IN VITRO EFFECT OF CEFTAZIDIME CONCENTRATION AND DURATION OF EXPOSURE

ON ERADICATION AND RESISTANCE DEVELOPMENT OF *KLEBSIELLA PNEUMONIAE*

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ผลของความเข้มข้นและช่วงเวลาที่เชื้อสัมผัสกับยาเซฟทาซิดีมที่มีต่อการกำจัด
และพัฒนาการดื้อยาของเชื้อเครบเซลลานิวโมนิเอในหลอดทดลอง

นางสาว ณัฏฐิณี ศรีประชา

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AND RESISTANCE DEVELOPMENT OF *KLEBSIELLA*

PNEUMONIAE

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ณัฏฐิณี ศรีประชา : ผลของความเข้มข้นและช่วงเวลาที่เชื้อสัมผัสกับยาเซฟทาซิดีมที่มีต่อการกำจัด และพัฒนาการดื้อยาของเชื้อเครบเซลลานิวโมนิเอในหลอดทดลอง. (*IN VITRO* EFFECT OF CEFTAZIDIME CONCENTRATION AND DURATION OF EXPOSURE ON ERADICATION AND RESISTANCE DEVELOPMENT OF *KLEBSIELLA PNEUMONIAE*) อาจารย์ที่ปรึกษา: รศ.ศิริภรณ์ ฟุ้งวิทยา, อาจารย์ที่ปรึกษาร่วม : ศ.พญ.นลินี อัศวโภคี, จำนวนหน้า: 83 หน้า. ISBN 974-17-5358-6.

การดื้อยา ceftazidime ของเชื้อ *Klebsiella pneumoniae* อันเนื่องมาจากการสร้างเอนไซม์ชนิด extended-spectrum-ß-lactamase(ESBL)มาทำลายยาถือเป็นปัญหาที่สำคัญทางคลินิกเนื่องจากเชื้อสายพันธุ์นี้ก่อให้เกิดโรคติดเชื้อที่รุนแรงหลายโรค การวิจัยครั้งนี้ต้องการศึกษาผลของความเข้มข้นและช่วงเวลาที่เชื้อสัมผัสกับยา ceftazidime ที่มีต่อการกำจัดและพัฒนาการดื้อ ยาโดยการสร้างเอนไซม์ ESBL ของเชื้อ *Klebsiella pneumoniae* 3 สายพันธุ์ที่มีความไวต่อยา ceftazidime ต่างกัน 3 ระดับ คือ สายพันธุ์ไวมาก(KN 246;MIC=0.125 µg/ml), สายพันธุ์ไวปานกลาง(KN 012;MIC=0.5 µg/ml), และสายพันธุ์ไวน้อย(KN280; MIC=2 µg/ml) การศึกษาในส่วนของการกำจัดเชื้อจะใช้วิธี time-kill study โดยใช้ความเข้มข้นตั้งแต่ 1MIC-8MICและความเข้ม ข้นของระดับยาในร่างกายจากการให้ขนาด 1 กรัมทุก 8 ชั่วโมงทางหลอดเลือดดำซึ่งมีความเข้มข้นสูงสุด (C max), ความเข้มข้น เฉลี่ย(C average) และความเข้มข้นต่ำสุด (C min) เท่ากับ 70, 35, และ 4 µg/ml ตามลำดับเพื่อศึกษาผลของความเข้มข้นต่อการ กำจัดเชื้อ ส่วนผลของช่วงเวลาที่เชื้อสัมผัสยาต่อการกำจัดเชื้อดูจากผลการกำจัดเชื้อในแต่ละช่วง half-life ที่เชื้อได้สัมผัสยาพบว่า สายพันธุ์ไวมาก(KN 246) ต้องใช้ความเข้มข้นในระดับ 4MIC และ C min และตลอด 4 ช่วงhalf-lifeที่เชื้อสัมผัสกับยาสามารถ กำจัดเชื้อได้ ส่วนสายพันธุ์ไวปานกลาง(KN 012)และสายพันธุ์ไวน้อย(KN 280) ความเข้มข้น 8MICและ C min ไม่แสดงการกำจัด เชื้อได้เพียงแต่ลดจำนวนเชื้อลงเท่านั้นแต่ C max และ C average สามารถกำจัดเชื้อได้และในช่วงเวลาที่เชื้อสัมผัสยามากกว่า 2 และ 1 half-life ความเข้มข้นของยาจะลดลงจนไม่สามารถกำจัดเชื้อสายพันธุ์ไวปานกลาง(KN 012)และสายพันธุ์ไวน้อย(KN 280) ได้ตามลำดับ ในส่วนการศึกษาพัฒนาการดื้อยาของเชื้อทั้ง 3 สายพันธุ์ตามวิธี daily passage พบว่าสายพันธุ์ไวน้อย(KN 280) เกิดการดื้อยาได้เร็วกว่าสายพันธุ์ไวปานกลาง(KN 012) และสายพันธุ์ไวมาก(KN 246) โดยการดื้อยาเกิดขึ้นหลังเชื้อสัมผัสยาเป็น เวลา 9, 18, และ 24 วันตามลำดับและเมื่อนำ resistant mutants มาทดสอบการผลิตเอนไซม์ ESBL โดยวิธี double-disk method พบว่าทั้ง 3 สายพันธุ์มีการสร้างเอนไซม์ชนิดนี้ซึ่งเป็นกลไกที่ทำให้เชื้อ *Klebsiella pneumoniae* ดื้อต่อยา ceftazidime นอกจากนั้นเมื่อนำสายพันธุ์ไวน้อย(KN280)มาสัมผัสยาในความเข้มข้นเริ่มต้น C max แล้วลดระดับยาลงครึ่งหนึ่งทุกช่วง half-life เลียนแบบระดับยาในร่างกายพบว่าเชื้อจะถูกกำจัดอย่างหมดสิ้นเมื่อถึง dose ที่ 5 สำหรับช่วงความเข้มข้นที่เป็น mutant selection window ของสายพันธุ์ไวน้อย(KN 280) จะกว้างกว่าสายพันธุ์ไวปานกลาง(KN012) และสายพันธุ์ไวมาก(KN246) เมื่อ พิจารณาค่า mutant prevention concentration ประกอบกับความเข้มข้นของยาตลอดช่วงเวลาการรักษาพบว่าขนาดยา 1 กรัม ทุก 8 ชั่วโมงจะมีประสิทธิภาพในการกำจัดและป้องกันการคัดเลือกเชื้อดื้อยาสำหรับสายพันธุ์ไวมาก(KN246)เท่านั้น สำหรับสาย พันธุ์ไวปานกลาง(KN 012) ขนาดยานี้ไม่เหมาะสมจึงแนะนำให้ใช้ high dosage regimen ในขณะที่สายพันธุ์ไวน้อย(KN 280) ทั้งขนาดรักษาและ high dosage regimen ไม่สามารถกำจัดและป้องกันการคัดเลือกเชื้อดื้อยาได้จึงควรพิจารณาให้ยากลุ่ม aminoglycoside หรือ fluoroquinolone ร่วมในการรักษาหรือเปลี่ยนใช้ยาที่มีฤทธิ์แรงกว่าเช่นกลุ่ม carbapenem หรือยาร่วม กลุ่มเบตาแลคแทม-สารต้านเอนไซม์เบตาแลคแทมเมสแทน จากผลการทดลองนี้ได้เสนอขนาดและช่วงเวลาการให้ยาที่เหมาะ สมในการนำมาใช้ทางคลินิก

ภาควิชา เภสัชวิทยา   ลายมือชื่อนิสิต _____

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NATTINEE SRIPRACHA : *IN VITRO* EFFECT OF CEFTAZIDIME CONCENTRATION AND DURATION OF EXPOSURE ON ERADICATION AND RESISTANCE DEVELOPMENT OF *KLEBSIELLA PNEUMONIAE.* THESIS ADVISOR :ASSOC. PROF. SIRIPORN FUNGWITTHAYA, THESIS COADVISOR : PROF. NALINEE ASWAPOKEE, 83 pp. ISBN 974-17-5358-6

K. pneumoniae producing extended- spectrum-ß-lactamase (ESBL) organisms confer resistance to ceftazidime caused important clinical problems from serious infections. The present study aimed to evaluate the effect of ceftazidime concentration and duration of exposure on eradication and resistance development by 3 strains of ESBL producing *K. pneumoniae* which included highly susceptible strain(KN246:MIC=0.125 µg/ml), moderately susceptible strain(KN012:MIC=0.5 µg/ml), and less susceptible strain(KN280:MIC=2 µg/ml). In the study on the effect of concentration on eradication, 1MIC-8MIC and serum drug level following administer therapeutic dose: 1 g q 8 hr IV (Cmax, Caverage, and Cmin = 70, 35, and 4 µg/ml) were used. The study on the effect of duration of exposure on the bacterial eradication when the organisms exposed to ceftazidime 4 half-life (8hr) were performed. The results from time kill study demonstrated that the highly susceptible strain(KN246) required concentration at 4MIC and Cmin and exposed to ceftazidime 4 half-life (8hr) to exhibit bactericidal property. For moderately susceptible strain(KN012) and less susceptible strain(KN280), concentration at 8MIC and Cmin exhibited bacteriostatic property whereas Cmax and Caverage had bactericidal property. Furthermore, when duration of exposure more than 2 and 1 half-life (4 and 2 hr), decreasing concentration did not have bactericidal property for moderately susceptible strain (KN012) and less susceptible strain(KN280), respectively. Regarding to study resistance development by daily passage method, results demonstrated that resistance occurred in less susceptible strain(KN280) faster than moderately susceptible strain(KN012) and highly susceptible strain (KN246). Resistance occured when organisms exposed to ceftazidime at day 9, 18, and 24, respectively. From double disk method, resistance mechanisms of these 3 strains were ESBL production. Additionally, when less susceptible strain(KN280) exposed to Cmax at the beginning and decreased concentration at every half-life which simulated pharmacokinetic achievable drug level, organisms was eradicated at the fifth dose. Less susceptible strain(KN280) had range of mutant selection window broader than moderately susceptible strain(KN012) and highly susceptible strain(KN246). When considered mutant prevention concentration and ceftazidime concentration during treatment time, the results demonstrated that the therapeutic dose could eradicate and prevent selection of resistant mutants only for highly susceptible strain(KN246). Whereas, moderately susceptible strain(KN012) required high dosage regimen. For less susceptible strain(KN280), both therapeutic dose and high dosage regimen did not appropriate therefore, combination therapy with aminoglycoside or fluoroquinolone or used more potent drugs such as carbapenem or ß-lactam- ß-lactamase inhibitor combination would be considered. The results obtained suggest that the concentration and duration of exposure of ceftazidime are appropriate for clinical application.

Department of Pharmacology   Student's signature   .

Field of study Pharmacology   Advisor's signature   .

Academic year 2003   Co-advisor's signature   .

ACKNOWLEDGEMENTS

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°C = degree Celsius

AUC = Area under the curve

BA24 = Bacteriolytic area of 24 hours

CFU = Colony forming unit

E. coli = *Escherchia 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

K. pneumoniae = *Klebsiella pneumoniae*

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

NCCLS = The National Committee for Clinical Laboratory Standards

NSS = Normal saline solution

P. aeruginosa = *Pseudomonas aeruginosa*

PBP = Penicillin binding protein

CHAPTER I

INTRODUCTION

The β-lactam drugs are the most widely used for the management of many bacterial infections. Since the mechanism of action of β-lactam antibiotics is specific to bacterial cell wall, they are therefore highly safe antibiotics for treatment of the infection caused by bacteria in human. As a result, a large number of β-lactam modified antibiotics have been developed and available in health center until to the present era. The current of β-lactam antibiotics are classified into six groups as their core β-lactam ring structure (Figure 1-1).
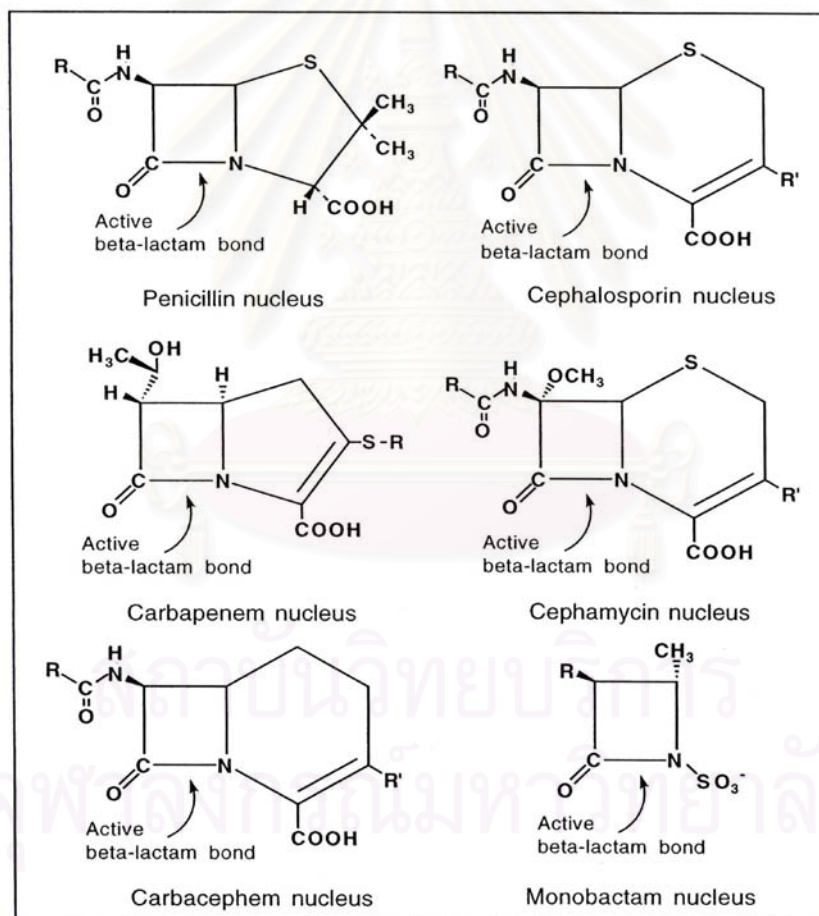
Figure 1-1 Basic structure of penicillin, cephalosporin, carbapenem, cephamycin, carbacephem and monobactam.

They are bactericidal agents that inhibit bacterial cell wall synthesis. The targets of β-lactam drugs are penicillin-binding proteins (PBPs), the membrane bound enzyme that are required for the biosynthesis of the bacterial cell wall (Zhao et al.,1999). PBPs catalyze the final steps of the polymerization (transglycosylation) and cross-linking (transpeptidation) of peptidoglycan, an essential component of the bacterial cell wall.

Resistance to β-lactam drugs are three mechanisms : (1) structurally altered PBPs target sites ; (2) β-lactamase production; and (3) reduced outer membrane permeability. (Sader and Gales, 2001).Amongs gram- negative bacteria, β-lactamase production is the most common mechanism of resistance. The type of β-lactamase have been classified into several schemes, but a generally accepted classification scheme is the one established by Amber (1980). This classification arranges the β-lactamases into four groups according to β-lactam molecular weight.(Table 1-1)

Table 1-1 Classification schemes for bacterial β-lactamases

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

Table 1-1 (continue)

Structural class (Ambler)	Functional group (Bush)	Preferred substrates	Inhibition by clavulanate	Representative enzyme
Zincβ-lactamase				
B	3	Most β-lactams, including carbapenems	-	L1 from *Xanthomonas maltophilia*, CcrA from *Bacteroides fragilis*

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

 - , negligible inhibition

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

Class A,C, and D comprise evolutionarily distinct groups of serine enzyme and class B enzymes utilize a zinc ion to attack the β-lactam ring.

Phases of the reaction of catalyzing the β-lactam antibiotics by serine β-lactamase include (i) reversible non-covalent binding of the β-lactamase and the β-lactam ring, (ii) rupture of the β-lactam ring, which becomes covalently acylated on to the active site serine. (iii) hydrolysis of the acyl enzyme to reactive the β-lactamase, splitting the amide bond, and liberate the inactivated drug molecule. As a result, the antibiotics can no longer inhibit bacterial cell wall synthesis (Figure 1-2).
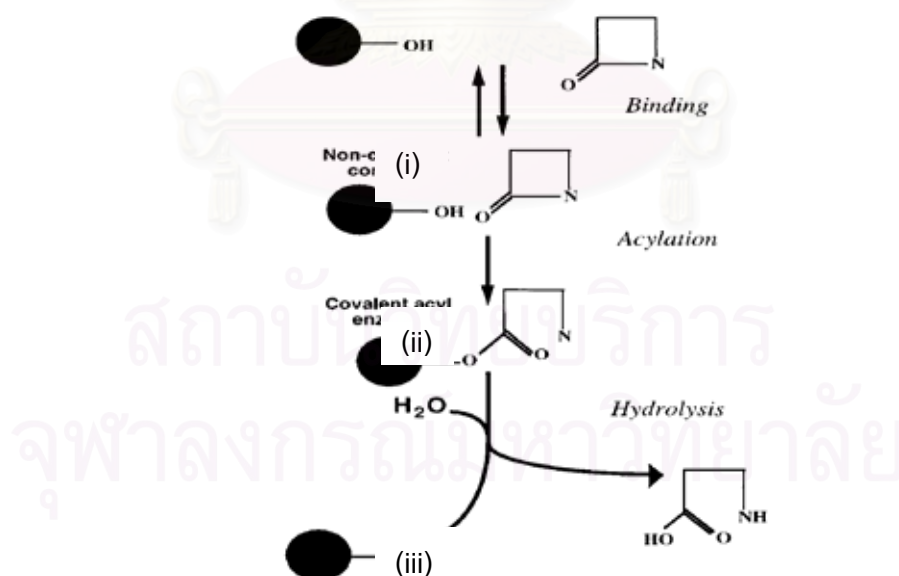
(Modified from Livermore, 1995)

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

In some gram –negative bacteria notably *Bacteriodes fragilis* and *Klebsiella pneumoniae* produce class A chromosome β-lactamase.Control of production of this enzyme is readily derepressed, so that high β-lactamase levels are produced. In addition to these chromsomally mediated enzyme, gram-negative bacteria may harbor plasmids the code for many types of powerful β-lactamase such as TEM-1, TEM-2 and SHV-1 (Greenwood, 1986). TEM-1 predominates in *Escherichia coli* while SHV-1 predominates in *K. pneumoniae*. These enzymes are expressed constitutively and give resistance to ampicillin, carbenicillin, and ticarcillin (Livermore, 1991).

The Extended-Spectrum-ß-Lactamase (ESBL) enzymes (Bush group 2be) are plasmid-mediated enzymes capable of hydrolyzing and inactivating penicllin, oxyimino cephalosporins (ceftazidime, cefotaxime, ceftriazone etc.) and aztreonam (Martinez, et al., 1996). However, other ß- lactams such as cephamycin, , imipenem, and β-lactam-β-lactamase inhibitor combinations remain sensitive in the presence of an ESBL(Aswapokee, 1997). These enzymes evolve from common TEM-1 and SHV-1 penicillinase through point mutations in regions important for β-lactam binding and/or hydrolysis (Jacoby and Medeiros, 1991).

ESBL producing organisms are among the fastest growing problems in the area of infectious diseases. The most common ESBL-producing organisms are *K.pneumoniae* and *E. coli.* (Nathisuwan et al., 2001). Study of the Division of infectious disease, Faculty of Medicine, Siriraj hospital in 1997 demonstrated that *K. pneumoniae* is the third most frequent causes of clinically antibiotic resistance problems (Aswapokee, 1997). The presence of ESBLs in *K.pneumoniae* poses an important challenge in clinical practice, since these organisms cause of serious infections such as bactremia, pneumonia, urinary tract infection, and nosocomial infection in febrile neutropenic patients and confer resistance to other antibiotics such as aminoglycosides and trimethoprim-sulfamethoxazole hence they become multi-drug resistance (MDR) and spread in hospital by cross-transmission.

A recent national microbiological surveillance program demonstrated an alarming increase prevalence of ceftazidime-resistant *K.pneumoniae* during a 4-year period from 3.6% in 1990 to 14.4% in 1993 (Itokazu et al., 1996). Furthermore data from the study of the Division of infectious disease, Faculty of Medicine, Siriraj hospital in 1992 demonstrated that

37 % of *K.pneumoniae* were ESBL producing strains. These strains act against third-generation cephalosporins which have highest MIC to ceftazidime, monobactam, aminoglycoside and fluoroquinolone. Therefore it is becoming an increasing problems for clinicians in treatment. High cost and high mortality rate will occur (Tiengrim, 2002).

Ceftazidime is the third- generation cephalosporin. It is active against gram negative bacteria in family enterobacteriaceae. It is more active against *Pseudomonas aeruginosa* than other third- generation cephalosporins such as cefoperazone (Chamberland et al., 1992). It is not absorbed from the GI tract and must be given parenterally ;IM or IV. The dosage can be varied widely, according to the nature and severity of the infection. For the treatment of moderately severe *K. pneumoniae* infection , the drug has been given in dosage of 1 g every 8 hours for 7-10 days; IV. The serum half-life of the drug is approximately 2 hours. The pharmacokinetic achievable concentration; C max, C average and C min are 70 , 35 , and 4 µg/ml, respectively. (AHFS Drugs, 2001).

