Pattern 3 consisted of 1 strains (3.33%) which was susceptible to 4 antimicrobial agents. One strains (3.33%) was also susceptible to 3 antimicrobial agents and was classified as pattern 4. The other 13 strains (43.33%) which were susceptible to 2 different antimicrobial agents were classified as pattern 5 to 7. The highest number of strains (10 strains, 33.33%) which were pattern 8 was susceptible only to colistin.

Table 4.1 Susceptibility patterns (antibiograms) of 30 *P. aeruginosa* strains against 10 antimicrobial agents

Pattern	Susceptible antimicrobial agent	No. of susceptible agent	No. of strains (%)
1	FEP CAZ GM NN CIP TZP CL AK	8	4 (13.33)
2	FEP CAZ TZP CL AK	5	1 (3.33)
3	FEP CAZ CL AK	4	1 (3.33)
4	CAZ CL AK	3	1 (3.33)
5	CIP CL	2	1 (3.33)
6	TZP CL	2	8 (26.67)
7	IPM CL	2	4 (13.33)
8	CL	1	10 (33.33)

FEP= cefepime, CAZ= ceftazidime, GM= gentamicin, NN= tobramycin, CIP= ciprofloxacin, TZP= piperacillin-tazobactam, IPM= imipenem, RA= rifampin, CL=colistin, AK=amikacin

In Table 4.2 it was shown that all strains were resistant to rifampin whereas susceptible to colistin. For gentamicin and tobramycin, 86.67% of tested strains were resistant to both of antimicrobial agents. Eighty percent of these strains were resistant to ciprofloxacin, while 76.67% were resistant to imipenem, cefepime, ceftazidime and amikacin. The other 13.33% were resistant to piperacillin-tazobactam.

Table 4.2 Antimicrobial activity of 10 antimicrobial agents against 30 strains of *P. aeruginosa* as tested by disk diffusion method.

Antimicrobial agents	Susceptible		Resistant	
	No. of strains	%	No. of strains	%
amikacin	7	23.33	23	76.67
cefepime	6	20	24	80
ceftazidime	7	23.33	23	76.67
ciprofloxacin	5	16.67	25	83.33
colistin	30	100	0	0
gentamicin	4	13.33	26	86.67
imipenem	4	13.33	26	86.67
piperacillin-tazobactam	13	43.33	17	56.66
rifampin	0	0	30	100
tobramycin	4	13.33	26	86.67

Minimum inhibitory concentrations (MICs) determined by E-test method

The MIC_{50} and MIC_{90} of amikacin, imipenem, ciprofloxacin, piperacillin-tazobactam and cefepime against all 30 strains were shown in Table 4-3. It was found that most of the pathogens (80%) were resistant to ciprofloxacin when compared to the other tested agents. The MIC_{50} and MIC_{90} of ciprofloxacin were both >32 µg/ml which were the high level of resistance (susceptibility breakpoint ≤ 1 µg/ml). In addition, the MIC_{50} and MIC_{90} of imipenem and cefepime were ≥ 32 µg/ml and ≥ 256 µg/ml, respectively. Whereas, the MIC_{50} of amikacin and piperacillin-tazobactam were 48 µg/ml and 96 µg/ml with the same MIC_{90} of ≥ 256 µg/ml. It was also shown that 21 strains (70%) were resistant to imipenem, 23 strains (76.67%) to cefepime, 11 strains (36.67%) to piperacillin-tazobactam and 9 strains (30%) to amikacin.

Table 4-3 MIC_{50} and MIC_{90} of each tested antimicrobial agents

	Amikacin		Imipenem		Ciprofloxacin		Piperacillin-tazobactam		Cefepime	
	No.	%	No.	%	No.	%	No.	%	No.	%
S	7	23.33	3	10	5	16.67	12	40	4	13.33
I	14	46.67	6	20	1	3.33	7	23.33	3	10
R	9	30	21	70	24	80	11	36.67	23	76.67
MIC_{50}	48		>32		>32		96		>256	
MIC_{90}	>256		>32		>32		>256		>256	

S= Susceptible, I= Intermediate, R= Resistant

The result from this part of studies showed that 19 strains (63.33%) were the multidrug-resistant (MDR) strains of *P. aeruginosa* because they were resistant to at least 3 out of 5 of the tested agents, while 11 strains (36.67%) were non-MDR strains. Among the MDR strains, 6 strains (20%) were resistant to 3 agents, 10 strains (33.33%) were resistant to 4 agents and 3 strains (10%) were resistant to all antimicrobial agents tested (Table 4-4). The distributions of multidrug-resistant strains of *P. aeruginosa* according to the number of antimicrobial agents were shown in Figure 4-1.

Table 4-4 Distribution of *P .aeruginosa* according to the number of antimicrobial agents that they were resistant

No. of antimicrobial agent	Resistant strains	% of strains	Total strains	%
0 drug	1	3.33	11 (Non-MDR)	36.67
1 drug	5	16.67		
2 drugs	5	16.67		
3 drugs	6	20	19 (MDR)	63.33
4 drugs	10	33.33		
5 drugs	3	10		
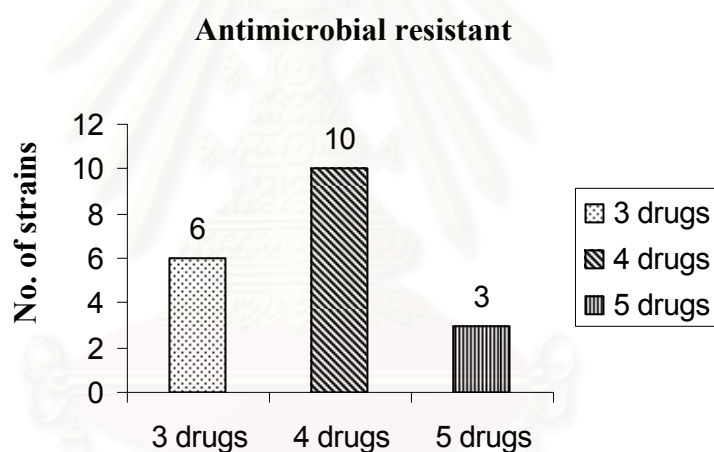
Figure 4-1 Distribution of multidrug-resistant strains of *P. aeruginosa* according to the number of antimicrobials to which they were resistant.

Metallo-β-lactamase production in imipenem resistant strains

The detection of metallo-β-lactamase enzyme production was performed in all 30 strains. The negative reaction for metallo-β-lactamase production was observed from the double disk method.
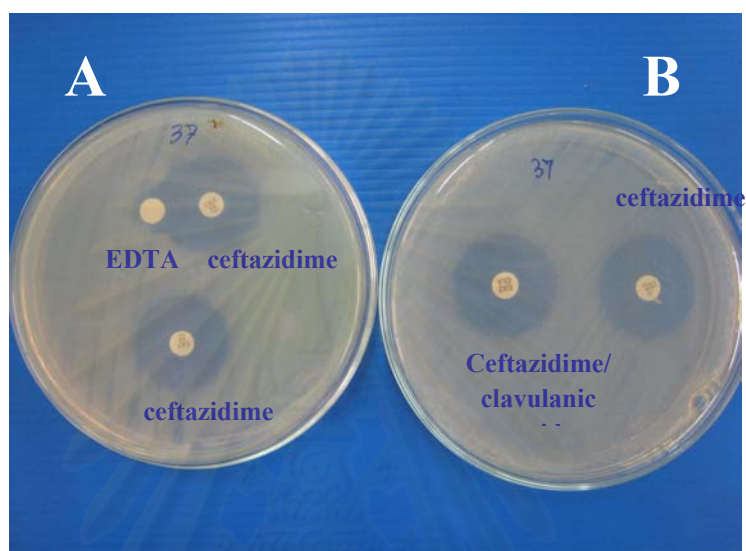
Figure 4.2 (A), Negative for metallo-β-lactamase production by the double disk method in imipenem-resistant strains. (B), negative for β-lactamase inhibition by clavulanic acid (strain No.37).

Minimum inhibitory concentration (MICs) of Imipenem and Colistin determined by Broth macrodilution method

MICs of imipenem and colistin from macrodilution method (Figure 4-3) when tested with all 30 strains of *P. aeruginosa* were shown in Table 4-5. The 26.67% of the tested organism were imipenem resistant strains while none of the strains were resistant to colistin (susceptible breakpoint $\leq$4 µg/ml). MICs range of imipenem were 4 to 16 µg/ml (susceptible breakpoint $\leq$4 µg/ml) but the MICs range of colistin were 0.5 to 4 µg/ml. The MIC_{50} and MIC_{90} of imipenem were 8 µg/ml and 16 µg/ml, respectively, whereas both MIC_{50} and MIC_{90} of colistin were 2 µg/ml.

Table 4-5 MIC distribution and MIC_{50}, MIC_{90} of imipinem and colistin by broth macrodilution method

	Imipenem			colistin	
	MIC range	% (No.)		MIC range	% (No.)
	0.03	0		0.03	0
	0.06	0		0.06	0
	0.12	0		0.12	0
	0.25	0		0.25	0
	0.5	0		0.5	6.67 (2)
	1	0		1	40 (12)
	2	0	$MIC_{50, 90}$	2	46.67 (14)
	4	23.33 (7)		4	6.67 (2)
MIC_{50}	8	50 (15)		8	0
MIC_{90}	16	26.67 (8)		16	0
	32	0		32	0
	64	0		64	0
	128	0		128	0
	256	0		256	0

Figure 4.3 MIC of imipenem against one strain of *P. aeruginosa* was determined by broth macrodilution method.

Synergy study

The result from the checkerboard synergy study in 30 strains of *P. aeruginosa* were shown in Table 4-6 and Table 4-7. The synergistic interaction between imipenem and colistin were evaluated from the Fractional inhibitory concentration (FIC) index.

The combination was fully synergy against 20 strains (66.67%) [strain No. 2, 3, 6, 7, 9, 10, 11, 12, 13, 16, 17, 19, 20, 21, 22, 24, 25, 27, 28 and 30], partial synergy in 3 strains (10%) [strains No. 5, 8 and 32], additive in 7 strains (23.33%) [strains No. 1,,4,,14,,15, 18, 29 and 36]. No indifference or antagonistic activities were observed.
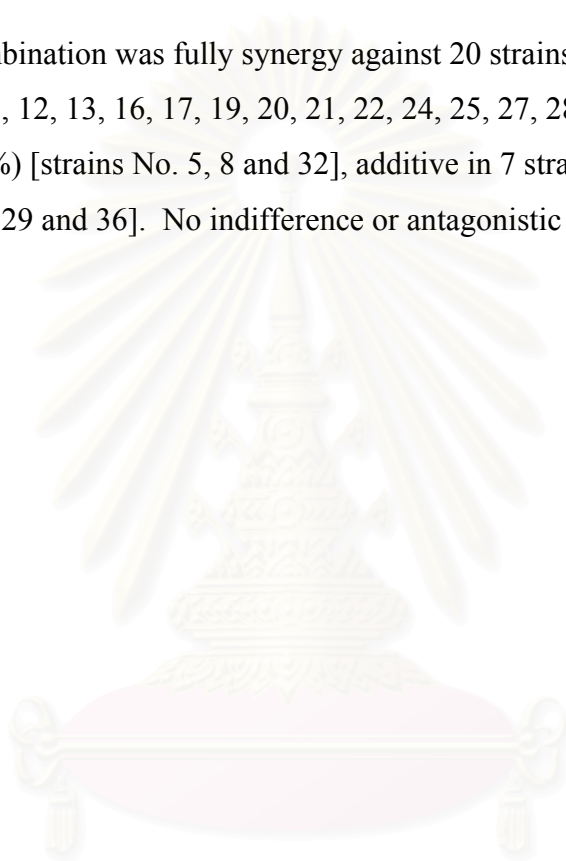
Table 4-6 MICs of imipinem, colistin and FIC index were determined by checkerboard method

Strains No.	IMP		CL		FIC index	Interpreted[b]
	MIC[a]	Interpreted	MIC[a]	Interpreted		
1	16	R	1	S	1	A
2	16	R	0.5	S	0.091	S
3	4	S	2	S	0.375	S
4	8	I	2	S	1	A
5	16	R	1	S	0.75	P
6	8	I	2	S	0.375	S
7	16	R	1	S	0.25	S
8	8	I	0.5	S	0.75	P
9	8	I	1	S	0.25	S
10	16	R	2	S	0.375	S
11	16	R	1	S	0.375	S
12	8	I	2	S	0.375	S
13	8	I	1	S	0.313	S
14	8	I	2	S	1	A
15	4	S	1	S	1	A
16	4	S	1	S	0.5	S
17	4	S	1	S	0.5	S
18	8	I	1	S	1	A
20	8	I	1	S	0.5	S
21	16	R	2	S	0.5	S
22	8	I	2	S	0.5	S
25	8	I	2	S	0.5	S
29	4	S	2	S	1	A
30	16	R	4	S	0.188	S
31	8	I	2	S	0.5	S
32	8	I	4	S	0.75	P
33	4	S	1	S	0.313	S
34	8	I	2	S	0.313	S
36	4	S	2	S	1	A
37	8	I	2	S	0.375	S

[a] MICs from broth macrodilution method; [b] S= synergy, P=partial synergy, A=additive, IMP=imipenem, CL=colistin

Table 4-7 Combination effect of imipenem and colistin against 30 strains of *P. aeruginosa*

	Number of strains	% of strains
synergy	20	66.67
partial synergy	3	10
additive	7	23.33
indifference	0	0
antagonist	0	0

Time kill study

Time-kill study was performed to evaluate the bactericidal activity of the combination between imipenem and colistin at various concentrations. In this study, imipenem resistant strains were tested, strains including number 1, 2, 5, 7, 10, 11, 21 and 30 as shown in Table 4-8. The concentrations of imipenem and colistin chosen in the study were shown in Table 4-9.

Table 4-8 Characteristic of the chosen strains in the time kill study

Strains No.	No. of antimicrobial resistant[a]	FIC index	Synergy study[b]
1	1	1	A
2	1	0.091	S
5	1	0.75	P
7	3	0.25	S
10	5	0.375	S
11	4	0.375	S
21	5	0.5	S
30	3	0.188	S

[a] determined by E-test, [b]S= synergy, P=partial synergy, A=additive

Table 4-9 The concentration of imipenem and colistin chosen for the assessment of bactericidal activity by time kill method

Strains No.	Combination therapy				Monotherapy			
	imipenem (µg/ml)		colistin (µg/ml)		colistin (µg/ml)			
	1xMIC	2xMIC	0.25xMIC	0.5xMIC	1xMIC	2xMIC	4xMIC	8xMIC
1	16	32	0.25	0.5	1	2	4	8
2	16	32	0.12	0.25	0.5	1	2	4
5	16	32	0.25	0.5	1	2	4	8
7	16	32	0.25	0.5	1	2	4	8
10	16	32	0.5	1	2	4	8	16
11	16	32	0.25	0.5	1	2	4	8
21	16	32	0.5	1	2	4	8	16
30	16	32	1	2	4	8	16	32

Combination Therapy

In this study, 8 strains of imipenem-resistant *P. aeruginosa* were included, the mean ($\pm$SD) log decrease of viable cell count and bacteriolytic area for 24 hours (BA_{24}) by the combination of imipenem and colistin were shown in Figure 4-4 and Table 4-10.

Imipenem 1xMIC alone was shown to have bactericidal activity (-3 log CFU/ml or 99.9% killing) at 6th hour after the organism was exposed to the drug (BA_{24} = 217.64$\pm$55.70 log CFU/ml*h), while 2xMIC was shown to have bactericidal activity at 4^{th} hour without any bacterial regrowth until 24^{th} hour. The BA_{24} of 2xMIC of imipenem = 253.22$\pm$48.91 log CFU/ml*h which was no significantly different from that of 1xMIC ($p>0.05$) [Table 4-10].

Even though, colistin at 0.25xMIC and 0.5xMIC did not show any bactericidal activity during the time of study but BA_{24} of 0.25xMIC (77.75$\pm$63.09 log CFU/ml*h) was significantly different ($p<0.05$) when compared to the BA_{24} of 0.5xMIC (131.69$\pm$68.47 log CFU/ml*h).

