ฤทธิ์ต้านเชื้อของอิมิพีเน็มร่วมกับโคลิสตินในหลอดทดลองต่อ *ACINETOBACTER BAUMANNII* ที่ดื้อต่อยาหลายชนิด

นางสาวสกุลทิพย์ พนาภักดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IN VITRO ANTIBACTERIAL ACTIVITY OF IMIPENEM IN COMBINATION WITH COLISTIN AGAINST MULTI-DRUG RESISTANT *ACINETOBACTER BAUMANNII*

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Thesis Title	*IN VITRO* ANTIBACTERIAL ACTIVITY OF IMIPENEM IN COMBINATION WITH COLISTIN AGAINST MULTI-DRUG RESISTANT *ACINETOBACTER BAUMANNII*
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สกุลทิพย์ พนาภักดี : ฤทธิ์ด้านเชื้อของอิมิพีเน็มร่วมกับโคลิสตินในหลอดทดลองต่อ *ACINETOBACTER BAUMANNII* ที่ดื้อต่อยาหลายชนิด. (*IN VITRO* ANTIBACTERIAL ACTIVITY OF IMIPENEM IN COMBINATION WITH COLISTIN AGAINST MULTI-DRUG RESISTANT *ACINETOBACTER BAUMANNII*) อ.ที่ปรึกษา: รศ. ศิริภรณ์ ฟุ้งวิทยา, อ.ที่ปรึกษาร่วม: รศ.ดร. พิณทิพย์ พงษ์เพ็ชร, 94 หน้า.

จุดประสงค์ในการวิจัยครั้งนี้เพื่อศึกษาฤทธิ์ด้านเชื้อของ imipenem ร่วมกับ colistin ต่อ *A. baumannii* ที่ดื้อต่อยาหลายชนิดจำนวน 30 สายพันธุ์ จากการทดลองพบว่า *A. baumannii* ทั้ง 30 สายพันธุ์ เป็นสายพันธุ์ที่ดื้อต่อยาหลายชนิด และดื้อต่อ imipenem ทั้งหมดทุกสายพันธุ์ โดยมีค่า MIC ของ imipenem อยู่ในช่วง 8-128 มคก/มล แต่ยังคงมีความไวต่อ colistin ซึ่งค่า MIC ของ colistin อยู่ในช่วง 0.5-2 มคก/มล เมื่อตรวจการสร้างเอนไซม์ metallo-beta-lactamase ไม่พบการสร้างเอนไซม์ชนิดนี้ในทุกสายพันธุ์ และเมื่อประเมินฤทธิ์ร่วมโดยวิธี checkerboard ใน *A. baumannii* ทั้ง 30 สายพันธุ์ พบว่าการให้ imipenem ร่วมกับ colistin จำนวน 30 สายพันธุ์ ให้ผลเสริมฤทธิ์กัน (synergy) 27 สายพันธุ์ และเสริมฤทธิ์กันบางส่วน (partial synergy) 3 สายพันธุ์ เมื่อนำสายพันธุ์ที่ให้ผลเสริมฤทธิ์กันจำนวน 15 สายพันธุ์มาศึกษาต่อเพื่อประเมินผลฆ่าเชื้อโดยวิธี time kill พบว่าการให้ยา imipenem เดี่ยวๆ ที่ความเข้มข้น 32 มคก/มล ซึ่งเป็นความเข้มข้นที่เท่ากับความเข้มข้นเฉลี่ยของยาในเลือดเมื่อให้ในขนาดที่ใช้ในการรักษา สามารถฆ่าเชื้อได้ 99.9% จำนวน 1 สายพันธุ์ (6.67%) ที่เวลา 8 ชั่วโมงหลังจากได้รับยา และ 1 สายพันธุ์ (6.67%) ที่เวลา 24 ชั่วโมงหลังจากได้รับยา การให้ colistin เดี่ยวๆที่ความเข้มข้น 1/16 MIC ไม่มีฤทธิ์ฆ่าเชื้อในทุกสายพันธุ์ที่นำมาทดสอบ ในขณะที่ colistin เดี่ยวๆที่ความเข้มข้น 1/4 MIC สามารถฆ่าเชื้อได้เพียง 90.0% ที่เวลา 4, 6 และ 8 ชั่วโมงเป็นจำนวน 1 ถึง 2 สายพันธุ์ แต่อย่างไรก็ตามการใช้ imipenem เดี่ยวๆ และcolistin เดี่ยวๆที่ความเข้มข้น 1/16 MIC และ 1/4 MIC เชื้อจะกลับเจริญขึ้นได้อีก (regrowth) ที่เวลา 24 ชั่วโมงเป็นจำนวน 7 (46.67%), 15 (100%) และ 15 (100%) สายพันธุ์ตามลำดับ สำหรับการให้ imipenem (32 มคก/มล) ร่วมกับ colistin ความเข้มข้น 1/16 MIC สามารถฆ่าเชื้อได้ 99.9% จำนวน 2 สายพันธุ์ (13.33%) ที่เวลา 8 ชั่วโมง และ 4สายพันธุ์ (26.67%) ที่เวลา 24 ชั่วโมงหลังจากได้รับยา เมื่อให้ imipenem (32 มคก/มล) ร่วมกับ colistin ความเข้มข้น 1/4 MIC สามารถฆ่าเชื้อได้ 99.9% จำนวน 1 สายพันธุ์ (6.67%) ที่เวลา 2 ชั่วโมง และ 10 สายพันธุ์ (66.67%) ที่เวลา 24 ชั่วโมงหลังจากได้รับยา นอกจากนี้การใช้ imipenem ร่วมกับ colistin ที่ความเข้มข้น 1/16 MIC และ 1/4 MIC จะมีเชื้อกลับเจริญได้อีกที่เวลา 24 ชั่วโมง เป็นจำนวน 4 สายพันธุ์ (26.67%) และ 2 สายพันธุ์ (13.33%) ตามลำดับ จำนวนเชื้อที่ถูกฆ่าภายใน 24 ชั่วโมงเมื่อให้ imipenem ร่วมกับ colistin (1/16MIC) [จำนวนเชื้อที่ถูกฆ่าภายใน 24 ชั่วโมง = 158.12 log CFU/ml'h] ไม่แตกต่างจากการให้ imipenem เดี่ยวๆอย่างมีนัยสำคัญทางสถิติ [จำนวนเชื้อที่ถูกฆ่าภายใน 24 ชั่วโมง = 127.66 log CFU/ml'h] ในขณะที่การให้ imipenem ร่วมกับ colistin (1/4 MIC) [จำนวนเชื้อที่ถูกฆ่าภายใน 24 ชั่วโมง = 193.12 log CFU/ml'h] จะแตกต่างจากการให้ imipenem เดี่ยวๆ และการให้ imipenem ร่วมกับ colistin (1/16 MIC) อย่างมีนัยสำคัญทางสถิติ นอกจากนั้นผลของการฆ่าเชื้อจะสังเกตได้จากการเปลี่ยนแปลงทางสัณฐานวิทยาและผนังเซลล์ถูกทำลายหลังสัมผัส imipenem (32 มคก/มล) ร่วมกับ colistin (1/4 MIC) มากกว่าเมื่อให้ imipenem (32 มคก/มล) เดี่ยวๆ และ colistin (1/4 MIC) เดี่ยวๆ จากผลการทดลองแสดงเห็นว่าการให้ยา imipenem ร่วมกับ colistin สามารถแสดงฤทธิ์ในการฆ่าเชื้อได้ดีกว่าการให้ยาเดี่ยวๆ เมื่อเปรียบเทียบในด้านจำนวนเชื้อที่ถูกฆ่า, จำนวนสายพันธุ์ที่ถูกฆ่าและความเร็วในการฆ่าเชื้อ ดังนั้นการใช้ imipenem ร่วมกับ colistin จึงเป็นอีกทางเลือกหนึ่งในการรักษาโรคติดเชื้อ *A. baumannii* สายพันธุ์ที่ดื้อต่อยาหลายชนิด

สาขาวิชา.....เภสัชวิทยา.....ลายมือชื่อนิสิต.....

ปีการศึกษา.....2550.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: *Acinetobacter baumannii* / IMIPENEM / COLISTIN / COMBINATION

SAKULTHIP PANAPAKDEE : *IN VITRO* ANTIBACTERIAL ACTIVITY OF IMIPENEM IN COMBINATION WITH COLISTIN AGAINST MULTI-DRUG RESISTANT *ACINETOBACTER BAUMANNII*. THESIS ADVISOR : ASSOC. PROF. SIRIPORN FUNGWITTHAYA, THESIS COADVISOR : ASSOC. PROF. PINTIP PONGPECH, 94 pp.

The purpose of present study is to determine the *in vitro* antibacterial activity of imipenem in combination with colistin against 30 strains of multi-drug resistant *A. baumannii*. Thirty *A. baumannii* strains that were found to be multi-drug resistant strains and all strains were resistant to imipenem (MIC range 8-128 μg/ml) but susceptible to colistin (MIC range 0.5-2 μg/ml). The metallo-β-lactamase activity could not be detected in all strains. Checkerboard method served to determine the activity of imipenem in combination with colistin. Combination of the imipenem with colistin showed synergistic effect against 27 strains (90%) and partial synergistic effect against 3 strains (10%) of *A. baumannii* strains tested. In the time kill study using 15 *A. baumannii* strains resulted in synergistic effect. Imipenem 32μg/ml, the concentration was equal to the mean serum level of imipenem at the therapeutic dose showed bactericidal activity (99.9% killing) in 1 strain (6.67%) at 8 hours of growth and 1 strain (6.67%) at 24 hours of growth, 1/16MIC of colistin alone showed no antibacterial activity against all strains tested, whereas 1/4MIC of colistin alone showed bacteriostatic activity (90.0% killing) in 1 to 2 strains at 4, 6 and 8 hours of growth. However, the regrowth was observed in 7 (46.67%), 15 (100%) and 15 (100%) strains at 24 hours by imipenem alone, 1/16MIC and 1/4MIC of colistin alone, respectively. The combination of imipenem (32μg/ml) plus 1/16MIC of colistin showed bactericidal activity against 2 strains (13.33%) at 8 hours of growth and 4 strains (26.67%) at 24 hours of growth. The combination of imipenem (32μg/ml) plus 1/4MIC of colistin showed bactericidal activity against 1 strain (6.67%) at 2 hours of growth and against 10 strains (66.67%) at 24 hours of growth. In addition, the combination of imipenem (32μg/ml) plus 1/16MIC and 1/4MIC of colistin showed regrowth against 4 strains (26.67%) and 2 strains (13.33%) at 24 hours of growth, respectively. The amount of bacteria killed by the combination of imipenem plus 1/16 MIC of colistin [BA_{24} = 158.12 log CFU/ml·h] were not significantly higher than those killed by imipenem alone [BA_{24} = 127.66 log CFU/ml·h] while the amount of bacteria killed by the combination of imipenem plus 1/4MIC of colistin [BA_{24} = 193.72 log CFU/ml·h] were significantly higher than those kill by imipenem alone and the combination of imipenem plus 1/16MIC of colistin. In addition, the bactericidal effect was observed by morphological akteration and cell wall destruction after exposure to combination of imipenem (32μg/ml) plus colistin (1/4 MIC) more than exposure to imipenem alone (32μg/ml) and colistin alone (1/4 MIC). The results obtained suggested that antibacterial activity of the combination of imipenem and colistin were higher than antibacterial activity of single drug alone. It is concluded that the combination of imipenem and colistin could be promising alternative in the treatment of infection due to multi-drug resistant *A. baumannii*.

Field of study....PHARMACOLOGY..... .Student's signature Sakulthip Panapakdee

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Co-advisor's signature Pintip Pongpech

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LIST OF ABBREVIATIONS

^{o}C	= degree Celsius
A. baumannii	= *Acinetobacter bauamannii*
AUBKC	= Area under the bacterial killing curve
BA24	= Bacteriolytic area of 24 hours
CFU	= colony forming unit
CLSI	= Clinical and Laboratory Standards Institute
E. coli	= *Escherichia coli.*
ESBL	= extended-spectrum β-lactamase
et al.	= et alii (and other peoples)
FIC	= Fractional inhibitory concentration
Fig	= Figure
g	= gram
hr	= hour
L	= Liter
Log	= decimal logarithm
MBL	= metallo-β-lactamase
MDR	= multi-drug resistant
MHA	= Meuller-Hinton agar
MHB	= Meuller-Hinton broth
MIC	= Minimum inhibitory concentration
min	= minute
ml	= milliliter
mm	= millimeter
mol	= mole

M	= Molar
NCCLS	= The National Committee for Clinical Laboratory Standards
nm	= nanometer
NSS	= Normal saline solution
OXA	= Oxacillin-hydrolyzing β-lactamase
P. aeruginosa	= *Pseudomonas aeruginosa*
PBP	= Penicillin binding protein
TSA	= Tryptic soy agar
µg	= microgram
µl	= microliter

CHAPTER I

INTRODUCTION

Acinetobacter baumannii are gram-negative coccobacilli, glucose non-fermentative, non-fastidious, strictly aerobe, non motile, catalase-positive and oxidase-negative (Von Graevenitz, et al., 1995). This genus is commonly found in the environment particularly in soil and water. It can also be found on the skin and distal urethra of healthy people. Skin colonization of patient plays an important role in the subsequent contamination of the hand of hospital staff during contacts, thereby contributing to the spread of the organism. High colonization rates of the skin, throat, respiratory system or digestive tract, of various degrees of importance, have been documented in several outbreaks (Getchel-Whith, Donowitz and Groschel, 1989). *A. baumannii* can colonize multiple body sites of hospitalized patients, and survive for a long time on in animate surfaces (Jawad, Heritage and Snelling, 1996). Both characteristics contribute, at least in part, to the prominent role of *A. baumannii* in nosocomial infections, including ventilator-associated pneumonia, surgical site infections, urinary tract infection, wound infection and septicemia, involving mostly patients with impaired host defenses (Borgogne-Berezin, 2001). Whereas *A. baumannii* has itself a quite high level of naturally occurring antibiotic resistance, it may acquire additional resistance traits as a source of multi-drug resistance. A major contributing factor in the emergence of resistant *A. baumannii* is the acquisition and transfer of antibiotic resistance via plasmid and transposons.

A. baumannii has become resistant to almost all antimicrobial agents that are currently available, including the aminoglycosides, quinolones and broad-spectrum beta-lactams (Bergogne-Berezin and Towner, 1996). The number of multi-drug resistant strains of *A. baumannii* is increasing, resulting in great problems for choosing the proper treatment. In 2002, 57 % of *A. baumannii* isolated from infected hospitalized patients in Siriraj Hospital were multi-drug resistant and the mortality rate of such patients was higher than 50% (Keerasuntonpong, Samakeepanich and Tribuddharat, 2003).

The carbapenems, imipenem and meropenem, are among the drugs of choice in the treatment of these multi-drug resistant *A. baumannii* infections (Navon-

Venezia, Ben-Ami and Carmeli, 2005). However, carbapenem resistance is increasingly, recognized as a threat to the effective treatment of these infections (Corbella et al., 2000). Resistance to these potent β-lactams may be due to impaired permeability resulting from altered outer membrane proteins or to alterations in the penicillin binding proteins. However the carbapenem-hydrolyzing β-lactamase (carbapenemase), a group that includes the metallo-β-lactamase (MBLs) and some oxacillinase, are also recognized as imported contributors to carbapenem resistance in *A. baumannii* (Bou et al., 2000; Fernandez-Cuenca et al., 2003; Urban, Segal-Maurrer and Rahal, 2003). In Thailand, the data from the National Antimicrobial Resistance Surveillance Thailand (NARST) showed that the prevalence of imipenem resistant *A. baumannii* from in 2002-2006 were 18%, 29%, 43%, 49% and 55%, respectively. Thus, the antimicrobial resistance of the *A. baumannii* may cause the complication in the treatment of infections and cause the adverse clinical outcomes and increase the treatment costs for patients.

Presently, treatment options for infection caused by members of *A. baumannii* are limited. Colistin has become one of the most commonly used antibiotic for the treatment of infections caused by multi-drug resistant gram-negative bacteria including multi-drug resistant *A. baumannii*. Its favorable property is the rapid bacterial killing, a narrow spectrum against all these pathogens. However the use of this agent has been limited because of the concerns about poor pharmacokinetics and nephrotoxicity (Evans, Feola and Rapp, 1999; Falagas and Kasiakcu, 2005; Li et al., 2005). Combinations of agents that exhibit synergy or even partial synergy could potentially reduce toxicity and improve the treatment of patients caused by resistant organisms. Thus, combination therapy is the other choice in the treatment *A. baumannii* aiming at decreasing emergence of resistant strains and increasing bacterial killing. Several previous studies have demonstrated the synergistic activity of meropenem and sulbactam against clinical isolates of *A. baumannii* (Kiffer et al., 2005). In addition, several non-traditional antibiotics, such as colistin, doxycycline and rifampin, have been tested against multi-drug resistant strains of these bacteria and synergistic effects have been determined for the combinations of these antimicrobial agents (Timurkaynak et al., 2006).

Therefore, the purpose of the present study is to determine the susceptibility of *A. baumannii* from clinical specimens to 10 antibiotics (cefepime, gentamicin, tobramycin, ceftazidime, ciprofloxacin, piperacillin/tazobactam, rifampin, imipenem, amikacin and colistin) by Kirby-Bauer disk diffusion method. The minimal inhibitory concentration (MICs) of 5 antimicrobial agents (cefepime, amikacin, ciprofloxacin, imipenem and piperacillin/tazobactam) against the tested organisms determined by E-test method were used to detected multi-drug resistant strains. The MICs of the two antimicrobial agents (imipenem and colistin) against all isolates were also determined by the standard agar dilution method. At the same time, the combined antibacterial activities of colistin plus imipenem against multi-drug resistant *A. baumannii* were also determined by the checkerboard method and determine the bactericidal effect of the combination of imipenem plus colistin against multi-drug resistant *A. baumannii* by time kill method. Another aim of this study is to detect metallo-β-lactamase activity by disk diffusion method in imipenem resistant *A. baumannii* and examine the morphological changes in *A. baumannii* treated with imipenem, colistin and the combination by scanning electron microscope.

CHAPTER II

LITERATURE REVIEWS

1. *Acinetobacter baumannii*

Acinetobacter baumannii (*A. baumannii*) is aerobic Gram negative coccobacilli, strictly aerobic, non-fastidious, glucose-non-fermentative, non-motile, catalase-positive and oxidase-negative (Von Graevenitz et al., 1995). It is ubiquitous in nature; they can be recovered easily from soil or water, and have also been found frequently in animal and human host (Henriksen et al., 1976). *A. baumannii* is now recognized as significant nosocomial pathogen, particularly for the subset of critically-ill patients requiring mechanical ventilation in hospital intensive care units. However, *A. baumannii* infections can also be acquired outside the healthcare setting. Colonization of hospital personal and contamination of a patient's environment were reported as the most important predisposing factors. In several cases, multi-drug resistant *A. baumannii* was isolated from bed linen and also from curtains that separated patients on large wards. Their contribution to nosocomial infection has increased over the past three decades and many outbreaks of hospital infection involving acinetobacters have been report worldwide. According to data from the National Nosocomial Infections Surveillance System, *Acinetobacter spp*. were isolated in 1% of all nosocomial infections from 1990 to 1992 (Emori and Gaynes, 1993). Although prevalent in nature and regarded generally as commensals of human skin and the human respiratory tract, acinetobacters have also been implicated as the cause of serious infectious disease such as pneumonia, endocarditis and septicaemia, involving mostly patients with impaired host defenses (Bergogne-Berezin and Towner, 1996).

Resistance to almost all antibiotics in *A. baumannii* is a critical challenge. The number of multi-drug resistant *A. baumannii*, which is an opportunistic pathogen mainly in immunocompromised patients that may cause pneumonia, bacteremia infection in burn wounds, meningitis and urinary tract infections has been on the increase globally and it is now regarded as one of the most difficult nosocomially acquired Gram-negative pathogens to treat and control (Jain and Danziger, 2004; Livermore, 2004).

2. Antibiotic Resistance Problems

A. baumannii is notoriously associated with outbreaks, facilitated by resistance to disinfectants and desiccation. Until the 1970s, most isolates were susceptible to a wide range of antibiotics (Bergogne-Berezin, 2001). Subsequently, *A. baumannii* has shown a remarkable propensity to develop resistance to virtually every antibiotic class (Hanwood et al., 2002).

Resistant mechanism of *A. baumannii* is often attributed to impermeability or the presence of a β-lactamase alone but in reality, these factors work together so that for any given external β-lactam concentrations, the periplasmic β-lactam concentration maintains a steady-state level, the magnitude of which determines the extent of PBP poisoning. Reducing permeability through porin loss or increased β-lactamase activity reduces the steady-state periplasmic drug concentrations and thereby reduces PBP inactivation (Amyes and Young, 1996; Sato and Nakae, 1991; Dance, Navia and Ruiz, 2002).

One of the most worrying antibiotic resistance problems in *A. baumannii* is the increasing trend of carbapenem resistance, since carbapenems are often used as antibiotics of last resort. In 1991, a nosocomial outbreak of imipenem resistant *A. baumannii* occurred in a surgical ICU in the USA (Go, Urban and Burns, 1994). From 1999 to 2003, carbapenem resistant *A. baumannii* have been identified in China (Wang et al., 2003). The SENTRY antimicrobial surveillance program reports surveillance during 1997 to 1999 showed that 11% of *A. baumannii* was resistant to the carbapenems while the prevalence of carbapenem resistance in *A. baumannii* isolated across Latin America in 2001 was estimated to be 25% (Jones et al., 1999; Sader et al., 2004). During 2005, carbapenem resistant rates for *A. baumannii* were around 40% in 12 Colombian tertiary-care hospitals (Miranda et al., 2006). In Thailand, the data from National Antimicrobial Resistance Surveillance Thailand (NARST) during the year 2002 to 2006 showed that imipenem susceptibility was decreased from 91% in the year 2001 to only 42% in the year 2002. An increase in the prevalence of antimicrobial-resistant *A. baumannii* has been presented in Table 2-1.

Table 2-1 Antimicrobial susceptibility of *A. baumannii* isolates from 32 hospitals in Thailand during the year 2001-2006 (Modified from http://narst.dmsc.moph.go.th).

