กลไกการดื้อยาหลายชนิดพร้อมกันของ *Pseudomonas aeruginosa*
ที่แยกได้จากสัตว์เลี้ยงและผู้ป่วย

นางสาวกานต์ชนา พูนสุข

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ ที่ส่งผ่านทางบัณฑิตวิทยาลัย
The abstract and full text of theses from the academic year 2011 in Chulalongkorn University Intellectual Repository (CUIR)
are the thesis authors' files submitted through the University Graduate School.

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

MECHANISMS OF MULTIDRUG RESISTANCE IN *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES FROM COMPANION ANIMAL AND HUMAN PATIENTS

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Miss Kanchana Poonsuk

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Veterinary Public Health

Department of Veterinary Public Health
Faculty of Veterinary Science
Chulalongkorn University

Academic Year 2014

Copyright of Chulalongkorn University

Thesis Title	MECHANISMS OF MULTIDRUG RESISTANCE IN *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES FROM COMPANION ANIMAL AND HUMAN PATIENTS
By	Miss Kanchana Poonsuk
Field of Study	Veterinary Public Health
Thesis Advisor	Associate Professor Rungtip Chuanchuen, D.V.M., M.Sc., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

_______________________________________ Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M., Ph.D.)

THESIS COMMITTEE

_______________________________________ Chairman

(Associate Professor Alongkorn Amonsin, D.V.M., Ph.D.)

_______________________________________ Thesis Advisor

(Associate Professor Rungtip Chuanchuen, D.V.M., M.Sc., Ph.D.)

_______________________________________ Examiner

(Associate Professor Suphachai Nuanualsuwan, D.V.M., M.P.V.M., Ph.D.)

_______________________________________ Examiner

(Taradon Luangtongkum, D.V.M., Ph.D.)

_______________________________________ External Examiner

(Associate Professor Chanwit Tribuddharat, M.D., Ph.D.)

กานต์ชนา พูนสุข : กลไกการดื้อยาหลายชนิดพร้อมกันของ *Pseudomonas aeruginosa* ที่แยกได้จากสัตว์เลี้ยงและผู้ป่วย (MECHANISMS OF MULTIDRUG RESISTANCE IN *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES FROM COMPANION ANIMAL AND HUMAN PATIENTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร. รุ่งทิพย์ ชวนชื่น, 147 หน้า.

เชื้อ *Pseudomonas aeruginosa* เป็นเชื้อที่มีความสามารถในการดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกันทั้งแบบ intrinsic resistance และแบบ acquire resistance ซึ่งในปัจจุบันการศึกษากลไกการดื้อยาหลายชนิดพร้อมกัน (multidrug resistance; MDR) ของเชื้อดังกล่าวครอบคลุมเพียงแค่เชื้อสายพันธุ์ที่ใช้ในห้องปฏิบัติการและไม่มีข้อมูลของเชื้อที่ก่อให้เกิดปัญหาในทางคลินิกมากนัก การศึกษานี้จึงมีจุดประสงค์เพื่อทำความเข้าใจกลไกการดื้อยาในระดับโมเลกุลที่จำเป็นในการดื้อต่อยาหลายชนิดพร้อมกันในเชื้อที่ก่อให้เกิดปัญหาในทางคลินิก โดยศึกษากลไกหลักในการดื้อยาปฏิชีวนะหลายชนิดพร้อมกันสองกลไกสำคัญ ได้แก่ integrons และ multidrug efflux system (Mex) ที่อยู่ในกลุ่ม Resistance-Nodulation-Cell Division (RND) ในเชื้อที่แยกได้จากมนุษย์และสัตว์ป่วยจำนวน 114 สายพันธุ์ ผลการศึกษาสำคัญหนึ่งที่พบคือ พบการปรากฏของ class 1 integrons ที่มียีนดื้อยาในระดับสูงถึงร้อยละ 69.3 ในเชื้อที่แยกได้จากผู้ป่วยที่เข้ารับการรักษาในโรงพยาบาลทั้งหมด 101 ตัวอย่าง โดยพบ gene cassette ที่ไม่เคยมีรายงานมาก่อนจำนวน 5 ลักษณะ ได้แก่ *aacA7-cmlA, aadB-bla*$_{OXA-10}$*-aadA1, aadB-arr-2-cmlA- bla*$_{OXA-10}$*-aadA1, aadB-cmlA-aadA1* และ *aadB-cmlA- bla*$_{OXA-10}$*-aadA15* คิดเป็นร้อยละ 50.5 ของเชื้อที่ใช้ในการศึกษา ปัจจุบันมีรายงานระบบ Mex ที่เกี่ยวข้องกับการขับออกของยาปฏิชีวนะทั้งสิ้น 6 ระบบ จากยีนในกลุ่ม RND ทั้งหมด 12 operon ได้แก่ MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK, MexVW และ MexXY ซึ่งระบบทั้งหมดมีบทบาทสำคัญในการทำให้เกิดการดื้อข้ามระหว่างยาปฏิชีวนะแต่ละชนิด ผลการศึกษาเด่นที่พบจากการศึกษาเชื้อจากมนุษย์และสัตว์จำนวน 43 สายพันธุ์ คือพบการแสดงออกของระบบ Mex อย่างน้อย 3 ระบบในเชื้อสายพันธุ์เดียวกัน แสดงให้เห็นว่าการดื้อยาหลายชนิดพร้อมกันในเชื้อที่ก่อให้เกิดปัญหาทางคลินิกเป็นผลร่วมกันของระบบ Mex หลายระบบ และพบการแสดงออกของระบบ MexCD-OprJ, MexEF-OprN, MexJK และ MexVW ซึ่งระบบดังกล่าวไม่พบการแสดงออกในสภาวะปกติ ทั้งนี้การศึกษานี้ได้พัฒนาวิธี resistance phenotypic marker ร่วมกับ multiplex RT-PCR เพื่อตรวจการแสดงออกของระบบ Mex 4 ชนิดที่มีความสำคัญทางคลินิกด้วย ผลการศึกษาทั้งหมดแสดงให้เห็นถึงความซับซ้อนของการแสดงออกของระบบ Mex และการควบคุมกลไกดังกล่าวในเชื้อ *P. aeruginosa* ที่ก่อให้เกิดปัญหาในทางคลินิก ทั้งยังแสดงให้เห็นว่าการดื้อยาหลายชนิดพร้อมกันเป็นผลมาจากการทำงานร่วมกันของกลไกในหลายระบบและควรพิจารณาถึงผลจากระบบ Mex ที่ไม่พบการแสดงออกในสภาวะปกติ การศึกษาสนับสนุนให้เห็นว่าการใช้ยาปฏิชีวนะอย่างสมเหตุสมผล การเฝ้าระวังการดื้อยาปฏิชีวนะและการให้ความรู้แก่ประชาชน มีความจำเป็นในการกำหนดแผนนโยบายเพื่อควบคุมและป้องกันการดื้อยาปฏิชีวนะ

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต ....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

ปีการศึกษา 2557

5275965631 : MAJOR VETERINARY PUBLIC HEALTH
KEYWORDS: ANIMAL / MECHANISMS OF MULTIDRUG RESISTANCE / PATIENT

KANCHANA POONSUK: MECHANISMS OF MULTIDRUG RESISTANCE IN *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES FROM COMPANION ANIMAL AND HUMAN PATIENTS. ADVISOR: ASSOC. PROF. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D., 147 pp.

Pseudomonas aeruginosa has been notoriously known for its intrinsic and acquired resistance to multiple antimicrobial agents simultaneously. So far, mechanisms underlining multidrug resistance (MDR) in P. aeruginosa have been extensively studied but mostly, in laboratory stains. Such information is still limited in the clinical isolates. Therefore, the goal of this dissertation was to understand genetic mechanisms responsible for MDR phenotype in P. aeruginosa clinical isolates. We examined two major MDR mechanisms, including integrons and multidrug efflux systems (Mex) of the Resistance-Nodulation-Cell Division (RND) family, in the P. aeruginosa clinical isolates from animals and humans (n=114). One of the major findings was the high prevalence of class 1 integrons containing resistance genes cassette (69.3%) in the P. aeruginosa human isolates admitted to a hospital (n=101). Five novel resistance gene cassettes including aacA7-cmlA, aadB-bla_{OXA-10}-aadA1, aadB-arr-2-cmlA- bla_{OXA-10}-aadA1, aadB-cmlA-aadA1 and aadB-cmlA- bla_{OXA-10}-aadA15 were identified (50.5%). Up to date, there are up to 12 RND efflux operons found on P. aeruginosa chromosome, of which six Mex systems (i.e. MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK, MexVW and MexXY) have been shown to extrude antimicrobial substrates and play a role in cross-resistance among antimicrobials. The predominant finding was the simultaneous expression of at least 3 Mex systems in a single P. aeruginosa isolate from either humans or animals (n=43), suggesting that expression of several Mex systems concomitantly contributes to multiple drug resistance in the clinical isolates. Role of normally silent Mex systems including MexCD-OprJ, MexEF-OprN, MexJK and MexVW were highlighted. Method for simultaneous detection of four-clinically important Mex systems were developed using combination of resistance-phenotypic markers and multiplex RT-PCR. In conclusion, the results demonstrated an intriguing and complex picture of expression and regulation of the Mex systems in the P. aeruginosa clinical isolates. The observations demonstrated that multiple pathways existing to participate in MDR phenotypes and contribution of normally silent Mex systems should not be underestimated. In addition, control/prevention strategies for antimicrobial resistance need to be encouraged e.g. responsible therapeutic use of antimicrobials, routine antimicrobial resistance monitoring and public education.

Department:	Veterinary Public Health	Student's Signature ....
Field of Study:	Veterinary Public Health	Advisor's Signature ....
Academic Year:	2014	

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Assoc.Prof.Dr.Rungtip Chuanchuan for all of her support, kindly guidance, kindly and encouragement. I truly appreciate her patience and help on everything during my study. She is not only my Ph.D. advisor but also my life advisor.

I would like to express my thanks to my dissertation committee member, Assoc.Prof.Dr. Alongkorn Amonsin, Assoc.Prof.Dr. Suphachai Nuanualsuwan, Assoc.Prof.Dr. Chanwit Tribuddharat and Dr. Taradon Luangtongkum for their advice.

My sincere thank also goes to Assist.Prof.Dr. Benjamas Patamalai, Assist.Prof.Dr. Suthep Ruangwises and Assist.Prof.Dr. Thanis Damrongwattanapokin for their support during my courses of study.

I would like to express my sincere appreciation to Prof.Dr.Tung T. Hoang for his kindly support during my work at University of Hawaii at Manoa, Honolulu, Hawaii, USA.

I would also like to express my thanks to late Assoc.Prof.Dr.Thongchai Chalermchaikit who introduce me to antimicrobial resistance and public health field.

I am thankful to graduate students and staff in Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, my friends in Dr. Tung Hoang's laboratory, University of Hawaii, Staff of Center for Antimicrobial Resistance Monitoring in Foodborne Pathogens (in cooperation with WHO) for their help and support during my research.

I would like to thank Royal Golden Jubilee Ph.D. program and East-West Center for financial support.

Finally, my deepest gratitude goes to my family for their unconditional love and support. Special thanks to my father, Assoc.Prof.Kriengsak Poonsuk who inspired me to the fascinating field of Veterinary Science and encouraged me with his enthusiastic work.

CONTENTS

Page

THAI ABSTRACT iv

ENGLISH ABSTRACT v

ACKNOWLEDGEMENTS vi

CONTENTS vii

CHAPTER I 2

1.1 Importance and Rationale 3

1.2 Keywords 6

1.3 Literature Review 7

1.3.1 General characteristic of *P. aeruginosa* 7

1.3.2 Pathogenesis of *P. aeruginosa* 7

1.3.3 Antimicrobial resistance in *P. aeruginosa* 8

1.3.4 Genetics of antimicrobial resistance in *P. aeruginosa* 9

1.4 Research Objectives 19

1.5 Research Outline 20

1.6 Research Benefits 21

CHAPTER II 22

2.1 Abstract 23

2.2 Introduction 24

2.3 Materials and Methods 25

2.3.1 Bacterial isolates and antimicrobial susceptibility testing 25

2.3.2 PCR, DNA purification and DNA sequencing 26

2.3.3 Conjugation experiments 27

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

2.4 Results 28

2.4.1 Antimicrobial resistance profile 28

2.4.2 Class 1 integrons analysis 29

2.4.3 Transfer of class 1 integrons 30

2.5 Discussion 33

CHAPTER III 37

3.1 Abstract 38

3.2 Introduction 39

3.3 Materials and Methods 42

3.3.1 Bacterial strains, plasmids and growth conditions 42

3.3.2 Antimicrobial susceptibility testing 44

3.3.3 General DNA techniques 44

3.3.4 Construction of unmarked chromosomal Δ(mexXY) mutants 44

3.3.5 PCR, reverse transcription (RT)-PCR and DNA sequencing 46

3.3.6 Quantitative real-time PCR (qRT-PCR) 48

3.4 Results 48

3.4.1 Aminoglydoside susceptibility in *P. aeruginosa* isolates from canine and feline infections 48

3.4.2 Effects of mexXY loss on AMG resistance 49

3.4.3 Quantitation of mexXY and PA5471 50

3.4.4 Sequencing analysis of *mexZ*, *rplY* and *galU* 50

3.4.5 Expression of *nuoG* 52

3.4.6 The presence of AMG-modifying enzyme encoding genes 52

Page

3.5 Discussion 54

CHAPTER IV 58

4.1 Abstract 59

4.2 Introduction 60

4.3 Materials and Methods 62

4.3.1 Bacterial isolates 62

4.3.2 Antimicrobial susceptibility testing 62

4.3.3 PCR, RT-PCR, and DNA sequencing 63

4.3.4 Statistical analysis 64

4.4 Results 66

4.4.1 Antimicrobial susceptibilities 66

4.4.2 The presence of AMG-modifying enzyme-encoding genes 67

4.4.3 Expression of MexY and NuoH 67

4.4.4 Mutation in *rplY* and *galU* 68

4.4.5 Associations between resistance genes and phenotypes 68

4.5 Discussion 71

CHAPTER V 76

5.1 Abstract 77

5.2 Introduction 78

5.3 Materials and methods 81

5.3.1 Bacterial isolates and growth conditions 81

5.3.2 MIC determination 82

5.3.3 PCR and DNA sequencing 82

Page

5.3.4 RNA purification and cDNA synthesis 83

5.3.5 Quantitative real-time PCR 87

5.4 Results 87

5.4.1 Antimicrobial susceptibilities and the presence of AMG-modifying enzyme genes 87

5.4.2 Quantitative expression of *mexB*, *mexD*, *mexF*, *mexK*, *mexW*, *mexY* and *oprD* genes 88

5.4.3 Alterations in regulatory genes 89

5.5 Discussion 92

CHAPTER VI 99

6.1 Abstract 100

6.2 Introduction 101

6.3 Materials and Methods 103

6.3.1 Bacterial isolates and growth conditions 103

6.3.2 Antimicrobial susceptibility testing 104

6.3.3 RNA extraction and cDNA synthesis 104

6.3.4 Multiplex RT-PCR 105

6.3.5 PCR amplification and DNA sequencing 106

6.4 Results 106

6.4.1 Resistance phenotypes associated with the Mex efflux pumps 106

6.5 Discussion 109

6.6 Conclusions 111

CHAPTER VII 113

7.1 Conclusion 120

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Page

7.2 Research limitation and suggestion for further investigations 120

REFERENCES 122

VITA 147

LIST OF TABLES

Table 1 Antimicrobial susceptibilities of the *P. aeruginosa* (*n=101*) and *A. baumannii* strains (*n=176*) 29

Table 2 Characteristics of class 1 integrons in the *P. aeruginosa* (*n*=101) and *A.baumannii* strains (*n*=176)........ 31

Table 3 Bacterial strains and plasmids used in this study 43

Table 4 Primers used in this study........ 47

Table 5 MICs, transcription level of *mexY* and PA5471 and mutations in the *P. aeruginosa* clinical isolates and their corresponding Δ*mexXY* derivatives........ 53

Table 6 Primers used in this study........ 65

Table 7 AMG-modifying enzyme genes and resistance patterns in *P. aeruginosa* (*n*=100)........ 69

Table 8 AMG-resistance mechanisms in *P. aeruginosa* (*n*=30)........ 70

Table 9 Primers used in this study........ 84

Table 10 Antimicrobial susceptibilities of the *P. aeruginosa* clinical isolates (*n*=13)........ 85

Table 11 Expression of the Mex systems and mutations in the corresponding regulatory regions in *P. aeruginosa* isolates (*n=13*)........ 91

Table 12 Primers used in this study 105

Table 13 Phenotypic and genotypic properties of the *P. aeruginosa* reference strains... 107

Table 14 Antimicrobial susceptibility and expression of the RND efflux determined by Multiplex RT-PCR in the *P. aeruginosa* clinical isolates........ 107

LIST OF FIGURES

Figure 1 The structure of class 1 integrons. 10

Figure 2 a) Organization of 6 clinically important *mex* operons and repressor genes, b) Structure of Mex systems...15

Figure 3 Research outline 20

Figure 4 Distribution of minimum inhibitory concentration (MIC) of amikacin, gentamycin, neomycin, streptomycin, spectinomycin and tobramycin for 100 *Pseudomonas aeruginosa* isolates. 66

Figure 5 PCR amplicons of 4 clinically important Mex systems generated by RT-PCR...108

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF ABBREVIATIONS

A	adenine
aac	aminoglycoside acetyl transferase
aad	aminoglycoside adenylyl transferase
ADP	adenosine diphosphate
Ala	alanine
AMG	aminoglycoside
Amk	amikacin
AMR	antimicrobial resistance
ant	aminoglycoside nucleotidyltransferase
aph	aminoglycoside phosphoryltransferase
Arg	arginine
arr	rifampin ADP-ribosyltransferase
Asp	aspartic acid
Atm	aztreonam
bla	β-lactamase
bp	base pair(s)
C	cytosine
°C	degree(s) Celcius
Car	carbenicillin
catB	chloramphenicol acetyltransferase B
Cef	ceftaxidime
cDNA	complimentary deoxyribonucleic acid(s)
CF	cystic fibrosis
Chp	choramphenicol
Cip	ciprofloxacin
CLSI	Clinical and laboratory standards institude

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

cmlA	chloramphenicol resistance protein
DNA	deoxyribonucleic acid(s)
dfrA	dihydrofolate reductase A
dhfr	dihydrofolate reductase
dNTP	deoxyribonucleoside triphosphate(s)
E.	*Escherichia*
EPI	efflux pump inhibitor(s)
G	guanine
e.g.	exampla gratia, for example
ERIC	enterobacterial repetitive intergenic consensus
Ery	erythromycin
galU	UTP-glucose-1-phosphate uridylyltransferase (PA2023)
Gen	gentamycin
Glu	glutamic acid
h	hour(s)
His	histidine
i.e.	id est, that is
Ile	isoleucine
Imp	imipenem
Kan	kanamycin
kb	kilobase(s) or 1000 bp
LB	Luria Bertani
Leu	leucine
MDR	multidrug resistance/ resistant
Met	methionine
Mex	multidrug efflux
mg	milligram(s)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	minimal inhibitory concentrations
min	minute(s)
ml	milliliter(s)
mM	milimolar(s)
mRT-PCR	multiplex reverse transcription polymerase chain reaction
NCCLS	National committee for clinical laboratory standards
Neo	neomycin
Nor	norfloxacin
nuoG	type I NADH dehydrogenase subunit G
oprD	outer membrane porin D
orf	open reading frame
PAβN	phenylalanine-argenine-β-napthylamine
PCR	polymerase chain reaction
Phe	phenylalanine
Pip	piperacillin
Pro	proline
qac	quaternary ammonium compound
qRT-PCR	quantitative reverse transcription polymerase chain reaction
R	resistance/ resistant
RNA	ribonucleic acid
RND	resistance-nodulation-cell division
rplY	50S ribosomal protein L25
rpm	round per minutes
rpsL	30S ribosomal protein S12

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

RT-PCR	reverse transcription polymerase chain reaction
s	second(s)
SD	standard deviation
Ser	serine
Spc	spectinomycin
Str	streptomycin
T	thymine
Tet	tetracycline
Thr	threonine
tnpA	transposes A
Tob	tobramycin
Tri	trimethoprim
Val	valine
μg	microgram(s)
μl	microliter(s)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

1.1 Importance and Rationale

Pseudomonas aeruginosa is one of the most common opportunistic pathogens associated with nosocomial infections (Chung et al., 2011). The pathogen is infamous for its multidrug resistance (MDR) characteristics that cause the difficulty in treatment and eventually therapeutic failure. Emerging of MDR *P. aeruginosa* strains in humans has been increasing worldwide and resulted in several nosocomial outbreaks (Masoud-Landgraf et al., 2012). Likewise, the prevalence of MDR *P. aeruginosa* has increased in animals (Harada et al., 2012). Regardless of the infected host species, MDR *P. aeruginosa* have caused the increased morbidity, mortality and cost of treatment (Tenover, 2006). New generation of antipseudomonal drugs have been continuously developed. Unfortunately, *P. aeruginosa* could develop resistance so efficiently that resistance will be reported not long after introduction of new drugs.

Infections of MDR *P. aeruginosa* in humans have been reported over decades and increasingly founded among nosocomial infections. A study in India demonstrated that incidence of MDR *P. aeruginosa* in hospitals was 66% in 2011 (Nagaveni et al., 2011). In the United states, 12% of *P. aeruginosa* isolated from intensive care units of 13 hospitals were resistant to multiple drugs (Kathryn J. Eagye,

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2012). In Thailand, MDR *P. aeruginosa* infections are common and recognized as a major pathogen of hospital-acquired infections. The study of Monitoring Antimicrobial Resistance Trends (SMART) in Asia-Pacific regions, including Thailand showed that more than 90% of *P. aeruginosa* from intra-abdominal infections in 2007 were resistant to ertapenem, imipenem, amikacin, cefepime, cefotaxime, cefoxitin, ceftaxidime, ceftriaxone, ciprofloxacin, levofloxacin, ampicillin-sulbactam and piperacillin-tazobactam (Hawser et al., 2009).

Similarly, infections of MDR *P. aeruginosa* in animals are increasingly reported in many species and treatment failure is common. The MDR *P. aeruginosa* strains are frequently isolated form dogs with otitis, dermatitis and chronic renal diseases (Mekic et al., 2011, Zamankhan Malayeri et al., 2010). A study in Japan showed that the dog and cat *P. aeruginosa* isolates exhibited resistance to enrofloxacin, cefotaxime and gentamicin at the rate of 31.5%, 17.8% and 4.1% respectively (Harada et al., 2012). It was also expected that *P. aeruginosa* isolated from cow mastitis exhibited resistance to β-lactams, aminoglycosides, chloramphenical, tetracycline, sulfonamide and trimethoprim (Ohnishi et al., 2011). However, there is only a limited data of MDR *P. aeruginosa* isolated from animals in Thailand.

P. aeruginosa is naturally resistant to a wide range of antimicrobials due to its unique outer membrane (StratevaYordanov, 2009). This pathogen can acquire antimicrobial resistance by obtaining resistance determinants via horizontal transmission from other microorganisms and/or by chromosomal mutations due to exposure to selection pressure. Horizontal transfer is most common route for spreading of resistance determinants in bacteria. Among these, class 1 integrons and

aminoglycoside-modifying enzymes are most common in Gram-nagative bacteria and previously reported in *P. aeruginosa* (Stalder et al., 2012). Class 1 integrons are mobile genetic elements that harbor multiple resistance gene cassettes in variable region and these resistance gene cassettes could be co-selected by a single antibiotic (Gaze et al., 2005). In addition, Multidrug Efflux Systems (Mex) have been shown to play an important role in multiresistance in *P. aeruginosa* (Poole, 2001). The Mex systems are chromosomally located and therefore, vertically transmitted. Their expression is usually due to mutations in their regulatory genes. The Mex systems can extrude drug molecules out of cells, resulting in insufficient intracellular drug concentration. They can simultaneously pump out a variety of drugs that are not structurally related and promote cross-resistance between antimicrobial groups (Navon-Venezia et al., 2005).

Like other bacteria, *P. aeruginosa* uses many mechanisms to become resistant to antimicrobials. Resistance to a single drug may be mediated by multiple resistance mechanisms. It is evident that the presence of class 1 integrons, amonoglycoside-modifying enzymes (Boonkerd et al., 2009, Colinon et al., 2010, Girlich et al., 2002) and overexpression of the Mex systems (Beinlich et al., 2001, Ohnishi et al., 2011) play an important role in multidrug resistance. Most studies of MDR mechanisms were conducted in *P. aeruginosa* laboratory strains. Such studies are still limited in the clinical isolates.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

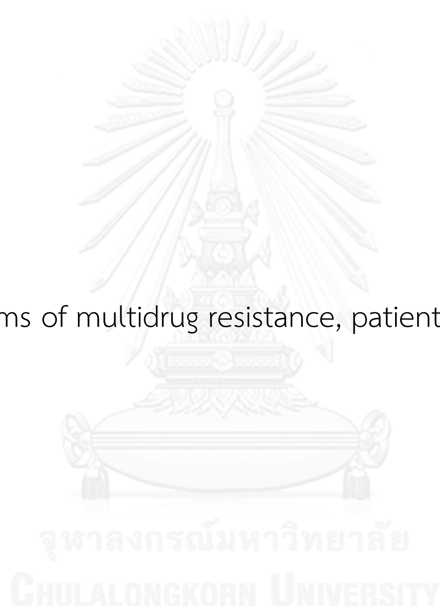
Understanding of molecular basis of resistance is necessary for implementation of prevention and control strategies of MDR bacteria. Such data is fundamental to set up antimicrobials prudent use protocol, suggest new therapeutic

guideline, define a novel molecular diagnostic tool and clarify the reliability of new therapies (Chung et al., 2011, de BentzmannPlesiat, 2011). Therefore, this study was investigated two- common mechanisms including integrons, aminoglycoside-modifying enzymes (i.e. representative for horizontally-transferred mechanisms) and the Mex systems (i.e. a representative of vertically-transferred mechanisms) underlying multidrug resistance in *P. aeruginosa* clinically isolated from human and animal patients.

1.2 Keywords

Keywords:

Animal, mechanisms of multidrug resistance, patient, *Pseudomonas aeruginosa*


จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.3 Literature Review

1.3.1 General characteristic of *P. aeruginosa*

P. aeruginosa is a gram-negative, non-fermentative bacilli and classified to Pseudomonadaceae family. The microorganism is oxidase positive, alkaline producer, motile and able to grow in aerobic condition at 37˚C and 42˚C (Boyd et al., 2008). *P. aeruginosa* can produce water-soluble pigments e.g. pyocyanin and fluorescin. The pyocyanin is formed only by *aeruginosa* species, while fluorescin can produce by other species of *Pseudomonas* (FluitSchmitz, 1999). The green-blue appearance of pyocyanin surrounding colonies is a typical characteristic of *P. aeruginosa*. *P. aeruginosa* is an ubiquitus microorganism, since it can live in environment, animals or humans.

1.3.2 Pathogenesis of *P. aeruginosa*

P. aeruginosa is a well-known opportunistic pathogen inflicting diseases in both animals and humans. In animals, *P. aeruginosa* infection has been reported in many species including dogs, cats, horses, dairy cows, snakes and aquatic animals. The infections affect many organs such as ear, skin, respiratory tract, udder or vital organs (E. L. Westman, 2010).

In humans, *P. aeruginosa* is usually associated with nosocomial infection or infection in immunocompromised patients. The pathogen is commonly found in patients with burn, surgerical wound and chronic wound. Contaminated devices such as catheter, mechanical ventilation in intensive care units or a common daily used

device such as contact lens are likely to be a vehicle for transfer an organism to human (Rosenberger et al., 2012). The invasion of bacterium to host's epithelium relies on production of several toxins, enzymes and metabolites (de BentzmannPlesiat, 2011). Uncontrolled infection of *P. aeruginosa* in patients leads to septicemia, septic shock and eventually, death.

1.3.3 Antimicrobial resistance in *P. aeruginosa*

Drugs of choice for *P. aeruginosa* treatmant are usually in the groups of β-lactams, carbapenems, fluoroquinolones and aminoglycosides. However, treatment of *P. aeruginosa* is challenged because resistance to antipseudomonal drugs frequently occurs. Particular concern has been raised because the microorganism can resist multiple drugs in the same time.

The problems caused by MDR *P. aeruginosa* have been reported all over the world. In humans, the MDR *P. aeruginosa* strains are frequency reported in Europe, North America and Asia (Lee et al., 2012, Masoud-Landgraf et al., 2012, Rosenberger et al., 2012). The prospective surveillance of ten Asian countries in 2008-2009 showed high prevalence of MDR bacteria including *P. aeruginosa* (Chung et al., 2011). In Thailand, MDR *P. aeruginosa* has been reported in hospitals since 1999 and the isolates were resistant to expand-spectrum cephalosporins and aztreonam at high level (Girlich et al., 2002). In 2006, 16% of *P. aeruginosa* isolates from regional hospitals in the north of Thailand were resistant to imipenem (Boonkerd et al., 2009).

In animals, the studies in dogs in Japan, Croatia and Iran reported *P. aeruginosa* isolates with resistance to β-lactams, fluoroquinolone, aminoglycosides,

macrolides, chloramphenicol and rifampin (Harada et al., 2012, Mekic et al., 2011, Zamankhan Malayeri et al., 2010). In United States, the MDR strains were isolated from dogs and snakes (Beinlich et al., 2001). A study in France reported the presence of MDR *P. aeruginosa* isolated from a snake and found evidence of cross-contamination between the snake and its owner (Colinon et al., 2010). A study of *P. aeruginosa* isolates from cow in Japan showed that the isolates exhibited low susceptibilities to ampicillin, cefazolin, cefuroxime, cefmetazole, ceftiofur, chloramphenicol, kanamycin, oxytetracycline, sulfamethoxaxole-trimethoprim and minocycline (Ohnishi et al., 2011).

1.3.4 Genetics of antimicrobial resistance in *P. aeruginosa*

Multidrug resistance in *P. aeruginosa* is multifactorials and usually a result of combinations between intrinsic resistance and acquired resistance (Lee et al., 2012). These resistance mechanisms could be associated with horizontal and vertical transmission (Nagaveni et al., 2011). The common horizontally-transferred mechanism is integrons and that of the vertically-transferred mechanisms are the Mex systems.

1.3.4.1 Integrons

Integrons are mobile genetic elements that can contain antimicrobial resistance gene cassettes and integrate on to bacterial chromosome or plasmid. Conjugative plasmids carrying integrons can transmit horizontally between bacteria.

1.3.4.1.1 Class 1 integrons

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Class 1 Integrons is a mobile genetic element composing of three main sections. First, the *intl1* gene located on 5'conserved segment (5'CS) encodes integrase enzyme for integration of integrons. The 3'CS carries two resistance genes encoding resistance to sulfonamide (*sul1*) and quaternary ammonium compounds (*qacEΔ1*) (Gaze et al., 2005, Kazama et al., 1998, Kucken et al., 2000). The last section is variable region (Mesaros et al.) located between 5'CS and 3'CS. The VR contains resistance gene cassette of variable numbers, leading to resistance in multiple antimicrobial agents. The structure of class 1 integrons is shown in figure 1.