Several previous studies have demonstrated that third- generation cephalosporins are the inducer of ESBL production (Aswapokee, 1994). Whether ceftazidime not only the substrate of ESBL but also induce ESBL production and select resistant mutants in *K.pneumoniae*. The present study aims to investigate effect of ceftazidime concentration and duration of exposure on eradication and resistance development by ESBL production of *K.pneumoniae.* This study hypothesized that the selection of strains with increase levels of resistance to a drug will occur at a particular drug concentration and duration of exposure (selective compartments). One prediction of this hypothesis is that there would be a range of concentrations, corresponding to the maximum differences in bacterial growth and killing rates, at which selection would be the most intense; we call this range of concentrations a " selective window"(Maria, 2000 and Martinez , 2000). The results from this study provide appropriate dosage regimens of ceftazidime for treatment *K.pneumoniae* infections with highest efficacy and not have resistance development.

CHAPTER II

LITERATURE REVIEW

1. The Cephalosprins

　　　　Many cephalosporins are now in clinical use. They have a wide range of activity against different species of bacteria. It appears that modification at position 7 of the ß-lactam ring are associated with alteration in antibacterial activity and that substitution at position 3 of the dihydrothiazine ring are associated with change in the metabolism and the pharmacokinetic properties of the drugs. (Table 2-1)

Table 2-1 Structure of cephem nucleus and the cephalosporins

(modified from Hardman and Limbard, 2000)

COMPOUND (TRADE NAMES)	R₁	R₂
Second-generation (cont.) Ceforanide (PRECEF)		
Third-generation Cefotaxime (CLAFORAN)		
Cefpodoxime proxetil§ (VANTIN)		
Ceftizoxime (CEFIZOX)		
Ceftriaxone (ROCEPHIN)		
Cefoperazone (CEFOBID)		
Ceftazidime (FORTAZ, others)		
Fourth-generation Cefepime (MAXIPIME)		

Cephem nucleus

1.1 <u>Antimicrobial activity</u>

Cephalosporins may be classified into 4 generations, base on antimicrobial activity and ß-lactamase stability. The first-generation agents; cephalothin and cefazolin were active againsts gram-positive bacteria and relatively modest activity against gram-negative microorganisms. The second-generation agents have somewhat increased activity against gram-negative bacteria and including some agents (cefoxitin, cefotetan and cefmetazole) with antianaerobe activity against the B. fragilis group. The third-generation agents are less active than first-generation agents against gram-positive cocci, but they are much more active against the enterobacteriaceae including ß-lactamase producing strains. A subset of the third-generation agents (ceftazidime and cefoperazone) active against P. aeruginosa, and the fourth-generation with a spectrum similar to the third but having increased stability to hydrolysis by ß-lactamase (Kucer A, et al., 1997).

1.2 <u>ß-lactamase susceptibility</u>

The cephalosprins, however, have variable susceptibility to ß-lactamase. For example of the first-generation agents, cefazolin is more susceptible to hydrolysis by ß-lactamase from *S. aureus* than in cephalothin. The second-generation agents, cefoxitin and cefuroxime more resistant to hydrolysis by the ß-lactamase produced by gram-negative bacteria than first-generation cephalosporins. Third-generation cephalosporins are susceptible to hydrolysis by inducible, chromsomally encoded ß-lactamase. Induction of the enzymes by treatment of infections due to aerobic gram-negative bacilli with second or third- generation cephalosporins and/or imipenem may result in resistance to all third-generation cephalosprins. Fourth generation such as cefepime, are poor inducer of chromosomally mediated ß-lactamase and less susceptible to hydrolysis by these enzymes than are the third-generation agents.

<u>2. Ceftazidime</u>

Ceftazidime contains an aminothiazolyl side chain at positon 7 and a pyridine substituent at position 3 of the cephalosporin nucleus. Ceftazidime also contains a carboxypropryl oxyimino group in the side chain.
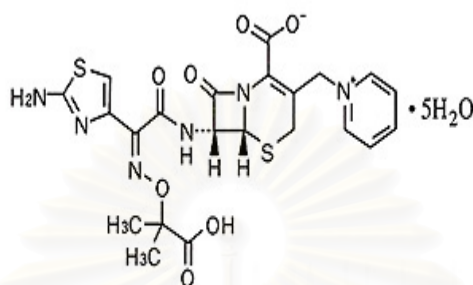
Fig 2-1 Structure of ceftazidime

Its activity against the enterobacteriaceae is vary similar to another third-generation cephalosprins, but its major distinguish feature is excellent activity against *P. aeruginosa*.

Some of the enterobacteriaceae, such as *Enterobacter* spp*., Citrobacter* spp*., Proteus vulgaris, Providencia* spp*., Morganella morganii, Hafnia and Serratia* spp*.,* harbor low levels of chromosomally mediated ß-lactamases (Figure 2-2). The enzyme in these organisms are said to be " repressed" but they can start overproducing these ß-lactamases or become " derepressed" by one of two mechanisms. The first involves exposure of wild type bacteria to an enzyme inducer, such as cefoxitin, another ß-lactamase stable cephalosporin, or some other ß-lactam antibiotics. The second mechanism involves spontaneous chromosomal mutation to a stably " derepressed" state, when again these enzymes are over produced. Then these enzymes can hydrolyze ceftazidime and the organisms become resistant to the drug as well as to other third-generation cephalosporins, such as cefotaxime. The bacteria with this type of ß-lactam resistance can become widespread in a hospital or a special unit if ceftazidime is widely used. Partial return to ceftazidime sensitivity may occur when the use of ceftazidime is restricted (Kucer. et al., 1997)

The plasmid-mediated extended-spectrum ß-lactamases (Bush group 2be), which can hydrolyze cefotaxime and other third-generation cephalosporins, including ceftazidime (Table 2-2), were first detected in the mid-1980s in Europe, but now they have spread to many countries, including the UK and USA. Initially they were mainly found in K. pneumoniae and E. coli, but they soon spread to some other enterobacteriaceae. These ß-lactamases are single mutations from the previously well known plasmid-mediated TEM-1, TEM -2, and SHV-1 enzymes (Bush group 2b), which did not hydrolyze cefotaxime, cetazidime and. Some of these enzyme hydrolyze only third-generation cephalosporins, but not cephamycins and are inhibited by ß-lactamases inhibitors (Kucer, et al., 1997).

11

β-Lactamases and Their Distribution in Nature

Microbial β- lactamases

Gram-positive bacteria

Gram-negative bacteria

Chromosomal

Plasmid

Plasmid

Chromosomal* (Multipurpose)

Chromosomal* (Multipurpose)

Bacillus spp. (penicillinases)

Staphylococcus aureus
Staphylococcus epidermidis
Staphylococcus haemolyticus

Zn²⁺ stimulated enzyme

Hydrolyzes all β-lactams

(penicillinases Primarily)

Nocardia

Mycobacterium

Chromosomal

Plasmid

Cephalosporinases

Broad spectrum

Cefuroximases

All β-lactams

Broad spectrum

Carbenicillinase

Oxacillinase

Cefotaximase

Inducible

Constitutive

Klebsiella*

Xanthomonas maltophilia

Enterobacter
Citrobacter freundii
Serratia marcescens
Pseudomonas aeruginosa
Morganalla morganii
Providencia

Enterobacter
Citrobacter freundii
Acinetobacter
Bacteroides*

Bacteroides spp*
Legionella spp*

Proteus vulgaris
Pseudomonas cepacia

TEM-1,-2, SHV-1*

Enterobacteriaceae
Haemophilus

Pseudomonas

Enterobacteriaceae
Escherichia coli

TEM-3 to –8, SHV-2 to 5*

Enterobacteriaceae

Klebsiella

(Modified from Chambers, 2000)

* = inhibited by clavulanic acid, sulbactam and tazobactam

Figure 2-2   Diagrammatic representation of β-lactamases

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

Table I. Molecular and phenotypic classifications of β-lactamases

Structural class (Ambler)[6]	Functional group (Bush et al.)[3]	Richmond Sykes class[4]	Substrate preference[a]							Inhibition[b]		
			penicillin	carbenicillin	oxacillin	cephaloridine	sefotaxime	aztreonam	imipenem	clavulanate	aztreonam	EDTA
Serine β-lactamases												
A	2a	NL	+++	+	-	±	-	-	-	++	-	-
	2b	II and III	+++	+	+	++	-	-	-	++	-	-
	2be	III and IV[c]	+++	+	+	++	+	+	-	++	-	-
	2br	NL	+++	+	+	+	-	-	-	-	-	-
	2c	II and V	++	+++	+	+	-	-	-	+	-	-
	2e	Ic	++	++	-	++	+	++	-	++	-	-
	2f	NL	++	+	?	+	+	++	++	+	-	-
C	1	I, except Ic	++	+	-	+++	+	-	-	-	++	-
D	2d	V	++	+	+++	+	-	-	-	V	-	-
Undetermined[d]	4[d]	NL	++	++	++	V	V	-	-	-	-	-
Zinc β-lactamases												
B	3	NL	++	++	++	++	++	-	++	-	-	++

[a] Activity: +++, preferred substrate (highest V_{max}); ++, good substrate; +, hydrolysed; ±, barely hydrolysed; -, stable; V, varies within group; ?, uncertain.
[b] Inhibition: ++, strong inhibitor of all members of class; +, moderate inhibition; V, inhibition varies within the class; -, negligible inhibition.
[c] K1 enzyme of *K. oxytoca* was placed in Richmond & Sykes Class IV and Bush group 2be; however, most Bush 2be enzymes are mutants of TEM and SHV.
[d] None of Bush's group 4 enzymes has yet been sequenced. They are assumed to be serine types because they lack carbapenemase activity and are not inhibited by EDTA. NL: not listed.
For full details of these classifications, see Richmond & Sykes,[4] Ambler,[6] Bush *et al.*[3] and Frère.[24]

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3. Exteneded-Spectrum ß-lactamase (ESBL)

The ESBL enzymes are plasmid-mediated enzymes capable of hydrolyzing and inactivating penicillins, oxyiminocephalosporins (cefotaxime, ceftazidime, ceftriaxone etc.), and aztreonam. These enzymes are the result of mutation of TEM-1, TEM-2 and SHV-1, all of which are ß-lactamase enzymes commonly found in the enterobacteriaceae family. Normally TEM-1, TEM-2 and SHV-1 enzymes confer high-level resistance to early penicillins and low-level resistance to first-generation cephalosporins(Medeiros, 1997).The wide spread use of third-generation cephalosporins and aztreonam is believed to be the major cause of the mutations in these enzymes that have led to the emergence of ESBLs.(Rice et al.,1990 ; Naumovski et al.,1992 ; Meyer et al.,1993 and Brun-Buisson et al.,1995).

3.1 Classification of plasmid-mediated ESBL

The ESBL enzymes can be further classified into a variety of groups as shown in table 2-3

Table 2-3 Type of plasmid-mediated extended-spectrum ß-lactamase enzymes

Type	Classification	Prefered Substrates	ß-lactamase Inhibitors	Location
TEM and SHV	Bush:Class 2be Ambler:Class A	Oxyiminocephalosporins and other ß-lactams except cephamycin and carbapenems	Susceptible	Worldwide
Inhibitor resistant TEMS	Bush:Class 2br Ambler:Class A	Penicillins	Resistant	Worldwide
PER	Bush:Class 2be Ambler:Class D	Oxyiminocephalosporins and other ß-lactams except cephamycin and carbapenems	Susceptible	Turkey,South America
OXA	Bush:Class 2d Ambler:Class D	Oxyiminocephalosporins, Cephamycin, and other ß-lactams except carbapenems	Resistant	Turkey
Plasmid mediated AmpC	Bush:Class 1 Ambler:Class C	Oxyiminocephalosporins, Cephamycin, and other ß-lactams except carbapenems	Resistant	Worldwide
Plasmid mediated carbapenemases	Bush:Class 3 Ambler:Class B	All ß-lactams, including carbapenems	Resistant	Japan, Singapore, Italy; not yet reported in U.S.

(modified from Medeiros, 1997 and Bush et al., 1999)

3.2 ESBL detection method

Testing for the presence of ESBL using several techniques such as double-disk test, E-test, and dilution test (recommended by the NCCLS, 2003 guildeline).

3.2.1. Double disk approximation test

In this test, the organism is swabbed onto a Mueller-Hinton agar plate. Susceptibility disk containing amoxicillin-clavulanate is placed in the center of the plate, and disks containing one of the oxyimino-β-lactam antibiotics are placed 30 mm (center to center) from the amoxicillin-clavulanate disk. As shown in Fig. 2-3 A enhancement of the zone of inhibition of the oxyimino-β-lactam caused by the synergy of the clavulanate in the amoxicillin-clavulanate disk is a positive result (Jarlier et al., 1988).

3.2.2. E-test ESBL

Several commercial manufacturers have developed ESBL detection tests that can be used along with MIC test methods already in place in the clinical laboratory. E-test ESBL strips (AB Biodisk, Solna, Sweden) are two-sided strips that contain a gradient of ceftazidime on one end and ceftazidime plus clavulanate on the other end. As shown in Fig. 2-3 B a positive test for an ESBL is a ≥ 3 twofold dilution reduction in the MIC of ceftazidime in the presence of clavulanic acid. This test was shown to be more sensitive than the double-disk approximation test in detecting ESBLs in clinical isolates (Cormican et al.,1996). This method is convenient and easy to use, but it is sometimes difficult to read the test when the MICs of ceftazidime are low because the clavulanate sometimes diffuses over to the side that contains ceftazidime alone as shown in figure 2-3 C (Vercayteren et al., 1997).

3.2.3.Dilution test

NCCLS recommends an initial screening by testing for growth in a broth medium containing 1 µg/ml of one of five expanded-spectrum β-lactam antibiotics. A positive result is to be reported as suspicious for the presence of an ESBL (NCCLS,2000). This screen is then followed by a phenotypic confirmatory test that consists of determining MICs of either ceftazidime or cefotaxime with and without the presence of clavulanic acid (4 µg/ml). A decrease in the MIC of ≥ 3 twofold dilutions in the presence of clavulanate is indicative of the presence of an ESBL. If an ESBL is detected, the strain should be reported as nonsusceptible to all expanded-spectrum cephalosporins and aztreonam regardless of the susceptibility testing result .
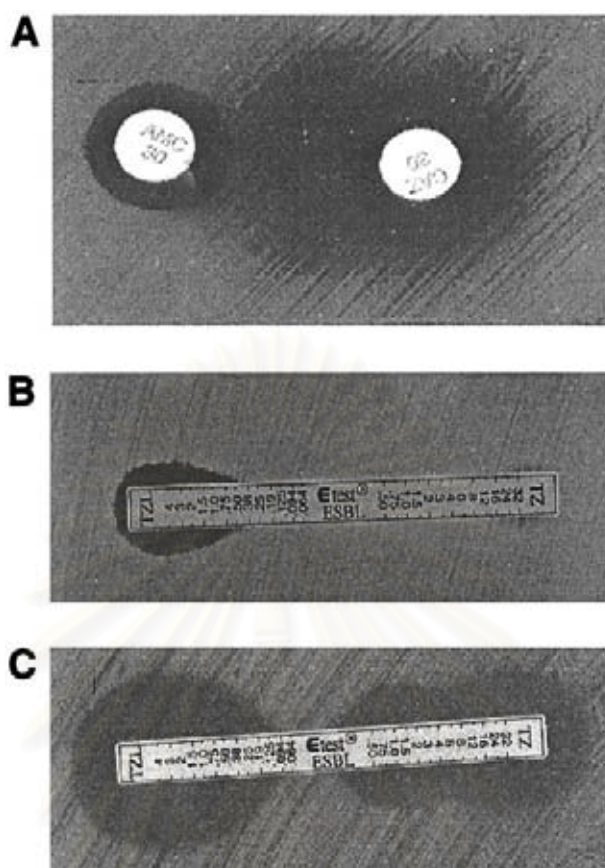
FIG. 2-3 Double-disk diffusion and Etest ESBL detection tests.

(A) The double-disk diffusion ESBL detection test as suggested by Jarlier et al. is shown. A disk containing amoxicillin-clavulanate (AMC) is placed in proximity to a disk containing ceftazidime (CAZ) or another oxyimino-cephalosporin. The clavulanate in the amoxicillin-clavulanate disk diffuses through the agar and inhibits the β-lactamase surrounding the ceftazidime disk. Enhancement of the zone of the ceftazidime disk on the side facing the amoxicillin-clavulanate disk is interpreted as a positive test.

(B) Etest ESBL strip (AB Biodisk, Solna, Sweden). The zone of inhibition is read from two halves of the strip containing ceftazidime alone (TZ) or ceftazidime plus clavulanate (TZL). A reduction in the MIC of ceftazidime of ≥ 3 two fold dilutions in the presence of clavulanate is interpreted as a positive test.

(C) The Etest ESBL strip is sometimes difficult to interpret with weak enzyme producers such as the strain expressing TEM-12 shown in this panel. The clavulanate from the ceftazidime plus clavulanate half of the strip diffuses into the agar and interferes with the reading of the MICs for the half of the strip containing ceftazidime alone.

4.Anibiotic use and resistance selection

Daily clinical experience strongly suggests that there is certain link between the use of antibiotics and the selection resistance. For instance, the rise in antibiotic resistance among *Streptococcus pneumoniae* isolates appears to be related to the total consumption of antibiotics (Baquero, 1996).

An important question is whether the use of antibiotics influences bacterial evolution. There are two features essential to the understanding of bacterial evolution under antibiotic pressure are the selective process leading to proliferation of resistant organisms and the less-well-known factor that influences this process, the so-called random genetic drift.

4.1. Selective Pressure

Selective pressure is a general concept that refers to the many factors that create an environmental landscape and allow organisms with novel mutations or newly acquired characteristics to survive and proliferate. In the most Darwinian sense of the term, selective pressure permits the expression of differences in fitness in such a landscape, resulting in the differential proliferation of resistant organisms. Organisms resistant to antibiotics were resistant before antibiotics were used but were not able to differentially proliferate; thus, both survival and proliferation are essential.

Selective Pressure has been defined recently by Tenover and McGowan,1996. In their view, selective pressure refers to environmental conditions, including not only the use of antibiotics but also any other environmental factors such as how the patients are linked by epidemiological features, other drugs used, or environmental pollutants. But the important factor from the use of antibiotic on resistance selection is antibiotic concentration and duration of exposure.

Antibiotic concentration

The concentration of the selector has an important role in the rate of mutation to antibiotic resistance. At low selector concentrations, mutations in any of those genes can effectively protect the bacteria from the action of the antibiotic and thus be selectable. However, once the antibiotic concentration rises, the number of selectable mutants decreases. At certain antibiotic concentrations, combination of mutations in more than one gene might be required to provide the resistance phenotype ,so that at high selector

concentrations, a sharp decrease in the mutation rate will occur. Another important point is that the probability that a specific type of mutant will emerge is expected to have a maximum at one particular antibiotic concentration close to the MIC for the organism(Baquero and Negri., 1997). For instance, a specific antibiotic concentration may be sufficient to decrease the growth rate or to suppress the original ancestor population but may not be sufficient to affect the resistant variant population. Beyond this concentration, antibiotic concentrations may be able to reduce or suppress in an equivalent way the growth of both susceptible and variant populations, and therefore, no selection for the variant is expected to occur. The same applies when the antibiotic concentration is below the level to which both populations are susceptible. Therefore, the selection of a particular antibiotic-resistant variant may happen only in a narrow range of drug concentrations that define a selective window. The conclusion is that the observed mutation rate is very sensitive to changes in drug concentration, and different rates and types of mutants may be obtained in a discontinuous way along the range of concentrations. On the other hand, as the selective effect of the drug may depend (as for ß-lactam antibiotics) on the time of exposure, this period of time may be critical to yield one or another mutation rate (Martinez and Baquero., 2000).

4.2.Random Genetic Drift

Random Genetic Drift is a mechanism (providing something at random can be called a mechanism) that has remained largely unconsidered as a factor in the evolution of antibiotic resistance. The Darwinian definition considers random drift as the fluctuations in frequency of variations (variants) that have no adaptive significance or are otherwise equally fit. To a certain extent, this reflects the "survival of the luckiest" in opposition to the true selective process of "survival of the fittest. It can be considered that random genetic drift may occur preferentially in critical situations, e.g., when by chance a given individual organism or small population of organisms survives under circumstances that have eliminated neighboring organisms. In particular, catastrophic events leading to mass extinctions may produce this effect, and it is clear that the use of active antibiotics produces mass extinctions in bacterial population. It is evident that a nonoriented mechanism such as genetic drift may produce quite unexpected results.

4.3 In vitro selection of variant TEM ß-lactamase

Recent advances in the study of the genetics of bacterial resistance to antibiotics have provided the elements to reevaluate the evolutive and clinical importance of mechanisms of low-level resistance. A good example is the evolution of TEM-1 ß-lacta-mase, probably the most widespread bacterial enzyme involved in clinical resistance to antibiotics. As is well known, TEM-1 is considered a broad-spectrum enzyme because it hydrolyzes penicillins and some cephalosporins. Unexpectedly, molecular variants of TEM-1 (or the very similar TEM-2) recently acquired the ability to catalyze the hydrolysis of newly available cephalosporins such as cefotaxime and ceftazidime. These variants, termed extended-spectrum ß -lactamases, emerged and disseminated probably as a result of the introduction of new ß -lactam antibiotics in the therapeutic armamentarium.

