EFFECTS OF AMIKACIN, CEFTAZIDIME, AND FOSFOMYCIN COMBINATION AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA*

MANAT SITTHICHAI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE IN PHARMACY
(CLINICAL PHARMACY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2006

ISBN–974-04-7877-8

Thesis
entitled

EFFECTS OF AMIKACIN, CEFTAZIDIME, AND FOSFOMYCIN COMBINATION AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA*

Manat Sitthichai
.....
Mr. Manat Sitthichai
Candidate

Preecha Montakantikul
.....
Assist.Prof. Preecha Montakantikul,
Pharm.D., BCPS.
Major-Advisor

Sumarn Saraya
.....
Assoc. Prof. M.L. Sumarn Saraya,
Ph.D. (Pharmacokinetics)
Co-Advisor

จิระชัย วัยวรวุฒิ
.....
Jirachai Waiwarawooth,
M.D. (Diplomate Thai Board of Internal Medicine) (Diplomate Thai Board of Internal Medicine Infectious Disease)
Co-Advisor

.....
Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies

.....
Prof. Ampol Mitrevej,
Ph.D. (Pharmaceutics)
Chairman
Master of Science in Pharmacy
Programme in Clinical Pharmacy
Faculty of pharmacy

Thesis
entitled

EFFECTS OF AMIKACIN, CEFTAZIDIME, AND FOSFOMYCIN COMBINATION AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA*

was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Master of Science in Pharmacy (Clinical Pharmacy)
on
November 1, 2006

Manat Sitthichai
Mr. Manat Sitthichai
Candidate

Cheardchai Soontornpas
Assist.Prof. Cheardchai Soontornpas,
Ph.D. (Biopharm. Sc.)
Chairman

Preecha Montakantikul
Assist.Prof. Preecha Montakantikul,
Pharm.D., BCPS.
Member

จิระชัย วัยวรุธ
Jirachai Waiwarawooth, M.D.
(Diplomate Thai Board of Internal Medicine) (Diplomate Thai Board of Internal Medicine Infectious Disease)
Member

Sumarn Saraya
Assoc. Prof. M.L. Sumarn Saraya,
Ph.D. (Pharmacokinetics)
Member

Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Ampol Mitrevej
Prof. Ampol Mitrevej,
Ph.D. (Pharmaceutics)
Dean
Faculty of pharmacy
Mahidol University

ACKNOWLEDGMENTS

I would like to express my deepest and sincere gratitude to Assistant Professor Dr. Preecha Montakantikul, my advisor and Associate Professor Dr. M.L. Sumarn Saraya, my co-advisor for their guidance, invaluable advice, great attention and assistance with consultant encouragement throughout my study.

I am deeply grateful to Doctor Jirachai Waiwarawooth, my co-advisor for his helpful guidance, suggestion, comment and wonderful vision.

I would like to express my greatest appreciation and sincere gratitude to Associate Professor Dr. Busba Chindavijak for her valuable advice and in pharmacokinetic principle. Also, I am extremely grateful to Assistant Professor Dr.Chulaluk Komoltri for her helpful guidance in statistical approaches to this work.

I wish to thank Assistant Professor Dr. Cheardchai Soontornpas for his kindness in the external examiner of thesis defense.

I would like to thank all staff and members of Microbiology Unit, Pathology Department, Chonburi Hospital for their supports and fulfillment throughout my work.

This research was kindly supported by Thai Meiji Pharmaceutical., LTD.

Finally, I would like to express my warmest thanks and sincere gratitude and appreciation to my dear parents, sisters and brothers for their love, care, pushing up, cheerfulness, devoting and encouragement throughout my life.

Manat Sitthichai

EFFECTS OF AMIKACIN, CEFTAZIDIME, AND FOSFOMYCIN COMBINATION AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA*

MANAT SITTHICHAI 4737149 PYCP/M

M.Sc. in Pharm (CLINICAL PHARMACY)

THESIS ADVISORS: PREECHA MONTAKANTIKUL, Pharm.D., BCPS., M.L.SUMARN SARAYA, Ph.D. (Pharmacokinetics),
JIRACHAI WIWARAWOOTH, M.D. (Diplomate Thai Board of Internal Medicine, Diplomate Thai Board of Internal Medicine Infectious Disease)

ABSTRACT

The objective of this study was to determine the *in vitro* effects of triple combination; amikacin, ceftazidime, and fosfomycin on multidrug-resistant *Pseudomona aeruginosa* (MDR-PA) clinical isolates and to determine the optimal dosage regimen of this combination by using pharmacokinetic/pharmacodynamic (PK/PD) approach. This study, designed as an experimental study, was performed at the Department of Microbiology, Faculty of Pharmacy, Mahidol University during July 2005 to March 2006. The study comprised 34 MDR-PA clinical isolates from patients admitted at Chonburi Hospital. They showed high level resistance with the minimum inhibitory concentration at 50% (MIC_{50}) and MIC_{90} of amikacin, ceftazidime, and fosfomycin, at > 256 µg/mL, > 256 µg/mL, and > 1024 µg/mL, respectively. The combination of amikacin and ceftazidme can decrease the MIC of either amikacin or ceftazidime within 14 isolates (41.18%). According to the fractional inhibitory concentration (FIC) index, there was 1 synergism (2.94%) and 33 additivities (97.06%) with the mean FIC index 1.61±0.53. More importantly, none of them showed antagonistic activities.

The agar well method demonstrated that the addition of fosfomycin to the combination of amikacin and ceftazidime could increase the number of susceptible isolates. For instance, the higher concentration of fosfomycin provided the trends of greater activities of the triple combination. In addition, concentration of fosfomycin which was used in combination with amikacin and ceftazidime should be at least its MIC to optimize antimicrobial interaction. Throughout, the calculated dosage regimen based on PK/PD data resulted in extremely huge dosage. However, if the strain had MIC against amikacin, ceftazidime, and fosfomycin equal to or less than 80 µg/mL, 50 µg/mL, and 200 µg/mL, respectively, this combination would be a candidate for treatment of MDR-PA infections. In conclusion, the study demonstrated that this triple combination might be of benefit to overcome the organism.

KEY WORDS : AMIKACIN/CEFTAZIDIME/FOSFOMYCIN/COMBINATION/ MULTIDRUG-RESISTANT/*PSEUDOMONAS AERUGINOSA*.

110 P. ISBN 974-04-7877-8

ผลการใช้ อะมิกาซิน และเซฟตาซิดีม ร่วมกับ ฟอสโฟไมซิน ในการต้านเชื้อซูโดโมนาส แอรูจิโนสา ที่ดื้อยาหลายขนาน (EFFECTS OF AMIKACIN, CEFTAZIDIME, AND FOSFOMYCIN COMBINATION AGAINST MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA*)

มานัส สิทธิชัย 4737149 PYCP/M

ภ.ม. (เภสัชกรรมคลินิก)

คณะกรรมการควบคุมวิทยานิพนธ์: ปรีชา มนทกานติกุล, Pharm.D., BCPS., มล.สุมาลย์ สาระยา, Ph.D. (Pharmacokinetics), จิระชัย วัยวราวุธ, M.D. (Diplomate Thai Board of Internal Medicine, Diplomate Thai Board of Internal Medicine Infectious Disease)

บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินผลทางห้องปฏิบัติการของการใช้ อะมิกาซิน และเซฟตาซิดีม ร่วมกับฟอสโฟไมซิน ในการต้านเชื้อซูโดโมนาส แอรูจิโนสาที่ดื้อยาหลายขนาน และใช้หลักเภสัชจลนศาสตร์/เภสัชพลศาสตร์ เพื่อหาข้อกำหนดการบริหารยา (dosage regimen) ที่เหมาะสมที่สุดในการรักษาโรคติดเชื้อดังกล่าว โดยทำการทดลองที่ภาควิชาจุลชีววิทยา คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล ตั้งแต่ กรกฎาคม พ.ศ. 2548 ถึง มีนาคม พ.ศ. 2549 กับเชื้อจากผู้ป่วยที่เข้ารับการรักษาที่โรงพยาบาลชลบุรี ทั้งหมด 34 สายพันธุ์ ที่มีการดื้อยาในระดับสูง โดยมีค่าความเข้มข้นต่ำสุดที่ยับยั้งการเจริญของเชื้อได้ร้อยละ 50 และ ร้อยละ 90 (MIC_{50} และ MIC_{90}) ของ อะมิกาซิน เซฟตาซิดีม และ ฟอสโฟไมซิน เป็น > 256 µg/mL > 256 µg/mL และ > 1024 µg/mL ตามลำดับ ผลการให้ยาร่วมกันระหว่าง อะมิกาซิน และเซฟตาซิดีม นั้นสามารถลด MIC ของเชื้อลงได้ 14 สายพันธุ์ (ร้อยละ 41.18) มีดัชนี fractional inhibitory concentration (FIC) เฉลี่ย 1.61±0.53 โดยจัดเป็น synergism 1 ครั้ง (ร้อยละ 2.94) additivity 33 ครั้ง (ร้อยละ 97.06) ทั้งนี้ไม่พบการต้านฤทธิ์กัน

การทดสอบให้ ฟอสโฟไมซิน ร่วมกับ อะมิกาซิน และ เซฟตาซิดีม ด้วยวิธี agar well พบว่าจำนวนสายพันธุ์ที่ถูกยับยั้งมีแนวโน้มเพิ่มขึ้น และการเพิ่มความเข้มข้นของ ฟอสโฟไมซิน ที่ให้ร่วมกับ อะมิกาซิน และ เซฟตาซิดีม พบว่าช่วยเพิ่มฤทธิ์ของสูตรยาสามรายการดังกล่าว โดยได้ผลการยับยั้งเชื้อที่ดีเมื่อใช้ความเข้มข้นของ ฟอสโฟไมซิน ที่ไม่น้อยกว่าค่า MIC ของ ฟอสโฟไมซิน การศึกษาในครั้งนี้สรุปว่าการใช้หลักการเภสัชจลนศาสตร์/เภสัชพลศาสตร์ เพื่อหาข้อกำหนดการบริหารยามักได้ขนาดยาที่ใช้สูงมาก อย่างไรก็ตามหากเป็นเชื้อที่มีค่า MIC ของ อะมิกาซิน เซฟตาซิดีม และ ฟอสโฟไมซิน น้อยกว่าหรือเท่ากับ 80 µg/mL 50 µg/mL และ 200 µg/mL ตามลำดับ สูตรยาดังกล่าวอาจเป็นทางเลือกในการรักษาโรคติดเชื้อซูโดโมนาส แอรูจิโนสาที่ดื้อยาหลายขนานได้

110 หน้า ISBN 974-04-7877-8

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF TABLES (cont.)

Table **Page**

LIST OF FIGURES

Figures **Page**

LIST OF ABBREVIATIONS

MDR	Multidrug-resistant
MDR-PA	Multidrug-resistant *Pseudomonas aeruginosa*
ICU	Intensive care unit
EPO	Efflux pump overexpression
FQ	Fluoroquinolone
FIC	Fractional inhibition concentrations
E test	Epsilometer test
PK	Pharmacokinetic
PD	Pharmacodynamic
MIC	Minimum inhibitory concentration
T>MIC	Time above MIC
C_{max}/MIC	Maximum concentration above MIC
PAE	Post-antibiotic effect
LD	Loading dose
MD	Maintenance dose
CI	Continuous infusion
S	Salt factor
F	Bioavailability factor
C_0	Desired target concentration
$C_{max,ss}$	Maximum concentration at steady state
C_{ss}	Concentration at steady state
Vd	Volume of distribution
k	Elimination rate constant
k_0	Rate of drug infusion (mg/hr)
t'	Infusion time
τ	Dosing interval

LIST OF ABBREVIATIONS (cont.)

TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
MHA	Meuller Hinton Agar
MIC_{mono}	MIC of each antimicrobial agent
MIC_{combo}	MIC of amikacin in the combination with ceftazidime
CFU	Colony forming unit
µg	Microgram
mg	Miligram
mL	Milliliter
L	Liter
min	Minute
hr	Hour
mm	Millimeter
AMK	Amikacin
CTZ	Ceftazidime
FMC	Fosfomycin
CPF	Ciprofloxacin
IMP	Imipenem
MRP	Meropenem
PIP/TAZO	Piperacillin/Tazobactam
AC	Amikacin plus ceftazidime
ACF	Amikacin plus ceftazidime plus fosfomycin
S	Sensitive
R	Resistant

CHAPTER I
INTRODUCTION

Antimicrobial resistance in *Pseudomonas aeruginosa* is one of the leading causes of severe infections and associated with significant morbidity, mortality, and health-care costs especially with multidrug-resistant *P. aeruginosa* (MDR-PA) isolates. It has inherent resistance to many drug classes and its ability to acquire resistance to all relevant treatments, via mutation or acquisition of exogenous resistant determinants. They can be mediated resistance by several mechanisms; degrading enzymes, reduced permeability, active efflux and target modification.

Nowadays, there are few data on the incidence of MDR-PA. In the USA, the proportion of MDR-PA (resistant to over three agents: ceftazidime, piperacillin, imipenem gentamycin and amikacin) increased from 12.8 to 20.8% between 1997 and 2000 (1). In the SENTRY surveillance program, the rates of MDR-PA were found to reflect geographic differences, being higher in Latin America (8%), lower in Europe (c.5%), and even lower in North America and Asia-Pacific region (<2%) (2). Also in Thailand, the rates were difference in each institution, Piriykitphaiboon found the incidence of MDR-PA increased from 5 to 29% in 1994 to 1996 (3) and the incidence increased from 47 to 55% in 1999 to 2001 in tertiary care hospital (4). Also in Chonburi Hospital, the incidence of MDR-PA was 18% in 2004 (5).

The rational for combination therapy is essentially to reduce the chances of selection of resistant mutants during therapy, as well as to exploit the potential synergistic activity of some agents. The preferred combination remains antipseudomonal penicillins with aminoglycosides, as synergism between these drugs has been demonstrated by *in vitro* studies and the results of several clinical studies point out the superiority of similar regimens as opposed to monotherapy for the treatment of *P. aeruginosa* bacteremia, especially in neutropenic patients. Some clinical studies have demonstrated the superiority of combination therapy (6, 7, 8). In particular, three drugs combination have been demonstrated efficacy higher than

two drugs combination *in vitro*, using the number of growth-inhibited strains as variable, one of the most effective regimen was the combination of amikacin, ceftazidime, and aztreonam. In addition, some studies demonstrated synergistic effects of fosfomycin against *P. aeruginosa* such as ceftazidime, imipenem, cefepime, aztreonam, meropenam, and cefpirome

This study was designed in two parts; part I was the *in vitro* effects of triple combination; amikacin, ceftazidime, and fosfomycin on MDR-PA clinical isolates from Chonburi Hospital. The second part was the determination of optimal dosage regimen of triple combination; amikacin, ceftazidime, and fosfomycin in the treatment of MDR-PA infections using population pharmacokinetic approach.

Objectives

1. To determine the *in vitro* effects of triple combination; amikacin, ceftazidime, and fosfomycin on MDR-PA clinical isolates.
2. To determine the optimal dosage regimen of triple combination; amikacin, ceftazidime, and fosfomycin in the treatment of MDR-PA infections using population pharmacokinetic approach.

Expected Outcomes and Benefits

1. The triple combination of amikacin, ceftazidime, and fosfomycin show the synergism or additivity interaction without antagonism interaction.
2. Demonstrates the recommended dosage regimen of triple combination; amikacin, ceftazidime, and fosfomycin in the treatment of MDR-PA infections.

CHAPTER II
LITERATURE REVIEW

I *Pseudomonas aeruginosa*

1. Microbiology

Pseudomonas aeruginosa is a non-fermentative aerobic gram negative bacilli belonging to the bacterium family *Pseudomonadaceae* that can produce two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin. The latter is produced abundantly in media with low-iron content and functions in iron metabolism in the bacterium. *P. aeruginosa* normally lives in moist environments. It has minimal nutrition requirements while being able to use several organic compounds for growth. Its optimum temperature for growth is 37 Celsius, and it is able to grow at temperatures as high as 42 Celsius. It is tolerant to wide variety of physical condition, including temperature. It is resistant to high concentration of salts and dyes, weak antiseptics, and many commonly used antimicrobial agents. They are common inhabitants of soil and water. In fact, *P. aeruginosa* is an opportunistic pathogen, meaning that it exploits some host break in the host defense to initiate an infection (9).

The typical *P. aeruginosa* in nature might be found in a biofilm, attached to some surface or substrate, or in planktonic form, as a unicellular organism. The mucoid exopolysaccharide produced by *P. aeruginosa* is a repeating polymer of mannuronic acid referred to as alginate. Alginate slime forms the matrix of the *P. aeruginosa* biofilm which anchors the cells to their environment and, in medical situation; it protects the bacteria from the host defenses such as lymphocytes, phagocytes, the cilia action of the respiratory tract, antibodies and complements. Biofilm mucoid strains of *P. aeruginosa* are also less susceptible to antimicrobial agents than their plantonic counterparts. Mucoid strains are most often isolated from

patients with cystic fibrosis and they are usually found in post mortem lung tissues from such individuals (9).

P. aeruginosa produces three other soluble proteins involved in the invasion, a cytotoxin and hemolysins. The cytotoxin is a pore forming protein. It was originally named leukocidin because of its effect on neutrophils, but it appears to be cytotoxic for most eukaryotic cells. Of the two hemolysins, one is a phospholipase and the other is a lecithinase. They appear to act synergistically to break down lipids and lecithin. The cytotoxin and hemolysins contribute to invasion through their cytotoxic effects on eukaryotic cells (9).

P. aeruginosa is typically an opportunistic pathogen that seldom causes disease in healthy subjects. Normally, for an infection to occur, some disruption of the physical barriers (skin or mucous membranes), or by-passing of them (e.g., by urinary catheters, endotracheal tubes or other invasive devices), and or an underlying dysfunction of the immune defense mechanisms, such as neutropenia, is necessary (2, 10). Nosocomial infections caused by *P. aeruginosa* most frequently involve the respiratory tract, the urinary tract and wounds. *P. aeruginosa* is amongst the leading causes of nosocomial pneumonia, especially in mechanically ventilated patients. Overall, these patients have a much higher probability of developing nosocomial pneumonia, with *P. aeruginosa* being the most frequent cause. Mortality rates ranging from 40% to more than 60% have been reported in bacteremic, nosocomial pneumonia and in ventilator associated pneumonia. Nosocomial urinary tract infections caused by *P. aeruginosa* are usually related to catheterization or other invasive procedures, and may be complicated by bacteremia (2).

P. aeruginosa is primarily a nosocomial pathogen. The frequency with which it causes disease is reliably estimated from annual surveillance data collected by the National Nosocomial Infection Surveillance (NNIS) system of the Centers for Disease Control and Prevention (CDC). According to these data, collected between 1986 and 1998, *P. aeruginosa* was the second most common cause of nosocomial pneumonia (14% of isolates), the third most common cause of urinary tract infection (7%), the fourth most common cause of surgical site infection (8%), the seventh most frequently isolated pathogen from the bloodstream (2%), and the fifth most common

isolate (9%) overall from all sites. Patients with cystic fibrosis, neutropenia, or multiple devices are at the greatest risk of *P. aeruginosa* infection (11). In clinical practice, *P. aeruginosa* is one of the leading causes of severe infections and associated with significant morbidity, mortality, health-care costs and is often difficult to treat especially multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) isolates (2, 12, 13).

2. Mutidrug-resistant *P. aeruginosa* (MDR-PA)

Multidrug-resistant (resistant to at least three different classes of antimicrobial agents) *P. aeruginosa* (MDR-PA) strains were first reported in patients with cystic fibrosis, and dissemination of these resistant organisms has been reported among hospitalised patients. *P. aeruginosa* isolates resist to carbapenems, or all antimicrobial agents available for clinical use (Pandrug-resistant *P. aeruginosa*; PDR-PA), have been reported to cause nosocomial infections and outbreaks among patients hospitalised in ICUs or burn units (14).

Definition of MDR-PA is varying according to the authors or institutions. Lang et al. (15) defined as resistance all of antimicrobial agents in two of the following three antimicrobial agents classes: (1) β–lactams, including piperacillin, aztreonam and imipenem (2) aminoglycosides, including amikacin, gentamycin and tobramycin (3) fluoroquinolones, and particularly ciprofloxacin, whereas Jung et al. (16) defined as resistance to at least three of four drugs including ceftazidime, imipenem, ciprofloxacin, and tobramycin. In this study, MDR-PA is defined in accordance with Jung et al, but used amikacin instead of tobramycin in susceptibility testing.

3. Mechanism of acquired resistance

The mechanism of resistance that bacteria use to evade the effect of antimicrobial agents fall in to three categories, including (i) removal of antimicrobial agents through the membrane pumps, (ii) the accumulation of mutations that decrease uptake or the affinity of the target for the antimicrobial agents, and (iii) the destruction or sequestration of the antimicrobial agents (17). *P. aeruginosa* is intrinsically resistant to many antimicrobial agents, including most β-lactams, the older quinolones,

Table 2.1 Major resistance mechanisms to antipseudomonal drugs (10)

Class	Agents	Resistance Mechanisms/Comments
Penicillin	Ticarcillin, Carbenicillin, Piperacillin	Derepression of chromosomal β-lactamase. Overexpression of the MexAB-OprM multidrug efflux pump due to a NalB mutation. Specific plasmid-mediated β –lactamases.
Cephalosporin	Ceftazidime, Cefoperazone, Cefepime, Cefpirome	Derepression of chromosomal β-lactamase. Overexpression of the MexAB-OprM multidrug efflux pump due to a NalB mutation. For the fourth generation cephalosporins cefepime and cefpirome, overexpression of the MexCD-OprJ multidrug efflux pump due to an NfxB mutation.
Aminoglycoside	Gentamicin, Tobramycin, Amikacin	Overexpression of the MexXY efflux pump in impermeability type-resistance due to a mutation in the regulatory gene MexZ. Plasmid-mediated production of modifying enzymes.
Quinolone	Ciprofloxacin	Target site mutations in the GyrA (or sometimes the GyrB) topoisomerase subunit; Overexpression of multidrug efflux pumps due to NalB, NfxB or NfxC mutations.
Polymyxin	Colistin	Outer membrane LPS changes due to PhoP/PhoQ regulatory mutations. No evidence this occurs in the clinic.
Carbapenem	Imipenem, Meropenem	Loss of specific outer membrane porin channel, OprD; Reduction in levels of OprD due to an NfxC mutation that also upregulates multidrug resistance due to MexEF-OprN; For meropenem overexpression of the MexAB-OprM multidrug efflux pump due to a NalB mutation

chloramphenicol, tetracycline, macrolides, trimethoprim–sulfamethoxazole and rifampin.

3.1 Fluoroquinolones

Acquired resistance to fluoroquinolones can be due either to mutations that cause the up-regulation of efflux systems, including MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM, or to mutations of the topoisomerase targets (*gyr*A and also *par*C). Both mechanisms are responsible for cross-resistance to all fluoroquinolones, whereas an efflux mutant also typically exhibit a decreased susceptibility to other drugs, while only quinolones susceptibility is affected in targets mutants (2, 10, 18).

3.2 Beta-lactams

Resistance to antipseudomonal β-lactams is common and can result from one or more of several different mechanisms. Mutations leading to the increased production of the AmpC β-lactamase can occur at frequencies of 10^{-7}-10^{-9}, and may result in decreased susceptibility to overt resistance (depending on the amount of enzyme, mutant phenotype and β-lactam compound) to those compounds that are normally active as they do not induce AmpC production, but that are not entirely stable to the enzyme (penicillins, cephems and monobactams). Three different mutant phenotypes have been described, including a moderate-level constitutive production, a high-level constitutive production and a moderate-level basal with hyperinducible production. The mutational events leading to the deregulation of AmpC production in *P. aeruginosa* have been identified only in part, but they appear to be substantially different from those usually responsible for the same phenomenon in *Enterobacteriaceae*. Moreover, in *P. aeruginosa*, multiple mutational pathways could be responsible for these phenotypes, which would explain the phenotype heterogeneity and notable tendency to segregate similar mutants.

The MexB protein is a broad-spectrum pump, located in the cytoplasmic membrane: the OprM protein is a pore-forming protein that provides an exit portal through the outer membrane: and the MexA protein physically links these components. The primary role of OprD protein (initially called D2 porin) is in the

passive uptake of basic amino acids across the outer membrane, but it form pores that are also permeable to carbapenems, through not other β-lactams (19).

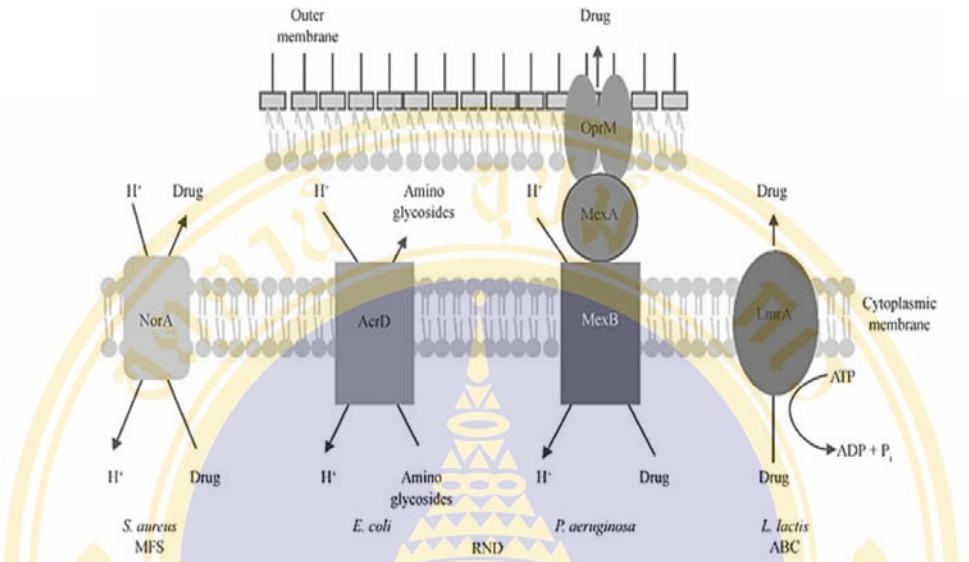


Figure 2.1 Three-component efflux pump. The pump itself (MexB, MexD, or MexF, according to the system) lies in the cytoplasmic membrane and is attached via a linker lipoprotein (MexA, MexC, or MexE) to the exit portal (OprM, OprJ, or OprN). Efflux system components appear in large roman type; other membrane components appear in small italic type (18).

Mutations leading to a decreased amount of the OprD porin can occur at relatively high frequency (10^{-7}) and result in resistance to imipenem and reduced susceptibility to meropenem. Loss of OprD production can be due to deletions, substitutions or insertions that cause inactivation of the *oprD* gene, but OprD production can also decrease following regulatory mutations that cause both the down-regulation of OprD and the up-regulation of the MexEF–OprN efflux system. In the former case, only susceptibility to imipenem is affected, while, in the latter case, susceptibility to quinolones is also decreased.

