การศึกษาเปรียบเทียบฤทธิ์ฆ่าเชื้อและผลต่อการเปลี่ยนแปลงรูปร่างของเชื้อแบคทีเรียกรัมลบ ในหลอดทดลองของยา Cefpirome, Cefepime, Imipenem และ Meropenem เมื่อใช้เดี่ยวและใช้ร่วมกัน

นาย พิสิฐ เขมาวุฒฒ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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COMPARATIVE IN-VITRO STUDY OF KILLING ACTIVITIES AND MORPHOLOGICAL CHANGES OF CEFPIROME, CEFEPIME, IMIPENEM AND MEROPENEM ALONE AND IN COMBINATION AGAINST GRAM NEGATIVE BACTERIA

Mr. Phisit Khemawoot

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy
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Thesis Title	Comparative In-vitro Study of Killing Activities and Morphological Changes of Cefpirome, Cefepime, Imipenem and Meropenem Alone and in Combination against Gram Negative Bacteria.
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พิสิฐ เขมาวุฒฒ์ : การศึกษาเปรียบเทียบฤทธิ์ฆ่าเชื้อและผลต่อการเปลี่ยนแปลงรูปร่างเชื้อแบคทีเรียกรัมลบในหลอดทดลองของยา Cefpirome, Cefepime, Imipenem และ Meropenem เมื่อใช้เดี่ยวและใช้ร่วมกัน (COMPARATIVE IN-VITRO STUDY OF KILLING ACTIVITIES AND MORPHOLOGICAL CHANGES OF CEFPIROME, CEFEPIME, IMIPENEM AND MEROPENEM ALONE AND IN COMBINATION AGAINST GRAM NEGATIVE BACTERIA) อาจารย์ที่ปรึกษา : รศ.ศิริภรณ์ ฟุ้งวิทยา, อาจารย์ที่ปรึกษาร่วม : ศ.พญ.นลินี อัศวโภคี, 78 หน้า. ISBN 974-03-1513-5.

ยาปฏิชีวนะกลุ่มเบต้าแลคแตม จัดเป็นยาที่สามารถฆ่าเชื้อแบคทีเรียโดยออกฤทธิ์ยับยั้งเอนไซม์ที่ใช้ในการสังเคราะห์ผนังเซลล์ (Penicillin binding proteins, PBPs) ทำให้เกิดความผิดปกติของรูปร่างเซลล์แบคทีเรียและส่งผลให้เซลล์แตกตายในที่สุด การวิจัยครั้งนี้ต้องการหาความสัมพันธ์ของการยับยั้ง PBPs ที่ส่งผลถึงการเปลี่ยนแปลงรูปร่างเซลล์และฤทธิ์ฆ่าเชื้อจากยาเบต้าแลคแตม 4 ชนิด คือ cefpirome, cefepime, imipenem และ meropenem ในการกำจัดเชื้อ *P. aeruginosa*, *E. cloacae* และ *E. coli* ที่ไวต่อยาทดสอบทั้ง 4 ชนิดด้วยวิธี time kill จากการทดลองพบว่า cefpirome และ cefepime สามารถออกฤทธิ์ฆ่าเชื้อ *E. coli* ได้ในความเข้มข้นตั้งแต่ 4MIC ทว่ายาทั้ง 2 ชนิดกลับต้องใช้ความเข้มข้นมากกว่า 32MIC ในการออกฤทธิ์ฆ่าเชื้อ *E. cloacae* และยังสามารถตรวจพบ regrowth ของ *E. cloacae* ได้ภายในระยะเวลา 24 ชั่วโมงแม้ในความเข้มข้นที่ยาสามารถออกฤทธิ์ฆ่าเชื้อแล้วก็ตาม เมื่อตรวจสอบการเปลี่ยนแปลงรูปร่างเซลล์ของแบคทีเรียทั้ง 2 ชนิดจากยา cefpirome และ cefepime พบว่ามีความคล้ายคลึงกัน คือ ยาทั้ง 2 ชนิดทำให้เกิดการเปลี่ยนแปลงจากเซลล์แบคทีเรียรูปแท่งไปเป็น filamentous cells ในทุกความเข้มข้นและในกรณีของ cefepime ที่ความเข้มข้นมากกว่า 32MIC สามารถตรวจพบการเปลี่ยนรูปเป็น bulge cells เพิ่มเติม ซึ่งส่งผลถึงฤทธิ์ฆ่าเชื้อที่เพิ่มขึ้นจากค่า bacteriolytic area ที่เพิ่มขึ้นด้วย สำหรับยาในกลุ่ม carbapenems พบว่าทั้ง imipenem และ meropenem แสดงฤทธิ์ฆ่าเชื้อ *E. coli* ที่ความเข้มข้น 1MIC-2MIC และต้องเพิ่มความเข้มข้นเป็น 4MIC ในกรณีของ *P. aeruginosa* เมื่อตรวจสอบการเปลี่ยนแปลงรูปร่างเซลล์จากยาทั้ง 2 ชนิดพบว่า *E. coli* เปลี่ยนแปลงจากเซลล์รูปแท่งไปเป็น ovoidal cells นอกจากนี้ยังสามารถตรวจพบลักษณะ filamentous cells เพิ่มเติมในกรณีของ meropenem ที่ความเข้มข้นมากกว่า 32MIC สำหรับการทดสอบผลเสริมฤทธิ์กันในการกำจัดเชื้อ *E. coli* จากการใช้ยาเบต้าแลคแตมที่สามารถจับกับ PBPs ได้ต่างชนิดกันโดยใช้ cefpirome จับกับ PBP3 และ imipenem จับกับ PBP2 พบว่าเกิดการเสริมฤทธิ์กันจากการใช้ยาร่วมที่ 1/4MIC ทว่ายังสามารถตรวจพบ regrowth ของ *E. coli* ได้แม้จะเกิดการเสริมฤทธิ์กันแล้วก็ตาม ดังนั้นการที่ยาสามารถจับกับ PBPs ได้หลายชนิดนั้นทำให้ยาสามารถออกฤทธิ์เปลี่ยนแปลงรูปร่างเซลล์ของเชื้อแบคทีเรียได้เพิ่มขึ้นและยังส่งผลต่อฤทธิ์กำจัดแบคทีเรียให้เพิ่มขึ้นด้วย

ภาควิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2544 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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KEY WORDS : PBPs / BACTERICIDAL ACTIVITY / MORPHOLOGICAL CHANGE / GRAM NEGATIVE BACTERIA

PHISIT KHEMAWOOT : COMPARATIVE IN-VITRO STUDY OF KILLING ACTIVITIES AND MORPHOLOGICAL CHANGES OF CEFPIROME, CEFEPIME, IMIPENEM AND MEROPENEM ALONE AND IN COMBINATION AGAINST GRAM NEGATIVE BACTERIA. THESIS ADVISOR : ASSOC. PROF. SIRIPORN FUNGWITTHAYA, THESIS COADVISOR : PROF. NALINEE ASWAPOKEE, 78 pp. ISBN 974-03-1513-5.

The β-lactam antibiotics are generally regarded as the bactericidal agents. The mechanism of action is inhibiting of the enzymes in late stage of peptidoglycan synthesis namely Penicillin binding proteins (PBPs). The inhibitions of PBPs cause morphological changes lead to bacteriolysis and cell death. The relationship among PBPs, morphological changes and bactericidal activities by the β-lactam antibiotics has been evaluated in this research. Cefpirome, Cefepime, Imipenem and Meropenem were tested against susceptible strain of *P. aeruginosa*, *E. cloacae* and *E. coli* by time kill method. Cefpirome and Cefepime demonstrated bactericidal properties to *E. coli* from 4MIC, whereas *E. cloacae* showed regrowth to both drugs in concentration range from ¼MIC-128MIC after 24 hours of exposure. For morphological changes, both drugs established the filamentous cells on both Enterobacteria, which related to PBP3 binding as primary target of Cephalosporins. Interestingly, Cefepime above 32MIC manifested filamentous with bulbous cells that correlated to PBP3 and PBP2 binding with an increase of bactericidal property. Imipenem and Meropenem exerted bactericidal property to *E. coli* above concentration 1MIC-2MIC, whereas *P. aeruginosa* required concentration up to 4MIC for this property after 24 hours of exposure. For morphological changes, both drugs established ovoidal cells that related to PBP2 binding as primary target of Carbapenems while Meropenem required concentration above 32MIC to establish the filamentous with bulbous cells that connected to PBP2 and 3 binding in *E. coli*. For synergy determination, the combination between Cefpirome (PBP3 attacker) and Imipenem (PBP2 attacker) at 1/4MIC and 2 MIC was done in *E. coli*. The synergism was detected in 1/4MIC combination, whereas the regrowth was observed in both combinations after 24 hours of exposure. Thus, it can be concluded that drug's concentrations attacked to many types of essential PBPs, can increase bactericidal properties and morphological changes of the susceptible bacteria.

Department of Pharmacology Student's signature
Field of study Pharmacology Advisor's signature
Academic year 2001 Co-advisor's signature

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

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
BA24	= Bacteriolytic area of 24 hours
CFU	= Colony forming unit
D-ala	= D-alanine
DNA	= Deoxyribonucleic acid
E. cloacae	= *Enterobacter cloacae*
E. coli	= *Escherichia coli*
e.g.	= exempli gratia (for example)
enz	= enzyme
et al.	= et alii (and other peoples)
etc.	= et cetera (and other similar things)
Fig	= Figure
g	= gram
hr	= hour
ID_{50}	= Interfering dose 50%
KR3	= Killing rate of the first 3 hours
L	= Liter
log	= decimal logarithm
MBC	= Minimum bactericidal concentration
MHA	= Meuller-Hinton agar
MHB	= Meuller-Hinton broth
MIC	= Minimum inhibitory concentration
min	= minute
ml	= milliliter
mm	= millimeter

mol = mole

NAG = N-acetylglucosamine

NAM = N-acetylmuramic acid

NCCLS = The National Committee for Clinical Laboratory Standards

NSS = Normal saline solution

P. aeruginosa = *Pseudomonas aeruginosa*

PBP = Penicillin binding protein

PCM = Phase contrast microscopy

Ser = Serine

UDP = Uridine diphosphate

μm, um = micrometer

UMP = Uridine monophosphate

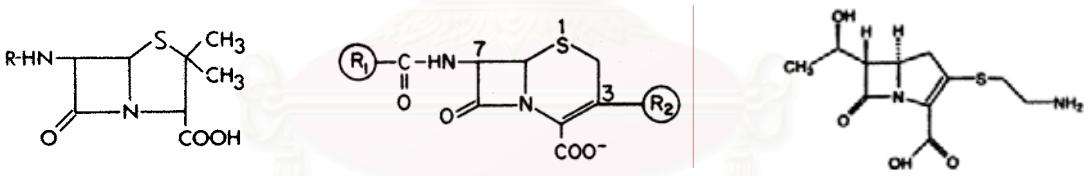
USA = The United States of America

UTP = Uridine triphosphate

CHAPTER I

INTRODUCTION

β-Lactam modified antibiotics have expanded widely over the past 50 years with the introduction of penicillins and cephalosporins. In the late 1970s, a new class of β-Lactam, the carbapenems was identified. They are differentiated from penicillins and cephalosporins by a methylene replacement for sulfur in the five-membered ring structure (Chambers, 2000). This modification results in smaller compounds than previously discovered drugs, which will be advantage to the permeability property into bacterial periplasmic space (Cornaglia, et al., 1992). Furthermore, all conventional β-lactams contain an acyl amino side chain while carbapenems have a hydroxyethyl side chain. This side chain in the conventional β-lactams is in *cis* configuration, whereas hydroxyethyl side chain of carbapenems is in *trans* configuration. The *trans* configuration of hydroxyethyl side chain is responsible for the β-lactamase stability of these compounds (Chambers, 2000).



Penicillin Cephalosporin Carbapenem

Figure 1-1 Basic structures of penicillin, cephalosporin and carbapenem.

The β-lactams are the most frequently prescribed antibiotics worldwide. Therefore, the resistance to these agents has become a major problem especially in the past two decades after the introduction of extended-spectrum cephalosporins, carbapenems and β-lactamase inhibitor/β-lactam drug combinations. Among the variety of pathogens that can provide resistance to β-lactam antibiotics, the most important are gram negative bacilli. In gram negative bacilli, the action of β-lactam antibiotics depend on 3 factors:

1) The penetration of β-lactams into periplasmic space
2) The destruction of β-lactams by β-lactamases in periplasmic space
3) The affinity of β-lactams to killing sites (Penicillin binding proteins, PBPs) in periplasmic space (Pitout, Sanders, and Sanders, 1997).

To reach target PBPs, β-lactam antibiotics must penetrate through the outer membrane of gram negative bacteria via protein pores called porins. The hydrophilic nature and molecular size of these drugs preclude the passage through hydrophobic portions of the outer membrane into periplasmic space (Nakaido, 1988). After penetration into periplasmic space, the β-lactams may be destroyed by particular destructing enzymes called β-lactamases. Thus, the remaining β-lactams will attack PBPs for the drug action as killing targets (Figure 1-2A). The covalent binding to PBPs interferes with synthesis of cell wall and ultimately leads to cell death (Livermore and William, 1996). Therefore, the resistance to β-lactam antibiotics may arises through one or more of the following mechanism (Pitout, et al., 1997):

1) One or more PBP(s) may change resulting in an altered target with either reduced or no binding to antibiotics (Figure 1-2B)
2) The porins in the outer membrane may change resulting in an decreased ability of drug to penetrate the membrane and reach PBPs (Figure 1-2C)
3) The organism may produce one or more β-lactamases to inactivate the antibiotics (Figure 1-2D).

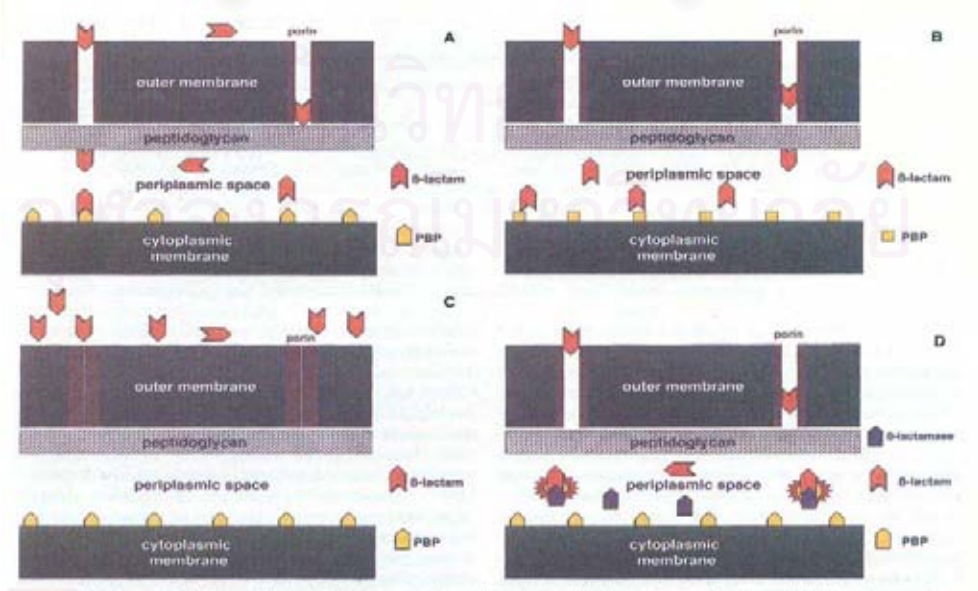


Figure 1-2 Resistance to β-lactam antibiotics. In the gram-negative cell, β-lactam antibiotics must enter through porins in the outer membrane, traverse the periplasmic space, and attach to their target penicillin-binding proteins (PBPs) located on the outer aspect of the cytoplasmic membrane (**A**). Resistance may arise through modification of the targets of the drugs, the PBPs (**B**); alterations in porin proteins that impede drug penetration into the cell (**C**); or the production of drug-inactivating enzymes, the β-lactamases (**D**).

Among the mechanisms in gram negative bacilli that can provide resistance to β-lactam antibiotics, the production of β-lactamases is the most important factor in clinical situation. This resistance may be potentiated by the alteration in the target of the drugs (PBPs) and through alterations in outer membrane permeability of the microorganisms to the drugs (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 *Klebsella pneumoniae*

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

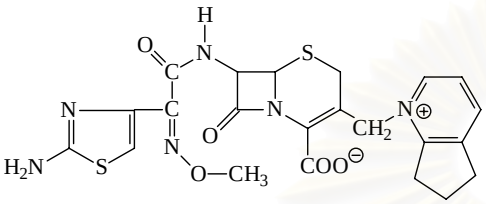
III. Decrease concentration of β-lactam antibiotics inside cell

A. Restrict its entry (loss of porins)

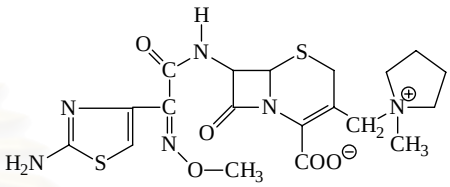
B. Pump it out (efflux mechanism)

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

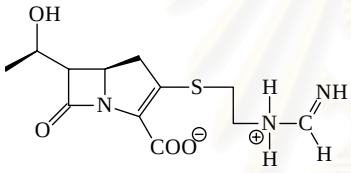
During several years, the zwitterions β-lactam antibiotics have been developed in order to circumvent these mechanisms of resistance. Because of the zwitterions compounds demonstrate an excellent permeability through bacterial outer membrane (Nakaido, Liu, and Rosenberg, 1990). Hence, a new class of β-lactams called the 4th generation cephalosporins (cefepime and cefpirome) and some carbapenems (imipenem and meropenem) were developed to be zwitterions compounds (Figure 1-3).



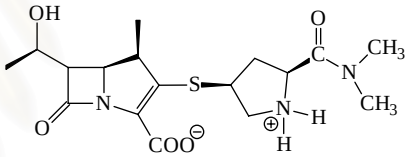
Cefpirome



Cefepime



Imipenem



Meropenem

Figure 1-3 The structures of cefpirome, cefepime, imipenem and meropenem.
(Modified from Nakaido, et al., 1990; Fung-Tomc, et al., 1995)

These drugs demonstrate the resemblance in rapid permeability rate and high stability on β-lactamases hydrolysis. Nevertheless, the difference between them is the PBPs binding affinity. Cefpirome is excellently bound to PBP3 only, whereas cefepime is dominantly bound to both PBP3 and PBP2 in *Enterobacteria* (Pucci, et al., 1991). Similarly, imipenem is excellently bound to PBP2 while meropenem is dominantly bound to PBP2 and PBP3 of gram negative bacteria (Sumita, Fukasawa, and Okuda, 1990). Therefore, the distinction in PBP binding among drugs may cause different antibacterial properties, which will be investigated in this research.

CHAPTER II

LITERATURES REVIEW

1. Bacterial Morphology and Cell Wall Synthesis

Cells from animals, plants and fungi are eukaryotes, whereas bacteria and the blue green algae belong to the prokaryotes. The prokaryotes are lack of nuclear membrane, other organelles such as 80s ribosome and the existence of the peptidoglycan cell wall surround the membrane to protect it against the environment. These and other distinguishing features are shown in table 2-1.

Table 2-1 Major Characteristics of Eukaryotes and Prokaryotes

CHARACTERISTC	EUKARYOTE	PROKARYOTE
Major groups	Algae, fungi, protozoa, plants, animals	Bacteria
Size (approximate)	>5 μm	0.5 to 3 μm
Nuclear structures		
Nucleus	Classic membrane	No nuclear membrane
Chromosomes	Strands of DNA	Single, circular DNA
	Diploid genome	Haploid genome
Cytoplasmic structures		
Mitochondria	Present	Absent
Golgi bodies	Present	Absent
Endoplasmic reticulum	Present	Absent
Ribosomes (sedimentation coefficient)	80S (60S + 40S)	70S (50S + 30S)
Cytoplasmic membrane	Contains sterols	Does not contain sterols
Cell wall	Is absent or is composed of chitin	Is a complex structure containing protein, lipids, and peptidoglycans
Reproduction	Sexual and asexual	Asexual (binary fission)
Movement	Complex flagellum, if present	Simple flagellum, if present
Respiration	Via mitochondria	Via cytoplasmic membrane

From Holt S. In Slots J, Taubman M, editors: *Contemporary oral microbiology and immunology,* St Louis, 1992, Mosby.

Bacteria can be distinguished from one another by their morphology, metabolic, antigenic and genetic characteristics. Gram stain is a powerful methodology to cleave the two major classes of bacteria and initiate therapy. Bacteria that are heat fixed onto a slide and stained with crystal violet; this stain is precipitated with gram iodine and then the excess stain is removed by washing with the acetone-based decolorizer. A counterstain, safranin, is added to stain any decolorized cells. This takes time less than 10 minutes. For gram positive bacteria will turn purple because the crystal violet stain gets trapped in a thick, crosslinking structure of the peptidoglycan layer which surround the cell. Gram negative bacteria have a thin peptidoglycan layer that does not retain crystal violet stain, thus the cells must be counterstained with safranin and turned red

(Murray, et al., 1998). The differentiation between these characteristics led to the different action of β-lactam antibiotics to both of them. Gram negative cell walls are more complex than gram positive cell walls both structurally and chemically. Structurally, a gram negative cell wall contains two layers external to the cytoplasmic membrane. Immediately external to the cytoplasmic membrane is a thin peptidoglycan layer, which accounts for only 5% to 10% of the gram positive cell wall by weight (Figure 2-1).