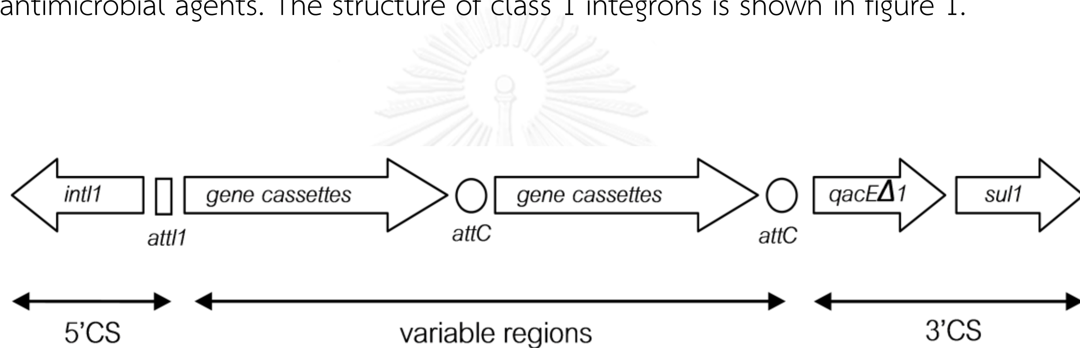


Figure 1 The structure of class 1 integrons. 5'CS; 5' conserved segment, 3'CS; 3' conserved segment, *Intl1*; integrase 1 encoding gene, *qacEΔ1*; quaternary ammonium compounds resistance gene and *sul1*; sulfonamide resistance gene

1.3.4.1.2 Class 2 and class 3 integrons

Class 2 and class 3 integrons share the similar features with class 1 integrons but use different integrase enzymes encoded by *intl2* and *intl3* genes, respectively. Detection of *intl2* and *intl3* are used for differentiation of class 2 and class 3 integrons. Class 2 integrons with *dfrA1-sat1-aadA1* cassette was reported in *P. aeruginosa* isolates in China (Xu et al., 2009). Until now, class 3 integrons was found only in *Klebsiella pneumoniae* (Correia et al., 2003)

1.3.4.2 Aminoglycoside-modifying enzymes

Aminoglycoside-modifying enzyme encoding genes are non chromosomally-encoded resistance determinants. The major aminoglycoside-modifying enzymes mediated aminoglycoside resistance are aminoglycoside acetyltransferase (*aac*), aminoglycoside phosphoryl transferase (*aph*) and aminoglycoside nucleotidyl transferase (*ant*) (Poole, 2005). Aminoglycoside-modifying enzymes modify aminoglycoside structure and change them to an inactive form. Aminoglycoside-modifying enzyme encoding genes are usually present on plasmid and transferred horizontally among bacteria. Previous studies revealed the common occurrence of these genes in *P. aeruginosa* (ShahidMalik, 2005, Vaziri et al., 2011).

1.3.4.3 Multidrug efflux systems

The whole genome sequence of *P. aeruginosa* suggests the presence of 12 multidrug efflux systems in Resistance-Nodulation-Cell-Division (RND) family on chromosome. Among the characterized systems, six multidrug effluxes (Mex) have been reported to be associated with antibiotic resistance. The typical structure of Mex systems consists of 3 parts including RND transporter protein located on inner cell membrane, membrane fusion protein located in periplasmic space and outer membrane protein located on outer cell membrane. The pumps extrude drug out of the cells, resulting in insufficient level of drugs to bind at target sites. Most Mex proteins are encoded by genes on chromosome that can be transmitted vertically among *P. aeruginosa* isolates. Six Mex systems that are involved in resistance to

clinically-important antibiotics are as follows and their organization and structure are shown in figure 2.

1.3.4.3.1 MexAB-OprM

MexAB-OprM efflux system causes intrinsic resistance due to its constitutive expression in wild-type. Transcription of *mexAB-oprM* operon is regulated by its local repressor gene, *mexR*. Studies reported that overexpression of *mexAB-oprM* associated with mutation on *mexR* (Adewoye et al., 2002) and *nalC* (Sadeghifard et al., 2012). The MexAB-OprM is composed of three components; the inner membrane protein (MexB), the membrane fusion protein (MexA) and the outer membrane protein (OprM). Overexpression of *mexAB-oprM* results in resistance to β-lactams, fluoroquinolones, tetracycline, macrolides, chloramphenicol, sulphonamides, trimethoprim and novobiocin.

1.3.4.3.2 MexCD-OprJ

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The MexCD-OprJ efflux system is normally silent in *P. aeruginosa* wild-type. The *mexCD-oprJ* operon is regulated by its negative regulatory gene, *nfxB* located upstream. Mutation(s) on the regulatory gene can promote overexpression of MexCD-OprJ (Chuanchuen et al., 2001, Jeannot et al., 2008). Topological structure of MexCD-OprJ consists of an inner membrane protein (MexD), membrane fusion protein (MexC) and outer membrane protein (OprJ). Acquired expression of this system promotes resistance to fluoroquinolones, macrolides, tetracycline, chloramphenicol, trimethoprim, novobiocin and some β-lactams.

1.3.4.3.3 MexEF-OprN

The MexEF-OprN efflux is not normally expressed in wild-type. Transcription of *mexEF-oprN* operon is regulated by its positive regulator, *mexT* that is controlled by its regulator, *mexS* (Kohler et al., 1999, Sobel et al., 2005). Mutations on *mexT* inhibit expression of the system (Llanes et al., 2011). Three components of MexEF-OprN comprise of MexF as an inner membrane transporter, MexE as a membrane fusion protein and OprN as an outer membrane protein. Expression of this Mex system confers resistance to fluoroquinolones, chloramphenicol, trimethoprim and carbapenem.

1.3.4.3.4 MexJK

MexJK efflux is not normally expressed in wild-type. The expression of *mexJK* operon is regulated by the adjacent transcriptional repressor, *mexL*, located divergently upstream to the operon (Chuanchuen et al., 2005). The structure of system is composed of the membrane fusion protein (MexJ) and the inner membrane protein (MexK). It can borrow OprM from MexAB-OprM to complete its function as multidrug efflux system. To date, the only two known antibiotic substrates of MexJK efflux are tetracycline and erythromycin (Chuanchuen et al., 2002).

1.3.4.3.5 MexVW

MexVW efflux system is normally silent in wild-type *P. aeruginosa*. The system has MexW as an inner membrane protein, MexV as a membrane fusion

protein (Li et al., 2003). There is not the presence of regulatory gene closed to the operon. Expression of MexVW efflux confers resistance to fluoroquinolones, tetracycline, chloramphenicol and erythromycin.

1.3.4.3.6 MexXY

The MexXY efflux system is constitutive expressed in wild-type and the expression was also shown to be inducible. Transcription of *mexXY* operon is regulated by the transcriptional repressor genes, *mexZ*. Mutation on *mexZ* or *mexZ-mexX* intergenic region and high expression level of *PA5471* gene are associated with overexpression of MexXY (Islam et al., 2004). The two components of MexXY system are the membrane fusion protein (MexX) and the inner membrane protein (MexY). MexXY utilizes outer membrane component, OprM from the MexAB-OprM efflux system to complete the function. Up to date, the MexXY-OprM is the only RND efflux system that has been reported to extrude aminoglycosides in *P. aeruginosa*. The other substrates of MexXY system are fluoroquinolone, tetracycline and macrolides.

CHULALONGKORN UNIVERSITY

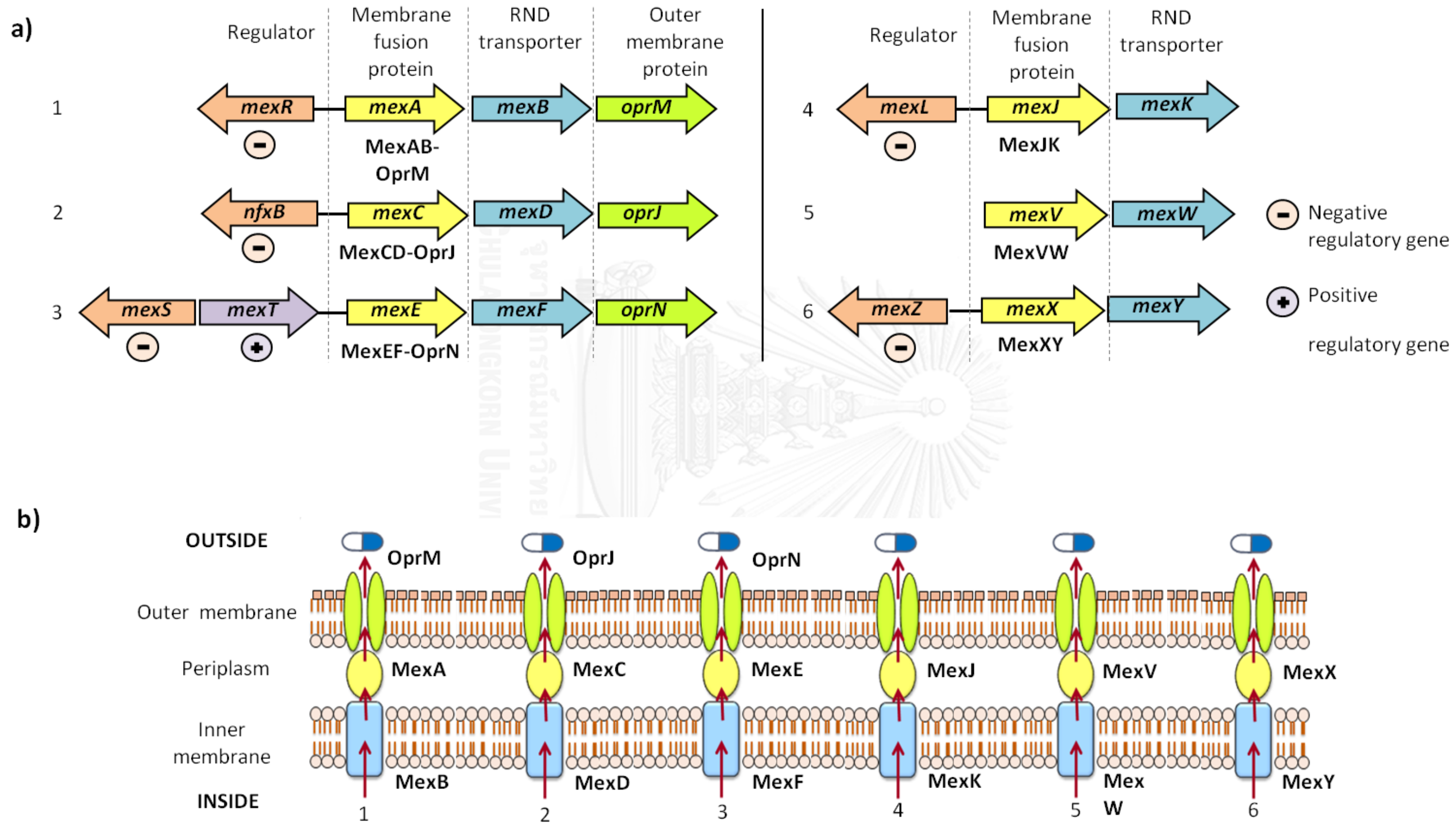


Figure 2 a) Organization of 6 clinically important *mex* operons and repressor genes, b) Structure of Mex systems

1.3.4.4 Chromosomally-encoded aminoglycoside resistance mechanisms

Besides expression of *mexXY*, a study revealed the presence of three chromosomally-encoded aminoglycoside resistance mechanisms in *P. aeruginosa* (El'Garch et al., 2007, Islam et al., 2009). These three genes encode different traits. Two of them (i.e. *galU* and *nuoG*) associated with aminoglycosides uptake and the others, *rplY*, is associated with target alteration. The *galU* gene encodes UTP-glucose-1-phosphate uridylyltransferase, which functions in biosynthesis and degradation of surface polysaccharides and lipopolysaccharides (LPS). The *nuoG* gene encodes NADH-quinone oxidoreductase chain G, which functions in electron transport. The *rplY* gene encodes ribosomal protein L25 that is the target site of aminoglycosides and functions in ribosomal proteins synthesis and modification. Mutations or changes of expression of these genes lead to reduced aminoglycoside sensitivity.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.3.4.5 Carbapenem resistance mechanisms

Carbapenems is a group of antimicrobials currently known as the last line of drug in treatment of bacterial infections, especially *P. aeruginosa*. This group of drugs is a class of β-lactam antimicrobials and has broad-spectrum activitiy. Drugs in this group include imipenem, meropenem, ertapenem, doripenem, panipenem and biapenem. Penem antimicrobials inhibit cell wall synthesis and have activity against non-fermentative Gram-negative bacilli.

Carbapenem resistance may occur by synergistic effects of many mechanisms (Gutierrez et al., 2007, Jwu-Ching Shu, 2012, Rodriguez-Martinez et al., 2009, Wang et al., 2010). Carbapenem resistance in *P.aeruginosa* could be due to production of carbapenem-hydrolyzing enzymes, loss of outer membrane protein OprD and expression of multidrug efflux system.

Many studies suggested that reduction of *oprD* expression is the main mechanism for decreased carbapenem susceptibility (Gutierrez et al., 2007, Jwu-Ching Shu, 2012, Rodriguez-Martinez et al., 2009, Wang et al., 2010). OprD is the specific porin, which facilitates imipenem uptake (Kolayli et al., 2004). Loss of OprD reduces portal of entry of drugs and underline cause of treatment failure when infection with carbapenem resistant *P. aeruginosa* occurs (Rodriguez-Martinez et al., 2009).

Four multidrug efflux systems previously reported to be involved in carbapenem resistance are MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. Overexpression of MexAB-OprM increases MIC of meropenem but not affects

imipenem efficacy (Li et al., 1995, Masuda et al., 2000). MexCD-OprJ and MexXY-OprM also cause the reduction on meropenem susceptibility (Masuda et al., 2000). Accompany of efflux systems to resist to carbapenem resistance was found in clinical isolates (Maniati et al., 2007, Vettoretti et al., 2009). The most commonly observed mechanism is the coordination of loss of OprD and overexpression of MexEF-OprN.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.4 Research Objectives

1.4.1 To investigate the characteristics and transferability of integrons in *P. aeruginosa* clinically isolated from companion animal and human patients

1.4.2 To investigate the characteristics of aminoglycoside-modifying enzymes in *P. aeruginosa* clinically isolated from companion animal and human patients

1.4.3 To investigate the characteristics and contribution of the Mex systems in *P. aeruginosa* clinically isolated from companion animal and human patients

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.5 Research Outline

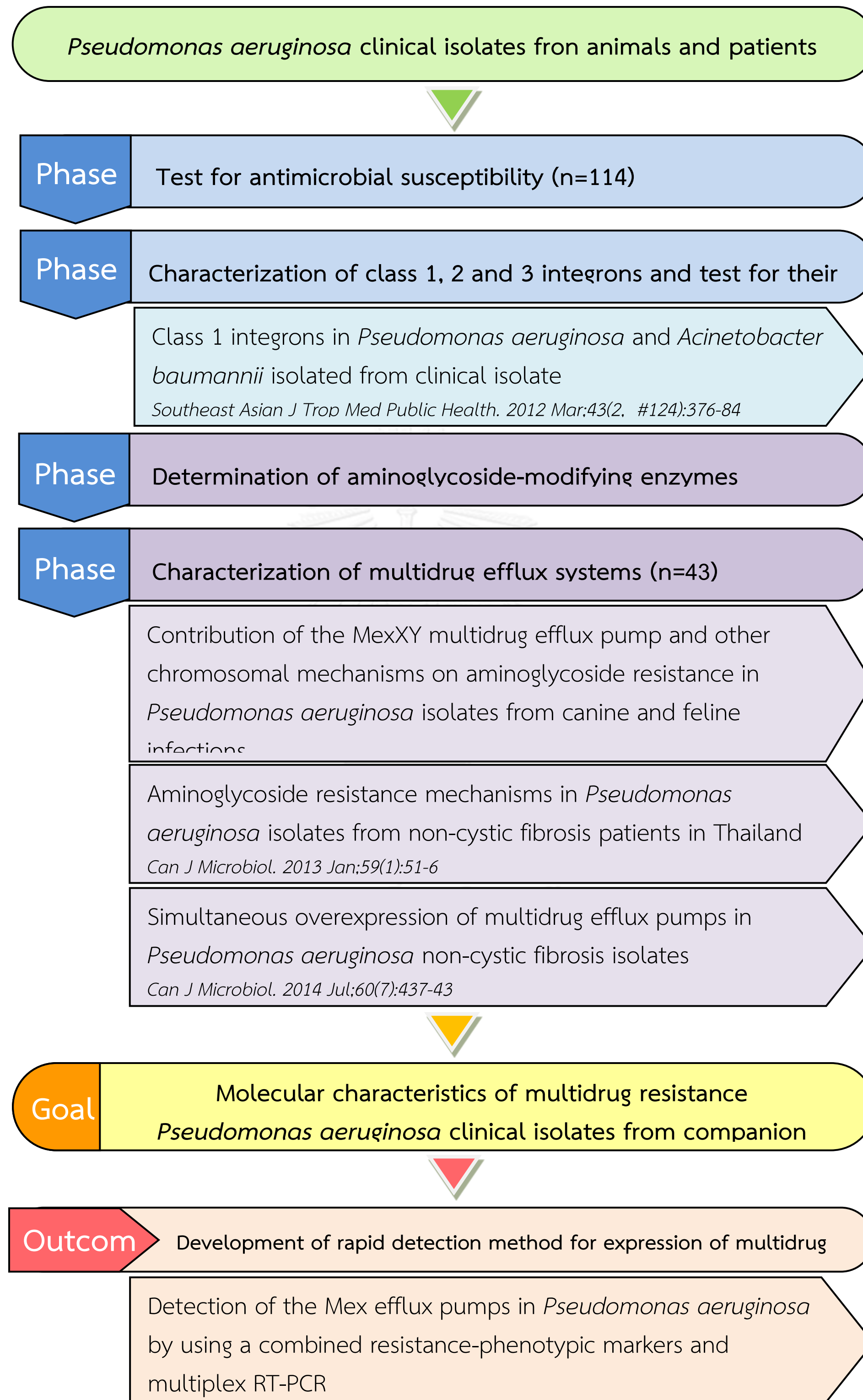


Figure 3 Research outline

1.6 Research Benefits

1.6.1 Novel knowledge

- Genetic data on mechanisms underlying multidrug resistance in *P. aeruginosa* clinical isolates from humans and animals in Thailand.

1.6.2 Application of knowledge

- Data on antimicrobial resistance phenotypes and genotypes can be partly used for risk assessment of antimicrobial resistance in *P. aeruginosa.*

- Antimicrobial-resistance profiles and data of resistance mechanisms can be applied for suggestion of the prudent use of antimicrobials in human and veterinary medicine.

- The *P. aeruginosa* isolates overexpressing Mex systems can be used in future experiments; for example, characterization of Mex function, new drug development and production of new diagnostic tools.

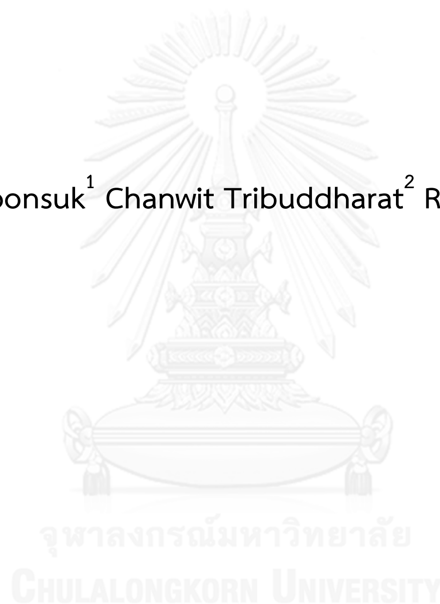
- Data can be included as part of antimicrobial resistance surveillance in *P. aeruginosa* in Thailand.

CHULALONGKORN UNIVERSITY

CHAPTER II

Class 1 integrons in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from clinical isolates

Kanchana Poonsuk[1] Chanwit Tribuddharat[2] Rungtip Chuanchuen[1]



[1]Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

[2]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Southeast Asian Journal of Tropical Medicine and Public Health (2012)

Class 1 integrons in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from clinical isolates

2.1 Abstract

Resistance to various antimicrobial agents is an increasing problem in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections. In this study, the roles of integrons were examined in 101 *P. aeruginosa* isolates and 176 *A. baumannii* isolates from patients. The frequencies and characteristics of class 1, 2 and 3 integrons were investigated and the horizontal transfer of integrons was assessed. Among these isolates, class 1 integrons with a resistance gene cassette were detected in 69.3% of *P. aeruginosa* and 31.8% of *A. baumannii* isolates, but class 2 and 3 integrons were not found. Five novel gene cassette arrays were identified in *P. aeruginosa*: *aacA7-cmlA, aadB-*$bla_{OXA\text{-}10}$*-aadA1*, aadB-*arr-2-cmlA-* $bla_{OXA\text{-}10}$*-aadA1, aadB-cmlA-aadA1 and aadB-cmlA-* $bla_{OXA\text{-}10}$*-aadA15*. The integrons found in *A. baumannii* isolates in this study were previously reported. Horizontal transfer of some class 1 integrons was detected in both *P. aeruginosa* (2/70) and *A. baumannii* (5/57). These data confirm the high prevalence of class 1 integrons with a variety of gene cassette combinations among multidrug-resistant *P. aeruginosa* and *A. baumannii* clinical isolates.

Keywords: *Acinetobacter baumannii*, class 1 integrons, multidrug resistance, *Pseudomonas aeruginosa*

2.2 Introduction

Pseudomonas aeruginosa and *Acinetobacter baumannii* are recognized as common nosocomial pathogens. They are clinically significant due to their multidrug-resistant (MDR) phenotypes leading to therapeutic failures. Several resistance mechanisms have been identified in these two pathogens, including acquisition of resistance-encoding genes through mobile genetic elements (Seward, 1999, Xu et al., 2009). These elements include integrons able to integrate and mobilize the gene cassettes, most of which contain resistance-encoding genes (FluitSchmitz, 1999). To date, nine classes of integrons have been recognized, among which class 1 integrons are the most prevalent among *P. aeruginosa* and *A. baumannii* (Gu et al., 2007). Of particular concern are class 1 integrons frequently located in plasmids and transposons. These have the ability to undergo horizontal transfer and contribute to rapid dissemination of antibiotic resistance genes among bacterial isolates not limited to *P. aeruginosa* and *A. baumannii* (FluitSchmitz, 1999).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Resistance to various antibiotics is common among *P. aeruginosa* and *A. baumannii* isolates in many parts of the world, including Thailand. However, there is currently a relative paucity of data on integron-associated gene cassettes among MDR *P. aeruginosa* and *A. baumannii* strains, particularly in most developing countries. This study was conducted to characterize antibiotic susceptibilities and class 1 integrons among *P. aeruginosa* and *A. baumannii* isolates. The presence of class 2 and 3 integrons was also investigated.

2.3 Materials and Methods

2.3.1 Bacterial isolates and antimicrobial susceptibility testing

A hundred and one *P. aeruginosa* isolates and 176 *A. baumannii* isolates were randomly selected from the stock of Siriraj hospital, Bangkok Thailand. All the strains were obtained from a variety of clinical specimens collected during 2001-2008. All bacterial strains were identified by using the VITEK GNI card (bioMérieux Vitek, Inc., Hazelwood, Mo.) and the API 20NE system (bioMérieux, Inc.). Only one colony was collected from each positive clinical sample. The genetic relatedness of these isolates was not tested. All the isolates were tested for their minimum inhibitory concentrations (MICs) of 15 antimicrobial agents including amikacin (Amk), aztreonam (Atm), carbenicillin (Car), ceftaxidime (Cef), chloramphenicol (Chp), ciprofloxacin (Cip), erythromycin (Ery), gentamicin (Gen), kanamycin (Kan), neomycin (Neo), piperacillin (Pip), streptomycin (Str), spectinomycin (Spc), tetracycline (Tet) and trimethroprim (Tri) using a two-fold agar dilution method according to the CLSI guidelines (CLSI) (NCCLS, 2002). The *P. aeruginosa* ATCC27853 type strain was used as a control. Multidrug resistance was defined as resistance to at least 6 different antimicrobial agents (Gu et al., 2007).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.3.2 PCR, DNA purification and DNA sequencing

Template DNA used for PCR was prepared as previously described (Levesque et al., 1995). All *P. aeruginosa* and *A. baumannii* isolates were screened for the presence of the integrase genes of the *intI1*and *intI2* and *intI3* genes using the following primer pairs: for *intI1*, int1LF (5′-CAG GAG ATC GGA AGA CCT-3′) and int1LR (5′-TTG CAA ACC CTC ACT GAT-3′); for *intI2*, (5′-GGC AGA CAG TTG CAA GAC AA -3′) and (5′-AAG CGA TTT TCT GCG TGT TT-3′) and for *intI3*, (5′-CCG GTT CAG TCT TTC CTC AA-3′) and (5′-GAG GCG TGT ACT TGC CTC AT-3′) (Chuanchuen et al., 2007, Ekkapobyotin et al., 2008). Inserted-gene cassettes were analyzed using PCR with a conserved segment primer set: 5’CS-GGCATCCAAGCAGCAAG and 3’CS-AAGCAGACTTGACCTGA (Levesque et al., 1995). The PCR amplicons were purified using QIAQuick Gel Extraction kit (Qiagen, Hilden, Germany) and submitted for nucleotide sequencing at Macrogen Inc. (Seoul, South Korea). The resulting DNA sequence was analyzed using the BLAST algorithm software available at http://www.ncbi.nlm.nih.gov. Positive controls for the *intI1*, *intI2* and *intI3* genes were *Pseudomonas aeruginosa* P90 (Chuanchuen et al., 2007), *Salmonella* Paratyphi B var Java (van Essen-Zandbergen et al., 2007) and pAV3.5 (Xu et al., 2007), respectively.

2.3.3 Conjugation experiments

Possible conjugal transfer of *integrons* was investigated using biparental mating (Maniati et al., 2007). The *P. aeruginosa (n=70)* and *A. baumannii (n=57)* isolates carrying class 1 integrons with resistance gene cassettes were used as donors. Rifampicin-resistant *E.coli* MG1655 derivatives were recipients. Transconjugants were selected on Luria-bertani (LB) agar (Difco, BD Diagnostic Systems, MD, USA) supplemented with 32 µg/ml of rifampicin and one of the following antibiotics: streptomycin (80 µg/ml), gentamycin (100 µg/ml), and trimethroprim (10 µg/ml). Transconjugants were confirmed to be *E. coli* by growth on MacConkey agar (Difco) and transfer of class 1 integrons was confirmed using PCR as described above. The biparental mating procedure was repeated on two separate occasions for each donor-recipient combination yielding no transconjugants. All experiments were carried out at Biosafety Level 2.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.4 Results

2.4.1 Antimicrobial resistance profile

The MIC value and resistance rates of all the isolates tested are shown in Table 1. All the *P. aeruginosa* and *A. baumannii* isolates were resistant to at least six antimicrobial agents. All *P. aeruginosa* strains were resistant to chloramphenicol, erythromycin, kanamycin, neomycin, spectinomycin, tetracycline and trimethoprim. Eighty-six isolates (85.2%) were resistant to ceftaxidime, while resistance rates to all other antibiotics tested were above 90%. All the *A. baumannii* isolates were absolutely resistant to spectinomicin and trimethoprim. Resistance rates to amikacin and carbenicillin were 88.6% and 86.4%, respectively. Resistance rates to all other antibiotics were greater than 90%. The resistance phenotypes of *P. aeruginosa* and *A. baumannii* could be arranged into 14 and 30 patterns, respectively (data not shown). The most common resistance pattern in both *P. aeruginosa* (78.8%) and *A. baumannii* (81.2%) was Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri. Among *P. aeruginosa*, the other resistance patterns with more than one isolates were: Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Str-Spc-Tet-Tri (2%), Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (2%) and Atm-Chp-Cip-Ery-Kan-Neo-Str-Spc-Tet-Tri (2%). Among *A. baumannii* other resistance pattern were Amk-Atm-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1.7%) and Atm-Cef-Chp-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1.7%).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 1 Antimicrobial susceptibilities of the *P. aeruginosa* (*n=101*) and *A. baumannii* strains (*n=176*)

Antibiotics	*P. aeruginosa*		*A. baumannii*	
	Range of MIC (μg/ml)	% resistance	Range of MIC (μg/ml)	% resistance
Amk	8->2048	92.1	<8->2048	88.6
Atm	<1->256	96.0	16->256	97.2
Car	16->2048	94.1	16->2048	86.4
Cef	4->256	81.2	8->2048	90.9
Chp	128-512	100	64-512	99.4
Cip	<0.25->256	99.0	0.25-256	94.3
Ery	>512	100	<2->2048	97.2
Gen	<1->256	95.0	<8->2048	94.3
Kan	256->256	100	<8->2048	95.6
Neo	64->256	100	<8->2048	92.0
Pip	<2->256	90.1	32->1024	95.5
Spc	256->256	100	32-2048	100
Str	4->256	99.0	<8->2048	97.2
Tet	64->256	100	<8->2048	94.9
Tri	128->256	100	16->1024	100

Amk, amikacin; Atm, aztreonam; Car, carbenicillin; Cef, ceftaxidime; Chp, chloramphenicol; Cip, ciprofloxacin; Ery, erythromycin; Gen, gentamicin; Kan, kanamycin; Neo, neomycin; Pip, piperacillin; Str, streptomycin; Spc, spectinomycin; Tet, tetracycline and Tri, trimethroprim

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.4.2 Class 1 integrons analysis

Ninety-six *P. aeruginosa* isolates (95%) were positive to *intl1*, of which 70 isolates (69.3%) harbored resistance gene cassettes. Twelve integron profiles (IPs) were defined based on the number and the size of the PCR amplicons obtained (Table 2). The most frequently-identified gene cassette array was *aadB-cmlA-aadA1* (37.5%) in IP-XI. Two distinct class 1 integrons containing the *aacA4* and *aacA7-cmlA* cassette arrays were found in two *P. aeruginosa* isolates (IP-V).

The *intI1* gene amplicons were obtained from 69 *A. baumannii* isolates (39.2%). Fifty-seven isolates (32.4%) carried class 1 integrons with inserted-resistance gene cassettes that were classified into 13 IPs. Among class 1 integrons-carrying isolates, the most common gene cassette combination was *dfrA1-orfC* in IP-I and *bla*$_{VEB-1}$-*aadB-arr-2-cmlA-bla*$_{OXA-10}$-*aadA1* (18.8%) in IP-VI. The presence of a complete *aadA1* gene was additionally tested in all 13 *A. baumannii* strains carrying the gene array *aac(6')I1-aadA1-IS26-tnpA-IS26-aadA1* (IP-VI) and the gene was detected in only two isolates. Coexistence of empty class 1 integrons and gene cassette-containing integrons was found in 8 *A. baumannii* strains. Among these isolates, five strains carried two class 1 integrons (IPs-X and XI) and the others carried three class 1 integrons (IPs-XII and XIII). None of the *P. aeruginosa* or *A. baumannii* strains was found to carry the *intI2* and *intI3* genes.

2.4.3 Transfer of class 1 integrons

Among the *P. aeruginosa* isolates, two class 1 integrons carrying the gene cassette arrays *dfrA1-orfC* (IP-IV) and *aadB-cmlA-aadA1* (IP-XI) in the variable regions were conjugally transferred. Five *A. baumannii* strains could horizontally transfer class 1 integrons, including class 1 integrons with the *aac(6')I1-aadA1* array in IP-XI and four empty class 1 integrons in IP-X – XIII. The latter included the empty integrons from two *A. baumannii* isolates in IP-XI and one of each empty integrons from the isolate in IPs-XII and XIII.