Mutations leading to the up-regulation of efflux systems (MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM) can variably result in decreased susceptibility to full resistance (depending on the system, the level of up-regulation and the compound) to antipseudomonal β-lactams. Up-regulation of the MexAB–OprM system, which is usually caused by mutations that inactivate the *mexR* regulatory gene, decreases susceptibility to virtually all antipseudomonal β-lactams,

Table 2.2 Extended-spectrum metallo–β-lactamases found in *Pseudomonas aeruginosa* (18)

Enzyme(s)	Country where strain was found	Encodement site	Associated phenotype, by drug						Inhibition by	
			Carb-Tic	Pip-Azl	Czid	Cpm-Cpr	Atm	Imi-Mero	Clv	Taz
PER-1	Turkey (mostly), Italy, France, Belgium	Plasmids or chromosome	R	R	R	R	R	S	Strong	Weak
OXA-11, -14, -16, -19, -28	Turkey (OXA-11, -14, and -16); France (OXA-19 and -28)	Integrins in plasmids or chromosome	R	R	R	R	R	S	Weak	Weak
OXA-15	Turkey	Plasmids	R	R	R	R	R	S	Weak	Weak
IMP-1/-8	Japan (I9MP-1); Canada (IMP-7)	Integrins in plasmids or chromosome	R	R	R	R	S	r/R	No	No
VIM types	Italy (VIM-1), France, Greece, Korea (VIM-2), Taiwan (VIM-8)	Integrins in plasmids or chromosome	R	R	R	R	S	r/R	No	No

NOTE. Azl, azlocillin; Carb, carbenicillin, Clv, clavulanate; Cpm, cefepime; Cpr, cefpirome; Imi, imipenem; Czid, ceftazidime; Mero, meropenem; Pip, piperacillin; r, reduced susceptibility; R, frank resistance, which may vary in its distinction from "r," according to the breakpoints adopted, the permeability of the strain, and the amount of enzyme produced; S, susceptible; Taz, tazobactam; Tic, ticarcil

Table 2.3 Mutational resistances in *Pseudomonas aeruginosa* (18)

Mechanism	Mutation site	Effect on strain, according to antipseudomonal drug								
		Fq	Carb-Tic	Pip-Azl	Czid-Atm	Cpm-Cpr	Imi	Mero	Agl	Pm
Reduced affinity										
Of topoisomerase II	*gyrA*	r/R	—	—	—	—	—	—	—	—
Of topoisomerase IV	*parC*	r/R	—	—	—	—	—	—	—	—
Derepression of AmpC										
Partial	*ampD*	—	R	R	R	R	—	—	—	—
Total	*ampD* + other	—	R	R	R	R	—	—	—	—
Up-regulation										
Of MexAB-OprM	*nalB* at *mexR*; *nalC* at other	R/R	R	r/R	r/R	r/R	—	r	—	—
Of MexCD-OprJ	*nfxB*	r/R	r/R	r/R	r/R	R	—	r	—	—
Of MexEF-OprN	*nfxC* at *mexT*	r/R	r/R	r/R	r/R	r/R	r	r	—	—
Of MexXY-OprM		r/R	r/R	r/R	r/R	r/R	—	r	r	—
Reduced aminoglycoside transport		—	—	—	—	—	—	—	r/R	—
Loss of OprD	*oprD*; *nfxC* at *mexT*	—	—	—	—	—	R	r	—	—
Membrane changes		—	—	—	—	—	—	—	—	R

NOTE. Agl, aminoglycosides; Azl, azlocillin; Atm, aztreonam; Carb, carbenicillin, Czid, ceftazidime, Cpm, cefepime; Cpr, cefpirome; Fq, fluoroquinolone; Imi, imipenem; Mero, meropenem; Pip, piperacillin; Pm, polymyxin; r, reduced susceptibility; R, frank resistance, which may vary in its distinction from "r," according to the breakpoints adopted; Tic, ticarcillin.

except imipenem, while up-regulation of the MexCD–OprJ and MexXY–OprM systems only affects susceptibility to some cephems (cefpirome, cefepime, cefoperazone) and to meropenem. On the other hand, the MexEF–OprN system does not efflux β-lactams, but the up-regulation of this system indirectly affects carbapenem susceptibility due to concomitant down-regulation of OprD.

Finally, the acquisition of secondary β-lactamase genes by horizontal transfer can be responsible for acquired β-lactam resistance, the spectrum of which reflects the substrate specificity of the acquired enzyme. From this point of view, the acquired β-lactamases found in *P. aeruginosa* can belong to three different groups: (i) narrow-spectrum active site-serine enzymes of molecular classes A and D (e.g., PSE-1, PSE-4 and some OXA-type enzymes) that efficiently degrade the antipseudomonal penicillins and cefoperazone, but have no significant activity against the other antipseudomonal cephems, monobactams or carbapenems; (ii) extended-spectrum active site-serine enzymes of molecular classes A and D (e.g., PER-1, VEB-1, GES-1, GES-2, various OXA-type enzymes and, although rarely, also TEM- and SHV-type extended-spectrum variants) that, in addition to penicillins, can also degrade the antipseudomonal cephems and monobactams but not carbapenems (the GES-2 enzyme also has a modest carbapenemase activity that can confer resistance to imipenem when associated with impermeability-mediated resistance mechanisms; and (iii) metalloenzymes of molecular class B (e.g., the enzymes of the IMP, VIM, SPM and GIM type) that efficiently degrade virtually all the antipseudomonal β-lactams except monobactams. Members of the last two groups are the most worrisome from the clinical standpoint due to their broad substrate profiles; and although, in most cases, isolates producing these are still found sporadically or may cause small outbreaks, similar enzymes are progressively disseminating in the clinical setting and are currently included amongst the emerging β-lactamases of increasing clinical importance. In some cases, multiple acquired β-lactamases can be found in clinical isolates, broadening the spectrum of β-lactam resistance. In particular, the simultaneous presence of an extended-spectrum β-lactamase and a metallo-β-lactamase can result in a phenotype of resistance to all the antipseudomonal β-lactams (2, 10, 18).

Recently, two papers have examined the interplay of β-lactamase and efflux in determining resistance. The conclusion from these studies are that the susceptibility of *P. aeruginosa* to some β-lactams (e.g. ceftazidime, cefepime, piperacillin, aztreonam) is more strongly influence by efflux, whereas susceptibility to others (imipenem, panipemen) is more strongly affected by the presence of β–lactamase, while the third group (ceftriaxone, meropenem, moxalactam) is influenced only by knockout of both efflux and β–lactamase. In contrast, knockout of efflux has no apparent effect (10).

3.3 Aminoglycosides

Most large studies have indicated that around 10% of *P. aeruginosa* isolates are aminoglycoside resistant (10). Acquired resistance to aminoglycosides, can be due to the production of aminoglycoside-modifying enzymes encoded by horizontally acquired resistance determinants, or by mutations that reduce aminoglycoside accumulation in the bacterial cell. The most prevalent aminoglycoside-modifying enzymes found in *P. aeruginosa* are the acetyl-transferases AAC(6′)-II (resistance to gentamicin, tobramycin and netilmicin), AAC(3)-I (resistance to gentamicin), AAC(3)-II (resistance to gentamicin, tobramycin and netilmicin) and AAC(6′)-I (resistance to tobramycin, netilmicin and amikacin), and the adenylyl-transferase ANT(2′)-I (resistance to gentamicin and tobramycin). Reduced aminoglycoside uptake could be due to mutations causing lipopolysaccharide changes or up-regulation of efflux systems based on the MexXY linker-pump module. Unlike resistance mediated by modifying enzymes, the spectrum of which can be variable depending on the nature of the enzyme, resistance mediated by efflux systems tends to be broad spectrum (2). Although emzyme-mediated aminoglycoside reistance is observed, at least 50% and up to 90% of isolates appears to carry the ‘impermeability’ type resistance. Interestingly, overexpression of MexXY alone from cloned gene did not result in resistance. Another relevant of aminoglycoside resistance is adaptive resistance. Such resistance is reversible, after a post-antimicrobial effect, upon removal of selective pressure. The mechanism of adaptive resistance is not understood. However, Hancock et al. have demonstrated that the two-component regulatory system, PhoP-PhoQ, which responds to divalent cation concentration, can

regulate susceptibility to aminoglycosides as well as to polymyxin B and some cationic antimicrobial peptides (10).

3.4 Polymyxins

Acquired resistance to polymyxins has been occasionally described in *P. aeruginosa* isolates from cystic fibrosis patients treated for long periods with the nebulized drug, and seems to be related to mutations causing changes in the outer membrane architecture (2, 18).

3.5 Fosfomycin

The low toxicity and broad-spectrum bactericidal activity of fosfomycin have resulted in its increased clinical use, which intern has resulted in fosfomycin-resistant strains. Acquired resistance to fosfomycin may be attributed to either mutation in the uptake and transport mechanisms or the presence of fosfomycin-modifying enzyme whose product has no antibacterial activity. There are four difference fosfomycin-modifying enzyme have been described including, FosA, Fos B, FosC and FosX that confer resistance to fosfomycin by adding glutathione, L-cysteine, ATP, or a hydrozyl group respectively, to the oxirane ring of fosfomycin, rendering it inactive. The best characterized one is FosA, which has high specificity for fosfomycin with the presence of a metal cofactor (Mn^{2+} and K^{+}). Currently, there are no inhibitors to combat FosA-mediated fosfomycin resistance. To develop effective FosA inhibitors, it will be essential to understand the catalytic mechanism and, consequently, the amino acids determinants of structure and activity (17, 20).

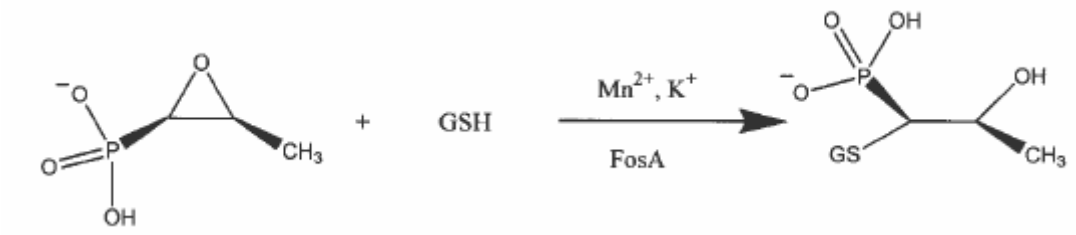


Figure 2. 2 FosA catalyzes the addition of fosfomycin to glutathione (GSH) to fosfomycin (17)

4. Emergence of MDR-PA

Although the simultaneous emergence of multiple mutants is highly unlikely, sequential emergence is possible and is facilitated by the fact that infections caused by a strain resistant to certain antimicrobial agents are thus treated with a different antimicrobial agent. Moreover, in some cases, a single mutational event can compromise multiple drugs with different mechanisms (e.g., mutations leading to the up-regulation of efflux systems that cause resistance to both fluoroquinolones and β-lactams). On the other hand, acquired resistance genes are often carried on genetic elements (such as plasmids, transposons or integrons) in which several resistance determinants are clustered, so that an MDR phenotype can be acquired in a single step upon acquisition of the element. All of this accounts for the remarkable potential of *P.* aeruginosa to evolve rapidly towards multidrug resistance (2).

Kriengkauykiat et al. (12) studied the contribution of efflux pump overexpression (EPO) in levofloxacin-resistant strains. They found that, EPO was present in the majority (61%) of fluoroquinolone (FQ)-resistant strains compared to only 9% of FQ-susceptible strains and the efflux pump overexpressed phenotype is most frequently encounter in the presences of coresistant to piperacillin/tazobactam (85%) followed by ceftazidime (76%). It notable that many of FQ-resistant strains are associated with cross-resistance to structurally unrelated antimicrobial agents. More importantly, they have been provided support between FQ-resistant and multidrug resistance through phenotypic confirmation of EPO. Considering, increased exposure through widespread prescribing of the fluoroquinolones will undoubtedly promote the selection for strains overexpressing efflux pumps leading to increasing the prevalence of FQ-resistant and MDR-PA.

Previous treatment with antimicrobial agents that are characterized by high antipseudomonal activity and prolonged antimicrobial treatment are both recognized risk factors for the emergence of drug-resistant *P. aeruginosa* (21). Also Defez et al (1) determine the risk factors for nosocomial infection with MDR-PA among all hospitalized patients and among those with nosocomial infection due to *P. aeruginosa*. They found that, in addition to urinary catheterization, nasogastric feeding is an important risk factor in MDR-PA infection. Indeed, an imbalance in gut flora,

modifications to the mucous membranes due to the use of nasogastric feeding and the selection pressures exerted by antimicrobial agents were implicated in the occurrence of this infection. In the multivariate analysis, exposure to β-lactams [OR = 2.5 (1.0–6.3)] or fluoroquinolones [OR = 4.1 (1.5–11.7)] was linked to nosocomial infection due to MDR-PA. The other risk factors were age (older patients had an increased risk), transfer from another care unit, being bedridden, urinary catheterization or nasogastric feeding. Cao et al (22) demonstrated that patients with nosocomial infections due to MDR-PA were more likely to have been exposed to imipenem/meropenem 15 days before the isolation (OR, 44.8; 95% CI, 9.16–219), and to have been treated with mechanical ventilation 48 hours found multiple logistic regression analysis (OR, 8.2; 95% CI, 1.65–40.7).

5. Epidemiology of MDR-PA

Nowadays, there are few data on the incidence of MDR-PA. In the USA, between 1997 and 2000, the proportion of MDR-PA (resistant to over three agents: ceftazidime, piperacillin, imipenem gentamycin and amikacin) increased from 12.8 to 20.8 % (1). In the SENTRY surveillance program, MDR-PSA isolates, defined as being resistant to piperacillin, ceftazidime, imipenem, and gentamicin. Their distribution by geographic region and body site is shown in Table 2.4. The respiratory tract was still the most frequent site of isolation (46.1%), and the bloodstream was the second most frequent source of MDR-PSA isolates. Latin America was the region with the greatest number of MDR-PSA isolates. The rates of occurrence of MDR-PSA by geographic region were as follows: Latin America, 8.2%; Europe, 4.7%; United States, 1.2%; Asia-Pacific, 1.6%; and Canada, 0.9%. Approximately 56% of MDR-PA strains isolated in Latin America were collected from just 1 Brazilian medical center. If this site were excluded, the frequency of occurrence of MDR-PSA in Latin America would be reduced to only 3.6%, i.e., less than the rate recorded for Europe (11). In Japan, the frequency of MDR strains among the 3233 clinical isolates, according to the clinical sources from which they were isolated. Of the 3233 strains, 89 (2.8%) were MDR. The most frequent source of MDR strains was urine (42 strains; 47.2%), followed by the respiratory tract (sputum and pharyngeal mucus, 17 strains; 19.1%), pus (13 strains; 14.6%), blood (8 strains; 9.0%), cerebrospinal fluid (1 strain; 1.1%), and others (8 strains; 9.0%), as shown in Table 2.5 and Table 2.6 (23).

Table 2.4 Occurrence of the 218 MDR-PA strains by body site of isolation [a]

Country or region, year (no. of isolates tested)	Occurrence by site of infection: no. of MDR-PSA isolates (no. of medical centers [b])			
	Blood	Respiratory	Wound	Urine
Asia-Pacific				
1997 (n = 8)	1 (1)	2 (2)	5 (3)	0
1998 (n = 7)	1 (1)	6 (1)	0	0
Canada				
1997 (n = 3)	0	3 (2)	0	0
1998 (n = 2)	0	1 (1)	0	1 (1)
1999 (n = 0)	0	0	0	1 (1)
Europe				
1997 (n = 6)	3 (2)	1 (1)	1 (1)	1 (1)
1998 (n = 48)	19 (8)	15 (5)	12 (4)	2 (2)
1999 (n = 24)	5 (4)	16 (5)	2 (1)	1 (1)
Latin America				
1997 (n = 23)	3 (3)	13 (5)	4 (2)	3 (2)
1998 (n = 31)	10 (4)	16 (4)	2 (2)	3 (3)
1999 (n = 36)	11 (3)	11 (5)	7 (3)	7 (2)
United States				
1997 (n = 12)	2 (2)	7 (4)	2 (1)	1 (1)
1998 (n = 8)	0	4 (4)	1 (1)	3 (2)
1999 (n = 10)	3 (3)	6 (4)	0	1 (1)

NOTE. *P. aeruginosa* isolates were defined as multiresistant (MDR-PA) if they were resistant to piperacillin (MICs, >64 mg/mL), ceftazidime (>16 mg/mL), imipenem (>8 mg/mL), and gentamicin (>8 mg/mL).

[a] Data were obtained from participant medical centers in the SENTRY Antimicrobial Surveillance Program.

[b] This indicates the number of medical centers contributing MDR-PA in the respective region.

Also in Thailand, the rates were difference in each institution, Piriykitphaiboon found that the incidence of MDR-PA in creased from 5 to 29% in 1994 to 1996 (3) and the incidence increased from 47-55 % in 1999 to 2001 in tertiary care hospital (4).

Table 2.5 Frequency of isolation and PFGE patterns of multi-drug-resistant *Pseudomonas aeruginosa* isolates from seven regions of Japan (23)

Region (no. of isolates)	No. of MDR isolates (%)
Hokkaido (224)	2 (0.9)
Tohoku (439)	17 (3.9)
Kanto (1341)	39 (2.9)
Chubu (340)	3 (0.9)
Kansai (126)	6 (4.8)
Chugoku/Shikoku (372)	10 (2.7)
Kyushu/Okinawa (391)	12 (3.1)
Total (3233)	89 (2.8)

Multi-drug-resistant (MDR), resistant to imipenem, gentamicin, and ciprofloxacin

Table 2.6 The frequency of multi-drug-resistant *Pseudomonas aeruginosa* strains according to source of clinical specimen (23).

Specimen	No. of isolates	Percentage
Urine	42	47.2
Sputum/Pharyngeal mucosa	17	19.1
Pus	13	14.6
Blood	8	9.0
Cerebrospinal fluid	1	1.1
Other	8	9.0
Total	89	100

Multi-drug-resistant (MDR); resistant to imipenem, gentamicin, and ciprofloxacin. There were 89 MDR isolates among the 3233 isolates

6. Treatment options

As *P. aeruginosa* can be a lethal pathogen and the precocity of therapy is critical to the outcome of the infection, empirical regimens adequate for *P. aeruginosa* coverage should always be initiated prior to receipt of the results of cultures and susceptibility testing when infection by this species is suspected. For the selection of empirical regimens, several aspects should be considered, including: (i) the nature and source (nosocomial vs. community-acquired) of the infection; (ii) information concerning the epidemiology of resistance phenotypes in the individual setting; (iii) pharmacokinetic parameters; (iv) underlying risk factors (e.g., length of hospitalisation, ICU admissions, previous antimicrobial therapy) and diseases; and (v) hospital prescription policies (2).

Despite the fact that *P. aeruginosa* has high intrinsic resistance to antimicrobial agents, a number of drugs are available for treatment of infection. The major classes that have been used with success include aminoglycosides (such as gentamicin and tobramycin), semisynthetic penicillins (such as carbenicillin, ticarcillin and piperacillin) third generation cephalosporins (including ceftazidime and cefoperazone), quinolones (such as ciprofloxacin) and carbapenems (including meropenem and imipenem) (2, 10). An understanding of the principle and specific mechanisms that pathogens use to evade drugs is important in providing a basis for rational design of new and improved therapeutic options (17).

In addition to the conventional antimicrobial agents listed above, newer agents are being developed to counter the problem of antimicrobial resistance. Small cationic peptides, either natural or synthetic, are active against many strains of *P. aeruginosa*. These agents are present throughout nature (including within human neutrophils) and provide hope for treating infections caused by strains that are resistant to currently available drugs. Human testing is underway, but these novel agents are not yet available for routine clinical use. Another approach, being pursued by Microcide Pharmaceuticals (US) is the development of inhibitors of efflux pumps (e.g. MC-207) as a method of potentiating the activity of fluoroquinolones, and possible other drugs. Other agents to be considered are those which have not been thought to have useful antipseudomonal activity, e.g. the macrolides, but appear to improve the

prognosis of patients with chronic *P. aeruginosa* pulmonary inections. Such agents may indeed have a slow bactericidal effect on this organism (10, 24).

In vitro activity of tigecycline against enteric Gram-negative bacteria, it was found to be highly active against *Enterobacteriaceae*, regardless of the presence or absence of ESBLs. Specifically, tigecycline was very active with MIC_{90} values of ≤2 mg/L, and >99% of all of the tested strains were inhibited with ≤4 mg/L of tigecycline. Uniformly reduced activity was observed for tigecycline against *P. mirabilis* and indole-positive *Proteus spp*. In *P. mirabilis*, reduction of *in vitro* activity of tigecycline is associated with the AcrAB multidrug efflux pump. Additionally, *P. aeruginosa* is not reliably inhibited by tigecycline (24).

II. Antimicrobial combinations

1. Rational for the use of antimicrobial combinations

P. aeruginosa is a virulent organism that is susceptible to only a limited number of antimicrobial agents. For *P. aeruginosa,* antimicrobial resistance is an increasing problem. Infections caused by *P. aeruginosa* are difficult to cure and often require combination therapy (25). Antimicrobial monotherapy is usually recommended for urinary tract infections caused by *P. aeruginosa*, with the exception of upper tract infections complicated by abscess formation, or for infections in neutropenic patients, or whenever there is a suspicion of bacteremia. On the other hand, combination therapy with at least two different antipseudomonal agents is normally recommended for the treatment of severe *P. aeruginosa* infections, such as endocarditis, nosocomial pneumonia and bacteremia (2).

The rational for combination therapy is essentially to reduce the chances of selection of resistant mutants during therapy, as well as to exploit the potential synergistic activity of some agents. The preferred combination remains β-lactams with aminoglycosides, as synergism between these drugs has been demonstrated by in *vitro* studies and results of several clinical studies point to the superiority of similar regimens as opposed to monotherapy for the treatment of *P. aeruginosa* bacteremia,

especially in neutropenic patients. However, some clinical studies have cast doubt on the actual superiority of combination therapy (2).

2. Definitions of antimicrobial combinations

Despite differences in experimental methods and criteria used to define quantitatively the results of antimicrobial combinations, there is general agreement on qualitative definition of synergism and antagonism. Synergy is a positive interaction; the combine effect of the drugs being examined is significantly greater than the expected result, based on their independent effects when the drugs are used separately. (Table 2.7) Antagonism is a negative interaction; the combine effect of the drugs being examined is significantly less than their independent effects when the drugs are used separately (26).

Many of the problems involved in assessing antimicrobial combinations result from uncertainly about the expected result with combinations in which there is no significant interaction between the antimicrobials being tested, whether that result be described as additivity, indifferance, or autonomy. Additivity is the basis of the checkerboard system and assumes that the result observed with more than one drug should be the sum of the separate effects of the drugs being tested if those drugs do not interact with one anther. Autonomy (or indifference) is based on the idea that only one metabolic pathway can be growth rate-limiting for an organism at a time. Based on this observation, autonomy suggests that the combined effect of drugs that do not interac with one another should be simply the effect of the more (most) active drug alone (26).

3. Laboratory methods used to assess the activity of antimicrobial combinations

The accurate prediction of clinically relevant antimicrobial synergy based upon the results of *in vitro* testing has been a goal of researchers for some time. There are, in fact, examples of such a correlation existing. Combinations of antimicrobial agents which have been shown to be synergistic *in vitro* have been associated with a more favorable clinical outcome in neutropenic patients with gram-negative sepsis and in the treatment of enterococcal endocarditis. Increased bactericidal activity in patient serum has also been documented with antimicrobial combinations which are synergistic *in vitro* (27).

3.1 Checkerboard

The checkerboard method is the technique that has been used most frequently to assess antimicrobial combination *in vitro*, presumably because (a) its rational is easy to understand, (b) the mathematics necessary to calculate and interpret the result are simple, (c) it can be readily perform in clinical laboratories using microdilution systems that are obtainable commercially, and (d) it have been the suggested an advantage of synergistic therapy in the treatment of neutropenic patients with Gram-negative septicemia. The term "checkerboard" refers to the pattern formed by multiple dilutions of the two antimicrobials being tested, in concentration equal to, above, and below their MICs for the organism being tested. The concentration tested foe each antimicrobial typically rang from four or five dilutions below the MIC to twice the MIC (or higher of antagonism is suspected), using two fold dilutions of each antimicrobial. Limitations of this technique, unless each of the tubes (or wells) without obvious macroscopic evidence of growth is sampled to determine bactericidal activity, it yields only inhibitory data. This lack of bactericidal data is a serious limitation, because the organisms most frequently submitted for such testing are from patients with infections that most clinicians believe should be treated with bactericidal therapy. Its usually performed provides only all-or-none response and in thus incapable of measuring the graded responses necessary to define dose-response curves. Finally, because the results are usually examined only at one point in time, this method typically provides a static, rather than a dynamic, view of antimicrobial interaction (26).

3.2 Time-kill curves

In contrast to the checkerboard technique, which typically provides only inhibition data, the killing-curves technique measures the bactericidal activity of the combination being tested. For this reason, it is presumably more relevant for clinical situations in which bactericidal therapy is desirable. The other major advantage of killing curves over the checkerboard technique is that they provide the dynamic picture of antimicrobial action and interaction over time (base on serial colony counts), as opposed to the checkerboard technique, which is usually examined only once (after 16 to 24 hours of incubation). The major disadvantage of these method is that the repetitive sampling necessary for each of the flasks being tested and the

Table 2.7 Quantitative definition of results with antimicrobial combinations** (26).

Checkerboard	Kinetic Methods
Assumptions	
Assumes linear (or identical) dose response curves for all drugs tested	Do not assume linear or identical dose response
Additivity	
The result with two drugs is equal to the sum of the results for each of the drugs uesd separately, as defined by $FIC_A + FIC_B = 1.0$	The result with two drugs is equal to the combined activity of each drugs uesd separately, as defined by $g_{(A+B)} = [g_{(A)} \times g_{(B)}] / g_{(0)}$
Autonomy (indifference)	
The result with two drugs dose not significantly differ from the result with the most effective drug alone $FIC_A + FIC_B = FIC_A$ or FIC_B	The result with two drugs is equals the of the more active drugs used alone $g_{(A+B)} = g_{(A)}$ or $g_{(B)}$
Antagonism	
The result with two drugs is significantly less than the additive response $FIC_A + FIC_B > 1.0$	The result with two drugs is significantly less than the additive response $g_{(A+B)} > [g_{(A)} \times g_{(B)}] / g_{(0)}$
Synergism	
The result with two drugs is significantly greater than the additive response $FIC_A + FIC_B \leq 1.0$	The result with two drugs is significantly greater than the additive response $g_{(A+B)} < [g_{(A)} \times g_{(B)}] / g_{(0)}$

** FIC_A and FIC_B are the fractional inhibition concentrations of drugs A and B (the amount of each drug necessary to inhibit growth when combined with the other, devided by its minimum inhibitory concentration

** $g_{(0)}$, $g_{(A)}$, $g_{(B)}$ and $g_{(A+B)}$ represent the growth constants observed in the absence of antimicrobials $g_{(0)}$, with drug A alone $g_{(A)}$, with drug B alone $g_{(B)}$, and with both drug A and B $g_{(A+B)}$.

multiple colony counts required seriously limit the number of antimicrobial combinations that can be tested. For this reason, it is essential that the concentrations that are tested be chosen with the knowledge of antimicrobial concentrations that are achievable at the site(s) of infection (26).

3.3 Diffusion method

A variety of methods have been explored to assess, primarily in qualitative fashion, interactions of antimicrobials as they diffuse through agar plates seed with a test organism. One major advantage of the diffusion method is that commercially produced antimicrobial-impregnated disks and Mueller-Hinton agar plates may be employed. Paper strips soaked in antimicrobial solutions are used in some modifications of this method. Therefore, it is not necessary to make up a series of flasks or agar plates containing different antimicrobial concentrations. The other major advantage of these techniques is that they are easy to perform in the laboratory. As noted, that it may be difficult to distinguish additive from synergistic interaction using this technique.