Year	Percentage of susceptible *Acinetobacter baumannii*				
	Imipenem	Amikacin	Gentamicin	Ceftazidime	Ciprofloxacin
2001	91	41	35	36	41
2002	78	41	36	37	40
2003	69	39	34	34	35
2004	55	38	33	34	35
2005	48	40	32	32	33
2006	42	35	31	29	28

In *A. baumannii* carbapenem resistance, various mechanisms of resistance to carbapenems are likely to present including the decreased in the outer-membrane permeability caused by the loss or reduced expression of porin, the overexpression of multi-drug efflux pumps, the alterations in penicillin binding proteins and the production of carbapenemase (Clark, 1996; Gehrlein et al., 1991; Chu, Afzal-Shah and Houang, 2001; Riccio, Fransceschini and Boschi, 2001).

3. The β-lactamases

The β-lactamases are the major defense of Gram-negative bacteria against β-lactam antibiotics. These enzymes cleave the amide bond of the β-lactam ring thus inactivating the antibiotics.

The fundamental relationships of β-lactamase enzymes to one another are best reflected by the Ambler classification (and numbering) scheme, which is based upon amino acid sequence similarity, rather than by their phenotypic properties as defined

in the Bush-Jacoby-Medeiros classification system (Bush, Jacoby and Medeiros, 1995; Ambler, Coulson and Frere, 1991). The Bush's scheme classifies β-lactamases according to the substrate profiles, inhibitor profiles and physical characteristic such as molecular weight and isoelectric points (Bush, Jacoby and Medeiros, 1995). This divides the enzymes into four groups (1 through 4).

However, the classification scheme proposed by Ambler is also commonly used. β-lactamases can be divided into four evolutionary distinct molecular classes (A, B, C and D), each with distinct sequence motifs (Ambler, Coulson and Frere, 1991) as shown in Table 2-2.

Class A and class C β-lactamase are the most common and have a serine residue at the active site, as do class D β-lactamase. Class B comprises the metallo-β-lactamase (MBLs), requiring divalent cation, usually zinc, as metal cofactors for enzyme activity.

Table 2-2 Classification of β-lactamases (Ambler, Coulson and Frere, 1991).

Molecular class and functional mechanism	Types that are normally chromosomal and ubiquitous in species or group	Types that are normally plasmid-, transposon- or integron-mediated
Class A Serine β-lactamases	SHV-1, LEN-1 and K1 in *Klebsiella* spp.; chromosomal cefuroximases of *Proteus vulgaris*; chromosomal β-lactamases of *Bacteroides* spp.	Staphylococcal penicillinase; TEM, SHV, VEB, PER and CTX-M penicillinases and ESBLs; KPC, IMI/NMC and SME carbapenemases
Class B Metallo-β-lactamases	L1 enzyme of *Stenotrophomonas maltophilia*; chromosomal enzymes of some *Chryseobacterium* spp. and *Aeromonas* spp.; CcrA enzyme found in 1–3% of *Bacteroides fragilis* isolates.	IMP, VIM and SPM types
Class C Serine β-lactamases	Chromosomal AmpC enzymes of *Escherichia coli*, *Shigella* spp., *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp. and *Serratia* spp.	CMY-1, LAT-1, BIL, MOX, ACC, FOX and DHA types[a]
Class D Serine β-lactamases	Chromosomal (along with other β-lactamases) in *Acinetobacter* spp. (OXA-51-like); *P. aeruginosa* (OXA-50) and some *Aeromonas* spp. (e.g. OXA-12).	Most OXA types, excluding those detailed here as chromosomal

Carbapenemase (Classes A, B and D β-lactamase)

Carbapenemases are a diverse group of β-lactamase. In the Ambler functional classification scheme, carbapenemase are located in three class, namely class A such as KPC enzyme, class B (metallo- β-lactamases) and class D (OXA-type β-lactamases) as shown in Table2-3. They are currently uncommon, but are sources of considerable concern. They are active not only against oxyimino-cephalosporins and cephamycins but also against carbapenems (Nordmann and Poirel, 2002).

In 1989, Bush further classified metallo-β-lactamases into a separate group (group3) according to their functional properties and remain the recommended referencing system for β-lactamases generally (Bush, 1989).

Table 2-3 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), amino-penicillins (amoxicillin and ampi-cillin), carboxypenicillins (carbeni-cillin and ticarcillin), ureidopenicillin (piperacillin), narrow-spectrum cephalosporins (cefazolin, cepha-lothin, 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-cephalo-sporins (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 (ce-fotetan, cefoxitin, and others)	0	C
Carbapenemase	IMP family, VIM family, GIM-1, and SPM-1	Substrates of the expanded-spec-trum 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.

Based on molecular studies, two types of carbapenem-hydrolyzing enzymes have been described: serine enzymes possessing a serine moiety at the active site and metallo-β-lactamase (MBLs), requiring divalent cations, usually zinc, as metal cofactors for enzyme activity (Bush, Jacoby and Medeiros, 1995; Frere, 1995).

The serine carbapenemases are invariably derivatives of class A or class D β-lactamases and usually mediate carbapenem resistance in Enterobacteriaceae or *Acinetobacter spp*. OXA class D carbapenemases have been identified in *A. baumannii* collected in UK (OXA-23), Spain (OXA-24, 25 and 40), Belgium (OXA-26) and Singapore (OXA-27). These enzymes hydrolyze carbapenems poorly but are able to confer resistance and are only partially inhibited by clavulanic acid. A few class A enzymes, notably the plasmid-mediated KPC enzymes, are effective carbapenemases as well (Poirel et al., 2004). Clones of *K. pneumoniae* and *E. cloacae* with KPC enzymes have spreaded in multiple hospitals around NewYork since 2003, where they have presented severe treatment problems, causing up to 47% mortality (Aubron et al., 2005).

The first indication of metallo-β-lactamase was with the discovery of *P. aeruginosa* strain GN17203 in Japan in 1988. In 1999, an identical gene was found in *Serratia marcescens* strain Tn9106 isolated from a urinary tract infection at Aichi Hospital in Okazaki, Japan.

Metallo-β-lactamases (MBLs) are classified as Ambler class B and possess a very broad substrate profile, including expanded-spectrum cephalosporin and carbapenem. All MBLs hydrolyze imipenem, but their ability to achieve this varies considerably and the rate of hydrolysis may or may not correlate with the bacterium's level of resistance to carbapenems, in addition these enzymes are resistant to the inhibitory activity of clavulanic acid. MBLs possess a distinct set of amino acid that define the finite architecture of the active site which coordinates the zinc ions. The zinc ions in turn usually coordinate two water molecules necessary for hydrolysis. Thus, these enzymes possess the characteristic hallmark of being universally inhibited by EDTA as well as other chelating agents of divalent cations, a quint essential feature of MBLs that correlates with their mechanistic functions (Rasmussen and Bush, 1997).

The recently reported situation in Korea, where 14.2% of imipenem-resistant *A. baumannii* isolates produced MBLs, is a disturbing revelation. Moreover, this survey encompassed 28 hospitals and MBL-producing isolates were found in 60.7% of Korean hospitals (Lee et al., 2003).

4. Treatment of *Acinetobacter baumannii* infections

A. baumannii infections may pose treatment difficulty as nosocomial isolates are typically resistant to a wide variety of antimicrobials. This problem is compounded by the increasing rates of resistance to broad-spectrum antibiotics detected in *A. baumannii*). The carbapenems, imipenem and meropenem are amoung the drug of choice for the treatment of these multi-drug resistant *A. baumannii* infections. Despite the rising threat of multi-drug resistant *A. baumannii*, no new class of drugs has been introduced since the advent of imipenem in the early 1980s, and none are expected to appear for commercial use in the near future. New approaches are clearly required to prevent the propagation of drug resistant mutants.

Although metallo-enzyme inhibitors may be used *in vitro*, no metallo-β-lactamase inhibitors are available for treating patients. The association of other antibiotic molecules such as aminoglycosides may be limited due to the coresistance mechanisms.

Drug combination, most commonly those involving a β-lactam and either an aminoglycoside or fluoroquinolone, have long been considered to constitute optimal antibacterial treatment for *A. baumannii* infection. Theoretical advantages of combining two drugs with synergistic activity *in vitro* include enhanced clinical efficacy and the prevention of emergence of resistant strains.

Sulbactam is often used for the treatment of MDR *A. baumannii*, usually as ampicillin/sulbactam. Most studies have investigated only the ampicillin/sulbactam combination, since sulbactam alone is not available commercially in many countries. In 1996, a prospective observational study follow 79 patients with *A. baumannii* bacteraemia. Ampicillin-sulbactam was used in eight patients, with a cure rate of 88% (Cisneros, Reyes and Pachon, 1996). Corbella et al. treated 42 patients with non-life-threatening multi-drug resistant *A. baumannii* infections, including seven bacteraemias, with sulbactam alone and in combination with ampicillin (1 g every 8 h.); 39 improved or were cured with no major adverse effects. In this study, killing curves showed that sulbactam was bacteriostatic, and no synergy was observed between ampicillin and sulbactam (Corbella, et al., 2000). Unfortunately, emergence of resistance to sulbactam has been noted in imipenem-resistant strains of

A. baumannii, leaving the polymyxins (colistimethate and polymyxinB) as the only treatment alternative (Wood and Reboli, 1993). Colistin was used in the 1960s and 1970s, but was abandoned because of adverse side effects, and the discovery of the safer antimicrobials. In 2002, Jimenez-Mejias et al. reported a case of meningitis caused by multi-drug resistant *A. baumannii* which was treated successfully with intravenous colistin sulphomethate sodium (5 mg/µg/day) (Jimenez-Mejias et al., 2002).

5. Carbapenem (Imipenem)

The carbapenems are β-lactam antimicrobial agent with an exceptionally broad spectrum of activity. They have the broadest spectrum of activity within the β-lactam class and exhibit *in vitro* bactericidal activity against numerous pathogens, including Gram-positive and Gram-negative aerobes and anaerobes. Carbapenems are β-lactams that contain a fused β-lactam ring and a 5-membered ring system that differ from penicillins in having a carbon atom replacing the sulphur at position 1 and an unsaturated bond between C2 and C3 in the five membered ring structure. The broad spectrum of activity of carbapenems is associated with their intrinsic resistance to nearly all β-lactamase. This β-lactamase stability is due to the trans α-1 hydroxyethyl substituent at the 6 position of carbapenems; this is unique when compared with the side chains of penicillins and caphalosporins, which have cis configurations (Zhanel, Johanson and Embil, 2005).

The first carbapenem to be discovered was thienamycin in the mid 1970s, a compound produced by the soil organism *Streptomyces cattleya* (Zhanel, Johanson and Embil, 2005). The unstable nature of this molecule led to the development of an N-formimidoyl derivative called imipenem. Its chemical name is (5R,6S)-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, nonhygroscopic crystalline compound with a molecular weight of 317.37. It is sparingly soluble in water and slightly soluble in methanol. Its empirical formula is $C_{12}H_{17}N_3O_4S \cdot H_2O$, and its structure formula is:

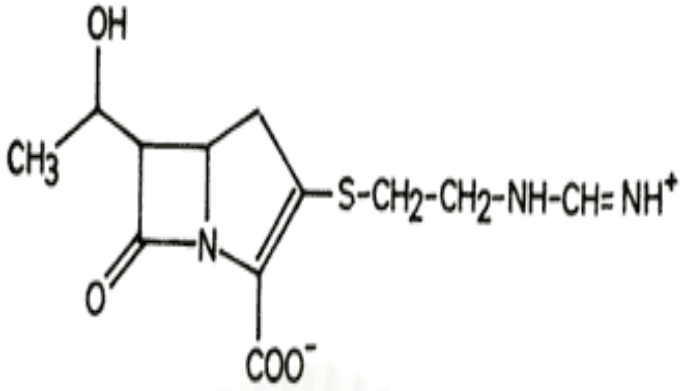

Figure 2-1 Structure of Imipenem

Imipenem is not absorbed orally. When given paranterally, it is degraded by a naturally occurring enzyme renal dehydropeptidase presents in the proximal renal tubules of mammals; therefore it is used in combination with cilastatin in 1:1 ratio. Cilastatin is the inhibitor of the enzyme dehydropeptidase and has no intrinsic antibacterial activity (Norrby, 1995).

The activity of imipenem is excellent *in vitro* for a wide variety of both Gram-positive and Gram-negative bacteria including *Haemophilus influenzae*, *Neisseria meningitis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and Enterobacteriaceae. It is not active against *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* and *Stenotrophomonas maltophilia* (Tsuji, Ishii and Chno, 1998).

Mechanism of action

Imipenem acts as an antimicrobial through inhibiting cell wall synthesis of various Gram positive and Gram negative bacteria. Imipenem must diffuse across the outer membrane of the Gram negative cell, using pores formed by porin proteins, and then cross the periplasm (which can contain any type of β-lactamase) before reaching its PBPs (penicillin-binding proteins) targets, which lie on the outer surface of the cytoplasmic membrane.

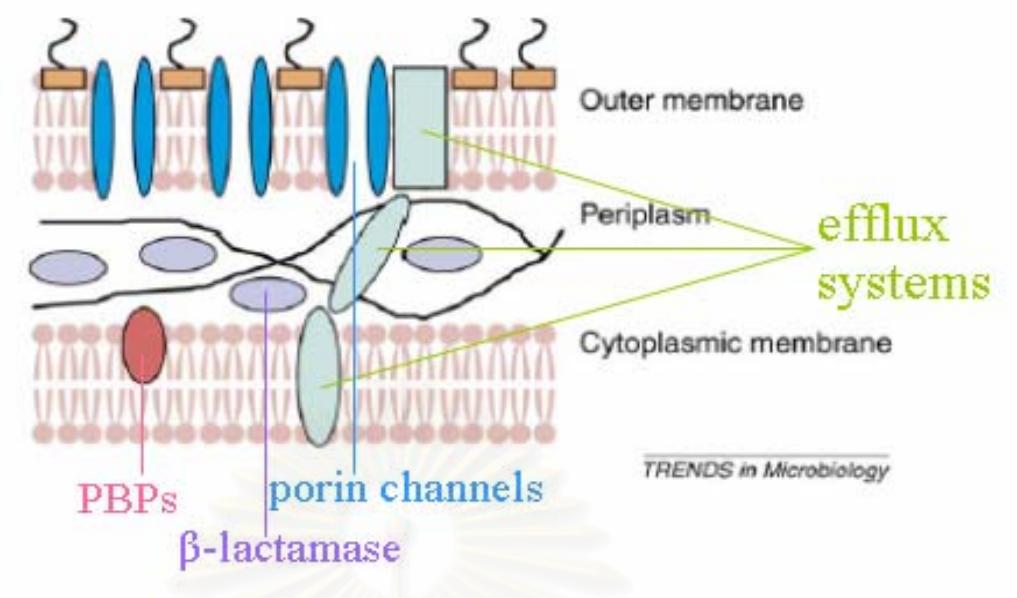

Figure 2-2 Diagram of the Gram-negative cell envelope across which β-lactams must diffuse to reach PBPs. The outer membrane is traversed by porin channels and exit portals for efflux systems; the periplasmcontains β-lactamase and linker proteins for efflux systems. The cytoplasmic membrane contains efflux pumps and the PBPs that are targeted by β-lactams (Modified from Livermore and Woodford, 2006).

Several distinct PBPs found in any bacterium are usually species specific and vary in their abilities to react with different β-lactam antibiotics. The binding of the β-lactam molecule to the PBPs prevents bacteria from completing transpeptidation (cross-linking) of peptidoglycan strands, thus preventing the synthesis of an weak bacteria cell wall cause death of the microorganisms.

The PBPs vary in their affinities for different β-lactam antibiotic binds, affects the morphologic response of the bacterium to the agent. In susceptible Gram-negative bacteria, imipenem binds preferentially to PBP2, followed by PBP1a and PBP1b and has weak affinity for PBP3 (Livermore, Sefton and Scott, 2003).

Dosage and administration

Imipenem/cilastatin administered by intravenous infusion 500 mg doses resulted in mean maximum plasma concentrations (C_{max}) at the end of infusion of 30-35 mg/l. Both imipenem and cilastatin have similar half-life of approximately 1 hour. In the presence of cilastatin, 60-70% of imipenem is excreted unchanged in the urine (Buckley, Brogden and Barradell, 1992).

6. Colistin (Polymyxin E)

Colistin is an old antimicrobial belonging to the polymyxin family. The polymyxins are cyclic basic polypeptide that consist of the five chemically different compounds (polymyxin A-E) and characterized by poor diffusibility, a molecular weight of approximately 1100. Only polymyxin B and polymyin E (also called colistin) are sufficiently non-toxic for therapeutic use and activity directed predominantly against Gram-negative aerobes. Colistin is a cyclic cation decapeptide linked to a fatty acid side chain and contain D-and L-amino acids, a heptapeptide ring, 2-4-diaminobytyric acid and a fatty acid attached through an amide bond (Katz and Demain, 1977; Storm, Rosenthal and Swanson, 1977).

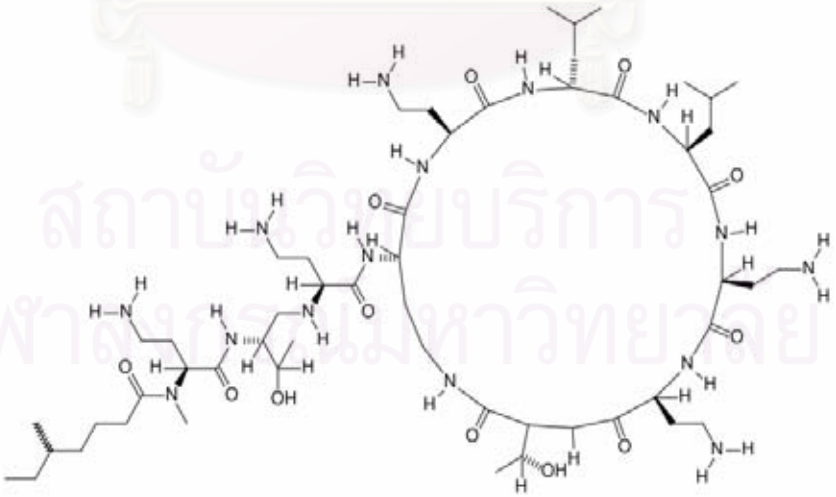

Figure 2-3 Structure of Colistin

Mechanism of action

The polymyxins are surface active amphipathic agent, which interact strongly with phospholipids within the cell membrane in the detergent like fashion to disrupt the structure of the cell membrane as shown in Figure 2-4 (Hoeprich, 1970; Evans, Feola and Rapp, 1999).

The initial association of colistin with the bacterial membrane occurs through interactions between the cationic polypeptide (colistin) and the anionic lipopolysaccharide within the outer membrane, after which it enters the periplasm of the cell and inserts into the cytoplasmic membrane. Colistin displaces magnesium and calcium (ion that normally stabilize the lipopolysaccharide molecule) from the negative changed lipopolysaccharide, leading to a loss of integrity of the membrane and an increase in the permeability of the cell envelope, leakage of cell content, and subsequently, cell death (Davis, Iannetta and Wedgewood, 1971; Schindler and Osborn, 1979).

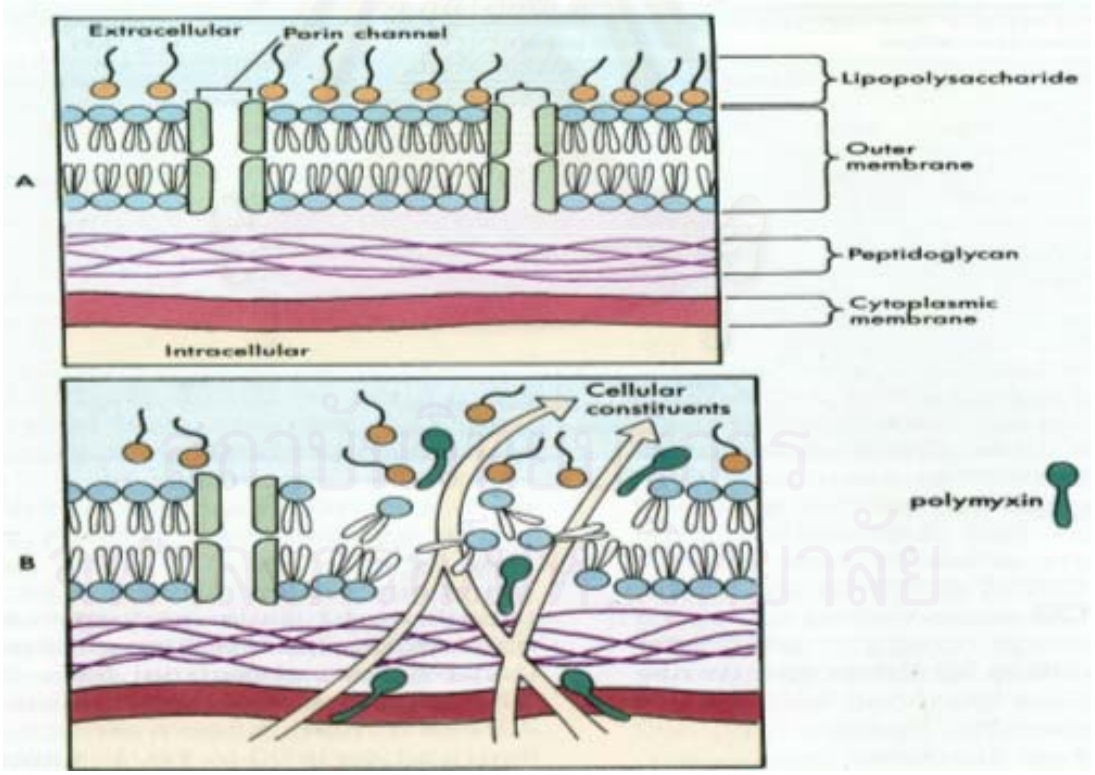

Figure 2-4 Mechanism of action of polymyxins. Microbial cell, A, absence and B, presence of polymyxin (Brody et al., 1994).

The actual killing may involve several targets in the cell, and it occurs within a very short time in the case of most polymyxin derivatives (in seconds to a few minutes) (Wu et al., 1999; Zhang et al., 2000). The bactericidal activity of colistin is due to a detergent effect on the cell membrane. Colistin must be hydrolyzed to release the active free base, which occurs at the body temperature and at a physiological pH in aqueous systems.

