การศึกษาคุณสมบัติทางเภสัชพลศาสตร์ของยารวมกลุ่มเบตาแลคแทม-สารต้านเอนไซม์เบตาแลคทาเมส ในหลอดทดลองต่อเชื้อแบคทีเรียแกรมลบซึ่งสร้างเอนไซม์เบตาแลคทาเมสที่มีความสำคัญทางคลินิก

นางสาว ดวงกมล อมรศักดิ์โสภณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2545

ISBN 974-17-2959-6

IN VITRO PHARMACODYNAMIC STUDIES OF β-LACTAM-β-LACTAMASE INHIBITOR COMBINATIONS AGAINST β-LACTAMASE PRODUCING CLINICALLY IMPORTANT GRAM-NEGATIVE BACTERIA

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy

Department of Pharmacology
Faculty of Pharmaceutical Sciences
Chulalongkorn University

Academic Year 2002
ISBN 974-17-2959-6

Thesis Title	*IN VITRO* PHARMACODYNAMIC STUDIES OF β-LACTAM-β-LACTAMASE INHIBITOR COMBINATIONS AGAINST β-LACTAMASE PRODUCING CLINICALLY IMPORTANT GRAM-NEGATIVE BACTERIA.
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ดวงกมล อมรศักดิ์โสภณ : การศึกษาคุณสมบัติทางเภสัชพลศาสตร์ของยารวมกลุ่มเบตาแลคแทม-สารต้านเอนไซม์เบตาแลคทาเมสในหลอดทดลองต่อเชื้อแบคทีเรียแกรมลบซึ่งสร้างเอนไซม์เบตาแลคทาเมสที่มีความสำคัญทางคลินิก. (*IN VITRO* PHARMACODYNAMIC STUDIES OF β-LACTAM-β-LACTAMASE INHIBITOR COMBINATIONS AGAINST β-LACTAMASE PRODUCING CLINICALLY IMPORTANT GRAM-NEGATIVE BACTERIA) อาจารย์ที่ปรึกษา: รศ. ศิริภรณ์ ฟุ้งวิทยา, อาจารย์ที่ปรึกษาร่วม : ศ.พญ.นลินี อัศวโภคี, จำนวนหน้า 112 หน้า. ISBN 974-17-2959-6.

ยารวมกลุ่มเบตาแลคแทม-สารต้านเอนไซม์เบตาแลคทาเมส จัดเป็นกลุ่มยาปฏิชีวนะที่ใช้สำหรับฆ่าเชื้อแบคทีเรียที่มีความสำคัญทางคลินิกหลายชนิดโดยเฉพาะเชื้อแบคทีเรียที่แสดงลักษณะการดื้อต่อยาปฏิชีวนะกลุ่มเบตาแลคแทมด้วยวิธีการสร้างเอนไซม์เบตาแลคทาเมส หน้าที่ของสารต้านเอนไซม์เบตาแลคทาเมสคือการแย่งจับกับเอนไซม์เบตาแลคทาเมส ส่งผลทำให้ยากลุ่มเบตาแลคแทมไม่ถูกทำลายและสามารถออกฤทธิ์ยับยั้งการสร้างผนังเซลล์ของแบคทีเรียต่อไปได้ การวิจัยครั้งนี้ต้องการศึกษาผลเสริมฤทธิ์กันระหว่างยากลุ่มเบตาแลคแทม และ สารต้านเอนไซม์เบตาแลคทาเมส 3 ชนิด โดยเลือกทดสอบกับเชื้อแบคทีเรียที่เหมาะสมกับยาแต่ละชนิดซึ่งใช้จริงทางคลินิกดังนี้ amoxicillin-clavulanic acid กับเชื้อ *Moraxella catarrhalis* และ *Haemophilus influenzae*; piperacillin-tazobactam กับเชื้อ *Klebsiella pneumoniae* และ *Pseudomonas aeruginosa*; cefoperazone-sulbactam กับเชื้อ *Pseudomonas aeruginosa* และ *Acinetobacter baumannii* ด้วยวิธี checkerboard technique และ time kill จากการทดลองพบว่า clavulanic acid ที่ความเข้มข้น 2 มคก/มล สามารถทำให้ค่า MIC ของ amoxicillin ลดลงถึง 64 เท่า เมื่อทดสอบกับเชื้อ *M. catarrhalis* และ *H. influenzae*, tazobactam ที่ความเข้มข้น 4 มคก/มล สามารถทำให้ piperacillin แสดงค่า MIC ต่อเชื้อ *K. pneumoniae* และ *P. aeruginosa* ลดลง 64 และ 4 เท่าตามลำดับ, sulbactam สามารถลดค่า MIC ของ cefoperazone ต่อเชื้อ *P. aeruginosa* ได้ 8 เท่า ที่ความเข้มข้น 8 มคก/มล ในขณะที่ *A. baumannii* ต้องการ sulbactam ถึง 32 มคก/มล เพื่อทำให้ค่า MIC ของ cefoperazone ลดลง นอกจากนี้เมื่อให้ยากลุ่มเบตาแลคแทม (amoxicillin, piperacillin, cefoperazone) ที่ความเข้มข้น 2 MIC ร่วมกับสารต้านเอนไซม์เบตาแลคทาเมสที่ระดับความเข้มข้นเฉลี่ยในร่างกาย (clavulanic acid 2 มคก/มล, tazobactam 4 มคก/มล, sulbactam 8 มคก/มล) พบว่ายารวมที่ระดับความเข้มข้นดังกล่าวสามารถกำจัดเชื้อแบคทีเรียได้มากกว่า 100 เท่า เมื่อเปรียบเทียบกับยากลุ่มเบตาแลคแทมเดี่ยวๆที่ความเข้มข้นเดียวกัน ยกเว้นกรณีของเชื้อ *A. baumannii* ซึ่งต้องการเพียง sulbactam ที่ความเข้มข้น 32 มคก/มล ในการกำจัดเชื้อโดยไม่ต้องอาศัย cefoperazone สำหรับการทดสอบ Post β-lactamase inhibitor effect (PLIE) พบว่า เฉพาะ amoxicillin-clavulanic acid และ piperacillin-tazobactam เท่านั้นที่แสดงค่า PLIE เมื่อทดสอบกับเชื้อ *H. influenzae* และ *P. aeruginosa* ตามลำดับ โดยค่า PLIE ดังกล่าวมีลักษณะแปรผันตามอิทธิพลของความเข้มข้นของสารต้านเอนไซม์เบตา-แลคทาเมส สำหรับการทดสอบการเหนี่ยวนำการสร้างเอนไซม์เบตาแลคทาเมสของสารต้านเอนไซม์เบตาแลคทาเมสทั้ง 3 ชนิด พบว่ามีเพียง clavulanic acid เท่านั้นที่แสดงผลการเหนี่ยวนำเอนไซม์ของเชื้อ *Enterobacter cloacae* ต่อยา cefuroxime เมื่อทดสอบด้วยวิธี double disk โดยการเหนี่ยวนำดังกล่าวสามารถทำให้ค่า MIC ของ cefuroxime เพิ่มขึ้นจาก 6 มคก/มล เป็น 32 มคก/มล (clavulanic acid 10 มคก/มล) เมื่อทดสอบด้วยวิธี agar dilution และผลจากการทดลองแสดงให้เห็นว่าขนาดของสารต้านเอนไซม์เบตาแลคทาเมสที่ให้ร่วมกับยากลุ่มเบตาแลคแทมนั้นเป็นปริมาณที่เหมาะสมสำหรับการนำมาใช้ทางคลินิก

ภาควิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2545 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

4476569933 : MAJOR PHARMACOLOGY
KEY WORDS : SYNERGISM / BACTERICIDAL ACTIVITY / POST β-LACTAMASE INHIBITOR EFFECT / β-LACTAMASE INDUCTION EFFECT / GRAM NEGATIVE BACTERIA

DUANGKAMON AMONSAKSOPON : *IN VITRO* PHARMACODYNAMIC STUDIES OF β-LACTAM-β-LACTAMASE INHIBITOR COMBINATIONS AGAINST β-LACTAMASE PRODUCING CLINICALLY IMPORTANT GRAM-NEGATIVE BACTERIA. THESIS ADVISOR : ASSOC. PROF. SIRIPORN FUNGWITTHAYA, THESIS COADVISOR : PROF. NALINEE ASWAPOKEE, 112 pp. ISBN 974-17-2959-6.

Combinations of the β-lactam and β-lactamase inhibitor are antibiotics extensively used in clinic for the treatment of infectious disease caused by the β-lactamase producing bacteria. The mode of action of β-lactamase inhibitor is regarded as irreversible, suicide inhibitors of the target enzyme resulting in persistent activity of β-lactams to inhibit bacterial cell wall synthesis, which leads to cell death. The present study aimed to evaluate the synergistic interaction between β-lactams and β-lactamase inhibitors on clinically important β-lactamase producing gram-negative bacteria by checkerboard technique and time kill method. Clavulanic acid at 2 μg/ml demonstrated synergy to amoxicillin against *Moraxella catarrhalis* and *Haemophilus influenzae* by reduction MIC of amoxicillin to 64 times. Similarly tazobactam at 4 μg/ml could reduce the MIC of piperacillin against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* to 64 and 4 times, respectively. The MIC of cefoperazone against *P. aeruginosa* was decreased 8 times when being combined with sulbactam 8 μg/ml whereas *Acinetobacter baumannii* required sulbactam 32 μg/ml to reduce the MIC of cefoperazone. Additionally, β-lactams (amoxicillin, piperacillin, cefoperazone) at 2 MIC in concomitant with β-lactamase inhibitors at average concentration (clavulanic acid at 2 μg/ml, tazobactam at 4 μg/ml, sulbactam at 8 μg/ml) demonstrated the antibacterial properties and synergistic activity by decreasing colony forming unit more than 100 fold comparing with the most active single drug except for *A. baumannii* that required sulbactam at least 32 μg/ml to show those properties. Regarding to post β-lactamase inhibitor effect (PLIE), amoxicillin-clavulanic acid and piperacillin-tazobactam manifested the time period of PLIE that correlated to concentration of β-lactamase inhibitors against *H. influenzae* and *P. aeruginosa*, respectively. Furthermore it found that one of three β-lactamase inhibitors, clavulanic acid, demonstrated β-lactamase induction effect by inducing *Enterobacter cloacae* to produce β-lactamase that destroyed cefuroxime as tested by double disks as well as agar dilution methods. The MIC of cefuroxime was increased from 6 μg/ml to 32 μg/ml on exposure to clavulanic acid 10 μg/ml. The results obtained suggest that the concentration of β-lactamase inhibitors and β-lactams under studies are appropriate for clinical application.

Department of Pharmacology ..... Student's signature .....

Field of study Pharmacology ..... Advisor's signature .....

Academic year 2002 ..... Co-advisor's signature .....

ACKNOWLEDGEMENTS

I am deeply indebted to the following individuals and institutions for their assistance in conducting this thesis.

- Professor Nalinee Aswapokee
- Associate Professor Siriporn Fungwitthaya
- Mrs. Surapee Tienkrim
- Mrs. Busaba Charoensook
- Ms. Kantima Sangsiriwut
- Glaxosmith Kline Co. Ltd.
- Pfizer Co. Ltd.
- Wyeth-Ayerst Co. Ltd.
- The Graduate School of Chulalongkorn University
- The Laboratory of Infectious Diseases and Preventive Medicine, Faculty of Medicine (Siriraj Hospital), Mahidol University
- The Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University

Furthermore, I am grateful to the committee members: Associate Professor Mayuree Tantisira and Associate Professor Pintip Pongpech for their valuable advice. Finally, I would like to express my appreciation to all staff members and students in the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their technical assistance and encouragement.

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LIST OF ABBREVIATIONS

% v/v = percent of volume by volume (ml/100ml)

% w/v = percent of weight by volume (g/100 ml)

°C = degree Celsius

aa = amino acid

AUC = Area under the curve

A. baumannii = *Acinetobacter baumannii*

BA24 = Bacteriolytic area of 24 hours

BL = β-lactam

BI = β-lactamase inhibitor

CFU = Colony forming unit

DAP = Diaminopimelic acid

D-ala = D-alanine

D-Glu = D-glutamate

E. cloacae = *Enterobacter cloacae*

e.g. = exempli gratia (for example)

enz. = enzyme

et al. = et alii (and other peoples)

etc. = et cetera (and other similar things)

FIC = Fractional inhibitory concentration

Fig = Figure

g = gram

hr = hour

H. influenzae = *Haemophilus influenzae*

IC_{50} = Inhibition concentration 50%

KR3 = Killing rate of the first 3 hours

K. pneumoniae = *Klebsiella pneumoniae*

L = Liter

L-ala = L-alanine

log = decimal logarithm

LPS = Lipopolysaccharide

MBC = Minimum bactericidal concentration

MHA = Meuller-Hinton agar

MHB = Meuller-Hinton broth

MIC = Minimum inhibitory concentration

min = minute

ml = milliliter

mm = millimeter

mol = mole

M. catarrhalis = *Moraxella catarrhalis*

NAG = N-acetylglucosamine

NAM = N-acetylmuramic acid

NCCLS = The National Committee for Clinical Laboratory Standards

nm = nanometer

NSS = Normal saline solution

P. aeruginosa = *Pseudomonas aeruginosa*

PAE = Post antibiotic effect

PBP = Penicillin binding protein

PLIE = Post β-lactamase inhibitor effect

UDP = Uridine diphosphate

μg = microgram

μm = micrometer

UMP = Uridine monophosphate

USA = The United States of America

UTP = Uridine triphosphate

CHAPTER I

INTRODUCTION

Since the discovery of the first antibiotic of penicillin over 40 years ago, the mortality rates of patients with infectious diseases have largely decreased. After a while, the bacterial resistance to antibiotics increased because of irrational use of antibiotic especially the β-lactam groups that are the most widely used for the management of many bacterial infections. Since the mechanism of action of β-lactam antibiotics is specific to bacterial cell wall, they are therefore highly safe antibiotics for treatment of the infection caused by bacteria in human. As a result, a large number of β-lactam modified antibiotics have been developed and available in health center until to the present era. The current of β-lactam antibiotics are classified into six groups as their core β-lactam ring structure (Figure 1-1).

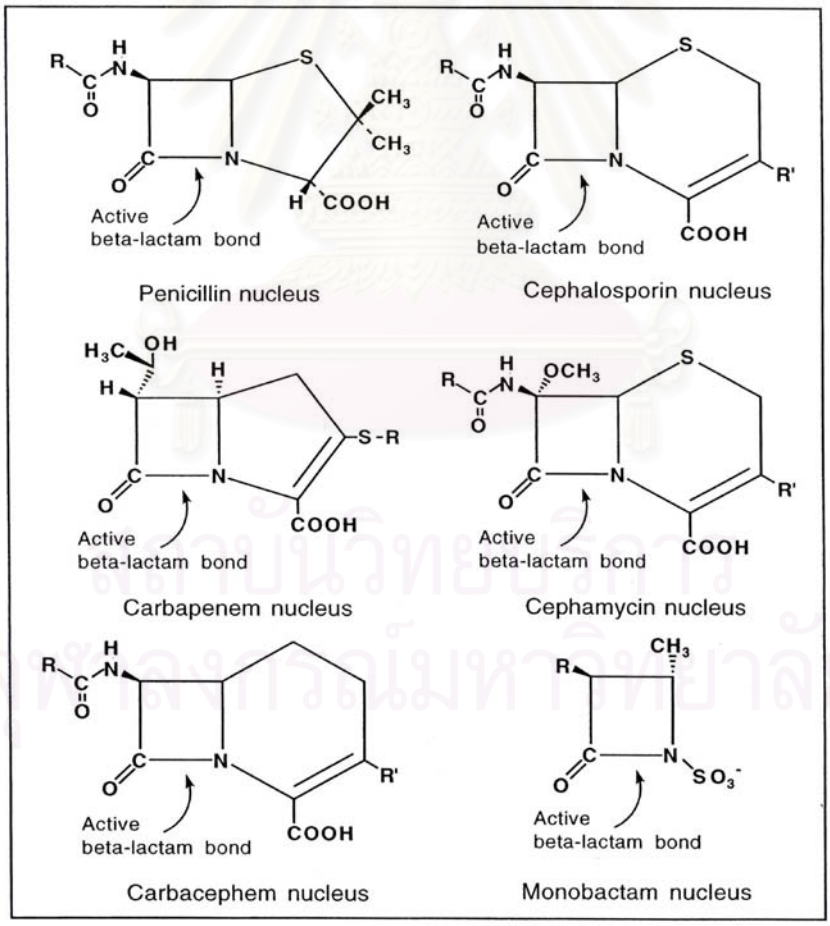


Figure 1-1 Basic structure of penicillin, cephalosporin, carbapenem, cephamycin, carbacephem and monobactam.

The β-lactam antibiotics are able to execute the bacteria since their β-lactam ring closely resembles the configuration of D-alanyl-D-alanine that is substrate for enzyme named Penicillin Binding Protein (PBP); PBPs are vital enzymes in the synthesis of peptidoglycan layer. β-lactams inhibit bacterial cell wall synthesis by penetration into periplasmic space and irreversible affix to PBP. As a result, PBP cannot function as active enzyme. After all, the cell wall synthesis can no longer continue causing bacterial death eventually. Nonetheless, the extravagant β-lactams performing has been important motive of the bacterial resistance that is becoming an increasing problem for clinicians, in both hospital and community settings. The mechanisms account for clinically significant bacterial resistance to β-lactam antibiotics consisting of 3 types as shown in Table 1-1.

Table 1-1 The mechanisms of resistance to β-lactam antibiotics in clinical situation.

I. Alter target sites (PBPs, Penicillin binding proteins)

A. Decrease affinity of PBPs for β-lactam antibiotics

1. Modify existing PBPs
   a. Create mosaic PBPs, e.g., Insert nucleotides obtained from neighboring bacteria, e.g., penicillin resistant *Streptococcus pneumoniae*
   b. Mutate structural gene of PBPs, e.g., ampicillin resistant β-lactamase negative *Haemophilus influenzae*
2. Import new PBPs, e.g., mecA in methicillin resistant *Staphylococcus aureus*

II. Destroy β-lactam antibiotics

A. Increase production of β-lactamase

1. Acquire more efficient promoter
   a. Mutate existing promoter
   b. Import new one
2. Deregulate control of β-lactamase production
   a. Mutate regulator gene, e.g., ampD in "stably derepressed" *Enterobacter cloacae*

B. Modify structure of resident β-lactamase

1. Mutate its structural gene, e.g., extended spectrum β-lactamases in *Klebsiella pneumoniae*

C. Import new β-lactamases with different spectrum of activity

III. Decrease concentration of β-lactam antibiotics inside cell

A. Restrict its entry (loss of porins)

B. Pump it out (efflux mechanism)

(Modified from Opal, Mayer, and Medeiros, 2000)

Of these three mechanisms, PBP alterations are the most important mechanisms of resistance in gram-positive bacteria. On the contrary, β-lactamase destruction of antibiotics is predominant in gram-negative species. The types of β-lactamases have been classified into several schemes, but a generally accepted classification scheme is the one established by Amber (1980). This classification arranges the β-lactamases into four groups according to β-lactam molecular weight.

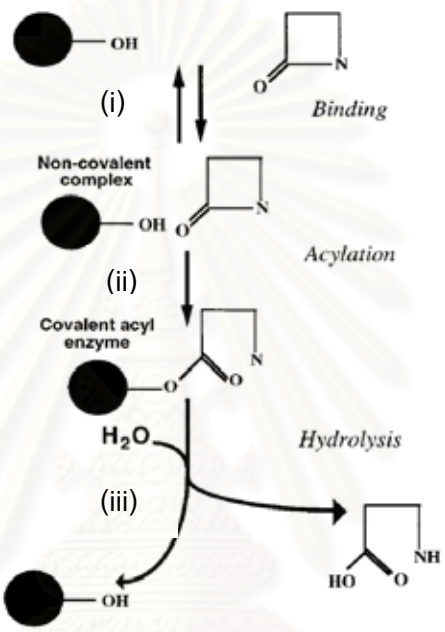
Table 1-2 Classification schemes for bacterial β-lactamases

Structural class (Ambler)	Functional group (Bush)	Preferred substrates	Inhibition by clavulanate	Representative enzyme
Serine β-lactamase				
A	2a	Penicillins	+ +	Penicillinases from gram-positive bacteria
	2b	Penicillins, cephalosporins	+ +	TEM-1, TEM-2, SHV-1
	2be	Penicillins, narrow-spectrum and extended-spectrum cephalosporins, monobactams	+ +	TEM-3 to TEM-26, SHV-2 to SHV-6, *Klebsiella oxytoca* K1
	2br	Penicillins	-	TEM-30 to TEM-36, TRC-1
	2c	Penicillins, carbenicillin	+	PSE-1, PSE-3, PSE-4
	2e	Cephalosporins	+ +	Inducible cephalosporinases from *Proteus vulgaris*
	2f	Penicillins, cephalosporins, carbapenems	+	NMC-A from *Enterobacter cloacae*, Sme-1 from *Serratia marcescens*
C	1	Cephalosporins	-	AmpC enzymes from gram-negative bacteria; MIR-1
D	2d	Penicillins, cloxacillin	±	OXA-1 to OXA-11, PSE-2 (OXA-10)
Undetermined	4	Penicillins	-	Penicillinase from *Pseudomonas cepacia*
Zinc β-lactamase				
B	3	Most β-lactams, including carbapenems	-	L1 from *Xanthomonas maltophilia*, CcrA from *Bacteroides fragilis*

+ +, Strong inhibitor of all members of class, +, moderate inhibition, ±, inhibition varies within the class, - , negligible inhibition

(Modified from Williams, 1999 and Bush et al., 1995)

Phases of the reaction of catalyzing the β-lactam antibiotics by β-lactamase include (i) reversible non-covalent binding of the β-lactamase and the β-lactam ring, (ii) rupture of the β-lactam ring, which becomes covalently acylated on to the active site serine. (iii) hydrolysis of the acyl enzyme to reactive the β-lactamase, splitting the amide bond, and liberate the inactivated drug molecule. As a result, the antibiotics can no longer inhibit bacterial cell wall synthesis (Figure 1-2).



(Modified from Livermore, 1995)

Figure 1-2. Action of a serine β-lactamase to β-lactam antibiotic

Nevertheless, in the present there are new drugs developed to nurse the infectious diseases that resistance to β-lactam antibiotics caused by β-lactamase producing bacteria. The drug's group bestowed in the most clinical therapeutic is β-lactam-β-lactamase inhibitor (BL-BI). The BL-BI is combination between β-lactam that is not qualified to kill the bacteria right now with β-lactamase inhibitor that enable to bind irreversible to the β-lactamase. As the result, BL-BI combinations are efficient to destroy the bacteria that resisted to β-lactams.

The β-lactamase inhibitors are structurally related to β-lactam antibiotics, retaining the amide bond of the β-lactam ring of the parent compound, but with a modified side chain. These structural features enable the inhibitors to bind irreversibly as suicide substrates to the β-lactamases, rendering them inactive. There are three β-lactamase inhibitors currently used in clinical practice namely clavulanic acid, sulbactam and tazobactam (Figure 1-3).

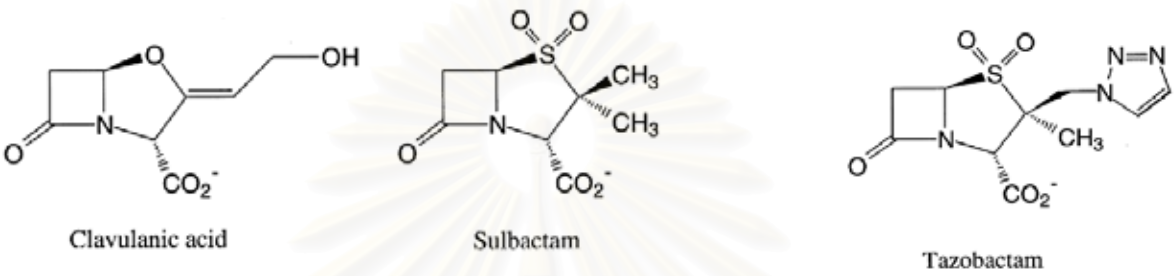


Figure 1-3. Molecular structures of β-lactamase inhibitors

β-lactamase inhibitors are not only able to inhibit the β-lactamase capacity, but they also exhibit β-lactamase induction effect notably Amp C that is β-lactamase categorized in cephalosporinase group. Thus, the medical team should carefully practice the BL-BI combinations. Nowadays, there are five currently available BL-BI combinations, which are drug of choice for the treatment infectious diseases caused by β-lactamase producing bacteria as shown in table 1-3.

Table 1-3. β-Lactam-β-lactamase inhibitors for clinical use

β-lactam	β-lactamase inhibitor	Administration route	Combination (mg.) (BL:BI)
Ampicillin	Sulbactam	Parenteral and oral	1000:500
Cefoperazone	Sulbactam	Parenteral only (not available in the USA)	2000:1000 500:500 (Thailand)
Piperacillin	Tazobactam	Parenteral only	4000:500
Ticarcillin	Clavulanic acid	Parenteral only	3000:100
Amoxicillin	Clavulanic acid	Parenteral and oral (only oral form available in the USA)	1000:200, 500:100 250:125, 500:125

BL = β-lactam, BI = β-lactamase inhibitor

In order to use the β-lactam and β-lactamase inhibitor combination to treat infectious diseases effectively, both pharmacokinetics and pharmacodynamics should be considered. Pharmacokinetics determines the time course of drug concentration in serum meanwhile pharmacodynamics is important to the relationship between serum concentration and the pharmacological and toxicological effects of drugs. Additionally, there are many factors influence the activity and pharmacodynamics of BL-BI combinations as follows:

1. Potency of both β-lactam and β-lactamase inhibitor agents
2. Pharmacokinetics of the β-lactamase inhibitors.
3. Type and quantity of β-lactamases produced by the target bacteria
4. Potential for the β-lactamase inhibitors to induce expression of chromosomal cephalosporinases.

Several studies over the past decade have demonstrated that β-lactam antibiotics exerted the least post antibiotic effect (PAE) referring to persistent suppression of bacterial growth following exposure to an antimicrobial agent for gram-negative bacteria. Moreover, there were many evidences to imply diverse effects in the direction of PAE value such as type and concentration of antimicrobial agent, type of bacteria, exposure time etc. On the other hand, the recent researches by Thorburn and colleagues (1996) and Murbach and colleagues (2001) have demonstrated the phenomenon of continuing suppression of bacterial growth after briefly contact to β-lactamase inhibitor agents termed the post β-lactamase inhibitor effect (PLIE). Hence, The present study aims to investigate the appropriate ratio and concentration of each BL-BI combinations for its clinically important organisms and to compare the β-lactamase induction effect in each β-lactamase inhibitor by quantitative determination. In addition to study the phenomenon of a PLIE along with consider the influence of β-lactamase inhibitor concentration to PLIE. All research questions are established in order to clarify various impacts to the activity of BL-BI combinations to improve and provide appropriate dosage regimens for utilizing the clinical practice in the future.

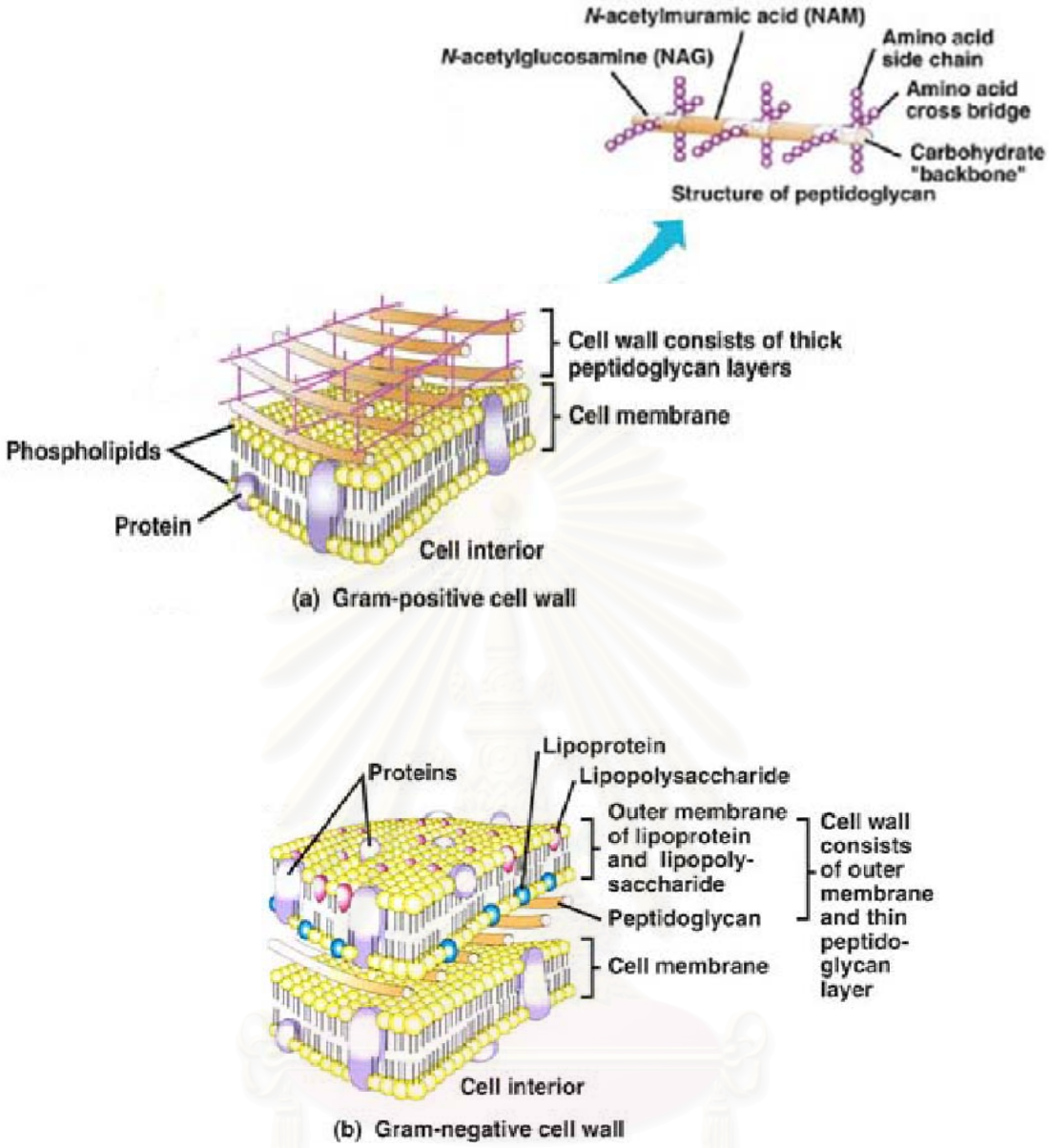
CHAPTER II

LITERATURES REVIEW

1. Bacterial Cell Wall Synthesis

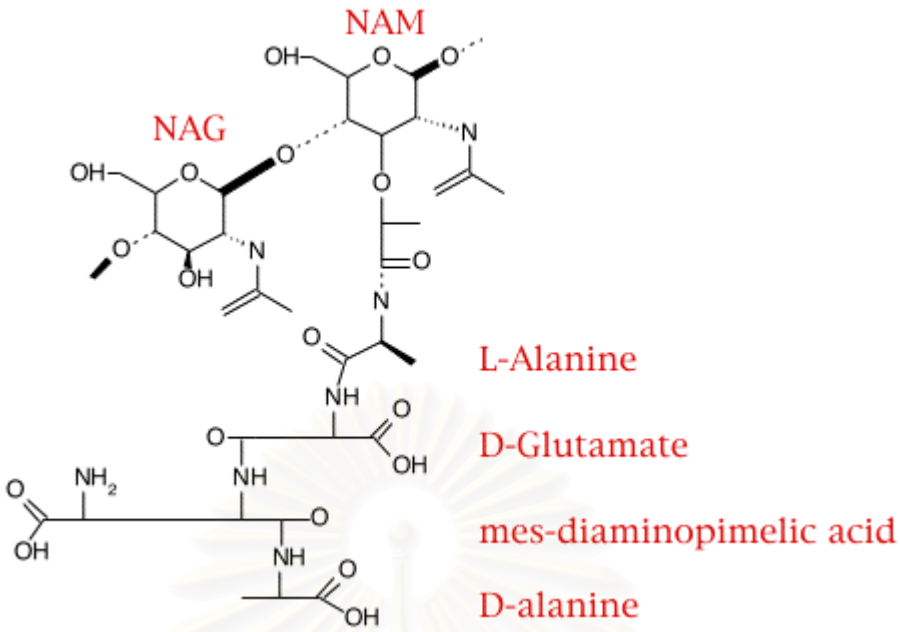
The most important function of the bacterial cell wall is to provide a semipermeable barrier through which only desired substances may pass, to provide a barrier against osmotic stress and to prevent digestion by host enzyme. Bacteria can be distinguished from one another by their morphology (size, shape, and staining characteristics) metabolic, antigenic, and genetic characteristics. Nevertheless, gram strain is a powerful, easy test that allows clinicians to distinguish between the two major classes of bacteria as gram-positive and gram-negative bacteria (Murray, et al., 1998).

The cell walls of gram-positive (Figure 2-1a) and gram-negative (Figure 2-1b) bacteria differ considerably. In gram-positive bacteria the peptidoglycan layer is about 25 nm and contains an additional polysaccharide called teichoic acid. About 60-90 percent of the cell wall is peptidoglycan, and the material is so abundant that gram-positive bacteria are able to retain the crystal violet-iodine complex in gram staining. By contrast, the cell wall of gram-negative bacteria has no teichoic acid, and its peptidoglycan layer is only about 3 nm thick. The wall is enclosed by an outer membrane not found in gram-positive bacteria. The membrane consists of two rows of molecules: an inner row of phospholipid; and an outer row of lipopolysaccharide (LPS) not found in any other living thing (McKane and Kandel, 1996). Peptidoglycan, an important chemical constituent of the cell wall in bacteria, is a very large molecule composed of alternating units of two amino-containing carbohydrates, *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), joined by cross-bridges of four amino acids (D-glutamate, mes-diaminopimelic acid, D-alanine, L-alanine) as illustrated in figure 2-1a and figure 2-2. The chemical composition of the bacterial cell wall differs significantly from that of the mammalian lipid bilayer, and as such provides multiple targets for the development of specific bactericidal agent.



(Modified from Alcamo, 2000)

Figure 2-1 A comparison of the cell walls of gram-positive and gram-negative bacteria (a) The cell wall of a gram-positive bacterium is composed of peptidoglycan layers combined with teichoic acid molecules. The structure of peptidoglycan is shown as units of NAG and NAM joined laterally by amino acid cross-bridges and vertically by side chains of four amino acids. (b) In gram-negative cell wall, the peptidoglycan layer is much thinner, and there is no teichoic acid. Moreover, an outer membrane closely over lines the peptidoglycan layer so that the membrane and layer comprise the cell wall.