In the combination between imipenem 1xMIC and colistin 0.5xMIC, bactericidal activity was observed at 2nd hour without any bacterial regrowth during the time of study. BA_{24} of the combination of imipenem 1xMIC and colistin 0.5xMIC were 269.26$\pm$38.76 log CFU/ml*h. There was not significantly difference ($p>0.05$) from the BA_{24} of imipenem 1xMIC. Whereas the bactericidal activity of the combination between imipenem 1xMIC and colistin 0.25xMIC was observed during the 2^{nd} hour to the 8^{th} hour. The regrowth of the pathogens were observed after that. At 24^{th} hour, bacteriostatic activity (-2 log CFU/ml) was observed in 2 bacterial strains. BA_{24} of these combination, between imipenem 1xMIC and colistin 0.25xMIC, were 261.59$\pm$38.19 log CFU/ml*h. There was not significantly difference between the BA_{24} value of imipenem 1xMIC and the combination of imipenem 1xMIC and colistin 0.5xMIC (p-value = 0.05).
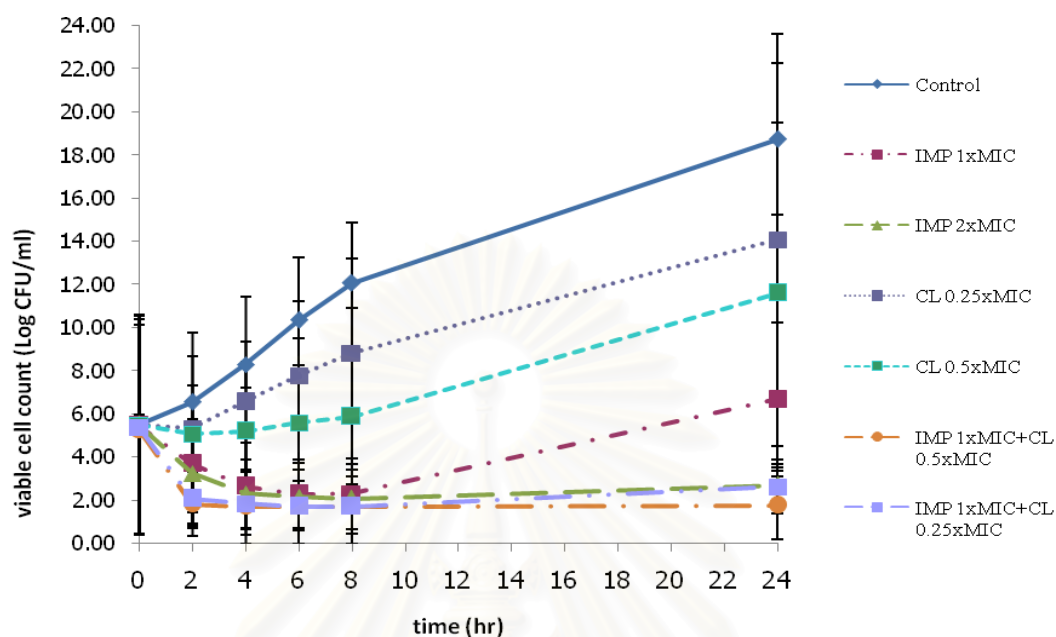
Figure 4-4 Time killing curve showing the antibacterial activity of the combination against 8 strains of imipenem-resistant *P. aeruginosa*.

Table 4-10 Mean $\pm$SD of log change viable cell counts at various time intervals, $AUBKC_{0-24}$ and BA_{24} of *P. aeruginosa* (8 strains)

Condition	Log change in average viable cell counts					$AUBKC_{0-24}$	BA_{24}
	$\Delta 2$	$\Delta 4$	$\Delta 6$	$\Delta 8$	$\Delta 24$		
Control	1.02$\pm$0.80	2.78$\pm$1.21	4.85$\pm$1.11	6.54$\pm$1.23	13.23$\pm$3.37	314.40$\pm$39.77	-
Imipenem 1xMIC	-1.78$\pm$0.79	-2.80$\pm$0.59	-3.17$\pm$0.80	-3.16$\pm$0.76	1.21$\pm$3.25	96.77$\pm$29.20	217.64$\pm$55.70[a]
Imipenem 2xMIC	-2.26$\pm$0.69	-3.21$\pm$0.72	-3.37$\pm$0.77	-3.45$\pm$0.68	-2.81$\pm$1.92	61.18$\pm$17.86	253.22$\pm$48.91
Colistin 0.25xMIC	-0.17$\pm$1.02	1.07$\pm$1.88	2.30$\pm$2.55	3.32$\pm$3.03	8.58$\pm$4.48	236.65$\pm$42.38	77.75$\pm$63.09[b]
Colistin 0.5xMIC	-0.43$\pm$1.58	-0.30$\pm$2.15	0.09$\pm$3.11	0.37$\pm$3.37	6.10$\pm$3.84	182.71$\pm$61.94	131.69$\pm$68.47
Imipenem 1xMIC + Colistin 0.5xMIC	-3.47$\pm$0.69	-3.59$\pm$0.46	-3.59$\pm$0.46	-3.59$\pm$0.46	-3.52$\pm$0.43	45.14$\pm$1.71	269.26$\pm$38.76[c, d]
Imipenem 1xMIC + Colistin 0.25xMIC	-3.33$\pm$0.97	-3.56$\pm$0.58	-3.69$\pm$0.39	-3.69$\pm$0.39	-2.77$\pm$1.22	52.81$\pm$11.46	261.59$\pm$38.19[e]

[a]= $p>0.05$ compared to activity of imipenem 2xMIC, [b]=$p<0.05$ compaied to activity of colistin 0.5xMIC, [c, d and e]= $p>0.05$ compaied to activity of imipenem 1xMIC, $AUBKC_{0-24}$= Area under bacterial killing and regrowth curve for 24 hours, BA_{24}= Bacteriolytic area for 24 hours

Numbers of strains which were killed at various time intervals were shown in Table 4-11. The 99.9% killing (-3 log CFU/ml)was observed at 2nd hour in 1 strain (strain No.5) after exposed to 1xMIC of imipenem and 99.9% killing in 5 strains (strain No.5, 7, 11, 21 and 30) was observed at 8th hours. At 24th hour, 99.9% killing were observed in only 2 strains (strains No.5 and 11). When the concentration of imipenem was increased to 2xMIC, at 2nd hour, 99.9% killing was found in 2 strains (strain No. 5 and 11), and in 7 strains (strain No.1, 5, 7, 10, 11, 21 and 30) at 8th hour (87.5%). At 24th hour, the 99.9% killing was observed in only 6 strains (strains No.1, 2, 5, 11, 21 and 30) with the regrowth of 2 strains (strain No.7 and 11).

For colistin, in the concentration of 0.25xMIC, 99.9% killing could not be observed during time of study. Only one strain (No.30) was killed at the level of 90% killing (-1 log CFU/ml) at 2nd hour and one strain (strain No.2) shown regrew at 8th hours. At 24th hours, all strains were regrown. Whereas, colistin 0.5xMIC showed 99.9% killing during the 2nd to 8th hours in strains No.30. At 24th hours, all strains were shown to be regrown.

The combination between imipenem 1xMIC and colistin 0.5xMIC shown 99.9% killing in 6 strains (strains No.1, 2, 5, 10, 11, 21 and 30) at 2nd hours and increased to 7 strains (87.5%, strain No.1, 2, 5, 10, 11, 21 and 30) at 24th hours while only 99% killing without bacterial regrowth was observed in the last strain (strain No.7) at 24 hours.

The combination between imipenem 1xMIC and colistin 0.25xMIC shown 99.9% killing in 6 strains (strain No.5, 7, 10, 11, 21 and 30) but the number of strains were decreased to 5 strains (strain No.2, 5, 7, 11 and 21) at 24th hours with the regrowth of 2 strains (strain No.10 and 30).

Table 4-11 Reduction of *P. aeruginosa* (8 strains) viable cell counts at various time intervals.

condition	No. of strains to be killed at time point																
	2 hr			4 hr			6 hr			8 hr				24 hr			
	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3	R	-1	-2	-3	R
IMP 1xMIC	4	1	1	1	3	4	-	3	5	1	2	5	-	-	-	2	6
IMP 2xMIC	3	3	2	-	3	5	1	1	6	-	1	7	-	1	-	6	2
CL 0.25xMIC	-	1	-	-	1	-	-	1	-	1	-	-	1	-	-	-	8
CL 0.5xMIC	-	-	1	1	1	1	-	2	1	3	-	1	-	-	-	-	8
IMP 1xMIC + CL 0.5xMIC	-	2	6	-	1	7	-	1	7	-	1	7	-	-	1	7	-
IMP 1xMIC + CL 0.25xMIC	1	1	6	-	1	7	-	-	8	-	-	8	-	2	1	5	2

-1 = 90% of viable reduction versus initial inoculums; -2 = 99% of viable reduction versus initial inoculums, -3 and -4= 99.9 % of viable reduction versus initial inoculums, R= regrowth

Colistin monotherapy

The bactericidal activities of various concentrations of colistin were shown in Figure 4-5 and Table 4-12. The mean log viable cell count at various time were also shown in Table 4-12.

Colistin 1xMIC showed bacteriostatic activity during the 2nd hour until the 8th hours. The BA_{24} of colistin 2xMIC were 213.45 ± 57.74 log CFU/ml*h. The 4xMIC also showed bactericidal activity during the 2nd hour to 8th hours. In contrary, at 24th hours, only colistin 4xMIC showed the bacteriostatic activity while colistin 2xMIC did not have any activity left. Both concentrations exhibited the BA_{24} of 233.87 ± 44.48 and 258.80 ± 43.86 log CFU/ml*h, respectively. Colistin 8xMIC showed bactericidal activity during the 2nd to the 24th hour ($BA_{24} = 267.87 \pm 37.65$ log CFU/ml*h). There were no significantly difference ($p>0.05$) among the BA_{24} at different concentrations.
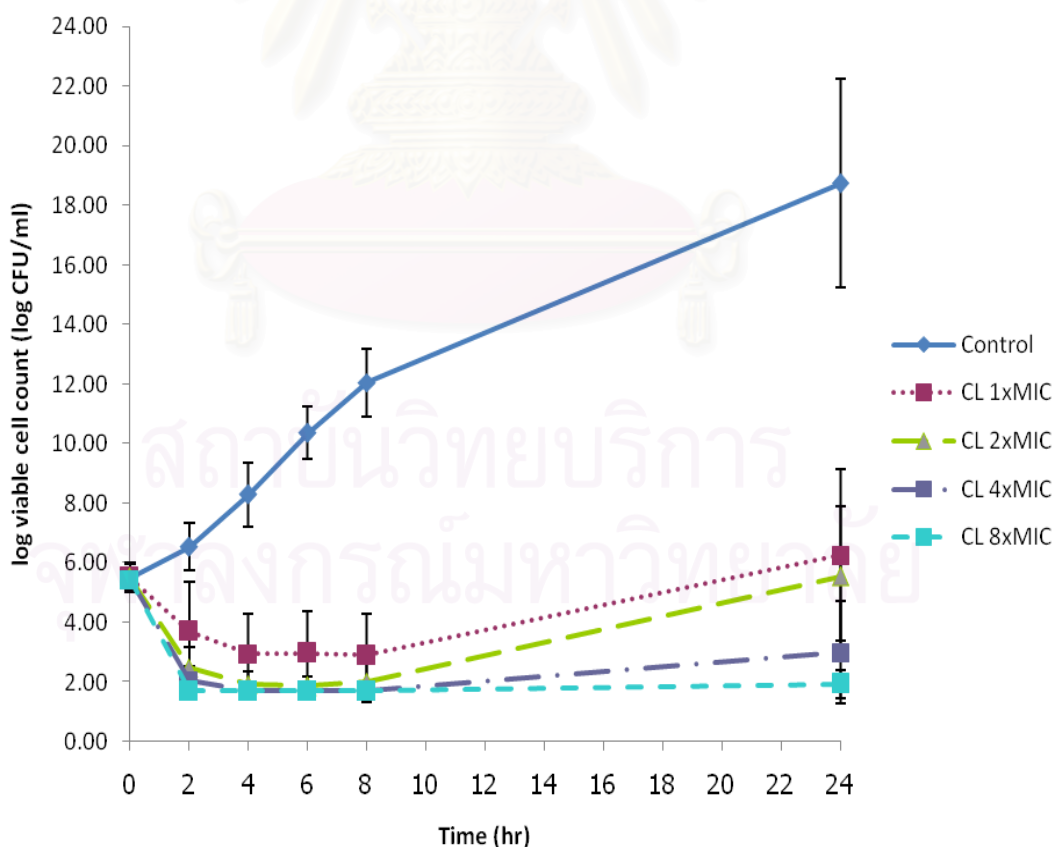
Figure 4-5 Time killing curve average of 8 strains of *P. aeruginosa* by various concentrations of colistin

Table 4-12 Mean $\pm$SD of log change viable cell counts at various time intervals, AUBKC$_{0-24}$ and BA$_{24}$ from average of 8 strains *P. aeruginosa*

Condition	Log change in viable cell counts					AUBKC$_{0-24}$	BA$_{24}$
	Δ2	Δ4	Δ6	Δ8	Δ24		
Control	1.02+0.80	2.78+1.21	4.85+1.11	6.54+1.23	13.23+3.37	314.40+39.77	-
Colistin 1xMIC	-1.79+1.48	-2.55+1.36	-2.53+1.65	-2.60+1.66	0.76+3.18	100.95+38.53	213.45+57.74[a,b,c]
Colistin 2xMIC	-3.04+0.91	-3.58+0.77	-3.65+0.63	-3.51+0.68	0.04+2.48	80.53+21.74	233.87+44.48[d,e]
Colistin 4xMIC	-3.38+0.51	-3.74+0.41	-3.74+0.41	-3.74+0.41	-2.45+1.55	55.61+13.89	258.80+43.86[f]
Colistin 8xMIC	-3.75+0.40	-3.75+0.40	-3.75+0.40	-3.75+0.40	-3.50+0.50	46.53+3.91	267.87+37.65

[a]= *p>0.05* compared to activity of colistin 2xMIC, [b]= *p>0.05* compared to activity of colistin 4xMIC, [c]= *p>0.05* compared to activity of colistin 8xMIC, [d]= *p>0.05* compared to activity of colistin 4xMIC, [e] = *p>0.05* compared to activity of colistin 8xMIC, [f]= *p>0.05* compared to activity of colistin 8xMIC, AUBKC$_{0-24}$= Area under bacterial killing and regrowth curve for 24 hours, BA$_{24}$= Bacteriolytic area for 24 hours

Numbers of strains killed at various time intervals were shown in Table 4-13. The 99.9% killing was observed at 2nd hour in 2 strain (25%, strain No.21 and 30) when exposed to colistin 1xMIC. The 99.9% killing of 5 strains (strain No.7, 10, 11, 21 and 30) was observed at 8th hour while at 24th hour, 99.9% killing was observed in only one strain (No. 11). The regrowth were observed at 24th hour in 5 strains (strain No. 2, 5, 7, 10 and 21). When the concentration of colistin was increased to 2xMIC, at 2nd hour 99.9% killing was found in 4 strains (strain No. 5, 11, 21 and 30), in 6 strains (strain No.5, 7, 10, 11, 21 and 30) at 8th hour, and at 24th hours the 99.9% killing was observed in only 2 strains (strains No. 7 and 30) with the regrowth of 6 strains (strain No.1, 2, 5, 10, 11 21 and 30).

For colistn 4xMIC and 8xMIC, shown 99.9% killing of the 6 strains (strain No. 5, 7, 10, 11, 21 and 30) and the 7 strains (87.5% strain No.1, 5, 7, 10, 11, 21 and 30), respectively, were observed at 2nd hour. Whereas, 99.9% killing were found in 7 strains (75%, strain No.1, 5, 7, 10, 11 21 and 30) by both concentrations at 8th hour. At 24th, the 99.9% killing was observed in 4 strains (strain No. 1, 7, 11 and 30) by 4xMIC, and in 6 strains (75%, strains No. 5, 7, 10, 11, 21 and 30) by 8xMIC. Bacterial regrowth was observed in only 2 strains (strain No. 5 and 21) when exposed to colistin 4xMIC, excepted in colistin 8xMIC.