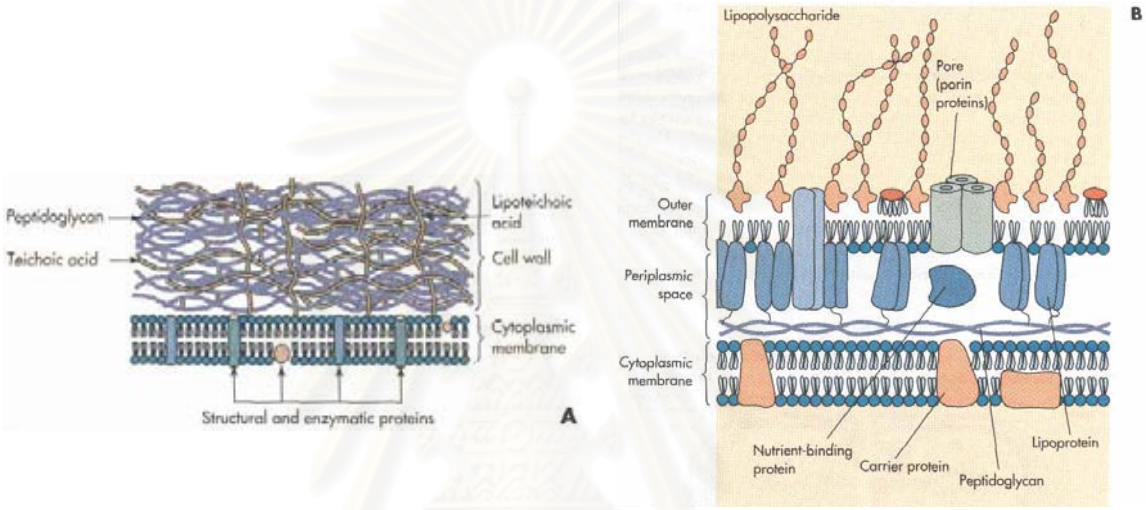


Figure 2-1 Comparison of the gram positive and gram negative bacterial cell wall. A, A gram positive bacterium has a thick peptidoglycan layer that contains teichoic and lipoteichoic acids. B, A gram negative bacterium has a thin peptidoglycan layer and an outer membrane that contains lipopolysaccharide, phospholipid and proteins. The periplasmic space between the cytoplasmic and outer membranes contains transport, degradative and cell wall synthetic proteins. The outer membrane is joined to the cytoplasmic membrane at adhesion points and is attached to the pepetidoglycan by lipoprotein links.

(Modified from Murray, et al., 1998)

External to the peptidoglycan layer is the outer membrane, which is unique to gram negative bacteria. They are like a sac around the bacteria that maintain the bacterial structure and a permeability barrier to large molecules. They also provide

protection from adverse environmental conditions such as the digestive systems of host, which is important for *Enterobacteriaceae* organism. This membrane consists of 2 major components: the phospolipid bilayer and the transmembrane proteins. A group of these proteins are known as **porins** because they formed pores that allow the diffusion of hydrophilic molecules less than 700 daltons in size through the membrane. The outer membrane and the porin channels allow passage of metabolites and small hydrophilic antibiotics, but it is a barrier for large or hydrophobic antibiotics and proteins such as lysozyme. Consecutively, the area between the internal surface of the outer membrane and the external surface of the cytoplasmic membrane is referred to the **periplasmic space**. This space is actually a compartment containing a variety of hydrolytic enzymes, which are important to the breakdown of large macromolecules for metabolism. These enzymes typically include proteases, phosphatases, lipases, nucleases and carbohydrate degrading enzymes. In the case of pathogenic gram negative species, many of the lytic virulence factors such as collagenases, hyaluronidases, proteases and **β-lactamases** are in the periplasmic space. This space also contains the peptidoglycan layer, which important for the rigidity of the bacterial cell wall (Bryan and Godfrey, 1991).

The peptidoglycan is the component that made up of polymer subunits. Synthesis of peptidoglycan occurs on the outside bacterial cytoplasm, whereas the precursors and subunits of the final structure are assembled in a factory like setting on the inside. They attach to a conveyor belt like structure, brought to the surface and then attached to the preexisting structure outside. For bacteria, the molecular conveyor belt like structure is a large hydrophobic phospholipid called bactroprenol (undecaprenol, C55 isoprenoid). The precursors must be activated with high energy bonds (e.g. phosphates) or other means to power the attachment reactions occurring outside cytoplasm. The peptidoglycan is a rigid structure that produce from linear polysaccharide chains cross-linked by peptide bonds (Livermore and William, 1996). This polysaccharide is made up of repeating disaccharides (monomer) of N-acetylglucosamine (NAG, G) and N-acetylmuramic acid (NAM, M). Figure 2-2,

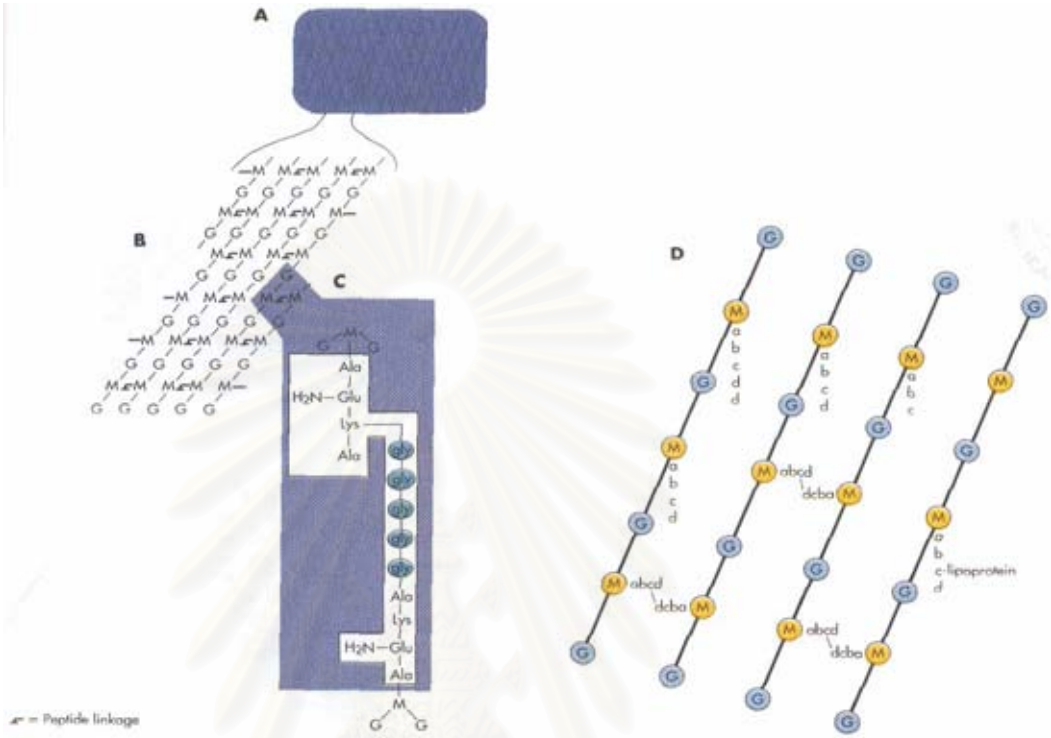


Figure 2-2 General structure of the peptidoglycan component of the cell wall. **A,** The peptidoglycan forms a meshlike layer around the cell. **B,** The peptidoglycan mesh consists of a polysaccharide polymer that is cross-linked by peptide bonds. **C,** Peptides are cross-linked through a peptide bond between the terminal D-alanine (D-ala) from one chain and a lysine (lys) (or another diamino amino acid) from the other chain. A pentaglycine bridge (gly_5) expands the cross-link in *S. aureus* (as shown). **D,** Representation of the *E. coli* peptidoglycan structure. Diaminopimelic acid, the diamino amino acid in the third position of the peptide, is directly linked to the terminal alanine of another chain to cross-link the peptidoglycan. Lipoprotein anchors the outer membrane to the peptidoglycan. M, *N*-Acetylmuramic acid; G, *N*-acetylglucosamine; *Glu*, glucosamine; *gly*, glycine. (**D** based on Joklik KJ et al, editors: *Zinsser microbiology*, Norwalk, Conn, 1988, Appleton & Lange.)

(Modified from Murray, et al., 1998)

The cellular procedures of peptidoglycan synthesis from the monomers consist of 3 steps. First, inside the cell that glucosamine is enzymatically converted into NAM and then energetically activated by a reaction with uridine triphosphate (UTP) to produce uridine diphosphate-N-acetylmuramic acid (UDP-NAM). The UDP-NAM is assembled in a series of enzymatic steps to be: UDP-NAM-aa-aa-aa-Dala-Dala (UDP-NAM-pentapeptide). The UDP-NAM-pentapeptide is attached to the bactroprenol in the cytoplasmic membrane through a pyrophosphate link with the release of uridine diphosphate (UDP). Consequently, NAG is added to make the disaccharide building block of the peptidoglycan to be NAG-NAM-pentapeptide. Second, the bactroprenol molecule translocates the NAG-NAM-pentapeptide precursor to the outside of the

cytoplasmic membrane. The NAG-NAM-pentapeptide is then attached to a peptidoglycan chain using the pyrophosphate link between itself and the bactroprenol as energy to drive the reaction. The pyrophosphobactroprenol is converted back to a phosphobactroprenol and recycled. Third, outside the cytoplasm near the membrane surface, peptide chains from adjacent glycan chains are cross-linked to each other by a peptide bond exchange (transpeptidation). The transpeptidation is occured 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 2-3). 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. Both of these enzymes are called **penicillin-binding proteins** (**PBPs**) because they are targets for penicillin and other β-lactam antibiotics. Penicillin and related β-lactam antibiotics resemble the transition state, the conformation of the Dala-Dala unit when bound to these enzymes (Further information available in chapter II topic 2). Different PBPs are used for extending the peptidoglycan, creating a septum for cell division, and curving the peptidoglycan mesh (Murray, et al., 1998).

The peptidoglycan is constantly being synthesized and degraded. **Autolysins** such as lysozyme are important for determining bacterial shape and continue to degrade the peptidoglycan even if peptidoglycan synthesis is inhibited. Inhibition or synthesis of the cross-linking of the peptidoglycan does not stop the autolysins and their action on the bacterial structure is leads to lysis and cell death. New peptidoglycan synthesis does not occur during starvation, which leads to a weakening of the peptidoglycan layer and a loss in the dependability of gram stain. An understanding of the biosynthesis of peptidoglycan is essential in medicine because these reactions are unique to bacterial cells and hence can be inhibited with little or no adverse effect on human cells. A number of developed antibiotics target one or more steps in this pathway (Spratt, 1975).

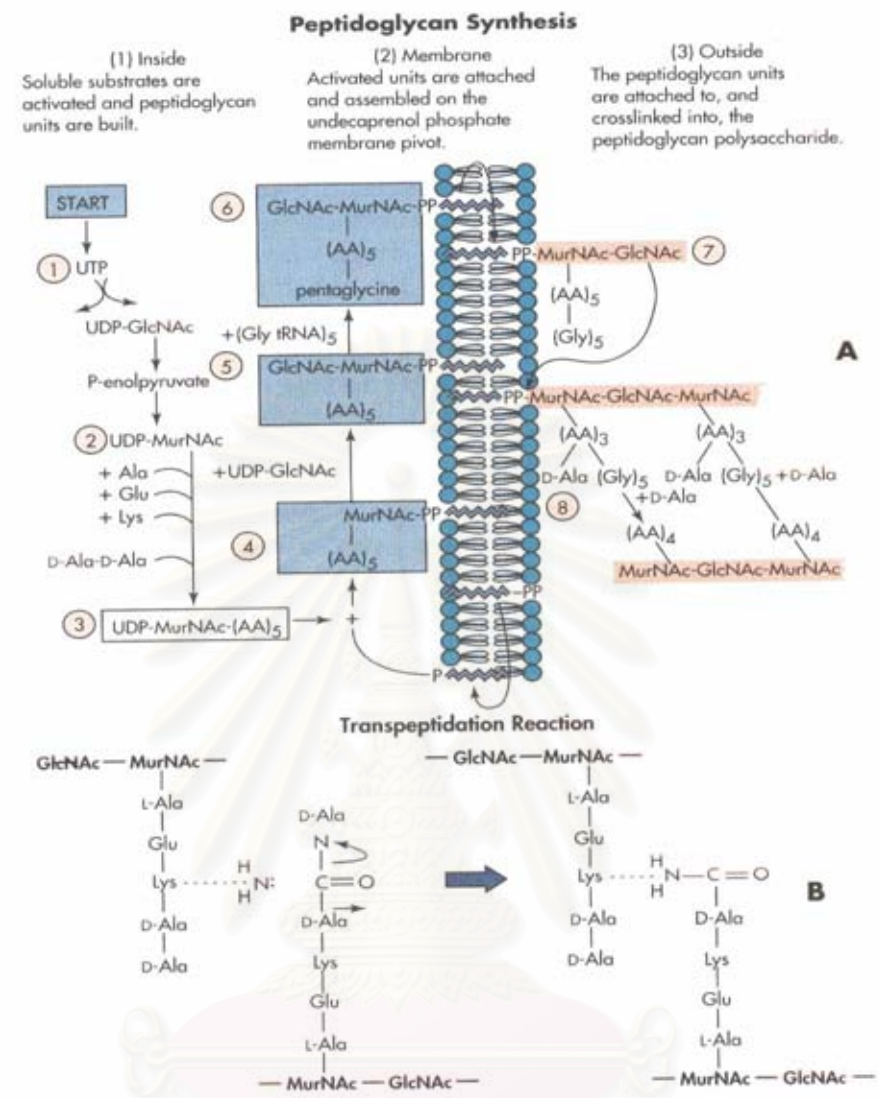


Figure 2-3 Peptidoglycan synthesis. A, Peptidoglycan synthesis occurs in three phases. (1) Peptidoglycan is synthesized from prefabricated units constructed and activated for assembly and transport inside the cell. (2) At the membrane, the units are assembled onto the undecaprenol phosphate conveyor belt and assembly is completed. (3) The unit is translocated to the outside of the cell, where it is attached to the polysaccharide chain and the peptide is crosslinked to finish the construction. B, The crosslinking reaction is a transpeptidation. One peptide bond (produced inside the cell) is traded for another (outside the cell) with the release of D-alanine, D-alanine transpeptidase, carboxypeptidase. These enzymes are the targets of β-lactam antibiotics and are called penicillin binding proteins (PBPs)

(Modified from Murray, et al., 1998)

2. Mechanism of β-Lactam Antibiotics

The cellular targets for β-lactam antibiotics are the enzymes that catalyze synthesis of the cross-linked peptidoglycan of the cell wall. These enzymes are known as penicillin binding proteins, which give identical pattern of 7 PBPs in *Escherichia coli*. They are designated in order of descending molecular weight: 1a, 1b, 2, 3, 4, 5, and 6. The higher molecular weight molecules are thought to be essential for bacterial function, which consist of PBP1-3 in *Escherichia coli* (Spratt, 1975). The physiological role of PBP1-3 are catalyze peptidoglycan transglycosylase and transpeptidase reactions, which is important for the synthesis and cross-linking of the peptidoglycan, respectively.

The ability of β-lactams to inhibit the D-alany-D-alanine transpeptidases (PBP1-3) and carboxypeptidases (PBP4-6) depends on conformational similarity between the amide bond (O=C-N) of the β-lactam ring and the peptide link of D-alanyl-D-alanine. Penicillins and other β-lactams have been proposed to act as analogs of acyl-D-alanyl-D-alanine, a position postulated from the structural similarities of stereomodels of the different molecules (Bryan and Godfrey, 1991). Considerable effort was put into comparing the bond angles of these structures and showing how they resembled one another (Figure 2-4).

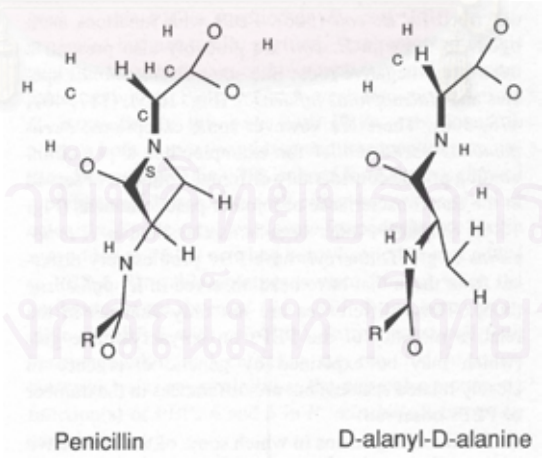


Figure 2-4 Dreiding stereomodels of penicillin and D-alanyl-D-alanine.

(Modified from Bryan and Godfrey, 1991)

Under natural conditions the enzymes performing the transpeptidation 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 releases of the free enzyme. Treatment of the bacteria with β-lactam antibiotics would interfere with this process. A penicilloylated enzyme complex, formed following interaction of the enzyme with penicillin, would act as a competitor to the formation of the normal acylated enzyme. Studies on the interaction of both the normal substrate and β-lactams with the PBPs of *Escherichia coli* have suggested that an active site serine is the residue responsible for binding (Bryan and Godfrey, 1991). More recent studies have suggested that penicillin and the normal substrate may bind to different residues within the active center. The consequence of this competition is interference with the normal cross-linking of the cell wall, resulting in disruption of cell wall synthesis and eventually cell death (Figure 2-5).

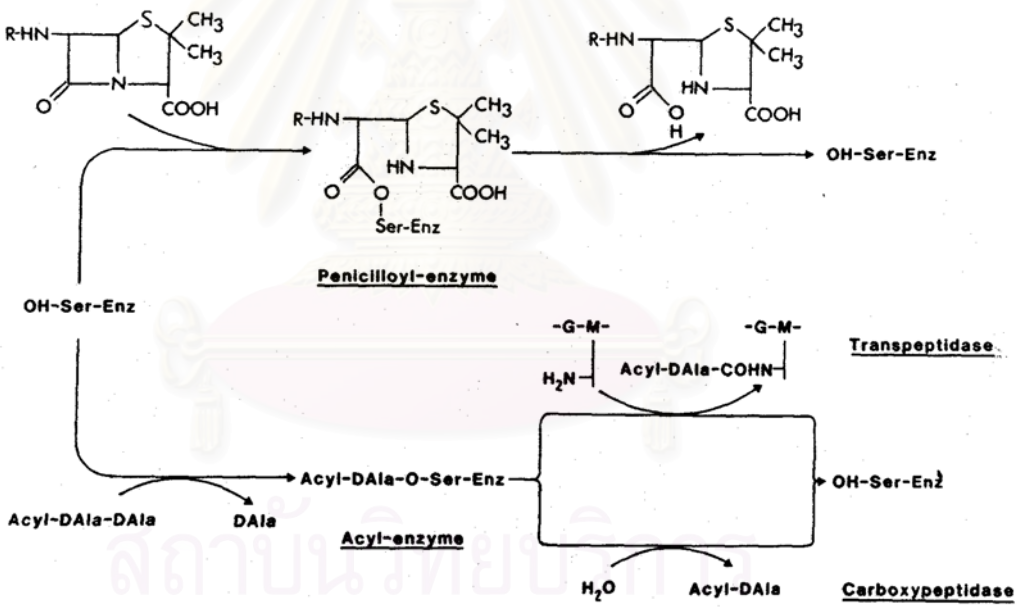


Figure 2-5 Proposed mechanism for the reaction of penicillin-sensitive enzymes with penicillin or acyl-D-Ala-D-Ala. A nucleophile in the enzyme active site (shown as a serine residue) reacts either with acyl-D-Ala-D-Ala or penicillin to yield either acyl-D-alanyl-enzyme or penicilloyl-enzyme intermediates. Serine has been established as the residue to which the substrate or inhibitor binds in the carboxypeptidases (PBP-5) of *Bacillus subtilis*, and *Bacillus stearothermophilus* (152) and certain carboxypeptidase/transpeptidases from streptomycetes (45). Whether a similar situation exists in the high molecular weight PBPs remains to be established. Subsequent reaction of the acyl-D-alanyl-enzymes with a suitable amino acceptor peptide results in formation of a cross-link and release of the enzyme (transpeptidase). Alternatively, reaction with water results in the release of acyl-D-alanine and enzyme (carboxypeptidase). Although both functions are shown for the same enzyme individual proteins tend to favour one reaction, i.e., they are D,D-carboxypeptidases with inefficient transpeptidase activity or the reverse may be the case. The penicilloyl-enzyme is more stable than the acyl-D-alanyl-enzyme and consequently the antibiotic residue is not transfered to an amino acceptor. Certain of these complexes do, however, react slowly with water to release penicilloic acid and active enzyme. In other cases further degradation of the penicillin nucleus occurs.

(Modified from Hayes and Ward, 1986)

Classification of the penicillin-sensitive enzymes as PBPs and subsequent study of these proteins resulted in the formation of a model of activity based upon the behavior of *E. coli* cells grown in the presence of different β-lactams. The PBP1A is encoded by the *pon*A gene, has been proposed to be one of the major targets for cefsulodin, and its inhibition in combination with inhibition of PBP 1B results in the cessation of cell elongation and cellular lysis. The enzymatic activities associated with PBP 1A are suggested to be both a **transglycosylase** and a **transpeptidase**. Both of these enzymatic activities also have been shown to be associated with PBP1B, and the extensive amino-acid sequence homology between the two PBPs implies a similar function for both enzymes (Livermore and William, 1996). This postulate is further supported by the observation that the two enzymes are interchangeable in peptidoglycan synthesis. PBP1B was one of the first higher molecular weight PBPs to be purified for enzymatic studies, probably due to its stability throughout the isolation process. Most of the higher molecular weight PBPs loss much of their enzymatic activity when subjected to the stress of the affinity chromatography that is used to extract them.