Antibiotic-inactivating extended-spectrum ß -lactamases differ from TEM-1 in one to five amino acid substitutions within the enzyme sequence (Jacoby and Medeiros, 1991). For instance, TEM-10 differs from TEM-1 in the replacements of Arg164 by Ser and Glu240 by Lys, which increases the cefotaxime MIC from 0.03 mg/mL to 1 mg/mL (Baquero, 1995). Apparently, enzymes like TEM-10 have evolved under cefotaxime pressure from previous TEM-1 variants with a single amino acid substitution; for instance, TEM-12 (with only the Arg164 replacement) is a likely ancestor of TEM-10. This may imply that strains harboring TEM-12, despite the very low increase in the MIC of certain cephalosporins (0.06–0.12 mg/mL for cefotaxime, compared with 0.03 mg/mL for TEM-1), were indeed selected during therapy and proliferated sufficiently to develop another point mutation, leading to a new enzyme with more effective resistance (TEM-10).

The hypothesis that low-level-resistant variants (such as TEM-12) have been selected in vivo implies the occupation (together with the wild strain, TEM-1) of a particular body compartment where the antibiotic concentration is selective for the variant. This concentration could not be higher than that tolerated by the variant but not lower than that required to inhibit the fully susceptible wild strain. As concentrations that inhibit the variant and the wild strain may be extremely close, the selective range of concentrations may be extremely narrow. In fact, this phenomenon can be described as antibiotic concentration–dependent selection.

This process was recently reproduced under in vitro conditions in a model where wild-type and variant *E. coli* subpopulations were mixed in culture medium and challenged with different antibiotic concentrations(Baquero, 1997). The predominant subpopulation (90% of total cell number) contained the TEM-1 ß-lactamase. The minority subpopulation contained the same enzyme, but with a single amino acid replacement (Ser164 instead of Arg164) obtained by directed mutagenesis; thus the enzyme was called TEM-12, and the susceptibility of the *E. coli* strain was slightly reduced.

In mixed cultures, TEM-12 was selected over TEM-1 at very low cefotaxime concentrations, ranging from 0.006 mg/mL to 0.06 mg/mL. As expected on the basis of the concept of concentration-dependent selection, at slightly higher concentrations (0.12 mg/mL), the wild subpopulation harboring TEM-1 remained dominant. Of course, the TEM-1 subpopulation was also reduced in number at 0.12 mg/mL; however, its final predominancy reflects that, in the absence of selection, most surviving cells belonged to the wild-type subpopulation.

Because these results depended on the composition of the original mixed population, concentration-dependent selection can be related with frequency-dependent selection (Levin, 1988).

<u>5. Mutant selection window and Mutant prevention concentration</u>

5.1 The mutant selection window (MSW)

The mutant selection window is an antimicrobial concentration range extending from the minimal concentration required to block the growth of wild-type bacteria up to that required to inhibit the growth of the least susceptible, single-step mutant (Drlica., 2003).

The lower boundary of the window is the lowest concentration that blocks the growth of the majority of drug-susceptible cells, since below that concentration the mutant cells do not have a growth advantage. The lower boundary can be approximated by the MIC for half the cells in the population (MIC $_{(50)}$); however, inhibition of 99% of the cells (MIC$_{(99)}$) is a more suitable boundary since it is measured more accurately. Placing MIC near the lower boundary of the selection window contradicts traditional medical teaching, in which resistant mutants are thought to be enriched selectively at concentrations below MIC (Ambrose, et al., 2002; Ho, et al., 2001; and Schentag., 2001) . This distinction is important because traditional dosing recommendations to exceed MIC (Heffelfinger, et al., 2000) are likely to place drug concentrations inside the selection window where they will enrich resistant mutant subpopulations. Whereas low drug concentrations do not enrich resistant mutants, they do allow pathogen population expansion; consequently, low drug doses indirectly foster the generation of new mutants that will be enriched by subsequent antimicrobial challenge.

The upper boundary is also called the mutant prevention concentration (MPC) which is the drug concentration that blocks the growth of the least susceptible, single-step mutant. Above this concentration, cell growth requires the presence of two or more resistance mutations. Since two concurrent mutations are expected to arise rarely, few mutants will be amplified selectively when a susceptible population is exposed to drug concentrations that exceed the upper boundary.(Zhao, et al., 1997; Ng,1996, Pan, et al., 1996 ;and Iseman, 1994).

Depiction of the mutant selection window in term of pharmacokinetic profiles (Figure2-4) provides a framework for considering initial stages in the development resistance.
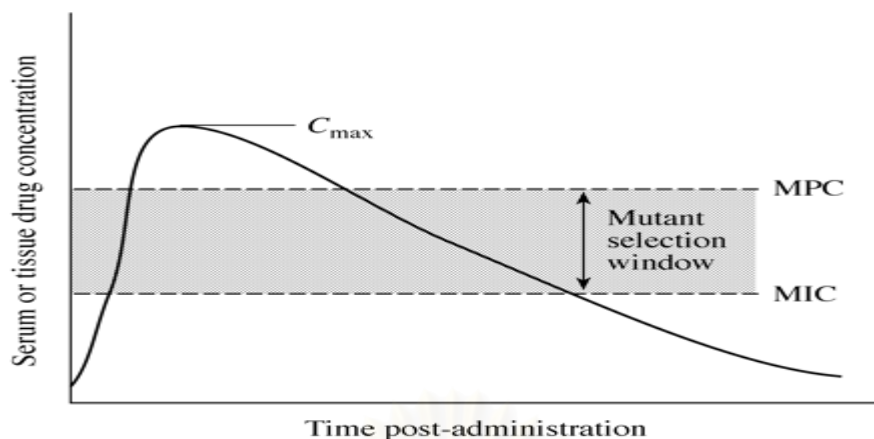
Figure 2-4. Pharmacodynamic depiction of the mutant selection window. A hypothetical pharmacokinetic profile is shown in which MIC and MPC are arbitrarily indicated. Double-headed arrow indicates the mutant selection window.

5.2 Mutant prevention concentration (MPC)

The MPC was defined as the lowest drug concentration that prevented bacterial colony formation from a culture containing $\geq 10^{10}$ bacteria (Zhao and Drlica, 2001).

The choice of 10^{10} cells is based on several considerations. First, 10^{10} is large enough for mutant subpopulations to be present for testing. Second, infections rarely contain more than 10^{10} organisms. Third, testing more cells is often logistically difficult.

5.2.1 The measurement of MPC

The measurement of MPC is performed in two general ways.

In one, cells are applied to multiple agar plates at several antimicrobial concentrations such that the total number of cells tested for a given drug concentration exceeds 10^{10}. When narrow concentration increments are used, isolated colonies can be found and counted to show that their number progressively approaches zero as drug concentration increases (mutant selection curves become steeper as MPC is approached).

In a second method, more than 10^{10} cells are placed on single agar plates that differ in drug concentration by two-fold increments. This method, which allows large numbers of isolates to be surveyed, often gives confluent growth or no growth owing to the large concentration increment. With some bacteria, the large inoculum may affect the apparent susceptibility. Correction factors for inoculum effects can be obtained by carrying out the

same experiment with smaller inocula distributed to many more plates (Blondeau, et al., 2001).

For both methods, growth at antimicrobial concentrations below MPC is confirmed by retesting colonies for growth on agar containing the selecting concentration of drug. To assure that the mutants are stable, they are grown on drug-free agar prior to retesting.

Consideration of the mutant selection window leads to the suggestion that antimicrobial concentrations between $MIC_{(99)}$ and MPC enrich mutant subpopulations selectively (standard MIC is often quantitatively similar to $MIC_{(99)}$). Such conditions may suppress most infections especially when host defences effectively eliminate pathogens(Tillotson, 2001 and Fogarty et al., 2001). However, when large numbers of patients are treated at concentrations inside the selection window, susceptibility decreases gradually. Eventually a point is reached at which the antimicrobial agent becomes ineffective. According to these ideas, restricting the development of resistance requires that antimicrobial concentrations at the site of infection be kept above MPC. If that cannot be done for a given agent–pathogen combination, the agent should be used as part of a combination therapy involving agents with different targets. Such an approach is likely to be required for plasmid-borne resistance.

Whether exceeding the MPC is sufficient to restrict the development of resistance requires clinical testing. Such tests are important because numerical considerations, such as mutation frequencies and relevant drug concentrations, could depend significantly on whether the microbes are growing on agar plates or in host organisms. Moreover, fluctuations in antimicrobial pharmacokinetics could require dosing adjustments to make MPC an effective threshold. Animal and clinical studies now seem justified, since a mutant selection window can be measured for many pathogen–antimicrobial combinations (Lu et al., 2003).

CHAPTER III

MATERIALS & METHODS

MATERIALS

1. <u>Microorganisms, Chemicals and Reagents</u>

 1.1 Microorganisms

The bacterial strains used throughout this study were *K. pneumoniae*. These bacteria were clinically isolated from patients in Siriraj Hospital during May-July year 2002. Microorganisms were susceptible to ceftazidime as tested by disk susceptibility method, which was described in the National Committee for Clinical Laboratory Standards (NCCLS), 2003. Additionally, microorganisms were resist to ampicillin but were susceptible to amoxicillin/calvulanic acid due to SHV-1 ß-lactamase which are commonly found in *K. pneumoniae* and not produce enzyme Extended-Spectrum-ß-Lactamase (ESBL) as tested by double disk diffusion method which was modified from Livermore., 1995 and Bradford.,2001. The determination of the minimum inhibitory concentration (MIC) against all clinical isolates by agar dilution method was performed according to the recommendation in NCCLS, 2003. These clinical isolates represented high, moderate, and low susceptible to ceftazidime were randomly sampling and were then examined by nitrocefin disk test to confirm β-lactamase producing ability.

 1.2 Chemicals

- Standard powders

 Ceftazidime were kindly supplied by Glaxo SmithKline. Working standard solutions were prepared immediately prior to use, as specified by the manufacturers before dilute with test broth.

- Susceptibility disks

 Ampicillin (10 μg), amoxicillin/clavulanic acid (20 μg /10 μg), ceftazidime (30 μg), cefepime (30 μg), and cefotaxime (30 μg) disks were purchased from Oxoid (Oxoid Chemicals, England). These disks were used to determine susceptibility

pattern and evaluate interaction of antimicrobial agent combination by disk

susceptibility method and double disks method, respectively.

Nitrocefin disk from BBL chemicals (Beckton Dickinson, USA) were used to

confirm β-lactamase producing ability.

Reagents

- Mueller-Hinton Agar (MHA) and Mueller-Hinton Broth (MHB) were purchased

from Oxoid (Oxoid Chemicals, England) used as the susceptibility test medium.

- MacConkey Agar was purchased from Oxoid (Oxoid Chemicals, England) used

as the media to culture *K. pneumoniae.*

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

working solution.

- Sterile normal saline solution (NSS) was chosen as the diluent of the inoculum

in turbidity adjusting processes to quantity the precise numbers of bacteria by

spectrophotometer at the wavelength 625 nanometer. This NSS also applied as

the diluent of specimens in colony counting procedures of time kill method.

- A $BaSO_4$ 0.5 McFarland standard

To standardize the inoculum density for a susceptibility test, BaSO4 turbidity

standard, equivalent to a 0.5 McFarland standard should be used. A $BaSO_4$ 0.5

McFarland standard may be prepared as follows:

- A 0.5 ml aliquot of 0.048 mol/L $BaCl_2$ (1.175 % w/v $BaCl_2$. $2H_2O$) was added
  to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a
suspension.

- The correct density of the turbidity standard should be verified by using a
  spectrophotometer with a 1-cm light path and matched cuvette to determine
  the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the
  0.5 McFarland standard.

- The barium sulfate suspension should be transferred in 4 to 6 ml aliquots into
  screw-cap tubes of the same size as those used in growing or diluting the
  bacterial inoculum.

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

- The barium sulfate turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appear, the standard should be replaced.

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

2. <u>Laboratory Equipment</u>

   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, 2003).

   - Cotton plugs were applied for glass equipment that contains inoculum and others to keep sterile environment in the containers throughout the research.

   - Aluminum foil was chosen to keep sterility in potentiation with cotton plugs.

   2.2 Steriled Glass Equipment

   - Petri dishes were practiced as agar containing plate for culture microorganisms in the whole processes such as subculture, susceptibility testing and colony counting.

   - Erlenmeyer flasks were used for the media preparation, sterile water and sterile NSS before autoclaving.

   - Cylinders were picked to measure the gross quantity of water and liquid media in preparing procedures.

   - Glass tubes were used throughout the experiments such as in the preparation of the standard solution, dilute inoculum and specimen, etc.

   - Pipettes, used in experiment divided into 2 types

     1. Glass pipettes were chosen to measure media, inoculum, drugs and solvent as general equipment processes.

2. Micropipette was used for calibrate specimens in colony counting procedures from time kill method.

2.3 General Equipment

- Chemical spoons were used as equipment to spoon and adjust the chemical powders in the weighing processes.

- The loops used in this experiment were of 2 types

    1. General loop was selected for streaking bacteria in general procedures such as subculture, inoculum preparation, etc.

    2. Standard loop was picked as measuring equipment to calibrate the specimen in time kill method before streaking specimen in solid media for colony counting process.

- Ruler was chosen for measuring the clear zone in disk susceptibility method performed by the NCCLS, 2003.

- Tube rack was 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, diluent, inoculum and others throughout the experiment for sterile condition in the research.

- Refrigerators were used to maintain bacteriostatic condition between research process and also preserved media before using in all experiments.

- Incubator was used to provide the appropriate environmental condition for bacterial growth throughout the procedures such as subculture, disk susceptibility process, inoculum preparation, etc.

- Water bath shaker was chosen to apply appropriate bacterial growth condition of liquid media that simulate human body temperatures in the time kill method.

- Hot air ovens were used to keep drying and sterilize all glass equipment before using.

3.2 General Instruments

- Chemical scale was selected for weighing media and standard powder of antimicrobial agent in preparing procedures of both test media and working standard solutions.

- Spectrophotometer, A-JUST™ turbidity meter of Abbott Laboratories, U.S.A., was applied to adjust turbidity of the inoculum to equivalent with 0.5 McFarland standard solution and 1.0 McFarland standard solution.

- Mechanical vortex mixer was used to mix 0.5 McFarland standard, inoculum and specimen, which result to homogeneity of suspension before using for further procedures in the experiment.

METHODS

1. Disk diffusion test to determine susceptibility pattern of *K. pneumoniae* to the ceftazidime (NCCLS, 2003).

2. Double disks method to detect enzyme Extended- Spectrum β-Lactamase (ESBL) production (Livermore., 1995 and Bradford.,2001).

3. Agar Dilution Method to determine minimum inhibitory concentration (MIC). (NCCLS, 2003).

4. Nitrocefin disk test to detect enzyme SHV-1 β- lactamase production of selected organisms (NCCLS,2003; Livermore and Williams, 1996).

5. Time kill method to investigate bactericidal activity of ceftazidime to *K.pneumoniae.* (Firsov, et al., 1997).

6. Study effect of ceftazidime concentrations and durations of exposure on resistant development of *K. pneumoniae.* (Chan, et al., 1999)

7. Determine mutant prevention concentration (MPC) (Blondeau, et al.,2001 and Allen, Kaatz and Rybak., 2003)

1. <u>Procedures for Performing the Disk Diffusion Test (NCCLS, 2003)</u>

1.1 Preparation of Agar Plate

1.1.1   MHA(Oxoid Chemicals, England)were prepared from a commercially available dehydrated base according to the manufacturer's instructions.

1.1.2   Immediately after autoclaving, allow it to cool in a 45 to 50 $^{\circ}$C water bath.

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

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

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

1.2 Inoculum Preparation

   Growth Method

1.2.1   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 Muller-Hinton broth(Oxoid Chemicals, England).

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

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

1.3 Inoculation Test Plates

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

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

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

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

1.4   Application of Disks to Inoculated Agar Plates

1.4.1   Ceftazidime disks were dispensed onto the surface of the inoculated agar plate. They must be pressed down to ensure complete contact with the agar surface.

1.4.2   The plates were inverted and placed in an ambient air incubator set to 37°C within 15 minutes after the disks were applied in ambient air.

1.5   Reading Plates and Interpreting Results

1.5.1   After 24 hours of incubation, the plates were examined. If the plates were satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disk. Zones were measured to the nearest whole millimeter by using a ruler, which was held on the back of the inverted petri plate. The petri plate was held a few inches above a black, nonreflecting background and illuminated with reflected light.

1.5.2   The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored. However, discrete

colonies growing within a clear zone of inhibition should be subculture, re-identified, and retest.

1.5.3 The size of the inhibition zone were interpreted by referring to the NCCLS, 2003 and the organisms were reported as either susceptible, intermediate, or resistant to the agents that have been tested (Tables 3-1).

Table 3-1 Zone diameter interpretive standards breakpoints for Enterobacteriaceae (NCCLS, 2003)

Drug	Disk content	Zone diameter (mm)		
		R[a]	I[b]	S[c]
Ceftazidime	30 μg	≤14	15-17	≥18
Ampicillin	10 μg	≤13	14-16	≥17
Amoxicillin/clavulanic	20/10 μg	≤13	14-17	≥18

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

2. Double disks method (Livermore; 1995, Bradford; 2001)

The resistance to ampicillin is due primarily to the production of penicillinase type β- lactamase SHV-1 in *K. pneumoniae*. Theses β- lactamase are inhibited by clavulanic acid and belong to the functional Bush group 2b. (Bush, Jacoby and Mederios; 1995). *K. pneumoniae* strains with a positive β- lactamase SHV-1 production must resistance to ampicillin but susceptible to amoxicillin/clavulanic acid as tested by disk susceptibility method modified from NCCLS, 2003.

Enzyme Extended- Spectrum- β Lactamase (ESBL) belong to the functional Bush group 2be and are derived from functional Bush group 2b enzymes TEM-1, TEM-2 and SHV-1.These enzymes are capable of hydrolyzing the oxyimino cephalosporins and aztreonam and are inhibited by clavulanic acid. Then ESBL production must show enhanced zone of inhibition between amoxicillin/clavulanic acid and oxyimino cephalosporin disks (the clavulanic acid diffuses out from amoxicillin/ clavulanic acid disk and inhibit ESBL produced by the organisms) as tested by double disk method modified from Livermore; 1995, Bradford; 2001.

2.1 Preparation of Agar Plate as mentioned at 1.1 page 28

2.2 Inoculum Preparation as mentioned at 1.2 page 29

2.3 Inoculation Test Plates as mentioned at 1.3 page 29

2.4 Application of Disks to Inoculated Agar Plates

    2.4.1 Amoxicillin/clavulanic acid disk was placed in the middle of inoculated plate. Ampicillin, ceftazidime, cefotaxime, and cefepime disks were placed around in distance from amoxicillin/clavulanic acid disk 20-30 mm from center to center. Each disk must be pressed down to ensure complete contact with the agar surface. 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 ambient air incubator set to 37°C within 15 minutes after the disks were applied in ambient air for 24 hours before measuring the shape zones of inhibition.

    2.4.3 Reading Plates and Interpreting Results

        2.4.3.1 After 24 hours of incubation, each plate was examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be clear and there will be a confluent lawn of growth.

        2.4.3.2 The size of inhibition zone of ampicillin and amoxicillin/clavulanic acid were interpreted by referring to NCCLS, 2003 (Table 3-1) if organisms resist to ampicillin but susceptible to amoxicillin/ clavulanic acid production of SHV-1 β- lactamase is inferred.

        2.4.3.3 Detection enzyme ESBL production by looking for enhanced zone between amoxicillin/clavulanic acid and cephalosporin disks as shown in figure 3-1.
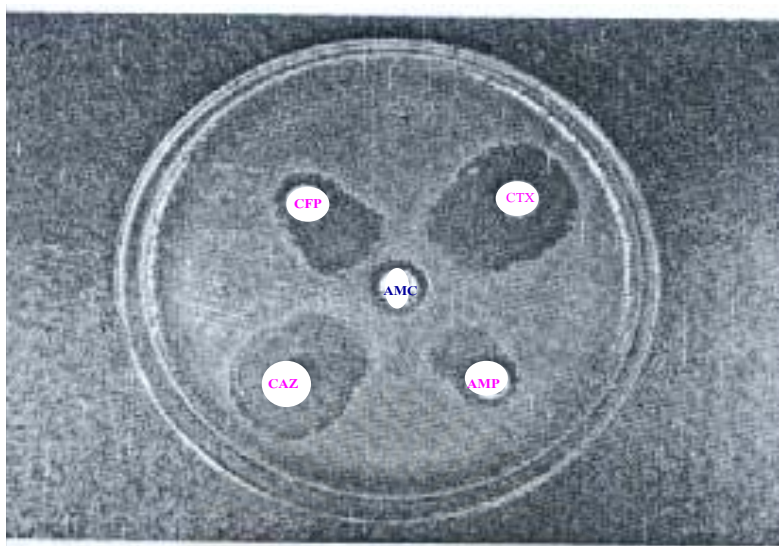
Figure 3-1 Assessment of enzyme ESBL production with double disks technique

(Amc-Amoxicillin/clavulanic acid, AMP-Ampicillin, CAZ-Ceftazidime,

CTX- Cefotaxime, CFP-Cefepime)

3. **Agar dilution method (NCCLS, 2003)**

3.1   Preparation of agar dilution plates

3.1.1   1 ml of each appropriate two fold dilution concentration (0.15-80 µg/ml of

ceftazidime solutions are pipetted into glass, flat-bottomed Petri dishes.

3.1.2   MHA were prepared from a commercially available dehydrated base

according to the manufacturer's instructions.