Table 2 Characteristics of class 1 integrons in the *P. aeruginosa* (*n=101*) and *A. baumannii* strains (*n=176*)

IP	Integron Size bp)	Gene cassette[e]	No. of isolates (%)[a]	Resistance pattern
P. aeruginosa				
I	0.8	*aacA4*	3 (3.1)	Atm-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Str-Spc-Tet-Tri (1) Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1) Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1)
II	1.3	*aadA6*	4(4.2)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Str-Spc-Tet-Tri (1) Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (3)
III	1.3	*aadA6-orfD*	2(2.1)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (2)
IV	1.3	*dfrA1-orfC* [c]	1(1.0)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1)
V	0.8, 1.8	*aacA4*, *aacA7-cmlA*	2(2.1)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (2)
VI	1.8	$bla_{IMP\text{-}14}$-*aac(6')*	1(1.0)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1)
VII	2.0	$bla_{PSE\text{-}1}$-*aadA2*	7(7.3)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (7)
VIII	2.0	$bla_{IMP\text{-}15}$-*dhfr-aac(6')*	1(1.0)	Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Str-Spc-Tet-Tri (1)
IX	2.5	*aadB*-$bla_{OXA\text{-}10}$-*aadA1*	7(7.3)	Amk-Atm-Car-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (5) Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (2)
X	2.5	*aadB-arr-2-cmlA*-$bla_{OXA\text{-}10}$-*aadA1*	1(1.0)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1)
XI	3.0	*aadB-cmlA-aadA1* [c]	36(37.5)	Amk-Chp-Cip-Ery-Gen-Kan-Neo-Str-Spc-Tet-Tri (1) Amk-Atm-Car-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (2) Amk-Atm-Car-Chp-Cef-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (33)
XII	3.5	*aadB-cmlA*-$bla_{OXA\text{-}10}$-*aadA15*	5(5.2)	Amk-Atm-Car-Chp-Cef-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (5)

IP	Integron Size bp)	Gene cassette[e]	No. of isolates (%)[a]	Resistance pattern
A. baumannii				
I	1.2	*dfrA1-orfC*	13(18.8)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (11) Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Pip-Str-Spc-Tet-Tri (2)
II	1.6	*aac(6')I1-aadA1*	4(5.8)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (4)
III	1.9	*dfrA12-orfF-aadA2*	1(1.4)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1)
IV	2.5	*aacC1-orfX-orfY-aadA1a*	1(1.4)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1)
V	3.0	*aacC1-orfX -orfX′-orfY-aadA1a*	3(4.3)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (3)
VI	2.3	*aac(6')I1-aadA1-IS26-tnpA-IS26-aadA1*	9(13.0)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (9)
VII	5.5	bla_{VEB-1}*-aadB-arr-2-cmlA-*bla_{OXA-10}*-aadA1*	13 (18.8)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (13)
VIII	1.9, 2.5	*dfrA12-orfF-aadA2, aacC1-orfX-orfY-aadA1a*	4 (5.8)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (4)
IX	2.2, 3.0	*aacA4-catB8-aadA1, aacC1-orfX-orfX′-orfY-aadA1a*	1(1.4)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1)
X	0.15[b], 1.6	*aac(6')I1-aadA1*[c]	2(2.9)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (2)
XI	0.15[d], 3.0	*aacC1-orfX -orfX′-orfY-aadA1a*	3(4.3)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (3)
XII	0.15[d], 1.9, 2.5	*dfrA12-orfF-aadA2, aacC1-orfX-orfY-aadA1a*	2(2.9)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (2)
XIII	0.15[d], 2.2, 3.0	*aacA4-catB8-aadA1, aacC1-orfX-orfX′-orfY-aadA1a*	1(1.4)	Amk-Atm-Car-Cef-Chp-Cip-Ery-Gen-Kan-Neo-Pip-Str-Spc-Tet-Tri (1)

[a] Total number of isolates used; 96 for *P. aeruginosa* and 69 for *A. baumannii*

[b] Class 1 integrons without any inserted-gene cassette in variable region

[c] Capable of horizontal transfer.

[d] Empty integrons conjugally transferred

[e] Antimicrobial resistance-encoding genes: *aacA4*, *aac(6')* and *aac(6')I1* amikacin, kanamycin and tobramycin; *aacC1* and *aacA7 ,* gentamicin; *aadA1, aadA2* and *aadA6*, streptomycin and spectinomicin; *aadB*, gentamicin, kanamicin and tobramycin; bla_{PSE-1}, β-lactams; bla_{OXA-10}, oxacillin; bla_{IMP-14}, imipenem and meropenem; *cmlA* and *catB8*, chloramphenicol; *dfrA1* and *dfrA12*, trimethoprim

2.5 Discussion

All *P. aeruginosa* and *A. baumannii* isolates in this study are multidrug resistant; this high resistance rates are in agreement with previous studies (Gu et al., 2007, Seward, 1999). These findings were according to our expectations since the pathogens have been infamous for their highly-intrinsic resistance to antibiotics. Resistance to amikacin, piperacillin and ceftzidime is of special concerns since these are important drugs of choice for treating *P. aeruginosa* and *A. baumannii* infections. In most cases, infections with these two pathogens did not respond well to antibiotic. The emergence of resistant *P. aeruginosa* and *A. baumannii* strains reduces the antibiotic treatment options leading to an increased possibility of treatment failure.

In this study, genes conferring resistance to aminoglycosides were frequently found; the most common gene cassettes belong to the *aad* and *aac* families. The most frequent aminoglycoside-resistance gene cassettes found in class 1 integrons from *P. aeruginosa* was *aadB*, and among *A. baumannii* was *aadA1.* The widespread presence of aminoglycosides resistance gene cassettes can be explained by the extensive use of drugs in this class for treatment.

Among *P. aeruginosa*, two Metallo-β-lactamase gene**s** bla_{IMP-14} and bla_{IMP-15} were identified in combination with different genes, *aac(6′)* and *dhfr-aac(6′)*, respectively. Both bla_{IMP-14}-*aac(6′)* and bla_{IMP-15} -*dhfr-aac(6′)* gene cassette arrays were previously described in class 1 integrons in Thailand (GenBank accession no.AY553332 and AY553333, respectively). The bla_{IMP-15} gene cassette was previously

identified in carbapenem-resistant *P. aeruginosa* strains, but with a different gene cassette array (Garza-Ramos et al., 2008). Five gene cassette combinations identified in this study, including *aacA7-cmlA* , *aadB-bla*$_{\text{OXA-10}}$-*aadA1*, *aadB-arr-2-cmlA-bla*$_{\text{OXA-10}}$-*aadA1*, *aadB-cmlA-aadA1* and *aadB-cmlA-bla*$_{\text{OXA-10}}$-*aadA15* have never been previously reported from *P. aeruginosa*, even though all these genes have been demonstrated in other settings and different orders (Girlich et al., 2002, Gu et al., 2007). A similar gene cassette array *aadB-arr-2-cmlA-bla*$_{\text{OXA-10}}$-*aadA1* was described in *P. aeruginosa* clinical isolates in Thailand (Girlich et al., 2002). The difference was the lack of *bla*$_{\text{VEB-like}}$ in the array *aadB-arr-2-cmlA-bla*$_{\text{OXA-10}}$-*aadA1* newly discovered in this study. This cassette array could be a result of homologous-recombination exchange of gene cassettes between two class 1 integrons or Intl1-mediated site specific recombination (Partridge et al., 2002).

The gene cassette arrays identified in *A. baumannii* have been previously found worldwide, for example, the *aacA4-catB-aadA1*, *dfrA12-orfF-aadA2* and *aacC1-orfX-orfY-aadA1a* arrays were demonstrated in the clinical isolates from China (Gu et al., 2007) and Taiwan (Lee et al., 2009). The latter was recently found in class 1 integrons from Australia (Zong et al., 2008). The most common gene cassette array identified in *A. baumannii* in this study, *bla*$_{\text{VEB-1}}$-*aadB-arr-2-cmlA- bla*$_{\text{OXA-10}}$-*aadA1*, was previously characterized in *P. aeruginosa* isolates (Girlich et al., 2002). The *dfrA1-orfC* array was found in both *P. aeruginosa* and *A. baumannii*. This gene cassette combination has been previously identified in other bacteria: *Salmonella* spp. (Hsu et al., 2006) and *Proteus mirabilis* (Boyd et al., 2008). The *bla*$_{\text{PSE-1}}$-*aadA2* array found in *P. aeruginosa* was previously identified in *P. mirabilis* (Boyd et al., 2008). The presence of the identical gene arrays in different bacterial species or in the same

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

species from different geographical areas suggested the efficient horizontal transfer of class 1 integrons. This notion was confirmed by the presence of class 1 integrons located on conjugative plasmids in this study. In addition, some empty integrons was conjugally transmitted when streptomycin was used as a selective pressure, suggesting the expression of other streptomycin-resistance encoding determinants located elsewhere on the same plasmids. This observation highlights the important role of conjugative R-plasmids on dissemination of resistance among bacteria.

In addition to the *aacC1-orfX-orfY-aadA1a* array, a similar cassette combination with additional *orfX*, *aacC1-orfX-orfX'-orfY-aadA1a* was observed among *A. baumannii* isolates. This gene cassette array has been previously identified; it has been suggested that the second copy of *orfX* may be captured by site-specific recombination mechanisms (Turton et al., 2005). The *aac(6')/1-aadA1-IS26-tnpA-IS26-aadA1* array was first described in the patient isolates from South Korea (Han et al., 2008). As *aadA1* was expected to be inactivated by IS26 insertion, all nine strains carrying this array were resistant to spectinomycin and streptomycin. However, only two isolates contained a complete *aadA1* gene, indicating the existence of unidentified mechanisms encoding in resistance to both aminoglycosides in the other isolates.

Class 1 integrons devoid of gene cassettes were found commonly among the *intl1*-positive isolates in this collection (i.e 53.6% in *A.baumannii* and 27.1% in *P. aeruginosa*). The empty variable regions in these integrons were available to capture

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

the new coming gene cassette (s) for further horizontal dissemination even though their sources are still ambiguous.

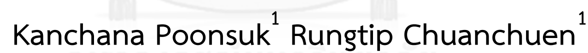
Class 2 integrons have also been described among *P. aeruginosa* (Xu et al., 2009) and *A. baumannii* (Seward, 1999) but no class 3 integrons have been reported among these pathogens. The absence of class 2 and 3 integrons among the isolates in the present study indicated that these two genetic elements did not play a role in antimicrobial resistance among these bacteria. Resistance gene cassettes present in class 1 integrons cannot cover all resistance phenotypes in both pathogens, indicating the existence of other resistance mechanisms not tested. Several resistance mechanisms have been reported among *P. aeruginosa* and *A. baumannii*, such as multidrug efflux systems (Marchand et al., 2004, Schweizer, 2003); however, these were not pursued in this study.

In conclusion, the results confirm the high prevalence of class 1 integrons and their important role in the dissemination of antimicrobial-resistance genes among the *P. aeruginosa* and *A. baumannii* isolates in this study. Clinical use of antibiotics may increase a selective pressure for the MDR strains and for horizontal gene transfer. This could be a serious threat to the efficacy of antibiotics used for treating infections caused by not only *P. aeruginosa* and *A. baumannii,* but also other clinically-significant pathogens.

CHAPTER III

Contribution of the MexXY multidrug efflux pump and other chromosomal mechanisms on aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from canine and feline infections

Kanchana Poonsuk[1] Rungtip Chuanchuen[1]



[1]Department of Veterinary Public Health, Faculty of Veterinary Science,
Chulalongkorn University, Bangkok, Thailand 10330

Journal of Veterinary Medical Science (2012) DOI DN/JST.JSTAGE/jvms/12-0239

Contribution of the MexXY multidrug efflux pump and other chromosomal mechanisms on aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from canine and feline infections

3.1 Abstract

As study of multidrug efflux pumps is a crucial step for development of efflux pump inhibitors for treatment of *Pseudomonas aeruginosa* infection, the objective of this study was to examine the contribution of the MexXY multidrug efflux systems and other chromosomal mechanisms in aminoglycoside (AMG) resistance in *P. aeruginosa* isolated from dogs and cats. Thirteen *Pseudomonas aeruginosa* isolates from canine and feline infections were examined for contribution of the MexXY multidrug efflux pump and four other chromosomally-encoded genes including PA5471, *galU*, *nuoG* and *rplY* to AMG resistance. All the isolates were resistant to multiple AMGs and expressed *mexXY*. Deletion of *mexXY* caused 2- to 16-fold reduction in AMG MICs. Overproduction of MexXY did not fully account for the observed AMG resistance. No good correlations were detected between MexXY transcription level and AMG MICs. While no mutations were found in *mexZ*, PA5471 expression varied and its impact on MexXY expression and AMG resistance is diverse. No mutations were found in *galU*. Only two isolates carried a single base change G-367-T in *rplY*. Complete transcription of *nuoG* was detected in all the isolates. In conclusion, the MexXY multidrug efflux pump plays a role in AMG resistance in the dog and cat *P. aeruginosa* isolates, while disruption of *nuoG*, *rplY* and *galU* did not have a significant impact. These results indicate the existence of uncharacterized AMG-resistance mechanisms.

Keyword: aminoglycoside resistance, *mexXY*, *Pseudomonas aeruginosa*

CHULALONGKORN UNIVERSITY

3.2 Introduction

Pseudomonas aeruginosa is infamously known as a common cause of chronic and recurrent infections in both humans and animals, of which the most-notably diseases in dogs and cats include otitis externa/media, urinary tract infection and pyoderma (Gatoria et al., 2006, Hariharan et al., 2006, Petersen et al., 2002). Treatment of *P. aeruginosa* infection is challenging because the pathogen intrinsically exhibits and efficiently develops high resistance to several antimicrobials structurally and functionally unrelated, leading to multidrug resistance (Chuanchuen et al., 2001, Schweizer, 2003).

As many antipseudomonal drugs are available for therapeutic use in dogs and cats, aminoglycosides (AMGs) are considered a vital component of antipseudomonal chemotherapy (Poole, 2005) due to their efficacy, safety and reasonable price. However, panaminoglycoside resistance e.g. gentamicin, spectinomycin, streptomycin, amikacin has been increasingly reported in the *P. aeruginosa* dog and cat isolates (Mekic et al., 2011, Pedersen et al., 2007, Rubin et al., 2008). Such resistance has been clarified to be predominantly due to a poorly understood mechanism namely "impermeability resistance" as a consequence of diminished drug uptake and/or accumulation (Poole, 2005, Westbrock-Wadman et al., 1999). The MexXY efflux system, a multidrug efflux pump in the resistance-nodulation-cell-division (RND) family is involved in the reduced level of AMG accumulation implicated in both impermeability-type and adaptive-type AMG resistance (Hocquet et al., 2003, Poole, 2005, Poole, 2005). The MexXY efflux pump is encoded by the *mexXY* operon and evidently serves as the major AMG-resistance mediating system in *P. aeruginosa*

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

clinical isolates. This system additionally confers resistance to nonaminoglycosides, including tetracycline, erythromycin and fluoroquinolones (Masuda et al., 2000). Inhibition of the MexXY efflux pump appears to be a promising approach for restoring the activity of the existing antimicrobials and improving the *P. aeruginosa* infection (Tegos et al., 2002). In addition to MexXY, chromosome-encoded genes including *mexZ*, PA5471, *nuoG, rplY* and *galU* have a cumulative contribution to AMG MICs in the *P. aeruginosa* laboratory strain (El'Garch et al., 2007).

MexZ is the TetR family transcriptional regulator that binds to the *mexZ-mexX* intergenic region located between positions -104 to -66 bp and represses transcription of the *mexXY* operon (Matsuo et al., 2004). Recently, PA5471-dependent overexpression of MexXY was demonstrated. The PA5471 product modulates transcription of MexXY by binding to MexZ and reduced MexZ DNA-binding ability (Yamamoto et al., 2009). The combination amino acid substitution in MexZ and overexpression of PA5471 causes increased *mexXY* expression, leading to AMG resistance. Individual disruption of *nuoG, rplY* and *galU* led to gradual increases in AMG MICs in PAO1. The *nuoG* gene belongs to the *nuoABDEFGHIJKLMN* operon and encodes type I NADH dehydrogenease required for the synthesis of protonmotive force (Taber et al., 1987). Inactivation of *nuoG* results in impaired membrane energy leading to decreased AMG uptake. The *rplY* gene encodes the L25-ribosomal protein that is an AMG-target site, therefore, mutations in this region contribute to resistance to AMGs (El'Garch et al., 2007). The *galU* gene encodes UDP-glucose pyrophosphorylase essential for the synthesis of LPS-outer core. The disrupted *galU* gene leads to the production of the A and B band-deficient LPS that adversely affect AMG binding and impairs their outer membrane uptake

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

(DeanGoldberg, 2002). However, it was recently shown that inactivation of *nuoG, rplY* and *galU* did not play an important role in AMG resistance in the *P. aeruginosa* cystic fibrosis (CF) isolates (Islam et al., 2009).

Up to date, efflux pump inhibitors for combating antimicrobial resistance caused by multidrug efflux pumps have been researched only for medical treatment in humans (Tegos et al., 2002) . These molecules could be discovered and/or developed as a novel strategy for treatment of *P. aeruginosa* infection in dogs and cats. In this case, study of contribution and functions of multidrug efflux pumps in antimicrobial resistance is a crucial step to achieve the new therapeutic goal. However, role of MexXY in AMG resistance has been systematically studied in *P. aeruginosa* from CF patients (Islam et al., 2004, Vogne et al., 2004, Wolter et al., 2004). In contrast, such information is still limited in the *P. aeruginosa* veterinary isolates. We previously examined contribution of the MexXY efflux pump in AMG resistance in the isolates from cow mastitis (Chuanchuen et al., 2008). However, there have been no published data on the MexXY efflux pump of the *P. aeruginosa* isolates from dogs and cats and different types of antimicrobials are used in food-producing and companion animals. Therefore, the aim of this study was to investigate the involvement of MexXY in AMG resistance in the *P. aeruginosa* clinical isolates from dogs and cats. Additional-chromosomal mechanisms including PA5471, *nuoG, rplY* and *galU* were also examined.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids and growth conditions

All the bacterial strains and plasmids used in this study are shown in Table 1. Thirteen *P. aeruginosa* clinical isolates were obtained from the strain collection of the Veterinary Diagnostic Laboratory (VDL), Faculty of Veterinary Science, Chulalongkorn University. They were originated from samples that were collected from dogs and cats at Small Animal Hospital during 2005-2010 and submitted for bacterial isolation at the VDL. These dogs and cats were from different households.

Isolation of *P. aeruginosa* was performed using standard methods (KingPhillips, 1985). Briefly, the bacteria were isolated on blood agar. The colonies with hemolytic zone and pyocyanin- nonfluorescent bluish pigment were collected and further confirmed to be *P. aeruginosa* by using biochemical tests. Only a single *P. aeruginosa* colony was taken from each positive sample at only one time. The bacterial isolates were grown either on Luria Bertani (LB) agar (Difco, BD Diagnostic Systems, MD, USA), in LB broth (Difco), in Mueller-Hinton broth (MHB; Difco) with adjusted concentrations of Ca^{2+} and Mg^{2+} and on Mueller-Hinton agar (MHA; Difco). Antibiotics used in selective medium were as follows: ampicillin, 100-150 μg/ml and gentamicin, 15 μg/ml for *Escherichia coli* and carbenicillin, 200 μg/ml and gentamicin, 50 μg/ml for *P. aeruginosa*. *E. coli* SM10 was a donor in conjugation experiment.

Table 3 Bacterial strains and plasmids used in this study

Strain		Relevant properties	Source or reference
P. aeruginosa	PAO1	Wild type expressing MexAB-OprM	(WatsonandHolloway, 1978)
	PA049	PAO1Δ(*mexXY*)::*FRT*	(Chuanchuen et al., 2008)
	PA3579	PAO1 with Δ*mexZ*	(Westbrock-Wadman et al., 1999)
	PAO267	PA3579 with Δ(*mexAB-oprM*)	(Chuanchuen et al., 2001)
	PAO280	PA267 with Δ(*mexXY*)	(Chuanchuen et al., 2001)
	Clinical isolates		
	PAJ226 and PAJ245	Pus from wounds in a cat	This study
	PAJ228 and PAJ230	Feline nasal cavity	This study
	PAJ227, PAJ229 and PAJ230	Urine from a cat	This study
	PAJ233 and PAJ239	Pus from wounds in a dog	This study
	PAJ232, PAJ234 and PAJ240	Canine otitic ears	This study
	PAJ235	Urine from a dog	This study
	Δ(*mexXY*) mutants		
	PAJ262	PAJ226Δ(*mexXY*)::*FRT*	This study
	PAJ264	PAJ227Δ(*mexXY*)::*FRT*	This study
	PAJ266	PAJ229Δ(*mexXY*)::*FRT*	This study
	PAJ268	PAJ230Δ(*mexXY*)::*FRT*	This study
	PAJ270	PAJ232Δ(*mexXY*)::*FRT*	This study
	PAJ272	PAJ234Δ(*mexXY*)::FRT	This study
	PAJ274	PAJ239Δ(*mexXY*)::FRT	This study
	PAJ277	PAJ240Δ(*mexXY*):: Gm^r-*FRT*	This study
	PAJ276	PAJ245Δ(*mexXY*)::FRT	This study
E. coli	SM10	Donor for biparental mating *thi-1 thr leu tonA lacy supE recA*::RP4-2-Tc::*Mu* (Km^r)	(de LorenzoandTimmis, 1994)
Plasmid	pUCP20	Ap^r; a cloning vector	(West et al., 1994)
	pPS1221	Ap^r, Gm^r: pEX18Ap containing Δ(*mexXY*) ::FRT-*Gm^r* fragment	(Chuanchuen et al., 2002)
	pAMR1	Ap^r, pUCP20 carrying *mexX*	(Westbrock-Wadman et al., 1999)

Abbreviations: Ap^r, ampicillin resistance; Gm^r, gentamycin resistance, Km^r, kanamycin resistance

จุฬาลงกรณ์มหาวิทยาลัย CHULALONGKORN UNIVERSITY

3.3.2 Antimicrobial susceptibility testing

Antimicrobial susceptibilities was examined by determination of minimum inhibitory concentrations (MICs) using the standard two-fold microdilution technique according to Clinical and Laboratory Standards Institute guidelines (CLSI)(CLSI, 2002). The MIC breakpoints used in this study were from CLSI, when available (Table 5). However, the specific-CLSI breakpoints for *P. aeruginosa* are not available for all antimicrobials (i.e. streptomycin, neomycin and tobramycin). Therefore, the CLSI interpretive breakpoints for the Enterobacteriaceae and those in the published data were used for the antimicrobials lacking the CLSI breakpoints (Rubin et al., 2008). Experiments were performed in triplicates and repeated independently twice. *P. aeruginosa* ATCC 27853 and wildtype PAO1 were used as quality control organisms.

3.3.3 General DNA techniques

Chromosomal DNA was extracted and purified using the QIAamp mini kit (Qiagen, Hilden, Germany). Plasmid DNA was prepared using QIAprep® Mini-spin kit (Qiagen). Selected DNA fragments were purified from agarose gels using QIAquick Gel Extraction kit (Qiagen).

3.3.4 Construction of unmarked chromosomal Δ(mexXY) mutants

pPS1221 was used as a source for Δ(*mexXY*) mutant alleles (Chuanchuen et al., 2002) to delete chromosomal *mexXY* operon by using Flp/*FRT* recombinase-mediated excision technology as previously described (Chuanchuen et al., 2001,

Hoang et al., 1998). Gene replacement at the *mexXY* operon was confirmed by PCR and DNA sequencing analyses. Resistance phenotypes of the parents and their corresponding Δ(*mexXY*) mutant derivatives were confirmed by complementation analyses using pAMR-1 (Westbrock-Wadman et al., 1999).

3.3.5 PCR, reverse transcription (RT)-PCR and DNA sequencing

PCR amplifications were performed using KAPATaq ReadyMix DNA polymerase (Kapabiosystems, Boston, MA, USA) by following the manufacturer's protocol. All the primer pairs used for PCR amplification are listed in Table 4. The DNA fragments encompassing *mexZ* and the *mexZ-mexX* intergenic region, *rplY* and *galU* were PCR-amplified from genomic DNA template. All the AMG-modifying enzyme encoding genes were amplified using whole cell DNA templates.

Reverse transcription (RT)-PCR was performed to detect transcription of *mexY* and *nuoH.* Total RNA isolation of *P. aeruginosa* was carried out using Qiagen RNeasy Mini kit (Qiagen) and treated with RNase-free DNaseI (Invitrogen, CA, USA). One-µg DNase treated RNA was reversed transcribed to single stranded-cDNA using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) with the reverse primers specific to *mexY* (i.e. mexYRTdown) and *nuoH* (i.e.nuoHL823-L) and then, the cDNA obtained was used as the template for PCR amplification using the specific primer pairs as described above.

For nucleotide sequencing analyses, the PCR products were purified using the QIAQuick Gel Extraction kit (Qiagen) and submitted for sequencing at Macrogen Inc. (Seoul, South Korea) using the PCR primers. The nucleotide sequence of each purified PCR product was determined on both strands and the DNA-sequencing results were compared with PA2023 and PA2020 available at the Pseudomonas Genome Project (http://pseudomonas.com) for *galU* and *mexZ*, respectively (2011).

Table 4 Primers used in this study

Gene/region	Primer	Sequence (5′-3′)	Reference
Mutation			
mexZ -mexZX intergenic region	mexZXup	CGCAGAATTCCGGCGTCCGC	(Chuanchuen et al., 2008)
	mexZXdown	GCAAGCTTCTGCACATCAGCGAG	
rplY	rplYF144-U	ATCGCCCGAACGCTGGT	(Islam et al., 2009)
	rplYF144-L	ATGCCGGGTCTGGTCGTATTC	
galU	galUF14o-U	CGAGCGCAGCCTGATTAGACT	(Islam et al., 2009)
	galUR1121-L	ACAGCTCAGGTAGGGCGGATA	
RT-PCR			
mexY [a]	mexYRTUP	AGCTACAACATCCCCTA	(Chuanchuen et al., 2008)
	mexYRTdown	AGCACGTTGATCGAGAAG	
nuoH	nuoHF657-U	GCAGGAACTGGCGGACGG	(Islam et al., 2009)
	nuoHL823-L	GGTCTTGGCGGCGAAGTAGAA	
qRT-PCR			
PA5471	PA5471-U	CGACATCGGCTGTGGCA	(Islam et al., 2009)
	PA5471-L	AGTCGCTCCAGGTCTCGTC	
rpsl	rpsl-realtimeup	CGGCACTGCGTAAGGTATG	(Chuanchuen et al., 2008)
	rpslrealtime down	CCCGGAAGGTCCTTTACACG	
AMG-Modifying enzymes			
aadA1	aadA1-F	CTCCGCAGTGGATGGCGG	(ChuanchuenandPadungtod, 2009)
	aadA1-R	GATCTGCGCGCGAGGCCA	
aadA2	aadA2-F	CATTGAGCGCCATCTGGAAT	(ChuanchuenandPadungtod, 2009)
	aadA2-R	ACATTTCGCTCATCGCCGGC	
aadB	aadB-F	CTAGCTGCGGCAGATGAGC	(Chuanchuen et al., 2008)
	aadB-R	CTCAGCCGCCTCTGGGCA	
aac(3')-Ia	aac3Iaup	CTGACCAAGTCAAATCCATGCGGG	(Chuanchuen et al., 2008)
	aac3Iadown	CCACTGCGGGATCGTCACCG	
aac(6')-IIa	aac6IIaup	AGAGCGATGGCGGAAGAGTCC	(Chuanchuen et al., 2008)
	aac6IIAdown	ATCCTGCCTTCTCATTGCAGCG	
aac(6')-IIb	aac6IIbup	CCGAAGAAGGAGTGACGCCG	(Chuanchuen et al., 2008)
	aac6IIbdown	GCGCAAACCGTTCACCAACGG	
aph(3')-IIb	aph3IIbup	GAACGAAACCCAGAGCGACGG	(Chuanchuen et al., 2008)
	aph3IIbdown	CAATCGATGAAGCCGCTGAAGC	
ant(2")-Ia	ant2Iaup	TGGAGCAGCAACGATGTTACGC	(Chuanchuen et al., 2008)
	ant2Iadown	CCACTGGTGGTACTTCATCGG	
strA	strA-F	TGGCAGGAGGAACAGGAGG	(ChuanchuenandPadungtod, 2009)
	strA-R	AGGTCGATCAGACCCGTGC	
strB	strB-F	GCGGACACCTTTTCCAGCCT	(ChuanchuenandPadungtod, 2009)
	strB-R	TCCGCCATCTGTGCAATGCG	

[a]Also used for qRT-PCR of *mexY*

3.3.6 Quantitative real-time PCR (qRT-PCR)

Transcription level of the *mexY* and PA5471 genes was assessed by qRT-PCR as previous described with some modifications (Chuanchuen et al., 2008, Islam et al., 2009). The cDNA was synthesized from 1 µg of total RNA as describe above and quantified using KAPA SYBR® FAST qPCR kit (Kapabiosystems). PCR assays were done in triplicates for all the genes. The Ct values from two separate experiments (SD< 0.1) were used to estimate the average cDNA copy numbers for each sample and the *rpsL* gene was used as internal control. The average *mexY* and PA5471 cDNA copy number was normalized with that of of *rpsL* and the transcription levels were identified as fold change-ratios to that of the reference strain PAO1.

3.4 Results

3.4.1 Aminoglydoside susceptibility in *P. aeruginosa* isolates from canine and feline infections

All of the *P. aeruginosa* isolates were examined for their AMG susceptibility (Table 5). All exhibited resistance to spectinomycin and most isolates showed intermediate resistance to streptomycin, kanamycin and neomycin. Only few isolates were resistant to gentamicin (5 isolates) and tobramycin (3 isolates). None of the isolates were resistant to amikacin.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.4.2 Effects of mexXY loss on AMG resistance

To further assess the participation of MexXY in AMG resistance in all isolates, the *mexXY* operon was deleted to generate unmarked Δ(*mexXY*)::*FRT* mutants and the impact on AMG resistance was evaluated. However, construction of unmarked Δ(*mexXY*)::FRT mutants was completed in only eight isolates. In addition, the Gm^r-*FRT* cassette was not successfully excised in one isolates (i.e. PAJ240), resulting in the Δ(*mexXY*):: Gm^r-*FRT* marked mutant (i.e. PAJ277) where the association of MexXY to gentamicin resistance was not assessed.

The effect of MexXY loss on AMG resistance varied among the isolates in this collection. Deletion of *mexXY* resulted in two- to 16 fold decline in AMG MICs in all nine isolates. Constitutive expression of MexXY from pAMR-1 fully restored the AMG susceptibilities, confirming the influence of Δ(*mexXY*) on all the AMG MICs observed (data not shown). The loss of *mexXY* reduced the MICs of all AMGs (four- to 16-fold) in three isolates (i.e. PAJ227, PAJ229 and PAJ230) and six of the seven AMGs in two isolates (i.e. PAJ226 and PAJ245). The absence of MexXY had only moderate effects (two- to fourfold decline in MIC) on AMG resistance in many strains. For example, the MICs for all AMGs tested of PAJ277 were only two- to fourfold reduced from those of the parents PAJ240. When consider individual AMG substrates, consequences of *mexXY* loss were different for the same substrate in different *P. aeruginosa* host strain. For instance, *mexXY* loss generated only a marginal effect on streptomycin susceptibility in two isolates (PAJ232 and PAJ240) but resulted in 16-fold decreased streptomycin MIC in three isolates (i.e. PAJ229, PAJ230 and PAJ239).

3.4.3 Quantitation of mexXY and PA5471

To evaluate the association between *mexXY* expression and AMG resistance, *mexXY* expression was initially measured by RT-PCR and their transcription was observed in all clinical isolates (data not shown). Then the relative transcription level of *mexXY* was assessed. The Ct values of the *rpsL* internal control from different isolates were comparable with each other in all qRT-PCR experiments. The *mexXY* operon was found to overproduce three to 191-fold higher than PAO1 in 11 isolates. A decreased transcription of *mexXY* was detected in one isolate (i.e. PAJ232, 0.03 fold less than PAO1). The last isolate i.e. PAJ233 produced MexXY at the level equivalent to that of PAO1.

Relative expression of PA5471 was measured by qRT-PCR in all isolates. Of all the isolates tested, PA5471 was upregulated in 10 isolates and its transcription level varied from two- to 86-fold. The variability of PA5471 transcription level was observed among the isolates with comparable MexXY expression. For instance, PAJ234, PAJ228 and PAJ245 that expressed 6-fold MexXY produced PA5471 two- to 13-fold. Similarly, the strains with the similar PA5471 transcription level (i.e. PAJ227, PAJ230 and PAJ239) did not express MexXY at the same level. The strains with higher PA5471 expression produced neither higher *mexXY* transcriptional level nor higher AMG MICs than those with less PA5471 expression. This is best illustrated with PAJ233 that was not more resistant to AMGs than PAJ288 and PAJ235.