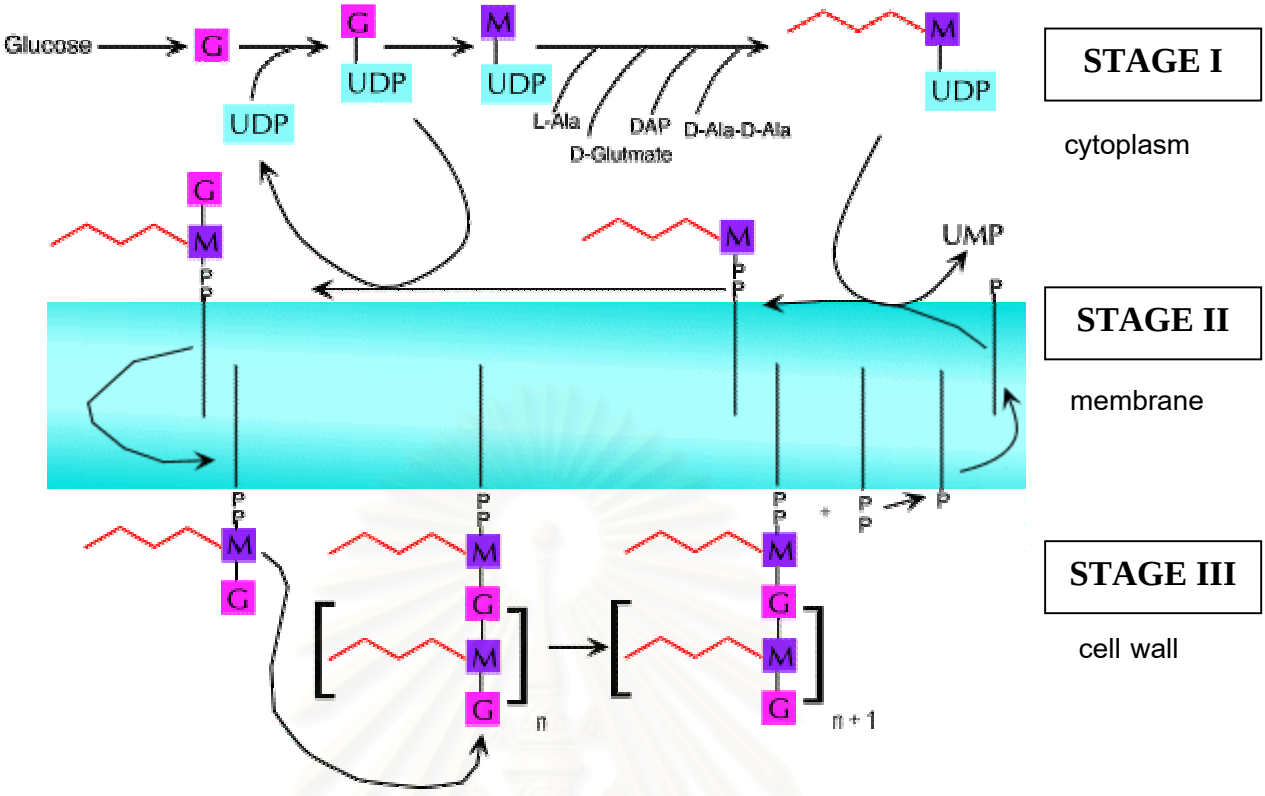


(Modified from Paustian, 2003)

Figure 2-2 The chemical structure of peptidoglycan

- The biosynthesis of peptidoglycan

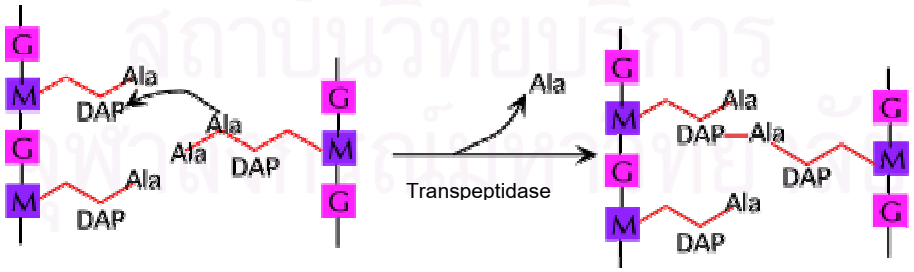
Synthesis of peptidoglycan can be divided into three stages according to where the reactions take place. The first series of reaction, the formation of building block (UDP-acetylmuramyl-pentapeptide) that make up peptidoglycan taken place in the cytoplasm. In the second stage, the precursor unit is carried from inside the cell membrane to outside. During this process, UDP-acetylmuramyl-pentapeptide and UDP-acetylglucosamine are linked covalently to the preexisting cell wall (with the release of the uridine nucleotide). The third stage of process involves the completion of the cross-link. This is accomplished by a transpeptidation reaction that taken place entirely outside the cell membrane (Figure 2-3). The transpeptidase itself is membrane bound. The terminal glycine residue of the pentaglycine bridge is linked to the fourth residue of the pentapeptide (D-alanine), releasing the fifth residue (D-alanine) (Figure 2-4)



(Modified from Paustian, 2003)

Figure 2 -3 Diagram of the biosynthesis of peptidoglycan including 3 stages.

M= *N*-acetylmuramic acid (NAM); G= *N*-acetylglucosamine (NAG); UDP= Uracil diphosphate; L-Ala= L-Alanine; D-Glu= D-Glutamate; DAP= diaminopimelic acid; D-Ala= D-Alanine.

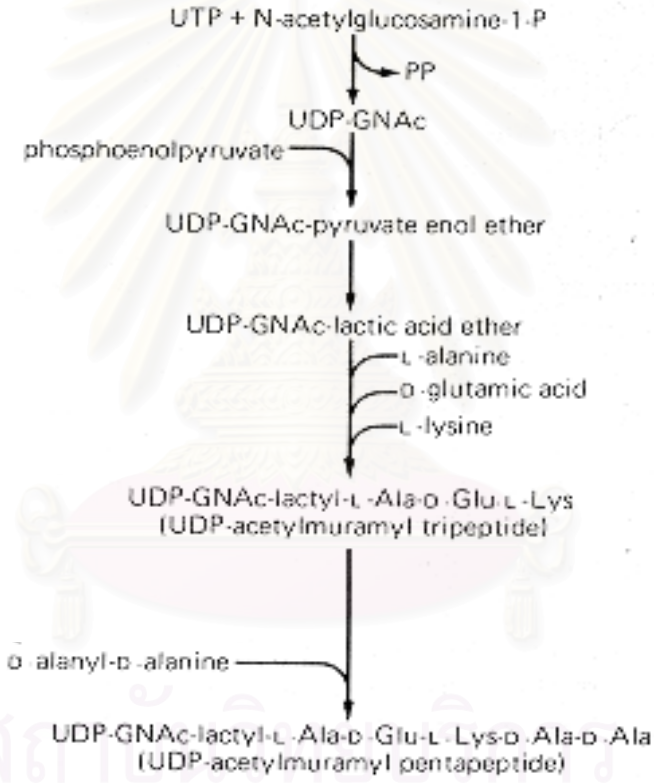


(Modified from Paustian, 2003)

Figure 2-4 Transpeptidation of peptidoglycan chains.

STAGE I : PRECURSOR FORMATION.

Synthesis of the monomers of peptidoglycan begins with glucose, which is readily converted into *N*-acetylglucosamine (NAG). Synthesis begins by activating NAG with the addition of uracil diphosphate (UDP), which serves as a carrier of the growing peptidoglycan during its synthesis in the cytoplasm. Phosphoenol pyruvate is then added to UDP-NAG and this is then converted into UDP-NAM. Next, the UDP-NAM-peptide is formed by four sequential additions of the appropriate amino acids (L-alanine, D-glutamic acid, L-lysine and finally two D-alanines) as shown in figure 2-5.

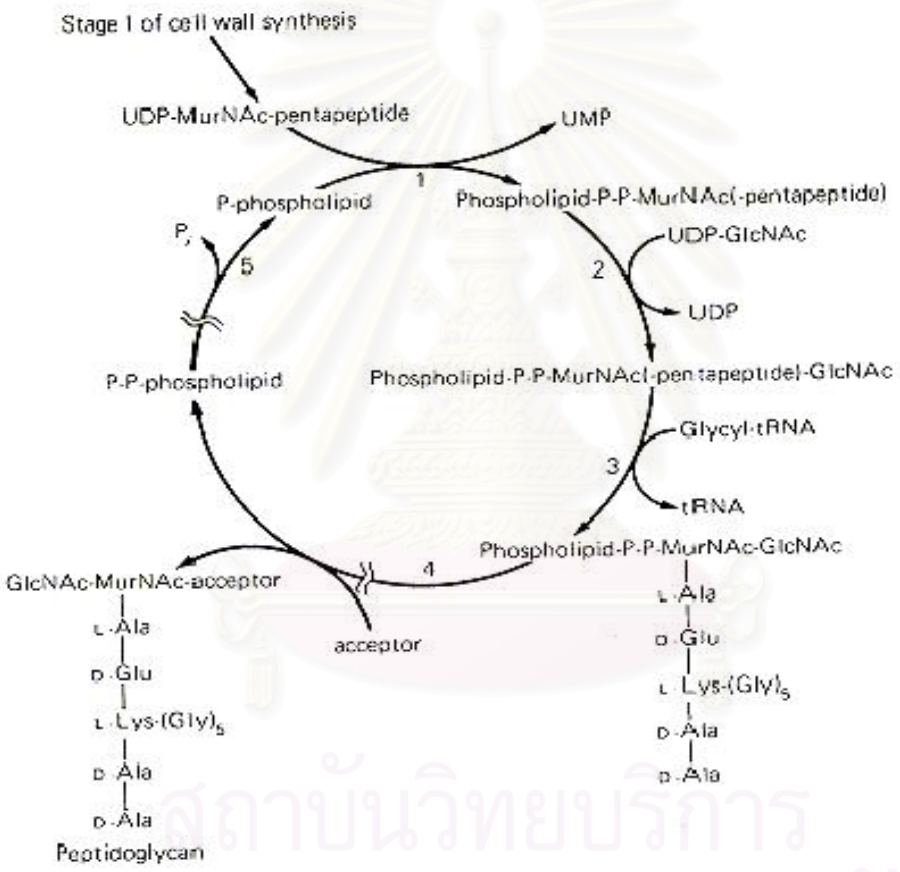


(Modified from Scholar and Pratt, 2000)

Figure 2-5 The sequence of reactions comprising the first stage of peptidoglycan synthesis in *S. aereus*.

STAGE II : FORMATION OF A LINEAR PEPTIDOGLYCAN.

In the second stage, the two uridine nucleotide UDP-acetylmuramyl pentapeptide and UDP-*N*-acetylglucosamine are linked together to form a linear polymer. During the stage, the peptidoglycan precursor units are attached to the cell membrane. In the first reaction, the sugar pentapeptide becomes attached by a pyrophosphate bridge to a phospholipid bound to the cell membrane. Then a second sugar derived from UDP-*N*-acetylglucosamine is added to form a disaccharide (-pentapeptide)-p-p-phospholipid.

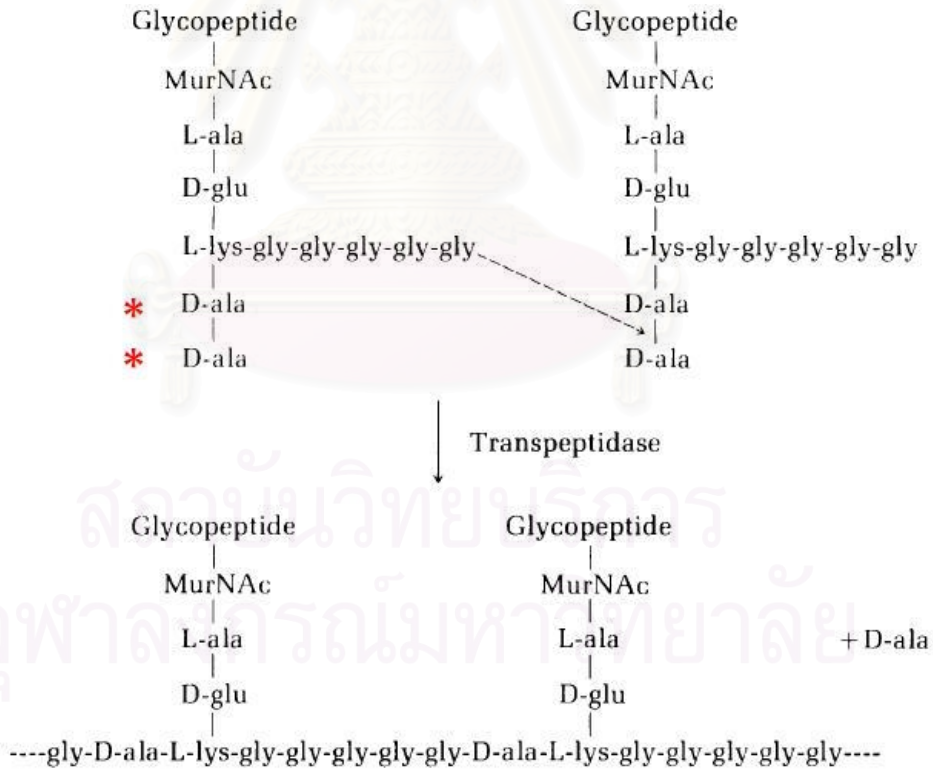


(Modified from Strominger et al, 1967)

Figure 2-6 The second stage of cell wall synthesis in *S. aureus*. An ATP-requiring amidation of glutamic acid that occurs between reaction 2 and reaction 3 has been omitted.

STAGE III : CROSS-LINKING OF THE PEPTIDOGLYCAN.

The terminal reactions in cell wall synthesis take place outside the cell. At this stage, the glycopeptide polymers become cross-linked to each other by means of a transpeptidation reaction. The transpeptidation is occurred between the free amine of the amino acid in the 3rd position of the pentapeptide and the D-alanine at the fourth position of the outer peptide chain, result to the releasing of terminal D-alanine of the precursor (Figure 2-7). This step requires no additional energy because peptide bonds are traded. The cross-linking reaction is catalyzed by membrane bound transpeptidases. Related enzymes, DD-carboxypeptidases, remove extra terminal D-alanines, which limit the extent of cross-linking. Thus, each polypeptide side chain of each repeating unit becomes covalently linked to the side chains in two neighboring peptidoglycan strands. Both of these enzymes are called penicillin-binding proteins (PBPs) because they are targets for penicillin and other β-lactam antibiotics.



(Modified from Strominger et al, 1967)

Figure 2-7. The third stage of cell wall synthesis in *S. aureus*: cross-linking of peptidoglycan polymers by the joining of the peptide side chains with the elimination of D-alanine.

2. Mechanism of β-Lactam Antibiotics

Penicillins and cephalosporins are the most common of the β-lactam antibiotics, which are similar in structure and activity (Figure 2-8). The basic penicillin structure is composed of a thiazolidine ring attached to a four member (β-lactam) ring. Whereas, the cephalosporin structure contains a dihydrothiazine ring joined to β-lactam ring. Other β-lactam antibiotics also used clinically include the carbapenem, cephamycin, carbacephem and monobactam, which have a similar basic structure with a β-lactam ring. The four-member ring is somewhat strained, and a number of important ring-opening reactions take place here.

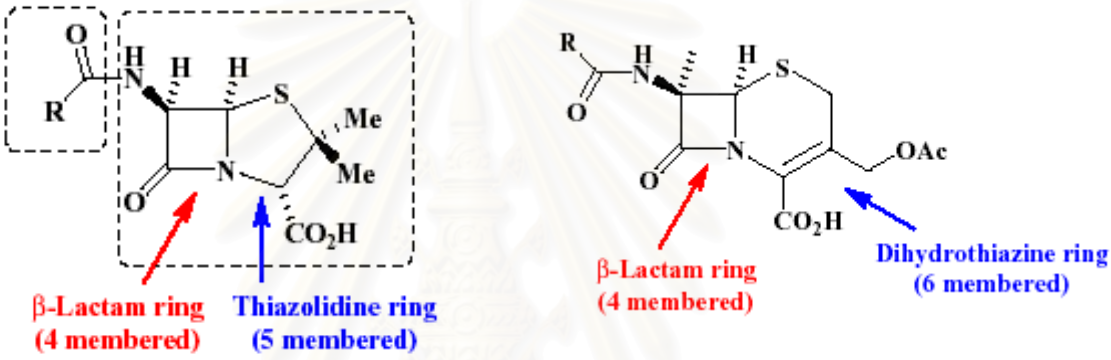
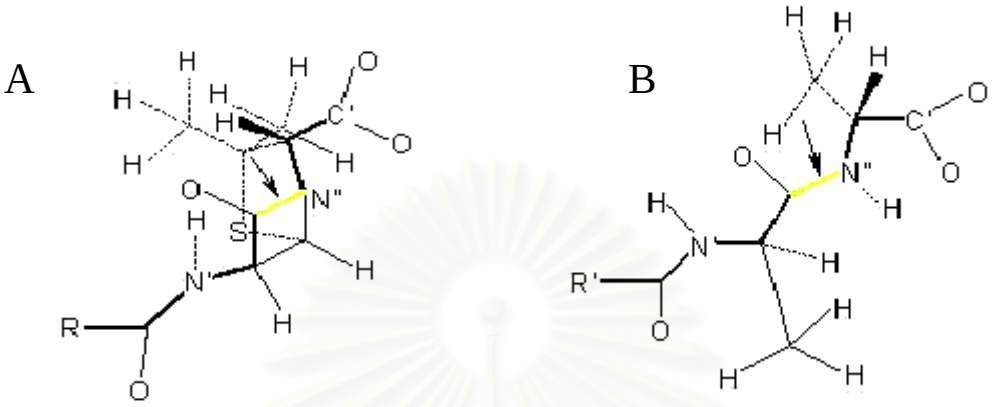


Figure 2-8 Basic structure of penicillins and cephalosporins

The principle mechanism of β-lactam antibiotics in killing microorganism is the inhibition of transpeptidase, which is the vital enzyme in cross-linking of the peptidogycan. β-lactams are proposed to act either as a transition-state analogue or as an allosteric inhibitor because the nitrogen-carboxyl (N-CO) bond of their ring is similar in structure and position to the peptide D-alanyl-D-alanine bond that is cleaved in the transpeptidation reaction as illustrated in figure 2-9. However, there are slight differences between the two molecules in conformation that are differences in bond angles and lengths.

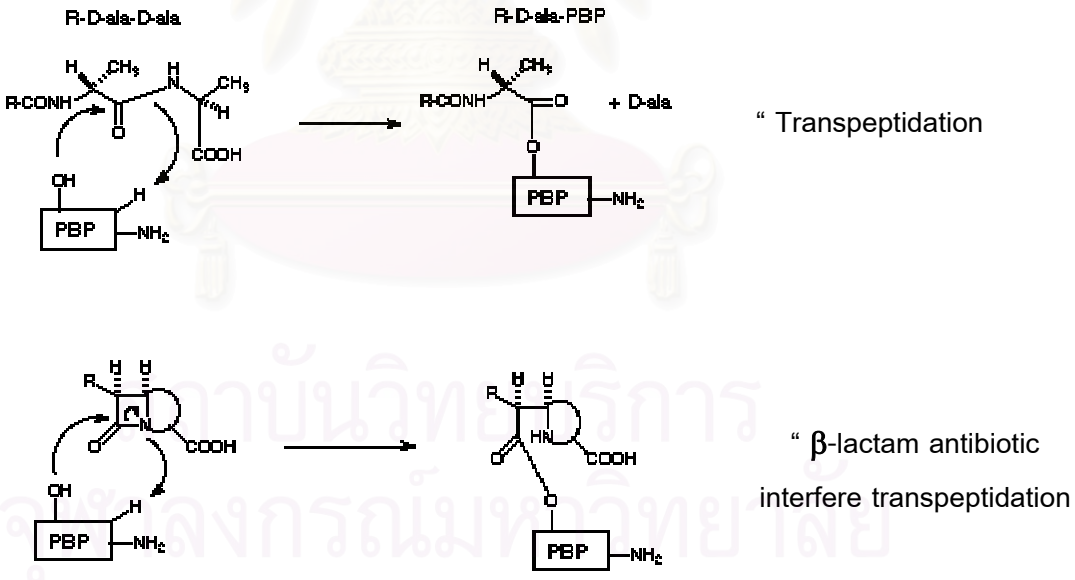
Under natural conditions the PBP enzymes performing the transpeptidation reaction and/or carboxypeptidation reactions, react with acyl-D-alanyl-D-alanine to form an acyl-D-alanyl-enzyme complex, with the elimination of the terminal D-alanine. The complex would then interact with a free amino group on another peptide side-chain, resulting in cross-linking of the two chains and release of the free enzyme (Bryan and Godfrey, 1991). Treatment of the bacteria with a β-lactam antibiotic would interfere with this process of cell wall synthesis. A penicilloylated enzyme complex, formed following interaction of the enzyme

with penicillin, would act as a competitor to formation of the normal acylated enzyme (Figure 2-10). The consequence of this competition is interference with the normal cross-linking of the cell wall, resulting in disruption and eventual death of the bacterial cell.



(Modified from Strominger et al, 1967)

Figure 2-9 Stereomodels of penicillin (A) and of the D-alanyl-D-alanine end of the peptidoglycan strand (B). The arrows indicate the N-CO bond in the β–lactam ring of the penicillin and the N-CO bond in the D-alanyl-D-alanine end of the peptidoglycan strand.



(Modified from Scholar and Pratt, 2000)

Figure 2-10 Proposed mechanism of transpeptidase inhibition by penicillin. Penicillin occupies the D-alanyl-D-alanine substrate site of transpeptidase, the reactive four-membered (β-lactam) ring is broken by cleavage at the N-CO bond, and the antibiotic becomes linked to the enzyme by a covalent bond.

Classification of the penicillin-sensitive enzymes as penicillin binding protein (PBP) and subsequent study of these proteins resulted in the formation of a model of activity based on the behavior of each bacterial strain in the presence of different β-lactams. *E. coli* was the first microorganism elucidated the different functions of PBPs by Spratt,1975. Inhibition of PBP1A and 1B by a β-lactam results ultimately in cellular lysis. Inhibition of PBP2 results in the formation of spherical cells that eventually lysis, suggesting that PBP2 has a role in an initial step in cell elongation and in determining the rod shape. Inhibition of PBP3 leads to the formation of filaments, indicating that it is important for the ordered process of cross wall formation and cell division. PBPs 4,5, and 6 were originally thought to be nonessential to the bacterium (Bryan and Godfrey, 1991). The functions performed by PBPs in other species have not been well defined but presumably also are distinct.

No single PBP species is the target of β-lactam antibiotics, which produce their lethal effect on bacteria by inactivation of multiple PBPs simultaneously. The lethal effect in both gram-positive and gram-negative organisms appears to be cell cycle-dependent, with inhibition of PBPs leading to disruption of a crucial event probably at the time of cell division. Unopposed action of autolysins occurring when PBPs are inhibited by β-lactam antibiotics may also contribute to the antibacterial effect in some organisms.

Table 2-1 Properties of PBPs from *E. coli* and theirs response with β–lactam antibiotics

PBP	Molecular weight	Molecules/cell	Morphological changes after occupied by β-lactams
1A	91	230	Spheroplasting cells
1B	86.5	81.5	
2	66	20	Ovoidal cells
3	60	50	Filamentous cells
4	49	110	-
5	42	1800	-
6	40	5700	-

(Modified from Spratt, 1975; Hayes and Ward, 1986)

3. Mechanism of bacterial resistance to β-lactam antibiotics

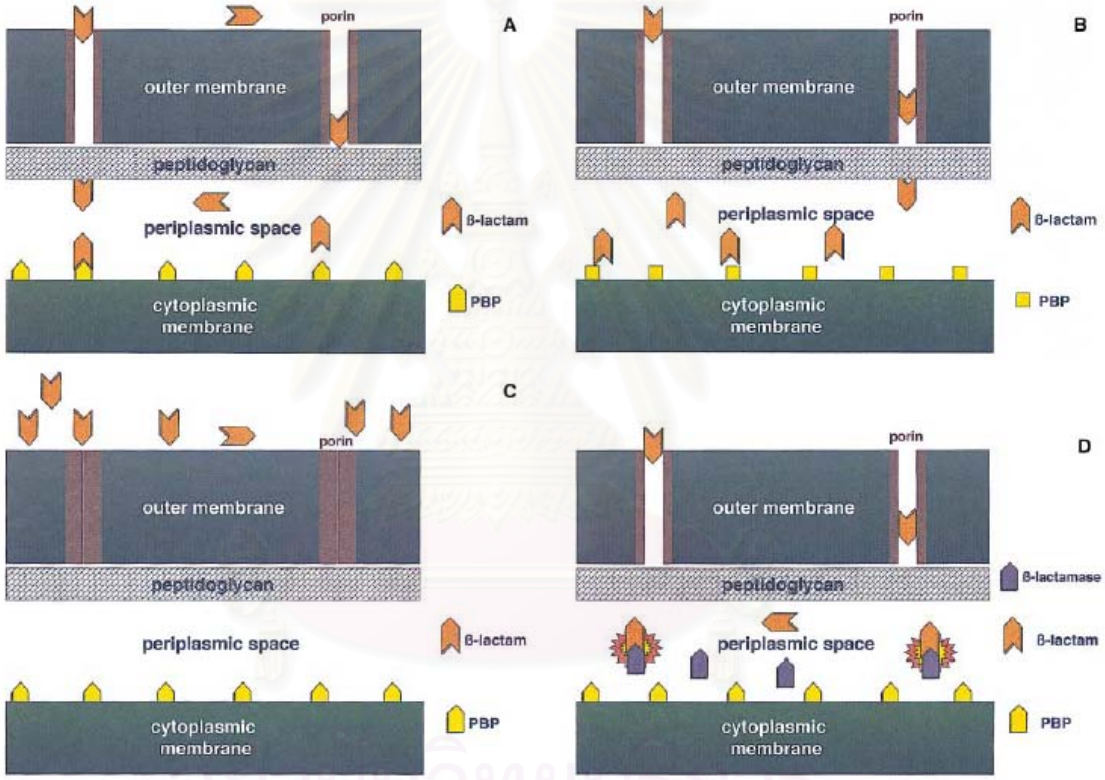
β-Lactam antibiotics are the most varied and widely used of all the groups of antimicrobial agents. Starting from the discovery of benzylpenicillin in 1928 and its first clinical use in 1940. β-Lactams account for 50% of all systemic antimicrobial use because they have low toxicity and the availability of so many derivatives. β-Lactam antibiotics exert their antimicrobial effect by interfering with cell wall biosynthesis in the susceptible bacterial cell. This is accomplished by the drugs attaching covalently to their targets, the penicillin-binding proteins (PBPs). The PBPs are diverse enzymes involved in cell wall synthesis, and are anchored in the cytoplasmic membrane of the bacterium. The site at which β-lactam antibiotics bind to PBPs is located on the portion of the PBP that extends into the periplasmic space of gram-negative bacteria. Covalent binding to PBPs interferes with synthesis of cell wall and ultimately leads to cell death (Figure 2-11A). The resistance to β-Lactam antibiotics has been found after a while penicillin had been used in human and this evidence is becoming a significant problem for clinician. Resistance to β-lactam antibiotics arises through one or more of the following mechanisms:

(1) The target sites for the β-lactams are the PBPs in the cytoplasmic membrane. Modification in one or more PBPs may influence their binding affinity for β-lactam antibiotics and hence the sensitivity of the altered bacterial cell to inhibition by these antibiotics (Figure 2-11B). Such a mechanism is responsible for penicillin resistance in pneumococci, methicillin resistance in staphylococci, and for an increasing number of bacteria with intrinsic resistance to the β-lactams, such as gonococci, enterococci, and *Haemophilus influenzae*.

(2) The outer cell membrane of gram-negative bacteria provides an efficient barrier to the penetration of β-lactam antibiotics to their target PBPs in the bacterial cell membrane. β-lactams must generally pass through the hydrophilic porin protein channels in the outer membrane of gram-negative bacteria to reach the periplasmic space. Alteration in the porins in the outer membrane may manipulate in a decreased ability of drug to penetrate through the membrane and reach PBPs (Figure 2-11C). The permeability barrier of the outer membrane is a major factor in the resistance of *Pseudomonas aeruginosa* to many β-lactam antibiotics.

(3) The organism may produce one or more β-lactamases that catalyze the hydrolysis of the β-lactam ring, splitting the amide bond. As a result, the antibiotics can no longer inhibit bacterial cell wall synthesis (Figure 2-11D). β-lactamase production has been widely reported among the Enterobacteriaceae, *Haemophilus influenzae*, *Moraxella* spp., and *Pseudomonas aeruginosa.*

In gram-negative organisms, the interplay between two or more of these mechanisms plays an important role in determining resistance to an antibiotic. However, the production of β-lactamase enzyme is the most frequent and most efficient mechanism of resistance to β-lactams, which are now seen in a wide variety of clinically important bacteria.



(Modified from Pitout, et al., 1997)

Figure 2-11 Resistance to β-lactam antibiotics in the gram-negative cell,

(A) β-lactam antibiotics must enter through porins in the outer membrane, transverse the periplasmic space, and attach to their target penicillin-binding proteins (PBPs) located on the outer aspect of the cytoplasmic membrane.

(B) Resistance may arise through modification of the targets of the drugs.

(C) The PBPs alterations in porin proteins that impede drug penetration into the cell.

(D) The production of drug-inactivating enzymes, the β-lactamases.

- Basic Science of β-lactamase

β-lactamase is an enzyme discovered in almost type of gram-negative bacteria. The main function of this enzyme is destroying penicillins and cephalosporins by a serine ester hydrolysis mechanism (Figure 2-12) and a few use zinc ion to attack the β-lactam ring. Although all β-lactamases catalyze the same reaction, a number of different types of these enzymes have been isolated and characterized. They have been classified according to several schemes based on :

1. The location of genes encoding β-lactamases

The location of genes encoding β-lactamases may be an innate part of the chromosome, or are encoded on plasmids. Chromosomal β-lactamases are universal in a specific bacterial species, whereas the presence of those encoded by plasmids is variable, and they are transferable between bacterial species. Further genetic mobility may be provided by transposons, which can carry β-lactamase genes from plasmids to chromosomes. More rarely, chromosomal β-lactamase genes may escape onto plasmids. This mobility is important since it allows the possibility of to spread resistance genes through several bacterial communities. The β-lactamase characteristic in each microorganism is shown in Figure 2-13.

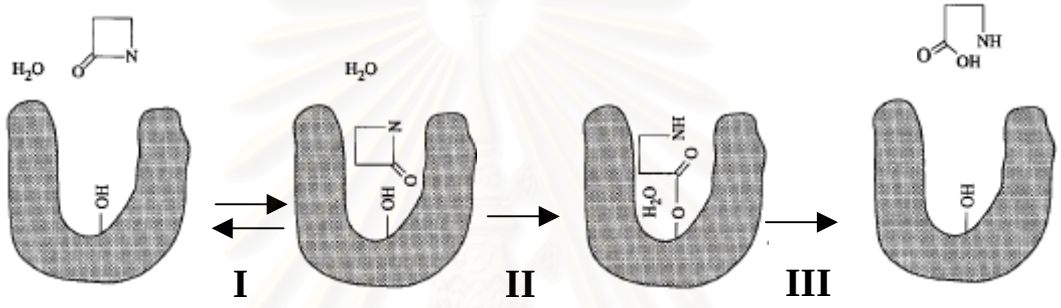
2. The β-lactamase production in the bacterial cell (Aswapokee, 1994)

- *Constitutive β-lactamase* : These enzymes, frequently found in extracellular of gram-positive bacteria, can be extremely produced by microorganism as a general rule without inducer.

- *Inducible β-lactamase* : these enzymes are fairly generated and existed into periplasmic space of microorganism because bacteria has mechanism for limitation β-lactamase producing (repression). However, these enzymes can be extra produced in condition induced by inducer and this phenomenon named “derepression“. Moreover, the derepression can be separated into temporary derepression and stable derepression.

3. The biochemical characteristic of β-lactamase

The first scheme that achieved wide acceptance was proposed by Richmond & Sykes. This scheme was based on whether an enzyme hydrolyzed penicillin more or less rapidly than cephaloridine and whether its activity was inhibited by cloxacillin and/or p-chloromercuribenzoate. The latter classification proposed by Bush, which is based on their substrate preference and their susceptibility to inhibition by clavulanate. Moreover, the advance in molecular biology now allow classification on the basis of amino sequence as proposed by Amber (Table 2-2)



(Modified from Livermore, 1998)

Figure 2-12 Mode of action of serine β-lactamases. The -OH group shown in the enzyme structure is on the side chain of the active-site serine. Phases of the reaction are:

(I) Reversible non-covalent binding of the β-lactamase and β-lactam ring;

(II) Rupture of the β-lactam ring, which becomes covalently acylated on to the active-site serine; and

(III) Hydrolysis of the acyl enzyme to reactivate the β-lactamase and liberate the inactivated drug molecule.

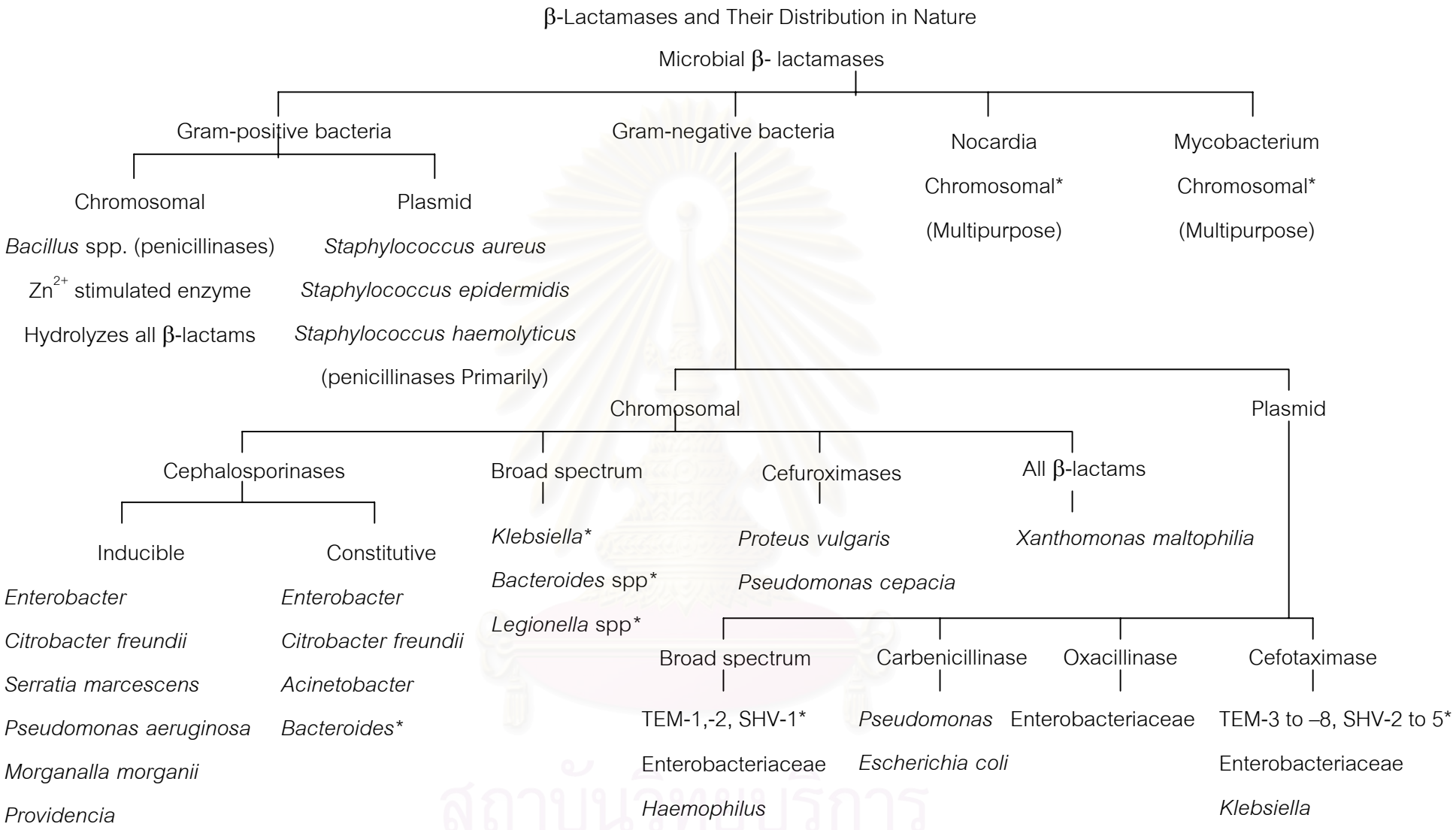


Figure 2-13 Diagrammatic representation of β-lactamases.

Table 2-2 Molecular and phenotypic classifications of β-lactamases (Modified from Livermore,1998)

Structural class (Ambler)	Functional group (Bush *et al.*)	Richmond Sykes class	Substrate preference[a]							Inhibition[b]		
			penicillin	carbenicillin	oxacillin	cephaloridine	cefotaxime	aztreonam	imipenem	clavulanate	aztreonam	EDTA
Serine β-lactamases												
A	2a	NL	+++	+	–	±	–	–	–	++	–	–
	2b	II and III	+++	+	+	++	–	–	–	++	–	–
	2be	III and IV[c]	+++	+	+	++	++	++	–	++	–	–
	2br	NL	+++	+	+	+	–	–	–	–	–	–
	2c	II and V	++	+++	+	+	–	–	–	+	–	–
	2e	Ic	++	++	–	++	++	++	–	++	–	–
	2f	NL	++	+	?	+	+	++	++	+	–	–
C	1	I, except Ic	++	+	–	+++	+	–	–	–	++	–
D	2d	V	++	+	+++	+	–	–	–	V	–	–
Undetermined[d]	4[d]	NL	++	++	++	V	V	–	–	–	–	–
Zinc β-lactamases												
B	3	NL	++	++	++	++	++	–	++	–	–	++

[a] Activity: +++, preferred substrate (highest V_{max}); ++, good substrate; +, hydrolysed; ±, barely hydrolysed; –, stable; V, varies within group; ?, uncertain.
[b] Inhibition: ++, strong inhibitor of all members of class; +, moderate inhibition; V, inhibition varies within the class; –, negligible inhibition.
[c] K1 enzyme of *K. oxytoca* was placed in Richmond & Sykes Class IV and Bush group 2be; however, most Bush 2be enzymes are mutants of TEM and SHV.
[d] None of Bush's group 4 enzymes has yet been sequenced. They are assumed to be serine types because they lack carbapenemase activity and are not inhibited by EDTA.
NL: not listed.