3.1.3   Immediately after autoclaving, allow it to cool in a 56 $^{\circ}$C water bath

and then pour 9 ml of the freshly prepared and cooled medium into

plates that contain 1 ml of ceftazidime solution.

3.1.4   The agar and ceftazidime solution were mixed thoroughly.

3.1.5   The agar dilution plates are allowed to solidity at room temperature, and

used immediately.

3.2   Inoculum Preparation

3.2.1   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 test broth medium.

3.2.2   The broth culture was incubated at 37°C until it achieved or exceeded the

turbidity of 0.5 McFarland standard (usually 2 to 6 hours).

    3.2.3   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 result in a suspension containing approximately 1 to 2 x 10^8 CFU/ml. A-JUST™ turbidity meter of Abbott Laboratories, U.S.A. is a photometric device used to perform this step propriety.

    3.2.4   The 0.5 McFarland suspension should be diluted 1: 10 in sterile broth or saline to obtain a concentration of 10^7 CFU/ml.

    3.2.5   Inoculumn replicators deposit approximately 1 to 2 µl on the agar surface. The final inoculumn on the agar will then be approximately 10^4 CFU per spot.

3.3   Inoculating agar dilution plates

    3.3.1   The tubes containing the adjusted and diluted bacterial suspension (10^7 CFU/ml) should arranged in order in a rack. An aliquot of each well-mixed suspension is placed into the corresponding well in the replicator inoculumn block.

    3.3.2   The agar plates are marked for orientation of the inoculumn spots.

    3.3.3   A 1 µl of each inoculumn is applied to the agar surface by the use of an inocula-replicating device.

    3.3.4   A growth-control plate (no antimicrobial agent) is inoculated first and then, starting the lowest concentration, the plates containing the different ceftazidime concentrations are inoculated. A second growth control plate Is inoculated last to ensure that there was no contamination or significant antimicrobial carry-over during the inoculation.

3.4   Incubating agar dilution plates

The inoculated plates are allowed to stand at room temperature until the moisture in the inoculumn spots has been absorbed into the agar until the spots are dry, but no more than 30 minutes. The plates are inverted and incubated at 37 $^\circ$C for 24 hours.

3.5   Determining agar dilution end points

The plates should be placed on a dark nonreflecting surface to determine

the end points. The MIC is recorded as the lowest concentration of
antimicrobial agent that completely inhibits growth, disregarding a single
colony or a faint haze caused by the inoculum.

4. **β-Lactamase Detection (Nitrocefin-disk Test) NCCLS,2003; Livermore and Williams, 1996).**

The selected microorganisms were confirmed to produce β-lactamase by
nitrocefin-based test as mentioned in the NCCLS,2003; Livermore and Williams, 1996.

4.1 Apply sterile water on nitrocefin disk until it wet.

4.2 The top of 1-2 well- isolated colonies were touched with a loop and transferred
on nitrocefin disk.

4.3 β-lactamase activity was indicated by color changing from yellow to red color.
This usually appears within 1 to 2 minutes.

5. **Bactericidal Activity Test by Time Kill Method (Firsov, et al., 1997).**

5.1 Prepare ceftazidime concentrations at MIC, 2MIC, 4MIC, 8MIC and Cmax,
Caverage , Cmin (70, 35, 4 µg/ml) that referred to pharmacokinetic achievable
concentration from previously published articles (AHFS Drugs information, 2001;
http:// www. Drugs.com/ Fortaz) to study effect of concentration on
eradication and prepare concentrations at every half-life 0, 2, 4, 6, 8 hours (70,
35, 17, 8, 4 µg/ml) to effect of duration of exposure on eradication.

5.2 Dilute the standardized inoculum to obtain the final bacterial quantity 5×10^5
CFU/ml into working media and control tubes containing broth without
antimicrobial agents on water bath shaker at 37°C

5.3 Collect the samples to detect for colony forming unit at the time 0,1,2,4,6 and 24
hours after microorganism exposed to drug in each concentration including the
control group.

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

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

5.6   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 $\geq$ 3 logCFU/ml at 24 hours of exposure. The regrowth is defined as an increase of $\geq$ 2 logCFU/ml after $\geq$ 6 hours. (Amsterdam, 1996; Pankuch, Jacobs and Appelbaum, 1994; Satta, et al., 1995).The quantitative evaluation of antimicrobial effect was calculated as in the published article (Firsov, et al.,1997).

The Quantitative Evaluation of Antimicrobial Effect

1. The following parameters were estimated by extrapolation of the killing curves as shown in Figure 3-2.

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

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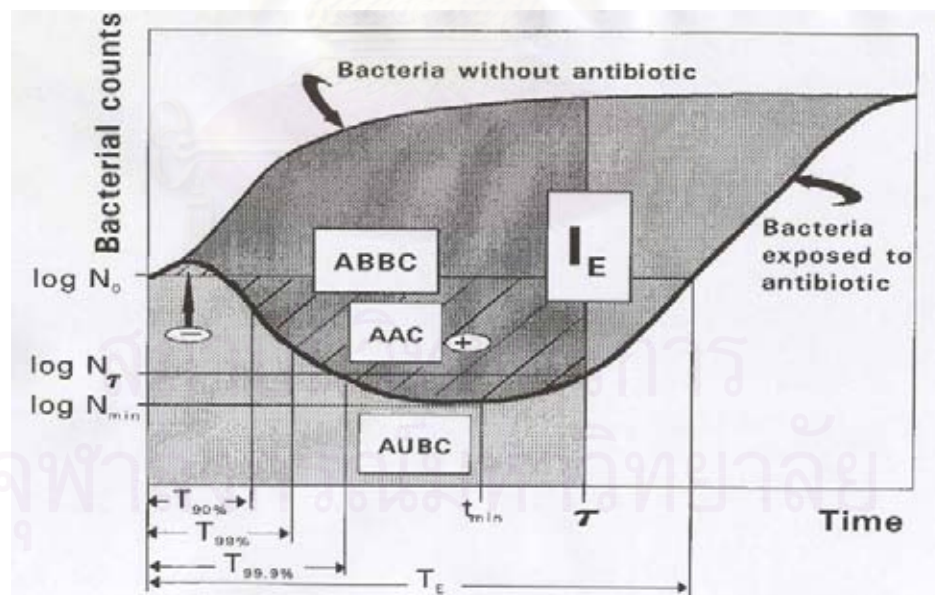

Figure 3-2   Parameters for quantifying bacterial killing and regrowth curve and the antimicrobial effect.

(Modified from Firsov, et al., 1997)

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

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

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

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)

.

6. Study on the effect of concentration and duration of exposure on resistant development of *K. pneumoniae* to ceftazidime

Method A (modified from Chan, et al., 1999)

Method A was microbiology method or daily passage that *K. pneumoniae* strains were exposed to the constant ceftazidime concentration every 24 hr.

6.1 Inoculum preperation

6.1.1 At least three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 10 ml of a test broth medium.

6.1.2 The broth culture was the adjusted   to obtain turbidity optically comparable to that of the 1 McFarland standard. This result in a suspension containing approximately 3×10^8 CFU/ml and incubated overnight (18-24 hours).

6.1.3 After an overnight incubation ; cultures were then concentrated by

centrifugation (3,000 x g) for 30 minutes to change suspending bacterial cells to be sediments to yield concentration $\geq 10^{10}$ CFU/ml.

6.2   Glass tubes ; each containing 5 ml of Mueller-Hinton broth and were initially inoculated with approximately 10^{10} CFU/ml at ceftazidime concentration 1/2MIC, MIC, 2MIC, 4MIC, 8MIC, 16MIC, 32MIC and 64MIC.

6.3   The tubes were incubated at 37 $^{\circ}$C for 24 hours.

6.4   For each daily passage; an inoculum in each tubes were centrifuged at 3000 x g for 30 minutes and then were applied on ceftazidime containing agar plates for detect resistant colonies.

6.5   Determine minimum inhibitory concentration (MIC) for resistant colonies until MIC $\geq$ 32 µg/ml that was resistant breakpoint as referred in NCCLS,2003 or until 30 daily passages.

6.6   Inoculumn was re-inoculated into 5 ml of Mueller-Hinton broth containing constant ceftazidime concentration and the culture was again incubated overnight at 37°C

Method B

Method B or drug administration cycle simulated pharmacokinetic achievable concentration that less susceptible strain (KN280) was exposed to C max at the beginning and decreased a half at every half-life (2 hr) and exposed to C max again every 8 hr(dosing interval).

6.1   Inoculum preparation as mentioned in 6.1 page 37.

6.2   Glass tube ; containing 2 ml of Mueller-Hinton broth and were initially inoculated with approximately 10^{10} CFU/ml at ceftazidime concentration 70 µg/ml (C max) and then incubated 2 hours (half-life).

6.3   After incubated; filled Mueller-Hinton broth 2 ml in this tube for diluted cultures to have ceftazidime concentration 35 µg/ml and incubated 2 hours.

6.4   Make procedures like this pattern by filling Mueller-Hinton broth amount equal in the tube every 2 hours until 8 hours (dosing interval). An inoculum in the tube were centrifuged at 3000 x g for 30 minutes and then were applied on McConkey agar plates and determinined MIC for growth colonies.

6.5   Determine minimum inhibitory concentration (MIC) for growth colonies every

dosing intervals until organisms were eradicated or until MIC $\geq$ 32 µg/ml that was

resistant breakpoint as referred in NCCLS,2003 or until 30 dosing intervals.

7. Determination of the mutant prevention concentration (MPC)

(Modified from Blondeau, et al.,2001 and Allen, Kaatz and Rybak., 2003)

7.1   Inoculum preperation

7.1.1   At least three to five well-isolated colonies of the same morphological

type were selected from an agar plate culture. The top of each colony was

ouched with a loop, and the growth was transferred into a tube

containing 10 ml of a test broth medium.

7.1.2   The broth culture was the adjusted   to obtain turbidity optically comparable

to that of the 1 McFarland standard. This result in a suspension containing

approximately 3 x 10^{8} CFU/ml and incubated overnight (18-24 hours).

7.1.3   After an overnight incubation ; cultures were then concentrated by

centrifugation (14000 x g) for 5 minutes to change suspending bacterial

cells to be sediments to yield concentration $\geq$ 10^{10} CFU/ml.

7.2   Aliquot 100 µl containing $\geq$ 10^{10} CFU were applied to McConkey containing

ceftazidime concentration two-fold dilution plates.

7.3   Inoculated plates were incubated at 37 $^{\circ}$C for 96 hours and then screened for

growth.

7.4   MPC was recorded as the lowest concentration completely inhibiting bacterial

growth.

CHAPTER IV

RESULT

1. <u>Susceptibility testing</u>

   Microorganisms was resistant to ampicillin but susceptible to amoxicillin/clavulanic acid due to SHV-1 ß-lactamase which are commonly found in *K.pneumoniae* and not produce ESBL.

   Agar dilution method was used to assess the MIC for clinical strains of *K. pneumoniae* to ceftazidime. The MIC for the three selected strains are lower than the susceptible level of interpretive guidelines (NCCLS, 2003) that were highly, moderately and less susceptible as shown in table 4-1 and confirmed ß-lactamase production by nitrocefin disk test .

Table 4-1 The MICS of ceftazidime to selected *K.pneumoniae* strains.

K.pneumoniae strains NO.	MIC (µg/ml)	Susceptible level	Nitrocefin test
KN 246	0.125	High	Positive
KN 012	0.5	Moderate	Positive
KN 280	2	Low	Positive

2. <u>In vitro effect of ceftazidime at difference concentration on eradication of</u> <u>K.pneumoniae</u>

   Time kill study was exercised   to study this effect.

   2.1 <u>At concentration 1MIC-8MIC</u>

   Ceftazidime had bactericidal property at 4MIC-8MIC for highly susceptible strain (KN246) and the regrowth were detected at every concentration as shown in figure 4-1 A. Whereas, every concentration did not have bactericidal property for moderately susceptible strain (KN012) and less susceptible strain (KN280). The regrowth of moderately susceptible strain

(KN012)   was suppressed when expose to ceftazidime 8MIC while less susceptible strain (KN280) needed concentration at 4MIC to suppress it.(Figure4-1 B and C).

The antimicrobial effect quantitatively evaluated in bacterial killing and regrowth curves demonstrated that, highly susceptible strain (KN246) exhibited T99.9% at 5.80 hr and 5.95 hr when expose to concentration at 4MIC and 8MIC respectively except concentration at 1MIC and 2MIC exhibited T99.9% at >24 hr. For moderately susceptible strain (KN012)   and less susceptible strain (KN280) when exposed to every concentration   exhibited T99.9% at >24 hr. Bacteriolytic area increased when exposed to increasing concentration for three stratins. (Table4-2)

2.2 At concentration C max, C average and C min

C max and C average had bactericidal property for every strains . C min had bactericidal property only for highly susceptible strain (KN246) whereas had bacteriostatic property for moderately susceptible strain (KN012)   and less susceptible strain (KN280) (Figure 4-2) .Every concentration suppressed regrowth of every strains.

The antimicrobial effect quantitatively evaluated in bacterial killing and regrowth curves demonstrated that bacteriolytic area of C max more than C average and C min respectively. (Table 4-3).
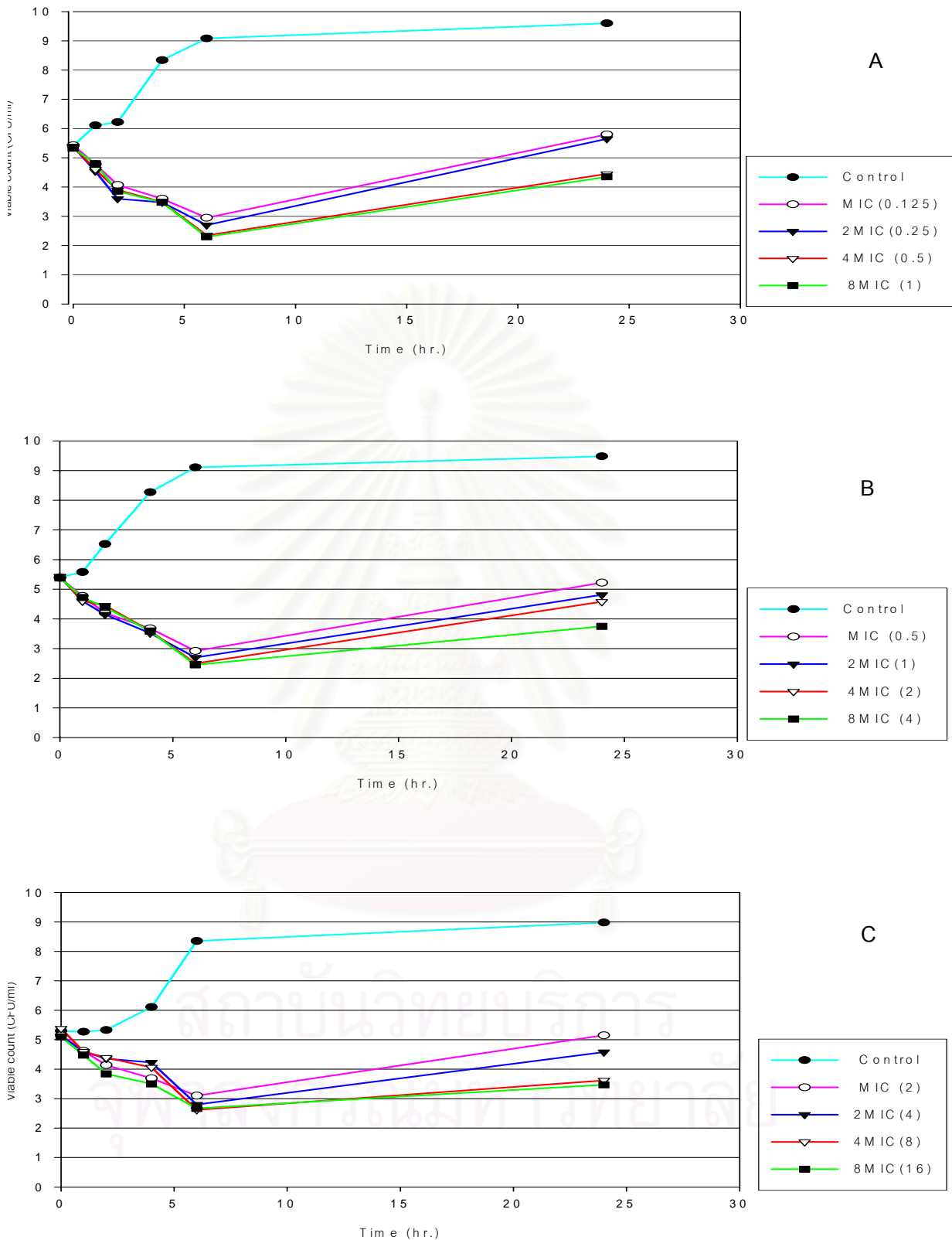
Figure 4-1. Time kill curves of different ceftazidime concentrations (1MIC-8MIC) against

highly susceptible strain: KN 246 (A), moderately susceptible strain: KN 012 (B), and

less susceptible strain: KN 280 (C).

Table 4-2. Antibacterial effect measures for each strain of *Klebsiella pneumoniae* with each ceftazidime concentration (1, 2, 4, 8 MIC)

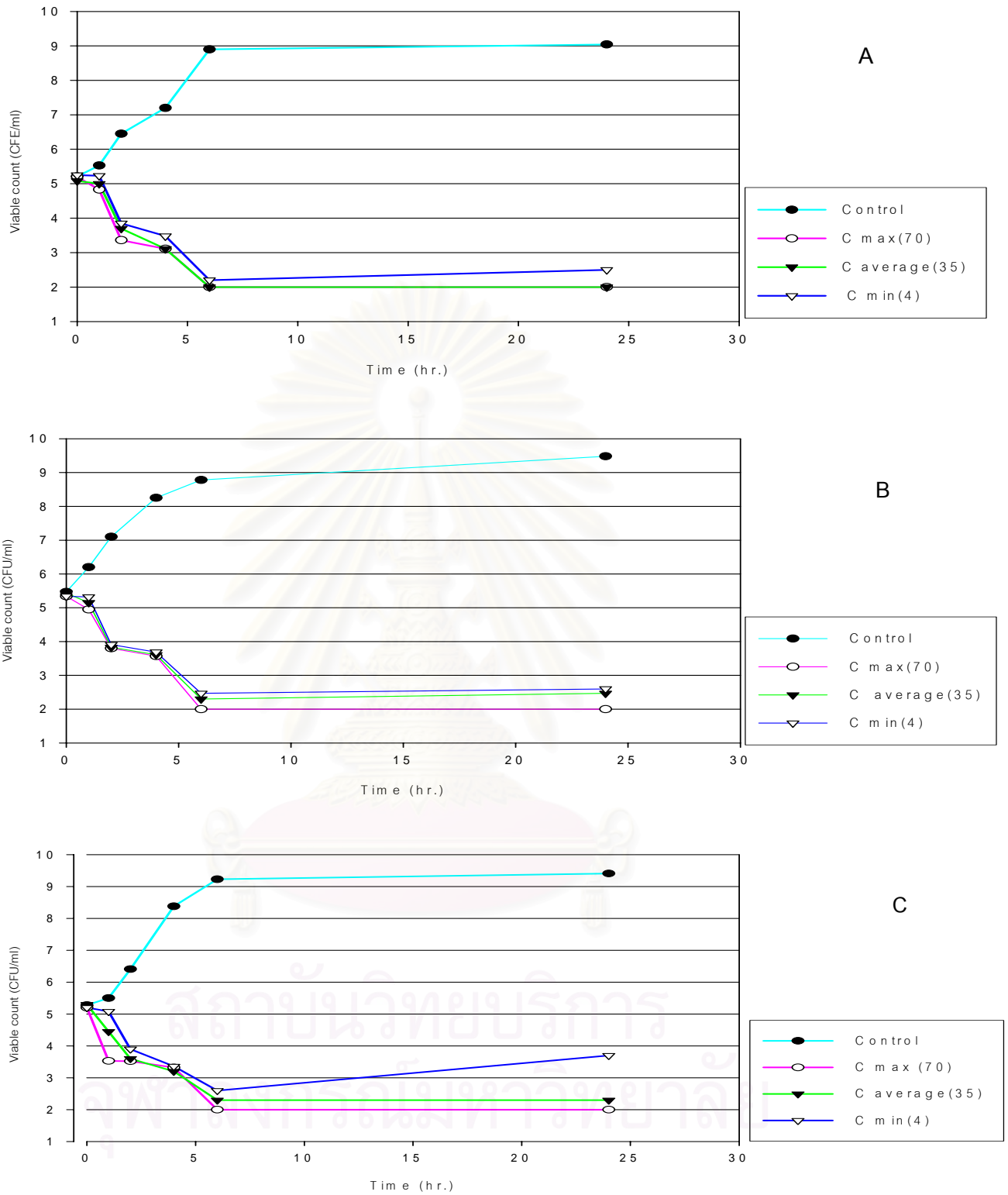| *K. Pneumoniae* strain (MIC;µg/ml)	Conc. (MIC)	Conc. (ug/ml)	Viable count (log CFU/ml) Δ max	Δ24	AUBKC 0-24 (logCFU/ml.h)	Bacteriolytic area (logCFU/ml.h)	T 99.9% (hr.)	T min (hr.)
N 246 (0.125)	1	0.125	2.45	2.85	102.47	109.25	>24	6
	2	0.25	2.70	2.95	97.40	114.32	>24	6
	4	0.5	3.05	2.10	83.72	128.01	5.80	6
	8	1	3.10	2.05	82.16	129.56	5.95	6
N 012 (0.5)	1	0.5	2.48	2.30	97.32	114.08	>24	6
	2	1	2.70	2.11	91.06	120.34	>24	6
	4	2	2.90	2.08	87.74	123.36	>24	6
	8	4	2.95	1.30	79.61	137.79	>24	6
N 280 (2)	1	2	2.20	2.05	98.16	94.40	>24	6
	2	4	2.50	1.78	91.52	101.64	>24	6
	4	8	2.68	1.00	81.06	111.44	>24	6
	8	16	2.63	0.80	77.71	114.79	>24	6