CHULALONGKORN UNIVERSITY

3.4.4 Sequencing analysis of *mexZ*, *rplY* and *galU*

The involvement of *mexZ* and the *mexZ-mexX* intergenic region in *mexXY* expression was examined by DNA sequencing in all isolates. The *mexZ* sequences in all the *P. aeruginosa* isolates tested were homologous to that of PAO1 while five isolates (i.e. PAJ226, PAJ240, PAJ228, PAJ235 and PAJ238) contained nucleotide changes in the *mexZ-mexX* intergenic region. A replacement of A at position -112 (relative to position 1 being the A of the *mexX* start codon) with G was most commonly found. Expression of MexXY in three isolates carrying only A(-112)G varied from three to 24-fold compared to PAO1. One isolate contained up to 6 single base mutations (i.e. PAJ228) and its MexXY transcription level was comparable to that of the isolates carrying two single base mutations (i.e. PAJ235). The PAJ227 strain with highest MexXY transcription level contained no mutation within the *mexZ-mexX* intergenic region. Some isolates with the *mexZ-mexX* intergenic region indistinguishable from that of PAO1 (i.e. PAJ234 and PAJ245) had MexXY expression comparable to that of the strains carrying mutations in the region (i.e. PAJ228 and PAJ235).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

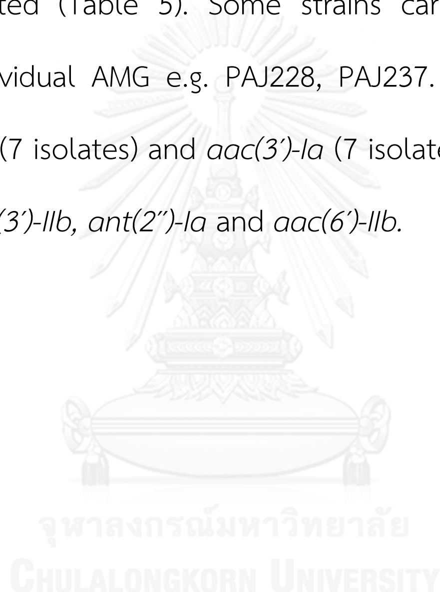
The *galU* and *rplY* genes were sequenced in all isolates*.* The resulting DNA sequences showed that the nucleotide sequence of *galU* in all isolates was identical to that of PAO1. Only two isolates (i.e. PAJ226 and PAJ245) contained a G-367-T single point mutation in *rplY*, leading to an Ala-123-Ser amino acid substitution in RplY.

3.4.5 Expression of *nuoG*

Transcription of *nuoH* located downstream of *nuoG* was investigated by using RT-PCR in all isolates. Expression of *nuoH* was observed in all the isolates

3.4.6 The presence of AMG-modifying enzyme encoding genes

Most isolates except PA227, PA238, PA239 and PA240 were positive to AMG-resistance genes tested (Table 5). Some strains carried several genes encoding resistance to an individual AMG e.g. PAJ228, PAJ237. The genes most commonly identified were *aadB* (7 isolates) and *aac(3′)-Ia* (7 isolates). None of the isolates were found to contain *aph(3′)-IIb, ant(2′′)-Ia* and *aac(6′)-IIb.*


จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Strains	MIC(breakpoint) (μg/ml)[a]							Transcription level		Mutations		AMG-modifying enzyme
	AMK(66)	GEN(8)	KAN(66)	NEO(10)	STR(66)	SPC(128)	TOB(8)	*mexY*	PA5471	*mexZ-mexX*[b,c]	*rplY*	
PAO1	8	1.6	66	16	16	512	1	1	1	-	-	-
PA069	2	0.2	32	6	8	512	0.5					-
PAJ226	8	2	256	32	32	2068	8	26	6	A(-112)-G	G-367-T(Ala-123-Ser)	*aadB*, *aac(3')-Ia*, *strAB*
PAJ262	0.5	<0.25	32	16	6	128	1					ND
PAJ227	2	6	8	6	66	2068	6	191	16	-	-	-
PAJ266	0.25	1	2	0.5	16	256	0.5					-
PAJ229	8	6	256	128	128	1026	2	6.7	1	-	-	*aadB*, *aac(3')-Ia*
PAJ266	1	0.5	66	8	8	128	0.5					ND
PAJ230	8	6	128	16	66	1026	2	26	16	-	-	*aadA2*, *aac(3')-Ia*
PAJ268	1	0.5	32	6	6	256	0.25					ND
PAJ232	6	6	128	16	32	1026	128	0.03	28	-	-	*aadA1*, *aadB*, *aac(3')-Ia*
PAJ270	2	1	66	8	32	256	16					ND
PAJ236	8	8	128	128	512	2068	2	6	13	-	-	*aadB*, *aac(6')-IIa*
PAJ272	1	1	66	16	128	256	1					ND
PAJ239	8	2	128	8	128	1026	1	21	2	-	-	-
PAJ276	2	0.5	66	0.5	8	256	0.5					-
PAJ260	16	8	256	128	128	2068	6	3	1	A(-112)-G	-	-
PAJ277	2	ND	66	32	66	512	1					-
PAJ265	6	6	256	32	66	2068	0.5	6	2	-	G-367-T(Ala-123-Ser)	*aadA2*, *aac(3')-Ia*
PAJ276	0.5	<0.25	66	8	8	128	0.25					ND
PAJ228[d]	6	256	128	8	512	1026	256	6	12	C(-11)-G, G(-57)-A, A(-88)-T, G(-99)-T, G(-106)-C, A(-112)-G	-	*aadB*, *aadA1*, *aadA2*, *aac(3')-Ia*, *strAB*
PAJ233[d]	8	8	128	32	66	1026	1	1	86	-	-	*aadB*, *aac(3')-Ia*
PAJ235[d]	16	128	256	16	512	1026	6	8	19	G(-57)-A, A(-112)-G	-	*aadA2*, *strAB*
PAJ238[d]	8	6	128	32	512	1026	1	21	1	A(-112)-G	-	-

Table 5 MICs, transcription level of *mexY* and PA5471 and mutations in the *P. aeruginosa* clinical isolates and their corresponding Δ*mexXY* derivatives

Abbreviations and concentration ranges (in parenthesis): AMK, amikacin (1-2048 μg/ml); GEN, gentamicin (1-256 μg/ml); KAN, kanamycin (1-2048 μg/ml); NEO, neomycin (1-256 μg/ml); STR, streptomycin (1-2048 μg/ml); SPC, spectinomycin (1-2048 μg/ml); TOB, tobramycin (1-128 μg/ml); ND, not detected

[a]Values in boldface indicate MICs for the parent strains that are at least 4-fold different from those for the corresponding Δ*mexXY* derivatives

[b]*mexZ-mexX* intergenic region

[c]Negative number indicates the positions of base pair changes relative to the first nucleotide of the *mexX* ATG start codon

[d]Deletion of the *mexXY* operon was not successful.

3.5 Discussion

In the present study, the *P. aeruginosa* isolates from canine and feline infections were resistant to panaminoglycosides. High resistance rates to spectinomycin, streptomycin, kanamycin and neomycin were observed, in agreement with a previous study (Petersen et al., 2002). This may be not surprising since these antibiotics are commonly prescribed in dogs and cats due to their relatively-less expensive cost. In contrast, lack of amikacin resistance and low resistance rates to gentamicin and tobramycin were observed. Both gentamicin and amikacin are antibiotics of last resort due to their common side effects of kidney damage and hearing loss. Similarly, tobramycin produces side effects and is not used long-term. This limited usage could be an explanation for such low resistance rates observed.

Diverse contribution of MexXY to AMG resistance among the dog and cat isolates in this study was similar to that previously reported in the *P. aeruginosa* veterinary isolates (Chuanchuen et al., 2008). The loss of *mexXY* caused up to four- to 16-fold reduction in MICs of several AMGs in many isolates, suggesting the significant contribution of the MexXY efflux pump in panaminoglycoside resistance. The moderate effects of *mexXY* loss on AMG resistance in several strains suggested the existence of other AMG-resistance determinants that were not characterized in this study. Such marginal effect of *mexXY* loss on resistance to clinically-important AMGs was previously observed in the cow isolates (Chuanchuen et al., 2008) and could create clinical impact by preventing AMGs from reaching their optimal concentrations, especially where the antibiotic penetration is impeded**.** Impact of

MexXY loss on a single AMG was different in different *P. aeruginosa* host strains. This is mostly due to the different combination of AMG-resistance mechanisms additionally expressed and compromised *mexXY* loss in each individual strain.

In addition, most isolates harbored AMG-modifying enzyme encoding genes confirming the existence of additional AMG-resistance mechanisms in the *P. aeruginosa* strains. However, the contribution level of these genes to AMG resistance is still unclear. Further investigations are required to disclose their significance level.

Expression of *mexXY* and its association with AMG resistance varied. The strains with the highest *mexXY* expression (i.e. PAJ227) did not show greater resistance to AMGs that those with much less *mexXY* expression (i.e. PAJ234, PAJ240, PAJ228, and PAJ235). The strain with a decreased transcription of *mexXY* was resistant up to four AMGs. Taken together, the data supported that *mexY* transcription was not associated with AMG resistance. This phenomenon was previously observed in the animal and human isolates (Chuanchuen et al., 2008, Sobel et al., 2003). Therefore, high *mexXY* expression level is not an absolute marker for high AMG resistance level among the *P. aeruginosa* clinical isolates.

The predominant nucleotide change on MexZ, A(-112)G, was located outside of the MexZ-binding domain but still situated in the predicted promoter of *mexX* located between positions -88 and -133 (Chuanchuen et al., 2008). It was found that the presence and the number of single base mutations were not correlated to the MexXY transcription level and the absence of mutation within the *mexZ-mexX* intergenic region did not guarantee the low MexXY transcription level. Based on the

observations, it is unlikely that the mutations in the *mexZ-mexX* intergenic region have substantial impacts on MexZ functioning as a *mexXY* repressor.

The *P. aeruginosa* strains with comparable MexXY transcription level expressed PA5471 at different level (two- to 13 folds) and MexXY transcription level among the strains with comparable PA5471 transcription level was quite different. Therefore, there was a lack of good correlation between PA5471 expression level and *mexXY* transcription level. Two isolates i.e. PAJ233 and PAJ232 overexpressed PA5471 upto 86 and 28 fold, respectively but did not overproduce MexXY, in agreement with a previous study in the CF isolates (Islam et al., 2009). In *vice versa*, three isolates with PA5471 expression level comparable to that of PAO1 (i.e. PAJ229, PAJ240 and PAJ238) had elevated-MexXY expression (three-to 21 fold). These results suggest the existence of additional regulatory mechanisms that manipulate *mexXY* expression in the isolates. The strains with higher PA5471 expression produced neither higher *mexXY* transcriptional level nor higher AMG MICs than those with less PA5471 expression. This is best illustrated with PAJ233 that was not more resistant to AMGs than PAJ288 and PAJ235. Hence, the prominent PA5471 expression cannot be a conclusive indicator of high *mexXY* expression and high AMG resistance.

Sequencing analyses revealed the presence of single point mutation, A G-367-T, only in *rplY* from two isolates. This nucleotide change may have an impact on AMG resistance; however, its actual effect on AMG MICs was not investigated in this study. Due to its location downstream of *nuoG*, expression of *nuoH* was examined to confirm the complete transcription of *nuoG*. Expression of *nuoH* was observed in all the isolates, indicating the *nuoG*-complete transcription and suggesting that the

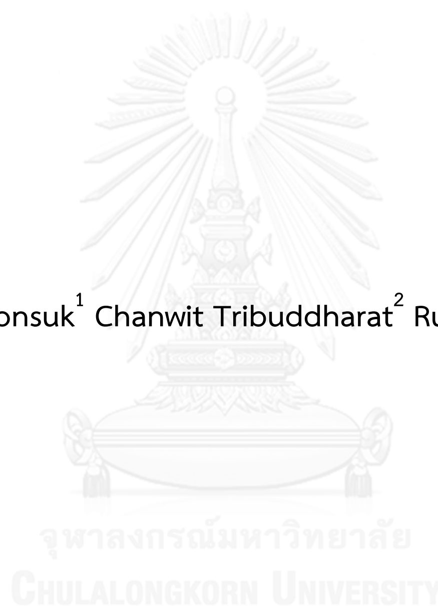
alteration of this gene did not play a role in AMG resistance among the veterinary strains in this study. Altogether, changes of *nuoG, rplY* and *galU* did not significantly contribute to AMG resistance level in the dog and cat isolates, similar to a previous observation in the human clinical isolates (Islam et al., 2009).

In conclusions, the results obtained in this study showed the contribution of the MexXY efflux pump in AMG resistance in the *P. aeruginosa* isolates from canine and feline infections. MexXY expression alone cannot explain the whole AMG resistance observed. The presence of MexXY-regulatory mechanisms additional to *mexZ* and PA5471 is suggested. The effects of *nuoG*, *rplY* and *galU* on AMG resistance were insignificant in AMG resistance of the *P. aeruginosa* dog and cat isolates. Further studies are warranted to elucidate the whole picture of MexXY-regulatory machinery in the AMG-resistant mutants in this study. Furthermore, the systematic surveillance of antimicrobial resistance that is usually performed in the pathogens from humans and livestocks is required for those from companion animals. The prudent guideline for antimicrobial use should be elaborated for pet animals to prevent and control the emergence and dissemination of the multidrug-resistant *P. aeruginosa* strains.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER IV

Aminoglycoside resistance mechanisms in *Pseudomonas aeruginosa* isolates from non-cystic fibrosis patients in Thailand



Kanchana Poonsuk[1] Chanwit Tribuddharat[2] Rungtip Chuanchuen[1, 3]

[1]Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
[2]Department of Microbiology, Faculty of Medicine, Mahidol University, Bangkok, Thailand
[3]Center for antimicrobial resistance Monitoring in Foodborne Pathogens (in *cooperation with WHO*); Global Foodborne Infections Network: South-East Asia and Western Pacific Region, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

Canadian Journal of Microbiology (2013) DOI 10.1139/cjm-2012-0465

Aminoglycoside resistance mechanisms in *Pseudomonas aeruginosa* isolates from non-cystic fibrosis patients in Thailand

4.1 Abstract

This study aimed to examine aminoglycosides (AMGs) resistance mechanisms, including the AMG-modifying enzyme genes, *mexXY*, *rplY*, *nuoG*, and *galU*, in the *Pseudomonas aeruginosa* non-cystic fibrosis (CF) isolates in Thailand. One hundred *P. aeruginosa isolates* from non-CF patients were examined for susceptibility to AMGs and for the presence of 10 AMG-modifying enzyme genes. Thirty randomly selected isolates were tested for transcription of *mexXY* and *nuoG* and mutations in *rplY* and *galU*. All the *P. aeruginosa* isolates exhibited simultaneous resistance to at least 4 AMGs. High resistance rates to amikacin (92%), gentamicin (95%), streptomycin (99%), and tobramycin (96%) were observed, and all isolates were resistant to kanamycin, neomycin, and spectinomycin. Nine AMG-modifying enzyme genes were detected, including *aadA1* (84%), *aadB* (84%), *aadA2* (67%), *ant(2″)-Ia* (72%), *strA-strB* (70%), *aph(3')-IIb* (57%), *aac(3')-Ia* (40%), and *aac(6')-IIa* (27%). None of the isolates harbored *aac(6')-IIb*. Of 30 isolates tested, all but 1 isolate expressed MexXY. Two isolates did not express *nuoG*. Six isolates carried an amino acid change in RplY, but none of the isolates harbored mutation in galU. The results indicated that the AMG-modifying enzyme genes were widespread among the *P. aeruginosa* non-CF isolates. The MexXY efflux pump and inactivation for *rplY* played a role in AMG resistance but disruption of *nuoG* or *galU* did not.

Keywords: aminoglycoside-modifying enzymes, aminoglycoside resistance, MexXY, *Pseudomonas aeruginosa*, *rplY* gene

4.2 Introduction

Pseudomonas aeruginosa represents the most frequent cause of lung infection in cystic fibrosis (CF) that is a genetic disease common within the caucasian population (Costerton, 2001, Pier, 1998). While CF varies in prevalence among different racial and ethnic groups, the disease is rare in native Africans and Asians (Hamosh et al., 1998). Concurrently, the pathogen is the most well-known cause of hospital-acquired infections, particularly in immunocompromised and burn patients, worldwide including Thailand. *P. aeruginosa* is notorious for resistance to various antibiotics and considered the ultimate superbugs. Several antibiotics are currently available for treatment of *P. aeruginosa* infections. Among these, aminoglycosides (AMGs) are potent bacteriocidal antibiotics that are a vital component for the antipseudomonal combinations and mostly used in synergy with β-lactams. Two major drawbacks with AMG use include host toxicity and bacterial resistance. Their nephro-, oto- and audiotoxicity appear to be lessen by increasing dosing intervals and adjusting administration routes e.g. aerosolization in lung infection (Turnidge, 2003). However, resistance to AMGs has been increasing and become an important issue in *P. aeruginosa* therapy (Kim et al., 2008, Over et al., 2001, Vaziri et al., 2011).

Resistance to AMGs typically occurs via drug inactivation, impermeability and active efflux pumps (Poole, 2005). Among these mechanisms, the inactivation by plasmid- or chromosome-encoded modifying enzymes, traditionally including AMG phosphoryltransferase (APH), AMG acetyltransferase (AAC) and AMG nucleotidyltransferase (ANT) is the most predominant (VakulenkoMobashery, 2003).

An individual *P. aeruginosa* could co-harbors multiple modifying enzyme genes, resulting panaminoglycoside resistance phenotype (Vaziri et al., 2011).

In addition to enzymatic mechanisms, nonenzymatic AMG resistance mechanisms including the *mexXY* multidrug efflux system, *nuoG, rplY* and *galU* have been identified on *P. aeruginosa* chromosome (El'Garch et al., 2007). Of 12 pseudomonal multidrug efflux pumps, the MexXY-OprM efflux pump is the only member of Resistance-Nodulation-Cell division family that has been known to export AMGs (Aires et al., 1999, Masuda et al., 2000). Inactivation of four genes including *nuoG, rplY* and *galU* gradually increases AMG resistance due to reduced proton motif force, modification of AMG target and impaired AMG binding and uptake in the laboratory strain, respectively (DeanGoldberg, 2002).

Despite the problems, AMGs are still a pivotal antibiotic for treatment of a severe *P. aeruginosa* infection. Since the distribution of AMG-resistance genes and other AMG-resistance dynamically changes depending on different selection pressure and strains variation, the study of resistance rate and relevant mechanisms is periodically required. AMG-resistance mechanisms have been extensively studied in *P. aeruginosa* but mostly conducted in the CF isolates. This study aimed to determine the occurrence of AMG-modifying enzyme genes and the involvement of enzyme-independent mechanisms in AMG resistance in the non-CF *P. aeruginosa* isolates

4.3 Materials and Methods

4.3.1 Bacterial isolates

One hundred *P. aeruginosa* isolates were randomly selected from the stock of Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand. They were originated from respiratory tract (32%), urogenital tract (21%), wound and pus (28%) and others (i.e cerebrospinal fluid, catheter and blood) (15%) of patients admitted in the hospital. The clinical samples were collected, as part of routine clinical practice, from patients initially receiving empirical treatment with β-lactams-AMG combinations (i.e. ceftaxidine/gentamicin and/or imipenem/amikacin) and admitted at the hospital. All the samples were submitted for bacterial diagnosis and antimicrobial susceptibility test for clinical treatment at Clinical Microbiology Laboratory. One sample was obtained from each patient. The VITEK GNI card (bioMérieux Vitek, Inc., Hazelwood, Mo.) and the API 20NE system (bioMérieux, Inc.) were used to identify all the isolates. A single isolate was collected from each positive sample that was the first sample from each patient and stored at -80°C in 20% glycerol. The clonal relatedness of the isolates was not assessed.

4.3.2 Antimicrobial susceptibility testing

Antimicrobial susceptibilities to seven AMGs, including amikacin, gentamicin, kanamycin, neomycin, streptomycin and spectinomycin were determined by using determination minimal inhibitory concentration (MIC) in our previous study (Poonsuk et al., 2012). Susceptibility to tobramycin was additionally performed in this study

using two-fold agar dilution method (CLSI, 2008). *P. aeruginosa* ATCC 27853 and PAO1 were used as control strains.

4.3.3 PCR, RT-PCR, and DNA sequencing

PCR-template DNA were whole cell boiled lysates prepared as previously described (Levesque et al., 1995). All PCR primers are listed in Table 6. PCR amplifications were performed using KAPA Taq ReadyMix (KAPAbiosystem, MA, USA) according to the manufacturer's instruction. The *galU* and *rplY* genes were amplified using primer pairs/rplYF902-L and galUF14o-U/galUR1121-L, respectively. All PCR products were purified from agarose gel using Nucleospin® ExtractII (Mccherey-Nagel, Düren, Germany) and sent for nucleotide sequencing at Macrogen Inc. (Seoul, South Korea). The resulting-DNA sequence were compared with PA2023 and PA4671 available at the Pseudomonas Genome Project (http://pseudomonas.com) for *galU* and *rplY*, respectively (Winsor et al., 2011).

The presence of 10 AMG-modifying enzyme encoding genes were determined using PCR with the following primers: *aadA1*, aadA1-F/aadA1-R; *aadA2*, aadA2-F/aadA2-R; *aadB*, aadB-F/aadB-R; *aac(3')-Ia*, aac3Iaup/aac3Iadown; *aac(6')-IIa*, aac6IIaup/aac6IIadown; *aac(6')-IIb*, aac6IIbup/aac6IIbdown; *aph(3')-IIb*, aph3IIbup and down and *ant(2")-Ia,* ant2Iaup/down.

Total RNA was isolated using Total RNA Extraction Mini Kit (RBC Bioscience, New Taipei City, Taiwan) and DNA contaminants were removed by treatment with RNase-free DNaseI (Fermentas, Ontario, Canada). cDNA was synthesized from one µg of total RNA using ImProm-II™ Reverse Transcriptase (Promega, WI, USA) with the

reverse primer nuoHL823-L. The conventional PCR were performed using nuoHF657-U and nuoHL823-L as described above.

4.3.4 Statistical analysis

Statistical analysis was carried out using Fisher’s exact test in STATA software Version 8.0 (STATA Corp., College Station, TX, USA). A *P* value of <0.05 was considered statistically significant. Odd ratios for significant associations were calculated. OR>1 represents positive associations and OR<1 represents negative associations.

Table 6 Primers used in this study

Gene	Primer	Sequence (5′-3′)	Reference
rplY	rplYF144-U rplYF144-L	ATCGCCCGAACGCTGGT ATGCCGGGTCTGGTCGTATTC	Islam et al., 2009
galU	galUF14o-U galUR1121-L	CGAGCGCAGCCTGATTAGACT ACAGCTCAGGTAGGGCGGATA	Islam et al., 2009
mexY	mexYRTUP mexYRTdown	AGCTACAACATCCCCTA AGCACGTTGATCGAGAAG	Chuanchuen et al., 2008
nuoH	nuoHF657-U nuoHL823-L	GCAGGAACTGGCGGACGG GGTCTTGGCGGCGAAGTAGAA	Islam et al., 2009
aadA1	aadA1-F aadA1-R	CTCCGCAGTGGATGGCGG GATCTGCGCGCGAGGCCA	Chuanchuen and Padungtod, 2009
aadA2	aadA2-F aadA2-R	CATTGAGCGCCATCTGGAAT ACATTTCGCTCATCGCCGGC	Chuanchuen and Padungtod, 2009
aadB	aadB-F aadB-R	CTAGCTGCGGCAGATGAGC CTCAGCCGCCTCTGGGCA	Chuanchuen and Padungtod, 2009
aac(3')-Ia	aac3Iaup aac3Iadown	CTGACCAAGTCAAATCCATGCGGG CCACTGCGGGATCGTCACCG	Chuanchuen et al., 2008
aac(6')-IIa	aac6IIaup aac6IIAdown	AGAGCGATGGCGGAAGAGTCC ATCCTGCCTTCTCATTGCAGCG	Chuanchuen et al., 2008
aac(6')-IIb	aac6IIbup aac6IIbdown	CCGAAGAAGGAGTGACGCCG GCGCAAACCGTTCACCAACGG	Chuanchuen et al., 2008
aph(3')-IIb	aph3IIbup aph3IIbdown	GAACGAAACCCAGAGCGACGG CAATCGATGAAGCCGCTGAAGC	Chuanchuen et al., 2008
ant(2")-Ia	ant2Iaup ant2Iadown	TGGAGCAGCAACGATGTTACGC CCACTGGTGGTACTTCATCGG	Chuanchuen et al., 2008
strA	strA-F strA-R	TGGCAGGAGGAACAGGAGG AGGTCGATCAGACCCGTGC	Chuanchuen and Padungtod, 2009
strB	strB-F strB-R	GCGGACACCTTTTCCAGCCT TCCGCCATCTGTGCAATGCG	Chuanchuen and Padungtod, 2009

4.4 Results

4.4.1 Antimicrobial susceptibilities

All the *P. aeruginosa* isolates were simultaneously resistant to at least four AMGs, while most exhibited high AMG resistance level (fig. 4). Eighty-six percent were resistant to all AMGs tested. One hundred percent of the isolates were resistant to kanamycin (MIC range 256->256 μg/ml), neomycin (MIC range 64->256), and spectinomycin (MIC range 256->256). Resistance rates to amikacin, gentamicin, streptomycin and tobramycin (MIC ranges 8-2048 μg/ml, 1->256 μg/ml, 4->256 μg/ml and 8->256 μg/ml) were 92%, 95%, 99% and 96%, respectively.

Figure 4 Distribution of minimum inhibitory concentration (MIC) of amikacin, gentamycin, neomycin, streptomycin, spectinomycin and tobramycin for 100 *Pseudomonas aeruginosa* isolates.

4.4.2 The presence of AMG-modifying enzyme-encoding genes

All the *P. aeruginosa* strains were screened for 10 AMG-modifying enzyme encoding genes (Table 7). All but one isolate were positive to at least one AMG-resistance genes. An individual isolates carried multiple AMG-modifying enzyme encoding genes up to nine genes. While most strains harbored *aadA1* (84%) and *aadB* (84%), none of them were positive to *aac(6')-IIb*. The *aadA2*, *ant(2")-Ia*, *strA-strB* genes were present at the similar rate, 67%, 72% and 70%, respectively. The prevalence of the other AMG-modifying enzyme genes tested were as follows: *aph(3')-IIb* (57%), *aac(3')-Ia* (40%) and *aac(6')-IIa* (27%), respectively. The AMG-modifying enzyme encoding genes were arranged in to 53 patterns, of which the most common pattern were *aac(3')-Ia*, *aph(3')-IIb*, *ant(2")-Ia*, *aadB*, *aadA1, aadA2*, *strA-strB* (9%) and *aac(3')-Ia*, *ant(2")-Ia*, *aadB*, *aadA1*, *aadA2*, *strA-strB* (9%). In most cases, the presence of each resistance gene matched to the resistance phenotype. However, PA190 (gentamicin MIC=4 μg/ml) was positive to *aadB, ant(2")-Ia*, and *aac(6')-IIa* and susceptible to gentamicin.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.4.3 Expression of MexY and NuoH

Thirty *P. aeruginosa* isolates were randomly selected and tested for expression of *mexY* and *nuoH* (Table 8). All but one isolate expressed MexY. Transcription of *nuoH* was detected in 28 isolates. Both isolates without *nuoH* expression were resistant up to six AMGs.

4.4.4 Mutation in *rplY* and *galU*

Thirty *P. aeruginosa* isolates that were assessed for *mexY* and *nuoH* expression were further examined for the presence of mutations in *rplY* and *galU*. Nucleotide sequencing analysis revealed that six isolates were found to contain a single nucleotide change G367-T in *rplY*, leading to Ala123-Ser in RplY. No mutations were found in *galU*.

4.4.5 Associations between resistance genes and phenotypes

When the associations between resistance genes and phenotypes were statistically analyzed, the strong-positive associations ($P<0.05$) were observed between *ant(2")-Ia* and gentamicin resistance phenotype (OR=11.83) and *aadA*2 and amikacin resistance phenotype (OR= 17.77). The associations between resistance genes were also tested. The positive associations ($P<0.05$) were between gene pairs *aadB/aadA1*(OR=6.40), *aadA1/aadA2* (OR=4.42), *aadA2/strA-strB*(OR=2.8) and *ant(2")-Ia/strA-strB*(OR=2.8). Only *aac(6')-IIa* exhibited negative association ($P<0.05$) with *aadB* (OR=0.29).

Table 7 AMG-modifying enzyme genes and resistance patterns in *P. aeruginosa* (*n*=100)

AMG-modifying enzyme encoding gene (No. of isolates)[a]	Resistance phenotype (No. of isolates)
aac(3')-Ia, aac(6")-IIa, aph(3')-IIb, ant(2')-Ia, aadB, aadA1, aadA2, strA-strB(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
aac(3')-Ia, aac(6")-IIa, ant(2')-Ia, aadB, aadA1, aadA2, strA-strB(3)	AMK-GEN-KAN-NEO-STR-SPC-TOB(3)
aac(3')-Ia, aph(3')-IIb, ant(2')-Ia, aadB, aadA1, aadA2, strA-strB(9)	AMK-GEN-KAN-NEO-STR-SPC-TOB(9)
aac(3')-Ia, aph(3')-IIb, ant(2')-Ia, aadB, aadA1, strA-strB(3)	AMK-GEN-KAN-NEO-STR-SPC-TOB(3)
aac(3')-Ia, ant(2')-Ia, aadB, aadA1, aadA2, strA-strB(9)	AMK-GEN-KAN-NEO-STR-SPC-TOB(9)
aac(6")-IIa, aph(3')-IIb, ant(2')-Ia, aadB, aadA1, strA-strB(3)	AMK-GEN-KAN-NEO-STR-SPC-TOB(3)
aph(3')-IIb, ant(2')-Ia, aadB, aadA1, aadA2, strA-strB(5)	AMK-GEN-KAN-NEO-STR-SPC-TOB(5)
aac(6")-IIa, aph(3')-IIb, aadB, aadA1, aadA2(3)	GEN-KAN-NEO-STR-SPC-TOB(1)
	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
aph(3')-IIb, ant(2")-Ia, aadB, aadA1, aadA2(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
aph(3')-IIb, ant(2')-Ia, aadB, aadA1, strA-strB(3)	AMK-GEN-KAN-NEO-STR-SPC-TOB(3)
aph(3')-IIb, aadB, aadA1, aadA2, strA-strB(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
ant(2')-Ia, aadB, aadA1, aadA2, strA-strB(7)	AMK-GEN-KAN-NEO-STR-SPC-TOB(7)
aac(3')-Ia, ant(2')-Ia, aadB, aadA1(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
aph(3')-IIb, ant(2')-Ia, aadB, aadA1(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB(1)
	GEN-KAN-NEO-STR-SPC-TOB(1)
aph(3')-IIb, aadB, aadA1, strA-strB(2)	KAN-NEO-STR-SPC(1)
	GEN-KAN-NEO-STR-SPC-TOB(1)
aph(3')-IIb, aadB, aadA2, strA-strB(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
aadB, aadA1, aadA2, strA-strB(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
aph(3')-IIb, ant(2')-Ia, aadB(2)	GEN-KAN-NEO-STR-SPC-TOB(1)
	AMK-GEN-KAN-NEO-STR-SPC-TOB(1)
aadB, aadA1, aadA2(2)	AMK-KAN-NEO-STR-SPC-TOB(1)
	AMK-GEN-KAN-NEO-STR-SPC-TOB(1)
aadB, aadA1(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)

[a]Only the patterns with at least two isolates are shown.