The pharmacologic action of colistin may account for the low levels of bacterial resistance to it. *In vitro,* colistin has shown excellent activity against a variety of Gram-negative, including those which are resistant to the other classes of antimicrobial.

Dosage and administration

Oral absorption is very poor, so the drug is usually given intramuscularly or intravenously. Dosage recommendations of colistin are based in limited data, and many pharmacokinetic and pharmacodynamic indices have not been studied. After intravenous or intramuscularly administration, it penetrates most tissues, but shows poor penetration of the blood brain barrier and excretion is mainly renal.

There are two forms of colistin available commercially: colistin (sulfate) mainly for topical use and colistin methanesulfonate (sodium) (also known as colistimethate sodium) for parenteral use. Both colistin sulfate and colistimethate sodium may be administered by nebulization. The basic chemistry, pharmacology, clinical applications, pharmacokinetics and pharmacodynamics of these two forms are different, and colistin methanesulfonate is a nonactive prodrug, and after perenteral administration, colistin is formed *in vitro* and *in vivo* (Li et al., 2001; Li et al., 2005).

In aqueous solutions, the colistin methanesulfonate is hydrolyzed and forms a complex mixture of partially sulfomethylates and forms a complex mixture of partially sulfomethylated derivatives and colistin (McMillan and Pattison, 1969).

The reported incidence of adverse reaction after colistin administration has generally limited the widespread use of the polymyxins (Falagas and Kasiakcu, 2005). However, colistin has increasingly been used for the treatment of infection caused by

multi-drug-resistant *Acinetobacter spp.* with relative success (Gernacho-Montero et al., 2003). In addition, Kallel et al showed that colistin therapy was clinically effective and safe in the treatment of nosocomial infection caused by multi-drug resistant *A. baumannii* (Kallel et al., 2006).

Recently the studies on MDR *A. baumannii* isolated in Thailand have been performed. *In vitro* activity of polymyxin B and colistin against 100 clinical isolates of multi-drug resistant *A. baumannii* collected from the patients hospitalized at Siriraj Hospital from 2002 and 2003 revealed that all isolates were susceptible to polymyxin B and colistin (Tribuddharat et al., 2003). In addition, in 2006 Koomanachai et al showed a good clinical outcome and less overall mortality in patients who received colistin for treatment of the multi-drug resistant *A. baumannii*. However, nephrotoxicity was also the found in 30.8% of the patients receiving colistin. Some patients in this group who developed nephrotoxicity also had other contributing factors. Thus, colistin was clamed to have safe and effective effect in the treatment of infections caused by multi-drug-resistant *A. baumannii* in Thai adult patients. (Koomanachai et al., 2007).

7. The study on the activity of the combined antimicrobial agents

Synergy is one of the most common reasons for using combination antimicrobial therapy. It is still unsettled as to which *in vitro* method could be the best predictor of the clinical outcome, because there has often been a discrepancy between the conclusions obtained using different tests. Empirical combination antimicrobial therapy is usually used to expand the antibacterial spectrum and to reduce the selection of resistant mutants during treatment and prevention of resistant subpopulations of bacteria. Antimicrobial synergism is defined as an interaction between two or more agents that results in an effect greater than expected from the sum of their independent effects. Conversely, combinations may be considered antagonistic if a combination of antimicrobials exerts an effect less than that observed when each agent is considered independently. Combinations of antimicrobials that are neither synergistic nor antagonistic may be termed indifferent if the agents appear

to work similarly alone or in combination, or additive if effect of a combination simply reflect addition of each of their respective activities.

The checkerboard method is the technique that has been used most frequently to assess antimicrobial combinations *in vitro*. The term “checkerboard” refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of two antimicrobials being tested, in combinations equal to, above and below their minimal inhibitory concentrations (MICs) for the organisms being tested. The concentrations tested for each antimicrobial typically range from four or five dilution below the MIC to twice the MIC (or higher if antagonism is suspected), using two fold dilutions of each antimicrobial. The dilutions of the antimicrobials being tested are usually performed in Mueller-Hinton broth or another suitable broth for bacterial studies, so that the drug-containing solutions can be mixed with drug-free medium to produce the final concentrations.

The bactericidal activity of antibiotics can be assessed *in vitro* by sequential sampling and counting viable bacteria in broth following the addition of the tested antimicrobial agent. This method is often termed the 'time-kill curve' and, if combinations of antibiotics are used, it is a recognized means of detecting *in vitro* synergy or antagonism between antimicrobial agents. Combination of two antimicrobials may increase or decrease the rate of killing relative to that observed with either antibiotic alone.

Sader et al demonstrated synergistic activity between cefepime and ampicillin/sulbactam among 30 of 34 partially resistant *Acinetobacter spp*. isolates, with no antagonistic interactions (Sader, Huynh and Jones, 2003). Chang et al found that imipenem plus amikacin demonstrated synergy against 36% of 22 isolateds of *A. baumannii* causing bacteremia and partial synergy against 50% of the isolates (Chang et al., 1995). Ko et al have demonstrated synergism of meropenem and sulbactam (the latter in 8 µg/ml) against a specific *A. baumannii* clone through a time kill study (Ko et al., 2004). Another study showed that synergy testing of meropenem and sulbactam performed by the checkerboard method with isolates having elevated MICs, especially resistant to one or the other agent, revealed synergism or partial synergism in 37 of 48 isolates (Kiffer et al., 2005).

Yoon et al showed that the double combinations of polymyxin B plus imipenem and polymyxin B plus rifampin against multi-drug resistant *A. baumannii* were bactericidal against seven out of eight isolates tested (Yoon et al., 2004). Timurkarynak et al showed the *in vitro* effect of non-traditional antimicrobials combination against multi-drug-resistant strains of *A. baumannii*. There has been a report on the combinations of colistin plus meropenem and colistin plus azithromycin were synergistic against three of these five strains each, the colistin plus doxycycline combination was partially synergistic against four of the *A. baumannii* strains (Timurkarynak et al., 2006). Therefore, investigations of combination therapy have become increasingly important as the prevalence of multi-drug-resistant pathogens in patients continues to rise. The use of such combinations has resulted in varying clinical outcomes (Gleeson, Petersen and Mascola, 2005; Saballs et al., 2006). Thus, reinforcing the need for caution when translating *in vitro* testing results to clinical practice.

CHAPTER III

MATERIAL & METHODS

1. Microorganisms

1.1 Clinical isolates

The bacterial isolates used throughout this study were 30 isolates of *Acinetobacter baumannii* which were clinically isolated from the patients at Siriraj Hospital between January and December 2006. Speciation was performed by using API 20 NE (Bio Merieux Inc.,France). RAPD was used for molecular typing of the strains as shown in Table A-6 in Appendices. *Escherichia coli* ATCC25922 was used as the control strain.

2. Chemicals

- Standard powders

Standard powder of colistin (Potency = 437.8 µg/mg) was kindly provided from Atlantic Pharmaceutical Co., Ltd, Thailand. Working standard solutions were prepared immediately prior to use, as specified by the manufacturers.

Imipenem and cilastatin for injection 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

Antimicrobial disks were cefepime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), rifampin (5 µg), piperacillin/tazobactam (100 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), imipenem (10 µg) and colistin (10 µg). All of the disks, which were purchased from BBL chemicals (USA) were used to determine inhibition zone.

-E-test strips

E-test strips containing cefepime (0.016-256µg/ml), amikacin (0.016-256 µg/ml), piperacillin/tazobactam (0.016-256 µg/ml), ciprofloxacin (0.002-32 µg/ml)

and imipenem (0.002-32 μg/ml) (AB BIODISK Solna, Sweden) were used for the determination of the minimum inhibitory concentration (MIC) of the tested.

3. Media and Reagents

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

- Tryptic Soy Agar (TSA) (BBL chemicals, USA) were used as the culture media for *A. baumannii* and *E. coli* ATCC25922.

- Sterile water was used as the solvent for the chemical powders.

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

4. Disk diffusion test

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 the other antimicrobial agents.

4.1 Preparation of Media

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

4.1.2 Immediately after autoclaving, allow it to cool in a 45 to 50^{o}C water bath.

4.1.3 Pour the freshly prepares and cooled medium into glass, flat-bottomed petri-dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 25 ml for plates with a diameter of 100 mm.

4.1.4 The agar medium should be allowed to cool at room temperature and all prepared plates must be examined sterility by incubating at 37^{o}C for 24 hours.

4.1.5 Unless the plates were used the same day, stored in a refrigerator (2 to 8^{o}C) and should be used within 7 days after preparation.

4.2 Inoculum Preparation

4.2.1 The well-isolates colony of each 18 hours *A. baumannii* from clinical specimen and *E. coli* ATCC25922 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 result in a suspension containing approximately 1 to 2 x 10^{8} CFU/ml.

4.3 Inoculation Test Plates

4.3.1 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab dipped into the adjusted suspension. The swab should be rotates several time and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.

4.3.2 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^{o} each time to ensure an even distribution of inoculum.

4.3.3 The lid may be left agar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the antibiotic disks.

4.4 Application of Disks to Inoculated Agar Plates

4.4.1 The antibiotic disks were applied to the surface of the medium with sterile forceps. Each disk must be pressed down to ensure complete contact with the agar surface. They must be distributed evenly so that they are no closer than 24 mm from center to center. Because some of the drugs diffuse almost instantaneously, a disk should not be relocates once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.

4.4.2 The plate were inverted and incubated at 37^{o}C for 24 hours before measuring the zones of inhibition.

4.5 Reading Plates and Interpreting Results

4.5.1 After 24 hours of incubation, each plate was examined. The resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. The diameters of zones of inhibition, including the diameter of the disk was measured with digital sliding venier caliper.

4.5.2 The size of the inhibition zone 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 (Tables3-1).

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Table 3-1 Zone diameter interpretive standards breakpoints for *A. baumannii* and *E. coli* ATCC 25922 (NCCLS, 2004).

Drug	Disk content	Zone diameter (mm)			
		A. baumannii			*E.coli*
		R[a]	I[b]	S[c]	ATCC 25922
Amikacin	30 μg	≤14	15-16	≥17	19-26
Gentamicin	10 μg	≤12	13-14	≥15	19-26
Tobramycin	10 μg	≤12	13-14	≥15	18-26
Ceftazidime	30 μg	≤14	15-17	≥18	25-32
Rifampin	5 μg	≤16	17-19	≥20	8-10
Ciprofloxacin	5 μg	≤15	16-20	≥21	30-40
Piperacillin/tazobactam	100/10 μg	≤17	18-20	≥21	24-30
Cefepime	30 μg	≤14	15-17	≥18	29-35
Imipenem	10 μg	≤13	14-15	≥16	26-32
Colistin	10 μg	≤8	9-10	≥11	11-15

[a]Resistant, [b]Intermediate, [c]Susceptible

5. MICs Determination by E-test method

E-test Method was performed according to CLSI, 2006. (*E. coli* ATCC25922 was also includes in this as the control strains). The minimal inhibitory concentration (MICs) of 5 broad-spectrum antibiotics (cefepime, amikacin, ciprofloxacin, imipenem and piperacillin/tazobactam) against all 30 isolates of *A. baumannii* were determined in order to screen for multi-drug resistant strain. The method was briefly described as follow;

5.1 Preparation of Media

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

5.1.2 Immediately after autoclaving, allow it to cool in a 45^{o} to 50^{o}C water bath.

5.1.3 Pour the freshly prepared and cooled medium into glass, flat-bottomed. Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 25 to 30 ml for plates with a diameter of 100 mm.

5.1.4 The agar medium should be allowed to cool to room temperature and all prepares plates must be examined sterility by incubating at 37^{o} C for 24 hours.

5.1.5 Unless the plates were used the same day, stored in a refrigerator (2 to 8^{o}C) and should be used within 7 days after preparation.

5.2 Inoculum Preparation

5.2.1 The well-isolates colony of each 18 hours *A. baumannii* from clinical specimen and *E. coli* ATCC25922 were selected from Tryptic soy agar (TSA) plates and transferred to a tube containing 7 ml normal saline solution (NSS).

5.2.2 The suspension was adjusted to match the turbidity of the 0.5 McFarland standard solution. This result in a suspension containing approximately 1 to 2 x 10^{8} CFU/ml.

5.3 Inoculation Test Plates

5.3.1 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab dipped into the inoculum suspension and remove excess fluid by pressing it against the inside wall of the test tube. Carefully streak the entire agar surface three times, rotating the plate approximately 90 degree each time to evenly distribute the inoculum. This will remove excess inoculum from the swab.

5.3.2 Allow excess moisture to be absorbed for about 10 to 15 minutes so that the surface is completely dry before applying the E-test strips.

5.4 Application of E-test strips to Inoculated Agar plates

5.4.1 Using forceps remove the required number of E-test strips and place them on dry clean surface.

5.4.2 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 cannot be moved because of instantaneous release of antibiotic into the agar.

5.4.3 The plate were inverted and incubated at 37^{o}C for 18 hours.

5.5 Reading Plates and Interpreting Results

5.5.1 The MICs were recorded as the lowest concentration of antimicrobial agent that completely inhibits growth. Read the MIC value where the edge of the inhibition ellipse intersects the side of the strip. When growth occurs along the entire strip i.e. no inhibition ellipse is seen, report the MIC as greater than (>) the higher value on the scale. When the inhibition ellipse is below the strip i.e. it does not intersect the strip, report the MIC as less than (<) the lowest value on the scale.

5.5.2 E-test generates MIC values from a continuous scale and can give results in between conventional two-fold dilutions. An E-test MIC value which falls between two-fold dilutions must be rounded up to the next upper two-fold value before categorization.

5.5.3 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) (CLSI, 2006)

Antibiotic	Minimum Inhibitory Concentration [MICs] (μg/ml)				
	A. baumannii			*E. coli*	*P .aeruginosa*
	S[a]	I[b]	R[c]	ATCC25922	ATCC27853
Imipenem	≤ 4	8	≥16	0.064-0.25	1.0-4.0
Amikacin	≤16	32	≥64	0.5-4.0	1.0-4.0
Ciprofloxacin	≤1	2	≥4	0.004-0.015	0.125-0.5
Piperaceillin/tazobactam	≤16	32-64	≥128	1.0-4.0	1.0-8.0
Cefepime	≤8	16	≥32	0.016-0.064	1.0-4.0

[a]Susceptible, [b]Intermediate, [c]Resistant

5.6 Screening for multi-drug resistant strains

An isolate was considered to be multi-drug resistant when E-test showed it to be resistant to three or more of the following broad-spectrum agents: cefepime, amikacin, ciprofloxacin, piperacillin/tazobactam and imipenem.

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

A double disk diffusion test was contructed for detection of metallo-beta lactamase-producing gram-negative bacteria. Two Kirby-Bauer disks containing ceftazidime and filter disk containing a metallo-β-lactamase inhibitor were used in this test. The EDTA disk were used in this study, because these agent have been reported to block metallo-β-lactamase (Payne et al., 1994). When the bacterium produces this enzyme, a distinct growth inhibitory zone appeared between the Kirby-Bauer disk containing ceftazidime and the filter disk containing EDTA.

6.1 Preparation of Media

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

6.1.2 Immediately after autoclaving, allow it to cool in a 45 to 50^{o}C water bath.

6.1.3 Pour the freshly prepared and cooled medium into glass, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 25 to 30 ml for plates with a diameter of 100 mm.

6.1.4 The agar medium should be allowed to cool to room temperature and all prepares plates must be examined sterility by incubating at 37^{o} C for 24 hours.

6.1.5 Unless the plates were used the same day, stored in a refrigerator (2 to 8^{o}C) and should be used within 7 days after preparation.

6.2 Inoculum Preparation

6.2.1 The well-isolates colony of each 18 hours imipenem resistant *A. baumannii* strains were selected from Tryptic soy agar (TSA) plates and transferred to a tube containing 7 ml normal saline solution (NSS).

6.2.2 The suspension was adjusted to match the turbidity of the 0.5 McFarland standard solution. This result in a suspension containing approximately 1 to 2 x 10^{8} CFU/ml.

6.3 Inoculation Test Plates

6.3.1 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab dipped into the inoculum suspension and remove excess fluid by pressing it against the inside wall of the test tube. Carefully streak the entire agar surface three times, rotating the plate approximately 90 degree each time to evenly distribute the inoculum. This will remove excess inoculum from the swab.

6.3.2 Allow excess moisture to be absorbed for about 10 to 15 minutes so that the surface is completely dry before applying the ceftazidime disk and EDTA disk.

6.4 Application of Disks to Inoculated Agar plates

6.4.1 Two commercially supplied Kirby-Beuer disks, each containing 30µg of cetazidime were then placed on the plates. The distance between the two ceftazideme disks was kept at about 4 to 5 cm, and filter disk was places

near one of the ceftazidime disks within a center-to-center distance of 1.0 to 2.5 cm.

6.4.2 5µl of 500mM EDTA was dropped to filter disk on the agar.

6.4.3 The plate were inverted and incubated at 37^{o}C for 18 hours.

6.5 Reading Plates and Interpreting Results

A positive result will show the growth-inhibitory zone between the two disks expanded as shown in Figure 3-1, while no change is evident around the two double disks containing ceftazidime with or without EDTA for negative result.

Figure 3-1 Assessment of metallo-β-lactamase with double disks technique.

7. Agar Dilution MIC of imipenem and colistin determinations (NCCLS, 2004)

Agar dilution method was performed according to NCCLS, 2004 in order to determine minimal inhibitory concentration (MIC) of imipenem and colistin against all tested isolates.

7.1 Preparation of agar dilution plates

7.1.1 The two-fold dilution of imipenem solution (0.03-256 µg/ml) and colistin solution (0.03-256 µg/ml) were prepared. Because final volume in each plate consisted of 2.5 ml of each dilution antimicrobial agent and 22.5 ml of MHA. Thus antimicrobial concentrations used in initial (stock)

solutions should be prepared ten-fold in greater than the desired final concentration.

7.1.2 MHA was prepared from a commercially available dehydrated base according to the manufacturer's instructions.

7.1.3 Immediately after autoclaving, allow it to cool in a 55^{o}C water bath and then pipetted 2.5 ml of each dilution into MHA 22.5 ml.

7.1.4 The agar and antimicrobial agent solution were mixed thoroughly and then pour into plates.

7.1.5 The agar dilution plates were allowed to solidity at room temperature, and used immediately.

7.2 Inoculum preparation

7.2.1 The well-isolates colony of each 18 hours *A. baumannii* from clinical specimen and *E. coli* ATCC25922 were selected from Tryptic soy agar (TSA) plates and transferred to a tube containing 7 ml normal saline solution (NSS).

7.2.2 The suspension was adjusted to match the turbidity of the 0.5 McFarland standard solution. This result in a suspension containing approximately 1 to 2 x 10^{8} CFU/ml.

7.2.3 The 200 µl-inoculum suspension was pipetted into inoculum replicators.

7.3 Inoculating agar dilution plates

7.3.1 The agar plates were marked for orientation of the inoculum spots.

7.3.2 A 1 µl. of each inoculum was applied to the agar surface by the use of an inocula-replicating device. The final inoculum on the agar will then be approximately 10^{4} CFU per spot.

7.3.3 A growth-control plate (no antimicrobial agent) was inoculated first and then, starting the lowest concentration, the plates containing the different concentrations were inoculated.

7.4 Incubating agar dilution plates

The inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots have been absorbed into the agar until the spots were dried, but no more than 30 minutes. The plates were inverted and incubated at 37^{o}C for 24 hours.

7.5 Determining agar dilution end points

7.5.1 The MICs were recorded as the lowest concentration of antimicrobial agent that completely inhibits growth, disregarding a single colony or a faint haze caused by the inoculum.

7.5.2 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) (NCCLS, 2004)

Drug	Minimum Inhibitory Concentration [MICs] (µg/ml)			
	A. baumannii			*E. coli*
	S[a]	I[b]	R[c]	ATCC25922
Imipenem	≤ 4	8	≥16	0.06-0.25
Colistin	<2	-	≥4	0.25-1

[a]Susceptible, [b]Intermediate, [c]Resistant

8. Combination effect of imipenem and colistin by Checkerboard Microdilution Panel Method

Checkerboard method was performed according to NCCLS, 2004; Moody, 2004. All isolates were test to determine the combination effect of imipenem and colistin. The concentrations tested for colistin were 0.06, 0.125, 0.25, 0.5, 1, 2 and 4 µg/ml and for imipenem were 0.03, 0.06, 0.125, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 µg/ml.

8.1 Preparing Test Broth

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

8.1.2 The medium concentrations used in the initial solutions were prepared four-fold in greater than the desired final concentration.

8.2 Preparing Diluted Antimicrobial Agents

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

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

8.3 Broth Dilution Testing

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

8.3.1 Optimally, within 15 minutes the adjusted inoculum suspension should be diluted in broth so that after inoculation, each tube contained approximately $5x10^5$ CFU/ml.

8.3.2 The final volume of 200 µl in each well consisted of 50 µl of MHB, 50 µl of broth for imipenem, 50 µl of broth for colistin and 50 µl of broth containing a suspension of the organism was obtained.

8.3.3 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.

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128

Imipenem

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

8.4 Reading plates and Interpreting Results

8.4.1 After 16-24 hours, each tube was examined to determine MIC, the MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes as detected by the unaided eye. The amount of growth in the tubes containing the antibiotic should be 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.

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

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

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

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

8.4.4 The fractional inhibitory concentration index (FICI) or ∑FIC for this combinations was calculates according to the following formula.

FIC index (∑FIC) = FIC of imipenem + FIC of colistin

8.4.5 FIC index results for each combination were defined as :

Synergy : decrease in the MIC of each agent was ≥ 4-fold (∑FIC ≤ 0.5).

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

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

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

Antagonist : increase in the MIC of both agents was ≥ 4-fold (∑FIC ≥ 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.

9. Determination of bactericidal activity of the combination between imipenem and colistin by time kill method

The antibacterial activity of the combination was performed according to time kill method by NCCLS, 1999. 15 isolates were test to determined bactericidal activity of the combination of imipenem plus colistin against multi-drug resistant *A. baumannii*. The selected drugs and bacteria in time kill method must be correlated with checkerboard method to define MICs as describe previously.