The Epsilometer, or E test, is a relatively new agar diffusion method for performing antimicrobial susceptibility testing. The antimicrobial concentrations on one side and an interpretation scale of the antimicrobial agent on the other side. The strip is placed onto the surface of an agar plate inoculated with bacteria and incubated overnight, and an elliptical zone of inhibited growth is formed around the strip. The MIC is read at the intersection of the zone with the strip. If the use of the E strip could be standardized for synergy testing and subsequent results could be demonstrated to be similar to those obtained by established methods, this new method of performing synergy tests would represent an attractive alternative to its labor intensive predecessors. Further, this method could be performed on a routine basis in a clinical microbiology laboratory (27).

A number of methods used to detect *in vitro* synergy between antimicrobial agents have been described; however, the checkerboard and time-kill curve methods are the most widely used techniques. The checkerboard method is a relatively easy test to perform; however, it is merely a gauge of inhibitory activity. The time-kill method of synergy testing assesses bactericidal activity but is time-consuming and labor-intensive. Several studies have compared results generated by the checkerboard

and time-kill methods. While some studies have shown excellent agreement between these methods, most have not, and controversy about the comparability of results generated by these techniques exists. Each method has its advocates (27).

A reliable *in vitro* test that could accurately predict the *in vivo* synergy of antimicrobial combinations has been sought for many years. The two most extensively used *in vitro* methods for detecting synergy, checkerboard and time-kill, have yielded mixed results in pertinent evaluations. Further, their respective results, when used in parallel to evaluate synergy in comparative studies, have often been at odds. This is not entirely surprising, as the two methods measure different phenomena. The checkerboard technique, based upon MICs, reflects the inhibition of bacterial growth, whereas the time-kill methodology measures the extent of killing. The checkerboard method has been questioned as an appropriate technique by some investigators but is vigorously defended by others (27, 28).

Although it appears that some investigators have found the time-kill method to be a reliable predictor of *in vivo* synergy, it is not without problems. Shortcomings of the time-kill method include the effect of inoculum size, the difficulties in interpretation of results because relatively few antimicrobial concentrations are examined, and the reliance on the reading at one time point (usually 24 h) as the sole determinant of the interaction. Another obvious disadvantage is that time-kill experiments are labor-intensive and time-consuming. Moreover, a review of the relevant literature reveals that, in addition to using different definitions of synergy, investigators employ a variety of concentrations (clinically achievable concentrations and fractions of the MIC) and various bacterial inocula. The former is a serious limitation, as when one does not include a concentration of one antimicrobial agent that does not affect the growth curve, it is difficult to distinguish between synergy and additivity. Finally, the timing of the colony count determination has varied from one investigation to the next and it has been shown, for example, that different synergy results are obtained at 24 and 48 h (27).

Bonapace et al (28) evaluated the E test method to assess synergy and compared it to the checkerbosrd and time-kill tests. The agreement between the E test method and checkerboard test (63%) and between E test and time-kill test (72%) was higher than agreement between checkerboard test and time-kill test (51%). When

disagreement occurred between E test method and either the checkerboard test or time-kill test, the E test method was generally more conservative. These results are similar to previously published comparison of the checkerboard, time-kill and E test. In the study of White et al (27) the agreement between E test method and checkerboard test was 75%, whereas the agreement between E test method and time-kill test ranged from 63% to 75%. The results of the E test method for detecting synergy evaluated in this study appear to agree fairly well with results from checkerboard and time-kill testing despite differences in endpoints (inhibition compared with killing) and media (broth compared with agar) (27). Additionally, antagonism was not detected using the E test method in the study. Furthermore, when antagonist was observed using the time-kill test in that study, additivity/indifference was reported using the E test method. As far as the new method in concerned, the E test method is less labor intensive and easy to perform. Although it appears to be more conservative than either the checkerboard or time-kill test in detecting synergy (28).

4. Mechanisms of antimicrobial combinations resulting in synergism (26)

There are presently four well-established mechanisms of antimicrobial interaction that produce synergism:

1. Sequential inhibition of common biochemical pathway (e.g., TMP plus SMZ, amdinocillin plus a β- lactam, and vancomycin plus a β- lactam).

2. Inhibition of β- lactamase or decreased production of β- lactamase (e.g., clavulanic acid or sulbactam plus penicillin or chloramphenicol plus penicillin).

3. Sequential inhibition of cell wall synthesis (a variation of the sequential inhibition of common biochemical pathway) (e.g., amdinocillin or vancomycin plus a β- lactam).

4. Use of β- lactams or other agents acting on the cell wall to permit increased entry of aminoglycosides (e.g., penicillin, carbenicillin, vancomycin, or imipenem plus a streptomycin or gentamycin).

5. *In vitro,* synergistic effects of antimicrobial combinations against MDR-PA

Combinations of aminoglycosides and antipseudomonal penicillins have been well investigated. Synergistic effects found in combination of amikacin with ceftazidime, meropenem, imipenem, cefepime, cefoperzone/sulbactam, or aztreonam. Inaddition, fosfomycin exhibited high efficacy rate in 66.7-76.7% (29) when combined with cefepime, aztreonam, meropenam, imipenam, ceftazidime, piperacillin, gentamycin, or levofloxacin, and was active against 92% of *P. aeruginosa* O12 isolates in combination with amikacin (30). Alvarez-Lerma et al. (31) studied efficacy and tolerability of piperacillin/tazobactam versus ceftazidime in combination with amikacin for treating nosocomial pneumonia in intensive care patients. They found that most of severe adverse effects were related to an increase in serum creatinine level in both combinations, which was probably or possibly due to the use of aminoglycosides. Interestingly, combination of β- lactams plus aminoglycosides have been used less commonly in recent years due to the prevalence of acute renal insufficiency in critically ill patients and concerns on the part of the medical house staff regarding perceived toxicity associated with the aminiglycosides (16).

Whereas, combination of antipseudomonal penicillins and fluoroquinolones have been showed difference degree of synergistic effects. Fish et al.(32) demonstrated *in vitro* synergy in ~60-80% of tested *P. aeruginosa* , which similar to, or higher than, those reported previously with these agents (~25-75%). In MDR-PA strains the results shown to be less synergistic effect (33, 34). More importantly, Kriengkauykiat et al. (12) noted that widespread prescribing of the fluoroquinolones will undoubtedly promote the selection for strains overexpressing efflux pumps leading to increasing the prevalence of FQ-resistant and MDR-PA. (FQ-resistant strains are associated with cross-resistance to structurally unrelated antimicrobial agents).

6. *In vivo,* synergistic effects of antimicrobial combinations against MDR-PA

In vivo, Dubois et al. (6) studied the efficacy of cefepime (6gm/day) combination with amikacin (15mg/kg body weight day) in patients who were colonized and/or infected with *P. aeruginosa* P12 strains, the treatment led to the elimination of multiresistant pathogen, demonstrated high efficacy of combination therapy. Mirakhur et al. (7) used fosfomycin in combination with other antimicrobial agents to treat 30 pulmonary exacerbations who colonized with *P. aeruginosa*, mainly resistance strains. They found that, the remaining patients showed clinical resolution of their chest exacerbations (mean FEV1% predicted; pre 41.1 vs. post 49.4, $p<0.001$) and only one patient developed nausea and fosfomycin treatment was withdrawn. Sobieszczyk et al. (8) conducted a retrospectively reviewed the clinical and microbiological efficacy and safety profile of polymyxin B intravenous and/or aerosolized in combination with imipenem, meropenem, amikacin, tobramycin, cefepime, quinolone, ampicillin/salbactam or aztreonam for treatment of multidrug-resistant gram-negative respiratory tract infections, *Acinetobacter baumanii* was the most frequently isolated organism (55%) followed by *P. aeruginosa* (41%) and Alcaligenes xylosoxidants (1%), all isolates were found to susceptible to polymyxin B. They found 76% of the patients had a favorable response, 41% achieved microbiological clearance and 52% survived their hospitalization, with minimal nepho- or neurotoxicity, yet end-of treatment mortality following polymyxin B therapy was only 21%. This is a higher clinical success rate that seen in recently published studies.

7. Fosfomycin used in the combinations therapy against MDR-PA

Fosfomycin is a phosphoenolpyruvate analogue produced by *Streptomyces* that irreversibly inhibits enolpyruvate tranferase, which prevent the formation of N-acetyl-muramic acid, an essential element of the peptidoglycan cell wall (35, 36). It can be used with other antimicrobial agents because it exhibits no cross-resistance due to their differing mechanisms of action (35, 36, 37). It shows excellent penetration in to body tissues and fluids and is practically free of toxicity. Because of the frequent occurrence of resistant mutants, its use in combination has been recommended (39). There are data demonstrated synergistic effects of fosfomycin against *P. aeruginosa*

such as ceftazidime, imipenem (40), cefepime, aztreonam, meropenam (29), and cefpirome (38). Otherwise, it has been reported that fosfomycin protects the kidneys by stabilizing the lysosome membrane and inhibiting drug uptake by renal tubular epithelial cells (7, 37, 39, 41, 42). Yoshiyama et al. (37) suggested that the role of fosfomycin in alleviating nephrotoxicity is dose dependent. A similar mechanism may account for protection against aminoglycoside-related ototoxicity (7). So, fosfomycin can be candidate in combination therapy with other antimicrobial agents.

Tessier et al (39) suggest that fosfomycin in combination therapy with ciprofloxacin, amikacin, or imipenem could be a good alternative for treating *P. aeruginosa* infections, even against some strains that exhibit low-level resistance to fosfomycin.

8. Triple combinations against MDR-PA

In 1984, Takahashi and Kanno (43) found synergistic activities *in vitro* of combinations of piperacillin, cefoperazone, and cefsulodin with fosfomycin against 80.0, 85.0, and 82.6% of the strains test, respectively. The mean fractional inhibitory concentration; FIC index were 0.48, 0.42, and 0.46, respectively (synergism indicated by an FIC index of $\leq$0.5). The synergistic activities of these combinations were enhanced by the addition of tobramycin, 0.25 mcg/mL, the mean of FIC index were 0.30, 0.24, and 0.27, respectively. They propose that these combinations may be useful for severe *P. aeruginosa* infections. In 1999, Oie et al. (44) evaluated the *in vitro* effects of various combinations of five types of widely used antipseudomonal drugs (piperacillin, meropenem, cefatzidime, aztreonam, and amikacin) against six *P. aeruginosa* strains that were resistant to each antimicrobial agent. Statistical comparison of the combination effects of antimicrobial agents against the strains used showed a significant difference between the combination of two drugs and those of three drugs ($p<0.01$), by the chi-square test, using the number of growth inhibited strains as variable. And in 2003, Oie et al. (34) evaluated the combined effects of antimicrobial agents by agar incorporation inhibitory test and by time-kill tests on seven geographically and epidemiologically distinct isolates of MDR-PA. All strains were resistant to aztreonam, piperacillin, meropenem, cefatzidime, cefoperazone/sulbactam, amikacin and ciprofloxacin. By agar incorporation inhibitory test, among the three-drug combinations, the combination of piperacillin, ceftazidime

and amikacin, and that of cetazidime, aztreonam and amikacin were the most effective. In time-kill tests, the three-drug combination of cetazidime, aztreonam and amikacin was the most effective.

III. Optimization of antimicrobial therapy using Pharmacokinetic and Pharmacodynamic parameters

Among the documented misuses contributing to drug resistance are inappropriate dosage regimens (dose, dosage interval, duration of treatment, route and conditions of administration). Rational antimicrobial therapy requires dosage regimens to be optimized, not only to guarantee clinical efficacy, but also to minimize the selection and spread of resistant pathogens (45).

Pharmacokinetic (PK) and pharmacodynamic (PD) characteristic both influence dosing regimens of antimicrobials. For two decades, the focus has been on pharmacokinetic characteristics—— what the body dose to the drug and the overall disposition of the drug in the body. This reflected most often by the serum concentration profile over time. Of particular interest as well is the penetration of drug in to sites of infection. Medications are administered, however, for their pharmacodynamic characteristics — what the drug does in the body. Susceptibility of the pathogen to the drug, determined by measuring the MIC, is a reflection of the potency of a drug. However, to be able to understand the application or relevance of drug dose to efficacy, we have to integrate pharmacokinetic characteristic with pharmacodynamic characteristic. The increasing occurrence of antimicrobial resistant pathogens complicates the integration of pharmacokinetic and pharmacodynamic, and has an impact on treatment approaches to respiratory tract infections. Clearly, as pathogens become more resistant to antimicrobials, the efficacy of standard dosing regimens may be reduced (46).

1. Pharmacokinetic and Pharmacodynamic terminology according to the ISAP (47)

The International Society for Anti-Infective Pharmacology (ISAP) is an interdisciplinary scientific society that promotes the study of the PK and the PD of antimicrobials so as to improve dosing regimens. Since the foundation of the ISAP in 1991, the study and use of pharmacokinetic and pharmacodynamic principles in antimicrobial therapy has increased greatly. Variation in the definition of pharmacological indices used in published materials has caused confusion. In order to avoid this, the ISAP published a paper about the PK/PD terminology for anti-infective drugs, in which the PK and PD parameters and the PK/PD indices are defined.

Time>MIC (to be written as T>MIC)

Definition: the cumulative percentage of time over a 24 h period that the drug concentration exceeds the MIC. Note: if the period is other than 24 h, this should be stated explicitly. T>MIC (the expression $t_{c>MIC}$ would be more accurate) is mainly used to predict the efficacy of time-dependent antimicrobials (e.g. β-lactams, glycopeptides, macrolides, clindamycin and oxazolidinones). Drugs that belong to these classes show no or little enhancement of the effect with an increase in antimicrobial concentration.

Peak/MIC (C_{max}/MIC) (ratio)

Definition: the peak level divided by the MIC. In the literature, C_{max}/MIC is also denoted as peak/MIC, inhibitory quotient (IQ) or inhibitory rate (IR). This index is used to predict or describe the antibacterial effect of concentration-dependent antimicrobials. Aminoglycosides and quinolones show such an enhanced activity with increasing concentrations.

AUC/MIC

Definition: the area under the concentration–time curve over 24 h divided by the MIC. If a subscript indicating another time period is not present, the AUC is assumed to be the 24 h value at steady state. Note: For all practical purposes, the expression AUC/MIC should be used to show PK/PD relationships involving the AUC and MIC.

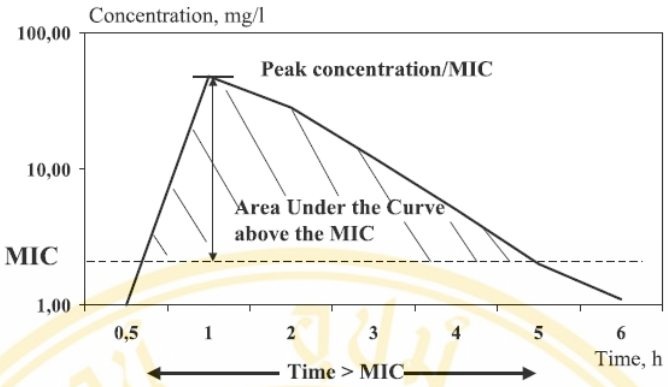
2. Patterns of antimicrobials activity

a. Time dependent killing

Time dependent killing refers to the time it takes for a pathogen to be killed by exposure to an antimicrobial (Figure 2.3). The goal of time dependent killing is to optimize the duration of exposure. With time-dependent killing, post-antimicrobial effects; PAE (persistence of antimicrobial action after the antimicrobial is removed) are minimal. Time-dependent killing is characteristic of β-lactams (penicillins, cephalosporins, monobactams, and carbapenems), macrolides, clindamycin, and oxazolidinones (47, 48).

The major pharmacokinetic/pharmacodynamic parameter that correlates with the clinical and bacteriologic efficacy of these drugs is the time for which the serum concentration exceeds the MIC of the pathogen. β-lactams are the most commonly used antimicrobials in clinical practice, especially in treating infections of the upper and lower respiratory tract. In animal models of human infection, different classes of β-lactams are require different times above the MIC for net and maximum bactericidal activity. As would be expected, the required time above the MIC varies, depending on the pathogen, infection site, and drug, but is generally 40-50% of the dosing interval. Similar time above MIC are required to achieve 80% or greater rates of bacteriologic cure in otitis media and in sinusitis caused by *Hemophilus influinzae* and *Streptococcus peumoniae* with β-lactams (46). For instance, carbapenems require 20% and 40% of T>MIC for bacteriostatic and bactericidal activity, whereas cephalosporin require 35-40% for bacteriostatic and 60-70% for maximum bactericidal activity. Penicillins generally achieve bactericidal exposures at 50% T > MIC (49).

Nishida *et al*. evaluated the activity of 3 cephalosporins against *E. coli* using an in *vitro* model. By varying drug concentration and exposure time they demonstrated that longer exposure time at or above the MIC resulted in greater bactericidal effect up to a point of maximal killing. Maximal effect was seen at concentrations 1 to 4 times the MIC, with no further significant reduction in bacterial counts when concentrations exceed 4 times the MIC (48).



Fiugure 2.3 PK parameters used for correlation with effect *in vivo*. The curve illustrates a random serum concentration curve of an antimicrobial administered either orally or intramuscularly (50).

To optimize the T > MIC, continuous infusion of β-lactams has been investigated. Bodey et al. compare carbenicillin 30 g/day plus intermittent intravenous cefamandole (3 g given every 6 hours) with carbenicillin plus continuous infusions of cefamandole (12 g/day) in febrile patients with cancer. In patients remaining profoundly granulocytopenic (i.e. absolute neutrophil count < 1000 cells/mm^3), the continuous infusion was significantly more effective than intermittent infusion; 65 vs 21 % of patients achieved clinical cure ($p = 0.03$) (48).

While earlier comparison between continuous infusion and intermittent infusion of β-lactams reported similar clinical and microbiological outcomes. Krueger et al. through the use of Monte Carlo simulation with MYSTIC surveillance data reported that continuous infusion of meropenam provided a better likelihood of attaining a bacterial exposure against *P. aeruginosa* compare with the intermittent administration (83% vs 64%). Tan et al. also found that continuous infusion of cefepime 4 g over 24 hours provided a much higher cumulative fraction of response of 65-82% ($p< 0.001$) compare with standard dose administration of 2 g every 12 hours (4-38%) against *P. aeruginosa* isolates from their institution. (49)

Data from several small open label trials in critically ill patients suggest that in the presence of a concomitant aminoglycoside, continuous infusion ceftazidime 3–4g/day results in a greater T>MIC, similar microbiological eradication rates, clinical cure rates and adverse drug effects, and lower treatment costs, than intermittent ceftazidime 2g intravenously every 8 hours (51).

Unfortunately, there are some realized limitations of continuous infusion β-lactams, including drug stability and compatibility problems, the risk of delayed tissue equilibration at the site of infection (which can be avoided with the use of an initial loading dose), susceptibility to an inoculum effect, the lack of a well-defined goal steady-state concentration/MIC ratio, and the potential need to monitor serum concentrations (if lower total daily doses are used) to ensure adequate concentrations are maintained in the presence of pharmacokinetic variability (51).

b. Concentration dependent killing

The goal of concentration dependent killing is to maximize concentration and attain the highest possible antimicrobial concentration at the site of infection. With concentration-dependent killing, prolonged post antimicrobial activity, persisting even when concentration are below MICs, is also often present. Concentration dependent killing is characteristic of aminoglycosides, quinolones, azalides (azithromycin), and ketolides (46). Aminoglycoside, because of the potential nephrotoxicity and ototoxicity associated with the use of these agents, pharmacokinetic monitoring is routinely used to maintain aminoglycoside concentrations within the therapeutic rang. However, after the decades of monitoring, and multitude of studies designed to assess concentration-effect relationships for both efficacy and toxicity, the ability to achieve concentration that will ensure efficacy without causing toxicity continues to elude clinicians (48).

The major pharmacodynamic parameters that correlate with clinical and bacteriologic efficacy of these drugs are the AUC/MIC ratio, or the C_{max}/MIC ratio, base on free or unbound serum concentration values. So, again, the MIC remains a primary correlate of pharmacodynamic potency when correlates with the appropriate parameter. The parameters that correlate with the clinical and bacteriologic efficacy are 24-h AUC/MIC ratios of ≥25-30 in immunocompetent patients, ≥100-125 in immunocompromised patients, and peak/MIC ratios of ≥10-12. Pharmacodynamic breakpoints can therefore be determined by the formula AUC÷25 for immunocompetent patients, or AUC÷125 for immunocompromised patients.

Table 2.8 Pharmacodynamic parameters that correlate with efficacy (49)

	Cmax/MIC	AUC/MIC	T>MIC
Examples	Aminoglycosides Fluoroquinolones	Azithromycin Fluoroquinolones Telithromycin	β-lactams (penicillins, cephalosporins, carbapenems) Macrolide(clarithromycin, erythromycin) Oxazolidinones (linezolid)
Organism kill	Concentration dependent	Concentration dependent	Time dependent
Therapeutic goal	Maximize exposure	Maximize exposure	Optimize duration of exposure

Table 2.9 Pharmacodynamic breakpoints established in the literature (49)

Antimicrobial class	PD parameter that best describe activity	Breakpoints for clinical or microbiological efficacy
β-lactams	T>MIC	Penicillins: 50% Cephalosporins: 50-70% Carbapenems: 40%
Aminoglycosides	Cmax/MIC	≥10-12*
Fluoroquinolones	AUC/MIC	Gram-negative bacteria: total AUC/MIC ≥125 Gram-positive bacteria AUC/MIC ≥ 30

* or ≥8 (51)

Aminoglycosides are subject to adaptive resistance – a short-term decrease or down- regulation in drug uptake and subsequent reduction in bactericidal activity after prolonged exposure low drug concentrations. *In vitro* data has shown that C_{max}/MIC ratios of ≥8 are required to prevent regrowth and the emergence of resistant isolates from *in vitro*, animal and human studies, the theoretical goal of aminoglycoside administration regimens may be give higher dose less frequently to take advantage of their concentration-dependent killing and PAE, and to prevent adaptive resistance. Thus, this concept has led to the development of the high-dose extended-interval administration regimens that have been used clinically. Both C_{max}/MIC and AUC24/MIC may predict the efficacy of aminoglycosides; however, animal models have shown AUC24/MIC to be more predictive of efficacy (51).

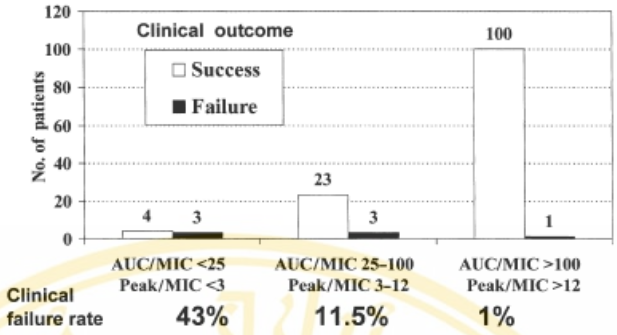
Although some meta-analyses have shown either statistically significant increases in microbiological efficacy, clinical efficacy and overall response, or statistically significant reductions in nephrotoxicity, there appears to be no clinically important differences between aminoglycosides administered using conventional or extended-interval regimens (51).

3. The pharmacokinetic/pharmacodynamic approach to a rational dosage regimen for antimicrobials

The risk of under- or overdosing is evident, as variability in the pharmacokinetic and pharmacodynamic parameters in themselves leads to considerable diversity in pharmacokinetic/pharmacodynamic (PK/PD) breakpoints. It is difficult to reduce the complex situation existing in the body during an infection simply to only one value. Thus, it is far too optimistic to generalize the PK/PD indices down to one universal PK/PD index for all antimicrobials, species and infection sites, which guarantees a clinical cure. Dealing with PK and PD aspects of antimicrobial therapy helps to determine the correct dosage, but for the clinical application of PK/PD breakpoints, further investigations are necessary. The breakpoints have to be verified in prospective clinical trials that include a large number of patients (47).

nnnnnnnn

In a unique study of 134 hospitalized patients with bacteriologically proven respiratory tract, skin or complicated urinary tract infections treated with 500 mg of intravenous levofloxacin daily for 5-14 days, Preston SL (52) correlated clinical outcome with steady-state pharmacokinetics on day 3 of treatment with levofloxacin MICs of the pathogen from the corresponding patient. The study showed that patients with AUC/MIC ratios >100 or peak/MIC ratios >12 had a 1% rate of clinical failure, those with AUC/MIC ratios of 25-100 or peak/MIC ratios of 3-12 had a 12% rate of clinical failure, and those with AUC/MIC ratios of <25 or peak/MIC ratios of <3 had a 43% rate of clinical failure (Figure 2).



Fiugure 2.4 Correlation of PK/PD of levofloxacin and clinical outcome in 134 hospitalized patients with respiratory tract, skin or complicated urinary tract infections treated with levofloxacin 500 mg once daily for 5-14 days. (46, 52)

A more sophisticated method to estimate the volume of distribution, elimination rate constant and clearance of antimicrobial for a particular patient would be to use population pharmacokinetic models. In these models, clearance or elimination rate is related to parameters such as renal function and the volume of distribution is described as a function of bodyweight or lean body mass. Individual pharmacokinetic parameters and serum concentrations can be predicted accurately from population pharmacokinetics (53).

From Mouton et al. (53, 54) studied it was clear that, although there was variation between groups of patients, the concentrations reached during continuous infusion for most drugs do not differ between patients by more than a factor of two provided there is no renal insufficiency. If time above the MIC is taken as the pharmacodynamic parameter for β-lactams, it seems to be more important to know the MIC (which is usually determined to within ±1 dilution) for the infecting bacterium than the precise pharmacokinetic characteristics of the individual patient. The reported MIC for the bacterium and calculated pharmacokinetic parameters in the patient can then both be used to determine the dose necessary to obtain the desired concentration.

CHAPTER III
METERIALS AND METHODS

I. Materials

1. Bacterial strains
   - *Pseudomonas aeruginosa* ATCC 27853
   - Clinical isolates of *Pseudomonas aeruginosa* (MDR-PA) from Chonburi Hospital
2. Antimicrobial agents
   - Amikacin sulfate (Sigma, USA)
   - Ceftazidime pentahydrate (Sigma, USA)
   - Fosfomycin disodium (Sigma, USA)
3. Chemicals and Reagents
   - Sodium chloride (Carlo Erba)
   - α-D-Glucose-6-phosphate monosodium salt (Calbiochem, Germany)
   - E test strips (AB Biodisk ; Solna, Sweden)
     - Amikacin (0.016-256 µg/mL)
     - Ceftazidime (0.016-256 µg/mL)
     - Fosfomycin (0.064-1024 µg/mL)
4. Culture Media
   - Cetrimide Agar (Difco, USA)
   - Cysteine Tryptone Fluid Medium (Difco, USA)
   - Meuller Hinton Agar (Difco, USA)
   - Trypticase Soy Agar (Soybean-Casein Digest Agar, USP; Difco, USA)
   - Trypticase Soy Broth (Soybean-Casein Digest Medium; Difco, USA)

5. Instruments
    - Autoclave (MLS-3020, Sanyo)
    - Hot air oven (UL50, Memmert)
    - Laminar cabinet class II (Holten Lamin Air, Denmark)
    - Incubator (BE 600, Memmert)
    - Micropipette (Gilson, France)
    - Vortex mixer (VX 100, Labnet)

II. Methods

1. Definition of terms

The terms used throughout the study were defined as follows:-

Minimum inhibitory concentration (MIC) is defined as the lowest antimicrobial concentration inhibiting visible growth after 18-hours incubation at 37° C (55).