Figure 4-2. Time kill curves of different ceftazidime concentrations (C max, C average, and C min) against highly susceptible strain: KN 246 (A), moderately susceptible strain: KN 012 (B), and less susceptible strain: KN 280 (C).
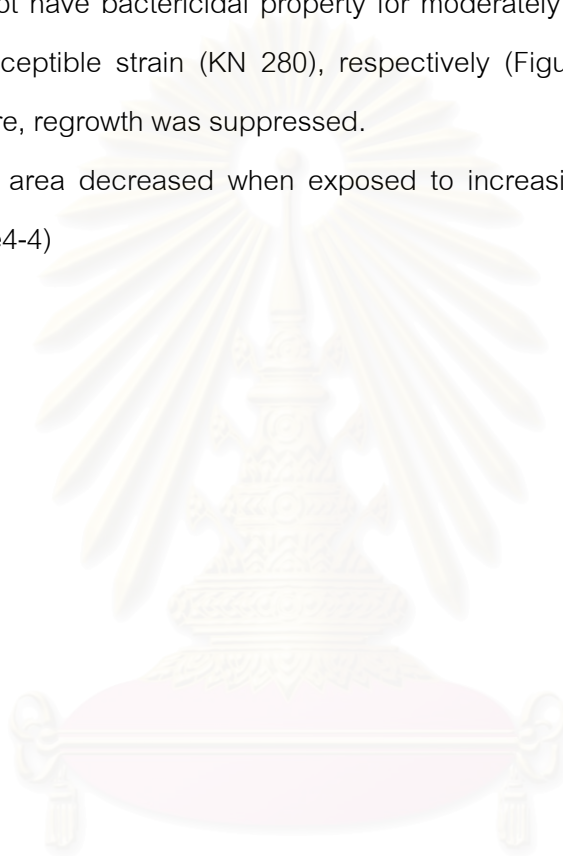
Table 4-3. Antibacterial effect measures for each strain of *Klebsiella pneumoniae* with each ceftazidime concentration (C max, C ave, C min)

K. Pneumoniae strain (MIC;µg/ml)	Conc.	Conc. (ug/ml)	Viable count (log CFU/ml) Δ max	Δ 24	AUBKC 0-24 (logCFU/ml.h)	Bacteriolytic area (logCFU/ml.h)	T 99.9% (hr.)	T min (hr.)
N 246 (0.125)	C max	70	3.20	0.00	56.14	206.73	5.62	6
	C ave	35	3.08	0.00	56.70	206.17	5.84	6
	C min	4	3.00	0.30	64.41	198.46	5.90	6
N 012 (0.5)	C max	70	3.34	0.00	57.86	151.43	5.39	6
	C ave	35	3.09	0.17	61.22	148.07	5.73	6
	C min	4	2.87	0.13	67.41	141.88	>24	6
N 280 (2)	C max	70	3.20	0.00	56.88	154.94	5.62	6
	C ave	35	3.00	0.00	62.60	149.25	6.00	6
	C min	4	2.30	0.80	82.02	129.83	>24	6

3. In vitro effect of ceftazidime at difference exposure times (0 t1/2- 4 t1/2) on eradication of *K. pneumoniae.*

Highly susceptible strain (KN246) exposed to ceftazidime 4 half-life (8 hr) to exibit bactericidal property and regrowth was suppressed as shown in figure 4-3 A. When duration of exposure more than 2 and 1 half-life (4 and 2 hr), decreasing concentration did not have bactericidal property for moderately susceptible strain (KN 012) and less susceptible strain (KN 280), respectively (Figure 4-3 B and C). All durations of exposure, regrowth was suppressed.

Bacteriolytic area decreased when exposed to increasing exposure times for three stratins. (Table4-4)
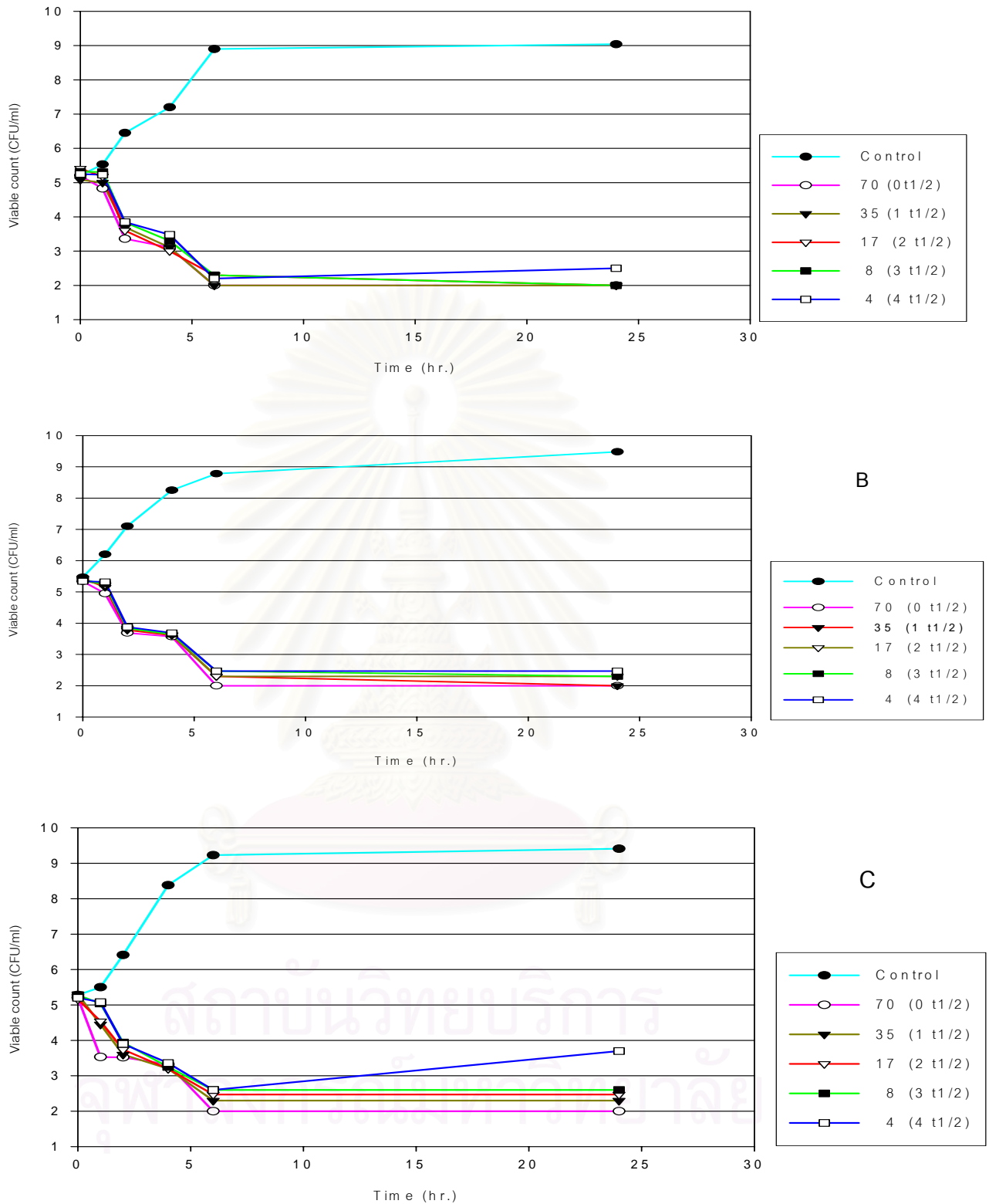
Figure 4-3. Time kill curves of ceftazidime at different exposure times (0 t1/2- 4 t1/2) against highly susceptible strain: KN 246 (A), moderately susceptible strain: KN 012 (B), and less susceptible strain: KN 280 (C).

Table4-4. Antibacterial effect measures for each strain of *Klebsiella pneumoniae* with ceftazidime concentration at 0,1,2,3,4 half-life

K. Pneumoniae strain (MIC;µg/ml)	Conc. (t1/2)	Viable count (log CFU/ml)		AUBKC 0-24 (logCFU/ml.h)	Bacteriolytic area (logCFU/ml.h)	T 99.9% (hr.)	T min (hr.)
		Δ max	Δ 24				
N 246 (0.125)	70(0)	3.20	0.00	56.14	206.73	5.62	6
	35(1)	3.08	0.00	56.70	206.17	5.84	6
	17(2)	3.11	0.30	59.61	203.26	5.79	6
	8(3)	3.02	0.30	60.60	202.27	5.96	6
	4(4)	3.00	0.30	64.41	198.46	5.90	6
N 012 (0.5)	70(0)	3.34	0.00	57.86	151.43	5.39	6
	35(1)	3.09	0.17	61.22	148.07	5.73	6
	17(2)	3.09	0.30	63.98	145.31	5.28	6
	8(3)	2.90	0.13	69.71	143.53	>24	6
	4(4)	2.87	0.13	67.41	141.88	>24	6
N 280 (2)	70(0)	3.20	0.00	56.88	154.97	5.62	6
	35(1)	3.00	0.00	62.60	149.25	6.00	6
	17(2)	2.68	0.43	65.97	145.88	>24	6
	8(3)	2.68	0.00	69.06	142.79	>24	6
	4(4)	2.30	0.80	82.02	129.83	>24	6

4. In vitro effect of ceftazidime concentration and duration of exposure on resistant development of *K. pneumoniae*

There were 2 methods to study ceftazidime concentration and duration of exposure on resistant development of *K. pneumoniae* ; method A was microbiology method or daily passage that *K. pneumoniae* strains were exposed to the constant ceftazidime concentration every 24 hr whereas method B or administration cycle simulated pharmacokinetic achievable concentration that less susceptible strain (KN280) was exposed to C max at the beginning and decreased a half at every half-life and exposed to C max again every 8 hr(dosing interval).

4.1 Effect of ceftazidime concentrations and duration of exposure on resistant development of less susceptible strain (KN 280) ; MIC = 2 µg/ml (method A)

Ceftazidime at 64 MIC did not select resistant mutants and organisms were eradicated at day 5 (Figure 4-4). At 1/2MIC-32MIC had efficacy to select resistant mutants as shown in table 4-5. Concentration at 32 MIC used duration of exposure 9 days to develop resistance as same as 1MIC-16MIC but these concentrations reduced susceptibility at day 8 before increased MIC to resistance breakpoint (32 µg/ml) at day 9. (Figure 4-5 to 4-10). Concentration at 1/2MIC reduced susceptibility at day 7 that faster than the others but used more duration of exposure to develop resistance (Figure 4-11). It demonstrated that low concentration reduced susceptibility before high concentration and used more steps to develop resistance .Furthermore double disk method showed resistance mechanism of KN280 to ceftazidime were ESBL production as shown in figure 4-12.

Table 4-5 Effect of ceftazidime concentration and duration of exposure on selection

resistant mutants of less susceptible strain (KN280) ; MIC = 2 µg/ml

Exposure ceftazidime		Selection resistant mutants	
Conc. (µg/ml)	Fold of MIC	Cycle increase in MIC	Increasing MIC (µg/ml)
128	64	ND	ND
64	32	9	32*
32	16	8	8
		9	32*
16	8	8	16
		9	32*
8	4	8	16
		9	32*
4	2	8	8
		9	32*
2	1	8	8
		9	32*
1	1/2	7	8
		9	16
		11	32*

ND = not detected (not changable MIC and organisms were eradicated at cycle 5)

  * = resistant breakpoint as referred in NCCLS, 2003 and ESBL positive for resistant

   mutants

Fig 4-4 Not changeble MIC when KN 280 expose to ceftazidime concentration 128ug/ml (64MIC) and organisms were eradicated at day 5
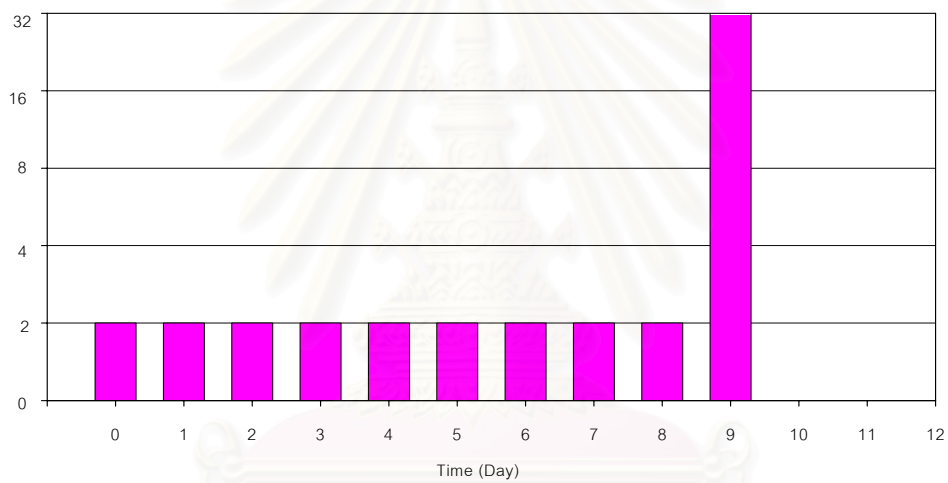
Fig 4-5 Increasing MIC when KN 280 expose to ceftazidime concentration 64 ug/ml (32MIC)
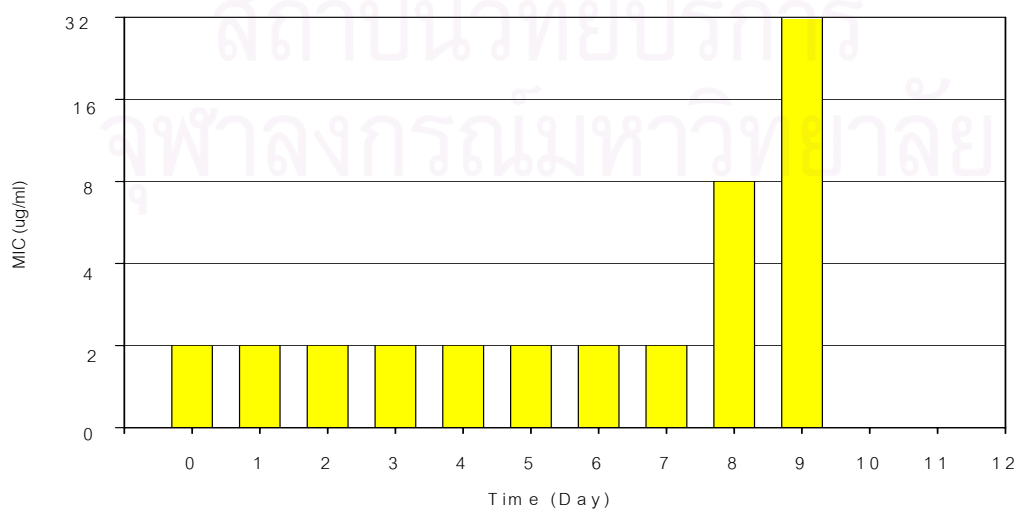
Fig 4-6 Increasing MIC when KN 280 expose to ceftazidime concentration 32ug/ml (16MIC)
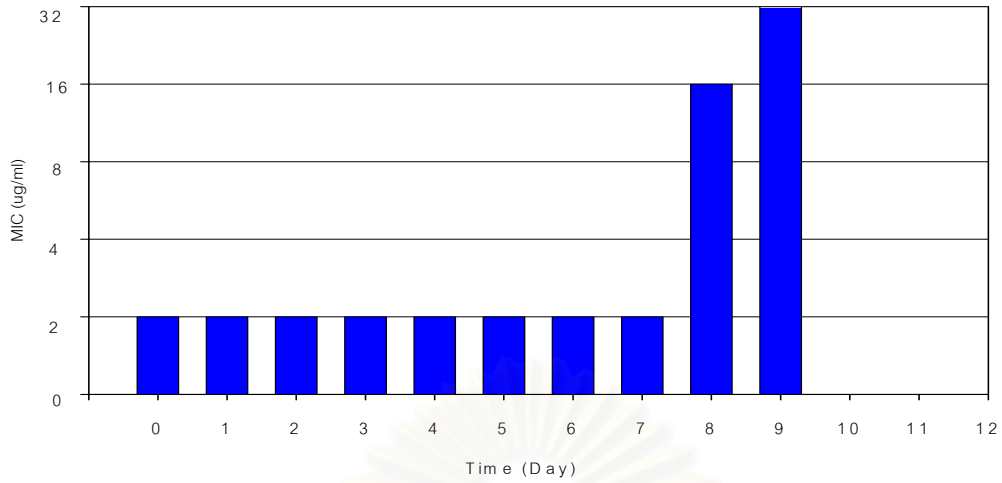
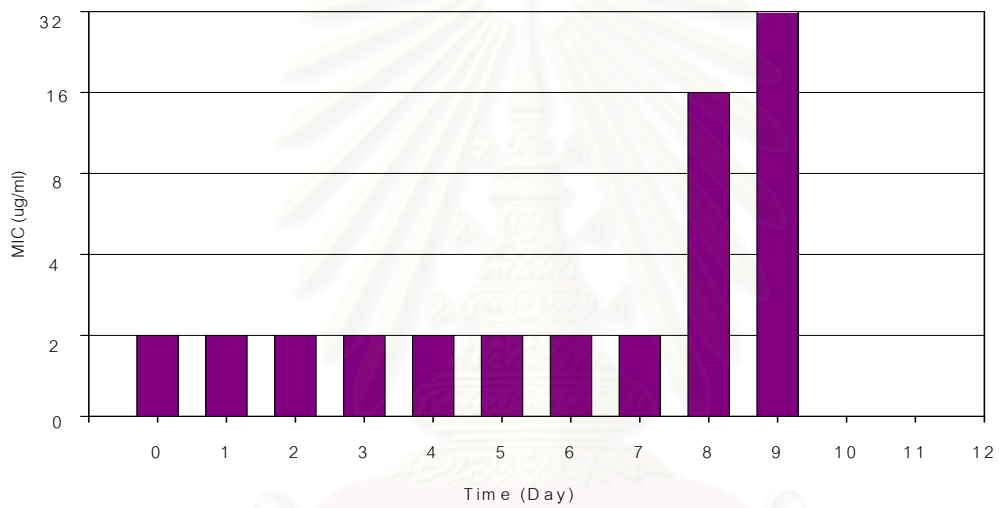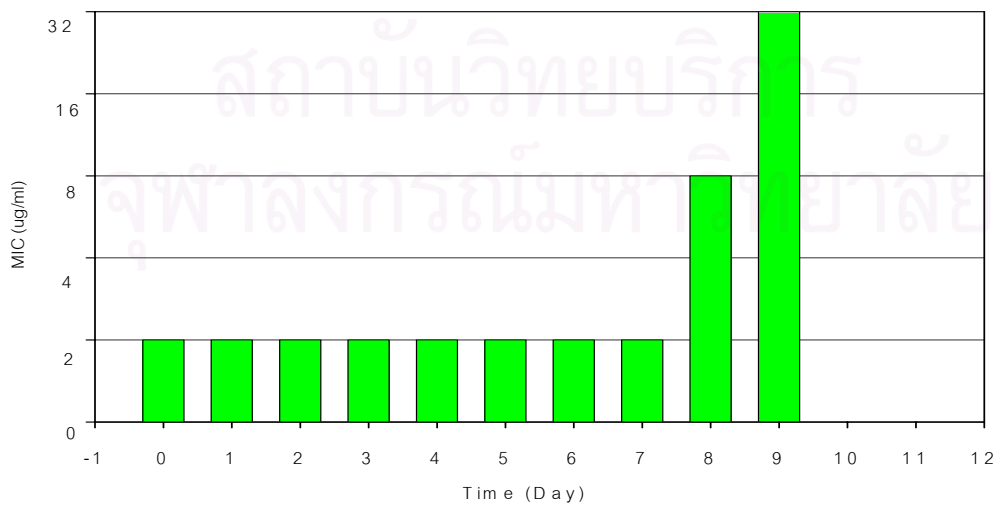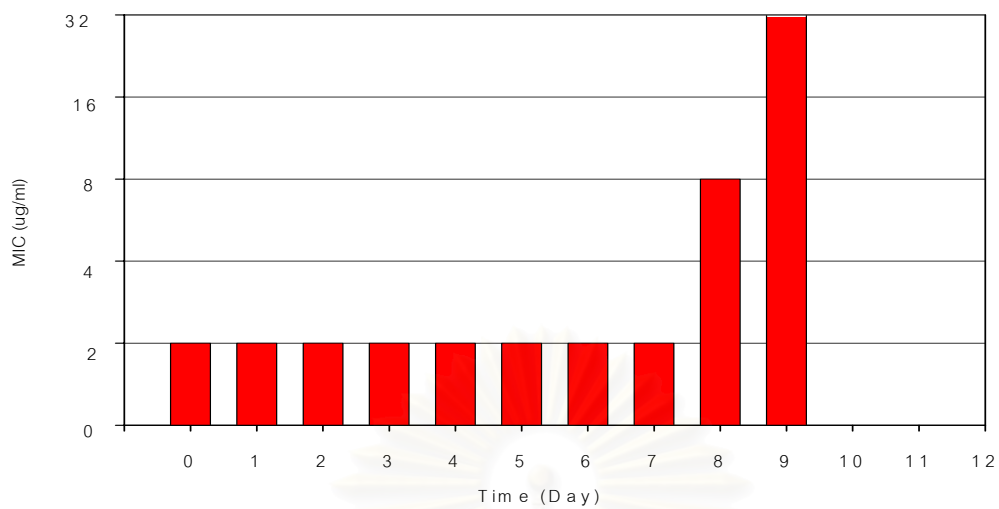Fig 4-7 Increasing MIC when KN 280 expose to ceftazidime concentration 16ug/ml (8MIC)