4. Role of β-Lactamase Inhibitors

Protection of a labile β-lactam with a β-lactamase inhibitor provides an alternative strategy for overcoming β-lactamases. The combination of ampicillin and oxacillin were occasionally used against *P.aeruginosa* urinary tract infection as early as 1963, based on the reasoning that oxacillin should inhibit the organism's Amp C enzyme, which otherwise destroys the ampicillin. This combination was not very effective, probably because oxacillin penetrates *P.aeruginosa* poorly or is pumped out, and the strategy was dropped with the development of carbenicillin. Interest reawakened in the mid-1970s, when several class of inhibitor were found in rapid succession, including clavulanic acid, penicillanic acid sulphones, halogenated penicillanic acids, olivanic acids and various penems. Of the inhibitor classes, only clavulanic acid and penicillanic acid sulphones have been developed into clinical used in the current era.

Clavulanic acid was destined to become the first β-lactamase inhibitor to enter clinical use. The discovery of clavulanic acid further stimulated the search for other β-lactamase inhibitors, and eventually led to the development of the penicillanic acid sulphones, sulbactam and tazobactam which are now available clinically. Each inhibitor is available only as a fixed-combination preparation that includes an active β-lactam antibiotic as the companion agent. There are minor differences in potency, activity, and pharmacology among the β-lactamase inhibitors, and clinically they can be considered therapeutically equivalent. The antibacterial activity of the β-lactam-β-lactamase inhibitor combination is determined by the spectrum of the companion β-lactam antibiotic.

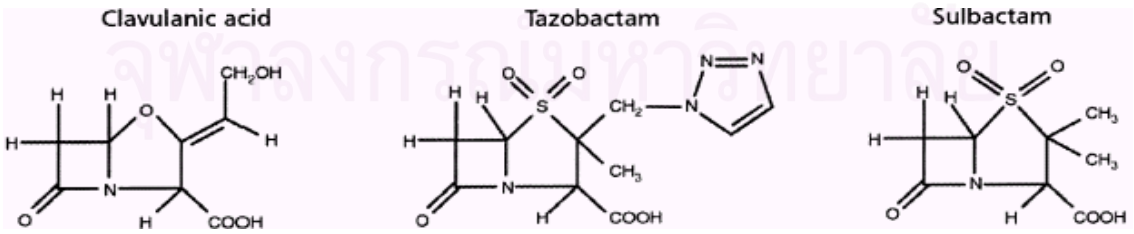


Figure 2-14 Structural formula of clavulanic acid, tazobactam and sulbactam

- Type of β-lactamase inhibitors

- *Clavulanic Acid*

Clavulanic acid is a naturally occurring weak antimicrobial agent found initially in cultures of *Streptomyces clavuligerus*. This agent acts primarily as a "suicide inhibitor" by forming an irreversible acyl enzyme complex with the β-lactamases, leading to loss of activity of the enzyme. Clavulanic acid acts synergistically with various penicillins and cephalosporins against β-lactamase-producing gram negative bacteria. Currently, clavulanic acid is available for clinical use in a 1:2 and 1:4 combination with oral amoxicillin and in a 1:15 and 1:30 parenteral combination with ticarcillin. The pharmacologic parameters of amoxicillin and ticarcillin are not significantly altered when the drug is combined with clavulanic acid. Amoxicillin-clavulanate is moderately well absorbed from the gastrointestinal tract, with a half-life in serum of about 1 h. for each component. One-third of a dose is metabolized, while the remainder is excreted unchanged in the urine. The drug is widely distributed to various body tissue and fluids, but it penetrates uninflamed meninges very poorly.

Adverse reactions are similar to those reported for amoxicillin or ticarcillin used alone. Nausea, vomiting, abdominal cramps, and diarrhea occur in 5 to 10% of patients taking amoxicillin-clavulanate. The incidence of allergic skin reaction is similar to that of amoxicillin alone.

- *Sulbactam*

Sulbactam is a semisynthetic 6-desaminopenicillin sulfone with weak antibacterial activity. It acts synergistically with penicillins and cephalosporins against β-lactamases-producing gram negative bacteria. For clinical use, sulbactam is combined with ampicillin and cefoperazone as a parenteral preparation in a 1:2 ratio and 1:1, 1:2 ratio, respectively. The pharmacologic properties of the drugs are not affected by each other in these combinations. Ampicillin-sulbactam penetrates well into body tissues and fluids, including peritoneal and blister fluids. It enters the CSF in the presence of impaired renal function, dosage adjustments are similar for the two drugs.

The most common side effects of the ampicillin-sulbactam combination are nausea, diarrhea, and skin rash. Transient eosinophilia and elevation of serum transaminases have been reported. Adverse reactions attributed to ampicillin may also occur with the use of ampicillin-sulbactam.

- *Tazobactam*

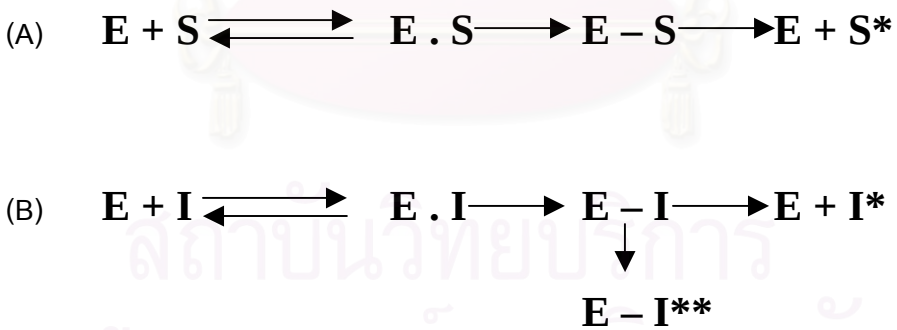
Tazobactam is a penicillanic acid sulphone derivative structurally related to sulbactam. Like clavulanic acid and sulbactam, tazobactam acts as a suicidal β-lactamase inhibitor and binds to bacterial PBP1 or PBP2. Despite having very poor intrinsic antibacterial activity by itself, it is comparable to clavulanate and sulbactam in lowering the MICs by up to 20-fold for many organisms when combined with various β-lactams against β-lactamases-producing organisms. Of the penicillin-β-lactamases inhibitor combinations, piperacillin-tazobactam is the most active (twofold to eightfold lower MICs) against β-lactamases-producing aerobic and anaerobic gram-negative bacilli. Tazobactam is administered parenterally as a 1:8 combination with piperacillin. The two drugs do not affect each other's metabolism or pharmacokinetics. High concentrations of both agents are achieved in the intestinal mucosa, lungs, and skin, with relatively poor distribution to muscle, fat, prostate, and CSF (in the absence of inflamed meninges). With a half-life in serum of about 1 h, tazobactam is eliminated mainly via the renal route and is not affected by hepatic failure.

The major adverse effects of the piperacillin-tazobactam combination are similar to those of piperacillin alone and include diarrhea, skin rash, and allergic reactions. Mild elevation in transaminase levels in serum is encountered in about 10% of patients.

- Mechanism of β-lactamase inhibitors

The means by which clavulanic acid, sulbactam and tazobactam function as inhibitors of bacterial β-lactamases has been studied in detail with active-site serine β-lactamases. The data show similarities in the modes of action of the three agents and may be regarded as irreversible, suicide inhibitors of the target enzyme.

Most clinically important β-lactamases have a serine hydroxyl group at the active site, which forms a non-covalent complex with the β-lactam-carbonyl bond of the β-lactam substrate. An acylation reaction follows with the formation of an acyl-enzyme and opening of the β-lactam ring. In the case of β-lactamase-labile antibiotics the acyl-enzyme complex hydrolyzes rapidly to liberate free enzyme and the antibacterial inactive product (Figure 2-15A). In the case of a suicide inhibitor, the acyl-enzyme intermediate is comparatively stable and may react slowly to yield hydrolyses inhibitor and reactivated enzyme, or achieve stability by further reaction with the enzyme (Figure 2-15B) Such reactions are possible because the hydrolysis of the β-lactam moiety of clavulanic acid or the penicillanic acid sulphones unmasks reactive groups that can form stable covalent bonds at the active site.



(Modified from Sutherland, 1995)

Figure 2-15 Models for inhibition of β-lactamase with (A) β-lactamase-labile substrate (penicillin), and (B) with a β-lactamase suicide inhibitor ; E = enzyme ; S = substrate ; I = inhibitor ; E.S = noncovalent complex ; E-S = acyl-enzyme complex ; S* = hydrolyzed (inactive product) ; E-I** = permanently inactivated enzyme ; I* = hydrolyzed inhibitor

- Spectrum of Inhibition of β-lactamases

β-lactamase inhibitors are most effective against β-lactamases produced by *S.aureus*, *H. influenzae*, *M. catarrhalis*, *Bacteroided* spp., and some Enterobacteriaceae. Chromosomal β-lactamase of *Serratia* spp., *C. freundii*, *Enterobacter* spp., *P. aeruginosa*, but some Enterobacteriaceae are not inhibited by β-lactamase inhibitors.

β-lactamase inhibitors are most active against plasmid-encoded β-lactamases, the most common of which is TEM-1, so called for the initials of the original patient from whom the *E. coli* β-lactamase containing isolate was derived. There are also TEM-2; oxacillin-hydrolyzing enzymes OXA-1, -2, and –3; sulfhydro-inhibited enzymes SHV-1 and HMS; and PSE-1, -2, -3, and –4, originally thought to be enzymes found only in *Pseudomonas* but now found occasionally in *E. coli*. All of these plasmid enzymes are inhibited, as are the new cefotaxime-ceftazidine-hydrolyzing enzymes TEM-3 through –27 and SHV-2, -3, -4, -5, -7, and –8 (Table 2-3).

Inhibition of chromosomal β-lactamase inhibitors is variable. The most important chromosomal β-lactamase, which generally are of the Richmond-Sykes class I type are present in *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Proteus*, *Pseudomonas* and *Serratia*. These are inducible enzymes that are not inhibited by β-lactamase inhibitors at clinically useful concentrations. β-lactamases are produced constitutively by some *Enterobacter*, *C. freundii*, and *Aeromonas* spp., and these are not inhibited. However, chromosomal β-lactamases of *Legionella* and *Bacteroides* are inhibited by β-lactamase inhibitors, as are some other chromosomally mediated β-lactamases, such as the class IV enzymes produced by *Klebsiella*.

Table 2-3 Inhibition of β-lactamases by β-lactamase inhibitors

β-lactamase	Name	Organisms	Inhibited by Clavulanate, Sulbactam and Tazobactam
Plasmid		*Staphylococcus aureus*	Yes
Plasmid	TEM-1	*Escherichia coli*	Yes
		Haemophilus	
		Neisseria gonorrhoeae	
		Salmonella	
		Shigella	
Plasmid	TEM-2	*Escherichia coli*	Yes
Plasmid	TEM-3 to –8	*Klebsiella*	Yes
Plasmid	SHV-1	*Klebsiella*	Yes
Plasmid	SHV-2 to 5	*Enterobacteriaceae*	Yes
Plasmid	OXA-1, -2, -3	*Escherichia coli*	Variable
Plasmid	PSE-1, -2, -3	*Pseudomonas*	Variable
Chromosomal	Type1a*	*Enterobacter*	No
		Morganella	
		Citrobacter	
		Serratia	
Chromosomal	Type Id*	*Pseudomonas*	No
Chromosomal	Type IV*	*Klebsiella*	Yes
Chromosomal		*Bacteriodes*	Yes
Chromosomal		*Legionella*	Yes
Chromosomal		*Branhamella*	Yes

* Classification based on Richmond MM, Sykes RB.

(Modified from Chambers, 2000)

- Factor Influencing the Activity of β-lactam-β-lactamase Inhibitor Combinations (Lister, 2000)

Currently, five β-lactam-β-lactamase inhibitor combinations are in medical used : ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam and cefoperazone-sulbactam. Many factors influence the activity and pharmacodynamics of the β-lactam-β-lactamase inhibitor combinations in the clinical setting and make them different from each other.

1. *Potency of the β-lactam* : As general rule, the more potent the β-lactam, the less protection it requires from the β-lactamase inhibitor.

2. *Potency of the β-lactamase inhibitor* : The potency of a β-lactamase inhibitor is influenced by the number of molecules that are hydrolyzed before inactivation of a β-lactamase molecule is achieved or may be determined from the concentration of β-lactamase inhibitor required to achieve 50% inhibition of enzyme activity (IC_{50}). The β-lactamase inhibitors illustrate different IC_{50} in each type of β-lactamase produced from microorganism (Table 2-4).

3. *Type of β-lactamase Producing* : When inhibitory activities of the β-lactamase inhibitors are evaluated against a panel of various β-lactamases, it is evident that individually the drugs interact differently with different enzymes. The impact of β-lactamase type on potency of inhibitor combinations is not simply a function of inhibitor activity. Just as important with some strains are the enzyme's substrate specificity and differences in rates of hydrolysis.

4. *Amount of β-lactamase Produced* : The level of β-lactamase produced by the target bacteria is an important factor influencing the efficacy of the β-lactam-β-lactamases inhibitor combinations. Once enzyme level increases sufficiently to provide β-lactam resistance, the inhibitory activity of β-lactamase inhibitor can store the activity of β-lactam.

5. *Pharmacokinetics of the β-lactamase inhibitor* : The pharmacokinetic of β-lactamase inhibitors are the vital factor governing the overall antibacterial effect. Generally, with recommended dosages, the length of time that antibacterial activity is maintained over the dosing interval is determined by the amount of time β-lactamase inhibitor concentrations remain above a critical level necessary to protect the β-lactam sufficiently.

Table 2-4 β-lactamase inhibitory activity of clavulanic acid, tazobactam and sulbactam

β-lactamase class	Organism	IC_{50} (mg/ml)		
		Clavulanic acid	Tazobactam	Sulbactam
Ia	*Enterobacter cloacae*	>50	0.93	5.0
Ib	*Escherichia coli*	>50	2.9	7.6
Ic	*Bacteroides fragilis*	0.006	0.03	0.04
Id	*Pseudomonas aeruginosa*	>50	0.97	2.9
III (TEM-1)	*Escherichia coli*	0.055	0.028	1.7
III (SHV-1)	*Escherichia coli*	0.035	0.14	13.0
IV	*Klebsiella pneumoniae*	0.011	0.047	3.8
IV	*Klebsiella oxytoca*	0.047	0.038	4.5

(Modified from Coleman et al., 1989)

6. *Induction of Chromosomal Cephalosporinases* : There were evidences demonstrated the ability of clavulanic acid to induce chromosomal cephalosporinases of *P.aeruginosa* and antagonize the activity of ticarcillin, induction was dose dependent (Lister, Gardner, and Sanders, 1999). In contrast, cephalosporinase induction is not a problem associated with tazobactam or sulbactam.

5. Therapy with Combined Antimicrobial Agents

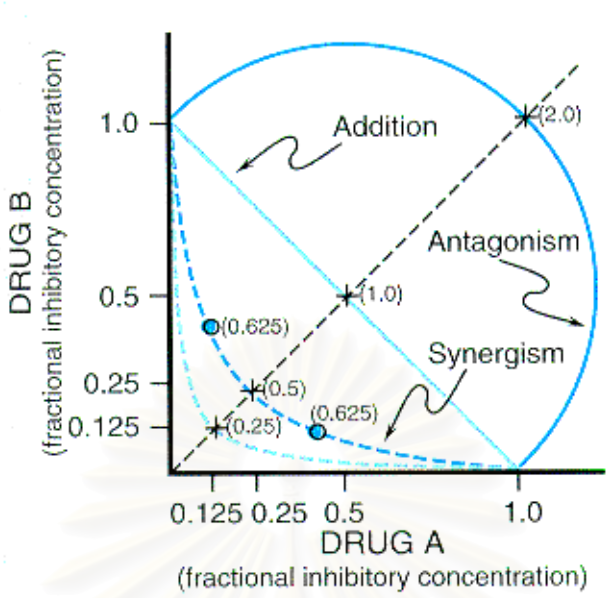
The simultaneous use of two or more antimicrobial agents has a certain rational and is recommended in specifically defined situation. However, selection of an appropriate combination requires an understanding of the potential for interaction between the antimicrobial agents. Such interactions may have consequences for both the microorganism and the host. Since the various classes of antimicrobial agents exert different actions on the microorganism, one drug has the potential to either enhance or inhibit the effect of the second. Similarly, combinations of drugs that rationally used to cure infections may have additive or supraadditive toxicities.

- Method of testing antimicrobial activity of drug combination

To predict the potential therapeutic efficacy of combinations of antibiotics, methods have been developed to quantify their effects on bacterial growth in vitro. Two distinctly different methods are used

1. Checkerboard method :

This method employs serial twofold dilutions of antibiotics in broth inoculated with a standard number of the test microorganism. Inhibition of bacterial growth is quantified after 18 hours of incubation. This test determines whether the MIC of one drug is reduced, unchanged, or increased in the presence of another drug. Synergism is defined as inhibition of growth with a combination of drugs when their concentrations are less than or equal to 25% of the MIC of each drug acting alone. This implies that one drug is affecting the microorganism in such a way that it becomes more sensitive to the inhibitory effect of the other. If one-half of the inhibitory concentration of each drug is required to produce inhibition, the result is called additive (fractional inhibitory concentration [FIC] index =1), suggesting that the two drugs are working independently of each other. If more than one-half of the MIC of each drug is necessary to produce the inhibitory effect, the drugs are said to be antagonistic (FIC index >1). When the drugs are tested for a variety of proportionate drug concentrations, such as with the checkerboard technique, an isobologram may be constructed. Synergism is shown by a concave curve, the additive effect by a straight line, and antagonism by a convex curve. A potential limitation of this method is that its end-point is growth inhibition, not killing, and consequently synergism may not indicate enhanced bactericidal effect.



(Modified from Chambers and Sande, 1996)

Figure 2-16 Effect of combinations of two antimicrobial agents to inhibit bacterial growth

The effects are expressed as isobologram and fractional inhibitory concentration (FIC) indices. The FIC index is equal to sum of the values of FIC for the individual drugs:

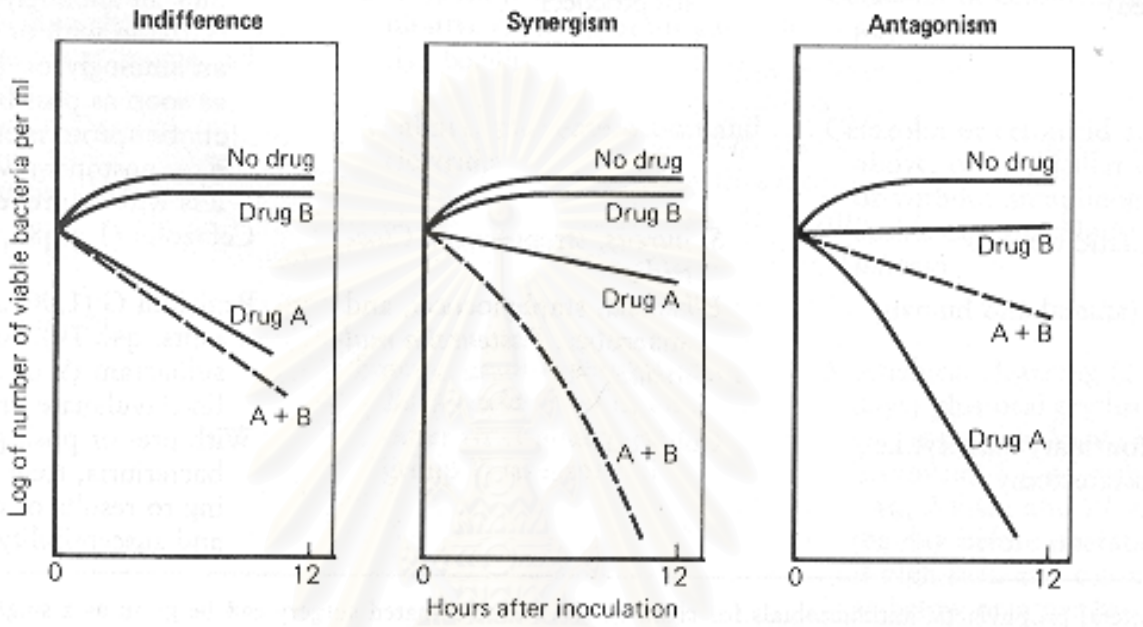
$$\text{FIC index} = \frac{\text{(MIC of A with B)}}{\text{(MIC of A alone)}} + \frac{\text{(MIC of B with A)}}{\text{(MIC of B alone)}}$$

Points on concave isobolograms (FIC index <1) are indicative of synergistic interaction between the two agents, and points on convex isobolograms (FIC index >1) represent antagonism. The nature of the interaction is adequately revealed by testing combinations lying along the black dashed line.

2. Time-kill study :

This method for evaluating drug combinations involves quantitation of their rate of bactericidal action. Identical cultures are incubated simultaneously with antibiotics added single or in combination. If a combination of antibiotics is more rapidly bactericidal than either drug alone, the result is termed synergism. Moellering (1986) has recommended that the minimal criterion for synergism should be the observation of a 100-fold additional

decrease in the number of microorganisms counted at any one time. If the bactericidal rate of the combination is less than that for either drug alone, antagonism is said to occur. If the bactericidal rate is as rapid as that for the more bactericidal drug, the result is called indifference. (Chambers and Sande, 1996)



(Modified from Scholar and Pratt, 2000)

Figure 2-17 Patterns of response to therapy with two antibiotics. The response of bacteria suspended in growth medium to exposure to drug A or B alone is represented by the solid line. The dashed lines represent the responses to simultaneous administration of the two drugs

6. Pharmacodynamic of β-Lactam-β-Lactamase inhibitor combination

The pharmacology of antimicrobial therapy can be divided into two distinct components. The first of these components is pharmacokinetics utilized to determine the drug concentrations in serum. Pharmacodynamics is the relationship between serum concentration and the antimicrobial effect at the site infection. Antimicrobial pharmacodynamic properties are determined from minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC). Both parameters have been the major parameters used to quantify the activity of an antibacterial drug against the infection pathogen. Although these parameters are good predictors of the potency of the drug-organism interaction, they do not provide any information on the time course of antimicrobial activity. Therefore bactericidal activity is proposed to obtain the information on killing rate and whether increasing antimicrobial concentration can enhance this rate as well as the persistent effects of antibacterial agents named the postantibiotic effect (PAE) defined as microorganism growth inhibitory effects after exposure to an antimicrobial agent. They are important parameters giving much better description of the time course of antimicrobial activity than those provided by the MIC and MBC.

Bactericidal activity

Bactericidal activities are classified into 2 groups based on the relationship between antimicrobial concentration and killing rate. The first group is concentration dependent killing, the higher the drug concentration the greater the rate and extent of killing (e.g., aminoglycosides and fluoroquinolones). The second group is time-dependent activity that has little relationship to the magnitude of drug concentration, as long as the concentrations are above a minimally effective level. Saturation of the killing rate occurs at low multiples of the MIC. Concentrations above these values do not kill the organisms any faster or more extensively. This is a common characteristic of β-lactams. These properties suggest that maintaining β-lactam concentration at or above the MIC of the infecting organism should optimize antibacterial effect.

Persistent Effects

PAE refers to the persistent suppression of bacterial growth following exposure to an antimicrobial. PAE can be considered the time it takes for an organism to recover from the effects of exposure to an antimicrobial. All antibacterials produce PAE in vitro when susceptible gram-positive bacteria are exposed to antibiotics. Prolonged PAE for gram-negative bacteria are observed after exposure to antibacterials that are inhibitors of protein synthesis or nucleic acid synthesis. In contrast, short PAE or no PAE are observed for gram-negative bacteria after exposure to β-lactam antibiotics. However, the recent evidences have demonstrated the phenomenon of continuing suppression of bacterial growth after briefly contact to β-lactamase inhibitor agents termed the post β-lactamase inhibitor effect (PLIE). The mechanism of the PLIE observed in vitro remains hypothesis that after β-lactamase inhibition by β-lactam-β-lactamase inhibitor or β-lactamase inhibitor during the pre-exposure period, followed by the elimination of β-lactamase inhibitor, the surviving bacteria might require a “latency period” to synthesize a sufficient level of β-lactamase. During this period the β-lactams still present can fully exert its antibiotics activity and inhibits bacterial growth until the β-lactamase concentration is again sufficient to hydrolyze β-lactam and allows bacterial regrowth.

The differences in pharmacodynamics activity have implications for optimal dosage regimens. The results of more recent evidences suggest that additional studies are needed to further correlate pharmacokinetic/pharmacodynamic parameters for many antibacterials with therapeutic efficacy in a variety in human infections.

CHAPTER III

MATERIALS & METHODS

MATERIALS

1. Microorganisms, Chemicals and Reagents

1.1 Microorganisms

The bacterial strains used throughout this study were *Haemophilus influenzae, Moraxella (Branhamella) catarrhalis, Acinetobacter baumannii, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Enterobacter cloacae*. These bacteria were clinically isolated from patients in Siriraj Hospital during year 2001-2002. Susceptibility patterns of all microorganisms except for *E. cloacae* were highly to moderately susceptible to β-lactam - β-lactamase inhibitor combination and resistant to β-lactam alone as tested by disk susceptibility method, which was described in the National Committee for Clinical Laboratory Standards (NCCLS), 2000. The selected microorganisms, drawn from 20 clinical isolates by simple random sampling to collect 1 strain of each species, were examined by nitrocefin-based test to confirm β-lactamase producing ability. Subsequently, they were utilized in bactericidal activity test and post β-lactamase inhibitor effect determination by time kill method. *E. cloacae* was bacterial strain performed in quantitative β-lactamase induction effect study thus the antagonistic effect between β-lactam disk and β-lactamase inhibitor disk must be shown in the selected strain as tested by double disks method, which was modified from the NCCLS, 2000; Eliopoulos and Moellering, 1996; Lister, Gardner, and Sanders, 1999; Hejnar, Kolar, and Hajek, 1999.

1.2 Chemicals

- Standard powders

  Four β-lactam and three β-lactamase inhibitor standard powders were tested : Amoxicillin trihydrate, lithium clavulanate were kindly supplied by GlaxoSmithKline; cefoperazone dihydrate, sulbactam by Pfizer; piperacillin monohydrate, tazobactam by Wyeth-Ayerst and cefuroxime was purchased from Sigma. Working standard

solutions were prepared immediately prior to use, as specified by the manufacturers before dilute with test broth.

- Susceptibility disks

Ampicillin (10 μg), amoxicillin/clavulanic acid (20 μg /10 μg), cefoperazone (75 μg), cefoperazone/sulbactam (75μg/30 μg), piperacillin (100μg), piperacillin/tazobactam (100 μg/10 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), cefepime (30 μg), cefpirome (30 μg), cefpodoxime (10 μg) and cefotaxime (30 μg) disks were purchased from Oxoid (Oxoid Chemicals, England) and BBL chemicals (Beckton Dickinson, USA). Clavulanic acid (0.5 μg), clavulanic acid (2 μg), clavulanic acid (4 μg), clavulanic acid (10 μg) clavulanic acid (20 μg) and clavulanic acid (50 μg) disks were prepared by laboratory. These disks were used to determine susceptibility pattern and evaluate interaction of antimicrobial agent combination by disk susceptibility method and by double disks method, respectively. Cefuroxime (E-test) was purchased from AB BIODISK Solna, Sweden used to determine minimum inhibitory concentration (MIC) in β-lactamase induction effect study.

1.3 Reagents

- Mueller-Hinton Agar (MHA) and Mueller-Hinton Broth (MHB) were purchased from Oxoid (Oxoid Chemicals, England) used as the test medium for all bacterial strains except for *H. influenzae.*

- Haemophilus Test Agar (HTA) and Haemophilus Test Broth (HTB) has been specifically combination of MHA or MHB, yeast extract and Haemophilus test medium supplement purchased from Oxoid (Oxoid Chemicals, England) used as the test medium for *H. influenzae*.

- MacConkey Agar was purchased from Oxoid (Oxoid Chemicals, England) used as the media to culture *P. aeruginosa, K. pneumoniae, E. cloacae*, and *A. baumannii*.

- Blood agar was prepared from blood agar base purchased from Oxoid (Oxoid Chemicals, England) and human blood by used as the media to culture *M. catarrhalis.*

- Chocolate agar has been specifically combination of GC medium base, hemoglobin powder and vitox purchased from Oxoid (Oxoid Chemicals, England) used as the media to culture *H. influenzae.*

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

- Sterile normal saline solution (NSS) was chosen as the diluent of the inoculum in turbidity adjusting processes to quantity the precise numbers of bacteria by spectrophotometer at the wavelength 625 nanometer. This NSS also applied as the diluent of specimens in colony counting procedures of time kill method.

- A $BaSO_4$ 0.5 McFarland standard

To standardize the inoculum density for a susceptibility test, BaSO4 turbidity standard, equivalent to a 0.5 McFarland standard should be used. A $BaSO_4$ 0.5 McFarland standard may be prepared as follows:

- A 0.5 ml aliquot of 0.048 mol/L $BaCl_2$ (1.175 % w/v $BaCl_2$. $2H_2O$) was added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.
- The correct density of the turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standard.
- The barium sulfate suspension should be transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum.
- These tubes should be tightly sealed and stored in the dark at room temperature.
- The barium sulfate turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appear, the standard should be replaced.
- The barium sulfate standards should be replaced or their densities verified monthly.

2. Laboratory Equipment

2.1 Disposable Equipment

- Cotton swabs were used to take and streak standard inoculum onto the solid media before impregnated the disks as performed in the disk susceptibility method (NCCLS, 2000).
- Cotton plugs were applied for glass equipment that contains inoculum and others to keep sterile environment in the containers throughout the research.
- Aluminum foil was chosen to keep sterility in potentiation with cotton plugs.

2.2 Steriled Glass Equipment

- Petri dishes were practiced as agar containing plate for culture microorganisms in the whole processes such as subculture, susceptibility testing and colony counting.
- Erlenmeyer flasks were used for the media preparation, sterile water and sterile NSS before autoclaving.
- Cylinders were picked to measure the gross quantity of water and liquid media in preparing procedures.
- Glass tubes were used throughout the experiments such as in the preparation of the standard solution, dilute inoculum and specimen, etc.
- Pipettes, used in experiment divided into 2 types
  1. Glass pipettes were chosen to measure media, inoculum, drugs and solvent as general equipment processes.
  2. Micropipette was used for calibrate specimens in colony counting procedures from time kill method.

2.3 General Equipment

- Chemical spoons were used as equipment to spoon and adjust the chemical powders in the weighing processes.
- The loops used in this experiment were of 2 types
  1. General loop was selected for streaking bacteria in general procedures such as subculture, inoculum preparation, etc.
  2. Standard loop was picked as measuring equipment to calibrate the specimen in time kill method before streaking specimen in solid media for colony counting process.

- Ruler was chosen for measuring the clear zone in disk susceptibility method performed by the NCCLS, 2000.
- Tube rack was used as shelf to hold a large number of tubes, both in broth macrodilution procedures and time kill procedures.

3. Laboratory Instruments

3.1 Temperature Controlling Instruments

- Autoclave was used to sterilize equipment, media, diluent, inoculum and others throughout the experiment for sterile condition in the research.
- Refrigerators were used to maintain bacteriostatic condition between research process and also preserved media before using in all experiments.
- Incubator was used to provide the appropriate environmental condition for bacterial growth throughout the procedures such as subculture, disk susceptibility process, inoculum preparation, etc.
- Water bath shaker was chosen to apply appropriate bacterial growth condition of liquid media that simulate human body temperatures in the time kill method.
- Hot air ovens were used to keep drying and sterilize all glass equipment before using.

3.2 General Instruments

- Chemical scale was selected for weighing media and standard powder of antimicrobial agent in preparing procedures of both test media and working standard solutions.
- Spectrophotometer, A-JUST™ turbidity meter of Abbott Laboratories, U.S.A., was applied to adjust turbidity of the inoculum to equivalent with 0.5 McFarland standard solution and 1.0 McFarland standard solution.
- Mechanical vortex mixer was used to mix 0.5 McFarland standard, inoculum and specimen, which result to homogeneity of suspension before using for further procedures in the experiment.

METHODS

1. Disk Diffusion Test to determine susceptibility pattern of gram-negative bacteria to the β-lactam alone and β-lactam /β-lactamase inhibitor combination.
2. Nitrocefin-Base Test to detect β-lactamase producing in selected bacteria.
3. Broth Macrodilution Method (Checkerboard Technique) to evaluate synergistic interaction between β-lactam and β-lactamase inhibitor in addition to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).
4. Time Kill Method to investigate bactericidal activity of β-lactam -β-lactamase inhibitor combination to selected microorganism.
5. Time Kill Method to determine the post β-lactamase inhibitor effect (PLIE) of β-lactamase inhibitor to selected microorganism.
6. Double Disks Method and Agar Dilution Method to quantify β-lactamase induction effect of β-lactamase inhibitor to *Enterobacter cloacae* strain.

1. Procedures for Performing the Disk Diffusion Test

1.1 Preparation of Agar Plate

1.1.1 MHA and HTB were prepared from a commercially available dehydrated base according to the manufacturer's instructions.

1.1.2 Immediately after autoclaving, allow it to cool in a 45 to 50 °C water bath.

1.1.3 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.

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

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

1.2 Inoculum Preparation

1.2.1 Growth Method

1.2.1.1 At least three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4 to 5 ml of a test broth medium.

1.2.1.2 The broth culture was incubated at 37°C until it achieved or exceeded the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours).

1.2.1.3 The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard. This result in a suspension containing approximately 1 to 2 x 10^8 CFU/ml. A-JUST™ turbidity meter of Abbott Laboratories, U.S.A. is a photometric device used to perform this step propriety.

1.2.2 Direct Colony Suspension Method

1.2.2.1 This approach is the recommended method for testing the fastidious organisms such as *H. influenzae*.

1.2.2.2 As a convenient alternative to the growth method, the inoculum can be prepared by making a direct broth or saline suspension of isolated colonies selected from a 18- to 24-hour chocolate agar plate. The suspension was adjusted to match the 0.5 McFarland turbidity standard. This suspension will contain approximately 1 to 2x10^8 CFU/ml.

1.3 Inoculation Test Plates

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

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

1.3.3 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 drug-impregnated disks.

1.4 Application of Disks to Inoculated Agar Plates

1.4.1 The predetermined battery of antimicrobial disks was dispensed onto the surface of the inoculated agar plate. 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 drug diffuses almost instantaneously, a disk should not be relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.

1.4.2 The plates were inverted and placed in an ambient air incubator set to 37°C within 15 minutes after the disks were applied in ambient air. With the exception of *H. influenzae* and *M. catarrhalis,* the plates should be incubated at 37°C in an atmosphere of 5%CO_2 for 16-18 hours before measuring the zones of inhibition.

1.5 Reading Plates and Interpreting Results

1.5.1 After 16 to 18 hours of incubation, each plate was examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disk. Zones were measured to the nearest whole millimeter by using a ruler, which was held on the back of the inverted petri plate. The petri plate was held a few inches above a black, nonreflecting background and illuminated with reflected light.

1.5.2 The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored. However, discrete colonies growing within a clear zone of inhibition should be subculture, re-identified, and retest.

1.5.3 The size of the inhibition zone were interpreted by referring to the NCCLS, 2000 and the organisms were reported as either susceptible, intermediate, or resistant to the agents that have been tested (Tables 3-1 to 3-4).

Table 3-1 Zone diameter interpretive standards breakpoints for Enterobacteriaceae

Drug	Disk content	Zone diameter (mm)		
		R[a]	I[b]	S[c]
Piperacillin	100 μg	≤17	18-20	≥21
Piperacillin/Tazobactam	100/10 μg	≤17	18-20	≥21

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

Table 3-2 Zone diameter interpretive standards breakpoints for *P. aeruginosa* and *Acinetobacter* spp.