Table 4-13 Reduction of *P. aeruginosa* (8 strains) viable cell counts at various time
intervals.

condition	No. of strains to be killed at time point															
	2h			4h			6h			8h				24h		
	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3	R	-1	-2	-3	R
CL 1xMIC	2	1	2	2	1	4	1	2	4	2	-	5	-	-	1	1	5
CL 2xMIC	1	3	4	1	-	7	-	1	7	-	2	6	-	-	-	2	6
CL 4xMIC	-	2	6	-	1	7	-	1	7	-	1	7	-	-	2	4	2
CL 8xMIC	-	1	7	-	1	7	-	1	7	-	1	7	-	-	2	6	-

-1 = 90% of viable reduction versus initial inoculums; -2 = 99% of viable reduction versus initial inoculums; -3 and -4 = 99.9

% of viable reduction versus initial inoculums, R= regrowth

Morphological cell structure change in *P. aeruginosa* after exposed to imipenem, colistin and the combination of the drugs observed by scanning electron microscope

The morphological changes of the MDR *P. aeruginosa* strain No.10 [imipenem-resistant strain which was killed by the combination of the drugs in the time kill study at 2nd hour] after exposed to imipenem 1xMIC (16 μg/ml), colistin 0.5xMIC (1 μg/ml) and the combination between imipenem 1xMIC and colistin 0.5xMIC for 2 hours as shown in Figure 4-6. These observation were made under an scanning electron microscope. The normal morphological structure of bacteria was observed in control cell (Figure 4-6A). Imipenem alone (at 1xMIC) exhibited minor morphological alterations. No cell destruction was observed, but only abnormal forms were visible (Figure 4-6B). However, colistin alone (at 0.5xMIC) had a profound effect on the surface of bacteria (Figure 4.6C). The combination of both agents could cause the abnormal forms of bacterial cell with the roughly spherical surface outpouching (Figure 4.6D) and produced numerous protrusion on the surface of cell. After that, cell lysis was observed (Figure 4.7).
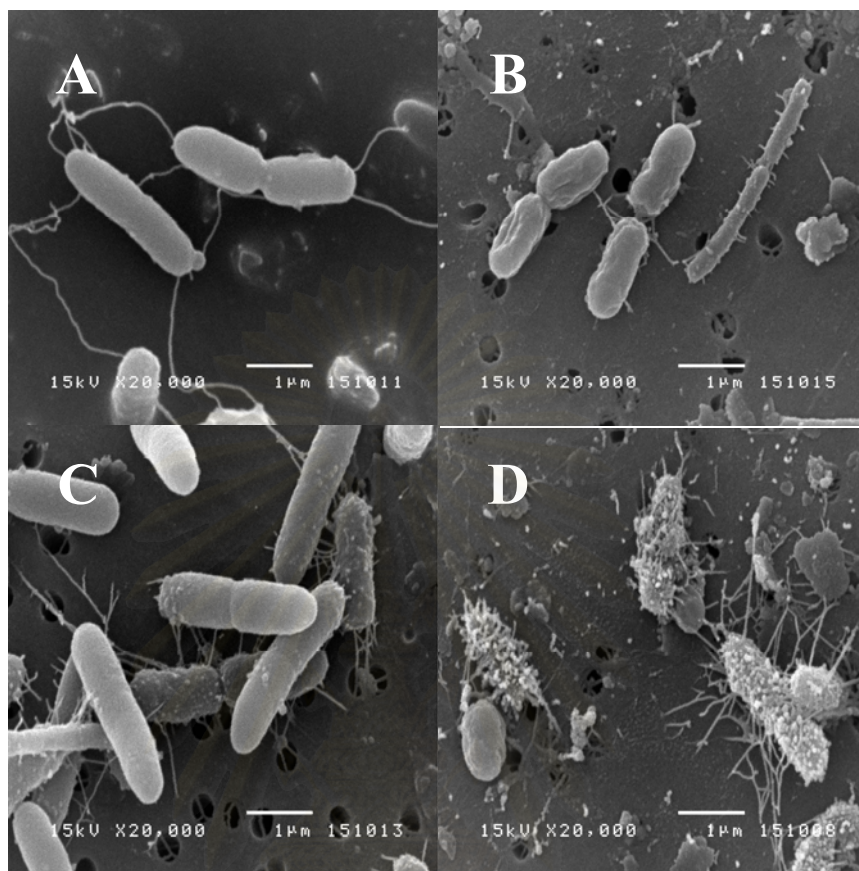
Figure 4-6 Scanning electron micrographs of *P. aeruginosa* No. 10 after exposed to (A) no antibiotic, (B) imipenem 1xMIC (16 μg/ml), (C) colistin 0.5xMIC (1 μg/ml) and (D) combination between imipenem 1xMIC (16 μg/ml) and colistin 0.5xMIC (1 μg/ml) for 2 hours.
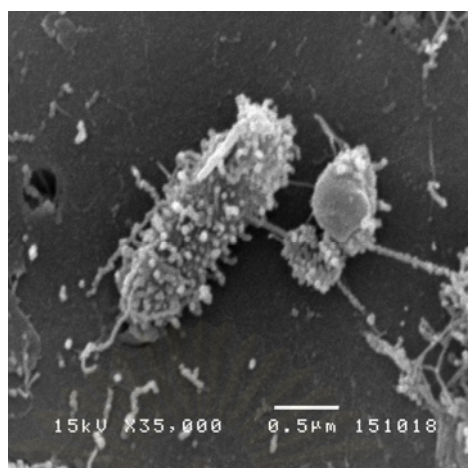
Figure 4-7 Scanning electron micrographs of *P. aeruginosa* strain No. 10 after exposed to the combination between imipenem 1xMIC (16 µg/ml) and colistin 0.5xMIC (1 µg/ml) for 2 hours.

CHAPTER V

DISCUSSION AND CONCLUSION

P. aeruginosa continues to be a dangerous pathogen causing infections which are difficult to treat because of the organism's intrinsic resistance to many antimicrobials and its propensity to develop resistance during therapy. The emergence and rapid spread of multidrug-resistant (MDR) strains are of great concern for clinicians in the treatment of the with confirmed or suspected *P.aeruginosa* infections.

All *P.aeruginosa* clinical isolates in this study were highly resistant to almost all antimicrobial agents which were commonly used in the treatment of *P. aeruginosa* infection except for colistin (100% susceptible). Even though, the data from United States Today Surveillance Studies in 2001-2003 have shown that the rate of MDR strains was 9.9% (James *et al.*, 2005) but the recovery rate of MDR *P. aeruginosa* in this study was as high as 63.3% of the isolates. The increase in the incidence of MDR *P.aeruginosa* leads to the decrease in the number of effective antimicrobial agents in the treatment of these organisms including carbapenem which has been recently the effective agent for the treatment of these organisms.

The mechanisms involved the carbapenem resistance have been attributed to the alternation in membrane permeability such as a decrease or a leak of porinD2, the multidrug efflux pump and/or the carbapenem-hydrolyzing-β-lactamases (Troillet *et al.*, 1997). The carbapenem hydrolyzing β-lactamases can be divided in 2 groups, Class A or Bush-Jacoby-Medeiros Group 2f, which has serine at the active site; and Class B or Bush-Jacoby-Medeiros Group 3a and 3b (metallo-β-lactamases), which have been identified mainly in Japan (Troillet *et al.*, 1997). The lack of porinD2, multidrug efflux pump and hydrolysis by class A or B β-lactamases will produce greater resistance to imipenem as compared with meropenem, cause for concern in therapy of critically ill patients. In this study metallo-β-lactamase could not be detected, thus, imipenem resistance strains might use the other resistant mechanisms described above.

Nowadays multidrug-resistance (MDR) in gram-negative bacteria is a serious growing problem worldwide. New therapeutic are need urgently, but current research suggests that there is no particularly promising antimicrobial on the horizon for the near future and it takes quite along time to develop a new drug. Consequently, old drugs have recently been brought back into use for the treatment of MDR bacteria as a stopgap measure until new antimicrobial can be developed (Wood *et al*., 2003; Markou *et al*., 2003: Timurkaynak *et al*., 2006).

Colistin is an old antimicrobial belongs to the polymyxin family. Despite of the effectiveness for against most gram-negative bacteria including *A. baumannii* and *P. aeruginosa*, the use of colistin have been limited because of concerns about poor pharmacokinetics, neurological and nephrotoxicity (Lin *et al*., 2005; Kasiakou and Michalopoulos, 2005; Falagas *et al*., 2005 and 2006). However, the recent studies have reported the safe use of colistin and the good outcome when use monotherapy or in the combination (Hamer, 2000; Linden *et al*., 2003; Michalopoulos *et al*., 2004; Berlana *et al*., 2005; David *et al*., 2005; Reina *et al* 2005; Colar *et al*., 2006; Koomanachai *et al*., 2006; Timurkaynak *et al*., 2006; Papagalopoulos *et al*., 2007).

Moreover, many studies reported that the combination therapy showed the effective against MDR pathogens (Yu *et al*., 1987; Korvick *et al*., 1992; Yiong *et al*., 1996; Bustamante, Wharton and Wade, 1990, Visalli *et al*., Erdem *et al*., 2002; Oie *et al*, 2003). Bustamante *et al*. reported that the combination between ciprofloxacin and imipenem showed the synergic effect in 36% of tested isolates (*P. aeruginosa*). In addition, Nakamura *et al*. demonstrated that the combination of meropenem plus aminoglycoside showed the synergic effect on *P.aeruginosa in vitro*. These investigators proposed that the activity occurred by enhancement of the entry of aminoglycoside into the bacterial cell. With respect to synergy, it is suggested that colistin probably causes rapid permeabilization of the outer cell membrane, which allows enhanced penetration of the other antibiotic in the combination (Gunderson *et al*., 2003; Yoon *et. al*., 2004; Tascini *et al*., 2004). In addition, the combination might be preventing emergence of resistant bacterial and decrease the toxicity of colistin monotherapy. The present study has shown *in vitro* synergistic effect between

colistin and imipenem against clinical isolates of *P. aeruginosa* and is the first study performed in our country. This study proposed that colistin enhance the entry of imipenem into the bacterial cell and increase the binding of PBPs (site of action of imipenem) by disrupting the cytoplasmic membrane of bacteria.

Eight strains of imipenem-resistant *P. aeruginosa* were used to determine the bactericidal activity in the time kill study. Despite the fact that 5 out of the 8 imipenem-resistant *P. aeruginosa* were MDR strains, all of the isolates were low level resistant to imipenem with the MIC_{90} of 16 µg/ml and very susceptible to colistin with the MIC_{90} of 2 µg/ml. Although the colistin-imipenem combination showed the promising results that the synergy effect with FIC index 0.091-0.5 were observed in 20 out 30 strains (16 strains were imipenem-resistant strains, and 4 strains were imipenem-susceptible strains) and no antagonist was observed. The partial synergy and additive effect were also observed in 3 and 7 strains, respectively. The combination could decrease the MIC of colistin to 1-2 times when compare with the MIC of colistin alone. Therefore, this study suggested that the combination of imipenen and colistin may be used in the treatment of MDR *P.aeruginosa* infection leading to the decrease of the colistin toxicity.

The time-kill was performed in order to quantitative of the combined drug on the rate of bactericidal action at different time intervals. The present study showed that the combination of 1xMIC of imipenem (16µg/ml) and 0.5xMIC of colistin is the most effective in the treatment of MDR *P.aeruginosa*. The bactericidal activity and the regrowth could not be observed during the time of study (24 hour), while the combination of imipenem 1xMIC plus colistin 0.25xMIC showed the bactericidal activity at the 2nd hour. However, the bacterial regrowth was at the 24th hour in 2 stains. The same results were observed in imipenem 1xMIC alone (6 strains) and 2xMIC alone (2 strains). It is suggestive that the treatment of MDR *P. aeruginosa* could be done by using imipenem 1xMIC combined with colistin 0.5xMIC because of the best *in vitro* efficacy with the lower dose of imipenem from the therapeutic dose to 1 time (from 33 µg/ml, peak concentration in plasma average) leading to the decrease of the patients-care cost. In the other hand, the combination showed bactericidal

activity at 2nd hour after exposed, and continuous to 24th hour. Thus, the combination might be give once daily.

Bactericidal activity at 24 hours (BA$_{24}$) of colistin alone was not significant different from those in colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC of colistin alone. High concentration of colistin showed a good bactericidal effect because colistin was the concentration-dependent antimicrobial agent. The nephrotoxicity was also correlated to the high concentration of colistin. In this study, colistin 8xMIC was shown to be the most effective concentration because the bactericidal activity was observed at 2nd hour and continued to 24th hour while the bacterial regrowth did not occur. The result agreed with the previous study by Song *et al*, 2007 who showed the bactericidal activity of colistin against carbapenem resistant *A. baumannii* at all concentration 4-8xMIC. The use of colistin monotherapy might need very high concentration (8xMIC) which at the same time may increase the high nephrotoxicity. Therefore, the combination should be more appropriate than colistin monotherapy because the dose of colistin could be decreased to 16 times leading to the lower incidence of the nephotoxicity.

The study of the morphological change of MDR *P.aeruginosa* after exposed to the combination of imipenem 1xMIC and colistin 0.5xMIC as compared to the drug alone (imipenem or colistin) showed the abnormal cell form with roughly spherical surface and the bactericidal lysis when the bacteria were in the combined drugs. In accordance with the literature, colistin normally acts on the cytoplasmic membrane and cause the damage to the osmotic barrier which leads to the leakage of the intracellular contents (Hancock *et al.*, 1999). Whereas, imipenem and colistin alone showed only abnormal cell form when compared with the growth in control media (no antibiotic). Therefore, the combination therapy could enhance the effect of drug alone.

In conclusion, these studies showed that *P. aeruginosa* were still low level imipenem resistance with the MIC$_{90}$ of 16 μg/ml. Thus, the concentration of 2xMIC of imipenem (equal to peak concentration in plasma average when administered with

the normal dose) showed the good effectiveness. However, the combination could enhance the bactericidal activity against MDR *P. aeruginosa* without any regrowth within 24 hours of study. The morphology change confirmed the synergistic effect of the combination in bacterial killing. Moreover, high concentration of colistin monotherapy is needed in the therapy of MDR *P. aeruginosa*, while lower colistin concentration in the combination may be as effective, leading to the decrease in the nephrotoxic effect due to the high dose of colistin. In addition, the combination of imipenem and colistin might be given once daily (colistin followed by imipenem), while impenem monotherapy in the therapeutic dose (500 mg) have to be administered every 6 hours because of the best bactericidal activity of the concentration.

Therefore, the combination of imipenem and colistin could be the promising alternative for treatment of infection due to MDR *P.aeruginosa* and deserves future *in vitro* and *in vivo* assessment. The efficacy of combination of both agents needs to be confirmed in clinical trial.

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APPENDICES

Table A-1 Susceptibility of 37 clinical isolates *P. aeruginosa* to 10 antimicrobial agents by disk diffusion method.

Isolates No.	Cefepime (FEP-30)		Ceftazidime (CAZ-30)		Gentamicin (GM-10)		Tobramycin (NN-10)		Ciprofloxacin (CIP-5)		Pip/tazo (TZP-100/10)		Imipenem (IMP-10)		Rifampin (RA-5)		Colistin (CL-10)		Amikacin (AK-30)	
1	24.10	S	24.04	S	19.20	S	21.24	S	30.43	S	25.04	S	11.01	R	9.53	R	12.72	S	22.10	S
2	25.89	S	23.86	S	17.70	S	21.78	S	29.31	S	25.09	S	10.29	R	6.59	R	13.11	S	21.97	S
3	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	21.16	S	NZ	R	NZ	R	14.46	S	11.05	R
4	23.63	S	22.53	S	20.11	S	24.17	S	23.15	S	24.40	S	12.61	R	NZ	R	14.18	S	25.13	S
5	25.08	S	24.38	S	15.16	S	20.81	S	30.03	S	23.73	S	12.73	R	NZ	R	13.92	S	18.23	S
6	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	20.05	S	11.51	R	NZ	R	13.77	S	11.24	R
7	NZ	R	NZ	R	NZ	R	NZ	R	27.52	S	14.84	I	9.79	R	14.08	R	14.36	S	NZ	R
8	11.31	R	NZ	R	NZ	R	NZ	R	NZ	R	16.54	I	11.21	R	NZ	R	11.70	S	7.58	R
9	17.20	I	24.14	S	NZ	R	NZ	R	NZ	R	14.78	I	11.49	R	NZ	R	13.71	S	20.22	S
10	11.42	R	NZ	R	NZ	R	NZ	R	14.21	R	14.81	I	11.25	R	NZ	R	12.64	S	NZ	R
11	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	14.41	I	11.64	R	NZ	R	12.64	S	14.05	R
12	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	18.66	S	12.23	R	NZ	R	13.31	S	11.36	R
13	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	14.73	I	12.08	R	NZ	R	13.60	S	11.94	R
14	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	20.28	S	14.32	I	NZ	R	13.32	S	12.15	R
15	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	14.76	I	20.18	S	NZ	R	14.02	S	10.66	R
16	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	16.85	I	25.08	S	NZ	R	15.38	S	8.46	R
17	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	14.94	I	16.86	S	NZ	R	14.03	S	10.13	R
18	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	13.33	R	12.48	R	NZ	R	13.37	S	11.24	R
20	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	19.51	S	13.39	I	NZ	R	13.30	S	12.14	R
21	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	16.58	I	9.68	R	NZ	R	12.92	S	NZ	R
22	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	16.68	I	12.65	R	NZ	R	13.61	S	11.66	R
25	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	12.95	R	12.31	R	NZ	R	13.34	S	13.63	R
29	20.41	S	27.40	S	NZ	R	NZ	R	19.94	I	18.54	S	10.28	R	NZ	R	11.09	S	19.17	S
30	NZ	R	NZ	R	NZ	R	NZ	R	11.61	R	14.22	I	8.71	R	NZ	R	12.04	S	NZ	R
31	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	22.21	S	13.14	I	NZ	R	12.32	S	12.66	R
32	NZ	R	NZ	R	NZ	R	NZ	R	10.59	R	11.82	R	8.73	R	NZ	R	12.04	S	NZ	R

Table A-1 (continuous) Susceptibility of 37 clinical isolates *P. aeruginosa* to 10 antimicrobial agents by disk diffusion method.