Resistance is defined by NCCLS includes bacteria that are not inhibited by the usually achievable systemic concentrations of the agent with normal dosage schedule and/or fall in the MIC ranges where specific resistance mechanisms are likely and clinical efficacy has not been reliable in treatment studies (55).

Multidrug-resistant *Pseudomonas aeruginosa*; MDR-PA is defined as resistant to at least three of four drugs including ceftazidime, imipenem, ciprofloxacin, and amikacin (16).

Synergism is defined as positive interaction; the combined effect of drugs being examined is significantly greater than the expected result, based on their independent effects when the drugs are used separately (26).

Additivity is defined as the combined effect of drugs being equal to the sum of separate effects of the drugs being tested (26).

Antagonism is defined as negative interaction; the combined effect of drugs being examined is significantly less than their independent effects when they are tested separately (26).

Fractional inhibitory concentration index; FIC is defined as the index used to interpret the interaction of combined drugs and calculated as follows:

FIC index = FIC of drug A +FIC of drug B

FIC of drug A = MIC of drug A in combination / MIC of drug A alone

FIC of drug B = MIC of drug B in combination / MIC of drug B alone

Synergy is defined as an FIC index ≤ 0.5, additivity is defined as an FIC index >0.5 but of ≤ 4, and antagonism is defined as an FIC index > 4 (28).

Monotherapy is defined as the patient's infection had been treated simultaneously with only one antimicrobial drug (56).

Combination therapy is defined as the patient's infection had been treated simultaneously with two or more antimicrobial drugs (56).

2. Study design

This study was designed as experimental study.

3. Study population

Subjects were recruited according to the following criteria:

Inclusion criteria

1. MDR-PA clinical isolates from patients who were admitted at Chonburi Hospital
2. MDR-PA clinical isolates which were resistant to at least three of four drugs including ceftazidime, imipenem, ciprofloxacin, and amikacin

Exclusion criteria

1. The clinical isolates which did not show the fluorescent pigment pyoverdin or the blue pigment pyocyanin on cetrimide agar.
2. The clinical isolates which were grown with other isolate(s) (contaminated).

Sample size

We enrolled 34 MDR-PA clinical isolates for sample size.

4. Period of study

Study period was during July 2005 to March 2006.

5. Steps of investigation

Part 1 *In vitro* effects of the triple combination of amikacin, ceftazidime, and fosfomycin on 41clinical isolates of MDR-PA from Chonburi Hospital.

1. Confirmation of *P. aeruginosa*

1.1 The clinical isolates from Chonburi Hospital were streaked to trypticase soy agar (TSA) plate in four directions (Figure 3.1).

1.2 Plates were incubated for 18 hours at 37 ^{o}C.

1.3 Pure cultures (isolated colonies) were obtained.

1.4 Streaked the isolated colonies to cetrimide agar (CM) in four directions.

1.5 Plates were incubated for 18 hours at 37 ^{o}C.

1.6 The colony of *P. aeruginosa* showed the fluorescent pigment pyoverdin or the blue pigment pyocyanin.

1.7 During the study, the isolates were sub-cultured weekly.

1.8 Three to five well-isolated colonies were transferred into a tube containing 10 mL of cysteine tryptone fluid medium as the MDR-PA stock cultures which can be kept up to 3 months)

Figure 3.1 Preparation of the isolated colonies of clinical MDR-PA isolates.

2. Determination of the MIC of antimicrobial (MIC_{mono}) using E test method (duplicate) (27, 28, 57).

2.1 Prepared the isolated colonies from clinical isolates of MDR-PA. (the same as 1.1-1.3)

2.2 Prepared the inoculum of MDR-PA clinical isolates.

2.2.1 Three to five well-isolated colonies were transferred into a tube containing 5 ml of trypticase soy broth (TSB).

2.2.2 The broth culture was incubated at 37°C until it nearly achieved the turbidity of the 0.5 McFarland turbidity (usually 2 to 6 hours)

2.2.3 The turbidity of the actively growing broth culture was adjusted with sterile saline to obtain turbidity optically comparable to that of the 0.5 McFarland turbidity. This resulted in a suspension containing approximately 3 to 5×10^8 CFU/mL (Figure 3.2). To aid comparison, compare the test and 0.5 McFarland turbidity against a white background with a contrasting black lines card.

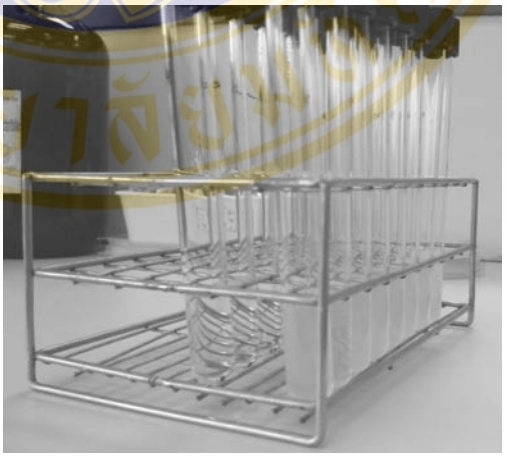


Figure 3.2 Preparation of the inoculum of clinical isolates of MDR-PA.

2.3 Inoculated the test plate.

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

2.3.2 The dried surface of a Meuller-Hinton Agar (MHA) plate was inoculated by spreading the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° angle each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.

2.3.3 The lid may be left ajar 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 strips.

NOTE: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

2.4 Prepared the E test strips of amikacin (0.016-256 µg/mL), ceftazidime (0.016-256 µg/mL) and fosfomycin (0.064-1024 µg/mL).

2.4.1 Removed the package, storage container from the -20 ^{o}C freezer and allowed it to reach room temperature for 30 minutes.

2.4.2 Cut along the broken line across the top of blister compartment.

2.4.3 Tipped the strips slightly out of the compartment and removed with the forceps.

2.5 Applied the E test strips to inoculated agar plates.

2.5.1 The predetermined battery of antimicrobial strips was dispensed onto the surface of the inoculated agar plate. Each strip must be pressed down to ensure complete contact with the agar surface. Ordinarily, no more than 2 strips should be placed on one 90 mm plate (Figure 3.3). Because some of the drug diffused almost instantaneously, a strip should not be relocated once it had come into contact with the agar surface.

2.5.2 The plates were placed in an incubator set to 37°C for 18 hours, within 15 minutes after the strips are applied.

2.5.3 Interpreted the MIC at which the ellipse intersected the scale imprinted on the strip.

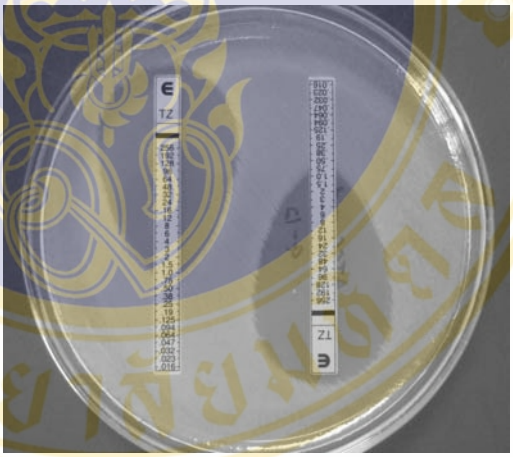


Figure 3.3 Determination of the MIC of antimicrobial (MIC_{mono})

3. Determination of the MIC between amikacin with ceftazidime (MIC_{combo}) using E test strips (27, 28, 57).

3.1 Prepared the isolated colonies from the clinical isolates of MDR-PA. (the same as 1.2-1.3)

3.2 Prepared the inoculum of clinical isolates of MDR-PA. (the same as 2.2)

3.3 Inoculated of the test plate. (the same as 2.3)

3.4 Prepared the E test strips of amikacin (0.016-256 µg/mL) and ceftazidime (0.016-256 µg/mL) (AB Biodisk; Solna, Sweden). (the same as 2.4)

3.5 Applied the E test strips to inoculated agar plates.

3.5.1 Placed E test strip of amikacin and ceftazidime on the MHA in a cross formation (Figure 3.4). [90 ^{o}angle at the intersection between their respective MICs]

3.5.2 The plates were placed in an incubator set to 37°C for 18 hours, within 15 minutes after the strips were applied.

3.5.3 Interpreted the MIC_{combo} at which the ellipse intersected the scale imprinted on each E test strip.

Figure 3.4 Determination of the MIC between amikacin with ceftazidime (MIC_{combo})

4. Determination of the effects of the addition of fosfomycin to combination of amikacin and ceftazidime using agar well method (triplicate) (58-61).

4.1 Prepared the isolated colonies from MDR-PA clinical isolates. (the same as 1.2-1.4)

4.2 Prepared the inoculum of MDR-PA clinical isolates. (as same as 2.2)

4.3 Inoculated the test plate.

4.3.1 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension to that of the 0.5 McFarland turbidity. Poured the inoculum and gently mixed it with melted MHA (45 ^{o}C),

4.3.2 Then poured the mixtures into the plate (140mm).

4.3.3 The dried surface of a MHA plate was made the 6 mm well by sterile borer.

4.3.4 Filled up the antimicrobial solution in each well (Figure 3.5).

4.3.4.1 Three groups of antimicrobial regimen were used.

4.3.4.1.1 One antimicrobial (amikacin, ceftazidime, and fosfomycin).

4.3.4.1.2 Two antimicrobials (amikacin plus ceftazidime).

4.3.4.1.3 Three antimicrobials (amikacin plus ceftazidime plus fosfomycin).

4.3.4.2 Concentrations of antimicrobial used in the combinations.

4.3.4.2.1 Amikacin and ceftazidime; used at the concentration equal to MIC_{combo} of these two antimicrobials.

4.3.4.2.2 Fosfomycin; used at the concentration equal to MIC_{mono} and at the concentration below and above MIC_{mono} 1 dilution.

4.4 The plates were placed in an incubator at 37°C for 18 hours, within 15 minutes after the strips were applied.

4.5 Measured and recorded the diameters of the inhibition zones.

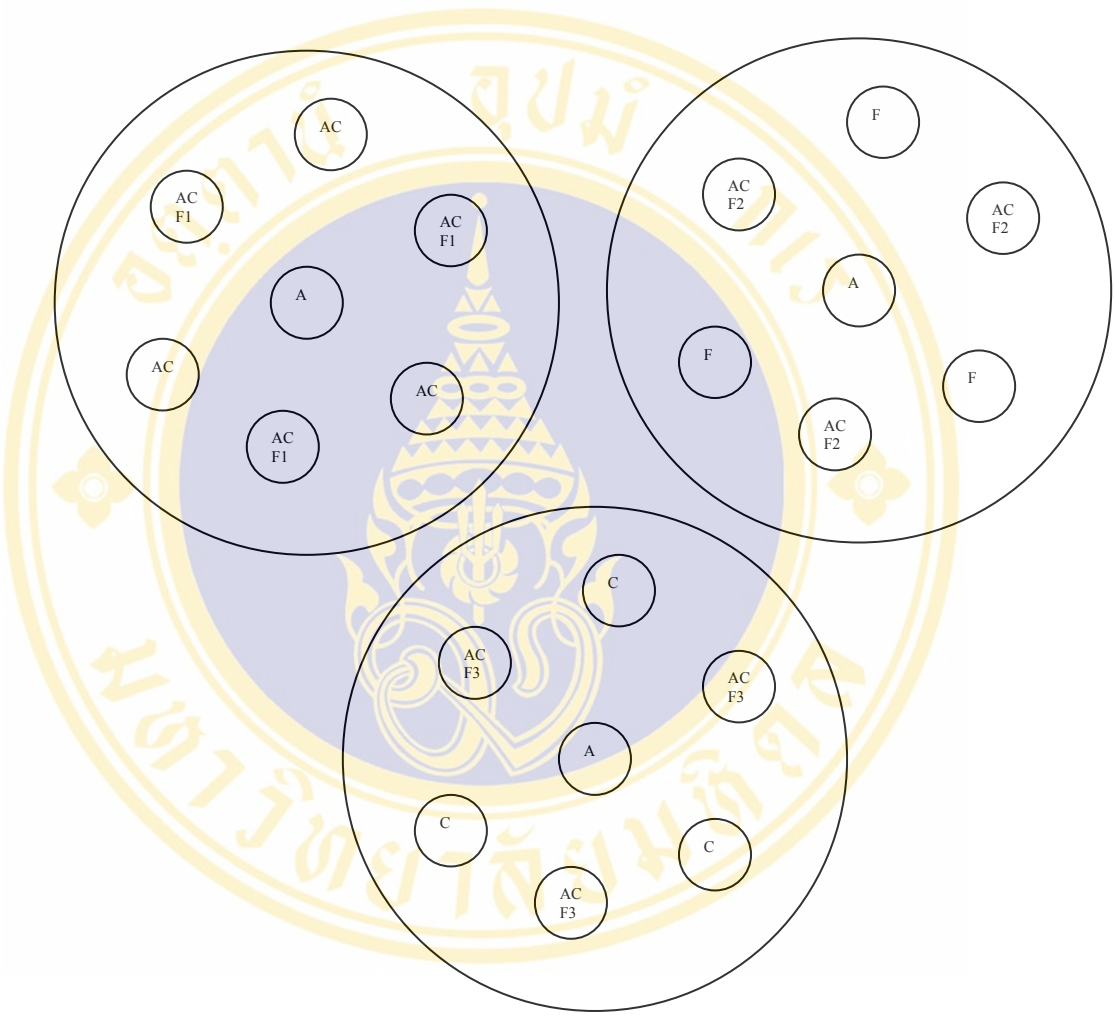


Figure 3.5 Determination of inhibition zones from antimicrobial regimen

NOTE

A=Amikacin, C= Ceftazidime, F=Fosfomycin, AC= amikacin plus ceftazidime,
ACF1= amikacin plus ceftazidime plus fosfomycin (below MIC 1 dilution),
ACF2= amikacin plus ceftazidime plus fosfomycin (at MIC concentration),
ACF3= amikacin plus ceftazidime plus fosfomycin (above MIC 1 dilution)

Part II Optimization of antimicrobial therapy using pharmacokinetic and pharmacodynamic parameters

1. The pharmacokinetic/pharmacodynamic approach to a rational dosage regimen for antimicrobials
   1.1 Reviewed patterns of antimicrobials activity of amikacin, ceftazidime, and fosfomycin
   1.2 Reviewed the PK/PD of amikacin, ceftazidime, and fosfomycin
2. Selection the infusion model which were fitted with antimicrobials property of amikacin, ceftazidime, and fosfomycin
3. Determination of dosage regimen to optimize the surrogate marker
   3.1 Selection an infusion method
   3.2 Determination of loading dose
   3.3 Determination of maintenance dose

The ability of antimicrobial dosing regimen to meet the pharmacokinetic/ pharmacodynamic parameter required for efficacy against emerging resistant bacteria needs to be considered in designing effective antimicrobial regimens and in selecting suitable empirical therapy.

For some drugs, maintenance of a consistent plasma concentration is advantageous because of desire to achieve a consistent effect. To maintain consistent plasma drug concentrations, continuous intravenous infusion are often used. Continuous infusion can be though of as the administration of small amounts of drug at infinitely time small dosing intervals. The plasma drug concentrations resulting from the continuous infusion of drug are determined by rate of drug input (infusion rate, k_0), volume of distribution (Vd), and drug clearance. The relationship among these parameters is demonstrated in equation 5 (62).

Table 3.1 Equation for loading dose and maintenance dose calculation (62)

Intravenous infusion	Equation	
Bolus model; $t' \leq$ 1/6 time of it half-life		
Loading dose (LD)	$LD = \frac{C_0 \times Vd}{S \times F}$	(Equation 1)
Maintenance dose (MD)	$MD = \frac{C_{max,ss} \times Vd \times (1 - e^{-k\tau})}{S \times F}$	Equation 2)
Infusion model; $t' >$ 1/6 time of it half-life		
Loading dose (LD)	$\frac{LD}{t'} = \frac{C_0 \times k \times Vd}{S \times F \times (1 - e^{-kt'})}$	(Equation 3)
Maintenance dose (MD)	$\frac{MD}{t'} = \frac{C_{max,ss} \times k \times Vd \times (1 - e^{-k\tau})}{S \times F \times (1 - e^{-kt'})}$	(Equation 4)
Continuous infusion (CI)		
Rate of drug infusion (k_0)	$k_0 = S \times F \times V_d \times k \times C_{ss}$	(Equation 5)

When:

S	=	salt factor
F	=	bioavailability factor = 1(for intravenous administration)
LD	=	loading dose (mg)
MD	=	maintenance dose (mg)
C_0	=	desired target concentration (mg/L)
$C_{max,ss}$	=	C_{max} at steady state (mg/L)
C_{ss}	=	concentration at steady state (mg/L)
Vd	=	volume of distribution (L)
k	=	elimination rate constant (hr^{-1})
k_0	=	rate of drug infusion (mg/hr)
t'	=	infusion time (hr)
τ	=	dosing interval (hr)

6. Data analysis

Descriptive statistics were used to summarize the antimicrobial susceptibility, the MIC of amikacin, ceftazidime, and fosfomycin, (MIC_{mono}), and the MIC of amikacin in combination with ceftazidime (MIC_{combo}).

The Kruskal-Wallist H and Mann-Withney U in the program SPSS for Windows released 10.0 were used to test the significant differences of mean inhibition zones, among 7 antimicrobial regimens, within clinical isolates and between regimens, respectively. Inaddition, The Chi-Square test was used to test significant differences of the number of inhibited isolates. The significant difference was then taken at p-value less than 0.05 for all statistic values.

CHAPTER IV
RESULTS

The study was performed in two parts. The study was performed at the Applied Microbiology and Virology Laboratory, Department of Microbiology, Faculty of Pharmacy, Mahidol University during July 2005 to March 2006. The results were presented as follows:

Part I *In vitro* effects of the triple combination on clinical isolates of MDR-PA from Chonburi Hospital

1. Antimicrobial susceptibility of 41 clinical isolates of MDR-PA from Chonburi Hospital

The antimicrobial susceptibilities of 41 clinical isolates of MDR-PA from Chonburi Hospital were conducted by Microbiology Unit, Department of Pathology, Chonburi Hospital by disk diffusion method. Table 4.1 summarized the contribution of amikacin, ceftazidime, ciprofloxacin, imipenem, meropenem, fosfomycin and piperacillin/tazobactam resistance to MDR phenotypes for clinical isolates of *P. aeruginosa.* By the definition of MDR-PA in this study that the culture must resist to at least three of four drugs including amikacin, ceftazidime, imipenem and ciprofloxacin. So, all isolates were MDR-PA, except the isolates number 3, 19, 24, 25, and 27, were sensitive to both ciprofloxacin and imipenem. So these 5 isolates were excluded from the study. The sources of these 36 MDR-PA isolates were shown in Table 4.2, 16 isolates (44.44%) from sputum or tracheal secretion, 10 isolates (27.77%) from wound and pus, 7 isolates (19.44%) from urine, and 3 isolates (8.33%) from blood.

Table 4.1 Antimicrobial susceptibility of 41 clinical isolates of MDR-PA from Microbiology Unit, Department of Pathology, Chonburi Hospital

Isolate no.	AMK[a]	CTZ[b]	CPF[c]	IMP[d]	MRP[e]	FMC[f]	PIP/TAZO[g]
1	R[i]	R	R	R	R	R	S[h]
2	R	R	R	R	R	S	S
3	R	R	S	S	S	NA[j]	S
4	R	R	R	R	R	R	S
5	R	R	R	R	R	R	S
6	R	R	R	R	R	R	S
7	R	R	R	R	R	R	S
8	R	R	R	NA	R	R	R
9	R	R	R	S	S	S	R
10	R	R	R	S	S	S	R
11	R	R	R	R	R	R	R
12	R	R	R	S	S	S	S
13	R	R	R	S	R	S	R
14	R	R	R	R	R	R	S
15	R	R	R	R	R	R	R
16	R	R	R	S	S	S	S
17	R	R	R	R	R	R	R
18	R	R	R	R	R	R	S
19	R	R	S	S	S	NA	S
20	R	R	R	S	S	S	S
21	R	R	R	S	S	NA	S
22	R	R	R	R	R	R	R
23	R	R	R	R	R	S	S
24	R	R	S	S	S	NA	S
25	R	R	S	S	S	NA	S
26	R	R	R	R	R	NA	R
27	R	R	S	S	S	S	S
28	R	R	R	R	R	NA	R
29	R	R	R	R	R	R	R
30	R	R	R	R	R	NA	R
31	R	R	R	S	S	NA	S
32	R	R	S	R	R	NA	S
33	R	R	R	R	R	NA	R
34	R	R	R	S	S	NA	S
35	R	R	R	S	S	NA	S
36	R	R	R	S	S	NA	R
37	R	R	R	S	R	NA	S
38	R	R	R	S	S	NA	S
39	R	R	R	S	S	NA	S
40	R	R	R	S	S	NA	S
41	R	R	R	S	S	NA	S

[a] AMK = Amikacin, [b]CTZ = Ceftazidime, [c]CPF = Ciprofloxacin, [d]IMP = Imipenem, [e]MRP = Meropenem, [f]FMC = Fosfomycin, [g]PIP/TAZO = Piperacillin/Tazobactam, [h] S = sensitive, [i]R = resistant,[j] NA = not available

Table 4.2 Frequency of MDR-PA strains according to source of clinical specimen

Specimen	No. of isolates	Percentage
Blood	3	8.33
Urine	7	19.45
Wound and pus	10	27.77
Sputum/ tracheal secretion	16	44.45

The antimicrobial susceptibility rate among of 36 MDR-PA was shown in Table 4.3. All isolates (100%) were resistant to amikacin and ceftazidime and only one isolate (2.78%) of 36 isolates was sensitive to ciprofloxacin. Eight isolates (38.10%) of 21 isolates were sensitive to fosfomycin, 14 (38.89%) of 36 isolates were sensitive to meropenem, 16 (45.71%) of 35 isolates were sensitive to imipenem, and 22 (61.11%) of 36 isolates were sensitive to piperacillin/tazobactam.

Table 4.3 Antimicrobial susceptibility rate among of 36 MDR-PA

Antimicrobial agents	Susceptibility rate (no.)	Isolate number
Amikacin	0% (0)	-
Ceftazidime	0% (0)	-
Ciprofloxacin	2.78% (1)	32
Fosfomycin	38.10% (8)	2, 9, 10, 12, 13, 16, 20, 23
Meropenem	38.89% (14)	9, 10, 12, 16, 20, 21, 31, 34-36, 38-41
Imipenem	45.71% (16)	9, 10, 12, 13, 16, 20, 21, 31,34-41
Piperacillin/Tazobactam	61.11% (22)	1, 2, 4, 5, 6, 7, 12, 14, 16, 18, 20, 21, 23, 31, 32, 34, 35, 37-41

Table 4.4 Contribution of antimicrobial susceptibility among of 36 MDR-PA

No. of antimicrobials which isolates were resistant	Total % of isolates (no.)	Isolate number.
7	13.89 (5)	11, 15, 17, 22, 29
6	33.33 (12)	1, 4-8, 14, 18, 26, 28, 30, 33
5	8.33 (3)	2, 13, 23
4	13.89 (5)	9, 10, 32, 36, 37
3	30.56 (11)	12, 16, 20, 21, 31, 34, 35, 38-41

The contribution of resistance to individual antimicrobials to MDR phenotypes among *P. aeruginosa* clinical isolates was demonstrated in Table 4.5. Interestingly, there were 54.29% and 61.11% of the tested isolates which resistance emerged to imipenem and meropenem, respectively. Certainly, these resistant isolates were also resistant to amikacin and ceftazidime.

Table 4.5 Contribution of resistance of 36 MDR-PA among seven antimicrobials by disk diffusion method

No. of antimicrobials to which isolates were resistant	% of MDR-PA isolates which resistant to (no.)						
	AMK[a]	CTZ[b]	CPF[c]	IMP[d]	MRP[e]	FMC[f]	PIP/ TAZO[g]
7	100 (5)	100 (5)	100 (5)	100 (5)	100 (5)	100 (5)	100 (5)
6	100 (12)	100 (12)	100 (12)	100 (11)	100 (12)	100 (8)	41.67 (5)
5	100 (3)	100 (3)	100 (3)	66.67 (2)	100 (3)	0 (0)	33.33 (1)
4	100 (5)	100 (5)	100 (5)	20 (1)	40 (2)	0 (0)	60 (3)
3	100 (11)	100 (11)	100 (11)	0 (0)	0 (0)	0 (0)	0 (0)

[a] AMK = Amikacin, [b]CTZ = Ceftazidime, [c]CPF = Ciprofloxacin, [d]IMP = Imipenem,
[e]MRP = Meropenem, [f]FMC = Fosfomycin, [g]PIP/TAZO = Piperacillin/Tazobactam

Interestingly, all 14 isolates which were sensitive to meropenem were also sensitive to imipenem whereas 2 isolates (isolate number 13 and 37) which susceptible to imipenem but showed resistant to meropenem. Among isolates which resistant to both imipenem and meropenem, there were 2 isolates (isolate number 2 and 23) were susceptible to fosfomycin. Table 4.4 gave the contribution of antimicrobial susceptibility among of 36 MDR-PA. Besides 36 MDR-PA clinical isolates, there were 30.56% (11 of 36) of isolates resistant to 3 antimicrobials, 13.89% (5 of 36) of isolates resistant to 4 antimicrobials, 8.33% (3 of 36) of isolates resistant to 5 antimicrobials, 33.33% (12 of 36) of isolates resistant to 6 antimicrobials, and 13.89% (5of 36) of isolates resistant to all antimicrobials. The number of antimicrobials which their almost showed resistant to were 6 (33.33%) and 3 (30.56) agents. In addition, there were 5 (13.89%) isolates showed resistant to all of 7 antimicrobials.

2. The MIC of each antimicrobial agent (MIC_{mono}) determined by E test method.

A total 36 clinical isolates of MDR-PA from Chonburi Hospital and *P. aeruginosa* ATCC 27853, the control strain were studied for MIC. E test method was used to determine the MIC of each strain to amikacin, ceftazidime, and fosfomycin. The MIC was defined as the concentration at which the ellipse intersected the concentration scale imprinted on the E test strip and interpreted according to the manufacturer's guidelines. Reading the MIC value after 18 hours in ambient air of incubation at 37^{o} C. The MIC_{mono} of each antimicrobial agent was conducted in duplicate fashion. For the isolate which no inhibition ellipse was seen, the MIC was reported as greater than the highest value on the E test scale. When the inhibition ellipse was below the strip, the MIC was reported as less than the lowest value on the E test scale. According to the manufacturer guideline, the MICs determining the susceptibility threshold for the different antimicrobials were as follows: amikacin, ≤16 µg/mL; ceftazidime, ≤8 µg/mL; and fosfomycin, ≤64 µg/mL.