9.1 Imipenem concentration was prepared to 32µg/ml (AHFS Drugs, 2001) which referred to the mean serum concentrations of the drug at therapeutic dose and prepare concentration to 1/4 MIC and 1/16 MIC of colistin. Antimicrobial concentrations used in initial (stock) solutions were prepared ten fold greater than desired final concentration.

9.2 A 1 ml of each drug was pipetted into Mueller Hinton broth (MHB) for prepares working media before adding the standardized inoculum (final volume of working media = 9). As the result, there had been 6 groups were control (no antimicrobial agents), imipenem alone, colistin 1/16MIC alone, colistin 1/4MIC alone, imipenem combined with colistin 1/16MIC and imipenem combined with colistin 1/4MIC.

9.3 Inoculum which 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.

9.4 A 1 ml of inoculum was pipetted to working media and incubated at 37^{o}C in a shaking water bath.

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

9.6 A 0.5 ml of the collected sample was dilutes ten fold in NSS and 20 µl of each dilution was dropped on TSA plates which were then incubated at 37^{o}C for 18 hours.

9.7 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.

9.8 Killing curve were constructed by Microsoft Excel 2002 at each time interval. The $\log_{10}$ change of the viable cell counts compared to the starting inoculum was determined.

9.8.1 The results were analyzed by determining the number of strains which yield changes in the $\log_{10}$ 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 of it reduced the original inoculum size by

≥3 log10 CFU/ml (≥99.9% killing) at each of the time periods or bacteriostatic if the inoculum size was reduced by 0-3 $\log_{10}$ CFU/ml. The regrowth was defined as an increase of ≥2 log CFU/ml after ≥6 hours (Amterdam, 1996; Pankuch, Jacobs and Appelbaum, 1994).

9.8.2 The quantitative evaluation of antimicrobial effect was calculates as in the published article (Firsov et al., 1997).

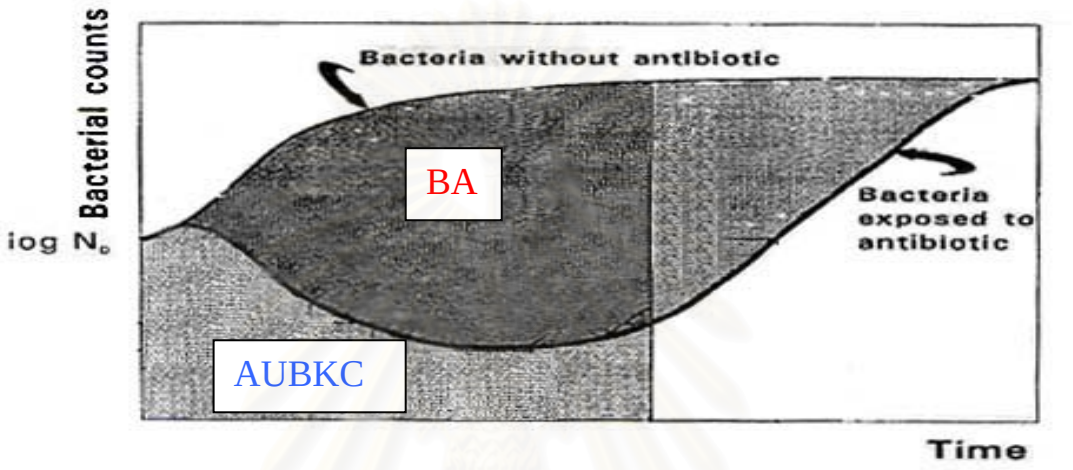

Figure 3-3 Parameters for quantifying bacterial killing and regrowth curve and the antimicrobial effect (Modified from Firsov et al., 1997).

The following parameters were calculated by various methodologies as follow:

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

Bacteriolytic area for 24 hours (BA24) = the area between control growth curve and the bacterial killing and regrowth curves ($AUBKC_{0-24}$ of the control growth curve substracted by $AUBKC_{0-24}$ of the bacterial killing and regrowth curves)

Statistic analysis

Student's t-test was used to compared the Log change of viable cell counts,$AUBKC_{0-24}$ and BA_{24}, which expressed their mean value (± SD) values. Any value of P below 0.05 was considered as significant.

10. Determination of the morphological cell structure change of *A. baumannii* 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 *A. baumannii* treat with imipenem, colistin and combination at the second hours by time kill method. Hence, the selected concentration of drugs and bacteria strain in scanning electron microscopy must be correlated with agar dilution method and time kill method to define MIC and detect for killing activities, respectively.

10.1 Imipenem concentration was prepared to 32µg/ml (AHFS Drugs, 2001) which referred to the mean serum concentrations of the drug at therapeutic dose and prepare concentration to 1/4 MIC of colistin. Antimicrobial concentrations used in initial (stock) solutions were prepared ten fold greater than desired final concentration.

10.2 A 1 ml of each drug was pipetted into Mueller Hinton broth (MHB) for prepares working media before adding the standardized inoculum (final volume of working media = 9). As the result, there had been 4 groups were control (no antimicrobial agents), imipenem alone, colistin 1/4MIC alone and imipenem combined with colistin 1/4MIC.

10.3 Inoculum which 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.

10.4 A 1 ml of inoculum was pipetted to working media and incubated at 37^{o}C in a shaking water bath.

10.5 Collect specimens at zero and second hours of exposure to detect the morphological changes.

10.6 The specimens were centrifuged at low speed centrifugation (3000 x g) for 10 minutes to change suspending bacterial cells to be sediments (This procedure conducted at 4^{o}C to keep bacteriostatic condition).

10.7 Fix specimens in 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.2 for 2 hours.

10.8 Rinse specimens twice in phosphate buffer for 5 min/each and once in distilled water for 10 minuate.

10.9 Dehydrate specimens with a graded series of ethanol (30%, 50%, 70%, 90% 5 min/each and absolute ethanol 3 times, 5 min/time).

10.10 Critical point dry (Critical point dryer, Balzer model CPD 020), mount and coat with gold (Sputter coater, Balzers model SCD 040).

10.11 Observe under a Scanning electron microscope (JEOL, model JSM-5410LV).

CHAPTER IV

RESULT

1. Susceptibility test

1.1 Disk diffusion test

From disk diffusion test according to NCCLS(2004), all *A. baumannii* isolate were resistant to imipenem, piperacillin/tazobactam and gentamicin, while 99.67%, 76.67%, 56.67% and 46.67% were resistant to ciprofloxacin, ceftazidime, rifampin and amikacin, respectively. For cefepime and tobramycin, 70% of the tested organisms were resistant to both of drugs. All *A. baumannii* were susceptible to colistin as shown in Table 4-1 and Table A-1 in Appendices.

Table 4-1 *In vitro* activity of imipenem, amikacin, ciprofloxacin, ceftazidime, cefepime, piperacillin/tazobactam, tobramycin, gentamicin, rifampin and colistin against 30 strains of *A. baumanniii* as tested by disk diffusion method.

Drug	No. of isolates (% susceptibility)		
	Resistant	Intermediate	Susceptible
Amikacin	14 (46.67)	9 (30)	7 (23.33)
Cefepime	21 (70)	5 (16.67)	4 (13.33)
Ceftazidime	23 (76.67)	4 (13.33)	3 (10)
Ciprofloxacin	29 (96.67)	1 (3.33)	0
Colistin	0	0	30 (100)
Gentamicin	30 (100)	0	0
Imipenem	30 (100)	0	0
Piperacillin/tazobactam	30 (100)	0	0
Rifampin	17 (56.67)	12 (40)	1 (3.33)
Tobramycin	21 (70)	4 (13.33)	5 (16.67)

Fifty percent of strains were defined by resistance to all antimicrobial agents except colistin. One out of 30 strains (3.33%) were resistant to 6 drugs, 3 strains (10%) to 7 drugs, 11strains (36.67%) to 8 drugs and 15 strains (50%) to 9 drugs. The number of bacterial strains which were resistant to at least 6 antimicrobial agents were shown in Table 4-2.

Table 4-2 Number of resistant bacterial strains to at least 6 antimicrobial agents

No. of antimicrobial agents	No. of resistant strains (%)
6	1/30 (3.33%)
7	3/30 (10%)
8	11/30 (36.67%)
9	15/30 (50%)

1.2 MICs Determination by E-test method

The MIC_{50}, MIC_{90} of imipenem, ciprofloxacin, piperacillin/tazobactam, cefepime and amikacin against all 30 strains of *A. baumannii* were shown in Table 4-3. The MICs of imipenem, ciprofloxacin and piperacillin/tazobactam indicated that all *A. baumannii* strains were resistant to these 3 agents. The MIC_{50} and MIC_{90} of imipenem as well as those of ciprofloxacin were the same (>32 µg/ml) while the MIC_{50} and MIC_{90} of piperacillin/tazobctam were >256 µg/ml. In addition, only 1 strain (3.33%) of the tested pathogens was susceptible to cefepime and 11 strains (36.67%) were susceptible to amikacin. The MIC_{50} and MIC_{90} of cefepime were 32 and >256 µg/ml while, MIC_{50}, MIC_{90} of amikacin were 24 and 256 µg/ml, respectively.

One strain of *A. baumannii* (3.33%) were resistant to amikacin, imipenem, ciprofloxacin and piperacillin/tazobactam, 11 strains (36.67%) were resistant to imipenem, cefepime, ciprofloxacin and piperacillin/tazobactam and 18 strains (60%) were resistant to the five tested antimicrobials including; imipenem, cefepime, ciprofloxacin, piperacilllin/tazobactam and amikacin. (Raw data of susceptibility testing by disk diffusion method and E-test method were shown in Table A-2 in Appendices.)

Table 4-3 MIC_{50} and MIC_{90} of imipenem, ciprofloxacin, piperacillin/tazobactam, cefepime and amikacin against 30 strains of *A. baumannii* and percentage of susceptibility determined by E-test method.

	Imipenem	Ciprofloxacin	Pip/tazo	Cefepime	Amikacin
MIC_{50} (µg/ml)	>32	>32	>256	32	24
MIC_{90} (µg/ml)	>32	>32	>256	>256	256
S (%)	0	0	0	3.33	36.67
I (%)	0	0	0	33.33	46.67
R (%)	100	100	100	63.33	16.67

R= resistant, I= intermediate, S= susceptible

It was shown that all *A. baumannii* tested strains were considered to be the multi-drug resistant because they were resistant to three or more of the broad-spectrum antimicrobial agents. The distribution of multi-drug resistant strains of *A. baumannii* according to the number of antibiotics to which they were resistant was shown in Figure 4-1.

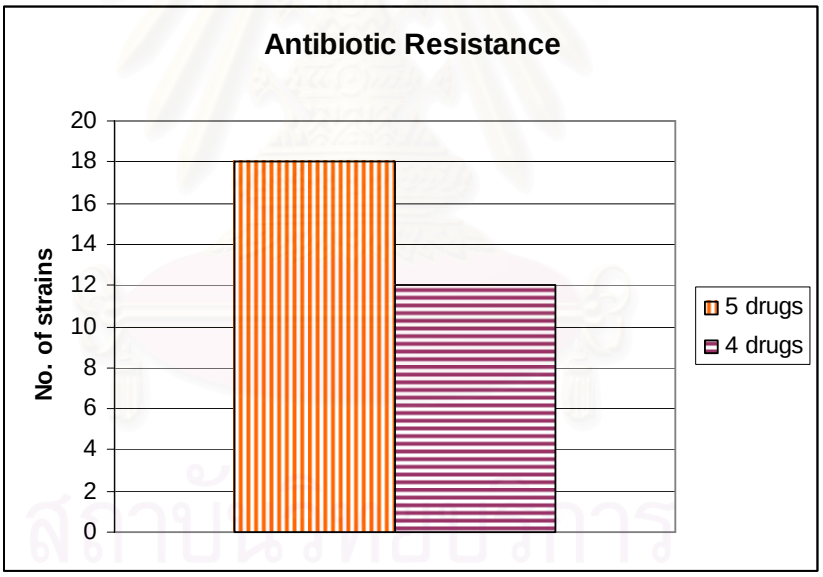

Figure 4-1 Distribution of the multi-drug resistant strains of *A. baumannii* according to the number of antibiotics.

Detection of Metallo-β-lactamase activity.

From double disk synergy test, all 30 strains of imipenem-resistant *A. baumannii* presence a negative reaction indicated that all the tested microorganisms do not produce metallo-β- lactamase.

1.3 MIC determination by Agar dilution method

The range of MICs observed, as well as the MIC_{50}, MIC_{90} and percentage of susceptible strains of imipenem and colistin among the 30 strains were shown in Table 4-4. All strains were resistant to imipenem. MICs of imipenem ranged from 8 to128 µg/ml (susceptibility breakpoint ≤ 4µg/ml). The MIC_{50} and MIC_{90} of imipenem were 32 and 64µg/ml, respectively. While, all strains were susceptible to colistin. MICs of colistin ranged from 0.5-2 µg/ml (susceptibility breakpoint ≤ 2 µg/ml). The MIC_{50} and MIC_{90} colistin were 1 and 2 µg/ml, respectively. Figure 4-2 to 4-3 showed assessment MICs against *A. baumannii* by agar dilution method of imipenem and colistin.

Table 4-4 *A. baumanniii* isolate (n = 30) MIC distribution (%) with MIC_{50} and MIC_{90} for imipenem and colistin by Agar dilution method

	Imipenem			Colistin	
	MIC range	% (no. of strains)		MIC range	% (no. of strains)
	0.5	0		0.5	3.33 (1)
	1	0	MIC_{50}	1	53.33 (16)
	2	0	MIC_{90}	2	43.33 (13)
	4	0		4	0
	8	3.33 (1)		8	0
	16	13.33 (4)		16	0
MIC_{50}	32	66.67 (20)		32	0
MIC_{90}	64	13.33 (4)		64	0
	128	3.33 (1)		128	0
	>256	0		>256	0

* unit of MIC = µg/ml.

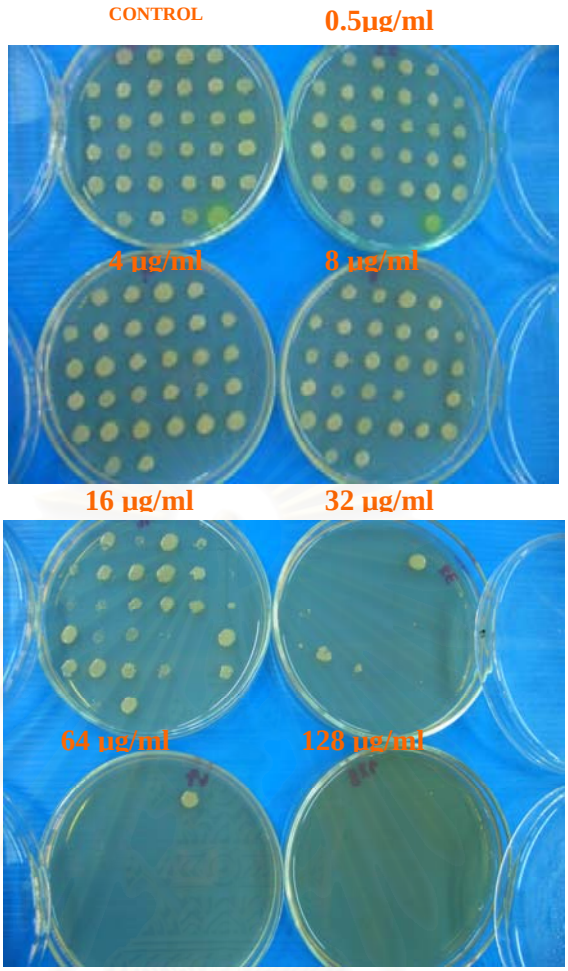

Figure 4-2 Assessment of MICs of imipenem against 30 strains of *A. baumannii* by agar dilution method.

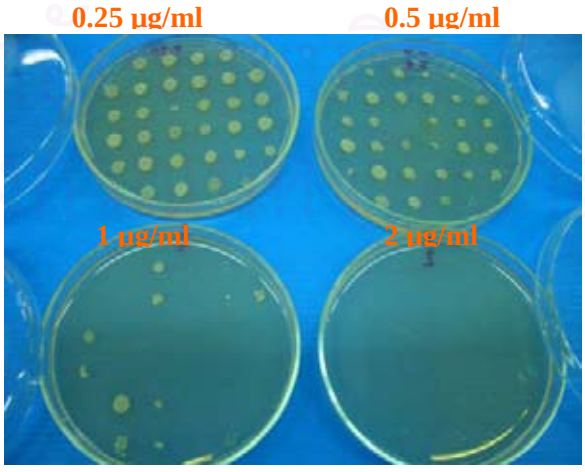

Figure 4-3 Assessment of MICs of colistin against 30 strains of *A. baumannii* by agar dilution method.

5. Synergist test (Raw data of checkerboard were shown in Figure A-1 to A-30 of imipenem plus colistin in Appendices.)

Checkerboard method was used to assess the MIC and the synergistic activity of two antimicrobial agent combinations against 30 strains of multi-drug resistant *A. baumannii*. The synergistic interactions between imipenem plus colistin in this study were not only assessed from the MIC value but also were evaluated from the fractional inhibitory concentration (FIC) index that were modified from checkerboard result as described in chapter III (method section).

The FIC index calculated from imipenem-colistin combination to 30 strains *A. baumannii* were between 0.0319-0.750 as shown in Table 4-6. The combination of imipenem plus colistin showed the synergistic effect in 27 strains (90%) and the partial synergistic effect in 3 strains(10%), strain no.14, 16 and36 [FIC index interpretive as followed: FICI $\leq$ 0.5, synergistic; > 0.5 FICI < 1, partially synergistic; FIC = 1, additive; > 1 FICI < 4, indifferent; and FICI $\geq$ 4, antagonistic]. The antibacterial effect of the combination of imipenem plus colistin against 30 strains of multi-drug resistant *A. baumannii* tested by checkerboard method were shown in Table 4-5.

Table 4-5 Effect of the combination of imipenem and colistin against 30 strains of multi-drug resistance *A.baumannii*.

Effect	Combination [number (%) of isolates]
Synergism	27 (90%)
Partial synergy	3 (10%)
Additive	-
Indifference	-
Antagonist	-

Table 4-6 Results obtained with antibiotic combination and susceptibility testing by checkerboard method.

Strain no.	Imipenem		Colistin		FIC index	Interprete
	MIC	Interprete	MIC	Interprete		
2	64	R	2	S	0.1870	Synergy
6	32	R	1	S	0.1500	Synergy
7	64	R	1	S	0.5000	Synergy
8	32	R	1	S	0.0678	Synergy
9	64	R	1	S	0.0639	Synergy
11	64	R	2	S	0.0339	Synergy
14	64	R	1	S	0.7500	Partial Syn
15	64	R	2	S	0.1270	Synergy
16	32	R	1	S	0.6250	Partial Syn
18	64	R	2	S	0.0925	Synergy
19	32	R	2	S	0.0925	Synergy
20	32	R	1	S	0.3750	Synergy
22	32	R	1	S	0.1278	Synergy
23	16	R	1	S	0.0756	Synergy
25	32	R	1	S	0.0756	Synergy
27	64	R	1	S	0.0756	Synergy
28	64	R	1	S	0.3750	Synergy
29	16	R	1	S	0.1356	Synergy
30	32	R	1	S	0.0910	Synergy
31	16	R	2	S	0.0675	Synergy
32	16	R	2	S	0.0756	Synergy
34	64	R	1	S	0.3700	Synergy
35	64	R	2	S	0.5000	Synergy
36	32	R	2	S	0.6250	Partial Syn
37	64	R	1	S	0.0639	Synergy
38	32	R	1	S	0.5000	Synergy
39	32	R	1	S	0.0638	Synergy
40	64	R	1	S	0.1225	Synergy
41	64	R	1	S	0.0639	Synergy
42	64	R	2	S	0.0319	Synergy

R = resistant, I = intermediate, S = susceptible ; Partial syn = Partial synergy

6. Time kill studies (Raw data were shown in Appendices Table A-4 to A-5.)

The bactericidal activity of combination of imipenem plus colistin against 15 strains of multi-drug resistant *A. baumannii* tested by time kill method. To be selected for this study, the concentration was equal to the mean serum level of imipenem at the therapeutic dose and concentration of colistin were 1/16MIC and1/4MIC, cause the checkerboard study showed the majority of 15 strains tested

resulted in synergism with concentration of colistin 1/16MIC to 1/4MIC combine with concentration of imipenem were 8-16 µg/ml. In addition, random 15 strains of MDR *A. baumannii* had to present synergistic effect (strain no.8, 9, 11, 19, 22, 23, 25, 27, 29, 30 32, 37, 39, 41 and 42), these strains must have been not clonally related at all (previously identification with a genotypic method).

The mean $\log_{10}$ decrease of viable cell count and bacteriolytic area for 24 hours (BA_{24}) by the combination of imipenem plus colistin were shown in Figure 4-4 and Table 4-6.

Imipenem alone was shown to have bacteriostatic activity during the 8 hour of growth. 1/16MIC and 1/4MIC of colistin alone showed no antibacterial activity during the time of study. The combination of imipenem plus 1/16MIC of colistin were shown bacteriostatic activity during the time of study but bactericidal activity was observed when combined of imipenem plus 1/4MIC of colistin at 24 hour of growth (Table 4-7).

The amount of bacteria killed (BA_{24}) by the combination of imipenem plus 1/16 MIC of colistin were significantly higher than those killed by 1/16MIC of colistin alone but not significantly higher than those killed by imipenem alone while the amount killed by the combination of imipenem plus 1/4MIC of colistin were significantly higher than those kill by 1/4MIC of colistin alone, imipenem alone and the combination of imipenem plus 1/16MIC of colistin (Table 4-7).

Number of the strains killed at various time intervals and the amount of bacteria killed were shown in Table 4-8. Imipenem alone shown 90% killing (≥1 log CFU/ml were reduced) in 1 strain (6.67%), 6 strains (40%), 3strains (20%) and 2 strains (13.33%) at 2, 4, 6, and 24 hour of growth, respectively. The 99% killing (≥2 log CFU/ml were reduced) 1 strain (6.67%), 5 strains (33.33%), 6 strains (40%), 7 strains (46.67%) and 3 strains (20%) at 2, 4, 6, 8 and 24 hour of growth, respectively. While 99.9% killing (≥3 log CFU/ml were reduced)1 strain (strain no.29) was observed at 8 hour of growth but regrowth of 7 strains (strain no.8, 9, 22, 23, 27, 37 and 42) were observed at 24 hours.