Table 8 AMG-resistance mechanisms in *P. aeruginosa* (n=30)

Group	AMG-resistance mechanism[a]			No.(%)	AMG-modifying enzyme encoding gene[b,c]	AMG-resistance pattern[b]
	mexY	*nuoH*	*rplY*			
I	+	+	-	23(76.7)	*aac(3')-Ia, aph(3')-IIb, ant(2")-Ia, aadB, aadA1, aadA2, strA-strB*(3)	AMK-GEN-KAN-NEO-STR-SPC-TOB(3)
					aac(3')-Ia, aph(3')-IIb, ant(2")-Ia, aadB, aadA, strA-strB(3)	AMK-GEN-KAN-NEO-STR-SPC-TOB(3)
					aph(3')-IIb, ant(2")-Ia, aadB, aadA1,aadA2, strA-strB(3)	AMK-GEN-KAN-NEO-STR-SPC -TOB(3)
					aac(3')-Ia, aph(3')-IIb, ant(2")-Ia, aadB, aadA1, aadA2	AMK-GEN-KAN-NEO-STR-SPC-TOB
					aac(3')-Ia, aac(6')-IIa, aph(3')-IIb, ant(2")-Ia, aadB, aadA1	GEN-KAN-NEO-STR-SPC-TOB
					aac(6')-IIa, aph(3')-IIb, aadA1,aadA2, strA-strB	AMK-GEN-KAN-NEO-STR-SPC-TOB
					aac(6')-IIa, aph(3')-IIb, aadB, aadA1, strA-strB	AMK-GEN-KAN-NEO-STR-SPC-TOB
					aph(3')-IIb, aadB, aadA1,aadA2, strA-strB	AMK-GEN-KAN-NEO-STR-SPC-TOB
					aac(6')-IIa, aph(3')-IIb, aadB, aadA1,aadA2(3)	GEN-KAN-NEO-STR-SPC-TOB
					aph(3')-IIb, aadB, aadA1, strA-strB	AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
					aac(6')-IIa, aph(3')-IIb, ant(2")-Ia, aadB	AMK-GEN-KAN-NEO-STR-SPC-TOB
					aph(3')-IIb, ant(2")-Ia, aadB, aadA1	AMK-GEN-KAN-NEO-STR-SPC-TOB
					aac(3')-Ia, aph(3')-IIb, aadB	GEN-KAN-NEO-STR-SPC-TOB
					aph(3')-IIb, ant(2")-Ia, aad (2)	AMK-KAN-NEO-STR-SPC-TOB
						AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
II	+	+	Ala123-Ser	5(16.7)		AMK-GEN-KAN-NEO-STR-SPC-TOB(2)
					aph(3')-IIb, aadB, aadA2, strA-strB(2)	AMK-GEN-KAN-NEO-STR-SPC-TOB
					aph(3')-IIb, aadB, aadA1, aadA2	KAN-NEO-STR-SPC
					aph(3')-IIb, aadB, aadA1, strA-strB	AMK-GEN-KAN-NEO-SPC-TOB
III	+	-	-	1(3.3)	*aph(3')-IIb*	AMK-GEN-KAN-NEO-STR-SPC-TOB
IV	-	-	Ala123-Ser	1(3.3)	*aac(6')-IIa, aph(3')-IIb, aadA1, aadA2*	AMK-GEN-KAN-NEO-STR-SPC-TOB
					aph(3')-IIb, aadA2, strA-strB	

Abbreviation: AMI, amikacin; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; STR, streptomycin; SPC, spectinomycin; TOB, tobramycin

[a] +, expressed; -, not expressed

[b] The patterns represented by only one isolate are shown without number.

[c] Each gene encodes AMG resistance as follows: *aadA1*, streptomycin and spectinomicin; *aadA2*, streptomycin and spectinomicin; *aadB*, gentamicin, kanamicin and tobramicin; *aac(3')-Ia* gentamicin; *aac(6')-IIa*, gentamicin and tobramicin; *aac(6')-IIb*, gentamicin and tobramicin; *aph(3')-IIb*, gentamicin, kanamycin and neomycin; and *ant(2")-Ia*, gentamicin, kanamycin and tobramicin

4.5 Discussion

The *P. aeruginosa* strains in this study exhibited multiresistance to AMGs with high resistance level. Overall, AMG resistance rates in this study were higher than those in previous studies (Kim et al., 2008, Over et al., 2001, Vaziri et al., 2011). Such high resistance rate and level could be explained by prior exposure to AMGs in empirical combination therapy.

Most isolates exhibited resistance phenotype consistent to AMG-modifying enzymes observed, suggesting the regular expression of the genes. Interestingly, PA190 (gentamicin MIC=4 μg/ml) carried up to three gentamicin-resistance encoding genes but was still susceptible to the antibiotic based on MIC determination. The exact reason for the sensitive phenotype of this strain is still unknown. However, similar observations were previously demonstrated in *Staphylococcus epidermidis* susceptible to oxacillin and/or gentamicin (Martineau et al., 2000) and *S. aureus* susceptible to oxacillin and/or erythromycin (Martineau et al., 2000). Few explanations were suggested to explain these phenomena, including the regulation of gene expression, lack of host factors required for the phenotypic expression and the borderline of MIC breakpoints (Martineau et al., 2000)*a*, 2000*b*). In this study, *P. aeruginosa* isolates contained multiple-AMG resistance genes, inconsistent with previous studies reporting that the majority of resistant isolates harbored only a single AMG-modifying gene (Miller et al., 1997). However, the findings could explain the panaminoglycoside resistance patterns of the strains in this collection. The most frequent resistance genes were *aadA1* (84%) and *aadB* (84%). Both *aadA1* and *aadB*

genes were commonly found in foodborne pathogens, particularly *Salmonella enterica* and *Escherchia coli* (ChuanchuenPadungtod, 2009, SundeNorstrom, 2006), indicating their wide circulation among different bacterial species The existence of the identical genes in different bacterial hosts from different sources suggests their localization of transferable element e.g. plasmid, transposon. However, the horizontal transfer was not tested in this study.

The *ant(2")-I* and *aac(6')-II* genes represent the most common AMG-modifying enzyme genes in *P. aeruginosa* (Poole, 2005, Vaziri et al., 2011). In this study, *ant(2")-Ia* was detected at high rate (72%) that is much higher than that was reported in U.S.A. and Europe (Miller et al., 1997). Of two *aac(6')-II* genes tested, only *aac(6')-IIa* gene was found (27%). The reason for such dominance remains unclear and may be associated with the type of AMGs used in the country. The *aac(6')-II* gene was prevalent in Iran (36%)(Vaziri et al., 2011) and Europe (32.5%) (Miller et al., 1997). Conversely, a Korean nationwide study showed the absence of *aac(6')-II* among the *P. aeruginosa* isolates (Kim et al., 2008). The variation was suggested to be a result of different types of AMGs used in different counties, difference in bacterial strains and different-geographical presence of AMG-modifying genes (Vaziri et al., 2011).

Statistical analyses revealed the strong positive associations between some resistance gene pairs. The possible explanation could be co-localization of the genes on the same genetic elements or the distribution of the same clonal isolates. However, genetic relatedness and plasmids were not analyzed in this study. Strong positive association of *ant(2")-I* and gentamicin resistance phenotype was also observed, supporting its predominance in the *P. aeruginosa* clinical isolates. In

contrast, *aac(6')-IIa* exhibited negative association with *aadB*. The latter may be explicated by an unstable existence of the genes on plasmids in the same incompatibility groups.

The varied impact of MexXY on AMG resistance has been previously demonstrated in the *P. aeruginosa* human and animal isolates (Chuanchuen et al., 2008, Sobel et al., 2003, Vogne et al., 2004). In this study, almost all of the isolates (*n*=29) expressed MexY. This is corresponded to a previous study showing that high proportion of CF strains (17/20) overproduced MexXY and the expression was associated with AMG resistance (Islam et al., 2009). Another study in the CF isolates also demonstrated a dramatic increase in MexXY production in the AMG resistant strains (Vogne et al., 2004). However, the exact contribution and expression of MexXY was not assessed in the present study. The only strain without MexXY expression (Gr. IV) was resistant to all AMGs tested, indicating that MexXY may not participate in AMG resistance in this isolate. Vice versa, a substantial MexXY production was previously detected in an AMG-susceptible CF isolate (Vogne et al., 2004). Taken together, the data supports that the MexXY efflux system does not always play a crucial role in AMG resistance in each individual *P. aeruginosa* clinical isolate and its significance in AMG resistance varies. The MexXY-non producer carried an Ala123-Ser amino acid change in Rpyl and three modifying enzymes i.e. *aph(3')-IIb, aadA2, strA-strB* that are associated with its AMG resistance. However, these resistance determinants could not explain whole AMG-resistance phenotypes of the strain, suggesting the presence of uncharacterized AMG-resistance mechanisms.

The *nuoG* gene resides in the *nuoABDEFGHIJKLMN* operon and its complete transcription was investigated by determination of *nuoH* transcription. The mRNA production of *nuoH* was not observed in two isolates (Gr. III and IV), in agreement with a previous study reporting that only one of 40 CF isolate had disrupted *nuoG* (Islam et al., 2009). While protonmotive force in these two strains may be damaged due to the disrupted *nuo* operon, one isolate expressed MexXY. In this case, the MexXY pumps in the latter may not fully function.

Six out of 30 strains (Gr. II and IV) carried an Ala123-Ser amino acid substitution in RplY that has never been previously reported in the *P. aeruginosa* isolates. This observation disagreed with a previous report in the CF isolate, where no mutations were found in *rplY* (Islam et al., 2009). This variation may be attributed to the strain variation and difference in selection pressure generated by different AMG use.

No mutations were observed in *galU* in all examined isolates, in agreement with a previous study (Islam et al., 2009). Taken together, inactivation of *galU* is unlikely to play a role in AMG resistance in either the CF or non-CF P. aeruginosa isolates.

The unequal number of isolates used for screening AMG-resistance genes (n=100) and assessing the non-enzymatic AMG resistance mechanisms (n=30) should be also noted. The observations in the latter may not represent the contribution of the same mechanisms in the isolates not tested. Even though the participation of *galU*, *nuoG* and *rplY* in AMG resistance appeared insignificant, the impact of MexXY

and AMG-resistance genes may not be precisely compared in this study. *P. aeruginosa* normally combines several mechanisms to become resistant to an antibiotic. While MexXY partly contributes to AMG resistance (Chuanchuen et al., 2008, Sobel et al., 2003) and synergizes the effect of AMG-modifying enzyme (Morita et al., 2012), its influence is variable in different strains. Due to existence of other as-yet uncharacterized mechanisms, the contribution of each AMG-resistance mechanism cannot be directly from resistance phenotypes or MIC values. Therefore, further investigations are required to evaluate the actual effect of these mechanisms to AMG susceptibility e.g. determination of gene expression and deletion of individual genes by allelic exchange.

In conclusion, the AMG-modifying enzyme genes were widespread in the non-CF *P. aeruginosa*. Of the isolates tested, most strains expressed MexXY. Two isolates did not produce the *nuoG* mRNA. Six carried an amino acid change in RplY but none of the isolates harbored mutation in *galU*. Further studies are required to elucidate uncharacterized AMG-resistance mechanisms in the *P. aeruginosa* non-CF isolates.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER V

Simultaneous overexpression of multidrug efflux pumps in *Pseudomonas aeruginosa* non-cystic fibrosis clinical isolates

Kanchana Poonsuk[1] Chanwit Tribuddharat[2] Rungtip Chuanchuen[1, 3]

[1]Department of Veterinary Public Health, Research Unit in Microbial Food Safety and Antimicrobial Resistance, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

[2]Department of Microbiology, Faculty of Medicine, Mahidol University, Bangkok, Thailand.

Canadian Journal of Microbiology (2014) DOI 10.1139/cjm-2014-0239

Simultaneous overexpression of multidrug efflux pumps in *Pseudomonas aeruginosa* non-cystic fibrosis clinical isolates

5.1 Abstract

The purpose of this study was to examine expression and regulation of six multidrug efflux systems including MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK and MexVW in 13 non-cystic fibrosis (CF) clinical isolates of *Pseudomonas aeruginosa.* These isolates displayed high level of resistance to many clinically-important antibiotics. Some isolates simultaneously overexpressed up to four different Mex systems as determined by quantitative real-time RTPCR. None of the isolates overexpressed MexCD-OprJ and only one isolate overproduced MexJK. All the isolates overexpressed MexXY, while overexpression of MexEF-OprN and MexVW was common. DNA sequencing analysis of regulatory genes showed that no clear correlation could be established between i) the presence of mutations ii) the type of mutations iii) the expression level of the Mex systems and iv) resistance to antibiotic substrates. The results suggest that the concomitant overexpression of some Mex systems may superimpose their antimicrobial drug efflux capabilities, contributing to the multidrug resistance phenotype in the *P. aeruginosa* non CF clinical isolates. The existence of uncharacterized regulators for the Mex systems was signified.

Keywords: Multidrug resistance, Multidrug Efflux System, *Pseudomonas aeruginosa*

5.2 Introduction

As a leading cause of nosocomial infections, *Pseudomonas aeruginosa* is the most common cause of morbidity and mortality in patients with cystic fibrosis (CF) that is most common within the Caucasian population. In non-white populations, *P. aeruginosa* is the most well-known cause of hospital-acquired infections, particularly in immunocompromised individuals, patients undergoing chemotherapy and those with burn injuries.

P. aeruginosa is infamous for exhibiting a high level resistance to a variety of structurally unrelated antimicrobial agents, which is mainly attributed to expression of multidrug efflux systems. The *P. aeruginosa* genome contains at least 12 structural genes for multidrug efflux pumps belonging to the Resistance Nodulation/Cell Division (RND) family (Lister et al., 2009). To date, ten of the RND systems (i.e. MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK, MexGHI-OpmD, MexPMQ-OpmI, MexMN, MexVW and TriABC) have been characterized and only the first four of these efflux pumps have been shown to be of clinical relevance (Lister et al., 2009).

Evidence showed that two Mex systems, MexAB-OprM and MexXY, play a key role in the natural resistance of *P. aeruginosa* (Lister et al., 2009). MexAB-OprM is produced constitutively in *P. aeruginosa* wildtype. In *nalB* mutants, mutations in the neighboring repressor gene, *mexR,* result in overproducing of MexAB-OprM (Srikumar et al., 2000). In contrast, the *nalC* and *nalD* mutants harbor intact *mexR* and mutations in *nalC* and *nalD* genes respectively (Cao et al., 2004, Sobel et al., 2005). Of all characterized RND multidrug efflux pumps, MexAB-OprM confers resistance to

the broadest range of substrates e.g. β-lactams, chloramphenicol, tetracycline, macrolides, novobiocin, fluoroquinolones, sulpamethoxazole and trimetroprim (Lister et al., 2009). MexXY is somewhat unique in *P. aeruginosa*. The pump is constitutively expressed as a result of mutations occurring inside or outside of the repressor *mexZ* gene (Llanes et al., 2004) and is inducible by ribosome inhibitors (e.g. tetracycline, erythromycin and chloramphenicol) (Masuda et al., 2000). MexXY is the only Mex system that plays a crucial role in intrinsic resistance to aminoglycosides (AMGs) and additionally provides resistance to other drugs including tetracycline, macrolides and fluoroquinolones (Masuda et al., 2000).

MexCD-OprJ is typically silent in wild-type strains with overexpression in the *nfxB*-type mutants and contributes to resistance to some β-lactams, fluoroquinolones, macrolides and tetracycline (Morita et al., 2001). Transcription of MexCD-OprJ can be detected in wild-type strains but is insufficient to confer intrinsic resistance. Similarly, MexEF-OprN is normally quiescent in wild-type cells. Overexpression of MexEF-OprN is observed in the *nfxC*-type multidrug resistant mutants, causing resistance to imipenem, fluoroquinolones, trimethroprim and tetracycline (Kohler et al., 1997). MexEF-OprN is a unique RND pump that is modulated by MexT, a LysR-type transcriptional activator encoded by *mexT* located upstream of the operon in the same orientation in *P. aeruginosa* (Maseda et al., 2000). MexEF-OprN is usually co-regulated with OprD. This coregulation is mediated by MexT that upregulate MexEF-OprN and also downregulates OprD at both the transcriptional and posttranscriptional levels (Kohler et al., 1997). Mutants with up-regulated MexEF-OprN and down-regulated OprD exhibit resistance to multiple drugs including carbapenems and the decreased OprD (not overexpressed MexEF-OprN) is a

major contributor to imipenem resistance (Kohler et al., 1997, Ochs et al., 1999). MexJK is not active in wild-type cells, with expression following mutations in the *mexL* repressor. Of all the RND pumps in *P. aeruginosa,* this system has the most limited substrates (i.e. triclosan, erythromycin and tetracycline) (Chuanchuen et al., 2002).

Additional RND efflux pumps i.e. MexGHI–OpmD and MexVW have been reported (Aendekerk et al., 2002, Li et al., 2003). However, their expression and contribution to resistance in clinical isolates has yet to be elucidated. MexGHI–OpmD is expressed in wild-type. It was originally shown to mainly confer vanadium resistance (Aendekerk et al., 2002). Later, this Mex system was shown to mediate resistance to ethidium bromide, acriflavine, rhodamine 6G, and norfloxacin (Morita et al., 2001). The later is only an antibiotic substrate currently known so far. MexVW is silent in wild-type *P. aeruginosa* and its substrate profile includes chloramphenicol, erythromycin, fluoroquinolones, and tetracycline (Li et al., 2003). No genes for regulatory protein exist in the upstream region of the *mexGHI-OpmD* and *mexVW* efflux operons.

Current data has accumulated on expression of MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY in the CF clinical isolates of *P. aeruginosa*. Still, little is known about expression of other Mex systems and limited knowledge exists for the non-CF clinical isolates. In this study, we aim to examine the expression of six RND efflux systems that would have an effect on susceptibilities to multiple antibiotics in the non-CF clinical isolates of *P. aeruginosa*.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

5.3 Materials and methods

5.3.1 Bacterial isolates and growth conditions

Thirteen *P. aeruginosa* clinical isolates from different non-CF patients admitted in Siriraj Hospital, Bangkok, Thailand between 2004 and 2008 were selected because of their high resistance to multiple drugs. The bacterial strains were isolated from sputum (PAJ107, PAJ147, PAJ151, PAJ189, PAJ191 and PAJ208), wound (PAJ111 and PAJ127), pus (PAJ197 and PAJ207) and urine (PAJ166, PAJ172 and PAJ214). The samples were collected from patients admitted to the hospital for routine bacterial identification and antimicrobial susceptibility testing. The complete history of antibiotic therapy was not revealed but most patients received initial empirical antibiotic therapy including β-lactams-AMG combinations (i.e. imipenem/amikacin and (or) ceftaxidine/gentamicin). The VITEK GNI card (bioMérieux Vitek, Inc., Hazelwood, Mo.) and the API 20NE system (bioMérieux, Inc.) was used to identify all the isolates. A single colony was collected from each positive sample. The clonality of all the isolates was examined by using ERIC PCR (Woods et al., 1993) and all were confirmed to be nonrepetitive strains (data not shown). The wild-type *P. aeruginosa* strain PAO1, constitutively producing MexAB-OprM, was used as the reference control throughout the study (WatsonHolloway, 1978). *P. aeruginosa* mutants constitutively overexpressing MexCD-OprJ (PAO200-2) (Chuanchuen et al., 2001), MexFF-OprN (PAO-7H) (Kohler et al., 1997), MexXY (PAO267) (Chuanchuen et al., 2001) and MexJK (PAO238-1)(Chuanchuen et al., 2002) were included as controls in gene expression experiments. All the *P. aeruginosa* strains were grown on Luria Bertani (LB) agar, LB

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

broth (Difco, BD Diagnostic Systems, MD, USA) or in Mueller-Hinton broth (MHB; Difco).

5.3.2 MIC determination

MICs of 17 antimicrobials including amikacin (Amk), aztreonam (Atm), carbapenem (Car), ceftaxidime (Cef), chloramphenicol (Chp), ciprofloxacin (Cip), erythromycin (VakulenkoMobashery), gentamicin (Gen), imipenem (Imp), kanamycin (Kan), neomycin (Neo), piperacillin (Pip), spectomycin (Spc), streptomycin (Str), tetracycline (Tet), tobramycin (Tob) and trimethoprim (Tri) were determined by using two-fold both microdilution method (CLSI, 2008). *P. aeruginosa* ATCC 27853 and wild-type PAO1 were used as control strains.

5.3.3 PCR and DNA sequencing

Genomic DNA template was prepared from overnight culture using QIAamp Mini kit (Qiagen, Hilden, Germany). Nucleotide sequence information for PCR primer design (Table 9) was obtained from the *Pseudomonas* geneome project (http://www.pseudomonas.com) (Winsor et al., 2011). The coding sequences of *mexR*, *nfxB*, *mexT*, *mexL* and *mexZ* and the corresponding intergenic regions were amplified. PCR amplifications were conducted using KAPA Taq ReadyMix (KAPAbiosystem, Boston, MA, USA) according to instruction of manufacturer. The PCR products were gel-purified using Nucleospin® ExtractII (Mccherey-Nagel, Düren, Germany) and sequenced on both strands with the PCR primers at 1st BASE Pte Ltd. (Gemini Singapore Science ParkII, Singapore). The resulting-DNA sequences were compared with the corresponding sequences of PAO1 available at the *Pseudomonas*

Genome Project. All the isolates were screened by PCR for the presence of 10 AMG-modifying enzyme encoding genes using whole cell DNA template as previous described (ChuanchuenPadungtod, 2009, Poonsuk et al., 2013).

5.3.4 RNA purification and cDNA synthesis

Total RNA was isolated from exponential-phase cultures (A_{540}~1) using Total RNA Extraction Mini Kit (RBC Bioscience, New Taipei City, Taiwan) and subsequently treated with RNase-free DNaseI (Fermentas, Ontario, Canada). The RNA concentration was measured by spectrophotometry at 260 nm wavelength (Nanodrop 1000, Thermo Fisher Scientific, DE, USA). Primers used for determination of the *mexB, mexD, mexF, mexK, mexW* and *oprD* transcription level were designed by Primer3 software available at http://frodo.wi.mit.edu/primer3 (Table 2).The primers for *mexY* and *rpsL* genes were previously described (Chuanchuen et al., 2008). One µg of total RNA was used to synthesize cDNA by reverse transcription using ImProm-II™ Reverse Transcriptase (Promega, WI, USA) with the relevant reverse primers (Table 9). The cDNA was stored at -20°C until used.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 9 Primers used in this study

Gene	Primer	Sequence (5'-3')	Reference
qRT-PCR			
mexB	mexBRTup	ATCTACCGGCAGTTCTCC	This study
	mexBRTdown	CGATCACCACGTAGATCAG	
mexD	mexDRTup	CTACCCTGGTGAAACAGC	This study
	mexDRTdown	AGCAGGTACATCACCATCA	
mexF	mexFRTup	CATCGAGATCTCCAACCT	This study
	mexFRTdown	GTTCTCCACCACCACGAT	
mexK	mexKup	ATGCTACGGCCTTCTACC	This study
	mexKdown	CCACGTAGTTGTCGATGC	
mexW	mexWup	GCCCTGTTCAAGGAGTTC	This study
	mexWdown	GGTGTAGCGATTCAGGTAGT	
mexY	mexYRTup	AGCTACAACATCCCCTA	(Chuanchuen et al., 2008)
	mexYRTdown	AGCACGTTGATCGAGAAG	
oprD	oprDup	TCCAAGACCATGCTGAAG	This study
	oprDdown	GCTGAGGTTATCGGTGATT	
rpsL	rpsLrealtimeup	CGGCACTGCGTAAGGTATG	(Costerton, 2001)
	rpslrealtimedown	CCCGGAAGGTCCTTTACACG	
Mutation			
mexR	mexRUp	GCATCCCAGGAAGTCGAG	This study
	mexRDown	AGCTCGGTATTCAGGGTCAC	
nfxB	nfxBup	CACCGTCAGGACCTCCAC	This study
	nfxBdown	GCCGGTGAGGACTGATCTT	
mexT	mexT1up	CAGTTCGAAGCCGAGACC	This study
	mexT1down	AATAGTCGTCGAGGGTCAGC	
	mexT2up	ATCTCCACCGCCATGAGTC	This study
	mexT2down	AGCGGTTGTTCGATGACTTC	
mexL	mexLup	AAAGGCCTGGCTCACCTC	This study
	mexLdown	GCCTACTGGGTCGAGCACT	
mexZ	mexZ2013up	CGCTGGTGATGCCGATAG	This study
	mexZ2013down	GCCTGTCGGTGCTCTACATC	

Table 10 Antimicrobial susceptibilities of the *P. aeruginosa* clinical isolates (*n*=13)

Strain	MIC																	AMG-modifying enzyme gene[a]
	Car	Pip	Cef	Atm	Amk	Gen	Kan	Neo	Str	Spc	Cip	Tet	Ery	Chp	Tri	Tob	Imp	
PAO1	128	8	4	1	8	1.6	128	32	32	256	0.5	32	512	32	256	4	1	ND
PAJ107	>2048	256	4	64	>2048	>256	>256	128	>256	>256	64	>256	>512	>512	256	>256	128	AMG-1
PAJ111	2048	512	8	32	2048	>256	>256	>256	>256	>256	64	>256	>512	>512	>256	>256	128	AMG-2
PAJ127	>2048	256	16	>256	>2048	>256	>256	128	>256	>256	64	>256	>512	>512	>256	>256	32	AMG-1
PAJ147	>2048	128	>256	>256	1024	>256	>256	128	>256	>256	64	>256	>512	>512	>256	>256	64	AMG-3
PAJ151	2048	512	4	32	1024	>256	>256	128	>256	>256	64	>256	>512	>512	>256	>256	16	AMG-1
PAJ166	>2048	256	256	>256	512	256	>256	>256	>256	>256	64	>256	>512	>512	>256	>256	16	AMG-4
PAJ172	>2048	256	>256	>256	256	256	>256	256	>256	>256	128	>256	>512	>512	>256	>256	4	AMG-4
PAJ189	1028	16	64	>256	32	16	>256	256	>256	>256	0.5	>256	>512	>512	>256	>256	16	AMG-5
PAJ191	>2048	256	>256	>256	32	64	>256	>256	>256	>256	16	>256	>512	>512	256	>256	>128	AMG-6
PAJ197	>2048	256	>256	>256	512	>256	>256	256	>256	>256	64	>256	>512	>512	>256	>256	64	AMG-2
PAJ207	128	8	16	16	512	16	>256	>256	>256	>256	64	128	>512	>512	>256	>256	128	AMG-7
PAJ208	128	8	16	>256	32	>256	256	128	>256	>256	1	>256	>512	>512	128	8	128	AMG-6
PAJ214	>2048	128	32	>256	32	>256	>256	>256	>256	>256	32	128	>512	128	>256	>256	16	AMG-8
Breakpoint	512	128	32	32	64	16	16	16	32	32	4	16	8	32	4	16	16	AMG-1

Amk, amikacin; Atm, aztreonam; Car, carbenicillin; Cef, ceftaxidime; Chp, chloramphenicol; Cip, ciprofloxacin; Ery, erythromycin; Gen, gentamicin; Imp, imipenem; Kan, kanamycin; Neo, neomycin; Pip, piperacillin; Spc, spectomycin; Str, streptomycin; Tet, tetracycline; and Tri trimethoprim

[a]AMG-1, *aac(3')-Ia, aph(3')-IIb, ant(2")-Ia, aadB, aadA1, aadA2, strA-strB*

AMG-2, *aph(3')-IIb, ant(2")-Ia, aadB, aadA1, aadA2, strA- strB*

AMG-3, *aac(3')-Ia, aph(3')-IIb, ant(2")-Ia, aadB, aadA1, aadA2*

AMG-4, *aac(3')-Ia, aph(3')-IIb, ant(2")-Ia, aadB, aadA1, strA-strB*

AMG-5 *aac(6')-IIa, aph(3')-IIb, aadB, aadA1, aadA2*

AMG-6, *aph(3')-IIb, aadB, aadA1, strA-strB*

AMG-7, *aph(3')-IIb, ant(2")-Ia, aadB, aadA1*

AMG-8, *aac(6')-IIa, aph(3')-IIb, ant(2")-Ia, aadB*

[c]Each gene encodes AMG resistance as follows: *aadA1*, streptomycin and spectinomicin; *aadA2*, streptomycin and spectinomicin; *aadB*, gentamicin, kanamicin and tobramicin; *aac(3')-Ia* gentamicin; *aac(6')-IIa*, gentamicin and tobramicin; *aac(6')-IIb*, gentamicin and tobramicin; *aph(3')-IIb*, gentamicin, kanamycin and neomycin; and *ant(2")-Ia*, gentamicin, kanamycin and tobramicin

5.3.5 Quantitative real-time PCR

Real-time PCR assays were performed with cDNA originated from one µg of total RNA using KAPA SYBR® FAST qPCR kit (Kapabiosystems) (Chuanchuen et al., 2008, Poonsuk et al., 2013). The amplifications were conducted in triplicates for each gene. The Ct values from three separate experiments (SD< 0.1) were used to estimate the average cDNA copy numbers for each sample and the ribosomal *rpsL* gene was chosen as a reference housekeeping gene. The average cDNA copy number of each gene was normalized with that of *rpsL* and the expression level were presented as fold change-ratios to that of PAO1. The representative amplicons of real-time PCR products were submitted for sequencing and the melting curve analysis was subsequently performed after PCR amplification. The target genes in overproducing mutants and clinical isolates were considered as overexpressed when their transcription levels were at least 3 fold more than those of the corresponding genes in PAO1 (Islam et al., 2004).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

5.4 Results

5.4.1 Antimicrobial susceptibilities and the presence of AMG-modifying enzyme genes

All the clinical isolates of *P. aeruginosa* in this study were resistant to at least 12 antimicrobials tested. All were highly resistant (MIC≥128 µg/ml) to kanamycin, neomycin, streptomycin, spectinomycin, tetracycline, erythromycin, chloramphenicol and trimethoprim. Most strains exhibited high resistance levels (MIC≥128 µg/ml) to

carbenicillin, piperacillin, aztreonam, amikacin, and tobramycin. The MICs for gentamycin, ciprofloxacin and imipenem varies greatly, ranging from 6->256 μg/ml, 0.5-64 μg/ml and 4-128 μg/ml, respectively.

All the isolates were simultaneously positive to at least four AMG-modifying enzyme encoding genes (up to seven genes), of which the most commonly found genes were *aadB* (100%) and *aph(3')-IIb* (100%) (Table 10). None of the isolates were found to contain *aac(6')-IIb*. In all except one isolate, the resistance genes were present where the corresponding resistance phenotypes were observed. PAJ208 was susceptible to tobramycin and found to contain *aadB*. The *aadB* gene originated from the latter was submitted for DNA sequencing and no mutations were found (data not shown).

5.4.2 Quantitative expression of *mexB*, *mexD*, *mexF*, *mexK*, *mexW*, *mexY* and *oprD* genes

All the clinical isolates in this study simultaneously overexpressed at least two Mex systems, of which two isolates expressed concurrently up to four efflux pumps (i.e. PAJ166, MexEF-OprN/MexJK/MexVW/MexXY; PAJ208, MexAB-OprM/MexEF-OprN/MexVW/MexXY).

Most isolates simultaneously expressed three Mex systems, of which the strains with overexpression of MexEF-OprN/MexVW/MexXY (i.e. PAJ127, PAJ151 and PAJ191) were most common.

Four clinical isolates (i.e. PAJ107, PAJ197, PAJ207 and PAJ208) produced MexB 3.1 to 6.2 fold more than PAO1. All the isolates overproduced MexY (3.9 to 5,580 fold).

Eight isolates were *nfxC*-type mutants overproducing MexEF-OprN (3.6-26.2 folds), while overexpression of MexW was common (n=10) and its transcription level varied from 4.3 to 583.6 fold. None of the clinical isolates produced MexCD-OprJ and all except one isolate (i.e. PAJ166) did not overexpress MexK. Ten isolates produced 4.3-29.9 fold more OprD mRNA than PAO1. The relationship between MexF and OprD expression varied. Among 10 OprD-overproducing stains, two isolates (i.e. PAJ127 and PAJ172) produced a comparable amount of MexF and OprD. Three isolates (i.e. PAJ197, PAJ191 and PAJ207) with OprD-down regulation overexpressed MexF (9.9-26.2 fold). In contrast, two strains with upreguation of OprD (i.e PAJ107 and PAJ111) did not produce MexF. The concomitant overexpression of both MexF and OprD was observed in some isolates (PAJ151, PAJ166 and PAJ208).

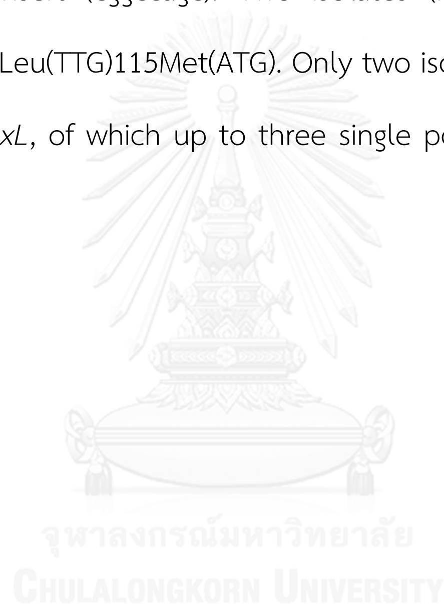
จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

5.4.3 Alterations in regulatory genes

Nucleotide sequencing analyses showed that only two isolates had mutations in *mexR* (i.e. PAJ208, Val(GTG)126Glu(GAG, #19) and PAJ214, Thr(ACC)130Ser(TCC)). PAJ208 is a *nalB* mutant overexpressing 4.4 fold more MexB mRNA than PAO1, while the others did not overproduce MexB. No changes in *mexR* were found in three other strains with elevated levels of MexB (i.e. PAJ107, PAJ197 and PAJ207). None of the isolates carried mutations in *nfxB*.