Table 4.6 The MIC of each antimicrobial agent (MIC_{mono}) against MDR-PA determined by E test method

Isolate no.	MIC_{mono} (µg/mL)											
	Amikacin				Ceftazidime				Fosfomycin			
	1	2	mean	I[a]	1	2	Mean	I	1	2	Mean	I
1	>256	>256	>256	R[b]	>256	>256	>256	R	>1024	>1024	>1024	R
2	>256	>256	>256	R	>256	>256	>256	R	384	384	384	R
4	>256	>256	>256	R	>256	>256	>256	R	>1024	>1024	>1024	R
5	128	96	112	R	>256	>256	>256	R	48	48	48	S[e]
6	>256	>256	>256	R	>256	>256	>256	R	>1024	>1024	>1024	R
7	>256	>256	>256	R	>256	>256	>256	R	>1024	>1024	>1024	R
8	>256	>256	>256	R	>256	>256	>256	R	>1024	>1024	>1024	R
9	6	6	6	S[e]	64	48	56	R	6	6	6	S[c]
10	>256	>256	>256	R	>256	>256	>256	R	192	192	192	R
11	>256	>256	>256	R	48	48	48	R	>1024	>1024	>1024	R
12	128	128	128	R	>256	>256	>256	R	24	24	24	S
13	>256	>256	>256	R	>256	>256	>256	R	96	96	96	R
14	12	12	12	S[e]	>256	>256	>256	R	>1024	>1024	>1024	R
15	1.5	2	1.75	S[e]	48	48	48	R	48	48	48	S[e]
16	12	12	12	S[e]	64	48	56	R	>1024	>1024	>1024	R
17	>256	>256	>256	R	>256	>256	>256	R	>1024	>1024	>1024	R
18	>256	>256	>256	R	>256	>256	>256	R	>1024	>1024	>1024	R
20	128	192	160	R	>256	>256	>256	R	32	32	32	S
21	192	128	160	R	>256	>256	>256	R	32	24	28	S
22	>256	>256	>256	R	24	24	24	R	>1024	>1024	>1024	R
23	>256	>256	>256	R	>256	>256	>256	R	384	384	384	R[f]
26	>256	>256	>256	R	>256	>256	>256	R	128	128	128	R
28	4	3	3.5	S[e]	32	24	28	R	192	128	160	R
29	>256	>256	>256	R	96	96	96	R	48	48	48	S[e]
30	>256	>256	>256	R	>256	>256	>256	R	64	96	80	R
31	96	96	96	R	>256	>256	>256	R	>1024	>1024	>1024	R
32	96	128	112	R	>256	>256	>256	R	>1024	>1024	>1024	R
33	48	48	48	R	>256	>256	>256	R	>1024	>1024	>1024	R
34	48	64	56	R	>256	>256	>256	R	>1024	>1024	>1024	R
35	96	64	80	R	>256	>256	>256	R	>1024	>1024	>1024	R
36	128	128	128	R	>256	>256	>256	R	>1024	>1024	>1024	R
37	128	128	128	R	>256	>256	>256	R	>1024	>1024	>1024	R
38	64	64	64	R	>256	>256	>256	R	>1024	>1024	>1024	R
39	>256	>256	>256	R	>256	>256	>256	R	>1024	>1024	>1024	R
40	>256	>256	>256	R	>256	>256	>256	R	>1024	>1024	>1024	R
41	96	96	96	R	>256	>256	>256	R	>1024	>1024	>1024	R
C[d]	1.5	2	1.75	S	1	1	1	S	3	3	3	S

[a] I = Interpretation of susceptibility test, [b] R = resistant or intermediate resistant, when MIC > 16 µg/mL for amikacin, > 8 µg/mL for ceftazidime, and > 64 µg/mL for fosfomycin, [c] S = sensitive, when MIC ≤ 16 µg/mL for amikacin, ≤ 8 µg/mL for ceftazidime, and ≤ 64 µg/mL for fosfomycin., [d] C = control; *P. aeruginosa* ATCC 27853, [e] the susceptibility changed from originally resistant to susceptible, [f] the susceptibility changed from originally sensitive to resistant

The MICs of amikacin, ceftazidime, and fosfomycin of the clinical isolates from E test method were reported in Table 4.6. The MIC of reference strain, *P. aeruginosa* ATCC 27853, was 1.75 µg/mL, 1.00 µg/mL, and 3 µg/mL, within normal rang according to the NCCLS, for amikacin, ceftazidime, and fosfomycin, respectively. From the definition of MDR-PA which described previously, isolate number 9 and 16 were excluded from the study because there were susceptible to amikacin and resistant to only ceftazidime and ciprofloxacin. So, there were 34 MDR-PA would be analyzed. Among these 34 isolates, there were 3 (8.82%) isolates and 6 (17.65%) isolates which sensitive to amikacin and fosfomycin, respectively and none of isolates were susceptible to ceftazidime.

The MIC_{50} of amikacin, ceftazidime, and fosfomycin, were > 256 µg/mL, > 256 µg/mL, and > 1024 µg/mL, respectively. Also, the MIC_{90} of these three agents were equal to their MIC_{50}, data were shown in Table 4.7. Among the antipseudomonal agents tested, fosfomycin showed the highest MIC_{90} (>1024 µg/mL).

Table 4.7 MIC_{50} and MIC_{90} of amikacin, ceftazidime, and fosfomycin

Antimicrobial agent	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Amikacin	> 256	> 256
Ceftazidime	> 256	> 256
Fosfomycin	> 1024	> 1024

Table 4.8 showed the maximum and minimum MICs of the tested agents, the maximum MIC of amikacin, ceftazidime, and fosfomycin were > 256 µg/mL, > 256 µg/mL, and > 1024 µg/mL, respectively. In addition, the minimum MIC of amikacin, ceftazidime, and fosfomycin were 1.75 µg/mL, 24 µg/mL, and 24 µg/mL, respectively.

Table 4.8 Maximum and minimum MIC of amikacin, ceftazidime, and fosfomycin

Antimicrobial agent	Minimum MIC (µg/mL)	Maximum MIC (µg/mL)
Amikacin	1.75	> 256
Ceftazidime	24.00	> 256
Fosfomycin	24.00	> 1024

3. The MIC of amikacin in the combination with ceftazidime (MIC_{combo}) determined by E test method

In the MIC_{combo} determination, the MIC_{mono} of amikacin and ceftazidime were used to determine the MIC of amikacin and ceftazidime (MIC_{combo}) by place each E test strip on the Meuller Hinton Agar (MHA) in a cross formation between their respective MICs. When the mean MIC_{mono} was not found within the value imprint on the E test strip, the upper near value was used to represent the MIC_{mono} of each antimicrobial agent. Results of MIC_{combo} of amikacin and ceftazidime by E test, *in vitro* synergy, were presented in Table 4.9. Of the 34 isolates, we found the decreased of the MIC of either amikacin or ceftazidime within 14 isolates (41.18%) (isolate number 5, 11, 12, 14, 15, 20, 21, 22, 28 29, 32, 34, 37and 39. The mean MIC decreased of amikacin and ceftazidime were 2.31 times (1.33 to 5.33 times) and 2.36 times (1.33 to 5.33 times), respectively. Isolate number 28 showed the most decreased of the MIC (5.33 times) for both antimicrobial agents. Isolate number 5, 12, 32, and 34 showed the least decreased of the MIC (1.33 times) for amikacin. While isolate number 5, 21, 32, 37, and 39 showed the least decreased of the MIC (1.33 times) for ceftazidime. Interestingly, the susceptibility to ceftazidime changed from resistant to sensitive in 2 isolates (5.88%) (isolate number 22 and 28). Despite, these were not found with amikacin and fosfomycin.

In this study, the FIC index of tested regimen ranging from 0.38 to 2.0. The enhanced activities of these combinations on the 34 isolates were shown in Table 4.9. The reference strain, *P. aeruginosa* ATCC 27853, showed additivity activities with fractional inhibitory concentration (FIC) index 0.76. Among 34 MDR-PA isolates, there were 1 synergistic activity (2.94%) and 33 additivity activities (97.06%). More importantly, none of them showed antagonistic activities in the combination of amikacin and ceftazidime. The frequencies of the antimicrobial interaction were shown in Table 4.10 and Figure 4.1.

Table 4.9 MIC_{combo} of amikacin and ceftazidime against MDR-PA determined by E test method

Isolate no.	MIC of amikacin(µg/mL)			MIC of ceftazidime(µg/mL)			FIC[a]	AI[b]
	mono	combo	decreased (time)	mono	combo	decreased (time)		
1	>256	>256	-	>256	>256	-	2.00	A[e]
2	>256	>256	-	>256	>256	-	2.00	A
4	>256	>256	-	>256	>256	-	2.00	A
5	**128**[c]	**96**	**1.33**	**>256**	**192**	**1.33**	**1.50**	**A**
6	>256	>256	-	>256	>256	-	2.00	A
7	>256	>256	-	>256	>256	-	2.00	A
8	>256	>256	-	>256	>256	-	2.00	A
10	>256	>256	-	>256	>256	-	2.00	A
11	**>256**	**96**	**2.67**	**48**	**24**	**2.00**	**0.88**	**A**
12	**128**	**96**	**1.33**	**>256**	**128**	**2.00**	**1.25**	**A**
13	>256	>256	-	>256	>256	-	2.00	A
14	**12**	**4**	**3.00**	**>256**	**96**	**2.67**	**0.71**	**A**
15	**2**[c]	**0.75**	**2.67**	**48**	**12**	**4.00**	**0.63**	**A**
17	>256	>256	-	>256	>256	-	2.00	A
18	>256	>256	-	>256	>256	-	2.00	A
20	**192**[c]	**96**	**2.00**	**>256**	**128**	**2.00**	**1.00**	**A**
21	**192**[c]	**96**	**2.00**	**>256**	**192**	**1.33**	**1.25**	**A**
22	**>256**	**96**	**2.67**	**24**	**8**[g]	**3.00**	**0.71**	**A**
23	>256	>256	-	>256	>256	-	2.00	A
26	>256	>256	-	>256	>256	-	2.00	A
28	**4**[c]	**0.75**	**5.33**	**32**[c]	**6**[g]	**5.33**	**0.38**	**S**[f]
29	**>256**	**96**	**2.67**	**96**	**32**	**3.00**	**0.71**	**A**
30	>256	>256	-	>256	>256	-	2.00	A
31	96	96	-	>256	>256	-	2.00	A
32	**128**[c]	**96**	**1.33**	**>256**	**192**	**1.33**	**1.50**	**A**
33	48	48	-	>256	>256	-	2.00	A
34	**64**[c]	**48**	**1.33**	**>256**	**>256**	**-**	**1.75**	**A**
35	96[c]	96	-	>256	>256	-	2.00	A
36	128	128	-	>256	>256	-	2.00	A
37	**128**	**64**	**2.00**	**>256**	**192**	**1.33**	**1.25**	**A**
38	64	64	-	>256	>256	-	2.00	A
39	**>256**	**128**	**-**	**>256**	**192**	**1.33**	**1.25**	**A**
40	>256	>256	-	>256	>256	-	2.00	A
41	96	96	-	>256	>256	-	2.00	A
[d]C	**2**[c]	**0.75**	**2.67**	**1**	**0.38**	**2.63**	**0.76**	**A**

[a] FIC = Fractional inhibitory concentration index,

[b]AI = antimicrobial interaction, [c] use the value at upper near mean MIC,

[d] C = *P. aeruginosa* ATCC 27853, [e] A = additivity, [f]S = synergy,

[g] the susceptibility changed from resistant to susceptible

Table .4.10 Antimicrobials interaction between amikacin and ceftazidime

Antimicrobial interaction	FIC[1] index	Total % of isolates (no.)	Isolate no.
Synergism	≤0.5	2.94 (1)	28
Additivity	>0.5 to ≤4.0	97.06 (33)	all isolates except no. 28
Antagonism	> 4.0	0 (0)	-

[1] FIC = Fraction inhibitory concentration

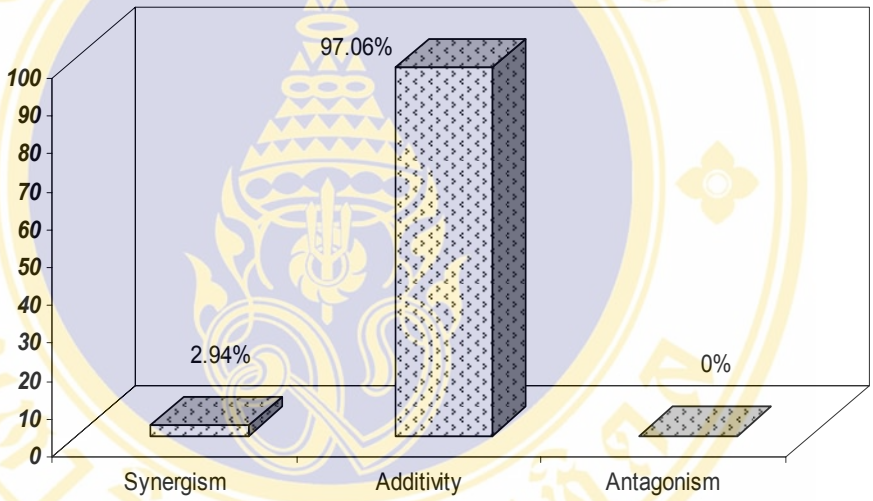


Figure 4.1 Antimicrobials interaction between amikacin and ceftazidime

4. The effects of the addition of fosfomycin to the combination of amikacin and ceftazidime using agar well method

The agar well diffusion method was used to determine the effect of the addition of fosfomycin into the combination of amikacin and ceftazidime by measuring the inhibition zone diameter. In the present study, amikacin and ceftazidime were used at the concentration of MIC_{combo} of each antimicrobial agent (Table 4.9). Since, we did not determine the MIC_{combo} of fosfomycin when used in the combination with amikacin or ceftazidime, three concentrations of fosfomycin were empirically used; at MIC_{mono} and varied its concentration in two fold dilution manner (one dilution above and one dilution below the MIC_{mono} of fosfomycin) in order to screen the trend of antimicrobial interaction when added fosfomycin into the combination of amikacin and ceftazidime.

Seven antimicrobial regimens were tested including, amikacin, ceftazidime, fosfomycin, amikacin plus ceftazidime, amikacin plus ceftazidime plus fosfomycin (1 dilution below MIC), amikacin plus ceftazidime plus fosfomycin (at MIC), and amikacin plus ceftazidime plus fosfomycin (1 dilution above MIC). The first-four regimens were used as the control to compare inhibition zone diameter with the last-three regimens. Each experiment was carried out in triplicate and the diameters of the inhibition zone were recorded. The mean inhibition zones were shown in Table 4.11.

There were no inhibition zones in 13 (38.24%) isolates (isolate number 5, 7, 10, 17, 26, 31, 32, 33, 35, 36, 39, 40, and 41). It should be stated that all seven antimicrobial regimen have no effect against these strains and there were 21 (61.76%) isolates which some regimens have the activity against them. In order to detect enhanced killing effects, Kruskal-Wallist H and Mann-Withney U were used to test the significant differences of mean inhibition zones within isolates and between regimens, respectively. The p-value from Kruskal-Wallist H test was shown in Table 4.12. Eleven isolates (32.35%); isolate number 1, 2, 8, 13, 22, 23, 29, 30, 34, 37, and 38 were demonstrated significant difference at p-value 0.008, 0.009, 0.004, 0.006, 0.029, 0.024, 0.028, 0.003, 0.003, 0.004, and 0.003, respectively. These represented, there were any pair(s) of antimicrobial regimens had significant difference of the mean inhibition zone among respective isolates.

Table 4.11 Antimicrobial activities of 7 antimicrobial regimens

Isolate no.	Mean inhibition zone (mm)							p-value
	A[a]	C[b]	F[c]	AC[d]	ACF1[e]	ACF2[f]	ACF3[g]	
1	17.0	0.0	0.0	17.2	16.7	16.5	17.0	0.008[h]
2	0.0	22.0	0.0	22.3	20.8	22.5	22.0	0.009[h]
4	18.3	22.7	23.3	21.2	25.0	20.0	25.7	0.147
5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.000
6	21.3	19.3	21.3	19.7	20.3	21.3	19.8	0.884
7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.000
8	0.0	0.0	0.0	11.7	0.0	0.0	12.3	0.004[h]
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.000
11	21.8	25.0	23.5	22.5	26.0	25.0	22.8	0.202
12	24.0	23.5	26.0	28.5	27.5	25.5	28.5	0.096
13	18.0	27.0	0.0	27.3	29.0	28.3	27.7	0.006[h]
14	19.5	19.5	21.3	22.8	20.5	20.0	21.5	0.286
15	25.0	22.0	23.0	24.5	25.0	25.5	21.5	0.243
17	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.000
18	17.0	19.0	18.0	19.7	19.3	18.0	18.0	0.625
20	23.5	22.0	22.3	20.5	24.3	25.8	22.0	0.144
21	21.2	19.7	19.3	20.3	17.7	21.0	20.8	0.188
22	22.2	22.2	28.7	26.8	21.3	29.2	30.2	0.029[h]
23	0.0	0.0	17.5	16.3	18.7	18.8	18.8	0.024[h]
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.000
28	10.0	0.0	13.0	16.0	0.0	18.5	16.7	0.104
29	17.7	23.3	28.0	18.7	25.0	28.3	25.3	0.028[h]
30	0.0	0.0	0.0	23.5	0.0	0.0	26.0	0.003[h]
31	0.0	0.0	0.0	0.00	0.0	0.0	0.00	1.000
32	0.0	0.0	0.0	0.00	0.0	0.0	0.00	1.000
33	0.0	0.0	0.0	0.00	0.0	0.0	0.00	1.000
34	0.0	0.0	0.0	10.33	0.0	0.0	10.33	0.003[h]
35	0.0	0.0	0.0	0.00	0.0	0.0	0.00	1.000
36	0.0	0.0	0.0	0.00	0.0	0.0	0.00	1.000
37	0.0	0.0	0.0	12.67	0.0	0.0	12.33	0.004[h]
38	0.0	0.0	0.0	9.33	0.0	0.0	9.83	0.003[h]
39	0.0	0.0	0.0	0.00	0.0	0.0	0.00	1.000
40	0.0	0.0	0.0	0.00	0.0	0.0	0.00	1.000
41	0.0	0.0	0.0	0.00	0.0	0.0	0.00	1.000
C[i]	20.7	20.3	23.2	22.0	20.5	22.8	21.8	0.811

[a]A=Amikacin, [b]C= Ceftazidime, [c]F=Fosfomycin, [d]AC= amikacin plus ceftazidime,

[e]ACF1= amikacin plus ceftazidime plus fosfomycin (below MIC 1 dilution),

[f]ACF2= amikacin plus ceftazidime plus fosfomycin (at MIC concentration),

[g]ACF3= amikacin plus ceftazidime plus fosfomycin (above MIC 1 dilution),

[h] Significant differences from Kruskal-Wallist H test,

[i]C = *P. aeruginosa* ATCC 27853

Table 4.12 Comparison of the inhibition zone diameter among 7 antimicrobial regimens

Antimicrobial regimen [a]		p-value*of isolate no.										
		1	2	8	13	22	23	29	30	34	37	38
AC	A	0.317	0.034[c]	0.037[c]	0.034[c]	0.127	0.037[c]	1.000	0.037[c]	0.034[c]	0.037[c]	0.034[c]
	C	0.034[c]	0.653	0.037[c]	0.317	0.127	0.037[c]	0.376	0.037[c]	0.034[c]	0.037[c]	0.034[c]
	F	0.034[c]	0.034[c]	0.037[c]	0.034[c]	0.513	0.513	0.050	0.037[c]	0.034[c]	0.037[c]	0.034[c]
	ACF1	0.099	0.043[c]	0.037[b]	0.072	0.046[c]	0.072	0.050	0.037[c]	0.034[b]	0.037[b]	0.034[b]
	ACF2	0.105	0.637	0.037[b]	0.099	0.376	0.814	0.046[b]	0.037[c]	0.034[b]	0.037[b]	0.034[b]
	ACF3	0.317	0.114	0.637	0.456	0.275	0.127	0.050	0.184	1.000	0.637	0.239
ACF1	A	0.114	0.034[c]	1.000	0.037[c]	0.825	0.034[c]	0.050	1.000	1.000	1.000	1.000
	C	0.034[c]	0.105	1.000	0.037[c]	0.825	0.034[c]	1.000	1.000	1.000	1.000	1.000
	F	0.034[c]	0.034[c]	1.000	0.037[c]	0.046[b]	0.817	0.077	1.000	1.000	1.000	1.000
	AC	0.099	0.043[b]	0.037[c]	0.072	0.046[b]	0.072	0.050	0.037[b]	0.034[c]	0.037[c]	0.034[c]
	ACF2	0.637	0.046[b]	1.000	0.346	0.046[b]	0.507	0.105	1.000	1.000	1.000	1.000
	ACF3	0.114	0.034[b]	0.034[b]	0.105	0.046[b]	0.817	0.827	0.037[b]	0.034[b]	0.034[b]	0.034[b]
ACF2	A	0.121	0.037[c]	1.000	0.034[c]	0.050	0.037[c]	0.046[c]	1.000	1.000	1.000	1.000
	C	0.037[c]	0.500	1.000	0.034[c]	0.050	0.037[c]	0.105	1.000	1.000	1.000	1.000
	F	0.037[c]	0.037[c]	1.000	0.034[c]	0.827	0.376	0.817	1.000	1.000	1.000	1.000
	AC	0.105	0.637	0.037[c]	0.099	0.376	0.814	0.046[c]	0.037[b]	0.034[c]	0.037[c]	0.034[c]
	ACF1	0.637	0.046[c]	1.000	0.346	0.046[c]	0.507	0.105	1.000	1.000	1.000	1.000
	ACF3	0.121	0.121	0.034[b]	0.197	0.376	0.827	0.268	0.037[b]	0.034[b]	0.034[b]	0.034[b]
ACF3	A	1.000	0.025[c]	0.034[c]	0.034[c]	0.050	0.037[c]	0.050	0.037[c]	0.034[c]	0.034[c]	0.034[c]
	C	0.025[c]	1.000	0.034[c]	0.114	0.050	0.037[c]	0.658	0.037[c]	0.034[c]	0.034[c]	0.034[c]
	F	0.025[c]	0.025[c]	0.034[c]	0.034[c]	0.658	0.658	0.814	0.037[c]	0.034[c]	0.034[c]	0.034[c]
	AC	0.317	0.114	0.637	0.456	0.275	0.127	0.050	0.184	1.000	0.637	0.239
	ACF1	0.114	0.034[c]	0.034[c]	0.105	0.046[c]	0.817	0.827	0.037[c]	0.034[c]	0.034[c]	0.034[c]
	ACF2	0.121	0.121	0.034[c]	0.197	0.376	0.827	0.268	0.037[c]	0.034[c]	0.034[c]	0.034[c]

[a]A=Amikacin, C= Ceftazidime, F=Fosfomycin, AC= amikacin plus ceftazidime,

ACF1= amikacin plus ceftazidime plus fosfomycin (below MIC 1 dilution),

ACF2= amikacin plus ceftazidime plus fosfomycin (at MIC concentration),

ACF3= amikacin plus ceftazidime plus fosfomycin (above MIC 1 dilution);

[b] significant smaller than another regimen;

[c] significant larger than another regimen;

* Mann-Withney U test

Accordingly, Mann-Withney U test was used to indicate the pair of antimicrobial regimen(s) which had significant difference of the mean inhibition zone; p-value was shown in Table 4.11 and Table 4.12. The contribution of the mean inhibition zone different of 7 antimicrobial regimens against those 11 MDR-PA isolates was shown in Table 4.13.

Table 4.13 Characteristics of inhibition zone among tested strains

Characteristics	Isolate no.	p-value*	Total % of isolate (no.)
AC[d]			
Larger than A[a], C[b], and F[c]	8, 30, 34, 37, and 38	≤ 0.037	45.45 (5)
ACF1[e, #]			
Larger than A, C, and F	13	0.037	45.45 (5)
Larger than AC	8, 34, 37, and 38	≤ 0.037	
ACF2[f, @]			
Larger than A, C, and F	13	0.034	72.73 (8)
Larger than AC	8, 29, 34, 37, and 38	≤ 0.046	
Larger than ACF1	2 and 22	0.046	
ACF3[g]			
Larger than A, C, and F	8, 30, 34, 37 and 38	≤ 0.037	63.64 (7)
Larger than ACF1	2, 8, 22, 30, 34, 37 and 38	≤ 0.046	
Larger than ACF2	8, 30, 34, 37 and 38	≤ 0.037	

[a]A=Amikacin, [b]C= Ceftazidime, [c]F=Fosfomycin, [d]AC= amikacin plus ceftazidime,

[e]ACF1= amikacin plus ceftazidime plus fosfomycin (below MIC 1 dilution),

[f]ACF2= amikacin plus ceftazidime plus fosfomycin (at MIC concentration),

[g]ACF3= amikacin plus ceftazidime plus fosfomycin (above MIC 1 dilution),

[#] ACF1 was smaller than AC in isolate number 2, 22, and 30,

[@] ACF2 was smaller than AC in isolate number 30

* Mann-Withney U test

The two-antimicrobials combination regimen, amikacin plus ceftazidime (AC) had the mean inhibition zones significantly larger than mono-antimicrobial regimen, amikacin (A), ceftazidime (C), and fosfomycin (F) in 5 isolates (45.45%) ($p \leq 0.037$).

While three-antimicrobials combination of amikacin plus ceftazidime plus fosfomycin (below MIC 1 dilution); ACF1, provided the inhibition zone larger than ones of A, C, and F in isolate number 13 (9.09%) and larger than AC in 4 isolates (36.36%); isolate number 8, 34, 37, and 38. So, there were 5 (45.45%) isolates showed the active activities of ACF1 regimen against these isolates. In the contrary, ACF1 showed the smaller size than ones of AC in 3 (27.27%) isolates; isolate number 2, 22, and 30.

Throughout, the triple combinations of amikacin plus ceftazidime plus fosfomycin (at MIC concentration); ACF2 showed the inhibition zone larger than A, C, and F in 1 isolate number 13 (9.09%). Also, they showed the larger zone than ones of AC in 5 isolates (45.45%); isolate number 8, 29, 34, 37, and 38. In the contrary, isolate number 2 and 22 (18.18%) showed the inhibition zone larger than ones of ACF1. Then, there were totally 8 (72.73%) isolates showed the preferred activities against these strains.

Finally, the inhibition zone of amikacin plus ceftazidime plus fosfomycin (above MIC 1 dilution); ACF3, were larger than ones of all mono-antimicrobial regimens in 5 isolates (45.46%); isolate number 8, 30, 34, 37 and 38. Seven isolates (63.64%); isolate number 2, 8, 22, 30, 34, 37 and 38 showed the inhibition zone larger than ones of ACF1. Also, the inhibition zone of ACF3 was larger than ones of ACF2 in 5 (45.45%) isolates; isolate number 8, 30, 34, 37 and 38. As a consequence, ACF3 almost showed the inhibition zone larger than ones of each antimicrobial alone, double combination of AC, or triple combination of ACF1 or ACF2 in total of 7 (63.64%) isolates.

For instance, the double combination of AC and triple combination of ACF1, ACF2, and ACF3 provided the numbers of antimicrobial coverage on MDR-PA isolates within 5, 5, 8, and 7 isolates, respectively (Table 4.13). Thoroughly, the antimicrobial combination, either double combination of amikacin and ceftazidime or triple combination of amikacin, ceftazidime, and fosfomycin, showed larger size of inhibition zone than mono-antimicrobial regimen. Among the triple combination, regimen of ACF3 showed greater activity. Also, the ACF2 showed greater activity than ACF1.