Colistin 1/16MIC alone showed no antibacterial activity in all strains tested and regrowth of 15 strains (100%) were observed at 24 hours.

Colistin 1/4MIC alone showed only 90% killing (≥1 log CFU/ml were reduced) against 1 strain (strain no.11), 2 strains (strain no.11 and 32) and 1 strain (strain no.32) at 4, 6 and 8 hours of growth, respectively but regrowth all strains were observed at 24 hours (Table 4-8).

The combination of imipenem plus 1/16MIC of colistin showed 90% killing in 5 strain (33.33%), 7 strains (46.67%), 6 strains (40%), 4 strains (26.67%) and 2 strains (13.33%) at 2, 4, 6, 8, 24 hour of growth, respectively. The 99% killing were observed in 3 strains (20%), 6 strains (40%), 7strains (46.67%), 7strains (46.67%) and 4 strains (26.67%) at 2, 4, 6, 8, 24 hour of growth, respectively. While 99.9% killing (≥3 log CFU/ml were reduced) against 2 strains (13.33%) were observed at 8 hour of growth and against 4 strains (26.67%) at 24 hour of growth. In addition, regrowth of 4 strains (26.67%) [strain no.9, 22, 23 and 37] were observed at 24 hours (Table4-8).

The combination of imipenem plus 1/4MIC of colistin showed 90% killing were observed in 9 strains (60%), against 7 strains (46.67%), 2 strains (13.33%), 2 strains (13.33%) and 1 strain (6.67%) at 2, 4, 6, 8, 24 hour of growth, respectively. The 99% killing were observed in 2 strains (13.33%), 7 strains (46.67%), 9 strains (60%), 8 strains (53.33%) and 2 strains (13.33%) at 2, 4, 6, 8, 24 hour of growth, respectively. While 99.9% killing were observed in 1 strain (6.67%), 1 strain (6.67%), 4 strains (26.67%), 4 strains (26.67%) and against 10 strains (66.67%) at 2, 4, 6, 8 and 24 hours, respectively. However, regrowth of 2 strains (13.33%) [strain no.22 and 23] were observed at 24 hours (Table4-8).

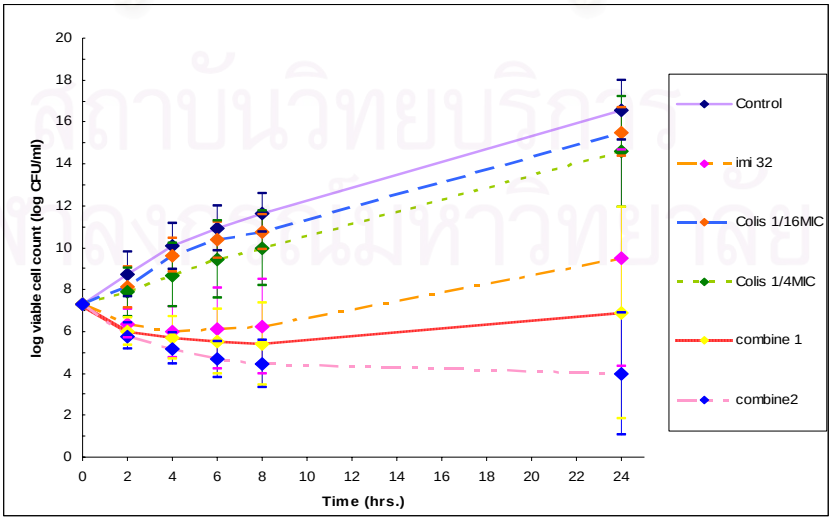

Figure 4-4 Time kill curve showing the antibacterial activity of the combination of imipenem plus colistin against 15 strains multi-drug resistant *A. baumannii*.

Table 4-7 Mean log change viable counts at various time interval, $AUBKC_{0-24}$ and BA_{24} in 15 strains of MDR *A. baumannii*.

Condition	Mean(±SD) log change viable cell counts					Mean(±SD) $AUBKC_{0-24}$	Mean(±SD) BA_{24}
	Δ2	Δ4	Δ6	Δ8	Δ24		
Control	1.40±0.59	2.74±0.95	3.61±0.99	4.34±0.92	9.24±1.29	304.08±24.63	-
Imipenem [32]	-0.91±0.60	-1.31±1.11	-1.15±0.76	-1.05±1.99	2.23±4.89	176.42±64.94	127.66±58.61
Colistin 1/16	0.82±0.64	2.34±0.77	3.09±0.76	3.48±0.83	8.24±1.15	284.38±19.03	19.70±10.75
Colistin1/4	0.58±0.82	1.40±1.23	2.14±1.56	2.69±1.54	7.31±2.36	265.82±44.18	38.27±33.74
Imi+Colis1/16	-1.32±0.77	-1.61±0.95	-1.79±1.28	-1.89±1.66	-0.40±4.65	145.99±61.67	158.12±55.12 [a ,b]
Imi+Colis1/4	-1.54±0.74	-2.12±0.81	-2.63±0.80	-2.87±0.98	-3.34±2.71	110.36±35.07	193.72±36.81 [c, d, e]

[a]p> 0.05 compared to activity of imipenem alone , [b] p< 0.05 compared to activity of colistin 1/16MIC
[c]p< 0.05 compared to activity of imipenem alone , [d] p< 0.05 compared to activity of colistin 1/4MIC
[e] p< 0.05 compared to activity of the combination of imipenem plus colistin 1/16MIC
Δ = Mean log change viable cell counts at 2, 4, 6, 8 and 24 hours, respectively
$AUBKC_{0-24}$ = Area under bacterial killing and regrowth curves for 24 hours
BA_{24} = Bacterolytic area for 24 hours

Table 4-8 Reduction of *A. baumannii* viable cell counts at various time intervals.

Antimicrobial agent	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
Imipenem [32]	1	1	-	6	5	-	3	6	-	-	7	1	-	2	3	1	7
Colistin1/16MIC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
Colistin1/4MIC	-	-	-	1	-	-	2	-	-	1	-	-	-	-	-	-	15
Imi+Colis1/16	5	3	-	7	6	-	6	7	-	4	7	2	-	2	4	4	4
Imi+Colis1/4	9	2	1	7	7	1	2	9	4	2	8	4	-	1	2	10	2

(-1 = 90% of viable reduction versus initial inoculum; -2 = 99% of viable reduction versus initial inoculum; -3 , -4 = 99.9 % of viable reduction versus initial inoculum, R= regrowth)
Imi = imipenem, Colis = colistin

Antibacterial activities were observed from the time kill study (Figure 4-4). The comparative activities between the combinations of various MIC levels of colistin and imipenem were summarized as followed.

1. Imipenem alone and the combination of imipenem plus colistin 1/16MIC

The number of bacteria killed by the combination of imipenem plus colistin 1/16MIC [BA_{24} = 158.12 log CFU/ml·h] were not significantly higher than the number killed by imipenem alone [BA_{24} = 127.66 log CFU/ml·h] ($p>0.05$) as shown in Table 4-7. However, the number of strains showed bacteriostatic activity during the time of study by combination of imipenem plus colistin 1/16MIC were higher than

the number of strains showed bacteriostatic activity by imipenem alone. In addition, the number of strains killed to the level of ≥3 log CFU/ml at 8 hour by the combination of imipenem plus 1/16MIC of colistin (2 strains, strain no. 29 and 42) were higher than those killed by imipenem alone (1 strain, strain no.29). The number of strains killed to the level of ≥3 log CFU/ml at 24 hour by the combination of imipenem plus 1/16MIC of colistin (4 strains, strain no.11, 19, 39 and 42) were higher than those killed by imipenem alone (1 strain, strain no.19) and the number of bacteria regrowth by imipenem alone were 7 strains (strain no.8, 9, 22, 23, 27, 37 and 42) whereas, combination of imipenem and colistin1/16MIC were 4 strains (strain no.9, 22, 23 and 37) at 24 hour of growth. The time that cells were reduced to the level of ≥3 log CFU/ml by the combination of imipenem plus 1/16MIC of colistin (2 strains, at 8 hours) were similar to the killing time by imipenem alone (1 strain, at 8 hour) [Table 4-8].

2. Imipenem alone and the combination of imipenem plus colistin 1/4 MIC

The number of bacteria killed by the combination of imipenem plus colistin 1/4MIC [BA_{24} = 193.72 log CFU/ml·h] were significantly higher than the number killed by imipenem alone [BA_{24} = 127.66 log CFU/ml·h] ($p<0.05$) as shown in Table 4-7. In addition, the number of strains killed to the level of ≥3 log CFU/ml at 24 hour by the combination of imipenem plus colistin1/4MIC (10 strains, strain no. 9, 11, 19, 25, 27, 29, 37, 39, 41 and 42) were higher than these killed by imipenem alone (1 strain, strain no. 19) and the number of bacteria regrowth by imipenem alone were 7 strains (strain no.8, 9, 22, 23, 27, 37 and 42) whereas, combination of imipenem and colistin 1/4 MIC were 2 strains (strain no. 22 and 23) at 24 hour of growth. The time that cells were reduced to the level of ≥3 log CFU/ml by the combination of imipenem plus colistin 1/4 MIC (1 strain, at 2 hour) were faster than the killing time by imipenem alone(1strain, at 8 hour) [Table 4-8].

3. Combination of imipenem plus colistin 1/16 MIC and combination of imipenem plus colistin 1/4 MIC

The number of bacteria killed by the combination of imipenem plus colistin1/16MIC [BA_{24} = 158.12logCFU/ml·h] were significantly higher than the

number killed by the combination of imipenem plus colistin 1/4 MIC [BA_{24} = 193.72 log CFU/ml·h] ($p<0.05$) as shown in Table 4-7. The number of strains killed to the level of ≥3 log CFU/ml by combination of imipenem plus colistin 1/16MIC (13.33% at 8 hour and 26.67% at 24 hour) was shown in Table 4-8. The time that cells were reduced to the level of ≥3 log CFU/ml by the combination of imipenem plus colistin 1/4MIC (1 strain at 2 hour) were faster than the killing time by the combination of imipenem plus colistin 1/16MIC (2strains at 8 hour) [Table4-8].

7. Determination of the morphological cell structure change of *A. baumannii* by scanning electron microscope.

The morphological changes of the multi-drug resistant *A. baumannii* strain no.29 after the exposure to imipenem at 32 µg/ml and 1/4MIC of colistin alone and in combination for 2 hour are shown in Figure 4-5 to 4-6. These observations were made under an scanning electron microscope. As shown for control cell (Figure 4-5A) had a much smoother surface. No morphological change could be noted after the cells were incubated for 2 hour with colistin alone (Figure 4-5B) which was the same as that observed without antibiotic (control cell). Imipenem alone exhibited moderate morphological alteration and cell wall destruction was observed (Figure 4-5C). When the cells were exposed to imipenem in combination with colistin, roughly spherical surface outpouchings became obvious and produced numerous protrusion on the surface of cell and some of this particular material appeared to be released from the cell (Figure 4-5D). After that, cell lysis was observed from the sites considered to be fragile (Figure 4-6).

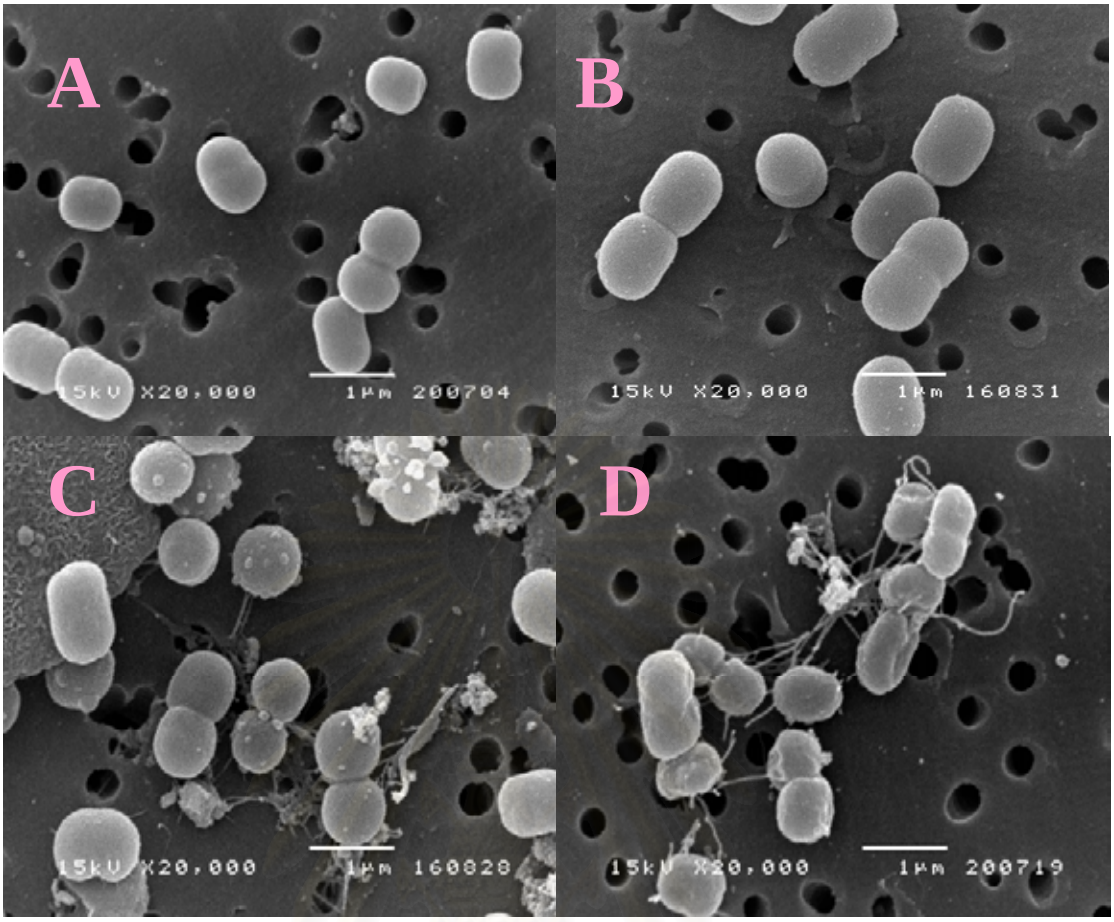

Figure 4-5 Scanning electron micrographs of *A. baumannii* strain no.29 exposed for 2 hours (A) no antibiotic, (B) after exposure to colistin 1/4MIC [0.25µg/ml], (C) imipenem [32µg/ml] and (D) combination of imipenem plus colistin. Each bar indicates 1µm.

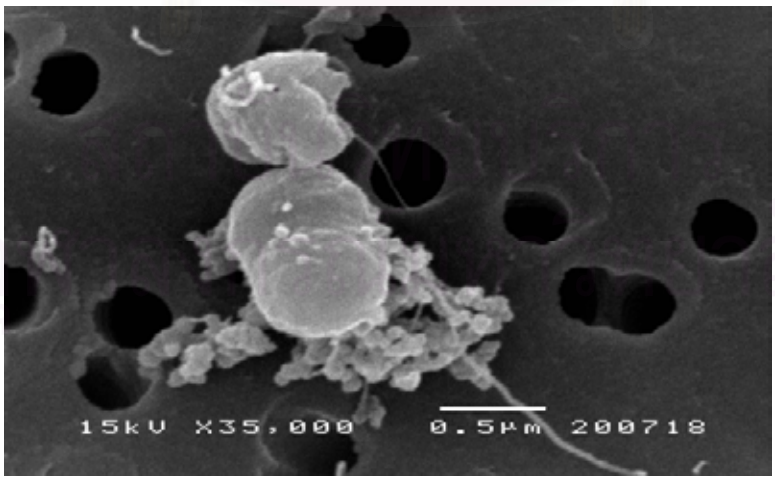

Figure 4-6 Scanning electron micrographs of *A. baumannii* strain no.29 exposed for 2 hours after exposure to combination of imipenem plus colistin. Each bar indicates 0.5µm.

CHAPTER V

DISCUSSION & CONCLUSION

The emergence and rapid spread of multi-drug resistant *A. baumannii* strains are of a great concern worldwide. Imipenem was one of the most potent agents for treatment of those infections caused by multi-resistant strains. The increasing prevalence of imipenem resistance limits therapeutic options and leads to outbreaks of carbapenems resistant strains.

Our result showed that all 30 strains of *A. baumannii* were resistant to imipenem by the disk diffusion method, E-test method and agar dilution method. In addition, the study revealed that very few antimicrobial agents were effective for *A. baumannii*. Even the wildly used agents like amikacin, ceftazidime, cefepime, ciprofloxacin and piperacillin/tazobactam were not active. This problem may be due to multi-drug resistant (MDR) nature of the organism. It is known that *A. baumannii* produced several enzymes (beta-lactamases, aminoglycoside-modifying enzymes) and these strains may use the resistant mechanisms such as porin reduction, alternation of PBPs and multi-drug efflux pump.

In our study, metallo-β-lactamase could not be detected in all imipenem-resistant *A. baumannii* strains indicating that these strains might use the other resistant mechanisms to imipenem such as production of OXA carbapenemase, alteration of PBPs and multi-drug efflux pump. However, the result of this study showed that colistin remained the most active drug against 30 strains of *A. bauamnnii*. No colistin resistant strains were detected, with MICs ranging from 0.5-2 μg/ml (MIC_{50}, 1 μg/ml and MIC_{90}, 2 μg/ml). Whereas MIC_{50} and MIC_{90} to imipenem were high (32 and 64 μg/ml, respectively).

Use of colistin as parenteral therapy has been limited because of its poor pharmacokinetics and nephrotoxicity. Therefore, the use of polymyxin has been unpopular over the past several decades when other safer anti-Gram negative bacteria have been available. The reported incidence of adverse reactions after colistin administration has generally limited the widespread use of the polymyxins (Falagas and Kasiakcu, 2005). However, several reports published during the period 1999 to 2003 revealed that colistin were effective and safe for treatment of patients infected

with MDR Gram-negative bacteria. Therefore, colistin should be considered for therapy of patient infected with MDR *A. baumannii* in Thailand.

Combinations of agents that exhibit synergy, partial synergy or even additive activity could potentially reduce toxicity, prevent the emergence of bacterial resistance and improved outcomes for patients infected with Gram-negative bacteria known to develop resistant on single therapy. In study on Gram negative bacilli, combinations of a β-lactam and amikacin which were synergistic *in vitro* have been associate with significantly better outcomes than those achived with nonsynergistic regimens. Thus, with respect to synergy, it is suggested that colistin probably causes rapid permeabilization of the outer cell membrane, which allows enhanced penetration by and activity of the other antibiotic in the combination. Therefore, this is the first study which has been performed by checkerboard method using imipenem combination with colistin against 30 strains of MDR *A. baumannii*. The study of synergism interaction between imipenem plus colistin against 30 *A. baumannii* strains showed synergistic action and partial synergistic action against 27 strains (90%) and 3 strains (10%), respectively. Therefore, the checkerboard results showed that concentration of imipenem combined with colistin may be used in treat infectious disease caused by MDR *A. baumannii*. For example, it was shown that concentration was equal to the mean serum level of imipenem (32μg/ml) could lower the MIC of colistin against MDR *A. baumannii* to 4-16 folds. The results from this part of study indicated the possible use of the combination of imipenem and colistin in order to inhibit or kill the MDR *A. baumannii*. This finding was similar to the result from the study of Timurkaynak et al, who reported that the synergy effect between colistin and meropenem was seen in two out of five strains of multi-drug resistant *A. baumannii* (Timurkaynak et al., 2006).

The second study detected a synergistic bactericidal effect using time-kill method. This method for evaluating drug combinations involves quantitation of their rate of bactericidal action at different time interval. Identical cultures are incubated simultaneously with antibiotics added singly or in combination. If a combination of antibiotics is more rapidly bactericidal than either drug alone, the result is termed synergism (Chambers and Sande, 1996). In this study, antibacterial activity with the concentration equal to the mean serum level of imipenem alone at the therapeutic dose and in combination with 1/16MIC and 1/4MIC of colistin were determined by time

kill method. The results demonstrated that imipenem plus colistin had antibacterial activity against MDR *A. baumannii*. In addition, it was demonstrated that imipenem alone still has antibacterial activity against some strains. It might be the concentration of imipenem used in this study was higher or equal to MICs of imipenem in strains tested. The concentration of colistin used in this study was equal to 1/4MIC and 1/16MIC. Thus, it was shown that the better bactericidal and bacteriostatic activities against these strains were obtained when 1/4MIC of colistin plus imipenem was used than when 1/16MIC of colistin was used as shown in the number of cell killed, the number of strains killed and the time that the bacteria were killed. In this study, all combination activity of imipenem plus colistin showed antibacterial activity greater than colistin alone. In addition, after 6 hour of growth, 1/16MIC and 1/4MIC of colistin alone showed the regrowth to all strains. This could be explained that colistin probably cause rapid permeabilization of the outer membrane, which allows enhanced penetration of imipenem. Therefore, this outcome suggested that we could possibly use the combination of imipenem plus colistin in the treatment of infectious disease caused by MDR strains. However, our results need to be examined further by the *in vivo* studies in order to obtain more conclusive evidence.

The morphological structures of *A. baumannii* treated with imipenem, colistin and with both drugs combination were examined by scanning electron microscopy. Exposure to each of these agents for 2 hours led to the morphological changes. Since most β-lactam antibiotic bind to more than one PBP, the morphological changes induced by these antibiotic depend on the sum of the individual inhibitory effects. Whereas the morphological alteration of the cells exposed to colistin 1/4MIC for 2 hours was the same as that observed without antibiotic (control cell). When the cell was exposed to imipenem plus colistin for 2 hours, alls became spheroplast-like structure and produced numerous protrusions or blebs on the surface of cell. Of interest, the synergistic combinations also seemed to inhibit certain morphological change normally seen with the individual antibiotics.

Conclusion

This study suggested that infections due to multi-drug resistant *A. baumannii* strain, might be treated with the combination of antimicrobials. This *in vitro* study

demonstrated that the combinations of colistin and imipenem show synergy and partial synergy actively against MDR *A. baumannii*. Thus, the combination of imipenem plus colistin could be promising alternative for the treatment of infection due to MDR *A. baumannii* strains. Although the safety associated with elevated dosages for colistin are not known, such approaches may improve the outcomes in patient with MDR *A. baumannii* infection. However, in vitro data must be validated by assessing the clinical performance of combinations of antimicrobial agents before specific recommendations to modify existing treatment.