Sequencing results showed that deletion of 72 nucleotides from nucleotide positions 250 to 321 was common in *mexZ* (n=8) and only one isolate (i.e. PAJ214) had a single base pair change. Three isolates had identical *mexZ* sequence to PAO1. Despite several attempts, PCR amplification of *mexZ* in PAJ191 with the highest MexX expression level (5,880 fold) was unsuccessful.

Analyses of *mexTs* in all the clinical isolates revealed that these *mexTs* did not have an 8-bp insert (cggccagc). Two isolates (i.e. PAJ107 and PAJ111) has additional mutations Leu(TTG)115Met(ATG). Only two isolates (i.e PAJ208 and PAJ214) had mutations in *mexL*, of which up to three single point mutations were found in PAJ214.



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 11 Expression of the Mex systems and mutations in the corresponding regulatory regions in *P. aeruginosa* isolates (*n=13*)

Strain	Transcription level							Regulatory mutation[a]				
	MexB	MexD	MexF	MexK	MexW	MexY	OprD	*mexR*	*nfxB*	*mexT*[b]	*mexL*	*mexZ*[d]
PAO1	1	1	1	1	1	1	1	-	-	(+)	-	-
PAO200-2	ND	83.8	ND	ND	ND	ND	ND	ND	Leu(CTC)264Pro(CCC)[e]	ND	ND	ND
PAO7H	ND	ND	35.9	ND	ND	ND	2.2	ND	ND	(-)	ND	ND
PAO238-1	ND	ND	ND	4.2	ND	ND	ND	ND	ND	ND	Ala(GCC)141Asp(GAC)[f]	ND
PAO267	ND	ND	ND	ND	ND	66.2	ND	ND	ND	ND	ND	Δ*mexZ*[e]
PAJ107	6.2	1.4	0.9	0.28	198.5	1,180.3	29.9	-	-	(-)[c], Leu(TTG)115Met(ATG)	-	Δ72bp
PAJ111	0.7	0.03	1.5	0.84	242.2	76.7	245	-	-	(-)[c], Leu(TTG)115Met(ATG)	-	-
PAJ127	1.5	0.01	3.56	0.09	462.2	128	4.5	-	-	(-)[c]	-	Δ72bp
PAJ147	0.3	0.3	2.5	0.08	191.83	30.6	6.3	-	-	(-)[c]	-	Δ72bp
PAJ151	2.5	0.1	8.6	0.18	383.6	393.1	29.6	-	-	(-)[c]	-	Δ72bp
PAJ166	1.2	0.2	10.4	4.1	85.73	36.9	28.4	-	-	(-)[c]	-	Δ72bp
PAJ172	1.4	0.2	3.2	0.09	1.6	49.3	4.3	-	-	(-)[c]	-	Δ72bp
PAJ189	0.4	0.1	2.5	0.67	7.5	4.0	7.6	-	-	(-)[c]	-	-
PAJ191	1.9	0.3	11.9	0.82	37.4	5,880	1.3	-	-	(-)[c]	-	NA
PAJ197	3.1	0.1	9.9	1.11	1.72	208.1	1.4	-	-	(-)[c]	-	Δ72bp
PAJ207	4.1	0.5	26.2	1.41	2.15	3.9	1.8	-	-	(-)[c]	-	Δ72bp
PAJ208	4.4	0.8	11.8	1.25	4.3	37	6.4	Val(GTG)126Glu	-	(-)[c]	Ser(TCC)4Pro(CCC)	-
PAJ214	1.8	1.1	2.7	1.69	32.6	123.5	6.7	Thr(ACC)130Ser(TCC)	-	(-)[c]	Ser(TCC)4Pro(CCC), Ser(TCC)6Phe(TTC), His(163Arg(CGC)	Ala(GCC)175Val(GTC)

NA No amplifications

ND Not determined

[a]- identical to the PAO1 sequence

[b](+), with or (-), without insertion of 8 nucleotides (cggccagc) between nucleotides 104 and 105 of *mexT*

[c]with additional mutation Phe(TTC)-129-Ile(ATC)

[d]Δ72bp deletion of 72 nucleotides from nucleotide positions 250 to 321 of *mexZ*

[e]identified by Chuanchuen et al. 2001

[f]identified by Chuanchuen et al. 2002

5.5 Discussion

Since the Mex systems have a wide range of antibiotic substrates, up to 17 antibiotics were included for susceptibility testing to cover drugs from most antibiotic categories and those commonly prescribed for treatment of respiratory tract infection in Thailand. All the *P. aeruginosa* clinical isolates in this study exhibited decreased susceptibilities to multiple classes of antibiotics. Regardless, this indicated the expression of one or several nonenzymatic resistance mechanisms and was suggestive of multidrug efflux pump expression.

One of the major findings was the concomitant expression of Mex systems in the non-CF isolates of *P. aeruginosa*. Coexpression of Mex systems has been demonstrated in the CF clinical isolates, for example, MexAB-oprM/MexXY (Hocquet et al., 2006, Llanes et al., 2011), MexXY/MexJK (Hocquet et al., 2006), MexAB-oprM/MexCD-OprJ (Llanes et al., 2011) and MexAB-oprM/MexEF-OprN (PumbwePiddock, 2000). Based on our knowledge, this is the first report of a single *P. aeruginosa* clinical isolate with up to four Mex systems simultaneously expressed.

Most isolates had similar MexAB-OprM expression level compared with PAO1, in agreement with previous studies in both CF and non-CF clinical isolates (Islam et al., 2004, Llanes et al., 2011). One of two strains with impaired *mexR* did not overexpress MexAB-OprM, suggesting other regulatory mechanisms for downregulation of the pump. In contrast, three of four MexAB-OprM overexpressing strains (i.e. PAJ107, PAJ197 and PAJ207) lacked mutations in MexR and may be *nalC*-

or *nalD*-type mutants. However, involvement of *nalC* and *nalD* in MexAB-OprM was not pursued. Regardless, these results confirmed that *mexAB-oprM* expression is not always regulated by its own upstream *mexR* but instead, is modulated by multiple regulatory controls (Evans et al., 2001).

All the clinical isolates in this study produced MexXY at quite variable levels, mostly resulted from partial deletions of *mexZ*. Despite strong MexXY overexpression ($\geq$100 fold) in up to 6 isolates, there was no clear correlation between the transcriptional level of MexY and particular type of *mexZ* mutations, similar to previously observed results in the *P. aeruginosa* clinical isolates (Llanes et al., 2004). Surprisingly, we were unable to PCR-amplify *mexZ*-coding sequence in PAJ191 with the highest MexY mRNA. Different primer pairs and cycling conditions were used to amplify different portions of the gene (data not shown). Despite several attempts, no specific PCR amplicons were obtained. It is possible that there is a partial deletion or rearrangement of chromosome, resulting in truncated MexZ in this isolate. Further investigations to examine the regulatory mechanisms of MexXY expression in the isolate are warranted. Three MexXY-expressing isolates had an intact MexZ, suggesting existence of additional regulatory mechanisms besides inactivation of MexZ that is in agreement with previous studies in both CF and non-CF clinical isolates (Islam et al., 2004, Poonsuk et al., 2013).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

None of the isolates overexpressed MexCD-OprJ, in agreement with previous studies showing that MexCD-OprJ expressing strain is quite rare in CF and non-CF clinical isolates (Llanes et al., 2011). Similarly, only one MexJK producer was found (i.e. PAJ166, 4.1 fold), indicating its infrequent expression in clinical setting. To date,

overexpression of MexJK was reported only in a few non-CF clinical isolates additionally overexpressing MexXY and has never been shown in the CF clinical isolates (Hocquet et al., 2006). Interestingly, the MexJK-overexpressing strain lacked *mexL* mutation while those with mutant-MexL (i.e. PAJ208 and PAJ214) did not produce MexJK. Again, this furthers highlight the complexity of Mex-regulatory system.

In this study, function of MexT as a transcriptional activator was confirmed in two strains (i.e. PAJ107 and PAJ111) with a single-base-pair mutation in *mexT,* producing inactive MexT and leading to low MexF mRNA. Due to sequence variations of *mexT*, MexT regulates MexEF-OprN expression via different pathways in different wild-type *P. aeruginosa* strains (Maseda et al., 2000). In comparison to PAO1, all the *nfxC*-type mutants lacked an 8-bp insertion in *mexT*. This may result from additional mutation (s) or deletion (s) in *mexT* that reversed MexT from the inactive to active form, leading to overproduction of MexEF-OprN (Maseda et al., 2000). Some studies illustrated MexEF-OprN expression associated with mutations in MexS and in this case, active MexT is required (Sobel et al., 2005). Another study demonstrated that MexEF-OprN was controlled by MvaT independent of MexT and MexS (Westfall et al., 2006). A more recent study revealed a more complex picture of MexEF-OprN regulatory mechanism (s) (Wolter et al., 2008). The study revealed the existence of uncharacterized-regulatory mechanism (s) that is not involved in *mexT*, *mexS* or *mvaT* in a levofloxacin-spontaneous mutant derivative of a *P. aeruginosa* clinical isolate (Wolter et al., 2008). However, role of other genes in regulation of MexEF-OprN was not examined in the present study.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Numerous reports have demonstrated downregulation of *oprD* expression in *nfxC*-type mutants (Kohler et al., 1997, Ochs et al., 1999). The *nfxC*-type mutants are resistant to imipenem due to the *concerted* decrease in *OprD, as observed in* three imipenem-resistant isolates (i.e. PAJ91, PAJ197 and PAJ207) (Ochs et al., 1999). However, the links between MexEF-OprN, OprD and imipenem resistance were more complicated in the present study where the intimate coregulation was not always observed. The discrepancy was previously demonstrated (Wolter et al., 2008). Two *mexT* mutants with downregulated MexEF-OprN (i.e. PAJ107 and PAJ111) overexpressed OprD (29.9 and 24.5 fold, respectively), confirming that MexEF-OprN and OprD expression in the clinical isolates is MexT dependent (Sobel et al., 2005). Despite high OprD expression, both PAJ107 and PAJ111 MexEF-OprN remained resistance to impenem, indicating the presence of other imipenem resistance mechanism (s) that can compromise the elevated OprD expression level. Some strains (i.e. PAJ127 and PAJ172) produce MexEF-OprN and OprD at the same level, suggesting existence of alternative pathway of MexEF-OprN regulation without synchronized OprD regulation. PAJ172 was also susceptible to imipenem (MIC=4 µg/ml) and this could result from an increased-outer membrane permeability mediated by its high amount of OprD channel.

Expression of MexVW was originally studied by expressing the efflux pump genes from a plasmid in a laboratory mutant (Δ*mexAB-OprM*Δ*mexCD-OprJ*Δ*mexEF-OprN*Δ*mexXY*) and its role in clinical isolates remains unclear (Li et al., 2003). Interestingly, most clinical isolates in this collection overexpressed MexVW at high level. The observations underscored the significance of MexVW in the non-CF isolates

even though its actual contribution to resistance in clinic was uncertain and not assessed.

When considering resistance to the antibiotic substrates specific for individual Mex system, there was not a link between expression level of each Mex system and antimicrobial-resistance level. For example, PAJ208 the only tobramycin-susceptible isolate showed higher MexXY expression level than tobramycin-resistant isolates. Concurrently, four amikacin-susceptible isolates with the same MIC value (32 μg/ml) produced quite different MexXY mRNA (i.e. PAJ189, 4.0 fold; PAJ191, 5,880 fold; PAJ208, 37 fold and PAJ214, 123.5 fold).

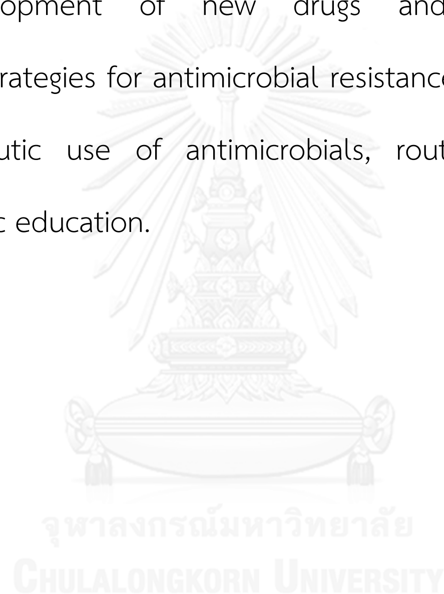
In addition to MexXY expression, an individual AMG-resistant *P. aeruginosa* isolate in this study harbored multiple modifying enzymes, in agreement with previous studies (Poonsuk et al., 2013). Of the two AMG-modifying enzyme encoding genes most commonly found in this strain collection, *aph(3')-IIb* is chromosomally encoded (Hachler et al., 1996). The *aadB* gene and the other less-common genes (i.e. *ant(2")-Ia, aadA1, aadA2* and *strA-strB*) are frequently associated with mobile elements, especially class 1 integrons and plasmid, that facilitate their widespread movement (Bunny et al., 1995, Gilleland et al., 1989, Poonsuk et al., 2013). These AMG-modifying enzymes are partly involved in the high resistance level observed, although their actual contributions were not assessed. In most cases, the presence of AMG-modifying enzyme genes was well corresponded with the resistance phenotype. The exception was observed in PAJ208 a tobramycin-susceptible isolate carrying an intact *aadB* and the reason for non-corresponding resistance phenotype in the strain remains unclear.

Additional example is PAJ207 that was susceptible to aztreonam, carbenicillin and piperacllin but produced higher levels of MexAB-OprM expression than those exhibiting high resistance to the antibiotics. The phenomenon was also observed in PAJ208 susceptible to carbenicillin and piperacllin. *P. aeruginosa* possesses redundant β-lactam-resistance mechanisms and intrinsic resistance to antibiotics in this class is due to interplay between chromosomal β-lactamases and MexAB-OprM (Masuda et al., 1999). Therefore, β-lactam-resistance in MexAB-OprM-deficient isolates most likely resulted from β-lactamases produced by β-lactamase-encoding genes located elsewhere on the chromosome.

Fluoroquinolones are a good substrate for many Mex systems (Li et al., 2003, Llanes et al., 2011, Masuda et al., 2000). Unexpectedly, PAJ189 and PAJ208 is susceptible to ciprofloxacin (MIC=0.5 and 1 μg/ml, respectively) despite coexpression of Mex systems capable of extruding fluoroquinolones (i.e. PAJ189, MexVW/MexXY; PAJ208, MexEF-OprN/MexVW/MexXY). At this point, the reasons underlying the observations are still unclear. Ciprofloxacin resistance in *P. aeruginosa* is mainly due to the synergy between slow uptake and efficient efflux of the drug molecules (Berlanga et al., 2004, Llanes et al., 2011). Therefore, it is most likely that other factor (s) must exist to overcome the efflux capacity of the Mex systems e.g. increased cell wall permeability to ciprofloxacin, overproduction of porins, and increased intracellular accumulation of ciprofloxacin.

In conclusion, the results demonstrated an intriguing and complex picture of expression and regulation of the Mex systems in the *P. aeruginosa* non-CF clinical isolates as previously seen in the CF isolates. Clearly, multiple pathways exist to

participate in the regulation of the Mex systems and contribution of normally silent Mex systems should not be underestimated. To date, the most important therapeutic challenge for *P. aeruginosa* infection is still its multidrug-resistance phenotype, mainly due to overexpression of RND/Mex systems. The *P. aeruginosa* clinical isolates resistant to multiple drugs and coexpressing *simultaneously* many Mex systems is quite common. In a therapeutic viewpoint, efflux pump inhibitors with the broad activity against the Mex pumps would be more clinically useful. In addition to development of new drugs and efflux pump inhibitors, control/prevention strategies for antimicrobial resistance need to be encouraged e.g. responsible therapeutic use of antimicrobials, routine antimicrobial resistance monitoring and public education.



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER VI

Detection of The Mex Efflux Pumps in *Pseudomonas aeruginosa* by Using a Combined Resistance-Phenotypic Markers and Multiplex RT-PCR

Kanchana Poonsuk[1] Rungtip Chuanchuen[1]

[1]Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

Open Journal of Medical Microbiology (2014) DOI 10.4236/ojmm.2014.43018

Detection of The Mex Efflux Pumps in *Pseudomonas aeruginosa* by Using a Combined Resistance-Phenotypic Markers and Multiplex RT-PCR

6.1 Abstract

The aim of this study was to detect the expression of 4 clinically-important efflux pumps in the Resistance-Nodulation-Cell Division (RND) family including MexAB-OprM, MexXY, MexCD-OprJ and MexEF-OprN in *Pseudomonas aeruginosa* using a combination of resistance-phenotypic markers and multiplex RT-PCR (mRT-PCR).The antibiotic substrates specific for each Mex systems were used as phenotypic markers including carbenicillin, MexAB-OprM; erythromycin, MexCD-OprJ; norfloxacin and imipenem, (MexEF-OprN) and gentamicin, MexXY-OprM). The methods were validated with reference strains with known genotypes of the Mex systems and the potential applicability in clinical practice was tested with clinical isolates. The results for the reference strains support that the combination of resistance phenotype and mRT-PCR is a potential-attractive method for diagnosis of efflux-mediated resistance in *P. aeruginosa*. Further development to make it more practical for clinical use and study in a larger number of clinical isolates is required.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Keywords: Multidrug Efflux Pumps, Multiplex RT-PCR, *Pseudomonas aeruginosa*, Resistance-phenotypic marker

6.2 Introduction

Pseudomonas aeruginosa , a common cause of nosocomial infections, is infamous for its resistance to multiple drugs)Aksamit, 1993(that is mainly attributed to the synergy between the low outer membrane permeability and the expression of multidrug efflux systems, particularly in the Resistance-Nodulation-Cell Division (RND) family)Lister et al., 2009(. Most RND-type drug efflux operons are chromosomally encoded. It is now generally accepted that the RND multidrug efflux systems function as tripartite systems consisting of a cytoplasmic membrane-associated RND transporter (e.g. MexB, MexD, MexF, MexY), periplasmic membrane fusion protein (MFP) (e.g. MexA, MexC, MexE and MexX and an outer membrane protein (e.g. OprM, OprJ, and OprN) (PooleSrikumar, 2001). The *P. aeruginosa* genome contains at least 12 structural genes for the RND efflux systems, of which four are clinically-important (i.e. MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY))Lister et al., 2009(. Due to their constitutive expression, MexAB-OprM and MexXY contribute to intrinsic resistance to many antibiotics)Lister et al., 2009(. MexCD-OprJ and MexEF-OprN do not express wild-type cells, but can overexpress after the acquisition of regulatory mutations, resulting in acquired multidrug resistance)Kohler et al., 1997, Morita et al., 2001(. Coexpression of Mex systems has been reported in the *P. aeruginosa* clinical isolates where its variable impact on antibiotic susceptibility has been observed (Aendekerk et al., 2002, Sevillano et al., 2006, Wolter et al., 2004).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Diagnosis of efflux-mediated resistance generates data that is helpful for both routine clinical analysis (e.g. rationalizing the antibiotic selection and dose) and

epidemiological studies (e.g. monitoring the existing and prevalent resistance mechanisms) (Mesaros et al., 2007). Efflux pump inhibitors (EPIs) have been under investigation as an alternative to the development of new antibiotics for treatment of *P. aeruginosa* infection (Lomovskaya et al., 2001). As yet, no EPIs are approved for clinical use. However, detection methods for efflux-mediated resistance should be concurrently developed in preparation for the new treatment protocol. Phenotypic-based methods usually yield vague outcomes due to the possible existence of other resistance mechanisms, the simultaneous overexpression of variable Mexs and the difficulty in assessing the pumps conferring low or moderate resistance level (Mesaros et al., 2007). Detection of the Mex systems has long relied on western blot analysis using monoclonal or polyclonal antibodies. The technique is complicated and time consuming and the antibodies specific for the Mex protein are not commercially available. Quantitative realtime RT-PCR (realtime qRT-PCR) is a rapid method for measuring gene expression. However, many probes are required for simultaneous detection of many Mex genes, resulting in increased cost. In a previous study, realtime qRT-PCR was applied to measure the Mex expression level but limited to that of only 2 Mex systems (i.e. MexAB-OprM amd MexXY) (Yoneda et al., 2005). In contrast, quantitative RT-PCR (qRT-PCR) is less expensive but laborious. The disadvantage may be resolved by concomitantly detecting of multiple Mex genes using multiplex-qRT-PCR (mRT-PCR). The latter allows us to easily observe the amplification products and is feasible in the laboratory where a realtime PCR machine is not available. Recently, the combined phenotypic and genotypic methods were used for detecting the expression of all 4 clinically-important Mex systems (Mesaros et al., 2007). However, two different PCR-based methods were

used for detecting each two systems in the same sample. So far, none of these new diagnostic methods are commercially available.

In this study, we have combined antibiotic-resistance-phenotypic markers and mRT-PCR for detecting the expression of MexAB-OprM, MexXY, MexCD-OprJ and MexEF-OprN in *P. aeruginosa*. The methods were validated in reference strains with known genotypes of the Mex systems and the potential applicability in clinical practice was tested with clinical isolates.

6.3 Materials and Methods

6.3.1 Bacterial isolates and growth conditions

The *P. aeruginosa* reference strains including PAO1 (WatsonHolloway, 1978), PAO200(Chuanchuen et al., 2001), PAO200-2, PAO238, PAO7H1A, PAO255 (Chuanchuen et al., 2001), PAO267 and PAO280 (Chuanchuen et al., 2001, Chuanchuen et al., 2002) and clinical isolates used in this study are listed in Table 13 and 14. The reference strains were selected as carrying known-RND efflux pumps expressed. The clinical isolates were randomly selected from our strain collection. Each of them was isolated from different patients admitted at Siriraj Hospital, Bangkok, Thailand in previous studies (Poonsuk et al., 2013). The clonality of the clinical isolates was examined by using ERIC PCR (Woods et al., 1993) and all were confirmed to be nonrepetitive strains (data not shown). All the *P. aeruginosa* strains were grown on Luria Bertani (LB) agar, LB broth (Difco, BD Diagnostic Systems, MD, USA) or in Mueller-Hinton broth (MHB; Difco). All the bacterial cultures in LB broth

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

were incubated at 37°C, with agitation at 120 rpm and under aerobic condition for 12 h. All the bacteria in LB agar and MHB were grown with aeration at the same temperature and period of time.

6.3.2 Antimicrobial susceptibility testing

MICs of antibiotics tested including carbapenem (Car), erythromycin (VakulenkoMobashery), imipenem (Imi), norfloxacin (Nor) and gentamicin (Gen), were determined by using two-fold both microdilution method in the presence and absence of Phe-Arg-β-naphthylamide (PAβN), a broad spectrum EPI, at the concentration of 50 µg/ml (Sigma Aldrich, St. Louis, MO, USA) (CLSI, 2013). All antibiotics were purchased from Sigma Aldrich. *P. aeruginosa* ATCC 27853 (REF) and wild-type PAO1 were used as control strains.

6.3.3 RNA extraction and cDNA synthesis

The *P. aeruginosa* cells grown in LB were harvested at 12 h of growth (A_{540}~5.5) by centrifugation. The cells were immediately used for RNA extraction using Total RNA Extraction Mini Kit (RBC Bioscience, New Taipei City, Taiwan) and subsequently treated with RNase-free DNaseI (Fermentas®, Mainz, Germany) as suggested by the manufacture's instruction. The absence of genomic DNA residuals was determined by PCR. Synthesis of cDNA was performed by reverse transcription using ImProm-II™ Reverse Transcriptase (Promega, WI, USA). Each 5 µl RNA-primer mixture contained 0.5 µg of free DNA-RNA, 10 pmol of each reverse primer (mexBMRTdown, mexDRTdown, mexFRTdown and mexYMRTdown). The mixture was

incubated at 70°C for 5 min, quickly chilled at 4°C for 5 min and hold on ice. The reverse transcription PCR reaction was performed in a 20 µl volume containing 6 µl of the RNA-primer mixture, 4 µl of Improm-II™ 5X Reaction Buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (10 mM each), 1 µl of Improm-II™ Reverse Transcriptase and nuclease free water added to 20 µl. The PCR cycles were as follows: annealing for 5 min at 25°C, extension for 45 min at 45°C and heat-inactivation for 15 min at 70°C. The cDNA was stored at -20°C until used. All the primers were designed by Primer3 software available at http://frodo.wi.mit.edu/primer3 (Table 12).

Table 12 Primers used in this study

Target	Primer	Sequence (5´-3´)	Amplicon size (bp)	Source
mexB	mexBMRTup	5´-ACTTCTTCAGCTTCAAGGAC-3´	155	This study
	mexBMRTdown	5´-GAGCATGAGGAACTTGTTG-3´		
mexD	mexDRTup	5´-CTACCCTGGTGAAACAGC-3´	250	This study
	mexDRTdown	5’ AGCAGGTACATCACCATCA-3´		
mexF	mexFRTup	5´-CATCGAGATCTCCAACCT-3´	350	This study
	mexFRTdown	5´-GTTCTCCACCACCACGAT-3´		
mexY	mexYMRTup	5´-GCTACAACATCCCCTATGAC-3´	445	This study
	mexYMRTdown	5´-AACTGGCGGTAGATGTTG-3´		

6.3.4 Multiplex RT-PCR

cDNA from each bacterial strain was individually used as DNA template in mRT-PCR, as well as the mixture of cDNA from all reference strains. All the mRT-PCR reactions were carried out in a 30 µl volume containing 5 µl cDNA (100-2000 ng/ml), 10 pmol of each primer, 15 µl of KAPATaq ReadyMix DNA polymerase (Kapabiosystems, MA, USA) and nuclease free water added to 30 µl. The PCR

amplication was performed according to the following cycles: one predenaturation for 5 min at 95°C and 30 cycles of denaturation for 45 s at 95°C, annealing for 45 s at 54°C and extension for 30 s at 72°C, followed by final extension for 10 min at 72°C.

6.3.5 PCR amplification and DNA sequencing

All the conventional PCR amplifications were conducted using KAPA Taq ReadyMix (KAPAbiosystem) according to the manufacturer's instruction. The PCR amplicons from either conventional PCR or mRT-PCR were gel-purified using Nucleospin® ExtractII (Mccherey-Nagel, Düren, Germany) and submitted for sequencing with the PCR primers at 1st BASE Pte Ltd. (Gemini Singapore Science ParkII, Singapore). The DNA sequences obtained were compared with the corresponding sequences of PAO1 available at the *Pseudomonas* Genome Project (Winsor et al., 2011)

6.4 Results

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

6.4.1 Resistance phenotypes associated with the Mex efflux pumps

Five antibiotics were used as resistance-phenotypic markers for 4 clinically-important Mex systems tested in this study: carbenicillin, MexAB-OprM; erythromycin, MexCD-OprJ; norfloxacin and imipenem, MexEF-OprN and gentamicin, MexXY-OprM. The MIC values for these antibiotic markers in the presence and absence of PAβN are shown in Table 13 and 14. For the reference strains overexpressing a Mex pump, the addition of Phenylalanine-Arginine β-Naphthylamide (PAβN) reduced the MIC values for the corresponding antibiotic markers 2 to 128 folds. The lowest-reduction (2 fold)

was observed for imipenem MIC in PAO7H1A overexpressing MexEF-OprN. Most of the clinical isolates exhibited higher resistance level to all antibiotics tested than PAO1 did.

Table 13 Phenotypic and genotypic properties of the *P. aeruginosa* reference strains

Strain	Relevant genotype	Expressed efflux	Antibiotic marker	MIC -PAβN	MIC +PAβN	MIC Reduction fold	Efflux determined by Multiplex RT-PCR
PAO1	Wild-type	MexAB-OprM	Carbenicillin	128	16	8	MexAB-OprM
PAO200	PAO1Δ(*mexAB-oprM*)	None		1	1	0	None
PAO1	Wild-type	MexAB-OprM	Erythromycin	512	256	2	Wild-type
PAO200	PAO1Δ(*mexAB-oprM*)	None		8	8	-	None
PAO200-2	PAO200*nfxB*	MexCD-OprJ		512	4	128	MexCD-OprJ
PAO238	PAO200-2Δ(*mexCD-oprJ*)	None		32	4	8	None
PAO7H1A	PAO7H[a]Δ(*mexAB-oprM*)	MexEF-OprN	Imipenem	8	4	2	MexEF-OprN
PAO255	PAO7H1AΔ(*mexEF-oprN*)	None		1	0.5	2	None
PAO7H1A	PAO7HΔ(*mexAB-oprM*)	MexEF-OprN	Norfloxacin	2	0.06	33	MexEF-OprN
PAO255	PAO7H1AΔ(*mexEF-oprN*)	None		<0.06	<0.06	-	None
PAO267	PAO3579[b] with Δ(*mexAB-oprM*)	MexXY	Gentamicin	2	0.03	64	MexXY
PAO280	PAO267 with Δ(*mexXY*)	None		2	0.25	8	None

[a]PAO7H, overproduced MexEF-OprN (Kohler et al., 1997)
[b]PAO3579, PAO1Δ(*amr*) (Westbrock-Wadman et al., 1999)

Table 14 Antimicrobial susceptibility and expression of the RND efflux determined by Multiplex RT-PCR in the *P. aeruginosa* clinical isolates

Clinical isolate	Relevant genotype	MIC Car -PAβN	MIC Car +PAβN	MIC Ery -PAβN	MIC Ery +PAβN	MIC Imi -PAβN	MIC Imi +PAβN	MIC Nor -PAβN	MIC Nor +PAβN	MIC Gen -PAβN	MIC Gen +PAβN	Efflux determined by Multiplex RT-PCR
PAJ114	-	16384	8192	256	32	256	32	64	16	512	256	AB
PAJ128	-	16384	4096	256	64	256	32	128	16	512	256	AB, CD, XY
PAJ147	-	16384	4096	256	4	64	32	128	16	512	256	AB, CD, XY
PAJ197	-	16384	8192	256	64	>256	16	64	32	256	128	AB, EF, XY
PAJ207	-	128	32	256	64	>256	256	8	1	16	8	AB, EF, XY
PAJ212	-	8192	1024	256	64	256	32	128	16	>16384	1024	AB, XY
PAJ215	-	8192	4092	256	64	256	32	16	16	>16384	4092	AB, XY

\- Not known

AB, MexAB-OprM; CD, MexCD-OprJ; EF, MexEF-OprN, XY, MexXY-OprM

6.4.2 Expression of the Mex efflux pumps determined by mRT-PCR

For the reference strains, the expression of the Mex efflux pumps is shown in Table 13. When the mixture of cDNA from all the reference strains was used for template, PCR amplicons of all 4 Mex systems was obtained (Figure 5) and DNA sequencing analyses confirmed their specificity. By using mRT-PCR, MexB, MexD, MexF and MexY expression was detected in PAO1, PAO200-2, PAO7H1A and PAO267, respectively. None of the Mex expression was observed in PAO200, PAO238, PAO255 and PAO280 that are null mutant derivatives.

All the clinical isolates produced MexAB-OprM and MexXY as determined by mRT-PCR. Four isolates expressed 3 Mex systems including PAJ128 and PAJ147 (overexpressing MexAB-OprM, MexCD-OprJ and MexXY) and PAJ197 and PAJ207 (overexpressing MexAB-OprM, MexEF-OprN and MexXY). All the isolates were resistant to imipenem and norfloxacin but MexEF-OprN expression was observed in 2 isolates (PAJ197 and PAJ207). The similar result was observed for erythromycin resistance.

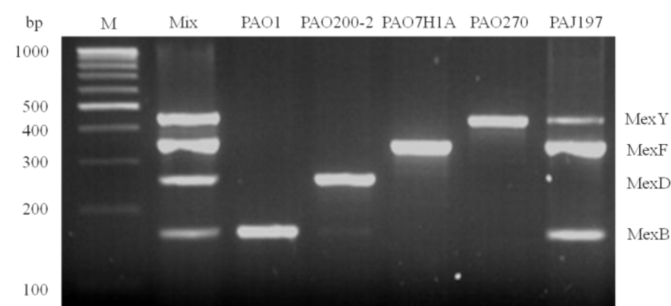


Figure 5 PCR amplicons of 4 clinically important Mex systems generated by RT-PCR. Lane 1) M, molecular weight marker; Lane 2) Mix, The mixture of cDNA from PAO1, PAO200-2, PAO7H1A and PAO27 was used as template. The size of *mexB* (155 bp), *mexD* (250 bp), *mexF* (350 bp) and *mexY* (445 bp). Lane 3-6) band for *mexB* (155 bp), *mexD* (250 bp), *mexF* (350 bp) and *mexY* (445 bp) PAO1 overexpressing MexB; PAO200-2 overexpressing MexD; PAO7H1A overexpressing MexF and PAO270 overexpressing MexY respectively. Lane 7, PAJ197 a clinical isolate overexpressing MexB/MexF and MexY.