Part II Optimization of antimicrobial therapy using pharmacokinetic and pharmacodynamic parameters

The pharmacokinetic/pharmacodynamic approach to a rational dosage regimen for antimicrobials

Selection of an appropriate antimicrobials and dosage regimen requires careful consideration of both organism- and patient-specific factors, including site of infection, impact of the inoculum effect and possible consequences of resistant bacterial subpopulations. Using above concepts, could be designed dosage regimens to optimize the important pharmacokinetic surrogates identified for given class of antimicrobial agents (48). Clearly, as pathogens become more resistant to antimicrobial agents, the efficacy of standard dosing regimen may be reduced. This increases the need for newer regimens and newer antimicrobials. The population pharmacokinetic parameter estimates of amikacin, ceftazidime, and fosomycin were shown in Table 4.14. These parameters were used in the determination of optimal dosage regimen for each drug.

Table 4.14 Population pharmacokinetic parameter estimates (63-67)

Population parameters	Amikacin	Ceftazidime	Fosfomycin
Patients	Thai patients	Critically ill	Ventriculitis
Sex (male/female)	40/31	9/3	2/4
Age[1](years)	60.2±18.9	57±12	53±8
Weigh[1]t (kg)	53.7±10.7	-	71±9
Volume of distribution[1]; Vd (L)	18.0±4.18	18.9±9.0	26.3±9.7
Elimination rate constant[1], k (hr^{-1})	0.208±0.07	0.268±0.205	-
Clearance[1,2]; Cl (L/hr)	3.92±2.09	-	5.0±2.0
Half life[1,3] (hr)	4.02±2.26	3.48±1.61	4.0±0.5
Maximum dose (gm/day)	1.5	6	16
Infusion time ; t' (hr)	0.5-1.0	> 0.5	1.0
Dosing interval; τ (hr)	24	24	4

[1]Data presented in mean ±SD, [2]$Cl = k \times Vd$, [3]Half-life =0.693/k

Since, tested isolates demonstrated a very high MIC_{50} and MIC_{90} against amikacin, ceftazidime, and fosfomycin at > 256 μg/mL, > 256 μg/mL, and > 1024 μg/mL, respectively. These represented high resistant strains which were difficult to treat with standard dosing regimen. Therefore, these isolates were excluded from the calculation to reduce the MICs which were used to determine the optimal dosage regimen for each drug. The pharmacodynamic parameters of these isolates were shown in Table 4.15.

Table 4.15 Pharmacodynamic parameters of the tested isolates[1]

Pharmacodynamic parameters	Amikacin (n=20)[2]	Ceftazidime (n=13)	Fosfomycin (n=13)
MIC_{50} (mg/L)	96	128	80
MIC_{90} (mg/L)	96	192	384

[1] Not including the isolates with showed the MIC greater than the highest value on the E test scale

[2] No. of participant isolates in each antimicrobials group

Antimicrobial regimen;

For **amikacin**, concentration dependent, significant post-antibiotic effect (PAE) appears to be better suited to less frequent administrations at higher doses; extended dosing intervals (68, 69). Desired target concentration at 8 times above MIC (C_{max}/MIC ≥8) is required (49). To determine the loading dose and maintenance dose, we considered its half life and infusion time. An infusion model (Table 3.1) was used in the calculation because the infusion times (60 minutes) longer than one sixth of its half life (4.02 hours/6 = 40.2 minutes).

Intravenous infusion (iv infusion);

Loading dose; $$\frac{LD}{t'} = \frac{C_0 \times k \times Vd}{S \times F \times (1 - e^{-kt'})}$$

; $C_o = 8 \times MIC_{50}$, S = 1, F = 1, Vd =18.0 (L), k = 0.208 (hr^{-1}), t' = 1 hr

$$LD = \frac{8 \times 96\,(mg/L) \times 0.208\,(hr) \times 18\,(L) \times 1\,(hr)}{(1 - e^{(-0.208)(1)})}$$

$$= 15{,}311.50 \text{ mg}$$

rounded to 15,320 mg intravenous infusion in 60 minutes.

The extended intervals high dose regimen (iv infusion);

Maintenance dose; $\frac{MD}{t'} = \frac{C_{max,ss} \times k \times Vd \times \left(1 - e^{-k\tau}\right)}{S \times F \times \left(1 - e^{-kt'}\right)}$

; $C_{max,ss} = 8 \times MIC_{50}$, S = 1, F = 1, Vd =18.0 (L), k = 0.208 (hr^{-1}), t' = 1 hr, τ = 24 hr

$$MD = \frac{8 \times 96\,(mg/L) \times 0.208\,(hr) \times 18\,(L) \times (1 - e^{(-0.208)(24)}) \times 1\,(hr)}{(1 - e^{(-0.208)(1)})}$$

$$= 15{,}207.50 \text{ mg}$$

rounded to 15,210 mg intravenous infusion in 60 minutes every 24 hours

For **ceftazidime**, time dependent, it requires 35- 40% for bacteriostasis and 60-70% for maximum bactericidal activity. Continuous infusion resulted in greater T>MIC than an intermittent intravenous. For instance, the antimicrobial level should be 1- 4 times greater than their MIC (48); therefore, the patient should receive the initial loading dose to avoid the risk of delayed time to steady state concentration at the site of infection. When both loading bolus injection and continuous infusion are given, the net effect should be a fairly steady plasma concentration. Before the constant intravenous infusion has reached steady state, the bolus loading dose has produced a nearly steady state drug concentration, and when the drug from the loading dose is almost eliminated, the constant intravenous infusion should be about at steady state (62). To determine the loading dose, we considered its half life and infusion time. Because the infusion times (30 minutes) less than one sixth of its half life (3.48 hours/6 = 34.8 minutes), a bolus model was used in the calculation (Table 3.1).

Intravenous infusion (iv infusion)

Loading dose; $LD = \frac{C_0 \times Vd}{S \times F}$

; C_o = MIC_{50}, S = 1, and F = 1, Vd =18.9 (L)

$$LD = 128 \text{ (mg/L)} \times 18.9 \text{ (L)}$$

$$= 2{,}419.20 \text{ mg}$$

rounded to 2,500 mg intravenous infusion in 30 minutes (do not exceed 35 minutes).

or given 5,000 mg intravenous infusion in 30 minutes if $C_o = 2 \times MIC_{50}$

Continuous intravenous infusion (CI)

Rate of drug infusion (k_0);

$$k_0 = S \times F \times V_d \times k \times C_{ss}$$

; C_{ss} = MIC_{50}, S = 1, and F = 1, Vd =18.9 (L), k = 0.268 (hr^{-1})

$$k_0 = 18.9\,(L) \times 0.268\,(hr^{-1}) \times 128\,(mg/L)$$

$$= 648.35 \text{ mg/hr}$$

rounded to 650 mg/hr

or given 1,300 mg/hr if $C_{ss} = 2 \times MIC_{50}$

For **fosfomycin**, time dependent, it is a broad-spectrum antibiotic with a wide therapeutic range and characteristic pharmacological properties. Fosfomycin penetrates excellently into various tissues and cerebrospinal fluid and is frequently administered in combination with other antimicrobial agents (70). To determine the loading dose and maintenance dose, we considered its half life and infusion time. An infusion model (Table 3.1) was used in the calculation because the infusion times (60 minutes) longer than one sixth of its half life (4.00 hours/6 = 40 minutes).

Intravenous infusion (iv infusion)

Loading dose; $$\frac{LD}{t'} = \frac{C_0 \times k \times Vd}{S \times F \times (1 - e^{-kt'})}$$

; C_o = MIC_{50}, k = 0.173 hr^{-1}, t' = 1 hr, Vd = 26.3 (L)

$$LD = \frac{80\,(mg/L) \times 0.173\,(hr^{-1}) \times 26.3\,(L) \times 1(hr)}{(1 - e^{(-0.173)(1)})}$$

$$= 2{,}291.24 \text{ mg}$$

rounded to 2,300 mg intravenous infusion in 1 hour (do not less than 40 minutes).

or given 4,600 mg intravenous infusion in 1 hour if $C_o = 2 \times MIC_{50}$

Intermittent intravenous infusion (iv infusion)

Desired concentration to optimize T>MIC more than 50%

$$C_o = \frac{C_t}{e^{-kt''}}$$

; $C_t = \text{MIC}_{50}$, $k = 0.173\ (\text{hr}^{-1})$, $\tau = 4$ hr, $t'' = \frac{\tau}{2} = 2$ hr

$$C_o = \frac{80\ (\text{mg/L})}{e^{(-0.173)(2)}}$$

$$= 113.07 \text{ mg}$$

rounded to 120 mg

or given 230 mg if $C_t = 2 \times \text{MIC}_{50}$

Maintenance dose; $$\frac{MD}{t'} = \frac{C_{\text{max,ss}} \times k \times Vd \times \left(1 - e^{-k\tau}\right)}{S \times F \times \left(1 - e^{-kt'}\right)}$$

; $C_{\text{max,ss}} = C_o$, S = 1, and F = 1, $Vd = 26.3$ (L), $k = 0.173\ (\text{hr}^{-1})$, $t' = 1$ hr, $\tau = 4$ hr

$$MD = \frac{120\ (\text{mg/L}) \times 0.173\ (\text{hr}^{-1}) \times 26.3\ (\text{L}) \times (1 - e^{(-0.173)(4)})}{(1 - e^{(-0.173)(1)})}$$

$$= 1{,}716.46 \text{ mg}$$

rounded to 1,800 mg intravenous infusion in 1 hour every 4 hours

or given 3,500 mg intravenous infusion in 1 hour if $C_t = 2 \times \text{MIC}_{50}$

The MIC_{50} and MIC_{90} of amikacin and ceftazidime which used in the calculation was their $\text{MIC}_{\text{combo}}$, in Table 4.8. For fosfomycin, the MIC_{mono}, in Table 4.8 were used, because we did not determine the $\text{MIC}_{\text{combo}}$ of fosfomycin when used in the combination. Then, the recommended dosage regimen for amikacin, ceftazidime, and fosfomycin were demonstrated in Table 4.16, Table 4.17, and Table 4.18, respectively.

Table 4.16 Recommended amikacin regimens

Dosage	Times MIC_{50} of Amikacin		
	$1 \times MIC_{50}$	$2 \times MIC_{50}$	$8 \times MIC_{50}$
Target concentrations (mg/L)	96	192	768
Loading dose (mg)	1915[1]	3830[1]	15320[1]
Maintenance dose (mg)	1900[2]	3800[2]	15210[2]
Total dose / day (mg)	1900[3]	3800[3]	15210[3]

[1] Intravenous infusion, [2] Intravenous infusion every 24 hr, [3] Exceed maximum dose per day

Table 4.17 Recommended ceftazidime regimens

Dosage	Times MIC_{50} of Ceftazidime		
	$1 \times MIC_{50}$	$2 \times MIC_{50}$	$4 \times MIC_{50}$
Target concentrations (mg/L)	128	256	512
Loading dose (mg)	2500[1]	5000[1]	10000[1]
Maintenance dose	650 mg/hr[2]	1300 mg/hr[2]	2600 mg/hr[2]
Total dose / day (mg)	15600[3]	31200[3]	62400[3]

[1] Intravenous infusion, [2] Continuous infusions; CI, [3] Exceed maximum dose per day

Table 4.18 Recommended fosfomycin regimens

Dosage	Times MIC_{50} of Fosfomycin		
	$1 \times MIC_{50}$	$2 \times MIC_{50}$	$4 \times MIC_{50}$
Target concentrations (mg/L)	80	160	320
Loading dose (mg)	2300[1]	4600[1]	9200[1]
Maintenance dose (mg)	1800[2]	3500[2]	7000
Total dose / day (mg)	10800	21000[3]	42000[3]

[1] Intravenous infusion, [2] Intermittent infusions; CI, [3] Exceed maximum dose per day

It was notable that the recommended dosage regimen almost utilized total dose of the antimicrobials exceeds the maximum dose per day. On the other hand, the maximum standard dose of amikacin, ceftazidime, and fosfomycin provided the plasma concentrations which overcame 7 (20.59%) isolates, 4 (11.77%) isolates, and 11 (32.35%) isolates, respectively. Data were shown in Table 4.19.

Table 4.19 Maximum standard antimicrobial regimen

Description	Amikacin	Ceftazidime	Fosfomycin
Standard dose	1500 mg	250 mg/hr	3000 mg
Administration	Infusion 30 min q 24 hr	Continuous infusion	Infusion 1 hr q 4 hr
Plasma concentration	83.90 mg/L	49.36 mg/L	209.74 mg/L
No. of coverage isolate (%)	7 (20.59%)	4 (11.77%)	11 (32.35%)
Isolate no.	14, 15, 28, 33-35, 38	11, 15, 22, 28, 29	5, 10, 12, 13, 15, 20, 21, 26, 28-30

CHAPTER V
DISCUSSION

Part I *In vitro* effects of the triple combination on clinical isolates of MDR-PA from Chonburi Hospital

The emergence of serious infections due to MDR Gram-negative organisms poses a contemporary therapeutic challenge. Much attention has been paid to the therapeutic option. New class of antimicrobials active against *P. aeruginosa* will not be available for therapy within the next 5–7 years. Most new quinolones under the development are likely to show some degree of cross-resistance to ciprofloxacin. Carbapenems, considered as the antimicrobials of choice for these organisms, become less useful subsequent to the rapid increase in carbapenem-resistant strains. Other therapeutic alternatives to treat infections caused by these organisms include the aminoglycosides and the polymyxin classes. Resistance rates to aminoglycosides are also usually elevated and the polymyxins (polymyxin B and colistin) should be avoided when possible, secondary to toxicity concerns (71).

The rational for combination therapy is essential to reduce the chances of selection of resistant mutants during therapy, as well as to exploit the potential synergistic activity of some agents. The preferred combination remains β-lactams with aminoglycosides, as synergism between these drugs has been demonstrated by *in vitro* studies and results of several clinical studies pointed to the superiority of similar regimens as opposed to monotherapy for the treatment of *P. aeruginosa* bacteremia, especially in neutropenic patients. However, some clinical studies had cast doubt on the actual superiority of combination therapy (2). For β-lactam and fluoroquinolone combinations against *P. aeruginosa*, synergism and indifference had generally demonstrated and, antagonism has occasionally been reported in a small percentage of isolates (72).

In 1984, Takahashi and Kanno (43) found synergistic activities of combinations of piperacillin, cefoperazone, and cefsulodin with fosfomycin which were enhanced by the addition of tobramycin. Also, Jittaropas et al (73) found the superiority of three drugs combination to two drugs as shown by a further reduction of a Fraction inhibitory concentration (FIC) index. In 1999, Oie et al. (44) found significantly different in growth-inhibited strains between the combination of two drugs and those of three drugs against isolates of *P. aeruginosa* ($p<0.01$). In 2003, Oie et al (34) found the most effective regimens were the combination of piperacillin, ceftazidime and amikacin, and that of cetazidime, aztreonam and amikacin. These proposed that these combinations might be useful for severe *P. aeruginosa* infections. In addition, fosfomycin offered as an added bonus, protection against aminoglycoside nephro- and oto-toxicity (7, 39). Furthermore, amikacin was the most active aminoglycosides when was combined with fosfomycin (39). Determination of the *in vitro* effects of triple combination; amikacin, ceftazidime, and fosfomycin on clinical isolates of MDR-PA should be stated.

1. Antimicrobial susceptibility of 41 clinical isolates of MDR-PA from Chonburi Hospital

Agar disk diffusion assay or Kirby-Bauer method is one of the most reliable and routinely used methods for determining the antimicrobial susceptibilities of bacteria in the clinical microbiological laboratories. This method is simple to perform, easy to interpret and flexible with respect to test antimicrobial agents. It has been used to classify a microorganism as susceptible, intermediate resistant, and resistant to antimicrobial agents by interpreting the result with zone diameter interpretative standards and equivalent MIC breakpoint recommended by NCCLS (74).

Forty-one MDR-PA clinical isolates were used as tested strains. The most frequent site of these MDR-PA isolates were sputum and trachea secretion (44.45%). The results were similar to the Global SENTRY Antimicrobial Surveillance Program; 1997–1999, the distribution of *P. aeruginosa* isolates by body site was generally the same for all regions, the respiratory tract was the most frequent source of *P. a*eruginosa isolates (46.1%). While bloodstream was the second most frequent source of MDR-PA isolates (11). In this study, bloodstream was the least frequent source of

infection (8.33%). In addition, an epidemiology of nosocomial infections caused by *P.* aeruginosa was reported mostly frequently in the respiratory tract (2).

Mechanisms of *P. aeruginosa* resistance to amikacin, ceftazidime, carbapenems, and fosfomycin varies considerably. Ceftazidime less active than amikacin against our *P. aeruginosa* strains. This might reflect the extensive use of ceftazidime in this hospital. *P. aeruginosa* resistance to ceftazidime and carbapenems might be due to various mechanisms, including selection of class C chromosomal β-lactamase-derepressed mutants, acquisition of a plasmid- or transposon-mediated extended spectrum β-lactamases, and loss of functional porins that cause low outer membrane permeability (75). The acquisition of metallo-β-lactamase is possibly responsible for resistance of these isolates because these enzymes (e.g., the enzymes of the IMP, VIM, SPM and GIM type) efficiently degrade all the antipseudomonal β-lactams (including carbapenems) except monobactams (2, 10, 18).

In present study, there were 2 (5.56%) isolates; isolate number 13 and 37, which susceptible to imipenem but showed resistant to meropenem. Some study suggested that meropenem behaves as a substrate of MexAB-OprM pump because of the presence of a hydrophobic side chain at position 2, whereas imipenem and ER-35786 contain strongly charged, hydrophilic side-chains, cannot become a substrate. However, the correlation between resistance and efflux may not be simple, because the influx of carbapenems is affected by the levels of OprD (76). *In vitro* studies, all carbapenems selected loss of OprD as a first mechanism of resistance. However, in an OprD-deficient background, meropenem was able to select MexAB-OprM overproducers as a secondary resistance mechanism. In addition, among isolates which resistant to both imipenem and meropenem, there were 2 isolates (isolate number 2 and 23) susceptible to fosfomycin, these promised the utilities of fosfomycin in the combination therapy.

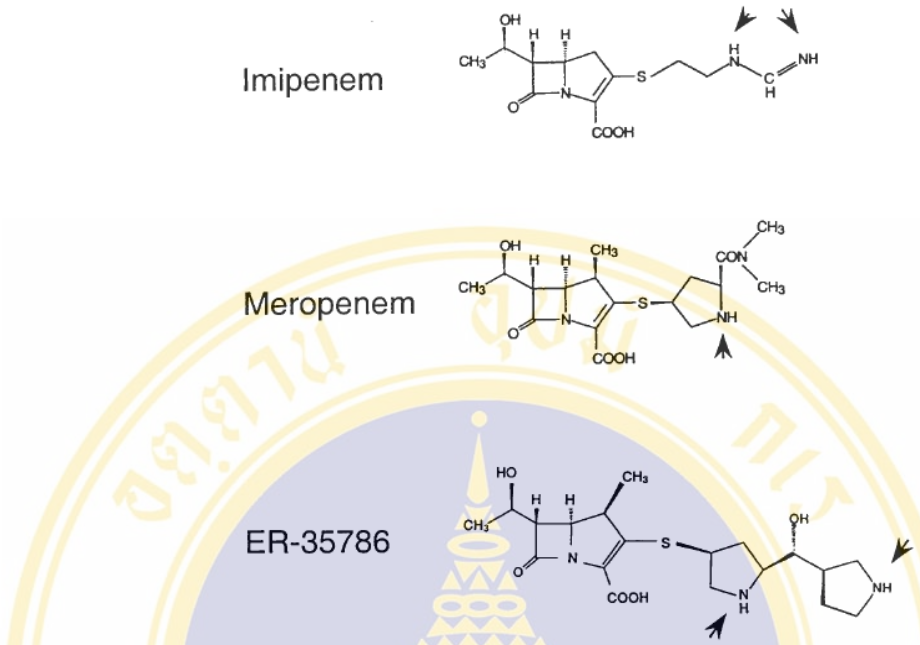


Figure 5.1 Chemical structures of the three carbapenems studied. Arrowheads indicate nitrogen atoms which can be charged positively (76)

Resistance to aminoglycosides was attributable either to plasmid-carried genes encoding enzymes for aminoglycoside modification, or to impairment or loss of transport mechanisms that can prevent the drug from reaching its target sites (75). Resistance of tested isolates to both ceftazidime and amikacin might also be attributed to plasmid-carried genes encoding enzymes for hydrolysis of ceftazidime and for aminoglycoside modification (77).

Of these MDR-PA isolates, they showed high level of resistance, it seems to be inappropriate to treat the MDR-PA infections by monotherapy of the tested agents because the use of each antimicrobial agent had low susceptibility against these organisms. In our study, the susceptible agent against these isolates ranging from piperacillin/tazobactam, imipenem, meropenem, and fosfomycin, respectively. While amikacin, ceftazidime, and ciprofloxacin showed less active against such isolates. Then, piperacillin/ tazobactam, carbapenams (imipenem and meropenem), and fosfomycin considered the antimicrobials of choice for these organisms.

To limits the emergence of carbapenems and piperacillin/tazobactam resistance, its use should be reserved for infections when the infecting strain was resistant to the first-line drugs. The combination therapy promised to reduce the chances of selection of resistant mutant and the data from *in vitro* studies and results of several clinical studies supported the uses of combination therapy in the treatment of *P. aeruginosa* infections. To optimal clinical outcomes, it appropriate to treat these infections with two or more antimicrobial agents.

2. The MIC of each antimicrobial agent (MIC_{mono}) determined by E test method.

The Epsilometer, or E test, is a relatively new agar diffusion method for performing antimicrobial susceptibility testing. Rather than a fixed concentration, a continuous gradient of an antimicrobial agent, in a range that corresponds to 15 two-fold dilutions, is presented on a strip. The technique is less labor-intensive than determining the MICs by the standard microdilution broth method. Despite limitation of the budgets, we determined the MIC only three antimicrobial agents which comprised in the combination. And the regimen which promised to be the effective regimen against MDR-PA was the combination of amikacin, ceftazidime, and fosfomycin.

The MIC_{mono} of amikacin, ceftazidime, and fosfomycin against MDR-PA were shown in Table 4.5. Most of these clinical isolates were resistant to the tested antimicrobials. They demonstrated higher MIC_{50} and MIC_{90} than the strains were tested in other studies (39, 78). It should be stated that the MDR-PA strains which used in our study were highly resistant and might be more difficult to treat. The results were similar to the susceptibility from disk diffusion at Chonburi Hospital.

Antimicrobial susceptibility rate among of MDR-PA isolates by disk diffusion and E test were shown in Table 5.1. It showed a similar result, there were a very low susceptibility rates against all three antimicrobials. Fosfomycin were the most active antimicrobials while; ceftazidime showed the less active against MDR-PA. It was remarkable that no therapeutic agent that was routinely tested inhibited >50% of MDR-PA strains.

Table 5.1 Antimicrobial susceptibility rate among of MDR-PA isolates

Antimicrobial agents	Susceptibility rate (no.)	
	Disk diffusion	E test
Amikacin	0% (0)	8.83% (3)
Ceftazidime	0% (0)	0% (0)
Fosfomycin	38.10% (8)	17.65% (6)

The contributions of MDR-PA resistance by E test method were shown in Table 5.2. The clinical isolates of MDR-PA were classified in to 3 groups according to the susceptibility testing to those of three antipseudomonal agents which were used to test antimicrobials interaction; amikacin, ceftazidime, and fosfomycin. Most of them, 76.47% (26 of 34) of isolates were resistant to all of three antimicrobial agents. The second, there were 20.59% (7 of 34) of isolates which were resistant to 2 antimicrobial agents; ceftazidime and either amikaicin or fosfomycin. The last group, there were 2.94% (1 of 34) of isolates which were resistant to only ceftazidime. Importantly, all isolates (100%) were resistant to ceftazidime. Therefore, our population had high risk of treatment failure from mono- or double antimicrobial combination. Even, triple combination may result in treatment failure; however, if there is synergistic or additive effect, the triple combination might still be benefit.

TABLE 5.2 The contributions of MDR-PA resistance by E test method

No. of agents to which isolates were resistant	Total % of isolates (no.)	% of MDR-PA isolates which resistant to (no.)		
		Amikacin	Ceftazidime	Fosfomycin
1	2.94 (1)	0 (0)	100 (1)	0 (0)
2	20.59 (7)	71.43 (5)	100 (7)	28.57(2)
3	76.47 (26)	100 (26)	100 (26)	100 (26)

In addition, in some isolates, the susceptibility of fosfomycin seems not to be correlated with the susceptibility of both amikacin and ceftazidime. Besides, fosfomycin is a unique broad-spectrum bactericidal antimicrobials chemically unrelated to any other known antimicrobial agents and is not cross-resistant to other antimicrobial agents and adds benefit of conferring renal and auditory protection from use of concomitant aminoglycoside (7, 36-38). Thus, it seems to be an effective antimicrobial agent, as part of combination intravenous therapy, for the treatment of pseudomonal infection as same as Watine and Okazaki studies demonstrated (29, 30).

3. The MIC of amikacin in the combination with ceftazidime (MIC_{combo}) determined by E test method

Bonapace et al (28) evaluated the E test method to assess synergy and compared it to the checkerboard and time-kill tests. The results of the E test method appeared to agree fairly well with the results from checkerboard and time-kill testing (27). Similary, the study of Balke et al. (79) was evaluated the performance of the E test in the assessment of synergy in comparison to the standard agar dilution checkerboard susceptibility test and determined the activity of two antimicrobial combinations against 163 MDR-PA cystic fibrosis isolates. The agreement between the checkerboard and the E test susceptibility test method was excellent (>90%). They conclude that the E test is a valuable and practical method for routine microbiological diagnostics improving the antimicrobial options in the treatment of CF patients chronically infected with *P. aeruginosa.* Therefore, we used E test to assess antimicrobial interaction between amikacin and ceftazidime.

To our knowledge, the strains which were used in this study showed high-level resistance to amikacin, ceftazidime, and fosfomycin. Therefore, double combination of antimicrobials might not be benefit. However, some investigators reported enhanced killing effect of amikacin combination with β-lactam (including ceftazidime) on 39 to 50% of MDR-PA isolates. These were independent of the strain MIC level to β-lactam or amikacin (77). Therefore, our study chose to determine the effects of amikacin and ceftazidime combinations against MDR-PA isolates by E test. The natures of synergism, additivity, or antagonism were determined on the basis of the calculated FIC index as described in chapter 3.

The present study demonstrated very low synergistic activities between amikacin and ceftazidime, the mean of the FIC index were 1.61±0.53. Only one (2.94%) isolate out of 34 isolates showed synergism. Other *in vitro* study demonstrated a higher number of isolates with synergistic activities between amikacin and ceftazidime (70.80%) or amikacin and meropenem (40.00%) (75). Because of all tested isolates in this study were MDR-PA which were resistant to either amikacin or ceftazidime with higher MIC_{50} and MIC_{90} (≥256 µg/mL).

Therefore, they showed lower percentage of synergistic activities. This finding was different from the study by Giamarellos-Bourboulis et al. whereas the tested isolates in previous studies were more susceptible and might be attributed to either the different methodology applied or the adaptation of different mechanisms of resistance over the a period of times. More importantly, in this study did not find any antagonistic activity between amikacin and ceftazidime, similar to previous studies (34, 39, 56). The possible reason was our isolates were resistant to several groups of different mechanism of action of antimicrobials. Therefore, combination might not be able to overcome the resistance while other previous studies might have isolates with less resistant.

Isolate number 28 showed, the MIC at 3.5 µg/mL (sensitive) to amikacin and 28 µg/mL (resistant) to ceftazidime, synergistic activity between amikacin and ceftazidime. Furthermore, this isolate demonstrated the highest decreased of the MIC (5.33 times) for both agents. Whereas in the other isolates which showed additivity interaction usually demonstrated the high level resistant to either amikacin or ceftazidime.