Fig 4-8 Increasing MIC when KN 280 expose to ceftazidime concentration 8ug/ml (4MIC)

Fig 4-9 Increasing MIC when KN 280 expose to ceftazidime concentration 4ug/ml (2MIC)

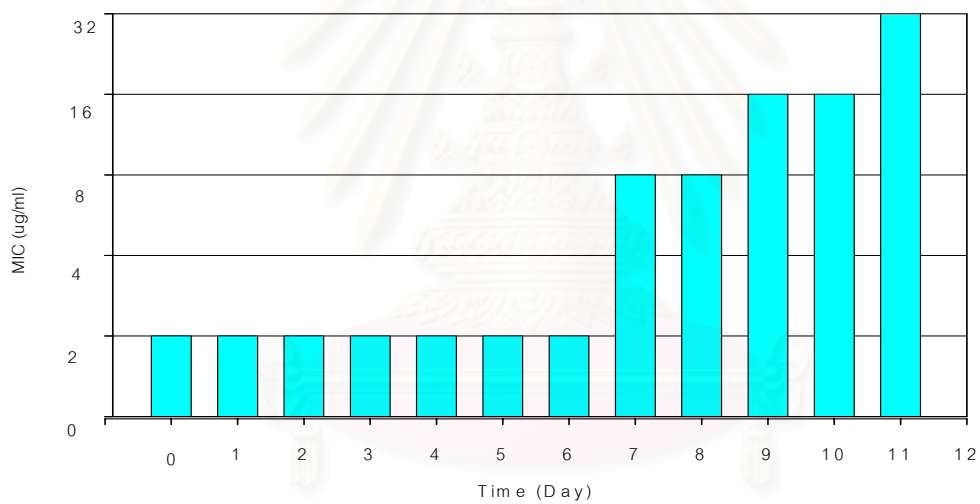Fig 4-10 Increasing MIC when KN 280 expose to ceftazidime concentration 2ug/ml (1MIC)

Fig 4-11 Increasing MIC when KN 280 expose to ceftazidime concentration 1ug/ml (1/2MIC)
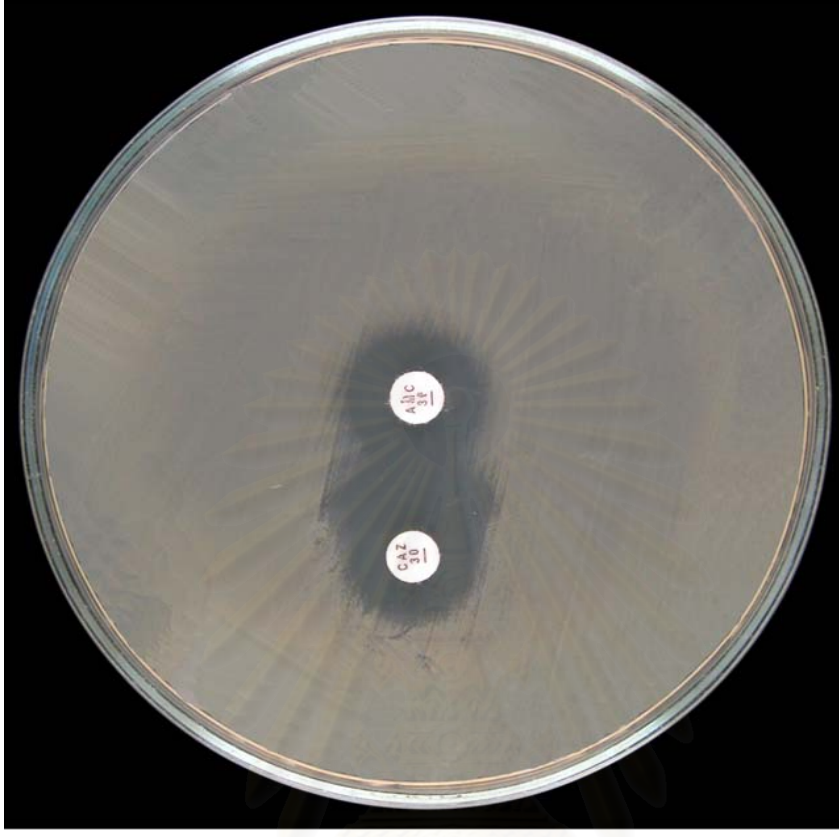
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Fig 4-12 Assessment of ESBL production with double disks technique of original less susceptible strain; KN280 (A) and resistant mutants (B)

4.2 Effect of ceftazidime concentrations and duration of exposure on resistant development of moderately susceptible strain (KN 012) ; MIC = 0.5 µg/ml (method A)

Ceftazidime with 32MIC-64MIC did not select resistant mutants and organisms were eradicated at day 5 and 4, respectively. Concentration at 1/2MIC-16MIC had efficacy to select resistant mutants as shown in table 4-6. Concentration at 2MIC-16MIC reduced susceptibility at day 7 (Figure 4-15 to 4-18) .Concentration at 8MIC and 16MIC increased MIC to resistance breakpoint (32 µg/ml) at day 19 and 18, respectively that faster than 2MIC and 4MIC that MIC = 32 µg/ml at day22. Similarly, concentration at 1/2MIC and 1MIC increased MIC to resistance breakpoint (32 µg/ml) at day 22 but reduced susceptibility at day 6 (Figure 4-19 and 4-20). It demonstrated that low concentration reduced susceptibility before high concentration and used more steps to develop resistance. Furthermore double disk method showed resistance mechanism of KN012 to ceftazidime were ESBL production as shown in figure 4-21.

Table 4-6 Effect of ceftazidime concentration and duration of exposure on selection

resistant mutants of moderately susceptible strain (KN012) ; MIC = 0.5 µg/ml

Exposure ceftazidime		Selection resistant mutants	
Conc. (µg/ml)	Fold of MIC	Cycle increase in MIC	Increasing MIC (µg/ml)
32	64	ND[1]	ND[1]
16	32	ND[2]	ND[2]
8	16	7	8
		18	32*
4	8	7	4
		14	16
		19	32*
2	4	7	4
		10	8
		17	16
		22	32*
1	2	7	2
		9	4
		15	8
		18	16
		22	32*
0.5	1	6	2
		10	4
		13	8
		20	16
		22	32*
0.25	1/2	6	1
		7	4
		15	8
		19	16
		22	32*

ND[1] = not detected (not changable MIC and organisms were eradicated at cycle 4)

ND[2] = not detected (not changable MIC and organisms were eradicated at cycle 5)

 *   = resistant breakpoint as referred in NCCLS, 2003 and ESBL positive for resistant mutants
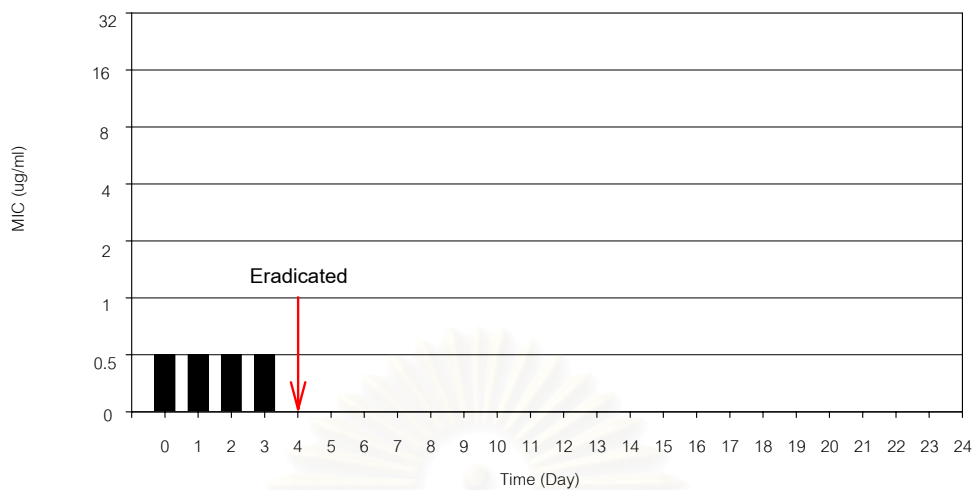
Fig 4-13 Not changeable MIC when KN012 expose to ceftazidime concentration 32 ug/ml (64MIC) and organisms were eradicated at day 4
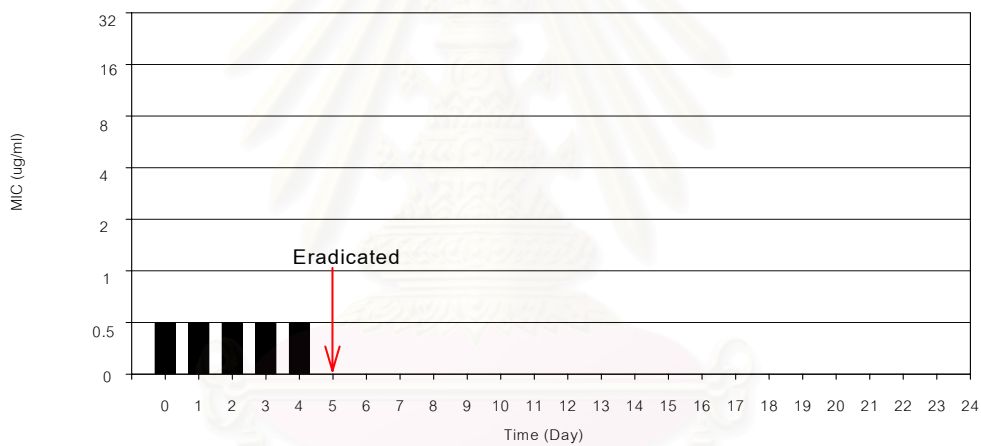

Fig 4-14 Not changeable MIC when KN012 expose to ceftazidime concentration 16 ug/ml (32MIC) and organisms were eradicated at day 5
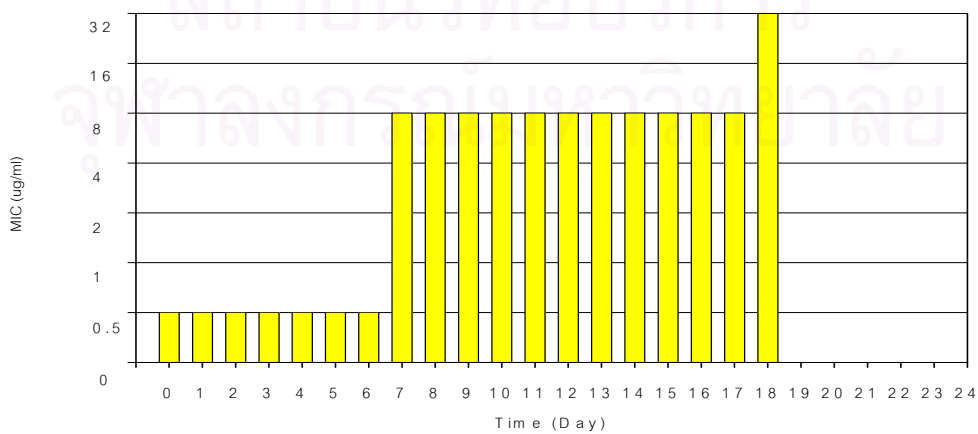

Fig 4-15 Increasing MIC when KN 012 expose to ceftazidime concentration 8ug/ml (16MIC)
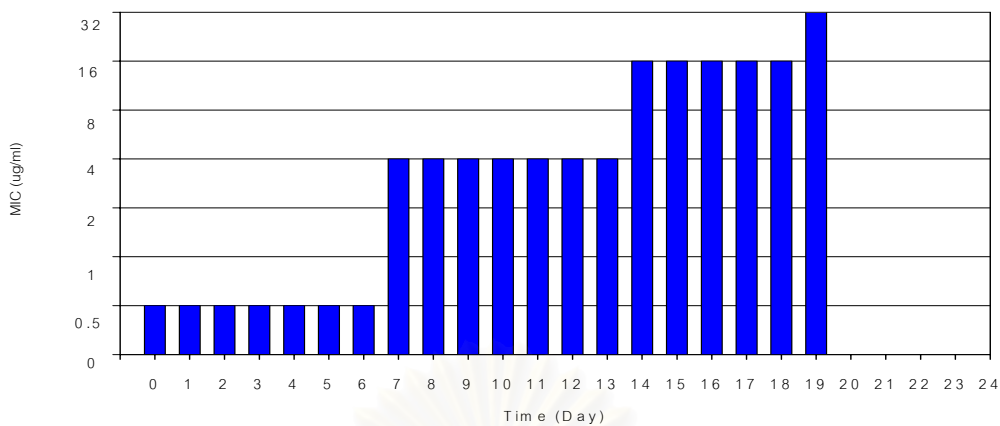
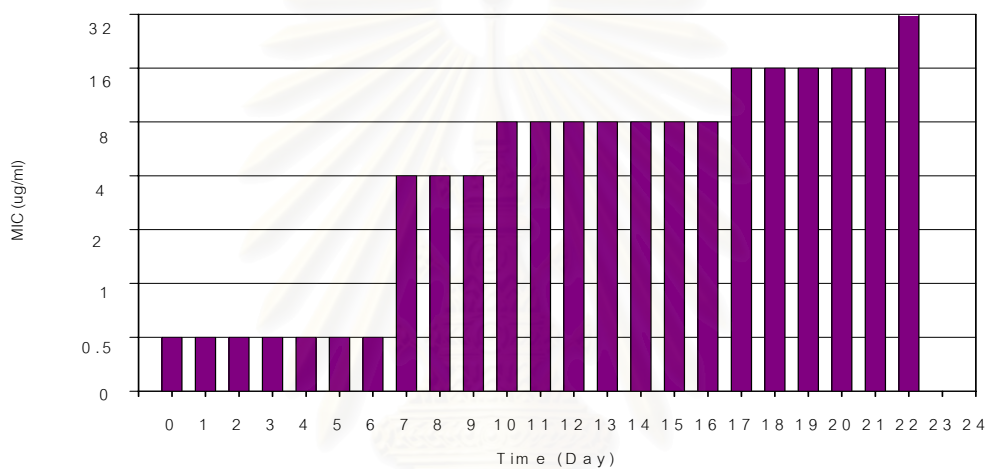Fig 4-16 Increasing MIC when KN 012 expose to ceftazidime concentration 4ug/ml (8 MIC)

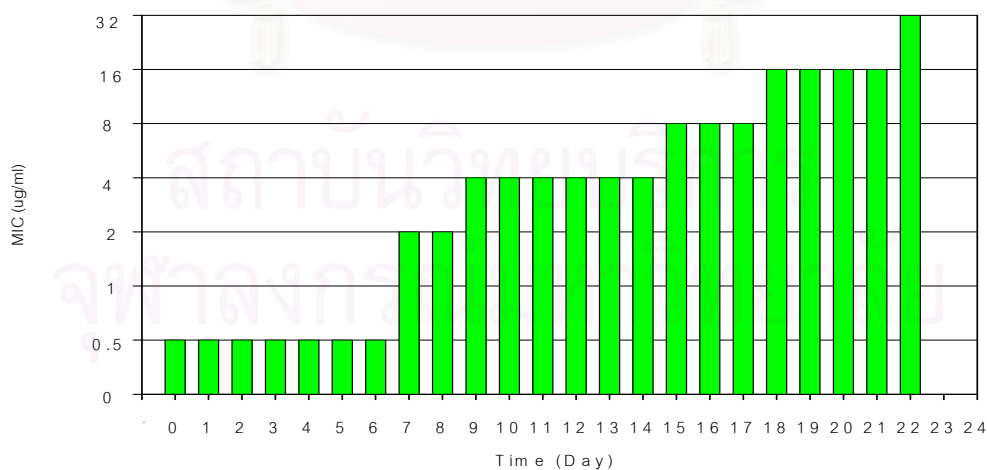Fig 4-17 Increasing MIC when KN 012 expose to ceftazidime concentration 2 ug/ml (4MIC)

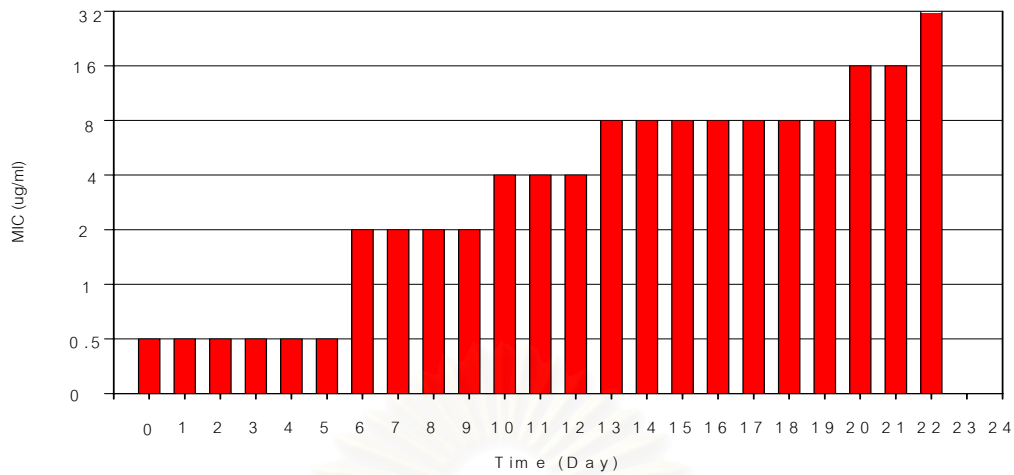Fig 4-18 Increasing MIC when KN 012 expose to ceftazidime concentration 1 ug/ml (2MIC)

Fig 4-19 Increasing MIC when KN 012 expose to ceftazidime concentration 0.5 ug/ml (1MIC)