Drug	Disk content	Zone diameter (mm)			Comment
		R[a]	I[b]	S[c]	
Piperacillin	100 μg	≤17	-	≥18	For *P.aeruginosa*
		≤17	18-20	≥21	For *Acinetobacter* spp.
Piperacillin/Tazobactam	100/10 μg	≤17	-	≥18	For *P.aeruginosa*
		≤17	18-20	≥21	For *Acinetobacter* spp.
Cefoperazone	75 μg	≤15	16-20	≥21	-
Cefoperazone/Sulbactam	75/30 μg	≤15	16-20	≥21	-

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

Table 3-3 Zone diameter interpretive standards breakpoints for *H. influenzae*

Drug	Disk content	Zone diameter (mm)		
		R[a]	I[b]	S[c]
Ampicillin	10 μg	≤18	19-21	≥22
Amoxicillin/Clavulanic acid	20/10 μg	≤19	-	≥20

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

Table 3-4 Zone diameter interpretive standards breakpoints for *M.catarrhalis*[d]

Drug	Disk content	Zone diameter (mm)		
		R[a]	I[b]	S[c]
Ampicillin	10 μg	≤13	14-16	≥17
Amoxicillin/Clavulanic acid	20/10 μg	≤13	14-17	≥18

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

[d]Not determined in the NCCLS,2000. Data from Acar and Goldstein, 1996.

2. β-Lactamase Detection (Chromogenic Cephalosporins: Nitrocefin-Based Test)

The selected microorganisms were confirmed to produce β-lactamase by nitrocefin-based test as mentioned in the NCCLS,2000; Livermore and Williams, 1996.

2.1 A 0.5 mmol/liter of nitrocefin solution was prepared by dissolving 2.58 mg of nitrocefin in 0.5 ml of dimethylsulfoxide (DMSO) and then diluting with 9.5 ml of 0.1 mol/liter phosphate buffer, pH 7.0. This solution was stable for 10 days at 4 °C in a foil-wrapped bottle.

2.2 Colonies of test isolates were scraped from nutrient agar plates and applied on a glass slide or on the lid of a petri dish, and 20-μl of nitrocefin solution was dropped directly to colonies.

2.3 β-lactamase activity was indicated by color changing from yellow to red color. This usually appears within 1 to 2 minutes but may take longer in some bacterial strain.

3. Broth Macrodilution Procedures (Checkerboard Technique)

3.1 Preparing Test Broth

3.1.1 MHB and HTB were recommended as the medium of choice for the susceptibility testing of commonly isolated, rapidly growing aerobic and fastidious organisms such as *H. influenzae*, respectively.

3.1.2 The pH of each batch of MHB and HTB should be checked with a pH meter after the medium was prepared; the pH should be between 7.2 and 7.4 at room temperature.

3.2 Preparing Diluted Antimicrobial Agents

3.2.1 Sterile 13- x100-mm test tubes should be used to conduct the test.

3.2.2 The tubes can be closed with cotton plugs.

3.2.3 A control tube containing broth without antimicrobial agent was used for each organism tested.

3.2.4 The twofold dilutions of β-Lactam and β-Lactamase inhibitor were prepared volumetrically in the broth. Because final volume of 1.0 ml in each tube consisted of 0.5 ml of broth containing antimicrobial agents (0.25 ml of broth for β-Lactam and 0.25 ml of broth for β-Lactamase inhibitor) and 0.5 ml of broth containing a suspension of the organism to be tested. Thus antimicrobial concentrations used in the initial (stock) solutions should be prepared four-fold in greater than the desired final concentration. The concentrations tested for each antimicrobial typically range from 4 to 5 dilutions below the MIC to twice the MIC or higher.

3.2.5 A series of antimicrobial solutions containing four times the desired final concentrations were taken to produce the desired range of drug concentration by adding an aliquot of those solution to each tube in the appropriate row or column (as shown in Figure 3-1)

3.3 Broth Dilution Testing

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

3.3.1 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.

3.3.2 Within 15 minutes after the inoculum has been diluted, 0.5 ml of the adjusted inoculum was added to each tube already containing 0.5 ml of antimicrobial agent in the dilution series and the positive control tube containing only broth, and each tube was mixed. This results in a 1:4 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculum.

3.3.3 The inoculated macrodilution tubes should be incubated at 37°C for 16 to 20 hours in an ambient air incubator. When testing in *H. influenzae*, incubation should proceed for 20 to 24 hours in ambient air before interpreting result.

3.4 Reading plates and Interpreting Results

3.4.1 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 tubes (no antibiotics) and the negative-control tubes (no organism) used in each set of tests when determining the growth end points.

3.4.2 Susceptibility pattern of selected organisms to tested drugs by broth macrodilution method was interpolated by referring to the NCCLS, 2000 (shown in table 3-5).

3.4.3 Collect 10 μl of sample from all tubes showed clear broth and then inoculated the samples on appropriate solid media for 16 to 18 hours at 37°C in ambient air incubator. With the exception of *H. influenzae* and *M. catarrhalis,* the plates should be incubated at 37°C in an atmosphere of 5%CO_2 for 16-18 hours before determine the MBC by detection from the quantity of survival bacteria.

3.4.4 The criteria to define MBC is the decreasing in colony forming unit from the origin point $\geq$ 99.9% (Schoenknecht et al., 1985).

3.4.5 The results of checkerboard study were interpreted by the pattern they form on the isobologram (Figure 3-2) and the fractional inhibitory concentration index (FIC index) calculated as a mathematical restatement of isobologram (Table 3-6).

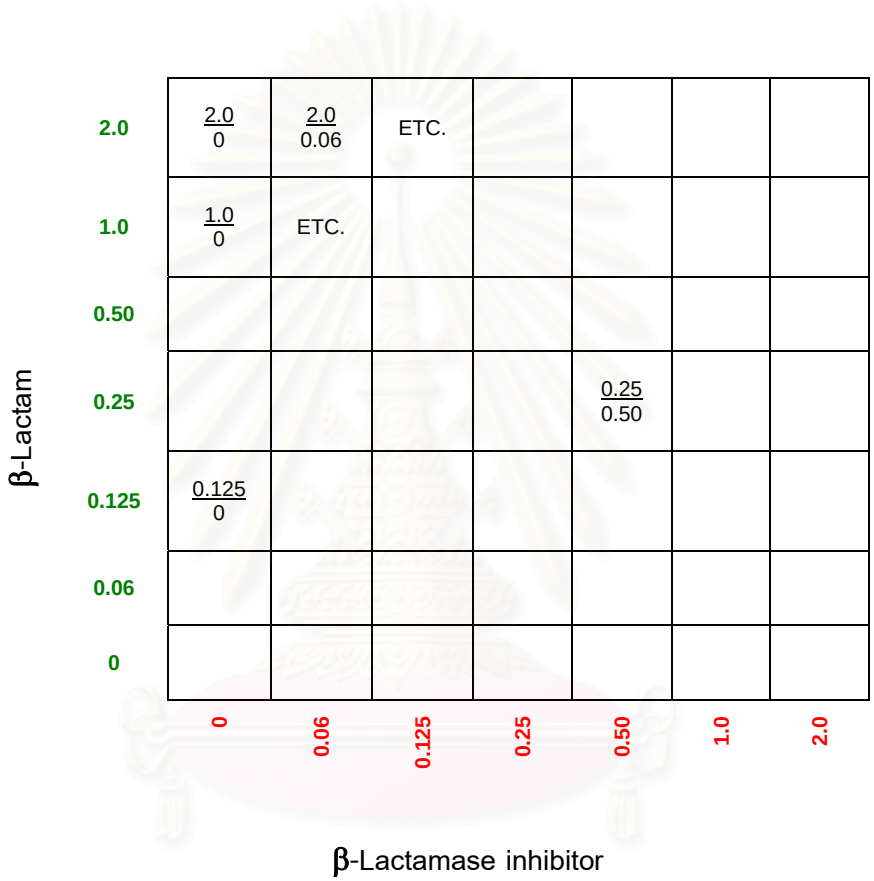


Figure 3-1 Checkerboard technique. In the checkerboard, serial dilutions of two drugs are preformed using drug concentrations proportional to the MICs of the drugs being tested. (Modified from Eliopoulos and Moellering, 1996)

Table 3-5 The MIC interpretive standards (μg/ml) for susceptible bacteria (data from the NCCLS, 2000)

Organisms / Drugs	*Haemophilus influenzae*	*Moraxella catarrhalis*[a]	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
Ampicillin	≤ 1	≤ 0.25	-	-	-
Amx/Cla*	≤ 4/2	≤ 8/4	-	-	-
Cefoperazone	-	-	≤ 16	≤ 16	-
Cpz/Sul**	-	-	≤ 16	≤ 16	-
Piperacillin	-	-	-	≤ 64	≤ 16
Pip/Tzb***	-	-	-	≤ 64/4	≤ 16/4

* =Amoxicillin/Clavulanic acid, ** =Cefoperazone/Sulbactam, *** =Piperacillin/Tazobactam

[a]Not determined in the NCCLS,2000. Data from Amsterdam, 1996.

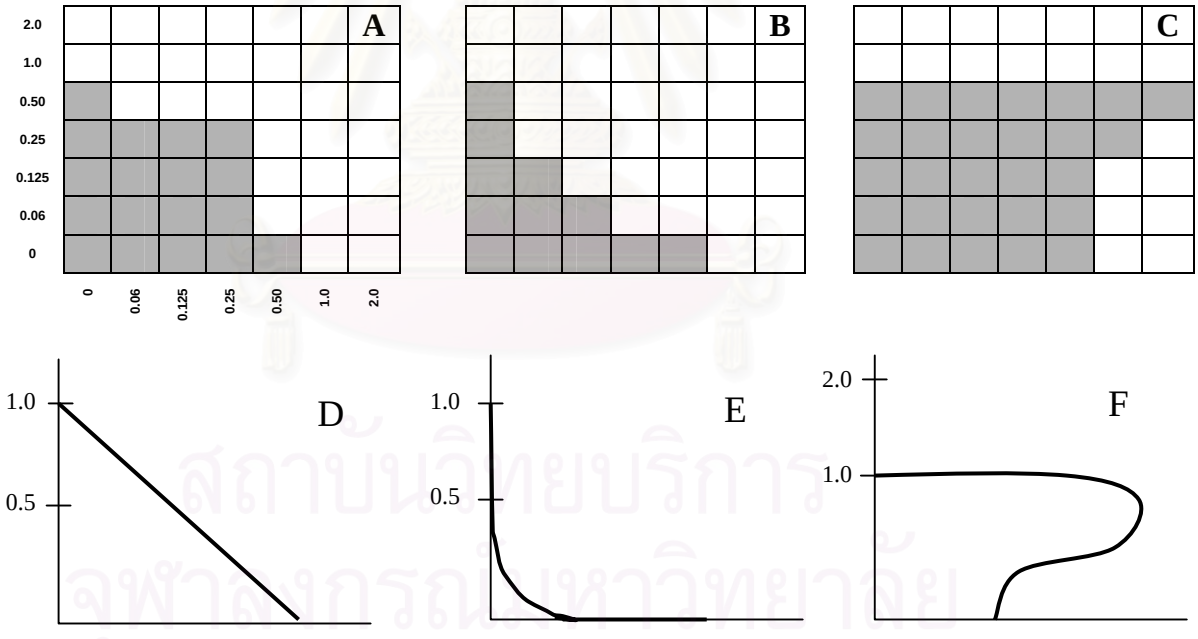


Figure 3-2 Assessment of antimicrobial combinations with the checkerboard method. A, B, and C. Results of testing combinations of two drugs. Shading, visible growth. Concentrations are expressed as multiples of the MIC. D, E, F. Isobolograms (plotted on an arithmetic scale) that represent the results of checkerboards shown in A, B, and C, respectively. A and D. Additive effect. B and E. Synergism. C and F. Antagonism. (Modified from Eliopoulos and Moellering, 1996)

Table 3-6 Calculation of the fractional inhibitory concentration (FIC) Index for combination of two antimicrobials and quantitative definitions of results with antimicrobial combination.

Quantitative Definition	Equation
Additive The result with two drugs is equal to the sum of the results for each of the drugs used separately	$0.5 < FIC_A + FIC_B > 1$
Autonomy (indifference) The result with two drugs does not significantly differ from the result with the most effective drug alone	$1 < FIC_A + FIC_B < 2$
Antagonism The result with two drugs is significantly less than the additive response	$FIC_A + FIC_B \geq 2$
Synergism The result with two drugs is significantly greater than the additive response	$FIC_A + FIC_B \leq 0.5$
$\frac{(A)}{(MIC_A)} + \frac{(B)}{(MIC_B)} = FIC_A + FIC_B = \text{FIC index}$	

(A) is the concentration of drug A in a tube that is the lowest inhibitory concentration in its row. (MIC_A) is the MIC of the organism to drug A alone. FIC_A is the fractional inhibitory concentration of drug A. (B), MIC_B , and FIC_B are defined in the same fashion for drug B. (modified from Eliopoulos and Moellering, 1996; European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Disease (ESCMID), 2000)

4. Bactericidal Activity Test by Time Kill Method

The selected drugs and bacteria in time kill method must be correlated with broth macrodilution method to define MIC as describe previously. The range of β-Lactamase inhibitor concentrations to conduct the time kill method should be pharmacokinetic achievable concentration from previously published articles (Joly-Guillou, et al., 1995; Craig, 1998). The standardized inoculum for the time kill method should be prepared by growing microorganisms or suspending colonies directly to the turbidity of the 0.5 McFarland standard which equivalent to bacterial quantity 1 to $2x10^8$ CFU/ml.

4.1 Prepare concentrations to 1/4MIC, 2MIC and 8MIC of the β-lactam.

4.2 Prepare the β-lactamase inhibitor concentrations to C_{min}, $C_{average}$ and C_{max}. ($C_{average}$ and C_{max} were referred to pharmacokinetic achievable concentration of each drug whereas C_{min} was minimum concentration of β-lactamase inhibitor that can reduce the MIC of accompanied β-lactam to the break point of interpretive guideline the NCCLS, 2000 determined from the results of the checkerboard technique).

4.3 Combine both drugs in the specific concentration into MHB or HTB for prepare working media before adding the standardized inoculum. As the result, the concentration ratio between β-lactam and β-lactamase inhibitor for testing consisted of ¼ MIC:C_{min}, ¼ MIC:$C_{average}$, ¼ MIC:C_{max}, 2 MIC:C_{min}, 2 MIC:$C_{average}$, 2 MIC:C_{max} , 8 MIC:C_{min}, 8 MIC:$C_{average}$, and 8 MIC:C_{max}.

4.4 Dilute the standardized inoculum to obtain the final bacterial quantity 1 to 2 x 10^7 CFU/ml into working media and control tubes containing broth without antimicrobial agents on water bath shaker at 37°C.

4.5 Collect the samples to detect for colony forming unit at the time 0,1,2,3,6 and 24 hours after microorganism exposed to drug in each concentration including the control group.

4.6 Inoculate the samples on appropriate solid media for 16 to 18 hours at 37°C to detect for colony forming units. With the exception of *H. influenza*e and *M. catarrhalis*, the plates should be incubated at 37°C in an atmosphere of 5% CO_2 for 20-24 hours.

4.7 Calculate the quantity of survival bacteria in each group to obtain the killing curves data.

4.8 Killing curves were constructed by Microsoft Excel 97. The criteria to define the bactericidal property is the decreasing in colony forming unit from the origin point ≥ 3 logCFU/ml at 24 hours of exposure. The regrowth is defined as an increase of ≥ 2 logCFU/ml after ≥ 6 hours. (Amsterdam, 1996; Pankuch, Jacobs and Appelbaum, 1994; Satta, et al., 1995). The criteria to define the synergism is the decreasing of colony forming unit in combination groups compare with the most active single drug ≥ 2 logCFU at 24 hours. (Chalkey and Koornhof, 1985; Navashin, et al., 1989; Satta, et al., 1995; White, Burgess, et al., 1996; Mayer and Nagy, 1999; Bonapace,

et al., 2000). The quantitative evaluation of antimicrobial effect was calculated as in the published article (Firsov, et al., 1997).

The Quantitative Evaluation of Antimicrobial Effect

1. The following parameters were estimated by extrapolation of the killing curves as shown in Figure 3-3.

   $T_{90\%}$ = The time to reduce the initial inoculum 10 fold

   $T_{99\%}$ = The time to reduce the initial inoculum 100 fold

   $T_{99.9\%}$ = The time to reduce the initial inoculum 1000 fold

   T_E = The time shift between the normal growth and the regrowth curves

   T_{min} = The time to reach the minimum number of bacteria resulting from exposure to antibiotic

   N_{min} = The minimum number of bacteria resulting from exposure to antibiotic

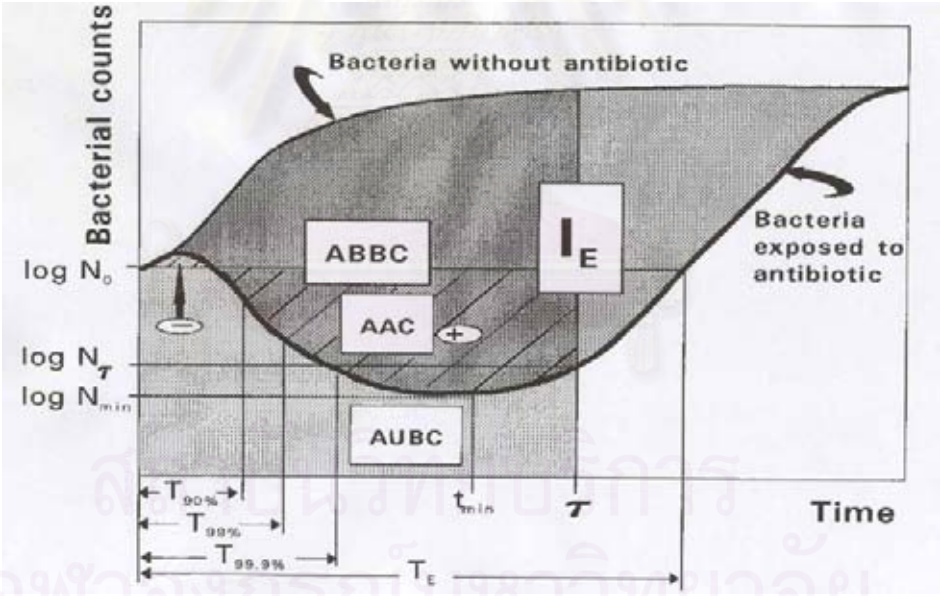


Figure 3-3 Parameters for quantifying bacterial killing and regrowth curve and the antimicrobial effect.

(Modified from Firsov, et al., 1997)

2. The special parameter *T* is the time at the end of administration period that usually mimicked the dosing interval. This data referred to the registered monograph of each agent, which were approved by the Food and Drugs Administration of Thailand. The N_T was determined by extrapolation of the killing curves as shown in figure 3-3.

   T = The time at the end of the administration period that usually mimicked the dosing interval

   N_T = The number of viable counts at the end of administration period that usually mimicked the dosing interval

3. The following data were computed from the difference of viable counts in various times.

   ΔlogCFU 3 hours, ΔlogCFU 24 hours = The difference between the number of viable counts at time zero versus the number of viable counts after exposed to antimicrobial for 3 hours and 24 hours, respectively

   ΔlogN (single drug - combination) = The difference between the number of viable counts in combination groups versus the most active single drug at 24 hours

4. The following parameters were calculated by various methodologies as followed:

   Killing rate of the first 3 hours (KR3) = The differential parameter between the number of viable counts at time zero minus the number of viable counts after exposed to antimicrobial for 3 hour, and then divided by time

   AUC 24 hours = Area under the control growth curve or the bacterial killing and regrowth curves that calculated by the trapezoidal rule which is generally accepted as standard method to determine the AUC for the pharmacokinetic model

   Bacteriolytic area for 24 hours (ABBC, BA24) = The area between control growth curve and the bacterial killing and regrowth curves (AUC24 of the control growth curve subtracted by AUC24 of the bacterial killing and regrowth curve)

5. Post β-lactamase Inhibitor Effect (PLIE) by Time Kill Method

The post β-lactamase inhibitor effect was experiment to clarify persistent phenomenon of the bacteria after briefly exposed to β-lactamase Inhibitor. The PLIE can be assessed either after pre-exposure with β-lactamase inhibitor alone or after pre-exposure with the β-lactam-β-lactamase inhibitor combination. Hence, the two methods of PLIE determination were studied in this research. The selected drugs and bacteria in post β-lactamase inhibitor effect must be correlated with broth macrodilution method and time kill method to define MIC and detect for bactericidal activities, respectively.

5.1 Prepare the β-lactamase inhibitor concentrations to C_{min}, $C_{average}$ and C_{max} into MHB or HTB for prepare working media before adding β-lactam and the standardized inoculum. ($C_{average}$ and C_{max} were referred to pharmacokinetic achievable concentration of each drug whereas C_{min} was minimum concentration of β-lactamase inhibitor that can reduce the MIC of accompanied β-lactam to the break point of interpretive guideline the NCCLS, 2000 by determine from result of checkerboard technique).

5.2 Dilute the β-lactam to take final concentration of 2 MIC into working media for testing PAE and PLIE (method1) not including PLIE (method 2).

5.3 The standardized inoculum was prepared by growing microorganisms or suspending colonies directly to the turbidity of the 0.5 McFarland standard which equivalent to bacterial quantity 1 to $2x10^8$ CFU/ml.

5.4 Dilute the standardized inoculum to obtain the final bacterial quantity 1 to 2×10^7 CFU/ml into working media and control tubes containing broth without antimicrobial agents on water bath shaker at 37°C for 2 hours.

5.5 Collect specimens at zero and second hours of exposure to calculate the quantity of survival bacteria.

5.6 At the end of the pre-exposure time the β-lactam and β-lactamase inhibitor were removed by dilution method with free broth to determine PAE and removed only the β-lactamase inhibitor with broth containing β-lactam to determine PLIE. The summarized procedures were shown in Table 3-7.

5.7 After the drug removal procedure, the tubes were maintained in the water bath shaker at 37°C for 24 hours.

5.8 Collect the samples to detect for colony forming unit at the time 0,2,4,6,8 and 24 hours after drug removal in each concentration including the control group.

5.9 Inoculate the samples on appropriate solid media for 16 to 18 hours at 37°C to detect for colony forming units. With the exception of *H. influenza*e and *M.catarrhalis*, the plates should be incubated at 37°C in an atmosphere of 5% CO_2 for 20-24 hours.

5.10 Calculate the quantity of survival bacteria in each group to obtain the killing curves data.

5.11 Killing curves were constructed by Microsoft Excel 97. The definition the PAE and PLIE is the period time that increase in colony forming unit from the origin point equal 1 $\log_{10}$ CFU/ ml. The killing curves were plotted and the duration of PAE and PLIE were calculated as below equations.

- Calculation of Post-antibiotic Effects (PAE)

The counts of CFU/ml are tabulated, and the duration of PAE is calculated by the equation: PAE = T - C. T is the time required for the CFU count in the PAE test culture to increase 1 $\log_{10}$ above the count observed immediately after drug removal. C is the time required for the count of the untreated control culture to increase by 1 $\log_{10}$ above the count observed immediately after completion of the same procedure used on the test culture for drug removal.

- Calculation of Post-β-lactamase inhibitor Effects (PLIE)

The counts of CFU/ml are tabulated, and the duration of PLIE is calculated by the equation: PLIE (method 1) = Total delay - (delay growth + PAE) and PLIE (method 2) = Total delay – Delay growth. Total delay is the time required for the CFU count in the PLIE test culture to increase 1 $\log_{10}$ above the count observed immediately after β-lactamase inhibitor removal. Delay growth is the time required for the CFU count of the culture post-exposed with β-lactam agent to increase by 1 $\log_{10}$ above the count observed immediately after completion of the same procedure used on the test culture for drug removal.

Table 3-7 The summarized procedure of post-β-lactamase inhibitor effects (PLIE) determination. (Modified from Murbach et al., 2001; Thorburn et al., 1996)

Term	Pre-exposure 2 hours with suitable broth containing	Remove antimicrobial agent by dilution with suitable broth containing
Delay growth	Free drug	β-lactam (2MIC)
PAE	β-lactam (2MIC) and β-lactamase inhibitor (C_{min},$C_{average}$,C_{max})	Free drug
PLIE (method 1)	β-lactam (2MIC) and β-lactamase inhibitor (C_{min},$C_{average}$,C_{max})	β-lactam (2MIC)
PLIE (method 2)	β-lactamase inhibitor (C_{min},$C_{average}$,C_{max})	β-lactam (2MIC)

6. Double Disks Method and Agar Dilution Method.

E. cloacae strain for β-lactamase induction effect study must show blunting zone between β-lactam-β-lactamase inhibitor disk (amoxicillin-clavulanic acid, piperacillin-tazobactam, cefoperazone-sulbactam) and β-lactam disk (ceftazidime, ceftriaxone, cefuroxime, cefepime, cefpirome, cefpodoxime, cefotaxime) as tested by double disks method modified from the NCCLS, 2000; Eliopoulos and Moellering, 1996; Lister, Gardner, and Sanders, 1999; Hejnar, Kolar, and Hajek, 1999 to confirm that selected strain was able to be induced to produce β-lactamase after exposure to β-lactamase inhibitor. Subsequently, the couples of β-lactamase inhibitor and β-lactam antibiotic showing positive result were further tested by method A modified from time-kill method and method B modified from published article (Bongaerts and Roelofs-Willemse, 1998) to quantify the β-lactamase induction effect.

6.1 Double disks Method

6.1.1 Preparation of Agar Plate

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

6.1.1.2 Immediately after autoclaving, allow it to cool in a 45 to 50 °C water bath.

6.1.1.3 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.

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

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

6.1.2 Inoculum Preparation

6.1.2.1 At least three to five well-isolated colonies of the same morphological type of *E. cloacae* strain were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4 to 5 ml of a test broth medium.

6.1.2.2 The broth culture was incubated at 37°C until it achieved or exceeded the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours).

6.1.2.3 The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard.

6.1.3 Inoculation Test Plates

6.1.3.1 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab should be rotated several times and

pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.

6.1.3.2 The dried surface of an agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of agar was swabbed.

6.1.3.3 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 drug-impregnated disks.

6.1.4 Application of Disks to Inoculated Agar Plates

6.1.4.1 The predetermined battery of antimicrobial disks was dispensed onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure complete contact with the agar surface. They must be distributed approximately 15-20 mm from center to center. Because some of the drug diffuses almost instantaneously, a disk should not be relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.

6.1.4.2 The plates were inverted and placed in an ambient air incubator set to 37°C within 15 minutes after the disks were applied in ambient air. for 16-18 hours before measuring the shape zones of inhibition.

6.1.5 Reading Plates and Interpreting Results

6.1.5.1 After 16 to 18 hours of incubation, each plate was examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be clear and there will be a confluent lawn of growth.

6.1.5.2 Both of two sides of zones of β-lactam disk were observed to compare the difference zone width and modified shape as judged by the unaided eye. The induction effect was determine from the D-

shape of inhibition zone produced by β-lactam disk as located near to β-lactam-β-lactamase inhibitor disk as shown in figure 3-4

6.2 β-lactamase induction effect study (method A)

6.2.1 Prepare the β-lactamase inhibitor previous selected from double disks method to obtain concentrations to C_{min}, $C_{average}$ and C_{max} as referring to pharmacokinetic achievable concentration of each drug into MHB for prepare working media before adding the standardized inoculum.

6.2.2 Prepared the standardized inoculum by growing microorganisms method to the turbidity of the 1.0 McFarland standard which equivalent to bacterial quantity $3x10^8$ CFU/ml.

6.2.3 Dilute the standardized inoculum to obtain the final bacterial quantity 1×10^8 CFU/ml into working media and control tubes containing broth without β-lactamase inhibitor on water bath shaker at 37°C for 24 hours.

6.2.4 Collect the sample at the time 0,3,6 and 24 hours after microorganism exposed to drug in each concentration including the control group to determining MIC to β-lactam previous selected by agar dilution method as follows:

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

6.2.4.2 Immediately after autoclaving, allow it to cool in a 45 to 50 °C in a water bath.

6.2.4.3 Appropriate dilution of β-lactam solutions were added to molten test agars.

6.2.4.4 The agar and antimicrobial solution were mixed thoroughly and the mixture was poured into petri dishes on a level surface to result in an agar depth of 4 mm. The agar medium was allowed to cool to room temperature.

6.2.4.5 Prepare the standardized inoculum to 0.5 McFarland standard from collected sample in section 6.2.4 and diluted to obtain the final bacterial quantity 1 to 2×10^7 CFU/ml.

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

6.2.4.7 A 1- to 2 μL of each inoculum was applied to the agar surface by the use of an inocula-replication device.

6.2.4.8 The inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots has been absorbed into the agar, but no more than 30 minutes. The plates were inverted and incubated at 37 °C for 16 to 20 hours.

6.2.4.9 The MIC is the lowest concentration of β-lactam antibiotic that completely inhibits growth of the organism on the agar as detected by the unaided eye.

6.2.4.10 β-lactamase induction effect by β-lactamase inhibitor was interpolated from the alteration of MIC and susceptibility pattern by referring to break point in the NCCLS,2000.

6.3 Induction effect test (method B)

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

6.3.2 Immediately after autoclaving, allow it to cool in a 45 to 50 °C in a water bath.

6.3.3 Appropriate dilutions of β-lactamase inhibitor previous selected from double disks method were added to molten test agars.

6.3.4 The agar and antimicrobial solution were mixed thoroughly and the mixture was poured into petri dishes on a level surface to result in an agar depth of 4 mm. The agar medium was allowed to cool to room temperature.

6.3.5 Prepared the standardized inoculum by growing microorganisms to the turbidity of the 0.5 McFarland standard which equivalent to bacterial quantity 1 to $2x10^8$ CFU/ml.

6.3.6 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab should be rotated several times and pressed

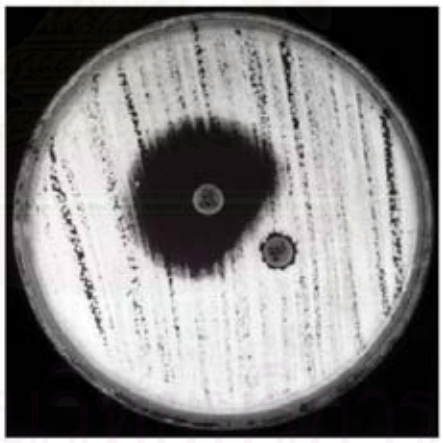
firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.

6.3.7 The dried surface of an agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of agar was swabbed.

6.3.8 β-lactam strip was applied onto the surface of the inoculated agar plate.

6.3.9 The plates were inverted and placed in an ambient air incubator set to 37°C within 15 minutes after the strip was applied in ambient air for 16-18 hours before determine the MIC.

6.3.10 β-lactamase induction effect by β-lactamase inhibitor was interpolated from the alteration of MIC and susceptibility pattern by referring to break point in the NCCLS,2000.



(Modified from Livermore and Brown, 2001)

Figure 3-4 Assessment of β-lactamase induction effect with double disks technique

CHAPTER IV

RESULT

Broth macrodilution method (checkerboard technique) was used to assess the MIC and the synergistic activity of three antimicrobial agent combinations including amoxicillin-clavulanic acid against *Moraxella catarrhalis* and *Haemophilus influenzae*; piperacillin-tazobactam against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*; cefoperazone-sulbactam against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The MICs of all single drugs were higher than the resistance level in the interpretive guidelines from NCCLS, 2000. In contrary, all tested combined drugs demonstrated that the MICs against all microorganisms tested were lower than the susceptible level in the interpretive guidelines from NCCLS, 2000. All drugs manifested the MBC which were similar to the MIC as shown in table 4-1. When comparing the MIC of β-lactam alone and β-lactam combined with β-lactamase inhibitor, it was shown that clavulanic acid at the level of 2 μg/ml could reduce the MIC of amoxicillin against *M. catarrhalis* and *H. influenzae* by 64 times as shown in figure 4-1 and 4-2. Similarly tazobactam at the level of 4 μg/ml could reduce the MIC of piperacillin against *K. pneumoniae* and *P. aeruginosa* to 64 and 4 times, respectively (Figure 4-3 and 4-4). The MIC of cefoperazone against *P. aeruginosa* was decreased 8 times when being combined with 8 μg/ml of sulbactam (Figure 4-5). However, as high as 32 μg/ml of sulbactam were required to reduce the MIC of cefoperazone from >128 μg/ml to 0.015 μg/ml (Figure 4-6).

The synergistic interactions between β-lactam and β-lactamase inhibitor in this study were not only assessed from the MIC value but also were evaluated from the graph shape plotted on the isobologram and the fractional inhibitory concentration (FIC) index that were modified from checkerboard result as described in chapter III (method section). The graph shape of all antimicrobial agent combinations in tested microorganism, except for cefoperazone-sulbactam to *A. baumannii*, were in the concave curve and were defined as the synergism effect. Whereas the straight curve (additive pattern) was displayed in the study on the cefoperazone-sulbactam against *A. baumannii* as shown in figure 4-7. Nevertheless, the average of FIC index calculated from amoxicillin-clavulanic acid combination to *M. catarrhalis*

and *H. influenzae*; piperacillin-tazobactam combination to *K. pneumoniae*; cefoperazone-sulbactam combination against *P. aeruginosa* were equal or lower than 0.5 (0.35, 0.40, 0.33 and 0.50, respectively). On the contrary, the mean of FIC index of piperacillin-tazobactam against *P. aeruginosa* and cefoperazone-sulbactam against *A. baumannii* were between 0.5-1.0 (0.60 and 0.92 respectively). Furthermore, the FIC index calculated at each concentration of β-lactamase inhibitor (Table 4-2) demonstrated that the concentrations of clavulanic acid were 0.008-2 μg/ml against *M. catarrhalis* and *H. influenzae*; those of tazobactam were 0.125-16 μg/ml against *K. pneumoniae*, 4-16 μg/ml against *P. aeruginosa*; those of sulbactam were 2-32 μg/ml against *P. aeruginosa* and 32 μg/ml against *A. baumannii* were equal or less than 0.5.

Table 4-1 The MICs and MBCs of selected β–lactam-β–lactamase inhibitor combinations to tested gram-negative bacteria.