Although only one synergy had found in our study. We still able to determine several additivity interactions. The well-established mechanisms of antimicrobial interaction between β- lactams and aminoglycosides, ceftazidime acting on the cell wall to permit increased entry of amikacin (26). Therefore, when using amikacin in combination with ceftazidime, the MIC decreased in either amikacin or ceftazidime within 14 (41.18%) isolates in our study (Table 4.8). Despite high MIC of ceftazidime when used in the combination, the susceptibility changed from resistant to sensitive in 2 (8%) isolates; isolate number 22 and 28. While not found with amikacin.

Among the tested isolates which were sensitive to amikacin, even though they were resistant to ceftazidime, there were 33.33% (1 of 3; isolate number 28) of isolates showed synergistic activity and 66.67 % (2 of 3; isolate number 14 and 15) of isolates showed additivity. These exploited the potential option of amikacin could be candidate in the combination regimen against the strains with susceptible or had low level resistant to amikacin. Interstingly, when the tested strain showed the MIC_{mono} of ceftazidime < 256 µg/mL, all of them (isolate number 11, 15, 22, 28, and 29) demonstrated synergy or additivity interaction with amikacin. In the other hand, the isolates which showed MIC_{mono} of amikacin > 256 µg/mL and MIC_{mono} of ceftazidime ≤ 96 µg/mL or the strains which showed MIC_{mono} of ceftazidime > 256 µg/mL and MIC_{mono} of amikacin ≤ 192 µg/mL, were presented synergy or additivity interaction, with FIC index ≤1.00, between amikacin and ceftazidime (isolate number 11, 14, 15, 20, 22, 28, and 29). This represent that it will represent synergy or additivity interaction when at least one of antimicrobials in the combination were susceptible or showed low level resistant against the MDR-PA isolates.

4. The effects of the addition of fosfomycin to the combination of amikacin and ceftazidime using agar well method

Nowadays, there were checkerboard and time-kill curve used to investigate antimicrobial interaction between two or more drugs combination. Otherwise, there was no any standardized quantitative method to investigate antimicrobial interaction between three drugs combination, so we used agar well (qualitative) method in our study because it has the advantage of showing both inhibition and control growth (outside the inhibitory zone) of fastidious organisms on the same plate. It was the technique which usually uses to screen antimicrobial activity from herbal or other natural products. Inhibition zone diameter was measured to the nearest millimeter (mm) in triplicate fashion. In this study, this technique was applied to detect antimicrobial interaction by comparing the mean inhibition zones diameter which was produced by each antimicrobial regimen. The inhibition zone diameter represents the activity of each antimicrobial regimen against MDR-PA isolates. When inhibition zone around the well of combination regimen larger than inhibition zone around mono-antimicrobial regimen, the effect was described as an enhanced killing effect, and when it was smaller, the effect was described as an antagonism. Although, a

larger inhibition zones around the combination regimen excludes significant antagonism, it may be impossible to distinguish between additivity and synergistic activity. Although, it yields only qualitative information about antimicrobial interactions (26), the advantages of this technique were its simplicity and the use of readily available materials.

Among 11 (32.35%) out of 34 MDR-PA isolates which showed significant difference of the mean inhibition zone, their characteristics of inhibition zone among the tested regimens were shown in Table 4.12. For instance, the double combination of amikacin and ceftazidime (AC) provided the activities cover the number of the MDR-PA isolates within 5 isolates equal to the triple combination of amikacin plus ceftazidime plus fosfomycin (below MIC 1 dilution); ACF1 but lower than the triple combinations of amikacin plus ceftazidime plus fosfomycin (at MIC concentration); ACF2, and combination of amikacin plus ceftazidime plus fosfomycin (above MIC 1 dilution); ACF3 within 8, and 7 isolates, respectively. These demonstrated that AC combination had the activities covered the number of MDR-PA isolates which was differed from ACF2, and ACF3 combinations, but not statistical significant ($p=0.385$). These showed the tendency of the addition of fosfomycin to the combination of amikacin and ceftazidime could increase the number of susceptible isolates. It might be due to fosfomycin has a unique chemically structure unrelated to any other known antimicrobial agents and is not cross-resistant to other antimicrobial agents.

Interestingly, focusing on the triple combination, although the number of isolates with significant difference of inhibition zone of ACF2 similar with one of ACF3. However, the inhibition zone of ACF3 was lager than ones of ACF2 and the inhibition zone of ACF2 was lager than ones of ACF1. For instance, the higher concentration of fosfomycin provided the trends of greater activities of the triple combination.

The number of the MDR-PA isolates which were covered by ACF1 equal to ones of AC (within 5 isolates). In addition, ACF1 showed smaller inhibition zone than the double combination of AC (Table 4.12). These suggested that it should not be used fosfomycin at concentration lower than its MIC in the combination with amikacin and ceftazidime. To provide greater antimicrobial interaction, optimal concentration

of fosfomycin in the triple combination with amikacin and ceftazidime should be equal to or more than its MIC.

Another interesting point to confirm the above idea. Although, the inhibition zone of AC was larger than ones of ACF2 in isolates number 30, however ACF3 (the higher fosfomycin concentration regimen) showed the similar inhibition zone with AC. These demonstrated the similar activity between AC and ACF3 regimen. Then, there was no antagonistic activity, *in vitro*, between fosfomycin and the combination of amikacin and ceftazidime. The results were similar to the previous studies (Takahashi, Tessier). These promised the uses of amikacin, ceftazidime, and fosfomycin combination against MDR-PA isolates.

Despite, there were no correlation between the results from the detection of synergy by E test method and agar well; it might be due to the difference of method. However, an enhanced killing effect of amikacin and ceftazidime by adding fosfomycin promised the treatment option against MDR-PA infection with the triple combination.

Part II Optimization of antimicrobial therapy using pharmacokinetic and pharmacodynamic parameters

The pharmacokinetic/pharmacodynamic approach to a rational dosage regimen for antimicrobials

According to literature reviewed, the inappropriate dosage regimens can mainly contribute to drug resistance in the clinical settings. Rational antimicrobial therapy requires dosage regimens to be optimized, not only to guarantee clinical efficacy, but also to minimize the selection and spread of resistant pathogens (45).

Antimicrobials dosage regimen;

The population pharmacokinetic parameter estimates of amikacin, ceftazidime, and fosomycin were used in the determination of optimal dosage regimen for each drug. The recommended dosage regimens were demonstrated in Table 4.15-Table 4.17. Even though the MIC_{50} of the tested isolates was used in the determination, most of them resulted in the total daily dose of all antimicrobials exceed the maximum standard dose, except fosfomycin. Because these organisms were MDR-PA which their resistance probably emerged via various mechanisms then many classes of antimicrobials became the substrate. This might be responsible to high resistant level of the tested strains from Chonburi Hospital.

For amikacin, the extended intervals high dose regimen was used because it takes advantage of their concentration dependent killing and prolonged post-antibiotic effect (PAE), and to prevent adaptive resistance (51, 69, 70, 80). In addition, it has been shown no difference in renal toxicity compared with traditional every-8-hour administration (49). The *in vitro* data have shown that C_{max}/MIC ratio of ≥ 8 is required to prevent regrowth and the emergence of resistant and outcomes data from human studies have demonstrated the importance of high C_{max}/MIC ratio. One retrospective study demonstrated that 92% of patient with C_{max}/MIC >8 achieved clinical cure compared with 12.5% of patients with C_{max}/MIC ≤ 4 (49).

Therefore, the target concentration of amikacin should be more than 8 times the MIC. This might not be possible in the high resistant populations because the large dose amikacin is required to overcome the infected pathogen. The extremely

larger dose might not be appreciable to the clinicians. They might be much concern to antimicrobials toxicities, especially nephrotoxicity from amikacin which was dose dependent (37, 41). In addition, to avoid the larger dose, amikacin should be administered not longer than 40 minutes in the clinical practice because there was elimination of antimicrobials in the later period of times.

Ceftazidime, unlike aminoglycosides, demonstrates concentration independent killing, achieving maximal killing at concentrations of four or five times above the MIC for the organism (65). While one study demonstrated the maximal effect was seen at concentrations of 1 to 4 times the MIC, with no further significant reduction in bacterial counts when concentrations exceed 4 times the MIC (48). Severe infections caused by *P. aeruginosa* require emergency treatment, usually by intermittent infusion of β-lactams. In view of pharmacodynamic and pharmacokinetic properties of β-lactams, continuous infusion has been advocated as an alternative to intermittent dosing to promote maximal bactericidal effect.

While earlier comparison between continuous infusion and intermittent infusion of β-lactams reported similar clinical and microbiological outcomes, Tam et al. found that continuous infusion of β-lactams provided a much higher cumulative fraction of response of 65-81% ($p<0.001$) compared with standard dose administration of 2 gm every 12 hours (4-38%) against *P. aerugunosa* isolates from their institution (49). To our knowledge, ceftazidime has no significant post-antibiotic effect (PAE). However, Giamarellos-Bourboulis et al. (81) found a prolonged PAE reaching the mean PAE (±SE) of 3.10 ± 0.71 hours after exposure to the combinations of amikacin with ceftazidime.

Thus, it would appear that the major determinant for bactericidal activity is the time for which the serum drug concentrations remain above the MIC and not the magnitude of antibiotic concentrations. Continuous intravenous administration produces a relatively constant concentration of antibiotic that can maintain above the MIC (82). However, after a continuous infusion of drugs is begun, five drug half-lives are needed to achieve steady state. If an immediate effect is desired, a loading dose is administered at the initiation of the infusion so that the therapeutic range is maintained from the outset (62). Therefore, to optimize the T > MIC of ceftazidime, continuous infusion was recommended in the treatment of these MDR-PA infections.

In spite of reports of Konishi and Pickering pointing to aminoglycoside–β-lactam incompatibilities. However, Servais et al found physically and chemically compatible with amikacin, isepamicin, tobramycin, and gentamycin as same as fluconazole, ketamine, sufentanil, valproic acid, furosemide, uradipil, and the standard amino acid solutions (VAMIN). In addition, they also checked for intactness of those drugs and found no evidence of significant effect after 1 hour of contact at 25°C; this lack of aminoglycoside-ceftazidime interaction. Finally, the amount of pyridine liberated will remain lower than the USP limit of 1.1 mg/mL for pharmaceutically acceptable ceftazidime solutions (based on published data showing that a 10% degradation of ceftazidime in a 5.8% solution over 24 hours is associated with the liberation of only; 0.5 mg of pyridine/mL. Then, the recommendations for taking 90% stability as a limit, it clearly appears that ceftazidime must be kept at temperatures not higher than 25°C, whatever its concentration in the 4 to 12% (wt/vol) range. As reported earlier, the pH of the solutions appeared critical and could not exceed 10 even at 4°C (83).

In general, fosfomycin exerts time-dependent bacterial killing effect with wide therapeutic range (35, 70). Tissue concentrations of fosfomycin were identical to plasma levels in healthy volunteers, and differed only moderately in critically ill patients and diabetes (84). The intermittent dosing regimen was used in the calculation. Therefore, we calculated the desired target concentration of fosfomycin which were used to provide T>MIC more than 50% of the dosing interval.

In general, the goal plasma concentration of the antimicrobials is recommended ranging from 1 to 5 times of its MIC (48). Although, in our study, we used the desired concentration within 1 time of their MIC it still provided a very high dose. These demonstrated that even the pharmacokinetic/ pharmacodynamic approach was used to optimize the surrogate markers; a huge dose of antimicrobials resulted from the calculation might not be appropriate. These might be the limitation of the pharmacokinetic/pharmacodynamic approaches in institute with the high level of resistant. However, the recommended dosage regimen might be useful in special population such as the critical ill patients who were infected with more resistant organism. In patients with highly resistant Gram-negative organisms, antimicrobial concentration achieved during infusions would never result in bactericidal or even

bacteriostatic activity. To avoid this potential clinical problem it would be prudent to establish the range of MICs for the Gram-negative organisms in individual institutions in order to calculate appropriate loading and infusion doses (85).

In conclusion, the data from our study demonstrated the role of this triple combination might be benefit to overcome the organism by using *in vitro* study. However, the calculated dosage regimen based on PK/PD data resulted in extremely huge dose of antimicrobials then the health care professionals might be considering the treatment option for individual patient. In addition, if the infected strain showed the MIC against amikacin, ceftazidime, and fosfomycin within ≤ 80 μg/mL, ≤ 50 μg/mL, and ≤ 200 μg/mL, respectively, this combination would be candidate for treatment of MDR-PA infections. An ongoing surveillance of microbial pathogens and their resistant profiles were important to guide future antimicrobial chemotherapy and the development of antimicrobial agents. Independent of the controversy that concerns the need for monotherapy versus combination therapy for *P. aeruginosa* infections, antimicrobial resistance of *P. aeruginosa* has reached a level in most regions of the world such that empirical therapy against this organism may require the initial use of 2 or more agents, until susceptibility testing results are known. In the era of increasing antimicrobial resistance, and with very few promising drugs on the horizon targeting Gram-negative bacilli, it is important to seek ways to reduce the rate of resistance development and effectively use existing antimicrobial currently on hospital formularies. Utilization of these triple combination therapies should be explored further given the limited choices in the therapy currently available.

Limitation of study

The study had some limitations as follows:-

1. This *in vitro* study was conducted in 34 isolates from infected patients who admitted at Chonburi Hospital. This number of isolates may not be a good representative for all MDR-PA isolates in this region. Then, results of the present study represented only the susceptibilities of populations at Chonburi Hospital.
2. Nowadays, there was not standardized E test method to determine the effects of triple antimicrobial combination. Then, concentrations of antimicrobials used in agar well method are the MIC_{combo} of amikacin and ceftazidime together with MIC_{mono} fosfomycin from E test method. It is not the exact concentration at MIC of amikacin and cefatzidime in the combination with fosfomycin (MIC_{triple}).
3. Because the population pharmacokinetic parameter estimates of ceftazidime and fosfomycin were used in the determination of optimal dosage regimen is not from Thai patients. In addition, parameters of amikacin included only patients in Smut songkram Province. Therefore, these parameters may not be a good representative of Thai patients.

CHAPTER VI
CONCLUSION

In this study, we aimed to determine the *in vitro* effects of triple combination; amikacin, ceftazidime, and fosfomycin on MDR-PA clinical isolates and to determine the optimal dosage regimen of this combination by using population pharmacokinetic approach. This study was performed at Department of Microbiology, Faculty of Pharmacy, Mahidol University during July 2005 to March 2006. The antimicrobial susceptibilities of 34 MDR-PA clinical isolates from Chonburi Hospital ranging from piperacillin/tazobactam, imipenem, meropenem, and fosfomycin, respectively. While amikacin, ceftazidime, and ciprofloxacin showed less active against such isolates. In addition, they showed high level of resistance with the MIC_{50} and MIC_{90} of amikacin, ceftazidime, and fosfomycin, were > 256 μg/mL, > 256 μg/mL, and > 1024 μg/mL, respectively. It seems to be inappropriate to treat the MDR-PA infections by monotherapy of the tested agents. The combination of amikacin and ceftazidme can decreased of the MIC of either amikacin or ceftazidime within 14 isolates (41.18%). According to FIC index there were 1 synergistic activity (2.94%) and 33 additivity activities (97.06%) with the mean of the FIC index 1.61±0.53. More importantly, none of them showed antagonistic activities.

As yet, the agar well diffusion method was used to determine the effect of the addition of fosfomycin into the combination of amikacin and ceftazidime by measuring the inhibition zone diameter. The p-value from Kruskal-Wallist H test demonstrated 11 isolates (32.35%) had significant difference of the mean inhibition zone. Then, Mann-Withney U was used to test the significant differences of mean inhibition zones between 7 antimicrobial regimens. The AC combination had the activities covered the number of MDR-PA isolates which was less than from ACF2, and ACF3 combinations. These showed the tendency of the addition of fosfomycin to the combination of amikacin and ceftazidime could increase the number of susceptible isolates. Focusing on the triple combination, the inhibition zone of ACF3 was larger

than ones of ACF2 and the inhibition zone of ACF2 was larger than ones of ACF1, respectively. For instance, the higher concentration of fosfomycin provided the trendsof greater activities of the triple combination. In addition, the number of the MDR-PA isolates which were covered by ACF1 equal to ones of AC and ACF1 showed smaller inhibition zone than AC. These suggested that to provide greater antimicrobial interaction, optimal concentration of fosfomycin in the triple combination with amikacin and ceftazidime should be equal to or more than its MIC.

In conclusion, the data from our study demonstrated the role of this triple combination might be benefit to overcome the organism by *in vitro* study. The calculated dosage regimen based on PK/PD data resulted in extremely huge dose of antimicrobials then the health care professionals might be considering the treatment option for individual patient. In addition, if the infected strain showed the MIC against amikacin, ceftazidime, and fosfomycin within ≤ 80 μg/mL, ≤ 50 μg/mL, and ≤ 200 μg/mL, respectively, this combination would be candidate for treatment of MDR-PA infections.

REFERENCES

1. Defez C, Fabbro-Perray P, Bouziges N, Gouby A, Mahamat A, Daure`s JP, et al. Risk factors for multidrug-resistant *Pseudomonas aeruginosa* nosocomial infection. Journal of Hospital Infection 2004;57:209-16.
2. Rossolini GM, Mantengoli E et al. Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. Clinical Microbiology Infection 2005;11(4):17-32.Clin Microbiol Infect
3. Piriykitphaiboon S. Antimicrobial drug resistance of *Pseudomonas aeruginosa* in Sappasitthiprasong Hospital. Medical Journal Ubon Ratchathani Hospipal. 1997;(3):173-9. Taken from: Thai index medicus - indexed for TJDEX (Abstract no.46567)
4. Naveen G, Santosh S, Aparna Y. Multidrug resistance and MIC of *Pseudomonas aeruginosa* in burn wound infections. Journal of Infectious Diseases and Antimicrobial Agents. 2003;20(3):139-143. Taken from: Thai index medicus - indexed for TJDEX (Abstract no.68809)
5. โรงพยาบาลชลบุรี. (2547). รายงานความไวของแบคทีเรียต่อยาต้านจุลชีพในโรงพยาบาลชลบุรีปี 2547. เอกสารไม่ตีพิมพ์.
6. Dubois V, Arpin C, Melon M, Melon B, Andre C, Frigo C, et al. Nosocomial outbreak due to a multiresistant strain of P12: Efficacy of cefepime-amikacin therapy and analysis of ß–lactam resistance. Journal of Clinical Microbiology 2001;39(6):2072-8.
7. Mirakhur A, Gallagher MJ, Ledson MJ, Hart CA, Walshaw MJ, et al. Fosfomycin therapy for multiresistant *Pseudomonas aeruginosa* in cyctic fibrosis. Journal of Cystic Fibrosis 2003; 2: 19-24.
8. Sobieszczyk ME , Furuya EY, Hay CM, Pancholi P, Della-Latta P, Hammer SM, et al. Combination therapy with polymyxin B for the treatment of multidrug-resistant gram-negative respiratory tract infections. Journal of Antimicrobial Chemotherapy 2004;54:566-9.

9. Kenneth Todar University of Wisconsin-Madison Department of Bacteriology. (2004). *Pseudomonas aeruginosa.* [Online] Available: http://textbookofbacteriology.net/pseudomonas.html. [2005, January 31]
10. Hancock REW, Speert DP. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. Drug Resistance Updates 2000;3:247-55.
11. Gales AC, Jones RN, Turnidge J, Rennie R, Ramphal R, et al. Characterization of *Pseudomonas aeruginosa* isolates: occurrence rates, antimicrobial susceptibility patterns, and molecular typing in the global SENTRY antimicrobial surveillance program, 1997–1999. Clinical Infectious Diseases 2001;32(suppl 2):S146-S155.
12. Kriengkauykiat J, Porter E, Lomovskaya O, Wong-Beringer A. Use of an efflux pump inhibitor to determine the prevalence of efflux pump–mediated fluoroquinolone resistance and multidrug resistant in *Pseudomonas aeruginosa*. Antimicrobial Agents and chemotherapy 2005;49:565-70.
13. Micek ST, Lloyd AE, Ritchie DJ, Reichley RM, Fraser VJ, Kollef MH. *Pseudomonas aeruginosa* bloodstream infection: importance of appropriate initial antimicrobial treatment. Antimicrobial Agents and chemotherapy 2005;49:1306-11.
14. Wang CY, Jerngl JS, Cheng KY, Lee LN, Yu CJ, Hsueh PR, et al. Pandrug-resistant *Pseudomonas aeruginosa* among hospitalised patients:clinical features, risk-factors and outcomes. Clinical Microbiology and Infection 2001;12:63-8.
15. Lang BJ, Aaron SD, Ferris W, Macdonald NE. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with multiresistant strains of *Pseudomonas aeruginosa*. American Journal Respiratory Critical Care Medicine 2000;162:2241-5.
16. Jung R, Fish DN. Surveillance of multi-drug resistant *Pseudomonas aeruginosa* in urban tertiary-care teaching hospital. Journal of Hospital Infection 2004;57:105-11.

17. Pakhomova S, Rife CL, Armstrong RN, Newcomer ME. Structure of fosfomycin resistance protein FosA from transposon Tn2921. Protein Science 2004;13:1260–1265.
18. Livermore DM. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa* :our worst nightmare?. Clinical Infectious Diseases 2002;37:26-32.
19. Livermore DM. Of Pseudomonas, porins, pumps, and carbapenams. Journal of Antimicrobial Chemotherapy 2001;47:247-50.
20. Beharry Z and Palzkill T. Functional analysis of active site residues of the fosfomycin resistance enzyme FosA from *Pseudomonas aeruginosa*. The Journal of Biological Chemistry 2005;280:17786-91.
21. Paramythiotou E, Lucet JC, Timsit JF, Vanjak D, Paugam-Burtz C, Trouillet J-L, et al. Acquisition of multidrug-resistant *Pseudomonas aeruginosa* in patients in intensive care units:role of antibiotics with antipseudomonal activity. Clinical Infectious Diseases 2004;38:670-7.
22. Cao B, Wang H, Sun H, Zhu Y, Chen M. Risk factors and clinical outcomes of nosocomial multi-drug resistant *Pseudomonas aeruginosa* infections. Journal of Hospital Infection 2004; 57:112–8.
23. Tsuji A, Kobayashi I, Oguri T, Inoue M, Yabuuchi E, Goto S. An epidemiological study of the susceptibility and frequency of multiple-drug-resistant strains of *Pseudomonas aeruginosa* isolated at medical institutes nationwide in Japan. Journal of Infection and Chemotherapy 2005; 11: 64-70.
24. Pankey GA. Tigecycline. Journal of Antimicrobial Chemotherapy 2005;56:470–480.
25. Harris A, Torres-Viera C, Venkataraman L, DeGirolami P, Samore M, Carmeli Y. Epidemiology and clinical outcomes of patients with multiresistant *Pseudomonas aeruginosa*. Clinical Infectious Diseases 1999;28:1128–33.
26. Eliopoulos GM and Moellering RC. Antimicrobial combinations. In: Lorian V (ed). Antibiotics in laboratory medicine. Baltimore: Williams & Wilkins, 1996:330-96.

27. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different *in vitro* methods of detecting synergy; time-kill, checkerboard, and Etest. Antimicrobial Agents and Chemotherapy 1996;40:1914-18.

28. Bonapace CR, White RL, Friedrich LV, Bosso JA. Evaluation of antibiotic synergy against *Acinetobacter baumannii:* a comparison with Etest, time-kill, and checkerboard methods. Diagnostic Microbiology and Infectious. Disease 2000;38:43-50.

29. Okazaki M, Susuki K, Asano N, Araki K, Shukuya N, Egami T, et al. Effectiveness of fosfomycin combined with other antimicrobial agents against multidrug-resistant *Pseudomonas aeruginosa* isolates using the efficacy time index assay. Journal of Infection and Chemotherapy 2002; 8: 37-42.

30. Watine J, Bourrel C, Dubourdieu B, Gineston JL, Bories P, Durand M, et al. Susceptibility of mutirsistant serotype O12 *Pseudomonas aeruginosa* to fosfomycin in combination with other antibiotics. Pathologie Biologie. 1994;42(4):293-5. Taken from: PubMed - indexed for MEDLINE (Abstract no. 7808781).

31. Alvarez-Lerma F, Insausti-Ordenana J, Jorda-Marcos R, Maravi-Poma E, Torres-Marti A, Nava J, et al. Efficacy and tolerability of piperacillin/tazobactam versus ceftazidime in association with amikacin for treating nosocomial pneumonia in intensive care patients: a prospective randomized multicenter trial. Intensive Care Medicine 2001;27:493-502.

32. Fish DN, Choi MK, Jung R. Synergic activity of cephalosporins plus fluoroquinolones against *Pseudomonas aeruginosa* with resistance to one or both drugs. Journal of Antimicrobial Chemotherapy 2002;50:1045-9.

33. Erdem I, Kaynar-Tascioglu J, Kaya B, Goktas P. The comparison of *in vitro* effect of imipenem or meropenem combined with ciprofloxacin or levofloxacin against multidrug-resistant *Pseudomonas aeruginosa* strains. International Journal of Antimicrobial Agents 2000;20:384-6.

34. Oie S, Uematsu T, Sawa A, Mizuno H, Tomita M, Ishida S, et al. *In vitro* effects of combination of antipseudomonal agents against seven strains of mutidrug-resistant *Pseudomonas aeruginosa*. Journal of Antimicrobial Chemotherapy 2003; 52:911-4.
35. Grif K, Dierich MP, Pfaller K, Miglioli PA, Allerberger F. *In vitro* activity of fosfomycin in combination with various antistaphylococcal substances. Journal of Antimicrobial Chemotherapy 2001; 48: 209-17.
36. Nakazawa H, Kikuchi Y, Honda T, Isago T, Nozaki M. Enhancement of antimicrobial effects of various antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) by combination with fosfomycin Journal of Infection and Chemotherapy 2003; 9: 304-9.
37. Yoshiyama Y, Yazaki T, Wong PC, Beauchamp D, Kanke M. The effect of fosfomycin on glycopeptide antibiotic-induced nephrotoxicity in rat. Journal of Infection and Chemotherapy 2001;7:243-6.
38. Zeitlinger MA, Marsik C, Georgopoulos A, Muler L, Heinz G, Joukhadar C. target site bacterial killing of cefpirome and fosfomycin in critically ill patients. International Journal of Antimicrobial agents 2001; 21: 562-67.
39. Tessier F and Quentin C. *In vitro* activity of fosfomycin combined with ceftazidime, imipenem, amikacin, and ciprofloxacin against *Pseudomonas aeruginosa*. European Journal of Microbiological Infectious Disease 1997;16:159-62.
40. Chin NX, Neu NM, Neu HC. Synergy of fosfomycin with beta-lactam antibiotics against Staphylococci and aerobic Gram-negative bacilli. Drug Experimental Clinical Research 1986;12:943-7.
41. Sterner G, Walder M, Ode B, Reduced nephrotoxicity of gentamicin in uremic rats using a combination with fosfomycin. Proceeding of the 14th Intenational Congress of Chemotherapy. Kyoto,1985:2636-7.
42. Tanaka K, Umezu Y, Sayama S, Komutsu T, Satoh R, Hayashi H. Protective effects of fosfomycin on cisplatin-induced renal toxicity in the mouse. Ibid:2626-7.