A bifunctional mode of action also has been proposed for PBP2. In conjunction with the *rod*A gene product, PBP2 has both transglycosylase and transpeptidase activities (Bryan and Godfrey, 1991). The specific effects of inhibition of PBP2 on the cell cylcle in *Escherichia coli*, have been aided by this protein's unique affinity for the mecillinam as well as the two β-lactams, imipenem and clavulanate. PBP2 has also been proposed to play a role in the induction of the chromosomal β-lactamases. This observation fits well with the unique binding affinity of PBP2 for imipenem, which is a potent inducer of β-lactamases at the subMIC concentrations, conditions under which PBP2 is the major target. The inhibition of PBP2 causes the oviodal cells of the bacteria. The PBP3 is product of the *pbp*B gene. It demonstrates both transglycosylase and transpeptidase activities and required for septum formation during cellular division. Inhibition of this PBP with β-lactam antibiotics results in the formation of filaments in *E. coli* (Spratt, 1975). Filaments so formed are resolved upon removal of the antibiotic, and septation resumes at newly sites near the cell poles (Table 2-2).

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

PBP	Symbol	Molecular weight	Molecules/cell	Morphological changes after occupied by β-lactams
1A	*ponA*	91	230	Spheroplasting cells with rapid lysis
1B	*ponB*	86.5-81.5		
2	*rodA*	66	20	Ovioidal cells
3	*pbpB*	60	50	Filamentous cells
4	*dacB*	49	110	-
5	*dacA*	42	1800	-
6	*dacC*	40	5700	-

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

The nonessential penicillin-binding proteins (PBPs 4,5 and 6) of *E. coli* comprise a group of proteins that account for > 80% of the bound penicillin in bacterial membranes. The majority of this binding activity is provided by PBP5. The enzymatic activities associated with this group of proteins are basically those of D-alanine carboxypeptidases: the enzymes that inhibit the extent of the cross-linking of peptidoglycan. PBP4, product of the *dac*B gene has been proposed to have three different cellular functions. The first, proposed by several groups, is that of an endopeptidase hydrolyzing the cross-links in mature peptidoglycan. The second function proposed for PBP4 is as a secondary peptidase catalyzing the formation of additional cross-linking in newly assembled peptidoglycan. The third position is that the protein acts as a transpeptidase, acting in the formation of diamino-pimelyl-diaminopimelic acid cross-links in the cell wall. In vitro, and possibly in vivo, PBP5 and PBP6 catalyze the D-alanine carboxypeptidase reaction. Deletion or mutants in either *dac*A (PBP5) or *dac*C (PBP6) show little if any morphological variation, suggesting that their functions are dispensable and may compensate each other. The loss of one or the other of these proteins does not compromise the cell, and both mutant types produce peptidoglycan with the normal degree of cross-linking (Bryan and Godfrey, 1991). The D,D-carboxypeptidase activity produced by either PBP5 or PBP6 may compensate for the activity produced by either PBP5 or PBP6 for the activity missing in the mutant strain. The activities of the different PBPs in other gram negative bacteria are not as well described as those in *E. coli*. All *Entrobacteriaceae* so far examined appear to have

PBPs with profile and function similar to those described for *E. coli*. PBPs with functions analogous to those in *E. coli* are probably present in other gram-negative rods, like some *Pseudomonas* species.

3. Selective Binding of β-Lactam Antibiotics to PBPs of Gram Negative Bacteria

As already known that the essential PBPs are lethal targets of β-lactam antibiotics. However, the selective binding of β-lactam antibiotics to PBPs of gram negative bacteria may differ among drugs and bacterial species. Therefore, many investigations have been carried out with a wide range of organisms, to determine the affinity of PBPs for β-lactams. The acceptable value to determine the affinity of PBPs with β-lactams is the **interferance doses 50** (**ID_{50}**). The ID_{50} is the concentration of the β-lactams required to reduce the binding of radiolabeled penicillin by 50% in each PBP (Hayes and Ward, 1986). The majority of these data were undertaken by pharmaceutical companies in an attempt to obtain a greater understanding of the structure activity relationships between the PBPs and β-lactams. This data provide valuable information relating to the identification of β-lactam killing targets (Tables 2-3 and 2-4).

Table 2-3 Competition of β-lactam antibiotics for the PBPs of *Escherichia coli*[a]

Antibiotics	ID_{50} for PBP (ug/ml)							MIC[b] (ug/ml)	
	1A	1B	2	3	4	5	6		
Penicillin G	0.5	3.0	0.8	0.9	1.0	24	19	16	(1.6)
Ampicillin	1.4	3.9	0.7	0.9	2	140	9	3.2	(0.5)
Mecillinam	>500	>500	0.04	>500	>500	>500	>500	0.05	(0.01)
Aztreonam	0.5	70	>100	0.1	>100	>100	>100	0.1	-
Cefsulodin	0.47	3.7	>250	>250	>250	>250	>250	25	(6.4)
Cefotaxime	0.05	0.7	5.0	<0.05	30	>50	>50	0.08	(0.01)
Cefpirome	-----1.4-----		7.0	0.03	10	-	-	0.02[c]	-
Cefepime	-----1.5-----		0.25	0.03	16	-	-	0.01[c]	-
Imipenem	0.18	0.81	0.013	17.3	0.02	0.9	4.3	0.06[c]	-
Meropenem	0.82	0.58	0.011	0.72	0.04	2.4	22	0.015[c]	-

[a]Data from Hayes and Ward, 1986; Kitzis, Acar, and Gutmann, 1989; Pucci, et al., 1991.

[b]Numbers in parentheses indicate MIC against *E. coli* DC2, a permeability mutant of *E. coli*.

[c]Numbers in parentheses indicate MIC against *E. coli* K12, a betalactamase deficient of *E. coli*.

In *E. coli*, PBP4, 5 and 6 do not appear to be essential for growth and thus their interaction with β-lactams is not believed to be of major significance for the killing action of these antibiotics. The remaining proteins (PBP1A, 1B, 2 and 3) have been described previously as the primary lethal targets. The binding of a β-lactam leading to the inactivation of any of these proteins is potentially lethal and results in a physiological response characteristic of a particular antibiotic. This response, which is often manifested by a morphological change, is dependent upon the PBP binding profile of the β-lactam and the concentration of antibiotic used (Hayes and Ward, 1986). For example, ampicillin bind preferentially to their primary targets PBP2 and 3 at similar concentration (ID_{50} = 0.7 ug/ml and 0.9 ug/ml, respectively). This compound induces bulge formation (inhibition of PBP2) in the center of cells in addition to filamentation (inhibition of PBP3) at their lowest effective concentration. However, the rapid killing effect of this antibiotic probably results from the inhibition of PBP 1A and 1B which causes spheroplasting and occurs at slightly higher concentrations of the antibiotics (ID_{50} = 3-4 ug/ml).

The inhibition of bacterial growth by β-lactam antibiotics that have similar affinities for the target proteins is likely to result from the simultaneous inactivation of more than one of the lethal targets. The multiple inactivation of these essential PBPs will often lead to a population of cells showing a mixed morphological response at the supraMIC level of β-lactams. In some cases, The rationale for the selection of synergistic combination of β-lactams has been based on multiple PBP inactivation. Satta and colleagues (1995) have reported the increasing of bactericidal activity of cefsulodin (PBP1 specific), mecillinam (PBP2 specific) and aztreonam (PBP3 specific) in combination of each other for eradicate *E. coli*. They have shown that the binding affinities of β-lactams to PBP1A and 1B are thought to be the most important factor for the lytic effect of these compounds. Interestingly, several investigators reported PBP1A is more sensitive to β-lactams than PBP1B but the activity of PBP1B is of greater important than that of PBP 1A in cell wall elongation and initiation of the β-lactams induced lytic effect in *E. coli*.

However, the majority of β-lactam antibiotics used in clinical practice today inhibit PBP3 as their primary lethal target. The filaments resulting from PBP3 inhibition continue to grow for 4-6 generations before deformation and collapse but not complete lysis. It seems probable that during this time the significant interaction of β-lactams with others essential PBPs may occur and eventually initiate cell lysis. This secondary effect will be dependent on the other target proteins. For example, in a study of the interaction of cefotaxime with the PBPs of *E. coli*, Chase, Fuller, and Reynolds (1981) showed that the loss of viability and lysis of these organisms occurred only under conditions where there was significant binding to PBP1A in addition to PBP3. At supraMIC concentration many of the filament forming β-lactams have the potential to induce rapid lysis by inhibition of PBP1A and1B.

In other gram negative bacteria, the binding of β-lactam antibiotics to the PBPs has been studied. The binding of a range of β-lactam antibiotics to *Pseudomonas aeruginosa* PBPs is shown in table 2-4. The PBP profile of *P. aeruginosa* is very similar, but not identical to that of *E. coli*. The preferential binding of β-lactam antibiotics to *P. aeruginosa* PBPs is resemble to that found in *E. coli*, however their absolute affinity for the antibiotics may differ. Consequently, the concentration of β-lactams which required to elicit a particular morphological response in *P. aeruginosa* and *E. coli* may differ as a result of the differences in PBP affinities, permeability or β-lactamases hydrolysis. Accordingly, the competitive data for the binding of β-lactams to the PBPs of the *Enterobacteriaceae* is similar to that of *E. coli* (Bellido, Pèchere, and Hancock, 1991). On the basis of these results, it may suggest that the PBPs in these bacteria probably perform similar roles in cell wall elongation, shape and division that have been determined for PBP1A, 1B, 2 and 3 of *E. coli.*

Table 2-4 Competition of β-lactam antibiotics for the PBPs of *Pseudomonas aeruginosa*[a]

Antibiotics	ID_{50} for PBP (ug/ml)							MIC[b] (ug/ml)	
	1A	1B	2	3	4	5	6		
Ampicillin	<0.25	0.9	0.5	1.3	0.25	>250	190	160	-
Mecillinam	>100	>100	0.1	>100	>100	>100	>100	>128	-
Aztreonam	ND	ND	>100	0.1	>100	-	>100	0.2	-
Cefsulodin	19	2.0	>250	0.3	39	>250	>250	0.8	(0.03)
Cefotaxime	0.05	0.1	-	-	5.0	>200	0.5	16	-
Ceftazidime	0.8	6.0	25	0.1	6.4	50	50	0.5	(0.05)
Cefpirome	0.06	0.50	>25	<0.0025	0.17	-	-	0.5[c]	-
Cefepime	0.035	0.75	>25	<0.0025	0.04	-	-	0.06[c]	-
Imipenem	0.08	0.22	0.06	0.06	0.08	0.12	-	2.0[c]	-
Meropenem	0.14	0.06	0.025	0.01	0.2	>11	-	0.5[c]	-

[a]Data from Hayes and Ward, 1986; Kitzis, et al., 1989; Pucci, et al., 1991.

[b]Numbers in parentheses indicate MIC against *P. aeruginosa* 799161, a permeability mutant of *P. aeruginosa*.

[c]Numbers in parentheses indicate MIC against *P. aeruginosa* SC8329, a betalactamase deficient of *P. aeruginosa*

4. Effect of β-Lactam Antibiotics to Bacteria

The relationships among PBP inhibitions, morphological changes and bactericidal action have been discussed at a biochemical level, but gross effects should also be mentioned. The bactericidal action of β-lactams is concentration-independent above the MIC unless an increase in PBP target occurs. At supraMIC level, the bactericidal activities of β-lactam antibiotics to gram negative bacilli depend on 2 major factors: growth rate and osmotic pressure (Livermore and William, 1996; Greenwood, 1997). The chemostat studies show that rapidly growing cells are killed more rapidly than slowly growing ones but that the proportion of cells killed per generation is virtually constant at all growth rates (Figure 2-6).

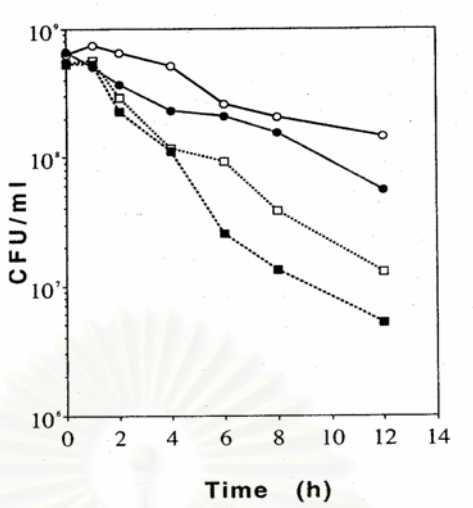


Figure 2-6 Killing of *P. aeruginosa* strain M2297 by ceftazidime (10 μg/mL). Cultures were grown in a chemostat with doubling times of 2.3 hours (■), 2.9 hours (□), 7.7 hours (●), and 12 hours (○). Note that the bactericidal rate increases with increasing growth rate but that the reduction in cell count per generation time is roughly constant.

(Modified from Livermore and Williams, 1996)

Accordingly, the bactericidal effect of β-lactam antibiotics can be quantitatively prevented by providing sufficient osmotic protection in gram negative bacilli. One consequence of the osmotic basis of lethality in gram negative bacilli is that those bacteria, notably *Proteus spp.* and *Haemophilus influenzae* that exhibit a naturally low intracellular osmolality, are much less susceptible to the osmotic lysis than *Escherichia coli*. This postulate is encouraged by the decrease of bactericidal activity of β-lactam antibiotics in treatment of upper urinary tract infection. These drugs can not lyse the susceptible bacteria in kidney, which contains the extreme level of drug and high extracellular osmolality.

However, the nature of the lethal event in gram positive cocci appears to be different. Since these bacteria posses a much thicker and tougher peptidoglycan layer than that present in the gram negative cell wall. Therefore, the much greater damage has to be inflicted before death of the cell ensues. One of the first events that occurs on exposure of gram positive cocci to β-lactam antibiotics is a release of lipoteichoic acid, an event which appears to trigger a suicide response of autolytic dismantling of the peptidoglycan (Bryan and Godfrey, 1991).

Interestingly, even though a great deal of bacterial populations are killed after exposed to β-lactam antibiotics but some are survive even on prolong exposure to an optimal bactericidal concentration, about 1 in 10^5 bacteria usually survive. These persisters have not acquired resistance, since most of their immediate progeny are killed on reexposure to antibiotics. How persisters arise is not known, but clearly that they are bacteria caught in a particular metabolic state which prevents the β-lactam drugs from achieving its lethal effect. Persisters may be cells in which the peptidoglycan coat exists transiently as a complete covalently linked macromolecule. In order to grow, a cell in this state has to use autolytic enzymes to create holes in the peptidoglycan where new cell wall building blocks can be added. If β-lactam antibiotics inhibit such enzymes, then these cells would be trapped in a state in which they could not grow and therefore could not be killed until the antibiotic is removed (Greenwood, 1997).

The understanding in the basis response of bacteria to β-lactam antibiotics suggests the optimal dosing of the administered drugs. The facts that β-lactam antibiotics demonstrate the concentration-independent activity unless the increase in PBP target occurs may be the useful information to clinical practice. Therefore, the present studies were undertaken to compare bactericidal activities and effect on morphological changes of the β-lactam antibiotics with different PBP binding property, namely cefpirome, cefepime, imipenem and meropenem either alone and in combination against gram negative bacteria.

CHAPTER III

MATERIALS & METHODS

MATERIALS

1. Microorganisms, Chemicals and Reagents

1.1 Microorganisms

The bacterial strains used throughout this study were *Escherichia coli*, *Enterobacter cloacae* and *Pseudomonas aeruginosa*. These bacterials were clinical isolates from the patients in Siriraj Hospital during year 2001. All microorganisms were highly to moderately susceptible to the drugs to be studied (cefepime, cefpirome, imipenem and meropenem) as tested from disk susceptibility method, which was described by the National Committee for Clinical Laboratory Standards (2000) or NCCLS, 2000. The selected microorganisms were sampled from 20 clinical isolates by simple random sampling to collect 1 strain of each specie for further studies in the broth macrodilution test and the time kill test.

1.2 Chemicals

Cefepime, cefpirome, cilastatin-free imipenem and meropenem disks were purchased from Oxoid (Oxoid Chemicals, England) and BBL chemicals (Beckton Dickinson, USA). These disks were used to determine the susceptibility patterns in the disk susceptibility method according to NCCLS, 2000. Cefepime, cefpirome, cilastatin-free imipenem and meropenem standard powders were kindly provided as the generous gifts from Bristol Myers Squibb (Syracuse, USA), Aventis Pharma (Frankfurt, Germany), Merck Sharp and Dohme (New Jersey, USA) and AstraZeneca (Wilmington, USA), respectively. Working solutions were prepared immediately prior to use, as specified by the manufacturers before adding to the working media.

1.3 Reagents

- Meuller Hinton Agar (MHA) and Meuller Hinton Broth (MHB) were purchased from Oxoid (Oxoid Chemicals, England). All media were prepared according to

the method of NCCLS, 2000 (both the disk susceptibility method and the broth macrodilution method).

- Steriled water was used as solvent of the chemical powders to develop the working solution and culture media.
- Steriled normal saline solution (NSS) was chosen to be used 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.
- 7.4% Formaldehyde solution was selected to fix the bacterial cells before determining the morphological changes in time kill method by phase contrast microscopy.

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 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 plate containing agar for culture microorganisms in the whole processes such as subculture, susceptibility testing and colony counting.
- Erlenmayer 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 loops were 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 NCCLS, 2000.
- Racking tubes were used as shelf to hold a large number of tubes, both in broth macrodilution procedures and time kill procedures.

3. <u>Laboratory Instruments</u>

3.1 Temperature Controlling Instruments

- Autoclave was used to sterilize equipment, media, diluents, 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.
- Shaker bath was chosen to apply appropriate bacterial growth condition of liquid media that mimic 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 antibiotics in preparing procedures of both working media and working antibiotic solutions.
- Spectrophotometers were applied to adjust turbidity of the inoculum to equivalent with 0.5 McFarand standard solution (the preparation of 0.5 McFarland standard is described in method)
- 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.

3.3 Instruments in Phase Contrast Microscopy Technique.

- Phase contrast microscopy is chosen to detect the morphological changes of bacterial cells both before and after exposure to antibiotics.
- Centrifuge is applied to precipitate the bacterial cells in specimens before detection of the morphological changes by phase contrast microscopy.
- Glass slides are used to prepare and fix the bacterial cells before the detection of morphological changes by phase contrast microscopy.

METHODS

1. <u>Turbidity Standard for Inoculum Preparation</u>

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

1.1 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.

1.2 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.

1.3 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.

1.4 These tubes should be tightly sealed and stored in the dark at room temperature.

1.5 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.

1.6 The barium sulfate standards should be replaced or their densities verified monthly.

2. <u>Procedures for Performing the Disk Diffusion Test</u>

2.1 Preparation of Agar Plate

2.1.1 Meuller-Hinton agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions.

2.1.2 Immediately after autoclaving, allow it to cool in a 45 to 50 °C.

2.1.3 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.

2.1.4 The agar medium should be allowed to cool to room temperature and, stored in a refrigerator (2 to 8 °C).

2.1.5 Plates should be used within 7 days after preparation and a representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35 °C for 24 hours.

2.2 Inoculum Preparation

2.2.1 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 suitable broth medium.

2.2.2 The broth culture was incubated at 35°C until it achieved or exceeded the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours).

2.2.3 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. This resulted in a suspension containing approximately 1 to 2 x 10^8 CFU/ml for *E. coli* ATCC 25922. To perform this step properly, a photometric device can be used.

2.3 Inoculation Test Plates

2.3.1 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.

2.3.2 The dried surface of a Meuller Hinton 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.

2.3.3 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.

2.4 Application of Disks to Inoculated Agar Plates

2.4.1 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.

2.4.2 The plates were inverted and placed in an incubator set to 35°C within 15 minutes after the disks were applied in ambient air. Because of the interpretive standards were developed by using ambient air incubation, and CO_2 will significantly alter the size of the inhibitory zones of some agents.

2.5 Reading Plates and Interpreting Results

2.5.1 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.

2.5.2 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.

2.5.3 The size of the inhibition zone were interpreted by referring to NCCLS, 2000 and the organisms were reported as either susceptible, intermediate, or resistant to the agents that have been tested (Tables 3-1 and 3-2).

Table 3-1 Zone diameter interpretive standards breakpoints for Enterobacteriaceae

Drug	Disk content	Zone diameter (mm)			Comment
		R[a]	I[b]	S[c]	
Imipenem	10 ug	≤13	14-15	≥16	-
Meropenem	10 ug	≤13	14-15	≥16	-
Cefepime	30 ug	≤14	15-17	≥18	-
Cefpirome	30 ug	≤14	15-17	≥18	Not determined in NCCLS, 2000 Data from Jones, et al., 1984; Jones, Thronsberry, and Barry, 1986.

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

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

Drug	Disk content	Zone diameter (mm)			Comment
		R[a]	I[b]	S[c]	
Imipenem	10 ug	≤13	14-15	≥16	-
Meropenem	10 ug	≤13	14-15	≥16	-
Cefepime	30 ug	≤14	15-17	≥18	-
Cefpirome	30 ug	≤14	15-17	≥18	Not determined in NCCLS, 2000 Data from Jones et al., 1984; Jones, et al., 1986.

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

3. Broth Macrodilution Procedures

3.1 Meuller Hinton Broth Medium

3.1.1 MHB was recommended as the medium of choice for the susceptibility testing of commonly isolated, rapidly growing aerobic or facultative

organisms. MHB demonstrates good batch to batch reproducibility for susceptibility testing and yields satisfactory growth of most pathogens.

3.1.2 The pH of each batch of MHB 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 (25°C).

3.2 Preparing and Storing Diluted Antimicrobial Agents

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

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

3.2.3 The tubes can be closed with cotton plugs.

3.2.4 The final twofold dilutions of antibiotic were prepared volumetrically in the broth. A minimum final volume of 1 ml of each dilution was needed for the test. A single pipette can be used for measuring all diluents and then for adding the stock antimicrobial solution to the first tube. A separate pipette should be used for each remaining dilution in that set. Because there will be a 1:2 dilution of the drugs when an equal volume of inoculum was added, the antimicrobial dilutions were often prepared at twice the desired final concentration.

3.3 Broth Dilution Testing

A standardized inoculum for the macrodilution broth method may be prepared by growing microorganisms to the turbidity of the 0.5 McFarland standard.