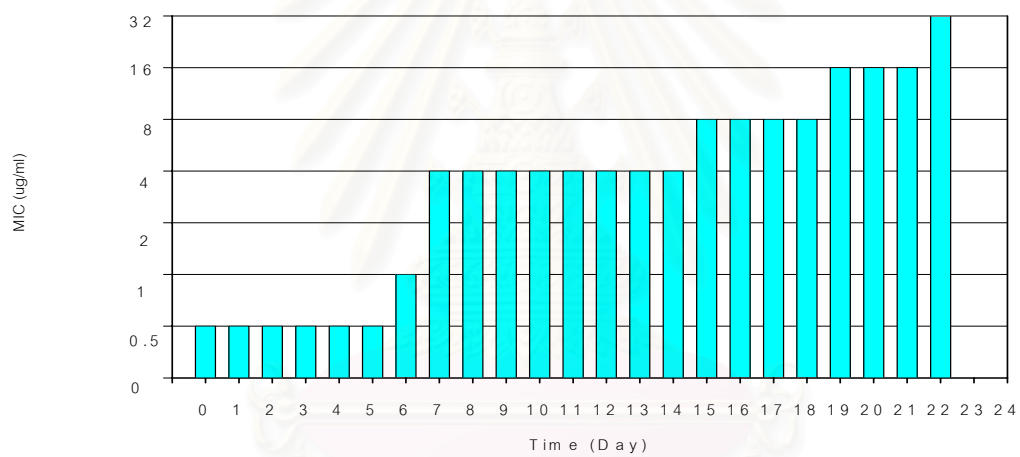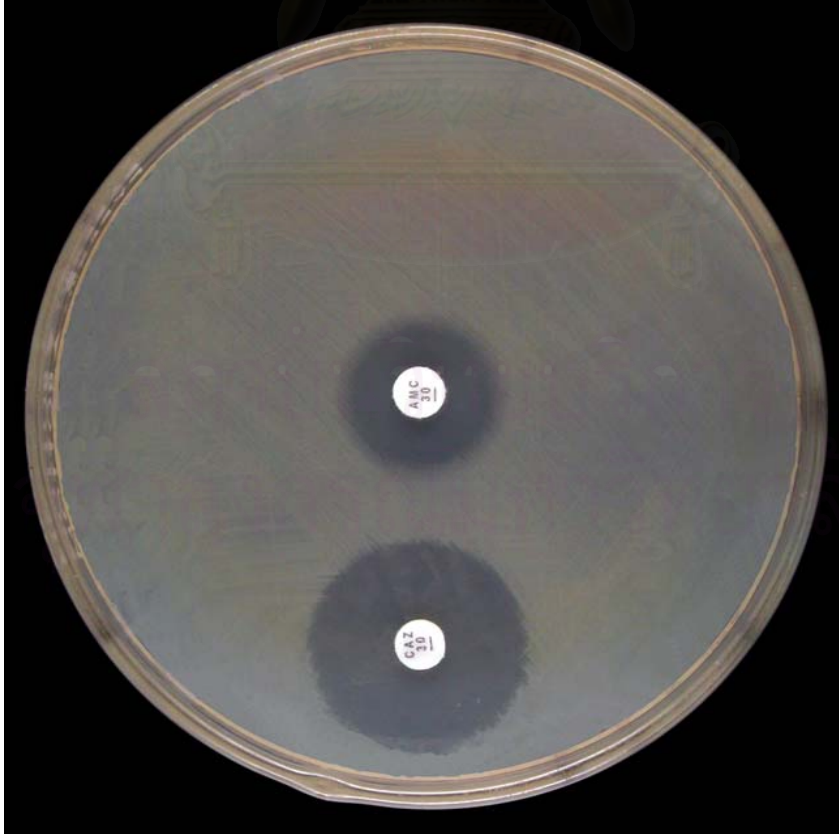

Fig 4-20 Increasing MIC when KN 012 expose to ceftazidime concentration 0.25 ug/ml (1/2MIC)
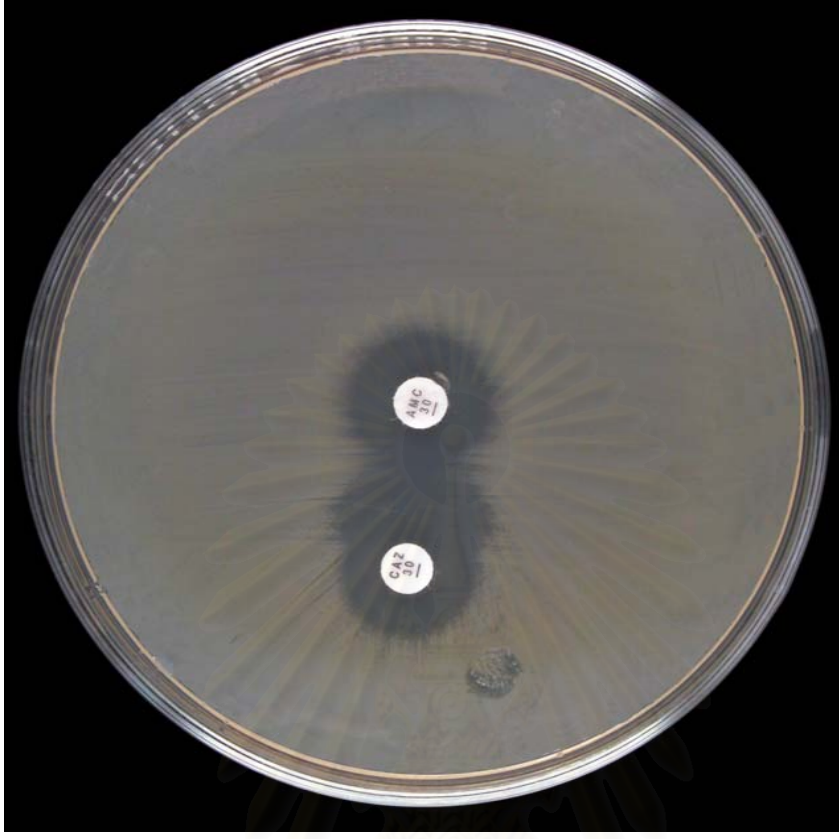
Fig 4-21 Assessment of ESBL production with double disks technique of original moderately susceptible strain;KN012 (A) and resistant mutants (B)

4.3 Effect of ceftazidime concentrations and duration of exposure on resistant development of highly susceptible strain (KN 246) ; MIC = 0.125 μg/ml (method A)

Ceftazidime 32MIC-64MIC did not select resistant mutants and organisms were eradicated at day 5 and 4, respectively. Concentration at 1/2MIC-16MIC had efficacy to select resistant mutants as shown in table 4-7. Every concentrations reduced susceptibility at day 5. Concentration at 4MIC, 8MIC and 16MIC increased MIC to the resistance breakpoint (32 μg/ml) at day 24 (Figure 4-24 to 4-26) whereas concentration at 1MIC and 2MIC occurred at day 25 (Figure 4-27 and 4-28) and concentration at ½ MIC occurred at day 26 (Figure 4-29).It was demonstrated that the low concentration used more steps to develop resistance than high concentration. Furthermore double disk method showed resistance mechanism of KN246 to ceftazidime were ESBL production as shown in figure 4-30.

Table 4-7 Effect of ceftazidime concentration and duration of exposure on selection resistant mutants of highly susceptible strain (KN246) ; MIC = 0.125 µg/ml

Exposure ceftazidime		Selection resistant mutants	
Conc. (µg/ml)	Fold of MIC	Cycle increase in MIC	Increasing MIC (µg/ml)
8	64	ND[1]	ND[1]
4	32	ND[2]	ND[2]
2	16	5	8
		13	16
		24	32*
1	8	5	4
		13	8
		19	16
		24	32*
0.5	4	5	2
		9	4
		13	8
		18	16
		24	32*
0.25	2	5	1
		12	2
		16	4
		19	8
		21	16
		25	32*
0.125	1	5	0.5
		8	1
		13	2
		15	4
		19	8
		22	16
		25	32*

ND[1] = not detected (not changable MIC and organisms were eradicated at cycle 4)

ND[2] = not detected (not changable MIC and organisms were eradicated at cycle 5)

* = resistant breakpoint as referred in NCCLS, 2003 and ESBL positive for resistant mutants.

Table 4-7 (Continued) Effect of ceftazidime concentration and duration of exposure on selection resistant mutants of highly susceptible strain (KN246) ; MIC = 0.125 µg/ml

Exposure ceftazidime		Selection resistant mutants	
Conc. (µg/ml)	Fold of MIC	Cycle increase in MIC	Increasing MIC (µg/ml)
0.06	1/2	5	0.5
		8	1
		10	2
		14	4
		16	8
		22	16
		26	32*

 * = resistant breakpoint as referred in NCCLS, 2003 and ESBL positive for resistant mutants.
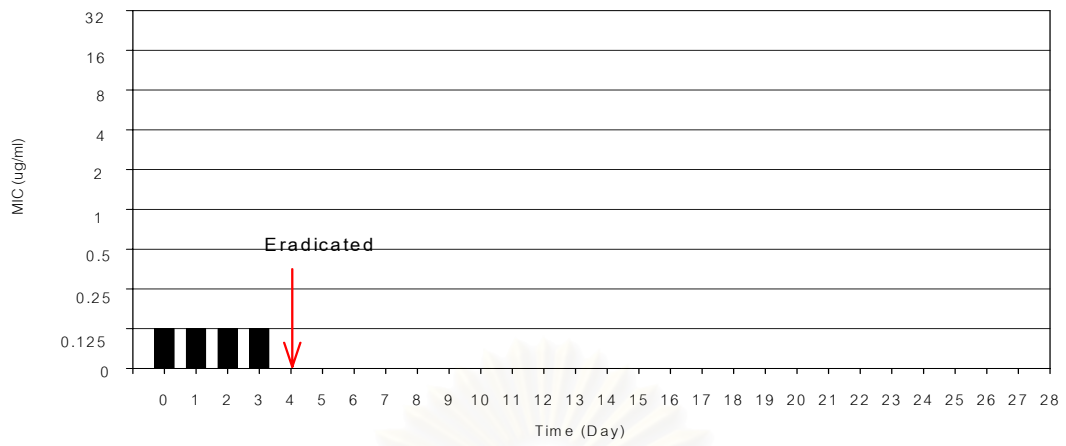
Fig 4-22 Not changeable MIC when KN 246 expose to ceftazidime concentration 8 ug/ml (64MIC)

Fig 4-23 Not changeable MIC when KN 246 expose to ceftazidime concentration 4 ug/ml (32MIC)

Fig 4-24 Increasing MIC when KN 246 expose to ceftazidime concentration 2 ug/ml (16MIC)

Fig 4-25 Increasing MIC when KN 246 expose to ceftazidime concentration 1 ug/ml (8MIC)

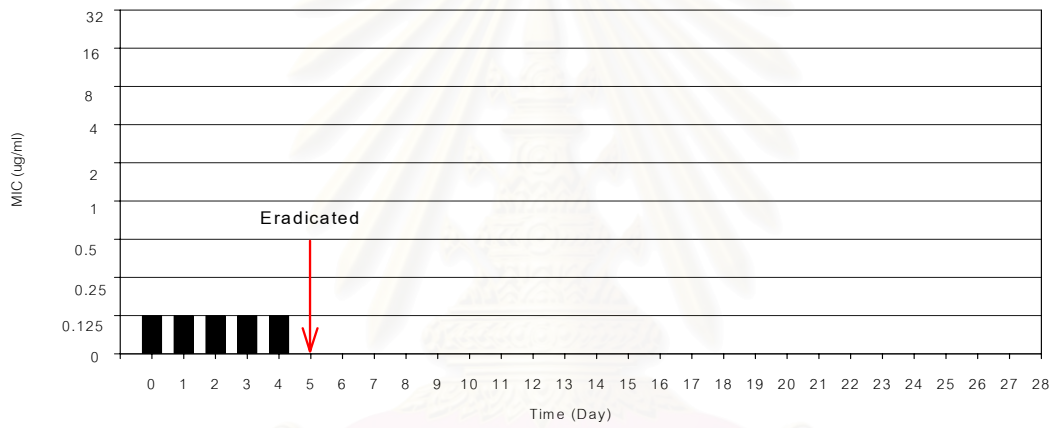Fig 4-26 Increasing MIC when KN 246 expose to ceftazidime concentration 0.5 ug/ml (4MIC)
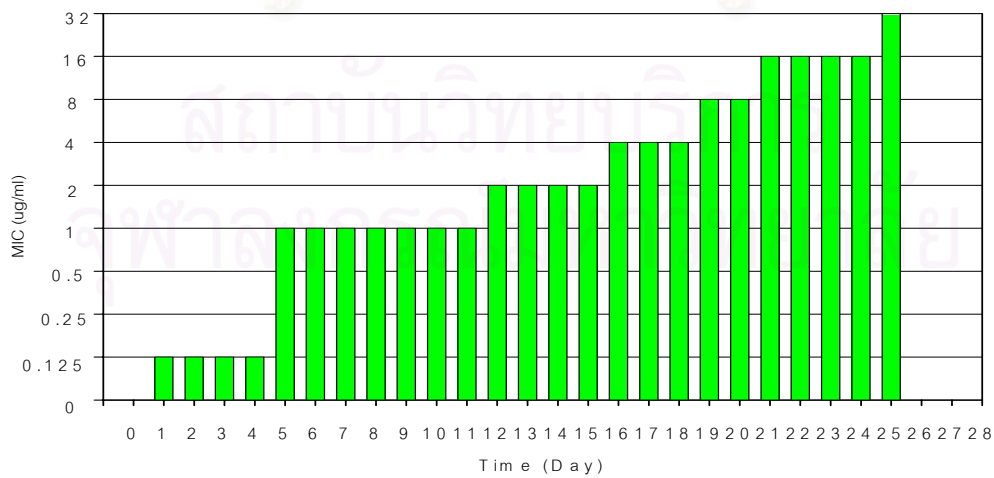
Fig 4-27 Increasing MIC when KN 246 expose to ceftazidime concentration 0.25 ug/ml (2MIC)
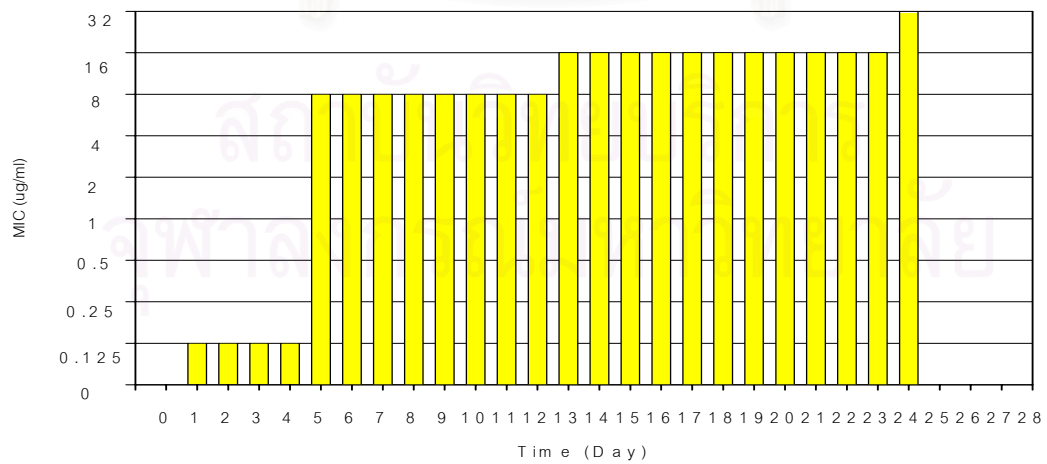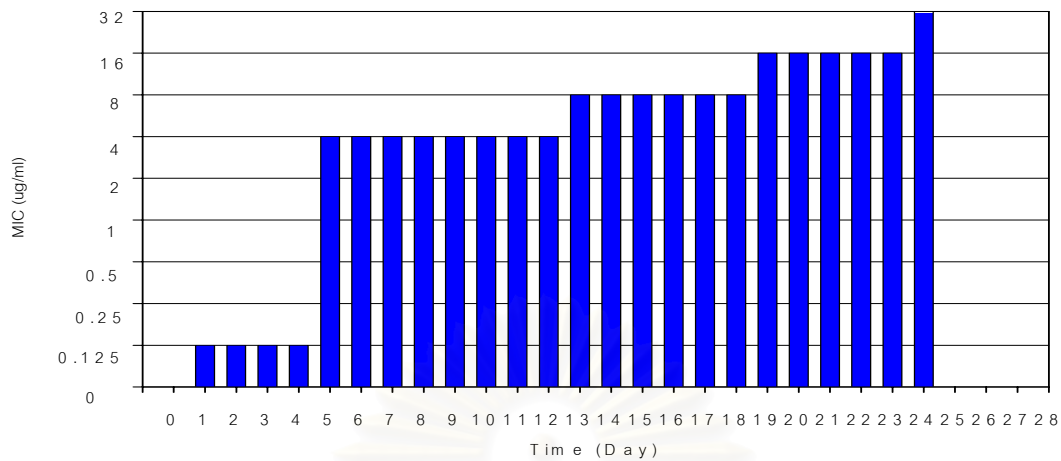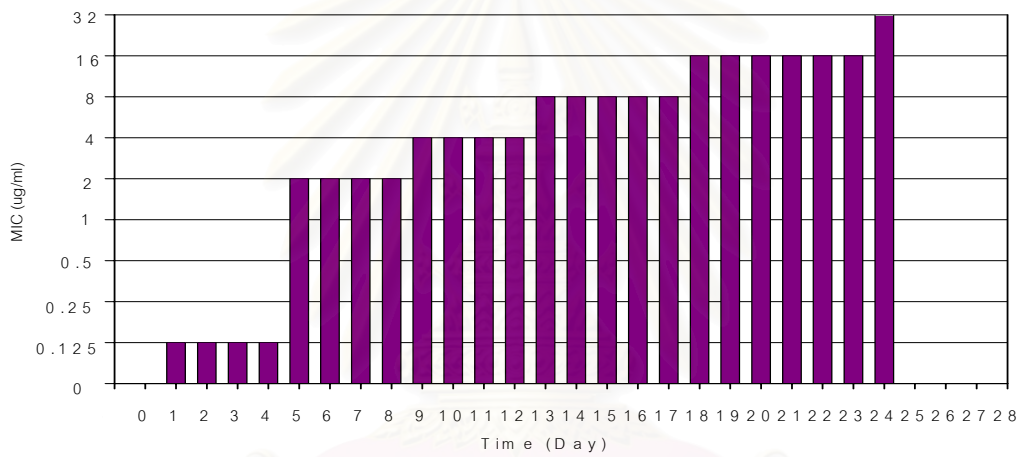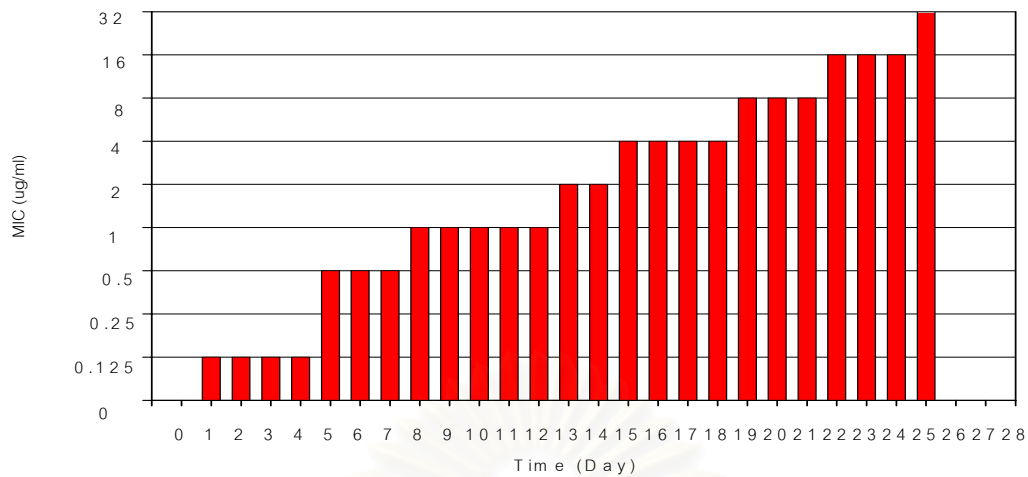
Fig 4-28 Increasing MIC when KN 246 expose to ceftazidime concentration 0.125 ug/ml (1MIC)
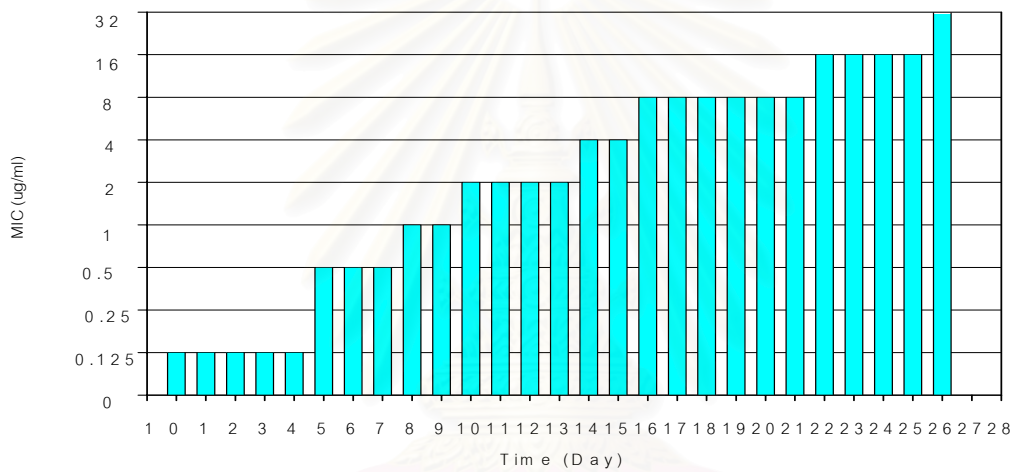
Fig 4-29 Increasing MIC when KN 246 expose to ceftazidime concentration 0.06 ug/ml 1/2MIC)
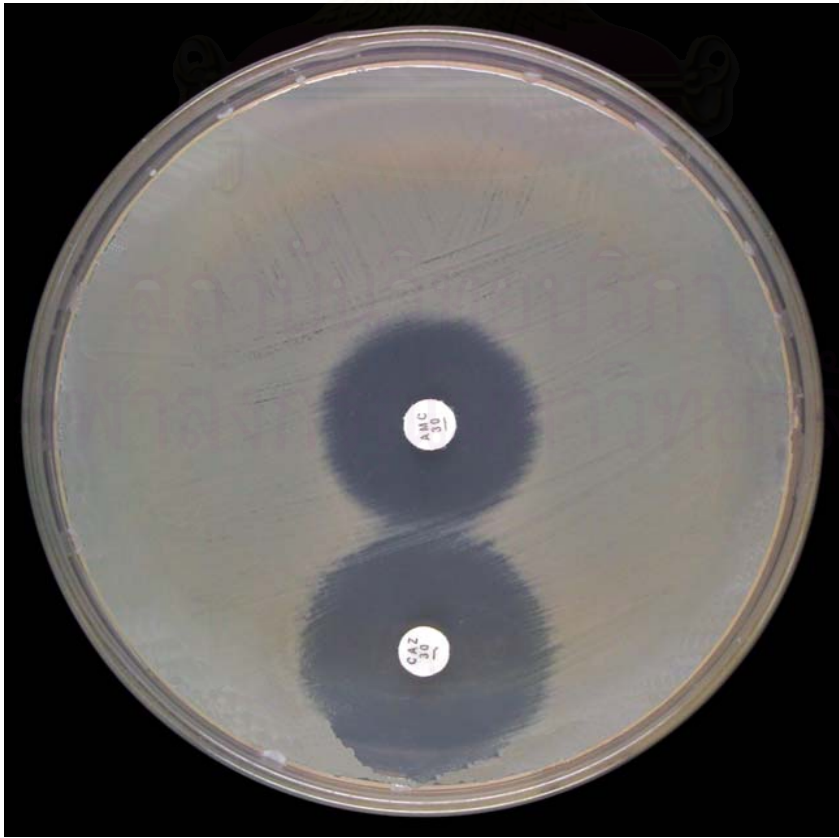
Fig 4-30 Assessment of ESBL production with double disks technique of original highly susceptible strain ;KN246 (A) and resistant mutants (B)

4.4 Effect of ceftazidime concentrations and duration of exposure on resistant development of less susceptible strain (KN 280) ; MIC = 2 µg/ml (method B)

Method B which simulated administration cycle was demonstrated that less susceptible strain (KN280) was eradicated at the fifth dose as shown in table 4-8.