Microorganism	Antibiotic	MIC (μg/ml)	Antibiotic	MIC (μg/ml)	MBC (μg/ml)
M. catarrhalis	Amx	8	Amx:cla	0.125:2	0.125:2
H. influenzae	Amx	16	Amx:cla	0.25:2	0.25:2
K. pneumoniae	Pip	128	Pip:taz	2:4	2:4
P. aeruginosa	Pip	128	Pip:taz	32:4	32:4
P. aeruginosa	Cpz	>128	Cpz:sul	16:4	32:4
A. baumannii	Cpz	>128	Cpz:sul	0.015:32	0.015:32

Amx = amoxicillin, Pip = piperacillin, Cpz = cefoperazone, cla = clavulanic acid, taz= tazobactam, sul = sulbactam

32	32/0.004	32/0.008	32/0.015	32/0.03	32/0.06	32/0.125	32/0.25	32/0.5	32/1	32/2	32/4	32/8
16	16/0.004	16/0.008	16/0.015	16/0.03	16/0.06	16/0.125	16/0.25	16/0.5	16/1	16/2	16/4	16/8
8	8/0.004	8/0.008	8/0.015	8/0.03	8/0.06	8/0.125	8/0.25	8/0.5	8/1	8/2	8/4	8/8
4	4/0.004	4/0.008	4/0.015	4/0.03	4/0.06	4/0.125	4/0.25	4/0.5	4/1	4/2	4/4	4/8
2	2/0.004	2/0.008	2/0.015	2/0.03	2/0.06	2/0.125	2/0.25	2/0.5	2/1	2/2	2/4	2/8
1	1/0.004	1/0.008	1/0.015	1/0.03	1/0.06	1/0.125	1/0.25	1/0.5	1/1	1/2	1/4	1/8
0.5	0.5/0.004	0.5/0.008	0.5/0.015	0.5/0.03	0.5/0.06	0.5/0.125	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8
0.25	0.25/0.004	0.25/0.008	0.25/0.015	0.25/0.03	0.25/0.06	0.25/0.125	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8
0.125	0.125/0.004	0.125/0.008	0.125/0.015	0.125/0.03	0.125/0.06	0.125/0.125	0.125/0.25	0.125/0.5	0.125/1	0.125/2	0.125/4	0.125/8
0.06	0.06/0.004	0.06/0.008	0.06/0.015	0.06/0.03	0.06/0.06	0.06/0.125	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8
0.03	0.03/0.004	0.03/0.008	0.03/0.015	0.03/0.03	0.03/0.06	0.03/0.125	0.03/0.25	0.03/0.5	0.03/1	0.03/2	0.03/4	0.03/8
0.015	0.015/0.004	0.015/0.008	0.015/0.015	0.015/0.03	0.015/0.06	0.015/0.125	0.015/0.25	0.015/0.5	0.015/1	0.015/2	0.015/4	0.015/8
0.008	0.008/0.004	0.008/0.008	0.008/0.015	0.008/0.03	0.008/0.06	0.008/0.125	0.008/0.25	0.008/0.5	0.008/1	0.008/2	0.008/4	0.008/8
	0.004	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8

Amoxicillin

Clavulanic acid

Figure 4-1 The synergism result (checkerboard) of amoxicillin-clavulanic acid against *Moraxella catarrhalis*
shadow zone : visible microorganism growth, white zone : no microorganism growth

32	32/0.004	32/0.008	32/0.015	32/0.03	32/0.06	32/0.125	32/0.25	32/0.5	32/1	32/2	32/4	32/8
16	16/0.004	16/0.008	16/0.015	16/0.03	16/0.06	16/0.125	16/0.25	16/0.5	16/1	16/2	16/4	16/8
8	8/0.004	8/0.008	8/0.015	8/0.03	8/0.06	8/0.125	8/0.25	8/0.5	8/1	8/2	8/4	8/8
4	4/0.004	4/0.008	4/0.015	4/0.03	4/0.06	4/0.125	4/0.25	4/0.5	4/1	4/2	4/4	4/8
2	2/0.004	2/0.008	2/0.015	2/0.03	2/0.06	2/0.125	2/0.25	2/0.5	2/1	2/2	2/4	2/8
1	1/0.004	1/0.008	1/0.015	1/0.03	1/0.06	1/0.125	1/0.25	1/0.5	1/1	1/2	1/4	1/8
0.5	0.5/0.004	0.5/0.008	0.5/0.015	0.5/0.03	0.5/0.06	0.5/0.125	0.5/0.25	0.5/0.5	0.5/1	0.5/2	0.5/4	0.5/8
0.25	0.25/0.004	0.25/0.008	0.25/0.015	0.25/0.03	0.25/0.06	0.25/0.125	0.25/0.25	0.25/0.5	0.25/1	0.25/2	0.25/4	0.25/8
0.125	0.125/0.004	0.125/0.008	0.125/0.015	0.125/0.03	0.125/0.06	0.125/0.125	0.125/0.25	0.125/0.5	0.125/1	0.125/2	0.125/4	0.125/8
0.06	0.06/0.004	0.06/0.008	0.06/0.015	0.06/0.03	0.06/0.06	0.06/0.125	0.06/0.25	0.06/0.5	0.06/1	0.06/2	0.06/4	0.06/8
0.03	0.03/0.004	0.03/0.008	0.03/0.015	0.03/0.03	0.03/0.06	0.03/0.125	0.03/0.25	0.03/0.5	0.03/1	0.03/2	0.03/4	0.03/8
0.015	0.015/0.004	0.015/0.008	0.015/0.015	0.015/0.03	0.015/0.06	0.015/0.125	0.015/0.25	0.015/0.5	0.015/1	0.015/2	0.015/4	0.015/8
0.008	0.008/0.004	0.008/0.008	0.008/0.015	0.008/0.03	0.008/0.06	0.008/0.125	0.008/0.25	0.008/0.5	0.008/1	0.008/2	0.008/4	0.008/8
	0.004	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8

Amoxicillin

Clavulanic acid

Figure 4-2 The synergism result (checkerboard) of amoxicillin-clavulanic acid against *Haemophilus influenzae*
shadow zone : visible microorganism growth, white zone : no microorganism growth

128	128/0.125	128/0.25	128/0.5	128/1	128/2	128/4	128/8	128/16	128/32	128/64
64	64/0.125	64/0.25	64/0.5	64/1	64/2	64/4	64/8	64/16	64/32	64/64
32	32/0.125	32/0.25	32/0.5	32/1	32/2	32/4	32/8	32/16	32/32	32/64
16	16/0.125	16/0.25	16/0.5	16/1	16/2	16/4	16/8	16/16	16/32	16/64
8	8/0.125	8/0.25	8/0.5	8/1	8/2	8/4	8/8	8/16	8/32	8/64
4	4/0.125	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64
2	2/0.125	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64
1	1/0.125	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64
0.5	0.5/0.125	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	0.25/0.125	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
	0.125	0.25	0.5	1	2	4	8	16	32	64

Piperacillin

Tazobactam

Figure 4-3 The synergism result (checkerboard) of piperacillin-tazobactam against *Klebsiella pneumoniae* shadow zone : visible microorganism growth, white zone : no microorganism growth

128	128/0.125	128/0.25	128/0.5	128/1	128/2	128/4	128/8	128/16	128/32	128/64
64	64/0.125	64/0.25	64/0.5	64/1	64/2	64/4	64/8	64/16	64/32	64/64
32	32/0.125	32/0.25	32/0.5	32/1	32/2	32/4	32/8	32/16	32/32	32/64
16	16/0.125	16/0.25	16/0.5	16/1	16/2	16/4	16/8	16/16	16/32	16/64
8	8/0.125	8/0.25	8/0.5	8/1	8/2	8/4	8/8	8/16	8/32	8/64
4	4/0.125	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64
2	2/0.125	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64
1	1/0.125	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64
0.5	0.5/0.125	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	0.25/0.125	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
	0.125	0.25	0.5	1	2	4	8	16	32	64

Piperacillin

Tazobactam

Figure 4-4 The synergism result (checkerboard) of piperacillin-tazobactam against *Pseudomonas aeruginosa* shadow zone : visible microorganism growth, white zone : no microorganism growth

Cefoperazone														
128	128/0.015	128/0.03	128/0.06	128/0.125	128/0.25	128/0.5	128/1	128/2	128/4	128/8	128/16	128/32	128/64	128/128
64	64/0.015	64/0.03	64/0.06	64/0.125	64/0.25	64/0.5	64/1	64/2	64/4	64/8	64/16	64/32	64/64	64/128
32	32/0.015	32/0.03	32/0.06	32/0.125	32/0.25	32/0.5	32/1	32/2	32/4	32/8	32/16	32/32	32/64	32/128
16	16/0.015	16/0.03	16/0.06	16/0.125	16/0.25	16/0.5	16/1	16/2	16/4	16/8	16/16	16/32	16/64	16/128
8	8/0.015	8/0.03	8/0.06	8/0.125	8/0.25	8/0.5	8/1	8/2	8/4	8/8	8/16	8/32	8/64	8/128
4	4/0.015	4/0.03	4/0.06	4/0.125	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.015	2/0.03	2/0.06	2/0.125	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.015	1/0.03	1/0.06	1/0.125	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.015	0.5/0.03	0.5/0.06	0.5/0.125	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	0.25/0.015	0.25/0.03	0.25/0.06	0.25/0.125	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
0.125	0.125/0.015	0.125/0.03	0.125/0.06	0.125/0.125	0.125/0.25	0.125/0.5	0.125/1	0.125/2	0.125/4	0.125/8	0.125/16	0.125/32	0.125/64	0.125/128
0.06	0.06/0.015	0.06/0.03	0.06/0.06	0.06/0.125	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
0.03	0.03/0.015	0.03/0.03	0.03/0.06	0.03/0.125	0.03/0.25	0.03/0.5	0.03/1	0.03/2	0.03/4	0.03/8	0.03/16	0.03/32	0.03/64	0.03/128
0.015	0.015/0.015	0.015/0.03	0.015/0.06	0.015/0.125	0.015/0.25	0.015/0.5	0.015/1	0.015/2	0.015/4	0.015/8	0.015/16	0.015/32	0.015/64	0.015/128
	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128

Sulbactam

Figure 4-5 The synergism result (checkerboard) of cefoperazone-sulbactam against *Pseudomonas aeruginosa* shadow zone : visible microorganism growth, white zone : no microorganism growth

Cefoperazone														
128	128/0.015	128/0.03	128/0.06	128/0.125	128/0.25	128/0.5	128/1	128/2	128/4	128/8	128/16	128/32	128/64	128/128
64	64/0.015	64/0.03	64/0.06	64/0.125	64/0.25	64/0.5	64/1	64/2	64/4	64/8	64/16	64/32	64/64	64/128
32	32/0.015	32/0.03	32/0.06	32/0.125	32/0.25	32/0.5	32/1	32/2	32/4	32/8	32/16	32/32	32/64	32/128
16	16/0.015	16/0.03	16/0.06	16/0.125	16/0.25	16/0.5	16/1	16/2	16/4	16/8	16/16	16/32	16/64	16/128
8	8/0.015	8/0.03	8/0.06	8/0.125	8/0.25	8/0.5	8/1	8/2	8/4	8/8	8/16	8/32	8/64	8/128
4	4/0.015	4/0.03	4/0.06	4/0.125	4/0.25	4/0.5	4/1	4/2	4/4	4/8	4/16	4/32	4/64	4/128
2	2/0.015	2/0.03	2/0.06	2/0.125	2/0.25	2/0.5	2/1	2/2	2/4	2/8	2/16	2/32	2/64	2/128
1	1/0.015	1/0.03	1/0.06	1/0.125	1/0.25	1/0.5	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
0.5	0.5/0.015	0.5/0.03	0.5/0.06	0.5/0.125	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	0.25/0.015	0.25/0.03	0.25/0.06	0.25/0.125	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
0.125	0.125/0.015	0.125/0.03	0.125/0.06	0.125/0.125	0.125/0.25	0.125/0.5	0.125/1	0.125/2	0.125/4	0.125/8	0.125/16	0.125/32	0.125/64	0.125/128
0.06	0.06/0.015	0.06/0.03	0.06/0.06	0.06/0.125	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
0.03	0.03/0.015	0.03/0.03	0.03/0.06	0.03/0.125	0.03/0.25	0.03/0.5	0.03/1	0.03/2	0.03/4	0.03/8	0.03/16	0.03/32	0.03/64	0.03/128
0.015	0.015/0.015	0.015/0.03	0.015/0.06	0.015/0.125	0.015/0.25	0.015/0.5	0.015/1	0.015/2	0.015/4	0.015/8	0.015/16	0.015/32	0.015/64	0.015/128
	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128

Sulbactam

Figure 4-6 The synergism result (checkerboard) of cefoperazone-sulbactam against *Acinetobacter baumannii* shadow zone : visible microorganism growth, white zone : no microorganism growth

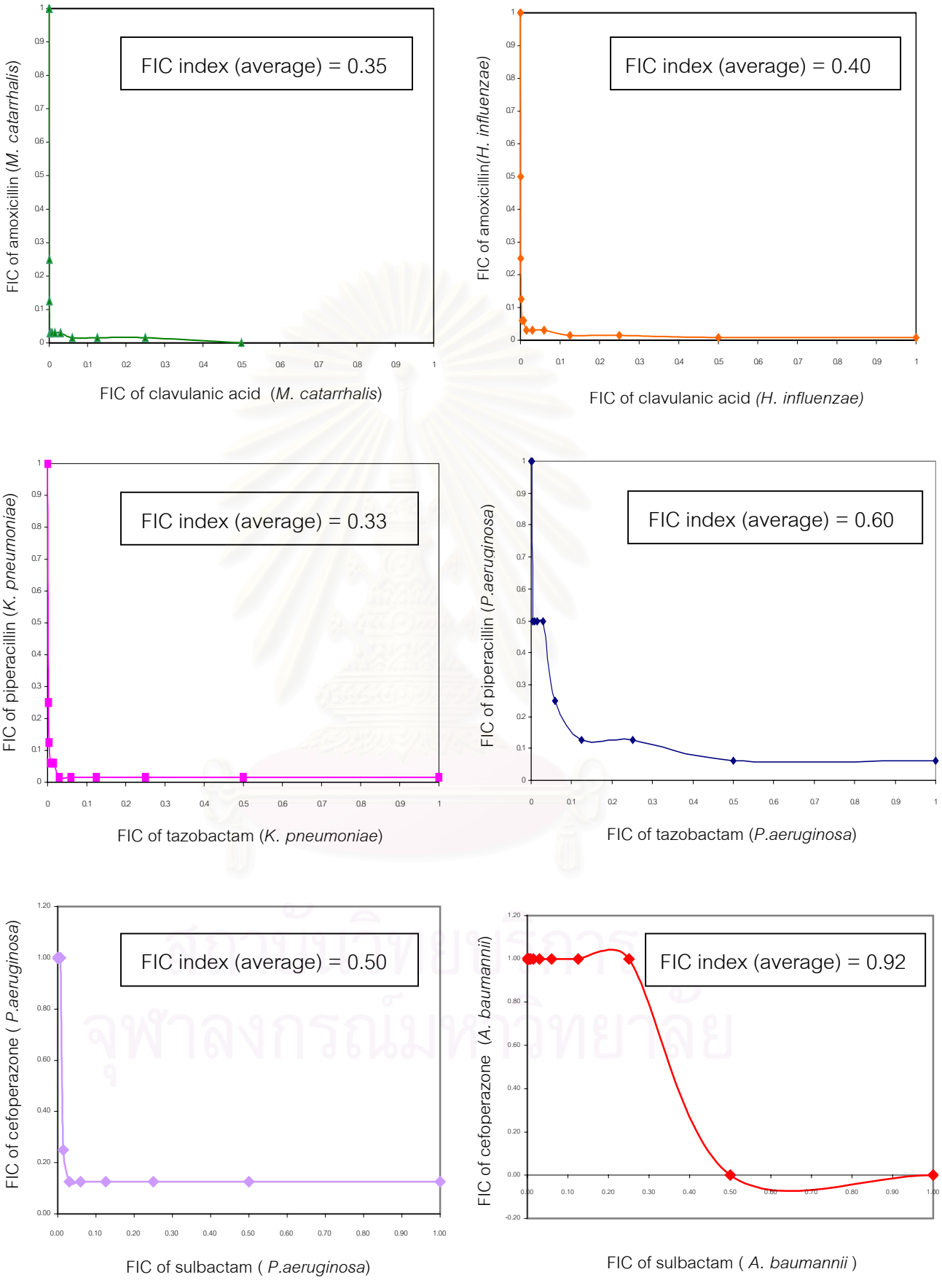


Figure 4-7 The isobologram and FIC index (average) of β-lactam-β-lactamase inhibitor combinations against gram-negative bacteria

Table 4-2 The calculation FIC index of β-lactam plus β-lactamase inhibitor and MICs of β-lactam at each concentration of β-lactamase inhibitor (shade : the concentrations of β-lactamase inhibitor that showed FIC index below than 0.5)

Concentration of clavulanic acid (μg/ml)	8	4	2	1	0.5	0.25	0.125	0.06	0.03	0.015	0.008	0.004	0
MIC of amoxicillin (μg/ml) to *M. catarrhalis*	0	0.125	0.125	0.125	0.25	0.25	0.25	0.25	0.25	1	2	8	8
FIC index	1	0.516	0.266	0.141	0.094	0.062	0.047	0.039	0.035	0.13	0.251	1.00	1.00

Concentration of clavulanic acid (μg/ml)	8	4	2	1	0.5	0.25	0.125	0.06	0.03	0.015	0.008	0.004	0
MIC of amoxicillin (μg/ml) to *H. influenzae*	0.125	0.25	0.25	0.5	0.5	0.5	1	1	2	4	8	16	16
FIC index	1.008	0.52	0.266	0.156	0.094	0.062	0.08	0.07	0.13	0.252	0.50	1.00	1.00

Concentration of tazobactam (μg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	0
MIC of piperacillin (μg/ml) to *K. pneumoniae*	2	2	2	2	2	2	8	8	16	32	128
FIC index	1.02	0.52	0.27	0.14	0.08	0.05	0.08	0.07	0.13	0.25	1.00

Concentration of tazobactam (μg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	0
MIC of piperacillin (μg/ml) to *P.aeruginosa*	8	8	16	16	32	64	64	64	64	128	128
FIC index	1.06	0.56	0.38	0.25	0.31	0.53	0.52	0.51	0.50	1.00	1.00

Concentration of sulbactam (μg/ml)	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0
MIC of cefoperazone (μg/ml) to *P.aeruginosa*	16	16	16	16	16	16	32	128	>128	>128	>128	>128
FIC index	1.13	0.63	0.38	0.25	0.19	0.16	0.27	1.01	>1	>1	>1	>1

Concentration of sulbactam (μg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	0.06	0
MIC of cefoperazone (μg/ml) to *A. baumannii*	0	0.015	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
FIC index	1.00	0.50	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1

Time-kill study was exercised to compare the bactericidal activity of the combined drugs in various concentrations between β-lactam and β-lactamase inhibitor to each microorganism. The concentrations of β-lactam and β-lactamase inhibitor chosen in the study were shown in table 4-3. Amoxicillin alone did not have antibacterial activity against *M. catarrhalis* and amoxicillin merely displayed bacterial growth inhibition being with clavulanic acid at minimum concentration of 0.03 μg/ml (Figure 4-8). However, the bactericidal property was demonstrated in amoxicillin at 2 MIC when combined to clavulanic acid at 2 and 4 μg/ml. Thus, the activity was not increased even concentration of amoxicillin was greater to 8 MIC (Figure 4-9 and 4-10). Furthermore, the antibacterial activity of amoxicillin obtained was not different when clavulanic acid at either 2μg/ml or 4μg/ml was brought together with amoxicillin at supra MIC level (Figure 4-11 to 4-13). The regrowth of *M. catarrhalis* was suppressed if amoxicillin above 2 MIC was combined with clavulanic acid 2μg/ml and 4μg/ml. Regarding, the antimicrobial effect in drug combination was quantitatively evaluated from bacterial killing and regrowth curves as described in Firsov, et al., 1997. The synergisms between amoxicillin-clavulanic acid against *M. catarrhalis* were demonstrated in the combination between 2 MIC or 8 MIC of amoxicillin and 2 and 4 μg/ml of clavulanic acid by 4.33 - 4.88 logCFU/ml decreasing at 24 hours in comparison to the most active single drug (amoxicillin 2MIC, 8MIC). The KR3 calculated from amoxicillin alone at the concentration of 1/4MIC-8MIC against *M.catarrhalis* was -0.37 to -0.25 logCFU/hr.ml and the highest KR3 which was 0.99 logCFU/hr.ml was obtained when amoxicillin was combined with clavulanic acid (Table 4-4).

In case of *H. influenzae*, the pattern of bactericidal of amoxicillin-clavulanic acid obtained from time-kill curve (Figure 4-14 to 4-19) were the same as that of amoxicillin and clavulanic acid combination against *M. catarrhalis* as mentioned above. The antimicrobial effect in drug combination quantitatively evaluated as described by Firsov, et al., 1997 demonstrated that the combinations of amoxicillin at 2MIC to 8MIC and clavulanic acid manifested their KR3 at –0.27 to -0.57 logCFU/hr.ml (-0.30 logCFU/hr.ml for amoxicillin without clavulanic acid) to *H. influenzae*. Additionally, the LogN24 of amoxicillin combined with clavulanic acid at 2 and 4 μg/ml were less than that amoxicillin alone (4.29 – 4.94 logCFU/ml). The BA24 of amoxicillin alone was less than amoxicillin in combination; amoxicillin demonstrated BA24 more than 75 logCFU.hr/ml in concomitant with clavulanic acid at either 2 or 4 μg/ml, whereas amoxicillin alone could not express this value (Table 4-5).

Table 4-3 The concentrations of β–lactam and β–lactamase inhibitor chosen in the assessment of bactericidal activity by time kill method

Microorganism	BL	MIC (μg/ml)	¼ MIC (μg/ml)	2 MIC(μg/ml)	8MIC (μg/ml)	BI	C_{min} (μg/ml)	$C_{average}$(μg/ml)	C_{max} (μg/ml)
M. catarrhalis	Amx[a]	0.125	0.03	0.25	1	Cla	0.03	2	4
H. influenzae	Amx[a]	0.25	0.06	0.5	2	Cla	0.06	2	4
K. pneumoniae	Pip[b]	2	0.5	4	16	Taz	0.25	4	32
P. aeruginosa	Pip[b]	32	8	64	128	Taz	0.25	4	32
P. aeruginosa	Cpz[c]	16	4	32	128	Sul	4	8	64
A. baumannii	Cpz	0.015	0.015*	16*	128*	Sul	32	8	64

Amx = amoxicillin, Pip = piperacillin, Cpz = cefoperazone, Cla = clavulanic acid, Taz= tazobactam, Sul = sulbactam

BL = β-lactam ,BI= β-lactamase inhibitor,

a = MIC of amoxicillin as combined with clavulanic acid 2 μg/ml, b = MIC of piperacillin as combined with tazobactam 4 μg/ml, c = MIC of cefoperazone as combined with sulbactam 8 μg/ml

* = the concentration of cefoperazone to *A. baumannii* determined from breakpoint of interpretive guideline by referring the NCCLS, 2000

C_{min} = the minimum concentration of β-lactamase inhibitor that can reduce the MIC of accompanied β-lactam to the breakpoint of interpretive guideline the NCCLS, 2000

$C_{average}$ = the average concentration of β-lactamase inhibitor after taking available dosage of β-lactamase inhibitor (Joly-Guillou, et al., 1995; Gilbert, Moellering, and Sande, 2001)

C_{max} = the peak concentration of β-lactamase inhibitor after taking available dosage of β-lactamase inhibitor (Joly-Guillou, et al., 1995; Gilbert, Moellering, and Sande, 2001)

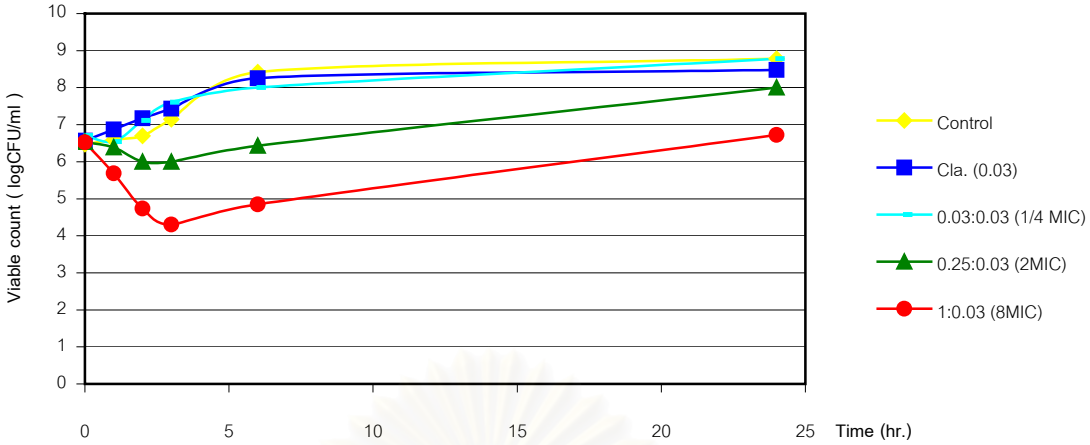


Figure 4-8 The killing curves of amoxicillin-clavulanic acid against *Moraxella catarrhalis* at concentration of 0.03:0.03, 0.25:0.03, and 1:0.03

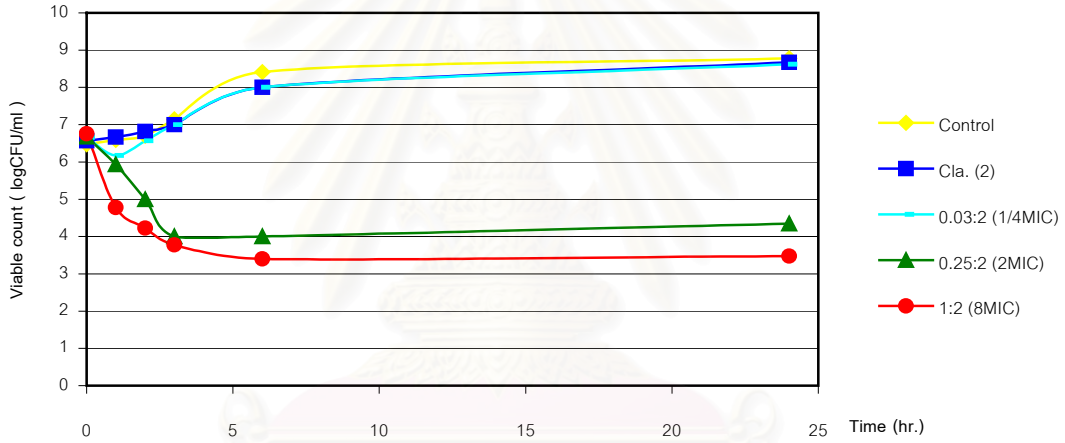


Figure 4-9 The killing curves of amoxicillin-clavulanic acid against *Moraxella catarrhalis* at concentration of 0.03:2, 0.25:2, and 1:2

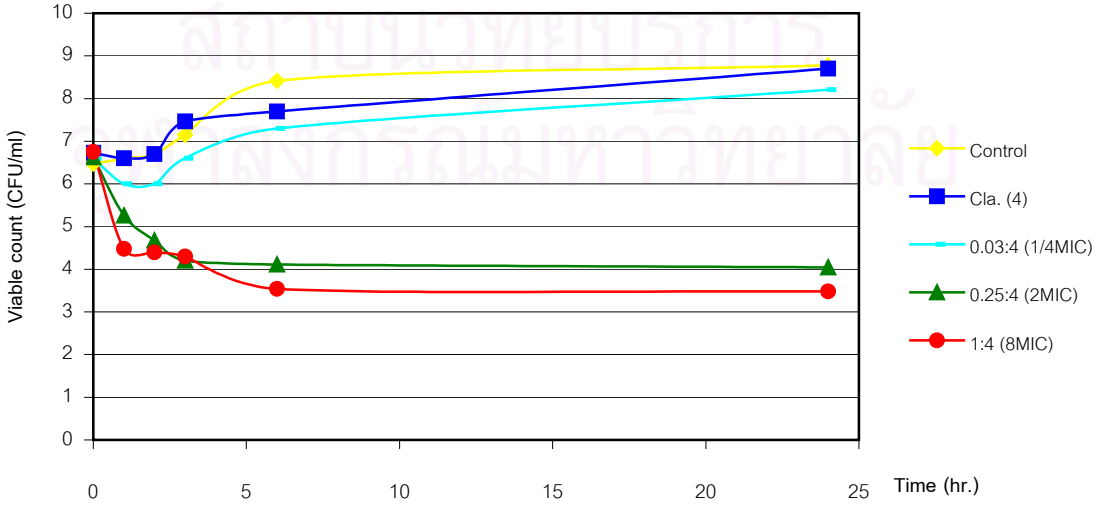


Figure 4-10 The killing curves of amoxicillin-clavulanic acid against *Moraxella catarrhalis* at concentration of 0.03:4, 0.25:4, and 1:4

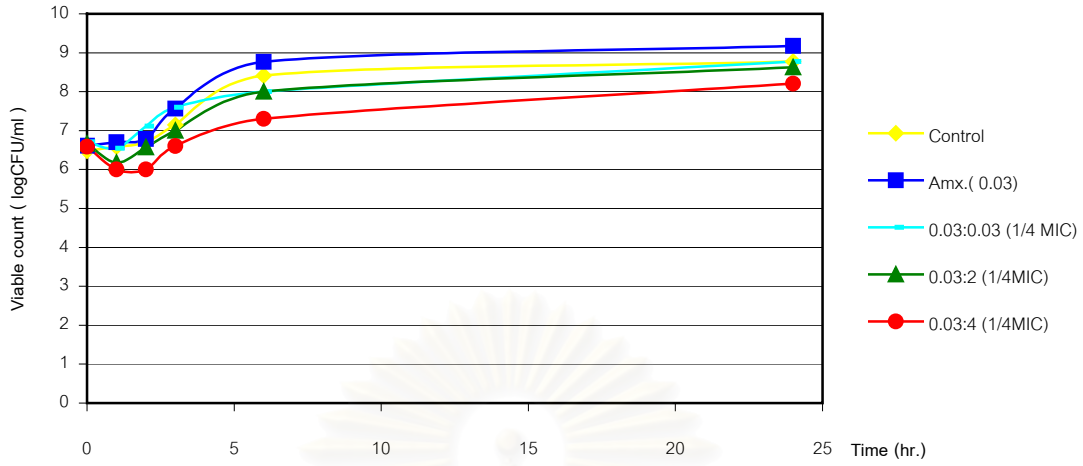


Figure 4-11 The killing curves of amoxicillin-clavulanic acid against *Moraxella catarrhalis* at concentration of 0.03:0.03, 0.03:2, and 0.03:4

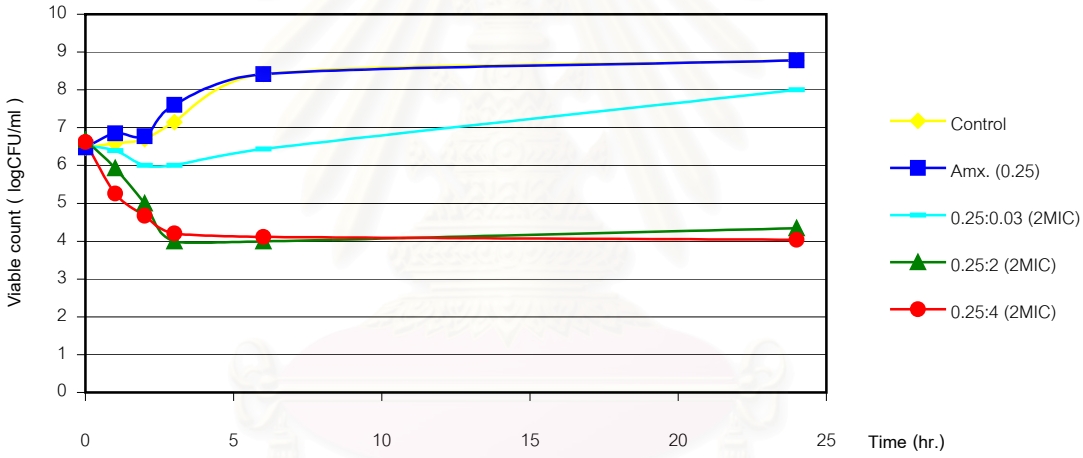


Figure 4-12 The killing curves of amoxicillin-clavulanic acid against *Moraxella catarrhalis* at concentration of 0.25:0.03, 0.25:2, and 0.25:4

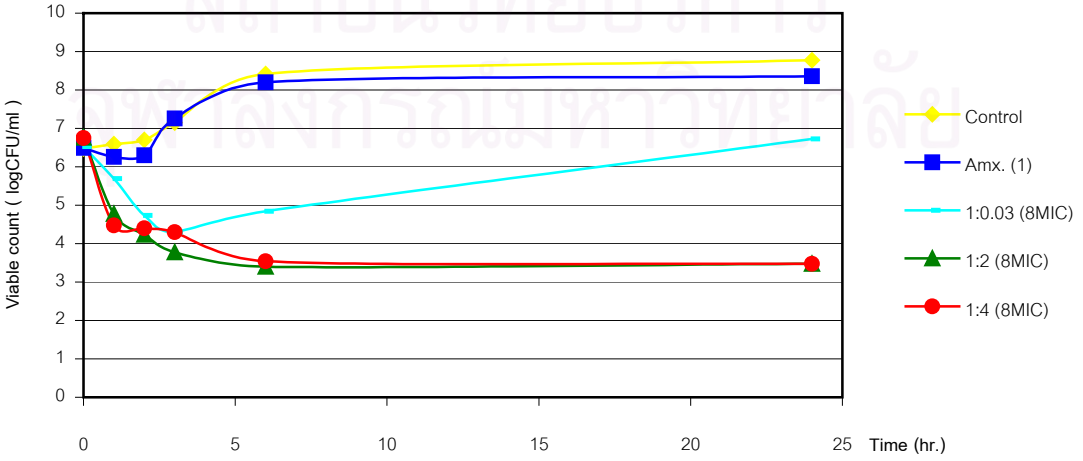


Figure 4-13 The killing curves of amoxicillin-clavulanic acid against *Moraxella catarrhalis* at concentration of 1:0.03, 1:2, and 1:4

Table 4-4 The killing kinetics and regrowth parameters of amoxicillin and clavulanic acid against *Moraxella catarrhalis*.