3.3.1 Optimally, within 15 minutes of preparation, the adjusted inoculum suspension should be diluted in water so that after inoculation, each tube contains approximately $5x10^5$ CFU/ml. The dilution procedure to obtain this final inoculum varies according to the method of delivery of the inoculum to the individual tube and according to the organism being tested, and it must be calculated for each situation.

3.3.2 Within 15 minutes after the inoculum has been standardized as described above, 1 ml of the adjusted inoculum was added to each tube already containing 1 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 1:2 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculum.

3.3.3 The inoculated macrodilution tubes should be incubated at 35°C for 16 to 20 hours in an ambient air incubator.

3.3.4 The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the tubes 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 growth-control tubes (no antibiotics) used in each set of tests when determining the growth end points.

3.3.5 Define MIC of selected organisms to tested drugs by Broth macrodilution method (shown in table 3-3)

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

Organisms / Drugs	*Escherichia coli*	*Enterobacter cloacae*	*Pseudomonas aeruginosa*
Cefepime	≤ 8	≤ 8	≤ 8
Cefpirome*	≤ 8	≤ 8	≤ 8
Meropenem	≤ 4	≤ 4	≤ 4
Imipenem	≤ 4	≤ 4	≤ 4

*Not determined in NCCLS, 2000. Data from Jones, et al., 1984; Jones, et al., 1986.

4. Time Kill Method (Single Drug)

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 antibacterial concentrations to conduct the time kill method should be pharmacokinetic achievable concentration from previously published articles (Clissold, Todd, and Campoli-Richards, 1987; Nakayama, et al., 1992; Auwera and Santella, 1993; Drusano and Hutchison, 1995; Craig, 1998; Mouton, et al., 2000). The standardized inoculum for

the time kill method should be prepared by growing microorganisms to the turbidity of the 0.5 McFarland standard which equivalent to bacterial quantity 1 to $2x10^8$ CFU/ml.

4.1 Vary the drug concentrations ranging from 1/4MIC to 128MIC as two fold dilution steps into MHB for prepare working media before adding the standardized inoculum

4.2 Dilute the standardized inoculum to obtain the final bacterial quantity 1 to 2×10^7 CFU/ml into working media and control tubes on shaker bath at 37°C.

4.3 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.

4.4 Select specimen at the second hours after bacteria exposed to drug in every concentration to observe morphological changes by phase contrast microscopy and compare with control.

4.5 Inoculate the samples on appropriate solid media for 16 to 18 hours at 37°C to detect for colony forming units.

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

4.7 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, 1991; Pankuch, Jacobs and Appelbaum, 1994; Satta, et al., 1995).

5. Phase Contrast Microscopy

The phase contrast microscopy was chosen to determine morphological changes of the bacteria after exposed to drug at the second hours by time kill method. Hence, the selected drugs and bacteria in phase contrast microscopy must be correlated with broth macrodilution method and time kill method to define MIC and detect for killing activities, respectively.

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

5.2 The specimens were centrifuged at low speed centrifugation (3000xg) for 10 minutes to change suspending bacterial cells to be sediments (This procedure conducted at 4°C to keep bacteriostatic condition).

5.3 Resuspend the sediments and fixed with 7.4% formaldehyde solution to inhibit the growth and cell division of resuspended bacteria.

5.4 Bacterial cells were examined and photographed under phase contrast microscopy to detect the morphological changes compare with control at 100x solution with the oil immersion.

5.5 Refer the morphological evidence to PBP subtypes that were attacked by different drugs in different concentrations.

5.6 Criteria for morphological changes in correlate with PBP subtypes are shown in table 3-4

Table 3-4 Effects of β-lactam antibiotics on growth of gram negative bacilli predicted by their relative affinities for PBPs

Relative affinities of binding protein 1,2, and 3 for a β-lactam antibiotics	Morphological effect produced by three arbitrary concentrations of a β-lactam antibiotics		
	Low concentration	Medium concentration	High concentration
1 > 2 or 3	Lysis	Lysis	Lysis
2 > 1 > 3	Ovoid cells	Lysis	Lysis
2 > 3 > 1	Ovoid cells	Filaments with bulges	Lysis
2 >> 1 or 3	Ovoid cells	Ovoid cells	Ovoid cells
3 > 1 > 2	Filaments	Lysis	Lysis
3 > 2 > 1	Filaments	Filaments with bulges	Lysis

(Greenwood and O'Grady, 1973; Spratt, 1975; Greenwood, 1986; Hayes and Ward, 1986; Sumita, et al., 1990; Pucci, et al., 1991)

6. Time Kill Method (Synergy Detection)

The selected drug concentrations and bacteria were chosen to detect for synergy by time kill method; must be correlated with the unique killing activity and morphological changes by time kill method (single drug) and phase contrast microscopy as described previously.

6.1 Select cefpirome (act as PBP3 binder) to combine with imipenem (act as PBP2 binder) at 1/4MIC and 2MIC. The 1/4MIC was represented as the model of unsaturated PBP and the 2MIC was represented as the model of saturated PBP. Thus, they will be 7 investigated groups that consist of:

- control
- cefpirome 1/4MIC - imipenem 1/4MIC - combination 1/4MIC
- cefpirome 2MIC - imipenem 2MIC - combination 2MIC

6.2 Add the selected drugs in the specific concentration to MHB for preparing working media in the shaker bath at 37°C.

6.3 Add the standardized inoculum into working media and control to give final amount of *E. coli* quantity equivalent to 1 to 2 x 10^7 CFU/ml.

6.4 Collect specimens and detect for colony forming unit at the time 0,1,2,3,6 and 24 hours after microorganism exposed to drugs (both control and antibiotic added group).

6.5 Collect specimens at the time 0,1,2,3,6 and 24 hours after bacteria exposed to drugs in every group to observe morphological changes by phase contrast microscopy compare with the control.

6.6 Calculate the quantity of survival bacteria in each group to perform the killing curves data after incubating the specimens in appropriate solid media for 16 to 18 hours at 37°C.

6.7 Killing curves were constructed by Microsoft Excel 97 and **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)

6.8 Evaluate the killing rate combine with morphological evidence to detect for synergism and determine drugs action on PBPs.

7. <u>The Quantitative Evaluation of Antimicrobial Effect</u> (Firsov, et al., 1997)

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

$T_{90\%}$ = 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

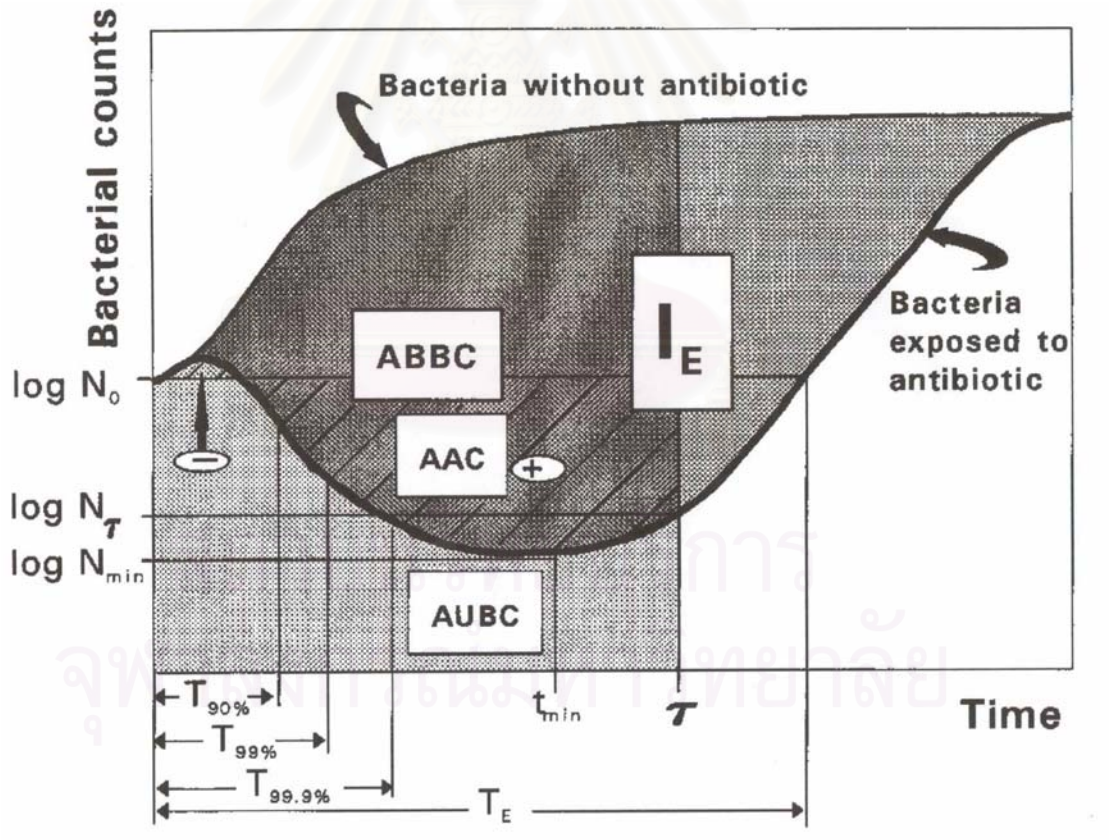


Figure 3-1 Parameters for quantitating bacterial killing and regrowth curve and the antimicrobial effect.

(Modified from Firsov, et al., 1997)

7.2 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 3-1.

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

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

ΔlogCFU 2 hours = The difference between the number of viable counts at time zero versus the number of viable counts after exposed to antimicrobial for 2 hours

ΔlogCFU 3 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

ΔlogCFU 6 hours = The difference between the number of viable counts at time zero versus the number of viable counts after exposed to antimicrobial for 6 hours

7.4 The following parameters were calculated by various methodologies as follow:

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)

CHAPTER IV

RESULTS

Broth macrodilution method, the MICs of cefpirome, cefepime, imipenem and meropenem for the selected strains were presented in table 4-1. Most of drugs had the lowest MIC for *E. coli, E. cloacae* (MIC range from 0.015-0.25 ug/ml) and *P. aeruginosa* (MIC range from 0.5-2.0 ug/ml). Furthermore, most of drugs manifested the MBC equal to MIC except for cefpirome against *E. cloacae* that exhibited MBC higher than MIC for 4 fold.

Time kill method, the different drug's concentrations are tested against Enterobacteria (1/4MIC-128MIC) and *P. aeruginosa* (1/4MIC-8MIC). The 4^{th} generation cephalosporins demonstrated bactericidal property to *E. cloacae* above 32MIC, whereas the regrowth were detected at supraMBC level as shown in figures 4-1 and 4-2. In case of carbapenems against *P. aeruginosa*, they established the MBC larger than MIC for 4 fold which equal to the concentration to suppress regrowth as shown in figures 4-3 and 4-4. Similarly, *E. coli* required the drug's concentration to inhibit regrowth and express MBC at resembled value. The 4^{th} generation cephalosporins required concentration 4MIC-8MIC to show the bactericidal property while carbapenems wanted 1MIC-2MIC to exhibit this property. Moreover, the 4^{th} generation cephalosporins could inhibit regrowth at 4MIC-32MIC, whereas carbapenems suppressed regrowth at 1MIC-2MIC (figures 4-5 to 4-8).

For the quantitative evaluation of the antimicrobial effect from bacterial killing and regrowth curves, the killing rate of the first 3 hours (KR3) and $T_{99.9\%}$ were the reflection of initial killing and the bacteriolytic area at 24 hours (BA24) was the expression of total killing of the drugs to bacteria. The KR3 from cefpirome 2MIC-64MIC against *E. cloacae* was 1.21-1.38 logCFU/hr.ml and increasing to 1.75 logCFU/hr.ml at 128MIC. Similarly, cefepime 2MIC-64MIC manifested their KR3 at 0.99-1.37 logCFU/hr.ml and elevated to 1.74 logCFU/hr.ml at 128MIC. Therefore, the $T_{99.9\%}$ of cefpirome was shorter than cefepime around 0.5-1.0 hour ($T_{99.9\%}$ of cefpirome 2MIC-64MIC = 1.44-1.82 hr and $T_{99.9\%}$ of cefepime 2MIC-64MIC = 1.77-3.06 hr). However, the

BA24 of cefpirome was less than cefepime, cefpirome demonstrated BA24 more than 50 logCFU.hr/ml at 32MIC, whereas cefepime 8MIC could express this value (tables 4-1 and 4-2).

In case of carbapenems against *P. aeruginosa*, The KR3 from imipenem 1MIC-8MIC was slightly less than meropenem at the same concentrations (imipenem 1MIC-8MIC = 0.75-0.92 logCFU/hr.ml and meropenem 1MIC-8MIC = 0.90-1.07 logCFU/hr.ml). Thus, the $T_{99.9\%}$ of imipenem 1MIC-8MIC was slightly more than meropenem 1MIC-8MIC (3.40-3.90 hour versus 2.58-3.60 hour, respectively). Interestingly, the BA24 of both drugs exhibited the similar value from 4MIC, which range from 65-80 logCFU.hr/ml as shown in table 4-3 and 4-4. For *E. coli*, the KR3 of the 4th generation cephalosporins 1MIC-16MIC was resemble at 1.4 logCFU/hr.ml and increase more than 1.5 logCFU/hr.ml above 32MIC. Moreover, the $T_{99.9\%}$ of both drugs was less than 1 hour since 64MIC. Accordingly, these drugs demonstrated the BA24 more than 80logCFU.hr/ml at 32MIC as shown in tables 4-5 and 4-6. In case of carbapenems against *E. coli*, they expressed BA24 more than 80 logCFU.hr/ml from 2MIC-4MIC and more than 100 logCFU.hr/ml from 16MIC-32MIC. The KR3 from carbapenems was larger than 1.5 logCFu/hr.ml from 1MIC and the $T_{99.9\%}$ was less than 1 hr at 4MIC as shown in tables 4-7 and 4-8.

Phase contrast microscopy, the morphological changes of *E. cloacae* after exposed to the 4th generation cephalosporins were resemble between drugs. Both of them change the rod-shaped bacteria to filamentous cells at the lowest concentration (1/4MIC). Nevertheless, the complete filamentous cells are detected at 1/2MIC for both drugs. Moreover, the bulge formations were observed from cefepime above 32MIC and the collapsing cells were occurred by cefpirome at the same concentration as shown in figures 4-9 and 4-10. Similarly, the carbapenems affected *P. aeruginosa* from the lowest concentration. The bacterial morphology was modified from rod-shape to filamentous cells. However, the complete filamentous formations were occurred at 1MIC of imipenem and 1/2MIC of meropenem. Furthermore, the ovoidal cells were detected at 4MIC of imipenem and a special morphological change called filamentous with bulbous cells could be observed in meropenem 1/2MIC-4MIC as shown in figures 4-11. For morphological changes of *E. coli*, they expressed various morphological changes from

different drugs as follow. The 4th generation cephalosporins changed the rod-shaped bacteria to filamentous cells at the lowest concentration (1/4MIC). However, the complete filamentous formation was detected at 1MIC for both drugs. Moreover, cefepime and cefpirome above 32MIC produced the bulge cells and collapsing cells, respectively (figures 4-12 and 4-13). Conversingly, the carbapenems transformed the rod-shaped bacteria to ovoidal cells at the lowest concentration (1/4MIC) and the complete ovoidal cells were observed at 1/2MIC of imipenem and 1MIC of meropenem. Furthermore, the filamentous cells were detected in meropenem 32MIC or above (figures 4-14 and 4-15).

For synergy detection, we chose imipenem 1/4MIC as the unsaturated model of PBP2 in combination with cefpirome 1/4MIC as the unsaturated model of PBP3. Moreover, imipenem 2MIC was chosen as the saturated model of PBP2 in combination with cefpirome 2MIC as the saturated model of PBP3. The morphological changes of 1/4MIC combination showed incomplete ovoidal cells and incomplete filamentous cells at 2 hours of exposure. Accordingly, the morphological changes of 2MIC combination exhibited complete ovoidal cells and complete filamentous cells as shown in figure 4-18. For KR3, they were not any differences between two combinations. The 1/4MIC combination and 2MIC combination expressed killing rate at 1.45 logCFU/hr.ml and 1.47 logCFU/hr.ml, respectively. Furthermore, these killing rates were not differ from imipenem alone at 2MIC which showed killing rate at 1.45 logCFU/hr.ml. However, they had the difference in $T_{99.9\%}$: the $T_{99.9\%}$ of 1/4MIC combination was 2.04 hour, $T_{99.9\%}$ of 2MIC combination was 1.58 hour and the $T_{99.9\%}$ of imipenem 2MIC was 1.15 hour as shown in table 4-9. For synergy determination, the synergism was detected at 1/4MIC combination with 4.32 logCFU/ml decreasing at 24 hours more than the most active single drug (imipenem 1/4MIC). However, the regrowth was detected in both combinations as shown in figure 4-16.

Furthermore, a special time kill procedure was conducted to determine the difference between the saturation model of PBP2 and PBP3 which resulted in the distinction of killing kinetics and regrowth parameters as follow:

Drugs	PBPs status
imipenem 1/4MIC+cefpirome 1/4MIC	(unsaturation model of PBP2 and PBP3)
meropenem 4MIC	(saturation model of PBP2 but not PBP3)
meropenem 32MIC	(saturation model of PBP2 and PBP3)
imipenem 2MIC+cefpirome 2MIC	(saturation model of PBP2 and PBP3)
cefepime 128MIC	(saturation model of PBP2 and PBP3 and may be PBP1)

It was found that the KR3 did not differ among these groups which ranging between 1.45-1.55 logCFU/hr.ml except for meropenem 4MIC which demonstrated killing rate at 1.33 logCFU/hr.ml ($p<0.05$). Accordingly, the $T_{99.9\%}$ among groups were between 1.5-1.7 hour except for the 1/4MIC combination that exhibited $T_{99.9\%}$ at 2.04 hr ($p<0.05$) and meropenem 32MIC was 0.96 hr ($p<0.05$). However, the greatest bacteriolytic area of 24 hours expressed by cefepime 128MIC which was 101.82 logCFU.hr/ml ($p<0.05$ compared with the other groups that range between 65-85 logCFU.hr/ml). Moreover, the regrowth was detected in every group except for cefepime 128MIC as shown in table 4-10 and figure 4-17.

Table 4-1 The MICs and MBCs of selected β–lactam antibiotics to tested bacteria.

Drugs	Bacteria	MIC (ug/ml)	MBC (ug/ml)
Cefpirome	*Escherichia coli* 271	0.12	0.12
Cefpirome	*Enterobacter cloacae* 113	0.06	0.25
Cefepime	*Escherichia coli* 271	0.06	0.06
Cefepime	*Enterobacter cloacae* 113	0.06	0.06
Imipenem	*Escherichia coli* 271	0.25	0.25
Imipenem	*Pseudomonas aeruginosa* 396	2	2
Meropenem	*Escherichia coli* 271	0.015	0.015
Meropenem	*Pseudomonas aeruginosa* 396	0.5	1