Isolates No.	Cefepime (FEP-30)		Ceftazidime (CAZ-30)		Gentamicin (GM-10)		Tobramycin (NN-10)		Ciprofloxacin (CIP-5)		Pip/tazo (TZP-100/10)		Imipenem (IMP-10)		Rifampin (RA-5)		Colistin (CL-10)		Amikacin (AK-30)	
	Inhibition Zone (mm)
33	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	20.96	S	10.10	R	NZ	R	13.26	S	11.80	R
34	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	20.91	S	10.18	R	6.69	R	13.91	S	11.13	R
36	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	10.31	R	20.32	S	NZ	R	15.48	S	NZ	R
37	18.67	S	23.70	S	NZ	R	NZ	R	12.63	R	16.40	I	11.89	R	NZ	R	14.90	S	18.45	S

S=susceptible, I=intermediate, R=resistant, NZ=no zone

Table A-2 The Disk diffusion result of *P. aeruginosa* 30 clinical isolates to 10 antimicrobial agent separate by RAPD type

RAPD type	Isolate No.	Antibiogram										No. of antimicrobial resistance
		Cefepime (FEP-30)	Ceftazidime (CAZ-30)	Gentamycin (GM-10)	Tobramycin (NN-10)	Ciprofloxacin (CIP-5)	Pip/tazo (TZP-100/10)	Imipenem (IMP-10)	Rifampicin (RA-5)	Colistin (CL-10)	Amikacin (AK-30)	
1	1	S	S	S	S	S	S	R	R	S	S	2
2	2	S	S	S	S	S	S	R	R	S	S	2
	6	R	R	R	R	R	S	R	R	S	R	8
	12	R	R	R	R	R	S	R	R	S	R	8
	13	R	R	R	R	R	I	S	R	S	R	7
	14	R	R	R	R	R	S	I	R	S	R	7
	16	R	R	R	R	R	I	R	R	S	R	8
	18	R	R	R	R	R	R	R	R	S	R	9
	20	R	R	R	R	R	S	I	R	S	R	7
	22	R	R	R	R	R	I	R	R	S	R	8
	25	R	R	R	R	R	R	R	R	S	R	9
	31	R	R	R	R	R	S	I	R	S	R	7
	34	R	R	R	R	R	S	R	R	S	R	8
3	3	R	R	R	R	R	S	R	R	S	R	8
4	4	S	S	S	S	S	S	R	R	S	S	2
5	5	S	S	S	S	S	S	R	R	S	S	2
6	7	R	R	R	R	S	I	R	R	S	R	7
7	8	R	R	R	R	R	I	R	R	S	R	8
8	9	I	S	R	R	R	I	R	R	S	S	5
9	10	R	R	R	R	R	I	R	R	S	R	8
10	11	R	R	R	R	R	I	R	R	S	R	8
	15	R	R	R	R	R	I	S	R	S	R	7
	17	R	R	R	R	R	I	S	R	S	R	7
11	21	R	R	R	R	R	I	R	R	S	R	8
12	29	S	S	R	R	I	S	R	R	S	S	4
13	30	R	R	R	R	R	I	R	R	S	R	8

Table A-2 (continuous) The Disk diffusion result of *P. aeruginosa* 30 clinical isolates to 10 antimicrobial agent separate by RAPD type

RAPD type	Isolate No.	Antibiogram										No. of antimicrobial resistance
		Cefepime (FEP-30)	Ceftazidime (CAZ-30)	Gentamicin (GM-10)	Tobramycin (NN-10)	Ciprofloxacin (CIP-5)	Pip/tazo (TZP-100/10)	Imipenem (IMP-10)	Rifampin (RA-5)	Colistin (CL-10)	Amikacin (AK-30)	
14	32	R	R	R	R	R	R	R	R	S	R	9
15	33	R	R	R	R	R	S	R	R	S	R	8
16	36	R	R	R	R	R	R	S	R	S	R	8
17	37	S	S	R	R	R	I	R	R	S	S	5

S=susceptible, I=intermediate, R=resistant

Table A-3 The minimum inhibitory concentrations (MICs) to broad-spectrum agents against 30 strains of *P. aeruginosa*

Isolates No.	MIC (µg/ml)										No. of resistance
	Amikacin		Imipenem		Ciprofloxacin		Piperacillin-tazobactam		cefepime		
*Ps.*1	3	S	>32	R	0.125	S	8	S	2	S	1
*Ps.*2	2	S	>32	R	0.125	S	4	S	3	S	1
*Ps.*3	64	R	>32	R	>32	R	48	S	>256	R	4
*Ps.*4	2	S	12	I	0.5	S	8	S	4	S	-
*Ps.*5	6	S	>32	R	0.19	S	4	S	1.5	S	1
*Ps.*6	32	I	>32	R	>32	R	>256	R	>256	R	3
*Ps.*7	>256	R	>32	R	0.5	S	96	I	>256	R	3
*Ps.*8	128	R	>32	R	>32	R	96	I	32	R	4
*Ps.*9	6	S	8	I	4	R	96	I	12	I	1
*Ps.*10	>256	R	>32	R	8	R	>256	R	48	R	5
*Ps.*11	32	I	>32	R	>32	R	128	R	>256	R	4
*Ps.*12	48	I	>32	R	>32	R	96	I	>256	R	3
*Ps.*13	48	I	>32	R	>32	R	>256	R	>256	R	4
*Ps.*14	48	I	8	I	>32	R	32	S	>256	R	2
*Ps.*15	48	I	6	I	>32	R	96	I	>256	R	2
*Ps.*16	64	R	3	S	>32	R	64	S	>256	R	3
*Ps.*17	48	I	6	I	>32	R	96	I	>256	R	2
*Ps.*18	32	I	>32	R	>32	R	>256	R	>256	R	4
*Ps.*20	48	I	12	I	>32	R	64	S	>256	R	2
*Ps.*21	>256	R	>32	R	>32	R	>256	R	>256	R	5
*Ps.*22	32	I	>32	R	>32	R	>256	R	>256	R	4
*Ps.*25	48	I	>32	R	>32	R	>256	R	>256	R	4
*Ps.*29	>256	R	4	S	2	I	0.75	S	16	I	1
*Ps.*30	12	S	>32	R	8	R	>256	R	32	R	4
*Ps.*31	32	I	16	R	>32	R	64	S	>256	R	3
*Ps.*32	>256	R	>32	R	12	R	>256	R	>256	R	5
*Ps.*33	48	I	>32	R	8	R	64	S	>256	R	3
*Ps.*34	48	I	16	R	>32	R	64	S	>256	R	3
*Ps.*36	>256	R	4	S	>32	R	>256	R	>256	R	4
*Ps.*37	6	S	>32	R	4	R	96	I	16	I	2

S=susceptible, I=intermediate, R=resistant

Table A-4 Metallo-β-lactamase production by double disk method, EDTA and ceftazidime disk were used. Ceftazidime/clavulanic acid were used to determine inhibition zone compare with ceftazidime if the inhibition zone which greater than ceftazidime disk showed that clavulanic acid were β-lactamase inhibitor (metallo-β-lactamase enzyme were not inhibited by clavulanic acid).

Isolate No.	MBL production[a]	Inhibition Zone (mm)	
		CAZ[b]	CAZ/clav[c]
1	-	24.78	25.28
2	-	24.26	24.41
3	-	NZ	21.32
4	-	24.77	24.95
5	-	25.21	25.18
6	-	NZ	15.03
7	-	NZ	14.41
8	-	NZ	10.55
9	-	24.49	23.62
10	-	NZ	15.62
11	-	NZ	18.01
12	-	NZ	17.47
14	-	NZ	22.85
16	-	NZ	18.98
18	-	NZ	14.37
19	-	NZ	14.94
20	-	NZ	16.01
21	-	NZ	19.49
22	-	NZ	16.41
23	-	NZ	16.51
24	-	NZ	16.65
25	-	NZ	13.58
26	-	NZ	17.18
27	-	NZ	18.20
28	-	NZ	15.36
29	-	28.90	28.74
30	-	16.01	10.59
31	-	NZ	15.10
32	-	NZ	13.21
33	-	NZ	15.99
34	-	NZ	16.26
35	-	NZ	16.59
37	-	24.18	24.90

[a] = not detected, [b] = ceftazidime , [c] = ceftazidime /clavulanic acid, NZ = no zone

Table A-5 The minimum inhibitory concentrations (MICs; µg/ml) of imipenem and colistin by broth macrodilution method, checkerboard method and FIC index.

Isolated No.	IMP		CL		FIC index	
	Broth	Checkerboard	Broth	Checkerboard		
1	16	8	1	0.5	1	Additive
2	16	16	0.5	1	0.09125	Synergy
3	4	4	2	1	0.375	Synergy
4	8	8	2	0.5	1	Additive
5	16	16	1	0.5	0.75	Partial synergy
6	8	8	2	1	0.375	synergy
7	16	16	1	1	0.25	Synergy
8	8	16	0.5	1	0.75	Partial synergy
9	8	8	1	1	0.25	Synergy
10	16	16	2	1	0.375	Synergy
11	16	8	1	1	0.375	Synergy
12	8	8	2	2	0.375	Synergy
13	8	8	1	2	0.3125	Synergy
14	8	4	2	2	1	Additive
15	4	4	1	1	1	Additive
16	4	2	1	0.5	0.5	Synergy
17	4	4	1	2	0.5	Synergy
18	8	8	1	2	1	Additive
20	8	4	1	1	0.5	Synergy
21	16	16	2	1	0.5	Synergy
22	8	8	2	2	0.5	Synergy
25	8	8	2	2	0.5	Synergy
29	4	4	2	4	1	Additive
30	16	16	4	2	0.1875	Synergy
31	8	4	2	2	0.5	Synergy
32	8	8	4	2	0.75	Partial synergy
33	4	8	1	2	0.3125	Synergy
34	8	8	2	2	0.3125	Synergy
36	4	2	2	1	1	Additive
37	8	8	2	1	0.375	Synergy

IMP=imipenem, CL=colistin

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Table A-6 Viable cell count (log CFU/ml) at the following time of *P. aeruginosa* (8 strains)

Isolated No.	Viable cell count (log CFU/ml) at the following time					
	0 h	2 h	4 h	6 h	8 h	24 h
*Ps.*1						
CONTROL	5.72	6.35	10.08	10.95	13.68	22.76
IMP 1xMIC	5.80	5.00	4.08	3.65	3.44	7.20
IMP 2xMIC	5.76	4.34	3.74	3.11	2.76	2.10
CL 0.25xMIC	5.76	5.18	5.30	5.12	4.73	23.57
CL 0.5xMIC	5.70	5.68	5.18	5.08	4.64	14.90
IMP 1xMIC+CL 0.5xMIC	5.70	1.70	1.70	1.70	1.70	2.24
IMP 1xMIC+CL 0.25xMIC	5.24	3.76	2.76	1.70	1.70	3.04
*Ps.*2						
CONTROL	4.54	6.48	8.98	10.95	12.44	17.44
IMP 1xMIC	4.70	3.80	2.54	2.70	2.74	7.48
IMP 2xMIC	4.74	2.68	2.40	2.90	2.74	1.70
CL 0.25xMIC	4.78	4.72	6.63	9.99	12.74	12.60
CL 0.5xMIC	4.78	4.63	5.89	8.90	10.70	11.63
IMP 1xMIC+CL 0.5xMIC	4.70	2.63	1.70	1.70	1.70	1.70
IMP 1xMIC+CL 0.25xMIC	4.76	2.54	1.70	1.70	1.70	1.70
*Ps.*5						
CONTROL	5.72	7.81	8.72	9.86	11.97	20.70
IMP 1xMIC	5.60	2.51	2.30	1.70	1.70	2.48
IMP 2xMIC	5.57	2.30	1.70	1.70	1.70	1.70
CL 0.25xMIC	5.68	5.83	8.89	9.72	9.80	16.90
CL 0.5xMIC	5.48	5.74	7.85	8.78	8.97	16.58
IMP 1xMIC+CL 0.5xMIC	5.60	1.70	1.70	1.70	1.70	1.70
IMP 1xMIC+CL 0.25xMIC	5.54	1.70	1.70	1.70	1.70	1.70
*Ps.*7						
CONTROL	5.16	5.35	7.57	10.74	11.24	14.70
IMP 1xMIC	5.02	3.48	1.70	1.70	1.70	9.78
IMP 2xMIC	5.07	2.30	1.70	1.70	1.70	4.06
CL 0.25xMIC	4.92	5.97	7.17	8.90	9.74	13.89
CL 0.5xMIC	5.03	5.83	4.86	4.60	4.40	13.58
IMP 1xMIC+CL 0.5xMIC	4.57	1.70	1.70	1.70	1.70	1.70
IMP 1xMIC+CL 0.25xMIC	4.93	1.70	1.70	1.70	1.70	1.70
*Ps.*10						
CONTROL	5.72	5.63	6.44	9.08	10.10	16.51
IMP 1xMIC	5.48	4.00	2.78	2.68	2.81	6.08
IMP 2xMIC	5.80	3.94	2.83	2.35	2.24	7.00
CL 0.25xMIC	5.89	5.78	5.63	9.13	10.28	11.83
CL 0.5xMIC	5.78	5.81	4.44	3.70	3.80	6.99
IMP 1xMIC+CL 0.5xMIC	5.44	1.70	1.70	1.70	1.70	1.70
IMP 1xMIC+CL 0.25xMIC	5.86	1.70	1.70	1.70	1.70	4.86

Table A-6 (continuous) viable cell count (log CFU/ml) at the following time of *P. aeruginosa* (8 strains)

Isolated No.	Viable cell count (log CFU/ml) at the following time					
	0 h	2 h	4 h	6 h	8 h	24 h
*Ps.*11						
CONTROL	5.83	6.70	7.98	11.01	11.93	20.70
IMP 1xMIC	5.78	3.11	2.44	1.70	1.70	2.00
IMP 2xMIC	5.88	2.86	1.88	1.70	1.70	1.70
CL 0.25xMIC	5.63	5.95	8.63	7.93	8.35	14.10
CL 0.5xMIC	5.68	5.94	7.78	8.88	8.63	13.40
IMP 1xMIC+CL 0.5xMIC	5.80	1.70	1.70	1.70	1.70	1.70
IMP 1xMIC+CL 0.25xMIC	5.76	1.70	1.70	1.70	1.70	1.70
*Ps.*21						
CONTROL	5.76	6.70	8.13	9.11	11.78	14.18
IMP 1xMIC	5.70	3.85	2.70	2.48	2.44	10.68
IMP 2xMIC	5.44	3.95	1.70	1.70	1.70	1.70
CL 0.25xMIC	5.44	5.76	6.57	8.01	9.93	10.68
CL 0.5xMIC	5.54	5.18	3.54	3.00	3.90	8.95
IMP 1xMIC+CL 0.5xMIC	5.10	1.70	1.70	1.70	1.70	1.70
IMP 1xMIC+CL 0.25xMIC	5.44	1.70	1.70	1.70	1.70	1.70
*Ps.*30						
CONTROL	5.68	7.30	8.48	11.19	13.35	22.97
IMP 1xMIC	5.60	3.68	2.70	1.70	1.88	7.68
IMP 2xMIC	5.86	3.68	2.48	2.00	2.00	1.70
CL 0.25xMIC	5.83	3.40	3.70	3.48	4.89	8.98
CL 0.5xMIC	5.95	1.70	2.00	1.70	1.88	6.74
IMP 1xMIC+CL 0.5xMIC	5.40	1.70	1.70	1.70	1.70	1.70
IMP 1xMIC+CL 0.25xMIC	5.60	1.70	1.70	1.70	1.70	4.54
Average [a]						
CONTROL	5.52±0.44	6.54±0.80	8.30±1.07	10.36±0.88	12.06±1.14	18.74±3.50
IMP 1xMIC	5.46±0.39	3.68±0.72	2.65±0.67	2.29±0.72	2.30±0.66	6.67±3.10
IMP 2xMIC	5.51±0.41	3.26±0.81	2.30±0.72	2.14±0.58	2.07±0.46	2.71±1.92
CL 0.25xMIC	5.49±0.42	5.32±0.89	6.56±1.72	7.79±2.31	8.81±2.75	14.07±4.52
CL 0.5xMIC	5.49±0.40	5.06±1.43	5.19±1.99	5.58±2.89	5.86±3.12	11.60±3.68
IMP 1xMIC+CL 0.5xMIC	5.29±0.46	1.82±0.33	1.70±0.00	1.70±0.00	1.70±0.00	1.77±0.19
IMP 1xMIC+CL 0.25xMIC	5.39±0.39	2.06±0.75	1.83±0.38	1.70±0.00	1.70±0.00	2.62±1.37