Parameter\Conc	Control	Amx			Cla			Amx:Cla (1/4 MIC)			Amx:Cla (2 MIC)			Amx:Cla (8 MIC)		
		¼ MIC (0.03)	2 MIC (0.25)	8 MIC (1)	Cmin (0.03)	Caverage (2)	Cmax (4)	0.03:0.03	0.03:2	0.03:4	0.25:0.03	0.25:2	0.25:4	1:0.03	1:2	1:4
T90%	-3.78	-	-	-	-	-	-	-	-	-	-	1.28	0.74	1.18	0.50	0.44
T99%	-9.40	-	-	-	-	-	-	-	-	-	-	2.33	2.11	2.46	1.04	0.88
T99.9%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.16	5.17
T*E*	-	-	-	2.20	-	-	2.04	1.30	2.21	2.98	7.15	>24	>24	22.07	>24	>24
Tmin	0.00	0.00	0.00	1.00	0.00	0.00	1.00	1.00	1.00	2.00	3.00	6.00	24.00	3.00	6.00	24.00
LogNmin	6.48	6.61	6.48	6.26	6.57	6.57	6.60	6.54	6.18	6.00	6.00	4.00	4.04	4.30	3.40	3.48
6logNmin	0.00	0.00	0.00	0.24	0.00	0.00	0.13	0.16	0.48	0.59	0.53	2.67	2.58	2.22	3.36	3.27
T	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
LogN*T*	8.46	8.81	8.46	8.22	8.28	8.07	7.81	8.09	8.07	7.40	6.61	4.04	4.11	5.05	3.41	3.54
LogN24	8.78	9.18	8.78	8.36	8.48	8.67	8.70	8.78	8.62	8.20	8.00	4.34	4.04	6.72	3.48	3.48
6Log $N_{(single\ drug\ -combination)}$	-	-	-	-	-	-	-	- 0.30	-0.14	0.28	0.48	4.33*	4.66*	1.64	4.88*	4.88*
6logN3	-0.67	-0.96	-1.12	-0.76	-0.86	-0.43	-0.73	-0.89	-0.34	-0.01	0.53	2.67	2.42	2.22	2.98	2.45
6logN24	-2.30	-2.56	-2.30	-1.87	-1.91	-2.10	-1.97	-2.07	-1.96	-1.61	-1.47	2.33	2.58	-0.20	3.28	3.27
Killing rate3	-0.22	-0.32	-0.37	-0.25	-0.29	-0.14	-0.24	-0.30	-0.11	0.00	0.18	0.89	0.81	0.74	0.99	0.82
AUC24	198.18	206.53	199.43	191.71	195.17	192.81	190.72	195.22	191.68	179.00	167.19	103.33	101.22	133.68	86.92	89.36
Bacteriolytic area24		-8.35	-1.25	6.47	3.01	5.37	7.46	2.96	6.50	19.18	31.00	94.85	96.97	64.50	111.27	108.82

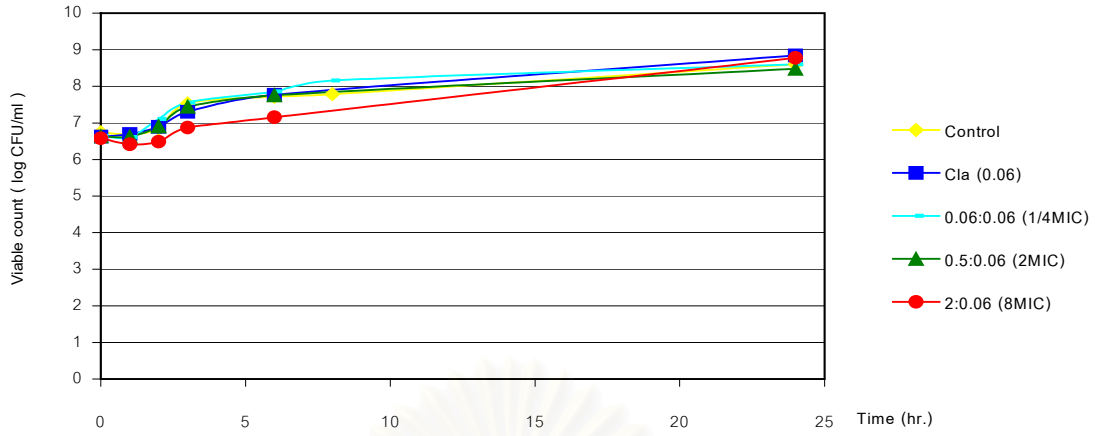


Figure 4-14 The killing curves of amoxicillin-clavulanic acid against *Haemophilus influenzae* at concentration of 0.06:0.06, 0.5:0.06, and 2:0.06

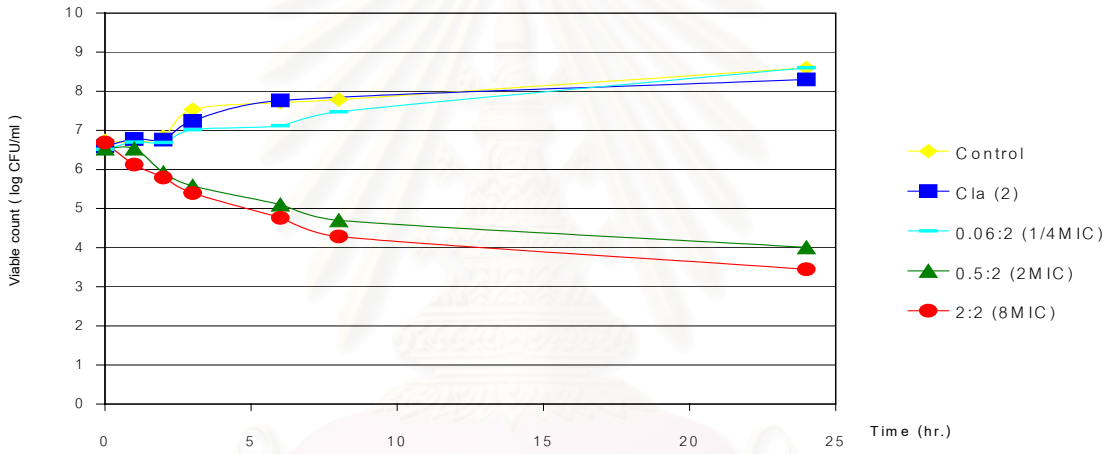


Figure 4-15 The killing curves of amoxicillin-clavulanic acid against *Haemophilus influenzae* at concentration of 0.06:2, 0.5:2, and 2:2

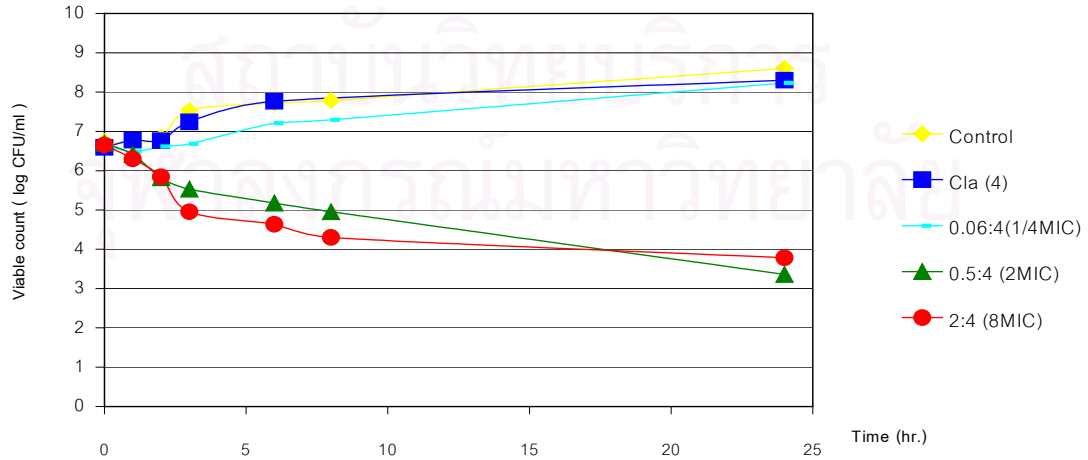


Figure 4-16 The killing curves of amoxicillin-clavulanic acid against *Haemophilus influenzae* at concentration of 0.06:4, 0.5:4, and 2:4

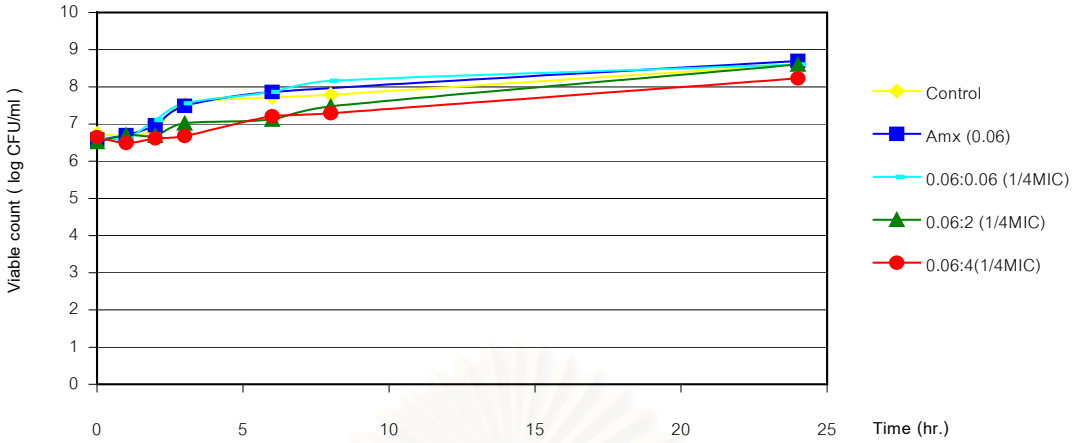


Figure 4-17 The killing curves of amoxicillin-clavulanic acid against *Haemophilus influenzae* at concentration of 0.06:0.06, 0.06:2, and 0.06:4

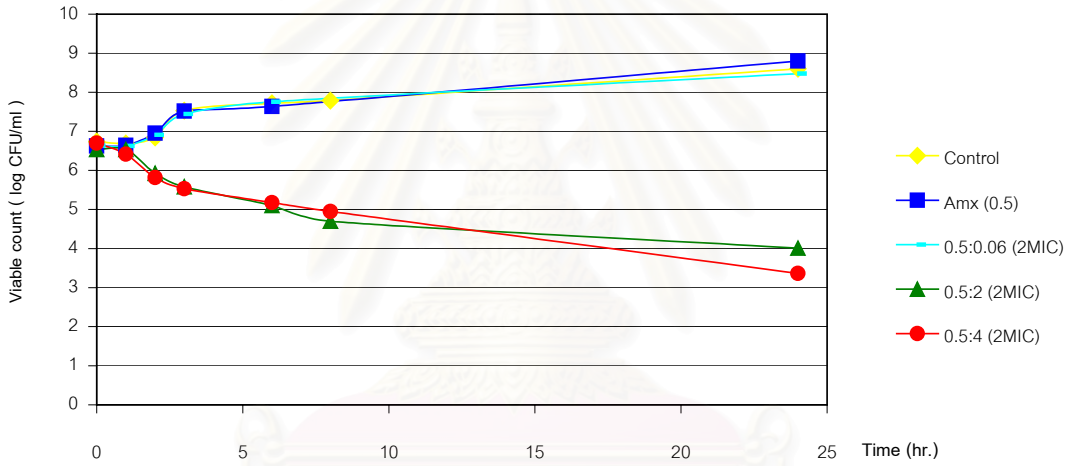


Figure 4-18 The killing curves of amoxicillin-clavulanic acid against *Haemophilus influenzae* at concentration of 0.5:0.06, 0.5:2, and 0.5:4

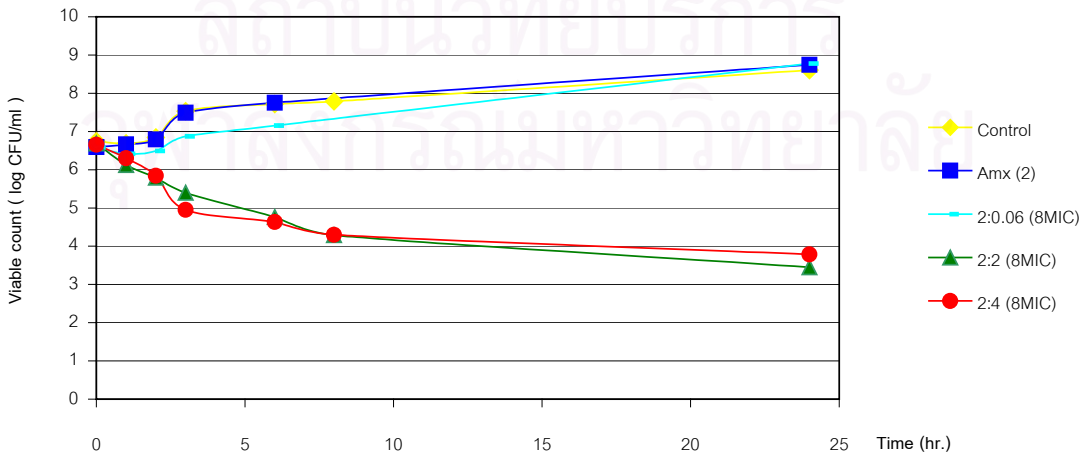


Figure 4-19 The killing curves of amoxicillin-clavulanic acid against *Haemophilus influenzae* at concentration of 2:0.06, 2:2, and 2:4

Table 4-5 The killing kinetics and regrowth parameters of amoxicillin and clavulanic acid against *Haemophilus influenzae*.

Parameter\Conc	Control	Amx			Cla			Amx:Cla (1/4 MIC)			Amx:Cla (2 MIC)			Amx:Cla (8 MIC)		
		¼ MIC (0.06)	2MIC (0.5)	8MIC (2)	Cmin (0.06)	Caverage (2)	Cmax (4)	0.06:0.06	0.06:2	0.06:4	0.5:0.06	0.5:2	0.5:4	2:0.06	2:2	2:4
T90%	-6.57	-	-	-	-	-	-	-	-	-	-	3.31	2.41	-	2.26	2.22
T99%	-	-	-	-	-	-	-	-	-	-	-	11.94	10.52	-	6.30	5.81
T99.9%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19.43	-
T*E*	1.31	-	-	-	-	-	-	1.06	-	2.43	1.03	>24	>24	2.23	>24	>24
Tmin	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	1.00	24.00	24.00	1.00	24.00	24.00
LogNmin	6.69	6.58	6.62	6.59	6.62	6.59	6.69	6.61	6.52	6.49	6.62	4.01	3.36	6.41	3.45	3.79
6logNmin	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.15	0.01	2.52	3.34	0.16	3.24	2.87
T	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
LogN*T*	7.79	7.96	7.76	7.86	7.89	7.82	7.25	8.16	7.48	7.29	7.83	4.70	4.95	7.33	4.29	4.30
LogN24	8.60	8.70	8.80	8.74	8.85	8.30	8.30	8.60	8.60	8.23	8.48	4.01	3.36	8.78	3.45	3.79
6Log N (single drug -combination)	-	-	-	-	-	-	-	0.10	-0.30	0.07	0.32	4.29*	4.94*	-0.04	4.85*	4.51*
6logN3	-0.79	-0.91	-0.90	-0.90	-0.68	-0.65	-0.43	-0.91	-0.50	-0.04	-0.81	0.95	1.17	-0.30	1.29	1.70
6logN24	-1.86	-2.12	-2.18	-2.15	-2.22	-1.71	-1.61	-1.96	-2.08	-1.59	-1.84	2.52	3.34	-2.20	3.24	2.87
Killing rate3	-0.26	-0.30	-0.30	-0.30	-0.23	-0.22	-0.14	-0.30	-0.17	-0.01	-0.27	0.32	0.39	-0.10	0.43	0.57
AUC24	190.14	192.79	191.31	191.79	192.70	187.54	180.42	194.07	184.59	179.30	189.45	113.99	111.07	184.04	104.11	105.96
Bacteriolytic area24		-2.65	-1.17	-1.64	-2.56	2.60	9.72	-3.93	5.55	10.84	0.69	76.15	79.07	6.10	86.03	84.19

The killing activity to *K. pneumoniae* was not illustrated by piperacillin in the absence of tazobactam at minimum level of 0.25 μg/ml however this activity was obtained when piperacillin at 2 MIC was combined with tazobactam at 4, or 32 μg/ml. Moreover, the antibacterial activity became greater when the concentration of piperacillin was amplified to 8 MIC (Figure 4-20 to 4-22). On the other hand, the concentration of tazobactam between 4 and 32 μg/ml in combination with piperacillin at supra MIC did not show the different bacterial killing property as shown in figure 4-23 to 4-25. The regrowth of *K. pneumoniae* was suppressed when being exposed to combination between piperacillin at 8 MIC and tazobactam at 32 μg/ml. The quantitative evaluation in antimicrobial effect demonstrated that the KR3 of 2MIC of piperacillin alone was less than that of concentration of piperacillin combined with tazobactam (piperacillin 2MIC-8MIC = -0.78 to –0.24 logCFU/hr.ml and increased to = 0.64-0.69 logCFU/hr.ml when piperacillin 2MIC and 8MIC was combined with tazobactam 4 μg/ml and to be 0.61-0.81 logCFU/hr.ml as combined with tazobactam 32 μg/ml). The synergism activity to *K. pneumoniae* of piperacillin was detected at 8 MIC in combination with tazobactam 4 and 32 μg/ml with 3.39 – 4.04 logCFU/ml decreasing at 24 hours more than the most active single drug. Similarly, the BA24 of those combinations increased approximately 100 logCFU.hr/ml (Table 4-6).

Piperacillin at high concentration (4MIC) demonstrated bacterial growth suppression in *P. aeruginosa*. Antibacterial activity of piperacillin was clearly expressed being combined with tazobactam at 4 and 32 but not 0.25 μg/ml. Moreover, the increased level of piperacillin generated the greater killing activity in the exponential phase of *P. aeruginosa* (Figure 4-26 to 4-28). Whereas, the concentration of tazobactam between 4 and 32μg/ml combined with piperacillin at supra-MIC displayed the comparable antibacterial activity (Figure 4-29 to 4-31). The regrowth of *P. aeruginosa* could be suppressed when piperacillin above 2 MIC was combined with tazobactam above 4μg/ml. The antimicrobial effect quantitatively evaluated in table 4-7 demonstrated that the synergism interaction between piperacillin and tazobactam was observed when piperacillin at supra MIC was combined with tazobactam 4 and 32 μg/ml (LogN24 of piperacillin combination was less than piperacillin alone around 2.13 – 2.66 logCFU/ml). The KR3 of piperacillin alone was less than piperacillin combinations approximately 0.40 logCFU/hr.ml (piperacillin alone = -0.04 to 0.25 logCFU/hr.ml and piperacillin 2MIC-4MIC combined with tazobactam 4 and 32μg/ml = 0.41-0.65 logCFU/hr.ml).

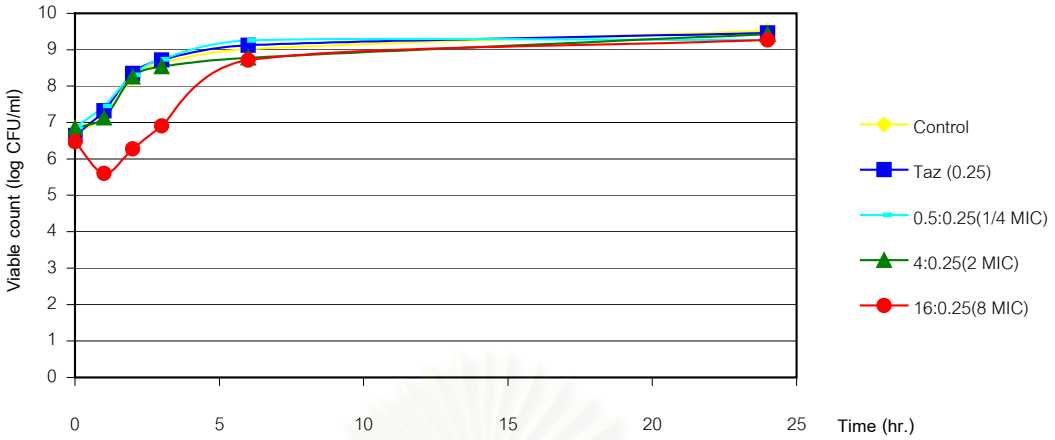


Figure 4-20 The killing curves of piperacillin-tazobactam against *Klebsiella pneumoniae* at concentration of 0.5:0.25, 4:0.25, and 16:0.25

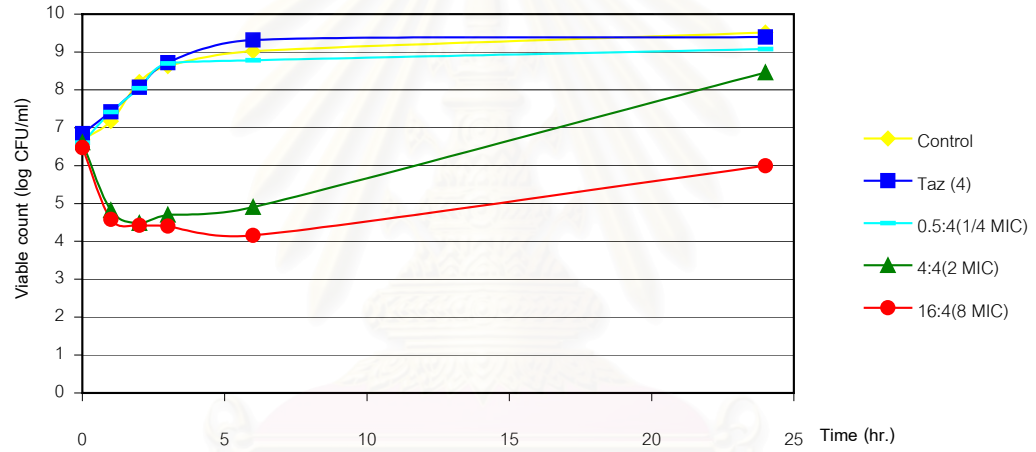


Figure 4-21 The killing curves of piperacillin-tazobactam against *Klebsiella pneumoniae* at concentration of 0.5:4, 4:4, and 16:4

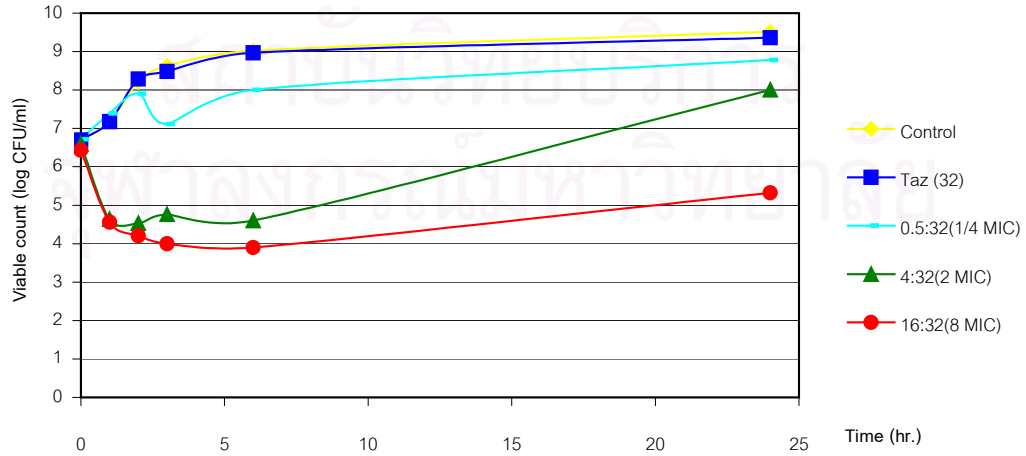


Figure 4-22 The killing curves of piperacillin-tazobactam against *Klebsiella pneumoniae* at concentration of 0.5:32, 4:32, and 16:32

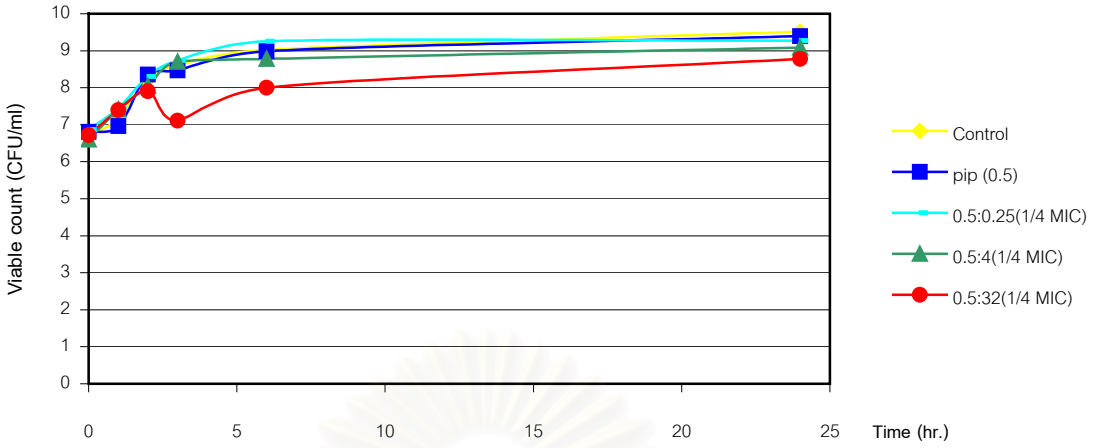


Figure 4-23 The killing curves of piperacillin-tazobactam against *Klebsiella pneumoniae* at concentration of 0.5:0.25, 0.5:4, and 0.5:32

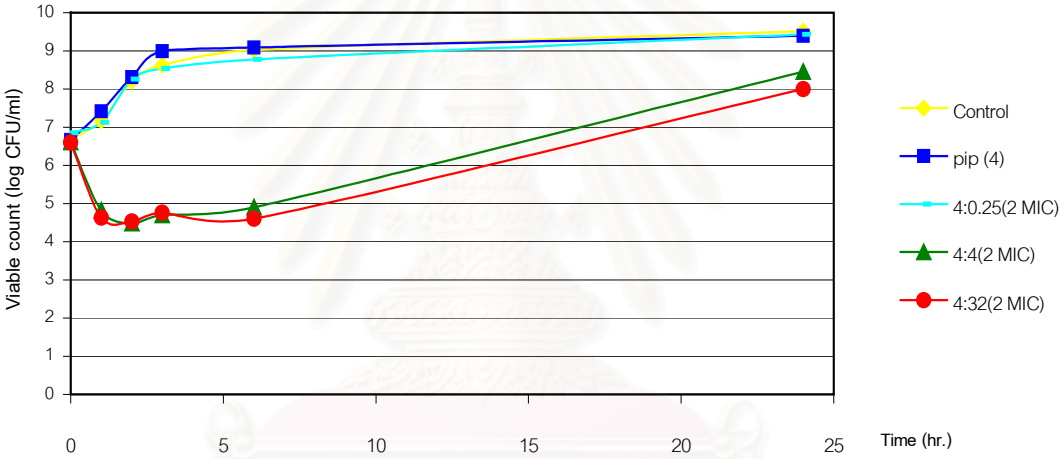


Figure 4-24 The killing curves of piperacillin-tazobactam against *Klebsiella pneumoniae* at concentration of 4:0.25, 4:4, and 4:32

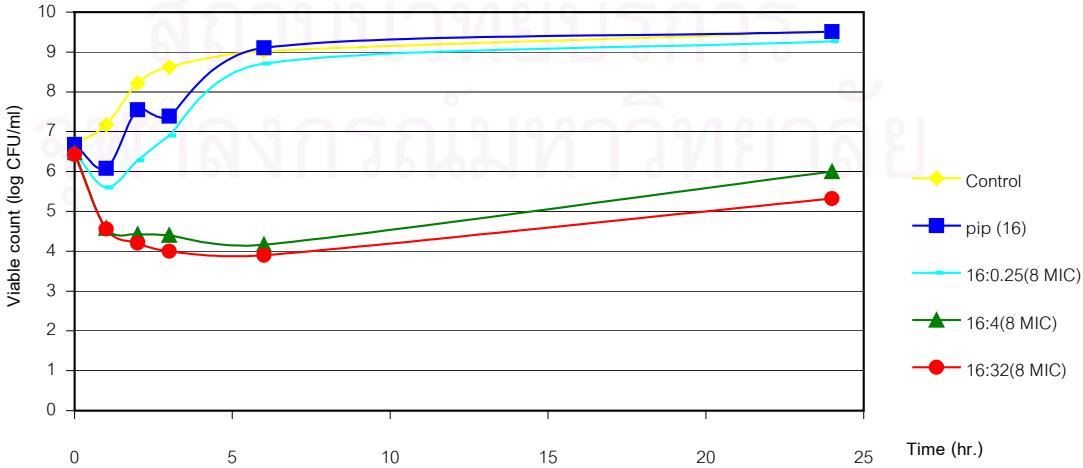


Figure 4-25 The killing curves of piperacillin-tazobactam against *Klebsiella pneumoniae* at concentration of 16:0.25, 16:4, and 16:32

Table 4-6 The killing kinetics and regrowth parameters of piperacillin and tazobactam against *Klebsiella pneumoniae.*

Parameter\Conc	Control	Pip			Taz			Pip:Taz (1/4 MIC)			Pip:Taz (2MIC)			Pip:Taz (8MIC)		
		¼ MIC (0.5)	2MIC (4)	8MIC (16)	Cmin (0.25)	Caverage (4)	Cmax (32)	0.5:0.25	0.5:4	0.5:32	4:0.25	4:4	4:32	16:0.25	16:4	16:32
T90%	-1.49	-	-	-	-	-	-	-	-	-	-	0.56	0.51	-	0.53	0.53
T99%	-3.38	-	-	-	-	-	-	-	-	-	-	1.62	1.40	-	1.67	1.43
T99.9%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T*E*	-	-	-	1.41	-	-	-	-	-	-	-	14.65	16.54	2.32	>24	>24
Tmin	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.00	2.00	1.00	6.00	6.00
LogNmin	6.68	6.81	6.66	6.08	6.64	6.85	6.70	6.87	6.60	6.72	6.85	4.48	4.53	5.60	4.16	3.90
6logNmin	0.00	0.00	0.00	0.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.12	2.06	0.88	2.32	2.53
T	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
LogN*T*	9.08	9.04	9.12	9.15	9.16	9.32	9.01	9.26	8.81	8.09	8.85	5.30	4.98	8.77	4.37	4.06
LogN24	9.51	9.40	9.40	9.51	9.46	9.39	9.36	9.27	9.08	8.78	9.43	8.46	8.00	9.27	6.00	5.32
6Log N (single drug -combination)	-	-	-	-	-	-	-	0.13	0.31	0.58	-0.03	0.93	1.36	0.19	3.39*	4.04*
6logN3	-1.95	-1.66	-2.33	-0.71	-2.08	-1.87	-1.78	-1.86	-2.10	-0.40	-1.69	1.91	1.84	-0.43	2.07	2.43
6logN24	-2.84	-2.59	-2.73	-2.83	-2.82	-2.55	-2.66	-2.40	-2.48	-2.06	-2.58	-1.85	-1.41	-2.79	0.48	1.11
Killing rate3	-0.65	-0.55	-0.78	-0.24	-0.69	-0.62	-0.59	-0.62	-0.70	-0.13	-0.56	0.64	0.61	-0.14	0.69	0.81
AUC24	216.33	214.65	217.05	212.95	217.38	218.69	214.15	217.27	210.04	195.89	212.95	149.60	142.30	203.84	118.75	108.86
Bacteriolytic area24		1.68	-0.72	3.38	-1.05	-2.36	2.19	-0.94	6.29	20.45	3.39	66.73	74.03	12.49	97.59	107.47

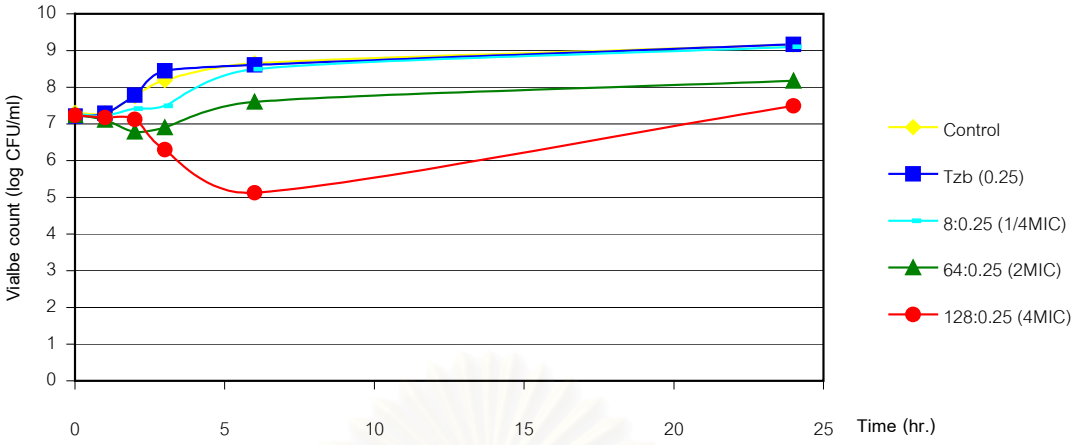


Figure 4-26 The killing curves of piperacillin-tazobactam against *Pseudomonas aeruginosa* at concentration of 8:0.25, 64:0.25, and 128:0.25

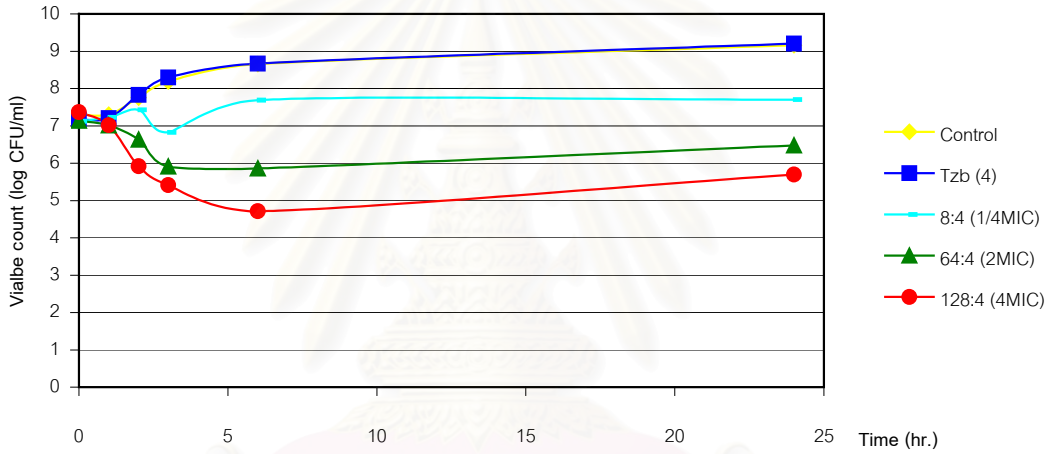


Figure 4-27 The killing curves of piperacillin-tazobactam against *Pseudomonas aeruginosa* at concentration of 8:4, 64:4, and 128:4

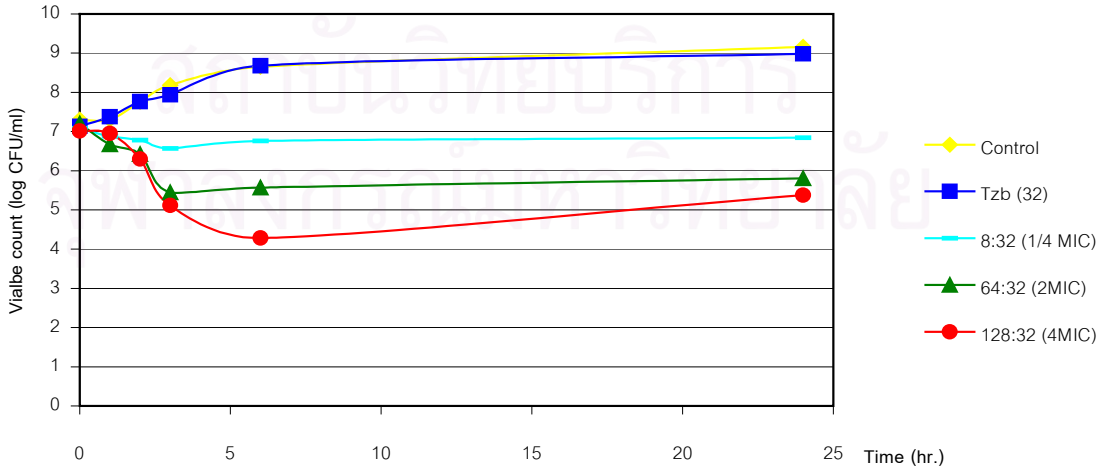


Figure 4-28 The killing curves of piperacillin-tazobactam against *Pseudomonas aeruginosa* at concentration of 8:32, 64:32, and 128:32

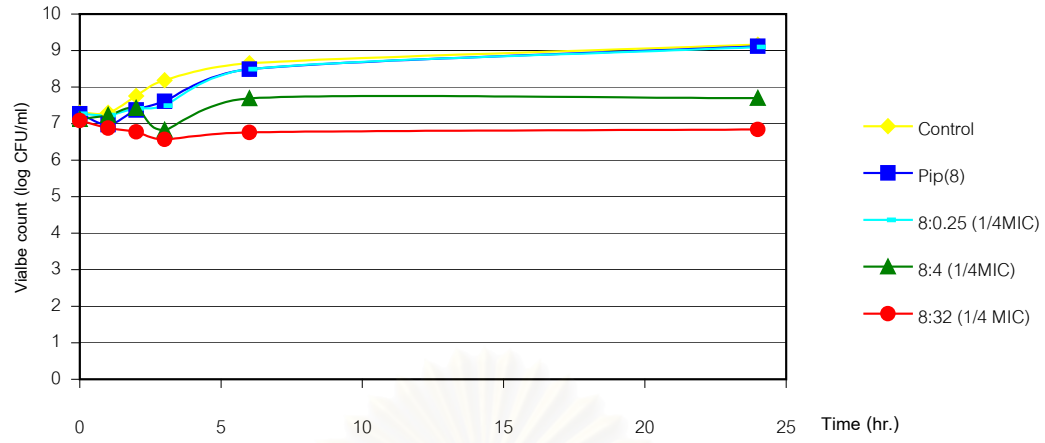


Figure 4-29 The killing curves of piperacillin-tazobactam against *Pseudomonas aeruginosa* at concentration of 8:0.5, 8:4, and 8:32

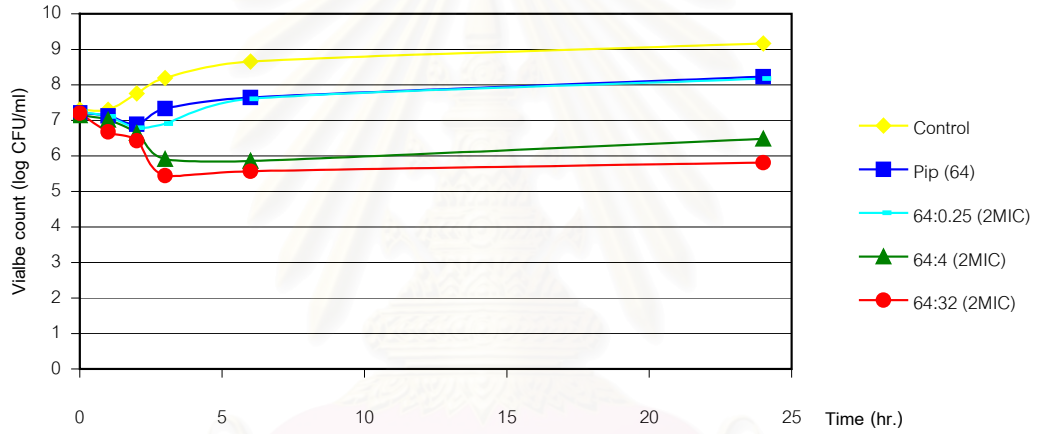


Figure 4-30 The killing curves of piperacillin-tazobactam against *Pseudomonas aeruginosa* at concentration of 64:0.5, 64:4, and 64:32

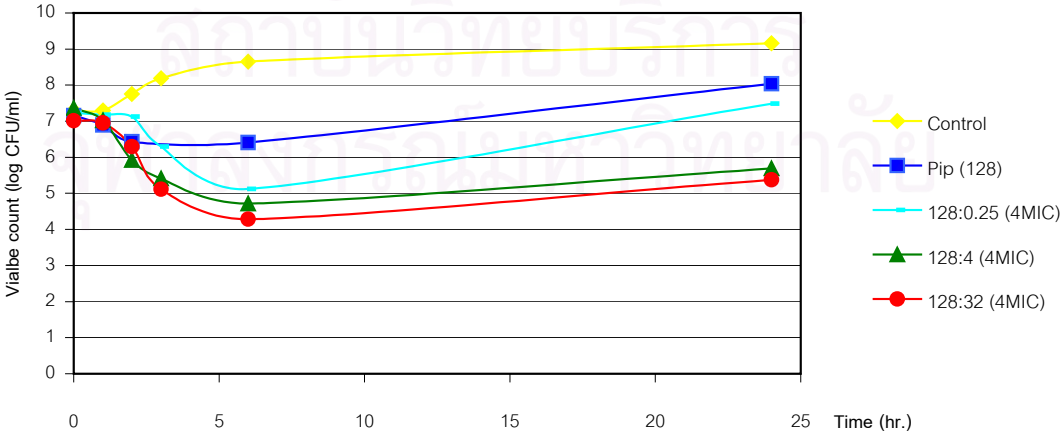


Figure 4-31 The killing curves of piperacillin-tazobactam against *Pseudomonas aeruginosa* at concentration of 128:0.5, 128:4, and 128:32

Table 4-7 The killing kinetics and regrowth parameters of piperacillin and tazobactam against *Pseudomonas aeruginosa.*

Parameter\Conc	Control	Pip			Taz			Pip:Taz (1/4MIC)			Pip:Taz (2MIC)			Pip:Taz (4MIC)		
		1/4MIC (8)	2MIC (64)	4MIC (128)	Cmin (0.25)	Caverage (4)	Cmax (32)	8:0.25	8:4	8:32	64:0.25	64:4	64:32	128:0.25	128:4	128:32
T90%	-3.71	-	-	-	-	-	-	-	-	-	-	2.68	2.23	3.18	1.59	2.24
T99%	-	-	-	-	-	-	-	-	-	-	-	-	-	5.72	3.17	3.32
T99.9%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T*E*	1.00	1.72	2.73	14.28	-	-	-	1.21	4.01	>24	4.46	>24	>24	22.02	>24	>24
Tmin	1.00	1.00	2.00	6.00	0.00	0.00	0.00	1.00	3.00	3.00	2.00	6.00	3.00	6.00	6.00	6.00
LogNmin	7.30	6.98	6.89	6.41	7.21	7.17	7.13	7.23	6.83	6.57	6.79	5.86	5.45	5.12	4.72	4.28
δlogNmin	0.00	0.30	0.33	0.75	0.00	0.00	0.00	0.04	0.29	0.51	0.46	1.28	1.76	2.11	2.65	2.73
T	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
LogN*T*	8.71	8.56	7.71	6.59	8.67	8.73	8.71	8.56	7.69	6.77	7.67	5.93	5.59	5.39	4.82	4.41
LogN24	9.16	9.12	8.23	8.04	9.17	9.20	8.98	9.10	7.70	6.85	8.18	6.48	5.81	7.49	5.69	5.38
δLog N (single drug -combination)	-	-	-	-	-	-	-	0.02	1.42	2.13*	0.05	1.75	2.42*	0.55	2.35*	2.66*
δlogN3	-0.89	-0.34	-0.12	0.76	-1.24	-1.13	-0.81	-0.22	0.29	0.51	0.34	1.23	1.76	0.93	1.95	1.90
δlogN24	-1.86	-1.84	-1.02	-0.88	-1.96	-2.04	-1.85	-1.83	-0.58	0.24	-0.93	0.66	1.40	-0.26	1.68	1.64
Killing rate3	-0.30	-0.11	-0.04	0.25	-0.41	-0.38	-0.27	-0.07	0.10	0.17	0.11	0.41	0.59	0.31	0.65	0.63
AUC24	208.39	204.43	186.61	169.46	208.55	209.11	206.58	204.33	181.91	162.92	184.74	148.83	138.32	151.73	128.22	120.38
Bacteriolytic area24		3.97	21.79	38.93	-0.16	-0.72	1.81	4.06	26.49	45.47	23.66	59.56	70.08	56.66	80.18	88.01

Cefoperazone alone at 8 MIC could not eradicate *P. aeruginosa* but the antibacterial property of cefoperazone was demonstrated if either cefoperazone 2 MIC or 8 MIC was combined to sulbactam at 4μg/ml. As well as both concentrations of cefoperazone (2MIC and 8MIC) in combination with sulbactam showed comparable antibacterial activity (Figure 4-32 to 4-34). Likewise, the altered concentrations of sulbactam (4,8, and 64 μg/ml) could not produce the different killing activity of cefoperazone (Figure 4-35 to 4-37). The regrowth of *P. aeruginosa* was suppressed when being exposed to combination between cefoperazone at 2 to 8MIC and sulbactam above 8 μg/ml. The antimicrobial effect quantitatively evaluated in table 4-8 demonstrated that the synergism of cefoperazone-sulbactam combination against *P.aeruginosa* was expressed at 2MIC-8MIC of cefoperazone combined with sulbactam 4-32 μg/ml by 2.39 - 3.80 logCFU decreasing at 24 hours more than the most active single drug. Accordingly, the KR3 of combinations between cefoperazone at supra MIC and sulbactam at 4 to 32 μg/ml was calculated to 0.46 - 0.64 logCFU/hr.ml (the KR3 of cefoperazone alone was -0.027 to -0.23 logCFU/hr.ml). Additionally, those drug combinations demonstrated the BA24 more than 70 logCFU.hr/ml.