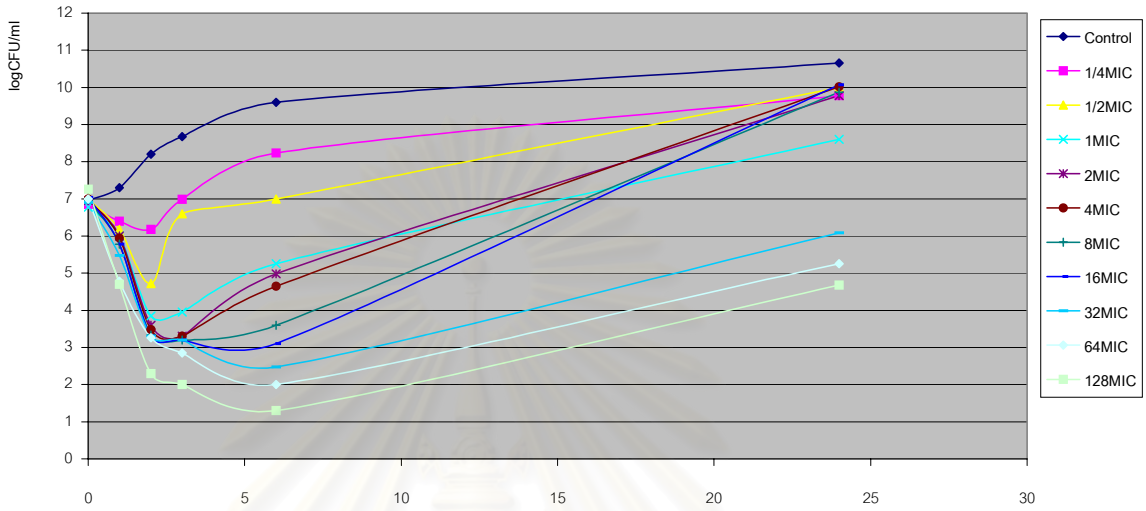


Figure 4-1 The killing curves of cefpirome against *Enterobacter cloacae*

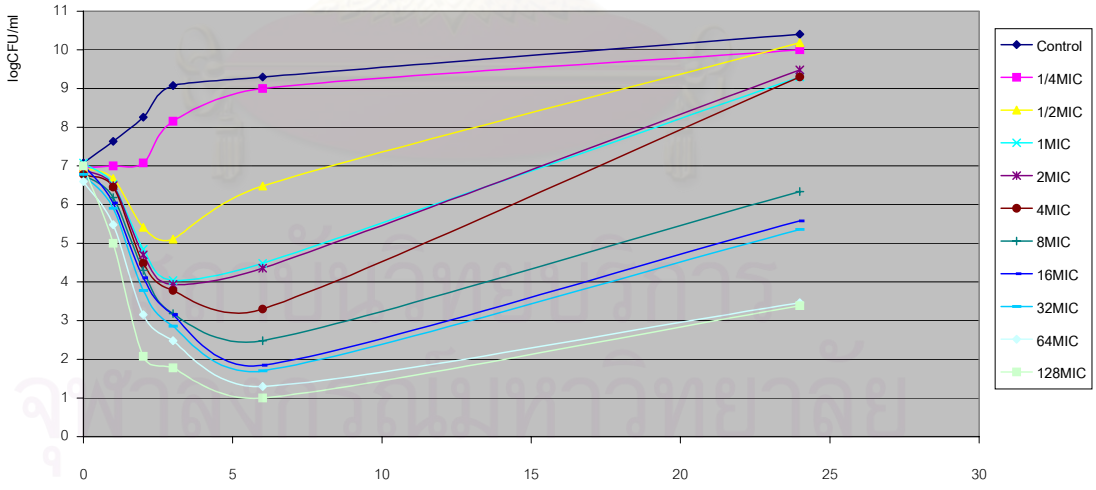


Figure 4-2 The killing curves of cefepime against *Enterobacter cloacae*

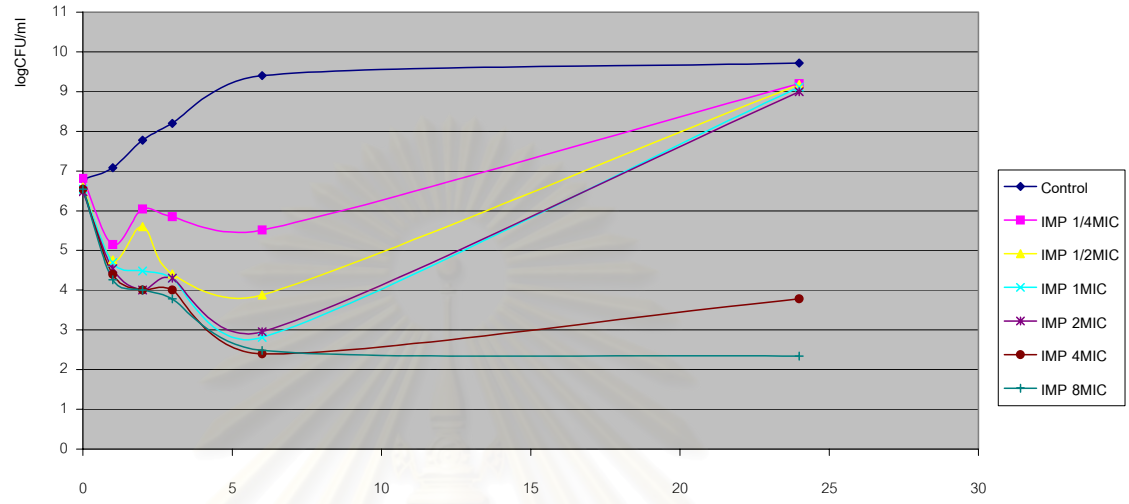


Figure 4-3 The killing curves of imipenem against *Pseudomonas aeruginosa*

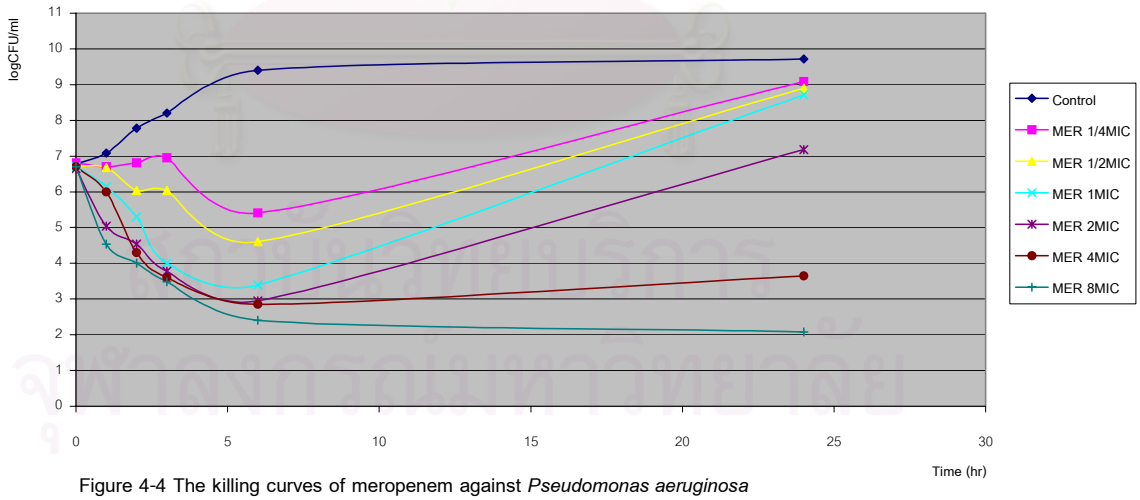


Figure 4-4 The killing curves of meropenem against *Pseudomonas aeruginosa*

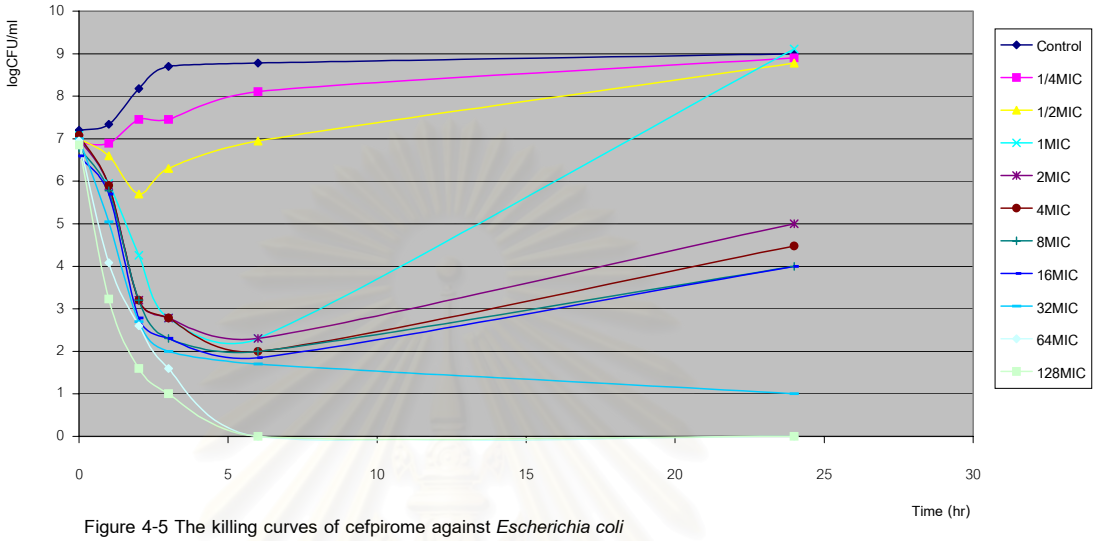


Figure 4-5 The killing curves of cefpirome against *Escherichia coli*

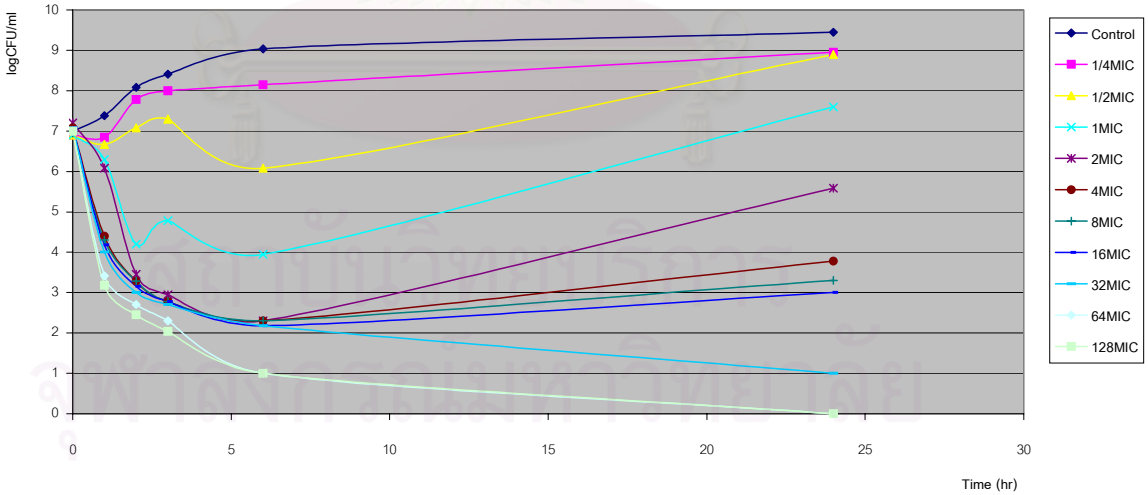


Figure 4-6 The killing curves of cefepime against *Escherichia coli*

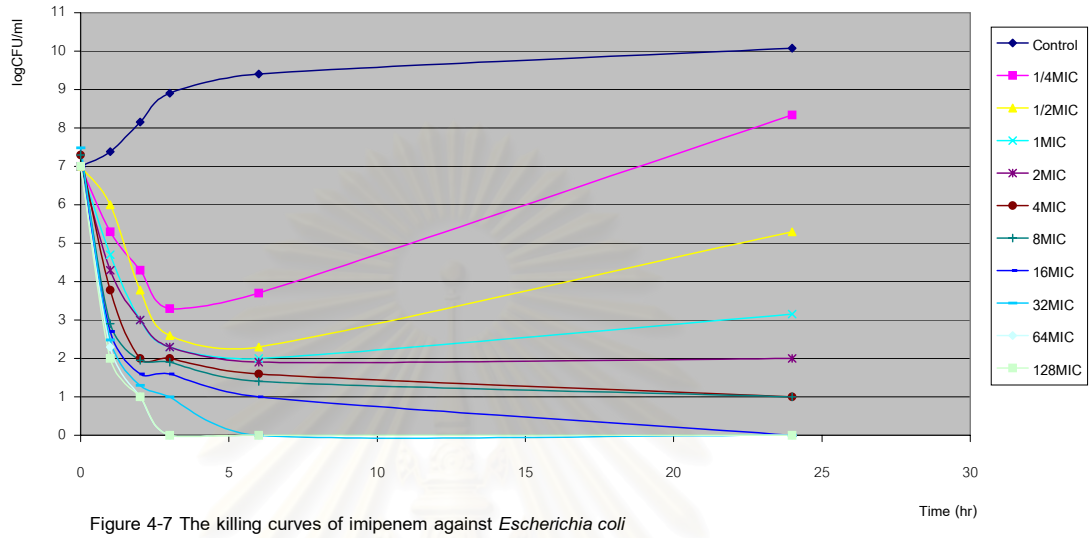


Figure 4-7 The killing curves of imipenem against *Escherichia coli*

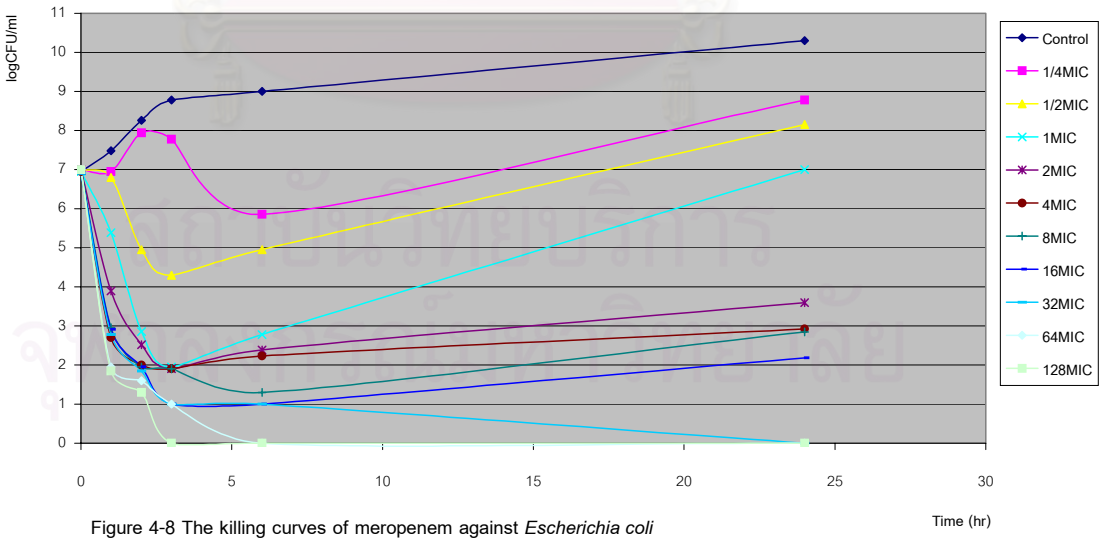


Figure 4-8 The killing curves of meropenem against *Escherichia coli*

Table 4-2 The killing kinetics and regrowth parameters of cefpirome against *Enterobacter cloacae*

Parameter\Conc	Control	1/4MIC	1/2MIC	1MIC	2MIC	4MIC	8MIC	16MIC	32MIC	64MIC	128MIC
T90%	-1.72	-	1.13	1.13	1.03	0.96	0.88	0.84	0.74	0.43	0.4
T99%	-3.36	-	1.66	1.53	1.42	1.38	1.33	1.3	1.27	0.87	0.8
T99.9%	-11	-	-	2.1	1.82	1.74	1.69	1.66	1.66	1.44	1.18
T*E*	-	2.83	6	12.9	13.2	13.8	15.7	16.3	>24	>24	>24
Tmin	0	2	2	2	3	3	3	6	6	6	6
LogNmin	6.95	6.18	4.72	3.85	3.3	3.3	3.2	3.11	2.48	2	1.3
δlogNmin	0	0.67	2.28	2.93	3.65	3.7	3.75	3.93	4.42	5	5.96
T	8	8	8	8	8	8	8	8	8	8	8
logN*T*	9.78	8.46	7.34	5.7	5.56	5.28	4.28	3.85	2.81	2.28	1.6
δlogN2	-1.25	0.67	2.28	2.93	3.35	3.52	3.61	3.74	3.6	3.74	4.95
δlogN3	-1.73	-0.15	0.4	2.82	3.65	3.7	3.75	3.84	3.7	4.15	5.26
δlogN6	-2.65	-1.39	0	1.52	1.98	2.37	3.35	3.93	4.43	5	5.95
Killing rate3	-0.58	-0.05	.13*	0.94	1.21	1.23	1.25	1.28	1.23	1.38	1.75*
AUC24	123.03	110.81	110.65	103.13	103.87	105.51	102.61	104.04	67.51	59.25	52.47
Bacteriolytic area24	0	12.22*	12.38*	19.9	19.16	17.52	20.42	18.99	55.52*	63.78*	70.56*

*p<0.05

Table 4-3 The killing kinetics and regrowth parameters of cefepime against *Enterobacter cloacae*

Parameter\Conc	Control	1/4MIC	1/2MIC	1MIC	2MIC	4MIC	8MIC	16MIC	32MIC	64MIC	128MIC
T90%	-1.7	-	1.56	1.3	1.35	1.37	1.25	1	1.07	0.92	0.52
T99%	-3	-	-	1.85	1.84	1.82	1.73	1.5	1.53	1.36	1
T99.9%	-18	-	-	2.9	3.06	3	2.42	2.05	2	1.77	1.33
T*E*	-	0	8	16	14.9	16.6	>24	>24	>24	>24	>24
Tmin	0	0	3	3	3	6	6	6	6	6	6
logNmin	7.08	6.95	5.11	4.04	3.93	3.3	2.48	1.85	1.7	1.3	1
ϭlogNmin	0	0	1.84	3.04	2.97	3.48	4.3	4.86	5.08	5.3	6
T	8	8	8	8	8	8	8	8	8	8	8
logN*T*	9.43	9.18	6.95	5	4.9	3.88	2.85	2.18	2	1.45	1.23
ϭlogN2	-1.18	-0.12	1.54	2.23	2.2	2.3	2.48	2.93	3	3.46	4.92
ϭlogN3	-2	-1.19	1.84	3.04	2.97	3	3.6	3.9	3.92	4.12	5.22
ϭlogN6	-2.22	-2.05	0.48	2.6	2.54	3.48	4.3	5.2	5.08	5.3	6
Killing rate3	-0.67	-0.4	0.61	1.01	0.99	1	1.2	1.3	1.31	1.37	1.74*
AUC24	120.75	115.36	111.05	99.93	101.19	98.54	70.66	63.61	60.86	42.63	40.34
Bacteriolytic area24	0	5.39*	9.7*	20.82	19.56	22.21	50.09	57.17	59.89	78.12*	80.41*

*p<0.05

Table 4-4 The killing kinetics and regrowth parameters of imipenem against *Pseudomonas aeruginosa*

Parameter\Conc	Control	1/4MIC	1/2MIC	1MIC	2MIC	4MIC	8MIC
T90%	-2	0.5	0.45	0.55	0.48	0.44	0.39
T99%	-4	-	2.7	1.25	1.05	0.9	0.83
T99.9%	>24	-	-	3.74	3.9	3.5	3.4
T*E*	-	12.7	15.5	17	16.7	>24	-
Tmin	0	1	6	6	6	6	24
logNmin	6.78	5.15	3.88	2.81	2.95	2.4	2.34
ɓlogNmin	0	1.66	2.72	3.73	3.53	4.14	4.2
T	6	6	6	6	6	6	6
logN*T*	9.4	5.51	3.88	2.81	2.95	2.4	2.48
ɓlogN2	-1	0.77	1	2.07	2.48	2.54	2.54
ɓlogN3	-1.43	0.97	2.19	2.24	2.18	2.54	2.77
ɓlogN6	-2.62	1.31	2.73	3.73	3.52	4.15	4.07
Killing rate3	-0.48	.32*	0.73	0.75	0.73	0.85	0.92*
AUC24	114.85	101.44	98.31	97.1	95.29	47.87	37.73
Bacteriolytic area24	0	13.41*	16.54*	17.75*	19.56	66.98*	77.12*

*p<0.05

Table 4-5 The killing kinetics and regrowth parameters of meropenem against *Pseudomonas aeruginosa*

Parameter\Conc	Control	1/4MIC	1/2MIC	1MIC	2MIC	4MIC	8MIC
T90%	-2	4.2	3.4	1.6	0.6	0.45	0.43
T99%	-4	-	4.7	2.47	1.8	1.33	0.9
T99.9%	>24	-	-	3.6	3.2	2.86	2.58
T*E*	-	13.6	15.3	17.4	21.7	>24	-
Tmin	0	6	6	6	6	6	24
logNmin	6.78	5.41	4.6	3.4	2.95	2.85	2.08
∂logNmin	0	1.4	2.1	3.3	3.7	3.85	4.62
T	8	8	8	8	8	8	8
logN*T*	9.48	5.7	4.98	3.9	3.34	2.93	2.3
∂logN2	-1	0	0.66	1.4	2.11	2.4	2.7
∂logN3	-1.43	-0.14	0.66	2.7	2.88	3.1	3.22
∂logN6	-2.62	1.4	2.1	3.3	3.7	3.85	4.3
Killing rate3	-0.48	-0.05	.22*	0.9	.96*	1.03*	1.07*
AUC24	114.85	102.86	99.28	93.75	78.58	46.96	36.12
Bacteriolytic area24	0	11.99*	15.57*	21.1	36.27	67.89*	78.73*

*p<0.05

Table 4-6 The killing kinetics and regrowth parameters of cefpirome against *Escherichia coli*

Parameter\Conc	Control	1/4MIC	1/2MIC	1MIC	2MIC	4MIC	8MIC	16MIC	32MIC	64MIC	128MIC
T90%	2.03	-	1.58	0.9	0.94	0.9	1	1	0.52	0.33	0.26
T99%	>24	-	-	1.5	1.34	1.3	1.4	1.37	1.02	0.66	0.52
T99.9%	>24	-	-	2.1	1.67	1.64	1.73	1.67	1.42	1.06	0.78
T*E*	-	1.06	6.3	18.8	>24	>24	>24	>24	-	-	-
Tmin	0	1	2	6	6	6	6	6	24	24	24
logNmin	7.2	6.89	5.7	2.3	2.3	2	2	1.85	1	0	0
ϭlogNmin	0	0.01	1.3	4.78	4.7	5.08	4.78	4.75	6	6.95	6.85
T	12	12	12	12	12	12	12	12	12	12	12
logN*T*	7.85	8.43	7.59	4.45	3.02	2.74	2.6	2.52	2.46	0	0
ϭlogN2	-0.97	-0.54	1.3	2.82	3.8	3.88	3.57	3.82	4.3	4.35	5.24
ϭlogN3	-1.49	-0.54	0.7	4.3	4.22	4.3	4.48	4.3	5	5.35	5.85
ϭlogN6	-1.57	-1.21	-0.05	4.78	4.7	5.08	4.78	4.76	5.3	6.95	6.85
Killing rate3	-0.5	-0.18	.23*	1.43	1.41	1.43	1.49	1.43	1.67	1.78*	1.95*
AUC24	108.72	104.27	99.72	95.05	57.5	52.75	47.69	43.71	26.67	11.98	10.16
Bacteriolytic area24	0	4.45*	9*	13.67*	51.22	55.97	61.03	61.41	82.05*	96.74*	98.56*

*p<0.05

Table 4-7 The killing kinetics and regrowth parameters of cefepime against *Escherichia coli*

Parameter\Conc	Control	1/4MIC	1/2MIC	1MIC	2MIC	4MIC	8MIC	16MIC	32MIC	64MIC	128MIC
T90%	-1.85	-	-	1.22	0.93	0.37	0.34	0.32	0.3	0.25	0.23
T99%	-5.44	-	-	1.61	1.35	0.7	0.69	0.65	0.6	0.5	0.47
T99.9%	>24	-	-	-	1.7	1.2	1.18	1.05	0.98	0.7	0.64
T*E*	-	1.1	1.6	20.5	>24	>24	>24	>24	-	-	-
Tmin	0	1	6	6	6	6	6	6	24	24	24
logNmin	7	6.85	6.08	3.95	2.3	2.3	2.3	2.18	1	0	0
δlogNmin	0	0.05	0.82	2.95	4.9	4.73	4.65	4.9	6.08	7	7
T	12	12	12	12	12	12	12	12	12	12	12
logN*T*	9.26	8.43	7.9	5.08	2.3	2.74	2.6	2.41	1.78	0.6	0.48
δlogN2	-1.08	-0.88	-0.18	2.7	3.76	3.8	3.72	3.93	4.08	4.3	4.55
δlogN3	-1.41	-1.1	-0.4	2.12	4.25	4.3	4.18	4.3	4.38	4.7	4.96
δlogN6	-2.04	-1.2	0.82	2.95	4.9	4.78	4.48	4.9	4.9	6	6
Killing rate3	-0.47	-0.37	-0.13	0.71	1.42	1.43	1.39	1.43	1.46	1.57	1.65*
AUC24	112.16	105.32	102.03	84.77	63.22	45.93	41.76	39.16	31.21	19.52	18.92
Bacteriolytic area24	0	6.84*	10.13*	27.39*	48.94	66.23	70.4	73	80.95*	92.64*	93.24*