[a] = Mean ±SD, IMP= imipenem, CL= colistin

Table A- 7 Killing rate of *P. aeruginosa* (8 strains) by imipenem, colistin and the combination of imipenem and colistin

Isolated No.	Time (hr) for 3 log killing	Time (hr) for regrowth
Ps.1		
IMP 1xMIC	-	24
IMP 2xMIC	8	-
CL 0.25xMIC	-	24
CL 0.5xMIC	-	24
IMP 1xMIC+CL 0.5xMIC	2	-
IMP 1xMIC+CL 0.25xMIC	6	-
Ps.2		
IMP 1xMIC	-	24
IMP 2xMIC	24	-
CL 0.25xMIC	-	8
CL 0.5xMIC	-	24
IMP 1xMIC+CL 0.5xMIC	4	-
IMP 1xMIC+CL 0.25xMIC	4	-
Ps.5		
IMP 1xMIC	2	-
IMP 2xMIC	2	-
CL 0.25xMIC	-	24
CL 0.5xMIC	-	24
IMP 1xMIC+CL 0.5xMIC	2	-
IMP 1xMIC+CL 0.25xMIC	2	-
Ps.7		
IMP 1xMIC	4	24
IMP 2xMIC	4	24
CL 0.25xMIC	-	24
CL 0.5xMIC	-	24
IMP 1xMIC+CL 0.5xMIC		-
IMP 1xMIC+CL 0.25xMIC	2	-
Ps.10		
IMP 1xMIC	-	24
IMP 2xMIC	6	24
CL 0.25xMIC	-	24
CL 0.5xMIC	-	24
IMP 1xMIC+CL 0.5xMIC	2	-
IMP 1xMIC+CL 0.25xMIC	2	24
Ps.11		
IMP 1xMIC	4	-
IMP 2xMIC	2	-
CL 0.25xMIC	-	24
CL 0.5xMIC	-	24
IMP 1xMIC+CL 0.5xMIC	2	-
IMP 1xMIC+CL 0.25xMIC	2	-

Table A- 7 (continuous) Killing rate of *P. aeruginosa* (8 strains) by imipenem, colistin and the combination of imipenem and colistin

Isolated No.	Time (hr) for 3 log killing	Time (hr) for regrowth
*Ps.*21		
IMP 1xMIC	4	24
IMP 2xMIC	4	-
CL 0.25xMIC	-	24
CL 0.5xMIC	-	24
IMP 1xMIC+CL 0.5xMIC	2	-
IMP 1xMIC+CL 0.25xMIC	2	-
*Ps.*30		
IMP 1xMIC	6	24
IMP 2xMIC	4	-
CL 0.25xMIC	-	24
CL 0.5xMIC	2	24
IMP 1xMIC+CL 0.5xMIC	2	-
IMP 1xMIC+CL 0.25xMIC	2	24

IMP= imipenem, CL= colistin

Table A-8 Change in viable cell count (log CFU/ml) at the following time and kinetic parameters ($AUBKC_{0-24}$, BA_{24}) of *P. aeruginosa* (8 strains)

Isolated No.	Change in viable cell counts (log CFU/ml) at the following time					$AUBKC_{0-24}$	BA_{24}
	$\Delta 2$	$\Delta 4$	$\Delta 6$	$\Delta 8$	$\Delta 24$		
*Ps.*1							
CONTROL	0.63	4.36	5.23	7.96	17.04	365.66	-
IMP 1xMIC	-0.80	-1.72	-2.14	-2.36	1.40	119.79	245.87
IMP 2xMIC	-1.42	-2.02	-2.65	-3.00	-3.66	69.74	295.92
CL 0.25xMIC	-0.58	-0.46	-0.64	-1.03	17.81	268.10	97.56
CL 0.5xMIC	-0.02	-0.52	-0.62	-1.06	9.20	198.55	167.11
IMP1xMIC+CL 0.5xMIC	-4.00	-4.00	-4.00	-4.00	-3.46	49.13	316.53
IMP1xMIC+CL 0.25xMIC	-1.48	-2.48	-3.54	-3.54	-2.20	61.30	304.36
*Ps.*2							
CONTROL	1.93	4.43	6.41	7.90	12.90	308.83	-
IMP 1xMIC	-0.90	-2.15	-2.00	-1.96	2.78	107.26	201.57
IMP 2xMIC	-2.06	-2.34	-1.84	-2.00	-3.04	58.95	249.88
CL 0.25xMIC	-0.06	1.85	5.21	7.96	7.82	262.93	45.90
CL 0.5xMIC	-0.15	1.11	4.12	5.92	6.85	232.94	75.89
IMP1xMIC+CL 0.5xMIC	-2.07	-3.00	-3.00	-3.00	-3.00	45.63	263.20
IMP1xMIC+CL 0.25xMIC	-2.22	-3.06	-3.06	-3.06	-3.06	45.53	263.30
*Ps.*5							
CONTROL	2.09	3.00	4.14	6.25	14.98	331.79	-
IMP 1xMIC	-3.09	-3.30	-3.90	-3.90	-3.12	53.73	278.06
IMP 2xMIC	-3.27	-3.88	-3.88	-3.88	-3.88	45.85	285.94
CL 0.25xMIC	0.15	3.21	4.04	4.12	11.23	277.94	53.85
CL 0.5xMIC	0.26	2.37	3.30	3.49	11.11	263.56	68.23
IMP1xMIC+CL 0.5xMIC	-3.90	-3.90	-3.90	-3.90	-3.90	44.68	287.12
IMP1xMIC+CL 0.25xMIC	-3.85	-3.85	-3.85	-3.85	-3.85	44.62	287.17
*Ps.*7							
CONTROL	0.19	2.41	5.58	6.08	9.54	271.27	-
IMP 1xMIC	-1.54	-3.32	-3.32	-3.32	4.76	112.29	158.99
IMP 2xMIC	-2.77	-3.37	-3.37	-3.37	-1.01	64.24	207.03
CL 0.25xMIC	1.05	2.25	3.99	4.82	8.97	247.77	23.50
CL 0.5xMIC	0.80	-0.17	-0.43	-0.63	8.55	183.86	87.42
IMP1xMIC+CL 0.5xMIC	-2.88	-2.88	-2.88	-2.88	-2.88	43.65	227.62
IMP1xMIC+CL 0.25xMIC	-3.23	-3.23	-3.23	-3.23	-3.23	44.01	227.27
*Ps.*10							
CONTROL	-0.09	0.72	3.36	4.38	10.79	270.98	-
IMP 1xMIC	-1.48	-2.70	-2.80	-2.66	0.60	98.34	172.64
IMP 2xMIC	-1.85	-2.97	-3.44	-3.55	1.20	100.23	170.75
CL 0.25xMIC	-0.11	-0.26	3.24	4.40	5.94	234.16	36.82
CL 0.5xMIC	0.03	-1.34	-2.08	-1.98	1.21	123.76	147.23
IMP1xMIC+CL 0.5xMIC	-3.74	-3.74	-3.74	-3.74	-3.74	44.52	226.47
IMP1xMIC+CL 0.25xMIC	-4.16	-4.16	-4.16	-4.16	-1.00	70.23	200.75

Table A-8 (continuous) Change in viable cell count (log CFU/ml) at the following time and kinetic parameters ($AUBKC_{0-24}$, BA_{24}) of *P. aeruginosa* (8 strains)

Isolated No.	Change in viable cell counts (log CFU/ml) at the following time					$AUBKC_{0-24}$	BA_{24}
	Δ2	Δ4	Δ6	Δ8	Δ24		
Ps.11							
CONTROL	0.87	2.15	5.18	6.10	14.87	330.16	-
IMP 1xMIC	-2.66	-3.34	-4.08	-4.08	-3.78	51.57	278.59
IMP 2xMIC	-3.01	-4.00	-4.18	-4.18	-4.18	47.63	282.53
CL 0.25xMIC	0.33	3.00	2.30	2.72	8.47	238.60	91.56
CL 0.5xMIC	0.27	2.10	3.20	2.95	7.72	235.71	94.45
IMP1xMIC+							
CL 0.5xMIC	-4.10	-4.10	-4.10	-4.10	-4.10	44.87	285.29
IMP1xMIC+							
CL 0.25xMIC	-4.06	-4.06	-4.06	-4.06	-4.06	44.84	285.32
Ps.21							
CONTROL	0.94	2.37	3.35	6.02	8.42	273.04	-
IMP 1xMIC	-1.85	-3.00	-3.22	-3.26	4.98	131.11	141.93
IMP 2xMIC	-1.49	-3.74	-3.74	-3.74	-3.74	49.03	224.02
CL 0.25xMIC	0.32	1.13	2.57	4.49	5.24	220.91	52.13
CL 0.5xMIC	-0.37	-2.00	-2.54	-1.64	3.41	135.75	137.30
IMP1xMIC+							
CL 0.5xMIC	-3.40	-3.40	-3.40	-3.40	-3.40	44.17	228.87
IMP1xMIC+							
CL 0.25xMIC	-3.74	-3.74	-3.74	-3.74	-3.74	44.52	228.53
Ps.30							
CONTROL	1.62	2.80	5.51	7.67	17.29	363.47	-
IMP 1xMIC	-1.93	-2.90	-3.90	-3.73	2.07	100.04	263.43
IMP 2xMIC	-2.18	-3.38	-3.86	-3.86	-4.16	53.76	309.71
CL 0.25xMIC	-2.42	-2.13	-2.35	-0.94	3.15	142.82	220.65
CL 0.5xMIC	-4.26	-3.95	-4.26	-4.08	0.79	87.55	275.92
IMP1xMIC+							
CL 0.5xMIC	-3.70	-3.70	-3.70	-3.70	-3.70	44.47	318.99
IMP1xMIC+							
CL 0.25xMIC	-3.90	-3.90	-3.90	-3.90	-1.06	67.44	296.03
Average [a]							
CONTROL	1.02+0.80	2.78+1.21	4.85+1.11	6.54+1.23	13.23+3.37	314.40+39.77	-
IMP 1xMIC	-1.78+0.79	-2.80+0.59	-3.17+0.80	-3.16+0.76	1.21+3.25	96.77+29.20	217.64+55.70
IMP 2xMIC	-2.26+0.69	-3.21+0.72	-3.37+0.77	-3.45+0.68	-2.81+1.92	61.18+17.86	253.22+48.91
CL 0.25xMIC	-0.17+1.02	1.07+1.88	2.30+2.55	3.32+3.03	8.58+4.48	236.65+42.38	77.75+63.09b
CL 0.5xMIC	-0.43+1.58	-0.30+2.15	0.09+3.11	0.37+3.37	6.10+3.84	182.71+61.94	131.69+68.47
IMP1xMIC+							
CL 0.5xMIC	-3.47+0.69	-3.59+0.46	-3.59+0.46	-3.59+0.46	-3.52+0.43	45.14+1.71	269.26+38.76
IMP1xMIC+							
CL 0.25xMIC	-3.33+0.97	-3.56+0.58	-3.69+0.39	-3.69+0.39	-2.77+1.22	52.81+11.46	261.59+38.19

[a] = Mean ±SD, $AUBKC_{0-24}$ =Area under bacterial killing and regrowth curve for 24 hours, BA_{24}= Bacteriolytic area for 24 hours

Table A-9 Viable cell count (log CFU/ml) of colistin in the various concentrations at
the following time of *P. aeruginosa* (8 strains)

Isolated No.	Viable cell counts (log CFU/ml) at the following time					
	0 h	2 h	4 h	6 h	8 h	24 h
*Ps.*1						
CONTROL	5.72	6.35	10.08	10.95	13.68	22.76
CL 1xMIC	5.78	5.52	4.63	4.11	4.06	6.02
CL 2xMIC	5.90	3.11	2.60	2.57	3.65	5.68
CL 4xMIC	5.78	3.10	1.70	1.70	1.70	2.60
CL 8xMIC	5.68	1.70	1.70	1.70	1.70	2.83
*Ps.*2						
CONTROL	4.54	6.48	8.98	10.95	12.44	17.44
CL 1xMIC	4.63	2.83	3.85	5.74	5.40	11.90
CL 2xMIC	4.54	2.74	2.65	2.18	1.70	8.95
CL 4xMIC	4.65	2.10	1.70	1.70	1.70	1.70
CL 8xMIC	4.63	1.70	1.70	1.70	1.70	1.70
*Ps.*5						
CONTROL	5.72	7.81	8.72	9.86	11.97	20.70
CL 1xMIC	5.74	3.98	3.13	3.26	4.06	6.19
CL 2xMIC	5.80	2.54	1.70	1.70	1.70	6.20
CL 4xMIC	5.83	2.24	1.70	1.70	1.70	6.06
CL 8xMIC	5.68	1.70	1.70	1.70	1.70	2.57
*Ps.*7						
CONTROL	5.16	5.35	7.57	10.74	11.24	14.70
CL 1xMIC	4.95	2.30	1.70	1.70	1.70	4.60
CL 2xMIC	4.97	2.74	1.70	1.70	1.70	1.70
CL 4xMIC	5.01	1.70	1.70	1.70	1.70	1.70
CL 8xMIC	5.08	1.70	1.70	1.70	1.70	1.70
*Ps.*10						
CONTROL	5.72	5.63	6.44	9.08	10.10	16.51
CL 1xMIC	5.89	5.98	4.81	3.05	2.40	8.20
CL 2xMIC	5.80	3.54	1.70	1.70	2.18	6.93
CL 4xMIC	5.60	1.70	1.70	1.70	1.70	3.54
CL 8xMIC	5.51	1.70	1.70	1.70	1.70	1.70
*Ps.*11						
CONTROL	5.83	6.70	7.98	11.01	11.93	20.70
CL 1xMIC	5.83	5.18	2.10	2.48	1.70	2.72
CL 2xMIC	5.85	1.70	1.70	1.70	1.70	5.93
CL 4xMIC	5.72	2.24	1.70	1.70	1.70	1.70
CL 8xMIC	5.88	1.70	1.70	1.70	1.70	1.70
*Ps.*21						
CONTROL	5.76	6.70	8.13	9.11	11.78	14.18
CL 1xMIC	5.35	2.18	1.70	1.70	2.18	6.88
CL 2xMIC	5.48	1.70	1.70	1.70	1.70	6.48
CL 4xMIC	5.48	1.70	1.70	1.70	1.70	4.95
CL 8xMIC	5.57	1.70	1.70	1.70	1.70	1.70
*Ps.*30						
CONTROL	5.68	7.30	8.48	11.19	13.35	22.97
CL 1xMIC	5.83	1.70	1.70	1.70	1.70	3.60
CL 2xMIC	5.78	1.70	1.70	1.70	1.70	2.57
CL 4xMIC	5.48	1.70	1.70	1.70	1.70	1.70
CL 8xMIC	5.60	1.70	1.70	1.70	1.70	1.70

Table A-9 (continuous) Viable cell count (log CFU/ml) of colistin in the various concentration at the following time of *P. aeruginosa* (8 strains)

Isolated No.	Viable cell counts (log CFU/ml) at the following time					
	0 h	2 h	4 h	6 h	8 h	24 h
Average [a]						
CONTROL	5.52±0.44	6.54±0.80	8.30±1.07	10.36±0.88	12.06±1.14	18.74±3.50
CL 1xMIC	5.50±0.48	3.71±1.68	2.95±1.34	2.97±1.42	2.90±1.42	6.26±2.89
CL 2xMIC	5.51±0.50	2.47±0.71	1.93±0.43	1.87±0.33	2.00±0.69	5.56±2.35
CL 4xMIC	5.44±0.41	2.06±0.49	1.70±0.00	1.70±0.00	1.70±0.00	2.99±1.71
CL 8xMIC	5.45±0.40	1.70±0.00	1.70±0.00	1.70±0.00	1.70±0.00	1.95±0.47

[a] = Mean ±SD, CL= colistin

Table A-10 Change in viable cell counts (log CFU/ml) of colistin in the various concentration at the following time and kinetic parameters ($AUBKC_{0-24}$, BA_{24}) of *P. aeruginosa* (8 strains)