6.5 Discussion

For phenotype detection, the antibiotics used for phenotypic markers are specific substrates for each of 4 clinically-important Mex systems based on previous studies (Hocquet et al., 2003, Kohler et al., 1997, Masuda et al., 2000, Murata et al., 2002). For MexEF-OprN, imipenem was also included as an indirect indicator of pump as previously suggested (Mesaros et al., 2007). In the up-regulated MexEF-OprN mutant strain, resistance to carbapenems is a result of down-regulated OprD that happens concomitantly with the elevated-expression of MexEF-OprN (Kohler et al., 1997, Ochs et al., 1999). In the present study, the addition of PAβN caused a 2 fold-reduction of imipenem MIC in both PAO7H1A overexpressing only MexEF-OprN and its isogenic-null mutant PAO255. Similarly, PAβN also reduced the imipenem MIC in all clinical isolates either with or without MexEF-OprN expression. These observations suggest the possible existence of uncharacterized Mex systems that are also inhibited by PAβN in these strains. However, the OprD expression and its actual involvement of MexEF-OprN in the MexEF-OprN-overespressing isolates were not examined. Several studies showed that the synchronized expression and carbapenem resistance was not always observed in clinical isolates (Wolter et al., 2008). It is evident by our observation that some isolates with high imipenem MICs (e.g.PAJ128 and PAJ147) did not produce MexEF-OprN. Taken together, these data confirmed that imipenem is not a good-indirect phenotypic marker for MexEF-OprN.

In the laboratory reference strains, a good agreement was observed between the RND-efflux genotype and the Mex expression determined by mRT-PCR in all the reference strains, (Table 2). For example, only expression of MexD was detected in

PAO200-2 that is a spontaneous *nfxB* derivative of PAO200. The good correlation was also identified between resistance phenotype and the Mex expression. For the instance, PAO1 constitutively producing MexAB-OprM exhibited high carbenicillin MIC. PAO200-2 overexpressing MexCD-OprJ was highly resistant to erythromycin.

Unlike the laboratory reference strains, clinical isolates have diverse genetic backgrounds, resulting in diverse phenotypes. Therefore, it cannot simply use resistance phenotype to predict the expressed-Mex efflux pumps. The discrepancy may be overcome by increasing the number of antibiotic markers to cover all the possible Mex systems. This will markedly cause increasing cost and thus, decreasing the attraction of the method. The addition of a specific Mex inhibitor is of interest but such inhibitors are still not commercially available (Mesaros et al., 2007). Transcription of MexB and MexY was detected in all the clinical isolates, is in agreement with the fact that these efflux systems are always expressed at basal level. The good correlation of resistance phenotype and MexCD-OprJ was observed in two isolates (PAJ128 and PAJ147) and that of MexEF-OprN was observed in PAJ197 and PAJ207. This may not be surprising because MexCD-OprJ and MexEF-OprN are expressed in regulatory mutants and their contribution to antibiotic markers may vary. The addition of PAβN revealed that the contribution of the Mex systems in resistance level varied in clinical isolates and suggested the existence of other enzymatic or non-enzymatic resistance mechanisms. The marginal effect (i.e. 2 fold reduction) of the Mex systems on antibiotic susceptibility was observed in some isolates. It could prevent antibiotics from reaching their optimal concentrations in target organs, especially where the antibiotic concentrations are hindered (e.g. in pus,

biofilms, lung tissues), and therefore is still of clinical importance (Poonsuk et al., 2013).

A comment could be made that the usefulness of mRT-PCR may be less in comparison to realtime qRT-PCR because the Mex expression level was not quantified. Several studies showed no correlation between the level of transcription and resistance in *P. aeruginosa* clinical isolates from either animals or humans (Islam et al., 2004, PoonsukChuanchuen, 2012). Therefore, the measurement of expression level is not always essential for routine diagnosis. Importantly, mRT-PCR could be easily performed in most clinical laboratories, especially those without a sophisticated realtime PCR machine. Still, it cannot be disputed that MIC determination is a gold standard method for assessing susceptibility of *P. aeruginosa* before choosing antibiotic treatment. For better treatment regimen, mRT-PCR will allow optimal antibiotic choices, especially antibiotics available for use in combination with EPIs.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

6.6 Conclusions

The results in this study support that the combination of resistance phenotype and mRT-PCR is a potential-attractive method for diagnosis of efflux-mediated resistance in *P. aeruginosa*. mRT-PCR is rapid and specific for detection of the Mex systems. However, further development to make it more practical for clinical use and study in a larger number of clinical isolates is still required. The appropriate antibiotics that can be specifically used for the Mex pumps need to be explored. Although the knowledge from this study requires more research before the

application in clinical analysis, it is a useful tool for epidemiological studies of the prevalent Mex systems without delay.

CHAPTER VII

GENERAL DISCUSSION

General Discussion

Pseudomonas aeruginosa is an opportunistic pathogen, which is notoriously known for its difficulty in treatment. The pathogen is a leading cause of chronic and recurrent infections in both humans and animals. It represents the most frequent cause of lung infection in cystic fibrosis (CF) that is common within the caucasians. Among non-white population including Thais, the pathogen is the most well-known cause of hospital-acquired infections, particularly in immunocompromised and burn patients. In animals, *P. aeruginosa* causes the most-notably diseases in dogs and cats i.e. otitis externa/media, urinary tract infection and pyoderma.

P. aeruginosa exhibits intrinsic resistance to several antimicrobials simultaneously and potentially acquires resistance to additional antimicrobial drugs. Such multidrug resistance ability limits antimicrobial choice for treatment and could lead to treatment failure. *P. aeruginosa* is usually considered multidrug resistance when the bacterium is resistant to at least three different groups of antimicrobials simultaneously (Falagas et al., 2006, Magiorakos et al., 2012) and this property is outstanding among the clinical isolates.

As seen in other bacteria, mechanisms underlining multidrug resistance phenotype in *P. aeruginosa* are complex and evidences showed that multiple resistance mechanisms are involved in resistance to a single drug (Islam et al., 2009). Resistance mechanisms that have been identified in *P. aeruginosa* include limitation of drug uptake via impermeability of cell wall and cell membrane, alteration of drug targets, enzymatic modification of drug structure and increased drug extrusion by efflux pump (Beinlich et al., 2001, Islam et al., 2004). Among these mechanisms, class

1 integrons and Multidrug efflux (Mex) systems are predominant in *Enterobacteriaceae and Pseudomonadaceae* (Delmar et al., 2014). Integrons is a mobile genetic element that is capable of capturing gene cassette (s) in variable region. Several resistance gene cassettes could be inserted, contributing to multidrug resistance. Integrons are usually carried on plasmid, of which some may be horizontal transferred. Therefore, acquisition of integrons could mediate acquired resistance to the pathogen. In contrast, the Mex systems are usually chromosomal encoded. Mex system confers resistance to multiple drugs that are not structurally related. The substrates of Mex are not limited to antibiotics but include metal or disinfectants. When *P. aeruginosa* exposes to substrates, Mex systems may be overexpressed as a result of regulatory mutations. Some Mex system is also induced to express (Kohler et al., 1997, Kohler et al., 1997, Masuda et al., 2000). Thus, expression of Mex system could promote cross-resistance between antimicrobials, leading to treatment failure (Baker-Austin et al., 2006). Understanding of multidrug resistance mechanisms in clinical strains is critical for development of novel drugs, diagnostic tools and control and prevention strategies for antimicrobial resistance in *P. aeruginosa*. Up to date, class 1 integrons have been studied in *P. aeruginosa*. However, such data is still limited in Thailand. Concurrently, role of Mex systems in multidrug resistance has been extensively studied but rather limited in laboratory strains and the CF isolates. Knowledge on Mex systems in clinical isolates is few and that is much less in Thailand. Therefore, this dissertation was aimed to investigate multidrug resistance mechanisms including integrons, and the Mex systems in *P. aeruginosa* clinically isolated from human and animal patients.

Our research highlighted high resistance level to multiple antibiotics of clinical importance in *P. aeruginosa* clinical isolates. This suggests the extensive use of antimicrobial agents for bacterial treatment in animals and humans. It also confirms that responsible use of antimicrobials should be encouraged. The *P. aeruginosa* clinical isolates from human and animal patients possessed both class 1 integrons with resistance gene cassettes and Mex system (Poonsuk et al., 2012, Poonsuk et al., 2014), supporting the cumulative effect of these resistance mechanisms in antimicrobial resistance in *P. aeruginosa*. However, impact of each system on antimicrobial resistance was not measured and could be a topic of future study.

The human isolates in this study showed high prevalence of class 1 integrons containing resistance gene cassette (69.3%) and five novel resistance cassette arrays (*aacA7-cmlA, aadB-bla*$_{OXA\text{-}10}$-*aadA*1, *aadB*-arr-2-cmlA- *bla*$_{OXA\text{-}10}$-aadA1, aadB-cmlA-aadA1 and aadB-cmlA- *bla*$_{OXA\text{-}10}$-aadA15) were discovered (50.5%) (Poonsuk et al., 2012). This could be a results of different antibiotic selective pressure in hospitals in different countries and also confirms that class 1 integrons could serve as an indicator for emergence and evolution of AMR.

One of the major findings in this study is the simultaneous expression of Mex systems in a MDR *P. aeruginosa*. In the human study, a single isolate could simultaneously overexpressed up to four different Mex systems and all the isolates overexpressed MexXY (Poonsuk et al., 2014). Importantly, expression of normally silent MexEF-OprN and MexVW was common, suggesting that contribution of these quiescent Mex systems should not be underestimated. However, there was no clear correlation between the presence and type of mutations, expression level of the

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Mex systems and resistance to antibiotics. Concomitant overexpression of many Mex systems superimposes their antimicrobial drug efflux capabilities in the *P. aeruginosa* non CF clinical isolates. Regulation of these Mex systems is not straightforward and multiple pathways apparently exist to participate in the regulation of the Mex systems.

The Mex systems in the dog and cat isolates exhibit the similar-sophisticated contribution and regulation. All simultaneously overexpressed up to three different Mex systems. The most profound observation was the prevalent overexpression of MexEF-OprN, a distinctive Mex pump, reflecting genetic diversity of the *P. aeruginosa* isolates from different geographical region and also different antibiotic exposure in different hospital settings.

Complexity of regulatory system of Mex expression was demonstarted in both *P. aeruginosa* isolates from animals and humans (PoonsukChuanchuen, 2012, Poonsuk et al., 2014). Previous studies were suggested regulatory change due to amino acid substitution in efflux regulatory genes lead to overexpression of systems. Nevertheless, there are some isolates overexpressing Mex but lack of amino acid change in their corresponding regulatory genes, indicating existence of uncharacterized regulatory pathway.

Aminoglycosides is one of the common components in antimicrobial combination for treatment of *Pseudomonas* infection, while MexXY is the only Mex system that has been shown to play a role in AMG resistance. Impact of MexXY on AMG MICs was evaluated in the dog and cat isolates by knockout of *mexXY* operon

(PoonsukChuanchuen, 2012). Construction of Δ*mexXY* mutants revealed variation of MexXY effect on AMG MIC level and suggested the involvement of other mechanisms (e.g. AMG-modifying enzymes, target alteration, membrane integrity and mutation in *galU*) in AMR resistance. However, examination of MexXY contribution in AMG resistance in *P. aeruginosa* isolated from human by gene knockout technique was not successful. This was because the strains in this collection exhibited high resistance level to multiple drugs and the antibiotic-resistance counterselectable markers are not available in our laboratory.

In this study, combination of resistance phenotypic markers and multiplex RT-PCR was shown to be useful for rapid detection of Mex expression, leading to better antimicrobials selection and reduction of treatment period and cost. This will also support the promotion of responsible use of antimicrobials.

Expression of Mex systems has been proven to be a major multidrug resistance mechanism in *P. aeruginosa* clinical isolates (PoonsukChuanchuen, 2012). Each Mex system confers resistance to multiple antimicrobials and some Mex systems have the overlapped substrates. Identification of Mex system expression may provide data that may be applied for selection of appropriated antimicrobials. The knowledge could be used for developing tool for rapid identification of Mex systems prior to selecting an antibiotic for treatment. The antimicrobials that are suggested to be avoided in the *P. aeruginosa* clinical isolates expressing Mex systems are listed (Figure 6).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

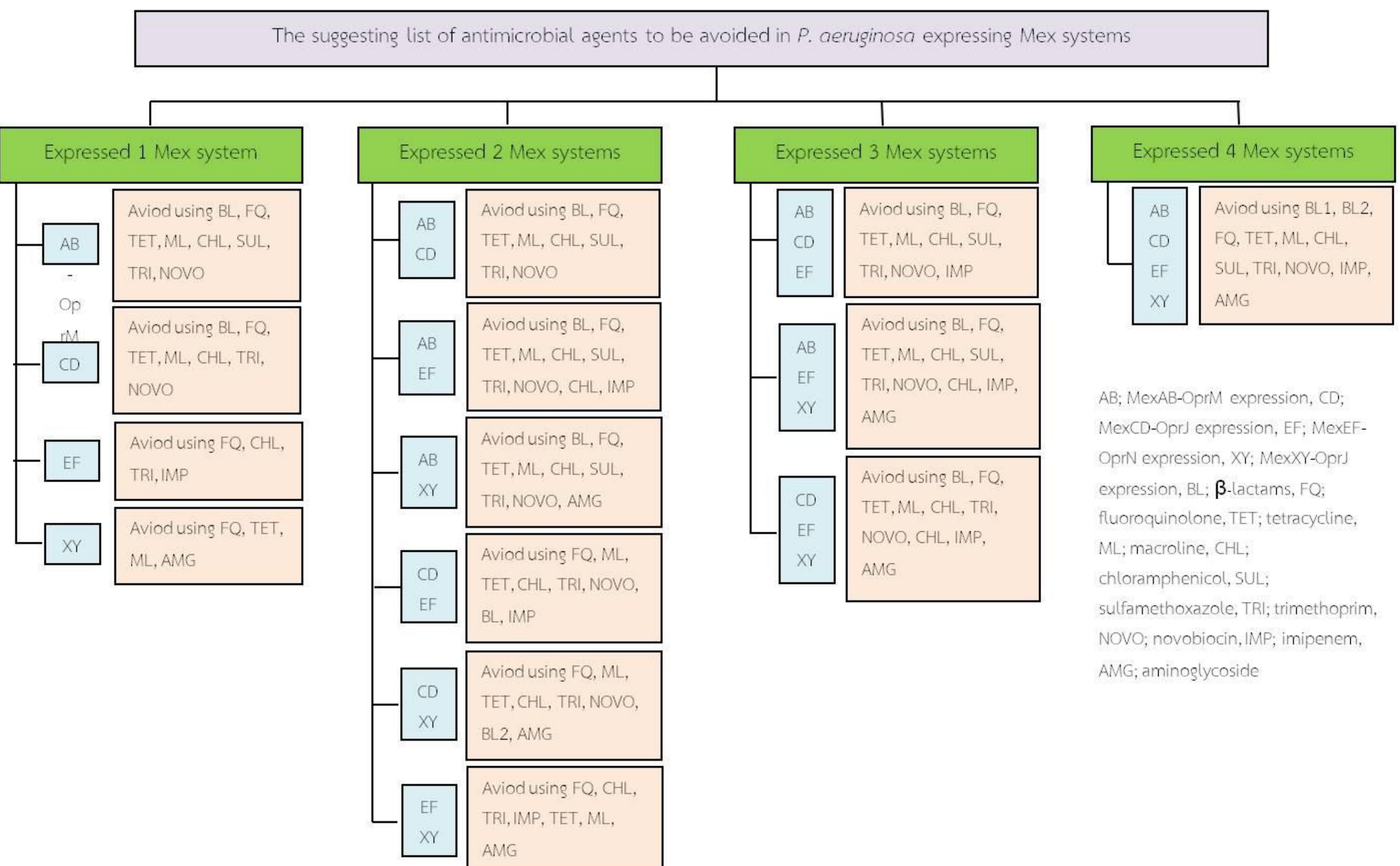


Figure 6 Suggestting list of antimicrobial agents to be avoid in *P. aeruginosa* expressing Mex systems

7.1 Conclusion

This study demonstrated the widespread of multidrug resistance among *P. aeruginosa* clinical isolate from animals and humans. It is evident that multidrug resistance in *P. aeruginosa* could occur via both clonal (Mex systems) and horizontal (class 1 integrons). These raised the need for implementation of control and prevention of antimicrobial resistance in *P. aeruginosa* and also other bacterial pathogens e.g. responsible therapeutic use of antimicrobials, routine antimicrobial resistance monitoring and public education. It points out that the systematic surveillance of antimicrobial resistance and prudent guideline for antimicrobial use in the human, livestock and companion animals should be encouraged.

7.2 Research limitation and suggestion for further investigations

- Antimicrobial susceptibility profile in a bacterial population could dynamically change upon type of antimicrobials exposed, duration and time of exposure, environment and the bacterial physiological condition. Therefore, AMR monitoring is needed to be routinely performed to obtain the current status of resistance trend.

- Using of Efflux Pump Inhibitor (EPI) is a novel therapeutic choice of *P. aeruginosa* infection. Since many Mex systems simultaneously expressed in a clinical isolate, study of broad spectrum EPI will be more useful.

- Detection methods for Mex expression using a combined resistance-phenotypic markers and multiplex RT-PCR was tested in a small group of bacteria. Further evaluation of sensitivity and specificity in a larger bacterial population is required.

- The study of genetic relatedness of *P. aeruginosa* should be performed to identify the potential sources of the epidemiological clones.

REFERENCES

REFERENCES

Adewoye, L., Sutherland, A., Srikumar, R. and Poole, K. 2002. The mexR repressor of the mexAB-oprM multidrug efflux operon in Pseudomonas aeruginosa: characterization of mutations compromising activity. J Bacteriol. 184(15): 4308-4312.

Aendekerk, S., Ghysels, B., Cornelis, P. and Baysse, C. 2002. Characterization of a new efflux pump, MexGHI-OpmD, from *Pseudomonas aeruginosa* that confers resistance to vanadium. Microbiology. 148: 2371-2381.

Aires, J.R., Koehler, T., Nikaido, H. and Plesiat, P. 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. 43: 2624-2628.

Aksamit, T.R. 1993. Pseudomonas pneumonia and bacteremia in the immunocompromised patient. In: Pseudomonas aeruginosa: The opportunist - pathogenesis and disease.ed. R.B.J. Fick (ed). Boca Raton, Ann Arbor, London, Tokyo: CRC Press. 177-188.

Baker-Austin, C., Wright, M.S., Stepanauskas, R. and McArthur, J.V. 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14(4): 176-182.

Beinlich, K.L., Chuanchuen, R. and Schweizer, H.P. 2001. Contribution of multidrug efflux pumps to multiple antibiotic resistance in veterinary clinical isolates of Pseudomonas aeruginosa. FEMS Microbiol Lett. 198(2): 129-134.

Berlanga, M., Montero, M.T., Hernandez-Borrell, J. and Vinas, M. 2004. Influence of the cell wall on ciprofloxacin susceptibility in selected wild-type Gram-negative and Gram-positive bacteria. Int J Antimicrob Agents. 23(6): 627-630.

Boonkerd, N., Pibalpakdi, P., Tiloklurs, M. and Niumsup, P.R. 2009. Class 1 integron containing metallo beta-lactamase gene blaIMP-1 in carbapenem-resistant Pseudomonas aeruginosa in Thailand. J Infect Chemother. 15(4): 257-261.

Boyd, D.A., Shi, X., Hu, Q.H., Ng, L.K., Doublet, B., Cloeckaert, A. and Mulvey, M.R. 2008. *Salmonella* genomic island 1 (SGI1), variant SGI1-I, and new variant

SGI1-O in *Proteus mirabilis* clinical and food isolates from China. Antimicrob. Agents Chemother. 52(1): 340-344.

Boyd, D.A., Shi, X., Hu, Q.H., Ng, L.K., Doublet, B., Cloeckaert, A. and Mulvey, M.R. 2008. Salmonella genomic island 1 (SGI1), variant SGI1-I, and new variant SGI1-O in proteus mirabilis clinical and food isolates from China. Antimicrob Agents Chemother. 52(1): 340-344.

Bunny, K.L., Hall, R.M. and Stokes, H.W. 1995. New mobile gene cassettes containing an aminoglycoside resistance gene, aacA7, and a chloramphenicol resistance gene, catB3, in an integron in pBWH301. Antimicrob Agents Chemother. 39(3): 686-693.

Cao, L., Srikumar, R. and Poole, K. 2004. MexAB-OprM hyperexpression in NalC-type multidrug-resistant Pseudomonas aeruginosa: identification and characterization of the nalC gene encoding a repressor of PA3720-PA3719. Mol Microbiol. 53(5): 1423-1436.

Chuanchuen, R., Beinlich, K., Hoang, T.T., Becher, A., Karkhoff-Schweizer, R.R. and Schweizer, H.P. 2001. Cross-resistance between triclosan and antibiotics in Pseudomonas aeruginosa is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ. Antimicrob Agents Chemother. 45(2): 428-432.

Chuanchuen, R., Beinlich, K., Hoang, T.T., Becher, A., Karkhoff-Schweizer, R.R. and Schweizer, H.P. 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. Antimicrob. Agents Chemother. 45: 428-432.

Chuanchuen, R., Gaynor, J.B., Karkhoff-Schweizer, R. and Schweizer, H.P. 2005. Molecular characterization of MexL, the transcriptional repressor of the mexJK multidrug efflux operon in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 49(5): 1844-1851.

Chuanchuen, R., Khemtong, S. and Padungtod, P. 2007. Occurrence of *qacE/qacED1* genes and their correlation with class 1 integrons in *Salmonella enterica*

isolates from poultry and swine. Southeast Asian J. Trop. Med. Public. Health. 38: 855-862.

Chuanchuen, R., Narasaki, C.T. and Schweizer, H.P. 2002. The MexJK efflux pump of Pseudomonas aeruginosa requires OprM for antibiotic efflux but not for efflux of triclosan. J Bacteriol. 184(18): 5036-5044.

Chuanchuen, R., Narasaki, C.T. and Schweizer, H.P. 2002. The MexJK efflux pump of *Pseudomonas aeruginosa* requires OprM for antibiotic efflux but not for efflux of triclosan. J. Bacteriol. 184: 5036-5044.

Chuanchuen, R. and Padungtod, P. 2009. Antibiotic Resistance Genes in *Salmonella enterica* Isolates from Poultry and Swine. J. Vet. Med. Sci. 70: 1349-1355.

Chuanchuen, R. and Padungtod, P. 2009. Antimicrobial resistance genes in Salmonella enterica isolates from poultry and swine in Thailand. J Vet Med Sci. 71(10): 1349-1355.

Chuanchuen, R., Wannaprasat, W., Ajariyakhajorn, K. and Schweizer, H.P. 2008. Role of the MexXY multidrug efflux pump in moderate aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from Pseudomonas mastitis. Microbiol. Immunol. 52(8): 392-398.

Chuanchuen, R., Wannaprasat, W., Ajariyakhajorn, K. and Schweizer, H.P. 2008. Role of the MexXY multidrug efflux pump in moderate aminoglycoside resistance in Pseudomonas aeruginosa isolates from Pseudomonas mastitis. Microbiol Immunol. 52(8): 392-398.

Chung, D.R., Song, J.H., Kim, S.H., Thamlikitkul, V., Huang, S.G., Wang, H., So, T.M., Yasin, R.M., Hsueh, P.R., Carlos, C.C., Hsu, L.Y., Buntaran, L., Lalitha, M.K., Kim, M.J., Choi, J.Y., Kim, S.I., Ko, K.S., Kang, C.I. and Peck, K.R. 2011. High prevalence of multidrug-resistant nonfermenters in hospital-acquired pneumonia in Asia. Am J Respir Crit Care Med. 184(12): 1409-1417.

CLSI. 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard-Second Edition. NCCLS document M31-A2. PA, USA: CLSI. 84 pp.

CLSI. 2008. Performance Standards for Antimicrobial Susceptibility Testing: Eighteenth Informational Supplement. CLSI document M100-S18. Wayne, PA, USA: Clinical Laboratory Standards Institute. pp.

CLSI. 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Approved standard-Fourth Edition VET01-A4. Wayne, PA, USA: Clinical and Laboratory Standards Institute. pp.

Colinon, C., Jocktane, D., Brothier, E., Rossolini, G.M., Cournoyer, B. and Nazaret, S. 2010. Genetic analyses of Pseudomonas aeruginosa isolated from healthy captive snakes: evidence of high inter- and intrasite dissemination and occurrence of antibiotic resistance genes. Environ Microbiol. 12(3): 716-729.

Correia, M., Boavida, F., Grosso, F., Salgado, M.J., Lito, L.M., Cristino, J.M., Mendo, S. and Duarte, A. 2003. Molecular characterization of a new class 3 integron in Klebsiella pneumoniae. Antimicrob Agents Chemother. 47(9): 2838-2843.

Costerton, J.W. 2001. Cystic fibrosis pathogenesis and the role of biofilms in persistent infections. Trends Microbiol. 9: 50-52.

de Bentzmann, S. and Plesiat, P. 2011. The Pseudomonas aeruginosa opportunistic pathogen and human infections. Environ Microbiol. 13(7): 1655-1665.

de Lorenzo, V. and Timmis, K.N. 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol. 235: 386-405.

Dean, C.R. and Goldberg, J.B. 2002. Pseudomonas aeruginosa galU is required for a complete lipopolysaccharide core and repairs a secondary mutation in a PA103 (serogroup O11) wbpM mutant. FEMS Microbiol Lett. 210(2): 277-283.

Delmar, J.A., Su, C.C. and Yu, E.W. 2014. Bacterial multidrug efflux transporters. Annu Rev Biophys. 43: 93-117.

E. L. Westman, J.M.M., and J. S. Lam. 2010. Pseudomonas. In: Pathogenesis of Bacterial Infections in Animals 4thed. J.F.P. Carlton L. Gyles, J. Glenn Songer, Charles O. Thoen (ed), Vol. 1. Ames, Iowa, USA: Blackwell Publishing. 443-468.

Ekkapobyotin, C., Padungtod, P. and Chuanchuen, R. 2008. Antimicrobial resistance of Campylobacter coli isolates from swine. Int J Food Microbiol. 128(2): 325-328.

El'Garch, F., Jeannot, K., Hocquet, D., Llanes-Barakat, C. and Plesiat, P. 2007. Cumulative effects of several nonenzymatic mechanisms on the resistance of Pseudomonas aeruginosa to aminoglycosides. Antimicrob Agents Chemother. 51(3): 1016-1021.

El'Garch, F., Jeannot, K., Hocquet, D., Llanes-Barakat, C. and Plesiat, P. 2007. Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. 51(3): 1016-1021.

Evans, K., Adewoye, L. and Poole, K. 2001. MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. J. Bacteriol. 183: 807-812.

Falagas, M.E., Koletsi, P.K. and Bliziotis, I.A. 2006. The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) Acinetobacter baumannii and Pseudomonas aeruginosa. J Med Microbiol. 55(Pt 12): 1619-1629.

Fluit, A.C. and Schmitz, F.J. 1999. Class 1 integrons, gene cassettes, mobility, and epidemiology. Eur. J Clin. Microbiol. Infect. Dis. 18(11): 761-770.

Fluit, A.C. and Schmitz, F.J. 1999. Class 1 integrons, gene cassettes, mobility, and epidemiology. Eur J Clin Microbiol Infect Dis. 18(11): 761-770.

Garza-Ramos, U., Morfin-Otero, R., Sader, H.S., Jones, R.N., Hernandez, E., Rodriguez-Noriega, E., Sanchez, A., Carrillo, B., Esparza-Ahumada, S. and Silva-Sanchez, J. 2008. Metallo-beta-lactamase gene $bla_{(\text{IMP-15})}$ in a class 1 integron, In95, from *Pseudomonas aeruginosa* clinical isolates from a hospital in Mexico. Antimicrob. Agents Chemother. 52(8): 2943-2946.

Gatoria, I.S., Saini, N.S., Rai, T.S. and Dwivedi, P.N. 2006. Comparison of three techniques for the diagnosis of urinary tract infections in dogs with urolithiasis. J Small Anim. Pract. 47(12): 727-732.

Gaze, W.H., Abdouslam, N., Hawkey, P.M. and Wellington, E.M. 2005. Incidence of class 1 integrons in a quaternary ammonium compound-polluted environment. Antimicrob Agents Chemother. 49(5): 1802-1807.

Gilleland, L.B., Gilleland, H.E., Gibson, J.A. and Champlin, F.R. 1989. Adaptive resistance to aminoglycoside antibiotics in Pseudomonas aeruginosa. J Med Microbiol. 29(1): 41-50.

Girlich, D., Naas, T., Leelaporn, A., Poirel, L., Fennewald, M. and Nordmann, P. 2002. Nosocomial spread of the integron-located veb-1-like cassette encoding an extended-pectrum beta-lactamase in *Pseudomonas aeruginosa* in Thailand. Clin. Infect. Dis. 34(5): 603-611.

Girlich, D., Naas, T., Leelaporn, A., Poirel, L., Fennewald, M. and Nordmann, P. 2002. Nosocomial spread of the integron-located veb-1-like cassette encoding an extended-pectrum beta-lactamase in Pseudomonas aeruginosa in Thailand. Clin Infect Dis. 34(5): 603-611.

Gu, B., Tong, M., Zhao, W., Liu, G., Ning, M., Pan, S. and Zhao, W. 2007. Prevalence and characterization of class I integrons among *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates from patients in Nanjing, China. J. Clin. Microbiol. 45(1): 241-243.

Gutierrez, O., Juan, C., Cercenado, E., Navarro, F., Bouza, E., Coll, P., Perez, J.L. and Oliver, A. 2007. Molecular epidemiology and mechanisms of carbapenem resistance in Pseudomonas aeruginosa isolates from Spanish hospitals. Antimicrob Agents Chemother. 51(12): 4329-4335.

Hachler, H., Santanam, P. and Kayser, F.H. 1996. Sequence and characterization of a novel chromosomal aminoglycoside phosphotransferase gene, aph (3')-IIb, in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 40(5): 1254-1256.

Hamosh, A., FitzSimmons, S.C., Macek, M., Jr., Knowles, M.R., Rosenstein, B.J. and Cutting, G.R. 1998. Comparison of the clinical manifestations of cystic fibrosis in black and white patients. J. Pediatr. 132(2): 255-259.

Han, H.L., Jang, S.J., Park, G., Kook, J.K., Shin, J.H., Shin, S.H., Kim, D.M., Cheon, J.S., Moon, D.S. and Park, Y.J. 2008. Identification of an atypical integron carrying an IS26-disrupted *aadA1* gene cassette in *Acinetobacter baumannii*. Int. J. Antimicrob. Agents. 32(2): 165-169.

Harada, K., Arima, S., Niina, A., Kataoka, Y. and Takahashi, T. 2012. Characterization of Pseudomonas aeruginosa isolates from dogs and cats in Japan: current status

of antimicrobial resistance and prevailing resistance mechanisms. Microbiol Immunol. 56(2): 123-127.

Hariharan, H., Coles, M., Poole, D., Lund, L. and Page, R. 2006. Update on antimicrobial susceptibilities of bacterial isolates from canine and feline otitis externa. Can. Vet. J. 47(3): 253-255.

Hawser, S.P., Bouchillon, S.K., Hoban, D.J., Badal, R.E., Hsueh, P.R. and Paterson, D.L. 2009. Emergence of high levels of extended-spectrum-beta-lactamase-producing gram-negative bacilli in the Asia-Pacific region: data from the Study for Monitoring Antimicrobial Resistance Trends (SMART) program, 2007. Antimicrob Agents Chemother. 53(8): 3280-3284.

Hoang, T.T., Karkhoff-Schweizer, R.R., Kutchma, A.J. and Schweizer, H.P. 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene. 212: 77-86.

Hocquet, D., Nordmann, P., El Garch, F., Cabanne, L. and Plesiat, P. 2006. Involvement of the MexXY-OprM efflux system in emergence of cefepime resistance in clinical strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 50(4): 1347-1351.