*p<0.05

Table 4-8 The killing kinetics and regrowth parameters of imipenem against *Escherichia coli*

Parameter\Conc	Control	1/4MIC	1/2MIC	1MIC	2MIC	4MIC	8MIC	16MIC	32MIC	64MIC	128MIC
T90%	-1.8	0.56	0.47	0.42	0.34	0.27	0.22	0.16	0.18	0.19	0.17
T99%	-3.3	1.25	1	0.85	0.7	0.53	0.41	0.42	0.36	0.39	0.36
T99.9%	-21.6	2.3	1.8	1.36	1.18	0.83	0.62	0.63	0.54	0.58	0.54
T*E*	-	20	>24	>24	>24	-	-	-	-	-	-
Tmin	0	3	5.3	6	6	24	24	24	6	3	3
logNmin	7	3.3	2.28	2	1.9	1	1	0	0	0	0
ϭlogNmin	0	3.7	4.72	5	5.1	6.3	6.3	7	7.48	7	7
T	8	8	8	8	8	8	8	8	8	8	8
logN*T*	9.48	4.23	2.6	2.11	1.89	1.59	1.48	1	0	0	0
ϭlogN2	-1.15	2.7	3.22	4	4	5.3	5.35	5.4	6.18	6	6
ϭlogN3	-1.9	3.7	4.4	4.7	4.7	5.3	5.4	5.4	6.48	7	7
ϭlogN6	-2.4	3.3	4.7	5	5.1	5.7	5.82	6	7.48	7	7
Killing rate3	-0.63	1.23*	1.47*	1.57	1.57	1.77	1.8	1.8	2.16*	2.33*	2.33*
AUC24	118.14	89.19	59.79	39.69	31.17	25.2	23.72	17.74	10	8.34	8.2
Bacteriolytic area24	0	28.95*	58.35*	78.45	86.97	92.94	94.42	100.4	108.14*	109.80*	109.94*

*p<0.05

Table 4-9 The killing kinetics and regrowth parameters of meropenem against *Escherichia coli*

Parameter\Conc	Control	1/4MIC	1/2MIC	1MIC	2MIC	4MIC	8MIC	16MIC	32MIC	64MIC	128MIC
T90%	-1.6	4.8	1.47	0.68	0.3	0.27	0.24	0.23	0.22	0.19	0.18
T99%	-5.4	-	1.96	1.18	0.6	0.5	0.45	0.45	0.43	0.36	0.35
T99.9%	-19.3	-	-	1.54	0.95	0.78	0.68	0.68	0.68	0.53	0.52
T*E*	-	14	17.5	23.7	>24	>24	>24	>24	-	-	-
Tmin	0	6	3	3	3	3	6	3	24	24	24
logNmin	6.95	5.86	4.3	4.3	1.9	1.9	1.3	1	0	0	0
ϭlogNmin	0	1.14	2.7	2.7	5.1	5.05	5.7	5.9	6.9	7	7
T	12	12	12	12	12	12	12	12	12	12	12
logN*T*	9.43	6.85	6.04	4.2	2.8	2.3	1.78	1.38	0.7	0	0
ϭlogN2	-1.3	-0.94	2.05	4.1	4.48	4.95	5.05	4.95	5.06	5.4	5.7
ϭlogN3	-1.82	-0.78	2.7	5	5.1	5.05	5.1	5.9	5.9	6	7
ϭlogN6	-2.05	1.14	2.05	4.18	4.62	4.72	5.7	5.9	5.9	7	7
Killing rate3	-0.61	-0.26	0.9*	1.67	1.7	1.68	1.7	1.97	1.97	2	2.33*
AUC24	119.12	102.4	90.48	74.88	43.18	36.64	34.84	27.72	17.13	9.68	8.3
Bacteriolytic area24	0	16.72*	28.64*	44.24*	75.94	82.48	84.28	91.4	101.99*	109.44*	110.82*

*p<0.05

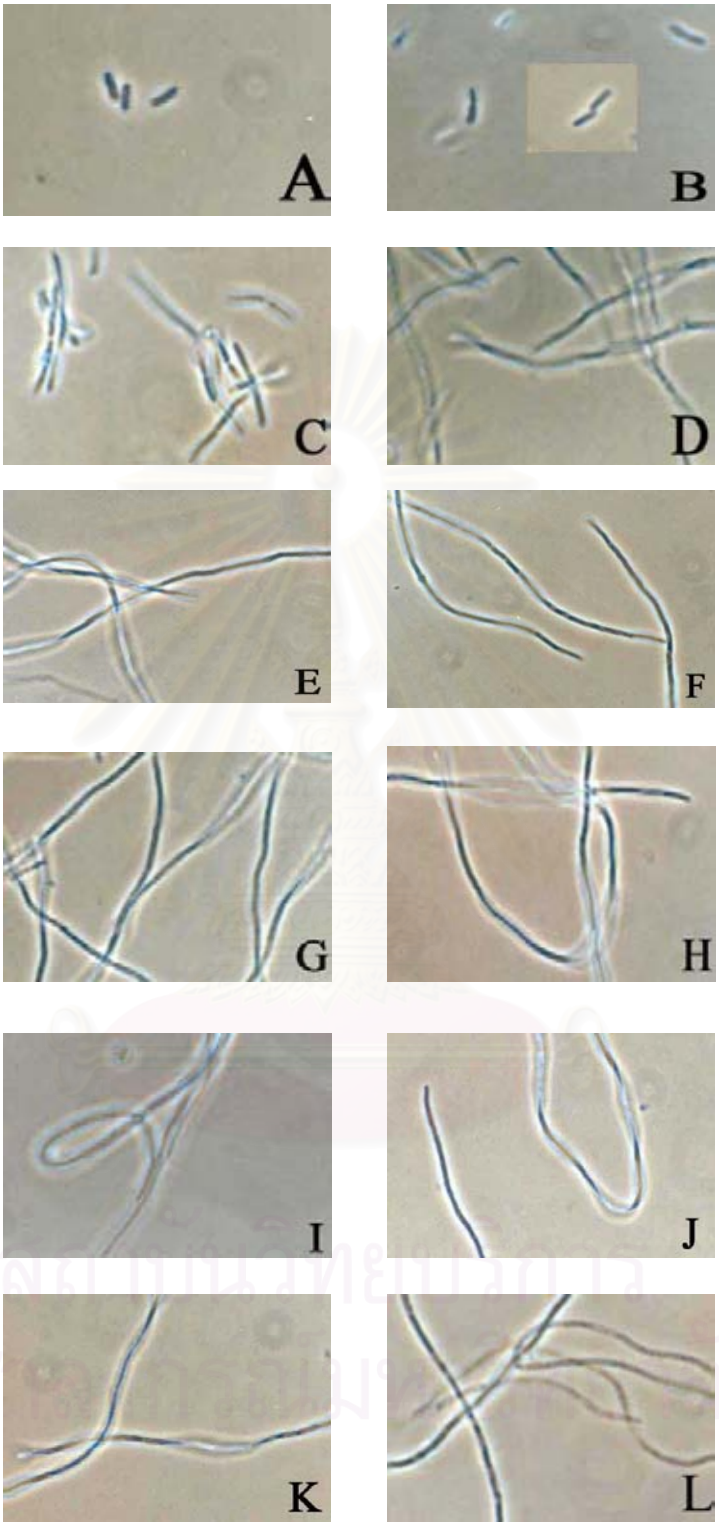


Figure 4-9 The morphological changes of *Enterobacter cloacae* after exposed to cefpirome concentration range from ¼MIC-128MIC under phase contrast microscopy (100x solution); A.control-to, B.control-t2, C.1/4MIC-t2, D.1/2MIC-t2, E.1MIC-t2, F.2MIC-t2, G.4MIC-t2, H.8MIC-t2, I.16MIC-t2, J.32MIC-t2, K.64MIC-t2, L.128MIC-t2.

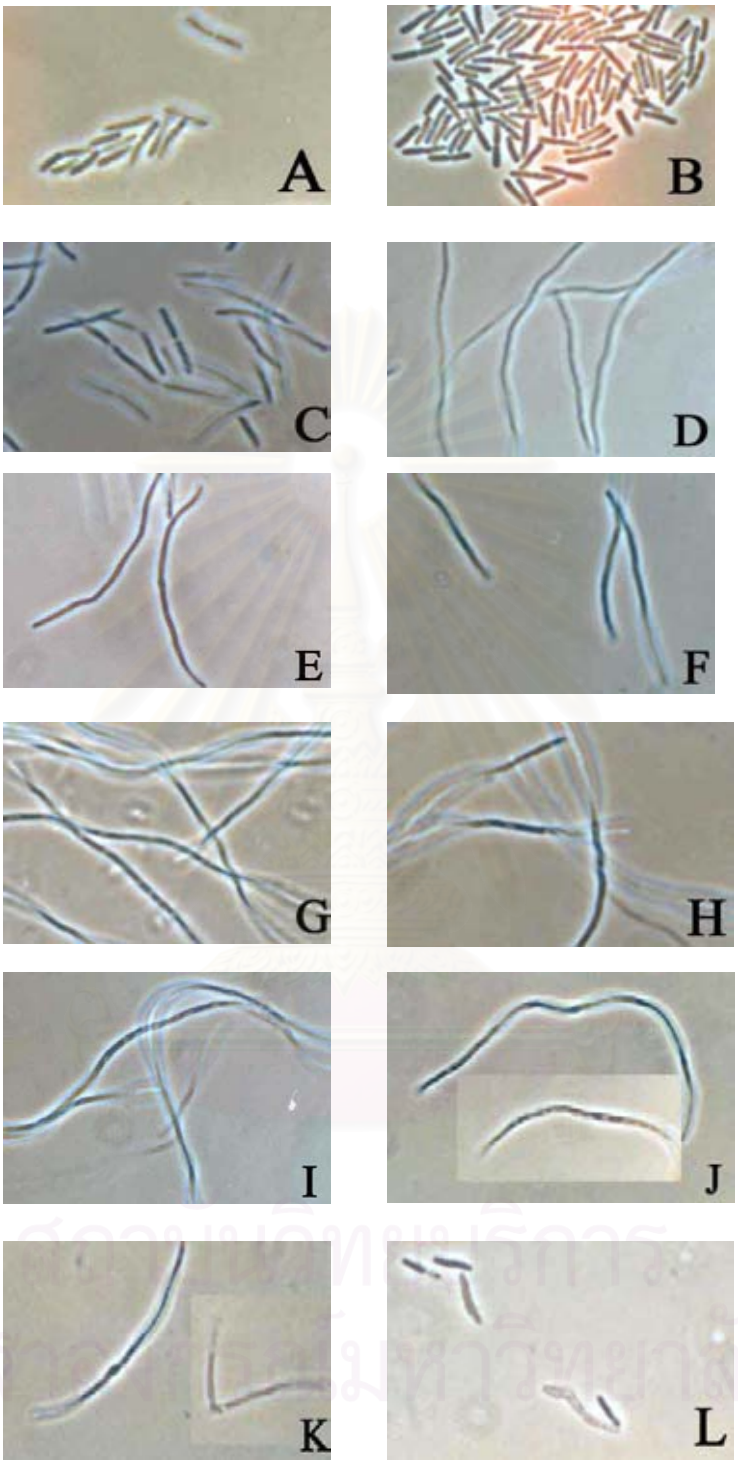


Figure 4-10 The morphological changes of *Enterobacter cloacae* after exposed to cefepime concentration range from ¼MIC-128MIC under phase contrast microscopy (100x solution); A.control-to, B.control-t2, C.1/4MIC-t2, D.1/2MIC-t2, E.1MIC-t2, F.2MIC-t2, G.4MIC-t2, H.8MIC-t2, I.16MIC-t2, J.32MIC-t2, K.64MIC-t2, L.128MIC-t2.

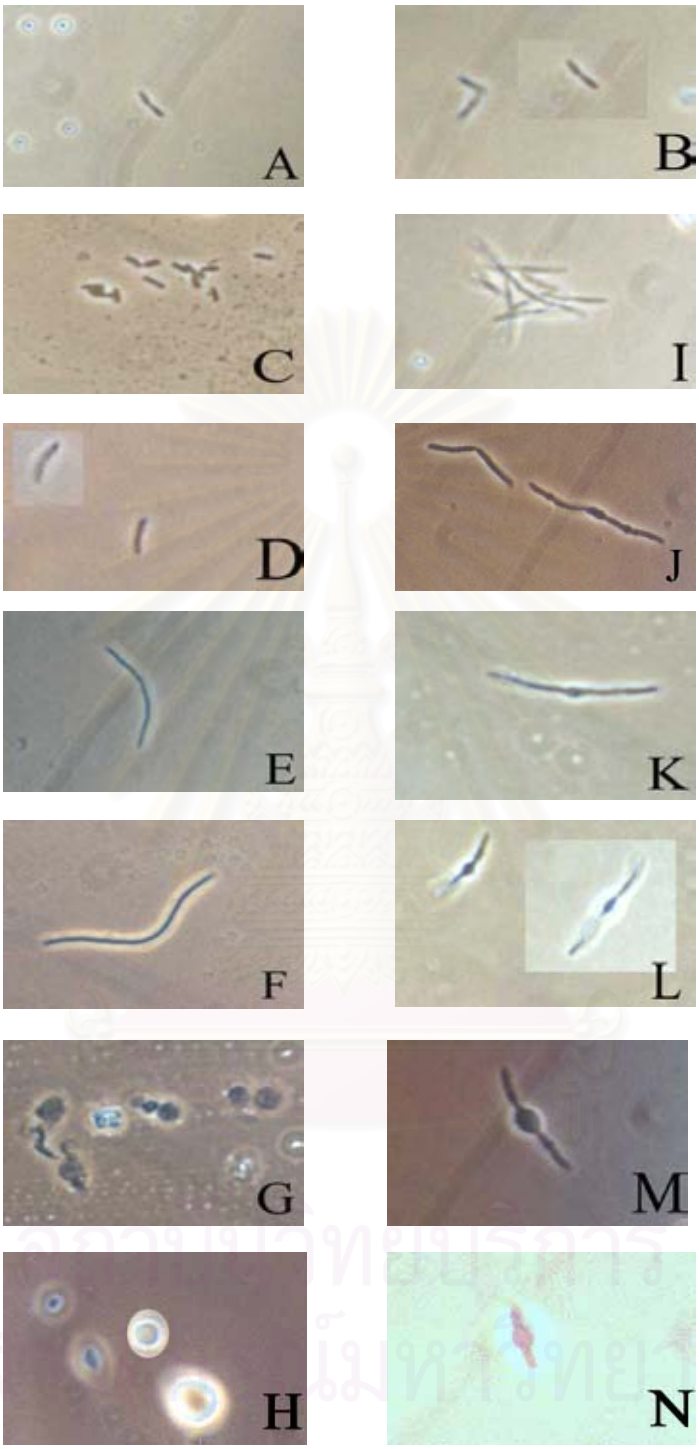


Figure 4-11 The morphological changes of *Pseudomonas aeruginosa* after exposed to imipenem and meropenem concentration range from 1/4MIC-8MIC under phase contrast microscopy (100x solution); A.control-to, B.control-t2, C.Imp1/4MIC-t2, D.Imp1/2MIC-t2, E.Imp1MIC-t2, F.Imp2MIC-t2, G.Imp4MIC-t2, H.Imp8MIC-t2, I.Mer1/4MIC-t2, J.Mer1/2MIC-t2, K.Mer1MIC-t2, L.Mer2MIC-t2, M.Mer4MIC-t2, N.Mer8MIC-t2.

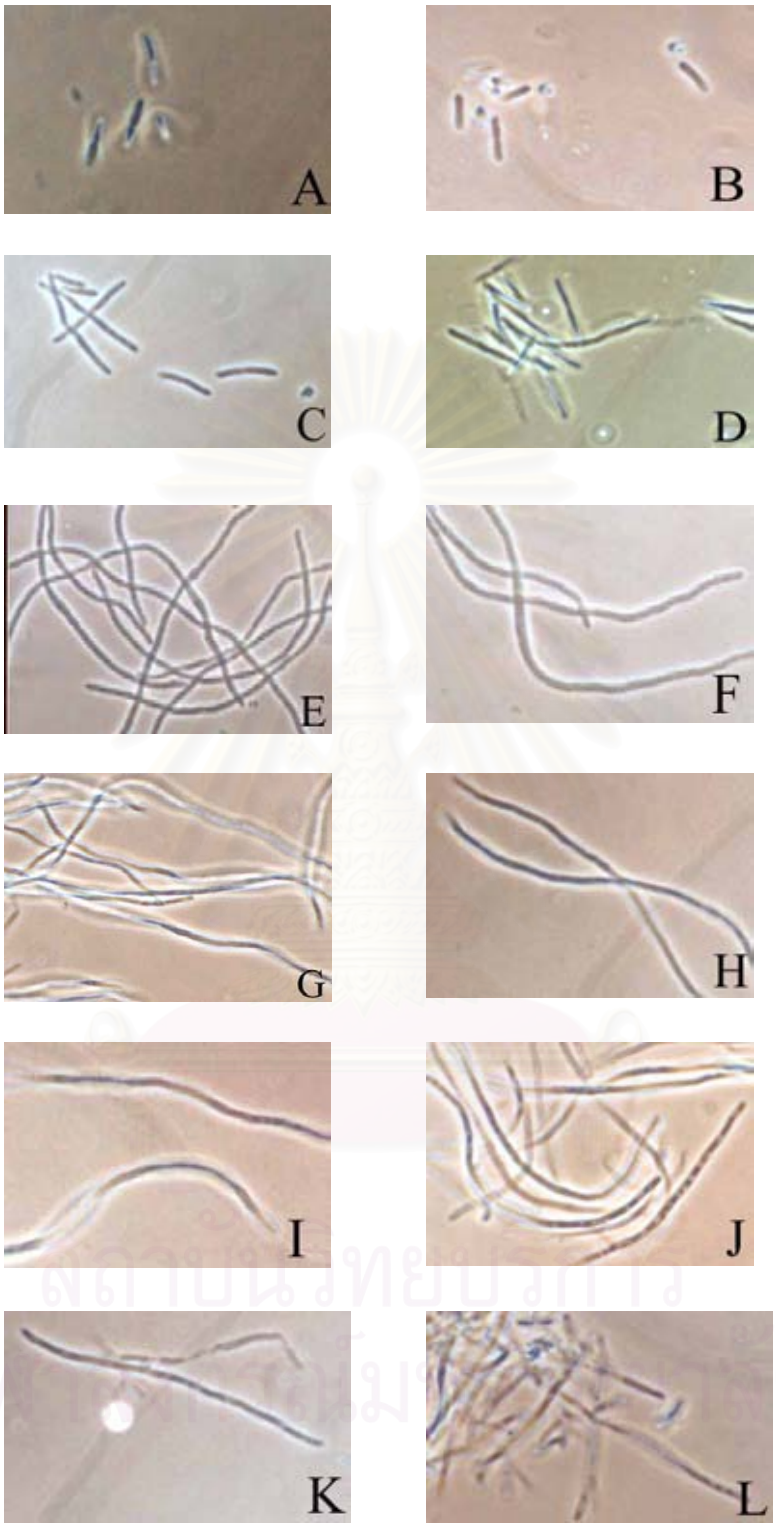


Figure 4-12 The morphological changes of *Escherichia coli* after exposed to cefpirome concentration range from ¼MIC-128MIC under phase contrast microscopy (100x solution); A.control-to, B.control-t2, C.1/4MIC-t2, D.1/2MIC-t2, E.1MIC-t2, F.2MIC-t2, G.4MIC-t2, H.8MIC-t2, I.16MIC-t2, J.32MIC-t2, K.64MIC-t2, L.128MIC-t2.