43. Takahashi K, Kanno H. Synergistic activities of combinations of β-lactams, fosfomycin, and tobramycin against *Pseudomonas aeruginosa.* Antimicrobial Agents and Chemotherapy 1984;26:789-91.
44. Oie S, Sawa A, Kamiya A, Mizuno H. In-vitro effects of a combination of antipseudomonal agents against multi-drug resistant *Pseudomonas aeruginisa*. Journal of Antimicrobial Chemotherapy 1999; 44:689-91.
45. Toutain PL, Del Castillo JRE, Bousquet-Melou A. The pharmacokinetic-pharmacodynamic approach to a rational dosage regimen for antibiotics. Research in Veterinary Science 2002;73:105-14.
46. Jacobs MR. Optimisation of antimicrobial therapy using pharmacokinetic and pharmacodynamic parameters. Clinical Microbiology and Infection 2001;7:589-96.
47. Barger A, Fuhst C, Wiedemann B. Pharmacological indices in antibiotic therapy. Journal of Antimicrobial Chemotherapy 2003; 52: 893-9.
48. Hyatt JM, McKinnon PS, Zimmer GS, Schentag JJ. Importance of pharmacokinetic/ pharmacodynamic surrogate marker to outcome; focus on antibacterial agents. Clinical Pharmacokinetic 1995;28:143-60.
49. DeRyke CA, Lee SY, Kuti JL, Nicolau DP. Optimizing dosing strategies of antibacterials utilizing pharmacodynamic principles; impact on the development of resistance. Drugs 2006;66:1-14.
50. Frimodt-Moller N. How predictive is PK/PD for antibacterial agents?. International Journal of Antimicrobial agents. 2002;19:333-9.
51. Slavik RS, Jewesson PJ. Selecting Antibacterials for Outpatient Parenteral Antimicrobial Therapy Pharmacokinetic-Pharmacodynamic Considerations. Clinical Pharmacokinetic. 2003; 42: 793-817.
52. Preston SL. The importance of appropriate antimicrobial dosing: pharmacokinetic and pharmacodynamic consideration. The annals of pharmacotherapy 2004;38:S14-S18.
53. Mouton JW, Vinks AATMM. Is continuous infusion of β-lactam antibiotics worthwhile?—efficacy and pharmacokinetic considerations. Journal of Antimicrobial Chemotherapy. 1996;38:5-15.

54. Mouton JW, Vinks AATMM, Punt NC. Pharmacokinetic-pharmacodynamic modeling of activity of ceftazidime during continuous and intermittent infusion. Antimicrobial Agents and Chemotherapy 1997;41:733-38.

55. Struelens MJ. The problem of resistance, In: Finch RG, Greenwood D, Rangnar Norrby S, Whitley RJ, editors. Antibiotic and chemotherapy. 8th ed. London: MPG Books; 2003. p. 25-47.

56. Chamot E, El Amari EB, Rohner P, Delden CV. Effectveness of combination antimicrobial therapy for *Pseudomonas aeruginosa* bacterimia. Antimicrobial Agents and Chemotherapy 2003;47:2756-64.

57. AB BIODISK. International product list [Online] Available: http://www.abbiodisk.com/pdf/pricelist/M0000013. pdf. [2005, July 16]

58. Wilson B, Abraham G, Manju VS, et al. Antimicrobial activity of Curcuma zedoaria and Curcuma malabarica tubers. Journal of Ethnopharmacology 2005;99:147-51. Mathew M, Vimala B, Sundaresan S,

59. Ervin FR, Bullock,JR WE. Simple assay for clindamycin in the presence of aminoglycoside. Antimicrobial Agents and Chemotherapy. 1974;6:831-5.

60. Zaidi MA, Crow Jr SA. Biologically active traditional medicinal herbs from Balochistan, Pakistan. Journal of Ethnopharmacology. 2005;96:331-34.

61. Jimenez-Diaz R, Ruiz-Barba J, Cathcart DP, Holo H, Nes IF, Sletten KH, et al. Purification and partial amino acid sequence of Plantaricin S, a bacteriocin produced by Lactobacillus plantarum LPCO10, the activity of which depends on the complementary action of two peptides. Applied and Environmental Microbiology 1995;61;4459-63.

62. DiPiro JT, Spruill WJ, Blouin RA, et al. Relationships of pharmacokinetic parameters and intravenous infusion. Concepts in clinical pharmacokinetics. American Society of Health-System Pharmacist 2002:63-77.

63. Arayachaichan A. Population pharmacokinetics of amikacin in Thai patients [M.S. Thesis in Pharmacy]. Bangkok: Faculty of Graduate Studies, Mahidol University; 2003.

64. Benko A, Cappelletty DM, Kruse JA, Rybak MJ. Continuous infusion versus intermittent administration of ceftazidime in critically ill patients with suspected Gram-negative infections. Antimicrobial Agents and Chemotherapy 1996;40:691-5.

65. Pfausler B, Spiss H, Dittrich P, Zeitlinger M, Schmutzhar E, Joukhadar C. Concentrations of fosfomycin in the cerebrospinal fluid of neurointensive care patients with ventriculostomy-associated ventriculitis. Journal of Antimicrobial Chemotherapy 2004; 53: 848–52.

66. Gahart BL and Nazareno AR. Intravenous medication. Mosby, 2005:69-72, 258-61.

67. Thomson MICROMEDEX. Fosfomycin [CD-ROM]. MICROMEDEX(R) Healthcare Series. Thomson Healthcare 2005.

68. McGrath BJ, Lamp KC, Rybak MJ. Pharmacodynamic effects of extended dosing intervals of imipenem alone and in combination with amikacin against *Pseudomonas aeruginosa* in an *In Vitro* Model. Antimicrobial Agents and Chemotherapy 1993;37:1931-7.

69. Staneva M, Markova B, Atanasova I, Terzivanov D. Pharmacokinetic and pharmacodynamic approach for comparing two therapeutic regimens using amikacin. Antimicrobial Agents and Chemotherapy 1994;38:981-5.

70. Sauermann R, Karch R, Langenberger H, Kettenbach J, Mayer-Helm B, Petsch M, et al. Antibiotic abscess penetration: fosfomycin levels measured in pus and simulated concentration-time profiles. Antimicrobial Agents and Chemotherapy 2005;49:4448-54.

71. Sader HS, Jones RN. Comprehensive *in vitro* evaluation of cefepime combined with aztreonam or ampicillin/sulbactam against multi-drug resistant *Pseudomonas aeruginosa* and *Acinetobacter spp*. International Journal of Antimicrobial agents 2005;25:380-4.

72. Song W, Woo HJ, Kim JS, Lee KM. *In vitro* activity of β-lactams in combination with other antimicrobial agents against resistant strains of *Pseudomonas aeruginosa*. International Journal of Antimicrobial Agents 2003;21:8-12.

73. Jittaropas Y, Rikitomi N, and Matsumoto K. Comnination effects of norfloxacin, gentamycin, and betalactams on betalactamase resistant *Pseudomonas aeruginosa*. Bulletin of the Department of Medical Services 1987;12:379-83.

74. National Committee for Clinical Laboratory Standards. Performance standard for antimicrobial susceptibility testing; Twelfth Information Supplement. NCCLS Document M100-S12, 2002.

75. Karahoc B, Gerceker AA. *In vitro* activities of various antibiotics, alone and in combination with amikacin against *Pseudomonas aeruginosa*. International Journal of Antimicrobial agents 2001;18:567-70.

76. Kohler T, Michea-Hamzehpour M, Epp SF et al. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. Antimicrobial Agents and Chemotherapy 1999;42:424-7.

77. Giamarellos-Bourboulis EJ, Grecka P, Giamarellou H. Comparative *in vitro* interactions of ceftazidime, meropenam, and imipenam with amikacin on mutiresistant *Pseudomonas aeruginosa*. Diagnostic Microbiological Infectious Diseases 1997;29:81-6.

78. Maslow MJ, Simberkoff MS, Rahal JJ. Clinical efficacy of a synergistic combination of cefotaxime and amikacin against multiresistant and Serratia infections. Journal of Antimicrobial Chemotherapy 1985;16:227-34.

79. Balke B, Hogardt M, Schmoldt S, Hay L, Weissbrodt H, Hiiussler S. Evaluation of the Etest for the assessment of synergy of antibiotic combinations against multi-resistant *Pseudomonas aeruginosa* isolates from Cystic Fibrosis patients Journal of Cystic Fibrosis. 2005; 4: S34-S58.

80. Burkhardt O, Lehmann C, Madabushi R, Kumar V, Derendorf H, Welte T. Once-daily tobramycin in cystic fibrosis: better for clinical outcome than thrice-daily tobramycin but more resistance development?. Journal of Antimicrobial Chemotherapy 2006;58(4):822-9.

81. Giamarellos-Bourboulis EJ, Kentepozidis N, Antonopoulou A, Plachouras D, Tsaganos T, Giamarellou H. Postantibiotic effect of antimicrobial

combinations on multidrug-resistant *Pseudomonas aeruginosa*. Diagnostic Microbiological Infectious Diseases 2005;51:113-7.

82. Robaux MA, Dube L, Caillon J, Bugnon D, Kergueris MF, Navas D, et al. *In vivo* efficacy of continuous infusion versus intermittent dosing of ceftazidime alone or in combination with amikacin relative to human kinetic profiles in a rabbit endocarditis model. Journal of Antimicrobial Chemotherapy 2001; 47:617-22.
83. Servais H, Tulkens PM. Stability and compatibility of ceftazidime administered by continuous infusion to intensive care patients Antimicrobial Agents and Chemotherapy 2001;45:2643-7.
84. Gattringer R, Meyer B, Heinz G Guttmann C, Zeitlinger M, Joukhadar C. Single-dose pharmacokinetics of fosfomycin during continuous venovenous haemofiltration. Journal of Antimicrobial Chemotherapy 2006;58:367-71.
85. Lipman J, Gomersall CD, Gin T, Joynt GM, Young RJ. Continuous infusion ceftazidime in intensive care: a randomized controlled trial. Journal of Antimicrobial Chemotherapy 1999; 43:309-11.

APPENDIX

APPENDIX A

Reading Sharp and Hazy MIC end points

Background

Depending on their mode of action and levels achieved at various infection sites, antibiotics may have a bacteriostatic or bactericidal action or a combination of both. However, antibiotics are usually classified as bacteriostatic or bactericidal agents based on their mode of action.

Bacteriostatic drugs inhibit the growth and replication of microorganisms. The final eradication of the pathogen is accomplished by the immune system of the host.

Bactericidal drugs actively kill and thus effectively eradicate the pathogenic organisms.

MIC endpoint criteria

The inhibition of growth is measured by MIC determinations while killing by MBC testing. However, the static or cidal mode of action of drugs also affects the outlook of MIC end points. The following guidelines can be used to select the MIC end point.

1. Bactericidal drugs: End points are usually sharp and the MIC should be read at the point of complete inhibition of growth including microcolonies, hazes and isolated colonies.
2. Bacteriostatic drugs may give trailing end points such as hazy zone edges and microcolonies across 1-3 dilutions. The MIC should be read at the so-called 80% to 90% inhibition i.e. the first point of significant inhibition as judged by the naked eye.

   Etest Reading Guide, technical guides, CIS 007 enclosures 1, 2, 3 illustrate different types of results and end point selection criteria.

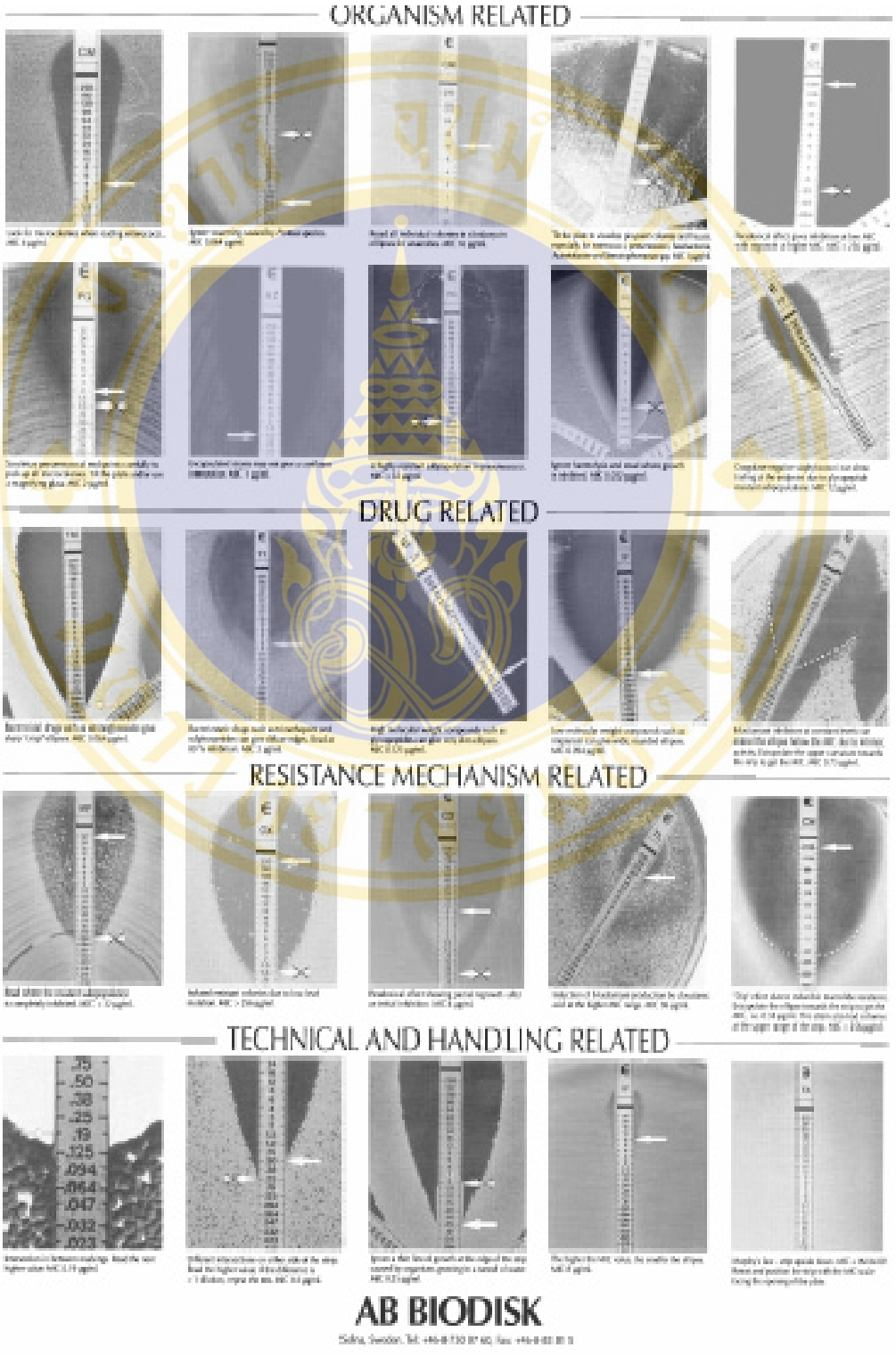
Table 1. Overview of antibiotic classification as bactericidal and bacteriostatic agents

BACTERICIDAL	BACTERICIDAL	BACTERIOSTATIC
Interference at cell wall/ cell membrane level	**Interference at DNA/ RNA level**	**Interference with protein synthesis**
Penicillins	Quinolones	Lincosamides(clindamycin)
Cephalosporins	Metronidazole	Tetracyclines
ß -lactamase inhibitor	Rifamycins (rifampin)	Streptogramins(quinupristin/
(Clavulanic acid)	Flucytosine	dalfopristin)
Monobactams		Oxazolidinones (linezolid)
(aztreonam)		Chloramphenicol
Carbapenems (imipenem)	**Interference with**	Macrolides
Glycopeptides	**protein synthesis**	Azalides (azithromycin)
(vancomycin)	Aminoglycosides	Fusidic acid
Fosfomycin		
Colistin, polymyxin		**Interference at cell membrane**
Polyene (amphotericin B)		**Level**
Isoniazid		Azole
Ethionamide		
Ethambutol		**Interference with folic acid**
		Synthesis
		Sulfonamides
		Trimethoprim

Important observation: For clinical laboratories in the USA, information in this document does not in any way imply indications outside those clinically cleared.

APPENDIX B

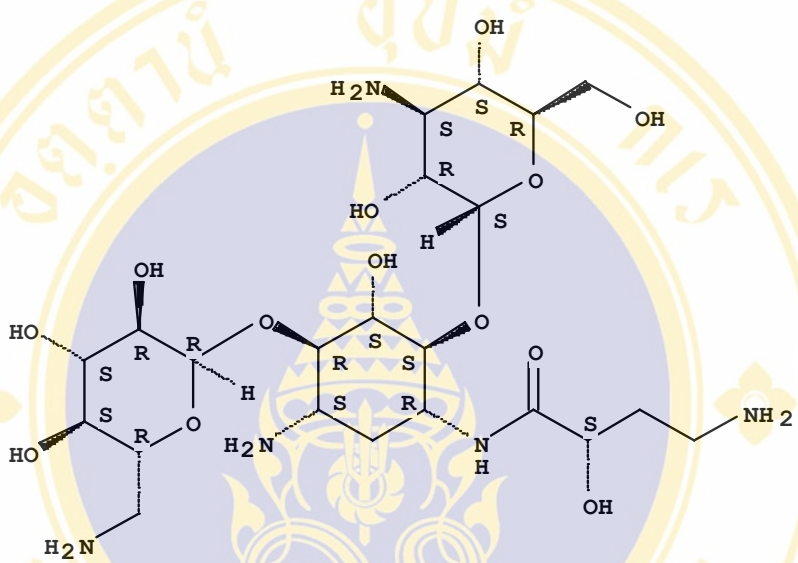
E test reading guide



APPENDIX C

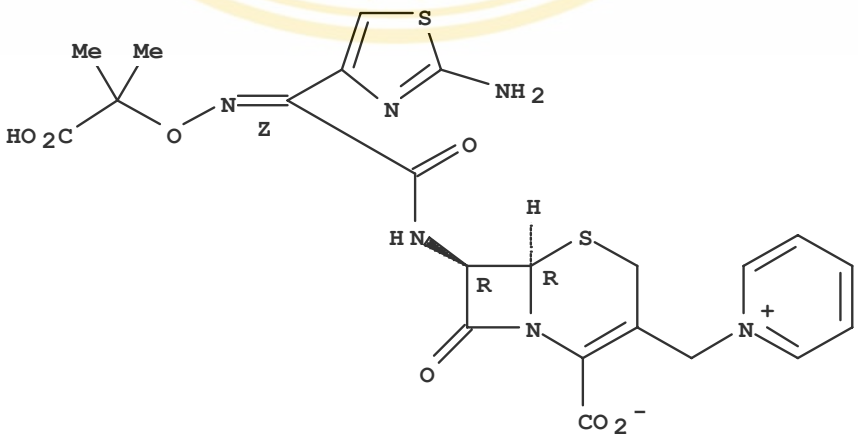
Antimicrobials structure

1. Amikacin



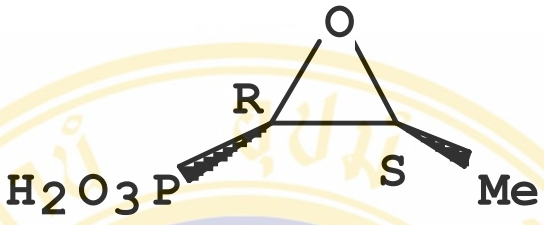
Formula: C22 H43 N5 O13

2. Ceftazidime



Formula: C22 H22 N6 O7 S2

3. Fosfomycin



Formula: C3 H7 O4 P

APPENDIX D

MIC distribution

Table 2. MIC_{mono} distribution among 34 MDR-PA clinical isolates

Order	Amikacin		Ceftazidime		Fosfomycin	
	MIC[1]	Isolate no	MIC[1]	Isolate no	MIC[1]	Isolate no
1	1.75	15	24	22	24	12
2	3.5	28	28	28	28	21
3	12	14	48	11	32	20
4	48	33	48	15	48	5
5	56	34	96	29	48	15
6	64	38	>256	1	48	29
7	80	35	>256	2	80	30
8	96	31	>256	4	96	13
9	96	41	>256	5	128	26
10	112	5	>256	6	160	28
11	112	32	>256	7	192	10
12	128	12	>256	8	384	2
13	128	36	>256	10	384	23
14	128	37	>256	12	>1024	1
15	160	20	>256	13	>1024	4
16	160	21	>256	14	>1024	6
17	>256	1	>256	17	>1024	7
18	>256	2	>256	18	>1024	8
19	>256	4	>256	20	>1024	11
20	>256	6	>256	21	>1024	14
21	>256	7	>256	23	>1024	17
22	>256	8	>256	26	>1024	18
23	>256	10	>256	30	>1024	22
24	>256	11	>256	31	>1024	31
25	>256	13	>256	32	>1024	32
26	>256	17	>256	33	>1024	33
27	>256	18	>256	34	>1024	34
28	>256	22	>256	35	>1024	35
29	>256	23	>256	36	>1024	36
30	>256	26	>256	37	>1024	37
31	>256	29	>256	38	>1024	38
32	>256	30	>256	39	>1024	39
33	>256	39	>256	40	>1024	40
34	>256	40	>256	41	>1024	41

[1] (μg/mL)

Table 3. MIC_{combo} distribution among 34 MDR-PA clinical isolates

Order	Amikacin		Ceftazidime		Fosfomycin	
	MIC[1]	Isolate no	MIC[1]	Isolate no	MIC[1, 2]	Isolate no
1	0.75	15	6	28	24	12
2	0.75	28	8	22	28	21
3	4	14	12	15	32	20
4	48	33	24	11	48	5
5	48	34	32	29	48	15
6	64	37	96	14	48	29
7	64	38	128	12	80	30
8	96	5	128	20	96	13
9	96	11	192	5	128	26
10	96	12	192	21	160	28
11	96	20	192	32	192	10
12	96	21	192	37	384	2
13	96	22	192	39	384	23
14	96	29	>256	1	>1024	1
15	96	31	>256	2	>1024	4
16	96	32	>256	4	>1024	6
17	96	35	>256	6	>1024	7
18	96	41	>256	7	>1024	8
19	128	36	>256	8	>1024	11
20	128	39	>256	10	>1024	14
21	>256	1	>256	13	>1024	17
22	>256	2	>256	17	>1024	18
23	>256	4	>256	18	>1024	22
24	>256	6	>256	23	>1024	31
25	>256	7	>256	26	>1024	32
26	>256	8	>256	30	>1024	33
27	>256	10	>256	31	>1024	34
28	>256	13	>256	33	>1024	35
29	>256	17	>256	34	>1024	36
30	>256	18	>256	35	>1024	37
31	>256	23	>256	36	>1024	38
32	>256	26	>256	38	>1024	39
33	>256	30	>256	40	>1024	40
34	>256	40	>256	41	>1024	41

[1] (μg/mL), [2] MIC_{mono}

APPENDIX E

Standard solution

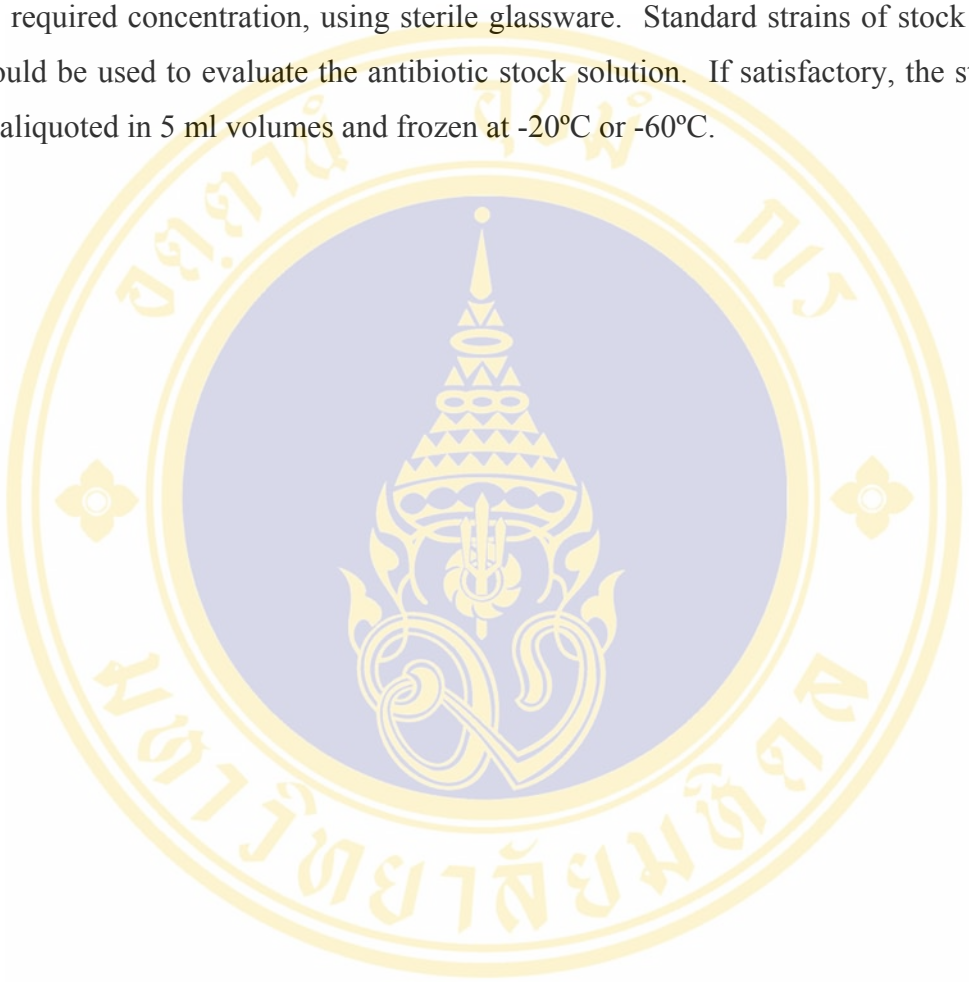
Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. A $BaSO_4$ 0.5 McFarland standard may be prepared as follows:

1. A 0.5-ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 . 2H_2O$) is added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.
2. The correct density of the turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 McFarland standard.
3. The Barium Sulfate suspension should be transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum.
4. These tubes should be tightly sealed and stored in the dark at room temperature.
5. The barium sulfate turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appear, the standard should be replaced. Latex particle suspensions should be mixed by inverting gently, not on a vortex mixer
6. The barium sulfate standards should be replaced or their densities verified monthly.

Preparation of antimicrobials stock solutions

Antimicrobials may be received as powders or tablets. It is recommended to obtain pure antibiotics from commercial sources, and not use injectable solutions. Powders must be accurately weighed and dissolved in the appropriate diluents to yield the required concentration, using sterile glassware. Standard strains of stock cultures should be used to evaluate the antibiotic stock solution. If satisfactory, the stock can be aliquoted in 5 ml volumes and frozen at -20ºC or -60ºC.



BIOGRAPHY

NAME	Mr. Manat Sitthichai
DATE OF BIRTH	5 August 1975
PLACE OF BIRTH	Phetchaburi, Thailand
INSTITUTE ATTENDED	Silpakorn University, 1994-1998: Bachelor of Science (Pharmacy) Mahidol University, 2004-2006: Master of Science in Pharmacy (Clinical Pharmacy)
POSITION & OFFICE	1998-2000, Department of pharmacy Chaophraya Abhaibhubejhr Hospital Prachin Buri, Thailand 2000-present, Department of pharmacy Samut Sakhon Hospital Samut Sakhon, Thailand Position: Pharmacist