Isolated No.	Change in viable cell counts (log CFU/ml) at the following time					$AUBKC_{0-24}$	BA_{24}
	$\Delta 2$	$\Delta 4$	$\Delta 6$	$\Delta 8$	$\Delta 24$		
Ps.1							
CONTROL	0.63	4.36	5.23	7.96	17.04	365.66	-
CL 1xMIC	-0.26	-1.15	-1.67	-1.72	0.24	118.99	246.67
CL 2xMIC	-2.79	-3.30	-3.33	-2.25	-0.23	100.78	264.88
CL 4xMIC	-2.68	-4.08	-4.08	-4.08	-3.18	54.88	310.78
CL 8xMIC	-3.98	-3.98	-3.98	-3.98	-2.85	53.80	311.86
Ps.2							
CONTROL	1.93	4.43	6.41	7.90	12.90	308.83	-
CL 1xMIC	-1.80	-0.78	1.11	0.77	7.27	173.26	135.57
CL 2xMIC	-1.80	-1.89	-2.37	-2.85	4.41	106.61	202.22
CL 4xMIC	-2.56	-2.95	-2.95	-2.95	-2.95	44.53	264.31
CL 8xMIC	-2.93	-2.93	-2.93	-2.93	-2.93	43.70	265.13
Ps.5							
CONTROL	2.09	3.00	4.14	6.25	14.98	331.79	-
CL 1xMIC	-1.76	-2.61	-2.49	-1.68	0.45	112.54	219.26
CL 2xMIC	-3.25	-4.10	-4.10	-4.10	0.41	82.60	249.19
CL 4xMIC	-3.59	-4.13	-4.13	-4.13	0.23	80.89	250.91
CL 8xMIC	-3.98	-3.98	-3.98	-3.98	-3.10	51.75	280.04
Ps.7							
CONTROL	0.19	2.41	5.58	6.08	9.54	271.27	-
CL 1xMIC	-2.65	-3.26	-3.26	-3.26	-0.35	68.46	202.81
CL 2xMIC	-2.23	-3.27	-3.27	-3.27	-3.27	46.13	225.15
CL 4xMIC	-3.31	-3.31	-3.31	-3.31	-3.31	44.09	227.19
CL 8xMIC	-3.38	-3.38	-3.38	-3.38	-3.38	44.16	227.12
Ps.10							
CONTROL	-0.09	0.72	3.36	4.38	10.79	270.98	-
CL 1xMIC	0.09	-1.08	-2.84	-3.49	2.31	120.79	150.19
CL 2xMIC	-2.25	-4.10	-4.10	-3.62	1.13	94.70	176.28
CL 4xMIC	-3.90	-3.90	-3.90	-3.90	-2.06	59.44	211.54
CL 8xMIC	-3.81	-3.81	-3.81	-3.81	-3.81	44.59	226.39
Ps.11							
CONTROL	0.87	2.15	5.18	6.10	14.87	330.16	-
CL 1xMIC	-0.65	-3.73	-3.35	-4.13	-3.11	62.38	267.78
CL 2xMIC	-4.15	-4.15	-4.15	-4.15	0.08	78.76	251.40
CL 4xMIC	-3.48	-4.02	-4.02	-4.02	-4.02	45.88	284.28
CL 8xMIC	-4.18	-4.18	-4.18	-4.18	-4.18	44.95	285.21
Ps.21							
CONTROL	0.94	2.37	3.35	6.02	8.42	273.04	-
CL 1xMIC	-3.18	-3.65	-3.65	-3.18	1.52	91.09	181.96
CL 2xMIC	-3.78	-3.78	-3.78	-3.78	1.01	82.84	190.21
CL 4xMIC	-3.78	-3.78	-3.78	-3.78	-0.52	70.60	202.45
CL 8xMIC	-3.88	-3.88	-3.88	-3.88	-3.88	44.65	228.39
Ps.30							
CONTROL	1.62	2.80	5.51	7.67	17.29	363.47	-
CL 1xMIC	-4.13	-4.13	-4.13	-4.13	-2.23	60.13	303.34
CL 2xMIC	-4.08	-4.08	-4.08	-4.08	-3.20	51.85	311.61
CL 4xMIC	-3.78	-3.78	-3.78	-3.78	-3.78	44.55	318.92
CL 8xMIC	-3.90	-3.90	-3.90	-3.90	-3.90	44.68	318.79

Table A-10 (continuous) Change in viable cell counts (log CFU/ml) of colistin in the various concentrations at the following time of each strains of *P. aeruginosa*.

Isolated No.	Change in viable cell counts (log CFU/ml) at the following time					$AUBKC_{0-24}$	BA_{24}
	$\Delta 2$	$\Delta 4$	$\Delta 6$	$\Delta 8$	$\Delta 24$		
Average [a]							
CONTROL	1.02+0.80	2.78+1.21	4.85+1.11	6.54+1.23	13.23+3.37	314.40+39.77	-
CL 1xMIC	-1.79+1.48	-2.55+1.36	-2.53+1.65	-2.60+1.66	0.76+3.18	100.95+38.53	213.45+57.74
CL 2xMIC	-3.04+0.91	-3.58+0.77	-3.65+0.63	-3.51+0.68	0.04+2.48	80.53+21.74	233.87+44.48
CL 4xMIC	-3.38+0.51	-3.74+0.41	-3.74+0.41	-3.74+0.41	-2.45+1.55	55.61+13.89	258.80+43.86
CL 8xMIC	-3.75+0.40	-3.75+0.40	-3.75+0.40	-3.75+0.40	-3.50+0.50	46.53+3.91	267.87+37.65

[a] = Mean ±SD, $AUBKC_{0-24}$ =Area under bacterial killing and regrowth curve for 24 hours, BA_{24}= Bacteriolytic area for 24 hours

Antimicrobial resistance
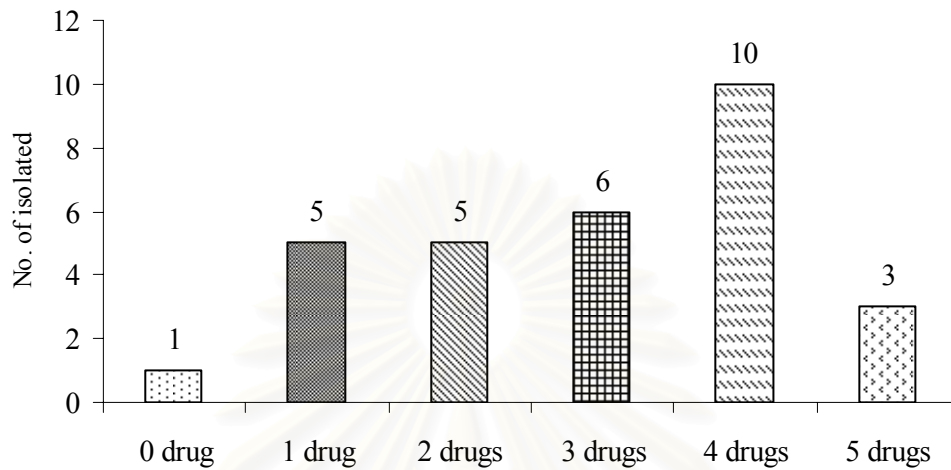
Figure A-1 Distribution of antimicrobial resistance of *P. aeruginosa* according to the number of antimicrobial to which they showed resistance.
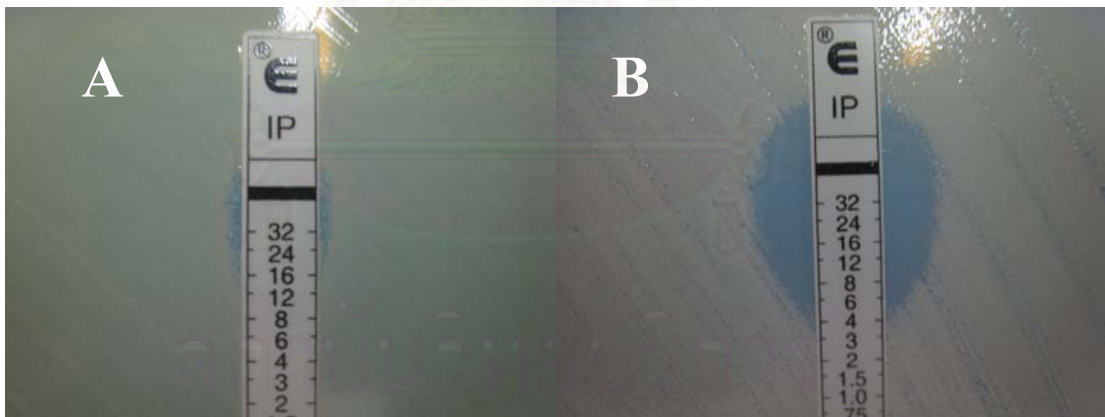
Figure A-2 (A) imipenem-resistance strains (No.1). (B) imipenem-susceptible strains (No.16), determined by E-test.

i m i p e n e m	64										
	32										
	16										
	8										
	4	▓	▓	▓							
	2	▓	▓	▓	▓						
	1	▓	▓	▓	▓						
	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32

colistin

Figure A-3 checkerboard result of *P. aeruginosa* strain No.1 were showed FIC index=1, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

i m i p e n e m	64										
	32										
	16										
	8	▓									
	4	▓	▓	▓	▓						
	1	▓	▓	▓	▓						
	0.5	▓	▓	▓	▓						
	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32

colistin

Figure A-4 checkerboard result of *P. aeruginosa* strain No.2 were showed FIC index=0.09125, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

i m i p e n e m	16										
	8										
	4										
	2	▓	▓								
	1	▓	▓	▓							
	0.5	▓	▓								
	0.25	▓	▓	▓	▓						
	C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64

colistin

Figure A-5 checkerboard result of *P. aeruginosa* strain No.3 were showed FIC index=0.375, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
64											
32											
16											
8											
4											
2											
1											
C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32

colistin

Figure A-6 checkerboard result of *P. aeruginosa* strain No.4 were showed FIC index=1, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
64											
32											
16											
8											
4											
2											
1											
C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32

colistin

Figure A-7 checkerboard result of *P. aeruginosa* strain No.5 were showed FIC index=0.75, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
64											
32											
16											
8											
4											
2											
1											
C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32

colistin

Figure A-8 checkerboard result of *P. aeruginosa* strain No.6 were showed FIC index=0.375, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
64												
32												
16												
8	▓	▓										
4	▓	▓	▓	▓								
2	▓	▓	▓	▓								
1	▓	▓	▓	▓	▓							
C	▓	▓	▓	▓	▓	▓						

colistin

Figure A-9 checkerboard result of *P. aeruginosa* strain No.7 were showed FIC index=0.25, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
64												
32												
16												
8												
4	▓	▓	▓									
2	▓	▓	▓	▓	▓							
1	▓	▓	▓	▓	▓							
C	▓	▓	▓	▓	▓							

colistin

Figure A-10 checkerboard result of *P. aeruginosa* strain No.8 were showed FIC index=0.75, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
32												
16												
8												
4	▓	▓	▓	▓								
2	▓	▓	▓	▓								
1	▓	▓	▓	▓								
0.5	▓	▓	▓	▓	▓	▓						
C	▓	▓	▓	▓	▓	▓						

colistin

Figure A-11 checkerboard result of *P. aeruginosa* strain No.9 were showed FIC index=0.25, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
64											
32											
16											
8											
4											
2											
1											
C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64

colistin

Figure A-12 checkerboard result of *P. aeruginosa* strain No.10 were showed FIC index=0.375, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
64											
32											
16											
8											
4											
2											
1											
C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	32	64

colistin

Figure A-13 checkerboard result of *P. aeruginosa* strain No.11 were showed FIC index=0.375, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
32											
16											
8											
4											
2											
1											
0.5											
C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64

colistin

Figure A-14 checkerboard result of *P. aeruginosa* strain No.12 were showed FIC index=0.375, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
32												
16												
8												
4	▨	▨	▨	▨								
2	▨	▨	▨	▨	▨							
1	▨	▨	▨	▨	▨							
0.5	▨	▨	▨	▨	▨							
C	▨	▨	▨	▨	▨							

colistin

Figure A-15 checkerboard result of *P. aeruginosa* strain No.13 were showed FIC index=0.3125, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
32												
16												
8												
4												
2	▨	▨	▨	▨	▨							
1	▨	▨	▨	▨	▨	▨						
0.5	▨	▨	▨	▨	▨	▨						
C	▨	▨	▨	▨	▨	▨						

colistin

Figure A-16 checkerboard result of *P. aeruginosa* strain No.14 were showed FIC index=1, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
16												
8												
4												
2	▨	▨	▨	▨	▨							
1	▨	▨	▨	▨	▨	▨						
0.5	▨	▨	▨	▨	▨							
0.25	▨	▨	▨	▨	▨							
C	▨	▨	▨	▨	▨							

colistin

Figure A-17 checkerboard result of *P. aeruginosa* strain No.15 were showed FIC index=1, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
16												
8												
4												
2												
1												
0.5												
0.25												
C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	

colistin

Figure A-18 checkerboard result of *P. aeruginosa* strain No.16 were showed FIC index=0.5, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
16												
8												
4												
2												
1												
0.5												
0.25												
C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	

colistin

Figure A-19 checkerboard result of *P. aeruginosa* strain No.17 were showed FIC index=0.5, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
32												
16												
8												
4												
2												
1												
0.5												
C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	

colistin

Figure A-20 checkerboard result of *P. aeruginosa* strain No.18 were showed FIC index=1, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem		colistin									
64											
32											
16											
8											
4											
2											
1											
C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32

colistin

Figure A-21 checkerboard result of *P. aeruginosa* strain No.20 were showed FIC index=0.5, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem		colistin									
64											
32											
16											
8											
4											
2											
1											
C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64

colistin

Figure A-22 checkerboard result of *P. aeruginosa* strain No.21 were showed FIC index=0.5, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem		colistin									
32											
16											
8											
4											
2											
1											
0.5											
C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64

colistin

Figure A-23 checkerboard result of *P. aeruginosa* strain No.22 were showed FIC index=0.5, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
32											
16											
8											
4											
2											
1											
0.5											
C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64

colistin

Figure A-24 checkerboard result of *P. aeruginosa* strain No.25 were showed FIC index=0.5, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
16											
8											
4											
2											
1											
0.5											
0.25											
C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64

colistin

Figure A-25 checkerboard result of *P. aeruginosa* strain No.29 were showed FIC index=1, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem											
64											
32											
16											
8											
4											
2											
1											
C	0.12	0.25	0.5	1	2	4	8	16	32	64	32

colistin

Figure A-26 checkerboard result of *P. aeruginosa* strain No.30 were showed FIC index=0.1875, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem		colistin									
32											
16											
8											
4											
2											
1											
0.5											
C	0.12	0.25	0.5	1	2	4	8	16	32	64	128

Figure A-27 checkerboard result of *P. aeruginosa* strain No.31 were showed FIC index=0.5, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.
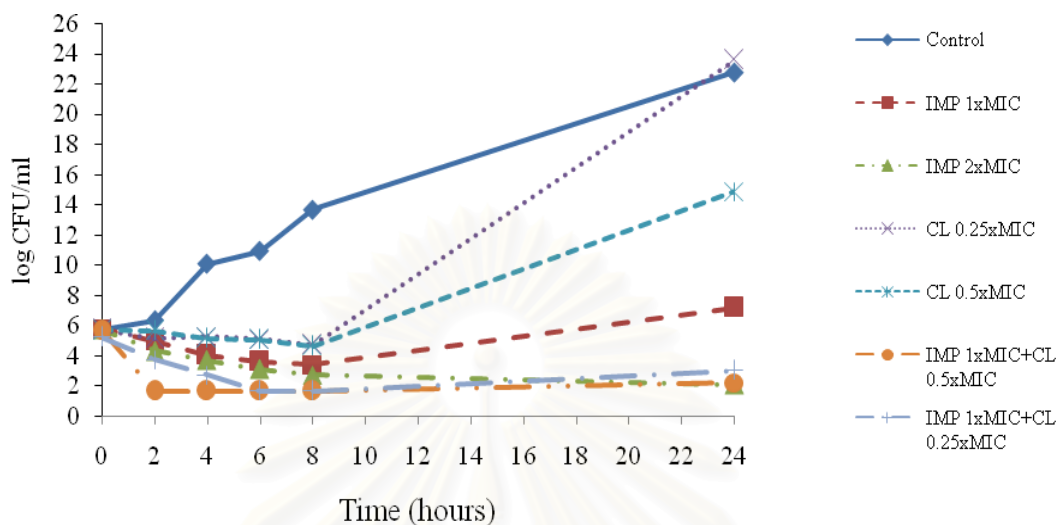
imipenem		colistin									
64											
32											
16											
8											
4											
2											
1											
C	0.12	0.25	0.5	1	2	4	8	16	32	64	128

Figure A-28 checkerboard result of *P. aeruginosa* strain No.32 were showed FIC index=0.75, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem		colistin									
32											
16											
8											
4											
2											
1											
0.5											
C	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32

Figure A-29 checkerboard result of *P. aeruginosa* strain No.33 were showed FIC index=0.3125, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
32												
16												
8												
4												
2												
1												
0.5												

colistin

Figure A-30 checkerboard result of *P. aeruginosa* strain No.34 were showed FIC index=0.3125, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
16												
8												
4												
2												
1												
0.5												
0.25												

colistin

Figure A-31 checkerboard result of *P. aeruginosa* strain No.36 were showed FIC index=1, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.