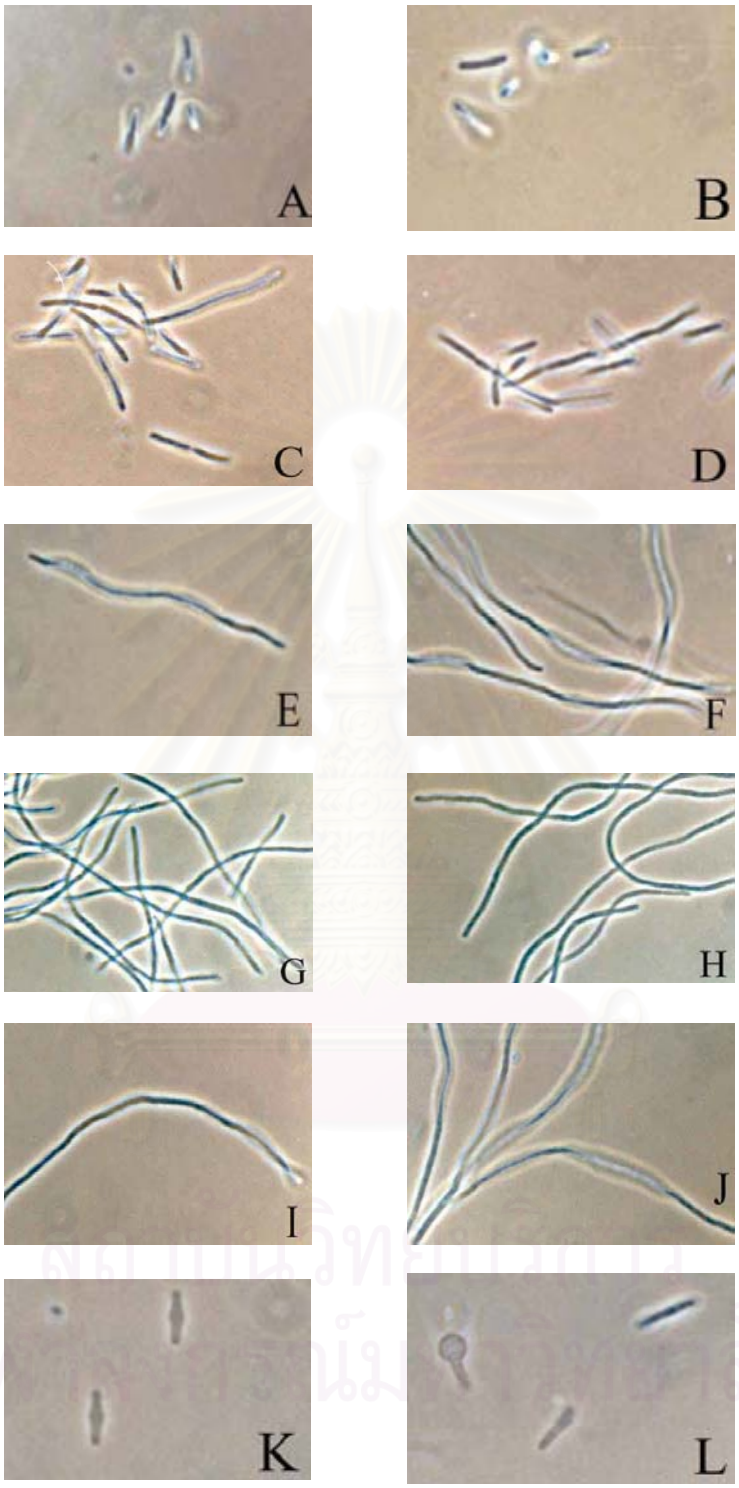


Figure 4-13 The morphological changes of *Escherichia coli* after exposed to cefepime concentration range from ¼MIC-128MIC under phase contrast microscopy (100x solution); A.control-to, B.control-t2, C.1/4MIC-t2, D.1/2MIC-t2, E.1MIC-t2, F.2MIC-t2, G.4MIC-t2, H.8MIC-t2, I.16MIC-t2, J.32MIC-t2, K.64MIC-t2, L.128MIC-t2.

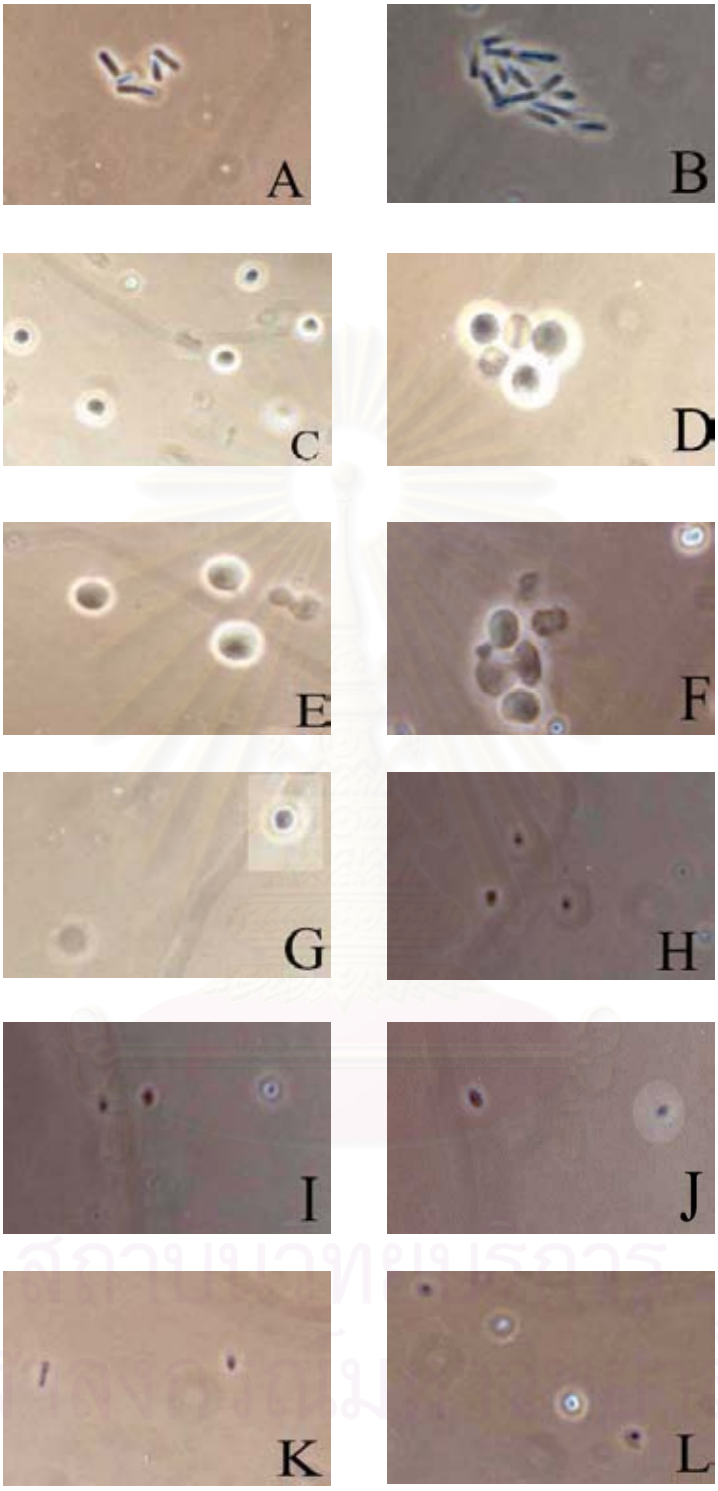


Figure 4-14 The morphological changes of *Escherichia coli* after exposed to imipenem concentration range from ¼MIC-128MIC under phase contrast microscopy (100x solution); A.control-to, B.control-t2, C.1/4MIC-t2, D.1/2MIC-t2, E.1MIC-t2, F.2MIC-t2, G.4MIC-t2, H.8MIC-t2, I.16MIC-t2, J.32MIC-t2, K.64MIC-t2, L.128MIC-t2.

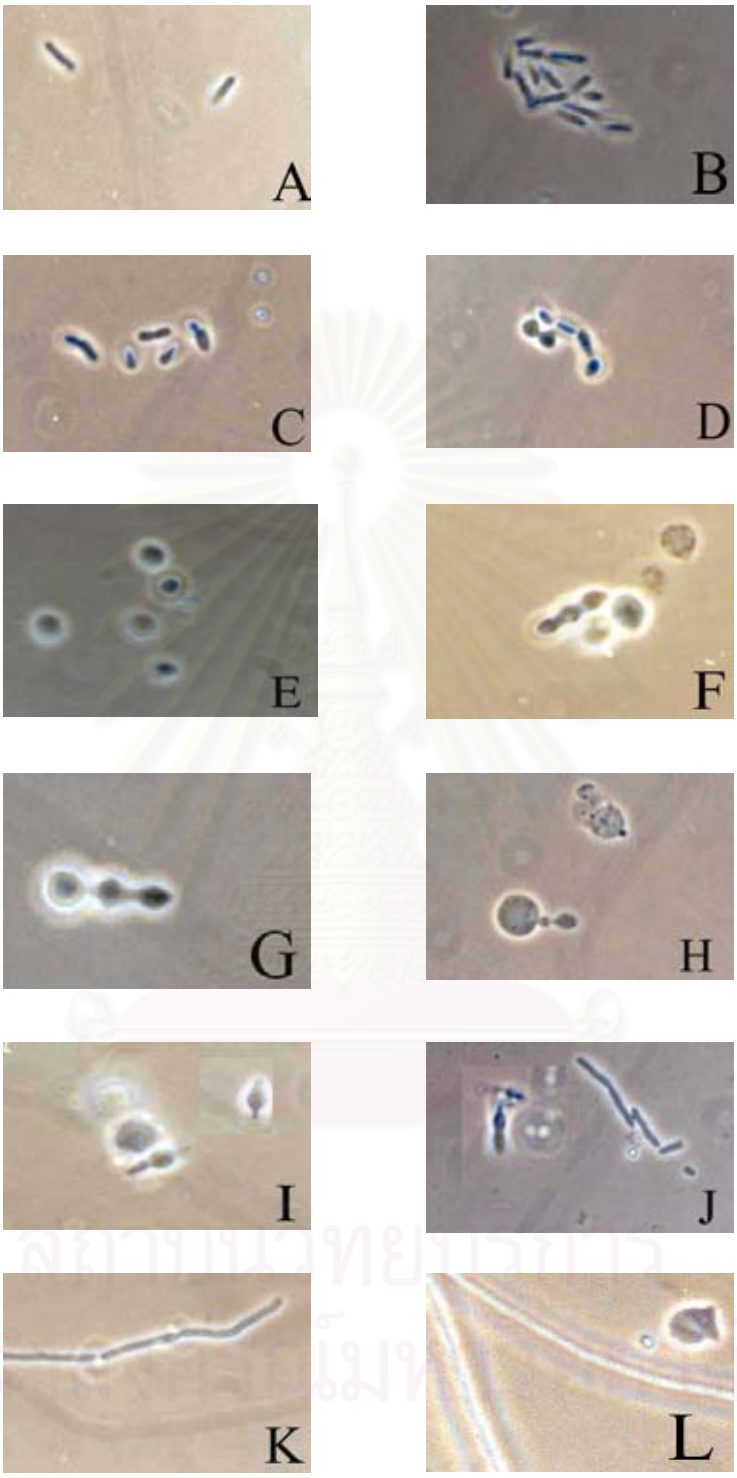


Figure 4-15 The morphological changes of *Escherichia coli* after exposed to meropenem concentration range from ¼MIC-128MIC under phase contrast microscopy (100x solution); A.control-to, B.control-t2, C.1/4MIC-t2, D.1/2MIC-t2, E.1MIC-t2, F.2MIC-t2, G.4MIC-t2, H.8MIC-t2, I.16MIC-t2, J.32MIC-t2, K.64MIC-t2, L.128MIC-t2.

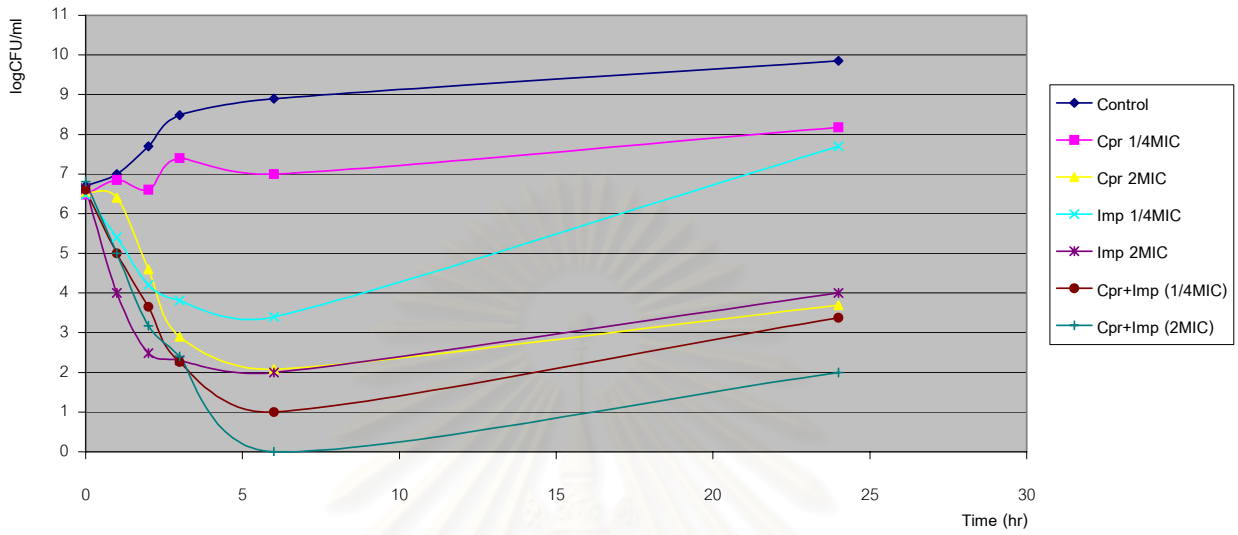


Figure 4-16 The killing curves of synergy detection between the combination of cefpirome and imipenem against *Escherichia coli*

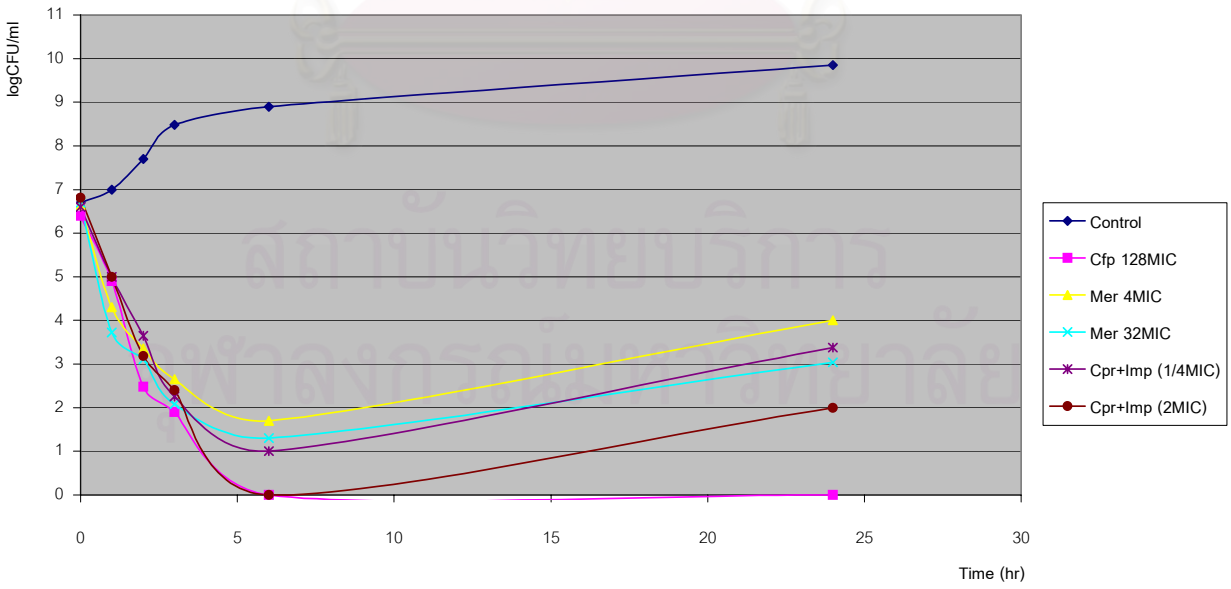


Figure 4-17 The killing curves of PBP2-3 attackers against *Escherichia coli*

Table 4-10 The killing kinetics and regrowth parameters of the combination between imipenem and cefpirome against *Escherichia coli*

Parameter\Conc	Control	CPRQ	IMPQ	CPR2	IMP2	COMQ	COM2
T90%	2	-	0.94	1.5	0.65	0.75	0.75
T99%	4.2	-	1.72	2	0.82	1.26	1.08
T99.9%	21	-	4	2.63	1.15	2.04	1.58
T*E*	-	2.2	19	>24	>24	>24	>24
Tmin	0	0	6	6	6	6	6
logNmin	6.7	6.48	3.38	2.08	2	1	0
∂logNmin	0	0	3.1	4.46	4.65	5.66	6.81
T	8	8	8	8	8	8	8
logN*T*	9.02	7.11	3.81	2.23	2.18	2.2	0.08
∂logN2	-1	-0.12	2.27	1.94	4.18	2.95	3.64
∂logN3	-1.78	-0.92	2.66	3.64	4.35	4.35	4.41
∂logN6	-2.2	-0.52	3.1	4.46	4.65	5.6	6.81
Killing rate3	-0.59	-0.31	0.87	1.21	1.45	1.45	1.47
AUC24	114.34	95.91	83.36	46.8	46.45	41.52	29.17
Bacteriolytic area24	0	18.43*	30.98	67.54	67.89	72.82	85.17*

*p<0.05

Table 4-11 The killing kinetics and regrowth parameters of the PBP2-3 attackers against *Escherichia coli*

Parameter\Conc	Control	CFP128	MER4	MER32	COMQ	COM2
T90%	-2	0.3	0.3	0.27	0.75	0.76
T99%	-4.1	1.22	0.73	0.58	1.27	1.08
T99.9%	-21	1.58	1.67	0.96	2.04	1.58
T*E*	-	-	>24	>24	>24	>24
Tmin	0	24	6	6	6	6
logNmin	6.7	0	1.7	1.3	1	0
ϭlogNmin	0	6.4	4.95	5.4	5.6	6.81
T	12	12	12	12	12	12
logN*T*	9.26	0	2.4	1.81	1.76	0.48
ϭlogN2	-1	3.92	3.29	3.59	2.95	3.64
ϭlogN3	-1.78	4.49	4	4.62	4.35	4.41
ϭlogN6	-2.2	6.4	4.95	5.4	5.6	6.81
Killing rate3	-0.59	1.5	1.33*	1.54	1.45	1.47
AUC24	114.34	12.52	47.29	37.5	41.52	29.17
Bacteriolytic area24	0	101.82*	67.05	76.84	72.82	85.17

*p<0.05

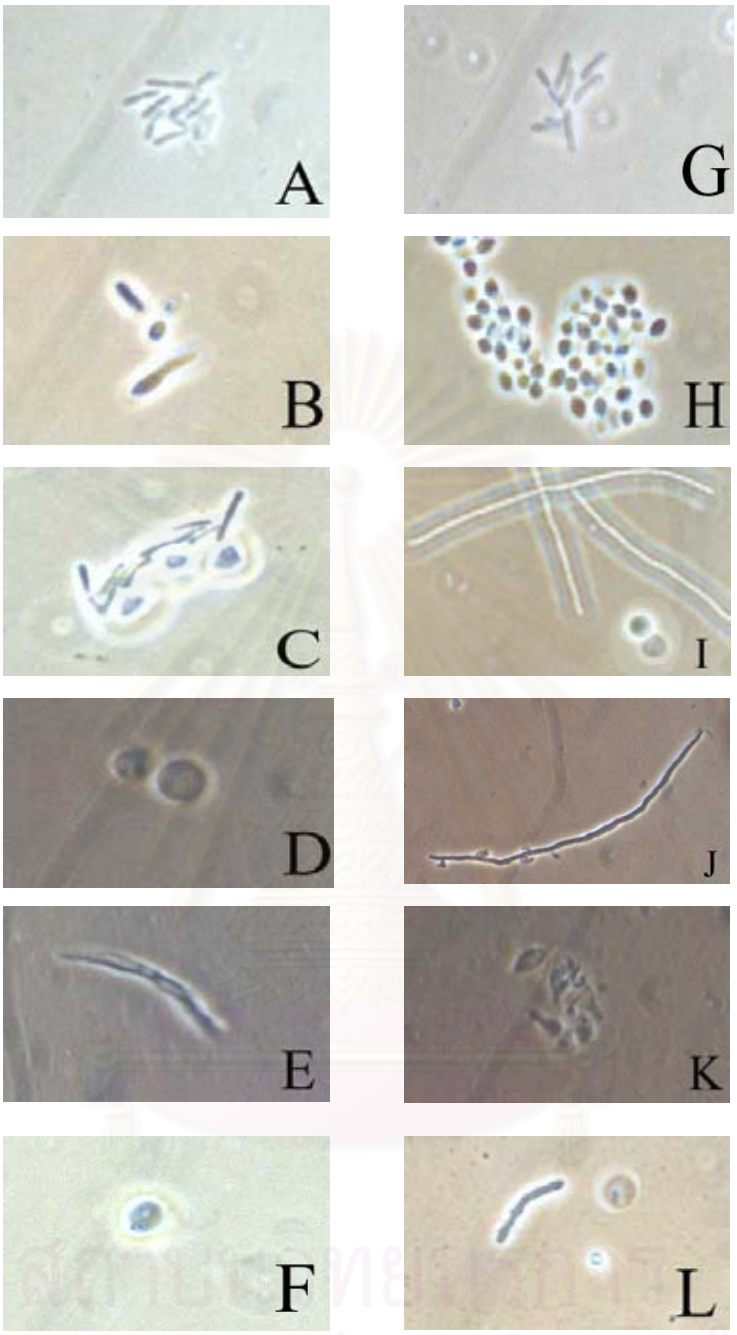


Figure 4-18 The morphological changes of *Escherichia coli* after exposed to the combination of cefpirome and imipenem at ¼MIC and 2MIC under Phase contrast microscopy (100X solution) in various times; A.1/4MIC-t0, B.1/4MIC-t1, C.1/4MIC-t2, D.1/4MIC-t3, E.1/4MIC-t6, F.1/4MIC-t24, G.2MIC-to, H.2MIC-t1, I.2MIC-t2, J.2MIC-t3, K.2MIC-t6, L.2MIC-t24.

CHAPTER V

DISCUSSION & CONCLUSION

DISCUSSION

The regrowth and elevation of MBC for *E. cloacae* after exposed to the 4th generation cephalosporins might imply the bacteriostatic property of these drugs to this specie (Amsterdam, 1991). However, these drugs could exhibit their bactericidal property during initial phase of exposure before subtracted by the regrowth in late phase. Therefore, the BA24 of these microorganisms to the 4th generation cephalosporins were less than *E. coli* for wide range. At subMIC, the BA24 demonstrated the concentration dependent manner, thus the slope occurred in the dose response curve was due to the unsaturated status of the primary killing target. Consequently, the BA24 at supraMIC level (1MIC-10MIC) manifested the concentration independent manner, the flat dose response was presented in this range which implied the saturated status of the primary killing target. Furthermore, the dose response curves exerted their slope again at 10MIC-50MIC followed with plateau at 50MIC-128MIC (figure 5-1). This event could be explained by the same as above but it might result from the secondary killing site binding.