Further studies should be performed to examine the role of colistin and decreased colistin dosing strategies for the management of serious multi-drug resistant *A. baumannii* and evaluated forms of therapy optimization might help identify for patients with these infection.

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APPENDICES

Table A-1 Antimicrobial Susceptibility of 30 *A. baumannii* by disk diffusion method.

	Rifampin		Gentamicin		Imipenem		Ciprofloxacin		Ceftazidime		Tobramycin		Pip/Tazo		Cefepime		Colistin		Amikacin	
	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion
2	14.88	R	NZ	R	NZ	R	NZ	R	NZ	R	14.28	I	NZ	R	NZ	R	15.10	S	16.84	S
6	15.53	R	NZ	R	7.63	R	NZ	R	9.57	R	8.43	R	8.82	R	10.58	R	14.04	S	10.39	R
7	14.26	R	NZ	R	NZ	R	NZ	R	10.51	R	NZ	R	11.28	R	18.14	S	16.35	S	10.79	R
8	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	16.71	S	14.25	R	NZ	R	15.46	S	15.55	I
9	19.25	I	NZ	R	NZ	R	NZ	R	9.43	R	NZ	R	11.00	R	15.60	I	16.69	S	14.15	I
11	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	14.32	I	13.37	R	NZ	R	16.38	S	14.60	I
14	16.65	I	NZ	R	7.52	R	NZ	R	10.40	R	NZ	R	10.17	R	14.21	I	14.99	S	NZ	R
15	16.90	I	NZ	R	NZ	R	NZ	R	8.90	R	NZ	R	11.52	R	17.39	I	15.22	S	10.27	R
16	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	15.08	S	12.26	R	NZ	R	15.86	S	13.94	R
18	18.72	I	NZ	R	7.80	R	NZ	R	8.91	R	NZ	R	9.52	R	16.09	I	16.53	S	17.46	S
19	18.35	I	NZ	R	9.36	R	NZ	R	NZ	R	NZ	R	11.53	R	19.11	S	15.60	S	NZ	R
20	18.72	I	NZ	R	8.12	R	NZ	R	NZ	R	NZ	R	NZ	R	9.12	R	17.79	S	9.53	R
22	11.84	R	NZ	R	NZ	R	NZ	R	20.40	S	15.23	S	13.47	R	20.79	S	16.41	S	12.08	R
23	18.19	I	NZ	R	6.66	R	NZ	R	8.48	R	NZ	R	9.32	R	17.42	I	17.90	S	NZ	R
25	14.88	R	NZ	R	7.00	R	NZ	R	NZ	R	9.42	R	10.02	R	NZ	R	16.19	S	17.76	S

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

Table A-1 (continue) Antimicrobial Susceptibility of 30 *A. baumannii* by disk diffusion method.

	Rifampin		Gentamicin		Imipenem		Ciprofloxacin		Ceftazidime		Tobramycin		Pip/Tazo		Cefepime		Colistin		Amikacin	
	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion	Zone Diameter (mm.)	Interpretion
27	15.37	R	NZ	R	11.05	R	NZ	R	17.03	I	NZ	R	10.71	R	10.08	R	15.48	S	12.45	R
28	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	13.94	I	14.32	R	NZ	R	15.07	S	15.43	I
29	14.32	R	12.14	R	11.05	R	NZ	R	14.36	R	17.71	S	12.37	R	21.61	S	14.79	S	19.94	S
30	14.90	R	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	8.40	R	NZ	R	17.23	S	14.47	I
31	15.75	R	NZ	R	8.90	R	18.86	S	15.30	I	NZ	R	9.45	R	NZ	R	15.74	S	8.48	R
32	NZ	R	NZ	R	10.36	R	NZ	R	NZ	R	NZ	R	11.70	R	NZ	R	16.80	S	NZ	R
34	NZ	R	7.64	R	NZ	R	NZ	R	NZ	R	17.21	S	14.36	R	NZ	R	16.63	S	14.49	I
35	17.17	I	NZ	R	6.60	R	NZ	R	19.57	S	NZ	R	14.58	R	10.21	R	17.79	S	14.34	I
36	18.78	I	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	7.50	R	NZ	R	16.67	S	19.24	S
38	15.48	R	11.51	R	7.72	R	NZ	R	9.73	R	NZ	R	9.85	R	NZ	R	16.16	S	16.81	I
37	18.12	I	NZ	R	8.08	R	NZ	R	18.19	S	NZ	R	11.19	R	14.02	R	17.11	S	17.05	S
39	22.68	S	NZ	R	7.55	R	NZ	R	16.86	I	NZ	R	9.52	R	11.65	R	15.77	S	17.45	S
40	16.85	I	NZ	R	NZ	R	NZ	R	17.35	I	NZ	R	12.73	R	9.56	R	16.58	S	11.77	R
41	16.68	I	NZ	R	NZ	R	NZ	R	12.33	R	NZ	R	7.80	R	NZ	R	15.80	S	15.08	I
42	NZ	R	NZ	R	NZ	R	NZ	R	NZ	R	14.33	I	NZ	R	NZ	R	18.02	S	13.12	R

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

Table A-2 Broad-spectrum antimicrobial Susceptibility of 30 *A. baumannii* strains by disk diffusion method and E-test method.

	Imipenem				Cefepime				Ciprofloxacin				Pip/Tazo				Amikacin			
	Zone Diameter (mm)	Interpretion	MIC	Interpretion	Zone Diameter (mm)	Interpretion	MIC	Interpretion	Zone Diameter (mm)	Interpretion	MIC	Interpretion	Zone Diameter (mm)	Interpretion	MIC	Interpretion	Zone Diameter (mm)	Interpretion	MIC	Interpretion
2	NZ	R	>32	R	NZ	R	128	R	NZ	R	>32	R	8.46	R	>256	R	16.84	S	6	S
6	7.63	R	>32	R	10.58	R	32	R	NZ	R	>32	R	8.82	R	>256	R	10.39	R	48	I
7	NZ	R	>32	R	18.14	S	24	I	NZ	R	>32	R	11.28	R	>256	R	10.79	R	48	I
8	NZ	R	>32	R	NZ	R	>256	R	NZ	R	>32	R	14.25	R	64	R	15.55	I	24	I
9	NZ	R	>32	R	15.60	I	32	R	NZ	R	>32	R	11.00	R	>256	R	14.15	I	256	R
11	NZ	R	>32	R	NZ	R	>256	R	NZ	R	>32	R	13.37	R	128	R	14.60	I	32	I
14	7.52	R	>32	R	14.21	I	32	R	NZ	R	>32	R	10.17	R	>256	R	NZ	R	24	I
15	NZ	R	>32	R	17.39	I	24	I	NZ	R	>32	R	11.52	R	>256	R	10.27	R	48	I
16	NZ	R	>32	R	NZ	R	>256	R	NZ	R	>32	R	12.26	R	64	R	13.94	R	24	I
18	7.80	R	>32	R	16.09	I	24	I	NZ	R	>32	R	9.52	R	192	R	17.46	S	8	S
19	9.36	R	>32	R	20.02	S	12	I	NZ	R	>32	R	11.53	R	>256	R	NZ	R	>256	R
20	8.12	R	>32	R	9.12	R	96	R	NZ	R	>32	R	NZ	R	>256	R	9.53	R	32	I
22	NZ	R	>32	R	20.97	S	6	S	NZ	R	>32	R	13.47	R	64	R	12.08	R	24	I
23	6.66	R	>32	R	17.42	I	16	I	NZ	R	>32	R	9.32	R	>256	R	NZ	R	4	S
25	7.00	R	>32	R	NZ	R	128	R	NZ	R	>32	R	10.02	R	>256	R	17.76	S	8	S
27	11.05	R	>32	R	10.08	R	16	I	NZ	R	>32	R	10.71	R	>256	R	12.45	R	16	S
28	NZ	R	>32	R	NZ	R	>256	R	NZ	R	>32	R	14.32	R	64	R	15.43	I	24	I

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

Table A-2 (continue) Broad-spectrum antimicrobial Susceptibility of 30 *A. baumannii* strains by disk diffusion method and E-test method.

	Imipenem				Cefepime				Ciprofloxacin				Pip/Tazo				Amikacin			
	Zone Diameter (mm)	Interpretion	MIC	Interpretion	Zone Diameter (mm)	Interpretion	MIC	Interpretion	Zone Diameter (mm)	Interpretion	MIC	Interpretion	Zone Diameter (mm)	Interpretion	MIC	Interpretion	Zone Diameter (mm)	Interpretion	MIC	Interpretion
29	11.05	R	>32	R	21.61	S	24	I	NZ	S	>32	R	12.37	R	>256	R	19.94	S	6	S
30	NZ	R	>32	R	NZ	R	32	R	NZ	R	>32	R	8.40	R	>256	R	14.47	I	32	I
31	8.90	R	>32	R	NZ	R	192	R	18.86	I	12	R	9.45	R	>256	R	8.48	R	192	R
32	10.36	R	>32	R	NZ	R	48	R	NZ	R	>32	R	11.70	R	>256	R	NZ	R	>256	R
34	NZ	R	>32	R	NZ	R	>256	R	NZ	R	>32	R	14.36	R	>256	R	14.49	I	16	S
35	6.60	R	>32	R	10.21	R	12	I	NZ	R	>32	R	14.58	R	>256	R	14.34	I	24	I
36	NZ	R	>32	R	NZ	R	128	R	NZ	R	>32	R	7.50	R	>256	R	19.24	S	3	S
37	8.08	R	>32	R	14.02	R	12	I	NZ	R	>32	R	11.19	R	>256	R	16.81	I	4	S
38	7.72	R	>32	R	NZ	R	64	R	NZ	R	>32	R	9.85	R	>256	R	17.05	S	6	S
39	7.55	R	>32	R	11.65	R	16	I	NZ	R	>32	R	9.52	R	>256	R	17.45	S	4	S
40	NZ	R	>32	R	9.56	R	12	I	NZ	R	>32	R	12.73	R	>256	R	11.77	R	32	R
41	NZ	R	>32	R	NZ	R	32	R	NZ	R	>32	R	7.80	R	>256	R	15.08	I	24	I
42	NZ	R	>32	R	NZ	R	>256	R	NZ	R	>32	R	NZ	R	>256	R	13.12	R	48	I

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

Table A-3 Raw data of susceptibility testing by agar dilution method.

Isolate No.	Imipenem		Colistin	
	MIC (µg/ml)	Interpretion	MIC(µg/ml)	Interpretion
2	32	R	1	S
6	32	R	2	S
7	128	R	2	S
8	32	R	2	S
9	32	R	1	S
11	32	R	2	S
14	32	R	2	S
15	32	R	1	S
16	32	R	2	S
18	16	R	2	S
19	16	R	2	S
20	32	R	1	S
22	32	R	0.5	S
23	32	R	1	S
25	32	R	1	S
27	32	R	1	S
28	64	R	2	S
29	32	R	1	S
30	32	R	1	S
31	32	R	1	S
32	8	I	1	S
34	32	R	1	S
35	64	R	1	S
36	64	R	2	S
37	64	R	2	S
38	32	R	1	S
39	16	R	1	S
40	32	R	1	S
41	16	R	2	S
42	32	R	2	S

R= resistant, I= intermediate, S= susceptible

Table A-4 Log viable cell counts at various time points of *A. baumannii* strains

Strain no.	Antimicrobial agents	Log viable count (log CFU/ml) at time point					
		1	2	4	6	8	24
A 8	Control	7.013	8.512	9.161	10.085	11.954	17.286
	Imipenem 32µg/ml	7.09	6.389	6.386	7.238	7.544	10.079
	Colistin (1/16MIC)	7.09	7.274	9.328	9.929	11.297	14.677
	Colistin (1/4MIC)	7.013	7.338	8.097	9.262	10.17	15.185
	Imipenem + Colistin (1/16)	7.097	6.371	6.09	5.7	5.146	5.097
	Imipenem + Colistin (1/4)	7.13	6.371	6.09	5.29	4.966	4.286
A 9	Control	7.942	9.403	10.13	10.367	11.225	15.097
	Imipenem 32µg/ml	7.875	6.942	6.415	7.377	9.09	14.021
	Colistin (1/16MIC)	7.929	8.212	9.462	10.474	10.778	13.097
	Colistin (1/4MIC)	7.778	8.491	9.332	10.45	10.477	14.033
	Imipenem + Colistin (1/16)	8.097	6.653	6.013	5.42	5.107	9.813
	Imipenem + Colistin (1/4)	7.829	6.574	5.903	5.238	4.929	2.845
A 11	Control	6.795	7.602	7.942	9.223	9.978	14.813
	Imipenem 32µg/ml	6.72	6.13	5.358	5.072	6.308	6.352
	Colistin (1/16MIC)	6.512	7.013	8.053	9.124	9.544	14.829
	Colistin (1/4MIC)	6.65	6.09	5.428	5.19	7.072	7.439
	Imipenem + Colistin (1/16)	6.65	5.76	5.348	5.013	4.829	2.544
	Imipenem + Colistin (1/4)	6.9	5.76	5.322	4.813	4.403	1.699
A 19	Control	5.929	6.72	8.218	8.677	9.444	12.274
	Imipenem 32µg/ml	5.829	4.942	4.079	3.628	3.19	1.699
	Colistin (1/16MIC)	5.76	6.312	8.274	8.589	9.013	13.439
	Colistin (1/4MIC)	5.829	6.312	7.653	8.267	8.423	12.19
	Imipenem + Colistin (1/16)	5.796	4.954	4.079	3.274	3.274	1.699
	Imipenem + Colistin (1/4)	5.823	4.813	3.966	3.29	2.989	1.699
A 22	Control	8.274	10.061	10.17	11.021	11.041	16.279
	Imipenem 32µg/ml	8.114	7.049	6.107	7.297	8.318	14.544
	Colistin (1/16MIC)	8.033	8.889	9.602	10.079	10.439	15.358
	Colistin (1/4MIC)	8.021	8.7	9.477	9.903	10.358	15.09
	Imipenem + Colistin (1/16)	8.212	6.212	5.502	6.572	8.124	15.097
	Imipenem + Colistin (1/4)	8.23	6.061	5.415	6.403	7.318	12.829
A 23	Control	7.297	9.916	11.377	12.522	13.053	17.371
	Imipenem 32µg/ml	7.286	7.328	9.312	10.199	10.217	16.398
	Colistin (1/16MIC)	7.362	9.262	10.29	11.267	11.328	16.474
	Colistin (1/4MIC)	7.389	9.14	10.267	11.25	11.255	16.498
	Imipenem + Colistin (1/16)	7.394	6.875	8.439	8.829	9.17	16.279
	Imipenem + Colistin (1/4)	7.406	6.061	5.585	4.348	4.916	7.307

Table A-4 (continue) Log viable cell counts at various time points of *A. baumannii* strains

Strain no.	Antimicrobial agents	Log viable count (log CFU/ml) at time point					
		1	2	4	6	8	24
A 25	Control	6.829	9	10.297	11.542	12.097	16.966
	Imipenem 32µg/ml	6.796	6.262	6.19	5.17	4.439	4.238
	Colistin (1/16MIC)	6.778	8.455	10.041	11.267	11.367	15.403
	Colistin (1/4MIC)	6.942	8.829	9.813	10.491	11.17	15.352
	Imipenem + Colistin (1/16)	6.76	6.185	5.813	4.86	4.342	4.079
	Imipenem + Colistin (1/4)	6.966	5.829	5.23	4.531	4.146	3.279
A 27	Control	6.916	7.778	11.14	11.699	12.061	17.58
	Imipenem 32µg/ml	6.86	6.286	6.218	6.155	6.021	15.318
	Colistin (1/16MIC)	6.889	7.574	10.367	10.929	11.631	16.554
	Colistin (1/4MIC)	6.845	7.512	8.212	9.431	11.389	16.535
	Imipenem + Colistin (1/16)	6.889	6.328	5.889	5.398	5.114	4.436
	Imipenem + Colistin (1/4)	6.929	6.255	5.875	5.322	5.013	2.574
A 29	Control	8.4	9.989	11.176	11.301	12.199	17.529
	Imipenem 32µg/ml	8.412	6.352	6.053	5.415	5.238	5.648
	Colistin (1/16MIC)	8.348	9.428	10.124	10.916	10.903	16.414
	Colistin (1/4MIC)	8.407	9.428	10.267	10.76	10.415	16.297
	Imipenem + Colistin (1/16)	8.371	6.29	6.146	5.389	5.29	5.645
	Imipenem + Colistin (1/4)	8.412	6.185	5.829	5.212	5.072	4.033
A 30	Control	7.053	8.653	11.033	12.297	12.42	17.225
	Imipenem 32µg/ml	7.041	7	5.889	5.225	4.989	5.574
	Colistin (1/16MIC)	7.23	9.176	10.574	11.243	11.371	16.544
	Colistin (1/4MIC)	7.146	8.217	10.041	11.185	11.407	16.529
	Imipenem + Colistin (1/16)	7.107	6.989	5.889	5.176	4.942	5.23
	Imipenem + Colistin (1/4)	7.14	6.358	5.338	5.09	4.377	5.594
A 32	Control	6.845	7.544	10.42	11.061	11.916	16.942
	Imipenem 32µg/ml	6.796	6.176	4.845	4.574	4.185	6.146
	Colistin (1/16MIC)	6.813	7.155	10.23	10.279	11.161	16.386
	Colistin (1/4MIC)	6.76	6.225	6.033	5.643	5.312	10.398
	Imipenem + Colistin (1/16)	6.76	5.107	4.829	4.358	4.352	6.021
	Imipenem + Colistin (1/4)	6.813	5.161	4.72	4.23	3.978	4.17
A 37	Control	8.114	10.176	10.978	11.989	12.185	17.212
	Imipenem 32µg/ml	8.217	6.796	7.17	9.989	10.114	16.459
	Colistin (1/16MIC)	8.061	9.217	10.204	11.185	11.238	16.525
	Colistin (1/4MIC)	8.199	9.19	10.19	10.942	11.17	16.602
	Imipenem + Colistin (1/16)	8.061	5.256	6.262	8.72	9.338	16.217
	Imipenem + Colistin (1/4)	7.978	4.477	3.628	3.097	2.813	1.699

Table A-4 (continue) Log viable cell counts at various time points of *A. baumannii* strains

Strain no.	Antimicrobial agents	Log viable count (log CFU/ml) at time point					
		1	2	4	6	8	24
A 39	Control	7.966	9.079	10.13	11.274	12.06	17.204
	Imipenem 32µg/ml	7.875	7.013	5.677	5.398	5.262	5.286
	Colistin (1/16MIC)	7.86	8.061	10.041	11.053	11.455	15.538
	Colistin (1/4MIC)	8.013	7.114	8.072	9.875	11.114	15.538
	Imipenem + Colistin (1/16)	8.021	6.041	5.512	5.322	5.061	4.021
	Imipenem + Colistin (1/4)	7.875	6.021	5.477	5.17	4.954	2.677
A 41	Control	7.225	7.86	10.053	10.394	11.243	17.338
	Imipenem 32µg/ml	7.1	5.352	5.061	4.829	4.29	6.097
	Colistin (1/16MIC)	7.204	7.1	9.19	10.021	10.4	16.428
	Colistin (1/4MIC)	7.124	7	9.161	9.916	10.348	16.033
	Imipenem + Colistin (1/16)	7.14	5.301	5.13	4.76	4.238	5.646
	Imipenem + Colistin (1/4)	7.14	5.267	5.061	4.279	4.021	3.25
A 42	Control	7.17	8.512	8.677	10.531	11.954	17.212
	Imipenem 32µg/ml	7.23	5.512	4.796	4.439	4.285	14.83
	Colistin (1/16MIC)	7.318	8.394	8.529	9.161	9.398	15.079
	Colistin (1/4MIC)	7.23	8.428	8.338	8.942	9.544	15.217
	Imipenem + Colistin (1/16)	7.097	5.377	4.367	3.86	2.72	1.699
	Imipenem + Colistin (1/4)	7.097	5.377	4.367	3.86	2.72	1.699

Table A-5 Log change viable counts at various times points and kinetic parameters of 15 *A. baumannii* strains

Strain no.	Antimicrobial agents	viable log change					AUBKC 0-24	Bacteriolytic Area
		Δ2	Δ4	Δ6	Δ8	Δ24		
A8	Control	1.499	2.148	3.072	4.941	10.273	308.403	-
	Imipenem 32µg/ml	-0.701	-0.704	0.148	0.454	2.989	195.644	112.759
	Colistin (1/16MIC)	0.184	2.238	2.839	4.207	7.587	279.241	29.162
	Colistin (1/4MIC)	0.325	1.084	2.249	3.157	8.172	269.417	38.986
	Imipenem + Colistin(1/16)	-0.726	-1.007	-1.397	-1.951	-2	130.509	177.894
	Imipenem + Colistin (1/4)	-0.759	-1.04	-1.84	-2.164	-2.844	121.614	186.789
A9	Control	1.461	2.188	2.425	3.283	7.155	289.543	-
	Imipenem 32µg/ml	-0.933	-1.46	-0.498	1.215	6.146	243.321	46.222
	Colistin (1/16MIC)	0.283	1.533	2.545	2.849	5.168	266.003	23.54
	Colistin (1/4MIC)	0.713	1.554	2.672	2.699	6.255	270.881	18.662
	Imipenem + Colistin(1/16)	-1.444	-2.084	-2.677	-2.99	1.716	168.736	120.807
	Imipenem + Colistin (1/4)	-1.255	-1.926	-2.591	-2.9	-4.984	110.38	179.163
A11	Control	0.807	1.147	2.428	3.183	8.018	264.635	-
	Imipenem 32µg/ml	-0.59	-1.362	-1.648	-0.412	-0.368	147.428	117.207
	Colistin (1/16MIC)	0.501	1.541	2.612	3.032	8.317	259.42	5.215
	Colistin (1/4MIC)	-0.56	-1.222	-1.46	0.422	0.789	163.226	101.409
	Imipenem + Colistin(1/16)	-0.89	-1.302	-1.637	-1.821	-4.106	102.705	162.409
	Imipenem + Colistin (1/4)	-1.14	-1.578	-2.087	-2.497	-5.201	91.909	172.726