imipenem	C	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
64												
32												
16												
8												
4												
2												
1												

colistin

Figure A-32 checkerboard result of *P. aeruginosa* strain No.37 were showed FIC index=0.375, gray zone: visible microorganism growth, white zone: no microorganism growth were observed.
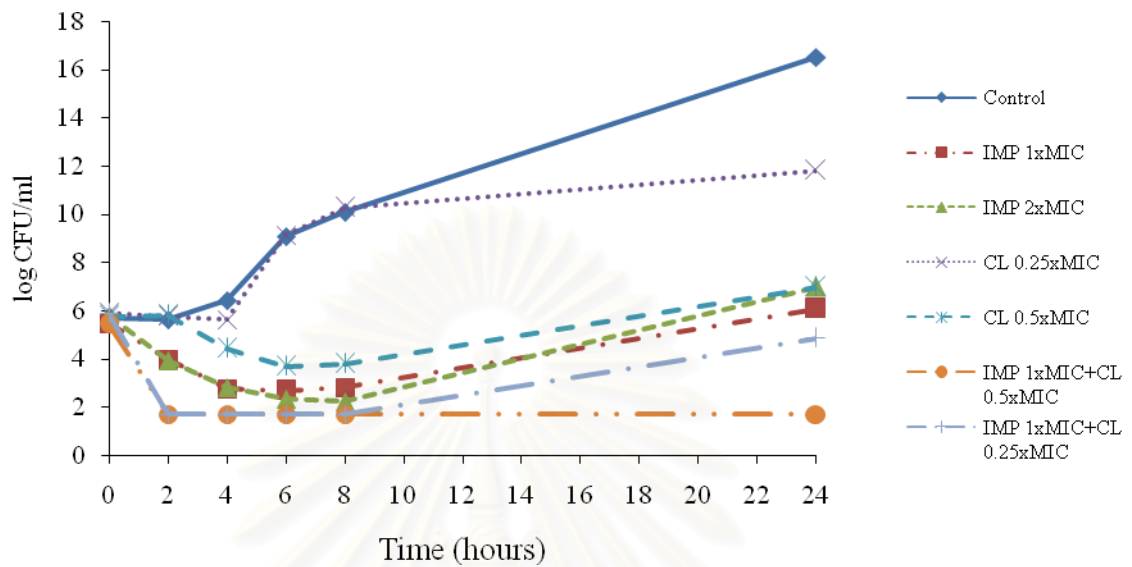
Figure A-32 Time-killing curve of imipenem 1xMIC, imipenem 2xMIC, combination of imipenem 1xMIC plus colistin 0.25xMIC and imipenem 1xMIC plus colistin 0.5xMIC against *P. aeruginosa* strain No.1.
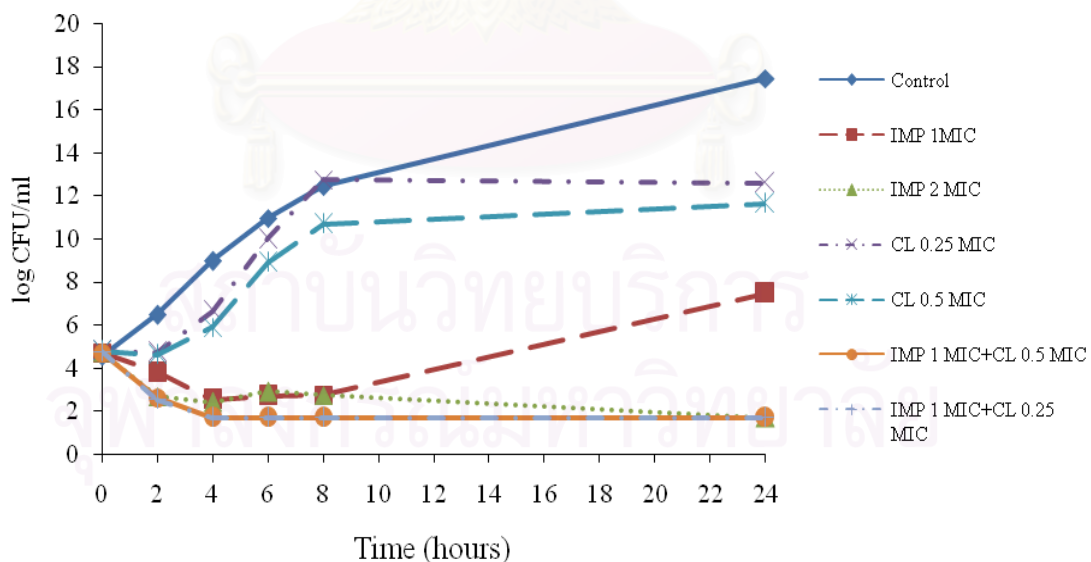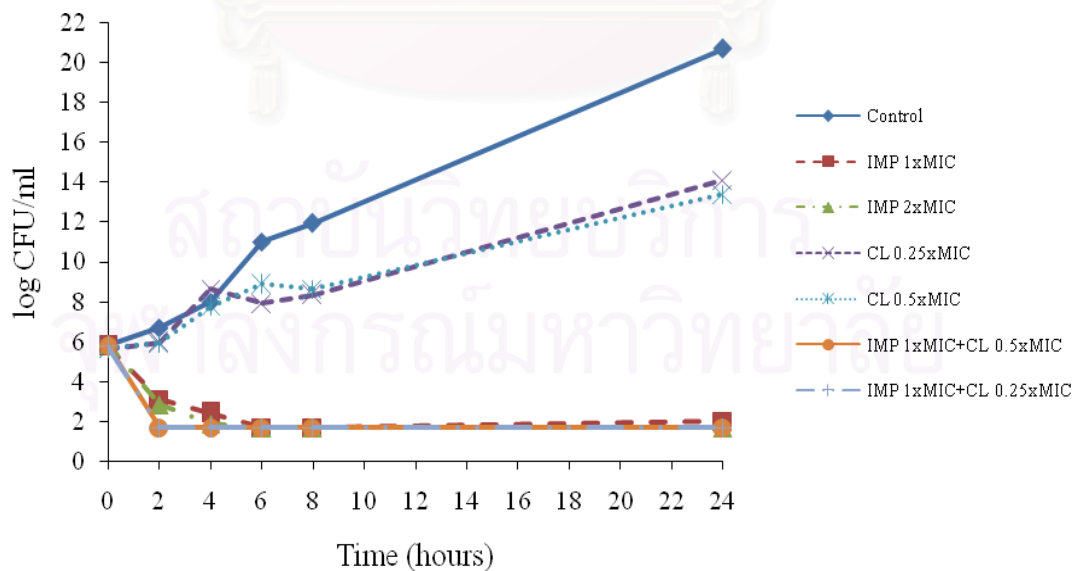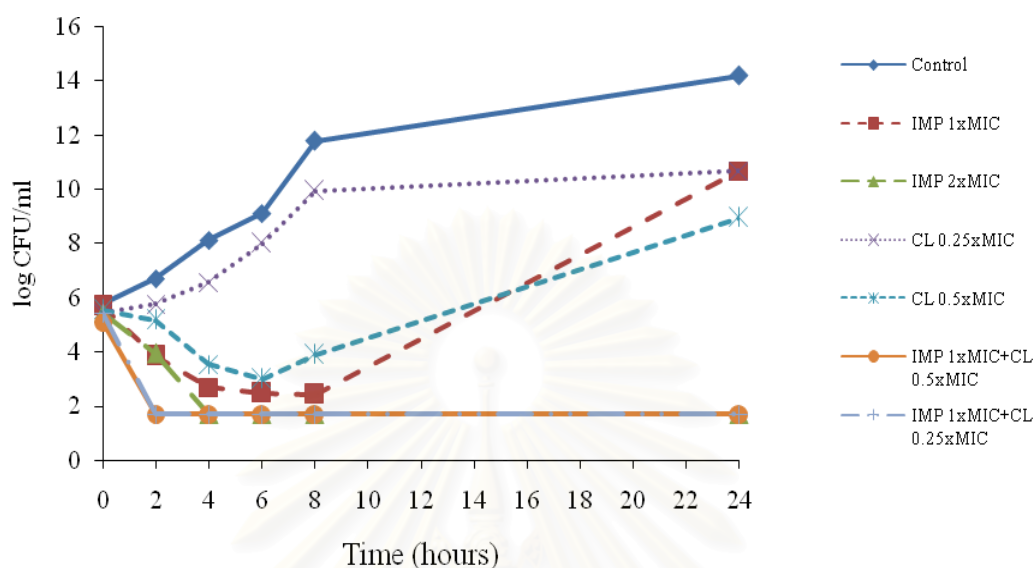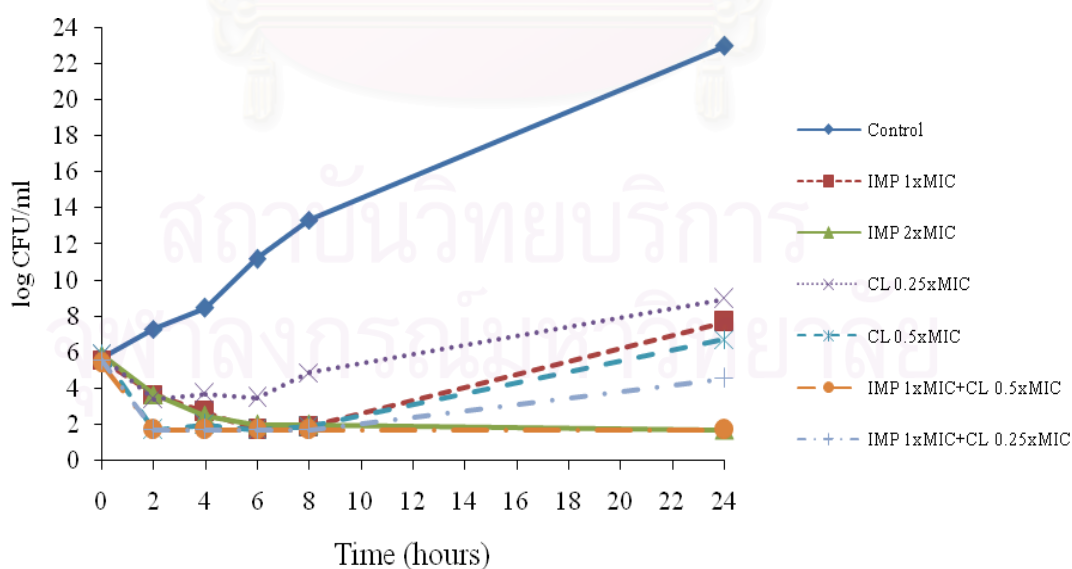

Figure A-33 Time-killing curve of imipenem 1xMIC, imipenem 2xMIC, combination of imipenem 1xMIC plus colistin 0.25xMIC and imipenem 1xMIC plus colistin 0.5xMIC against *P. aeruginosa* strain No.2.
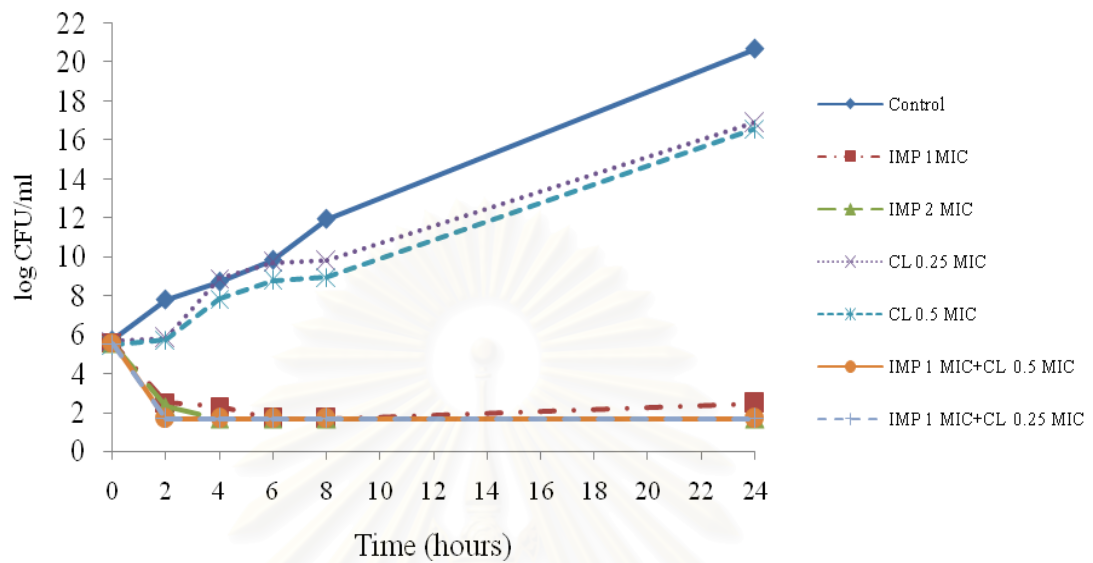
Figure A-34 Time-killing curve of imipenem 1xMIC, imipenem 2xMIC, combination of imipenem 1xMIC plus colistin 0.25xMIC and imipenem 1xMIC plus colistin 0.5xMIC against *P. aeruginosa* strain No.5.
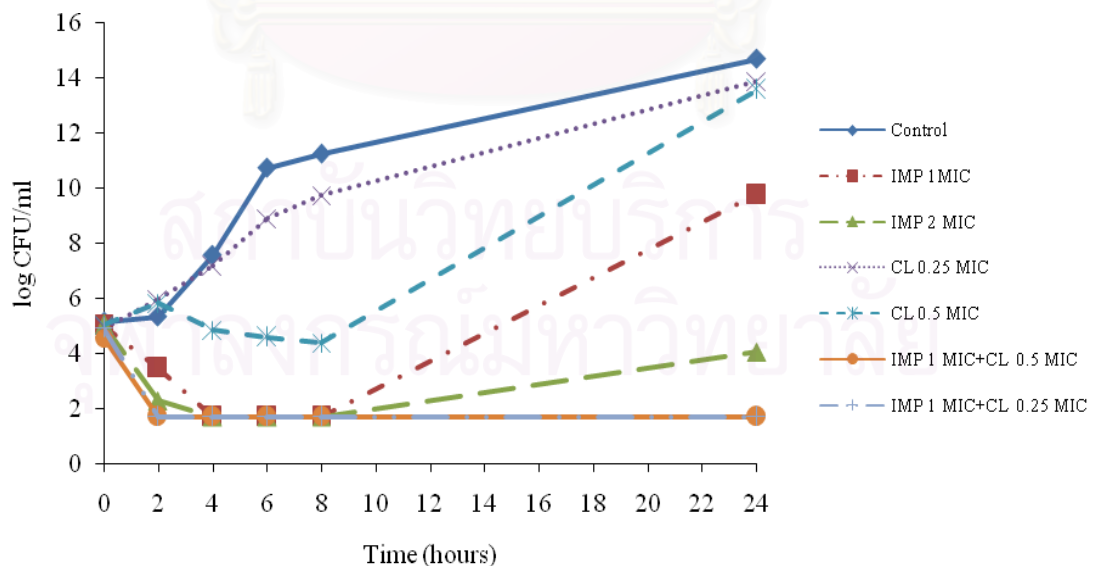
Figure A-35 Time-killing curve of imipenem 1xMIC, imipenem 2xMIC, combination of imipenem 1xMIC plus colistin 0.25xMIC and imipenem 1xMIC plus colistin 0.5xMIC against *P. aeruginosa* strain No.7.

Figure A-36 Time-killing curve of imipenem 1xMIC, imipenem 2xMIC, combination of imipenem 1xMIC plus colistin 0.25xMIC and imipenem 1xMIC plus colistin 0.5xMIC against *P. aeruginosa* strain No.10.

Figure A-37 Time-killing curve of imipenem 1xMIC, imipenem 2xMIC, combination of imipenem 1xMIC plus colistin 0.25xMIC and imipenem 1xMIC plus colistin 0.5xMIC against *P. aeruginosa* strain No.11.

Figure A-38 Time-killing curve of imipenem 1xMIC, imipenem 2xMIC, combination of imipenem 1xMIC plus colistin 0.25xMIC and imipenem 1xMIC plus colistin 0.5xMIC against *P. aeruginosa* strain No.21.