The correlation of the KR3 and drug's concentrations resembled with the relationship between BA24 and drug's concentrations. Both of them established slope at subMIC level, which impled to the concentration dependent manner as a result of unsaturated status of the primary killing target. Consequently, the plateau of the dose response curve was exhibited at supraMIC level (1MIC-64MIC) which was due to the saturated status of the primary killing target. Furthermore, the slope was manifested again above 64MIC that related to the concentration dependent manner, which caused by the unsaturated status of the secondary killing target (figure 5-2). These relationships reconciled with the morphological changes; the incomplete filamentous cells are detected within subMIC level that resulted from the unsaturated status of PBP3 in occupied by cephalosporins. Afterwards, the complete filamentous cells were observed at supraMIC (1MIC-32MIC) as a result of the saturated status of PBP3. Furthermore, the

bulge formation was noticed in cefepime 64MIC-128MIC that was due to the PBP2 binding as the secondary killing target (Spratt, 1975; Pucci, et al., 1991).

However, these morphological changes were not detected at 24 hours of exposure. The appearance of normal rod-shaped *E. cloacae* occurred instead of the filamentous or bulbous cells. This observation might explain by the destruction of the drugs in bacterial periplasmic space from the enzyme called cephalosporinase. Opal and colleagues (2000) have stated the occurrence of *E. cloacae* that can produce the cephalosporinase from the stimulation of cephalosporins. This explanation agreed with the double disk synergy that conducted in this research (data not shown). The double disks synergy method could not detect this enzyme because of the cephalosporinases were not susceptible to clavulanate that applied in this methodology (Bush, 1988; Jarlier, et al., 1988). Therefore, the further investigation shall be required to detect the evidence and expression of cephalosporinase in this specie in Thailand.

The appropriate concentration of carbapenems to eradicate *P. aeruginosa* was 4MIC or above. This concentration exerted the MBC without regrowth at 24 hours observational period. Accordingly, the BA24 of this concentration was significantly higher than the less concentration ($p<0.05$) as shown in figure 5-3.

This event agreed with the morphological changes at 4MIC that exhibited the filamentous with bulbous cells, which related to PBP3 and PBP2 binding, respectively. (Spratt, 1975; Sumita, et al., 1990) However, the relationship between the drug's concentrations and KR3 did not relate to this observation. They demonstrated the plateau of dose response curve at 1MIC-8MIC, which implied to the concentration independent manner of this parameter (figure 5-4). Therefore, the increasing of the drug concentrations above the MIC could not elevate the KR3. Conversingly, both of drugs manifested the slope of KR3 at 1/4MIC-1MIC that implied to the concentration dependent manner of this parameter at subMIC level. Furthermore, imipenem expressed the KR3 more than meropenem at subMIC. This event resulted from the rapid permeability rates of imipenem, which this property associated, with the compact molecular size of this drug (Cornaglia, et al., 1992). Therefore, the acceleration of drug input might determine the amount of drug that bound to the unoccupied killing site (PBP) at subMIC level. Conversingly, meropenem at supraMIC level (1MIC-8MIC)

demonstrated the higher KR3 than imipenem. This appearance could be explained by the saturated status of the PBP. At supraMIC, the saturation of the primary target PBP occurred, therefore the permeability was not an important factor to determine the KR3. The most important factor was the quantitative binding of the drug to the essential PBPs. Meropenem could attack to both PBP2 and PBP3 at 1/2MIC (the filamentous with bulbous cells were detected by PCM) in comparison with imipenem that attacked to PBP3 only (the filamentous cells were detected by PCM). Therefore, the higher KR3 of meropenem might result from this explanation.

Interestingly, the filamentous formation was detected at the lowest concentration of carbapenems, which implied that these drugs attacked PBP3 as the primary target. This discovery was contrast to many previous articles that determined the PBP2 as the primary target of *P. aeruginosa* (Sumita, et al., 1990; Yang, Bhachech, and Bush, 1995). However, this discovery resembled to Trautmann and colleagues in 1998. They have stated that the clinical isolates of *P. aeruginosa* from Germany established the filamentous cells more than spheroplasting cells after exposed to 1/2MIC of meropenem at 2 hours by PCM. This report reconciled with the evidence of Kitzis and colleagues in 1989, they have determined that the clinical isolates of *P. aeruginosa* from France exhibited the ID_{50} of PBP3 lower than PBP2 in occupied with meropenem. Therefore, it might hypothesize that the *P. aeruginosa* from clinical isolate of Siriraj Hospital preferably represented their PBP3 as the primary target in the appearance of carbapenems which will be useful for the clinical application in the future.

The appropriate concentrations of the 4th generation cephalosporins to eradicate *E. coli* were different from carbapenems. These events might result from the diversity of drug's action and permeation into bacterial periplasmic space (Livermore and William, 1996). Carbapenems manifested more rapids killing rate than cephalosporins. This appearance might due to the smaller molecular size of carbapenems than the 4th generation cephalosporins, which resulted in the rapid permeability into bacterial periplasmic space for drug's action (Nakaido, et al., 1990; Cornaglia, et al., 1992). Therefore, the amount of the drugs at subMIC, which permeated into periplasmic space and occupied to the essential PBPs, might be the most important factor to determine the bactericidal action of these drugs (Bryan and Godfrey, 1991). This event resulted in the

concentration dependent manner of the dose response curve. However, all of drugs expressed the concentration independent manner of the killing rate at 1MIC-32MIC. This appearance might result from the saturated status of the primary killing site by the drug at supraMIC level. Furthermore, the concentration dependent manner of the killing rate reversed again at 64MIC-128MIC, which was due to the secondary killing site binding (figures 5-5 and 5-6).

Accordingly, this manner agreed with the morphological changes. The incomplete filamentous cells occurred at subMIC which caused by the unsaturated status of PBP3 (primary killing site) and the complete filamentous cells were detected at 1MIC-32MIC as a result of the saturated status of PBP3 by cephalosporins (Spratt, 1975). Moreover, the bulbous formations were observed at 64MIC-128MIC of cefepime, which related to the PBP2 binding. However, this result was contrast to previously report by Pucci and colleagues in 1991, they have stated that the bulge formations of *E. coli* K12 could occur from 10MIC of cefepime. This circumstance might result from the β-lactamase deficient of the microorganisms used in their research. Because the clinical isolated microorganism could produce the β-lactamase at low level in the periplasmic space (Aswapokee, 1994). Therefore, the clinical isolated *E. coli* might require the higher drug's concentration to interact with PBP in their periplasmic space compared with *E. coli* K12, the β-lactamase defective strain.

Consequently, the morphological changes of *E. coli* from carbapenems resembled to many previously reports. Carbapenem could produce the oval shaped bacteria at the lowest concentration, which could imply that the PBP2 was the primary killing target of the carbapenems (Sumita, et al., 1990; Satta, et al., 1995). Furthermore, the filamentous formations were observed in *E. coli* from 32MIC of meropenem that due to the PBP3 binding as the secondary target. However, this event did not happen with cefpirome and imipenem because of these drugs were PBP3 and PBP2 specific attacker in *E. coli* (Sumita, et al., 1990; Pucci, et al., 1991). Therefore, the combination between them might express the excellent bactericidal activity in *E. coli*.

For synergy detection, the 1/4MIC combination, which was the unsaturated model of PBP2 and PBP3, demonstrated the synergism between drugs. Nevertheless, the 2MIC combination, which was the saturated model of PBP2 and PBP3, exhibited

only additive effect but not synergy. This event reconciled with Satta and colleagues in 1995. They have not found the synergism between the saturated model of PBP2 (mecillinam) in combination with the unsaturated and saturated model of PBP3 (aztreonam) to eradicate *E. coli* DC2, a permeability defective strain. Moreover, the morphological changes of the combination between cefpirome and imipenem should be mentioned. They did not demonstrate the intrinsic property of PBP2 and PBP3 attacker in the same cell of bacteria, which resulted in the disappearance of the mixed morphological changes such as filamentous with bulbous cells in *E. coli*. Only either the filamentous cells or ovoidal cells were detected. It might hypothesize that the drugs were separate or interfere to act each other. Therefore, further investigation at the cellular level shall be required to determine the interaction of combination between β-lactams to eradicate gram negative bacteria.

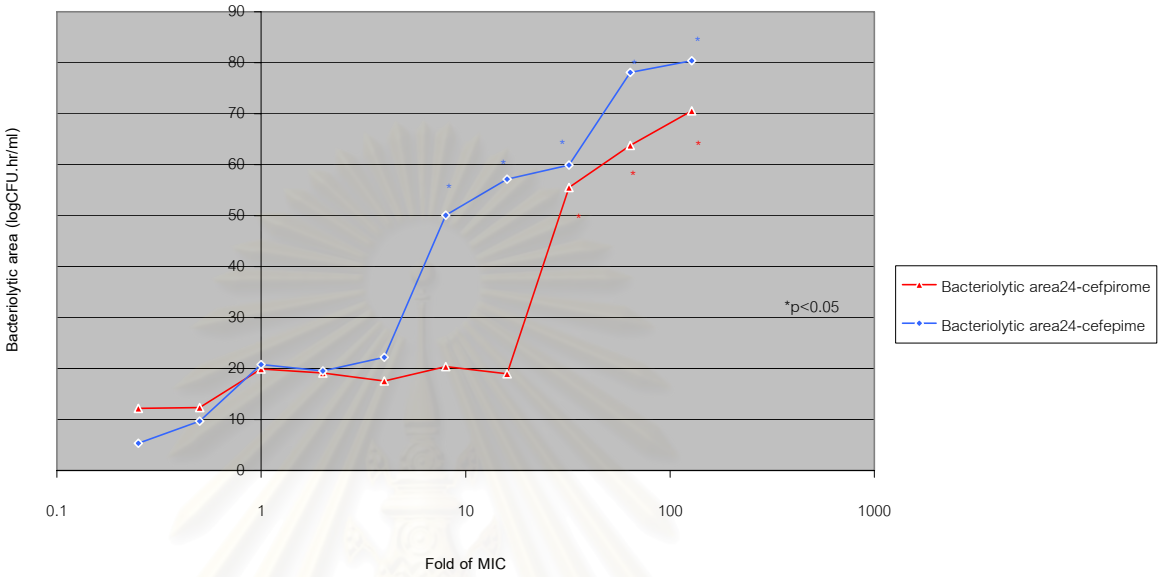


Figure 5-1 The relationship between bacteriolytic area of Enterobacter cloacaae and drug concentrations

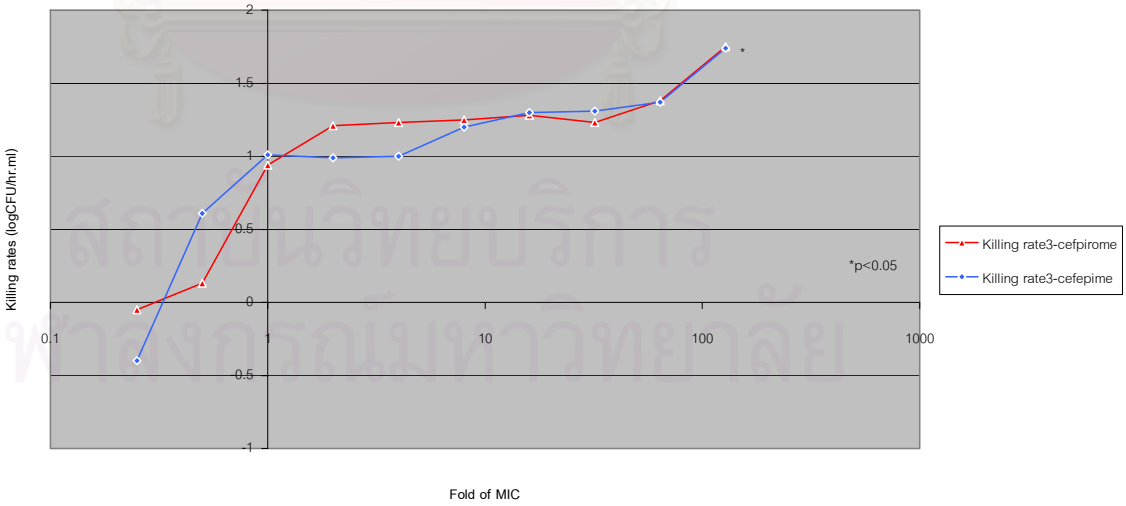


Figure 5-2 The relationship between killing rates of *Enterobacter cloacae* and drug concentrations

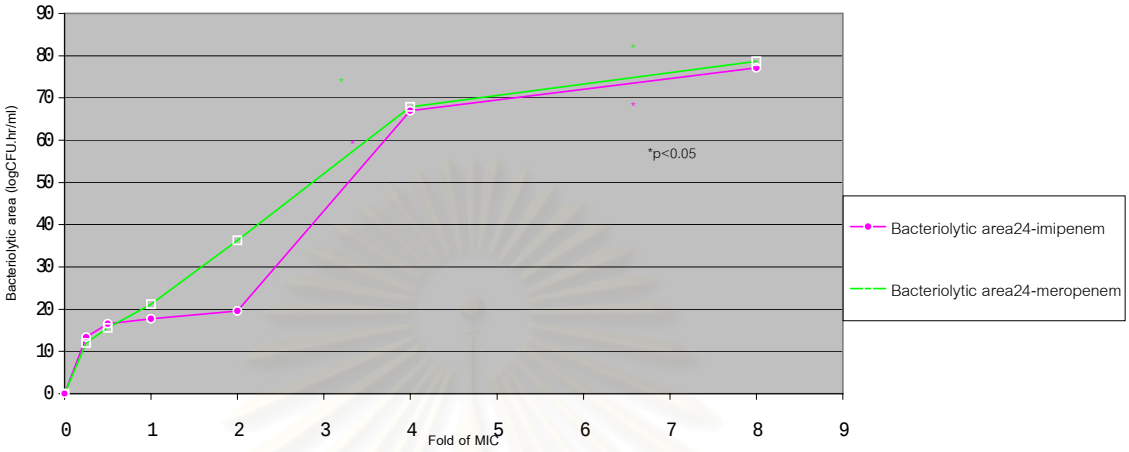


Figure 5-3 The relationship between bacteriolytic area of *Pseudomonas aeruginosa* and drug concentrations

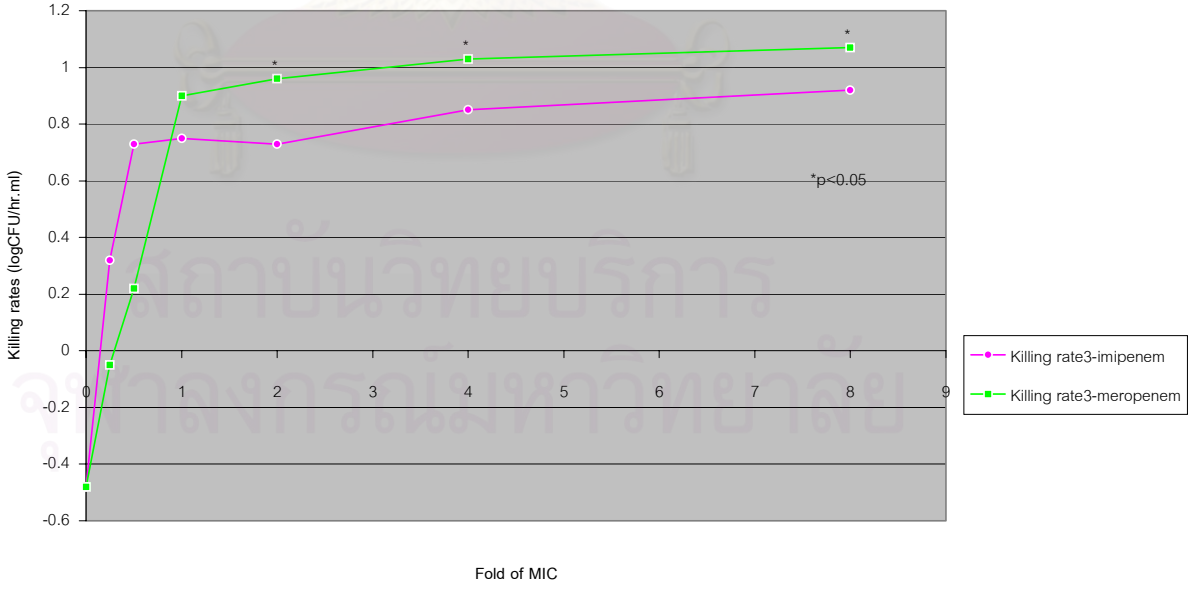


Figure 5-4 The relationship between killing rates of *Pseudomonas aeruginosa* and drug concentrations

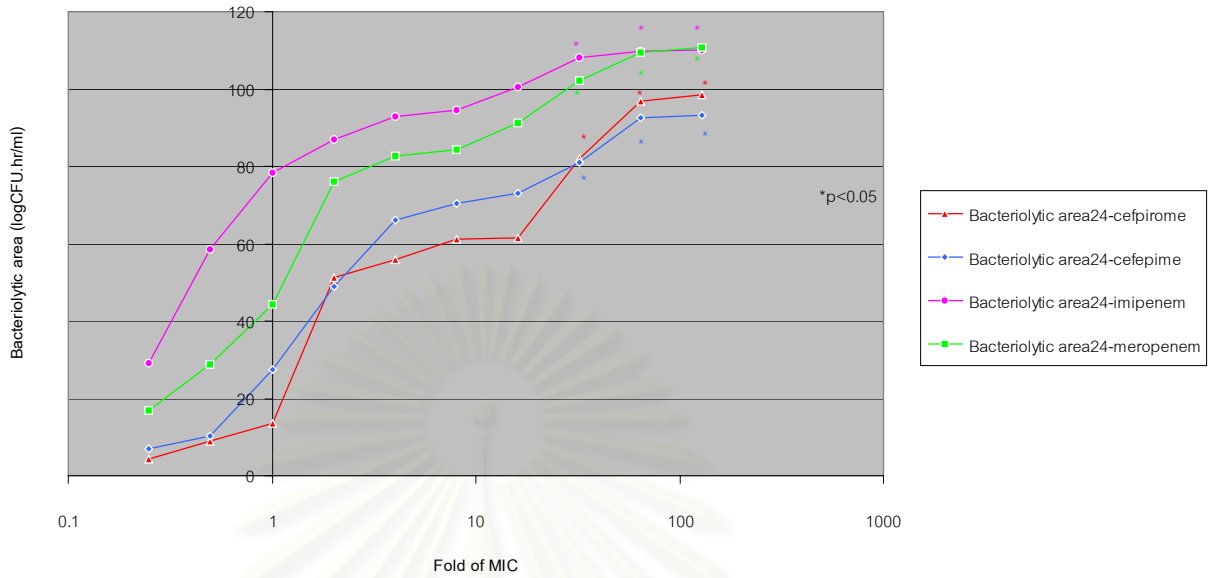


Figure 5-5 The relationship between bacteriolytic area of *Escherichia coli* and drug concentrations

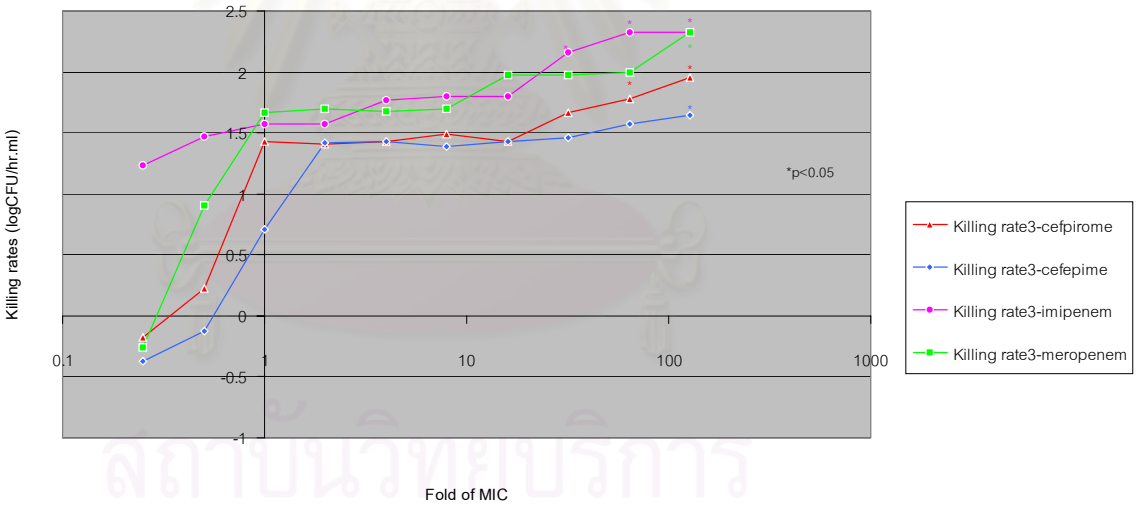


Figure 5-6 The relationship between killing rates of *Escherichia coli* and drugs concentrations

CONCLUSION

β-Lactam modified antibiotics are generally regarded as the concentration independent bactericidal agents at supraMIC level. The concentration independent manner results from saturated status of the primary killing site binding by these drugs. However, our research demonstrated as if an increase in killing targets occurred (PBP), these agents can express the concentration dependent manner to susceptible bacteria with increasing of the bactericidal properties and morphological changes. Furthermore, the resemblance of this manner among 3 species that conducted in our research may apply to other gram negative bacteria in response to β-lactam antibiotics. Therefore, further investigation shall be required to encourage this hypothesis that conflicts to many previous reports by several researchers, which determined the flat dose response is the major characteristic of β-lactams to eradicate gram negative bacteria.

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