Table A-5 (continue) Log change viable counts at various times points and kinetic parameters of 15 *A. baumannii* strains

Strain no.	Antimicrobial agents	viable log change					AUBKC $_{0-24}$	Bacteriolytic Area
		Δ2	Δ4	Δ6	Δ8	Δ24		
A19	Control	0.791	2.289	2.748	3.515	6.345	236.347	-
	Imipenem 32µg/ml	-0.887	-1.75	-2.201	-2.639	-4.13	73.429	162.918
	Colistin (1/16MIC)	0.552	2.514	2.829	3.253	7.679	240.739	-4.392
	Colistin (1/4MIC)	0.483	1.824	2.438	2.594	6.361	223.62	12.727
	Imipenem + Colistin(1/16)	-0.842	-1.717	-2.522	-2.522	-4.097	73.468	162.879
	Imipenem + Colistin (1/4)	-1.01	-1.857	-2.533	-2.834	-4.124	70.454	165.893
A22	Control	1.787	1.896	2.747	2.767	8.005	300.379	-
	Imipenem 32µg/ml	-1.065	-2.007	-0.817	0.204	6.43	240.234	60.145
	Colistin (1/16MIC)	0.856	1.569	2.046	2.406	7.325	281.988	18.391
	Colistin (1/4MIC)	0.679	1.456	1.882	2.337	7.069	278.123	22.256
	Imipenem + Colistin(1/16)	-2	-2.71	-1.64	-0.088	6.885	238.676	61.703
	Imipenem + Colistin (1/4)	-2.169	-2.815	-1.827	-0.912	4.599	212.482	87.897
A23	Control	2.619	4.08	5.225	5.756	10.074	331.372	-
	Imipenem 32µg/ml	0.042	2.026	2.913	2.931	9.112	284.101	47.271
	Colistin (1/16MIC)	1.9	2.928	3.905	3.966	9.112	302.744	28.628
	Colistin (1/4MIC)	1.751	2.878	3.861	3.866	9.109	301.982	29.39
	Imipenem + Colistin(1/16)	-0.519	1.045	1.435	1.776	8.885	268.442	62.93
	Imipenem + Colistin (1/4)	-1.345	-1.821	-3.058	-2.49	-0.099	142.094	189.277

Table A-5 (continue) Log change viable counts at various times points and kinetic parameters of 15 *A. baumannii* strains

Strain no.	Antimicrobial agents	viable log change					AUBKC $_{0-24}$	Bacteriolytic Area
		Δ2	Δ4	Δ6	Δ8	Δ24		
A25	Control	2.171	3.468	4.713	5.268	10.137	313.108	-
	Imipenem 32µg/ml	-0.534	-0.606	-1.626	-2.357	-2.558	115.895	197.213
	Colistin (1/16MIC)	1.677	3.263	4.489	4.589	8.625	291.831	21.277
	Colistin (1/4MIC)	1.887	2.871	3.549	4.228	8.41	288.554	24.564
	Imipenem + Colistin(1/16)	-0.575	-0.947	-1.9	-2.418	-2.681	112.186	200.922
	Imipenem + Colistin (1/4)	-1.137	-1.736	-2.435	-2.82	-3.687	101.692	211.416
A27	Control	0.862	4.224	4.783	5.145	10.664	317.339	-
	Imipenem 32µg/ml	-0.574	-0.642	-0.705	-0.839	8.458	220.911	96.428
	Colistin (1/16MIC)	0.685	3.478	4.04	4.742	9.665	301.74	15.599
	Colistin (1/4MIC)	0.667	1.367	2.586	4.544	9.69	291.936	25.403
	Imipenem + Colistin(1/16)	-0.561	-1	-1.491	-1.775	-2.453	123.633	193.706
	Imipenem + Colistin (1/4)	-0.674	-1.054	-1.607	-1.916	-4.355	107.542	209.797
A29	Control	1.589	2.776	2.901	3.799	9.129	323.355	-
	Imipenem 32µg/ml	-2.06	-2.359	-2.997	-3.174	-2.764	136.378	186.977
	Colistin (1/16MIC)	1.08	1.776	2.568	2.555	8.066	298.723	24.632
	Colistin (1/4MIC)	1.021	1.86	2.353	2.008	7.89	293.428	29.927
	Imipenem + Colistin(1/16)	-2.081	-2.225	-2.982	-3.081	-2.726	136.791	186.564
	Imipenem + Colistin (1/4)	-2.227	-2.583	-3.2	-3.34	-4.379	120.776	202.579

Table A-5 (continue) Log change viable counts at various times points and kinetic parameters of 15 *A. baumannii* strains

Strain no.	Antimicrobial agents	viable log change					AUBKC $_{0-24}$	Bacteriolytic Area
		Δ2	Δ4	Δ6	Δ8	Δ24		
A30	Control	1.6	3.98	5.244	5.367	10.172	320.599	-
	Imipenem 32µg/ml	-0.041	-1.152	-1.816	-2.052	-1.467	132.762	187.837
	Colistin (1/16MIC)	1.946	3.344	4.013	4.141	9.314	303.907	16.692
	Colistin (1/4MIC)	1.071	2.895	4.039	4.261	9.383	300.927	19.672
	Imipenem + Colistin(1/16)	-0.118	-1.218	-1.931	-2.165	-1.877	129.533	191.066
	Imipenem + Colistin (1/4)	-0.782	-1.802	-2.05	-2.763	-1.546	124.857	195.742
A32	Control	0.699	3.575	4.216	5.071	10.097	307.675	-
	Imipenem 32µg/ml	-0.62	-1.951	-2.222	-2.611	-0.65	124.819	182.856
	Colistin (1/16MIC)	0.342	3.417	3.466	4.348	9.573	293.678	13.997
	Colistin (1/4MIC)	-0.535	-0.727	-1.117	-1.448	3.638	173.554	134.121
	Imipenem + Colistin(1/16)	-1.653	-1.931	-2.402	-2.408	-0.739	122.684	184.991
	Imipenem + Colistin (1/4)	-1.652	-2.093	-2.583	-2.835	-2.643	104.197	203.478
A37	Control	2.062	2.864	3.875	4.071	9.098	321.761	-
	Imipenem 32µg/ml	-1.421	-1.047	1.772	1.897	8.242	278.825	42.936
	Colistin (1/16MIC)	1.156	2.143	3.124	3.177	8.464	302.615	19.146
	Colistin (1/4MIC)	0.991	1.991	2.743	2.971	8.403	302.189	19.572
	Imipenem + Colistin(1/16)	-2.805	-1.799	0.659	1.277	8.156	262.315	59.446
	Imipenem + Colistin (1/4)	-3.501	-4.35	-4.881	-5.165	-6.279	69.291	252.47

Table A-5 (continue) Log change viable counts at various times points and kinetic parameters of 15 *A. baumannii* strains

Strain no.	Antimicrobial agents	viable log change					AUBKC $_{0-24}$	Bacteriolytic Area
		Δ2	Δ4	Δ6	Δ8	Δ24		
A39	Control	1.113	2.164	3.308	4.094	9.238	315.104	-
	Imipenem 32µg/ml	-0.862	-2.198	-2.477	-2.613	-2.589	133.697	181.407
	Colistin (1/16MIC)	0.201	2.181	3.193	3.595	7.678	293.569	21.535
	Colistin (1/4MIC)	-0.899	0.059	1.862	3.101	7.525	282.465	32.639
	Imipenem + Colistin(1/16)	-1.98	-2.509	-2.699	-2.96	-4	119.488	195.616
	Imipenem + Colistin (1/4)	-1.854	-2.398	-2.705	-2.921	-5.198	107.213	207.891
A41	Control	0.635	2.828	3.169	4.018	10.113	303.73	-
	Imipenem 32µg/ml	-1.748	-2.039	-2.271	-2.81	-1.003	124.97	178.76
	Colistin (1/16MIC)	-0.104	1.986	2.817	3.196	9.224	284.85	18.88
	Colistin (1/4MIC)	-0.124	2.037	2.792	3.224	8.909	280.674	23.056
	Imipenem + Colistin(1/16)	-1.839	-2.01	-2.38	-2.902	-1.494	120.832	182.898
	Imipenem + Colistin (1/4)	-1.873	-2.079	-2.861	-3.119	-3.89	98.543	205.187
A42	Control	1.342	1.507	3.361	4.784	10.042	307.892	-
	Imipenem 32µg/ml	-1.718	-2.434	-2.791	-2.945	7.6	193.929	113.963
	Colistin (1/16MIC)	1.076	1.211	1.843	2.08	7.761	264.7	43.192
	Colistin (1/4MIC)	1.198	1.108	1.712	2.314	7.987	266.278	41.614
	Imipenem + Colistin(1/16)	-1.72	-2.73	-3.237	-4.377	-5.398	79.926	227.966
	Imipenem + Colistin (1/4)	-1.72	-2.73	-3.237	-4.377	-5.398	72.377	235.515

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-1 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.2
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-2 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.6
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/132	4/64	4/128	4/256
2	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128	2/256
1	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
0.5	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128	0.5/256
0.25	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128	0.25/256
0.12	0.125/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128	0.12/256
0.06	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128	0.06/256
0	0.25	0.5	1	2	4	8	16	32	64	128	256
	Imipenem										

Figure A-3 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.7
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-4 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.8
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-5 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.9
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-6 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.11
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-7 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.14
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-8 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.15
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-9 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.16
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-10 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.18
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-11 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.19
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-12 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.20
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin												
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-13 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.22
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin												
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-14 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.23
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin												
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-15 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.25
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin												
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-16 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.27
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin												
4	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/132	4/64	4/128	4/256
2	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128	2/256
1	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
0.5	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128	0.5/256
0.25	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128	0.25/256
0.12	0.125/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128	0.12/256
0.06	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128	0.06/256
0	0.25	0.5	1	2	4	8	16	32	64	128	256
	Imipenem										

Figure A-17 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.28
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-18 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.29
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-19 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.30
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-20 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.31
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.06	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64
2	2/0.06	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64
1	1/0.06	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64
0.5	0.5/0.06	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64
0.25	0.25/0.06	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64
0.12	0.125/0.06	0.12/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64
0.06	0.06/0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64
0	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64

Imipenem

Figure A-21 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.32
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128

Imipenem

Figure A-22 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.34
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128

Imipenem

Figure A-23 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.35
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/132	4/64	4/128	4/256
2	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128	2/256
1	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
0.5	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128	0.5/256
0.25	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128	0.25/256
0.12	0.125/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128	0.12/256
0.06	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128	0.06/256
0	0.25	0.5	1	2	4	8	16	32	64	128	256

Imipenem

Figure A-24 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.36
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin												
4	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/132	4/64	4/128	4/256
2	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128	2/256
1	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
0.5	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128	0.5/256
0.25	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128	0.25/256
0.12	0.125/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128	0.12/256
0.06	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128	0.06/256
0	0.25	0.5	1	2	4	8	16	32	64	128	256
	Imipenem										

Figure A-25 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.37
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-26 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.38
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-27 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.39
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-28 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.40
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-29 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.41
Shadow zone : visible microorganism growth, white zone : no microorganism growth

Colistin											
4	4/0.12	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.12	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.12	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.12	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8	0.5/16	0.5/32	0.5/64	0.5/128
0.25	0.25/0.12	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8	0.25/16	0.25/32	0.25/64	0.25/128
0.12	0.125/0.12	0.12/0.25	0.12/0.5	0.12/1	0.12/2	0.12/4	0.12/8	0.12/16	0.12/32	0.12/64	0.12/128
0.06	0.06/0.12	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8	0.06/16	0.06/32	0.06/64	0.06/128
0	0.12	0.25	0.5	1	2	4	8	16	32	64	128
	Imipenem										

Figure A-30 The synergism result (checkerboard) of imipenem plus colistin against *A. baumannii* strain no.42
Shadow zone : visible microorganism growth, white zone : no microorganism growth

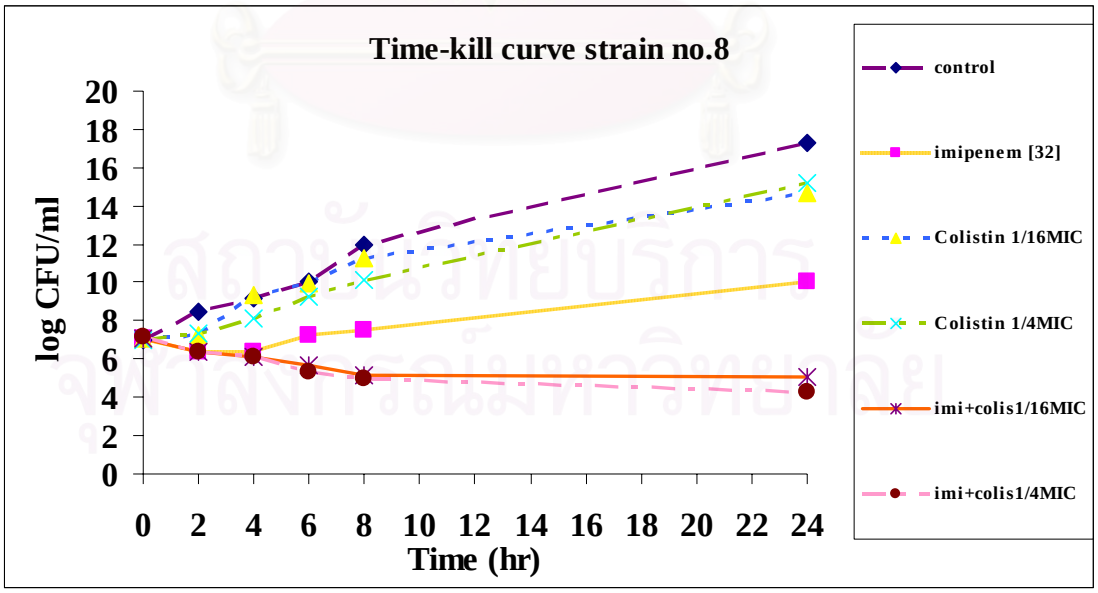

Figure A -31 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no. 8.

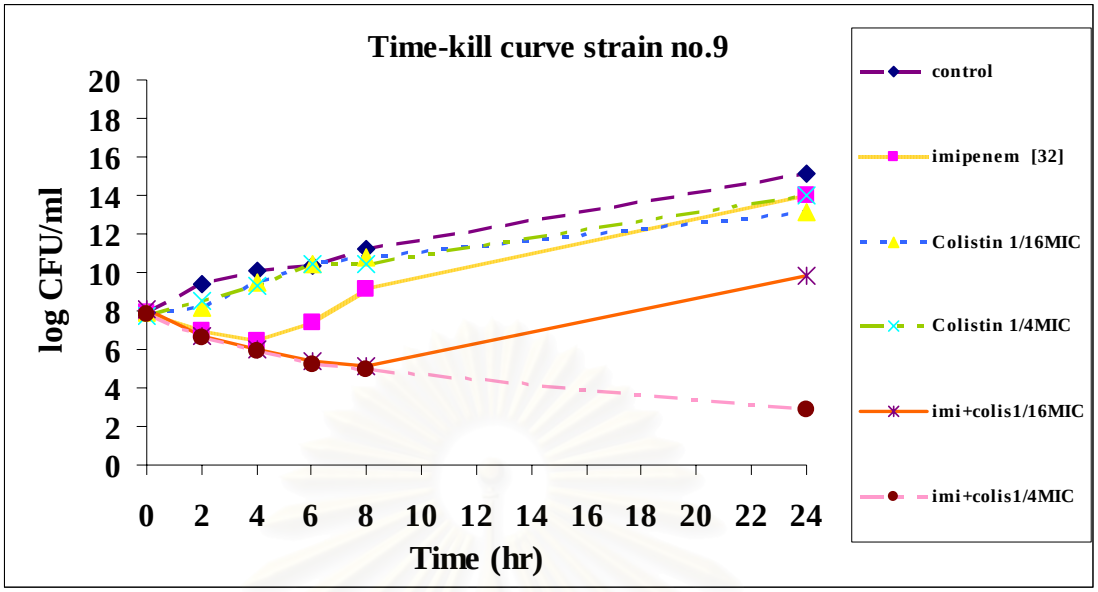

Figure A -32 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.9.

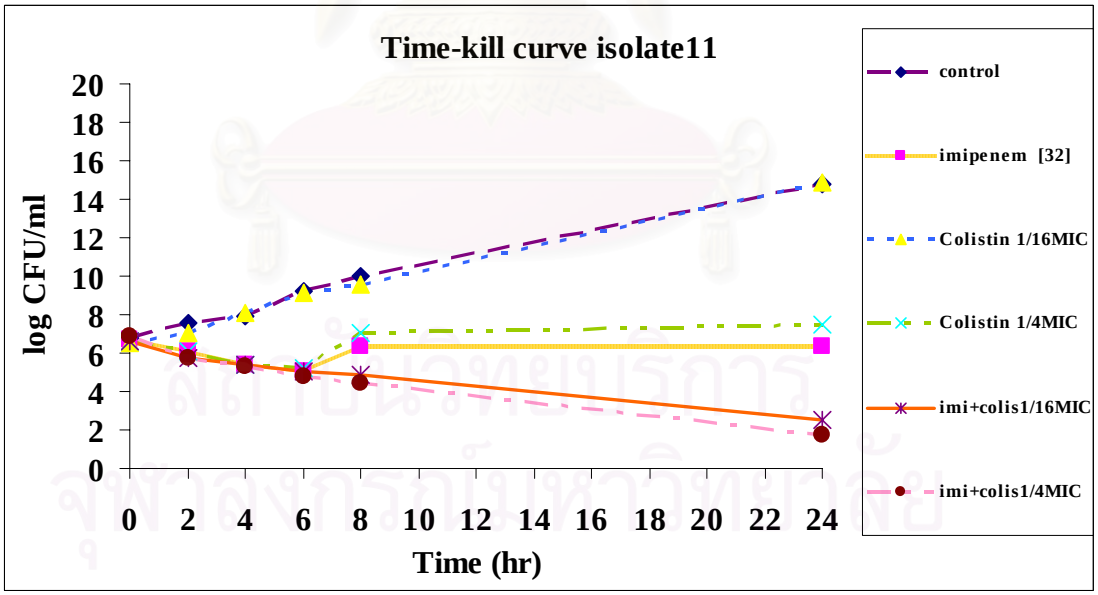

Figure A -33 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.11.

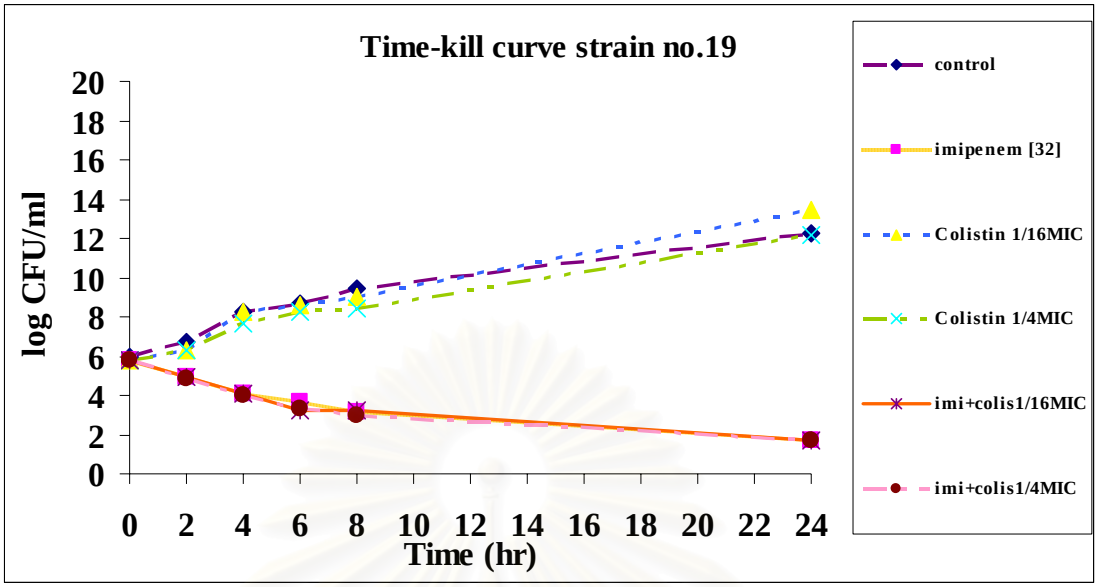

Figure A -34 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.19.

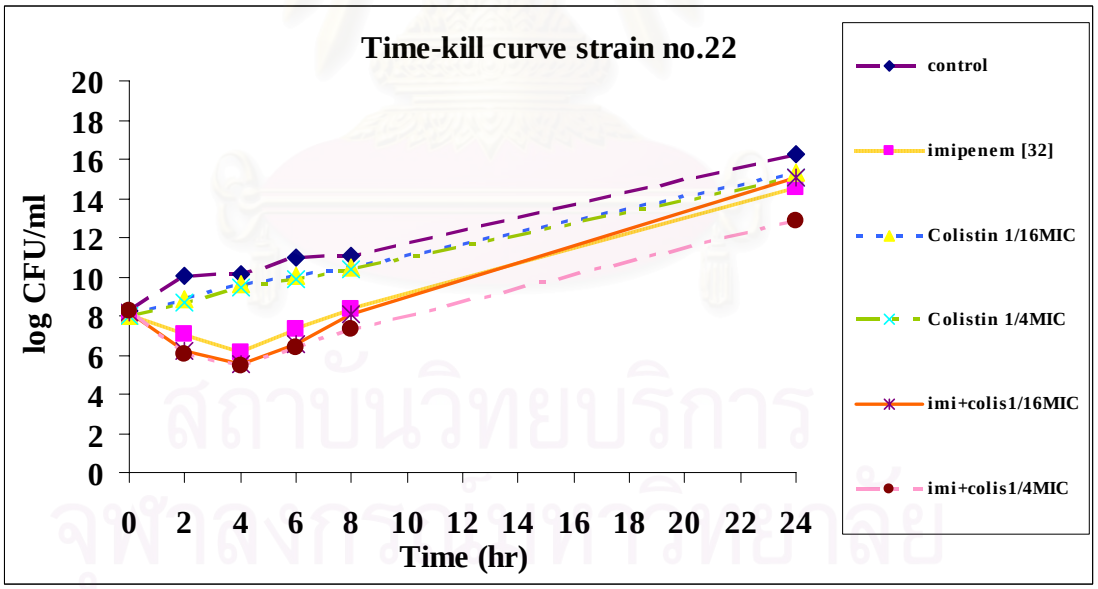

Figure A -35 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.22.