Figure A-39 Time-killing curve of imipenem 1xMIC, imipenem 2xMIC, combination of imipenem 1xMIC plus colistin 0.25xMIC and imipenem 1xMIC plus colistin 0.5xMIC against *P. aeruginosa* strain No.30.
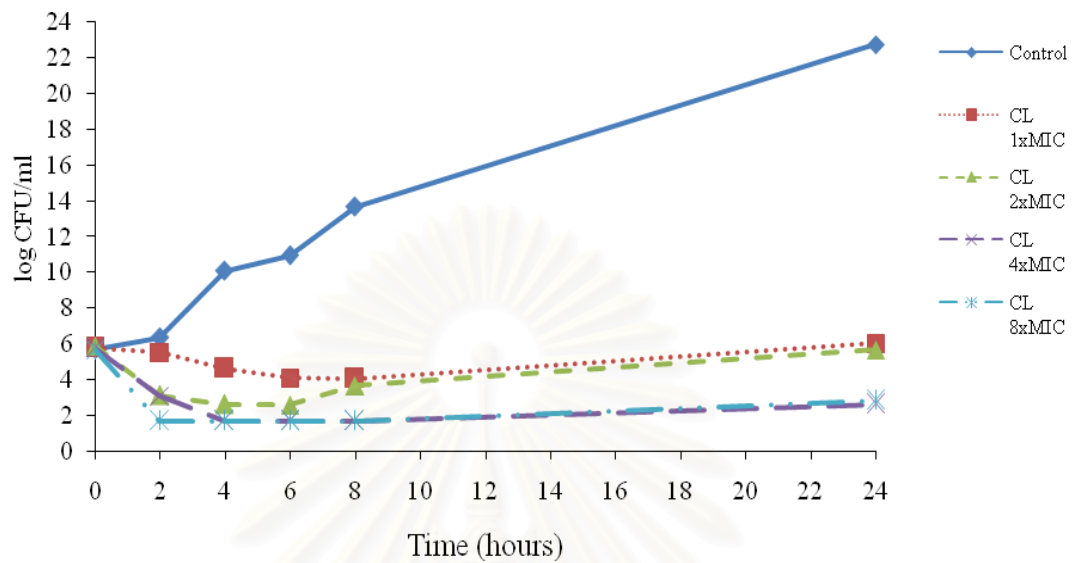
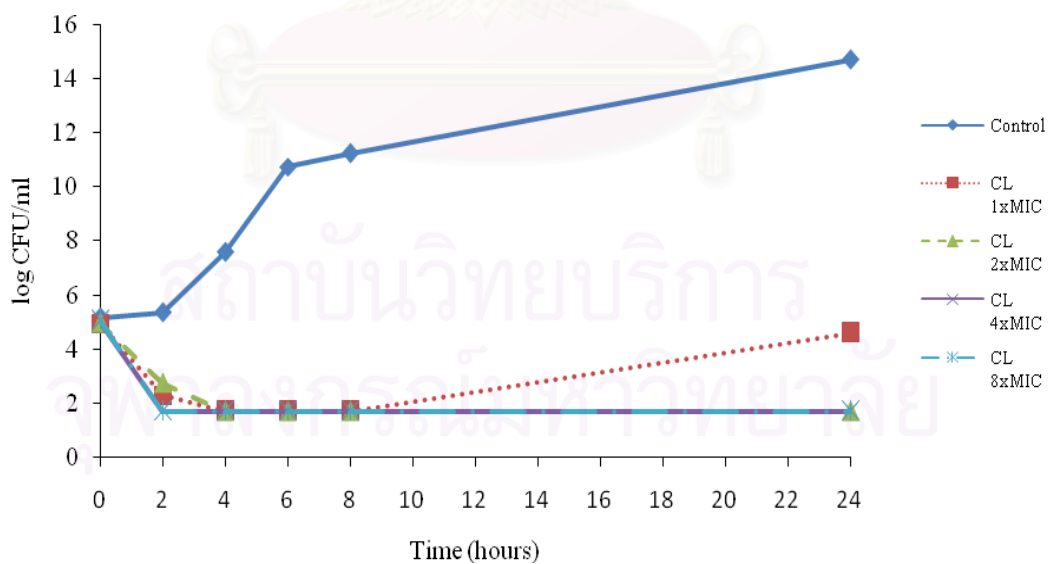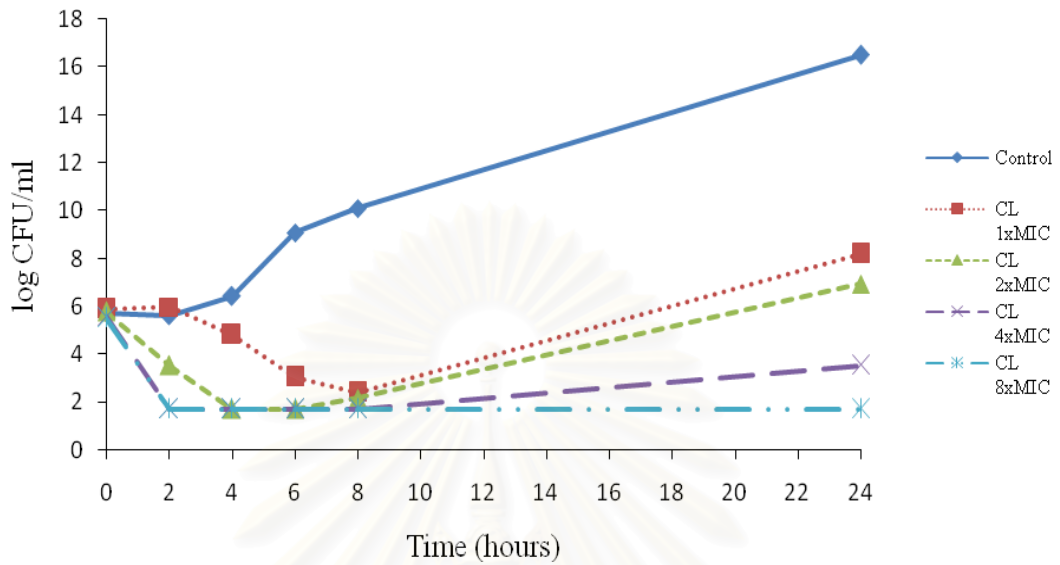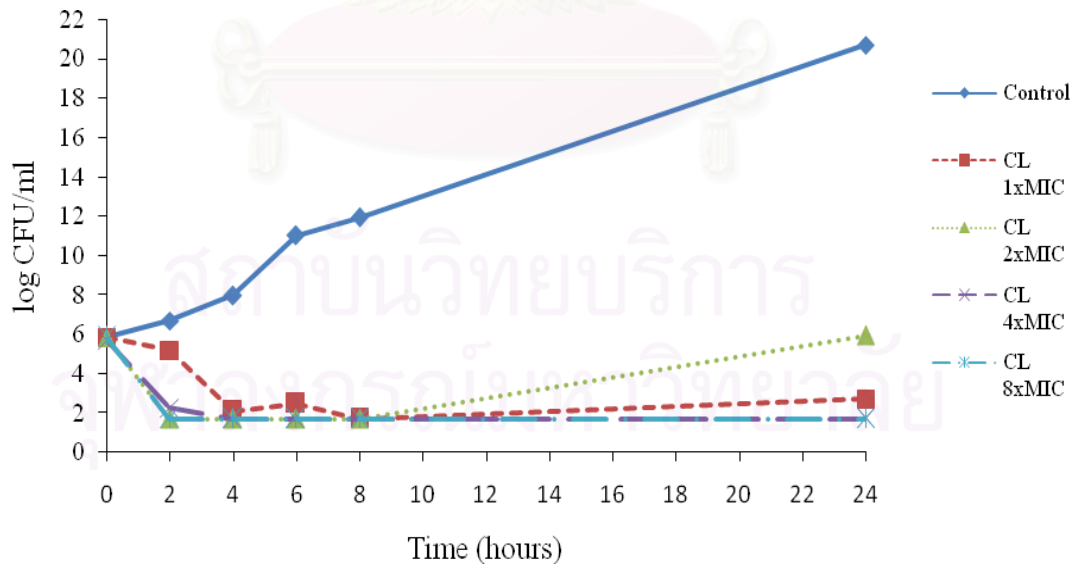Figure A-40 Time-killing curve of colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC

against *P. aeruginosa* strain No.1.
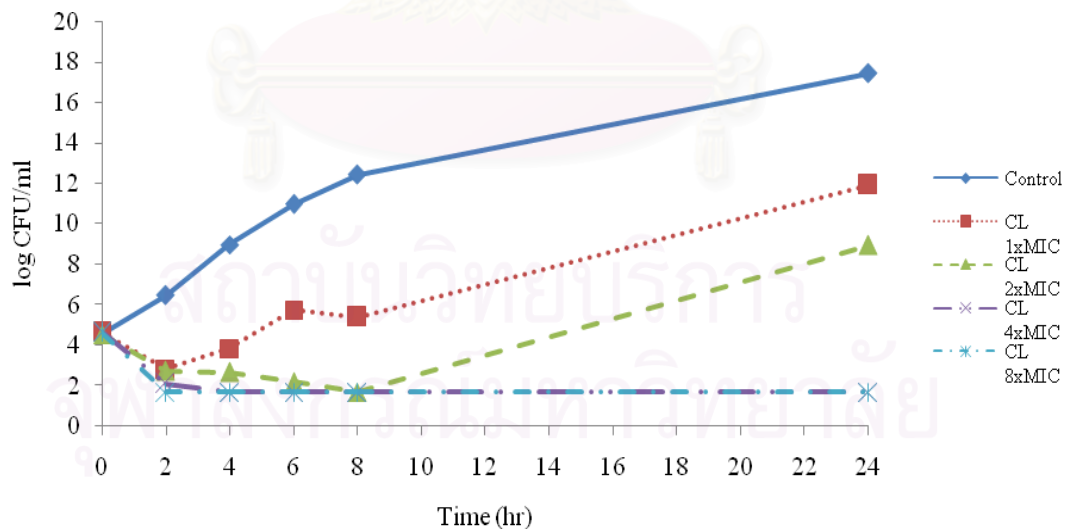
Figure A-41 Time-killing curve of colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC

against *P. aeruginosa* strain No.2.
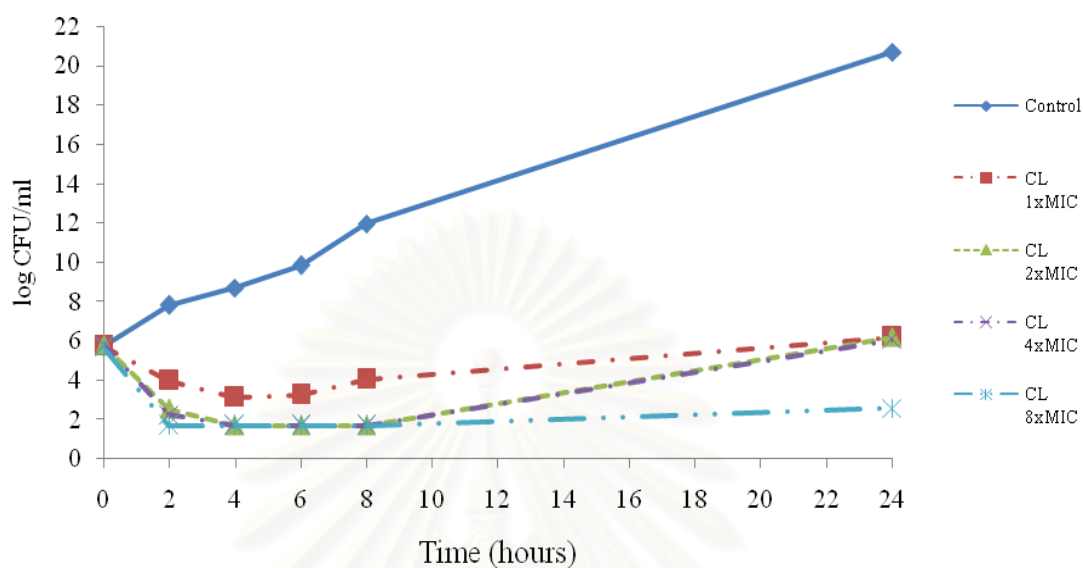
Figure A-42 Time-killing curve of colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC against *P. aeruginosa* strain No.5.

Figure A-43 Time-killing curve of colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC against *P. aeruginosa* strain No.7.
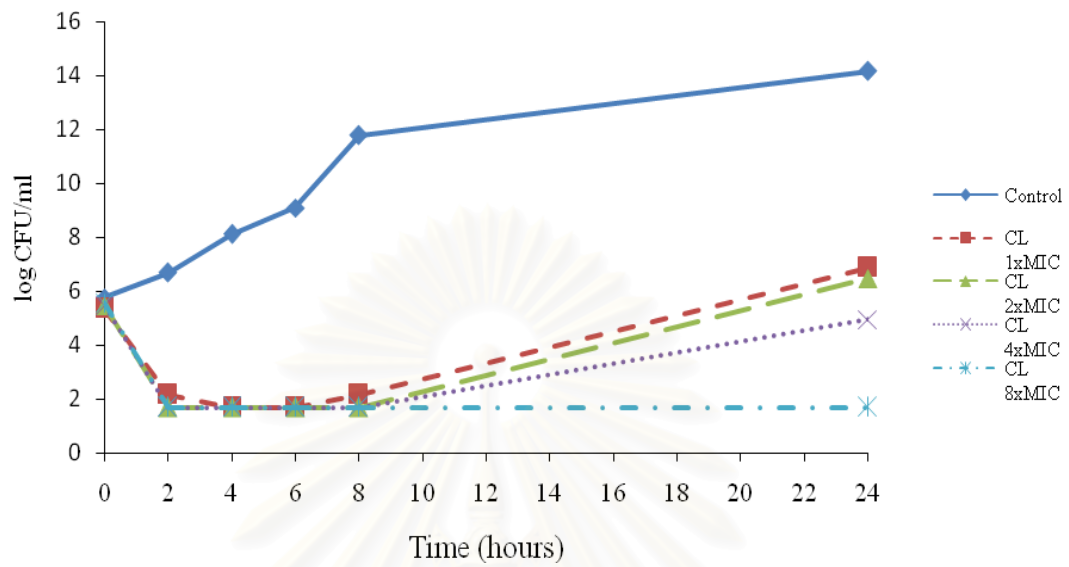
Figure A-44 Time-killing curve of colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC
against *P. aeruginosa* strain No.10.

Figure A-45 Time-killing curve of colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC
against *P. aeruginosa* strain No.11.

Figure A-46 Time-killing curve of colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC against *P. aeruginosa* strain No.21.
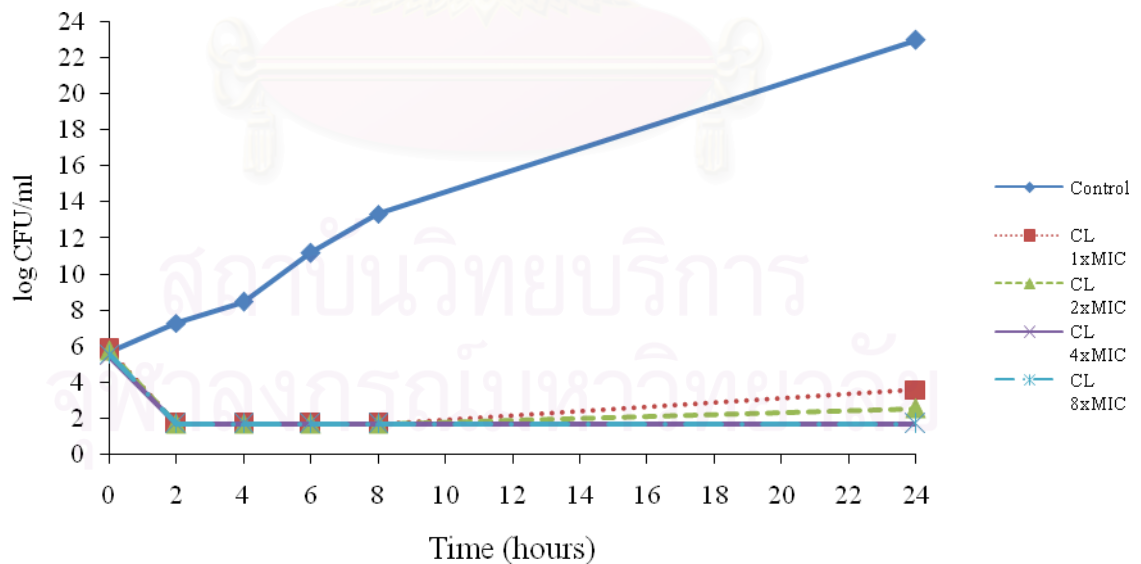

Figure A-47 Time-killing curve of colistin 1xMIC, 2xMIC, 4xMIC and 8xMIC against *P. aeruginosa* strains No.30.

BIOGRAPHY

Miss Sirinat Phanuwong was born in August 11, 1978 in Buriram, Thailand. My graduated with a Bachelor of Pharmacy in 2002 from the Faculty of Pharmaceutical Science, Khonkaen University, Thailand. After graduation, I worked as a pharmacist in Jacharat Hospital, Nakhornratchasima, Thailand, for 3 years.