Interestingly, there was no antibacterial property against *A. baumannii* exerted by cefoperazone alone (128 μg/ml) whereas sulbactam alone at 32 μg/ml could show this property (Figure 4-38 to 4-40). The antibacterial activity of sulbactam could be superior developed when sulbactam was 64 μg/ml (Figure 4-41 to 4-43). The regrowth of *A. baumannii* was suppressed when being exposed to combination between cefoperazone at any concentration and sulbactam above 64 μg/ml. The antimicrobial effect quantitatively evaluated in table 4-9 showed that there was no synergism characteristic was observed in combination between cefoperazone and sulbactam (the colony forming unit at 24 hours of combinations between cefoperazone and sulbactam was not different from that of sulbactam alone). Correspondingly, the KR3 and BA24 calculated of sulbactam alone were comparable with that of sulbactam in combination with cefoperazone against *A.baumannii*.

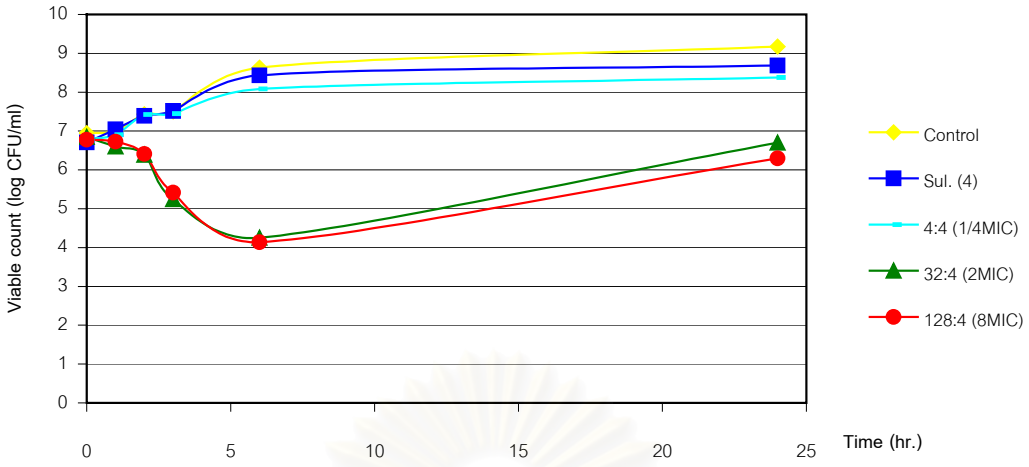


Figure 4-32 The killing curves of cefoperazone-sulbactam against *Pseudomonas aeruginosa* at concentration of 4:4, 32:4, and 128:4

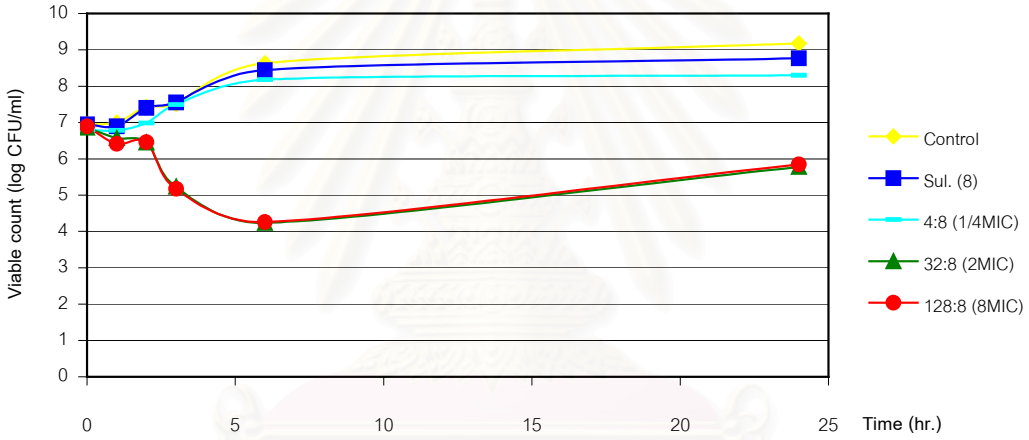


Figure 4-33 The killing curves of cefoperazone-sulbactam against *Pseudomonas aeruginosa* at concentration of 4:8, 32:8, and 128:8

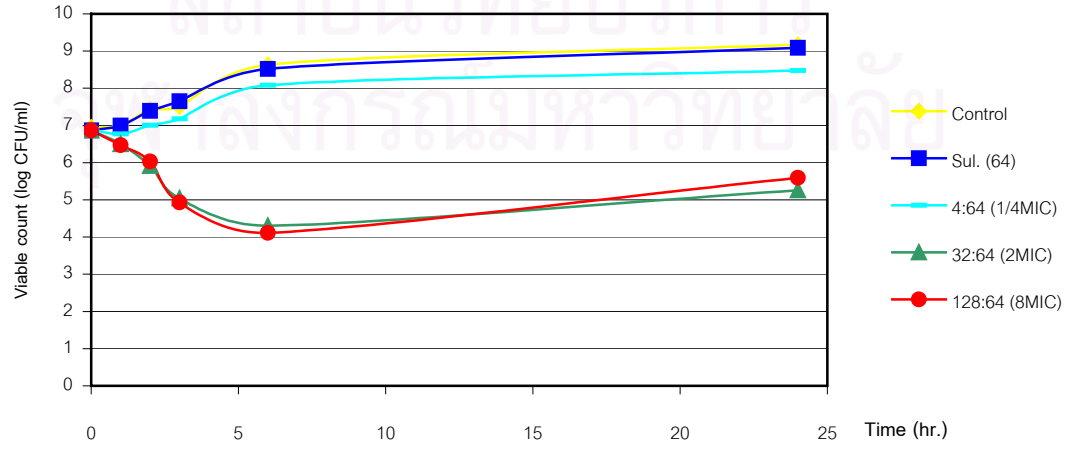


Figure 4-34 The killing curves of cefoperazone-sulbactam against *Pseudomonas aeruginosa* at concentration of 4:64, 32:64, and 128:64

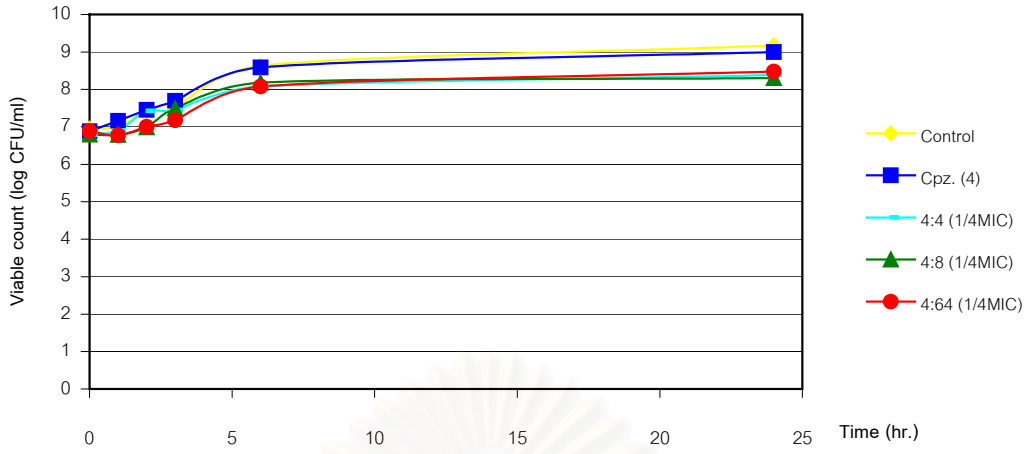


Figure 4-35 The killing curves of cefoperazone-sulbactam against *Pseudomonas aeruginosa* at concentration of 4:4, 4:8, and 4:64

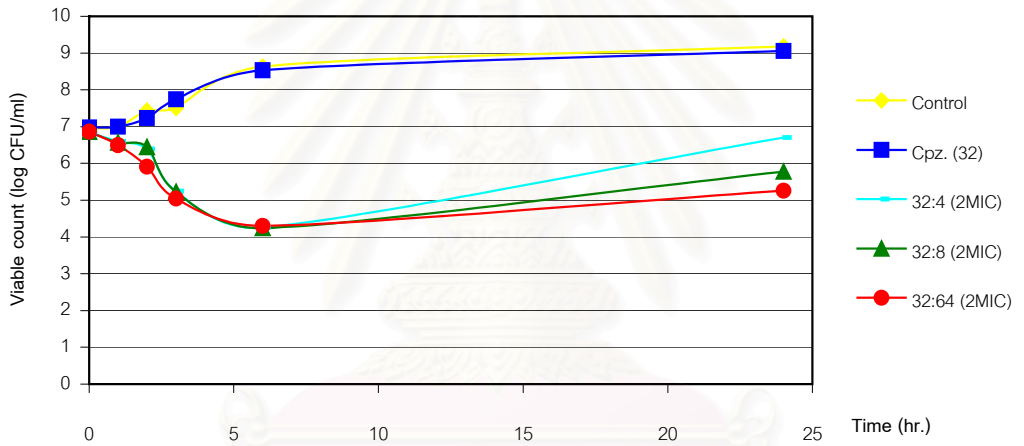


Figure 4-36 The killing curves of cefoperazone-sulbactam against *Pseudomonas aeruginosa* at concentration of 32:4, 32:8, and 32:64

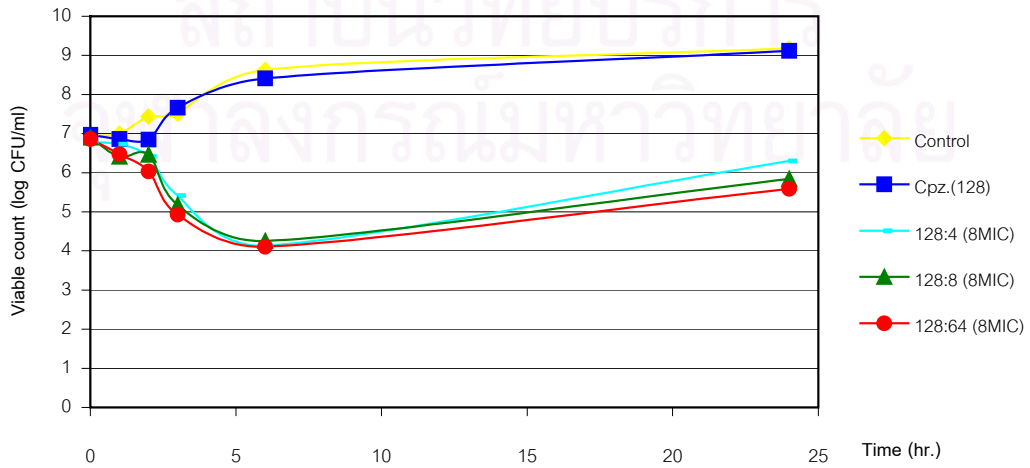


Figure 4-37 The killing curves of cefoperazone-sulbactam against *Pseudomonas aeruginosa* at concentration of 128:4, 128:8, and 128:64

Table 4-8 The killing kinetics and regrowth parameters of cefoperazone and sulbactam against *Pseudomonas aeruginosa.*

Parameter\Conc	Control	Cpz			Sul			Cpz:Sul (1/4MIC)			Cpz:Sul (2MIC)			Cpz:Sul (8MIC)		
		1/4MIC (4)	2 MIC (32)	8MIC (128)	Cmin (4)	Caverage (8)	Cmax (64)	4:4	4:8	4:64	32:4	32:8	32:64	128:4	128:8	128:64
T90%	-4.18	-	-	-	-	-	-	-	-	-	2.47	2.48	2.06	2.63	2.44	2.51
T99%	-16.47	-	-	-	-	-	-	-	-	-	4.21	4.12	3.76	4.49	3.91	3.26
T99.9%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T*E*	-	-	-	2.15	-	1.12	-	-	1.05	1.52	>24	>24	>24	>24	>24	>24
Tmin	0.00	0.00	0.00	2.00	0.00	1.00	0.00	0.00	1.00	1.00	6.00	6.00	6.00	6.00	6.00	6.00
LogNmin	6.95	6.89	6.97	6.85	6.71	6.89	6.87	6.79	6.79	6.77	4.26	4.24	4.30	4.14	4.26	4.11
6logNmin	0.00	0.00	0.00	0.12	0.00	0.06	0.00	0.00	0.01	0.12	2.59	2.62	2.56	2.64	2.64	2.75
T	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
LogN*T*	8.69	8.64	8.59	8.49	8.46	8.49	8.58	8.11	8.19	8.12	4.53	4.41	4.41	4.38	4.44	4.27
LogN24	9.18	9.00	9.06	9.11	8.69	8.77	9.09	8.38	8.30	8.48	6.70	5.78	5.26	6.30	5.85	5.59
6Log N (single drug -combination)	-	-	-	-	-	-	-	0.31	0.47	0.52	1.99	2.99*	3.80*	2.39*	2.92*	3.50*
6logN3	-0.56	-0.81	-0.77	-0.69	-0.81	-0.61	-0.78	-0.65	-0.69	-0.29	1.60	1.63	1.81	1.37	1.72	1.93
6logN24	-2.23	-2.11	-2.09	-2.14	-1.98	-1.82	-2.22	-1.59	-1.50	-1.59	0.15	1.08	1.60	0.48	1.05	1.27
Killing rate3	-0.19	-0.27	-0.26	-0.23	-0.27	-0.20	-0.26	-0.22	-0.23	-0.10	0.53	0.54	0.60	0.46	0.57	0.64
AUC24	206.16	204.65	204.30	201.97	199.56	200.54	204.40	192.87	192.75	192.74	131.95	123.45	118.43	127.51	124.06	119.27
Bacteriolytic area24		1.51	1.86	4.19	6.60	5.62	1.77	13.30	13.41	13.43	74.22	82.71	87.73	78.65	82.10	86.90

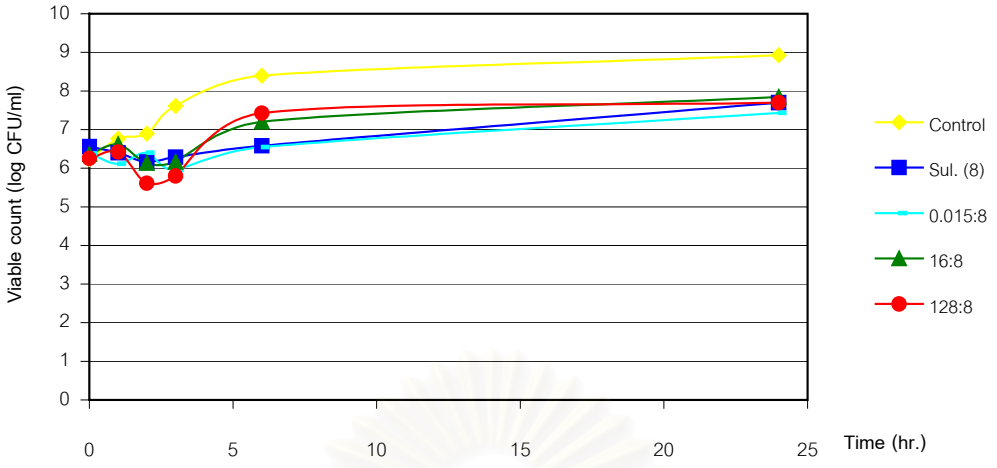


Figure 4-38 The killing curves of cefoperazone-sulbactam against *Acinetobacter baumannii* at concentration of 0.015:8, 16:8, and 128:8

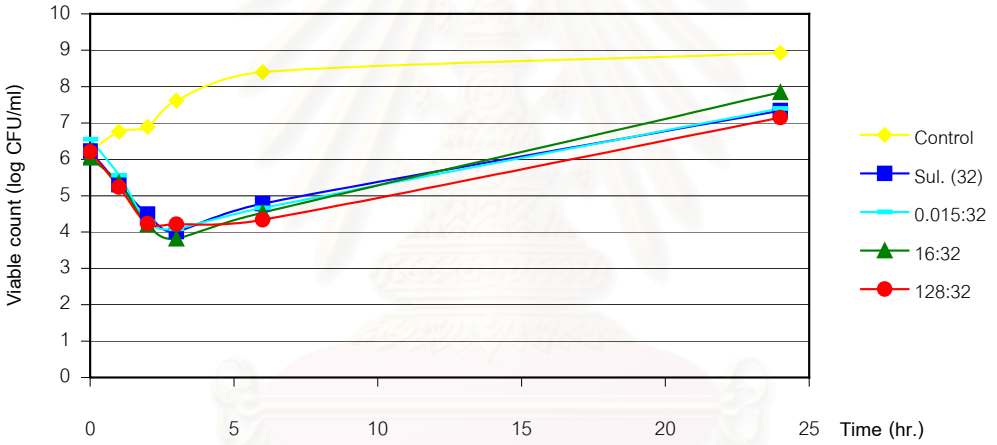


Figure 4-39 The killing curves of cefoperazone-sulbactam against *Acinetobacter baumannii* at concentration of 0.015:32, 16:32, and 128:32

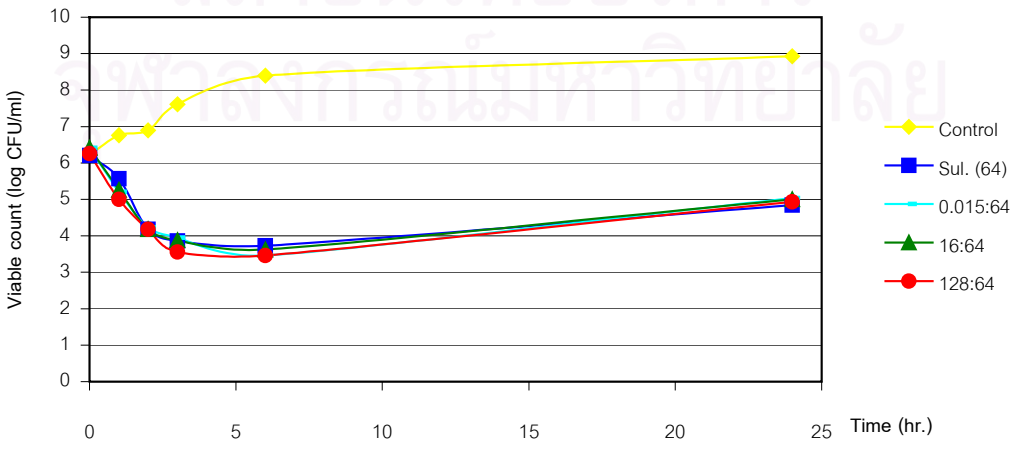


Figure 4-40 The killing curves of cefoperazone-sulbactam against *Acinetobacter baumannii* at concentration of 0.015:64, 16:64, and 128:64

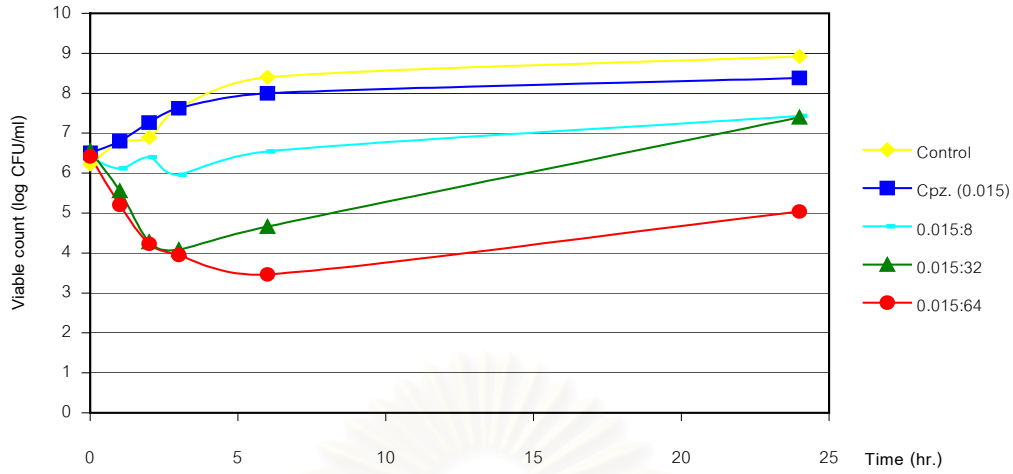


Figure 4-41 The killing curves of cefoperazone-sulbactam against *Acinetobacter baumannii* at concentration of 0.015:8, 0.015:32, and 0.015:64

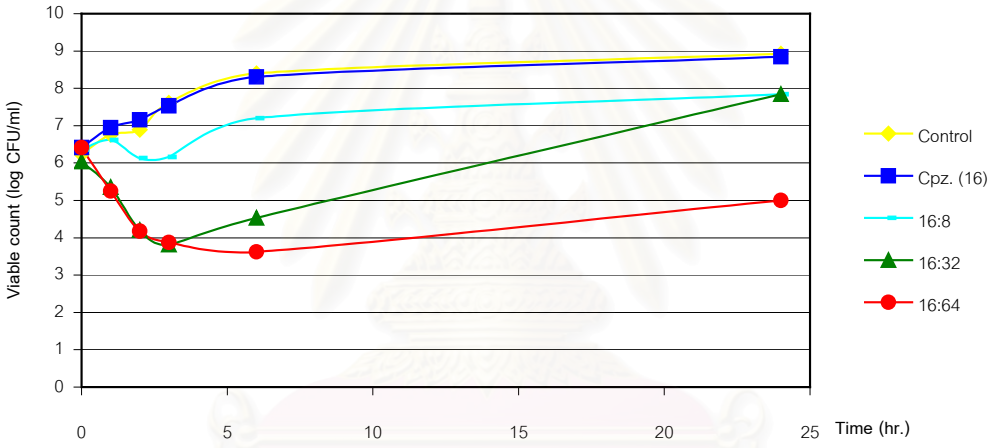


Figure 4-42 The killing curves of cefoperazone-sulbactam against *Acinetobacter baumannii* at concentration of 16:8, 16:32, and 16:64

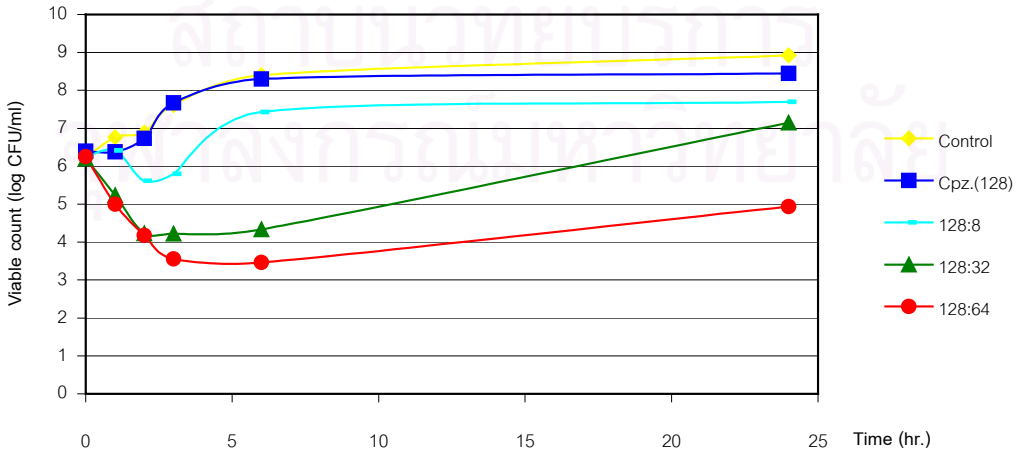


Figure 4-43 The killing curves of cefoperazone-sulbactam against *Acinetobacter baumannii* at concentration of 128:8, 128:32, and 128:64

Table 4-9 The killing kinetics and regrowth parameters of cefoperazone and sulbactam against *Acinetobacter baumannii.*

Parameter\Conc	Control	Cpz (Break point)			Sul			Cpz:Sul			Cpz:Sul			Cpz:Sul		
		(0.015)	(16)	(128)	Caverage (8)	Cmin (32)	Cmax (64)	0.015:8	0.015:32	0.015:64	16:8	16:32	16:64	128:8	128:32	128:64
T90%	-2.47	-	-	-	-	1.09	1.27	-	1.00	0.83	-	1.73	0.87	-	1.03	0.79
T99%	-5.35	-	-	-	-	2.54	1.98	-	1.78	1.81	-	2.42	1.79	-	2.00	1.90
T99.9%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T*E*	-	-	-	1.06	5.79	16.20	>24	5.17	18.48	>24	3.58	14.19	>24	3.85	17.91	>24
Tmin	0.00	0.00	0.00	1.00	2.00	3.00	6.00	3.00	3.00	6.00	2.00	3.00	6.00	2.00	3.00	6.00
LogNmin	6.23	6.80	6.41	6.38	6.16	4.01	3.72	5.96	4.08	3.46	6.13	3.82	3.62	5.61	4.22	3.46
6logNmin	0.00	-0.29	0.00	0.02	0.40	2.22	2.48	0.42	2.48	2.95	0.23	2.22	2.79	0.65	1.98	2.80
T	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
LogN*T*	8.46	8.04	8.36	8.32	6.70	5.07	3.85	6.64	4.97	3.64	7.27	4.90	3.78	7.46	4.65	3.63
LogN24	8.92	8.38	8.85	8.45	7.70	7.34	4.85	7.43	7.40	5.04	7.85	7.85	5.00	7.70	7.15	4.93
6Log N (single drug -combination)	-	-	-	-	-	-	-	0.27	-0.06	-0.19	-0.15	-0.51	-0.15	0.00	0.19	-0.08
6logN3	-1.38	-1.12	-1.12	-1.27	0.27	2.22	2.34	0.42	2.47	2.47	0.20	2.22	2.54	0.46	1.98	2.70
6logN24	-2.69	-1.88	-2.43	-2.05	-1.14	-1.11	1.36	-1.05	-0.84	1.38	-1.48	-1.80	1.41	-1.44	-0.95	1.32
Killing rate3	-0.46	-0.37	-0.37	-0.42	0.09	0.74	0.78	0.14	0.82	0.82	0.07	0.74	0.85	0.15	0.66	0.90
AUC24	200.49	191.98	199.14	194.84	166.80	137.25	103.28	163.22	136.82	102.23	174.44	138.43	103.43	174.04	130.88	100.18
Bacteriolytic area24		8.51	1.35	5.65	33.69	63.25	97.21	37.28	63.67	98.26	26.05	62.07	97.06	26.45	69.61	100.31

The time period of persistent suppression in bacterial growth (PLIE) was evaluated two types containing PLIE method1 and PLIE method2 as tested by time kill method. PLIE method1 was following briefly pre-exposure to β-lactam-β-lactamase inhibitor combination and post-exposure to β-lactam alone while PLIE method2 was pre-exposure with β-lactamase inhibitor alone and post-exposure to β-lactam alone. The phenomenon of PLIE was observed in *H. influenzae,* after pre-exposure to clavulanic acid being combined with amoxicillin as shown in figure 4-44 and after pre-exposure to clavulanic acid alone as shown in figure 4-45. The PLIE of clavulanic acid to *H. influenzae* calculated in table 4-10 demonstrated the long duration of suppression more than 18 hours when the organism was incubated with clavulanic acid at 2 and 4 μg/ml in both PLIE method1 and PLIE method2. In contrast the PAE of combination between amoxicillin and clavulanic acid was not observed in *H. influenzae*. The PLIE study on tazobactam against *P. aeruginosa* was shown that tazobactam could suppress the bacterial growth after pre-exposure to tazobactam in combination with piperacillin (Figure 4-46) and after pre-exposure to tazobactam alone (Figure 4-47). The PLIE of tazobactam to *P. aeruginosa* displayed in table 4-10 demonstrated that PLIE duration of tazobactam was longer when concentration of tazobactam was amplified. Moreover, the calculation of PLIE from method1 was more prolong than that of PLIE from method 2 (PLIE of tazobactam at 0.025, 4 and 32 μg/ml from method 1 were 0.78, 3.33 and 7.75 hours, respectively while method 2 were 0.14, 1.68 and 6.25 hours, respectively). While the PAE of combination between piperacillin and tazobactam was not observed in *P. aeruginosa*. Conversely, sulbactam did not show persistence effect to *A. baumannii* both in PLIE method 1 and method 2 (Figure 4-48 and 4-49). The PLIE of sulbactam to *A.baumannii* tabulated in table 4-10 demonstrated that PLIE method 1 displayed short duration while PLIE method 2 was negative period. Similarly the PAE of combination between cefoperazone and sulbactam was observed in negative measure against *P. aeruginosa*.