Hocquet, D., Vogne, C., El Garch, F., Vejux, A., Gotoh, N., Lee, A., Lomovskaya, O. and Plesiat, P. 2003. MexXY-OprM efflux pump is necessary for a adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. 47(4): 1371-1375.

Hsu, S.C., Chiu, T.H., Pang, J.C., Hsuan-Yuan, C.H., Chang, G.N. and Tsen, H.Y. 2006. Characterisation of antimicrobial resistance patterns and class 1 integrons among *Escherichia coli* and *Salmonella enterica* serovar Choleraesuis strains isolated from humans and swine in Taiwan. Int. J. Antimicrob. Agents. 27(5): 383-391.

Islam, S., Jalal, S. and Wretlind, B. 2004. Expression of the MexXY efflux pump in amikacin-resistant isolates of *Pseudomonas aeruginosa*. Clin. Microbiol. Infect. 10(10): 877-883.

Islam, S., Jalal, S. and Wretlind, B. 2004. Expression of the MexXY efflux pump in amikacin-resistant isolates of Pseudomonas aeruginosa. Clin Microbiol Infect. 10(10): 877-883.

Islam, S., Oh, H., Jalal, S., Karpati, F., Ciofu, O., Hoiby, N. and Wretlind, B. 2009. Chromosomal mechanisms of aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Clin. Microbiol. Infect. 15(1): 60-66.

Islam, S., Oh, H., Jalal, S., Karpati, F., Ciofu, O., Hoiby, N. and Wretlind, B. 2009. Chromosomal mechanisms of aminoglycoside resistance in Pseudomonas aeruginosa isolates from cystic fibrosis patients. Clin Microbiol Infect. 15(1): 60-66.

Jeannot, K., Elsen, S., Kohler, T., Attree, I., van Delden, C. and Plesiat, P. 2008. Resistance and virulence of Pseudomonas aeruginosa clinical strains overproducing the MexCD-OprJ efflux pump. Antimicrob Agents Chemother. 52(7): 2455-2462.

Jwu-Ching Shu, J.-H.C., Leung-Kei Siu, An-Jing Kuo, Shu-Huan Huang, Lin-Hui Su, Tsu-Lan Wu. 2012. Interplay between mutational and horizontally acquired resistance mechanisms and its association with carbapenem resisance amongst exensively drug-resistant *Pseudomonas aeruginosa* (XDR-PA). International Journal of Antimicrobial Agents. 39: 217-222.

Kathryn J. Eagye, M.A.B., David P. Nicolau. 2012. *Pseudomonas aeruginosa* is not just in the intensive care unit anymore: Implications for emperical therapy. Crit Care Med. 40(2): 1329-1332.

Kazama, H., Hamashima, H., Sasatsu, M. and Arai, T. 1998. Distribution of the antiseptic-resistance genes qacE and qacE delta 1 in gram-negative bacteria. FEMS Microbiol Lett. 159(2): 173-178.

Kim, J.Y., Park, Y.J., Kwon, H.J., Han, K., Kang, M.W. and Woo, G.J. 2008. Occurrence and mechanisms of amikacin resistance and its association with beta-lactamases in *Pseudomonas aeruginosa*: a Korean nationwide study. J. Antimicrob. Chemother. 62(3): 479-483.

King, A. and Phillips, I. 1985. Pseudomonads and related bacteria. In: Identification and identification of microorgansims of medical and veterinary importanceed. C.H. Collins and J.M. Grange (ed). New York: Academic Press. 1-11.

Kohler, T., Epp, S.F., Curty, L.K. and Pechere, J.C. 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of Pseudomonas aeruginosa. J Bacteriol. 181(20): 6300-6305.

Kohler, T., Michea-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L.K. and Pechere, J.C. 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of Pseudomonas aeruginosa. Mol Microbiol. 23(2): 345-354.

Kohler, T., Michea-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L.K. and Pechere, J.C. 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. 23: 345-354.

Kohler, T., Michea-Hamzehpour, M., Plesiat, P., Kahr, A.L. and Pechere, J.C. 1997. Differential selection of multidrug efflux systems by quinolones in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 41(11): 2540-2543.

Kolayli, F., Karadenizli, A., Savli, H., Ergen, K., Hatirnaz, O., Balikci, E., Budak, F. and Vahaboglu, H. 2004. Effect of carbapenems on the transcriptional expression of the oprD, oprM and oprN genes in Pseudomonas aeruginosa. J Med Microbiol. 53(Pt 9): 915-920.

Kucken, D., Feucht, H. and Kaulfers, P. 2000. Association of qacE and qacEDelta1 with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria. FEMS Microbiol Lett. 183(1): 95-98.

Lee, S.K., Park, D.C., Kim, M.G., Boo, S.H., Choi, Y.J., Byun, J.Y., Park, M.S. and Yeo, S.G. 2012. Rate of isolation and trends of antimicrobial resistance of multidrug resistant pseudomonas aeruginosa from otorrhea in chronic suppurative otitis media. Clin Exp Otorhinolaryngol. 5(1): 17-22.

Lee, Y.T., Huang, L.Y., Chen, T.L., Siu, L.K., Fung, C.P., Cho, W.L., Yu, K.W. and Liu, C.Y. 2009. Gene cassette arrays, antibiotic susceptibilities, and clinical characteristics of *Acinetobacter baumannii* bacteremic strains harboring class 1 integrons. J. Microbiol. Immunol. Infect. 42(3): 210-219.

Levesque, C., Piche, L., Larose, C. and Roy, P.H. 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. 39(1): 185-191.

Li, X.Z., Nikaido, H. and Poole, K. 1995. Role of mexA-mexB-oprM in antibiotic efflux in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 39(9): 1948-1953.

Li, Y., Mima, T., Komori, Y., Morita, Y., Kuroda, T., Mizushima, T. and Tsuchiya, T. 2003. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 52(4): 572-575.

Li, Y., Mima, T., Komori, Y., Morita, Y., Kuroda, T., Mizushima, T. and Tsuchiya, T. 2003. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in Pseudomonas aeruginosa. J Antimicrob Chemother. 52(4): 572-575.

Lister, P.D., Wolter, D.J. and Hanson, N.D. 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin. Microbiol. Rev. 22(4): 582-610.

Llanes, C., Hocquet, D., Vogne, C., Benali-Baitich, D., Neuwirth, C. and Plesiat, P. 2004. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrob. Agents Chemother. 48(5): 1797-1802.

Llanes, C., Kohler, T., Patry, I., Dehecq, B., van Delden, C. and Plesiat, P. 2011. Role of the MexEF-OprN efflux system in low-level resistance of Pseudomonas aeruginosa to ciprofloxacin. Antimicrob Agents Chemother. 55(12): 5676-5684.

Lomovskaya, O., Warren, M.S., Lee, A., Galazzo, J., Fronko, R., Lee, M., Blais, J., Cho, D., Chamberland, S., Renau, T., Leger, R., Hecker, S., Watkins, W., Hoshino, K., Ishida, H. and Lee, V.J. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob. Agents Chemother. 45: 105-116.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T. and Monnet, D.L. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant

bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 18(3): 268-281.

Maniati, M., Ikonomidis, A., Mantzana, P., Daponte, A., Maniatis, A.N. and Pournaras, S. 2007. A highly carbapenem-resistant *Pseudomonas aeruginosa* isolate with a novel blaVIM-4/blaP1b integron overexpresses two efflux pumps and lacks OprD. J. Antimicrob. Chemother. 60(1): 132-135.

Maniati, M., Ikonomidis, A., Mantzana, P., Daponte, A., Maniatis, A.N. and Pournaras, S. 2007. A highly carbapenem-resistant Pseudomonas aeruginosa isolate with a novel blaVIM-4/blaP1b integron overexpresses two efflux pumps and lacks OprD. J Antimicrob Chemother. 60(1): 132-135.

Marchand, I., Damier-Piolle, L., Courvalin, P. and Lambert, T. 2004. Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. Antimicrob. Agents Chemother. 48(9): 3298-3304.

Martineau, F., Picard, F.J., Grenier, L., Roy, P.H., Ouellette, M. and Bergeron, M.G. 2000. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. . J Antimicrob Chemother. 46(4): 527-534.

Martineau, F., Picard, F.J., Lansac, N., Menard, C., Roy, P.H., Ouellette, M. and Bergeron, M.G. 2000. Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Antimicrob Agents Chemother. 44(2): 231-238.

Maseda, H., Saito, K., Nakajima, A. and Nakae, T. 2000. Variation of the *mexT* gene, a regulator of the MexEF-OprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. 192: 107-112.

Masoud-Landgraf, L., Badura, A., Eber, E., Feierl, G., Posch, J., Zarfel, G., Zach, M. and Marth, E. 2012. Molecular epidemiology of Pseudomonas aeruginosa in cystic fibrosis patients from Southeast Austria. Wien Klin Wochenschr. 124(7-8): 262-265.

Masuda, N., Gotoh, N., Ishi, C., Sakagawa, E., Ohya, S. and Nishino, T. 1999. Interplay between chromosomal β-lactamase and the MexAB-OprM efflux system in intrinsic resistance to β-lactams in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 43: 400-402.

Masuda, N., Sagagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H. and Nishino, T. 2000. Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 44: 2242-2246.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H. and Nishino, T. 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 44: 3322-3327.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H. and Nishino, T. 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 44(12): 3322-3327.

Matsuo, Y., Eda, S., Gotoh, N., Yoshihara, E. and Nakae, T. 2004. MexZ-mediated regulation of mexXY multidrug efflux pump expression in Pseudomonas aeruginosa by binding on the mexZ-mexX intergenic DNA. FEMS Microbiol Lett. 238(1): 23-28.

Mekic, S., Matanovic, K. and Seol, B. 2011. Antimicrobial susceptibility of Pseudomonas aeruginosa isolates from dogs with otitis externa. Vet Rec. 169(5): 125.

Mekic, S., Matanovic, K. and Seol, B. 2011. Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolates from dogs with otitis externa. Vet. Rec. 169(5): 125.

Mesaros, N., Glupczynski, Y., Avrain, L., Caceres, N.E., Tulkens, P.M. and Van Bambeke, F. 2007. A combined phenotypic and genotypic method for the detection of Mex efflux pumps in Pseudomonas aeruginosa. J Antimicrob Chemother. 59(3): 378-386.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Mesaros, N., Glupczynski, Y., Avrain, L., Caceres, N.E., Tulkens, P.M. and Van Bambeke, F. 2007. A combined phenotypic and genotypic method for the detection of Mex efflux pumps in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 59(3): 378-386.

Miller, G.H., Sabatelli, F.J., Hare, R.S., Glupczynski, Y., Mackey, P., Shlaes, D., Shimizu, K. and Shaw, K.J. 1997. The most frequent aminoglycoside resistance mechanisms--changes with time and geographic area: a reflection of aminoglycoside usage patterns? Aminoglycoside Resistance Study Groups. Clin. Infect. Dis. 24 Suppl 1: S46-62.

Morita, Y., Komori, Y., Mima, T., Kuroda, T., Mizushima, T. and Tsuchiya, T. 2001. Construction of a series of mutants lacking all of the four major *mex* operons for multidrug efflux pumps or possessing each one of the operons from *Pseudomonas aeruginosa* PAO1: MexCD-OprJ is an inducible pump. FEMS Microbiol. Lett. 202: 139-143.

Morita, Y., Tomida, J. and Kawamura, Y. 2012. Primary mechanisms mediating aminoglycoside resistance in the multidrug-resistant Pseudomonas aeruginosa clinical isolate PA7. Microbiology. 158(Pt 4): 1071-1083.

Murata, T., Kuwagaki, M., Shin, T., Gotoh, N. and Nishino, T. 2002. The substrate specificity of tripartite efflux systems of *Pseudomonas aeruginosa* is determined by the RND component. Biochem. Biophys. Res. Commun. 299(2): 247-251.

Nagaveni, S., Rajeshwari, H., Oli, A.K., Patil, S.A. and Chandrakanth, R.K. 2011. Widespread Emergence of Multidrug Resistant Pseudomonas aeruginosa Isolated from CSF Samples. Indian J Microbiol. 51(1): 2-7.

Navon-Venezia, S., Ben-Ami, R. and Carmeli, Y. 2005. Update on Pseudomonas aeruginosa and Acinetobacter baumannii infections in the healthcare setting. Curr Opin Infect Dis. 18(4): 306-313.

NCCLS. 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard-Second Edition. NCCLS document M31-A2. PA, USA: NCCLS. 84 pp.

Ochs, M.M., McCusker, M.P., Bains, M. and Hancock, R.E. 1999. Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. Antimicrob. Agents Chemother. 43(5): 1085-1090.

Ohnishi, M., Sawada, T., Hirose, K., Sato, R., Hayashimoto, M., Hata, E., Yonezawa, C. and Kato, H. 2011. Antimicrobial susceptibilities and bacteriological characteristics of bovine Pseudomonas aeruginosa and Serratia marcescens isolates from mastitis. Vet Microbiol. 154(1-2): 202-207.

Over, U., Gur, D., Unal, S. and Miller, G.H. 2001. The changing nature of aminoglycoside resistance mechanisms and prevalence of newly recognized resistance mechanisms in Turkey. Clin. Microbiol. Infect. 7(9): 470-478.

Partridge, S.R., Collis, C.M. and Hall, R.M. 2002. Class 1 integron containing a new gene cassette, *aadA10*, associated with Tn1404 from R151. Antimicrob. Agents Chemother. 46(8): 2400-2408.

Pedersen, K., Pedersen, K., Jensen, H., Finster, K., Jensen, V.F. and Heuer, O.E. 2007. Occurrence of antimicrobial resistance in bacteria from diagnostic samples from dogs. J. Antimicrob. Chemother. 60(4): 775-781.

Petersen, A.D., Walker, R.D., Bowman, M.M., Schott, H.C., 2nd and Rosser, E.J., Jr. 2002. Frequency of isolation and antimicrobial susceptibility patterns of *Staphylococcus intermedius* and *Pseudomonas aeruginosa* isolates from canine skin and ear samples over a 6-year period (1992-1997). J. Am. Anim. Hosp. Assoc. 38(5): 407-413.

Petersen, A.D., Walker, R.D., Bowman, M.M., Schott, H.C., 2nd and Rosser, E.J., Jr. 2002. Frequency of isolation and antimicrobial susceptibility patterns of Staphylococcus intermedius and Pseudomonas aeruginosa isolates from canine skin and ear samples over a 6-year period (1992-1997). J Am Anim Hosp Assoc. 38(5): 407-413.

Pier, G.B. 1998. *Pseudomonas aeruginosa*: a key problem in cystic fibrosis. ASM News. 64: 339-347.

Poole, K. 2001. Multidrug efflux pumps and antimicrobial resistance in Pseudomonas aeruginosa and related organisms. J Mol Microbiol Biotechnol. 3(2): 255-264.

Poole, K. 2005. Aminoglycoside resistance in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 49(2): 479-487.

Poole, K. 2005. Aminoglycoside resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 49(2): 479-487.

Poole, K. 2005. Efflux-mediated antimicrobial resistance. J. Antimicrob. Chemother. 56(1): 20-51.

Poole, K. and Srikumar, R. 2001. Multidrug efflux in Pseudomonas aeruginosa: components, mechanisms and clinical significance. Curr Top Med Chem. 1(1): 59-71.

Poonsuk, K. and Chuanchuen, R. 2012. Contribution of the MexXY Multidrug Efflux Pump and Other Chromosomal Mechanisms on Aminoglycoside Resistance in <i>Pseudomonas aeruginosa</i> Isolates from Canine and Feline Infections. J Vet Med Sci.

Poonsuk, K. and Chuanchuen, R. 2012. Contribution of the MexXY Multidrug Efflux Pump and Other Chromosomal Mechanisms on Aminoglycoside Resistance in Pseudomonas aeruginosa Isolates from Canine and Feline Infections. J Vet Med Sci.

Poonsuk, K., Tribuddharat, C. and Chuanchuen, R. 2012. Class 1 in tegrons in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from clinical isolates. Southeast Asian J Trop Med Public Health. 43(2): 376-384.

Poonsuk, K., Tribuddharat, C. and Chuanchuen, R. 2012. Class 1 integrons in Pseudomonas aeruginosa and Acinetobacter baumannii isolated from clinical isolates. Southeast Asian J Trop Med Public Health. 43(2): 376-384.

Poonsuk, K., Tribuddharat, C. and Chuanchuen, R. 2013. Aminoglycoside resistance mechanisms in Pseudomonas aeruginosa isolates from non-cystic fibrosis patients in Thailand. Can J Microbiol. 59(1): 51-56.

Poonsuk, K., Tribuddharat, C. and Chuanchuen, R. 2014. Simultaneous overexpression of multidrug efflux pumps in Pseudomonas aeruginosa non-cystic fibrosis clinical isolates. Can J Microbiol. 60(7): 437-443.

Pumbwe, L. and Piddock, L.J. 2000. Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 44: 2861-2864.

Rodriguez-Martinez, J.M., Poirel, L. and Nordmann, P. 2009. Molecular epidemiology and mechanisms of carbapenem resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 53(11): 4783-4788.

Rosenberger, L.H., LaPar, D.J. and Sawyer, R.G. 2012. Infections caused by multidrug resistant organisms are not associated with overall, all-cause mortality in the surgical intensive care unit: the 20,000 foot view. J Am Coll Surg. 214(5): 747-755.

Rubin, J., Walker, R.D., Blickenstaff, K., Bodeis-Jones, S. and Zhao, S. 2008. Antimicrobial resistance and genetic characterization of fluoroquinolone resistance of *Pseudomonas aeruginosa* isolated from canine infections. Vet. Microbiol. 131(1-2): 164-172.

Sadeghifard, N., Valizadeh, A., Zolfaghary, M.R., Maleki, M.H., Maleki, A., Mohebi, R., Ghafourian, S. and Khosravi, A. 2012. Relationship between the Presence of the nalC Mutation and Multidrug Resistance in Pseudomonas aeruginosa. Int J Microbiol. 2012: 575193.

Schweizer, H.P. 2003. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet. Mol. Res. 2(1): 48-62.

Sevillano, E., Valderrey, C., Canduela, M.J., Umaran, A., Calvo, F. and Gallego, L. 2006. Resistance to antibiotics in clinical isolates of *Pseudomonas aeruginosa*. Pathol. Biol. (Paris). 54(8-9): 493-497.

Seward, R.J. 1999. Detection of integrons in worldwide nosocomial isolates of *Acinetobacter* spp. Clin. Microbiol. Infect. 5(6): 308-318.

Shahid, M. and Malik, A. 2005. Resistance due to aminoglycoside modifying enzymes in Pseudomonas aeruginosa isolates from burns patients. Indian J Med Res. 122(4): 324-329.

Sobel, M.L., Hocquet, D., Cao, L., Plesiat, P. and Poole, K. 2005. Mutations in PA3574 (nalD) lead to increased MexAB-OprM expression and multidrug resistance in

laboratory and clinical isolates of Pseudomonas aeruginosa. Antimicrob Agents Chemother. 49(5): 1782-1786.

Sobel, M.L., McKay, G.A. and Poole, K. 2003. Contribution of the MexXY Multidrug Transporter to Aminoglycoside Resistance in *Pseudomonas aeruginosa* Clinical Isolates. Antimicrobial agents and chemotherapy. 47(10): 3202-3207.

Sobel, M.L., McKay, G.A., Poole, K., Department of, M. and Immunology, Q.s.U.K.O.K.L.N.C. 2003. Contribution of the MexXY Multidrug Transporter to Aminoglycoside Resistance in *Pseudomonas aeruginosa* Clinical Isolates. Antimicrobial agents and chemotherapy. 47(10): 3202-3207.

Sobel, M.L., Neshat, S. and Poole, K. 2005. Mutations in PA2491 (mexS) promote MexT-dependent mexEF-oprN expression and multidrug resistance in a clinical strain of Pseudomonas aeruginosa. J Bacteriol. 187(4): 1246-1253.

Srikumar, R., C.J., P. and Poole, K. 2000. Influence of mutations in the *mexR* repressor gene on expression of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol. 182: 1410-1414.

Stalder, T., Barraud, O., Casellas, M., Dagot, C. and Ploy, M.C. 2012. Integron involvement in environmental spread of antibiotic resistance. Front Microbiol. 3: 119.

Strateva, T. and Yordanov, D. 2009. Pseudomonas aeruginosa - a phenomenon of bacterial resistance. J Med Microbiol. 58(Pt 9): 1133-1148.

Sunde, M. and Norstrom, M. 2006. The prevalence of, associations between and conjugal transfer of antibiotic resistance genes in *Escherichia coli* isolated from Norwegian meat and meat products. J. Antimicrob. Chemother. 58(4): 741-747.

Taber, H.W., Mueller, J.P., Miller, P.F. and Arrow, A.S. 1987. Bacterial uptake of aminoglycoside antibiotics. Microbiol Rev. 51(4): 439-457.

Tegos, G., Stermitz, F.R., Lomovskaya, O. and Lewis, K. 2002. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemother. 46(10): 3133-3141.

Tenover, F.C. 2006. Mechanisms of antimicrobial resistance in bacteria. Am J Med. 119(6 Suppl 1): S3-10; discussion S62-70.

Turnidge, J. 2003. Pharmacodynamics and dosing of aminoglycosides. Infect. Dis. Clin. North Am. 17(3): 503-528, v.

Turton, J.F., Kaufmann, M.E., Glover, J., Coelho, J.M., Warner, M., Pike, R. and Pitt, T.L. 2005. Detection and typing of integrons in epidemic strains of *Acinetobacter baumannii* found in the United Kingdom. J. Clin. Microbiol. 43(7): 3074-3082.

Vakulenko, S.B. and Mobashery, S. 2003. Versatility of aminoglycosides and prospects for their future. Clin. Microbiol. Rev. 16(3): 430-450.

Vakulenko, S.B. and Mobashery, S. 2003. Versatility of aminoglycosides and prospects for their future. Clin Microbiol Rev. 16(3): 430-450.

van Essen-Zandbergen, A., Smith, H., Veldman, K. and Mevius, D. 2007. Occurrence and characteristics of class 1, 2 and 3 integrons in *Escherichia coli*, *Salmonella* and *Campylobacter* spp. in the Netherlands. J. Antimicrob. Chemother. 59(4): 746-750.

Vaziri, F., Peerayeh, S.N., Nejad, Q.B. and Farhadian, A. 2011. The prevalence of aminoglycoside-modifying enzyme genes (aac (6')-I, aac (6')-II, ant (2")-I, aph (3')-VI) in Pseudomonas aeruginosa. Clinics (Sao Paulo). 66(9): 1519-1522.

Vettoretti, L., Floret, N., Hocquet, D., Dehecq, B., Plesiat, P., Talon, D. and Bertrand, X. 2009. Emergence of extensive-drug-resistant Pseudomonas aeruginosa in a French university hospital. Eur J Clin Microbiol Infect Dis. 28(10): 1217-1222.

Vogne, C., Aires, J.R., Bailly, C., Hocquet, D. and Plesiat, P. 2004. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrob. Agents Chemother. 48(5): 1676-1680.

Wang, J., Zhou, J.Y., Qu, T.T., Shen, P., Wei, Z.Q., Yu, Y.S. and Li, L.J. 2010. Molecular epidemiology and mechanisms of carbapenem resistance in Pseudomonas aeruginosa isolates from Chinese hospitals. Int J Antimicrob Agents. 35(5): 486-491.

Watson, J.M. and Holloway, B.W. 1978. Chromosome mapping in Pseudomonas aeruginosa PAT. J Bacteriol. 133(3): 1113-1125.

Watson, J.M. and Holloway, B.W. 1978. Chromosome mapping in *Pseudomonas aeruginosa*. J. Bacteriol. 133: 1113-1125.

West, S.E., Schweizer, H.P., Dall, C., Sample, A.K. and Runyen-Janecky, L.J. 1994. Construction of improved Escherichia-Pseudomonas shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in Pseudomonas aeruginosa. Gene. 148(1): 81-86.

Westbrock-Wadman, S., Sherman, D.R., Hickey, M.J., Coulter, S.N., Zhu, Y.Q., Warrener, P., Nguyen, L.Y., Shawar, R.M., Folger, K.R. and Stover, C.K. 1999. Characterization of a Pseudomonas aeruginosa efflux pump contributing to aminoglycoside impermeability. Antimicrob Agents Chemother. 43(12): 2975-2983.

Westbrock-Wadman, S., Sherman, D.R., Hickey, M.J., Coulter, S.N., Zhu, Y.Q., Warrener, P., Nguyen, L.Y., Shawar, R.M., Folger, K.R. and Stover, C.K. 1999. Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside resistance. Antimicrob. Agents Chemother. 43: 2975-2983.

Westfall, L.W., Carty, N.L., Layland, N., Kuan, P., Colmer-Hamood, J.A. and Hamood, A.N. 2006. mvaT mutation modifies the expression of the Pseudomonas aeruginosa multidrug efflux operon mexEF-oprN. FEMS Microbiol Lett. 255(2): 247-254.

Winsor, G.L., Lam, D.K., Fleming, L., Lo, R., Whiteside, M.D., Yu, N.Y., Hancock, R.E. and Brinkman, F.S. 2011. Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. Nucleic Acids Res. 39(Database issue): D596-600.

Winsor, G.L., Lam, D.K., Fleming, L., Lo, R., Whiteside, M.D., Yu, N.Y., Hancock, R.E. and Brinkman, F.S. 2011. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res. . 39: D596-600.

Wolter, D.J., Hanson, N.D. and Lister, P.D. 2008. Novel Mechanism of *mexEF-oprN* Efflux Pump Overexpression in *Pseudomonas aeruginosa* without co-regulation of *oprD* Expression. Abstr. 48th Intersci. Conf. Antimicrob. Agents Chemother. Washibgton, D.C.

Wolter, D.J., Smith-Moland, E., Goering, R.V., Hanson, N.D. and Lister, P.D. 2004. Multidrug resistance associated with *mexXY* expression in clinical isolates of

Pseudomonas aeruginosa from a Texas hospital. Diagn. Microbiol. Infect. Dis. 50(1): 43-50.

Woods, C.R., Versalovic, J., Koeuth, T. and Lupski, J.R. 1993. Whole-cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. J Clin Microbiol. 31(7): 1927-1931.

Xu, H., Davies, J. and Miao, V. 2007. Molecular characterization of class 3 integrons from *Delftia* spp. J. Bacteriol. 189(17): 6276-6283.

Xu, Z., Li, L., Shirtliff, M.E., Alam, M.J., Yamasaki, S. and Shi, L. 2009. Occurrence and characteristics of class 1 and 2 integrons in Pseudomonas aeruginosa isolates from patients in southern China. J Clin Microbiol. 47(1): 230-234.

Xu, Z., Li, L., Shirtliff, M.E., Alam, M.J., Yamasaki, S. and Shi, L. 2009. Occurrence and characteristics of class 1 and 2 integrons in *Pseudomonas aeruginosa* isolates from patients in southern China. J. Clin. Microbiol. 47(1): 230-234.

Yamamoto, M., Ueda, A., Kudo, M., Matsuo, Y., Fukushima, J., Nakae, T., Kaneko, T. and Ishigatsubo, Y. 2009. Role of MexZ and PA5471 in transcriptional regulation of mexXY in Pseudomonas aeruginosa. Microbiology. 155(Pt 10): 3312-3321.

Yoneda, K., Chikumi, H., Murata, T., Gotoh, N., Yamamoto, H., Fujiwara, H., Nishino, T. and Shimizu, E. 2005. Measurement of *Pseudomonas aeruginosa* multidrug efflux pumps by quantitative real-time polymerase chain reaction. FEMS Microbiol. Lett. 243(1): 125-131.

Zamankhan Malayeri, H., Jamshidi, S. and Zahraei Salehi, T. 2010. Identification and antimicrobial susceptibility patterns of bacteria causing otitis externa in dogs. Vet Res Commun. 34(5): 435-444.

Zong, Z., Lu, X., Valenzuela, J.K., Partridge, S.R. and Iredell, J. 2008. An outbreak of carbapenem-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemase in western China. Int. J. Antimicrob. Agents. 31(1): 50-54.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX

APPENDIX

Part of this dissertation has been published or present in the conferences as follow:

List of international publications

Poonsuk, K, Tribuddharat, C and Chuanchuen, R. 2012. Class 1 Integrons in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* Isolated from Clinical Isolates. Southeast Asian J Trop Med Public Health. 43:376-384.

Poonsuk, K and Chuanchuen, R. 2012. Contribution of the MexXY Multidrug Efflux Pump and Other Chromosomal Mechanisms on Aminoglycoside resistance in *Pseudomonas aeruginosa* Isolates from Canine and Feline Infections. J Vet Med Sc.i 74(12):1575-82. DOI DN/JST.JSTAGE/jvms/12-0239

Poonsuk, K, Tribuddharat, C and Chuanchuen, R. 2013. Aminoglycoside resistance mechanisms in *Pseudomonas aeruginosa* isolates from non-cystic fibrosis patients in Thailand. Can J Microbiol. 59(1):51-6. DOI 10.1139/cjm-2012-0465

Poonsuk, K., C. Tribuddharat and R. Chuanchuen. Simultaneous Expression of Multidrug Efflux Pumps in *Pseudomonas aeruginosa* Clinical Isolates. Can J Microbiol. 60(7):437-43. DOI 10.1139/cjm-2014-0239

Poonsuk, K and Chuanchuen, R. 2014. Detection of The Mex Efflux Pumps in *Pseudomonas aeruginosa* by Using a Combined Resistance-Phenotypic Markers and Multiplex RT-PCR. OJMM. 4(3). DOI: 10.4236/ojmm.2014.43018

Poonsuk, K and Chuanchuen, R. 2014. The multidrug-resistant *Pseudomonas aeruginosa* clinical isolates from dogs and cats expressed three multidrug efflux systems simultaneously. TJVM (submitted).

List of international conferences

Poonsuk, K, Pagdepanichkij, S and Chuanchuen, R. Contribution of the Multidrug Effflux System MexXY-OprM in Aminoglycosides Resistance *Pseudomonas aeruginosa* Clinical Isolates from Dogs and Cats. The 37th International Conference on Veterinary Science (ICVS), Bangkok, Thailand. February 29 – March 2, 2012.

Poonsuk, K. and Chuanchuen, R. 2014. MexEF-OprN, a Prominent Resistance Mechanism in Multidrug Resistance *Pseudomonas aeruginosa* Isolate from Dogs and Cats. 18th Federation of Asian Veterinary Association (FAVA) Congress. Marina Bay Sands, Singapore. November 28-30, 2014

<u>List of local conferences</u>

Poonsuk, K and Chuanchuen, R. 2011. Contribution of the MexXY-oprM efflux system in aminoglycosides resistance in the *Pseudomonas aeruginosa* clinical isolates from canine and feline. Proceeding of the RGJ Seminar Series LXXX “Innovations for Animal Health and Production II, Bangkok, Thailand. April 20, 2011

Poonsuk, K and Chuanchuen, R. 2012. Expression of the Multidrug efflux system MexCD-OprJ and MexEF-OprN in *Pseudomonas aeruginosa* isolates from animals and humans. RGJ Seminar Series LXXXVII "From Life Sciences to One Health", Bangkok, Thailand. May 17, 2012.

Poonsuk, K, Tribuddharat, C and Chuanchuen, R. 2013. Aminoglycoside resistance mechanisms in *Pseudomonas aeruginosa* from non-cystic fibrosis patients. RGJ-Ph.D. Congress XIV “Basic Research for Sustainable Development”, Chonburi, Thailand. April 5 – 7, 2013

VITA

Miss Kanchana Poonsuk was born on April, 10, 1985 in Bangkok, Thailand. She obtained Doctor of Veterinary Medicine from Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2009. She has been worked in Center for Antimicrobial Resistance Monitoring in Foodborne Pathogens (in cooperation with WHO) and WHO Global Foodborne Infections Network: South-East Asia and Western Pacific Region (GFN) before enrolled her Doctor of Philosophy Program in Veterinary Public Health, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. While study Ph.D. program, she received the scholarship from Thailand Research Fund (TRF) in Royal Golden Jubilee (RGJ) Ph.D. Program and participate research at University of Hawaii at Manoa as a recipient of Affiliate Scholar Award, East-West Center, Honolulu, Hawaii, United State. During her Ph.D. study, she had 6 international publications and 5 proceedings.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University