Table 4-8 Antibacterial effect of ceftazidime to less susceptible strain (KN280)

Dose	Viable count (CFU)
1	1.57×10^{4}
2	5×10^{1}
3	1×10^{1}
4	5
5	0

5. Measurement of mutant prevention concentration

The values of MPC of three strains were shown in table 4-9. It demonstrated that MPC could be valued in the descending order KN280 > KN012 > KN246 as a result MPC/C max ratio have a similar order. When considered MPC/MIC ratio that refered to range of selection resistant mutants concentrations (Figure 4-32) ; KN 280 was 64 while KN 012 and KN 246 were 32.

Table 4-9 Mutant prevention concentration of *K. pneumoniae strains.*

K. pneumoniae Strain NO.	MIC (µg/ml)	MPC (µg/ml)	MPC/MIC	C max (µg/ml)	MPC/C max
KN 280	2	128	64	70	1.83
KN 012	0.5	16	32	70	0.23
KN 246	0.125	4	32	70	0.057

Figure4-31 Mutant selection window of less susceptible strain;KN280 (A), moderately susceptible strain;KN012 and less susceptible strain;KN280(C)
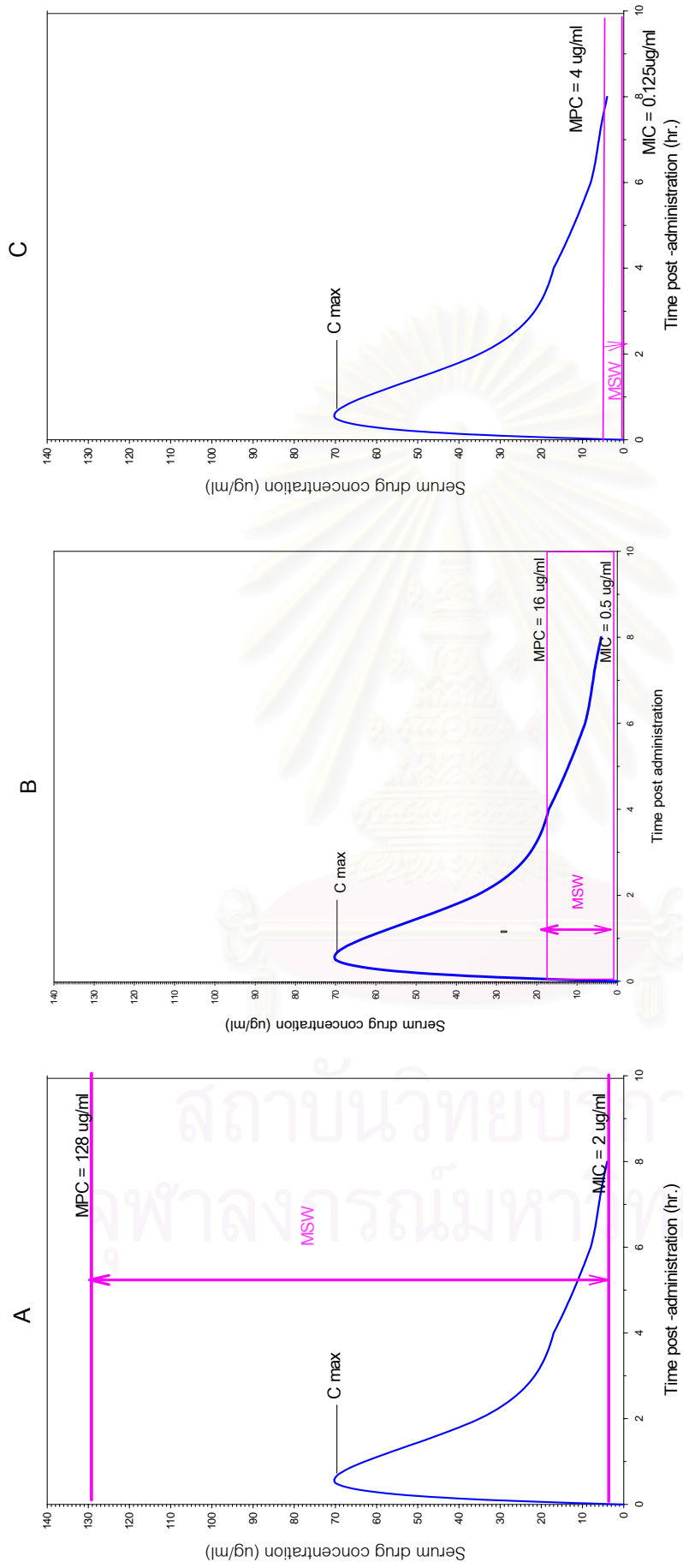
CHAPTER V

DISCUSSION & CONCLUSION

DISCUSSION

Regarding to the effect of ceftazidime concentration on eradication of *K.pneumoniae* by time-kill method, the results demonstrated that highly susceptible strain (KN246) required four times of the MIC to exhibit bactericidal property. Concentration above 4MIC did not kill the organisms any faster or more extensively. Which according to their patterns of killing activity of ß-lactam, maximum killing is usually achieved at 3-4 times the MIC. (Turnidge, 1998). Whereas moderately (KN012) and less (KN280) susceptible strain, concentration at 1MIC-8MIC did not have bactericidal activity. In case of pharmacokinetic achievable concentrations after administered the therapeutic does of 1 g, the results showed that Cmax (70 µg/ml) and Caverage (35 µg/ml) had bactericidal property for all strains. But Cmin (4 µg/ml) had bactericidal property only for highly susceptible strain (KN246). According to the results from the study with the concentration of 1MIC-8MIC be described above, at the concentration of 8MIC of the moderately susceptible strain(KN012) and at concentration of 2MIC of less susceptible strain(KN280) which equal to Cmin did not have bactericidal property then should aware to keep drug levels more than Cmin during treatment time.

Furthermore, when considered the effect of the duration of exposure on eradication, we found that highly susceptible strain (KN246) was eradicated when exposed to ceftazidime all 4 half-life (8 hr),while moderately (KN012) and less (KN280) susceptible strain were not eradicated when exposed to ceftazidime at more than 2 half-life (4 hr) and 1 half-life (2 hr), respectively due to decreased drug concentration.

Consequently, the present results as described above it might imply that the highly susceptible strain(KN246) required concentration at 4MIC-8MIC to be eradicated and the therapeutic dose of 1 g every 8 hr provided appropriate concentrations and duration of exposure to eradicate this strain. The moderately (KN012) and the less (KN280) susceptible strain(KN280) should keep drug levels more than C min during treatment time and should not expose to ceftazidime more than 4 hr and 2 hr, therefore

decreased dosing interval and high dosage regimen be considered. However, recommended dose provided T>MIC 100% of the dosing interval but data from animal models suggested that maximum bacterial killing of β-lactam drugs is achieved when T>MIC is 60-70% of the dosing interval for gram-negative bacilli (Craig, 1998).

Regarding to the study on the effect of the concentration and the duration of exposure on resistant development by microbiology method (method A) or daily passage that *K.pneumoniae* exposed to ceftazidime concentration at 1/2MIC-64MIC . At each passage, the overnight culture was concentrated by centrifugation and the sediment was re-inoculated into MHB containing at constant concentration and again incubated. The results demonstrated that range of selection concentrations of less susceptible strain(KN280) were 1/2MIC-32MIC, moderately(KN012) and less (KN280) susceptible strain were1/2MIC-16MIC. Interestingly, all strains in the high concentration could select resistant mutants at faster than low concentration which might be explained that low concentration (weak selective pressure) selected low-level resistant mutants (small increase in MIC) whereas high concentration selected high-level resistant mutants (high increase in MIC). According to the previous study ; the stepwise-selection of ESBL in *E.coli* strains under cefotaxime exposure (Baquero, 2001) that manifested TEM-12 ;low-level resistant mutants were selected at low cefotaxime concentration while TEM-10; high-level resistant mutants were selected at higher concentration indicating that the low concentration used more steps to develop resistance than high concentration. As a result, it might implied that we should avoid high concentration especially supra MIC in clinical treatment due to selection resistant mutants occurred easily according to pharmacodynamic of β-lactam that is concentration –independent activity.

Comparing the effect of the duration of exposure on the resistance in case of reducing susceptibility, the results manifested that highly susceptible strain(KN246) occurred at day 5 before moderately susceptible strain(KN012) at day 6 and less susceptible strain(KN280) at day 7 . It might be explained that MIC values of original strain of highly susceptible strain(KN246) lower than both strains so changing MIC values could occur easily. In case of develop MIC values of resistant mutants to resistant breakpoint the results manifested that less susceptible strain(KN280) occurred at day 9

which faster than moderately susceptible strain(KN012) at day 18 and highly susceptible strain(KN246) at day 24 .It might be explained that less susceptible strain(KN280) used less steps to develop resistance than both strains. Furthermore, the MIC of third-generation cephalosporins that likely to express an ESBL is 2 µg/ml as referred by NCCLS,2003 then less susceptible strain(KN280) which had MIC value 2 µg/ml could emerge resistance faster than other strains. However, increasing MIC (reducing susceptibility) which not equal 32 µg/ml it effected to clinical outcome.

Although, this method provided range of selection concentrations which can be applied to the administer drug concentration outside the selection window such as less susceptible strain(KN280) required concentration of more than 32 MIC while moderately (KN012) and highly(KN246) susceptible strain required concentration more than 16 MIC to prevent selection resistant mutants. However, method A could not provide duration of exposure that selected resistant mutants to apply an appropriate dosage regimen in clinical treatment due to duration of exposure in this method was 24 hr which more than dosing interval of therapeutic dose. However, it implied that *K.pneumoniae* required long duration of exposure to create more selective pressure to develop resistance.Then, short duration of β-lactam in clinical treatment was appropriated. Conceivably, the number of passage required for each antibiotic to select resistance depends on the following factors; (1) the potential of the antibiotic to induce development of resistance mechanisms in bacteria; (2) the intrinsic ability of the bacterial species as well as individual strains to develop the resistance mechanisms; (3) the antimicrobial activity of the test anibiotic; and (4) the ability of the antibiotic itself to withstand the resistance mechanism (Chan et al' 1999).

Consequently, method B that simulated pharmacokinetic achievable drug levels or administration cycle was used to study the less susceptible strain (KN280). At the beginning, less susceptible strain(KN280) exposed to C max and drug concentration were diluted into half every 2 hr (half-life) for 8 hr (dosing-interval) and repeated exposure to C max again. As a result, less susceptible strain(KN280) was eradicated at the fifth dose which comfirmed that less susceptible strain(KN280) required high ceftazidime concentration and high frequency of dosing interval to suppress selection resistant mutants. However , method B did not provide selective environment like in

human body due to many factors such as host defence systems which usually eliminate mutant bacteria especially after growth of susceptible pathogens is blocked by antibiotic.Morever, elimination of drug that flactuated drug concentration caused selection property differed from exposed to the constant concentrations all time. Therefore further investigation by using in vitro pharmacokinetic model should be confirmed these results.

Regarding to the measurement of MPC which represented a concenptual concentration threshold for restricting the development of resistance. MPC has been a therapeutically useful parameter; its value must below the serum and tissue drug concentration attained following administration all treatment time (Drlica, 2003). In case of less susceptible strain (KN280); MPC value was 128 µg/ml (64MIC) and C max of therapeutic dose was 70 µg/ml (Figure 5-1 A) as a result, this therapeutic dose could not prevent selection resistant mutants. Even if increasing dose to 2 g that C max was 170 µg/ml and after the first half-life(2 hr) , serum concentration belowed MPC value that selection   resistant mutants had occurred (Figure 5-1 B). Therefore less susceptible strain(KN280) should considered combination therapy with aminoglycoside or fluoroquinolone or changed ceftazidime to the others such as carbapenem or ß-lactam-ß-lactam inhibitior combination (Nathisuwan et al., 2001). Continuous infusion may be useful for this strain if calculated concentration more than MPC, however, it should be considered the last choice. Moderately susceptible strain(KN012); MPC value was 16 µg/ml (32MIC) when administered the therapeutic dose, at 6 hr serum concentration was 8 µg/ml that bellowed MPC value (Figure 5-2 A) then should decrease dosing interval or increase dose to 2 g which concentration bellowed MPC values almost dosing interval (Figure 5-2 B). Whereas with the highly susceptible strain(KN246), the therapeutic dose could prevent selection of resistant mutants because MPC value was 4 µg/ml that equal to C min.

Regarding to resistance mechanisms of evaluated *K.pneumoniae* strains was ESBL production as referred by double disk method. Ceftazidime was an inducer that induced the ESBL production by induced mutation from SHV-1 β-lactamase. After that, selected resistant mutants could survive and overgrow. As a result, ceftazidime not only an inducer but also a selector. Low concentration induced the ESBL production and

gradually accumulated mutation until appropriate duration of exposure and then selected resistant mutants. Whereas high concentration induced ESBL production and selected resistant mutants suddenly due to high selective pressure. Similarly, less susceptible strain (KN280) when exposed to the ceftazidime at the concentration of 64 µg/ml, resistance emerged at day 9 with no steps to develop resistance while lower concentration had more steps to develop resistance.

Besides the ESBL production, it might use the other resistant mechanisms of *K.pneumoniae* to ceftazidime such as the decreased in the porin production or increase efflux of ceftazidime (Bush, 2001) and vertical genetic transfer (Aswapokee, 1997) which were not detected in this study.
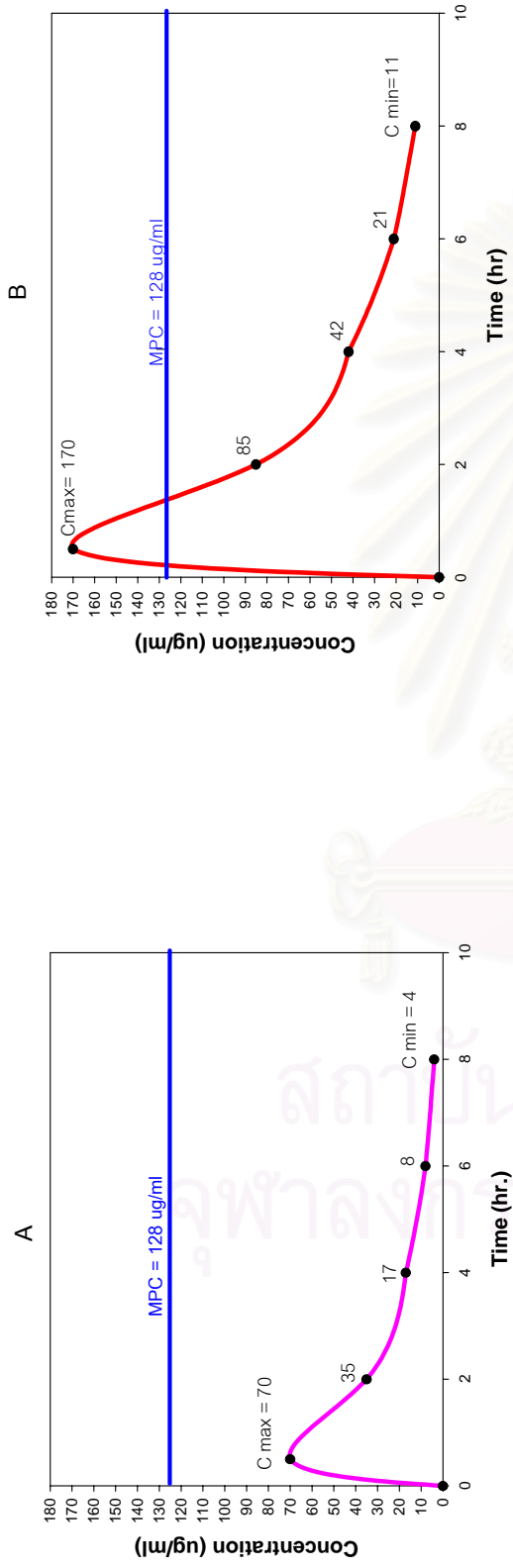
Fig 5.1 Correlations of MPC and serum drug concentration following administer dose 1 g (A) and 2 g (B) for less susceptible strain (KN280)
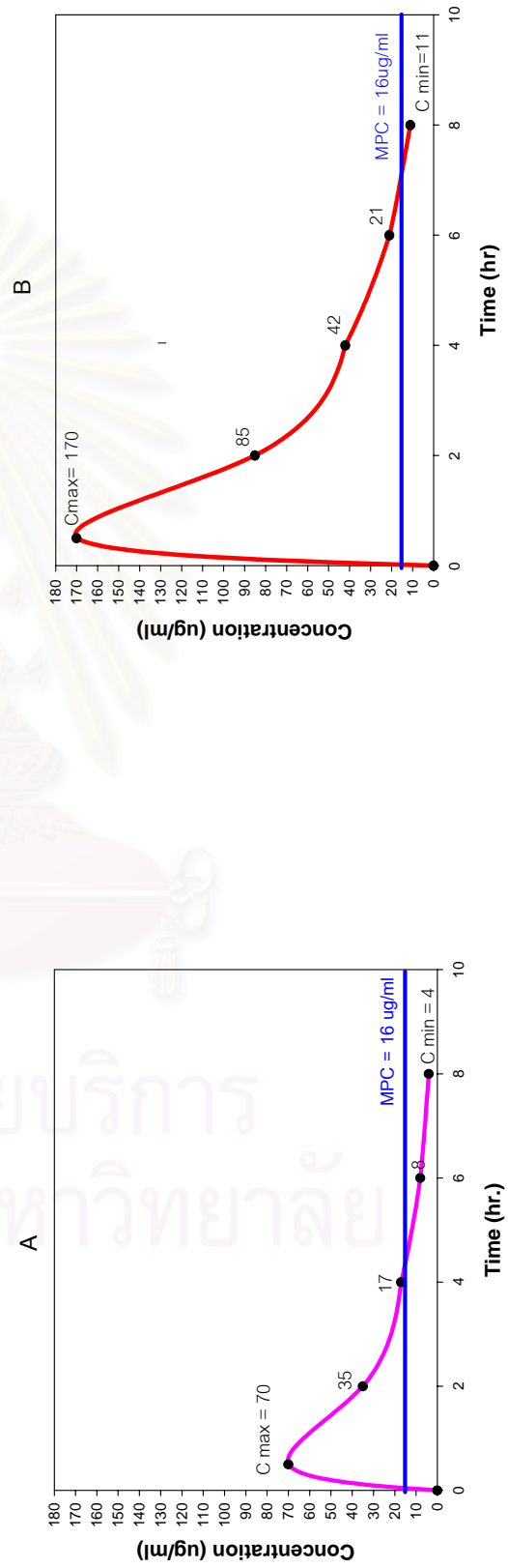

Fig 5.2 Correlations of MPC and serum drug concentration following administer dose 1 g (A) and 2 g (B) for moderately susceptible strain (KN012)

Conclusion

From the study on the effect of ceftazidime concentrations and duration of exposure on eradication and resistant development of ESBL-producing *K.pneumoniae* provides appropriate dosage regimen to eradicate organisms and prevent selection resistant mutants. For highly susceptible strain(KN 246 : MIC=0.125 µg/ml); therapeutic dose has enough potency to eradicate and prevent selection resistant mutants whereas moderately susceptible strain(KN012 : MIC=0.5 µg/ml) require high dosage regimen. Conversely, less susceptible strain(KN280 : MIC=2 µg/ml) dose 1-2 g cannot eradicate and prevent selection resistant mutants.Therefore, combination therapy with aminoglycoside or fluoroquinolone or using more potent drugs such as carbapenem or ß-lactam- ß-lactamase inhibitor combination are considered.
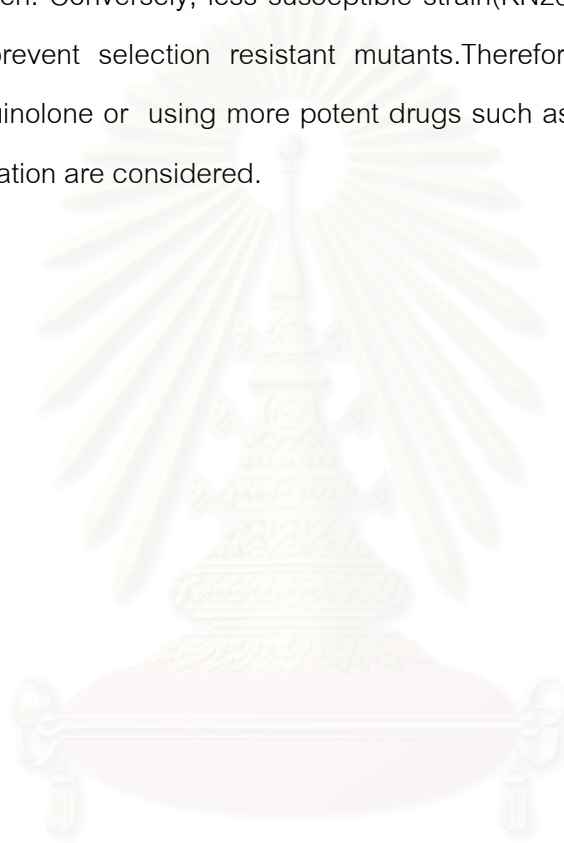
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