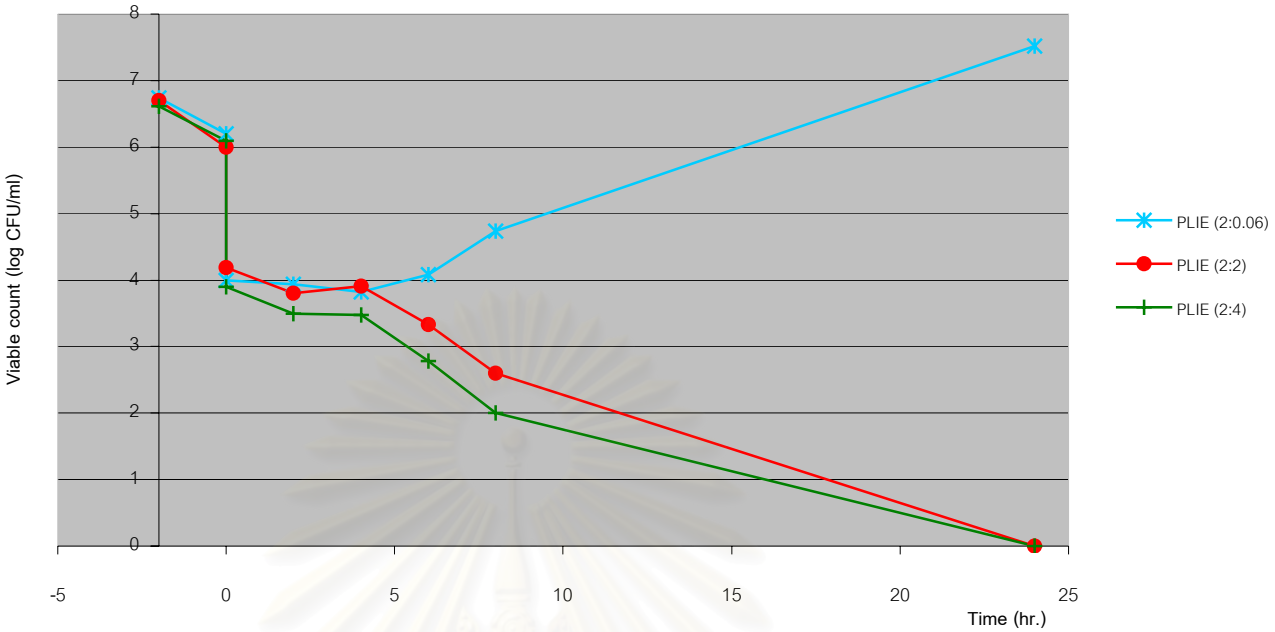


Figure 4-44 The killing curves of PLIE (method1) between the combination of amoxicillin and clavulanic acid against *Haemophilus influenzae*

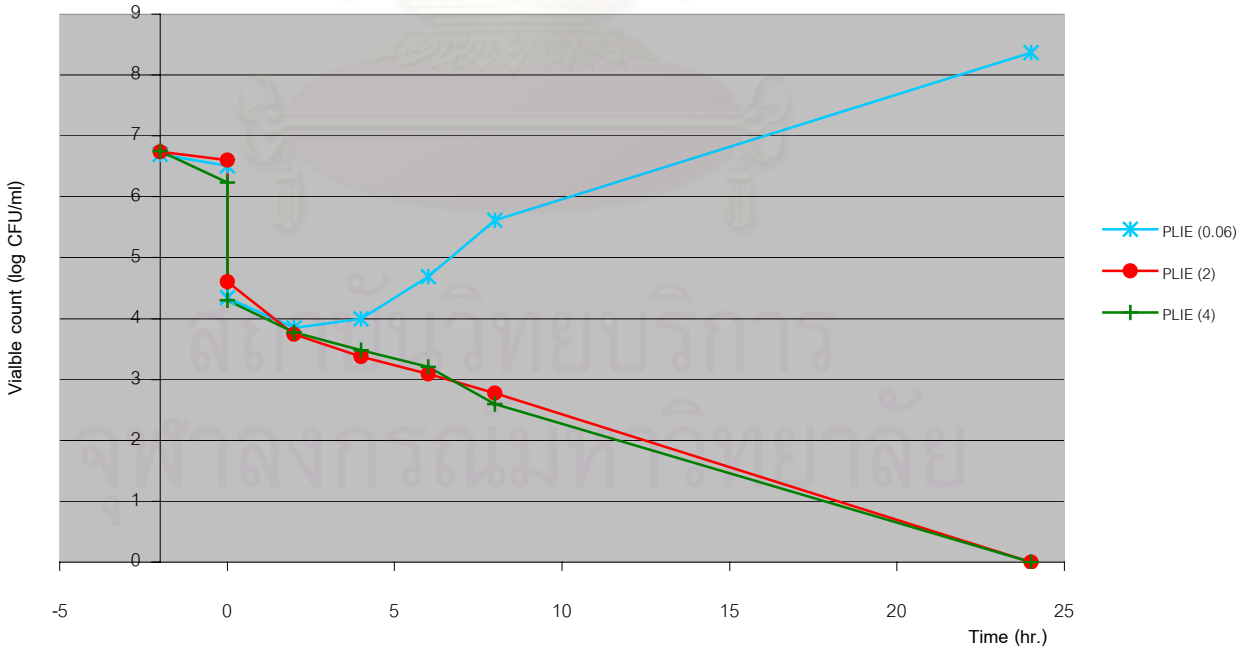


Figure 4-45 The killing curves of PLIE (method2) between the combination of amoxicillin and clavulanic acid against *Haemophilus influenzae*

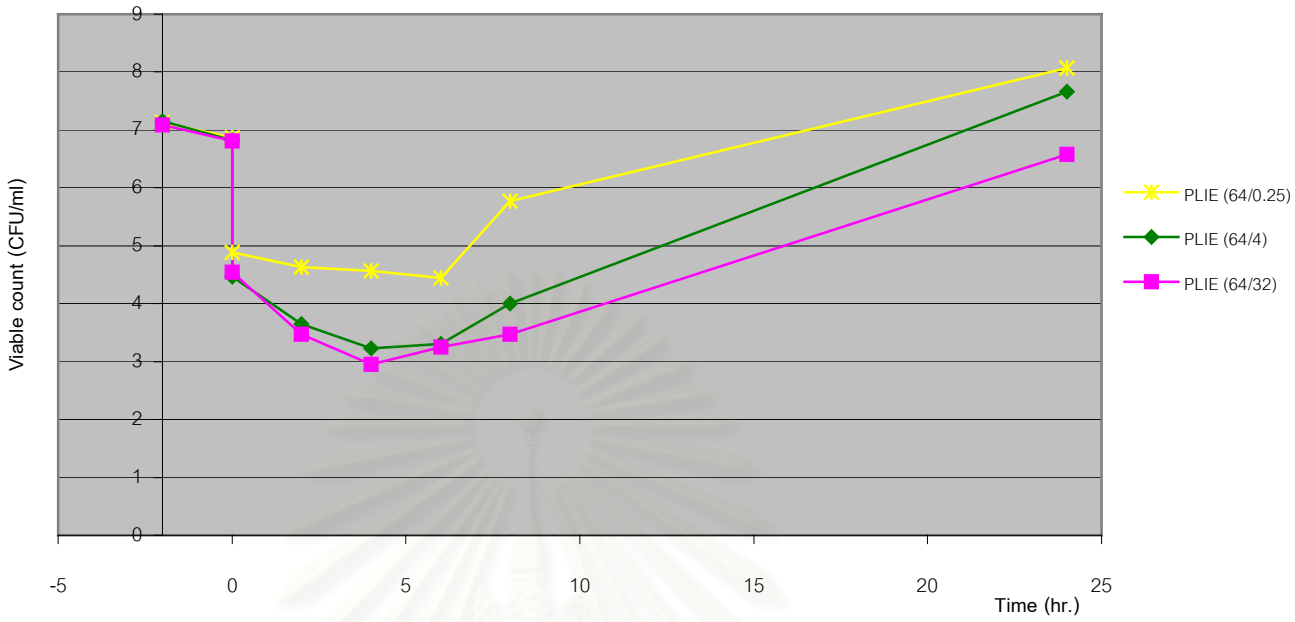


Figure 4-46 The killing curves of PLIE (method1) between the combination of piperacillin and tazobactam against *Pseudomonas aeruginosa*

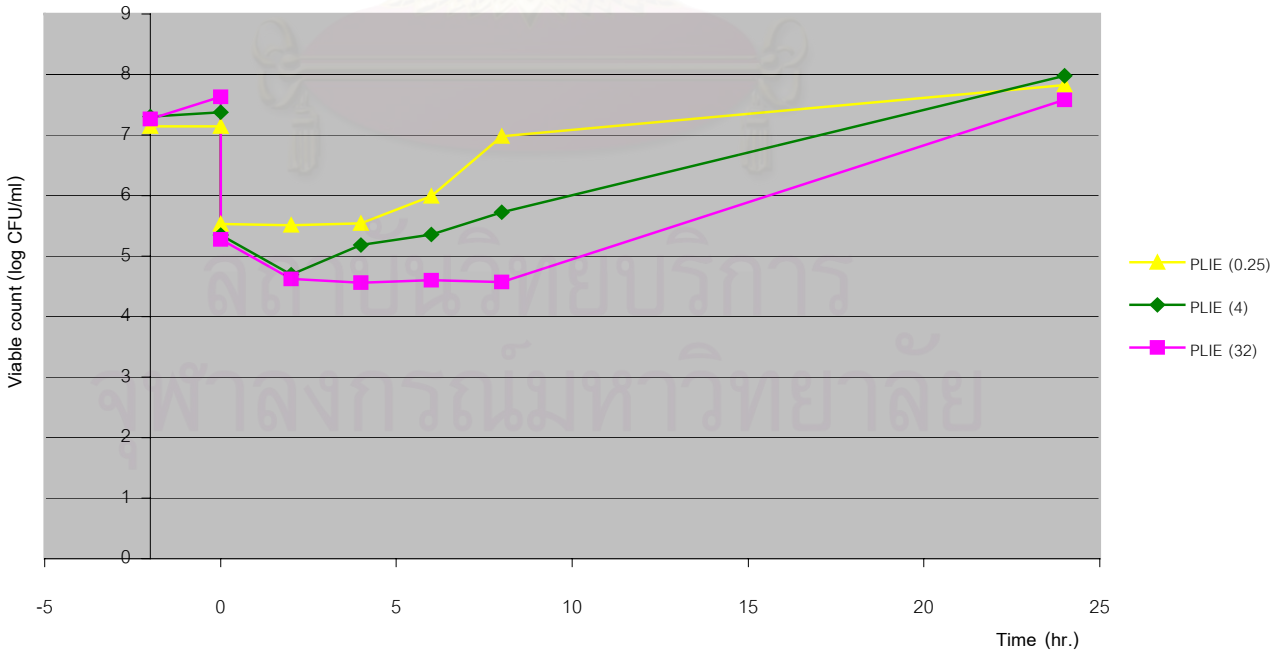


Figure 4-47 The killing curves of PLIE (method2) between the combination of piperacillin and tazobactam against *Pseudomonas aeruginosa*

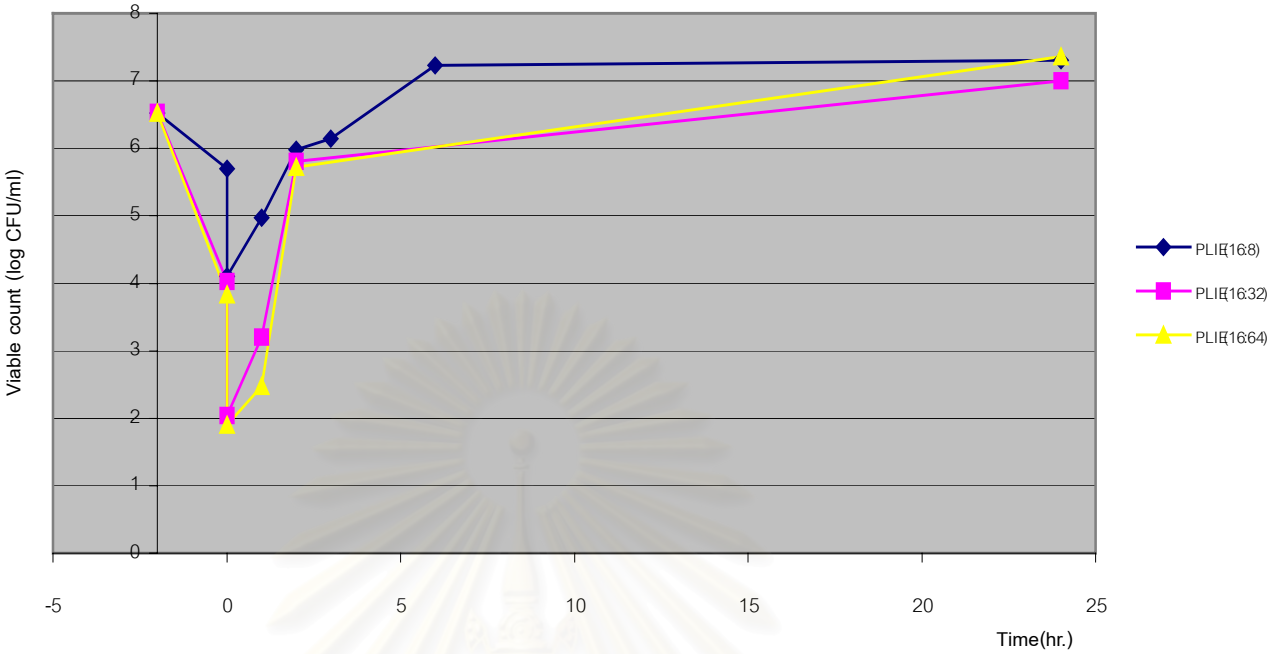


Figure 4-48 The killing curves of PLIE (method1) between the combination of cefoperazone and sulbactam against *Acinetobacter baumannii*

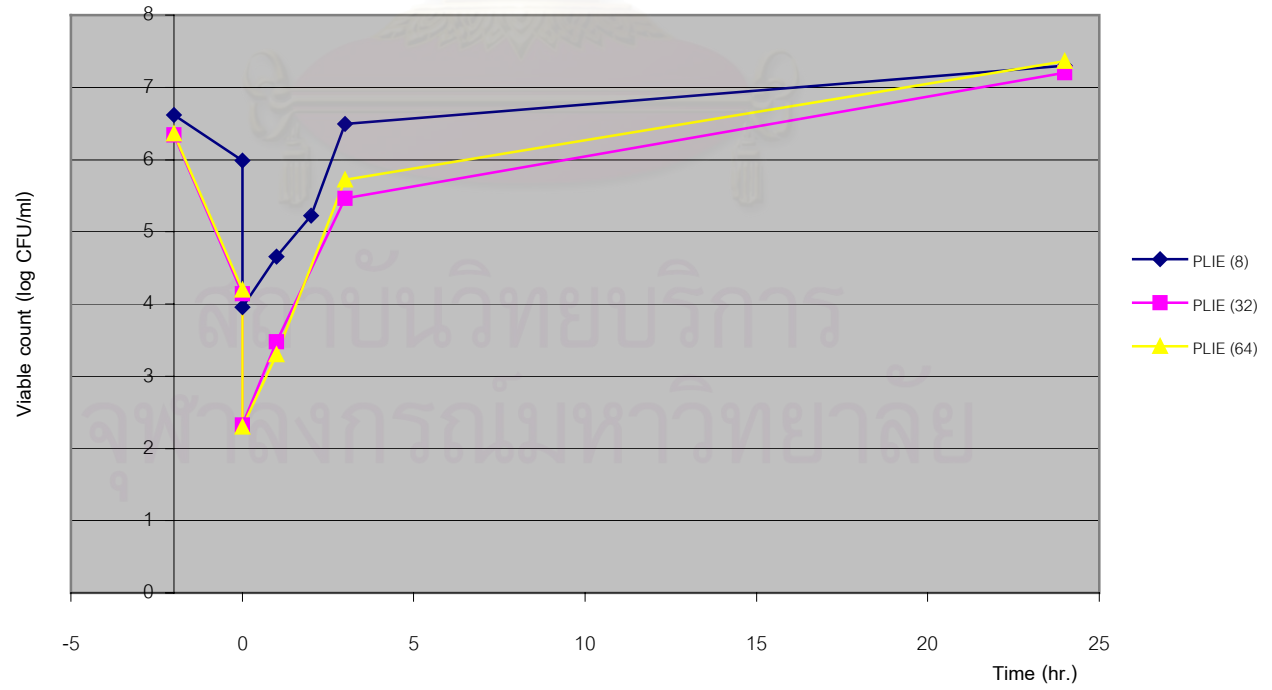


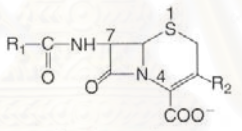
Figure 4-49 The killing curves of PLIE (method2) between the combination of cefoperazone and sulbactam against *Acinetobacter baumannii*

Table 4-10 Result of post-β-lactamase inhibitor effect (PLIE) in a range of ratio between β-lactam and β-lactamase inhibitor against tested microorganism.

Microorganism	Antimicrobial agent	Ratio (BL:BI)	Ratio (BL:BI) (μg/ml)	PAE (hr.)	PLIE (method1) (hr.)	PLIE (method2) (hr.)
H. influenzae	Amoxicillin:Clavulanic acid	2MIC : Cmin	2:0.06	0.07	3.50	1.43
		2MIC : Caverage	2:2	-0.58	>18	>18
		2MIC : Cmax	2:4	-1.04	>18	>18
P. aeruginosa	Piperacillin:Tazobactam	2MIC : Cmin	64:0.25	0.72	0.78	0.14
		2MIC : Caverage	64:4	0.14	3.33	1.68
		2MIC : Cmax	64:32	0.00	7.75	6.25
A.baumannii	Cefoperazone:Sulbactam	2MIC : Cmin	16:8	-2.41	0.58	-1.43
		2MIC : Caverage	16:32	-2.42	0.46	-2.14
		2MIC : Cmax	16:64	-2.57	0.71	-2.00

Negative PAE and negative PLIE mean no phenomenon of PAE and PLIE

β-lactamase induction effect by β-lactamase inhibitor was screened from double disks method. It was found that clavulanic acid could induce *E. cloacae* strain to increasingly produce β-lactamase that destroyed antibacterial activity of cefuroxime only. While other test β-lactams shown in figure 4-50 was not hydrolyzed by β-lactamase produced from *E.cloacae*. Furthermore, the narrow clear zone of cefuroxime was demonstrated when high concentration of clavulanic acid disk (10, 20 and 50 μg/disk) was closely located to cefuroxime disk as shown in figure 4-51. After that the various concentrations of clavulanic acid was taken to expose with *E. cloacae* for 24 hours by broth method before the MIC determination of cefuroxime by agar dilution namely method A. The MICs of cefuroxime measured to *E. cloacae* after exposure to clavulanic acid were 8 μg/ml as same as the untreated control culture (Table 4-52). On the other hand, the MICs of cefuroxime to *E. cloacae* being exposed clavulanic acid determined by E-test as described in method B (Figure 4-2) performed significant dissimilarity between the MIC of untreated control culture (6 μg/ml) and that of test culture exposed to 10 μg/ml of clavulanic acid (32 μg/ml) as shown in table 4-52.



Cephem nucleus

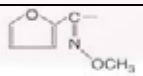
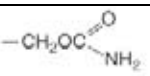
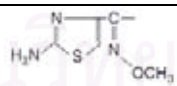
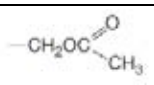
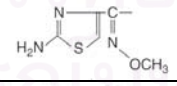
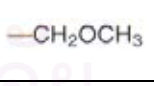
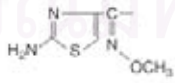
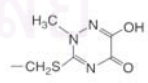
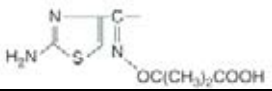
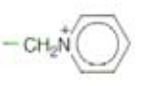
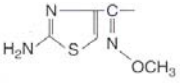
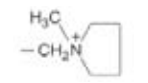
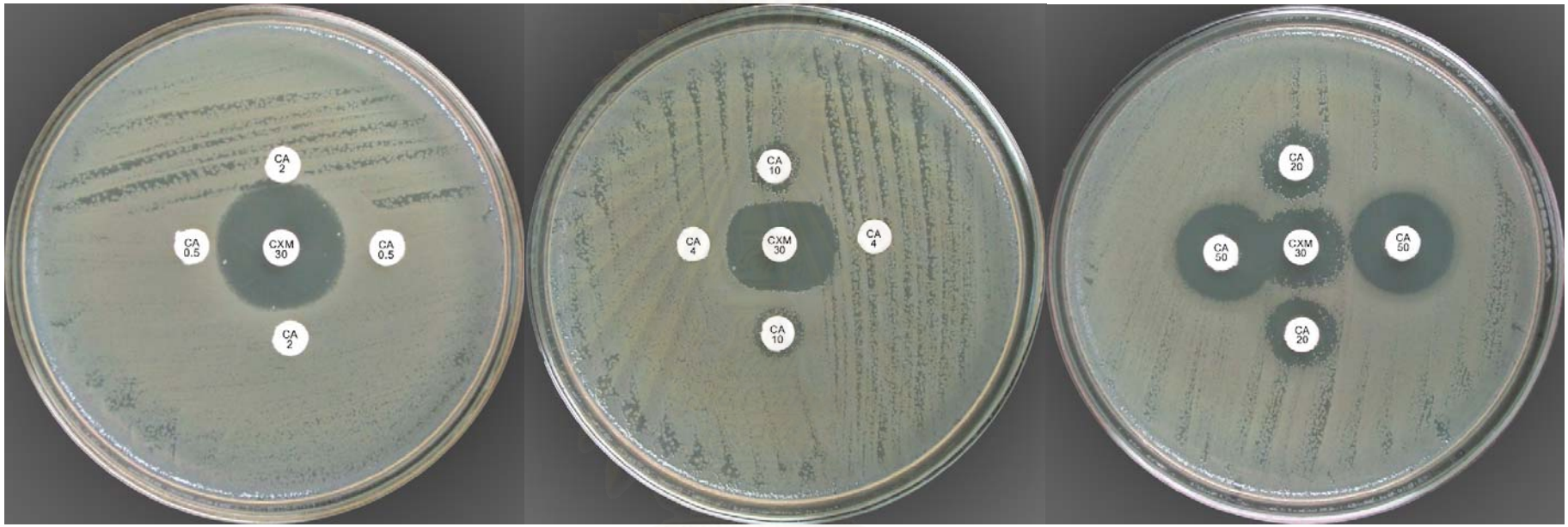
COMPOUND (TRANDNAME)	R1	R2
Second generation		
CEFUROXIME		
Third generation		
CEFOTAXIME		
CEFPODOXIME		
CEFTRIAXONE		
CEFTAZIDIME		
Forth generation		
CEFEPIME		

Figure 4-50 Structure of cephalosporin groups



A **B** **C**

Figure 4-51 Growth of *Enterobacter cloacae* strain with positive production of inductive β-lactamase as exposure to clavulanic acid (A) 0.5 and 2 μg/disk, (B) 4 and 10 μg/disk, (C) 20 and 50 μg/disk by double disks method. Positive outcome = deformation of inhibitory zones around the cefuroxime disk.

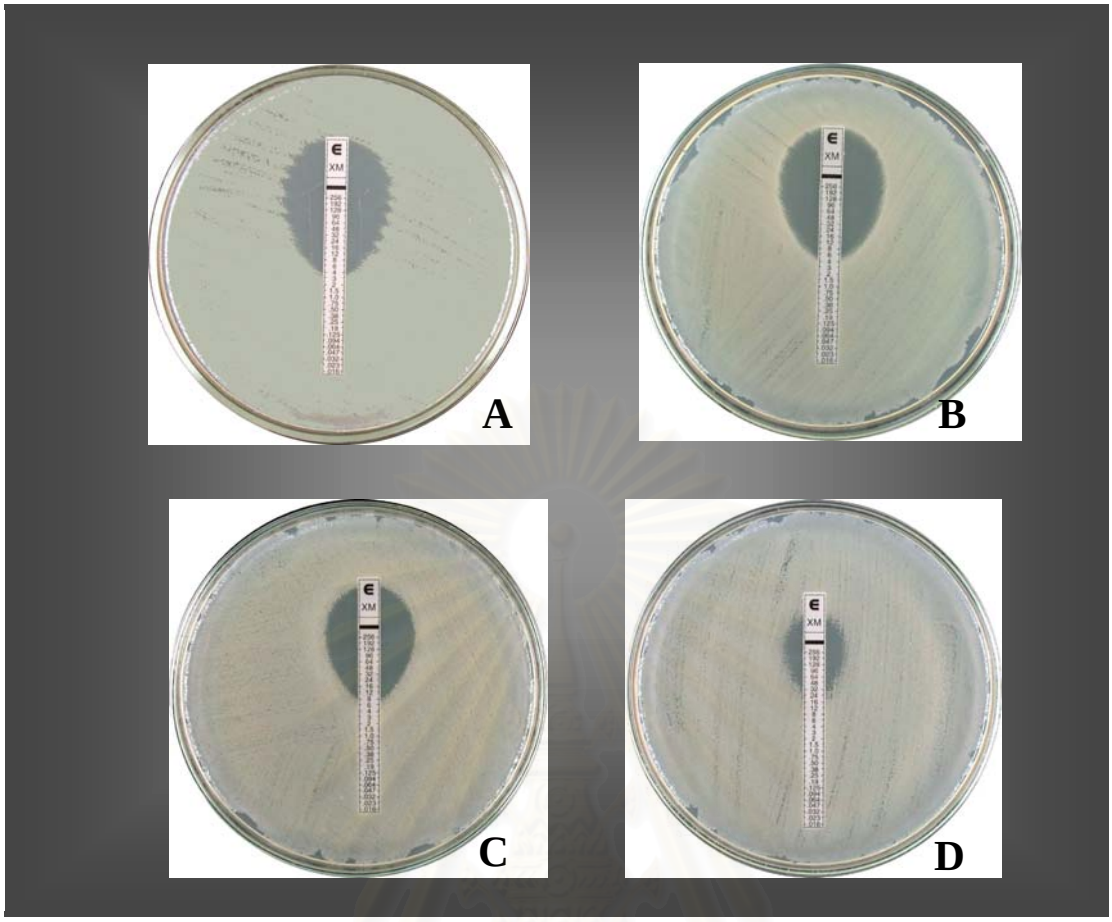


Figure 4-52 Difference susceptibility patterns of cefuroxime against *Enterobacter cloacae* strain after exposure to various concentrations of clavulanic acid as tested by method B. The clavulanic acid concentrations were: (A) none, (B) 2 μg/ml, (C) 4 μg/ml, and (D) 10 μg /ml.

Table 4-11 Summary of the observed inducibility determined from MIC as tested by method A and B, and interpolate the susceptibility patterns of cefuroxime against *Enterobacter cloacae* strain by referring to the break point in the NCCLS,2000.

Type	method	control	CLA 0.5 μg/ml	CLA 2 μg/ml	CLA 4 μg/ml	CLA 10 μg/ml	CLA 20 μg/ml	CLA 50 μg/ml
MIC of CXM (μg/ml)	A	8	8	8	8	8	8	8
Susceptibility pattern		S	S	S	S	S	S	S
MIC of CXM (μg/ml)	B	6	6	6	8	32	ND	ND
Susceptibility pattern		S	S	S	S	R	-	-

S = susceptibility, R= resistance, ND= no detectable

CHAPTER V

DISCUSSION & CONCLUSION

DISCUSSION

- **Amoxicillin-clavulanic acid**

Investigation of the synergism interaction between amoxicillin and clavulanic acid against β-lactamase producing *Moraxella catarrhalis* and *Haemophilus influenza*e was performed by checkerboard method. Clavulanic acid at the concentration 2 μg/ml could reduce the MIC of amoxicillin by 64 times. The synergistic activity between amoxicillin and clavulanic acid was also illustrated by the shape of graph plotted on isobologram and the FIC index (average). The isobologram pattern showed concave curve and FIC index (average) calculated was lower than 0.5. That meant antibacterial activity of the combination between amoxicillin and clavulanic acid was greater than the sum of the results for each of the drugs used separately. Furthermore, if consideration to concentration of clavulanic acid that provided the FIC index to be lower than 0.5 as in the definition of synergism effect (Chambers and Sande, 1996), it was found that the range of concentrations of clavulanic acid taken in that criterion was 0.08 – 4 μg/ml in both *M. catarrhalis* and *H. influenza*e. Those concentrations were containing 2 and 4 μg/ml that were the average and peak concentration of clavulanic acid in serum after oral administration clavulanic acid 125 mg (AHFS, 2001 and Cilberet, Moellering, Sande, 2001). Therefore, the checkerboard results suggested that concentration of clavulanic acid currently combined in amoxicillin was the appropriate concentration for treatment of infectious disease caused by β-lactamase producing *M. catarrhalis* and *H. influenza*e.

Regarding to the antibacterial activity of amoxicillin and clavulanic acid combination at various concentrations as tested by time-kill method. The results demonstrated that the bactericidal effect of amoxicillin alone at the supra MIC level was promoted in concentration independent characteristic to β-lactamase producing *M. catarrhalis* and *H. influenza*e when it was combined with clavulanic acid at above 2μg/ml. However, if the antibacterial activity of amoxicillin at 2 MIC was compared with the combined between clavulanic acid 2 μg/ml and 4

μg/ml, it was shown that antibacterial activity of amoxicillin in concomitant to clavulanic acid 4 μg/ml did not display higher than that of amoxicillin being combined with clavulanic acid 2 μg/ml. Thus, this outcome suggested that the amount of β-lactamase produced from *M. catarrhalis* and *H. influenza*e was probably so minimal because they could be destroyed by only 2 μg/ml of clavulanic acid.

Pharmacokinetic property of clavulanic acid in the previous research has demonstrated that concentration of clavulanic acid was fallen below the minimum β-lactamase inhibitory concentration in-vitro within the 1-3 hour following administration. However, PLIE determination in this study illustrated that clavulanic acid at 2 and 4 μg/ml could perform long duration (> 18 hours). Implying that the surviving bacteria should require a latency period approximately 18 hours to synthesize a sufficient level of β-lactamase and within this period the presence of amoxicillin could fully exert antibiotic activity and inhibit bacterial growth until the β-lactamase concentration was again sufficient to hydrolyze amoxicillin and allowed bacterial regrowth. That was way explained bacterial activity of amoxicillin in the absence of clavulanic acid in serum.

Regarding β-lactamase induction effect, merely clavulanic acid exhibited positive result against *E. cloacae* by induction of the β-lactamase production that could destroy cefuroxime (2^{nd} generation cephalosporin) as screened by double disks method. Among of the cephalosporins tested included ceftazidime, ceftriaxone, cefpodoxime, cefotaxime as 3^{rd} generation and cefipime, cefpirome as 4^{th} generation, only cefuroxime exhibited this phenomenon. This might be the effect from the structure of cefuroxime particularly at the 7^{th} position, which is the position for β-lactamase attack and β-lactam ring stability. The substitute of cefuroxime at 7^{th} position is furanyl ring and methoxyiminoacyl group while the 7^{th} position of the other compounds are thiazolyl ring and methoxyiminoacyl group (Figure 4-50). Thus, the furanyl ring of cefuroxime might provide less protective property from binding to β-lactamase produced by *E. cloacae* than the others. Nevertheless, when the study was conducted with concentrations mimicking serum levels of clavulanic acid (2, 4, 10 and 20 μg/ml) after administration of 125 mg. (oral) and 100, 200 mg. (intravenous), it was demonstrated that the β-lactamase induction to destroy cefuroxime was not observed in *E. cloacae* exposed to cefuroxime and clavulanic acid in the different time (method A). However,

when cefuroxime and clavulanic acid were administrated together at the same time (method B), MIC of cefuroxime against *E. cloacae* was higher than the breakpoint value and the level of resistance was related to the concentration of clavulanic acid used during the exposure step. This might be due to the fact that clavulanic acid could transiently induce β-lactamase production by *E. cloacae* because this occurrence disappeared immediately as no existed clavulanic acid.

- **Piperacillin-tazobactam**

The study of synergism interaction between piperacillin and tazobactam against β-lactamase producing *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was performed by checkerboard method. It was shown that 4 μg/ml of tazobactam could lower the MIC of piperacillin against *K. pneumoniae* to 64 folds and the MIC of piperacillin against *P. aeruginosa* was decreased by 4 times. The synergistic activity between piperacillin and tazobactam was also illustrated by the shape of graph plotted on isobologram and the FIC index (average). The similar outcome from graph plotted on isobologram and FIC index (average) showing the synergism between piperacillin and tazobactam against *K. pneumoniae* and *P. aeruginosa*. That meant antibacterial activity of the combination between piperacillin and tazobactam was greater than that of the sum of the results for each of the drugs used separately. Furthermore, if consideration to concentration of tazobactam that provided the FIC index lower than 0.5 as definition of synergism effect (Chambers and Sande, 1996), it was found that the range of concentrations of tazobactam taken in that criterion contained 0,125 – 16 μg/ml in *K. pneumoniae* and 4 – 16 μg/ml for *P. aeruginosa*. Those concentrations were containing 4 μg/ml that were the average concentration of tazobactam in serum after the intravenous administration of 500 mg. tazobactam (AHFS, 2001; Cilberet, Moellering, Sande, 2001; Perry and Markham, 1999). Therefore, the checkerboard results suggested that concentration of tazobactam currently combined with piperacillin was the appropriate concentration for the treatment of infectious disease caused by β-lactamase producing *K. pneumoniae* and *P. aeruginosa*.

Antibacterial activity of piperacillin and tazobactam combination at various concentrations as tested by time-kill method. The results demonstrated that piperacillin at supra-MIC could reduce the colony forming unit of *K. pneumoniae* and *P. aeruginosa* by 100

times within 6 hours when piperacillin was combined with 4 and 32 μg/ml of tazobactam. However, if comparison the antibacterial activity of piperacillin at 2 MIC as combined between tazobactam 4 μg/ml and 32 μg/ml. It was shown that antibacterial activity of piperacillin in concomitant to tazobactam 32 μg/ml did not display higher than that of amoxicillin being combined with tazobactam 4 μg/ml. Thus, this outcome suggested that the amount of β-lactamase produced from *K. pneumoniae* and *P. aeruginosa* was probably so minimal because they can be destroyed by only 4 μg/ml of tazobactam.

Pharmacokinetic property of tazobactam in the previous research has demonstrated that concentration of tazobactam was fallen below the minimum β-lactamase inhibitory concentration in-vitro within the 4-6 hour following administration. However, PLIE determination in this study illustrated that tazobactam at 32 μg/ml could perform bacterial suppressed duration (6.25 and 7.75 hours). Implying that the surviving bacteria should require a latency period approximately 7 hours to synthesize a sufficient level of β-lactamase and within this period the presence of piperacillin could fully exert antibiotic activity and inhibit bacterial growth until the β-lactamase concentration was again sufficient to hydrolyze piperacillin and allowed bacterial regrowth. That was way explained bacterial activity of piperacillin in the absence of tazobactam in serum. Furthermore, the PLIE calculated from pre-exposure to tazobactam alone (method 2) against *P. aeruginosa* was shorter than that observed after pre-exposure to piperacillin and tazobactam combination (method1). As well as the PLIE determination seemed proportional to the concentration of tazobactam used during the pre-exposure step. This might be due to the colony- forming unit of organism counted immediately after remove piperacillin in pre-exposure to piperacillin and tazobactam was smaller than system pre-exposure with tazobactam alone. Thus the less quantity of surviving bacteria in pre-exposure to piperacillin and tazobactam required a latency period to produce a sufficient level of β-lactamase longer than a full amount of living bacteria in pre-exposure to tazobactam without piperacillin. No β-lactamase induction effect was observed in tazobactam against *E.cloacae* as tested by double disks method.

- Cefoperazone-sulbactam

The study of synergism interaction between cefoperazone and sulbactam against β-lactamase producing *Pseudomonas aeruginosa* was performed by checkerboard method. Sulbactam at 8 μg/ml could lower the MIC of cefoperazone against *P. aeruginosa* by 8 folds. On the other hand, the MIC of piperacillin against *Acinetobacter baumannii* decreased from upper 128 μg/ml to 0.015 μg/ml when combined with 32 μg/ml of sulbactam. The synergistic activity between cefoperazone and sulbactam was also illustrated by the shape of graph plotted on isobologram and the FIC index (average). The similar outcome from graph plotted on isobologram and FIC index (average) showing the synergism between cefoperazone and sulbactam against *P. aeruginosa* but displayed additive pattern to *A. baumannii*. That meant antibacterial activity of combination between cefoperazone and sulbactam was greater than that of the sum of the results for each of the drugs used separately when tested *P. aeruginosa*. While antibacterial property in *A. baumannii* of cefoperazone-sulbactam was not different from the sum of the results for each of the drugs used separately. Furthermore, if consideration to concentration of sulbactam that provided the FIC index lower than 0.5 as definition of synergism effect (Chambers and Sande, 1996). It found that the range of concentrations of sulbactam taken in that criterion contained 2 – 32 μg/ml in *P. aeruginosa* and 32 μg/ml for *A. baumannii*. The concentrations of tazobactam in *P. aeruginosa* were containing 8 μg/ml that were the average concentration of sulbactam in serum after the intravenous administration sulbactam 500 mg. (AHFS, 2001 and Cilberet, Moellering, Sande, 2001). Therefore, the checkerboard results suggested that concentration of sulbactam currently combined in cefoperazone was the appropriate concentration for treatment of infectious disease caused by β-lactamase producing *P. aeruginosa*. Conversely, the concentration of sulbactam is not enough for treatment of infectious disease caused by β-lactamase producing *A. baumannii*.

Antibacterial activity of cefoperazone and sulbactam combination at various concentrations as tested by time-kill method. The results demonstrated that cefoperazone at supra-MIC could reduce the colony forming unit of *P. aeruginosa* by 100 times within 6 hours when cefoperazone was combined with 4, 8 and 64 μg/ml of sulbactam. However, if comparison the antibacterial activity of cefoperazone at 2 MIC as combined among three concentrations of sulbactam (4, 8 and 64 μg/ml). It was shown that antibacterial activity of

cefoperazone in concomitant to sulbactam 64 μg/ml did not display higher than that of amoxicillin being combined with sulbactam 4 and 8 μg/ml. Thus, this outcome suggested that the amount of β-lactamase produced from *K. pneumoniae* was probably so minimal because they can be destroyed by only 4 μg/ml of sulbactam. Interestingly, when comparison the antibacterial activity in *A. baumannii* between sulbactam without cefoperazone and sulbactam in concomitant to cefoperazone was not difference. This result demonstrated that sulbactam could not inhibit β-lactamase produced from *A. baumannii* but take action as antibiotic to *A. baumannii*. The consequence corresponds to the previous study described by Joly-Guillou, et al (1995) that sulbactam can attach to PBP2 of *A. baumannii*. Therefore, this outcome suggested that we could use sulbactam alone in high concentration for treatment of infectious disease caused by β-lactamase producing *A. baumannii*.

Pharmacokinetic property of sulbactam in the previous research has demonstrated that concentration of sulbactam was fallen below the minimum β-lactamase inhibitory concentration in-vitro within the 4-6 hour following administration. However, PLIE determination in this study illustrated that sulbactam could not perform bacterial suppressed duration as tested to *A. baumannii*. Implying that the surviving bacteria could rapidly grow up after sulbactam was removed and cefoperazone could not suppress their regrowth. This might be explained due to sulbactam could not inhibit β-lactamase produced from *A. baumannii* meantime the level of β-lactamase was still in high concentration and could destroy cefoperazone at all. No β-lactamase induction effect was observed in sulbactam against *E.cloacae* as tested by double disks method.

CONCLUSION

In vitro pharmacodynamic study of effects of different combination of β-lactam-β-lactamase inhibitor in present study suggests that the dosages of clinically available β-lactam-β-lactamase inhibitor combinations are appropriate for the treatment of infectious diseases caused by β-lactamase producing microorganism. Furthermore, sulbactam alone in high concentration can be taken into treatment of infectious disease caused by *Acinetobacter baumannii*. Regarding the time interval of amoxicillin-clavulanic acid, the alternative administration of amoxicillin-clavulanic acid is taking amoxicillin-clavulanic acid combination two times a day in the morning and evening while in lunch can take only amoxicillin without clavulanic acid. Additionally, we can use any antibiotics immediately after discontinue amoxicillin-clavulanic acid.

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