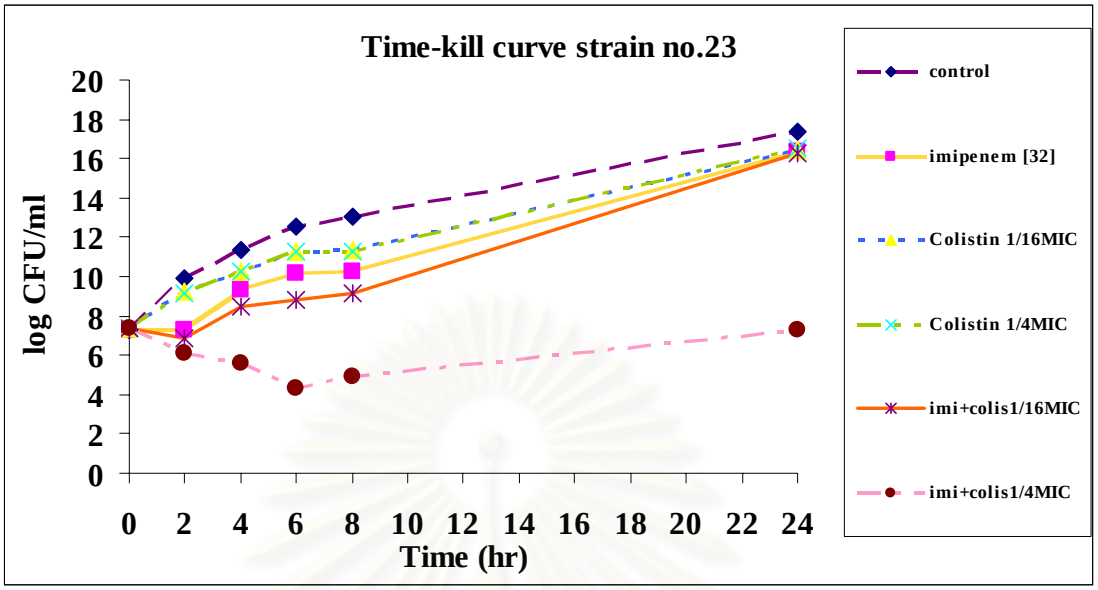

Figure A -36 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.23.

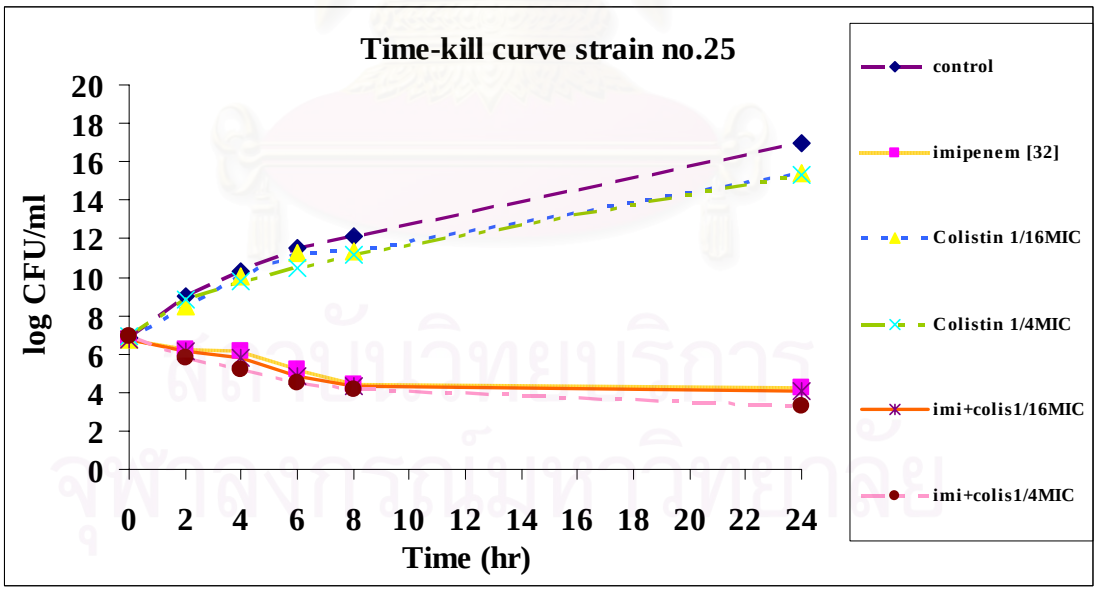

Figure A -37 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.25.

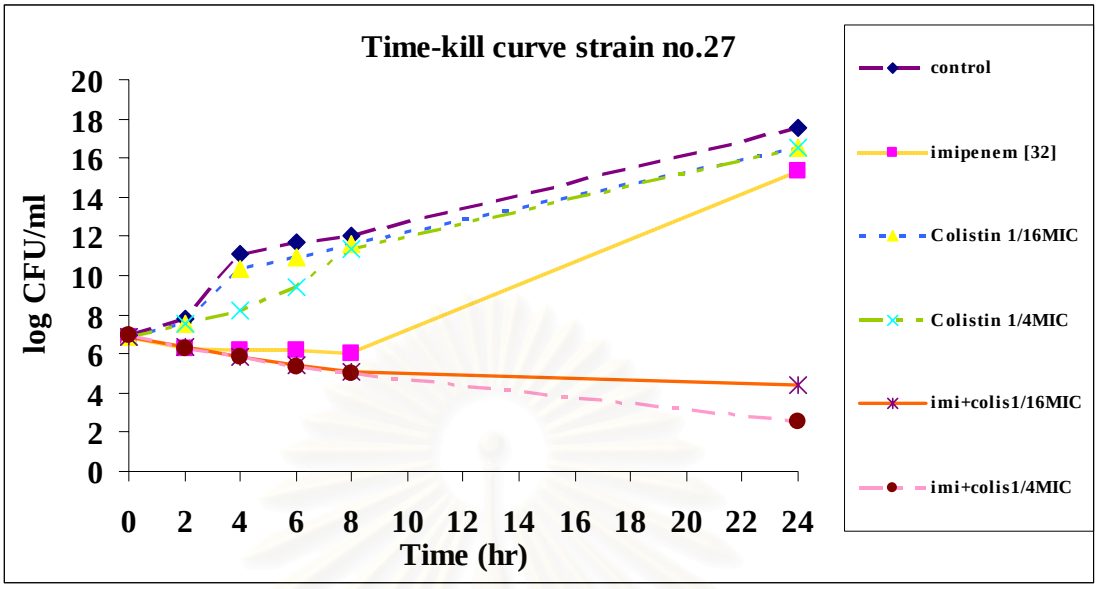

Figure A -38 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.27.

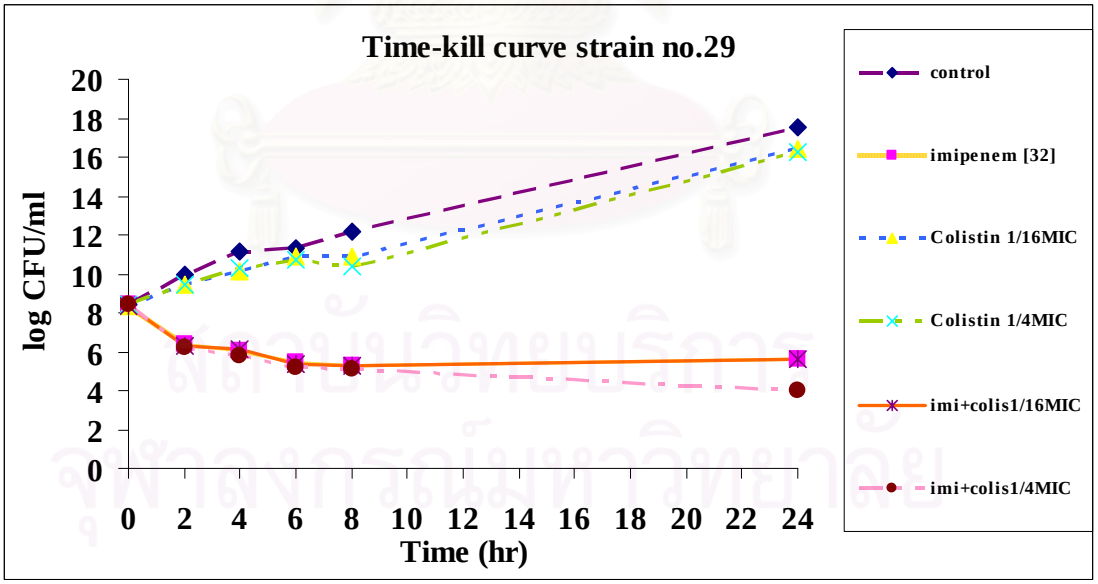

Figure A -39 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.29.

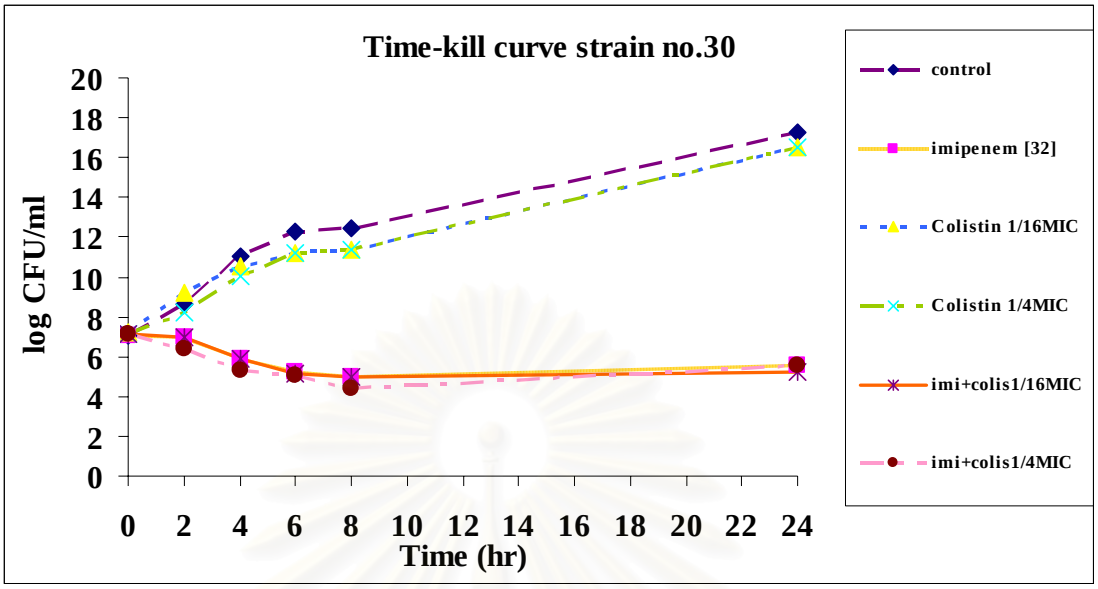

Figure A -40 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.30.

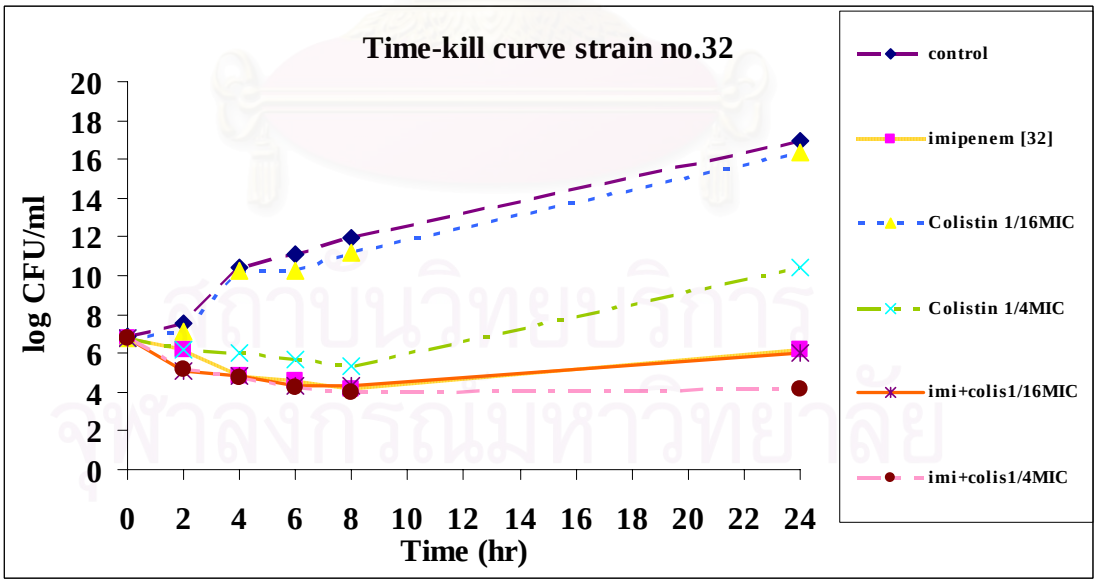

Figure A -41 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.32.

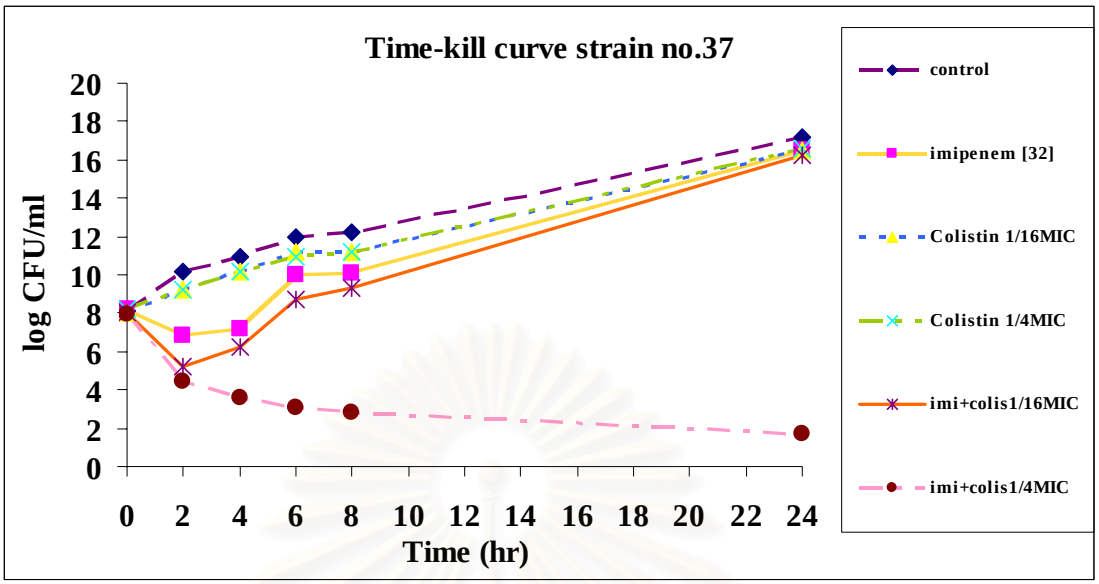

Figure A -42 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.37.

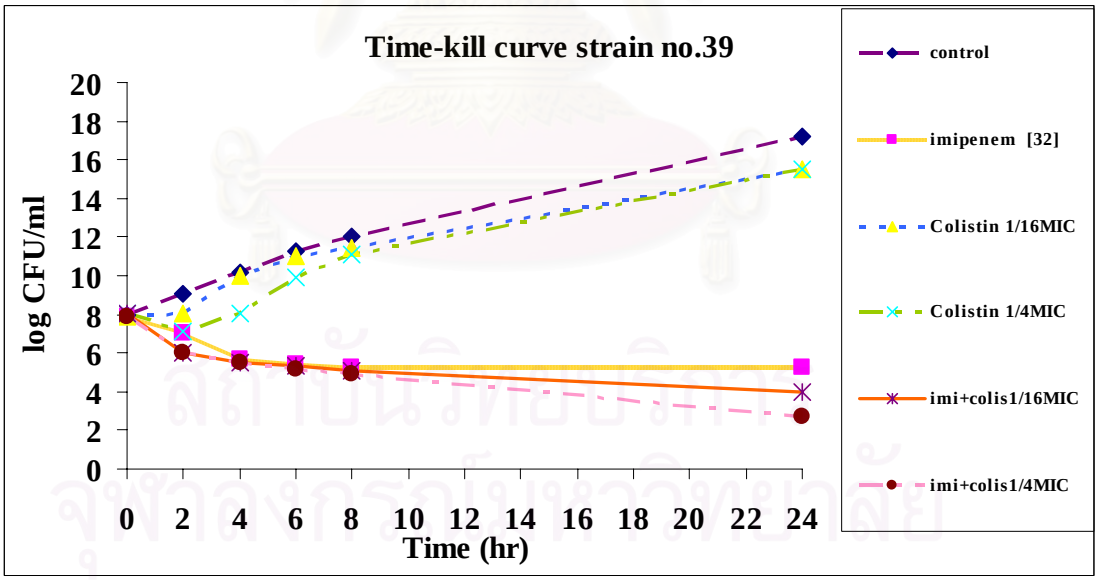

Figure A -43 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.39.

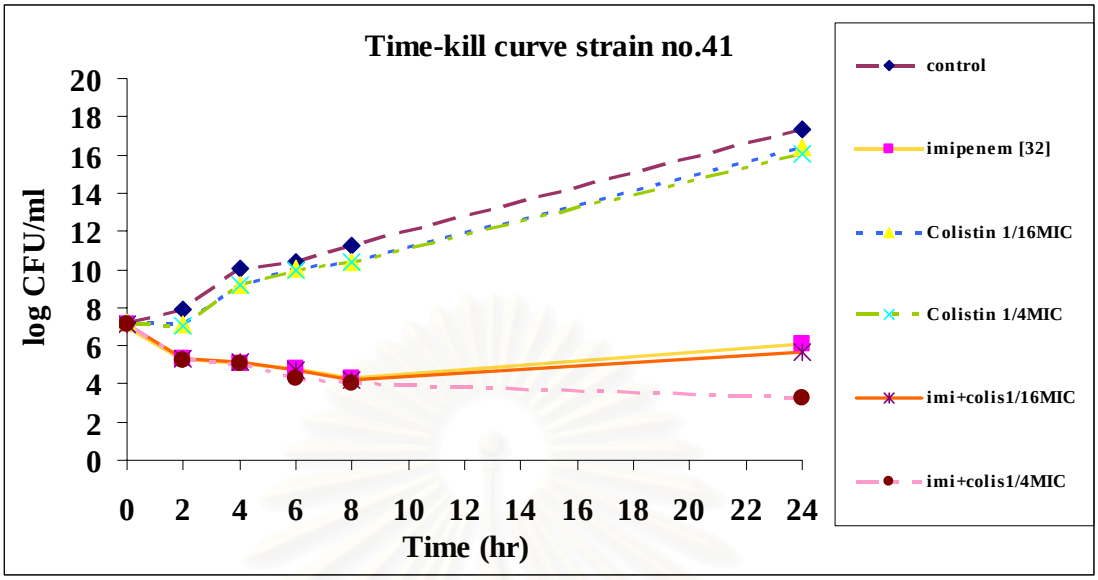

Figure A -44 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.41.

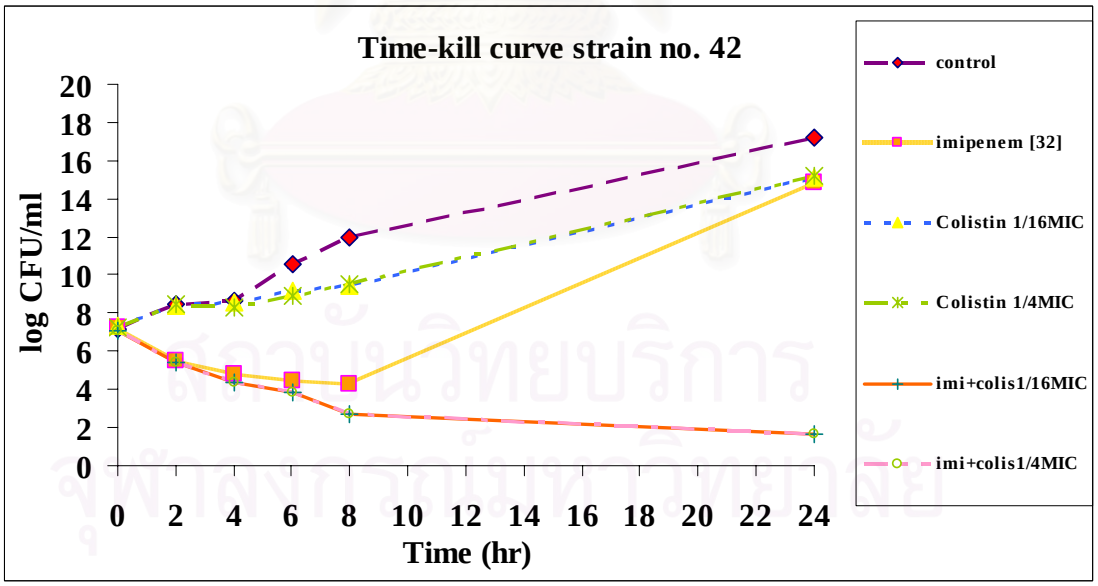

Figure A -45 Time kill curves showing the antibacterial activity of the combination of Imipenem plus Colistin 1/16MIC and Imipenem plus Colistin 1/4MIC against *A. baumannii* strain no.42.

Table A-6 Raw data of RAPD type in 30 strains *A. baumannii*.

RAPD type	Strain No.
1	2 6 7 15 20 23 25 27 35 38 40
2	8 9 11 14 16 18 19 22 28 31 34 36 42
3	29
4	30 41
5	37 39
7	32

BIOGRAPHY

My name is Sakulthip Panapakdee. I was born in 21 September 1983 in Bangkok. I have graduated with the bachelor degree in Microbiology from Kasetsart University since 2004. I have enrolled for the Master's degree in Pharmacology (Inter-Department), Faculty of Graduate School, Chulalongkorn University since June 2005.